A potential strategy to treat liver fibrosis

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Chapter 7

Summarizing discussion, conclusions and future perspectives

Liver fibrosis is a progressive disease that ultimately leads to cirrhosis and this occurs when the ongoing injury and the subsequent excessive production of extracellular matrix are not stopped (1). The cause of liver fibrosis can be very diverse: alcoholism, chronic infection by virus Hepatitis B or C, diabetes, obesity, as well as autoimmune diseases. A considerable number of people are affected by liver fibrosis, presently being the 9th leading cause of death in the western world. Since most of the proclaimed antifibrotic therapies have failed in clinical testing, liver transplantation remains the only relevant and successful treatment. This invasive procedure however constitutes severe risks for the patient, and is associated with high costs and lack of donor organs. Consequently, liver fibrosis is an unmet medical need and novel antifibrotic therapies will have a huge impact on the future treatment of liver diseases (2).

Recent insights in the molecular mechanisms of liver fibrosis have provided novel approaches in mechanistically-based antifibrotic strategies. One of the most important findings is the recognition of activated hepatic stellate cells (HSC) as major collagen-producing cells in the injured liver, and their identification as a major target cell for antifibrotic therapy (3;4). In the present PhD research project we exploited this knowledge and studied the possibility of treating liver fibrosis by targeting antifibrotic drugs to these HSC. By using a cell-selective delivery of drugs in the body, we can improve drugs that exhibit unacceptable side-effects that limit their clinical applicability. To deliver these potential antifibrotic drugs to HSC, mannose-6-phosphate-modified human serum albumin (M6PHSA) was employed as a drug carrier (5). Although this technology has been applied for various drugs in recent years as a novel therapeutic approach in the liver fibrosis field, not all promising antifibrotic drugs possess appropriate chemical groups for their conjugation to M6PHSA. In the present study, we therefore explored a new linker technology for the preparation of HSC-directed conjugates of antifibrotic drugs, which allowed the targeting of a broader range of drugs. We show that the application of the platinum-based linkage system ULS™ as drug linker, permitted a straightforward and rapid method for the development of drug-M6PHSA conjugates, and afforded the investigation of three new products in the chosen liver fibrosis models. We hypothesized

that cell-specific distribution of these products and the fact that they accumulate inside the target stellate cells will improve the efficacy of the conjugated drugs greatly, and will also prevent undesired side effects.

Chapter 1 presents the scope of the present thesis. **Chapter 2** briefly reviews the function of the liver and the problems associated with liver fibrosis. In the same chapter, we also describe the pharmacological actions of the drugs employed in the present thesis. **The selection of the antifibrotic drug candidates was guided by two main criteria:**

1. The candidate drug should be a promising antifibrotic agent to treat liver fibrosis or acts on a crucial HSC process in this respect, but may exhibit severe side effects in other tissues than the liver. The candidate drug may not only exert antifibrotic actions in HSC but also profibrotic actions in other cell types, so that cell-selective drug targeting will confine its activity to HSC. Pentoxifylline (PTX, chapter 3) is a representative of this latter class of compounds, while losartan (chapter 4&5) can be regarded a compound with a safety risk in fibrotic patients, despite its well-documented safety in hypertensive patients. The third compound (PTKI, chapter 6), a PDGF kinase inhibitor with great similarity to the anticancer agent Gleevec (Novartis), is an experimental compound of which the safety profile has not been investigated. Yet, the widespread involvement of PDGF receptor systems in various physiological processes implies that its inhibition will evoke intra or extrahepatic side effects, and that this drug may strongly benefit from targeting.

2. The candidate drug lacks appropriate chemical groups that allow its conjugation to the M6PHSA carrier via the common covalent coupling reactions. Yet, it should have a chemical structure that enables a coordinative bond with the ULS linker. These specifications are discussed in the second part of chapter 2.

In chapter 3, we describe the development of the first generation of ULS-based conjugates, PTX-M6PHSA, and we demonstrate that the application of the ULS linker is straightforward and safe, despite its cisplatin-related structure. Several

chemical and biological characteristics of the product were examined in various *in vitro* assays, to demonstrate the feasibility of platinum-based linkage chemistry for drug delivery purposes. We investigated conjugate stability and biodegradability, safety, and pharmacological efficacy, and concluded that the conjugate possessed unique drug-releasing properties. We also observed promising antifibrotic effects of PTX-M6PHSA *in vitro*. The production of collagen was reduced notably and we observed striking changes in the morphology of HSC, which were not observed after treatment with free PTX. Although the observed changes in morphology suggest that the cells may detach and die "*anoikis*", we did not observe this in our apoptosis or viability assays. An elimination of fibrogenic HSC by PTX-M6PHSA may be a beneficial effect during liver fibrosis, apart from antifibrotic effects by reducing collagen deposition. We therefore concluded that PTX-M6PHSA is a promising candidate for further evaluation as an antifibrotic drug.

We also showed that advanced liver fibrosis can be successfully treated with losartan-M6PHSA, the second novel conjugate presented in this thesis. Chapters 4 & 5 describe the development, characterization and *in vivo* properties of losartan-M6PHSA. The effect of losartan-M6PHSA was extensively studied in two different rat models of liver fibrosis. Importantly, this novel drug targeting preparation appeared to be far more effective than free losartan, despite the lower dosage of losartan-M6PHSA administered, compared to free losartan. This observation indicated that drug targeting may enhance the intrinsic efficacy of antifibrotic agents. This improvement was attributed to an altered biodistribution of the drug in the organism and we demonstrated an enhanced delivery of our construct to HSC. We examined several steps in the cellular handling of the conjugate: First, after binding to M6P/IGFII receptor, losartan-M6PHSA was internalized via the endocytotic route (5). Degradation of the conjugate will therefore occur in the lysosomes, the compartment to which M6PHSA is routed after internalization (6). We postulate that drug release occurs in multiple steps, in which the albumin is first degraded by lysosomal enzymes, followed by actual release of the drug from ULS in other intracellular compartments, e.g. in the cytosol. This chemical bond between the drug and the *ULS* can not be degraded by proteinases or low pH present

within the lysosomes. More likely, release of the drug will occur by competitive agents that bind to the ULS and displace the drug. Glutathione may be such a compound (7). The cytosol contains high levels of glutathione, so we hypothesize that this compound competitively displaces losartan from the platinum linker, a process that we have already demonstrated *in vitro* for PTX-M6PHSA. Since the intracellular drug release rate was shown to be relatively slow, a local depot of losartan is formed in the target cells that will generate continuous drug levels at the target site.

The mechanism of action of losartan-M6PHSA during liver fibrosis is unknown, and pharmacodynamic differences with the parent drug can not be excluded. The effect of losartan is mediated by binding to the angiotensin II type 1 receptor (AT1). Interaction of losartan with this AT1 receptor after binding of losartan-M6PHSA to the M6P/IGFII receptor may occur via receptor cross-talk in the dynamic plasma membrane. In this context, the so- called "rafts", enriched domains where various receptors reside (8), or the dynamic membrane invaginations in the early endosomes may facilitate such a process. In this case, we propose a model where cell-membrane associated losartan-M6PHSA might bind to other putative receptors on the plasma membrane. Another possibility is that Losartan-M6PHSA may act as an antagonist of the AT1 receptor of a neighbouring cell.

Subsequently, the involvement of angiotensin II (Ang II) in the modulation of TGF-β ligand expression may be important for the antifibrotic action of losartan (9). Ang II increased TGF-β gene expression in a dose-dependent manner in activated HSCs in vitro (10). TGF-β plays a crucial role in HSC activation and thereby regulates the production of matrix molecules during liver fibrosis. The increase of intrahepatic levels of TGF-β, induced by bile duct ligation, was attenuated in AT1 receptor knockout mice (11). Inhibition of the release of Ang II by losartan therefore can markedly suppress the development of liver fibrosis (12;13). So, Ang II seems to be an important pro-fibrotic factor. Thus, the selective delivery of losartan to the HSC by means of losartan-M6PHSA may also interfere in the TGF-β cascade and therefore account for the observed antifibrotic effect.

Drug targeting of antifibrotic agents to the liver has been frequently investigated in the bile duct ligation (BDL) model. The extrahepatic obstruction of the bile duct leads to an accumulation of bile salts and other molecules that provoke an inflammatory and fibrogenic process in the liver and bile duct proliferation (14). In the CCl_4 model of liver fibrosis, the injury is induced by administration of CCl_4 and phenobarbital and the pathogenesis of fibrosis in this model is completely different from the BDL model. BDL is a very rapid and progressive model (15), while the CCl_4 model of liver fibrosis is relatively slow. Consequently, the CCl_4 model may better reflect human fibrotic disease, in which the slow fibrogenic process can be ongoing for several years before the disease becomes manifest and treatment is initiated.

The demonstration of the efficacy of losartan-M6PHSA in both the BDL model and the CCl_4 model of liver fibrosis thus strengthens its potential as a new antifibrotic modality.

In Chapter 6, we present studies showing that the delivery of a PDGF-kinase-inhibiting drug to HSC may be a novel strategy to attenuate liver fibrogenesis.

Targeting the PDGF signaling cascade represents a promising antifibrotic approach since PDGF is regarded as the most potent mitogen for HSC in vitro, and it plays an important role in the transformation of HSC into myofibroblast-like cells in vivo (16). Recent studies have reported antifibrogenic effects of the BCR-ABL kinase inhibitor imatinib (Gleevec), which potently inhibits PDGF-BB kinase (17;18). Kinase inhibitors represent an important and challenging class of novel therapeutics. They seem especially suitable for specific interference with the activation or transformation of cells induced by growth factors or inflammatory mediators. The HSC-selective carrier M6PHSA was therefore equipped with the PDGF receptor tyrosine kinase inhibitor (PTKI, a compound closely related to imatinib) by means of the ULS-based linkage strategy. PTKI-M6PHSA induced antifibrogenic effects in cultured HSC and in fibrotic liver slices, and also displayed significant antifibrotic effects after a single dose in the BDL model of liver fibrosis. However, the administration of multiple dosages did not lead to a significant enhancement of the effect. Thus, while PTKI-M6PHSA proved most effective in vitro, losartan-M6PHSA was the most effective compound in animal models. Of note, there is

an evident increase of PDGF receptor expression in the BDL model of liver fibrosis from day 10 to day 14. This receptor system apparently could not be efficiently blocked by the dose of the targeted PTKI that we administered. We suggest that a higher dose could improve the effect of PTKI via an effective blockade of the PDGF receptor kinase, which is so abundantly expressed in activated HSC.

The observed differences in effectiveness may be also explained by the complex nature of liver fibrosis and HSC activation. Especially if the causative agent of liver fibrosis is not removed, specific inhibition of PDGF-R in HSC may only temporarily affect HSC proliferation (19). Chronic treatment regimens might allow enough time for the activation of alternative pathways and cytokines that may compensate for the inhibition of the PDGF cascade (20). Thus, only inhibiting PDGF would not be sufficient to stop HSC proliferation, activation and subsequent production of extracellular matrix constituents. Parallel pro-fibrogenic mechanisms, such as TGF-β-mediated activation, could be responsible for triggering the fibrogenic process within the liver despite a complete blockade of the PDGF receptor pathway.

Future perspectives.

Our strategy offers multiple opportunities for the development of novel HSC- directed therapeutics. For instance, the coupling of different classes of kinase inhibitors that presented problems with conjugation to M6PHSA-like carriers in the past. This strategy profoundly changes the body distribution of these drugs and thus leading to enhanced efficacy and reduced side-effects which are important especially for the long-term treatment with this kind of drugs.

An interesting new concept that may originate from the studies with PTKI-M6PHSA is the use of so-called "cocktail treatments". Such combination therapies are already common in anticancer research, but could also be applicable to liver fibrosis.

What cocktail would be the most effective one, can only be speculated but a combined inhibition of TGF-β and PDGF cascades might significantly contribute to an effective inhibition of hepatic fibrosis (21). Kinase inhibiting drugs may represent a valuable asset in such an approach. In a similar linkage strategy as described for the other drugs in this thesis, a TGF-β receptor kinase inhibitor (ALK5 inhibitor, 3-(Pyridin-2-yl)-4-(4-quinonyl)-1H-pyrazole) was conjugated to a renal targeting system (Prakash, manuscript in preparation), and this approach is also applicable to the M6PHSA carrier. Combined administration of the ALK5-inh-M6PHSA conjugate with PTKI-M6PHSA can be explored to counteract PDGF and TGF kinase cascades in HSC, but other rational combinations of inhibitors are feasible as well. Interestingly, the combination of two antifibrotic agents intervening in PDGF signaling (imitanib) and the renin-angiotensin pathway (the angiotensin converting enzyme inhibitor, perindopril) has recently been investigated and proved more effective than inhibition of either pathway alone (22). The participation of angiotensin II (Ang II) in modulating TGF-β expression has been already discussed. Ang II is a potent inducer of TGF-β synthesis in cultured HSCs in vitro (10) and intervention with the renin-angiotensin system (RAS) appears to be very effective if this is combined with an inhibition of the mitogenic action of PDGF. In this respect, a combination therapy with losartan-M6PHSA and PTKI-M6PHSA seems an attractive approach, and both conjugates are currently available in our department. Thus, targeting of both intracellular cascades in the HSC could be the eligible approach in future investigations for an effective anti-fibrotic therapy.

Another possibility is to target anti-angiogenic drugs to the fibrotic liver, in order to prevent the development of hepatocarcinoma in patients with liver fibrosis. It has been already reported that neovascularization is increased during the development of liver fibrosis, and suppression of angiogenic signaling attenuates liver fibrogenesis (23). The ongoing destruction of healthy liver tissue and tissue remodeling during liver fibrosis constitutes a serious risk factor for the development of liver cancer (24). Among others, liver fibrosis is associated with hypoxia, which is one of the strongest inducers of vascular endothelial growth factor (VEGF)(25). The elevated expression of VEGF and other

growth factors favors the progression of tumor growth and angiogenesis. Delivery of a VEGF receptor kinase inhibitor to the fibrotic liver thus seems an interesting approach, and the delivery of anti-angiogenic agents to endothelial cells was shown to be a feasibile option in our lab. We have recently developed drug-carrier conjugates of the VEGF-receptor kinase inhibitor PTK787 (vatalanib) and RGD-equipped albumins (Temming, manuscript in preparation). The presently discussed ULS-based conjugation strategy was also applied for preparation of these PTK787-HSA conjugates. Another interesting aspect of PTK787 is that it also inhibits the PDGR receptor kinase, apart from its VEGF receptor kinase inhibition. Therefore, the delivery of PTK787 to the fibrotic liver by means of a PTK787-M6PHSA conjugate would interfere with the formation of new blood vessels and simultaneously affect the proliferative actions of PDGF on HSC, thus combining antiangiogenic activity with antifibrotic actions.

Another quite relevant question is which specific pathways need to be blocked at specific phases of the fibrotic process. Various profibrotic pathways become activated at different time points during the progression of liver fibrosis (26), so the timing of an appropriate therapy is crucial for its efficacy. Antifibrotic agents can either serve as a preventive therapy for the development of fibrosis in newly affected areas, or they may act in areas where the fibrosis is already established. A specific treatment might evolve in a personalized therapy, in which the disease progression and therapeutic response should both be closely monitored in time. Future experiments may therefore focus on the activation of kinase pathways and expression profiles of profibrotic factors by inspecting biopsies from patients suffering from liver fibrosis. The availability of appropriate biomarkers will enable disease monitoring and the proper selection of antifibrotic drugs that subsequently can be delivered specifically to the fibrotic liver by means of drug conjugates.

Apart from developing a novel linker technology, we also have developed a very promising compound, losartan-M6PHSA, which showed antifibrotic effects in two different models of liver fibrosis. The optimization of losartan-M6PHSA conjugates should be part of future research, including the investigation of long-term administration

and safety before the clinical trials. In addition, it would be interesting to explore the application of losartan-M6PHSA to treat the portal hypertension occurring in liver fibrosis. Cirrhotic patients suffer from portal hypertension which, as a consequence, leads to systemic hypotension. The specific delivery of losartan to the liver would avoid its hypotensive actions in peripheral vascular beds, and by this reduce the risk of a hypotensive shock. Testing of this hypothesis in an animal model of advanced liver fibrosis, including measurement of both systemic and portal blood pressure seems one of the most challenging ideas. In addition, follow-up studies of losartan-M6PHSA in different animal models of liver fibrosis are required to validate its therapeutic applicability in this disease.

Furthermore, we have demonstrated that ULS-based products are not-toxic per se, as demonstrated by *Alamar blue* studies, *caspase 3/7* activation and TUNEL assays *in vitro*. In sight of a prolonged administration of ULS conjugates to treat liver fibrosis the question that remains unanswered is the toxicity of cisplatinum after drug release. There are various reasons that validate its potential application in therapy. Compared to the high administration of cisplatinum in antitumour therapy, the dose that it would be administered to stop liver fibrosis is rather low. First, because the fibrogenesis in the liver is developed during ten to twenty years and the therapy would aim to a monthly administration of a potent antifibrotic drug targeted to HSC, which would prevent the formation of extracellular matrix and scar tissue. Secondly, ULS's reactive groups are covered with the targeted drug and the carrier. After degradation of the carrier and release of the drug, ULS linker would be readily detoxified with many intracellular molecules that are available in the cell containing amino or thiol groups. Taking into consideration that this is one of the reasons explaining the cell-resistance phenomenon to cisplatinum in anticancer therapy, the ULS strategy remains applicable. In any case, a favorable treatment for the patients with liver fibrosis would prevail always considering the suitable balance of the beneficial treatment versus possible side effects. In this regard, more extensive safety testing is required during further development of this technology.

Conclusions

The research presented in this thesis demonstrates that platinum coordination chemistry can be employed for the conjugation of potential drug candidates to target the fibrotic liver. Drug-ULS conjugates display adequate stability and bioreversibility, and a unique slow release profile of the coupled drug upon prolonged incubation. This feature can be attributed to the platinum-coordination bond and has been observed for all tested compounds. We demonstrated that our approach of HSC-directed drug targeting is effective in animal models of liver fibrosis, both when examining hepatic drug levels and the pharmacological outcome. Eventually, the present work may generate a versatile approach for linking various antifibrotic agents and other classes of drugs to targeting devices, leading to effective conjugates that can stop or reverse the ongoing process of liver fibrosis.

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Cafe Central with its gothic vaults and quality coffee is probably the most famous coffeehouse in Vienna. At the turn of the century, it was a meeting place for intellectuals such as Victor Adler and Otto Bauer, writer Peter Altenberg and Leon Trotsky.

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