Alantolactone Attenuates Renal Fibrosis via Inhibition of Transforming Growth Factor β/Smad3 Signaling Pathway

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**Background:** Renal fibrosis is characterized by the accumulation of extracellular matrix proteins and interstitial fibrosis. Alantolactone is known to exert anticancer, anti-inflammatory, antimicrobial and antifungal effects; however, its effects on renal fibrosis remains unknown. Here, we investigated whether alantolactone attenuates renal fibrosis in mice unilateral ureteral obstruction (UUO) and evaluated the effect of alantolactone on transforming growth factor (TGF) signaling pathway in renal cells.

Methods: To evaluate the therapeutic effect of alantolactone, cell counting kit-8 (CCK-8) assay, histological staining, Western blot analysis, and real-time quantitative polymerase chain reaction were performed in UUO kidneys in vivo and in TGF-β-treated renal cells in vitro.

Results: Alantolactone (0.25 to 4 µM) did not affect the viability of renal cells. Mice orally administered 5 mg/kg of alantolactone daily for 15 days did not show mortality or liver toxicity. Alantolactone decreased UUO-induced blood urea nitrogen and serum creatinine levels. In addition, it significantly alleviated renal tubulointerstitial damage and fibrosis and decreased collagen type I, fibronectin, and α-smooth muscle actin (α-SMA) expression in UUO kidneys. In NRK-49F cells, alantolactone inhibited TGF-β-stimulated expression of fibronectin, collagen type I, plasminogen activator inhibitor-1 (PAI-1), and α-SMA. In HK-2 cells, alantolactone inhibited TGF-β-stimulated expression of collagen type I and PAI-1. Alantolactone inhibited UUO-induced phosphorylation of Smad3 in UUO kidneys. In addition, it not only decreased TGF-β secretion but also Smad3 phosphorylation and translocation to nucleus in both kidney cell lines.

Conclusion: Alantolactone improves renal fibrosis by inhibiting the TGF-β/Smad3 signaling pathway in obstructive nephropathy. Thus, alantolactone is a potential therapeutic agent for chronic kidney disease.

Keywords: Fibrosis; Transforming growth factors; Ureteral obstruction.

INTRODUCTION

Renal fibrosis is the final progressive manifestation of chronic kidney disease leading to end-stage renal disease, it is characterized by the activation of interstitial myofibroblasts and excessive accumulation of extracellular matrix (ECM) proteins. Transforming growth factor β (TGF-β) plays a crucial role in these processes [1-5]. TGF-β expression is upregulated in various experimental and human renal diseases, where it induces the production of ECM proteins, including collagen type I and fibronectin, and renal tissue fibrosis [6-8]. TGF-β interacts with TGF-β receptors and activates intercellular mediators, known as Smad3 [5,9]. Phosphorylation of Smad3 by TGF-β has been observed.
in diabetic nephropathy [10,11], obstructive nephropathy [12], and drug toxicity-related nephropathy [13,14]. Smad3 mediates TGF-β-induced ECM deposition and renal fibrosis [5].

Alantolactone (AL) is extracted from \textit{Inula} species, which is traditionally used as a Chinese herbal medicine against allergic contact dermatitis [15], and is an effective anti-tumoral [16], anti-inflammatory [17], and antioxidant agent [18]. Previous studies have reported its anti-inflammatory effects on the kidneys in mice with streptozotocin-induced diabetes [19], protective effects against neuronal cell death and amnesia in mice [20], and anti-fibrotic effects in pulmonary fibrosis [21]. However, its functional role in renal fibrosis has not been investigated \textit{in vivo} and \textit{in vitro}.

In this study, we investigated the effect of AL on Smad3 inhibition and acceleration of renal interstitial fibrosis in unilateral ureteral obstruction (UUO)-induced renal fibrosis.

**METHODS**

**Animals and UUO model**
Male C57BL/6 8-week-old mice, weighing 25±2 g, were purchased from the Koatech Technology Corporation (Pyeongtaek, Korea). All experiments were approved by the Animal Care and Use Committee of Daegu Gyeongbuk Institute of Science and Technology (DGIST-IACUC-19052105-01). Mice were bred under standard room temperature (22°C±2°C) and humidity (60%±10%) conditions with a 12-hour light/dark cycle. Animals were randomly divided into the following three groups: control (CON, n=7), UUO (n=7), UUO with 5 mg/kg AL treatment (UUO/AL, n=7). AL (Carbosynth, Compton, UK) was dissolved in a filtered solution (1.25% dimethyl sulfoxide [DMSO], 2% polyethylene glycol [PEG] 400, 0.5% Tween 20 in saline), which was administered orally by gavage in equivalent volumes to CON and UUO mice once per day for 15 days, including 5 days of pretreatment. The UUO surgery was performed as previously described [22]: briefly, anesthetized mice underwent laparotomy to ligate the left with sterilized 5–0 silk for 10 days. Fibrosis-induced kidneys were fixed in a 10% formalin solution (Sigma-Aldrich, Burlington, MA, USA) or stored in liquid nitrogen for use as experimental samples.

**Blood analysis**
The serum of mice, separated using centrifugation of blood, was used to measure the levels of serum creatinine (Scr), blood urea nitrogen (BUN), alanine aminotransferase and aspartate aminotransferase using an automatic biochemical analyzer (Cobas Integra 800, Roche, Basel, Switzerland).

**Cell culture**
Human proximal tubule cell line (HK-2) and rat fibroblast cell line (NRK-49F) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HK-2 cells were grown in keratinocyte serum-free medium (Gibco, Waltham, MA, USA), supplemented with 50 µg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor. NRK-49F cells were cultured in Dulbecco’s modified Eagle medium/nutrient mixture F-12 medium (Gibco) with 5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Welgene, Gyeongsan, Korea). Cells were cultured in an atmosphere of 5% CO₂ at 37°C in a humidity chamber and treated with 5 and 2 ng/mL of TGF-β1 (R&D Systems, Minneapolis, MN, USA), respectively, for the indicated time periods.

**Western blot analysis**
Total protein from cells and kidney tissues were obtained by homogenization in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing cOmplete, Mini Protease Inhibitor Cocktail (Roche), and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Samples with an equal protein concentration were electrophoresed on Mini-PROTEAN Gels (Bio-Rad, Hercules, CA, USA), and then electrotransferred onto polyvinylidene fluoride membranes (Bio-Rad). After blocking with 5% bovine serum albumin (Sigma-Aldrich) for 1 hour, the membranes were immunoblotted overnight at 4°C with one of the following primary antibodies: anti-collagen type I (1:500) and anti-fibronectin (1:1,000) from Abcam (Cambridge, UK); anti-vimentin (1:1,000), anti-α-smooth muscle actin (α-SMA; 1:1,000), anti-plasminogen activator inhibitor-1 (PAI-1; 1:500) from BD Biosciences (Franklin Lakes, NJ, USA); and anti-phospho-Smad3 (1:500), anti-phospho-Smad3 (1:500), anti-phospho-signal transducer and activator of transcription 3 (STAT3; 1:1,000), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000), and anti-β-actin (1:1,000) from Santa Cruz Biotechnology (Dallas, TX, USA). The membranes were subsequently treated horseradish peroxidase (HRP)-linked secondary antibody (1:3,500). Protein expression was measured by visualizing the proteins bands using ChemiDoc XR5+ (Bio-Rad) and performing densitometric analysis using the Image J program 1.53e (National Institutes of Health, Bethesda, MD, USA).
mRNA expression analysis using real time-quantitative polymerase chain reaction

An RNA Extraction Kits (Takara, Tokyo, Japan) was used to extract total RNA. cDNA was synthesized using a Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) and, mRNA levels were detected using an SYBR Green polymerase chain reaction (PCR) Master Mix kit (Applied Biosystems) on an ABI 7500 quantitative polymerase chain reaction (qPCR) system. The qPCR conditions and primer sequences were designed using the Primer Express Software ver. 3.0 (Thermo Fisher Scientific). The sequences of all the primers are provided in Table 1.

Histological analysis and immunohistochemical staining

Sirius red and Masson's trichrome (Sigma-Aldrich) and immunohistochemical staining (Thermo Fisher Scientific) were performed on paraffin sections (4 μm thick) according to the manufacturer's instructions. Kidney sections were deparaffinized using xylene and rehydrated using graded ethanol series. Tubulointerstitial collagen deposition was assessed using Sirius red or Masson's trichrome staining. For Sirius red staining, the slides were immersed for 18 hours in saturated picric acid with 0.1% Sirius red F3BA, and 0.01N hydrochloric acid for 2 minutes. For Masson’s trichrome staining, slides were treated with Bouin's solution and incubated sequentially with hematoxylin for 10 minutes, Biebrich scarlet-acid fuchsin for 5 minutes, phosphotungstic acid/phosphomolybdic acid for 10 minutes, aniline blue for 15 minutes, and 1% acetic acid for 5 minutes. Immunohistochemical staining was performed with anti-collagen type I (1:200), anti-fibronectin (1:200, Abcam), anti-α-SMA (1:300), anti-p-Smad3 (1:200, Santa Cruz Biotechnology), and DAB staining kit (Roche). The kidney sections were dehydrated with a graded series of ethanol and xylene and mounted on glass slides for microscopic investigation. After staining, sections from each kidney of five different animals were selected and the images (×200) were taken using Leica Microscope (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite V3.8 software (Leica Microsystems). Quantification of the aniline blue (collagen, blue), Sirius red (collagen fiber, red), and immunostaining (brown color) positive areas was performed using computer-based morphometric analysis.

Immunofluorescence staining

HK-2 and NRK-49F cells, seeded on 8-well glass slides (Millepore, Burlington, MA, USA) were treated with AL or TGF-β1. For fixation, the glass slides were incubated with 2% paraformaldehyde for 2 hours at room temperature (20°C to 25°C). After washing the cells with phosphate-buffered saline (PBS), they were permeabilized for 2 minutes using 0.3% Triton X-100 (Sigma-Aldrich) in PBS. Cells were blocked with CAS-Block Histochemical Reagent (Thermo Fisher Scientific) for 1 hour, and then incubated overnight with anti-p-Smad3 antibody (1:100, Abcam) at room temperature. The slides were washed three times for 5 minutes each with PBS and then incubated with Alexa Fluor 488 anti-rabbit antibody for 1 hour in the dark. After staining the nuclei with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) for 5 minutes, the cells were washed three times with distilled water prior to mounting with a fluorescent mounting medium (Sigma-Aldrich). Immunofluorescence was detected using a laser scanning confocal microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany) and analyzed using the ZEN 2.6 (blue edition) software.

Statistical analysis

Data are expressed as the mean ± standard error of the mean of three independent experiments. Statistical analysis was performed using Student's t-test. Statistical significance was set at *P*<0.05.

Table 1. Sequences of real time-quantitative polymerase chain reaction primers for collagen type I, fibronectin, α-SMA, and PAI-1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
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<tr>
<td>GAPDH</td>
<td>Forward AGTTTGTCCTCTGCGGACCTCAGAGTTCA, Reverse CAGGAAATGAGCTTGACAAAGTTG</td>
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<tr>
<td>Rat collagen type I</td>
<td>Forward GCGATGGCCGTGATGGC, Reverse TCGGCTCCCGGTTCCTTGA</td>
</tr>
<tr>
<td>Rat fibronectin</td>
<td>Forward CATGCTTTAGGGCGAACCA, Reverse CATCTACATTGCGGAGGTATGG</td>
</tr>
<tr>
<td>Rat α-SMA</td>
<td>Forward ATGGCTCCCCGCTGATGAA, Reverse ACAGCCTGGGAGCATTCA</td>
</tr>
<tr>
<td>Human and rat PAI-1</td>
<td>Forward GGACACCTCCAGTGCTTGA, Reverse ACAGCCTGGGAGCATTCA</td>
</tr>
<tr>
<td>Human collagen type 1</td>
<td>Forward GAGGCGGACAGGAGG, Reverse CGTITGCGCAGGAGCATC</td>
</tr>
</tbody>
</table>
RESULTS

Toxicity of alantolactone

To examine the cell toxicity of AL, NRK-49F and HK-2 cells were incubated with the indicated concentrations of AL and their viability was measured using the cell counting kit-8 (CCK-8) assay. AL did not affect the cell viability at 0.25 to 4 µM but at 10 µM the viability of both the renal cell lines was less than 90% (Fig. 1A and B). Therefore, we used 1, 2, and 4 µM of AL for the subsequent in vitro experiments. In vivo toxicity of AL, was assessed in mice orally administered 5 or 10 mg/kg of AL daily for 15 days; these mice did not exhibit mortality, any unusual behavior, and liver toxicity (Supplementary Fig. 1).

Alantolactone attenuate UUO-induced renal dysfunction and renal fibrosis

As shown in Fig. 1C and D, the levels of Scr and BUN were increased in the UUO group. Administration of AL in UUO mice led to lower Scr and BUN levels compared with those in the UUO mice that were not treated.

A comparison of results for the UUO and UUO/AL groups (Fig. 2A-C) showed that UUO kidneys exhibited significantly increased tubular atrophy and tubulointerstitial fibrosis. Conversely, UUO-induced renal fibrosis was markedly decreased in the kidneys of AL-treated mice. Masson’s trichrome and Sirius red staining showed that interstitial collagen deposition in UUO kidneys increased significantly, whereas less intense staining was observed in the kidneys of AL-treated mice. Im-

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**Fig. 1.** Effects of alantolactone (AL) on the cell viability of renal cells and on renal function in mice with unilateral ureteral obstruction. (A, B) Serum-starved rat fibroblast cell line (NRK-49F) and human proximal tube cell line (HK-2) cells were incubated with the indicated concentration of AL for 24 hours and the viability was assessed using the cell counting kit-8 (CCK-8) assay. Quantification of data from three independent experiments was done, and values are shown as the mean ± standard error of the mean. (C, D) Analysis of blood urea nitrogen (BUN) and serum creatine (Scr) levels. *P < 0.05 vs. 0 µM; **P < 0.05 and ***P < 0.01 vs. control (CON); ^P < 0.05 and ^P < 0.01 vs. unilateral ureteral obstruction (UUO).
Alantolactone ameliorates renal fibrosis

Fig. 2. Alantolactone (AL) alleviates unilateral ureteral obstruction (UUO)-induced renal fibrosis. Mice received AL (5 mg/kg) by oral gavage for 5 days before UUO and for 10 additional days thereafter. (A) Images showing the staining of kidney sections with Masson’s trichrome and Sirius red and immunostaining for collagen type I, fibronectin, and α-smooth muscle actin (α-SMA) at 20× magnification. (B, C, D, E, F) Quantification of fibrotic scores or positively stained areas using computer-based morphometric analysis. Data in all the bar graphs were normalized against the control (n=1) and are expressed as a fold increase relative to the control. (G, H, I, J) Representative band (two or three cases) obtained in the Western blot analysis of UUO kidney lysates for collagen type I, fibronectin, and α-SMA. Expression levels of these proteins in different groups were quantified using densitometry and normalized with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and against the control group (n=1). Data in all the bar graphs are expressed as a fold increase relative to the control. *P<0.01 vs. control (CON); †P<0.05 and ‡P<0.01 vs. UUO (n=7 for each group); ††P<0.05 vs. control (CON); ‡‡P<0.05 and ‡‡‡P<0.01 vs. UUO. (Continued to the next page)
Fig. 3. Alantolactone inhibits the expression of transforming growth factor β (TGF-β)-induced renal fibrotic factors in renal fibroblasts and renal proximal tubule cells. Serum-starved rat fibroblast cell line (NRK-49F) and human proximal tubule cell line (HK-2) cells were incubated with 2 ng/mL (NRK-49F) or 5 ng/mL (HK-2) TGF-β for 24 hours in the presence of alantolactone (0 to 4 μM). (A, B, C, D) Real-time quantitative polymerase chain reaction (qPCR) analysis of the expression of collagen type I, fibronectin, plasminogen activator inhibitor-1 (PAI-1), and α-smooth muscle actin (α-SMA) in TGF-β-stimulated NRK-49F cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. (E) Quantification analysis of collagen type I, fibronectin, PAI-1, and α-SMA bands obtained using Western blot analysis of lysates of TGF-β-stimulated NRK-49F cells. (F, G) Real-time qPCR analysis of the expression of collagen type I and PAI-1 in TGF-β-stimulated HK-2 cells. GAPDH mRNA was used as an internal control. (H) Quantification of collagen type I and PAI-1 bands obtained using Western blot analysis of lysates of TGF-β-stimulated HK-2 cells. Data represent mean±standard error of the mean values from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control (TGF-β [–]); #P<0.05, ##P<0.01, and ###P<0.001 vs. TGF-β. (Continued to the next page)
Alantolactone ameliorates renal fibrosis

Fig. 3. Continued.

Fig. 4. Alantolactone (AL) inhibits Smad3 phosphorylation in unilateral ureteral obstruction (UUO) kidneys and renal cells. Mice received (5 or 10 mg/kg per day) by oral gavage for 5 days before UUO and for 10 additional days thereafter. (A) Images showing immunostaining of kidney sections for phospho-Smad3 (p-Smad3) at 20× magnification and quantification of positively stained areas using computer-based morphometric analysis. All data were normalized against the control (n=1) and are expressed as a fold increase relative to the control. (B) Representative bands (two or three cases) obtained in the Western blot analysis of UUO kidney lysates for p-Smad3 (upper). Expression levels of p-Smad3 in different groups were quantified using densitometry and normalized with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and against the control (n=1). Data in all bar graphs are expressed as a fold increase relative to the control (lower). aP<0.01 vs. control (CON); bP<0.01 vs. UUO (n=7 for each group); cP<0.01 vs. UUO.
Fig. 5. Alantolactone (AL) inhibits the transforming growth factor β (TGF-β)/Smad3 signaling pathway in renal cells. Serum-starved rat fibroblast cell line (NRK-49F) and human proximal tubule cell line (HK-2) cells were incubated with 2 ng/mL (NRK-49F) or 5 ng/mL (HK-2) TGF-β for 24 hours in the presence of AL (0 to 4 μM). (A, B) Measurement of TGF-β secretion in TGF-β-stimulated NRK-49F and HK-2 cells with or without 2 μM AL treatment. (C, D) Western blot analysis of protein expression of p-Smad3 in TGF-β-stimulated NRK-49F and HK-2 cells with or without 2 μM AL treatment. (E) Immunofluorescence staining of p-Smad3 in TGF-β-stimulated NRK-49F and HK-2 cells. Quantification of Western blot data is from three independent experiments; data are shown as the mean ± standard error of the mean. (B) Immunofluorescence staining of p-Smad3 (red in NRK-49F; green in HK-2) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclear counterstaining (blue) in TGF-β-stimulated NRK-49F and HK-2 cells with or without 2 μM AL treatment (20× magnification). *P<0.01 and #P<0.001 vs. control (CON); 4P<0.01 vs. TGF-β; 5P<0.01 vs. control (TGF-β [-]); *P<0.05 and 6P<0.001 vs. TGF-β.
munohistochemical staining showed that the expression of collagen type I, fibronectin, and α-SMA was increased in UUO kidneys. In contrast, the expression of all the fibrotic factors was decreased in AL-treated UUO kidneys (Fig. 2A and D-F). Similar results were also observed in immunoblot analysis of kidney tissue lysates. The expression of these fibrotic factors proteins was significantly increased in UUO kidneys and decreased in AL-treated UUO kidneys (Fig. 2G-J).

**Alantolactone inhibits the expression of TGF-β-induced renal fibrotic factors in renal fibroblasts and renal proximal tubule cells**

Next, the effects of AL on TGF-β-stimulated profibrotic target gene inhibition and on protein expression in NRK-49F and HK-2 cells were evaluated. Real-time qPCR analysis showed that AL dose-dependently inhibited the expression of TGF-β-stimulated collagen type I, fibronectin, PAI-1, and α-SMA mRNAs in NRK-49F cells (Fig. 3A-D). In addition, AL effectively inhibited the expression of TGF-β-stimulated profibrotic factor proteins in NRK-49F cells (Fig. 3E). AL also inhibited TGF-β-stimulated expression of collagen type I and PAI-1 mRNA and protein in HK-2 cells (Fig. 3F-H).

**Alantolactone ameliorates renal fibrosis by inhibition of Smad3 phosphorylation in UUO kidneys and renal cells**

TGF-β regulates renal fibrotic genes and matrix proteins through Smad signaling, and subsequently contributes to renal fibroblast activation and tubulointerstitial fibrosis. To elucidate the inhibitory mechanism of renal fibrosis, we investigated whether AL regulated Smad3, which is activated in UUO kidneys and TGF-β-treated renal cells. In the UUO kidneys, phospho-Smad3 levels increased compared with those in CON kidneys and were significantly decreased in AL-treated UUO kidneys (Fig. 4A). In addition, the protein expression in UUO kidneys was significantly inhibited by AL administration (Fig. 4B). As shown in Fig. 5A and B, AL inhibited TGF-β secretion in cultured renal fibroblasts and tubular epithelium-like cells. Immunoblot analysis showed that AL inhibited TGF-β-stimulated Smad3 phosphorylation as well as Smad3 translocation (Fig. 5E) to nucleus in both the renal cells (Fig. 5C and D).

**DISCUSSION**

This study is the first to demonstrate that AL, a natural compound produced by *Inula helenium*, effectively ameliorated renal fibrosis during obstructive nephropathy. AL prevented the expression of renal interstitial fibrotic factors including type I collagen, fibronectin, α-SMA, and PAI-1 in UUO kidneys and TGF-β-treated renal cells. This inhibitory effect of AL on renal fibrosis proceeds by first decreasing the secretion of TGF-β and thereby sequentially inhibiting phosphorylation and localization of Smad3.

Pharmacokinetic studies have shown that the absorption of AL is via passive diffusion, and that its intestinal absorption is good [23]; the highest concentration of AL was achieved in the small intestine and feces clearance was shown to be the dominant elimination pathway for the lactones [24]. However, AL has a rapid onset and does not cause significant damage to normal animal tissues and organs [24-26]. Several studies have shown that AL exhibits multiple pharmacological activities [15-18]. We focused on the anti-fibrotic effect of AL in the study. To demonstrate the anti-fibrotic effects of AL on renal fibrosis, we used the UUO model with oral gavage administration. Zhu et al. [19] administered 5 or 10 mg/kg per 2 days AL by oral gavage for 8 weeks to confirm its protective effect on streptozotocin-induced renal inflammation. In several studies, the anticancer effects of AL were observed after administering AL at concentration ranging from 5 to 50 mg/kg to mouse or rats [25,27-29]. Based on the pharmacokinetic characteristics described in several reports and our preliminary studies, we selected 5 mg/kg of AL to demonstrate the anti-fibrotic effect in the UUO-induced renal fibrosis model.
cally inhibited Smad3 phosphorylation in UUO-induced fibrotic kidneys and ameliorated renal fibrosis in vivo. In vitro, AL inhibited TGF-β-stimulated Smad3 phosphorylation and localization into the nucleus in renal cells as well as significantly reduced TGF-β secretion.

AL is well known as a selective STAT3 inhibitor and its beneficial effects in many diseases, including glioblastoma, liver cancer, breast cancer, and inflammatory disease, have been focused upon in several studies [27,28,35,36]. We also confirmed that alantolactone inhibited TGF-β- or UUO-stimulated STAT3 phosphorylation in vivo and in vitro (Supplementary Fig. 2A and B). In addition, AL inhibited the expression of vimentin, an epithelial-mesenchymal transition (EMT) factor, and F4/80, a macrophage marker, in UUO kidneys (Supplementary Fig. 2C and D). These results show the potential of AL to inhibit EMT and inflammation, and studies on these aspects are ongoing. Qin et al. [37] showed that the potential of herb-drug interactions rises when they are co-administered with other drugs. Specifically, it has been suggested that AL might exhibit herb-drug interactions when co-administered with drugs that are CYP3A4 and CYP2C19 substrates [37]. Clinical studies are warranted to further investigate the interactions of drugs co-administered with AL.

The in vivo and in vitro results of the current study suggest that AL ameliorates renal fibrosis by regulating the TGF-β/Smad3 signaling pathway. This study is the first to demonstrate that AL inhibits renal fibrosis and the expression of its related factors in an animal model of obstructive nephropathy induced by UUO. In cultured renal cells, AL effectively inhibited the expression of renal fibrotic factors via downregulating TGF-β secretion, and Smad3 phosphorylation and translocation into the nucleus. Because oral administration of AL effectively attenuated renal fibrosis, it could be proposed as a therapeutic agent for fibrotic kidney disease. The use of AL for treatment of human kidney disease deserves further investigation.

SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at https://doi.org/10.4093/dmj.2022.0231.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: K.M.L.
Acquisition, analysis, or interpretation of data: Y.J.H., K.M.L., G.S.J.
Drafting the work or revising: Y.J.H., K.M.L., G.S.J.
Final approval of the manuscript: K.M.L.

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None

REFERENCES

8. Roberts AB, Heine UI, Flanders KC, Sporn MB. Transforming


Supplementary Fig. 1. Mice received alantolactone (5 or 10 mg/kg per day) by oral gavage for 5 days before unilateral ureteral obstruction (UUO) and for 10 additional days thereafter. Blood biochemical parameters, (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) in animals treated with alantolactone were assessed to determine liver toxicity of alantolactone. CON, control.
Supplementary Fig. 2. Serum-starved rat fibroblast cell line (NRK-49F) cells were incubated with 2 ng/mL transforming growth factor β (TGF-β) for 24 hours in the presence of alantolactone (AL; 0 to 4 μM). (A) Western blot analysis of protein expression of phospho-signal transducer and activator of transcription 3 (p-STAT3) in TGF-β-stimulated NRK-49F cells. Quantification of Western blot data is from three independent experiments. Data are shown as the mean ± standard error of the mean. Mice received AL (5 mg/kg per day) by oral gavage for 5 days before unilateral ureteral obstruction (UUO) and for 10 additional days thereafter. (B, C) Representative results of Western blot analysis of kidney lysates for p-STAT3 and vimentin in UUO kidneys. (D) Image showing the immunostaining of kidney sections for F4/80 at 20× magnification. (B-D) Quantification of fibrotic scores or positively stained areas using computer-based morphometric analysis. Data in all bar graphs were normalized against the control (n=1) and expressed as a fold increase relative to the control. t-STAT3, total-signal transducer and activator of transcription 3. aP<0.001 vs. control (TGF-β –); bP<0.05 and cP<0.01 vs. TGF-β; dP<0.05 and eP<0.01 vs. UUO; fP<0.01 vs. control (CON); gP<0.05 vs. UUO (n=7 for each group).