Therapeutic effects of the traditional medicinal plant Ipomoea stolonifera for the treatment of liver diseases
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Chapter 6

General discussion and future perspectives

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Over the past decades, numerous studies have addressed the treatment of chronic liver diseases, identifying a growing number of promising molecules and therapeutic interventions in experimental models, although the step towards systematic clinical studies has yet to be made. In the final chapter of this thesis, we will discuss the results of our studies relative to the current state of knowledge on liver inflammation and hepatic fibrosis.

**Liver inflammation**

Programed cell death is an essential process in normal physiological processes and tissue homeostasis. However, dysregulation of cell death, in particular the apoptotic program is a sign of stress, injury or infection and is linked to tissue damage and disease pathogenesis. In liver diseases, different forms of cell death occur, depending on the liver disease (as discussed in Chapter 1). In addition to hepatocytes, cell death also affects non-hepatocytic cell types, like cholangiocytes [1, 2], hepatic stellate cells [3] and sinusoidal endothelial cells [4, 5]. However, hepatocyte death is a key event in the progression of liver diseases. Although hepatocyte cell death can be considered as a normal and beneficial event to remove damaged or malignant cells, excessive and prolonged hepatocyte cell death leads to loss of liver function, chronic inflammation, fibrosis and, because of the continuous regeneration, hepatocellular carcinoma Therefore, the contribution of cell death to the progression of liver diseases is cell-, stage- and context-specific [6]. From animal studies it has been concluded that acute liver failure is characterized by both severe apoptosis and necrosis; cholestatic liver diseases and some drug-induced liver failure, like acetaminophen (APAP, paracetamol) intoxication are characterized mainly by necrotic cell death [6-8] and cell death in NASH is characterized by moderate apoptosis, although human studies also demonstrated necroptosis [9]. In many liver diseases both necrosis and apoptosis occur simultaneously, e.g. in ALD and NAFLD [10-14].

Massive cell death is a hallmark of acute liver diseases and leads to a dramatic loss of liver function. Therefore, specific interventions targeted to prevent or attenuate this massive cell death may be very effective in these acute conditions. Caspase inhibitors like IND-6556 and GS-9450 have been investigated and effectively block the apoptotic cascade and reduce liver injury in experimental models [15-18]. Unfortunately, inhibition of caspase-dependent apoptosis may lead to caspase-independent apoptosis, necroptosis or necrosis and therefore caspase inhibitors have not achieved widespread application in clinical hepatology [6]. Initially, cell death was considered to be the result of inflammation, e.g. as a result of the generation of reactive oxygen species, however, cell death can also precede, trigger or amplify the inflammatory response directly by disrupting epithelial barriers and triggering immune responses [19]. Hence, inhibition of inflammation is considered an effective strategy in reducing liver injury. Inflammation is a complex process in which numerous cell types, organelles and signaling factors are involved,
e.g. Fas/FasL, TNF/TNF-receptor, Damage-Associated Molecular Patterns (DAMPs), chemokines, MAP-kinases, transcription factors like Nuclear Factor-κB (NF-κB), mitochondrial pathways etc. NF-κB has emerged as a central regulator of inflammatory activity. Inhibition of NF-κB or NF-κB target genes is considered an attractive strategy for anti-inflammatory therapy and caffeic acid, curcumin, resveratrol and silymarin have been used as NF-κB inhibiting interventions [20]. Alternatively, since TNF is both a NF-κB-dependent gene in inflammatory cells as well as an inducer of NF-κB in target cells, anti-TNF therapy is also considered as an anti-inflammatory intervention. However, all TNF-antagonists currently used, e.g. Infliximab, have also been associated with drug-induced liver injury [21, 22]. This is probably due to the lack of target-cell specificity of TNF-antagonists: TNF antagonists will also prevent NF-κB activation in epithelial cells like hepatocytes and in these cell types, NF-κB acts as a survival factor [23]. Interestingly, TNF-R knockout mice were protected from hepatic steatosis and fibrosis [24]. In addition to TNFα/NF-κB antagonists, other compounds have been evaluated as anti-inflammatory agents in liver inflammation. E.g. glycyrhizin, used as inhibitor of both DAMP and HMGB-1 showed a beneficial effect in HCV and HCC [25-27]. Depletion of Kupffer cells is also effective in the prevention of inflammation and subsequent liver injury [28-30]. To summarize, therapy may be aimed to directly inhibit cell death of hepatocytes and/or indirectly via inhibition of inflammatory pathways. Furthermore, anti-inflammatory interventions may have opposite effects in different cell types, therefore, cell-specific targeting may be necessary to achieve optimal results. In view of these considerations, the raw extract of Ipomoea stolonifera may be a promising candidate as discussed below.

In Chapter 2, we demonstrate that the raw extract of Ipomoea stolonifera and its purified components profoundly reduce caspase-3 activation in hepatocytes exposed to the hydrophobic bile acid glycochenodeoxycholic acid (GCDCA). Since activation of caspase-3 is a late event in the apoptotic pathway and invariably leads to cell death, the prevention of caspase-3 activation demonstrates a truly protective effect of BE-IS, the raw extract of Ipomoea stolonifera and its components. In addition to the cytoprotective effect, the anti-inflammatory properties of BE-IS and its components were also assessed. Hepatocyte inflammation was induced by a combination of three inflammatory cytokines (TNFα, IL-1β and IFN-γ). Our results provide evidence for the anti-inflammatory action of BE-IS and some of its components. Of note, the anti-inflammatory effect is independent of the cytoprotective effect, since the GCDCA-induced model of hepatocyte death is not related to inflammation [31]. BE-IS contains multiple bioactive components and has been demonstrated to have therapeutic effects in the management of various liver diseases [32]. Very recently, we further investigated the synergistic effect and dose optimization of the five main components of BE-IS used in Chapter 2, in vivo and in vitro [33]. It was demonstrated that esculetin, curcumin and hesperetin are the principal constituents having synergistic effects. Combined, our findings demonstrate that BE-IS (or a selected combination of its main components) has a superior effectiveness over single-agent therapy with respect to cytoprotection and attenuation of inflammation. On the other
hand, the single components should also be evaluated in more detail. E.g. curcumin has been demonstrated to be effective in attenuating inflammation via inhibition of TNFα, NF-κB and COX-2 [34-36]. Additional studies in relevant in vivo models will be needed to assess the potency of these natural compounds against liver inflammation.

Liver macrophages (resident macrophages or Kupffer cells) and hepatic leukocytes (NK cells and NKT cells) play a crucial role in the first line of defense against external and internal pathogens and irritants. These cells can rapidly produce copious amounts of cytokines after activation, which enables them to respond quickly and subsequently help to remove invading pathogens, toxins and food antigens. However, if this defense system is inadequately activated, severe inflammation, ultimately resulting in septic shock and multiple organ failure may occur, with cells of the innate immune system contributing to and amplifying the liver injury [37-39]. Hence, it is hypothesized that drugs that affect immune function may be beneficial in the treatment of liver diseases. In the liver, various bacterial stimuli can activate Kupffer cells, e.g. lipopolysaccharide (LPS), the cell wall component of gram negative bacteria. LPS directly activates Kupffer cells and leads to the production of large amounts of cytokines, such as interleukins, chemokines etc. Of these cytokines, TNFα plays a crucial role, since it plays a key role in liver inflammation and, under specific conditions, is also a potent inducer of apoptosis [40].

In Chapter 3, we used two in vivo models, e.g. lipopolysaccharide (LPS)-induced and Concanavalin A (Con A)-induced fulminant hepatitis, to analyze the anti-inflammatory properties of one of the purified components from Ipomoea stolonifera raw extracts, namely hesperetin. These two models differ with respect to the initiating mechanism and the main cell types and cytokines involved in the onset and perpetuation of fulminant hepatitis. E.g., unlike LPS-induced hepatitis, Con A-induced liver injury is dependent on T-cell mediated phenomena [41] and cannot be induced in athymic nude mice. Con A is therefore considered to be a model for human autoimmune hepatitis [42]. Con A is an activator of NKT cells. After Con A injection, NKT cells produce large amounts of cytokines such as IFNγ, IL-4, IL-13, TNFα and GM-CSF [43]. Con A-induced cytokines, such as TNFα and IFNγ, have a direct role in the induction of widespread hepatocyte apoptosis [18]. IFNγ, a signature cytokine of NK/NKT cells inhibits hepatocyte proliferation and regeneration in liver injury [44]. Moreover, NKT cells cause hepatocyte cell death by releasing pro-apoptotic FasL [45]. LPS or endotoxin is a cell wall product of Gram-negative bacteria and a very potent activator of macrophages and inflammation. Exposure of macrophages, including Kupffer cells, to LPS leads to increased generation of inflammatory cytokines like TNFα, IL-1 and IL-6, as well as chemokines and reactive oxygen species. Uncontrolled exposure to LPS/endotoxin, as happens in sepsis, can lead to septic shock and death. Since LPS is also a potent inducer of IFNγ, Kupffer cells are also able to activate liver NK and NKT cells [46].

Our data provide compelling evidence for the hepatoprotective effects of hesperetin
in LPS-induced and Con A-induced fulminant hepatitis, which is underscored by its ability to reduce TNFα-depended apoptosis via the JNK pathway and dramatically repress IFNγ expression. The clinical efficacy of anti-TNF therapy has been established in chronic inflammatory diseases like inflammatory bowel disease (IBD), and rheumatoid arthritis [47]. Some studies have suggested that Con A-induced hepatitis can be inhibited by treatment with antibodies against IFNγ or IL-4 [48, 49]. In another study, LPS-induced hepatic injury in Propionibacterium acnes-primed mice was completely blocked by the combination of anti-IFNγ and anti-TNFα therapy and only partially blocked by inhibition of only one pathway [42]. Therefore, hesperetin, which is able to inhibit both IFNγ and TNFα holds great promise for the treatment of inflammatory diseases, including liver failure. Hesperetin is abundant in citrus fruits and its daily consumption can be high [50, 51]. In chapter 3, we demonstrated the remarkably protective effect of orally ingested hesperetin in fulminant hepatitis. Given the bioavailability of hesperetin when taken orally and the proven safety and tolerability, hesperetin is a promising candidate for the treatment of both acute and chronic liver diseases.

Liver fibrosis

Liver fibrosis is a dynamic and, at least to a certain extent, reversible process which involves many liver cell types and numerous mediators (described in detail in Chapter 1). Treatment of chronic liver diseases, including liver fibrosis has been hampered by the lack of effective therapeutic interventions. Early liver fibrosis, with absence of extensive ECM crosslinking and marked angiogenesis can still reverse to near-normal architecture when the underlying cause is successfully treated or removed (e.g. viral eradication or cessation of alcohol abuse). Chronic inflammation inevitably leads to liver fibrosis and is characterized by injury and damage of hepatocytes, persistent activation of inflammatory cells, including Kupffer cells and increased production of ECM by activated hepatic stellate cells, regardless of the etiology. Many of these effects are caused by inflammatory cytokines, chemokines and oxidative stress, resulting in death (apoptosis, necrosis) of the functional hepatocytes. Therefore, an effective treatment for liver fibrosis should block the action of these noxious factors and/or cell death of hepatocytes [52, 53], and should reduce the inflammatory state of macrophages, enhance the anti-oxidant status of hepatocytes and reduce steatosis through lipogenic pathways [54-59]. Importantly, therefore, drugs that address more than one single pathogenic pathway will be more efficient than drugs that target only a single pathway [60].

To determine the efficacy of esculetin as an antifibrotic drug, in Chapter 4, we studied the direct effect of esculetin on cultured rat HSC, and subjected mice to the established chronic model of CCl₄-induced fibrosis, where mice were given esculetin treatment during the final 2 weeks of a 4 week CCl₄ treatment. This study revealed multiple antifibrotic properties of esculetin, including suppression of hepatic stellate cell activation, profibrotic TGF-β/Smad signaling and induction of fibrogenolysis by enhancing the MMP/TIMP-1 balance. Additionally, the anti-oxidant capacity of the
liver is improved by increasing the hepatic GSH/GSSG ratio.

Indeed, many different aspects are involved in the process of fibrogenesis, e.g. organ inflammation, myofibroblast activation, scar tissue formation and fibrosis resolution. Therefore, in theory, molecular targets on any of this aspect will affect fibrosis progression. Ideally, the more targets, the better. In our study, esculetin seems to be such a promising anti-fibrotic agent that is multifaceted in various ways. HSC play a crucial role in hepatic fibrogenesis. HSC transdifferentiate into hepatic myofibroblasts and the excretion of excessive amount of ECM is the key event in fibrogenesis. Thus, suppression of proliferation and activation of hepatic myofibroblasts is a major focus of anti-fibrotic therapy. Esculetin strongly suppresses HSC proliferation and the expression of markers of fibrosis (collagen 1a1 and α-Sma). Among different signaling events, the TGF-β/Smad pathway is a key profibrotic pathway associated in liver fibrosis. In our study, esculetin significantly inhibits Tgf-β expression and downstream Smad2/3 phosphorylation, while Pparγ and Pdgfr-β expression were not changed.

An interesting option to accelerate the resolution of fibrosis is to increase matrix degradation, a process that is controlled by balancing the level of matrix metalloproteinases (MMPs) and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), in particular myofibroblast-derived TIMP-1 [61]. Increasing the activity of MMPs and/or antagonizing TIMPs will benefit matrix degradation as demonstrated by Iredale et al. and Issa et al. [3, 62] in seminal studies on the resolution of liver fibrosis in rats by decreasing the production of TIMP-1. In addition, it has been postulated that reducing TIMP activity in the liver may actually have the dual advantage of promoting matrix degradation and inducing apoptosis of highly fibrogenic HSCs [63]. In our study, CCl4-induced expression of Mmp2 and Mmp13 were not changed by esculetin, while Mmp9 was only moderately reduced. Most pronounced, however, was the strong suppression of CCl4-induced TIMP-1 upon esculetin treatment. Thus, the esculetin-increased MMP/TIMP-1 balance favors fibrogenolysis.

Our results suggest that the anti-fibrotic action of esculetin acts, at least in part, through inhibition of lipoxigenases, e.g. 5-LO and/or 12/15-LO. This is in line with our earlier observations that activation of RORα by melatonin or a synthetic agonist (SR1078) suppresses 5-LO expression in HSC and thereby suppresses cell proliferation and expression of Collagen1a1 and Acta2 (encoding αSma) [64]. In a similar way, esculetin suppressed HSC proliferation and expression of those markers of fibrosis. Lipoxigenases (LOXs) are dioxygenases that catalyze the formation of corresponding hydroperoxides from polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, to produce inflammatory lipid intermediates that directly or indirectly affect cellular function and survival [65]. Human and mouse express six LOX isoforms, which are defined by the site of oxygenation, either at carbon position 5, 12, or 15 [66]. Kupffer cells, the resident macrophages of liver, express Alox5 and Alox12/15, while hepatocytes appear to be devoid of these enzymes [67-69]. Kupffer
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cells participate in the initiation of the inflammatory cascade leading to liver fibrosis by virtue of various proinflammatory factors, such as cytokines and leukotrienes, the latter being produced via the LOX pathway [70-72]. Thus, this pathway plays a direct role in HSC activation and possibly also modulating the extracellular matrix (ECM) in the liver [71]. Although the exact role of LOXs in liver fibrosis need to be established, inhibitors of 5-LO and/or 12-15-LO have been demonstrated to prevent hepatocellular injury during inflammation and liver fibrosis [67, 73, 74].

As we found that esculetin effectively blocks the progression of liver fibrosis even under continued liver toxicity conditions induced by CCl₄, we next aimed to analyze its therapeutic potential in short treatment periods through 2 different administration routes, namely via intraperitoneal or intravenous injections. A key consideration in the design of our study in Chapter 5 was that esculetin treatment was started while liver injury and the development of fibrosis were already ongoing, most closely paralleling the situation in patients with (chronic) liver disease. Esculetin treatment was given only for 1 week, either in the 2nd, 3rd or 4th week of a 4-week CCl₄ protocol.

Remarkably, short early treatment of esculetin effectively suppressed liver injury (serum AST/ALT levels) and the progression of liver fibrosis. Intravenously administered esculetin was most effective in reducing serum transaminase (ALT/AST), HSC activation markers (Collagen 1a1 and α-SMA expression), as well as collagen deposition. So apparently, esculetin provides a potent and long-lasting therapeutic effect against CCl₄-induced liver injury and fibrosis in mice.

In the studies described in Chapter 4 and 5, esculetin was administered either i.p. or i.v. is this is expected to exert the strongest therapeutic effect. Since esculetin is present in many medicinal plants, it also relevant now to determine whether it has similar therapeutic properties as a nutritional supplement. Several studies have analyzed the safety and therapeutic properties of oral intake of esculetin. Long term (over months) dietary intake of esculetin is well-tolerated by mice and rats, with minimal side effects [75-77]. Moreover, Qin pi, the dried branch or stem bark of Fraxinus rhynchophylla that is rich in esculetin, is used in traditional medicine to treat patients with chronic bronchitis and bacillary dysentery in children [75]. In rodents, ingested esculetin is rapidly absorbed with peak plasma levels appearing within minutes after intake. Subsequently, esculetin rapidly disappears from the blood and accumulates in the liver and kidneys [78]. Thus, esculetin may hold promise in preventing liver injury and/or liver fibrosis as a nutritional supplement.

Future perspectives

Alternative and/or complementary therapy for the treatment of acute and chronic diseases has received increased attention and recognition over the past few decades. Various herbal extracts, as well as purified compounds from those extracts, show medical benefits in liver diseases and include phenolic compounds, terpenoids, alkaloids and flavonoids [79-81]. Thus, natural products that are contained in
millions of botanicals and herbs are a rich source for drug development. Approximately 20% to 30% of patients with chronic liver diseases use herbal products [82, 83] and sales of e.g. silymarin reaches $180 million in Germany alone [84]. This thesis aimed to identify the therapeutic compounds from one such medicinal herb, *Ipomoea stolonifera*, and provide insight in the mechanisms by which it suppresses fulminant liver inflammation and liver fibrosis.

First, a natural product needs to demonstrate therapeutic potential in a particular disease, but also safety and effectiveness of traditional medicine products needs to be established, e.g. for hesperetin, esculetin and the raw extract of BE-IS in our study. Such compounds may be used therapeutically when the disease is already established, but perhaps even more interesting is their potential to prevent disease development altogether, as our studies on esculetin demonstrate. Ideally, such compounds should be included in the diet as a supplement to prevent liver injury and fibrosis. Future experiments should reveal such hepatoprotective action of esculetin when given as a dietary supplement before liver injury is induced.

Although clear therapeutic actions have been assigned to several herbal extracts, their composition is highly dependent on environmental conditions, such as climate, season and the location where the herb grows. Therefore, the same “product” can vary significantly from batch to batch [85, 86]. Consequently, the taxonomic identity of the source and the chemical identity, purity and stability of the constituents may vary. This is clearly unacceptable when used as a therapeutic and even as a dietary supplement. Standardization of the extracts and quality and safety control are therefore urgently needed, but not in place yet. Approval of each new submission must therefore be based on a thorough evaluation of its composition, its safety and toxicological profile, pharmacokinetics and metabolism and identification of its active ingredients [87, 88]. Ultimately, the challenge will be in the identification of the active constituents of the extracts and the controlled reconstitution of the most effective mixture of these purified components.
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