Novel strategies targeting hepatic stellate cells to reverse liver fibrosis

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INTRODUCTION AND AIM OF THE THESIS

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Chapter 1

The liver has a unique regenerative response towards injuries. After removal of the harmful source, the mass, architecture and function of liver tissue can be entirely restored. However, in chronic conditions when the injury is long-lasting and persistent, the healing response shifts to fibrogenesis where scar-forming connective tissue gradually disrupts the liver function leading to cirrhosis and increasing the risk for liver cancer. Cirrhosis, as the terminal stage of progressive liver fibrosis, is the 12th most common cause of death worldwide. Over a million patients die from cirrhosis every year, which is 2.2% of all deaths annually [1,2]. Liver fibrosis develops in response to a variety of triggers, such as viral hepatitis, excess alcohol consumption, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis, hemochromatosis, as well as hereditary diseases, including progressive familial intrahepatic cholestasis (PFIC), Wilson’s disease, haemochromatosis and α1-anti-trypsin deficiency. In the final stages of cirrhosis, the liver may fail with systemic complications, such as portal hypertension-induced variceal bleeding, ascites, hepatic encephalopathy, bacterial infection and often hepatocellular carcinoma (HCC). Although there is no medicine to directly target (advanced) fibrosis, accumulating clinical and experimental evidence show that liver fibrosis can be halted and even reversed using combination therapies [3–6]. The common ground for all therapeutic approaches consists of attenuating pathological mechanisms and simultaneously promoting liver regeneration.

Liver fibrosis begins with deposition of connective tissue (components of the extracellular matrix (ECM), particularly collagen I, collagen III and fibronectin) in and around inflamed or damaged areas [7]. The source of the abnormal ECM are myofibroblasts. Exposure to a variety of triggers, including inflammatory molecules and oxidative stress, myofibroblasts, in particular Hepatic Stellate Cells (HSC), undergo cell-intrinsic changes to become ‘activated’. Activated myofibroblasts are migratory, contractile and proliferative and mostly derive from two mesenchymal origins, Hepatic Stellate Cells and portal myofibroblasts. Extracellular signals from other cells, such as macrophages, hepatocytes, liver sinusoidal endothelial cells, natural killer (T) cells, platelets and B cells, promote HSC activation. The external stimuli from other cells are mainly paracrine factors, such as platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ) and other growth factors together with cytokines and chemokines [8]. The function and morphology of HSC-derived myofibroblasts are very similar to those that originate from portal myofibroblast. Thus, the therapeutic strategies that aim to block the trans-differentiation from the mesenchymal phenotype to the fibroblast phenotype are similar and will target both types. However, HSCs appear to be the primary target for direct treatment of liver fibrosis, as they are the major source of myofibroblasts (over 80%) in rodent models of liver fibrosis, including chronic CCl₄ exposure, bile duct ligation, the 3,5-diethoxycarbonyl-1,4-dihydrocollidin diet and Mdr2-knockout mice [9].
Quiescent Hepatic Stellate Cells (qHSCs)

In healthy conditions, HSCs are in a ‘quiescent’ state populating about 15% of all cells in the liver. qHSCs are located in the subendothelial space of Disse between hepatocytes and sinusoids that carry nutrient- and oxygen-rich blood to the hepatocytes [10,11]. HSCs are relatively small in size compared to hepatocytes and characterized by large cytoplasmic lipid droplets. The HSC lipid droplets are specialized organelles for storage and metabolism of retinoids (e.g. vitamin A) and store about 80-90% of total retinoids of the liver and about 50-80% of total retinoids in the body. HSCs are responsible for maintaining stable levels of circulating retinyl in blood at around 2 µmol/L in humans and 1-1.5 µmol/L in rodents[1]. Most retinoids in the cytoplasmic droplets are stored in the form of retinyl esters, predominantly as retinyl palmitate. The composition and size of these lipid droplets are dependent on dietary intake. Besides retinoids, other lipids, such as triglycerides, phospholipids, cholesterol and free fatty acids, are abundantly deposited in HSC lipid droplets [11,12].

The uptake, transport and storage of dietary vitamin A is a complex process. In the intestinal epithelium, dietary carotenes are converted to retinyl esters and transported to the liver via circulation within chylomicrons. Hepatocytes take up the chylomicrons and hydrolyze retinyl esters to retinyl, which is distributed from the hepatocyte to the circulation bound to the retinyl binding protein 4 (RBP4). HSCs absorb retinyls from the blood and convert it back to retinyl esters and store it in lipid droplets [13–16]. As HSC activation is trigged, cells rapidly lose their lipid droplets, including the vitamin A content, while transdifferentiating into myofibroblasts. As a consequence, vitamin A deficiency is associated with advanced liver fibrosis [13].

Vitamin A homeostasis in HSC is achieved by enzymes that esterify and hydrolyse retinyl and retinyl esters, respectively. Lecithin retinyl acyltransferase (LRAT) and acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) are responsible for retinyl esterification [12,17]. Two esterases, adipose triglyceride lipase/patatin-like phospholipase domain containing 2 (ATGL/PNPLA2) and adiponutrin (ADPN/PNPLA3), have been identified as retinyl ester hydrolases (REH) in HSC [18–20]. LRAT appears to be essential for hepatic retinyl esterification, as LRAT-null mice are prone to develop vitamin A deficiency (VAD) [21,22]. For retinyl ester hydrolysis, PNPLA3 seems to play a rate-limiting role, as specific PNPLA3 gene variants in humans are associated with hepatic retinyl ester accumulation and serum retinyl levels[20]. In contrast, ATGL-null mice have normal hepatic vitamin A levels implying that ATGL, while being an established REH, is not essential for maintaining hepatic retinoid metabolism [18]. The expression of vitamin A-associated enzymes, such as LRAT or PNPLA3, rapidly drops during HSC activation, along with
the disappearance of lipid droplets. There is a third REH, hormone-sensitive lipase (HSL), which is dominantly expressed in adipose tissue, but is also detected in the liver [23,24]. So far, hepatic HSL is reported to be expressed only in hepatocytes, where it mainly hydrolyses cholesteryl esters to cholesterol [24,25]. Nevertheless, the role of HSL in retinoid metabolism in HSC has not been studied in detail yet.

**Key factors in HSC activation and liver fibrogenesis**

The extracellular matrix (ECM) is a network of proteins that provides micro-scaffolds for cells supporting cellular adhesion, migration, differentiation and proliferation. In the liver, ECM is present in the Glisson's capsule, portal tracks, central veins and in the perisinusoidal space of Disse [26]. Collagens (I, III, IV, and V), fibronectin, laminin and proteoglycans are the predominant proteins of ECM. Collagen types I, III, and V are predominantly expressed in the portal and central areas, whereas collagen IV is localized mainly in basement membranes [27]. Fibrotic liver is highly enriched in collagen I and III (7) and about 80% is produced by HSCs, in particular collagen I [28].

Matrix metalloproteinase proteins (MMPs) are responsible for degradation of ECM proteins and, together with tissue Inhibitors of metalloproteinases (TIMPs), have fundamental roles in ECM remodeling during liver fibrosis and in fibrosis resolution. While activated HSC are the prime producers of ECM proteins, in particular collagen I, III and IV, they also express MMPs and TIMPs. Especially the expression of TIMP-1, is up-regulated in activated HSCs, thereby reducing MMP activities and promoting the accumulation of ECM (29,30). MMP-2, MMP-9, and MMP-13 are among highly-expressed proteins in activated HSCs. MMP-2, MMP-8 and MMP-9 activities are significantly elevated in alcoholic liver disease and can function as serum markers of disease severity [31]. MMP-13 activity can be used for diagnosis of alcoholic liver cirrhosis [29]. Besides induced ECM production, HSC activation is characterized by elevated expression of alpha-smooth muscle actin (α-SMA). α-SMA is a cytoskeleton protein that has a major role in mobility and contractility of activated HSC [32]. The contractile force of α-SMA is stronger than other actin isoforms in fibroblastic cells [33].

Transforming growth factor β1 (TGF-β1) is a key mediator of HSC activation upon liver. TGF-β1 is a multifunctional cytokine that regulates a variety of cellular functions, including proliferation, differentiation, apoptosis and migration [34]. During HSC activation, TGF-β1 binds to its receptors and forms a complex that directly activates Smad signalling leading to overexpression of profibrotic genes. Smad2 and Smad3 are the two major downstream regulators that mediate TGF-
β1-induced tissue fibrosis, while Smad7 serves as a negative feedback regulator of TGF-β1/Smad pathway and suppresses TGF-β1-mediated fibrosis [35,36].

PDGF is one of the most potent mitogens that stimulates HSC activation. Upon binding to its receptors at the membrane of HSC, PDGF triggers a signalling cascade that leads to upregulation of collagen synthesis, as well as MMP-2, MMP-9 and TIMP-1, the latter of which prevents ECM degradation [37,38]. PDGF-B and PDGF-D are the most fibrogenic isoforms of PDGF and cause PDGFR-β autophosphorylation that activates the extracellular signal-regulated kinase (ERK)1/2, C-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and protein kinase (PK)B/Akt pathways leading to further proliferation and activation of HSCs and damage to hepatocytes [37,39].

Leukotrienes are pro-inflammatory mediators that increase microvascular permeability and causes leukocyte infiltration in inflamed areas in the fibrotic liver [40]. Leukotrienes are synthesized from essential fatty acids (EFA), e.g. arachidonic acid, by the enzyme arachidonate 5-lipoxygenase, also known as 5-lipoxygenase (5-LO) (36). The inhibition of 5-LO suppresses renal fibrosis and progression of chronic kidney disease by preventing the production of leukotrienes [41]. Moreover, 5-LO, is one of the main contributors of lipid peroxidation that further increases tissue inflammation. Besides lipoxygenases, cyclooxygenases and cytochrome P450 enzymes oxidize EFAs, however, lipoxygenases contribute most prominently to the generation of lipid peroxides [42].

**Therapeutic approaches to halt and/or reverse liver fibrosis**

So far, the only effective way to stop or cure liver fibrosis is to eliminate the pathological stimulus that causes chronic liver damage and/or inflammation. The stimulus can be long-term exposure of a toxic agent, fatty diet, alcohol consumption, viral or bacterial infection. Clinical studies have shown that therapies aiming at elimination of the primary disease cause, e.g. antivirals in viral hepatitis, can regress liver fibrosis, even at advance stages [43–45]. Consequently, treatment against HCV infections [46–48], alcohol abstinence [49] and weight loss (50) have been reported to be effective to reverse liver fibrosis.

However, in most cases of chronic liver disease, it is challenging or even currently impossible to eliminate the primary cause of disease. As an alternative approach, anti-inflammatory drugs and/or immunosuppressants are commonly used. Inflammation is a key factor that drives liver fibrosis and is regulated by proinflammatory and anti-inflammatory factors secreted from
Kupffer cells and other recruited immune cells, such as bone marrow–derived macrophages, neutrophils and T lymphocytes. Chronic inflammation leads to progressive hepatocyte damage and activation of HSCs and portal myofibroblasts [51]. Common anti-inflammatory drugs are corticosteroids, such as prednisone and prednisolone, and are used to suppress cytokine production through transcriptional regulation [52–54]. Zileuton, a 5-lipoxygenase inhibitor, blocks leukotriene-mediated pathways and can be considered for clinical use [55–57]. Pentoxifylline (PTX) is another anti-inflammatory drug that suppresses Tumor Necrosis Factor-alfa (TNF-α) synthesis [58]. Recent studies report celecoxib [59], azathioprine [47] and rapamycin [60,61] as suppressors of fibrogenesis through their potent immunomodulatory properties. Antioxidative agents, such as taurine [47,62] and vitamin E [63] reduce oxidative stress and thereby suppress inflammation and resultant fibrogenesis.

HSCs develop fibrotic structures by producing abnormal extra cellular matrix (ECM) depositions, as well by secreting inflammatory cytokines that recruit immune cells to the inflamed area and promote local inflammation. Thus, suppressing activation and promoting apoptosis or senescence of HSCs are alternative approaches to halt fibrosis [64]. Inactivation of HSCs have been achieved in experimental models by inhibiting the principal activation mechanisms, including the TGF-β1/Smad- (35,36,65) and PDGF-[37] signaling pathways. Some cytokines and growth factors, such as Insulin-like growth factor-1 [66] and interleukin-22 [67], induce senescence in HSCs while IFN-α (68) and IFN-γ [10,69] are candidates for induction of HSC apoptosis.

Promoting hepatocyte regeneration is an important strategy to preserve liver function during fibrosis. Ursodeoxycholic acid (UDCA) is a hepatoprotective bile acid used for treatment of various chronic liver diseases, including primary biliary cirrhosis (PBC), NAFLD and intrahepatic cholestasis of pregnancy (ICP) [70,71]. UDCA protects hepatocyte and cholangiocytes by replacing toxic bile acids, inhibition of apoptosis induced by toxic bile acids and stimulation of bile secretion. Hepatocyte growth factor (HGF) has been shown to reduce apoptosis and promote angiogenesis and liver regeneration by stimulating hepatocyte motility and mitogenesis [47,72,73]. Moreover, bone-marrow-derived cells and mesenchymal cells can stimulate proliferation of hepatocytes by producing mitogens, including HGF [72,74,75]. Although the impact of HGF on hepatocyte regeneration has been established in many experimental models of liver diseases, no clinical research is done to further develop HGF into treatment for liver fibrosis, in particular due to the potential risk of inducing cancer.
Complementary and alternative medicine for the treatment of liver fibrosis

Besides developing novel pharmaceutical compounds to target liver fibrosis, natural products may also hold potent anti-fibrotic properties. Melatonin is a hormone produced by the pineal gland and controls the sleep-wake cycle. However, melatonin can also be produced in other tissues, including the liver, and acts as an endogenous antioxidant [76]. Recent studies have revealed that a variety foods and drinks, such as vegetables, cereals, fruits, nuts, seeds, grapes, red wine and beer, contain considerable amounts of melatonin [77–80]. Melatonin is available as supplementary medication, in particular to control the day-night sleep rhythm. Hepatoprotective effects of melatonin have been shown in various liver diseases including, hepatic steatosis [81,82], hepatitis [83], fibrosis [84] and hepatocellular carcinoma [85]. The therapeutic effects of melatonin are mostly attributed to its ability to suppress oxidative stress, inflammatory signaling, hepatocyte apoptosis, neutrophil infiltration and regulation of profibrogenic genes [86,87]. Melatonin reduces the expression of 5-LO [88] and attenuates liver fibrosis in several animal models including, bile duct ligation (BDL)-induced cirrhosis [89], thioacetamide (TAA)-induced fibrosis [90] and carbon tetrachloride (CCl4)-induced fibrosis [91]. However, a direct role of melatonin on HSC activation has not been established yet.

Anti-fibrotic compounds may also be found in plants used in traditional medicine. Esculetin is present in many herbs, including Cortex fraxini, Artemisia capillaries, Citrus limonia, Euphorbia lathyris and Fraxinus rhynchophylla [92,93]. Esculetin has been used in China for treatment of liver and gallbladder diseases for centuries. Esculetin has anti-inflammatory and anti-tumor properties. It down-regulates the expression of MMP-1 in cartilage, while it also inhibits the production of pro-inflammatory cytokines, such as TNF-α, in co-cultured adipocytes and macrophages [93]. Furthermore, esculetin is a potent anti-oxidant that ameliorates mitochondrial damage induced by nitric oxide radicals [94], hepatic apoptosis induced by CCl4 [92] and DNA damage caused by lipid hydroperoxide-induced oxidative stress [95]. Moreover, esculetin has anti-hepatitis B virus activity [96] and inhibits 5-LO and 12-LO in leukocytes [97–99]. Other herbal compounds that exhibit anti-fibrotic activities include salvianolic acid B [100], oxymatrine [101], tetrandrine [102], glycyrrhetinic acid [103] and silymarin [104–106]. In all these examples, the pharmacological effects include scavenging free radicals and modulating the inflammatory pathways. In addition to natural compounds, there are drugs that are not preliminary developed for targeting liver fibrosis, yet could theoretically have such therapeutic effect. Verapamil for instance, a calcium channel blocker that is used to treat high blood pressure, has hepatoprotective properties. In NAFLD, verapamil reduces hepatic lipid droplet accumulation, insulin resistance and
steatohepatitis [107]. The combination of verapamil and silymarin improves liver fibrosis and significantly reduces mRNA and protein level of α-SMA [6]. Hydroxycarbamide, also known as hydroxyurea, is an antiproliferative agent, which inhibits DNA synthesis and cell cycle replication by blocking the enzyme ribonucleotide reductase [108]. Hydroxyurea is widely used to treat chronic myelogenous leukemia [109], cervical cancer [109], polycythemia [110] and sickle-cell disease [111]. Cellular absorption of hydroxyurea is an active transport carried out by solute care transporters including multi-specific organic cation transporter (OCTN1), which is expressed by HSCs[112]. Hydroxyurea has not yet been explored as anti-fibrotic agent.

The aim of this thesis

In this thesis, we explored the therapeutic effects of melatonin, esculetin and hydroxyurea on HSC activation in order to establish their potential as anti-fibrotic drugs. Additionally, we investigated the role of HSL in vitamin A homeostasis and activation of HSCs.

HSL is the dominant retinyl ester hydrolyser in adipose tissue. However, its expression and function in liver are less known and the current data is limited to its cholesterol hydrolysing activity in hepatocytes. Giving the fact that HSC lipid droplets abundantly accumulate retinyl esters, in Chapter 2, we analysed HSL expression and function in quiescent and activated HSCs.

Numerous studies have examined the therapeutic properties of melatonin in a variety of liver injury models. Yet, its direct effect on HSC activation has not been explored. For possible use of melatonin in a treatment for liver fibrosis, it is essential to fully understand its potential impact on the main contributors of fibrogenesis, the activated HSC. Thus, Chapter 3 of this thesis explores the direct impact of melatonin on HSC activation and its 5-LO- associated inhibitory mechanism.

As stated earlier, the contribution of 5-LO in promoting inflammation is well-established. We hypothesized that inactivating 5-LO using esculetin reduces HSC activation and ameliorates liver fibrosis. In Chapter 4, we therefore studied the direct effect of esculetin on HSC activation and proliferation in vitro and in vivo.

In Chapter 5, we investigated the possibility of using hydroxyurea as an anti-fibrotic drug. For this, the effect of hydroxyurea on HSC activation and hepatocyte preservation and regeneration was investigated. Finally, Chapter 6 provides a summary of relevant findings and results of all experimental chapters followed by a general discussion that highlights possibilities for future research and perspectives of therapeutics for liver fibrosis.
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