Renal-specific delivery of antifibrotic drugs using lysozyme
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Chapter 7

Local Inrarenal Inhibition of TGF-beta Signaling for the Treatment of Renal Fibrosis

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Abstract

During renal insult, transforming growth factor-beta (TGF-β) activates tubular epithelial cells and fibroblasts eventually leading to fibrosis. TGF-β acts through its type-I receptor known as Activin Receptor Like Kinase (ALK)-5. We hypothesized that local blockade of ALK5 within kidneys might be a promising approach for the treatment of renal fibrosis. We examined the efficacy of an ALK5 inhibitor (3-(Pyridin-2-yl)-4-(4-quinonyl)-1H-pyrazole; TKI) in normal rat kidney-(52E) tubular cells. TKI significantly inhibited the expression of albumin-induced monocyte chemotactant protein (MCP)-1, tissue inhibitor of metalloproteinase (TIMP)-1, and alpha-smooth muscle actin (α-SMA) genes and TGF-β1-induced procollagen-Iα1 and TIMP-1 genes. To achieve tubular cell-specific delivery, we conjugated TKI to lysozyme (LZM) using the Universal Linkage System (ULS™). TKI-LZM showed its activity by inhibiting TGF-β1-induced procollagen-Iα1 gene expression in cultured human renal tubular cells. In vivo, after a single intravenous dose of TKI-LZM, it accumulated rapidly and provided a local depot for at least 3 days in tubular cells. We evaluated the efficacy of TKI-LZM in the unilateral ureteral obstruction model in rats. A single dose of TKI-LZM inhibited renal MCP-1 gene expression significantly after 3 days. This was associated with a marked reduction in inflammatory macrophages and inhibition of the profibrotic marker α-SMA. In contrast, free drug did not alter MCP-1 gene expression, although it also diminished the influx of immune cells and α-SMA expression. We conclude that ALK5 is a promising target for intervention in renal fibrosis, and that renal delivery of an ALK5 inhibitor may serve as a novel therapeutic strategy in renal fibrosis.
Introduction

Transforming growth factor–beta (TGF-β), a multifunctional cytokine, plays a crucial role in the pathogenesis of renal fibrosis (1). During renal injury, the active form of TGF-β is either locally produced by resident or infiltrated cells, or systemically derived. Irrespective of its source, TGF-β can activate tubular epithelial cells causing their transformation into fibroblasts through epithelial-mesenchymal transition (2). Eventually, resident and epithelial cells derived fibroblasts are transformed into myofibroblasts and produce extracellular matrix (ECM) proteins such as collagens (types I, III, IV, V and VI) and fibronectin (3). TGF-β binds to its type-II receptor, which subsequently associates with the type-I receptor known as Activin Receptor Like Kinase 5 (ALK5) (4). ALK5 activates downstream Smad and mitogen-activated protein kinase (MAPK) pathways. Both Smad and MAPK participate in the development of renal fibrosis by regulating ECM deposition (5). Blockade of ALK5 in renal tubular cells therefore seems an attractive therapeutic strategy to prevent or attenuate tubulointerstitial fibrosis.

Recently, ALK5 inhibitors have been developed and evaluated for the inhibition of TGF-β action in various in vitro systems (6). These inhibitors bind to the ATP-binding pocket of the kinase domain of the TGF-β type I receptor and inhibit the subsequent activation of downstream pathways (6,7). Recent studies have demonstrated beneficial effects of ALK5 inhibitors on lung, kidney and liver fibrosis in animal models (8-10). However, relatively high and multiple doses had to be administered to achieve therapeutic effects. Moreover, systemic administration of an ALK5 inhibitor may elicit immunological side-effects, since TGF-β plays an essential role in immune tolerance via the regulation of lymphocyte proliferation and differentiation (11). Administration of TGF-β–inhibiting compounds is therefore not without risk of severe side effects and, the development of TGF-β blocking drugs has been a challenge for several decades. In order to minimize the side effects and enhance efficacy in target cells, we now aim at inhibiting TGF-β locally within the kidney.

We have developed a renal drug delivery system which is also applicable to the presently studied class of kinase inhibitors (12,13). Using platinum-based linking technology, the so-called Universal Linkage System (ULS™), organic drug molecules are coupled to the renal carrier protein lysozyme (LZM), providing drug-LZM conjugates that accumulate efficiently in the kidneys (14). In the present study, we applied this technology to a TGF-β receptor Kinase Inhibitor (TKI) which inhibits ALK5 and several other kinases including p38 MAPkinase (6). We first evaluated the capacity of TKI to inhibit inflammatory and fibrotic signaling events in cultured renal tubular cells. Next, we conjugated TKI to LZM, characterized the obtained TKI-LZM conjugate in renal tubular cells and studied its pharmacokinetics in healthy rats. Finally, we evaluated the therapeutic potential of TKI-LZM in the unilateral ureteral obstruction (UUO) model for renal fibrosis.
Chapter 7

Materials and Methods

The ALK5 inhibitor [3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole, (TKI) was purchased from Calbiochem (Darmstadt, Germany). Human recombinant TGF-β1 was purchased from Roche Diagnostics, Mannheim, Germany. Rhodamine-ULS was purchased from Kreatech Biotechnology, Amsterdam, The Netherlands.

Synthesis of TKI-ULS-lysozyme and Rhodamine-ULS-lysozyme

TKI was conjugated to LZM using ULS via platinum coordinative linkages. Since drug-ULS adducts react readily with methionine residues, the renal carrier LZM was enriched with surface-exposed methionine groups as described before (14). A freshly prepared solution of ULS-mono-nitrate-mono-chloride (20.5 mM, dissolved in DMF) was added in an equimolar amount to TKI (9.98 mg, 36.6 µmol) and reacted at 37°C overnight. TKI-ULS product was characterized by HPLC, Pt-NMR and LC-MS analyses.

TKI-LZM was synthesized by reacting TKI-ULS (3.5 µmol, 7 mg/ml) to methionine-LZM (0.7 µmol, 10 mg/ml in 0.02 M tricine/sodium nitrate buffer, pH 8.5). ESI-MS analysis confirmed the formation of TKI-LZM conjugate. The amount of conjugated drug was quantified after displacing TKI using sodium dithiocarbamate (Merck, Darmstadt, Germany). TKI-LZM was also characterized for its stability and drug-release properties. TKI-LZM (100 µg/ml) was incubated in PBS, 5 mM glutathione (GSH) in PBS, or serum at 37°C for 24h. Then, samples were immediately processed for HPLC analysis as described below.

To synthesize Rhodamine-ULS-LZM, Rhodamine-ULS (2.1 µmol, in DMF) was reacted to methionine-LZM (0.7 µmol) and purified according to the same protocol as described for TKI-LZM. The eventually provided red-colored conjugate was characterized by ESI-MS.

HPLC analysis of TKI

TKI was analysed by reverse-phase HPLC on a Waters system (Waters, Milford, MA, USA). Separations were achieved with a C18 reversed-phase SunFire™ column (Waters). TKI was monitored at 320 nm after eluting with the mobile phase (water-acetonitrile-trifluoroacetic acid, 91:9:0.1, v/v/v; pH 2.0). TKI eluted at the retention time of 5.8 min. The peak heights were measured to quantify the drug concentrations. To determine TKI in serum and urine samples, 100 µl of the sample was extracted with 3 ml of diethylether twice. After evaporating the organic layer, the residue was reconstituted in 100 µl of mobile phase and 25 µl of it was injected into HPLC. To estimate TKI in tissue homogenates, 100 µl of the sample was treated with 200 µl of methanol, vortex and centrifuged to precipitate proteins. 50 µl of the clear supernatant was injected into HPLC.
Cell Experiments

Effects of TKI and TKI-LZM in renal tubular cells

NRK-52E (normal rat kidney) tubular epithelial cells were kindly provided by Prof. Russel, University of Nijmegen, The Netherlands. Cells were cultured in DMEM medium (BioWhittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS, BioWhittaker), 4 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 ng/ml). Human kidney tubular cells (HK-2) were obtained from ATCC (Manassas, VA) and grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 ng/ml). To examine the effects of TKI and TKI-LZM, NRK-52E or HK-2 cells were treated with different compounds as described in the figure legends and processed for the mRNA determination by quantitative real-time RT-PCR as described below.

Uptake study of Rhodamine-ULS-LZM in NRK-52E and HK-2 cells

Since some renal tubular cell lines may lose their property to internalize proteins, we compared the protein uptake property of NRK-52E and HK-2 cells. In a subconfluent layer, cells were incubated with Rhodamine-ULS-LZM (100 µg/ml per well) for 2 h at 37°C. To compete for receptor-mediated endocytosis, cells were preincubated for 15 min with 100 fold excess of LZM. Cells were washed with cold PBS 3 times, dried and examined under the fluorescent microscope to observe uptake of the conjugate.

Animal Experiments

Normal male Wistar rats (220-240 g) were obtained from Harlan (Zeist, The Netherlands). All experimental protocols for animal studies were approved by the Animal Ethics Committee of the University of Groningen.

Pharmacokinetic studies with TKI-ULS-LZM and Rhodamine-ULS-LZM

An intravenous dose of the TKI-LZM conjugate (20 mg/kg dissolved in 5% glucose) was administered. At indicated time points, animals were sacrificed and blood samples were collected by heart puncture and organs were isolated after gently flushing with saline through abdominal aorta. Urine samples were collected using metabolic cages. Kidneys were weighed, homogenized (1:3 w/v, PBS) and then stored at -80°C. Part of the organs was processed for immunohistochemical analysis. To measure drug levels, samples were treated with 0.5 M potassium thiocyanate (serum, kidney homogenates) or 0.1 M sodium dithiocarbamate (urine) at 80°C for 24h to release TKI from linker and then measured by HPLC as explained above. Localization of TKI-LZM in the kidneys was detected by anti-LZM immunostaining on cryostat sections as described earlier (14).

To investigate the cellular handling of drug-ULS-LZM lysozyme in the kidneys, rats were injected intravenously with Rhodamine-ULS-LZM conjugate (20 mg/kg dissolved in
5% glucose) and sacrificed at 1h or 24h after administration. Localization of the conjugate was determined by fluorescence microscopy at cryosections.

**Effect of TKI-LZM in rats with unilateral ureteral obstruction**

The efficacy of TKI-LZM was studied in the Unilateral Ureteral Obstruction (UUO) model which is characterized by a significant induction of TGF-β production during the process of renal fibrosis. Animals were divided into 4 groups: normal rats (n=4) and UUO animals treated with either vehicle (5% glucose, n=5), TKI-LZM (25 mg/kg equivalent to 630 µg/kg TKI, n=5) or free, unconjugated TKI (630 µg/kg, n=5). TKI was dissolved in 20% hydroxylpropyl-β-cyclodextrin in water with 5% DMSO, whereas TKI-LZM was dissolved in 5% glucose. To allow unhindered uptake of the products in the kidneys, rats were injected intravenously with either of these compounds 2 h before the ureteral obstruction. Left kidneys and ureter were exposed via a flank-incision under anesthesia (2% isoflurane in 2:1 O₂/N₂O, 1 L.min⁻¹), after which the ureter was ligated at 2 sites with 4-0 silk near the hilum. After 3 days, animals were sacrificed and kidneys were gently flushed and harvested. Kidney cortex pieces were snap-frozen to isolate RNA. Kidney pieces were fixed in 4% formalin solution in PBS to make paraffin-embedded sections. Immunohistochemical stainings for the profibrotic marker alpha-smooth muscle actin (α-SMA) and monocytes/macrophages (ED-1) were performed on paraffin-embedded sections.

**Immunohistochemistry**

Commercially available antibodies for α-SMA (clone 1A4, Sigma, MO, USA) and ED-1 (Serotec, Oxford, UK) were used. A robotic DAKO Autostainer (DAKO Corporation, Carpinteria, California) was used for immunohistochemistry by the standard procedure as described earlier in detail (15). The extent of interstitial α-SMA expression and interstitial macrophage influx were determined in 30 fields per section by computerized morphometry as described previously (15). All morphometric measurements were performed by an observer in a blinded manner.

**Determination of mRNA expression**

Gene expression levels for the following genes were measured by real-time quantitative PCR using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers for the rat species were obtained from Sigma-Genosys (Haverhill, UK). The rat primers for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), monocyte chemoattractant protein-1 (MCP-1), procollagen-Ια1 and tissue inhibitor of metalloproteinase-1 (TIMP-1) have been reported earlier (14) whereas primers for α-SMA were 5'-GACACCAGGGAGTGATGGTT-3' and 5'-GTTAGCAAGGTCGGATGTC-3'. The TaqMan primers for Human species were obtained from Applied Biosystems (Assay–On–Demand). The cDNA amplification was
performed and the threshold cycle number (Ct) was calculated for each gene and then relative gene expressions were calculated after normalization to the expression of GAPDH.

**Statistical analysis**

The statistical analyses were performed using Student’s t-test with \( p < 0.05 \) as the minimal level of significance. Results are presented as mean ± SEM. Pharmacokinetic analysis of the serum TKI concentrations was performed using the Multifit program (Department of Pharmacokinetics and Drug Delivery, University of Groningen, The Netherlands).

**Results**

**Synthesis and characterization of TKI-ULS-LZM**

The synthesis scheme of TKI-LZM is shown in Fig. 1. TKI was efficiently coupled with ULS, yielded 77.3% of the TKI-ULS product. The analyses by HPLC, Pt-NMR and electrospray mass spectrometry (ESI-MS) confirmed a 1:1 coupling ratio of TKI and ULS. Subsequently, TKI-LZM conjugate was synthesized and characterized by ESI-MS of the whole conjugate and by HPLC analysis of the coupled drug. On average, 1-2 TKI molecules were attached per molecule of protein.

![Synthesis scheme of TKI-ULS-LZM](image)

**Figure 1** Synthesis scheme of TKI-ULS-lysozyme conjugates. LZM, lysozyme; ULS, Universal Linkage System.
We found that the conjugate remained stable in PBS and serum since no drug was released after 24h at 37°C. In contrast, 4% of the coupled drug was released upon incubation with GSH, a likely intracellular ligand exchange molecule for platinum.

**Cell Experiments**

**Effect of TKI in NRK-52E cells**

To investigate fibrotic events in renal tubular cells (NRK-52E), we treated the cells with natural activators such as albumin (BSA, 30 mg/ml) or TGF-β1 (10 ng/ml). Albumin stimulated the gene expression of the inflammation marker MCP-1 by 35-fold, which in turn could be inhibited by 82% with TKI (10 µM) (Fig. 2A).

![Graph](image)

**Figure 2.** Effects of TKI on albumin or TGF-β1-induced gene expressions of MCP-1 (A), procollagen-Iα1 (B), TIMP-1 (C) and α-SMA (D) in NRK-52E renal tubular cells. Cells were grown to 80% confluency in 12-well plates and then deprived from serum for 24h. Thereafter, the cells were preincubated for 2 h with TKI (10 µM) and then activated with either BSA (30 mg/ml) or TGF-β1 (10 ng/ml) for 24h. mRNA expressions were determined by quantitative real-time RT-PCR. Data represent the mean ± SEM for at least 3 different experiments. Differences versus control are presented as †p<0.05, ††p<0.01 and †††p<0.001. Differences versus other groups are *p<0.05, **p<0.01, ***p<0.001.
Furthermore, albumin induced fibrosis markers such as TIMP-1 and \( \alpha \)-SMA significantly, while procollagen–I\( \alpha \)1 was not elevated significantly (Fig. 2B-D). Treatment with TKI reduced the expression of these genes to levels below basal expression, as was also observed in non-activated cells that were treated with the drug. Similarly, TKI inhibited TGF-\( \beta \)1–induced gene expressions of procollagen–I\( \alpha \)1 and TIMP–1 significantly (Fig. 2B-D). MCP-1 expression was not elevated by TGF-\( \beta \)1 treatment.

**Renal uptake study of Rhodamine-ULS-LZM**

Intracellular delivery of TKI by TKI-LZM is dependent on receptor-mediated endocytosis from the medium. We evaluated cellular uptake of drug-LZM conjugates in NRK-52E and HK-2 cells using Rhodamine-ULS-LZM. We found that proximal tubular cell of human origin (HK-2 cells) internalized the conjugate, while NRK-52E cells showed no uptake (Fig. 3A). To demonstrate that receptor–mediated uptake of the conjugate was involved, we competed for receptor binding and internalization with a high amount of non-modified LZM, which clearly reduced the uptake of the fluorescent protein.

**Effect of TKI and TKI-LZM in HK-2 cells**

Next, we evaluated the inhibitory effect of TKI-LZM in HK-2 cells. After incubating the cells with TGF-\( \beta \)1 for 24h, gene expression of procollagen–I\( \alpha \)1 was 10-fold increased which was reduced over 90% after treatment with TKI-LZM conjugate, comparable to the inhibition produced by free TKI (10 \( \mu \)M). At lower concentrations, the conjugate inhibited procollagen–I\( \alpha \)1 expression to a lesser extent whereas the equivalent amount of free drug still reduced it by 75% (Fig. 3B). Treatment with the LZM carrier alone did not affect profibrotic signaling. From these results, we concluded that the conjugate was capable to deliver pharmacologically active drug.
fold induction (procollagen Iα1)

Figure 3. (A) Fluorescent photomicrographs (a, c, d, f) showing lysosomal uptake in HK-2 cells and NRK-52E cells after incubating with Rhodamine-ULS-lysozyme (100 µg/ml) at 37°C for 2 h. For competition studies, lysozyme (10 mg/ml) was added 15 min before adding Rhodamine-ULS-lysozyme. Light photomicrographs (b, e) demonstrate the confluency of the cells in culture plate. (B) Effects of TKI and TKI-lysozyme on procollagen-Iα1 gene expression in HK-2 cells. Cells were grown to 80% confluency and then deprived from serum for 24 h. TKI-lysozyme (8 or 80 µg/ml are equivalent to 1 or 10 µM TKI, respectively) or methionine-modified lysozyme (LZM, 8 or 80 µg/ml) was added at the time of serum deprivation whereas TKI (1 or 10 µM) was added 1 h before adding TGF-β1 to the cells. mRNA expressions were determined by quantitative real-time RT-PCR. Data represent the mean ± SEM for at least 3 experiments. Differences versus control is presented as †††p<0.001. Differences versus other groups are **p<0.01 and ***p<0.001.

Animal Experiments

Pharmacokinetics of the TKI-LZM conjugate

We optimized the sampling protocol for the pharmacokinetic studies with TKI-LZM on the basis of our previous studies with drug-LZM conjugates (14). The chosen time points allowed reliable estimation of the pharmacokinetic parameters by curve-fitting in a multi-compartment model. The serum-disappearance curve of TKI-LZM followed a two-compartment pharmacokinetic model (Fig. 4A). The pharmacokinetic data obtained from the serum-disappearance curve were as follows: distribution half-life (t1/2α), 0.14 ± 0.03 h; elimination (t1/2β) half-life, 22.3 ± 18.7 h; initial volume of distribution (V1), 5.2 ± 0.8 ml; initial serum clearance (CLinit), 21.6 ± 2.8 ml/h. Only carrier-bound TKI was detected in the serum, while free drug was absent. Furthermore, similar to other drug-LZM conjugates,
TKI-LZM accumulated efficiently in the kidneys within 1h following the intravenous injection (Fig. 4B). The accumulation of the conjugate in proximal tubular cells was confirmed by immunohistochemical staining for LZM on kidney sections (Fig. 4C). Urine levels of TKI (Fig. 4D) indicated a prolonged and continuous excretion of the drug which corresponded to the continuous release profile in the kidneys.

**Figure 4.** Serum (A), renal (B) and urine (D) levels of TKI-lysozyme. Symbols represent the levels of TKI as % of injected dose at each time point. The continuous line represents the pharmacokinetic data-fit curve (two-compartment model). Panel (C) shows the localization of the conjugate in tubular cells at t=1h by anti-LZM immunohistochemical staining. Magnification, 200x.

The renal drug disappearance curve of the previous figure demonstrated that TKI-LZM conjugate resided in the kidneys for several days and released slowly. Since we were
interested in the fate of the drug-ULS part of the conjugates after their uptake by tubular cells, we injected Rhodamine-ULS-LZM as a model drug-LZM conjugate. We found that Rhodamine-ULS-LZM was accumulated in the tubular cells rapidly within 1h after intravenous injection (Fig. 5A and B) which was well correlated with anti-LZM staining. While the carrier protein was hardly detectable at t=24h, Rhodamine was still detectable in a vesicular pattern (Fig. 5C and D). From these results, we concluded that the conjugate resided primarily in the lysosomal compartment after degradation of the protein backbone, from which it released the drug slowly to the surrounding environment.

Figure 5. Localization of Rhodamine-ULS-lysozyme conjugate in the kidney. Light photomicrograph showing anti-LZM immunohistochemical staining on kidney sections t=1h (A) and t=24h (C) after administration of a single dose of Rhodamine-ULS-lysozyme (20 mg/kg) at magnification 100×. Panel (B) and (D) show the fluorescence photomicrographs of rhodamine fluorescence in kidney sections at t=1h and t=24h, respectively at magnification, 100×. “g” indicate the localization of glomeruli in the non-fluorescent space in B and D. Note the pattern of the localization of anti-lysozyme staining and rhodamine fluorescence. Lysozyme was degraded after 24h while rhodamine was still present in the tubules.

Effect of TKI and TKI-LZM in UUO rats

Proof-of-concept for the efficacy of TKI-LZM was investigated in the UUO model for renal fibrosis. Ureteral ligation causes the induction of inflammatory and fibrotic processes, which rapidly progress to end-stage renal disease. Since the renal accumulation of TKI-LZM and prolonged release of TKI will provide continuous local drug levels, we administered only a single dose of the conjugate 2 h before ureteral obstruction. Three days
later, ureter obstructed animals had significantly increased expressions of the inflammation marker MCP-1 and the fibrosis marker procollagen-IIα1 in comparison to normal rats (Fig. 6). Moreover, treatment with TKI-LZM substantially reduced the MCP-1 gene expression but did not inhibit procollagen-IIα1 expression. In contrast, a single dose of free TKI did not lower the expression either MCP-1 or procollagen-IIα1.

In line with the lowered production of MCP-1, which is a chemoattractant for macrophages, we also detected significant reduced levels of infiltrated macrophages upon TKI-LZM treatment (ED-1 immunostaining, Fig 7). However, in contrast to the gene expression study, we also found a reduction of ED-1 immunostaining with free TKI. Moreover, the expression of the fibrosis marker α-SMA was also significantly decreased by TKI-LZM or TKI treatments, which indicates potential antifibrotic activity of both TKI and TKI-LZM (Fig. 8).

**Figure 6.** Gene expression levels of MCP-1 (A) and procollagen-IIα1 (B) in renal cortex of normal rats and vehicle–treated, TKI-LZM–treated and TKI–treated rats after Unilateral Ureteral Obstruction (UUO). Each bar represents the mean ± SEM for n=5, except for normal rats (n=4). Differences between normal rats and vehicle-treated UUO rats are presented as ††p<0.01 and †††p<0.001. Differences versus other groups are *p < 0.05.

**Discussion**

In the present study, we demonstrate the potential effects of the ALK5 inhibitor TKI in cultured renal tubular cells and the unilateral ureteral obstruction (UUO) rat model, which stresses the importance of the TGF-β pathway in fibrotic signaling in renal interstitial fibrosis. We furthermore demonstrate that renal-specific inhibition of this pathway is affecting fibrotic events. For local deliver of TKI to proximal tubular cells in the kidney, we have developed a novel renal-specific conjugate. Our results indicate that inhibition of
fibrotic and inflammatory kinase pathways can be an interesting strategy to treat tubulointerstitial fibrosis.

TKI was conjugated to the low molecular weight protein LZM via the coordinative linker ULS. Our in vitro results as well as the in vivo pharmacokinetic data demonstrated that TKI-LZM remained stable in serum and accumulated in the targeted tubular cells in the kidney. Since thiols can compete for the drug-platinum linkage, GSH is a likely candidate to act as an intracellular release-triggering ligand (16), which is supported by our studies. Thus, we propose that TKI-LZM forms a depot in the lysosomes of tubular cells, from

**Figure 7.** Representative photomicrographs of the immunohistochemical staining for ED-1 positive cells (infiltrated macrophages) in normal rats (A) and 3 days after Unilateral Ureteral Obstruction (UUO) (B-D) rats in vehicle–treated (B), TKI-LZM–treated (C) and TKI–treated animals (D). Arrows indicate the immunolocalization of ED-1 in tubulointerstitial area. Magnification, 200×. Panel (E) shows the number of ED-1 positive cells per interstitial field. Each bar represents the mean ± SEM for n=5, except for normal rats (n=4). Differences between normal rats and vehicle-treated UUO rats are presented as †††p<0.001. Differences versus other groups are *p < 0.05.
which TKI is released during a period of several days. It is very unlikely that a single dose of free TKI will provide similar renal drug levels during the investigated time period of several days.

![Figure 8.](image)

**Figure 8.** Representative photomicrographs of the immunohistochemical staining of α-SMA in normal rats (A) and 3 days after Unilateral Ureteral Obstruction (UUO) (B-D) rats in vehicle-treated (B), TKI-LZM-treated (C) and TKI-treated animals (D). Magnification, 200×. Panel (E) shows the semi-quantification of α-SMA positive area per interstitial field. Each bar represents the mean ± SEM for n=5, except for normal rats (n=4). Differences between normal rats and vehicle-treated UUO rats are presented as †††p<0.001. Differences versus other groups are *p < 0.05.

Activation of tubular cells is a crucial event during renal injury which can lead to chemoattraction, inflammation, and eventually fibrosis. Tubular cell activation can be caused by many factors such as filtered proteins, cytokines and oxidative stress during renal injury which initiates multiple events in the cells. In the present study, TKI inhibited
proinflammatory and profibrotic events induced by either albumin or TGF-β1 in cultured rat kidney tubular cells. The inhibited genes are highly relevant since they are upregulated during renal fibrosis as demonstrated in multiple studies (10,17,18). In addition, TKI-LZM demonstrated its activity in human kidney tubular cells, which are capable of internalizing proteins, by inhibiting the TGF-β1–induced expression of procollagen-Iα1. Such an effect can only take place after internalization and processing of the conjugate into active drug, since less than 2% of the coupled drug was released from the conjugate upon prolonged incubation at 37°C in cell culture medium (data not shown).

To evaluate the efficacy of TKI-LZM conjugate on renal fibrosis, we chose the UUO rat model since this model is well known to induce renal fibrosis and associated with abundant expression of TGF-β and its receptors (19,20). Vielhauer et al demonstrated that the expression of MCP-1 also increased continuously in time following ureteral obstruction (21), which is confirmed in the present study. Moreover, inhibition of MCP-1 using gene therapy or MCP-1 antagonists has been proven to be beneficial for the treatment of renal fibrosis in UUO (22,23). We now demonstrate local inhibition of MCP-1 in the kidneys with a single dose of TKI-LZM, and a substantial reduction of renal macrophage influx. In addition, the effectiveness of the conjugate on renal fibrosis was evidenced by the reduced interstitial α-SMA protein expression. Unexpectedly, however, a single intravenous dose of free TKI also reduced the influx of macrophages and α-SMA expression. Importantly, free drug distributed throughout the body and the observed effects are not likely due to a local renal action. Moreover, free TKI inhibited macrophage infiltration without affecting renal MCP-1 expression, underlining the different pharmacological profiles of TKI-LZM and free drug. A possible explanation is that free TKI probably exerted anti-inflammatory activity in immune cells, thereby also affecting the UUO-inflicted renal inflammation and fibrosis. Since TKI is a very potent compound, as evidenced in our in vitro studies, the relatively low dose given prior to UUO may have been sufficient to inhibit early inflammatory and pre-fibrotic events. Yet, the pharmacokinetic data of TKI-LZM clearly illustrate its renal accumulation and prolonged drug release, making it more likely that the conjugate exerted its activity locally in the kidney.

In conclusion, the present study shows that targeting of a TGF-β kinase inhibitor to tubular cells is in potential a promising approach for the treatment of renal fibrosis. Cell-specific delivery of such compounds can successfully be accomplished using this approach that affords a sustained release of drugs for several days. Moreover, prolonged inhibition of TGF-β action locally may serve as a novel therapeutic intervention for chronic diseases such as tubulointerstitial fibrosis.

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