Pharmacological Inhibition of Apical Sodium-Dependent Bile Acid Transporter Changes Bile Composition and Blocks Progression of Sclerosing Cholangitis in Multidrug Resistance 2 Knockout Mice

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Deficiency for multidrug resistance 2 (mdr2), a canalicular phospholipid floppase, leads to excretion of low-phospholipid “toxic” bile causing progressive cholestasis. We hypothesize that pharmacological inhibition of the ileal, apical sodium-dependent bile acid transporter (ASBT), blocks progression of sclerosing cholangitis in mdr2−/− mice. Thirty-day-old, female mdr2−/− mice were fed high-fat chow containing 0.006% SC-435, a minimally absorbed, potent inhibitor of ASBT, providing, on average, 11 mg/kg/day of compound. Bile acids (BAs) and phospholipids were measured by mass spectrometry. Compared with untreated mdr2−/− mice, SC-435 treatment for 14 days increased fecal BA excretion by 8-fold, lowered total BA concentration in liver by 65%, reduced total BA and individual hydrophobic BA concentrations in serum by >98%, and decreased plasma alanine aminotransferase, total bilirubin, and serum alkaline phosphatase levels by 86%, 93%, and 55%, respectively. Liver histology of sclerosing cholangitis improved, and extent of fibrosis decreased concomitant with reduction of hepatic profibrogenic gene expression. Biliary BA concentrations significantly decreased and phospholipids remained low and unchanged with treatment. The phosphatidylcholine (PC)/BA ratio in treated mice corrected toward a ratio of 0.28 found in wild-type mice, indicating decreased bile toxicity. Hepatic RNA sequencing studies revealed up-regulation of putative anti-inflammatory and antifibrogenic genes, including Ppara and Igf1, and down-regulation of several proinflammatory genes, including Ccl2 and Lcn2, implicated in leukocyte recruitment. Flow cytometric analysis revealed significant reduction of frequencies of hepatic CD11b+ F4/80+ Kupffer cells and CD11b+Gr1+ neutrophils, accompanied by expansion of anti-inflammatory Ly6C− monocytes in treated mdr2−/− mice. Conclusion: Inhibition of ASBT reduces BA pool size and retention of hydrophobic BA, favorably alters the biliary PC/BA ratio, profoundly changes the hepatic transcriptome, attenuates recruitment of leukocytes, and abrogates progression of murine sclerosing cholangitis. (HEPATOLOGY 2015; 00:000-000)

Chronic fibrosing cholangiopathies, such as progressive familial intrahepatic cholestasis type 3 (PFIC3), biliary atresia, or primary sclerosing cholangitis (PSC), carry high morbidity and mortality owing to complications from progressive cholestasis and fibrosis. Cholestasis in these conditions often results from impaired hepatocellular and bile duct epithelial excretion of bile constituents, primarily bile acids (BAs),
phospholipids (PLs), and cholesterol, and from ductal obstruction of bile flow. Bile duct epithelial cells become injured, which leads to up-regulation of adhesion molecules, secretion of proinflammatory cytokines and chemokines, and, ultimately, recruitment of leukocytes to the portal tract driving fibro-obliteration of the biliary tree, as previously reviewed for cholestatic disorders and their animal models.\(^1\)\(^2\) Formation and retention of “toxic” BA likely play important roles in perpetuating the disease process.\(^3\) For instance, hydrophobic BA species are known to trigger hepatobiiliary inflammation and cause hepatocyte death by apoptosis.\(^4\) Further evidence for the pathogenic role of BAs in cholangiopathies originates from genome-wide association studies on patients with PSC, which identified not only genes linked to the human leukocyte antigen complex, but also GPBAR1 encoding the BA receptor, TGR5, as possible disease genes.\(^5\) Among many functions, TGR5 has been implicated in regulating production of proinflammatory cytokines by macrophages.\(^6\)

The multidrug resistance gene 2 knockout (KO) mouse (mdr2\(^{-/-}\)) represents an established animal model for chronic cholestatic disorders, especially PFIC3,\(^7\) and may recapitulate some aspects of the pathogenesis of PSC.\(^8\) These mice display absence of PL from bile,\(^9\) which disrupts formation of mixed micelles and subsequently leads to “toxic bile,” triggering an inflammatory cascade.\(^10\) This model has been extensively used for preclinical studies of novel therapies for chronic cholestasis, targeting anticholestatic, -inflammatory, and fibrogenic pathways.\(^11\)\(^12\)

BA homeostasis is tightly regulated through \textit{de novo} synthesis from cholesterol in the liver, efficient reuptake of BAs in the distal small intestine, and feedback regulation of hepatic \textit{de novo} synthesis through the BA/farnesoid X receptor (FXR)/fibroblast growth factor (FGF)\(^19\) (Fgf15 in mice)–signaling pathway. BAs act through FXR in ileal enterocytes and induce expression of the hormone, FGF19/15, which subsequently binds to the receptor complex, FGFR4-β-Klotho, on the surface of hepatocytes, resulting in repression of cholesterol 7α-hydroxylase (Cyp7a1)–mediated BA synthesis. Less than 5% of the circulating BA pool typically escapes reabsorption and is eliminated in feces on a daily basis. The majority of BAs are absorbed by active transport in the terminal ileum, mediated by the \textit{ABCB4}-encoded apical sodium-dependent bile acid transporter (ASBT). The compound, SC-435, is a specific, nonabsorbable inhibitor of ASBT and BA reuptake in the distal ileum.\(^13\) Consequently, we hypothesized that inhibiting the enterohpatic circulation of BAs by pharmacologically blocking ASBT with SC-435 would diminish the BA pool size and attenuate progression of sclerosing cholangitis in the mdr2\(^{-/-}\) mouse model of chronic cholestasis.

\textbf{Material and Methods}

\textbf{Mice.} Mdr2\(^{-/-}\) mice in BALB/cJ background were a generous gift from Prof. Lammert (Homburg University, Homburg, Germany) and wild-type (WT) BALB/cJ mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were bred in-house and
kept in conventional conditions. Thirty-day-old, female mdr2+/− and WT mice were treated with 0.006% SC-435 admixed to high-fat breeder chow (Mouse diet 5020 with 9% fat; Cincinnati Lab Supply, Cincinnati, OH) for 14 days until time of harvest, providing approximately 11 mg/kg/day of the compound. Control mice were treated under identical conditions, but received Lab 5020 chow without the compound. All protocols were approved by the animal care and use committee of the Cincinnati Children’s Research Foundation (Cincinnati, OH).

**Fecal BA.** Mice were single-housed on raised, wire-bottom cages for 48 hours before collection of all fecal material, which was rehydrated with water equal to 2 times the total fecal weight, overnight at 4°C. BAs were extracted with 50% (v/v) t-butanol/water (v/v) for 45 minutes at 37°C, then centrifuged at 2,000g for 15 minutes. BA concentration was determined using the Diazyme Total Bile Acids Universal (enzymatic; Diazyme Laboratories, Poway, CA) kit and read on a BioTek Synergy H1 Plate Reader (Bio-Tek Instruments Inc, Winooski, VT).

**Plasma and Serum Biochemistries.** Plasma total bilirubin and alanine aminotransferase (ALT) levels were determined as reported before. Serum alkaline phosphatase (ALP) concentration was measured using DISCRETPAK reagents from Catachem (C174-0C; Catachem, Inc., Bridgeport, CT).

**Analysis of BA, PL, Cholesterol and C4 Concentrations by Liquid Chromatography/Mass Spectrometry.** Quantitative analysis of BA, PL, and cholesterol was carried out using a Waters API triple-quadrupole mass spectrometer interfaced with an Aquity UPLC system (Aquity, Milford, MA). Serum and biliary BAs were extracted on a reverse-phase/solid-phase cartridge. Liver tissue was exhaustively extracted with organic solvents before aqueous dilution and solid-phase extraction. BAs in biological samples were quantified against curves constructed for the 15 most common endogenous BAs based on a stable-isotope dilution analysis with single-ion recording mass spectrometry (MS). PLs were extracted by the Folch procedure and quantified using phosphatidylcholine (PC) standards based on MS with multiple reactions monitoring function. Total PL was calculated based on the summation of 13 major PC species detected in serum or bile. Bile samples were treated with alkaline hydrolysis to measure the total cholesterol concentrations by an isotope dilution atmospheric pressure chemical ionization liquid chromatography MS method. Plasma 7α-hydroxy-4-cholesten-3-one ([3H2, 25,26,26,27,27.27]-7α-hydroxy-4-cholesten-3-one; D=4) as the internal standard.

**Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was isolated from liver tissue, reverse transcribed, and messenger RNA (mRNA) concentration of TNFα was determined by SYBR green quantitative polymerase chain reaction (qPCR). SMA (Acta2) and TIMP1 genes were amplified using TaqMan Universal Master Mix II, with the probes, Mm01546133_m1 and Mm00441818_m1, respectively, and run on an Applied Biosystems 7900H Fast Real Time PCR System (Applied Biosystems, Foster City, CA). The ΔΔCt method was used for computation of relative mRNA concentrations, and data were normalized to mouse HPRT expression.

**Liver Histology, Ultrastructure, and Ki67 Immunohistochemistry.** Liver histology was blindly assessed by S.K.S. for inflammation, necrosis, bile duct proliferation, and fibrosis on a 1-4+ scale, as previously used in a model of obstructive cholangiopathy. Masson’s trichrome-stained liver slides were scanned using a Aperio AT2 Digital Whole Slide scanner (Leica Microsystems Inc., Buffalo Grove, IL) at a resolution of 50,000 pixels per inch (0.5 μm per pixel). Pixels were classified as fibrosis (blue), liver cells (red), or background (white) by means of an image analysis algorithm, as described previously. For electron microscopy, liver tissue was fixed in 3% buffered glutaraldehyde, subjected to ultrathin sectioning, and examined with a Hitachi H7650 transmission electron microscope (Hitachi, Tokyo, Japan). Immunohistochemistry (IHC) for Ki67 was performed on paraffin-embedded liver sections, using anti-mKi67 (clone SP6) after heat antigen retrieval with 1 M of Na citrate (pH = 6).

**RNA Sequences and ToppGene Unbiased Pathway Analysis.** Total hepatic RNA was isolated from the caudate liver lobe. RNA sequencing (RNA-seq) libraries were prepared with the Illumina TruSeq RNA preparation kit and sequenced on the Illumina Hi-Seq 2000 (Illumina, San Diego, CA). Sequences were aligned to the reference genome with TopHat and processed with Cufflinks, which quantifies each transcript in each sample using reference annotations produced by the University of California Santa Cruz UCSC. Differentially expressed genes with a fold change of >2.0 and P < 0.05 between SC-435-treated and control animals were submitted to pathway enrichment analysis with ToppFun application from the ToppGene Suite, which uses unbiased methods to assess pathway enrichment. Raw and normalized data are accessible through the National Center for Biotechnology Information’s

**Flow Cytometry.** Livers were perfused with 10 mL of RPMI (Gibco, Grand Island, NY) with 1 μg/mL of collagenase D (Roche Diagnostics, Indianapolis, IN) before mechanical disruption with a Miltenyi Biotec disintegrator (Miltenyi Biotec, Cambridge, MA). Single-cell preparation and staining was performed, as described before,14 using the following antibodies from BioLegend (anti-mCD3-Brilliant violet 421, anti-mCD19-Brilliant violet 605; BioLegend, San Diego, CA), eBioscience (anti-mF4/80-PE, anti-mCD11b-PerCP-Cy5.5, anti-mGR1-APC-eFluor 780; eBioscience, San Diego, CA), and BD-Pharmingen (anti-mLy6C-PE-Cy7; BD-Pharmingen, Bedford, MA). Data were acquired on a BD Canto III and analyzed using FlowJo software (version 7.6.5; Tree Star, Inc., Ashland, OR).

**Statistical Analysis.** Values are expressed as mean ± standard error of the mean and statistical significance was determined by unpaired t test, with a significance set at P < 0.05. One-way analysis of variance (ANOVA) with post-hoc Tukey’s multiple comparison test was used to assess statistical significance between more than two groups.

**Results**

**SC-435 Increased Fecal BA Excretion and Lowered Serum BA Levels.** Compared to age- and gender-matched control mice receiving chow without the compound, 48-hour fecal BA excretion after 14 days of treatment with SC-435 was increased by 8.6-fold in mdr2−/− and by 3.2-fold in mdr2+/+ mice (Fig. 1A). Increased fecal BA losses were associated with reduction of total BA concentration in liver tissue by 65% (Fig. 1B) and a decrease in serum total BA levels by 98.9% in SC-435-treated compared to control mdr2−/− mice (Fig. 1C). The major BA species, taurocholic (TCA) and tauro-β-muricholic acid (TMCA), which constituted 95.9% of serum BA in control mdr2−/− mice, were both significantly reduced after treatment with SC-435 (Fig. 1D). Importantly, the more hydrophobic BAs, taurochenodeoxycholate (TCDDCA) and taurodeoxycholate (TDCA), which were significantly elevated in control mdr2−/− compared to mdr2+/+ mice (1,260- and 13-fold, respectively) were significantly reduced by treatment with SC-435.

**SC-435 Exerted Anticholestatic, Anti-Inflammatory, and Antifibrogenic Effects.** Altered BA homeostasis was associated with profound changes in the sclerosing cholangitis phenotype in mdr2−/− mice. Whereas control mdr2−/− mice started to lose weight after day 7 of the treatment period, those treated with SC-435 maintained weight in a similar manner to mdr2+/+ controls (Fig. 2A). Furthermore, biomarkers of hepatocellular injury (plasma ALT concentration) and cholestasis (plasma total bilirubin and serum ALP concentrations), which were all increased in untreated mdr2−/− mice compared to mdr2+/+ controls, were reduced in SC-435-treated mdr2−/− mice by 86%, 93%, and 55%, respectively (Fig. 2B).
Consistent with the change in plasma biochemistries, liver histology of sclerosing cholangitis featuring periportal inflammation and fibrosis, as depicted in representative hematoxylin and eosin (H&E)- and trichrome-stained sections in Fig. 3A, was significantly improved by SC-435 treatment. Review of histology, using a previously validated sclerosing cholangitis scoring system, revealed reduced scores for periportal inflammation, bile duct proliferation, necrosis, and fibrosis in SC-435-treated KO mice compared to controls (Fig. 3B). Reduction in fibrosis score was consistent with results from Aperio-based image analysis of trichrome-stained liver sections. Percentage fibrosis of total liver tissue was reduced from 4.7 to 1.6 with SC-435 treatment (Fig. 3C). Histological data were corroborated by targeted gene expression studies for signature genes of sclerosing-cholangitis–associated hepatic inflammation (TNFα) and fibrosis (SMA and TIMP1). mRNA expressions for TNFα, SMA, and TIMP1 were significantly down-regulated in SC-435 versus control mdr2−/− mice (Fig. 3D).

**SC-435 Treatment Upregulated Genes of BA Synthesis and Hepatoprotective Pathways, and Down-Regulated Proinflammatory Genes.** To further probe the mechanism of action by which disruption of the enterohepatic circulation of BAs blocked progression of sclerosing cholangitis in mdr2−/− mice, we performed RNA-seq studies on total liver RNA purified from SC-435-treated and control mice of both genotypes. There were 497 genes that were >2-fold up-regulated by SC-435 treatment in mdr2−/− mice, of which 32 (6.4%) were also up-regulated in SC-435-treated mdr2+/+ mice, when compared to the corresponding untreated genotype. In contrast, 332 genes were down-regulated in mdr2−/− mice, of which only three (0.9%) were also down-regulated in mdr2+/+ mice (Fig. 4A). In an unbiased analysis, many genes related to cholesterol and BA metabolism were significantly up-regulated by SC-435 treatment, whereas genes in pathways related to leukocyte recruitment and function were generally down-regulated when compared to untreated mdr2−/− mice (Fig. 4B). Most notably, Cyp7a1 and Hmgcr, encoding the rate-limiting enzymes of BA and cholesterol synthesis, respectively, were up-regulated by SC-435 in mice of both genotypes. Several genes encoding proteins previously implicated in protection from cholestatic injury were also up-regulated in SC-435-treated mdr2−/− mice. For instance, these included Ppara encoding the transcription factor Pparα, Car1/Car3/Car5a encoding isoforms of carbonic anhydrase, Aqp8 encoding the water channel aquaporin 8, and Igf1 encoding insulin growth factor 1 (Fig 4C). Among the down-regulated genes in treated mdr2−/− mice, a few were related to BA and cholesterol metabolism, including Nr0b2/SHP, the downstream messenger molecule of Fxr, and Abcb1a/Mdr1 and Abcg5/Abcg5 encoding canalicular transporters for BA and cholesterol, respectively. The majority of genes down-regulated after SC-435 treatment were proinflammatory and fibrogenic. The highest fold changes in differential gene expression were observed for the genes Ccl2, Cxcl1, and Lcn2 (Fig. 4C). Tgfb1 mRNA expression was reduced by 97% in SC-435-treated versus control mdr2−/− mice (P = 0.001). Expression data for candidate genes, which are transcriptionally regulated by BAs through Fxr-signaling, are listed in the Supporting Table 1. For instance, SC-435 treatment was associated with increased hepatic expression of Fgf15, but did not significantly alter expression of Abcb11/Bsep or Abcc2/Mrp2 in mdr2−/− mice.

**SC-435 Treatment Profoundly Altered Bile Composition.** Consistent with hepatic up-regulation of Cyp7a1, the rate-limiting enzyme of BA synthesis, upon treatment with SC-435, plasma levels of the sterol...
intermediate, C4, a surrogate marker of BA synthesis, were increased by 3-fold in mdr2−/− and by 10-fold in mdr2+/+ mice (Fig. 5A). We next examined whether this increased synthesis resulted in higher concentrations of BA in bile, the compartment in which low-phospholipid, nonmicellar bile directly injures bile duct epithelium.9,10 As expected, biliary PC concentration in mdr2−/− mice was reduced to 0.29% of concentrations found in bile of mdr2+/+ controls and did not significantly change upon treatment with SC-435 (Fig. 5B). However, SC-435 treatment reduced significantly the biliary concentrations of BA and cholesterol by 98.8 and 97.6%, respectively, in these mice. Therefore, despite persistence of low biliary phospholipid levels, the biliary PC/BA ratio was markedly increased in SC-435-treated mdr2−/− mice (mean, 0.05 ± 0.02), although still lower than WT control mice (mean, 0.28 ± 0.07), and the average coefficient of 0.30 reported for most mammals under physiological conditions.22 Using the Wang and Carey phase diagram to describe the influence of biliary composition on cholesterol crystallization,23,24 control mdr2−/− mice plot in the 2 phase (liquid crystal) zone A (predicting precipitation of cholesterol), whereas SC-435-treated mdr2−/− mice, as with WT animals, plot in the 1 phase micellar zone, predicting micellar solubilization of cholesterol. Collectively, these findings suggest that SC-435 attenuates progression of sclerosing cholangitis in mdr2−/− mice by altering bile composition to reduce the “toxicity” of nonmicellar bile. Therefore, we next examined evidence for diminished injury to cholangiocytes in SC-435-treated mdr2−/− mice.

**SC-435 Treatment Reduced Cholangiocyte Injury.** Typically, acute injury to cholangiocytes is followed by regeneration, which can be labeled by the proliferation marker, Ki67.25 Proliferation of cholangiocytes was significantly increased in control mdr2−/−, compared
to mdr2+/+, mice by IHC for Ki67. Reduced frequency of Ki67+ cells among cholangiocytes of interlobular bile ducts in SC-435-treated versus control mdr2−/− mice suggested reduced bile duct epithelial injury in these animals (Fig. 6A). Previous studies in mdr2−/− mice revealed that injury to cholangiocytes allows leaking of BA into the periductal space, which was associated with disruption in basement membranes surrounding the interlobular bile ducts. Marked improvement in ultrastructure of basement membrane of the interlobular bile duct was observed in SC-435-treated mdr2−/− mice. In treated mice, 70% of interlobular bile ducts had continuous and intact basement membrane, as opposed to only 40% in untreated mdr2−/− mice. In diseased bile ducts, the basement membrane was discontinuous and irregular to being absent in some instances (n = 10 bile ducts/group; Fig. 6B).

SC-435 Treatment Reduced Recruitment of Kupffer Cells and Neutrophils to the Liver and Promoted Expansion of Anti-Inflammatory Monocytes. Diminished leukocyte recruitment and function was an additional mechanism of action of SC-435, indicated by the global hepatic gene expression studies. A significant reduction in frequencies of F4/80+CD11b+ Kupffer cells (KCs) and Gr1+CD11b+ neutrophils, respectively, was observed by flow cytometry (FCM) in SC-435-treated
versus control mdr2–/– mice. Frequency of F4/80-CD11b
monocytes in mdr2–/– mice was increased
upon SC-435 treatment, attributable to a rise in the
anti-inflammatory subset of Ly6C– monocytes in liver,
whereas the proportion of proinflammatory and -fibro-
genic Ly6C+ monocytes was reduced (Fig. 7).

Discussion

We have demonstrated that SC-435, a small-molecule
inhibitor of ASBT, increased fecal BA excretion, leading
to a dramatic reduction of liver and serum concentra-
tions of BAs and biomarkers of hepatocellular and cho-
lestatic injury in female mdr2–/– mice. This response
was rapid, occurring within 14 days of treatment. Com-
pared to control mice, body mass wasting was pre-
vented, and progression of hepatic inflammation and
fibrosis was attenuated. The mechanisms of action of
SC-435 may be pleiotropic, and some are supported by
our data, including a (1) reduction of the total BA pool
size and circulating hydrophobic BA, (2) decrease of bili-
ary BA and cholesterol concentrations rendering bile
less hepatotoxic, and (3) reduction of cholestasis-
associated inflammatory responses.

SC-435 has been reported to enhance fecal BA excre-
tion in noncholestatic rodents. 26 Although ASBT is
expressed in the terminal ileum of mdr2–/– in similar
concentration to that of mdr2+/+ mice,27 it was
unknown whether enterohepatic reuptake of BA is
depressed under cholestatic conditions in these mice,
thus conferring resistance to treatment with an ASBT
inhibitor. Under our experimental conditions, SC-435
treatment resulted in an 8-fold increase in fecal BA
excretion, with a concomitant 90-fold reduction of
serum BA concentrations and reduction of plasma ALT,
total bilirubin, and serum ALP levels. Furthermore,
fibrosis was diminished, as assessed by image analysis of
trichrome-stained liver sections.

We propose several mechanisms of action by which
treatment with SC-435 exerts its anticholestatic, -inflam-
matory, and fibrogenic effects in the mdr2–/– mouse
model of chronic cholestasis. Based on the physicochem-
ical properties of SC-435 having minimal intestinal
absorption, we focused our investigations on SC-435-
mediated regulation of BA synthesis and composition of
the hepatic and biliary pool and its downstream conse-
quences. SC-435 treatment rapidly lowered serum total
and hydrophobic BA concentrations. Hydrophobic BAs
have previously been linked to pathogenesis of sclerosing
cholangitis, especially in female mdr2–/– mice,28 and
were shown to stimulate hepatocyte apoptosis through
nonspecific detergent effects,29 activation of death recep-
tors,30 and induction of oxidative damage causing
mitochondrial dysfunction.31 Which of these pathways
is predominant in mdr2–/– mice is unknown.
Global hepatic gene expression studies provided further insights into effects of SC-435 treatment on putative mechanisms of action. Consistent with reduction of the BA pool size resulting in a state of low transhepatic BA flow, differential gene expression suggested decreased activation of Fxr, that is, the expression of Shp, a repressor of BA synthesis pathways, was diminished resulting in increased expression of the rate-limiting enzyme (\textit{Cyp7a1}) for BA synthesis. Importantly, genes encoding proteins with potentially protective properties during cholestasis were also up-regulated in SC-435-treated mdr2–/– mice, including \textit{Ppara}, which may promote alternative BA elimination/detoxification and reduce inflammation,\textsuperscript{3} \textit{Car3} and \textit{Aqp8}, involved in bicarbonate-rich hydrocholeresis reducing the toxic effects of hydrophobic BA on biliary epithelium,\textsuperscript{32,33} and \textit{Igf1}, reported to exert antifibrogenic properties in cholestasis.\textsuperscript{12} In addition, of the 332 down-regulated genes in SC-435-treated mdr2–/– mice, 95 play a role in inflammation and fibrosis, most prominently \textit{Ccl2}/Mcp1 and \textit{Lcn2}/lipocalin 2. Mcp1 is expressed by bile duct epithelial cells upon exposure to BA\textsuperscript{34} and has been linked to activation of hepatic stellate cells in biliary atresia and cystic fibrosis.\textsuperscript{35} Lipocalin 2 is secreted by hepatocytes and involved in leucocyte recruitment and was found to be a sensitive biomarker of liver injury.\textsuperscript{36}

Many changes in the hepatic gene expression profile appear consistent with what would be expected to occur when severe cholestasis is alleviated, including Fxr inactivation and reduction of proinflammatory gene expression, and might be operative in any cholestatic disorder in which enterohepatic BA uptake is disrupted. Other results of our studies may have more specific bearing for the pathogenesis of sclerosing cholangitis in mdr2–/– mice, in particular, our findings on the changes in bile composition. We propose that reduction of biliary BA and cholesterol concentrations, which are determinants of bile solubility and cholangiocyte injury from precipitated bile microcrystals in mdr2–/– mice,\textsuperscript{9,10} were critical for attenuation of the sclerosing cholangitis phenotype. We speculate that increased cholehepatic shunting, reported to be the result of bile duct proliferation in mdr2–/– mice,\textsuperscript{27} and down-regulation of genes encoding the canalicular BA and cholesterol transporters, Mdr1 and Abcg5, respectively, contribute to this change in bile
composition despite activation of de novo hepatic BA synthesis after treatment with SC-435. Our findings of decreased frequency of Ki67-expressing cholangiocytes and improved liver ultrastructure with intact basement membranes surrounding interlobular bile ducts may indicate reduced BA leakage and support this mechanism of action of SC-435 in mdr2−/− mice.

Finally, we validated results of gene expression studies, which revealed decreased hepatic expression of chemokines recruiting leukocytes to the liver by showing that SC-435 treatment decreased the frequency of KCs, neutrophils, and inflammatory monocytes accompanied by expansion of anti-inflammatory Ly6C− monocytes. Interestingly, this pattern of changes in hepatic leukocyte composition has previously been reported during treatment with a Tgr5 receptor agonist in a model of steatohepatitis.37 This raises the possibility that some of the effects of SC-435 on hepatic inflammation are mediated by Tgr5, as previously described for metabolic effects observed in treatment with anionic resins, an alternative method of disrupting enterohepatic circulation of BA. These studies suggested that binding of BA by resins resulted in spillover of BA into the colon, which led to Tgr5-dependent intestinal release of glucagon-like peptide 1 (Glp-1) exerting glycemic control in the liver.38 Whether ASBT inhibitors affect Tgr5
signaling in the colon and modulate secretion of GLP-1 requires further investigations.

In summary, this preclinical study demonstrates the potential of pharmacological inhibition of ASBT in halting progression of fibrosing cholangiopathies during the early phase of the disease process and identifies several mechanisms of action, which may operate synergistically.

References


Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27973/suppinfo.