

PhD Thesis

**Role of Ligand-activated Nuclear Receptors in the Pathogenesis and
Treatment of Sclerosing Cholangitis**

submitted by

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Dedicated to my parents

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1. ZUSAMMENFASSUNG

Chronisch biliäre Erkrankungen (Cholangiopathien) wie die primär sklerosierende Cholangitis (PSC) und die primär biliäre Zirrhose (PBC) sind chronisch cholestatische Lebererkrankungen mit begrenzten Therapieoptionen und können ohne wirksame Behandlung zu Leberversagen oder Lebertransplantation führen. Die derzeit verfügbare medikamentöse Therapie mit Ursodeoxycholsäure (UDCA) ist bei PSC – im Gegensatz zu PBC, wo zwei Drittel der PatientInnen ansprechen – nicht effektiv und ist in erhöhter Dosierung sogar mit negativen Effekten behaftet. Es besteht daher ein großes Interesse an der Entwicklung neuer effizienterer Medikamente zur Behandlung chronischer Cholangiopathien, insbesondere der PSC. Da Cholestase mit Entzündung, Fibrose sowie erhöhter systemischer und intrahepatischer Gallensäurekonzentration in der Leber assoziiert ist, kann die pharmakologische Hemmung der Gallenwegsentzündung sowie medikamentöse Aufrechterhaltung der Gallensäurehomöostase in der Behandlung der sklerosierenden Cholangitis von Nutzen sein. Daher war das übergeordnete Ziel dieser Arbeit zu erforschen, ob die Aktivierung von PPAR γ und der Gallensäuren-Rezeptoren FXR und TGR5 eine entscheidende Rolle in der Pathogenese und der Behandlung von sklerosierenden Cholangitis spielen kann.

Im ersten Teil dieser Thesis wurde die Hypothese aufgestellt, dass der natürlicher PPAR γ Agonist Curcumin durch Hemmung der aktivierten Cholangiozyten als auch pro-fibrogenen Zellen eine therapeutische Wirkung in einem Tiermodell (*Mdr2*^{-/-} Mausmodell) der sklerosierenden Cholangitis könnte. Es konnte erstmals gezeigt werden, dass Curcumin wichtige zelluläre Vorgänge bei biliären Erkrankungen beeinflusst: entzündungsbedingte Veränderungen von Cholangiozyten (durch PPAR γ Aktivierung) sowie die Aktivierung der portalen Myofibroblasten, als einer Zellpopulationen mit zentraler Rolle in der biliären Fibrose (durch Hemmung der ERK1/2 Signaltransduktionswegs), was zur Hemmung der portalen Entzündung und Leberfibrose führt.

Im zweiten Teil dieser Thesis stellten wir die Hypothese auf, dass die FXR Aktivierung über eine Reduktion der intrahepatischen potentiell toxischen Gallensäurekonzentration und/oder direkte entzündungshemmende Wirkung in den Hepatozyten, Makrophagen und Cholangiozyten Leberschäden bei der sklerosierenden Cholangitis verringern kann. Darüber hinaus könnte die Aktivierung von TGR5 durch cAMP-vermittelte Stimulation des Gallenflusses und biliärer Bicarbonatsekretion sowie

über direkte entzündungshemmende Wirkung in den Kupffer Zellen und Cholangiozyten eine therapeutische Wirkung haben. Therapeutische Mechanismen von selektiven FXR- und TGR5-Agonisten sowie eines dualen FXR/TGR5-Agonisten wurden im *Mdr2*^{-/-} Modell der sklerosierender Cholangitis untersucht. Lediglich der duale FXR/TGR5-Agonist zeigte eine therapeutische Wirkung auf die Leberschädigung durch die Induktion der wichtigsten hepatoprotektiven Mechanismen: FXR-vermittelten Stimulation der Bicarbonat-reichen Gallensekretion (durch Induktion des hepatozellulären Carboanhydrase 14), bei deutlicher Senkung der biliären Gallensäuresekretion (durch Hemmung der endogenen Gallensaureproduktion). Erstmals wurde anhand dieser Ergebnisse die FXR-abhängige Stimulation der Bicarbonat-Sekretion als wichtiger Schutzmechanismus gegenüber der Gallensäuretoxizität im Gallengang gezeigt.

Zusammen, zeigen die im die Rahmen dieser Thesis beschriebene Ergebnisse neue zelluläre und molekulare Ziele für die Behandlung von sklerosierenden Cholangitis. Die Beeinflussung der selektiven und spezifischen Signalübertragungswege in verschiedenen Zellpopulationen der Leber könnte wichtige Implikationen für die Behandlung von cholestatischen Lebererkrankungen wie der sklerosierende Cholangitis darstellen.

2. SUMMARY

Chronic cholangiopathies such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) are chronic cholestatic liver diseases with limited therapeutic options. Without efficient treatment, cholangiopathies progress rapidly, leading to the liver failure and need for liver transplantation. Although two thirds of PBC patients show biochemical response after standard therapy with ursodeoxycholic acid (UDCA), treatment in PSC is inefficient at recommended dose and is associated with increased risk of serious adverse effects at higher dose. Thus, there is an urgent need for novel and efficient pharmaceutical therapy in chronic cholangiopathies, especially in PSC. Since cholestasis is associated with liver inflammation, fibrosis and increased systemic and intrahepatic bile acid concentrations, targeting molecular mechanisms regulating inflammation and bile acid homeostasis may be beneficial to treat sclerosing cholangitis. Nuclear receptors play a critical role in inflammation, fibrogenesis and bile homeostasis. Peroxisome-activated receptor gamma (PPAR γ) has anti-inflammatory effects through inhibition of NF- κ B signaling pathway and may inhibit pro-fibrogenic cell activation. Nuclear bile acid receptor farnesoid X receptor (FXR) regulates bile acid homeostasis, bile secretion and inflammation, whereas membrane-bound G-protein coupled bile acid receptor TGR5 may induce protective mechanisms by cAMP-mediated bicarbonate production and inhibiting inflammation in Kupffer cells. Therefore, the overall aim of this thesis was to explore the hypothesis that activation of PPAR γ or the bile acid receptors FXR and TGR5 may play a critical role in the pathogenesis and treatment of sclerosing cholangitis.

In the first part of thesis we hypothesized that PPAR γ natural agonist curcumin may be beneficial in sclerosing cholangitis *via* inhibition of activated cholangiocyte phenotype as well as pro-fibrogenic cell activation. Curcumin decreased portal inflammation and biliary fibrosis in the *Mdr2*^{-/-} model of sclerosing cholangitis by targeting the main cellular events in cholangiopathies: in cholangiocytes curcumin inhibited reactive cholangiocyte phenotype and portal inflammation through PPAR γ -mediated inhibition of chemoattractant molecule production, whereas inhibition of main pro-fibrogenic cell (portal myofibroblast) proliferation was ERK1/2-mediated.

In the second part of thesis we hypothesized that FXR activation may decrease portal inflammation *via* reduction of intrahepatic and systemic concentrations of potentially toxic bile acids and/or direct anti-inflammatory effects in hepatocytes,

macrophages and cholangiocytes. In addition, TGR5 activation in cholangiocytes may be beneficial through cAMP-mediated stimulation of bile flow and biliary bicarbonate secretion as well as *via* direct anti-inflammatory effects in Kupffer cells and cholangiocytes. The role of FXR and TGR5 activation was studied in the *Mdr2*^{-/-} model of sclerosing cholangitis by using a selective FXR agonist or TGR5 agonists and a dual FXR/TGR5 agonist. Only the dual FXR/TGR5 significantly reduced portal inflammation and biliary fibrosis in *Mdr2*^{-/-} mice *via* FXR-mediated promotion of bicarbonate-rich bile secretion (by induction of hepatocellular carbonic anhydrase 14) and simultaneous reduction of biliary bile acid export (*via* inhibition of endogenous bile acid synthesis), thus resulting in the production of less toxic bile. These findings showed for the first time FXR-dependent stimulation of biliary bicarbonate secretion in hepatocytes.

Together, this thesis discovered novel cellular and molecular targets for treatment of sclerosing cholangitis. Targeting important signaling mechanisms such as PPAR γ and ERK1/2 in cholangiocytes and portal myofibroblasts (cell populations involved in portal inflammation and fibrosis) as well as FXR-mediated induction of bicarbonate secretion and modifications of bile acid homeostasis by hepatocytes provide novel possibilities to treat cholestatic liver diseases such as sclerosing cholangitis in human.

3. ABBREVIATIONS

AE2/Ae2: anion exchanger 2,
AE3/Ae3: anion exchanger 3,
ALT: alanine aminotransferase,
ALP: alkaline phosphatase,
 α -SMA: alpha smooth muscle actin,
BA: bile acid,
BADGE: bisphenol A diglycidyl ether,
BSEP/Bsep: bile salt export pump,
BW: body weight,
CA: cholic acid,
CA14/Ca14: carbonic anhydrase 14,
CBDL: common bile duct ligation,
CFTR: cystic fibrosis transmembrane conductance regulator
CTGF: connective tissue growth factor
Co: control diet-fed mice,
Colla1: collagen, type I, alpha 1,
Colla2: collagen, type I, alpha 2,
Cyp7a1: cholesterol 7 alpha-hydroxylase,
FCS: fetal calf serum,
FXR/Fxr: farnesoid X receptor,
HDL: high density lipoproteins,
H&E staining: hematoxylin and eosin staining,
HP: hydroxyproline,
HSC: hepatic stellate cell,
IL-1: interleukin 1
IL-6: interleukin 6
K8/18: keratin 8/18,
K19: keratin 19,
LW: liver weight,
Mcp-1: monocyte chemotactic protein 1,
Mdr2: multidrug resistance protein 2 (canalicular phospholipid export pump),
MFB: myofibroblast,
Mrp2: multidrug resistance associated protein 2,

Mrp3: multidrug resistance associated protein 3,
Mrp 4: multidrug resistance associated protein 4,
Ntcp: Na⁺/taurocholate co-transporting polypeptide,
NR: nuclear receptor,
PBC: primary biliary cirrhosis,
PC: phosphatidylcholine,
PL: phospholipid,
PSC: primary sclerosing cholangitis,
RU: relative units,
SW: spleen weight,
TG: triglycerides,
TNF α /Tnf- α : tumor necrosis factor alpha,
UDCA: ursodeoxycholic acid
VCAM-1/Vcam-1: Vascular cell adhesion molecule-1,
VPAC-1/Vpac-1: Vasoactive intestinal peptide receptor-1,
WT: wild type

4. INTRODUCTION

4. 1. Introduction to functional liver anatomy

The liver plays a critical role in processes of digestion, nutrient and energy homeostasis, detoxification and removal of toxins as well as synthesis of proteins and hormones, etc. The multifunctional activity of liver is possible due to its specific architecture and coordinated action of several highly differentiated cell types. Up to 70% of the liver is presented by hepatocytes, polarized parenchymal cells, which are localized in plates radiating from the central vein (Alberts *et al.*, 1994; Wright *et al.*, 1979). Hepatocyte polarity is determined by structural and functional diversity of different cell parts. The basolateral/sinusoidal membrane of hepatocyte is the part of cell surface which is oriented towards the blood sinusoid. Due to the lack of *lamina basal*, this part of hepatocytes is in direct contact with the so called "space of Disse", a space between hepatocytes and endothelial cells lining blood sinusoids. Fenestration of endothelial cells in liver makes the barrier between hepatocytes and sinusoidal blood permeable for fluids and molecules but not for the cells (Jones *et al.*, 1980). In normal liver, Kupffer cells, important players in immune response and phagocytosis and the quiescent stellate cells, important players in wound healing and fibrogenesis are localized in space of Disse (Wright *et al.*, 1979). The apical/canalicular membranes of 2 adjacent hepatocytes from neighboring plates form the ***bile canaliculus***, a small channel which constitutes the first part of bile transport system. Tight junctions form a barrier between the hepatocytes to prevent the bile leakage. Bile canaliculi further connect with canal of Hering, an intermediate part lined with the progenitor cells, which further form the bile ductules. Bile ductules join from different liver parts into collecting bile ducts and carry bile to the gallbladder. Bile ducts are lined with the cholangiocytes, a monolayer of epithelial cells which form up to 5% of total liver cell population (Tavoloni, 1987).

Liver receives blood supply from two routes: venous blood is delivered to liver from intestine *via* portal vein, whereas arterial blood is delivered by hepatic artery. Both these circulation systems are branching in the liver to deliver nutrients and oxygen to each liver lobule. In the sinusoids, portal and arterial blood is mixed and passes toward the central vein, which then returns hepatic blood into main circulation.

The area of liver where bile ductules, lobular branches of portal vein and hepatic artery are localised is known as portal tract/field.

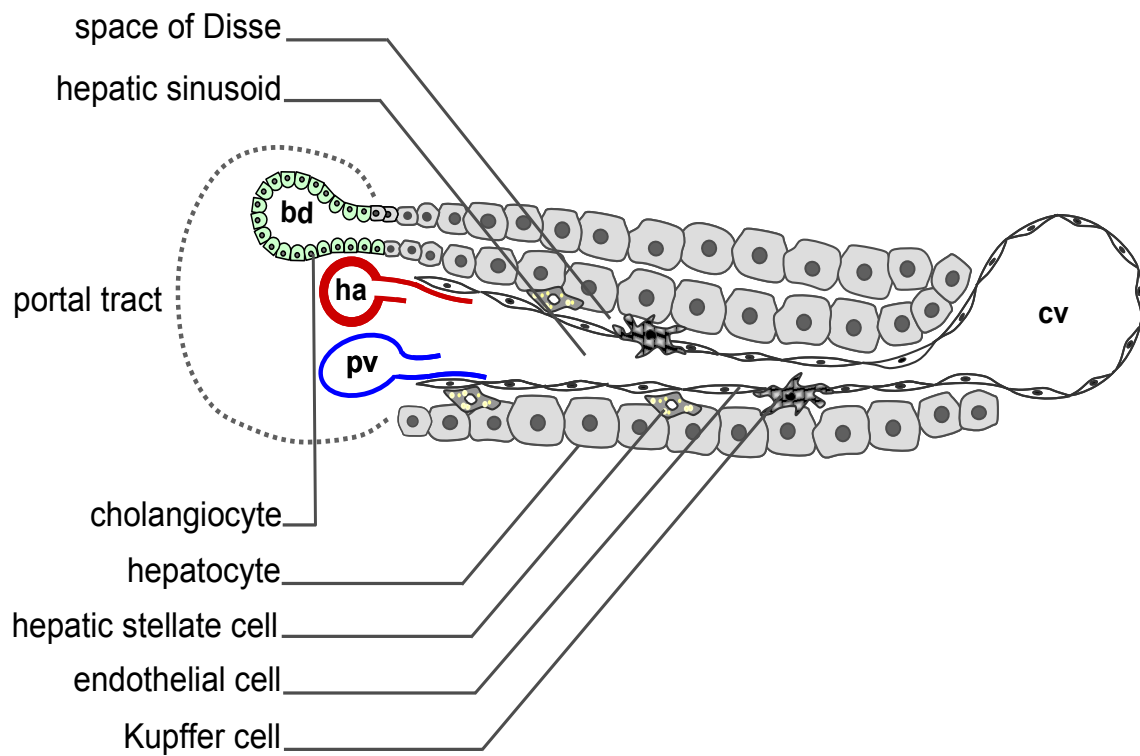


Figure 1. Schematic illustration of liver histological structure. *bd*: bile ductile, *cv*: central vein, *ha*: hepatic arteriol, *pv*: portal venule.

4.2. Bile acids

Synthesis of bile acids (BA) and bile production are fundamentally important liver functions. BAs are amphipathic substances, which molecule contains a hydrophobic side, hydrophilic side and a negative charge at one end (Hofmann *et al.*, 2008). In humans, the major primary BAs are cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas in mice, a more hydrophilic muricholic acid (MCA) is presented as the main primary BA. Synthesis of BAs from cholesterol takes place in hepatocytes (Russell, 2003) by two pathways: the classic (or neutral) pathway and alternative (or acidic) pathway. Reactions of BA synthesis involve at least 17 enzymes that are localized in different cell compartments: endoplasmic reticulum, mitochondria, cytosol and peroxisomes. The initial and rate-limiting step of cholesterol enzymatic conversion is the cholesterol 7- α -hydroxylase (Cyp7a1) in neutral and sterol 27-hydroxylase in acidic pathway. As a result of neutral pathway, two primary BAs are synthesized: CA and CDCA. Sterol 12 α -hydroxylase (Cyp8b1) (Russell, 2003) is an important enzyme involved in both the pathways and determines the ratio of cholate and chenodeoxycholate synthesis thus regulating the biliary BA hydrophobicity index.

Newly-synthesized BAs are being conjugated with either glycine or taurine (Falany *et al.*, 1994), excreted into bile canaliculus and passaged into gallbladder, where they are stored. Following food consumption, gallbladder contraction releases bile into intestine (Hofmann, 1999b). In the small intestine, BAs are efficiently reabsorbed through intestinal BA uptake transporter ASBT (Meier *et al.*, 2002), whereas in the colon, passive absorption of remaining BAs takes place. Re-absorbed BAs thus return to the liver *via* portal vein and are re-secreted into bile. Only a small part of BAs is being lost *via* feces, which is then compensated by *de novo* synthesis. This efficient recycling of BAs is known as enterohepatic circulation (Figure 2) (Hofmann, 1999a; Meier *et al.*, 2002)

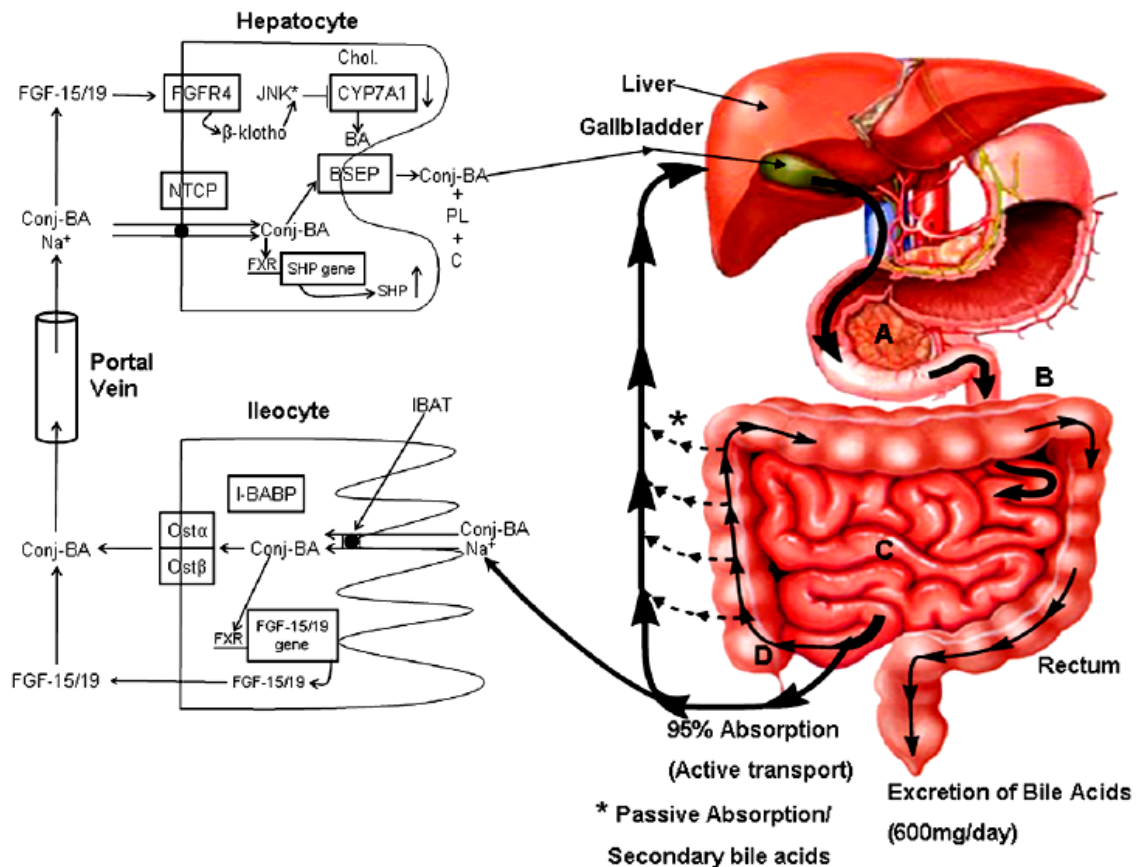


Figure 2. Schematic illustration of BA homeostasis.

Figure from (Hylemon et al., 2009). Conjugated bile acids (Conj-BA) are actively transported from the canalicular side of the hepatocyte by the bile salt export pump (BSEP; ABC B11) along with phospholipids (PL; ABC 4) and cholesterol (C; ABC G5/G8). Conjugated bile acids are actively transported into ileocytes by the sodium-dependent intestinal bile acid transporter (IBAT). Inside the ileocyte, bile acids induce the synthesis of fibroblast growth factor 19 (FGF-15) (in rodents, Fgf19). Bile acids and FGF-15/19 are transported back to the liver via the portal blood. Conjugated bile acids are actively transported into the hepatocyte primarily by the Na⁺/taurocholate cotransporting polypeptide (NTCP). FGF-15/19 binds to and activates hepatic fibroblast growth factor receptor 4 (FGFR4), which in turn activates the JNK signaling pathway, which in turn downregulates the gene encoding cholesterol 7 α -hydroxylase (CYP7A1), inhibiting bile acid synthesis. Figure legend adapted from (Hylemon et al., 2009).

Physiological functions of BAs include stimulation of bile flow, biliary PL and cholesterol excretion as well as absorption of lipids and lipid-soluble vitamins. Importantly, BA synthesis is an important mechanism for cholesterol elimination from

circulation. The function of BAs as very potent and important signaling molecules is introduced in further chapters of this thesis.

4.3. Cholestasis: role of BAs as mediators of liver damage

Liver damage is often accompanied with the development of **cholestasis**, a clinical condition known as impairment of bile flow and clinically manifesting with jaundice, *pruritus* and lipid malabsorption. Pathogenetic mechanisms leading to cholestasis may include genetic or acquired alterations of bile secretion at the hepatocellular or cholangiocellular level.

Detergent properties of BAs are critical for solubilization and absorption of dietary fat. However, in high concentrations or in situations of defective protective mechanisms, BAs can solubilize lipid layers of cell membranes and subcellular organelles. Cholestasis is associated with increased intrahepatic and systemic BA concentrations (Lindblad *et al.*, 1977), (Setchell *et al.*, 1997). Independent of cholestasis causing factor, increased intracellular concentrations of potentially toxic BAs may initiate the hepatocellular apoptosis and damage (Guicciardi *et al.*, 2002). Hepatotoxic effects of hydrophobic BAs have been confirmed by induction of hepatocyte death and apoptosis in cell culture experiments (Faubion *et al.*, 1999), (Reinehr *et al.*, 2003), (Graf *et al.*, 2002), (Allen *et al.*, 2010). Formation of mixed micelles of BAs with phospholipids and cholesterol on the canalicular membrane of hepatocytes is considered to serve as one of the essential protective mechanisms against the detergent properties of high concentrations of excreted BAs (Hofmann, 2009; Moschetta *et al.*, 2000; Velardi *et al.*, 1991). Another important protective mechanism is secretion of biliary HCO_3^- , which regulates the intracellular pH, bile alkalization and fluidization. In addition, inhibition of BA synthesis or uptake as well as stimulation of their detoxification and elimination may also serve to protect against the detergent BA concentrations in cholestatic liver diseases.

4.4. General concepts of bile formation

Bile formation is an important excretory function of the liver which serves not only for digestion of nutrients but also has a critical role in eliminating certain endogenous as well as exogenous substances and toxins from the body. Besides these physiological functions, antibacterial and immunomodulatory role of bile have been described in the intestine

(Hofmann, 2007). In physiological conditions, bile mostly consists of water, in which the organic and inorganic components are presented either in dissolved form or in form of suspension (Esteller, 2008). In human bile the main organic constituents of final bile are bile acids (BA) (~ 67%), which are mainly presented in form of bile salts (BS), phospholipids (PL) (~22%), cholesterol (~5%), proteins (~5%) and biliary pigments, whereas inorganic fraction is mainly presented by ions such as HCO_3^- , Cl^- , Na^+ , K^+ , Ca^{2+} , etc. (Esteller, 2008).

4.4.1. Generation of bile flow

Bile flow is defined as process of bile production per unit of time and liver weight. Initially, bile flow was considered to be a result of osmotic forces (Sperber, 1959). With the studies on taurocholate secretion and bile flow in dog, it became clear that bile flow was associated with canalicular BA secretion (Preisig *et al.*, 1962). Further research has demonstrated that BA secretion was not the only factor determining canalicular bile flow and role of other biliary constituents in promoting canalicular fluid secretion was suggested (Boyer *et al.*, 1970; Erlinger *et al.*, 1970). By using mannitol clearance tests in late 60s, the role of cholangiocytes in formation of final bile was discovered (Forker, 1967; Wheeler *et al.*, 1968). These reports uncovered the main concepts in physiology of bile flow:

- i) bile flow is dependent on excretion of main biliary constituents into bile canaliculus
- ii) canalicular secretion of various substances and fluids does not reflect the final bile composition which is drained into gallbladder.

BA-dependent and BA-independent bile flow

Based on selective mechanisms contributing to bile production at the hepatocellular or cholangiocellular level, 3 different fractions may be identified:

1. BA-dependent canalicular fraction (BADFc), where the driving force is BA secretion against a 100-1000-fold concentration gradient into bile canaliculus, followed by the facilitated water efflux (Erlinger *et al.*, 1974; Suchy *et al.*, 2006). There is a linear correlation between canalicular BA secretion and the amount of water following BAs (7-25 ml/mmol) (Esteller, 2008) through the water transporters aquaporins and tight junctions. The main transporters performing this

function are bile salt export pump (Bsep) and multidrug resistance protein 2 (Mrp2) (Trauner *et al.*, 2003). BADFc accounts for approximately 35% of basal bile flow in humans (Esteller, 2008) and is known to vary among different species (Sperber, 1959), (Erlinger *et al.*, 1974).

2. BA-independent canalicular fraction (BAIFc) is a fraction of bile secreted in bile canaliculi independently of BA osmotic force. This fraction is mainly attributed to the transporter-mediated secretion of HCO_3^- (Hardison *et al.*, 1978), (Van Dyke *et al.*, 1982) or glutathione (Ballatori *et al.*, 1989) from hepatocytes. Major transporters here are HCO_3^- transporter anion exchanger 2 (Ae2) and the glutathione transporter Mrp2. BAIFc reaches up to 40 % of basal bile secretion in humans (Erlinger, 1982) and up to 60% in rodents (Boyer, 1971), (Erlinger *et al.*, 1974).
3. Ductular bile flow, in which the secretory and reabsorptive function of bile duct lining cells, cholangiocytes, is critical. Thanks to functional activity of cholangiocytes, primary canalicular bile undergoes a process of alkalization and fluidization during its passage through biliary tree (Banales *et al.*, 2006). As stated earlier, cholangiocytes regulate biliary HCO_3^- homeostasis through apically localized Ae2 (Boyer, 1996), (Kanno *et al.*, 2001), (Masyuk *et al.*, 2000). Importantly, an additional $\text{Na}^+/\text{HCO}_3^-$ co-transporter Slc4a4 has been identified in mouse cholangiocytes to play role in ductular HCO_3^- homeostasis (Uriarte *et al.*, 2010). Ductular bile flow is considered to be BA-independent.

4.4.2. Hepatocellular transporters

With the unique demonstration of functional bipolarity of hepatocytes in 1924 (Cramer *et al.*, 1926), new concepts evolved addressing the cellular and molecular compartments involved in the complex process of bile production. Our current knowledge is based on discovery of several proteins that are capable of transporting endogenous and exogenous compounds in/out of hepatocytes and cholangiocytes. Differential expression of specific transport proteins in two polar surfaces of hepatocytes enables well-coordinated hepatocellular uptake of diverse molecules from blood sinusoids and export into bile canaliculus. Importantly, hepatocytes also express so-called alternative transporters, which are localized on the basolateral membrane and are of importance for the export of some metabolites and BAs into sinusoidal blood.

Hepatocellular uptake transporters

Hepatic sinusoids are lined with the fenestrated endothelial cells, through which the protein-bound compounds can easily pass to reach the basolateral surface of hepatocytes. In this thesis, basolateral uptake transporters for BAs will be introduced. Currently, there are two basolateral uptake transporters known: sodium-dependent Na⁺-taurocholate co-transporting polypeptide NTCP/Ntcp (SLC10A1/Slc10a1) (Meier *et al.*, 1997; Meier *et al.*, 2002) and members of sodium-independent BA/organic anion exchange transporter organic anion-transporting polypeptide OATP/Oatp (Sl21a) family (Kullak-Ublick *et al.*, 1997; Kullak-Ublick *et al.*, 2002). While Ntcp shows high substrate specificity for glycine and taurine-conjugated BAs, OATPs perform the uptake of wide range of amphipathic organic compounds, which in addition to conjugated and unconjugated BSs, include the steroids, thyroid hormone, bilirubin, peptides and several drugs (Kullak-Ublick *et al.*, 2000). In rodent liver, the following representatives of Oatp family have been identified on the basolateral membrane: Oatp1, Oatp2 and Oatp4.

Canalicular transporters

Canalicular transporters are critical for secretion of biliary constituents from hepatocytes into bile canaliculus, a process which takes place against the concentration gradient and requires energy released from ATP (Higgins, 1992), (Walker *et al.*, 1982). The majority of canalicular transport proteins mediating this process belong to the superfamily of ATP Binding Cassette (ABC) proteins. Altered expression or function of these transporters results in impaired bile formation and can manifest in form of diverse cholestatic syndromes, e.g. progressive familial intrahepatic cholestasis (PFIC) syndromes.

Bile salt excretion is mainly mediated by a member of Abcb family protein bile salt export pump BSEP/Bsep (ABCB11/Abcb11) (Gerloff *et al.*, 1998). In contrast to other BS transporters, Bsep is expressed only in hepatocytes and has high substrate specificity for BAs. Deficiency of this transporter manifests in humans in form of progressive familial intrahepatic cholestasis type 2 (PFIC 2 also known as Byler Syndrome) (Strautnieks *et al.*, 1998), (Jansen *et al.*, 1999). PFIC2 is characterized by dramatic reduction of BA secretion into bile, accumulation of intrahepatic BAs, early onset of intrahepatic cholestasis, liver injury and progression to fibrosis, cirrhosis and end-stage liver disease usually in childhood (Jonker *et al.*, 2011). Interestingly, two milder cholestatic syndromes have been described in humans to be associated with the BSEP

mutations: benign recurrent intrahepatic cholestasis type 2 (BRIC2) and intrahepatic cholestasis of pregnancy (ICP). BRIC2 is characterized by intermittent episodes of cholestasis without progression to liver injury and fibrosis (van Mil *et al.*, 2004). ICP usually manifests in third trimester of pregnancy with *pruritus*, elevated serum liver tests, and is associated with an increased risk of intrauterine fetal death and prematurity (Pauli-Magnus *et al.*, 2004). Importantly, inhibition of Bsep activity by several drugs and bacterial lipopolysaccharide (LPS) is an important mechanism for development of drug-induced liver injury and sepsis-induced cholestasis respectively (Kullak-Ublick *et al.*, 2004). However, Bsep mutation resulted in persistent but not progressive cholestasis in mice (Wang *et al.*, 2001), making this murine model less representative for human cholestasis.

In addition to Bsep, canalicular BA export may be mediated by a group of multidrug resistance proteins (MRPs) which mediate organic anion transport and belong to Abcc family: MRP1, MRP2, MRP3 and MRP6. These transporters share similar structural features, are expressed in many tissues and show low substrate specificity. Despite Mrp1 (Abcc1) is localized on basolateral membrane and Mrp2 (Abcc2) shows canalicular localization (Paulusma *et al.*, 1996), (Buchler *et al.*, 1996), these two transporters show similar substrate specificity: glutathione-, sulphate- or glucuronide-conjugated BSs and drugs (Schinkel *et al.*, 2003). Except liver, Mrp2 is also expressed in the intestine and kidney. Functional activity of Mrp2 is critical for biliary glutathione excretion and BA-independent bile flow.

In humans, MRP2 mutations manifest in form of Dubin-Johnson syndrome, a condition characterized by conjugated hyperbilirubinemia (Paulusma *et al.*, 1997), (Kartenbeck *et al.*, 1996).

In contrast to Mrp2, Mrp3 (Abcc3) and Mrp6 (Abcc6) are localized on the basolateral membrane of hepatocytes and are less important for glutathione transport. These transporters gain importance in cholestatic conditions, where their stimulation serves for alternative BA elimination into blood sinusoids (Hirohashi *et al.*, 1999) (Hirohashi *et al.*, 2000) (Zollner *et al.*, 2006), (Zollner *et al.*, 2003).

Canalicular PC excretion is mediated by a PC-specific floppase, the multidrug resistance-associated protein 2 (*Mdr2/Abcb4* in rodents, *MDR3/ABCB4* in humans) which is located on the hepatocyte canalicular membrane (Buschman *et al.*, 1992), (Smit *et al.*, 1994), (Ruetz *et al.*, 1994). MDR3/Mdr2 translocates PC from the inner leaflet of cellular

membrane to the outer leaflet in from of PC microvesicle budding (Crawford *et al.*, 1995), thus positioning PC on the outer surface of canalicular membrane. Further PC release from the canalicular membrane is facilitated by formation of mixed micelles *via* insertion of hydrophobic BA anions. High concentrations of hydrophobic BAs (mM) were reported to be toxic to the cell membranes (Rust *et al.*, 2009). Formation of mixed micelles of BAs with PC and cholesterol on the canalicular membrane of hepatocytes is considered to be one of essential protective mechanisms against the detergent properties of high concentrations of excreted BAs (Hofmann, 2009; Moschetta *et al.*, 2000; Velardi *et al.*, 1991). Therefore, altered expression or function of MDR3/Mdr2 transporter may initiate liver damage due to detergent properties of free non-micellar bound BAs in the bile. Deficiency of MDR3 transporter in humans manifests in form of hereditary cholestasis with high serum γ -glutamyl transpeptidase levels classified as progressive familial intrahepatic cholestasis type 3 (PFIC3) (Deleuze *et al.*, 1996). In addition, recent studies showed increased prevalence of cholestatic liver diseases such as gallstone disease and intrahepatic cholestasis of pregnancy in families of PFIC 3 patients (Jacquemin *et al.*, 1999; Jacquemin *et al.*, 2001; Lucena *et al.*, 2003).

Canalicular cholesterol excretion is mediated by the members of Abcg family Abcg5 and Abcg8, which are presented as half-transporters. In contrast to full transporters of ABC family (which consist of two sets of 6 transmembrane domains and consequently 2 ATP-binding motifs), half-transporters consist of only 6 transmembrane domains and have a single ATP-binding domain. These transporters are believed to heterodimerize to form a functional transport pump. Abcg5/Abcg8 is expressed in the liver and intestine. In the liver, Abcb5/Abcg8 are critical for biliary cholesterol excretion (Yu *et al.*, 2002b) and its mutation causes sitosterolemia in humans, a disease that is characterized by impaired biliary sterol secretion, increased absorption of plant sterols from intestine and their accumulation in the circulation.

Canalicular HCO_3^- export is another important mechanism to protect cell membranes from high concentrations of potentially toxic BAs (Beuers *et al.*, 2010). Biliary HCO_3^- excretion is mediated by a electroneutral Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchange transporter Anion exchanger 2 AE2/Ae2 (SLC4A2/Slc4a2) (Benedetti *et al.*, 1991), (Alper, 2006; Romero *et al.*, 2004). This transporter is presented in 3 major isoforms (Ae2a, Ae2b1, Ae2b2) and is widely expressed in various tissues. Importantly, Ae2 is highly expressed

in both hepatocytes and cholangiocytes and is critical for regulation of intracellular pH, bile alkalization and fluidization. Gene deficiency of Ae2 in mice results in increased perinatal mortality and growth retardation (Recalde *et al.*, 2006), whereas mature *Ae2a,b*^{-/-} mice showed pronounced biochemical, histological and immunological features of PBC (Salas *et al.*, 2008). In addition, increased hepatic Ae2 expression was shown to be an efficient mechanism for beneficial effects of UDCA and glucocorticoid therapy in PBC patients (Arenas *et al.*, 2008).

Excretion of numerous drugs from the liver is mediated by Mdr1a and Mdr1b, transporters highly expressed in liver, small intestine and kidney (Thiebaut *et al.*, 1987), (Brady *et al.*, 2002). The function of these transporters is critical for excretion of drugs and resistance against these drugs. In contrast to rodents, Mdr1a and Mdr1b are presented as a single transporter MDR1 (ABCB1) in humans which performs the same function. Main transporters involved in hepatocellular BA transport are illustrated in Figure 3.

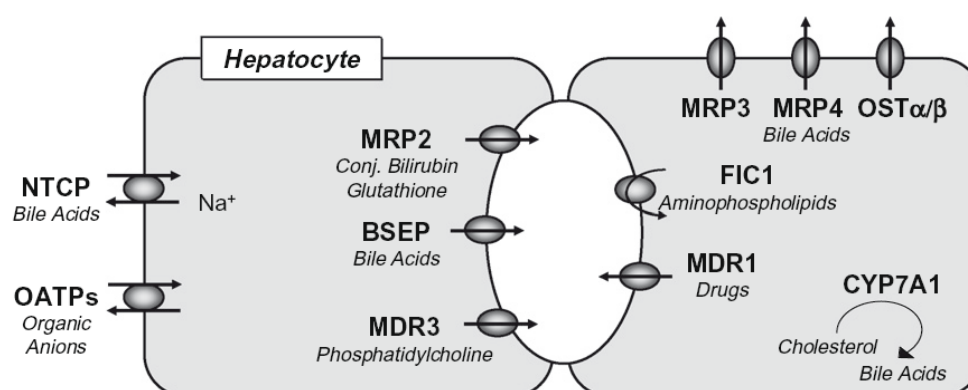


Figure 3: Illustration of main hepatocellular bile acid transporters.

Figure adapted from (Wagner *et al.*, 2009a).

Table 1 summarizes the main hepatocellular and cholangiocellular export transporters involved in bile formation.

Table 1: List of the main transporters involved in canalicular and ductular bile flow.

Table modified after (Esteller, 2008).

Hepatocellular canalicular transporters	
Transporter abbreviation	Function
ABCG5/ABCG8 Abcg5/Abcg8*	Cholesterol export
AE2/SLC4A2 Ae2/Slc4a2*	Cl ⁻ /HCO ₃ ⁻ exchange transporter, acid loader
AQP8 Aqp8*	Water channel
BSEP/ABCB11 Bsep/Abcb11*	Bile salt export
Cl-channel	Cl ⁻ export
MRP2/ABCC2 Mrp2/Abcc2*	Glutathione, dihydroxi-BA and non-BA organic anion export
MDR1 Mdr1*	Export of lipophylic cations
MDR3/ABCB4 Mdr2/Abcb4*	Phospholipid export
NHE3/SLC9A3	Na ⁺ /H ⁺ exchanger, acid extruder
Cholangiocellular apical transporters	
AE2/SLC4A2 Ae2/Slc4a2*	Cl ⁻ /HCO ₃ ⁻ exchange transporter, acid loader
ASBT/SLC10A2 Asbt/Slc10a2*	Uptake of BA s and NA ⁺
AQP1 Aqp1*	Water channel
CFTR Cfr*	Cl ⁻ channel, exports Cl ⁻
NBCe/NBC4/SLC4A5 Slc4a5*	Na ⁺ /HCO ₃ ⁻ symporter, acid extruder
Slc4a4 only in mice	Na ⁺ /HCO ₃ ⁻ symporter, acid extruder

* transporter abbreviation in rodents

4.5. Chronic Cholangiopathies

Cholangiopathies represent a large group of hereditary or acquired cholestatic liver diseases which are characterized by progressive inflammation and scarring of bile ducts with subsequent development of biliary fibrosis and cirrhosis (Lazaridis *et al.*, 2004; Michaels *et al.*, 2008).

4.5.1. Etiology and pathogenesis of chronic cholangiopathies

Multiple etiological factors including impaired immunity, genetic and developmental disorders, infectious agents, ischemia or toxic agents may cause bile duct damage and lead to development of cholangiopathies as presented in the Table 2.

Table 2. Classification of cholangiopathies dependent on their etiology. Table adapted from (Lazaridis *et al.*, 2004)

Etiology		Disease
<i>Hereditary/ developmental</i>		Biliary Atresia
		Cystic Fibrosis (CF)
		Progressive Familial Intrahepatic Cholestasis (PFIC)
		α -1-antitrypsin deficiency
		Allagile Syndrome
<i>Acquired</i>	immunological	Primary Biliary Cirrhosis (PBC)
		Primary Sclerosing Cholangitis (PSC)
		Graft vs. Host Disease (GVHD)
		Sarcoidosis
		Autoimmune Cholangitis
	infectious	Bacterial Ascending Cholangitis,
		CMV,
		HCV,
		HIV,
		Retrovirus Type 3,
		Parasites,
		Fungi,
		Protozoa
	toxic	Paraquat, Chlorpromazine, Floxuridine, Antibiotics
	neoplastic	Histiocytosis X
		Mb. Hodgkin
		Cholangiocellular Carcinoma (CCC)
	vascular/ischemic	Hepatic artery stenosis after liver transplantation
<i>Non-classified</i>		Idiopathic Ductopeny in Adults
<i>Cholangiopathies</i>		Cholangiopathies in Burns and critically ill patients

Primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) are acquired immune-mediated diseases of bile ducts which cause and pathogenetic factors are poorly understood. PSC is more frequently diagnosed in 25-45 years aged men and is often associated with inflammatory bowel disease (IBD). PBC is believed to be auto-immune disease, even if genetic and environmental factors are considered to play certain role. In contrast to PSC, PBC occurs mainly in females at the age of 35-60 years and often in persons with positive family history.

Formation of pro-inflammatory cytokines and intracellular adhesion molecules are important mediators of inflammatory response in cholestatic liver diseases (Gujral *et al.*, 2004), (Yamashiki *et al.*, 1998), (Neuman *et al.*, 2002). Pathophysiologically, development of cholangiopathies is linked with phenotypic and functional modifications of bile duct lining epithelial cells: cholangiocytes, which is characterized by induced proliferation and acquired production of adhesion molecules, pro-inflammatory and pro-fibrogenic mediators (Alvaro *et al.*, 2008; Alvaro *et al.*, 2007; Lazaridis *et al.*, 2004). Activated cholangiocytes may attract inflammatory and fibrogenic cells to site of damage, thus representing not only the victims but also the active mediators of disease progression (Sedlacek *et al.*, 2001).

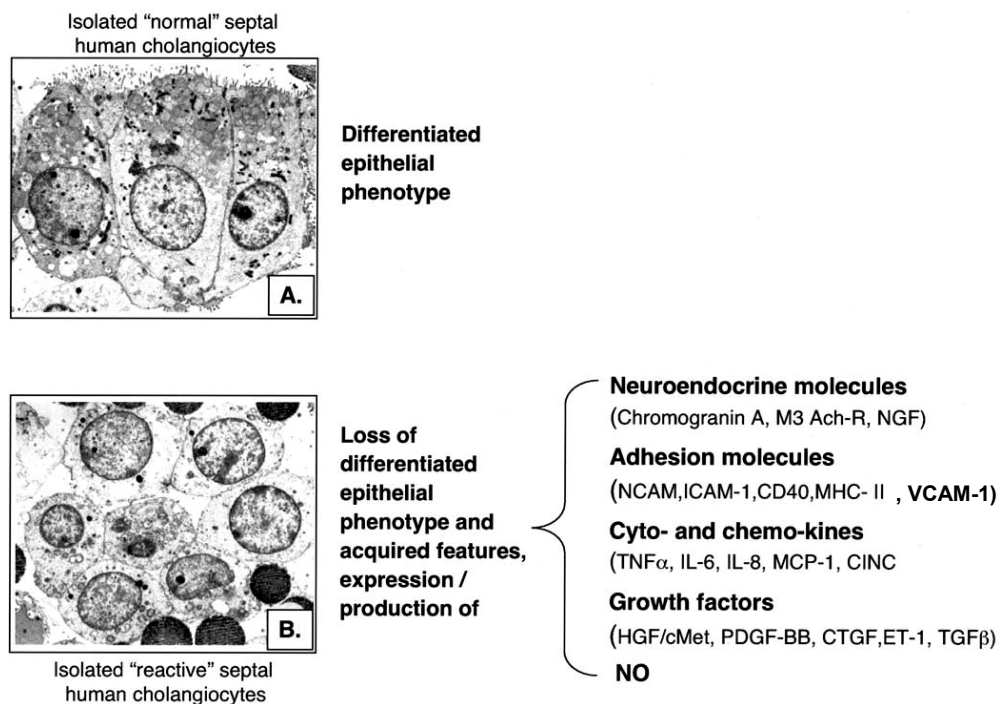


Figure 4. Illustration of normal and reactive cholangiocyte phenotype.

Activated cholangiocytes lose markers of differentiation and acquire new pro-inflammatory features such as production of inflammatory mediators, growth factors and adhesive molecules. Original picture by (Lazaridis et al., 2004) is used with minor modification.

Persisting injury and cholestasis results in development of biliary fibrosis (Ramadori *et al.*, 2004a; Ramadori *et al.*, 2004b), a process characterized by deposition of scarring tissue around damaged bile ducts and portal fields. The central players in fibrogenesis are activated portal myofibroblasts (MFBs) and hepatic stellate cells (HSC). In chronic cholangiopathies, production of pro-inflammatory cytokines and adhesion molecules by activated cholangiocytes initiates the activation of pro-fibrogenic cells such as portal MFBs and HSCs. Morphologically, activated stellate cells are characterized by loss of Vitamin A/retinol stores that are typical for quiescent stellate cells, acquisition of moving ability, prominent endoplasmic reticulum (Friedman *et al.*, 1989; Friedman *et al.*, 1985; Kent *et al.*, 1976; Maher *et al.*, 1990). Functionally, activated myofibroblasts and stellate cells are characterized stimulated proliferation as well as production of extracellular matrix (ECM) proteins such as collagens type I, type III, and IV, laminin, fibronectin and proteoglycans. However, interaction of epithelial cells with the mesenchymal cells is not one-side directed. Activated fibrogenic and inflammatory cells may activate/maintain the reactive phenotype of epithelial cells by production of stimulatory mediators and growth factors.

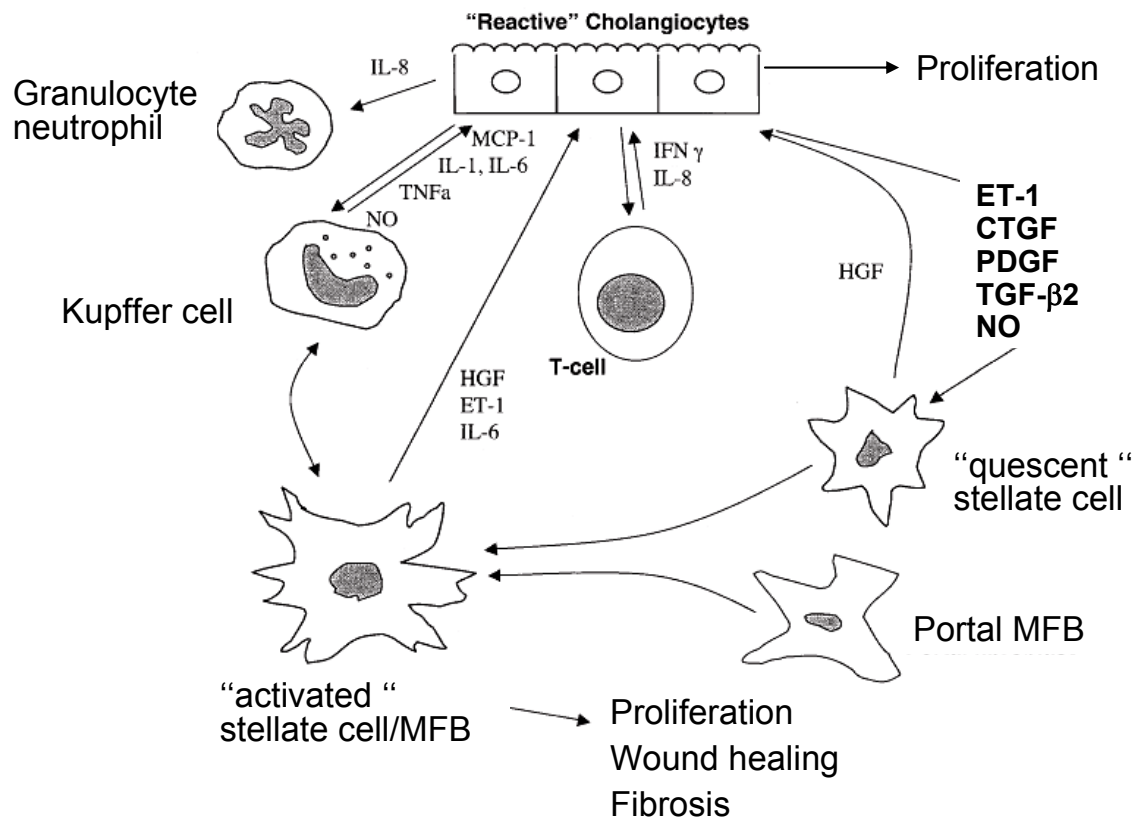


Figure 5: Schematic illustration of cellular interactions in cholangiopathies.

CTGF: connective tissue growth factor, ET-1: endothelin 1, HGF: human growth factor, IL-1: interleukin 1, IL-6: interleukin 6, MCP-1: monocyte chemotactic protein 1, PDGF: platelet-derived growth factor, TGF-β: transforming growth factor beta, TNF-α: tumor necrosis factor alpha, NO: nitric oxide. Figure from (Lazaridis et al., 2004) with modification.

Complications of chronic cholangiopathies usually include cirrhosis, end-stage liver disease, portal hypertension, frequent infections, cholangiocellular carcinoma (CCC), osteoporosis and colon cancer. Without therapeutic intervention, cholangiopathies progress rapidly and lead to end-stage liver disease with eventual need for liver transplantation. Since cholangiopathies have broad etiological diversity and poorly understood patho-physiology, treatment of these diseases is associated with many limitations. However, the ultimate goal is to reduce hepatic inflammation, stop the development of biliary fibrosis and possibly postpone the need for liver transplantation. Currently available medical therapies to slow the progression of cholangiopathies show limited efficacy. Despite standard therapeutic agent ursodeoxycholic acid (UDCA) improved biochemical parameters and clinical symptoms in PBC patients at early-stage, one third of PBC patients have been recognized as incomplete responders (Leuschner *et*

al., 2000). Moreover, in PSC patients, where disease progresses rapidly and leads to ultimate liver failure (Ishibashi *et al.*, 2007; Lazaridis *et al.*, 2004), UDCA was inefficient to improve the disease course at standard doses (15mg/kg/day) and had deleterious effects at higher doses (30mg/kg/day) (Lindor *et al.*, 2009). American Association of the Study of Liver advises against use of UDCA in its treatment guidelines, although guidelines from European Association of the Study of Liver still include and option for UDCA as standard therapeutic in PSC. Thus, there is an urgent need for effective therapies in chronic cholangiopathies, especially in PSC.

4.5.2. *Mdr2*^{-/-} mouse as a murine model for sclerosing cholangitis.

As mentioned above, biliary PC excretion is essential for preventing bile toxicity via formation of mixed micelles with BAs and cholesterol. In line with MDR3 deficiency in human, *Mdr2*^{-/-} (*Abcb4*^{-/-}) mice spontaneously develop chronic cholestatic liver disease, characterized by increased serum liver enzymes, proliferation of bile ducts, sclerosing cholangitis and biliary type of liver fibrosis (Fickert *et al.*, 2004; Mauad *et al.*, 1994; Popov *et al.*, 2005). Importantly, *Mdr2*^{-/-} mice show significant alterations of bile composition as presented by absent PL, dramatically reduced cholesterol and glutathione output, whereas BA output remains unchanged. Spontaneous development of liver disease in *Mdr2*^{-/-} mice with biochemical and histological features of chronic cholangiopathies, established this mouse model as suitable and reliable model for studies of cholangiopathy, in particular sclerosing cholangitis (Trauner *et al.*, 2007) (Trauner *et al.*, 2010), (de Vree *et al.*, 1998). Therefore, *Mdr2*^{-/-} mouse model has been used as in this thesis as accepted model for chronic cholangiopathies to address the pathogenetic and new therapeutic approaches.

4.5.3. Novel therapeutic strategies

Since pathogenesis of chronic cholangiopathies is poorly understood, the ultimate goal is to reduce portal inflammation and stop biliary fibrosis and postpone the need for liver transplantation. In this context, BA-mediated as well as BA-independent pathogenetic and hepatoprotective mechanisms may be important therapeutic targets. In search for novel pharmaceutical approaches the role of bile acid (BA) receptors and their modulators is intensively studied. In this thesis, potential therapeutic effects of BA-mediated nuclear

and membrane receptor activation as well as non BA-mediated nuclear receptor PPAR γ was studied to better understand the pathophysiology of cholangiopathies and to address the role of these signaling mechanisms as novel therapeutic interventions for treatment of sclerosing cholangitis.

4.6. Nuclear receptors (NRs)

NRs belong to the superfamily of ligand-dependent transcriptional factors, which play an important role in gene regulation. Natural ligands for NRs include lipophilic molecules such as hormones, fatty acids, vitamins and BAs (Tobin *et al.*, 2006), (McEwan, 2009). Several nuclear receptors have been reported to have critical role in inflammation, carcinogenesis, cholestasis and metabolic diseases. In the liver, NRs comprise an important role in regulating BA transporters, synthesis and hepatic inflammation, highlighting critical importance of NRs in both physiological and pathological conditions (Elfaki *et al.*, 2009; Trauner *et al.*, 2005; Wagner *et al.*, 2009b). Ligands for NRs have been used for treatment of liver diseases and signs of cholestasis long before their molecular mode of action was discovered (Tobin *et al.*, 2006), (Huang *et al.*, 2004), (Anderson *et al.*, 2008).

4.7. Bile acid-dedicated nuclear receptor farnesoid X receptor

Besides their important role in digestion, BA have been recently identified as highly potent signaling molecules with promising therapeutic actions (Thomas *et al.*, 2008). Multiple actions of BAs in different tissues are now known to be mediated through activation of BA-specific receptors. Despite BA were reported to activate number of various receptors, two BA-specific receptors have been described: the nuclear BA receptor farnesoid X receptor (FXR: NR1H4) and the membrane-bound BA receptor TGR5 (also called GPBAR1 or M-BAR/BG37).

Especially important is BA signaling through the nuclear receptor farnesoid X receptor (FXR; NR1H4 also known as BAR) (Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang *et al.*, 1999). FXR was the first NR for which the BAs have been identified as endogenous ligands. Its cDNA was first cloned in 1995 from mouse and rat (Seol *et al.*, 1995), (Forman *et al.*, 1995). FXR belongs to the class 2 NRs which require heterodimer formation with the retinoid X receptor (RXR) and binds DNA with an inverted element

separated by 1 nucleotide, inverse repeat 1 (Laffitte *et al.*, 2000), (Modica *et al.*, 2006). FXR is highly expressed in the liver, intestine, kidney and adrenals (Forman *et al.*, 1995). Two isotypes of FXR have been identified: FXR α and FXR β . Whereas both FXR α and FXR β are functionally active in rodents, only FXR α is functional in humans and FXR β is present as a pseudogene. Most potent FXR α activator is the primary BA CDCA and its conjugates (Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang *et al.*, 1999), whereas hydroxymetabolites of CDCA such as MCA are not efficient for FXR activation.

FXR controls BA homeostasis and bile formation

In the liver, FXR plays critical role in controlling the systemic and intracellular BA load. Interestingly, BA-mediated FXR activation controls the feedback regulation of BA synthesis by repressing *Cyp7a1* transcription (Lu *et al.*, 2000), (Goodwin *et al.*, 2000), the rate-limiting enzyme in BA synthesis. This repression was shown to be mediated through both hepatic activation of SHP and intestinal upregulation of fibroblast growth factor *Fgf15* and its human homologue FGF19, an intestinal factor that has been identified in 2005 to be induced in response to FXR activation. FGF19/Fgf15 is transported with the portal blood to liver, where it binds to the fibroblast growth factor receptor 4 (FGFR4/Fgfr4) and inhibits CYP7A1/*Cyp7a1* transcription (Inagaki *et al.*, 2005). These findings established the important role of FXR in regulating the BA homeostasis through functional gut-liver axis (figure 2).

FXR plays also important role in biliary excretion of BAs (Wagner *et al.*, 2009b) and other biliary constituents. Specifically, FXR directly induces gene expression of the bile salt export pump (Bsep) and phospholipid transporter Mdr2 (Abcb4), thus contributing to the regulation of BA-dependent bile flow. From the other side, FXR activation efficiently reduces BA uptake by downregulating the sinusoidal BA uptake transporter NTCP (Denson *et al.*, 2001) in rabbit and alternative export transporters OST α and OST β , which are highly expressed in the liver and intestine (Boyer *et al.*, 2006), (Dawson *et al.*, 2005), (Rao *et al.*, 2008).

Role of FXR in inflammation.

FXR activation was demonstrated to interfere with the NF- κ B inflammatory signaling pathway, thus inhibiting the production of pro-inflammatory mediators in the hepatocytes. Moreover, FXR activation reduced the intestinal inflammation in a model of IBD

(Gadaleta *et al.*, 2010), whereas pro-inflammatory cytokines repressed FXR activation in experimental IBD (Gadaleta *et al.*, 2011). In addition, FXR ligands were able to inhibit bacterial overgrowth and regulate the genes involved in anti-microbial action in the intestine without having bacteriostatic effects (Inagaki *et al.*, 2006).

4.8. BA-dedicated membrane receptor TGR5

In addition to the nuclear BA receptor FXR, a membrane-bound BA-specific receptor TGR5 (also called GPBAR1 or M-BAR/BG37) was discovered in 2002 (Kawamata *et al.*, 2003; Maruyama *et al.*, 2002) and new metabolic functions of BAs were uncovered. TGR5 belongs to the rhodopsin-like subfamily of G-protein coupled receptors (GPCRs) and meanwhile became a founder of new BA-receptor subclass of GPCRs. The highest TGR5 expression was reported in gallbladder, whereas liver, brown adipose tissue, intestine and some regions of central nervous system showed low TGR5 expression (Vassileva *et al.*, 2006; Watanabe *et al.*, 2006). In liver, sinusoidal endothelial cells, Kupffer cells and intrahepatic bile ducts showed positive TGR5 staining, whereas hepatocytes and the quiescent stellate cells were negative (Keitel *et al.*, 2008; Keitel *et al.*, 2007; Keitel *et al.*, 2010b). Despite intensive studies, the functional role of TGR5 activation in response to BAs still remains poorly understood.

Several studies showed ***protective role of TGR5*** activation in inflammation. First functional reports on TGR5 activation were in macrophages and described the anti-inflammatory impact of TGR5 activation by BAs (Kawamata *et al.*, 2003; Keitel *et al.*, 2008). Besides this, TGR5 activation in sinusoidal endothelial cells (SECs) suggested important role of this receptor in nitric oxide (NO) production via c-AMP-dependent induction of endothelial nitric oxide synthetase (eNOS) (Keitel *et al.*, 2007). BA-mediated activation of TGR5 was also reported to reduce diet-induced obesity and insulin resistance by increasing the energy expenditure in brown adipose tissue (BAT) through cAMP-dependent induction of iodothyronine deiodinase 2 (D2), an enzyme that is involved in thyronine-to-triiodothyroxine transformation (Watanabe *et al.*, 2006).

Since Tgr5 is expressed on the cilia of bile ducts, its activation by luminal BAs is very likely have anti-inflammatory effects in cholangiocytes and to stimulate bile flow and biliary HCO₃⁻ secretion. This effect would be beneficial in conditions of increased bile toxicity, such as increased biliary cholesterol or BA content.

However, Tgr5-deficient mice showed normal development and no signs of growth abnormalities or any organ failure on control diet.

4.9. Peroxisome proliferator-activated receptor gamma (PPAR γ)

Hepatic stellate cells express all three classes of nuclear receptor peroxisome proliferator-activated receptor (PPAR): PPAR α , PPAR γ and PPAR δ . Similar to FXR, PPARs form a heterodimer with RXR to bind specific DNA sequence of target genes, called peroxisome proliferator hormone response elements (PPRE).

Free fatty acids and eicosanoids are endogenous ligands for PPARs. Ligand binding depends on the specific shape of ligand-binding domain, indicating that recruitment of selective co-activators and co-repressors can stimulate or inhibit target gene transcription. PPAR γ (NR1C3) also called a glitazone receptor is highly expressed in various tissues and regulates fat and glucose homeostasis. Importantly, PPAR γ expression was found to be dramatically repressed after stellate cell activation. Moreover, activated stellate cell phenotype was reversed by PPAR γ activation (Hazra *et al.*, 2004b; Hellemans *et al.*, 2003; Marra *et al.*, 2000; Miyahara *et al.*, 2000; Yang *et al.*, 2006), establishing an important role of this NR in process of fibrogenesis. From the other side, activation of PPAR γ is an efficient mechanism to inhibit the inflammation in different tissues (Straus *et al.*, 2007). Specifically, anti-inflammatory effects of PPAR γ are mediated by inhibition of NF- κ B and AP-1-mediated pathways and decreased production of intra- and extra-cellular adhesion molecules. These properties of PPAR γ make it very attractive for treatment of chronic cholestatic liver diseases with portal inflammation and fibrosis. Indeed, PPAR γ agonists showed beneficial effects in PBC patients by reducing the serum liver enzymes, IgM and AMA levels (Dohmen *et al.*, 2004; Iwasaki *et al.*, 2007; Kanda *et al.*, 2003; Kurihara *et al.*, 2000; Miyaguchi *et al.*, 2000; Nakai *et al.*, 2000). However, serious side effects including hepatotoxicity, pancreatitis and rhabdomyolysis were observed. Therefore, targeting PPAR γ by natural ligands with diminished toxicity may be a promising therapeutic approach for hepatic inflammation and fibrosis.

5. MAJOR AIMS OF THE THESIS

The aim of the research described in this thesis was to better understand the pathophysiology of cholangiopathies and to study the role of nuclear receptors PPAR γ and FXR as well as membrane BA receptor TGR5 as novel therapeutic strategies for chronic cholestatic liver diseases such as sclerosing cholangitis. In this context, the following hypotheses have been addressed:

- PPAR γ activation by natural and non toxic PPAR γ agonists may promote the reversal of activated stellate cell phenotype and stop the progression of biliary fibrosis. In addition, PPAR γ activation in cholangiocytes may repress the activated cholangiocyte phenotype and reduce portal inflammation in sclerosing cholangitis.
- Transcriptional regulation of BA homeostasis by intestinal and hepatic FXR activation may decrease intrahepatic and systemic concentrations of potentially toxic BAs and improve liver damage in cholestasis. Moreover, direct FXR activation may have anti-inflammatory effects on hepatocytes as well as cholangiocytes and therefore is likely to inhibit portal inflammation in sclerosing cholangitis.
- Expression of membrane-bound BA receptor TGR5 on the cilia of bile ducts may stimulate the secretory functions of cholangiocytes by luminal BAs and will promote bile flow and biliary HCO $_3^-$ secretion through cAMP-mediated pathways. In addition, TGR5 activation in Kupffer cells and cholangiocytes may directly inhibit hepatic inflammation in sclerosing cholangitis.

To address these hypotheses, peroxisome proliferators-activated receptor gamma (PPAR γ), BA-dedicated NR farnesoid X receptor (FXR) and the membrane-bound BA receptor TGR5 were studied as therapeutic targets in *Mdr2*^{-/-} mouse model of sclerosing cholangitis *in vivo*. In addition, potential molecular and cellular mechanisms involved in pathogenesis of chronic cholestasis were highlighted *in vitro*.

In part 1 the potential therapeutic effects of BA-independent anti-inflammatory and anti-fibrotic effects of PPAR γ were studied by using a natural compound curcumin in *Mdr2*^{-/-} model.

In part 2 the role of BA-dedicated nuclear receptor FXR and membrane receptor TGR5 was addressed in *Mdr2*^{-/-} mouse model of sclerosing cholangitis as well as complementary model of total biliary obstruction: (common bile duct ligation).

6. MATERIALS AND METHODS

Materials and Methods are reused from (Baghdasaryan et al., 2010) and (Baghdasaryan et al., 2011) with modifications.

Animal experiments. *Mdr2*^{-/-} mice of the FVB/N background were obtained from Jackson Laboratory (Bar Harbor, ME) and housed with a 12:12-hour light/dark cycle with water and a control mouse diet (SSNIFF, Soest, Germany) *ad libitum*. *Fxr*^{+/+} (C57BL6/J background) mice were from Himberg, Austria. *Fxr*^{-/-} mice were obtained from Dr. Frank Gonzalez (NIH, Bethesda, MD) (Sinal *et al.*, 2000). *Tgr5-Tg* mice were kindly provided by Dr. J. Auwerx (Thomas *et al.*, 2009). Male *Tgr5-Tg* mice were cross-bred with female C57BL6/J mice to generate sufficient amount of heterozygous *Tgr5-Tg* and *Tgr5*^{+/+} littermates. The experimental protocols were approved by the local Animal Care and Use Committee according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the US National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985).

Feeding protocols are described separately for each experimental part.

Food intake, liver/body weight and spleen/body weight measurements. Food intake, general behavior, activity and body weight (BW) were monitored each second day. Liver weight (LW) and spleen weight (SW) were normalized to the BW at time point of harvesting and the % ratios were calculated (LW/BW and SW/BW respectively).

Serum liver biochemistry. Blood was collected at harvesting. Samples were left for 10 min at room temperature and centrifuged for 15 min at 4500 rpm. Serum samples were stored at -80°C until analysis. Assays for serum total bilirubin, cholesterol, triglycerides, alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were performed by a Hitachi 917 analyzer (Boehringer Mannheim, Mannheim, Germany). For serum BA measurements Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) was used.

Liver histology. For conventional light microscopy (hematoxylin and eosin (H&E) and Sirius red staining), livers were fixed in 4% neutral buffered formaldehyde solution for 24

h and embedded in paraffin. Paraffin-embedded livers were cut into sections of 2 μ M thickness. Tissue slides were first heated at 65°C for 40 min, followed by deparafinization with 2 changes of xylol solution (approx. 8 min. each) followed by dehydration with 2 changes of 100 % ethanol (2 min. each). Slides were further put into subsequent solutions of 90%, 70% and 2 changes of 50% ethanol and washed briefly in distilled water. Slides were stained in solution of Hematoxylin nach Gill for 5 min, followed by wash in tap water. After 5 sec. of Kalialaun solution slides were differentiated in 0.6% HCl with further bluing in 30% ammonia solution. Each previous step was followed by the washing in running tap water for approx 5 min. Slides were counterstained in eosin-phloxin and 90% ethanol solution (1:1). Slides were finally dehydrated in subsequent solutions of 90% ethanol and 100% ethanol (twice each), cleared in butyl acetate and mounted with the xylol-based mounting media entellan.

For Sirius red staining, deparafinization and dehydration of slides was performed as described for H&E. Slides were stained with Celestine blue for 7 min, followed by the staining with Hämalaun nach Mayer. After short differentiation in 0.6% HCl and washing under the tap water slides were stained in Picro-Siriusred for 30 min. with subsequent dehydration in 100% ethanol (3x), cleaning in xylol and mounting with entellan.

Immunohistochemistry. Detection of cholangiocyte cytoskeleton marker keratin 19 (K19), vascular cell adhesion molecule-1 (Vcam-1), proliferation marker Ki-67, mononuclear inflammatory marker CD-11b, macrophage marker F4/80 and lymphocyte markers CD4 and CD8 was performed in cryo-preserved liver samples as described (Fickert *et al.*, 2004). Liver tissue was preserved immediately after animal harvesting in pre-cooled 2-methyl-butan solution and stored in liquid nitrogen. Slices were cut 3-4 μ M thick and fixed with pre-cooled formalin, methanol, and acetone solutions (for K19, Cd4, CD8 and CD11b stainings). Rat K19 antibody (dilution 1:500, Troma-III, Developmental Studies Hybridoma Bank, Iowa City, IA) was used to detect the cholangiocellular mass. To detect the mononuclear inflammatory cells, a rat anti-CD11b (dilution 1:50, BD Pharmingen) was used, whereas for lymphocytes anti-CD4 (dilution 1:30, BD Pharmingen) and anti-CD8 (dilution 1:10, BD Pharmingen) antibodies were used.

For Vcam-1 and F4/80 stainings, tissue fixation in pre-cooled acetone (-20°C) was used. Rat anti-Vcam-1 antibody (dilution 1:50, BD Pharmingen) was used to detect the reactive cholangiocyte phenotype and rat monoclonal anti-F/80 antibody (dilution 1:200, AbDSerotec) was used in to detect the macrophages. As secondary antibody, a polyclonal

rabbit anti-rat biotinylated immunoglobulin (dilution 1:100, Dako, Glostrup, Denmark) was used. Negative controls were stained together with the experimental slides without incubation with the specific primary antibody.

Determination of hepatocyte proliferation. Polyclonal rabbit anti-Ki-67 antibody (dilution 1:500, Novocastra, Newcastle upon Tyne, UK) was used to detect Ki-67 protein in paraffin embedded liver sections. β -amino-9-ethyl-carbazole (AEC; Dako, Glostrup, Denmark) has been used to detect binding of the antibody in ABC system (Dako). Number of Ki-67–positive nuclei in 30 high-power fields per animal in 5 animals per group was counted to assess the hepatocellular proliferation.

Determination of hepatic hydroxyproline content. To quantify liver fibrosis, hepatic hydroxyproline (HP) was measured from standardized liver lobe, weighing approximately 200 mg, as described previously (Jamall *et al.*, 1981). The liver lobe was first homogenized in 6 n HCl solution, after which the homogenate was incubated in sealed glass tube at 110°C for 24 h for hydrolysis. The hydrolyzates were filtered using a filter paper and the filtrate was neutralized by using a citrate acetate buffer containing 2.2% NaOH. As standards, titrated concentrations of pure hydroxyproline (Sigma Aldrich, Germany) have been used. Samples and standards were incubated with Chloramin-T solution (20 min at room to) followed by 12 min incubation with perchloric acid and 20 min incubation with the dimethylbenzaldehyde solution at 60°C after cooling, absorbance was measured at 560 nm.

Messenger RNA analysis and polymerase chain reaction. RNA isolation, complementary DNA synthesis, and real-time polymerase chain reaction were performed as described previously (Fickert *et al.*, 2006).

Western blotting for hepatic keratin 19, Vcam-1, hepatocellular transporters Ntcp, Bsep, Mrp2, Mrp3, Mrp4 and β -actin. Total protein was isolated from frozen liver homogenates as described (Fickert *et al.*, 2006). Protein concentrations were determined using a Bradford kit (BioRad, Richmond, CA, USA). Western blotting for K19 and Vcam-1 was performed by using monoclonal rat anti-K19 antibody (dilution 1:5000, Troma-III, Developmental Studies Hybridoma Bank, Iowa City, IA) and polyclonal rat Vcam-1 antibody (dilution 1:30, PharMingen, san Diego, CA,) as previously described

(Fickert *et al.*, 2006). For hepatocellular transporters, preparation of liver membranes, detection of immuno-complexes as well as densitometric quantification of band intensities, was performed as described (Fickert *et al.*, 2006; Fickert *et al.*, 2001). Polyclonal antibodies against Ntcp, Bsep, Mrp2, Mrp3 (dilution 1:1000, provided by Dr. D. Keppler, German Institute of Cancer Research, Heidelberg, Germany), Mrp2 and Mrp3 (dilution 1:1000, provided by Dr. John D. Schuetz, University of Tennessee, Memphis, TN) were used.

Determination of bile flow and biliary composition. To distinguish between the primary effects of treatment on bile and the secondary changes due to improved liver injury, bile flow and biliary composition were analyzed after short-term (7days) feeding protocol. Mice were anesthetized (10 mg of avertin intraperitoneally), abdominal cavity was opened, common bile duct was ligated and gallbladder was cannulated. After a 10-minute equilibration period, bile was collected in pre-weighed tubes for 30 minutes. Bile flow was determined gravimetrically and normalized to LW. Samples were kept frozen at -20°C until analysis. Bile flow and output of biliary phospholipids (PLs), BAs, cholesterol and glutathione were determined as previously described (Fickert *et al.*, 2006). Biliary PL concentration was determined using a commercial kit (Phospholipid B; Wako, Neuss, Germany). Biliary cholesterol was measured by enzymatic colorimetric test using the cholesterol liquicolor kit (HUMAN, Weisbaden, Germany).

For biliary HCO₃⁻ measurements, bile was collected for additional 30 minutes in separate tubes under oil. Biliary HCO₃⁻ content was determined immediately after collection by using an automatic blood gas analyzer (AVL 995 hp, AVL, Graz, Austria; COBAS Mira plus, Roche) by measurement of pH and total carbon dioxide (CO₂) pressure.

Measurement of serum, hepatic and biliary BA composition.

(Measurement of different BAs was performed by Prof. Aldo Roda and Stefania Vecchiotti in the laboratory of Prof. A. Roda).

Endogenous BAs and INT-compounds were measured in bile samples by high performance liquid chromatography (HPLC) as previously described (Roda *et al.*, 1995)

Common Bile Duct Ligation (CBDL) experiments. CBDL was performed on C57BL6/J mice after 5 days of pre-feeding either a chow diet or a diet, supplemented with the INT-747, the INT-777 or the INT-767 (0.03% w/w) as described (Gujral *et al.*, 2004).

Experimental feeding was continued for additional 3 days and mice were sacrificed on day 4 after CBDL. Serum liver enzymes, liver histology and gene expression were performed as described above.

Cell culture experiments

Isolation, culture and experiments with portal MFBs. Portal MFBs were isolated as described (Uchio *et al.*, 2002). Briefly, the biliary tree was isolated from 12-week-old *Mdr2*^{-/-} mice by collagenase and pronase digestion. Portal tract residues were allowed to adhere in Petri dishes and cultured in DMEM medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin/streptomycin. After 2-3 days an extensive outgrowth and adherence of portal MFBs was observed around biliary structures. Purification of portal MFBs from other cells was obtained through serial passages and cell type analysis was made by immunofluorescence staining for hepatocellular cytoskeleton marker K8/18, activated MFB marker α -SMA and nuclear marker DAPI. The majority of isolated cell population was α -SMA-positive and K8/18-negative, demonstrating sufficient purity (data not shown). Cells from passage 8-20 were used for experiments. Cell viability was tested by trypan blue dye exclusion after 24 h of curcumin incubation. At concentrations of 10 and 20 μ M curcumin did not induce MFB death, while 30 μ M was toxic (Figure 5A); therefore, 10 and 20 μ M concentrations were subsequently used for *in vitro* experiments. In proliferation studies, portal MFBs were stained for proliferation marker Ki-67.

Isolation of nuclear and cytoplasmic proteins. Nuclear and cytoplasmic proteins were isolated by using a Pierce kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Rockford, IL, USA). Western blotting for NF- κ B and pERK was performed by using polyclonal rabbit antibodies against NF- κ B (dilution 1:2000) (NeoMarkers, Fremont, CA, USA) and pERK1/2 (dilution 1:1000) (Cell Signaling Technology, Inc, Danvers, MA, USA). Specific binding was detected by secondary anti-rabbit antibody (dilution 1:3000) (Cell Signaling Technology, Inc, Danvers, MA, USA). Equal protein loading was confirmed by Coomassie blue staining of gels and Ponceau S staining of membranes.

Cholangiocyte culture experiments with curcumin. Cholangiocarcinoma cell line (HuCCT1) was obtained from Japan Health Sciences Foundation Resources Bank, Osaka,

Japan. Cells were incubated in RPMI culture medium (PAA Laboratories GmbH, Pasching, Austria) with 10% FCS and used for experiments at 80% confluence. TNF- α and troglitazone were from Sigma-Aldrich Chemie, GmbH, Steinheim, Germany. Bisphenol A diglycidyl ether (BADGE) was obtained from Sigma-Aldrich, Vienna, Austria. Cytotoxicity of curcumin was tested by trypan blue dye exclusion method.

In vitro experiments for FXR and TGR5 signaling. RAW264.7 and HepG2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Mouse primary immortalized cholangiocyte cell line (BEC) was described elsewhere (Yahagi et al., 1998) and obtained from Dr. T. Toyota. Mouse primary portal myofibroblasts (MFB) were isolated from *Mdr2*^{-/-} mouse livers as described (Uchio et al., 2002). All cell lines were cultivated in 10% fetal calf serum (FCS) and 1% penicillin-streptomycin-containing DMEM (PAA Laboratories GmbH, Pasching, Austria) culture medium in humidified incubator with 5%CO₂ at 37°C. INT-767 concentrations were determined for each cell line using the MTT cell viability assay. Pro-inflammatory cytokine expression was induced in RAW264.7 cells by 6 h incubation with LPS (100 ng/ml) in 0% FCS. To study anti-inflammatory effects of INT-767, cells were pre-incubated with INT-767 (5 μ M) overnight followed either by INT-767 incubation alone or by simultaneous LPS and INT-767 incubation for 6 h. In HepG2 and BEC cells pro-inflammatory cytokine expression was induced by 24 h incubation with human and mouse TNF- α respectively (10 ng/ml) in 0% FCS. To study the INT-767 anti-inflammatory effects cells were pre-incubated with INT-767 (10 μ M) overnight followed either by INT-767 incubation alone or by simultaneous TNF- α and INT-767 incubation for 24 h. To induce Col1a1 pro-fibrogenic gene expression portal MFBs were incubated with 10% FCS-containing medium. To study the INT-767 anti-fibrotic effects cells were first pre-incubated with INT-767 (5 μ M) in 0% FCS overnight followed either by INT-767 incubation alone or by simultaneous INT-767 and 10% FCS incubation for 24 h.

Statistical Analysis. Results were evaluated using SPSS software (Release 14.0, 2005, SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using one-way ANOVA test followed by Mann-Whitney. Data are reported as means of 5 animals per group (unless otherwise noted) $\pm$ SD. A P value $\leq$ 0.05 was considered significant.

7. STUDY PART 1

The results of study part 1 have been published in:

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Curcumin improves sclerosing cholangitis in *Mdr2*^{-/-} mice by inhibition of cholangiocyte inflammatory response and portal myofibroblast proliferation.

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This chapter is adapted from (**Baghdasaryan *et al.*, 2010**)

Summary

Chronic cholangiopathies have limited therapeutic options and represent an important indication for liver transplantation. Curcumin, the yellow pigment of the spice turmeric, has pleiotropic actions and attenuates hepatic damage in animal models of chemically-induced liver injury. Whether curcumin has beneficial effects in cholangiopathies is unknown.

Potential anti-cholestatic, anti-inflammatory and anti-fibrotic mechanisms of curcumin were explored *in vivo* in *Mdr2*^{-/-} mice as a murine model of chronic cholangiopathy; as well as *in vitro* in a cholangiocyte cell line (HuCCT1) and portal myofibroblasts (MFBs) isolated from *Mdr2*^{-/-} mice.

Liver damage, cholestasis and fibrosis were reduced in *Mdr2*^{-/-} mice after curcumin feeding. Moreover, curcumin inhibited cholangiocyte proliferation and expression of activation marker vascular cell adhesion molecule-1 (Vcam-1) in *Mdr2*^{-/-} mice. Curcumin - similar to PPAR γ synthetic agonist troglitazone - directly inhibited TNF- α -induced inflammatory activation of cholangiocytes *in vitro*, whereas these beneficial effects of curcumin were largely blocked by a PPAR γ synthetic antagonist. In addition, curcumin blocked proliferation and activation of portal MFBs by inhibiting ERK1/2 phosphorylation, thus contributing to reduced fibrogenesis.

These results show that curcumin may have multiple targets in liver including PPAR γ activation in cholangiocytes and inhibition of ERK1/2 signaling in MFBs, thereby modulating several central cellular events in a mouse model of cholangiopathy. Targeting these pathways may be a promising therapeutic approach for treatment of cholangiopathies.

Introduction

In cholestatic liver diseases, cholangiocytes may undergo phenotypic and functional modifications, characterized by production of pro-inflammatory and pro-fibrogenic mediators, thus contributing to propagation and progression of liver diseases (Alvaro *et al.*, 2008; Alvaro *et al.*, 2007; Lazaridis *et al.*, 2004). Therefore, it is attractive to hypothesize that targeting the bile duct inflammatory phenotype may be beneficial in treatment of cholangiopathies.

Curcumin, a non-steroidal yellow pigment found in rhizomes of the perennial herb *Curcuma longa*, has been used for centuries in Ayurvedic medicine to cure a wide range of gastrointestinal disorders, and is a component of the spice turmeric (Aggarwal *et al.*, 2006; Goel *et al.*, 2008a; Jain, 1994; Maheshwari *et al.*, 2006; Oppenheimer, 1937; Rasyid *et al.*, 1999). In rodent models of chemically induced liver damage and fibrosis such as carbon tetrachloride and thioacetamide-induced liver cirrhosis, curcumin was shown to have anti-inflammatory, anti-oxidative and anti-fibrotic properties (Fu *et al.*, 2008; Reyes-Gordillo *et al.*, 2008; Reyes-Gordillo *et al.*, 2007; Zhao *et al.*, 1996). Curcumin was shown to activate PPAR γ , a NR that has strong anti-inflammatory effects. Moreover, blocking JNK signalling by curcumin was shown to inhibit inflammation-mediated alterations of hepatobiliary transporter gene expression in hepatocytes (Li *et al.*, 2002). However, to date no study has addressed the effects of curcumin on bile duct inflammation and liver damage in cholangiopathies. We therefore designed a longitudinal study to explore potential molecular mechanisms of curcumin in *Mdr2*^{-/-} mice serving as a genetic model of progressive cholangiopathy with biliary fibrosis (Fickert *et al.*, 2004; Mauad *et al.*, 1994; Popov *et al.*, 2005).

This study demonstrates that curcumin reduces liver damage, cholangitis and biliary fibrosis in *Mdr2*^{-/-} mice. Beneficial effects of curcumin were mediated by acting on multiple targets: inhibiting the inflammatory phenotype of bile duct epithelial cells through PPAR γ activation and blocking proliferation/activation of portal myofibroblasts (MFBs) through inhibition of ERK1/2 signaling.

Feeding protocol of control and curcumin-supplemented diets. Curcumin was gifted by Sabinsa Corporation (Sabinsa Utah, Payson, UT, USA) in form of Curcumin C3 complex, where content of curcuminoids reached 96.24%, of which 72.1% was curcumin, 21.98% demethoxy curcumin and 5.91% bisdemethoxy curcumin. Curcumin was mixed to the chow diet (4% w/w) and stored after γ -irradiation (Harlan Teklad, Madison, WI, USA). Chow diet was obtained from Harlan Teklad (Harlan Teklad, Madison, WI, USA). Feeding of control and curcumin-enriched diets was started in $Mdr2^{-/-}$ mice at the age of 4 weeks. To discriminate the effects of curcumin on hepatic lesions during the disease progression experimental feeding was continued for either 4 or 8 weeks. Schematic illustration of feeding protocol in $Mdr2^{-/-}$ mice is presented in figure 6. Potential toxicity of curcumin was studied in age-matched $Mdr2^{+/+}$ mice after 4 weeks of feeding.

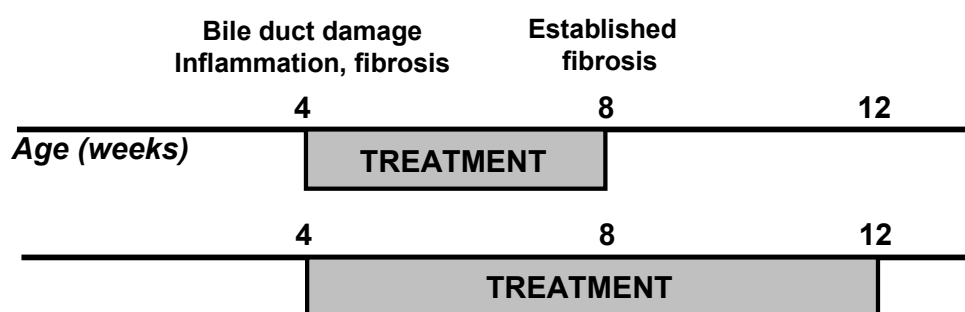


Figure 6. Schematic illustration of feeding protocols in $Mdr2^{-/-}$ mice.

Experimental feeding (chow or curcumin-enriched diets) in $Mdr2^{-/-}$ mice was initiated at the age of 4 weeks, when the ductular proliferation and fibrosis showed high activity and was continued for 4 or 8 weeks. Feeding of age-matched $Mdr2^{+/+}$ mice followed the same experimental protocol.

Results

Long-term curcumin feeding is well-tolerated and reduces liver injury in *Mdr2*^{-/-} mice. Survival rate (100%), body weight gain and food consumption (average: 4-5 g/day) were undistinguishable between chow-fed and curcumin-enriched diet-fed *Mdr2*^{+/+} as well as *Mdr2*^{-/-} mice after 4 and 8 weeks, demonstrating that long-term dietary curcumin enrichment was well tolerated in mice (Figure 7). Serum liver enzymes (ALT and ALP) were significantly lower in *Mdr2*^{-/-} mice after 4 weeks of curcumin feeding compared with untreated controls (Figure 8). In line with reduced serum liver tests, curcumin decreased histological features of sclerosing cholangitis and ductular proliferation in *Mdr2*^{-/-} mice after 4 weeks of feeding (Figure 3), demonstrating beneficial role of curcumin on cholestatic phenotype in *Mdr2*^{-/-} mice.

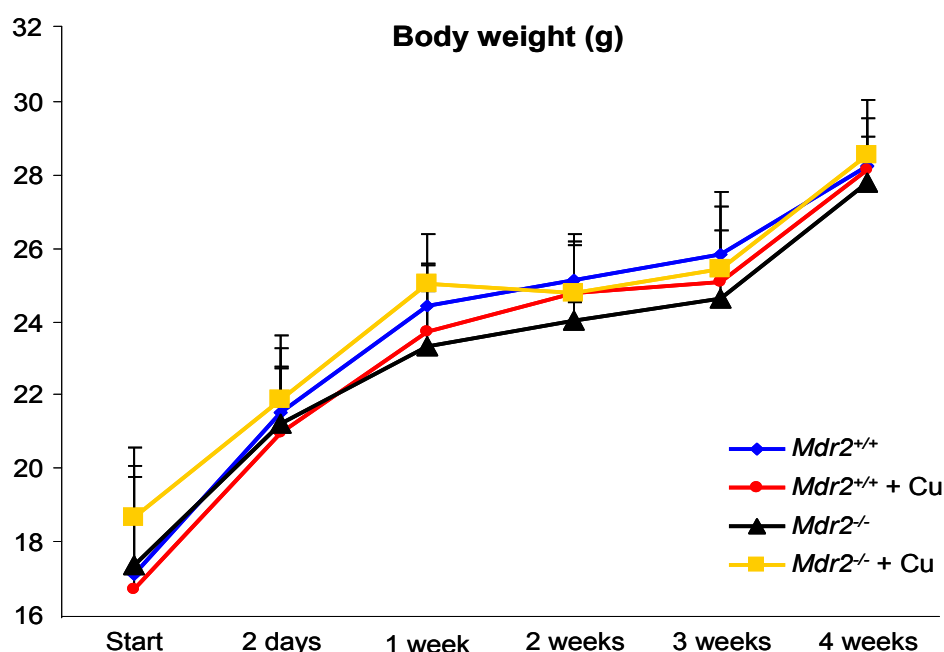


Figure 7. Gain of body weight in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice during 4 weeks of control diet or curcumin-supplemented diet feeding.

Curcumin feeding does not influence body weight in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice. Values are presented as means of 5 mice per group $\pm$ SD.

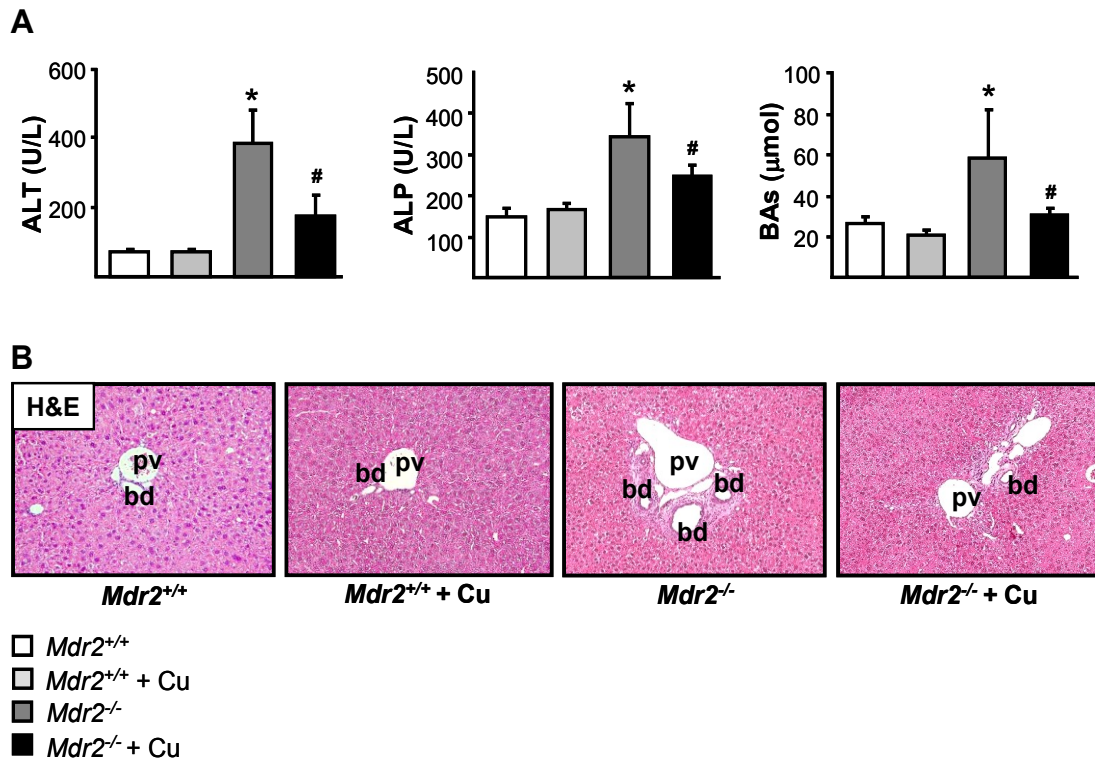


Figure 8. Curcumin reduces liver damage and cholestasis in $Mdr2^{-/-}$ mice.

(A) Serum parameters of liver injury (ALT) and cholestasis (ALP and BAs) were measured in $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mice after 4 weeks of feeding either a chow or curcumin-enriched diets. Compared with age-matched $Mdr2^{+/+}$ mice $Mdr2^{-/-}$ mice have significantly higher serum ALT, ALP and BA levels. Curcumin feeding significantly reduces serum parameters of liver injury in $Mdr2^{-/-}$ mice ($Mdr2^{-/-} + Cu$). Values are presented as means of 5 animals per group $\pm$ SD. * $P < 0.05$ $Mdr2^{-/-}$ vs. $Mdr2^{+/+}$, # $P < 0.05$ $Mdr2^{-/-} + Cu$ vs. $Mdr2^{-/-}$. (B) Liver histological picture (H&E staining) remains unchanged between chow-fed and curcumin-fed $Mdr2^{+/+}$ mice ($Mdr2^{+/+}$ and $Mdr2^{-/-} + Cu$). Representative histological picture of age-matched $Mdr2^{-/-}$ mouse shows portal field infiltration with inflammatory cells, bile duct proliferation and biliary fibrosis. Curcumin decreases signs of sclerosing cholangitis in $Mdr2^{-/-}$ mouse after 4 weeks (original magnification 10-fold). ALT: alanine aminotransferase, ALP: alkaline phosphatase, BAs: bile acids, bd: bile duct, pv: portal vein.

Sirius red staining demonstrated anti-fibrotic effects of curcumin, which were further confirmed by decreased hepatic HP content and inhibited *Colla1* gene expression (Figure 9).

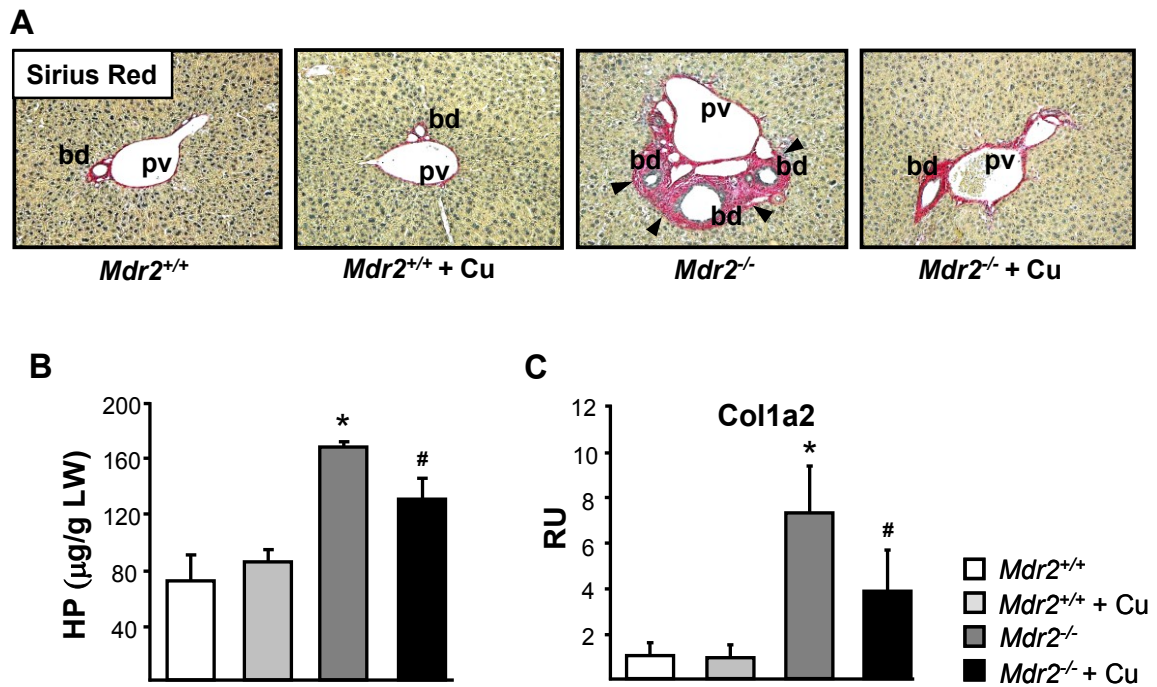


Figure 9. Hepatic fibrosis is reduced in $Mdr2^{-/-}$ mice by curcumin.

(A) Representative pictures of Sirius red stained livers. Chow-fed and curcumin-fed $Mdr2^{+/+}$ mice ($Mdr2^{+/+}$ and $Mdr2^{+/+} + Cu$ respectively) show no biliary fibrosis. In contrast, untreated $Mdr2^{-/-}$ mouse shows pronounced hepatic fibrosis as presented by collagen deposition (red) around bile ducts (arrowheads). Curcumin-treated $Mdr2^{-/-}$ mouse ($Mdr2^{-/-} + Cu$) demonstrates reduced collagen deposition after 4 weeks compared with age matched untreated littermate (original magnification x20). (B) Hepatic hydroxyproline (HP) content in $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mice after chow or curcumin-enriched diet feeding. (C) Curcumin feeding significantly inhibits hepatic *Col1a2* mRNA levels in $Mdr2^{-/-}$ mice. Gene expression is normalized to 18s house-keeping gene and mean value in $Mdr2^{+/+}$ mice is expressed as 1. Values are presented as means of 4-5 mice per group $\pm$ SD. * $P < 0.05$ $Mdr2^{-/-}$ vs. $Mdr2^{+/+}$, # $P < 0.05$ $Mdr2^{-/-} + Cu$ vs. $Mdr2^{-/-}$, bd: bile duct, pv: portal vein.

However, prolonged curcumin feeding for 8 weeks did not show further reduction in serum ALT and ALP (Figure 10). Untreated $Mdr2^{-/-}$ mice showed progression of fibrosis with the age as determined by higher HP levels (243 ± 45.8 at 12 weeks vs. 166 ± 3.1 $\mu\text{g/g LW}$ at 8 weeks). However, hepatic HP content did not differ between 4 and 8 weeks of curcumin feeding (139 ± 12 vs. 129.6 ± 15 $\mu\text{g/g LW}$, n.s.) (Figure 10C), despite more pronounced reduction of relative HP levels after 8 weeks (42.7% reduction after 8-weeks

vs. 21.9% reduction after 4 weeks of experimental feeding). These findings suggest that curcumin feeding is less likely to have an effect on fibrosis resolution, and reduces progression of hepatic fibrosis in *Mdr2*^{-/-} mice *via* inhibition of collagen production.

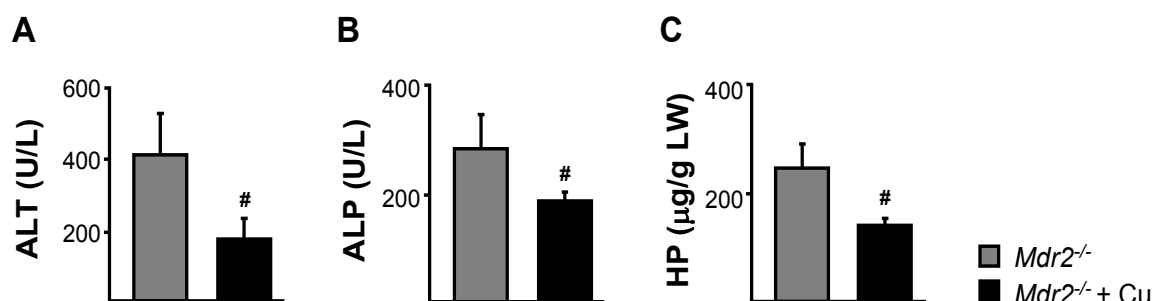


Figure 10. Prolonged curcumin feeding for 8 weeks reduces liver damage and fibrosis in *Mdr2*^{-/-} mice.

Serum liver enzymes (A, B) and hepatic hydroxyproline (C) were measured in *Mdr2*^{-/-} mice to quantify liver damage and fibrosis after 8 weeks of curcumin feeding. Curcumin reduces serum liver enzymes as well as fibrosis in *Mdr2*^{-/-} mice after 8 weeks of feeding. Values are presented as means of 4 animals per group $\pm$ SD. [#] $P < 0.05$ *Mdr2*^{-/-} + Cu vs. *Mdr2*^{-/-}. ALT: alanine aminotransferase, ALP: alkaline phosphatase, HP: hydroxyproline.

Curcumin promotes biliary glutathione output and stimulates BA-independent bile flow in *Mdr2*^{-/-} mice. Altered bile formation is critical for development of liver disease in *Mdr2*^{-/-} mice (Smit *et al.*, 1993; Trauner *et al.*, 2008). Direct effects of curcumin on bile flow and composition were next addressed in 4-week-old *Mdr2*^{+/+} and *Mdr2*^{-/-} mice after a short-term (7 days) feeding. Curcumin did not modify biliary output of BAs, PL or cholesterol, but significantly increased glutathione output and consequently the bile flow in *Mdr2*^{+/+} mice (table 3). Interestingly, biliary glutathione output was significantly lower in untreated *Mdr2*^{-/-} mice compared with age-matched *Mdr2*^{+/+} mice. In line with *Mdr2*^{+/+} mice, curcumin significantly increased and restored biliary glutathione output and stimulated bile flow in *Mdr2*^{-/-} mice. However, hepatic content of total glutathione was not significantly altered by curcumin feeding (data not shown), indicating, that curcumin is less likely to influence glutathione synthesis. Untreated *Mdr2*^{-/-} mice showed almost absent PL and decreased cholesterol output, which remained unchanged after curcumin feeding. Together, these findings show that curcumin did not

modify BA toxicity, but significantly increased BA-independent bile flow by increasing glutathione output.

Table 3. Bile flow and output of main biliary constituents in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice after curcumin feeding.

Parameter	<i>Mdr2</i> ^{+/+}	<i>Mdr2</i> ^{+/+} + Cu	<i>Mdr2</i> ^{-/-}	<i>Mdr2</i> ^{-/-} + Cu
Bile flow (μl/g LW/min)	1.3 ± 0.2	1.7 ± 0.2 ^{\$}	1.2 ± 0.2	1.8 ± 0.2 [#]
Bile acids (nmol/g LW/min)	28.1 ± 6.5	38.2 ± 10.1	27.4 ± 1.7	30.1 ± 14.4
Cholesterol (nmol/g LW/min)	0.6 ± 0.2	0.6 ± 0.1	0.1 ± 0.0*	0.2 ± 0.0*
Glutathione (nmol/g LW/min)	4.0 ± 0.7	8.4 ± 1.4 ^{\$}	1.2 ± 0.3*	4.9 ± 1.1 [#]
Phospholipids (nmol/g LW/min)	4.5 ± 0.7	4.7 ± 1.1	0.3 ± 0.1*	0.5 ± 0.1*

Mdr2^{+/+} + Cu: curcumin-fed *Mdr2*^{+/+} mice, *Mdr2*^{-/-} + Cu: curcumin-fed *Mdr2*^{-/-} mice. Values are means of 5 animals per group ± SD. * $P < 0.05$ *Mdr2*^{-/-} and *Mdr2*^{-/-} + Cu vs. *Mdr2*^{+/+}, # $P < 0.05$ *Mdr2*^{-/-} + Cu vs. *Mdr2*^{-/-}, \$ $P < 0.05$ *Mdr2*^{+/+} + Cu vs. *Mdr2*^{+/+}.

BA synthesis and transport are not altered by curcumin in *Mdr2*^{-/-} mice. Since serum BA levels were significantly decreased by curcumin, next BA homeostasis was studied to address whether beneficial effects of curcumin might be due to changes in BA homeostasis. Expression of main basolateral BA transporter, Ntcp, as well as canalicular BA export transporters Bsep, Mrp2 and Mrp3 remained unchanged by curcumin on protein level (Figure 11A), reflecting unchanged biliary BA output. Moreover, gene expression of *Cyp7a1*, the rate limiting enzyme in the neutral pathway of BA synthesis was also remained unchanged after curcumin feeding in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice (Figure 11B), proving that curcumin does not influence BA homeostasis in mice.

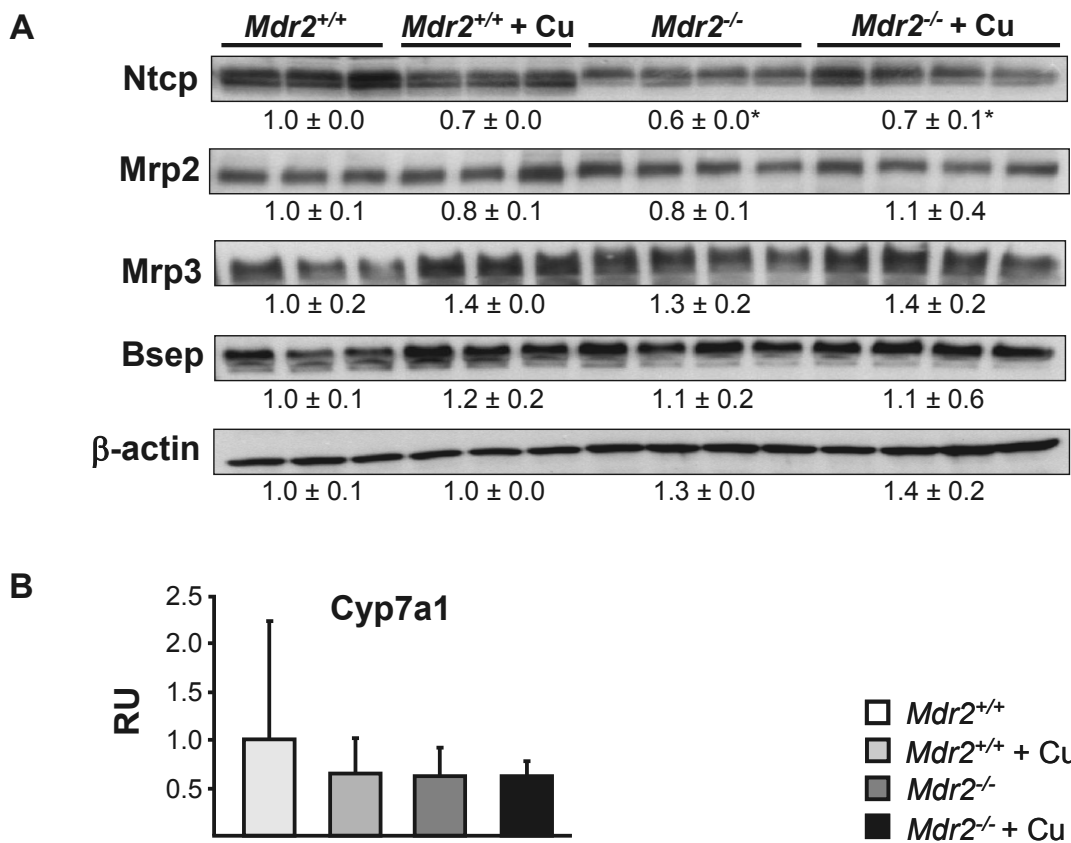


Figure 11. Bile acid transport and synthesis are not altered by curcumin.

(A) Key hepatocellular transporters were detected in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice by Western blotting in liver membranes. No significant changes of Ntcp, Bsep, Mrp2 and Mrp3 were detected by curcumin in *Mdr2*^{-/-} mice (*Mdr2*^{-/-} + Cu). Mean densitometry data of untreated *Mdr2*^{+/+} mice was accepted as 1. Values are presented as means of 3-4 animals per group. β-actin protein levels were used as a protein loading control. * $P < 0.05$ *Mdr2*^{-/-} and *Mdr2*^{-/-} + Cu vs. *Mdr2*^{+/+}. (B) Curcumin does not significantly modify mRNA levels of Cyp7a1 in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice. Expression levels are normalized to 18s mRNA and mean value of *Mdr2*^{+/+} is expressed as 1. Values are presented as means of 5 animals per group ± SD. Bsep: bile salt export pump, Cyp7a1: cholesterol 7α-hydroxylase, Mrp2: multidrug resistance associated protein 2, Mrp3: multidrug resistance associated protein 3, Ntcp: Na⁺/taurocholate cotransporting polypeptide.

Proliferation of activated portal myofibroblasts is inhibited by curcumin via repression of ERK signaling. Liver fibrosis in *Mdr2*^{-/-} mice is mainly localized in portal fields and the portal myofibroblasts (MFBs) represent major fibrogenic cells in this model. To address the cellular and molecular mechanisms behind the anti-fibrotic effects of curcumin, portal MFBs were next isolated from *Mdr2*^{-/-} mice. Purity of isolated primary cell population is presented in figure 12.

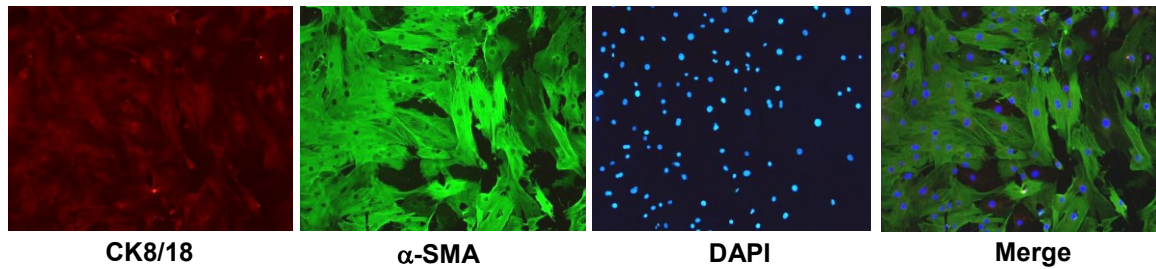


Figure 12. Purity of isolated primary myofibroblasts.

Immunofluorescent staining for activated myofibroblast marker α -SMA, hepatocyte/cholangiocyte cytoskeleton marker K8/18 and nuclear marker DAPI is presented. Isolated cell population contained mainly α -SMA-positive and K8/18-negative cells.

Portal MFBs as well as hepatic stellate cells (HSC) gain high activity and proliferating rate upon contact with the plastic *in vitro*. To reduce portal MFB activation and to synchronize the isolated cells *in vitro*, 0% FCS culture medium was used, whereas 10% FCS was used as a stimulating agent for proliferation and activation. Curcumin did not show cytotoxic effects on primary MFBs at concentrations of 10 and 20 μ M (figure 13).

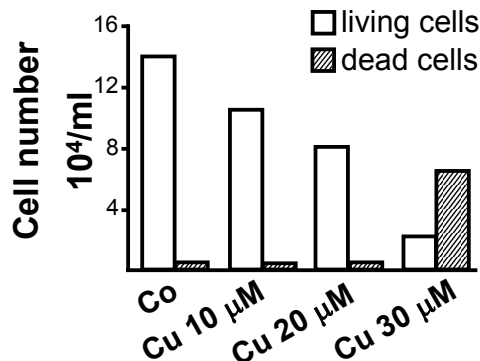


Figure 13. Curcumin is not cytotoxic in primary MFBs at concentrations of 10 and 20 μ M. Portal Myofibroblasts (MFB) were isolated from *Mdr2*^{-/-} as described in Materials and Methods. Cell survival was determined by Trypan blue dye exclusion after 24 h of incubation with curcumin *in vitro*. Curcumin did not influence viability of primary MFBs at concentrations of 10 and 20 μ M, whereas 30 μ M was toxic. Values are presented as absolute number of living and dead cells calculated per 1 mL of cell suspension.

Proliferation and fibrogenic phenotype of portal MFBs was stimulated by 10% FCS as reflected by increased number of Ki-67-positive cells and induced *Colla1* and α -SMA

gene expression (figures 14 and 15). Interestingly, curcumin inhibited 10% FCS-stimulated proliferation of portal MFBs at concentrations of 10 and 20 μ M (Figure 13).

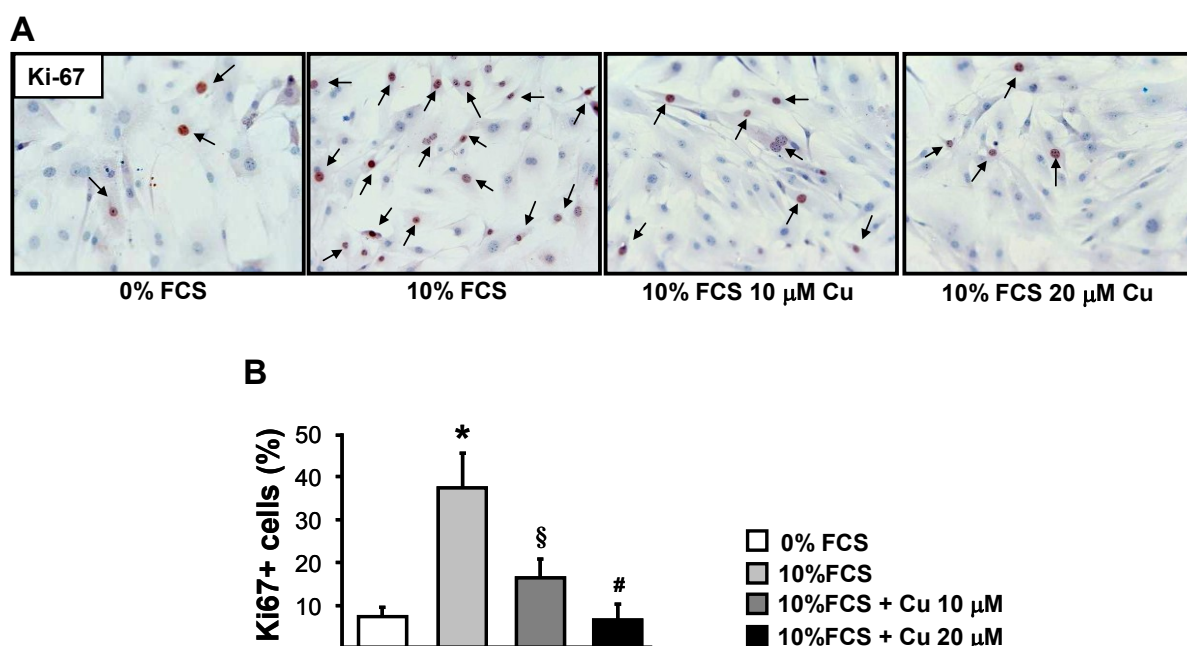


Figure 14. Curcumin reduces primary MFB proliferation and inhibits pro-fibrogenic gene expression in primary MFBs.

Curcumin reduces MFB proliferation in vitro. Cells were first synchronized in 0% FCS containing medium and further grown on sterile cover slips using 0% FCS, 10% FCS (to induce proliferation) and curcumin at concentrations of 10 and 20 μ M in 10 % FCS medium for 24 h. **(A)** Immunohistochemical staining for proliferation marker Ki-67 was used and **(B)** number of Ki-67-positive nuclei was counted in 10 randomly chosen power fields in triplicate for each condition. The mean value of Ki-67-positive vs. total cell number ratio (%) was calculated. Compared with 0 % FCS condition 10% FCS stimulates MFBs proliferation, whereas curcumin (10 and 20 μ M) reduces 10% FCS-induced MFB proliferation in concentration-dependent manner.

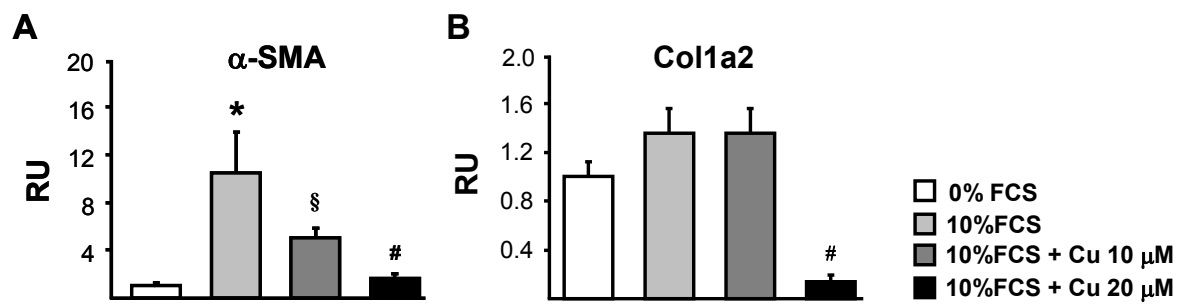


Figure 15. Effects of curcumin on pro-fibrogenic gene expression.

(A) and (B) Curcumin inhibits *Col1 α 2* and α -SMA gene expression. Expression levels are normalized to 18s house-keeping gene and levels of controls are expressed as 1. Data are presented as means $\pm$ SD. * $P < 0.005$ 10% FCS vs. 0% FCS, § $P < 0.005$ 10% FCS + Cu 10 μ M vs. 10% FCS, # $P < 0.005$ 10% FCS + Cu 20 μ M vs. 10% FCS.

As next, PPAR γ (NR1C3), NF- κ B and ERK signaling were studied as mechanisms known to mediate proliferation and activation of fibrogenic cells (Chen *et al.*, 2008; Hazra *et al.*, 2004b; Hellerbrand *et al.*, 1998a; Hellerbrand *et al.*, 1998b; Marra *et al.*, 2000). Loss of PPAR γ expression has been reported to accompany the activation of fibrogenic cells and is associated with increased ability to modulate the recruitment and activation of inflammatory cells by expression of monocyte chemotactic protein 1 (Mcp-1) (Marra *et al.*, 1998; Marra *et al.*, 2000). Isolated primary MFBs showed preserved basal *Ppar γ 1* expression, which remained unchanged after incubation with curcumin (data not shown). Moreover, expression of *Mcp-1*, which has been reported to be altered by PPAR γ ligands in fibrogenic cells (Marra *et al.*, 2000), remained unchanged by curcumin in MFBs (figure 16A) despite significant inhibition of 10% FCS-induced *Col1 α 2* and α -SMA gene expression (figure 15). These findings suggested that inhibition of fibrogenic phenotype in MFBs by curcumin might be PPAR γ -independent. Interestingly, curcumin-mediated reduction of α -SMA content in *Mdr2*^{-/-} mice was associated with decreased cytosolic pERK1/2 content compared with untreated littermates (figure 16B). In line with these data, curcumin dramatically decreased cytoplasmic as well as nuclear pERK1/2 content (figure 16C) in cultures of isolated portal MFBs, whereas nuclear NF- κ B (p65) remained unchanged, indicating that inhibition of ERK1/2 signaling pathway could mediate anti-proliferative effects of curcumin in primary MFBs.

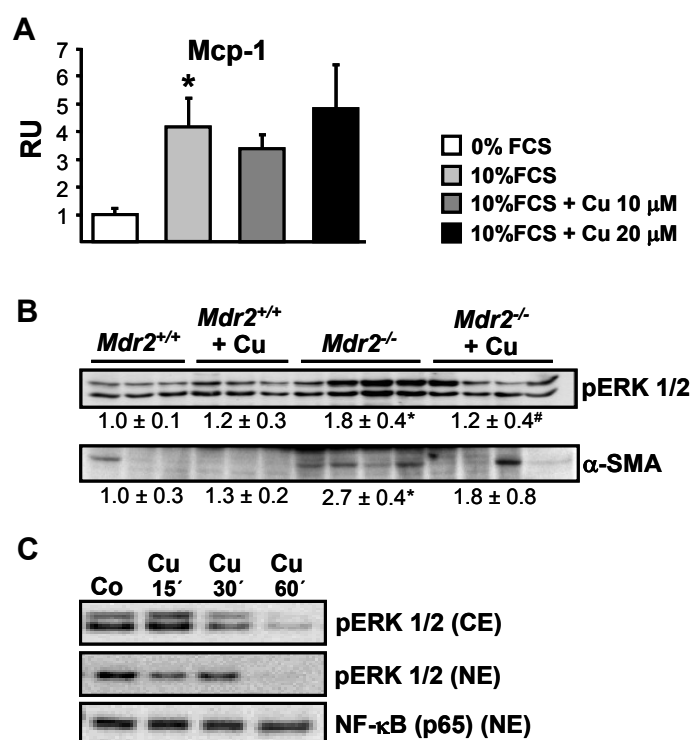


Figure 16. Curcumin represses ERK signaling in portal MFBs.

(A) Curcumin does not inhibit expression of PPAR γ target gene *Mcp-1* in primary MFBs. (B) Western Blotting was performed to detect hepatic content of pERK1/2 and α -SMA in *Mdr2*^{+/+} and *Mdr2*^{-/-} mice after feeding either on chow or curcumin-enriched diet (*Mdr2*^{+/+} + Cu and *Mdr2*^{-/-} + Cu respectively). Protein was isolated from frozen liver homogenates as described in Materials and Methods. Curcumin feeding significantly decreased cytoplasmic pERK1/2 content in livers of *Mdr2*^{-/-} mice. Densitometry data are expressed as means of 3-4 mice per group and mean value of untreated *Mdr2*^{+/+} mice is expressed as 1. * $P < 0.05$ *Mdr2*^{-/-} vs. *Mdr2*^{+/+}, # $P = 0.05$ *Mdr2*^{-/-} + Cu vs. *Mdr2*^{-/-}. (C) Cytoplasmic and nuclear proteins were isolated from MFBs after incubation with 10% FCS and 20 μ M curcumin and analyzed for pERK1/2 and NF- κ B (p65) by Western blotting. Nuclear translocation of NF- κ B is not influenced; whereas ERK phosphorylation is inhibited by curcumin in portal MFBs. Representative graph from 2 independent experiments performed in duplicate for each condition. α -SMA: alpha smooth muscle actin, CE: cytoplasmic extracts, Co: control, Cu: curcumin, FCS: Fetal Calf Serum, *Mcp-1*: monocyte chemotactic protein 1, NE: nuclear extracts.

Curcumin attenuates the reactive cholangiocyte phenotype by activating the nuclear receptor PPAR γ . Reactive cholangiocyte phenotype was decreased by curcumin

as determined by reduction of cholangiocyte cytoskeleton marker K19 in immunohistochemical staining and Western blotting (figure 17) as well as decreased immunohistochemical signal for Vcam-1 (figure 18).

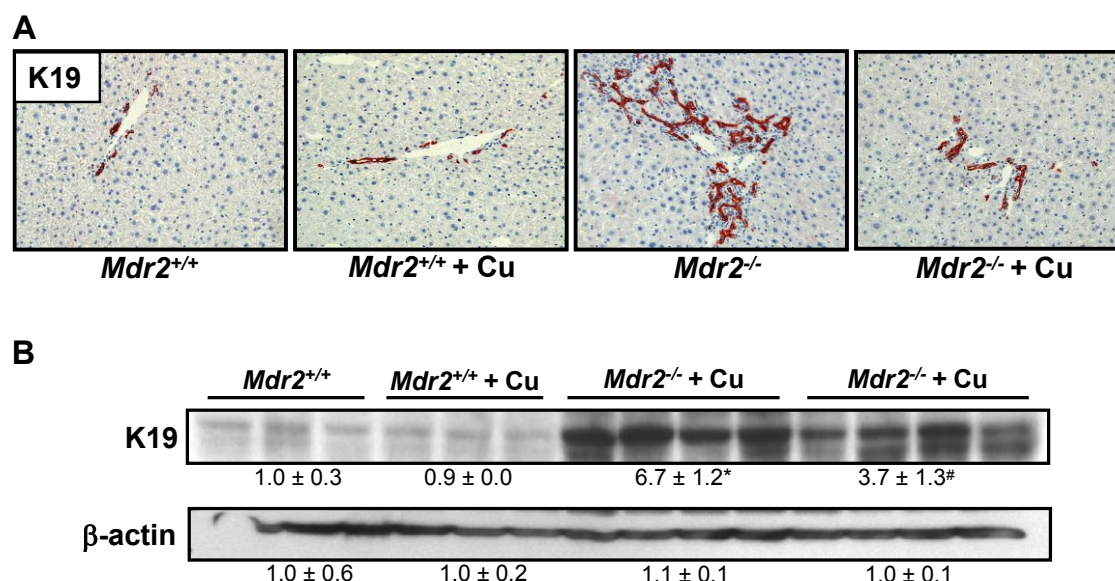


Figure 17. Cholangiocyte proliferation is inhibited by curcumin in *Mdr2*^{-/-} mice.

(A) Cholangiocytes were detected by immunohistochemical staining for cholangiocyte cytoskeleton marker K19. Representative picture of untreated *Mdr2*^{-/-} mouse shows pronounced ductular proliferation (red) compared with *Mdr2*^{+/+} mice. Curcumin feeding decreased ductular proliferation in *Mdr2*^{-/-} mice (*Mdr2*^{-/-} + Cu). K19 staining remained unchanged in *Mdr2*^{+/+} mice after curcumin feeding. **(B)** Quantification of bile duct proliferation. Total protein was analyzed for K19 content by Western blotting. Curcumin-treated *Mdr2*^{-/-} showed significantly less K19 protein levels compared with control diet-fed age-matched littermates. Densitometry data are presented as means of 3-4 animals per group ± SD. Mean value of control diet-fed *Mdr2*^{+/+} mice was accepted as 1. Protein content in *Mdr2*^{+/+} + Cu, *Mdr2*^{-/-} and *Mdr2*^{-/-} + Cu is expressed as fold change relative to *Mdr2*^{+/+} mice. * $P < 0.05$ *Mdr2*^{-/-} vs. *Mdr2*^{+/+}, # $P = 0.05$ *Mdr2*^{-/-} + Cu vs. *Mdr2*^{-/-}.

Pro-inflammatory cytokines such as *Tnf-α* may stimulate *Vcam-1* gene expression (Kelly *et al.*, 2007; Osborn *et al.*, 1989). Interestingly, despite curcumin inhibited *Vcam-1* expression, it failed to inhibit increased *Tnf-α* gene expression in *Mdr2*^{-/-} mice (figure 18). These findings suggested that beneficial effects of curcumin on cholangiocyte reactive activation are not likely to be *Tnf-α* mediated. Indeed, curcumin significantly increased the gene expression of *Pparγ*, the nuclear receptor known to have critical role in inflammation, suggesting that inhibition of *Vcam-1* production in cholangiocytes by

curcumin could be Ppar γ -mediated. In line with these findings, incubation of the cholangiocyte cell line HuCCT1 (expressing functionally active PPAR γ) with TNF- α induced *VCAM-1* gene expression, which was abrogated by curcumin and specific PPAR γ agonist troglitazone (figure 18D). In addition, pre- and co-incubation with the PPAR γ synthetic antagonist BADGE restored curcumin-mediated inhibition of *VCAM-1* gene expression (figure 18E). These data therefore indicate that, that reduction of cholangiocyte reactive activation by curcumin may be PPAR γ -mediated.

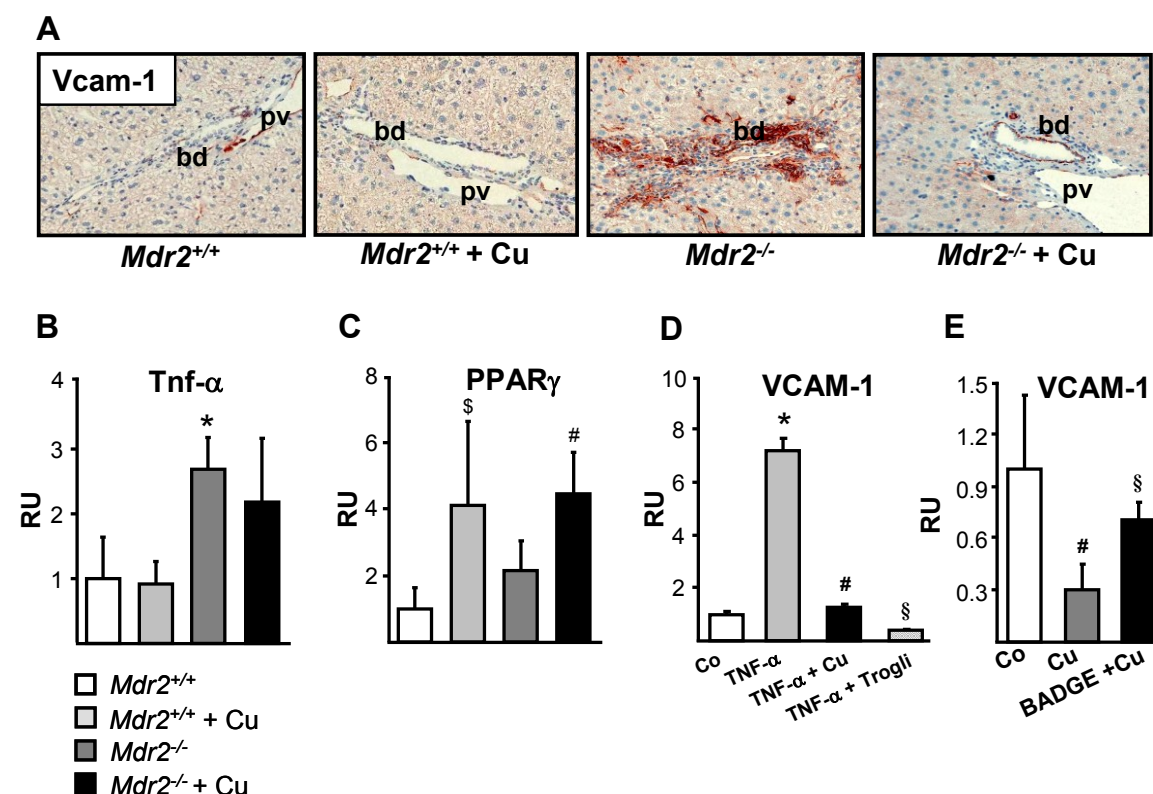


Figure 18. Curcumin decreases Vascular Cell Adhesion Molecule 1 (VCAM-1) production in bile duct epithelial cells.

(A) Reactive cholangiocyte phenotype was detected by immunohistochemical staining for *Vcam-1*. Representative histological picture shows highly positive *Vcam-1* staining in bile ducts of untreated *Mdr2*^{-/-} mice (red), whereas bile ducts from chow- and curcumin-fed *Mdr2*^{+/+} mice stain negative for *Vcam-1*. Curcumin reduced *Vcam-1* staining in bile ducts of *Mdr2*^{-/-} mice after 4 weeks of feeding (*Mdr2*^{-/-} + Cu). bd: bile duct, pv: portal vein. (B) Gene expression of pro-inflammatory cytokine *Tnf- α* was significantly increased in *Mdr2*^{-/-} compared with *Mdr2*^{+/+} mice and remained unchanged after curcumin feeding. (C) Baseline levels of PPAR γ gene expression were undistinguishable between untreated *Mdr2*^{+/+} and *Mdr2*^{-/-} mice, whereas curcumin induces PPAR γ expression in both the genotypes (*Mdr2*^{+/+} + Cu and *Mdr2*^{-/-} + Cu respectively). B and C: Expression

levels are normalized to 18s gene expression and mean value of $Mdr2^{+/+}$ is expressed as 1. Values are means of five mice per group $\pm$ SD. * $P < 0.05$ $Mdr2^{-/-}$ vs. $Mdr2^{+/+}$, # $P < 0.05$ $Mdr2^{-/-}$ + Cu vs. $Mdr2^{+/+}$ and $Mdr2^{-/-}$, § $P < 0.05$ $Mdr2^{+/+}$ + Cu vs. $Mdr2^{+/+}$. **(D)** Curcumin blocks TNF- α -stimulated induction of VCAM-1 gene expression in cholangiocytes. HuCCT1 cells were incubated either with TNF- α alone (to induce VCAM-1 expression, TNF- α (10 ng/ml) and curcumin (10 μ M) simultaneously or a PPAR γ specific agonist troglitazone (100 μ M) for 24 h. Curcumin as well as troglitazone inhibited TNF- α induced VCAM-1 expression in the HuCCT1 cholangiocyte cell line. **(E)** Inhibition of VCAM-1 expression in HuCCT1 cholangiocytes by curcumin is partially restored by PPAR γ synthetic antagonist BADGE. HuCCT1 cells were pre-incubated with BADGE (50 μ M) for 12 h and further incubated either with curcumin alone (10 μ M) or simultaneous curcumin and BADGE for 24 h. D and E: Representative experiments performed in triplicate for each condition are presented. Values are means $\pm$ SD. * $P < 0.005$ TNF- α vs. Co, # $P < 0.005$ TNF- α + Cu vs. TNF- α , § $P < 0.005$ TNF- α + Trogli vs. TNF- α , E: # $P < 0.05$ Cu vs. Co, § $P < 0.05$ BADGE + Cu vs. Cu. Co: control, Cu: curcumin, Trogli: troglitazone.

Curcumin significantly reduces portal inflammation as well as hepatocellular proliferation in $Mdr2^{-/-}$ mice. Portal inflammation in $Mdr2^{-/-}$ mice originates due to production of adhesion molecules and inflammatory cytokines by bile duct cells and activated MFBs. Untreated $Mdr2^{-/-}$ mice showed pronounced infiltration of portal fields with CD11b-positive cells, reflecting the neutrophil granulocytes (figure 19) and CD4 – positive cells, reflecting the lymphocytes (figure 20A). In line with inhibited Vcam-1 production and portal MFB activation, curcumin significantly reduced CD11b and CD4 staining in portal fields of $Mdr2^{-/-}$ mice (figures 19 and 20). However, CD8 positive cells were not detectable in $Mdr2^{-/-}$ mice at this time point independent of treatment condition (figure 20B). Together with unchanged *Tnf- α* , *Il-1 β* and *Tgf- β* expression (figure 18B and data not shown), these data suggest that inhibition of cholangiocellular Vcam-1 production was sufficient to reduce inflammatory cell infiltration in $Mdr2^{-/-}$ mice.

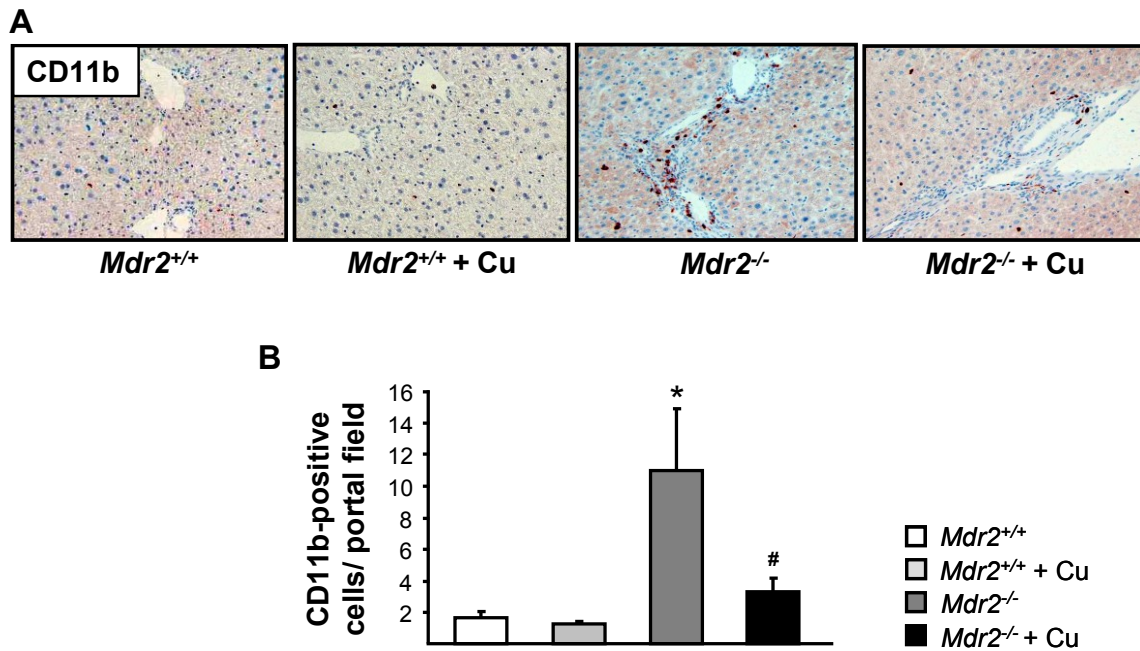


Figure 19. Recruitment of neutrophil granulocytes is reduced in portal fields of $Mdr2^{-/-}$ mice after curcumin feeding.

(A) Representative immunohistochemical staining shows reduced amount of portal CD11b-positive cells in curcumin-treated $Mdr2^{-/-}$ mice ($Mdr2^{-/-} + Cu$). (B) Quantification of neutrophil granulocytes by counting the number of CD11-positive cells in 20 portal fields per mouse in 4-5 mice per group. Values are means of 4 animals per group $\pm$ SD. * $P < 0.05$ $Mdr2^{-/-}$ vs. $Mdr2^{+/+}$, # $P < 0.005$ $Mdr2^{-/-} + Cu$ vs. $Mdr2^{-/-}$.

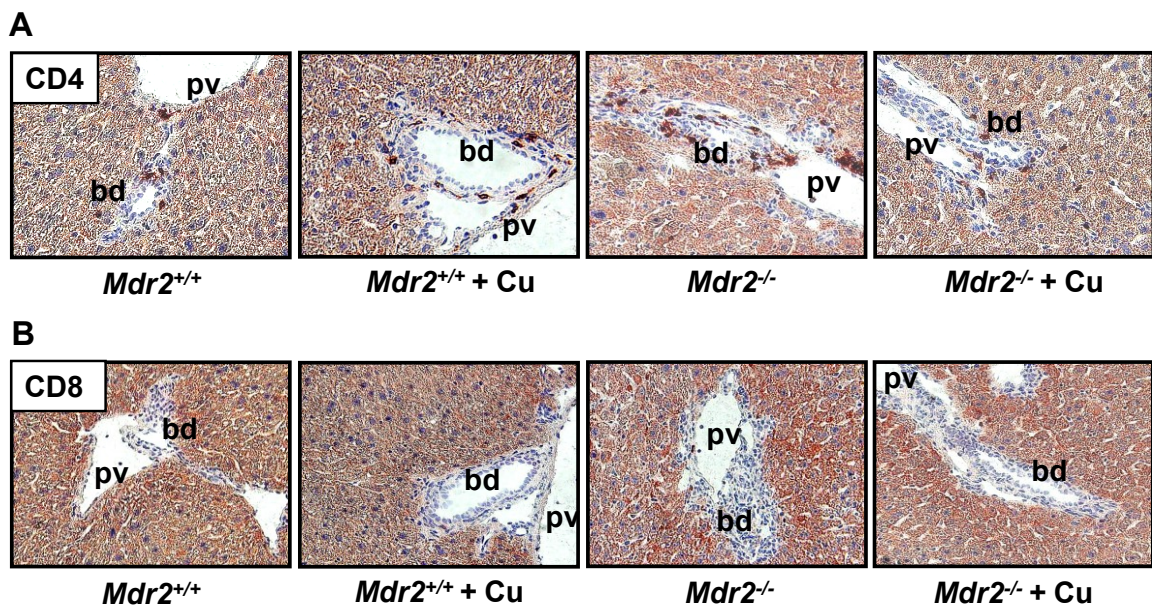


Figure 20. Curcumin reduces recruitment of lymphocytes to the portal fields of $Mdr2^{-/-}$ mice.

(A) Representative staining for lymphocyte marker CD4 demonstrates increased accumulation of CD4 positive cells in the portal field of $Mdr2^{-/-}$ compared with $Mdr2^{+/+}$ mouse. 4-week curcumin feeding reduced CD4 positive lymphocyte accumulation in portal fields of $Mdr2^{-/-}$ mouse. No pronounced differences were observed in curcumin-fed ($Mdr2^{+/+} + Cu$) compared with chow-fed $Mdr2^{+/+}$ mice. (B) No CD8 positive cells were detected in the portal fields of $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mice independent of treatment. bd: bile duct, pv: portal vein.

In addition, curcumin significantly reduced hepatocellular proliferation in $Mdr2^{-/-}$ mice reflecting the overall reduced hepatocellular damage (figure 21A, B).

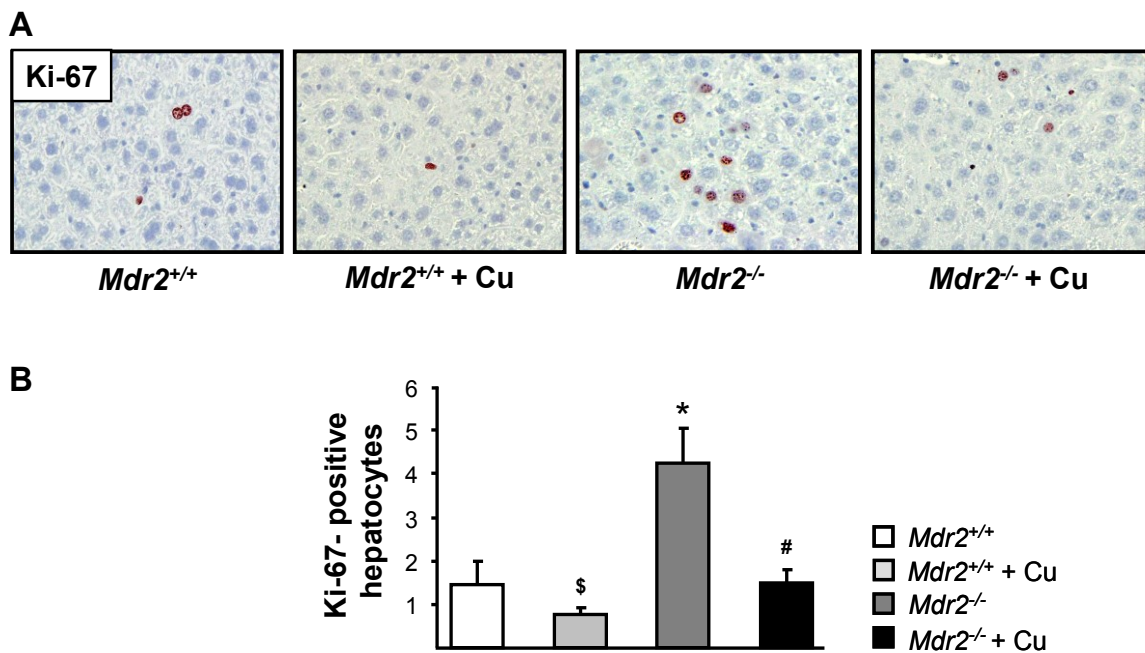


Figure 21. Curcumin significantly reduces hepatocellular proliferation in $Mdr2^{-/-}$ mice. (A) Immunohistochemical staining for proliferation marker Ki-67 (red) in $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mouse after 4 weeks of experimental feeding. $Mdr2^{-/-}$ mouse demonstrates increased number of Ki-67-positive hepatocytes compared with $Mdr2^{+/+}$. Curcumin feeding reduces Ki-67 staining in $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mice ($Mdr2^{+/+} + Cu$ and $Mdr2^{-/-} + Cu$ respectively) (original magnification x20). (B) Quantification of hepatocellular proliferation by counting the Ki-67-positive hepatocytes in 30 high-power fields per mouse in sections of 5 animals per group. Values are means of 5 animals per group $\pm$ SD. * $P < 0.05$ $Mdr2^{-/-}$ vs. $Mdr2^{+/+}$, # $P < 0.05$ $Mdr2^{-/-} + Cu$ vs. $Mdr2^{-/-}$, \$ $P < 0.05$ $Mdr2^{+/+} + Cu$ vs. $Mdr2^{+/+}$.

8. STUDY PART 2

The results of this study have been published in:

***Hepatology*. 2011 Oct;54(4):1303-12**

Dual FXR/TGR5 agonist INT-767 reduces liver injury in the *Mdr2*^{-/-} (*Abcb4*^{-/-}) mouse cholangiopathy model by promoting biliary HCO₃⁻ output.

Anna Baghdasaryan, Thierry Claudel, Judith Gumhold, Dagmar Silbert, Luciano Adorini, Aldo Roda, Stefania Vecchiotti, Frank J. Gonzalez, Kristina Schoonjans, Mario Strazzabosco, Peter Fickert, Michael Trauner

This chapter is adapted from (**Baghdasaryan *et al.*, 2011**)

Summary

Chronic cholangiopathies have limited therapeutic options and represent an important indication for liver transplantation. The nuclear farnesoid X receptor (FXR) and the membrane G protein-coupled receptor TGR5 regulate bile acid homeostasis and inflammation. Therefore, we hypothesized that activation of FXR and/or TGR5 could ameliorate liver injury in *Mdr2*^{-/-} (*Abcb4*^{-/-}) mice, a model of chronic cholangiopathy. Hepatic inflammation, fibrosis, as well as bile secretion and key genes of BA homeostasis were addressed in *Mdr2*^{-/-} mice fed either a chow diet or a diet supplemented with the FXR agonist INT-747, the TGR5 agonist INT-777, or the dual FXR/TGR5 agonist INT-767 (0.03% w/w). Only the dual FXR/TGR5 agonist INT-767 significantly improved serum liver enzymes, hepatic inflammation and biliary fibrosis in *Mdr2*^{-/-} mice, while INT-747 and INT-777 had no hepatoprotective effects. In line, INT-767 significantly induced bile flow and biliary HCO₃⁻ output, as well as gene expression of carbonic anhydrase 14, an important enzyme able to enhance HCO₃⁻ transport, in an Fxr-dependent manner. In addition, INT-767 dramatically reduced bile acid synthesis via induction of ileal *Fgf15* and hepatic *Shp* gene expression, thus resulting in significantly reduced biliary bile acid output in *Mdr2*^{-/-} mice. Conclusion: This study shows that FXR activation improves liver injury in a mouse model of chronic cholangiopathy by reduction of biliary BA output and promotion of HCO₃⁻-rich bile secretion.

Introduction

BAs have critical role in regulating their own homeostasis, lipid and glucose metabolism as well as inflammation (Thomas *et al.*, 2008). In the liver, FXR is mostly detected in hepatocytes, whereas cholangiocytes showed weak FXR expression (Fickert *et al.*, 2009). In contrast, TGR5 is highly expressed in the biliary epithelium, sinusoidal endothelial cells and Kupffer cells (Keitel *et al.*, 2009; Keitel *et al.*, 2008; Keitel *et al.*, 2007; Keitel *et al.*, 2010b). FXR activation inhibits BA synthesis (Inagaki *et al.*, 2005; Kim *et al.*, 2007) and has anti-inflammatory effects in atherosclerosis (Guo *et al.*, 2006), inflammatory bowel disease (Gadaleta *et al.*, 2010) and experimental cholestasis (Liu *et al.*, 2003), while TGR5 activation, via cAMP-mediated pathways, reduces pro-inflammatory cytokine production in macrophages (Kawamata *et al.*, 2003) and Kupffer cells (Keitel *et al.*, 2008). In addition, FXR and TGR5 mutations have been identified in intra-hepatic cholestasis of pregnancy (Van Mil *et al.*, 2007) and PSC (Hov *et al.*), respectively, emphasizing that these receptors are attractive novel therapeutic targets.

We therefore hypothesized that selective FXR activation by INT-747 (Pellicciari *et al.*, 2002), selective TGR5 stimulation by INT-777 (Pellicciari *et al.*, 2009), and/or dual FXR/TGR5 activation by INT-767 (Rizzo *et al.*, 2010) could exert beneficial therapeutic mechanisms on liver inflammation and fibrosis in mice lacking the phospholipid (PL) flippase Mdr2 (*Mdr2*^{-/-} or *Abcb4*^{-/-}) with sclerosing cholangitis (Fickert *et al.*, 2004; Mauad *et al.*, 1994; Popov *et al.*, 2005). In this study we have identified the dual FXR/TGR5 agonist INT-767 as a novel promising treatment in a mouse model of chronic cholangiopathy and characterized the underlying molecular and cellular mechanisms.

Feeding protocols for pharmacological FXR and TGR5 activation.

INT-747, INT-777 and INT-767 compounds were synthesized by Intercept Pharmaceuticals, New York, USA (figure 22). Eight week-old male *Mdr2*^{-/-} mice received either a chow diet or a diet supplemented with the FXR agonist INT-747, the TGR5 agonist INT-777 and the dual FXR/TGR5 agonist INT-767 (0.03% w/w) for 4 weeks. In addition, low-dose (0.01% w/w) INT-747-supplemented diet was tested. Since low-dose INT-747 did not significantly alter liver injury (data not shown), we used 0.03% (w/w) INT-747-supplemented diet in all subsequent experiments.

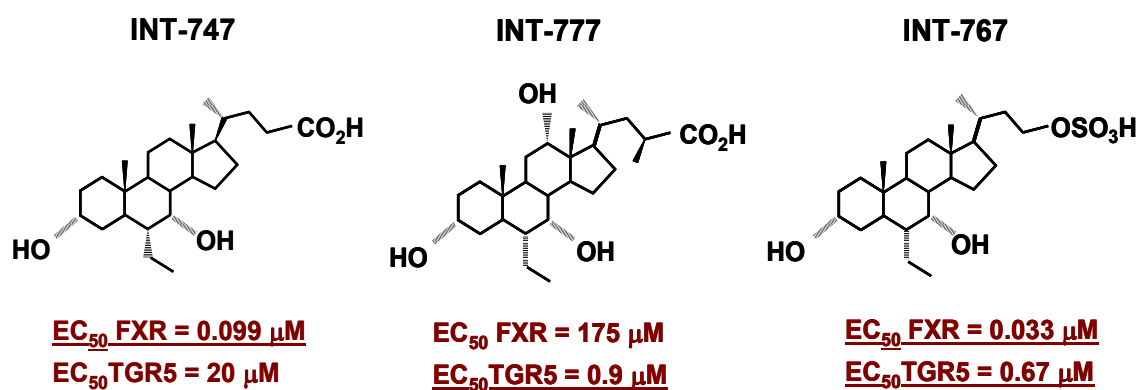


Figure 22. Chemical structures of INT-747, INT-777 and INT-767 compounds with respective EC₅₀ for FXR and TGR5.

Results

Dual FXR/TGR5 agonist INT-767 decreases liver injury in *Mdr2*^{-/-} mice.

Feeding of the selective FXR agonist INT-747, the selective TGR5 agonist INT-777 or the dual FXR/TGR5 agonist INT-767 did not influence survival rate as well as BW gain in *Mdr2*^{-/-} mice (figure 23).

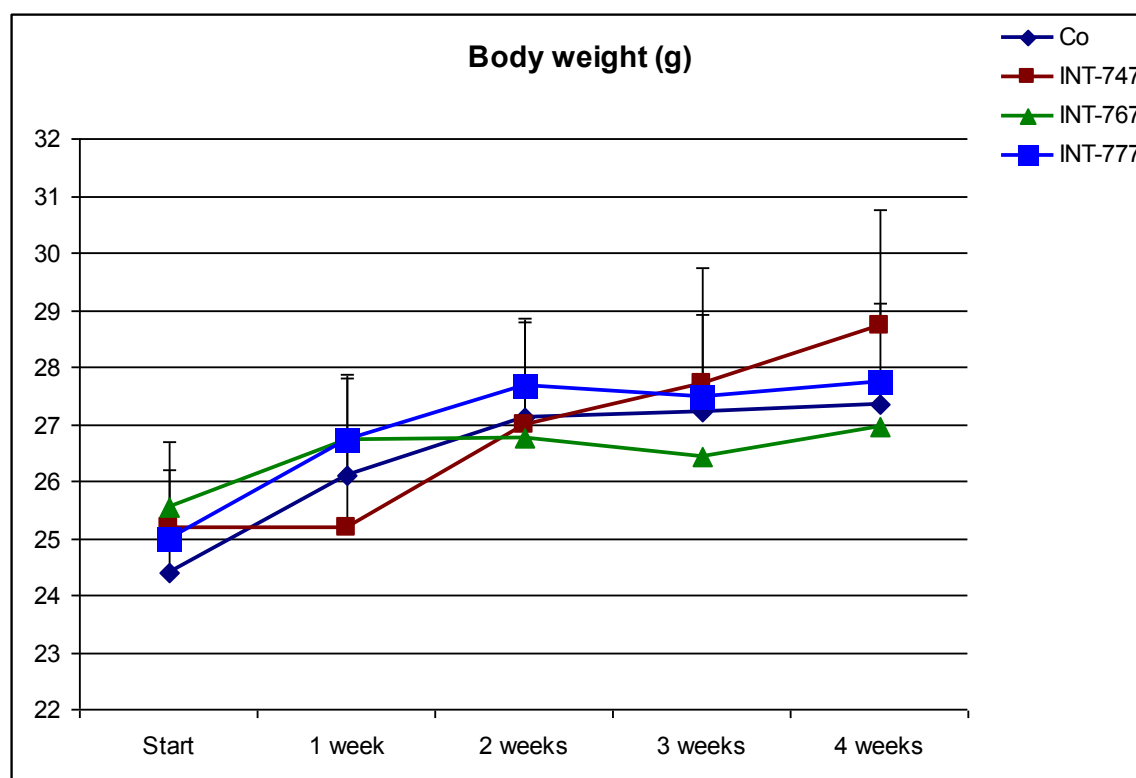


Figure 23. Gain in body weight was not altered by INT-747, INT-777 and INT-767 in *Mdr2*^{-/-} mice after 4 weeks of experimental feeding.

Means of 6 mice/group $\pm$ SD are presented. Co: chow-fed littermates.

Interestingly, INT-767 significantly reduced, whereas INT-747 and INT-777 increased serum ALT levels in *Mdr2*^{-/-} mice (figure 24A). However, all treatments increased serum ALP (figure 24A) and LW/BW ratio (figure 25A). In line with ALT levels, histological examination (H&E staining) of INT-767-treated *Mdr2*^{-/-} mouse livers showed less portal inflammation and bile duct proliferation (figure 24B). In contrast, INT-747 aggravated liver damage in *Mdr2*^{-/-} mice, as reflected by increased bile duct proliferation, portal tract expansion (figure 24B) and single cell necrosis with lobular inflammation (figure 25B), whereas no evident alterations were detected by INT-777. Together, these findings establish the beneficial role of INT-767 in *Mdr2*^{-/-} mice.

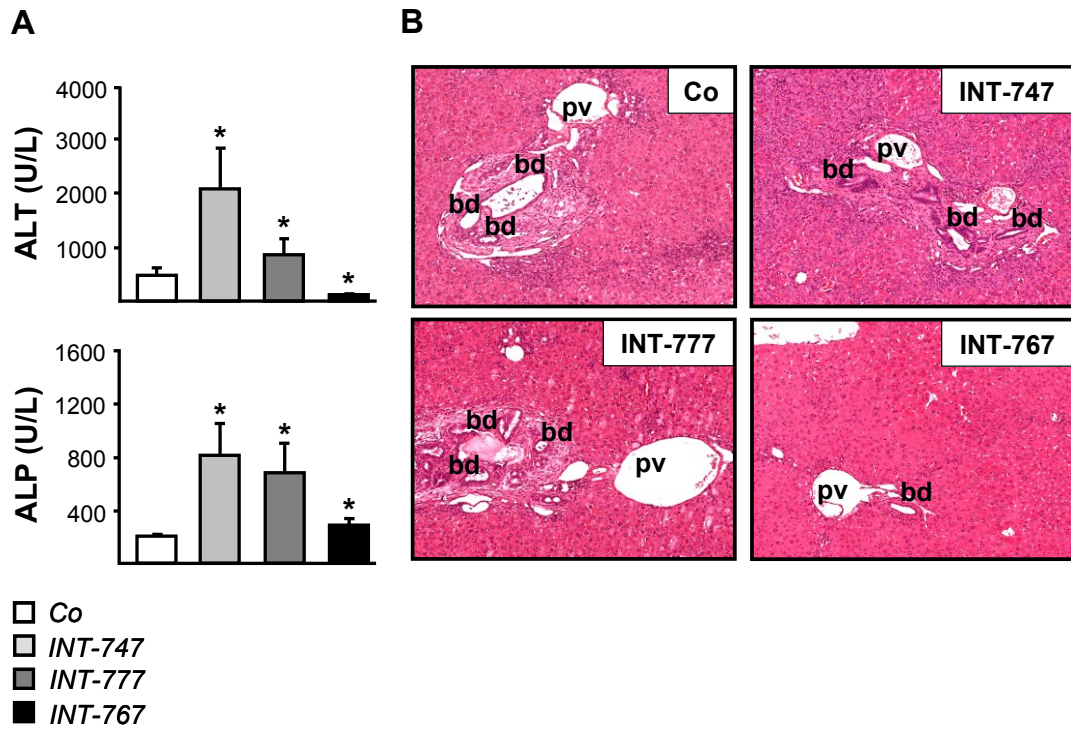


Figure 24. Dual FXR/TGR5 agonist INT-767 improves liver injury in *Mdr2*^{-/-} mice.

Mdr2^{-/-} mice were fed either on a chow diet or a diet supplemented with the FXR agonist INT-747, the TGR5 agonist INT-777 or the dual FXR/TGR5 agonist INT-767 for 4 weeks.

(A) INT-747 and INT-777 increased serum ALT and ALP levels, while INT-767 significantly reduced ALT. Means of 6 mice/group $\pm$ SD are presented. * $P < 0.05$ INT-747, INT-767 and INT-777 vs. controls. **(B)** Representative histological pictures of H&E stained livers. Bile duct proliferation and portal infiltration of inflammatory cells is reduced by INT-767. INT-747-fed *Mdr2*^{-/-} mouse showed increased bile duct proliferation, expansion of biliary tract and accumulation of inflammatory cells. No obvious alterations were detected in INT-777-fed *Mdr2*^{-/-} mouse liver. Co: chow-fed littermates, pv: portal vein, bd: bile duct.

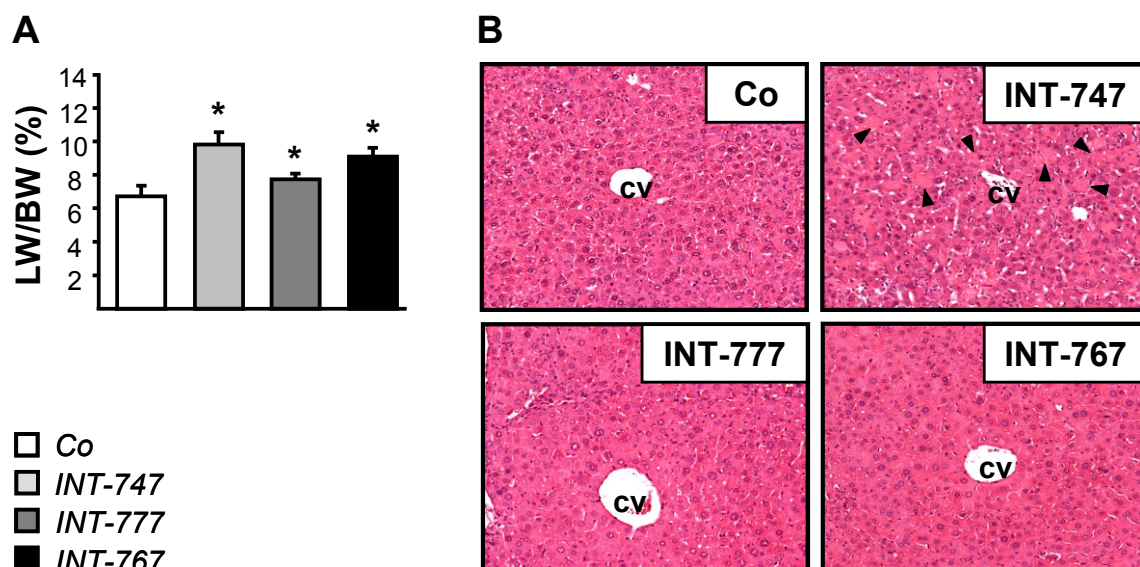


Figure 25. LW/BW ratio is increased by INT-747, INT-777 and INT-767, whereas only INT-747 induces single cell necrosis in *Mdr2*^{-/-} mice.

(A) All INT compounds increased the liver/body weight ratio (LW/BW) in *Mdr2*^{-/-} mice. Values are presented as means of 4-5 animals per group $\pm$ SD. * INT-747, INT-777 and INT-767 vs. controls. **(B)** Illustration of liver damage in H&E staining. INT-767-treated mouse liver shows no hepatocellular necrosis or inflammatory cell accumulation within the liver parenchyma. In contrast, INT-747 induced the hepatocellular necrosis (arrows) and inflammatory cell infiltration in the liver parenchyma. Magnification 20x. Co: chow-fed *Mdr2*^{-/-} mice, cv: central vein.

INT-767 suppresses hepatic inflammation, reactive cholangiocyte activation and fibrosis in *Mdr2*^{-/-} mice. Portal inflammation was next characterized by measurement of the macrophage marker *F4/80*, pro-inflammatory cytokines *Tnf- α* and *Il-1 β* as well as immunohistochemical staining for mononuclear inflammatory cells (CD11b) and macrophages (F4/80). INT-767 treatment reduced *F4/80*, *Tnf- α* and *Il-1 β* mRNA levels (figure 26) as well as number of CD-11b- and F4/80-positive cells (figure 27). In contrast, INT-747 increased *Il-1 β* mRNA levels (figure 26) and portal CD-11b- and F4/80-positive cell accumulation (figure 27).

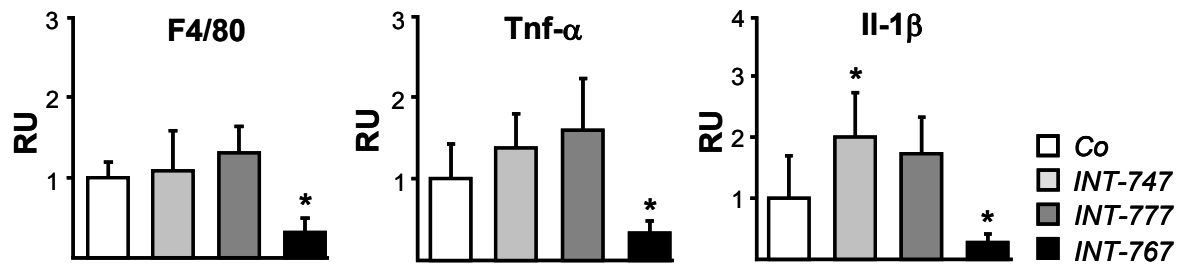


Figure 26. INT-767 reduces hepatic inflammation in *Mdr2*^{-/-} mice.

INT-767 inhibited gene expression of macrophage marker *F4/80* and pro-inflammatory cytokines *Tnf-α* and *Il-1β* in *Mdr2*^{-/-} mice, whereas INT-747 induced *Il-1β* gene expression. Gene expression levels are normalized to 36b4 house-keeping gene and the mean expression value of untreated *Mdr2*^{-/-} mice is accepted as 1. Values are means of 4-5 animals per group $\pm$ SD. Co: chow-fed *Mdr2*^{-/-} mice.

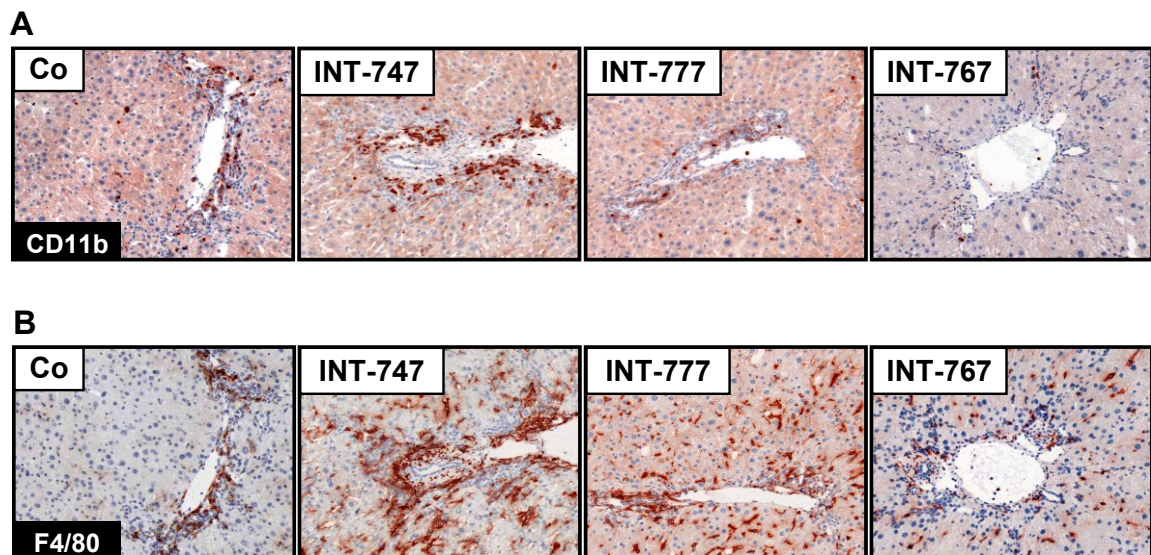


Figure 27. Portal inflammation is reduced in *Mdr2*^{-/-} mice after INT-767 feeding.

(A) Accumulation of mononuclear inflammatory cells is presented by immunohistochemical staining for CD11b. Portal infiltration of CD-11b-positive cells was reduced by INT-767, while increasing by INT-747 and remaining unchanged by INT-777. (B) Macrophage infiltration is presented by immunohistochemical staining for *F4/80* marker. INT-767 decreased, while INT-747 increased portal macrophage infiltration in *Mdr2*^{-/-} mice. No obvious change of portal inflammatory cell infiltration was evident in INT-777-fed mice (magnification x20). Co: control diet-fed *Mdr2*^{-/-} mice.

In addition, INT-767 reduced reactive cholangiocyte phenotype, as reflected by significantly lowered mRNA levels of cholangiocyte cytoskeleton marker *K19* and inflammatory marker *Vcam-1* and reduced portal *K19* and *Vcam-1* signal in immunohistochemistry (figure 28). INT-747 increased *Vcam-1* and *Mcp-1* mRNA levels and induced *Vcam-1* staining in cholangiocytes, inflammatory cell infiltrates and periportal hepatocytes; whereas INT-777 increased only *Mcp-1* mRNA levels (figure 28).

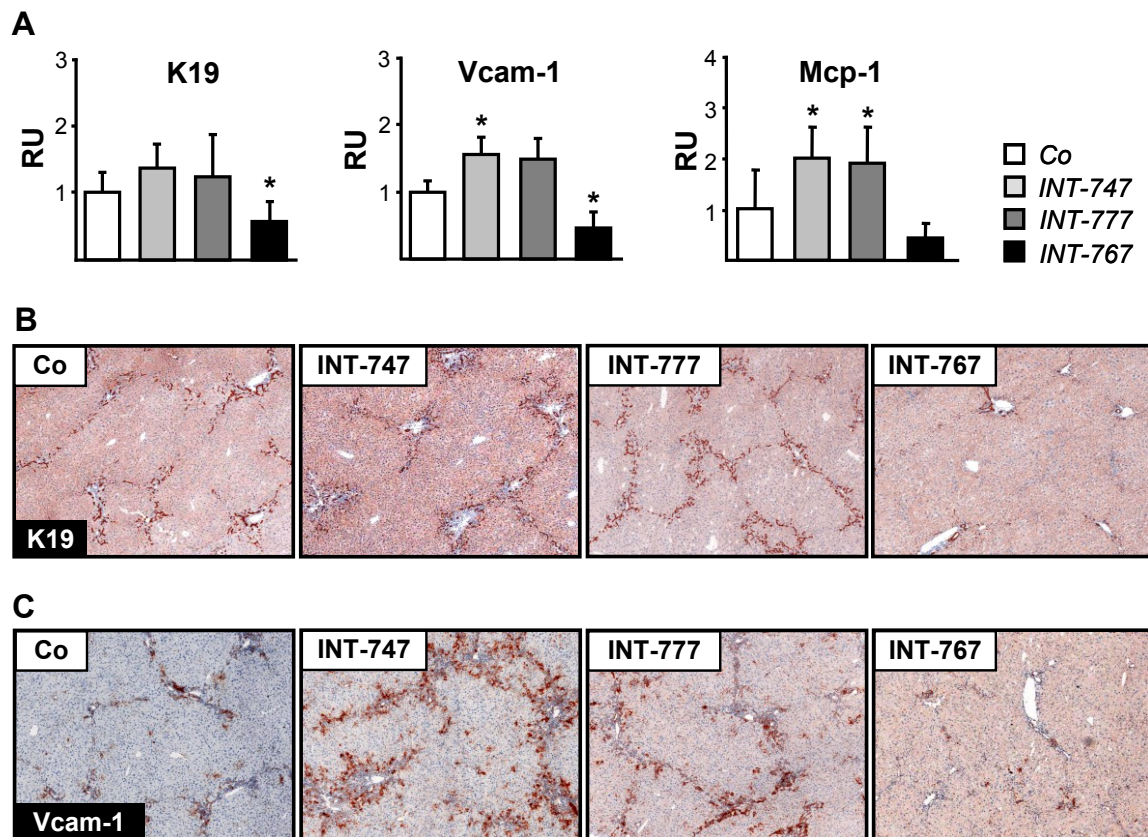


Figure 28. INT-767 reduces bile duct reactive phenotype in *Mdr2*^{-/-} mice.

(A) INT-767 inhibits gene expression of cholangiocyte cytoskeleton marker *K19* as well as inflammatory markers *Vcam-1* and *Mcp-1*. INT-747 and INT-777 induced *Mcp-1* gene expression, whereas only INT-747 induced *Vcam-1*. Expression levels are normalized to 36b4. Means of 6 animals per group $\pm$ SD. Mean value in chow-fed *Mdr2*^{-/-} mice is accepted as 1. * $P < 0.05$ INT-747, INT-767 and INT-777 vs. controls (Co). **(B)** Immunohistochemical staining for *K19*. Representative picture shows reduced bile duct proliferation in INT-767-treated *Mdr2*^{-/-} mouse. **(C)** Reactive cholangiocyte phenotype is demonstrated by immunohistochemical staining for *Vcam-1*. Compared with untreated *Mdr2*^{-/-}, INT-767-treated *Mdr2*^{-/-} mouse liver showed reduced cholangiocellular *Vcam-1*

staining. In contrast, INT-747 and INT-777 increased *Vcam-1* staining in cholangiocytes, inflammatory cells and peri-portal hepatocytes.

In line with hepatic inflammation, liver fibrosis was reduced in INT-767-treated *Mdr2*^{-/-} mice as shown by less collagen deposition in Sirius red staining (figure 29A). These findings were further confirmed by reduced HP content, inhibited *Col1a1* gene expression and reduced SW/BW ratio (figure 29B, C, D). In contrast, INT-747 increased HP, *Col1a1* mRNA as well as SW/BW ratio, whereas liver fibrosis remained unchanged in INT-777-fed mice.

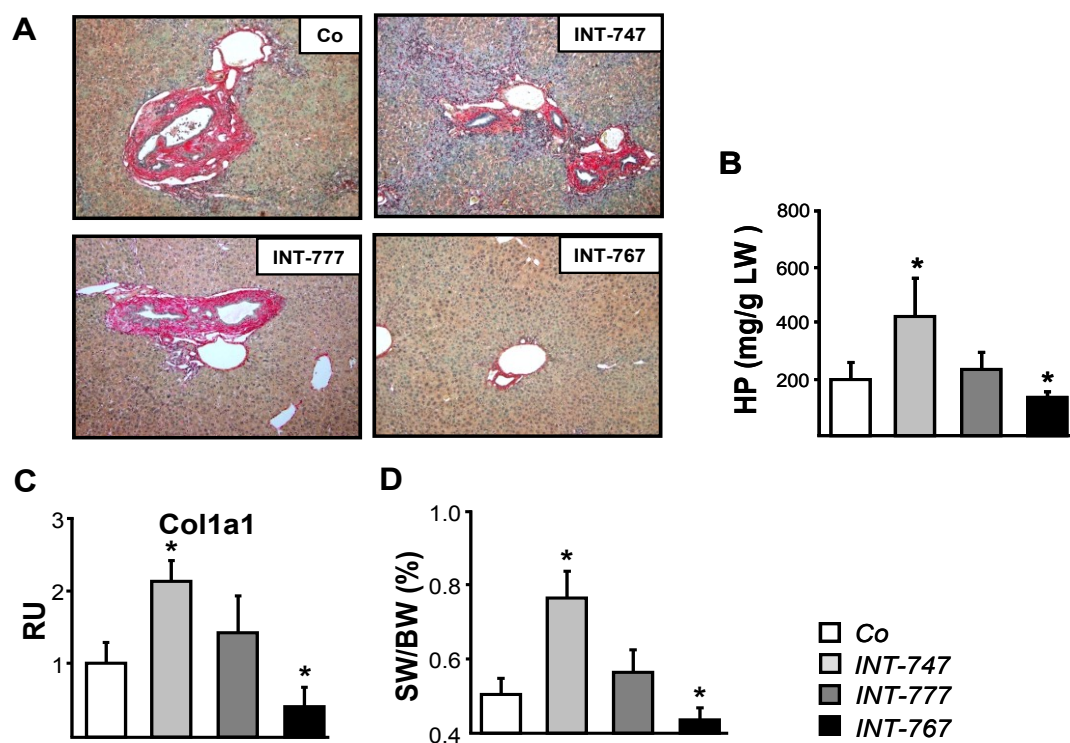


Figure 29. INT-767 significantly decreases hepatic fibrosis.

(A) Illustration of liver fibrosis in *Mdr2*^{-/-} mice by Sirius red staining. INT-767-treated *Mdr2*^{-/-} mouse liver showed less biliary fibrosis, whereas INT-747 feeding increased biliary fibrosis as well as collagen deposition within the liver lobule. INT-777 did not influence liver fibrosis in *Mdr2*^{-/-} mice. (B), (C) INT-767 lowered hepatic hydroxyproline (HP) levels as well as *Col1a1* gene expression in *Mdr2*^{-/-} mice, whereas INT-747 increased and INT-777 did not modify HP or *Col1a1* gene expression. (D) Spleen weight was normalized to body weight and % ratio is presented (SW/BW). Values are presented as means of 6 mice/group $\pm$ SD. * $P < 0.05$ INT-747 and INT-767 vs. control diet-fed *Mdr2*^{-/-} mice (Co).

Next, macrophage, cholangiocyte and hepatocyte cell lines and isolated primary MFBs were used to address the anti-inflammatory and anti-fibrotic effects of INT-767 *in vitro*. Surprisingly, INT-767 did not abrogate LPS-induced pro-inflammatory gene expression in RAW264.7 macrophages (figure 30A), *Tnf- α* -induced *Vcam-1* gene expression in BEC bile duct epithelial cells (figure 30B) and *TNF- α* -induced *CYP7A1* gene expression in HepG2 cells despite pronounced *CYP7A1* inhibition (figure 30C).

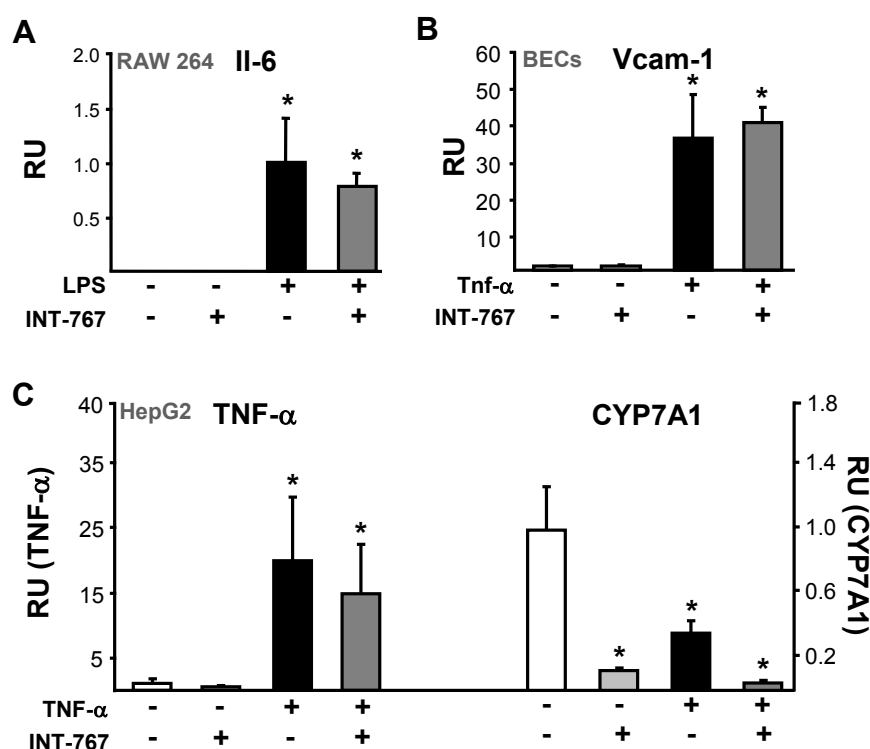


Figure 30. INT-767 has no direct anti-inflammatory effects on RAW264.7 macrophage, BEC cholangiocyte and HepG2 hepatocyte cell lines.

(A) RAW264.7 macrophage cell line was activated by LPS (100 ng/ml) incubation for 6 h. Impact of INT-767 was studied in cells pre-incubated overnight with INT-767 (5 μ M) followed either by INT-767 incubation alone or by simultaneous LPS and INT-767 incubation for 6 h. Non-stimulated cells showed undetectable expression of pro-inflammatory cytokine *Il-6*. LPS dramatically induced *Il-6* gene expression in RAW264.7 cells. LPS-induced *Il-6* expression was not ameliorated by INT-767. Values are expressed as means of 3 samples per group $\pm$ SD. Expression levels are normalized to *36b4* gene and mean expression value of LPS-stimulated cells is accepted as 1. (B) *Vcam-1* gene expression was stimulated in BEC cholangiocyte cell line by *TNF- α* (10 ng/ml) incubation for 24 h. Impact of INT-767 was studied in cells pre-incubated overnight with INT-767 (10 μ M) followed by either INT-767 incubation alone or by simultaneous *Tnf- α* and INT-767 incubation for 24h. INT-767 had no impact on *Tnf- α* -induced *Vcam-1* gene

expression in BEC cholangiocyte cell line. **(C)** Pro-inflammatory cytokine expression was stimulated in HepG2 hepatocyte cell line by human TNF- α (10 ng/ml) incubation for 24h. Impact of INT-767 was studied in cells pre-incubated overnight with INT-767 (10 μ M) followed by either INT-767 incubation alone or simultaneous TNF- α and INT-767 incubation for 24 h. TNF- α gene expression was significantly induced in HepG2 by TNF- α incubation and remained unchanged after INT-767 incubation. CYP7A1 gene expression was significantly inhibited by INT-767, TNF- α as well as simultaneous INT-767 and TNF- α incubation. **B.** and **C.** Values are expressed as means of 3 samples per group $\pm$ SD. Expression levels are normalized to 36b4 gene and mean value of non-stimulated cells is accepted as 1. * $P < 0.05$ vs. non-stimulated controls. RU: relative units.

Furthermore, INT-767 did not inhibit 10% FCS-induced *Colla1* gene expression in isolated portal MFBs *in vitro* (figure 31). Collectively, these findings show that INT-767 reduced portal inflammation, cholangiopathy and fibrosis in *Mdr2*^{-/-} mice without any anti-inflammatory effects on macrophages, cholangiocytes or hepatocytes or anti-fibrotic effects in portal MFBs.

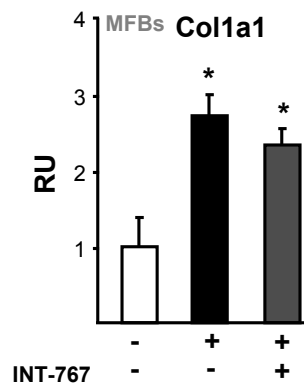


Figure 31. INT-767 has no impact on pro-fibrogenic gene expression in primary MFBs.

Primary MFBs were isolated from 8-week-old *Mdr2*^{-/-} mice and cultivated as described in M&M. 10% FCS stimulates *Colla1* pro-fibrogenic marker expression in primary MFBs. INT-767 did not inhibit 10% FCS-induced *Colla1* gene expression in primary MFBs. Data are presented as means of 3 samples per group $\pm$ SD. Expression levels are normalized to 36b4 gene and mean value of non-stimulated cells is accepted as 1. * $P < 0.05$ 10% FCS condition vs. 0% FCS controls. RU: relative units.

INT-767 inhibits BA synthesis by induction of ileal *Fgf15* and hepatic *Shp* gene expression. BA synthesis was dramatically repressed by FXR agonist INT-747 and the dual FXR/TGR5 agonist INT-767 as reflected by repressed *Cyp7a1* and *Cyp8b1* gene expression (figure 32A, B). In addition, both INT-747 and INT-767 stimulated *Fgf15* gene expression (figure 32C). However, only INT-767 decreased serum BA levels and induced hepatic *Shp* gene expression (figure 32D, E).

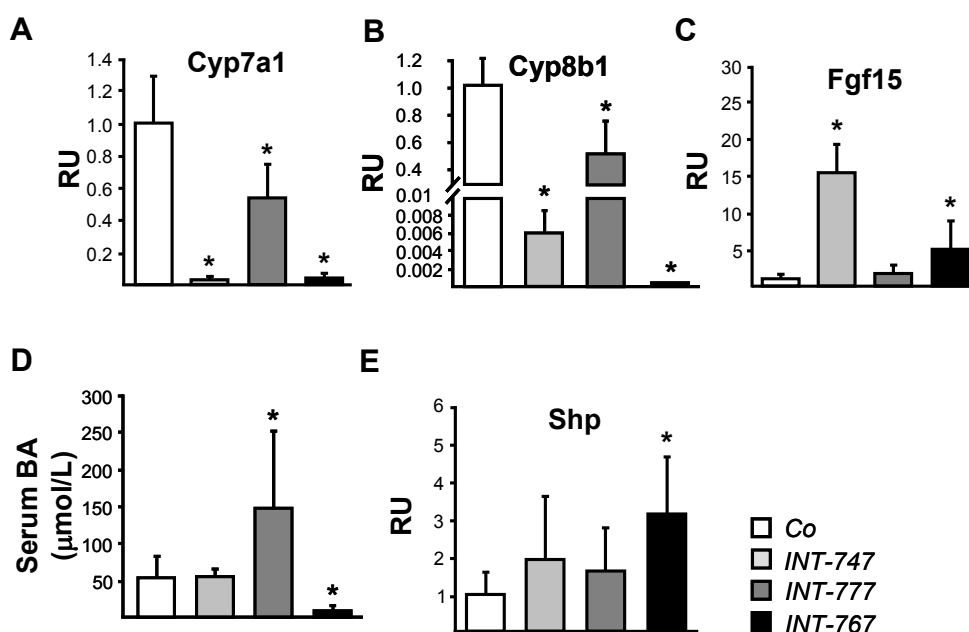


Figure 32. INT-747 and INT-767 inhibit BA synthesis in *Mdr2*^{-/-} mice.

(A), (B), (C) Both INT-747 and INT-767 dramatically inhibited BA synthesis genes *Cyp7a1* and *Cyp8b1*, while inducing ileal *Fgf15* gene expression. (D) Only INT-767 significantly decreased, whereas INT-777 increased serum BA levels in *Mdr2*^{-/-} mice. Values are presented as means of 6 animals per group $\pm$ SD. (E) *Shp* gene expression is induced only by INT-767. Gene expression levels are normalized to 36b4 house-keeping gene and the mean expression value of untreated *Mdr2*^{-/-} mice is accepted as 1. * $P < 0.05$ INT-747, INT-767 and INT-777 vs. controls. Co: control diet-fed *Mdr2*^{-/-} mice.

BA uptake transporter *Ntcp* was repressed by INT-747 and INT-767 at mRNA and protein levels (figure 33A, B). Only INT-767 increased Bsep protein levels (figure 33C). No significant alteration of *Mrp2*, *Mrp3* and *Mrp4* transporters were detected by any treatment (figure 33C). Together, these results establish that apart from inhibited BA synthesis INT-767 ameliorates liver injury in *Mdr2*^{-/-} mice by additional protective mechanism.

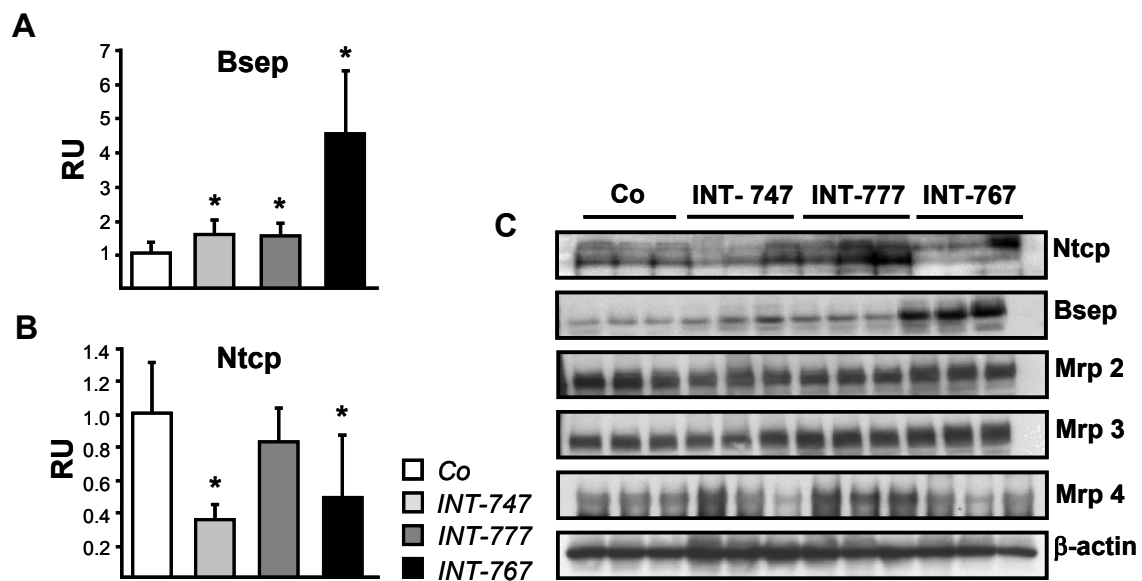


Figure 33. INT-767 inhibits *Ntcp*, while increasing *Bsep* expression on mRNA and protein levels.

Mdr2^{-/-} mice received either a chow diet or diet supplemented with the FXR agonist INT-747, the TGR5 agonist INT-777 or the dual FXR/TGR5 agonist INT-767 for 4 weeks. (A), (B) Gene expression of hepatic *Ntcp* and *Bsep* are presented. All INT compounds increased *Bsep*, whereas *Ntcp* was repressed only by INT-747 and INT-767. Values are presented as means of 4-5 animals per group $\pm$ SD. Expression levels are normalized to 36b4 gene and mean value of untreated *Mdr2*^{-/-} controls is presented as 1. * $P < 0.05$ INT-747, INT-767 and INT-777 vs. controls. RU: relative units. (C) Hepatic protein levels of BA uptake transporter *Ntcp* and export transporters *Bsep*, *Mrp2*, *Mrp3* and *Mrp4* are presented. *Ntcp* protein levels were reduced after INT-767 feeding, whereas there was only a tendency to reduction after INT-747 feeding. Only INT-767 increased hepatic *Bsep* protein levels. No alterations of *Mrp2* and *Mrp3* were detected after INT-747, INT-777 or INT-767 feeding in *Mdr2*^{-/-} mouse livers, whereas *Mrp4* protein levels were slightly reduced by INT-767. Protein levels of randomly depicted livers (3 per group) are presented, β -actin protein levels served as loading control.

INT-767 stimulates HCO₃⁻-rich bile flow and inhibits BA output in *Mdr2*^{-/-} mice. Since bile composition was dramatically altered in *Mdr2*^{-/-} mice (Smit *et al.*, 1993), we further hypothesized that INT-767-mediated beneficial effects might result from alterations of bile flow and composition. Indeed, INT-767 significantly increased bile flow and bicarbonate output in *Mdr2*^{-/-} mice, while reducing biliary BA output (figure 34).

In contrast, bile flow and biliary composition remained unchanged after INT-747 and INT-777 feeding. These findings therefore prove that only INT-767 impacted on bile secretory function and improved liver injury in *Mdr2*^{-/-} mice.

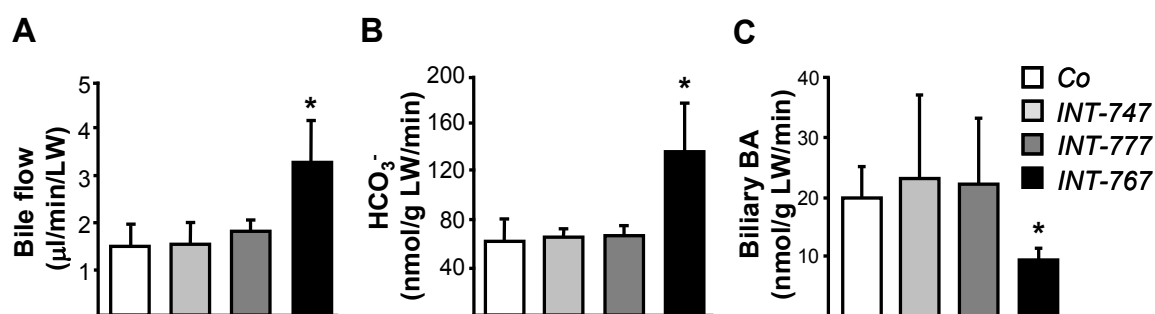


Figure 34. INT-767 stimulates HCO₃⁻-rich bile flow in *Mdr2*^{-/-} mice.

Bile flow and composition were measured in *Mdr2*^{-/-} mice after 7 days of feeding either a chow or a diet supplemented with INT-747, INT-777 or INT-767. **(A)** INT-767 increased bile flow and **(B)** biliary HCO₃⁻ output. **(C)** Biliary BA output was significantly decreased by INT-767. Neither INT-747 nor INT-777 modified bile flow, biliary HCO₃⁻ or BA output in *Mdr2*^{-/-} mice. Values are presented as means of 4-5 mice per group ± SD. * *P* < 0.05 INT-767 vs. controls. Co: control diet-fed *Mdr2*^{-/-} mice.

INT-767-mediated alterations of bile flow and composition are Fxr-dependent. Since INT-767 compound is a strong FXR and TGR5 activator, we next addressed the role of these receptors in INT-767-induced choleresis by using *Fxr*^{-/-} mice. Bile flow and biliary HCO₃⁻ output, increased by INT-767, was abolished in *Fxr*^{-/-} mice (figure 35A, B), whereas INT-747 and INT-777 had no impact on bile flow or biliary HCO₃⁻ output. By using a genetic model of Tgr5 over-expression (*Tgr5-Tg* mice), we further confirmed that bile flow and biliary HCO₃⁻ output are not controlled by Tgr5 *in vivo* (figure 35C, D).

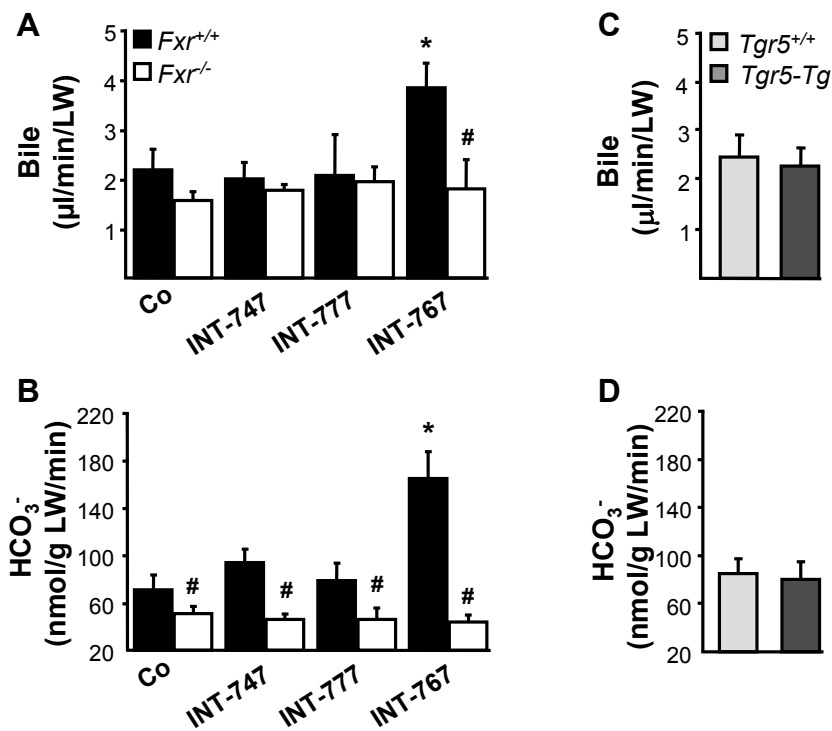


Figure 35. Induction of HCO₃⁻-rich choleresis by INT-767 is Fxr-, but not Tgr5-dependent.

(A), (B) Bile flow and biliary HCO₃⁻ output were measured in Fxr^{+/+} and Fxr^{-/-} mice after 7 days of feeding on a chow or INT-747-, INT-777- or INT-767-supplemented diet. (A) INT-767 induced only in Fxr^{+/+} mice, whereas INT-747 and INT-777 did not change bile flow in both Fxr^{+/+} and Fxr^{-/-} mice. (B) Only INT-767 induced biliary HCO₃⁻ output in Fxr^{+/+}, but not in Fxr^{-/-} mice. Data are presented as means of 4-5 mice per group ± SD. * $P < 0.05$ INT-767-fed Fxr^{+/+} vs. Fxr^{+/+} controls. Co: control diet-fed Fxr^{+/+} and Fxr^{-/-} mice respectively. (C) and (D) Bile flow and biliary HCO₃⁻ output is undistinguishable between age- and sex-matched Tgr5-Tg and Tgr5^{+/+} mice. Data are presented as means of 4-5 mice per group ± SD.

In line with BA synthesis inhibition, INT-767 decreased biliary BA and consequently cholesterol and PL output (figure 36A, B, C) in an Fxr-dependent manner. Intriguingly, INT-777 decreased biliary PL and cholesterol output in Fxr^{+/+} mice (figure 36B, C). To rule out a differential absorption of INT-compounds between Fxr^{+/+} and Fxr^{-/-} mice, we further studied biliary enrichment of INT-compounds. INT-767 concentration was higher in Fxr^{-/-} compared with Fxr^{+/+} mice, whereas INT-747 and INT-777 concentrations did not differ between genotypes (figure 36D). However, the highest biliary enrichment was detected by INT-747. Altogether these findings confirm that INT compounds were

absorbed and that alterations in bile flow and biliary composition by INT-767 were Fxr-dependent.

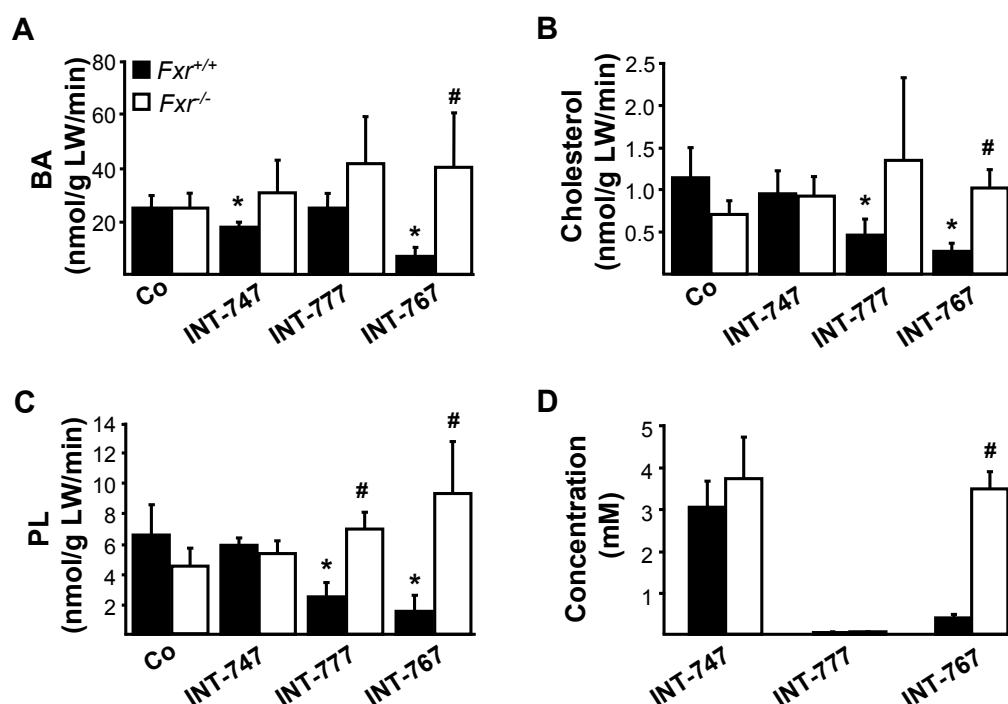


Figure 36. INT-767 reduces biliary output of endogenous BAs in Fxr-dependent manner.

(A), (B), (C) Biliary BA, cholesterol and PL output was reduced in $Fxr^{+/+}$ mice by INT-767, whereas INT-747 decreased only biliary BA output and INT-777 reduced biliary PL and cholesterol output. Values are presented as means of 4-5 animals per group $\pm$ SD. * $P < 0.05$ INT-767 and INT-777 vs. controls. (D) Biliary concentrations of respective INT-compounds in pooled bile samples. Co: control diet-fed $Fxr^{+/+}$ and $Fxr^{-/-}$ mice respectively.

Interestingly, glutathione output remained unchanged by all 3 compounds in both genotypes (figure 37).

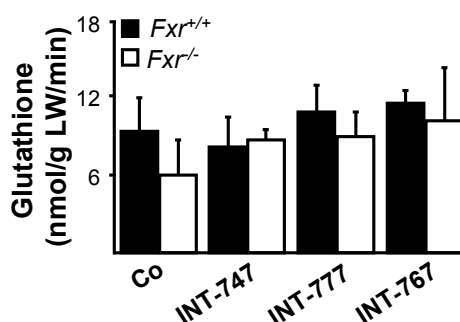


Figure 37. Biliary glutathione output is undistinguishable between $Fxr^{+/+}$ and $Fxr^{-/-}$ mice after INT-747, INT-777 and INT-767 feeding.

Biliary glutathione output was measured in $Fxr^{+/+}$ and $Fxr^{-/-}$ mice after 7 days of chow diet, or a diet supplemented with INT-747, INT-777 or INT-767 as described in M&M. Data are presented as means of 4-5 mice per group $\pm$ SD. Co: chow-fed $Fxr^{+/+}$ and $Fxr^{-/-}$ mice respectively.

INT-767-induces HCO_3^- -rich choleresis via induction of hepatic carbonic anhydrase 14 gene expression. Fxr -mediated induction of HCO_3^- -rich choleresis by INT-767 suggested a transcriptional mechanism. In human gallbladder epithelium FXR was shown to induce the HCO_3^- -rich secretion via induction of VPAC-1 (Chignard *et al.*, 2005), the receptor for vasoactive intestinal peptide, an important regulator of secretory functions in this epithelium. However, INT-767 decreased hepatic *Vpac-1* mRNA levels in $Mdr2^{-/-}$ as well as $Fxr^{+/+}$ mice, (figure 38), showing that *Vpac-1* is unlikely to be responsible for HCO_3^- -rich secretion by INT-767 in mice.

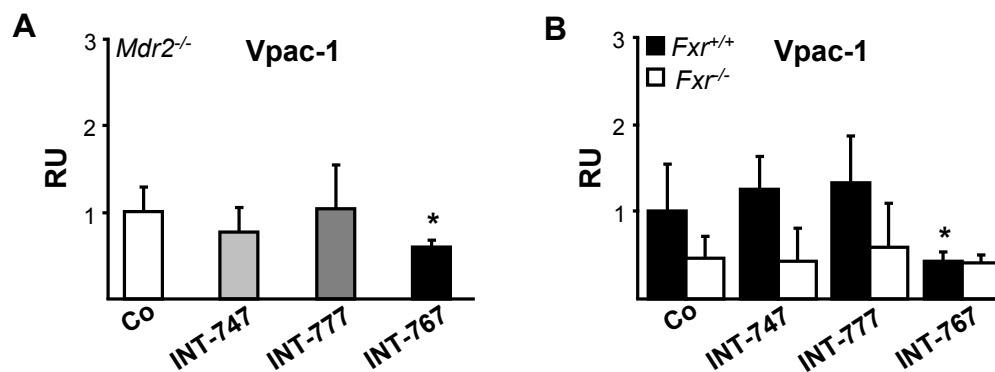


Figure 38. INT-767 inhibits gene expression of vasoactive intestinal peptide (VIP) receptor *Vpac-1*.

(A) *Vpac-1* gene expression in livers of $Mdr2^{-/-}$ mice. INT-767 inhibited *Vpac-1* gene expression in $Mdr2^{-/-}$ mice. (B) *Vpac-1* gene expression in $Fxr^{+/+}$ and $Fxr^{-/-}$ mice after 1 week of experimental feeding. INT-767 inhibited *Vpac-1* gene expression in $Fxr^{+/+}$, but not in $Fxr^{-/-}$ mice. Values are presented as means of 5-6 animals per group $\pm$ SD. Expression levels are normalized to 36B4 house-keeping gene. * $P < 0.05$ INT-767 vs. controls. Co: control diet-fed $Fxr^{+/+}$ and $Fxr^{-/-}$ respectively, RU: relative units.

Canalicular HCO_3^- secretion in hepatocytes is mediated via the anion exchanger Ae2 (Aranda *et al.*, 2004; Benedetti *et al.*, 1991; Benedetti *et al.*, 1994; Martinez-Anso *et al.*, 1994), whereas mouse cholangiocytes in addition to Ae2 display the $\text{Na}^+/\text{HCO}_3^-$ co-

transporter *Slc4a4* (Uriarte *et al.*, 2010). *Ae2* as well as *Slc4a4* gene expression remained unchanged in *Mdr2*^{-/-}, *Fxr*^{+/+} and *Fxr*^{-/-} mice (figure 39).

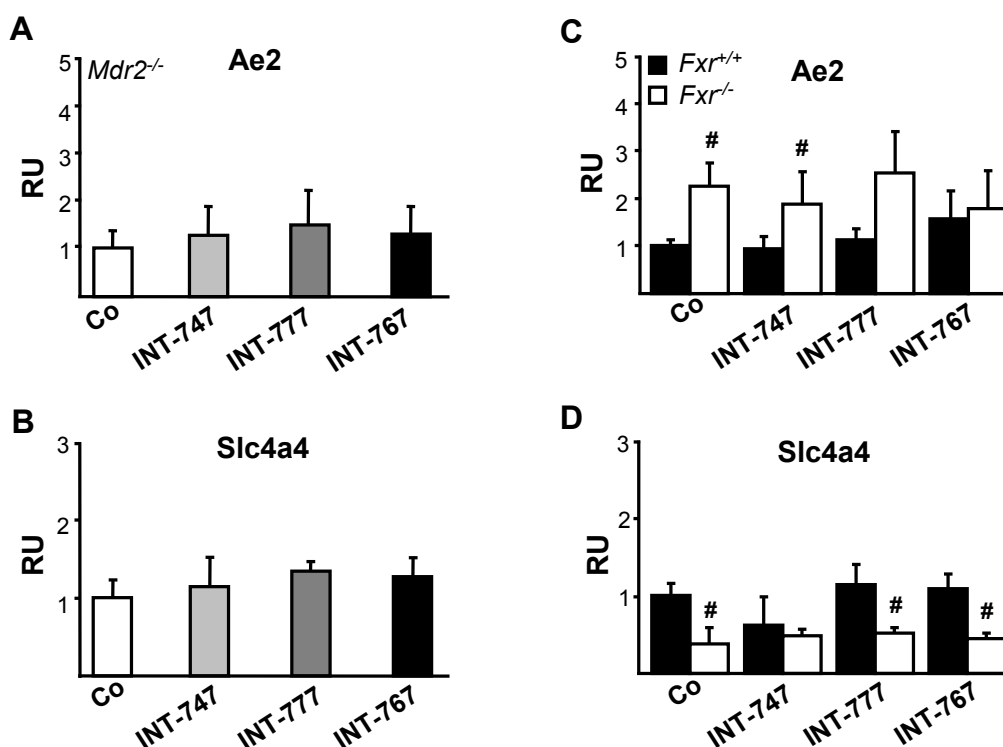


Figure 39. INT-767 does not influence gene expression of HCO_3^- export transporters *Ae2* and *Slc4a4*.

(A), (B) Gene expression of HCO_3^- transporters in livers of *Mdr2*^{-/-} mice is presented. Both *Ae2* and *Slc4a4* gene expression remained unchanged after INT-747, INT-777 and INT-767 feeding in *Mdr2*^{-/-} mice. (C), (D) *Ae2* and *Slc4a4* gene expression in *Fxr*^{+/+} and *Fxr*^{-/-} mice after 1 week of experimental feeding. Compared with *Fxr*^{+/+} mice *Fxr*^{-/-} mice had significantly increased *AE2* and low *SLC4A4* gene expression. However, none of INT compounds had an impact on *AE2* and *SLC4A4* expression in *Fxr*^{+/+} and *Fxr*^{-/-} mice. Values are presented as means of 5-6 animals per group $\pm$ SD. Expression levels are normalized to 36B4 house-keeping gene. # $P < 0.05$ *Fxr*^{-/-} vs. *Fxr*^{+/+}. Co: control chow-fed *Fxr*^{+/+} and *Fxr*^{-/-} respectively, RU: relative units.

Ae2 function requires intracellular HCO_3^- , generated via the basolateral input through $\text{Na}^+/\text{HCO}_3^-$ co-transporter *Slc4a5* and/or by generation through the carbonic anhydrase (CA) pathway. Since none of INT compounds altered *Slc4a5* gene expression in *Mdr2*^{-/-} mice (data not shown), we studied the regulation of CAs by INT-767. INT-767 significantly increased the gene expression of hepatocellular membrane-bound *CA14* (Parkkila *et al.*, 2002) in *Mdr2*^{-/-} and *Fxr*^{+/+}, but not in *Fxr*^{-/-} mice (figure 40A, B).

Furthermore INT-767 directly induced *CA14* gene expression in human HepG2 hepatocytes (figure 40C). Collectively, these findings indicate that INT-767 induced HCO_3^- -rich choleresis *via* Fxr-dependent induction of the membrane-bound *CA14* in hepatocytes.

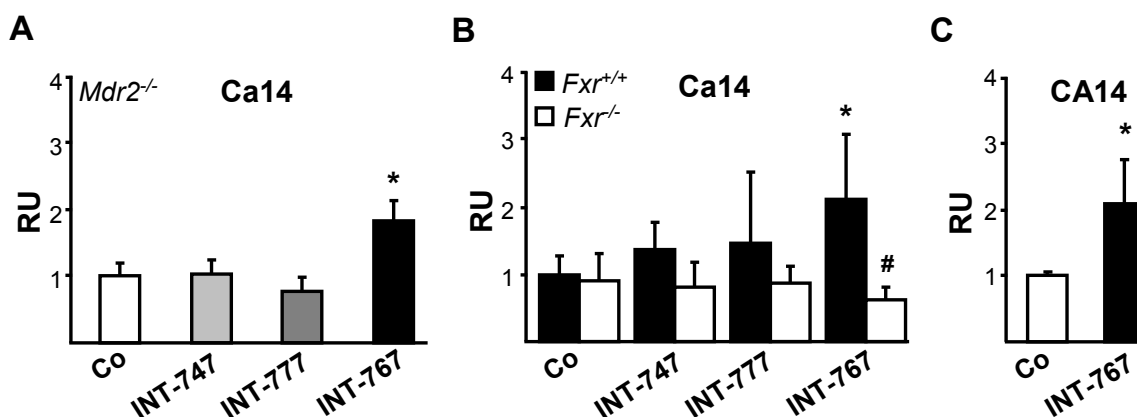


Figure 40. INT-767 significantly induces carbonic anhydrase 14 (CA14) gene expression via Fxr.

(A) Only INT-767 significantly stimulated hepatic *Ca14* gene expression in *Mdr2*^{-/-} mice. (B) INT-767 specifically induced *Ca14* gene expression in *Fxr*^{+/+} mice, whereas no alterations were detected in *Fxr*^{-/-} mice. Values are presented as means of 5-6 animals per group $\pm$ SD. (C) INT-767 (10 μ M) significantly induced *CA14* gene expression in HepG2 cells. Values are presented as means of 3 samples per group $\pm$ SD. Expression levels are normalized to 36B4. * $P < 0.05$ INT-767 vs. controls. Co: cells incubated in control medium, RU: relative units.

Fxr activation by INT-767 stimulates hepatocellular proliferation. Since LW/BW ratio increased by all the treatments in *Mdr2*^{-/-} mice (figure 25A), we further quantified hepatocyte proliferation by staining for the proliferation marker Ki-67 and counting Ki-67-positive nuclei. Both INT-747 and INT-767 increased hepatocellular proliferation in *Mdr2*^{-/-} (data not shown) and *Fxr*^{+/+} mice, but not in *Fxr*^{-/-} mice (figure 41), demonstrating that induction of hepatocellular proliferation by INT-767 was FXR-mediated.

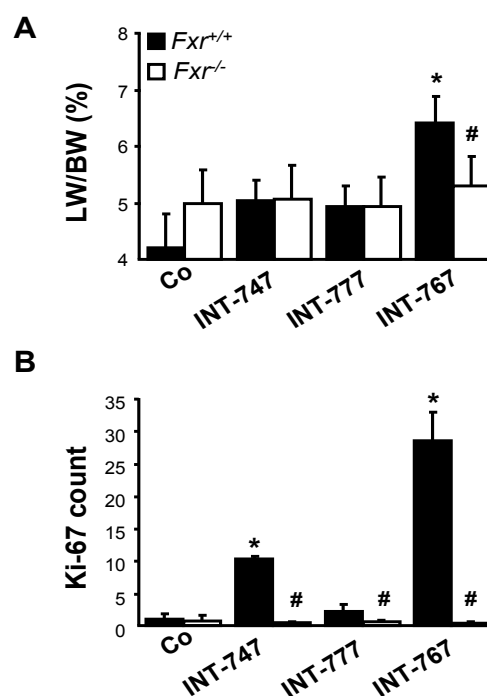


Figure 41. INT-767 increases LW/BW ratio as well as hepatocyte proliferation in FXR-dependent manner.

Fxr^{+/+} and *Fxr*^{-/-} mice received either a chow diet, a diet supplemented with the INT-747, the INT-777 or the INT-767 for 7 days. **(A)** LW/BW ratio was increased by INT-767 in *Fxr*^{+/+} mice, while remaining unchanged in *Fxr*^{-/-} mice. No significant change in LW/BW ratio was detected by INT-747 and INT-777 in FXR-WT and FXR-KO mice. Values are presented as means of 5-6 mice per group $\pm$ SD. **(B)** Hepatocellular proliferation was addressed by immunohistochemical staining for Ki-67 proliferation marker. Amount of Ki-67-positive hepatocytes is presented. Both INT-747 and INT-767 significantly increased Ki-67-positive hepatocytes in *Fxr*^{+/+} mice, but not in *Fxr*^{-/-} mice. Ki-67-positive hepatocytes were counted in 30 randomly chosen power fields per mouse. Values are presented as means of counts in 5 mice per group $\pm$ SD. * $P < 0.05$ INT-747 and INT-767 vs. controls.

INT-767 shows moderate protection in a model of total biliary obstruction. Finally, impact of INT-747, INT-777 and INT-767 feeding was addressed in the common bile duct ligation (CBDL), a model of total biliary obstruction. INT-747 and INT-767 increased size and amount of bile duct infarcts as well as LW/BW ratio in CBDL mice (Figure 42A, B). However, only INT-767 significantly decreased SW/BW ratio in CBDL mice (Figure 42B).

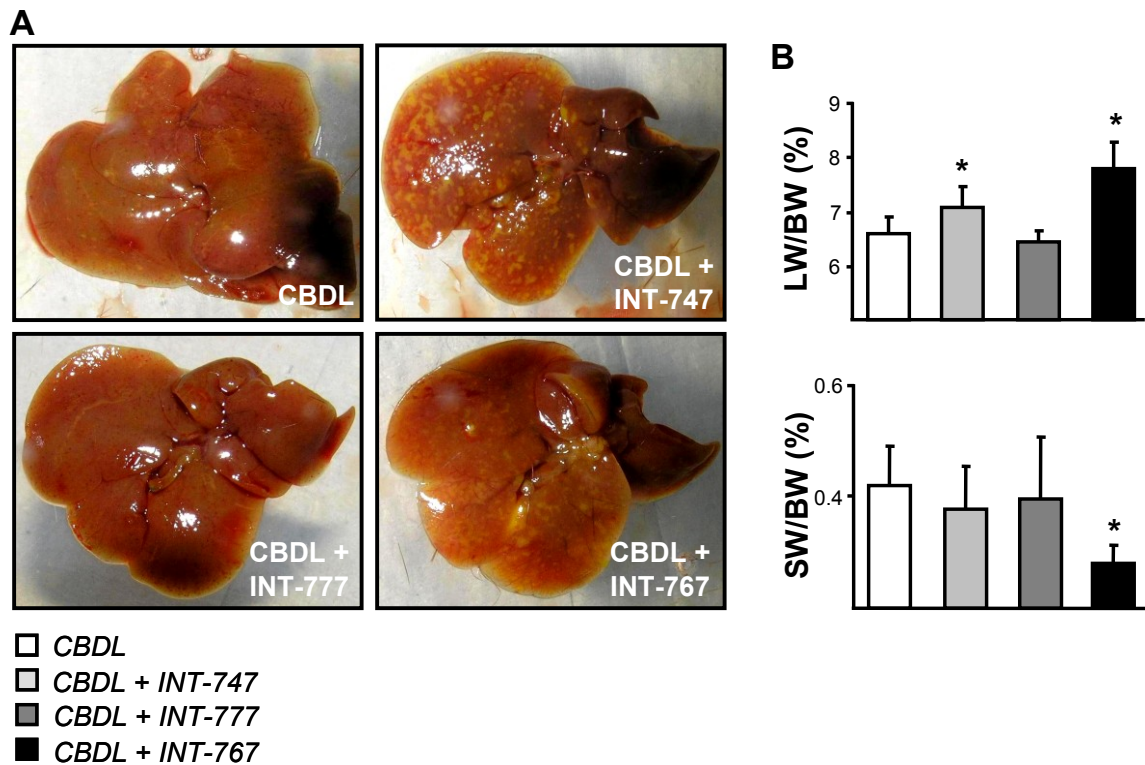


Figure 42. INT-747 and INT-767 induce bile infarcts and increase LW/BW, while only INT-767 decreases SW/BW in model of common bile duct ligation (CBDL).

CBDL was performed in male C57BL6/J mice after 5 days of pre-feeding either a control chow diet or a diet supplemented with INT-747, INT-777 or INT-767 (0.03% w/w). Experimental feeding was continued for additional 3 days (M&M) and mice were sacrificed at day 4 after CBDL. **(A)** Macroscopic images of livers are presented. Both INT-747 and INT-767 increased size and amount of bile infarcts in CBDL, whereas no alterations were detected by INT-777. **(B)** LW/BW and SW/BW ratio is presented. INT-747 and INT-767 increased, whereas INT-777 had no impact on LW/BW ratio in CBDL model. SW/BW was significantly decreased by INT-767. Values are presented as means of 5 animals per group. * $P < 0.05$ INT-747 and INT-767 vs. CBDL controls. BW: body weight, LW: liver weight, SW: spleen weight.

Moreover, despite increased amount and size of bile infarcts, serum ALT showed a trend to reduction in INT-767-fed CBDL mice, whereas no alteration of ALP was detected (figure 43A). Although histological examination of H&E stained livers revealed bile infarcts in all the groups, only INT-747 increased infiltration of inflammatory cells within the portal fields (figure 43B).

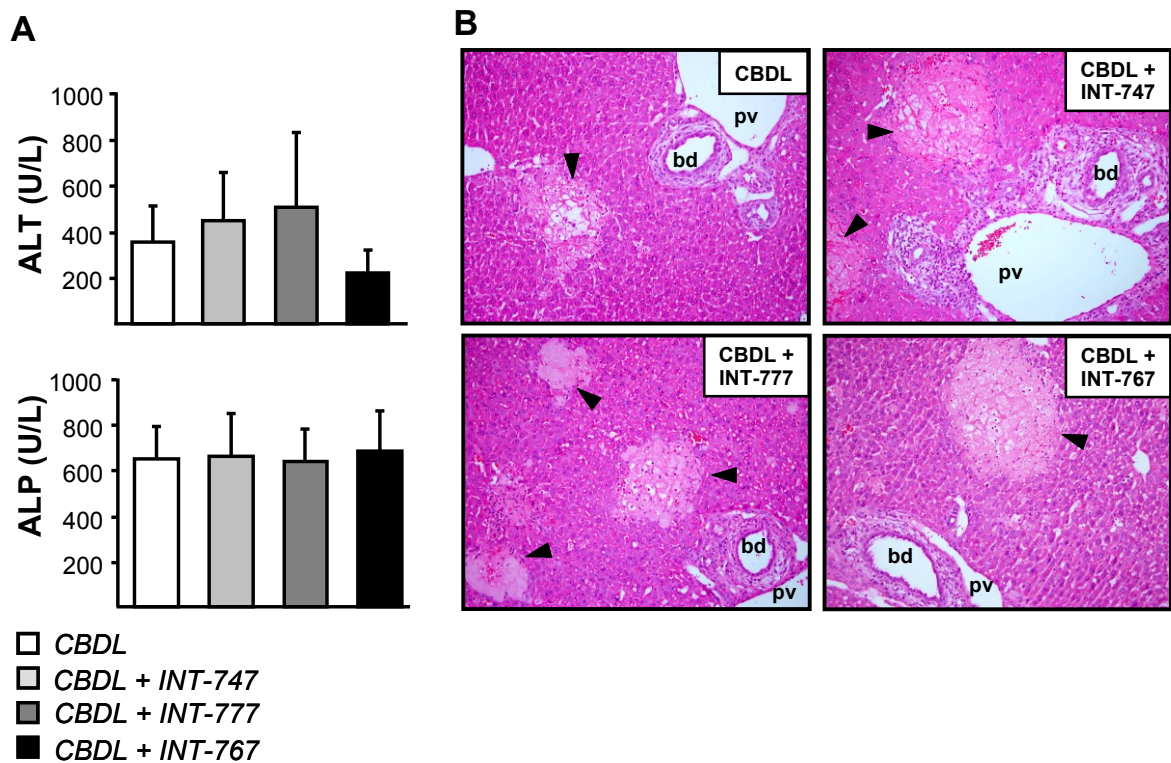


Figure 43. INT-767 has moderate impact on serum ALT in CBDL model.

CBDL was performed as described in M&M. **(A)** Serum parameters of liver damage and cholestasis. Only INT-767 showed tendency to reduce serum ALT levels. Serum ALP levels remained unchanged by INT-747, INT-777 and INT-767. **(B)** Representative pictures of H&E stained livers. Bile infarcts were present in all the groups (arrowheads). However, INT-747-fed mouse liver shows more inflammatory cell infiltrates in portal fields. Magnification: 10-fold.

In line with serum ALT levels, hepatic expression of pro-inflammatory genes TNF- α and IL-1 β decreased in INT-767-fed CBDL mice (figure 44A). Furthermore, histological examination showed less CD-11b- and F4/80-positive cells around bile infarcts in INT-767-fed CBDL mice (figure 44B).

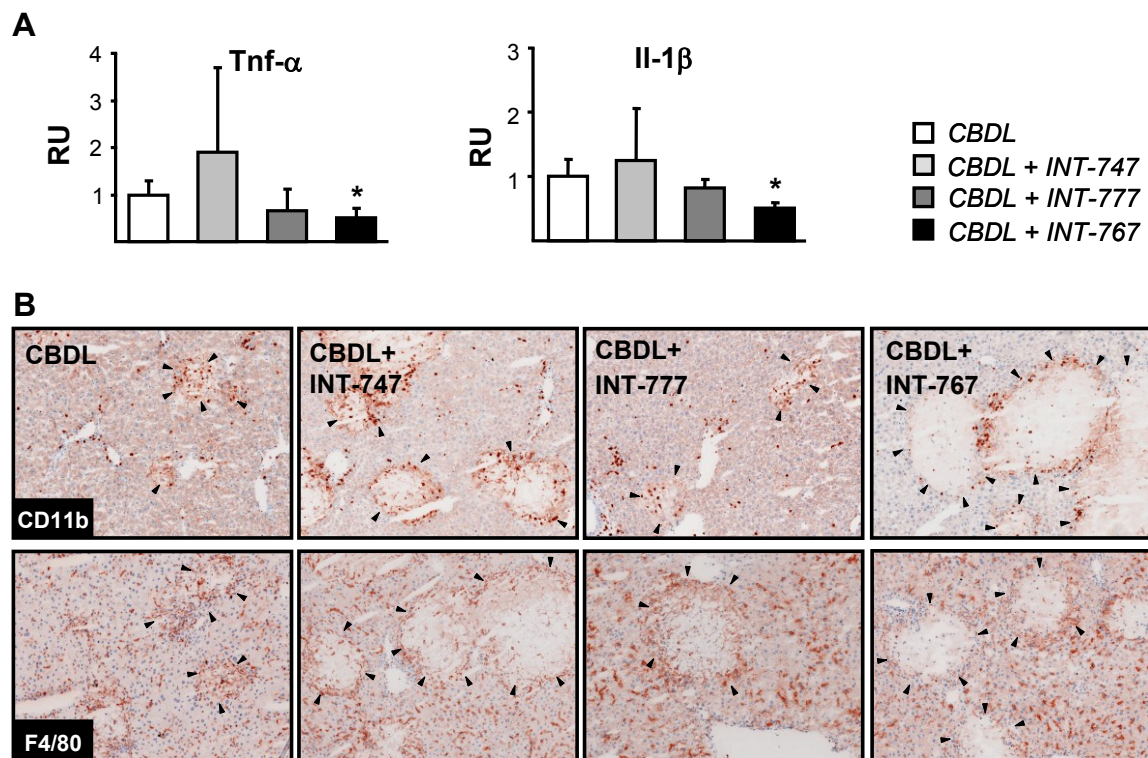


Figure 44. INT-767 reduces liver inflammation in common bile duct ligation model of obstructive cholestasis.

(A) Pro-inflammatory cytokine gene expression was measured in mouse livers after 3 days of CBDL and experimental feeding with INT-747, INT-777 and INT-767 (5 days pre-feeding prior to CBDL). INT-767-treated mice showed significantly reduced *Tnf-α* and *Il-1β* gene expression. Values are presented as means of 5 animals per group. Expression levels are normalized to 36B4 house-keeping gene and mean values of CBDL controls is expressed as 1. * $P < 0.05$ INT-767 vs. controls. RU: relative units. **(B)** Immunohistochemical stainings for mononuclear inflammatory cells (CD11b) and macrophages (F4/80). Control CBDL mouse liver showed CD11b- and F4/80-positive cells around bile infarcts (pointed by black arrowheads). Despite increased size of bile infarcts, INT-767-treated liver showed less CD11b-positive and F4/80-positive cell accumulation around bile infarcts as well as within liver parenchyma. Liver infiltration with CD-11b- and F4/80-positive cells is increased in by INT-747, while remaining unchanged by INT-777. Original magnification: x10.

However, *K19* and *Vcam-1* gene expression remained unchanged in CBDL mice after INT-747, INT-777 and INT-767 feeding (figure 45). Together, these data prove that despite increased bile infarcts, INT-767 had a moderate protective role even in a model of

total biliary obstruction, most likely due to reduced BA and increased HCO_3^- content in the bile.

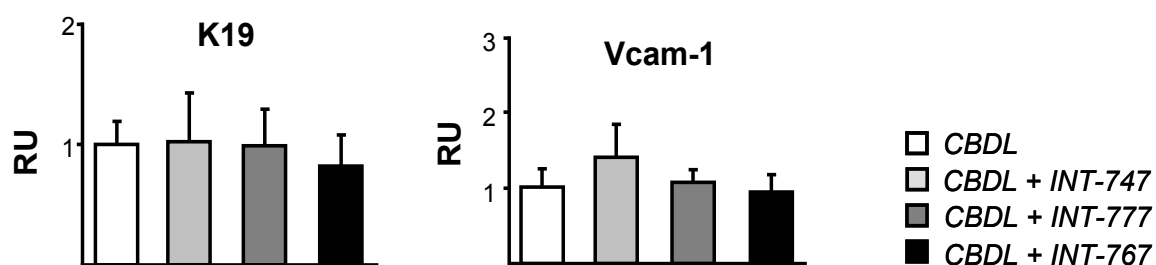


Figure 45. INT-767 does not alter cholangiocyte proliferation and Vcam-1 gene expression in CBDL mice.

CBDL was performed as described in M&M. A. Bile duct proliferation was quantified by measurement of cholangiocyte cytoskeleton marker K19. K19 gene expression remained unchanged by INT-747, INT-777 and INT-767 in mice after CBDL. B. Cholangiocyte reactive activation was quantified by measurement of Vcam-1 gene expression. None of INT-compounds modified Vcam-1 gene expression in CBDL mice. Values are presented as means of 4-5 mice per group $\pm$ standard deviation. Expression levels are normalized to 36B4 house-keeping gene. RU: relative units.

9. DISCUSSION

Discussion is reused from (Baghdasaryan et al., 2010) and (Baghdasaryan et al., 2011) with modifications.

Chronic cholangiopathies include a wide spectrum of diseases ranging from congenital genetic defects (Alagille syndrome, cystic fibrosis, *MDR3* mutations, etc) to autoimmune disorders (PSC, PBC, acute/chronic allograft rejection, graft vs. host disease) and are characterized by progressive disease course and limited therapeutic options. Phospholipids are one of the main bile constituents, accounting for up to 22% in human bile (Esteller, 2008). *Mdr2*^{-/-} mice lacking biliary PL excretion have been characterized as a suitable mouse model for spontaneous development of sclerosing cholangitis (Fickert *et al.*, 2004; Mauad *et al.*, 1994; Popov *et al.*, 2005). Bile duct damage and fibrosis in *Mdr2*^{-/-} mice start early after birth and evolve over time to a full-blown phenotype at the age of 8 weeks. Formation of ‘toxic bile’ due to increased concentrations of non-micellar bound BAs is considered to play a central role in initiation and propagation of bile duct injury in *Mdr2*^{-/-} mice (Trauner *et al.*, 2008).

The aim of this thesis was to study novel pharmaceutical therapies in sclerosing cholestasis. To this purpose, anti-inflammatory and antifibrotic effects of the NR PPAR γ and the BA-dedicated NR FXR and the BA-dedicated membrane-bound receptor TGR5 were studied in the *Mdr2*^{-/-} mouse model of sclerosing cholestasis.

Part 1 demonstrates beneficial effects of the natural compound curcumin in bile duct injury and biliary fibrosis in *Mdr2*^{-/-} model and proposes potential molecular and cellular targets of curcumin in this model. This study demonstrates that curcumin simultaneously targets important pathogenic steps in cholangiopathies such as MFB proliferation (via pERK1/2 inhibition) and cholangiocellular *Vcam-1* expression (in a PPAR γ -dependent fashion).

Curcumin is an extract from turmeric which has been used as traditional medicine for treatment of various gastrointestinal disorders (Aggarwal *et al.*, 2006; Goel *et al.*, 2008b; Jain, 1994; Maheshwari *et al.*, 2006; Oppenheimer, 1937; Rasyid *et al.*, 2002), (Sharma *et al.*, 2001). Interestingly, the early data on curcumin use date back to 1934 when pronounced choleretic effects after intravenous curcumin injection were observed by using the choledochal fistula in rabbits and analyzing duodenal juice in humans

(Henning NaK, 1934). Shortly thereafter, curcumin was successfully used to treat cholestatic jaundice in patients (Oppenheimer, 1937). Acute choleretic effects of curcumin were further confirmed in bile fistula and isolated perfused rat liver models (Chen *et al.*, 2006; Deters *et al.*, 1999). Interestingly, curcumin was also shown to reduce gallstone formation in mice after lithogenic diet feeding (Hussain *et al.*, 1992). However, the impact of curcumin on bile composition and chronic cholangiopathies has not yet been explored.

Since curcumin feeding decreased liver damage and serum parameters of cholestasis in *Mdr2*^{-/-} mice, we first explored whether curcumin modulates bile flow and composition. Interestingly, the protective action of curcumin in *Mdr2*^{-/-} mice was not paralleled by changes of main biliary constituents, indicating that its beneficial effects may not be related to changes of bile micellization. These data are consistent with unchanged expression of BA excretion (Bsep, Mrp2, Mrp3), uptake (Ntcp) or synthesis (*Cyp7a1*) systems suggesting that curcumin improved serum BA levels indirectly via reduced liver damage in *Mdr2*^{-/-} mice. In line with early reports in humans (Henning NaK, 1934; Oppenheimer, 1937) and rats (Deters *et al.*, 1999) curcumin feeding increased bile flow both in WT and *Mdr2*^{-/-} mice. Moreover, biliary glutathione output was increased in *Mdr2*^{-/-} as well as WT mice without changes in total hepatic glutathione content showing that glutathione synthesis is not induced by curcumin. Together with unchanged expression of canalicular Mrp2, mediating glutathione export into bile, we speculate that differences in biliary glutathione output may result from reduced biliary damage and glutathione degradation by gamma-glutamyl transpeptidase in *Mdr2*^{-/-} mice.

Bile ducts in cholangiopathies show increased proliferation and acquisition of new properties such as expression of adhesion molecule *VCAM-1*, a gene induced in epithelial cells in response to the pro-inflammatory cytokine TNF- α . Curcumin reduced Vcam-1 staining in cholangiocytes of *Mdr2*^{-/-} mice, while expression of stimulatory pro-inflammatory cytokines *Tnf- α* , as well as *Il-1 β* and *Tgf- β* remained unaffected. In addition, curcumin completely inhibited TNF- α –induced *VCAM-1* expression in human cholangiocarcinoma cell line *in vitro*. These findings support the concept that primary inflammation and activation of cholangiocytes is an important event in the pathogenesis of cholangiopathies. Targeting cholangiocyte inflammation might represent an important strategy to cure bile duct diseases despite preserved exposure to the causative damaging factor and pro-inflammatory mediators. Previous reports in other cells have shown that curcumin may act as a PPAR γ activator (Jacob *et al.*, 2007; Lin *et al.*, 2008; Xu *et al.*,

2003; Zheng *et al.*, 2004; Zheng *et al.*, 2007; Zhou *et al.*, 2007). Since curcumin-mediated inhibition of *VCAM-1* expression in HuCCT1 cells was restored by PPAR γ specific antagonist BADGE, it is attractive to speculate that inhibition of both basal and TNF- α -induced *VCAM-1* expression in cholangiocytes by curcumin may be at least in part mediated by PPAR γ . Previous studies have shown reduction of bile duct proliferation and biliary fibrosis by PPAR γ agonists in bile duct-ligated rats (Marra *et al.*, 2000), (Marra *et al.*, 2005). In line with these studies our data suggest that PPAR γ activation in cholangiocytes may inhibit a central pathogenetic event in cholangiopathies, namely acquisition of the cholangiocyte reactive phenotype (Alvaro *et al.*, 2007; Lazaridis *et al.*, 2004).

Curcumin reduced liver fibrosis in *Mdr2*^{-/-} mice after both 4 and 8 weeks of feeding. Although relative reduction of hepatic HP levels was more pronounced after prolonged feeding (42.7% reduction after 8 weeks vs. 21.9% reduction after 4 weeks), the absolute HP levels did not differ significantly between 4 and 8 weeks of feeding, suggesting that curcumin inhibits progression of fibrosis rather than stimulating resolution of established fibrosis. A central event towards fibrosis is activation of HSCs and their transformation to MFB-like cells in response to pro-inflammatory cytokines and chemokines (Bataller *et al.*, 2005), (Friedman *et al.*, 2000). In biliary fibrosis, the subpopulation of portal MFBs contributes greatly to fibrosis (Cassiman *et al.*, 2002). Several studies reported that activation of HSCs and their transformation to MFB-like cells with fibrogenic properties was associated with diminished level and trans-activating ability of PPAR γ , while NF- κ B activation was increased (Hazra *et al.*, 2004b), (Eng *et al.*, 2000; Hazra *et al.*, 2004a; Mann *et al.*, 2002). Additionally, incubation of activated HSCs with PPAR γ agonists was shown to revert the fibrogenic phenotype of these cells by inhibiting collagen production, proliferation, migration, and production of the pro-inflammatory chemokine Mcp-1 (Marra *et al.*, 1998; Marra *et al.*, 2000), (Galli *et al.*, 2000; Galli *et al.*, 2002; Miyahara *et al.*, 2000). The importance of PPAR γ in maintaining the quiescent state of HSCs was further demonstrated by its ectopic expression in activated stellate cells resulting in inhibition of their extracellular matrix producing ability (Hazra *et al.*, 2004b). Curcumin reduced proliferation as well as fibrotic activity of portal MFBs isolated from *Mdr2*^{-/-} mice. In contrast to previous reports, demonstrating that curcumin reduced the activated phenotype of primary HSCs by increasing PPAR γ expression and activity (Xu *et al.*, 2003; Zheng *et al.*, 2004), (Zhou *et al.*, 2007), (Kunnumakkara *et al.*, 2008) exposure of portal MFBs to curcumin did not induce

PPAR γ 1 gene expression. Moreover, curcumin did not modify *MCP-1* expression, a marker of activated MFB-like cells (Marra *et al.*, 1998), (Maher *et al.*, 1998) known to be inhibited by PPAR γ ligands (Marra *et al.*, 1998). Based on these findings, we suggest that curcumin had a very modest influence on PPAR γ activation in MFBs perhaps due to the lack of specific PPAR γ co-factors.

Inflammatory cells play a key role in the pathogenesis of PSC (O'Mahony *et al.*, 2006), (Chapman *et al.*, 2008). Moreover, direct interaction of HSCs with CD8 positive lymphocytes was suggested to play an important role in liver fibrosis (Safadi *et al.*, 2004), (Muhanna *et al.*, 2007). Interestingly, decreased fibrosis in curcumin-treated *Mdr2*^{-/-} mice correlated with reduced CD11b and CD4 positivity. However, CD8 positive cells were virtually undetectable in control and curcumin-treated *Mdr2*^{-/-} mice at the age of 8 weeks. Besides its ability to activate PPAR γ , curcumin was also shown to modulate NF- κ B and ERK signaling pathways in HSCs *in vitro* resulting in reduction of their activation and proliferation (Xu *et al.*, 2003). Unchanged nuclear NF- κ B content after curcumin incubation is consistent with the lack of PPAR γ activation in portal MFBs. However, the reduced proliferation and activation of MFBs was associated with inhibition of ERK1/2 phosphorylation both *in vitro* and *in vivo*, suggesting that direct inhibition of ERK signaling could contribute to the reduction of biliary fibrosis by curcumin.

Reduced hepatocellular proliferation reflected by decreased Ki-67 staining is in line with improved liver injury in *Mdr2*^{-/-} mice. Since liver regeneration occurs in response to liver damage, it is tempting to speculate that these anti-proliferative effects may result indirectly from pleiotropic effects of curcumin on different liver cell types. Moreover, anti-proliferative effects of curcumin have been reported in several cancer cells (Kunnumakkara *et al.*, 2008), suggesting that curcumin might also have direct anti-proliferative effects in hepatocytes.

Collectively, curcumin decreased liver injury and hepatic fibrosis in *Mdr2*^{-/-} model through several signaling mechanisms in different hepatic cell populations. Activation of PPAR γ signaling in cholangiocytes may inhibit production of pro-inflammatory cytokines by activated cholangiocytes, thus reducing attraction of inflammatory cells into portal filed. In addition, curcumin may inhibit proliferation and activation of main pro-fibrogenic cells in biliary fibrosis: portal MFBs via inhibition of ERK1/2 signaling pathway in this cell population.

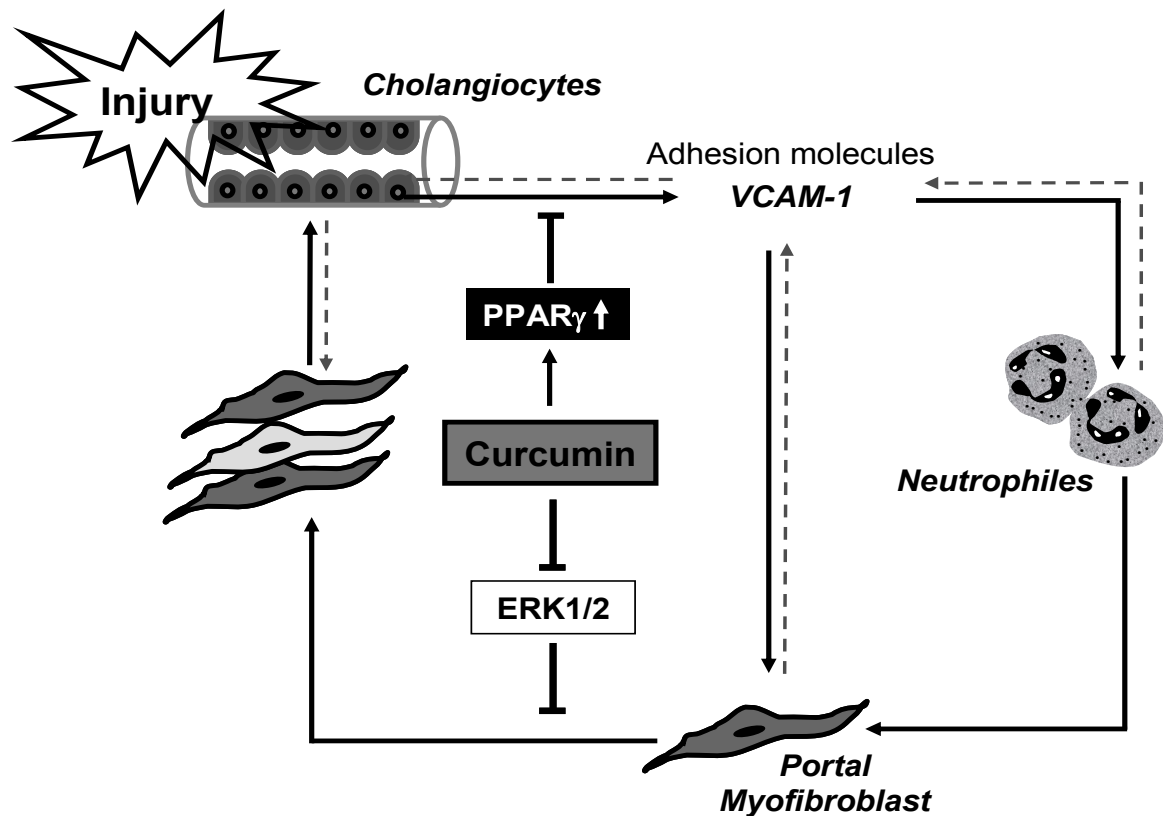


Figure 44. Potential cellular and molecular targets of curcumin in *Mdr2*^{-/-} mice. Our data suggest that curcumin may have pleiotropic actions on several critical steps involved in the pathogenesis of liver damage and biliary fibrosis in *Mdr2*^{-/-} mice. By activation of PPAR_γ in cholangiocytes, curcumin may block inflammatory activation and Vcam-1 expression in cholangiocytes, resulting in decreased attraction of neutrophil granulocytes as well as myofibroblasts in the portal fields. In addition, by inhibiting the ERK1/2 signaling curcumin may decrease proliferation of portal myofibroblasts the major fibrogenic cells in *Mdr2*^{-/-} mice, thus contributing to reduction of liver fibrosis.

In part 2 of this thesis, the therapeutic role of BA signaling through the nuclear BA receptor FXR and the membrane BA receptor TGR5 was addressed in a mouse model of chronic cholangiopathy. Results described in this part of the thesis show that the novel dual FXR/TGR5 agonist INT-767 reduced bile toxicity by decreasing biliary BA output and inducing HCO₃⁻-rich choleresis in an FXR-dependent manner.

BAs are important signaling molecules with hormonal actions (Thomas *et al.*, 2008). TGR5 and FXR polymorphisms (Hov *et al.*, ; Van Mil *et al.*, 2007) further support the importance of BA signaling in human biliary diseases. Liver injury in *Mdr2*^{-/-} mice is considered to evolve due to detergent properties of non-micellar bound free biliary BAs (Fickert *et al.*, 2004; Trauner *et al.*, 2008), suggesting an important role for BA signaling in modulating biliary pathophysiology. Surprisingly, only the dual FXR/TGR5 agonist INT-767 was hepatoprotective as shown by reduced serum ALT, decreased hepatic inflammation, ameliorated reactive cholangiocyte phenotype and biliary fibrosis in *Mdr2*^{-/-} mice. However, INT-767 had no direct anti-inflammatory effects in macrophages, cholangiocytes or hepatocytes and no direct anti-fibrotic effects in primary MFBs. These findings led us speculate that INT-767 might improve liver injury *via* impact on bile physiology. Indeed, *via* FXR activation, INT-767 inhibited BA synthesis (by ileal *Fgf15* and hepatic *Shp* induction), thus resulting in decreased biliary BA output, while significantly increasing bile flow and HCO₃⁻ output.

HCO₃⁻ formation and secretion into bile is considered to be protective mechanism that regulates intercellular pH, contributes to bile alkalization and sustains BA-independent bile flow (Beuers *et al.*, 2010). Since INT-767 increased HCO₃⁻-rich choleresis *via* FXR, we focused on transcriptional regulations of genes involved in HCO₃⁻ transport and production. FXR was shown to increase biliary HCO₃⁻ secretion in human gallbladder epithelium *via* VPAC-1 induction (Chignard *et al.*, 2005). However, in our experiments *Vpac-1* expression decreased by INT-767 in *Mdr2*^{-/-} and *Fxr*^{+/+} mice, indicating that other mechanisms contribute to the INT-767-stimulated HCO₃⁻ secretion. In addition, these findings suggest differential species and/or organ specific VPAC-1 regulation by FXR. Biliary HCO₃⁻ export is mediated by electroneutral HCO₃⁻/Cl⁻ exchange transporter AE2 in hepatocytes (Martinez-Anso *et al.*, 1994; Meier *et al.*, 1985) and Ae2 and Slc4a4 in cholangiocytes (Uriarte *et al.*, 2010). Nor *Ae2* neither *Slc4a4* gene expression was altered by INT-767, showing that alternative mechanism was taking place. HCO₃⁻ secretion can be facilitated by induction of CAs which *via* formation of functional complexes with AEs form so called HCO₃⁻ transport metabolon (McMurtrie *et al.*, 2004;

Sterling *et al.*, 2002; Vince *et al.*, 1998). This mechanism was reported to maximize the HCO_3^- flux in several organs (Casey *et al.*, 2009; Morgan *et al.*, 2007; Svichar *et al.*, 2009). More specifically, the sub-group of membrane-bound or extracellular CAs facilitate AEs and HCO_3^- transport to buffer the extracellular fluids (Parkkila *et al.*, 2002; Pastorekova *et al.*, 1997; Saarnio *et al.*, 2001; Svichar *et al.*, 2009). In addition, the role of membrane-bound CAs was suggested to propagate the HCO_3^- umbrella at the apical surface of cholangiocytes (Beuers *et al.*, 2010). Interestingly, the expression of Ca4, an isoform expressed in apical membrane of cholangiocytes (Parkkila *et al.*, 1996) was undetectable and remained unchanged in vivo and in vitro (BECs) by INT-767, while expression of cholangiocellular basolateral Ca9 (Pastorekova *et al.*, 1997), (Saarnio *et al.*, 2001) increased by INT-767 in *Fxr*^{+/+} but not in *Mdr2*^{-/-} mice and remained unchanged in BECs (data not shown). CA14 is a membrane-bound enzyme expressed in hepatocytes (Parkkila *et al.*, 2002) with its catalytic domain on extra-cellular surface of hepatocytes, suggesting an important role for this enzyme in bile secretion. Interestingly, INT-767 induced *CA14* gene expression in *Mdr2*^{-/-} mice. Moreover, this induction was reproduced in *FXR-WT*, while being abolished in *FXR-KO* mice. Finally, we could show that INT-767 significantly induced *CA14* mRNA levels in human hepatocytes. The functional and physical interaction of CA14 with $\text{Cl}^-/\text{HCO}_3^-$ exchanger AE3 was proven to be an efficient mechanism to facilitate HCO_3^- transport in mouse brain (Casey *et al.*, 2009). Of note, AE2 and AE3 share COOH-terminal loop, where the interaction with extracellular CA14 takes place (Casey *et al.*, 2009). Therefore, it is tempting to speculate that INT-767 *via* FXR-dependent induction of *CA14* expression in hepatocytes promotes the formation of a HCO_3^- transport metabolon involving CA14 and AE2. The driving force for HCO_3^- transport is high HCO_3^- concentration on the active site of transporter and low HCO_3^- concentration on the opposite site of membrane. Since CA14 has a high catalytic activity, in addition to enhanced the HCO_3^- transport through a complex formation with the AE transporter, it can catalyze the hydration HCO_3^- with H^+ into CO_2 and H_2O and thus increase the trans-membrane HCO_3^- gradient to further enhance the rate of HCO_3^- transport by AE2. These findings suggest that INT-767 by induction of membrane-bound CAs, can promote HCO_3^- -driven bile secretion on hepatocyte canalicular membrane. However, role of FXR in regulation of hepatocellular *CA14* and cholangiocellular CA genes deserves further studies.

In contrast to INT-767, the selective FXR agonist INT-747 was found to deteriorate liver injury and fibrosis in *Mdr2*^{-/-} model. Whereas low-dose INT-747 did not

modify liver damage in *Mdr2*^{-/-} mice, high dose (0.03% w/w) aggravated it, despite induced *Fgf15* and efficient inhibition of BA synthesis. Interestingly, INT-747 did not induce hepatic *Shp* gene expression, suggesting that in contrast to INT-767, which efficiently activates FXR in the intestine and the liver, INT-747 is less likely to have hepatic activity in mice. However, *NTCP* gene expression was inhibited by INT-747, which may reflect increased inflammation and pro-inflammatory cytokine expression by this compound (Zimmerman *et al.*, 2006). In addition, INT-747 had no impact on *CA14* gene expression *in vivo* and *in vitro* (data not shown), another important mechanism which contributes to the beneficial effects of INT-767. Together, these findings point out to a differential regulation of FXR-dependent genes by INT-747 and INT-767. *In vitro* EC₅₀ of INT-767 is approximately 10-fold higher compared with EC₅₀ of INT-747 for FXR, indicating that INT-767 is a stronger FXR activator in even very low concentrations. Ligand binding to FXR can favor conformations that in turn allow only specific cofactors recruitment depending on the DNA binding sequence, therefore resulting in selective modulation of gene expression. Our findings suggest that INT-767 acts as a specific bile acid receptor modulator or BARM, in a way similar to other BAs such as LCA or other synthetic modulators (Costantino *et al.*, 2005; Downes *et al.*, 2003; Dussault *et al.*, 2003; Yu *et al.*, 2002a)

BA hydrophobicity is another important factor directly linked to BA detergent properties (Heuman *et al.*, 1991a; Heuman *et al.*, 1991b; Moschetta *et al.*, 2001). Hydrophobic BAs were toxic to hepatocyte at high concentrations (Rust *et al.*, 2009). Endogenous BAs and INT-747 are hydrophobic BAs, whereas INT-767 compound is hydrophilic (Rizzo *et al.*, 2010). Of note, INT-747 showed highest biliary enrichment in *Mdr2*^{-/-} mice. We believe that accumulation of the hydrophobic INT-747 in the liver without stimulation of hepatoprotective mechanisms may act as an additional important factor for initiation and promotion of liver damage in *Mdr2*^{-/-} mice. INT-767 in contrast reduces bile toxicity and prevents further progression of liver injury, *via* a strong inhibition of endogenous BA synthesis, replacing hydrophobic BAs with the hydrophilic INT-767 and inducing HCO₃⁻-rich bile secretion. Nevertheless, preliminary data from human trials reported a beneficial effect of INT-747 on serum liver enzyme levels in PBC patients (Kowdley *et al.*, 2011a), (Kowdley *et al.*, 2011b), even if high-dose INT-747 (50 mg/kg) was associated with higher rate of side effects such as hyperbilirubinemia, itching and PBC flare. It is therefore tempting to speculate that INT-767 and/or INT-767 derivatives will constitute a new leading approach to treat cholangiopathies such as PSC

(resembling phenotypically to the *Mdr2*^{-/-} model), whereas INT-747 will be of more interest in metabolic diseases or in PBC. However, FXR-mediated stimulation of bile flow may be deleterious in cases of obstructive cholestasis (Stedman *et al.*, 2006).

Since TGR5 signals through cAMP signaling pathway, an important regulator of chloride channel CFTR, and was reported to be localized on cholangiocyte apical surface (Keitel *et al.*, 2010b), we expected increased bile flow and HCO₃⁻ output by the selective TGR5 agonist INT-777. Surprisingly, INT-777 did not influence bile secretory function as reflected by unchanged bile flow and biliary HCO₃⁻ output in *Mdr2*^{-/-}, *Fxr*^{+/+} and *Fxr*^{-/-} mice, despite its ability to activate TGR5 *in vitro* and *in vivo* (Pellicciari *et al.*, 2009; Thomas *et al.*, 2009). In addition to pharmaceutical TGR5 activation, by using *TGR5-TG* mice we could confirm that TGR5 over-expression also had no impact on bile secretion. However, INT-777 decreased biliary PL and cholesterol output in *FXR-WT* mice in the presence of unchanged BA concentrations. Therefore, INT-777 by promoting imbalanced bile composition may initiate deleterious effects similar to what is observed in *Mdr2*^{-/-} mice. These findings are consistent with a previous report showing, that *Tgr5*^{-/-} mice had higher biliary PL compared with *Tgr5*^{+/+} mice and were protected from gallstone development upon lithogenic diet feeding (Vassileva *et al.*, 2006). Moreover, gallbladder smooth muscle function was shown to be inhibited by hydrophobic BAs through TGR5 activation (Lavoie *et al.*, 2010), indicating that Tgr5 activation may not be beneficial for disorders of gallbladder and biliary tree. Altogether, these data suggest that despite a beneficial effect of TGR5 activation in diabetes, TGR5 is unlikely to be beneficial in cholangiopathies and diseases with impaired bile composition as well as gallbladder function. However, the failure of INT-777 to improve disease progression in *Mdr2*^{-/-} does not rule out the possibility that another TGR5 activator might help to delay or cure the disease.

Un-desirable effects of TGR5 activation have been reported in several extrahepatic organs. Specifically, development of acute reflux-induced pancreatitis was directly associated with BA-mediated Tgr5 activation in pancreatic acini (Perides *et al.*, 2010). TGR5 activation promoted production of reactive oxygen species in astrocytes (Keitel *et al.*, 2010a) and activated AKT signaling pathways in cardiomyocytes (Desai *et al.*, 2010). Finally, TGR5-activation by BAs was linked to increased hepatocellular apoptosis (Yang *et al.*, 2007) through activation of c-Jun N-terminal kinase (JNK) signaling pathways, indicating that Tgr5 might have a critical role in cells death and cancer development.

The role of biliary HCO_3^- secretion as an important hepatoprotective mechanism in portal inflammation has been shown previously in *Mdr2*^{-/-} mice after treatment with norUDCA, which represents a side-chain shortened modification of UDCA and undergoes cholehepatic shunting, thereby inducing HCO_3^- -rich bile flow (Fickert *et al.*, 2006). Moreover, liver biopsies and blood mononuclear cells from PBC patients showed decreased AE2 expression (Medina *et al.*, 1997; Prieto *et al.*, 1993). In line with this findings, Ae2 deficiency in mice resulted in formation of anti-mitochondrial antibodies and other features of PBC (Salas *et al.*, 2008). Interestingly, therapeutic effects of UDCA were associated with restored AE2 expression (Medina *et al.*, 1997; Prieto *et al.*, 1999). Together with the literature data, our findings underline critical role of biliary HCO_3^- and suggest that that induction of HCO_3^- -rich bile secretion by promotion of bicarbonate transport metabolon formation with Ca14, may serve as another efficient mechanism to reduce bile toxicity and decrease portal inflammation.

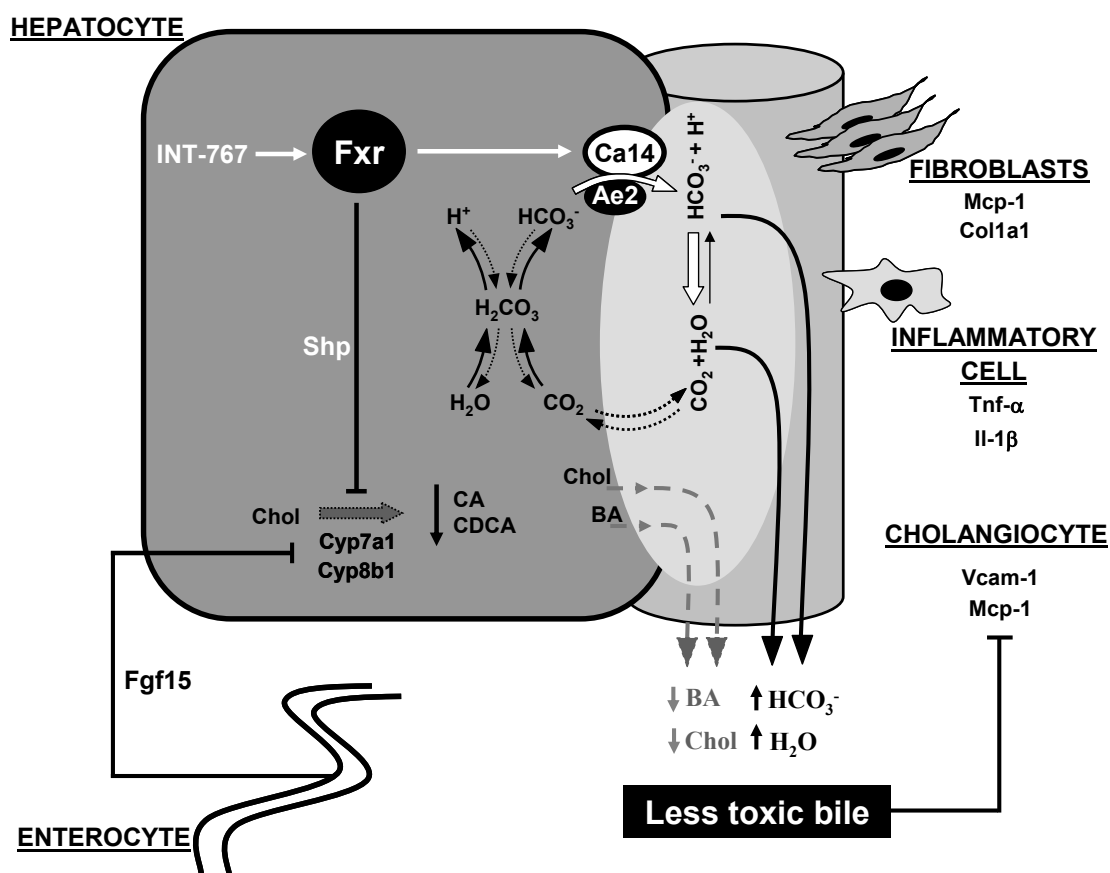


Figure 45. Proposed model of INT-767-mediated beneficial effects in *Mdr2*^{-/-} mice. INT-767 activates FXR in ileum and in the liver. By induction of ileal *Fgf15* and hepatic *Shp*, INT-767 dramatically inhibits endogenous BA synthesis, resulting in a significant reduction of biliary output of hydrophobic BAs and cholesterol. In addition, INT-767 via *Fxr* activation induces expression of hepatocellular membrane-bound *Ca14*, which in turn promotes HCO_3^- output and HCO_3^- -rich choleresis due to i) complex formation with HCO_3^- transporter *Ae2* and ii) hydration of HCO_3^- with H^+ into CO_2 and H_2O , thus increasing the local trans-membrane HCO_3^- gradient to further enhance the rate of HCO_3^- transport. Since cell membranes are easily permeable for CO_2 , generated CO_2 in turn can enter the cells where it is rehydrated to H^+ and HCO_3^- thus contributing to HCO_3^- recycling. Collectively, INT-767 by reducing concentration of detergent BAs and increasing HCO_3^- -rich choleresis contributes to less reactive alterations of bile duct epithelium and results in decreased portal inflammation and fibrosis.

Collectively, the findings described in this thesis have important implications for understanding the pathophysiology of cholangiopathies and design of novel efficient therapeutic strategies for human cholangiopathies. Specifically, combined targeting of cholangiocyte and MFB activation by inducing $\text{PPAR}\gamma$ activation and repressing the ERK1/2 signaling represents an attractive strategy to treat or delay the progression of chronic cholangiopathies and liver fibrosis. In addition, FXR activation by specific and highly potent agonists such as INT-767 can have critical role in reducing bile toxicity by inducing bile flow, decreasing BA output and increasing HCO_3^- output resulting in repression of hepatic inflammation as well as biliary fibrosis.

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APPENDIX

Publication list

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