Cell-specific targeting of renal fibrosis

Poosti, Fariba

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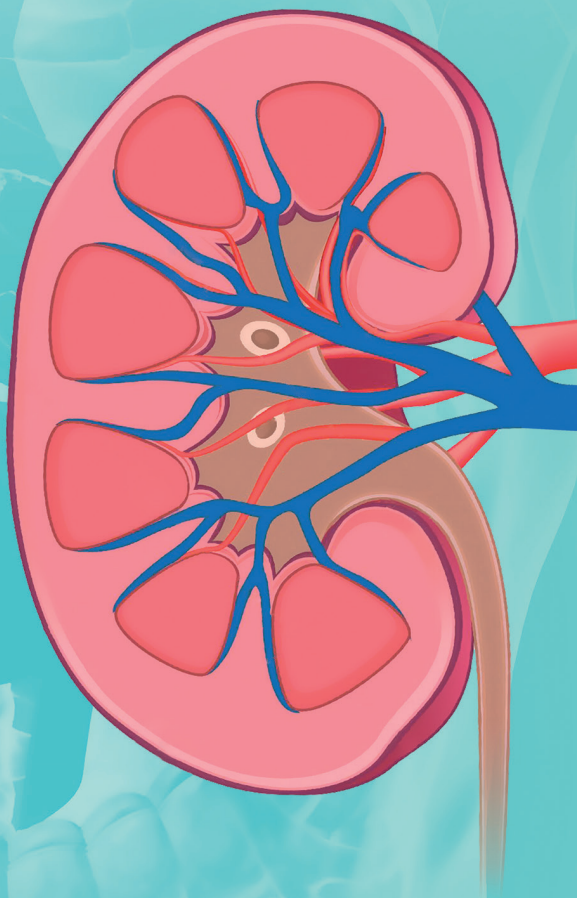
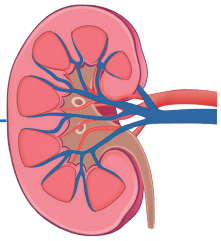


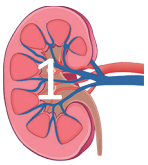
General introduction
&
Scope of the thesis



Renal fibrosis is the most prominent hallmark of chronic kidney disease (CDK). CKD can progress to end stage renal disease (ESRD), a life-threatening condition with currently no successful treatment available. The only available options at the moment for ESRD patients are dialysis or kidney transplantation (1). ESRD is characterized by severely impaired renal function which results from tubular damage and interstitial fibrosis (2). Despite many experimental and clinical studies aiming at anti-fibrotic drug discovery and development, the nephrology field is still suffering from a lack of clinically approved effective therapeutic strategies to halt or reverse renal fibrosis (2). As a consequence, there is a high risk of mortality in CKD patients worldwide (2-4). Hence, the development of novel therapeutic strategies is imperative.

The myofibroblast plays a key role in the development and progression of renal fibrotic lesions, and is therefore under intensive research as a target for anti-fibrotic therapy. In order to achieve this goal, a large number of investigations have been performed to advance our knowledge on the origin of myofibroblasts (1, 5, 6). Nonetheless, their precise cellular origin has been a matter of debate for a long time. To date, several cell populations have been suggested as precursors of renal myofibroblasts such as resident fibroblasts, bone marrow-derived cells including circulating fibrocytes, pericytes, tubular epithelial cells (via epithelial-to-mesenchymal transition, EMT), endothelial cells (via endothelial-to-mesenchymal transition, EndMT), and perivascular $Gli1^{+}$ progenitors cells (mesenchymal stem cell-like cells) (6-10). However, recent studies show that epithelial cells, endothelial cells and leukocytes are not important contributors, whereas pericytes, resident fibroblasts, and perivascular mesenchymal stem cells seem to be the main source of cells that differentiate into myofibroblasts in kidney disease (9, 11-12). Importantly, it seems that the interstitial myofibroblasts can arise from various precursors, and is dependent on the type of renal injury and thus highly context-dependent (12, 13).

The complex interplay between different cell types in the tubulointerstitial micro-environment of the kidney also has impact on the initiation and progression of renal fibrosis, e.g. by promoting the differentiation and activation of above-mentioned precursor cells toward matrix-producing myofibroblasts. The renal vasculature is highly efficient in regulating renal tissue homeostasis by providing oxygen and nutrients to the cells, and also by removal of toxic metabolites. Any disturbances in both blood and/or lymphatic capillary circulation can have effects on kidney homeostasis due to the reciprocal interactions between endothelial cells with other cell types in the kidney microenvironment (14, 15). Therefore, in order to halt the progression of renal fibrosis, not only targeting the key cells is important, but also normalizing the hostile microenvironment is vital. A disturbed microcirculation can activate blood/lymphatic endothelial cells, stimulate angiogenesis and lymphangiogenesis, induce microvascular rarefaction, reorganize the influx and/or efflux of inflammatory cells, and also promote extracellular matrix remodeling.



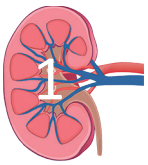
Collectively, these changes can stimulate a variety of processes which eventually result in pro-inflammatory and pro-fibrotic responses. Thus, the kidney microvasculature is another potential target in order to prevent the activation of myofibroblasts, and thereby inhibit the formation of dysfunctional scar tissue.

Another important aspect in the development of novel therapeutic agents to target renal fibrosis is that current conventional drugs require high systemic concentrations in order to be effective in the kidney. However, such high dosages induce many unwanted side effects in off-target organs. Even if the drug ends up in the kidney, it might not accumulate in target cells (16, 17). Moreover, impaired renal function can alter the distribution of the therapeutic agents to the kidney. To overcome these shortcomings, targeted drug delivery is being used to deliver the intended amount of drugs to the specific target cells, and by this way not only increasing the therapeutic index, but also at the same time reducing the adverse systematic side effects in off-target organs or cells.

SCOPE OF THE THESIS

The work described in this thesis aims to develop cell-specific therapy for the treatment of renal fibrosis, as well as to further explore the pathophysiological role and potential therapeutic value of renal lymphatic vessels and lymphangiogenesis in renal disease.

In *chapter 2*, an overview of renoprotective effects of protein kinase inhibitors in chronic kidney disease is provided, as well as future directions in this exciting field of research that may lead to the development of highly specific pharmacological interventions. Activated proximal epithelial cells in the kidney have proved to be central triggers of inflammatory and fibrotic processes in a wide range of renal diseases. Based on that, in *chapter 3* targeted inhibition of the Rho kinase pathway in proximal tubular cells was studied in order to reduce inflammation and/or lymphangiogenesis in an acute renal allograft rejection model. The Rho kinase inhibitor Y27632 was coupled to lysozyme (Y27632-lysozyme) providing a kidney-specific conjugate that can release its drug to proximal tubular cells. A novel targeted drug-delivery carrier (PPB: PDGFRβ-recognizing peptide) for the delivery of the anti-fibrotic cytokine IFNγ to PDGFRβ-expressing myofibroblasts was studied in *chapter 4*. This compound was tested *in vitro* as well as *in vivo* in the unilateral ureteral obstruction (UUO) mouse model. In *chapter 5,* a modification of the approach described in chapter 4 was used. In order to further reduce systemic side effects, the efficacy of a targeted (to PDGFRβ using PPB) IFNγ-mimetic was tested *in vivo*. In this mimetic the IFNγR-binding domain of IFNγ was removed in order to reduce the proinflammatory effects of IFNγ. Furthermore, in *chapter 6*, an *ex vivo* model of renal fibrosis, which can provide a step forward towards the development of anti-fibrotic compounds, is explored. We developed an *ex vivo* model of renal fibrosis by using precision-cut kidney slices. In this model, early onset of renal fibrosis and efficacy of the anti-fibrotic cytokine IFNγ was evaluated. Renal fibrosis appears to be associated with lymphangiogenesis although it is not known yet whether this is a response to renal damage and fibrosis, or actually precedes fibrosis. Therefore, a time-course of renal interstitial lymphangiogenesis and its association with others markers of renal damage was investigated in an established rat proteinuria model, as described in *chapter 7*. In *chapter 8* the obtained results are summarized and future perspectives of anti-fibrotic therapeutic approaches are discussed.



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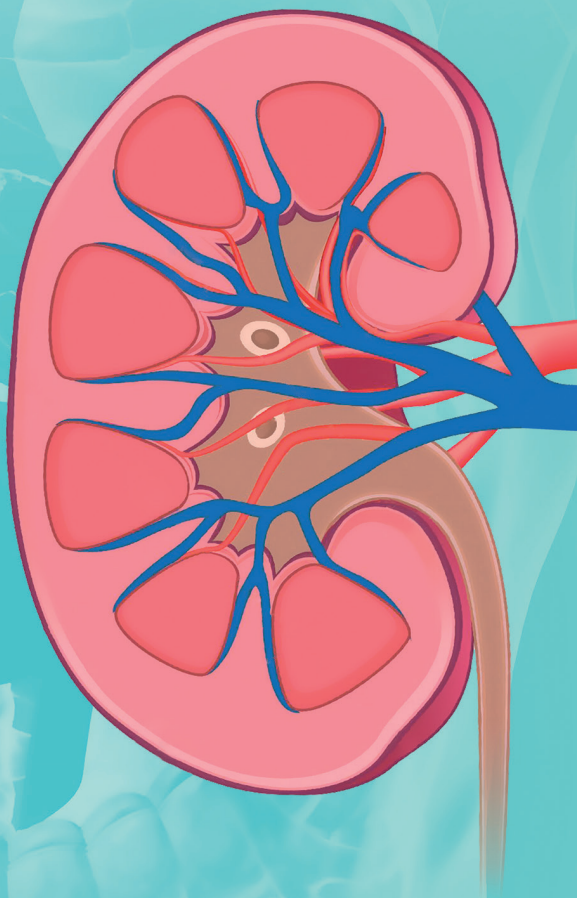
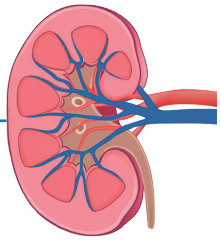


Targeted renal delivery of protein kinase inhibitors for the treatment of chronic kidney disease



Fariba Poosti[1], Jan-Luuk Hillebrands[1], Harry van Goor[1], Martin H. de Borst[2]

[1]Department of Pathology and Medical Biology, Division of Pathology, and [2]Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen and University of Groningen, Groningen, The Netherlands

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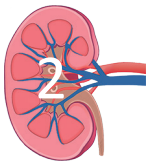
ABSTRACT

The increasing prevalence of end-stage renal disease urges novel therapeutic strategies for the treatment of chronic kidney disease. As protein kinases play a pivotal role in renal inflammation and fibrosis, specific protein kinase inhibitors have been demonstrated to be renoprotective in experimental studies. However, since protein kinases are also involved in key physiological mechanisms such as cell differentiation, cell growth and proliferation, these beneficial effects have been associated with serious side effects, limiting their clinical applicability.

However, the possibility to selectively deliver a drug to cells with a particular phenotype (*i.e.* cells expressing a cell-specific protein to which drugs can be targeted) has increased the potential of protein kinase inhibitors in chronic kidney disease. Several studies have reported renoprotective effects of protein kinase inhibitors specifically delivered to fibrotic cells, or to specific cell types such as proximal tubular epithelial cells or mesangial cells. An overview of these studies will be provided, as well as future directions in this exciting field of research that may lead to novel highly specific pharmacological intervention strategies.

INTRODUCTION



Progressive loss of renal function, ultimately resulting in end-stage renal disease (ESRD), is a major health problem worldwide. In the United States, the incidence of ESRD, adjusted for age, gender and race, increased from 200 per million in 1990 to 354 per million in 2007 (www.usrds.org). Although the incidence of ESRD may have stabilized in recent years (1), the adjusted ESRD prevalence is still rising from 800 per million in 1990 to 1665 per million in 2007 in the US. In other parts of the Western world, the numbers of patients are slightly lower but similar trends exist (2); as a result, the worldwide ESRD population will soon exceed 2 million patients (3). The increasing prevalence of ESRD is caused on one hand by the rising prevalence of diabetes and hypertension in the Western world (4). On the other hand the all-cause mortality of CKD patients has declined from 250 deaths per 1000 patient years in 1995 to 200 deaths per 1000 patients years in 2007 (adjusted for race, gender, hospitalization, and comorbidities; www.usrds.org). Mortality due to cardiovascular disease is generally remarkably increased in patients with ESRD compared with subjects with normal renal function (5), but this has improved as well over the last decade. Thus, prevalence of ESRD risk factors/determinants has increased, and mortality has decreased.

Besides being a urgency from a medical perspective, ESRD forms an enormous financial burden: from 1991 to 2007, total costs of medical care for ESRD patients increased from $9 billion to $35 billion in the United States (www.usrds.org). These data support the urgency of novel therapies that may avoid chronic kidney disease (CKD) to become ESRD.

The availability of powerful anti-proteinuric interventions – *i.e.* blockers of the renin-angiotensin-aldosterone system (RAAS) – has slowed the rising incidence of end-stage renal disease. In spite of this state-of-the-art pharmacological treatment, residual proteinuria due to resistance to anti-proteinuric therapy is common. Furthermore, animal experiments have suggested that, especially under conditions of a low circulating volume, RAAS blockade may induce pre-fibrotic renal lesions (6). On the other hand, animal studies indicate that under certain conditions, glomerular sclerosis is reversible (reviewed in (7)), yielding hope to find a modality of treatment able to provide regression of renal lesions. In auto-immune-mediated renal disorders such as SLE or Wegener's disease, as well as in kidney transplantation, novel therapies (e.g. mycophenolate mofetil) have been proven successful but at the expense of side effects. Together, these findings indicate that new treatment modalities, either as a substitution of current therapy or as addition on top of current therapy, are urgently required.

Inhibition of intracellular signaling molecules such as protein kinases may be a powerful intervention in CKD. Due to their involvement in various cellular processes such as proliferation and cytokine production, protein kinases play a role in chronic renal inflammation and fibrosis, processes critically involved in (the progression of) renal damage. This article will first discuss the potential role of various protein kinases (e.g. mitogen-activated protein kinases, Rho kinase, and growth factor receptor kinases) in the progression of renal damage. Next, the therapeutic potential of protein kinase inhibitors

in renal disease will be discussed. First studies on systemic treatment with protein kinase inhibitors in (experimental) renal disease will be addressed. As it turns out that systemic blockade of protein kinases yields a high risk of side-effects and poor tolerability, organ-specific or even cell type-specific targeting of protein kinase inhibitors has become available. Thus, a significant part of this review will be dedicated to protein kinase inhibitors that are chemically modified or coupled to achieve target-organ specific delivery of the drug. As clinical experience with these types of compounds is limited, the majority of studies summarized here are in experimental models of renal damage. However, these preclinical data show promising effects, both in terms of efficacy and of reduced side effects.

Protein kinases in chronic kidney disease (CKD)

The protein kinase superfamily consists of several subtypes, which can be divided in serine/threonine-specific protein kinases, such as mitogen-activated protein (MAP) kinases and Rho kinase (ROCK), and thyrosine kinases which are primarily growth factor receptors such as platelet-derived growth factor receptor (PDGFR) or epidermal growth factor receptor (EGFR). By their capacity to phosphorylate other proteins, protein kinases are crucial signal transduction molecules. A major group of protein kinase target molecules are transcription factors. Through regulation of transcription factor activity, protein kinases can modulate the expression of many genes and thereby affect virtually all types of cellular mechanisms, either with beneficial or with deleterious effects.

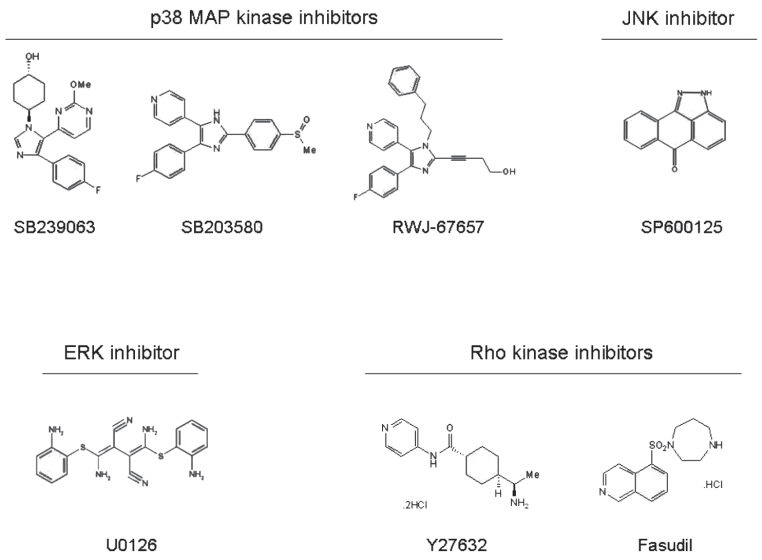
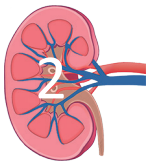


Figure 1: Structural formulas of some widely used protein kinase inhibitors: p38 MAP kinase inhibitors SB239063, SB203580, and RWJ-67657; the JNK inhibitor SP600125; the ERK inhibitor U0126; and Rho kinase inhibitors Y27632 and fasudil.

MAP kinases

The potential role of the MAP kinase p38 in CKD has been subject of many studies. The most widely used p38 MAP kinase inhibitors are presented in Figure 1. In experimental models, pharmacological inhibition of p38 reduced renal inflammation and fibrosis both in experimental models of kidney disease characterized by inflammation such as anti-GBM glomerulonephritis (8), crescentic glomerulonephritis (9), angiotensin II-mediated renal damage (10-12), or chronic allograft nephropathy (13), and in nephrotic models such as adriamycin nephropathy (14) and puromycin-aminonucleoside (PAN) nephrosis (15). In human renal disease, it was documented that activation of p38, both in intrinsic renal cells and in infiltrating leukocytes, was strongly present in biopsies from patients with glomerulonephritis, and correlated with renal dysfunction and histopathology (16). Similar results were found for diabetic nephropathy (17). These findings suggest that p38 plays a role in renal inflammation. As p38 regulates a wide variety of genes involved in cell proliferation, growth, differentiation, and apoptosis, there could be several mechanisms of renoprotection involved. Probably, in the injured kidney, several pathogenic pathways are activated such as pro-inflammatory (e.g. through MCP-1, IL-6), pro-fibrotic, and pro-apoptotic pathways. As p38 is a crucial modulator of many of these pathways, this could explain renoprotective effects of p38 inhibition



Clinical experience with p38 MAP kinase inhibitors is limited to a small number of trials, indicating reduced inflammatory reponse in healthy volunteers (18, 19) and, more recently, in COPD patients (20). Studies in Crohn's disease (21) and rheumatoid arthritis (22, 23) have been negative. However, p38 also plays a role in many physiological processes. To some extent, many pathways involved in renal damage are in fact physiological mechanisms that have become deregulated. It could therefore be expected that p38 MAP kinase inhibition might also deteriorate renal damage instead, as repair mechanism could also be blocked by this intervention. In addition, cross-talk exists between MAP kinase families. Ohashi et al reported that p38 inhibition deteriorates renal damage in a model of renal fibrosis, probably through reactive ERK activation and enhanced renal cell proliferation (24).

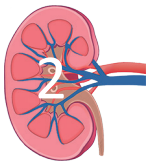
A crucial next step would be to identify renal cells in which p38 plays a pathogenic role in renal disease. Several *in vitro* experiments, enabling to exclusively study the role of p38 in a particular cell type, in different renal cell types and with various readout parameters have been performed. Table 1 summarizes effects of p38 activation in several renal cell types. This table identifies mesangial cells, tubular epithelial cells, podocytes and fibroblasts as potential target cells for specific delivery of p38 inhibitors. The subject of cell type-specific drug delivery will be discussed in more detail below. Table 1 also indicates that p38 is mainly involved in the regulation of inflammation, e.g. through MCP-1 gene expression, and apoptosis. As most types of renal disease are characterized by chronic inflammation finally resulting in fibrosis and increased apoptosis, these processes, modulated by p38 MAP kinase, are targets for intervention in renal disease.

Table 1: Overview of renal cell types in which p38 MAP kinase is expressed, and putative effects of p38 activation in respective cell types.

Renal cell type	Possible role of p38	Reference
Mesangial cells	MCP-1 expression	[134]
	Collagen expression	[135]
	Apoptosis	[136]
	Nitric oxide production	[137]
Tubular epithelial cells	MCP-1 expression	[138]
	Epithelial mesenchymal transition	[65]
	TGF-beta and collagen I expression	[139]
	Apoptosis	[140]
	Angiotensinogen production	[141]
Podocytes	Apoptosis [137]	[142]
	MCP-1 production	[143]
Fibroblasts	Extracellular matrix production	[144]
	ICAM-1 induction	[145]

Besides p38 MAP kinase, other MAP kinases like ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) are activated in and have been associated with the severity of human renal disease (25-27). We have found that JNK may play an important role in renal inflammation by regulation of MCP-1 (monocyte chemoattractant protein-1) gene expression in renal tubular epithelial cells (26). JNK activates the transcription factor c-Jun, which is part of the AP-1 (activator protein-1) complex that is involved in regulation of many genes. The gene encoding MCP-1, which is a crucial chemokine attracting inflammatory cells to sites of renal injury, contains multiple binding sites for the AP-1 complex (28). Other studies confirmed that JNK may play a role in the inflammatory response to (tubular) renal damage in cultured tubular cells (e.g. HK-2 cells (25)), experimental models (anti-GBM glomerulonephritis (29), ischemia/reperfusion (30)), and in human transplantation (31). Under pathological conditions, JNK may also play a role in tubular cell apoptosis, as indicated by studies in JNK knockout mice (32). Given the data summarized here, tubular epithelial cells are the renal cell types that would be most suitable for specific delivery of JNK inhibitors. Alternatively, specific JNK inhibition in macrophages might be a good approach, as infusion of $JNK^{-/-}$ macrophages in macrophage-depleted mice with renal damage provided renoprotection (29, 33). JNK may be involved in the inflammatory response through effects on other cell types as well, as documented for podocytes (inflammatory cytokines released by macrophages cause podocyte injury mediated by JNK and p38) (34), endothelial cells and renal interstitial fibroblasts (where activated JNK is found in animals with glomerulonephritis (35)), and mesangial cells (36).

Similar to p38, systemic treatment with JNK inhibitors may have important side effects. A recent study indicated that db/db mice (a widely used animal model for type 2 diabetes mellitus) treated with a JNK inhibitor as well as JNK2 knockout mice made diabetic by streptozotocin injection both displayed reduced insulin resistance but, surprisingly, increased albuminuria (37). These findings indicate that a particular protein kinase inhibitor may not be beneficial under all circumstances, and that besides the target organ or tissue, also the underlying disease must be taken into account.



While p38 and JNK are considered key players in inflammation, fibrosis and apoptosis, the MAP kinase ERK is involved mainly in cell growth and differentiation. Therefore, it is not surprising that activated ERK is abundantly found in the developing (embryonic) kidney (38). The Ras-Raf/MEK/ERK pathway is under intense investigation as a candidate target for renal cell carcinoma (39). The Raf kinase inhibitor sorafenib is currently registered in the US and in Europe for the treatment of advanced renal cell carcinoma. In addition, systemic treatment with an (upstream) inhibitor of ERK reduced inflammation and apoptosis in experimental models of renal damage such as cisplatin nephrotoxicity (40) and angiotensin II-mediated renal damage (11). Although renal targeting of ERK inhibitors might be a suitable approach in CKD, more experience with systemic administration in experimental models of CKD would be required.

Rho kinase (ROCK)

Rho kinase (ROCK) is a protein kinase belonging to the family of serine-threonine kinases. An overview of the main cellular functions of ROCK is provided in Figure 2. ROCK is a downstream effector protein of the small GTPase Rho, which is one of the major regulators of the cytoskeleton. A major function of ROCK is stabilization of actin filaments to maintain integrity of the cytoskeleton (41). In line with this, ROCK is involved in cytokinesis (42) and cell cycle control (among others through Cdc42) (43). Besides regulation of the cytoskeleton, ROCK is also involved in infiltration of inflammatory cells (44, 45). Recent studies demonstrated that mainly ROCK1 is involved in recruitment of neutrophils and macrophages, at least in part through regulation of the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) (46). Thirdly, ROCK plays an important role in regulation of the vascular tone. The binding of vasopressors (e.g. angiotensin II) to G protein-coupled receptors (e.g. the angiotensin II type 1 (AT1) receptor) leads to vascular smooth muscle contraction through a Ca^{2+}-dependent pathway and through Rho kinase (47). The Ca^{2+}-dependent pathway causes smooth muscle contraction and hypertension through activation of phospholipase C, increased protein kinase C activity, and phosphorylation of myosin II regulatory chain [48]. The ROCK pathway increases Ca^{2+} sensitization of smooth muscle contraction (49) (*i.e.* an increase of the contractile response to an increase in intracellular calcium) and modulates the phosphorylation of myosin light chain. Through (deregulation of) these processes, Rho kinase may be involved in the development of hypertension, both in situations of disturbed (50) and normal renal function (51, 52). Fourthly, inhibition of the Rho kinase pathway has pleiotropic effects, as investigated most deeply in studies on

the protective effects of statins. Statins prevent the synthesis of other important factors of the cholesterol biosynthetic pathway, including farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) that are downstream from L-mevalonic acid (53). These factors are involved in post-translational modification of proteins such as nuclear lamins, Ras, Rho, Rac and Rap (54). In particular, by inhibiting mevalonate synthesis, statins prevent membrane targeting of Rho and its subsequent activation of ROCK (55). Indeed, many pleiotropic effects of statins are modulated by Rho kinase, as demonstrated in *in vitro* studies (56-58). A recent study by Liu et al confirmed that simvastatine reduces Rho kinase activity in dyslipidemic human subjects without cardiovascular disease (59). Treatment with ezetimibe similarly reduced LDL cholesterol but did not affect Rho kinase. The renoprotective role of ROCK inhibitors has been demonstrated in several experimental models of renal disease. Anti-inflammatory and anti-fibrotic effects of the ROCK inhibitor Y-27632 were shown in the unilateral ureteral obstruction (UUO) model (60) and ischemia/reperfusion renal injury (61). In an experimental model of nephrotic syndrome PAN nephrosis), Y-27632 was renoprotective as well (62). In an experimental model of kidney transplantation, Y-27632 reduced chronic allograft nephropathy (63). Given the important effects of ROCK downstream of G protein-coupled receptors such as the AT1 receptor, it is not surprising that ROCK inhibition is also beneficial in angiotensin II-mediated renal damage (64). *In vitro* studies elucidated an additional important renoprotective mechanism by showing that ROCK inhibition reduces EMT of tubular epithelial cells, induced by angiotensin II (65).

Besides monotherapy, specific intervention in the ROCK pathway has also been performed in combination with “classical” renoprotective therapy, namely blockade of the renin-angiotensin-aldosterone system. A recent study reported on renoprotective effects of dual therapy with the ROCK inhibitor fasudil and an ACE inhibitor (66). Studies with combinations of kinase inhibitors and shRNAs demonstrated that, as opposed to inhibition of a single kinase, combined inhibition of either ROCK and ZEB1/2 or ROCK and TGFbeta type I receptor kinase, respectively, were successful to reverse the process of epithelial-to-mesenchymal transformation (EMT) in tubular epithelial cells (67).

Although Y-27632 is the most widely used Rho kinase inhibitor in experimental studies, the only clinically available Rho kinase inhibitor is fasudil. The oral formulation of fasudil has demonstrated effectiveness in patients with angina pectoris (68, 69), after previous studies had demonstrated that fasudil reduces coronary artery spasm (70, 71). Other formulations of fasudil include an intravenous form which is under investigation for the treatment of ischemic stroke (72). Organ-specific therapy (besides renal targeting) with Rho kinase inhibitors in patients has been applied in patients with pulmonary hypertension (fasudil as aerosol) (73) and in patients with ocular disease to reduce intra-ocular pressure (SNJ-1656 as topical agent) (74, 75).

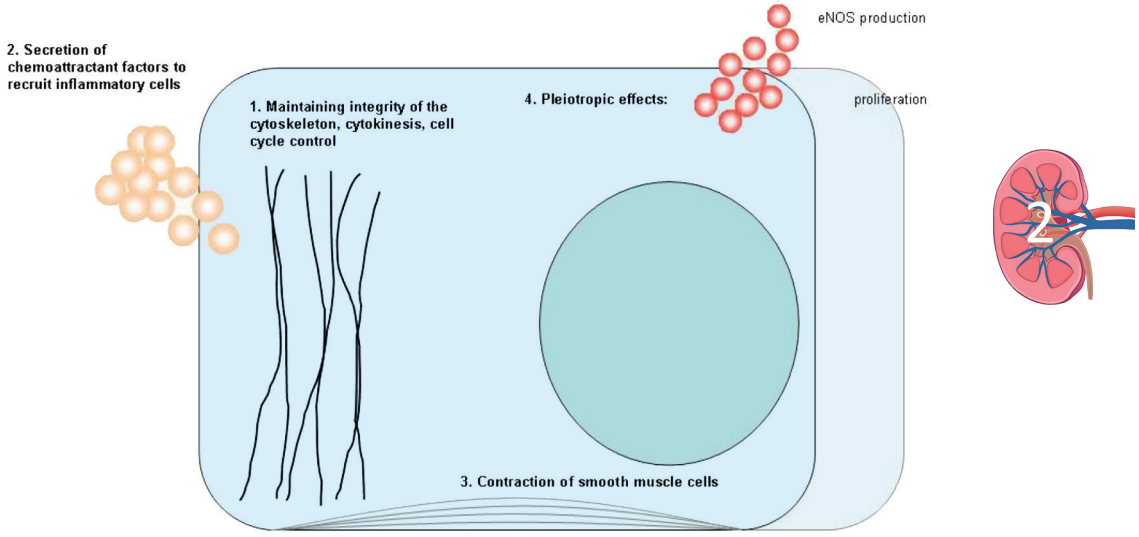


Figure 2: Figure summarizing possible cellular effects of Rho kinase: 1. Maintaining integrity of the cytoskeleton (and, consequently, cytokinesis and cell proliferation); 2. Secretion of chemoattractants such as MCP-1; 3. Contraction of smooth muscle cells, thereby regulating vascular tone; 4. Pleiotropic effects such as eNOS secretion and cell proliferation. For more in-depth discussion of the effects of Rho kinase refer to the text.

Growth factor receptor kinases

Growth factor receptor kinases are mainly, but not solely, tyrosine kinases. Tyrosine kinases can be divided into two subfamilies: the receptor tyrosine kinases, of which more than 50 are currently known, and non-receptor or cytoplasmic tyrosine kinases. In recent years, tyrosine kinases have been under intense investigation as targets for treatment of various types of cancer, including hematologic malignancies. Several excellent review papers on this subject have been published elsewhere (76-79).

A limited number of recent studies addressed the possible renoprotective effects of tyrosine kinase inhibitors. A selective inhibitor of spleen tyrosine kinase (Syk) demonstrated remarkably strong reduction of renal fibrosis, with regression of existing lesions, in association with strong anti-inflammatory effects (*i.e.* reduction of glomerular macrophage and CD8+ cell numbers, and renal MCP-1 and IL-1beta) (80). Previously, the same Syk inhibitor (R788) had shown renoprotection in a mouse model of lupus nephritis (81).

Imatinib, a selective tyrosine kinase inhibitor blocking among others the PDGFR, reduced renal inflammation and fibrosis in a rat model of anti-GBM nephritis (82) as well as in lupus nephritis (83). In transgenic rats overexpressing renin (Ren2), characterized by activation of the RAAS, treatment with imatinib reduced renal microvascular hypertrophy and perivascular fibrosis, and furthermore displayed cardioprotective effects (84).

Studies of the renoprotective effect of vascular endothelial growth factor (VEGF) inhibition have been conflicting. Specifc inhibition of VEGF by a pan-VEGF receptor tyrosine kinase inhibitor, SU5416, reduced albuminuria in a mouse model of type 2 diabetic nephropathy (db/db) (85), although studies in a model of glomerulonephritis suggested that blockade of VEGF may also increase proteinuria through loss of nephrin in podocytes (86). In line with the latter result, early studies on VEGF in renal disease were based on the concept that VEGF improves repair of the glomerular capillary network (87, 88). In experimental kidney transplantation, preliminary studies suggest that deregulation of VEGF may play a role in the development of chronic allograft nephropathy (CAN), although additional studies are required to further elucidate the possible role of VEGF in CAN (89).

The epidermal growth factor receptor (EGFR) may also play a role in renal damage, as the EGFR may link urinary albumin to the activation of ERK and increased expression of inflammatory cytokines such as IL-8 (90). A recent study demonstrated that EGFR inhibition reduces renal damage in the ischemia/reperfusion model (91) and in the L-NAME model characterized by nitric oxide (NO) deficiency-induced hypertension (92). Given the rapidly growing availability of EGFR inhibitors that have been developed for studies and for clinical application in cancer, future studies on renoprotective effects of EGFR inhibitors in other models are expected in the near future.

Transforming growth factor-beta (TGF-beta) as well as its receptors TGF-beta type I receptor, also known as activin receptor-like kinase 5 (ALK5), and TGF-beta type II receptor, which act as serine/threonine kinases, have been extensively targeted to treat fibrotic renal damage. Compounds targeting the TGF-beta pathway that have demonstrated renoprotective effects include a soluble TGF-beta type II receptor that binds TGF-beta1 (93), TGF-beta neutralizing antibodies (94), and small-molecule ALK5 inhibitors such as SB-431542 (95) *in vitro*, SB-525334 in PAN nephritis (96), SD-208 in ischemia/reperfusion injury (97), IN-1130 in UUO (98), and GW788388 in diabetic nephropathy (98, 99). As for the Rho kinase pathway, also the TGF-beta pathway has been targeted in combination with RAAS inhibition, resulting in powerful anti-fibrotic effects in rats with established diabetic nephropathy, in which progression of renal damage was halted (100).

Renal targeting of protein kinase inhibitors

As summarized above, renoprotective effects of protein kinase inhibition have been demonstrated in a wide range of experimental studies, both *in vitro* and *in vivo*. Yet, the clinical application of protein kinase inhibitors in clinical studies has been extremely limited. Probably, the limited initiation of phase I studies with protein kinase inhibitors in CKD patients, as well as the lacking progress towards clinical application of these agents, is related to side-effects. As clarified in the first part of this review, protein kinases have diverse physiological functions throughout the body (e.g. proliferation, differentiation) that cannot be inhibited (chronically) without serious side effects. It can be expected that if serious side effects are noted in early clinical studies, no data will be published and further development of the research on this agent will probably be cancelled. In addition, the

severity of side effects that is acceptable in CKD patients may well be different from that in cancer patients, in whom no (curative) treatment possibilities exist.

An important strategy to reduce side effects and to increase efficacy of a certain drug is to deliver this drug specifically to the cells that play a pathogenetic role by specific targeting. For example, the mannose-6-phosphate/insulin-like growth factor-II receptor (M6P/IGFIIR) is expressed not only by several types of tumor cells (101), but also by fibrogenic (*i.e.* damaged) renal cells [102]. Targeting of the antifibrinogenic drug mycophenolic acid (MPA) to fibrogenic cells using M6P-human serum albumin resulted in renoprotection in a model of RAAS-mediated renal damage (Ren2) (102). Similarly, the platelet-derived growth factor (PDGF) receptor has been used for targeting, allowing compounds to be delivered only to fibrotic cells (103); this approach has also allowed specific targeting of anti-cancer drugs to tumor cells (104). Moreover, targeting of myofibroblasts through gene therapy directed against alpha-smooth muscle actin has been proposed (105). Another elegant solution to reduce side effects observed in systemic treatment with a given drug is to selectively deliver the drug to the target organ, where it is released. Indeed, several drugs have now been chemically modified to allow specific targeting to the kidney. Renal targets in the kidney include cells in the glomerulus (the filtering unit of the nephron), tubular epithelial cells (mainly involved in water and electrolyte homeostasis) and interstitial cells (e.g. fibroblasts). As any of these cell types may contribute to progression of renal damage, they could be relevant candidate targets for protein kinase inhibitors.

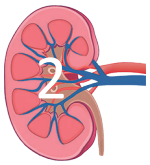


Table 1: Overview of drugs that have been coupled to carriers to achieve selective delivery to the kidney

Drug	Carrier	Targeted cell type	Model	Renal effects	Ref
Doxorubicin	Immunoliposomes/OX7 F(ab')	Mesangial	Healthy rats	Selective injury (proof of principle)	[106]
Actinomycin D	IBCA nanoparticles	Mesangial	Healthy rats	Renal accumulation of the drug	[108]
Dexamethasone	Immunoliposomes/Ab(Esel)	Endothelial	Anti-GBM	Reduction of albuminuria	[110]
Naproxen	Lysozyme	Tubular epithelial	Healthy rats	Renal accumulation of the drug	[118]
Lisinopril	Lysozyme	Tubular epithelial	Healthy rats	Reduced ACE activity, no effect on systemic BP	[121]
SB202190	Lysozyme	Tubular epithelial	I/R	Reduced pre-fibrotic markers	[122]
TKI	Lysozyme	Tubular epithelial	UUO	Reduced inflammation and tubular activation	[123][146]
Y27632	Lysozyme	Tubular epithelial	I/R	Reduced inflammation and fibrosis	[61]
Prednisolone	LMWC	Tubular epithelial	Healthy rats	Renal accumulation of the drug	[124]

IBCA, Isobutylcyanoacrylate; Esel, E-selectin; GBM, glomerular basement membrane; ACE, angiotensin I converting enzyme; BP, blood pressure; SB202190, p38 MAP kinase inhibitor; I/R, ischemia/reperfusion; TKI, TGF-beta receptor kinase inhibitor; UUO, unilateral ureteral obstruction; Y27632, Rho kinase inhibitor; LMWC, 50% N-acetylated low molecular weight chitosan.

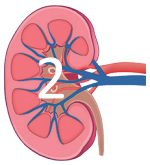
In the glomerulus, mesangial cells can be targeted using immunoliposomes decorated with OX7 F(ab') fragments (106, 107). Within the kidney these fragments bind to the Thy1.1 antigen that is specifically expressed by mesangial cells. Tuffin et al demonstrated that the

use of immunoliposomes to deliver doxorubicin indeed selectively damaged mesangial cells. Another type of nanoparticles, isobutylcyanoacrylate nanoparticles, have also been used to target a drug (in this case actinomycin D) to mesangial cells (108). However, no reports on mesangial delivery of protein kinase inhibitors are available yet.

A similar approach was used to target endothelial cells: in this case, immunoliposomes were decorated with anti-E-selectin antibodies (Ab(Esel)), which bind to inflamed endothelium (109). Efficacy of Ab(Esel) liposomes delivery was investigated by developing dexamethason-loaded Ab(Esel) liposomes. These liposomes reduced albuminuria in a model of anti-GBM nephritis without binding to nontargeted renal microvasculatures (110).

Although the glomerulus has previously been considered the pathophysiologically most relevant subunit of the nephron, research of the last decade has clearly underlined a crucial role of tubulointerstitial damage in the pathogenesis of progressive renal damage (111). Tubulointerstitial damage, which may or may not be secondary to glomerular injury (112), has become an important target for pharmaceutical intervention (113). In order to target tubular epithelial cells, glomerular filtration is the first step to reach the tubuli. It has been demonstrated that particles with a hydrodynamic diameter below 5–7 nm are rapidly cleared by renal filtration and urinary excretion (114). However, most particulate drug carriers have a size in the 10–200 nm range and thus renal targeting to tubular cells has not been studied with these systems. Rather, kidney-selective proteins and small synthetic polymers have been used for renal tubular cell targeting (as reviewed in (115)). Among these, one of the most used carrier for drug targeting to tubular epithelial cells is lysozyme. Lysozyme is a low-molecular weight protein that is filtered by the glomerulus and subsequentially taken up by tubular epithelial cells via megalin-mediated endocytosis, where it is degraded in lysosomes (116, 117). If coupled to lysozyme, a compound is thereby released locally in tubular epithelial cells or in the tubular lumen. Using this approach, the non-steroidal anti-inflammatory drug (NSAID) naproxen (118) and the ACE inhibitor captopril (119, 120) have been targeted to renal tubular epithelial cells. Lysozyme-captopril resulted in longer-lasting ACE inhibition as compared to the free drug, whereas systemic blood pressure was not affected by the coupled drug, as opposed to the free drug (121). In a recent study, we demonstrated that renal targeting of a p38 MAP kinase inhibitor not only reduced p38 activation in renal tubular epithelial cells, but this compound also reduced the renal pre-fibrotic marker alpha-smooth muscle actin, confirming local efficacy of the drug (122). The SB202190-lysozyme complex remained stable in serum but was dissociated, releasing the drug, in kidney homogenates (122). Similarly, a TGF-beta receptor kinase inhibitor was coupled to lysozyme, and tested for specific renal effects (123). Of note, we found that a single dose of the coupled drug inhibited the activation of tubular cells and fibroblasts in UUO (unilateral ureteral obstruction) rats and reduced renal inflammation. In contrast, free TGF-beta kinase receptor inhibitor at an equimolar (low) dosage exhibited little effects. Finally, we have coupled the Rho kinase inhibitor Y27632 to lysozyme (61). Y27632-lysozyme strongly reduced renal inflammation and fibrosis in a rat model of ischemia/reperfusion. As

opposed to the free drug, the coupled drug did not affect systemic blood pressure. Lysozyme-immunostaining confirmed selective localization of the conjugate in renal tubular epithelial cells. Together, these studies indicate that of low molecular weight proteins, lysozyme has been mostly used as carrier to target drugs specifically to the kidney. Lysozyme-coupled drugs have been demonstrated efficacy in several models of renal damage, without systemic (side-)effects as in the respective free drug. Thus, lysozyme-based drug targeting strategies could very well make it closer to clinical application, although extensive safety studies are lacking to date.



Similar to lysozyme, randomly 50% N-acetylated low molecular weight chitosan (LMWC) is another low molecular weight carrier that could be used to target drugs to tubular epithelial cells. Yuan et al have demonstrated selective accumulation of prednisolone in the kidney when prednisolone was covalently coupled to LMWC (124); the tubular uptake was megalin-dependent (125).

Besides tubular epithelial cells, also interstitial cells have been targeted. Interstitial fibroblasts are an important target for anti-fibrotic therapy, since these cells produce large amounts of extracellular matrix – one of the characteristic components of interstitial fibrosis (126). Yet, studies targeting interstitial fibroblasts are limited to gene therapy (not delivery of compounds) and data are preliminary (127-129). Gene targeting to the kidney has been reviewed excellently elsewhere (130, 131) and will not be discussed in detail here. Nevertheless, it is anticipated that selective drug targeting to renal interstitial fibroblast may be within reach within the coming decade.

CONCLUSIONS

The rising prevalence of ESRD urges the development of new therapies to reduce or even halt the progression of renal damage. One of the most promising classes of drugs that have been developed in recent years are protein kinase inhibitors. Yet, these compounds have been used mainly in haemato-/oncological clinical trials, and are available in the clinic for a limited number of patients with progressive malignant diseases. However, as the tolerability and safety of these compounds is improving, these drugs may become available to patients with other types of disease (e.g. renal disease) as well.

As discussed above, inhibitors of MAP kinases, Rho kinase and growth factor receptors may play a role in the development and/or progression of renal damage. Specific inhibitors of several of these protein kinases have been developed, most of which have demonstrated renoprotective effects in animal models. However, the translation to clinical practice has been impeded by poor tolerability and side-effects of protein kinase inhibitors (132, 133). Yet, the development of more sophisticated compounds, as well as organ-specific or even cell type-specific delivery of drugs, is expected to improve efficacy while reducing systemic side effects. Indeed, these improvements have been reported in animal studies.

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Although the clinical safety of locally delivered compounds themselves should be taken into account – many linker constructs for example contain platinum – the experimental data as summarized above are promising.

Where should we go from here? Now that the proof of principle for selective drug targeting without detectable systemic side effects has been demonstrated for several targeted drugs in several experimental models of renal disease, the next step is to transfer this experience to the clinic. Obviously, some multi-protein kinase inhibitors that have been in clinical trials and approved for haemato-oncological indications could be relatively easily tested for other indications as well, although the balance between efficacy and safety will remain a major issue. Whether or not organ-specific and/or cell type-specific drug delivery will turn out to be a clinically feasible therapy within the coming years remains a question. The positive results obtained in recent years in experimental models of renal damage, combined with the urgent need for (adjunct) therapies in CKD patients, may however speed up the development of pharmacological therapies specifically directed towards the kidney. This should allow halted progression or even repair of tissue damage, without systemic side-effects.

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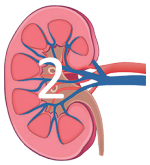

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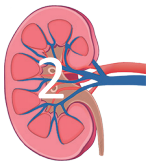

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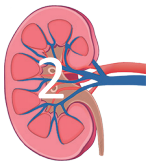

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