Curcumin improves sclerosing cholangitis in Mdr2<sup>−/−</sup> mice by inhibition of cholangiocyte inflammatory response and portal myofibroblast proliferation

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ABSTRACT

Background and aim 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.

Methods Potential anticholestatic, anti-inflammatory and antifibrotic mechanisms of curcumin were explored in vivo in Mdr2<sup>−/−</sup> 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<sup>−/−</sup> mice.

Results Liver damage, cholestasis and fibrosis were reduced in Mdr2<sup>−/−</sup> mice after curcumin feeding. Moreover, curcumin inhibited cholangiocyte proliferation and expression of activation marker vascular cell adhesion molecule 1 in Mdr2<sup>−/−</sup> mice. Curcumin—similar to PPARγ 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 PPARγ synthetic antagonist. In addition, curcumin blocked proliferation and activation of portal MFBs by inhibiting ERK1/2 phosphorylation, thus contributing to reduced fibrogenesis.

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

Chronic cholangiopathies such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis are characterised by progressive inflammation and subsequent development of biliary fibrosis and cirrhosis.¹ ² Since the effectiveness of currently available medical therapies to slow the progression of cholangiopathies is limited, there is an urgent need for novel and effective medical treatment strategies. Importantly, cholangiocytes may undergo phenotypic and functional modifications, characterised by production of pro-inflammatory and profibrogenic mediators, thus contributing to propagation and progression of liver diseases.³ ⁵ Therefore, it is attractive to hypothesise 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.⁶ ¹¹ 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, antioxidative and antifibrotic properties.¹² ¹⁵ Moreover, blocking c-Jun N-terminal kinase (JNK) signalling by curcumin was shown to inhibit inflammation-mediated alterations of hepatobiliary transporter gene expression in hepatocytes.¹⁶ 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 multidrug-resistant protein 2 knockout (Mdr2<sup>−/−</sup>) mice serving as a genetic model of progressive cholangiopathy with biliary fibrosis.¹⁷ ⁵¹

We herein demonstrate that curcumin reduces liver damage, cholangitis and biliary fibrosis in Mdr2<sup>−/−</sup> mice most likely by acting on multiple targets: inhibiting the inflammatory phenotype of bile duct epithelial cells through PPARγ activation and blocking proliferation/activation of portal myofibroblasts (MFBs) through inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) signalling.

MATERIALS AND METHODS

Animal experiments

Mdr2<sup>−/−</sup> mice (FVB/N background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed with a 12:12 hour light/dark cycle with water and a mouse diet (Harlan Teklad, Madison, WI, USA) ad libitum. Male mice were used exclusively. 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 US National Academy of Sciences (National Institutes of Health publication 86–23, revised 1985).

Feeding protocols

Feeding of control and curcumin-enriched (4% wt/wt) diets in Mdr2<sup>−/−</sup> mice was performed for 4 and 8 weeks starting at the age of 4 weeks, a time point...
when the principal features of bile duct disease and biliary fibrosis are already present and show high activity.\textsuperscript{17, 22} Curcumin was kindly gifted by Sabinsa Corporation in form of Curcumin C3 complex (Sabinsa Utah, Payson, UT, USA), which contained 96.24% curcuminoids, of which 72.1% was curcumin, 21.98% demethoxy curcumin and 5.91% bisdemethoxy curcumin. Wild type (WT) mice of the same age received a control diet (Harlan Teklad, Madison, WI, USA).

**Food intake, body weight/liver weight measurement**
Food intake was controlled at days 1, 3 and 7 during the first week, and further monitored once a week, whereas gain in body weight (BW) was monitored daily. Liver weight (LW) was compared to the BW and the percentage ratio was calculated (LW/BW).

**Routine serum biochemistry**
Blood was collected at harvesting and centrifuged for 15 min at 4500 rpm. Serum was stored at \(-80^\circ\text{C}\) until analysis. Assays for alanine aminotransferase and alkaline phosphatase were routinely measured, whereas serum bile acid (BA) concentration was determined by using Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) in a Hitachi 917 analyser (Boehringer Mannheim, Mannheim, Germany).

**Liver histology**
For conventional light microscopy, livers were fixed in 4% neutral buffered formaldehyde solution for 24 h and embedded in paraffin. Sections were cut 2 \(\mu\text{m}\) thick and stained with haematoxylin and eosin (H&E) or Sirius red.

**Determination of hepatic hydroxyproline content**
To quantify liver fibrosis, hepatic hydroxyproline (HP) was measured from standardised liver lobe as described previously.\textsuperscript{25}

**Immunohistochemistry**
Detection of proliferation marker Ki-67, cholangiocyte cytoskeleton marker keratin 19 (K19), vascular cell adhesion molecule-1 (VCAM-1), neutrophil granulocyte marker CD-11b and lymphocyte markers CD4 and CD8 was performed as described.\textsuperscript{17, 18}

**Western blotting for hepatic K19 and hepatocellular transporters Ntcp, Bsep, Mrp2 and Mrp3**
Total proteins from liver homogenates were isolated as described\textsuperscript{24} and protein concentrations were determined using a Bradford kit (BioRad, Richmond, CA, USA). Western blotting for K19 was performed as previously described.\textsuperscript{18} For hepatocellular transporters, preparation of liver membranes, detection of immunocomplexes as well as densitometric quantification of band intensities, was performed as described.\textsuperscript{25, 26}

**Measurement of bile flow and composition**
Bile flow and output of biliary phospholipids (FLs), BAs, cholesterol and glutathione were determined as previously described.\textsuperscript{18} Experimental feeding was initiated in 4-week-old Mdr2\(^{−/−}\) and WT mice. After 7 days of feeding either a control or a curcumin-enriched diet, mice were anaesthetised (10 mg of avertin intraperitoneally), abdominal cavity was opened, common bile duct was ligated and gallbladder was cannulated. After a 10 min equilibration period, bile was collected in pre-weighed tubes for 30 min. Bile flow was determined gravimetrically and normalised to LW. Samples were kept frozen at \(-20^\circ\text{C}\) until analysis.

**Messenger RNA analysis and Polymerase Chain Reaction (PCR)**
RNA isolation, complementary DNA synthesis, and real-time PCR were performed as described previously.\textsuperscript{27}

**Isolation, culture and experiments with portal myofibroblasts**
Portal MFBs were isolated as described.\textsuperscript{28} 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% foetal 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...
RESULTS
Curcumin significantly reduces liver damage and cholestasis in Mdr2^-/- mice
We first addressed whether curcumin-enriched diet was well tolerated by Mdr2^+/+ (WT) and Mdr2^-/- mice. Survival rate (100%) and food consumption (average: 4–5 g/day) did not differ between study groups. LW/BW ratio was increased in 8-week-old Mdr2^-/- mice compared with age-matched WT mice and remained unchanged after curcumin feeding (data not shown). Curcumin feeding reduced sclerosing cholangitis, ductular proliferation (figure 1A), serum liver enzymes (alanine aminotransferase and alkaline phosphatase) and BA levels in Mdr2^-/- mice (figure 1B) after 4 weeks of feeding. Compared with 4-week-feeding, prolonged curcumin feeding for 8 weeks did not result in a further reduction of serum liver enzymes (figure 3B,C). Taken together, these data demonstrated beneficial effects of curcumin on cholestatic phenotype in Mdr2^-/- mice.

Hepatic fibrosis in Mdr2^-/- mice is significantly reduced by curcumin
Biliary fibrosis, determined by Sirius red staining, measurement of hepatic HP levels and Col1α2 mRNA expression, was reduced in Mdr2^-/- mice after 4 weeks of curcumin feeding (figure 2A–C). With age untreated Mdr2^-/- mice showed progression of fibrosis as determined by increased hepatic HP levels (243±45.8 at 12 weeks vs 166±3.1 μg/g LW at 8 weeks). Prolonged curcumin feeding (figure 3D) resulted in a more pronounced relative reduction of HP levels (42.7% after 8-week-feeding vs 21.9% reduction after 4-week-feeding), but did not further decrease HP levels compared with 4-week-feeding (159±12 vs 129.6±15 μg/g LW, not significant). Taken together, these findings suggest that curcumin feeding had no effect on resolution, but rather reduced progression of hepatic fibrosis in Mdr2^-/- mice via inhibition of collagen production.

Curcumin increases bile flow and biliary glutathione output in Mdr2^-/- mice
Since liver disease in Mdr2^-/- mice primarily results from toxic bile composition due to increased concentrations of non-micellar...
bound BAs, we next addressed whether curcumin modified bile formation and output of main biliary constituents. To distinguish between direct effects on bile flow and liver healing properties of curcumin, 4-week-old WT and Mdr2<sup>−/−</sup> mice were fed short-term (7 days) either a control or a curcumin-enriched diet. In WT mice, curcumin increased bile flow and biliary glutathione output, but did not modify biliary BA, PL and cholesterol output (table 1). Compared with WT mice, biliary PL and cholesterol output was dramatically reduced in Mdr2<sup>−/−</sup> mice and remained unchanged after curcumin feeding. In addition, curcumin increased bile flow in Mdr2<sup>−/−</sup> mice and restored altered biliary glutathione output (table 1). However, total hepatic glutathione content did not differ between the groups (data not shown), suggesting that curcumin had no impact on hepatic glutathione synthesis. Collectively, these data show that curcumin increased bile flow and biliary glutathione output, but did not modify bile toxicity as reflected by unchanged biliary PL, cholesterol and BA output.

**Therapeutic effects of curcumin in Mdr2<sup>−/−</sup> mice are neither mediated by changes of bile acid transport nor synthesis**

Reduction of serum BA levels in curcumin-treated Mdr2<sup>−/−</sup> mice may reflect alterations of BA transport and synthesis. Neither expression of the main sinusoidal BA importer (Ntcp) nor canalicular BA/anion exporters (Bsep, Mrp2, Mrp3) were altered by curcumin feeding in Mdr2<sup>−/−</sup> mice (figure 4A). Expression of Cholesterol 7 α-hydroxylase (Cyp7a1), the rate limiting enzyme for BA synthesis also did not differ significantly between the groups (data not shown), suggesting that curcumin had no impact on hepatic glutathione synthesis. Collectively, these data show that curcumin increased bile flow and biliary glutathione output, but did not modify bile toxicity as reflected by unchanged biliary PL, cholesterol and BA output.

**Curcumin reduces proliferation of activated portal myofibroblasts and inhibits ERK signalling**

Since activated portal MFBs represent an important source for the extracellular matrix in Mdr2<sup>−/−</sup> mice, we next studied direct antibiotic mechanisms of curcumin in portal MFBs. Since isolated MFBs as well as hepatic stellate cells (HSCs) demonstrate high proliferation rate and activity upon contact with the plastic, we used 0% FCS to inhibit MFB activation and synchronise the cells and 10% FCS to stimulate MFB activation. Compared with 0% FCS condition, 10% FCS stimulated proliferation as well as expression of fibrogenic markers in portal MFBs (figure 5B,C), while curcumin at 10 and 20 μm, two doses shown to be nontoxic (figure 5A), reduced 10% FCS-induced proliferation of portal MFBs (figure 5B). We next studied PPARγ mediated by changes of bile acid transport nor synthesis.

**Figure 3** Curcumin feeding for 8 weeks reduces liver damage and fibrosis in Mdr2<sup>−/−</sup> mice. A. Schematic illustration of feeding protocols in Mdr2<sup>−/−</sup> mice. Experimental feeding in Mdr2<sup>−/−</sup> mice was initiated at the age of 4 weeks, when the ductular proliferation and fibrosis showed high activity and lasted for 4 or 8 weeks. Feeding of age matched wild type mice followed the same experimental protocol. B. Serum liver enzymes and hepatic hydroxyproline were measured in Mdr2<sup>−/−</sup> mice to quantify liver damage and fibrosis after 8 weeks of curcumin feeding. Curcumin reduces serum liver enzymes ALT and AP as well as fibrosis in Mdr2<sup>−/−</sup> mice after 8 weeks of feeding. Values are presented as means±SD from four animals per group. *p<0.05 Mdr2<sup>−/−</sup>+Cu vs Mdr2<sup>−/−</sup>. ALT, alanine aminotransferase; AP, alkaline phosphatase; HP, hydroxyproline.

**Figure 4** Transport of biliary constituents and bile acid synthesis are not affected by curcumin in Mdr2<sup>−/−</sup> mice. A. Liver membranes for Western blotting were isolated from wild type (WT) and Mdr2<sup>−/−</sup> mice fed either a control or a curcumin-enriched diet for 4 weeks. Curcumin feeding does not influence levels of key hepatocellular transporters in Mdr2<sup>−/−</sup> mice (Mdr2<sup>−/−</sup>+Cu). Densitometry data are expressed as fold change relative to WT group. Values are means from three to four animals per group. β-actin protein levels were used as a protein loading control. *p<0.05 Mdr2<sup>−/−</sup> and Mdr2<sup>−/−</sup>+Cu vs WT. B. Cyp7a1 gene expression does not differ between WT, WT+Cu, Mdr2<sup>−/−</sup> and Mdr2<sup>−/−</sup>+Cu groups. Expression levels are normalised to 18s gene expression and values of WT are expressed as 1. Values are presented as means±SD from five animals per group. Ntcp, Na<sup>+</sup>/taurocholate cotransporting polypeptide; Mrp2, multidrug resistance associated protein 2; Mrp3, multidrug resistance associated protein 3; Bsep, bile salt export pump; Cyp7a1, cholesterol 7 α-hydroxylase.
Table 1  Curcumin effects on bile flow and composition in wild type and Mdr2<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>WT + Cu</th>
<th>Mdr2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Mdr2&lt;sup&gt;−/−&lt;/sup&gt; + Cu</th>
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<tr>
<td>Bile flow (μL/g LW/min)</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.2†</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.2†</td>
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<tr>
<td>Bile acids (nmol/LW/min)</td>
<td>29.1 ± 6.5</td>
<td>38.2 ± 10.1</td>
<td>27.4 ± 1.7</td>
<td>30.1 ± 14.4</td>
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<tr>
<td>Cholesterol (nmol/LW/min)</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
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<tr>
<td>Glutathione (nmol/LW/min)</td>
<td>4.0 ± 0.7</td>
<td>8.4 ± 1.4†</td>
<td>1.2 ± 0.3</td>
<td>4.9 ± 1.1</td>
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<tr>
<td>Phospholipids (nmol/LW/min)</td>
<td>4.5 ± 0.7</td>
<td>4.7 ± 1.1</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
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†p < 0.05 Mdr2<sup>−/−</sup> and Mdr2<sup>−/−</sup> + Cu vs WT.
‡p < 0.05 Mdr2<sup>−/−</sup> + Cu vs Mdr2<sup>−/−</sup>.
#p < 0.05 WT + Cu vs WT.

Values are means ± SD, n = 5 per group.

Curcumin inhibits proliferation and activation of portal myofibroblasts. A. Portal myofibroblasts (MFBs) were isolated from Mdr2<sup>−/−</sup> mice as described in Materials and methods and cytotoxic effect of curcumin was tested after 24 h incubation at concentrations of 10, 20 and 30 μm by using trypan blue dye exclusion method. Absolute number of living as well as dead cells was counted and calculated per 1 ml of cell suspension. At concentrations of 10 and 20 μm curcumin does not induce MFB cell death, whereas 30 μm is toxic. B. Influence of curcumin on MFB proliferation was assessed by immunohistochemical staining for Ki-67. Cells were first synchronised in 0% FCS containing medium and further grown on sterile cover slips using 0% FCS (to reduce proliferation), 10% FCS (to induce proliferation) and curcumin at concentrations of 10 and 20 μm in 10% FCS medium for 24 h. Number of Ki-67-positive nuclei was counted in 10 randomly chosen power fields in triplicate for each condition and the mean values of Ki-67-positive versus total cell number ratio (%) was calculated. Compared with 0% FCS condition 10% FCS stimulates MFB proliferation, while incubation with curcumin (10 and 20 μm) reduces 10% FCS-induced proliferation of MFBs in a concentration-dependent manner. C. Curcumin effects on profibrogenic gene expression were studied in 10% FCS-activated MFBs after 24 h incubation (as described in B). Curcumin inhibits Col1α2 and α-SMA gene expression, whereas Mcp-1 expression remains unchanged. Expression levels are normalised to 18s gene expression and levels of wild type (WT) are expressed as 1. B and C: Data are presented as means ± SD. *p < 0.005 10% FCS vs 0% FCS, †p < 0.005 10% FCS + Cu 10 μm vs 10% FCS, ‡p < 0.005 10% FCS + Cu 20 μm vs 10% FCS. D. Cytoplastic and nuclear proteins were isolated from MFBs after incubation with 10% FCS and 20 μm curcumin and analysed for pERK1/2 and NF-κB (p65) by Western blotting. Curcumin does not modify nuclear translocation of NF-κB, but inhibits ERK phosphorylation in portal MFBs. Representative data from two independent experiments performed in duplicate for each condition are shown. E. Proteins were isolated from livers of wild type (WT) and Mdr2<sup>−/−</sup> mice fed either a control or a curcumin-enriched diet for 4 weeks and analysed for pERK and α-SMA by Western blotting. Curcumin significantly reduces cytoplasmic pERK1/2 in livers of Mdr2<sup>−/−</sup> mice. Densitometry data are expressed as fold change relative to WT mice. Values are presented as means ± SD. *p < 0.05 Mdr2<sup>−/−</sup> vs WT, †p < 0.05 Mdr2<sup>−/−</sup> + Cu vs Mdr2<sup>−/−</sup>; Co, control; Cu, curcumin; FCS, Foetal Calf Serum; Col1α2, collagen, type I; α-Z, α-SMA; α-smooth muscle actin; Mcp-1, monocyte chemotactic protein 1; CE, cytoplasmic extracts; NE, nuclear extracts.
described as reactive phenotype.\textsuperscript{5, 17} Curcumin-treated Mdr2\textsuperscript{-/-} mice showed decreased staining for K19 as well as significantly decreased hepatic K19 content (figure 6A,B) compared with untreated Mdr2\textsuperscript{-/-} mice. In addition, bile ducts of untreated Mdr2\textsuperscript{-/-} mice were highly positive for VCAM-1 expression compared with WT mice, whereas curcumin feeding reduced VCAM-1 staining in bile ducts of Mdr2\textsuperscript{-/-} mice (figure 7A). VCAM-1 gene expression can be stimulated by pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α).\textsuperscript{36, 37} TNF-α expression was significantly increased in Mdr2\textsuperscript{-/-} mice but remained unchanged after curcumin treatment (figure 7B). In contrast, PPARγ expression increased in both curcumin-treated WT and Mdr2\textsuperscript{-/-} mice (figure 7C), suggesting that inhibition of production in bile duct lining cells by curcumin could be PPARγ-mediated. In line with our in vivo findings, incubation of the cholangiocyte cell line HuCCT1 (expressing functionally active PPARγ) with TNF-α induced VCAM-1 expression, which was blocked by curcumin as well as specific PPARγ agonist troglitazone (figure 7D). Moreover, curcumin mediated inhibition of VCAM-1 expression in cholangiocytes was largely restored by pre-incubation and co-incubation with the PPARγ synthetic antagonist BADGE (figure 7E). Together, these findings suggest that curcumin reduced bile duct damage by inhibiting cholangiocyte activation via PPARγ signalling.

**Curcumin significantly reduces recruitment of inflammatory cells as well as hepatocellular damage and proliferation in Mdr2\textsuperscript{-/-} mice**

Expression of adhesion molecules and inflammatory cytokines by bile duct cells and activated MFBs stimulates an inflammatory response in the portal tract of Mdr2\textsuperscript{-/-} mice as shown by increased CD11b levels (figure 8A). Curcumin feeding in Mdr2\textsuperscript{-/-} mice resulted in pronounced reduction of CD11b staining (figure 8A,B). In addition, compared with WT mice Mdr2\textsuperscript{-/-} mice demonstrated increased staining of lymphocyte marker CD4 in portal tracts, which was reduced by curcumin (figure 9A). However, CD8 positive cells were not detectable in Mdr2\textsuperscript{-/-} mice at this time point independent of treatment condition (figure 9B). Interestingly, expression of TNF-α, IL-1β and TGF-β did not differ between curcumin-treated and untreated Mdr2\textsuperscript{-/-} mice (figure 7B and data not shown), suggesting that inhibition of VCAM-1 production by cholangiocytes was sufficient to reduce infiltration by neutrophil granulocytes. Since hepatocellular proliferation is a consequence of liver damage, we next studied hepatocellular proliferation by immunohistochemical staining for the proliferation marker Ki-67. Mdr2\textsuperscript{-/-} mice showed increased amount of Ki-67-positive hepatocytes compared with WT, while curcumin reduced Ki-67-staining (figure 10A, B).

**DISCUSSION**

This study demonstrates that the natural compound curcumin improves bile duct injury and biliary fibrosis in a mouse model of chronic cholangiopathy. We demonstrate that curcumin targets important pathogenic steps in cholangiopathies such as MFB proliferation (via pERK1/2 inhibition) and cholangiocellular VCAM-1 expression (in a PPARγ-dependent fashion). Taken together, curcumin reduces portal inflammation and fibrosis in the Mdr2\textsuperscript{-/-} mouse liver.

Chronic cholangiopathies include a wide spectrum of diseases ranging from autoimmune disorders (PSC, primary biliary cirrhosis, acute/chronic allograft rejection, graft vs host disease) to congenital genetic defects (Alagille syndrome, cystic fibrosis, MDR3 mutations, etc). Long-term beneficial effects of the one established medical therapy, ursodeoxycholic acid, are unclear, and liver transplantation often remains the ultimate therapeutic solution.\textsuperscript{38–41} Curcumin, an extract from turmeric has been used as traditional medicine for treatment of various gastrointestinal disorders.\textsuperscript{6–11} 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 analysing duodenal juice in humans.\textsuperscript{42} Shortly thereafter, curcumin was successfully used to treat cholestatic jaundice in patients.\textsuperscript{10} Acute choleretic effects of curcumin were further confirmed in bile fistula and isolated perfused rat liver models.\textsuperscript{43, 44} Interestingly, curcumin was also shown to reduce gallstone formation in mice after lithogenic diet feeding.\textsuperscript{45} However, the impact of curcumin on bile composition and chronic cholangiopathies has not yet been explored.

Mdr2\textsuperscript{-/-} mice lacking biliary PL excretion have been characterised as a suitable mouse model for spontaneous development of sclerosing cholangitis.\textsuperscript{17} Bile duct damage and fibrosis in

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**Figure 6** Curcumin feeding significantly reduces bile duct proliferation in Mdr2\textsuperscript{-/-} mice. A. Representative immunohistochemical staining for cholangiocyte cytoskeleton marker K19 (red) shows increased ductular proliferation in Mdr2\textsuperscript{-/-} mouse compared with wild type (WT). Immunohistochemical staining for K19 does not differ between chow-fed (WT) and curcumin-fed wild type (WT+Cu) mouse, whereas 4-week curcumin-treated Mdr2\textsuperscript{-/-} mouse (Mdr2\textsuperscript{-/-}+Cu) demonstrates reduced K19 staining. B. Total protein was isolated from livers and analysed by Western blotting for K19 content. Mdr2\textsuperscript{-/-}+Cu mice show significantly decreased K19 protein levels compared with control diet-fed age-matched littermates. Densitometry data are expressed as fold change relative to WT mice. Values are presented as means ± SD. *p<0.05 Mdr2\textsuperscript{-/-} vs WT, **p=0.05 Mdr2\textsuperscript{-/-}+Cu vs Mdr2\textsuperscript{-/-}.
Mdr2\(^{-/-}\) mice start early after birth and evolve over time to a full-blown phenotype at the age of 8 weeks.\(^{17,22}\) To study the potential action of curcumin on progression of liver injury in a mouse model of sclerosing cholangitis we designed 4-week and 8-week feeding protocols (figure 3A) in Mdr2\(^{-/-}\) mice starting from the time of weaning (age of 4 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.\(^{20}\) 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 micellisation. 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, rodents, and humans, 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 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-1β and TGF-β remained unaffected. In addition, curcumin completely inhibited TNF-α-induced VCAM-1 expression in cholangiocytes 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 biliary 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 PPARγ activator. Since curcumin-mediated inhibition of VCAM-1 expression in HuCCT1 cells was restored by PPARγ 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 PPARγ. Previous studies have shown reduction of bile duct proliferation and biliary fibrosis by PPARγ agonists in bile duct-ligated rats. In line with these studies our data suggest that PPARγ activation in cholangiocytes may inhibit a central pathogenetic event in cholangiopathies, namely acquisition of the cholangiocyte reactive phenotype.

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...
Figure 11 Hypothetical mechanisms of curcumin action in Mdr2<sup>−/−</sup> 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<sup>−/−</sup> mice. By activation of PPARγ 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 signalling curcumin may decrease proliferation of portal myofibroblasts the major fibrogenic cells in Mdr2<sup>−/−</sup> mice, thus contributing to reduction of liver fibrosis.68 69 However, CD8 positive cells were virtually undetectable in both control and curcumin-treated Mdr2<sup>−/−</sup> mice at the age of 8 weeks.

Reduced hepatocellular proliferation reflected by decreased Ki-67 staining is in line with improved liver injury in Mdr2<sup>−/−</sup> mice. Since liver regeneration occurs in response to liver damage, it is tempting to speculate that these antiproliferative effects may result indirectly from pleiotropic effects of curcumin on different liver cell types. Moreover, antiproliferative effects of curcumin have been reported in several cancer cells,30 suggesting that curcumin might also have direct antiproliferative effects in hepatocytes.

In conclusion, we demonstrate that curcumin is beneficial in a genetic mouse model of cholangiopathy and biliary fibrosis, and propose potential molecular and cellular targets of curcumin in this model (figure 11). Collectively, our findings have important implications for understanding the pathophysiology of cholangiopathies and suggest that combined targeting of cholangiocyte and MFB activation could represent a central strategy to treat or delay the progression of chronic cholangiopathies and liver fibrosis.

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Competing interests None.

Contributors AB: Performed the experiments, data collection, wrote the manuscript. KL: Data collection, technical support. PF, TC, AK: Critical revision of the article, experimental help. AF, JG, DS, KL: Data collection, technical support.

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