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Research article

A novel GSK3β inhibitor 5n attenuates acute kidney injury

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ABSTRACT

Acute kidney injury (AKI) is a clinical syndrome with high morbidity and mortality caused by various factor. The specific strategies for AKI are still lacking. GSK3β is widely expressed in the kidneys. In acute models of injury, GSK3β promotes the systemic inflammatory response, increases the proinflammatory release of cytokines, induces apoptosis, and alters cell proliferation. We screened a series of 3-(4-pyridyl)-5-(4-sulfamido-phenyl)-1,2,4-oxadiazole derivatives which are recognized as new GSK3β inhibitors, and found that 5n had the least toxicity and the best cell protection. We then tested the anti-inflammatory and reno-protective effect of 5n in cisplatin-treated tubular epithelial cells. 5n had anti-inflammation effect indicated by phosphor–NF–κB detection. Finally, we found that 5n ameliorated renal injury and inflammation in cisplatin-induced AKI mouse model. Silencing GSK3β inhibited cell injury and inflammation induced by cisplatin. We found that GSK3β interacted with PP2Ac to modulate the activity of NF-κB. In conclusion, 5n, the novel GSK3β inhibitor, protects against AKI via PP2Ac-dependent mechanisms which may provide a potential strategy for the treatment of AKI in clinic.

1. Introduction

Acute kidney injury (AKI) is characterized by decreased glomerular filtration rate (GFR), urine output(UOP) and increased serum creatinine, serum blood urea nitrogen (BUN) level [1,2]. This clinical syndrome with high morbidity and mortality is caused by various factors, with an incidence that varies from 5.0% to 7.5% in hospitalized patients and that reaches up to 50–60% in critically ill patients [3,4]. AKI can result in a broad spectrum of renal outcomes ranging from full recovery to ESRD [5]. Over the last decade, it has been observed that many cases of AKI lead to development of chronic kidney disease (CKD).

AKI is commonly caused by nephrotoxic agents, sepsis, ischemia-reperfusion (I/R) induced injury and obstructive nephropathy. The pathogenesis of AKI is complex, involving the following numerous overlapping processes: inflammation, oxidative stress, impaired hemodynamics and oxygen delivery, impaired mitochondrial function, impaired repair and fibrosis, tubular epithelial cell apoptosis and impaired cellular metabolism [6,7]. Cisplatin that is an effective chemotherapeutic agents [8] is commonly used to induced AKI in patients. Cisplatin is hindered by dose-limiting nephrotoxicity and causes acute kidney injury (AKI) in 30% of patients [9]. At present,

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there is no therapy to treat cisplatin-induced AKI, and hydration is thought to be the only pretreatment strategy. When the patients developed severe AKI, kidney replacement treatment is suggested [10]. Nowadays we lack effective treatment to reduce the direct damage of AKI, and actively explore safe and efficient treatment strategies.

Glycogen synthase kinase-3 (GSK3), with two highly conserved isoform GSK3α and GSK3β [11], is an active serine-threonine kinase which participates in cellular processes such as glycogen synthesis, cell cycle, and neurogenesis [12]. Moreover, GSK3 is considered to play a role in the pathogenesis of several diseases involving neurological/neurodegenerative disease, diabetes mellitus; inflammatory diseases, rheumatoid arthritis and different types of cancer [13,14], as well as acute kidney injury (AKI) and chronic kidney disease (CKD) [15,16]. In our previous research [17], we designed and synthesized a series of chemical entities that inhibiting GSK3β. One of the compounds was found to improve the cognitive impairment in the mouse model of Alzheimer. In the present study, we further investigate the function of GSK3β in AKI and explore the medium of GSK3β to regulate the inflammation, and use brand new GSK3β inhibitor to confirm the prospect of GSK3β as therapeutic target to cure AKI.

2. Material and method

2.1. Compounds

A series of 3-(4-pyridyl)-5-(4-sulfamido-phenyl)-1,2,4-oxadiazole derivatives as novel GSK-3β inhibitors were designed and synthesized in the laboratory and used in the experiment.

2.2. Cell culture

The human kidney tubular epithelial cell line HK2 was provided by Prof. Hui Yao Lan (The Chinese University of Hong Kong). cell were planted in 6-well plates and cultured in DMEM/F12 medium with 5% FBS at 37°and a 5% carbon dioxide incubator. Before processing, cell were starved in DMEM/F12 medium without FBS for 12 h, then HK2 cells were treated with cisplatin (20 μM) for 24 h in the same culture conditions. Cells were lysed and gathered for following experiment.

2.3. Animal model of cisplatin-induced acute kidney injury

Male C57BL/6 mice (at 6–8weeks of age with body weight of 20–22g) were provided by the Experimental Animal Center, Anhui Medical University. All animal procedures were approved by the Animal Experimentation Ethics Committee of the Anhui Medical University (LLSC20232206) and conducted by the Guide for the Care and Use of Laboratory Animals, eighth edition. Male C57BL/6 mice were intraperitoneally injected with cisplatin (20 mg/kg) for 3 days. As for therapeutic experiment, mice were intraperitoneally injected compound or inhibitor 24 h before the modeling. Mice were divided into these groups Control group: Mice received the same amount of saline as control; Model group: mice received cisplatin for 3 days; Treatment Group: Mice received compound or inhibitor for 24 h, then received cisplatin for 3 days. Mice were sacrificed by exsanguination under anesthesia with inhaled 5% isoflurane in room air. Mice were killed under anesthesia while kidney tissues and blood samples were collected for further experiments, including renal function, histology, and molecular analysis.

2.4. BUN and SCr level detection

Samples of blood were harvested from mice with or without intraperitoneal injection of 20 mg/kg cisplatin after 3 days. The levels of creatinine and BUN in blood samples were measured using the Creatinine and BUN Assay Kit (Nanjing, China) according to the manufacturer's instructions.

2.5. Methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay

HK2 cells were seeded in 96-well plates. For detecting the toxicity, we set a series of concentration of the compounds (0.5, 1, 2, 4, 8, 16, 32, 64 μM) and used DMSO as control. The compounds were added for 24 h. For detecting the therapeutic effect, we set control group (DMSO), model group (cisplatin 20 μM), and treatment group (cisplatin + compounds, 0.5–64 μM). Cell viability was measured by the MTT assay according to the manufacturer's instructions.

2.6. Transfection

GSK3β was knocked down by transfection with siRNA (jima Co.Ltd., China) and Lipofectamine TM 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions as previously described.

2.7. Western blot analysis

The HK2 cells and renal tissue were lysed by using RIPA-Buffer (Beyotime, Jiangsu, China). We collected the samples and followed Western blot to detect following indicator in protein level. The primary antibodies included: p-GSK3β(Ser-9)(CST; #5558; 1:1000), GSK3β(CST; #12456; 1:1000), p-P65 (Santa Cruz; #52401; 1:1000), P65 (Santa Cruz; #8008; 1:1000), KIM-1 (CST; #14971; 1:1000),

p-PP2Ac (Thermo Fisher; PA5-36874), PP2Ac (CST; #2259; 1:1000), TNF-α (Santa Cruz; sc-515765; 1:1000), β-actin (Bioss; 0061R; 1:2000). Total protein was loaded in 10% SDS-PAGE and transferred onto nitrocellulose membranes. Then membranes were blocked with 5% milk for 2 h. Membranes were incubated with primary antibodies overnight at 4 °C and treated with secondary antibodies matched for 1.5–2 h. Signals were detected with Licor/Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE, USA) and the intensities of bands were quantified by using the Image J software (NIH, Bethesda, MD, USA).

2.8. Histology and immunohistochemistry

Tissue were collected and fixed in 4% paraformaldehyde overnight. Fixed samples were embedded in paraffin and sectioned at 4 μM. We used PAS staining to detect the level of injury of mice kidney. The degree of tubular damage including tubular dilation, glycogen accumulation, and cast formation was scored by three experienced renal pathologist without knowing the group. The rating criteria are as follows: 0 = normal; 1 = 10%; 2 = 10–25%; 3 = 26–50%; 4 = 51–75%; 5 = 75–95%; 6 = more than 96%. For immunohistochemistry, the kidney sections were treated with 0.01 M sodium citrate buffer (pH 6.0) by a microwave-based antigen retrieval technique for 20 min at 95 °C was used followed by 10 min 3% H_2O_2 to block endogenous peroxidase activity, incubated with rabbit anti-KIM1, anti-TNF-α, and anti-p-GSK3β antibodies for 24 h at 4 °C and secondary antibodies for 30 min at 37 °C. After staining with DAB, the slides were visualized with microscope (Leica, Bensheim, Germany).

2.9. Co-immunoprecipitation (Co-IP) between GSK3β and PP2Ac

HK2 cells were planted in 6-well plates, and were divided into control group and model group (Cisplatin, 20 μM). Cells were stimulated by cisplatin for 24 h. Cells were lysed with NP-40 for 0.5 h in 4 °C, then HK2 cells were centrifuged (3000 rpm, 4 °C, 0.5 h). Primary anti-GSK3β or IgG were added and incubated at 4 °C for 2 h. After washing 3 times, samples were centrifuged (3000 rpm, 4 °C, 0.5 h) and then incubated with Agarose beads overnight in the Low temperature shaker (4 °C). Then lysate was centrifuged. The supernatant was removed and washed for 3 times by NP-40. Later we extracted the protein and used Western blot to analyze the connection between GSK3β and PP2Ac.

2.10. Cellular thermal shift assay (CETSA)

CETSA is a novel, stringent, label-free, biophysical assay that measures physical target engagement by drugs in cells and tissue samples directly [18]. Cells were treated with or without 5n after which, RIPA lysis buffer was added. Total protein was quantified using a protein assay kit (Beyotime, Jiangsu, China), and samples adjusted to similar final concentrations. Equal aliquots were placed in different PCR tubes, and samples were denatured for 11 min at varying temperatures in the PCR instrument (Eppendorf, Germany). The samples were freeze-thawed three times using liquid nitrogen, and centrifuged; the supernatants were analyzed using Western blot.

2.11. RNA extraction and real-time PCR

Total RNA of tissues and HK2 cells were extracted by using RNeasy isolation kit (Qiagen, USA) and RNA concentration was detected using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Then the RNA was reverse transcribed into to cDNA by using reverse transcription kit (Bio-Rad, Hercules, CA, USA) with Real Master Mix (TOYOBO, Japan). Real-time PCR was performed using

Table 1
Primer sequences used in Real-time PCR.

Mouse		
Genes	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
β-actin	CATTGCTGACAGGATGCAGAA	ATGGTGCTAGGAGCCAGAGC
IL-1β	GCTTCAGGCAGGCAGTAT	ACAAACCGCTTTTCCATCT
KIM-1	CAGGGAAGCCGCAGAAAA	GAGACACGGAAGGCAACCAC
Human		
Genes	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**
TNF-α	CCCAGGGACCTCTCTCTAATCA	GCTACAGGCTTGTCACTCGG
IL-6	CGGGAACGAAAGAGAAGCTCTA	CGGGAACGAAAGAGAAGCTCTA
MCP-1	AGCAGCAAGTGTCCCAAAGA	GGTGGTCCATGGAATCCTGA
β-actin	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG
GSK3β	ACCGAGAACCACCTCCTTTG	TGTGGTTACCTTGCTGCCAT
IL-1β	ACTACAGCAAGGGCTTCAGG	CATATCCTGTCCCTGGAGGT
KIM-1	CTGCAGGGAGCAATAAGGAG	TCCAAAGGCCATCTGAAGAC

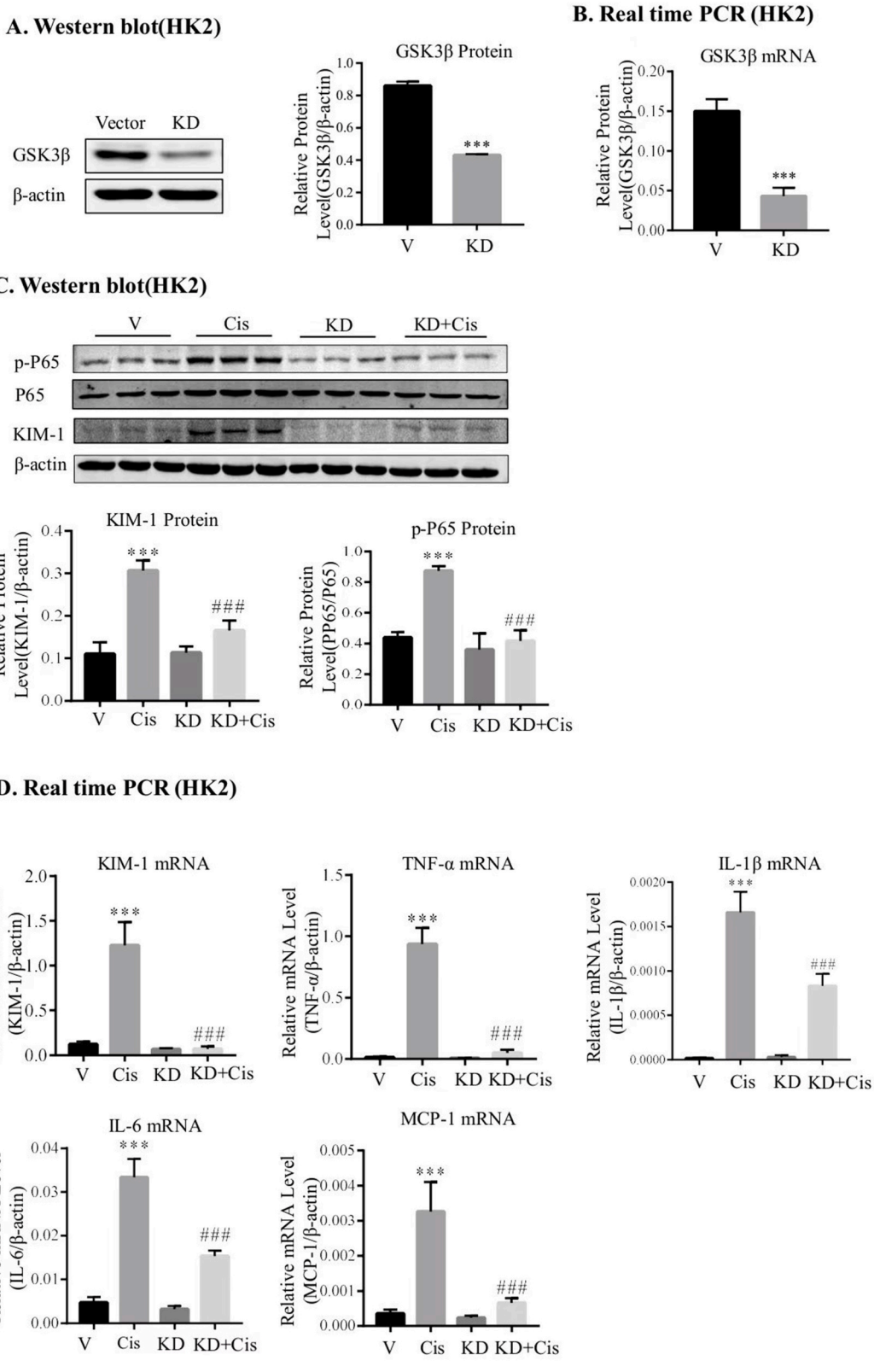

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Fig. 1. Knocked-down GSK3β repressed the injury and inflammation of AKI in HK2. (A) (B)Western blot and Real-time PCR results of knocked-out GSK3β in HK2 (C)Western blot analysis and quantitative data of p-P65, P65 and KIM-1 in HK2.(D) Real-time PCR statistics of KIM-1 and inflammation factors(TNF-α, IL-1β, IL-6 and MCP-1) in HK2. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the vector. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the Cis group. V, vector control. Cis, cisplatin group. KD, GSK3β knocked-down group. KD + Cis, cisplatin + knocked down group. Data represent the mean ± SEM.

the SYBR Green super mix with Opticon2 (Bio-Rad, Hercules, CA), we detected KIM-1, TNF-α, IL-1β, IL-6, IL-8, GSK3β and β-actin. Primer sequences are listed in Table 1.We evaluated the mRNA expression which were normalized to that of β-actin.

2.12. Statistical analyses

Data were analyzed by a two-sample *t*-test or one-way analysis of variance (ANOVA) Journal Pre-proof followed by Tukey's post hoc tests using GraphPad Prism 7 software. All the other data use a one-way ANOVA with Bonferroni's multiple comparisons test. Significance was indicated as followed: *P < 0.05; **P < 0.01; ***P < 0.001; #P < 0.05; ##P < 0.01; ###P < 0.001. Data represent the mean ± SEM.

3. Results

3.1. Knock down of GSK3β ameliorated the injury and inflammation in HK2 cells

In order to further explore the role and mechanism of GSK3β in kidney, we used siRNA to knockdown GSK3β in HK2, with protein and mRNA shown in Fig. 1A and B, respectively. Furthermore, it was shown that the knockdown of GSK3β significantly reduced the protein levels of KIM-1 and phosphorylated P65 in cisplatin-treated HK2 cells, which indicates the improvement of HK2 injury and

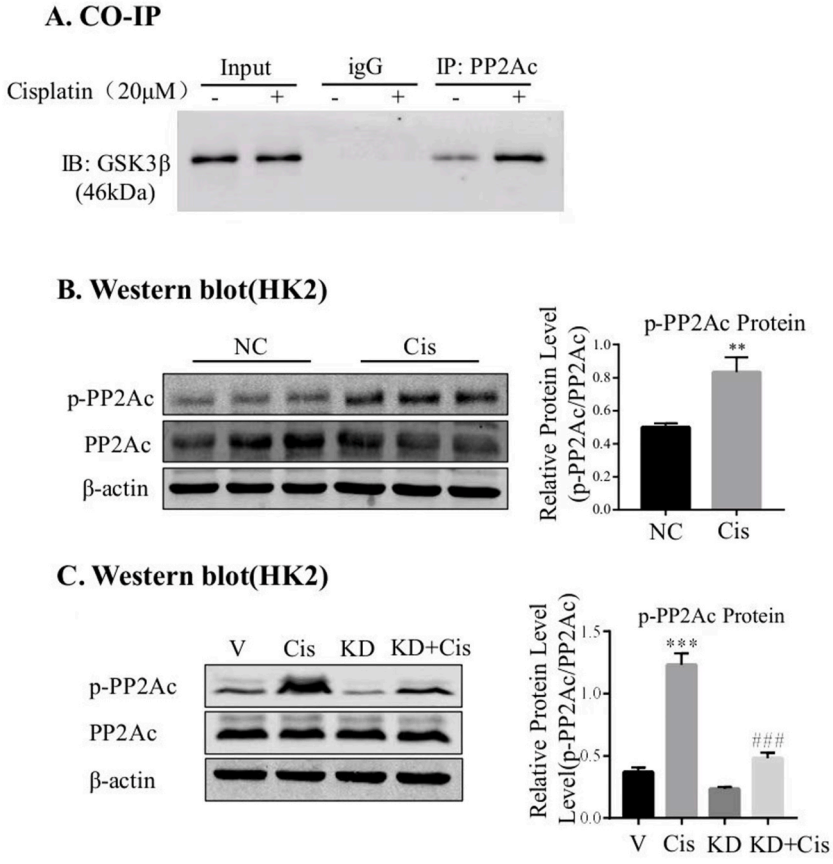


Fig. 2. Mechanism of how GSK3β modulating activity of NF-κB pathways. (A)CO-IP assay showed that GSK3β is combined with PP2Ac in normal HK2, and the affinity was enhanced by cisplatin. (B)Western blot results of p-PP2Ac, PP2Ac in cisplatin-induced AKI in HK2. (C)Western blot results showed that knocked-down of GSK3β inhibits the phosphorylation of PP2Ac. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the vector. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the Cis group. V, vector control. Cis, cisplatin group. KD, GSK3β knocked-down group. KD + Cis, cisplatin + knocked down group. Data represent the mean ± SEM.

inflammation (Fig. 1C). Real-time qPCR showed that compared to Cis group, knockdown of GSK3β can reduce the mRNA level of KIM1 and inflammatory factors TNF-α, IL-1β, IL-6 and MCP-1 (Fig. 1D).

3.2. *GSK3β regulate NF-κB pathway mediated by PP2Ac*

To explore the role of GSK3β in regulating NF-κB, we searched for a prospective protein that may have relationship between GSK3β

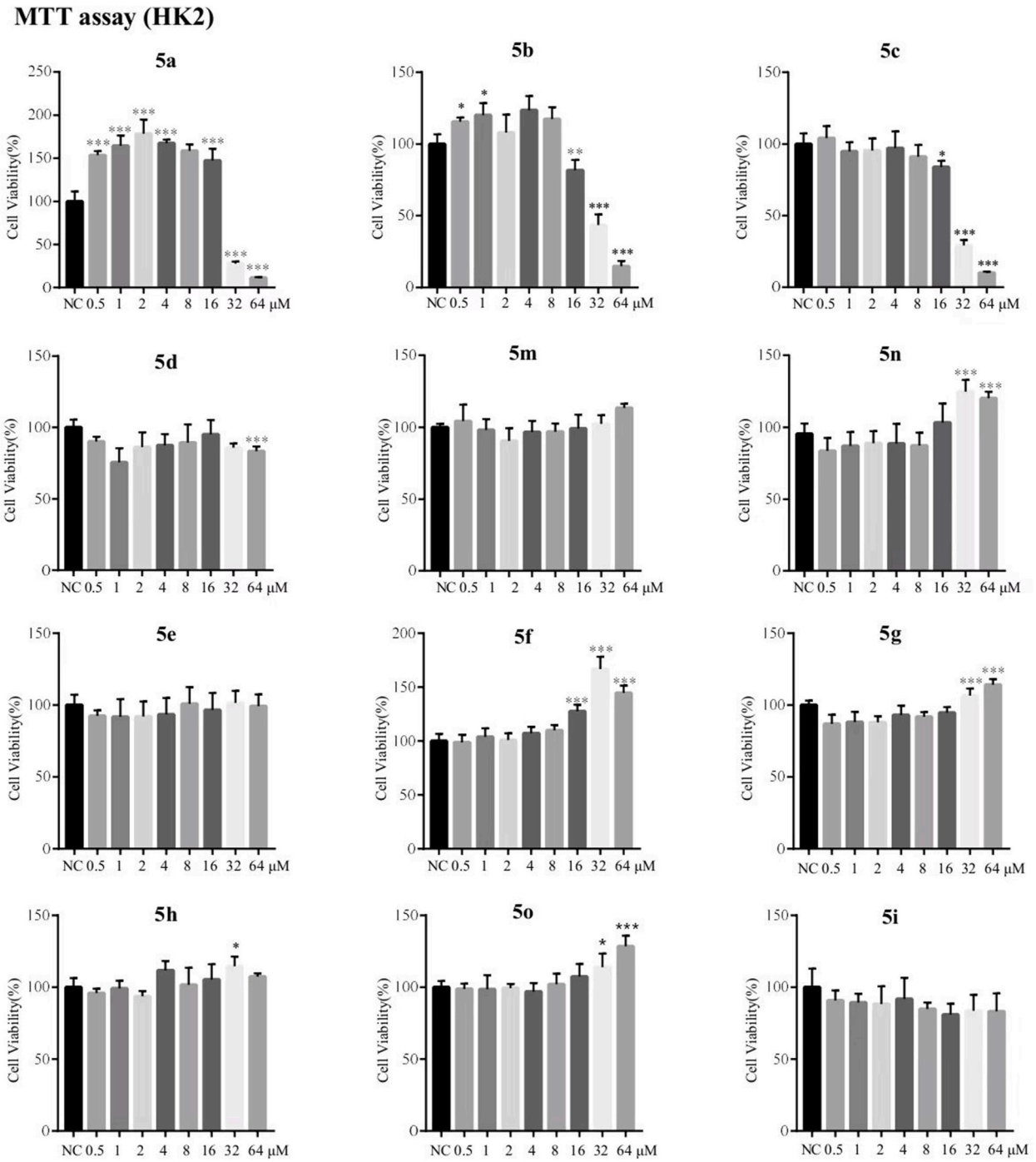


Fig. 3. MTT assay showed that among 12 derivatives, 5a-c had cell toxicity in the range of concerntration, 5d-n showed low toxicity in the range of concerntration.

Figs. 3 and 4. Selection of therapeutic compound to improve acute kidney injury.

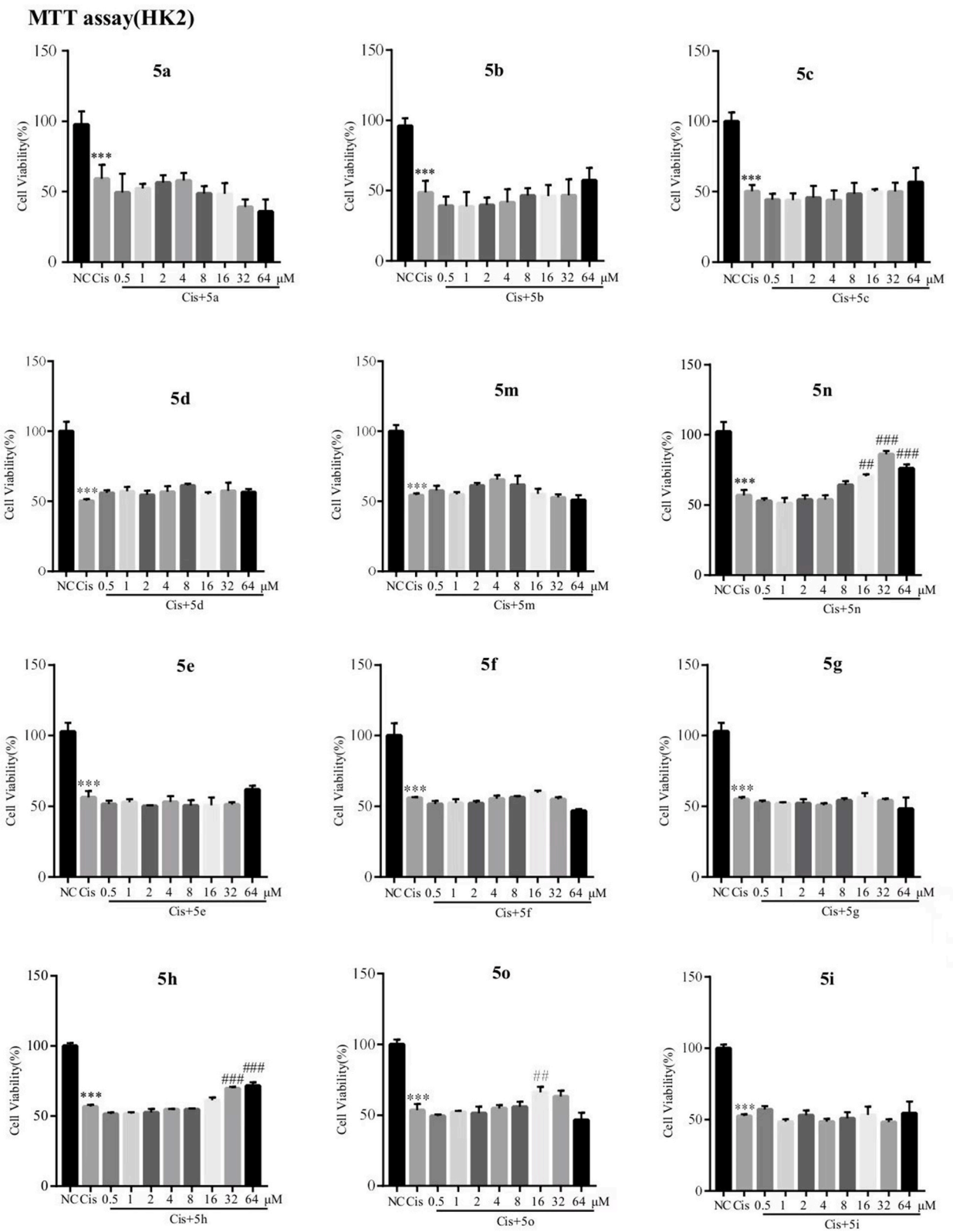
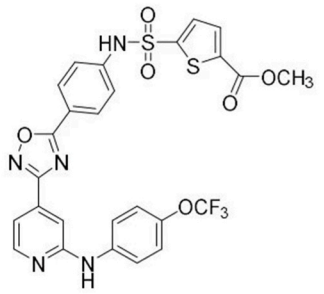
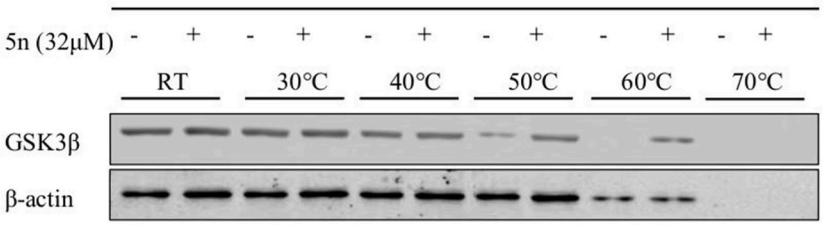


Fig. 4. MTT assay showed that 5n had the best renal protection in normal HK2 and 32 μM was the suitable concerntration.*P < 0.05, **P < 0.01, ***P < 0.001 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the Cis group. NC, control group. Data represent the mean ± SEM.

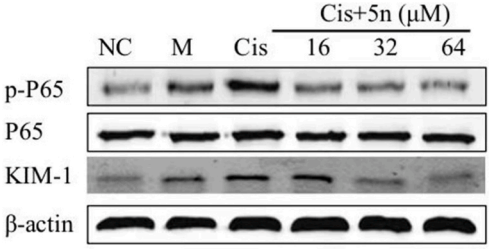
A. Molecular Formula of 5n

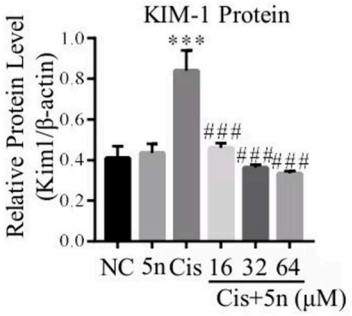


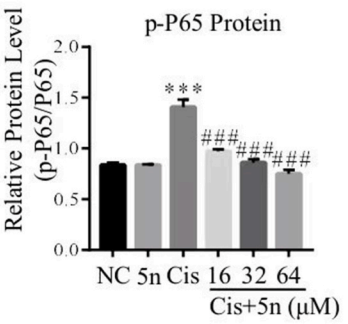
B. CETSA



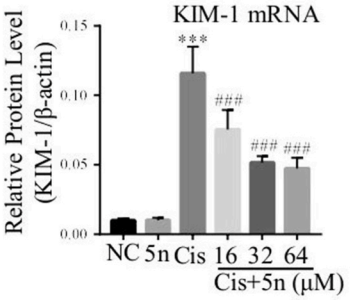
C. Western blot(HK2)

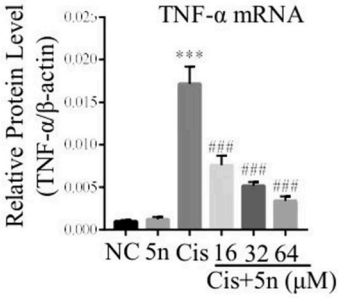


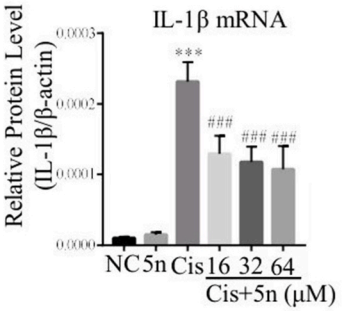


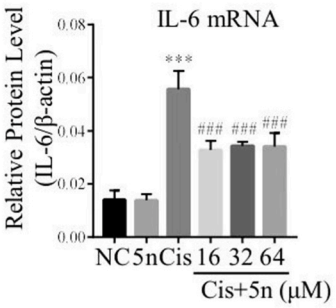


D. Real time RCR(HK2)









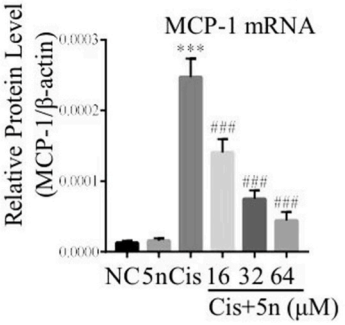


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Fig. 5. 5n reduced direct injury and inflammation in cisplatin induced AKI in HK2 (A)Molecular formula of M6. (B)CETSA assay showed that 5n is combined with GSK3β, which performing thermal stability in HK2. (C)Western blot and quantitative data of KIM-1, p-P65 and P65 in HK2. (D)Real-time PCR statistics of KIM-1 and inflammation factors(TNF-α, IL-1β, IL-6 and MCP-1). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the Cis group. NC, control group. 5n, 5n group. Cis, cisplatin group. Data represent the mean ± SEM.

and NF-κB signal pathway. To verify this hypothesis, we made use of CO-IP assay to detect whether GSK3β binds to PP2Ac. The CO-IP result demonstrated that GSK3β binds to PP2Ac in HK2, and cisplatin promote their connection (Fig. 2A, supplementary data A). Western blot results showed that PP2Ac phosphorylated protein levels were elevated after cisplatin stimulation (Fig. 2B). Next, we studied whether GSK3β can modulate the phosphorylation of PP2Ac. Silencing GSK3β could reduce the phosphorylation of PP2Ac induced by cisplatin (Fig. 2C). Accordingly, we conclude that GSK3β activates the NF-κB pathway by combining with PP2Ac and promotes the phosphorylation.

3.3. Selection of therapeutic compound to improve acute kidney injury by repressing GSK3β

In the present study, the potential GSK3β inhibitors were used in treating cisplatin-induced AKI. After the screening by MTT assay (Figs. 3 and 4), compound 5n (with the chemical structure shown in (Fig. 5A) was shown with minor toxicity and higher protective effect, compared with other compounds. In order to further verify the interaction between 5n and GSK3β protein, we performed acellular thermal shift assay (CETSA) that enabled us to evaluate target engagement. The result showed that in the control group (DMSO, 16 μL), GSK3β entirely degraded at 60 °C, whereas 5n increased the thermal stability of GSK3β (degradation temperature at over 60 °C) (Fig. 5B), which indicated that 5n directly binds to the GSK3β protein. Similarly, the CETSA test results show that 5n cannot bind to GSK3α (supplementary data B). Western blot results showed that 5n could directly inhibit the expression of GSK3β protein (supplementary data C). We found intriguing results, low doses of 5n do not affect GSK3α protein expression, high dose of 5n can inhibit GSK3α (supplementary data D).

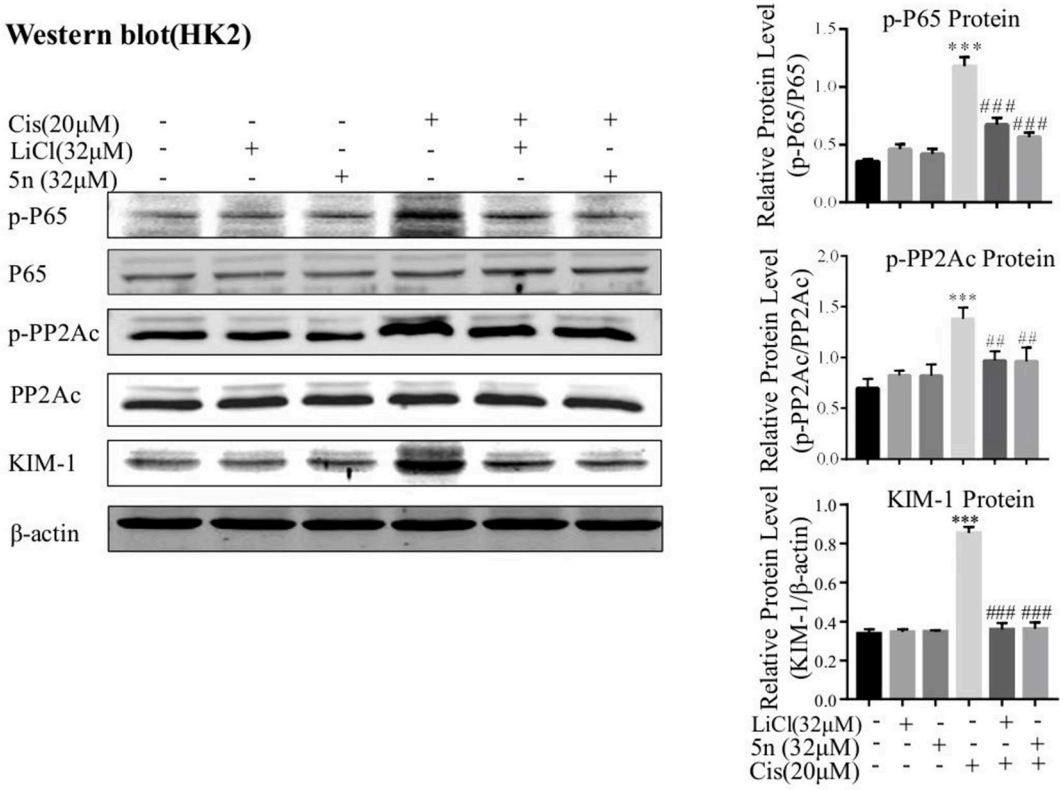

Fig. 6. 5n has better anti-inflammation effect than LiCl. Western blot results showed that 5n had the same anti-phosphorylation effect as LiCl, and 5n reduced activity of NF-κB better than LiCl. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the CIS group. NC, control group. Data represent the mean ± SEM.

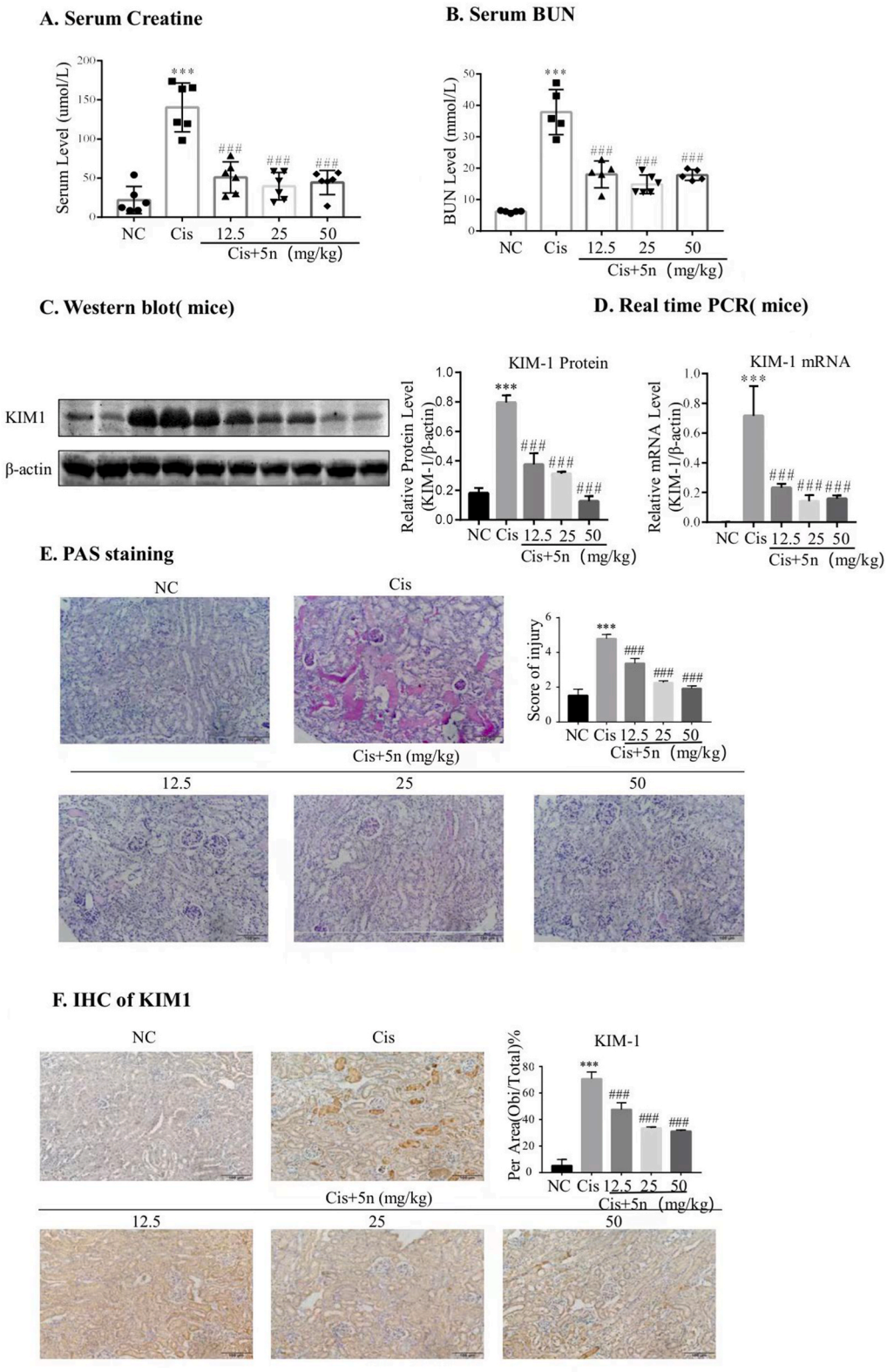

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Fig. 7. 5n reduced the decline of renal function and injury in cisplatin induced AKI in mice kidney. (A)(B)Renal function tests. Results of BUN, and SCr indicated that 5n ameliorated the decline of renal function induced by cisplatin. (C)(D) 5n alleviates the protein and RNA levels of KIM-1 in kidney tissue with cisplatin induced AKI. (E)PAS stain analysis showed that 5n reduced the damage of AKI in mice. (F)The immunohistochemical analysis and quantitative data of KIM-1. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the Cis group. NC, control group. CIS, cisplatin group. Data represent the mean ± SEM.

3.4. 5n alleviates acute kidney injury and inflammatory responses in vitro

To specifically study the therapeutic effect of 5n on cisplatin-induced AKI *in vitro*, a series of concentration (16, 32, 64 μM) to verify the protective effect of 5n. Western blot showed that the injury factor KIM-1 decreased in treatment group (Fig. 5C), as well as the inflammatory signal p-P65. Real-time qPCR also showed 5n protected against inflammatory response as evidenced by decreased TNF-α, IL-1β, IL-6 and MCP-1 expression levels (Fig. 5D). Then we compared the effect between 5n and LiCl (typical inhibitors of GSK3β) on cisplatin-induced HK2 injury. Western blot showed that 5n has similar anti-inflammatory effect with LiCl (Fig. 6).

3.5. 5n alleviates acute kidney injury and inflammatory responses in vivo

Further, compound 5n (12.5, 25, 50 mg/kg) was used to treat AKI in C57/BL6 mice induced by cisplatin (20 mg/kg i.p.). Serum BUN and SCr decreased in the treatment group (Fig. 7A and B). The protein level and mRNA level of KIM-1 in renal tissue of mice with AKI after 5n injection were decreased compared with those in the cisplatin induced AKI model group (Fig. 7C and D). The immunohistochemical staining and quantitative analysis of KIM-1 showed the same conclusion (Fig. 7F). PAS staining showed that 5n inhibited cisplatin-induced renal glycogen accumulation and tubular necrosis (Fig. 7E). Western blot results show that 5n significantly reduced the protein level of p-P65 in cisplatin treatment (Fig. 8A). In addition, immunohistochemical and quantitative analysis showed that 5n reduced TNF-α positive signals in injured kidney (Fig. 8B). Real-time qPCR results showed that 5n significantly downregulated mRNA levels of TNF-α, IL-1β, IL-6 and MCP-1 which were induced by cisplatin (Fig. 8C). These evidence indicate that 5n can reduce cisplatin induced NF-κB signaling activation and inflammatory cytokine release, thereby reducing kidney injury.

4. Discussion

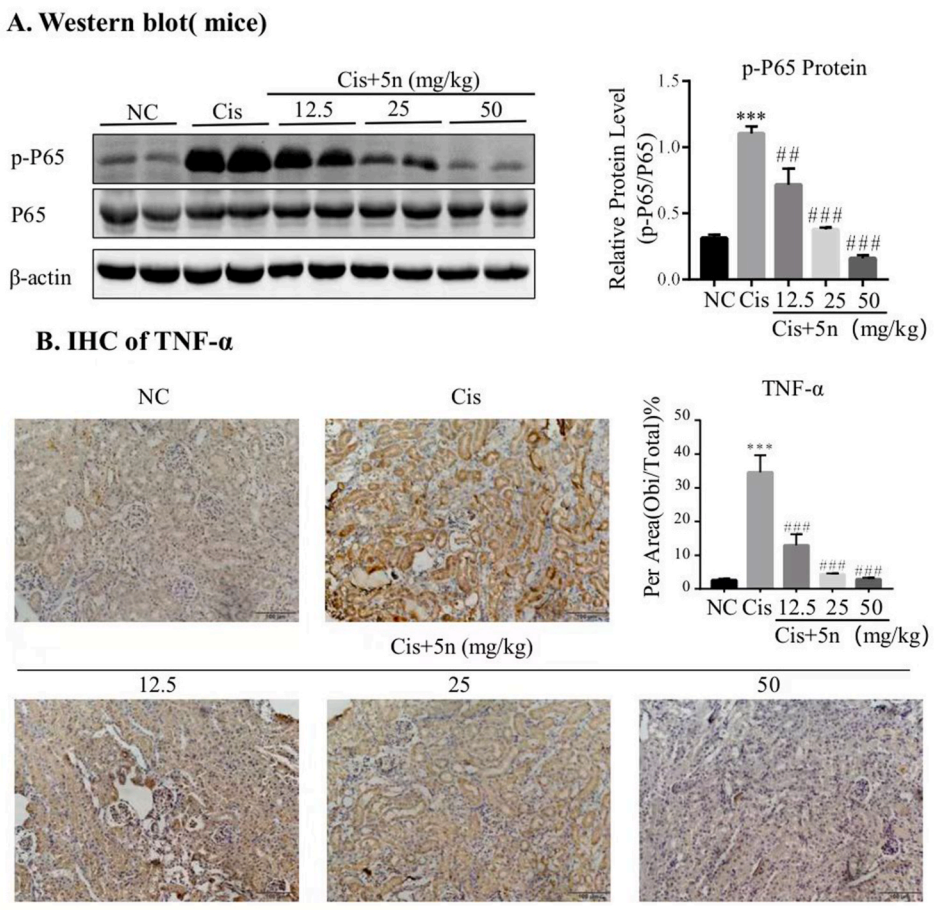
Glycogen synthase kinase 3 beta (GSK3β) is a constitutively activated Ser/Thr protein kinase that regulates glycogen metabolism, gene expression, and apoptosis [19,20]. Unlike most kinases, GSK3β has constitutive activity in cells, and a variety of extracellular stimuli act by inhibiting GSK3β activity [21]. It is believed that phosphorylation of GSK3β at Tyr216 residues lead to activation, while phosphorylation at Ser9 induces inactivation [22]. Some studies have shown that GSK3β is upregulated in numerous disease states, including neurodegeneration [23], diabetes [24], inflammatory conditions [25], and some cancers [26].

GSK3β has been confirmed widely expressed in the kidney [27]. In recent years, a large number of studies have shown that GSK3β is involved in pathogenesis of diverse kidney diseases, such as glomerular disease [28], acute kidney injury (AKI) [29,30], diabetic nephropathy [31], and chronic kidney disease (CKD) [15,16]. Activation or suppression of GSK-3β regulates a variety of cellular responses, including apoptosis, oxidative stress, inflammation, cytoskeleton [32], mitochondria permeability transition [33], cell proliferation, autophagy, senescence signaling [34] and so on.

In acute models of injury, GSK3β promotes the systemic inflammatory response, increases the inflammatory release of cytokines, induces apoptosis, and alters cell proliferation. GSK3β plays a critical role in AKI by promoting tubular epithelial cell apoptosis, inflammation and fibrosis, and suppressing repair [35]. New research describes the relationship between GSK3β and inflammation [13]. GSK3β acts as a potent inducer of inflammation. As a reflection of the impact of GSK3β on inflammatory diseases, the application of the GSK3β activity index (i.e., the ratio of total to Ser9-phosphorylated GSK3β) has been proposed as a new diagnostic and prediction tool [25,36]. The different activity of GSK3β depends on various sites on the enzyme including phosphorylation [37], ubiquitination [38] and methylation [39]. Among the numerous sites of GSK3β, phosphorylation of Ser9 is essential to modulate its kinase activity [40]. The phosphorylation site Ser9 is recognized as the key regulation of kinase activity. However research about how GSK3β works in AKI was still insufficient. Research showed that GSK3β inhibits tubular regeneration in acute kidney injury by a FoxM1-dependent mechanism [41].

After cisplatin stimulation of cultured murine tubular epithelial cells, p-GSK3β(Ser9) protein level reached its highest level at 2 h and decreased at 24 h. Moreover, in cultured renal tubular cells, cisplatin exposure led to transient repression of GSK3β activity followed by a prolonged upregulation of activity [29]. In another study, the results showed that the expression of both GSK3β and phosphorylated GSK3β (Ser9) was elevated in cisplatin-induced AKI kidney tissue [42].

In addition, multiple *in vitro* and *in vivo* studies have observed that increased phosphor-GSK3β Ser9 levels correlate with reduced apoptosis and kidney damage in various AKI and CKD models. GSK3β promotes the release of TNF-α, IL-1β and IL-6 in neurodegenerative diseases and lung injury [43,44]. The first studies examining the role of GSK3β in AKI [45] , GSK3β is a known regulator of NF-κB activity. In a model of severe acute pancreatitis-induced AKI, GSK3β inhibition reduced NF-κB activity and pro-inflammatory factors [46]. Renal proximal tubule-specific GSK3β knockout mice showed resistance to mercuric chloride-induced renal injury [30]. Inhibitors for GSK3β including lithium exhibited renoprotective effects on acute kidney injury caused by diclofenac, cisplatin, ischemia, and lipopolysaccharide in rats and mice. In our study, we found that silencing GSK3β reduced direct cell damage and the release of inflammatory cytokines *in vitro* model of cisplatin-induced acute kidney injury. Silencing GSK3β also inhibited activity of



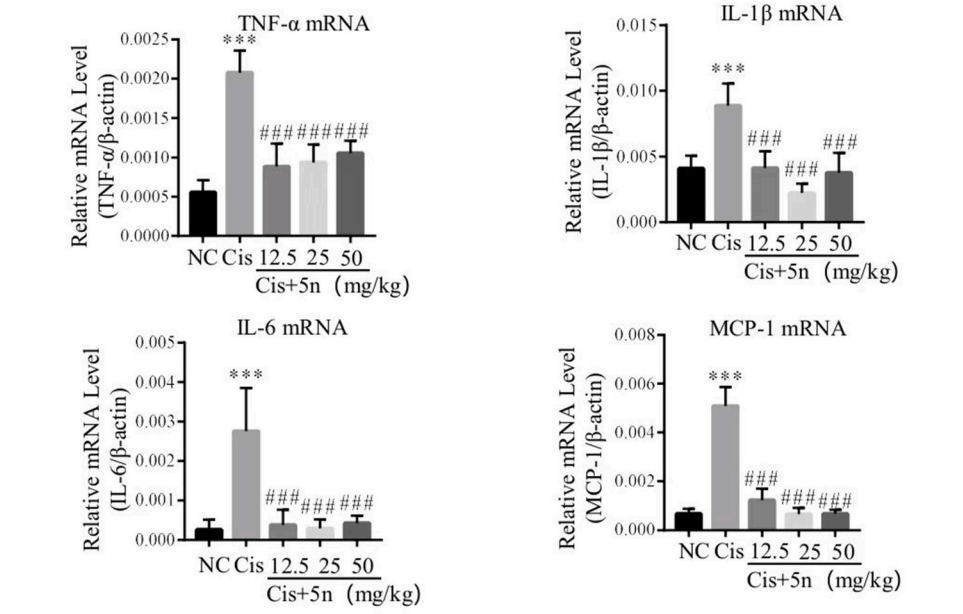


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Fig. 8. 5n reduced inflammation in cisplatin induced AKI in mice kidney. (A)Western blot analysis and quantitative data of p-P65, P65 in mice. (B) The immunohistochemical analysis and quantitative data of TNF-α. (C)Real-time PCR statitics of TNF-α, IL-1β, IL-6, MCP-1 in cisplatin-induced mice kidney. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the CIS group. NC, control group. Cis, cisplatin group. Data represent the mean ± SEM.

NF-κB pathway. These results are consistent with other studies of kidney removal.

Protein phosphatase 2A (PP2A) is a major cellular serine-threonine phosphatase, and consists of a catalytic subunit C (PP2Ac), a structural subunit A (PP2Aa), and a greatly variable regulatory subunit B (PP2Ab). The PP2A subunit PP2Ac is known to regulate a variety of cellular processes including signal transduction, cell differentiation, and apoptosis [47]. Recent research showed that PP2Ac plays an essential role in modulating the NF-κB pathway. PP2Ac is one of the main phosphatases that represses activity of NF-κB pathway. Nizamutdinova et al. [48] reported that continuous PP2Ac phosphorylation at Tyr307 (that is, its inactivation) led to dysregulated IKK or IκB, initiating sustained NF-κB nuclear translocation in HG-cultured cardiomyocytes. It has been shown that the determinant of whether PP2Ac is activated depends on the upstream kinase and the negative factor. PP2Ac modulates the downstream TRAF, IKK, P65, and MEKK3 to inhibit NF-κB pathway [49–52]. Research shows that PP2Ac could negatively regulate the HG-induced IKK-IκBα–NF–κB signaling pathway in H9C2 cardiomyocytes [53]. It is fascinating to note that PP2A is a substrate for GSK3β in multiple cell types, such as HEK293 cells and N2a cells, when activated, GSK3β can negatively regulate PP2Ac activity [54,55]. In diabetic cardiomyopathy, GSK3β regulated the activity of PP2Ac and showed anti-inflammatory effect [53]. Therefore, activated GSK3β might lead to PP2Ac phosporylation and, subsequently, to sustained activation of NF-κB signaling.

However, the mechanism of how GSK3β regulate PP2Ac in kidney remains unclear. In our study, PP2Ac was phosphorylated by cisplatin-induced inhibition, suggesting inhibition of enzyme activity. For exploring the relationship between PP2Ac and GSK3β, we detected the phosphorylation of PP2Ac in GSK3β knocked-down HK2 cells. Evidence showed that down-regulation of GSK3β repressed the phosphorylation of PP2Ac induced by cisplatin. CO-IP assay additionally proved that GSK3β is binded with PP2Ac in normal HK2 cells, and the affinity was enhanced in cisplatin induced AKI. These studies showed that GSK3β mediates the injury and inflammation in HK2 cells through the phosphorylation process of PP2Ac.

GSK3 acts as a potent driver of inflammation, rendering GSK3 inhibitors a promising target of anti-inflammatory research [56,57]. The modulation of GSK3 (especially GSK3β) activity via natural compounds [58] or the design of pharmacologically applicable inhibitors [59] is still a promising target for various therapeutic approaches. In several preclinical and clinical trials, the efficacy and safety of pharmacological GSK3 inhibitors for different clinical purposes are or have been addressed [60]. Systemic pharmacological GSK3 inhibition, or proximal tubule-specific GSK3β gene deletion can significantly reduce tubular injury, accelerate regeneration and suppress renal fibrosis following AKI in mouse models [41]. Isoform nonspecific pharmacologic inhibitors of GSK3β such as LiCl, TDZD-8, SB216763, SB415286, or BIO reduced apoptosis of renal tubular epithelial cells. Lithium is a naturally selective inhibitor of GSK3β [61], which accelerated recovery of renal function, promoted repopulation of renal tubular epithelium, and improved kidney repair in murine models of cisplatin- and ischemia/reperfusion-induced AKI [29].

We synthesized a series of derivatives containing oxadiazole ring and pyridine ring structures [17,62]. All of these derivatives were proved to be inhibitory against GSK3β and most of them demonstrated a certain neuroprotective effect on nerve injury model, especially decreasing the neuroinflammation [17]. 12 derivatives, among these agents, were assayed and we confirmed that 5n is the prospective derivative to suppress injury and inflammation in cisplatin-induced AKI. Even though 5n has the best therapeutic effect, it is not the most inhibitory among all of the derivatives, there were still underlying mechanisms about the activity and the anti-inflammation effect of GSK3β.

The present study demonstrated that the knockdown of GSK3β resulted in the protective effect against cisplatin-induced renal injury *in vitro*. Mechanistically, we found that GSK3β was able to bind to PP2Ac and likely promote the phosphorylation of PP2Ac, causing activation of NF-κB pathways and damage to renal cells. Next, we found that the GSK3β inhibitor 5n mitigated cisplatin-induced AKI. 5n is the least toxic of the series of compounds we screened and has the best therapeutic effect. The combination between 5n and GSK3β was confirmed as well. Initially, 5n was shown to ameliorate injury and inflammation induced by cisplatin-induced AKI *in vivo* and *in vitro*, suggesting it to be an essential regulator and promising therapeutic target.

There are still some shortcomings in this study. In this study, we mainly studied the function and mechanism of GSK3β in cisplatin-induced AKI by silencing or inhibiting GSK3β. In this study, only LiCl was used and no other inhibitors of GSK3β were used to compare the protective effect of 5n on cisplatin-induced AKI. In addition, the molecular mechanism of GSK3β regulating PP2Ac still needs to be studied and explored in further experiments.

5. Conclusions

It was observed that GSK3β participates in the pathway of cisplatin-induced nephrotoxicity. GSK3β inhibitor 5n was showed moderate anti-inflammatory effect *in vivo* and *in vitro*. Moreover, 5n significantly attenuated cisplatin-induced renal toxicity in HK-2 cells and mice.

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Ethics declarations

This study was reviewed and approved by the Animal Experimentation Ethics Committee of the Anhui Medical University, with the approval number: LLSC20232206.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Yu-ting Cai: Investigation, Data curation. **Zeng Li:** Resources, Project administration. **Yue-yue Wang:** Methodology. **Chao Li:** Software, Formal analysis. **Qiu-ying Ma:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29159.

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