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Review

Renal targeting of kinase inhibitors

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ABSTRACT

Activation of proximal tubular cells by fibrotic and inflammatory mediators is an important hallmark of chronic kidney disease. We have developed a novel strategy to intervene in renal fibrosis, by means of locally delivered kinase inhibitors. Such compounds will display enhanced activity within tubular cells and reduced unwanted systemic effects. In our approach kinase inhibitors are linked to the renal carrier lysozyme using a platinum-based linker that binds drugs via a coordinative linkage. Many kinase inhibitors contain aromatic nitrogen atoms able to bind to this linker without the need of prior derivatization. The resulting drug-lysozyme conjugates are rapidly filtered in the glomerulus into the tubular lumen and subsequently reabsorbed via the endocytic pathway for clearance of low-molecular weight proteins. An important property of the formed conjugates is their *in vivo* stability and the sustained drug release profile within target cells.

This review summarizes the state-of-the-art of drug targeting to the kidney. Furthermore, we will highlight recent results obtained with kinase inhibitor-lysozyme conjugates targeted to different kinases, i.e. the transforming growth factor (TGF)-beta-receptor kinase, p38 MAPkinase and Rho-associated kinase. Both *in vitro* and *in vivo* results demonstrated their efficient tubular uptake and beneficial therapeutic effects, superior to treatment with free kinase inhibitors. These proof-of-concept studies clearly indicate the feasibility of drug targeting for improving the renal specificity of kinase inhibitors.

Contents

Abbreviations: α-SMA, alpha-smooth muscle actin; ALK5, activin-receptor like kinase; CKD, chronic kidney disease; CTGF, connective tissue growth factor; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transdifferentiation; ESRD, end-stage renal disease; G4D, generation 4 dendrimer; HK2 cells, human kidney proximal tubule cells; HPMA, *N*-(hydroxypropyl)methylacrylamide; I/R, ischemia–reperfusion; KSCN, potassium thiocyanate; LMWC, low molecular weight chitosan; LZM, lysozyme; MCP-1, monocyte chemoattractant protein-1; p38MAPK, p38 mitogen activated protein kinase; PAMAM, polyamidoamine; PDGF, platelet derived growth factor; PVD, poly(vinylpyrrolidone-co-dimethyl maleic acid); PVP, polyvinylpyrrolidone; ROCK, Rho-associated kinase; SOD, superoxide dismutase; TGF-β1, transforming growth factor beta-1; TIMP-1, tissue inhibitor of metalloproteinase-1; TKI, TGF-β receptor kinase inhibitor; TNF-α, tumor necrosis factor-alpha; ULS, Universal Linkage System; UUO, unilateral ureteral obstruction.

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1. Introduction

The number of patients with chronic kidney disease (CKD) has markedly increased during the last decades (Iseki, 2005; Levey et al., 2005; Ryan et al., 2007). Partly, the increase in CKD incidence relates to the increased incidence of diabetes, systemic hypertension, and obesity (Iseki, 2005; Cignarelli and Lamacchia, 2007). In addition to this, patients develop diabetes and hypertension more frequently and at younger age, which also leads to an increased incidence of CKD (Koopman et al., 2005; Rubenstein, 2005; Burns et al., 2007). Lastly, since worldwide ageing of the population takes place, the incidence of CKD is expected to further increase (El Nahas and Bello, 2005). In many cases CKD results in renal fibrosis, characterized by inflammation and fibrosis of glomeruli and/or tubulointerstitium (Liu, 2006).

Increased insight in the pathology of renal diseases along with the development of potent antifibrotic agents may lead to improved therapies for CKD. Kinase inhibitors targeted to fibrotic signaling cascades may serve as such antifibrotic agents. However, kinase inhibitors can also display many other undesired actions within the body. A more focused action in the kidney may improve their potential effectivity for CKD. This article provides (1) a short overview of different strategies used for targeted renal drug delivery, (2) explains how kinase inhibitors can be of benefit for the treatment of renal fibrosis and how they can be targeted to the kidney, and (3) addresses future potential application areas of renal drug delivery and the linkage technology used by our group to couple the kinase inhibitors to the kidney-targeted carrier systems.

2. Targeted renal drug delivery

Each kidney consists of millions of nephrons, which are the functional part of the kidney. Each nephron consists of a glomerulus, located in Bowman's capsule, and a tubular system composed of a proximal tubule, the loop of Henle and a distal tubule ending in the collecting duct. The blood is filtered in the glomerulus, where the primary urine is formed. Along the tubule, the primary urine is concentrated and many compounds are either reabsorbed or secreted from or into the urine, before it finally ends-up in the bladder.

When targeting drugs to the kidney, it is essential to identify which part of the nephron or cell type needs to be targeted. Important considerations in this are which cell types are involved in the disease pathology, the mechanism of action of the drug and the renal handling of the drug carrier system. Different targeting approaches have been investigated for the delivery of drugs to either the glomerulus or renal tubuli.

2.1. Targeting to the glomerular cells

The glomerulus is in direct contact with the bloodstream. Glomerular cells can hence be targeted by intravenously administered particulate carrier systems that do not filter into the urine. Both endothelial cells which line up the vessel walls and mesangial cells can be reached from the bloodstream, because the glomerular endothelium is fenestrated and lacks a basement membrane between the glomerular capillaries and the mesangial cells. Tuffin et al. (2005, 2008) investigated the possibilities to target mesangial cells with immunoliposomes decorated with OX7 F(ab′) fragments, which bind Thy 1.1. antigen expressed by this cell type. When tested in rats, a rapid clearance of the particles from the bloodstream was observed together with a significant accumulation in mesangial cells. As a proof-of-concept, tissue specific damage in the glomeruli was inflicted with OX7-immunoliposomes loaded with doxorubicin. Recently, immunoliposomes have also been used for the delivery of anti-inflammatory drugs to the glomerular endothelial cells (Asgeirsdottir et al., 2007). Successful targeting to glomerular endothelial cells was achieved by immunoliposomes decorated with anti-E-selectin antibodies, which bind to inflamed endothelium. When evaluated in a glomerulonephritis model, this type of dexamethasone-immunoliposomes inhibited proinflammatory gene expression in the glomeruli and reduced renal damage, while systemic glucocorticoid activity was diminished (Asgeirsdottir et al., 2008).

2.2. Targeting to tubular cells

Targeting of drugs to the proximal tubular epithelial cells in the kidney can be relatively easily achieved from the tubular lumen, since there is no endothelial layer between the epithelial cells and the tubular fluid. Furthermore, proximal epithelial cells express high levels of internalizing receptors at their luminal membrane, which are able to take up a broad variety of the compounds that have been filtered in the glomerulus into the urine. This reabsorptive capacity of proximal tubular cells opposes the loss of valuable endogenous molecules into the urine. The efficiency of this system is for instance exemplified by the absence of glucose and proteins in the urine of normal subjects.

Glomerular filtration is the gateway to the tubular lumen, which sets a limit to the maximum size of drug carriers applicable to tubular cell targeting. It has been proven that particles with a hydrodynamic diameter below 5–7 nm are rapidly cleared by renal filtration and urinary excretion (Choi et al., 2007). As most particulate drug carriers have a size in the 10–200 nm range, renal drug targeting to tubular cells has not been studied with these systems. Rather, renal-selective proteins and small synthetic polymers have been used for renal tubular cell targeting.

One of the best-studied carriers for tubular cell drug targeting is the low molecular weight protein lysozyme. Previously, it has been shown that low molecular weight proteins with different p*I* such as cytochrome *C*, aprotinin and lysozyme are extensively accumulated in the kidney in proximal tubular epithelial cells (Haas et al., 1993). Later studies have revealed that the tubular accumulation is mediated via the megalin receptor (Christensen and Verroust, 2002). Fig. 1 shows the principle of drug targeting to the kidneys using lysozyme. After glomerular filtration lysozyme is recognized by the megalin receptor at the luminal membrane of the proximal tubular epithelial cells. Binding results in internalization and routing to the lysosomes, where degradation of lysozyme occurs. Drugs attached to lysozyme are released during this process and may act intracellularly or be transferred to the tubular lumen of the kidney. This approach has been applied successfully for delivery of drugs like the non-steroidal anti-inflammatory drug naproxen and the angiotensin converting enzyme inhibitor captopril (Haas et al., 1997; Kok et al., 1999; Prakash et al., 2005b). We will discuss this technique more extensively in a later part of this review, in relation to its application as a carrier for kinase inhibitors.

Polymeric carriers that have been described for renal drug delivery are anionized derivatives of polyvinylpyrrolidone (PVP) (Kamada et al., 2003; Kodaira et al., 2004), and low molecular weight chitosan (LMWC) (Yuan et al., 2007). Derivatives of another type of polymer, *N*-(hydroxypropyl)methylacrylamide (HPMA), may also end-up in the kidneys when its size is below the glomerular threshold and its modification enhances interactions with renal cells (Kissel et al., 2002; Ghandehari, 2008). Lastly, dendritic polymers have been used for renal imaging and may also be of use as carrier systems for tubular drug targeting.

In vivo studies in mice have shown that low molecular weight anionized PVP derivates accumulate extensively in the proximal

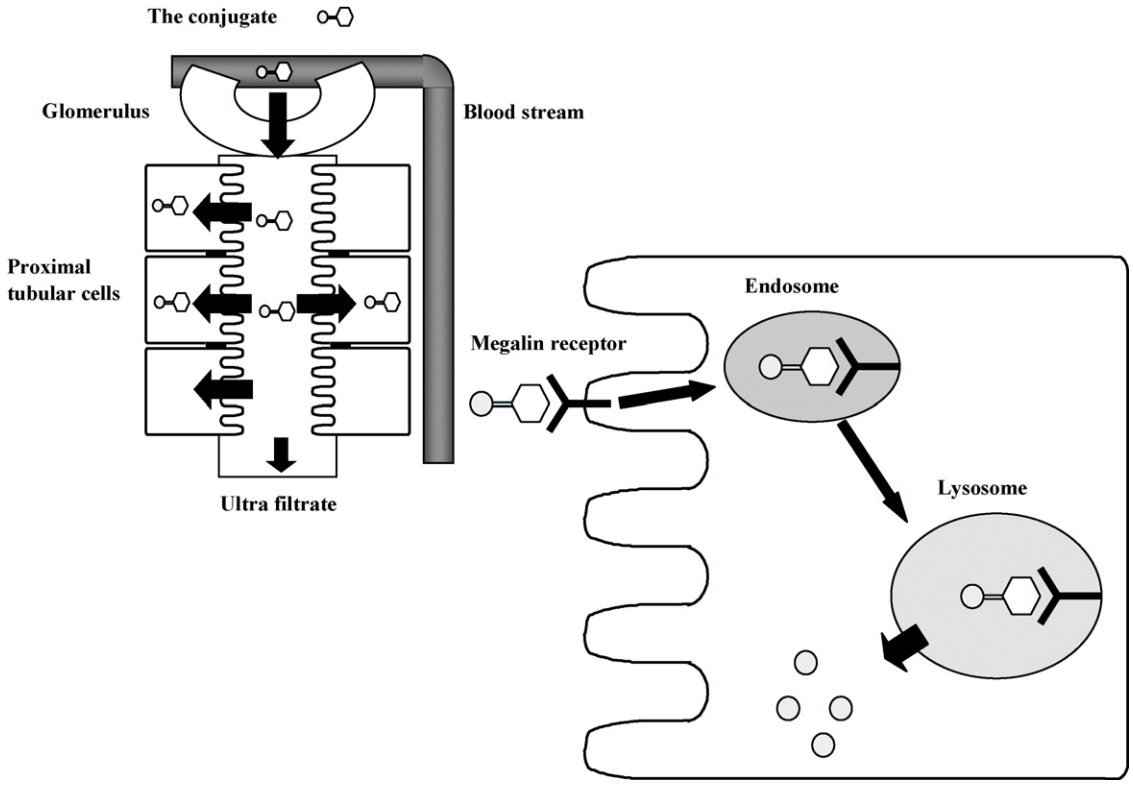

Fig. 1. Principle of drug delivery to the kidneys with lysozyme conjugates. Nephrons are the functional part of the kidney, each consisting of a glomerulus and the renal tubuli. The glomerulus is the basic filtration unit of the kidney, responsible for the formation of a plasma ultra filtrate. In the first part of the tubule (also called the proximal tubule), reabsorption of many endogenous compounds like amino acids, glucose and proteins occurs. The property of proximal tubular cells to reabsorb small molecules is used in our delivery approach to target kinase inhibitors to the kidney. In short, after glomerular filtration drug-LZM conjugates specifically accumulate in proximal tubular epithelial cells by recognition of the megalin receptor, which is responsible for reabsorption of proteins. The megalin receptor is expressed in high density at the luminal membrane of the cells. Free drug will be released intracellularly from the conjugate after lysosomal degradation of the drug-LZM conjugate.

tubular epithelial cells (Kodaira et al., 2004). In contrast, normal neutral PVP does not accumulate in the tubular cells and is excreted in the urine. Of special importance is the type of anionic groups introduced in the PVP polymers. While carboxylated PVPs showed relatively high renal accumulation, sulfonated PVPs showed only little renal accumulation. The highest renal accumulation was obtained with 20% carboxylated PVP, of which 30% of the injected dose accumulated in the kidney. Both non-carboxylated and 100% carboxylated PVP hardly accumulated in the kidneys. Another carboxylated PVP, poly(vinylpyrrolidone-co-dimethyl maleic acid) (PVD) also showed extensive kidney accumulation (Kamada et al., 2003). Intravenous administration of PVD in mice resulted in a remarkable accumulation of almost 80% in the proximal tubular cells 24 h after administration. Approximately 60% of the administered PVD had been eliminated in the urine at 4 days after its administration, indicating that PVD stays in the body for a long time. PVD was used as a carrier for the delivery of the protein drug superoxide dismutase (SOD). The PVD-SOD conjugate showed therapeutic effects in an acute renal failure model. Until now, it has not been investigated whether this type of carrier can be applied for the delivery of small molecule drugs.

LMWC which had been randomly acetylated for 50% was used to deliver prednisolone to the renal tubular cells (Yuan et al., 2007). Delivery of prednisolone using LMWC with a molecular weight of 19 kDa resulted in the highest renal accumulation. A maximum accumulation of approximately 15% was obtained 15 min after intravenous administration in mice, which is comparable to the accumulation observed for low molecular weight proteins (as depicted in Fig. 4). The mechanism by which LMWC is being taken up by the renal tubular cells is unknown. Despite the efficient renal targeting by prednisolone-LMWC, the amount of drug in the kidneys decreased quickly, indicating the rapid release of the delivered drug from the carrier and its rapid elimination or redistribution from the renal tissue.

Another polymer for which accumulation in the kidney has been reported is HPMA. Kissel et al. (2002) investigated the *in vivo* biodistribution of biotinylated HPMA in rats and compared this with the biodistribution of non-biotinylated HPMA. It was found that the biodistribution profiles of 26 kDa polymers were comparable except for the kidneys, which showed an increased uptake of biotinylated HPMA at day 7 after intravenous injection. The accumulation of biotin-HPMA in proximal tubular cells was 33 times higher as compared to normal HPMA. Of note, for both polymers, the highest accumulation was found in spleen, liver and tumor. Biotin-HPMA showed a renal uptake of 4.7% of the initial dose per gram, while in case of HPMA this percentage was only 0.14%. Assuming that the weight of both kidneys in rats is about 0.9% of total body weight (de Cassia da Silveira and de Oliveira Guerra, 2007), the total accumulation of biotin-HPMA as percentage of the injected dose in both kidneys was 8.5%. The renal accumulation may be explained by the presence of a biotin transporter on the brush border of proximal tubular cells (Baur and Baumgartner, 1993), which binds the renally filtered polymers.

More recently, renal accumulation of RGD-decorated HPMA was also reported (Ghandehari, 2008). Although developed for the purpose of targeting to tumor endothelium, RGDfK-HPMA 43 kDa showed extensive renal accumulation, far exceeding the attained levels in the tumor tissue. Similar to biotinylated HPMA, a prolonged retention was found which lasted for days, indicating the slow renal degradation of these polymers.

Although dendrimers have not been investigated for renal drug targeting yet, they have been successfully applied for the purpose of magnetic resonance imaging (MRI) to detect structural and functional abnormalities of the kidneys (Kobayashi et al., 2004). Dendrimers that were used for the development of renal MRI contrast agents are polyamidoamine (PAMAM) and diaminobutane. PAMAM-based macromolecular MRI contrast agents with a hydrodynamic diameter of less than 8 nm were rapidly cleared from the circulation by glomerular filtration and subsequently excreted by the kidneys or taken up by the renal tubuli (Kobayashi et al., 2003, 2004). Both dendrimers of the 4th (G4D) and 5th generation were used to prepare gadolinium loaded contrast agents. When examined in mice, a rapid and extensive accumulation in the kidneys was found, with highest renal tissue levels obtained with G4D (Kobayashi et al., 2001). Although the uptake by proximal tubular cells was not studied directly, indirect evidence was obtained by simultaneous injection of G4D contrast agent and lysine, which competes for megalin binding. An increased urinary excretion of G4D was observed.

2.3. Gene targeting to the kidney

Also in the field of gene delivery, attention has been given to the targeting of the glomerulus and renal tubuli. This topic has been reviewed excellently by others and we will not elaborate on this subject (see for instance Han et al., 2000; van der Wouden et al., 2004; Isaka, 2006). Examples of targeting approaches and systems that have been used for renal gene delivery are liposomes, adenoviruses, and the administration of naked plasmids or antisense oligonucleotides. Besides the choice between vectors, also the route of administration plays an important role in the renal targeting of the different cell types in the kidney (van der Wouden et al., 2004).

3. Renal fibrosis

Renal fibrosis is the result of activation of renal cells by inflammatory cytokines (e.g. IL-1β and TNF-α) and proteinuria, leading to the production of chemokines, adhesion molecules and other mediators, leading to tubulointerstitial inflammation (Eddy, 2000; Strutz and Neilson, 2003). Besides inflammation, also epithelial-to-mesenchymal transition (EMT) of proximal tubular epithelial cells into myofibroblasts occurs (Strutz and Neilson, 2003). Stimulation of fibroblasts by cytokines and growth factors (e.g. TGF-β, CTGF, PDGF, and EGF) leads to the excessive formation of extracellular matrix and the replacement of nephrons by scar tissue (Eddy, 2000; Strutz and Neilson, 2003).

Although the kidneys have an overcapacity of nephrons, it is not infinite and loss of kidney function eventually can lead to the development of end-stage renal disease (ESRD). Patients with ESRD need renal replacement therapy, by dialysis or transplantation, which is a high burden for the patient and very expensive (El Nahas and Bello, 2005).

One of the approaches in the treatment of renal fibrosis is prevention by treatment of the underlying cause, e.g. hypertension or diabetes mellitus. However, since renal diseases are often diagnosed at a late stage, it is also important to develop other therapeutic possibilities to arrest the progression of renal fibrosis, or even to reverse it. Activation of proximal tubular epithelial cells leading to the onset of different signaling pathways plays an important role in the progression of renal fibrosis. Kinases play an important role in these signaling pathways, and offer an interesting target in the treatment of renal fibrosis (Sharpe and Hendry, 2003; Hayashi et al., 2006; Liu, 2006; Burns et al., 2007). Both *in vitro* studies with proximal tubular cells and studies in animal models have shown the beneficial effects of kinase inhibitors in renal fibrotic disease (Francois et al., 2004; Zhang et al., 2005; Prakash et al., 2006, 2008a,b; De Borst et al., 2007). We therefore have investigated the specific targeting of kinase inhibitors to the proximal tubular cells. Targeted delivery of kinase inhibitors has several potential benefits. Firstly, high concentrations of kinase inhibitors in the proximal tubular epithelial cells will result in an enhanced efficacy within the kidneys. Secondly, specific targeting will reduce unwanted systemic effects. Until now kinase inhibitors are only clinically used for the treatment of cancer (Madhusudan and Ganesan, 2004; Ross et al., 2004; Keri et al., 2005). Kinase inhibitors are considered targeted drugs. However, this targeted feature refers to their specific mode of action (Fabian et al., 2005), rather than to targeted delivery. Kinase inhibitors are not cell- or tissue specific, and will thus act in both unhealthy and healthy cells in which the molecular target is expressed and activated. Kinases play an important role in many physiological processes in the human body (Harper and LoGrasso, 2001; Adams and Sweatt, 2002; Vlahos et al., 2003) and, consequentially, their inhibition in healthy cells may result in adverse effects. Recently, it has been shown that tyrosine kinase inhibitors are associated with cardiotoxicity (Kerkela et al., 2006; Force et al., 2007), bleeding, and neurological complications (Eskens and Verweij, 2006). An example of such an inhibitor is the well-known breakpoint cluster region-Abelson (BCR-ABL) tyrosine kinase inhibitor imatinib (Kerkela et al., 2006; Force et al., 2007). One of the techniques used to prevent cardiac toxicity of imatinib is structural reengineering, which yielded a derivative with an altered kinase inhibitory profile (Fernandez et al., 2007). This new compound showed decreased cardiac side effects but maintained the antitumor properties of imatinib. Adverse reactions can be divided in on-target and off-target side effects. Once the toxicity of the kinase inhibitor is directly related to the intended pharmacological response it is called an 'on-target' side effect. Off-target side effects are related to inhibition of off-target kinases or to other non-kinase dependent mechanisms (Temming et al., 2008). Structural reengineering is a valid approach for drug molecules in which the toxicity can be dissected from the intrinsic activity of the compound but cannot be used to reduce toxicity directly associated with the pharmacological activity. In the latter case, cell-specific targeting of kinase inhibitors is an attractive approach.

4. Design and synthesis of kinase-inhibitor-lysozyme conjugates

When using a carrier system for the specific targeting of kinase inhibitors to the proximal tubular cells, it is important to use a carrier which is biodegradable. If a polymeric carrier is poorly degradable, its lysosomal accumulation may result in toxicity of the carrier. Proteins are natural polymers which display excellent lysosomal biodegradability, while in case of many synthetic polymers a lack of biodegradability is a concern. We therefore applied the low molecular weight protein lysozyme as tubular cell-specific carrier for the delivery of kinase inhibitors.

Kinase inhibitors were linked to lysozyme using the platinum(II)-based Universal Linkage System (ULS^{TM}) as a new coupling agent (Prakash et al., 2006, 2008a; Fretz et al., 2007). ULS binds to thioether groups (present in methionine residues of the proteinaceous carrier) and to aromatic nitrogens. Many kinase inhibitors contain an aromatic nitrogen atom, and can therefore be coupled to ULS via a coordinative bond without prior derivatization steps. An important property of platinum–ligand bindings is that coordinative bindings dissociate very slowly, thereby providing adequate stability of the conjugates (Prakash et al., 2006; Gonzalo et al., 2007; Reedijk, 2008). On the other hand, the platinum

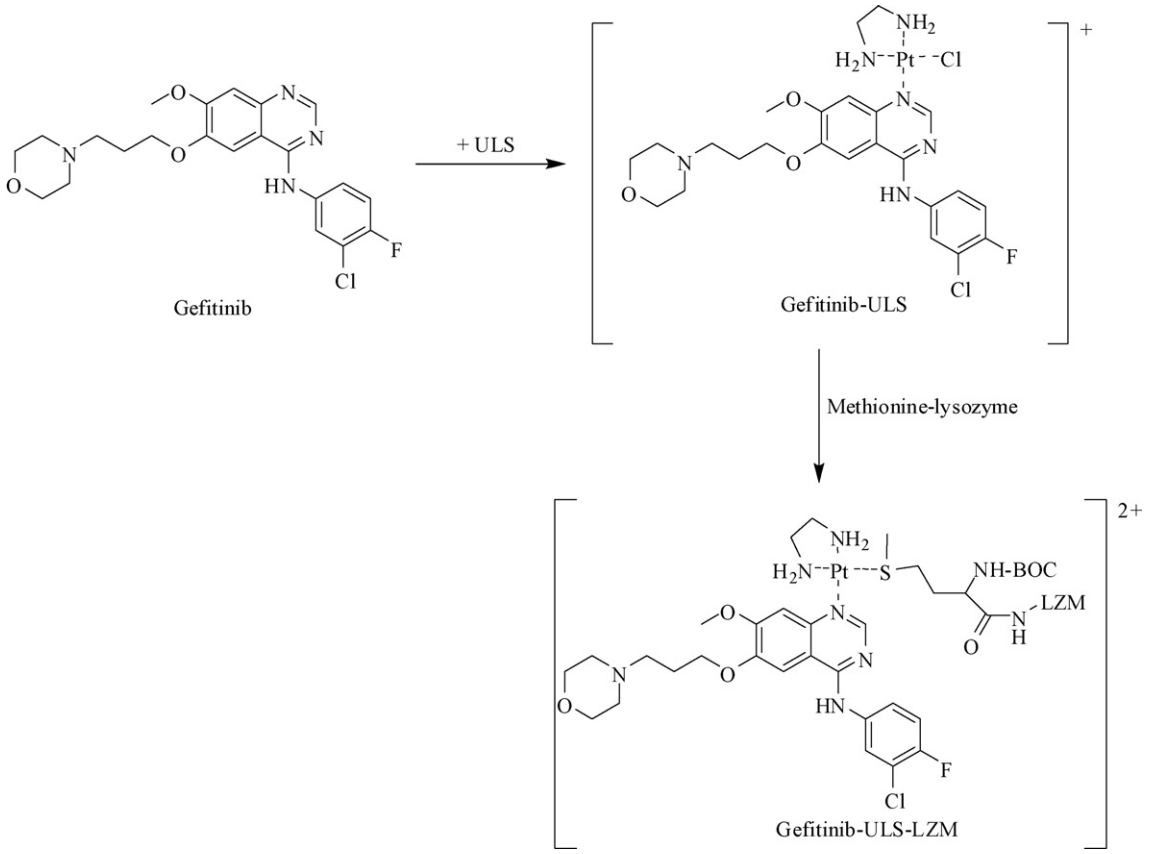

Fig. 2. Reaction scheme of the linkage of gefitinib to the platinum linker, and its subsequent conjugation to lysozyme by reaction to methionine residues in lysozyme. Gefitinib was reacted with ULS in DMF at 37 °C for 9 h. During this reaction the platinum atom in ULS bound the aromatic nitrogen atom present in gefitinib. After characterization and purification of gefitinib-ULS, gefitinib-LZM was synthesized by reacting gefitinib-ULS to methionine-LZM for 24 h at 37 °C and pH 8.5. The platinum atom in ULS bound the thioether present in the methionine residue of LZM.

coordination bonds are also bioreversible and can intracellularly release the conjugated drug via competitive displacement with glutathione (Gonzalo et al., 2006; Temming et al., 2006b).

Fig. 2 shows the linkage of the kinase inhibitor gefitinib (Iressa®) to the platinum linker, and the subsequent conjugation of gefitinib-ULS to lysozyme. Typically, these conjugates are synthesized by coupling the drug to the platinum linker at a slight excess of the drug. Consumption of the parent drug is detected by HPLC analysis of small aliquots of the reaction mixture. Once the reaction has been completed, the remaining traces of non-reacted linker were precipitated by addition of NaCl, yielding the poorly water-soluble ULS-dichloride linker. Gefitinib-ULS was synthesized using equimolar amounts of ULS and gefitinib and subsequently coupled to methionine groups in lysozyme by overnight incubation at 37 °C.

Although lysozyme contains two methionine residues, the protein does not bind drug-ULS readily since these residues are buried in the core of the protein. We therefore introduced surface-exposed methionine residues into lysozyme by chemical derivatization of lysyl residues with methionine-NHS. Such a chemically derivatized methionine-lysozyme (met-LZM) carrier prototype can be replaced in the future by a recombinantly produced protein with surface-accessible Pt(II) targets. Typically, the chemical derivatization of LZM yielded a carrier equipped with approximately one extra methionine residue, as determined by mass spectrometry (Prakash et al., 2006).

HPLC analysis of gefitinib-LZM provided a broad peak (Fig. 3B), likely consisting of different topological isomers. Those isomers result from the ability of methionine-NHS to bind to different lysine residues in lysozyme, and also have been observed for other types of lysozyme-derivatives (Salmain et al., 2001; Masuda et al., 2005; Teske et al., 2007).

Incubation with an excess of KSCN, a known ligand for platinum, resulted in drug release from the conjugate by competitive displacement (Fig. 3C). This result clearly demonstrates that binding of gefitinib to LZM via ULS is reversible. Similar results were obtained for other kinase inhibitor conjugates. On the other hand, no release of the drug was observed upon incubation of drug-ULS-LZM conjugates in PBS for up to 24 h and at 37 °C. This indicates adequate stability of the conjugates during storage (Prakash et al., 2006; Temming et al., 2006a,b; Gonzalo et al., 2007).

5. Biological stability drug-ULS-LZM conjugates

The biological stability of drug-ULS-LZM conjugates has been studied by *in vitro* incubation of conjugates in either rat serum or kidney homogenates, or by incubating the conjugates in 5 mM glutathione (Gonzalo et al., 2006; Prakash et al., 2006). Typically, less than 2% release of the drug was observed upon incubation in serum for up to 24 h at 37 °C (Prakash et al., 2006, 2008a). On the other hand, slow-release of the coupled drug was observed in the presence of competing glutathione or in the presence of kidney homogenate (at pH 5.0 and 7.4), suggesting that the drug can be released once the conjugates have been reabsorbed by proximal tubular cells (Gonzalo et al., 2006; Prakash et al., 2006).

Adequate stability in the circulation can be expected on the basis of two observations: firstly, the *in vitro* drug release rate in serum is much slower than the rate at which the conjugates will be accumulated in proximal tubular cells. While the $t_{1/2}$ of drug

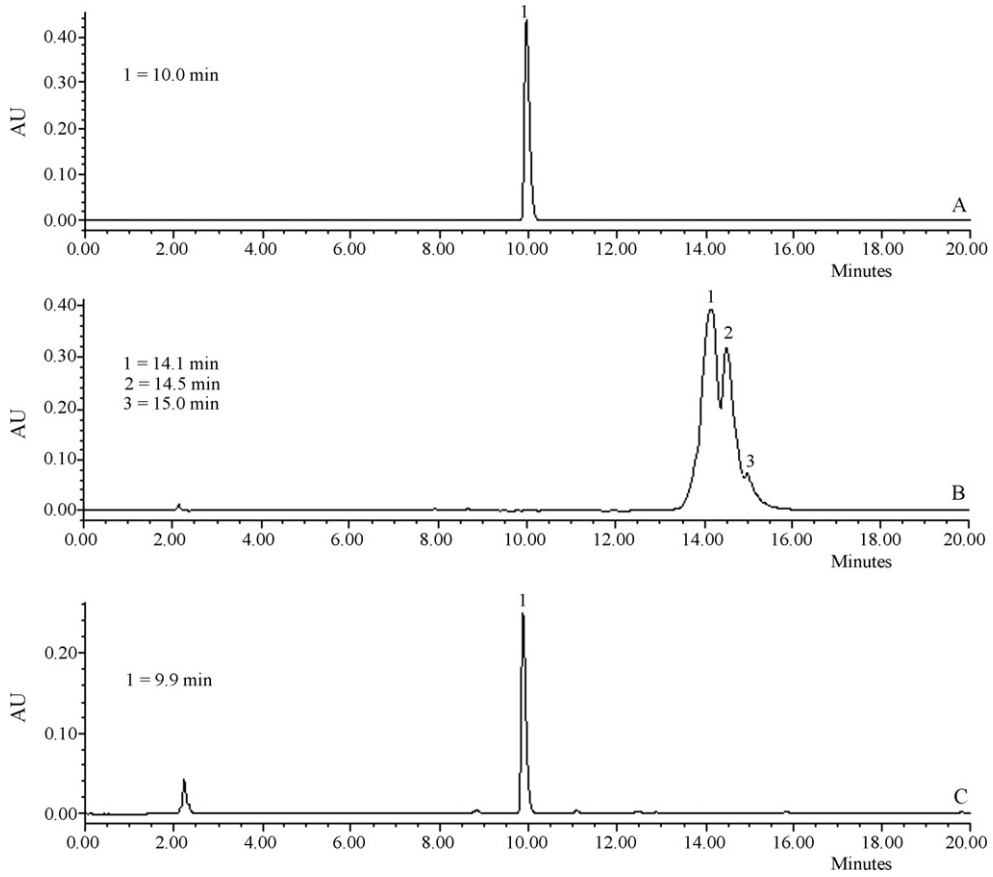

Fig. 3. HPLC chromatograms of free gefitinib (A), gefitinib-ULS-LZM (B) and gefitinib-ULS-LZM after incubation with an excess of KSCN at 80 °C (C). Compared to free gefitinib (A) the conjugate gives a broad peak, consisting of different topological isomers (B). This results from the ability of methionine-NHS to bind to different lysine residues in lysozyme. Subpart B also shows there is no free gefitinib present after synthesizing the conjugate, as shown by the absence of a peak at a retention time of approximately 10 min. Neither is gefitinib-ULS present, which gives peaks at 8.7, 10.4 and 11.9 min (data not shown). It furthermore can be concluded that binding of gefitinib to LZM via ULS is reversible, as shown by subpart C which shows the disappearance of the gefitinib-LZM peak and reappearance of the parent drug after competitive drug displacement with KSCN. Samples were separated on a C_4 reversed-phase column, using a water–acetonitrile–trifluoroacetic acid gradient, and monitored at a wavelength of 270 nm.

release is in the order of days, drug-lysozyme conjugates disappear from the circulation with a $t_{1/2}$ of 25 min, providing maximal renal accumulation within 2 h (Kok et al., 1999; Prakash et al., 2005b). Secondly, the drug release rate in kidney homogenate was much higher than the release in serum, in line with the different levels of thiols in both matrices (Prakash et al., 2006). We confirmed these expectations in our pharmacokinetic studies with drug-LZM conjugates prepared with three different kinase inhibitors, directed to either p38 MAPkinase, TGF-β type 1 receptor kinase (ALK5) or Rho-associated kinase (ROCK) (Prakash et al., 2006, 2008a,c). These conjugates showed similar distribution and clearance profiles to the lysozyme carrier, and accumulated in the kidneys at about 20% of the injected doses (Fig. 4) in the first hours (1–6 h) post injection (Prakash et al., 2006). Furthermore, we observed a prolonged residence of the delivered drug within the kidneys, as evidenced by the persistence of renal drug levels of the p38MAPK inhibitor SB202190 for several days after a single dose administration (Prakash et al., 2006). Pharmacokinetic data of the free kinase inhibitors are not available and we have not performed in depth pharmacokinetic studies for each of the individual test drugs, due to the large amounts needed of these costly compounds. However, the direct intravenous administration of one of these compounds, SB202190, showed that less than 0.5% of the free drug accumulated in the kidneys (Prakash et al., 2005a). The renal accumulation of the SB-LZM conjugate therefore greatly exceeded the accumulation of the free drug, indicating the feasibility of our targeting approach.

The biodistribution of drug-lysozyme conjugates to other organs has also been investigated. Immunostaining for drug-lysozyme conjugates showed the absence of the conjugate in liver, spleen and lungs (Prakash et al., 2008a). The preferential renal accumulation can be explained by the rapid glomerular filtration of low

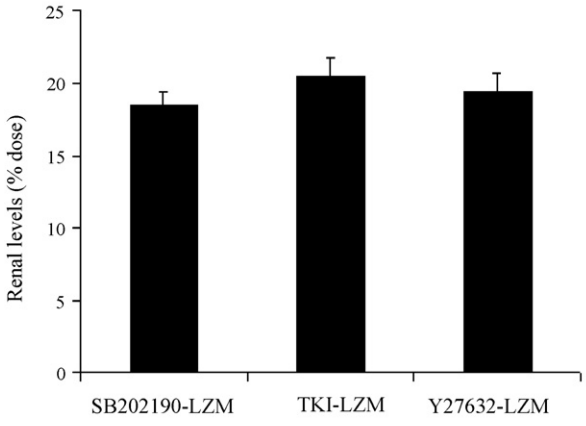

Fig. 4. Renal accumulation levels of drug-LZM conjugates. Single dose intravenous injection in rats with three different conjugates resulted in comparative renal accumulation. Shown values are the average values of renal C_{max}, as calculated from the renal concentrations between 1 and 6 h (mean ± S.E.M.) (Prakash et al., 2006, 2008a,c).

molecular weight proteins, and the efficient tubular reabsorption via megalin. Although megalin is predominantly expressed by the proximal tubular cells in the kidney and much lower levels of expression are found in other cell types, such as epithelial cells of the small intestine (Christensen and Verroust, 2002), this does not largely diminish the renal accumulation of lysozyme. A plausible explanation is that the megalin receptor is expressed at the luminal side of the epithelial cells of the small intestine making it not accessible for intravenously administered lysozyme.

6. *In vitro* and *in vivo* effects of drug-LZM conjugates

The above-mentioned conjugates directed to profibrotic cascades, TKI-LZM, SB202190-LZM and Y27632-LZM, have been investigated for efficacy *in vitro* in cultured kidney cells and *in vivo* in rats.

The first conjugate, TKI-LZM, is a TGF-β type I receptor kinase inhibitor. TGF-β plays a pivotal role in renal fibrosis by inducing synthesis of matrix proteins and by decreasing their degradation. It furthermore induces tubular epithelial-to-mesenchymal transdifferentiation (EMT) and the proliferation of fibroblasts (Fan et al., 1999; Bottinger and Bitzer, 2002; Razzaque et al., 2002; Laping, 2003; Docherty et al., 2006). Recent *in vitro* and *in vivo* experiments demonstrated the inhibitory effects of TKI-LZM on the MCP-1 mRNA expression and on the fibrosis marker α-SMA (Prakash et al., 2008a). We furthermore demonstrated that TKI-LZM effectively inhibited the phosphorylation of Smad2, a downstream target of the activated TGF-β receptor, in HK2 cells (Fig. 5). We found a reduction in TGF-β1-induced phosphorylation of Smad2 of approximately 50% when treated with TKI-LZM. In case of an equimolar concentration of unbound TKI a reduction of approximately 95% was found. An explanation for the relative higher potency of the free drug is that TKI-LZM needs to be internalized and processed by the cells to release free drug from the conjugate. In contrast, unbound TKI can readily pass the cell membranes by passive diffusion because of its relatively high lipophilicity (calculated log *P* is 2.85). The pharmacological superiority of TKI-LZM can therefore not be demonstrated

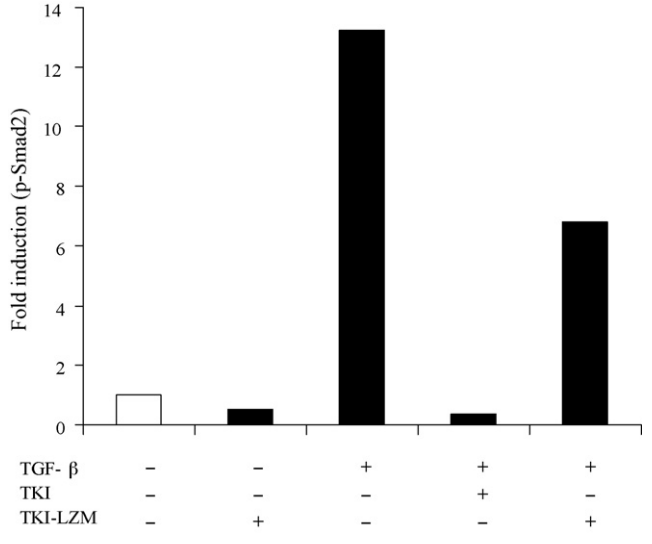

Fig. 5. Effects of TKI and TKI-LZM on TGF-β1-induced phosphorylation of Smad2. Cells were grown to 80% confluency in a 6-well plate and then deprived from serum for 24 h. Cells were preincubated for 24 h with TKI-LZM (10 μM) or for 30 min with TKI (10 μM) and then activated with 10 ng/ml TGF-β1 for 30 min. Phospho-Smad2 levels were detected by anti-phospho-Smad2 Western blotting (Cell Signaling Antibody #3101S) directed to phosphorylated amino acids in Smad2 (Ser465 and Ser467) and normalized to resting cells by β-actin levels (white bar).

Table 1
In vivo inhibitory effects of drug-LZM conjugates on profibrotic markers

Animal model + conjugate	Gene expression	Immunohistochemical markers
UUO + TKI-LZM	MCP-1 ↓	α-SMA ↓ Macrophage influx ↓ Vimentin ↓
I/R + SB202190-LZM		α-SMA ↓ p-p38 ↓
I/R + Y27632-LZM	MCP-1 ↓↓ Procollagen Iα1 ↓ TGF-β1 ↓ TIMP-1 ↓ α-SMA ↓↓	Macrophage influx ↓ Vimentin ↓

In vivo experiments are performed in the unilateral ureteral obstruction (UUO) rat model and ischemia–reperfusion (I/R) rat model. Mentioned profibrotic markers in this table are elevated in both animal models. The symbol ↓ means drug treatment resulted in a significant decrease in gene expression or immunohistochemical staining up to 50%, the symbol ↓↓ means a decrease of 50–90%. All results reported in this table are against non-treated control animals. UUO rats were injected intravenously with a single dose of 25 mg/kg TKI-LZM (equivalent to 630 μg/kg TKI). Rats were sacrificed after 3 days. Treatment with TKI-LZM resulted in a decrease in the gene expression level of MCP-1 and a decrease in α-SMA, macrophage influx and vimentin (Prakash et al., 2008a). Treatment of I/R rats with a single intravenous injection of 32 mg/kg SB202190-LZM (equivalent to 752 μg/kg free SB202190) resulted in a decrease in α-SMA and p-p38 4 days after administration (Prakash et al., 2006). In case of Y27632-LZM I/R rats were daily treated with an intravenous injection of 20 mg/kg conjugate (equivalent to 555 μg/kg Y27632) for four days. Treatment resulted in a decrease in gene expression levels of MCP-1, Procollagen Iα1, TGF-β1, TIMP-1 and α-SMA and in a decrease of vimentin and macrophage influx (Prakash et al., 2008c).

in vitro, but requires *in vivo* testing in which local delivery provides increased concentrations of TKI in the kidneys.

The second investigated drug-LZM conjugate is SB202190-LZM, which targets the p38MAPK pathway. *In vitro* research in HK2 cells demonstrated a reduction in TGF-β1 induced procollagen-Iα1 mRNA expression. Single dose administration of SB202190-LZM in a unilateral renal ischemia–reperfusion rat model resulted in a reduction of intrarenal p38MAPK phosphorylation and α-SMA protein expression (Prakash et al., 2006).

The third conjugate investigated, prepared with the Rho kinase inhibitor Y27632, has also been evaluated in the unilateral renal ischemia–reperfusion rat model (Prakash et al., 2008c). Upon daily treatment for four days, Y27632-LZM improved histological parameters as well as gene-expression levels of fibrotic and inflammatory markers. The collective results of the three conjugates have been summarized in Table 1. In all studies the reported effects were compared to untreated control animals. Inhibitory effects were detected on gene expression levels of profibrotic and inflammatory mediators and genes related to extracellular matrix production, and on immunohistochemical markers of fibrosis and EMT. Furthermore a reduction in the number of infiltrated macrophages was observed, indicating that renal inflammation had been inhibited. No antifibrotic effects of lysozyme were observed in control experiments with cultured tubular cells, while kinase inhibitor-lysozyme conjugates are clearly effective. This illustrates that the observed effects can be attributed to the attached drug, rather than to the carrier itself. Windt et al. (2004) investigated the antifibrotic effects of lysozyme, and a captopril-lysozyme conjugate *in vivo* in adriamycin-induced proteinuric rats. It was found that captopril-LZM had antifibrotic effects, while lysozyme alone had no effects.

We also investigated the safety of the applied platinum-linker. Obviously, this is a serious concern in light of the information available on the nephrotoxicity of cisplatin-based cytostatics. Cisplatinum exerts its effect by cross-linking DNA, which is the result of the availability of free reactive sites at the platinum atom

(Temming et al., 2008). No toxic effects were observed when studying the acute toxicity of SB202190-LZM in rats (Prakash et al., 2006).

7. Future perspectives

Drug targeting may play an important role in the development of more specific agents for the treatment of all kinds of renal diseases. Renal drug targeting aims for a higher efficacy of the drug along with avoidance of unacceptable side effects. Different drug targeting approaches are attractive, depending on the type of kidney cells that are involved in the pathogenesis of the disease. Liposomal carriers seem promising systems for glomerular targeting. For drug targeting to the proximal tubular cells, carboxylated PVP derivates, 4th generation dendrimers and small proteins like lysozyme are promising carrier systems. These carrier systems exploit the rapid glomerular filtration of medium-sized macromolecules and give a high renal accumulation. The advantage of proteinaceous carriers is that they are efficiently degraded in the lysosomes of the target cells.

Although not described extensively in this article, targeted gene delivery also offers interesting possibilities for inhibiting renal diseases processes. As compared to other organs, an advantage of targeting to the kidneys is that the proximal tubular cells are able to reabsorb different substances including nucleic acids.

This paper discussed a new type of kinase inhibitor-lysozyme conjugates for the delivery of kinase inhibitors to tubular cells in the kidney. These products may find future application in the treatment of renal fibrosis and other types of kidney diseases. Besides kinase inhibitors, other types of drugs can be linked via the ULS linker technology to lysozyme, as long as the drugs contain an aromatic nitrogen atom or other donor group that can coordinate to platinum. Also other carriers than lysozyme can be used, directed to the kidney cells or to other types of target cells outside the kidney, providing that the linker can be reacted to the carrier system. We already have explored two other classes of kinase-inhibitor-carrier conjugates, either directed to angiogenic endothelial cells in tumors or to hepatic stellate cells in the liver (Temming et al., 2006a; Gonzalo et al., 2007). In the near future, we will explore the feasibility of targeting kinase inhibitors to tumor cells, by means of monoclonal antibody carriers.

8. Conclusion

Renal drug targeting is an interesting technique for improving the treatment of renal diseases for which no adequate therapies exist, such as renal fibrosis. Both glomerular and tubular targeting strategies have been developed successfully. A great part of the kidney consists of specialized tubular cells, able to reabsorb endogenous compounds from the tubular lumen by receptor-mediated uptake. The unique properties of this cell type offer good opportunities for tubular cell-specific drug delivery, exemplified in this manuscript by the megalin-mediated uptake of lysozyme-drug conjugates. This targeting approach makes it possible to specifically deliver kinase inhibitors, but also other drugs, to the kidney.

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