A potential strategy to treat liver fibrosis
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Chapter 2

General Introduction:
Potential strategies to treat liver fibrosis

There, inside, you filter and apportion
You separate and divide,
You multiply and lubricate
You raise and gather
the threads and the grams of life...
from you I hope for justice:
I love life: Do not betray me! Work on!
Do not arrest my song.

Ode to the liver,
**Liver**

In Greek mythology, Prometheus was punished by the gods for revealing fire to humans and he was chained to a rock where an eagle, Ethon, pecked out parts of his liver, which would grow to a complete organ again overnight. Curiously enough, the liver is the only human internal organ that actually can regenerate itself, a characteristic which apparently already was known to the Greeks. In fact, the liver is capable of natural regeneration of lost tissue: as little as 25% of remaining liver can regenerate into a whole liver again (1).

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**Figure 1. Liver with the right and left lobe.** (Obtained from Anatomy of the Human Body. Fig. 1085 Henry Gray, 1918.)

The adult human liver normally weighs between 1.3 - 3.0 kilograms, and is a soft, pinkish-brown "boomerang shaped" organ. It is the largest internal organ within the human body. The liver is essential in keeping the body functioning properly. It plays an important role in the clearance of compounds from the blood (metabolism, excretion), produces immune proteins to control infections and directly removes germs and bacteria (innate immune system) and synthesizes proteins that regulate blood clotting and various other physiological processes. Furthermore, the liver produces and excretes bile fluid which is required for food digestion and absorption of fats and fat-soluble vitamins (2). You cannot live without a proper functioning liver. Due to this complex spectrum of functions it is not yet possible to produce an artificial organ capable of replacing all functions of the liver, and the attempts to do so are outside the reach of science in the foreseeable future.
Liver cells network

Classically, the liver was seen as being divided in hexagonal lobules formed by parenchymal hepatocytes that constitute around 80% of the total liver volume, and also by three different nonparenchymal cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC, formerly known as fat-storing cells, Ito cells, lipocytes or vitamin A-rich cells). The hepatocytes are important for the high metabolic activity of the liver and the secretion of compounds into the bile (3;4). The fenestrated endothelium plays an important role in the filtration of compounds from the blood to the hepatocyte surface. Like Kupffer cells, SECs have a huge endocytic capacity for many ligands including glycoproteins and several components of the extracellular matrix (ECM). SEC are also active in the secretion of cytokines and other mediators of cellular activity (5;6).

Kupffer cells are local tissue macrophages located in the sinusoids space with a pronounced endocytic and phagocytic capacity. Kupffer cells control the early phase of liver inflammation, and thus play an important part in the innate immune defense system (7). High exposure of Kupffer cells to bacterial products, especially endotoxin (lipopolysaccharide, LPS), leads to the production of inflammatory mediators and ultimately to liver injury. Moreover, during liver injury and inflammation, Kupffer cells secrete enzymes and cytokines that may damage hepatocytes, and they are active in the remodeling of extracellular matrix (8).

Hepatic stellate cells (HSC) reside in the perisinusoidal space. In the normal liver, HSC are characterized by storing vitamin A and they control the turnover of extracellular matrix, and the regulation of the contractility of sinusoids. Acute damage to hepatocytes induces transformation of the quiescent HSC into activated myofibroblast-like cells and the latter cells play a key role in the development of inflammatory fibrotic responses (9;10). Activated HSC transdifferentiate into proliferative, fibrogenic, and contractile myofibroblasts that initiate further cell proliferation and increased deposition of extracellular matrix (ECM) components in the process of wound healing (2;11). During chronic liver injury, the excessive ECM replaces the functional liver, influencing the
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function of remaining cells and forms a solid mechanical scaffold for cell adhesion and migration. This matrix consists of collagens, glycoproteins, proteoglycans, glycosaminoglycans and molecules that are bound specifically to the ECM, such as certain growth factors/cytokines, matrix metalloproteinases (MMPs) and enzymes such as tissue transglutaminase and procollagen propeptidases (12). It is a finely tuned ecosystem which is dysbalanced during chronic liver injury.

Figure 2. Activation of Hepatic Stellate Cells during liver fibrogenesis.

A. Hepatic sinusoid with hepatocytes as parenchymal cells, and the non-parenchymal cells: the hepatic stellate cells with the vitamin-A droplets (**HSC**) in the space of Disse, the endothelial cells (**EC**) and the Kupffer cells (**KC**) in the sinusoid.

B. During liver injury, the activation of HSC leads to cell proliferation producing extracellular matrix components (**ECM**). Figure adapted from Friedman, J Hepatol 2003.

What is liver Fibrosis?

Liver fibrosis is a reaction to chronic liver injury, and it is characterized by an excessive accumulation of extracellular matrix proteins including collagen. It is a common process during the majority of chronic liver diseases (13). All liver cell types play their specific role in liver fibrosis, and there is much evidence now of cross-talk between the different cell types through the release of a wide variety of key mediators, e.g. nitric oxide, interleukins, chemokines, growth factors and reactive oxygen species (ROS) (4). The cooperation between liver cells is better understood due to the knowledge gained in the last decades on liver fibrosis and other liver diseases.
The fibrogenic process resembles a continuous wound-healing, yet accumulation of huge amounts of extracellular matrix results in scarring of the tissue and disturbance of blood supply. The progressive necrotic areas lead to even more inflammation and tissue damage that needs to be repaired (12). If the liver injury persists, an exacerbation of fibrosis may lead to the development of cirrhosis (14). Various etiological factors can be responsible for a perpetuation of the fibrogenic process including alcohol consumption, exposure to various drugs and toxic chemicals, viral hepatitis, metabolic syndrome, autoimmune disease and hereditary disorders of metabolism (15). Chronic liver injury finally leads to cirrhosis and all its complications, portal hypertension, and ultimately liver failure. Liver transplantation, an extremely costly procedure, is currently the only remedy in this condition (16).

Central to the liver fibrogenesis is the activation of HSC (17). HSCs are activated by inflammatory and fibrogenic cytokines such as TGF-β, angiotensin II (18), and PDGF-BB (19). Cellular changes accompanying HSC activation include morphological changes such as the appearance of the cytoskeletal protein smooth muscle α -actin (α-SMA), a loss in the cellular vitamin A stores, and an increase in the appearance of rough endoplasmic reticulum. An increase in DNA synthesis and cellular proliferation also occurs following HSC activation. The pattern of gene expression changes and a dramatic increase in types I and III collagens production occurs (20). TGF-β is the most potent fibrogenic cytokine described for the HSC activation (21) and the receptor expression for this cytokine is largely increased following HSC activation. At the same time, HSC proliferate under the influence of growth factors. Platelet derived growth factor (PDGF-BB) is regarded as the most potent mitogen for HSCs. The PDGF receptor expression on HSC are also increased during the liver injury (22;23), leading to a continuous proliferation of these cells.

The perpetuation of the activated phenotype of HSC is caused by the ongoing cytokine production and remodeling of extracellular matrix (ECM) (24;25). The collagen production and the cytokines secreted by activated HSC as well as autocrine and paracrine stimulation of other liver cell types (injured hepatocytes, Kupffer cells) contribute to the
aggravation of the fibrotic process. After prolonged chronic injury, the liver contains high levels of the matrix proteins collagen and elastin and of other structural glycoproteins, proteoglycans and pure carbohydrates, *i.e.* hyaluronan (26).

In conclusion, two major features render the HSC the key fibrogenic cell. Firstly, a dramatic increase in the synthesis and deposition of extracellular matrix proteins produced by activated HSC and, secondly, the increased proliferation rate of HSCs which strongly amplifies the number of fibrogenic cells (27).

Moreover, HSC activation is associated with an increase in cell contractility, which leads to increased portal pressure via the constriction of individual sinusoids and contraction of the cirrhotic liver as a whole (28).

**Epidemiology of liver fibrosis**

Chronic liver disease is responsible for over 1.4 million deaths annually according to data from the World Health Organization Mortality Database (WHO, World Health Report 2005; http://www.who.int/en/) and in the western world this disease is among the top ten of disease-related causes of death (CDC, National Center for Health Statistics, 2005). Overall there has been reported a 13% increase in the death rate from liver-related disease per year (29). Of the liver-related deaths, 77% were associated with viral hepatitis, 14% with alcohol abuse, and 9% with hepatocellular carcinoma (30). Many etiological factors cause fibrosis and eventually lead to cirrhosis. It has been estimated that excessive alcohol consumption is a major contributor in 41-95 percent of deaths from cirrhosis in some countries (31). The level and duration of alcohol consumption are important determinants in the development of liver pathology. As the primary site for detoxification of alcohol and its metabolites, the liver can go through the following pathological stages: fatty liver, alcoholic hepatitis, fibrosis and cirrhosis.

Because of the high rates of liver disease, liver transplantation is now considered a standard therapy for patients with end-stage liver disease, regardless of the cause. Currently, about 5,000 liver transplants are performed yearly in the United States at more than 120 medical centers. As a consequence of the limited supply of livers, there are more
than 17,000 persons on the liver transplant waiting list and at least 1,500 will die annually while waiting (http://liverplan.niddk.nih.gov).

The concept of Liver fibrosis reversion

Centuries after the greeks suggested it in their mythology, the regeneration of the damaged liver was finally catalogued by Perez-Tamayo in 1979 (32). In this study, the first evidence for reversibility of fibrosis and cirrhosis in animal models and human was presented. This has stimulated researchers to search for potential antifibrotic drugs that could definitely reverse liver fibrosis. Emerging antifibrotic therapies aim at inhibiting the accumulation of fibrogenic cells or preventing the deposition of extracellular matrix proteins. Although various antifibrotic agents are effective in experimental models of liver fibrosis, to date their efficacy and safety in humans have not been established (33-35). On the other hand, evidence of fibrosis regression has been documented after treatment of patients with antivirals for Hepatitis B (36) and Hepatitis C (37) in clinical trials. These studies and studies in experimental animal models have improved the understanding of mechanisms of extracellular matrix (ECM) production and degradation. It appeared that liver scar tissue can be resorbed in fact (38). The accumulated ECM can be degraded through the action of matrix metalloproteinases, enzymes that digest components of the ECM (39). The existence of tissue inhibitors of these matrix metalloproteinases (TIMPs) partly explains the excessive accumulation of ECM in fibrosis and influence the dynamic process of synthesis and degradation. Interestingly, activated HSC also play a vital role in orchestrating matrix degradation during liver fibrogenesis. Apart from their participation in the synthesis of large amounts of extracellular matrix, simultaneously they increase TIMP-1 levels resulting in decreased matrix degradation. When fibrosis regresses, TIMP-1 levels decline and degradation of ECM increases. This effect is associated with removal of activated stellate cells through apoptosis (40;41). In contrast, sustained TIMP-1 expression inhibits protease activity and blocks apoptosis of activated stellate cells (42). Thus, it is implicit that fibrosis is associated with the massive deposition of extracellular matrix (ECM), increased levels of inhibitors of matrix metalloproteinases and collagenases
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(TIMP), and also a significant collagen cross-linking by tissue transglutaminase activity (43).

In conclusion, the fibrogenic process can be viewed as a dynamic balance between matrix degradation and production so that even advanced fibrosis or cirrhosis seem to be reversible.

Need for antifibrotic therapies

Many studies in the past two decades have shed considerable light on the mechanisms of liver fibrosis, with particular emphasis on stellate cell biology, and have led to an increased enthusiasm for treating hepatic fibrosis (44). There is tremendous activity in the field of drug development for this purpose and ongoing testing of potential antifibrotic agents (45). Nevertheless, efficient and well-tolerated antifibrotic drugs are still lacking, and current treatment of hepatic fibrosis is limited to the withdrawal of the harmful agent and transplantation. In situations in which dealing with the underlying process is not possible, interference with the liver fibrosis process is essential. A large number of these approaches have been validated in cultured cells and in animal models, and clinical trials are underway or anticipated for a growing number of molecules (46).

A successful antifibrotic strategy does not need to eradicate hepatic fibrosis entirely, because the liver has an enormous functional reserve. Instead, any therapeutical approach that sufficiently attenuates fibrosis progression to prevent the development of cirrhosis and/or hepatocellular carcinoma will be viewed as a success (47).

Several antifibrotic therapies have been tried ending with poor or mediocre success (48). The problems with many potentially antifibrogenic drugs are, among others, the lack of cell specificity of the drugs in vivo with the occurrence of extrahepatic side effects. For such drugs, a cell-specific delivery may prove beneficial. The acquired high specificity of a locally delivered compound would permit the long-term treatment that is required for a chronic liver disease.

During the past years, several projects concerning target-cell specific antifibrotic therapies have been started in our department (Table 1). Several promising therapeutic approaches
have been encompassed in our drug delivery strategies, either targeting hepatic inflammation (dexamethasone, naproxen, losartan)(49) or intracellular signaling and transcriptional pathways involved in stellate cell activation and ECM turnover (Pentoxifylline, gleevec, kinase inhibitors)(50;51), or provoking apoptosis of activated cells (doxorubicin, gliotoxin)(52;53). Other strategies include the delivery of anti-inflammatory agents like IL-10. The near future will learn which of the chosen drugs will be most effective, or which other (combination of) targeted therapies can be envisioned. In the next section, three drugs will be more extensively discussed: pentoxifylline, losartan and the PDGF receptor tyrosine kinase inhibitor (imatinib). These compounds have been subject of drug targeting strategies in the present thesis.


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Table 1. Drugs in development for antifibrotic approaches in our group.

<table>
<thead>
<tr>
<th>Main mechanism</th>
<th>Agent</th>
<th>Studies (Ref)</th>
<th>In our department</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenuate HSC activation</td>
<td>Pentoxifylline</td>
<td>JCR 2006 (54)</td>
<td>T. Gonzalo</td>
</tr>
<tr>
<td>Reduce inflammation</td>
<td>Interleukin-10</td>
<td>Pharm Res. 2004 (55)</td>
<td>H. Rachmawati</td>
</tr>
<tr>
<td></td>
<td>Losartan</td>
<td>In preparation (56)</td>
<td>T. Gonzalo</td>
</tr>
<tr>
<td></td>
<td>Prostaglandin</td>
<td>In preparation</td>
<td>W. Hagens</td>
</tr>
<tr>
<td>Promoting apoptosis of HSC</td>
<td>Gliotoxin</td>
<td>Liver Int. 2006 (60)</td>
<td>W. Hagens</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiproliferative to HSC</td>
<td>PDGF R inhibitor</td>
<td>Submitted (57)</td>
<td>T. Gonzalo</td>
</tr>
<tr>
<td></td>
<td>Micophenolic acid</td>
<td>J Hepatol. 2005 (58)</td>
<td>R. Greupink</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>JPET 2006 (59)</td>
<td>R. Greupink</td>
</tr>
<tr>
<td>Reduction inflammation and epithelial-mesenchymal transformation</td>
<td>p38 MAP kinase inhibitor</td>
<td>JPET 2006 (61)</td>
<td>J. Prakash</td>
</tr>
<tr>
<td></td>
<td>TGF-b kinase inhibitor</td>
<td>In preparation</td>
<td>J. Prakash</td>
</tr>
</tbody>
</table>

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Antifibrotic drugs: Pentoxifylline, losartan and PDGF

Tyrosine Kinase Inhibitor (PTKI)

Pentoxifylline

Pentoxifylline (PTX) is a phosphodiesterase inhibitor that is clinically useful for the treatment of disorders of vascular perfusion and cerebrovascular diseases due to its favorable effect as a peripheral vasodilator (62). The antifibrogenic effect of PTX on activated hepatic stellate cells has been extensively reported and demonstrated (63-65). Although its mechanism of action remains unclear, it has been suggested that PTX reduces the transdifferentiation of HSC to myofibroblasts and inhibits HSC proliferation (11;66;67).

Figure 3. Proposed antifibrotic mechanism of Pentoxifylline action in the cell.
Figure adapted from references (Raetsch,2002; Duncan 1995; Rodriguez-Barbero 2002; Chen 1999) (11,68-70). Abbreviations: PTX, pentoxifylline; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; CTGF, connective tissue growth factor; TGF-β, transforming growth factor β; ERK, extracellular-regulated kinase; P38 MAP kinase, mitogen-activated kinase; ECM, extracellular matrix.
PTX also may reduce the fibrogenic effect of TGF-β on HSC by interference with p38 MAP kinase and ERK1/2 pathways, thereby decreasing hepatic procollagen type 1 mRNA expression (71). In addition, PTX interferes with cAMP involved in inflammatory signaling (72). In another study it was shown that PTX blocks NF-kB, one of the important mediators of HSC, thus preventing the activation and proliferation of HSC induced by carbon tetrachloride, a rat model of liver fibrosis. In vitro studies with fibroblasts have shown that PTX potently reduces cell proliferation, stimulates interstitial collagenase activity, and suppresses the synthesis, secretion, and deposition fibrillar collagens type I and III, proteoglycans, and fibronectin (73-75). Moreover, Lee et al demonstrated that PTX downregulates hepatic procollagen type I expression in the bile duct ligation model of liver fibrosis (76).

Yet, despite beneficial effects in HSC, profibrotic effects of PTX on Kupffer cells have been reported (11), as well as many effects in other cell types (77,78). Cell-selective targeting of PTX to HSC seems therefore required to create favorable effects in HSC, while avoiding the profibrotic effects in Kupffer cells and effects in other organs (79). Taking into account the antifibrotic properties attributed to PTX, we have developed a drug targeting conjugate for the delivery of PTX to HSC. In chapter 2 we describe the development of the novel HSC-directed conjugate PTX-M6P/HS.

Losartan

Losartan is an orally active, nonpeptide angiotensin II (Ang II) receptor antagonist. It was the first of a new class of drugs introduced for the treatment of hypertension and renal disease. These angiotensin receptor blockers bind competitively and selectively to the Ang II type 1 (AT1) receptor, thereby blocking Ang II-induced physiological effects (80).

Systemic hypertension is a complex pathophysiological state that is primarily manifested as chronic high blood pressure. It is a major risk factor for stroke, ischemic heart disease, peripheral vascular disease, and progressive renal damage (81). It is well established that a hyperactive renin angiotensin system (RAS) plays a key role in the development and maintenance of human primary hypertension. This disorder contributes to at least 10% to 30% of all cases of hypertension by some estimation (82).
RAS blockers are reliable and affordable, and their short duration of action makes them an excellent drug of choice if reversal of their activity is required. On the other hand, as with most antihypertensive drugs, their effects are short-lived having to be administered on a frequent basis with a risk of significant side effects (83).

Recent experimental studies indicate that the RAS also plays an important role in liver fibrogenesis (84,85). Hepatic stellate cells (HSC) are the main target cell type for the pathogenic effects of Ang II in liver fibrosis. In the normal human liver, HSC do not express AT1 receptors nor do they secrete Ang II. Following chronic liver injury however, HSC transform into myofibroblast-like cells which express both AT1 receptors and generate mature Ang II, which exerts an array of pro-inflammatory and profibrogenic actions (86-89). These pathogenic effects can be prevented largely by AT1 receptor antagonists, as has been demonstrated in different models of experimentally-induced liver fibrosis (90-92). Based on these data, RAS inhibitors like Angiotensin Converting Enzyme (ACE) inhibitors or Ang II receptor blockers are currently considered as novel antifibrotic therapies to treat liver fibrosis. Preliminary clinical data suggest that AT1 receptor blockers may attenuate the fibrogenic process (93). The use of AT1 receptor blockers would be particularly useful in conditions characterized by a rapid progression of fibrosis (i.e. acute alcoholic hepatitis and severe hepatitis C virus reinfection after liver transplantation). However, in patients with advanced fibrosis, the use of angiotensin antagonists may be hampered by undesirable effects on the arterial pressure, especially since patients with cirrhosis are generally associated with low systemic blood pressure. Indeed, the use of the AT1 receptor blocker losartan in patients with advanced fibrosis was associated with the risk of hypotensive shock syndrome (94).

We hypothesized that targeting of losartan to HSC could be an effective strategy to attenuate hepatic fibrosis. Therefore, we have coupled losartan to M6PHSA, a stellate cell-selective carrier, resulting in losartan-M6PHSA. In addition, this strategy would overcome side effects such as reduction of blood pressure. Details about this novel approach are explained in chapters 3 and 4 in the present thesis.
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**PDGF Receptor tyrosine kinase inhibitors**

The fundamental role that protein tyrosine kinases appear to play in liver fibrogenesis and many other diseases, has made them attractive therapeutic targets (95), and has provided the rationale for the development of specific inhibitors of these enzymes.

PDGFR-β is a receptor tyrosine kinase that consists of an extracellular ligand binding domain connected to a cytoplasmic domain, responsible for intracellular signal transduction. Extracellularly, the PDGFRβ receptor binds via the beta subunits only the isoform PDGF-B (96,97). The binding induces activation of the receptor kinases by formation of receptor dimers that catalyze the transfer of the γ-phosphate of ATP to tyrosine residues in protein substrates. This leads to a wide variety of cell responses, e.g., differentiation, proliferation, migration, angiogenesis and survival (98).

![Diagram of PDGF Receptor tyrosine kinase inhibitors](image)

**Figure 4.** Proposed mechanism of action of PDGFR-β kinase inhibitors interfering with a wide variety of cell responses. (Adapted from Aaronson 1991).
Activated HSC display high levels of PDGFR-β receptor and release PDGF-BB cytokines during liver fibrosis (99). It has been demonstrated that PDGFR-β receptors are upregulated on the cell surface of hepatic stellate cells during fibrosis (100-102). The fibrogenic process is encouraged by platelet-derived growth factor (PDGF-BB), identified as the most potent mitogen for HSC (103). Several isoforms of PDGF have been described in activated HSC have been found. Dimers formed by disulphide bond are PDGF-AA, AB, BB, CC, or DD polypeptide chains (104). The expression of the PDGF isoforms is differentially regulated by PDGF-BB itself and by TGF-β1, two mediators that are produced by HSC themselves during liver fibrogenesis (105;106). New drugs that inhibit PDGF-β kinase activity have emerged in recent years.

Imatinib mesylate (Gleevec, STI571) was initially designed for use in chronic myeloid leukemia (CML) as an anti-tumor drug. Imatinib is active against a number of related tyrosine kinases that are mutated in cancer (107). Since imatinib also inhibits PDGFR-β kinase activity, it may also be applied as a drug for liver fibrosis.

Other strategies targeting the PDGF pathway involve the use of antibodies against PDGFR-β kinase or a soluble receptor blocking the natural binding of PDGF cytokine (108). Blockade of PDGF-BB interaction with its tyrosine kinase receptor may represent a promising approach for therapeutic intervention in hepatic fibrosis.

We have developed a new construct, PTKI-M6PHSA, in which a PDGF Tyrosine Kinase Inhibitor (PTKI), related to imatinib mesylate, is coupled to our stellate cell-carrier M6PHSA. In the present thesis, we tested the impact of PTKI-M6PHSA on liver fibrogenesis in vitro and in vivo. Chapter 5 describes this approach.
Drug targeting technology

The first idea of drug targeting was proposed by Ehrlich in the nineteenth century. He presented the idea of “the magic bullet” that can bind selectively to specific types of cells in a manner similar to that of the key and lock approach. Scientists have ever since worked on the principle of drug targeting based on this idea of specifically delivering drugs to diseased cells.

Classical drug molecules: actions in whole body

- Side-effects
- toxicity limits effect
- limited therapeutic effect

Drug targeting approach: locally acting drugs

- no serious side-effects
- improved therapeutic effect

Drug

Drug-carrier

Figure 5. Classical drug administration versus the drug targeting approach.

Drug targeting is defined as selective drug delivery to specific physiological sites, organs, tissues, or cells where the pharmacological activity of the chosen drug is required (109). In principle, a drug that distributes throughout the whole body after its administration may cause side effects at sites other than the pathological tissues. Although that may not create a problem for the majority of drugs, side-toxicity is one of the limiting events for the therapeutic use of, for example, cytotoxic agents. Due to these adverse reactions, dose limitations may prevent effective treatment. Therefore, selective delivery into the target
tissue may allow a higher drug concentration at or in the target cells or even in specific compartments of the target cells, and thus improve the therapeutic index/safety of such compounds.

Drug targeting may be classified into two general strategies: passive and active targeting. Passive targeting is a strategy whereby the physicochemical properties of carrier systems increase the target/nontarget ratio of the quantity of drug delivered to the target tissues, organs, or cells. In this way, targeting of drugs would avoid side effects by preventing major distribution to a particular organ or cell type.

Carriers included in this category are synthetic polymers, some natural polymers such as albumin, liposomes, micro (or nano) particles, and polymeric micelles. Chemical factors such as hydrophilicity and positive/negative charge and physical factors such as size and mass greatly influence the passive targeting efficiency. For example, the cardiotoxicity of doxorubicin can be decreased by including it in a liposomal formulation (110). However, it should be stressed that drugs in such preparations should maintain their intrinsic anti-tumor efficacy and also should not exhibit side effects specifically related to the liposomal formulations such as liver (macrophage) toxicity.

Active targeting employs specific receptor interactions to increase the delivery of drugs to a target site where the pharmacological effect of the drug is required. The incorporation of a homing devices, or site-directed ligands, redirects the construct to specific binding sites on cell membranes. These interactions include antigen-antibody and ligand-receptor binding. The homing devices can be carbohydrate ligands, functional groups bearing antibodies or other peptide ligands. These homing devices may be coupled to drug carriers like antibodies, or other proteinaceous carriers like albumin or transferrin, polymers, liposomes or nanoparticles.

**Drug Targeting approach to Stellate cells**

Targeting of drugs to hepatic stellate cells (HSC) currently represents a challenge for the scientists involved in the design of a treatment for liver fibrosis. As explained in detail in
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the previous section, HSC are a crucial target for pharmacological intervention of liver fibrosis. Various HSC-specific carriers have been developed in our group (79).

Figure 6. Picture of Losartan-ULS-M6PHSA conjugate targeting to the Hepatic Stellate cell (star-like cell) via the M6P/IGFII receptor upregulated during liver injury.

The magic bullet concept can now be applied also for targeting of losartan, among other drugs, as illustrated in figure 6. In this schematic model, the drug delivery preparation is composed of three parts: drug, linker and carrier. The applied linkage system is ULS, which will be explained later in this thesis, and the carrier is M6PHSA, which binds to the M6P/IGFII-receptor on activated HSC.

Types of carrier

In figure 7 various types of drug carriers are depicted. The type of water-soluble polymeric carrier also includes apart from the chemically prepared carriers, naturally occurring
polymers. Emulsions comprise small oil droplets stabilized with a monolayer of an amphiphilic substance on the surface. Nanospheres are solid small particles made from natural or synthetic polymers. A major difference between droplets in emulsions and nanospheres is the status of the interior: liquid for emulsions and solid for nanospheres. A liposome is a vesicle made up with a lipid bilayer that mimics cellular membranes. Polymeric micelles are an assembly of amphiphilic polymers (typically comprising multiples, i.e. 10 to 100 of polymeric chains) with a spherical inner core and an outer shell. Combination strategies are nowadays being employed, for example, antibody-targeted liposomes, bispecific antibody-mediated viral vectors, ligand-peptide modified plasma proteins, recombinant proteins, etc. In the present thesis, a modified human serum albumin protein is being employed as a carrier.

![Diagram of different types of carriers utilized for drug targeting.](image)

**Figure 7. Different types of carriers utilized for drug targeting.**

It is generally known that nano-sized carriers are a prerequisite for efficient drug targeting. Carrier systems of 200 nm diameter or smaller are used for drug targeting, and larger systems are subjected to nonspecific capture in the reticuloendothelial system (111). On the other hand, small drug carriers have a short circulation time in the blood stream due to renal filtration. Therefore, drug carriers with a diameter from 10 nm to 200 nm are used in drug targeting approaches. These carriers are not largely cleared by renal filtration or by the reticulo-endothelial system which allows a large amount of delivery to the target sites.
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M6PHSA as a soluble carrier protein

Albumin is the most abundant plasma protein, and has a biological half-life of 19 days. It consists of a single chain of 585 amino acids organized in a tridimensional structure in a helical conformation. The helices are bound by 17 disulfide bridges, leaving only one free thiol (Cys34) (112).

Albumin is biodegradable and therefore biocompatible and contains many different functional groups, i.e. -NH₂ of the lysine residues or methionine, which can be used for conjugation of the homing device, the linker, or the drug. In addition, due to its size and charge, it is not cleared from the blood by renal filtration.

In our strategy, albumin was modified with sugar mannose-6-phosphate groups on its surface resulting in M6PHSA (113). M6PHSA has been shown to specifically interact with mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptors expressed on the surface of hepatic stellate cells. Due to stellate cell proliferation during liver fibrosis and a concomitant increase in M6P/IGF II receptor expression on this cell type (100), the disease process itself may selectively direct the carriers to the diseased tissue. This targeting strategy may largely contribute to the increased therapeutic concentration of drug in the target tissue (114).

Linkage between drug and carrier

The concept of the “magic bullet” is very intuitive and appealing and looks as if it can be easily realized through simply coupling a drug to a carrier. However, the development of drug targeting constructs is a delicate process and several difficulties have to be overcome during the synthesis of the conjugate. The linkage between the drug and the carrier system is a crucial element of the conjugate, as it controls both the stability of the conjugate and the efficiency of drug release or rate, and eventually, the final therapeutic effect (115). For bioconjugates, the nature of the linker between the pharmacologic agent and the carrier often dictates the degree of successful delivery and its outcome. Over decades, investigators have therefore invested great efforts to find the appropriate linkage system.
Linkages used in bioconjugation to proteins

The release of the drug from the carrier is decisive for its pharmacological activity. Various types of biodegradable linkages have been developed for coupling drugs to proteins. Amide linkages can be used to conjugate a drug containing carboxylic acid or amino groups, like mycophenolic acid to M6PHSA (58).

**Figure 8.** Drugs used in the present thesis, linkers available for coupling these drugs, and M6PHSA carrier utilized for Stellate cell-directed drug targeting. PTKI can be conjugated only via ULS linker. Losartan can be conjugated via Ester or ULS linkage and Pentoxifylline can be coupled via ULS or Schiff base linkage. ULS reacts with aromatic nitrogens of the depicted ring (PTKI), tetrazole ring (losartan) or xanthine moiety (pentoxifylline).
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The amide linkage however is not easily split within the target cell. In addition, the ester linkers may be used to conjugate carboxylic acid groups and hydroxyl groups of drugs, like in the case of losartan (figure 8). Also mycophenolic acid could be conjugated to M6PHSA via an ester linkage. However this resulted in less drug molecules coupled to the carrier as compared to the amide-based conjugate which allowed higher drug loading (58).

Yet, as anticipated, the conjugate synthesized via the ester linkage proved more effective than with the amide linkage since active drug could be released from the ester linkage. This example illustrates the crucial role that the linkage plays in drug targeting conjugates efficiency. The Schiff base hydrazone linkers may form an imino bond between a carbonyl group of the drug molecule and a hydrazine functionality of the spacer. Such linkages have been employed with doxorubicin, streptomycin and chlorambucil (116-119). Pentoxifylline could be also form a Schiff base linkage via its carbonyl group (figure 8). The disulfide bond is a covalent linkage which arises as a result of the oxidation of two sulfhydryl (SH) groups of cysteines or other SH-containing material (120). It can be formed with drug molecules that contain free thiol groups or, alternatively, with drug derivatives in which a free thiol group has been introduced. For instance, in liver-directed conjugates, gliotoxin was conjugated to M6PHSA using a disulfide bond (121). A disadvantage of the disulfide linkage is its relative instability in the bloodstream. Disulfide bonds can be degraded by reducing enzymes or disrupted chemically by thiol-disulfide exchange with free thiol compounds such as glutathione (122). Another type of linker is based on polymers. Multiple drug molecules can be covalently attached to a single functional group of the carrier when polymeric bridges are used.

The Universal Linkage System (ULS)

As can be appreciated, the finding of the appropriate linkage system is a critical step during the synthesis of a promising drug carrier construct. Some of the linkers modalities lack stability in plasma (ester linker), enzymes at non-targeted sites may cleave the linkage or simply they are not able to react with the drug or the chosen carrier system (115).

A major problem in the synthesis of drug conjugates is that the majority of drugs cannot be coupled using traditional linking procedures since they lack the appropriate functional
groups (i.e., carbonyl, amino, hydroxyl or thiol groups). In the present thesis, we have developed conjugates with a new linker technology that allows the coupling of a broader spectrum of drugs. The ULS (Universal Linker System) is a platinum-based linker that has been previously applied in Life Sciences for the conjugation of different types of reporter molecules to DNA and proteins (123;124;128;129).

The ULS linker technology is based on platinum coordination and shows a cis geometry of the coupled drug and carrier (Figure 8). The importance of this coordination chemistry is based on the stability and kinetically slow release properties of platinum complexes to nucleic acids and proteins (125). In general, platinum is found to react with S-donors such as methionine and the Cys34 residues of albumin, the latter being the most abundant free thiol group in blood plasma (126). Furthermore, it has been reported extensively that platinum forms coordination bonds with aromatic Nitrogen groups in DNA, which is kinetically favored over the reaction with Oxygen groups (127). The most important feature of the ULS platinum linker in drug delivery derivatives is that the strength of the drug-linker bond is stable enough to reach the target yet reversible enabling the release of the drug in the target tissue or cells.

In the presently discussed drug targeting conjugates, different antifibrotic drugs were linked to ULS and subsequently to the carrier protein M6PHSA. ULS linker bound to aromatic nitrogens in the drug molecules. Furthermore, ULS may react with the thiocarbonyl group between the M6P group and the albumin core protein, since it is known that cisplatinum readily reacts with sulfur containing ligands. However, a reaction of ULS with other functional groups in the protein like methionine or cysteine residues or even hydroxyl or amine side chains may also occur. In chapter 2, the possible sites for a reaction between ULS and M6PHSA are depicted. In the same chapter, characteristics of drug release from ULS linker are explained in detail. In chapter 3, 4 and 5, we will present data of drug targeting to HSC, employing the ULS linker during in vivo studies after single or multiple administrations of the conjugates.
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Martini Tower in Groningen.