Transforming growth factor-β in the pathogenesis of breast cancer metastasis and fibrosis

Petersen, Maj

Chapter 5

Oral administration of GW788388, a kinase inhibitor of the TGF-β type I and type II receptors, reduces renal fibrosis in db/db mice.

MSc. Maj Petersen [*], MSc. Midory Thorikay[*], Dr. Martine Deckers[*], Maarten van Dinther[*], Dr. Eugène T. Grygielko[§], Dr. Françoise Gellibert[†], Dr. Anne-Charlotte Degouville[†], Dr. Stéphane Huet[†], Prof. Peter Ten Dijke[*1], and Dr. Nicholas J. Laping[§]

[*] *Department of Molecular Cell Biology, Leiden University Medical Centre, Leiden, The Netherlands,*
[†] *GlaxoSmithKline, Les Ulis, France,*
[§] *GlaxoSmithKline Pharmaceuticals, King of Prussia, Pennsylvania*

Published in *Kidney Int.* December 2007.

[1] To whom correspondence should be addressed

Abstract

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 epithelialmesenchymal 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.

Abbreviations

ActRII, activin type II Receptor; ALK, activin receptor-like kinase; caALK, constitutively active ALK; β-gal, β-galactosidase; BMP, bone morphogenic protein; BMPRII, BMP type II receptor; BRE, BMP responsive element; COL, collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GW788388, 4-(4-(3-(Pyridin-2-yl)-1H-pyrazol-4-yl) pyridin-2-yl)-N-(tetrahydro-2Hpyran-4-yl) benzamide; MMP, matrix metalloproteinase; NMuMG, namru murine mammary gland; PAI-1, plasminogen activator inhibitor 1; PAS, picric acid stain; PSmad, phosphorylated Smad; RCC4, renal cell carcinoma; RT-PCR, reverse transcriptase polymerase chain reaction; SB431542, 4-(5-benzo(1,3) dioxol- 5-yl-4-pyridin-2-yl-1H -imidazol-2-yl)-benzamide; Smad, small phenotype and mothers against DPP related protein; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; TIMP, Tissue inhibitor of metalloproteinase; VHL, von Hippel Lindau.

Introduction

Diabetic nephropathy leads to end-stage renal failure in 20 to 30% of patients with type 1 or type 2 diabetes mellitus[1].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, 9, 10]. These factors include fibronectin (FN), type I collagen (COL-1), type III collagen (COL-III), type IV collagen (COL-IV) and laminin [11]. TGF-β further stimulates ECM accumulation through enhancing expression of connective tissue growth factor, which in turn induces FN and COL-III expression [12]. Also, activated TGF-β suppress the activity of matrix metalloproteinases [13] through increased expression of tissue inhibitor of metalloproteinases and plasminogen activator inhibitor 1 (PAI-1) [14]. 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 cellcell contact. The cells undergo cytoskeletal remodelling and gain mesenchymal markers essential for cell-ECM association. The net result being enhanced cell motility and invasiveness [15, 16]. 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 [17].

TGF-β and the superfamily members, activins 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 [18]. In endothelial cells, however, TGF-β signals via ALK1 and ALK5 [19]. In contrast, BMP signals through ALK2, ALK3, or ALK6 and activin, and nodal through ALK4 and ALK7 [20, 21]. 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 DPPrelated protein (Smad)2, and Smad3. For TGF-β/ALK1 and BMP, the signal is via phosphorylation of the R-Smads, Smad1, 5, and 8 [22]. 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 [23].

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 [24, 25]. 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 [26]. 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, 27, 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 pro-collagen 1a(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/kg 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 IC50 for GST-ALK5 of 0.018 $\pm$ 0.08 TGF-μM [32]. To test the specificity of GW788388, we performed an *in vitro* kinase assay on full length receptors. Human embryonic kidney 293T cells were transiently transfected with expression plasmids encoding constitutively active ALK (caALK) 5, TβRII, BMP type II receptor (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 the 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 hours. GW788388 showed no toxicity in Namry murine mammary gland (NMuMG) (Figure 1c), MDA-MB-231, renal cell carcinoma (RCC)4, or 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).

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 mitogenactivated protein kinases (data not shown). Thus, GW788388 selectively inhibits TGF-β and activin Smad signalling and not the

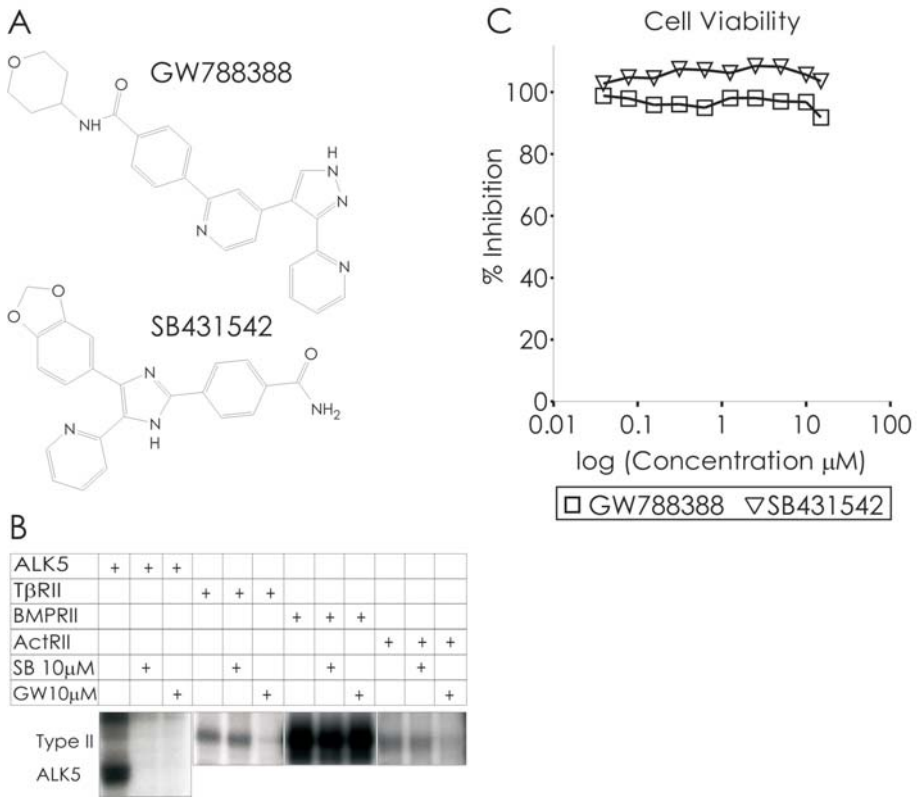

Figure 5.1: GW788388 inhibits both ALK5 and TβRII. (A) Chemical structures of GW788388, 4-(4-(3-(Pyridin-2-yl)-1H-pyrazol-4-yl) pyridin-2-yl)-N-(tetra-hydro-2Hpyran- 4-yl) benzamide and SB431542, 4-(5-benzo(1,3) dioxol- 5-yl-4-pyridin-2-yl-1H -imidazol-2-yl)-benzamide. (B) Effect of GW788388 and SB431542 on autophosphorylation of caALK5, TβRII, ActRII and BMPRII kinase activity. HEK293T cells were transfected with plasmids encoding full length actively signalling receptors. These were immunoprecipitated and the *in vitro* kinase assays were performed with gamma ^{32}P-labelled ATP in the presence of 10 μM GW788388 (GW) or 10 μM SB431542 (SB). (C) Cell viability assay. NMuMG cells were treated with dilutions of GW788388 (squares) and SB431542 (triangles) for 72 hours. Viability was measured with MTS assay. Data is presented as % inhibition compared to vehicle control. Bars represent mean $\pm$s.e.m.

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

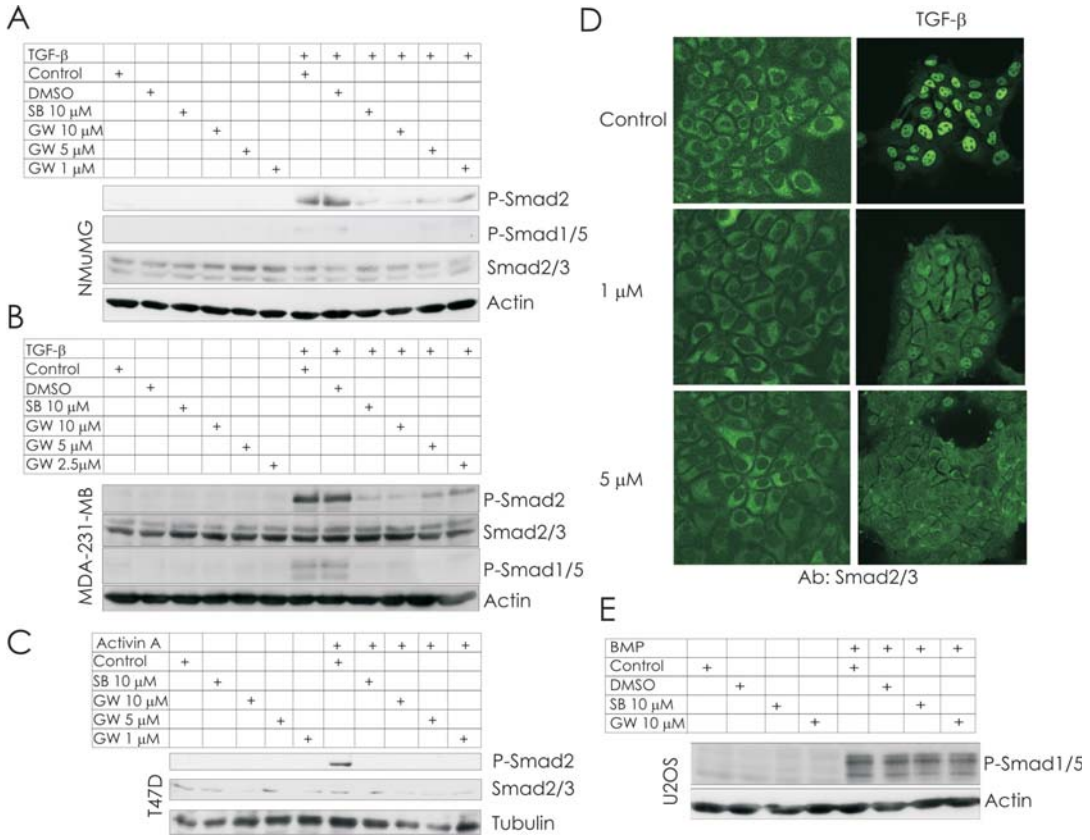

Figure 5.2: GW788388 inhibits TGF-β-induced Smad activation dose-dependently. NMuMG (A) and MDA-MD-231 cells (B) were treated with GW788388 (GW) or SB431542 (SB) in the presence or absence of TGF-β for 1 hour. Protein expression of PSmad2, PSmad1/5 and Smad2/3 was analysed by western blot analysis. β-Actin served as a loading control. (C) Immunofluorescent staining of Smad 2/3 in NMuMG cells treated with vehicle or GW788388 $\pm$ TGF-β for 1 hour. Images were captured with confocal microscopy. (D) Western blot analysis of U2OS cells treated with GW788388$\pm$BMP6 for 1 hour. Control denotes non treated cells and DMSO was used as vehicle.

[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 BMPinduced 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.

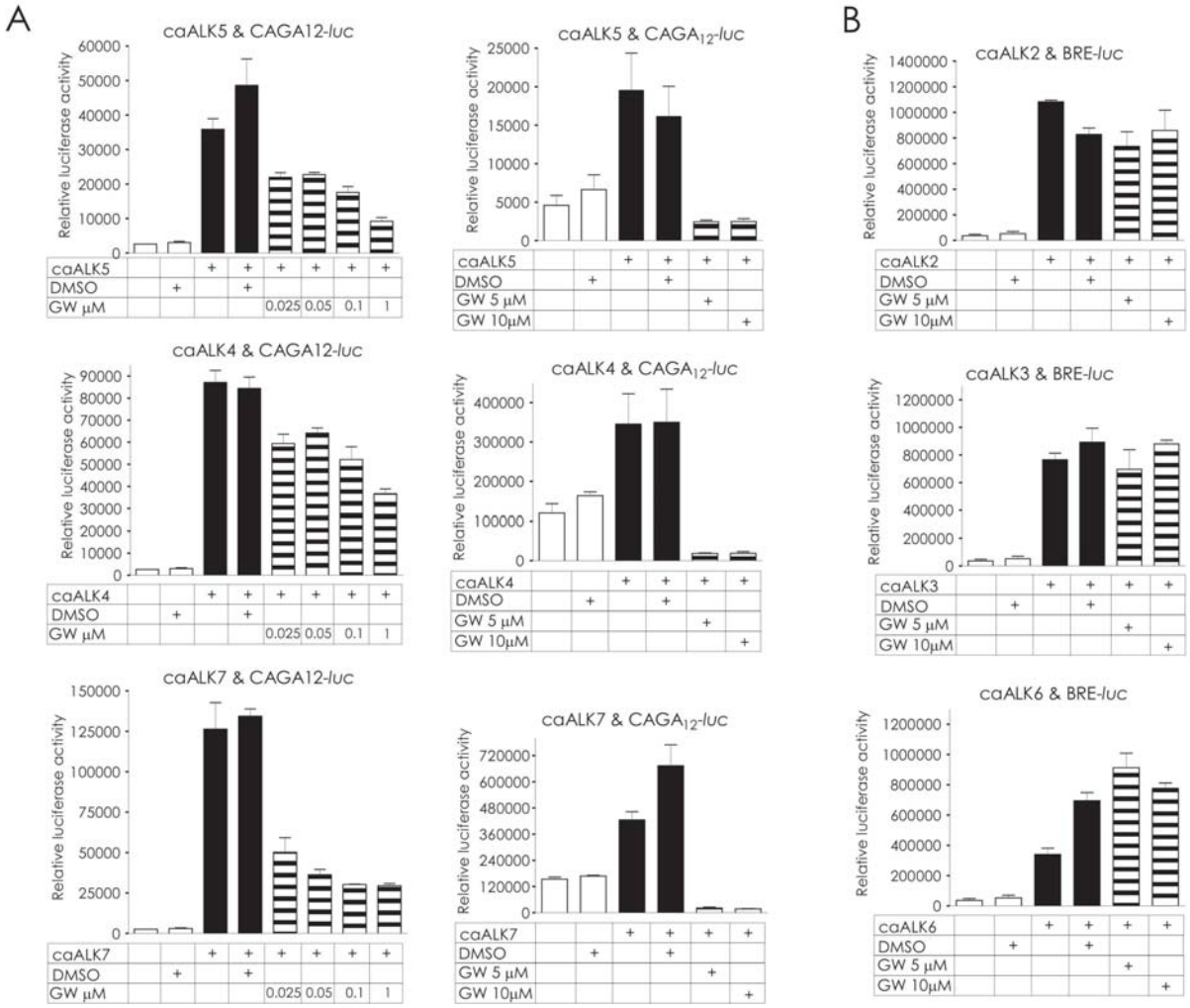

Figure 5.3: GW788388 inhibits ALK5, ALK4 and ALK7 in a dose-dependent manner and has no effect on ALK2, ALK3 and ALK6. (a) U2OS cells were transfected with caALK4, caALK5 or caALK7 together with the TGF-β specific luciferase reporter construct CAGA12-luc. Cells were treated with doses of GW788388 (GW) or vehicle. (b) U2OS cells were transfected with caALK2, caALK3 or caALK6 together with the BMP responsive BRE-luciferase reporter. Measurements are presented as luciferase activity normalised to β-gal activity. Error bars indicate mean $\pm$s.e.m. of three measurements, one representative experiment is shown.

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 4a, increasing concentrations of GW788388 inhibits TGF-β-induced growth inhibition. These re-

sults 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 RT-PCR and western blot analysis. We found that GW788388 could prevent the TGF-β-induced up-regulation of CTGF, 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 signaling in several *in vitro* models. We next sought to examine the effects of GW788388 *in vivo*. First, we compared the i.v. pharmacokinetic profiles of GW788388 compared to SB431542, in Sprague-Dawley rats. Clearance was 34 $\pm$ 12.2 ml/min/kg for GW788388 versus 37.5 $\pm$ 13.5 ml/min/kg for SB431542. The half-life of GW788388 was 4.1 $\pm$ 1.8 hours versus 28.5 $\pm$ 16.1 minutes for SB431542 (data not shown). Hence, GW788388 is far more

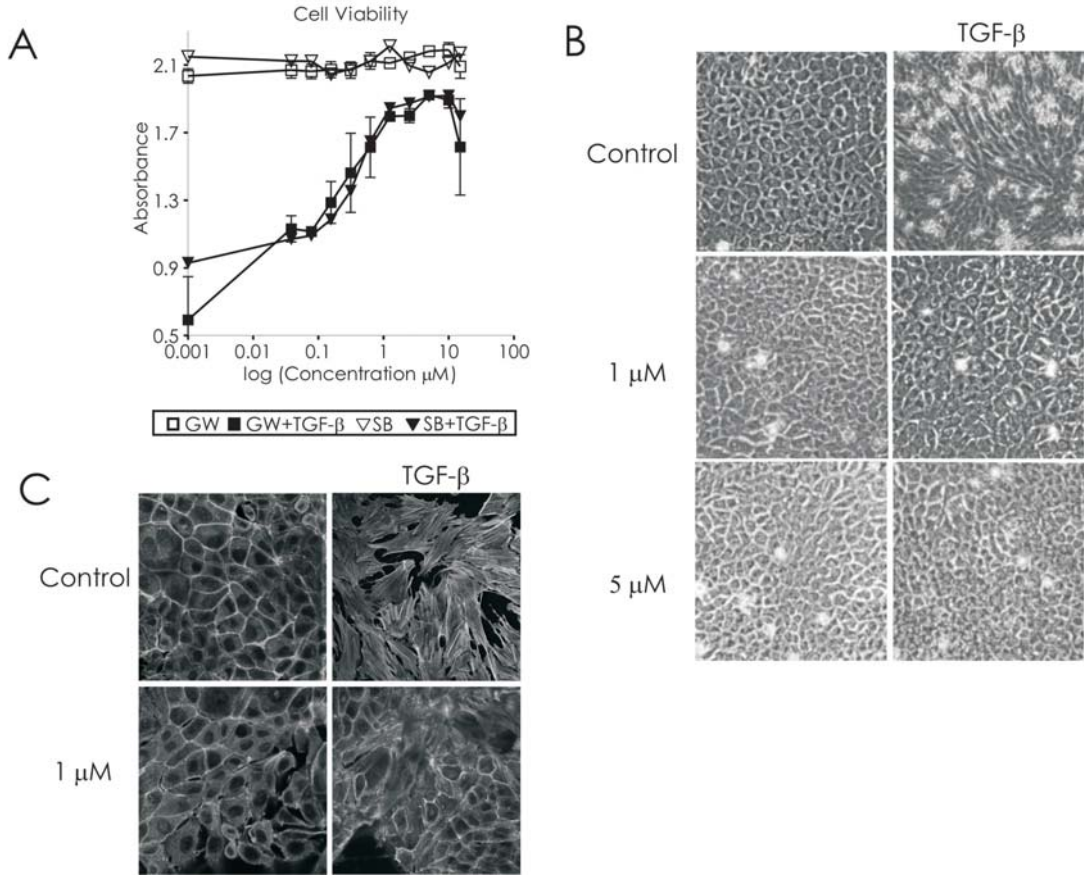

Figure 5.4: GW788388 inhibits TGF-β-mediated EMT and apoptosis. NMuMG cells were treated with GW788388, the vehicle control DMSO and TGF-β where indicated, for 48 hours. (A) NMuMG cell proliferation measured after 72 hours drug stimulation with dilution series of GW788388 (GW) (squares) and SB431542 (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. (C) Immunofluorescent staining of actin stress fibre formation. Images were captured with confocal microscopy.

suitable for *in vivo* applications 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 advance stage renal disease, significant glomerular changes and elevated albuminuria [40]. Mice were treated for 5 weeks with oral administration of 2 mg/kg/day of GW788388. 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/day resulted in a reduced collagen staining (Figure

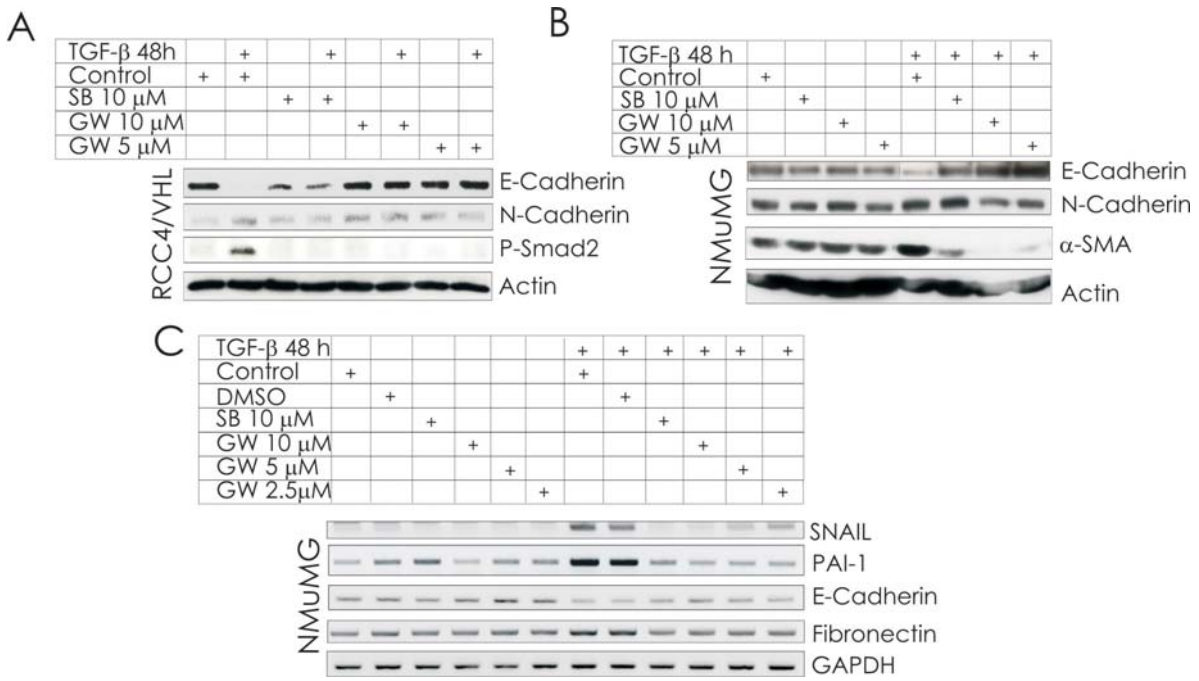

Figure 5.5: TGF-β-induced EMT is inhibited by GW788388. Western blot analysis of epithelial and mesenchymal protein markers in RCC4/VHL (A) and NMuMG (B) cells after 48 hours drug and TGF-β stimulation. Control is DMSO treated cells. β-Actin was used as a loading control. (C) RT-PCR semiquantitative 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 hours. GAPDH was included as loading control. Control depicts non treated cells and DMSO vehicle treated cells.

7a right panel). Glomerulopathy was assessed independently on PAS 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 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 to their lean littermates (Figure 7d). Treatment with 2 mg/kg/day 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.

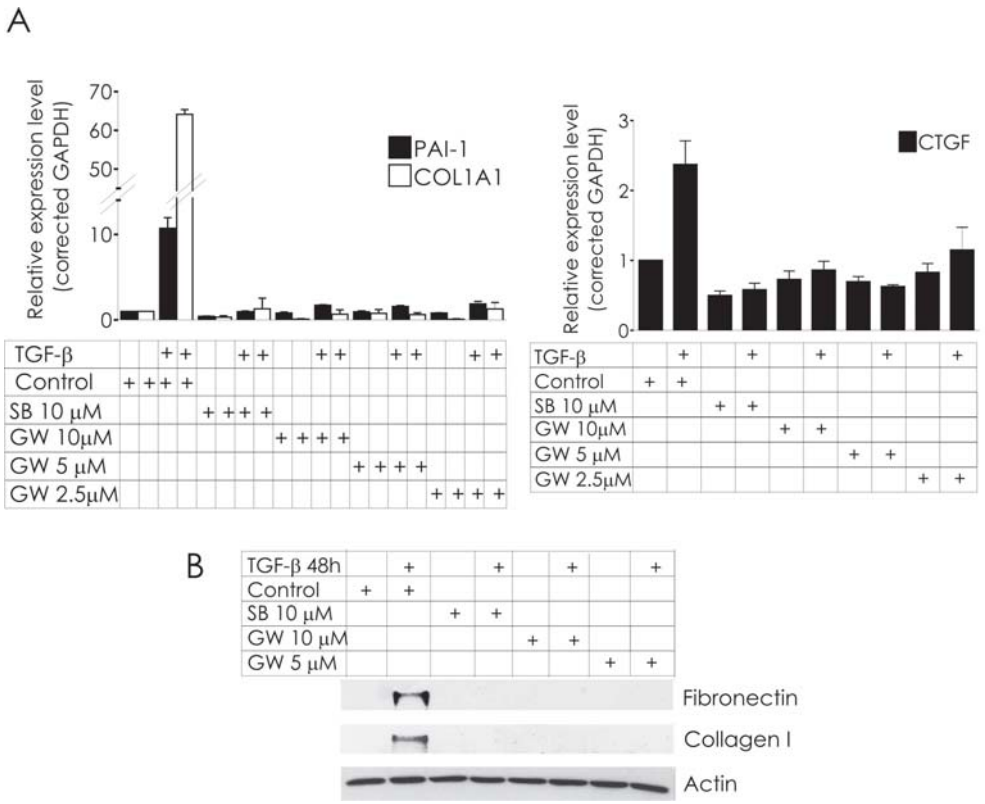

Figure 5.6: GW788388 inhibits the TGF-β-induced fibrotic response *in vitro*. (A) The effect of GW788388 (GW) on TGF-β-induced mRNA expression of the ECM genes PAI-1, COL-1αI and CTGF were analysed by real time Q-PCR. RNA was extracted from RCC4/VHL renal epithelial cells stimulated with drug $\pm$ TGF-β for 48 hours. GAPDH was used as a reference housekeeping gene. Results are presented as means $\pm$ SD 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.

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 the type I and the 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. These results encourage further studies to therapeutically target the TGF-β

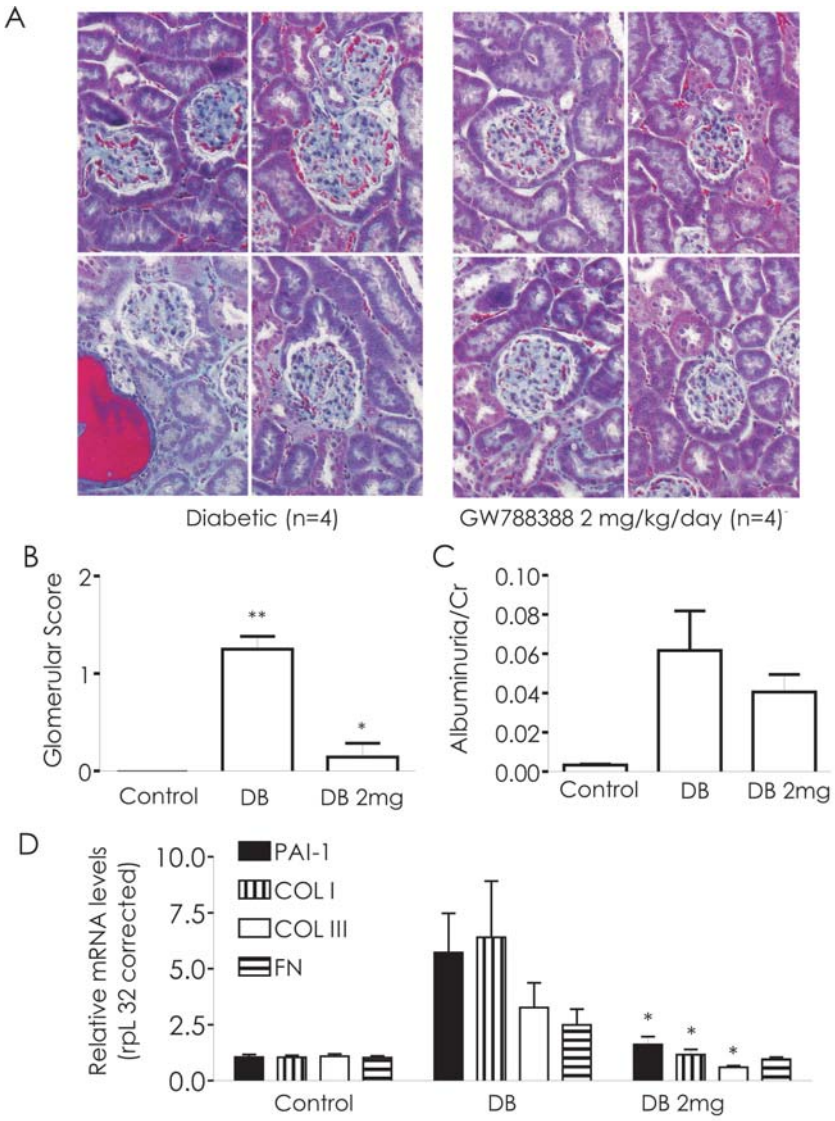

Figure 5.7: GW788388 attenuates renal fibrosis in db/db mice. GW788388 was orally administered to db/db mice for 5 weeks at 2 mg/kg/day. (A) Masson's Trichrome stained kidney sections. Representative images are shown for db/db control (left panel) and db/db mice treated with 2 mg/kg/day GW788388 (right panel). Blue stain indicates heavy collagen presence indicative of glomerulosclerosis. (B) Glomerulopathy blinded scores of PAS stained kidney sections. 40 tufts were scored/animal and the mean score ± s.d were tabulated for each animal. $^{**}P{\leq}0.001 versus lean control (n = 10), ^{*}P{\leq}0.01$ versus vehicle treated db/db mice (DB) (n=12). 2 mg/kg/day dose of GW788388 (2mg DB) (n=7). (C) Urinary albumin levels corrected for creatinine excretion. Lean controls (n=10), db/db control (DB) (n=11) and treated with 2 mg/kg/day (2mgDB) (n=6). (D) GW788388 reduced the expression of TGF-β-induced extracellular matrix target genes *in vivo*. RNA extracted from kidneys and analysed by real time PCR of lean controls (n=11), db/db control mice (n=12) and mice treated with 2 mg/kg/day (n=7). Bars represent mean ± s.e.m.

pathway in order to treat renal diseases.

GW788388 was identified as an orally active ALK5 inhibitor with much improved *in vivo* properties compared to SB431542 [24, 32]. In order to study the specificity of the compound we performed an *in vitro* kinase assay with ^{32}P-ATP. We found that GW788388 potently inhibits both the ALK5 and the TβRII kinase receptor activity but not the BMPRII. This contrasts what is seen for the related inhibitor, SB431542 [19, 22]. 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 i.e. 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 also reduce the TβRII kinase activity.

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 up-regulation 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 [27]. In our hands, GW788388 could prevent the TGF-β-mediated up-regulation of CTGF, 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, collagen I, and collagen III by GW788388. These results are in agreement with the oral application of GW788388 to rats with chemical induced liver fibrosis [32] and with db/db mice treated with neutralizing 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. This suggests that longer treatments 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. To address this hypothesis, treatment would need to

be started earlier before any albuminuria is observed. 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 preservation of glomerular morphology and function.

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 anti-fibrotic treatment will be to balance the disease related fibrotic actions against the immune modulatory and tumour suppressor functions of TGF-β. Also, all ALK5 kinase inhibitors reported to date inhibits the kinase activity of the ALK4 and ALK7 [20, 24, 33, 43, 46] and GW788388 to some extend the ActRII. Long-term treatment may therefore affect activin and nodal dependent signalling.

In summary, we have demonstrated that GW788388 can inhibit TGF-β 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 was 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 up-regulation 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, the human osteosarcoma U2OS and the monkey kidney COS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin and 50 μg/ml streptomycin. The human renal carcinoma cell line (RCC4) stably transfected with plasmid expressing the von Hippel Lindau (VHL) protein, was maintained in medium as above with neomycin. The murine breast epithelial cells, NMuMG were maintained in DMEM as above with 10 mg/ml insulin [44]. Cell lines were cultured at 37° in 5% CO_2. SB431542 was from Tocris. Compounds were dissolved in DMSO. We used 5 ng/ml TGF-β3, 100 ng/ml BMP6 and 50 ng/ml activin a. Antibodies recognizing phosphorylated Smad2 (PSmad2) and phosphorylated Smad 1/5 (PSmad1/5) are described in [47] and TβRII antibody in [18]. Smad2/3, N-cadherin and E-cadherin antibodies were from BD transduction laboratories. Collagen type I antibody from Southern Biotechnology. FN antibody from Abcam. β-Actin (AC-15), α-smooth muscle actin (1A4) and FLAGM2 antibodies from Sigma. 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 done as previously reported [19, 36, 37, 38, 39, 48]. RNA extraction RT-PCR and Q-PCR were described in [32, 44]. For detailed description of these methods see Supplementary Methods.

Histopathology

Kidneys were fixed in 10% formalin. Sections were stained with picric acid stain (PAS) and Masson's Trichrome at Research Pathology Services Incorporated (New Britain, PA). Stained sections were submitted to Pathology Associates, Incorporated 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). Male C57BLKS/J^{Lepr} db/db mice were used as a model for type 2 diabetes mellitus [40] (Jackson laboratory). Animals received GW788388 at 2 mg/kg/day mixed with powdered rodent chow, water *ad libitum*. After 5 weeks of drug treatment, a 24 hour 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 or fixed for histology. 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 $\pm$ 1.2 nM and urine concentration 0.9 $\pm$ 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$\leq$0.05 was considered to be statistically significant. Mean is presented either as $\pm$ s.e.m or $\pm$ s.d.

Disclosure

Authors from GlaxoSmithKline disclose a duality of interest.

Acknowledgement

We thank Dr. Lars van der Heide for critical reading of the manuscript, Susanne M. Kooistra for the initial studies. This work was supported by the following grants from the European Commission: The EpiplastCarcinoma Marie Curie RTN (project 005428), the BRECOSM sixth framework program (project 503224) and the Dutch Cancer Society grant (NKI-2001-2481).

References

[1] A.J. Collins, G. Hanson, A. Umen, C. Kjellstrand, and P. Keshaviah. Changing risk factor demographics in end-stage renal disease patients entering hemodialysis and the impact on long-term mortality. *Am.J.Kidney Dis.*, 15(5):422–432, May 1990.

[2] W.A. Border and N.A. Noble. Evidence that TGF-β should be a therapeutic target in diabetic nephropathy. *Kidney Int.*, 54(4):1390–1391, October 1998.

[3] K. Sharma, F.N. Ziyadeh, B. Alzahabi, T.A. McGowan, S. Kapoor, B.R. Kurnik, P.B. Kurnik, and L.S. Weisberg. Increased renal production of transforming growth factor-β1 in patients with type II diabetes. *Diabetes*, 46(5):854–859, May 1997.

[4] T. Yamamoto, T. Nakamura, N.A. Noble, E. Ruoslahti, and W.A. Border. Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc.Natl.Acad.Sci.U.S.A*, 90(5):1814–1818, March 1993.

[5] S. Tsakas and D.S. Goumenos. Accurate measurement and clinical significance of urinary transforming growth factor-β1. *Am.J.Nephrol.*, 26(2):186–193, 2006.

[6] G. Wolf, S. Chen, and F.N. Ziyadeh. From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes*, 54(6):1626–1634, June 2005.

[7] F.N. Ziyadeh, B.B. Hoffman, D.C. Han, Iglesias-De La Cruz MC, S.W. Hong, M. Isono, S. Chen, T.A. McGowan, and K. Sharma. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-β antibody in db/db diabetic mice. *Proc.Natl.Acad.Sci.U.S.A*, 97(14):8015–8020, July 2000.

[8] T. Nakamura, D. Miller, E. Ruoslahti, and W.A. Border. Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor-β 1. *Kidney Int.*, 41(5):1213–1221, May 1992.

[9] F.N. Ziyadeh, K. Sharma, M. Ericksen, and G. Wolf. Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β. *J.Clin.Invest*, 93(2):536–542, February 1994.

[10] A.C. Poncelet and H.W. Schnaper. Sp1 and Smad proteins cooperate to mediate transforming growth factor-β 1-induced α2(I) collagen expression in human glomerular mesangial cells. *J.Biol.Chem.*, 276(10):6983–6992, March 2001.

[11] D.S. Goumenos, A.C. Tsamandas, S. Oldroyd, F. Sotsiou, S. Tsakas, C. Petropoulou, D. Bonikos, A.M. El Nahas, and J.G. Vlachojannis. Transforming growth factor-β(1) and myofibroblasts: a potential pathway towards renal scarring in human glomerular disease. *Nephron*, 87(3):240–248, March 2001.

[12] S.N. Wang, M. DeNichilo, C. Brubaker, and R. Hirschberg. Connective tissue growth factor in tubulointerstitial injury of diabetic nephropathy. *Kidney International*, 60(1):96–105, July 2001.

[13] S. Sagar, N. Gibbons, E. Valderrama, and P.C. Singhal. Glomerular metalloprotease activity modulates the development of focal segmental glomerulosclerosis. *Clin.Nephrol.*, 44(6):356–361, December 1995.

[14] M. Laiho, O. Saksela, and J. Keski-Oja. Transforming growth factor-β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. *J.Biol.Chem.*, 262(36):17467–17474, December 1987.

[15] R. Kalluri and E.G. Neilson. Epithelial-mesenchymal transition and its implications for fibrosis. *J.Clin.Invest*, 112(12):1776–1784, December 2003.

[16] M.A. Huber, N. Kraut, and H. Beug. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr.Opin.Cell Biol.*, 17(5):548–558, October 2005.

[17] M. Zeisberg and R. Kalluri. The role of epithelial-to-mesenchymal transition in renal fibrosis. *J.Mol.Med.*, 82(3):175–181, March 2004.

[18] P. Franzen, P. ten Dijke, H. Ichijo, H. Yamashita, P. Schulz, C-H. Heldin, and K. Miyazono. Cloning of a TGF β type I receptor that forms a heteromeric complex with the TGF β type II receptor. *Cell*, 75(4):681–692, November 1993.

[19] M.J. Goumans, G. Valdimarsdottir, S. Itoh, F. Lebrin, J. Larsson, C. Mummery, S. Karlsson, and P. ten Dijke. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. *Mol.Cell*, 12(4):817–828, October 2003.

[20] P. ten Dijke, H. Yamashita, H. Ichijo, P. Franzen, M. Laiho, K. Miyazono, and C-H. Heldin. Characterization of type I receptors for transforming growth factor-β and activin. *Science*, 264(5155):101–104, April 1994.

[21] P. ten Dijke, H. Yamashita, T.K. Sampath, A.H. Reddi, M. Estevez, D.L. Riddle, H. Ichijo, C-H. Heldin, and K. Miyazono. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J.Biol.Chem.*, 269(25):16985–16988, June 1994.

[22] P. ten Dijke, O. Korchynskyi, G. Valdimarsdottir, and M.J. Goumans. Controlling cell fate by bone morphogenetic protein receptors. *Mol.Cell Endocrinol.*, 211(1-2):105–113, December 2003.

[23] P. ten Dijke and C.S. Hill. New insights into TGF-β-Smad signalling. *Trends Biochem.Sci.*, 29(5):265–273, May 2004.

[24] J.F. Callahan, J.L. Burgess, J.A. Fornwald, L.M. Gaster, J.D. Harling, F.P. Harrington, J. Heer, C. Kwon, R. Lehr, A. Mathur, B.A. Olson, J. Weinstock, and N.J. Laping. Identification of novel inhibitors of the transforming growth factor β1 (TGF-β1) type 1 receptor (ALK5). *J.Med.Chem.*, 45(5):999–1001, February 2002.

[25] R.S. Muraoka-Cook, I. Shin, J.Y. Yi, E. Easterly, M.H. Barcellos-Hoff, J.M. Yingling, R. Zent, and C.L. Arteaga. Activated type I TGFβ receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncogene*, 25(24):3408–3423, June 2006.

[26] N.J. Laping, E. Grygielko, A. Mathur, S. Butter, J. Bomberger, C. Tweed, W. Martin, J. Fornwald, R. Lehr, J. Harling, L. Gaster, J.F. Callahan, and B.A. Olson. Inhibition of transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity: SB-431542. *Mol.Pharmacol.*, 62(1):58–64, July 2002.

[27] W.A. Border, N.A. Noble, T. Yamamoto, J.R. Harper, Y. Yamaguchi, M.D. Pierschbacher, and E. Ruoslahti. Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature*, 360(6402):361–364, November 1992.

[28] K. Sharma, Y. Jin, J. Guo, and F.N. Ziyadeh. Neutralization of TGF-β by anti-TGF-β antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes*, 45(4):522–530, April 1996.

[29] W.A. Border, S. Okuda, L.R. Languino, M.B. Sporn, and E. Ruoslahti. Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β 1. *Nature*, 346(6282):371–374, July 1990.

[30] D.C. Han, B.B. Hoffman, S.W. Hong, J. Guo, and F.N. Ziyadeh. Therapy with antisense TGF-β1 oligodeoxynucleotides reduces kidney weight and matrix mRNAs in diabetic mice. *Am.J.Physiol Renal Physiol*, 278(4):F628–F634, April 2000.

[31] L.M. Russo, E. Del Re, D. Brown, and H.Y. Lin. Evidence for a role of transforming growth factor (TGF)-β1 in the induction of postglomerular albuminuria in diabetic nephropathy: Amelioration by soluble TGF-β type II receptor. *Diabetes*, 56(2):380–388, February 2007.

[32] F. Gellibert, A.C. de Gouville, J. Woolven, N. Mathews, V.L. Nguyen, C. Bertho-Ruault, A. Patikis, E.T. Grygielko, N.J. Laping, and S. Huet. Discovery of 4-4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl-n-(tetrahydro-2h- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-β type I receptor inhibitor. *J.Med.Chem.*, 49(7):2210–2221, April 2006.

[33] S.B. Peng, L. Yan, X.L. Xia, S.A. Watkins, H.B. Brooks, D. Beight, D.K. Herron, M.L. Jones, J.W. Lampe, W.T. McMillen, N. Mort, J.S. Sawyer, and J.M. Yingling. Kinetic characterization of novel pyrazole TGF-β receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry*, 44(7):2293–2304, 2005.

[34] E.T. Grygielko, W.M. Martin, C. Tweed, P. Thornton, J. Harling, D.P. Brooks, and N.J. Laping. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-β type I receptor kinase in puromycin-induced nephritis. *J.Pharmacol.Exp.Ther.*, 313(3):943–951, June 2005.

[35] J.A. Moon, H.T. Kim, I.S. Cho, Y.Y. Sheen, and D.K. Kim. IN-1130, a novel transforming growth factor-β type I receptor kinase (ALK5) inhibitor, suppresses renal fibrosis in obstructive nephropathy. *Kidney Int.*, 70(7):1234–1243, October 2006.

[36] A. Nakao, M. Afrakhte, A. Moren, T. Nakayama, J.L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N-E. Heldin, C-H. Heldin, and P. ten Dijke. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. *Nature*, 389(6651):631–635, October 1997.

[37] S. Dennler, S. Itoh, D. Vivien, P. ten Dijke, S. Huet, and J.M. Gauthier. Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.*, 17(11):3091–3100, June 1998.

[38] O. Korchynskyi and P. ten Dijke. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J.Biol.Chem.*, 277(7):4883–4891, February 2002.

[39] E. Piek, A. Moustakas, A. Kurisaki, C-H. Heldin, and P. ten Dijke. TGF-β type I receptor/ALK-5 and smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J.Cell Sci.*, 112 (Pt 24):4557–4568, December 1999.

[40] K. Sharma, P. McCue, and S.R. Dunn. Diabetic kidney disease in the db/db mouse. *Am.J.Physiol Renal Physiol*, 284(6):F1138–F1144, June 2003.

[41] E.M. Zeisberg, O. Tarnavski, M. Zeisberg, A.L. Dorfman, J.R. McMullen, E. Gustafsson, A. Chandraker, X. Yuan, W.T. Pu, A.B. Roberts, E.G. Neilson, M.H. Sayegh, S. Izumo, and R. Kalluri. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat.Med.*, 13(8):952–961, August 2007.

[42] B.S. DaCosta, C. Major, N.J. Laping, and A.B. Roberts. SB-505124 is a selective inhibitor of transforming growth factor-β type I receptors ALK4, ALK5, and ALK7. *Mol.Pharmacol.*, 65(3):744–752, March 2004.

[43] G.J. Inman, F.J. Nicolas, J.F. Callahan, J.D. Harling, L.M. Gaster, A.D. Reith, N.J. Laping, and C.S. Hill. SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol.Pharmacol.*, 62(1):65–74, July 2002.

[44] M. Deckers, M. van Dinther, J. Buijs, I. Que, C. Löwik, G. van der Pluijm, and P. ten Dijke. The tumor suppressor Smad4 is required for transforming growth factor β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer Res.*, 66(4):2202–2209, February 2006.

[45] A. Boutet, C.A. De Frutos, P.H. Maxwell, M.J. Mayol, J. Romero, and M.A. Nieto. Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney. *EMBO J.*, 23:5603–13, November 2006.

[46] E. Reissmann, H. Jornvall, A. Blokzijl, O. Andersson, C. Chang, G. Minchiotti, M.G. Persico, C.F. Ibanez, and A.H. Brivanlou. The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev.*, 15(15):2010–2022, August 2001.

[47] U. Persson, H. Izumi, S. Souchelnytskyi, S. Itoh, S. Grimsby, U. Engström, C-H. Heldin, K. Funa, and P. ten Dijke. The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.*, 434(1-2):83–87, August 1998.

[48] T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J.Immunol.Methods*, 65(1-2):55–63, December 1983.

Supplementary information

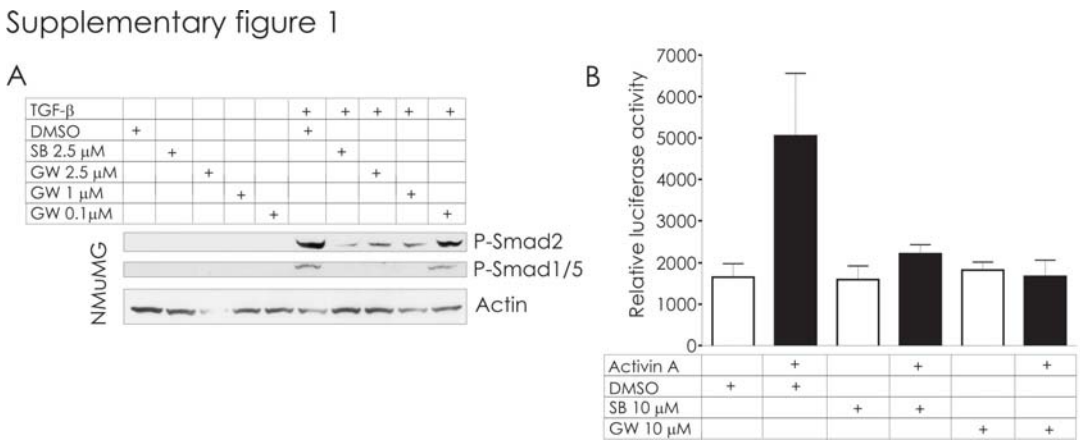

Figure 5.9: Supplementary Figure S1. TGF-β-induced Smad2 phosphorylation and activin-induced CAGA-luciferase activity are inhibited in a concentration-dependent manner. (A) Western blot analysis of Smad2 phosphorylation in NMuMG cells after 1 hour TGF-β stimulation. Smad2 phosphorylation is blocked with nanomolar concentrations of GW788388. (B) Activin a-induced CAGA-luciferase activity is inhibited by SB431542 and GW788388. Transiently transfected U2OS cells were stimulated with activin overnight and luciferase activity measured, corrected for β-gal activity. Error bars denote ± s.e.m of three measurements.

Supplementary methods

Transfection and reporter gene assay.

Transient transfection of cells were carried out using Lipofectamine (Invitrogen) [39]. Human constitutive active ALK (caALK) 1, 2, 3, 4, 5, 6, and -7 plasmids and the CAGA12 or the BRE luciferase reporter constructs have previously been described [36, 37, 38]. We measured on the Wallac 1420 VICTOR3.

RNA extraction, RT-PCR and Q-PCR.

Cells were treated with GW788388, SB431542 or DMSO and TGF-β the day after plating. Total RNA was isolated using the RNeasy kit (Qiagen). RT-PCR was performed for PAI-1, SNAIL, FN, E-cadherin and glyceraldehyde-3- phosphate (GAPDH) as previously described [44]. RNA was extracted from mouse kidneys and Q-PCR was performed using the ABI Prism 6700 Workstation [32]. RpL-32 or GAPDH were used as housekeeping genes.

In vitro kinase assay, cell viability assay and western blotting.

The kinase assay was performed as described in [7]. In brief, cells were transfected with caALK5, TβRII, BMP type II receptor (BMPRII) or the Activin type II receptor (ActRII). Immunoprecipitated receptors challenged with 14.8 kBq/ml [γ-32P]-ATP and 10 μM compounds.

Cell viability and proliferation assays were done according to the manufactures instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, The Netherlands). Viability and proliferation were measured after 72 hours drug treatment in the presence or absence of TGF-β. Western blot analyses were performed using standard techniques [39].

Immunofluorescence.

Cells were seeded on coverslips and treated with GW788388 and TGF-β where indicated, for 1 or 48 h as previously described [39, 44]. Images were captured with confocal microscopy.

Histopathological scoring.

Stained sections were independently assessed for glomerular changes. Scoring was done on approximately 40 glomerular tufts (representing essentially all of the glomeruli present in a typical section) from each animal using the following categorical criteria for each glomerulus: 0 - well-defined glomerular tuft with essentially no significant accumulations of PAS positive mesangial matrix; 1 - slight, focal increases in PAS positive mesangial matrix or thin bands of increased PAS positive matrix running along the core of the glomerular tuft; 2 - Multiple small foci of increased mesangial matrix or thicker more dense bands of matrix; 3 - Increased matrix to the extent that some lobules of a glomerular tuft can be considered sclerotic. Mesangial cells may be increased in these areas; 4 - significant increases in mesangial matrix and frequently increased numbers of mesangial cells involving the entire glomerular tuft.

Pharmacokinetic profiling *in vivo*.

Intravenous pharmacokinetics profiles were determined in Sprague-Dawley rats, using a crossover design on four separate study days. Femoral vein catheters implanted for infusion of test compounds at least three days prior to the start of the study. On day one, animals received GW788388 (6 μmol/kg target dose) or SB431542 (2 μmol/kg target dose) by 30 minutes i.v. infusion (4 mL/kg). The dose (pH = 3.5), contained 3% DMSO prepared in 20% aqueous encapsin (Cerestar USA Inc., Hammond, IN). For SB431542 the dose solution was prepared in 10% PEG 400 and isotonic saline (pH = 3.5-4.0) and contained 1.0% DMSO.