ABC transporters as therapeutic targets for liver fibrosis

Rehman, Atta ur

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ABC transporters as therapeutic targets for liver fibrosis

Atta ur Rehman

*In the name of **ALLAHA***

The Most Beneficent, The Most Merciful;

I owe my deepest gratitude to ALLAHA for His blessings,

enabling me to complete this

dissertation.

university of
groningen

ABC transporters as therapeutic targets for liver fibrosis

PhD thesis

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by

Atta ur Rehman

born on 8 March 1980
in Lahore, Pakistan

Supervisors:

Prof. K.N. Faber
Prof. A.J. Moshage

Assessment committee:

Prof. P.L.M. Jansen
Prof. K. Poelstra
Prof. P. Heeringa

To my parents and the friend…..

Paranimfen:

Ali Saeed
Manon Buist-Homan

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General introduction: Therapeutic targets to treat liver fibrosis

Atta ur Rehman and Klaas Nico Faber

Department of Gastroenterology and Hepatology,
University Medical Center Groningen,
University of Groningen

General Introduction

Liver

The liver is the second largest organ in the mammalian body and is involved in versatile functions like synthesis, secretion and metabolism of a variety of bio-molecules, including lipids, proteins, vitamins, carbohydrates and toxins. Anatomically, the human liver is divided in four lobes. The structural and functional unit of the liver is the lobule. Each lobule is composed of various cell types, which are organized in characteristic arrays of cells, in particular the hepatocytes, that allow the liver to perform its function optimally (Figure 1). The liver is a highly perfused organ. The hepatic artery and the portal vein supply the liver with blood, while it exits the liver via the central vein. The portal vein carries approximately 80% of the blood and brings nutrient-rich/oxygen-poor blood from the intestine, while the other 20% is highly oxygenated and enters through the hepatic artery. The portal vein and hepatic artery merge in the portal areas and blood flows alongside plates of hepatocytes exchanging nutrients and gases before exiting via the central vein [1].

A crucial function of the liver is the production of bile, which aids in the absorption and/or excretion of fatty compounds from the gastrointestinal tract, including cholesterol and fat-soluble toxins. The main constituents of bile are bile acids, phospholipids, cholesterol, bilirubin, inorganic salts and water [2]. The bile acids and phospholipids form mixed micelles and those are the main carriers for lipid-soluble compounds in bile and the small intestine. Bile is synthesized in the liver, secreted into the canaliculi, transported via bile ducts out of the liver and stored in the gall bladder. The bile ducts are composed of cholangiocytes that modify the composition of bile for optimal function [3]. Food intake triggers the contraction of the gallbladder and bile is released into the duodenum. In addition to the secretion of lipophilic compounds, bile also contains drug metabolites that after phase I modification and phase II conjugation are transformed to water-soluble compounds and secreted by hepatocytes to the bile for fecal excretion or to the blood for renal excretion [1-4].

The various liver cell types can be subdivided in parenchymal cells and mesenchymal cells. The parenchyma consists mainly of hepatocytes that make up approximately 80% of the liver volume and carry out the metabolic functions of the liver.

The mesenchymal compartment includes Kupffer cells (KCs, the liver-specific macrophages), dendritic cells (DCs), endothelial cells (ECs), cholangiocytes, portal myofibroblasts (PMFs), hepatic stellate cells (HSCs) and liver-specific progenitor (oval) cells (Figure 1).

Hepatocytes are polarized cells and are arranged in radial plates around a central vein. At the sinusoidal side, hepatocytes are in direct contact with blood via the basolateral membrane. The lateral sides of the neighboring hepatocytes are joined together separating the basolateral membrane from the apical –or canalicular- membrane[1,6]. The canalicular membranes of the hepatocytes together form small canaliculi that merge into bile ducts through which bile is transported from the hepatocytes to the gallbladder and small intestine [3]. The positional arrangement of the hepatocytes is optimal for controlling circulating levels of a great variety of biomolecules like proteins (e.g. albumin and clotting factors), carbohydrates (glucose), lipids (cholesterol) and vitamins (vitamin A, B, D, E and K) [1]. Moreover, hepatocytes are involved in the biotransformation of drugs and toxic compounds [7]. As such, hepatocytes may be regarded as a "filter" for toxins that are absorbed from the blood and excreted into the bile directly or after enzymatic modification [7]. The ultimate goal of biotransformation is to reduce the drug-induced toxic effects that cause liver damage and inflammation.
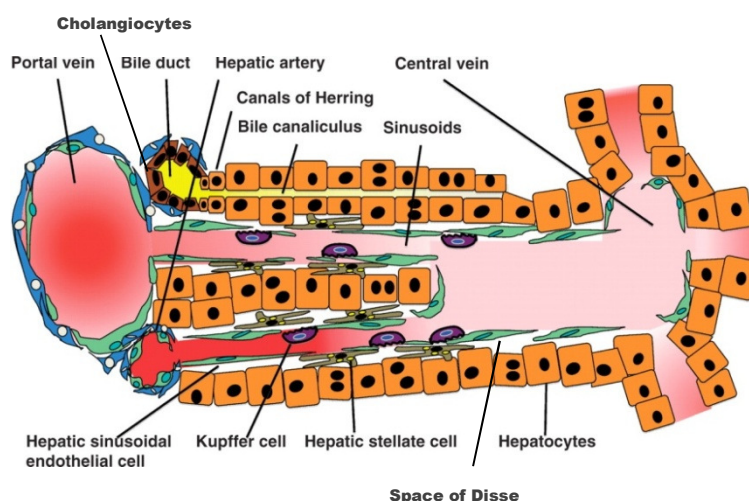
Figure 1. Microscopic features of the liver lobule. Spatial arrangement of liver cells in a lobule showing blood supply and bile canaliculi. The portal area is composed of a hepatic artery, portal vein and a bile duct. Blood flows from the portal area to the central vein. Bile canaliculi collect bile and drain into the bile duct. Hepatic stellate cells are present between the endothelial cells and hepatocytes (space of Disse). Adapted from [5].

Cholangiocytes form the epithelial lining of the bile duct through which bile flows from the canaliculi and is stored in the gall bladder. Cholangiocytes modify the composition and pH of bile in particular by secretion of bicarbonate ions and water [3,8].

Liver sinusoidal endothelial cells (LSEC) form the lining of the hepatic sinusoids that carry the blood through the liver. Endothelial cells are highly fenestrated allowing exchange of small molecules, including oxygen, in blood so they can reach the underlying hepatocytes. At the same time, LSEC form a physical barrier for large particles and specific cell types [9,10].

Hepatic stellate cells (HSCs) reside in the space of Disse, the space between the liver sinusoids and the hepatocytes. In healthy liver, HSCs are considered "quiescent" (qHSC) containing up to 80% of the body content of vitamin A, which is stored as retinyl-esters in large cytosolic lipid droplets, and maintain stable retinol levels in the blood [11,12]. These cells produce controlled amounts of extracellular matrix, which provides the embedding of other liver cells. HSCs have many characteristics of nerve cells, including specific nerve cell markers like glial fibrillar acidic protein, synemin, synaptophysin and nerve growth factor receptor p75 [13]. Moreover, qHSC can also act as antigen presenting cells thereby playing an immunomodulatory role [14]. Upon liver injury, HSCs become activated and transdifferentiate into proliferative myofibroblasts that cause liver fibrosis (see below).

The liver also contains "professional" immunomodulatory cells, such as macrophages, dendritic cells and natural killer cells. Kupffer cells (KCs) are the liver-specific macrophages and are located in the sinusoids in close contact with the endothelial cells. Kupffer cells are phagocytic in nature and clear the blood from worn-out blood cells, pathogens (bacteria, viruses) and other foreign substances that appear in the blood [15]. Dendritic cells are able to pass the fenestrated endothelium and enter the parenchyma, where they typically settle in the portal areas. Dendritic cells process antigenic materials and present them to lymphocytes bridging the innate and the adaptive immune system [1,10]

The portal area contains liver progenitor cells that are activated and proliferate during severe liver disease and replenish lost hepatocytes and cholangiocytes [5]. The portal area also contains myofibroblasts (PMFs) that are phenotypically distinct from

HSCs. They are devoid of vitamin A and Desmin that are present in HSCs [16,17]. Upon liver injury, PMF also become activated, motile and contractile and produce excessive amounts of extracellular matrix proteins (ECM), similar as HSC[17,18].

Taken together, the various liver cell types coordinate and perform highly specialized functions that make the liver a vital organ involved in metabolism, synthesis, secretion and biotransformation as well as an immune-competent organ. Therefore, a healthy liver is crucial for maintain homeostasis.

Liver diseases

Liver diseases can be subdivided in acute and chronic liver diseases depending on the time frame leading to symptoms of liver injury. In acute liver failure (ALF), the onset of disease takes place within hours to days, while chronic liver disease may develop over years to decades. In the United States, chronic liver diseases and cirrhosis claim 35,000 lives and 2,000 additional death with fulminant ALF every year [19]. Typically, ALF results from drug intoxication (paracetamol overdose, mushrooms, and party drugs) and viral infection (hepatitis A or B, it is uncommon in hepatitis C). Within a timeframe of hours to days, severe liver damage leads to a critical loss of functional liver tissue (over 80%), which develops into systemic inflammation and multiple organ failure. Patients are admitted to the intensive care unit and treatment is primarily aimed at treating symptoms, rather than the underlying cause. ALF patients often require liver transplantation to survive [20].

Chronic liver disease (CLD) develops with persistent viral infections (hepatitis B and C, cytomegalovirus (CMV), Epstein Barr Virus (EBV), or long-term intake of alcohol and drugs [21,22]. In addition, CLD include multiple forms of autoimmune hepatitis, including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) that develop slowly, but progressively to a stage where liver transplantation is the only therapeutic option [23-25].

Inherited genetic defects in transport processes of bile components may also result in various forms of chronic liver disease. For example, a defect in bile salt or phospholipid transport across the canalicular membrane (by the bile salt export pump (BSEP) or multidrug resistance protein 3 (MDR3), respectively) cause progressive liver disease and often requires liver transplantation at early age (within 1 or 2 years) [26].

Biliary atresia is a disease of the bile ducts caused by inflammation and obstruction of the bile duct. The accumulation of bile in the liver causes liver injury. The etiology of this disease is largely unknown and most patients require liver transplantation to survive.

A growing number of patients with liver disease are the result of the consumption of high-fat foods characteristic of the "Western" lifestyle. Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are steatotic liver diseases related to the excessive dietary fat intake without the involvement of alcohol. NASH, as opposed to NAFLD, is characterized by the presence of inflammation and slowly develops into chronic hepatitis, fibrosis, cirrhosis and ultimately liver failure. Additional complications like fluid retention, muscle wasting and intestinal bleeding may appear. Insulin resistance and diabetes are associated with NAFLD and NASH [27]. Weight control, dietary interventions and ultimately liver transplantation are the treatment options for patients suffering from NASH.

Liver injury is routinely assessed by biochemical parameters, in particular by determining serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin (PT), gamma-glutamyl-transpeptidase (GGT), albumin and bilirubin (conjugated and unconjugated). Bilirubin is the breakdown product of heme, which is abundantly present in red blood cells as cofactor of hemoglobin. Bilirubin is efficiently secreted into bile and increased serum levels therefore may indicate impairment in bile flow [3]. ALT and AST are intracellular enzymes released from damaged hepatocytes and therefore specific indicators of hepatocyte damage [28]. Albumin and PT are serum proteins synthesized by hepatocytes and are therefore markers for liver function. GGT is a membrane-bound enzyme located at the luminal side of bile duct epithelial cells (cholangiocytes) and hepatocytes [29]. Increased serum levels of GGT are considered to be a marker for bile duct injury in cholestasis, but its specificity as a liver damage marker is still unclear [30,31]. No specific serum markers for liver fibrosis are available yet and staining liver biopsies for collagen deposition is the golden standard to firmly establish the stage of liver fibrosis. In addition, liver fibrosis may be assessed non-invasively by transient elastography (Fibroscan) [32,33].

The liver has the unique capability to regenerate after an injury or surgical procedure. Loss of 50-70% liver tissue may be fully restored in a few days in mice or a few weeks in humans. The liver has a significant functional overcapacity: liver functions

only become compromised when more than 70-80% of liver tissue is lost. Although the functional overcapacity and ability to regenerate is a life-saving characteristic, it also means that clear symptoms of liver disease only appear when the liver disease is already in an advanced stage.

Liver fibrosis and resolution

Virtually all chronic inflammatory diseases lead to tissue damage and, as a result, to a wound-healing response characterized by re-establishment of the extracellular matrix that provides the proper support for tissue-specific cells. The control of synthesis and composition of extracellular matrix (ECM) proteins in a diseased liver is a complex mechanism in which various liver cells play a role and is described in detail in the following sections. Extracellular matrix (ECM) is composed of various structural proteins including, collagens, fibronectin, elastins and laminins. The composition of ECM is highly variable among tissues and during health and disease. ECM remodeling (synthesis, deposition and degradation) are strictly controlled by enzymes such as matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) secreted by various cell types [34]. MMPs are matrix degrading enzymes and in the liver the most relevant MMPs are MMP1 ,MMP3 , MMP9 , and MMP13 that degrades various types of collagens, fibronectin, laminins and proteoglycans [35]. The activity of MMPs, in turn, is regulated by TIMPs. The levels of TIMPs are increased during liver fibrogenesis leading to excessive deposition and diminished degradation of the ECM [34-37]. Consequently, the low-density ECM in the healthy liver, which is predominantly composed of collagen type IV and VI, is replaced by high-density ECM consisting of fibrillar collagens I, III and fibronectin [38]. The formation of thick fibrous tissue in the sinusoids impairs blood flow causing portal hypertension and limits the uptake and secretion of metabolites by hepatocytes resulting in decreased liver function [38-40].

When (acute) liver injury is cured, the natural balance between MMPs and TIMPs is restored and scar tissue is resolved resulting in the restoration of normal architecture and functional capacity of the liver. The reversibility of liver fibrosis is also evident from animal studies where fibrosis is resolved after cessation of the causative agent, for instance, in carbon tetrachloride (CCl_4) treated model of liver fibrosis in rodents [41]. Similarly, clearance of hepatitis B virus from infected patients also results in fibrosis resolution [42]. However, the resolution of liver fibrosis in patients is slow and complete

recovery may require years. Persistent liver injury and (mild) inflammation leads to fibrosis that may progress to cirrhosis, which is no longer reversible and increases the risk of hepatocellular carcinoma [40,43].

Cell signaling in liver fibrogenesis

Cytokines and growth factors

Initiation, perpetuation and resolution of liver fibrosis are dynamic processes. They involve growth factors, cytokines, hormones and eicosanoids (prostaglandins and leukotrienes) that are produced by various endogenous and liver-infiltrating cell types and have an autocrine or paracrine mode of action. In this section, we will discuss the involvement of these molecules in cell signaling in HSCs and other important liver cells that play a role in liver fibrosis.

Transforming growth factor-beta (TGF-β) is a well-characterized fibrogenic cytokine that induces the expression of collagens, TIMP-1 and plasminogen activator inhibitor type 1, and leads activation and transdifferentiation of HSCs into myofibroblasts. Kupffer cells and infiltrating macrophages are the main sources of TGF-β during liver injury [40,44]. Although TGF-β promotes proliferation of non-parenchymal cells, it suppresses hepatocyte division in the healthy liver [45]. However, in the (chronically) injured liver, hepatocytes become resistant to TGF-β and start to proliferate to restore liver cell mass [46]. TGF-β mediates its effect by binding to the extracellular domain of TGF-β receptor 1 or 2 and, subsequently, activates Sma and Mad Related (SMAD) proteins 2/3 that are attached to the intracellular domain of the TGF-β receptors. The activation (by phosphorylation) of Smad 2/3 can be inhibited by Smad7. Following activation, Smad 2/3 recruits Smad 4 and the protein complex is then translocated to the nucleus where it initiates the transcription of selective (TGF-β target) genes [40,47,48]. Under normal conditions, the effects of TGF-β are counteracted by a pseudo-TGF-β receptor named Bone morphogenetic protein (BMP) and Activin Membrane Bound Inhibitor (BAMBI) [49]. BAMBI is highly expressed in quiescent HSCs under normal conditions, which is suppressed upon liver injury by activation of Toll-like receptor-4, e.g. by lipopolysaccharide (LPS). In the absence of BAMBI, TGF-β is directed to the TGF-β receptor 1 or 2 and activates the SMAD signaling cascade [49,57]. Bone morphogenetic protein-7 (BMP-7) also suppresses the effect of TGF-β in HSCs and

prevents liver fibrosis [50]. TGF-β expression in HSC is also reduced by antagonists of the cannabinoid receptor [51,52]. In endothelial cells, low concentrations of TGF-β induce angiogenesis, but at high concentrations it actually suppresses angiogenesis [53]. TGF-β may also induce epithelial-mesenchymal transition (EMT) giving rise to myofibroblasts, although this phenomenon is still debated and putative mechanisms underlying this transition are largely unknown [54-56].
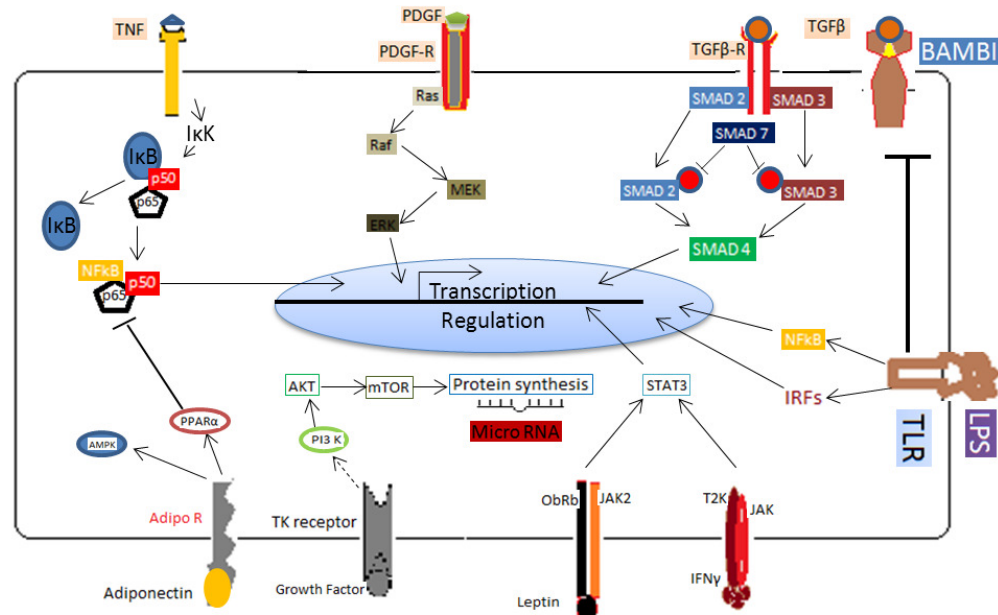
Figure 2. Cell Signaling in HSC via activation of different receptor thought biological ligands. Abbreviations: Tumor necrosis factor (TNF), Platelet derived growth factor (PDGF) receptor (R), Transforming growth factor beta (TGFβ), Sma and Mad related protein (smad), Lipopolysaccharide (LPS), Toll like receptor (TLR), Nuclear factor kappa B (NFkB), Interferon regulatory factors (IRF), Bone morphogenetic protein and Activin Membrane Bound Inhibitor (BAMBI), Interferon gamma (IFNγ), Janus kinase, Leptin receptor (ObRb), Phosphoinositide 3 kinase (PI3K), Protein kinase B (AKT), mechanistic target of rapamycin (mTOR), Adiponectin (Adipo), Peroxisome proliferator-activated receptor (PPAR), inhibitory Kappa B (IkB) and IkB kinase (IkK). Solid lines show activation and dotted line shows inhibition, blunt head shows inhibition of the binding of factors, arrows show activation.

LPS-mediated repression of BAMBI is not the only pathway by which TGF-β exerts its effects in promoting liver fibrosis. Several other factors downstream of LPS-TLR4 signaling result in the progression of liver fibrosis. In activated HSCs (aHSCs), LPS-mediated TLR4 activation leads to the translocation of Nuclear Factor kappaB (NFκB) to the nucleus and this induces transcription of pro-inflammatory cytokines, including Interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and RANTES [47]. TLR4 activation in endothelial cells leads to angiogenesis and sustained

exposure of endothelial cells to low concentrations of LPS results in the secretion of IL-10 thereby inactivating CD4^{+} cells and inducing tolerance against LPS [58]. The most significant role of LPS-TLR4 signaling is carried out by Kupffer cells in the early stages of liver fibrosis. LPS-TLR4 activation in KC leads to secretion of both TNF-α and TGF-β that has stimulatory effects on HSCs [57]. Hepatocytes are not much affected by LPS directly and therefore it is believed that LPS-induced TLR signaling in HSCs is the predominant pathway that promotes liver fibrosis after microbial infection [49]. Platelet-derived growth factor (PDGF) and vascular endothelia growth factors (VEGFs) are secreted by endothelial cells and are pro-mitogenic molecules. PDGF activates its transmembrane PDGF-receptor, tyrosine kinase (TK) and extracellular signal regulated protein kinase (ERK) pathway that regulate the transcription of the factors involved in cell proliferation and fibrogenesis. TGF-β and PDGF have synergistic effect on HSCs migration and expression of MMPs [59]. PDGF can also activate the PI3K-Akt signaling pathway and induces HSCs proliferation [60].
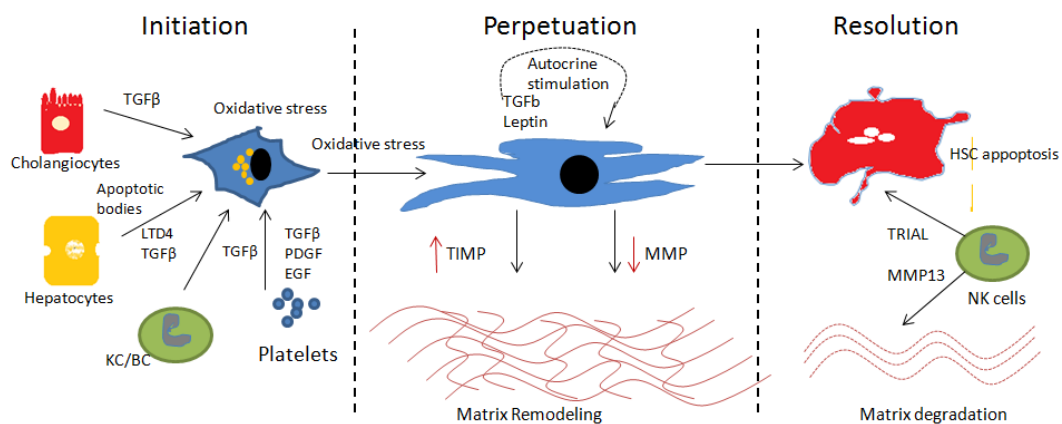
Figure 4. Interaction of Various cells following an injury. In the initiation phase dying hepatocytes activate KC and HSC release growth factors and inflammatory cytokines, Cholangiocytes also release TGFβ. During perpetuation phase HSCs become fully activated characterized by increased secretion of ECM and TIMPS while decrease secretion of MMP and net result is remodeling of ECM. During resolution phase macrophages secrete MMP that's degrades ECM followed by HSC apoptosis.

Janus kinase (Jak)-dependent activation of Signal Transducer and Activator of Transcription (STAT) has an important role in the growth and differentiation of almost every cell type in the liver. Jak is phosphorylated through the activation of specific receptors by cytokines like IL-6, IFN-gamma and leptin receptors. IL-6 binds to the membrane-bound surface receptor gp130 and mediates Stat3 activation [61]. In

hepatocytes, Stat3 activation enhances cell cycle progression thereby potentiating liver regeneration.

Leptin, PDGF, EGF and HGF mediate the activation of Stat3 in HSCs thereby inducing fibrosis. Leptin is a hormone synthesized by adipose tissue that regulates energy metabolism and also promotes the EMT that is mediated through Hedgehog signaling [62]. Leptin stimulates the expression of collagen 1a1 and enhances survival signals and proliferation of the HSCs [63-65]. In Kupffer cells Stat3 activation results in diverse effects that are dependent upon the cytokine profile and related regulatory intermediate proteins. For example, IL-6-gp130-mediated signaling results in activation of inflammatory pathways, while activation of the IL-10-IL10R-Stat3 pathway results in an anti-inflammatory response, which is carried out via silencing mediator of cytokine signaling-3 (SOCS3). SOCS3 inhibits the IL-6-mediated inflammatory signaling cascade by binding to gp130 [66,67].

Tumor Necrosis Factor-alpha (TNF-α) is produced by KCs in response to LPS. HSCs and hepatocytes express TNF-α receptors, which upon activation lead to the nuclear translocation of NFκB. NFκB is a pleotropic nuclear factor that regulates the expression of multiple anti-apoptotic (Bcl-2, Bcl-X_L and Bfl-1) and pro-apoptotic (Fas, FasL and DR4) proteins [68]. TNF-α also activates apoptosis-related caspases via fas associated death domain (FADD) attached to the intracellular side of the TNF-receptor. Therefore, TNF-α signaling has diverse effects depending on the cell type and the downstream activated signaling pathways. In HSCs, TNF-α-mediated NFκB activation enhances cell survival, whereas activation of caspases leads to apoptotic signals [69,70]. The balance between the pro-apoptotic and anti-apoptotic activity depends upon the basal levels of NFκB and its inhibitor IκB. In activated HSCs, the basal activity of NFκB increases due to a drop in activity of IκB-α, thereby preventing apoptosis and promoting proliferation. Hepatocytes express both TNFR1 and TNFR2. In chronic liver injury, TNFR1 leads to the activation of pro-apoptotic caspases via fas-associated death receptors thereby leading to loss of the hepatocytes [47].

Eicosanoids

Eicosanoids, e.g. prostaglandins and leukotrienes, are inflammatory mediators produced in response to endotoxin, enterotoxins, toxic viral proteins and mechanical or thermal injury. Eicosanoids are produced from arachidonic acid (AA) that, itself, is synthesized from membrane phospholipids by the action of phospholipase A_2 (PLA_2) [71,72]. AA is either converted into leukotrienes through the lipooxygenase pathway or into prostaglandins via the cyclooxygenase pathway. Kupffer cells and infiltrating macrophages synthesize LTA_4 through the subsequent action of 5-LO-activating protein (FLAP), and 5-lipoxygenase (5-LO), after which it is released into the extracellular space [72]. LTA_4 is taken up by the hepatocytes that convert LTA_4 to LTC_4 through the action of LTC_4 synthase. Next, LTC_4 is exported from hepatocytes and converted into LTD_4 and LTE_4 by membrane-bound gamma-glutamyl transpeptidase (γGT) and amino-peptidase, respectively [72]. LTD_4 and LTE_4 activate the G protein-coupled receptors CysLT1 and CysLT2 on target cells, such as Kupffer cells, sinusoidal endothelial cells and myofibroblasts, and induce Ca^{+2} mobilization, vasoconstriction, bronchoconstriction, vascular permeability and chemoattraction of neutrophils and eosinophils (Figure 3) [73]. Leukotrienes are degraded via peroxisomal β- and ω-oxidation and the metabolites are cleared via the urine [72]. Recent studies show that HSCs contain the complete leukotriene synthesis pathway, including 5-LO, FLAP and LTC_4 synthase and are able to produce LTC_4 (Figure 3) [74]. Leukotrienes induce lung fibrosis through binding to the CysLT receptors. CysLT receptor antagonists like montelukast and zafirlukast are used clinically to prevent bronchial asthma [75]. LTD_4 receptor inhibition by montelukast also represses the induction of fibrosis in a model of cholestasis-induced liver fibrosis [76]. Leukotrienes and prostaglandins have been reported to have opposite roles in fibrotic lung disease [77]. However, in the liver it has been shown that inhibition of COX-2 and 5-LO both attenuate inflammation and fibrosis via repression of the synthesis of prostaglandins and leukotrienes by Kupffer cells [78]. Prostaglandins, depending upon their type, have diverse cellular functions and play a key role in inflammation and allergic reactions. They inhibit apoptosis and immune response, but initiate cell proliferation and angiogenesis, e.g. PGE_2 inhibits collagen1a1 production by HSCs via inhibition of TGF-β [79]. This shows that the downstream effects of leukotrienes and prostaglandins are cell type- and tissue-specific. Collectively, these data show that leukotrienes and prostaglandins have a significant impact on the liver inflammation and onset of liver fibrosis.
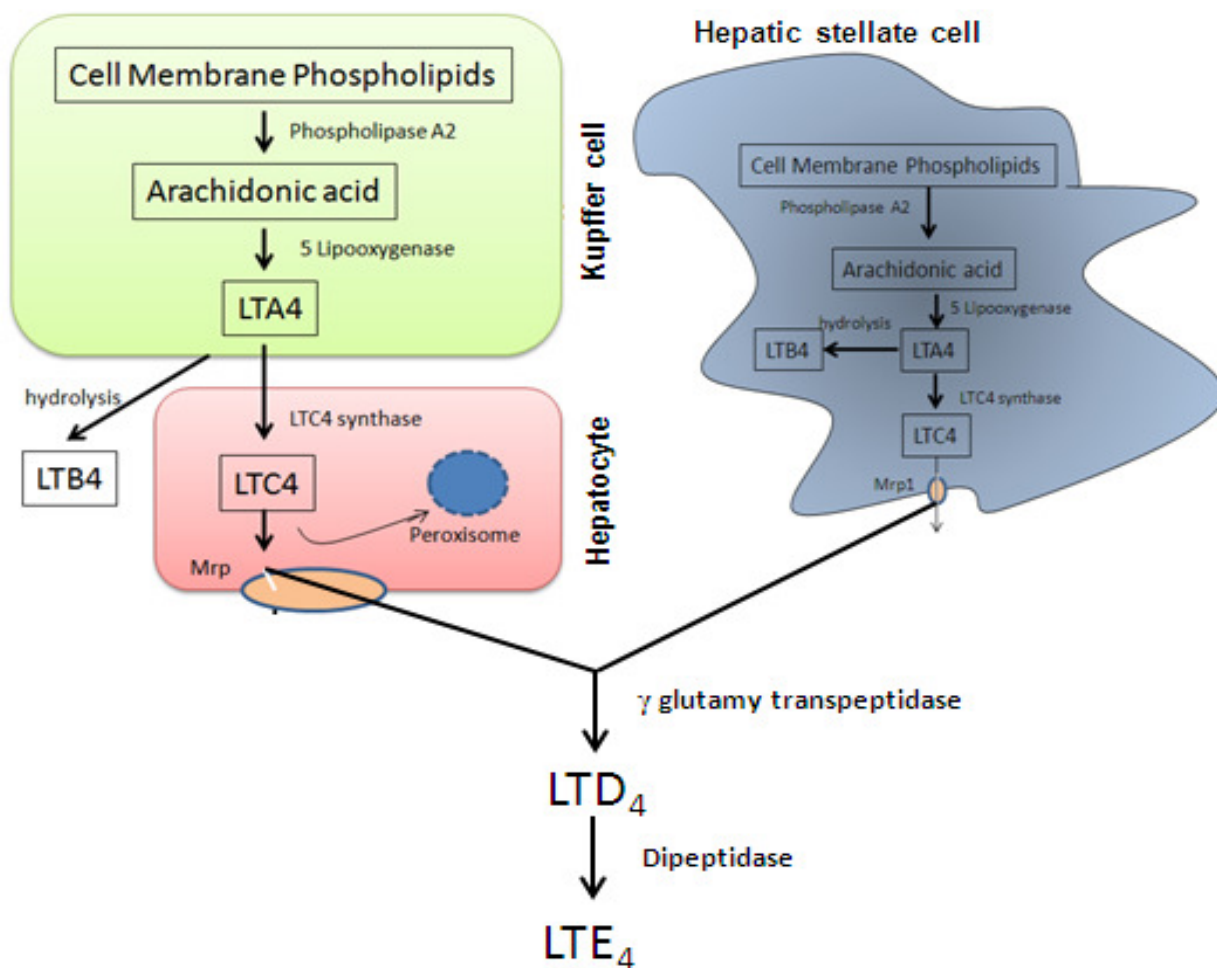
Figure 3. Leukotriene synthesis pathway in the liver. In the liver the inflammatory signal triggers the release of membrane phospholipids that are converted to arachidonic acid followed by the conversion to Leukotriene (LT) A_4. LTA_4 is secreted (by Kupffer cells) and taken up by hepatocytes. LTA_4 it is conjugated with glutathione in an enzymatic reaction driven by LTC_4 synthase. LTC_4 is either exported via Mrp1 or some related transporter to the exterior of the cells where it is converted to LTD_4 by extracellular enzyme gamma glutamyl transpeptidase and further to LTE_4 by dipeptidase. Leukotriene B_4 is synthesized by the hydrolysis of LTA_4 in the extracellular space.

Nuclear Receptors

Transdifferentiation of HSCs to a fibrogenic phenotype is associated with major metabolic and transcriptional changes. Also ligand-activated transcription factors of the nuclear receptor (NR) family are involved in this. The typical characteristic of HSCs transdifferentiation is the loss of the cytosolic lipid droplets that contain retinyl palmitate [11,12]. This phenomenon has generated significant interest in analyzing the roles of retinoic acid receptors (RARs) and Retinoid X receptor (RXR) in the activation process. Indeed, both natural and synthetic ligands of these nuclear receptors modulate proliferation and/or activation of HSCs. Activation of RXR repress proliferation, and

synthesis of collagen and fibronectin, while RAR activation also suppressed the expression of fibrosis markers, but did not affect HSCs proliferation [80]. In line with these observations, overexpression of RXR suppressed CCl_4-induced liver fibrosis in rats [81,82]. However, the use of vitamin A to suppress liver fibrosis is a controversial issue as hypervitaminosis A is also associated with liver injury and fibrosis. Recent studies on the role of NR in liver fibrosis have particularly focused on the Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ). Endogenous ligands of PPAR-γ are specific fatty acids, but this transcription factor is also activated by thiazolidinediones and prostaglandin-derivatives and initiate transcription of adipogenesis-associated genes. PPAR-γ expression is high in quiescent HSCs and sharply drops during transdifferentiation of HSCs. PPAR-γ ligands have been shown to attenuate HSCs activation and repress liver fibrosis [83-85].

Other studies have shown that the Farnesoid X receptor (FXR) is also involved in the activation of HSCs and fibrosis in mice [86,87]. FXR is known as the bile salt sensor and regulate bile salt synthesis and transport activities of the hepatocyte. Low levels of FXR are also detected in HSCs. A synthetic agonist of FXR was shown to induce the expression of PPAR-γ and thereby reduce the expression of TIMP1 and resolve liver fibrosis. However, recent studies show that human stellate cells hardly contain FXR [88] and thus may not play a role in liver fibrosis in humans.

Oxidative stress and its implication during liver fibrosis

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants [89]. The term pro-oxidant can be used for any substance that produces reactive oxygen species directly or indirectly. "Direct" pro-oxidants include transition metal ions like iron and copper. "Indirect" pro-oxidants include anticancer drugs or their metabolites. Normal metabolism also produces reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide, in particular in metabolically highly active organ like liver. However, this does not lead to oxidative stress as sufficient anti-oxidant capacity is present to neutralize these potentially toxic compounds. As a result of inflammation, ROS production is strongly increased during liver diseases. Pro-oxidants like superoxide anions, nitric oxide (NO) and hydrogen peroxide (H_2O_2) are produced by inflammatory and immune cells, including neutrophils and natural killer cells [89,92]. Cytokines and

growth factors that are released trigger a cascade of cellular events in the target cells resulting in oxidative stress and changes in the intracellular redox potential [93]. A disturbance in intracellular redox potential may cause damage to biomolecules like proteins, DNA and membrane lipids [94]. Depending upon the recovery mechanism and the level of redox shift, this may lead to activation of apoptotic signaling pathways and/or necrotic cell death [95,96].

Liver cells harbor several antioxidant mechanisms to cope with the endogenous and pathogen-induced ROS. These antioxidant mechanisms include antioxidant enzymes, antioxidant vitamins A, C and E and an efficient system for synthesis and recycling of the antioxidant peptide glutathione (GSH).

There are several types of antioxidant enzymes, e.g. superoxide dismutases (SOD), catalase and glutathione peroxidases. SODs are classified into three types based on their subcellular localization. Manganese-SOD (MnSOD/SOD1) is localized in mitochondria [97] and Copper/Zinc-SOD (Cu/Zn/SOD2) resides in the cytoplasm with a few reports claiming a substantial amount of CuZnSOD being present in peroxisomes [97,98]. Finally, extracellular-SOD (ecSOD/SOD3) is, as the name implies, secreted into the plasma or extracellular space [89]. Superoxides that are produced in the electron transport chain or in other metabolic pathways are highly reactive and therefore potentially toxic for the cell. The SODs convert superoxide anions into H_2O_2. H_2O_2 is less toxic than superoxide, but still has the ability to form free radicals. Therefore, H_2O_2 must be metabolized to prevent cell damage. Catalase is a peroxisomal enzyme and converts H_2O_2 to water and oxygen. Alternatively, H_2O_2 is detoxified by glutathione peroxidases (GPx) that convert it into water thereby forming oxidized glutathione (GSSG) [89]. Eight different isoforms of GPx exist with different subcellular locations, but GPx1 is the most dominant form in the liver. Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH acting as reducing agent.

The main antioxidant in the body is glutathione [99], which exists in either a reduced (GSH) or an oxidized (GSSG) state. Hepatocytes synthesize GSH, which is used both as an intracellular anti-oxidant and is exported to interstitial spaces and blood to prevent H_2O_2-induced tissue damage. Blood also contains relatively high levels of glutathione (approximately 1 mmol/L), over 90% of which is in the reduced state (GSH) [101]. The high cellular concentrations of glutathione (up to 5 mM in the liver) function

as a buffer, preventing reactive oxygen species (ROS) to react with other vital cellular components [100]. In enzymatic reactions, glutathione can donate a reducing equivalent ($H^+ + e^-$) to unstable reactive oxygen species. Consequently, glutathione becomes reactive itself. In the presence of glutathione peroxidase (GPx) GSH reacts with H_2O_2 to form water and oxidized glutathione (GSSG). Glutathione seems an important anti-oxidant for activated HSCs, since it has been reported that HSCs contain increased amounts of glutathione compared to quiescent HSCs [95,102].
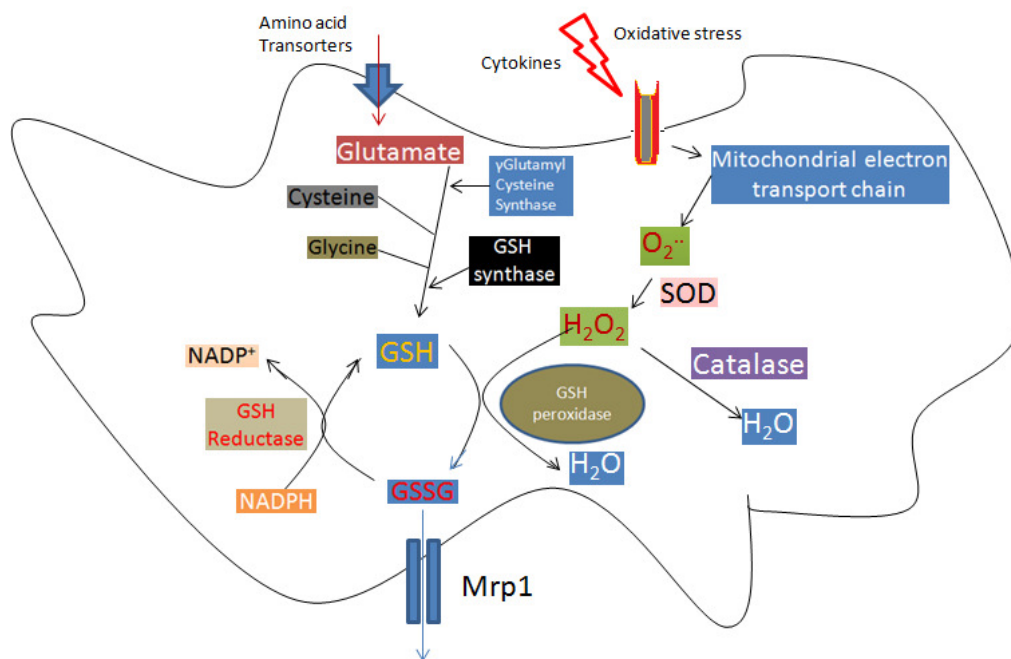
Figure 5. Mechanisms of neutralizing reactive oxygen species in HSC. Oxidative stress leads to the production of hydrogen peroxide (H_2O_2) due to super oxide dismutase activity. H_2O_2 is neutralized either by GSH peroxidase-catalyzed reaction to form disulfide Glutathione (GSSG) and water or catalase dependent reaction to produce H_2O and molecular Oxygen O_2.

GSH is not only acting as an antioxidant, but it is also essential for the biotransformation of endogenous molecules and drugs. Oxidized glutathione (GSSG), glutathione conjugates of drugs, prostaglandins and leukotrienes are exported from the cell via ABC transporters, such as multidrug resistance-associated proteins (MRP1, MRP2, MRP4, MRP6) or organic anion transporters, like OATP1 and OATP2 [103-108]. Inside cells, oxidized glutathione (GSSG) is recycled back to reduced glutathione (GSH) by glutathione reductase. Exported GSH/GSSG is catabolized to its constitutive amino acids by membrane bound gamma glutamyl transpeptidase (γGT) that is present on many cell types including cholangiocytes and endothelial cell [31].

ABC transporters and their putative role in Liver fibrosis

In liver disease, all liver cells are confronted with the same pathophysiological conditions. However, the cellular response towards these conditions is very different depending on cell type. Liver injury is characterized by damage to hepatocytes and subsequent decline in liver function. HSCs resist these harsh conditions and even become proliferative. One adaptation of the activated HSCs is the increased synthesis of glutathione that may protect these cells from the oxidative stress associated with liver damage [109]. A second mechanism to protect against cytotoxic conditions is the increased expression of specific substrate transporters that export harmful compounds from the cell [110]. The phenomenon of acquiring resistance to cytotoxic compounds is well known from studies on cancer. Cytostatic drugs are meant to kill the rapidly proliferating cancer cells, but often they become resistant by increasing the expression of drug transporters present in the cellular membrane that pump out these anticancer drugs [110]. These drug transporters belong to the superfamily of ATP binding cassette (ABC) transporters. The human genome encodes 48 different ABC transporters that, based on structural similarities, are subdivided in 7 subfamilies A to G. Only few of the ABC transporters are directly involved in multidrug resistance of cancers cells, and these include P-gp/MDR1 (ABCB1), MRP1 (ABCC1) and the breast cancer resistance protein (BRCP/ABCG2). Most ABC transporters function in normal physiological processes and transport endogenous substrates like lipids, bile acids, bilirubin, etc. Also the ABC transporters typically associated with drug resistance may be expressed in normal tissues including lung, brain, kidneys, intestine, liver and blood cells [111-114]. The main function of these exporters in these tissues is to protect the cells from endogenously produced toxic compounds and metabolites [113].

The best characterized ABC transporter is P-gp/MDR1 that is encoded by the *ABCB1* gene. It is a 170 kDa glycoprotein containing 1280 amino acids. Structurally, it consists of two homologous halves, each of which consists of 6 transmembrane domains and a cytosolic ATP binding domain. Humans only contain one MDR1 gene, while rodents have 2: Mdr1a and Mdr1b. MDR1 transports a great variety of structurally unrelated substrates across the plasma membrane, hence its characterization as a multidrug transporter [110]. Its closest homolog, MDR3 (Mdr2 in rodents) is not associated with multidrug resistance, but is a transporter of phospholipids, like

phosphatidylcholine and ethanolamine. It resides in the canalicular membrane of hepatocytes and transports phospholipids to the bile, where these form mixed micelles with bile salts. The bile salt/phospholipid-mixed micelles are the carriers for lipophilic compounds in the bile and intestine, so that they can either be efficiently absorbed or secreted via the feces [3,111].

Like MDR1/ABCB1, also members of the ABC subfamily C are associated with multidrug resistance. These are referred to as multidrug resistance-associated proteins (MRPs). This subfamily consists of 13 members and they transport organic anions, regulate chloride channels (CFTR) and act as ATP sensors (ABCC8 and ABCC9) [111]. ABCC1 (MRP1) is the founding member of the C-subfamily. Mrp1 is most prominently expressed in lung, testis, kidney and peripheral blood mononuclear cells [111]. Under healthy conditions, low levels of MRP1 are detected in the liver and intestine. MRP1 transports a great variety of compounds, including organic anions, reduced glutathione (GSH), oxidized glutathione (GSSG), cysteinyl leukotrienes, prostaglandins and glucuronide- and sulphate-conjugated hormones [105,115]. It also transports aflatoxin 1 and alkaloids (vincristine and vinblastine) [116]. The ratio GSH/GSSG is an indicator of the cellular redox status and MRP1 seems to play important role in maintaining the redox status by exporting the cellular GSH and GSSG [100].

ABC transporters are expressed by lymphocytes, natural killer cells and dendritic cells and have role in immune cell mediated inflammation as they are able to transport prostaglandins and leukotrienes. Multidrug resistance (-associated) protein-dependent initiation and perpetuation of inflammation has been demonstrated in *in vivo* studies for example exogenous treatment with leukotrienes in Mrp1 knockout mice that restored the migration of dendritic cells [117,118]. IL-6-induced expression of Mrp1 may be an adaptive response to enhance cytoprotection and leukotriene export [119]. Similarly, MRP1 expression is induced in the inflamed epithelium of patients with inflammatory bowel disease and this was shown to protect these cells from cytokine-induced apoptosis [121]. P-gp and Mrp1 are also associated with cytoprotection in hepatic progenitor cells. Mrp1 expression is enhanced in the progenitor cell compartment after partial hepatectomy [120]. The expression of Mrp1 is induced upon activation of HSCs and inhibiting its transport function by MK571 induced necrosis in these cells. Thus, Mrp1 may be an important facilitator in the development of liver fibrosis [122]. However, a direct role of

Mrp1 in cytoprotection of HSCs has not been demonstrated yet, as these experiments were performed with the pan-Mrp inhibitor MK571, which is also know to antagonize the CysLT receptors [123]. Still, a putative role of Mrp1 in stellate cell biology makes it a relevant target for detailed studies to delineate its role in liver fibrosis, including the identification the substrate(s) involved. Detailed knowledge about this may lead to the development of novel antifibrotic therapies.

Therapeutic targeting for liver fibrosis

Despite our increasing knowledge about the cellular processes leading to liver fibrosis, no effective treatment is available yet for this condition, other than removing the causative agent(s) and/or liver transplantation. There is ample evidence that liver fibrosis is, at least partly, reversible [40]. Liver fibrosis was shown to regress/resolve and scar tissue is replaced by functional liver mass, when the liver damaging conditions are eradicated [69,124]. However, when left untreated, liver fibrosis may progress to cirrhosis, which irreversibly disturbs the architecture of the liver and predisposes patients for liver cancer. It is therefore of eminent importance that anti-fibrotic therapy is started as early as possible. During the last decade major advancements have been made in understanding the pathogenesis of liver fibrosis and cirrhosis, including the contribution of the individual cell types and the intra- and intercellular signaling pathways. This now provides us with an array of molecular targets that are tested for their anti-fibrotic action [37,38,47,125]. Table 1 summarizes the current leads to develop therapies to treat fibrosis and highlights the potential therapeutic effects as well as the limitations.

For successful therapeutic interventions the accurate diagnosis and elimination of the causative factor is the most important factor without which the treatment of liver diseases would be inefficient. The primary focus should be the restoration of the parenchyma (the hepatocytes). This can be achieved by intake of antioxidants (vitamins) that result in reduction of oxidative stress-induced damage. However, this strategy has not been very successful yet. Although the gradual loss of hepatocytes can also be inhibited by the administration of either hepatocyte growth factor(s) or caspase inhibitors like VX-166 that prevent apoptosis in hepatocytes [126], however, this treatment has the potential threat of generalized inhibition of apoptosis and uncontrolled cell growth predisposing for cancer development.

Hepatic stellate cells and myofibroblast are fibrogenic cells that produce extracellular matrix, therefore they are the main target for the development of antifibrotic therapies. The inhibition of HSCs activation and induction of apoptosis/necrosis during liver fibrosis has been a main focus. The limitations in development of antifibrotic drugs are the potential toxic side effects when drugs that are meant to target HSCs are given systemically [127,128]. The drugs that show promising antifibrotic effects *in-vitro*, often show limited efficacy *in vivo* and/or give rise to unwanted cytotoxic and antiproliferative effects on other cells in or outside the liver. This problem can be circumvented by cell-specific targeting of drugs to hepatic stellate cells [127-129]. In the following section we discuss potential anti-fibrotic drugs that are tested without specific targeting to stellate cells, but their efficacy could even be improved using this strategy.

TGF-β/Smad- and PDGF/ERK-dependent signaling pathways have pronounced effect on HSCs activation and matrix production [125,130-134]. Due to their ubiquitous presence of TGF-β, all liver specific cells will be affected by these mediators when applied systemically. So, these pathways can only be modulated therapeutically when highly selective targeting to the hepatic myofibroblasts is achieved. TGF-β signaling inhibition can be achieved by inhibition of cannabinoid I receptor [51,52]. Similarly, Endothelin (ET) receptor A and B are expressed by stellate cells. The ET receptor inhibitor bosentan has anti-proliferative effect on HSCs [135], but the drawback of this compound is that it adversely inhibits the transport activity of BSEP and may cause cholestasis, in particular in genetically-prone patients [136]. NF-kB inhibition has been evaluated *in vitro* and *in vivo*. Inhibition of NF-kB sensitizes HSCs to apoptotic cell death, but this also leads to hepatocyte cell death [45,46]. Nerve growth factor (NGF)-dependent stimulation of P75 and adiponectin-dependent activation of AMPK can inhibit NF-kB signaling that promotes myofibroblast apoptosis [13,137]. Therefore, inhibiting this pathway can also be a promising therapy. Hedgehog signaling has been implicated in the activation of HSCs and EMT. Targeting this pathway attenuates liver fibrosis and *in vivo* studies are promising [138,139].

PPAR-γ expression in the HSC is associated with the quiescent phenotype. Activation of HSCs is accompanied by loss of PPAR-γ expression. A synthetic agonist of FXR induces the expression of PPAR-γ. Furthermore, synthetic agonists of PPAR-γ, such as pioglitazone and troglitazone, are already used as anti-diabetic drugs [83,140-142].

Table No. 1. Proposed therapeutic drug targets for fibrosis treatment

Target	Target	Drug	Advantage	Disadvantage	Ref
Hepatoprotectant / Antioxidants	Hepatocytes	Hepatic growth factor and analog Refanalin (in development)	Hepatocyte proliferation Prevent fibrosis in vivo	HSC proliferation, oncogenesis	[37]
	Caspase inhibitor	VX-166	Inhibits hepatocyte apoptosis, lowers hepatic damage	Cancer and other safety concern	[126]
Prevention Reversal of HSC Activation	HSC PPAR-γ agonist	Pioglitazone Troglitazone	Decrease HSC activation	Limited effects	[83,140-142]
	HSC FXR agonist	INT 747	Synthetic bile analog	Low expression of FXR in HSC. No data about toxicity yet	[88]
	HSC HMG-CoA reductase inhibitor	Atorvastatin (Lipitor) lowers cholesterol	Decreses, expression of pro-fibrotic cytokine and HSC proliferation	Limited effect on hepatic inflammation	[143]
	Renin angiotensin system	Losartan Telmisartan	Reduced fibrosis in CCL4 models. Decreases pro-fibrogenic response		[144,145]
	HSC Hedgehog signaling	GDC-0449	Prevents fibrosis in chronic model of fibrosis	Not yet tested	[138]
	Canabanoid receptor (CB)1 inhibitor	Rimonabant SR141716A	lowers TGFβ1	Not yet tested	[51,52]
Growth factors/ Cytokines	TGFβ	Neutralizing Antibodies, siRNA, Recombinanat smad Pirfenidone	Prevents HSC activation Reduce TGFβ Production	TGFβ receptors are ubiquitous	[146]
	PDGF and VEGF	Neutralizing, Antibodies, siRNA Sunitinib, Imatinib, mesylate	Antifibrotic in *in vivo*	Wide spread effects not effective in long term	[125,130-134]
	Endothelin A and B receptor antagonist	Bosentan	Regulates wound contraction and blood flow	Not yet tested	[135]
Inducing HSC apoptosis	TIMP and MMP	Mab against TIMP MMP9 Mutant MMP1	Induces apoptosis in HSC Scavanges TIMP1 Degrades Matrix	Not yet tested	[147-149]
	MRPs inhibition	MK571 (also LTD4 receptor antagonist)	Induces necrosis in HSC	Potential risk of liver carcinogenesis	[122]
	LTD4 receptors	Montelukast	Decreased TIMP, VEGF, TGFβ, and induced MMP-9	No reported trial for Liver fibrosis	[76]
Gene therapy	MicroRNA	miR29	Reduces collagen express	Gene therapy associated risk	[150]
Autologous cell therapy	Bone marrow cells transplantation	Induces expression of MMp9	Degrade Matrix		[151]
	Macrophages	Induces expression of MMp9	Degrade Matrix	Autoimmunity	[152]

So far, these drugs showed limited anti-fibrotic efficacy that could be due to limited expression of PPAR-γ in activated HSCs. Drugs that positively regulate the expression of PPAR-γ may therefore be considered as co-therapy. Angiotensin II receptor inhibitors (Losartan and Telmisartan) showed antifibrotic effects in patients with Hepatitis C [144,145]. Cholesterol lowering drug (Atorvastatin) reduced HSCs proliferation in the bile duct ligation (BDL) model of liver fibrosis [143]. As these drugs are already used in clinic they have potential for immediate availability for the treatment of liver fibrosis. Short hairpin microRNAs are small regulatory RNAs that post-transcriptionally control the expression of genes [153]. Recently, it has been shown that miRNA 29 represses the expression of collagen [150], however, the unavailability of the non-viral expression system and limited transfection efficiency are the main hurdles for their selection as therapeutic strategy.

Matrix protein synthesis and degradation are balanced dynamic processes in the healthy liver. This process is under strict control of MMPs, which degrade matrix proteins. The activity of MMPs is counteracted by tissue inhibitors of metalloproteases (TIMPs). MMPs can be utilized for the degradation of ECM during fibrosis. Macrophages secrete MMP13 and mediate th e resolution of fibrosis *in vivo*. Therefore, cell-based therapy (macrophage and bone marrow cell transplantation) can be explored as a therapy, but has the inherent potential of exacerbating the inflammation [151,152]. On the other hand, TIMP1 has an anti- apoptotic effect on HSCs.Therefore, an inhibitor (monoclonal antibody) of TIMP1 is considered as a therapeutic option.

Cysteinyl leukotrienes are also potential therapeutic targets for the regression of liver fibrosis [75,154]. Enhanced cellular levels of leukotrienes are associated with liver cirrhosis and lung fibrosis. In the liver, cysteinyl leukotrienes bind to LTD_4/LTC_4 receptors and induce contraction of myofibroblasts and activated HSCs, increasing portal hypertension. Furthermore, leukotrienes increase intestinal permeability resulting in enhanced levels of endotoxins in the blood [155,156]. The LTD_4 receptor antagonist montelukast is being assessed for its antifibrotic effects, but no data are available yet [57,157].

Reversing the activation state of hepatic myofibroblasts would be the ideal therapeutic effect of an antifibrotic drug. However, also selective killing of these cells can be considered as long as a portion of the (quiescent) HSCs remain unaffected to

repopulate the recovering liver. MK571 was shown to lead to HSCs necrosis, even in the absence of any other cytotoxic trigger [122]. This may be the result of inhibiting the multidrug resistance associated proteins (Mrp1). MK571 also acts as an antagonist of the LTD_4 receptor and may have off-target effects that cause necrosis. So, it first needs to be established which pathways are involved in MK571-mediated HSCs necrosis, before it can be further developed for a therapy to treat liver fibrosis.

Scope of the thesis

Chronic liver diseases lead to liver fibrosis, which may progress to cirrhosis and liver cancer and are associated with high morbidity and mortality. Liver hepatocytes die while hepatic stellate cells and myofibroblast become activated during liver disease. The aim of the work described in this thesis is to identify targets to develop therapies for liver fibrosis, specifically focusing on modulating the activation state of hepatic myofibroblasts.

In Chapter 2, we analyzed the role of the multidrug resistance-associated protein 1 (Mrp1/*Abcc1*) in the development of liver fibrosis *in vitro* and *in vivo*. Primary rat hepatic stellate cells (HSCs) and portal myofibroblasts (PMFs), as well as human LX-2 cells, were treated with pharmacological inhibitors of Mrp1 or Mrp1-specific RNA interference and their effects on the activation of these cell types were analyzed. Glutathione and leukotriene levels were manipulated to identify the Mrp1 substrate that modulates the activation of these cells. Finally, wild type and *Abcc1*$^{-/-}$ mice were exposed for 12 weeks to CCl_4 to establish the role of Mrp1 in liver fibrosis *in vivo*.

In order to establish the potential use of pharmacological inhibitors of Mrp1 for the treatment of liver fibrosis, we compared in chapter 3 the cytotoxic effect MK571 and reversan on hepatocytes, HSCs and portal PMFs in an *in vitro* model of liver disease. The various liver cell types were exposed to oxidative stress in combination with MK571 or reversan and necrotic cell death was quantified. Moreover, we quantified the intra-cellular levels of reduced and oxidized glutathione under these conditions and studied the specificity of the inhibitor to inhibit Mrp1-mediated transport.

In chapter 4, we analyzed the antioxidant defense mechanisms acquired by HSCs during the process of activation. Glutathione levels and expression of antioxidant enzymes were quantified in quiescent, activating and fully activated rat HSCs.

Glutathione depletion and repletion experiments, as well as inhibitors of anti-oxidant enzymes were used to determine their role in protecting HSCs from oxidative stress.

In Chapter 5, we analyzed the role of an intracellular ABC transporter, 70 kDa peroxisomal membrane protein (PMP70) in HSCs and PMFs activation. We analyzed the expression and cellular distribution of peroxisomal proteins in the process of HSC/PMF activation using immunofluorescence microscopy. PMP70 expression was inhibited by RNA interference or induced by recombinant production of PMP70-GFP and the effect on the expression of markers of fibrosis, including collagen1a1 and alpha-SMA, were determined.

In Chapter 6, we summarize the data presented in this thesis and discuss their relevance for liver fibrosis and the treatment of chronic liver diseases.

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Multidrug resistance associated protein 1(Mrp1) inhibition attenuates liver fibrosis in vitro and in vivo

Atta ur Rehman, Bojana Mikuš, Tjasso Blokzijl, Rutger Moderman, Catharina Reker-Smit*, Klaas Poelstra*, Han Moshage and Klaas Nico Faber

Department of Gastroenterology and Hepatology,
University Medical Center Groningen, University of Groningen
*Department of Pharmacokinetics, Toxicology and Targeting
University of Groningen

ABSTRACT

Background & Aims: Liver fibrosis invariably develops during chronic liver disease and predisposes for cirrhosis and liver cancer. Fibrosis is caused by myofibroblasts that produce excessive amounts of extracellular matrix proteins (ECM), which impair liver function. Hepatic stellate cells (HSCs) and portal myofibroblasts (PMFs) are important contributors to liver fibrosis and are therefore prime targets to develop anti-fibrosis therapy. HSCs express high levels of the Multidrug resistance-associated Protein 1 (Mrp1), a transporter of leukotriene C_4 (LTC_4) and glutathione, and this study aimed to determine its role in liver fibrosis.

Methods: Mrp1 expression/activity was modulated in primary rat HSCs, PMFs and the human hepatic stellate cell line LX-2 and the effect on cell survival and fibrogenic markers (alpha-Sma, Collagen1a1) was determined. Wild type and Mrp1 (*Abcc1*) knockout mice were exposed to CCl_4-induced liver fibrosis.

Results: Both pharmacological (Reversan) as well as genetic (siRNA) inhibition of Mrp1, repressed α-Sma and Col1a1 expression in rat HSCs, PMFs and LX-2 cells, without inducing cell death. Transient expression of Mrp1-GFP induced α-Sma expression. Glutathione depletion or supplementation did not change the expression of fibrogenic markers in HSCs and the anti-fibrotic action of reversan remained under these conditions. Inhibition of leukotriene synthesis (AA861) in HSCs was equally anti-fibrotic as inhibiting Mrp1, the LTC_4 transporter. α-Sma expression and Collagen deposition were induced in CCl_4-treated wild type mice, which was significantly reduced in CCl_4-treated Mrp1 knockout mice.

Conclusion: Inhibition of Mrp1 leads to suppression of liver fibrosis, most likely by preventing leukotriene export from hepatic myofibroblasts. MRP1 is therefore a highly relevant target for the treatment of liver fibrosis.

Key words; Hepatic stellate cells, Portal myofibroblasts, Fibrosis, MRP1, Leukotriene C_4

INTRODUCTION

Chronic liver diseases lead to a sustained healing process that causes liver fibrosis and may progress to cirrhosis and liver failure. The excessive production and deposition of extracellular matrix (ECM) proteins, including type I collagens and fibronectins, disturbs the architecture of the liver and impairs liver function. Currently, no effective therapy exists to treat liver fibrosis.

Hepatic stellate cells (HSCs) are important contributors to the development of fibrosis [1-4]. In the healthy liver, HSCs regulate retinol homeostasis and produce balanced amounts of extracellular matrix proteins to create the functional environment for the other liver cells. Cytokines and reactive oxygen species released upon liver injury activate HSCs, which transdifferentiate into proliferative, mobile and contractile myofibroblasts that produce excessive amounts of ECM proteins, while losing their retinol content [1,3]. In addition, hepatic myofibroblasts may originate from resident fibroblasts (PMFs) in the portal areas, through epithelial mesenchyme transition (EMT) and/or the influx of bone marrow-derived cells [3,5]. Irrespective of the origin, hepatic myofibroblasts express high levels of type I collagen and develop an extensive intracellular cytoskeleton of alpha-smooth muscle actin (α-SMA) that supports mobility and contractility of these cells [2,6]. Theoretically, an antifibrotic effect can be achieved either by selectively killing activated myofibroblasts or reversing the activation process in the fibrotic liver. Therefore, hepatic myofibroblasts are the prime therapeutic target to treat liver fibrosis.

Earlier, we showed that expression of the Multidrug resistance-associated protein type 1 (Mrp1) is induced upon activation of rat HSCs *in vitro* and *in vivo* [7]. MRP1 expression is also most prominent in activated human HSCs when compared to MRP2-6 and MDR1 [7]. MK571, a general inhibitor of MRP function, induced necrotic cell death in aHSCs and thus holds promise for future antifibrotic therapies [7]. MRP1 is an ATP-binding cassette (ABC) transporter with broad substrate specificity and is well-known for its adverse effect in the treatment of cancer [8,9]. Cytostatic drugs are effectively exported by MRP1, thereby preventing killing of cancer cells in drug therapy. However,

MRP1 also transports endogenous substrates, including reduced and oxidized glutathione [10]. Moreover, it is a high affinity transporter for leukotriene C_4 (LTC_4) [11].

LTC_4 is a proinflammatory signaling molecule that is produced by various cell types during liver injury, including Kupffer cells, infiltrating macrophages, hepatocytes and activated HSCs [12-15]. LTC_4 is synthesized from arachidonic acid (AA) through the sequential action of 5-LO-activating protein (FLAP), 5-lipoxygenase (5-LO), and LTC_4 synthase [16,17]. Like observed for MRP1, expression of 5-LO is induced upon HSCs activation and leads to the release of LTC_4 into the culture medium [15]. Once exported by Mrp1, LTC_4 is converted to LTD_4, which activates cysteinyl leukotriene receptor type 1 and/or 2 (cysLT(1)R and cysLT(2)R) [18,19]. Both endocrine and paracrine signaling lead to the activation of HSCs by LTC_4, as also observed for lung and skin fibroblasts [19,20]. In line with these mechanisms, LTC_4 treatment promotes collagen production in livers of monkeys and aggravates bile duct ligation-induced liver fibrosis [21]. The leukotriene synthesis and signaling pathway has therefore high potential to develop therapies to treat liver fibrosis. Indeed, pharmacological inhibition of 5-LO (using CJ13,610) reduced CCL_4-induced fibrosis in mice [22]. Moreover, the cysLT receptor-antagonist monteklast lowered BDL-induced fibrosis in rats [23]

MK571 is a general inhibitor of Mrp transporters, including Mrp1-6 [7,24-26], but also antagonizes the CysLT receptors [20,27]. The induction of necrosis in activated HSCs as observed earlier may therefore be a result of multiple actions of this compound. Given the broad range inhibitory actions, MK571 is prone to cause adverse side effects when used *in vivo*. To establish whether Mrp1 is a relevant therapeutic target to treat liver fibrosis, we studied the effects of the novel Mrp1 inhibitor reversan [28] and Mrp1-specific RNA interference on hepatic myofibroblasts *in vitro*. Moreover, we analyzed CCl_4-induced liver fibrosis in Mrp1 knockout mice.

MATERIALS AND METHODS

Animal: Specified pathogen free FVB/Ntac (wild type) and FVB.129P2-Abcc1atm1Bor N12 (MRP1 -/-) were purchased from Taconic (Taconic Europe A/S, Denmark) and housed under standard laboratory conditions and with free access to standard laboratory chow and water. The experiments were approved by the institutional committee for care and use of laboratory animals.

Induction of liver fibrosis: Liver fibrosis was induced in mice by an established protocol [29] with minor modifications, mainly the length of the treatment period, as the FVB mice are relative resistant to CCl_4-induced liver fibrosis. Mice were injected with intra-peritoneal administration of CCl_4 (Sigma) dissolved in corn oil (Sigma) for 12 weeks; first week 2 doses at a dose of 0.5 μl/gram of body weight (gbw), 2nd week 2 doses at 0.75 μl/gbw and for the last 10 weeks 2 doses per week at 1 μl/gbw dissolved in corn oil. Control animals were treated with corn oil only. Mice were terminated one day after the last injection. Blood was collected via cardiac puncture and liver specimens were put in paraformaldehyde (Sigma), snap frozen in isopentane and stored at -80ºC prior to use.

Cell isolation and culture conditions: Hepatic stellate cells (HSCs) were isolated from male wistar rats (500-600 g) using (12% w/v Nycodenz gradient centrifugation method as described previously [30]. Portal myfibroblast (PMFs) were isolated from male wistar rats (200-300 g) following the procedure described in [31]. The human hepatic stellate cell line (LX-2) was kind gift from Scott Friedman [32]. All cells were cultured at standard condition [30-32] with a slight modification that the HSCs medium did not contained nystatin immediately after isolation. All treatments on cells were carried out after overnight serum starvation in the absence of serum.

Experimental design: Culture-activated HSCs (passage 1-2) or PMFs (passage 2-5) were seeded at 80,000 cells per well of a 6-well plate or 40,000 cells/well of a 12-wells plate. For LX-2 cells (120,000/well or 50,000/well in 6- or 12-wells plate) were seeded at standard conditions [32]. LX-2 cells were activated in the presence of Tgf-β (100 pM) for 24 hours followed by treatment with Mrp1 inhibitor "reversan" (Tocris Bioscience, Bristol, UK). For identification of the MRP substrate responsible for antifibrotic effects, glutathione status was manipulated by GSH monoethyl ester (GSH-MEE; Sigma) to enhance intracellular glutathione or by buthionine sulphoximine (BSO; Sigma) to reduce intracellular GSH levels. Leukotriene synthesis was inhibited by using the 5-Lipo-oxygenase inhibitor, AA861 (Sigma). All the incubations were carried out for 24 hours, unless otherwise indicated for individual experiments.

MRP1 overexpression in LX-2: Human hepatic stellate cell line (LX-2) was transfected with MRP1-GFP expression plasmid described in [33] using Fugene (Promega) as transfection reagent according to manufacturer's instructions. EGFP-N1 (cytosolic GFP; BD Biosciences Clontech Palo Alto, CA) plasmid was used as control plasmid.

siRNA transfection: Inhibition of Mrp1 in rHSCs and LX2 was carried out using Mrp1-specific siRNA duplexes (sequence 5' CUC CAC CAG UAC UUU CAU A55 3'; Invitrogen, The Netherlands) using Oligofactmine (Invitrogen) as transfection reagent according to manufacturer instructions. Luciferase-specific siRNA (sequence 5' CGU ACG CGG AAU ACU UCG A55 3'; Invitrogen, The Netherlands) was used as oligonucleotide control.

RNA isolation, reverse-transcription-PCR (rtPCR) and real time-PCR: RNA was isolated using Tri Reagent (Sigma), reverse transcribed and analyzed for gene expression using real-time PCR system (7900HT Fast Real-time PCR system, Applied Biosystem) according to procedure described in [34]. Primers and probes used for human and rat ABC transporters are described in [7], and for mouse alpha-Sma, collagen1a1 and ABC transporters are listed in supplementary Table S1. Relative gene expression of the gene of interest was measured vs. control and normalized to18S using the ddCT method.

Micro RNA analysis: miR-29A (ID002112, Life Technologies) levels were analyzed as described [35] using the Taqman micro Assay and normalized to SnoRNA202 (ID001232, Life Technologies).

Mrp1 export activity Assay: Inhibition of Mrp activity by Mrp inhibitor (reversan) was carried out according to the protocol described in [7] with minor modifications. In short, cells were incubated with the Mrp inhibitor for 30 minutes and then loaded with CMFDA at a final concentration of 5 μM. After 45-60 minutes, cells were washed and refreshed with PBS containing appropriate inhibitor and photographs of cells were taken immediately using an Olympus CKX41 microscope. Cells were snap frozen at -70ºC for 10 minutes, followed by lysis in 1% triton X. 100 μl of the cell lysate was used to quantify the intracellular levels of CMFDA using excitation/ emission 480/535 on a Bio-tek FL600 microplate fluorescence reader.

Western blot: Following incubation with indicated reagents, cultured cells were harvested in cell lysis buffer as described in [34]. Liver tissue was homogenized in liquid nitrogen and lysed in buffer described in [34]. Proteins were quantified using the Bio-Rad Protein Assay system (Bio-Rad). Protein samples were separated by SDS-PAGE and analyzed by Western blotting according to established procedures [34]. Primary antibodies used are described in Supplementary Table S2. Respective HRP-conjugated secondary antibodies

were used. Protein signals were quantified using the chemidoc XRS system and band intensities were quantified using Imagelab software (Bio-Rad).

Immunocytochemistry and Immunoflourescence microscopy: Alpha-smooth muscle actin (α-SMA) immunostaining on mouse liver sections was carried out using the MOM kit (Vector labs) according to manufacturer protocol. Collagen 1a1 staining was carried out using goat anti-collagen1a1 according to the procedure described in [29]. Staining of collagen 1a1 was quantified by image analysis using the Cell^D analysis program (Olympus, Zoetermeer, The Netherlands). For *in vitro* culture, cells were fixed with 4% paraformaldehyde, labelled and analysed as described previously [36]. Primary antibody dilutions are listed in supplementary Table S2. Images were captured using a Leica SP2/AOBS confocal microscope at the UMCG Microscopy and Imaging Center (UMIC).

ALT and AST measurements: Liver damage markers ALT and AST were analyzed using standard protocol from the manufacture (SPINREACT, S.A. Santa Coloma, Spain).

LDH and Sytox green staining: Cell necrosis was determined by measuring relative LDH release from necrotic cells according to protocol described in [37] with minor modifications. Briefly, 100 µl of medium was loaded in 96-well flat bottom plates (Greiner bio-one) followed by addition of pyruvate and NADH. The rate (kinetic curve) of disappearance of NADH was measured (decrease in absorbance of 340 nm) for 30 minutes. LDH activity was measured using linear portion of the kinetic curve and compared to a standard curve of known concentrations of LDH. Necrosis was also determined by assessing Sytox Green (S-7020, Molecular probes) positive nuclei according to protocol described in [7].

Statistical analysis: Statistical analysis was performed using Prism5 software. Results are expressed as the mean value and standard deviation. Statistical significance was calculated using a Student's t-test, or one-way ANOVA with Bonferroni post-hoc analysis for correction for multiple comparisons. A p-value of <0.05 was considered to be statistically significant.
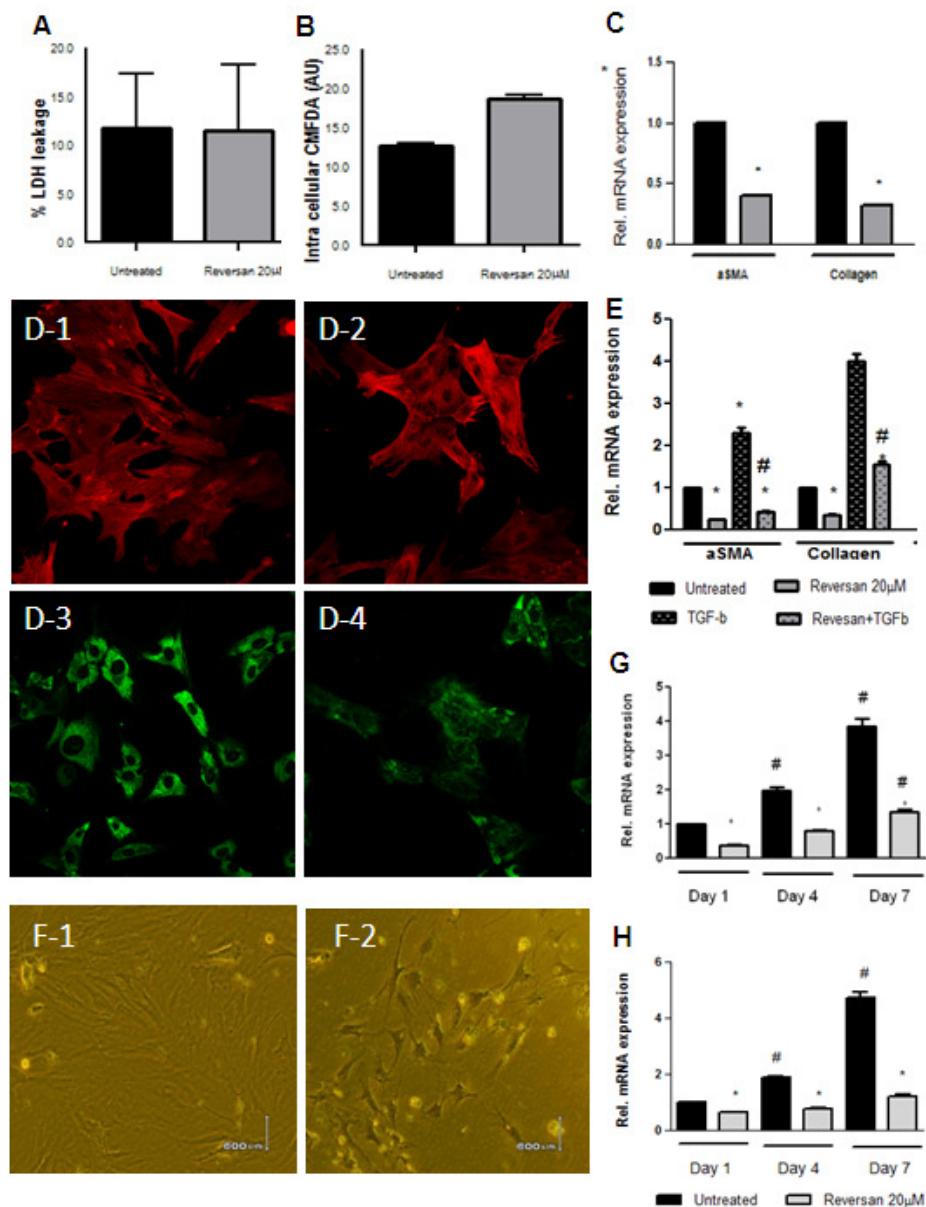
Figure 1. Expression of HSCs activation markers is supressed by the Mrp1 inhibitor reversan. A-D) Culture-activated primary rat HSCs (between 7-14 days after isolation) were exposed to 20 µM reversan and analysed for A) LDH release (indicator of necrosis) to the medium 24 hours after treatemnt; B) intracellular accumulation of the fluorescent Mrp1 substrate CMFDA as indicator off effective inhibition of Mrp1 after 30 minute; C) relative mRNA levels of the HSCs activation markers (α SMA and Collagen 1a1, normalized to 18 S as internal control) after 24 hours; D) protein expression of α SMA (untreated=D-1 and reversan=D-2) and Collagen 1a1 (untreated=D-3 and reversan=D-4) after 48 hours treatment, E) human LX-2 cells were treated for 24h with 20 µM reversan in the presence or absence of TGF-β and relative mRNA levels of α-SMA and Collagen 1a1normalized to 18S as internal control. F-H) Freshly isolated primary rat HSCs were cultured in the presence or absence of 20 µM Reversan and analysed for F) cellular morphology at day 7 and the relative mRNA levels of α-Sma (G) and Collagen 1a1 (H) after 1, 4 and 7 days in culture. Data are represented mean+/- SD of three independent experiment. * Significant difference (P<0.05) when compared to untreated control and # Significantly different (P<0.05) when compared to HSC at day 1.

RESULTS

The Mrp1 inhibitor reversan represses markers of liver fibrosis in activated primary rat HSC and human LX-2 cells

In order to obtain additional evidence for the vital role of Mrp1 in hepatic stellate cells (HSC), we exposed culture-activated primary rat HSC to reversan, a recently described inhibitor of Mrp1 [28]. In contrast to MK571 [7], reversan (20 μM) did not induce cell death in rHSCs (Figure 1A), while export of the Mrp1 substrate carboxymethyl fluorescein (CMF) was inhibited at these concentrations (Figure 1B). However, mRNA and protein levels of α-Sma and Collagen 1a1 were strongly repressed in rHSCs that were treated with reversan (Figure 1C and <u>D</u>). Similar results were obtained from the human hepatic stellate cell line (LX-2) after treatment with reversan, both under unstimulated and Tgf-β-stimulated conditions (Figure 1E). Likewise, reversan almost completely blocked the time-dependent induction of activation markers when freshly isolated (quiescent) rHSCs were culture-activated. After 7 days, they largely showed a quiescent phenotype (Figure 1F) and α−Sma and collagen 1a1 mRNA levels were hardly elevated (Figure 1G and H). Thus, in contrast to MK571, reversan does not induce significant levels of necrosis in rHSCs, while it is a potent inhibitor/antagonist of HSCs activation *in vitro*. Next, we performed siRNA-mediated repression of Mrp1 in activated rHSCs. A maximum suppression of 75% ($p<0.001$) of Mrp1 mRNA levels were obtained, which were accompanied with a significant reduction in α-Sma (-40%; $p<0.001$) and collagen1a1 (-12%; $p<0.001$) mRNA levels (Figure 2A). RNAi-treatment was slightly more effective in human LX-2 cells with a 82% ($p< 0.0001$) reduction in MRP1 mRNA levels, and led to a more robust reduction of α SMA (-62%; $p< 0.0001$) and Collagen 1a1 (-43% $p<0.0001$) (Figure 2B).

Mrp1 overexpression in human hepatic stellate cell line induces fibrosis markers

In a complementary approach, we transiently transfected LX-2 cells with a plasmid expressing GFP-tagged MRP1 [33] and studied the effect on fibrosis markers. LX-2 cells show an intermediate activation status and thus can be manipulated to become super-activated or suppressed. The expression of the various MRP-type transporters in LX-2 cells is highly comparable with primary rat and human HSCs (See Supplementary Figure S1 A). LX-2 cells were efficiently transfected (approximately 80%) and 72 h post-

transfection MRP1-GFP was readily detected by western blot analysis (Figure 3A, quantification in 3B) as
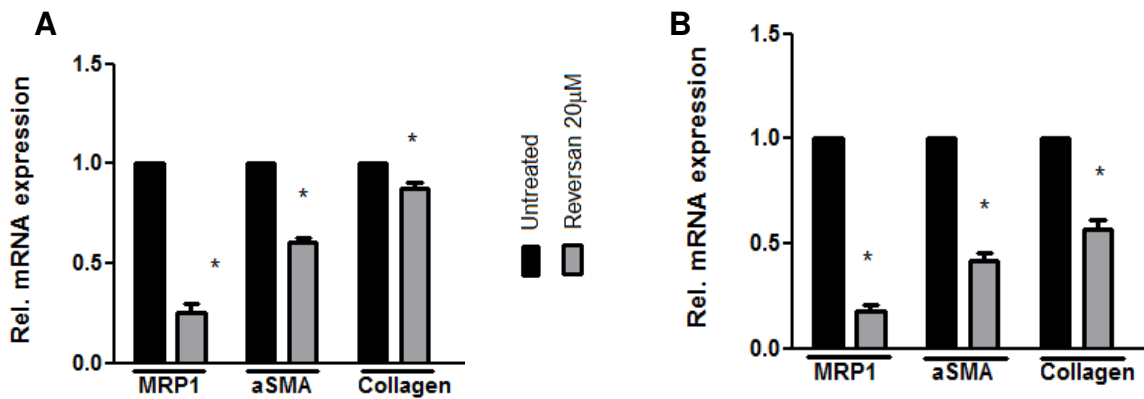
Figure 2. siRNA-mediated inhibition of Mrp1 leads to a decrease in markers of fibrosis in rat HSCs and human LX-2 cells. HSCs were isolated and culture-activated for 7 days. HSCs (A) or LX-2 cells (B) were treated with siRNA-Mrp1 and control cells were treated with siRNA-Luc, followed by determination of the relative mRNA levels of Mrp1, αSma and Collagen1a1 (normalized to 18 S). Data are presented as mean +/- SD of three different experiments. * Significantly different (P< 0.05) vs siRNA-Luc treated cells.
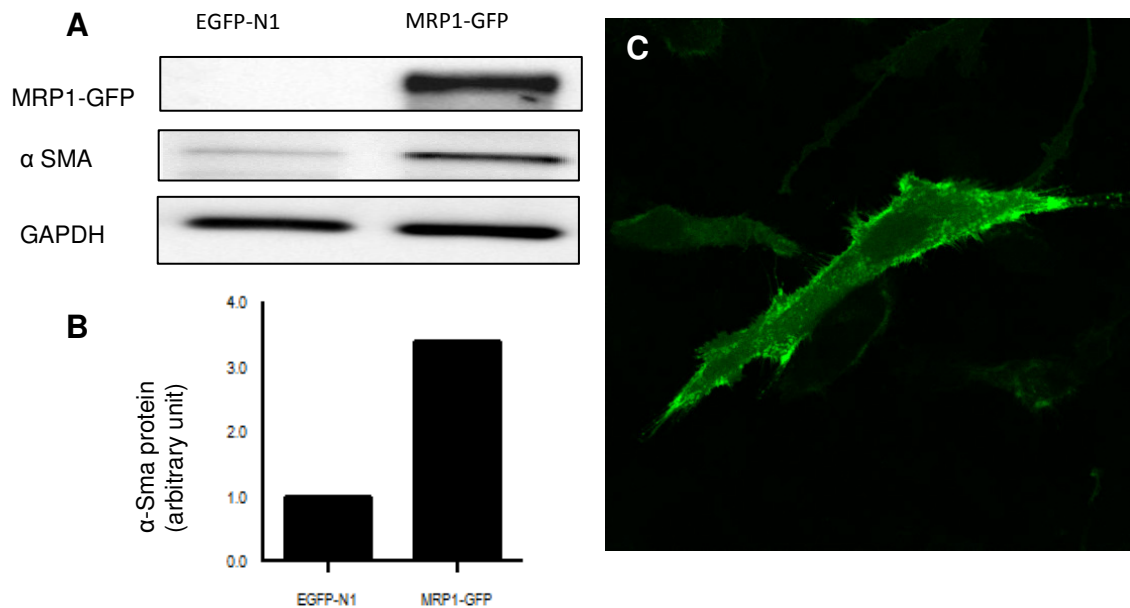
Figure 3. Mrp1 overexpression elevates αSma protein expression. Human LX-2 cells were transiently transfected with an Mrp1-GFP expression plasmid and control cells were treated with EGFP-N1. 72 h post-transfection, cells were analysed by Western blotting (A), and α-Sma protein levels were quantified in (B); GAPDH was used as a loading control) or (C) Fluorescence microscopy. A distinct membrane localization was detected for Mrp1-GFP in transfected LX-2 cell.

well as by fluorescence microscopy. MRP1-GFP staining was predominantly detected at the plasma membrane (Figure 3C). Overexpression of MRP1-GFP resulted in a significant induction of α-Sma expression compared to GFP-transfected control LX-2 cells (Figure 3A and B). Taken together, these data show that Mrp1 controls the activation of HSCs.

Reversan also represses fibrosis markers in portal myofibroblasts

Portal myofibroblasts (PMFs) are increasingly recognized as important contributors to liver fibrosis, together with HSCs[38,39]. Morphologically, PMFs are similar to HSCs, but are devoid of vitamin A-containing lipid droplets [40]. In culture, PMFs are highly proliferative, whereas HSCs senesce after 2-3 cell divisions. Activated primary rat PMFs show a comparable Mrp-expression profile as HSCs with Mrp1 being most dominantly expressed (Supplementary Figure S1 B). We also treated PMFs with reversan to determine whether Mrp1 also controls PMFs activation. Reversan (20 uM) treatment led to the accumulation the fluorescent substrate CMF, indicative of efficient inhibition of Mrp1 activity (Figure 4A). Similar as observed for HSCs, expression of α-SMA and Collagen 1a1 were reduced in reversan-treated PMFs (Figure 4B). These data imply that Mrp1 plays a substantial role in activation of various cell types that contribute to liver fibrosis.
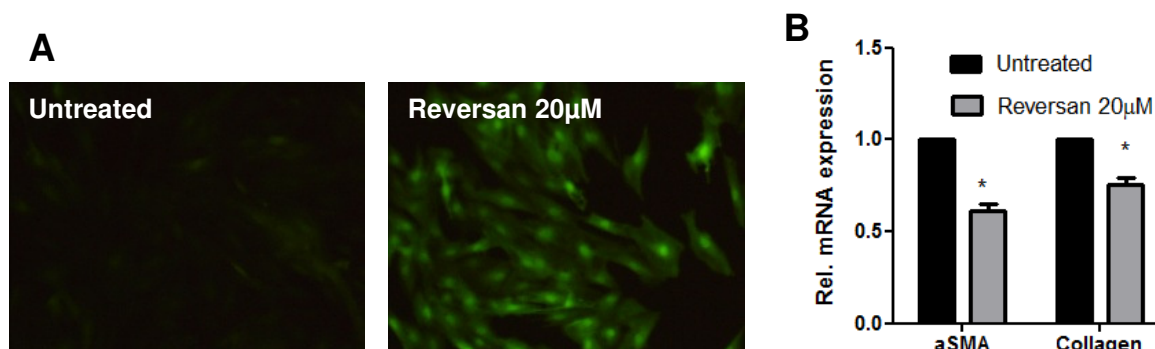
Figure 4. Reversan inhibits the expression of activation markers in primary rat portal myofibroblasts. Primary PMFs were isolated from rat liver and cultured-activated for at least 7 days. Fully-activated PMFs were treated with 20 μM reversan for 24 h and analysed for A) intracellular accumulation of the fluorescent Mrp1 substrate CMFDA as indicator of effective inhibition of Mrp1. B) relative mRNA levels of the activation markers α-SMA and Collagen 1a1 (normalized to 18 S as internal control). Values are mean+/- SD of three different experiments. * Significant difference (P<0.05) from the untreated control.

Leukotriene synthesis and export is a basal characteristic of LX-2 cells

High affinity substrates of Mrp1 are leukotriene C_4 (LTC_4) and glutathione (GSH) [41,42]. Earlier, we showed that GSH depletion did not affect the activation state of HSCs [34]. To establish whether endogenous production of cysteinyl leukotrienes induce HSCs activation, we inhibited LTC_4 synthesis by AA861 and compared its effect to the inhibition of Mrp1 with reversan. Exposure to reversan or AA861 resulted in a similar reduction of mRNA levels of α-Sma and Collagen 1a1, while co-exposure to AA861 and reversan had no added effect, indicating that they block a common pathway (Figure 5). This effect was independent of the GSH levels. GSH depletion (by BSO) and supplementation (by GSH- MEE) did not affect the reversan-mediated reduction in α-Sma and Collagen 1a1 expression (Fig. 5). These data indicate that leukotrienes, and most likely LTC_4, is a crucial Mrp1-transported compound involved in HSCs/PMFs activation.
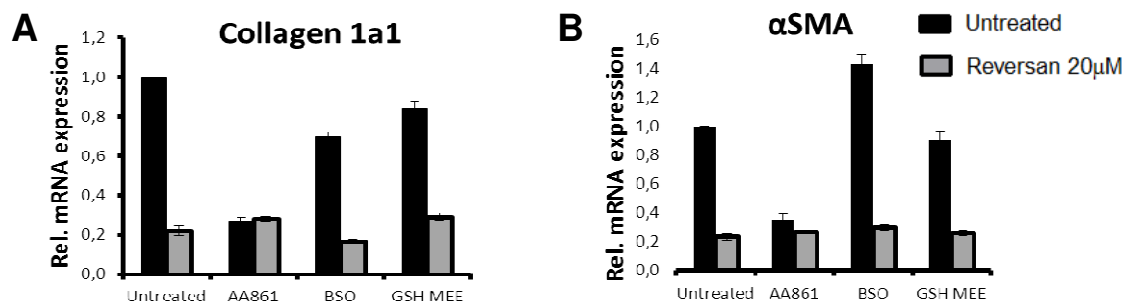
Figure 5. Inhibition of leukotriene synthesis and transport similarly suppress the expression of fibrosis markers in rat HSCs. Fully-activated primary rat HSCs were exposed for 24 h to reversan or AA861 (LO-5 inhibitor, preventing leukotriene synthesis) or both. Moreover, reversan treatment was combined with BSO (GSH depletion) or GSH-MEE (GSH supplementation) to determine the contribution of Mrp1 substrate GSH in the suppression of HSCs activation. Cells were harvested and analysed for relative mRNA levels of Collagen 1a1 (A) and α-Sma (B) and normalized to 18S as internal control. Representative data of 3 independent experiments are shown.

The absence of Mrp1 reduces CCl4-induced fibrosis in mice

To establish the role of Mrp1 in liver fibrosis *in vivo*, wild type and *Abcc1*^{-/-} (Mrp1 knock out) mice were exposed to CCl_4-induced fibrosis. Since the *Abcc1* knock out mouse is only available in the FVB background (which is relatively resistant to CCl_4-induced fibrosis), a 12-week treatment period was chosen. CCl_4 treatment induced hepatic Mrp1 expression in WT mice by 3.5-fold (Figure 6A). Compensatory induction of other hepatic
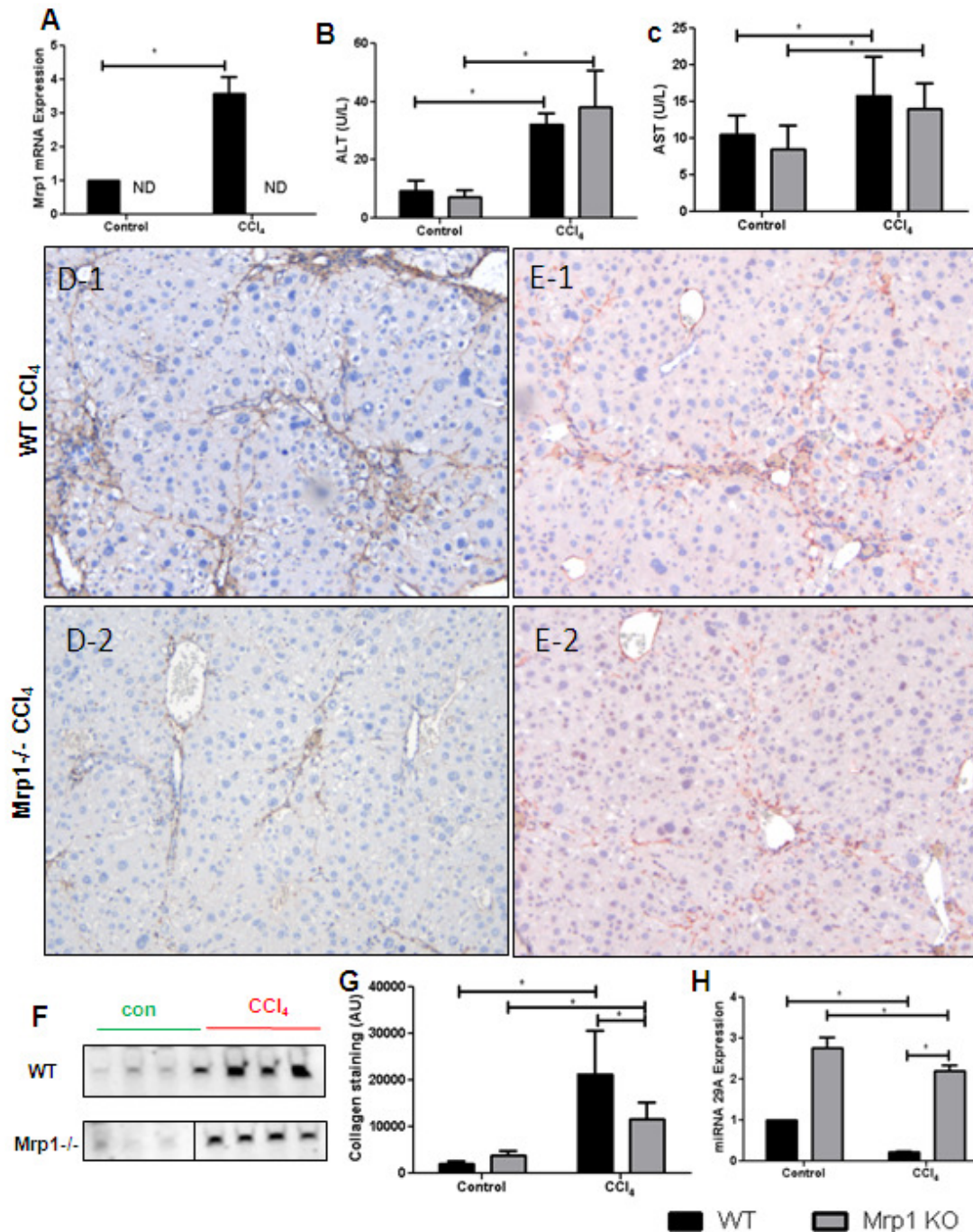
Figure 6. The absence of Mrp1 attenuates CCl₄-induced liver fibrosis in mice. *Abcc1* (Mrp1) [-/-] and WT (FVB) littermates were treated with CCl₄ for 12 weeks, sacrificed and analysed for A) hepatic Mrp1 mRNA expression, B,C) serum markers of liver damage ALT (B) and AST (C). Immunohistochemistry for Collagen1a1 (D) and α-Sma (E) was performed to compare the level of liver fibrosis in CCl₄-treated WT (D-1 and E-1) and *Abcc1*^{-/-} mice (D-2 and E-2). α-Sma protein levels were also analysed by Western blotting (F) and G shows the quantification of the Collagen 1a1 staining. H) shows the relative levels of hepatic miRNA 29A in the 4 experimental groups (normalized to SnoRNA202). Results are represented as mean with +/-SEM. * Significant difference using student t-test (P<0.05).

Mrp's due to the absence of Mrp1 was not observed. Moreover, the relative effect of CCl_4 on the expression of Mrp2-6 and Mdr1a/b was comparable in WT and *Abcc1$^{-/-}$* mice (Supplementary Figure S2). CCl_4 induced an increase in serum ALT and AST levels in WT and *Abcc1$^{-/-}$* mice to a similar extent (Figure 6B and C). As expected, the CCl_4 treatment led to induction of collagen deposition in the CCl_4-treated WT FVB mice (Figure 6D-1, quantification in G), and α-Sma protein compared to untreated control mice (Figure 6E-1 and F, upper blot). Collagen deposition (Figure 6, D-2, quantification in G) and αSma staining (Figure 6, E-2) and protein levels (Figure F, lower panel) were also increased in livers of CCl_4-treated *Abcc1$^{-/-}$* mice, however, to a significant lower level compared to CCl_4-treated WT mice. In addition, levels of miRNA29A, a miRNA that suppresses collagen 1A1 expression [43], are strongly reduced (>80%) in CCl_4-treated FVB WT mice, whereas basal levels in *Abcc1$^{-/-}$* mice are significantly higher (2.5-fold) and were only slightly reduced (-20%) when these animals are treated with CCl_4 (Figure 6H). Taken together, these data show that Mrp1 promotes liver fibrosis *in vivo*.

DISCUSSION

In this study we show that the ABC-transporter Mrp1 (*Abcc1*) is required for the activation of rat hepatic stellate cells, portal myofibroblasts and human LX-2 cells. The absence of Mrp1 in CCl_4-exposed mice represses liver fibrosis. The antifibrotic effect of blocking Mrp1 is independent of glutathione, one of the Mrp1 substrates that is dominantly present in HSCs [7]. Instead, preventing the synthesis of leukotriene C_4, a high-affinity substrate of Mrp1, led to comparable antifibrotic effect on *in vitro*-cultured HSCs and PMFs. These data indicate that Mrp1 plays and direct role in endocrine activation of HSCs/PMFs leading to liver fibrosis. In a previous study, we already proposed that Mrp1 might have a crucial role in HSCs functioning, as the expression of this transporter is increased upon activation of HSCs *in vitro* and treating activated HSCs with the Mrp inhibitor MK571 induced necrotic cell death [7]. The selective killing of activated HSCs could potentially clear the fibrotic liver from activated myofibroblasts and aid in reversal of fibrosis. MK571 is often referred to as a "selective" Mrp inhibitor, but it also inhibits the transport activity of several other Mrps [7,24-26]. Moreover, it also antagonizes the cysteinyl leukotriene receptors 1 and 2 [20,27]. Though the expression of either of the cysLT receptors in HSCs and/or PMFs has not been established yet, it has been shown that LTC_4 promotes liver fibrosis *in vivo* [21]. Moreover, lung myofibroblast

are activated by exogenously added LTC_4 [19,20,44], indirectly proving their presence in fibrogenic cells. Thus, MK571 may act through non-Mrp1 pathways to induce HSCs necrosis.

Reversan is a recently identified inhibitor of Mrp1, which does not inhibit Mrp2-5 [28], suggesting that it may be a more selective inhibitor than MK571. At concentrations that inhibit the transport activity of Mrp1, reversan did not induce significant cell death in activated HSCs and PMFs. Instead, it strongly repressed the expression of collagen 1a1 and α-Sma, the prototypical cellular markers of fibrosis. The different effects of MK571 and reversan suggest that the MK571-induced necrosis of activated HSCs is not due to blocking Mrp1-mediated transport. In line, we found that RNA interference-mediated reduction of Mrp1 expression caused a decrease in collagen1a1 and α-Sma expression, even though only partial reduction of Mrp1 was achieved. Together, these data show that specifically Mrp1 is crucial for the activation HSCs and PMFs, and is not compensated by other Mrp's, also because their expression in HSCs and PMFs is relative low compared to Mrp1. Off-target effects of MK571 cause HSCs necrosis. The expression level of Mrp1 tightly controls the activation state of hepatic myofibroblasts as artificial under- and overexpression directly affects the expression of the fibrosis markers α-Sma and Collagen1a1. This feature makes Mrp1 an interesting target for therapeutic approaches to treat liver fibrosis. Manipulation of cellular glutathione levels did not affect the expression of α-Sma and Collagen 1a1 in activated HSCs and PMFs, implying that this major substrate of Mrp1 does not regulate the activation process. In contrast, blocking leukotriene synthesis resulted in a similar repression of HSCs and PMFs activation as inhibition of Mrp1. This suggests that endogenous production and secretion of LTC_4 by hepatic myofibroblasts contributes to liver fibrosis via an endocrine signaling pathway. This is fully in line with the recent observation that HSCs express the key enzymes involved in LTC_4 synthesis [15]. Several Mrp's are able to transport LTC_4, but Mrp1 has by far the highest transport activity for it [25,41,45,46]. Fibroblasts in other tissues, like lung and skin, also produce LTC_4 [47]. While the presence of the LTC_4 biosynthetic pathway has been established for these fibroblasts, Mrp1 expression has not been established yet in these cell types.

Therapeutic targeting of Mrp1 may seem risky as it is expressed in various tissues, including lung, brain and intestine [48]. Still, whole body absence of Mrp1 does not lead

to a phenotype in mice under controlled laboratory conditions [8]. Only when animals are exposed to specific pro-inflammatory conditions, they may develop more severe symptoms [49]. In case of CCl_4-induced liver fibrosis, the opposite is true. The whole body absence of Mrp1 represses the development of liver fibrosis in mice, while general markers for liver damage are similar to those in WT animals. Thus, it seems feasible to treat liver fibrosis *in vivo*/patients with systemic application of selective Mrp1 inhibitors. As partial inhibition of Mrp1 may already induce reversal of liver fibrosis, pharmacological inhibitors may be used at conservative dosing that do not completely block essential anti-inflammatory functions of Mrp1. Our study indicates that reversan is superior over MK571. In line, reversan did not cause adverse effects in mice in mouse models of neuroblastoma [28].

Remarkably, the absence of Mrp1 was associated with an increase in miRNA29A. This miRNA is an inhibitor of collagen1a1 expression and thus represses fibrosis. Very little is known about the regulation of miRNAs levels including miRNA29A [43]. Our data could unveil a role of Mrp1 substrates, maybe even leukotrienes, in the regulation of miRNA29A.

Taken together, our results show that the ABC transporter Mrp1 promotes hepatic fibrogenesis, both *in vitro* and *in vivo*. Drug-targeted inhibition of Mrp1, for instance by reversan, may hold promise for therapeutic approaches to treat liver fibrosis in the future.

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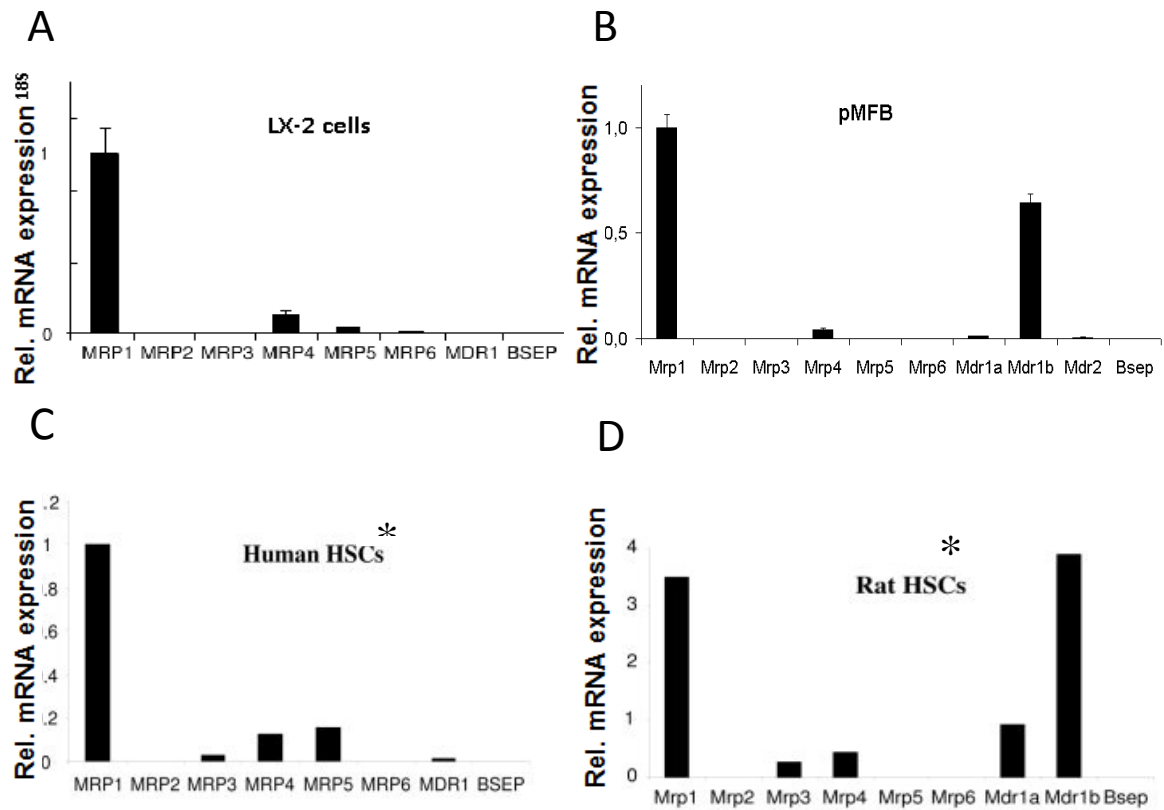
Figure S1. Expression of selected ABC transporters in rat HSCs, rat PMFs and human LX-2 cells. Fully activated primary HSCs and PMFs, as well as human LX-2 cells, were analysed for the relative mRNA levels of Mrp1-6, Mdr1a/b (MDR1 for LX-2), Mdr2 and Bsep (normalized to 18S). *Data for HSC are taken from Hannivoort *et al.*, 2008 and are only shown for comparison. The values are representative of three different experiments.
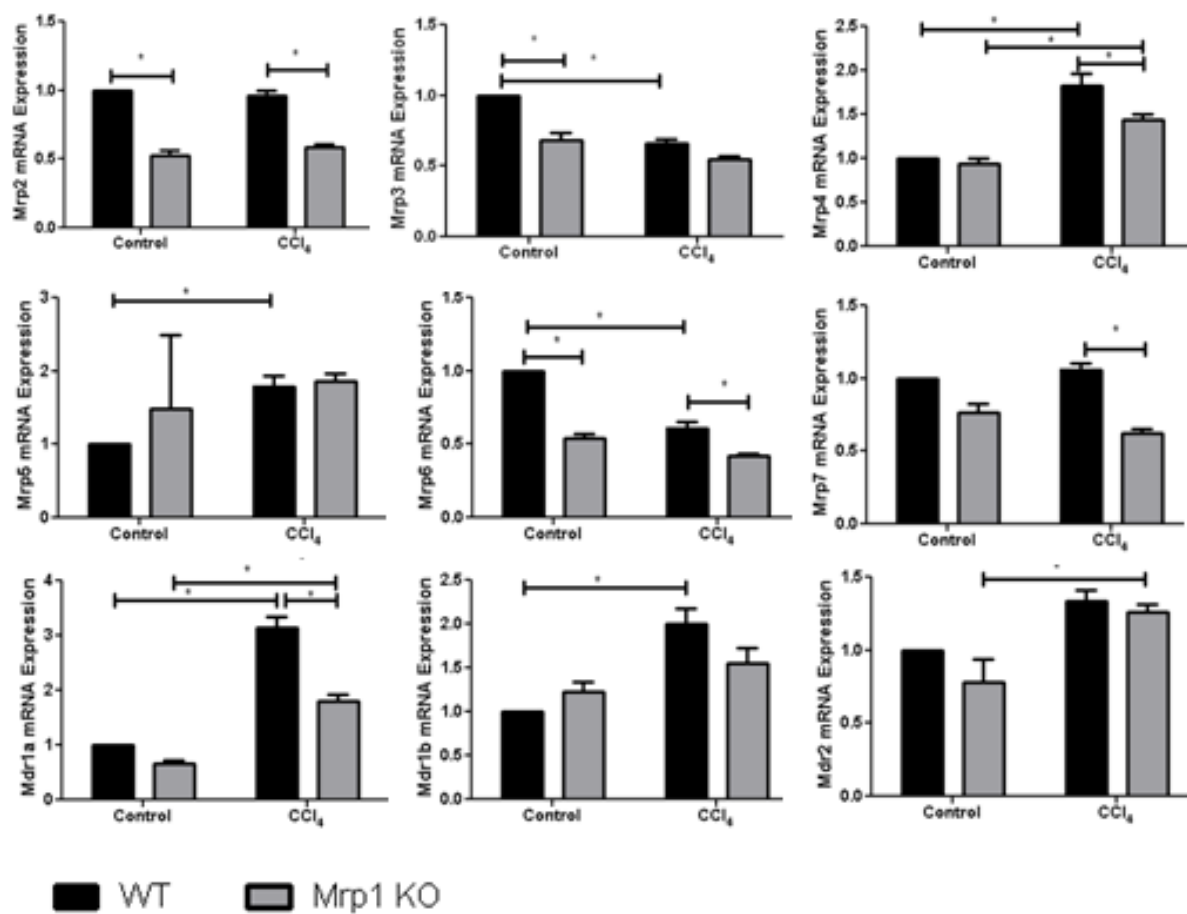
Figure S2. Expression of ABC transporters in whole liver homogenate of *Abcc1* (Mrp1) ⁻/⁻ and wild type after treatment with CCl₄. *Abcc1* (Mrp1) ⁻/⁻ and WT (FVB) littermates were treated with CCl₄ for 12 weeks, sacrificed and analysed for hepatic mRNA expression of Mrp2-7, Mdr1a/b and Mdr2. The expression in the untreated wild type group was set to 1 (18S/36 B4 were used as internal controls). Data represents the mean +/- SEM. * Significant difference using student t-test (P<0.05).

Supplementary Table S1. Sequence of primers and probes used in this study.

	Forward 5'- 3'	Reverse 5'-3'	Probe 5'-3'
18S	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
36B4	GCT TCA TTG TGG GAG CAG ACA	CAT GGT GTT CTT GCC CAT CAG	TCC AAG CAG ATG CAG CAG ATC CGC
Collagen 1a1 (human)	GGC CCA GAA GAA CTG GTA CAT C	CCG CCA TAC TCG AAC TGG AA	CCCCAAGGACAAGAGGCATGTCTG
Alpha Sma (human)	GGG ACG ACA TGG AAA AGA TCT G	CAG GGT GGG ATG CTC TTC A	CACTCTTTCTACAATGAGCTTCGTGTTGCCC
Collagen 1a1 (/mouse/rat)	TGG TGA ACG TGG TGT ACA AGG T	CAG TAT CAC CCT TGG CAC CAT	TCC TGC TGG TCC CCG AGG AAA CA
Alpha Sma (rat)	GCC AGT CGC CAT CAG GAA C	CAC ACC AGA GCT GTG CTG TCT T	CTT CAC ACA TAG CTG GAG CAG CTT CTC GA
Alpha Sma (mouse)	TTCGTGTGGCCCCTGAAG	GGACAGCACAGCCTGAATAGC	TTGAGACCTTCAATGTCCCCGCCA
Mrp1 (mouse)	TGA AAC AGA GAA GGA GGC TCC TT	AGG CAG TAA TCC CGG AAC TCT A	TGG CCC CAT TCA GGC CGT G
Mrp2 (mouse)	GGA TGG TGA CTG TGG GCT GAT	GGC TGT TCT CCC TTC TCA TGG	AGC TGC ATC GTC AGG AAT TTC CTC CAC A
Mrp3 (mouse)	TCC CAC TTT TCG GAG ACA GTA AC	ACT GAG GAC CTT GAA GTC TTG GA	CAC CAG TGT CAT CGG GCT ATG GC
Mrp4 (mouse)	GCC GAC ATC TAC CTC CTT GAT G	CGT GCA ACG CCT GAC AGA	CCC ACT TCT GCA TCG ACA GCG CT
Mrp5 (mouse)	CGG AGA ACA AGA TCG TTG AAT	CAG GGA AAG CCC CTC AAC TC	CCA AGA TGC TCT CGA AAC AGC AGC CC
Mrp6 (mouse)	CCA CAG GAT TGA CAG CAG AAG A	CGC AGG TAG CTC AGG TAT ATG GT	TCT TCA CCC GGC CAT ATC GCA CAC
Mrp7 (mouse)	GGG GCC ACT TAC AGG TTT GA	ATC GTG GCA TAG GAA GCA AAC T	AAC CAG CGA CTC TTG GAG CTG AAC CA
Mdr1a (mouse)	GCA GGT TGG CTA GAC AGG TTG T	GAG CGC CAC TCC ATG GAT AA	AGC AGC CAG AGT TCC ACC AGC ATG
Mdr1b (mouse)	GCT GGA CAA GCT GTG CAT GA	TGG CAG AAT ACT GGC TTC TGC T	CTT CCC CTC TTG ATG CTG GTG TTT GGA AAC
Mdr2 (mouse)	GCA GCG AGA AAC GGA ACA G	GGT TGC TGA TGC TGC CTA GTT	AAA GTC GCC GTC TAG GCG CCG T

Supplementary Table S2. Antibody dilutions for protein analysis in this study.

Antibody	Western Blot	Immunofluorescence	Company
Mouse α Alpha -SMA	1:2000	1:500	Sigma, St. Louis, MO, USA
Goat α Collagen type 1	1:2000	1:500	Southern Biotech Birmingham, AL 35260, USA
Rat α Mrp1	1:2000		SIGNETTM, Dedham, MA, USA
Mouse α GAPDH	1:10000		Calbiochem, La Jolla, CA, USA

In vitro analysis of the MRP1 inhibitors MK571 and reversan identifies reversan as potentially safest drug to treat liver fibrosis

Atta ur Rehman, Han Moshage and Klaas Nico Faber

Department of Gastroenterology and Hepatology
University Medical Center Groningen
University of Groningen

ABSTRACT

Introduction: Liver fibrosis is caused by chronic liver injury. It is associated with oxidative stress leading to hepatocyte death and activation of cells that produce the collagenous scar tissue, including hepatic stellate cells (HSCs) and portal myofibroblasts (PMFs). HSCs and PMFs express the multidrug resistance-associated protein 1 (Mrp1), which is essential for HSCs/PMF activation. MK571 and reversan are pharmacological inhibitors of Mrp's. MK571 is toxic for activated HSCs, while reversan suppresses HSCs activation without inducing cell death. Thus, liver fibrosis may be treated by pharmacological inhibition of Mrp1, but should avoid adverse effects on hepatocytes that express Mrp2-4 and Mrp6. Here, we analyzed the cytotoxicity of MK571 and reversan on HSCs, PMFs and hepatocytes exposed to oxidative stress.

Material and Methods: Primary rat HSCs, PMFs and hepatocytes were (co-)exposed to non-toxic concentrations of the superoxide anion donor menadione, MK571/Reversan and glutathione-monoethyl esther (GSH-MEE). The ratio oxidized-GSSG: reduced-GSH was determined and cell viability was analyzed by Sytox green nuclear staining and LDH leakage. Inhibition of MRP-mediated transport was studied using 5-chloromethylfluorescein diacetate (CMFDA) and cholyl-lysyl-fluorescein (CLF).

Results: Co-treatment of HSCs/PMFs with reversan and menadione led to cellular accumulation of oxidized GSSG and induced necrosis, which was prevented by GSH-MEE. Reversan with or without menadione, did not induce necrosis in hepatocytes. In contrast, MK571 induced hepatocyte necrosis that was further enhanced by co-exposure with menadione, which led to the cellular accumulation of GSSG, and was suppressed by GSH-MEE. MK571 inhibited the export of CMFDA and CLF from hepatocytes, while reversan did not.

Conclusion: MK571 blocks vital MRP-mediated export of oxidized glutathione from hepatocytes. Reversan did not affect hepatocyte viability, but only reduced HSCs/PMFs viability under oxidative stress conditions. Reversan may thus be applicable as drug to treat liver fibrosis, with less chance of adverse effects compared to MK571.

INTRODUCTION

Liver injury is followed by a wound-healing process, where hepatic myofibroblasts migrate to the site of tissue damage and produce new extracellular matrix (ECM) to promote liver regeneration [1,2]. Upon repair, the ECM is remodeled and the scar tissue is resolved [3-5]. However, in chronic liver disease the hepatic myofibroblasts produce excessive ECM leading to fibrosis, which may progress to cirrhosis where the liver architecture is irreversibly disrupted and can cause liver failure [6,7]. Moreover, cirrhosis predisposes for the development of liver cancer. Cytokines and growth factors induce the activation of hepatic stellate cells (HSCs) that transdifferentiate into hepatic myofibroblasts and become mobile, proliferative and secrete excessive amounts of ECM [8]. Hepatic myofibroblasts may also originate from the portal areas (PMFs), bone marrow, and endothelial cells through epithelial-mesenchym transition (EMT) [9-13]. The hepatic myofibroblasts survive and proliferate under pathological conditions where the functional liver cells (hepatocytes and cholangiocytes) are prone to apoptotic or necrotic death [6]. A variety of factors have been shown to stimulate myofibroblasts activation and/or transdifferentiation, including transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF), various cytokines and reactive oxygen species [6,8]. No therapeutic drugs to treat liver fibrosis are available yet.

The Multidrug Resistance-associated Protein 1 (MRP1) is an ATP Binding Cassette (ABC)-transporter which is well-known for its adverse effect in cancer therapy [14,15]. It transports a wide array of cytostatic drugs. Therefore cancer cells expressing MRP1 are resistant against such drugs [15,16]. Mrp1 also has an important role in normal cells. Hepatic expression of MRP1 is low, but is induced upon CCL_4-induced liver injury in rats [17] and mice (Rehman *et al.,* chapter 2). Mrp1 expression is predominantly found in myofibroblasts with very low levels in hepatocytes [17]. Also, MRP1 expression is strongly induced upon activation of HSCs *in vitro* [17]. Both pharmacological inhibition of Mrp1 (using reversan) and siRNA-mediated silencing of Mrp1 reduced the expression of activation markers, like collagen1a1 and alpha-smooth muscle actin (α-Sma), in HSCs and PMFs *in vitro* (Rehman *et al.*, Chapter 2). Moreover, CCl_4-treated *Abcc1*(Mrp1)$^{-/-}$ mice showed significantly lower amounts of collagen deposition compared to CCL_4-treated wild type mice, while serum transaminases (AST and ALT) were similar in both groups (Rehman *et al.,* Chapter 2).

Thus, whole body absence of Mrp1 represses the development of liver fibrosis, while parenchymal liver damage is not aggravated. These finding suggest that pharmacological inhibition of Mrp1 may by feasible to treat liver fibrosis, without major adverse effects. Several pharmacological inhibitors of Mrp1 activity are available, including MK571 and the more recently described reversan [14,18]. MK571 inhibits not only Mrp1, but also other close homologs like Mrp2, Mrp3 and Mrp4 [19,20]. Moreover, it is also known as an antagonist of the leukotriene D_4 receptor [21]. Reversan seems a more selective inhibitor of Mrp1 as it does not inhibit Mrp2, Mrp3, Mrp4 and Mrp5 in overexpressing MCDK cells [14]. Both inhibitors have been given to laboratory animals without causing major complications [14,22]. However, the potential adverse effects have not been studied with respect to the pathological conditions associated with liver disease.

Therefore, we studied the cytotoxic effects of MK571 and reversan on rat hepatocytes, HSCs and PMFs in the presence and absence of oxidative stress. We show that MK571 induces necrotic cell death in hepatocytes, especially when they are exposed to oxidative stress. In contrast, hepatocytes were unaffected by reversan even in the presence of menadione. Both inhibitors induced necrosis in menadione-treated HSCs and PMF. Our *in vitro* data support the notion that MK571 will potentially aggravate liver damage when used as drug therapy to treat liver fibrosis *in vivo*. In contrast, Reversan targets HSCs/PMFs and is tolerated by hepatocytes, which is an important prerequisite for further testing of this drug for the treatment of liver fibrosis *in vivo*.

MATERIALS AND METHODS

Animals: Specified pathogen-free male Wistar rats (220–250 g for hepatocytes and PMFs isolations and 400-500 g for HSCs isolations) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local committee for care and use of laboratory animals.

Isolation and culture of rat hepatocytes, hepatic stellate cells and portal myofibroblasts:
Primary rat hepatocytes [23] HSCs [24] and PMF [25] were isolated and cultured as described previously. Hepatocyte viability and purity were always more than 90%. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Experimental Design: Freshly-isolated primary hepatocytes, HSCs or PMFs were seeded in multi-well plastic culture plates (Greiner bio-one) and incubated overnight for attachment. HSCs and PMFs were treated with reversan (20µM; Tocris Bioscience, Bristol UK), while primary hepatocytes were treated with MK571 (50µM; cat No. RA-109; Enzo life sciences) and reversan alone or in combination with 20 µM menadione (Sigma). Cells were harvested after 18 to 20 hours treatment and processed for analysis. For glutathione supplementation, cells were loaded with 500 µM Glutathione monoethyl ester (GSH-MEE, Calbiochem) simultaneously with other treatments as indicated above. Each experimental condition was performed in duplicate and each experiment was repeated at least three time using cells from different isolations.

Quantification of necrosis: Cell necrosis was determined by Sytox green nuclear staining performed as described before [17] and by quantifying the relative amounts of LDH released to the culture medium according to procedure described before [26] with minor changes. In short, 20 to 50 µl of medium was taken in transparent 96-well flat bottom plates (Greiner bio-one). LDH buffer (0.08 M Tris and 0.2 M NaCl in distilled water adjusted to pH 7.0) was added to make up the volume to 100 µl followed by addition 100 µl of 3.24 mM pyruvate (Sigma) dissolved in LDH buffer and 100 µl of 0.68 mM NADH (sigma) dissolved in LDH buffer. The plate was scanned for absorption of 340 nm at 37^oC for 30 min at intervals of 1 min. The linear part of the kinetic curve was selected to determine the rate of disappearance of NADH, which is directly related to the LDH activity in the sample. The absolute units of LDH were then calculated from the standard curve of known concentration of LDH (Roche Diagnostics, Germany). For the total amount of LDH per well, cells were lysed in the cell lyses buffer containing HEPES 25 mM, $MgCl_2$ 5 mM, EDTA 5 mM, PMSF 2 mM, Pepstatine 10 ng/µl and Leupeptine 10 ng/µl and adjusted to pH 7. After three cycles of snap freezing and thawing, cell were centrifuged to pellet the cell debris. Cell necrosis was presented as a percent of total (medium and cellular) LDH released in the medium.

Quantification of reduced glutathione (GSH) and oxidized glutathione (GSSG): GSH and GSSG were quantified as described before [27] with minor modifications. Briefly, 20 to 50 µl of the supernatant (prepared as mentioned for LDH assay) was added to buffer A (125 mM $NaH_2PO_4.H_2O$ and 6.3 mM NaEDTA adjusted to pH 7.5 with NaOH) to a total volume of 100 µl in a transparent flat bottom 96-well plate. Next, 20 µl of 6 mM 5-5'-

dithiobis-2-nitrobenate (Sigma) dissolved in buffer A, 42 µl of 0.3 mM NADPH (Roche Diagnostics, Germany) dissolved in buffer A and 38 µl of glutathione reductase (Roche Diagnostics) dissolved to an enzyme activity of 5 units/ml in Buffer A. In order to measure GSSG, vinyl pyridine (Sigma) was added to the supernatant to block the reduced GSH. Cell lysates were always kept on ice and used for GSH and GSSG analysis directly. A curve of standard concentrations GSH and GSSG was used to quantify the cellular concentrations.

Intracellular accumulation of CMFDA and CLF: Intracellular accumulation of 5-chloromethylfluorescein diacetate (CMFDA) and cholyl-lysyl-fluorescein (CLF) was determined as described previously (Rehman *et al*., Chapter 2) with minor modification. Briefly, primary hepatocytes were incubated for 10 min with 50 µM MK571 or 20 µM reversan, after which they were loaded with 5 nM CMFDA (Invitrogen, Molecular probes, Oregon ,USA) or 2 nM CLF -a fluorescent bile salt [28]- and incubated for another 30 minutes. Medium was replaced by fresh medium that contained Mrp inhibitors and live cells were immediately analyzed for intra cellular fluorescence using an Olympus CKX41or Lieca DMI6000B microscope.

Statistical analysis: Statistical analysis was performed using Prism5 software. Results are expressed as the mean value with standard deviation. Statistical significance was calculated using a Student's t-test, or an one-way ANOVA with Bonferroni post-hoc analysis for correction for multiple comparisons. A corrected p value <0.05 was considered to be statistically significant.

RESULTS

Reversan induces necrosis in menadione-treated HSCs

As a model of oxidative stress-induced liver damage, we exposed cultured rat HSCs, PMFs and hepatocytes to menadione. Earlier, we showed that MK571 induces necrosis in HSCs even under unstimulated conditions [17], but this is unrelated to the inhibition of Mrp1 (Rehman *et al*., Chapter 2). In contrast, reversan suppresses the activation of HSCs and PMFs, while it did not induce necrosis in culture-activated HSCs within a time frame of 18 to 24 hours (Rehman *et al*., Chapter 2) and (Figure 1A). Co-treatment of primary rat HSCs with menadione and reversan led to significant detachment of the HSCs and increased the number of sytox green-positive cells (Figure 1A). While LDH release was

not increased in cells treated only with menadione or reversan, co-treatment led to a significant release of LDH to the medium (Figure 1B). Similar results were obtained for culture-activated PMFs (Supplementary Figure S1A and B). Previously, we showed that reversan reduces the expression of the activation markers Collagen 1a1 and α–SMA in culture-activated HSCs and PMFs (Rehman *et al*., Chapter 2). The new data imply that pharmacological inhibition of Mrp1 in a fibrotic liver, which is likely exposed to oxidative stress, also induces necrosis of HSCs and PMFs.
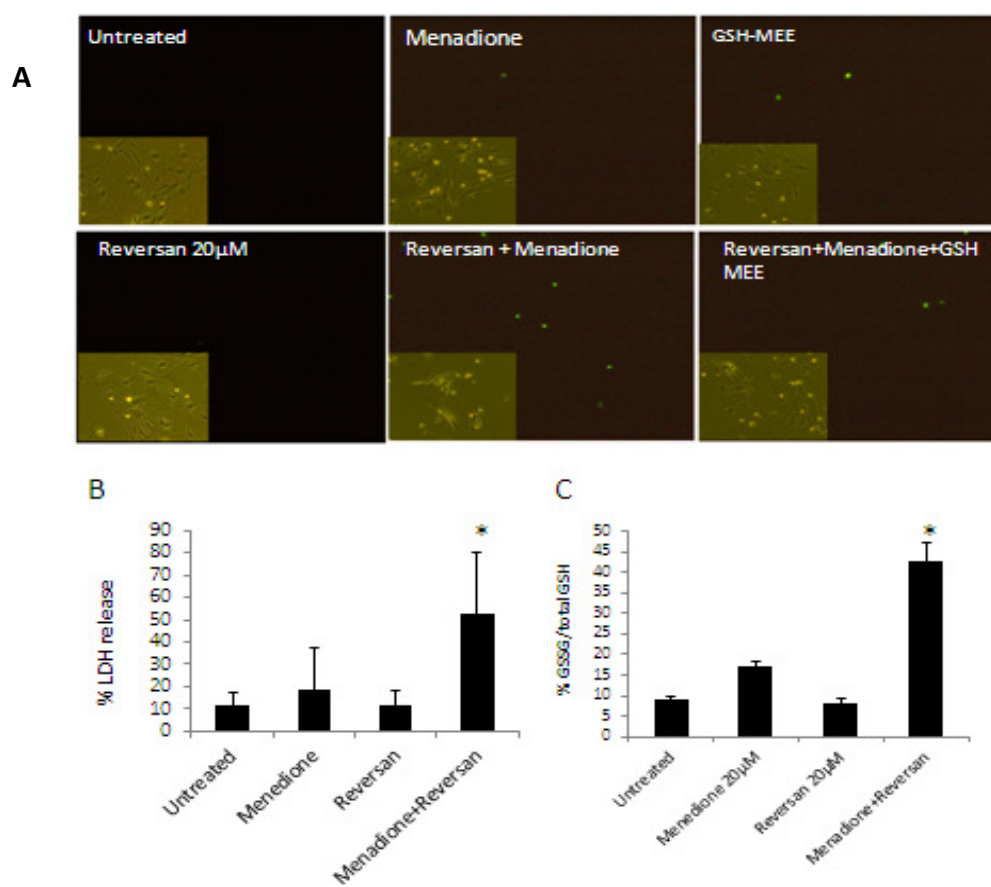
Figure 1. Co-treatment with reversan and menadione induces HSCs necrosis and the effect is reversible by GSH-MEE supplementation. Freshly–isolated HSCs were culture-activated for 7 days and, subsequently, treated for 18 to 24 hours with reversan (20 μM) with or without menadione (20μM) and with or without GSH-MEE (0.5 mM). A) Sytox green-positive nuclei identify necrotic HSC under the indicated conditions. Insets show the corresponding bright field images. (B) LDH activity was measured in total HSC protein extracts and the culture medium and the relative amount of LDH in the medium is given as a measure of necrosis. (C) Total glutathione and oxidized glutathione (GSSG) were quantified in total HSC extracts and the relative amount of GSSG over total-GSH is presented. * significantly different (p<0.001) from untreated and reversan treated cells.

MK571, but not reversan, induces necrosis in menadione-treated hepatocytes

MK571 is a pan-MRP inhibitor, whereas reversan is supposedly a more specific inhibitor of Mrp1 [14]. Hepatocytes constitute the functional liver tissue and a pharmaceutical therapy for liver fibrosis should prevent damage to these cells. Thus, we analyzed the toxic effects of MK571 and reversan on cultured rat hepatocytes in the
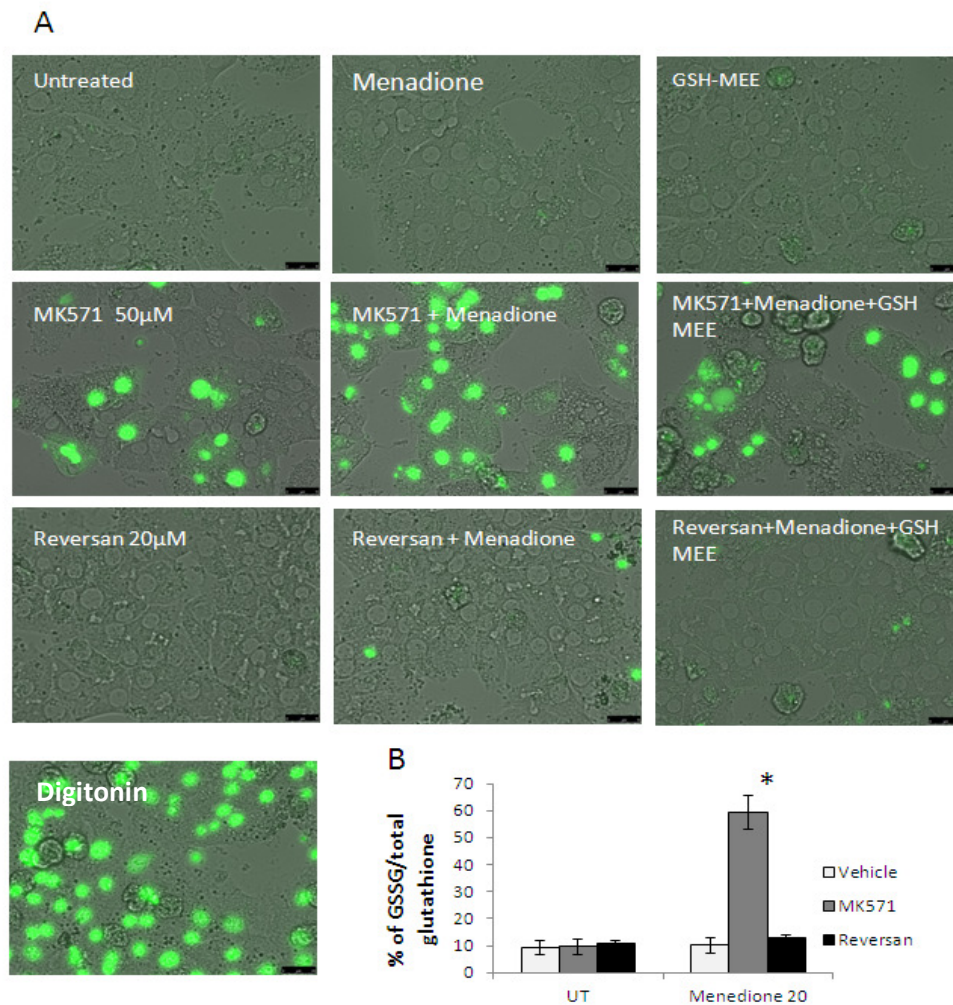
Figure 2. Differential effects of the Mrp1 inhibitors MK571 and Reversan on the viability primary rat hepatocytes exposed to menadione. Primary rat hepatocytes were treated for 18 to 24 hours with MK571 (50µM) or Reversan (20 µM) with or without menadione (20 µM) and with or without GSH-MEE (0.5 mM). A) Sytox green-positive nuclei identify necrotic hepatocytes under the indicated conditions. The green fluorescent signal is combined with the bright field images that reveal the presence of cells. Hepatocytes treated with digitonin are shown as positive control of necrosis. The pictures are representative of three independent experiments. (B) Total glutathione and oxidized glutathione (GSSG) were quantified in total hepatocyte extracts and the relative amount of GSSG over total-GSH is presented. * Significantly different (p<0.001) from the all other treatments.

absence and presence of oxidative stress. An 18 to 24 h exposure to 50 μM MK571 induced significant necrosis in rat hepatocytes, even in the absence of oxidative stress (Figure 2A). When co-treated with 20 μM menadione, almost all hepatocytes showed Sytox green-positive nuclei (Figure 2A), indicating massive necrosis under these conditions. In contrast, almost no necrosis was detected when hepatocytes were exposed to menadione alone. Moreover, co-treatment with 20 μM reversan did not lead to a significant increase in sytox green-positive nuclei in rat hepatocytes, nor in the absence or the presence of menadione (Figure 2A).

Differential effects of MK571 and reversan on the accumulation of oxidized glutathione in menadione-treated hepatocytes and HSCs

Reduced and oxidized glutathione (GSH and GSSG, respectively) are natural substrates for Mrp1 and Mrp2. Activated HSCs/PMFs typically express Mrp1, while rat hepatocytes express Mrp2. We analyzed the intracellular cellular % of GSSG versus total GSH of menadione-treated hepatocytes to determine whether blocking Mrp1/2 transporter function leads to the accumulation of GSSG (Figure 2B). Approximately 10% of the total glutathione pool exists in the oxidized (GSSG) form in cultured rat hepatocytes and HSCs. Treatment with MK571 or reversan alone did not increase the relative amount of GSSG. Also the treatment with menadione alone did not change the relative cellular amounts of GSSG versus total glutathione. However, when hepatocytes were co-treated with menadione and MK571, the relative amount of GSSG was dramatically increased amounting more than half of the total glutathione pool. Co-treatment with menadione and reversan did not cause a significant shift in the intracellular GSSG versus GSH in rat hepatocytes. In contrast, these conditions (menadione+reversan) led to an increase in GSSG levels in menadione-treated HSCs and PMFs (Figure 1C and Figure S1-C).

Glutathione supplementation reduces menadione-induced cell death when Mrp activity is blocked

We hypothesize that the intracellular accumulation of GSSG disturbs the intracellular redox potential following oxidative stress and reduced the viability of the cells. To examine this, we supplemented menadione/MK571- and menadione/reversan-treated cells with membrane-permeable glutathione mono ethyl ester (GSH-MEE) and

found that it largely prevented necrotic cell death of HSCs (Figure. 1A), hepatocytes (Figure 2A) and PMFs (Figure S1A) that were exposed to menadione and Mrp inhibitors. This direct comparison of MK571 and reversan shows that the latter is much less toxic towards hepatocytes under oxidative stress.

MK571, but not Reversan, blocks the export of CMFDA and CLF from hepatocytes

To obtain independent evidence for the higher selectivity of reversan towards Mrp1, we analyzed the accumulation of fluorescent substrates for Mrp1 and Mrp2 in rat hepatocytes exposed to either MK571 or reversan. Earlier, we showed that both MK571 and reversan block the export of the fluorescent substrate 5-chloro-methyl fluorescein diacetate (CMFDA) from activated HSCs and PMFs (Rehman *et al*., Chapter 2). CMFDA is transported by several Mrp transporters, including Mrp1-3 and 5. Since Mrp1 is the most prominently expressed MRP-type transporter in HSCs and PMFs, we related the intracellular accumulation of CMFDA to inhibition of this transporter. To confirm that reversan is truly selective for Mrp1, we exposed primary hepatocytes, which lack Mrp1 and express Mrp2, 3 and 4, to CMFDA in combination with MK571 or reversan (Fig. 3). Untreated hepatocytes hardly accumulate CMFDA indicative of efficient efflux of this substrate from these cells. MK571 treatment led to a strong accumulation of fluorescent CMFDA in primary rat hepatocytes, whereas reversan-treated hepatocytes showed very little amount of CMFDA-related fluorescence (Fig. 3). Notably, no significant amounts of CMFDA were detected in canalicular vacuoles that are formed between hepatocytes, indicating that CMFDA is predominantly transported by Mrp's present on the basolateral membrane, such as Mrp3 and/or 4. Recently, it was shown that the fluorescent bile acid cholyl-L-lysyl-fluorescein (CLF) is a high affinity substrate for Mrp2 (and not of the bile salt export pump-BSEP) [28,29]. In untreated cells, CLF accumulated in the canalicular region between adjacent hepatocytes. MK571 largely prevented the canalicular accumulation of CLF and led to a significant increase in the cytosolic accumulation of CLF. In contrast, reversan did not prevent the canalicular accumulation of CLF and only a minor increase in intracellular CLF accumulation was observed (Fig. 4). These results confirm that reversan does not inhibit the activity of canalicular Mrp2 and basolateral Mrp3/4.
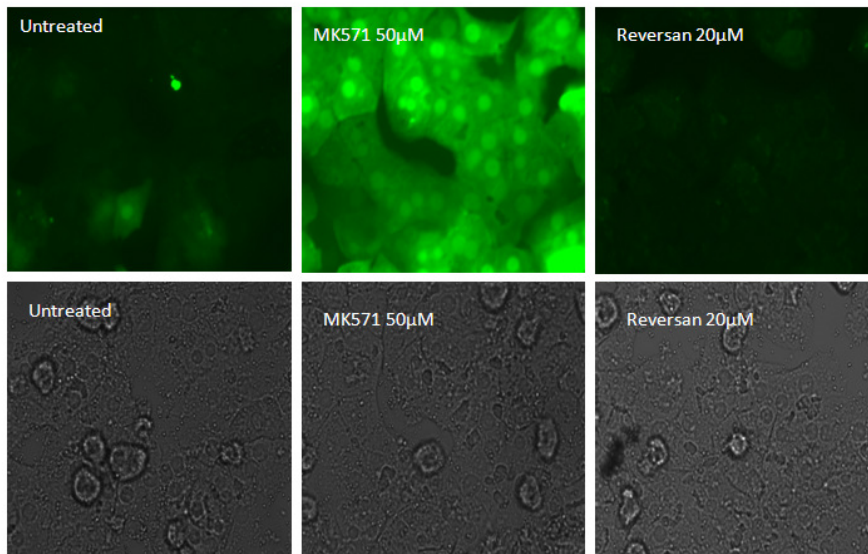
Figure 3. MK571 leads to the intracellular accumulation of CMFDA in primary rat hepatocytes, while reversan does not. (A) Primary rat hepatocytes were treated with MK571 (50μM) or Reversan (20 μM) for 10 minutes after which the cells were loaded with 2 μM CMFDA (fluorescent Mrp substrate) and incubated for an additional 30 minutes. Fluorescent images (top row) were taken using a Leica DMI6000B microscope. The bottom row shows the corresponding bright filed images. Each experiment was performed in duplicate. The pictures are representative of three independent experiments.
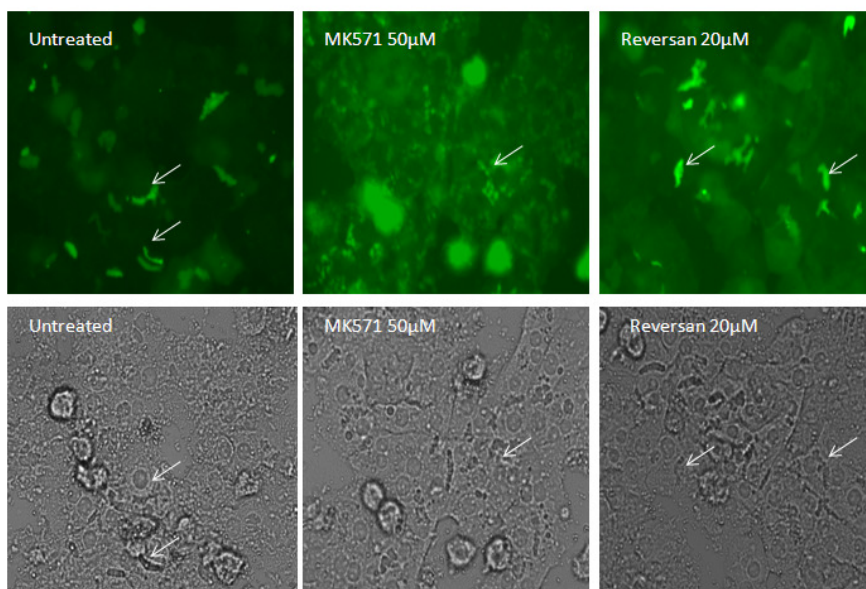
Figure 4. MK571 prevents hapatocanalicular transport of CLF, which accumulates intracellular while reversan does not. (A) Primary rat hepatocytes were treated with MK571 (50μM) or Reversan (20 μM) for 10 minutes after which the cells were loaded with 2 μM CLF (fluorescent Mrp2 substrate) and incubated for an additional 30 minutes. Fluorescent images (top row) were taken using a Leica DMI6000B microscope. The bottom row shows the corresponding bright filed images. Arrows point to canalicular vacuoles formed between hepatocytes. Each experiment was performed in duplicate. The pictures are representative of three independent experiments.

DISCUSSION

In this study, we show that the Mrp1 inhibitor reversan has no cytotoxic effect on menadione-exposed hepatocytes. This treatment induces necrosis in HSCs and PMFs that may enhance the anti-fibrotic action of reversan. In contrast, the pan-MRP inhibitor MK571 alone induces necrosis in hepatocytes, which is further enhanced by co-exposure to menadione. We show that reversan allows efficient efflux of endogenous (GSSG) and fluorescent MRP substrates from hepatocytes, while this is blocked by MK571. Collectively, these *in vitro* data indicate that MK571 is likely to aggravate oxidative stress-associated liver damage *in vivo*, while reversan may prevent adverse effects and specifically target fibrosis.

Previously, we found that reversan effectively blocked MRP1-mediated transport in HSCs and PMFs and reversed the activation process, as indicated by suppression of Collagen 1a1 and α-Sma expression (Rehman *et al*., chapter 2, submitted). Similar results were obtained with Mrp1-specific RNA interference *in vitro* and CCl$_4$-induced fibrosis was reduced in *Abcc1$^{-/-}$* (Mrp1) mice (Rehman *et al*., Chapter 2). Thus, Mrp1 is a potential target for the treatment of liver fibrosis. This observation paves the way for the use of pharmacological inhibitors of Mrp1 in the treatment of liver fibrosis, but requires a detailed analysis of potential adverse effects of such compounds, both *in vitro* and *in vivo*. Systemic application of Mrp1 inhibitors will block its function in all tissues. Mrp1 is ubiquitously expressed throughout the body, with relatively high expression levels in heart, lung, brain kidney and lymphocytes and minor amounts in the liver [30,31]. However, inflammation induces the expression of Mrp1, amongst others in the liver and intestine [32-34]. Mrp1 is a high-affinity transporter of the inflammatory signaling molecule leukotriene C$_4$ and, as a consequence, blocking or absence of Mrp1 reduces inflammation [35]. On the other hand, absence of Mrp1 may enhance tissue damage under inflammatory conditions as it aggravates dextran sodium sulfate (DSS)-induced colitis in mice [36], which is most likely the result of a cytoprotective role of Mrp1 in the intestinal epithelium [37]. Mrp1 also prevents passage of a variety of drugs through the blood-brain barrier and has been implicated in the clearance of neurotoxic lipid peroxidation products that characteristically accumulate in the brain and augments the onset of Alzheimer`s disease [38]. *Abcc1$^{-/-}$* mice that lack Mrp1 do not have an evident phenotype though they are hyper sensitive to a variety of anticancer drugs [35,39].

Abcc1^{-/-} mice developed significantly less liver fibrosis upon CCL₄ treatment compared to WT mice, while no adverse effects were observed (Rehman *et al.,* chapter 2). These data indicate that systemic inhibition of Mrp1 by drugs may be feasible as long it is not combined with other drugs that are Mrp1-substrates. Alternatively, inhibitors of Mrp1 may be targeted specifically to hepatic myofibroblasts in order to prevent extra-hepatic adverse effects [40,41].

It is crucial that the inhibition of Mrp1 is highly specific. In a comparative analysis of MK571 and reversan-induced toxic effects, we found that reversan did not induce significant necrosis or apoptosis in HSCs, however, reversan treatment sensitized the HSCs and PMFs in the presence of menadione and induced necrosis. Interestingly, reversan did not induced necrosis in primary hepatocytes in the absence or presence of menadione at same doses that were toxic for HSCs. Earlier, we showed that MK571 induces necrosis in HSCs [17]. While this may attenuate fibrosis, we show in this study that MK571 also induced necrosis in hepatocytes, especially in combination with menadione. These observations can be explained in the light of the specificity of MK571 and reversan, in combination with the specific Mrp-expression profile of HSCs and hepatocytes. MK571 inhibits the transport activity of various Mrp's, including Mrp1-5 [19,20,42], We confirm in this chapter that reversan [14] is a more specific inhibitor of Mrp1. Mrp1 is predominantly expressed in HSCs and PMFs, while Mrp2-4 and 6 are expressed in (cultured) hepatocytes. MK571 blocks the vital export of oxidized GSSG from menadione-exposed hepatocytes leading to necrotic cell death. Reversan did not change the cellular GSSG:GSH ratio in menadione-exposed hepatocytes, but did so in HSCs and PMFs. While the MK571 effect is likely to aggravate oxidative stress-inflicted liver damage, the antifibrotic action of reversan may actually be enhanced since it is selectively targeted the myofibroblasts. These results are in accordance with the earlier findings by Takahashi *et al.,* 2009 [43], who showed that Mrp1 inhibition enhanced menadione-induced necrosis in endothelial cells. In our study we confirmed that the induced sensitivity of the stressed cells is dependent upon glutathione status as it is reversed by the supplementation with glutathione (GSH-MEE), thus, strengthening our hypothesis that GSSG export is of vital importance for hepatocytes, HSCs and PMFs.

In the past, MK571 was selected as a candidate drug for the treatment of asthma due to its inhibitory effect on LTD₄ receptor. However, it was discontinued from clinical

trials at phase 2 due to the suspicion of inducing liver cancer [44,45]. Our result obtained with hepatocytes may explain this observation as prolonged use of MK571 my cause persistent damage to hepatocytes due to inhibition of MRPs present in this cell type.

In conclusion, our data show that reversan selectively targets fibrogenic cells in the liver, without adverse effects on hepatocytes. Further experiments need to show whether this inhibitor of Mrp1 can indeed repress liver fibrosis *in vivo* and is safer than MK571, which is toxic for hepatocytes.

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Supplementary Data
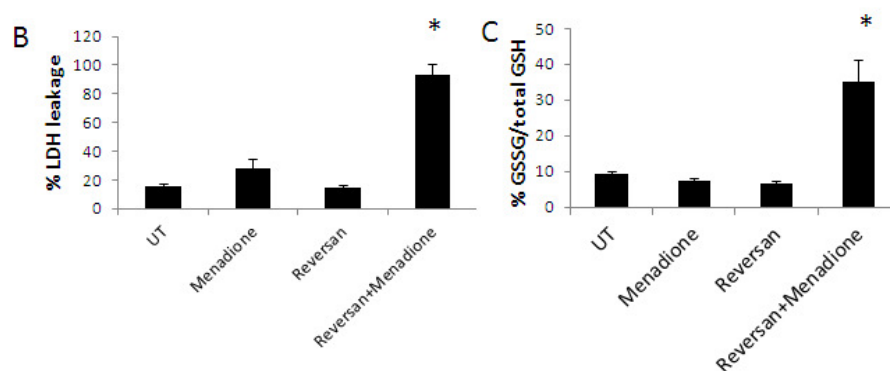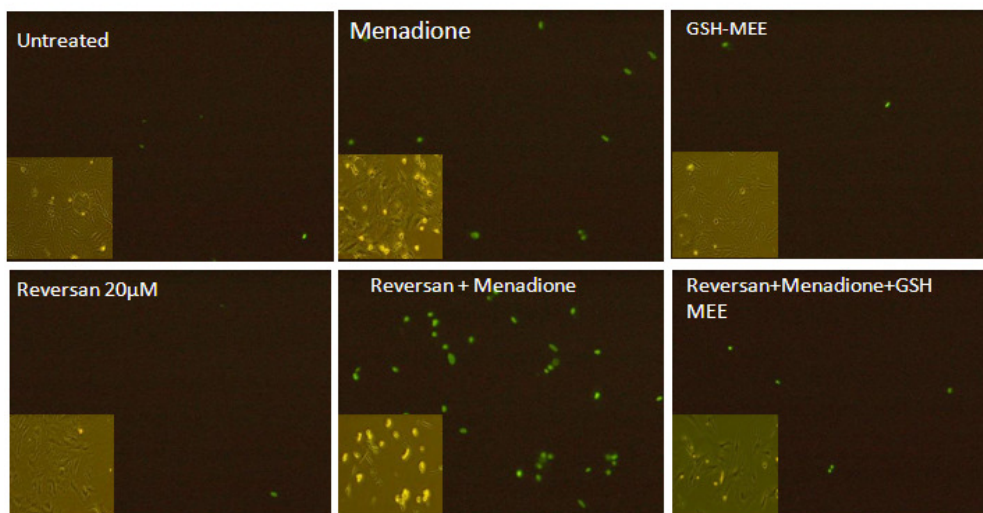
Figure S1. Co-treatment with reversan and menadione induces PMFs necrosis and the effect is reversible by GSH-MEE supplementation. Freshly–isolated PMFs were culture-activated for 7 days and, subsequently, treated for 18 to 24 hours with reversan (20 μM) with or without menadione (20μM) and with or without GSH-MEE (0.5 mM). A) Sytox green-positive nuclei identify necrotic PMFs under the indicated conditions. Insets show the corresponding bright field images. (B) LDH activity was measured in total PMFs protein extracts and the culture medium and the relative amount of LDH in the medium is given as a measure of necrosis. (C) Total glutathione and oxidized glutathione (GSSG) were quantified in total PMFs extracts and the relative amount of GSSG over total-GSH is presented. * significantly different (p<0.001) from untreated and reversan treated cells.

Glutathione and antioxidant enzyme serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death

Sandra Dunning[1], **Atta ur Rehman***[1], Marjolein H. Tiebosch*[1], Rebekka A. Hannivoort[1], Floris W. Haijer[1], Jannes Woudenberg[1], Fiona A.J. van den Heuvel[1], Manon Buist-Homan[1,2], Klaas Nico Faber[#1,2], Han Moshage[#1,2]*

(* and [#] indicate authors that contributed equally tot his study)

[1]Department of Gastroenterology and Hepatology
[2]Department of Laboratory Medicine
University Medical Center Groningen
University of Groningen

ABSTRACT

Background: In chronic liver disease, hepatic stellate cells (HSCs) are activated, highly proliferative and produce excessive amounts of extracellular matrix, leading to liver fibrosis. Elevated levels of toxic reactive oxygen species (ROS) produced during chronic liver injury have been implicated in this activation process. Therefore, activated hepatic stellate cells need to harbor highly effective anti-oxidants to protect against the toxic effects of ROS.

Aim: To investigate the protective mechanisms of activated HSCs against ROS-induced toxicity.

Methods: Culture-activated rat HSCs were exposed to hydrogen peroxide. Necrosis and apoptosis were determined by Sytox Green or acridine orange staining, respectively. The hydrogen peroxide detoxifying enzymes catalase and glutathione-peroxidase (GPx) were inhibited using 3-amino-1,2,4-triazole and mercaptosuccinic acid, respectively. The anti-oxidant glutathione was depleted by L-buthionine-sulfoximine and repleted with the GSH-analogue GSH-monoethylester (GSH-MEE).

Results: Upon activation, HSCs increase their cellular glutathione content and GPx expression, while MnSOD (both at mRNA and protein level) and catalase (at the protein level, but not at the mRNA level) decreased. Hydrogen peroxide did not induce cell death in activated HSCs. Glutathione depletion increased the sensitivity of HSCs to hydrogen peroxide, resulting in 35% and 75% necrotic cells at 0.2 and 1 mmol/L hydrogen peroxide, respectively. The sensitizing effect was abolished by GSH-MEE. Inhibition of catalase or GPx significantly increased hydrogen peroxide-induced apoptosis, which was not reversed by GSH-MEE.

Conclusion: Activated HSCs have increased ROS-detoxifying capacity compared to quiescent HSCs. Glutathione levels increase during HSC activation and protect against ROS-induced necrosis, whereas hydrogen peroxide-detoxifying enzymes protect against apoptotic cell death.

ABBREVIATIONS

3AT, 3-amino-1,2,4-triazole; BSO, L-buthionine-sulfoximine; dNTP, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GCL, glutamate cysteine ligase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSH-MEE, GSH-monoethylester; GSSG, oxidized glutathione; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HO-1, heme-oxygenase-1; HSC, hepatic stellate cell; MS, mercaptosuccinic acid; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulphonylfluoride; PPAR-γ, peroxisome proliferator-activated receptor gamma; RNA, ribonucleic acid; ROS, reactive oxygen species; α-SMA, α-smooth muscle actin ; SOD, super oxide dismutase; TGF-β, Transforming growth factor beta

INTRODUCTION

Oxidative stress is defined as the imbalance between pro-oxidants and anti-oxidants [1,2]. Under normal conditions, reactive oxygen species are detoxified by various enzymatic and non-enzymatic antioxidants. When pro-oxidants exceed the antioxidant capacity of the cell, oxidative stress is the result [1,2]. Prolonged oxidative stress in the liver is associated with liver fibrosis and cirrhosis [3-7]. Liver fibrosis is characterized by the loss of hepatocytes and the activation of hepatic stellate cells (HSCs) [3-5]. During the activation process quiescent HSCs transform into proliferating myofibroblast-like cells. Unlike quiescent HSCs, these activated cells lack retinoid-storing capacity, produce excessive amounts of connective tissue and proliferate [4,5].

Although generation of reactive oxygen species has been implicated in the activation of stellate cells and liver fibrosis [6-16], little is known about the role of the different antioxidant systems in activated HSCs. Several enzymes are able to generate hydrogen peroxide, e.g. NADPH-oxidases and xanthine oxidase [1,2]. In addition, hydrogen peroxide is generated in the detoxification of superoxide anions by superoxide dismutases like the cytosolic CuZn-SOD (SOD1) and the mitochondrial Mn-SOD (SOD2) [1,2]. Pathophysiological conditions often lead to increased hydrogen peroxide levels produced by inflammatory cells, e.g. neutrophils [1]. Hydrogen peroxide is detoxified by catalase that resides in peroxisomes or by cytosolic glutathione peroxidase. Glutathione peroxidase converts reduced glutathione (GSH) into oxidized glutathione (GSSG) [1,2]. To control the hydrogen peroxide level within the cell, the cell has to balance the activity of catalase and glutathione peroxidases relative to SODs. The aim of this study was to investigate the role of antioxidant systems in the resistance of stellate cells to hydrogen peroxide-induced toxicity.

MATERIAL AND METHODS

Animals: Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Hepatic stellate cell isolation and culture: Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and

collagenase-P (Roche, Almere, the Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (12% w/v) centrifugation as described previously [17]. Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 50 µg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 µg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, the Netherlands) in a humidified atmosphere containing 5% CO_2 at 37°C. For studying activation of hepatic stellate cells, cells were seeded, grown to confluency and harvested at the indicated time points. Primary HSC cultures were passaged via trypsinization, and then cultured in Iscove's medium with supplements as described above, except Nystatin. Prior to experiments, HSCs were serum-starved for 24 hours, unless indicated otherwise.

Experimental design: HSCs were culture-activated on tissue culture plastic for at least 7 days. The activated rat HSCs were exposed to oxidative stress induced by 0.2 or 1 mM hydrogen peroxide (Merck) or 20 or 50 µM menadione (Sigma-Aldrich, the Netherlands). The glutathione depleting compound L-buthionine-sulfoximine (BSO, Sigma-Aldrich, the Netherland) was used at 200 µM. The cell permeable glutathione donor GSH-monoethylester (GSH-MEE, Calbiochem, VWR, The Netherlands) was used at 5 mM. The glutathione peroxidase inhibitor mercaptosuccinic acid (MS, Sigma-Aldrich) was used at 10 mM and the catalase inhibitor 3-amino-1,2,4-triazole (3AT, Sigma-Aldrich) was used at 20 mM. The caspase-3 inhibitor (Z-DEVD-FMK004R & D Systems, Abingdon UK) was used at 0.05 µM. Inhibitors were added 30 minutes prior to exposure to hydrogen peroxide, with the exception of BSO, which was added 17-20 hours prior to exposure to hydrogen peroxide.

Glutathione assay: Glutathione and glutathione disulfide content were determined using a spectrophotometry-based assay as described previously [18,19]. HSCs were harvested in a lysis buffer composed of 25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM PMSF, 10 µg/µL pepstatin and 10 µg/µL leupeptin and then lysed by 3 cycles of snap-freezing in liquid nitrogen and thawing. Values were corrected for protein concentration, determined by the BioRad DC protein assay (Veenendaal, the Netherlands) according to the manufacturer's instructions.

RNA isolation: RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using random nonamers (Sigma-Aldrich) in a final volume of 50 µl. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C.

Quantitative Real-Time PCR: Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems, the Netherlands) initialized by 10 min at 95 °C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). Each sample was analyzed in duplicate. mRNA levels of 18S were used as an internal control. Reaction mixture contained qPCR mastermix plus-dTTP (Eurogentec, Maastricht, the Netherlands) supplemented with 900 nM sense and anti-sense primer and 200 nM labeled probe. The primers (Invitrogen) and probe (Eurogentec) used are listed in data Table 1. Relative gene expressions were calculated using the ΔΔCt method.

Western blot analysis: Western blot analysis was performed as described previously [19]. Equal amounts of protein were loaded on SDS-PAGE gels. Proteins were transferred using semi-dry electrophoretic transfer. Specific proteins were detected using primary antibodies: mouse anti-GAPDH (1:10,000, Calbiochem, VWR, the Netherlands CB1001), mouse anti-α-smooth muscle actin (1:2,000, Sigma Aldrich), rabbit polyclonal anti-Mn-superoxide dismutase (1:1,000, Stressgen, Enzo life Sciences, Antwerpen, Belgium, SOD-111), polyclonal rabbit anti-catalase (1:2,000, Calbiochem 219010), polyclonal rabbit anti-Pex-14 (1:2000, generous gift of Dr. M. Fransen, Leuven, Belgium), and polyclonal rabbit anti-β-actin (1:2000, Sigma-Aldrich A2066). Protein bands were detected using a Chemidoc XRS system (Bio-Rad).

Apoptosis and necrosis determination by Acridine orange and Sytox green/Hoechst 33342 nuclear staining: Cells were seeded in 12-well plates and treated as indicated. Apoptosis was determined by assessment of nuclear condensation using Acridine orange staining (Sigma-Aldrich) at 2.5 µg/mL. After 6 hours, the percentage of apoptotic cells was determined by dividing the number of condensed nuclei by the total number of nuclei per field, amplified with 100. Percentages are the mean of two randomly chosen fields per condition (magnification 200 x; 15 nuclei per field). To determine necrosis, HSCs were incubated with Sytox green nucleic acid staining (Invitrogen, Breda ,The Netherland) at 125 nM in combination with Hoechst 33342 (Roche, Almere, the Netherlands) at 5 µg/mL. Sytox green penetrates cells with leaky plasma membranes, a hallmark of necrotic

cells, but does not cross the plasma membranes of viable or apoptotic cells and has been validated before [19]. Hoechst 33342 crosses the plasma membrane of all cells. After 3 hours, the percentage of necrotic cells was determined by dividing the number of Sytox green positive nuclei by the number of Hoechst 33342 positive nuclei of the same field, amplified by 100. Two randomly-chosen fields were used to determine the average per condition (magnification200x; 15 nuclei per field). Cells were monitored using an Olympus CKX41 microscope at 450-490 nm.

Table 1. Oligonucleotide Primers and probes used for Real time-PCR

	Forward 5'- 3'	Reverse 5'-3'	Probe 5'-3'
18S	Cggctaccacatccaagga	Ccaattacagggcctcgaaa	cgcgcaaattacccactcccga
α-SMA	Gccagtcgccatcaggaac	Cacaccagagctgtgctgtctt	cttcacacatagctggagcagcttctcga
Catalase	Ggattatggcctccgagatct	Accttggtcaggtcaaatggat	atgccatcgccagtggcaattacc
Collagen type 1	Tggtgaacgtggtgtacaaggt	Cagtatcacccttggcaccat	tcctgctggtccccgaggaaaca
GCL	Gcccaattgttatggctttgagt	Cctcccgtgttctatcatctcacaga	actccccagcgacaatcaatgtctgacac
Gpx1	Ggacatcaggagaatggcaaga	Cgcacttctcaaacaatgtaaagttg	ttccctcaagtatgtccgacccggtg
HO-1	cacagggtgacagaagaggctaa	Ctggtctttgtgttcctctgtcag	cagctcctcaaacagctcaatgttgagc
CuZnSOD SOD1)	Caggacctcattttaatcctcactc	Gtctccaacatgcctctcttca	ccgctggaccgccatgtttctt
MnSOD (SOD2)	Caccgaggagaagtaccacga	Gaacttcagtgcaggctgaaga	cctgagttgtaacatctcccttggccag
TGF-β	Gggctaccatgccaacttctg	Gagggcaaggaccttgctgta	cctgcccctacatttggagcctgga

Immunofluorescence microscopy: Cells were fixed with 4% paraformaldehyde, labeled and analyzed as described previously [20]. Rabbit polyclonal antibodies against catalase (dilution 1:200; Calbiochem) or MnSOD (dilution 1:100, Stressgen) were use as primary antibodies, followed by secondary antibodies labeled with Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen), respectively. Images were captured with a Leica TCS SP2-AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany).

Proliferation assay: Proliferation of HSCs was determined using the Cell Proliferation ELISA kit (Roche, Almere, the Netherlands), a chemiluminescent ELISA-based detection of BrdU incorporation, according to the manufacturer's instructions.

Statistical analysis: Statistical analyses of data were performed using SPSS 14. Data are presented as mean ± standard deviation, unless otherwise indicated. Statistical differences between groups were calculated using the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney-U-test. p-values below 0.05 were considered significant.
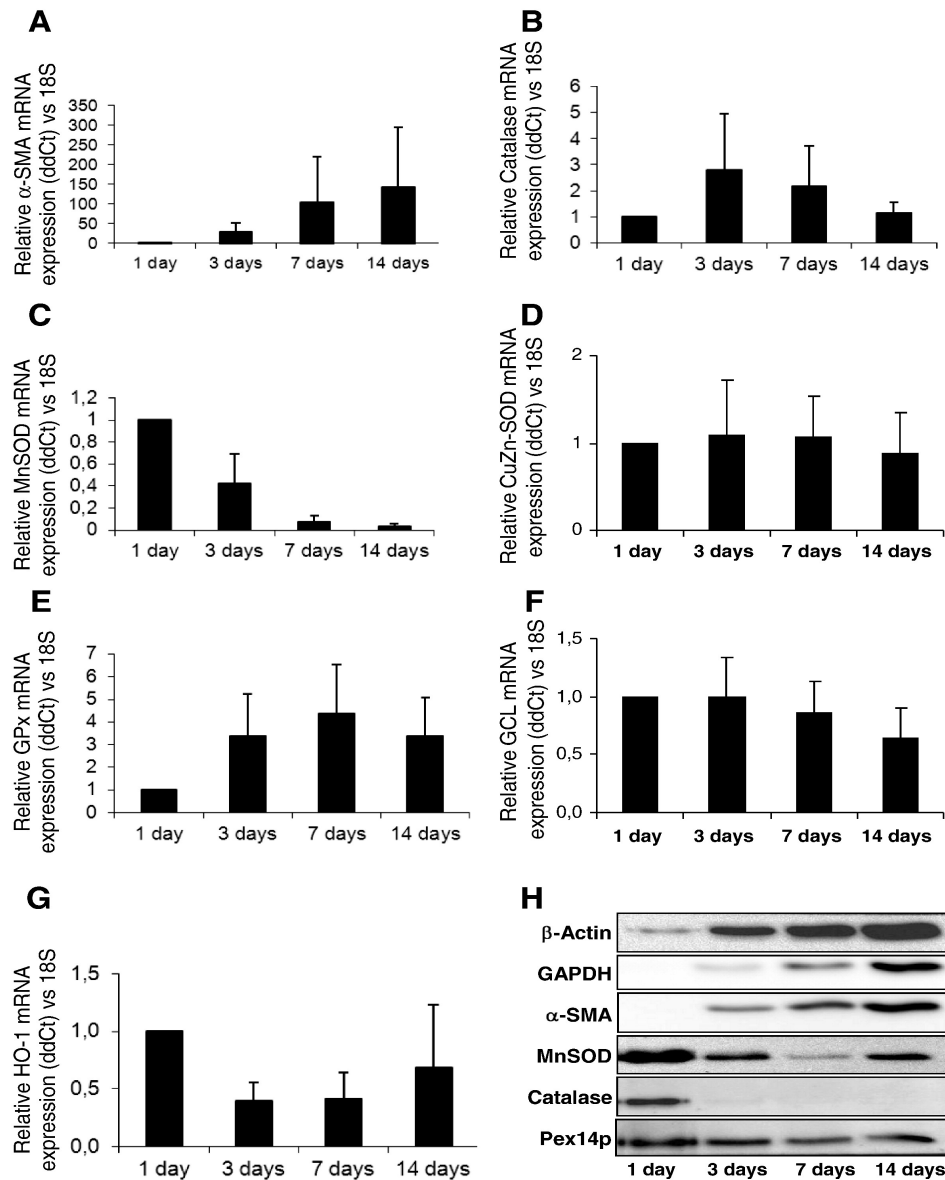
Figure 1. Expression of ROS-detoxifying enzymes during hepatic stellate cell activation. Primary rat HSC were culture-activated for 1, 3, 7 and 14 days and mRNA (A-G) and protein (H) levels of activation markers and anti-oxidant enzymes were quantified by RT-Q-PCR and western blotting, respectively. Alpha-SMA mRNA (A) and protein (H) levels progressively increased during HSC activation. Catalase mRNA levels (B) transiently increased, while the corresponding protein rapidly declined in total cell extracts after day 1 (H). Expression of Mn-SOD progressively decreased during HSC activation, both at mRNA (C) and protein (H) level, while CuZn-SOD levels did not change (D). The expression of GPx1 increased (E), while the expression of GCL did not change (F, H). The expression of HO-1 was low and not significantly changed during HSC activation (G). Pex14p was used as loading control for Western blot analysis, since β-actin and Gapdh protein levels strongly increased upon HSC activation (H), The Western blots are representative of four independent experiments. mRNA data was corrected for 18S and is presented as means ± SD. *Significant difference compared to quiescent HSCs at day 1, $p < 0.05$.

RESULTS

Glutathione peroxidase 1 expression is induced during activation of hepatic stellate cells

Marked changes in gene expression occur when quiescent HSCs transform into activated HSCs. Therefore, we first investigated the expression of various genes involved in the detoxification of reactive oxygen species during the activation process. As expected, alpha-smooth muscle actin (α-SMA), a marker for HSC activation, was strongly induced upon activation (Fig. 1A). mRNA levels of catalase were significantly induced after two days of culture, but decreased upon complete activation of stellate cells (Fig. 1B). Manganese superoxide dismutase (Mn-SOD) mRNA levels progressively declined upon activation (Fig. 1C), while the copper-zinc superoxide dismutase (CuZn-SOD) expression remained unaltered (Fig. 1D). Interestingly, mRNA expression of glutathione peroxidase 1 (GPx1) increased during activation of HSCs (Fig. 1E), while glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis, was not altered upon HSC activation (Fig. 1F). HO-1 expression in HSCs was very low and not altered upon HSC activation (Fig. 1F). HO-1 expression in HSCs was very low and although a trend towards reduced heme oxygenase-1 (HO-1) expression was observed, this did not reach statistical significance (Fig. 1G). The mRNA data were confirmed by analyzing the levels of the corresponding proteins by Western blotting (Fig. 1H). Alpha-smooth muscle actin, β-actin and Gapdh protein expression all increased upon HSC activation. In contrast, Mn-SOD protein levels sharply decreased during activation of HSC. Similarly, also catalase protein levels dropped immediately after day 1, which was not observed for the catalase mRNA levels. Despite the low protein levels of catalase and MnSOD as detected by Western blotting, both proteins remained detectable using immunocytochemistry and revealed a typical peroxisomal and mitochondrial location, respectively (Fig. 2). Given the large and unexpected difference in β-actin and Gapdh levels in quiescent versus activated HSC, we searched for alternative proteins to use as loading control for Western blot analysis. Pex14p, a protein involved in translocating proteins into peroxisomes, showed a stable signal relative to 18S mRNA level and total protein loaded for Western blot analysis (Figure 1H, bottom panel).

Activated hepatic stellate cells have a higher glutathione content than quiescent hepatic stellate cells

Total glutathione levels were determined in quiescent (1 day after isolation) HSCs and in fully activated (>7 days after isolation) HSCs. The total cellular glutathione content was increased 5.6-times upon activation of HSC from 0.18 µmol/µg protein in quiescent HSC
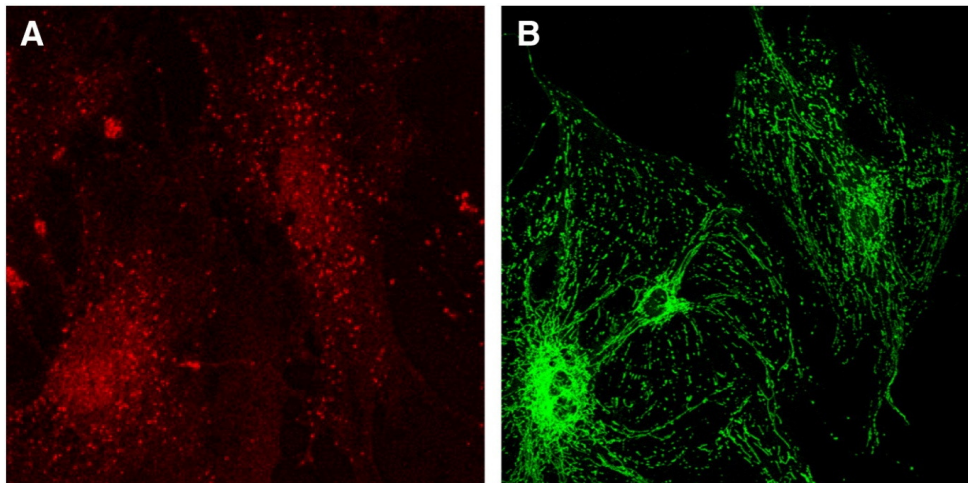
Figure 2. Catalase and Mn-SOD protein is still detectable in activated HSC. Fully activated HSC were analyzed for the presence and subcellular location of catalase (A) and MnSOD (B) using immunofluorescence microscopy. A punctate staining typical for a peroxisomal location was observed for catalase (red stain), while MnSOD staining (green) was restricted to mitochondria.
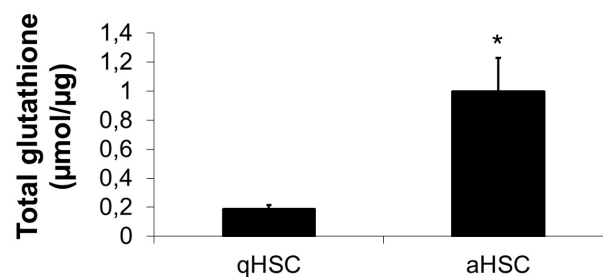
Figure 3. Total glutathione content is increased upon hepatic stellate cell activation. Total glutathione levels were quantified in quiescent (qHSC, 1 day in culture) and fully activated (aHSC, > 7 days in culture) HSC. Activated HSCs have approximately 5.6-fold higher glutathione levels than quiescent HSCs. Results are shown as mean ± S.E.M of four independent experiments, * significantly different from quiescent stellate cells, $p < 0.05$.

to 1.0 µmol/µg protein in activated HSC (Fig. 3), despite unchanged GCL mRNA and protein levels, the rate-limiting enzyme in the synthesis of glutathione (Fig. 1). Subsequent analysis revealed that 88% of the total glutathione content was in the reduced (GSH) form (mean of 3 different isolates of HSCs).

Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells

Pre-incubation of activated, serum-starved HSCs with BSO reduced total glutathione levels by 87% (Fig. 4A). No increase was observed in either necrotic or apoptotic cell death (data not shown) as well as no gross changes in cellular morphology was observed in BSO-treated HSC. Likewise, BSO treatment also reduced glutathione content by 88% in activated HSCs cultured in medium containing 20% FCS (data not shown), without visible morphological changes. To investigate whether glutathione depletion leads to increased oxidative stress in HSCs, we determined the mRNA level of the oxidative stress-responsive gene heme-oxygenase-1 (HO-1). Depletion of glutathione increased HO-1 mRNA levels 2.3-fold (Fig. 4B), which is only minor when compared to the induction of HO-1 by menadione or hydrogen peroxide (5-50 fold) [6,19]. In addition, mRNA expression of GCL, the rate-limiting enzyme in glutathione synthesis, was increased only 1.6-fold (Fig. 4B). In contrast, glutathione depletion had no effect on the
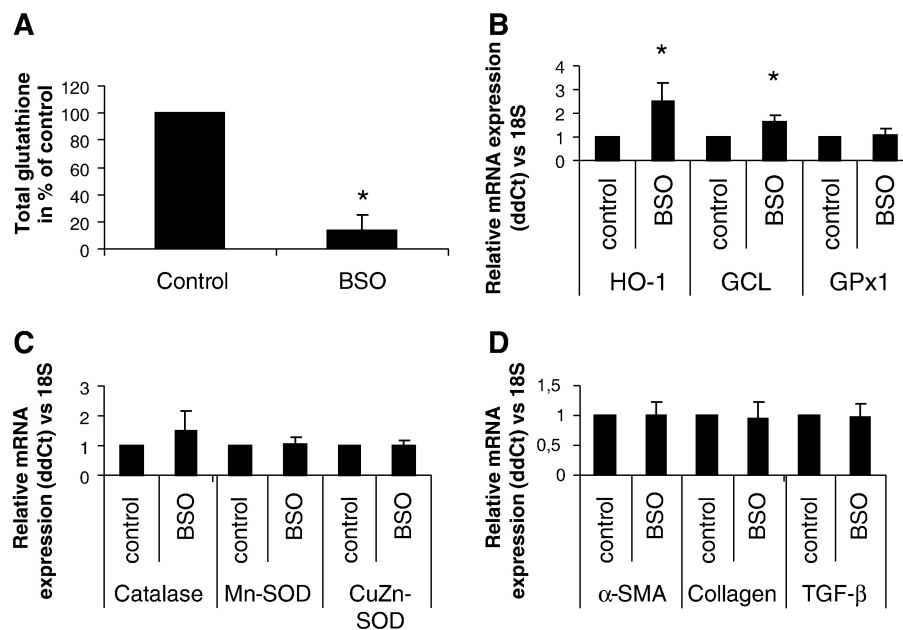
Figure 4. Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells. Fully-activated HSC were treated with BSO for 20 h and analyzed for total glutathione content (A) and mRNA levels of anti-oxidant enzymes (B,C) and activation markers (D). Pre-treating HSCs with BSO depleted cellular glutathione content by 87% in serum-starved HSCs (A). BSO treatment moderately induced HO-1 and GCL and had no effect on GPx1 (B) nor on the expression of the anti-oxidant enzymes catalase, Mn-SOD, and CuZn-SOD (C) or the expression of the HSC activation markers, α-SMA, collagen type 1 and TGF-β (D). Results are shown as mean ± st. dev. of at least four independent experiments, * significantly different from controls; $p < 0.05$.

expression of the hydrogen peroxide-detoxifying enzymes GPx1 and catalase and the superoxide dismutases Mn-SOD, and CuZn-SOD (Fig. 4B,C). These data indicate that glutathione depletion alone only minimally induces markers of oxidative stress. Furthermore, glutathione depletion did not change the expression of the known markers of stellate cell activation α-smooth muscle actin (α-SMA), collagen type 1, and TGF-β (Fig. 4D). Finally, glutathione depletion or glutathione supplementation, using GSH-MEE did not alter stellate cell proliferation (data not shown). Next, we analyzed whether glutathione depletion sensitizes HSCs for oxidative stress.

Depletion of glutathione increases sensitivity to hydrogen peroxide-induced necrosis

After depletion of glutathione with BSO, 35% and 75% of the cells became necrotic after a 3 hour exposure to 0.2 or 1 mM hydrogen peroxide, respectively (Fig. 5). This indicates that glutathione depletion greatly enhanced sensitivity to hydrogen peroxide-induced necrosis. Restoration of glutathione content, using GSH-MEE, almost completely reversed hydrogen peroxide-induced necrosis in BSO-treated HSCs (Fig. 5).
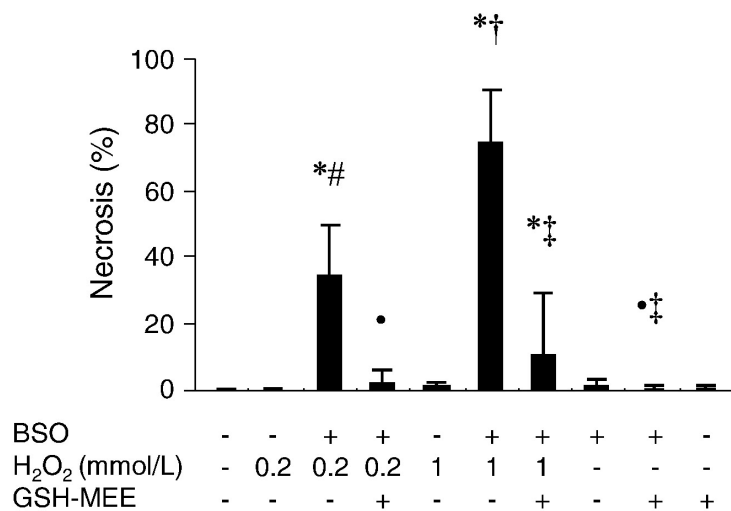
Figure 5. Glutathione depletion increases sensitivity of hepatic stellate cells to hydrogen peroxide-induced necrosis. Fully-activated HSCs were exposed for 3 h to H_2O_2 in the absence or presence of BSO and/or GSH-MEE and analyzed for necrotic cell death by Sytox green nuclear staining. BSO treatment followed by exposure to 0.2 mM or 1 mM hydrogen peroxide (H_2O_2) induced necrosis in 35% and 75% of the cells, respectively. Co-treatment with GSH-MEE almost completely prevented the induction of HSC necrosis by BSO+H_2O_2. Results are shown as mean ± st. dev. of four independent experiments. *Significant difference compared to control, $p < 0.05$; # Significant difference compared to 0.2 mM hydrogen peroxide, $p < 0.05$; •Significant difference compared to 0.2 mM hydrogen peroxide + BSO, $p < 0.05$; †Significant difference compared to 1 mM hydrogen peroxide, $p < 0.05$; ‡Significant difference compared to 1 mM hydrogen peroxide + BSO, $p < 0.05$.

Glutathione peroxidase and catalase protect against oxidative stress-induced apoptosis

The importance of the antioxidant enzyme GPx in the protection of activated HSCs against oxidative stress was investigated using the GPx inhibitor mercaptosuccinic acid (MS). Inhibition of GPx using mercaptosuccinic acid increased apoptosis in the absence of hydrogen peroxide and in the presence of 0.2 mM and 1 mM hydrogen peroxide (Fig. 6A). No effects on necrosis were observed under these conditions (data not shown). The importance of the antioxidant enzyme catalase in the protection of activated HSCs against oxidative stress was investigated using the catalase inhibitor 3-am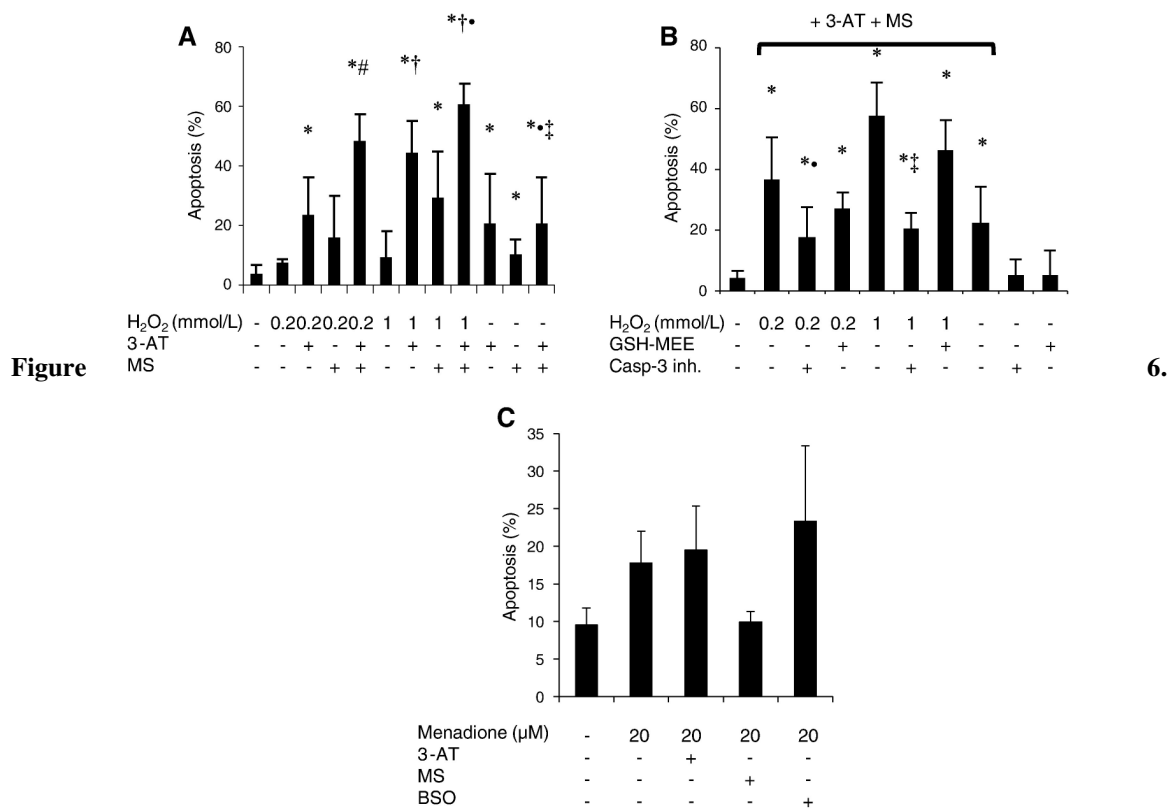ino-1,2,4-triazole (3-AT). This inhibitor significantly induced apoptotic cell death (18%) in HSCs after 6 hours, even in the absence of exogenous hydrogen peroxide (Fig. 6A). Cells treated with 0.2 or 1 mM hydrogen peroxide in the presence of the inhibitor catalase showed even higher levels of apoptosis, 22% an 44% respectively (Fig. 6A). No effects on necrosis were detected at this time point.

To investigate the importance of the hydrogen peroxide detoxifying enzymes catalase and GPx in the protection against superoxide anions that are converted into hydrogen peroxide by superoxide dismutases, we exposed activated stellate cells to the superoxide anion generator menadione. As described previously, menadione at 20 µM induced predominantly apoptotic cell death, which was reduced by the glutathione donor GSH-MEE [19]. Inhibition of catalase in the presence of menadione had no effect on apoptotic cell death, but slightly increased necrotic cell death, whereas inhibition of GPx did neither modulate apoptotic nor necrotic cell death (Fig. 6C). Prior reduction of cellular glutathione levels by BSO did not aggravate apoptotic cell death induced by 20 µM menadione. Menadione at 50µM resulted in detachment of cells and massive necrotic cell death.

Combined inhibition of glutathione peroxidase and catalase elevates apoptosis of HSCs

Inhibiting both GPx and catalase by cotreatment with MS and 3AT resulted in 22% apoptotic cells after 6 hours even in the absence of exogenous hydrogen peroxide (Fig. 6A). Exposing these cells to hydrogen peroxide induced apoptosis even further: to 49% in co-treatment with 0.2 mM hydrogen peroxide and to 62 % in co-treatment with 1

A

Apoptosis (%)

| H₂O₂ (mmol/L) | 3-AT | MS |

B

+ 3-AT + MS

Apoptosis (%)

| H₂O₂ (mmol/L) | GSH-MEE | Casp-3 inh. |

Figure **6.**

C

Apoptosis (%)

| Menadione (µM) | 3-AT | MS | BSO |

Inhibition of glutathione peroxidase and/or catalase induces apoptosis in activated hepatic stellate cells. Fully-activated HSCs were exposed for 6 h to H_2O_2 in the presence or absence of the catalase inhibitor 3-AT and/or GPx inhibitor MS (A), as well as in the presence or absence of GSH-MEE or the caspase-3 inhibitor Z-DEVD-FMK (B) and analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. 3-AT and MS treatment alone significantly increased the number of apoptotic HSC, which was further enhanced by cotreatment with H_2O_2 (A). Apoptosis induced by treatment with H_2O_2 in the presence of inhibitors of GPx and catalase is inhibited by a blocker of caspase-3 activity, but not by supplementing glutathione through GSH-MEE (B). Results are shown as mean ± stdev of at least four independent experiments. *Significant difference compared to control, $p<0.05$; ^Significant difference compared to MS, $p<0.05$; §Significant difference compared to 3AT, $p<0.05$; † Significant difference compared to 1 mM hydrogen peroxide, $p<0.05$; °Significant difference compared to MS + 3AT, $p<0.05$; #Significant difference compared to 0.2 mM hydrogen peroxide, $p<0.05$; •Significant difference compared to 0.2 mM hydrogen peroxide + 3-AT + MS, $p<0.05$; ‡Significant difference from 0.2 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$; ⁿSignificant difference from 1 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$. (C). Fully-activated HSCs were exposed for 9 h to 20µM menadione in the presence or absence of the catalase inhibitor 3-AT, the GPx inhibitor MS, as well as the glutathione depleting agent BSO analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. Menadione induced apotptocu cell death at 20 µM. 3-AT and MS did not significantly modulate apoptotic cell death by menadione. Moreover, depletion of glutathione using BSO did not aggravate menadione-induced apoptotic cell death. Results are shown as mean ± st. dev. of at least four independent experiments.

mM hydrogen peroxide (Fig. 6A). Increasing the glutathione content using the glutathione donor GSH-MEE did not change the cell viability under these conditions (Fig. 6B). Inhibition of caspase-3 partially decreased apoptosis of HSCs (Fig. 6B). Necrotic cell death was not significantly enhanced in the described conditions (data not shown).

DISCUSSION

Chronic liver injury is almost invariably accompanied by increased oxidative stress, activation of stellate cells and fibrogenesis [3-7]. Activated HSCs must be well protected against oxidative stress, since they survive and proliferate in the chronically injured liver. The oxidative stress in chronically injured liver is composed of several reactive oxygen species, including hydrogen peroxide and superoxide anions. In this study, we have investigated the resistance of hepatic stellate cells to hydrogen peroxide-induced injury. We demonstrate that this resistance is to a large extent due to a high intracellular glutathione content and increased expression of glutathione peroxidase in activated stellate cells. Although an increased glutathione content in activated stellate cells has been reported before [21], the implications have never been investigated in the context of oxidative stress-induced cell death.

Maher *et al*. showed, in addition to increased glutathione levels upon activation, an increase in the activity and mRNA level of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis [21]. We did not observe an induction of GCL mRNA upon stellate cell activation in our experiments. The increased cellular glutathione content is most likely due to a higher activity of GCL, especially since GCL activity is known to be regulated by the glutathione content [22]. It is unlikely that the increased glutathione content is due to reduced activity of the GSSG export pump Mrp1, since we have previously shown that the expression of this transporter is increased in activated stellate cells and contributes significantly to the survival of activated stellate cells. [23]

Upon HSC activation, the expression of the hydrogen peroxide detoxifying enzyme glutathione peroxidase 1 (GPx1) is increased. This increase may be an adaptive response to oxidative stress. Indeed, mice over-expressing GPx1 are better protected against oxidative stress and they survive concentrations of the oxidant paraquat that are lethal in wild type mice and even more harmful in GPx1 knockout mice [24,25]. Mice that overexpress GPx1 are also more resistant to oxidative stress due to myocardial

ischemia-reperfusion injury [26]. It should be noted that glutathione is essential for the activity of GPx, since GPx converts reduced glutathione into oxidized glutathione. This might explain the coordinated increase in cellular glutathione content and GPx expression during the activation process of hepatic stellate cells. Therefore, our data suggest that activated hepatic stellate cells may be more resistant against oxidative stress than quiescent stellate cells. Interestingly, upon activation the mRNA expression level of the mitochondrial superoxide anion converting enzyme Mn-SOD is reduced, both at the mRNA and at the protein level. This finding is partially at variance with previous reports that revealed an initial increase in MnSOD mRNA expression, followed by a steady decrease of MnSOD mRNA expression [27]. Disruption of the Mn-SOD gene, a known tumor suppressor gene, is lethal in mice, which is a direct result of mitochondrial dysfunction, leading to metabolic acidosis, ketosis and accumulation of lipids in the liver and skeletal muscle [28,29]. Characterization of the heterozygous Mn-SOD knockout mice revealed no compensatory increase in other ROS-detoxifying enzymes, like glutathione peroxidase, CuZn-SOD or catalase [30]. Since Mn-SOD is restricted to the mitochondria, changes in its activity may not affect other components of the antioxidant defense system in other cellular compartment like the cytoplasm[30]. At present it is not known how the activated stellate cells detoxify the reactive oxygen species generated in mitochondria. One possibility is that the residual MnSOD protein content, as determined by Western-blotting and immunofluorescence, is sufficient to detoxify ROS generated in mitochondria. The reduction in Mn-SOD mRNA expression during activation could be due to the strong reduction of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) that occurs during stellate cell activation (data not shown) and [31]. MnSOD expression is controlled by PPAR-γ: in PPAR-γ knockout mice, the expression of Mn-SOD is also reduced [32] and activation of PPAR-γ with the agonists rosiglitazone or rosuvastatin enhanced MnSOD activity and expression [33,34].

The catalase mRNA levels transiently increased during the activation process of HSC, which is largely in line which observations made by De Bleser et al. who analyzed catalase regulation during HSC activation by Northern blot analysis [27]. However, in contrast to the mRNA levels, the cellular catalase protein content sharply dropped 1 day after plating HSC. A typical peroxisomal staining of catalase remained detectable in fully activated HSC, indicating that some residual catalase protein was present in these cells. However, clearly, the catalase protein level was not an accurate reflection of the catalase

mRNA content. At present it is unclear why the catalase protein disappears from activated stellate cells, but this may be a result of a rapid and selective degradation of this antioxidant protein. The low catalase levels did not sensitize activated HSCs to acute H_2O_2-induced necrosis, indicating the presence of an alternative H_2O_2 scavenging mechanism(s) in activated HSCs. However, blocking catalase activity by 3-AT made HSCs highly sensitive for apoptotic cell death, which was further enhanced by exposure to 0.2 or 1.0 mM H_2O_2. A similar effect was observed after blocking GPx activity, indicating that these 2 proteins play a key role in regulating apoptotic cell death in HSCs.

HSCs increase their glutathione levels upon activation. However, glutathione depletion has no direct effect on stellate cell morphology [35] and in this study, we show that glutathione depletion also has no effect on the expression of markers of stellate cell activation, such as collagen type I, α-SMA and TGF-β or anti-oxidant genes like catalase, Mn-SOD, CuZn-SOD, and GPx1. Additionally, glutathione depletion did not affect the proliferation of hepatic stellate cells. The glutathione levels per se do not seem to be a key mechanism in developing the activated phenotype.

Although increased mRNA levels of GCL and HO-1 were observed after glutathione depletion, these increases were modest, indicating that depletion of glutathione alone does not exert significant oxidative stress on stellate cells. Induction of GCL mRNA levels after glutathione depletion has also been shown in lung epithelial cells [36], endothelial cells [37], and in rat liver *in vivo* [38]. Although HO-1 has been reported to inhibit HSC proliferation via p38 activation [39], we did not find an alteration in the proliferation rate of stellate cells after HO-1 induction due to glutathione depletion. Possibly, the induction of HO-1 by glutathione depletion in our study is too modest to have an effect on p38 phosphorylation and subsequent HSC proliferation. The induction of HO-1 by glutathione depletion was only 2.3-fold, whereas Li et al showed at least a 10-fold induction of HO-1 expression using 15-deoxy-delta-12,14-prostaglandin J2 [39,40].

Although glutathione depletion per sé had no effect on stellate cell viability, glutathione depletion increased the sensitivity of the cells to hydrogen peroxide-induced necrosis. Replenishing glutathione reduced necrotic cell death, without a shift towards apoptosis. Inhibition of the hydrogen peroxide-detoxifying activity by inhibitors of glutathione peroxidase or catalase induced HSC apoptosis, both in the absence and in the presence of exogenous hydrogen peroxide. Apoptotic cell death as a result of combined

inhibition of glutathione peroxidase and catalase was shown to be independent of glutathione content, but partially dependent on caspase-3 activity. The superoxide anion donor menadione dose-dependently induces apoptotic cell death in activated HSCs [19]. Inhibition of hydrogen peroxide detoxifying enzymes did not significantly modulate menadione-induced cell death, indicating that increased superoxide generation did not lead to a massive, superoxide dismutase-mediated, increase in hydrogen peroxide.

The observed difference in mode of cell death, necrosis after glutathione depletion and apoptosis after inhibition of hydrogen peroxide detoxifying enzymes, may be explained by the cellular redox state. Glutathione is the most important regulator of the cellular redox state [41,42]. Changes in the glutathione content will affect redox status and is known to influence activation of MAP-kinases, transcription factors and caspases [41-43]. In the presence of glutathione, caspases that require reduced cysteine-sulfhydryl groups in their catalytic site, can still be activated when the enzymes GPx and catalase are inhibited, because this inhibition is not likely to change the redox state of the cell. In contrast, in the absence of glutathione, e.g. after glutathione depletion, cells exposed to hydrogen peroxide cannot activate caspases and the apoptotic program and cell death is shifted towards necrosis. Such a shift from apoptotic to necrotic cell death has been reported before in hepatocytes exposed to superoxide anions [44].

In summary, our study reveals important changes in the defense against oxidative stress of hepatic stellate cells during activation. These changes are characterized by increased cellular glutathione content and GPx1 mRNA expression. Furthermore, we demonstrate that both glutathione and the hydrogen peroxide-converting enzymes GPx and catalase are important in the resistance against hydrogen peroxide-induced cell death. Our data suggests that activated hepatic stellate cells *in vivo* may acquire increased resistance to necrotic cell death, while remaining sensitive to apoptosis, providing an explanation for their survival in the fibrotic liver and their apoptotic clearance during reversal of fibrosis.

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The intracellular ABC-transporter PMP70 is required for generating the alpha-SMA cytoskeleton in hepatic myofibroblasts

Atta ur Rehman*, Jannes Woudenberg*, Floris W. Haijer, Bojana Mikuš, Fiona A.J. van den Heuvel, Krzysztof P. Rembacz, Han Moshage and Klaas Nico Faber

(*These authors contributed equally)

Department of Gastroenterology and Hepatology
University Medical Center Groningen
University of Groningen

ABSTRACT

Background: Liver fibrosis is a result of an exaggerated healing response to liver injury where proliferative, motile and contractile hepatic myofibroblasts produced excessive amounts of extracellular matrix proteins. These myofibroblasts may arise from difference cellular sources, including hepatic stellate cells (HSCs) and portal myofibroblasts (PMFs) that undergo drastic morphological and functional differentiation. Peroxisomes are multifunctional organelles that may adapt their number and specific function to the metabolic needs of the cell. We studied the expression, subcellular location and function of peroxisomal proteins in transdifferentiating HSCs and PMFs with emphasis on the ATP-binding cassette transporter 70 kDa peroxisomal membrane protein (PMP70).

Methods: Primary rat HSCs were analyzed after a 4 h-attachment period (quiescent-qHSCs) and after 1, 3, 7 and 14 days in culture (activated-aHSCs), as well as culture-activated PMFs and the human stellate cell line LX-2. Messenger RNA levels, protein expression and subcellular location of selected proteins were analyzed by quantitative PCR, western blotting and immunofluorescence microscopy, respectively. RNA interference and plasmid-encoded GFP-PMP70 were used to reduce and overproduce PMP70, respectively.

Results: HSCs harbour peroxisomes containing the peroxisomal markers Pex14p and catalase, expression of which do not strongly change during transdifferentiation. PMP70, a typical marker for peroxisomes in hepatocytes, resides in tubular strands that parallel the alpha-smooth muscle actin (αSMA) polymers in HSCs and PMFs. Silencing PMP70 strongly reduced the expression of αSMA and desmin, without affecting the expression of Collagen 1A1. GFP-PMP70 also localized to the tubular strands and stimulated αSMA expression in HSC, PMF and LX-2 cells.

Conclusions: PMP70 stimulates the production of the αSMA cytoskeleton in hepatic myofibroblasts, which renders these cells highly motile and contractile in the fibrotic liver.

INTRODUCTION

Liver fibrosis is the result of the excessive secretion of extracellular matrix proteins (ECM) by myofibroblasts, such as type I collagens and fibronectins [1]. The myofibroblasts start secreting increased levels of ECM in a healing response to liver injury, but with persistent inflammatory conditions the excessive ECM disturbs the architecture of the liver and compromises its function. Myofibroblasts may originate from various cell types that undergo drastic morphological and biochemical differentiation in response to liver injury. For long, hepatic stellate cells were considered the source of myofibroblasts in the fibrotic liver [2,3]. In the healthy liver, hepatic stellate cells are considered quiescent, reside in the space of Disse and contain large cytoplasmic liver lipid droplets that harbour the body reserves of vitamin A, stored as retinal palmitate [4]. In response to liver injury, the vitamin A-containing lipid droplets disappear and the cells become proliferative, contractile, motile and ECM overproducers [5]. There are also precursors of myofibroblasts residing in the portal areas [2,6,7]. While these portal myofibroblasts do not contain vitamin A, upon liver injury they do rapidly transdifferentiate into activated myofibroblasts with largely similar characteristics as activated HSCs [8]. Recent data suggest that also liver epithelial cells, e.g. hepatocytes and/or cholangiocytes, as well as bone marrow-derived cells may transdifferentiate into myofibroblasts and contribute to fibrosis progression [9-12]. Irrespective of the cellular origin, activated myofibroblasts have undergone drastic cellular and metabolic reprogramming. Hepatic stellate cells change from oxidative phosphorylation to glycolysis upon activation [13]. Besides increased ECM production, activated myofibroblasts contain a characteristic cytoskeletal network consisting of alpha-smooth muscle actin (α-SMA) that renders these cells motile and highly contractile [14].

Peroxisomes are versatile cellular organelles present in almost every eukaryotic cell that adapt their function and numbers upon the cellular needs. Peroxisomal enzymes play crucial roles in over 50 different metabolic pathways [15-18]. A common peroxisomal function shared by many eukaryotic organisms is the metabolism of lipids through α- or β-oxidation of either long chain fatty acids (LCFAs) or very LCFAs (VLCFAs) [19]. H_2O_2 is formed during these metabolic processes, which is detoxified by the peroxisomal enzyme catalase [20]. Peroxisomes are particularly enriched in the liver and kidney. Most of our knowledge about mammalian peroxisomes stems from research

on the liver, where peroxisomes are abundantly present in hepatocytes [17,21-23] and are, amongst others, involved in bile acid synthesis and fatty acid catabolism. The high metabolic activity of peroxisomes requires efficient metabolite transport across the peroxisomal membrane. The ATP-binding cassette (ABC) transporters Adrenoleukodystrophy protein (ALDP/*ABCD1* [24] and the 70 kDa Peroxisomal Membrane Protein (PMP70/*ABCD3* [25]) are such substrate transporters and supposedly transport VLCFAs and LCFAs into peroxisomes, respectively.

In contrast to hepatocyte peroxisomes, almost nothing is known about peroxisome function in liver myofibroblasts and how they may change their function and/or number during the transdifferentiation process. Peroxisomal function in HSCs is of particular interest, since activation of HSCs leads to the rapid loss of a large pool of fatty acids that may be metabolized through peroxisomal β-oxidation. Here, we analysed the expression of peroxisomal markers proteins in quiescent and transdifferentiating HSCs and studied their subcellular location in these cells. We specifically focussed on a novel function for PMP70 in establishing the αSMA network in activated myofibrobalasts.

MATERIALS AND METHODS

Animals: Specified pathogen-free male Wistar rats (220–250 g for hepatocyte isolations and 400-500 g for HSC isolations) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local Committee for Care and Use of laboratory animals.

Isolation and culture of rat hepatocytes, hepatic stellate cells and portal myofibroblasts: Hepatocytes[26], HSCs [27] and PMFs [6] were isolated and cultured as described previously. Hepatocyte viability and purity were always more than 90%. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

LX2: The immortalized human hepatic stellate cell line LX2 was a kind gift of Dr. Scott L. Friedman [28]. Cells were cultured as described in [28].

Plasmids: pEGFP-C1 (cytosolic GFP; BD Biosciences Clontech Palo Alto, CA), pDsRed-SKL [29] and PMP70-GFP [30] were used for transient transfections.

Transient transfections: Culture-activated HSC, PMF and LX-2 cells were transiently transfected using lipofectamine^{TM} 2000 (Invitrogen), Amaxa^{TM} Cell Line Nucleofector® Kit L (Lonza Cologne GmbH, Germany) and FuGENE® HD Transfection Reagent (Promega Madison, WI, USA), respectively. Cells were fixed for immunofluorescence 48 h after transfection.

PMP70 RNA interference (RNAi): Culture-activated HSC (t=7 days) or PMF were trypsinized and plated at a density of 1,500 cells/cm^2 in IMDM with Glutamax supplemented with 1% heat-inactivated fetal calf serum, 1 mmol/L sodium-pyruvate and 1x MEM non essential amino acids. Four hours after plating, cells were transfected with double-stranded siRNA duplexes (see supplementary Table S1) to silence PMP70 (siRNA-PMP70, Invitrogen). Control cells were transfected with oligonucleotides directed against luciferase (siRNA-luc, Invitrogen). Oligofectamine (Invitrogen) was used as a transfection reagent according to the manufacturer's instructions. PMF were harvested after 72 h for analysis. HSC were re-transfected with either siRNA-PMP70 or siRNA-luc after 3 days and 6 days. After 9 days, cell viability was measured in a cell viability assay. In parallel experiments, cells were either lysed for Q-PCR mRNA analysis or Western blot analysis or fixed with 4% paraformaldehyde for immunofluorescence.

Cell Viability Assay: The cell viability of siRNA-luc and siRNA-PMP70 transfected HSCs was determined by measuring cellular adenosine triphosphate (ATP) levels with the Cell Titer Glow assay (Promega) according to the manufacturer's instructions. In each experiment, measured ATP levels were corrected for the number of cells.

RNA isolation and Quantitative Polymerase Chain Reaction (Q-PCR): The isolation of total RNA, its conversion to cDNA and its analysis by Q-PCR was carried out as described previously [31]. Primers and probes used in this study are listed in supplementary Table S2. The expression of each gene of interest was normalized with respect to the endogenous control, 18S (ΔΔCt method).

SDS-PAGE and western blotting: Protein samples were separated by SDS-PAGE and analyzed by Western blotting according to established procedures [29]. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad Hercules, CA, USA) using bovine serum albumin as standard. All primary antibodies used are listed in supplementary Table S3. Horse radish peroxidase-conjugated secondary

antibodies (HRP-conjugated swine-anti rabbit, rabbit anti-goat and rabbit anti-mouse, Dako A/S, Glostrup, Denmark) and the Phototope®-HRP Western Blot Detection System (Cell Signalling technology Inc, Danvers, MA) were used for detection according to the manufacturers' protocols. The blots were exposed in a ChemiDoc XRS system (Bio-Rad).

Immunofluorescence microscopy: Cells were fixed with 4% paraformaldehyde, labeled and analyzed as described previously [29]. Primary antibody dilutions are listed in supplementary Table S3. Images were captured using Leica SP2/AOBS confocal microscope at the UMCG Microscopy and Imaging Center (UMIC).

Statistical analysis: All numerical results are reported as the mean of at least 3 independent experiments ± standard error of the mean (S.E.M). A Mann-Whitney *U* test was used to determine the significance of differences between experimental groups. A p-value smaller than 0.05 was considered to be statistically significant.

RESULTS

mRNA expression of peroxisomal genes in hepatocytes and transdifferentiating HSCs

We first compared the relative mRNA (Fig. 1A) and protein (Fig. 1B) levels of the peroxisomal markers Pex14p, catalase, PMP70 and ALDP in rat hepatocytes and fully activated hepatic stellate cells (aHSCs). Bile acid-CoA:amino acid N-acyltransferase (Baat), a peroxisomal protein required for bile salt synthesis [29], and alpha-smooth muscle actin (αSMA) were used as markers for hepatocytes and aHSCs, respectively. Pex14 transcript and protein levels, a protein involved in peroxisome biogenesis, were similar in hepatocytes and aHSCs, while catalase levels were much lower (10-fold) in aHSCs. ALDP transcript levels were 3.0-fold higher in aHSCs compared to hepatocytes, but ALDP protein levels appeared comparable in these cell types. PMP70 mRNA was predominant in hepatocytes, but significant levels (2.5-fold lower than in hepatocytes) were detected in aHSCs. Transcripts of genes involved in β-oxidation of VLCFAs (Acyl CoA Oxidase and MultiFunctional Protein) and peroxisome proliferation (Pex11a) were much lower in aHSCs compared to hepatocytes (Supplementary Fig. S1). We next determined the expression of peroxisomal marker genes during HSCs transdifferentiation (Fig. 1C). HSCs were analysed immediately after a 4h attachment period and 1, 3, 7 and 14 days after *in vitro* cultivation. αSMA expression became detectable after 1 day in

culture and steadily increased up to day 7, after which it did not further increase. Expression of PPARγ, a marker for quiescent HSCs, sharply dropped when the HSCs were culture-activated and remained low over 2 weeks. Pex14p levels slightly increased during transdifferentiation and returned to quiescent levels after 14 days. Catalase first dropped sharply in the first day of culture, then transiently increased during transdifferentiation after which levels dropped to approximately 25% of qHSCs. The expression of ALDP and PMP70 transiently increased during HSC activation. However, this increase was not significant.

Subcellular location of peroxisomal proteins in transdifferentiating HSCs

Immunofluorescence microscopy was used to analyse the subcellular location of Pex14p, catalase, ALDP and PMP70 in rat hepatocytes and aHSCs (Fig. 2). As expected, all 4 proteins showed a typical peroxisomal staining pattern in hepatocytes indicated by many discrete dots in the cytoplasm (Fig. 2, A1-A4). A clear dotted staining was also observed for Pex14p and catalase in aHSCs (Fig. 2, B1, B2; higher magnifications in C1 and C2), which were identified as peroxisomes in aHSC transfected with DsRed containing a peroxisomal targeting signal (DsRed-SKL; Supplementary Figure S2). In addition, a nuclear staining for these proteins was also detected. ALDP and PMP70 were predominantly detected in tubular strands diverting from the nucleus with variable amounts associated with peroxisomes (Fig. 2, B3, B4; higher magnifications in C3 and C4). Peroxisome-like dots were most evident after staining for ALDP, while the presence of PMP70-positive peroxisomes was highly heterogeneous between individual HSC (compare B-4 and D-1 and Suppl. Figure S2) and the tubular PMP70 staining was always observed. The morphological appearance of the ALDP- and PMP70-positive strands was different. Co-staining experiments revealed only partial co-localization of these proteins in these structures in aHSCs (Supplementary Fig. S3 D-F). This in sharp contrast to hepatocytes, where they strongly co-localize in peroxisomes (Supplementary Fig. S3 A-C). ALDP largely co-localized with calnexin, a marker for the ER (Supplementary Fig S4). The most remarkable finding of these experiments was that the PMP70-positive tubules paralleled the αSMA fibers (Fig. 2D1-D3). An identical location of PMP70 was detected in activated portal myofibroblasts (Fig 2, E1-E3), showing that this is a common feature of hepatic myofibroblasts that cause fibrosis.
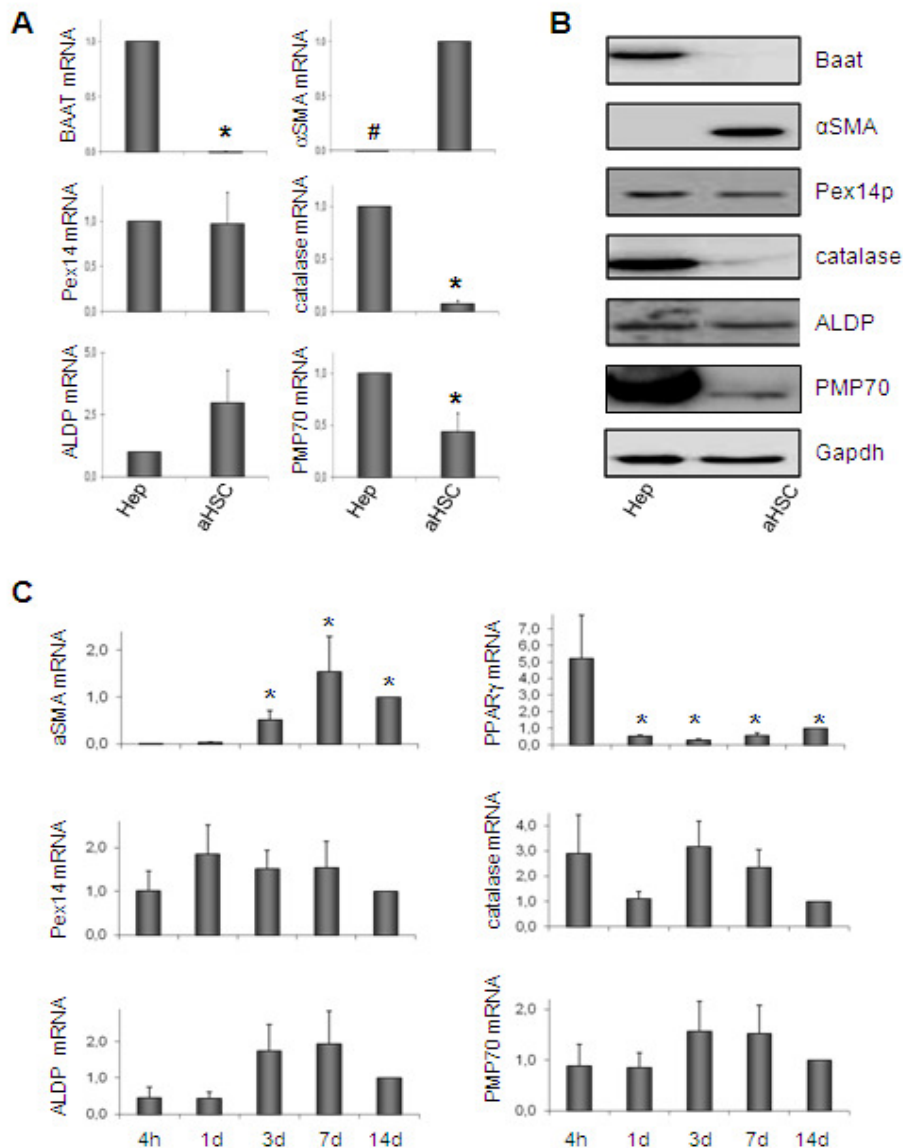
Figure 1. mRNA and protein expression of peroxisomal markers in hepatocytes and HSCs. Hepatocytes and HSCs were isolated using standard protocols. HSCs were activated in the presence of serum for 14 days (aHSCs). A) The relative mRNA expression (normalized to 18S) of the indicated genes was normalized to its expression in hepatocytes, except for αSMA, which was normalized to its expression in aHSCs (*Significant difference (p<0.05) when compared to hepatocytes, [#]significant difference (p<0.05) when compared to aHSCs). B) Equal protein amounts from hepatocytes and aHSCs were analyzed by western blotting using specific antibodies against the hepatocyte marker Baat, the activated stellate cell marker αSMA, and the peroxisomal proteins Pex14p, catalase, ALDP and PMP70. Gapdh expression was analyzed as a control for equal protein loading. C) Freshly isolated HSCs were cultured in the presence of serum for 4 hours, 1 day, 3 days, 7 days or 14 days. The relative mRNA expression (normalized to 18S) of each gene was normalized to its expression in aHSCs (t=14 days). *Significant difference (p<0.05) when compared to qHSCs (t= 4 hours).
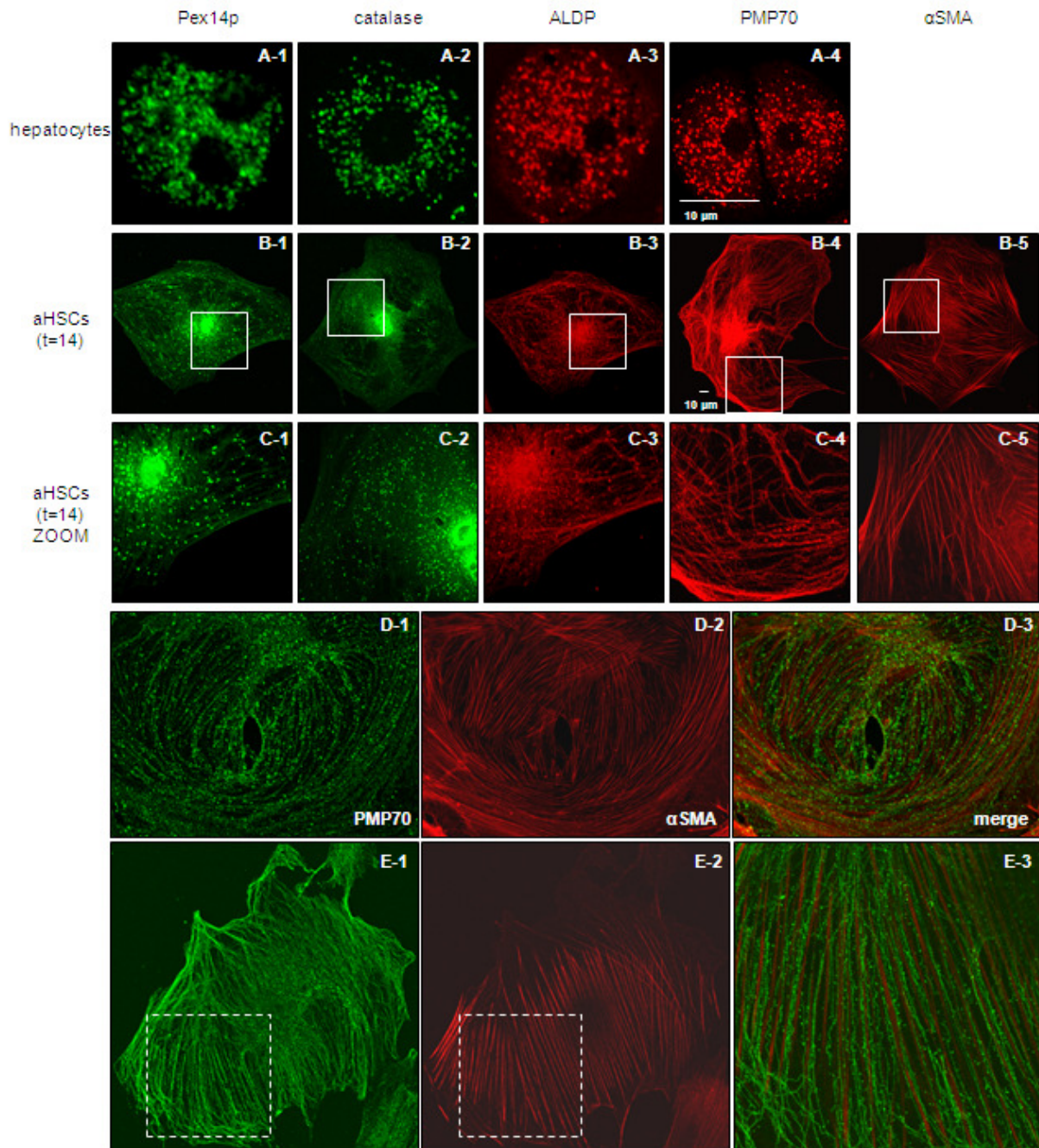
Figure 2. Subcellular location of peroxisomal proteins in hepatocytes, HSCs and PMF. Freshly isolated rat hepatocytes were fixed after a 4-hour attachment period (A) and freshly isolated HSCs were activated as described in Fig. 1 (B, zoomed images in C) followed by immunofluorescence microscopy to determine the subcellular location of Pex14p (column 1), catalase (column 2), ALDP (column 3), PMP70 (column 4) an αSMA (column 5). Co-immunofluorescent staining was performed for PMP70 (D-1, E-2) and the activation marker αSMA (D-2, E-2) in aHSC (D) and PMF (E). The merged images in D-3 and E-3 (higher magnification of box in E-1/E-2), show that the PMP70-tubules and the αSMA fibers run parallel through aHSCs, but do not exactly co-localize.

RNA interference-mediated silencing of PMP70 inhibits αSMA expression

The intimate association between the PMP70-positive tubules and the αSMA network prompted us determine the effect of down-regulating PMP70 on HSCs and PMFs activation. By applying RNA interference, PMP70 mRNA levels were reduced up to 80% in αHSCs (Fig. 3A), which was accompanied by similarly reduced PMP70 protein levels (Fig. 3C). Only minor effects were detected on the transcript levels of genes encoding other peroxisomal proteins (Fig. 3A) and the corresponding protein levels (Fig. 3C) and their subcellular location appeared unchanged (shown for Pex14p in Supplementary Fig. S5). Moreover, PMP70 silencing did not alter the viability of αHSCs (Fig 3D). In contrast, a strong (approximately 60%) reduction of αSMA mRNA and protein levels were detected in siRNA-PMP70-treated aHSCs (Fig. 3B and C). Also desmin mRNA and protein expression were significantly reduced, but less pronounced (approximately 40%). The mRNA levels of TGF-β and collagen-1a1 were not significantly changed in siRNA-PMP70-treated αHSCs (Fig. 3B). Essentially the same results were obtained when PMP70 expression was inhibited in PMFs (Supplementary Fig. S6). Immunofluorescence
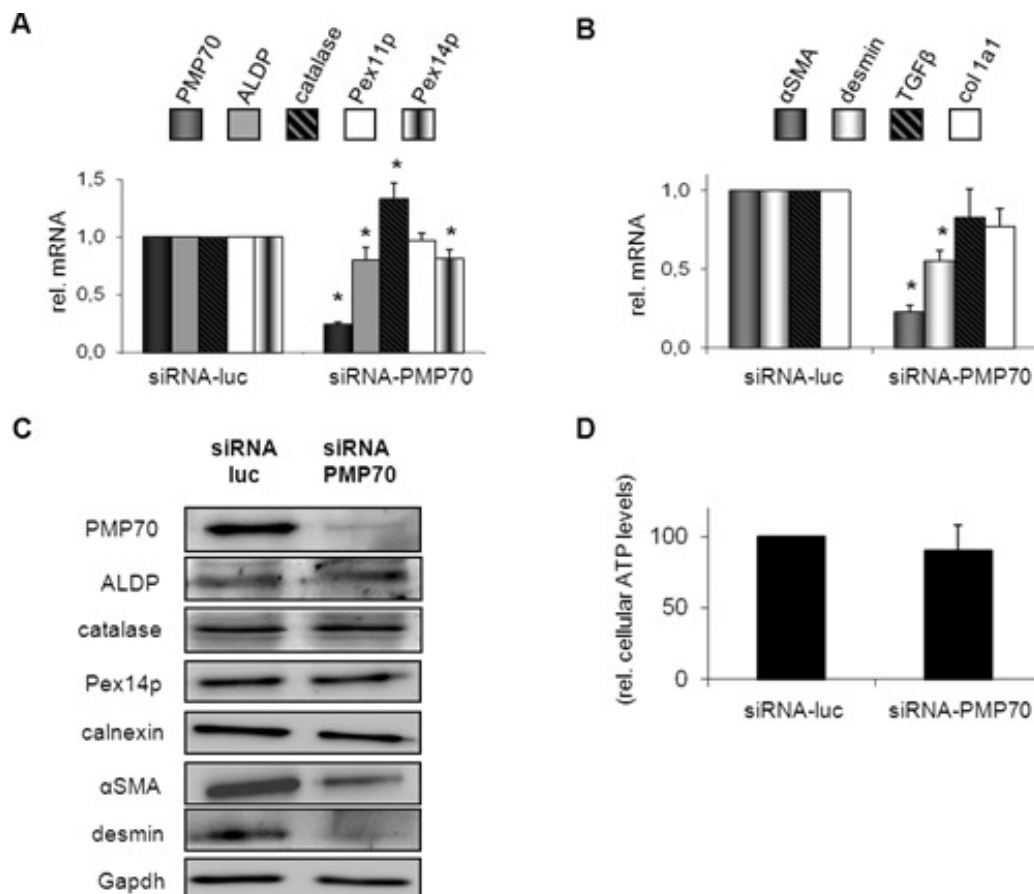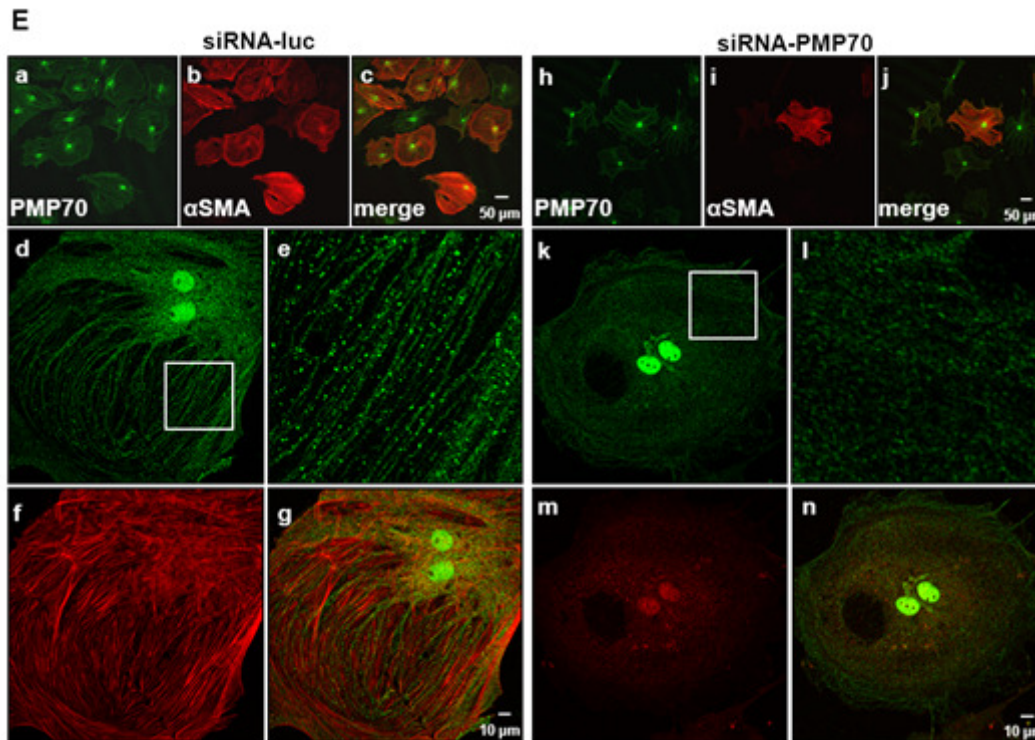
Figure 3. RNA interference-mediated silencing of PMP70 leads to a reduced expression of the activation markers αSMA and desmin in HSC. Primary rat HSCs were transiently transfected to silence PMP70 expression (siRNA-PMP70). Control cells were transfected with oligonucleotides directed against luciferase (siRNA-luc). After 3 and 6 days, HSCs were re-transfected with either siRNA-PMP70 or siRNA-luc. After 9 days, mRNA levels of PMP70, ALDP, catalase, Pex11p and Pex14p (A) or αSMA, desmin, TGF-β and collagen 1a1 (B) were analyzed by Q-PCR. The relative mRNA expression (normalized to 18S) of each gene was normalized to its expression after siRNA-luc transfection. Levels of selected proteins were analyzed by Western blotting, using antibodies against PMP70, ALDP, catalase, Pex14p, calnexin, αSMA and desmin. As a loading control, Gapdh expression was analyzed (C). The cell viability of siRNA-luc and siRNA-PMP70 transfected HSCs was determined by measuring cellular adenosine triphosphate (ATP) levels. In each experiment, measured ATP levels were corrected for the amount of cells and normalized to corrected ATP levels in siRNA-luc transfected cells (D). (E) The subcellular location of PMP70 (siRNA-luc: E-a, E-d and E-e; siRNA-PMP70: E-h, E-k and E-l) and the HSC activation marker αSMA (siRNA-luc: E-b and E-f; siRNA-PMP70: E-i and E-m) were analyzed by immunofluorescence microscopy. The merged images are shown in E-c and E-g (siRNA-luc) and E-j and E-n (siRNA-PMP70), respectively. * Significant difference ($p<0.05$) when compared to siRNA-luc treated control.

microscopy revealed a strong reduction in the number of αSMA-positive HSCs after silencing of PMP70 (Fig. 3E), which dropped from 92% in control-transfected (siRNA-LUC) HSCs (Fig. 3E a-c) to 38% in siRNA-PMP70 transfected HSCs (Fig. 3E h-j). The characteristic staining of PMP70 in tubular strands was absent in siRNA-PMP70-treated HSCs (compare Fig 3E d-g to 3E k-n) and coincided with the absence of αSMA staining.
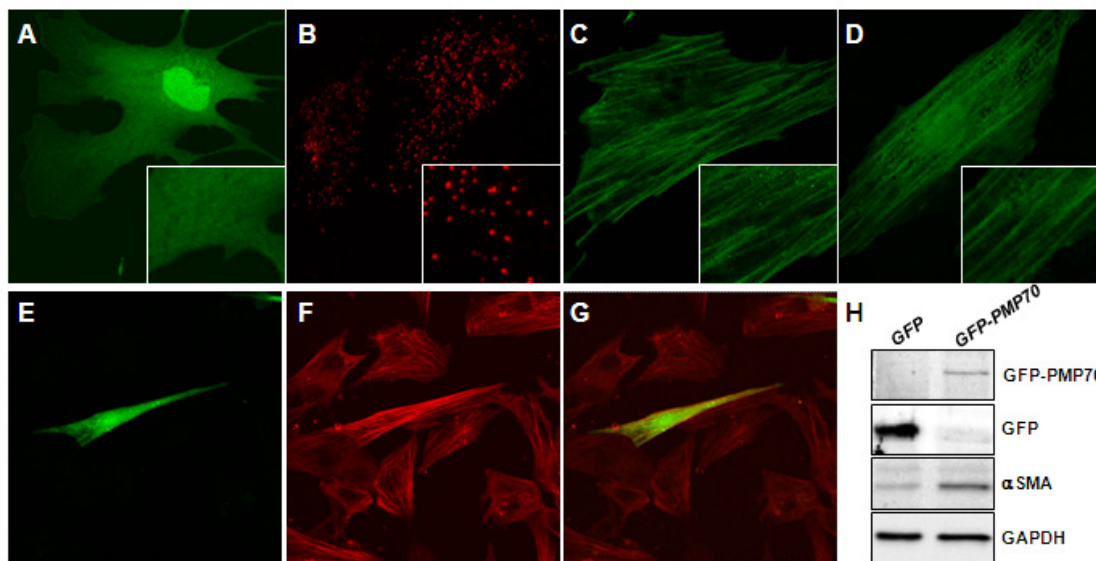
Figure 4. Recombinant PMP70-GFP accumulates in tubular structures and enhances aSma expression. HSC (A-C) and PMF (D-G) were transiently transfected with plasmids that encode cytosolic GFP (A), peroxisomal DsRed-SKL (B) or PMP70-GFP (C,D, E-F). Co-staining for PMP70-GFP (E) and aSma (F, overlay in G) reveals increased aSMA intensity in PMP70-GFP-positive PMF. H) Westernblot analysis of LX-2 cells transiently transfected with plasmids encoding GFP or PMP70-GFP and the effect on αSMA levels.

Transient expression of GFP-PMP70 stimulates αSMA expression

Complementary to these experiments, we performed overexpression of GFP-tagged PMP70 in HSC, PMF and LX-2 cells (Fig. 4). Similar to endogenous PMP70, transiently expressed GFP-PMP70 was predominantly detected in tubular strands in HSC and PMF (Fig. 4C and D, respectively; compare to cytosolic GFP (A) and peroxisomal DsRed-SKL (B)). The GFP-PMP70-transfected PMF showed an increased αSMA staining intensity compared to surrounding untransfected cells (Fig. 4 E-G). The transfection efficiency of primary HSCs and PMFs is low (<10%), which precluded confirmation of this effect by Western blot analysis. However, transfection efficiencies were much higher for the human stellate cell line LX-2 (>50%) and overexpression of GFP-PMP70 in these cells was accompanied by a significant increase in αSMA protein levels compared to control (GFP alone) transfected cells (Fig. 4H).

DISCUSSION

In this study, we show that the intracellular ABC transporters PMP70 is required for the production of the αSMA cytoskeleton in liver myofibroblasts, which is a characteristic feature of these cells causing liver fibrosis. PMP70 resides primarily in tubular strands that parallel the alpha-smooth muscle actin (αSMA) network. Suppression of PMP70 strongly decreased the expression of αSMA as well as desmin, while overproduction of GFP-PMP70 stimulated αSMA production. In contrast, other activation markers of activated myofibroblasts (TGF-β and collagen-1a1) did not respond to manipulation of PMP70 expression.

Peroxisomes are functionally and morphologically highly dynamic organelles and their function is cell- and condition-dependent. Moreover, the population of peroxisomes within one cell may be heterogeneous based on size and/or protein composition [23,32,33]. Peroxisomes may exist as individual spheres, but may also be interconnected forming a peroxisome reticulum [22,34]. The PMP70-containing tubular strands in hepatic myofibroblasts, however, seem to be an unprecedented morphological variation of a peroxisome, while the characteristic spherical peroxisomes were also present and contain catalase, Pex14p and ALDP. Small amounts of these typical peroxisomal markers were also found to colocalize with PMP70 in the tubular strands. Conversely, variable amounts of PMP70 were detected in the typical spherical peroxisomes containing Pex14p, catalase and ALDP, suggesting that the tubular strands are a specialized form of the peroxisome. At present, it is unknown how the cellular pools of the various "peroxisomal" proteins like PMP70, ALDP, Pex14p and catalase are differentially distributed among the spherical peroxisomes and the tubular strands. The PMP70-containing tubules have not been observed in other cell types, including skin fibroblasts, where both endogenous PMP70 and artificially expressed PMP70 reside in spherical peroxisomes [35]. We show that artificial expression of GFP-tagged PMP70 also accumulates in tubular strands in hepatic myofibroblasts, whereas this hybrid protein appears in peroxisomes in CHO [30] and HepG2 cells (data not shown). This demonstrates that the tubular strands are the genuine subcellular location of PMP70 in the hepatic myofibroblasts. We initiated subcellular fractionation experiments of cultured hepatic myofibroblasts to biochemically characterize the PMP70-containing tubules. However, it appears that organellar markers for peroxisomes, mitochondria, endoplasmic

reticulum as well as PMP70 all appear in the low-speed (500g) centrifugation step after osmotically-stabilized lysis of these cells (data not shown). The organelles are most likely attached to the cytoskeletal proteins preventing their purification from hepatic myofibroblasts by standard cell fractionation protocols.

Exogenous added arachidonic acid induces the formation of tubular peroxisomes in HepG2 cells [22]. Interestingly, it has recently been shown that hepatic stellate cells efficiently absorb exogenous arachidonic acid when culture-activated *in vitro* [36] and this may be (one of) the mechanism(s) that causes the formation of the PMP70-containing tubular strands. Arachidonic acid is the substrate for the synthesis of prostaglandins and leukotrienes by hepatic myofibroblasts and both have profound effects on activation of stellate cells *in vitro* and liver fibrosis *in vivo*. Prostaglandins, in particular prostaglandin E2, represses HSC activation [37], while leukotrienes promote fibrosis both in liver and lung [38,39]. Peroxisomes are involved in the degradation of prostaglandins [40,41] and leukotrienes [42]. Patients with Zellweger syndrome, a congenital disorder characterized by the absence of intact peroxisomes, secrete high levels of metabolic intermediates of prostaglandins [43] as well as leukotriene B4 and E4 [44-46] in the urine compared to healthy controls. Inhibition of PMP70 expression in HSC/PMF led to a specific reduction of αSMA and desmin, but not of collagen 1a1 or TGF-β. Such a selective reduction of αSma expression was also observed when prostaglandin synthesis was inhibited in human hepatic cell lines using the COX-2 inhibitor, NS-398, while collagen IV levels did not change [47] and Collagen 1a1 expression was actually increased [37]. These data suggest that stimulation of intracellular prostaglandin production may activate hepatic myofibroblasts. On the contrary, exogenously added PGE2 has a potent antifibrotic action [37]. Thus, the profibrotic effect of PGE2 is largely determined by the balance between its synthesis, cellular export and intracellular degradation. Earlier studies suggest that PMP70 transports long chain fatty acids and bile salt intermediates into peroxisomes to become metabolized through beta-oxidation, though solid evidence for these activities is not yet available [48,49]. Given the role of peroxisomes in prostaglandin and leukotriene breakdown and the selective effect of PMP70 expression on αSMA levels, it is now interesting to determine the possible role of PMP70 in balancing intracellular prostaglandin and/or leukotriene levels.

The PMP70-containing tubular strands lay parallel to the αSMA network. PMP70-containing peroxisomes have previously been shown to associate with microtubules [50], but this has only been studied in relation to peroxisome movement and division. Our data imply a direct role for PMP70 in the development of the αSMA cytoskeleton in hepatic myofibroblasts. Given its presumed role in substrate transport, this is most likely related to a role of PMP70 in PG and/or LT metabolism as described above. However, this remains to be established and alternative functions of PMP70 on the assembly of the αSMA network may be involved.

Our study does not provide evidence that peroxisomal activities are involved in the metabolism of fatty acids that are associated with the activation of hepatic stellate cells, as the activation process was not accompanied by increased expression of genes involved in β-oxidation, such as AOX and MFP (see also Supplemental Figure S7). This is in line with the recent observation that *in vitro*-activated HSCs specifically lose retinyl esters, but in fact increase the triacylglycerol species containing polyunsaturated fatty acids [36]. This implies that the initial stages of HSCs activation does not require increased metabolism of (very-)long chain fatty acids.

Taken together, our data show that the ABC transporter PMP70 controls the development of the αSMA cytoskeleton in hepatic myofibroblasts and is therefore a contributing factor in liver fibrosis. PMP70 is present in tubular strands that parallel the αSMA fibers.

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Supplementary data
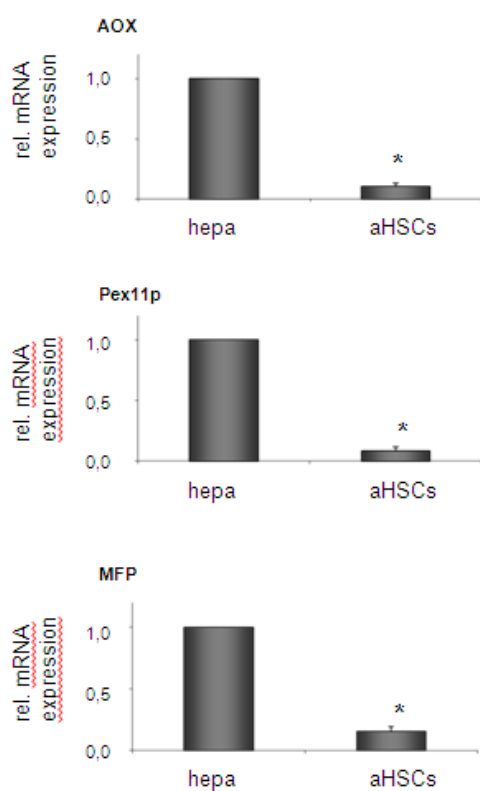
Figure S1. mRNA expression of the peroxisomal markers AOX, Pex11p and MFP in hepatocytes and aHSCs. Hepatocytes and HSCs were isolated using standard protocols (see materials and methods). HSCs were cultured in the presence of serum for 14 days (aHSCs). The relative mRNA expression (normalized to 18S) of each gene was normalized to its expression in hepatocytes (hepa). * Significant difference ($p<0.05$) when compared to hepatocytes.
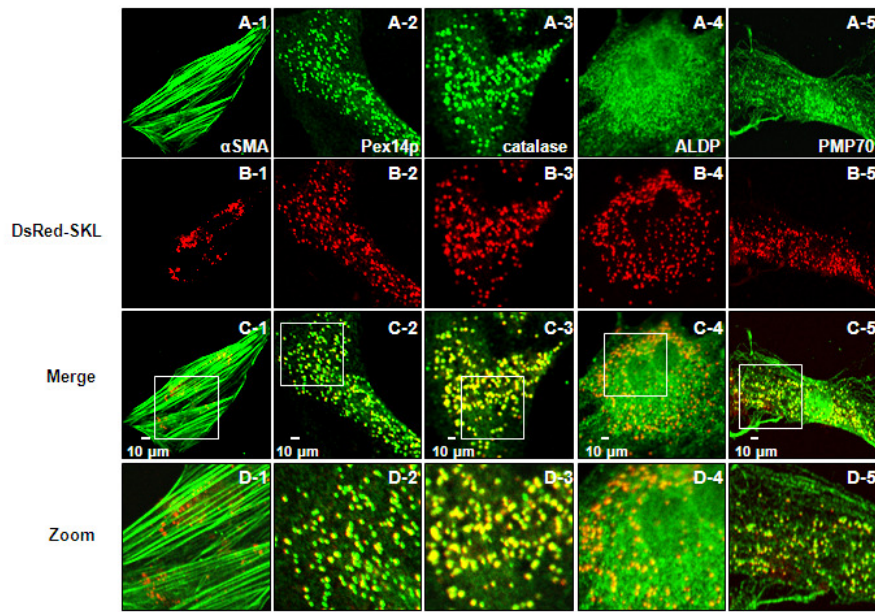
Figure S2. Co-localization studies of peroxisomal proteins with the peroxisomal marker DSRed-SKL in aHSCs. Activated HSCs were transfected with 4μg DSRed-SKL. Forty eight (48) hours after transfection, stellate cells were fixed and subjected to immunofluorescence microscopy to determine co-localization of αSMA (A), Pex14p (B), catalase (C), ALDP (D) and PMP70 (E)) with DSRed-SKL (row A-2 to E-2). Merged images are displayed in row A-3 to E-3 and higher magnifications or the regions of interest are shown in row A-4 to E-4.
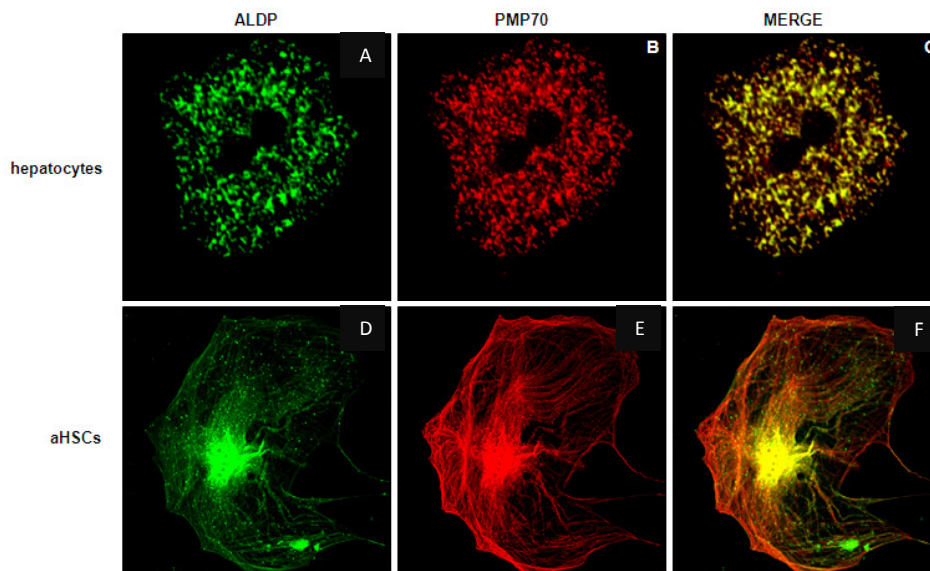
Figure S3. Subcellular location of ALDP and PMP70 in hepatocytes and aHSCs. Freshly isolated rat hepatocytes (A-C) were fixed after a 4h-attachment period and HSCs (D-F) after 14-day culture activation followed by immunofluorescence microscopy to determine the subcellular location of ALDP (A,D) and PMP70 (B,E). Merged images are shown in C and F.
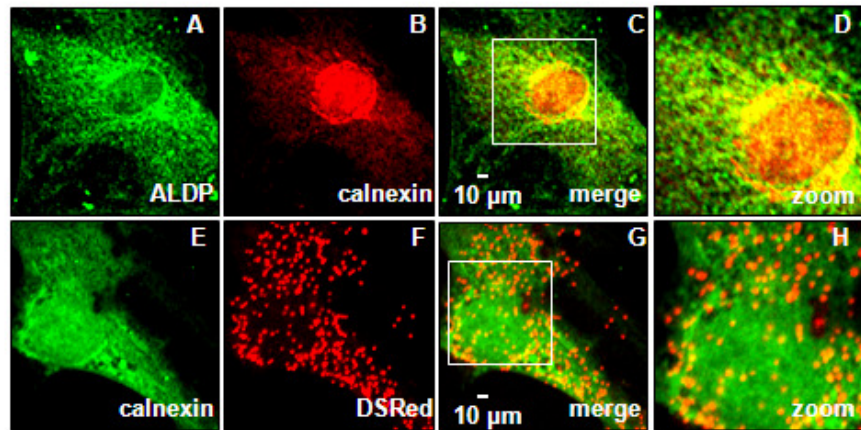
Figure S4. ALDP resides partly in the endoplasmic reticulum in aHSCs. Activated HSCs were processed for immunofluorescence microscopy to determine the co-localization of ALDP with calnexin (upper panels A-D). To determine co-localization between the (endogenous) ER marker calnexin and the (transfected) peroxisomal marker DSRed-SKL, aHSCs were transfected with 4μg DSRed-SKL, similarly as in Figure S2.
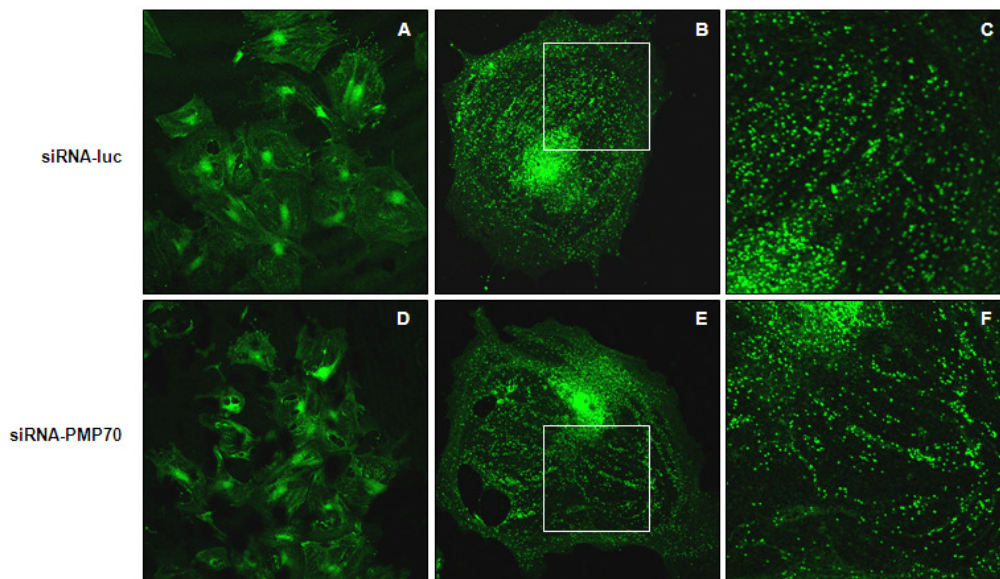
Figure S5. RNA interference-mediated silencing of PMP70 does not affect the sorting of the peroxisomal marker Pex14p. PMP70 expression was silenced as described in Fig. 3. The subcellular location of Pex14p (siRNA-luc: A-C; siRNA-PMP70: D-F) was analyzed by immunofluorescence microscopy. The zoomed images are shown in C (siRNA-luc) and F (siRNA-PMP70) respectively, showing that the subcellular location of Pex14p is not affected by RNA interference-mediated silencing of PMP70.
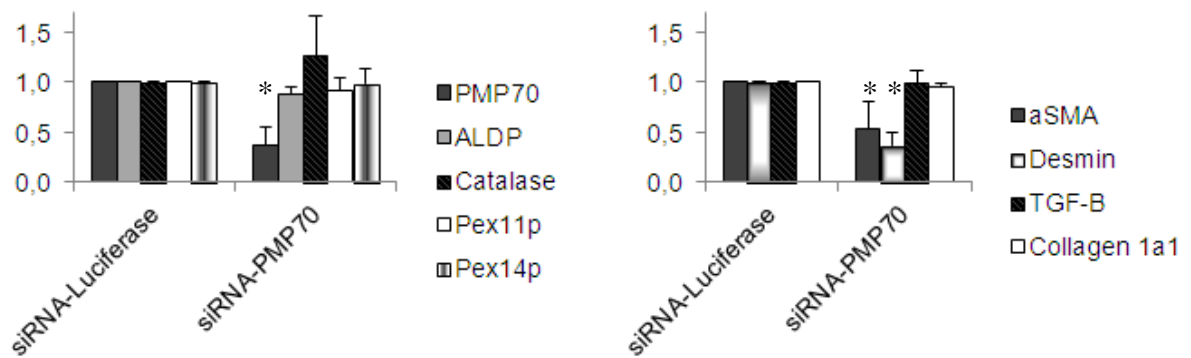
Figure S6. RNA interference-mediated silencing of PMP70 leads to a reduced expression of the activation markers αSMA and desmin in PMF.

Primary rat PMF were transiently transfected to silence PMP70 expression (siRNA-PMP70). Control cells were transfected with oligonucleotides directed against luciferase (siRNA-luc). After 72 h, mRNA levels of PMP70, ALDP, catalase, Pex11p and Pex14p (A) or αSMA, desmin, TGF-β and collagen 1a1 (B) were analyzed by Q-PCR. The relative mRNA expression (normalized to 18S) of each gene was normalized to its expression after siRNA-luc transfection.
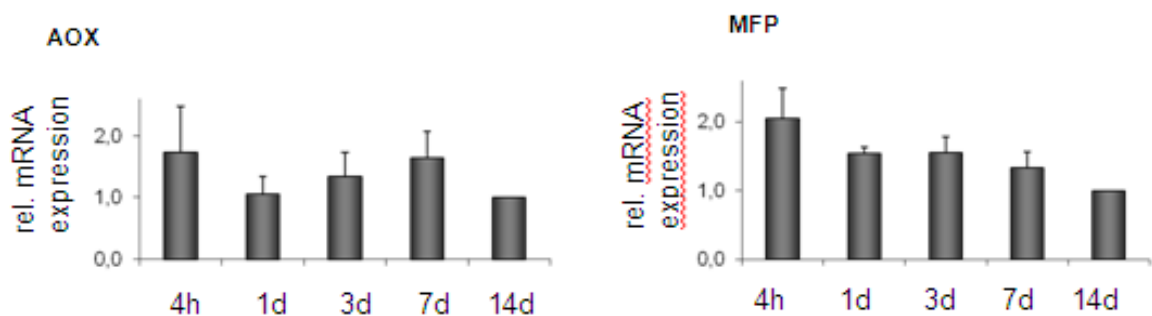
Figure S7. Transdifferentiating HSC do not show an (transient) increase in the expression of enzymes of LCFA β-oxidation. Freshly isolated HSC were cultured in the presence of serum for 4 hours, 1 day, 3 days, 7 days or 14 days. The relative mRNA expression (normalized to 18S) of Acyl CoA oxidae (AOX) and the Multifunctional Protein (MFP) was normalized to its expression in aHSCs (t=14 days). *Significant difference (p<0.05) when compared to qHSCs (t= 4 hours).

SUPPLEMNTARY TABLES

Table S1: Primers used for PMP70 RNA *interference*

RNA	Sense	Antisense
luciferase	5'-cuu acg cug agu acu ucg auu-3'	5'-ucg aag uac uca gcg uaa guu-3'
PMP70	5'-gag aca ggg uac uuc aua c-3'	5-gua uca agu acc cug ucu c-3'

Table S2: Sequences of Primers and Probes used for Real-time Quantitative PCR Analysis

Gene	Sense	Antisense	Probe
18S	5'-cgg cta cca cat cca agg a -3'	5'-cca att aca ggg cct cga aa-3'	5'FAM-cgc gca aat tac cca ctc ccg a-TAMRA3'
ALDP	5'-cat ctg gcc tgc tca tgg ta -3'	5'-ttc atg gct tct gag tct gac tct-3'	5'FAM-ccc cat cat cac agc cac tgg ct-TAMRA3'
AOX	5´-gcc acg gaa ctc atc ttc ga-3´	5´-cca ggc cac cac tta atg ga-3´	5´FAM-cca ctg cca cat atg acc cca aga ccc-TAMRA3´
Baat	5´-tgt aga gtt tct cct gag aca tcc taa-3´	5´-gtc caa tct ctg ctc caa tgc-3´	5´FAM-tgc caa ccc ctg ggc cca g-TAMRA3´
catalase	5'-gga tta tgg cct cc gaga tct-3'	5-acc ttg gtc agg tca aat gga t-3'	5'FAM-atg cca tcg cca gtg gca att acc-TAMRA3'
collagen 1a1	5'-tgg tga acg tgg tgt aca agg-3'	5'-cag tat cac cct tgg cac cat-3'	5'FAM-tcc tgc tgg tcc ccg agg aaa ca-TAMRA3'
desmin	5-tgg tac aag tcc aag gtt tca gac t-3'	5'-ctg gtg tcg gta ttc cat cat ct-3'	5'FAM-aag aac aac gat gcg ctg cgc c-TAMRA3'
MFP	5'-agg ttg gag cag gat gga ttg-3'	5'-ctt gct ggc att gct gaa gtc-3'	5'FAM-cgg aat cag ccc atg act ccc ga-TAMRA3'
Pex14p	5'-gct acc aca tca acc aac tgg at-3'	5'-gga act gtc tcc gat tca gaa ga-3'	5´FAM-tga gct caagtc aga aat caa ctc tct gaa agg ac-TAMRA3´
Pex11p	5´-gcc cgc cac tact ac tat ttc ct-3´	5´-tct gtc gcg tgc aac ttg tc-3´	5´FAM-cat atg cag caa gac ctc ata cag atc ccg -TAMRA3´
PMP70	5'-ctg gtg ctg gag aaa tca tca at-3'	5'-cca gat cga act tca aaa cta agg t-3'	5'FAM-tga tca tgt tcc ttt agc aac acc aaa tgg-TAMRA3'
PPARα	5´-cac cct ctc tcc agc ttc ca-3´	5´-gcc ttg tcc cca cat att cg-3´	5´FAM-tcc cca cca gta cag atg agt ccc ctg-TAMRA3´
PPARγ	5´-cac aat gcc atc agg ttt gg-3´	5´-gct ggt cga tat cac tgg aga tc-3´	5´FAM-cca aca gct tct cct tct cgg cct g-TAMRA3´
αSMA	5´-gcc agt cgc cat cag gaa c-3´	5´-cac acc aga gct gtg ctg tct t-3´	5´FAM-ctt cac aca tag ctg gag cag ctt ctc ga-TAMRA3´
TGF-β	5'-ggg cta cca tgc caa ctt ctg-3'	5'-gag ggc aag gac ctt gct gta-3'	5'FAM-cct gcc cct aca ttt gga gcc tgg a-TAMRA3'

Table S3: Antibody dilutions for protein analysis

Antibody	Western Blotting	Immunofluorescence	Company
Mouse α-ALDP	1:1000	1:100	Clone 1D6, Euromedex, Mundolsheim France
Rabbit α-Baat	1:2000		Generous gift of Prof. C. Falany, Birmingham, AL, USA [51]
Rabbit α-calnexin	1:2000	1:200	SPA 860D, Stressgen, MI, USA
Rabbit α-catalase	1:2000	1:200	Calbiochem, La Jolla, CA, USA
Mouse α-Cytochrome C	1:2000		BD Biosciences, Franklin Lakes, NJ, USA
Mouse α-desmin	1:1000		Sigma-Aldrich, St. Louis, MO, USA
Mouse α-Gapdh	1:10.000		Calbiochem, La Jolla, CA, USA
Rabbit α-Pex14p	1:2000	1:200	Generous gift of Dr. M. Fransen, Leuven, Belgium [52]
Rabbit α-PMP70	1:1000	1:200	Sigma-Aldrich, St. Louis, MO, USA
Mouse α-alphaSMA	1:2000	1:500	Sigma-Aldrich, St. Louis, MO, USA

General discussion and future prospects

Atta ur Rehman and Klaas Nico Faber

Department of Gastroenterology and Hepatology
University Medical Center Groningen
University of Groningen

Chronic liver diseases generally result in liver fibrosis [1-3]. Hepatic stellate cells (HSCs) and portal myofibroblasts (PMFs) are the key players in fibrogenesis. In response to inflammatory cytokines and oxidative stress, HSCs and PMFs become activated and transdifferentiate into proliferative motile myofibroblasts that produce excessive amounts of extracellular matrix proteins, such as collagens and fibronectins [2,4]. Liver fibrosis may progresses into cirrhosis, which strongly increases the risk for liver cancer. So far, there is no effective therapy for the treatment of liver fibrosis [5]. In this thesis we investigated the role of ABC-transporters in the development of liver fibrosis and their potential for being therapeutic targets.

In Chapter 2, we demonstrate that the multidrug resistance-associated protein 1 (Mrp1) is required for the activation of HSCs and PMFs and promotes CCl_4-induced liver fibrosis in mice. In chapter 3, we show that the Mrp1 inhibitor reversan has no cytotoxic effects on hepatocytes exposed to oxidative stress, while MK571 induces hepatocyte necrosis. We conclude that reversan is likely a safer drug to treat liver fibrosis *in vivo*. In Chapter 4, we show that glutathione and antioxidant enzymes serve complementary roles in protecting HSCs against oxidative stress. Finally in chapter 5, we show that the intracellular ABC transporter Peroxisomal Membrane Protein (PMP70) is required for the generation of the characteristic alpha-smooth muscle actin (αSma) network in HSCs and PMFs and thus plays a role in the activation of hepatic myofibroblasts.

The multidrug resistance-associated protein 1 is a transporter for glutathione, both reduced (GSH) and oxidized (GSSG), as well as for cysteinyl leukotriene C_4 ($CysLTC_4$) [6,7]. Previously, we have shown that the expression of Mrp1 is induced upon activation of HSCs and that treatment of activated HSCs with the Mrp-inhibitor MK571 rapidly induced necrosis in these cells [8]. Since Mrp1 is the most prominently expressed Mrp-type transporter on HSCs [8], this suggested that Mrp1 is essential for the viability of HSCs. Since MK571 inhibits several Mrps, as well as cysteinyl leukotriene receptors, it was essential to determine in more detail the role of Mrp1 in HSCs biology. Using a more specific inhibitor of Mrp1, reversan [9], as well as Mrp1-specific RNA interference and recombinant overexpression of Mrp1, we show in chapter 2 that Mrp1 is required for the activation of HSCs and PMFs and not so much for viability. In line with the *in vitro* data, we found that liver fibrosis is attenuated in CCl_4-treated Mrp1 (*Abcc1*) knockout mice compared to wild type mice receiving the same treatment. Reversan did not induce significant HSCs necrosis, but reduced the expression of αSma and collagen1a1 with no

apparent effect on cell morphology, which is in sharp contrast to the effects of MK571 [8]. The different effects of these two inhibitors of Mrp1 must be due to the off-target effects of MK571. MK571 has been shown to inhibit the transport activity of several Mrp's, including Mrp1-6 [8,10-12], while reversan does not block the export activity of Mrp2-5 [9]. However, the expression of Mrp2-5 is very low in HSCs and PMFs (chapter 2), so it is unlikely that one of the other Mrp transporters is crucial for the viability of HSCs. MK571 is also known to antagonize the LTD_4 receptor [10,11,13] that has been reported to promote activation of HSCs and fibrosis [14]. Moreover, exogenously added LTC_4 induced activation of HSCs and the use of an LTD_4 receptor antagonist led to reversal of fibrosis *in vivo* [15-17]. Thus, the MK571-induced necrosis of HSCs may be linked to inhibition of the LTD_4 receptor although this would need further experimental proof.

GSH and GSSG are natural substrates for Mrp1 and intracellular accumulation of one of these compounds may have a direct effect on HSCs activation. However, earlier studies already showed that manipulation of glutathione levels in HSCs did not affect markers of fibrosis [18,19]. Alternatively, Mrp1 may transport leukotriene C_4 (LTC_4) as it was previously shown that HSCs contain all the enzymes for leukotriene synthesis [20]. We showed that inhibitions of leukotriene synthesis had a similar inhibitory effect on fibrosis markers as the Mrp1 inhibitor reversan or Mrp1-targeted RNA interference. This suggests that the profibrotic effect of Mrp1 is due to the export of leukotrienes. This is in line with recent observations that exogenously added LTC_4 promotes collagen production by HSCs [20]. Moreover, HSCs efficiently accumulate arachidonic acid (AA) during the transdifferentiation phase [21]. AA is the precursor for leukotriene synthesis and it is now highly relevant to determine whether blocking Mrp1 indeed results in the intracellular accumulation of LTC_4. Our data show that Mrp1 is a potential drug target for the treatment of liver fibrosis, however, a potential drawback of pharmaceutical inhibitors of Mrp1 may be expression of Mrp1 in extrahepatic tissues, such as lung, brain and intestine [22]. Mrp1 knockout mice have no phenotype under controlled laboratory conditions [23], and we showed that in response to CCl_4, damage markers like ALT and AST were not different between Mrp1-ko and wild type mice. The absence of Mrp1 apparently does not lead to increased sensitivity towards inflammatory conditions as it is observed for DSS-induced colitis [24]. Moreover, Mrp1 levels are very low in hepatocytes, which make up over 80% of the liver, and Mrp1 also does not seem to play a protective role

during CCl$_4$-induced hepatocyte damage. Thus, pharmacological inhibition of Mrp1 may have great potential in the treatment of liver fibrosis.

In chapter 3 we addressed the issue of toxicities associated with Mrp1 inhibitors in greater detail. We compared the cytotoxic effects of two inhibitors of Mrp1, MK571 and reversan, on hepatocytes and HSCs in the absence and presence of oxidative stress. We found that reversan induced cell death in HSCs only in the presence of menadione. Previously, we showed that MK571 induces necrosis in activated HSCs even without further exposure to reactive oxygen species (ROS) [8]. Primary hepatocytes were also sensitive to MK571 treatment alone, but massive necrosis was induced when these cells were co-exposed to menadione. In sharp contrast, reversan did not induce detectable levels of cell death in hepatocytes, even if they were co-exposed to menadione. We confirmed that reversan is a more specific inhibitor of Mrp1 than MK571. Reversan did not block the Mrp-mediated export of CMFDA and fluorescent bile salts from primary rat hepatocytes, while it did prevent the Mrp1-mediated export of CMFDA from HSCs (Chapter 2). In contrast, MK571 prevented the export of CMFDA from both HSCs and primary rat hepatocytes. We also found that export of GSSG from cells is crucial for cellular resistance against oxidative stress. Enhanced expression of Mrp1 in activated HSCs may thus serve a dual role, namely 1) LTC$_4$ export to promote liver fibrosis in an autocrine signalling pathway and 2) GSSG export to protect HSCs from oxidative damage. Taken together, these data show that reversan has no adverse effects on hepatocytes, even under oxidative stress conditions, while MK571 has potent cytotoxic effects on these cells. Thus, for therapeutically targeting Mrp1 to treat liver fibrosis *in vivo*, reversan is likely a safer option than MK571.

In chapter 4, we showed that intracellular glutathione (GSH) levels (without proportional increase in oxidized glutathione (GSSG)) and the expression of the hydrogen peroxide neutralizing enzyme-glutathione peroxidase 1 (GPx1) are increased during HSCs activation. The sharp increase in reduced glutathione levels are surprizing as expression of Mrp1 is also increased during HSCs activation (chapter 2 of this thesis). Mrp1 transports GSSG much more efficiently than GSH [25,26] and the accumulation of GSH may be a result of strongly increased GSH synthesis, while concomitant produced GSSG is exported. However, the expression of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis, does not change during activation. Therefore, it is highly interesting whether HSCs express GSH importers. Such transporters have been

described for kidney, alveolar and intestinal epithelial cells and [27-30]. Alternatively, Mrp1 activity in activated HSCs may be predominantly consumed by high affinity substrates like leukotrienes, prostaglandins and/or GSSG and limits export of GSH [25].

A remarkable finding was the sharp decrease in mitochondrial superoxide dismutase (MnSOD) and peroxisomal catalase during activation of HSCs. Both proteins remained detectable by immunofluorescence microscopy, but still the levels of these antioxidant enzymes are probably 5-10 fold higher in quiescent HSCs. In contrast, GPx expression was significantly induced in activated HSCs. Both Gpx1 and catalase were found to be crucial for the protection of HSCs against H_2O_2- and menadione-induced oxidative stress. Chronic liver injury is almost invariably accompanied by increased oxidative stress, activation of stellate cells and fibrogenesis [31-34]. Activated HSCs must be well protected against oxidative stress, since they survive and proliferate in the chronically injured liver. The oxidative stress in chronically injured liver is composed of several reactive oxygen species, including hydrogen peroxide and superoxide anions. Mice overexpressing Gpx1 are better protected against oxidative stress, e.g. against chemical or myocardial ischemia-reperfusion induced injury and, conversely, GPx1 deficient mice are more prone to oxidative stress-induced injury [35,36]. Since GSH is required for the activity of Gpx1, the observed increase in total GSH and Gpx1 in activated HSCs may be a coordinated and adaptive response to oxidative stress. Superoxide dismutases like Mn-SOD and Cu,Zn-SOD prevent the toxicity induced by superoxide anions by converting them into H_2O_2. MnSOD is a known tumour suppressor gene and its disruption in mice is lethal due to mitochondrial dysfunction [37]. Surprisingly, MnSOD is decreased during activation of HSCs, both at the mRNA and protein level. This appears to be inconsistent with the described protective effects of Mn-SOD. MnSOD expression is under control of PPAR-γ [38] and PPAR-γ agonists enhances MnSOD activity [39]. Since PPAR-γ expression is strongly suppressed during HSCs activation, MnSOD levels may drop as a result of this. At present, it is not known whether the reduced levels of Mn-SOD in the activated HSCs are sufficient to detoxify reactive oxygen species generated in mitochondria or whether alternative mechanisms take over.

Catalase is a H_2O_2-neutralizing enzyme and resides in peroxisomes. While mRNA levels for catalase do not drop during HSCs activation, the corresponding protein became almost undetectable by Westernblotting. Only immunofluorence microscopy revealed a

typical peroxisomal staining of catalase. At present it is unclear how the catalase protein disappears from activated HSCs. One possibility is a rapid and selective intra peroxisomal degradation of this antioxidant protein. Peroxisomes contain a Lon protease that may be involved in this [40,41]. The expression of this protease in HSCs has not been studied yet. An alternative, and more provocative possibility would be that catalase is exported into the surrounding medium. Catalase activity is enhanced in plasma of patients suffering from fatty liver, acute alcoholic hepatitis and cardiac failure [42,43], but the source of catalase release is unknown. One could presume that plasma catalase originates from broken red blood cells (RBCs), but fatty liver and acute alcoholic hepatitis are not associated with RBCs disorders. Still, an export pathway for catalase is highly hypothetical. First experiments could be aimed at measuring the catalase activity in the medium harvested from transdifferentiating HSCs. Finally, we observed that glutathione depletion in HSCs sensitizes these cells to H_2O_2-induced cell death and replenishing glutathione reduces this effect. GSH is an important redox regulator in the cell and reacts with H_2O_2 to produce water and glutathione disulfide (GSSG). Therefore, we assume that oxidative stress changes the redox state of the cell in the absence of excess GSH, leading to cell death.

The Peroxisomal Membrane Protein 70 (PMP70) belongs to the D subfamily of ABC transporters that is traditionally called the peroxisomal ABC transporters [44]. The first identified member was PMP70 (*ABCD3*), which is highly abundant in liver and kidney, but is present in many different tissues and cell lines. The most intensively studied member of this subfamily is the Adrenoleukodystrophy protein (ALDP/*ABCD1*), mutations in which cause X-linked Adrenoleukodystrophy [45]. X-ALD leads to Progressive neurodegenerative decline, leading to a vegetative state without treatment. Biochemically, it is characterized by the impairment in breakdown of very-long chain fatty acids that accumulate in serum and other body fluids. The ABCD subfamily further contains ALD-related protein (ALDRP/*ABCD2*) and PMP70-related protein (P70R/*ABCD4*), the latter of which was recently shown not to reside in peroxisomes, but in lysosomes [46]. Mutations in *ABCD4* cause impairment in vitamin B12 metabolism. Thus, designating the ABCD subfamily as "peroxisomal" ABC transporters may need revision. This may be even more urgent as in chapter 5, we showed that PMP70 is predominantly localized in tubular structures that lay parallel to αSMA and is required for the expression of αSMA in HSCs and PMFs. Only low amounts of peroxisomal PMP70

were also observed, while the PMP70-stained tubules were consistently detected. siRNA-based suppression of PMP70 led to the disappearance of the tubular PMP70 staining and concomitant loss of the αSma network. Recombinant GFP-PMP70 was almost exclusively detected in tubular structures and increased αSma expression. Interestingly, other peroxisomal proteins like catalase and Pex14p were mainly observed in peroxisomes. ALDP expression is as least as high in HSCs as in hepatocytes and significant amounts were found in the endoplasmic reticulum. This staining pattern was clearly distinct from the PMP70 tubules. The PMP70 transporter is usually localized on the membrane of peroxisomes [44]. Though direct evidence is still lacking, PMP70 is thought to be involved in the import of long chain fatty acids into the peroxisomes. Moreover, a role in transport of bile acid intermediates into peroxisomes has been proposed for PMP70 [47,48]. Peroxisomes are also reported to be involved in the β- and ω-oxidation of leukotrienes and prostaglandins that play an important role in inflammation and allergy [49,50]. Metabolism of these molecules may be an additional pathway controlling HSC activation, as we found in chapter 2 that leukotriene synthesis and transport are important for autocrine signalling in these cells to promote liver fibrosis. So far, the import mechanism(s) of leukotrienes and prostaglandins into the peroxisome is/are unknown. The fact that PMP70 and ALDP show a largely non-peroxisomal localisation (Chapter 5), as well as the fact that catalase protein rapidly disappears from activated HSCs while transcription of the corresponding gene remains intact (Chapter 4), indicate that peroxisome biogenesis and/or function may be abnormal in these cells. PPARα is a nuclear receptor involved in the development of peroxisomes. HSCs express at best minor amounts of PPARα [51,52]. In line, we found that the PPARα agonist fenofibric acid did not induce the expression of peroxisomal proteins like AOX, MFP and Pex11p (data not shown). The presence of peroxisomal membrane proteins in the ER has been documented before [53] and accumulating evidence support that the ER may be the primary insertion site for peroxisomal membrane proteins from where they are sorted to peroxisomes [54]. The presence of ALDP may therefore be a result of a lower sorting efficiency of ALDP in HSCs as compared to hepatocytes, where an ER-location of ALDP is not evident. The presence of PMP70 in the tubular strands seems to be a truly different subcellular location than ever observed before. Pmp70 has been found in tubular peroxisomes [55,56], but these remain spherical in shape and do not extend throughout the cytoplasm. Unfortunately, subcellular fractionation experiments were unsuccessful to

further purify the PMP70-containing tubules for further characterisation. It would be interesting the analyse the effect of the overexpression of PPARα in HSCs on the subcellular location of ALDP and PMP70, which could show that PPARα-targets are insufficiently expressed in HSCs for normal peroxisome biogenesis as well as the effect on the αSma network. Such an experiment could also reveal whether leukotriene turnover in HSCs is effected when peroxisome biogenesis is manipulated in HSCs.

Future perspectives and concluding remarks

The research described in this thesis reveals that at least 2 ABC transporters play a role in hepatic myofibroblasts that cause liver fibrosis. Mrp1 expression is induced upon HSCs/PMFs activation and promotes expression of αSma and collagen 1a1. The absence of Mrp1 does not lead to adverse effects in mice, while chemically-induced fibrosis is attenuated. This paves way for the systemic application of Mrp1 antagonists as antifibrotic therapy. The pharmaceutical Mrp1 inhibitor Reversan is good candidate drug to test in animal models of liver fibrosis. Alternatively, Mrp1-specific antibodies that could bind the extracellular epitope of Mrp1 and inhibit its transport activity could be tested. The presence of HSCs in the space of Disse where they are in direct contact with the blood circulation may allow effective inhibition of Mrp1 by such antibodies.

The fact that partial inhibition of Mrp1 already leads to suppression of HSCs/PMFs activation may allow the use of low doses of such inhibitors while preserving Mrp1 function in other tissues, like lung, intestine and brain. Still, if higher levels are required for an anti-fibrotic effect, HSCs-specific targeting strategies may be explored to reduce the risk for adverse effects [57,58]. All in all, our data identify Mrp1 as an interesting target for the treatment of liver fibrosis, a condition that cannot be cured at this moment.

The role of PMP70 in liver fibrosis is less straight forward. PMP70-mediated RNA interference clearly inhibited αSma expression in HSCs and PMFs, but had no effect on the fibrogenic markers collagen1a1 and TGF-β. It remains to be tested what the effect of the absence of PMP70 would be on liver fibrosis *in vivo*. Though reported at scientific meetings, no details are known yet about the phenotype of the pmp70 knock out mouse. Such a mouse strain would be instrumental to analyse the role of PMP70 in liver fibrosis. Besides the potential clinical relevance of PMP70 in liver fibrosis, it is of high

interest to further characterize the tubular structures in HSCs to which it colocalizes. This will provide clues about its potential relationship with genuine peroxisomes in HSCs and how this affects the production of the αSma cytoskeleton.

In conclusion, the ABCs of liver fibrosis are beginning to emerge and hold promise for a better understanding and treatment of liver fibrosis.

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Summary /Samenvatting

Atta ur Rehman and Klaas Nico Faber

Dutch translation by Manon Buist-Homan

Department of Gastroenterology and Hepatology
University Medical Center Groningen
University of Groningen

Summary

Chronic liver diseases are a major health problem worldwide and include viral hepatitis, alcoholic liver disease, metabolic (fatty) liver disease, autoimmune (primary biliary cirrhosis, primary sclerosing cholangitis) liver diseases. Chronic liver diseases are characterized by liver fibrosis that is the result of an overactive healing response to liver injury. This leads to the accumulation of extracellular matrix proteins, predominantly collagen 1a1, that disrupts the liver architecture and impairs liver function. Liver fibrosis may progress to liver cirrhosis, which is an irreversible state of fibrosis and predisposes the patients to liver cancer and liver failure. Drug treatment for liver fibrosis is not available yet and liver transplantation is the only therapeutic option for patients with hepatic failure due to liver cirrhosis. Unfortunately, liver donors are scarce and many patients die before a suitable liver becomes available for transplantation. Thus, there is an urgent need for drug therapies to treat liver fibrosis.

Hepatic myofibroblasts (HMF) are responsible for the production of too much extracellular matrix proteins in the liver. HMF may originate from different intra- and extrahepatic cell types, but hepatic stellate cells (HSC) are believed to be the major source. In the healthy liver, HSC are the main storage site for vitamin A and they maintain stable blood levels of this vitamin for its function in a variety of processes in the body. Upon liver injury, HSC become activated and transdifferentiate to proliferative and mobile myofibroblasts that produce excessive amounts of extracellular matrix proteins, while losing their vitamin A stores. Thus, under disease conditions where the functional liver cells (hepatocytes) die, the HSC survive and in fact are stimulated to proliferate.

Multidrug resistance-associated proteins (Mrp's) belong to the super family of ATP-binding cassette (ABC) transporters and are well-documented for their role in protecting cancer cells to cytostatic drugs. However, these transporters also serve physiological functions and transport endogenously produced substances like glutathione and leukotrienes. Earlier, our group found that the expression of Mrp1 is strongly induced upon activation of HSC, both *in vitro* as well as in rats that were exposed to experimentally-induced liver fibrosis. In this thesis, we aimed to determine whether Mrp1 is important for the activation and/or survival of activated HSC and therefore may be a therapeutic target for the treatment of liver fibrosis.

In chapter 2 and 3, we show that pharmacological inhibition of Mrp1 with the highly specific inhibitor reversan reversed expression of fibrosis markers in HSC and had no toxic effect on the viability of hepatocytes. These results were also supported by *in vivo* studies, which showed that reduced amounts of extracellular matrix were produced in livers of Mrp1 knockout mice than in livers of WT mice, when treated with the fibrosis-inducing agent carbon tetrachloride

(CCL$_4$). Importantly, serum markers for liver injury were not different between the Mrp1-ko and WT mice after treatment with CCL$_4$, indicating that the fibrotic response to liver injury is truly suppressed in the absence of Mrp1. These observations suggest that Mrp1 is a potential target for treatment of liver fibrosis.

We also showed that the export of leukotrienes by Mrp1 may be the crucial activity of this transporter to induce activation in HSC. In contrast, glutathione does not seem to affect the activation and viability of HSC directly. During activation of HSC, cellular glutathione levels and glutathione peroxidase (GPx) expression are increased, while other antioxidant enzymes, e.g. catalase and Cu, Zn-superoxide dismutase (CuZnSOD), are largely unchanged (Chapter 4). Inhibition of glutathione synthesis or activity of GPx increased the sensitivity HSC for oxidative stress-induced necrosis and apoptosis, respectively. These data suggest that GSH levels do not directly control activation of HSC, but are important for the cytoprotection of HSC against reactive oxygen species.

Mrp-dependent export of GSH has been implicated in the regulation of intracellular redox environment, however, whether this plays a direct role in protecting HSC and/or hepatocytes against cell death is largely unknown. We found that inhibition of Mrp1 activity by reversan in HSC does not change the intracellular redox environment in the absence of reactive oxygen species. However, inhibition of Mrp1 in combination with exposure to reactive oxygen species inhibited the export of oxidized glutathione from HSC, leading to the intracellular accumulation of GSSG, ultimately causing HSC death. Hepatocytes do not express Mrp1 and the treatment of hepatocytes with reversan did therefore not reduce the viability of these functional liver cells either in the presence or absence of reactive oxygen species. In contrast, hepatocytes were highly sensitive to the general inhibitor of Mrp's, MK571, even in the absence of oxidative stress, most likely because it blocks Mrp2–mediated export of GSSG (chapter 3). Taken together, these data suggest that Mrp1 is a drug target for the treatment of liver fibrosis and the specific Mrp1 inhibitor reversan may be considered for further studies on its antifibrotic potential in chronic liver disease.

In chapter 5, we studied the function of another ABC transporter, the peroxisomal membrane protein (Pmp70), in HSC biology. Pmp70 is highly expressed in liver, but its function is still not well defined. It has been suggested to transport long chain fatty acids into peroxisomes for their breakdown through beta-oxidation. In addition, it may be involved in bile acid metabolism. Until now, its hepatic function has only been studied in hepatocytes, where it is localized to the peroxisome membrane. There is very little knowledge about the presence and function of peroxisomes in HSC. We found that HSC contain typical peroxisomes containing catalase and Pex14p, a protein involved in peroxisome biogenesis. Also Pmp70 is expressed in

HSC, albeit to a significant lower level than in hepatocytes. Using immunofluorescence microscopy, we found to our surprise that most of the Pmp70 in activated HSC was not present in peroxisomes, but rather in tubular structures that lay parallel to the characteristic alpha-smooth muscle actin (αSma) fibers. Artificially overexpressed Pmp70 resulted in enhanced expression of αSma, while siRNA-based inhibition of Pmp70 led to the disappearance of the αSma cytoskeleton. Manipulation of Pmp70 levels did not directly affect the production of collagen1a1. These data provide evidence for a novel function of Pmp70 in HSC where it is required for the generation of the αSma cytoskeleton and may therefore control the contractility and mobility of these cells in a fibrotic liver.

In conclusion, the studies presented in this thesis show that two ABC transporters, Mrp1 and Pmp70, serve different roles in the activation of HSC and are therefore potential drug targets for the treatment of liver fibrosis.

Samenvatting

Chronische leverziekten zoals virale hepatitis, alcoholisch leverziekten, stofwisselingziekten en auto-immuun leverziekten (primaire biliaire cirrose, primaire scleroserende cholangitis) zijn wereldwijd een groot gezondheidsprobleem. Chronische leverziekten worden gekarakteriseerd door leverfibrose als resultaat van een overactief helingsproces als respons op leverschade. Dit leidt tot accumulatie van extra cellulaire matrix eiwitten, voornamelijk collageen 1a1, wat de lever structuur vernielt en de lever functie schaadt. Leverfibrose kan zich ontwikkelen tot levercirrose, een onomkeerbare vorm van leverfibrose, wat lijdt tot leverkanker en leverfalen. Medicijnen voor leverfibrose zijn nog niet beschikbaar en levertransplantatie is de enige therapeutisch optie voor patiënten met leverfalen als gevolg van cirrose. Doordat er helaas te weinig leverdonoren zijn, sterven veel patiënten voordat er een geschikte lever beschikbaar komt voor transplantatie. Er zijn dus dringend therapieën nodig om leverfibrose te behandelen.

Hepatische myofibroblasten (HMF) zijn verantwoordelijk voor de productie van teveel extracellulaire matrix eiwitten in de lever. HMF kunnen afkomstig zijn van verschillende intra- en extrahepatische celtypes, maar er wordt verondersteld dat hepatische stellaat cellen (HSC) de belangrijkste bron is. In een gezonde lever, wordt de meeste vitamine A opgeslagen in de HSC, en zij zorgen voor stabiele bloed waardes van vitamine A voor de verschillende functies in het lichaam. Tijdens leverschade, worden HSC geactiveerd en differentiëren ze tot proliferatieve en mobiele myofibroblasten die grote hoeveelheden extracellulaire matrix eiwitten produceren terwijl ze hun vitamine A verliezen. Dus tijdens ziekte condities wanneer de functionele lever cellen (hepatocyten) dood gaan, overleven de HSC en worden zelfs gestimuleerd tot proliferatie.

Multidrug resistance-associated proteins (Mrp's) behoren tot de super familie van ATP-binding cassette (ABC) transporters en zijn goed beschreven voor hun rol in het beschermen van kanker cellen tegen cytostatica. Maar deze transporters hebben ook fysiologische functies en transporteren endogeen geproduceerde stoffen zoals glutathion en leukotrienen. Eerder heeft onze groep gevonden dat de expressie van Mrp1 sterk verhoogd is tijdens activatie van HSC, zowel *in vitro* als in ratten die blootgesteld zijn aan experimenteel geïnduceerde leverfibrose. In dit proefschrift wilden wij bepalen of Mrp1 belangrijk is voor de activatie of het overleven van geactiveerde HSC en daardoor een therapeutisch doelwit voor de behandeling van leverfibrose.

In hoofdstuk 2 en 3 laten we zien dat farmacologische remming van Mrp1 met de specifieke remmer, reversan, de expressie van fibrose markers in HSC omdraait en dat het geen toxisch effect heeft op de vitaliteit van hepatocyten. Deze resultaten worden ondersteund door *in vivo* studies die laten zien dat de productie van extracellulaire matrix is verminderd in levers van Mrp1 knockout muizen ten opzichte van wild type muizen als ze behandeld worden met het fibrose inducerende stof koolstof tetrachloride (CCL4). Belangrijk om te weten is dat serum markers voor leverschade niet verschillen tussen Mrp1-ko en WT muizen na behandeling met CCL4 wat betekent dat de fibrotische response op leverschade onderdrukt wordt als Mrp1 afwezig is. Deze observaties suggereren dat Mrp1 een potentieel doel is voor de behandeling van leverfibrose.

We laten ook zien dat de export van leuketrienen door Mrp1 een cruciale activiteit kan zijn van deze transporter om de activiteit van HSC te induceren. In tegenstelling lijkt glutathion geen direct effect te hebben op de activiteit en vitaliteit van HSC. Tijdens activatie van HSC is het cellulaire glutathion niveau en de glutathion peroxidase (GPx) expressie verhoogd terwijl andere antioxidant-enzymen zoals catalase en koper, zink-superoxide dismutase (CuZnSOD) onveranderd blijven (hoofdstuk 4). Het remmen van de glutathion synthese of activiteit van GPx verhoogt de gevoeligheid van HSC voor oxidatieve stress-geïnduceerde necrose en apoptose, respectievelijk. Deze gegevens suggereren dat het GSH niveau niet direct betrokken is bij de activatie van HSC maar belangrijk is voor het cyto-beschermende effect van HSC tegen reactive oxygen species.

Hoewel de Mrp afhankelijke export van GSH betrokken is bij de regulatie van de intracellulaire redox omgeving, is onbekend of dit een directe rol speelt in de bescherming van HSC en/of hepatocyten tegen celdood. We hebben gevonden dat remming van de Mrp1 activiteit door reversan in de afwezigheid van reactive oxygen species in HSC de intracellulaire redox status niet veranderd. Hoewel, de remming van Mrp1 in combinatie met het blootstellen aan reactive species oxygen remt de export van geoxideerd glutathion vanuit HSC wat leidt tot de intracellulaire ophoping van GSSG en uiteindelijk tot HSC dood. Mrp1 komt niet tot expressie in

hepatocyten en daardoor leidt de behandeling van hepatocyten met reversan niet tot een reductie in de vitaliteit van deze functionele lever cellen, zowel in de aanwezigheid als afwezigheid van reactive oxigen species. Hepatocyten zijn in tegenstelling erg gevoelig voor de algemene remmer van Mrp's, MK571, ook in de afwezigheid van oxidatieve stress, waarschijnlijk omdat het de Mrp2-gemedieerde export van GSSG blokkeert (hoofdstuk 3). Tezamen suggereert deze data dat Mrp1 een doel is voor de behandeling van leverfibrose en specifiek de Mrp1 remmer, reversan, kan worden gebruikt voor verder onderzoek naar zijn anti-fibrotische eigenschappen in chronische leverziekten.

In hoofdstuk 5 hebben we gekeken naar de functie van een andere ABC transporter, nl het peroxisomale membraan eiwit (Pmp70) in HSC. Pmp70 komt hoog tot expressie in de lever maar zijn functie is nog steeds niet bekend. Er wordt gesuggereerd dat het lange keten vetzuren transporteert de peroxisoom in voor hun afbraak door beta-oxidatie. Daarbij komt dat ze misschien betrokken zijn bij het galzuur metabolisme. Tot nu toe is alleen de hepatische functie van Pmp70 bestudeerd in hepatocyten, waar het gelokaliseerd is in de peroxisoom membraan. Er is weinig bekend over de aanwezigheid en functie van peroxisomen in HSC. Wij hebben gevonden dat HSC peroxisomen bevatten, die catalase en Pex14p, een eiwit betrokken bij peroxisomale biogenese, bevatten. Ook Pmp70 komt tot expressie in HSC, ofschoon in een significant lager niveau dan in hepatocyten. Met behulp van immunofluorescentie microscopie hebben we gevonden dat, tot onze verbazing, de meeste Pmp70 in geactiveerde HSC niet in de peroxisoom zit maar in tubulaire structuren die parallel liggen met de karakteristieke alpha-smooth muscle actine (αSma) strengen. Kunstmatige overexpressie van Pmp70 leidt tot verhoogde expressie van αSma, terwijl remming van Pmp70 door siRNA leidt tot de verdwijning van het αSma cytoskelet. Het manipuleren van Pmp70 levels had geen direct effect op de productie van collageen 1a1. Deze data levert bewijs voor een nieuwe functie van Pmp70 in HSC waar het nodig is voor het maken van het αSMA cytoskelet en daarmee zorgt voor de contractiliteit en mobiliteit van deze cellen in een fibrotische lever.

Tot slot, de studies gepresenteerd in dit proefschrift laten zien dat 2 ABC transporters, Mrp1 en Pmp70, verschillende rollen hebben in de activatie van HSC en daardoor mogelijke toepassing hebben in de behandeling van leverfibrose.

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List of Publication

1. *Sandra Dunning*,* **Atta ur Rehman****, Marjolein H. Tiebosch* and et al.,* **Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death.** Biochim Biophys Acta. 2013 Jul 16;1832(12):2027-2034

2. **Atta ur Rehman,** *Bojana Mikuš, Han Moshage and Klaas NicoFaber*. **Inhibition of MRP1 attenuates liver fibrosis in vitro and in vivo. (submitted)**Oral presentation; Vereniging voor Gastro-enterology (NVGE), 2012. Veldhoven, The Netherlands)

3. **Atta ur Rehman,** *Rutger Modderman, Bojana Mikuš, Han Moshage and Klaas NicoFaber* **The intracellular ABC-Transporter PMP70 is required for the characteristic alpha smooth muscle actin network in activated hepatic stellate cells. (In Preparation)**(Oral presentation in Open European Peroxisome Meeting (OEPM 2010) on 21 and 22 Oct 2010, Luntheren, The Netherlands.

4. **Atta ur Rehman,** *Bojana Mikuš, Han Moshage and Klaas NicoFaber* **Identification of the signaling pathways that reverse stellate cell activation after inhibition of the multidrug resistance-associated protein 1**. (PhD thesis), poster presentation in The liver meeting 2011, San Francisco (USA).

5. **Atta ur Rehman,** *Han Moshage and Klaas Nico Faber* **In vitro analysis of Mrp1 inhibitors identifies reversan as a potentially safe drug to treat liver fibrosis.** *(In Preparation)*Data is partially presented orally in Dutch Liver Retreat 11-12 Oct 2012 Spier, The Netherlands

6. **Atta ur Rehman,** *Floris Hijer, Han Moshage and Klaas Nico Faber* **Gamma Glutamyl Transpeptidase γGGT; a potential mediator of liver fibrosis and cirrhosis** *(In Preparation)*

7. **Atta ur Rehman,** *Rutger Modderman, Bojana Mikuš, Han Moshage and Klaas NicoFaber* **Pharmacological inhibition of human Multidrug Resistance-associated Proteins (MRPs) reverses liver fibrosis** *in vitro (* Oral presentation in Netherlands Vereniging voor Gastro-enterology (NVGE), 17-18 March 2011. Veldhoven, The Netherlands.

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9. **Atta ur Rehman,** *Rutger Modderman, Bojana Mikuš, Han Moshage and Klaas NicoFaber* **Pharmacological inhibition of human Multidrug Resistance-associated Proteins (MRPs) reverses liver fibrosis** *in vitro (* poster presentation *in* The Liver Meeting, Oct 29, 2010 AASLD in Boston (USA).