

Original Research

Salvianolic Acid C Inhibits the Epithelial-Mesenchymal Transition and Ameliorates Renal Tubulointerstitial Fibrosis

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Abstract

Background: Salvianolic acid C (SAC) is a natural compound derived from *Salvia miltiorrhiza* that can protect against renal diseases. The aims of this work were to explore the effect of SAC on kidney tubulointerstitial fibrosis and study the associated mechanism.

Methods: Models for unilateral ureteral obstruction (UUO) and aristolochic acid I (AAI) were established in mice to study renal tubulointerstitial fibrosis. Rat kidney fibroblasts (NRK-49F) and human kidney epithelial cells (HK2) were used as cellular models to evaluate the effects of SAC on kidney fibrosis. **Results:** Treatment with SAC for two weeks reduced the level of renal tubulointerstitial fibrosis in UUO- and AAI-induced fibrotic kidneys, as demonstrated by Masson's staining and Western blot. SAC inhibited extracellular matrix protein expression in NRK-49F cells and TGF- β -stimulated HK2 cells in dose-dependent fashion. Moreover, SAC inhibited the expression of epithelial-mesenchymal transition (EMT) factors in animal and cellular models of kidney fibrosis, as well as the EMT-related transcription factor snail. Furthermore, SAC inhibited the fibrosis-related signaling pathway Smad3 in the fibrotic kidneys of two mouse models and in renal cells. **Conclusions:** We conclude that SAC inhibits EMT and ameliorates tubulointerstitial fibrosis through involvement of the signaling pathway for transforming growth factor- β (TGF- β)/Smad.

Keywords: renal fibrosis; EMT; SAC; CKD

1. Introduction

Chronic kidney disease (CKD) is a major health issue worldwide because of its high prevalence, bad prognosis, and high medical burden [1]. CKD prevalence has been increasing recently and has now reached 14.3% worldwide [2]. Renal tubulointerstitial fibrosis occurs in all CKDs that progress to late-stage kidney disease [3]. This condition is characterized by pathological accumulation of extracellular matrix (ECM) proteins, including fibronectin and type 1 collagen [4]. Signaling through the TGF- β /Smad pathway is an important factor for renal fibrosis, whereby the Smad3 transcriptional factor regulates expression of fibrogenic genes [5,6].

During epithelial-mesenchymal transition (EMT), epithelial cells transform into a mesenchymal phenotype. Up-regulation of EMT genes, including N-cadherin and vimentin, represent hallmarks of renal tubulointerstitial fibrosis [7]. A major inducer of EMT is Snail, which plays a major part in organ fibrosis, including renal tubulointerstitial fibrosis [8,9].

Salvianolic acid C (SAC) is a phenolic acid extracted from *Salvia miltiorrhiza* [10]. The effect of different components of salvianolic acids on renal fibrosis has recently been studied. Salvianolic acid B exerts an anti-fibrotic ef-

fect on kidney diseases by regulating the HPSE/SDC1 axis and activating sirt1-mediated autophagy [11,12]. Salvianolic acid A ameliorates kidney fibrosis in 5/6 nephrectomized rats through inhibition of nuclear factor kappa-B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) signaling pathways [13]. Salvianolic acid A has a similar structure to SAC, and can be converted into SAC by heating and other conditions [14]. SAC inhibits inflammatory responses in several disease models [10,15,16]. Moreover, salvianolic acid A in combination with SAC can inhibit fibrosis in obstructive rat kidneys [17]. The action of SAC alone on kidney tubulointerstitial fibrosis remains unknown, however.

The goal of the current work was to study the effect of SAC on kidney tubulointerstitial fibrosis, as well as the associated mechanism.

2. Material and Methods

2.1 Mice

SPF grade C57BL/6 male mice were obtained from Shanghai Laboratory Animal Company (Shanghai, China) and housed at Shanghai University of Traditional Chinese Medicine in line with their rules and regulations. The animal ethics committee of the Shanghai Univer-



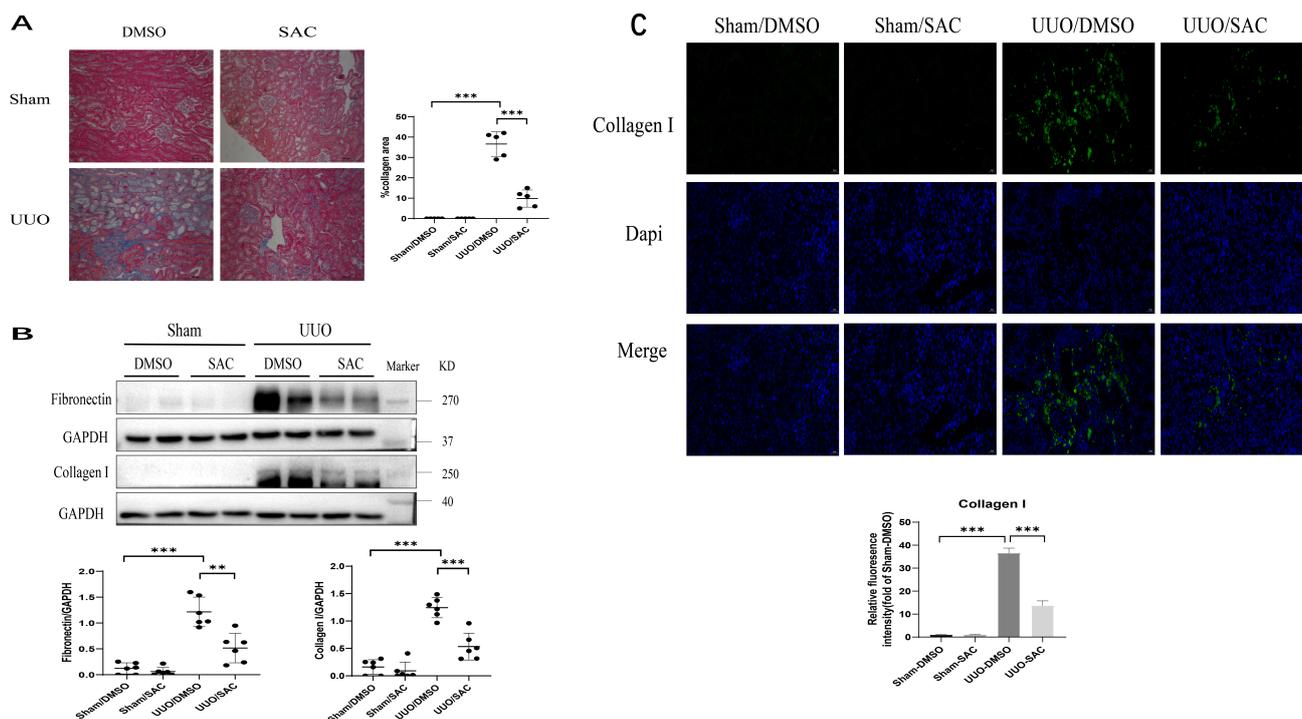


Fig. 1. SAC inhibits renal tubulointerstitial fibrosis in obstructed mouse kidneys. Sham or unilateral ureter obstruction (UUO) operation was performed on mice ($n = 6$), followed by two weeks of treatment with DMSO or salvianolic acid C (SAC). (A) Renal fibrosis was assessed by Masson's trichrome staining and then quantified. Bars = 100 μm . (B) The expression of fibronectin and collagen I were analyzed by Western blotting and then quantified. (C) Representative immunofluorescent images of collagen I staining in sham or UUO mouse kidneys. These were also quantified. DAPI (nuclei) = blue, collagen I = green. Bar = 20 μm . $N = 6$ in each experimental group. One representative of at least three independent experiments is shown. Data represents the mean \pm SD. $**p < 0.01$. $***p < 0.001$.

sity of Traditional Chinese Medicine approved this study (PZSHUTCM18111601).

2.2 Mouse Unilateral Ureteral Obstruction (UUO) Model

Male mice weighing from 18 to 22 g were anesthetized intraperitoneally with 8 mg/kg of pentobarbital before performing an abdominal incision on the left side. This was followed by either a sham operation or left ureteral ligation. Animals were assigned into 4 groups at random: (a) Sham + dimethyl sulfoxide (DMSO) ($n = 6$), (b) Sham + SAC ($n = 6$), (c) UUO + DMSO ($n = 6$), (d) UUO + SAC ($n = 6$). Intraperitoneal injections of DMSO or SAC (10 mg/kg body weight, TopScience, T3149, Shanghai, China) were administered after the surgery and for 14 consecutive days. Mice were sacrificed at day 14, with kidney tissue harvested for protein extraction and tissue embedding.

2.3 Mouse Model Induced by Aristolochic Acid I (AAI)

Mice were injected intraperitoneally with 5 mg/kg AAI (A9451, Sigma, St. Louis, MO, USA) in normal saline (NS) or with NS only once per week for 2 weeks (2 injections in total). One week after the second injection of AAI, the DMSO or SAC (10 mg/kg body weight, TopScience, T3149, Shanghai, China) was injected intraperitoneally and

daily for two weeks ($n = 6$). Mice were sacrificed after the treatment, and kidneys were collected for protein extraction and tissue embedding.

2.4 Cell Culture

Cells were grown as previously described by our group [18]. Normal rat kidney interstitial fibroblasts (NRK-49F) rat kidney interstitial fibroblast cells were obtained from the National Infrastructure Cell Line Resource, Chinese Academy of Medical Sciences (cat. 3111C0001CCC000413), which has been identified for the species by PCR and was negative for mycoplasma contamination. The cell line has been tested for the mycoplasma and been authenticated from the Chinese Academy of Medical Sciences. The cell line is originated from American Type Culture Collection, VA, USA (Cat. No. CRL1570). The culture medium for NRK-49F cells was Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin. NRK-49F cells were grown in 6-well plates until 60–70% confluence was reached, then starved in medium containing just 0.5% FBS for 12 h. After overnight starvation, fresh medium with 0.5% FBS was added and the cells treated with DMSO or with vari-

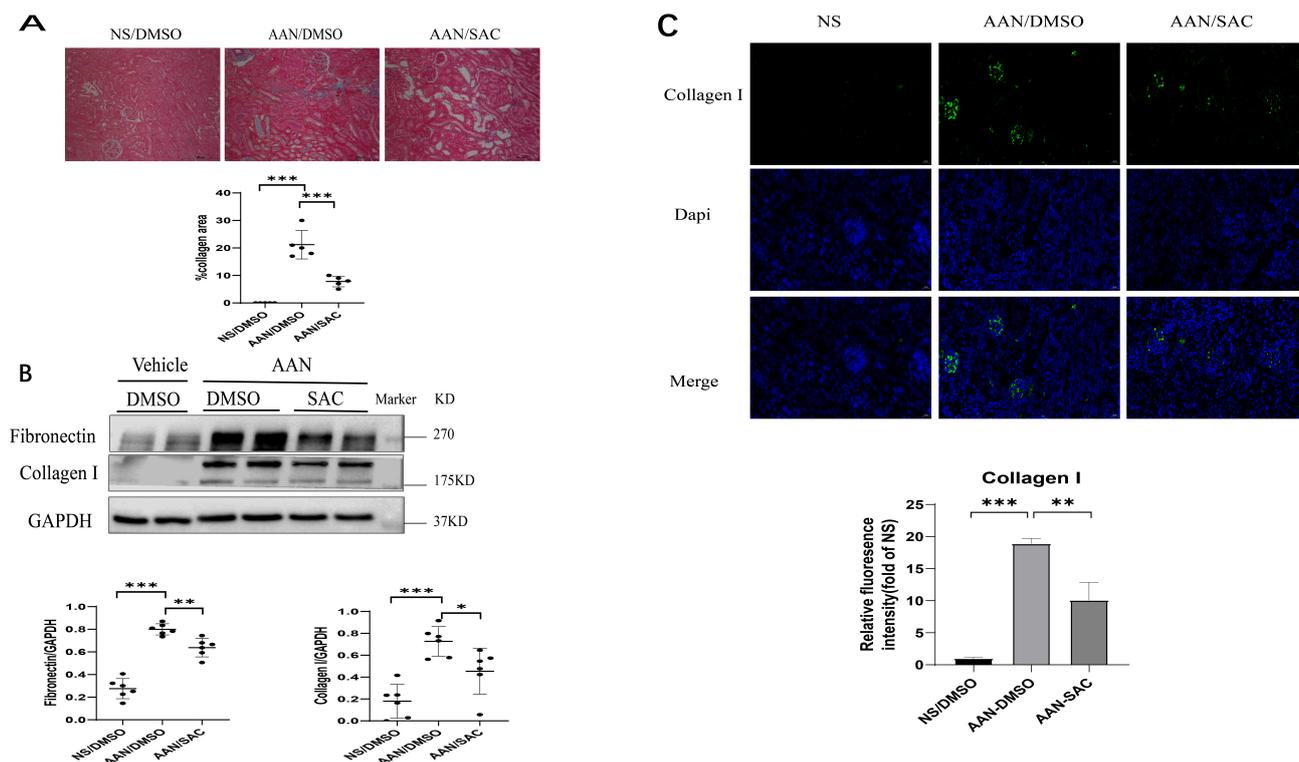


Fig. 2. SAC attenuates the development of renal tubulointerstitial fibrosis in mice with aristolochic acid nephropathy (AAN). Mice ($n = 6$) were injected intraperitoneally with aristolochic acid I (AAI) or normal saline (NS) once a week for two weeks. One week after the second injection of AAI or NS, mice were treated with DMSO or SAC (10 mg/kg) for two weeks. (A) Renal fibrosis was assessed by Masson's trichrome staining and then quantified. Bar = 100 μ m. (B) The expression of fibronectin and collagen I were analyzed by Western blotting and then quantified. (C) Representative immunofluorescent images of collagen I staining in control or AAN mouse kidneys. These were also quantified. DAPI (nuclei) = blue, collagen I = green. Bar = 20 μ m. $N = 6$ in each experimental group. One representative of at least three independent experiments is shown. Data represents the mean \pm SD. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

ous concentrations of SAC (TopScience, T3149, Shanghai, China). The kidney proximal tubular epithelial cell line Human Kidney 2 (HK2, cat. SCSP-511) was purchased from the Cell Bank, Shanghai Institute of Biological Sciences (Chinese Academy of Science), which has been validated by short tandem repeat profiling and was negative for mycoplasma contamination. The cells were grown in 6-well plates to 40–50% confluence, then starved overnight with DMEM/F12 containing just 0.5% FBS. The following day, fresh DMEM/F12 with 0.5% FBS was added, together with 2.5 ng/mL TGF- β (PeproTech, 100-21, Rocky Hill, NJ, USA) and different SAC concentrations for 48 h. The SAC concentrations used here were the same as those used in two previous publications [15,19].

2.5 Masson's Trichrome Staining

Staining with Masson's trichrome was carried out as described earlier [18]. Briefly, paraffin-embedded renal tissue sections (4 μ m) were cut and then stained using hematoxylin and ponceau, followed by incubation with phosphomolybdic acid. Kidney tissue was then stained using ani-

line blue and acetic acid and photographed using a Nikon 80i microscope (Nikon Eclipse 80i, Tokyo, Kanto Plain, Japan).

2.6 Western Blot

Proteins were obtained from the kidney tissue of mice as previously described [20]. The Bradford method was employed to measure protein concentrations. Samples were diluted with 5 \times SDS-PAGE loading buffer (P0015L, Beyotime Biotech, Nanjing, Jiangsu, China), separated by SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) by wet blotting. Primary antibodies used for protein detection were anti-snail (1:1000 dilution, A11794, Abclonal, Woburn, MA, USA), anti-fibronectin (1:5000, ab23750, Abcam, Cambridge, UK), anti-pSmad3 (1:1000, ET1609-41, HUABIO, Woburn, MA, USA), anti-Smad7 (1:1000, AB37036, Aboci, MD, USA), anti-collagen I (1:5000, ab260043, Abcam), anti-N-cadherin (1:500, sc-59987, Santa Cruz, Dallas, TX, USA), anti-vimentin (1:1000, ET1609-36, HUABIO), anti-GAPDH (1:5000, 60004-1-

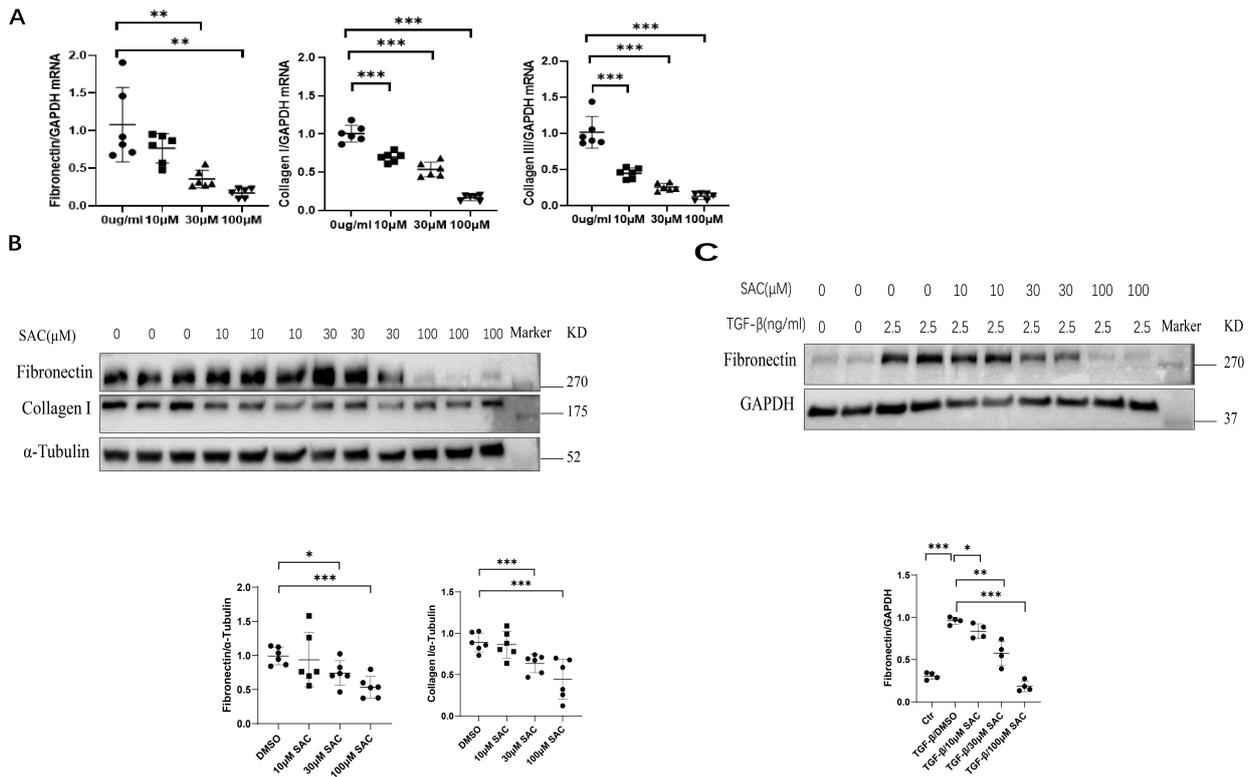


Fig. 3. SAC reduces ECM protein expression in renal cells. Rat renal fibroblasts (NRK-49F) were starved for 12 h, then treated for 24 h with different concentrations (10 μ M, 30 μ M, 100 μ M) of SAC. (A) The mRNA expression levels for fibronectin, collagen I and collagen III were analyzed by qPCR. (B) The expression of fibronectin and collagen I were analyzed by Western blotting and then quantified. (C) HK2 human renal epithelial cells were starved overnight, stimulated with TGF- β and then treated with various concentrations of SAC. Cell lysates were extracted and the expression of fibronectin was analyzed by Western blotting and then quantified. Data represents the mean \pm SD. One representative result of at least three independent experiments is shown. NS represents not significant. * p < 0.05. ** p < 0.01. * p < 0.001.**

Ig, Proteintech, Wuhan, Hubei, China), and anti- α -tubulin (1:1000, AF0001, Beyotime Biotech, Nanjing, Jiangsu, China). Primary antibodies were incubated overnight at 4 $^{\circ}$ C with membrane, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:1000, A0208 Beyotime Biotech, Nanjing, Jiangsu, China; or goat anti-mouse IgG, 1:1000, A0216 Beyotime Biotech, Nanjing, Jiangsu, China). Signal detection was achieved using enhanced chemiluminescence (BeyoECL Star, P0018A, Beyotime) and protein expression quantified with Image J1.8.0 (LOCI, University of Wisconsin, Madison, WI, USA).

2.7 Immunofluorescence

Immunofluorescence staining of renal tissue was carried out as described previously [21]. In short, paraffin-embedded tissue was cut into 4 μ m sections, blocked with 3% BSA, incubated overnight with anti-collagen I antibody (1:500) at 4 $^{\circ}$ C, rinsed in PBS, then incubated for 60 min with secondary antibody. Cell nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI) solution for 10 min and photographed using a Nikon 80i microscope (Tokyo,

Japan). Mean fluorescence intensity for each slide was analyzed using Image J and normalized to the control.

2.8 RNA Extraction and Quantitative RT-PCR

TRIzol (15596-018, Invitrogen, Carlsbad, CA, USA) was used to obtain total RNA from NRK-49F cells. This was reverse transcribed into cDNA using a Takara Prime-Script RT kit (RR0036A, Kyoto, Japan). Primer sequences for qPCR and specific for rat were: Fibronectin (F, forward), 5'-CCGAATCACAGTAGTTGCGG-3'; Fibronectin (R, reverse), 5'-GCATAGTGTCCGGACCGATA-3'; Collagen I (F), 5'-TCAAGATGGTGGCCGTTACT-3'; Collagen I (R), 5'-CATCTTGAGGTCACGGCATG-3'; Collagen III (F), 5'-ATGAATTGGGATGCAACTAC-3'; Collagen III (R), 5'-TCTAGTGGCTCATCATCACA-3'; GAPDH (F), 5'-TAAAGGGCCTCCTGGGCTACACT-3'; GAPDH (R), 5'-TTACTCCTTGGAGCCATGTAGG-3'. qPCR was performed using FastStart Universal SYBR Green Master (Rox) (Cat. 4913850001; Merck, Darmstadt, Germany) and StepOne Plus Sequence Detection System (Applied Biosystems, Foster City, CA, United States). Relative expression levels for each gene were analyzed using $2^{-\Delta\Delta Ct}$

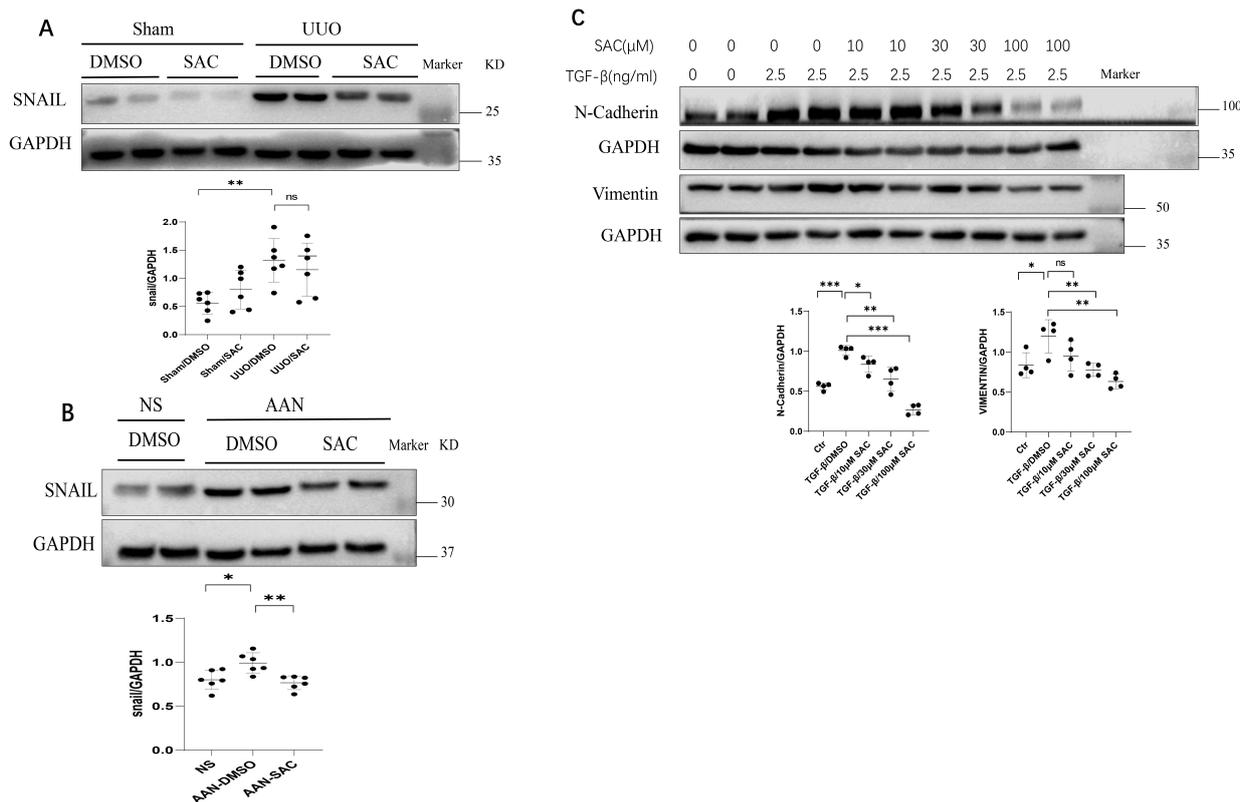


Fig. 4. SAC inhibits epithelial-mesenchymal transition in fibrotic kidneys and renal fibrotic cells. (A) The expression of Snail in sham or UUO kidneys ($n = 6$) was analyzed by Western blotting and then quantified. (B) The expression of Snail in NS or AAN kidneys ($n = 6$) was analyzed by Western blotting and then quantified. Data represents the mean \pm SD. (C) HK2 human renal epithelial cells were starved overnight, then stimulated with TGF- β and treated with various concentrations of SAC for 24 h. The expression of N-cadherin and of vimentin was analyzed by Western blotting and then quantified. One representative result of at least three independent experiments is shown. n.s. represents not significant. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

methods and normalized with GAPDH. Results are shown as the fold-change in mRNA expression.

2.9 Statistical Analyses

Results are given as mean \pm SD. Student t -test was used to compare two groups with GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA). A p -value < 0.05 was considered as representing statistical significance. Each experimental group had an $n = 6$. Representative results from at least three independent experiments are presented. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

3. Results

3.1 SAC Inhibits Renal Tubulointerstitial Fibrosis in Obstructed Mouse Kidneys

The effect of SAC on kidney tubulointerstitial fibrosis was first investigated *in vivo*. UUO is a classic mouse model for studying kidney fibrosis and was established here for this purpose. UUO mice received DMSO or SAC treatment for two weeks. Compared with sham operated mice, significant deposition of collagen was detected using Masson's trichrome staining in UUO kidneys. How-

ever, this was attenuated by two weeks of treatment with SAC (Fig. 1A). Western blot experiments showed that expression of two ECM proteins, fibronectin and collagen I, was up-regulated in mouse kidneys after UUO surgery, but was lowered by treatment with SAC (Fig. 1B). Elevated levels of collagen I expression in the interstitial areas of UUO kidneys relative to sham kidneys was also shown by immunofluorescent staining. Again, this was significantly attenuated by SAC treatment (Fig. 1C).

3.2 SAC Attenuates Kidney Tubulointerstitial Fibrosis in the Mouse AAN Model

The effects of SAC on anti-fibrosis were further studied in mice with chronic aristolochic acid nephropathy (AAN). Masson's trichrome revealed mild staining in the interstitial areas of AAN kidneys (Fig. 2A). Treatment with SAC for two weeks significantly reduced the positive areas shown by Masson's trichrome staining in AAN kidneys (Fig. 2A). Fibronectin and collagen I expression were increased in AAN kidneys relative to the control kidneys. SAC treatment was observed to reduce the expression of these ECM proteins (Fig. 2B). Up-regulation of collagen

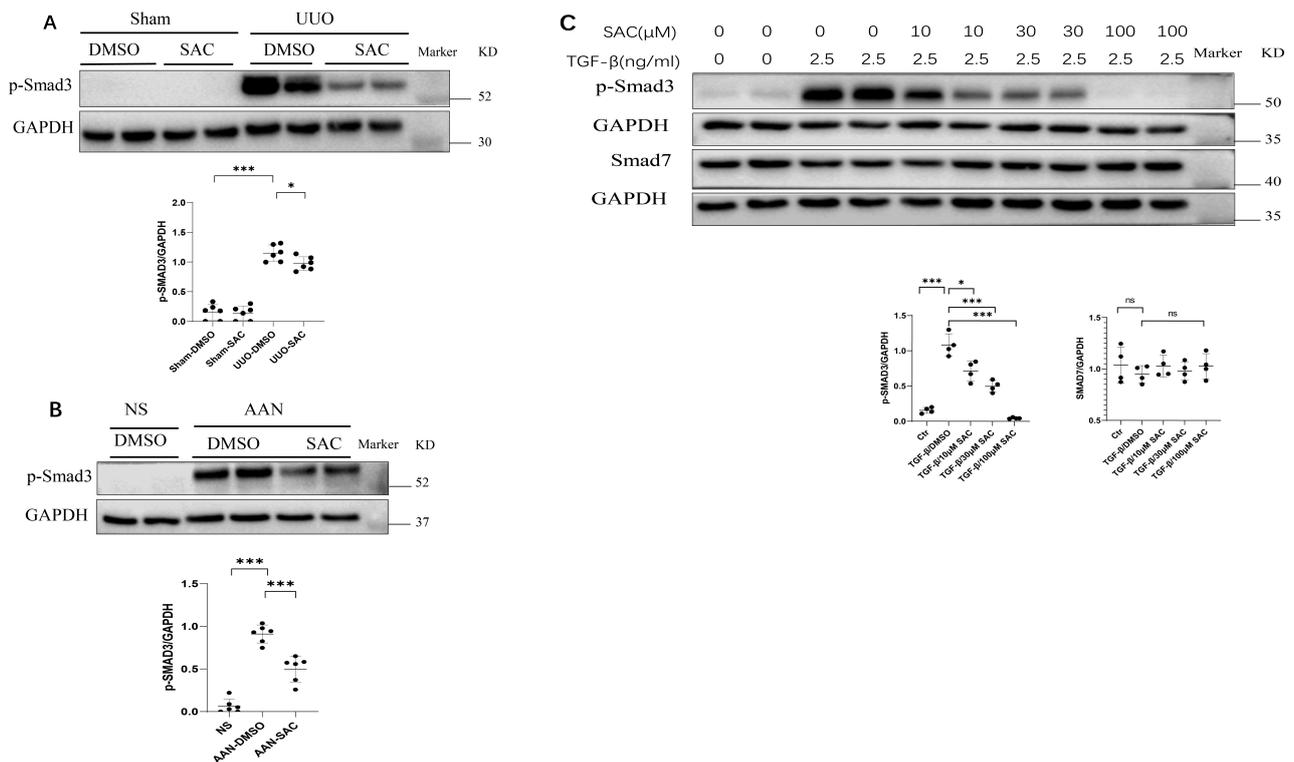


Fig. 5. SAC inactivates the Smad signaling pathway in fibrotic kidneys and renal fibrotic cells. (A) The expression of phosphorylated Smad3 (pSmad3) in sham or UUO kidneys ($n = 6$) was analyzed by Western blotting and then quantified. (B) The expression of pSmad3 in NS or AAN kidneys ($n = 6$) was analyzed by Western blotting and then quantified. (C) HK2 human renal epithelial cells were starved overnight, then stimulated with TGF- β and treated with various concentrations of SAC for 24 h. The expression of pSmad3 and of Smad7 was analyzed by Western blotting and then quantified. Data represents mean \pm SD. One representative result of at least three independent experiments is shown. n.s. represents not significant. * $p < 0.05$. *** $p < 0.001$.

I in the interstitial areas was further confirmed by immunofluorescent staining observed in AAN kidneys relative to control kidneys (Fig. 2C). SAC treatment significantly reduced positive areas of collagen I staining in AAN kidneys (Fig. 2C).

3.3 SAC Inhibits ECM Protein Expression in Renal Cells

Direct effects of SAC on kidney cells were further investigated *in vitro* by using the rat fibroblast cell line NRK-49F, as well as the human kidney proximal tubular epithelial cell line HK2. The mRNA levels of fibronectin, collagen I and collagen III in NRK-49F cells were reduced by SAC in dose-dependent fashion from 10 to 100 μ M (Fig. 3A). Western blot analysis showed that SAC also dose-dependently reduced fibronectin and collagen I protein expression in these cells from 30 μ M to 100 μ M (Fig. 3B). The dose-dependent (10 μ M to 100 μ M) inhibition of fibronectin expression by SAC was further shown in HK2 cells stimulated with TGF- β (Fig. 3C).

3.4 SAC Inhibits EMT in Fibrotic Kidneys and Renal Fibrotic Cells

The effect of SAC on EMT was evaluated *in vivo* as well as *in vitro*. Snail expression in UUO and AAN

kidneys was increased compared to their respective controls (Fig. 4A,B). However, treatment with SAC reduced renal expression of Snail in these mouse models. Increasing doses of SAC (10 μ M to 100 μ M) also progressively inhibited N-cadherin and vimentin expression in TGF- β -stimulated HK2 cells (Fig. 4C).

3.5 SAC Inhibits Smad3 Signaling in Fibrotic Kidneys and Renal Fibroblasts

We next evaluated the effects of SAC on Smad3 signaling *in vivo* and *in vitro*. Fig. 5A,B show that Smad3 phosphorylation was enhanced in fibrotic kidneys of UUO or AAN mice compared to their controls. SAC treatment decreased Smad3 phosphorylation (pSmad3) levels in UUO and AAN kidneys (Fig. 5A,B). Moreover, SAC inhibited pSmad3 in TGF- β -stimulated HK2 cells in a dose-dependent fashion from 10 μ M to 100 μ M. This correlated with up-regulated expression of the anti-fibrotic protein Smad7 (Fig. 5C).

4. Discussion

The beneficial effects of SAC were recently demonstrated in cisplatin-induced acute kidney injury [10]. The effects of SAC on kidney fibrosis, however, are still un-

known. To examine the role played by SAC in kidney tubulointerstitial fibrosis, we established the UUO and AAN mouse models. We showed that two weeks of treatment with SAC reduced collagen deposition in UUO or AAN kidneys, as observed with Masson's staining. Western blot and immunofluorescence methods revealed down-regulation of two ECM proteins (fibronectin and collagen I) following SAC treatment in these two mouse models. The anti-fibrotic effects of SAC on kidney cells were further studied using renal fibroblasts and TGF- β -stimulated kidney epithelial cells. Fibronectin and collagen I expression levels both decreased in a dose-dependent fashion after SAC treatment of renal fibroblasts and TGF- β -stimulated kidney epithelial cells. We conclude from these results that SAC protects against tubulointerstitial fibrosis in kidney diseases.

TGF- β /Smad3 is the classic pro-fibrotic signaling pathway [5,22]. The effect of phenolic acid extracts from *Salvia miltiorrhiza* on the TGF- β /Smad3 signaling pathway has been studied earlier. Zhang *et al.* [23] found that salvianolic acid A ameliorates kidney damage in rats with chronic kidney failure through inhibition of TGF- β signaling. Tao *et al.* [24] reported that salvianolic acid B delayed progression of liver fibrosis by inhibiting TGF- β signaling. Furthermore, inhibition of TGF- β signaling by salvianolic acid B reduces lung fibrosis [25]. Anti-fibrotic effects of salvianolic acid B in renal diseases are thought to be due to inactivation of the TGF- β /Smad pathways [26]. The present study showed for the first time that SAC inhibits TGF- β /Smad3 signaling. Smad7 was found to negatively regulate TGF- β /Smad-mediated pro-fibrotic signaling [27,28]. In our study, down-regulation of Smad7 by TGF- β stimulation was restored by SAC treatment. Thus, we conclude that SAC inhibits signaling by TGF- β /Smad via the inhibition of Smad3 and activation of Smad7.

A characteristic of kidney fibrosis is the upregulation of EMT markers [9]. The transcriptional factor Snail is an important molecular switch for the EMT program and has been involved in renal tubulointerstitial fibrosis [9]. Two studies in gastric cancer and renal fibrosis found that salvianolic acid B inhibits EMT [12,29]. In the present study, SAC was observed to reduce the expression of N-cadherin and vimentin in a dose-dependent fashion in TGF- β -stimulated kidney epithelial cells. Furthermore, SAC inhibition of snail was observed in animal models. We conclude that SAC is an inhibitor of EMT in renal fibrosis.

5. Conclusions

We conclude that SAC inhibits EMT and attenuates kidney tubulointerstitial fibrosis through the signaling pathway for TGF- β /Smad.

Availability of Data and Materials

The data used to support the findings of this research are available upon request.

Author Contributions

MW, DC and CY conceived and coordinated the study. MW wrote the paper. JL conducted the *in vitro* experiments. MW, DH and JL performed the animal experiments. JL performed and analyzed the Western blotting. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

The animal ethics committee of the Shanghai University of Traditional Chinese Medicine approved this study (PZSHUTCM18111601).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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