Oral administration of GW788388, an inhibitor of TGF-β type I and II receptor kinases, decreases renal fibrosis

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Progressive kidney fibrosis precedes end-stage renal failure in up to a third of patients with diabetes mellitus. Elevated intra-renal transforming growth factor-β (TGF-β) is thought to underlie disease progression by promoting deposition of extracellular matrix and epithelial–mesenchymal transition. GW788388 is a new TGF-β type I receptor inhibitor with a much improved pharmacokinetic profile compared with SB431542. We studied its effect *in vitro* and found that it inhibited both the TGF-β type I and type II receptor kinase activities, but not that of the related bone morphogenic protein type II receptor. Further, it blocked TGF-β-induced Smad activation and target gene expression, while decreasing epithelial–mesenchymal transitions and fibrogenesis. Using db/db mice, which develop diabetic nephropathy, we found that GW788388 given orally for 5 weeks significantly reduced renal fibrosis and decreased the mRNA levels of key mediators of extracellular matrix deposition in kidneys. Our study shows that GW788388 is a potent and selective inhibitor of TGF-β signalling *in vitro* and renal fibrosis *in vivo*.

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Diabetic nephropathy leads to end-stage renal failure in 20–30% of patients with type 1 or type 2 diabetes mellitus.[1] The multifunctional cytokine, transforming growth factor-β (TGF-β), is elevated in patients with diabetic nephropathy and is likely a prime mediator in the progression of renal disease.[2,3]

Specimens from patients with diabetic nephropathy show elevated TGF-β mRNA and protein levels in glomeruli and the tubulointerstitium.[4] Also, urinary and serum levels of TGF-β are significantly increased in diabetes patients.[5] In experimental animal models of type 1 and type 2 diabetes, similar patterns of increased TGF-β expression and secretion have been observed.[6] Nuclear accumulation of downstream TGF-β effector proteins was observed in diabetic kidneys. Furthermore, elevated levels of the TGF-β type II receptor (TβRII) have been reported in diabetic mice compared with non-diabetic controls.[7]

One of the mechanisms by which TGF-β induces fibrogenesis is through stimulation of extracellular matrix (ECM) proteins and inhibition of matrix degradation. Expression of key matrix components is enhanced upon TGF-β treatment, both in glomerular mesangial cells and renal tubular epithelial cells.[8–11] These factors include fibronectin (FN), type I collagen (COL-1), type III collagen (COL-III), type IV collagen (COL-IV) and laminin.[12] TGF-β further stimulates ECM accumulation through enhancing expression of connective tissue growth factor, which in turn induces FN and COL-III expression.[13] Also, activated TGF-β suppress the activity of matrix metalloproteinases[14] through increased expression of tissue inhibitor of metalloproteinases and plasminogen activator inhibitor 1 (PAI-1).[15] Thus, TGF-β promotes renal fibrogenesis by increasing the synthesis of ECM components and inhibiting matrix degradation.

An additional cellular pathomechanism whereby TGF-β promotes fibrosis is through the mediation of epithelial to mesenchymal transition (EMT), a process whereby polarised epithelial cells are transformed into highly migratory fibroblastoid cells. Epithelial cells lose polarity, epithelial markers, and cell–cell contact. The cells undergo cytoskeletal remodelling and gain mesenchymal markers essential for cell–ECM

association. The net result being enhanced cell motility and invasiveness.[16,17] In renal fibrosis, the pathological significance of tubular EMT has become increasingly recognised. Epithelia can contribute to the ECM overproduction by creating new fibroblasts through the induction of EMT.[18]

TGF-β and the superfamily members, activins nodal and bone morphogenic proteins (BMPs), signal through related type I and type II transmembrane serine/threonine kinase receptors. The kinases act in sequence, with the ligand-specific type I receptor acting as a substrate for the type II receptor. In most cell types, TGF-β signals via the TGF-β type I receptor also termed activin receptor-like kinase (ALK)5.[19] In endothelial cells, however, TGF-β signals via ALK1 and ALK5.[20] In contrast, BMP signals through ALK2, ALK3, or ALK6 and activin, and nodal through ALK4 and ALK7.[21,22] For TGF-β/ALK5 and activin, the signal is transduced into the cytoplasm through phosphorylation of the receptor-regulated Smads (R-Smads), Small phenotype and mothers against DPP-related protein (Smad)2, and Smad3. For TGF-β/ALK1 and BMP, the signal is via phosphorylation of the R-Smads, Smad1, 5, and 8.[23] Phosphorylated and activated R-Smads dissociate from the receptor complex and associate with Smad4 in a heteromeric manner. The activated complexes shuttle to and accumulate in the nucleus. Here they regulate expression of a large array of genes in a cell-type-specific and ligand dose-dependent manner.[24]

To directly address the therapeutic potential of TGF-β inhibitors in renal disease, small-molecule competitive antagonists of the ALK5 kinase activity have been developed. These inhibitors interact with the ATP-binding site, thereby preventing phosphorylation of Smad proteins.[25,26] The commonly used ALK5 inhibitor, 4-(5-benzo[1,3]dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide (SB431542), is an ATP competitive kinase inhibitor.[27] Another mechanism for abrogating TGF-β signalling has been through long-term treatment with monoclonal anti-TGF-β antibody. In diabetic rodents, this effectively prevented glomerulosclerosis and renal insufficiency.[7,11,28,29] Also, antisense TGF-β oligonucleotides were found to reduce kidney weight and expression of matrix components in diabetic mice.[30] Recently, a soluble fusion protein of the TβRII was reported to reduce albuminuria in a chemically induced model of diabetic nephropathy in rats.[31]

A limited number of studies have been reported on the use of small-molecule inhibitors of TGF-β signalling *in vivo*.[32,33] SB525334 was shown to significantly reduce procollagen 1α(I), in rat kidneys, in an induced model of nephritis.[34] Also the inhibitor IN-1130 reduced obstructive nephropathy in rats.[35] These data provide a strong foundation for using type I receptor kinase inhibitors in clinical testing.

Recently, 4-(4-[3-(Pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl)-*N*-(tetrahydro-2Hpyran-4-yl) benzamide (GW788388) was developed, as an alternative to the ALK5 inhibitor, SB431542, with better *in vivo* exposure (Figure 1). GW788388 is orally active and has a good pharmacokinetic profile, with an elimination half-life of 1.3 h and a systemic plasma clearance of 20 ml min^{-1} kg^{-1} in rats. It was previously shown to reduce the fibrotic response in a chemical induced model of fibrosis in rats and improve liver histology.[32]

In this study, we further characterised the potency and selectivity of this novel inhibitor, GW788388. We show that this compound effectively blocks both the ALK5 and to some extent the TβRII. In renal epithelial and cancer cell lines, we assess the inhibitory effects on TGF-β-mediated biological responses such as EMT and fibrogenesis. We examine the effect of blocking TGF-β signalling on renal fibrosis and kidney function in the db/db mouse model of spontaneous diabetic nephropathy, resembling the pathogenic phenotype observed in patients with type 2 diabetes mellitus. We show that GW788388 effectively inhibits TGF-β signalling *in vitro* and reduces renal fibrosis *in vivo*.

RESULTS

GW788388 is a selective inhibitor of ALK5 and TβRII

Structures of GW788388 and SB431542, two ATP competitive inhibitors of the kinase domain of ALK5, are shown in Figure 1a. In a biochemical binding assay, using the intracellular kinase domain of ALK5, GW788388 was found to have an IC_{50} for GST-ALK5 of 0.018 ± 0.08 μM.[32] To test the specificity of GW788388, we performed an *in vitro* kinase assay on full-length constitutively actively signalling receptors. Human embryonic kidney 293T cells were transiently transfected with expression plasmids encoding constitutively active ALK (caALK)5, TβRII, BMPRII, or activin type II receptor (ActRII). Receptors were immunoprecipitated and challenged with γ-^{32}P-labelled ATP and 10 μM of compounds. GW788388 potently inhibited autophosphorylation of ALK5, TβRII, and to some extent ActRII (Figure 1b). The compound had no effect on the BMP type II receptor kinase activity.

To address if GW788388 was cytotoxic, we treated cells with a dilution range of the compound and measured cell viability after 72 h. GW788388 showed no toxicity in Namru murine mammary gland (NMuMG) (Figure 1c), MDA-MB-231, renal cell carcinoma (RCC)4, and U2OS cells (data not shown) when treated with dilutions from 4 nM to 15 μM. Similar results were obtained with the SB431542 inhibitor (Figure 1c).

GW788388 inhibits TGF-β-induced Smad2 phosphorylation and Smad2/3 nuclear translocation

Since GW788388 could block the kinase activity of ALK5 and TβRII, we next studied the inhibitory effect on TGF-β, activin, and BMP-induced R-Smad phosphorylation and nuclear translocation. GW788388 inhibited TGF-β-induced Smad2 phosphorylation in a dose-dependent manner in NMuMG (Figure 2a; Figure S1a), MDA-231-MB (Figure 2b), and renal cell carcinoma (RCC4)/von Hippel Lindau (VHL) (data not shown). TGF-β-mediated Smad1/5 phosphorylation, which requires ALK5 and TβRII, was also inhibited by GW788388 (Figure 2a and b). In T47D cells, GW788388 and SB431542 inhibited the activin-induced phosphorylation of Smad2 (Figure 2c).

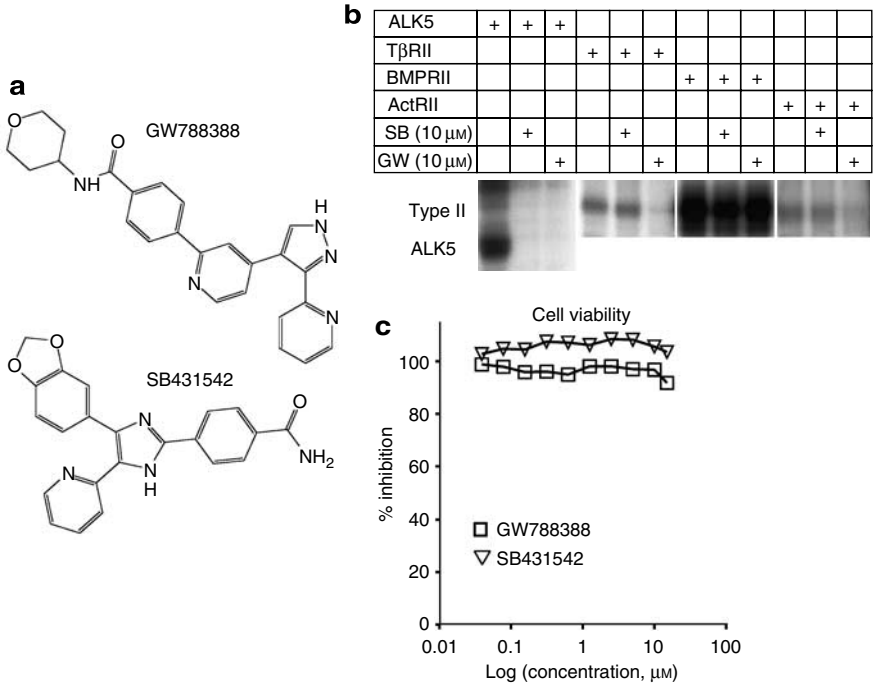

Figure 1 | GW788388 inhibits both ALK5 and TβRII. (**a**) Chemical structures of GW788388, 4-(4-[3-(Pyridin-2-yl)-1H-pyrazol-4-yl] pyridin-2-yl)-*N*-(tetrahydro-2Hpyran-4-yl) benzamide and SB431542, 4-(5-benzo[1,3] dioxol- 5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide. (**b**) Effect of GW788388 and SB431542 on autophosphorylation of caALK5, TβRII, ActRII, and BMP type II receptor kinase activity. Human embryonic kidney 293T cells were transfected with plasmids encoding full-length receptors. Type II receptors can signal independently of ligand. Receptors were immunoprecipitated and the *in vitro* kinase assays were performed with γ-^{32}P-labelled ATP in the presence of 10 μM GW788388 (GW) or 10 μM SB431542 (SB). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (**c**) Cell viability assay. NMuMG cells were treated with dilutions of GW788388 (squares) and SB431542 (triangles) for 72 h. Viability was measured with a modified MTS assay, measuring metabolically active cells.[49] Data are presented as % inhibition compared with vehicle control. Bars represent mean ± s.e.m.

Upon phosphorylation, R-Smads form complexes with Smad4 and accumulate in the nucleus. TGF-β-induced Smad2/3 nuclear translocation was dose-dependently inhibited when NMuMG cells were treated with GW788388 (Figure 2d). We tested if GW788388 inhibited the BMP-signalling cascade by analyzing the effect of the compound upon BMP-induced phosphorylation of Smad1/5. As shown in Figure 2e, GW788388 had no inhibitory effect on Smad1/5 phosphorylation by BMP. SB431542 was shown to have some inhibitory effect on the mitogen-activated protein kinase p38α at 10 μM.[25] We therefore tested whether GW788388 could inhibit sorbitol-activation of stress-induced kinases such as the mitogen-activated protein kinases p38 and ERK 1/2. GW788388 had no inhibitory effect on these mitogen-activated protein kinases (data not shown). Thus, GW788388 selectively inhibits TGF-β and activin Smad signalling and not the closely related BMP-signalling cascade.

GW788388 selectively inhibits ALK4, ALK5, and ALK7

To further confirm the selectivity of GW788388, U2OS cells were transiently transfected with expression plasmids encoding the constitutively active full-length receptors caALK2, caALK3, caALK4, caALK5, caALK6, and caALK7.[36] These mutationally active receptors signal independently of ligand and their type II receptors. They were cotransfected with the corresponding luciferase reporter constructs. The TGF-β-inducible reporter $CAGA_{12}$-Luc contains Smad-responsive elements from the PAI-1 promoter, which specifically bind Smad3/Smad4 and drive the luciferase reporter gene.[37] The BMP-inducible luciferase reporter, BMP-responsive element-Luc, contains a BMP-responsive elements from the inhibitor of DNA-binding 1 promoter.[38]

GW788388 inhibited the TGF-β response, very efficiently, by blocking signalling through caALK5, caALK4, and caALK7 in a dose-dependent manner (Figure 3a). The SB431542 inhibitor was used for comparison and similar results were obtained with all caALKs (data not shown). In agreement with the phosphorylation data, GW788388 had no inhibitory effect on the constitutively active BMP receptors (Figure 3b). In addition, TGF-β and activin (Figure S1b) but not BMP-induced reporter activity was blocked by GW788388 (data not shown). Thus, GW788388 is a selective inhibitor of the TGF-β type I receptors ALK5, ALK4, and ALK7.

GW788388 inhibits TGF-β-induced EMT and growth inhibition

In epithelial cells, the TGF-β-mediated growth inhibitory response and EMT are two cellular processes that have been extensively explored. The NMuMG cells are a widely used *in vitro* model system for studying these TGF-β-mediated responses.[39] To test if GW788388 could inhibit the TGF-β-induced growth inhibitory response, we measured cell proliferation with serial dilutions of GW788388. As shown in

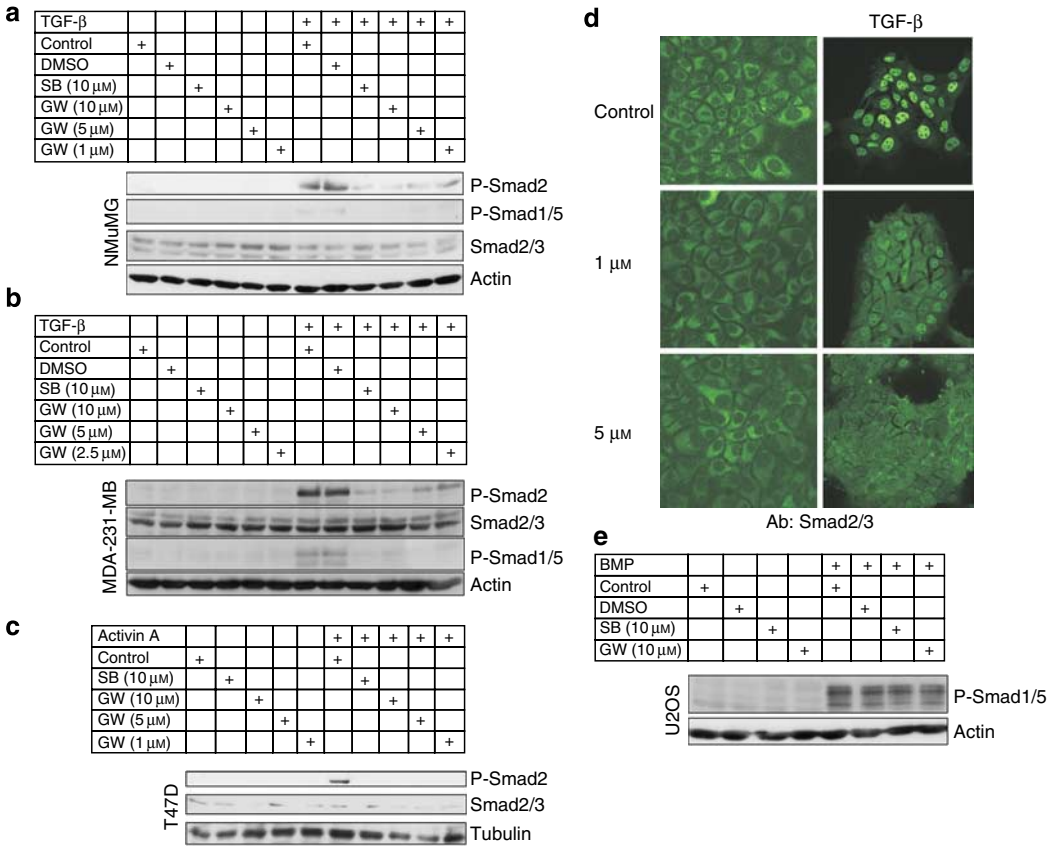

Figure 2 | GW788388 inhibits TGF-β-induced Smad activation dose-dependently. NMuMG (**a**) and MDA-MD-231 cells (**b**) were treated with GW788388 (GW) or SB431542 (SB) in the presence or absence of TGF-β for 1 h. Protein expression of PSmad2, PSmad1/5, and Smad2/3 was analyzed by western blot analysis. Actin served as a loading control. (**c**) T47D cells were treated with GW788388 or SB431542 and stimulated with activin A for 1 h. Protein expression of PSmad2 and Smad2/3 was analyzed by western blotting. (**d**) Immunofluorescent staining of Smad2/3 in NMuMG cells treated with vehicle or GW788388 ± TGF-β for 1 h. Images were captured with confocal microscopy. (**e**) Western blot analysis of U2OS cells treated with GW788388 ± BMP6 for 1 h. Control denotes non-treated cells and DMSO was used as vehicle.

Figure 4a, increasing concentrations of GW788388 inhibits TGF-β-induced growth inhibition. These results were further supported by light microscopy images, demonstrating detachment of NMuMG cells from the tissue culture surface in response to TGF-β treatment, suggestive of programmed cell death (Figure 4b). Thus, GW788388 blocked TGF-β-mediated growth arrest.

With phase-contrast microscopy, we observed the transition of the cells from an epithelial to a fibroblastoid phenotype upon stimulation with TGF-β for 48 h (Figure 4b). In cells stained by immunofluorescence with epithelial and mesenchymal markers, we observed actin stress fiber formation (Figure 4c), loss of E-cadherin expression at cell–cell junctions, and gain of N-cadherin expression (data not shown) in response to TGF-β treatment. This EMT response, mediated by TGF-β, was completely inhibited with GW788388 (Figure 4b and c).

We confirmed these visual observations by analyzing protein and mRNA expression by western blot analysis and semi-quantitative reverse transcriptase-polymerase chain reaction. In NMuMG and RCC4/VHL cells, we analyzed the changes in the expression of epithelial and mesenchymal markers after 48 h of TGF-β stimulation (Figure 5). E-cadherin protein expression was reduced upon TGF-β treatment, whereas the expression of the mesenchymal markers N-cadherin and α-smooth muscle actin was increased (Figure 5a and b). GW788388 attenuated these TGF-β-induced EMT responses. The inhibitory effect of GW788388 was also observed on mRNA levels of critical TGF-β target genes involved in EMT, such as E-cadherin, FN, and the transcriptional repressor of E-cadherin, SNAIL (Figure 5c). In conclusion, GW788388 blocks the TGF-β-induced growth inhibitory and EMT response.

GW788388 inhibits the TGF-β-induced fibrotic responses *in vitro*

Since GW788388 inhibited important TGF-β-induced target gene responses required for EMT, we sought to examine whether the drug also could inhibit TGF-β responses involved in ECM remodelling. We investigated this by quantitative reverse transcriptase-polymerase chain reaction and western blot analysis. We found that GW788388 could prevent the TGF-β-induced upregulation of connective tissue

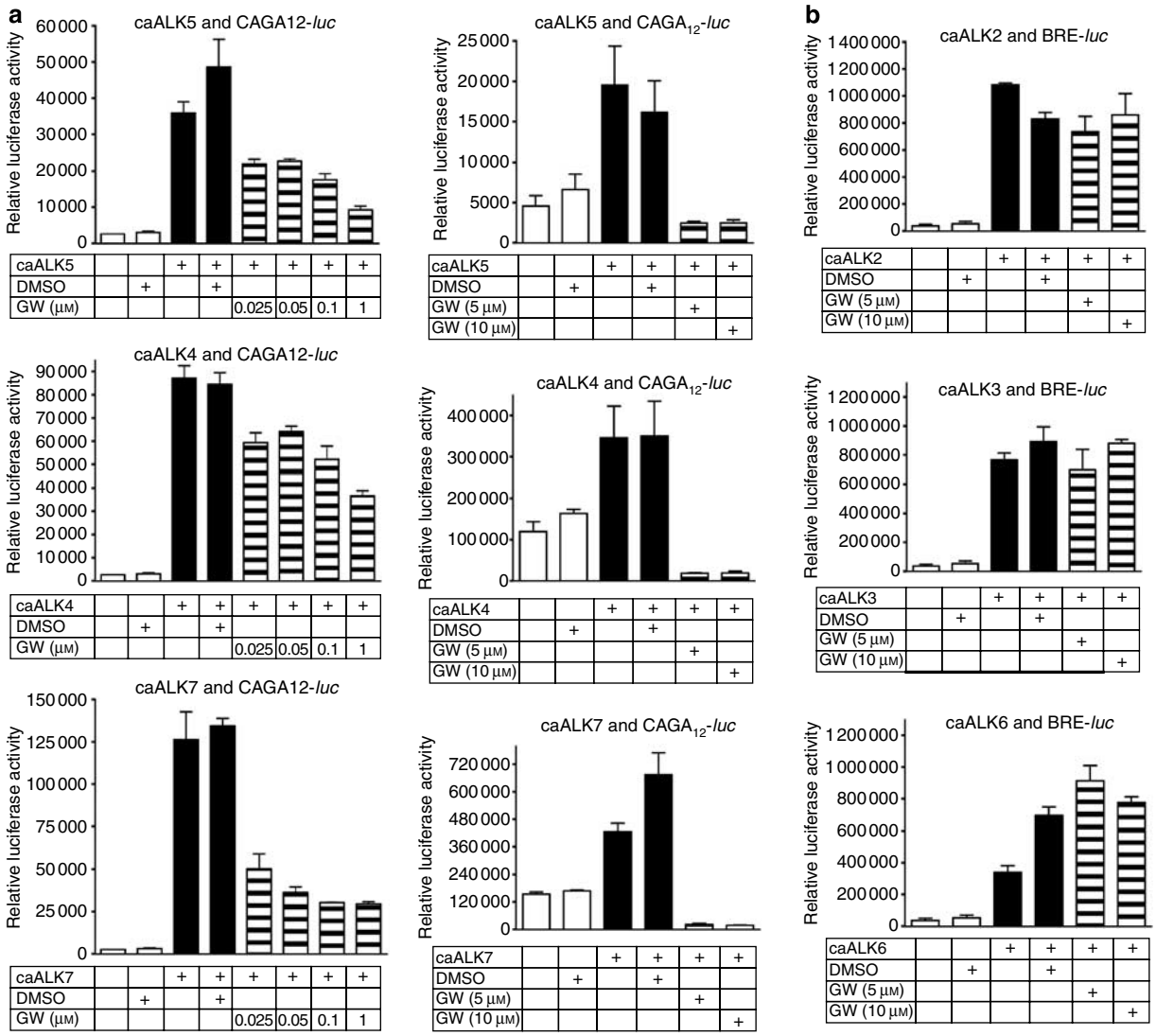

Figure 3 | GW788388 inhibits ALK5, ALK4, and ALK7 in a dose-dependent manner and has no effect on ALK2, ALK3, and ALK6. (**a**) U2OS cells were transfected with caALK4, caALK5 or caALK7 together with the TGF-β-specific luciferase reporter construct $CAGA_{12}$-luc. Cells were treated with doses of GW788388 (GW) or vehicle. (**b**) U2OS cells were transfected with caALK2, caALK3, or caALK6 together with the BMP responsive BMP-responsive element (BRE)-luciferase reporter. Measurements are presented as luciferase activity normalised to β-galactosidase activity. Error bars indicate mean ± s.e.m. of three measurements; one representative experiment is shown.

growth factor, PAI-1, and COL-I mRNA expression in the renal epithelial cells RCC4/VHL (Figure 6a) and FN in NMuMG cells (Figure 5c). On protein levels, we confirmed that GW788388 blocks the TGF-β-induced expression of COL-I and FN (Figure 6b). Thus, GW788388 inhibits the TGF-β-mediated expression of important players in fibrogenesis both on mRNA and protein levels.

GW788388 potently attenuates renal fibrosis *in vivo*

We have demonstrated that GW788388 is a potent inhibitor of TGF-β signalling in several *in vitro* models. We next examined the effects of GW788388 *in vivo*. First, we compared the intravenous pharmacokinetic profiles of GW788388 with SB431542 in Sprague–Dawley rats. Clearance was $34 \pm 12.2\,ml\,min^{-1}\,kg^{-1}$ for GW788388 versus $37.5 \pm 13.5\,ml\,min^{-1}\,kg^{-1}$ for SB431542. The half-life of GW788388 was 4.1 ± 1.8 h versus 28.5 ± 16.1 min for SB431542 (data not shown), making GW788388 far more suitable for *in vivo* use than SB431542.

The db/db mouse model of spontaneous diabetic nephropathy was chosen for further *in vivo* characterisation of GW788388. Six-month-old mice were used, with advanced-stage renal disease, significant glomerular changes, and elevated albuminuria.[40] Mice were treated with oral administration of $2\,mg\,kg^{-1}\,day^{-1}$ of GW788388 for 5 weeks. No adverse side effects were observed with the treatment. Figure 7a shows diabetic mouse kidneys stained with Masson's Trichrome stain. Collagen deposits are observed in blue. Robust collagen deposits were seen in glomeruli and minimal to mild glomeropathy was evident in most diabetic animals (left panel). Treatment with GW788388 at $2\,mg\,kg^{-1}\,day^{-1}$

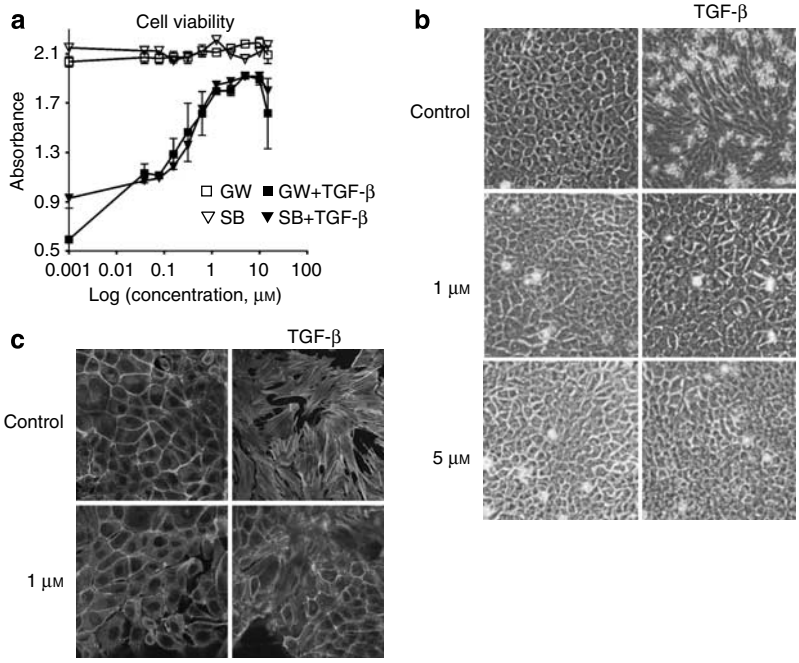

Figure 4 | GW788388 inhibits TGF-β-mediated EMT and apoptosis. NMuMG cells were treated with GW788388, the vehicle control DMSO, and TGF-β where indicated, for 48 h. (**a**) NMuMG cell proliferation measured after 72 h drug stimulation with dilution series of GW788388 (GW) (squares) and SB431542 (SB) (triangles) in the presence (closed symbols) or absence (open symbols) of TGF-β. Metabolically active cells were measured with a cell proliferation/viability assay. Bars represent means of three independent measurements ± s.e.m. (**b**) Phase-contrast images of TGF-β-induced EMT ± GW788388 after 48 h stimulation. (**c**) Immunofluorescent staining of actin stress fiber formation after 48 h drug and TGF-β stimulation. Images were captured by confocal microscopy.

resulted in a reduced collagen staining (Figure 7a, right panel). Glomerulopathy was assessed independently in picric acid stain-stained sections, scored blinded. Diabetic mice had significant glomerulopathy marked by mesangial matrix expansion, mesangial hypertrophy, proliferation, and glomerular basement membrane thickening. This was significantly reduced when treated with GW788388 (Figure 7b). Urinary albumin excretion was additionally measured and corrected for creatinine concentrations. In diabetic mice, urinary albumin levels were significantly elevated (Figure 7c). GW788388 appeared to decrease urinary albumin concentrations, although not statistically significant, suggesting that the underlying glomerular dysfunction persisted in the treated animals. To confirm that the observed changes, in glomerulopathy and the trend for reduced albuminuria, correlated with inhibition of TGF-β target genes *in vivo*, RNA was extracted from whole kidneys and the levels of matrix mRNAs examined. FN, COL-I, PAI-1, and COL-III expression levels were significantly increased in diabetic mice as compared with their lean littermates (Figure 7d). Treatment with 2 mg kg^{-1} day^{-1} of GW788388 significantly reduced the mRNA levels of PAI-1, COL-I, and COL-III to nearly the same levels as seen in the non-diabetic lean littermates. Taken together, these results indicate that GW788388 attenuates TGF-β signalling *in vivo* and effectively reduces hallmarks of fibrogenesis in mice suffering from late-stage diabetic nephropathy.

DISCUSSION

TGF-β is suggested to be a key factor in the generation of tissue fibrosis.[8,28,29,41] In the diabetic kidney, TGF-β plays an important role in early and late manifestations of nephropathy such as renal hypertrophy and mesangial matrix expansion.[7] These pathomechanisms result in destruction of functional renal tissue and eventually loss of renal function. Blocking TGF-β signalling is therefore considered a promising therapeutic approach in the treatment of renal disease. We studied a new TGF-β inhibitor, GW788388 (results are summarised in Figure 8). We show that GW788388 effectively inhibits TGF-β-mediated responses *in vitro* by blocking the kinase activity of both type I and type II receptors. Importantly, we show that oral administration of GW788388 to diabetic mice significantly reduces glomerulopathy in kidneys and attenuates expression of key components involved in fibrosis**.** This is consistent with a previous study showing that GW788388 reduced fibrosis markers in the kidney following puromycin-induced nephropathy.[33] These results encourage further studies to therapeutically target the TGF-β pathway in order to treat renal diseases.

GW788388 was identified as an orally active ALK5 inhibitor, with much improved *in vivo* exposure compared with SB431542.[25,32] In order to study the specificity of the compound, we performed an *in vitro* kinase assay with ^{32}P-labelled ATP. We found that GW788388 potently inhibits both the ALK5, TβRII and to some extent the ActRII kinase

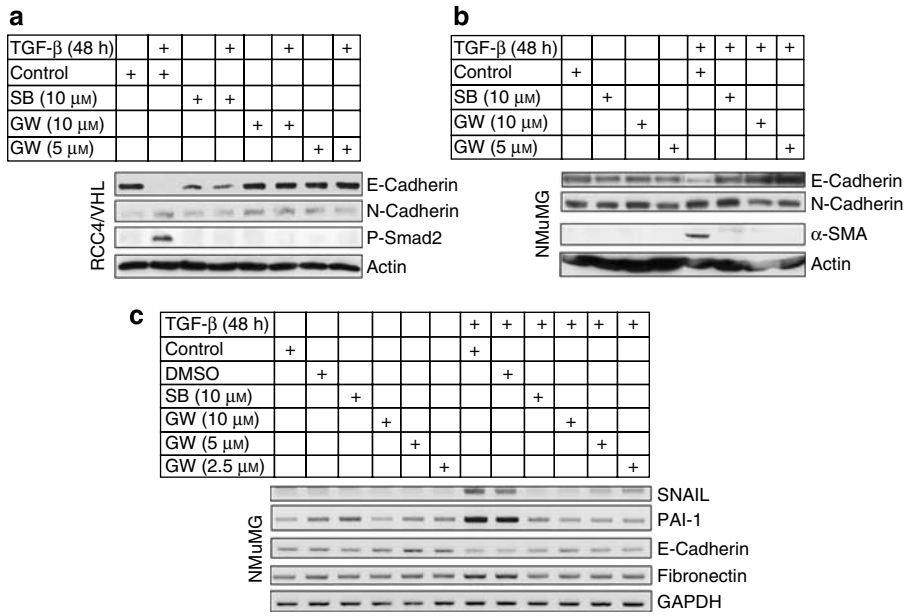

Figure 5 | TGF-β-induced EMT is inhibited by GW788388. Western blot analysis of epithelial and mesenchymal protein markers in RCC4/VHL (**a**) and NMuMG (**b**) cells after 48 h of drug and TGF-β stimulation. Control is DMSO-treated cells. β-Actin was used as a loading control. (**c**) Reverse transcriptase-polymerase chain reaction semi-quantitative analysis of SNAIL, PAI-1, E-cadherin, and FN in NMuMG cells after GW788388 (GW) or SB431542 (SB) treatment and TGF-β stimulation for 48 h. Glyceraldehyde-3-phosphate dehydrogenase was included as loading control. Control depicts non-treated cells and DMSO vehicle-treated cells.

receptor activities but not the BMP type II receptor. This contrasts what is seen for the related inhibitor, SB431542.[20,25] As a consequence of inhibiting the TGF-β receptors, we found that TGF-β-induced Smad2 phosphorylation and nuclear accumulation were potently blocked by GW788388. The specificity of GW788388 was further tested on all seven activated ALKs with reporter assays. We show that the compound could inhibit ALK5 along with the structurally similar receptors, that is, ALK4 and ALK7. GW788388 did not inhibit ALK2, ALK3, and ALK6. Previous studies using other TGF-β type I receptor inhibitors have shown similar results,[34,42,43] the main distinction being that GW788388 in addition targets the TβRII kinase activity. The additional activity against the TβRII kinase should not alter the selectivity profile or the potency of an ALK5 inhibitor as long as the ALK5 kinase binding is stronger than to the type II receptor.

TGF-β induces a growth inhibitory and an EMT response in NMuMG cells.[39,44] We show that GW788388 dose dependently inhibits these TGF-β responses. The TGF-β-mediated upregulation of target genes, involved in excess ECM deposition is well described. Treating renal epithelial cells with TGF-β mimics the fibrotic response seen in renal disease, where mRNA levels of PAI-1 and COL-I are increased by TGF-β treatment.[11] In our hands, GW788388 could prevent the TGF-β-mediated upregulation of connective tissue growth factor, PAI-1, and COL-I RNA levels, and FN and COL-I on protein levels in the renal epithelial cell line RCC4/VHL. Recently, SNAIL was described to directly induce renal fibrosis and strong expression was found in fibrotic human kidney sections.[45] With GW788388 we could block SNAIL mRNA expression in epithelial cells. Taken together, these data indicate that GW788388 selectively and efficiently inhibits TGF-β-mediated responses *in vitro*.

Since GW788388 inhibits important components in the TGF-β-induced fibrotic response in cell models, we hypothesised that GW788388 could reduce markers of fibrosis in a mouse model of diabetic nephropathy. Our aim was to examine if TGF-β receptor inhibition could be effective in older mice with established renal disease, as would be observed in patients presenting with impaired renal function. We show that oral administration of GW788388 for 5 weeks in 6-month-old db/db mice significantly attenuated glomerulopathy in mouse kidneys. This correlated with reduced mRNA expression of critical factors in ECM remodelling, namely PAI-1, COL-I, and COL-III, by GW788388. These results are in agreement with the oral administration of GW788388 to rats with chemically induced liver and renal fibrosis,[32] and with db/db mice treated with neutralising anti-TGF-β antibodies.[7]

Despite ALK5 inhibition having inhibitory effects on fibrogenesis and histological glomerulopathy, the effects on kidney function were not significant. Only a trend for a reduction in urinary excretion of albumin was observed. Longer treatments regimes may be necessary to reverse the effects of fibrosis. Moreover, it is not clear if ALK5 inhibition would address the underlying glomerular pathology leading to albuminuria in the first instance. Thus, for ALK5 inhibition alone to be fully effective against a change in glomerular permeability, we hypothesise that earlier and longer treatment periods are needed in order to inhibit tissue remodelling within the kidney and allow restoration and/or

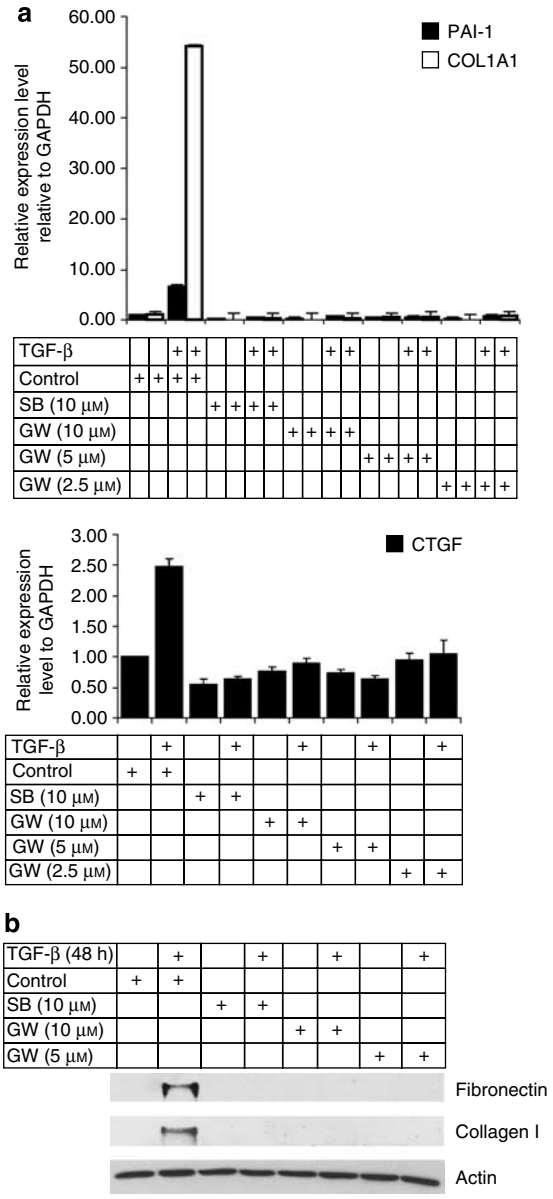

Figure 6 | GW788388 inhibits the TGF-β-induced fibrotic response *in vitro*. (**a**) The effect of GW788388 (GW) on TGF-β-induced mRNA expression of the ECM genes PAI-1, COL-1αI, and connective tissue growth factor (CTGF) was analyzed by real-time Q-PCR. RNA was extracted from RCC4/VHL renal epithelial cells stimulated with drug ± TGF-β for 48 h. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference housekeeping gene. Results are presented as means ± s.d. of three measurements; the experiment was repeated twice. (**b**) GW788388 inhibits TGF-β-induced FN and COL-I on protein level; β-actin was used as a loading control. Controls were treated with DMSO.

preservation of glomerular morphology and function. These results suggest that once renal fibrosis is established, restoration of function is likely to require prolonged therapy even with effective inhibition of TGF-β-mediated fibrogenic pathways.

All together, these data provide a strong foundation for using TGF-β receptor kinase inhibitors in a clinical setting. Renal disease progresses slowly and halting this process with a TGF-β receptor inhibitor will presumably require chronic treatment. However, TGF-β is a pleiotropic cytokine, which modulates a broad array of processes. The challenge in using TGF-β receptor inhibitors for antifibrotic treatment will be to balance the disease-related fibrotic actions against the immune modulatory, cardiovascular, and tumor suppressor functions of TGF-β. Also, all ALK5 kinase inhibitors reported to date inhibit the kinase activity of the ALK4 and ALK7,[22,25,33,43,46] and GW788388 to some extent the ActRII. Long-term treatment may therefore affect activin- and nodal-dependent signalling. Further investigation will be needed to gain insight to the effect of this inhibition.

In summary, we have demonstrated that GW788388 can inhibit TGF-β and activin signalling *in vitro* and attenuate renal fibrosis *in vivo* (Figure 8). By blocking the action of the ALK5 and TβRII kinase receptors, TGF-β-induced growth arrest, EMT, and ECM deposition were inhibited *in vitro*. Through oral administration of GW788388 to db/db mice for 5 weeks, we were able to reduce glomerulopathy and prevent the TGF-β-mediated upregulation of excess renal ECM deposition. Thus, we could reduce renal fibrosis in a mouse model for advanced diabetic nephropathy. Our results suggest that TGF-β receptor kinase inhibition should attenuate fibrogenesis and improve the fibrotic outcome for patients suffering from diabetic nephropathy. Whether prolonged or earlier treatment might restore or prevent declines in renal function and not just fibrosis remain to be determined.

MATERIALS AND METHODS

Cell culture and reagents

The human breast carcinoma MDA-MB 231 and T47D, the human osteosarcoma U2OS, and the monkey kidney COS cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. The human renal carcinoma cell line (RCC4) stably expressing the VHL protein under neomycin selection. The murine epithelial breast cells NMuMG were maintained in Dulbecco's modified Eagle's medium as above with 10 mg ml^{-1} insulin.[44] Cell lines were cultured at 37°C in 5% CO_2. SB431542 was from Tocris (Tocris Biosciences, Ellisville, MO, USA). Compounds were dissolved in dimethyl sulfoxide (DMSO). We used 5 ng ml^{-1} TGF-β3, 100 ng ml^{-1} BMP6, and 50 ng ml^{-1}, activin A. Antibodies recognising phosphorylated Smad (Psmad)2 and PSmad1/5 are described in Persson *et al.*[47] and TβRII antibody has been described in Franzen *et al.*[19] Smad2/3, N-cadherin, and E-cadherin antibodies were from BD Transduction Laboratories (Breda, The Netherlands); COL-I antibody was from Southern Biotechnology (Birmingham, AL, USA); FN antibody was from Abcam (Cambridge, UK); β-actin (AC-15), α-smooth-muscle actin (1A4), and FLAGM2 antibodies were from Sigma (St Louis, MO, USA); hemagglutinin antibody was from Roche.

Cellular assays, immunodetection, and RNA extraction

Immunofluorescence, western blotting, *in vitro* kinase assay, cell proliferation assays, transfection, and transcriptional reporter gene assay were performed as reported previously.[20,36–39,48] RNA extraction, reverse transcriptase-polymerase chain reaction, and Q-PCR were described in Gellibert *et al.*[32] and Deckers *et al.*[44] For detailed description see Supplementary Methods.

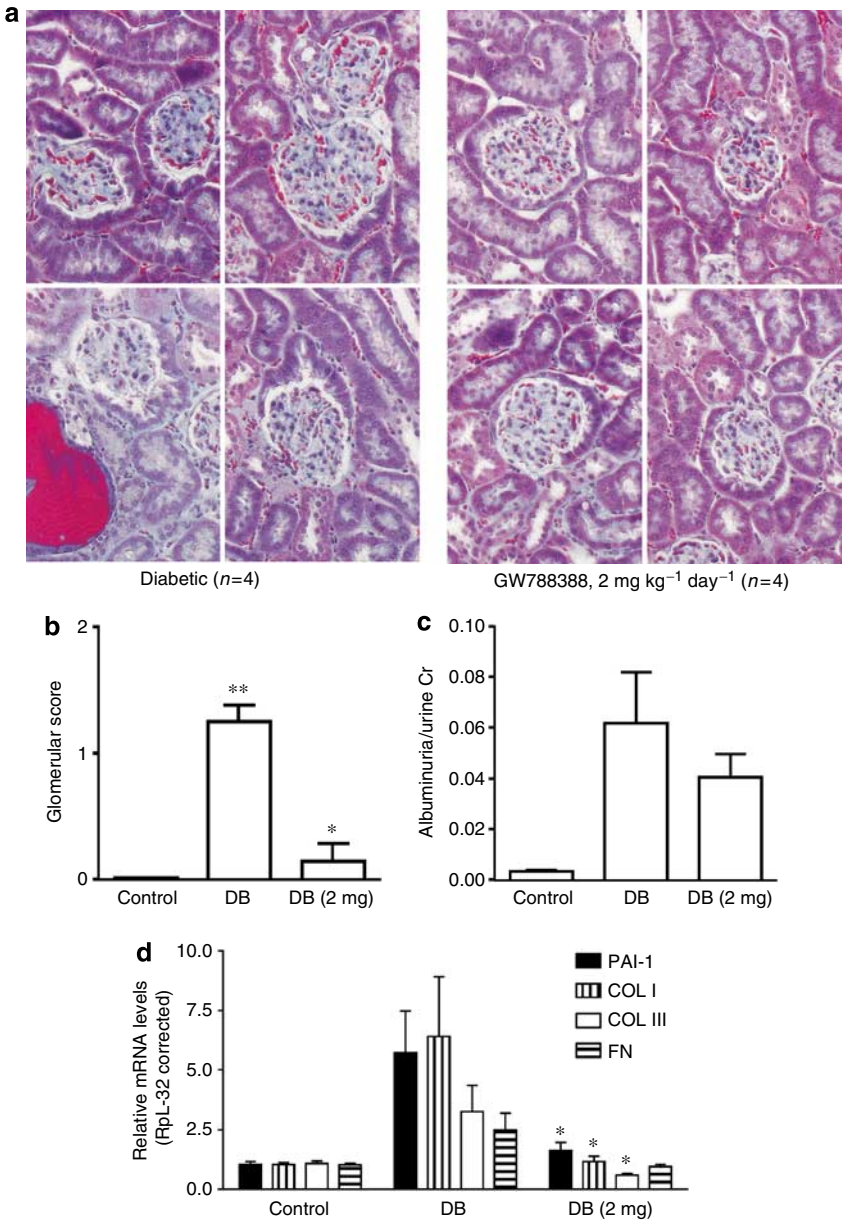

Figure 7 | GW788388 attenuates renal fibrosis in db/db mice. GW788388 was orally administered to 6-month-old male db/db mice for 5 weeks at a dose of 2 mg kg^{-1} day^{-1}. (**a**) Masson's Trichrome-stained kidney sections. Representative images are shown for db/db control mice (left panel) and db/db mice treated with 2 mg kg^{-1} day^{-1} GW788388 (right panel). Blue stain indicates heavy collagen presence indicative of glomerulosclerosis. (**b**) Glomerulopathy blinded scores of picric acid stain-stained kidney sections. Slides were reviewed blind, without knowledge of the study design, and 40 tufts were scored for each animal. The mean score and standard deviation were tabulated for each animal. $^{**}P<0.001$ versus lean control, $^{*}P<0.01$ versus vehicle-treated db/db mice (DB). A 2 mg kg^{-1} day^{-1} dose of GW788388 (2 mg DB) was administered. Numbers of mice analyzed in each group: lean controls ($n=10$), db/db control mice (DB) ($n=12$), and mice treated with 2 mg kg^{-1} day^{-1} (2 mg DB) ($n=7$). (**c**) Urinary albumin levels corrected for creatinine excretion. Lean controls ($n=10$), db/db control mice (DB) ($n=11$), and mice treated with 2 mg kg^{-1} day^{-1} (2 mg DB) ($n=6$). (**d**) GW788388 reduced the expression of TGF-β-induced ECM target genes *in vivo*. Total RNA was extracted from kidneys of lean controls ($n=11$), db/db control mice ($n=12$), and mice treated with 2 mg kg^{-1} day^{-1} ($n=7$). Expression of the following genes was analyzed by real-time quantitative PCR: PAI-1, collagen Iα1 (COL-I), collagen III (COL-III), and FN. $^{*}P<0.05$ versus db/db control group. Bars represent means ± s.e.m.

Histopathology

Kidneys were fixed in 10% formalin. Sections were stained with picric acid stain and Masson's Trichrome at Research Pathology Services (New Britain, PA, USA). Sections were submitted to Pathology Associates Inc. for assessment of glomerular changes. Scoring system is outlined in Supplementary Methods.

Animal experiments

Intravenous pharmacokinetics profiles were determined in Sprague–Dawley rats using a crossover design on four separate study days (see Supplementary Methods).

C57BLKS/J^{Lepr} db/db mice were used a model for type 2 diabetes mellitus[40] (Jackson Laboratory, Bar Habor, Maine, USA).

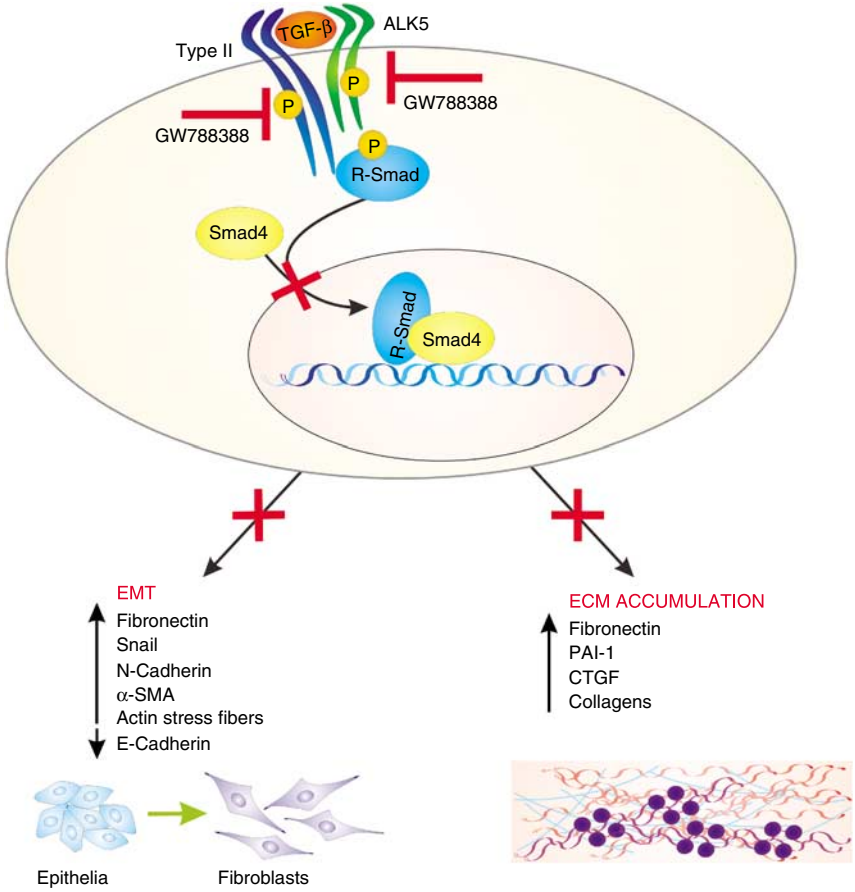

Figure 8 | Schematic representation of the inhibitory actions of GW788388 *in vitro*.

Animals received GW788388 at 2 mg kg^{-1} day^{-1} mixed with powdered chow, water *ad libitum*. After 5 weeks of drug treatment, a 24-h urine collection was performed by individual housing in metabolic cages. Albumin concentrations corrected for creatinine were determined (Nephrat II enzyme-linked immunosorbent assay kit). Kidneys were snap–frozen for RNA analysis. Plasma drug levels determined by HPLC/MS/MS. GW788388 was isolated from 50 µl of plasma (Sciex API 365). End plasma concentration was 10.4 ± 1.2 nM and urine concentration 0.9 ± 0.3 µM. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by US Institutes of Health (NIH Publication no. 85–23, revised 1996), and were approved by the Institutional Animal Care and Use Committee.

Statistical analysis

The experiment was completely randomised. One-way analysis of variance was performed with Bonferroni's multiple comparison test. $P<0.05$ was considered to be statistically significant. Mean is presented either as ± s.e.m. or ± s.d.

DISCLOSURE

Authors from GlaxoSmithKline disclose a duality of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. TGF-β-induced Smad2 phosphorylation and activin-induced CAGA-luciferase activity are inhibited in a concentration-dependent manner.

Supplementary Methods.

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