AASLD The Liver Meeting®

ABCB4/MDR2 HAPLOINSUFFICIENCY PREDISPOSES MICE TO BILIARY INJURY AND SECONDARY CHOLESTASIS AND DEFINES A TRANSLATIONAL MODEL OF TOXIC BILE INDUCED HEPATOBILIARY INJURY IN HUMAN CHOLANGIOPATHY

E. L. Bell[1], J. K. Truong[1], Y. Jo[1], B. Reddy[1], A. Kolpak[1], J. P. Miller[1], A. S. Garfield[1,2], and R. O. Hughes[1]

[1]Rectify Pharmaceuticals, Cambridge, MA [2]Present address: Rhythm Pharmaceuticals, Boston, MA

RECTIFY PHARMA

Introduction

Bile composition is tightly controlled to buffer the toxicity of monomeric bile acids (BA) within bile ducts, preventing biliary system injury and the development of cholangiopathies and cholestatic disease. The phospholipid floppase function of Abcb4/Mdr2 protein is necessary to buffer BA as demonstrated by the fact that loss of function leads to progressive familial intrahepatic cholestasis type 3 (PFIC3). The *Abcb4/Mdr2* KO mouse, a genetic model of PFIC3, has altered bile composition and serves as a model of toxic bile induced bile duct injury with sclerosing cholangitis[1]. In rare hepatobiliary diseases a two-hit hypothesis is possible, suggesting that the presence of a first hit genetic variant sensitizes the system to injury from a second environmental hit, thereby promoting development of hepatobiliary disease. Here we characterize a two-hit model utilizing the toxic bile of the *Abcb4/Mdr2* heterozygous mouse.

Aims

To generate a more clinically relevant model for BA induced bile duct damage that could support drug discovery and translational development, we asked if the altered bile composition in Abcb4/Mdr2 heterozygous animals can serve as the first hit in a two-hit model of hepatobiliary disease. The goal was to identify conditions that increased both alkaline phosphatase (ALP) and serum BA preferentially in the heterozygous (HET) compared to wild type (WT). We chose to stress the animals with two different diets; 1) 0.5% Cholic Acid (CA) diet or 2) 15% fat, 1.25% Cholesterol (CL), and 0.5% CA (Lithogenic diet).

Methods

Mice: Eight- to ten-week-old female FVB.129P2-Abcb4tm1Bor/J or FVB/NJ (strain# 001800) mice from Jackson Laboratories were used in all experiments with N=5-12. Studies were performed at Physiogenex (Toulouse, France) or Pharmaron (Beijing, China) in accordance with the Guide for the Care and Use of Laboratory Animals and French law or the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Pharmaron, respectively. The lithogenic diet is pre-made at Research Diet (D12336) and CA was pre-made at Dyets (D220612).

Serum and bile: Serum total bile acids were measured by microplate enzyme cycling assay from Abcam (AB239702) or using an enzyme cycling assay from Sjodax with Cannon clinical analyzer. Bile BA were measured using the Sjodax assay and phospholipids were determined with the LabAssay™ Phospholipid kit from FujiFilm. ALT, AST, and ALP were determined using Pentra assay kits with the Horiba Pentra 400 Chemical analyzer.

Gene expression: Frozen liver tissue was ground into powder using liquid N_2 and then genomic DNA-free RNA was extracted using Rneasy Plus mini Kit (Qiagen). Isolated RNA was converted to cDNA with High-Capacity RNA-to-cDNA Kit (Invitrogen). Using TaqMan® FAST Gene Expression Master Mix, 0.5uL of cDNA was analyzed using Thermo Fisher taqman probe sets. Relative expression was calculated using the ΔΔCt method using the average of Gapdh, Gusb, and Tbp as housekeeping genes

Cytokines: Liver tissues were homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitors in TissueLyser II with steel beads at 30Hz for 3 minutes. Samples were mixed at 4°C for 30 minutes, centrifuged and protein concentration of lysate determined with BCA. 300µg of liver tissue were added to V-PLEX Proinflammatory panel 1 mouse (MSD K15048D) and V-PLEX Cytokine panel 1 mouse (MSD K15245D) and manufacturer instructions were followed.

Histology: Liver was formalin-fixed for 24 hours, transferred to ethanol and then embedded in paraffin. Representative 4-µm thick sections were stained by single brightfield immunohistochemistry for CK19 (Abcam ab133496), CD11b (Abcam ab133357), VCAM (Abcam ab134047) or pico Sirius red. Whole liver section histomorphometric measurements were generated with the software from Visiopharm (Hoersholm, Denmark) using a semi-automated approach.

Statistical analysis: GraphPad Prism software was used to analyze data for statistical significance. Outliers were removed by ROUT method and then distribution was determined, and then two-tailed T-test was used to determine significance.

Altered bile composition

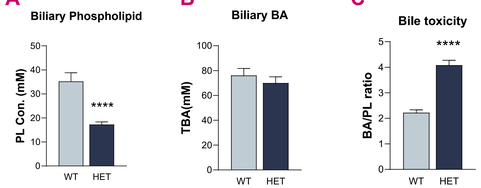

Figure 1. Abcb4/Mdr2 HET animals have altered bile composition. Loss of one copy of *Abcb4* results in decreased (A) bile PL without changing (B) bile BA levels. This leads to an increase in (C) the BA to PL ratio, likely impacting the generation of mixed micelles thereby increasing the "toxicity" of bile. This bile composition likely contains unbuffered BA, providing an environment conducive to biliary damage-induced cholestasis. HET animals are phenotypically normal and do not display any overt hepatobiliary phenotype[1].

Serum markers of cholestasis

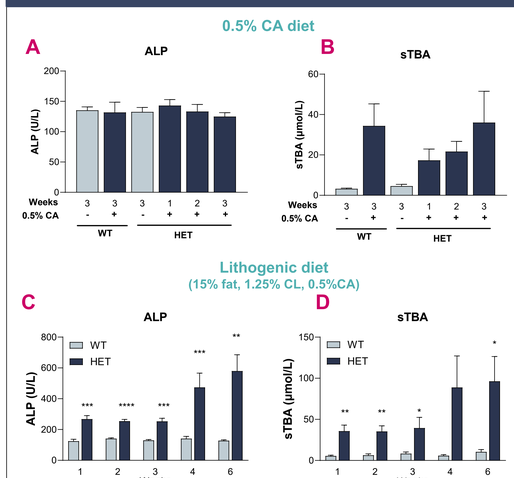

Figure 2. Peripheral markers of cholestasis are elevated in Abcb4/Mdr2 mice fed a lithogenic diet. WT and HET mice fed a diet containing 0.5% CA demonstrate no change in (A) ALP independent of time and genotype, while there is a time-dependent genotype-independent increase in (B) serum total BA. Feeding mice a lithogenic diet preferentially increases both (C) ALP and (D) sTBA in HET mice compared to WT mice. Levels of both markers are further elevated at 4 weeks. These data indicate 0.5% CA alone is not sufficient to induce hepatobiliary injury specific to HET mice. The addition of fat and CL to the CA in the diet is sufficient and, thus mice fed lithogenic diet were further characterized.

Serum markers of hepatotoxicity

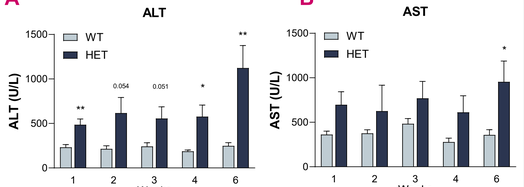

Figure 3. Peripheral markers of hepatotoxicity are elevated in Abcb4/Mdr2 HET mice Lithogenic diet increases (A) ALT and (B) AST in HET mice compared to WT mice.

Liver and gallbladder

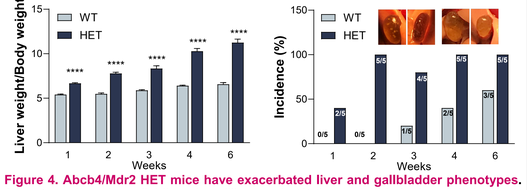

Figure 4. Abcb4/Mdr2 HET mice have exacerbated liver and gallbladder phenotypes. As early as 1 week on diet there is a difference in (A) liver weight to body weight between WT and HET mice. Furthermore, (B) crystal precipitates in the gallbladder were observed after 1 week in HET mice while it took 3 weeks in WT mice. These data indicate that changes in biliary PL predisposes to hepatobiliary phenotypes.

Ductular reaction

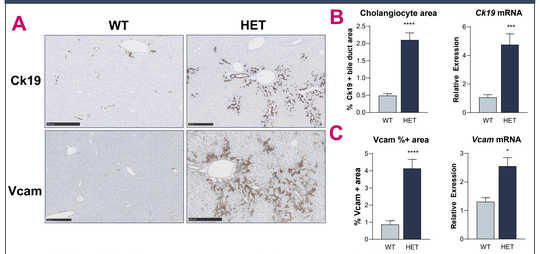

Figure 5. Abcb4/Mdr2 HET mice demonstrate increased Ck19 and Vcam compared to WT. IHC staining and quantitation of (A) Ck19 or (B) Vcam staining in WT and HET livers after 6 weeks on diet. (C) Ck19 (D) Vcam expression from livers of WT and HET mice confirms IHC quantitation of increased Ck19 and Vcam, indicating a preferential expansion of bile ducts in HET mice with dietary stress.

Cholangitis

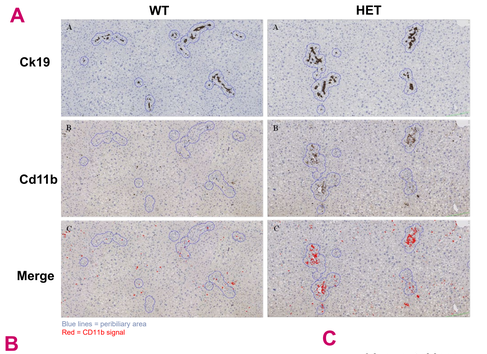

Figure 6. Indicators of cholangitis are elevated in Abcb4/Mdr2 HET mice after 6 weeks on diet. (A) IHC staining for leukocyte marker CD11b was colocalized to peribiliary space with Ck19 signal. The CD11b signal was quantified as (B left) peribiliary or (B right) parenchyma indicating the inflammation is peribiliary. Analysis of the liver for inflammatory signaling identified increases in (C) Cxcl-1, the mouse bioequivalent for IL-8 which is elevated in PSC patients and is a predictor of transplant free survival[2].

Fibrosis

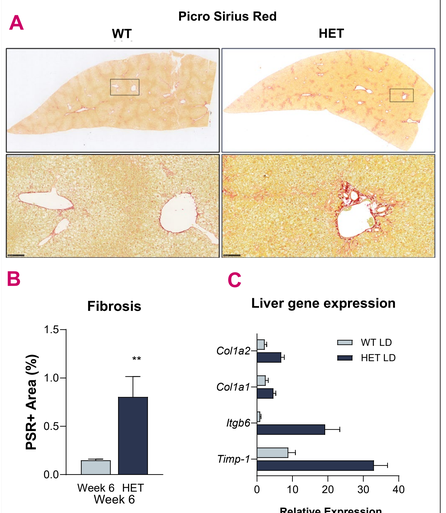

Figure 7. Abcb4/Mdr2 HET mice have elevated markers of fibrosis compared to WT mice. (A) Pico sirius red staining of WT and HET livers after 6 weeks on lithogenic diet indicates collagen deposition in the peri-biliary regions with initial parenchymal migration in HET mice while staining is normal in WT mice. (B) Quantitation of peribiliary and parenchymal staining demonstrates elevated levels in HET mice. (C) Gene expression analysis demonstrates elevated levels of fibrotic matrix expression *Col1a1* and *Col1a2* as well as matrix modifying genes *Itgb6* and *Timp-1*. Keeping animals on diet longer may promote more severe fibrosis.

Conclusions

The data presented here demonstrate that haploinsufficiency of the canalicular phospholipid floppase Abcb4/Mdr2 sensitizes animals to diet induced injury, generally phenocopying Mdr2 KO mice. The lack of an early phenotype in WT animals supports the notion that the altered bile composition contributes to development of cholangitis and cholestasis seen in a number of hepatobiliary diseases. Like PSC patients, this model presents with elevated levels of IL-8 as well as increased levels of Cd11b+ cells associated with bile ducts[3]. Thus, this serves as a useful tool for testing therapeutics impacting bile composition for the treatment of hepatobiliary diseases like PSC.

References

1. Smit JJM et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to complete absence of phospholipid from bile and to liver disease. Cell 75 (1993)
2. Vesterhus M et al. Novel serum and bile protein markers predict primary sclerosing cholangitis disease severity and prognosis. JHEP 66 (2017)
3. Zummer CL et al. A biliary immune landscape of primary sclerosing cholangitis reveals a dominant network of neutrophils and tissue-resident T cells. Sci Transl Med 13 (2021)