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Effects of mycophenolic acid–glucosamine conjugates on the base of kidney targeted drug delivery☆

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ABSTRACT

Mycophenolic acid has played an important role in treating immunosuppression and autoimmune diseases. Nevertheless, the agent needs a high dosage in treatment, following some side effects. To tackle this problem, in this study, mycophenolic acid–glucosamine conjugate (MGC), modified by 2-glucosamine, was synthesized to achieve kidney targeting and improved drug efficacy with a lower dosage. ^{1}H NMR, ^{13}C NMR and HRMS spectroscopy were used to verify the conjugate whose stability was good *in vitro*. The transport of MGC by human proximal renal tubular epithelial HK-2 cells was temperature-, time-, concentration-dependent and saturable, suggesting the involvement of carrier-mediated uptake. In addition, the cellular uptake of MGC dropped substantially with the inhibition of megalin receptor. The specific tissue distribution indicated the commendable renal-targeting capability of MGC. The concentration of MGC was improved in the kidney except for other tissues, about 6.76 times higher than that of MPA. Further, the bioavailability of MGC in plasma decreased as compared with mycophenolic acid. Moreover, therapeutic effect of MGC was enhanced significantly compared with MPA in the acute kidney injury model. All the findings suggested the potential of mycophenolic acid–glucosamine conjugate in kidney targeted drug delivery.

1. Introduction

In the present age, chronic kidney diseases (CKDs) have become a major health problem for its high incidence and low public awareness (Levey et al., 2005; Stenvinkel, 2010; Chen et al., 2005; Vassalotti et al., 2007). In developed countries like US (Coresh et al., 2003), Australia (Chadban et al., 2003), Japan (Iseki, 2003), and Europe (Hillege et al., 2001), approximately 6–11% population suffer from CKD-related symptoms. In contrast, the overall prevalence of CKD has already reached 10.8% in developing countries such as China. Not only does it make the government spend more money and energy on the treatment of CKDs, it also causes higher incidence of diabetes and hypertention (Zhang et al., 2012), which calls for the generation of effective and acceptable therapies.

Currently, transplantation and dialysis, the leading treatments, are known to have low patient compliance and high cost (Choi et al., 2000). Even though immunosuppressant mycophenolic acid (MPA) and its derivative, mycophenolate mofetil (MMF), clinically used medication, both show good therapeutic effects on the immunosuppression and autoimmune diseases, the process of treatment is often accompanied with myelosuppression, oral ulcers, gastrointestinal and other adverse reactions after long-term exposure (Pelletier et al., 2003; Knoll et al., 2003; Oellerich et al., 2000; Weber et al., 2002a, 2002b; Weng et al., 2011) for the exorbitant administration dosages, reaching 3 g/d for MMF. Most of toxicities and adverse effects are due to the extra renal side-effects. In terms of this condition, kidney-targeted drug delivery system may contribute to reducing the extra renal side-effects by specifically accumulating drugs in kidney with little concentration in other organs. Therefore, targeted delivery of MPA to the kidney will mostly likely result in a lower dosage and improved drug efficacy.

Accordingly, renal-targeted delivery systems have been developed in different strategies. For instance, lysozyme (Zhang et al., 2009; Prakash et al., 2008), long circulating cationic liposomes (Morimoto et al., 2007), 50% N-acetylated low molecular weight

Abbreviations: CKD, chronic kidney disease; MGC, mycophenolic acid–glucosamine conjugate; MPA, mycophenolic acid; LMWC, low molecular weight chitosan; MMF, mycophenolate mofetil; DMF, N,N-Dimethylformamide; DCM, dichloromethane; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylamiopryidine; EDTA, ethylene diamine tetraacetic acid; I/R, ischemia-reperfusion; CREA, serum creatine; BUN, blood urea nitrogen.

☆ Mycophenolic acid (PubChem CID: 446541); 2-glucosamine (PubChem CID: 45933887); dicyclohexylcarbodiimide (PubChem CID:10868); acetonitrile (PubChem CID:6342).

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chitosan (LMWC) (Yuan et al., 2007) and G3-C12 peptide (Geng et al., 2012) based drug delivery systems were designed to specifically deliver therapeutic agents to the kidney. However, in this research, a small molecule ligand would be discussed for its better druggability.

In the previous studies, we have found that the fundamental unit of LMWC, 2-glucosamine, could be applied as a promising carrier for kidney targeting (Lin et al., 2012). Specifically, 2-glucosamine possesses a homogeneous and well-defined molecular structure. Moreover, 2-glucosamine has a high degree of safety without the potential toxicity associated with macromolecular carriers (Okamoto et al., 2004). Additionally, 2-glucosamine is easy to be obtained in a large-scale directly from native chitin with limited processing time and cost.

Thus, we synthesized the mycophenolic acid–glusosamine conjugate (MGC) to investigate its therapeutic effects by achieving renal-targeting. Initially, we explored the mechanism for transport of MGC across the human proximal renal tubular epithelial cells *in vitro* and then compared the distribution of MGC with MPA *in vivo*. After that, bioavailability was investigated in kidney and plasma, respectively, which turned out an improved bioavailability in kidney. Eventually, the enhanced drug efficacy of MGC during the renal ischemia-reperfusion (I/R) injury therapy made the mycophenolic acid–glucosamine conjugate promised for renal targeted drug delivery.

2. Experimental procedures

2.1. Materials

2-Glucosamine was purchased from Kelong Chemical Reagent Factory (Chengdu, China). Mycophenolic acid was obtained from Hubei hengshuo Chemical Co. Ltd. (Henan, China) with purity of 98.0%. Acetonitrile (HPLC grade) was purchased from Kemiou (Tianjin, China). Watsons distilled water was used as water phase in LC–MS/MS system. All the other chemicals and reagents were of analytical grade and obtained commercially. TLC (silica gel GF254) was used to detect spots by UV radiation. Purification of intermediates and desired compound was achieved by column chromatography on silica gel. ^{1}H NMR and ^{13}C NMR were performed with an AMX-400 Bruker Spectrometer. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants are in units of hertz (Hz). HRMS spectroscopy was evaluated on Bruker microTOF-QII. Protein contents of cell lysate were estimated by the Pierce BCA protein assay reagent kit (Pierce, USA). The stained slides were viewed under upright Metallurgical microscope (Nikon Eclipse 80i, Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan). Apoptosis of kidney cells was identified by TUNEL assay using In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). Photomicrographs were taken with a microscope camera (Nikon Eclipse 80i, Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan).

Sprague-Dawley rats (male; body weight: 240 ± 20 g), provided by the West China Experimental Animal Center of Sichuan University (China), were maintained in a germ-free environment and allowed free access to food and water. All animal experiments were approved by 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).

2.1.1. Cell lines and cell culture

HK-2 cells (a human proximal renal tubular epithelial cell line) were cultured in DMEM with high glucose (GIBCO, USA) supplemented with 10% calf serum (Minhai, Gansu, China) and 100 YIU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. L929 cells (mouse fibroblast cell line) were cultured in RPMI 1640 (GIBCO, USA) supplemented with 10% calf serum (Minhai, Gansu, China) and 100 YIU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and the culture medium was changed every other day.

2.2. Methods

2.2.1. Synthesis of mycophenolic acid–glucosamine conjugation (MGC)

The synthetic routes of the intermediate 9 (Lin et al., 2012) and MGC were presented in Scheme 1 (Lin et al., 2012) and Scheme 2. Firstly, 2-glucosamine was chosen as the raw material and benzaldehyde was introduced in 1.2 e.q. NaOH aqueous solution, producing compound 2 by acetylation. Benzaldehyde was replaced by trichloroacetyl to obtain compound 3 which was stable in a low pH condition and could react with 2-bromoethanol in the presence of $BF_3{\cdot}Et_2O$ in anhydrous CH_2Cl_2. Then, the halogen of compound 4 was exchanged with azide in N,N-dimethylformamide (DMF) to produce compound 5, which was hydrolyzed to liberate the —OH and $—NH_2$ and the amino group could be protected again by Boc group, aimed at forming compound 8. Finally, reduction of azide under hydrogen atmosphere with Pd/C (10%) was carried out in methanol to yield the intermediate 9.

2.2.2. N-Boc-mycophenolic acid–glucosamine conjugation (N-Boc-MGC) (10)

To a solution of dry DMF 12 mL, intermediate 9 1.5 g (4.7 mmol) and mycophenolic acid 1.5 g (4.7 mmol) were added, with DCC 1.16 g and DMAP 6 mg as the catalytic conditions, which was followed by stirring 24 h at room temperature, then quenched by the addition of water. The resulting solution was extracted with n-butanol (15 ml × 3), and then the organic layer was washed with brine, dried and concentrated. The residual oil was purified by column chromatography on silica gel to obtain the N-Boc-mycophenolic acid–glusosamine conjugate as a light yellow solid.

2.2.3. Mycophenolic acid –glucosamine conjugation (MGC) (11)

To a solution of compound 10 0.874 g (1.4 mmol) in CH_2Cl_2 25 ml, CF_3COOH 5 ml was added, and stirred at room temperature for 2 h. The pH of the mixture was adjusted to 9 by saturated aqueous sodium hydrogen carbonate. Subsequently, CH_2Cl_2 was removed by reduced pressure and the water layer was extracted with n-butanol (15 ml × 3), the organic layer was washed with brine, dried and concentrated. The residual oil was purified by column chromatography on silica gel. Compound 11 (MGC) was obtained as a light yellow solid, m.p: 149 °C.

2.2.4. Sample preparation and LC–MS/MS analysis

2.2.4.1. Sample preparation. For cell uptake, 0.6 ml of acetonitrile was added to the broken cell pellets (0.24 ml), and vortexed for 5 min. The mixtures were centrifuged at 3000 rpm for 5 min, and the supernatants were analyzed by LC–MS/MS. For distribution, normal saline (0.2 ml) was mixed with 0.2 ml of plasma. Then 1 ml of acetonitrile was added to the above mixture and vortexed for 5 min. 2 ml of acetonitrile was added to the tissues homogenates (0.5 ml), and vortexed for 5 min. For plasma bioavailability, methanol (0.1 ml) was added to the plasma (0.1 ml), and vortexed for 2 min. Then acetonitrile (0.4 ml) was put into the mixture and vortexed for 5 min. The mixtures were centrifuged at 12,000 rpm for 10 min, and the supernatants were analyzed by LC–MS/MS.

2.2.5. LC–MS/MS analysis

2.2.5.1. Apparatus. The LC–MS/MS system consisted of an Agilent 1200 series RRLC system, which includes an SL autosampler,