

Organ- and Cell-Type Specific Delivery of Kinase Inhibitors: A Novel Approach in the Development of Targeted Drugs

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Abstract: During the past years, we have explored the cellular delivery of kinase inhibitors. Kinase inhibitors have selectivity for specific kinases but they lack cellular selectivity. This is exemplified by recent reports on cardiotoxicity of kinase inhibitors used in cancer treatment. We postulate that targeted cellular delivery of kinase inhibitors can improve their safety/toxicity profiles, as will be exemplified by recent published studies. Cell specific delivery of therapeutics is a quickly growing area of investigation. This innovative strategy employs carrier molecules that bind to receptors exposed on the surface of cell types involved in disease processes. Binding and receptor mediated internalization of the carrier facilitates local accumulation of the product in target cells. Upon systemic administration, this may create local drug depots in specific organs, while other tissues are avoided, thus favoring enhanced localized drug efficacy and reduced side-effects.

Synthesis of targeted kinase inhibitor-carrier conjugates was achieved using a new approach, in which kinase inhibitors were bound to a platinum(II) atom, the so-called Universal Linkage System (ULS). We review this novel linkage chemistry and demonstrate the applicability of ULS for drug targeting approaches aiming at angiogenic endothelial cells, hepatic stellate cells, and kidney tubular cells. We will review important issues like drug release mechanism, safety of the linker, and pharmacokinetics of the products in animals. Finally, we review the pharmacological efficacy of the cellular targeted drug conjugates in experimental animal models, especially in renal and liver fibrosis models.

Keywords: Signal transduction, kinase inhibitors, drug targeting, intracellular delivery, fibrosis, inflammation, angiogenesis.

INTRODUCTION

Kinases regulate cell function by transferring a phosphate group from ATP to recipient proteins, thereby influencing the activity, localization and overall function of the downstream substrate proteins. Phosphorylation occurs mainly at serine and threonine but also at tyrosine residues. Tyrosine kinases are often part of a cell surface receptor complex and are autophosphorylated upon binding of a ligand to the extracellular part of the receptor [1]. Serine-threonine kinases are regulators of the intracellular signaling networks. More than 500 kinases are encoded in the human genome, making kinases one of the largest families of related genes, with many kinases still uncharacterized [2]. Kinases are involved in virtually all physiological activities in the human body and have been identified as important therapeutic targets [3]. Most of the pharmaceutical firms have kinase inhibitors under development with furthest progress for kinase inhibitors targeting pathways involved in cancer.

Kinase inhibitors influence cellular signaling pathways by blockade of the phosphorylating activity of kinases, often by blockade of the ATP binding pocket of the protein [3, 4]. Kinase inhibitors are advertised as targeted agents by virtue of their selectivity for specific kinases. However, even fairly specific kinases are not acting solely at a single molecular target, as evidenced in studies using binding assays for small molecule-kinase interactions [5]. SB202190, a known

p38MAP kinase inhibitor, binds to the ATP pocket of several p38MAP kinases but also to JNK1, 2, 3, and EGFR to name only a few and even this assay covers only a fourth of the whole kinome. Similarly, other well-known kinase inhibitors display activity versus a range of pharmacological targets [6, 7]. Moreover, currently developed kinase inhibitors like sunitinib, dasatinib or sorafenib have been designed to target multiple kinases, in order to enhance efficacy and lower the possibility of drug resistance [8-11].

An often neglected aspect of kinase inhibitors is that they lack cellular selectivity. This would not be a problem if the targeted kinases were solely active in the pathophysiological process. However, this is hardly ever the case since kinases play pivotal roles in essential physiological processes. Potential detrimental effects of kinase inhibitors, especially with more chronic treatment, are illustrated by recently published reports on cardiotoxicity of tyrosine kinase inhibitors in cancer treatment [12, 13]. Several of the targeted kinases in cancer are also involved in cardiac tissue repair. Among others, inhibition of KIT within cardiac tissue aggravated the pathological remodeling of the heart post-myocardial infarction and subsequently prevented repair of cardiac tissue [13-15]. Cardiotoxicity has also been reported for kinase inhibitors directed to Her2-kinase, a well known target for EGF-receptor mediated signaling [16, 17]. The inhibition of VEGF signaling has led to bleeding and hypertension [18]. Thus, inhibition of cancer associated kinases in normal tissues instead of cancer cells can be a cause for adverse events. Since these toxicities relate to the intended pharmacological target of these drugs, they are also referred to as on-target side effects. Generally speaking, on-target side effects can be expected for many kinase inhibitors, since

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intuitively all kinases should have physiological tasks elsewhere in the body.

In addition, side-effects of drugs can relate to off-target toxicity, i.e. effects related to inhibition of off-target kinases or to other, non-kinase dependent mechanisms. In the former context, one should realize that the majority of kinases have not been identified yet and off-target kinase effects of kinase inhibitors are difficult to detect. Several off-target toxicities of kinase inhibitors may limit their clinical application. Among these, vomiting, hypophosphataemia, and hepatotoxicity have been reported for imatinib, while interstitial lung disease has been reported for erlotinib and gefitinib [13, 17].

In addition, some receptor tyrosine kinase inhibitors compromised viability and phagocytic capacity of macrophages, possibly affecting the immune defense [19].

Kinase inhibitors are mostly lipophilic small molecule drugs and, consequently, their distribution throughout the body is widespread, exposing both diseased tissues and normal tissues to the drug. We postulated that a targeted distribution of kinase inhibitors to disease-inflicted tissues could improve the balance between therapeutic and toxic effects. This concept is illustrated in Fig. (1). The conjugate is able to bind selectively to diseased cells due to the recognition of surface-exposed receptors (over) expressed on the cell mem-

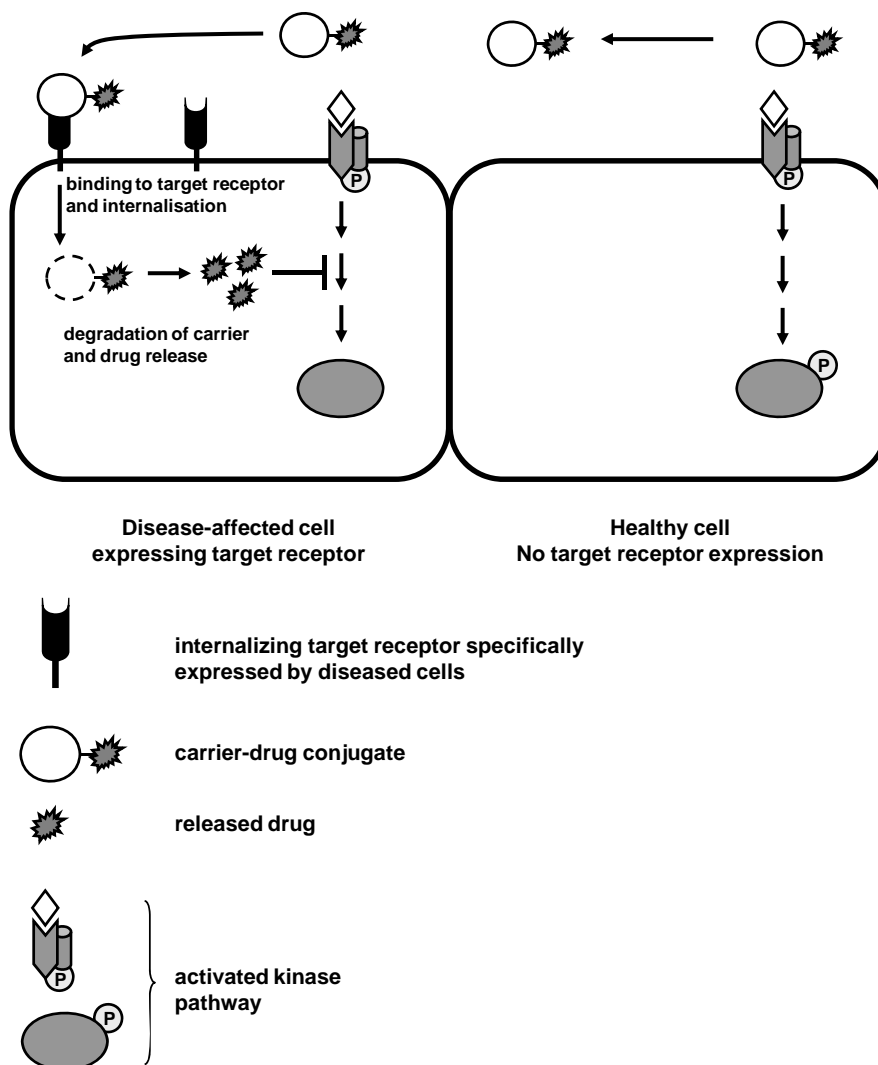


Fig. (1). Schematic representation of drug delivery approach for cell-type specific kinase inhibitors. Drug-carrier conjugates accumulate specifically in target cells by recognition of receptors on disease-afflicted target cells. The targeted receptor should be surface exposed and internalize upon binding of the conjugate to its receptor. Free drug will be released from the drug-carrier conjugate after lysosomal degradation of the conjugate. The depicted intracellular delivery approach ensures that the coupled drug (in this case, a kinase inhibitor) acts specifically in diseased cells, while normal cells are not affected by the drug.

brane. Receptor-mediated endocytosis will facilitate the accumulation of the conjugate in target cells. Subsequently, upon degradation of the bond between drug and carrier, the drug will be released into the cytosol and exerts its inhibitory effect on the targeted signaling pathway. In contrast, healthy cells will not accumulate the conjugate, thereby avoiding toxic inhibition of physiological processes.

Here we will give an overview of the challenges in the development of such tissue-specific kinase inhibitor preparations, preceded by a brief introduction of current concepts in drug targeting research. We will discuss the drug release mechanism, safety of the linker, and pharmacokinetics of kinase inhibitor drug targeting conjugates. More specifically, we will review the development of cellular targeted kinase inhibitors aimed at different target cell types, angiogenic endothelial cells, hepatic stellate cells (HSC) in liver fibrosis, and kidney tubular cells in renal fibrosis, respectively. Finally, we discuss the pharmacological efficacy of the targeted compounds in comparison to treatment with free, non-delivered kinase inhibitor in experimental models of the studied diseases.

TARGETED DRUG DELIVERY

Drug targeting aims at the accumulation of the drug in specific cell types or tissues in order to enhance drug efficacy, and to prevent accumulation in non-target tissue to reduce unwanted side effects [20]. Low molecular weight drugs, like most kinase inhibitors, distribute uniformly throughout the whole body, where they can cause side effects as already addressed in the previous section. The key to a successful drug targeting approach is the development of a suitable drug carrier which can override the tissue distribution of the drug, guiding it to specific cell types in which the desired action should occur.

Drug targeting strategies can be divided into two categories: passive and active targeting [21, 22]. In the case of passive targeting, the physicochemical properties of the carrier, like size and mass, are used to prevent the penetration of a drug into tissues and its clearance by liver or kidney. Several carrier systems (e.g. polymer-drug conjugates and long-circulating liposomes) have been developed to passively target drugs to tumor sites [23, 24]. The endothelial lining of tumor vasculature and at sites of inflammation is often leaky and this enhanced permeability allows extravasation of drug carrier system from the bloodstream into the underlying tissues, the so-called enhanced permeability and retention effect [25]. Using the active targeting strategy, the carrier differentiates between target and non-target cells by means of an appended targeting ligand, which binds to a receptor or epitope that is expressed selectively or at least over-expressed on the target cell. Targeting ligands have been developed to specifically recognize these receptors. To this end the ligands are either natural ligands like folate [26], or a moiety that resembles the binding epitope of the natural ligand such as various peptidic or carbohydrate ligands [27, 28], or monoclonal antibodies, which can recognize parts of the target receptor [29]. Optimally, the carrier has a long half-life to allow less frequent administration and a constant blood pool of drug targeting constructs ready for uptake by the target cell.

LIPOSOMAL DRUG DELIVERY

Several approaches have been developed over time, where drugs are either coupled to or encapsulated in a carrier system. Liposomes represent an established carrier system for both low and high molecular weight compounds, but encapsulation of kinase inhibitors remains a major challenge since most kinase inhibitors are hydrophobic small molecules. These properties cause difficulties with the encapsulation and particularly with retention within the liposomal carrier [30]. Alternatively, the incorporation of kinase inhibitor UCN-01 was achieved by the use of lipids with a high phase transition temperature [31]. UCN-01 liposomes gradually released the encapsulated drug upon intravenous administration to rats, providing less than 10% of liposomally encapsulated drug 24 h after administration. In addition to the above discussed normal liposomes, which do not bear a targeting ligand and thus only passively accumulate in target tissues, the use of immunoliposomes has been proposed for the delivery of kinase inhibitors, e.g. liposomes directed to E-selectin expressing inflamed endothelial cells [32].

DRUG DELIVERY VIA MACROMOLECULAR CONJUGATES

Drugs also can be directly attached to a carrier. Commonly applied approaches are drug-polymer conjugates, which can be further equipped with homing ligands that facilitate binding to target cells. Another popular approach is the conjugation of drug molecules to target-cell directed antibodies. In either case, the attachment of the drugs to the carrier is facilitated *via* a spacer or linker. Most linkers are based on the formation of disulfide-, ester-, and hydrazone linkages between the drug and carrier [33]. However, the formation of such bonds requires the presence of thiol, hydroxyl, keto or amine groups in the drug's structure. When such functional groups are not present, derivatives of the drug need to be synthesized. This may lead to inactivation of the compound or to an alteration in physicochemical or biological properties. The following section describes an alternative linking technique, in which kinase inhibitors can be linked without derivatization *via* a novel straightforward approach.

CONJUGATION VIA THE UNIVERSAL LINKAGE SYSTEM (ULSTM)

We explored a novel approach in which kinase inhibitors were reacted at a functional group, i.e. aromatic nitrogen atoms, that is present in most of the selected structures. Aromatic nitrogens can be found frequently in kinase inhibitors, as well as in other small molecule drug structures. The following synthesis approach is therefore readily applicable without extensive derivatization steps prior to the actual conjugation reaction. As exemplified in Fig. (2) for the derivatization of the well-known EGFR kinase inhibitor gefitinib (Iressa), we reacted the kinase inhibitor to the platinum(II)-based Universal Linkage System (ULSTM) and subsequently conjugated this intermediate to an antibody directed to EGFR, which is overexpressed by malignant cell types. The versatility of ULS in coupling of drugs was demonstrated by

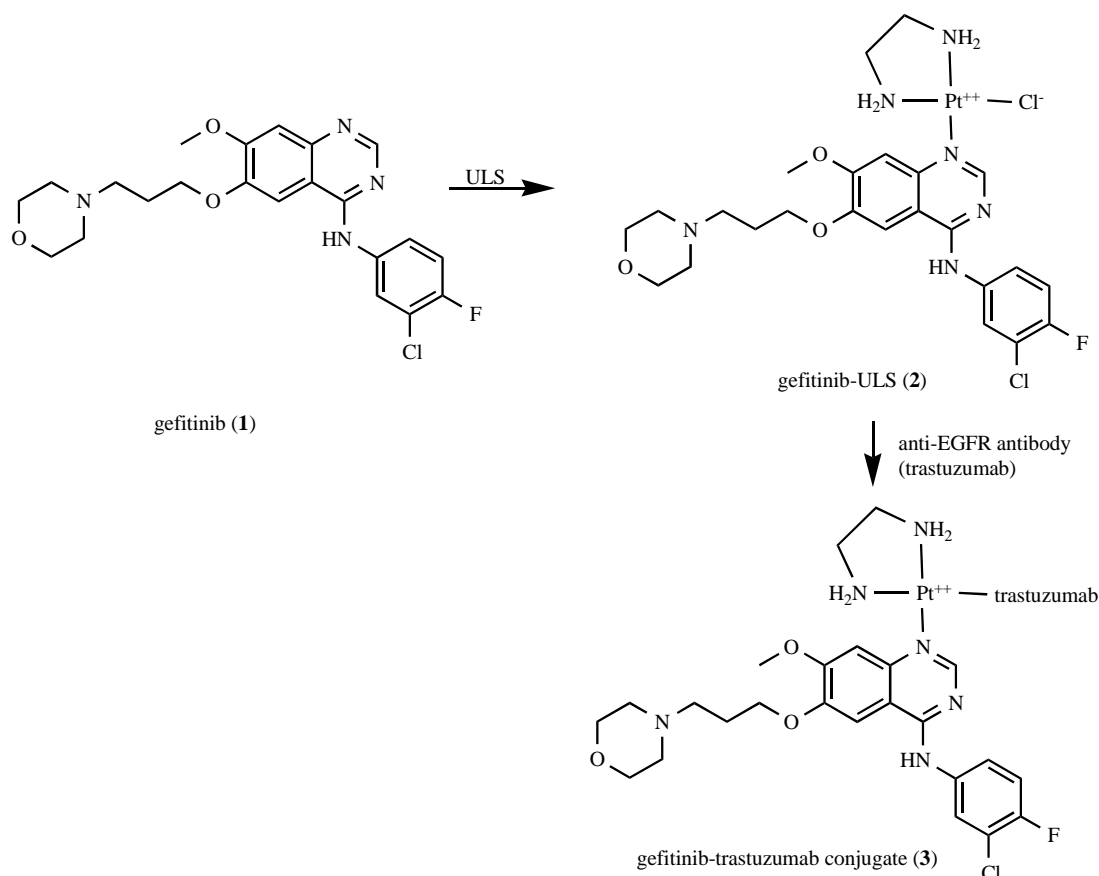


Fig. (2). Reaction scheme for the synthesis of cellularly directed kinase inhibitor conjugates, as exemplified by the conjugation of gefitinib (1) to the anti-EGFR antibody trastuzumab. First, the kinase inhibitor is reacted with the platinum(II) containing Universal Linkage System (ULS), which is subsequently reacted to methionine residues in the antibody. The linkage is of a coordinative nature and very stable in various media like PBS or serum. High intracellular levels of sulfur donor like glutathione accelerate the release of the drug, probably by competing for the platinum binding.

its use in combination with several other kinase inhibitors, as shown in Fig. (3).

The drug-ULS conjugates were used for further conjugation to different carrier systems, directed to different target cell types. In this manner, we were able to develop targeted conjugates directed to either angiogenic endothelium, liver stellate cells or to kidney tubular cells, intended for treatment of different diseases associated with the targeted cell types [34-37]. These approaches will be discussed below in detail. Typically, we used proteinaceous carriers which are biodegradable upon lysosomal uptake by the target cells. Methionine residues are believed to be the main binding site of ULS in proteins [38-41]. Due to the low abundance of these residues in proteins, the resulting conjugates display only mild modifications of the carrier with drug molecules, without gross disturbance of the three-dimensional structure. The highest achievable drug to carrier ratio is either limited by the number of available binding sites [34] or by the solubility of the resulting drug targeting conjugate [37]. Extensive modification of the otherwise soluble proteinaceous carriers with several hydrophobic drug groups may lead to aggregation and precipitation of the protein-drug conjugate, a problem that could be resolved by coupling of hydrophilic polyethylenglycol (PEG) groups [37, 42, 43].

The application of the ULS for coupling of kinase inhibitors can also be extended to (artificial) polymers. Dendrimers, for example, which first were modified with methionines, have successfully been modified with drug-ULS (unpublished data). The application of such polymer-drug conjugates will be investigated further in the future. The overall stability of such a polymeric carrier can be higher than for a proteinaceous carrier but eventually even a dendrimer will be metabolized and excreted. Whether this is possible without the formation of toxic metabolites still needs to be shown.

ULS AND DRUG RELEASE

The release rate of the drug from the carrier after the accumulation of the conjugate in target cells is furthermore determinant for whether a drug targeting approach is successful, and has great influence on intracellular drug levels over time. Drug linkers are designed to firmly hold on to the drug during storage and in the circulation, but to release the drug upon internalization at the target site. The stability and release profiles of drug-ULS conjugates were investigated in several publications [34, 36, 38]. Incubation of the products with serum or PBS led to very little release of the coupled drug, indicating adequate stability of the conjugates upon

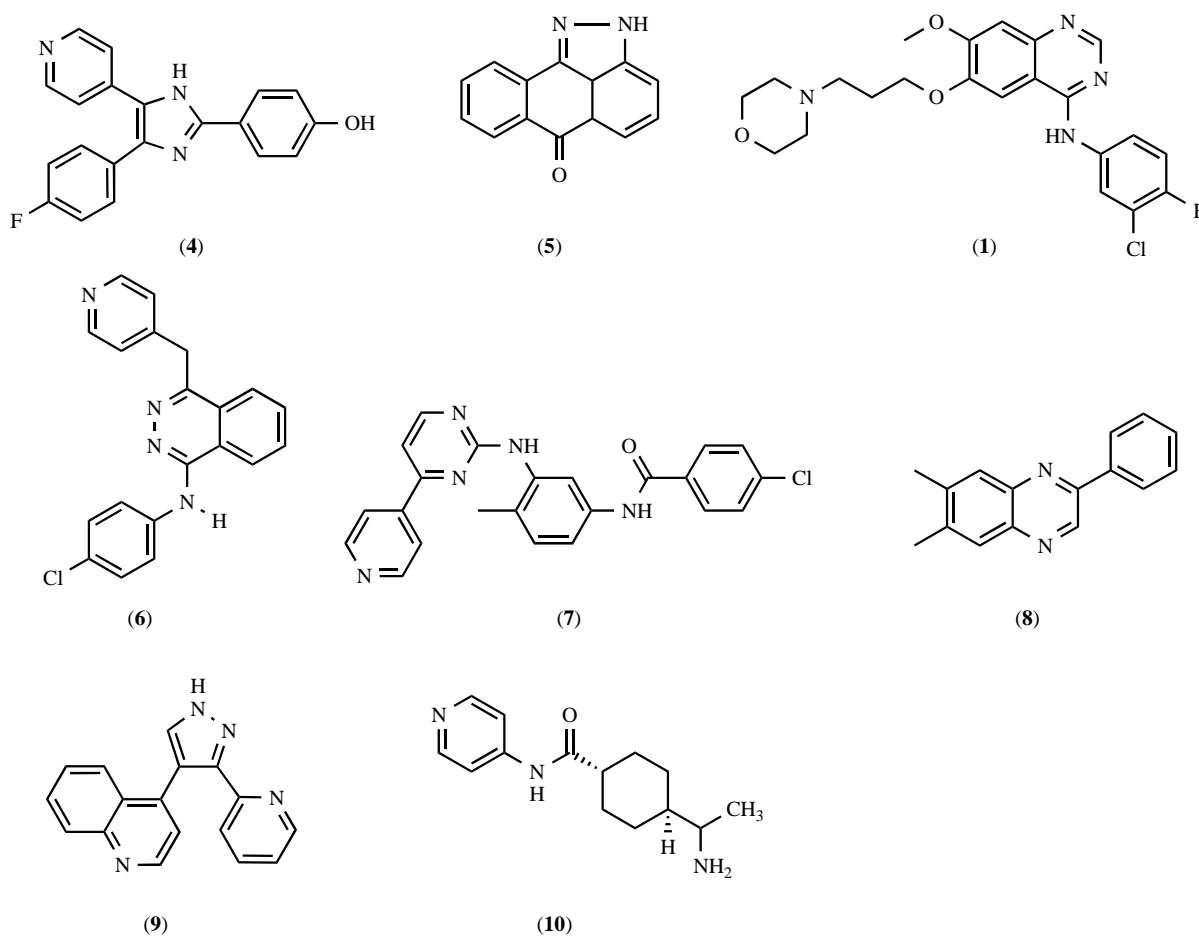


Fig. (3). Different signaling pathways can be inhibited by the investigated kinase inhibitors: MAPK signaling, i.e. p38 MAPK and JNK (SB202190 (4) and SP600125 (5)); EGFR signaling (gefitinib (1)); VEGF signaling (vatalanib/PTK787 (6)); PDGF signaling (PAP19 (7), AG1295 (8)); TGF- β signaling (ALK5 inhibitor 616451 (9)); and Rho/ROCK signaling (Y27632 (10)).

storage. Drug release was however markedly accelerated by addition of endogenous agents like glutathione, or by tissue homogenates, demonstrating that the coupled kinase inhibitors can be released from the carrier. We also demonstrated that carrier-bound kinase inhibitors remained stable in the circulation upon intravenous administration to animals [34, 35]. On the other hand, we observed a sustained-release of the drug from the carrier in the designated target tissues, which can be explained by the ligand exchange kinetics of platinum. Transition metals like platinum form coordinative bonds with ligands that are weaker than covalent bonds but the ligand exchange behavior of platinum-coordinated compounds is quite slow [44]. This gives them a high kinetic stability. Intracellular, glutathione is a likely candidate to displace drug from the drug-ULS complex. Furthermore, degradation of the protein carrier in the lysosomes can generate additional exchange ligands like methionine or cysteine residues. Sustained release is optimal for targeting of kinase inhibitors, since it can provide continuous levels of the free drug intracellularly during a prolonged period of time after a single dose. Intracellular concentrations of kinase inhibitors ideally need to be maintained above a certain threshold over a prolonged period of time to achieve an extended blockade of disease associated signaling pathways. It is furthermore important to stress that the drugs are released in their parental form without any modification. Other reversible drug

linkers often release a modified drug, which can adversely affect the activity of the drug.

ULS AND TOXICITY

Concerns that were raised about toxicity of ULS as a cisplatin-related compound have been addressed as well. Cisplatin toxicity involves cross-linking of DNA with the platinum atom, with the availability of free reactive sites at the platinum atom being essential for the toxicity [45]. Since all of the four coordinative sites of the platinum atom in ULS are fully occupied upon conjugation with the drug and carrier, it can therefore only display its cross-linking and anti-tumor features towards DNA after release of the drug and carrier. In view of the sustained intracellular drug release, we observed only slow formation of reactive platinum species in the target cell, that can be readily detoxified [46, 47]. The absence of acute toxicity of ULS-containing drug targeting conjugates upon incubation with either human endothelial cells [36], renal proximal tubular cells [34], or hepatic stellate cells (HSC) [38], provided evidence that indeed the released platinum is detoxified. Accordingly, no signs of acute platinum related toxicities were observed *in vivo* in different rat models of disease [34, 38]. Nephrotoxicity was especially scrutinized, due to the application of ULS in a renal targeting approach and the known nephrotoxicity of cisplatin related

compounds [48, 49]. However, the fate of the platinum linker in the body has not been fully elucidated yet and further *in vivo* studies will have to show how platinum conjugates are excreted from the body.

The following paragraphs highlight the results achieved with cellular targeted kinase inhibitors directed to specific cell types, by means of the different carrier systems employed.

DELIVERY OF KINASE INHIBITORS TO ANGIOGENIC ENDOTHELIUM

Endothelial cells play an important role in inflammatory disorders, as they control the recruitment of leukocytes into inflamed tissue and the formation of new blood vessels, called angiogenesis. Activation of endothelial cells can occur by for example tumor necrosis factor- α (TNF α), a process which is among others mediated *via* p38MAP kinase, or by vascular endothelial growth factor (VEGF) during angiogenesis [50-52]. The cell specific delivery of kinase inhibitors to activated endothelial cells is a promising strategy to counteract these processes, especially since the endothelium is easily accessible from the circulation. We developed kinase inhibitor conjugates using albumin as a carrier and cyclic RGD-peptide as a targeting ligand. The RGD-peptide introduced target cell specificity, since it binds to $\alpha_v\beta_3$ -integrin, which is overexpressed on activated and angiogenic

endothelium [53]. RGD-albumin carriers demonstrated high affinity binding to $\alpha_v\beta_3$ -expressing human endothelial cells and subsequent the drug carrier was internalized. The conjugates were prepared with either the p38 MAPK inhibitor SB202190 [36] or the VEGF kinase inhibitor vatalanib [37] and are schematically depicted in Fig. (4). The SB202190-conjugate inhibited the TNF α -induced expression of the proinflammatory genes E-selectin and IL-8, which also translated to a reduced IL-8 excretion. In chronic inflammatory diseases like arthritis, such conjugates might be able to prevent leukocyte recruitment and ongoing activation of endothelial and immune cells, without compromising the viability of the endothelial target cells.

RGD-albumin carriers for cell specific delivery of the VEGFR kinase inhibitor PTK787 (vatalanib) were prepared in different manners, either with or without PEGylation, resulting in conjugates that displayed the RGD-peptide in different densities [37]. These drug carriers demonstrated high binding affinity to $\alpha_v\beta_3$ -expressing cells correlated with the RGD-coupling density. Furthermore, the intracellularly delivered vatalanib reduced the activation of endothelial cells by VEGF, as measured by gene expression analysis of downstream nuclear receptors and transcription factors. The RGD-albumin carrier alone demonstrated no effect on gene expression or viability of human endothelial cells in the concentration range tested. Most likely, the effects of RGD-equipped carriers are dependent on the total concentration of

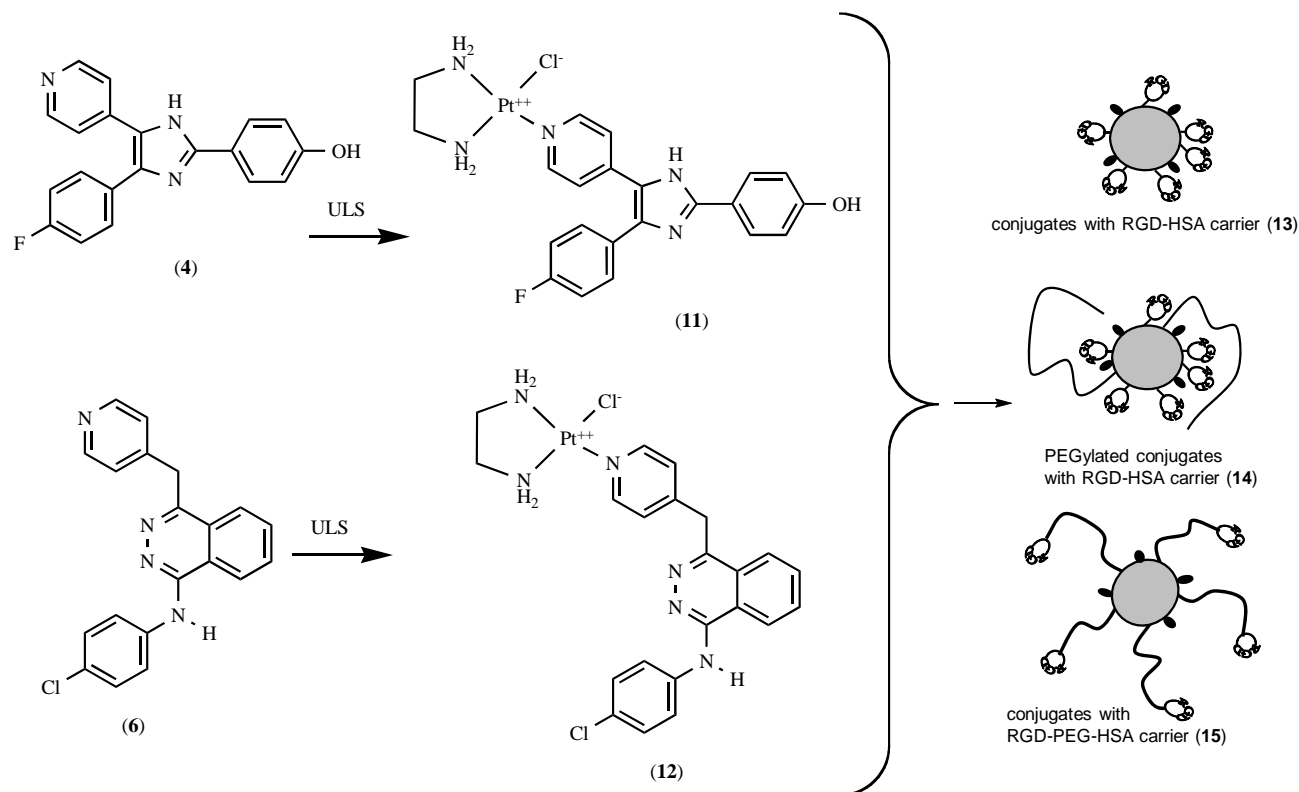


Fig. (4). Targeting of kinase inhibitors to angiogenic endothelium. The kinase inhibitors SB202190 (4) and vatalanib/PTK787 (6) were conjugated with ULS. Structures 11 and 12 display to which aromatic nitrogens the ULS was attached. Subsequently, 11 was further conjugated to an RGD-PEG-HSA carrier (15). 12 was conjugated to a range of different carriers (13, 14, 15), in which either binding and uptake properties or physicochemical properties were optimized by application of short or long spacers for the incorporation of cyclic RGD-peptides. The RGD-peptides facilitated binding and uptake into angiogenic endothelial cells.

the RGD peptide, and we have used relatively low concentrations (at which the drug is active). It was thus the intracellularly delivered PTK787 that inhibited the proangiogenic gene expression and not an effect of the RGD-carrier. The targeting approach might be expanded to inhibitors of NF κ B which plays a prominent role in inflammatory disease and cancer [54, 55] or to the well known VEGFR kinase inhibitors sunitinib and sorafenib.

DELIVERY OF KINASE INHIBITORS TO TUMOR CELLS

Cancer can also be attacked at the level of cancer cells. For this purpose several kinase inhibitors have been developed to block procarcinogenic signaling pathways. Often only a small fraction of systemically administered kinase inhibitor reaches a tumor, and gefitinib for example is known to induce side effects, such as skin toxicity and interstitial pneumonitis [17]. Targeting of this kinase inhibitor might prevent this. We have coupled gefitinib, an EGFR inhibitor, to the anti-EGFR antibody trastuzumab as depicted in Fig. (2). Trastuzumab and gefitinib demonstrated an additive effect *in vitro* and *in vivo* [56, 57] and the conjugation of both entities will ensure that the long half-life and tumor homing properties of the antibody are transferred to the small molecule kinase inhibitor.

DELIVERY OF KINASE INHIBITORS TO FIBROTIC LIVER / HEPATIC STELLATE CELLS

Chronic liver injury can lead to liver fibrosis, which is characterized by an excessive deposition of extracellular matrix proteins. The activation of HSCs and their transformation into fibroblasts is one of the hallmarks of liver fibrosis [58, 59]. Upon activation, HSCs change in morphology, proliferate, and start a vast production of collagen type I and III and a range of profibrotic cytokines. Transforming growth factor- β (TGF- β) has been identified as one of the key activators of HSC and platelet derived growth factor (PDGF-BB) is regarded as a driving force for proliferation [60, 61]. Inhibition of signal transduction initiated by these two growth factors is therefore regarded as an interesting strategy to counteract chronic liver fibrosis. We applied an HSC-selective carrier consisting of an albumin backbone modified with mannose-6-phosphate (M6P) targeting ligands, a carbohydrate that binds to the M6P/insulin-like growth factor-II receptor on activated HSC [62]. The M6PHSA carrier was subsequently modified using ULS with a potent inhibitor of PDGFR kinase, called PAP19 [63] as shown in Fig. (5). This PDGFR kinase inhibitor is closely related to the well known kinase inhibitor imatinib. PAP19-M6PHSA inhibited α -smooth muscle actin (α -SMA) and collagen 1A1 expression *in vitro* in isolated human HSCs and in precision-cut liver slices, comparably to free PAP19

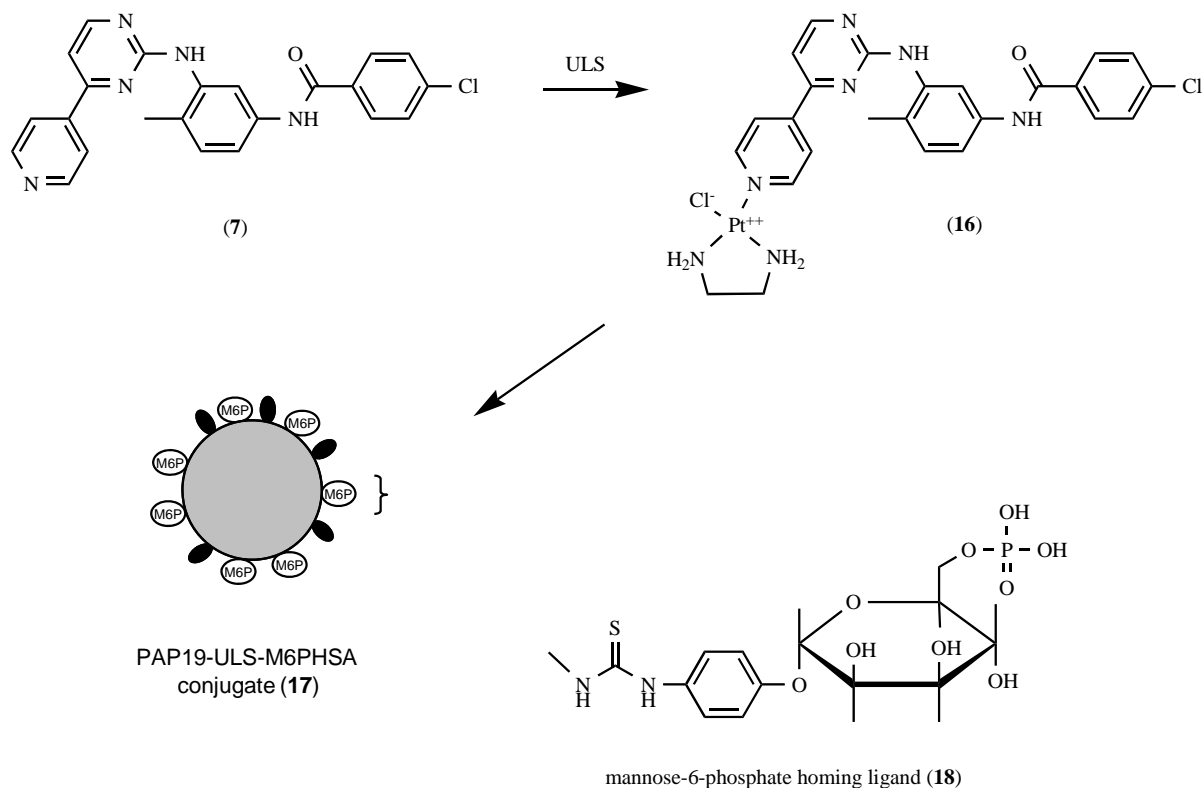


Fig. (5). Targeting of kinase inhibitors to hepatic stellate cells. PAP19 (7), an inhibitor of PDGF signaling, was conjugated with ULS as depicted. Thereafter, PAP19-ULS (16) was coupled to a mannose-6-phosphate-HSA resulting in the final conjugate (17). The mannose-6-phosphate (M6P) (18) equipped carrier M6PHSA is recognized and internalized by the M6P/insulin like growth factor receptor, which is highly upregulated by activated stellate cells in fibrotic livers. By these means, the PAP19-ULS-M6PHSA conjugate was able to attenuate the fibrotic process in bile duct ligated rats.

[35]. Upon administration to rats experiencing liver fibrosis inflicted by bile duct ligation, PAPI9-M6PHSA accumulated in the liver to 30 % of the injected dose and resided there for at least 48 h. This result indicates that the conjugate formed a sustained-release drug reservoir inside the target cells, from which free drug was generated continuously. The locally deposited PDGF kinase inhibitor attenuated collagen deposition at 24 and 48 h after injection, reduced portal bridging in fibrotic areas, and reduced the gene expression of the fibroblast marker α -SMA.

With such a powerful targeting strategy at hand other signaling pathways might come into focus. The inhibition of the TGF- β pathway for example might render the hepatic stellate cell quiescent again. TGF- β , however, is not only involved in the activation of stellate cells but also in the resolution of inflammation [64, 65] and it promotes or opposes the differentiation, survival, and proliferation of multiple cell lineages [66-68]. A long term systemic administration of TGF- β inhibitor might thus lead to serious adverse events, which can be avoided by local delivery of TGF- β inhibitors within the liver.

DELIVERY OF KINASE INHIBITORS TO RENAL FIBROSIS/ KIDNEY TUBULAR CELLS

Kidney tubular cells play a pivotal role in the etiology of renal fibrosis. Tubular cells become activated during renal injury and initiate fibrogenic processes which may lead to tubulointerstitial fibrosis and eventually to end stage renal disease [69]. Several recent papers underscore the potential of kinase inhibitors to treat renal fibrosis, as reviewed by Prakash and colleagues [70]. The complexity of the tubulointerstitial fibrotic process involves signaling *via* several important kinase pathways, including p38 MAPK, ERK, JNK, Rho-ROCK, TGF- β , PDGF, and NF κ B [70-72]. Kinase inhibitors intervening in these signal transduction pathways are already available and the cell-specific delivery of these inhibitors to proximal tubular cells is an interesting novel approach. To this end, we employed a low molecular weight protein as a renal-selective carrier, a concept that has been explored by us with other classes of drugs [73]. The low molecular weight protein of choice, lysozyme, is filtered in the glomeruli and reabsorbed efficiently from the urine by receptor mediated endocytosis *via* the megalin receptor [74].

Kinase inhibitors capable of intervening in different pathways involved in inflammation or the progression of fibrosis were conjugated to the renal carrier lysozyme *via* the above described approach employing ULS (Fig. (6)). First, the effect of free, non-conjugated inhibitors was investigated *in vitro* on renal tubular cells, which clearly demonstrated a reduction of profibrotic markers, as exemplified by the effect of the p38 MAPK inhibitor SB202190 on MCP-1, procollagen- $I\alpha$ 1 and TIMP-1 gene expression [34]. Similarly, the drug-lysozyme conjugates derived from SB202190 inhibited profibrotic signaling in cultured proximal tubular cells. Upon administration to rats, SB202190-lysozyme accumulated efficiently in proximal tubular cells of the kidneys, providing a depot of 20% of the injected dose within 1 h following the intravenous injection. This local reservoir of drug targeting conjugate inside the target cell continuously released SB202190, which resulted in effective levels of SB202190

for at least 3 days. Consequently, a single dosing in a unilateral renal ischemia-reperfusion rat model achieved a reduction of intrarenal p38MAPK phosphorylation and α -SMA protein expression, measured at day 4 after injury [34]. Animals treated with free SB202190 did not show reduced phospho-p38 MAPK levels in the kidneys nor altered α -SMA expression, which can be explained by the low dose of the p38 inhibitor. Generally, treatment protocols with kinase inhibitors rely on the administration of doses in the 10-100 mg/kg range [70]. In contrast, the SB202190-lysozyme conjugate was added in a dose of about 0.8 mg/kg of conjugated SB202190. Such a dose of the SB202190-lysozyme conjugate produced prolonged drug levels within the kidneys, as determined by HPLC analysis, but free SB202190 at the administered dose will not achieve therapeutic drug levels within the kidneys. When a higher dose of the free drug was administered for pharmacokinetic purposes, only 0.3% of the injected dose was detected in the urine and renal drug levels were below the level of detection [75]. The pharmacokinetic superiority of the conjugated versus the free drug encouraged us to investigate the renal targeting approach with other interesting kinase inhibitors.

Inhibition of the TGF- β type I receptor, also known as Activin Receptor Like Kinase 5 (ALK5), might prevent the conversion of tubular epithelial cells into fibroblasts. The ALK5 inhibitor 616451 (Calbiochem, hereafter referred to as TGF- β receptor kinase inhibitor or TKI) strongly inhibited the TGF- β induced gene expression of inflammatory and fibrosis related genes in renal tubular cells. Furthermore, TKI-lysozyme conjugate efficiently accumulated in the tubular cells of the kidney as demonstrated by immunohistochemical and pharmacokinetic studies, to a similar extent as observed for SB202190-lysozyme conjugate. When studied in the unilateral ureteral obstruction (UO) model of renal disease in rats, a single injection of TKI-lysozyme demonstrated superior efficacy in comparison to the free drug at the same dosage of 0.6 mg/kg drug. Tubular cell specific delivery of the kinase inhibitor resulted in a local inhibition of MCP-1, a substantial reduction of macrophage influx, and a reduced interstitial α -SMA protein expression. Unexpectedly, administration of the free drug reduced two out of three of these parameters as well, although these latter effects may relate to systemic anti-inflammatory effects of the free TKI¹ (unpublished data).

A third kinase inhibitor from which a renal specific drug-lysozyme conjugate was prepared is an inhibitor of the Rho-kinase (ROCK) pathway Y27632. The Rho kinase pathway plays a crucial role in structure and function of various kidney cells, e.g. tubular epithelial cells, mesangial cells, and podocytes [72]. Rho kinase inhibition was shown to attenuate the development of renal damage in several rat models of renal disease. Since coupling of the small molecule drug to the macromolecular carrier provides a conjugate bearing pharmacokinetic properties similar to the carrier, we were not surprised to observe a renal accumulation of approximately 20% of the injected dose, upon intravenous

¹ Prakash, J.; de Borst, M.; van Loenen-Weemaes, A. M.; Lacombe, M.; Opdam, F.; van Goor, H.; Meijer, D. K. F.; Moolenaar, F.; Poelstra, K.; Kok, R. J. Cell specific delivery of a Transforming Growth Factor-beta type I receptor kinase inhibitor to proximal tubular cells for the treatment of renal disease. submitted.

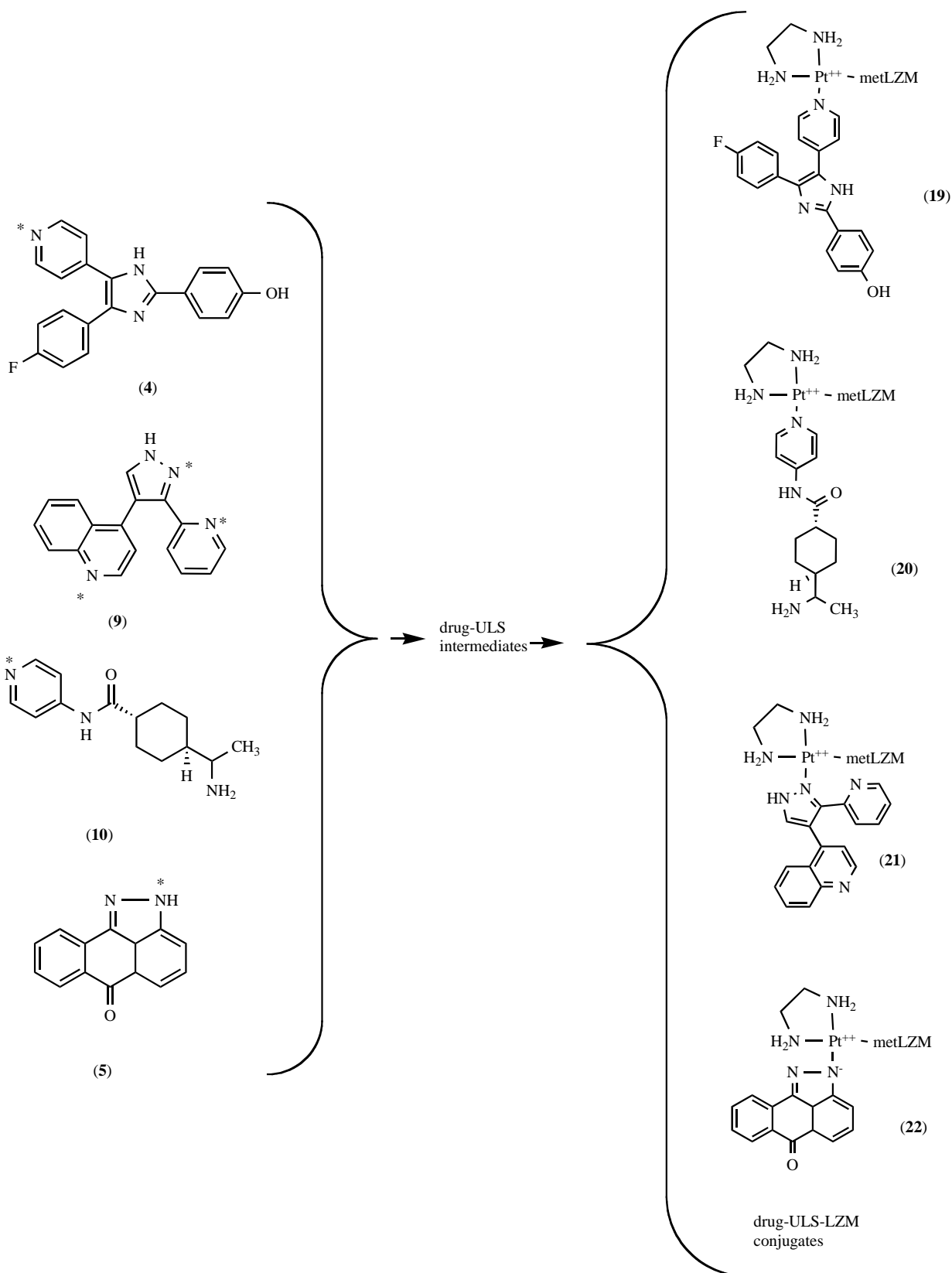


Fig. (6). Targeting of kinase inhibitors to proximal tubular cells. The ULS was applied to couple a range of kinase inhibitors (SB202190 (4), ALK5 inhibitor 616451 (9), Y27632 (10) and SP600125 (5)) to the carrier lysozyme (LZM) resulting in drug-LZM conjugates (19, 20, 21 and 22). Stars indicate possible binding sites for the ULS in each drug. The drug-ULS-lysozyme conjugates were evaluated in cultured cells and in rats. *In vivo*, the conjugates were filtered in the glomeruli and reabsorbed by proximal tubular cells *via* the megalin receptor. The released kinase inhibitors demonstrated efficient downregulation of various profibrotic genes and proteins over a prolonged period of time.

administration. The pharmacological potential of local renal ROCK inhibition was studied in the unilateral ischemia/reperfusion model in rats. Y27632-lysozyme downregulated the gene expression of a panel of inflammation and fibrosis related genes (among others, MCP-1, TGF- β 1, and α -SMA), and effectuated a clear reduction of macrophage influx and α -SMA disposition in the kidney. Especially impressive was the attenuated expression of the kidney injury marker-1 (KIM-1). Free Y27632 had no effect on renal inflammation and fibrosis markers² (unpublished data).

Clearly, these approaches have to be investigated further and in a more prolonged setting to evaluate their full potential as candidates for the treatment of renal fibrosis. Ongoing research will assess synthetic carriers like dendritic polymers. These can be tailored in size and charge in order to freely filter through the glomeruli and be deposited into the tubular cells [76]. One of the advantages of such polymeric carriers is the potential to achieve higher drug to carrier ratio's than with the lysozyme carrier, which typically bears one single drug molecule per carrier.

CONCLUDING REMARKS

During the past decades, targeted drug delivery has primarily focused on the delivery of cytostatic agents, and several tumor cell directed products are currently entering the clinic. Cell-specific drug targeting can also be of benefit for other target cell types and other potent drugs, such as kinase inhibitors, which present with on-target toxicity. Our approach of coupling kinase inhibitors *via* the universal linkage system can aid in the development of kinase inhibitor-carrier conjugates. The novel field of kinase inhibitor delivery may be of value in the treatment of cancer, but also serves a much wider therapeutic area of diseases in which activated signaling pathways have been identified, such as the discussed fibrotic disorders. One of the major advantages of linkage *via* ULS is that there is no need for extra modification of the drug molecule for efficient conjugation, and it also ensures that the drug is released in its unmodified form. The nature of the bond, a coordinative linkage to platinum(II), assures firm binding with high stability *in vitro* and *in vivo*. The conjugates did not dissociate upon the exposure to serum components, and adequately delivered the drug into the targeted cells/organs. The *in vivo* stability of the conjugates was demonstrated in different animal models dealing with different classes of drug-carrier conjugates. Lastly, the release rate of the drug within the target tissue is strikingly different from other types of drug-carrier conjugates, providing for prolonged release of the drug within the target cells. This release pattern allows for a continuous release of the drug which may ensure a continuous inhibition of the targeted kinase. Thus, the techniques here presented and drug targeting conjugates, but also other kinase inhibitor-carrier constructs, may prove potent therapeutics which display local activity within target cells or tissues.

² Prakash, J.; de Borst, M.; Lacombe, M.; Opdam, F.; Klok, P. A.; van Goor, H.; Meijer, D. K. F.; Moolenaar, F.; Poelstra, K.; Kok, R. J. Renal-specific delivery of ROCK inhibitor Y27632 inhibits ischemia/reperfusion-induced acute renal injury. submitted.

ABBREVIATIONS

α -SMA	=	alpha-smooth muscle actin
ALK5	=	Activin-Receptor like kinase
EGF(R)	=	epidermal growth factor (receptor)
EPR	=	enhanced permeability and retention
ERK	=	extracellular signal-regulated kinase
HSA	=	human serum albumin
HSC	=	hepatic stellate cell
IL-8	=	interleukin-8
JNK	=	c-jun N-terminal protein kinase
KIM-1	=	kidney injury marker-1
LZM	=	lysozyme
M6P	=	mannose-6-phosphate
MCP-1	=	monocyte chemoattractant protein-1
NF κ B	=	nuclear factor kappa-B
p38MAPK	=	p38 mitogen activated protein kinase
PDGF(R)	=	platelet derived growth factor (receptor)
RGD	=	arginine-glycine-aspartic acid
ROCK	=	Rho-associated kinase
TGF- β	=	transforming growth factor beta
TIMP-1	=	tissue inhibitor of metalloproteinase-1
TKI	=	TGF- β receptor kinase inhibitor
TNF α	=	tumor necrosis factor-alpha
VEGF(R)	=	vascular endothelial growth factor (receptor)
ULS	=	Universal Linkage System

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