Full Paper

Prednisolone-Glucose Derivative Conjugate: Synthesis, Biodistribution and Pharmacodynamics Evaluation

Xing Liu[1], Wenhao Li[1], Zhen Liang[1], Xuan Zhang[2], Yangming Guo[1], Tao Gong[1], and Zhirong Zhang[1]

[1] Key Laboratory of Drug Targeting and Drug Delivery Systems, West China School of Pharmacy, Sichuan University, Chengdu, China
[2] Shenzhen Salubris Pharmaceuticals Co., Ltd., Shenzhen, China

This study was aimed at synthesizing and evaluating a prednisolone-glucose derivative conjugate (PDG) that was expected to increase renal biodistribution without affecting pharmacological action and to decrease the systemic side effects of prednisolone. The PDG was designed and synthesized by tethering 6-amino-6-deoxy-D-glucose (a D-glucose derivative) to prednisolone and its chemical structure was confirmed by ^{1}H NMR, ^{13}C NMR, and LC-MS. This conjugate was then subjected to *in vitro* and *in vivo* evaluation like stability studies, biological distribution, pharmacodynamics, and systemic side effects studies. In these studies, PDG not only showed significant enhancement of renal target efficiency with high values of relative uptake efficiency (RE, 24.1), concentration efficiency (CE, 8.6), and kidney targeting index (KTI, 16.3), but retained the curative potency against minimal change nephrosis (MCN). In the systemic side effects study, no osteoporosis was observed in rats after the administration of PDG for 20 days, which exhibited limited side effects. Conclusively, our findings showed a pharmacologically active conjugate with the characteristics of renal targeting and limited systemic side effects. The results implied the potential of PDG as a promising therapeutic in the treatment of renal diseases.

Keywords: D-Glucose derivative / Minimal change nephrosis (MCN) / Prednisolone / Renal targeting / Systemic side effects

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Introduction

Most glucocorticoids, a type of highly potent anti-inflammatory and immunosuppressive drugs, need to be administrated continuously and/or repeatedly in the clinic due to their quick elimination. In addition, even their moderate dose administration may cause many systemic side effects, owing to their non-specific distribution to all organs, multiple physiological effects, and pharmacological actions, with the most serious being osteoporosis [1, 2]. Therefore, the development of a drug delivery system that could transport glucocorticoids to the desired site of action and reduce their biodistribution in normal tissues would be of great value. Prednisolone (PD) is one of the most widely used glucocorticoids in the clinic as an immunosuppressive drug for kidney transplants and nephritis treatment. Some renal target delivering systems of PD such as randomly 50% *N*-acetylated low molecular weight chitosan (LMWC) [3] and prednisolone succinate-glucosamine conjugate (PSG) [4] have been developed to improve its therapeutic efficacy and alleviate the systemic side effects. Both LMWC and PSG have achieved specific renal delivery and can be absorbed by renal epithelial cells. However, the structure of LMWC is so complicated that the precise quality control of this system is at present not feasible. As for PSG, the instability of the ester linkage *in vivo* limits its further application.

D-Glucose, the main energy of cells, is delivered through the bloodstream and transported to tissues by the glucose transporter (GLUT) families. And so far, two types of GLUT families in kidney have been identified [5–7]. One is the Na^{+}-dependent glucose transporters (SGLTs), which mediate the Na^{+}/glucose cotransport function in the kidney, the other is the Na^{+}-independent GLUTs, which serve to facilitate diffusion through the lipid bilayer [5]. In the mammalian kidney,

Correspondence: Zhirong Zhang and Tao Gong, Key Laboratory of Drug Targeting and Drug Delivery Systems, West China School of Pharmacy, Sichuan University, Sichuan 610041, China.
E-mail: zrzzl@vip.sina.com; gongtaoy@126.com
Fax: +86 28 85501615

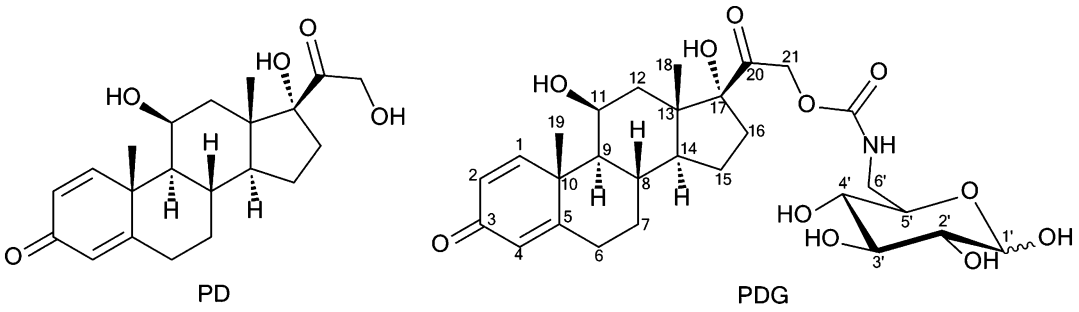


Figure 1. The structure of prednisolone (PD) and prednisolone-glucose derivative conjugate (PDG).

SGLTs contribute to the reabsorption of glucose that is filtered from the glomerulus in the process of urine formation. Studies have been shown that the modification of the C-6 position or to tether a substituent at the C-6 position of glucose has little influence on the affinity between the glucose derivative and GLUTs [6]. Herein, we propose an innovative covalent combination of PD and 6-amino-6-deoxy-D-glucose (a D-glucose derivative, see Fig. 1) for a potential SGLTs-mediated transportation. In addition, we also hope that this targeted transportation could enhance patient compliance and therapeutic efficacy of PD through the improved pharmacokinetics and biodistribution [7, 8].

In our study, PD was adopted as a model drug and a novel chemical conjugate (PDG) was synthesized and characterized. Then its *in vitro* stability in rat plasma and liver, kidney homogenate, biodistribution, systemic side effects, and curative potency against minimal change nephrosis (MCN) in rats were evaluated.

Results

In vitro stability of PDG

PDG showed high enzymolysis resistance *in vitro*, as the content of PDG did not decrease and no released PD was detected in both rat plasma and liver or kidney homogenate within 24 h (Fig. 2).

Biodistribution of PD and PDG in rat

The extraction recoveries of PDG in high, middle, and low concentrations ranged from 77 to 95% in plasma and different tissue homogenates, which were satisfying. PDG showed excellent renal targeting efficiency with 12- to 28-fold concentrations in the kidney compared with PD at different time points (Fig. 3). Its shorter elimination half-life in plasma led to a shorter MRT of 14.1 min (Table 1), which would contribute to decrease the non-targeted distribution of PDG. However, PDG had a slower renal elimination rate, with an MRT of 2.7 h in the kidney (Table 2); as a result, it could still be detected at a relatively high concentration at 2 h (51 nmol/g). On the contrary, PD suffered quicker elimination in the kidney, and was undetectable at 60 min. The results showed that PDG could be specifically delivered to the kidney quickly, with much lower concentration in organs (tissue) such as blood, heart, spleen, and lung than that of PD.

Preliminary study on pharmacodynamics of PDG

At the beginning of the pharmacodynamics study, no significant differences in the urinary protein content of rats were observed among each group. After 14 days, the urinary protein content of the normal group only slightly increased (6.56 mg); however, in the control group, it increased about nine-fold (78.31 mg; $p < 0.05$), which indicated a successful establishment of the rat pathologic model of MCN. Although the urinary protein content of the PD and PDG groups increased about 3.5-fold (23.50 mg) after 14 days, they were significantly lower than that of the control group ($p < 0.05$). And no obvious difference was observed between the PD and PDG groups on the 7th and 14th day, which suggested a similar therapeutic effect against MCN (Fig. 4).

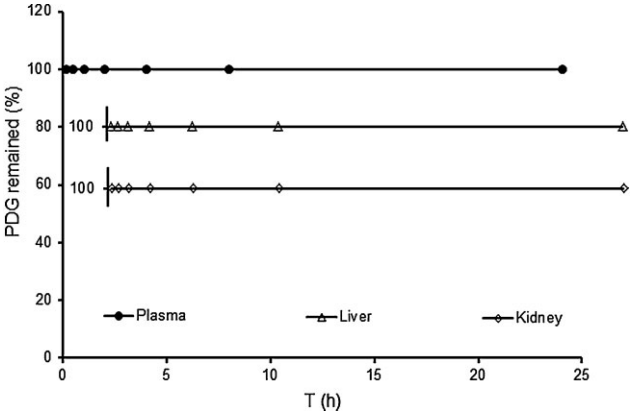


Figure 2. The *in vitro* stability of PDG in biological samples. PDG was incubated with fresh rat plasma (50%), liver or kidney homogenate (33%) for 24 h. All the mixtures were incubated at 37 ± 1°C in a shaking bath, and samples were taken at the predetermined time points. Results are means ± SD ($n = 3$).

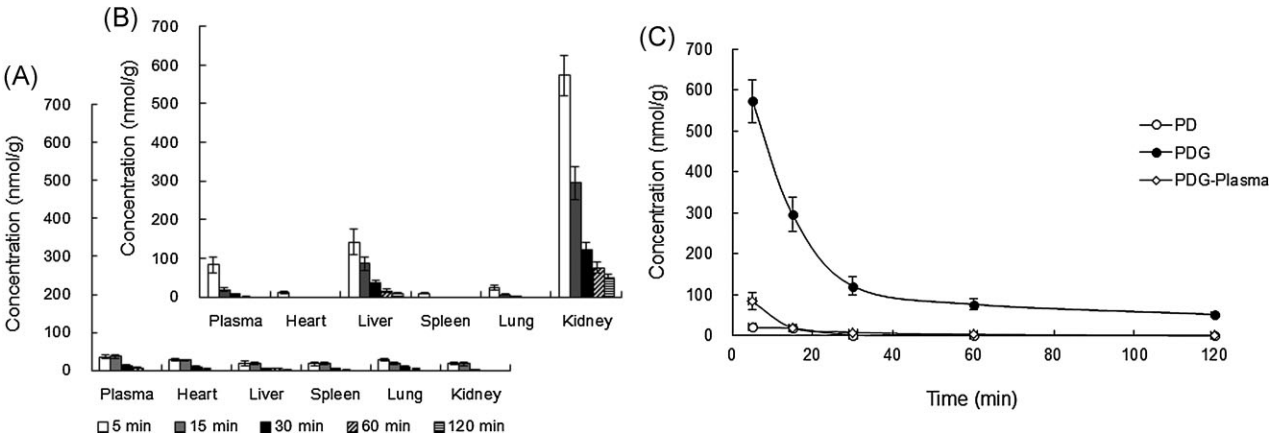


Figure 3. Biodistribution of PD (A), PDG (B), and the concentrations of PD and PDG in rat kidneys and plasma *versus* time curves (C) in Sprague–Dawley rats after intravenous injection (50 μmol/kg) within 2 h. Results are means ± SD ($n = 5$).

Table 1. Pharmacokinetic parameters of PD and PDG in rat plasma after intravenous injection of PD and PDG (50 μmol/kg).

	$T_{1/2}$ (min)	C_{max} (nmol/g)	MRT (min)	AUC_{0-t} (nmol/g h)
PD	22.2 ± 3.3	38.2 ± 4.5	25.4 ± 1.6	27.5 ± 1.7
PDG	5.3 ± 1.9*	83.5 ± 19.8*	14.1 ± 1.38*	23.7 ± 2.3

Results are means ± SD ($n = 5$).
* $p < 0.05$ with respect to PD.

Table 2. Pharmacokinetic parameters of PD and PDG in rat kidneys after intravenous injection of PD and PDG (50 μmol/kg).

	AUC_{0-t} (nmol/g h)	C_{max} (nmol/g)	MRT (min)	RE	CE	KTI
PD	10.6 ± 2.0	20.0 ± 3.1	11.4 ± 0.6	–	–	–
PDG	255.2 ± 29.2*	572.2 ± 53.1*	31.2 ± 1.2*	24.1	8.6	16.3

Results are means ± SD ($n = 5$).
* $p < 0.05$ with respect to PD.

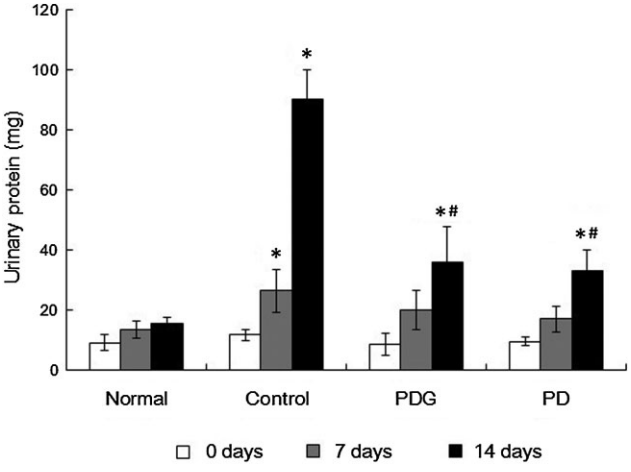


Figure 4. Urinary protein contents of rats in the various groups on day 0, the 7th day and 14th day (*$p < 0.05$ *vs.* the normal group; #$p < 0.05$ *vs.* the control group). Results are means ± SD ($n = 5$).

Serum analyses demonstrated that rats treated with PD and PDG were in better condition than those in the control group (see Fig. 5). Their biochemical indexes, including creatinine (CERA) and triglyceride (Tg), recovered to normal levels, which were similar with those in the normal group but significantly lower than those in the control group ($p < 0.05$). Blood urea nitrogen (BUN) content did not exhibit much variation among the groups. Although the albumin (ALB) levels of the rats in the PD and PDG groups did not recover to normal state, they were similar to each other. Conclusively, these data suggested that PDG kept the original potency of PD.

Adverse effects of PDG in normal rats

Studies on glucocorticoid-induced toxicity of PD and PDG were performed on male SD rats (120 ± 10 g) during a period of 20 days. The rats of the PD group grew much slower with smaller body weight (212.3 ± 10.2 g) and bone mineral

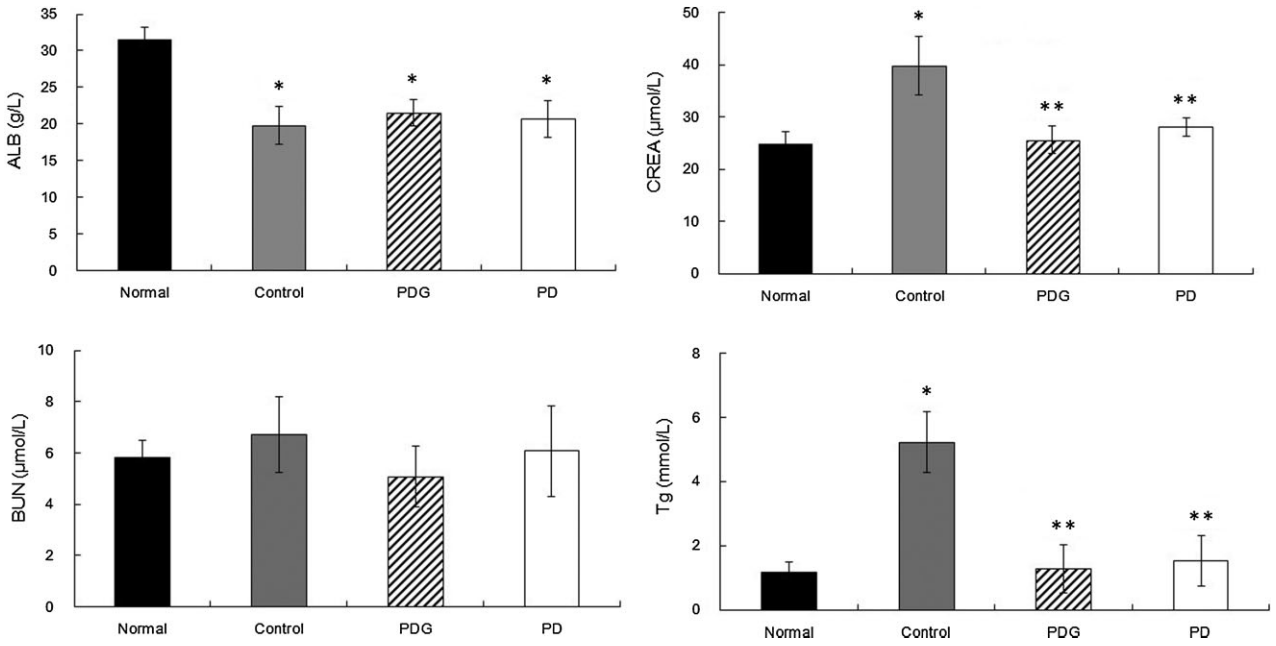


Figure 5. The serum biochemical indexes of rats in the various groups ($^{*}p < 0.05$ *vs.* the normal group; $^{**}p < 0.05$ *vs.* the control group). Results are means ± SD ($n = 5$).

density (BMD, 0.210 ± 0.003 g/cm^2) than those of the normal group ($p < 0.05$). In contrast, such influence on the rats of the PDG group was not observed as there was no significant difference in body weight and BMD between the PDG and the normal groups. However, the body weight and BMD of the rats in the PDG group were dramatically higher than those of the PD group, which indicated the limited glucocorticoid-induced side effects of PDG ($p < 0.05$; Fig. 6).

Discussion

In order to improve the therapeutic effects and alleviate the systemic side effects of some widely used drugs, researchers have turned to chemical modification as an effective approach to deliver them to the particular disease regions and to improve their biodistribution [9]. Nowadays, various transporter/receptor drug delivery systems have been

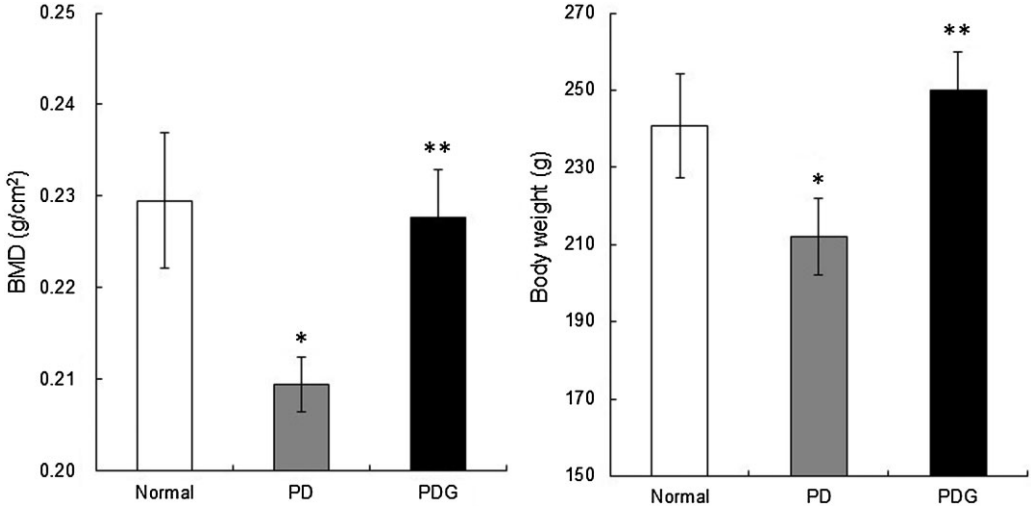


Figure 6. The BMD and body weight of the rats in various groups on the 21st day ($^{*}p < 0.05$ *vs.* the normal group; $^{**}p < 0.05$ *vs.* the PD group). Results are means ± SD ($n = 5$).

developed, which are expected to achieve targeted drug delivery, such as the glucosamine/megalin system [3, 10], the D-glucose/GLUT1 system [11], the peptide transport systems, and the amino acid transporters [12]. Although most of these systems could deliver drugs to the desired region, few had achieved the fundamental purpose of reducing the systemic side effects while maintaining the therapeutic effects. This may be due to several reasons: (i) The release mechanisms of drugs from macromolecular carriers are so complicated, including swelling, hydrolysis, erosion, and diffusion, as a result the drugs loaded on the carriers could not be totally released; (ii) the linkages between drugs and carriers are unstable *in vivo*, which results in their hydrolysis in circulation and low target efficiency; (iii) due to the active efflux systems, the released drug from the conjugates that do reach the desired regions might be transported back to the circulation and poor target efficiency happens; (iv) additionally, some carriers might have toxicity in tissues, which would notably limit their clinical application.

Several renal targeting delivery systems of PD have been developed [3, 4], but the issues mentioned above have not been totally solved. In this study, a stable chemical conjugate PDG was synthesized using 6-amino-6-deoxy-D-glucose as a ligand, which was coupled to PD by a carbamate bond [13, 14]. In contrast, two other 6-amino-6-deoxy-D-glucose-PD conjugates via ester linkage (succinic acid or *O*-phthalic acid) were hydrolyzed quickly in plasma *in vitro* and *in vivo*. There was no conjugate, but only PD could be detected within 3 min after intravenous administration, and their renal distribution was similar to PD (data not shown). Furthermore, the high stability of PDG could also avoid the secondary distribution of the released PD from the kidney to the blood circulation and accordingly evade the side effects. The evaluation of PDG was performed at different aspects, including performing a biodistribution study, analyzing the pharmacodynamics against MCN, and studying its adverse effects on normal rats. The results indicated a remarkable renal target efficiency of PDG with the enhancement of kidney distribution, accompanied by a decreased plasma distribution. Furthermore, the PDG administration could alleviate the gravity of MCN in rats with limited side effects.

It is well known that glucose in the glomerular filtrate can be efficiently reabsorbed by SGLT2 in the proximal convoluted tubule [15]. As a result, urine is essentially free from glucose in healthy individuals [16]. In our study, PDG showed a chemical structure with a glucose residue and a quick uptake process by the kidney. This phenomenon in experiments might provide us with the evidence that PDG might be an analogue of D-glucose. And the targeted accumulation of PDG in the kidney could be the result of an active transport process just like the reabsorption of the filtered glucose by SGLT2 [17, 18]. Currently, SGLT2 has been widely studied as the molecular target for the treatment of type 2 diabetes mellitus (T2DM) [15]. However, the renal-targeted delivery of drugs by glucose reabsorption in the kidney has not been reported until now, making the SGLT2 mediated renal targeting in this report a new method.

In biodistrubution study, PDG could still be detected at 2 h in the kidney, whereas it was undetectable at 15 min in the heart and the spleen, at 1 h in the lung and at 2 h in the plasma after intravenous injection. The reason may be that the reabsorbed PDG could not be released back into the circulation through the same way as the reabsorbed glucose released by the facilitative GLUTs [16]. This may be related with the fact that the glucose C-6 position modification prevented its transmembrane transport in cells [6]. Since PDG could neither be hydrolyzed *in vivo* nor be released into the circulation, it was ''locked'' in the kidney.

Minimal change nephrosis, originally named lipoid nephrosis, is mainly characterized by selective proteinuria, hypoalbuminemia, and hyperlipidemia. Glucocorticoids have been utilized for a long time in the treatment of MCN, and PD was the most widely used one [19]. In our study, MCN was induced in rats by a single intravenous administration of daunomycin hydrochloride. The successful establishment of the MCN rat model was confirmed both by the measurement of urinary protein contents and biochemical indexes (CREA, BUN, Tg, and ALB) of the rats. Remission was achieved after the treatment of PDG for 2 weeks with a curative effect similar to that of PD. The fact that the ALB level in both the PD and PDG groups did not recover to a normal state in 2 weeks just shows why the low cure rate of renal disease occurs in the clinic. Perhaps, the normalization of serum albumin would happen after a longer period of treatment [19].

In GC's clinical application, osteoporosis is one of their most common and serious side effects. The development of glucocorticoids inducing osteoporosis is a multifactorial process with complicated mechanisms [20, 21]. However, the nonspecific distribution of glucocorticoids might contribute to this pathological condition. In PDG's adverse effects study section, the intravenous injection of PD resulted in osteoporosis and growth inhibition in rats, whereas such side effects were not observed in the PDG group. Therefore, PDG's renal-targeted delivery and scarce distribution in non-targeted tissues may be the key issue of the limited side effects.

Conclusion

In our study, the novel synthesized PDG exhibited an ideal stability, excellent renal target efficiency, and the original therapeutic efficiency of PD but limited side effects. Thus, we conclude that PDG has the potential to be developed into a novel renal target delivery system of glucocorticoids.

Experimental

Materials and methods

Prednisolone (99.5% purity) was purchased from Henan Lihua Pharmaceutical CO., Ltd (Henan, China). All other chemicals and reagents were obtained commercially and were of analytical grade without further purification. Water was purified in an Aquapro water purification system (Chongqing, China). TLC (silica gel GF254) was used to detect spots by ultraviolet radiation. The purification of intermediates and the final synthesized compound were achieved by flash chromatography on silica gel. ^{1}H NMR and ^{13}C NMR analyses were performed using an AMX-400 Bruker spectrometer. Chemical shifts (δ units) and coupling constants (J values) are given in ppm and Hz, respectively. LCMS spectroscopy was performed on an Agilent 6410 (Agilent Technologies, Santa Clara, CA) utilizing electrospray ionization.

Chromatographic analysis was performed using an HPLC system (Waters, Milford, MA) equipped with a Waters Delta 600 pump, a Waters 2487 Dual λ absorbance detector, a Waters 600 controller, a model 100 column heater (CBL photoelectron technology) and a 20 μL injector loop. A Lichrospher column (C_8, 250 mm × 4.6 mm) was used to separate the samples and was protected by a Phenomenex guard column (4 mm × 3 mm, ODS).

All procedures involving animals were approved by the Sichuan University animal ethical experimentation committee according to the requirements of the National Act on the use of experimental animals (People's Republic of China). Sprague–Dawley rats were provided by the Laboratory Animal Center of Sichuan University and provided standard laboratory chow and water *ad libitum* until the time of the experiment.

Chemicals

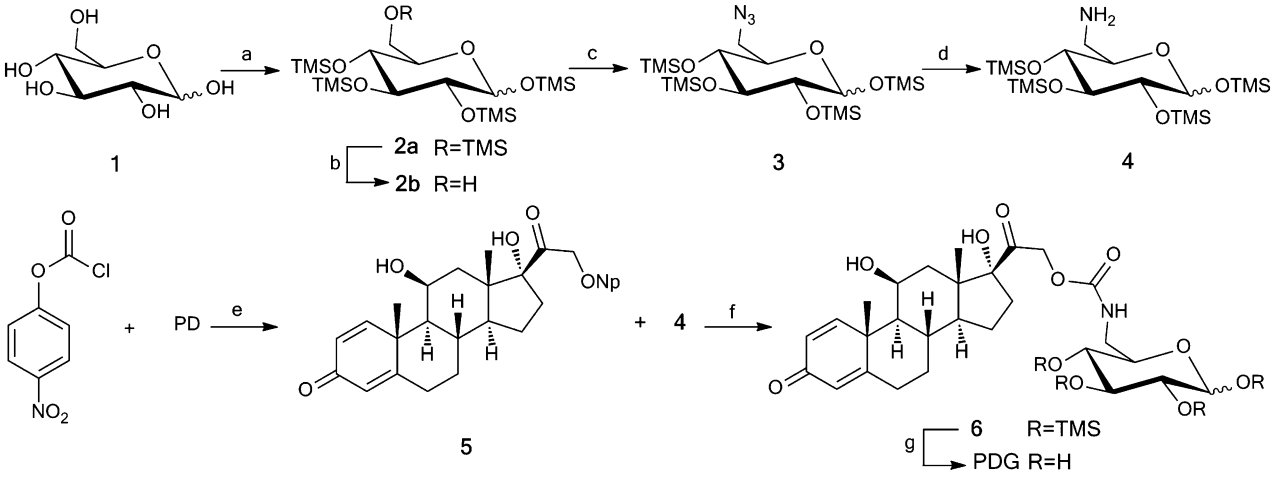
The synthetic route of PDG is outlined in Scheme 1. PDG was prepared by coupling prednisolone with the amino group of 6-amino-6-deoxy-D-glucose via a carbamate bond. **4** was obtained in five steps from a commercially available D-glucose (**1**) [22]: Per-TMS-protected D-glucose (**2a**) was prepared from **1** in a quantitative yield, and was selectively monodeprotected of the primary silyl ether to provide **2b**. The reaction of **2b** with diphenyl phosphorazidate (DPPA) and diisopropyl azodicarboxylate (DIAD) afforded **3**. To convert the azide into a primary amine group, **3** was stirred at room temperature under H_2 for 10 h which was catalyzed by Pd/C. PD and **4** were linked together by another three steps. Firstly, **5** was obtained by conversion of the prednisolone 21-hydroxyl group into 4-nitrophenyl carbonate, and the amino group of **4** was then appended by a carbamate bond to afford **6**. Finally the chemical conjugate PDG was obtained by deprotecting the silyl ether groups.

*1,2,3,4,6-Penta-O-trimethylsilyl-D-glucose (**2a**)*

D-Glucose (**1**, 360 mg, 2.00 mmol) was dissolved in anhydrous pyridine (20 mL) and cooled to 0°C. Hexamethyldisilazane (HMDS, 0.94 mL, 4.50 mmol) and trimethylchlorosilane (TMSCl, 1.89 mL, 14.95 mmol) were added sequentially. After that, the mixture was warmed to 30°C and stirred for another 10 h. Then the solvent was removed *in vacuo*, and white slurry was obtained as the resulting crude product. The material was suspended in petroleum ether (50 mL) and washed with water (30 mL). The aqueous layer was extracted with petroleum ether (3 × 20 mL). The organic layers were combined, washed with brine (3 × 30 mL) and dried with Na_2SO_4. Solvent evaporation provided viscous, colorless oil (98%) which was used without further purification in the next step. Yield 1.06 g (98%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.00 (d, 1H, J = 3.2 Hz, 1-H), 3.79–3.64 (m, 4H, 4-H, 5-H, 6-H_2), 3.40 (t, 1H, J = 8.8 Hz, 3-H), 3.34–3.32 (m, 1H, 2-H), 0.18–0.10 (m, 45H, 5 × $Si(CH_3)_3$). ESI–MS: m/z $[M+Na]^+$: 563.1.

*1,2,3,4-Tetra-O-trimethylsilyl-D-glucose (**2b**)*

2a (957 mg, 1.70 mmol) was dissolved in dichlormethane (DCM, 12 mL) and cooled to 0°C. Solution of AcOH (65 μL in 12 mL of MeOH) was added dropwise over 30 min. The solution was stirred for 8 h with the temperature below 5°C and it was quenched with saturated aqueous $NaHCO_3$ (2 mL) and then washed with water (15 mL), dried with Na_2SO_4 and concentrated under vacuum. The resulting residue was purified by silica gel chroma-



Scheme 1. Conditions and reagents: (a) TMSCl, HMDS, Py, 0°C to room temperature (rt); (b) AcOH, DCM/MeOH (1:1), 0–5°C; (c) DPPA, DIAD, PPh_3, THF, 0°C to rt; (d) H_2, Pd/C, MeOH, rt; (e) Py, DCM; (f) Et_3N, THF, rt; (g) TFA, DCM.

tography as colorless oil. Yield 0.60 g (75%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.00 (d, 1H, J = 3.2 Hz, 1-H), 3.81–3.65 (m, 4H, 4-H, 5-H, 6-H_2), 3.45 (t, 1H, J = 8.8 Hz, 3-H), 3.35–3.32 (m, 1H, 2-H), 0.18–0.10 (m, 36H, 4 × Si(CH_3)$_3$). ESI–MS: m/z [M+Na]$^+$: 491.1.

6-Azido-6-deoxy-1,2,3,4-tetra-O-trimethylsilyl-D-glucose (3)

Triphenylphosphine (PPh_3, 1.17 g, 4.47 mmol), followed by DIAD (0.90 g, 4.47 mmol) and then DPPA (1.23 g, 4.47 mmol) were added sequentially to a solution of **2b** (1.05 g, 2.24 mmol) in THF (10 mL) at 0°C. The mixture was allowed to warm to room temperature and stirred for another 2 h. Then it was concentrated under reduced pressure, and purified by silica gel chromatography. Yield 0.94 g (85%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.01 (d, 0.6H, J = 2.8 Hz, 1-H), 4.50 (d, 0.4H, J = 7.2 Hz, 1-H), 3.90–3.25 (m, 6H, 2-H, 3-H, 4-H, 5-H, 6-H_2), 0.18–0.14 (m, 36H, 4 × Si(CH_3)$_3$). ESI–MS: m/z [M+Na]$^+$: 516.1.

6-Amino-6-deoxy-1,2,3,4-tetra-O-trimethylsilyl-D-glucose (4)

10% Pd/C (20 mg) was added to a solution of **3** (211 mg, 0.43 mmol) in MeOH (5 mL). After having been stirred at room temperature under H_2 for 10 h, the reaction mixture was filtered through Celite. The solvent was removed by concentration *in vacuo* and then purified by silica gel chromatography. Yield 0.16 g. (79%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.98 (d,1H, J = 2.8 Hz, 1-H), 3.78 (t, 1H, J = 8.8 Hz, 4-H), 3.66 (m, 1H, 5-H), 3.33 (m, 1H, 2-H), 3.27 (t, 1H, J = 8.8 Hz, 3-H), 2.98 (dd, 1H, J_1 = 2.8 Hz, J_2 = 13.2 Hz, 6-H), 2.64 (dd, 1H, J_1 = 7.6 Hz, J_2 = 13.2 Hz, 6-H), 0.17–0.13 (m, 36H, 4 × Si(CH_3)$_3$). ESI–MS: m/z [M+H]$^+$: 468.1.

Prednisolone 21-(4-nitrophenyl carbonate) (5)

Prednisolone (PD, 500 mg, 1.39 mmol) and 4-nitrophenyl chloroformate (432 mg, 2.08 mmol) were dissolved in anhydrous DCM (15 mL) and cooled to 0°C. Pyridine (0.17 mL, 2.08 mmol) was dropped in slowly, after that the mixture was allowed to warm up and stirred for another 10 h at room temperature. Then DCM (30 mL) was added and the mixture was washed with 1 M HCl (3 × 30 mL) and brine (1 × 30 mL). The aqueous layer was re-extracted with DCM (3 × 25 mL) and the organic phases were combined and then dried with Na_2SO_4. Solvent was evaporated and the residue was purified with flash chromatography on silica gel giving the pure product as a white powder. Yield 0.61 g (84%). ^{1}H NMR ([D_6] DMSO, 400 MHz) δ 8.34 (d, 2H, J = 8.8 Hz, 2′-H, 6′-H), 7.56 (d, 2H, J = 8.8 Hz, 3′-H, 5′-H), 7.31 (d, 1H, J = 10.4 Hz, prednisolone 2-H), 6.15 (d, 1H, J = 10.4 Hz, prednisolone 1-H), 5.92 (s, 1H, prednisolone 4-H), 5.27 (d, 1H, J = 18.0 Hz, prednisolone 21-H), 4.94 (d, 1H, J = 18.0 Hz, prednisolone 21-H), 4.28 (s, 1H, prednisolone 11-H), 3.66–3.54 (m, 2H, prednisolone 6-H_2), 2.30–2.25 (m, 2H, prednisolone 16-H_2), 2.05–1.99 (m, 2H, prednisolone 15-H_2), 1.91–1.88 (m, 1H, prednisolone 9-H), 1.63–1.61 (m, 2H, prednisolone 12-H_2), 1.51–1.47 (m, 2H, prednisolone 7-H_2), 1.38 (s, 3H, prednisolone 18-H_3), 1.19–1.16 (m, 1H, prednisolone 8-H), 1.03–1.00 (m, 1H, prednisolone 14-H), 0.80 (s, 3H, prednisolone 19-H_3). ESI–MS: m/z [M+H]$^+$: 526.1.

Prednisolone 21-(6-carbamate-D-glucose) PDG

Prednisolone 21-(4-nitrophenyl carbonate) (**5**, 270 mg, 0.58 mmol) was dissolved in anhydrous THF (6 mL) and Et_3N (0.6 mL) was added. After 5 min, **4** (609 mg, 1.30 mmol) was introduced and the reaction was stirred overnight. Then ethyl acetate was added and the mixture was washed with brine (2 × 5 mL). The aqueous layers were re-extracted with ethyl acetate (2 × 10 mL). The organic layers were combined and dried with Na_2SO_4, followed by evaporation *in vacuo*. The crude product of **6** was obtained as colorless oil which was used without purification. Then it was dissolved in DCM (6 mL), and trifluoroacetic acid (TFA, 1.2 mL) was added dropwise into the reaction solution slowly. The solution was stirred for 2 h at room temperature. It was quenched with saturated aqueous $NaHCO_3$ (5 mL) and then extracted with DCM (3 × 10 mL). The organic layers were combined, washed with brine (15 mL) and dried with Na_2SO_4. Solvent was evaporated and the residue was purified with flash chromatography on silica gel giving the pure product as a white powder. Yield 0.20 g (62%). ^{1}H NMR ([D_6] DMSO, 400 MHz) δ 7.32 (d, 1H, J = 10.0 Hz, prednisolone 2-H), 6.15 (d, 1H, J = 10.4 Hz, prednisolone 1-H), 5.91 (s, 1H, prednisolone 4-H), 4.97–4.87 (m, 2H, prednisolone 21-H, 1-H), 4.69–4.64 (m, 1H, prednisolone 21-H), 4.28 (s, 1H, prednisolone 11-H), 3.63–3.58 (m, 1H, 6-H), 3.44–3.38 (m, 3H, prednisolone 6-H_2, 6H), 3.13–2.87 (m, 4H, 2-H, 3-H, 4-H, 5-H), 2.30–2.27 (m, 2H, prednisolone 16-H_2), 2.04–2.02 (m, 2H, prednisolone 15-H_2), 1.89–1.86 (m, 1H, prednisolone 9-H), 1.68–1.65 (m, 2H, prednisolone 12-H_2), 1.44–1.40 (m, 2H, prednisolone 7-H_2), 1.39 (s, 3H, prednisolone 18-H_3), 1.31–1.28 (m, 1H, prednisolone 8-H), 1.03–0.97 (m, 1H, prednisolone 14-H), 0.80 (s, 3H, prednisolone 19-H_3). ^{13}C-NMR ([D_6] DMSO, 400 MHz) δ 206.8 (C_{20}), 185.4 (C_3), 170.8 (C_5), 157.0 (C_1), 155.9 (OCONH), 127.2 (C_2), 121.8 (C_4), 97.2 (C_{17}), 93.3 (C'_1), 76.0 (C'_3), 72.9 (C'_4), 72.5 (C'_2), 71.8 (C'_5), 70.2 (C_{11}), 67.5 (C_{21}), 55.6 (C_9), 51.3 (C_{14}), 47.2 (C_{13}), 45.7 (C_{10}), 44.0 (C_{12}), 42.6 (C'_6), 34.2 (C_8), 33.2 (C_{16}), 31.5 (C_6), 31.1 (C_7), 23.7 (C_{15}), 21.0 (C_{19}), 16.7 (C_{18}). ESI–MS: m/z [M+H]$^+$: 566.5.

In vitro stability of PDG

A working solution of PDG (10.55 mg/mL) was prepared by dissolving PDG (21.10 mg) in methanol (2.0 mL). Then a 20 μL volume of the working solution was added to 5.0 mL of 50% rat plasma (diluted by 0.05 M PBS, v/v), 33% liver or 33% kidney homogenate (diluted by sterile saline, w/v) to investigate its enzymatic hydrolysis. The mixture was incubated at 37 ± 1°C under continuous shaking. Next, 200 μL of each incubated mixture was sampled at the predetermined time points, mixed with 40 μL trichloroacetic acid (20%, g/mL). After vortexing (5 min), it was centrifuged at 13,000 × g for 10 min. Aqueous supernatant (20 μL) was injected into the HPLC system to determine the concentration of PDG using the method described in the following section.

HPLC analysis

HPLC assay method was used to monitor the concentrations of PD and PDG in biological samples. A mixture of methanol/acetonitrile/acetate buffer solution (adjusted by acetic acid to pH 4.0) at a ratio of 97:15:95 by volume was used as the mobile phase. The flow rate was 1.0 mL/min, and the column effluent was monitored at 254 nm. The total analytical time was 12 min for the whole run. The obtained retention times were as follows: PD, 10 min; PDG, 8.5 min, which, respectively, possessed the limit of quantitation (LOQ) in biological samples of 0.02 and 0.03 μg/mL. Both PD and PDG in biological samples were completely separated under analytical conditions. And the standard curves for PD ranging from 0.03 to 10.30 μg/mL were linear ($r^2 > 0.99$). As for PDG, its linearity range for kidney was

10.04–150.57 μg/mL and for plasma, heart, liver, spleen, lung it was 0.20–40.17 μg/mL.

Biodistribution of PD and PDG in rat

Fifty male Sprague–Dawley rats (190 ± 10 g) were randomly divided into two groups which were treated with PD and PDG, respectively. For each preparation and sampling time point, five rats were treated with a single dose of PD/PDG (50 μmol/kg) through the caudal vein. The rats were killed at 5, 15, 30, 60, and 120 min after injection.

At the predetermined time point, blood samples were collected and placed into heparinized EP tubes. Rats were sacrificed and tissue samples, including heart, liver, spleen, lung, and kidney, were collected, flushed with cold saline and weighed immediately, followed by homogenization with twofold volume of 0.9% saline (g/mL). The plasma was separated by centrifugation at 9200 × *g* for 1 min. All biological samples were stored at −20°C before assay. An 0.5 mL aliquot of plasma or tissue homogenate was mixed with 0.1 mL of methanol and then 0.1 mL of trichloroacetic acid solution (20%, g/mL). After vortexing (5 min), it was centrifuged at 13,000 × *g* for 10 min. The supernatant (20 μL) was then injected into the HPLC system to determine the concentrations of PD and PDG using the method described above.

For the preparation of calibration curves of PD and PDG, working solutions of PD (72.1 μg/mL) and PDG (1.05 mg/mL) were prepared. Then they were diluted with methanol into other six different concentration solutions, respectively, before use. 0.1 mL of each working solution was added to the blank biological samples, which was followed by biological sample pretreatment. Lastly, the concentration of PD or PDG was detected by the HPLC. Their concentrations *versus* peak areas were recorded and these calibrations were subjected to analytical procedure to test the linearity of the method.

Preliminary study on pharmacodynamics of PDG

Minimal change nephrosis was employed as the pathological model, which was established in rats by a single injection of daunorubicin hydrochloride (12 mg/kg) through the caudal vein [23].

Experiments were performed on 20 male Sprague–Dawley rats (220 ± 20 g), which were randomly divided into four groups. In the normal group, rats were housed and fed without any treatment. Rats in the control group received a single dose of daunorubicin hydrochloride (12 mg/kg), whereas rats in the PD group and PDG group were given PD and PDG at a dose of 8.33 μmol kg^{-1} d^{-1} for 14 days separately after the administration of daunorubicin hydrochloride (12 mg/kg).

Each rat was housed in a metabolism cage, and its urine voided during 24 h was collected in tubes on the day before (day 0), the 7th day (day 7) after, and the 14th day (day 14) after the injection of daunorubicin hydrochloride. The urine volume was recorded and then it was mixed, centrifuged (850 × *g* for 5 min) and stored at −40°C for the urinary protein assay. Aliquots of the urine were sampled to analyze the contents of urinary protein by a urinary protein quantitation kit (Nanjing Jiancheng Bioengineering Institute).

On the 15th day, blood samples were collected, placed into EP tubes and left undisturbed at room temperature until clotted. Samples were then centrifuged at 850 × *g* for 10 min, and the serum obtained was immediately stored at −20°C until assay. The biochemical indexes including ALB, BUN, CREA, and Tg in the serum were monitored by a chemistry analyzer (Hitachi 7020, Japan).

Adverse effects of PDG in rats

Tests for adverse effects were performed on 15 male Sprague–Dawley rats (120 ± 10 g), which were randomly divided into three groups. In the normal group, rats were housed and fed normally, whereas the rats of the PD group and PDG group were treated with PD and PDG at a dose of 8.33 μmol kg^{-1} d^{-1} via caudal vein for 20 days. All rats were weighed every 3 days after the first administration, and the dosages were also changed according to the variance of their body weights. On the 21st day, rats were anesthetized with chloral hydrate (300 mg/kg, i.p.), and their femoral densities were analyzed by an iDXA instrument (GE Lunar, Madison, WI).

Statistical analysis

The area under the curve (AUC_{0-t}), maximal concentration (C_{max}), and mean retention time (MRT) in the biodistribution study were calculated by Data and Statistics software (DAS 2.0, Shanghai, China). The statistical analysis of the samples was performed using a Student's *t*-test with a *p*-value < 0.05 as the minimal level of significance.

The relative uptake efficiency (RE), concentration efficiency (CE), and kidney targeting index (KTI) were calculated to evaluate the kidney target efficiency of PDG. Values of RE, CE, and KTI were calculated as follows:

$$RE = \frac{(AUC_{0-t})_{PDG}}{(AUC_{0-t})_{PD}}$$

$$CE = \frac{(C_{max})_{PDG}}{(C_{max})_{PD}}$$

$$KTI = \frac{(AUC_{kidney}/AUC_{plasma})_{PDG}}{(AUC_{kidney}/AUC_{plasma})_{PD}}$$

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