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CD44-targeted hyaluronic acid-curcumin prodrug protects renal tubular epithelial cell survival from oxidative stress damage

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ABSTRACT

Based on the abnormally increased expression of CD44 receptors on renal tubule epithelial cells during ischemia/reperfusion-induced acute kidney injury (AKI), we developed a hyaluronic acid-curcumin (HA-CUR) polymeric prodrug targeting to epithelial cells and then relieving oxidative stress damages. The water solubility of HA-CUR was significantly enhanced and approximately 27-fold higher than that of CUR. Cellular uptake test showed HA-CUR was preferably internalized by H_2O_2-pretreated tubular epithelial (HK-2) cells compared with free CUR benefiting from the specific binding between HA and CD44 receptors. Biodistribution results further demonstrated the increased accumulation of HA-CUR in kidneys with 13.9-fold higher than that of free CUR. Pharmacodynamic studies indicated HA-CUR effectively ameliorated AKI, and the exact mechanism was that HA-CUR protected renal tubule epithelial cells from oxidative stress damage via inhibiting PtdIns3K-AKT-mTOR signaling pathway. Taken together, this study provides a new therapeutic strategy for the treatment of AKI based on the pathogenesis of the disease.

1. Introduction

Acute kidney injury (AKI) caused by sepsis, ischemia-reperfusion, and nephrotoxins is a major renal disease characterized by rapid loss of renal function leading to the accumulation of metabolic wastes imbalance of electrolytes and body fluid. Despite advances in clinical and basic studies over the past few decades, therapeutic outcomes of AKI are still poor, with gradually increasing morbidity, unavoidable high mortality, and unsatisfactory therapeutic options (Waikar, Curhan, Wald, McCarthy, & Chertow, 2006; Xue, Daniels, Star, Kimmel, & Eggers, 2006). The pathophysiology of AKI is associated with the complicated mechanisms, such as oxidative stress, inflammation, and vascular damage (Pabla & Dong, 2008; Price, Safirstein, & Megyesi, 2009; Sharfuddin & Molitoris, 2011). Pathologically, AKI is featured by serious and even lethal damage of renal tubules, leading to tubular dysfunction and cell death in the forms of necrosis and apoptosis (Berger, Bangen, Hammerich, Liedtke, & Floege, 2014; Sharples, 2007). Therefore, treatment of AKI starting from the pathogenesis of the disease to explore a new therapeutic approach is necessary and crucial.

CD44, a transmembrane proteoglycan, plays an important role as an adhesion molecule in cell-cell and cell-matrix interactions during organogenesis and wound repair (Kocak, Orug, Turhan, Ozcay, & Gonenc, 2009). It is involved with polymorphonuclear neutrophil migration, lymphocyte homing, and cancer metastases (Johnson & Ruffell, 2009). It has also been found that CD44 expression is upregulated on injured tubuloepithelial cells during renal ischemia-reperfusion injury (IRI) (Kocak et al., 2009; Rouschop, Roelofs, Claessen, da Costa Martins, & Zwaginga, 2005). The overexpression of CD44 on post-ischemic proximal tubule cells migration of neutrophils into the reperfused renal tissues (Declèves, Caron, Nonclercq, & Legrand, 2006).

Encouraged by these findings, we attempted to identify an efficient and safe cell-specific drug delivery system for the treatment of IRI-induced AKI. Drug targeting to the inflamed renal tissues enable the improved drug distribution, reduction of the required dose and ultimately enhanced therapeutic outcomes. Hyaluronic acid (HA), a biocompatible and biodegradable natural polysaccharide distributed in the extracellular matrix and synovial fluids, has attracted the keen interests due to its inherent property to specifically target CD44 receptors (Hayward, Wilson, & Kidambi, 2016; Yang, Iyer, Singh, Choy, & Hornicek, 2015). The abundance of carboxylic and hydroxyl groups make HA polymer backbone suitable to chemical modification to satisfy varied pharmaceutical requirements (Schanté, Zuber, & Herlin, 2011).

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Its high aqueous solubility also allows water-based reactions, and reduces and even avoids the use of harmful organic solvents.

Curcumin (CUR), an orange-yellow polyphenol, has shown remarkable potential as antioxidant, anti-inflammatory, anticancer, and several other therapeutic activities (Chongtham & Agrawal, 2016; Cai, Sun, Fang, & Fang, 2016; Nasra, Khiri, Hazzah, & Abdallah, 2017). Previous results also demonstrated CUR protected against renal ischemia-reperfusion injury (Awad & El-Sharif, 2011; Kaur, Kaur, Singh, & Pathak, 2016), and it was accordingly used as a model drug in this study. However, a major challenge for extensive use of CUR in clinical practice is its poor solubility leading to poor bioavailability in biological systems. Previous studies to address this issue of low bioavailability adopted various carriers, including liposomes, polymeric micelles, emulsion, polymers and nanospheres (Allijn, Schiffelers, & Storm, 2016; Zhu, Liu, Wu, & Xu, 2017; Zhao, Ma, Cao, & Yu, 2017). However, to access tubular cells, a carrier system needs to be filtered through the glomerulus. Glomerular filtration of compounds involves several limitations, including diameter (< 5.5 nm) and molecular weight (< 70 kDa) (Kok & Henning, 2010; Yuan et al., 2007). Based on these requirements, hyaluronic acid-curcumin (HA-CUR) polymeric prodrug probably be a promising pharmaceutical strategy and possesses the potential distinctive advantages over other formulations, as featured by enhanced water solubility, prolonged drug release, improved organ distribution and increased therapeutic outcomes.

To our knowledge, HA-based polymeric prodrug have never been used for anti-inflammatory therapies, nor has their specific targeted therapy mechanisms been systematically investigated. In this study, we designed a polymeric prodrug based on HA for active CD44-targeting CUR delivery to the inflamed renal tubular epithelial cells (Fig. 1A). HA with molecular weight of 4 kDa was used as polymer backbone due to lower molecular weight HA remaining slightly longer in circulation in comparison to higher molecular weight HA (Mattheolabakis, Milane, Singh, & Amiji, 2015). HA-CUR polymeric prodrug was synthesized via an esterification reaction, and its characteristics, including CUR grafting rate and in vitro drug release behavior, were examined in detail. The target efficacy of HA-CUR was investigated both *in vitro* and *in vivo*. The *in vivo* therapeutic efficacy was investigated in IRI-induced AKI murine model, as reflected by renal function, histopathological changes, production of pro-inflammatory cytokines, oxidative stress levels and expression of apoptotic related proteins. In addition, the specific mechanism of HA-CUR protects proximal tubule cells from oxidative stress damage was also uncovered.

2. Material and methods

2.1. Material

Curcumin (CUR) was purchased from Aladdin Bio-chem Technology Co. Limited (Shanghai, China). Hyaluronic acid (molecular weight: ~4 000 Da) was purchased from Freda Biochem Co., Ltd. (Shandong, China). Dicyclohexylcarbodiimide (DCC) and 4-Dimethylaminopyridine (4-DMAP) were purchased from Aladdin Bio-chem Technology Co. Limited (Shanghai, China). A CCK-8 assay kit was purchased from Beyotime Biotechnology Co. Limited (Shanghai, China). A TUNEL assay kit was obtained from Roche (Nutley, NJ, USA). All other solvents were of analytical or chromatographic grade.

2.2. Synthesis and characterization of HA-CUR

HA-CUR was synthesized by esterification reaction in the presence of DCC and 4-DMAP. Briefly, 800 mg HA, 100 mg DCC and 40 mg 4-DMAP were added into dimethylsulfoxide (DMSO)/H_2O (V/V, 1:1) mixture solution, and stirred for 2 h to carboxylic group of HA. Then, 73.6 mg CUR was added into this reaction system and stirred at 300 rpm for an extra 24 h at 60 °C to produce HA-CUR. After the reaction, the resultant solution was transferred into a dialysis bag (MWCO: 3.5 kDa) to dialyze for 48 h with frequent exchanges of deionized water to remove water-soluable byproducts. The dialyzed solution was centrifugated at 15 000 rpm to remove water-insoluble byproducts, followed by lyophilization.

The composition of HA-CUR was confirmed using a ^1H NMR spectrometer (AC-80, Bruker Bios pin. Germany). 20 mg/mL HA and HA-CUR were dissolved in deuterated H_2O, and CUR were dissolved in dimethylsulfoxide-d_6.

2.3. Solubility determination of HA-CUR

HA-CUR and CUR equilibrium solubility was determined in pH 7.4 PBS using shake flask method (Baka, Comer, & Takács-Novák, 2008). Briefly, an excess amount of HA-CUR and CUR was added into pH 7.4 PBS and sonicated until HA-CUR and CUR were fully dispersed, and then incubated in water bath shaker maintained at 37 ± 0.5 °C and shaken horizontally at 60 rpm for 72 h. The resultant mixtures were centrifuged at 10000 rpm for 10 min and the supernatant was diluted with methanol to be quantified using high performance liquid chromatography (HPLC) on an Agilent C18 (250 mm × 4.6 mm, 5 μm) column. Acetonitrile: 0.1% trifluoracetic acid (50/50 v/v), at pH 3.0, adjusted with triethylamine, was used as the mobile phase, and the detection wavelength was set as 427 nm with flow rate at 1.0 mL/min at 25 °C. The enhanced equilibrium solubility of CUR was calculated as the following formula:

$$\text{Relative rate (\%)} = C_{HA\text{-}CUR}/C_{CUR} \times 100\%$$

$C_{HA\text{-}CUR}$ representing equilibrium solubility of HA-CUR; C_{CUR} representing equilibrium solubility of CUR.

2.4. In vitro release of grafted CUR

In vitro drug release behaviors of HA-CUR in comparison to free CUR were investigated via the dialysis method (Yang, Liu, Yan, Zhou, & Xiong, 2016), and CUR was determined using HPLC assay. The release media included pH 7.4 PBS and pH 7.4 PBS containing 10% fetal bovine serum (FBS). The weighted free CUR and HA-CUR (equal CUR) were sealed in a dialysis membrane (MWCO: 3.5 kDa), and then immersed into 40 mL release medium, respectively. This experiment was carried out in an incubator shaker (HZ-8812S, Taicang, China) maintained at 37 ± 0.5 °C and shaken horizontally at 60 rpm. At predefined time points, the release medium was withdrawn and replaced with fresh medium. The collected CUR was qualified using HPLC assay from the standard CUR curve.

2.5. Cellular uptake

The cellular uptake of HA-CUR was investigated firstly. HK-2 cells were seeded into 24-well plates at a density of 5×10^4 cells per well, and incubated for 24 h. Cells were pretreated with 200 μmol/L H_2O_2 for 4 h and then incubated with free CUR or HA-CUR at a CUR concentration of 20 μg/mL for another 2 h, cells without H_2O_2-pretreatment as control. After washed with PBS and fixed with 4% paraformaldehyde solution, cells were visualized using laser scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Germany). The internalization of HA-CUR by HK-2 cells after 1, 2, and 4 h incubation was also investigated using flow cytometry (FC 500 MCL, Beckman Coulter, USA).

Then, the CD44 receptor-mediated internalization mechanism of HA-CUR by HK-2 cells was investigated, and the following cellular uptake assays were performed: (1) Cellular uptake of HA-CUR was investigated in different cell lines, including tubular epithelial (HK-2) cells and vein endothelial (HUVEC) cells. H_2O_2-pretreated HK-2 cells and HUVEC cells were respectively incubated with free CUR or HA-CUR at a CUR concentration of 20 μg/mL for 2 h, and then determined by