Original Article

# 7-Hydroxycoumarin protects against cisplatin-induced acute kidney injury by inhibiting necroptosis and promoting Sox9-mediated tubular epithelial cell proliferation

Wei-Feng Wu[a,b,1], Jia-Nan Wang[a,1], Zeng Li[a,1], Biao Wei[a], Juan Jin[c], Li Gao[a], Hai-Di Li[a], Jun Li[a], Hai-Yong Chen[b,*], Xiao-Ming Meng[a,*]

[a] The Key Laboratory of Major Autoimmune Diseases, Anhui Institute of Innovative Drugs, School of Pharmacy, Anhui Medical University, Hefei, Anhui 230032, China
[b] School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong and Department of Chinese Medicine, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China
[c] Department of Pharmacology, Key Laboratory of Anti-inflammatory and Immunopharmacology, Ministry of Education, Anhui Medical University, Hefei 230032, China

ARTICLE INFO

ABSTRACT

Background: 7-Hydroxycoumarin (7-HC), also known as umbelliferon, is commonly found in Chinese herbs (e.g. Eucommiae Cortex, Prunellae Spica, Radix Angelicae Biseratae). Previous laboratory studies have indicated that 7-HC has anti-inflammatory, anti-oxidative, and anti-tumor effects. Cisplatin is a widely used chemotherapeutic agent for cancer. Nephrotoxicity is one of the limiting side effects of cisplatin use.
Purpose: This study aimed to evaluate the renoprotective effect of 7-HC in a cisplatin-induced acute kidney injury (AKI) mouse model.
Methods: AKI was induced in male C57BL/6 mice (aged 6–8 weeks) by a single intraperitoneal injection of cisplatin at 20 mg/kg. The mice received 7-HC at 30, 60, and 90 mg/kg intraperitoneally before or after cisplatin administration. Renal function, necroptosis, and cell proliferation were measured. Mechanisms underlying the reno-protective effect of 7-HC were explored in renal tubular epithelial cells treated with or without cisplatin.
Results: In-vivo experiments showed that 7-HC significantly improved the loss in kidney function induced by cisplatin, as indicated by lower levels of serum creatinine and blood urea nitrogen, in AKI mice. Consistent herewith, cisplatin-induced tubular damage was alleviated by 7-HC as shown by morphological (periodic acid–Schiff staining) and kidney injury marker (KIM-1) analyses. We found that 7-HC suppressed renal necroptosis via the RIPK1/RIPK3/MLKL pathway and accelerated renal repair as evidenced by the upregulation of cyclin D1 in cisplatin-induced nephropathy. In-vitro experiments showed that knockdown of Sox9 attenuated the suppressive effect of 7-HC on KIM-1 and reversed the stimulatory effect of 7-HC on cyclin D1 expression in cisplatin-treated HK-2 cells, indicating that 7-HC may protect against AKI via a Sox9-dependent mechanism.
Conclusion: 7-HC inhibits cisplatin-induced AKI by suppressing RIPK1/RIPK3/MLKL-mediated necroptosis and promoting Sox9-mediated tubular epithelial cell proliferation. 7-HC may serve as a preventive and therapeutic agent for AKI.

## Introduction

Acute kidney injury (AKI) is characterized by a sudden decrease in renal function, which results in high morbidity and mortality and causes heavy socioeconomic burden (Mehta et al., 2016; Yang et al., 2019). Up to 20% of hospitalized patients suffer from AKI, accounting for approximately 1.7 million deaths per year (Guo et al., 2019). The most common pathophysiological feature of AKI is the death of tubular cells (Agarwal et al., 2016; Linkermann et al., 2014). The proliferation of resident cells, e.g. renal tubular epithelial cells, plays an important role in kidney repair (Agarwal et al., 2016; Berger and Moeller, 2014; Kumar, 2018). However, there is no cure for severe AKI apart from renal dialysis and renal replacement.

Necroptosis is a form of programmed, necrotic, inflammatory cell death (Agarwal et al., 2016; Linkermann et al., 2014). Recent studies indicated that activation of the RIPK1/RIPK3/MLKL pathway is key for

necroptosis induction in several animal AKI models by promoting the inflammatory response and cell death in the injured kidney (Linkermann, 2016; Xu et al., 2015). Renal tubular epithelial cells (TECs) play critical roles in intrinsic kidney repair after AKI (Humphreys et al., 2008). Sox9, a transcription factor found to be expressed in the tip of the ureteric bud, starting at an early stage (embryonic day [E]11) of renal development, has been shown to promote renal repair by accelerating TECs dedifferentiation and proliferation in the injured kidney (Agarwal et al., 2016; Kang et al., 2016; Kumar, 2018). Thus, the RIPK1/RIPK3/MLKL axis and Sox9 may be therapeutic targets for AKI.

7-Hydroxycoumarin (7-HC), also known as umbelliferon, is commonly found in Chinese herbs (e.g. Eucommiae Cortex, Prunellae Spica, Radix Angelicae Biseratae) and vegetables (e.g. carrot, coriander) (Ru et al., 2014; Vasconcelos et al., 2009). Recently, 7-HC has received an increased attention in the field of functional foods, nutritional supplements and medicines because of its extensive pharmacological and biological benefits. Previous studies have indicated that 7-HC has anti-inflammatory effects in myocardial injury, anti-oxidative activities in diabetic hepatic injury, and anti-tumor activities in colon cancer (Luo et al., 2018; Muthu et al., 2016; Vijayalakshmi and Sindhu, 2017; Yin et al., 2018; Yu et al., 2015).

In this study, we evaluated the potential protective effect of 7-HC in cisplatin-induced AKI mice and the underlying mechanism.

## Materials and methods

### Materials

Cisplatin was purchased from Sigma-Aldrich (Shanghai, China) and 7-HC (purity 99%) was purchased from YuanYe Biotechnology (Shanghai, China). Curcumin (purity >98%), IL-1β antibody and MCP-1 antibody used in this study was purchased from Abcam (Cambridge, MA, USA). Antibodies specific for RIPK1, RIPK3, KIM-1, TNF-α, Sox9 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-MLKL and anti-cleaved-caspase-3 and anti-cyclinD1 were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). Lipofectamine 3000 and Pierce™ LDH Cytotoxicity Assay Kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Protein Assay Kit and Cell Cycle and Apoptosis Analysis Kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Annexin V-FITC/PI double-staining apoptosis detection kit, creatinine assay kit (sarcosine oxidase), and urea assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Cisplatin-induced AKI mouse model

Male C57BL/6 mice (8 weeks of age) were purchased from the Laboratory Animal Center of Anhui province (Hefei, China). All animal experiments were approved by the Institutional Animal Experimentation Ethics Committee of Anhui Medical University. AKI was induced by a single intraperitoneal injection of 20 mg/kg cisplatin. Mice injected with saline served as a control. We set up two experiments to verify the preventive and treatment effects, respectively, of 7-HC. In the preventive experiment, mice received 7-HC at 30, 60, or 90 mg/kg intraperitoneally for three consecutive days. On the first day, 7-HC was given to mice 6 h prior to cisplatin administration. Curcumin (Abcam, 100 mg/kg) was used as a positive control. Curcumin, the major active component of the plant Curcuma longa, has been demonstrated to ameliorate cisplatin- induced acute kidney injury (Tan et al., 2019). In the treatment experiment, mice received cisplatin (20 mg/kg) on the first day, followed by the administration of 7-HC (90 mg/kg) for three consecutive days. All mice were euthanized on the fourth day. The kidney tissues and blood were collected for analysis.

### Renal function

Renal function was evaluated by measuring serum creatinine and blood urea nitrogen (BUN) using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

### Renal histology and immunohistochemistry

The kidney tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections (3–5 μm) were used for immunohistochemistry and periodic acid–Schiff (PAS) staining. Kidney tubulointerstitial injury was scored by determining the percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation as follows: 0 = normal; 1 = 1–10%; 2 = 10–25%; 3 = 26–50%; 4 = 51–75%; 5 = 75–95%; 6 = >96%. Ten consecutive low-power fields (20×) were scored per mouse. Immunohistochemical staining for KIM-1 was conducted as described previously (Meng et al., 2012), using an AxioVision 4 microscope (Carl Zeiss, Jena, Germany).

### Cell culture and treatments

HK-2 human kidney TECs (kindly provided by Prof. Hui Y Lan, Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, China) were cultured in HyClone™ DMEM/F12 supplemented with 5% FBS and 1% penicillin/streptomycin (15,140,122; Gibco) at 37 ℃ in a humidified atmosphere with 5% $CO_2$. For experiments, HK-2 cells were starved in culture medium with 0.5% FBS for 12 h, pre-treated with 7-HC at 0–80 μM for 6 h, and exposed to cisplatin (20 μM) in the medium for 24 h. Then, the cells were harvested and assayed for cell proliferation and apoptosis, cell necrosis, and inflammatory responses. Further, the cells were used for western blot analysis and real-time PCR. All experiments were conducted three or four times.

### Cell viability and cytotoxicity assays

HK-2 cells were treated with 7-HC (0–1280 μM) for 12 h, then with cisplatin (20 μM) for 24 h. An MTT assay for cell viability was conducted as previously described (Gao et al., 2016; Meng et al., 2018). Cytotoxicity was measured by a lactate dehydrogenase (LDH) cytotoxicity assay, which detects LDH released from the cytosol to the culture medium at the time of cell death, using a commercial kit per the manufacturer's instructions.

### Knockdown of Sox9 in HK-2 cells

HK2 cells were transfected with Sox9 Dicer-substrate siRNA (DsiRNA; Integrated DNA Technologies, Singapore) using Lipofectamine™ 3000 (Invitrogen). Negative control DsiRNA was used as a control. The experiment followed the manufacturer's instructions. Transfection efficiency was evaluated by western blotting.

### Apoptosis and cell-cycle analyses

Cell death and the cell cycle were evaluated by flow cytometry (Gao et al., 2016; Meng et al., 2018). The Annexin V-FITC/PI double staining apoptosis detection kit was employed to determine the cell death. The proliferation cell cycle was measured by the Cell Cycle and Apoptosis Analysis Kit. All experiments were performed according to the manufacturer's instruction.

### RNA extraction and RT-qPCR

Total RNA was extracted using the RNeasy Isolation Kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed in a total volume of

9 μl, including 2 μl cDNA solution, 4 μl Bio-Rad iQ SYBR Green supermix with Opticon 2 (Bio-Rad, Hercules, CA, USA), 2.4 μl nuclease-free water and 0.6 μl each primer. mRNA levels were determined by RT-qPCR as previously described (Meng et al., 2012), using the following thermal cycles: 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 58 °C for 20 s and 72 °C for 20 s. KIM-1, TNF-α, MCP-1, IL-1β, IL-6, and cyclin D1 were analyzed by RT-qPCR analysis. β-actin was used as an internal control. The primers used in this study are shown in **Supplementary Table 1**.

### Western blot analysis

Protein was extracted from kidney tissues or cells in ice-cold RIPA buffer (Beyotime, Jiangsu, China). Western blot analysis was carried out as described previously (Meng et al., 2012; Wang et al., 2019a). After blocking, membranes were incubated with primary antibodies against KIM-1, RIPK1, PIPK3, p-MLKL, cleaved caspase-3, cyclin D1, IL-1β, MCP-1, TNF-α and Sox9 at 4 °C overnight, and then incubated with an IRDye 800-conjugated secondary antibody (1:10,000, Rockland Immunochemicals, Gilbertsville, PA, USA) at room temperature for 2 h. An Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA) was used to detect immunocomplexes. Protein levels were quantified using the ImageJ software (NIH, Bethesda, MA, USA).

### Statistical analysis

Data are shown as the mean ± SEM and were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests using GraphPad Prism 5 software. A $p < 0.05$ was considered statistically significant.

## Results

### 7-HC attenuates the loss in renal function and renal tubular injury in cisplatin-induced AKI mice

Cisplatin significantly impaired renal function in mice, as demonstrated by the increase of serum creatinine and BUN (Fig. 1b and c). 7-HC suppressed the increase of Serum Creatinine and BUN in a dose-dependent manner, while curcumin (the positive control) also reduced the serum creatinine and BUN in the cisplatin treated mice. PAS staining revealed that 7-HC and curcumin significantly reduced tubular injury (Fig. 1d) and ameliorated tubular necrosis, tubular dilation, and cast formation (Fig. 1e) caused by cisplatin. mRNA and proteins levels of the kidney injury marker KIM-1 were significantly suppressed by treatment with 7-HC (Fig. 1f–h).

### 7-HC significantly attenuates inflammation and necroptosis in cisplatin-induced AKI mice

As shown in Fig. 2a–d, the inflammatory response (TNF-α and MCP-1) were increased in cisplatin-treated mice compared with saline-treated control mice, which was associated with the activation of NF-κB pathway, a canonical inflammation pathway. 7-HC significantly attenuated the cisplatin-induced inflammatory response dose-dependently (Fig. 2a–b) and inhibited the phosphorylation of the NF-κB p65 subunit in cisplatin-treated kidney (Fig. 2c and d).

Simultaneously, we found the cleaved caspase-3, a crucial mediator of apoptosis, was significantly activated in cisplatin-treated kidney while was attenuated by 7-HC (Fig. 2e and f). Next, we examined if the key signalling modulators in necroptosis, RIPK1, RIPK3 and p-MLKL, was involved. We found that cisplatin increased RIPK1, RIPK3, and p-MLKL protein levels, whereas 7-HC significantly suppressed the activation of the RIPK1/RIPK3/p-MLKL axis (Fig. 2g–j).

### 7-HC promotes cell proliferation in cisplatin-induced AKI mice

Interestingly, cyclin D1, a cell proliferation marker, was significantly increased in cisplatin-injured kidney, and even more upon treatment with 7-HC as demonstrated by western blot and RT-qPCR analyses (Fig. 3a–c). The result might indicate that the tissue repair is induced during cisplatin induces kidney injury and 7-HC promotes the tissue repair.

### 7-HC alleviates cisplatin-induced AKI in mice

To evaluate the therapeutic effect of 7-HC on cisplatin-induced AKI, cisplatin was administrated to mice 24 h before 7-HC treatment. 7-HC improved impaired kidney function (Serum Creatinine and BUN) in cisplatin-induced AKI mice (Fig. 4a and b). PAS staining revealed that 7-HC largely attenuated tubular injury (Fig. 4d) and alleviated tubular necrosis, tubular dilation, and cast formation (Fig. 4c) in cisplatin-induced AKI. Consistent herewith, 7-HC reduced the percentage of KIM-1 positive cells in injured kidney as shown in Fig. 4e and f.

### 7-HC reduces cell death in cisplatin-induced HK-2 cells

7-HC increased cell viability in a wide range of 10–320 μM, as shown by the MTT assay (Fig. 5a). 7-HC had no cytotoxic effect to cells in this concentration range (5–320 μM) as demonstrated by the LDH assay (Fig. 5b). In cisplatin-treated HK-2 cells, 7-HC dose-dependently restored cell viability at 2.5–160 μM (Fig. 5c), whereas it reduced cisplatin-induced cell death to the baseline at 80 μM (Fig. 5d). Based on these findings, 7-HC was used at 80 μM in following experiments.

### 7-HC suppresses cisplatin-induced cellular damage and inflammatory response

Consistent with our findings *in vivo*, 7-HC attenuated cellular damage as demonstrated by the dose-dependent reductions in KIM-1 mRNA and protein in cisplatin-injured HK-2 cells (Fig. 6a–c). Further, 7-HC suppressed the cisplatin-induced inflammatory response (MCP-1, TNF-α, and IL-1β) in cisplatin treated cells (Fig. 6d–j). 7-HC suppressed NF-κB p65 phosphorylation in cisplatin-treated HK-2 cells (Fig. 6g and k). In addition, 7-HC had no effects on KIM-1 and p-p65 in HK-2 cells compared to vehicle treated HK-2 cells as shown in the supplementary Fig. S1.

### 7-HC inhibits necroptosis in cisplatin-injured HK-2 cells

To further explore whether 7-HC protects against cisplatin-induced programmed cell death, cells were stained with PI/Annexin V and analyzed by flow cytometry. 7-HC (80 μM) inhibited cisplatin-induced necroptosis and apoptosis (Fig. 7a). Mechanistically, 7-HC reduced the RIPK1/RIPK3 levels and MLKL phosphorylation in cisplatin-treated HK-2 cells (Fig. 7b–e). Furthermore, cleaved caspase-3 was markedly decreased by 7-HC in cisplatin treated cells (Fig. 7f and g), indicating that 7-HC indeed inhibited cisplatin-induced necroptosis and apoptosis in HK-2 cells. Additionally, as shown in the supplementary Fig. 1, the addition of 7-HC did not induce necroptosis and apoptosis (RIPK1/RIPK3/p-MLKL/cle-caspase-3) in HK-2 cells.

### 7-HC promotes cell proliferation in cisplatin-induced HK-2 cells

Flow-cytometric analysis revealed that 7-HC significantly increased the cell population in the S and G2/M phases of the cell cycle as compared to control and cisplatin treatments (Fig. 8a). Western blot and RT-qPCR results indicated that cyclin D1 was significantly increased in response to 7-HC in HK-2 cells, regardless of cisplatin treatment (Fig. 8b–d).
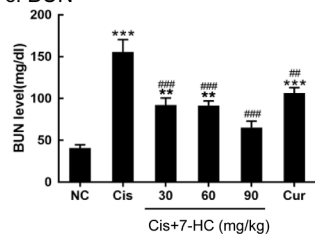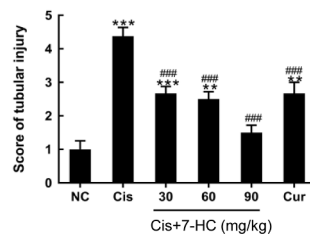
a. Chemical structure of 7-Hydroxycoumarin
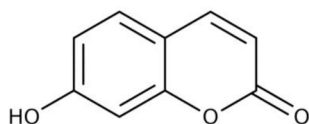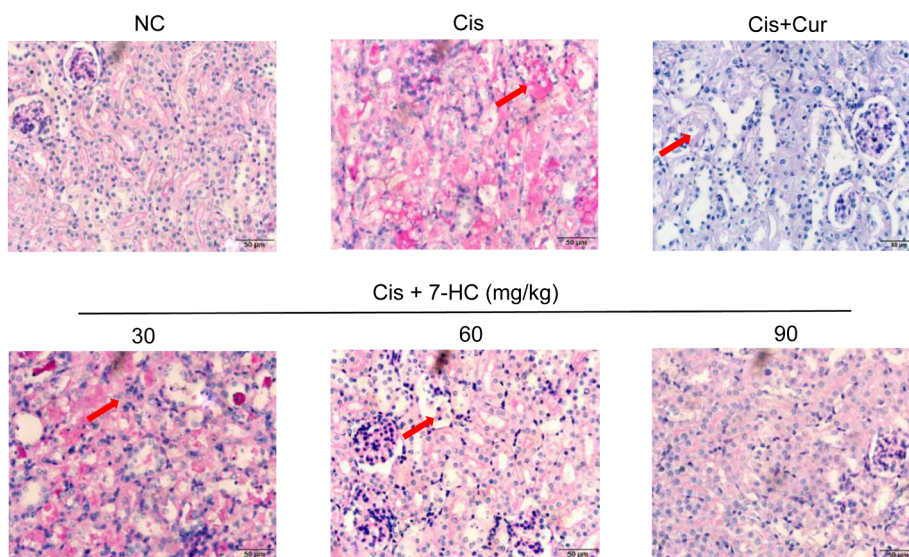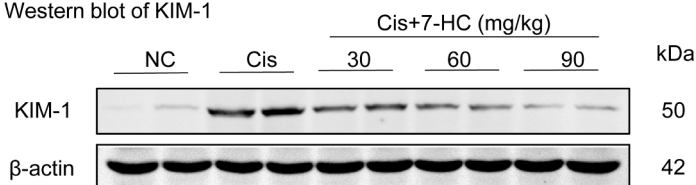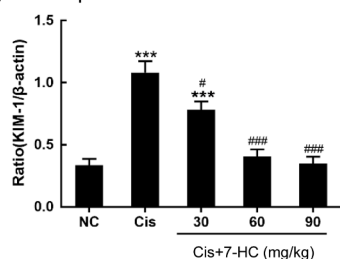


b. Serum Creatinine
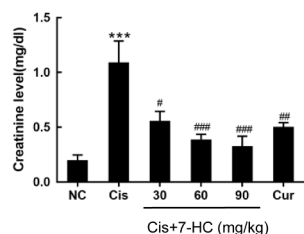


c. BUN



d. Tubular injury score



e. PAS Staining



f. Western blot of KIM-1
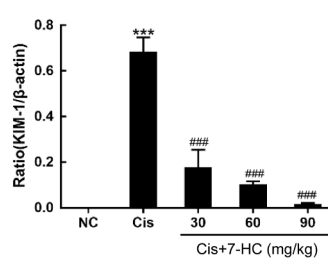


g. KIM-1 protein



h. KIM-1 mRNA



**Fig. 1.** 7-HC attenuates the loss in renal function and renal tubular injury in cisplatin-induced AKI mice. (a) Chemical structure of 7-Hydroxycoumarin. (b) Serum Creatinine levels. (c) BUN levels. (d) Tubular injury scores. (e) PAS staining of kidneys. (f, g) KIM-1 protein levels by Western blot analysis. (h) KIM-1 mRNA levels as determined by RT-qPCR. Data are the mean $\pm$ SEM from 6 to 8 mice. $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with AKI mouse model.
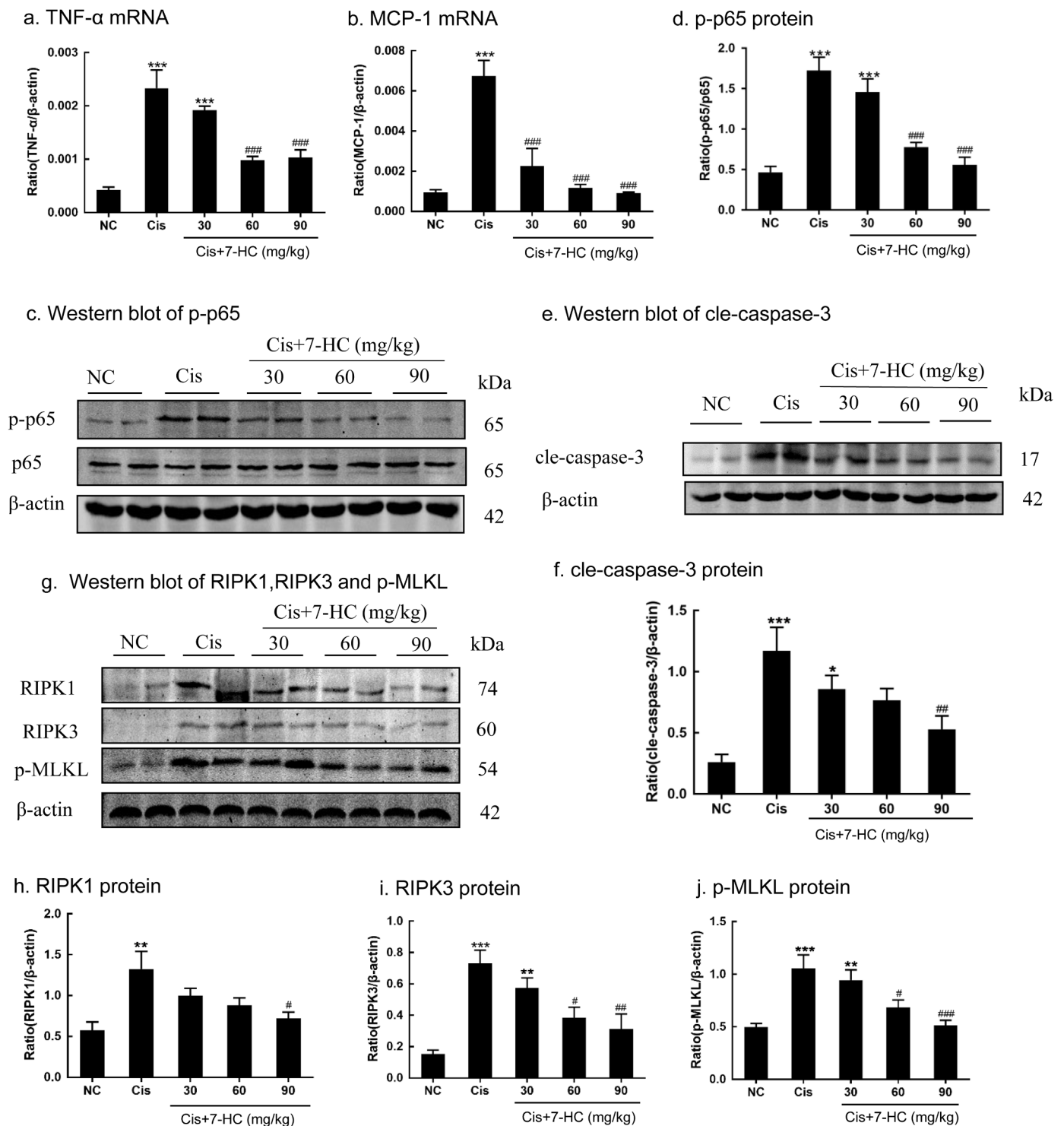
Fig. 2. 7-HC significantly attenuates necroptosis in cisplatin-induced AKI mice. (a, b) TNF-α and MCP-1 mRNA as determined by RT-qPCR. (c, d) Western blot and quantitative data for p-p65 protein. (e, f) Western blot and quantitative data of cleaved caspase-3. (g–j) Western blot and quantitative data for RIPK1, RIPK3, and p-MLKL. Data represent the mean ± SEM from 6 to 8 mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with AKI mouse model.

*7-HC ameliorates cisplatin-induced cell injury via a Sox9-dependent mechanism*

Sox9 is essential for cell proliferation. Thus, we examined whether 7-HC ameliorated cisplatin-induced cell injury by upregulating Sox9. Indeed, 7-HC dose-dependently increased Sox9 protein levels in HK-2 cells, regardless of cisplatin treatment (Fig. 9a and b). Consistent

herewith, administration of 30, 60, or 90 mg/kg of 7-HC to AKI model mice strongly increased Sox9 protein expression in the cisplatin-injured kidney (Fig. 9c and d).

Next, we knocked down Sox9 by transfecting HK-2 cells with Sox9 DsiRNA (Fig. 9e). 7-HC significantly reduced KIM-1 protein level but largely increased cyclin D1 protein levels in cisplatin-treated HK-2 cells compared with empty vector control (Fig. 9f–h). 7-HC-mediated
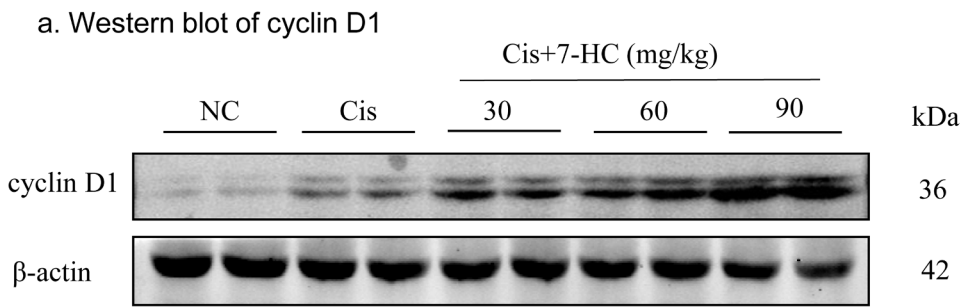
## a. Western blot of cyclin D1

### b. cyclin D1 protein

### c. cyclin D1 mRNA

### a. Serum Creatinine

### b. BUN

### d. Tubular injury score

### e. KIM-1 protein

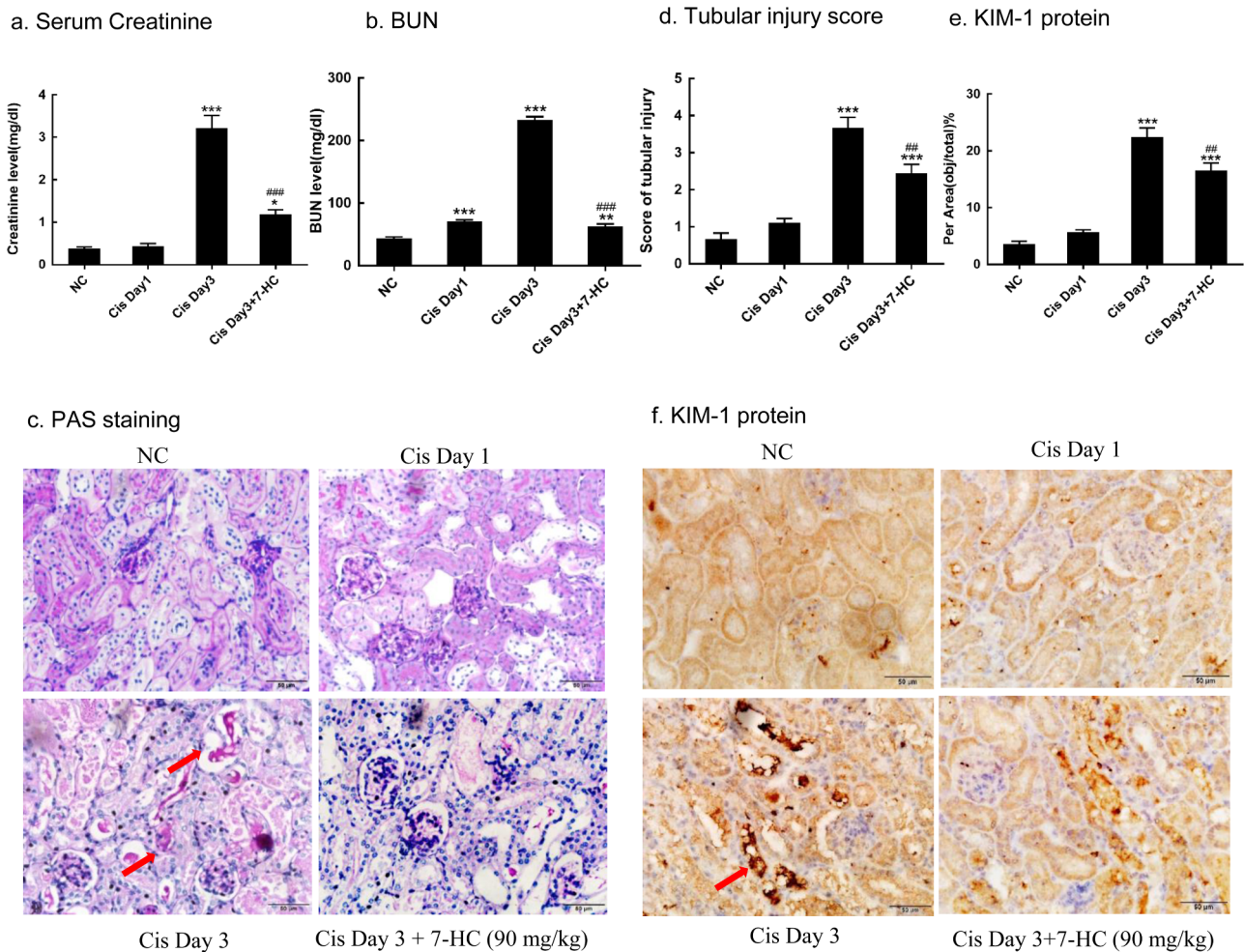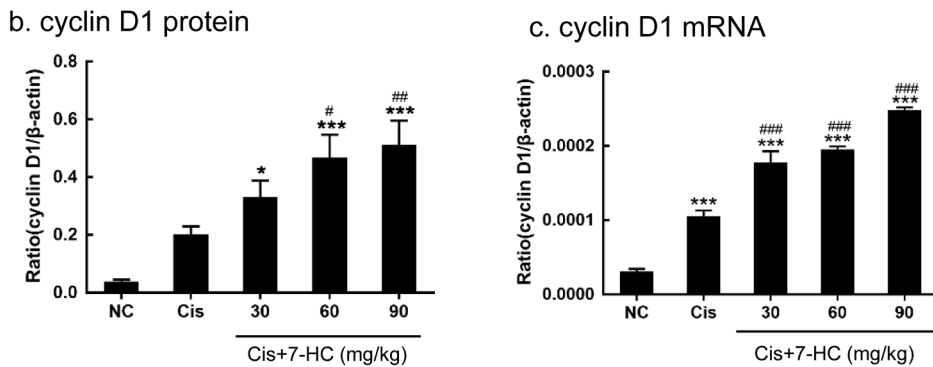### c. PAS staining

### f. KIM-1 protein

**Fig. 4.** 7-HC alleviates cisplatin-induced AKI in mice. (a) Serum Creatinine levels. (b) BUN levels. (c) PAS staining of kidneys. (d) Tubular injury scores. (e, f) Immunohistochemistry and quantitative data for KIM-1. Data represent the mean ± SEM from 6 to 8 mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with AKI mouse model.

## a. Cell viability with 7-HC



## b. Cytotoxicity with 7-HC



## c. Cell viability with 7-HC in cisplatin injured HK-2 cells



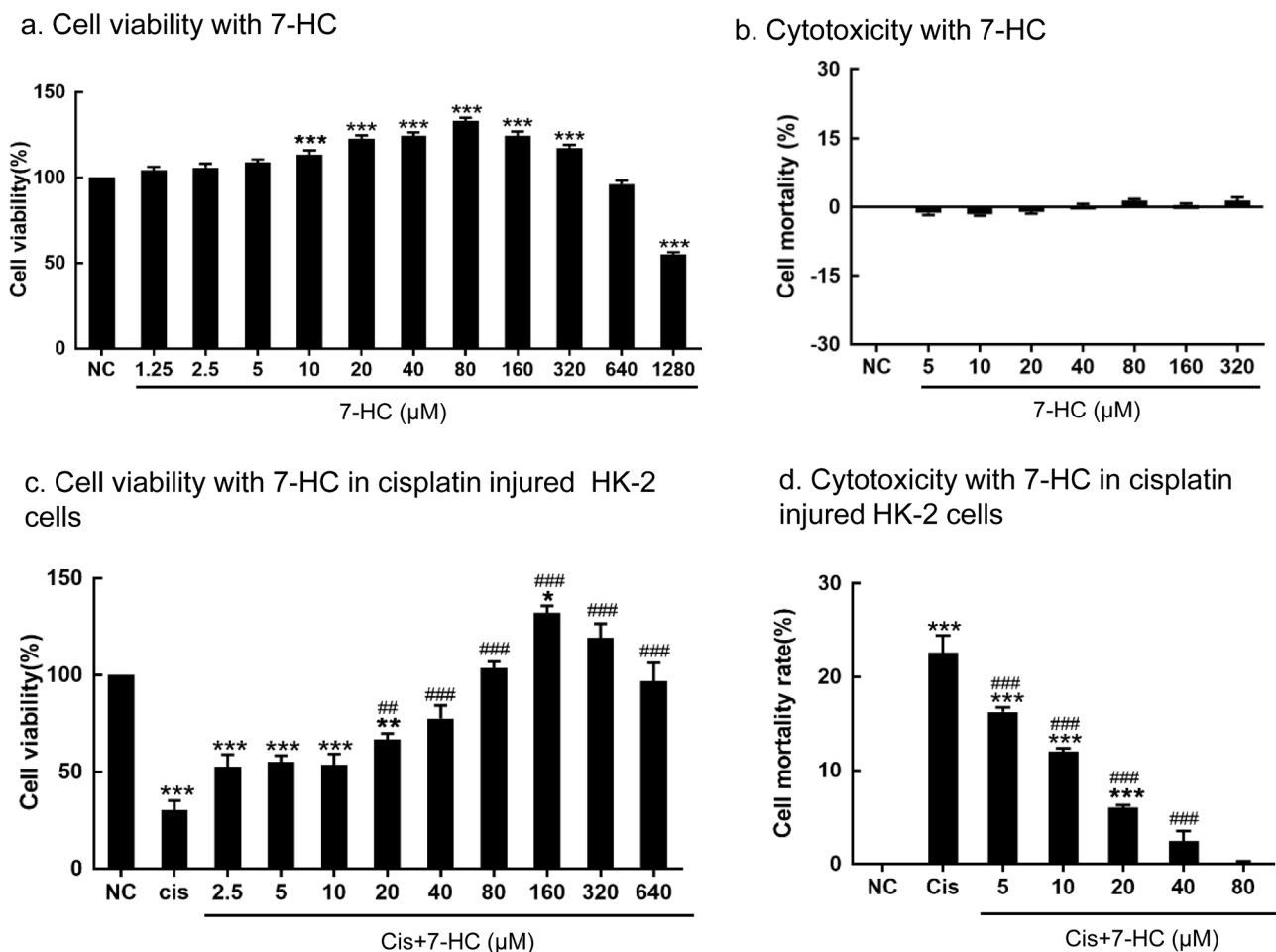## d. Cytotoxicity with 7-HC in cisplatin injured HK-2 cells



**Fig. 5.** 7-HC reduced cell death in cisplatin-induced HK-2 cells. (a) Effect of 7-HC at the indicated concentrations on the viability of HK-2 cells as determined by an MTT assay. (b) No cytotoxicity of 7-HC at the indicated concentrations in HK-2 cells as determined by LDH assay. (c) 7-HC restored cell viability at the indicated concentrations in cisplatin-injured HK-2 cells as determined by an MTT assay. (d) 7-HC at the indicated concentrations reduced deaths of cisplatin-injured HK-2 cells as determined by LDH assay. Data represent the mean ± SEM from 3 to 4 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with normal control. ##$p < 0.01$, ###$p < 0.001$ compared with cisplatin-treated cells.

suppression of KIM-1 and induction of cyclin D1 in the injured cells was attenuated when Sox9 was disrupted, suggesting that 7-HC ameliorated cisplatin-induced cell injury at least partially via a Sox9-dependent mechanism (Fig. 9f–h).

### Discussion

The present study demonstrated that 7-HC, a natural derivative of coumarin found in plants and Chinese herbs, has preventive and therapeutic effects on cisplatin-induced AKI by suppressing RIPK1/RIPK3/MLKL-mediated necroptosis and promoting Sox9-mediated tissue repair (via TECs proliferation) *in vivo* and *in vitro*.

AKI is a sudden kidney damage caused by sepsis, ischemia, or various exogenous nephrotoxins, resulting in loss of kidney function (Doi and Rabb, 2016). Cisplatin, a first-line chemotherapy reagent, shows kidney toxicity. The pathophysiological features of cisplatin-induced AKI include proximal tubular injury, oxidative stress, inflammation, and vascular injury in the kidneys (Gao et al., 2016; Yang et al., 2018), which is identical to the clinical manifestation of AKI in patients (Agarwal et al., 2016). Mechanistically, necroptosis and apoptosis are common pathogenic mechanisms in cisplatin-induced AKI (Linkermann et al., 2014). TECs proliferation plays a key role in kidney repair and kidney function recovery during AKI (Berger and Moeller, 2014). To date, specific and effective clinical therapies for cisplatin-induced AKI are limited.

Emerging evidence shows that 7-HC potentially suppresses inflammation and oxidative stress, while attenuating organ injuries induced by various stimuli. 7-HC has an anti-inflammatory effect in lipopolysaccharide-induced J774 macrophages (Timonen et al., 2011). Recent studies showed that 7-HC exerts antioxidant and anti-inflammatory activities in animal models of different disorder, including hyperammonemia (Germoush et al., 2018), ischemia reperfusion-induced myocardial injury (Luo et al., 2018), CCl4-induced liver fibrosis (Mahmoud et al., 2019), and diabetic liver injury (Yin et al., 2018).

In this study, we tested the hypothesis that 7-HC may attenuate cisplatin-induced AKI by limiting programmed cell death and accelerating renal repair. To the best of our knowledge, this is the first study to demonstrate that 7-HC has anti-necroptotic and pro-proliferative effects in cisplatin-induced nephropathy. We showed that 7-HC facilitates renal structural and functional repair, suppresses the inflammatory response, and promotes cell regeneration and proliferation in response to cisplatin.

Necroptosis is a recently identified form of programmed cell death associated with a strong inflammatory response. In cisplatin-induced AKI models, both necroptosis and apoptosis are detectable in renal proximal tubules. Deletion of RIPK3 and MLKL in mice blocked necroptosis induced by cisplatin in the kidneys, suggesting that necroptosis is RIPK3- and MLKL-dependent in AKI mice (Xu et al., 2015). We recently found that hsa-miR-500a-3P protects against renal injury by suppressing MLKL-mediated necroptosis in renal epithelial cells
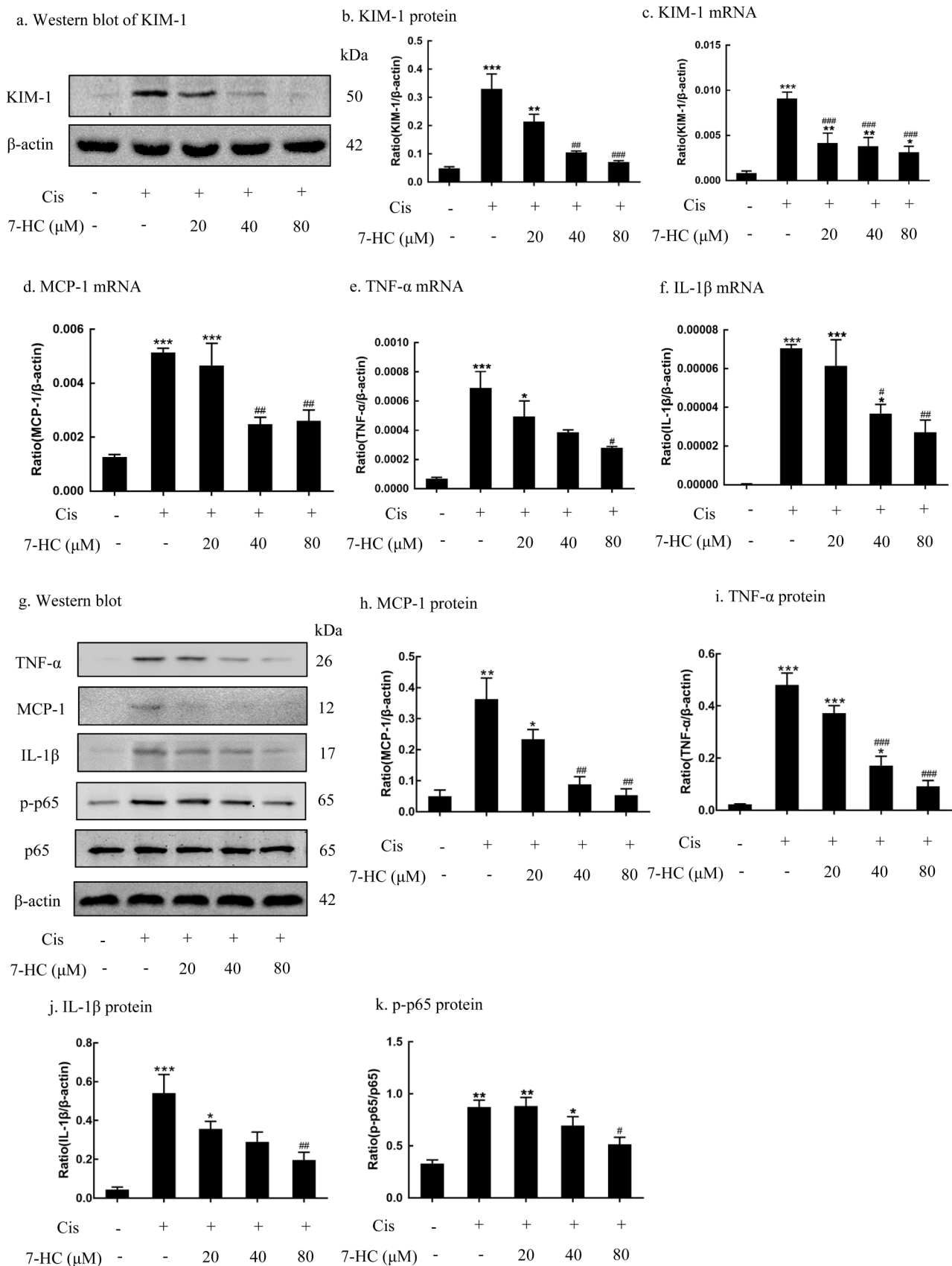
**Fig. 6.** 7-HC suppresses cisplatin-induced cellular damage and inflammatory response. (a, b) Western blot and quantitative data for KIM-1. (c-f) RT-qPCR analysis of KIM-1 and inflammation-related cytokines (MCP-1, TNF-α and IL-1β). Western blot analysis of inflammation-related cytokines MCP-1 (g, h), TNF-α (g, i) and IL-1β (g, j) and phosphorylated p65 (g, k). Data represent the mean ± SEM from 3 to 4 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$ compared with cisplatin-treated cells.

a. Flow cytometry



b. Western blot of RIPK1,RIPK3 and p-MLKL

c. RIPK1 protein



d. RIPK3 protein

e. p-MLKL protein



f. Western blot of cle-caspase-3
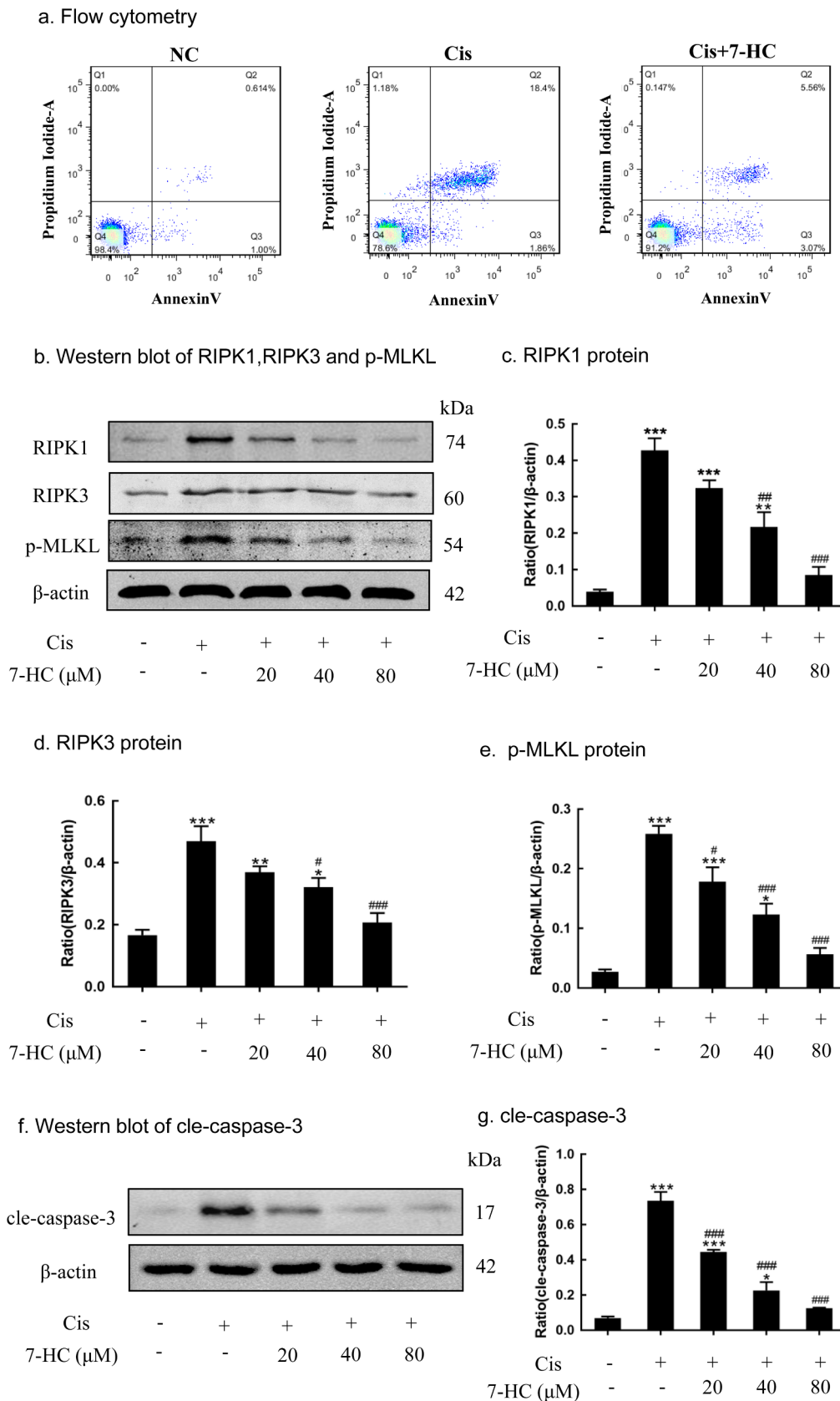
g. cle-caspase-3



**Fig. 7.** 7-HC inhibits necroptosis in cisplatin-induced HK-2 cells. (a) Flow-cytometric analysis of propidium (PI)/Annexin V-stained HK-2 cells. (b–e) Western blot and quantitative data for RIPK1, RIPK3, and phospho-MLKL in cisplatin-treated HK-2 cells. (f, g) Western blot and quantitative data for cleaved caspase-3 in HK-2 cells. Data represent the mean ± SEM from 3 to 4 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with cisplatin-treated cells.

## a. Cell cycle arrest

### NC

### Cis

### Cis+7-HC
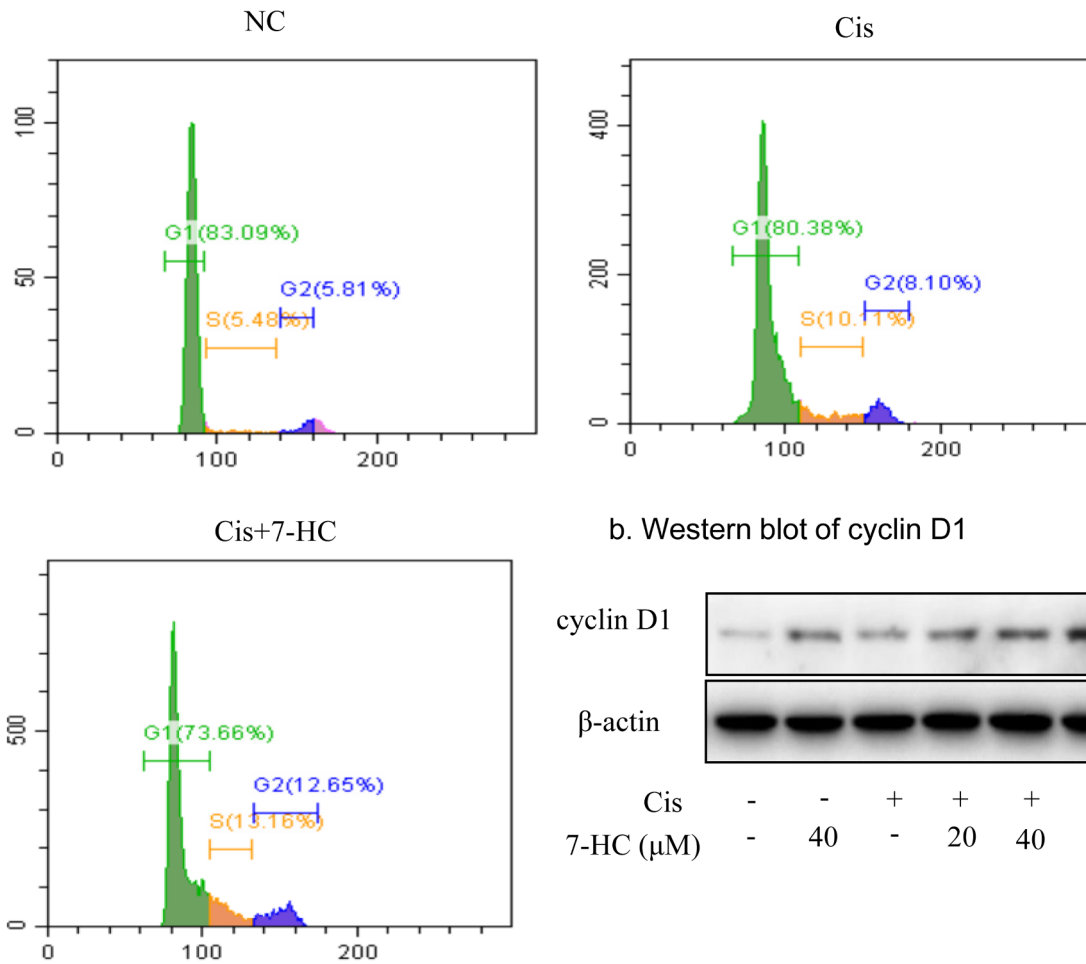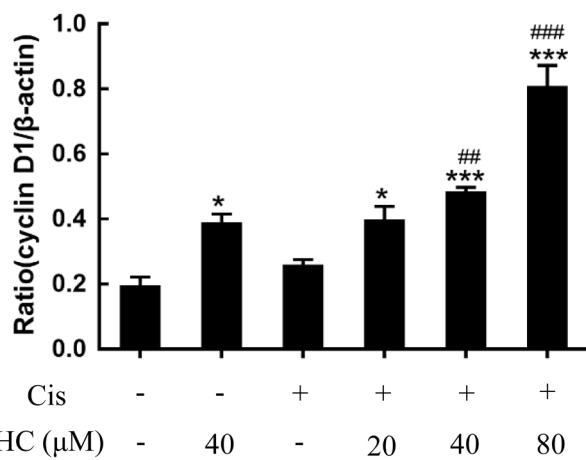
### b. Western blot of cyclin D1
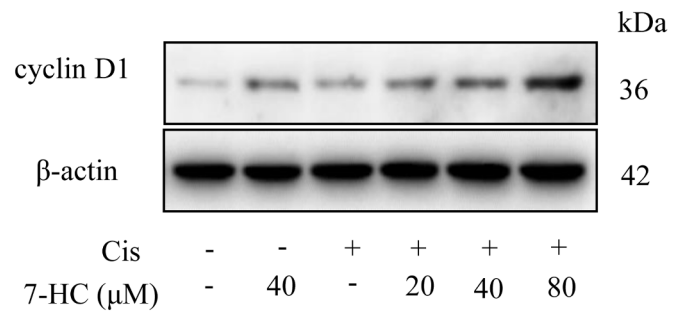


## c. cyclin D1 protein
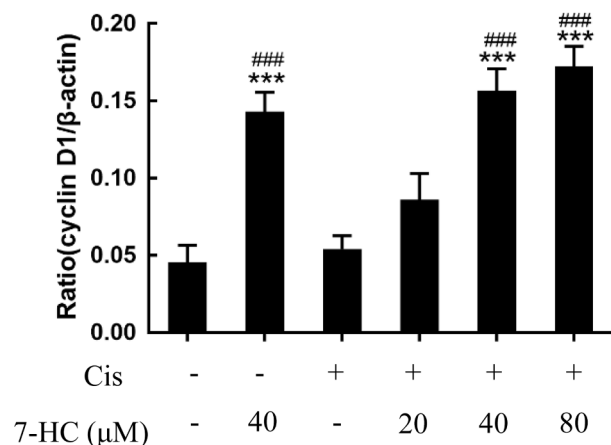
## d. cyclin D1 mRNA

**Fig. 8.** 7-HC promotes cell proliferation in cisplatin-induced HK-2 cells. (a) Flow-cytometric analysis of cell-cycle distribution in HK-2 cells. (b, c) Western blot analysis and quantitative data for cyclin D1 in cisplatin-treated HK-2 cells. (d) RT-qPCR analysis of cyclin D1 in cisplatin-treated HK-2 cells. Data represent the mean ± SEM from 3 to 4 independent experiments. *$p < 0.05$, ***$p < 0.001$ compared with normal control. ##$p < 0.01$, ###$p < 0.001$ compared with cisplatin-treated cells.

(Jiang et al., 2019). In addition, previous studies have demonstrated that the inflammatory response in AKI is associated with phosphorylation of NF-κB, a crucial inflammatory molecule, in the kidneys

(Linkermann, 2016; Xu et al., 2015). Given that programmed cell death plays a key role in AKI (Gao et al., 2018), we measured the suppressive effect of 7-HC on cisplatin-induced necroptosis and apoptosis. Flow-
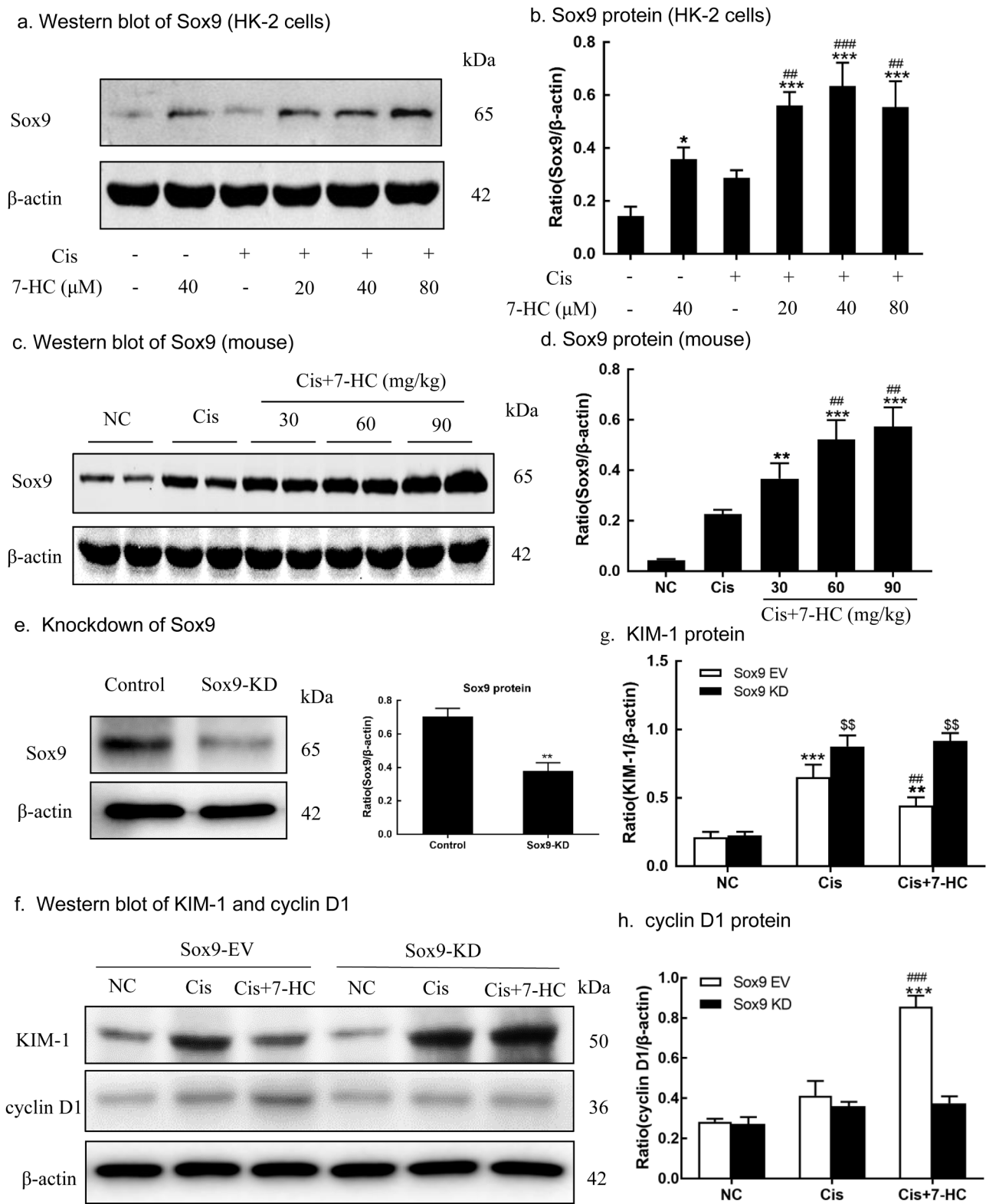
**Fig. 9.** 7-HC ameliorates cisplatin-induced cell injury via Sox9-dependent mechanism. (a, b) Western blot and quantitative data for Sox9 in cisplatin-treated HK-2 cells. (c, d) Western blot and quantitative data for Sox9 in cisplatin-induced AKI mice. (e) Sox9 protein levels in Sox9 knockdown HK-2 cells. (f-h) Western blot and quantitative data for KIM-1 and cyclin D1 in Sox9-silenced HK-2 cells. Data represent the mean ± SEM from 3 to 4 independent *in-vitro* experiments or from 6 to 8 mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control. $^{##}p < 0.01$, $^{###}p < 0.001$ compared with cisplatin-treated cells or model. $^{\$\$}p < 0.01$ compared with Sox9 knockdown control.

cytometric analysis demonstrated that 7-HC protects against cisplatin-induced necroptosis and apoptosis. Furthermore, the levels of RIPK1, RIPK3, p-MLKL, and cleaved caspase-3 were strongly reduced by 7-HC

treatment in cisplatin-treated mice and HK-2 cells, which was in line with the attenuation of NF-κB p65-mediated renal inflammation. To-gether, these findings suggest that 7-HC inhibits cisplatin-induced AKI

by suppressing programmed cell death and necroinflammation. However, it is unclear whether 7-HC directly targets key regulators of necroptosis-mediated cell death or indirectly regulates the RIPK1/RIPK3/MLKL pathway. Thus, the underlying mechanism remains to be elucidated.

Renal cell regeneration is critical for kidney repair after tissue injury (Humphreys et al., 2008). Noteworthy cell proliferation, particularly in injured renal tubules, can be detected after AKI (Agarwal et al., 2016). Among these proliferative cells, renal TECs play a dominant role in kidney regenerative repair, although a large number of epithelial cells die from AKI (Fu et al., 2017). Cyclin D1 promotes cell proliferation after AKI (Bao et al., 2014). We found that 7-HC upregulates cyclin D1 at both the protein and the mRNA level. Further, deletion of Sox9, a key protein controlling cell proliferation in AKI, inhibits tubular regeneration by arresting cells in G2/M phase of the cell cycle in AKI (Kang et al., 2016; Kumar, 2018). Notably, in our *in-vitro* and *in-vivo* experiments, 7-HC substantially enhanced Sox9 protein levels and switched the cell cycle from the G0/G1 to S and G2/M phases, which significantly promoted cell proliferation in the injured kidney. In HK-2 cells, 7-HC failed to reduce cisplatin-induced cell injury and promote cell proliferation when Sox9 was disrupted, indicating that 7-HC reduces cisplatin-induced AKI at least partially via a Sox9-dependent mechanism.

Interestingly, 7-HC shows anti-tumour activities. Weber et al. first reported the antineoplastic action of 7-HC on a human tumour cell line (Weber et al., 1998). Recent evidence suggests that 7-HC exhibits anti-cancer effects, including anti-proliferative and pro-apoptotic effects, on HepG2 hepatocellular carcinoma cells (Yu et al., 2015), colorectal cancer cells (Muthu et al., 2016), human oral carcinoma cells (Vijayalakshmi and Sindhu, 2017), and human renal cell carcinoma (Wang et al., 2019b). These findings suggest 7-HC may exert distinct effects in different cell types and disease conditions.

In conclusion, our study demonstrated that 7-HC substantially prevents and ameliorates cisplatin-induced AKI via multiple mechanisms, including suppression of RIPK1/RIPK3/MLKL-mediated necroptosis and promotion of Sox9-mediated tubular cell proliferation. Our findings highlight the preventive and therapeutic potential of 7-HC for treating AKI.

## Author contributions

XMM and HYC designed study and revised manuscript; WFW, JNW ZL and BW carried out experiments and wrote the manuscript; JJ and JL advised experimental design and data interpretation; LG and HDL analyzed the data and interpreted the results. All authors read and approved the manuscript.

## Declaration of Competing Interest

The authors declare no competing financial interest.

## Acknowledgments

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2020.153202.

## References

Agarwal, A., Dong, Z., Harris, R., Murray, P., Parikh, S.M., Rosner, M.H., ... Ronco, C., 2016. Cellular and molecular mechanisms of AKI. J Am Soc Nephrol 27 (5), 1288–1299. https://doi.org/10.1681/asn.2015070740.

Bao, H., Ge, Y., Wang, Z., Zhuang, S., Dworkin, L., Peng, A., Gong, R., 2014. Delayed administration of a single dose of lithium promotes recovery from AKI. J. Am. Soc. Nephrol. 25 (3), 488–500. https://doi.org/10.1681/asn.2013040350.

Berger, K., Moeller, M.J., 2014. Mechanisms of epithelial repair and regeneration after acute kidney injury. Semin. Nephrol. 34 (4), 394–403. https://doi.org/10.1016/j.semnephrol.2014.06.006.

Doi, K., Rabb, H., 2016. Impact of acute kidney injury on distant organ function: recent findings and potential therapeutic targets. Kidney Int. 89 (3), 555–564. https://doi.org/10.1016/j.kint.2015.11.019.

Fu, S., Tang, Y., Huang, X.R., Feng, M., Xu, A.P., Lan, H.Y., 2017. Smad7 protects against acute kidney injury by rescuing tubular epithelial cells from the G1 cell cycle arrest. Clin. Sci. 131 (15), 1955–1969. https://doi.org/10.1042/cs20170127.

Gao, L., Liu, M.M., Zang, H.M., Ma, Q.Y., Yang, Q., Jiang, L., ... Meng, X.M., 2018. Restoration of E-cadherin by PPBICA protects against cisplatin-induced acute kidney injury by attenuating inflammation and programmed cell death. Lab Invest 98 (7), 911–923. https://doi.org/10.1038/s41374-018-0052-5.

Gao, L., Wu, W.F., Dong, L., Ren, G.L., Li, H.D., Yang, Q., ... Meng, X.M., 2016. Protocatechuic aldehyde attenuates cisplatin-induced acute kidney injury by suppressing nox-mediated oxidative stress and renal inflammation. Front Pharmacol 7, 479. https://doi.org/10.3389/fphar.2016.00479.

Germoush, M.O., Othman, S.I., Al-Qaraawi, M.A., Al-Harbi, H.M., Hussein, O.E., Al-Basher, G., ... Mahmoud, A.M., 2018. Umbelliferone prevents oxidative stress, inflammation and hematological alterations, and modulates glutamate-nitric oxide-cGMP signaling in hyperammonemic rats. Biomed Pharmacother 102, 392–402. https://doi.org/10.1016/j.biopha.2018.03.104.

Guo, C., Dong, G., Liang, X., Dong, Z., 2019. Epigenetic regulation in AKI and kidney repair: mechanisms and therapeutic implications. Nat. Rev. Nephrol. https://doi.org/10.1038/s41581-018-0103-6.

Humphreys, B.D., Valerius, M.T., Kobayashi, A., Mugford, J.W., Soeung, S., Duffield, J.S., ... Bonventre, J.V., 2008. Intrinsic epithelial cells repair the kidney after injury. Cell Stem Cell 2 (3), 284–291. https://doi.org/10.1016/j.stem.2008.01.014.

Jiang, L., Liu, X.Q., Ma, Q., Yang, Q., Gao, L., Li, H.D., ... Meng, X.M., 2019. hsa-miR-500a-3P alleviates kidney injury by targeting MLKL-mediated necroptosis in renal epithelial cells. FASEB J 33 (3), 3523–3535. https://doi.org/10.1096/fj.201801711R.

Kang, H.M., Huang, S., Reidy, K., Han, S.H., Chinga, F., Susztak, K., 2016. Sox9-Positive progenitor cells play a key role in renal tubule epithelial regeneration in mice. Cell Rep. 14 (4), 861–871. https://doi.org/10.1016/j.celrep.2015.12.071.

Kumar, S., 2018. Cellular and molecular pathways of renal repair after acute kidney injury. Kidney Int. 93 (1), 27–40. https://doi.org/10.1016/j.kint.2017.07.030.

Linkermann, A., 2016. Nonapoptotic cell death in acute kidney injury and transplantation. Kidney Int. 89 (1), 46–57. https://doi.org/10.1016/j.kint.2015.10.008.

Linkermann, A., Chen, G., Dong, G., Kunzendorf, U., Krautwald, S., Dong, Z., 2014. Regulated cell death in AKI. J. Am. Soc. Nephrol. 25 (12), 2689–2701. https://doi.org/10.1681/asn.2014030262.

Luo, H., Fan, Z., Xiang, D., Jiang, Z., Zhang, W., Gao, L., Feng, C., 2018. The protective effect of umbelliferone ameliorates myocardial injury following ischemiareperfusion in the rat through suppression NLRP3 inflammasome and upregulating the PPAR-gamma. Mol. Med. Rep. 17 (2), 3404–3410. https://doi.org/10.3892/mmr.2017.8208.

Mahmoud, A.M., Hozayen, W.G., Hasan, I.H., Shaban, E., Bin-Jumah, M., 2019. Umbelliferone ameliorates CCl4-Induced liver fibrosis in rats by upregulating PPARgamma and attenuating oxidative stress, inflammation, and TGF-beta1/Smad3 signaling. Inflammation. https://doi.org/10.1007/s10753-019-00973-8.

Mehta, R.L., Burdmann, E.A., Cerda, J., Feehally, J., Finkelstein, F., Garcia-Garcia, G., ... Remuzzi, G., 2016. Recognition and management of acute kidney injury in the international society of nephrology 0by25 global snapshot: a multinational cross-sectional study. Lancet 387 (10032), 2017–2025. https://doi.org/10.1016/s0140-6736(16)30240-9.

Meng, X.M., Huang, X.R., Xiao, J., Chen, H.Y., Zhong, X., Chung, A.C., Lan, H.Y., 2012. Diverse roles of TGF-beta receptor II in renal fibrosis and inflammation *in vivo* and *in vitro*. J. Pathol. 227 (2), 175–188. https://doi.org/10.1002/path.3976.

Meng, X.M., Li, H.D., Wu, W.F., Tang, Ming-Kuen, P., Ren, L., Gao, L., ... Li, J., 2018. Wogonin protects against cisplatin-induced acute kidney injury by targeting RIPK1-mediated necroptosis. Lab Invest 98 (1), 79–94. https://doi.org/10.1038/labinvest.2017.115.

Muthu, R., Selvaraj, N., Vaiyapuri, M., 2016. Anti-inflammatory and proapoptotic effects of umbelliferone in colon carcinogenesis. Hum. Exp. Toxicol. 35 (10), 1041–1054. https://doi.org/10.1177/0960327115618245.

Ru, J., Li, P., Wang, J., Zhou, W., Li, B., Huang, C., ... Yang, L., 2014. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. J Cheminform 6 (13). https://doi.org/10.1186/1758-2946-6-13.

Tan, R.Z., Liu, J., Zhang, Y.Y., Wang, H.L., Li, J.C., Liu, Y.H., ... Wang, L., 2019. Curcumin relieved cisplatin-induced kidney inflammation through inhibiting Mincle-maintained M1 macrophage phenotype. Phytomedicine 52, 284–294. https://doi.org/10.1016/j.phymed.2018.09.210.

Timonen, J.M., Nieminen, R.M., Sareila, O., Goulas, A., Moilanen, L.J., Haukka, M., ... Aulaskari, P.H., 2011. Synthesis and anti-inflammatory effects of a series of novel 7-

hydroxycoumarin derivatives. Eur J Med Chem 46 (9), 3845–3850. https://doi.org/10.1016/j.ejmech.2011.05.052.

Vasconcelos, J.F., Teixeira, M.M., Barbosa-Filho, J.M., Agra, M.F., Nunes, X.P., Giulietti, A.M., ... Soares, M.B., 2009. Effects of umbelliferone in a murine model of allergic airway inflammation. Eur J Pharmacol 609 (1-3), 126–131. https://doi.org/10.1016/j.ejphar.2009.03.027.

Vijayalakshmi, A., Sindhu, G., 2017. Umbelliferone arrest cell cycle at G0/G1 phase and induces apoptosis in human oral carcinoma (KB) cells possibly via oxidative DNA damage. Biomed. Pharmacother. 92, 661–671. https://doi.org/10.1016/j.biopha.2017.05.128.

Wang, J.N., Liu, M.M., Wang, F., Wei, B., Yang, Q., Cai, Y.T., ... Meng, X.M., 2019a. RIPK1 inhibitor Cpd-71 attenuates renal dysfunction in cisplatin-treated mice via attenuating necroptosis, inflammation and oxidative stress. Clin Sci (Lond) 133 (14), 1609–1627. https://doi.org/10.1042/cs20190599.

Wang, X., Huang, S.S., Xin, X., Ren, Y., Weng, G.B., Wang, P., 2019b. The antitumor activity of umbelliferone in human renal cell carcinoma via regulation of the p110 gamma catalytic subunit of PI3K gamma. Acta Pharm. 69 (1), 111–119. https://doi.org/10.2478/acph-2019-0004.

Weber, U.S., Steffen, B., Siegers, C.P., 1998. Antitumor-activities of coumarin, 7-hydroxy-coumarin and its glucuronide in several human tumor cell lines. Res. Commun. Mol. Pathol. Pharmacol. 99 (2), 193–206.

Xu, Y., Ma, H., Shao, J., Wu, J., Zhou, L., Zhang, Z., ... Han, J., 2015. A role for tubular necroptosis in cisplatin-induced AKI. J Am Soc Nephrol 26 (11), 2647–2658. https://doi.org/10.1681/asn.2014080741.

Yang, Q., Ren, G.L., Wei, B., Jin, J., Huang, X.R., Shao, W., ... Lan, H.Y., 2019. Conditional knockout of TGF-betaRII /Smad2 signals protects against acute renal injury by alleviating cell necroptosis, apoptosis and inflammation. Theranostics 9 (26), 8277–8293. https://doi.org/10.7150/thno.35686.

Yang, Q., Wu, F.R., Wang, J.N., Gao, L., Jiang, L., Li, H.D., ... Meng, X.M., 2018. Nox4 in renal diseases: An update. Free Radic Biol Med 124, 466–472. https://doi.org/10.1016/j.freeradbiomed.2018.06.042.

Yin, J., Wang, H., Lu, G., 2018. Umbelliferone alleviates hepatic injury in diabetic db/db mice via inhibiting inflammatory response and activating nrf2-mediated antioxidant. Biosci. Rep. 38 (4). https://doi.org/10.1042/bsr20180444.

Yu, S.M., Hu, D.H., Zhang, J.J., 2015. Umbelliferone exhibits anticancer activity via the induction of apoptosis and cell cycle arrest in HepG2 hepatocellular carcinoma cells. Mol. Med. Rep. 12 (3), 3869–3873. https://doi.org/10.3892/mmr.2015.3797.