Therapeutic modulation of the bile acid pool by Cyp8b1 knockdown protects against nonalcoholic fatty liver disease in mice

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ABSTRACT: Bile acids (BAs) are surfactant molecules that regulate the intestinal absorption of lipids. Thus, the modulation of BAs represents a potential therapy for nonalcoholic fatty liver disease (NAFLD), which is characterized by hepatic accumulation of fat and is a major cause of liver disease worldwide. Cyp8b1 is a critical modulator of the hydrophobicity index of the BA pool. As a therapeutic proof of concept, we aimed to determine the impact of Cyp8b1 inhibition in vivo on BA pool composition and as protection against NAFLD. Inhibition of Cyp8b1 expression in mice led to a remodeling of the BA pool, which altered its signaling properties and decreased intestinal fat absorption. In a model of cholesterol-induced NAFLD, Cyp8b1 knockdown significantly decreased steatosis and hepatic lipid content, which has been associated with an increase in fecal lipid and BA excretion. Moreover, inhibition of Cyp8b1 not only decreased hepatic lipid accumulation, but also resulted in the clearance of previously accumulated hepatic cholesterol, which led to a regression in hepatic steatosis. Taken together, our data demonstrate that Cyp8b1 inhibition is a viable therapeutic target of crucial interest for metabolic diseases, such as NAFLD.—Chevre, R., Trigueros-Motos, L., Castaño, D., Chua, T., Corliano, M., Patankar, J. V., Sng, L., Sim, L., Juin, T. L., Carissimo, G., Ng, L. F. P., Yi, C. N. J., Eliathamby, C. C., Groen, A. K., Hayden, M. R., Singaraja, R. R. Therapeutic modulation of the bile acid pool by Cyp8b1 knockdown protects against nonalcoholic fatty liver disease in mice. FASEB J. 32, 3792–3802 (2018). www.fasebj.org

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Nonalcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids in hepatocytes (hepatic steatosis) (1). Twenty to forty percent of patients with NAFLD are affected by the clinically significant form, nonalcoholic steatohepatitis (NASH), which is characterized by inflammatory changes and fibrosis in the liver (1, 2). The prevalence of NAFLD is dramatically increasing worldwide, and NASH-related cirrhosis is expected to become the leading cause of liver transplantation in many developed countries in the near future (3, 4); however, no effective therapies exist for the treatment of NAFLD or NASH.

Bile acids (BAs) are the primary products of cholesterol catabolism. The hydrophilic/lipophilic balance of the BA pool is tightly controlled by different enzymes that modulate BA synthesis in the liver, as well as by the gut microbiome, and is the major regulator of cholesterol homeostasis and intestinal absorption of fat. Immediate products of BA synthesis pathways in humans are the

ABBREVIATIONS: BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; FXR, farnesoid X receptor; HCD, high-cholesterol diet; KD, knockdown; LDL-C, LDL cholesterol; MCA, muricholic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ORO, Oil-Red-O; siRNA, small interfering RNA

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primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), and the balance between their relative amounts determines the overall hydrophobicity of the BA pool (5, 6). The major regulator of the balance between CA and CDCA is sterol 12α-hydroxylase (CYP8B1), which adds a 12α-hydroxyl group to 7α-hydroxy-4-cholesten-3-one, thereby determining the relative amounts of 12α- and non-12α-hydroxylated BAs (7). In contrast to humans, CDCA in mice is largely converted to the more hydrophilic muricholic acid (MCA). Conjugation of primary BAs lowers their pKₐ and increases their solubility (8). In the gut, the microbiome also plays a critical role in BA pool remodeling by removing both conjugated moieties and hydroxyls to form more hydrophobic secondary BAs. Overall, these processes give rise to a complex assembly of surfactants with different hydrophobicity indexes and different abilities to absorb fat (8, 9). In addition to acting as surfactants, BAs also function as signaling molecules and are the physiologic ligands for nuclear farnesoid X receptor (FXR) (10–12), and the G protein–coupled BA receptor-1 (also known as TGR5) (13–15). As different BAs activate their receptors with different affinities (16), the complexity of the BA pool and the relative amounts of the different BAs determine the signaling properties of the BA pool.

In the last decades, BA sequestrants have been developed to modify the solubilization properties of the BA pool, and have demonstrated their efficacy in metabolic diseases and in lowering LDL cholesterol (LDL-C) (17). Moreover, BA receptors regulate the signaling pathways that are involved in myriad physiologic processes that are impaired in cardiometabolic disorders, such as lipid metabolism (18), glucose homeostasis (18, 19), energy expenditure (20), and hepatic inflammation (21). In this context, BAs emerged as potential therapeutic targets for the treatment of NAFLD (16, 22), and obeticholic acid (6-ethyl-CDCA), a semisynthetic FXR agonist, is currently developed to modify the solubilization properties of the BA pool.

CYP8B1 is responsible for the overall hydrophobicity index of the BA pool, regardless of the secondary modifications of BAs that take place within the intestinal tract. In BA feeding experiments in mice, overall hydrophilic/lipophilic balance of the bile salt pool, and not the individual fed BAs, per se, is the critical determinant of cholesterol absorption from the small intestine (9). Of note, in this study, feeding of 12α-BAs increased the absorption of cholesterol compared with that of non-12α-BAs (9). More recently, increased plasma levels of 12α-hydroxylated BAs have been associated with human insulin resistance (25), and insulin regulates hepatic lipid metabolism via modulation of BA levels (26). Moreover, abolished CA synthesis in different Cyp8b1−/− mouse models increased the global BA pool (27), reduced atherosclerosis (28), and improved insulin sensitivity (29). In addition, mouse and human studies demonstrated a critical role for Cyp8b1 and 12α-hydroxylated BAs in the regulation of lipid metabolism and fatty liver development (30–33). Of interest, the ratio of 12α- and non-12α-hydroxylated BAs is increased in patients with NAFLD (33), which suggests that a decrease in CYP8B1 may be associated with protection against NAFLD.

Recent advances have demonstrated the potential of RNAi targeting of the liver as a treatment strategy for metabolic diseases, such as NAFLD (34–36). Synthetic nanovectors are lipid- or polymer-based nanoscale particles that are used to deliver hydrophobic drugs, proteins, and nucleic acids—pDNA, RNA or small interfering RNA (siRNA)—in vivo (37). Gene-based methods have suffered from the insufficient control of dosage, risk of genomic integration, and antiviral immune responses. Modified siRNAs or antisense oligonucleotides in which one or more nucleotides are modified to increase their stealth properties represent an alternative therapeutic platform with great potential (35).

In this study, we aimed to establish a therapeutic proof of concept for the treatment of NAFLD by targeting hepatic Cyp8b1 by using nonviral delivery of modified siRNA in vivo to modulate the BA pool and increase its metabolically protective biologic properties. We achieved effective, long-term knockdown (KD) of hepatic Cyp8b1, which led to a profound remodeling of the BA pool. We demonstrate that this remodeling dramatically reduces lipid accumulation in the liver and inhibits NAFLD.

**MATERIALS AND METHODS**

**Animals and diets**

Female C57BL/6N mice age 2–5 mo were used in this study. Animals had ad libitum access to water and were fed with a standard chow diet (Altromin 1324 Mod) or a high-cholesterol diet (HCD; 0.5%; Harlan Diets, Madison, WI, USA) for 10–20 wk, beginning at age 2 mo. Mice were fed for 5 h before any procedures. Plasma for all experiments was isolated from blood that was drawn from the saphenous vein in EDTA-coated capillary tubes. Feces were collected continuously over 72 h. All experiments were approved by the Institutional Animal Care and Use Committee at the Agency for Science, Technology, and Research.

**In vitro siRNA screening**

HEK293T cells were maintained at 37°C in 5% CO₂ and DMEM (Sigma-Aldrich, St. Louis, MO, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare, Chalfont St. Giles, United Kingdom), 4 mM glutamine, and 1% penicillin/streptomycin. Twenty-four hours after seeding, cells were transiently cotransfected with a plasmid that encoded murine Cyp8b1 and 6 different siRNAs (Origene, Rockville, MD, USA; and Thermo Fisher Scientific, Waltham, MA, USA) by using Lipofectamine 2000 according to the manufacturer’s instructions (Thermo Fisher Scientific).

**In vivo inhibition of Cyp8b1**

Cyp8b1 siRNA (sense: CGGCACUAAGCGUUGCUUGUAtt, antisense: UAACACACGCUGUAACGC) or negative control siRNA (In Vivo Ready siRNA; Thermo Fisher Scientific) and Invivofectamine 3.0 (Thermo Fisher Scientific) complexes were prepared according to manufacturer instructions. Either PBS, control or Cyp8b1 siRNA-Invivofectamine solution (dose, 0.5 or 1...
mg siRNA/kg mouse) was administered via tail vein injection to wild-type mice for the indicated period of time.

**Gene expression analyses**

Total RNA was isolated from tissue homogenates by using an RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was generated by using random hexamers and a Superscript III First-Strand Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed by using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in an ABI QuantStudio 6 Flex Real-Time System. Reactions were performed in technical triplicates by using the following primers: mCyp8b1 (for in vivo studies): (forward) 5'-ATCGCTTAAAGCCGTGTCACG-3'; (reverse) 5'-AGCTGGGGAAGAGAAGGATGCC-3', mCyp7a1 (for cotransfection studies): (forward) 5'-CCTCTGAGAGGGTTTTTCG-3', (reverse) 5'-GGACCCGTAAGACTCACATCCC-3', mCyp7a1: (forward) 5'-AGCAGATTGCAATCAAGGAC-3', (reverse) 5'-GTACTGTGTGAGTCGCAACAG-3', mFgf6: (forward) 5'-CTCAATCCCTTGGAGGATC-3', (reverse) 5'-GGACAAAGGATCTTG-3', mSHP: (forward) 5'-TGGTCCCAAGGATGTACG-3'; (reverse) 5'-GCTCCAAAGCTGACAGTGTG-3', mActin: (forward) 5'-ATGCTCCCTCCGGGTATG-3', (reverse) 5'-CATAGAGCTTCTTGACCAAT-3'; mCbb3: (forward) 5'-TGTTGTGCTGTGGATCTGA-3', (reverse) 5'-TTGCTGTGGTAACTCAGAC-3'; mRpl37: (forward) 5'-GGAGTGGCAAGCTGATGCTG-3', (reverse) 5'-CTTGAACTCTGGTAGTGACT-3'; mFxr: (forward) 5'-ACTGCTGCAGATGACTG-3', (reverse) 5'-CATATATATCATGGATGG-3'; mCyp80a1 (for cotransfection studies): (forward) 5'-CTCGATCCGCTTTGGGAATTC-3', (reverse) 5'-CTCGATCCGCTTTGGGAATTC-3'; mHprt1: (forward) 5'-GGCTGCTTTCATTGCTTCAG-3', (reverse) 5'-GCTCCAAGACTTGACA-3'; mGapdh: (forward) 5'-AGTGTGAGTGGTCAATGG-3', (reverse) 5'-AGACTGAAGATCATGAACAG-3'; mRpl37: (forward) 5'-AGCAGAACTGGTACAG-3', (reverse) 5'-AGCAGAACTGGTACAG-3'; mRpl37.

**Western blot**

Mouse liver samples were homogenized in 300 μl RIPA buffer that was supplemented with complete protease inhibitors (Roche Diagnostics, Mannheim, Germany), and lysates were solubilized in Laemmli sample buffer. Proteins were resolved on a precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA), then transferred to PVDF membranes, blocked with 5% skim milk, and probed with anti-Cyp8b1 (clone P18; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-calnexin (Sigma-Aldrich) Abs overnight. Membranes were incubated in a rotor wheel for 1 h and spun at 15,000×g for 10 min. After incubation with horseradish peroxidase–conjugated secondary Abs (Santa Cruz Biotechnology), membranes were developed by using chemiluminescence (Bio-Rad). Relative densities of the proteins of interest vs. the housekeeping protein, calnexin, were calculated from the percent area under the curve generated by using ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Oral fat tolerance test**

Blood was drawn from unfed mice before and after—1, 2, and 4 h—intragastric gavage of 200 μl of corn oil. Plasma triglycerides were measured by using a LabAssay Triglyceride Kit (Wako Pure Chemicals, St. Louis, MO, USA).

**Liver histopathology**

For conventional light microscopy, livers from Cyp8b1 siRNA-treated or control mice were fixed in 4% paraformaldehyde for 36 h, deparaffinized, and embedded in paraffin. Sections (5 μm thick) were deparaffinized and subjected to routine hematoxylin and eosin staining or Sirius red staining. Lipid droplets in the liver were stained by using the lysosome Oil-Red-O (ORO) method. In brief, liver slices were washed in 1× PBS and fixed as previously described. Livers were sequentially cryoprotected in 15 and 30% sucrose until tissues sank. Thereafter, liver slices were included in labeled cryomolds using optimal cutting temperature compound (VWR Chemicals, Radnor, PA, USA) and cryosectioned in 12-μm sections (Micron HM525; Thermo Fisher Scientific). ORO stock solution was prepared by magnetic stirring of 2.5 g ORO (Sigma-Aldrich) in 400 ml of isopropanol for 2 h at room temperature. Bright-field images were captured with a light microscope (Axiovision; Zeiss, Oberkochen, Germany) at ×20 magnification, and lipid accumulation was quantified by use of ImageJ. For visualization of cholesterol crystals and macrophages, tissues, and slides were prepared according to the cryopreservation protocol previously described. For macrophages, antigen retrieval steaming in Dako Target Retrieval Solution S1699 (Dako, Carpinteria, CA, USA) was performed, and samples were blocked and stained overnight with the anti-F4/80 Ab (MCA497G, 1:400; Bio-Rad). Sections were then washed, incubated for 1 h with fluorescently tagged secondary Ab, and mounted with a water-soluble mounting medium after washing. Cholesterol crystals were detected by using polarized light signals as previously described (39).

**Flow cytometry**

Fifteen microliters of EDTA blood was washed in PBS before red blood cell lysis for 5 min on ice, followed by washing in PBS. Cells were stained for viability (Supplemental Table 1) for 30 min on ice, washed with PBS, and then blocked for 20 min in blocking buffer (1% rat serum; Sigma-Aldrich). Cells were then stained for 20 min with an Ab mix (Supplemental Table 1). After washing Abs with PBS, data were acquired on a LSR5 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data analysis was performed by using FlowJo (v.10 10.1; FlowJo, Ashland, OR, USA).

**Lipid extraction from liver and feces**

Mouse liver samples of feces (10 mg) were homogenized in 300 μl PBS, and lipids were extracted with equal volume of chloroform: isopropanol:NP-40 (7:11:0.1). After vortexing, samples were incubated in a rotor wheel for 1 h and spun at 15,000 g for 10 min. Organic phase was collected and air dried, and the lipid pellet was resuspended in isopropanol.

**BAs, lipids, and hepatic enzymes quantification**

BA levels in plasma were analyzed by LC-MS, and total BAs in feces were measured by using the Total Bile Acid Assay Kit (Cell Biolabs, San Diego, CA, USA) according to manufacturer instructions. Plasma alanine transaminase, aspartate transaminase, HDL cholesterol, LDL-C, and total cholesterol were measured by using Cobas c311 system (Roche Diagnostics). Liver and fecal content of cholesterol and triglycerides was measured from lipid extracts as previously described by using an Infinity Cholesterol Kit (Thermo Fisher Scientific) and LabAssay Triglyceride Kit, respectively.
Cox) test. Statistical significance was calculated by using Prism.

RESULTS

siRNA administration efficiently inhibits Cyp8b1 expression in vivo

Cyp8b1 is a critical modulator of the hydrophilic/lipophilic balance of the BA pool, which makes it a tightly regulated protein. Indeed, multiple signaling pathways and physiological conditions affect its expression, such as fasting, food intake, and circadian rhythms (40, 41), which allows levels of Cyp8b1 to double during the course of a single day (40). This high turnover of the Cyp8b1 protein is necessary to rapidly adapt the BA pool to changing energy intake and needs. Thus, inhibition of Cyp8b1 in vivo for therapeutic purposes by using transient methods may represent a challenge, as sustained inhibition may be required to achieve long-term alterations in the BA pool to impact metabolic phenotypes. To achieve long-term Cyp8b1 KD and BA pool remodeling, we first screened 6 different siRNAs that targeted Cyp8b1 (si1–6) in vitro, and identified 3 candidates that displayed similar levels of protein KD 48 h after transfection (Supplemental Fig. 1). Of the 3, we selected si1 (Fig. 1A and Supplemental Fig. 1) and evaluated its transfection efficiency in vivo by using Inivi- vofectamine 3.0, which promotes efficient KD in the liver without triggering significant inflammation (42). We administered either control or si1 siRNA (1 mg/kg i.v.) in wild-type mice, and found that hepatic Cyp8b1 protein expression was inhibited at d 2 postinjection (Fig. 1B).

To assess the kinetics of Cyp8b1 KD, we evaluated levels of Cyp8b1 RNA (Fig. 1C) and protein (Fig. 1D, E) at different time points and found that injection of 1 mg/kg si1 was sufficient to efficiently knock down Cyp8b1 protein by >75% for 4 d (Fig. 1D, E); however, as expected, Cyp8b1 expression rapidly increased to reach its initial mRNA and protein levels within 5 d postinjection (Fig. 1C–E). Cyp8b1+/− mice present milder BA pool remodeling compared with Cyp8b1−/− mice (32), which suggests that a high level of long-lasting KD is required to fully remodel the BA pool. To maintain long-term Cyp8b1 KD, we designed a multi-injection protocol, performing a second injection of 0.5 or 1 mg/kg si1 d 3 after the first injection of 1 mg/kg si1 (Fig. 2A–C). We observed dose-dependent KD in Cyp8b1 expression. The second administration of 0.5 mg/kg si1 allowed for a 50% KD at the protein level, but mRNA levels remained unaffected for up to 6 d after the first injection (Fig. 2A–C); however, the second injection of 1 mg/kg si1 achieved a 50% inhibition at the RNA level (Fig. 2A) and a >90% inhibition of Cyp8b1 at the protein level for up to 6 d after the first injection (Fig. 2B, C). Thus, we successfully designed a twice-per-week injection protocol that allowed for the maintenance of >90% KD of Cyp8b1 protein over longer-term studies.

Cyp8b1 silencing reduces 12α-hydroxylated BA production and promotes BA pool remodeling

As Cyp8b1 critically modulates the 12α-/non–12α-hydroxylated BA ratio, we first determined whether the 6-d KD of Cyp8b1 affected this balance. The injection protocol that used 1 mg/kg at d 0 and 3 led to a 59% reduction in plasma 12α-hydroxylated BAs (control: 0.831 μM; and Cyp8b1 siRNA: 0.341 μM) and a 69% decrease in the ratio of 12α-/non–12α-hydroxylated BA (Fig. 2D and Supplemental Fig. 2). Similar to Cyp8b1−/− mice (29), mice that were injected with Cyp8b1 siRNA demonstrated alterations in the composition of the BA pool (Supplemental Fig. 2), with significantly lower levels of CA and increased levels of MCAs. This indicates that our siRNA multi-injection protocol is sufficient to almost completely remodel the BA pool.

To evaluate the functional consequences of changes in the BA pool composition, we next assessed its signaling properties. Cyp7a1 is the rate-limiting enzyme in the BA synthetic pathway, and its expression is regulated in mice by the Fxr–Shp axis in the liver and by the intestinal secretion of Fgf15 (43, 44). Of interest, BA pool changes that are driven by Cyp8b1 KD were associated with a significant increase in Cyp7a1 transcript levels (Fig. 2E), recapitulating the phenotype described in Cyp8b1−/− mice (27). We found no significant changes in hepatic Fxr and Shp, and a mild increase in liver Fgfr4 (Fig. 2E), which is the hepatic receptor for Fgf15; however, we observed significant down-regulation in Shp, along with a trend toward a decrease in Fgf15 in the intestine (Fig. 2E).

Thus, our longer-term silencing of Cyp8b1 promoted the remodeling of the BA pool, along with changes in its signaling properties.

Cyp8b1 KD reduces intestinal lipid absorption and circulating LDL-C levels

To evaluate the potential therapeutic applicability of Cyp8b1 KD on metabolic diseases, such as NAFLD, we next investigated whether the silencing of Cyp8b1 affects the biologic properties of the BA pool. We performed oral fat tolerance testing after 6 d of silencing, and observed that Cyp8b1 KD significantly reduced the ability of the BA pool to facilitate the absorption of triglycerides in the digestive system (Fig. 3A). As long-term reductions in intestinal fat absorption may be beneficial against hepatic fat accumulation, we determined the impact of Cyp8b1 KD on fat absorption in a chronic setting by feeding mice an HCD for 15 wk, followed by an additional 3 wk of siRNA injections (2 injections/wk) during which mice continued on HCD. We observed a significant 16% increase in the fecal content of cholesterol in Cyp8b1 siRNA-treated mice (Fig. 3B), which suggested a decrease in intestinal cholesterol absorption. These changes were additionally potentiated by a significant increase in the dry weight of excreted feces.
which led to an increase in the overall excretion of cholesterol per day from 10.50 to 13.64 mg (P = 0.001). In addition, an increase in the expression of \( C_{yp7a1} \) should result in increased fecal BA content (27). Correlating with increased hepatic expression of \( C_{yp7a1} \) that was observed in Fig. 2E, fecal BA excretion was increased by 20% (Fig. 3D) in \( C_{yp8b1} \)-siRNA-treated mice. Of interest, these changes were associated with a decrease in plasma LDL-C levels (Fig. 3E), without a significant effect on HDL cholesterol levels (data not shown), which mimicked the LDL-C lowering effect of BA sequestrants in humans (17).

Taken together, our data indicate that siRNA-mediated \( C_{yp8b1} \) KD reduces fat absorption and increases both cholesterol catabolism and cholesterol excretion, which might lead to protection against NAFLD by lowering hepatic lipid accumulation.

**Therapeutic silencing of \( C_{yp8b1} \) inhibits NAFLD**

We next assessed \( C_{yp8b1} \) KD as a therapeutic proof of concept for the treatment of NAFLD. We fed mice a diet that was enriched in cholesterol (0.5% w/w; HCD) for 15 wk, followed by 4 additional wk of the HCD diet plus either control or si1 siRNA that targeted \( C_{yp8b1} \) (Fig. 4). We did not detect any changes in body weights after siRNA treatment (Supplemental Fig. 3). We observed a dramatic 52% reduction in liver steatosis, as assessed by hematoxylin and eosin staining (Fig. 4A). During tissue cryopreservation, liver slices from the siRNA-treated group sank in sucrose solution faster than did slices from the control group (median time decreased from 1015 to 137.5 min; \( P < 0.0001 \); Supplemental Fig. 4), which suggested a decrease in liver fat content. Indeed, livers that were treated with \( C_{yp8b1} \)-siRNA exhibited significantly lower liver cholesterol content (Fig. 4B) and lipid droplet area (Fig. 4C), which correlated with a significant decrease in liver triglycerides (Fig. 4D). At this time point and in this model, no significant fibrosis was observed in either the siRNA or control groups (Fig. 4E), and no changes in collagen could be detected between both groups (Fig. 4E). In addition, a significant decrease in the liver/body weight ratio was observed (Fig. 4F). We assessed the silencing of \( C_{yp8b1} \), as well as 12\( \alpha \) and non–12\( \alpha \)-BA levels and BA...
pool composition, at the end of the experiment, 3–4 d after mice received the final siRNA injection. Even after 1 mo of siRNA treatment, Cyp8b1 protein levels were still significantly decreased (by 55%) and resulted in significant remodeling of the BA pool (43% reduction in 12α/ non-12α-BA ratio; Supplemental Figs. 5 and 6, respectively). We detected no changes in hepatic macrophage content (Supplemental Fig. 7A), and mice did not present significant signs of general inflammation as assessed by quantification of circulating neutrophils (Supplemental Fig. 7B). Of interest, reduction in hepatic cholesterol was associated with significant inhibition in cholesterol crystal formation (Supplemental Fig. 7C and D), which has been associated with NASH in humans (39).

As Cyp8b1 siRNA-injected livers were almost devoid of steatosis, we hypothesized that this effect may not be exclusively attributed to a lack of de novo lipid accumulation, but may also be a result of increased removal of pre-established fat from the liver. To confirm our hypothesis, we compared hepatic steatosis before and after treatment with Cyp8b1 siRNA. We fed mice with HCD for 10 wk, and treated them for an additional 4 wk while on the HCD using our siRNA protocol. After 10 wk of HCD feeding, before beginning siRNA treatment, mice already presented nascent steatosis, which was visible by hematoxylin and eosin staining (Fig. 5A, B). Compared with mice before siRNA treatment, mice that were simultaneously treated with siRNA and HCD for an additional 4 wk presented significantly lower hepatic cholesterol content (Fig. 5C), which suggested that Cyp8b1 KD stimulated the clearance of preexisting cholesterol and was correlated with the increased cholesterol and BA excretion observed in Fig. 3B, D. In addition, compared with pre-siRNA treatment, a 35% decrease in hepatic steatosis was observed in the Cyp8b1 KD group (P = 0.026; Fig. 5B); however, in contrast to the significant reduction in hepatic cholesterol, no difference in hepatic triglycerides was observed after Cyp8b1 siRNA treatment compared with pre-siRNA treatment in the regression experiment (Supplemental Fig. 8). Plasma aspartate transaminase and alanine transaminase levels were within the normal range, similar to levels observed before treatment (Supplemental Fig. 9). Similarly, levels of plasma total and LDL-C were comparable to those observed before treatment (Supplemental Fig. 9). As a control, non-treated mice on HCD for an additional 4 wk continued...
Figure 3. Impact of Cyp8b1 KD on intestinal fat absorption and fecal lipid and BA excretion. A) Wild-type (WT) mice received intravenous injections of either Cyp8b1 siRNA (black circle) or control siRNA (white circle; 1 mg/kg) at d 0 and 3. Oral Fat gavage was performed at d 6 after the first injection, and fat absorption was assessed by measuring plasma triglyceride levels. Bars represent means ± SEM from n = 6 mice. B–E) WT mice fed HCD received injections (1 mg/kg, i.v.) of either Cyp8b1 siRNA (black bars) or control siRNA (white bars) twice a week. Fecal cholesterol content (B), weight of excreted feces (C), and fecal BA content (D) were assessed 3 wk after the first injection. E) Plasma LDL-C was assessed 4 wk after the first injection. Bars represent means ± SEM (n = 6–7 mice).

to accumulate liver cholesterol, liver triglycerides, and steatosis (Fig. 5 and Supplemental Fig. 8), which confirmed the results observed of our previous cohort (Fig. 4) that the silencing of Cyp8b1 significantly lowers hepatic fat accumulation. Thus, in our study, therapeutic silencing of Cyp8b1 not only lowered hepatic fat accumulation, but indeed reversed preexisting steatosis.

**DISCUSSION**

To determine the viability of long-term therapeutic inhibition of CYP8B1 in adulthood, and to assess whether this inhibition would result in beneficial effects on hepatic fat accumulation and NAFLD, driven by remodeling of the BA pool, we designed an RNAi-based therapeutic protocol to knock down Cyp8b1 in mice. We successfully achieved the long-term inhibition of hepatic Cyp8b1 in vivo, which resulted in remodeling of the BA pool and led to a significant and dramatic decrease in liver steatosis, driven by an increase in fecal excretion of lipids and BAs. Moreover, hepatic cholesterol content and steatosis were significantly lower after treatment compared with pre-treatment, which suggests that, in addition to reducing hepatic fat accumulation, Cyp8b1 inhibition resulted in regression of preexisting hepatic cholesterol. Our data demonstrate that long-term therapeutic inhibition of Cyp8b1 results in remodeling of the BA pool and may be an effective target to prevent and regress NAFLD.

Compared with the lifetime disruption of Cyp8b1 in Cyp8b1−/− mice, we determined in this study the dynamic effect of BA pool remodeling in adulthood over a short period of time. Our approach allowed us to evaluate the potential of transient modulation of Cyp8b1, which could be of interest for the development of therapeutics and for their translation to the clinic. Beyond a therapeutic proof of concept, our approach also allowed for investigation of the effects of the dynamic reshaping of the BA pool, independent of any compensatory mechanisms that occur in Cyp8b1−/− mice. Indeed, a recent study found that Cyp8b1+/- mice exhibit mild BA remodeling—significant changes in only HDCA and α-MCA, with no decrease, and in fact, a mild increase in CA—compared with their Cyp8b1+/- counterparts, associated with no changes in NAFLD development (32), which suggests that a robust reduction of Cyp8b1 is needed to sufficiently remodel the BA pool to observe a phenotype. Our findings emphasize that a reduction in CA levels by ~51% (Supplemental Fig. 6), as opposed to complete elimination of CA as in Cyp8b1−/− mice, is sufficient to decrease liver cholesterol content. By using our approach, we recapitulated the increase in hepatic Cyp7a1 expression and remodeling of the BA pool observed in adult Cyp8b1−/− mice after only 6 d of Cyp8b1 KD. The increase in Cyp7a1 expression, together with the increase in fecal BA excretion, suggest that BA synthesis and pool size may be increased in the face of Cyp8b1 KD. We quantified plasma BAs, which are a small fraction of the BA pool and may not accurately reflect the composition of the total BA pool. Quantification of total BA pool size is required to directly determine whether it is altered upon Cyp8b1 KD. Of note, BA pool remodeling directly affected fat absorption abilities, as observed in the oral fat tolerance test, also within 6 d of KD. Moreover, as BAs affect intestinal motility (45), the decrease in plasma triglycerides might also be a result of a decrease in intestinal transit time. In addition, our multi-injection protocol was robust enough to maintain these alterations during the 4 wk of Cyp8b1 inhibition and under chronic
HCD conditions. Indeed, we observed a 16% increase in cholesterol excretion after 3 wk of Cyp8b1 KD in mice that were fed HCD compared with the 50% increase in fecal neutral sterol content that resulted from the lifetime disruption of Cyp8b1 in Cyp8b1−/− mice (27). Despite the modest impact on intestinal fat absorption, Cyp8b1 inhibition reduced hepatic steatosis by 52%, which indicates that longer treatment protocols would have a higher impact on hepatic lipid accumulation and suggests great potential for the development of synthetic inhibitors that target Cyp8b1.

HCD feeding promotes NAFLD in rabbits (46). In addition, Cyp8b1−/− mice demonstrated reduced intestinal fat absorption when fed HCD (29). Thus, we used HCD (0.5% cholesterol) to elicit hepatic fat accumulation in our mouse model. By inhibiting Cyp8b1 in mice fed HCD, we conceptually aimed at having an impact at the level of dietary cholesterol absorption by modifying the hydrophilic/lipophilic balance of the BA pool (9), and at the level of cholesterol catabolism by increasing Cyp7a1 (27). In the context of NAFLD/NASH, establishing physiologically relevant animal models in vivo is challenging. Methionine-/choline-deficient diets promote fibrosis, but fail to recapitulate the complex metabolic environment of NAFLD (47). HCD, high-fat, and high-sucrose diets result in phenotypes that are more representative of the pathophysiology of NASH, but do not promote severe forms of liver fibrosis, at least in relatively short periods of time (47). Additional studies are required to validate the Cyp8b1-mediated reduction in fat accumulation in the liver as a versatile therapeutic strategy that targets different clinical stages of fatty liver disease.

Figure 4. Cyp8b1 KD lowers hepatic fat accumulation. Wild-type (WT) mice fed HCD for 15 wk received injections (1 mg/kg, i.v.) of either Cyp8b1 siRNA (black bars) or control siRNA (white bars) twice a week for an additional 4 wk. A) Liver steatosis was assessed by hematoxylin and eosin staining. B–D) Liver fat accumulation was evaluated by ORO staining (C) and by assessing cholesterol (B) and triglyceride (D) content in liver lipid extracts. E) Liver fibrosis was assessed by Sirius red staining for collagen fibers. F) Liver weights. Bars represent means ± SEM (n = 6–7 mice). Scale bars, 100 μm (A, E); 50 μm (C).
BA metabolism differs between humans and mice; thus, some consideration is needed before the direct translation of our findings to humans. One important difference between humans and mice is that, in humans, CDCA is a major primary BA, whereas in mice, CDCA is metabolized to MCA in the liver. CDCA is the most potent BA agonist for FXR, which suggests that, in humans, as opposed to mice, increased hepatic FXR signaling would be observed. In the intestine in humans, CDCA is modified to lithocholic acid, a less potent FXR agonist (16); however, in mice, tauro-β-MCA acts as an FXR antagonist (48). Thus, it is expected that increased FXR agonism would be observed in both the liver and the intestine of humans compared with mice. In our mouse model, we did not observe any changes in hepatic FXR signaling at the RNA level; however, we observed a decrease in intestinal Fxr activity as evidenced by a significant reduction in the Fxr-regulated genes, Shp and Fgf15 (27, 48), possibly caused by an increase in the Fxr antagonist, tauro-β-MCA. This decrease in intestinal Fxr activity correlates with the increase in Cyp7a1 in the liver. This is in agreement with previous studies that used tissue-specific Fxr knockout mice, which suggests that the intestinal Fxr–Fgf15 axis is more important for Cyp7a1 regulation compared with the liver Fxr–Shp axis (49).

Our study highlights the feasibility of nucleic acid therapy in targeting the BA pool. siRNA treatment for 4 wk led to a dramatic reduction in hepatic cholesterol accumulation and steatosis. The emergence of modified RNAs, siRNAs, or antisense oligonucleotides, along with new classes of delivery vectors, is promising (35). Their increased circulation time and enhanced targeting properties, which are associated with increased stability and decreased toxicity of new generations of nucleic acids, should improve the efficacy of therapies that target high-turnover proteins, such as CYP8B1. Indeed, a recent study that targeted proprotein convertase subtilisin/kexin type 9 in humans by using a single subcutaneous injection of siRNA conjugated to triantennary N-acetylgalactosamine carbohydrates to enhance hepatocyte targeting decreased plasma proprotein convertase subtilisin/kexin type 9 and LDL-C for 6 mo without promoting severe adverse effects (36).

Overall, we find here that the therapeutic inhibition of Cyp8b1 in mice results in remodeling of the BA pool, which alters both its signaling and physicochemical properties, and leads to the reduction and regression of NAFLD, modulated, in part, via a reduction in intestinal fat absorption. Thus, inhibition of CYP8B1 may be a valuable therapeutic target for the treatment of NAFLD.

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AUTHOR CONTRIBUTIONS

R. Chevre and L. Trigueros-Motos designed the study, designed, performed, and analyzed experiments, and...
made the manuscript; D. Castaño contributed to NAFLD study design, performed and analyzed experiments; T. Chua performed NAFLD sample analysis; L. Trigueros-Motos, M. Corliao, L. Sim, and L. Sng performed and quantified immunohistology experiments; J. V. Patankar contributed to experiment design and reviewed the manuscript; T. L. Juin helped with animal experiments; G. Carissimo and L. F. P. Ng performed and analyzed flow cytometry experiments; C. N. J. Yi and C. C. Eliathamby performed quantitative PCR analysis; A. K. Groen analyzed experiments and reviewed the manuscript; and M. R. Haylen and R. R. Singaraja designed experiments, supervised the study, and reviewed the manuscript.

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