Renal–Specific Delivery of ROCK Inhibitor Y27632 Inhibits Ischemia/Reperfusion–Induced Acute Renal Injury

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Submitted for publication
Abstract

Since the Rho kinase (ROCK) pathway plays an important role in the cytoskeletal reorganization and transdifferentiation of epithelial cells as well as in the infiltration of inflammatory cells, we hypothesized that ROCK blockade within the kidney might be beneficial for the treatment of renal fibrosis. We therefore coupled the well-known ROCK inhibitor Y27632 to the renal-specific carrier lysozyme (LZM) and evaluated the pharmacological properties of the resulting conjugate in an ischemia-reperfusion induced renal injury rat model. Y27632-LZM conjugate was prepared by reacting the drug with the platinum-based Universal Linkage System (ULS™) and subsequent reaction of the linker with reactive groups in LZM. Y27632-LZM conjugate was stable in serum but released drug upon incubation with glutathione in vitro. After intravenous injection in healthy rats, the conjugate was rapidly accumulated in the kidneys as determined by HPLC analysis of renal drug levels. Immunostaining for the localization of the carrier in kidneys demonstrated its accumulation in renal tubular cells. Next, we investigated the effects of Y27632-LZM in the unilateral I/R rat model. Rats were daily treated with either vehicle (5% glucose), Y27632-LZM conjugate or Y27632 for 4 days. We found that I/R-induced gene expression of inflammatory markers (MCP-1) and fibrotic markers (TGF-β1 and α-SMA) were significantly inhibited by Y27632-LZM after 4 days. In addition, Y27632-LZM also inhibited macrophage infiltration (ED-1 positive cells) and α-SMA protein expression as detected by immunostaining. In contrast, free Y27632 did not affect the gene expression and the protein expression of inflammatory and fibrotic factors. In conclusion, the renal-specific inhibition of ROCK with Y27632-LZM can be a promising approach to inhibit renal injury and fibrotic processes.
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Introduction

Renal ischemia–reperfusion (I/R) injury is the main cause of acute renal failure after major renal surgery, trauma and transplantation. During I/R injury, proximal tubular cells lose polarity and detach from the tubules. This tubular cell detachment is due to the cytoskeletal reorganization which is mainly regulated by RhoGTPases (1,2). RhoGTPases belong to the Ras superfamily of GTP–binding proteins and act by stimulating downstream Rho effectors such as Rho-associated coiled-coil forming protein kinase (ROCK). In addition to cytoskeletal regulation, RhoGTPases are involved in the infiltration of inflammatory cells (3,4). During I/R injury, infiltration of inflammatory cells into the tubulointerstitial space is an important process as these infiltrated cells potentiate inflammation and renal damage by generating several cytokines and growth factors (5). Moreover, activation of RhoGTPases leads to the transdifferentiation of renal tubular cells to fibroblasts which eventually lead to fibrosis (6). So, the RhoGTPase–mediated ROCK pathway plays a crucial role in the instigation of inflammation and fibrosis caused by I/R injury.

We hypothesized that blockade of Rho-ROCK pathway locally within kidneys may be an interesting approach to prevent I/R injury. Moreover, renal–specific delivery of signaling inhibitors is an attractive approach to investigate the renal–specific effects of these drugs, as it can avoid interactions with non-target cells in other organs and thereby decrease side-effects. Furthermore, drug delivery can enhance local drugs levels at the target site, thereby improving therapeutic efficacy. Several studies have shown the efficacy of a well-known ROCK inhibitor Y27632 in ischemia-reperfusion injury in different organs (7,8). Also, Y27632 exhibited beneficial effects in the unilateral ureteral obstruction model of renal fibrosis in mice (9). However, administration of ROCK inhibitors can also lead to systemic side-effects since the pathway is involved in the contraction of vascular smooth muscle cells (10,11) and in line with this, inhibition of ROCK with Y27632 at a dose of 1 mg/kg produced a 50% reduction in mean arterial pressure in rats (12). Renal-specific delivery of Y27632 may prevent such systemic side effects.

In earlier studies, we have shown that renal–specific drug delivery can be achieved using the low molecular weight protein lysozyme (LZM) as drug carrier (13). LZM filters through the glomerulus and is reabsorbed in the proximal tubule via the megalin receptor, thus accumulating in the lysosomal compartment of renal tubular cells (14,15). We have recently developed a new strategy for the coupling of drugs to carrier systems, the so-called Universal Linkage System (ULS™) which creates a platinum coordination bond with the drug. Using the p38 MAPkinase inhibitor SB202190, we have demonstrated that drug-ULS-LZM conjugates remain stable in serum but release drug slowly within the kidneys (16). In the present study, we considered the ROCK inhibitor Y27632 for renal targeting and conjugated Y27632 to LZM using the ULS linkage approach. We characterized the Y27632-LZM conjugate for its pharmacokinetics in healthy rats and evaluated the
therapeutic efficacy of ROCK inhibition in the unilateral ischemia-reperfusion (I/R) injury model in rats, four days after I/R damage.

Materials and Methods

Synthesis and Characterization of Y27632-ULS-LZM Conjugate

Y27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate] was purchased from Tocris, UK. Lysozyme was obtained from Sigma-Aldrich, St. Louis, MO. Cis-[Pt(ethylenediamine)nitrato-chloride] (ULS) was freshly prepared from cis-[Pt(ethylenediamine)dichloride as described elsewhere (17). Y27632 (2.95 µmol, 10 mg/ml in water) was basified with 1 M NaOH to pH 8 and reacted with cisULS (4.43 µmol) at 50°C overnight. Consumption of the starting drug and formation of the products was followed by HPLC and LC-MS. The reaction mixture was evaporated to dryness under reduced pressure, affording a pale yellow solid (yield 50 %) that consisted of the desired 1:1 reaction product. Y27632-ULS was subsequently reacted with lysozyme according to a similar protocol as described elsewhere (16). In brief, lysozyme (0.7 µmol) equipped with surface-exposed methionine groups by the Boc-L-methionine hydroxysuccinimide ester reagent, was dissolved in 0.02 M tricine/sodium nitrate buffer, pH 8.5. After addition of Y27632-ULS (3.5 µmol), the mixture was reacted at 37°C for 24 h. The product was purified by dialysis against water for 48 h, filtered, lyophilized and stored at -20°C. MS analysis confirmed the formation of Y27632-LZM conjugate. The amount of conjugated drug was quantified after competitive displacement of the drug from the conjugate by overnight incubation with 0.5 M potassium thiocyanate in PBS at 80°C, by HPLC analysis as described below. The absence of free drug in the preparation was investigated by HPLC analysis of freshly prepared dilutions of the conjugate in PBS.

Y27632-LZM was characterized for its stability and drug-release properties. The conjugate (100 µg/ml) was incubated in different media: 0.1 M PBS, 5 and 50 mM glutathione (reduced form) in PBS, and serum. After 24 h incubation at 37°C, 100 µl aliquots were taken and processed immediately for HPLC analysis of Y27632.

HPLC analysis of Y27632

Y27632 was analysed on a Waters liquid chromatograph (Waters, Milford, MA, USA) consisting of a 510 pump, an autoinjector 715, and a column oven set at 40°C (Waters, Milford, MA, USA) in combination with a Spectroflow 757 UV detector (Kratos, Kanagawa, Japan). Separations were performed on a C18 reversed-phase SunFire™ column (150 x 4.6 mm i.d., 5-µm particle size, Waters) preceded by a C18 guardPak precolumn (Waters), using a mobile phase of water-methanol-trifluoroacetic acid (86:14:0.1, v/v/v; pH 2.0) at a flow rate of 1 ml/min. The effluents were monitored at 270 nm. Y27632 eluted at a retention time of 5.7 min. Clear solutions were injected without pretreatment in the HPLC, while serum and kidney homogenates (200 µl) were extracted twice with 2 ml of diethyl
ether after adding 100 µl of 2 N NaOH. After freezing of the aqueous layer in liquid nitrogen, the organic layer was decanted and evaporated at 50°C. The residue was reconstituted in 200 µl of mobile phase, and 50 µl was injected into the HPLC. The peak heights were recorded and related to calibration curves in the corresponding biological matrices to quantify drug levels.

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

Pharmacokinetics of Y27632-LZM
Male Wistar rats (n=5) were injected intravenously with a single dose of the Y27632-LZM conjugate (20 mg/kg equivalent to 555 µg/kg of Y27632, dissolved in 5 % glucose). Injections were performed in the penile vein under inhalation anesthesia (2 % isoflurane in 2:1 O₂/N₂O, 1 L.min⁻¹). Animals were placed in metabolic cages for collection of urine, and sacrificed at 5, 30, 60, 120 and 360 min under anesthesia, n=1 per time point according to the protocol described earlier (16). Blood samples were collected by heart puncture and kidneys were isolated after gently flushing of the organs with saline through the abdominal aorta. Urine was collected from the urinary bladder and combined with the urine collected in the metabolic cages. Kidneys were weighed, homogenized (1:3 w/v, PBS) and then stored at −80°C. To determine Y27632 in serum or tissues, samples were incubated with 0.5 M potassium thiocyanate at 80°C for 24 h to release Y27632 from the linker followed by HPLC analysis as explained earlier. Anti-LZM immunostaining was performed on frozen kidney sections to detect the cellular localization of the conjugate in the kidneys as described below.

Effect of Y27632-LZM in unilateral renal Ischemia–Reperfusion (I/R) injury in rats
The pharmacological efficacy of Y27632-LZM was evaluated in the unilateral ischemia-reperfusion (I/R) rat model. Animals were divided into 4 groups: untreated normal rats (n=4), vehicle treated I/R rats (5% glucose, n=10), Y27632-LZM + I/R (20 mg/kg equiv. to 555 µg/kg Y27632, n=3), Y27632 + I/R (555 µg/kg, n=6). At 2 h prior to the ischemia procedure, rats were pre-injected with either of these compounds. Compounds were administered intravenously via the penis vein as described above. Animals were allowed to recover and placed back into the cages until the induction of renal ischemia. Animals were incised from the abdomen under anesthesia and the left renal artery and vein were clamped for 45 min to stop renal blood flow. Clamps were removed and reperfusion of the kidney was observed before closing of the wound. Rats were re-injected with the compounds at t=24, 48, and 72h after I/R. After 4 days, animals were sacrificed and blood samples were collected from the abdominal aorta. Kidneys were isolated after gentle perfusion with saline. Kidney pieces were snap-frozen in liquid nitrogen for mRNA
Determination of mRNA expression

Total RNA was isolated from renal cortex using Bio-Rad’s Aurum Total RNA Mini kit (Bio-Rad, Hercules, CA). RNA content was measured by a nanodrop UV-detector (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from similar amounts of RNA using the Superscript III first strand synthesis kit (Invitrogen, Carlsbad, CA). Gene expression levels for the following genes were measured by quantitative real-time RT-PCR (Applied Biosystems, Foster City, CA). The primers for rat species obtained from Sigma-Genosys (Haverhill, UK) were as follows: monocyte chemoattractant protein-1 (MCP-1; 5′-TCTCCACCCTAGCATGCAGGT-3′ and 5′-TTCCCTTATTGGGCAGCAC-3′, 255 bp), tissue inhibitor of metalloproteinase-1 (TIMP-1; 5′-GAGAGGCTGTGGATATGT-3′ and 5′-CAGCCACGACTATAGGTCT-3′, 334 bp), procollagen-Iα1 (5′-GAGCTGAGCCAGCAGATTGA-3′ and 5′-CCAGGTTGCAGCCTTGGTTA-3′, 145 bp), alpha smooth muscle actin (α-SMA; 5′-GACACCAGGGAGTGATGGTT-3′ and 5′-GTTAGCAAGGTCCAGATGTT-3′, 202 bp), TGF-β1 (5′-ATAACCCTGAGTGGCTGT and 5′-TGGGACTGATCCCATTGATT-3′, 153 bp) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5′-CGCTGGTGCTGATGTCGAC-3′ and 5′-CTGGGTCATGAGCCCTCC-3′, 179 bp).

SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used as a fluorescent probe for real-time RT-PCR. For each sample, 1 µl of cDNA was mixed with 0.4 µl of each gene-specific primer (50 µM), 0.8 µl DMSO, 8.4 µl water and 10 µl SYBR Green PCR Master Mix. The cDNA amplification was performed until 40 cycles followed by dissociation cycle. The final product was examined to provide a single peak in the dissociation curve. Finally, the threshold cycle number (Ct) was calculated for each gene and relative gene expressions were calculated after normalizing for the expression of the control gene GAPDH.

Histology and Immunohistochemistry

Cryostat kidney sections (4-µm thick) were cut with a Leica cryostat (Wetzlar, Germany). Sections were incubated with rabbit anti-LZM polyclonal antibody to detect the localization of Y27632-LZM, as described previously (16).

Anti-α-SMA and ED-1 immunohistochemical staining were performed on 4-µm thick paraffin-embedded sections. Sections were deparaffinized in xylene and rehydrated in alcohol and distilled water. Heat-induced antigen retrieval was achieved by incubation in 0.1 M Tris/HCl buffer (pH 9.0) overnight at 80°C. For immunohistochemistry, a DAKO Autostainer was used (DAKO Corp, Carpinteria, CA, USA). Sections were washed in PBS, treated with endogenous peroxidase blocking reagent containing 0.03% H2O2 / sodium azide for 5 min. Slides were then incubated with primary antibodies against α-SMA (clone
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1A4, Sigma, MO, USA) or the rat macrophage marker ED-1 (Serotec, Oxford, UK), diluted in PBS containing 1% bovine serum albumin (BSA) for 60 min at room temperature. Binding was detected by sequential incubation with peroxidase-labelled rabbit anti-mouse and goat anti-rabbit polyclonal antibodies (DAKO, Glostrup, Denmark), respectively, in the presence of 1% normal rat serum for 30 min. The peroxidase activity was visualised using 3,3’-diaminobenzidine tetrahydrochloride (DAB+, DAKO) for 10 min, and sections were mounted with Kaiser’s glycerin gelatin. Periodic acid Schiff’s base (PAS) staining was performed according to standard protocols to observe renal morphology. The extent of interstitial α-SMA expression and interstitial macrophage influx were determined by computerized morphometry. For that purpose, 30 interstitial rectangular fields per section were screened using a light microscope, equipped with a camera device connected to a computer system. Glomeruli and vascular areas were manually excluded. The amount of brown precipitate was measured and represented as either a percentage of the total selected area (α-SMA) or as estimated number of macrophages in the selected area. Averages were calculated from 30 fields per section, representing ultimate interstitial α-SMA and macrophage scores. All morphometry measurements were performed by a blinded observer.

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 ± standard error mean. Pharmacokinetic analysis of the serum Y27632 concentrations was performed using the Multifit program (Department of Pharmacokinetics and Drug Delivery, University of Groningen, The Netherlands).

Results

Synthesis and Characterization of Y27632-ULS-LZM

As illustrated in Fig. 1, Y27632 was first conjugated to ULS and then coupled to the carrier lysozyme. The final product was found to have a 1:1 drug to protein ratio when characterized for the amount of coupled drug by HPLC or by ESI-MS to analyze the intact conjugate. Prior studies with other drug-ULS-LZM conjugates (16) indicated that this type of conjugate is highly stable in buffers and serum, but releases the conjugated drug slowly upon ligand exchange with GSH. Stability studies with Y27632-LZM were in good agreement with these data. No release of the coupled drug was observed upon incubation in PBS or serum at 37°C. In contrast, addition of GSH to the medium afforded competitive release of drug that was concentration-dependent. Approximately 5% and 9% of the bound drug was released in 5 and 50 mM GSH, respectively, in 24 h. From these data, we postulated that drug release rates will be mainly governed by the applied ULS linkage, rather than by the coupled drug.
Figure 1. Synthesis of Y27632-ULS-lysozyme. ULS, Universal Linkage System; LZM, lysozyme.

**Pharmacokinetics of the Y27632-LZM conjugate**

In addition to *in vitro* stability studies, we also investigated the stability of Y27632-LZM after administration to rats. The serum-disappearance curve of Y27632-LZM, which followed a two-compartment pharmacokinetic model, is shown in Fig. 2A. Only carrier-bound Y27632 was detected in the serum, while free drug was absent. Furthermore, in accordance with other drug-LZM conjugates, Y27632-LZM accumulated efficiently in the kidneys within 30 min following the intravenous injection (Fig. 2B). The accumulation of the conjugate in kidneys was confirmed by anti-LZM immunohistochemical staining on kidney sections (Fig. 2C). A clear and selective accumulation of LZM in renal tubular cells was found.

**Effect of Y27632-LZM in the I/R Injury model in rats**

The antifibrotic efficacy of Y27632-LZM was evaluated in the unilateral I/R model. This animal model is characterized by an early inflammatory phase which finally leads to fibrosis. Typically, I/R treated rats display a mild stage of fibrosis after 4 days, which was confirmed in the present study. In comparison to normal rats, vehicle-treated I/R animals had a significant increase in the gene expression of the inflammation marker MCP-1 and of fibrosis markers α-SMA, TGF-β1, procollagen-Iα1 and TIMP-1 (Fig. 3). Moreover, immunohistochemical evaluation of the kidneys showed a significant increase of the number of macrophages in the tubulointerstitial space and the tubular lumen of I/R rats (Fig. 4). As illustrated by the PAS staining, tubular cells were lost from the renal tubules in I/R rats and abundant macrophage infiltration in the lumen was seen (Fig. 4B). These
results are in good agreement with the enhanced expression of MCP-1 since MCP-1 is an important chemotactic cytokine to attract monocytes and macrophages.

![Figure 2. (A) Serum levels and (B) renal levels of Y27632-LZM. Symbols represent the % dose of Y27632 at each time point; the continuous line represents the pharmacokinetic data-fit curve (two-compartment model). Panel (C) represents the localization of the conjugate in tubular cells at 30 min by anti-LZM immunohistochemical staining. Magnification, 200×.](image)

In addition, to determine the induction of fibrogenesis in this model we detected the expression of the fibroblast marker α-SMA in kidneys. In normal rats, only blood vessels were positive for α-SMA (Fig. 5A) whereas after I/R injury, there was a significant increase in α-SMA expression in the tubulointerstitial space of the renal cortex (Fig. 5B). These data confirm that tubulointerstitial fibrosis was initiated after 4 days of I/R injury. Finally, we observed changes in renal morphology by PAS staining (Fig. 4, 5, pink color) which clearly showed the dilation of tubules with loss of tubular brush border as well as tubular cells itself after I/R injury. Treatment with Y27632-LZM substantially improved almost all parameters, while free Y27632 showed no beneficial effects. The elevated gene expression of MCP-1, α-SMA and TGF-β1 was blunted by Y27632-LZM, whereas gene expression of procollagen-Iα1 and TIMP-1 was not reduced significantly (Fig 3). Furthermore, treatment with Y27632-LZM significantly reduced the influx of macrophages both in the tubular interstitium and lumen as indicated by ED-1 staining, which is in good agreement with the reduced renal expression of MCP-1 seen after administration of
Y27632-LZM. In contrast, free Y27632 did not reduce the expression of any of the studied genes and did not affect the number of ED-1 positive cells (Fig 4). In addition to the beneficial effect on inflammation, we found a significant reduction in α-SMA staining after Y27632-LZM treatment, whereas free drug did not show any effect on α-SMA expression (Fig 5). Finally but importantly, the conjugate treatment improved the renal morphology since there was less tubular dilation and more intact tubular cells. However, free drug hardly induced any improvement in morphology. Thus, drug delivery by means of Y27632-LZM considerably increased the renal activity of Y27632 against renal fibrosis.

**Figure 3.** Gene expression of MCP-1, TGF-β1, α-SMA and procollagen-Iα1 in renal cortex of normal rats (n=4) and vehicle-treated (n=10), Y27632-LZM-treated (n=3) and Y27632-treated (n=6) rats after unilateral ischemia-reperfusion injury. Data represents mean ± SEM. ††p<0.01 and †††p<0.001 represent the difference between normal rats and vehicle-treated I/R rats. Differences between other groups are indicated as *p < 0.05 and **p < 0.01.

**Discussion**

The Rho-ROCK system is an endogenous regulator of proliferation, migration and apoptosis of renal tubular cells (18). Moreover, the ROCK pathway participates in inflammatory and fibrotic processes by regulating infiltration of inflammatory cells and the transdifferentiation of epithelial tubular cells into fibroblasts (3,4,6). Since all of these processes are important aspects of fibrosis, ROCK inhibition is an attractive antifibrotic strategy. In the present study, we delivered the ROCK inhibitor Y27632 to the kidneys to
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confine its effects locally to the kidneys. Such a strategy will increase the cellular specificity of the drug for renal tubular cells, thereby avoiding other cells that respond to ROCK inhibition, like vascular smooth muscle cells. Activation of ROCK causes the contraction of vascular smooth muscles and inhibition of ROCK with Y27632 inhibits their contraction and decreases blood pressure in hypertensive rats (11,12,19).

![Figure 4](image)

**Figure 4.** Representative photomicrographs of the macrophage immunostaining (ED-1) in normal rats, n=4 (A) and 4 days after unilateral ischemia-reperfusion injury (B-D) rats. Vehicle−treated, n=10 (B); Y27632-LZM−treated, n=3 (C); Y27632−treated, n=6 (D) animals. Arrows indicate the immunolocalization of ED-1 positive cells (brown color) in tubulointerstitial area. PAS counterstaining depicts the renal morphology. Magnification, 200×. Panel (E) shows the number of ED-1 positive cells per interstitial field. †††p<0.001 represent the difference between normal rats and vehicle-treated I/R rats. Differences between other groups are indicated as **p < 0.01.
Coupling of Y27632 via ULS to LZM provided a serum-stable conjugate that accumulated rapidly into the kidneys. Furthermore, the pharmacokinetic profile of Y27632-LZM is quite similar to the earlier reported SB202190-ULS-LZM conjugate (16), both with respect to serum disappearance and the extent of renal accumulation (about 20% of the injected dose after 1-2 h). In the previous study, we demonstrated that SB202190-LZM provided continuous renal drug levels during a prolonged period of several days after its administration. Although we now examined the pharmacokinetics of Y27632-LZM only
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Until 6 h after its injection, it is fair to expect similar continuous drug release, in view of the similar release characteristics. Moreover, the in vitro release of Y27632 by glutathione suggests that drug will be released inside the target cells after the accumulation (16,20) where glutathione levels are relatively high. It is important to notice that Y27632 did not release from the conjugate in serum as determined by in vitro drug release study as well as by pharmacokinetic data in vivo. Thus, the renal delivery Y27632 can avoid systemic side-effects of the free drug such as the fall in blood pressure (12).

We investigated the effects of Y27632-LZM in I/R injury model, in which both inflammatory and fibrotic processes contribute to the renal pathology. In view of the continuous drug levels in the kidney after a single dose, we designed a protocol in which Y27632-LZM was administered once daily. Inhibition of MCP-1 gene expression and immune cells infiltration by Y27632-LZM indicated that ROCK pathway is an essential pathway in the regulation of inflammation during I/R injury. MCP-1 has shown to be a crucial gene during fibrogenesis (21,22). Moreover, local inhibition of ROCK with Y27632-LZM reduced the I/R-induced fibrotic genes (TGF-β1 and α-SMA) and the α-SMA expression which is a marker of fibroblast activation. This demonstrates the beneficial effects of Y27632-LZM on fibrotic signaling during I/R injury. Since the anti-inflammatory and anti-fibrotic effects were only observed after treatment with Y27632-LZM and not after administration of free drug, we inferred that these effects were due to the local inhibition of ROCK within tubular cells, rather than by systemic effects of the drug. Although the therapeutic efficacy of Y27632-LZM is obvious, explaining the mechanism of action is more difficult from the present data. The possible mechanism is that transformation of tubular cells into fibroblast is inhibited, combined with a reduced secretion of profibrotic and proinflammatory mediators, which lowered subsequent influx of immune cells. In addition to the beneficial effects on inflammatory and fibrotic signaling, the conjugate also improved the renal morphology significantly by preventing the loss of tubular cells. This can be explained by the studies showing that the blockade of ROCK within tubular cells inhibited the cytoskeletal reorganization induced by I/R injury which is the main cause for the loss of tubular cells (1,2). Local inhibition of ROCK within non-tubular cells in the kidney can not be excluded, since Y27632 might redistribute from the initial target cells after release from the carrier. Lastly, the absence of beneficial effects of free drug might be due to either rapid elimination of the drug and/or poor distribution to kidneys. Regardless of these considerations, however, the clear differences between renally delivered Y27632 and free drug clearly illustrate the improved efficacy of the novel compound.

In conclusion, the present study demonstrates that renal-specific delivery of ROCK inhibitor Y27632 can be successfully achieved using the Y27632-LZM conjugate. Blockade of ROCK pathway locally within kidneys with Y27632-LZM produced significant antifibrotic effects and can be a promising approach to treat renal injury.
**Acknowledgements**

Authors are grateful to Mrs. Annemieke M. van Loenen-Weemaes and Mrs. Catharina Reker-Smit from the Department of Pharmacokinetics and Drug Delivery and Mrs. Marian Bultuis and Mr. Mario van Dalen from the Department of Pathology for their technical assistance. Authors are also thankful to Mrs. Annie van Dam and Mrs. Margot Jeronimus-Stratingh from the Department of Analytical Biochemistry, University of Groningen, for their assistance in mass spectrometry analysis.

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