Chapter 9
Summary, Discussion and Future Perspectives
Summary and Discussion

Renal fibrosis is one of the major health problems in the world. It is characterized by deposition of extracellular matrix in kidneys which results into end-stage renal disease (ESRD) (1). In recent years, a tremendous increase in the incidence of ESRD world-wide has been reported. Diabetes and hypertension are found to be the main causes of ESRD. Moreover, other chronic renal diseases such as glomerulonephritis, cystic diseases and tubulointerstitial fibrosis also represent a large population of ESRD (2). Only available treatment for ESRD patients is either dialysis or renal transplantation which has increased the total public health burden enormously. In relation to this, an increase in the annual expenditure is expected from US$9 billion (1995) to US$28 billion by 2010 only in the USA (2,3). Therefore, there is an emergence to develop new therapies for the treatment of renal fibrosis.

Expanding research in this field has explored new pathways and mechanisms involved in the pathogenesis of renal fibrosis. Subsequently, new chemical entities modulating these pathways have been developed. However, most of these developed compounds possess unfavorable pharmacokinetics due to, among others, rapid body elimination or poor renal distribution. In addition, these compounds may exhibit several systemic side-effects by interfering with the normal physiological functions. As summarized in Chapter 1, the present thesis is focused on the development of a novel therapeutic system by which anti-fibrotic drugs can be delivered selectively to the kidneys. Renal-selective therapies aim to increase the therapeutic efficacy of drugs by increasing the amount of drugs locally within kidneys (4). In addition, renal targeting can avoid the interaction of the drug with other organs which can prevent the extra-renal side-effects of drugs.

To achieve renal selective delivery of drugs, we used low molecular weight protein (LMWP) lysozyme (LZM) as a drug carrier. In the kidneys, LZM filters freely through the glomerulus and is absorbed by proximal tubular cells via megalin receptor-mediated endocytosis. This has been demonstrated in our laboratory (5,6). Kok et al. have demonstrated that an angiotensin converting enzyme (ACE) inhibitor captopril could be targeted to kidneys using LZM. Moreover, renal-targeted captopril-LZM inhibited renal ACE activity effectively and selectively in comparison to non-targeted captopril after intravenous injection (7). In Chapter 2 and 3, we explored the possibility to administer captopril-LZM conjugate via the subcutaneous route. Such an administration route is more convenient to both patients and animals, and may prolong the action of the conjugate. We demonstrated that after subcutaneous administration, radiolabeled LZM accumulated gradually into the kidneys during 24 h and showed a better renal uptake profile in renal diseased (proteinuria) state in comparison to intravenous administration. Moreover, we found that subcutaneously administered captopril-LZM was accumulated slowly into kidneys and inhibited renal ACE activity gradually and selectively (8). Furthermore, chronic treatment with subcutaneously administered captopril-LZM in adriamycin-induced...
nephrotic rats displayed beneficial effects as it decreased proteinuria without affecting systemic blood pressure. In contrast, untargeted captopril did not reduce proteinuria but showed systemic side-effect by causing hypotension (9). From this study, we concluded that drug-LZM conjugates, in principle, can be administered subcutaneously. However, the rapid elimination of released captopril from the kidneys reduced the efficacy of the conjugate. Therefore, we focused our research at other drugs that could become more effective in the kidneys, either due to an improved pharmacokinetic profile (improved residence-time in the kidney) or to a more potent pharmacodynamic profile. In addition, we applied a novel type of drug linkage chemistry, the so-called Universal Linkage System (ULS™) which makes coordinative (non-covalent) bonds with the drug and the carrier protein. We succeeded in the coupling of the angiotensin II (AT II) type I receptor antagonist losartan to lysozyme using ULS. Our in vitro studies showed that losartan-LZM remained stable in serum while it released the drug in kidney homogenates at a slow rate, amounting to 20% release after 24h. When administered to rats, losartan-LZM disappeared from the circulation and accumulated into the kidneys in a manner similar to other drug-LZM conjugates. We subsequently evaluated the efficacy of the conjugate in the unilateral renal ischemia-reperfusion (I/R) injury model in rats for 4 days. We chose the I/R model to test the conjugate, since this model is a short-term model and induces inflammation and mild fibrosis by activating tubular cells, the target cell in our approach. In contrast to the earlier-used adriamycin-induced nephrosis model, it is a long-term model and represents both tubulointerstitial and glomerular fibrosis. Moreover, the I/R model showed less variation in-between animals than the adriamycin-induced nephrosis model. Although we only observed minor effectivity of losartan-LZM after multiple dosing for 4 days (data not shown), the combined results clearly demonstrated the feasibility of the applied drug delivery approach.

We also reviewed which other pathways and drugs play crucial roles in the pathogenesis of renal fibrosis, with the aim to establish which of the inhibitors might benefit from renal drug delivery (Chapter 4). Since our delivery strategy is focused at renal tubular cells, we especially studied the role of renal tubular cells in the pathological events. During renal injury, tubular cells are activated and produce several cytokines and growth factors which initiate inflammation thereby elicit a fibrotic process. Various signaling pathways such as mitogen-activated protein kinase (MAPK) and tyrosine kinase pathways participate in the activation of tubular cells and production of cytokines from tubular cells, and potent inhibitors of these cascades have been described in recent years. However, in these studies quite high dose of these inhibitors were administered (10-12). Since such potent compounds may exert side effects in other tissues beside the kidney, we hypothesized that renal selective delivery of kinase inhibitors might be an interesting approach to treat renal fibrosis. Moreover, local inhibition of a signaling pathway within tubular cells would provide an insight on the regulation of fibrosis by a specific pathway in these cells.
We first screened different kinase inhibitors on the basis of their efficacy in cultured renal tubular cells. Tubular cells were activated by natural activators such as albumin or transforming growth factor-beta1 (TGF-β1) and we evaluated the efficacy by gene expression for inflammation (MCP-1) and fibrotic (procollagen-Iα1, TIMP-1 and α-SMA) markers. Among different signaling inhibitors, we found that p38 MAPK inhibitor SB202190 and TGF-β1 receptor kinase inhibitor (ALK5 inhibitor; TKI) inhibited both inflammatory and fibrotic markers significantly. As described in Chapter 5, we studied the pharmacokinetics of SB202190 in rats to determine whether it efficiently distributes to kidneys (13). To measure SB202190 levels in different organs, we first developed a HPLC method that allows measurements of SB202190 after isolating it from different biological matrices with a liquid-liquid extraction method. The pharmacokinetics study demonstrated that SB202190 was rapidly eliminated from blood and poorly distributed to the kidneys. These results underlined the need to target SB202190 to the kidney for the treatment of renal fibrosis. In Chapter 6, we described the cell-specific delivery of SB202190 to renal tubular cells using LZM (14). We coupled SB202190 (SB) to LZM via two different linkages, i.e. a carbamate linkage and the ULS coordinative linkage. These were the only two available options to couple this drug to a protein. Carbamate linkage released the drug rapidly in serum which makes it unsuitable for renal targeting. However, SB-ULS-LZM conjugate remained stable in serum but released the drug in kidney homogenates slowly in vitro. Moreover, a single intravenous injection of the conjugate resulted into rapid accumulation in kidneys where it released drug slowly. Since ULS contains platinum which is known to cause nephrotoxicity, we examined the toxicity of the conjugate in renal tubular cells in vitro by cell-viability assays and in rats until 72 h by studying renal function, renal morphology and apoptosis in tubular cells. No sign of toxicity with the conjugate was observed both in vitro and in vivo. Finally, we investigated the effect of SB-ULS-LZM in human renal tubular cells in vitro. We found that application of conjugate to tubular cells in vitro substantially inhibited the TGF-β1–induced procollagen-Iα1 gene expression. In vivo, we tested the efficacy of SB-ULS-LZM in the unilateral I/R model. An important consideration in this respect is that p38 is highly activated in tubular cells in this model, which has been associated with renal inflammation and fibrosis. A single dose of SB-ULS-LZM displayed its activity after 4 days of I/R injury in rats by inhibiting phosphorylation of p38 and showed a significant effect on fibrosis by inhibiting α-SMA expression which is a marker for fibroblast activation, the crucial event during fibrogenesis. This study concluded that p38 MAPkinase inhibitor SB202190 can successfully be targeted to renal tubular cells where it is pharmacologically active. This targeted compound may therefore be used for the treatment of renal fibrosis.

During renal injury, TGF-β1 plays a pivotal role in the pathogenesis of renal fibrosis as it activates tubular cells. In response to TGF-β1, tubular cells transdifferentiate into fibroblasts via epithelial-mesenchymal transition process and further fibroblasts produces fibrogenic materials. TGF-β1 acts through its type I (Activin-Receptor Like Kinase-5, ALK5) and II receptors by activating (phosphorylating) Smad2/3 and MAPK pathways. We
hypothesized that blockade of TGF-β1 action locally within kidneys may provide an attractive means to treat renal fibrosis. In Chapter 7, we describe the studies in which we examined the effect of local inhibition of TGF-β in kidneys by renal-selective delivery of an ALK5 inhibitor (TKI) using LZM. Therefore, we conjugated TKI to lysozyme using the ULS™ strategy. Similar to the SB202190-ULS-LZM conjugate, TKI-LZM also accumulated rapidly in tubular cells and provided a local depot locally for 3 days. In addition, we conducted a study with rhodamine-ULS-LZM and proved that drug-ULS-LZM conjugate stays inside the tubular cells and releases the marker drug locally. Moreover, TKI-LZM displayed its activity by inhibiting TGF-β1–induced procollagen-Iα1 gene expression significantly in vitro in HK-2 cells. Subsequently, we evaluated the efficacy of TKI-LZM conjugate in vivo in the unilateral ureteral obstruction (UUO) rat model, since the involvement of TGF-β in this model has been well established in the literature. Of note, the UUO model displays advantages as it is a rapid model for tubulointerstitial fibrosis. However, the UUO model is not suitable for long-term studies with multiple dosing of the conjugates since blockade of glomerular filtration after ureteral ligation will result in blockade that prevent further accumulation of the conjugate in the kidney. Interestingly, a single dose of TKI-LZM administered at day 1 significantly inhibited MCP-1 gene expression, macrophage infiltration (ED-1 positive cells) and α-SMA protein expression after 3 days of ureteral ligation. Although we administered a low dose only once, we observed effects, which illustrate the potency of this compound. From this study, we concluded that blockade of the action of TGF-β within kidneys using the ALK5 inhibitor can be an important strategy for the treatment of renal fibrosis.

Recently, RhoGTPase mediated signaling has been recognized to play also an important role in the pathogenesis of renal fibrosis. RhoGTPases regulate cytoskeletal reorganization, transdifferentiation of tubular epithelial cells and neutrophil infiltration (15). RhoGTPases act by stimulating downstream Rho effectors such as Rho-associated coiled-coil forming protein kinase (ROCK). We investigated the consequence of ROCK blockade within the kidneys with our renal-targeting approach (Chapter 8). The well-known ROCK inhibitor Y27632 was conjugated to LZM using ULS and we studied the pharmacokinetics of Y27632-LZM conjugate in healthy rats. Similar to other drug-LZM conjugates, Y27632-LZM accumulated rapidly within the kidney. Next, we evaluated the efficacy of Y27632-LZM in the I/R rat model. Evaluation of Y27632-LZM in this model is relevant since the ROCK pathway regulates the cytoskeletal reorganization as well as the infiltration of inflammatory cells and both are affected during the disease. After 4 days therapy with Y27632-LZM, we found that Y27632-LZM substantially inhibited the gene expression of inflammatory (MCP-1) and fibrotic (TGF-β1 and α-SMA) genes. Moreover, it also significantly inhibited macrophages infiltration into kidneys. In contrast, free Y27632 did not reduce any of the parameters after 4 days of treatment. We conclude that local inhibition of the ROCK pathway within the kidneys enhances the activity of the
compound, and therefore it can also be explored as a treatment for renal inflammation and fibrosis.

**Conclusions and future perspectives**

The present thesis provides sufficient evidence for its overall technological aim, i.e. renal-specific delivery of anti-fibrotic compounds in rats with renal fibrosis. In this frame work, we employed different linkage techniques to couple drugs to LZM. The novel ULS linker showed superiority over other linkages as it is easy-to-use and provides good stability of conjugate in serum and slow release of drug in kidneys for several days. Moreover, for some drugs it is the only option to couple it to a protein. In vivo efficacy of drug-LZM conjugates was confirmed since they showed beneficial effects in the relevant animal models for renal fibrosis. Blockade of different pathways such as p38 MAPK, TGF-β1 receptor-I kinase and ROCK pathways within kidneys, using our renal-specific drug delivery approach, produced beneficial effects on both inflammation and fibrosis. Moreover, these studies also gave insight into the role of different pathways in the regulation of disease. For instance, in the I/R injury model, blockade of ROCK pathway with Y27632-LZM showed the most promising effects compared to the blockade of p38 MAPK or angiotensin II-mediated pathways. Since different doses and dosage schedules were used for Y27632-LZM and SB-ULS-LZM conjugates, more studies are required to confirm that inhibition of ROCK pathway is the most promising of the investigated approaches. Moreover, we the effectivity of TKI-LZM can not be compared directly to the other conjugates since we tested it in a different model of renal fibrosis. Therefore, it would be quite interesting to further examine the effecivity of different conjugates in different animal models.

For future studies, several steps can still be taken to improve the chosen renal drug targeting approach. Some potential limitations related to drug delivery using lysozyme, such as low drug to protein coupling ratio, possible immunogenicity and lower shelf-life, can presumably be solved by adopting polymers as renal drug carriers. For example, we have conducted pilot experiments with PAMAM dendrimers that filter freely through the glomerulus and are deposited into tubular cells (16). Coupling of drugs to dendrimers may enhance the drug loading to carrier and avoid immunogenicity. Pilot syntheses with this type of dendrimer were conducted successfully, and may lead to substantial improvement in the field of renal targeting.

Since our aim is to deliver drug specifically to tubular cells and subsequently intervene in renal fibrosis, we used a renal tubular cell culture system to screen for pharmacologically interesting drugs. Although we primarily used profibrotic activators, albumin and TGF-β, various other activating factors (IL-1β, TNF-α or angiotensin II) and activating conditions (hypoxia and high glucose) can be employed to mimic different renal disease activators and to activate different signaling pathways. A more extensive testing of kinase inhibitors and other anti-inflammatory and antifibrotic agents is needed to identify potential drug targets.
In the present study, we used gene expression analysis as a main tool to examine the efficacy of the drug and drug-LZM conjugates \textit{in vitro}. Yet, drugs may act by affecting one or more processes such as the expression of cytokines/growth factors, kinase activity, cell-size, cell migration or cell growth. Moreover, blockade of a signaling pathway may cause the activation of another pathway due to the cross-talks between signaling pathways. Therefore, different read-out systems such as determination of secreted cytokines in cell culture medium, kinase activity assays, proliferation assay and microarray techniques can also facilitate to select the most effective drug or a suitable combination of drugs. Furthermore, such read-out systems can also be employed to study the \textit{in vivo} effects of the conjugates.

Understandably, such extensive testing of antifibrotic compounds requires huge efforts and was not performed in selection of the presently studied compounds which were selected after reviewing of the available literature. In addition, chemical properties of the compounds need to be considered in view of drug linking technology and pharmacokinetic fate of the compounds. Nevertheless, the conjugates that have been prepared showed highly promising activity in both renal proximal tubular cells and in animal models of renal fibrosis, and thus may represent promising compounds in the management of renal fibrosis.

\textbf{References}


