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MECHANISMS OF INDIVIDUAL VARIATION IN GLUCURONIDATION, SULFONATION, AND AMIDATION: BISPHENOL A AND BILE ACIDS

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MECHANISMS OF INDIVIDUAL VARIATION IN GLUCURONIDATION, SULFONATION, AND AMIDATION: BISPHENOL A AND BILE ACIDS

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ABSTRACT

The role of conjugation of chemicals and endogenous compounds is an important mechanism in detoxification and metabolic signaling. Specifically, amidation, sulfonation, and glucuronidation are necessary functions of the human body. Exposure to environmental toxins such as bisphenols must be conjugated in order to be excreted from the body. Bisphenol A was originally developed as a synthetic estrogen, however later found use as a monomer within the plastics industry allowing for wide-spread exposure to BPA through food and drink containers. While most healthy individuals are capable of metabolizing and excreting bisphenol A, this metabolism can be impacted by diet and disease. Herein, the effects of obesity and fasting are shown to reduce the capacity of BPA glucuronidation in wild-type and ob/ob mice. In addition to environmental toxins, endogenous compounds such as bile acids can undergo sulfonation, amidation, and glucuronidation. Occasionally, drugs can cause drug-induced liver injury, often leading to cholestasis that can cause permanent liver injury if the drug is not stopped. The buildup of bile acids within the liver can then lead to liver damage because bile acids are amphiphilic compounds that act as strong detergents, capable of breaking down the hepatocellular membrane. In order to combat this build up toxic bile acids, conjugation occurs to increase the polarity and water solubility of the bile acids, thus leading to increased excretion. I proposed that drugs may inhibit the sulfonation of lithocholic acid (LCA), the most toxic bile acid, or inhibiting the amidation of other bile acids (the predominate form of bile acids) leading to drug induced liver injury. LCA sulfonation is catalyzed by human cytosolic sulfotransferase (SULT) 2A1. Bile acid amidation is catalyzed by human bile acid

coenzyme A synthetase/bile acid coenzyme A: amino acid N-acyltransferase (BACS/BAAT). In this study, 125 drugs were screened for inhibition of SULT2A1 and the BACS/BAAT enzymes. I successfully identified several compounds as potent SULT2A1 inhibitors. While only a single inhibitor of amidation was discovered, I developed a novel one step enzymatic assay to test for the inhibition of BACS and BAAT. While drugs can cause cholestasis as an unwanted effect, a class of drugs called fibrates are being studied for use in treatment of primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Fibrates are known peroxisome proliferator-activated receptor (PPAR) activators, and in this study, I proposed the use of fenofibrate in patients with PBC and PSC will reduce serum liver enzymes and bile acids. In both patient cohorts, we observed large decreases in serum bile acids, particularly conjugated bile acids, and the bile acid precursor 7α-hydroxy-4-cholesten-3-one (C4). In addition, we observed a shift from the classic synthesis pathway of bile acids towards an alternative pathway of chenodeoxycholic acid (CDCA) synthesis. These results suggest the downregulation of bile acid synthesis and the BACS/BAAT amidation system indicating a potential alternative treatment for cholestasis.

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PREFACE

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Bisphenol A Glucuronidation is Impaired in ob/ob Mice

Adam M Auclair, Angela L. Slitt and Roberta S. King

(Prepared for submission to Toxicology and Applied Pharmacology)

Bisphenol A glucuronidation is impaired in ob/ob mice

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ABSTRACT

Background

Bisphenol A (BPA) is a widely used monomer and known endocrine disruptor. Exposure to BPA is widespread and is metabolized by the liver through sulfonation and glucuronidation. BPA and obesity have been previously linked and thus BPA glucuronidation may be impaired in obese individuals.

Objectives

To determine the effects of fasting and obesity on BPA glucuronidation in mouse liver.

Methods

The capacity for BPA glucuronidation was determined in wild-type and in ob/ob mouse liver in both a fasted and fed state, and subsequently quantified by HPLC-UV.

Results

Compared to fed wild type mice (100%), BPA glucuronidation was reduced to 60% in wild type fasted mice, 39% in fed ob/ob mice, and 46% in fasted ob/ob mice. In addition, it was determined that two UGTs in mouse liver are responsible for BPA glucuronidation, a low K_m and a high K_m UGT.

Conclusion

These findings indicate that the capacity of BPA metabolism through glucuronidation is reduced in fasted and obese conditions in mice compared to wild type mice.

KEYWORDS
Bisphenol A, phase-II, Uridine 5'-diphospho-glucuronosyltransferase, obesity

Abbreviations

BPA	bisphenol A
BPA-G	bisphenol A-glucuronide
MRP	multidrug resistance-associated protein
SULT	sulfotransferase
UGT	uridine 5'-diphospho-glucuronosyltransferase
Srebf	sterol regulatory element binding transcription factor
NSAID	nonsteroidal anti-inflammatory drugs
UDPGA	uridine 5′-diphosphoglucuronic acid
HPLC	high pressure chromatography
Tris	tris(hydroxymethyl)aminomethane hydrochloride
EDTA	ethylenediaminetetraacetic acid
BHT	butylated hydroxytoluene

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INTRODUCTION

Bisphenol A (BPA), a synthetic industrial chemical within a larger group of bisphenols, is a potential endocrine disruptor with extensive exposure in humans. BPA was first synthesized in 1891 by Alexander Dianin (Dianin, 1891) and its estrogenic properties were later discovered in 1936 but considered a weak estrogen compared to other compounds (Dodds and Lawson, 1936). It was later identified as a monomer in polycarbonate plastics often used in plastic bottles, food cans, shampoo, lotion, soap, epoxy resins, and dental sealants with the dietary exposure being the primary source of human exposure (Shelby, 2008; Dodson *et al.*, 2012). In 2003, in a study of over 2,000 participants, 92.6% had detectable concentrations of BPA in urine (Calafat *et al.*, 2008).

Several studies have linked BPA to potential learning impairments (Mhaouty-Kodja *et al.*, 2018), dysregulation of the estrous cycle (Viguie *et al.*, 2018), and metabolic diseases including diabetes and obesity (Le Magueresse-Battistoni *et al.*, 2018). BPA and obesity had been associated as early as 2001 (Rubin *et al.*, 2001), but the mechanism of action was not well understood until recently. Ke and coworkers observed mice exposed to BPA for 10 months showed accumulation of hepatic triglycerides and cholesterol (Ke *et al.*, 2016). These effects were attributed to reprograming of the DNA methylation patterns of Srebf1 and Srebf2 (Sterol Regulatory Element Binding Transcription Factor). Another study has linked an increase in de novo fatty acid synthesis to BPA exposure, following a nonmonotonic dose-response curve with larger effects at low doses (Marmugi *et al.*, 2012). In 2012, a

study of approximately 3400 Chinese patients showed that high urinary BPA concentration was positively associated with increased obesity and insulin resistance (Wang *et al.*, 2012). Because of these findings, investigation into the metabolism of BPA in diseased states is warranted.

BPA is primarily metabolized in the liver through sulfate and glucuronidation conjugates which are then eliminated through urine or feces (Pritchett *et al.*, 2002; Hanioka *et al.*, 2008). BPA can exist as either a mono- or di-sulfate but both are minor metabolites compared to BPA-glucuronide (BPA-G) in both humans and rodents (Nishiyama *et al.*, 2002; Volkel *et al.*, 2002; Teeguarden *et al.*, 2015; Thayer *et al.*, 2015). In humans, urinary excretion is the predominate pathway of elimination, but in rodents, most BPA-G is eliminated through feces (Kurebayashi *et al.*, 2003) possibly because BPA-G shows high affinity for rodent mrp2, allowing for BPA-G to enter the bile duct (Mazur *et al.*, 2012). In humans, BPA-G shows affinity for MRP3 (multidrug resistance-associated protein) but not MRP2, accounting for species differences in elimination (Mazur *et al.*, 2012). In rodents, conjugated BPA has been shown to be deconjugated and undergo enterohepatic recirculation unlike humans (Sakamoto *et al.*, 2002; Doerge *et al.*, 2010) but two other studies have shown similar pharmacokinetics between primates and mice, suggesting that rodents are still a relevant model for BPA elimination (Tominaga *et al.*, 2006; Taylor *et al.*, 2011).

The sulfonation of BPA occurs through a class of phase-II detoxification enzymes called sulfotransferases. Sulfonation of BPA is predominately catalyzed by

sulfotransferase 1A1/1A3 (SULT1A1/1A3), and hepatic sulfonation of BPA has been shown to be reduced in diseased livers and obese mice (Yalcin *et al.*, 2016). The demonstration of sulfonation being decreased in obese mice suggests that BPA glucuronidation may also be altered in obesity. Indeed, test subjects with high BMI exhibited higher serum BPA levels than low BMI subjects after oral exposure to a test dose of BPA (Nishiyama *et al.*, 2002; Volkel *et al.*, 2002; Teeguarden *et al.*, 2015; Thayer *et al.*, 2015). Glucuronidation occurs through a class of enzymes called UDP-glucuronosyltransferases (UGT) which adds a glucuronide moiety to a variety of substrates such as NSAIDs (nonsteroidal anti-inflammatory drugs), opioids, anti-psychotics, anti-depressants, and contraceptives (Coffman *et al.*, 1998; Liston *et al.*, 2001; Edelman *et al.*, 2010). The UGT family is divided into two subgroups, UGT1 and UGT2 each containing several isoforms. In humans, BPA is believed to be metabolized by UGT2B15 and in rodents by ugt2B1 in rodents (Yokota *et al.*, 1999; Hanioka *et al.*, 2008). In a recent study, researchers knocked out the UGT2 family in mice, yet still observed BPA being glucuronidated, with only minor differences compared to wild-type (Fay *et al.*, 2015). The results of this study suggest that, in mice, the UGT1 family is partially responsible for BPA glucuronidation. Expression of the UGT2 family in rats has been shown to be tied to age, and specifically UGT2B15 in humans, which raises concerns of BPA exposure in pregnant females or young children (Coughtrie *et al.*, 1988; Divakaran *et al.*, 2014). The effects of obesity on UGT expression is filled with conflicting results, some demonstrating an increase in UGT activity, some reporting no change, and other showing decreases as reviewed by Merrell and Cherrington (Merrell and Cherrington, 2011). Herein, we describe the

decrease of BPA glucuronidation in liver tissue from obese mice, and show that more than one UGT may be responsible for glucuronidation.

MATERIALS AND METHODS

Chemicals

HPLC grade solvents and all reagents for microsomal preparation were purchased from Fisher Scientific (Waltham, MA). Bisphenol A and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Millipore Sigma (Burlington, MA). Bisphenol A glucuronide was purchased from Toronto Research Chemicals (North York, ON, Canada).

Animal treatment and fasting

Adult male 8 week old C57BL/6 (WT, n=8/group) and $Lep^{-/-}$ (B6.V-Lepob/J, ob/ob, n=8/group) (Jackson Laboratories, Bar Harbor, ME, USA) were ad libitum fed Harlan TekladLM-485 Mouse/Rat sterilizable diet. Fasted mice had food withheld for 24 hours immediately before sacrifice. Mice were housed in cages with corncob bedding, in a humidity, light and temperature-controlled environment within Fogarty Hall at the University of Rhode Island, Kingston, RI. IACUC approval was obtained prior to start of experiments. Livers were collected and snap frozen in liquid nitrogen and stored at -80^{o}C for further analysis.

Microsome isolation from mouse liver tissue

Approximately 150 mg of previously collected tissue was homogenized and prepared as previously reported (Yalcin *et al.*, 2013). Briefly, tissue was homogenized in sucrose/Tris/EDTA/BHT buffer and centrifuged to separate cytosolic and microsomal fractions. The microsomal pellet was resuspended by sonication in buffer containing 0.25M Sucrose, 10mM Tris-HCl (pH-7.8), 1mM EDTA, 20μM BHT and 20% glycerol. Protein concentrations were determined by Nano Drop ND1000 at 280 nm (Thermo Fisher Scientific, Waltham, MA)

BPA-glucuronidation assay

Glucuronidation assays for each liver microsome sample was performed in triplicate, with the average of the data being analyzed. The assay was optimized to ensure linearity with regards to all aspects. Mouse liver microsome (0.05 mg/mL) was incubated with 2 mM uridine diphosphate glucuronic acid (UDPGA) and various concentrations of BPA (1 to 50 μM) in 20 mM potassium phosphate (pH 7.0). Reaction was incubated at 37^{o}C for 15 min, then stopped by placing in boiling water for 30 sec, and centrifuging at 15,000 x g for 15 minutes to pellet the protein. BPA and BPA-G were quantified by HPLC with UV detection. 20 μl of reaction mixture was injected onto an Agilent Zorbax SB-Aq column (150 x 4.6 mm, 5 μm) (Santa Clara, CA) connected to a Hitachi LaChrome Elite HPLC with a L-2450 diode array detector (Tokyo, Japan). Mobile phase A consisted of 20 mM potassium phosphate buffer (pH 2.7), and mobile phase B consisted of acetonitrile. A linear gradient of B over 2 minutes from 20% to 50%, held for 6 minutes, then returned to starting conditions for

4 minutes at a flow rate of 1 mL/min was used to analyze BPA (6.6 min) and BPA-G (4.8 min) at 200 nm.

Statistical Analysis

Group comparisons were analyzed for statistical significance by ANOVA in GraphPad Prism 8 (La Jolla, CA). K_m and V_{max} were calculated with an n = 8, in triplicates per group with Prism 8's non-linear kinetics (Michaelis-Menten and two enzyme model) function. Best fit model was determined by Akaike criterion by comparing different models relative to each other.

RESULTS

BPA was separated from BPA-G as described in Methods. BPA had a retention time of 6.6 min, and BPA-G had a retention time of 4.8 min (Figure 1). A calibration curve of BPA-G from 0.1 μM to 10 μM was used to convert BPA-G peak area to concentration of BPA-G (μM). This curve was used to quantify BPA-G in the glucuronidation assay mixture with microsomal fraction from liver tissue of 32 mice (n=8 per group): wild type fed, wild type fasted, ob/ob fed, and ob/ob fasted. The data from various BPA concentrations were then put into GraphPad Prism to determine the best fit model by comparing Michaelis-Menten models and a two-enzyme model by Akaike criterion (Table 1). The two-enzyme model was determined to be the best fit to determine K_m and V_{max}. The kinetic parameters for the higher (10-50 μM BPA) could be elucidated, but the model was unable to determine the K_m and V_{max} for the other isoenzyme due to lack of data in the lower range (Figure 2, Table 2). The Lineweaver-

Burke plots for both the high and low K_m are shown to further demonstrate the two different isoforms present (Figure 3). A single enzyme Michaelis-Menten model of 1-2 μM BPA was used to determine approximate K_m and V_{max} for the lower range (Figure 4). BPA glucuronidation in wild type mice at 30 μM BPA averaged 449.7 pmol/min/mg and subsequently reduced to 60% in wild type fasted mice, 39% in fed ob/ob mice, and 46% in fasted ob/ob mice (Figure 5). Increased free fatty acids and triglycerides in both serum and the liver are characteristics of obesity. In order to better understand if glucuronidation is correlated to these characteristics, Pearson correlations were conducted with the calculated V_{max} values. The measured fatty acids and triglycerides were previously measured and published by our group (Figure 6) (Yalcin *et al.*, 2016). BPA glucuronidation demonstrates significant inverse correlation with liver triglycerides and serum free fatty acids (Figure 6A, 6D).

DISCUSSION

In this study we demonstrate the effects of obesity and fasting on wild type and ob/ob mice. With the use of the Akaike criterion and a Lineweaver-Burke plot, based on our results there are two different isoforms of Ugt capable of BPA glucuronidation. The lower K_m value could not be fully elucidated from the two-enzyme model as not enough data points were measured at such a low concentration. We thus used a typical Michaelis-Menten graph in order to better estimate the K_m of this Ugt to approximately 2 μM. The two-enzyme model was used in the 10-50 μM range to determine the higher K_m of 13.3 μM and 17.8 μM in WT and ob/ob mice respectively. This finding is supported by previous research where a UGT2 family mouse knockout

model was produced, but mice retained the majority of their capacity to conjugate BPA (Fay *et al.*, 2015). Human data demonstrated the highest activity of BPA glucuronidation by UGT2B15 at under 5 μM (80%) where at higher concentrations UGT1A9 becomes the predominate isoform for glucuronidation (Street *et al.*, 2017). While most studies examine the metabolism of BPA in rats, BPA is metabolized by different UGTs than that in mice (Ugt1a9, Ugt2b1) and humans (UGT1A9, UGT2B15). Thus, the potential Ugts of BPA glucuronidation are Ugt1a9 and Ugt2b1. While more studies using recombinant mouse enzymes or specific Ugt inhibitors would be needed to confirm the specific Ugts responsible for mouse glucuronidation, the identification of these would allow for better use of a mouse model in BPA metabolism.

Overall, we observed large reductions in Ugt activity in fasting and obese models compared to WT. These large reductions in capacity to metabolize BPA are concerning as BPA is a known endocrine disruptor. Reductions in conjugation of BPA will result in a large AUC of BPA. While fasting appeared to reduce conjugation in the WT mice, this reduction was no observed in the obese mice. Short term fasting is known to increase liver triglycerides and in mice can lead to hepatic steatosis (Guan *et al.*, 2009). This explains why there were smaller reductions between the fasted WT mice and the fed obese mice, as their livers were in a similar state. In addition, the addition of fasting did not promote anymore significant loss of conjugation as the livers were already in a fatty state.

While we observed a reduced capacity of BPA glucuronidation in this study, and reduced sulfonation in our previous work (Yalcin *et al.*, 2016), this does not fully agree with previous mRNA data showing an upregulation of Ugt1a9 in mRNA in obese and fasting states while Ugt2b1 is downregulated by obesity but upregulated in fasting (Xu *et al.*, 2012). UGT activity was also tested within the same study using acetaminophen, a known UGT1A9 substrate, and more closely matches our current data. The mRNA does not appear to correlate to the activity of the Ugts suggesting translational and post-translational issues could be at play. Additional studies with proteomics work on these livers could elucidate the potential mechanism in this system.

In summary, we report a significant decrease in BPA glucuronidation in obese and fasted mice and that two different isoforms are responsible for glucuronidation in mice. At low concentrations of BPA, one isoform is the predominate UGT, while at higher concentrations, a presumably less specific UGT can metabolize BPA. This suggests that obesity may increase BPA exposure through a reduction in conjugation resulting in an increase in AUC of BPA and its endocrine disruption.

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FIGURES

Figure 1. Separation on an Agilent Zorbax SB-Aq column. Detection was by UV at 200 nm. Bisphenol A-glucuronide was eluted with a mobile phase linear gradient of 20-50% acetonitrile in 20 mM potassium phosphate pH 2.7 as described in Methods.

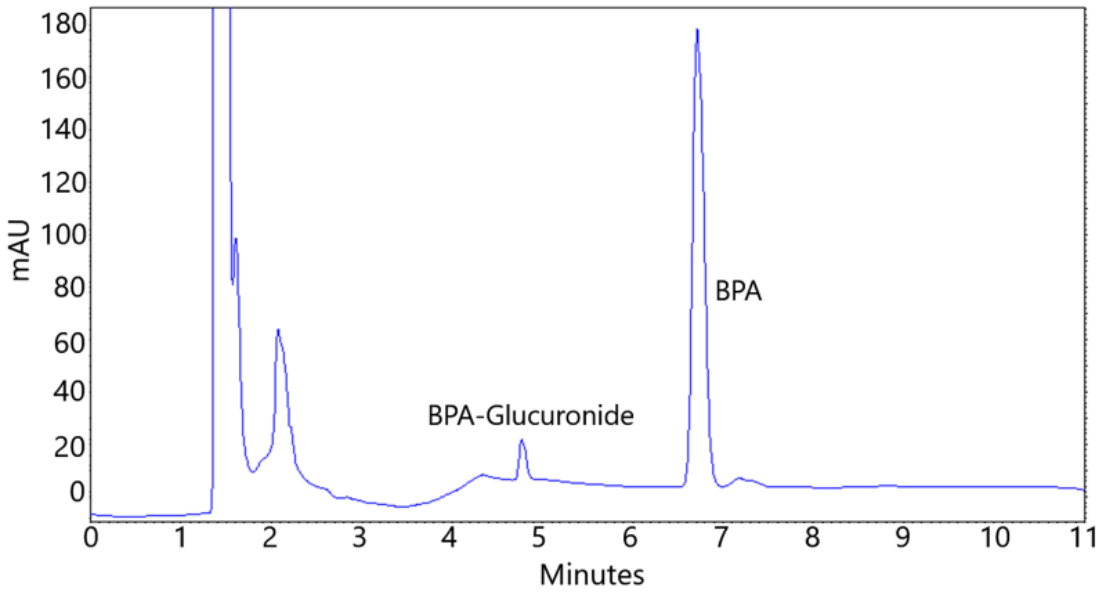


Figure 2. Two Enzyme Model (V/S). Evaluation of BPA glucuronidation rates over a range of BPA substrate concentrations (1 uM to 50 uM) to enable kinetic constant determination. All points were the average of 8 mice in triplicates. Best fit model was determined by Akaike criterion by comparing different models relative to each other.

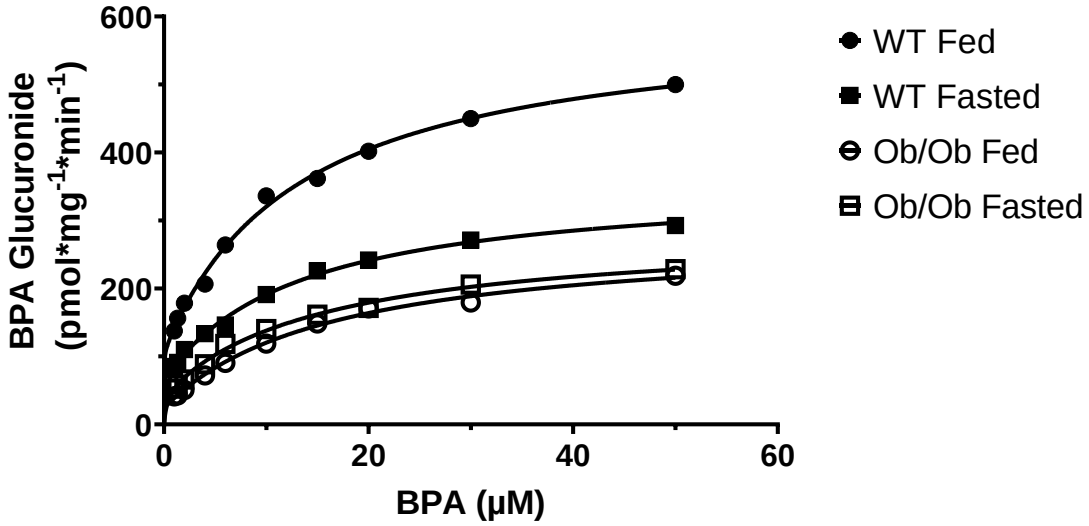


Figure 3. Lineweaver-Burke (1/v vs 1/s) plot. Indicates multiple isoforms contribute to BPA glucuronidation in mouse liver microsomes. All points were the average of 8 mice in triplicates.

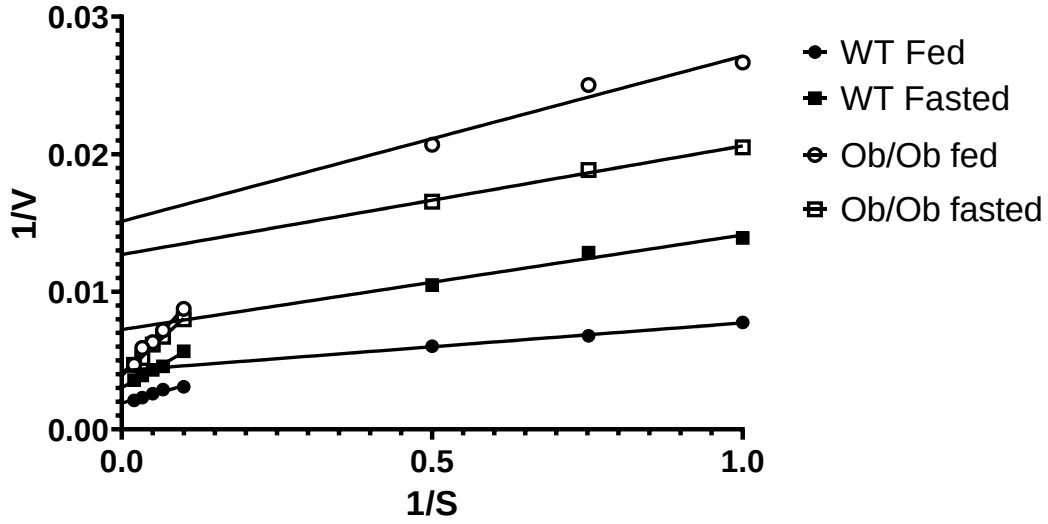


Figure 4. Michaelis-Menten Plot (1-2 uM) (V/S). Evaluation of BPA glucuronidation over a range of BPA from 1 uM to 2 uM to enable kinetic constant determination. All points were the average of 8 mice in triplicates.

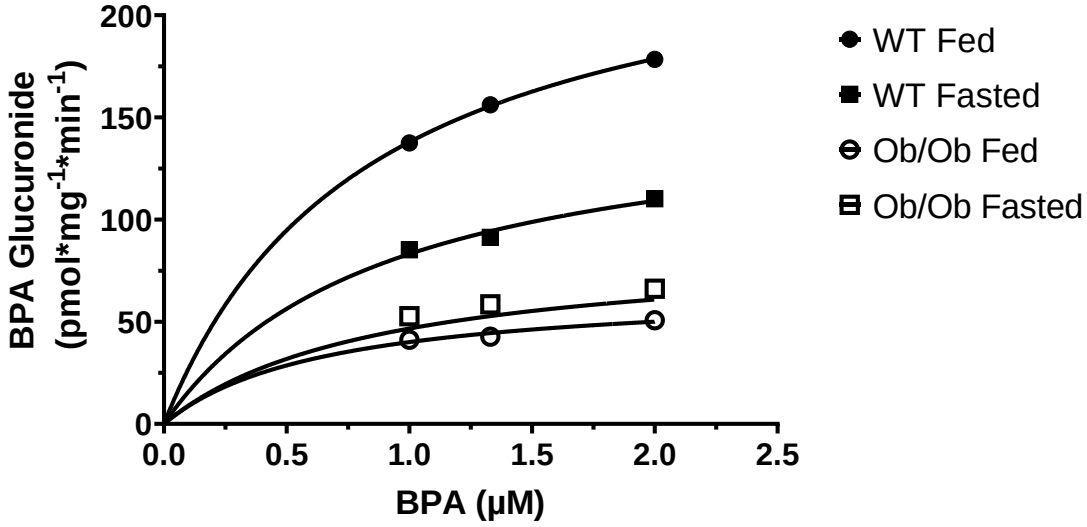


Figure 5. Comparison of mouse BPA glucuronidation activity in wild type (WT) versus obese (ob/ob). BPA glucuronidation activity was determined by incubating 30 µM BPA with mouse liver cytosol for 15 min in the presence of 2 mM UDPGA and 20 mM potassium phosphate pH 7.0. Different letters indicate statisical significance between the groups ($p<0.05$), with similar letters indicating not statisicially significant.

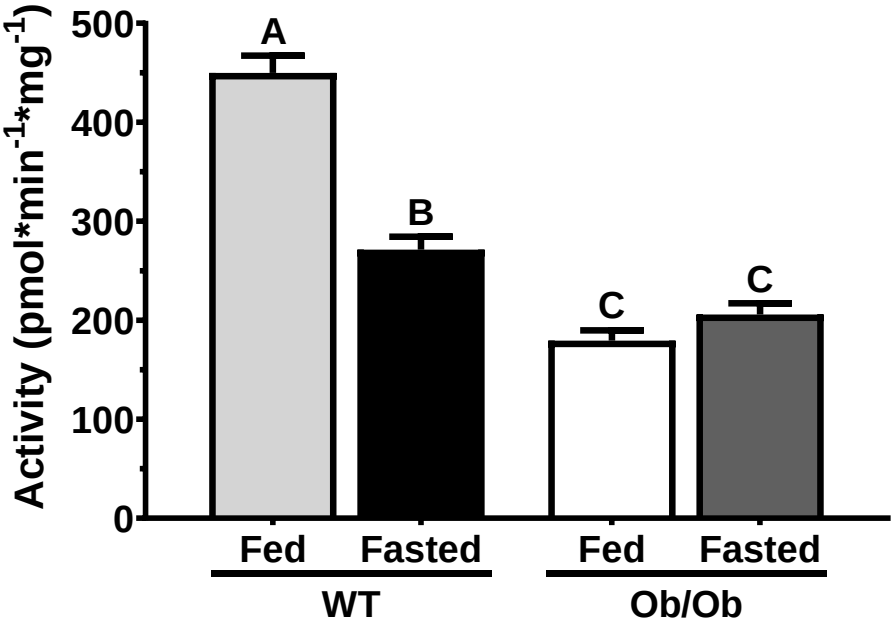
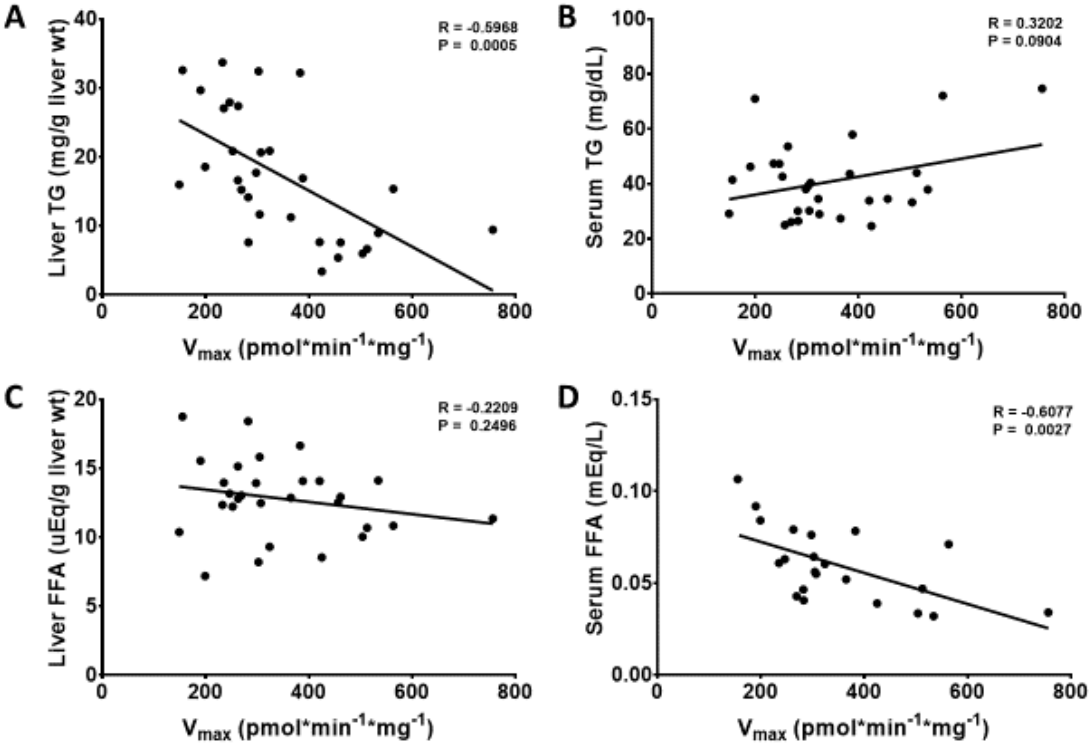


Figure 6. Correlation of BPA glucuronidation (V_{max}) in WT and ob/ob mice under both fed and fasted states (n=8/group) with **(A)** liver triglycerides (mg/g liver wt), **(B)** liver free fatty acids (uEq/g liver wt), **(C)** serum triglycerides (mg/dL), and **(D)** serum free fatty acids (mEq/L). Correlations were obtained in GraphPad Prism 8 by Pearson correlation. $P < 0.05$ were considered significant.



TABLES

Table 1. A test was run to determine the best fit model for the data by checking the Akaike criterion. The model with the lower AIC values are the best fit models for the data. $P < 0.05$ were considered significant.

	WT fed	WT fasted	Ob/Ob fed	Ob/Ob fasted
One Enzyme Model AIC	1995	1780	1449	1597
Two Enzyme Model AIC	1962	1756	1433	1585
Preferred Model	Two Enzyme	Two Enzyme	Two Enzyme	Two Enzyme
P Value	<0.0001	<0.0001	<0.0001	0.0004

Table 2. Calculated kinetic parameters of the two-enzyme model for the higher K_m isoform. V_{max} is expressed in pmol*min^{-1}*mg^{-1}. K_m is expressed in μM.

	10-50 uM BPA	
	V_{max}	K_m
C57Bl/6 fed	493.0	13.2
C57Bl/6 fasted	292.4	13.4
Ob/Ob fed	253.1	17.4
Ob/Ob fasted	236.9	16.3

CHAPTER 2

Evaluation of 125 Drugs as Inhibitors of Sulfotransferase 2A1 (SULT2A1), Bile Acid Coenzyme A Synthetase (BACS) and Bile Acid-CoA: Amino Acid N-Acyltransferase (BAAT)

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(Prepared for submission to Drug Metabolism and Disposition)

Evaluation of 125 Drugs as Inhibitors of Sulfotransferase 2A1 (SULT2A1), Bile Acid Coenzyme A Synthetase (BACS), and Bile Acid-CoA: Amino Acid N-Acyltransferase (BAAT)

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Running Title
125 Drugs as Potential Inhibitors of SULT2A1 and Amidation

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Abbreviations

BACS	bile acid coenzyme A synthetase
BAAT	bile acid coenzyme A: amino acid N-acyltransferase
Bsep	bile salt export pump
C4	7α-hydroxy-4-cholesten-3-one
CA	cholic acid
CDCA	chenodeoxycholic acid
CYP	cytochrome P450
DCA	deoxycholic acid
DILI	drug-induced liver injury
LCA	lithocholic acid
LCA-S	lithocholic acid sulfate
LC-MS	liquid chromatography-mass spectrometry
NHR	nuclear hormone receptors
NSAID	nonsteroidal anti-inflammatory drug
PAPS	3'-phosphoadenosine-5'-phosphosulfate
SULT	sulfotransferase
TCA	taurocholic acid
UGT	uridine 5'-diphospho-glucuronosyltransferase
VBDS	vanishing bile duct syndrome

ABSTRACT

Hepatic bile acid conjugation is a crucial function of human liver, and inhibition of bile acid metabolism can lead to potential liver injury through bile acid retention. Because drug-induced liver injury (DILI) is one of the most frequent reasons for drug withdrawal and mechanisms for DILI of some drugs are unknown, there is potential of DILI to be mediated through inhibition of bile acid amidation and sulfonation. We investigated 125 compounds for their potential inhibition of lithocholic acid sulfonation and cholic acid amidation. Enzymatic assays were developed for human sulfotransferase 2A1 (SULT2A1) and bile acid coenzyme A synthetase and bile acid coenzyme A: amino acid N-acyltransferase (BACS/BAAT). The two-step bile acid amidation was conducted in a single incubation mixture using human S9 fractions. Samples were analyzed by LC/MS/MS, and data were analyzed using non-linear regression to determine inhibition (IC50) values. Of 125 compounds tested for inhibition of LCA-sulfonation, nine had IC50 values less than 100 μM (range 0.73 – 99 μM), of which four are known to cause liver injury. Only one compound inhibited bile acid amidation (IC50 70 μM). Based on the results of this study, it does not appear inhibition of sulfonation or amidation is a significant cause of drug induced liver injury, but several new sulfotransferase 2A1 inhibitors were discovered, and a novel single-incubation amidation assay was developed.

INTRODUCTION

Hepatic bile acid synthesis, secretion, and conjugation are crucial functions of the human liver. Bile acids are amphiphilic molecules derived from cholesterol with important roles in metabolic signaling, absorption of lipids, cholesterol, fat and fat-soluble vitamins. However, over-accumulation of the detergent-like bile acids within the liver can be cytotoxic, leading to inflammation, fibrosis, cholestasis, cirrhosis, and liver failure. Excess bile acids have been shown to break down the cell membranes of hepatocytes (Scholmerich et al., 1984; Attili et al., 1986) and alter the membrane permeability transition of the mitochondria, leading to apoptosis or necrosis (Gores et al., 1998; Rolo et al., 2000).

The intrahepatic buildup of bile acids can be caused by several different mechanisms, but one of particular concern is as a side effect of drugs. The cause of this drug-induced liver injury (DILI) is often attributed to Bile Salt Export Pump (Bsep) inhibition, for example rifampicin, cyclosporin A, rifamycin, bosentan, and troglitazone leading to cholestasis in rats (Bohme et al., 1993; Stieger et al., 2000; Fattinger et al., 2001; Funk et al., 2001). Other DILI-associated drugs target the bile duct epithelium leading to vanishing bile duct syndrome (VBDS) also called drug-induced bile duct injury. VBDS refers to the destruction and disappearance of intrahepatic bile ducts which ultimately results in cholestasis (Reau and Jensen, 2008). While the effects of VBDS are sometimes permanent, often discontinuation of exposure to the drug causing VBDS can stop the progression and in some cases, reverse it (Ramos et al., 2002; Leeuwenburgh et al., 2008). Typically in humans, only

a subset of patients experience liver injury (Ortega-Alonso and Andrade, 2018) and once drug treatment is ceased, most patients' biomarkers return to normal, but some patients experience progressive and more permanent injury (Navarro and Senior, 2006).

Bile acid concentrations in the liver tissue are controlled by the combination of synthesis, secretion and conjugation. Bile acid synthesis occurs through one of two pathways, with the most common pathway being synthesis from cholesterol by the cytochrome P450 (CYP) enzymes, specifically cytochrome P450 7A1 (CYP7A1) to 7α-hydroxy-4-cholesten-3-one (C4), leading to the synthesis of cholic acid (CA) or chenodeoxycholic acid (CDCA) by CYP8B1 and CYP27A1, respectively (Chiang, 2004). The alternative pathway bypasses C4 and only produces CDCA by metabolizing cholesterol into 27-hydroxycholesterol via CYP7B1 (Chiang, 2004). Both CA and CDCA undergo conjugation to a polar group or reductive metabolism. Reductive metabolism into a secondary bile acid, i.e. deoxycholic acid (DCA) or lithocholic acid (LCA), is catalyzed by bacterial enzymes within the intestinal microflora (Hirano et al., 1981; Wells et al., 2000; Wells et al., 2003).

The majority of bile acids *in vivo* exist in a conjugated state which functions to alter the physiochemical properties by conjugation with taurine, glycine, sulfate or glucuronide, reducing hydrophobicity and toxicity through an increase in polarity (Trottier et al., 2011; Trottier et al., 2012). Amidation occurs through sequential action of two enzymes: bile acid coenzyme A synthetase (BACS) in the endoplasmic

reticulum, and bile acid coenzyme A: amino acid N-acyltransferase (BAAT) in the cytosol (Killenberg, 1978). The process of amidation adds an amine, i.e. taurine or glycine, to the carboxylic acid moiety of the unconjugated bile acid, enhancing aqueous solubility. Alternatively, glucuronidation adds a glucuronide to one of several sites containing an -OH moiety on the bile acid, resulting in a more hydrophilic and readily excreted compound. A specific uridine 5'-diphospho-glucuronosyltransferase (UGT) enzyme catalyzes the glucuronidation reaction, depending upon the particular -OH site of glucuronidation (Gall et al., 1999; Barbier et al., 2003; Trottier et al., 2006; Barbier et al., 2009). Lastly, sulfonation which is a minor reaction for most bile acids is the predominate conjugation mechanism of lithocholic acid (LCA), the most toxic bile acid. Sulfonation occurs through a class of cytosolic enzymes called sulfotransferases (SULT). The major bile acid sulfotransferase is SULT2A1, which almost exclusively sulfonates LCA (Huang et al., 2010).

The metabolic signaling function of bile acids has been shown in apoptosis, glucose homeostasis, cholesterol homeostasis, and as ligands for many nuclear hormone receptors (NHR) (Nguyen and Bouscarel, 2008; Carazo et al., 2017; Mueller et al., 2017). The levels of specific bile acids in the bile acid pool are tightly controlled by many transporters and NHRs. In addition, these bile acids are capable of altering the expression of many other NHRs and transporters within the system through their action as NHR ligands (Rodrigues et al., 2014). For example, bile acids binding to the nuclear receptors PXR, CAR or PPARα can cause an upregulation of SULT2A1,

thereby increasing bile acid sulfonation (Saini et al., 2004; Fang et al., 2005; Wunsch et al., 2015). During cholestasis, however, there is a large increase in circulating bile acids, with a 10-fold increase in sulfated bile acids, a 666-fold increase in amidated bile acids, and a shift from glyco- to tauro-bile acids (Humbert et al., 2012).

As DILI is one of the most frequent reasons for drug withdrawal, there is potential of DILI to be mediated through inhibition of bile acid amidation and sulfonation. We investigated the potential inhibition of 125 compounds on sulfonation (SULT2A1) and amidation (BACS/BAAT) of lithocholic acid and cholic acid.

METHODS

Chemicals

LC-MS grade acetonitrile, LC-MS grade formic acid, potassium phosphate (mono- and di-basic), adenosine triphosphate, and taurine were purchased from Fisher Scientific (Waltham, MA). 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was purchased from R&D Systems (Minneapolis, MN). Lithocholic acid sulfate was purchased from Santa Cruz Biotechnology (Dallas, Texas). Lithocholic acid, cholic acid, and taurocholic acid were purchased from Steraloids (Newport, RI). Coenzyme A, magnesium chloride, dithiothreitol, clomiphene, pooled mixed gender human cytosol (Product #C2988, Lot #SLBP8169V) were purchased from Millipore Sigma (St. Louis, MO). Pooled mixed gender human S9 (Product #H0620.S9, Lot #1610015) was purchased from Sekisui XenoTech, LLC (Kansas City, KS). Test compounds were provided by Pfizer Inc (Groton, CT) as 30 mM solution in DMSO with final assay concentrations of DMSO at 1% (Table 1).

Sulfonation Assay

SULT2A1-catalyzed LCA sulfonation activity was determined in the absence and presence of test compound in a total assay volume of 50 μL. In a microcentrifuge tube was placed 50 mM potassium phosphate (pH 7.0), 1 μM PAPS, 1 μM LCA, and various concentrations of test compound. The reaction was started by the addition of human liver cytosol (0.5 mg/mL) and placed in a shaking water bath at 37°C for 30 min and stopped by placing in boiling water for 30 sec. The mixture was centrifuged at 15,000 x g for 15 minutes to pellet the protein. Supernatant (10 μL) was then injected onto Waters XBridge C18 column (50 x 2.1 mm, 3.5 μm, Milford, MA) connected to a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) coupled to Sciex 4500 Q-Trap ESI (Sciex, Framingham, MA, USA). Mobile phase A consisted of water with 0.1% formic acid; mobile phase B consisted of acetonitrile with 0.1% formic acid. At a flow rate of 0.5 mL/min, the column was equilibrated with 20% B. Sample was injected, held for 1 min at 20% B, then increased linearly to 95% B over 4 min, then held for 2 min. The column was then re-equilibrated at 20% B for 2 minutes before the next injection. The mass spectrometer was operated in negative ion mode with the following settings: ISV, -4500; temperature, 500°C; DP, -65; EP, -10; CE, -65. LCA-S was measured through MRM mode (455.3→97.0) by comparing to a prepared calibration curve of LCA-S. Assays were performed in triplicate with the mean of the replicates being analyzed (SEM less than 10%). Clomiphene was used as a positive control for SULT2A1 inhibition.

Test compounds were first screened for % inhibition of LCA sulfonation at a single concentration (300 μM), and those with >10% inhibition (<90% activity remaining)

were further screened at four additional concentrations (100 μM, 10 μM, 1 μM, and 100 nM). Those observed to have an estimated IC_{50} value less than 100 μM were further analyzed at eight concentrations ranging from 300 μM to 100 nM.

Amidation Assay

Amidation activity was determined by measuring CA conjugation with taurine in the absence and presence of test compound in a total assay volume of 50 μL. In a microcentrifuge tube was placed 50 mM potassium phosphate (pH 7.0), 100 μM CA, 1 μM coenzyme A, 2 mM taurine, 5 mM $MgCl_2$, 5 mM ATP and various concentrations of test compound. The reaction was started by the addition of human liver S9 (0.3 mg/mL) and placed in a shaking water bath at 37°C for 60 min and stopped by placing in boiling water for 30 sec. The mixture was then centrifuged at 15,000 x g for 15 minutes to pellet the protein. The supernatant (10 μL) was then injected into Waters XBridge C18 column (50 x 2.1 mm, 3.5 μm, Milford, MA) connected to a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) coupled to Sciex 4500 Q-Trap ESI (Sciex, Framingham, MA, USA). Mobile phase A consisted of water with 0.1% formic acid; mobile phase B consisted of acetonitrile with 0.1% formic acid. At a flow rate of 0.5 mL/min, the column was equilibrated with 20% B. Sample was injected at 20% B, increased linearly to 50% B over 4 min, then increased to 90% B over 2 min and held for 2 min. The column was then re-equilibrated at 20% B for 2 minutes before the next injection. The mass spectrometer was operated in negative ion mode with the following settings: ISV, -4500; temperature, 400°C; DP, -50; EP, -10; CE, -117. TCA was measured through MRM mode (514.7→79.9) by comparing to a prepared calibration curve of TCA. Assays were performed in triplicate with the mean

of the replicates being analyzed (SEM less than 10%). No positive control was available for inhibition of CA-taurine amidation.

Test compounds were first screened for % inhibition of CA amidation at a single concentration (300 μM), and those with >10% inhibition (<90% activity remaining) were further screened at four additional concentrations (100 μM, 10 μM, 1 μM, and 100 nM). Those observed to have an estimated IC_{50} value less than 100 μM were further analyzed at eight concentrations ranging from 300 μM to 100 nM.

Statistical Analysis

Statistical Analysis was conducted using GraphPad Prism 7 software (La Jolla, CA). All concentrations values were first logged, and the concentrations of product were normalized between an inhibitor free reaction, and a reaction without cytosol or S9. The resulting data were then plotted using a non-linear fit and IC50 values were determined.

RESULTS

Bile Acid Sulfonation

The chromatographic conditions of LCA-S were optimized to ensure a swift run time with optimal peak shape, allowing for a retention time of 2.72 min (Figure 1A). The peak areas were converted to concentrations using a calibration curve. In order to account for day to day variability, the concentrations were normalized to an uninhibited reaction. Of the 125 compounds tested (Table 1) only nine showed inhibition below 100 μM, of which only four were known DILI compounds.

Clomiphene, the positive control had an IC50 value of 9.26 μM, similar to a previously reported value (Bamforth et al., 1992). Several other compounds were strong inhibitors with IC50 values below 10 μM (Table 2). All the compounds except clomiphene have never been reported as inhibitors, and benzbromarone was the only compound to exhibit an IC50 value below 1 μM leading to possibilities as a positive control in future studies (Figure 2). A structurally similar compound, benziodarone, also showed strong inhibition with an IC50 value of 5.76 μM. The only structural difference between the two compounds is replacement of two bromine atoms on the benzene ring for two iodine atoms. Other notable inhibitors include benztropine (1.27 μM), clemastine (5.00 μM), and phenoxybenzamine (7.87 μM).

Bile Acid Amidation

Taurocholic acid HPLC conditions were optimized to reduce run time and produce a sharp peak, producing a retention time of 3.02 min (Figure 1B). The peak areas were converted to concentrations using a calibration curve. In order to account for day to day variability, the concentrations were normalized to an uninhibited reaction. Very few enzyme incubations have been conducted with the BACS/BAAT system and all the previous work separated the two reactions (Solaas et al., 2000). We developed a single assay to include both enzymes during our screening process. All 125 compounds were tested, and only one compound showed inhibition below 100 μM, benoxaprofen, at 70.2 μM (Figure 3).

Comparison of SULT2A1 Inhibitors

The DILI classification of the nine sulfotransferase inhibitors were compared to the DILI classification of all drugs within the screening library. Within the library only

56% were classified as DILI, but in the drugs that were determined to be SULT2A1 inhibitors only 44% were classified as DILI (Figure 4). This would indicate that there does not appear to a connection between DILI and inhibition of SULT2A1. The compound library was then further compared with a variety of values previously published by colleagues (Aleo et al., 2017). Inhibition of SULT2A1 was compared to molecular weight, log D (pH 7.4), and polar surface area (Figure 5). These values were graphed in order to determine approximate properties of SULT2A1 inhibitors and the following ranges were identified: molecular weight, 277-576; LogD, 1.2-4.2; polar surface area, 12-116.

DISCUSSION

The search for a mechanism of causation for drug induced liver injury and ways to prevent it are an ongoing effort within the pharmaceutical industry. Only a small subset of patients typically experience liver injury (Ortega-Alonso and Andrade, 2018), and after drug treatment is ceased most patients' biomarkers return to normal. However, some patients experience progressive and more permanent injury (Navarro and Senior, 2006). Often in these liver injury situations, a buildup of bile acids occurs, capable of breaking down the hepatocellular membrane, potentially leading to permanent injury (Scholmerich et al., 1984; Attili et al., 1986). In this study, we proposed that the inhibition of SULT2A1 and BACS/BAAT may be responsible for the hepatoxicity and liver injury experienced by patients taking DILI-associated drugs. To test this hypothesis, human liver cytosol and S9 were incubated with the drug of interest and bile acids, searching for compounds that may be potent inhibitors. In this

study, we are the first to report SULT2A1 inhibition with several new compounds and a novel BACS/BAAT inhibitor.

While sulfonation is overall the least predominant form of bile acid conjugation, it is the predominate mechanism of detoxification of LCA, the most toxic bile acid (Huang et al., 2010). During the screening of 125 compounds, 9 inhibitors of SULT2A1 were found of which 4 were known DILI compounds. Most of these compounds were unknown inhibitors of SULT2A1. While there does not appear to be a general correlation between DILI and the inhibition of bile acid sulfonation, it is a potential mechanism of action of individual compounds. Zafirlukast, while not a strong inhibitor, has an unknown cause of the liver injury. The symptoms of zafirlukast liver injury are similar to those of cholestasis, and could potentially be linked to the inhibition of sulfonation. The information found from this experiment would warrant further investigation to determine the exact cause of zafirlukast hepatoxicity. Also, of note, was the compounds with IC50 values below that of clomiphene, the positive control. Our reported clomiphene IC50 values were within an acceptable range based on previous literature (Bamforth et al., 1992). These compounds would allow for future studies with more effective inhibition of SULT2A1. The inhibitors of SULT2A1 were graphed against the entire library of compounds with a variety of different properties to determine if the SULT2A1 inhibitors contain any similarities. The results in molecular weight, logD, and polar surface area do show unique constraints compared to the library. There is a relatively small window of molecular

weights and logD values that inhibit SULT2A1. This information will allow for future drug design for either avoiding inhibition or to intentionally inhibit SULT2A1.

In this current study, we demonstrate a novel method for BACS/BAAT screening. While other methods have split the reaction between the two enzymes, we developed a single assay (Shonsey et al., 2005a; Shonsey et al., 2005b). The purpose is because the end product of BACS is necessary for BAAT to occur, and inhibition at either step will prevent the amidation of cholic acid. In terms of a potential DILI mechanism through inhibition, splitting the reaction is not necessary. There was a recently published study that attempted to develop a one-step amidation reaction but their results could not be replicated (Ogimura et al., 2017). We also attempted to test their methods and their compounds in our assay. We saw no inhibition with the compounds at 300 μM.

While almost all compounds did not inhibit the BACS/BAAT pathway, we did find a single inhibitor benoxaprofen, which exhibited an IC50 of 70.2 μM. Benoxaprofen is a withdrawn nonsteroidal anti-inflammatory drug (NSAID). It is believed that the deaths were caused by bioaccumulation in the liver due to a toxic metabolite. While a weak inhibitor of amidation, it is possible that the inhibition of amidation could be a cumulative effect with the toxic metabolite leading to hepatotoxicity. Further studies are warranted to investigate this potential mechanism of action.

Only a handful of compounds appeared to inhibit the conjugation of bile acids but it is still possible that drug perturbations to the bile acid pool could be the cause of liver injury. The drugs still could be altering the conjugation of bile acids through downregulation of transcription or translation of the enzymes involved. In addition, alterations of the bile acid pool could still occur, but depend more on the excretion and reabsorption of bile acids. Follow up research involving human cell lines examining transport, and gene expression would help to further understand the mechanism of action of these compounds and their relationship to drug induced liver injury.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Auclair, Costales, King, Rodrigues.

Conducted experiments: Auclair

Contributed new reagents or analytic tools: Auclair, Costales, King, Rodrigues

Performed data analysis: Auclair

Wrote or contributed to the writing of the manuscript: Auclair, Costales, King, Rodrigues

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. The MRM chromatograms of LCA-S (A) and TCA (B) with retention times of 2.72 and 3.02 min, respectively. LCA-S was measured at the m/z transition 455.3→97.0 and TCA was measured at the m/z transition of 514.7→79.9.

Figure 2. IC50 curves of all compounds with IC50 values less than 100 μM, sorted by lowest to highest IC50, left to right. Calculated by using Graphpad Prism 7, using non-linear regression fit.

Figure 3. IC50 curve of benoxaprofen inhibition of the BACS/BAAT system. IC50 value of 70.2 μM with 95% confidence intervals from 45.6 μM – 107.8 μM.

Figure 4. The percentage of drugs tests that were classified as DILI vs non-DILI in the total screening (A) and of those that were SULT2A1 inhibitors (B).

Figure 5. Comparisons of the compounds SULT2A1 IC50 values vs molecular weight (A), logD at pH 7.4 (B), and polar surface area (C).

Table 1. Alphabetical list of 125 compounds tested for inhibition of SULT2A1 and BACS/BAAT. The bolded compounds are known DILI compounds.

Table 2. List of compounds with IC50 values less than 100 μM. The compound names in bold are known DILI compounds. Clomiphene was used as a positive control. All values are calculated from triplicates at each concentration.

Figure 1.

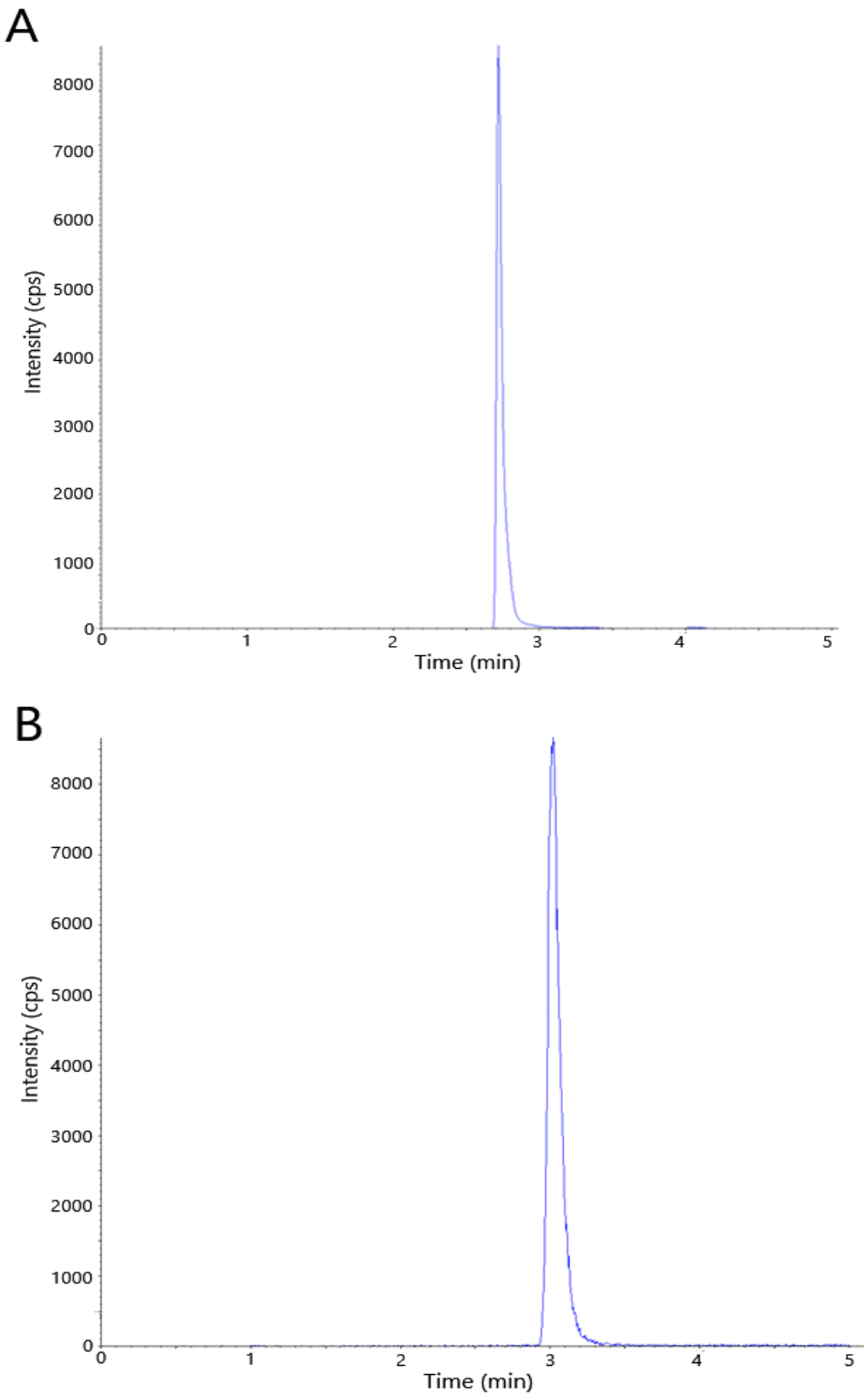
A
8000
7000
6000
5000
4000
3000
2000
1000
0
Intensity (cps)
0
1
2
3
4
5
Time (min)
B
8000
7000
6000
5000
4000
3000
2000
1000
0
Intensity (cps)
0
1
2
3
4
5
Time (min)

Figure 2.

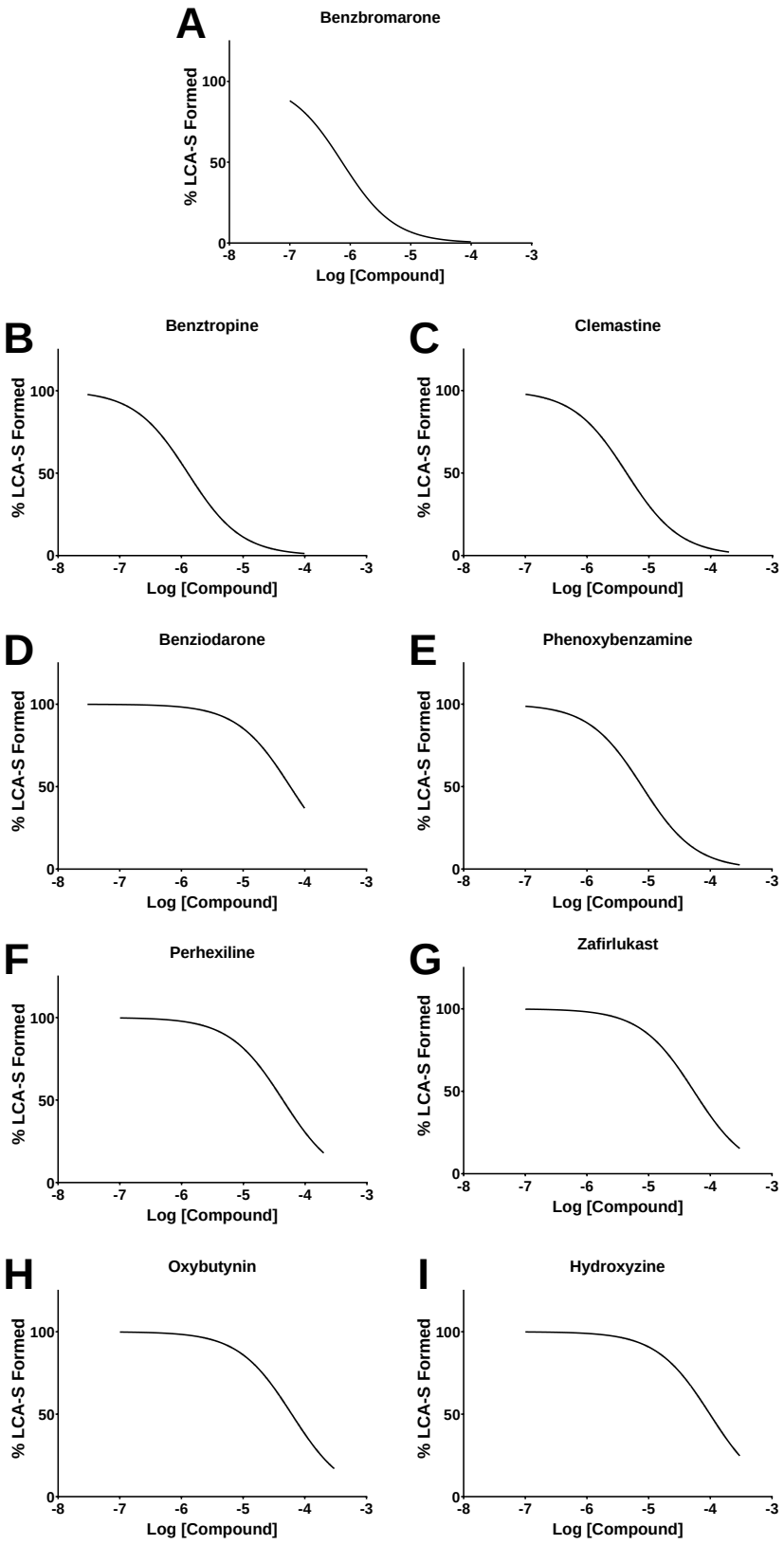
A
Benzbromarone
% LCA-S Formed
100
50
0
-8
-7
-6
-5
-4
-3
Log [Compound]
B
Benztropine
C
Clemastine
D
Benziodarone
E
Phenoxybenzamine
F
Perhexiline
G
Zafirlukast
H
Oxybutynin
I
Hydroxyzine

Figure 3.

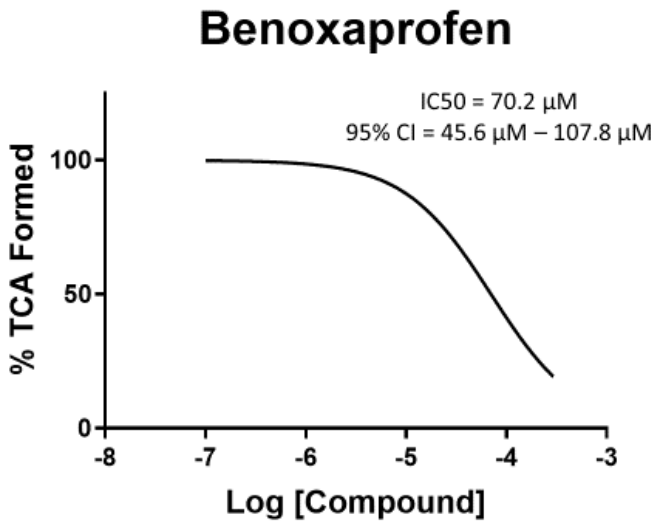
Benoxaprofen
IC50 = 70.2 µM
95% CI = 45.6 µM – 107.8 µM
% TCA Formed
100
50
0
-8
-7
-6
-5
-4
-3
Log [Compound]

Figure 4.

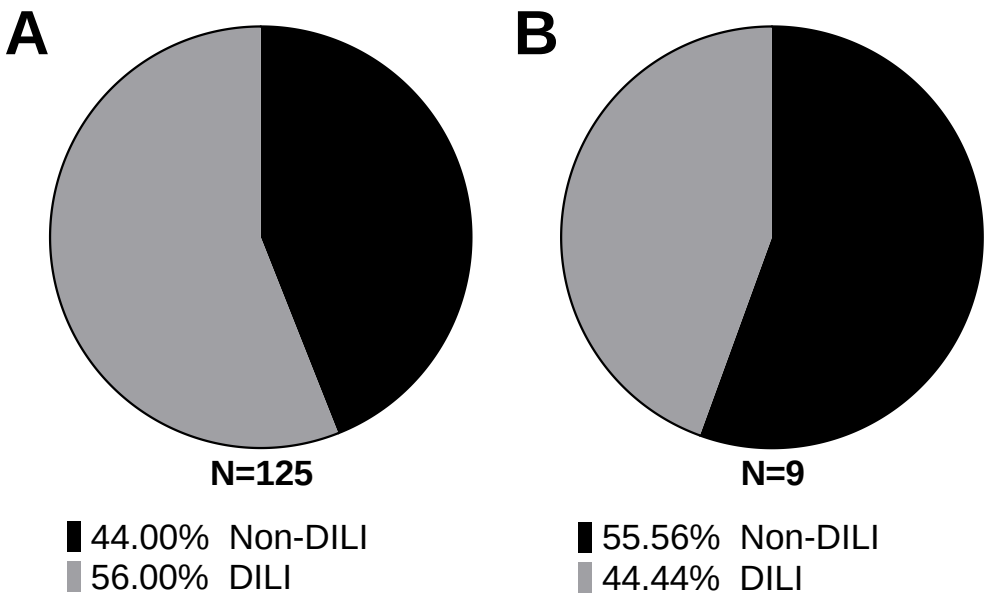

Figure 5.

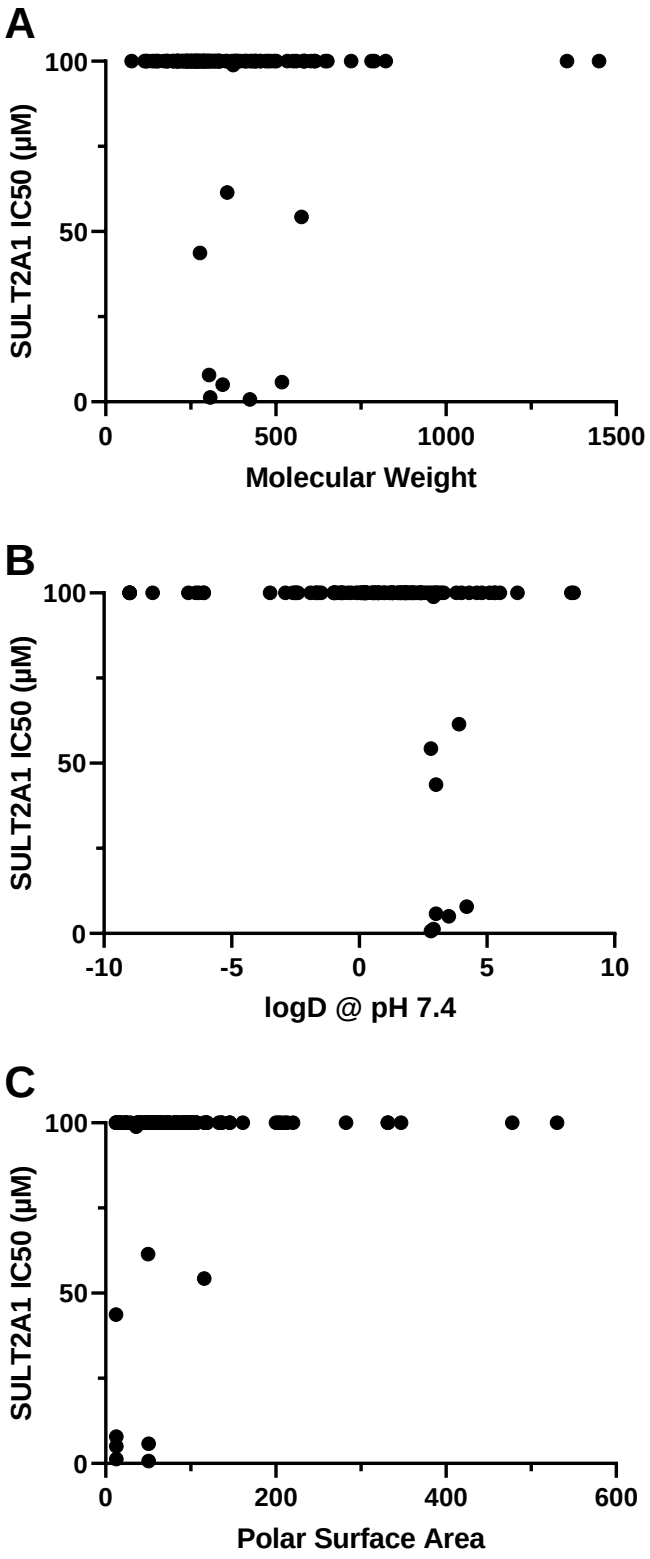
A
100
50
0
SULT2A1 IC50 (μM)
0
500
1000
1500
Molecular Weight
B
100
50
0
SULT2A1 IC50 (μM)
-10
-5
0
5
10
logD @ pH 7.4
C
100
50
0
SULT2A1 IC50 (μM)
0
200
400
600
Polar Surface Area

Table 1.

abacavir	chlorpheniramine	flutamide	**nefazodone**	**stavudine**
acetylsalicylic acid	**cinchophen**	fluvoxamine	**nevirapine**	streptomycin
albuterol	**ciprofloxacin**	folic acid	**niacin**	**sulfamethoxazole**
alendronate	clemastine	guanethidine	**nimesulide**	**sulindac**
ambrisentan	cyanocobalamin	**hydroxyurea**	**nitrofurantoin**	**tacrine**
amikacin	**dacarbazine**	hydroxyzine	**nomifensine**	**tamoxifen**
amineptine	deferoxamine	**indinavir**	**nortriptyline**	tamsulosin
aminosalicylicacid	**diclofenac**	iproniazid	**orlistat**	**tasosartan**
amiodarone	**didanosine**	**isoniazid**	oxybutynin	**tenofovir**
atropine	digoxin	isoproterenol	paliperidone	terazosin
benoxaprofen	dihydroergotamine	kanamycin	pamabrom	**ticrynafen**
benzarone	diphenhydramine	**ketoconazole**	paromomycin	**tipranavir**
benzbromarone	dobutamine	**labetalol**	**pazopanib**	**tolcapone**
benziodarone	doxylamine	**lamivudine**	penbutolol	tolterodine L-tartrate
benztropine	**ebrotidine**	**lapatinib**	**perhexiline**	trihexyphenidyl
betaine	ergocalciferol	**leflunomide**	phenazopyridine	triprolidine hcl
bicalutamide	**erythromycin estolate**	liothyronine	phenoxybenzamine	**troglitazone**
biperiden	esmolol	loratadine	phentolamine	**trovafloxacin**
bosentan	**ethambutol**	**mercaptopurine**	**pirprofen**	**valproicacid**
bromfenac	**etodolac**	metaproterenol	primaquine	vancomycin
brompheniramine	fenoprofen	**methimazole**	primidone	**verapamil**
busulfan	fexofenadine	**methotrexate**	procyclidine	**zafirlukast**
carbamazepine	fipexide	methysergide	protriptyline hcl	**zalcitabine**
carbetapentane	fluconazole	metronidazole	**rifampin**	**zidovudine**
carbidopa	fludrocortisone	minoxidil	**ritonavir**	**zimeldine**

Table 2.

Compound	SULT2A1 IC50, µM	95% CI lower, µM	95% CI upper, µM
clomiphene	9.27	5.74	14.81
benzbromarone	0.73	0.33	1.50
benztropine	1.27	0.37	4.57
clemastine	5.00	2.65	9.05
benziodarone	5.76	2.22	12.90
phenoxybenzamine	7.87	4.10	15.00
perhexiline	43.67	30.73	60.91
zafirlukast	54.29	20.24	144.00
oxybutynin	61.46	37.10	101.80
hydroxyzine	98.87	71.82	136.20

CHAPTER 3

Fenofibrate Reduces Bile Acid Toxicity During Cholestatic Liver Injury

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(Prepared for submission to Hepatology)

Fenofibrate reduces bile acid toxicity during cholestatic liver injury

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Keywords: PBC, PSC, Peroxisome proliferator-activated receptor alpha, cholestasis, fibrates

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Abbreviations

PBC	primary biliary cholangitis
PSC	primary sclerosing cholangitis
UDCA	ursodeoxycholic acid
PPAR	peroxisome proliferator-activated receptor
CYP	cytochrome P450
C4	hydroxy-4-cholesten-3-one
CA	cholic acid
CDCA	chenodeoxycholic acid
DCA	deoxycholic acid
LCA	lithocholic acid
BACS	bile acid coenzyme A synthetase
BAAT	bile acid coenzyme A: amino acid N-acyltransferase
UGT	uridine 5'-diphospho-glucuronosyltransferase
SULT	sulfotransferase
OCA	obeticholic acid
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ULN	upper limit of normal
Wy-14,643	pirinixic acid
GCDCA	glycochenodeoexycholic acid
GCA	glycocholic acid
GDCA	glycodeoxycholic acid
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TUDCA	tauroursodeoxycholic acid

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ABSTRACT

Cholestatic liver diseases, i.e. primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), result in the retention of bile acids, causing subsequent hepatotoxicity. Accumulation of toxic bile acids is a significant contributor of liver disease progression, often leading to cirrhosis, ultimately requiring a liver transplantation. Ursodeoxycholic acid (UDCA) and obeticholic acid are the only therapeutic options available, however they have limited efficacy or dose-limiting side effects, respectively. Alternative therapies are needed. The peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear receptor highly expressed in liver and it is a regulator of bile acid metabolism. Fenofibrate, a PPARα agonist, is FDA-approved to reduce elevated cholesterol. Clinical data demonstrates that adjunct fenofibrate improved abnormal liver biochemistries for patients with PBC who are not responding to UDCA, however the role of fenofibrate on bile acid metabolism during cholestasis remains unknown. We hypothesize that fenofibrate reduces bile acid toxicity during cholestasis via PPARα regulation. Adult patients with PBC or PSC taking UDCA (13-15 mg/kg/day) by mouth received adjunct fenofibrate (160 mg/day). Blood samples were used to measure serum liver enzymes and bile acid concentrations by standard clinical analyses and high-performance liquid chromatography and mass spectrometry, respectively. Treatment with adjunct fenofibrate reduced serum markers of cholestatic liver injury for patients with PBC or PSC. Addition of fenofibrate significantly reduced 7α-hydroxy-4-cholesten-3-one (C4), the bile acid precursor, as well as total, primary, and conjugated bile acids. Conclusions: Fenofibrate has anti-cholestatic actions through major reductions in toxic bile acids through taurine- and

glycine-conjugated pathways, suggesting a role of PPARα in bile acid amidation during cholestasis. Fenofibrate is a promising therapeutic option for patients with cholestasis and sub-therapeutic responses to UDCA monotherapy. These findings warrant further investigation into the role of fenofibrate in bile acid metabolism.

INTRODUCTION

Hepatic bile acid secretion and bile formation are essential functions of the mammalian liver. Bile acids are important endogenous steroid-derived molecules responsible for maintaining cholesterol homeostasis, with critical roles in the absorption of dietary lipids and fat-soluble vitamins. Bile acids are also integrally involved in lipid solubilization and metabolic signaling. Hepatic bile acid synthesis and biliary excretion constitute the main routes for cholesterol removal from the human body. While bile acids serve as endogenous detergents for the intestinal absorption of dietary cholesterol, due to their detergent properties, they also have the potential to be inherently cytotoxic. Accumulation of bile acids in the liver can be toxic and lead to inflammation, fibrosis, cirrhosis, and eventually liver failure. In particular, the hydrophobic nature of bile acids induces cellular injury by breaking down cell membranes of hepatocytes (1, 2). An excess of intracellular bile acids increases mitochondrial membrane permeability, leading to cellular apoptosis or necrosis (3, 4). This physiochemical property of bile acids can be reduced through conjugation pathways involving taurine, glycine, sulfates, or glucuronides.

In humans, bile acid synthesis occurs through one of two main pathways, with the most common pathway of synthesis occurring from cholesterol by the cytochrome P450 (CYP) enzymes, specifically Cytochrome P450 7A1 (CYP7A1) to 7α-hydroxy-4-cholesten-3-one (C4), the bile acid precursor, leading to the synthesis of cholic acid (CA) or chenodeoxycholic acid (CDCA) by CYP8B1 and CYP27A1, respectively (5). The alternative pathway of bile acid synthesis bypasses C4, thus only produces CDCA by metabolizing cholesterol into 27-hydroxycholesterol via CYP7B1 (5). Both CA and

CDCA undergo either conjugation to a polar group or metabolism into a secondary bile acid, i.e. deoxycholic acid (DCA) or lithocholic acid (LCA) by bacterial enzymes within the intestines (6-8). Amidation is one type of conjugation that occurs through sequential action of two enzymes: bile acid coenzyme A synthetase (BACS) in the endoplasmic reticulum or bile acid coenzyme A: amino acid N-acyltransferase (BAAT) in the cytosol (9). The process of amidation adds an amine, i.e. taurine or glycine, to the carboxylic acid moiety of the unconjugated bile acid, thereby enhancing aqueous solubility and decreasing toxicity. Alternatively, glucuronidation, a second form of conjugation, adds a glucuronide to one of several sites containing a hydroxyl moiety on the bile acid, resulting in a more hydrophilic and readily excreted compound. A specific uridine 5'-diphospho-glucuronosyltransferase (UGT) enzyme catalyzes the glucuronidation reaction; however, the specific UGT isoform involved depends on the site of glucuronidation. Lastly, sulfonation is a minor conjugation reaction that occurs predominately through sulfotransferase 2A1 (SULT2A1) and that sulfonates LCA almost exclusively (10).

The peroxisome proliferator-activated receptors (PPAR) are a group of nuclear hormone receptors that act as ligand-activated transcription factors involved in many cellular functions including lipid metabolism (11), fatty acid oxidation (12), and importantly bile acid metabolism (13). PPARα is the predominate isoform in the liver, where it plays a central role in maintaining cholesterol, lipid, and bile acid homeostasis (reviewed in (14)) by regulating many genes responsible for human bile acid metabolism and transport, including CYP7A1 (15, 16), CYP27A1 (17, 18) , CYP8B1 (19), UGT1A1, 1A3, 1A4, 1A6 (20), UGT2B4 (21), SULT2A1 (22), and the

human apical sodium-dependent bile salt transporter (23). The expression of many of these genes is altered during cholestatic liver diseases.

Cholestasis, including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), results from an impairment or disruption of bile flow that causes an intracellular accumulation of toxic bile constituents, notably bile acids. Current treatments for PBC include ursodeoxycholic acid (UDCA) or obeticholic acid (OCA) while there are no FDA-approved treatment options for PSC. Approximately 40% of patients with PBC have a sub-therapeutic response to UDCA, while patients taking OCA experience dose-limiting pruritus (24, 25). Furthermore, UDCA does not improve survival in patients with PSC, emphasizing urgent the need for alternative therapies. Fenofibrate belongs to the fibrate class of drugs and it is a PPARα agonist (26). While fenofibrate is FDA-approved to treat dyslipidemia, clinical data demonstrates that adjunct fenofibrate therapy improves liver function abnormalities in some patients with PBC and PSC who do not fully respond to UDCA monotherapy (27-31). The role of fenofibrate on bile acid metabolism during cholestasis, however, has not been evaluated. The results of our study show that fenofibrate reduces elevated liver enzymes during adult cholestasis, both PBC and PSC. Importantly, we present direct evidence that fenofibrate favorably regulates bile acid metabolism during cholestasis and that fenofibrate shifts the bile pool towards a less toxic composition in humans. Furthermore, these findings provide an integral role of PPARα regulation on bile acid metabolism during cholestatic liver diseases.

MATERIALS AND METHODS

Chemicals

Bile acids and deuterated internal standards (d4-CA, d4-CDCA, d4-DCA, and d4-LCA) were purchased from Steraloids Inc. (Newport, RI), C4 and d7-C4 were purchased from Toronto Research Chemicals (North York, ON, CA). Mass spectrometry (MS) grade solvents were purchased from Sigma-Aldrich (St. Louis, MO). A Synergi 4 μm Hydro-RP (100 x 2 mm) HPLC column and guard column were purchased from Phenomenex, Inc. (Torrance, CA).

Human Serum Samples

Serum or plasma from adult patients diagnosed with PBC (n=11) and PSC (n=9) who have received UDCA regimen (13-15 mg/kg/day) ± adjunct fenofibrate (160 mg/day) by mouth and have consented to have their blood samples de-identified and stored at the Yale University Liver Center Clinical Registry (New Haven, CT) were included in this study. These de-identified samples were determined to be exempt from the Institutional Review Board at Yale University (New Haven, CT) and the University of Rhode Island (Kingston, RI). Serum and plasma samples were used to measure liver enzymes and bile acid concentrations.

Liver Enzyme Levels

Serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were measured by standard clinical

enzymatic methods (Yale New Haven Hospital, New Haven, CT). Patients with serum ALP values > 1.5x upper limit of normal (ULN) were included for analysis.

Preparation of Serum Samples for High Performance Liquid Chromatography (LC)-MS/MS Analysis

Bile acid concentrations were measured by HPLC-MS/MS methodology. Samples were prepared by adding internal standards to serum**.** To this mixture, ice cold acetonitrile was added, vortexed and centrifuged at 10,000 x g to precipitate and pellet the protein. Supernatant was collected, evaporated under air, and reconstituted in methanol:water (50:50). A standard curve (1 nM to 5 µM) for each bile acid and C4 was prepared. To account for matrix effects of human serum, pooled (n=6) human serum (BioIVT, Westbury, NY) was charcoal stripped twice overnight with 0.25 mg of activated charcoal (Sigma-Aldrich) per mL of serum. The serum sample was then prepared in a similar manner as the patient samples.

HPLC-MS/MS Measurement of Bile Acids

Serum and plasma samples were analyzed using a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a Sciex 4500 Q-Trap ESI (Sciex, Framingham, MA, USA) under multiple reaction monitoring mode in a single HPLC-MS/MS method. Bile acids, their conjugates, and internal standards were measured in a single method, operated in negative ion mode. A volume of 15 µL of samples or standards were injected onto the column running a gradient method using 5 mM ammonium acetate in water acidified with 0.01% formic acid, 5 mM ammonium

acetate in methanol acidified with 0.01% formic acid, and acetonitrile at a flow rate of 0.6 mL/min over 18 mins (Figure 1). Parameters of the mass spectrometer for bile acid quantification have been previously described (32). C4 and d7-C4 were analyzed in positive mode using a gradient method, 5 mM ammonium acetate in water with 0.01% formic acid, 5 mM ammonium acetate in methanol over 8 mins. The ion source voltage was 5500, declustering potential of 21 V, entrance potential of 10 V, collision energy of 27 V, cell exit potential of 6 V, with mass transitions of 401.6→383.6 and 408.3→390.3 m/z for C4 and d7-C4 respectively (Figure 2). A standard curve (1 nM to 5 µM) for each bile acid and C4 was prepared and the lower limit of detection was 1.0 nM to 5.0 nM. Values below the limit of detection were imputed using R Studio (Boston, MA).

Data Analysis

Bile acid concentrations are reported as average (nM) ± standard error of the mean (SEM). Total bile acid concentrations represent the sum of all bile acids measured. Total primary bile acids consist of CA and CDCA along with all respective conjugated species, while secondary bile acids include DCA and LCA with their respective conjugated species. The sum of all unconjugated bile acids includes the hydroxylated bile acids and UDCA. Total conjugated bile acids consist of all glyco- and tauro-conjugated bile acids along with LCA-S. The glyco- and tauro-conjugates are the sum of all glyco- and tauro-conjugates of CA, CDCA, DCA, LCA, and UDCA. The data were analyzed by Mann-Whitney tests in GraphPad Prism 8 (La Jolla, CA). All tests were two sided and p-values < 0.05 were considered statistically significant.

RESULTS

Liver function enzymes

Serum ALP is a liver enzyme that serves as a marker of cholestatic liver injury (33, 34). Recent pilot studies have shown that patients with PBC and a sub-therapeutic response to UDCA therapy experienced reduced serum ALP levels after adjunct treatment with fibrates (29, 35-40). In the current study, all patients in the PBC and PSC groups receiving UDCA monotherapy had serum ALP > 1.5xULN. Following adjunct treatment with fenofibrate, serum ALP levels were reduced by 66% in the PBC (442.4 ± 156.4 to 197.4 ± 41.9 U/L, p=0.06) and 58% in the PSC cohort (705.4 ± 202.0 to 297.9 ± 103.5 U/L, p=0.05) (Figure 3A). Importantly, after treatment with fenofibrate, 30% of patients with PBC and 50% of patients with PSC had serum ALP values restored to normal ranges.

Serum ALT, AST, and total bilirubin, serve as general markers of liver injury. Patients with PBC receiving UDA monotherapy experienced mild elevations in their serum ALT and AST levels that were reduced by adjunct fenofibrate (63.5 ± 14.9 to 52.1 ± 10.2 U/L and 69.6 ± 17.6 to 52.5 ± 7.3 U/L, respectively.) shown in Figure 3B, 3C. In the PSC cohort, significant reductions in elevated serum ALT and AST were observed (125.1 ± 10.8 to 51.4 ± 10.5 U/L and 126.4 ± 21.6 U/L to 51.9 ± 10.3 U/L, respectively). Following adjunct fenofibrate therapy significantly reduced evaluated total bilirubin levels in the PSC cohort (Fig. 3D). These results demonstrate the therapeutic efficacy of adjunct fenofibrate for patients with PBC or PSC and are experiencing a sub-therapeutic response to UDCA.

Serum Bile Acid Concentrations in Patients with PBC

A hallmark feature of cholestatic liver diseases is elevated (toxic) serum bile acid levels, leading to increased hepatotoxicity (1, 2). To determine the effects of adjunct fenofibrate therapy on bile acid metabolism during cholestasis, adult patients with PBC (before fenofibrate n=11, after fenofibrate n=7) receiving UDCA (13-15 mg/kg/day) ± fenofibrate (160 mg/day) had their blood samples measured for bile acid concentration by LC-MS/MS. A summary of all bile acids measured in the current study is presented in Table 2. The addition of fenofibrate therapy for patients with PBC reduced the total bile acid concentrations by 47% (Figure 4A). In particular, the primary bile acids decreased by 53% (Figure 4B), and, interestingly, remained relatively unchanged as a percent of the total bile acid composition. The bile acid precursor C4, produced by CYP7A1, is the classical pathway of bile acid synthesis and then be enzymatically converted to CA or CDCA. The concentration was significantly reduced in fenofibrate-treated patients by 48% ($p = 0.008$, Figure 4F). In conjunction with the reduction in C4, a shift towards CDCA synthesis is observed as CDCA can then only be produced by the alternative pathway (Figure 4G).

In addition to a shift in bile acid composition favoring CDCA (over CA), a similar shift was observed in the ratio of glycine to taurine conjugates (Figure 4H). Adjunct fenofibrate treatment reduced total glycine- and taurine-conjugated bile acids by 41% and 53%, respectively (Figure 5Aand 6A), resulting in approximately a 70% shift favoring glycine- over taurine-conjugated bile acids (Figure 4H). Adjunct fenofibrate treatment reduced GCDCA, GCA, and GDCA by 34%, 58%, and 18% of the total bile acid pool (Figure 5B, 5C, 5D, and 5E). Similar to glycine-conjugates,

treatment with adjunct fenofibrate reduced the taurine-conjugates, specifically TCA and TCDCA by 70% and 45%, respectively (Figure 6A and 6B). Interestingly, the contribution of TCDCA reduced by half, however the contribution of TCDCA to the total bile acid pool remained unchanged, thereby supporting the shift towards CDCA (Figure 6E). Furthermore, the total amount of TDCA remained relatively unchanged, and thus the percent of TDCA within the bile acid pool increased accordingly by 2-fold (Figure 6D and 6E). Secondary bile acids exhibited a 16% decrease, resulting in a 1.5-fold increase in the relative percentage of secondary bile acids to the total bile acid pool (Figure 4C and 6G). Addition of fenofibrate also decreased the total percent of unconjugated bile acids by 40%, however, no major change in the contribution of unconjugated bile acids in the bile acid pool composition was observed (Figure 4E). Lastly for the bile acids which constituted less than 5% of the total bile acid pool, minor changes were observed in HCA (+102%), LCA-S (+38%), TLCA (+24%) and GLCA (-32%).

Serum Bile Acid Concentrations in Patients with PSC

To date, there are no FDA-approved therapies for PSC. As such, the use of adjunct fenofibrate during PSC is a potential therapy. A summary of all bile acids measured in patients with PSC is presented in Table 3. In the PSC cohort (before fenofibrate n= 9, after fenofibrate n=7), total bile acid concentrations were reduced by 72% following the addition of fenofibrate (Figure 7A). Importantly, the primary bile acids decreased by 81%, which reduced their contribution in the bile acid pool from 85% to 59% (Figure 7B and 7G). A large decrease in C4 concentration was also found

in the PSC group (-46%), which also resulted in a shift towards CDCA synthesis and corresponding change in ratio from 0.73:1 to 1.1:1 (CDCA:CA) (Figure 7F and 6G). This result may be explained by the 55% increase in absolute CDCA, resulting in a 5.6-fold increase in the relative contribution to the total bile acid pool.

In addition to adjunct fenofibrate therapy leading to a shift towards CDCA synthesis, and in a manner similar to the PBC cohort, the ratio of glycine to taurine conjugates shifted favoring glycine conjugated bile acids by 25% (Figure 7H), which is likely due to a 77% reduction in glycine-conjugates and 84% reduction in taurine-conjugated bile acids (Figure 8A and 9A). In particular, adjunct fenofibrate reduced GCDCA, GCA, and GDCA by 24%, 20%, and 18% of the total bile acid pool, respectively (Figure 8B, 8C, 8D, and 8E). Treatment with adjunct fenofibrate also reduced the taurine-conjugates, specifically TCA and TCDCA by 87% and 84%, respectively (Figure 9B and 9C). An unexplained effect of adjunct fenofibrate treatment was an increase in secondary bile acids by 42%, resulting in a 5-fold increase in their contribution to the bile acid pool (Figure 7C, G). Adjunct fenofibrate treatment also increased unconjugated bile acids by 9%, raising their relative contribution to the total bile acid pool to 33% (Figure 7E). Lastly, of the bile acids that constituted less than 5% of the total bile acid pool, changes in the following bile acids occurred: DCA (+103%), GDCA (+36%) (Figure 8D), TDCA (14%) (Figure 9D), HCA (+173%), HDCA (+20%), and LCA-S (+138%).

Similarities and differences in the bile acid composition between PBC and PSC cohorts

Both cohorts of patients included in this study had elevated bile acids while receiving UDCA monotherapy, before adjunct fenofibrate treatment. As hypothesized, the total bile acid pool and the composition of the bile acid pool decreased greatly following fenofibrate therapy, in both cohorts. Specifically, both patient groups had similar amounts of C4 present before fenofibrate and after adjunct fenofibrate therapy, the C4 levels were reduced by approximately the same percentage amounts (-44%). The reductions in C4 were also reflected in the shift away from CA towards CDCA in both cohorts. In the PSC cohort, 91% of the bile acids exist in a conjugated state while only 58% exist a conjugated state in the PBC cohort. In particular, conjugated bile acids were largely decreased by adjunct fenofibrate treatment, resulting in a shift towards glycine from taurine conjugation in patients with PBC and PSC. These changes were driven by a greater reduction in the glycine and taurine conjugated CAs, than their CDCA counterparts. In both patient cohorts, secondary bile acids constituted a rather small portion of the bile acid pool remaining below 12% and 3% before treatment in PBC patients and PSC patients, respectively. After treatment, in both groups, the secondary bile acids increased to 17% and 11% in PBC and PSC, respectively. LCA-S and HCA, while minor bile acids, were increased in both groups of patients by adjunct fenofibrate treatment.

Notable differences among the bile acids between the two cohort, however, were also found first, in the PBC cohort, primary bile acids constitute 45% of primary bile acids, whereas in the PSC cohort the primary bile acids constitute 85.0% of the

bile acid pool. Minor differences between the two groups, with the PSC cohort having increases in DCA, secondary bile acids, and unconjugated bile acids but decreases in patients with PBC.

CONCLUSIONS

Over the past several years the search for alternative therapies to treat cholestatic liver diseases has continued and the use of fibrates has emerged as a potential therapy. However, mainly for patients with PBC, leaving very few treatment options for patients with PSC and the disease often progresses to liver failure. The major effects of fibrates on cholestatic liver diseases is largely attributed to the activation of PPAR. In particular the PPARα isoform plays an important role in transcriptional activation of gene expression of bile acid metabolizing enzymes. In this study, we hypothesized that fenofibrate's anticholestatic effects could be mediated through detoxifying bile acids via PPARα regulation of bile acid metabolizing enzymes. To test this hypothesis, we studied patients with PBC or PSC receiving UDCA monotherapy before and after adjunct fenofibrate therapy. While the majority of previous pilot studies have shown the efficacy of fibrates in reducing elevated serum liver enzymes during cholestasis, the effects of fenofibrate on bile acid metabolism in cholestatic patients have not yet been reported, despite bile acid toxicity being a hallmark characteristic of cholestatic liver diseases. Results of this study demonstrate the reduction in total, primary, conjugated and unconjugated bile acids following adjunct fenofibrate treatments in patients with PBC and PSC.

Data from the current study shows that treatment with adjunct fenofibrate decreases all of the major biochemical biomarkers of cholestasis, in addition to normalizing ALP levels in 30% of patients with PBC and 50% of patients with PSC. The reductions in ALP are in agreement with recently published work using fibrates showing approximately 50% reductions in ALP, including one study involving adjunct bezafibrate in patients with PBC (39) and another study with bezafibrate and fenofibrate in patients with PSC (41). While the study conducted by Corpechot et al., showed anticholestatic effects of bezafibrate in patients with PBC, bezafibrate is not an FDA approved drug in the U.S and, furthermore, it is a non-selective PPAR agonist (42). In addition, most of these previous pilot studies did not report on serum bile acid concentrations except for C4, which was reduced by bezafibrate thus the need to examine bile acids in patients with cholestasis and treated with adjunct fenofibrate (39, 40).

The overall results from this study demonstrate that the use of fenofibrate effectively reduced serum bile acids in patients with PBC and PSC. The serum values of total bile acid concentrations for patients with PBC and PSC prior to fenofibrate treatment obtained in the current study are similar to those measured in previous studies (43). In the current study, the majority of the reductions in bile acids was a result of decreased conjugated bile acids and not the unconjugated forms of CA, CDCA, DCA, or LCA. Specifically, reductions in C4 concentrations by nearly 50% were observed in both cohorts, and these findings agree with previous literature reports that describe CYP7A1 mRNA downregulation in response to activation of PPARα via pirinixic acid (Wy-14,643), a PPARα agonist. This downregulation is

caused by a decrease in hepatocyte nuclear factor 4 alpha, a known CYP7A1 transcription factor (16). A direct effect of C4 reductions were observed through a reduction of CA in both cohorts after adjunct fenofibrate treatment. The alternative pathway of bile acid synthesis produces CDCA by bypassing via CYP27A1 and CYP7B1. This shift in bile acid synthesis may explain the observed increase in CDCA but not CA, as CA can only be synthesized through the traditional pathway. A shift towards the alternative pathway by fenofibrate is further supported in the decreased ratio of CA:CDCA in both patient cohorts after adjunct fenofibrate treatment. Together these findings suggest that either the alternate pathway could be upregulated or that the primary pathway through C4 was reduced, or a combination of the two occurred following adjunct fenofibrate therapy.

Typically, conjugation reduces bile acid toxicity and the goal for treatment during cholestasis includes reducing elevated bile acids and inducing a shift in the total bile acid pool towards conjugation to decrease the hydrophobicity. The results presented herein show a reduction in total bile acids was achieved by adjunct fenofibrate treatment as well as reductions in glycine- and taurine-conjugated bile acids, a result likely explained by the downregulation of BAAT mRNA and activity, a key enzyme involved in bile acid amidation which has been shown to be PPARα-dependent in mice (44). In combination with a downregulation of the amidation-mediated conjugation reactions, the decrease observed in conjugated bile acids by adjunct fenofibrate may also be partially accounted for by the reduction of unconjugated bile acid substrate necessary for the conjugation reaction to occur. In both patient controls, glycine conjugation was initially favored, and after fenofibrate

treatment a further shift towards glycine approaching the 3:1 (glycine:taurine) ratio observed in non-cholestatic patients (43) was observed. Additionally, sulfonation of LCA, while a relatively small conjugation pathway, in both patient cohorts, was found to be upregulated, a finding that is in agreement with previous report of a PPARα response element identified upstream of the SULT2A1 gene (22). Lithocholic acid, a monohydroxy bile acid, is the most toxic bile acid, thus sulfonation of this particular bile acid by adjunct fenofibrate treatment is clinically very important.

While this study showed significant reductions in clinical biomarkers of cholestasis and large reductions in many of the serum bile acids, there are limitations of this study. First, this study is a retrospective single-arm study examining the effects of adjunct fenofibrate vs. patients pre-treated with UDCA monotherapy. Second, the sample size was limited. For some patients, baseline blood samples were not available, and other patients have yet to have follow up post-fenofibrate therapy. Third, the lack of consistent time on treatment, measurement of biomarkers over time, and a washout period reduced the longitudinal aspect of this study. Lastly, more clinical endpoints need to measured, including pruritus, liver stiffness, stages of liver disease, or impact of adjunct therapy on liver transplant time. Additional studies of a prospective nature are necessary to address these needs, nevertheless our study provides compelling preliminary data to support larger clinical studies.

In conclusion, we present *in vivo* evidence that adjunct treatment with fenofibrate reduces serum liver enzyme abnormalities in patients with PBC and PSC. Importantly, this is one of the first to demonstrate adjunct fenofibrate therapy reduces bile acid synthesis during cholestasis. Importantly, the bile acid precursor C4 was

reduced after adjunct fenofibrate therapy, indicating that bile acid synthesis may be downregulated through PPARα activation in both PBC and PSC cohorts. Additionally, decreases in glycine- and taurine-conjugated bile acids are observed, with concentrations restoring patient serum values closer towards a 3:1 ratio of glycine to taurine, as observed in non-cholestatic patients (43). Collectively, the findings presented herein demonstrate that adjunct fenofibrate treatment reduces bile acid toxicity and, furthermore, suggests that PPARα is integrally involved in bile acid regulation during cholestasis. PPARα is a potential target for the treatment of cholestasis, for both PBC and PSC, especially for those patients who are non-responders to UDCA or OCA. While our study has a limited sample size, combined with the inherent large interindividual variation in endogenous serum bile acid concentrations, a larger prospective study is necessary. Nevertheless, the findings reported in this study suggest that liver diseases that are impacted by bile acid toxicity such as PBC and PSC and not therapeutically responding to UDCA monotherapy may benefit from adjunct fenofibrate therapy.

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Figure 1. A LC-MS chromatogram of 17 bile acids and 4 deuterated internal standards. Standards were spiked into double charcoal stripped pooled human serum and analyzed in negative ion mode.

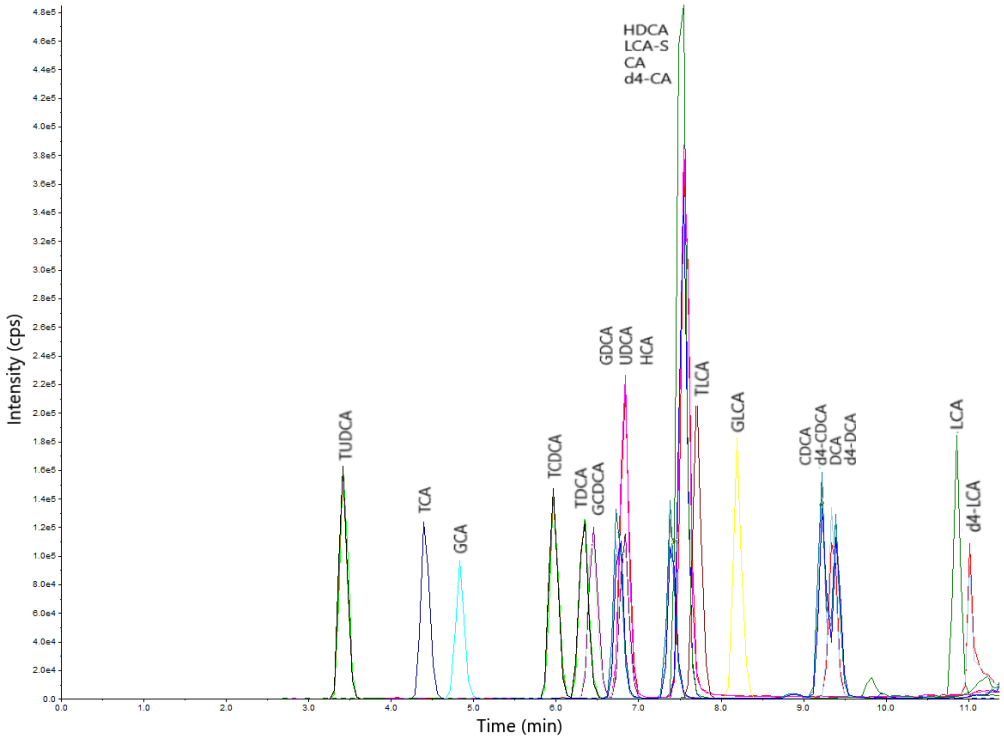

Figure 2. A LC-MS chromatogram of 7α-hydroxy-4-cholesten-3-one (C4) and d7-C4 internal standard. Standards were spiked into double charcoal stripped pooled human serum and analyzed in positive ion mode.

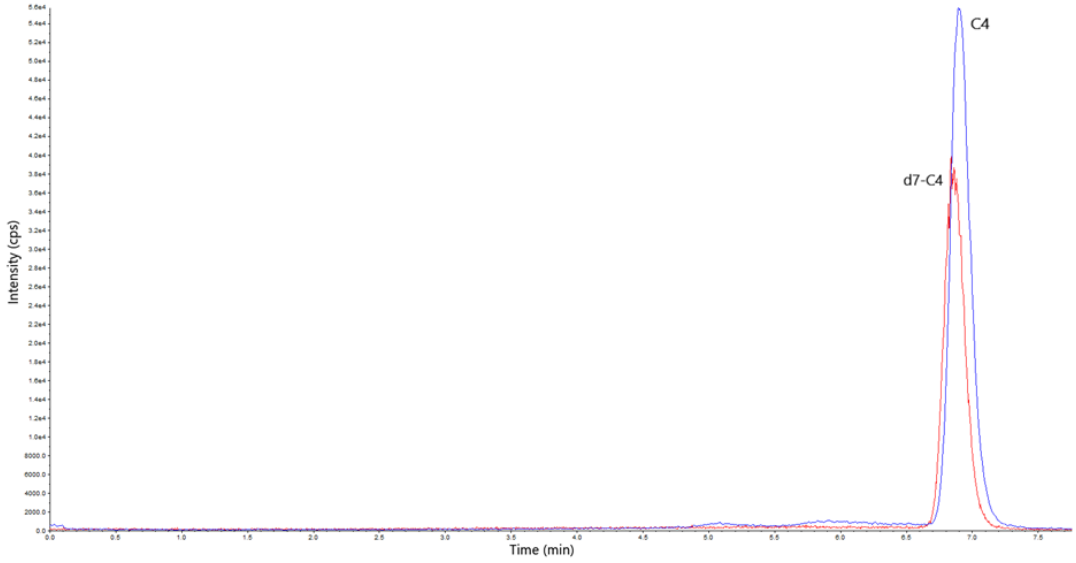

Figure 3. Treatment with fenofibrate reduces liver injury in patients with PBC and PSC. Serum levels of: (A) alkaline phosphatase, (B) alanine aminotransferase, (C) aspartate aminotransferase and (D) total bilirubin were measured by standard enzymatic methods. *P<0.05, **P<0.01 vs. before fenofibrate (UDCA monotherapy) levels.

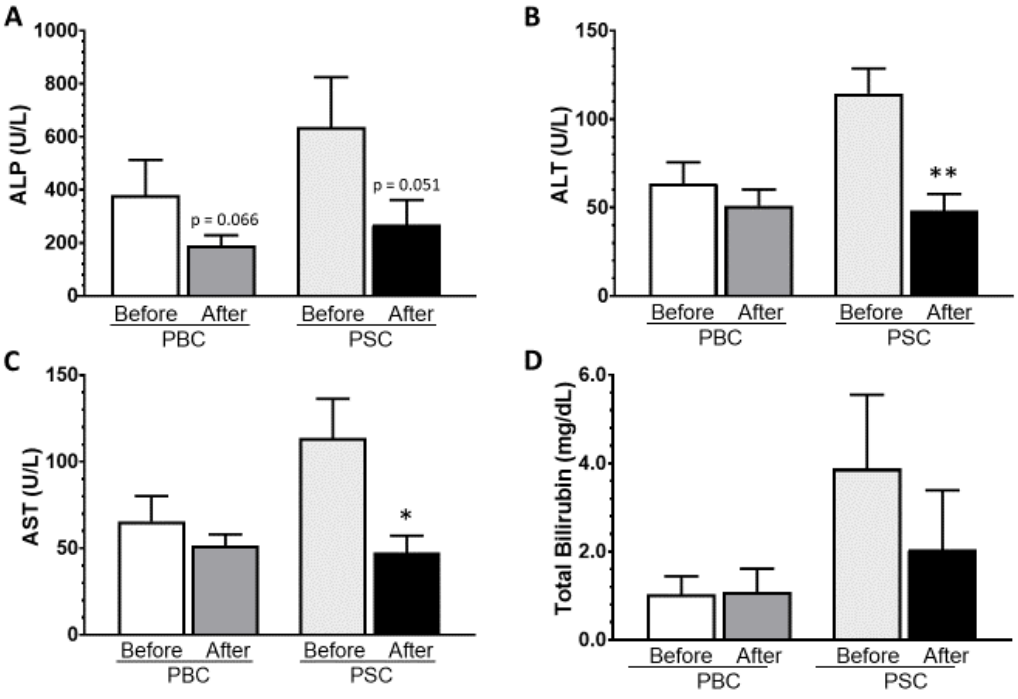

Figure 4. Treatment with adjunct fenofibrate alters serum bile acid concentrations in patients with PBC: (A) total bile acids, (B) primary bile acids (C) secondary bile acids, (D) conjugated bile acids, (E) unconjugated bile acids, and (F) C4 were measured by LC-MS/MS. Ratios of the bile acids and their conjugates are shown in (G) and total glycine and taurine conjugate shown in (H). Data are reported as average ± SEM; **P<0.01 vs. before fenofibrate (UDCA monotherapy) levels.

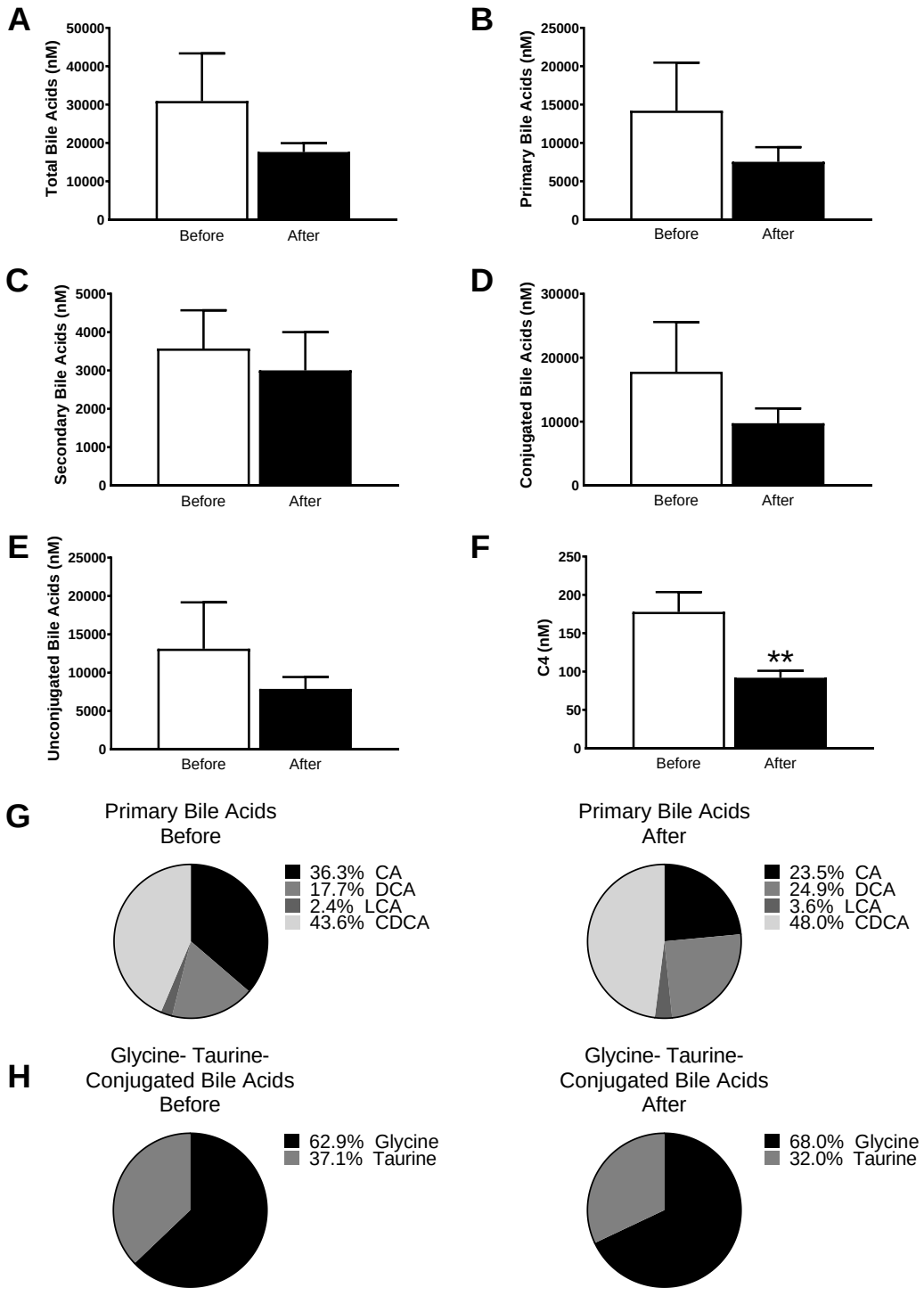

Figure 5. Treatment with fenofibrate alters serum glycine conjugated bile acid concentrations in patients with PBC: A) total glycine bile acids, B) GCA C) GCDCA, and D) GDCA were measured by LC/MS/MS. Ratios of glycine conjugated before (UDCA monotherapy) (E) and after (F) treatment with adjunct fenofibrate.

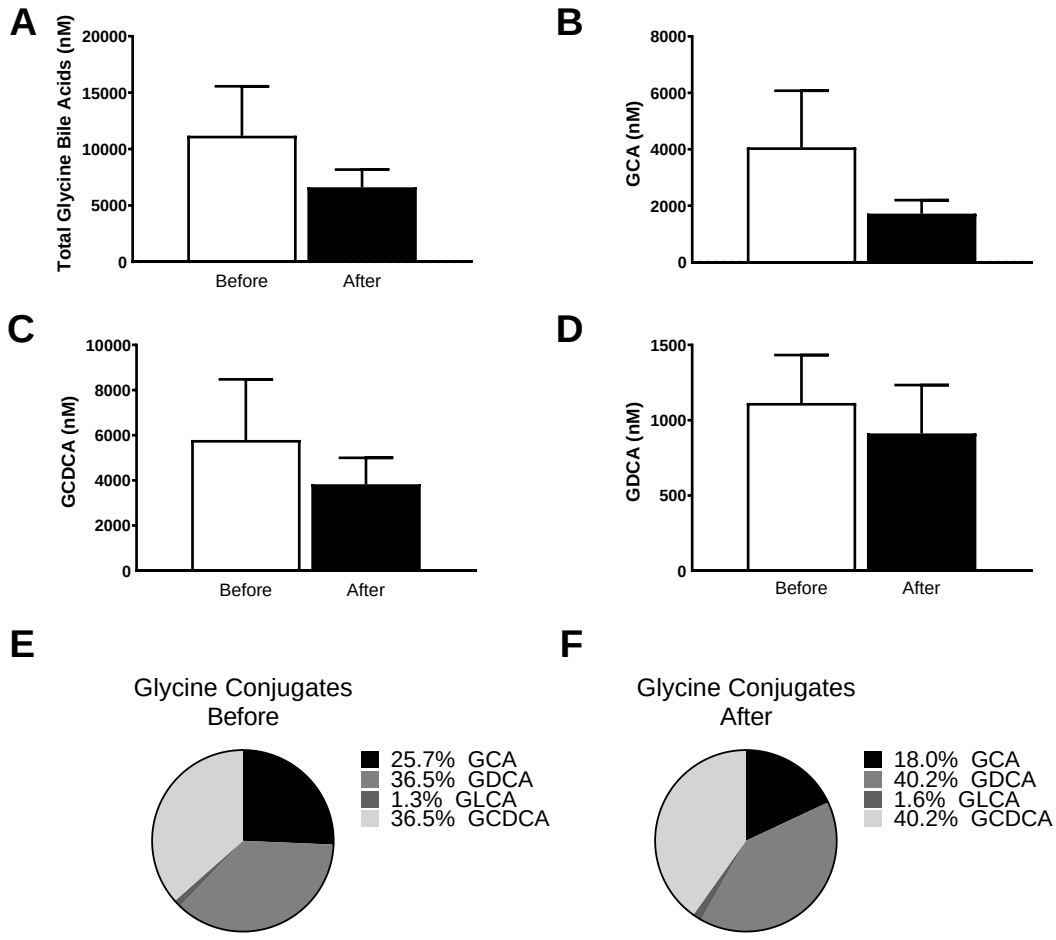

Figure 6. Treatment with adjunct fenofibrate alters serum taurine conjugated bile acid concentrations in patients with PBC: (A) total taurine bile acids, (B) TCA (C) TCDCA, and (D) TDCA were measured by LC-MS/MS. Ratios of taurine conjugated before (UDCA monotherapy) (E) and after (F) treatment with adjunct fenofibrate.

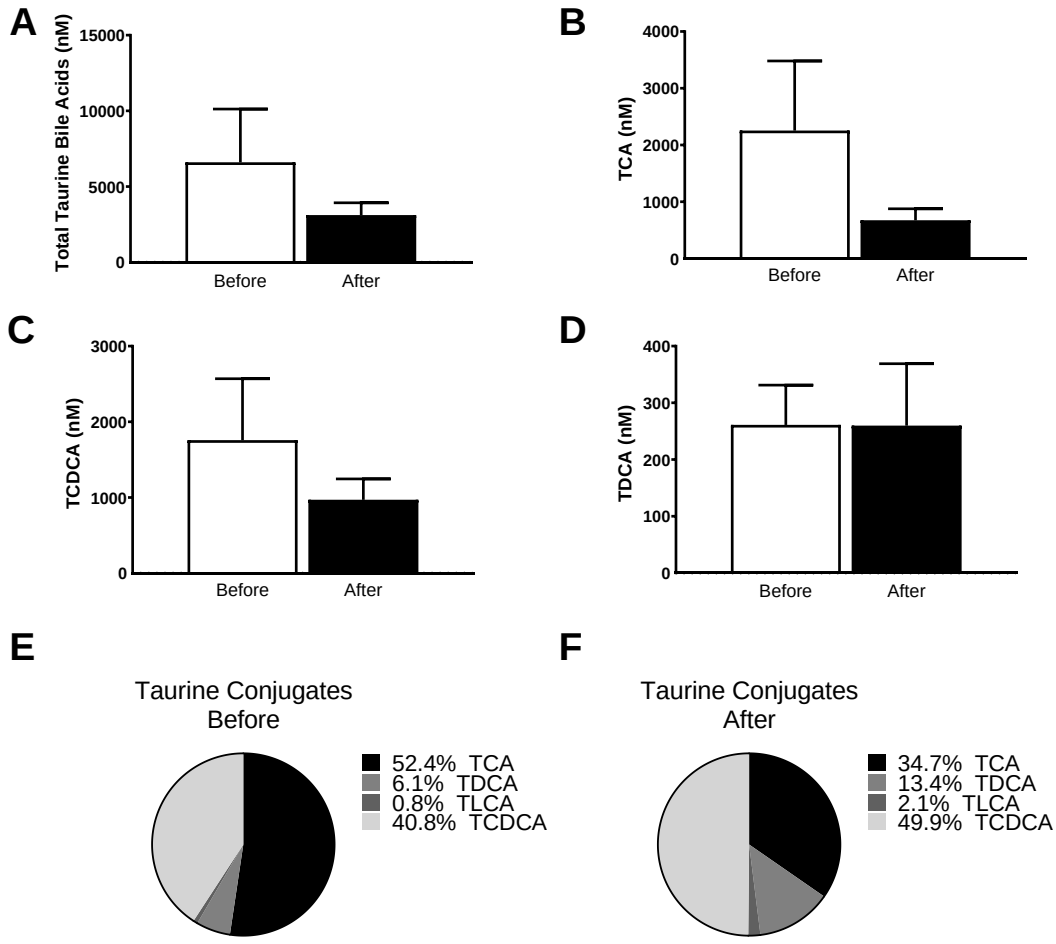

Figure 7. Treatment with fenofibrate alters serum bile acid concentrations in patients with PSC: (A) total bile acids, (B) primary bile acids (C) secondary bile acids, D) conjugated bile acids, (E) unconjugated bile acids, and (F) C4 were measured by LC-MS/MS. Ratios of the bile acids and their conjugates are shown in (G) and total glycine and taurine conjugate shown in (H).

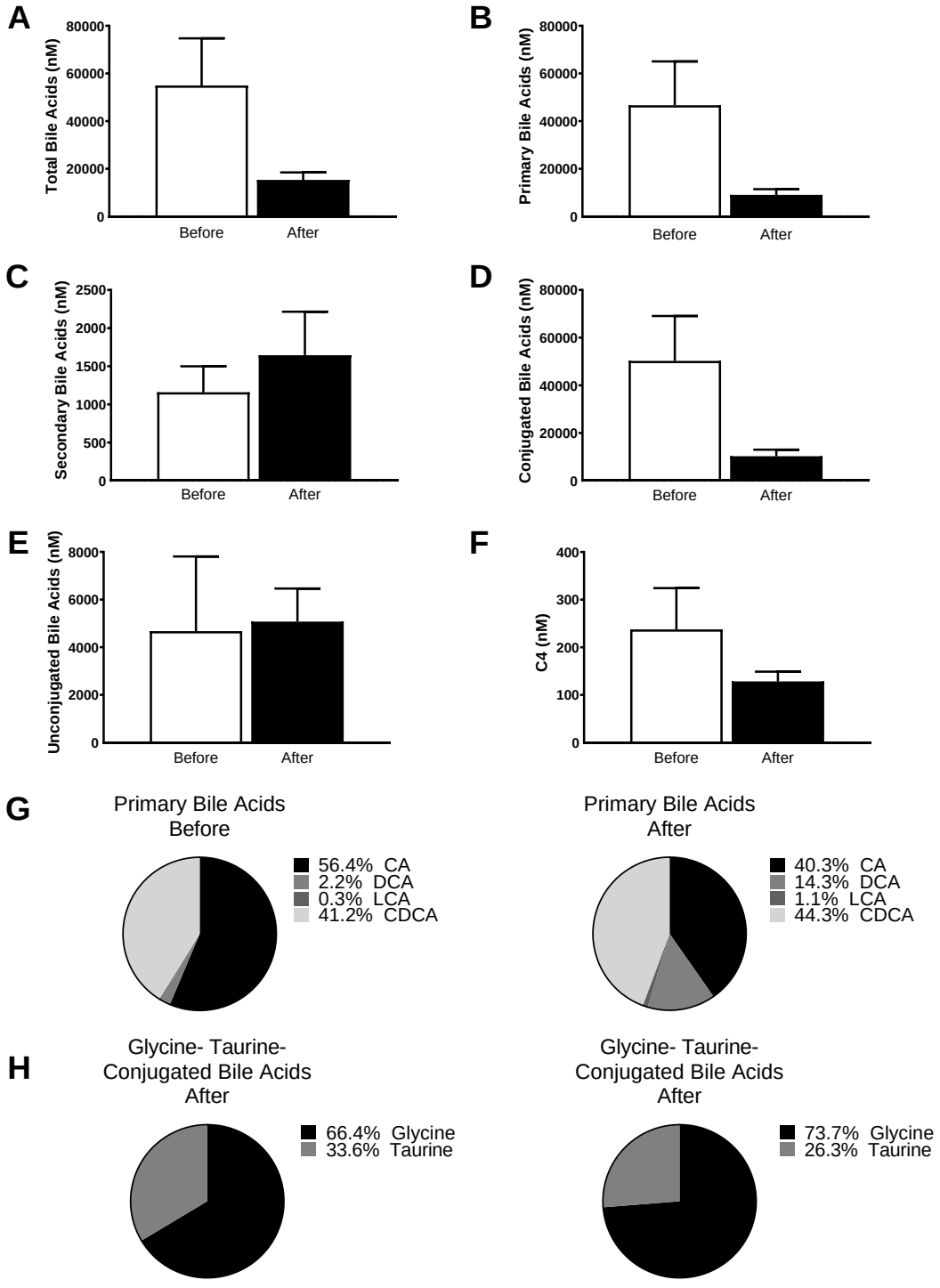

Figure 8. Treatment with fenofibrate alters serum glycine conjugated bile acid concentrations in patients with PSC: (A) total glycine bile acids, (B) GCA (C) GCDCA, and (D) GDCA were measured by LC/MS/MS. Ratios of glycine conjugated before (UDCA monotherapy) (E) and after (F) treatment with adjunct fenofibrate.

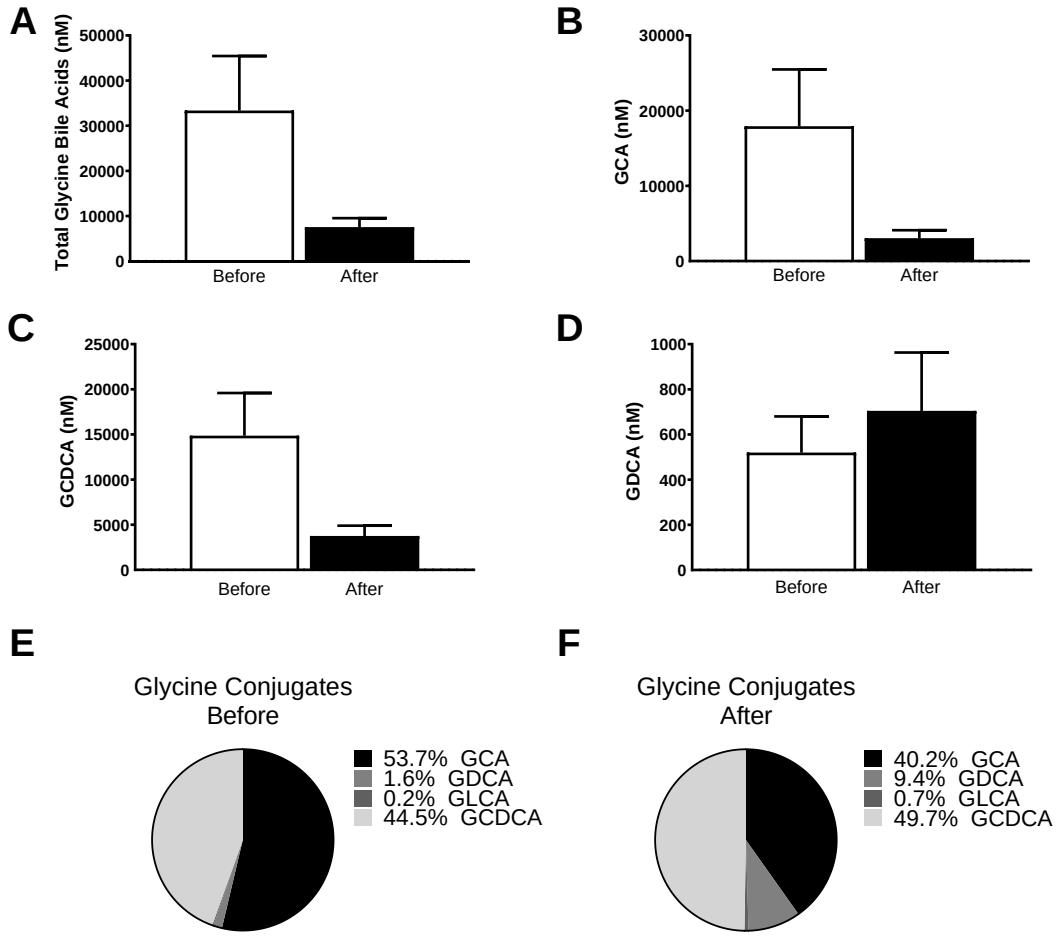

Figure 9. Treatment with fenofibrate alters serum taurine conjugated bile acid concentrations in patients with PSC: (A) total taurine bile acids, (B) TCA, (C) TCDCA, and (D) TDCA were measured by LC/MS/MS. Ratios of taurine conjugated before (UDCA monotherapy) (E) and after (F) treatment with adjunct fenofibrate.

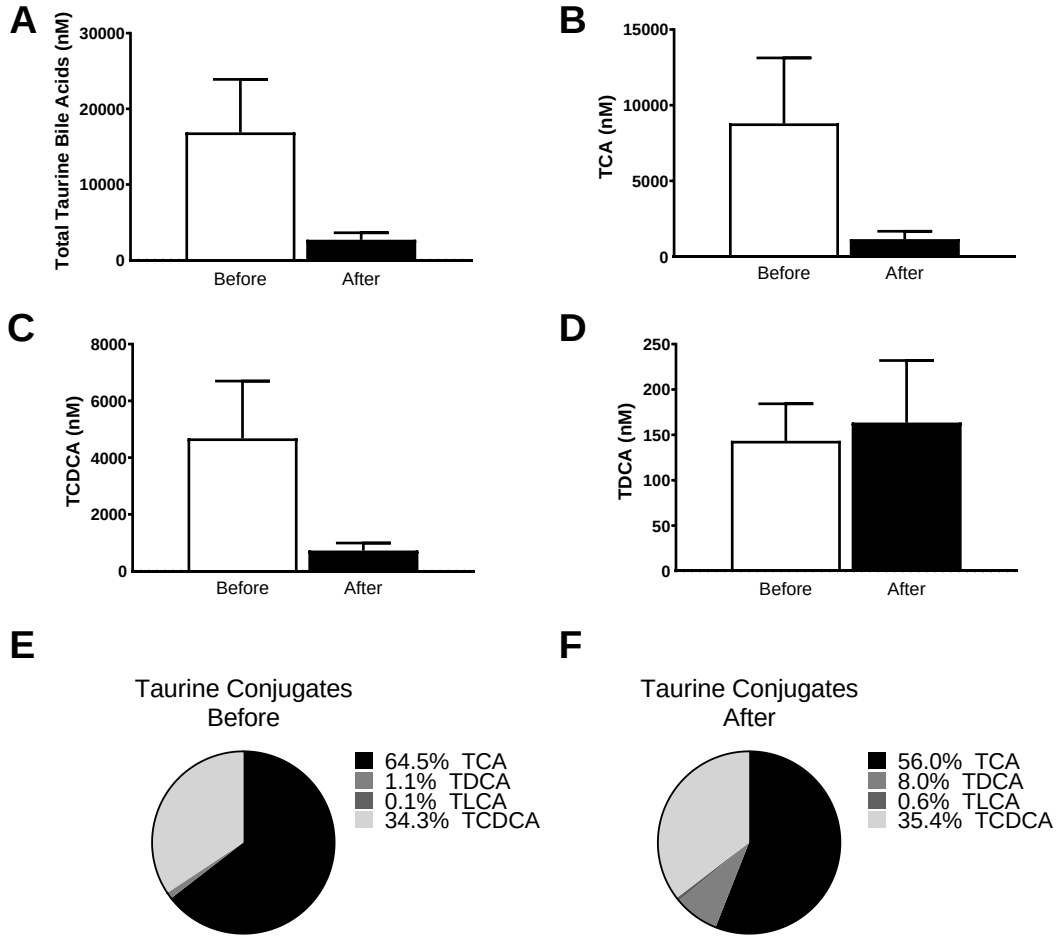

Table 1. Demographics of patients with cholestasis in both cohorts.

PBC Patients	Before (n=11)	After (n=9)
Age (Median)	54 (51-67)	58 (47-68)
Male	1	0
Female	10	9
White	8	6
Black	1	0
Hispanic	2	2
Unknown	1	2

PSC Patients	Before (n=9)	After (n=7)
Age (Median)	40 (24-54)	45 (33-66)
Male	8	5
Female	1	2
White	5	6
Black	3	0
Hispanic	0	0
Unknown	1	1

Table 2. Serum bile acid concentrations (nM ± SEM) in patients with PBC before (UDCA monotherapy) and after adjunct fenofibrate therapy. Statistical significance is indicated by *p < 0.05, **p < 0.01 versus before levels.

Bile Acids	PBC Before (Mean ± SEM)	PBC After (Mean ± SEM)
CA	102.15 ± 56.22	41.58 ± 23.10
CDCA	205.83 ± 77.41	263.48 ± 79.16
DCA	1393.65 ± 543.80	1107.00 ± 466.34
GCA	4066.77 ± 2009.31	1716.42 ± 479.81
GCDCA	5787.39 ± 2685.61	3823.73 ± 1176.24
GDCA	1114.27 ± 318.70	912.25 ± 321.21
GLCA	207.85 ± 43.63	153.60 ± 45.14
HCA	23.13 ± 9.05	46.78 ± 12.46
HDCA	366.31 ± 148.24	340.20 ± 202.12
LCA	172.07 ± 36.74	157.58 ± 33.49
LCA-S	20.55 ± 3.54	28.28 ± 10.08
TCA	2253.62 ± 1227.61	673.45 ± 203.24
TCDCA	1756.63 ± 812.84	968.96 ± 276.06
TDCA	260.99 ± 70.27	259.76 ± 109.15
TLCA	33.42 ± 6.77	41.36 ± 18.72
TUDCA	2293.15 ± 1617.56	1166.17 ± 332.05
UDCA	10851.73 ± 6037.62	5917.64 ± 1254.84
C4	177.70 ± 28.23	91.80 ± 9.14**
Total	30909.50 ± 12428.28	17618.23 ± 2330.57
Total Primary	14195.52 ± 6277.72	7534.39 ± 1910.95
Total Secondary	3569.11 ± 999.77	3000.02 ± 999.05
Total Unconjugated	13114.86 ± 6054.22	7874.25 ± 1558.22
Total Conjugated	17794.64 ± 7788.18	9743.97 ± 2320.69
Total Taurine	6597.81 ± 3522.25	3109.70 ± 816.65
Total Glycine	11176.27 ± 4387.25	6605.99 ± 1579.56

Table 3. Serum bile acid concentrations (nM ± SEM) in patients with PSC before (UDCA monotherapy) and after adjunct fenofibrate therapy. Statistical significance is indicated by *p < 0.05

Bile Acids	PSC Before (Mean ± SEM)	PSC After (Mean ± SEM)
CA	217.75 ± 206.94	100.08 ± 37.97
CDCA	167.40 ± 64.63	258.76 ± 78.27
DCA	271.77 ± 134.95	551.36 ± 249.57
GCA	17913.34 ± 8016.82	3027.81 ± 1072.87
GCDCA	14853.69 ± 5022.65	3747.66 ± 1148.19
GDCA	519.76 ± 169.80	704.45 ± 258.43
GLCA	71.32 ± 33.49	54.14 ± 22.71
HCA	9.88 ± 5.19	26.94 ± 8.12*
HDCA	92.09 ± 59.29	110.55 ± 32.20
LCA	38.14 ± 22.63	31.89 ± 8.93
LCA-S	6.29 ± 2.73	14.93 ± 5.84
TCA	8794.85 ± 4589.10	1146.90 ± 521.63
TCDCA	4676.26 ± 2145.08	725.38 ± 265.87
TDCA	143.32 ± 43.37	163.45 ± 68.46
TLCA	15.16 ± 7.54	12.67 ± 6.29
TUDCA	3242.15 ± 2086.89	636.14 ± 241.91
UDCA	3875.75 ± 3376.13	3991.68 ± 1168.19
C4	237.38 ± 92.08	128.36 ± 20.47
Total	54908.91 ± 21039.65	15304.81 ± 3166.69
Total Primary	46633.18 ± 19530.57	9033.53 ± 2391.28
Total Secondary	1157.84 ± 362.71	1643.47 ± 571.26
Total Unconjugated	4672.77 ± 3326.68	5071.27 ± 1392.51
Total Conjugated	50236.14 ± 19990.74	10233.54 ± 2772.53
Total Taurine	16871.73 ± 7444.46	2684.54 ± 931.31
Total Glycine	33358.12 ± 12815.23	7534.07 ± 1983.28