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Original Contribution

A low toxicity synthetic cinnamaldehyde derivative ameliorates renal inflammation in mice by inhibiting NLRP3 inflammasome and its related signaling pathways



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ABSTRACT

Uncontrolled inflammation is a leading cause of various chronic diseases. Cinnamaldehyde (CA) is a major bioactive compound isolated from the essential oil of the leaves of *Cinnamomum osmophloeum* kaneh that exhibits anti-inflammatory activity; however, the use of CA is limited by its cytotoxicity. Here, we synthesized three CA derivatives and identified 4-hydroxycinnamaldehyde-galactosamine (HCAG) as a low toxicity anti-inflammatory compound *in vitro* (HCAG IC₅₀ \gg 1600 μ M; CA IC₅₀=40 μ M) and *in vivo*. HCAG reduced pro-inflammatory mediator expression in LPS-activated macrophages by inhibiting MAPK and PKC- α/δ phosphorylation, decreasing ROS generation and reducing NF- κ B activation. HCAG also reduced NLRP3 inflammasome-derived IL-1 β secretion by inhibiting the ATP-mediated phosphorylation of AKT and PKC- α/δ . In a mouse model of LPS-induced renal inflammation, we observed reduced albuminuria and a mild degree of glomerular proliferation, glomerular sclerosis and periglomerular inflammation in the HCAG-treated mice compared with the vehicle-treated mice. The underlying mechanisms for these renoprotective effects involved: (1) inhibited NLRP3 inflammasome activation; (2) decreased superoxide anion levels and apoptosis; and (3) suppressed activation of NF- κ B and related downstream inflammatory mediators.

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1. Introduction

Inflammation is one of the most important host defense mechanisms in mammals and is also required for wound healing; however, uncontrolled inflammation is harmful to health. Many studies have shown that inflammation is a risk factor for metabolic diseases [1]. Recently, a caspase-1-containing multi-protein complex called the NLRP3 inflammasome was identified that controls the release of IL-1 β and IL-18 during the inflammatory processes [2]. A strong link between the NLRP3 inflammasome and the development of metabolic diseases is becoming increasingly evident [3,4]. For example, the NLRP3 inflammasome promotes renal inflammation and contributes to chronic kidney disease [5–10]. A

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.003 0891-5849/© 2015 Elsevier Inc. All rights reserved. recent study reported that peripheral blood mononuclear cells from chronic kidney disease patients showed higher expression of NLRP3 inflammasome components (NLRP3, caspase-1, and ASC) and products (IL-1 β and IL-18) compared with healthy subjects [11]. These results suggest that the NLRP3/IL-1 β pathway may be a potential therapeutic target for renal disease in humans.

Corticosteroids, some cytotoxic agents, and cyclosporine A are commonly used in the clinic to treat chronic kidney disease [12]; however, the prevention of the progression of renal lesions is accompanied by unsatisfactory side effects in the patients, which remains a major concern [13]. Thus, the development of new agents with sufficient therapeutic effects and negligible side effects is clinically important. Some small molecules or bioactive ingredients isolated from natural products ameliorated renal damage by inhibiting the NLRP3 inflammasome [14–16]. Recently, we isolated several small molecules from natural products and demonstrated their renal protective functions in various mouse

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chronic kidney disease models, including IgA nephropathy, focal segmental glomerulosclerosis, and lupus nephritis [17–22].

Cinnamaldehyde (CA) is a major bioactive compound isolated from the essential oil of leaves of Cinnamomum osmophloeum kaneh [23]. CA exhibits immune modulation properties in bacteriainfected zebrafish by enhancing the host's defenses against pathogen infection [24], in rat cerebral microvascular endothelial cells by decreasing IL-1\beta-induced COX-2 activity and PGE₂ production [25], and in TNF- α -treated endothelial cells by inhibiting the adhesion of monocytes to the endothelial cells [26]. Our previous study showed that CA inhibited cytokine secretion from lipopolysaccharide (LPS)-activated macrophages but was cytotoxic at concentrations $> 40 \mu M$ [23]. Additionally, CA induced caspase-3-dependent apoptosis of human hepatoma cells by enhancing ROS generation and disrupting mitochondrial function [27]. Notably, one study showed that food consumption and the body weights of rats and mice fed CA were reduced, suggesting the possibility of a side effect associated with CA [28]. Another study indicated that CA induced eryptosis of erythrocytes and hemolysis [29]. These results indicate that safety should be a concern when CA is used as an anti-inflammatory agent. Here, we report the development of a nontoxic synthetic CA derivative (4-hydroxycinnamaldehyde-galactosamine, HCAG) that inhibits the LPSinduced inflammation in macrophages and reduces the LPS-induced renal inflammation in mice.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed after approval by the Institutional Animal Care and Use Committee of The National Ilan University, Taiwan (Permit number: NIU 102-7) and were consistent with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Chemistries used in compound preparation

The goal of this study is to develop the nontoxic CA derivatives that can be used in ameliorating renal inflammation by inhibiting inflammation and NLRP3 inflammasome. To synthesize the CA derivatives, all reactions were conducted in dried glassware overnight in a 120 °C oven. All reagents were used as received from commercial suppliers unless otherwise stated. Dichloromethane, chloroform, and methanol were distilled over CaH₂ under nitrogen. Spectrograde chloroform-d and DMSO-d₆ were used as solvents. All NMR chemical shifts were reported as values in parts per million (ppm), and coupling constants (J) were given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; m, unresolved multiplet due to the field strength of the instrument; and dd, doublet of doublet. The purification was performed using preparative separations in flash column chromatography (Merck silica gel 60, particle size 230–400 mesh). Analytical TLC was performed on precoated plates (Merck silica gel 60, F254). Compounds analyzed on the TLC plates were visualized using UV light, I₂ vapor, or 2.5% phosphomolybdic acid in ethanol with heating.

2.3. Preparation of 4-(2,3,4,6-Tetra-O-acetyl-*D*-glucopyranosyloxy) benzaldehyde (2)

2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl 2,2,2-trichloroacetimidate **1** (9.85 g, 20 mmol) and 4-hydroxybenzaldehyde (3.66 g, 30 mmol) were dissolved in CH₂Cl₂ (100 ml); then, the reaction mixture was cooled to -20 °C. Boron trifluoride diethyl etherate (46%, 16 ml, 60 mmol) was added dropwise at this

temperature. The reaction mixture was stirred at -20 °C for 4 h and monitored by TLC. Saturated NaHCO₃ aqueous solution (150 ml) was added to the mixture. The aqueous layer was separated and extracted with CH₂Cl₂ (2 × 150 ml). The organic layers were combined, dried over MgSO₄ and concentrated. Purification by silica gel column chromatography yielded **2** (3.8 g, 42%) as a white solid.

2.4. Preparation of 4-(2,3,4,6-Tetra-O-acetyl-D-glucopyranosyloxy)-(E)-2-styryl-1,3-dioxolane (4)

4-(2,3,4,6-Tetra-O-acetyl-D-glucopyranosyloxy)benzaldehyde **2** (3.4 g, 7.5 mmol) was dissolved in THF (75 ml, potassium *tert*butoxide, 0.84 g, 7.5 mmol); then, (1,3-dioxolan-2-ylmethyl)triphenylphosphonium bromide **3** (3.95 g, 9.2 mmol) was added portionwise. The reaction mixture was heated to reflux for 10 h. After completion of the reaction, the mixture was added to water and extracted with CH₂Cl₂ (2 × 100 ml). The organic layers were combined, dried over MgSO₄ and concentrated. The residue was purified by silica gel column chromatography to yield **4** (2.5 g, 64%) as a white powder.

2.5. Preparation of 4-(*b*-glucopyranosyloxy)-(*E*)-3-(4-methoxyphenyl)acrylaldehyde (5)

Compound 4 (1.8 g, 3.4 mmol) was dissolved in dry MeOH (40 ml). Then, NaOMe in MeOH was added at 0 °C. The solution was stirred at room temperature for 3 h. The progress of this reaction was monitored by TLC. The reaction mixture was concentrated in a vacuum and purified by Sephadex LH-20 column chromatography to yield a pale yellow powder. The pale yellow powder (1.1 g) in CH₃OH (20 ml) was added to H₂O (10 ml) and HOAc (10 ml) at 0 °C. Then, the mixture was warmed to room temperature and stirred for 10 h. Saturated aqueous NaHCO₃ solution was added, and the aqueous layer was extracted with CH_2Cl_2 (2 × 50 ml). The organic layers were combined, dried over $MgSO_4$ and evaporated to give a yellow powder 5 (0.8 g, 77% in two steps); ¹H NMR (DMSO- d_6): 9.61 (d, I = 7.8 Hz, 1 H), 7.70 (d, *I*=8.4 Hz, 2 H), 7.67 (d, *I*=15.6 Hz, 1 H), 7.09 (d, *I*=8.4 Hz, 2 H), 5.18 (d, *I*=5.4 Hz, 1 H), 4.92 (d, *I*=7.8 Hz, 1 H), 4.87 (d, *I*=6.0 Hz, 1 H), 4.65 (t, J=5.4 Hz, 1 H), 4.51 (d, J=4.8 Hz, 1 H), 3.71-3.69 (m, 1 H), 3.62–3.56 (m, 5 H). ¹³C NMR (DMSO-*d*₆): 194.2, 159.8, 153.0, 130.5, 127.7, 126.7, 116.6, 100.5, 75.6, 73.2, 70.1, 68.1, 60.3.

2.6. Cell cultures

The murine macrophage cell lines RAW 264.7 and J774A.1 were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW 264.7 macrophages stably transfected with the NF- κ B reporter gene (RAW-BlueTM cells) were purchased from InvivoGen (San Diego, CA, USA). All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine (all from Life Technologies, Carlsbad, CA, USA) at 37 °C in a 5% CO₂ incubator. In the case of the RAW-BlueTM cells, 100 µg/ml of zeocin was added to the medium.

2.7. AlamarBlue[®] assay for cell viability

To evaluate the possible toxicity of CA derivatives toward macrophages, RAW 264.7 cells (5×10^3 cells in 0.1 ml medium) were incubated with or without samples for 24 h. The Alamar-Blue[®] assay was used to determine the cytotoxicity of the samples according to the protocol described in the manufacturer's instructions (AbD Serotec, Oxford, UK).

2.8. Western blotting

To detect the levels of protein expression and protein phosphorylation, Western blotting was adapted. Briefly, the samples were harvested, resuspended in sample buffer, and subjected to 12–15% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (EMD Millipore, Darmstadt, Germany). The membranes were blotted with primary antibodies and secondary HRP-conjugated antibodies, followed by ECL (EMD Millipore).

2.9. Inflammatory mediator expression

To evaluate the anti-inflammatory activity of HCAG, the levels of inflammatory mediator generated by LPS-activated macrophages were measured. For the NO generation and IL-6 and TNF- α secretion assay, RAW 264.7 macrophages (2 × 10⁵ cells in 0.25 ml medium) were incubated with or without the indicated concentrations of HCAG for 30 min and then treated with or without the addition of 0.1 µg/ml of LPS for 24 h. NO, IL-6, and TNF- α in the culture medium was measured by the Griess reaction and ELISA, respectively. For the proIL-1 β and NLRP3 protein expression assay, J774A.1 macrophages (2 × 10⁶ cells in 2 ml medium) were incubated with or without the indicated concentrations of HCAG for 30 min and then treated with or 0.1 µg/ml of LPS for 6 h. Protein expression levels of iNOS, proIL-1 β and NLRP3 in the cells were measured by Western blotting.

2.10. IL-1 β secretion and caspase-1 activation

To evaluate the inhibitory effect of HCAG on NLRP3 inflammasome, IL-1 β secretion and caspase-1 activation, the hallmarks of NLRP3 inflammasome activation, were measured. For the IL-1 β secretion and caspase-1 activation assays depicted in Fig. 3B. J774A.1 macrophages $(2 \times 10^5$ cells in 0.25 ml medium) were incubated with or without $0.1 \,\mu g/ml$ LPS for 5.5 h, followed by treatment with or without HCAG for 30 min. The cells were treated with or without 5 mM ATP for 0.5 h. IL-1 β in the culture medium was measured by ELISA, and activated caspase-1 (p10) and procaspase-1 (p45) in the cells were measured by Western blotting. For IL-1 β secretion (as shown in Fig. 3C and D), J774A.1 macrophages $(2 \times 10^5$ cells in 0.25 ml medium) were incubated for 5.5 h with or without 0.1 µg/ml LPS, followed by treatment with or without HCAG for 30 min. Then, the cells were treated with or without nigericin (10 μ M, 0.5 h), monosodium urate (MSU) (500 μ g/ml, 24 h), calcium pyrophosphate dehydrate (CPPD) crystals (100 µg/ml, 24 h), nanoparticles of silicon dioxide (nano SiO₂) (200 µg/ml, 24 h), alum crystals (500 µg/ml, 24 h), flagellin from S. typhimurium (FLA-ST) $(100 \mu g/ml, 24 h)$, or muramyl dipeptide (MDP) (100 ng/ml, 24 h). IL-1 β in the culture medium was measured by ELISA.

2.11. Phosphorylation levels of MAPKs, IKK- α , I κ B- α , PKC, and AKT

To investigate which signaling pathway is affected by HCAG, the phosphorylation levels of MAPKs, IKK- α , IKB- α , PKC- α , PKC- δ , and AKT in LPS- or ATP-activated macrophages were measured. For the phosphorylation levels of IKK- α and IKB- α (Fig. 4B), MAPKs (Fig. 4D), PKC- α and PKC- δ (Fig. 4E), RAW 264.7 macrophages (2 × 10⁶ cells in 2 ml medium) were incubated for 30 min with or without HCAG and then for 0–60 min with or without 0.1 µg/ml LPS. The phosphorylation levels of these proteins were analyzed by Western blotting. For the phosphorylation levels of AKT (Fig. 4G) and PKC- α (Fig. 4H) in Fig. 5, J774A.1 macrophages (2 × 10⁶ cells in 2 ml medium) were incubated for 5.5 h with 0.1 µg/ml LPS, then for 30 min with or without HCAG, and then for 0–60 min with or without 5 mM ATP. The phosphorylation levels of these proteins were analyzed by Western blotting.

2.12. ROS detection

ROS is an important signaling molecule mediating inflammatory response and NLRP3 inflammasome activation. We asked whether HCAG inhibits inflammatory response and NLRP3 inflammasome through reducing ROS level. Intracellular ROS production was measured by detecting the fluorescence intensity of 2',7'-dichlorofluorescein (the oxidation product of 2',7'-dichlorofluorescein diacetate, Molecular Probes, Eugene, OR, USA). For LPS-induced ROS generation (as shown in Fig. 4A), RAW 264.7 macrophages $(5 \times 10^3$ cells in 0.1 ml medium) were incubated with or without 50 µM HCAG, 10 mM N-acetyl cysteine (NAC), or DMSO (vehicle) for 30 min, with $2 \mu M 2'$,7'-dichlorofluorescein diacetate for 30 min, and then with or without 0.1 µg/ml LPS for 0-40 min. For ATP-induced general ROS generation (shown in Fig. 4F), [774A.1 macrophages $(5 \times 10^3 \text{ cells in } 0.1 \text{ ml medium})$ were incubated with $0.1 \,\mu g/ml$ LPS for 5.5 h, with or without 50 μM HCAG, 10 mM NAC, or DMSO for 30 min, with 2 μM 2',7'dichlorofluorescein diacetate for 30 min, and then with or without 5 mM ATP for 0-60 min. The fluorescence intensity of 2',7'-dichlorofluorescein was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm on a microplate absorbance reader (Bio-Tek, VT, USA). In each time point, the fluorescent intensity fold change of each group was calculated by comparison to control group (DMSO/PBS), as control group have a fold change of "one".

2.13. NF-*k*B reporter assay

NF-κB is one of the most important transcription factors regulating the inflammatory gene expression. We asked whether HCAG inhibits NF-κB activation in LPS-activated macrophages. RAW-BlueTM cells (2×10^5 cells in 0.25 ml medium) were incubated with or without HCAG for 30 min and then with or without 0.1 µg/ml LPS for 24 h. The medium (20μ l) from treated RAW-BlueTM cells was mixed with 200μ l of QUANTI-BlueTM medium (Invitrogen, Carlsbad, CA, USA) in 96-well plates and incubated at 37 °C for 15 min. The secreted embryonic alkaline phosphatase activity was assessed by measuring the optical density at 655 nm using a microplate absorbance reader (Bio-Tek, VT, USA).

2.14. Induction of LPS-induced renal inflammation

Experiments were performed on 8-week-old female C57/B6 mice (22–25 g body weights) obtained from the National Laboratory Animal Center, Taipei, Taiwan. LPS-induced renal inflammation was induced by injection of *E coli* LPS as described previously with mild modifications [30]. Briefly, LPS was administered intraperitoneally at a dose of 1 mg/kg body weight for 8 consecutive weeks. Two days prior to the first dose LPS injection, a daily dose of HCAG (100 mg/kg body weigh) or corn oil (vehicle) was administered via the intraperitoneal route until the sacrifice of the mice at week 8. The dose of HCAG used for the mice was extrapolated using an equation according to the dose employed for *in vitro* experiments as described in our previous study [31]. The equation is as the follows:

Dose of HCAG (mg/kg/day) = $40 \times (V \times P) \times O/W$

V is the volume of blood of a mouse on average (\sim 3 ml), *P* is the percentage of whole body water content of a mouse (\sim 70%), *O* is the optimal dose of HCAG used in cell models (100 mM; 0.031 mg/ml), *W* is the mouse body weight on average (\sim 25 g).Weekly urine samples were collected in metabolic cages. The concentration of urine albumin was determined by ELISA (Exocell, Philadelphia, PA,

USA); urine albumin levels were expressed relative to urine creatinine (Cr) levels measured using a kit from Wako Pure Chemical Industries (Osaka, Japan) as described previously [32]. Renal cortical tissues and blood samples were collected at week 8 and stored appropriately for further analysis. Serum levels of blood urea nitrogen (BUN) and Cr were measured using a BUN kit or Cr kit (both from Fuji Dry-Chem Slide, Fuji Film Medical, Tokyo, Japan) as described previously [33].

2.15. Renal histopathology

The tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin. A nephropathologist (A Chen) was assigned to examine one hundred glomeruli in a blinded way by light microscopy at the magnification of 400 with at least two renal tissue sections each. Scoring of the severity of individual renal lesions was performed as described previously [34]. The percentage (proportion) was determined for the following three major components: glomerular proliferation, glomerular sclerosis, or periglomerular inflammation.

2.16. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were incubated overnight at 4 °C with antibodies against CD3 (pan-T cells, BD Biosciences, CA, USA), F4/80 (monocytes/macrophages; Serotec, NC, USA), phospho-NF- κ B p65 (pNF- κ B p65; Cell Signaling, MA, USA), or IL-1 β (Serotec) diluted in DAKO antibody dilution buffer (DAKO, Denmark), followed by the incubation with horseradish peroxidase-conjugated second antibodies (DAKO) in the same buffer as described previously [20]. Scoring of the IHC results was performed using the Pax-it quantitative image analysis software (Paxcam, IL, USA) as described previously [20].

2.17. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Apoptosis staining was performed using ApopTag Plus Peroxidase in Situ Apoptosis Detection kit (Chemiconm Temecula, CA) according to the manufacturer's instructions. Scoring of the TUNEL results was performed using Pax-it quantitative image analysis software (Paxcam, IL, USA) as described previously [34].

2.18. Renal ROS detection

An *in situ* staining procedure with the fluorescent dye dihydroethidium (DHE) was performed in renal tissues and quantified by counting the percentage of total positive nuclei per kidney cross section using the Pax-it software (Paxcam) as described previously [20].

2.19. Renal NF-*k*B p65 activity assay

Renal NF-κB p65 activity was measured with the TransAM ELISA assay kit (Active Motif, CA, USA) according to the manufacturer's instructions using a microplate absorbance reader (Bio-Tek, VT, USA).

2.20. Renal MCP-1 and IL-6 assay

Renal cortical samples were homogenized in protease inhibitors (Roche Applied Science, USA) using a sterile razor blade. Both MCP-1 and IL-6 were measured using commercial ELISA kits (eBioscience, CA, USA) according to the manufacturer's instructions and a microplate absorbance reader (Bio-Tek).

2.21. Real-time polymerase chain reaction analysis

Renal cortex RNA was extracted using the TRIzol reagent and subjected to cDNA synthesis (Invitrogen, Carlsbad, CA, USA). The real-time PCR reaction was performed in the ABI Prism 7700 Seauence Detection System (Perkin Elmer Applied Systems, Foster City, CA, USA) using the SYBR Green I PCR kit (Perkin Elmer Applied Systems) to measure NLRP3, Caspase-1, IL-1B, IL-18, toll-like receptor 4 (TLR4) and MyD88 gene expression as described previously [35]. The following primers were used: NLRP3 (5'-CCCTTGGAGA-CACAGGACTC-3' and 5'-GAGGCTGCAGTTGTCTAATTCC-3'): Caspase-1 (5'-TCCGCGGTTGAATCCTTTTCAGA-3' and 5'-ACCACAATTGCT-GTGTGTGCGCA-3'): IL-1 β (5'-TGTAATGAAAGACGGCACACC-3' and 5'-TCTTCTTTGGGTATTGCTTGG-3'); IL-18 (5'-CTGTACAACCGG-AGTAATACGG-3' and 5'-TCCATCTTGTTGTGTCCTGG-3'); and TLR4 (5'-ACCAGGAAGCTTGAATCCCT-3' and 5'-TCCAGCCACTGAAGTTCT-GA-3′).

2.22. Flow cytometry

Splenocytes were double-stained with FITC-conjugated antibodies against mouse CD3, CD4, or CD19 (B cell marker) and phycoerythrin (PE)-conjugated anti-mouse CD69 antibodies (H1.2F3; marker of activated T and B cells) (BD Biosciences, CA, USA) and analyzed on a FACSCalibur (BD Biosciences) as described previously [34].

2.23. Statistical analysis

All values are reported as the mean \pm standard deviation (SD). The data were analyzed using a one-way ANOVA followed by a Scheffé test.

3. Results

3.1. Synthesis of (E)-3-phenyl-2-propenoyl- β - β - β -galactosamine (EPPG)

EPPG can be readily made in three steps from cinnamic anhydride; the synthetic strategy is demonstrated in Scheme 1. Cinnamic anhydride was prepared by treating the mixture of cinnamic acid and thionyl chloride under basic conditions according to known methods [36]. Subsequently, *N*-linked glycosylation was conducted using galactosamine and cinnamic anhydride at room temperature to produce a moderate yield of EPPG.

3.2. Synthesis of N-cinnamyl- β -D-galactosamine (NCAG)

The direct substitution of galactosamine to the allyl position of cinnamyl bromide is readily accomplished at room temperature using sodium methoxide in methanol to obtain NCAG (Scheme 2).

3.3. Synthesis of 4-(*p*-glucopyranosyloxy)-(*E*)-3-(4-methoxyphenyl) acrylaldehyde (5) (4-hydroxycinnamaldehyde-galactosamine, HCAG)

The strategy for the synthesis of 4-hydroxyphenylacrylaldehyde glycoside **5** is shown in Scheme 3; the strategy involves a three-step synthetic route from trichloroacetimidate derivative **1**. Trichloroacetimidate derivative **1** was prepared from 2,3,4,6-tetra-*O*-acetyl-*D*-glucopyranose according to Schmidt's trichloroacetimidate procedure [37]. BF₃-promoted glycosylation of **1** using 4-hydro-xybenzaldehyde afforded compound **2** with a 42% yield. Subsequently, compound **2** was treated with triphenyl-1,3-dioxolan-2-yl-methylphosphonium bromide **3** and potassium *tert*-butoxide in THF to produce a strong predominance of the *E* isomer of styryl-1,3-dioxolane glycoside **4**. Then, the acetyl groups were removed from



Scheme 3. Synthesis of HCAG.

styryl-1,3-dioxolane glycoside **4** using sodium methoxide in methanol, followed by neutralization of the reaction mixture with a mild acid to obtain HCAG with a 77% yield.

3.4. Effect of CA derivatives on cell viability

CA and the synthesized CA derivatives EPPG, NCAG, and HCAG (Fig. 1A) were evaluated to determine their cytotoxicity against the

mouse macrophage cell line RAW 264.7 after 24 h of treatment. CA exhibited high cytotoxicity against RAW 264.7 macrophages with an IC_{50} of 40 μ M. However, the cytotoxicity of HCAG was much lower than CA because the cell viability was not significantly reduced by HCAG at a concentration of 1600 μ M (Fig. 1B). The cytotoxicity of EPPG and NCAG was also lower than CA because the cell viability was not significantly reduced by EPPG and NCAG at a concentration of 100 μ M (data not shown).



Fig. 1. Chemical structure of CA and its derivatives and their effect on cell viability. (A) Chemical structure of CA and its derivative (B) Cell viability. The data are expressed as the means \pm SD of three separate experiments.

3.5. Effect of HCAG on LPS-induced inflammatory response

The anti-inflammatory activity of the CA derivatives was investigated in LPS-activated RAW 264.7 macrophages. NO levels were significantly reduced by HCAG in a dose dependent manner (Fig. 2A), while NO levels were slightly reduced by EPPG and were not affected by NCAG (data not shown). HCAG also decreased iNOS expression (Fig. 2B) and TNF- α (Fig. 2C) and IL-6 (Fig. 2D) secretion by LPS-activated macrophages. These results indicate that HCAG is a nontoxic cinnamaldehyde derivative that is able to inhibit inflammatory response in LPS-activated macrophages.

3.6. HCAG reduces nlrp3 inflammasome activation

Full activation of the NLRP3 inflammasome requires both a priming signal from LPS and an activation signal from a second stimulus (e.g., ATP); the former controls the expression of NLRP3 and proIL-1 β and the latter controls caspase-1 activation [38,39]. We found that proIL-1 β expression in HCAG-treated J774A.1 macrophages was reduced compared with the control cells, whereas NLRP3 expression was not affected (Fig. 3A). Additionally, ATP-induced IL-1 β secretion and caspase-1 activation (Fig. 3B) in LPS-primed J774A.1 macrophages were reduced by HCAG in a dose-dependent fashion. The inhibitory effect of HCAG on NLRP3 inflammasome was confirmed as HCAG reduced the induction of IL-1 β secretion by other NLRP3 inflammasome activators, including nigericin, monosodium urate crystal (MSU), calcium pyr-ophosphate dihydrate (CPPD), SiO₂ nanoparticles, and alum crystals (Fig. 3C). Next, we determined whether the effect of HCAG on



Fig. 2. Effect of HCAG on LPS-induced inflammatory mediator expression. (A) NO production. (B) iNOS expression. (C) TNF- α secretion. (D) IL-6 secretion. The data are expressed as the means \pm SD of three separate experiments. The Western blotting results are representative of results obtained in three separate experiments, and the histograms represent the change in the ratio relative to actin compared with the control group. * and *** indicate a significant difference at the level of *p* < 0.05 and *p* < 0.001, respectively, compared with LPS-treated group.



Fig. 3. Effect of HCAG on NLRP3 inflammasome activation. (A) Expression levels of NLRP3 and prolL-1 β . (B) IL-1 β secretion and caspase-1 activation induced by ATP. (C) IL-1 β secretion induced by Nigericin, MSU, CPPD, nano SiO₂, and Alum. (D) IL-1 β secretion induced by FLA-ST and MDP. The data are expressed as the means \pm SD of three separate experiments. The Western blotting results are representative of results obtained in three separate experiments, and the histograms represent the change in the ratio relative to p45 compared with the control group. * and *** indicate a significant difference at the level of *p* < 0.05 and *p* < 0.001, respectively, compared with the LPS-treated group (A) or LPS+ inducer group (B–D).

IL-1 β secretion was selective for the NLRP3-dependent inflammasome. HCAG was not selective for NLRP3-dependent inflammasome stimuli because HCAG also reduced IL-1 β secretion triggered by FLA-ST (flagellin from *S. typhimurium*) and muramyl dipeptide (MDP) (Fig. 3D); these stimuli were dependent on the NLRC4 and NLRP1 inflammasomes, respectively [2], and functioned independent of NLRP3.

3.7. Effect of HCAG on inflammatory signaling

ROS is one of the important signaling molecules mediating inflammatory signaling cascades in LPS-activated macrophages [23,39]. We asked whether HCAG is able to inhibit ROS generation in activated macrophages. We found that LPS-induced ROS generation in the HCAG-treated RAW 264.7 macrophages was reduced compared with the control cells (Fig. 4A). NAC (n-acetyl-cysteine), an anti-oxidant, was used as a positive control in inhibiting ROS generation. In addition, the fluorescent intensity of HCAG/PBS and

HCAG/LPS were not significantly different, indicating that HCAG is not only able to reduce ROS levels induced by LPS, but is also able to reduced endogenous ROS levels in untreated cells. NF-KB activation leads to the production of proIL-1 β and pro-inflammatory mediators [40]. Accordingly, we found that LPS-induced IKK- α and I κ B- α phosphorylation (Fig. 4B) and NF- κ B transcriptional activity (Fig. 4C) were reduced by HCAG. Additionally, mitogen-activated protein kinase (MAPK) and PKC regulate the expression of proIL- 1β and pro-inflammatory mediators [41,42]. We found that the phosphorylation levels of MAPK members p38, JNK1/2, and ERK1/ 2 (Fig. 4D) as well as PKC- α and PKC- δ (Fig. 4E) were reduced by HCAG. These results indicated that HCAG inhibited pro-inflammatory mediator expression and the priming of the NLRP3 inflammasome; these effects were associated with reduced ROS generation, NF- κ B activation, and MAPK and PKC- α/δ phosphorylation. Furthermore, ROS not only controls the priming step but also controls the NLRP3 inflammasome activation step [43]. ATP stimulates the phosphatidylinositol 3-kinase pathway and



Fig. 4. Effect of HCAG on inflammatory signaling. (A) ROS generation induced by LPS. (B) Phosphorylation levels of IKK- α and IkB- α induced by LPS. (C) Activation levels of NF- κ B induced by LPS. (D) Phosphorylation levels of MAPKs induced by LPS. (E) Phosphorylation levels of PKC- α/δ induced by LPS. (F) ROS generation induced by ATP. (G) Phosphorylation levels of PKC- α induced by ATP. The data are expressed as the means \pm SD of three separate experiments. The Western blotting results are representative of results obtained in three separate experiments, and the histograms represent the change in the ratio relative to action or corresponding total protein compared with the control group. * and *** indicate a significant difference at the level of *p* < 0.05 and *p* < 0.001 compared with the LPS group (B–E) or the ATP group (G and H).

subsequent AKT activation in a ROS-dependent manner, which is required for caspase-1 activation and IL-1 β secretion [44]. Therefore, we investigated whether HCAG regulated ROS generation and AKT activation during the NLRP3 inflammasome activation step. We found that ATP-induced ROS generation was significantly reduced by NAC, but not affected by HCAG (Fig. 4F). Additionally, phosphorylation levels of AKT (Fig. 4G) and PKC- α (Fig. 4H) in ATPactivated J774A.1 macrophages were reduced by HCAG.

3.8. HCAG improves clinical conditions and renal histopathology and reduces renal ROS production

LPS from bacteria can induce inflammatory cell infiltration in the kidney in a mouse model of accelerated lupus nephritis [45]. We evaluated the effects of HCAG on the prevention of the development

of LPS-induced renal inflammation (LPRI) in mice that received daily administration of HCAG for eight consecutive weeks. First, mice administered HCAG showed a stable increase in their body weight compared with the normal control group (data not shown), suggesting that the appetite and activity of the mice were not affected by the drug administration. Clinical assessment showed a significant reduction in proteinuria at weeks 2, 4, 6 and 8 in the HCAG-treated LPRI (HCAG+LPRI) mice compared with the vehicle-treated LPRI (vehicle+LPRI) disease control mice (Fig. 5A). Significantly lower BUN serum levels were observed in the HCAG+LPRI mice compared with the vehicle+LPRI mice, although there was no difference in the serum Cr levels (Fig. 5B) at week 8. Although the vehicle+LPRI mice exhibited glomerular proliferation and sclerosis and peri-glomerular inflammation at week 8, this effect was greatly inhibited in the HCAG+LPRI mice (Fig. 5C and D). In addition, significantly reduced



Fig. 5. Effect of HCAG on proteinuria, renal function, renal histopathology, renal ROS production, and renal apoptosis in the LPRI mouse model. (A) Proteinuria. (B) Serum levels of BUN and creatinine. (C) Histopathology of the kidney. H&E stain. The arrow indicates periglomerular mononuclear leukocyte infiltration. (D) Scoring of renal pathology. (E) Apoptosis detected by TUNEL assay. (F) Scoring for TUNEL assay. (G) ROS production detected by *in situ* DHE staining. (H) Scoring for DHE staining. Original magnification, $400 \times .$ The data are the mean \pm SEM for seven mice per group. *p < 0.05, **p < 0.01, **:p < 0.001. #: not detectable.

renal levels of apoptosis was observed in HCAG+LPRI mice, compared to those of vehicle+LPRI mice (Fig. 5E and F). Next, we measured renal ROS levels. As shown in Fig. 5G and H, significantly increased ROS production was observed in the renal tissues from the vehicle+LPRI mice compared with the normal control mice, but this effect was greatly suppressed in the HCAG+LPRI mice as demonstrated by *in situ* DHE staining.

3.9. HCAG decreases NLRP3 inflammasome activation and TLR4/ MyD88 expression in the kidney

We evaluated the effects of HCAG on: (1) the activation of the renal NLRP3 inflammasome, which has been implicated in the development and progression of renal inflammation [5,6], and (2) renal TLR4/MyD88 expression, which has been reported to



Fig. 6. Effect of HCAG on renal NLRP3 inflammasome activation and TLR4/MyD88 expression in the LPRI mouse model. (A) mRNA expression levels of NLRP3, caspase-1, IL-1 β , IL-18, TLR4, and MyD88. (B) IHC staining of IL-1 β . (C) Scoring for IL-1 β staining. Positively stained cells in the glomeruli were mainly podocytes and mesangial cells. Original magnification, 400 × . The data are the mean ± SEM for seven mice per group. *p < 0.05, **p < 0.01, ***p < 0.001.

promote renal inflammation and may represent a novel therapeutic target for the prevention of renal injury [6]. Although the mRNA expression levels of NLRP3, caspase-1, IL-1 β , IL-18, TRL4, and MyD88 in renal tissues were all significantly increased in the vehicle+LPRI mice compared with the normal controls, these effects were inhibited in the HCAG+LPRI mice (Fig. 6A). Additionally, IHC demonstrated that the vehicle+LPRI mice had significantly higher renal IL-1 β levels compared with the normal control mice; this effect was also significantly inhibited in the HCAG+LPRI mice (Fig. 6B and C).

3.10. HCAG decreases renal NF- κ B-mediated production of MCP-1 and IL-6

Renal production of NF- κ B-mediated pro-inflammatory cytokines such as MCP-1 and IL-6 has been shown to be a pathogenic factor for various types of glomerulonephritis [46,47]. As shown in Fig. 7A, vehicle+LPRI mice showed significantly increased renal phosphorylation levels of NF- κ B p65 by IHC, but this effect was significantly decreased in the HCAG+LPRI mice. This effect of HCAG was confirmed by the observation of significantly elevated levels of activated pNF- κ B p65 by ELISA (Fig. 7B); again, this effect was greatly decreased in the HCAG+LPRI mice. Significantly higher renal expression levels of MCP-1 and IL-6 were also observed in the vehicle+LPRI mice compared with the normal control mice, but this effect was inhibited in the HCAG+LPRI mice (Fig. 7C).

3.11. HCAG mitigates renal infiltration of macrophages and T cells

Infiltration of macrophages and T cells locally in the kidney plays a pivotal pathogenic role in the development of glomerular crescents of glomerulonephritis [21,48]. As shown in Fig. 8A and B,



Fig. 7. Effect of HCAG on renal NF-κB activation and MCP-1 and IL-6 expression levels in the LPRI mouse model. (A) Scoring of pNF-κB p65 staining. (B) Renal pNF-κB p65 activity measured by ELISA. (C) Renal MCP-1 and IL-6 protein levels measured by ELISA. The data are the mean ± SEM for seven mice per group. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

the vehicle+LPRI mice showed an intense infiltration of macrophages and CD3+T cells in the periglomerular region of the kidney, but few or no infiltrating macrophages and T cells were observed in the HCAG+LPRI mice.

3.12. HCAG inhibits T cell activation in the spleen

Systemic activation of T and B cells plays a crucial role in the pathogenesis of renal inflammation and fibrosis [49,50]. We examined whether inhibition of the activation of T or B cells may be

a mechanism of action involved in the beneficial effects of HCAG in the HCAG+LPRI mice. As shown in Fig. 9, increased activation of CD4⁺T cells in the spleen was observed in the vehicle+LPRI mice compared with the normal control mice; this effect was significantly suppressed in the HCAG+LPRI mice, as demonstrated by flow cytometry. Similarly, the HCAG+LPRI mice exhibited suppressed activation of CD3+T cells in the spleen, although the difference did not reach significance (p=0.07). Although the vehicle+LPRI mice showed higher levels of activation of CD19⁺B cells in the spleen compared with the normal control mice, there was no significant difference between the vehicle+LPRI mice and HCAG+LPRI mice.

4. Discussion

LPS-induced TLR4 activation in macrophages triggers ROS release, which leads to the activation of NF-kB and induces a rapid cytokine storm, including spikes in TNF α , IL-1 β , and IL-6 [51–53]. These cytokines, in turn, activate a large number of immune cells that produce more ROS and inflammatory factors and damage the surrounding tissue [54,55]. LPS not only activates macrophages but also promotes the activation of intrinsic renal cells, such as mesangial and tubular epithelial cells, and these contribute to the renal diseases [56,57]. LPS-induced VCAM-1 expression in mesangial cells was partially mediated through a TLR4/ROS-dependent pathway associated with recruitment of monocyte adhesion to kidney leading to the inflammatory responses in renal diseases [58]. In this study, we showed that HCAG could ameliorate renal lesions associated with glomerular proliferation, glomerular sclerosis, periglomerular inflammation, and renal levels of apoptosis in a mouse model of LPS-induced renal inflammation. Our data suggested that the beneficial effects of HCAG on LPS-induced renal inflammation were mainly due to the inhibition of renal ROS generation and NF-KB activation, as well as reduced inflammatory cytokine expression and NLRP3 inflammasome activation in the kidney (Fig. 10). We also demonstrated that HCAG was able to inhibit LPS-induced inflammatory response in macrophages; however, we have no solid evidences showing the direct effect of HCAG on the renal cells yet.

In addition, our previous studies demonstrated that renal ROS was increased in mice with renal disease, including IgA nephropathy [17,22,48], lupus nephritis [19], focal segmental glomerulosclerosis [20], and diabetic nephropathy [59]. Consistently, inhibition of renal ROS generation ameliorated the renal inflammation and injury in these mouse models of renal inflammation. Moreover, HCAG administration significantly inhibited the increase in ROS levels in renal tissues observed in the vehicle+LPRI mice (Fig. 5G and H). This result was consistent with our in vitro cell model finding that HCAG markedly inhibited ROS generation in LPS-activated macrophages (Fig. 4A). ROS is one of the key signaling molecules mediating the pro-inflammatory signaling cascades downstream of TLR4 [48,60,61]. Thus, the inhibition of ROS may be partially responsible for the reduced inflammation induced by HCAG. ROS is also involved in the activation process of the NLRP3 inflammasome [43]. The priming step of the NLRP3 inflammasome is regulated by ROS, which is required for NLRP3 expression [38]. HCAG did not affect NLRP3 expression in LPSactivated macrophages (Fig. 3A), although the antioxidant activity of HCAG in LPS-activated macrophages was observed (Fig. 4A). NLRP3 expression in LPS-activated macrophages is also regulated by NF- κ B [62] and the MAPK pathways [39]. We showed that NFκB (Fig. 4B and C) and MAPK (Fig. 4D) were inhibited and that the NLRP3 inflammasome activation was suppressed (Fig. 3B and C) by HCAG administration, although the expression level of the NLRP3 protein was not affected (Fig. 3A).



Fig. 8. Effect of HCAG on the renal infiltration of macrophages and T cells in the LPRI mouse model. (A) Staining of renal tissues for F4/80+ macrophages and CD3+ T cells. The arrows indicate positively stained cells. (B) Scoring of positively stained cells. Original magnification, $400 \times$. The data are the mean \pm SEM for seven mice per group. **p < 0.01, ***p < 0.001.



Fig. 9. Effect of HCAG on splenic T cell activation in the LPRI mouse model. Percentage of $CD3^+CD69^+$ cells in $CD3^+$ splenocytes, $CD4^+CD69^+$ cells in $CD4^+$ splenocytes and $CD19^+CD69^+$ cells in $CD19^+$ splenocytes. The data are the mean \pm SEM for seven mice per group. **p < 0.01, ***p < 0.001.



Fig. 10. A diagram of HCAG-mediated anti-inflammatory and renal protective effects.

The full activation of the NLRP3 inflammasome requires both a priming signal from LPS-stimulated TLR4 and an activation signal from ATP; the former controls the expression of NLRP3 and proIL- 1β and the latter controls caspase-1 activation. The up-regulation of ROS levels in ATP-treated macrophages resulted in the activation of the PI3K/AKT pathway, thereby promoting caspase-1 activation and IL-1 β secretion [44]. The inhibition of ATP-induced AKT phosphorylation may be responsible for the reduced caspase-1 activation and IL-1 β secretion induced by HCAG (Fig. 4G); however, HCAG did not inhibit ATP-induced ROS generation (Fig. 4F). These results suggest that HCAG acts upstream of AKT and downstream of ROS. Overproduction of mitochondrial ROS from damaged mitochondria promotes a mitochondrial permeability transition and facilitates the cytosolic release of mitochondrial DNA, which in turn stimulates the activation of the NLRP3 inflammasome [17,63]. Although HCAG inhibits NLRP3 inflammasome activation, the effect of HCAG on mitochondrial function requires further investigation. Interestingly, cinnamaldehyde induces apoptosis of cancer cells by inducing derangement of mitochondrial functions [64].

Mice with diabetic nephropathy exhibited significantly increased expression of TLR4 and its associated protein MyD88 in the kidney [65]. Interestingly, genetic deficiency of TLR4 ameliorated renal inflammation, fibrosis and podocytopathy [65,66]. TLR4-knockout mice were also protected from renal injury after LPS exposure [67], suggesting that TLR4 signaling may represent a potential therapeutic target for the prevention of renal injury [6]. In this study, we found that HCAG decreased renal TLR4 and MyD88 expression. This reduced expression may be responsible for the reduced renal injury induced by LPS. In our previous report, we demonstrated that CA inhibited TLR4-mediated signaling [23]; these findings were supported by another report [68].

Hit to lead is a stage in early drug discovery where small molecule hits undergo limited optimization through the synthesis of analogs to identify promising lead compounds with improved efficacy or reduced toxicity [69]. CA and its derivatives have been reported to possess anti-tumor activity [70], anti-bacterial activity [71], anti-tyrosinase activity [72], and anti-AP-1 and -NF- κ B activity [73,74]. In this study, we report for the first time that the glycosylated CA derivative possesses anti-inflammatory activity, and especially inhibits NLRP3 inflammasome activation. Importantly, the toxicity of HCAG was reduced more than one hundred-fold compared with CA (Fig. 1B). The reduced toxicity results in a huge increase in the HCAG therapeutic window and suggests that this molecule can treat disease effectively while staying within the safety range. Furthermore, our data suggest that HCAG may be helpful in ameliorating renal inflammation by targeting the interlinked inflammatory pathway.

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