**Avens Publishing Group**
J Integrative Med Ther
March 2017 Vol.:4, Issue:1


Open Access Research Article

Journal of Integrative Medicine & Therapy

# Salvianolic Acid a Ameliorates Cisplatin-Induced Nephrotoxicity in Mice

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**Submission:** 10 February, 2017
**Accepted:** 20 February, 2017
**Published:** 06 March, 2017



**Keywords:** Salvianolic acid A; Cisplatin; Nephrotoxicity

## Abstract

Cisplatin (DDP) was widely used for solid tumor chemotherapy. Unfortunately, nephrotoxicity is a frequent devastating adverse effect of DDP. Salvianolic acid A (SAA), a Chinese drug metamer, had protection effect against chronic kidney injury. However, the effect of SAA on nephrotoxicity that induced by DDP remains unclear. In this study, we investigated the effect of SAA on nephrotoxicity that induced by DDP. In addition, the underlying mechanism in this effect has been also explored. In this experiment, SAA was administered by oral gavage at doses of 17.6 mg/kg for 4 weeks after intraperitoneal DDP 20 mg/kg injection. The detection of serum creatinine, blood urea nitrogen (BUN) and urinary protein of 24 h was performed to evaluated renal function. The protein level of X-linked inhibitor of apoptosis protein (XIAP) and Survivin in was detected by Western blotting. The expression of fibronectin and collagen in kidney tissue was determined by immunofluorescence assay. In addition, the activity of p38 MAPK pathway in HK-2 cells was also evaluated. Our results showed that SAA decreased the level of creatinine, BUN and urea protein. The DDP-induced kidney damage was also ameliorated. The down-regulation of XIAP and Survivin in protein induced by DDP was restored by SAA treatment. Moreover, SAA impaired DDP-induced renal fibrosis through inhibiting the expression of fibronectin and collagen. In vitro, SAA could alleviate the increase phosphorylation level of p38 MAPK that induced by DDP in HK-2 cells. In summary, our results showed that SAA has a protective effect against DDP-induced nephrotoxicity. In addition, p38 MAPK signaling pathway might play a important role in this protection effect of SAA. SAA may be useful for preventing nephrotoxicity in cancer patients receiving DDP chemotherapy.

## Introduction

Cisplatin (cis-diamminedichloro-platinum, DDP) is one of the principal chemotherapeutic agents used for the treatment of solid tumor [1,2]. Unfortunately, nephrotoxicity is a frequent devastating adverse effect of DDP chemotherapy [3,4]. Renal tubular cells are particularly sensitive to DDP. Depending on its concentration, DDP induces necrosis and apoptosis in renal tubular cells, resulting in chronic kidney injury (CKI) and fibrosis [5,6]. Currently, there is no effective therapy for nephrotoxicity induced by DDP [7]. Therefore, it is essential to develop a new therapy for DDP-induced nephrotoxicity.

Salvianolic acid A (SAA), a Chinese drug metamer, was used commonly for CKI treatment. Its molecular formula is shown in Figure 1. Previous studies demonstrated that SAA had protection effect of kidney injury and anti-fibroplastic proliferation [8,9]. However, the effect of SAA on nephrotoxicity that induced by DDP remains unclear. Therefore, the aim of this study is to investigate the effect of SAA on nephrotoxicity that induced by DDP. In addition, the underlying mechanism in this effect has been also explored.

## Materials and Methods

### Animals

The animal experiments were conducted according to the Guidelines of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. C57BL/6 mice were purchased from the Shanghai Super-B&K Laboratory Animal Corporation [License number: SCXK (Hu) 2008-0016]. Male C57BL/6 mice, 8-10 weeks, weighting about 20-30 g, were randomly divided into 4 groups (n=6): (1) group control; (2) group DDP; (3) group SAA; (4) group SAA+DDP. The intraperitoneal injection of DDP (Sigma-Aldrich, St. Louis, MO, USA) was performed by dissolving DDP in a physiological solution. Mice in group DDP were received DDP a single injection of DDP (20 mg/kg). Mice in group SAA was given SAA (17.6/mg/kg/d) by intragastric administration for 4 weeks. Mice in group SAA+DDP was given SAA (17.6/mg/kg/d) by intra-gastric administration for 4 weeks after a single injection of DDP (20 mg/kg). Animals were sacrificed at 4 W after SAA administration. Kidneys were then perfused with PBS and collected.

### Measurement of Renal Function

Serum creatinine, blood urea nitrogen (BUN) and urinary protein

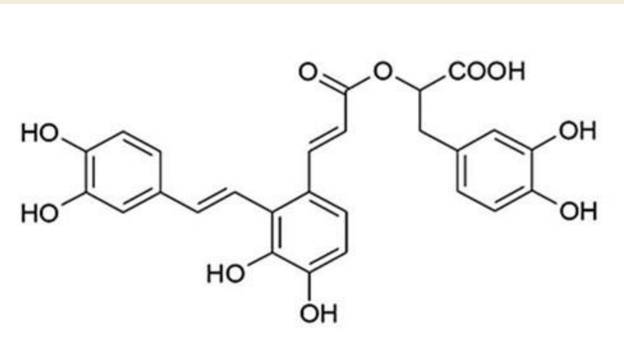


**Figure 1:** The molecular formula of salvianolic acid A.

ISSN: 2378-1343

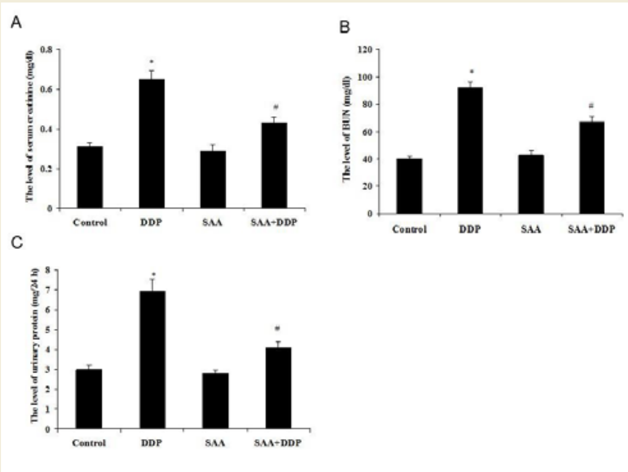


**Figure 2:** The effects of SAA on serum creatinine, BUN and urinary protein DDP-treated mice. (A), The level of serum creatinine of mice after DDP or (and) SAA treatment. (B), The level of BUN of mice after DDP or (and) SAA treatment. (C), The level of urinary protein of mice after DDP or (and) SAA treatment. *P<0.05 vs control; # P<0.05 vs DDP. n=6 each group. BUN=blood urea nitrogen; SAA= salvianolic acid A.

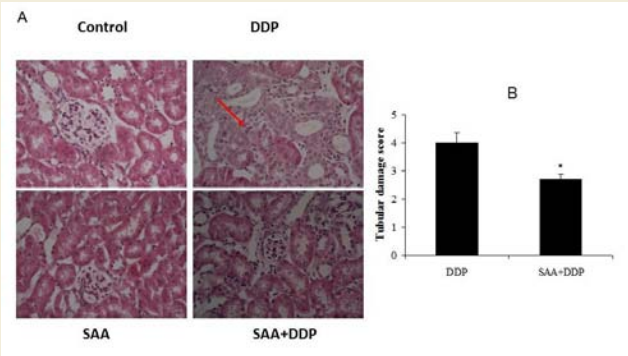


**Figure 3:** SAA protects kidney against DDP-induced injury. (A), Representative photomicrographs of hematoxylin and eosin staining for kidney sections of mice after DDP or (and) SAA treatment. (B), Quantitative histological assessment of tubular damage after DDP or (and) SAA treatment in mice. *P<0.05 vs DDP. n=6 each group. DDP=cisplatin; SAA= salvianolic acid A

Glomerular obvious swelling, hyperplasia and hypertrophy, irregular arrangement, mesangial cells grow thick, local conglutination and hardening, broadening apparent substrate membrane, malpighian tube cavity is not complete, free bubble or between adjacent particles, more protein type tube, inflammatory cells.

were measured by standard methods using a FUJI DRI-CHEM 3500i (Fuji Photo Film, Tokyo, Japan). For the urinary analysis, mice were housed individually in metabolic cages for 24 h urine collection.

**Renal morphology**

Kidney tissue was fixed in 10% buffered formalin, embedded in paraffin, and cut at 5 μm thick sections. After deparaffinization and rehydration, sections were stained with hematoxylin for 6 minutes and eosin for 30 minutes or sirius red for 6 hours. Kidney damage was examined and scored according to the percentage of damaged tubules: 0, no damage; 1, <25% damage; 2, 25-50% damage; 3, 50-75% damage; and 4, >75% damage.

**Immunofluorescence assay**

Kidney tissues were embedded, cut at 6 μm thickness, and mounted. After fixation and protein block, kidney sections were incubated with rabbit anti-fibronectin antibody (Sigma, USA) or rabbit anti-collagen I antibody (Abcam, USA) followed by Alexa 488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA). Slides were mounted with DAPI. Fluorescence intensity was analyzed using a microscope (Nikon Instruments Inc., USA). Quantitative evaluation of sections stained with antibodies to fibronectin and collagen I was performed using NIS-Elements Br 3.0 software.

**Western blotting**

Protein was extracted using RIPA buffer containing cocktail proteinase inhibitors and quantified with a Bio-Rad protein assay. For non-reducing conditions, 2-mercaptoethanol and dithiothreitol were excluded from the sample buffer. Protein samples were separated by to SDS-polyacrylamide gel, and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, USA). The membrane was blocked by protein block solution for 1 h at RT. The membrane was then washed with TBST (3×5 minutes), and was incubated for 24 h at 4 °C with 1/1000 dilution of XIAP antibodies (Abcam, USA), 1/1000 dilution of Survivin in antibodies (Abcam, USA), 1/1000 dilution of p-p38 and p38 MAPK antibodies (Abcam, USA), 1/5000 dilution of GAPDH antibodies respectively. After being washed with TBST (3×5 minutes), the followed by incubation with appropriate fluorescence-conjugated secondary antibodies. The proteins of interest were analyzed using an Odyssey IR scanner, and signal intensities were quantified using NIH Image/J software (National Institutes of Health, Bethesda, MD).

**Human proximal tubular cell culture**

Human proximal tubular cells (HK-2 cells) were purchased from Chinese Academy of Science (Shanghai, China). Cells were seeded in DMEM containing 10% fetal bovine serum (FBS), a 1% streptomycin-penicillin mixture in an atmosphere of 5% $CO_2$ and 95% air at 37 °C in a humidified incubator. HK-2 cells were treated in presence and absence of DDP 50 μM or (and) SAA 50 μM for 4 hours.

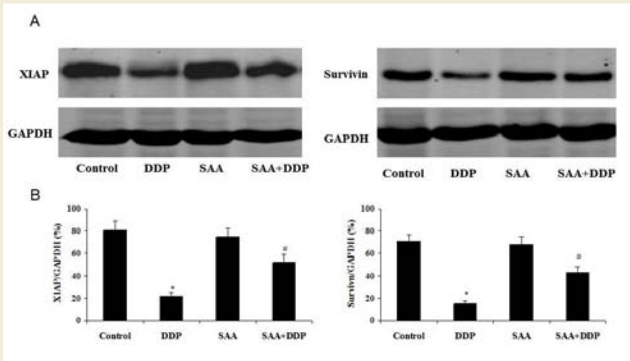


**Figure 4:** The effects of SAA on DDP-induced protein level of XIAP and Survivn. (A), Representative Western blotting of XIAP and Survivin in the kidneys of mice after DDP or (and) SAA treatment. (B), Quantitative analysis of XIAP and Survivin in the kidneys of mice *P<0.05 vs control, # P<0.05 vs DDP. n=6 each group. DDP=cisplatin; SAA= salvianolic acid A.

ISSN: 2378-1343

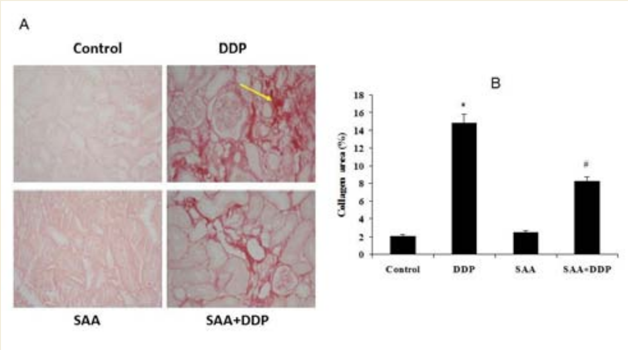


**Figure 5:** SAA reduces DDP-induced collagen deposition in kidney. (A), Representative photomicrographs of Sirius red staining for kidney sections of mice after DDP or (and) SAA treatment. (B), Quantitative analysis of interstitial collagen content in the kidneys of mice. *P<0.05 vs control; # P<0.05 vs DDP. n=6 each group. DDP=cisplatin; SAA= salvianolic acid A.

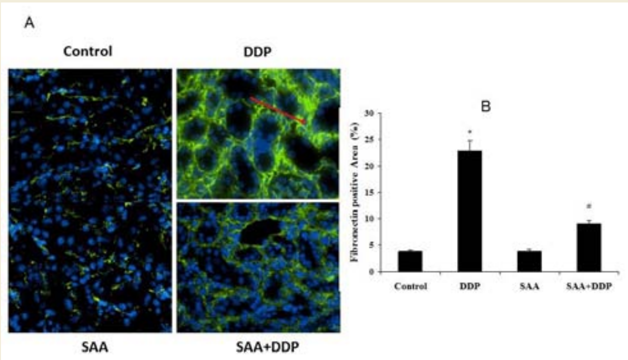


**Figure 6:** SAA reduces DDP-induced fibronectin expression in kidney. (A), Representative photomicrographs of fibronectin immunofluorescence staining in the kidneys of mice after DDP or (and) SAA treatment. (B), Quantitative analysis of fibronectin positive area in the kidneys of mice. *P<0.05 vs control, # P<0.05 vs DDP. n=6 per group. DDP=cisplatin; SAA= salvianolic acid A.

### Statistical analysis

Data analysis was performed by SPSS for Windows1 (v.16.0; SPSS Inc, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Multiple group comparisons were assessed by one-way analysis of variance (ANOVA). LSD test were used for post hoc comparisons. *P*<0.05 was considered statistically significant.

## Results

### SAA decreased the level of creatinine, BUN and urinary protein

As shown in Figure 2 the level of serum creatinine in group DDP was increased markedly as compared to group control. The level of serum creatinine in group SAA + DDP was lower than that in group DDP. Compared with group control, the level of BUN in group DDP was increased significantly. The level of BUN in group SAA+DDP was decreased significantly as compared with group DDP. The level of urinary protein in group DDP was increased markedly as compared to group control. The level of urinary protein in group SAA + DDP was lower than that in group DDP. These results indicated that SAA protects kidney against injury that induced by DDP.

### SAA ameliorated kidney tubular damage that induced by DDP

The kidney tubular damage was evaluated by hematoxylin and eosin (HE) staining. As shown in Figure 3, HE staining showed DDP-treated mice developed tubular injury as compared with mice in group control. The tubular injury that induces by DDP was attenuated by SAA treatment. The results indicated that SAA could ameliorate kidney tubular damage that induced by DDP treatment.

### SAA increased the protein level of anti-apoptosis

XIAP and Survivin in are anti-apoptosis proteins. They were determined by Western blotting. As shown in Figure 4, the expression of XIAP protein was decreased strongly after DDP treatment. The level of XIAP in group SAA+DDP was increased significantly as compared to group DDP. DDP treatment down regulated significantly the protein level of Survivin in. Compared with group DDP, the expression of Survivin in protein in group SAA+DDP was increased significantly. The results indicated that SAA ameliorates kidney tubular damage via up regulating XIAP and Survivin in.

### SAA attenuated renal fibrosis that induced by DDP

As shown in Figure 5, Sirius red staining showed that DDP-treated mice developed significant collagen deposition in the kidney tissue as compared with mice in group control. These fibrotic responses that induced by DDP were markedly decreased after SAA treatment. These results indicated that SAA could attenuated DDP-induced renal fibrosis.

### SAA decreased the expression of renal fibrosis-related protein

Renal fibrosis-related protein was determined by Western blotting. As shown in Figure 6, the expression of fibronectin was increased significantly after DDP treatment. The level of fibronectin in group SAA+DDP was decreased markedly as compared to group DDP. As shown in Figure 7, DDP treatment increased significantly the level of Collagen. Compared with group DDP, the expression of Collagen□protein was down-regulated significantly in group SAA+DDP. The results indicated that SAA attenuated renal fibrosis by downregulating fibronectin and Collagen.

### SAA down-regulated the activity of p38 MAPK signaling pathway

Figure 8 depict changes in the phosphorylation state of p38 MAPK

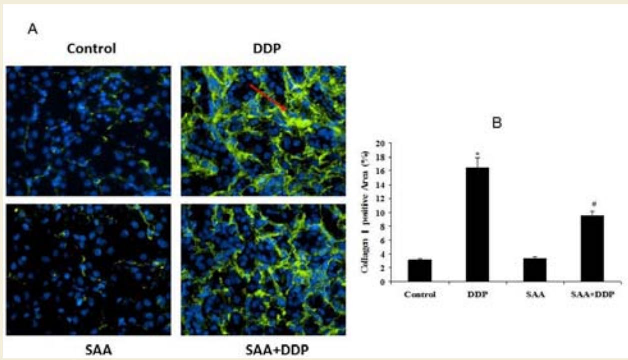


**Figure 7:** SAA reduces DDP-induced collagen I expression in kidney. (A), Representative photomicrographs of collagen I immunofluorescence staining in the kidneys of mice after DDP or (and) SAA treatment. (B), Quantitative analysis of collagen I positive area in the kidneys of mice. *P<0.05 vs control, # P<0.05 vs DDP. n=6 each group. DDP=cisplatin; SAA= salvianolic acid A.

ISSN: 2378-1343

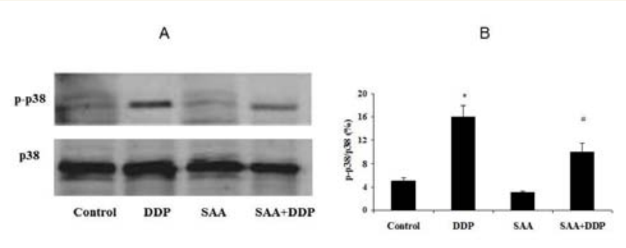


**Figure 8:** The effects of SAA on DDP-induced phosphorylation level of p38 MAPK in HK-2 cells. (A), Representative Western blotting of p38 MAPK in HK-2 cells after DDP or (and) SAA treatment. (B), Quantitative analysis of p38 MAPK in the kidneys of HK-2 cells *P<0.05 vs control, # P<0.05 vs DDP. n=6 each group. DDP=cisplatin; SAA= salvianolic acid A.

signaling pathway proteins elicited by DDP. The activity of p38 MAPK signaling pathway in HK-2 cells was evaluated by Western blotting. The phosphorylation level of p38 MAPK was increased markedly after HK-2 cells exposure to DDP. The phosphorylation level of p38 MAPK in group SAA+DDP was reduced significantly as compared to group DDP. These results indicated that SAA could alleviate the increase of p38 MAPK phosphorylation level that induced by DDP.

## Discussion

DDP has been successfully used as a chemotherapeutic agent in the treatment of solid tumors. However, nephrotoxicity is the primary toxic side effect of cisplatin administration and approximately 20-30% of patients result in renal dysfunction and kidney fibrosis after DDP treatment [10]. Therefore, finding an effective way to prevent DDP-induced nephrotoxicity is a critical issue. SAA has earlier been found to show anti-nociceptive and anti-cancer effects [11,12]. We studied for the first time the protective effects of SAA against DDP-induced nephrotoxicity. In this study, our results showed that the mice treated with DDP developed renal dysfunction, tubular damage and renal fibrosis. SAA treatment markedly attenuated DDP-induced these manifestations. In vitro experiment, we demonstrated that SAA significantly decreased the phosphorylation level of p38 MAPK, events that were induced in proximal tubular epithelial cells by treatment with DDP. These results suggested that SAA might be a good drug for preventing DDP-induced nephrotoxicity.

Apoptosis of renal tubular epithelial cells is a major cause of DDP-induced nephrotoxicity and kidney tubular injury. XIAP and Survivin in are 2 potent members of the inhibitor of apoptosis protein gene family. XIAP and Survivin in can bind directly to caspase-3, -7, and -9, to block their activities, and inhibit apoptosis [13,14]. Our results clearly showed a significant increase of creatinine, BUN and proteinuria in DDP-treated mice. Also, the protein level of XIAP and Survivin in kidney of DDP-treated mice were significant down-regulated. Moreover, we found that DDP-induced down-regulation of XIAP and Survivin in was substantially restored by SAA. These results indicated that the ameliorating effect of DAA on DDP-mediated nephrotoxicity and tubular damage was associated with up-regulation of XIAP and Survivin in.

Renal fibrosis is common consequence of CKI [15]. In this study, we found renal fibrosis in mice was induced by DDP treatment and SAA was able to protect kidney against DDP-induced renal fibrosis. However, the underlying mechanism of the anti-fibrosis effect of SAA remains unclear. Collagen I and fibronectin are two major components of extracellular matrix which are highly correlated the renal fibrosis [16,17]. To elucidate how SAA could protect kidney against fibrosis, we detected that the expression of collagen I and fibronectin in the tissue of kidney. We found that SAA attenuated the increase of collagen I and fibronectin that induced by DDP, suggesting that the anti-fibrosis effect of SAA was involved in decreasing the expression of collagen I and fibronectin.

It has been well established that p38 MAPK signaling pathway regulate the apoptosis and proliferation of tubular epithelial cells and play a important role in kidney tubular injury and fibrogenesis. Numerous studies documented that the activation of p38 MAPK pathway in the kidney promotes the upregulation of pro-apoptotic and pro-fibrotic factors, leading to tubular epithelial cells apoptosis and tubulo-interstitial fibrosis [18-20]. Our results showed that DDP treatment increased the activity of p38 MAPK signaling pathway. SAA significantly decreased the up-regulation of p38 MAPK pathway activity that induced by DDP. These results implicated that the effects of SAA attenuating DDP-induced chronic kidney injury may be through inhibiting p38 MAPK signaling pathway.

In summary, our results showed that SAA has a protective effect against DDP-induced nephrotoxicity. In addition, p38 MAPK pathway might play a important role in this protection effect of SAA. SAA may be useful for preventing nephrotoxicity in cancer patients receiving DDP chemotherapy.

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## Acknowledgements

This work was supported by National Nature Science Foundation of China (81173219) and Special Scientific Research in the Chinese Medicine Industry of the Ministry of Science and Technology (201007005).