Expert Opinion

Specific delivery of kinase inhibitors in nonmalignant and malignant diseases

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Introduction: Kinase inhibitors have been hailed as a breakthrough in the treatment of cancer. Extensive research is now being devoted to the development of kinase inhibitors as a treatment for many nonmalignant diseases. However, the use of kinase inhibitors in both malignant and nonmalignant diseases is also associated with side effects and the development of resistance. It may be worthwhile to explore whether cell-specific delivery of kinase inhibitors improves therapeutic efficacy and reduces side effects.

Areas covered: This review aims to provide an overview of the preclinical studies performed to examine the specific targeting of kinase inhibitors *in vitro* and *in vivo.* It gives an introduction to kinase signaling pathways induced during disease, along with the possible problems associated with their inhibition. It also discusses the studies on specific delivery and shows that altering the specificity of kinase inhibitors by targeting methods improves their effectivity and safety.

Expert opinion: Compared with the delivery of cytotoxic compounds, the specific delivery of kinase inhibitors has not yet been studied extensively. The studies discussed in this review provide an insight into methods used to target kinase inhibitors to different organs. The targeting of different kinase inhibitors has improved their therapeutic possibilities, but many questions still remain to be studied.

Keywords: cell-specific, fibrosis, kidney, kinase inhibitor, liver, tumor

Expert Opin. Drug Deliv. (2012) 9(1):59-70

1. Introduction

Protein kinase inhibitors have been the subject of intense research for the last two decades, leading to the approval of several kinase inhibitors for clinical use, mainly for the treatment of cancer. The first of the small-molecule kinase inhibitors in use was imatinib (Gleevec), which was approved as a treatment for chronic myelogenous leukemia by the FDA in 2001 [1] and, as such, was the first FDA-approved kinase inhibitor in clinical use. Herceptin, a Her2-blocking antibody (approved in 1998), was the first specific kinase inhibitor and it has powerful therapeutic effects in certain types of breast cancer [2]; however, this review focuses on the small-molecular kinase inhibitors. Up to 2009, approximately 10,000 possible kinase inhibitors had been patented and further research on the development of novel inhibitors is still thriving [3]. At the moment of writing this review, approximately 2400 clinical trials studying kinase inhibitors are in progress and registered on www.clinicaltrials.gov. Currently, interest is growing in the use of kinase inhibitors for indications other than cancer. Since signaling pathways are also deregulated in many nonmalignant diseases, inhibition of these pathways using kinase inhibitors promises to be a worthwhile strategy for the treatment of such diseases as well [4].

10.1517/17425247.2012.638625 © 2012 Informa UK, Ltd. ISSN 1742-5247

Article highlights.

- Kinase inhibitors are a potential treatment for both malignant and nonmalignant diseases.
- Though not directly toxic, kinase inhibitors elicit many side effects due to the ubiquitous expression of protein kinases and resistance to therapy can occur during treatment.
- Cell-specific delivery of various kinase inhibitors to tubular epithelial cells in kidneys, hepatic stellate cells in liver, endothelial cells of blood vessels and tumors has been achieved using a variety of drug delivery carriers.
- Tissue- or cell-specific delivery of these inhibitors can make them more specific to the diseased target tissue or cells and thereby improve their efficacy and reduce side effects.

This box summarizes key points contained in the article.

This review will first briefly describe the mechanism of action of small-molecule kinase inhibitors and then discuss some of the problems encountered with kinase inhibitor treatment, which give the rationale for specific targeting of these inhibitors. Although kinase inhibitors can be very potent drugs, they are not without side effects. These can either be caused by inhibition of target kinases in nontarget tissues or by off-target inhibition of other kinases. Furthermore, resistance to kinase inhibitors is increasingly becoming a problem in the clinic [5]. Increasing local drug levels in target cells or organs and decreasing drug levels elsewhere by specific targeting of kinase inhibitors may improve treatment options. Therefore, organ and/or cell-specific targeting of kinase inhibitors have been proposed in a number of studies. These studies will be discussed and different methods for specific delivery of kinase inhibitors will be examined.

2. Protein kinase inhibitors

Protein kinases are the phosphorylating enzymes present in all cells, although their expression varies from cell to cell and is dependent on the disease state. The transfer of a phosphate group from ATP to a protein, called phosphorylation, is a reversible process, which can cause activation or inactivation of the target protein, thus leading to a very diverse set of effects. To date, 518 protein kinases are known in human [6], although the function of some of these kinases is still unclear.

2.1 Protein kinase structure

Generally, each kinase consists of two lobes, an N-terminal and a C-terminal part, with the ATP-binding pocket located in the cleft between these lobes [1]. While all kinases have a structural similarity, the ATP-binding region in particular is very well conserved between different kinases. In most kinases, ATP can bind only when the protein kinase is in an active conformation. Conformational changes in the kinase protein induce changes between the active and inactive states of the kinase. Although these are commonly perceived as the only two possible conformations, kinases do not have one active and one inactive conformation. Instead there is a spectrum of conformations, some of which are active and others are not [7].

Protein kinases can also be classified into groups according to sequence comparison of the catalytic domains, combined with information on sequences outside of the catalytic domain and biological function. This results in a classification into nine groups of kinases, which can be further divided into families and subfamilies [6]. Kinases can also be characterized by the amino acid they commonly phosphorylate; the best-studied groups are the receptor tyrosine kinases and serine/threonine kinases. The currently approved inhibitors are all targeted toward members of these two groups [1].

2.2 Classification of protein kinase inhibitors

Kinase inhibitors comprise different types of molecules, which, in addition to their chemical properties, can also be classified according to mechanism of action, whether they compete with ATP, whether they target the active state of the kinase and whether the binding is reversible [8]. Apart from small-molecule inhibitors, monoclonal antibodies are also able to inhibit kinase proteins; however, this review will focus only on the small-molecule inhibitors.

The kinase inhibitors currently approved for use in the clinic are almost all ATP-competitive compounds [1] and exert their effects by blocking the ATP-docking site on a protein kinase directly or indirectly. Thus the transfer of a phosphate group from ATP by the kinase is blocked. ATP-competitive inhibitors can target both the active and the inactive state of the kinase [8]. Some ATP-competitive inhibitors bind directly in the ATP-docking site, which is a highly conserved structure, leading to high similarity in this region between different kinases. This explains a large part of off-target effects of these inhibitors [9], since they are able to block the ATP-docking site on several different kinases. The specificity of these inhibitors can be improved by fitting side groups that bind to hydrophobic pockets near the ATP-binding site, which are more variable between target kinases.

Non-ATP-competitive inhibitors block the functioning of the target kinase in an allosteric fashion by binding to a site other than the ATP-binding pocket, often altering the conformation of the protein kinase leading to its inactivation. It has been hypothesized that these inhibitors inherently would have a lower rate of off-target effects. Binding sites away from the ATP pocket display a higher degree of variation, and as such, inhibitors aimed at these sites should be more specific to a particular kinase. However, no such inhibitors have been approved yet [8].

2.3 Therapeutic use and limitations of kinase inhibitors

Kinases are involved in all basic cellular processes, such as proliferation, apoptosis, migration and invasion.

These processes are often dysregulated in disease, making them inviting druggable targets [10]. The versatility and the dependence of many homeostatic processes on kinase activity regulation also explain part of the potential for side effects that this class of drugs have. The kinase inhibitors currently in use in the clinic are approved as a treatment for different forms of cancer. However, there are a number of nonmalignant diseases in which kinase pathways are also deregulated and where kinase inhibitors may be a valuable treatment. Recent reviews have dealt with the use of tyrosine kinase inhibitors in several nonmalignant disorders, mainly proliferative diseases, such as lung fibrosis and cardiac hypertrophy [4,11]. Even though promising results with kinase inhibitors have been shown in (pre)clinical trials, the same problems facing kinase inhibitor treatments for cancer, such as side effects and resistance, may also occur with these treatments. The results of clinical trials performed to study the efficacy of p38-mitogen-activated protein kinase (MAPK) inhibitors as a treatment for rheumatoid arthritis show that even though potent effects were found in preclinical tests, these inhibitors did not have significant therapeutic effects in patients [12,13]. The reason in this case might be the redundancy of pathways in this disease. Inhibition of one pathway can then lead to the upregulation of others, which take over its function. The solution could be to switch to inhibitors of more upstream kinases or to inhibitors that inhibit multiple pathways [13], which could, however, also lead to more side effects.

Preclinical research has shown that several types of kinase inhibitors can reduce fibrogenesis in animal models. This would address an unmet medical need since fibrogenic or sclerotic diseases are generally very difficult to treat and there is no pharmacotherapy available for such diseases. An added difficulty in determining the clinical value of anti-fibrotic therapies is the disease process itself, where it may take decades for the injury to develop into a severe fibrosis with clinical symptoms. Although idiopathic pulmonary fibrosis generally evolves within 3 years, disease progression in other disease like liver or renal fibrosis may take decades [14,15]. To treat such a chronic disease, chronic treatment will be needed to evaluate the added value of a drug, necessitating a prolonged clinical trial. Moreover, adverse effects of drugs used for a long time as a treatment for a chronic disease, which may display in the first reversible, treatable phases only slight clinical symptoms, are not tolerated.

2.3.1 Side effects of kinase inhibitors

Although most inhibitors are targeted toward processes that are dysregulated in a diseased organ, many of these kinases or processes also can have essential functions in nonpathological processes. An example of this is formed by the experience with experimental MAPK/ERK kinase (MEK) inhibitors, which all cause the same dose-dependent side effect on the eye, pointing to an essential role of MEK in the regulation of vision [16].

The inhibitors in clinical use have been found to also exhibit side effects with varying degrees of severity [9,17]. One of the most severe side effects during treatment of patients with kinase inhibitors is the development of cardiac problems, although opinions are still divided as to the severity and rate of occurrence of these side effects [18,19]. Additionally, kinase inhibitors can cause a variety of other side effects, such as skin toxicity, hematological side effects, edema and nausea [17]. In comparison with conventional chemotherapy and in the context of relatively short-term treatment for cancer, these side effects are often seen as negligible. However, in the context of chronic treatment for other diseases, they may turn out to be dose limiting or even preclude the use of these inhibitors altogether.

In some cases, side effects might be avoided by inhibiting a kinase that functions further downstream in a signaling cascade, thus avoiding the inhibition of essential processes. However, this approach carries with it the danger that the inhibited kinase is redundant in the disease process [8]. The inhibitory effect on one kinase can be readily circumvented by activation of another kinase and thus the inhibition will not have the desired effect as a treatment.

2.3.2 Resistance to kinase inhibitors

Particularly in the field of cancer treatment, a new and growing concern is the fact that the inhibition of just one kinase may not be sufficient, since resistance to kinase inhibitors is increasingly seen in patients [5]. Many inhibitors currently in use are targeted to kinases that are malfunctioning because of a genetic mutation, for example, imatinib, targeted to the Bcr-Abl kinase [20]. It appears that it is also possible for the kinase gene once mutated to acquire extra mutations, which may confer resistance to the inhibitor on the tumor cells. Other causes for resistance include decreased drug bioavailability, amplification of oncogenes and continued activation of downstream pathways [5,21]. Resistance to kinase inhibitors may also be partly mediated through upregulation of efflux transporters, such as ATP-binding cassette transporters [22]. There is evidence that at higher concentrations kinase inhibitors can inhibit these transporters, while at lower doses they may be substrates for these transporters, which decreases the effectivity of the inhibitors [23,24]. However, the clinical relevance of this phenomenon still remains to be clarified [20]. Resistance to specific kinase inhibitors might be circumvented by the development of multitargeted inhibitors or the use of a combination therapy, applying several different kinase-specific inhibitors [25]. Additionally, there is evidence that the use of higher doses may prevent the development of resistance altogether [21]. However, higher doses or combination therapies obviously increase the risk of side effects. Since kinase inhibitors have only been in clinical use as a treatment for malignant diseases, so far there is no information available to assess how resistance would affect the use of kinase inhibitors in the treatment of nonmalignant diseases.

3. Specific delivery of kinase inhibitors

The problems described with the use of kinase inhibitors point to several possible improvements that may be achieved using specific delivery of kinase inhibitors. The first reasons for specific delivery would of course be to avoid side effects and increase the therapeutic levels of the drug in the target organ or designated cell type. This will increase the therapeutic window of the inhibitors. Additionally, a cell-specific action would prevent effects on neighboring cells. The change in pharmacokinetics might also be beneficial in the treatment of chronic diseases, by increasing the circulation time of the altered drug. Due to biological barriers, such as the blood–brain barrier, it is difficult to reach sufficiently high drug levels in some organs, and specific targeting may improve transport of kinase inhibitors over this barrier, which is essential for the treatment of glioblastoma or other central nervous system disorders. In addition, insight into the increasing problem of resistance induced by the upregulation of efflux pumps might also be gained by cell-specific targeting, since higher peak concentrations in the target cell may affect the mechanism of resistance [21,23,24]. As stated above, circumvention of redundancy in the signaling pathways can also be prevented by the use of upstream inhibitors. However, this in turn might affect essential physiological processes in non-target cells, which warrants the use of cell-specific delivery tools. A final possible advantage of cell-specific targeting is the option of combining several drugs within one carrier to inhibit several kinases at once in the target cell. Below we will discuss several malignant and nonmalignant syndromes in which cell- or organ-specific kinase inhibitor treatments have been studied (see also **Table 1**).

3.1 Liver fibrosis

Liver fibrosis is the outcome of almost all forms of chronic liver damage, including chronic hepatitis (type B and C), obesity-related fatty liver disease, alcohol abuse, autoimmune disorders, genetic disorders and others [26]. All these types of injury cause damage to hepatocytes, which results in the release of cytokines and cell debris, which activates other cell types in the liver. In the past few decades, the hepatic stellate cell (HSC) has been identified as the key cell type to drive the process of liver fibrosis, since this cell type produces large amounts of extracellular matrix and pro-fibrotic cytokines after activation [27,28]. This leads to malfunctioning of the liver and ultimately to end-stage cirrhosis and possibly to hepatocellular carcinoma.

The main target for cell-specific therapy in liver fibrosis is, therefore, the activated HSC, responsible for the majority of the production of extracellular matrix and pro-fibrotic cytokines [27]. This cell type overexpresses several receptors, a fact that has been used for the development of cell-specific carriers. One of these receptors is the mannose-6-phosphate/insulin-like growth factor II (M6P/IGFII)-receptor, which is highly upregulated on activated HSC after liver injury [29]. Inhibitors can be coupled to a modified albumin, mannose-6-phosphate human serum albumin (M6PHSA), which binds to the M6P/IGFII-receptor [30-32]. Previous studies with this carrier using non-kinase inhibitor drugs showed that use of this carrier results in improved efficacy of the coupled drug, compared with the corresponding free drug [33,34].

3.1.1 *PDGF-receptor-β inhibitor*

Platelet-derived growth factor (PDGF) is one of the most important pro-fibrotic cytokines [14], which in liver fibrosis signals mainly via the PDGF-receptor-β. This pathway can be inhibited by imatinib mesylate (Gleevec) and similar drugs [35,36]. However, these drugs have been linked to side effects, as discussed above, so specific delivery is necessary, especially during chronic treatment. A Gleevec-like drug, PAP19, which inhibits the PDGFRβ-receptor, has been shown to reduce fibrotic markers in HSC *in vitro* and has been targeted successfully to HSC in a rat model of cholestatic liver injury using the M6PHSA carrier. After a single treatment, the conjugate was very effective in reducing HSC activation and collagen deposition in rat livers. Liver drug levels were high and were sustained at least until 48 h after injection of a single dose [37] while showing a slow release of the drug from the carrier, which is particularly relevant for the treatment of chronic diseases. In contrast to this, free drug was accumulated in very low amounts in these livers and the drug was rapidly cleared, leading to low efficacy of this PDGFRβ-inhibitor.

3.1.2 *Rho-kinase inhibitor*

Rho-kinase is a downstream mediator of Rho-GTPase, an important regulator of contraction, migration and activation of HSC. Studies have shown that inhibition of Rho-kinase with the small-molecule inhibitor Y27632 results in inhibition of HSC activation *in vitro* and in a reduction of fibrosis *in vivo* [38-44]. Additionally, it may lead to a reduction in portal hypertension, an important complication of severe cirrhosis. However, this may be partly caused by the strong effects of Rho-kinase inhibition on vascular contraction and relaxation [45-47], which may also cause side effects in the form of a decrease in systemic vascular tension, which is a serious adverse effect in cirrhotic patients characterized by a decreased systemic blood pressure and associated clinical complications. Cell-specific targeting of Y27632 may be a valuable strategy to reduce side effects *in vivo* and to improve the efficacy of such drugs.

After coupling Y27632 to M6PHSA, an improved anti-fibrotic effect of the targeted conjugate compared with the free drug was shown in a chronic mice model for liver fibrosis, measured as reduced extracellular matrix deposition [48]. In an *ex vivo* model, it was shown that this conjugate did not affect vascular tone, while free drug at equimolar concentrations affected vascular tone severely. In an acute model for liver injury, the biodistribution of the conjugate was studied, and it was shown that high drug concentrations were achieved in

Table 1. An overview of studies examining the cell-specific delivery of several kinase inhibitors in different experimental models of malignant and nonmalignant diseases.

Disease	Pathway	Ref.	Delivery system	Effects *in vitro*	Effects *in vivo*
Liver fibrosis	PDGFR	[35]	M6PHSA	Anti-fibrotic effects on HSC	Liver accumulation in d10 BDL rats, sustained drug levels. Inhibits collagen and α-SMA in d10 BDL rats
	Rho-kinase	[46,47]	M6PHSA	Specific receptor binding. Anti-fibrotic effects in HSC	Binds to target cells. High drug concentration in liver during 48 h. Stronger anti-fibrotic effects than free drug
Renal fibrosis	p38 MAPK	[55]	Lysozyme	Inhibition of collagen gene expression in TGF-activated HK-2 cells	Accumulation in tubular cells during 3 days. Reduction of p38 phosphorylation and α-SMA expression after ischemia–reperfusion in rat kidney
	ALK5	[58,59]	Lysozyme	Inhibition of collagen gene expression in HK-2 cells	Accumulation in tubular cells during 3 days. Inhibition of activation of tubular cells and fibroblasts and reduced renal inflammation in unilateral urethra obstruction in rats. Stronger effects than equivalent free drug
	Rho-kinase	[60]	Lysozyme	–	Accumulation in kidney. Inhibition of tubular damage, Rho-kinase activation, inflammation and fibrogenesis after ischemia–reperfusion in rat kidney. Unconjugated drug did not have beneficial effects
Cardiovascular diseases	MAPK	[63]	RGD-albumin conjugate	Binds to HUVEC. Inhibits expression and secretion of pro-inflammatory cytokines in TNF-activated HUVEC	–
	c-Abl and PDGFR	[66]	Nanoparticles	Inhibition of cell proliferation and PDGFR phosphorylation	Reduction of neointima formation after graft surgery
Malignant diseases	PI3K	[69]	RGDS-conjugate	pAKT and pERK reduced in U937 and endothelial cells	Accumulation in tumor. Stronger reduction of tumor size in U87MG and PC3 tumor-bearing mice than untargeted inhibitor
		[70]	PEG-PE micelles, coupled to 2C5 mAb	Cytotoxic against U87MG, B16 and 4T1 cells	–
		[71]	Nanoparticles	Inhibition of Akt phosphorylation in B16 melanoma cells	Inhibition of endothelium proliferation and tubulogenesis in zebrafish xenotransplant model
	MAPK	[72]	Nanoparticles	Cytotoxic against B16 melanoma and lung carcinoma cells Inhibition of pERK in B16 cells	Stronger antitumor effect on B16-melanoma-bearing mice than free drug, particularly when both are combined with cisplatin

Schematic overview of the studies into specific delivery of kinase inhibitors, which are discussed in this review.
α-SMA: α-Smooth muscle actin; ALK5: Activin-like kinase 5 (TGF-receptor type I); BDL: Bile-duct ligation; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; HSC: Hepatic stellate cell; M6PHSA: Mannose-6-phosphate human serum albumin; PDGFR: Platelet-derived growth factor receptor; PEG-PE: Poly-ethylene glycol-phosphatidylethanolamine; PLGA: Poly(lactic-co-glycolic acid); p38 MAPK: p38-mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; TGF: Transforming growth factor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

Table 1. An overview of studies examining the cell-specific delivery of several kinase inhibitors in different experimental models of malignant and nonmalignant diseases (continued).

Disease	Pathway	Ref.	Delivery system	Effects *in vitro*	Effects *in vivo*
	EGFR	[75]	PLGA microspheres	Inhibition of EGFR activity in fibroblasts and carcinoma cells	–
		[76]	Liposomes	–	Three- to sixfold higher uptake of liposomal form than free drug in xenograft tumors
	VEGFR	[78]	RGD-albumin conjugate	Binds to angiogenic endothelium. Inhibits VEGF-induced gene expression in HUVEC	–
		[79]	APRPG-targeted liposomes	Inhibition of VEGF-induced endothelial cell proliferation	Inhibition of C26 tumor size in mice and prolonged survival. Effects stronger than untargeted liposomes with inhibitor

Schematic overview of the studies into specific delivery of kinase inhibitors, which are discussed in this review.
α-SMA: α-Smooth muscle actin; ALK5: Activin-like kinase 5 (TGF-receptor type I); BDL: Bile-duct ligation; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; HSC: Hepatic stellate cell; M6PHSA: Mannose-6-phosphate human serum albumin; PDGFR: Platelet-derived growth factor receptor; PEG-PE: Poly-ethylene glycol-phosphatidylethanolamine; PLGA: Poly(lactic-co-glycolic acid); p38 MAPK: p38-mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; TGF: Transforming growth factor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

the liver relative to free drug and that the conjugate remained in the liver for at least 48 h. The free drug was still released from the conjugate 48 h after a single injection [49]. Again, this is a favorable pharmacokinetic profile for a drug used against a chronic disease.

3.1.3 *Discussion*

The studies cited here reveal, though to a limited extent, that cell-specific delivery of kinase inhibitors to HSC in liver fibrosis is a potential strategy to improve the therapeutic efficacy of these drugs. This method not only yielded high drug levels in the target cells but also prolonged the drug release from the carrier for several days. Further research needs to be performed to assess the side effect profile and toxicity of both the carrier and linkers used in these studies. Preliminary evidence that kinase-specific side effects can be avoided is presented, but further research on the side effects after cell-specific targeting is needed.

3.2 Kidney fibrosis

Similar to liver diseases, in chronic kidney disease (CKD), proliferation and migration of fibroblasts to the site of injury is a key process in the pathogenesis of disease. Important causes for CKD are hypertension, diabetes and hyperlipidemia [50], leading finally to glomerulosclerosis and/or tubulointerstitial fibrosis [51]. Proximal tubular cells are activated and play an important role in inflammatory and fibrotic processes [52]. A common endpoint for all CKDs is tubulointerstitial fibrosis, which is influenced by several important cytokines, such as transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) [53]. These cytokines all activate kinase-regulated pathways. Since these growth factor signaling pathways are present in many cell types, enhancing the accumulation of drug within the kidney could make for a more effective treatment. The low-molecular-weight protein lysozyme is preferentially taken up by proximal tubular cells, the key cell involved in the pathogenesis of tubulo-interstitial fibrosis, forming a natural targeting system [54]. Uptake of this protein is mediated through the megalin receptor, which is abundantly present on proximal tubular cells, and regulates protein reabsorption. Using this protein, various kinase inhibitors were targeted to the diseased kidney and examined *in vitro* and in animal models.

3.2.1 *p38 MAPK inhibitor*

p38 MAPK plays a pivotal role in the activation of proximal tubular cells and also in the secretion of cytokines from these cells [55]. MAPK inhibitors, however, also cause immunosuppression, raising worries over side effects [56]. Coupling of the hydrophobic p38 MAPK inhibitor SB202190 to lysozyme yielded a conjugate that was specifically taken up in tubular cells and reduced inflammation and fibrosis in rat kidneys in a unilateral renal ischemia–reperfusion model [57].

This paper also compares different coupling methods for the kinase inhibitor to the protein and concludes that conventional linkage via a carbamate linker resulted in a conjugate that

rapidly released the drug in serum. A linkage via the novel Universal Linkage System (ULS™) resulted in a more stable conjugate, which released the drug in kidney homogenates but not in serum. This linker is a platinum-based linkage technology that facilitates the coupling of molecules directly to each other through the formation of a coordinative bond. The use of this linker was recently discussed in a review paper [58].

3.2.2 TGF-β type I receptor (ALK5) inhibitor

Since TGF-β1 is one of the most important pro-fibrotic cytokines, inhibition of its signaling pathway is potentially a very promising treatment. However, inhibition of TGF-β signaling can also deregulate the immune system, since TGF-β plays a role in the maintenance of immune tolerance [59]. One of the strategies to inhibit TGF signaling is the inhibition of its type-I receptor kinase, activin-like kinase 5 (ALK5). A small-molecule inhibitor of ALK5, TKI or LY-364947, conjugated to lysozyme, inhibited extracellular matrix markers in HK-2 cells [60,61]. In an *in vivo* model of rats with a unilateral ureteral obstruction, the conjugate inhibited activation of tubular cells and fibroblasts and reduced renal inflammation more potently than an equimolar dose of free drug. The conjugate accumulated in tubular cells and formed an intracellular depot, releasing drug for at least 3 days [61].

3.2.3 Rho-kinase inhibitor

As in liver fibrosis, in kidney disease Rho-kinase can regulate the cytoskeleton and the activation of fibroblasts. Additionally, the infiltration of inflammatory cells can be reduced by inhibition of Rho-kinase. In a kidney ischemia–reperfusion model in rats, lysozyme-conjugated Rho-kinase inhibitor reduced tubular damage more effectively than free drug [62], both on the mRNA and on the protein level. Inflammation and fibrogenesis were also strongly inhibited by this conjugate, but not by free Y27632.

3.2.4 Discussion

In contrast to liver targeting, renal tubular cell-specific targeting was achieved in a passive manner, which is not dependent on a disease-induced receptor. Therefore, the drug will be delivered to both diseased and healthy tubular cells. However, in the reported studies, no side effects were seen in the contralateral kidney when treated with targeted p38 MAPK and Rho-kinase inhibitors, indicating the safety of the therapy. Although a full toxicity profile has not been studied so far, no toxicity of the protein carrier and linker was found on liver and renal functions. In two different *in vivo* rat models, where the disease was induced due to tubular cell injury, targeting of two different kinds of kinase inhibitors inhibited the specific kinase pathways and induced superior beneficial effects compared with the free drugs. These data show a promising outcome of renal targeting, but more studies are needed in animal models where the treatment is performed on an established disease that might interfere with tubular cell uptake. Moreover, long-term safety studies will strengthen the clinical applicability of such a targeted product.

3.3 Cardiovascular diseases

Vascular endothelial cells play an important role in inflammatory diseases, since activated endothelial cells produce chemokines and cytokines, which, in combination with adhesion factors, can influence leukocyte recruitment and infiltration, thus aggravating the inflammation. Activation of endothelial cells is regulated by several kinase pathways [63]. Addressing inflammation by targeting the endothelium has the added advantage that endothelial cells can be readily reached by macromolecules, since they are in direct contact with the bloodstream. Activated endothelium expresses $\alpha_v\beta_3$ integrins, which can be used for specific targeting of drugs to these cells [64].

3.3.1 p38 MAPK inhibitor

Because p38 MAPK can play an important role in inflammatory diseases, an inhibitor of p38 MAPK was targeted to activated endothelial cells, using an RGD-HSA macromolecular carrier [65]. This carrier binds to the $\alpha_v\beta_3$ integrins, which are abundantly expressed on inflamed endothelium. The study showed abundant uptake of the conjugate into endothelial cells *in vitro* and inhibition of TNF-α-induced inflammatory cytokine expression, showing that the drug was still active in its conjugated form.

3.3.2 c-Abl and PDGF-receptor inhibitor

Neointima formation after vein graft operations can cause graft failure [66]. PDGF plays a central role in this process, since it causes the activation of vascular smooth muscle cells and infiltrating monocytes [63]. Imatinib mesylate (Gleevec), a multi-kinase inhibitor targeting the PDGF receptor and other kinases, has been shown to inhibit this process but only at doses above the usual clinical norm [67]. A nanoparticle formulation of this drug was used to treat veins *ex vivo* before graft surgery. This treatment suppressed neointima formation 28 days after grafting significantly, where free drug did not. The nanoparticle formulation inhibited cell proliferation *in vitro* and phosphorylation of PDGFβ-receptor [68].

3.3.3 Discussion

Endothelial targeting of kinase inhibitors so far has not been tested *in vivo*, so the superiority of this method compared with treatment with free drugs still has to be evaluated in practice. However, the specificity of this delivery method has been shown before, and uptake of the conjugated drug in endothelial cells was efficient in the studies cited above.

3.4 Malignant diseases

The most extensive field of cell-specific drug delivery is the field of tumor targeting. Because of the high cytotoxicity of most conventional chemotherapies and lack of efficacy of some other drugs in certain tumors, efforts have been made to target these drugs specifically to tumors. Although kinase inhibitor-based therapies are often regarded as targeted therapy in the cancer field, since kinase inhibitors are generally directed at a single kinase, or at least a limited number of

targets, tumor-targeted forms of kinase inhibitors are also studied. Some studies were mainly performed to improve poor pharmacokinetic characteristics of drugs by incorporation into micelles or nanoparticles. *In vivo* these particles were passively targeted to the tumor due to the enhanced permeability and retention (EPR) effect in tumors, where due to 'leaky' vessels nanoparticles can penetrate more easily into tumor tissue and are retained there because of passive entrapment, thus providing a mechanism for local accumulation of the drug [69]. Other studies have employed tumor-specific antibodies or other targeting ligands to enhance drug accumulation in the tumor even further.

3.4.1 PI3-kinase inhibitor

Phosphoinositide 3-kinase (PI3-kinase) plays an important role in different forms of cancer, since the pathway is prone to mutations that cause malignancies [70]. As there are many different downstream pathways from PI3-kinase, its inhibition can have broad effects. A pan-PI3-kinase inhibitor is hypothesized to affect proliferation, migration, metastasis, apoptosis and angiogenesis [71]. A vascular targeted prodrug of such an inhibitor was synthesized by coupling the drug LY294002 to an RGDS peptide, which binds to $\alpha_v\beta_3/\alpha_5\beta_1$ integrins. This targeted delivery system resulted in a higher accumulation of the drug in tumors and stronger antitumor and anti-angiogenic effects in U87MG and PC3 tumors in nude mice compared with control treatment with a sham-targeted drug [71].

The PI3-kinase pathway has also been targeted using tumor-cell-specific micelles [72]. This study showed that inclusion of the poorly soluble PI3-kinase pathway inhibitor DM-PIT-1 into micelles improved its solubility. By coupling these micelles to the 2C5 monoclonal antibody, which specifically binds cancer cell surface-bound nucleosomes, the pro-apoptotic effects of the drug were further enhanced in an *in vitro* cell viability experiment. Coupling of TRAIL to the micelle enhanced the pro-apoptotic effectivity even further.

A different study found that incorporation of a PI3-kinase inhibitor into nanoparticles, using an emulsion-solvent evaporation technique, resulted in nanoparticles capable of inhibiting Akt phosphorylation, downstream of PI3-kinase, in B16/F10 melanoma cells. Additionally, the drug-loaded nanoparticles were able to inhibit endothelial cell proliferation and tubulogenesis *in vitro* and angiogenesis in a zebrafish tumor xenotransplant model [73].

3.4.2 MAPK inhibitor

The MAPK pathway consists of several different kinases [74], all of which can be deregulated in tumors. RAS and RAF are particularly associated with gain of function mutations [75,76], which can lead to malignancy. Nanoparticles containing the MAPK inhibitor PD98059, which inhibits mediators downstream of RAS and RAF, were found to inhibit tumor cell proliferation *in vitro* in melanoma and lung carcinoma cells. In this study, nanoparticles were engineered from a hexadentate-polyD, L-lactic acid-co-glycolic acid polymer, which was conjugated chemically to the inhibitor. The nanoparticles were PEGylated to avoid clearance by macrophages. Treatment of tumor-bearing mice with the nanoparticles proved to be more effective in reducing tumor size than treatment with the corresponding free drug. This enhanced effect could also be found in a combination treatment with the cytotoxic drug cisplatin [74].

3.4.3 EGFR inhibitor

In contrast to some of the pathways discussed above, for the epidermal growth factor receptor (EGFR) pathway, several inhibitors are currently approved for use in the clinic. To date erlotinib, gefitinib [77] and the multi-inhibitors lapatinib and vandetanib [78,79] are FDA-approved drugs. Their use, however, is associated with side effects, and no specific delivery forms of these drugs are approved for use.

A new inhibitor of EGFR, AG1478, which is not yet approved for use in the clinic, was, therefore, encapsulated in poly(lactic-co-glycolic acid) microspheres. The microspheres were capable of releasing drug during up to 9 months *in vitro*, depending on the microsphere composition. The drug remained bioactive during this period and inhibited EGFR activity in several cell types [77]. This formulation could thus provide a slow-release form of the drug, avoiding peak concentrations that can often cause side effects.

Overexpression of certain kinase pathways in tumors can also be exploited to use as a marker to monitor therapy. By using an EGFR-binding kinase inhibitor and conjugating it to a radiolabel, the expression of EGFR in tumors can be measured before, during and after therapy with EGFR inhibitors. By incorporating this conjugate into liposomes, the tumor uptake was increased three- to sixfold [80], compared with tumor uptake of radiolabeled tracer alone, thus providing higher local concentrations.

3.4.4 VEGF inhibitor

An inhibitor of vascular endothelial growth factor receptor (VEGFR), which plays a crucial role in the development of new tumor vasculature, would be a potent way to target tumor angiogenesis. To improve efficacy and safety of these drugs, efforts have been made to target VEGFR pathway kinase inhibitors specifically to tumor vasculature. Tumor vascular endothelium expresses $\alpha_v\beta_3$ [81], which can bind RGD-like peptides. Conjugation of VEGFR kinase inhibitor PTK787 to RGD-albumin with and without PEG resulted in different conjugates that all bound to endothelial cells and inhibited VEGF-induced gene expression markers, such as nuclear receptors NR4A1 and NR4A3, in these cells [82]. Another study found that liposomes equipped with the targeting peptide APRPG proved to be a successful targeting mechanism for the VEGFR inhibitor SU1498 *in vivo*. Tumor microvessel density was decreased and survival time of tumor-bearing mice prolonged, as compared with untargeted drug [83].

3.4.5 Discussion

From the studies cited here, it can be concluded that few drugs are directly targeted to tumors, since many studies use

Box 1. Key points for further research.

- Comparative analysis of targeted and free drugs
- Search for cell-specific or disease-specific kinases
- Search for disease-specific receptors or other targets
- Comparative analysis of different carriers and/or targeting methods
- Combination therapies with different kinase inhibitors in one vehicle

the EPR effect and rely on passive targeting. In these studies, the prolonged circulation and lower peak concentrations may lead to a better ratio between effects and side effects, and thus to an increased effectivity of the inhibitor. Many of the available studies report only in *vitro* effects, or stability of the developed drug targeting conjugate, which makes it difficult to assess the advantage of these conjugates over their corresponding free drug. Although *in vivo* efficacy of targeted inhibitors seems to be higher than that of the free drugs in parallel experiments, no *in vivo* tests have yet been performed to assess the effect of targeting on side effects or resistance against tumor-specific targeted kinase inhibitors.

4. Conclusions

Kinase inhibitors have been seen as a promising new type of treatment for a broad spectrum of diseases for the last decades. This is a logical consequence of the fact that kinases are involved in many processes in health and disease, and inhibitors are relatively straightforward to design. However, to date new drugs have been approved only in the field of cancer in the 10 years since the first inhibitor. In this field as well, though kinase inhibitors are a breakthrough, there are problems associated with their use, such as development of resistance and side effects.

Cell- or organ-specific delivery of kinase inhibitors has been shown to be a successful way of increasing local drug concentrations, leading to improved effects in a variety of animal models in different diseases. This strategy thus provides a possible way to improve kinase inhibitors and circumvent problems of low efficacy or adverse effects seen during clinical trials of some of these inhibitors. This review shows that different delivery systems for kinase inhibitors have been tested *in vitro* and *in vivo*, particularly liposomes, proteins and nanoparticles. From this fairly varied overview of models and methods, it is not possible to draw a definitive conclusion about the relative benefit of one delivery system over the other. However, all studies together show that the field of kinase inhibitor delivery systems holds a great promise of improving the treatment of a variety of diseases with unmet medical needs.

5. Expert opinion

Up until now, the cell-specific delivery of kinase inhibitors has not been attempted as extensively as delivery of other drugs, perhaps because kinase inhibitors themselves were initially introduced as 'targeted therapy,' as they were thought to inhibit only one specific target. Various specific inhibitors have since been shown to inhibit multiple targets, and of course, inhibition of even a specific target in a nontarget cell can also cause side effects. If kinase inhibitors are to be used in (chronic) nonmalignant diseases, even a mild side effect profile might hinder their use. Targeted delivery of kinase inhibitors may be a valuable way to improve kinase inhibitors and to circumvent these problems.

Although as yet there is no consensus on the best delivery strategy to target kinase inhibitors to specific tissues or cells, the literature reviewed in this paper gives an overview of the methods tried to date. Most available options give an improved delivery and a slow-release pharmacokinetic profile. However, not all options have also been studied *in vivo*, which makes assessing the results more difficult. *In vivo* studies of drug delivery systems are essential to establish improved pharmacological and/or pharmacokinetic characteristics of the targeted drug versus the original drug. These characteristics cannot solely be determined in *in vitro* systems.

Specific targeting, as opposed to passive targeting using unlabeled liposomes or nanoparticles, seems to yield better results, regardless of the use of carrier. If kinase inhibitors are to be targeted to nonmalignant diseases, further research into disease- or cell-specific targets might also be warranted, since unlabeled nanoparticles cannot be used for these indications.

Inclusion in liposomes or nanoparticles may be a way to circumvent the problems of finding a convenient method to couple kinase inhibitors to the carrier. Kinase inhibitors are difficult to couple due to a lack of suitable functional groups in many inhibitors, so carrier systems that do not require covalent linkage of drug may be an advantage. However, suitable linkers are also available for conjugation, such as the ULS system described in several papers reviewed here. In this way, with the available knowledge in the drug delivery field, cell-specific delivery of kinase inhibitors is achievable. Newer approaches will undoubtedly benefit this field.

To obtain market approval for a targeted form of a kinase inhibitor for clinical studies, the carrier system is an important factor. Liposomes would in this case have the advantage of already being approved for clinical use, as, for example, Doxil. This might speed up the market approval of such a cell-specific targeted kinase inhibitor.

Finally, there are several areas of research still open, or as yet incomplete (**Box 1**). i) As mentioned above, only part of all targeted inhibitors has been tested *in vivo*. This should be done, since it is essential to compare the benefit to side effect ratio with that of the original free drug. ii) Another area that could be explored is a search for cell-specific or disease-specific kinases, that is, kinases that are abundantly upregulated in the diseased area. These might then, in combination with specific delivery techniques be used as a diagnostic tool, as well as a possible treatment. iii) Also further research into disease-specific receptors or other targets could highly increase the possibilities for

specifically targeted delivery of kinase inhibitors. An opportunity here would be the use of the monoclonal antibodies developed against some disease-specific kinases, which might conceivably also be used for targeting small-molecule kinase inhibitors. iv) Comparative analyses of different carriers for targeting would provide insight on the best carrier system for a particular disease. v) Furthermore, combination therapies of different kinase inhibitors using such systems should be exploited not only to improve the therapeutic efficacy of the treatment, but also to circumvent resistance.

In view of the revolution that kinase inhibitors provided when they were first introduced, any further improvement can yield powerful therapeutic effects. As this review shows, several possibilities for the delivery of kinase inhibitors have already been successfully explored, and many still remain open for further research, giving great opportunities for the development of specifically targeted therapies.

Declaration of interest

K Poelstra is a co-founder, shareholder (< 5%) and member of the Scientific Advisory Board of BiOrion Technologies BV, a company dedicated to the development of drug carriers, one of which is discussed in this paper. All other authors declare no conflict of interest.

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