GQ5 Hinders Renal Fibrosis in Obstructive Nephropathy by Selectively Inhibiting TGF-β–Induced Smad3 Phosphorylation

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ABSTRACT

TGF-β1, via Smad-dependent or Smad-independent signaling, has a central role in the pathogenesis of renal fibrosis. This pathway has been recognized as a potential target for antifibrotic therapy. Here, we identified GQ5, a small molecular phenolic compound isolated from the dried resin of Toxicodendron vernicifluum, as a potent and selective inhibitor of TGF-β1–induced Smad3 phosphorylation. In TGF-β1–stimulated renal tubular epithelial cells and interstitial fibroblast cells, GQ5 inhibited the interaction of Smad3 with TGF-β type I receptor (TβRI) by blocking binding of Smad3 to SARA, suppressed subsequent phosphorylation of Smad3, reduced nuclear translocation of Smad2, Smad3, and Smad4, and downregulated the transcription of major fibrotic genes such as α-smooth muscle actin (α-SMA), collagen I, and fibronectin. Notably, intraperitoneal administration of GQ5 in rats immediately after unilateral ureteral obstruction (UUO) selectively inhibited Smad3 phosphorylation in UUO kidneys, suppressed renal expression of α-SMA, collagen I, and fibronectin, and resulted in impressive renal protection after obstructive injury. Late administration of GQ5 also effectively attenuated fibrotic lesions in obstructive nephropathy. In conclusion, our results suggest that GQ5 hinders renal fibrosis in rats by selective inhibition of TGF-β1–induced Smad3 phosphorylation.

Renal fibrosis, characterized by massive interstitial myofibroblast activation and excessive matrix proteins accumulation, is the final common pathway of virtually all kinds of progressive CKD leading to ESRD.1–4 Mounting evidence has established a crucial role for TGF-β in mediating chronic inflammation, myofibroblast activation, and extracellular matrix (ECM) accumulation.4–7 TGF-β1 signals are transduced by transmembrane serine/threonine kinase receptors type I (TβRI) and type II (TβRII) and intracellular mediators known as Smads.8,9 Upon TGF-β1 stimulation, Smad2 and Smad3 recruit to TβRI by adaptor proteins such as Smad anchor for receptor activation (SARA)10 and are phosphorylated by TβRI. Phosphorylated Smads heteroligomerize with the common partner Smad4 and then translocate into the nucleus, where they control the transcription of TGF-β–responsive genes through interaction with specific cis-acting elements in the regulatory regions.11–13 Although both Smad2 and Smad3 are strongly activated in various experimental

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and human fibrotic kidney diseases, it is now well recognized that Smad3 is the key mediator of TGF-β1–induced ECM production and tissue fibrosis.14–16 Deletion of Smad3 suppresses fibrogenesis in a number of rodent models, including diabetic nephropathy,17 obstructive nephropathy,18,19 and drug toxicity–related nephropathy.20 On the other hand, conditional knocking out of Smad2 from kidney tubular cells significantly enhanced renal fibrosis via activating of Smad3 signaling.21 These findings indicate that Smad3 expression and/or phosphorylation might be a potential target for the intervention of renal fibrosis.

The potential of natural products as the candidates for drug discovery has been well recognized.22 Resina Toxicodendri is the dried resin secreted by Toxicodendron vernicifluum and has been used as an anti-inflammatory and antiscarring agent in traditional Chinese medicine for centuries. In this study, we isolated and purified the major component of Resina Toxicodendri, GQ5, a small molecular phenolic compound. We demonstrated that treatment with GQ5 significantly inhibited the progression of interstitial fibrosis in the unilateral ureteral obstruction (UUO) model. We also demonstrated that the antifibrotic effect of GQ5 might be mediated by selective inhibition of TGF-β1–induced Smad3 phosphorylation.

RESULTS

The Chemical Structure and Toxicity of GQ5

GQ5 isolated from Resina Toxicodendri is a HPLC-grade pure small molecular phenolic compound, 3-[{(Z)-pentadec-8-enyl}catechol. The chemical structure is shown in Figure 1. The detailed characterization and purity of this compound are presented in Supplemental Figures 1–3.

To test the toxicity of GQ5, rat proximal tubular cells (NRK52E) were incubated with indicated concentrations of GQ5. Cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments. GQ5, over a complete pharmacological relevant dose (0.25–4 μM), did not affect the viability or proliferation of the cells (Supplemental Figure 4).

In vivo toxicity studies showed that mice intraperitoneally injected with single dose of GQ5 up to 800 mg/kg did not die or develop any obvious adverse event during 7-day observation.

GQ5 Ameliorates Renal Interstitial Fibrosis after UUO

We initially investigated the effect of GQ5 on renal interstitial fibrosis in UUO, a typical model of renal interstitial fibrosis. In the preliminary study, GQ5 was administered right after operation via intraperitoneal injection at the dosage of 10, 20, 40, or 80 mg/kg per day (n=5 in each group). Rats were euthanized at day 7 after UUO. Real-time PCR and Western blot analyses demonstrated that 40 mg/kg of GQ5 significantly decreased the expression of α-smooth muscle actin (α-SMA), collagen I, and fibronectin (Supplemental Figure 5). Therefore, 40 mg/kg of GQ5 was used in the following experiments.

As shown in Figure 2, UUO rats exhibited marked interstitial inflammation and fibrosis in renal tissue stained with hematoxylin and eosin and Masson’s trichrome. Treatment with GQ5, either initiating right after or 7 days after operation, significantly reduced inflammatory cell infiltration and interstitial fibrosis score (Figure 2, A–C, Supplemental Figure 6A). Neither lymphocyte infiltration nor fibrogenesis could be detected in the contralateral kidneys (Supplemental Figure 6). Intervention with GQ5 also significantly inhibited the upregulation of α-SMA, collagen I, and fibronectin in UUO rats at both mRNA and protein levels (Figure 2, D–F), suggesting that treatment with GQ5 not only prevented renal fibrosis, but also ameliorated established renal fibrosis.

GQ5 Inhibits TGF-β1–Induced Smad3 Phosphorylation In Vitro

To further explore the mechanism underlying the antifibrotic effect of GQ5, we first examined whether GQ5 affected TGF-β1–induced activation of the Smads pathway in NRK52E and renal fibroblast (NRK49F) cells. As presented in Figure 3, A and B, incubation with TGF-β1 significantly induced phosphorylation of Smad2 and Smad3 in both NRK52E (Figure 3A) and NRK49F cells (Figure 3B). GQ5 treatment attenuated TGF-β1–induced Smad3 phosphorylation in a dose-dependent manner, but did not affect TGF-β1–induced Smad2 phosphorylation. The time course of TGF-β1–induced phosphorylation of Smad2 and Smad3 in the presence or absence of GQ5 is shown in Supplemental Figure 7. The inhibitory effect of GQ5 was almost undetectable in NRK52E and NRK49F cells in the absence of TGF-β1 stimulation.

To confirm the specific effect of GQ5 on Smad3, we further examined the expression of other components of the Smads pathway such as Smad4 and Smad7, as well as other downstream signaling of TGF-β1. As shown in Figure 3, C and D, GQ5 did not affect the protein expression of Smad4 or Smad7 (Figure 3C), nor the TGF-β1–induced phosphorylation of p38, ERK, or PI3K (Figure 3D). Taken together, these in vitro data indicated that GQ5 selectively inhibited TGF-β1–induced Smad3 phosphorylation.

GQ5 Selectively Inhibits Smad3 Phosphorylation In Vivo

We next examined whether GQ5 could inhibit Smad3 phosphorylation in UUO kidneys. As shown in Figure 4, A and B, phosphorylation of both Smad3 and Smad2 in renal tissue was significantly increased in UUO rats compared with sham controls. Treatment with GQ5 significantly inhibited Smad3 but not Smad2 phosphorylation. In line with our in vitro study, GQ5 did not affect the expression of Smad4 and Smad7 (Figure 4C), nor the phosphorylation of p38, PI3K, or ERK in UUO rats (Figure 4D).
GQ5 Inhibits the Transcription Activity of Smad Signaling

Upon TGF-β1 stimulation, phosphorylated Smad3 translocates into nuclei to regulate gene transcription.8,23,24 The effect of GQ5 on Smad3 signaling in response to TGF-β1 was further examined by Smad nuclear translocation and Smad3-responsive promoter assays. As shown in Figure 5A, immunofluorescence staining revealed that preincubating NRK52E cells with GQ5 significantly reduced the TGF-β1–induced Smad3 nuclear translocation. Treatment with GQ5 also reduced nuclear...
Figure 3. GQ5 selectively inhibits TGF-β1–induced Smad3 phosphorylation. NRK52E and NRK49F cells are preincubated with GQ5 for 1 hour before TGF-β1 (10 ng/ml) treatment. Cells are harvested 1 hour after TGF-β1 stimulation, and cell lysates are immunoblotted. (A) GQ5 selectively inhibits TGF-β1–induced Smad3 phosphorylation in NRK52E cells. (B) GQ5 selectively inhibits TGF-β1–induced Smad3 phosphorylation in NRK49F cells. (C) GQ5 does not affect Smad4 or Smad7 protein expression. (D) GQ5 does not affect TGF-β1–induced phosphorylation of p38, PI3K or ERK. Data are expressed as the mean±SD of three independent experiments. *P<0.05 in p-Smad3 expression in GQ5-treated cells (A and B; ANOVA). *P<0.05 versus untreated cells. Ctrl, control.
translocation of Smad2 and Smad4. Promoter assays, using a luciferase reporter system containing the promoter region of the human collagen I gene, also demonstrated that treatment with GQ5 significantly inhibited TGF-β1–induced Smad3-dependent collagen I promoter activity in a dose-dependent manner (Figure 5B).

Next, we tested the effect of GQ5 on the expression of TGF-β1–induced fibrotic genes such as α-SMA, collagen I, and fibronectin in both NRK52E and NRK49F cells. Treatment with GQ5 significantly inhibited TGF-β1–induced expression of α-SMA, collagen I, and fibronectin at both mRNA (Figure 5, C and D) and protein levels (Figure 5, E and F) in NRK52E (Figure 5, C and E) and NRK49F (Figure 5, D and F) cells, indicating that GQ5 significantly inhibited the downstream gene expression of TGF-β1/Smads signaling.

GQ5 Selectively Blocks the Interaction of Smad3 with TβRI

Upon TGF-β1 stimulation, TβRII phosphorylates TβRI, which binds to and phosphorylates Smad2 and Smad3. To test whether GQ5 affects TβRI-Smad3 interaction, cell lysates were harvested from NRK52E cells and coimmunoprecipitation analysis was performed. As shown in Figure 6, TβRII bound with TβRII, Smad2, and Smad3 upon TGF-β1 stimulation. Treatment with GQ5 significantly inhibited the interaction of Smad3 with TβRII, but did not affect the interaction of Smad2 with TβRII. Similar results were obtained from GQ5-treated UUO rats (Figure 7). These data suggest that GQ5 inhibited Smad3 phosphorylation by selectively blocking Smad3-TβRI interaction both in vitro and in vivo.

GQ5 Selectively Blocks the Interaction of Smad3 with SARA

To test the effect of GQ5 on Smad2/Smad3-SARA binding, NRK52E cells were preincubated with GQ5 and then stimulated with TGF-β1. Coimmunoprecipitation analysis showed that the interaction among Smad2/Smad3, TβRII, and SARA was significantly increased upon TGF-β1 stimulation. Treatment with GQ5 inhibited the interaction of SARA with Smad3, but did not affect the binding of SARA with Smad2 or TβRI (Figure 8). Similar results were obtained from GQ5-treated UUO rats (Supplemental Figure 8). These data suggest that GQ5 inhibited Smad3-TβRI interaction, probably through blocking SARA-Smad3 binding.

DISCUSSION

In this study, we demonstrated for the first time that GQ5, a small molecular phenolic compound extracted from Resina Toxicodendri, significantly attenuated renal fibrosis in UUO, by interfering TGF-β1/Smads signaling. GQ5 selectively inhibited TGF-β1–induced Smad3 phosphorylation via blocking the interaction of TβRI with Smad3, suggesting that GQ5 might be a potent inhibitor of TGF-β1/Smad3 signaling.

The obstructed kidneys at 7 days after ureteral ligation showed typical features of obstructive nephropathy such as inflammatory cell infiltration in the interstitium, tubular degeneration and atrophy, and interstitial fibrosis. Tubulointerstitial injury progressed in UUO kidneys at day 14. The morphologic changes were accompanied by increases in Smad3 phosphorylation and expression of α-SMA, collagen, and fibronectin. Considering an imperative role of Smad3 phosphorylation in transducing TGF-β1 signaling, it is predictable that inhibition of Smad3 phosphorylation by GQ5 leads to the preservation of tubular interstitial integrity. Our in vivo data indicated that targeted inhibition of Smad3 phosphorylation by GQ5 resulted in impressive renal protection after obstructive injury. Importantly, later administration of GQ5 7 days after UUO, when kidney injury was already established in this model, was also effective. To the best of our knowledge, this is the first study showing that selective inhibition of Smad3 activation is capable of ameliorating established renal fibrosis in vivo.

Activation of TGF-β1/Smads signaling plays a central role in the pathogenesis of tubulointerstitial fibrosis. TGF-β1 initiates its cellular response by binding with TβRII, which activates TβRI, resulting in phosphorylation of Smad2/Smad3 and their translocation into nuclei where they regulate the transcription of profibrotic genes. Our study demonstrated that treatment with GQ5 dramatically suppressed TGF-β1–induced Smad3 phosphorylation in both a cell-based assay and a UUO model. Treatment with GQ5 inhibited nuclear translocation of the Smads complex and the expression of Smads-driven genes including α-SMA, collagen I, and fibronectin. The inhibitory effect of GQ5 appears to be Smad3 specific because GQ5 did not affect Smad2 phosphorylation or the expression of Smad4 or Smad7. Of interest, GQ5 also blocked Smad2 and Smad4 nuclear translocation (Figure 5A), suggesting that Smad3 activation is obligatory for Smad2 and Smad4 entering into the nuclei upon TGF-β1 stimulation. Treatment with GQ5 did not influence the activation of other downstream signaling pathway of TGF-β1 such as p38, ERK, and PI3K, supporting the conclusion of a selective inhibition of GQ5 on Smad3.

More importantly, we demonstrated in vitro that GQ5 could inhibit TGF-β1–induced Smad3 phosphorylation in both renal tubular epithelial cells and interstitial fibroblasts, indicating that GQ5 might be able to hamper myofibroblast activation in addition to protecting renal tubular epithelial integrity. Several studies have indicated that the interstitial myofibroblast is the major cell component that produces ECM in UUO. However, a contribution of tubular epithelial cell injury to ECM accumulation in fibrotic kidneys cannot be excluded.

To explore the mechanisms by which GQ5 specifically inhibited Smad3 phosphorylation, we examined the effects of GQ5 on the interaction of Smad2/Smad3 with TGF-β receptors, a key event in TGF-β/Smads activation. Data from coimmunoprecipitation analysis revealed that GQ5 selectively blocked the binding of Smad3 with TβRI without
Figure 4. GQ-5 selectively inhibits Smad3 phosphorylation in UUO. Rats receive daily intraperitoneal injection of vehicle or GQ5 (40 mg/kg per day) right after (d1) or 7 days (d7) after UUO and are euthanized 14 days after UUO. (A and B) Immunohistochemical staining (A) and Western blots (B) are performed to analyze the expression of p-Smad3 and p-Smad2 in UUO rats. (C) Western blots show that GQ5 does not affect the expression of Smad4 or Smad7. (D) Western blots show that GQ5 does not affect the phosphorylation of p38, PI3K, or ERK. *P<0.05 versus vehicle (n=6 for each group).
Figure 5. GQ5 inhibits the transcription activity of phosphorylated Smad3. (A) NRK52E cells are preincubated with or without GQ5 (2.5 μM) for 1 hour before treatment with TGF-β1 (10 ng/ml) for 2 hour. Immunofluorescence staining reveals that GQ5 treatment inhibits TGF-β1–induced nuclei translocation of Smad3, Smad2, and Smad4. (B) NRK52E cells are cotransfected with p(CACA)-luc plasmid and PGL3, followed by TGF-β1 (10 ng/ml) stimulation for 24 hours in the absence or presence of indicated
interfering with the Smad2-TβRI interaction. Smad3 phosphorylation induced by interaction with TβRI has been recognized as a crucial step in TGF-β1/Smads signaling. Our previous studies demonstrate that disrupting the binding of Smad3 to TβRI inhibits the nuclear translocation of the Smad3 complex and suppresses the expression of target genes. Similarly, specific inhibition of Smad3 phosphorylation by small molecule inhibitor SIS3 significantly diminishes the upregulation of TGF-β1–induced ECM gene expression.

The mechanism underlying the blocking effect of GQ5 on the Smad3-TβRI interaction remains unclear at this stage. Interaction of TGF-β receptors with Smads is a complex biologic process involving several adaptor proteins such as SARA, embryonic liver fodrin (ELF), and kindlin-2. In this study, we found that GQ5 treatment selectively blocked the interaction of SARA with Smad3, and therefore decreased the binding of Smad3 with TβRI. This might be a potential mechanism underlying the inhibiting effect of GQ5 on Smad3 phosphorylation.

Despite the enormous unmet medical need, there are few intervention strategies available that specifically target the pathogenesis of renal fibrosis. Given the critical role of TGF-β/Smads signaling in fibrogenesis, the efforts for developing antifibrotic strategies are focusing on this signaling pathway. Molecules that inhibit TGF-β binding to its receptor are being developed including decorin, soluble chimeric TGF-β receptor, neutralizing antibodies against TGF-β, and small molecule inhibitors for TβRI activin receptor-like kinase 5, 6.

In conclusion, we have identified GQ5 as a potent small molecular inhibitor of TGF-β1–induced Smad3 phosphorylation. GQ5, through blocking the interaction of Smad3 with TβRI, inhibits activation of Smads signaling and attenuates interstitial fibrosis in UUO kidneys. These data suggest that targeted inhibition of Smad3 activation is a novel approach for antifibrotic intervention. Our studies also provide a promising lead compound for developing an effective antifibrotic remedy.

**CONCISE METHODS**

**GQ5 Preparation and Identification**
To isolate GQ5, the dried resins of _T. verniciflua_ (17 kg) (Yunnan Corporation of Materia Medica, Kunming, China) were extracted with 80% ethanol (3×20 liters) at room temperature. The extracts were concentrated under reduced pressure, and suspended in water followed by partition with ethyl acetate (3×5 liters). The extract (220 g) from ethyl acetate was subjected to a silica gel column (200–300 mesh, 12×150 cm, 2.5 kg; Qingdao Marine Chemical Inc., Qingdao, China), eluted with a gradient of CHCl₃/MeOH (100:0–80:20) to yield 10 fractions. Fraction 4 (15 g) was subjected to a MCI gel CHP 20P column (75–150 μm; Mitsubishi Chemical Industries, Tokyo, Japan), eluted with gradient aqueous acetonitrile (80:20–100:0) to yield fractions 4.1 and 4.2. Among them, fraction 4.2 (11.3 g) was filtrated on Sephadex LH-20 (CHCl₃/MeOH, 6:4; Amersham Pharmacia, Uppsala, Sweden) to yield GQ5 (10 g).

Spectroscopic and chemical methods were used to identify the structure of GQ5. The 1H-nuclear magnetic resonance (NMR) spectrum using a Bruker DRX-500 NMR spectrometer (Bruker Daltonics, Germany) indicated the diagnostic signals of one 1,2,4-trisubstituted benzene group, one methyl, and one aliphatic chain (Supplemental Figure 1A). The 13C-NMR and DEPT spectra revealed one methyl, five methine, and three quaternary carbons (two of them are oxygenated) (Supplemental Figure 1B). Mass spectra (electrospray ionization mass spectrometry) (API QSTAR Pulsar 1 spectrometer; AB SCIEX) and high-resolution electron ionization mass spectrometry (Autospec Premier P776 instrument; Waters) analyses indicated that the molecular formula of GQ5 was C₂₁H₃₄O₂ (Supplemental Figure 2). The NMR data of GQ5 (Supplemental Table 1) were in agreement with those of 3-[(Z)-pentadec-8-enyl] catechol. In addition, the position and geometry of the double bond in the side chain were confirmed by total chemical synthesis.

The purity of GQ5 was determined by analytic HPLC using an RP-18 column under 280, 254, 230, 225, and 210 nm, and then eluted by gradient aqueous MeOH (85%–100%, 0–20 minutes). Only one symmetric peak was found in all of the chromatograms in different detection conditions, indicating that GQ5 is an HPLC-grade pure compound (Supplemental Figure 3). Consistently, there was no impurity present in the 1H-NMR spectrum (Supplemental Figure 1A).

**Animal Model and Treatment**
Male Sprague-Dawley rats (body weight 200–250 g) and C57BL/6 mice (body weight 20–25 g) were purchased from the Animal Experiment Committee of the Southern Medical University and housed in the Nanfang Hospital Animal Center. UUO was performed using an
established protocol as described. To evaluate the effect of GQ5 on renal fibrosis, the rats were randomized into four groups (n=6 in each group): (1) sham operated rats, (2) UUO rats that received daily intraperitoneal injection of vehicle, (3) UUO rats treated with daily intraperitoneal injection of GQ5 right after UUO, and (4) UUO rats that received daily intraperitoneal injection of GQ5 7 days after UUO. GQ5 was dissolved in 5% propylene glycol (Amresco, Solon, OH). All of the rats were euthanized 14 days after UUO.

Morphologic and Immunohistochemical Analyses

Two-micrometer sections of paraffin-embedded kidney tissue were subjected to Masson’s trichrome or hematoxylin and eosin staining using commercial kits (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. Immunohistochemical staining was performed on 4-µm kidney sections as previously described. Briefly, the kidney sections were stained by anti-α-SMA (Sigma-Aldrich), anti-collagen I (Calbiochem, San Diego, CA), anti-fibronectin (Sigma-Aldrich), anti-CD4 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-CD8 (Santa Cruz Biotechnology) antibody, respectively, and then detected by the EnVision/HRP Kit (Dako, Carpinteria, CA).

Cell Culture and Treatment

NRK52E and NRK49F cells were cultured in DMEM-Ham’s medium (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% FBS (Gibco/Life Technologies). The cells that reached approximately 50% confluence were used for in vitro experiments. To test the effect of GQ5 on TGF-β1/Smads signaling, cells were serum starved for 12 hours and treated with indicated amount of GQ5 for 1 hour, followed by incubation with recombinant TGF-β1 (10 ng/ml; R&D Systems, Minneapolis, MN) for the indicated time period. GQ5 used in cellular experiments was dissolved in DMSO (Sigma-Aldrich).

MTT Assay

NRK52E cells were seeded into 96-well plates in a volume of 200 µl per well (1×10^5 cells/ml) and incubated for 24 hours to allow cells to attach. The cells were then incubated with the indicated amount of GQ5 for 1 hour. Cell viability was determined by addition of 20 µl of MTT at a concentration of 5 mg/ml. After incubation for 4 hours, the medium was removed and 150 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm by using an iMark Microplate Reader (Bio-Rad).

Real-Time RT-PCR

Total RNA was prepared from NRK52E and NRK49F cells or kidney tissues using TRIzol reagent, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). cDNA was synthesized using 2 µg of RNA by RT using AMV-RT and random primers at 42°C for 5 minutes. The sequences of the primer pairs are given in Supplemental Table 2. The pair of primer for analyzing type I collagen was a1 gene. The mRNA levels of various genes were calculated after normalizing with GAPDH by the comparative CT method.

Immunoprecipitation and Western Blot

Immunoprecipitation was performed as previously described. Briefly, cell or tissue lysates were prepared in RIPA buffer (1× PBS, pH 7.4, 0.5% sodium deoxycholate, 1% Triton, 0.5% SDS) with
protease inhibitor cocktail. Immunoprecipitates were obtained by incubating precleared lysates with indicated antibodies or normal IgG (as controls) overnight at 4°C, and then separated by SDS-PAGE gels. Western blot was performed as previously described.50–53 Brieﬂy, cells and kidney tissues were lysed in 500 μl of 1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA, pH 8.0, containing a 1:50 dilution of a protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice. Samples were centrifuged at 12,000g for 15 minutes and then heated at 95°C and separated on SDS-PAGE gels. Transferred membranes were immunoblotted with the following primary antibodies, respectively: anti-α-SMA and anti-fibronectin (Sigma-Aldrich); anti-collagen I (Calbiochem); anti-Smad7 and anti-TβRII (Santa Cruz Biotechnology); anti-p-Smad2, anti-p-Smad3, anti-Smad2, anti-Smad3, anti-Smad4, anti-TβRI, anti-p-p38, anti-p38, anti-p-PI3K, anti-PI3K, anti-p-ERK, and anti-ERK (Cell Signaling Technology, Beverly, MA); and anti-SARA (Santa Cruz Biotechnology). For measurement of collagen I, bands at 115 kD were analyzed. After extensive washing, the membranes were incubated with the secondary antibodies. Images were then detected by the Odyssey detector (LI-COR, Lincoln, NE).

Immunofluorescence Staining
Immunofluorescence staining was performed as previously described.54 Brieﬂy, the cells cultured on coverslips were ﬁxed, permeabilized with 0.5% Triton X-100, and incubated with the primary antibodies overnight at 4°C, followed by incubation with secondary antibodies conjugated with Alexa Fluor 488 or 588 (Invitrogen). Cells were counterstained with 4′,6-diamidino-2-phenylindole to visualize the nuclei. Images were taken by confocal microscopy (Olympus Corporation, Tokyo, Japan).

Smad3-Dependent Promoter Assay
NRK52E cells were transiently transfected with a Smad3 responsive promoter p(GAGA)12-luc (kindly provided by Professor H.Y. Lan, Chinese University of Hong Kong) as previously described.21 PGL3 basic plasmid was cotransfected into the cells as the control. The transfection procedure was performed using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were treated with GQ5 (0.1, 0.5, and 2.5 μM, respectively) for 1 hour, followed by incubation with TGF-β1 (10 ng/ml) for 24 hours. The luciferase activities of p(GAGA)12 were analyzed by a luciferase reporter gene assay kit (Roche Biochemical, Indianapolis, IN) according to the manufacturer’s instructions. The luciferase activity was normalized by protein concentration measured with the Lowry protein assay (Bio-Rad, Richmond, CA).

Statistical Analyses
Data are expressed as the mean±SD. Comparisons between two groups were conducted using the two-tailed t test. Comparisons between

Figure 7. GQ5 selectively blocks the interaction between TβRI and Smad3 in UUO. Rats receiving daily intra-peritoneal injection of vehicle or GQ5 (40 mg/kg per day) are euthanized 14 days after UUO. (A) Kidney homogenates are immunoprecipitated with anti-TβRI, followed by immunoblotting using antibodies against Smad3, Smad2, TβRII, and TβRI. (B) Kidney homogenates are immunoprecipitated with anti-Smad3, followed by immunoblotting using antibodies against Smad3, TβRII, and TβRI. (C) Kidney homogenates are immunoprecipitated with anti-Smad2, followed by immunoblotting using antibodies against Smad2, TβRII, and TβRI. *P<0.05 versus vehicle-treated UUO (n=6 for each group). IP, immuno-precipitation; IB, immuno-blotting.
Figure 8. GQ5 selectively blocks the interaction between SARA and Smad3. NRK52E cells are preincubated with or without GQ5 (2.5 μM) for 1 hour before treatment with TGF-β1 (10 ng/ml) for 30 minutes. Cell lysates are collected for immunoprecipitation. (A) Cell lysates are immunoprecipitated with anti-SARA, followed by immunoblotting using antibodies against Smad3, Smad2, TβRI, and SARA. (B) Cell lysates are immunoprecipitated with anti-Smad3, followed by immunoblotting using antibodies against SARA and Smad2. (C) Cell lysates are immunoprecipitated with anti-Smad2, followed by immunoblotting using antibodies against SARA and Smad2. Data are expressed as the mean±SD of three independent experiments. *P<0.05 versus GQ5-untreated cells under TGF-β1 stimulation. IP, immuno-precipitation; IB, immuno-blotting.

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REFERENCES


DISCLOSURES

We have applied three patents (one international patent and two Chinese patents) for the GQ5 small molecular phenolic compound. The request numbers are PCT/ CN2013/087585, 201210111642.X, and 201210111641.5.


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