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Molecular Mechanism of Altered Ezetimibe Disposition in Nonalcoholic Steatohepatitis

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Running Title: Ezetimibe disposition is altered in experimental NASH

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; EZE, ezetimibe; EZE-GLUC, ezetimibe glucuronide; MCD, methionine and choline deficient

Abstract

Ezetimibe (EZE) lowers serum lipid levels by blocking cholesterol uptake in the intestine. Disposition of EZE and its pharmacologically active glucuronide metabolite (EZE-GLUC) to the intestine is dependent on hepatobiliary efflux. Previous studies suggest that hepatic transporter expression and function may be altered during non-alcoholic steatohepatitis (NASH). The purpose of the current study was to determine whether NASH-induced changes in the expression and function of hepatic transporters result in altered disposition of EZE and EZE-GLUC. Rats fed a methionine-choline deficient (MCD) diet for 8 weeks were administered 10mg/kg EZE either by intravenous bolus or oral gavage. Plasma and bile samples were collected over 2 hours followed by terminal urine and tissue collection. EZE and EZE-GLUC concentrations were determined by LC-MS/MS. The sinusoidal transporter Abcc3 was induced in MCD rats which correlated with increased plasma concentrations of EZE-GLUC, regardless of dosing method. Hepatic expression of the biliary transporters Abcc2 and Abcb1 were also increased in MCD animals, but the biliary efflux of EZE-GLUC was slightly diminished while biliary bile acid concentrations were unaltered. The cellular localization of Abcc2 and Abcb1 appeared to be internalized away from the canalicular membrane in MCD livers, providing a mechanism for the shift to plasma drug efflux. The combination of induced expression and altered localization of efflux transporters in NASH shifts the disposition profile of EZE-GLUC toward plasma retention away from the site of action. This increased plasma retention of drugs in NASH may have implications on the pharmacologic effect and safety of numerous drugs.

Introduction

Non-alcoholic fatty liver disease (NAFLD), which is believed to be the most common liver disease in western society (Marra *et al.*, 2008), is a complex multi-faceted malady which originates as simple steatosis and may progress to the more severe non-alcoholic steatohepatitis (NASH). Recent estimates indicate that NAFLD affects 17-40% of adults with 40% of those patients often unknowingly afflicted by the more severe form, NASH (Ali *et al.*, 2009;McCullough, 2006). NAFLD is regarded as the hepatic component of the multi-symptom metabolic syndrome (Fan, 2008), of which 90% of NAFLD patients exhibit at least one clinical feature (Adams *et al.*, 2005). The presence of multiple features of the metabolic syndrome in NAFLD patients correlates with hepatic histological severity, with NASH patients exhibiting numerous symptoms (McCullough, 2006;Fan, 2008;Marchesini *et al.*, 2003). Due to the prevalence of metabolic syndrome in NAFLD patients and the multiplicity of symptoms (dyslipidemia, hyperinsulinemia, central adiposity, and hypertension), NAFLD patients may often be medicated for several symptoms. We have previously investigated the effect of NAFLD upon the disposition of acetaminophen (Lickteig *et al.*, 2007) and the function of uptake transporters (Fisher *et al.*, 2009a) in rodent models of simple steatosis and NASH. These studies have led us to hypothesize that NASH, in particular, causes significant disruption in hepatic metabolism and disposition of administered pharmaceuticals due to alterations in hepatic drug transporters. However, the precise mechanisms underlying these dispositional alterations in rodents and their manifestation in human NAFLD remain elusive.

Ezetimibe (EZE) is an orally administered cholesterol absorption inhibitor that acts primarily via inhibition of Niemann-Pick C1-like 1 (NPC1L1) at the villus tip of enterocytes of the small intestine (Garcia-Calvo *et al.*, 2005;Kosoglou *et al.*, 2005). EZE is quickly metabolized (~80%)

to a glucuronide metabolite (EZE-GLUC) within enterocytes, followed by delivery of parent drug and metabolite, via the portal vein, to the liver where additional EZE glucuronidation occurs (Kosoglou *et al.*, 2005). EZE and EZE-GLUC is then excreted into bile and returned to the small intestinal lumen for inhibition of NPC1L1 (Kosoglou *et al.*, 2005). Enterohepatic recycling of EZE and EZE-GLUC is thought to be an important determinant of the long half-life and efficacy of EZE as both parent and metabolite are pharmacologically active; however, EZE-GLUC is believed to be a more potent inhibitor of NPC1L1 and accounts for the vast majority of drug (80-90%) measured in biological compartments (Kosoglou *et al.*, 2005).

Studies in knockout animals have demonstrated the importance of several efflux drug transporters in the disposition of EZE and EZE-GLUC. In particular, Abcc2 and Abcb1 have been implicated as having a major role in the biliary excretion of EZE-GLUC (Oswald *et al.*, 2007;Oswald *et al.*, 2010;Oswald *et al.*, 2006b), while Abcc3 mediates sinusoidal efflux (de Waart *et al.*, 2009). Under normal physiologic conditions, EZE is eliminated primarily via feces in unconjugated form, which may be due to hydrolysis of the EZE-GLUC secreted into bile (Kosoglou *et al.*, 2005). However, in the absence of Abcc2, EZE disposition into the blood from the liver increases and leads to an increase in urinary excretion of EZE and EZE-GLUC (Oswald *et al.*, 2006b). Diminished biliary excretion of EZE-GLUC and concomitant increased urinary excretion may have an effect upon drug half-life and overall systemic exposure, necessitating the need for dosage adjustments. In the present study, we examined the effect of NASH on the disposition of EZE and its major glucuronide metabolite. EZE is administered orally to humans; however, the expression profile of drug transporters in the MCD has not been characterized. We therefore chose to dose both orally and intravenously to discern the hepatic contribution to EZE disposition in NASH. Additionally, we have conducted expression and localization analyses of major hepatic efflux drug transporters involved in the disposition of EZE and EZE-GLUC in the

methionine-choline deficient (MCD) diet rodent model of NASH to more clearly understand the potential effects of NAFLD upon clinical drug disposition.

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Materials and Methods

Materials. Ezetimibe (EZE- E975000) was obtained from Toronto Research Chemicals (Ontario, Canada) and was determined to 99.8% pure. Urethane, carboxymethylcellulose, ethanol, propylene glycol, polyethylene glycol (PEG), HPLC-grade methanol, HPLC-grade methyl *tert*-butyl ether, and β-glucuronidase (>5000 U) were obtained from Sigma-Aldrich (St. Louis, MO). Neutral-buffered formalin (10%) and HPLC-grade H_2O were acquired through Fisher Scientific (Pittsburgh, PA).

Animals. Male Sprague Dawley rats weighing 200-250 g were obtained from Harlan (Indianapolis, IN). All animals were acclimated in 12 hour light and 12 hour dark cycles in a University of Arizona AAALAC-certified animal facility for at least one week prior to experiments, and were allowed water and standard chow *ad libitum*. Housing and experimental procedures were in accordance with NIH guidelines for the care and use of experimental animals. Rats were fed a control, methionine-choline-deficient diet with methionine and choline re-supplemented (#518754) or a methionine-choline-deficient (MCD) diet (#518810) (Dyets Inc., Bethlehem, PA) for eight weeks. At the conclusion of eight weeks and prior to initiation of MTX experiments, control animals weighed an average of 400.006 ±6.895 g and MCD/NASH animals weighed an average of 194.36 ±2.283 g. Weight loss in the MCD model is a common and well-documented side effect (Schattenberg *et al.*, 2010;Fan *et al.*, 2009).

Ezetimibe Disposition Experiments. Following eight weeks of respective diet treatment, animals were administered a bolus dose of urethane (1.2g/kg w/v, i.p.). The femoral artery and vein were cannulated with PE-50 polyethylene tubing (Braintree Scientific, Braintree, MA), and the common bile duct was cannulated with PE-10 polyethylene tubing (Braintree Scientific, Braintree, MA) distal to the bile duct bifurcation. Animal core temperature was maintained

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throughout collection of bile with a TCAT-2V temperature monitor and heat pad (Physitemp Instruments Inc, Clifton, NJ). Animals were administered a 10 mg/kg dose (5 mL/kg) of EZE either orally (n=3-5, prepared in 0.25% carboxymethylcellulose) or intravenously through the femoral vein cannula (n=3-5, prepared in 10% ethanol, 40% propylene glycol, 30% PEG 400, and 20% sterile H_2O). Blood samples (100 µL) were drawn from the femoral artery cannula at 0, 2, 10, 20, 40, 60, 90, and 120 minutes. Bile samples were collected in chilled tubes at 15 minute intervals for 120 minutes following EZE administration. Terminal urine samples were collected via bladder puncture and collected in pre-weighed tubes. All samples were stored at -80°C until use. Following sample collections, animals were euthanized while still under anesthesia. After euthanasia, histological liver slices were collected and placed in 10% neutral-buffered formalin for 24 hours followed by 70% ethanol until paraffin embedding was performed by the University of Arizona Histology Service Laboratory. The remaining liver tissue was snap frozen in liquid nitrogen and stored at -80°C until further use. Small intestines were sectioned by forming a "Z" with the full length of the organ, differentiating the duodenum, jejunum, and ileum in order. Tissue samples for the duodenum and ileum were taken from the middle of the first and third arms of the "Z", flushed with sterile H_2O, snap frozen in liquid nitrogen, and stored at -80°C until use.

Sample Preparation. All reagents used were of HPLC-grade quality. Sample preparations were performed according to the methods of Oswald (Oswald *et al.*, 2006a;Oswald *et al.*, 2007) with slight modifications for smaller volumes. For determination of EZE, 25 µL plasma, urine, or bile was mixed with 2 µL hydroxychalcone internal standard solution. The mixture was then diluted with 0.4 mL H_2O. Samples were then extracted with 0.8 mL methyl *tert*-butyl ether for 15 minutes followed by centrifugation at 4000 rpm for 2 minutes. The organic layer was transferred to a clean tube. The extraction was then repeated and the resulting combined organic layers were evaporated under a gentle stream of N_2 at 50°C. The residue was then dissolved in 77.8%

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aqueous methanol. For determination of Total EZE (parent plus conjugated EZE), prior to the above described protocol, 90 µL H_2O and 10 µL β-glucuronidase (>5000 U) were added to 25 µL sample and incubated at 50°C for 60 minutes. After cooling, the above described protocol was resumed with dilution of the sample in 0.3 mL H_2O. For determination of EZE in liver tissue, 500 mg tissue was homogenized in 2.5 mL H_2O. Then, 0.1 mL homogenate was mixed with 0.2 mL H_2O and 2 µL internal standard solution. The protocol was then continued as described above for plasma, urine, and bile. To determine Total EZE in liver tissue, 0.1 mL of the prepared tissue homogenate was combined with 90 µL H_2O and 10 µL β-glucuronidase. The mixture was incubated for 60 minutes at 50°C. Then sample was the allowed to cool and diluted with 0.2 mL H_2O and 2 µL internal standard solution. Extractions were performed as described above.

Determination of Total EZE, EZE, and EZE-GLUC Concentrations. LC-MS/MS detection of Total EZE, EZE, and EZE-GLUC was conducted based upon the method of Oswald (Oswald *et al.*, 2006a) in the Arizona Laboratory for Emerging Contaminants. The LC-MS/MS system was composed of a Waters-Micromass Quattro Premier XE tandem mass spectrometer (Waters Corporation, Milford, MA) and an Acquity Ultra Performance LC with auto-sampler (Waters Corporation, Milford, MA) equipped with MassLynx 4.1 software (Waters Corporation, Milford, MA). The chromatography was performed with a gradient beginning at 20/80 (v/v) acetonitrile/water and ending at 80/20 (v/v) acetonitrile/water for 7 minutes with a flow rate of 0.25 mL/minute on an Acquity UPLC BH C18 1.7 µM (2.1 X 50 mm) column (Waters Corporation, Milford, MA). The mass spectrometer was used in multiple reaction monitoring mode (MRM) and equipped with an electrospray ionization source (ESI) in the negative mode. The *m/z* transitions monitored were as follows: EZE – 408 to 271 and internal standard – 223 to 117. Concentration of EZE was determined for all samples submitted. EZE-GLUC concentrations were calculated as the difference between Total EZE and EZE measurements.

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RNA Preparations. Total RNA was isolated from rodent liver and intestinal tissue using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's recommendations. RNA concentrations were determined by UV spectrophotometry, and the integrity of the RNA confirmed by ethidium bromide staining following agarose gel electrophoresis.

Protein Preparations. Whole cell lysate preparations of rodent liver and intestinal tissue were prepared from ~300mg tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25mL) at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer's recommendations.

Branched DNA Assay. Specific oligonucleotide probes for Abcc2, 3, Mdr1a,1b (Brady *et al.*, 2002;Cherrington *et al.*, 2002), and Ugt1a1 (Vansell *et al.*, 2002) were diluted in lysis buffer supplied by the Quantigene™ HV Signal Amplification Kit (Genospectra, Fremont, CA). Substrate solution, lysis buffer, capture hybridization buffer, amplifier and label probe buffer used in the analysis were all obtained from the Quantigene Discovery Kit (Genospectra, Fremont, CA). The assay was performed in 96-well format with 10 µg (liver) or 5 µg (intestine) total RNA added to the capture hybridization buffer and 50 µl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex™ 320 bDNA luminometer interfaced with Quantiplex™ Data Management Software Version 5.02.

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*Immunoblot Protein Analysis. W*hole cell lystate proteins (80 µg/well, liver and 40 µg/well, intestine) were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes overnight. The following mouse monoclonal antibodies were obtained from Abcam, Inc. (Cambridge, MA) and used to determine relative protein levels: Abcc3 (M_3II-9) and Abcb1 (C219). Abcc2 (M_2III-5) protein levels were determined using a mouse monoclonal antibody obtained from Kamiya Biomedical Company (Seattle, WA). Protein levels of Ugt1a1 were determined using a rabbit polyclonal antibody (Abcam, Inc. Camridge, MA). Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to total ERK (C-16 and C-14, Santa Cruz, CA).

Bile Acid Concentrations. Bile acids were measured in rodent bile samples at time = 0 of disposition studies. Bile samples were diluted 1 µL in 49 µL sterile-filtered saline. The Diazyme Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, CA) was used to determine total bile acid concentrations spectrophotometrically over a 1 minute interval at 405nm, per the manufacturer's instructions. The assay was calibrated using a manufacturer-provided standard 50 µmol/L bile acid calibrator solution.

Immunohistochemistry. Immunohistochemical staining for all proteins was performed on formalin-fixed, paraffin-embedded (FFPE) samples. Briefly, tissue sections were de-paraffinized in xylene and re-hydrated in ethanol, followed by antigen retrieval in citrate buffer (pH 6.0, Abcb1) or Tris-EDTA buffer (pH 9.0, Abcc2). Endogenous peroxidase activity was blocked with 0.3% (v/v) H_2O_2 in methanol for 20 minutes. Immunohistochemical staining for Abcb1 was performed with the MACH3 staining kit (Biocare Medical, Concord, CA) per the manufacturer's protocol. Samples were incubated in a primary antibody (antibodies described above) solution overnight at 4°C. Immunohistochemical staining for Abcc2 was performed with the MACH4 staining kit (Biocare Medical, Concord, CA) per the manufacturer's recommendations. Abcc2

antibody incubation was performed overnight at 4°C. All slides were imaged with a Nikon Eclipse E4000 microscope and a Sony Exwave DXC-390 camera.

Statistical Analysis. For disposition studies, a Student's t-test was used to determine significant differences between diet groups at all time points. All subsequent data was analyzed by the student's t-test to determine significant differences between diet groups. All analyses were performed with Stata10 software (Stata, College Station, TX), and a significance level of $p \leq 0.05$ was used for all data analyses.

Results

Hepatic Gene and Protein Expression in Diet-Induced NASH. mRNA levels of Abcc2, 3, Abcb1a, Abcb1b, and Ugt1a1 were determined by the branched DNA method of RNA quantification in Control and MCD rat livers, and the results shown in Figure 1. In MCD livers, Abcc3 mRNA levels were significantly increased (44.8 fold) from control. Additionally, Abcb1a and Abcb1b mRNA levels were both significantly elevated in MCD livers (3.8 and 17.3 fold, respectively). Abcc2 and Ugt1a1 were not altered at the transcriptional level.

Figure 2 shows relative protein levels of Abcc2, 3, Abcb1, and Ugt1a1 in control and MCD rat livers as determined by immunoblot analysis. Abcc2, 3, and Abcb1 efflux drug transporters were significantly elevated in MCD livers (2,2, 2.5, and 2.4 fold, respectively); however, no significant alterations in Ugt1a1 protein levels were observed.

EZE and EZE-GLUC Disposition in Diet-Induced NASH. EZE is clinically administered as a 10 mg oral dose; however, since the expression of drug transporters important to the disposition of EZE have not yet been evaluated in the MCD diet rodent model of NASH, we chose to dose EZE both orally and intravenously. This allowed for the separate determination of both the hepatic and intestinal contribution to EZE disposition in NASH. Figure 3 shows plasma concentrations of EZE, EZE-GLUC, and Total EZE following either oral or intravenous dosing over a 120 minute period. Total EZE and EZE-GLUC plasma concentrations were significantly elevated above control beginning at 40 minutes following oral dosing of EZE. This elevation above control persisted until conclusion of the experiment at 120 minutes. Following intravenous dosing of EZE, MCD animals exhibited significantly higher plasma concentrations of Total EZE throughout the study. Similarly, plasma concentrations of EZE-GLUC were significantly elevated

in MCD animals beginning at 10 minutes and continuing throughout the study. No significant changes in the sinusoidal efflux of EZE were observed between diet groups regardless of dosing method.

Biliary concentrations of EZE, EZE-GLUC, and Total EZE are shown in Figure 4. Total EZE and EZE-GLUC concentrations in MCD rodent bile were decreased from control in orally dosed animals; however, these results were not significant. Similarly, EZE-GLUC biliary concentrations were consistently lower in intravenously dosed MCD animals, but not to a significant extent. EZE biliary concentrations in intravenously dosed MCD animals were significantly decreased from control beginning at 30 minutes and continuing until 90 minutes.

Urinary samples were collected by bladder puncture at the conclusion of the 120 minute experiment. Urinary concentrations of EZE, EZE-GLUC, and Total EZE in control and MCD animals are shown in Figure 5. Total EZE and EZE concentrations were significantly higher in orally dosed MCD animals compared to control. Analysis of intravenously dosed animals revealed a significant increase in EZE, EZE-GLUC, and Total EZE urinary concentrations in MCD animals.

Hepatic tissue concentrations of EZE, EZE-GLUC, and Total EZE are shown in Figure 6. No significant alterations in tissue retention of Total EZE, EZE, or EZE-GLUC were observed for either oral or intravenously dosed animals.

Effect of Diet-Induced NASH on Bile Flow and Biliary Bile Acid Concentrations. Bile volume and flow rate throughout the 120 minute experiment was calculated assuming a specific gravity of 1.0, results are shown in Figure 7. Regardless of dosing, bile volume was unaltered in MCD animals. The bile flow rate, as normalized to body weight was elevated in MCD animals. Bile

acids concentration in bile samples of control and MCD diet-fed rats were determined spectrophotometrically by the enzyme cycling method using the Diazyme Total Bile Acids Kit (Diazyme Laboratories, Poway, CA). This analysis was performed in order to determine the functionality of bile acid excretion processes in the liver. Regardless of dosing method, total bile acid levels in bile were unaltered between diet groups.

Effect of Diet-Induced NASH on Intestinal Ugt1a1 Expression. mRNA expression of Ugt1a1 in control and MCD duodenum and ileum are shown in Figure 8. This analysis was conducted to determine if disruption of EZE metabolism in the gut of orally dosed animals underlies the effect of NASH on EZE disposition. However, no significant alterations in Ugt1a1 transcriptional regulation were detected in rodent intestine. Immunoblots and densitometric results of relative Ugt1a1 protein levels are shown in Figure 8. Similar to Ugt1a1 mRNA, no significant alterations between control and MCD were observed in Ugt1a1 protein.

Efflux Drug Transporter Localization in Diet-Induced NASH. Immunohistochemical staining of Abcc2 and Abcb1 in Control and MCD FFPE liver samples is shown in Figure 9 at 40X magnification. Staining of both Abcc2 and Abcb1 in MCD livers appears to be pulling away from the canalicular membrane (circled in red), suggesting that the transporters may be internalized in diet-induced NASH. Staining for both transporters appeared to be properly localized along the canalicular membrane in control livers.

Discussion

Despite the current prevalence and projected continuation in the growth of NAFLD, little information is available concerning the expression or function of drug metabolizing enzymes and transporters in human NAFLD patients. Recent studies within our laboratory have investigated drug metabolizing enzymes and transporters in both human samples and rodent models. These studies have included the identification of perturbations in cytochrome P450 (Fisher *et al.*, 2009b) and glutathione *S*-transferase (Hardwick *et al.*, 2010) enzyme expression and functionality in a population of human steatosis and NASH samples. Additionally, alterations in the efflux of APAP (Lickteig *et al.*, 2007) and uptake of bromosulfophthalein (Fisher *et al.*, 2009a) have been investigated in rodent models of NAFLD. However, information concerning the mechanisms behind these alterations in drug disposition and more importantly, how they may manifest clinically in the human disease is lacking. Nonetheless, it can be hypothesized that NAFLD has significant potential to alter the absorption, distribution, metabolism and elimination (ADME) of several pharmaceutical agents. Due to the pervasiveness of NAFLD within the general population and the propensity for these patients to be medicated for multiple symptoms of the metabolic syndrome, knowledge of the effect of NAFLD on ADME could be valuable in identifying patients at risk for ADRs.

In the current study, we have demonstrated that the MCD diet rodent model of NASH causes a significant increase in the amount of the pharmacologically active metabolite EZE-GLUC excreted into sinusoidal blood and away from its target site. However, due to inherent characteristics of the MCD model, NASH rodents presented with significantly lower total body weights prior to the initiation of disposition experiments. Due to the reduction in total body weight characteristic of the model, animals were dosed based upon body weight. Upon examination of the parent drug, EZE in the plasma of i.v. dosed animals, it appears that overall

drug exposure is not significantly different between control and NASH rodents leading us to conclude that the observed changes in body weight most likely have little effect upon the observed alterations in drug disposition. In contrast, specific alterations in exposure to the metabolite, EZE-GLUC, were observed in NASH rodents and we have proposed a possible mechanism for this observation. Generally, elevated plasma efflux of a drug results in increased systemic exposure to the drug in the form of retention within the systemic circulation. This could have profound effects upon toxicity in extrahepatic tissues such as the kidney. However, in the case of EZE increased efflux of EZE-GLUC into sinusoidal blood, as seen in NASH rodents, and diminished efflux into bile would mean that less drug is being delivered to the site of action in the small intestine. This could have implications for EZE efficacy and could possibly play a role in selecting the most appropriate therapeutic option for patients with NASH.

The plasma efflux of EZE-GLUC is similar to that of acetaminophen-glucuronide (APAP-GLUC) in MCD animals as demonstrated by Lickteig, *et al*. In particular, APAP-GLUC excretion into bile was diminished in MCD animals and plasma efflux was significantly increased. Evidence from Lickteig, *et al.*, and the current study suggest that changes in disposition of glucuronide metabolites of additional pharmaceutical agents is likely to occur in NASH patients. The conclusion made by Lickteig *et al.,* that increased plasma efflux of drug in the MCD model could be due to the combination of increased expression and affinity for Abcc3 (Lickteig *et al.*, 2007) is certainly plausible in the current study. de Waart and colleagues conducted inhibition experiments in membrane vesicles containing either Abcc3 or Abcc2 and discovered that EZE-GLUC is able to inhibit the transport of estradiol-17β-glucuronide more efficiently for Abcc3 in comparison to Abcc2 (de Waart *et al.*, 2009). Furthermore, studies have shown that Abcc3 preferentially transports glucuronide metabolites and there is evidence for higher affinity over Abcc2. Chu and colleagues have identified ethinylestradiol glucuronide as a higher affinity substrate for ABCC3 versus ABCC2 (Chu *et al.*, 2004). Further evidence implicating Abcc3 as

the major transporter of glucuronide conjugates stems from studies in Abcc3 knockout mice which exhibit significant hepatic retention of acetaminophen-glucuronide in comparison to wild-type animals without alterations to Abcc2 protein levels (Manautou *et al.*, 2005). Thus, the increased expression of Abcc3 in MCD rodents and preferential affinity for Abcc3 may help to explain, at least in part, the elevation in plasma efflux of EZE-GLUC.

Further evidence supporting the conclusion that NASH may cause a shift in the disposition profile of clinically relevant drugs is seen in the urinary excretion of Total EZE and EZE. Under normal physiological conditions, the majority of EZE is excreted via feces as parent drug due to hydrolysis of EZE-GLUC following excretion into bile (Kosoglou *et al.*, 2005). MCD animals exhibited higher concentrations of both Total EZE and EZE in the urine compared to controls. This finding is similar to results shown in Abcc2-deficient rats in which increased serum concentrations of EZE-GLUC resulted in increased renal excretion and decreased fecal excretion of EZE and EZE-GLUC (Oswald *et al.*, 2006b), indicating that the shift in the hepatic elimination of EZE from bile to plasma can have a significant influence on drug efficacy.

It is interesting to note that in the current study, diminished biliary concentrations of Total EZE and EZE-GLUC were observed in MCD animals. However, these results did not reach significance. Analysis of bile volume revealed no significant differences between control and MCD animals. However, the bile flow rate, which is a calculation of bile volume normalized to body weight, was elevated in MCD animals. This elevation in bile flow is reflective of the well-documented reduction in body weight that occurs due to the MCD diet (Fan *et al.*, 2009;Schattenberg *et al.*, 2010). Additionally, bile acid concentrations in the bile were not altered. These data suggest that diminishment of biliary drug excretion is not simply due to cholestatic conditions, and that instead, a more specific mechanism may be responsible. Immunohistochemical staining of Abcc2 and Abcb1, both of which play a major role in the biliary

excretion of EZE-GLUC and its repeated delivery to the site of action, revealed a unique mechanism of transport regulation. In the livers of MCD rodents, cellular localization of both Abcc2 and Abcb1 may be internalized away from the canalicular membrane, while localization of Abcg2 (an additional biliary transporter) remains unchanged, *data not shown*. Disrupted localization of specific transporters would make them unavailable for successful transport of drugs and their metabolites into bile, despite an induction of protein levels. Blocking of biliary transport by way of altered localization of efflux drug transporters could, in conjunction with increased expression of Abcc3 on the sinusoidal membrane, drive the shift from biliary to plasma efflux. Similarly, Mottino *et al.,* have shown that altered localization of Abcc2 during estradiol-17β-glucuronide-induced cholestasis results in reduction of the biliary concentration of Abcc2 substrates (Mottino *et al.*, 2005;Mottino *et al.*, 2002). Zhang and colleagues observed a similar phenomenon in sandwich-cultured rat hepatocytes. The biliary excretion of 5-(6)-carboxy-2',7'-dichlorofluorescein was diminished when Abcc2 was internalized (Zhang *et al.*, 2005). Of particular importance in the current study, is the possible internalization of not just one, but two efflux drug transporters on the canalicular membrane. While brightfield immunohistochemical staining suggests that localization of these transporters indeed appears to be disrupted in MCD livers, further investigation by confocal microscopy has been limited due to severe autofluorescence in the livers of MCD animals. However, several experiments have shown that internalization of Abcc2 alone can significantly disrupt biliary drug efflux, altered cellular localization of both Abcc2 and Abcb1 could have confounding effects upon biliary excretion in NASH. Additionally, the level of bile acids observed in the bile of control and MCD rodents indicates that bile acid secretion is intact in this disease model. This finding supports the conclusion that the alterations we have discovered in EZE disposition are likely due to expression and localization changes in specific transporters responsible for EZE disposition, rather than a general cholestatic phenomenon.

An additional explanation of the observed results of biliary excretion of EZE in MCD animals arises from studies in knockout animals. de Waart and colleagues have revealed a very complex interplay of efflux transporters in the disposition of EZE. Biliary excretion of EZE in Abcg2 (Bcrp) knockout mice revealed no significant alterations compared to wild-type 2 hours after dosing (de Waart *et al.*, 2009). Alternatively, Abcc2 knockout and Abcc2/Abcg2 double knockout mice exhibited a reduction in biliary excretion of EZE to 56% and 2.5% of controls, respectively (de Waart *et al.*, 2009). This indicates that though Abcg2 is not a major transporter of EZE-GLUC under normal circumstances, it may be capable of partially compensating for the loss of Abcc2, and as observed in the current study, Abcb1, as well. Though expression of Abcg2 was not evaluated in the current study, Lickteig *et al.,* found an elevation of Abcg2 protein in the MCD diet rodent model of NASH (Lickteig *et al.*, 2007) thus lending support to our observations that the discrepancy of EZE biliary excretion in NASH may be due to multiple overlapping substrate specificities, as well as, disruption in cellular localization of Abcc2 and Abcb1.

To further demonstrate the role of hepatic transporters and rule out a change in metabolism in the disposition of EZE, we examined expression of Ugt1a1 in the intestine and liver. Potential alterations of metabolism in the gut of orally-dosed animals could confound the disposition results acquired in the study. Similarly, changes in hepatic metabolism of intravenously dosed animals would diminish our ability to determine the effect of the liver on alterations of disposition in NASH. However, no significant alterations to Ugt1a1 at the mRNA or protein level were observed in the liver or intestine of MCD animals indicating that the alterations in EZE-GLUC plasma levels in MCD animals is not due to metabolism. As well, previous studies within our laboratory identified a reduction in uptake transport function in MCD animals (Fisher *et al.*, 2009a). We determined concentrations of EZE, EZE-GLUC, and Total EZE in hepatic tissue of

control and MCD animals to identify whether NASH affects uptake of EZE into hepatocytes. No significant changes in drug concentration within the hepatic tissue of MCD animals was observed, indicating that entry of drug into hepatocytes was not a confounding factor in the measured elevations of plasma drug concentrations in NASH.

In conclusion, the combination of altered cellular localization of the biliary efflux drug transporters Abcc2 and Abcb1, and the induction of the higher affinity sinusoidal efflux drug transporter, Abcc3, in rodent NASH drives a shift from primarily biliary efflux of EZE-GLUC to increased plasma concentrations and elevated urinary excretion. This plasma retention of drugs may have an implication on therapeutic efficacy and the potential risk of adverse drug reactions for many pharmaceuticals administered to patients with NASH.

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Authorship Contributions

Participated in research design: Hardwick, Fisher, and Cherrington.

Conducted experiments: Hardwick, Fisher, Street, and Canet.

Performed data analysis: Hardwick and Cherrington.

Wrote or contributed to the writing of the manuscript: Hardwick and Cherrington.

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Footnotes

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Figure Legends

Figure 1. Hepatic mRNA Expression in Diet-Induced NASH. mRNA levels in rats fed either a control or MCD diet for 8 weeks. mRNA levels were measured by the branched DNA assay and expressed as relative lights units (RLU) per 10 µg total RNA. The data are presented as mean ± SEM. Asterisk (*) indicate a significant difference from control with a significance level of $p \leq 0.05$.

Figure 2. Hepatic Protein Expression in Diet-Induced NASH. Protein levels in rats fed either a control or MCD diet for 8 weeks. Immunoblots are shown with Total ERK as control protein. Relative protein levels were determine by densitometric analysis and expressed as relative to Total ERK. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control with a significance level of $p \leq 0.05$.

Figure 3. Effect of Diet-Induced NASH on Plasma EZE, EZE-GLUC and Total EZE Concentrations. Following 8 weeks of control (▲) and MCD (□) diet feeding, EZE disposition experiments were conducted. Femoral artery and vein and bile duct cannulations were performed, and animals were administered either an oral or intravenous dose of 10 mg/kg EZE. Plasma samples were collected beginning 2 minutes after dose until 120 minutes. Concentrations of Total EZE and EZE were determined by LC-MS/MS, EZE-GLUC concentrations were calculated as the difference between Total EZE and EZE. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control for each time point with a significance level of $p \leq 0.05$.

Figure 4. Effect of Diet-Induced NASH on Biliary Concentrations of EZE, EZE-GLUC, and Total EZE. The experimental and analytical conditions were the same as described for Figure 3. Bile concentrations of EZE, EZE-GLUC, and Total EZE are shown in control (▲) and MCD (□) rodents. Following EZE dosing, bile was collected at 15 minute intervals over a 120 minute period. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control for each time point with a significance level of $p \leq 0.05$.

Figure 5. Effect of Diet-Induced NASH on Urinary Total EZE, EZE, and EZE-GLUC Concentrations. The experimental and analytical conditions were the same as described for Figure 3. Terminal urine was collected by bladder puncture 120 minutes after dosing. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control for each time point with a significance level of $p \leq 0.05$.

Figure 6. Hepatic Tissue Retention of Total EZE, EZE, and EZE-GLUC in Rodent NASH. The experimental and analytical conditions were the same as described for Figure 3. Liver tissue was snap frozen 120 minutes after dosing. Total EZE, EZE, and EZE-GLUC concentrations in liver tissue are shown. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control for each time point with a significance level of $p \leq 0.05$.

Figure 7. Effect of Experimental NASH on Bile Volume, Bile Flow and Bile Acid Excretion. The experimental and analytical conditions were the same as described for Figure 3. Bile volume, bile flow, and biliary bile acid concentrations are shown. Following EZE dosing, bile was collected at 15 minute intervals over a 120 minute period. Bile volume and bile flow was calculated assuming a specific gravity of 1.0, and the data expressed as µL/min and µL/min/kg, respectively, in control (▲) and MCD (□) rodents. Bile acid concentrations were determined

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spectrophotometrically by the Diazyme Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, CA) and expressed as µmol/L. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control for each time point with a significance level of $p \leq 0.05$.

Figure 8. Intestinal Ugt1a1 Expression in Rodent NASH. mRNA and relative protein levels of Ugt1a1 in rats fed either a control or MCD diet for 8 weeks. mRNA levels were measured by the branched DNA assay and expressed as relative light units (RLU) per 5 µg total RNA. Immunoblots are shown with Total ERK as control protein. Relative protein levels were determined by densitometric analysis and expressed as relative to Total ERK. The data are presented as mean ± SEM. Asterisks (*) indicate a significant difference from control with a significance level of $p \leq 0.05$.

Figure 9. Immunohistochemical Staining of Efflux Drug Transporters in Diet-Induced NASH. IHC staining of ABCC2 and ABCB1 in formalin-fixed paraffin-embedded control and MCD rodent liver samples is shown at 40X magnification. Antibody binding was detected by either the MACH 3 (Abcb1) or the MACH 4 method (Abcc2, Biocare Medical, Concord, CA). Color development was performed using Betazoid DAB (Biocare Medical, Concord, CA).

Figure 1

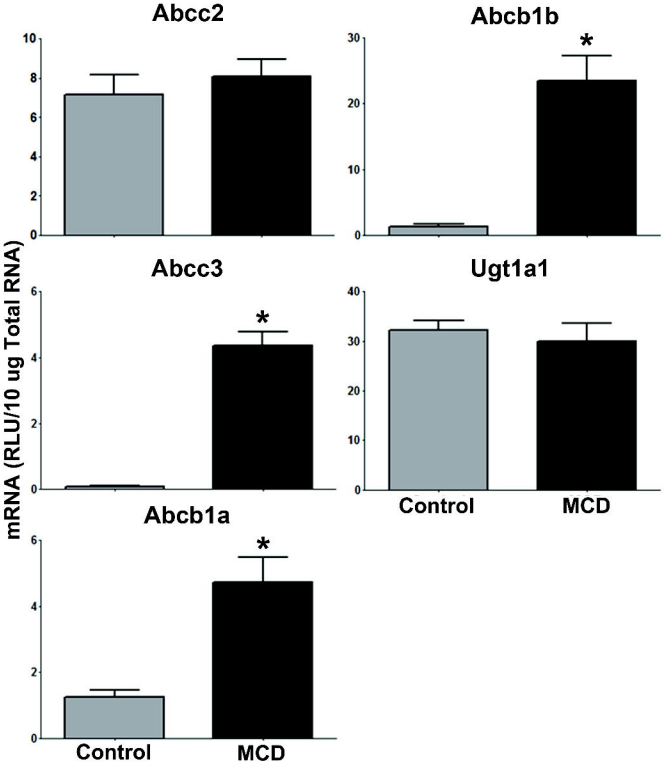
Abcc2
Abcb1b
Abcc3
Ugt1a1
Abcb1a
mRNA (RLU/10 ug Total RNA)
Control
MCD
Control
MCD

Figure 2

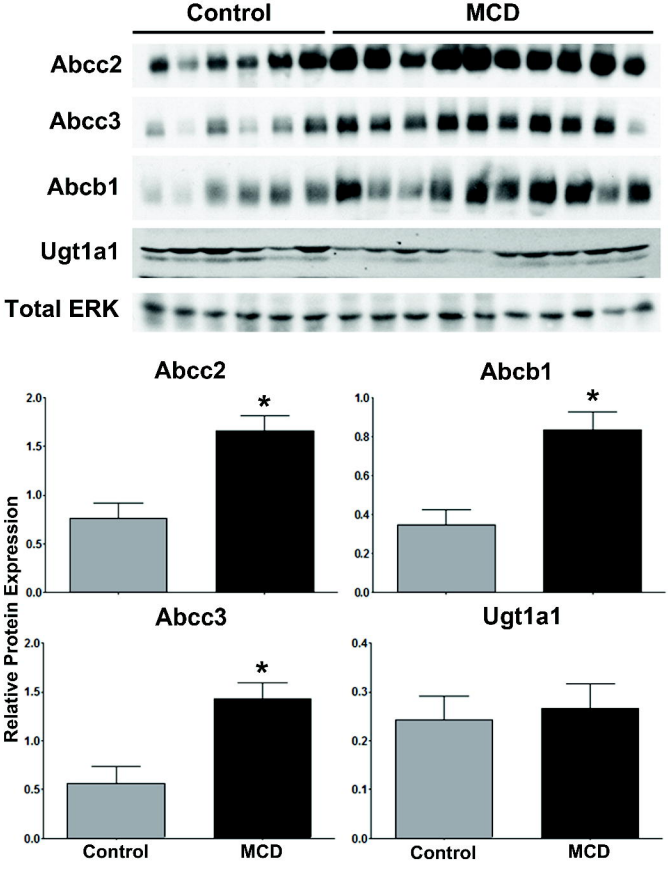
Control
MCD
Abcc2
Abcc3
Abcb1
Ugt1a1
Total ERK
Abcc2
Abcb1
Abcc3
Ugt1a1
Relative Protein Expression
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
0.4
0.3
0.2
0.1
0.0
*
Control
MCD

Figure 3

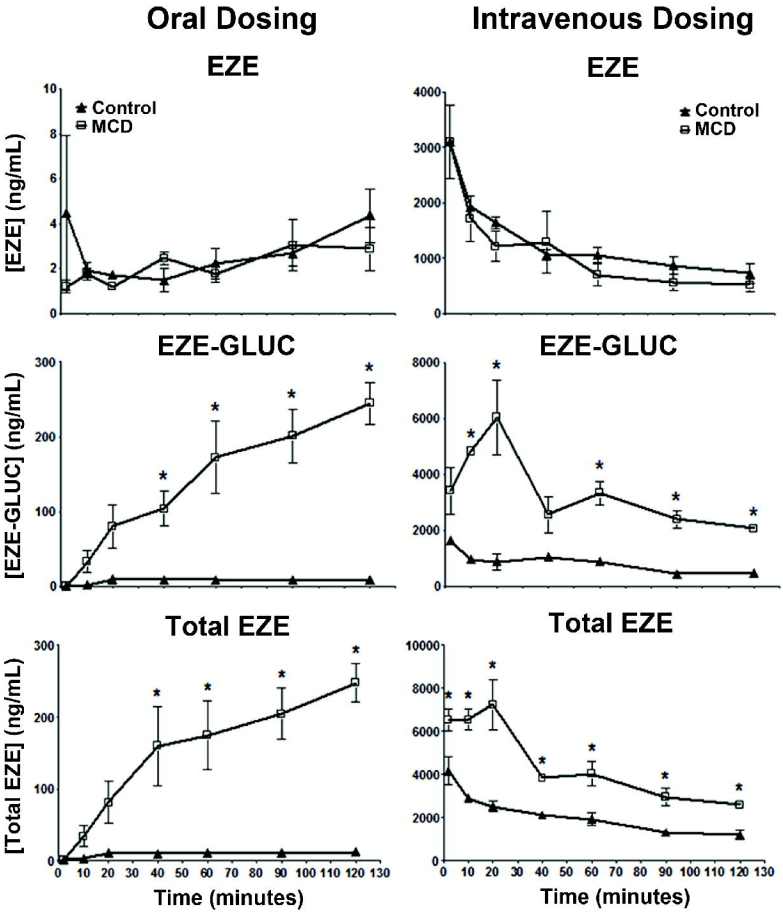
Oral Dosing
Intravenous Dosing
EZE
EZE
Control
MCD
Control
MCD
[EZE] (ng/mL)
EZE-GLUC
EZE-GLUC
[EZE-GLUC] (ng/mL)
Total EZE
Total EZE
[Total EZE] (ng/mL)
Time (minutes)
Time (minutes)

Figure 4

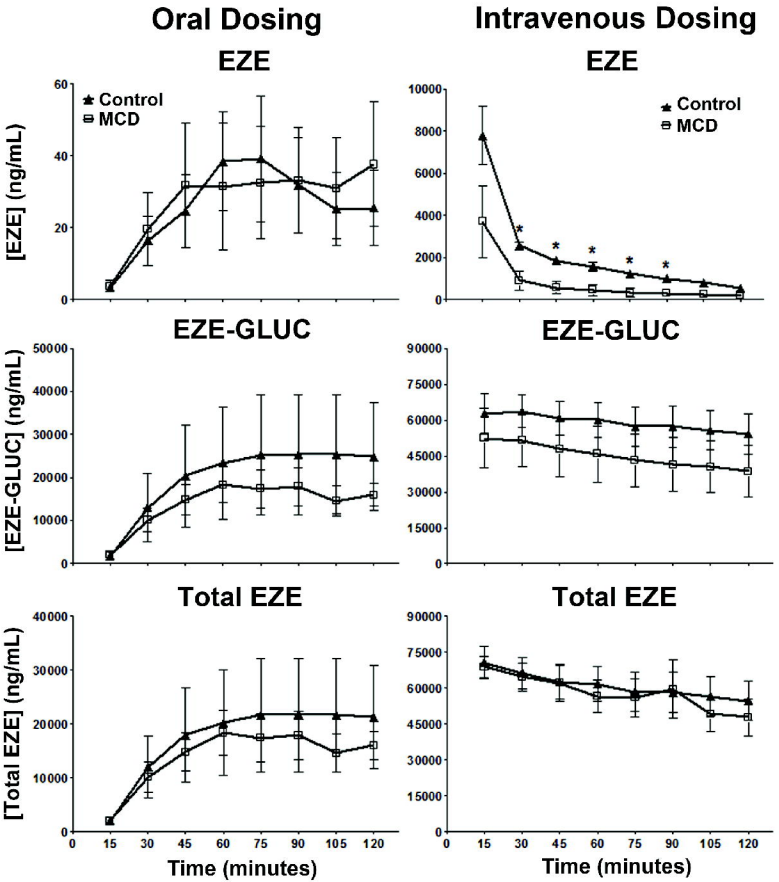
Oral Dosing
Intravenous Dosing
EZE
EZE
Control
MCD
Control
MCD
[EZE] (ng/mL)
EZE-GLUC
EZE-GLUC
[EZE-GLUC] (ng/mL)
Total EZE
Total EZE
[Total EZE] (ng/mL)
Time (minutes)
Time (minutes)

Figure 5

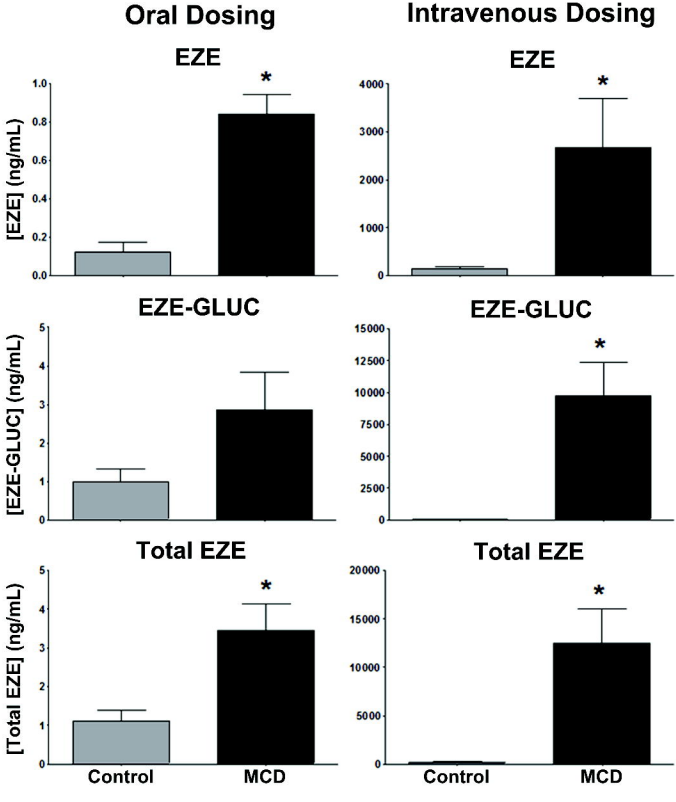
Oral Dosing
Intravenous Dosing
EZE
EZE
[EZE] (ng/mL)
1.0
0.8
0.6
0.4
0.2
0.0
4000
3000
2000
1000
0
*
*
EZE-GLUC
EZE-GLUC
[EZE-GLUC] (ng/mL)
5
4
3
2
1
0
15000
12500
10000
7500
5000
2500
0
*
*
Total EZE
Total EZE
[Total EZE] (ng/mL)
5
4
3
2
1
0
20000
15000
10000
5000
0
*
*
Control
MCD
Control
MCD

Figure 6

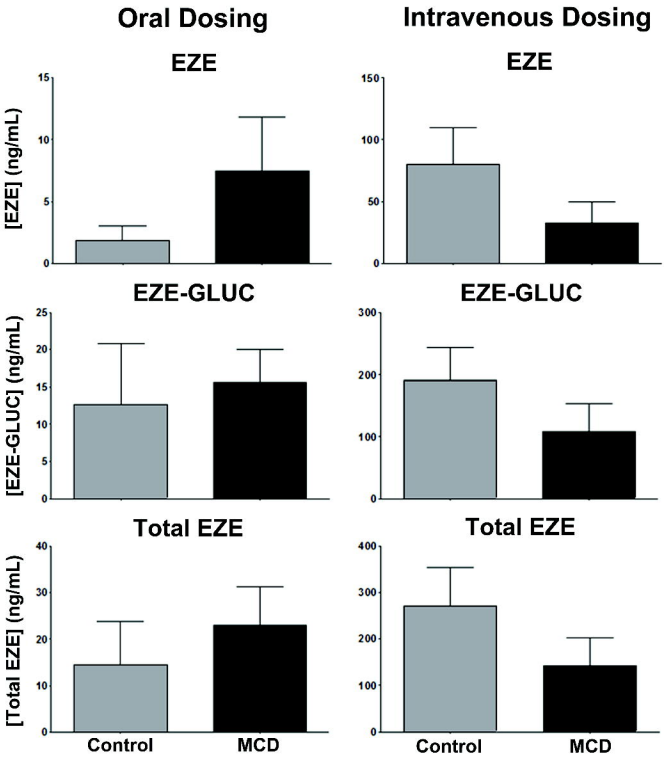
Oral Dosing
Intravenous Dosing
EZE
EZE
[EZE] (ng/mL)
15
10
5
0
150
100
50
0
EZE-GLUC
EZE-GLUC
[EZE-GLUC] (ng/mL)
25
20
15
10
5
0
300
200
100
0
Total EZE
Total EZE
[Total EZE] (ng/mL)
40
30
20
10
0
400
300
200
100
0
Control
MCD
Control
MCD

Figure 7

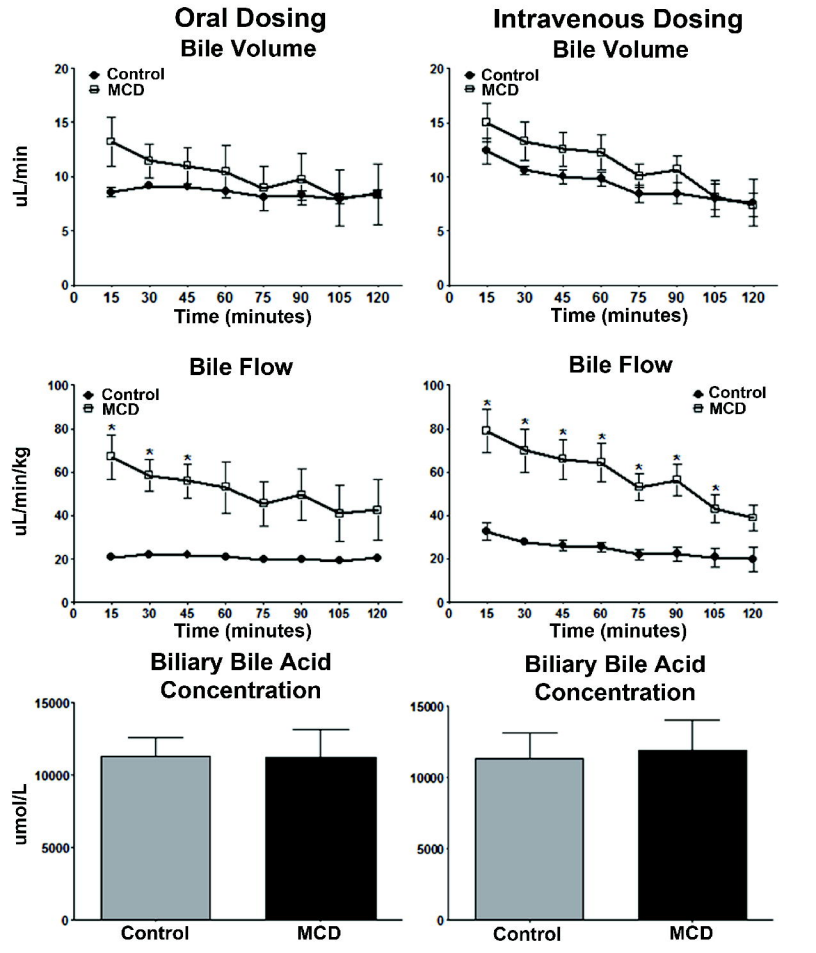
Oral Dosing
Bile Volume
Control
MCD
uL/min
Time (minutes)
Intravenous Dosing
Bile Volume
Control
MCD
Time (minutes)
Bile Flow
Control
MCD
uL/min/kg
Time (minutes)
Bile Flow
Control
MCD
Time (minutes)
Biliary Bile Acid Concentration
umol/L
Control
MCD
Biliary Bile Acid Concentration
Control
MCD

Figure 8

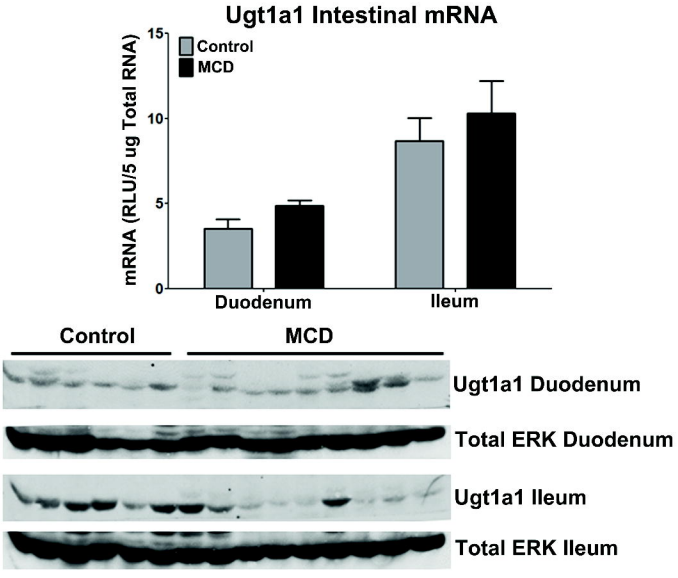
Ugt1a1 Intestinal mRNA
mRNA (RLU/5 ug Total RNA)
15
10
5
0
Control
MCD
Duodenum
Ileum
Control
MCD
Ugt1a1 Duodenum
Total ERK Duodenum
Ugt1a1 Ileum
Total ERK Ileum
Ugt1a1 Intestinal Protein
Relative Protein Expression
1.0
0.8
0.6
0.4
0.2
0.0
Control
MCD
Duodenum
Ileum

Figure 9