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M.-J.S. (mingja@ntu.
edu.tw)Antifibrotic effects of KS370G, a
caffeamide derivative, in renal
ischemia-reperfusion injured mice and
renal tubular epithelial cellsSung-Ting Chuang¹, Yueh-Hsiung Kuo^{2,3} & Ming-Jai Su¹¹Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan, ²Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 40402, Taiwan, ³Department of Biotechnology, Asia University, Taichung 41354, Taiwan.

Accumulating evidence suggests that renal tubulointerstitial fibrosis is a main cause of end-stage renal disease. Clinically, there are no beneficial treatments that can effectively reverse the progressive loss of renal functions. Caffeic acid phenethyl ester is a natural phenolic antifibrotic agent, but rapid decomposition by an esterase leads to its low bioavailability. In this study, we evaluated the effects of KS370G, a caffeic acid phenylethyl amide, on murine renal fibrosis induced by unilateral renal ischemia-reperfusion injury (IRI) and in TGF- β_1 stimulated renal tubular epithelial cells (NRK52E and HK-2). In the animal model, renal fibrosis was evaluated at 14 days post-operation. Immediately following the operation, KS370G (10 mg/kg) was administered by oral gavage once a day. Our results show that KS370G markedly attenuates collagen deposition and inhibits an IRI-induced increase of fibronectin, vimentin, α -SMA and TGF- β_1 expression and plasma TGF- β_1 levels in the mouse kidney. Furthermore, KS370G reverses TGF- β_1 -induced downregulation of E-cadherin and upregulation of α -SMA and also decreases the expression of fibronectin, collagen I and PAI-1 and inhibits TGF- β_1 -induced phosphorylation of Smad2/3. These findings show the beneficial effects of KS370G on renal fibrosis *in vivo* and *in vitro* with the possible mechanism being the inhibition of the Smad2/3 signaling pathway.

Tubulointerstitial fibrosis is a common chronic kidney disease with features characterized by tubular atrophy, myofibroblast accumulation and abnormal extracellular matrix (ECM) deposition^{1–3}. Epithelial-mesenchymal transition (EMT) is a process in which renal tubular epithelial cells under pathological conditions can phenotypically convert to fibroblast-like morphology in the tubulointerstitium. This process plays a critical role in the pathogenesis of tubulointerstitial fibrosis⁴. During the EMT process, a repression of epithelial cell adhesion molecules, like E-cadherin and an increase of mesenchymal cell markers, such as α -smooth muscle actin (α -SMA), are essentials for the structural integrity changes occurring in the renal epithelium⁵. Previous studies have shown that many growth factors are involved in renal interstitial fibrosis pathogenesis⁶. TGF- β_1 is one of the main growth factors that stimulate both EMT and ECM deposition through activating the downstream Smad signaling pathway^{7,8}. It is well accepted that TGF- β_1 mediates fibrosis by activating the phosphorylation of Smad2 and Smad3⁹.

Excessive accumulation of ECM proteins, including collagen and fibronectin, is also a key characteristic on renal fibrosis¹⁰. TGF- β_1 has been shown to stimulate the synthesis of ECM proteins and inhibit the degradation of collagen^{11,12}. In a unilateral ureteral obstruction (UUO) model, the obstructed kidneys have higher levels of TGF- β_1 thus inducing the transcription of genes that cause ECM protein accumulation^{13,14}. In addition, TGF- β_1 stimulates ECM proteins accumulation in renal cells by stimulating the expression of protease inhibitors, such as plasminogen activator inhibitor-1 (PAI-1)^{15,16}. PAI-1, a key physiological inhibitor of tissue and urokinase plasminogen activators and is considered to be the most important inhibitor of fibrinolysis^{16,17}. Recent studies show that PAI-1 directly promotes tissue fibrosis through increasing the migration of macrophages, transdifferentiating tubular epithelia, and myofibroblasts¹⁸.

There is much evidence indicating that polyphenolic compounds, such as resveratrol, curcumin and caffeic acid phenethyl ester (CAPE), possess anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-thrombotic, and

Figure 1 | KS370G regulates the expression of fibronectin and collagen deposition in a murine IRI model. (A) Western blot analysis of renal fibronectin expression in sham-operated (sham), ischemia-reperfusion injury (IRI), ischemia-reperfusion injury treatment with vehicle (Veh) and ischemia-reperfusion injury treatment with KS370G 10 mg/kg (K10), 14 days after IRI. Vehicle group was treated with RO water. (B) Quantitative results presented as mean $\pm$ SEM of the signal's optical density (n = 6 samples each group). (C) Representative images of Masson's trichrome staining and Picrosirius Red staining of renal cortex sections in sham, IRI, Veh and K10 groups. Bar = 50 μ m in all panels. (D and E) Quantitative results presented as mean $\pm$ SEM of the percentage of renal fibrosis area and collagen content. *P < 0.001 compared with sham group. #P < 0.001 compared with IRI and Veh groups. Original magnification $\times$ 200.

cardiovascular protective activities in various experimental models^{19–21}. CAPE is one of the major components of honeybee propolis which exhibits antioxidant, anti-inflammatory and anti-diabetic effects^{22,23}. However, rapid decomposition by esterases leads to CAPE's low bioavailability in vivo²⁴. Caffeic acid phenylethyl amide (KS370G), a caffeamide derivative, induces hypoglycemic effects in diabetic mice and is cardiovascular protective in pressure-overload mice hearts^{23,24}. However, it is not known whether KS370G has protective effects in renal fibrosis.

In this study, we investigated the effects of KS370G on renal fibrosis in mice using the IRI model and in human and non-human renal tubular epithelial cells (HK-2 and NRK52E) stimulated by TGF- β_1 . Our results reveal that KS370G inhibits renal fibrosis. We suggest that this inhibition is achieved by blocking the TGF- β /Smad signaling pathway.

Results

KS370G ameliorates fibronectin expression, renal interstitial fibrosis and collagen deposition in IRI kidneys. To examine the effect of KS370G on IRI-induced renal fibrosis, fibronectin, a typical marker

of fibroblast, and renal interstitial fibrosis and collagen deposition were measured. Western blot analysis shows that fibronectin expression increased in the IRI and Veh groups at day14 after the operation and that KS370G (10 mg/kg once a day) decreased fibronectin expression significantly after the IRI operation (Fig. 1A and 1B). Moreover, both Masson's trichrome staining and Picrosirius Red staining also indicate that renal interstitial fibrosis and collagen deposition were elevated in the IRI and Veh groups and KS370G treatment markedly reduced renal interstitial fibrosis and collagen deposition in IRI kidneys (Fig. 1C–1E).

KS370G inhibits α -SMA and vimentin protein expression in IRI kidneys. Next, we determined the effect of KS370G on the expression of myofibroblast activation markers, including α -SMA and vimentin. Western blot analysis shows that the expression of α -SMA and vimentin markedly increased in the IRI and Veh groups compared with sham group, suggesting that activation of myofibroblasts is stimulated following an IRI-induced injury. However, treatment with KS370G significantly decreases α -SMA and vimentin protein expression after the IRI operation (Fig. 2).

Figure 2 | KS370G regulates the expression of α -SMA and vimentin in a murine IRI model. (A) Western blot analysis of renal α -SMA and vimentin expression in sham-operated (sham), ischemia-reperfusion injury (IRI), ischemia-reperfusion injury treatment with vehicle (Veh) and ischemia-reperfusion injury treatment with KS370G 10 mg/kg (K10), 14 days after IRI. Vehicle group was treated with RO water. (B and C) Quantitative results presented as mean $\pm$ SEM of the signal's optical density ($n = 6$ samples each group). * $P < 0.005$ compared with sham group. # $P < 0.005$ compared with IRI and Veh groups.

KS370G reduces kidney tissue TGF- β_1 protein expression and plasma TGF- β_1 levels in IRI kidneys. Compared with the sham group, IRI and Veh groups increased the TGF- β_1 protein expression after the IRI operation. Treatment with KS370G significantly reduced TGF- β_1 protein expression (Fig. 3A and 3B). Similarly, ELISA results also indicate that plasma TGF- β_1 levels were increased in IRI and Veh groups compared with the sham group.

Figure 3 | KS370G regulates the expression of TGF- β_1 and plasma TGF- β_1 levels in a murine IRI model. (A) Western blot analysis of renal TGF- β_1 expression in sham-operated (sham), ischemia-reperfusion injury (IRI), ischemia-reperfusion injury with vehicle (Veh) or KS370G 10 mg/kg (K10) treatment groups. Vehicle group was treated with RO water. (B) Quantitative results presented as mean $\pm$ SEM of the signal's optical density ($n = 6$ samples each group). * $P < 0.01$ compared with sham group. # $P < 0.01$ compared with IRI and Veh groups. (C) ELISA assay analysis of plasma TGF- β_1 levels in sham, IRI, Veh and K10 groups. * $P < 0.05$ compared with sham group. # $P < 0.05$ compared with IRI and Veh groups.

Treatment with KS370G markedly decreased plasma TGF- β_1 levels after the IRI operation (Fig. 3C).

KS370G inhibits TGF- β_1 -stimulated EMT in NRK52E and HK-2 cells. We first evaluated the suitable dose of TGF- β_1 needed to induce the process of EMT in NRK52E cells. NRK52E cells were treated with different concentrations of TGF- β_1 (0, 2.5, 5 and 10 ng/ml) for 72 h. The expression of two well-known markers of EMT, E-cadherin and α -SMA, were analyzed in NRK52E cells. Western blot analysis shows that the protein level of E-cadherin was downregulated and α -SMA levels were upregulated in TGF- β_1 2.5 ng/ml treated cells, reaching a

Figure 4 | TGF- β_1 stimulates the expression of E-cadherin and α -SMA in NRK52E cells. (A) E-cadherin and α -SMA expression were determined by western blot of NRK52E cells cultured for 72 h in different concentration of TGF- β_1 . (B and C) Quantitative results presented as mean $\pm$ SEM of the signal's optical density for E-cadherin (B; $n = 5$) and α -SMA (C; $n = 5$). * $P < 0.05$ compared with control group.

maximal effect in TGF- β_1 5 ng/ml treated cells (Fig. 4). We therefore used 5 ng/ml of TGF- β_1 in NRK52E and HK-2 cells for 72 h in subsequent experiments.

Next, the effect of KS370G in preventing TGF- β_1 -stimulated EMT in NRK52E and HK-2 cells were examined. Western blot analysis shows that treatment with TGF- β_1 (5 ng/ml) in NRK52E cells for 72 h led to a marked decrease in E-cadherin expression and an increase in α -SMA expression. KS370G significantly prevented TGF- β_1 stimulated changes of the E-cadherin and α -SMA expression in NRK52E cells at concentrations ranging from 1 to 3 μ M (Fig. 5). Similar results were also obtained in HK-2 cells (Fig. 5). These results

suggest that KS370G prevents the loss of the epithelial marker E-cadherin and the de novo expression of myofibroblast marker α -SMA in both human and non-human renal epithelial cells stimulated by TGF- β_1 .

KS370G ameliorates TGF- β_1 -stimulated fibronectin and type I collagen expression in NRK52E and HK-2 cells. The ability of KS370G to decrease ECM proteins accumulation in NRK52E and HK-2 cells was examined. Western blot analysis shows that both fibronectin and type I collagen expression were significantly increased after TGF- β_1 treatment for 72 h. By contrast, KS370G attenuated fibronectin and type I collagen expression in a dose-dependent manner, especially at concentrations ranging from 0.3 to 3 μ M in NRK52E cells and 1 to 3 μ M in HK-2 cells (Fig. 6).

KS370G attenuates TGF- β_1 -stimulated PAI-1 expression in NRK52E and HK-2 cells. Western blot analysis indicates that PAI-1 expression was markedly elevated after TGF- β_1 stimulation for 72 h. KS370G significantly reduced TGF- β_1 -induced PAI-1 expression in both NRK52E and HK-2 cells at concentrations ranging from 1 to 3 μ M (Fig. 7).

KS370G blocks TGF- β_1 -stimulated phosphorylation of Smad2/3 in NRK52E cells. Western blot analysis shows that TGF- β_1 triggered the phosphorylation of Smad2/3 in NRK52E cells at the first 15 minutes of incubation and reached peak expression at 30 minutes. It then gradually decreased after prolonged TGF- β_1 stimulation (Fig. 8A). We chose 30 minutes to be the time point to investigate the regulatory role of KS370G on TGF- β_1 -induced Smad2/3 phosphorylation. KS370G inhibited the phosphorylation of Smad2/3 in a dose-dependent manner. Concentrations higher than 0.3 μ M significantly blocked Smad2/3 phosphorylation protein expression (Fig. 8B and 8C).

Discussion

This study was undertaken to address whether KS370G attenuates renal interstitial fibrosis *in vivo* and *in vitro* and to investigate the underlying mechanisms. Here, we show that IRI injury significantly induces the expression of fibronectin and collagen deposition, promotes myofibroblast activation and elevates plasma levels of TGF- β_1 and renal TGF- β_1 protein expression. Exposure to TGF- β_1 for 72 h in NRK52E and HK-2 cells induce a downregulation of E-cadherin and an upregulation of α -SMA. TGF- β_1 also increases ECM protein levels and PAI-1 expression in NRK52E and HK-2 cells. However, KS370G significantly reverses all of above changes *in vivo* and *in vitro* with the possible mechanism being through inhibiting the TGF- β_1 /Smad2/3 signaling pathway.

TGF- β_1 and its downstream signaling pathway were shown to play a critical role in activating cellular pathological mechanisms in renal tubulointerstitial fibrosis through the induction of interstitial cell activation and the expression of several pro-fibrotic genes²⁵. After ligand binding, the TGF- β_1 receptor, a transmembrane Ser/Thr kinase receptor, interacts with receptor-regulated Smads, such as Smad2/3. Phosphorylated Smads enter the nucleus, where they propagate TGF- β_1 signaling and regulate the promoter activities of TGF- β_1 target genes²⁶. Previous studies have examined the blockade of TGF- β_1 signaling as a means to attenuate renal fibrosis²⁷. Our results demonstrate that KS370G reduces TGF- β_1 induction and plasma TGF- β_1 levels in the IRI kidney. In addition, KS370G inhibits downstream Smad2/3 phosphorylation in NRK52E cells. The exact mechanism for the suppression effects of KS370G on renal TGF- β_1 production in the IRI mice model needs to be further elucidated.

Renal tubulointerstitial fibrosis is the final consequence of chronic kidney disease which leads to the destruction of the kidney's parenchyma and end-stage renal failure^{28,29}. Renal fibrosis is associated with tubular epithelial cells transition to mesenchymal cells via a process known as EMT³⁰. EMT is an important process in the patho-

Figure 5 | KS370G regulates the expression of E-cadherin and α -SMA in NRK52E and HK-2 cells induced by TGF- β_1 . (A and D) E-cadherin and α -SMA expression were determined by western blot of NRK52E and HK-2 cells cultured with different concentration of KS370G (0.1 to 3 μ M) for 72 h under TGF- β_1 stimulation. (B,C,E and F) Quantitative results presented as mean $\pm$ SEM of the signal's optical density for E-cadherin (B; n = 7) and α -SMA (C; n = 5) in NRK52E cells and E-cadherin (E; n = 3) and α -SMA (F; n = 3) in HK-2 cells. *P < 0.05 compared with control group. #P < 0.05 compared with TGF- β_1 (5 ng/ml) groups.

genesis of tubulointerstitial fibrosis and involves a loss of epithelial cell characteristics and an increase of mesenchymal cell markers stimulated by various profibrotic cytokines³¹. Therefore, blocking renal EMT may prevent renal fibrosis. TGF- β_1 is a well-known profibrotic cytokine in several renal diseases and plays a critical role in the renal EMT process². In this study, we used an IRI mice model and both human (HK-2) and non-human (NRK52E) renal epithelial cells stimulated by TGF- β_1 to examine the effects of KS370G on myofibroblast activation *in vivo* and renal EMT *in vitro*. We found that KS370G reduces upregulation of α -SMA and vimentin in the IRI kidney. KS370G also decreases α -SMA expression and increases E-

cadherin expression in HK-2 and NRK52E cells stimulated by TGF- β_1 . According to these results, we suggest that KS370G prevents renal fibrosis by inhibiting myofibroblast activation *in vivo* and TGF- β_1 -mediated renal EMT *in vitro*.

The abnormal ECM production in renal fibrosis is not only related to the overexpression of normal ECM, such as fibronectin, but also due to an accumulation of pathological ECM components, such as type I collagen³². These proteins are involved in the renal scarring process and are irreversibly deposited in renal fibrotic tissues²⁵. Increasing evidence indicates that TGF- β_1 expression is induced in human and animal renal fibrosis models and TGF- β_1 expression has

Figure 6 | KS370G regulates the expression of fibronectin and collagen I in NRK52E and HK-2 cells induced by TGF- β_1 . (A) Fibronectin and type I collagen expression were determined by western blotting of NRK52E and HK-2 cells cultured with different concentration of KS370G (0.1 to 3 μ M) for 72 h under TGF- β_1 stimulation. (B,C,E and F) Quantitative results presented as mean $\pm$ SEM of the signal's optical density for fibronectin (B; n = 5) and type I collagen (C; n = 5) in NRK52E cells and fibronectin (E; n = 3) and type I collagen (F; n = 3) in HK-2 cells. *P < 0.05 compared with control group. #P < 0.05 compared with TGF- β_1 (5 ng/ml) groups.

been seen as the main mediator in ECM protein accumulation in renal interstitial fibrosis and diabetic nephropathy^{33,34}. Our results show that renal fibronectin expression and collagen deposition are elevated in kidneys from IRI mice *in vivo* and that type I collagen and fibronectin levels increase in TGF- β_1 -stimulated cells *in vitro*. KS370G treatment beneficially attenuates ECM deposition both *in vivo* and *in vitro*. Normally, the ECM is continuously degraded. The pathogenic accumulation of ECM may also result from a loss in ECM degradation³². PAI-1, a main inhibitor of plasmin generation, inhibits ECM degradation and stimulates its accumulation, thereby con-

tributing to renal fibrotic disease^{35,36}. PAI-1 is also a prominent downstream target of the TGF- β_1 /Smad signaling pathway and is considered to be a contributor to fibrogenesis in several organs³⁷. It has been demonstrated that activation of TGF- β_1 signaling triggers a dramatic induction of Smad2/3 phosphorylation and PAI-1 protein expression in the obstructive kidney³⁸. PAI-1 deficiency ameliorates the fibrotic injury in a UUO model³⁶. A previous study also indicates that PAI-1 mRNA is also upregulated in NRK52E cells treated with TGF- β_1 ¹⁶. In this study, we have shown in HK-2 and NRK52E cells that KS370G treatment successfully inhibits TGF- β_1 -stimulated tar-

Figure 7 | KS370G reduces the expression of PAI-1 in NRK52E and HK-2 cells induced by TGF- β_1 . (A and C) PAI-1 expression were determined by western blotting of NRK52E and HK-2 cells cultured with different concentration of KS370G (0.1 to 3 μ M) for 72 h under TGF- β_1 stimulation. (B and D) Quantitative results presented as mean $\pm$ SEM of the signal's optical density in NRK52E cells (B; n = 5) and in HK-2 cells (D; n = 3). *P < 0.05 compared with control group. #P < 0.05 compared with TGF- β_1 (5 ng/ml) groups.

get gene expression, including matrix proteins and PAI-1. Our combined results suggest that KS370G attenuates renal interstitial fibrosis through both reducing ECM synthesis and elevating ECM degradation.

In conclusion, our study demonstrates that KS370G attenuates renal injury in an IRI animal model, preventing myofibroblast activation, ECM deposition and renal interstitial fibrosis. KS370G also inhibits renal EMT and ECM protein expression in NRK52E and HK-2 cells induced by TGF- β_1 . The possible mechanism involves the suppression of the TGF- β_1 /Smad2/3 pathway and the subsequent inhibition of PAI-1 expression.

Methods

Animals and experimental design. The investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and was approved by the Institutional Animal Care and Use Committee of the National Taiwan University. 7-week-old male ICR mice (BioLasco Taiwan Co., Ltd) were housed at National Taiwan University College of Medicine Experimental Animal Center, maintained in a temperature- and humidity-controlled ($22 \pm 1^\circ\text{C}$ and $60 \pm 5\%$) environment with a 12 h light-dark cycle and given free access to food and water. After 1 week of acclimatization, mice were randomly allocated into 4 groups: (1) sham-operation group (sham); (2) IRI-operation group (IRI); (3) IRI group with oral gavage of vehicle once a day (Veh) and (4) IRI group with oral gavage of KS370G 10 mg/kg once a day (K10).

To establish the unilateral IRI model, the mice were anesthetized with sodium pentobarbital (80 mg/kg intraperitoneal). The left renal artery and vein were identified through dorsal incisions and clamped for 30 minutes to stop renal blood flow. Reperfusion was visually confirmed upon releasing the clamps before wound closing. Sham animals were subjected to the same surgical procedure except the left renal artery and vein were not clamped.

KS370G (10 mg/kg) and vehicle (RO water) were administered from the day after the operation for 13 days. All animals were sacrificed on day 14 after IRI or sham operation. The kidneys were then rapidly removed, rinsed in ice-cold 0.9% NaCl solution. Blood samples were centrifuged at 10000 rpm for 5 minutes and the plasma was stored at -80°C until assaying.

Cell culture. NRK52E and HK-2 cells were purchased from American Type Culture Collection (ATCC). NRK52E cells were grown in DMEM supplemented with 5% FBS and HK-2 cells were grown in DMEM/F12 medium, supplemented with 10% FBS in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. NRK52E and HK-2 cells were incubated into 6-well plates at a density of 5×10^5 cells for 24 h and

then divided into the following six treatment groups: control, TGF- β_1 5 ng/ml, TGF- β_1 5 ng/ml + KS370G 0.1 μ M, TGF- β_1 5 ng/ml + KS370G 0.3 μ M, TGF- β_1 5 ng/ml + KS370G 1 μ M and TGF- β_1 5 ng/ml + KS370G 3 μ M. After another 72 h, cells were harvested and processed for western blot analysis.

Chemicals. KS370G was obtained from Professor Kuo's lab and was synthesized using an amide binding coupling method as previously described²³. Briefly, benzotriazol-1-yloxy-tris (dimethylamino) phosphonium hexafluorophosphate (BOP) (1.2 eq) in dichloromethane (CH_2Cl_2) (5 mL) was added to a mixture of caffeic acid (100 mg). To this solution, R-NH₂ (1.2 eq) and triethylamine (Et₃N) (0.08 mL) in dimethylformamide (DMF) (1.0 mL) were added. The mixture was stirred at 0°C for 30 min and then stirred at room temperature for 12 h. This reaction mixture was evaporated in vacuo, and the residue was partitioned between ethyl acetate (AcOEt) and H₂O. Successive washings of the AcOEt layer with 3N aqueous HCl and 10% NaHCO₃ (aq) were performed. The residue was dried over MgSO₄ and concentrated in vacuo. The residue was further purified by column chromatography with an eluting solution (CH_2Cl_2 -AcOEt 1 : 1, v/v) on silica gel (70–230 and 230–400 mesh, Merck 7734). The final product (82–88% yield) was recrystallized from AcOEt to obtain pure crystals. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 500 spectrometer. Electron impact mass spectrometries (EIMS) were determined on a Finnigan TSQ-46C mass spectrometer. IR spectra were recorded on a Nicolet Magna-IR 550 spectrophotometer.

Histological analysis. Kidney sections were immersion-fixed in 10% buffered formalin. Sections were embedded in paraffin, sliced into 4 μ m thick sections and mounted on glass slides. Deparaffinized and rehydrated sections were stained with Masson's trichrome or Picrosirius Red to investigate the level of renal fibrosis and the content of collagen *in vivo*. Tissue sections were examined using a microscope and photographed with a digital camera.

Plasma TGF- β enzyme-linked immunosorbent assay (ELISA). Plasma level of TGF- β_1 was measured using ELISA commercial kits (R&D systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instruction.

Western blot analysis. The protein expression in kidney tissue and two renal tubular epithelial cell lines were analyzed by western blotting. Equal amounts of protein samples were loaded on sodium dodecyl sulfate-polyacrylamide (SDS) gels for electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes and blotted with fibronectin (Cell Signaling, USA), α -SMA (abcam, UK), vimentin (Genscript, USA), E-cadherin (BD Biosciences, Canada), p-Smad2/3 (Cell Signaling, USA), Smad2/3 (Cell Signaling, USA), PAI-1 (Cell Signaling, USA), Collagen 1 (Santa Cruz, USA), β -actin (Santa Cruz, USA) and GAPDH (Santa Cruz, USA) primary antibodies, followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were detected using western

Figure 8 | KS370G inhibits the phosphorylation of Smad2/3 in NRK52E cells induced by TGF- β_1 . (A) Smad2/3 phosphorylation was determined by western blotting of NRK52E cells stimulated by TGF- β_1 (5 ng/ml) in a time course experiment. (B) Smad2/3 phosphorylation was determined by western blotting of NRK52E cells cultured with different concentration of KS370G (0.1 to 3 μ M) for 30 minutes under TGF- β_1 stimulation. (C) Quantitative results presented as mean $\pm$ SEM of the signal's optical density (n = 5). *P < 0.05 compared with control group. #P < 0.05 compared with TGF- β_1 (5 ng/ml) groups.

lightning series HRP chemiluminescent substrates and captured on light-sensitive X-ray film.

Statistical analysis. All data are shown as the mean $\pm$ S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Dunnett's post-hoc test. P < 0.05 is regarded as statistically significant.

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Author contributions

S.T.C. and M.J.S. conceived and designed the experiments; Y.H.K. synthesized the compound; S.T.C. performed the experiments; S.T.C., Y.H.K. and M.J.S. analyzed data; S.T.C. and M.J.S. wrote the manuscript; M.J.S. contributed reagents and materials, and all authors reviewed the manuscript.

Additional information

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