

## Original Article

# Protective effects of the AKT activator SC79 on renal ischemia-reperfusion injury

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**Abstract:** Background and Aims: SC79, an AKT activator, has been reported to protect experimental ischemia-elicited neuronal death, brain injury, and myocardiocyte hypoxia/reoxygenation (H/R) injury. However, the protection of SC79 from renal ischemia-reperfusion (I/R) injury and the precise mechanisms involved are unknown. Here, we investigated the effects of SC79 in renal tubular epithelial cells *in vitro* and in mouse kidney *in vivo* following hypoxia-reoxygenation (H/R) and renal I/R injury. Methods: The kidneys of Sprague-Dawley rats were subjected to 30 min of warm ischemia followed by 24 h of reperfusion. Murine renal tubular epithelial NRK-52E cells were subjected to hypoxia for 6 h and reoxygenation for 24 h. The NRK-52E cells and the renal I/R injury model were treated with SC79 and/or LY294002 at different times and concentrations. Serum creatinine (Cr) concentration, renal histology, cellular viability, and cell apoptosis were assessed. Levels of phospho-Akt, bad, Bim, bax, bcl-2, and bcl-XL in NRK-52E cells and renal tissues were determined by western blotting. Results: SC79 improved viability and inhibited apoptosis in NRK-52E cells following H/R. SC79 decreased serum Cr and markedly improved pathology and decreased cell apoptosis in kidneys following I/R. In addition, SC79 promoted the expression of phospho-Akt, bcl-2, and bcl-XL, and decreased the expression levels of bad, bax, and bim. PI3K inhibitor (LY294002) pre-treatment completely abolished these effects of SC79. Conclusions: The protective role of SC79 against H/R of NRK-52E cells or renal I/R injury is related to activation of phosphorylation of AKT, resulting in a decrease in the pro-apoptotic proteins bim, bax, and bad and an increase in the anti-apoptotic proteins bcl-2 and bcl-XL induced by cell H/R and renal I/R injury.

**Keywords:** Renal ischemia-reperfusion, Akt, SC79

## Introduction

Ischemia-reperfusion (I/R) injury is an important cause of renal damage that occurs during renal transplantation (RT) or trauma and other surgical procedures when the kidney is transiently deprived of oxygen and subsequently reoxygenated [1]. The identification of effective pharmacological agents could expand the available options for surgeons and enable the use of RT. Although animal models are frequently used for attenuating I/R injury in RT, many pharmacological agents have not become part of clinical routine. The development of protective pharmacological agents to reduce the negative effects of renal I/R injury is urgently needed.

Advances in molecular biology have suggested gene therapy as a promising approach for attenuating renal I/R injury [2-5]. However, the

toxicity of gene vectors, the low efficiency of gene transfection, and the uncertainty of protein expression after transfection limit the development of gene therapy. Although non-viral vectors may be associated with fewer toxic or immunological events, inefficient gene transfer remains a limiting factor [6].

The PI3K/Akt pathway has important biologic functions in cell proliferation, survival, and apoptosis. Activation of PI3K/Akt-dependent signaling has been demonstrated to result in the attenuation of I/R injury in the kidney and other organs [7, 8]. Activation of PI3K/Akt signaling enhances anti-apoptotic Bcl-2 and Bcl-xl protein expression, inhibits pro-apoptotic proteins such as Bax and Bad, and protects cells against apoptosis [9]. Despite the high demand for Akt activators for various therapeutic applications, efforts to identify these activators have been unsuccessful.

## SC79 protects rat renal I/R injury

SC79, a small molecule Akt activator, suppresses Akt membrane translocation while activating Akt in the cytosol [10]. It has shown cytoprotective effects in experimental ischemia-elicited neuronal death [10], early brain injury [11], UV radiation of retinal pigment epithelium [12], and *in vitro* oxygen and glucose deprivation/reoxygenation of myocardiocytes [13], but failed to reduce myocardial I/R injury [14], in contrast to previous studies using genetic models of cardiac AKT overexpression [15, 16].

In this study, we investigated the effect of SC79 on renal tubular epithelial NRK-52E cells following hypoxia-reoxygenation (H/R) *in vitro* and renal I/R injury *in vivo*. We observed that Akt was slightly activated during renal I/R or NRK-52E cell H/R. SC79 treatment significantly increased Akt phosphorylation levels and induced anti-apoptotic and down-regulated pro-apoptotic protein expression, improved pathological features, attenuated renal I/R injury, and protected NRK-52E cells from hypoxia-reoxygenation injury.

### Materials and methods

#### *Ethics statement*

The study was conducted in accordance with the ethical standards of the Declaration of Helsinki, and was approved by the institutional review board of Shandong Provincial Hospital Affiliated to Shandong University.

#### *Hypoxia-reoxygenation in primary hepatocytes*

The *in vitro* H/R model was established as previously described [17]. Briefly, NRK-52E cells were placed into serum-free Dulbecco's modified Eagle's medium and seeded at a density of  $4 \times 10^5$  cells/well in 6-well plates, which were equilibrated with 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub>. After hypoxia for 6 h, plates were returned to normal conditions for reoxygenation for 24 h.

#### *Renal I/R model in vivo*

Rats were fasted for 12 h but allowed free access to water before the induction of anesthesia. A model of 30 min of renal ischemia and 24 h of reperfusion was used according to previously described methods [18]. Following 24 h of reperfusion, blood and renal samples were collected for analysis.

#### *Experimental groups*

Rats or NRK-52E cells were randomly divided into four groups: sham, I/R, I/R+SC79, and I/R+SC79+LY294002. SC79 or saline was applied via intraperitoneal (i.p.) injection at a concentration of 0.04 mg/g body weight 0.5 h prior to ischemia followed by reperfusion ( $n = 6$ /per group). For the *in vitro* H/R model, NRK-52E cells were exposed to 8 µg/mL SC79 0.5 h prior to hypoxia following by reoxygenation for 24 h. NRK-52E cells or rats in the sham groups were injected with an equal volume of saline. The PI3K inhibitor LY294002 (Sigma-Aldrich, Shanghai, China; 1 mg/25 g body weight or 20 µM) was given to mice 15 min or 4 h before SC79 administration, according to a previously reported method [10].

#### *Measurement of renal function*

Serum was collected and stored at -70°C until analysis. Serum creatinine (Cr) was measured with an enzymatic Cr reagent kit according to the manufacturer's instructions (Thermo Fisher Scientific, Hangzhou, China).

#### *Histological score of kidney injury*

Kidney samples were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, transferred onto slides (4 µm), and subjected to hematoxylin and eosin (H&E) staining to assess histological injury. The pathological score ranged from 0 to 4 points based on a previously reported method [19].

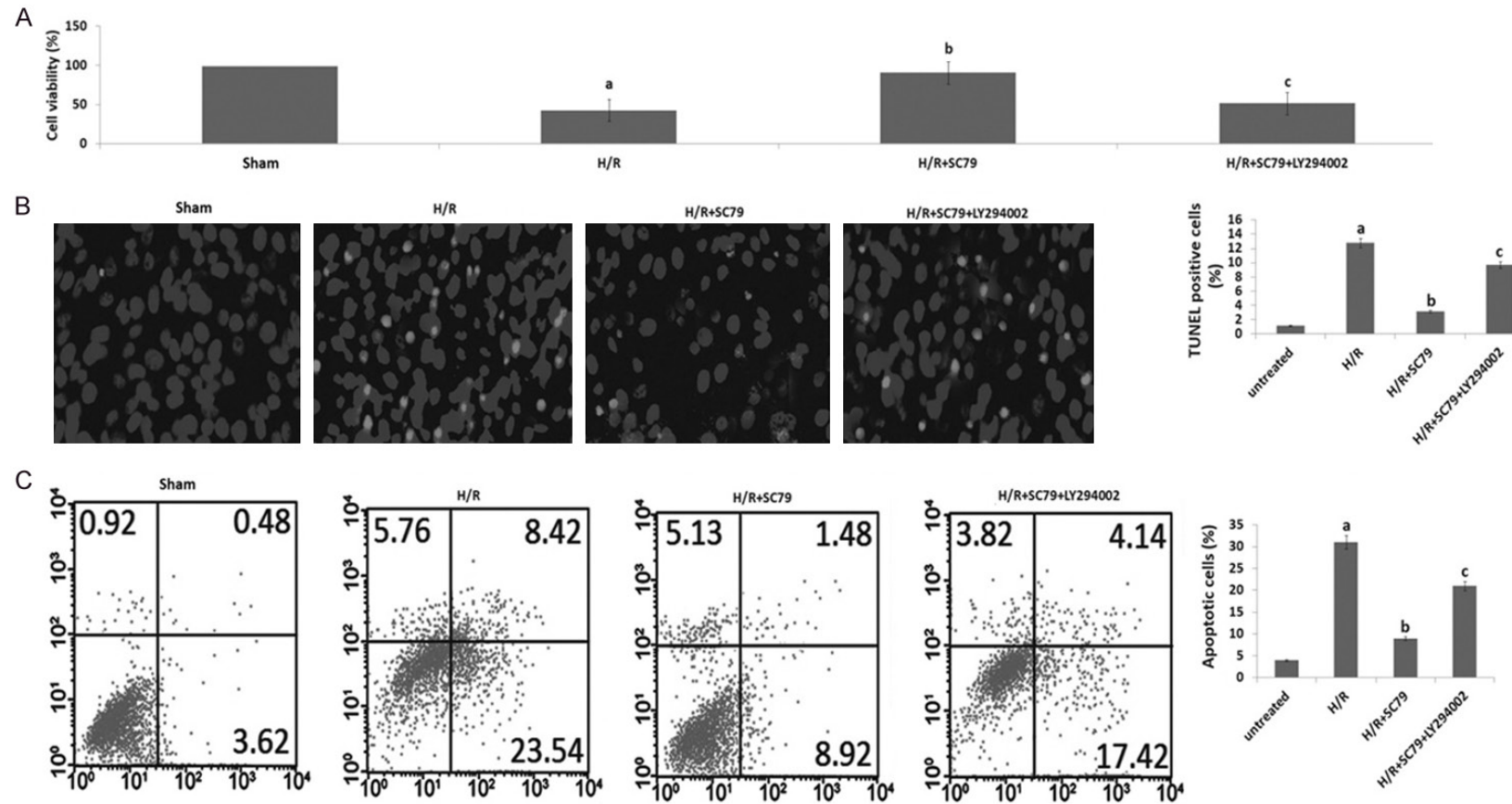
#### *Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining*

Formalin-fixed renal samples were embedded in paraffin, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using a fluorescent detection kit (Roche Diagnostics, Chengdu, China) following the manufacturer's instructions. The results of quantitative analysis were presented as percentage of TUNEL-positive cells in the kidney in each experimental group.

#### *Viability assay*

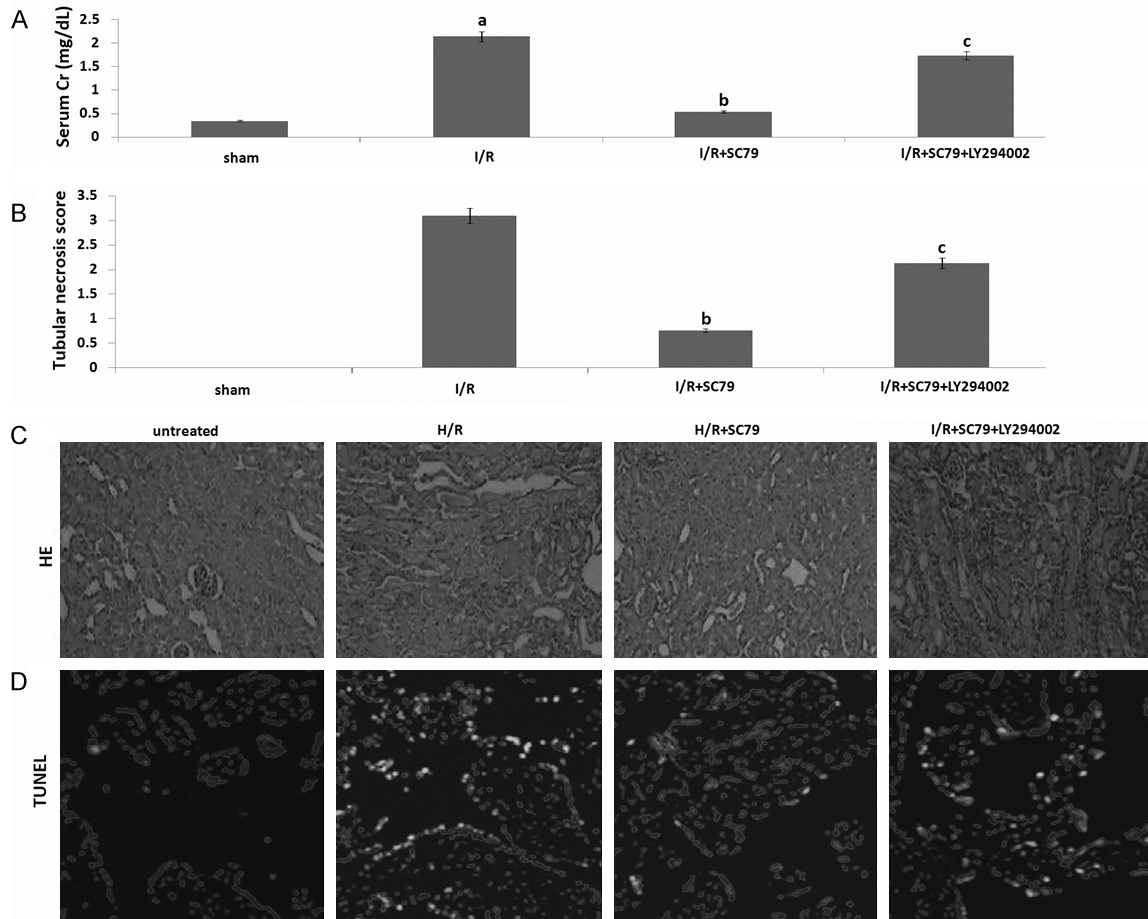
To study the viability of NRK-52E cells after different treatments, an *in vitro* toxicology MTT-based assay kit (Life Technologies Italia, Monza, Italy) was used. Cell viability was calculated relative to results in control cells (100% viable).

## SC79 protects rat renal I/R injury



**Figure 1.** SC79 protects NRK-52E cells from H/R injury *in vitro*. NRK-52E cells were subjected to hypoxia for 6 h and reoxygenation for 24 h. The NRK-52E cells that underwent H/R were treated with 8  $\mu\text{g}/\text{mL}$  SC79 0.5 h prior to hypoxia following by reoxygenation for 24 h, or treated with 20  $\mu\text{M}$  PI3K inhibitor LY294002 4 h before SC79 treatment. A. Cell viability was detected by MTT assay; B. Cell apoptosis was detected by TUNEL assay; C. Apoptosis was detected by flow cytometry using the FITC Annexin V Apoptosis Detection Kit. <sup>a</sup>P < 0.05 vs sham; <sup>b</sup>P < 0.05 vs H/R; <sup>c</sup>P < 0.05 vs H/R+SC79.

## SC79 protects rat renal I/R injury



**Figure 2.** SC79 protects renal I/R injury in vivo. Mice were subjected to 30 min of renal ischemia and 24 h of reperfusion. SC79 was applied by i.p. injection at a concentration of 0.04 mg/g body weight 0.5 h prior to ischemia followed by reperfusion. LY294002 (1 mg/25 g body weight or 20  $\mu$ M) was given to mice 15 min before SC79 administration. A. Blood collected 24 h after I/R was analyzed for serum Cr; B. Histogram of tubular necrosis scores; C. Renal tissues were collected and stained with H&E ( $\times$  200); D. TUNEL assay (apoptosis %). <sup>a</sup>P < 0.05 vs sham; <sup>b</sup>P < 0.05 vs I/R; <sup>c</sup>P < 0.05 vs I/R+SC79.

### *Annexin V/propidium iodide apoptosis detection by flow cytometry*

To evaluate the effects of SC79 on apoptosis, the number of apoptotic cells was measured by flow cytometry using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, Hangzhou, China) according to the manufacturer's instructions. Data were acquired using a BD FACSCalibur™ flow cytometer and analyzed using FlowJo software. All experimental conditions were tested in triplicate. All data are representative of three independent experiments.

### *Quantification of apoptosis in vitro*

NRK-52E cells cultured on coverslips were treated as described above. Then the cells were fixed with 4% paraformaldehyde (PFA) for 10 min and incubated with 50 nM NH<sub>4</sub>Cl to

extinguish PFA activity. Apoptosis was assessed by staining nuclei with DAPI followed by fluorescence microscopy analysis.

### *Western blotting*

The total cell lysates were collected from supernatants by centrifugation at 2000  $\times$  g for 5 min at 4°C. Western blotting analysis was performed as described previously [20]. The respective antigens were detected by antibodies against bcl-2, bcl-XL, bax, Bad, bim, cleaved caspase-3, anti-phospho-Akt (Thr-308/Ser-473) (sc-16646), and anti-Akt (sc-8312).  $\beta$ -actin was used as the loading control.

### *Statistical analysis*

All data represent the mean  $\pm$  standard error. Statistical comparisons between groups were

## SC79 protects rat renal I/R injury



**Figure 3.** Effects of SC79 on Akt activation and pro- and anti-apoptotic protein expression during 30-min/24-h H(I)/R. The effect of SC79 on the pro-apoptotic molecules Bax, bad, and Bim, and the anti-apoptotic molecules Bcl-2 and bcl-xL in renal tissue following I/R (A) and NRK-52E cells following H/R (B).

analyzed by Student's *t* test. A *p* value < 0.05 was considered significant.

### Results

#### *SC79 protects against H/R injury in NRK-52E cells in vitro*

We examined whether SC79 administration protects the NRK-52E cells from H/R-induced injury. H/R-treated NRK-52E cells are generally used to mimic renal I/R *in vivo*. The results show that SC79 pre-treatment (8 µg/mL 0.5 h before hypoxia) significantly increased cell viability (**Figure 1A**) and decreased cell apoptosis (**Figure 1B** and **1C**) compared with the untreated H/R groups, suggesting that SC79 protects NRK-52E cells from I/R injury *in vitro*. The level of cleaved caspase-3 in H/R-treated NRK-52E cells also treated with SC79 was significantly lower than in H/R-treated control NRK-52E cells (**Figure 3B**). This finding is consistent with results from the apoptotic assay. However, PI3K inhibitor (LY294002) pre-treatment completely abolished these effects of SC79, suggesting that the beneficial effects of SC79 are mediated by AKT activation (**Figure 1**).

#### *SC79 protects against renal I/R injury*

Renal I/R injury increased the serum Cr levels to  $2.14 \pm 0.13$  (vs sham,  $0.34 \pm 0.06$ ) mg/dL (*P*

< 0.05). The SC79-pretreated group ( $0.54 \pm 0.09$ ) showed significant reduction in the elevated Cr levels compared with the untreated I/R group (**Figure 2A**).

To investigate the effect of SC79 on I/R-induced renal tubular damage, kidney sections were stained with H&E. More serious damage was observed in the untreated I/R group. However, after pre-treatment with SC79, the damage was significantly reduced, and less histological damage was observed on H&E staining (**Figure 2C**). Similarly, the histological scores of renal injury were markedly elevated following I/R injury, but were reduced by SC79 administration (**Figure 2B**).

Apoptotic cells were detected using TUNEL staining. As shown in **Figure 2D**, mice with I/R injury had more TUNEL-positive cells, and pre-treatment with SC79 resulted in a significant decrease in the number of apoptotic cells compared with the untreated I/R group (*P* < 0.05). Meanwhile, the level of cleaved caspase-3 in the kidney treated with SC79 was significantly lower than in the untreated I/R group (**Figure 3A**). This finding is consistent with the results of the TUNEL assay. However, pretreatment with LY294002 abolished the effect of SC79 on the I/R-injured kidney, indicating that the protective effect of SC79 on I/R-injured kidney is due to AKT activation (**Figure 2**).

NRK-52E cells were subjected to hypoxia for 6 h and reoxygenation for 24 h. The NRK-52E cells that underwent H/R were treated with 8 µg/mL SC79 0.5 h prior to hypoxia following by reoxygenation for 24 h, or treated with 20 µM LY294002 4 h before SC79 treatment.

#### *SC79 protects the kidney against I/R injury via Akt activation*

Activation of PI3K/Akt signaling protects against H/R-induced cell injury [21]. In order to further elucidate the mechanism underlying the protective effect of SC79 against renal I/R

injury, we examined the phosphorylation of Akt in renal tissue. We found that within 24 h of reperfusion, the level of Akt phosphorylation in tissues was slightly increased (**Figure 3A**). Pre-treatment with SC79 resulted in a significant increase of Akt phosphorylation levels (**Figure 3A**).

We next evaluated the effect of SC79 on the pro-apoptotic molecules Bax, bad, and Bim, and the anti-apoptotic molecules Bcl-2 and bcl-xL in renal tissue following ischemia-reperfusion. The results showed that renal tissue I/R increased expression of the pro-apoptotic molecules Bax, bad, and Bim, and decreased expression of the anti-apoptotic molecules Bcl-2 and bcl-xL, and increased cleaved caspase-3 expression. SC79 administration effectively inhibited expression of Bax, bad, Bim, and cleaved caspase-3 and increased Bcl-2 and bcl-xL expression (**Figure 3A**). In addition, *in vitro* experiments showed that SC79 administration also increased Akt phosphorylation and Bcl-2 and bcl-xL levels, and effectively inhibited expression of Bax, bad, Bim, and cleaved caspase-3 (**Figure 3B**). However, pretreatment with LY294002 completely reversed the effect of SC79 on Akt phosphorylation and its downstream molecules (**Figure 3**).

Increasing evidence has shown that activation of PI3K/Akt signaling has important biological functions in protecting against H/R-induced cell injury [21]. Thus, activation of PI3K/AKT signaling has become a primary goal of therapeutic intervention for protection against I/R-induced liver injury. SC79 is a novel and safe small molecule compound, which can be used as a selective, highly efficient, and cell-permeable Akt activator [10]. SC79 protected against early brain injuries through the dual activities of antioxidation and anti-apoptosis [11]. In addition, SC79 has no significant side effects in experimental mice and human cells [10]. In the present study, we further investigated the potential therapeutic effects and mechanism of SC79 in renal I/R injury. Our results showed that the administration of SC79 significantly improved renal function, and reduced cell apoptosis in renal I/R injury models, suggesting the beneficial effect of SC79 against renal I/R injury.

The PI3K/Akt pathway was originally recognized to play a crucial role in regulating the

growth and survival of cells, and has recently been implicated in the protection of numerous organs against I/R injury by cell apoptosis [22-25]. In fact, activation of the PI3K/AKT signaling pathway plays a crucial role in the proliferation of renal tubular epithelial cells after I/R injury [26]. In this study, SC79 significantly decreased cell apoptosis and increased the cell proliferative effect in H/R-induced renal tubular epithelial cells. SC79 treatment also blocked cleavage of caspase-3 in injured kidney and renal tubular epithelial cells, and reduced the number of TUNEL-positive cells in the injured kidney. However, the PI3K inhibitor LY294002 significantly antagonized the proliferative and apoptotic effect of SC79 in H/R-induced renal tubular epithelial cells and attenuated renal I/R injury.

Previous studies showed that SC79 preferentially activated PI3K/Akt signaling, thereby protecting against H/R-induced cell injury. In this study, SC79 significantly upregulated phosphorylated levels of Akt and its downstream signaling molecules bcl-2 and bcl-XL, and downregulated its downstream signaling molecules bax, bad, and Bim, whereas the PI3K inhibitor LY294002 significantly antagonized the effect of SC79, suggesting that the anti-apoptotic effect of SC79 may be one of several mechanisms by which SC79 attenuated renal I/R injury.

In conclusion, our results demonstrate that SC79 ameliorated pathological alterations and improved renal function following I/R injury. The underlying mechanisms include suppression of renal tubular cell apoptosis, associated with the activation of PI3K/Akt signaling. Thus, SC79 may be an important therapeutic agent for the treatment of acute kidney injury.

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### Disclosure of conflict of interest

None.

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## SC79 protects rat renal I/R injury

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