

UC Irvine

UC Irvine Previously Published Works

Title

Novel inhibitors of the cellular renin-angiotensin system components, poricoic acids, target Smad3 phosphorylation and Wnt/ β -catenin pathway against renal fibrosis

Permalink

<https://escholarship.org/uc/item/59c4s0ws>

Journal

British Journal of Pharmacology, 175(13)

ISSN

0007-1188

Authors

Wang, Ming
Chen, Dan-Qian
Chen, Lin
[et al.](#)

Publication Date

2018-07-01

DOI

10.1111/bph.14333

Peer reviewed

Novel RAS inhibitors poricoic acid ZG and poricoic acid ZH attenuate renal fibrosis via Wnt/#-catenin pathway and targeted phosphorylation smad3 signaling

Ming Wang, Dan-Qian Chen, Lin Chen, Hui Zhao, Dan Liu, Zhi-Hao Zhang, Nosratola D. Vaziri, Yan Guo, Ying-Yong Zhao, and Gang Cao

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b00099 • Publication Date (Web): 31 Jan 2018

Downloaded from <http://pubs.acs.org> on February 13, 2018

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.



1 **Novel RAS inhibitors poricoic acid ZG and poricoic acid ZH attenuate renal fibrosis via Wnt/ β -catenin pathway**
2 **and targeted phosphorylation smad3 signaling**

3 Ming Wang^{†,@}, Dan-Qian Chen^{†,@}, Lin Chen[†], Dan Liu[†], Hui Zhao[†], Zhi-Hao Zhang[†], Nosratola D. Vaziri[‡], Yan Guo^{†,§},
4 Ying-Yong Zhao^{*,†,‡}, Gang Cao^{*,§}

5

6 [†] Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, School of Life
7 Sciences, Northwest University, No. 229 Taibai North Road, Xi'an, Shaanxi 710069, China

8 [‡] Division of Nephrology and Hypertension, School of Medicine, University of California Irvine, Irvine, California
9 92897, USA

10 [§] Department of Internal Medicine, University of New Mexico, Comprehensive Cancer Center, Albuquerque, New
11 Mexico, 87131, USA

12 [§] School of Pharmacy, Zhejiang Chinese Medical University, No. 548 Binwen Road, Hangzhou, Zhejiang 310053,
13 China

14 [@] Both the authors contributed equally to the research.

15

16 Corresponding authors:

17 Professor Ying-Yong Zhao, PhD, MD

18 School of Life Sciences, Northwest University, No. 229 Taibai North Road, Xi'an, Shaanxi 710069, China, Tel: +86 29
19 88305273; Fax: +86 29 88303572, E-mail: zyy@nwu.edu.cn; zhaoyybr@163.com

20 Scientific Reports, American Journal of Nephrology, Editorial Board

21 Professor Gang Cao, PhD

22 School of Pharmacy, Zhejiang Chinese Medical University, No. 548 Binwen Road, Hangzhou, Zhejiang 310053, China;
23 Tel/Fax: +86 571 87195895; E-mail: caogang33@163.com

24 **ABSTRACT:** Renal fibrosis is a common endpoint of the progression of chronic kidney disease (CKD). Suppressing
25 the development and progression of renal fibrosis is essential in the treatment of kidney disease. Our previous study
26 demonstrated that the ethyl acetate extract of the surface layer of *Poria cocos* exhibited beneficial anti-tubulointerstitial
27 fibrosis. In this study, we isolated new diterpene (PZF) and triterpenes (PZG and PZH) and examined their anti-fibrotic
28 effect. TGF- β 1 upregulated the collagen I protein expression in HK-2 cells and PZG and PZH treatment significantly
29 inhibited the upregulated collagen I expression (TGF group 0.59 ± 0.08 vs TGF+PZG group 0.36 ± 0.08 , $P < 0.01$;
30 TGF+PZH group 0.39 ± 0.12 , $P < 0.01$). Triterpenes, PZG and PZH, exhibited a stronger inhibitory effect on renal
31 fibrosis and podocyte injury than PZF. PZG and PZH further showed a stronger inhibitory effect on the activation of
32 renin-angiotensin system (RAS) caused by renal injury than PZF. Additionally, PZG and PZH markedly inhibited the
33 activation of Wnt/ β -catenin signaling, which plays an important role in fibrogenesis. Interestingly, PZG and PZH
34 suppressed TGF- β /Smad pathway by selectively inhibiting the phosphorylation of Smad3 through blocking the
35 interactions of SARA with TGF β 1 and Smad3. The analysis of the structure-activity relationship demonstrated that the
36 anti-fibrotic effect was closely associated with the first six-membered ring structure and the number of carboxyl groups
37 in this type of compounds. These novel tetracyclic triterpenoid compounds provided the potential lead compounds for
38 the research and development of anti-fibrosis drug and they possessed the potential to be utilized as RAS inhibitors.

39 **KEYWORDS:** Renin-angiotensin system; *Poria cocos*; poricoic acid; Wnt/ β -catenin pathway; TGF- β /Smad pathway

40

41 ■ INTRODUCTION

42 Renal fibrosis is the final pathways of progression of chronic kidney disease (CKD) to end stage renal disease (ESRD).
43 The characteristic of renal fibrosis is the increase of myofibroblasts, inflammatory cells infiltration, and excessive
44 extracellular matrix (ECM) accumulation in renal interstitium¹⁻³. Renal fibrosis is closely related to TGF- β /Smad
45 signaling pathway⁴. Transforming growth factor- β 1 (TGF- β 1) plays an irreplaceable role in the occurrence and the
46 development of renal fibrosis. TGF- β 1 is a potent cytokine that controls the transcription of many ECM genes. The

47 epithelial-mesenchymal transition (EMT), a critical process of tubulointerstitial fibrosis (TIF), is also driven by TGF- β 1
48 ^{5, 6}. Smad is a significant downstream gene of TGF- β 1 in the fibrogenesis. TGF- β 1 leads to fibrogenic effect by the
49 upregulation of Smad2 and Smad3 expression and the downregulation of Smad7 expression.

50 Renin-angiotensin system (RAS) and Wnt/ β -catenin pathways also play an important role in renal fibrosis ^{7, 8}. All of the
51 RAS components are present in the kidney tissues and activation of intrarenal RAS is a key component in the
52 development and progression of renal fibrosis ⁹. RAS consists of angiotensinogen (ATG), renin, angiotensin-converting
53 enzyme (ACE), angiotensin II (Ang II), angiotensin II type 1 receptor (AT1) and angiotensin II type 2 receptor (AT2).
54 Ang II is a key mediator in RAS-induced renal fibrosis. It stimulates TGF- β 1 expression in the kidney, or directly
55 phosphorylates Smad2 and Smad3 to promote fibrosis ¹⁰⁻¹². Although silent in adult kidney, Wnt/ β -catenin is reactive
56 after kidney injury. Wnt/ β -catenin signaling induces renal fibrosis through promoting downstream target genes
57 expression, most of which are fibrosis-related genes, including snail, twist, fibronectin, matrix metalloproteinase-7
58 (MMP-7), fibroblast specific protein-1 (Fsp-1), plasminogen activator inhibitor-1 (PAI-1) and RAS components ¹³.

59 Targeting inhibition of RAS/Wnt/ β -catenin axis achieved better anti-fibrotic effect than targeting a single pathway,
60 which indicated a new therapeutic strategy to mitigate renal fibrosis ⁸.

61 Recent studies demonstrated that small molecular weight natural products exhibited a good therapeutic effect on renal
62 fibrosis ¹⁴⁻¹⁹. Our previous studies showed that the ethanol extract of the surface layer of *Poria cocos* (SLPC) enhanced
63 diuresis and was highly effective in the treatments of CKD ^{20, 21}. Further investigation indicated that the ethyl acetate
64 extract of SLPC exhibited processes remarkable diuretic and anti-fibrotic effects ²²⁻²⁵. In the present study, we
65 investigated the renoprotective effect of three compounds with different skeletons isolated from the ethyl acetate extract
66 of SLPC. The three newly found and reported compounds include one new diterpene poricoic acid ZF (PZF) and two
67 new triterpenes poricoic acid ZG (PZG) and poricoic acid ZH (PZH). TGF- β 1 and Ang II were used to stimulate HK-2
68 cells and podocytes to induce renal injury. The treatment with PZF, PZG and PZH inhibited renal fibrogenesis, and the

69 anti-fibrotic effect of PZF, PZG and PZH were mediated by the suppression of RAS, TGF- β /Smad3 and Wnt/ β -catenin
70 signaling pathways.

71 ■ MATERIALS AND METHODS

72 **Chemicals and Instruments.** HPLC-grade methanol and acetonitrile was purchased from Baker Company (Baker Inc.,
73 USA). AR grade methanol, ethanol and acetonitrile were purchased from Tianjin Guangfu Fine Chemical Research
74 Institute (Tianjin, China). Chemicals were purchased from Thermo Fisher Scientific (New York, USA), unless indicated
75 otherwise. Ultra high purity water (UHP) was prepared by a Millipore-Q (France). DMEM-F12, DMEM and fetal
76 bovine serum were purchased from Gibco (Carlsbad, USA). Lipofectamine RNAiMAX was purchased from Invitrogen
77 (New York, USA). The recombinant human TGF- β 1 protein and recombinant human ANG II protein were purchased
78 from R&D system (New York, USA). The losartan (LOS) was purchased from Selleck (Shanghai, China). Other
79 chemicals were of analytical grade and their purity was above 99.5%.

80 Infrared (IR) spectrum was analyzed by using a Perkin Elmer FTIR system and Spectrum 2000 spectrophotometer
81 (Perkin Elmer, USA). ^1D and ^2D NMR spectra were measured by using Bruker AM-500 spectrometer (Bruker, Zurich,
82 Switzerland) with TMS as an internal standard. HRESI/MS were tested by an Agilent high-performance liquid
83 chromatography (HPLC)-Thermo Finnigan LCQ Advantage ion trap mass spectrometer (California, USA). Column
84 chromatography was carried out on silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Shandong China). HPLC
85 analysis and purification were performed on a Hanbon HPLC system equipped with a NP7000C serials pump and a
86 NU3000 serials detector (Hanbon, Jiangsu, China) at 210 nm, and the column used was a YMC-Pack C_{18} column (250
87 mm \times 4.6 mm i.d., 5 μm).

88 **Materials, extraction and isolation.** SLPC were collected in Yunnan Province (China) and authenticated by Fang M.
89 F. (Northwest University). The voucher specimen (16-09-12-01) was deposited in the herbarium of School of Life
90 Sciences, Northwest University. The dried and powdered SLPC (20 kg) was extracted with 95% ethyl alcohol (100 L \times
91 3) three times with at room temperature. The filtrates were combined and concentrated under reduced pressure until

92 elimination of ethyl alcohol to obtain crude extracts. Crude extracts (204 g) was fractionated by silica gel column
93 chromatography (60 × 10 cm) with the use of a gradient solvent system of CHCl₃/MeOH (from 100:0 to 1:1) to give six
94 fractions (FL1-FL6) according to TLC analysis. Fraction FL2 (12.0 g) was subjected to column chromatography using
95 silica gel with CHCl₃-MeOH (97:7) as a solvent system, and PZF (9 mg) was obtained. Fraction FL5 (24.1 g) was
96 fractionated by silica gel column chromatography with the use of a gradient solvent system of CHCl₃-MeOH (from 10:1
97 to 1:1) to obtain four fractions. The FL5-2 (2.7 g) was purified over reversed-phase HPLC with a gradient of
98 MeOH-H₂O (79:21 to 83:17 v/v) to yield PZG (7 mg, t_R=17.2 min). Fraction FL5-3 (1.3 g) was further purified over
99 reversed-phase HPLC (MeOH-H₂O, 75:25) to obtain PZH (6 mg, t_R=15.4 min).

100 **Cell culture and treatment.** Human kidney proximal epithelial cells (HK-2) and conditionally immortalized mouse
101 podocytes (MPC5) were employed to test the anti-fibrotic effect of PZF, PZG and PZH on Ang II and TGF-β1
102 stimulation. HK-2 cells were cultured in DMEM-F12 supplemented with 10% fetal bovine serum, and MPC5 was
103 cultured in DMEM containing 5.5 mM glucose with 10% fetal bovine serum. HK-2 cells were grown at 37°C with
104 5% CO₂, while MPC5 were grown at 33°C and differentiated at 37°C with 5% CO₂. The concentration of recombinant
105 human TGF-β1 protein was 2.5 ng/ml, and the concentration of recombinant human ANG II protein was 1.0 μM. The
106 10 μM of PZF, PZG and PZH were used to treat Ang II and TGF-β1-stimulated cells. The 10 μM of LOS was used to
107 treat HK-2 and MPC5. The cell viability of PZF, PZG and PZH were detected by CCK-8.

108 **Analysis of cell viability by CCK-8.** CCK-8 kit was purchased from EnoGene (Shanghai, China). The 1×10⁴ cells
109 were cultured in 96-well plates and treated with different concentrations of ZF, PZG and PZH (0, 1, 10, 50, 100 μM) for
110 24 hours. Before added 10 μl CCK-8, 100 μl/well fresh medium was added into plates. After 3 hours incubation, the
111 absorbance was detected at 450 nm on a microplate reader (Thermo, New York, USA). Six repeats were employed to
112 determine the cell viability of each concentration.

113 **Knockdown of Smad3 by siRNA.** HK-2 cell lines were grown to 60% confluence and the siRNA targeting human
114 Smad3 (5'-CCGCAUGAGCUUCGUCAAATT-3') and negative control (5'-UUCUCCGAACGUGUCACGUTT-3')

115 were transfected with 3 μ l of 10 μ M siRNA/well. Lipofectamine RNAiMAX was employed. The transfected cells were
116 measured by Western blot and qRT-PCR.

117 **Gene expression studies by qRT-PCR.** Total RNA was isolated using a High Pure RNA Isolation Kit (Tokyo, Japan)
118 according to the manufacturer's protocol. Total RNA was used for reverse transcription by a Transcriptor First Strand
119 cDNA Synthesis Kit (Roche, Germany). Real time quantifications of target genes were assessed using SYBR® Premix
120 Ex Taq™ II (Takara Bio, Japan). The housekeeping gene β -actin was used as the endogenous control. Samples were
121 amplified with a pre-cycling hold at 95°C for 30 seconds, 30 cycles of annealing and extension at 60 °C for 30 seconds
122 by using BioRad CFX 96 Touch™ system (Bio-Rad, USA).

123 **Western blot analysis.** Protein expression was performed by Western blot analysis as described previously^{26, 27}. Cells
124 were lysed in RIPA lysis buffer. Protein levels were measured by BCA Protein Assay Kit (23227, Thermo Scientific,
125 USA) and normalized by immunoblotting with α -tubulin, GAPDH or histone H3 expression. Total protein was
126 separated by SDS/PAGE and transferred to PVDF membranes (10600023, Amersham™ Hybond™, GE Healthcare,
127 USA). Membranes were blocked in 1 \times TBS with 0.1% Tween-20 (TBST) with 5% non-fat milk and then were
128 incubated with the primary antibodies. Finally, membranes were washed, incubated with goat anti-rabbit (1:5000,
129 ab6721, Abcam, USA), goat anti-mouse (1:5000, A21010, Abbkine, USA) or rabbit anti-goat (1:5000, A21110,
130 Abbkine, USA) secondary antibodies, and developed with ECL (RPN2232, GE Healthcare, USA).

131 The following primary antibodies were employed (dilution): AGT (1:200, sc-7419, Santa Cruz Biotechnology, CA),
132 renin (1:2000, 14291-1-AP, Proteintech, China), ACE (1:1000, sc-23908, Santa Cruz Biotechnology, CA), AT1 (1:800,
133 ab124505, Abcam, USA), Wnt1 (1:1000, ab85060, Abcam, USA), dephosphorylated active non-phosphorylated
134 β -catenin (1:2000, 05-665, Millipore, USA), β -catenin (1:1000, 610154, BD Transduction Laboratories, USA), collagen
135 I (1:5000, ab34710, Abcam, USA), alpha smooth muscle actin (α -SMA, 1:300, ab7817, Abcam, USA), fibronectin
136 (1:1000, ab2413, Abcam, USA), Snail1 (1:1000, ab180714, Abcam, USA), MMP-7 (1:400, ab5706, Abcam, USA),
137 Twist (1:2000, ab50581, Abcam, USA), Fsp-1(S100A4, 1:1000, ab197896, Abcam, USA), transforming growth factor

138 β receptor I (TGF β R1, 1:1000, ab31013, Abcam, USA), transforming growth factor β receptor II (TGF β R2, 1:2000,
139 ab186838, Abcam, USA), Smad2 (1:2000, 5339, CST, USA), p-Smad2 (1:2000, 3180, CST, USA), Smad3 (1:2000,
140 9523, CST, USA), p-Smad3 (1:2000, 9520, CST, USA), Smad4 (38454, CST, USA), Smad7 (1:500, sc-365846, Santa
141 Cruz, USA) and α -tubulin (Proteintech Company, China).

142 **Immunofluorescence staining.** Indirect immunofluorescence staining was performed using an established procedure⁸.
143 HK-2 cells were cultured on coverslip, and then fixed with fresh 4% paraformaldehyde solution. Normal goat serum
144 was used to reduce non-specific background staining. Cells were stained with primary antibody followed by treatment
145 with Alexa Fluor® 488 or 594-conjugated secondary antibody (Abcam, USA) for visualization. DAPI was employed to
146 localize nuclear. Cells were visualized by using a laser-scanning confocal microscope (FV1000, Olympus, Japan) with
147 FV10-ASW 4.0 VIEW.

148 **Co-immunoprecipitation (CO-IP) analysis.** After various treatments, HK-2 cells and podocytes were lysed by
149 M-PER™ Mammalian Protein Extraction Reagent (78501, Thermo Scientific, USA). After preclearing with protein
150 A/G (Immunoprecipitation Starter Pack, GE Healthcare, USA), cell lysates were incubated with anti-TGF β R1 (1:100),
151 anti-Smad2 (1:100) and anti-Smad3 (1:100) antibody overnight at 4°C, and then precipitated with protein A/G
152 overnight at 4°C. The precipitated complexes were analyzed by western blotting.

153 **Statistics analysis.** All results were analyzed as means \pm SD. GraphPad Prism software v 5.0 (San Diego, USA) was
154 used to calculate the statistical differences among different groups by one-way ANOVA. *P* values smaller than 0.05
155 were considered as statistically significant.

156 ■ RESULTS

157 Structure elucidation of isolated new compounds (PZF, PZG, PZH).

158 In this study, we isolated three novel compounds (PZF-PZH) from *P. cocos*. PZG and PZH were two novel triterpenes,
159 and PZH was found to possess a unique 5(6),8(9)-diene at B/C rings, all of which was identified for the first time in
160 triterpenes isolated from *P. cocos* (**Figure 1**). PZF was isolated as a yellow, amorphous powder. Its molecular formula,

161 $C_{30}H_{46}O_3$, was determined by HRESIMS molecular ion at m/z 453.1669 $[M-H]^-$ and ^{13}C NMR data. Its IR spectrum
162 revealed the absorption bands of the aliphatic long-chain (2924 cm^{-1}), carbonyl (1711 cm^{-1}) and aromatic ring (1639,
163 1449 cm^{-1}) groups. The 1H NMR spectrum of PZF indicated the presence of three protons in a trisubstituted aromatic
164 ring at δ_H 7.29 (d, $J = 8.2$ Hz), 7.12 (dd, $J = 8.1, 1.8$ Hz) and 6.95 (d, $J = 1.3$ Hz); two quaternary methyl groups at δ_H
165 1.49 and 1.21; two singlet methyl groups both at δ_H 1.23. The ^{13}C DEPT spectra of PZF revealed 30 carbon resonances,
166 belonging to five methyls, 12 methylenes, six methines, and seven aromatic/olefinic quaternary carbons. According to
167 the ^{13}C NMR spectrum, there were the typical signals of one carbonyl carbon at δ_C 176.4 corresponding to C-18, one
168 carboxyl carbon at δ_C 184.1 corresponding to C-20, and an isopropyl carbons at δ_C 34.3, 24.6 and 24.6 corresponding to
169 C-15, C-16 and C-17, respectively. A chain structure from C-1' to C-9' connected with C-18 was confirmed by the
170 important connectivities found in the heteronuclear multiple-bond connectivity (HMBC) spectrum (as shown in **Figure**
171 **1**) from H-19 (δ_H 1.49) to C-1' (δ_C 37.8), and from H-5 (δ_H 2.55) to C-18 (δ_C 176.4), and from H-1' (δ_H 1.84) to C-18
172 (δ_C 176.4). Furthermore, the HMBC signals from H-16, 17 (δ_H 1.23) to C-13 (δ_C 146.3) revealed that C-13 was
173 connected with the isopropyl group. The relative stereochemistry of PZF was assessed by its nuclear overhauser effect
174 pectroscopy (NOESY) spectrum (Fig. 1). The interactions between Me-19 (δ_H 1.49) and the protons at Me-21 (δ_H 1.21)
175 revealed that they were on the same side of the molecule, while the nuclear Overhauser effect (NOE) between H-2 (δ_H
176 1.41) and H-5 (δ_H 2.55) suggested that Me-30 and H-18 were on the same side. All of these data established the
177 structure of PZF as abieta-8,11,13-trien-18-pelargonyl-2-oic acid.

178 PZG was obtained as a white, amorphous powder. Its HRESIMS molecular ion at m/z 525.3176 $[M+Na]^+$ and ^{13}C NMR
179 data were consistent with the molecular formula $C_{30}H_{46}O_6$ (calcd 525.3192). The IR spectrum indicated the presence of
180 OH and C=C functional groups based on the absorptions at 3430 and 1644 cm^{-1} , respectively. The 1H NMR data (Table
181 1) showed six methyls at δ_H 1.05 (s), 1.11 (s), 1.36 (s), 1.36 (s), 1.51 (s) and 1.75 (s), two olefinic protons at δ_H 5.30 (t)
182 and 5.36 (br s), and an olefinic methylene at δ_H 4.85 (s) and 4.78 (s). The ^{13}C NMR data (Table 2) revealed 30 carbon
183 signals sorted by HSQC and DEPT. They included two carboxylic carbon at δ_C 177.0 and 179.2, six olefinic carbons at

184 δ_C 149.7, 142.3, 138.0, 120.8, 118.4 and 112.6, and one oxymethine carbon at δ_C 76.8. These data suggested that PZG
185 belonged to the group of 3,4-seco-lanosta-7,9(11)-diene type triterpenes. The hydroxyl group was located at C-25,
186 which was supported by the correlation from H-26, 27 (δ_H 1.36) to C-25 (δ_C 70.0), and from H-23 (δ_H 1.89) to C-25 (δ_C
187 70.0) in the HMBC experiment. The relative stereochemistry of PZG was assessed by its NOESY spectrum (Fig. 1).
188 The NOESY cross peaks of H-16 (δ_H 4.53) and Me-18 (δ_H 1.11), as well as interactions between H-16 (δ_H 4.53) and
189 H-20 (δ_H 2.98) suggested the α -orientation assignments of OH-16. Therefore, PZG was identified as
190 (20R)-16 α ,25-dihydroxy-3,4-seco-lanosta-4(28),7,9(11)-triene-3,21-dioic acid.

191 PZH was isolated as a white amorphous powder. It gave a $[M+Na]^+$ ion in the HRESIMS at m/z 521.3226, which was
192 consistent with a molecular formula of $C_{31}H_{46}O_5$ (calcd 521.3243). The IR spectrum indicated the presence of OH
193 (3430 cm^{-1}) and C=C (1650 cm^{-1} and 1644 cm^{-1}) functional groups. The ^1H NMR spectrum had seven methyls signals
194 at δ_H 1.64 (3H, s, Me-30), 1.41 (3H, s, Me-28), 1.38 (3H, s, Me-19), 1.34 (3H, s, Me-29), 1.17 (3H, s, Me-18), 1.01 (3H,
195 s, Me-27) and 1.00 (3H, s, Me-26), one olefinic proton at δ_H 6.56 (1H, s, H-6), and an olefinic methylene at δ_H 5.02 (s)
196 and 4.87 (s). The ^{13}C DEPT and ^1H NMR data and IR spectra of PZH indicated the presence of seven methyls, eight
197 methylenes, six methines and ten quaternary carbons (including one carboxylic carbon at δ_C 179.5 corresponding to
198 C-21). The presence of a tetrasubstituted double bond at C-8 and C-9 was supported by the correlation from H-30 (δ_H
199 1.64) to C-8 (δ_C 140.1), and from H-19 (δ_H 1.38) to C-9 (δ_C 162.9) in the HMBC experiment (**Figure 1**). Besides, the
200 correlation between H-29 (δ_H 1.34), H-19 (δ_H 1.38), H-28 (δ_H 1.41) and C-5 (δ_C 186.6), as well as the correlation
201 between H-6 (δ_H 6.56) and C-8 (δ_C 140.1) in the HMBC experiment suggested the location of a trisubstituted double
202 bond at C-5 and C-6. The NOESY cross peaks of H-16 (δ_H 4.48) and Me-18 (δ_H 1.17), as well as interactions between
203 H-16 (δ_H 4.48) and H-20 (δ_H 2.96) suggested the α -orientation assignments of OH-16. The ^{13}C NMR data of C-3 (δ_C
204 77.4) suggested the OH-3 was α -orientation, and it was supported by interactions between δ_H 4.60 (H-3) and δ_H 1.34
205 (H-29) in the NOESY experiment (Fig. 1). Hence, the structure of PZH was proposed as
206 (20R)-3 α ,16 α -dihydroxy-7-oxo-24-methyl-lanosta-5(6),8(9),24(31)-trien-21-oic acid.

207 **The toxicity of PZF, PZG and PZH on HK-2 cells.** To test the toxicity of PZF, PZG and PZH, the 0, 1, 10, 50 and
208 100 μM of PZF, PZG and PZH were incubated with HK-2 cells, respectively. CCK-8 method was used to test the value
209 of cell viability after 24 hours incubation. The values of cell viability were 0.98 ± 0.06 , 0.99 ± 0.05 , 0.99 ± 0.03 , 1.03 ± 0.06
210 and 1.05 ± 0.03 after incubation with the concentrations of 0, 1, 10, 50 and 100 μM of PZF, respectively. The values of
211 cell viability were 1.03 ± 0.04 , 0.99 ± 0.05 , 1.02 ± 0.03 , 0.98 ± 0.09 and 1.00 ± 0.07 after incubation with the concentrations
212 of 0, 1, 10, 50 and 100 μM of PZG, respectively. The values of cell viability were 1.02 ± 0.03 , 0.99 ± 0.05 , 0.98 ± 0.04 ,
213 1.01 ± 0.02 and 1.00 ± 0.06 after incubation with the concentrations of 0, 1, 10, 50 and 100 μM of PZH, respectively. In
214 conclusion, PZF, PZG and PZH showed no toxicity on HK-2 cells. The 10 μM of PZF, PZG and PZH was used for
215 subsequent experiments.

216 **PZF, PZG and PZH attenuated TIF and podocyte injury induced by TGF- β 1 and Ang II.** Collagen I, as one of
217 most important component of fibrosis, is an important indicator in renal fibrosis, so we chose collagen I as an indicator
218 for the determination of the inhibitory effect of the different concentrations of PZF, PZG and PZH in the
219 TGF- β 1-induced HK-2 cells. The compound concentrations of 1, 10, 50 and 100 μM were tested for collagen I protein
220 expression in HK-2 cells by induced TGF- β 1. As shown in **Figure 2A**, the concentration of 10 μM PZF, PZG and PZH
221 exhibited a strong anti-fibrotic effect. Hence, we selected the 10 μM of PZF, PZG and PZH for the subsequent
222 experiments.

223 We investigated the inhibitory effect of PZF, PZG and PZH on ECM accumulation, which was mainly expressed by
224 myofibroblasts. TGF- β 1 directly accumulates transcription of many ECM genes in HK-2 cells. Ang II directly
225 stimulates the expression of TGF- β 1, and directly stimulates Smads to induce renal fibrogenesis. In this study, both
226 TGF- β 1 and Ang II significantly upregulated the expression of ECM, including collagen I, fibronectin and α -SMA
227 (**Figure 2B-E**). E-cadherin is an epithelial cell marker and controls cell-cell junctions²⁸. E-cadherin can be viewed as
228 an inhibitor of EMT²⁹. The protein expression of E-cadherin was significantly downregulated in HK-2 cells by
229 inducing TGF- β 1 or Ang II (**Figure 2B-E**). The dysregulation caused by TGF- β 1 and Ang II were reversed by

230 triterpene PZG and PZH, but diterpene PZF hardly changed the dysregulation (**Figure 2B-E**). The upregulated mRNA
231 expressions of collagen I, fibronectin, α -SMA and vimentin were suppressed by PZG and PZH, while downregulating
232 E-cadherin mRNA expression induced by TGF- β 1 was reversed (**Figure 2F**). Interestingly, PZG exhibited a stronger
233 inhibitory effect than PZH. Additionally, immunofluorescent staining also showed that upregulated vimentin expression
234 were inhibited by PZG and PZH, whereas the expression of vimentin showed no fluctuation after given PZF, PZG or
235 PZH alone (**Figure 2K**).

236 Next, we explored the protective effect of PZF, PZG and PZH on podocyte injury. TGF- β 1 and Ang II stimulation
237 resulted in podocyte injury. The expression of podocyte-specific proteins, including podocin, nephrin, podocalyxin and
238 synaptopodin, were significantly decreased after TGF- β 1 or Ang II stimulation (**Figure 2G-J**). Triterpene compounds
239 PZG and PZH, rather than diterpene compound PZF, exhibited a protective effect on podocyte injury (**Figure 2G-J**). In
240 conclusion, triterpene PZG and PZH showed a good therapeutic effect on TIF and podocyte injury. The different effects
241 among triterpene compounds PZG, PZH and diterpene compound PZF were caused by the differences in the chemical
242 structures. Our results indicated that triterpene was the active compound of SLPC in the treatment of renal fibrosis and
243 podocyte injury. Interestingly, compared with the PZH, PZG showed stronger inhibitory effect on the TGF- β 1- and Ang
244 II-induced TIF and podocyte injury. The loss of first intact six-membered-ring led to hydroxyl group in PZH
245 transforming into carboxyl group in PZG. The increasing number of carboxyl group may be associated with the
246 bioactivity of triterpene compound.

247 **PZF, PZG and PZH inhibited the activation of RAS.** Since the inhibition of activated RAS was beneficial to
248 treatment of renal fibrosis and CKD ^{30, 31}, we examined the suppression of PZF, PZG and PZH on the activation of RAS
249 induced by TGF- β 1 and Ang II. TGF- β 1 stimulation upregulated the protein expression of RAS components in HK-2
250 cells. After PZG and PZH treatment, activated RAS was inhibited, while PZF showed no effect on activated RAS
251 (**Figure 3A,B**). Similarly, PZG and PZH significantly suppressed the activation of RAS caused by Ang II, while the
252 inhibitory effect of PZF was weak (**Figure 3C,D**). Next, we examined the inhibitory effect of PZF, PZG and PZH on

253 activated RAS in podocyte. TGF- β 1 stimulation activated RAS, and the expressions of RAS components were
254 increased. The significant decrease of RAS components were observed after PZG and PZH treatment, especially for
255 ACE and AT1 (**Figure 3E,F**). PZG and PZH were also obviously decreased abnormal activated RAS caused by Ang II,
256 whereas PZF showed a weak regulatory effect on this abnormality (**Figure 3G,H**).

257 **PZF, PZG and PZH suppressed activated Wnt/ β -catenin and its downstream target genes expression.**

258 Wnt/ β -catenin signaling is closely related to renal disease, especially for renal fibrosis³². Both TGF- β 1 and Ang II
259 stimulated the activation of β -catenin and the transcription of downstream target genes, resulting in upregulation of
260 downstream target genes. PZG and PZH significantly inhibited the upregulation of Wnt1, β -catenin, active
261 non-phosphorylated β -catenin, snail1, twist, MMP-7, PAI-1 and Fsp-1 expression induced by TGF- β 1 and Ang II.
262 However, PZF had no effect on Wnt/ β -catenin expression (**Figure 4A-D**). PZG and PZH also significantly inhibited the
263 upregulated mRNA expression of Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a and Wnt8a (**Figure 4E**). Immunofluorescent
264 staining also showed that PZG and PZH treatment have a significantly inhibitory effect on the β -catenin expression in
265 HK-2 cells induced by TGF- β 1 (**Figure 4F**). PZG and PZH performed better than PZF on the suppression of activated
266 Wnt/ β -catenin in renal epithelial cell. In podocyte, PZG and PZH attenuated the upregulating protein expression of
267 Wnt1, β -catenin and active β -catenin induced by TGF- β 1 and Ang II, and the increase of target genes, such as snail1,
268 twist, MMP-7, PAI-1 and Fsp-1, were reversed by PZG and PZH in podocytes (**Figure 4G-J**). PZG and PZH
269 suppressed the activation of Wnt/ β -catenin in HK-2 cells and podocytes.

270 **PZF, PZG and PZH selectively inhibits TGF- β /Smad3 pathway.** Since TGF- β /Smad signaling played an
271 irreplaceable role in the development and progression of renal fibrosis, we examined several important proteins in this
272 pathway. We found that TGF- β 1 and Ang II significantly induced the expressions of TGF β RII, p-Smad2, p-Smad3 and
273 Smad4, and significantly reduced Smad7 expression, which indicated activation of TGF- β /Smad signaling induced by
274 TGF- β 1 and Ang II. TGF- β 1 or Ang II promoted the phosphorylation of Smad2 and Smad3 and upregulated the
275 expression of p-Smad2 and p-Smad3, but did not change the expression of total Smad2 and Smad3. After PZH and PZG

276 treatment, the upregulation of p-smad3 was significantly inhibited. PZH and PZG only mitigated the upregulation of
277 p-Smad3, but showed no effect on the dysregulation of p-Smad2, Smad2, Smad3, Smad4 and Smad7 in HK-2 cells,
278 indicating Smad3 might be the intervention target of PZH and PZG (**Figure 5A-D**). Immunofluorescence staining
279 results demonstrated that PZH and PZG showed a stronger inhibitory effect on p-Smad3, but PZF have no effect on
280 p-Smad3 (**Figure 5E**). Similar results were observed in podocyte. TGF- β 1 and Ang II significantly upregulated
281 p-Smad2 and p-Smad3, but PZH and PZG treatment only inhibited the upregulation of p-Smad3 (**Figure 5F-I**). PZH
282 and PZG specifically attenuated increased p-Smad3 in HK-2 cells and podocytes, indicating PZH and PZG suppressed
283 the phosphorylation of Smad3 induced by TGF- β 1 and Ang II.

284 **PZF, PZG and PZH selectively interrupt the interactions of Smad3 with TGF β RI and SARA.** To further explore
285 the changes within TGF- β /Smad pathway, CO-IP were employed. First, we explored the interactions of TGF β RI and
286 Smad2/3, founding that PZG and PZH only disturbed the interaction between TGF β RI and p-Smad3 (**Figure 6A,B**).
287 The interactions of Smad2 with TGF β RI and TGF β RII was unaltered after PZF, PZG and PZH treatment (**Figure**
288 **6C,D**). Of note, PZG and PZH significantly weakened the interactions of Smad3 with TGF β RI and TGF β RII (**Figure**
289 **6E,F**). These results suggested that PZG and PZH specifically affected the interactions of Smad3 with TGF β RI and
290 TGF β RII, instead of Smad2.

291 Since after TGF- β 1 stimulation, TGF β RI and Smad2/3 were connected by adaptor proteins such as Smad anchor for
292 receptor activation (SARA)^{33, 34}, we explored the interactions of SARA with TGF β RI and Smad2/3. The interaction of
293 Smad3 with SARA was obviously weakened by PZG and PZH treatment, but PZG and PZH performed no effect on the
294 interaction of Smad2 with SARA (**Figure 6G-J**). As shown in **Figure 6K**, PZG and PZH specifically affected the
295 interaction between SARA and Smad3 to suppress the phosphorylation of Smad3 and downregulated the expression of
296 p-Smad3. These results indicated that Smad3 was the intervention target of PZF and PZH for suppressing TGF- β /Smad
297 pathway and retard renal fibrosis.

298 **PZF, PZG and PZH performed no anti-fibrotic effect after knockdown of Smad3.** According to our results, Smad3
299 was the main target of PZG and PZH to retard renal fibrosis. Specific Smad3 siRNA was employed to further confirm
300 the mechanism of PZG and PZH. We hypothesized that Smad3 was the primary target of PZG and PZH, which could
301 explain the results that once Smad3 decreased, PZG and PZH lost the intervention target and thus showed no effect on
302 renal fibrosis. First, we examined the inhibitory effect of specific Smad3 siRNA, founding that the protein expression of
303 Smad3 was significantly downregulated in HK-2 cells induced by specific Smad3 siRNA (**Figure 7A**). Moreover, PZF,
304 PZG and PZH had no influence on the process of RNAi, and would not upregulate Smad3 protein expression (**Figure**
305 **7B**). Interestingly, after knockdown Smad3, the anti-fibrosis of PZG and PZH significantly weakened, which provided
306 the evidence that Smad3 was the main intervention target of PZG and PZH to mitigate renal fibrosis (**Figure 7C,D**).

307 **Structure-activity correlation of PZF, PZG and PZH.** Our study demonstrated that PZG exhibited a stronger
308 inhibitory effect on the TGF- β 1- and Ang II-induced excessive ECM accumulation in HK-2 cells than PZH. According
309 to the classification of compound chemical structure, PZG and PZH belonged to secolanostane tetracyclic triterpenoid
310 compound and lanostane tetracyclic triterpenoid compound, respectively. The most important difference of two
311 tetracyclic triterpenoid compounds is that the first six-membered ring in the secolanostane compounds is opening-ring
312 chemical structure, but the first six-membered ring in lanostane compounds was intact six-membered ring chemical
313 structure. The loss of first intact six-membered-ring led to hydroxyl group in PZH transformed into carboxyl group in
314 PZG. Increasing number of carboxyl group may be associated with the bioactivity of triterpene compound. This result
315 indicated that compound skeleton has an important effect on this type of secolanostane and lanostane tetracyclic
316 triterpenoid compounds.

317 We compared the chemical structures of PZF and PZG. PZF and PZG both had chemical structures of three-membered
318 ring and one carboxyl group in the parent nucleus. PZF had no inhibitory effect on TGF- β 1- and Ang II-induced cell
319 injury, but PZG showed an inhibitory effect on TGF- β 1- and Ang II-induced cell injury. This result indicated that
320 carboxyl group in PZG plays a crucial role in this type of compound.

321 ■ DISCUSSION

322 Natural products have been widely used for the treatment of CKD and inhibition of renal fibrosis³⁵⁻³⁸. Our previous
323 study demonstrated that SLPC exhibited a good therapeutic effect on CKD. The ethanol extract of SLPC ameliorates
324 adenine-induced CKD by correcting the disturbance of amino metabolism and fatty acid metabolism²²⁻²⁴. On the basis
325 of biological activity screening, the ethyl acetate extract is the main active fraction. Diterpene compound PZF and
326 triterpene compounds PZG and PZH were isolated from SLPC. In the present study, we found that PZG and PZH
327 significantly attenuated renal fibrosis caused by inducing TGF- β 1 and Ang II. PZG and PZH exert their effect by
328 targeting the phosphorylation of Smad3, through inhibiting the interactions of Smad3 with TGF β RI with SARA. PZG
329 and PZH exhibited a strong inhibitory effect on the activation of RAS and Wnt/ β -catenin signalings (**Figure 8**).
330 However, PZF showed a weak effect, indicating triterpene components were the active component of SLPC, instead of
331 diterpene components. These important bioactivities may be associated with the first six-membered ring and the number
332 of carboxyl groups in secolanostane and lanostane tetracyclic triterpenoid compounds. This study provided activity
333 targets for anti-fibrosis drug research and development.

334 Regardless of the type of initial injury to the kidney, renal fibrosis is the common final pathway of CKD to ESRD³⁹.
335 The accumulation of ECM in the region between peritubular capillaries and tubules suppresses tubular function. ECM
336 resulted in tubules injury, tubular necrosis and interstitial fibrosis. Fibrosis in glomerular led to reduced filtration and
337 the loss of blood flow⁴⁰. With the increasing renal fibrosis area, the structural support of kidney was lost and kidney
338 volume was decreased, which led to the loss of renal function. Suppression the development and progress of renal
339 fibrosis is an indispensable component in CKD treatment. PZG and PZH showed excellent inhibitory effects on the
340 increase expression of collagen I, fibronectin, α -SMA and the decrease expression of E-cadherin, but PZF had no
341 influence on the dysregulation. Collagen I is an important component of fibrosis, and the decrease of collagen I is a vital
342 parameter to measure the anti-fibrotic effect. Fibronectin is the component of ECM, and PZG and PZH inhibited
343 fibronectin expression caused by inducing TGF- β 1 and Ang II in HK-2 cells and podocytes. α -SMA, the marker of

344 myofibroblasts, is mainly expressed in interstitium. After PZG and PZH treatment, α -SMA was downregulated, which
345 indicated the inhibition of EMT. PZG and PZH exhibited a good anti-fibrotic effect, and this may involved in several
346 signaling pathways, including RAS, TGF- β /Smad and Wnt/ β -catenin signaling.

347 RAS controls the blood pressure and fluid balance, and the activation of RAS is the major cause of hypertensive
348 nephropathy ⁴¹. CKD caused hypertension and hypertension further activated RAS ⁴². RAS inhibitors, especially for
349 angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II type 1 receptor blocker (ARB), were the first-line
350 therapy for CKD. ACEI and ARB antagonize Ang II to treat CKD. Ang II is a key mediator in RAS-induced renal
351 fibrosis ⁴³. It stimulates TGF- β 1 and Smads to promote renal fibrogenesis ⁵. TGF- β 1 and Ang II induce the activation of
352 RAS, while PZG and PZH suppress the activation of RAS by downregulating the expression of AGT, renin, ACE and
353 AT1. Our study provides strong evidence that PZG and PZH are novel RAS inhibitors.

354 Wnt/ β -catenin signaling pathway is an evolutionarily conserved signaling pathway that is regulated by the amount of
355 β -catenin ⁴⁴. Wnt/ β -catenin is silent in adult kidney tissue. However, Wnt/ β -catenin is reactivated in injured kidney,
356 especially in CKD ^{7, 45}. Wnt/ β -catenin plays an opposite role in CKD compared to the RAS. Wnt/ β -catenin is
357 advantageous in acute kidney injury for the restoration of kidney morphology and function ⁴⁶, but harmful in CKD ⁴⁷.

358 The continuous activation of Wnt/ β -catenin drives acute kidney injury to CKD ⁴⁶. The upregulation of Wnt1 ligand,
359 active β -catenin and β -catenin were observed after TGF- β 1 and Ang II stimulation, which indicated the activation of
360 Wnt/ β -catenin pathway. PZG and PZH efficiently suppressed activated Wnt/ β -catenin and its downstream target gene
361 expression. Upregulation of downstream target genes promoted renal fibrosis ⁸. Snail is a key transcription factor of
362 EMT and facilitates fibroblast migration, and was downregulated by PZG and PZH. MMP-7 is the component of ECM
363 and mediates the degradation of E-cadherin. MMP-7 was significantly inhibited by PZG and PZH. PAI-1 recruits
364 myofibroblasts, and PAI-1 was significantly inhibited by PZG and PZH. The expression of fibrotic marker, Fsp-1, was
365 also attenuated by PZG and PZH. PZG and PZH were better in the suppression of Wnt/ β -catenin and its downstream
366 target gene expression than PZH.

367 Undoubtedly, TGF- β /Smad plays a central and dominant role in the initiation and development of renal fibrosis ⁴⁸⁻⁵⁰.

368 The TGF- β isoforms spread all over every cell type in mammals. The ubiquitous intracellular signaling cascade Smad

369 family protein is the major pathway of TGF- β signaling in renal fibrogenesis. The active TGF- β 1 binds to TGF β RII,

370 then recruits and phosphorylates TGF β RI, which results in the phosphorylation of Smads ⁵¹. The binding between

371 TGF β RI and Smads is mediated by adaptor proteins, including SARA. Among several Smads, Smad3 is recognized as

372 the most important one during fibrogenesis ^{10, 14}, which directly connected with promoter region of collagens to trigger

373 their production ⁵². Smad7 is negative regulator of TGF- β /Smad signaling, and the expression of Smad7 is significantly

374 downregulated in CKD. The expression of Smad7 is a Smad3-dependent mechanism, and suppresses TGF- β 1 in turn ⁵³.

375 Activated Smad protein complexes transfers into the nucleus and engage the transcription of target genes ⁵⁴. In the

376 present study, we tested the expression of primary proteins, and found that PZH and PZG inhibited the upregulation of

377 p-Smad3 expression specifically, which indicated the phosphorylation of Smad3 were suppressed. CO-IP showed that

378 After PZG and PZH treatment, the binding between TGF β RI and Smad3 was weak. Since TGF β RI phosphorylated

379 Smad3 by the connection of SARA, the suppressed phosphorylation of Smad3 may result in broken connection. The

380 interaction of SARA with TGF β RI and Smad3 were simultaneously suppressed. Interestingly, PZG and PZH had no

381 effect on the phosphorylation of Smad2 and the interactions of SARA with TGF β RI and Smad2. In conclusion, PZG

382 and PZH targeted the phosphorylation of Smad3 by inhibiting the connection of SARA with TGF β RI and Smad3 in the

383 TGF- β /Smad pathway. When the expression of Smad3 subsided, PZG and PZH lost the intracellular target. This

384 provided potent evidence for elucidating anti-fibrotic mechanism of PZG and PZH.

385 In this study, we isolated the active components of renoprotective effect of SLPC and elucidated their anti-fibrotic

386 mechanism. Triterpenes, PZG and PZH, are the major active components of SLPC to treat retard renal fibrosis, instead

387 of diterpene PZF. PZG and PZH exhibited a stronger anti-fibrotic effect than PZF though inhibiting RAS,

388 Wnt/ β -catenin and TGF- β /Smad signaling pathways. PZG and PZH demonstrated strong potential to be developed as

389 new RAS inhibitors. PZG and PZH specifically inhibited the phosphorylation of Smad3 and mitigated renal fibrosis.

390 The anti-fibrotic effect is closely associated with the first six-membered ring structure and the number of carboxyl
391 groups in tetracyclic triterpenoid compounds. This study provides novel intervention targets for the development of
392 effective anti-fibrotic agent.

393 **Corresponding Author**

394 *(Y.-Y.Z.) Tel: +86-29-88305273. Fax: +86-29-88303572. E-mail: zyy@nwu.edu.cn, zhaoyybr@163.com. (G.C.)
395 Phone: +Tel/Fax: +86 571 87195895; E-mail: caogang33@163.com

396 **Funding**

397 This study was supported by the National Natural Science Foundation of China (Nos. 81673578, 81603271).

398 **Notes**

399 The authors declare that there is no conflict of interest.

400 **■ ABBREVIATIONS USED**

401 ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitor; AGT, angiotensinogen; AngII,
402 angiotensin II; ARB, angiotensin II type 1 receptor blocker; AT1, angiotensin II type 1 receptors; CKD, chronic kidney
403 disease; DEPT, distortionless enhancement by polarization transfer; ECM, extracellular matrix; EMT,
404 epithelial-to-mesenchymal transition; Fsp-1, fibroblast-specific protein 1; HSQC, heteronuclear single-quantum
405 coherence; LEF, lymphoid enhancer-binding factor; MMP-7, matrix metalloproteinase-7; NOESY, nuclear overhauser
406 effect spectroscopy; PAI-1, plasminogen activator inhibitor-1; PZC, poricoic acid ZC; PZD, poricoic acid ZD; PZE,
407 poricoic acid ZE; qRT-PCR, quantitative real-time PCR; RAS, renin-angiotensin system; siRNA, small interfering
408 RNA; SLPC, the surface layer of *Poria cocos*; TCF, T-cell factor; TGF- β , transforming growth factor- β ; TGF β RI,
409 transforming growth factor β receptor I; TGF β RII, transforming growth factor β receptor II; α -SMA, alpha smooth
410 muscle actin.

411 **■ REFERENCES**

- 412 1. LeBleu, V. S.; Taduri, G.; O'Connell, J.; Teng, Y.; Cooke, V. G.; Woda, C.; Sugimoto, H.; Kalluri, R., Origin and
413 function of myofibroblasts in kidney fibrosis. *Nat Med* **2013**, *19*, 1047-1053.
- 414 2. Han, S. J.; Noh, M. R.; Jung, J. M.; Ishii, I.; Yoo, J.; Kim, J. I.; Park, K. M., Hydrogen sulfide-producing
415 cystathionine gamma-lyase is critical in the progression of kidney fibrosis. *Free radical biology & medicine* **2017**, *112*,
416 423-432.
- 417 3. Zhao, Y. Y.; Wang, H. L.; Cheng, X. L.; Wei, F.; Bai, X.; Lin, R. C.; Vaziri, N. D., Metabolomics analysis reveals
418 the association between lipid abnormalities and oxidative stress, inflammation, fibrosis, and Nrf2 dysfunction in
419 aristolochic acid-induced nephropathy. *Sci Rep* **2015**, *5*, 12936.
- 420 4. Jin, Y.; Ratnam, K.; Chuang, P. Y.; Fan, Y.; Zhong, Y.; Dai, Y.; Mazloom, A. R.; Chen, E. Y.; D'Agati, V.; Xiong,
421 H.; Ross, M. J.; Chen, N.; Ma'ayan, A.; He, J. C., A systems approach identifies HIPK2 as a key regulator of kidney
422 fibrosis. *Nat Med* **2012**, *18*, 580-8.
- 423 5. Wolf, G., Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth
424 factor-beta pathway. *Kidney Int* **2006**, *70*, 1914-1919.
- 425 6. Bottinger, E. P.; Bitzer, M., TGF-beta signaling in renal disease. *Journal of the American Society of Nephrology*
426 **2002**, *13*, 2600-2610.
- 427 7. Chen, D. Q.; Cao, G.; Chen, H.; Liu, D.; Su, W.; Yu, X. Y.; Vaziri, N. D.; Liu, X. H.; Bai, X.; Zhang, L.; Zhao, Y.
428 Y., Gene and protein expressions and metabolomics exhibit activated redox signaling and wnt/beta-catenin pathway are
429 associated with metabolite dysfunction in patients with chronic kidney disease. *Redox biology* **2017**, *12*, 505-521.
- 430 8. Chen, L.; Chen, D. Q.; Wang, M.; Liu, D.; Chen, H.; Dou, F.; Vaziri, N. D.; Zhao, Y. Y., Role of
431 RAS/Wnt/beta-catenin axis activation in the pathogenesis of podocyte injury and tubulo-interstitial nephropathy. *Chem*
432 *Biol Interact* **2017**, *273*, 56-72.

- 433 9. Xu, Z.; Li, W.; Han, J.; Zou, C.; Huang, W.; Yu, W.; Shan, X.; Lum, H.; Li, X.; Liang, G., Angiotensin II induces
434 kidney inflammatory injury and fibrosis through binding to myeloid differentiation protein-2 (MD2). *Scientific Reports*
435 **2017**, *7*, 44911.
- 436 10. Yang, F.; Huang, X. R.; Chung, A. C.; Hou, C. C.; Lai, K. N.; Lan, H. Y., Essential role for Smad3 in angiotensin
437 II-induced tubular epithelial-mesenchymal transition. *The Journal of pathology* **2010**, *221*, 390-401.
- 438 11. Rodriguez-Vita, J.; Sanchez-Lopez, E.; Esteban, V.; Ruperez, M.; Egido, J.; Ruiz-Ortega, M., Angiotensin II
439 activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-beta-independent
440 mechanism. *Circulation* **2005**, *111*, 2509-17.
- 441 12. Remuzzi, G.; Perico, N.; Macia, M.; Ruggenenti, P., The role of renin-angiotensin-aldosterone system in the
442 progression of chronic kidney disease. *Kidney international. Supplement* **2005**, S57-65.
- 443 13. Zhou, L.; Li, Y.; Hao, S.; Zhou, D.; Tan, R. J.; Nie, J.; Hou, F. F.; Kahn, M.; Liu, Y., Multiple genes of the
444 renin-angiotensin system are novel targets of Wnt/beta-catenin signaling. *J Am Soc Nephrol* **2015**, *26*, 107-120.
- 445 14. Ai, J.; Nie, J.; He, J.; Guo, Q.; Li, M.; Lei, Y.; Liu, Y.; Zhou, Z.; Zhu, F.; Liang, M.; Cheng, Y.; Hou, F. F., GQ5
446 hinders renal fibrosis in obstructive nephropathy by selectively inhibiting TGF-beta-induced Smad3 phosphorylation. *J*
447 *Am Soc Nephrol* **2015**, *26*, 1827-1838.
- 448 15. Zhao, Y. Y.; Chen, H.; Tian, T.; Chen, D. Q.; Bai, X.; Wei, F., A pharmaco-metabonomic study on chronic kidney
449 disease and therapeutic effect of ergone by UPLC-QTOF/HDMS. *PLoS One* **2014**, *23*, e115467.
- 450 16. Ko, J.-W.; Shin, N.-R.; Park, S.-H.; Lee, I.-C.; Ryu, J.-M.; Kim, H.-J.; Cho, Y.-K.; Kim, J.-C.; Shin, I.-S., Silibinin
451 inhibits the fibrotic responses induced by cigarette smoke via suppression of TGF-beta 1/Smad 2/3 signaling. *Food and*
452 *Chemical Toxicology* **2017**, *106*, 424-429.
- 453 17. Zhao, Y. Y.; Zhang, L.; Mao, J. R.; Cheng, X. H.; R.C., L.; Zhang, Y.; Sun, W. J.,
454 Ergosta-4,6,8(14),22-tetraen-3-one isolated from *Polyporus umbellatus* prevents early renal injury in aristolochic
455 acid-induced nephropathy rats. *J Pharm Pharmacol* **2011**, *63*, 1581-1586.

- 456 18. Zhao, Y. Y.; Cheng, X. L.; Cui, J. H.; Yan, X. R.; Wei, F.; Bai, X.; Lin, R. C., Effect of
457 ergosta-4,6,8(14),22-tetraen-3-one (ergone) on adenine-induced chronic renal failure rat: a serum metabonomic study
458 based on ultra performance liquid chromatography/high-sensitivity mass spectrometry coupled with MassLynx i-FIT
459 algorithm. *Clin. Chim. Acta* **2012**, *413*, 1438-1445.
- 460 19. Zhao, Y. Y.; Shen, X.; Cheng, X. L.; Wei, F.; Bai, X.; Lin, R. C., Urinary metabonomics study on the protective
461 effects of ergosta-4,6,8(14),22-tetraen-3-one on chronic renal failure in rats using UPLC Q-TOF/MS and a novel MSE
462 data collection technique. *Process Biochem* **2012**, *47*, 1980-1987.
- 463 20. Zhao, Y. Y.; Cheng, X. L.; Wei, F.; Bai, X.; Lin, R. C., Application of faecal metabonomics on an experimental
464 model of tubulointerstitial fibrosis by ultra performance liquid chromatography/high-sensitivity mass spectrometry with
465 MS(E) data collection technique. *Biomarkers* **2012**, *17*, 721-729.
- 466 21. Feng, Y. L.; Lei, P.; Tian, T.; Yin, L.; Chen, D. Q.; Chen, H.; Mei, Q.; Zhao, Y. Y.; Lin, R. C., Diuretic activity of
467 some fractions of the epidermis of *Poria cocos*. *J Ethnopharmacol* **2013**, *150*, 1114-1118.
- 468 22. Zhao, Y. Y.; Lei, P.; Chen, D. Q.; Feng, Y. L.; Bai, X., Renal metabolic profiling of early renal injury and
469 renoprotective effects of *Poria cocos* epidermis using UPLC Q-TOF/HSMS/MSE. *J Pharm Biomed Anal* **2013**, *81-82*,
470 202-209.
- 471 23. Zhao, Y. Y.; Li, H. T.; Feng, Y. L.; Bai, X.; Lin, R. C., Urinary metabonomic study of the surface layer of *Poria*
472 *cocos* as an effective treatment for chronic renal injury in rats. *J Ethnopharmacol* **2013**, *148*, 403-410.
- 473 24. Zhao, Y. Y.; Feng, Y. L.; Bai, X.; Tan, X. J.; Lin, R. C.; Mei, Q., Ultra performance liquid chromatography-based
474 metabonomic study of therapeutic effect of the surface layer of *Poria cocos* on adenine-induced chronic kidney disease
475 provides new insight into anti-fibrosis mechanism. *PLoS One* **2013**, *8*, e59617.
- 476 25. Chen, D. Q.; Chen, H.; Chen, L.; Tang, D. D.; Miao, H.; Zhao, Y. Y., Metabolomic application in toxicity
477 evaluation and toxicological biomarker identification of natural product. *Chem Biol Interact* **2016**, *252*, 114-130.

- 478 26. Zhao, Y. Y.; Cheng, X. L.; Wei, F.; Bai, X.; Tan, X. J.; Lin, R. C.; Mei, Q., Intrarenal metabolomic investigation of
479 chronic kidney disease and its TGF-beta1 mechanism in induced-adenine rats using UPLC Q-TOF/HSMS/MS(E). *J.*
480 *Proteome Res.* **2013**, *12*, 2692–2703.
- 481 27. Chen, D. Q.; Chen, H.; Chen, L.; Vaziri, N. D.; Wang, M.; Li, X. R.; Zhao, Y. Y., The link between phenotype and
482 fatty acid metabolism in advanced chronic kidney disease. *Nephrology, dialysis, transplantation : official publication of*
483 *the European Dialysis and Transplant Association - European Renal Association* **2017**, *32*, 1154-1166.
- 484 28. Warrington, S. J.; Strutt, H.; Strutt, D., The Frizzled-dependent planar polarity pathway locally promotes
485 E-cadherin turnover via recruitment of RhoGEF2. *Development* **2013**, *140*, 1045-1054.
- 486 29. Andl, T.; Le Bras, G. F.; Richards, N. F.; Allison, G. L.; Loomans, H. A.; Washington, M. K.; Revetta, F.; Lee, R.
487 K.; Taylor, C.; Moses, H. L.; Andl, C. D., Concerted loss of TGFβ-mediated proliferation control and E-cadherin
488 disrupts epithelial homeostasis and causes oral squamous cell carcinoma. *Carcinogenesis* **2014**, *35*, 2602-2610.
- 489 30. Cao, W.; Xu, J.; Zhou, Z. M.; Wang, G. B.; Hou, F. F.; Nie, J., Advanced oxidation protein products activate
490 intrarenal renin-angiotensin system via a CD36-mediated, redox-dependent pathway. *Antioxidants & redox signaling*
491 **2013**, *18*, 19-35.
- 492 31. Breyer, M. D.; Susztak, K., The next generation of therapeutics for chronic kidney disease. *Nature Reviews Drug*
493 *Discovery* **2016**, *15*, 568-588.
- 494 32. He, W.; Tan, R. J.; Li, Y.; Wang, D.; Nie, J.; Hou, F. F.; Liu, Y., Matrix metalloproteinase-7 as a surrogate marker
495 predicts renal Wnt/beta-catenin activity in CKD. *Journal of the American Society of Nephrology : JASN* **2012**, *23*,
496 294-304.
- 497 33. Kong, J.; Du, J.; Wang, Y.; Yang, M.; Gao, J.; Wei, X.; Fang, W.; Zhan, J.; Zhang, H., Focal adhesion molecule
498 Kindlin-1 mediates activation of TGF-beta signaling by interacting with TGF-beta RI, SARA and Smad3 in colorectal
499 cancer cells. *Oncotarget* **2016**, *7*, 76224-76237.

- 500 34. Tsukazaki, T.; Chiang, T. A.; Davison, A. F.; Attisano, L.; Wrana, J. L., SARA, a FYVE domain protein that
501 recruits Smad2 to the TGFbeta receptor. *Cell* **1998**, *95*, 779-91.
- 502 35. Zhang, Z. H.; Vaziri, N. D.; Wei, F.; Cheng, X. L.; Bai, X.; Zhao, Y. Y., An integrated lipidomics and metabolomics
503 reveal nephroprotective effect and biochemical mechanism of Rheum officinale in chronic renal failure. *Sci Rep* **2016**,
504 *6*, 22151.
- 505 36. Zhang, Z. H.; Wei, F.; Vaziri, N. D.; Cheng, X. L.; Bai, X.; Lin, R. C.; Zhao, Y. Y., Metabolomics insights into
506 chronic kidney disease and modulatory effect of rhubarb against tubulointerstitial fibrosis. *Sci Rep* **2015**, *5*, 14472.
- 507 37. Wang, M.; Chen, L.; Liu, D.; Chen, H.; Tang, D. D.; Zhao, Y. Y., Metabolomics highlights pharmacological
508 bioactivity and biochemical mechanism of traditional Chinese medicine. *Chem Biol Interact* **2017**, *273*, 133-141.
- 509 38. Zhao, Y. Y., Metabolomics in chronic kidney disease. *Clin. Chim. Acta* **2013**, *422*, 59-69.
- 510 39. Cybulsky, A. V., Endoplasmic reticulum stress, the unfolded protein response and autophagy in kidney diseases.
511 *Nat Rev Nephrol* **2017**, *advance online publication*.
- 512 40. Sun, Y. B.; Qu, X.; Caruana, G.; Li, J., The origin of renal fibroblasts/myofibroblasts and the signals that trigger
513 fibrosis. *Differentiation; research in biological diversity* **2016**, *92*, 102-107.
- 514 41. Zhang, H.; Han, G. W.; Batyuk, A.; Ishchenko, A.; White, K. L.; Patel, N.; Sadybekov, A.; Zamlynyy, B.; Rudd, M.
515 T.; Hollenstein, K.; Tolstikova, A.; White, T. A.; Hunter, M. S.; Weierstall, U.; Liu, W.; Babaoglu, K.; Moore, E. L.;
516 Katz, R. D.; Shipman, J. M.; Garcia-Calvo, M.; Sharma, S.; Sheth, P.; Soisson, S. M.; Stevens, R. C.; Katritch, V.;
517 Cherezov, V., Structural basis for selectivity and diversity in angiotensin II receptors. *Nature* **2017**, *544*, 327-332.
- 518 42. Lichtnekert, J.; Kaverina, N. V.; Eng, D. G.; Gross, K. W.; Kutz, J. N.; Pippin, J. W.; Shankland, S. J.,
519 Renin-Angiotensin-Aldosterone System Inhibition Increases Podocyte Derivation from Cells of Renin Lineage. *Journal*
520 *of the American Society of Nephrology : JASN* **2016**, *27*, 3611-3627.
- 521 43. Ruster, C.; Wolf, G., Renin-angiotensin-aldosterone system and progression of renal disease. *Journal of the*
522 *American Society of Nephrology : JASN* **2006**, *17*, 2985-91.

- 523 44. Nusse, R.; Clevers, H., Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* **2017**,
524 *169*, 985-999.
- 525 45. Surendran, K.; Schiavi, S.; Hruska, K. A., Wnt-dependent beta-catenin signaling is activated after unilateral
526 ureteral obstruction, and recombinant secreted frizzled-related protein 4 alters the progression of renal fibrosis. *Journal*
527 *of the American Society of Nephrology : JASN* **2005**, *16*, 2373-84.
- 528 46. Xiao, L.; Zhou, D.; Tan, R. J.; Fu, H.; Zhou, L.; Hou, F. F.; Liu, Y., Sustained Activation of Wnt/beta-Catenin
529 Signaling Drives AKI to CKD Progression. *J Am Soc Nephrol* **2016**, *27*, 1727-1740.
- 530 47. Lin, X.; Zha, Y.; Zeng, X. Z.; Dong, R.; Wang, Q. H.; Wang, D. T., Role of the Wnt/beta-catenin signaling pathway
531 in inducing apoptosis and renal fibrosis in 5/6-nephrectomized rats. *Molecular medicine reports* **2017**, *15*, 3575-3582.
- 532 48. Meng, X. M.; Tang, P. M.; Li, J.; Lan, H. Y., TGF-beta/Smad signaling in renal fibrosis. *Frontiers in physiology*
533 **2015**, *6*, 82.
- 534 49. Meng, X. M.; Huang, X. R.; Chung, A. C.; Qin, W.; Shao, X.; Igarashi, P.; Ju, W.; Bottinger, E. P.; Lan, H. Y.,
535 Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis. *Journal of the American Society of Nephrology :*
536 *JASN* **2010**, *21*, 1477-87.
- 537 50. Meng, X. M.; Nikolic-Paterson, D. J.; Lan, H. Y., TGF-beta: the master regulator of fibrosis. *Nat Rev Nephrol*
538 **2016**, *12*, 325-338.
- 539 51. Xu, P.; Liu, J.; Derynck, R., Post-translational regulation of TGF- β receptor and Smad signaling. *FEBS Letters*
540 **2012**, *586*, 1871-1884.
- 541 52. Vindevoghel, L.; Lechleider, R. J.; Kon, A.; de Caestecker, M. P.; Uitto, J.; Roberts, A. B.; Mauviel, A.,
542 SMAD3/4-dependent transcriptional activation of the human type VII collagen gene (COL7A1) promoter by
543 transforming growth factor beta. *Proceedings of the National Academy of Sciences of the United States of America*
544 **1998**, *95*, 14769-74.

545 53. Liu, F. Y.; Li, X. Z.; Peng, Y. M.; Liu, H.; Liu, Y. H., Arkadia regulates TGF-beta signaling during renal tubular
546 epithelial to mesenchymal cell transition. *Kidney international* **2008**, *73*, 588-594.

547 54. Xie, F.; Zhang, Z.; van Dam, H.; Zhang, L.; Zhou, F., Regulation of TGF-beta Superfamily Signaling by SMAD
548 Mono-Ubiquitination. *Cells* **2014**, *3*, 981-93.

549

550

551

552

553

554

555

556

557

558

559

560

561

562

563

564

565

566

567

568 **Figure legends**

569 **Figure 1. Novel triterpenoids from SLPC.** (A) Chemical structures of compounds PZF-PZH. (B) Key HMBC
570 correlations for compounds PZF-PZH. (C) Key NOESY correlations for compounds PZF-PZH.

571 **Figure 2. PZF, PZG and PZH attenuated TIF and podocyte injury induced by Ang II and TGF- β 1.** (A)
572 Representative Western blot analyses revealed that the 10 μ M of PZF, PZG and PZH exhibited stronger suppression of
573 TGF- β 1-induced high expression of collagen I. (B) Representative Western blot analyses revealed that triterpene PZG
574 and PZH exhibited stronger suppression of TGF- β 1-induced TIF in HK-2 cells, and diterpene PZF hardly reversed the
575 abnormality. (C) Graphic presentation of collagen I, fibronectin, α -SMA and E-cadherin expression in different groups
576 as indicated. (D) Representative Western blot analyses revealed that PZG and PZH performed better inhibitory effect on
577 Ang II-induced TIF in HK-2 cells, and diterpene PZF was weaker. (E) Graphic presentation of collagen I, fibronectin,
578 α -SMA and E-cadherin expression in different groups as indicated. (F) The abnormal mRNA expression of collagen I,
579 fibronectin, α -SMA and vimentin were decreased by PZG and PZH, while down-regulated E-cadherin was increased
580 after PZG and PZH stimulation. (G) Representative Western blot analyses revealed that PZG and PZH exhibited
581 powerful inhibitory effect of TGF- β 1-induced podocyte injury, and PZF showed no inhibitory effect in MPC5. (H)
582 Graphic presentation of podocin, nephrin, podocalyxin and synaptopodin expression in different groups as indicated. (I)
583 Representative Western blot analyses revealed that PZG and PZH exhibited stronger suppression of podocyte injury
584 caused by Ang II, and PZF hardly reverse the injury in MPC5. (J) Graphic presentation of podocin, nephrin,
585 podocalyxin and synaptopodin expression in different groups as indicated. (K) Representative micrographs showed
586 vimentin expression in different groups in HK-2 cells. Each data represents the mean \pm SD for groups of different
587 group. #P<0.05; ##P<0.01 versus CTL. *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

588 **Figure 3. PZF, PZG and PZH inhibited the activation of RAS.** (A) Representative Western blot analyses revealed
589 that triterpene PZG and PZH down-regulated the activation of RAS in HK-2 cells after TGF- β 1 stimulation, but
590 diterpene PZF showed no effect. (B) Graphic presentation of AGT, renin, ACE and AT1 expression in different groups

591 as indicated. (C) Representative Western blot analyses revealed that Ang II up-regulated the protein expression of RAS
592 components, and PZG and PZH reversed the abnormal expression of AGT, renin, ACE and AT1 protein, but PZF
593 performed no effect in HK-2 cells. (D) Graphic presentation of AGT, renin, ACE and AT1 expression in different
594 groups as indicated. Each data represents the mean \pm SD for groups of different group. #P<0.05; ###P<0.01 versus CTL.
595 *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

596 **Figure 4. PZF, PZG and PZH suppressed activated Wnt/ β -catenin and its downstream target genes expression.**

597 (A) Representative Western blot analyses demonstrated that triterpene PZG and PZH decreased activated Wnt/ β -catenin
598 and its downstream targets after TGF- β 1 stimulation in HK-2 cells, but diterpene PZF showed no effect. (B) Graphic
599 presentation of Wnt1, β -catenin, active β -catenin, snail, twist, MMP-7, PAI-1 and Fsp-1 expression in different groups
600 as indicated. (C) Representative Western blot analyses showed that Ang II stimulated the accumulation of β -catenin and
601 active β -catenin, and PZG and PZH decreased the abnormal expression of Wnt/ β -catenin and its downstream targets,
602 but PZF performed no effect in HK-2 cells. (D) Graphic presentation of Wnt1, β -catenin, active β -catenin, snail, twist,
603 MMP-7, PAI-1 and Fsp-1 expression in different groups as indicated. (E) TGF- β 1 up-regulated Wnt1, Wnt2, Wnt3,
604 Wnt3a, Wnt7a and Wnt8a expression, and their abnormality were reversed after given PZG and PZH. (F)
605 Representative micrographs showed β -catenin expression in different groups in HK-2 cells. (G) Representative Western
606 blot analyses revealed that the inhibitory effect of PZG and PZH were obvious in TGF- β 1-induced the activation of
607 Wnt/ β -catenin, but PZF showed no effect in MPC5. (H) Graphic presentation of Wnt1, β -catenin, active β -catenin,
608 snail, twist, MMP-7, PAI-1 and Fsp-1 expression in different groups as indicated. (I) Representative Western blot
609 analyses indicated that PZG and PZH reversed the abnormal expression of Wnt1, β -catenin, active β -catenin, snail,
610 twist, MMP-7, PAI-1 and Fsp-1 protein caused by Ang II in MPC5, but PZF performed no effect in HK-2 cells. (J)
611 Graphic presentation of Wnt1, β -catenin, active β -catenin, snail, twist, MMP-7, PAI-1 and Fsp-1 expression in different
612 groups as indicated. Each data represents the mean \pm SD for groups of different group. #P<0.05; ###P<0.01 versus CTL.
613 *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

614 **Figure 5. PZF, PZG and PZH selectively inhibits TGF- β 1/Smad3 pathway.** (A) Representative Western blot
615 analyses showed that triterpene PZG and PZH exhibited stronger suppression of TGF- β 1-induced up-regulation of
616 TGF β RI and p-Smad3 in HK-2 cells, and diterpene PZF hardly reversed the abnormality. (B) Graphic presentation of
617 TGF β RI, TGF β RII, p-Smad2, Smad2, p-Smad3, Smad3, Smad4 and Smad7 expression in different groups as indicated.
618 (C) Representative Western blot analyses revealed that PZG and PZH performed better inhibitory effect on Ang
619 II-induced high expression of TGF β RI and p-Smad3 in HK-2 cells, and diterpene PZF was weaker. (D) Graphic
620 presentation of TGF β RI, TGF β RII, p-Smad2, Smad2, p-Smad3, Smad3, Smad4 and Smad7 in different groups as
621 indicated. (E) Representative micrographs show p-Smad3 expression in different groups in HK-2 cells. (F)
622 Representative Western blot analyses indicated that PZG and PZH exhibited powerful inhibitory effect of
623 TGF- β 1-induced activated TGF- β 1/Smad3 pathway, and PZF showed no inhibitory effect in MPC5. (G) Graphic
624 presentation of TGF β RI, TGF β RII, p-Smad2, Smad2, p-Smad3, Smad3, Smad4 and Smad7 expression in different
625 groups as indicated. (H) Representative Western blot analyses demonstrated that PZG and PZH exhibited stronger
626 suppression of activated TGF- β 1/Smad3 caused by Ang II, and PZF hardly reverse the injury in MPC5. (I) Graphic
627 presentation of TGF β RI, TGF β RII, p-Smad2, Smad2, p-Smad3, Smad3, Smad4 and Smad7 expression in different
628 groups as indicated. Each data represents the mean \pm SD for groups of different group. #P<0.05; ##P<0.01 versus CTL.
629 *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

630 **Figure 6. PZF, PZG and PZH selectively interrupt the interactions of Smad3 with TGF β RI and SARA in HK-2**
631 **cells.** (A) Representative Western blot analyses revealed that TGF- β 1 enhanced the interactions of TGF β RI and
632 TGF β RII with Smad2 and Smad3, while PZF, PZG specifically broke the interaction between TGF β RI and Smad3, and
633 PZF showed no effect. (B) Graphic presentation of representative Western blot in different groups as indicated. (C)
634 Representative Western blot analyses demonstrated that PZF, PZG and PZH performed no effect on the connection of
635 Smad2 with TGF β RI and TGF β RII. (D) Graphic presentation of representative Western blot in different groups as
636 indicated. (E) Representative Western blot analyses revealed that apart from PZF, PZG and PZH owned powerful

637 inhibitory effect on the interactions of Smad3 with TGF β RI and TGF β RII. (F) Graphic presentation of representative
638 Western blot in different groups as indicated. (G) Representative Western blot analyses indicated that PZF, PZG and
639 PZH affected the interactions of SARA with TGF β RI and Smad3, but hardly changed the interaction of SARA with
640 Smad2. (H) Graphic presentation of representative Western blot in different groups as indicated. (I) CO-IP
641 demonstrated that PZG and PZH only influenced the interaction between SARA and Smad3. (J) Graphic presentation of
642 representative Western blot in different groups as indicated. (K) PZG and PZH broke the interactions of SARA with
643 TGF β RI and Smad3. Each data represents the mean \pm SD for groups of different group. #P<0.05; ##P<0.01 versus
644 CTL. *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

645 **Figure 7. PZF, PZG and PZH performed no anti-fibrotic effect after knockdown of Smad3.** (A) Specific Smad3
646 siRNA evidently down-regulated protein expression of Smad3 in HK-2. Graphic presentation of β -catenin protein
647 expression in different groups as indicated. (B) PZF, PZG and PZH had no effect on the RNAi of Smad3. Graphic
648 presentation of β -catenin protein expression in different groups as indicated. (C) After knockdown Smad3, PZF, PZG
649 and PZH performed no effect on renal fibrosis. (D) Graphic presentation of collagen I, fibronectin, α -SMA and
650 E-cadherin expression in different groups as indicated. Each data represents the mean \pm SD for groups of different
651 group. #P<0.05; ##P<0.01 versus CTL. *P<0.05; **P<0.01 versus TGF- β 1 or Ang II stimulation.

652 **Figure 8. PZG and PZH inhibited EMT by suppressing the activation of RAS, Wnt/ β -catenin and TGF- β 1/Smad**
653 **pathways.** PZG and PZH selectively inhibited the phosphorylation of Smad3 to attenuate renal fibrosis. PZG and PZH
654 suppressed the activation of RAS and performed like the combination of ACEI and ARB. PZG and PZH decreased
655 Wnt1 and β -catenin protein expression.

656

657

658

659

660 **Table 1 Primers of Homo sapiens for qRT-PCR (5'→3') of EMT and Wnts genes**

Gene	Forward	Reverse	Product Size (bp)
Collagen I	TGTGCCACTCTGACTGGAAG	CGCCATACTCGAACTGGAATC	228
Fibronectin	CCACAGTGGAGTATGTGGTTAG	CAGTCCTTTAGGGCGATCAAT	104
α -SMA	GATGGTGGGAATGGGACAAA	GCCATGTTCTATCGGGTACTTC	94
Vimentin	GATTCACTCCCTCTGGTTGATAC	GTCATCGTGATGCTGAGAAGT	108
E-cadherin	CTTCTGCTGATCCTGTCTGATG	TGCTGTGAAGGGAGATGTATTG	144
Wnt1	CGGCGTTTATCTTCGCTATCA	GTAGTCACACGTGCAGGATT	95
Wnt2	CGGAATCTGCCTTGTGTTATG	TTGGATCACAGGAACAGGATTT	103
Wnt3	CTGACTTCGGCGTGTTAGT	CCTCGTTGTGTGCTTGTC	93
Wnt3a	CAAGATTGGCATCCAGGAGT	ATGAGCGTGCTCACTGCAAAG	173
Wnt7a	CGTGCTCAAGGACAAGTACA	GTACGACAGTGGCTTCTTGAT	103
Wnt8a	CAGTGAGAGCCACCATGAAA	CCTGGTCATACTTGGCCTTTAG	132
β -actin	ACAAGCCACAAGATTACAAG	ATCAGCAG TCTCATTCCAA	92

661

662

663

664

665

666

667

668 **Table 2 NMR data for PZF (500 MHz for ^1H and 125 MHz for ^{13}C , in pyridine)**

Position	C	H	HMBC (H to C)
1	39.0 (t)	1.51, 2.30 (m)	3, 4, 5, 10, 20
2	30.4 (d)	1.41 (m)	4, 10
3	35.3 (t)	2.54 (m)	4, 18, 19
4	48.0 (s)		
5	45.8 (d)	2.55 (m)	4, 7, 10, 18, 19, 21
6	19.4 (t)	1.63, 1.77 (m)	8, 18
7	30.8 (t)	2.88, 2.95 (m)	5, 8, 9, 14
8	135.5 (s)		
9	148.1 (s)		
10	37.6 (s)		
11	125.1 (d)	7.29 (d, $J = 8.2$ Hz)	3, 8, 10
12	124.7 (d)	7.12 (dd, $J = 8.1, 1.8$ Hz)	11, 13, 15
13	146.3 (s)		
14	127.7 (d)	6.95 (d, $J = 1.3$ Hz)	7, 9, 12, 15
15	34.3 (d)	2.81 (m)	12, 14
16	24.6 (q)	1.23 (s)	13, 15, 17
17	24.6 (q)	1.23 (s)	13, 15, 16
18	176.4 (s)		
19	17.5 (q)	1.49 (s)	3, 5, 1'
20	181.4 (s)		
21	25.7 (q)	1.21 (s)	1, 5, 10

1'	37.8 (t)	1.84, 2.08 (m)	19, 2'-6'
2'-6'	30.0 - 30.4 (t)	1.26 – 1.30	7'
7'	32.5 (t)	1.25	9'
8'	23.4 (t)	1.27	
9'	14.7 (q)	0.87 (m)	7', 8'

669

670

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686

687 **Table 3 NMR data for PZG and PZH (500 MHz for ^1H and 125 MHz for ^{13}C , in pyridine)**

Position	PZG		PZH	
	^{13}C	^1H	^{13}C	^1H
1	36.8 (t)	2.15, 1.93 (m)	33.1 (s)	1.73, 1.23 (m)
2	30.7 (t)	2.52 (dd, 10.7, 5.0)	23.8 (t)	2..30 (m)
3	177.0 (s)		77.4 (s)	4.60 (s)
4	149.7 (s)		42.8 (s)	
5	51.2 (d)	2.35 (s)	186.6 (s)	
6	28.9 (t)	2.56, 2.07 (m)	126.7 (d)	6.56 (s)
7	118.4 (d)	5.30 (t)	199.1 (s)	
8	142.3 (s)		140.1 (s)	
9	138.0 (s)		162.9 (s)	
10	39.3 (s)		43.6 (s)	
11	120.8 (d)	5.36 (br s)	30.3 (t)	1.27 (m)
12	37.5 (t)	2.71 (d, 17.9), 2.49 (m)	29.4 (t)	2.28, 2.13
13	46.0 (s)		46.9 (s)	
14	49.0 (s)		47.3 (s)	
15	44.1 (t)	2.43 (d, 8.7), 1.82 (m)	46.3 (t)	3.03, 2.96
16	76.8 (d)	4.53 (m)	76.1 (d)	4.48 (m)
17	58.0 (d)	2.87 (dd, 11.2, 6.0)	56.2 (d)	2.84 (m)
18	18.7 (q)	1.11 (s)	18.4 (q)	1.17 (s)
19	22.6 (q)	1.05 (s)	23.4 (q)	1.38 (s)
20	49.6 (d)	2.98 (dt, 11.2, 3.4)	49.0 (d)	2.96 (m)

21	179.2 (s)		179.5 (s)	
22	33.9 (t)	2.45 (m), 2.36 (s)	33.5 (t)	2.40, 2.54 (m)
23	23.8 (t)	2.10 (m), 1.89 (m)	23.8 (t)	2.30 (m)
24	45.3 (t)	1.85 (m), 1.72 (s)	156.4 (s)	
25	70.0 (s)		34.5 (d)	2.28 (m)
26	30.5 (q)	1.36 (s)	22.3 (q)	1.00 (s)
27	30.3 (q)	1.36 (s)	22.1 (q)	1.01 (s)
28	112.6 (t)	4.85, 4.78 (br s)	27.9 (q)	1.41 (s)
29	22.6 (q)	1.75 (s)	27.6 (q)	1.34 (s)
30	25.3 (q)	1.51 (s)	25.5 (q)	1.64 (s)
31			107.1 (t)	5.02, 4.87 (m)

688

689

690

691

692

693

694

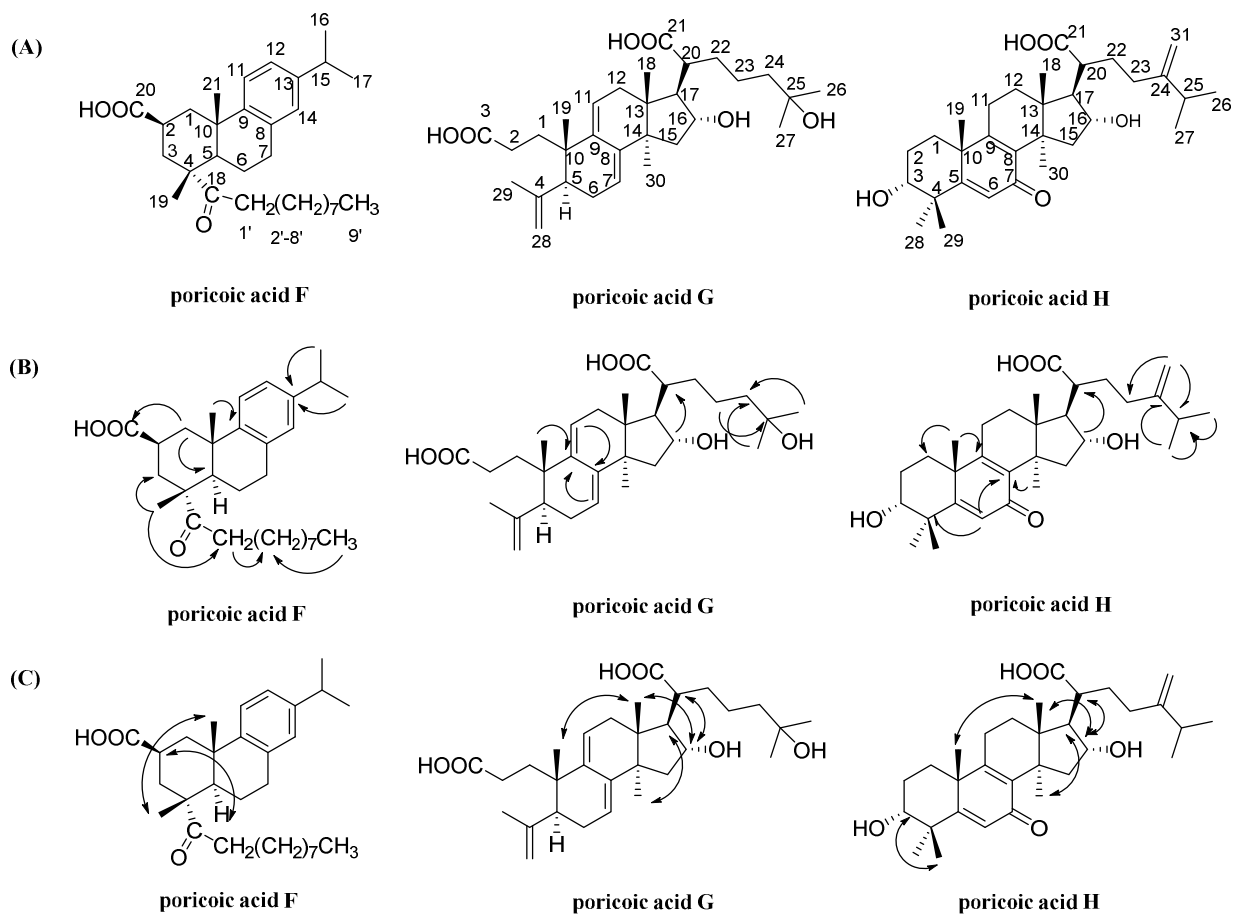
695

696

697

698

699

700 **Figure 1**

701

702

703

704

705

706

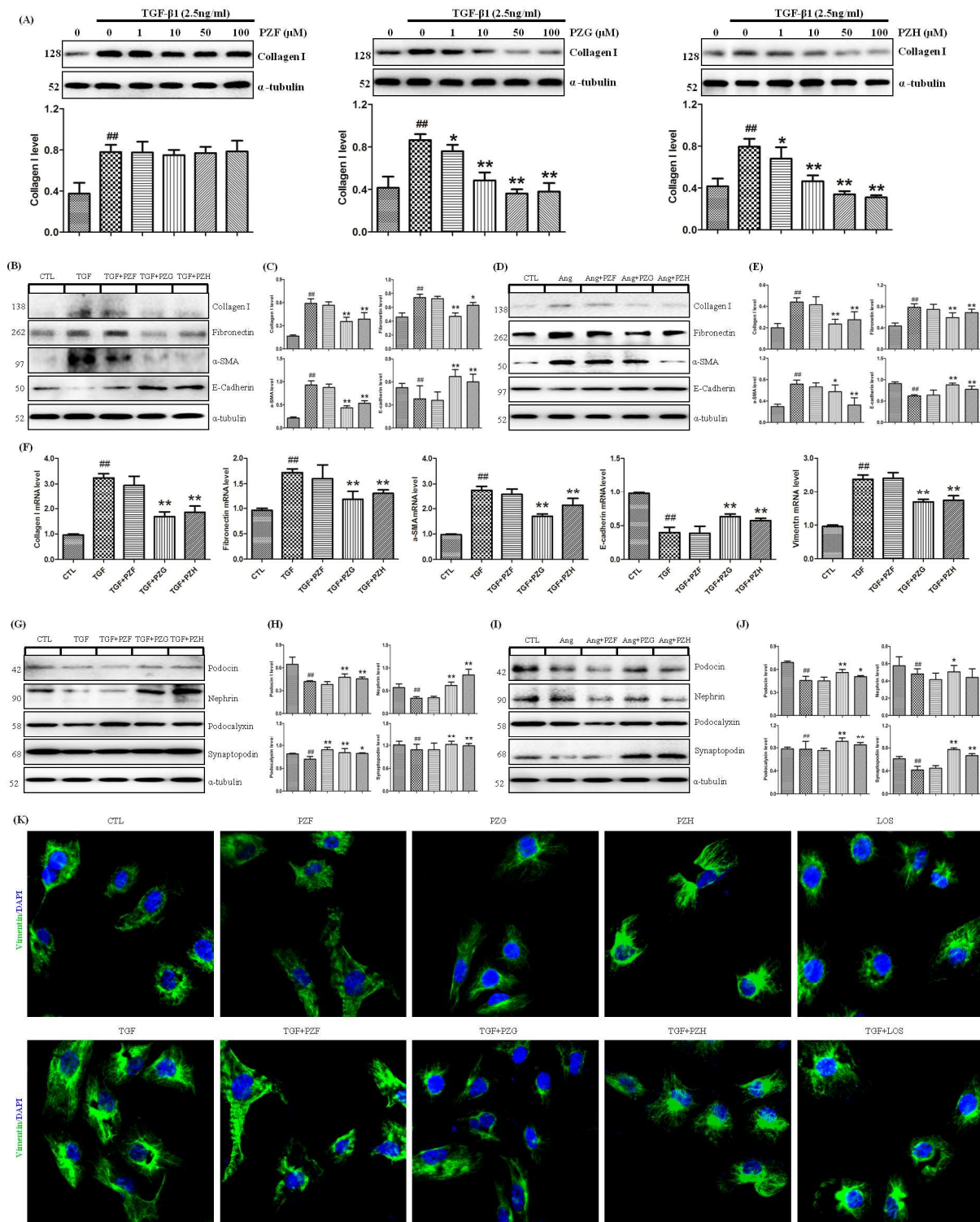
707

708

709

710

711

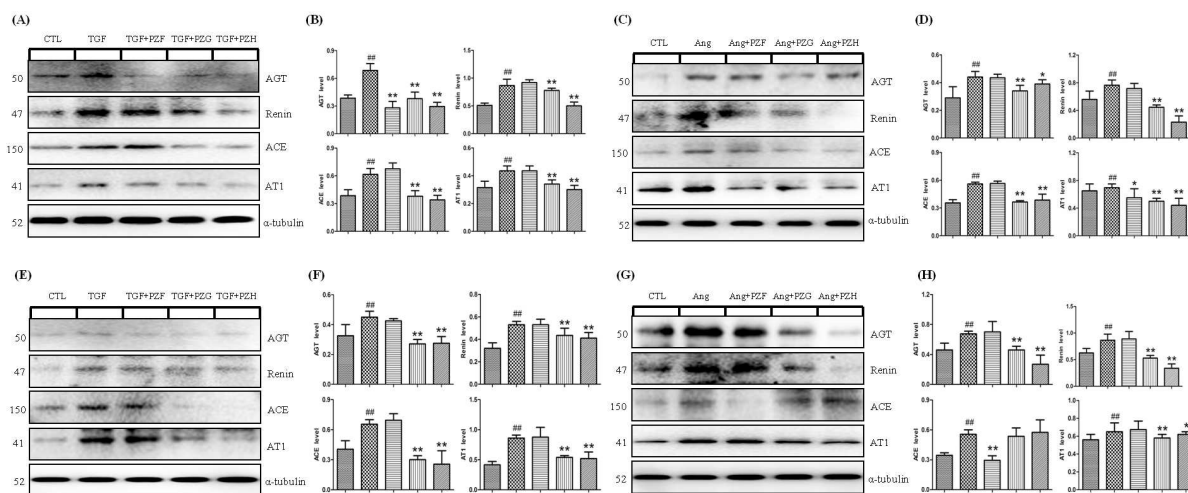
712 **Figure 2**

713

714

715

716

717 **Figure 3**

718

719

720

721

722

723

724

725

726

727

728

729

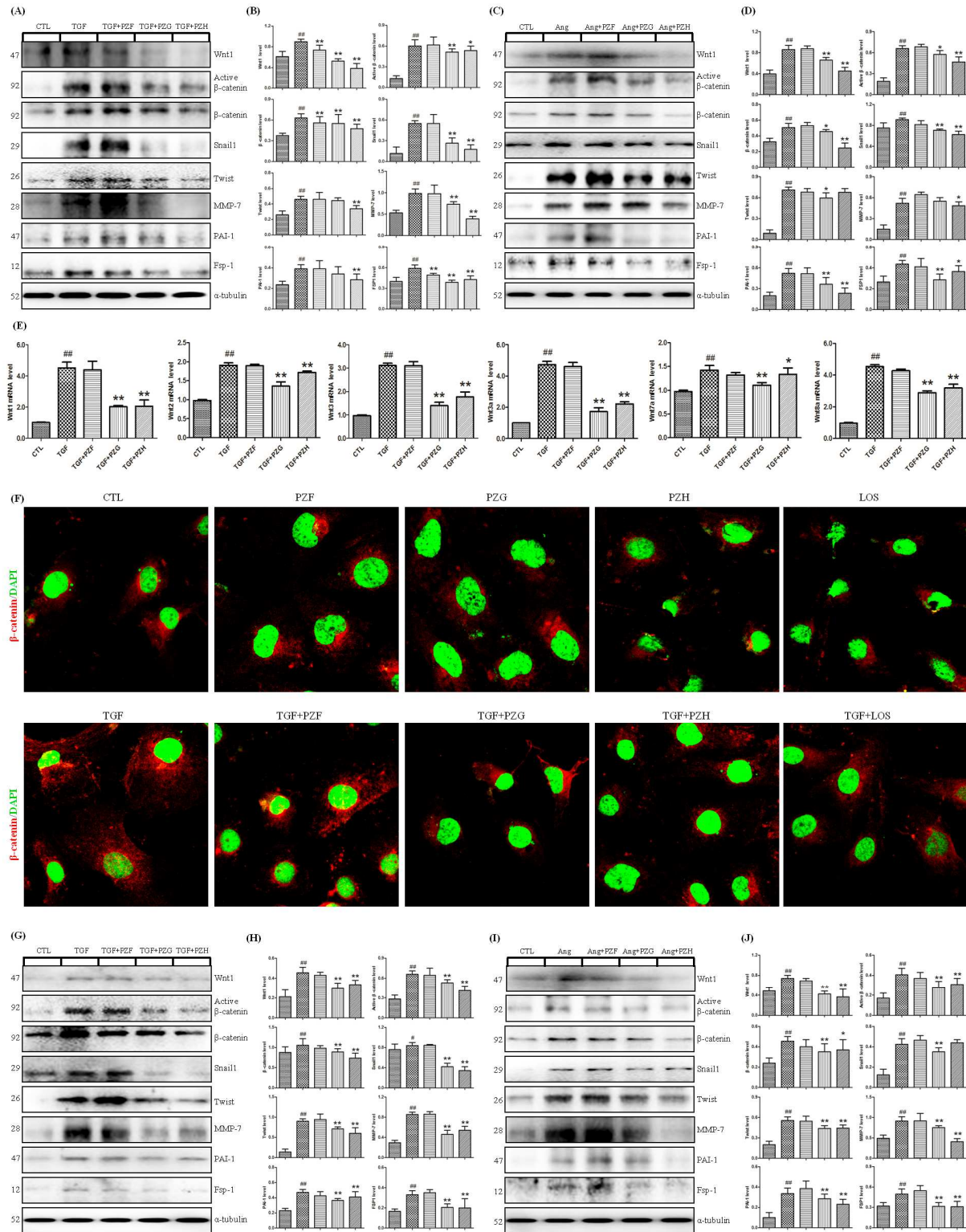
730

731

732

733

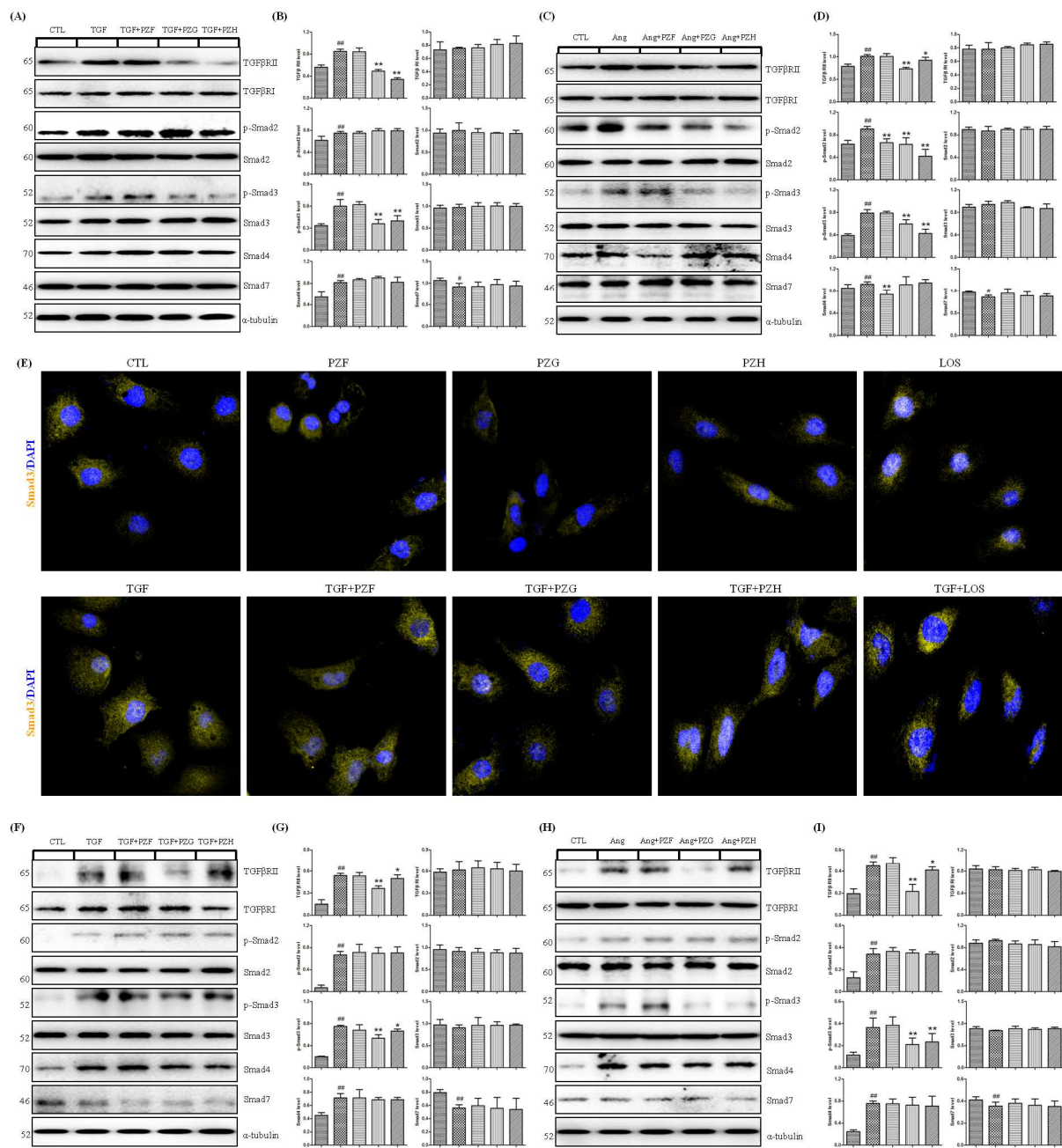
734

735 **Figure 4**

736

737

738

739 **Figure 5**

740

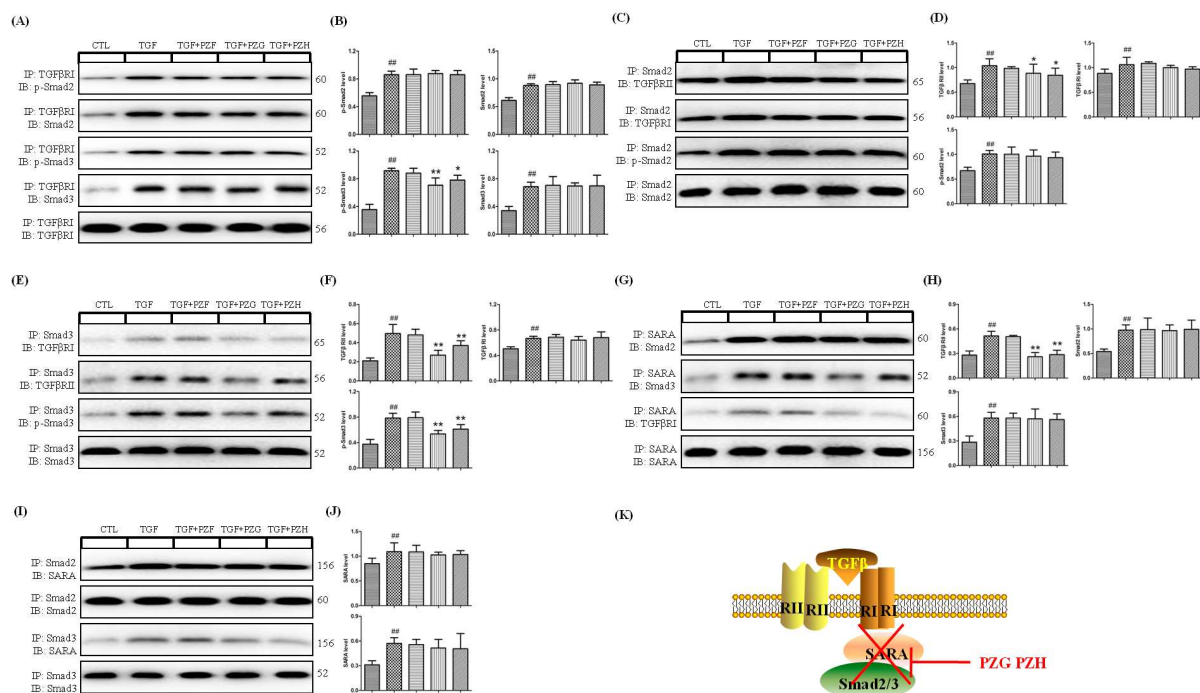
741

742

743

744

745

746 **Figure 6**

747

748

749

750

751

752

753

754

755

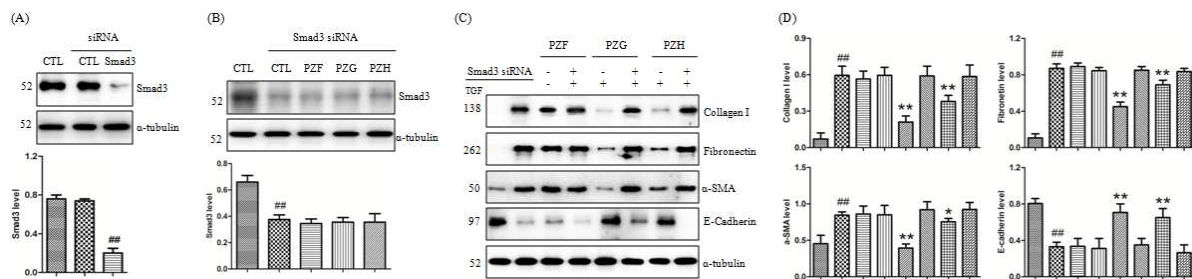
756

757

758

759

760

761 **Figure 7**

762

763

764

765

766

767

768

769

770

771

772

773

774

775

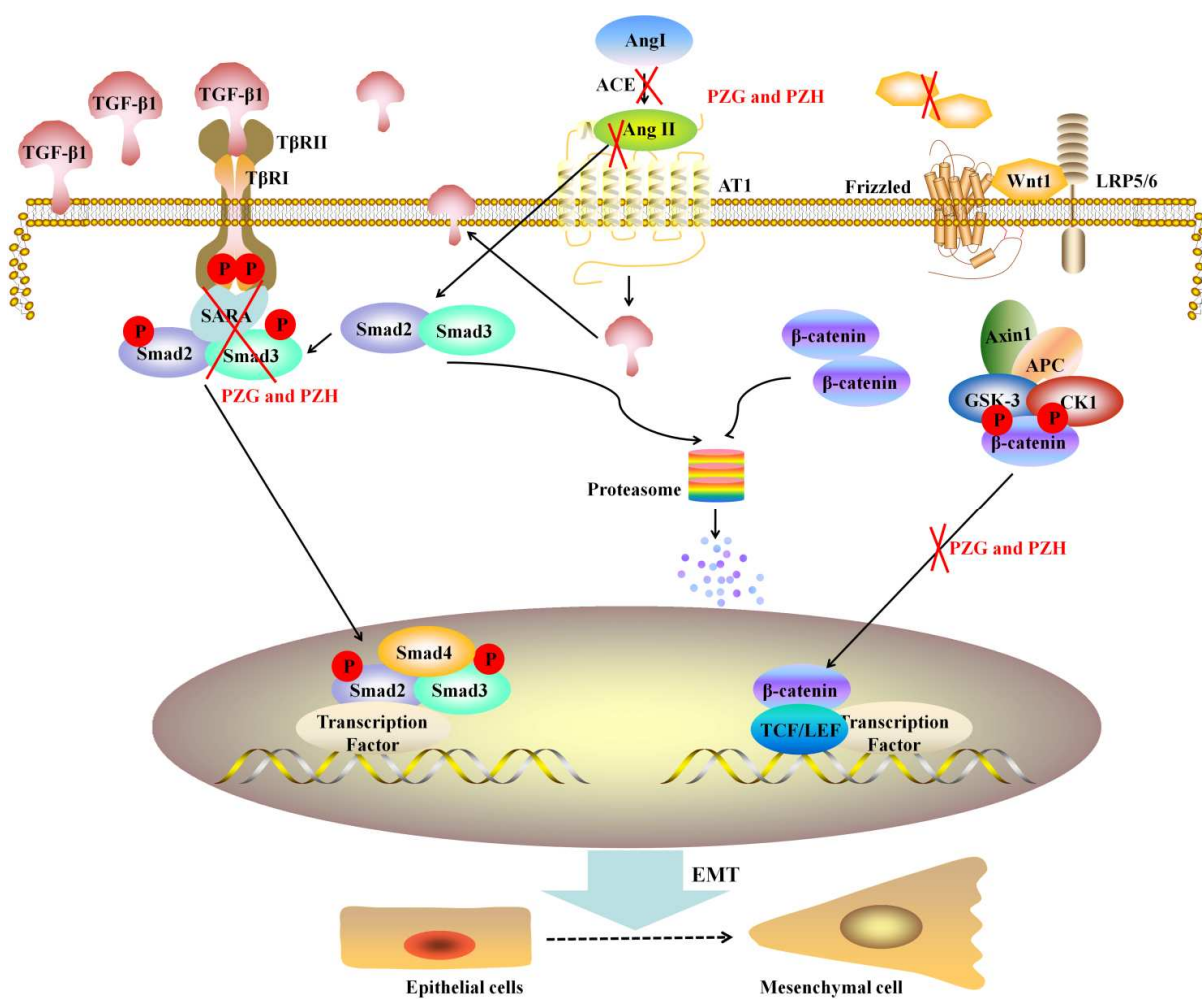
776

777

778

779

780

781 **Figure 8**

782

783

784

785

786

787

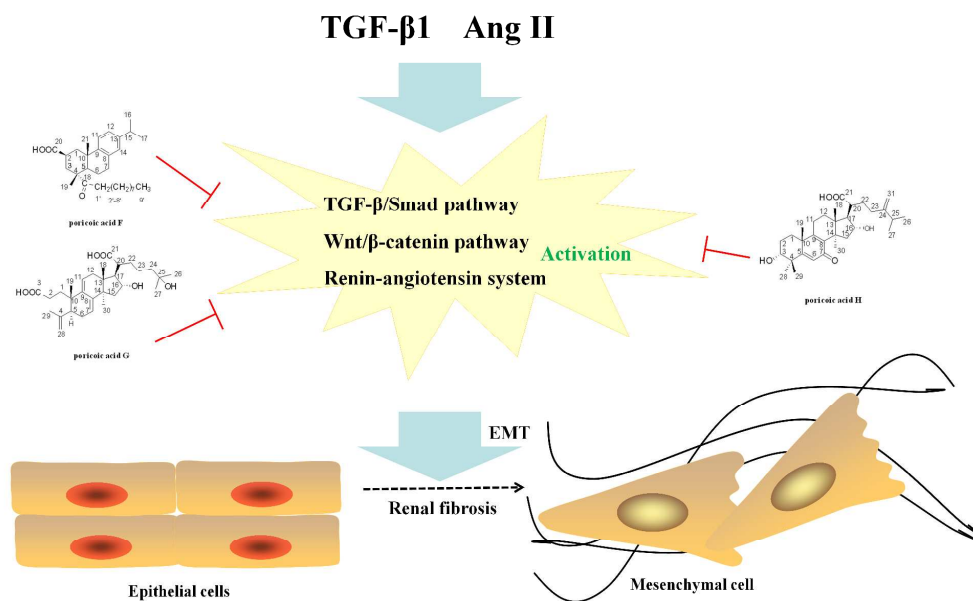
788

789

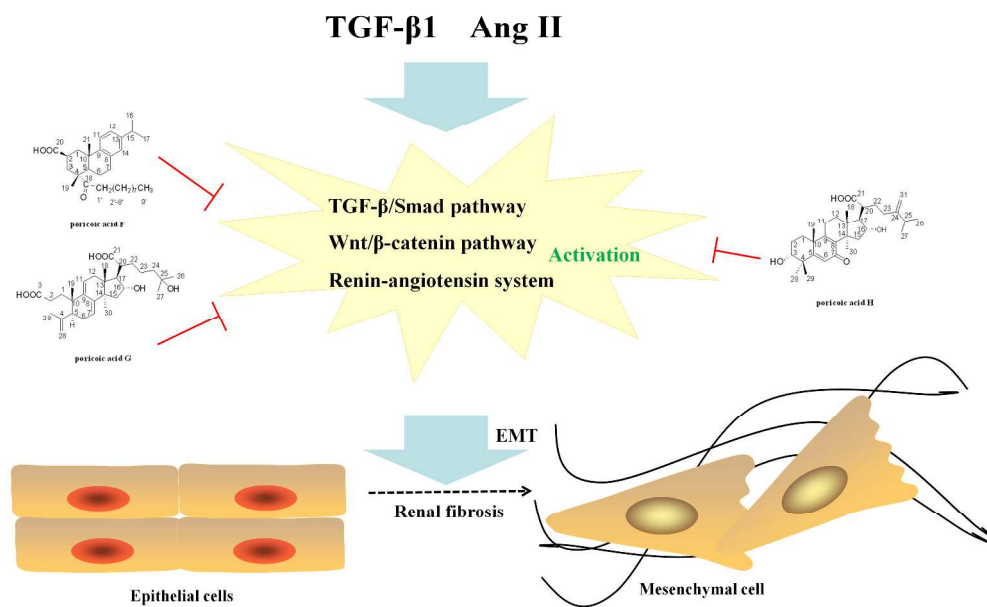
790

791

792 TOC graphic



793



1021x693mm (96 x 96 DPI)