Curriculum Vitae

Brandy Garzel

Department of Pharmaceutical Sciences
School of Pharmacy, University of Maryland Baltimore
20 Penn St, Baltimore, MD, 21201
Tel: 410 706 2123 Fax: 410 706 5017
Email: bgarzel1@umaryland.edu

DEGREE AND DATE CONFERRED: Ph.D. in Pharmaceutical Sciences, June 2017

EDUCATION

University of Maryland, Baltimore, MD
Ph.D. in Pharmaceutical Sciences **2017**
Dissertation: The Role of Bile Salt Export Pump (BSEP; ABCB11) Gene Repression in Drug-Induced Cholestatic Liver Injury

College of Notre Dame of Maryland, Baltimore, MD
B.A. Honors in Biology **2011**
Summa Cum Laude, University Honors, Departmental Honors

Karolinska Institute, Stockholm, Sweden
Semester Abroad: EuroScholars **Fall 2010**
Research performed in laboratory of Dr. Magnus Ingelman-Sundberg

THESIS

"Role of Bile Salt Export Pump (BSEP) in Drug-Induced Cholestatic Liver Injury"
Advisors: Dr. Hongbing Wang and Dr. Jean-Pierre Raufman; Committee: Drs. Peter Swaan, James E. Polli, Paul Shapiro, Yan Shu and Shiew-Mei Huang

In order to better understand the development of hepatic cholestasis, upon drug-treatment, the primary bile salt efflux transporter, bile salt export pump (BSEP) will be studied in relation to its gene expression and function. In contrast to the more common approach of studying drug-induced cholestasis through inhibition of BSEP function, primarily via inverted membrane vesicles, the effect of drugs on BSEP gene expression will be determined, and subsequent functional changes will be assessed in human primary hepatoycytes. Additionally, a mechanistic study using the type-two diabetes drug, metformin, will be performed, to better understand the pathway(s) involved in BSEP repression. The mechanistic study will investigate the role of AMPK, FXR, and NRF2.

ACADEMIC HONORS/AWARDS

Ruth L. Kirschstein National Research Service Award (F31) Predoctoral Fellowship, *National Institute of Health*	**August 2015-July 2017**
Travel Award, *Cellular and Molecular Mechanisms of Toxicity Gordon Research Conference*	**June 2015**
Travel Award, *American Association of Pharmaceutical Sciences*	**April 2015**
Chemistry Biology Interface Training Program Fellow, *National Institute of Health & University of Maryland*	**July 2014-June 2015**
Graduate Student of the Month, *Universisty of Maryland*	**April & October 2013**
Dean's List, *University of Maryland*	**Fall 2011-Summer 2017**
Sister Alma McNicholas Women Scientists Program, *College of Notre Dame of Maryland*	**Spring 2011**
Dean's List, *College of Notre Dame of Maryland*	**Fall 2009 – Spring 2011**
Notre Dame Award, *College of Notre Dame of Maryland*	**Fall 2009 – Spring 2011**
Chemistry Undergraduate Summer Research Award, *Saint Francis University*	**Summer 2008**
Dean's List, *Saint Francis University*	**Fall 2007 – Spring 2009**
Chemistry Department Fellowship Award, *Saint Francis University*	**Fall 2007 – Spring 2009**
Founders Award, *Saint Francis University*	**Fall 2007 – Spring 2009**

PUBLICATIONS

1. **Garzel, B**. et al (2017). "Metformin-mediated AMPK activation disrupts bile acid efflux through repression of BSEP." *Under Review.*

2. Li, L., Li, H., **Garzel, B.**, et al. (2015). "SLC13A5 is a novel transcriptional target of the pregnane x receptor and sensitizes drug-induced steatosis in human liver." Mol Pharmacol **97**(4):674-82. *Published*

3. **Garzel, B.**, H. Yang, et al. (2014). "The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity." Drug Metab Dispos **42**(3): 318-322. *Published*

4. Yang, H., **B. Garzel**, et al. (2014). "Metformin represses drug-induced expression of CYP2B6 by modulating the constitutive androstane receptor signaling." Mol Pharmacol **85**(2): 249-260. *Published*

MEETING PRESENTATIONS

Brandy Garzel. The Role of Bile Salt Export Pump Gene Repression in Drug-Induced Cholestasis. 2016 Experimental Biology, American Society for Pharmacology & Experimental Therapeutics (ASPET) Annual Meeting. San Diego, CA. April 2016. – *Invited Speaker*

Brandy Garzel, Hui Yang, Scott Heyward, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang. Mechanistic Insight into Metformin-Mediated Repression of the Bile Salt Export Pump (BSEP). 2016 Society of Toxicology Annual Meeting. New Orleans, LA. March 2016. – Poster

Brandy Garzel, Hui Yang, Scott Heyward, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang. Mechanistic Insight into Metformin-Mediated Repression of the Bile Salt Export Pump (BSEP). 2015 Cellular and Molecular Mechanisms of Toxicity Gordon Research Seminar and Conference. Hanover, NH. August 2015. – Poster

Brandy Garzel, Hui Yang, Scott Heyward, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang. Mechanistic Insight into Metformin-Mediated Repression of the Bile Salt Export Pump (BSEP). 2015 AAPS/ITC Joint Workshop on Drug Transporters. Baltimore, MD. April 2015. – Oral Presentation and Poster

Brandy Garzel, Hui Yang, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang . Bile Salt Export Pump (BSEP) Gene Repression in Drug-Induced Cholestatic Liver Injury. University of Maryland Graduate Research Conference. Baltimore, MD. April 2014. – *Oral Presenation*

Brandy Garzel, Hui Yang, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang . Bile Salt Export Pump (BSEP) Gene Repression in Drug-Induced Cholestatic Liver Injury. 2013 AAPS Annual Meeting and Exposition. San Antonio, TX. November 2013. – *Poster**

Brandy Garzel, Lei K. Zhang, Shiew-Mei Huang, James E. Polli, and Hongbing Wang . Contribution of Bile Salt Export Pump (BSEP) Gene Repression in Drug-Induced Cholestatic Liver Injury. Food and Drug Association, Office of Clinical Pharmacology XVII Annual Science Day Meeting. Silver Spring, MD. September 2012. – *Poster**

**Posters were also shown at CERSI Day (2012 and 2013) and UMB Science Day (2013)*

RESEARCH/WORK EXPERIENCE

Ruth L. Kirschstein National Research Service Award Pre-doctoral Fellow (**August 2015-present**); National Institute of Health, Bethesda, MD & University of Maryland, Baltimore, MD
- *Bile Salt Export Pump and Cholestasis*

Chemistry Biology Interface Research Training Fellow (**July 2014-June 2015**); National Institute of Health, Bethesda, MD & University of Maryland, Baltimore, MD
- *Bile Salt Export Pump and Cholestasis*

PhD Candidate (**2013-Present**); University of Maryland School of Pharmacy, Baltimore, MD
- *Bile Salt Export Pump and Cholestasis*

PhD Student (**2011-2013**); University of Maryland School of Pharmacy, Baltimore, MD
- *Bile Salt Export Pump and Cholestasis*

Intern/Sister Alma Women Scientist (**Spring 2011**); Johns Hopkins University School of Medicine, Baltimore, MD
- *Epigenetic histone modifications to p300*

Study Abroad Intern (**Fall 2010**), Karolinska Institute; Stockholm, Sweden
- *Epigenetic regulation of CYP3A4/5*

Summer Intern (**Summer 2010**), University of Maryland School of Pharmacy; Baltimore, MD
- *Transport function of MATE and OCTs*

Summer Intern (**Summer 2008**), Saint Francis University; Loretto, PA
- *Copper and zinc-induced protein-protein interactions*

LEADERSHIP EXPERIENCE

Graduate Student Assoc.- Graduate Council Representative	**Fall 2015-Spring 2016**
Rho Chi Member	**Spring 2013-Present**
Rho Chi Vice President of Graduate Affairs	**Fall 2013 – Spring 2014**
Pharmacy Graduate Student Assoc.- Secretary	**Fall 2013 – Spring 2014**
Student Mentor to Rotation Students	**Summer 2012 & 2013**
Big Sibling	**Fall 2012 – Fall 2014**
Graduate Student Assoc.- Pharmaceutical Sciences Representative	**Fall 2012- Spring 2015**
Pharmacy Graduate Student Assoc.- 2nd Year Representative	**Fall 2012- Spring 2013**
Delta Epsilon Sigma	**Spring 2011**
Kappa Gamma Pi	**Spring 2011**
Beta Beta Beta	**Spring 2009**
Student Government Association- Commuter Student Representative	**Fall 2008-Spring 2009**
Chemistry/Rural Outreach Chemistry for Kids (R.O.C.K.)- Assistant	**Fall 2007-Spring 2009**

SKILLS

- **TECHNICAL SKILLS**
  - **In-Vitro Studies**
    - Experience in working with HepG2, HEK-293, Huh7, LLC-PK1 immortalized cell lines
    - Experience using human and mouse primary hepatocyte cultures
    - Experience in working with HepaRG non-differentiated cell line
    - Designing and conducting relative luciferase assays
    - Designing and conducting radiolabeled transport (efflux) assays
    - Experience preforming transient transfections of protein expression plasmids
    - Experience performing adenoviral infections of expression plasmids
  - **DNA, mRNA, and Protein Studies**
    - Preforming DNA extraction/purification
    - Preforming SNP genotyping assay
    - Preforming RNA extraction/isolation using TRIzol
    - Preforming protein extraction and concentration determination using Bradford assay
    - Preforming reverse transcription of mRNA into cDNA coupled with quantitative PCR (qPCR) and normal PCR
    - Preforming protein detection via western blotting
  - **Bacteria Studies**
    - Executing transformation and purification of plasmids
    - Determining successful transformation with gel electrophoresis and sequencing
    - Performing restriction enzyme digestion of plasmid DNA

- **Creating a double-stable cell line**
  - Created a BSEP/NTCP stable cell line in HEK-293 cells

- **Instrumentation**
  - Experience in using the GLOMAX 20/20 Luminometer
  - Experience in using the GLOMAX 96 Microplate Luminometer
  - Experience in using ABI Prism 7000 Sequence Detection System
  - Experience in using Applied Biosystems StepOnePlus Real-Time PCR System
  - Experience in using Applied Biosystems ViiA 7 Real-Time PCR System
  - Experience in using spectrophotometer
  - Experience in using a scintillation counter

COMPUTER SKILLS

- Endnote
- GraphPad Prism
- SigmaPlot
- Adobe Photoshop/Acrobat
- MethylPrimer Express
- Chem/BioDraw
- Microsoft Office (Excel, Word, Powerpoint)

RELEVANT COURSEWORK

- Principles of Drug Discovery
- Principles of Drug Development
- Pharmaceutical Science Ethics
- Drug Transport and Metabolism
- Bioanalytical Methods
- Spectrometric Methods
- Pharmacokinetics
- Principles of Pharmacology
- Genetics (Undergraduate)
- Advanced Pharmacogenetics
- Pharmaceutical Science (PSC) Journal Club
- Chemistry/Biology Interface Journal Club

PROFESSIONAL MEMBERSHIPS

- Society of Toxicology
- American Association of Pharmaceutical Sciences
- American Association for the Advancement of Science
- Rho Chi National Honor Society- Omicron Chapter
- Kappa Gamma Pi Honor Society
- Delta Epsilon Sigma Honor Society

Abstract

Title of the dissertation: The Role of Bile Salt Export Pump (BSEP; ABCB11) Gene Repression in Drug-Induced Cholestatic Liver Injury

Author: Brandy N. Garzel, Ph.D. Candidate 2017

Dissertation directed by: Hongbing Wang, Ph.D.
Professor
School of Pharmacy
University of Maryland, Baltimore, USA

Drug-induced liver injury (DILI) is a persistent matter in the pharmaceutical field, with extensive research being dedicated to understanding the causes, risk factors, and potential biomarkers associated with it. While DILI manifests in a variety of ways and diseases, the most severe form is drug-induced cholestasis. Bile acids are amphipathic molecules responsible for the extraction of lipids from the diet, and while necessary for function, their amphipathic nature can cause toxicity if allowed to accumulate in tissue. Constant flow of bile acids through biliary tissue is facilitated by specific bile transporters, with the bile salt export pump (BSEP; ABCB11) dictating excretion of bile acids from the liver to the gall bladder. Disruption of BSEP function is a major contributor to both inherited and acquired cholestasis. The predominant mechanism of drug-induced cholestasis is direct inhibition of BSEP, however in the following work, the contribution of BSEP repression on BSEP function was investigated.

In the following studies, human primary hepatocytes (HPH) were used predominantly as a physiologically relevant system for studying drug transporter function and expression upon drug treatment. The functional transport assay employed was modified from the prototypical inhibition assays primarily used in cholestatic studies, and represents a novel approach to assess the cholestatic potential of BSEP repressors. Additional BSEP repressors were predicted from reported cases of drug-induced cholestasis combined with Bayesian modeling.

It was determined from our study, that compared to inhibition alone, BSEP inhibition combined with repression correlated with severe clinical manifestations of DILI. Mechanistically, activation of AMPK was found to repress basal BSEP expression, while known transcriptional regulators FXR and Nrf2 had no effect. Most importantly, under conditions which cause BSEP repression, bile acid efflux was also significantly reduced. Metformin was used as a model drug in our studies, however, a number of drugs associated with cholestasis do not inhibit BSEP directly. Employing a strategy to elucidate additional cholestatic BSEP repressors, tamoxifen was found to potently repress BSEP expression, indicating the potential discovery of additional repressors. Overall, these results confirm BSEP repression represents an understudied mechanism for drug-induced cholestasis.

The Role of Bile Salt Export Pump (BSEP; ABCB11) Gene Repression in Drug-Induced Cholestatic Liver Injury

by
Brandy N. Garzel

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

Dedicated to my wonderful, supportive husband Brandon

and our beloved son Britton

Acknowledgements

I would first like to thank my advisor, **Dr. Hongbing Wang**, for everything he has done over the past six years. Throughout this entire process, Dr. Wang has always given me the support and guidance necessary to become a successful researcher. Without his constant dedication and commitment to my success, and the success of my project, the following work would not have been possible. From the start, he has often told me how much potential he sees within me, and this encouragement has motivated me even during the hardest times. I am eternally grateful for his mentorship through my Ph.D. and the impact he has made on my future career.

I would also like to sincerely thank **Dr. Jean-Pierre Raufman** for joining Dr. Hongbing Wang two years ago as a co-mentor. He has been invaluable in the design and interpretation of *in vitro* data, specifically in its clinical extrapolation, and in understanding the significance of our findings. Additionally, Dr. Raufman has been very helpful in preparing my work for publication, and is always available when I have questions/requests. I am very thankful to have him included in the mentorship of my Ph.D. and truly believe my work has benefitted from his involvement.

In addition to my mentors, I would also like to graciously acknowledge the rest of my **Ph.D. thesis committee**, Dr. Shiew-Mei Huang, Dr. James E. Polli, Dr. Paul Shaprio, Dr. Yan Shu, and Dr. Peter Swaan. Their continued support throughout this process has contributed significantly to the success of my thesis, as well as my career.

Special thanks is given to my **past and current lab members**, Dr. Linhao Li, Dr. Hui Yang, Dr. Dauchuan Li, Dr. Zhihui Li, Dr. Duan Wang, Dr. Caitlin Lynch, William Hedrich, and Bryan Mackowiak, who have made working in the lab not only successful,

but enjoyable. I consider most of our lab like family, and the relationships we have made during the past six years will forever be cherished and taken with me as I move forward in my career.

I would like to express my utmost gratitude for **Dr. Caitlin Lynch**. She was a senior member in our lab when I first entered the Ph.D. program, and was vital to my learning and growth in the early years. Not only did I gain important research skills from her mentorship, but I also developed a life-long friendship, which is something very, very dear to me.

I would also like to thank **Dr. Linhao Li** specifically for his help during my Ph.D. He has been a constant mentor and friend and has been vital to the smooth operation of the lab since day one.

There have been numerous **colleagues and friends** made during the course of thesis, both within and outside of the University of Maryland Baltimore. Those which have had a significant impact on my life and work include Dr. Geoffrey Heinzl, Dr. Yewon (Joanna) Pak, Dr. Soundarya Vaithianathan, Dr. Julie Castaneda, Dr. Anika Lin Dzierlenga, Melissa Metry, Kristina San Juan, and Jessica Camacho. Their friendship has offered guidance, solace, and support throughout my Ph.D., something I will always be grateful.

I would like to thank my parents, **James and Brita Nichols**, for their love and support throughout my entire life. They have instilled in me a hard-working nature and love of learning, shaping my curious mind into the researcher I am today. Additionally, I would like to thank my husband's parents, **Gregory and Cynthia Garzel**, who have been incredibly loving and supportive as long as they have known me.

Finally, my deepest appreciation goes to my husband, **Brandon Garzel**. He has been my best friend, my partner, and my greatest supporter for over 13 years. I would not be the woman I am today without him. He has supported me in every way over the past six years, allowing me to pursue my goal of earning a Ph.D. His dedication and the immeasurable time and effort given have been invaluable to our family and myself. Words cannot express the love and respect I have for my husband, and how thankful I am to have him in my life, especially during these last six years.

Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Abbreviations

ABC	ATP Binding Cassette
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABCB11	ATP Binding Cassette Subfamily B Member 11
ABCB4	ATP Binding Cassette Subfamily B Member 4
ABCC2	ATP Binding Cassette Subfamily C Member 2
ABCC3	ATP Binding Cassette Subfamily C Member 3
ABCC4	ATP Binding Cassette Subfamily C Member 4
ABCG2	ATP Binding Cassette Subfamily G Member 2
ACC	Acetyl Coenzyme A Carboxylase
AICAR	5-Aminoimidazole-4-Carboxamide 1-β-D-Ribofuranoside
ALP	Alkaline Phosphatase
ALT	Alanine
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate-activated Protein Kinase
AMPKα	Adenosine Monophosphate-activated Protein Kinase Alpha Subunit
ANOVA	Analysis of Variance
AR	Adverse Reactions
AST	Aspartate Aminotransferases
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
ATP8B1	Adenosine Triphosphatase Phospholipid Transporting 8B1

AUC	Area Under Curve
BCA	Bicinchoninic Acid
BCRP	Breast Cancer Resistance Protein
BEI	Biliary Excretion Index
BRIC	Benign Recurrent Intrahepatic Cholestasis
BS1-1	Best-conformation Shared-pharmacophore 1-1
BSEP	Bile Salt Export Pump
BW	Box Warning
CA	Constitutively Active
CA*	Cholic Acid
CA-AMPK-GFP	Constitutively Active-Adenosine MonoPhosphate-Green Fluorescent Protein
CARB	Carbamazepine
CDCA	Chenodeoxycholic Acid
CF	Calcium Free
CIME	Cimetidine
CYP2B6	Cytochrome Family 2 Subfamily B Member 6
CYP3A4	Cytochrome Family 3 Subfamily A Member 4
CYP7A1	Cytochrome Family 7 Subfaimly A Member 1
DCA	Deoxycholic Acid
DILI	Drug-induced Liver Injury
DILIN	Drug-induced Liver Injury Network
D-PEN	D-penicillamine

EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinases
ERα	Estrogen Receptor Alpha
FAS	Fatty Acid Synthase
FCFP_6	Functional-Class Fingerprints Maximum Diameter 6
FDA	Food and Drug Administration
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCA	Glycocholic Acid
GCDCA	Glycochenodeoxycholic Acid
GFP	Green Fluorescent Protein
GGT	Gamma-glutamyl Transferase
HBSS	Hank's Balanced Salt Solution
HL	Human Liver
HLA	Human Leukocyte Antigen
Hmg-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPH	Human Primary Hepatocytes
IBUP	Ibuprofen
ICP	Intrahepatic Cholestasis of Pregnancy
LCA	Lithocholic Acid
LDC	Least DILI Concern
LOO	Leave-One-Out
LRH-1	Liver-Related Homolog-1

LTKB	Liver Toxicity Knowledge Base
MAPK	Mitogen-Activated Protein Kinase
MDC	Most DILI Concern
MDR1	Multi-Drug Resistance Protein 1
MDR3	Multi-Drug Resistance Protein 3
MRP2	Multi-Drug Resistance-Associated Protein 2
MRP3	Multi-Drug Resistance-Associated Protein 3
MRP4	Multi-Drug Resistance-Associated Protein 4
mTOR	Mechanistic Target Of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NIH	The National Institute of Health
NITR	Nitrofurantoin
NORT	Nortriptyline
NQO1	NAD(P)H Quinone Dehydrogenase 1
NR1H4	Nuclear Receptor Subfamily 1 Group H Member 4
NR2B1	Nuclear Receptor Subfamily 2 Group B Member 1
NRF2	Nuclear Factor Erythroid-Derived 2-Like 2
NTCP	Sodium-Taurocholate Cotransporter Polypeptide
OATPs	Organic Anion Transporter Proteins
OSTα/β	Organic Solute Transporter A And B Heterodimer
P450	Cytochrome P450
PCR	Polymerase Chain Reaction

PFIC	Progressive Familial Intrahepatic Cholestasis
pRL-TK	Renilla luciferase - Thymadine Kinase
QSAR	Quantitative Structure-Activity Relationship
ROC	Receiver Operating Characteristic
RT-PCR	Real Time - Polymerase Chain Reaction
RUCAM	Roussel Uclaf Causality Assessment method
S-2E	(S)-(+)-4-[1-(4-tert-Butylphenyl)-2-oxo-pyrrolidin-4-yl]methoxybenzoic acid
SD	Standard Deviation
SERM	Selective Estrogen Receptor Modulator
SFN	Sulforaphane
SHP	Small Heterodimer Partner
SLC10A1	Solute Carrier Family 10 Subfamily A Member 1
SLC21	Solute Carrier Family 21
SLCO	Solute Carrier Organic Anion Transporter Family
SREBP1	Sterol Regulatory Element Binding Protein 1
ST	Standard
SULI	Sulindac
TAM	Tamoxifen
TCA	Taurocholic Acid
TCDCA	Taurochenodeoxycholic Acid
TRIM	Trimethoprim
ULN	Upper Limit of Normal

VERA	Verapamil
WP	Warnings And Precautions

Chapter 1: Overview and Objectives

1.1 Drug-Induced Liver Injury

As the primary source for detoxification in the body, the liver is subjected to countless endogenous and xenobiotic chemicals daily (1). It is arguably the most exposed tissue to drugs; therefore, it is not surprising over 1000 drugs are reported to cause hepatotoxicity (2, 3). Of these, anti-microbials have the greatest incidence at 45.5%, with central nervous system agents coming in second at 15%. Additionally, immunomodulatory agents, analgesics, antihypertensive agents, antineoplastic agents and lipid-lowering agents are less commonly associated classes, yet they still make up 5.5%, 5%, 5%, 4%, and 3.4%, respectively (4). Drug-induced liver injury (DILI) is a growing problem in drug development, representing the leading cause for drug attrition in the United States (5, 6). In response, over the past fifteen years substantial research efforts have been made to better understand DILI. However, there is still much to be discovered regarding causes, risk factors, and potential biomarkers for prediction (7).

Hepatoxicity associated with drug treatment is referred to as drug-induced liver injury and is predominantly characterized by an increase of liver enzymes in serum (4, 8), which typically resolve when the drug is discontinued (4). Specifically, alanine/aspartate aminotransferases (ALT/AST) and alkaline phosphatase (ALP) are monitored, and when levels exceed the upper limit of normal (ULN), liver dysfunction is typically observed (9). Enzyme levels are considered individually, but also as computed into an R-value (ALT divided by ALP). The calculated R-value can indicate the pattern of hepatocellular injury present—$R > 5$ = hepatocellular; $5>R>2$ = mixed; $R<2$ = cholestatic (8, 10).

Moreover, elevated total bilirubin in serum (>2 x ULN) is a distinguishing feature of DILI, often ruling out other liver diseases. While elevated enzyme levels are a good first indicator of liver dysfunction, most diagnoses require additional consideration (11, 12).

1.1.1 Hy's Law

Hyman J. Zimmerman is credited for the observation that serum AST levels, in general, were not sufficient to predict acute liver failure or drug-induced hepatotoxicity (13, 14). It was dubbed "Hy's law" by Dr. Robert Temple, Deputy Center Director for Clinical Science at The Center for Drug Evaluation and Research of the FDA, in the 1980's (according to a statement he made in a 2006 publication), and has continued use through the present (15). Hy's Law predicts that elevations in both AST and bilirubin levels (jaundice), especially when no other cause for these elevations can be observed, will lead to at least a 1 in 10 risk of developing DILI (4).

1.1.2 Roussel Uclaf Causality Assessment Method (RUCAM)

Developed in 1993, the Roussel Uclaf Causality Assessment method (RUCAM) is one of the leading tools for determining the strength of association between liver injury and a suspected causative agent (16, 17). The method uses seven criteria, including: time to onset of DILI, recovery of liver function upon withdrawal of drug, risk factors, exclusion of other causes of liver injury, simultaneous use of other drugs, previous hepatotoxic reactions, and reaction to re-administration of drug, with each condition being assigned a point value (11, 18). Total point values possible for the RUCAM test range from -8 to 14, and higher values indicate a greater association with hepatotoxicity.

While some components and even the final score is considered to be imprecise, many still continue to use this method to help narrow down the cause for liver injury to be drug-related (18).

1.1.3 Clinical manifestation of DILI

Drug-induced liver injury manifests in a variety of ways, and parallels all forms of acute and chronic liver disease (19). The clinical presentation can range from nausea and abdominal pain (most frequently) to jaundice and pruritus; left undiagnosed, these can lead to acute liver injury and eventually hepatocellular death (10). Some of the diseases which DILI can mirror, their clinical presentation, and example causative agents are listed in Table 1.1 (10). Mimicking the clinical appearance of so many liver disorders confounds diagnosis of DILI and often requires thorough investigation of the drug, after elimination of these confounding liver diseases as a cause (20). In addition to searching literature for previous reports of hepatotoxicity, specifically with the same histological features, clinicians can consult databases which aim to include all relevant reports and data on drug-induced liver injury (21, 22). Accurate diagnosis and up-to-date reporting of DILI are crucial to the on-going efforts to understand, quickly identify, and possibly predict future cases of DILI.

1.2 Intrinsic versus idiosyncratic DILI

DILI can be classified into two categories based on mechanism, intrinsic (predictable) or idiosyncratic (unpredictable) drug induced liver injury (23, 24) (Table 1.2). Acetaminophen is the most commonly cited drug associated with intrinsic DILI;

Table 1.1: Examples of Liver Injury which DILI may resemble

Phenotype	Histological features	Example agents
Acute fatty liver with lactic acidosis	Microvesicular hepatic steatosis ± other tissue involvement	Didanosine, Fialuridine, Valproate
Acute hepatic necrosis	Collapse and necrosis of liver parenchyma	Isoniazid Niacin
Autoimmune-like hepatitis	Plasma cells & interface hepatitis with detectable autoantibodies	Nitrofurantoin, Minocycline
Bland cholestasis	Balloon hepatocytes with minimal inflammation	Anabolic steroids
Cholestatic hepatitis	Balloon hepatocytes with inflammation, predominance of serum alkaline phosphatase elevation (phenytoin, amoxicillin-clavulanate)	Phenytoin, amoxicillin-clavulanate
Fibrosis/cirrhosis	Hepatic collagenization with minimal inflammation	Methotrexate, Amiodarone
Immunoallergic hepatitis	Eosinophilic infiltrate	Trimethoprim-sulfamethoxazole
Nodular regeneration	Micro- or macroscopic liver nodules	Azathioprine, Oxaliplatin
Nonalcoholic fatty liver	Macro- and microsteatosis, hepatocyte ballooning and periportal inflammation	Tamoxifen
Sinusoidal obstruction syndrome	Inflammation with obliteration of central veins	Busulfan
Vanishing bile duct syndrome	Paucity of interlobular bile ducts	Sulfonamides, Beta-lactams

Table 1.1 Histological findings and associated diseases which mimic drug-induced liver injury. Several examples of liver injury which share similar symptoms and clinical manifestations with drug-induced liver injury. The phenotypes, histological features, and examples of drugs reported for each are listed. This table was modified from ref (Hayashi and Fontana, Seminars in Liver Disease (10))

while safe at therapeutic doses, it becomes toxic in overdose situations (or in patients with hepatic impairment) (23). Dose-dependent toxicity is a hallmark feature of intrinsic DILI, typically identified during drug development, in animal models with high frequency (24). These features lend to the predictability of intrinsic DILI and provide rationale for safe, therapeutic dosing in drugs which make it to market. In most cases however, preclinical observations of intrinsic DILI result in discontinuing development of the drug, preventing it from harming the general population (24). Altogether, even with a majority of acute liver failure cases being attributed to intrinsic DILI, it is manageable and potentially avoidable, making it the less severe of the two forms.

1.2.1 Idiosyncratic Drug-Induced Liver Injury

While DILI in general is undesirable and an obstacle pharmaceutical industries must address, idiosyncratic DILI (iDILI) is particularly troublesome to the pharmaceutical field as a whole. Due to the random nature in which it occurs, iDILI is not normally observed preclinically, and only becomes realized post-marketing (25). Unfortunately, the resources expended on a drug at this stage is much greater than in earlier stages of development, making it a costly issue for industry. Unforeseen toxicity also becomes a major concern for clinicians and patients, as well as the regulatory bodies, and requires additional monitoring to fully characterize such toxicity (26).

Post-approval, drugs become more accessible to the general population, broadening the number and type of individuals exposed. Since iDILI does not typically effect a specific patient population or adhere to dose-related toxicity, it is often not observed during clinical trials (24). A rare occurrence, with only 1 in 10000 individuals

Table 1.2: Intrinsic v. idiosyncratic DILI

Intrinsic	Idiosyncratic
Affects all individuals at some dose	Attacks only susceptible individuals
Clearly dose-related	Obscure relation to dose
Predictable latent period after exposure	Variable onset relative to exposure
Distinctive liver lesion	Variable liver pathology
Predictable using routine animal testing	Not predictable using routine animal testing

Modified from Roth and Ganey, 2010 (24).

suspected to develop iDILI (20, 27), it is very difficult to capture during development, when sample sizes are typically ~2500 individuals (22). This puts a greater emphasis on post-marketing surveillance of drugs, requiring both the pharmaceutical companies and regulatory agencies to monitor clinical reports associated with drug use (28, 29). When DILI is consistently observed, regulatory bodies and/or manufacturers can respond in a number of ways, which including making changes to the label (adding warnings and contraindications), issuing correspondences to clinicians (which may suggest regular liver monitoring), or in severe cases, withdrawing the drug from the market (5, 12).

1.2.2 Prediction of iDILI

Unpredictability is one of the greatest limitations in managing idiosyncratic DILI. A majority of research in the field continues to search for biomarkers or predictors of iDILI, or a common cause amongst drugs associated with it (12, 20). One of the greatest limitations in the prediction of iDILI centers on the numerous clinical manifestations of iDILI, making a one-size-fits-all solution unlikely. Categorizing the histopathological patterns of DILI into hepatocellular, cholestatic, or mixed allows common agents to be grouped together, providing a limited, but promising association to be utilized in prediction strategies (10). Furthermore, recent studies have discovered immunological responses associated with unpredictable drug-induced liver injury, prompting a new classification of iDILI which includes allergic and non-allergic type (12, 29). The exact role of the immune response is still under investigation, however several reports indicate drugs and/or their metabolites bind with liver proteins, producing an immune response from antibodies or cytotoxic T cells (28). As a result of the newly discovered

immunological connection to drug-induced liver injury, a genetic biomarker has emerged, with some success in predicting/diagnosing iDILI with regards to specific drugs (11, 12). Polymorphisms in the human leukocyte antigen (HLA) can predispose individuals to developing DILI upon treatment with particular drugs, specifically those which elicit an immune response (8, 30). Such innovative findings provide hope for even greater understanding in idiosyncratic drug-induced liver injury.

1.2.2.1 Liver toxicity databases

With an increasing number of drugs linked to drug-induced liver injury (over 1000 have been reported (11, 29)), a growing need has developed to collect information associated with it. Databases have been established to report cases of drug induced liver injury, including information about time of onset, dosage, clinical manifestation, and demographic information (22). The National Institutes of Health (NIH)-sponsored LiverTox database, Drug-Induced Liver Injury Network (DILIN), and Food and Drug Administration (FDA)-sponsored Liver Toxicity Knowledge Base (LTKB) are three highly consulted databases which compile reports of drug-induced liver injury. While their primary focus is to combine information about drug-induced liver injury in a centralized location, and not actively attempt to predict DILI, connections between drugs with common histological signatures, immunological responses, and genetic predispositions can become apparent. Additionally, these databases can aid in the diagnosis of iDILI, as previous hepatotoxicity associated with a drug can indicate to a clinician drug-induced liver injury may be in play, rather than other forms of liver disease.

Information contained in the liver toxicity databases can be also be used in the development of computational models for idiosyncratic drug-induced liver injury. As the databases contain a wealth of information regarding drug-induced liver injury, some studies have used text-mining strategies as a means to find connections, keywords, and/or indicators of DILI (31). Additionally, tracking hepatotoxic side-effects in the clinical reports found in databases can establish subgroups of drugs with common toxicities. Further categorization of these subgroups by looking for common chemical features can be utilized to generate quantitative structure-activity relationship (QSAR) models (32, 33). Overall, the establishment and maintenance of publicly accessible drug-induced liver toxicity databases provides numerous benefits in the battle against DILI.

1.3 Forms of drug-induced liver injury

While drug-induced liver injury can mimic any form of liver disease, these histopathological manifestations can also define the type of drug-induced liver injury experienced. There are five main classifications of DILI, as reported by the drug-induced liver injury network, and they include necrosis, cholestasis, steatosis, vascular injury patterns, and cytoplasmic alterations (34). Other studies or groups classify the pattern of DILI into acute hepatocellular injury (including necrosis), various forms of hepatitis, cholestasis, and vascular patterns of injury (35), however the overall features remain the same.

Many forms of drug-induced liver injury result in varying degrees of necrosis, ranging from individual hepatocyte necrosis, to entire zones (35). Cell death can be a precursor for other types of liver injury, such as acute liver injury. Inflammation, likely

through activation of the immune response, characterizes hepatitis, which can manifest in a variety of forms: acute hepatitis, chronic hepatitis, cholestatic hepatitis, granulomatous hepatitis, and steatohepatitis (35). Combined, the many forms of hepatitis may constitute the most common form of DILI injury, however when considered individually, cholestatic liver injury accounts for the most cases of DILI reported (36). Cholestatic liver injury (which includes mixed hepatocellular/cholestatic type) is the most severe form, with increasing occurrence.

1.4 Bile acids

Bile acids are important amphipathic molecules derived from the catabolism of cholesterol in the liver (37). There are several species of bile acids in humans, ranging from primary bile acids formed de novo, to their metabolites, the secondary and tertiary bile acids (38). The two primary bile acids are cholic acid (CA*) and chenodeoxycholic acid (CDCA), which are formed solely in the liver via the classical or alternative pathways of cholesterol catabolism (39, 40). Each pathway is initiated by a different metabolizing enzyme: the classical begins with cholesterol-7α-hydroxylase (CYP7A1), while the alternative pathway starts with mitochondrial sterol 27 hydroxylase (CYP27A1) (40). Conjugation of CA and CDCA in the liver with glycine or taurine form glyco- or tauro- cholic acid (GCA or TCA) and glyco- or tauro- chenodeoxycholic acid (GCDCA or TCDCA) and aids in their excretion from the liver, through the bile canaliculi, to the gallbladder (38, 41). Upon ingestion of food, bile acids are released from the gallbladder into the small intestine, where they can be deconjugated and dehydroxylated by the bacterial flora, creating the secondary bile acids deoxycholic acid (DCA; formed from CA) and lithocholic acid (LCA; formed from CDCA) (40, 42, 43). Finally,

transformation of lithocholic acid in the liver or intestine results in the formation of the tertiary bile acids sulfolithocholic acid and ursodeoxycholic acid (43).

The many roles of bile acids in the body include sequestration of water-insoluble lipids, regulation of glucose and lipid metabolism, excretion of endogenous metabolites and xenobiotics, and activation of signaling cascades by binding to receptors (44, 45). Many of these functions are interrelated, for example, activation of the nuclear receptor farnesoid-x-receptor (FXR) by CA or CDCA leads to the regulation of glucose and lipid metabolism (45, 46).

1.4.1 Feedback regulation of bile acids

The regulation of lipid metabolism by bile acids, specifically with their formation from the catabolism of cholesterol, is a feedback mechanism in response to bile acid levels. In the case of too many bile acids, the metabolizing enzyme CYP7A1 becomes repressed by bile acid-mediated activation of the FXR and its downstream target small heterodimer partner (SHP). SHP binds to the promoter of CYP7A1, along with the liver-related homolog-1 (LRH-1), to block further transcription of the gene, thus terminating the breakdown of additional cholesterol (2, 47). On the other hand, if there are not enough bile acids, FXR would cease to be activated, restoring CYP7A1 expression and activity to normal. Intestinal FXR is also very important in the regulation of bile acids, being activated by bile acids in the small intestines to induce the expression of fibroblast growth factor (FGF) 19. FGF19 binds to the liver FGF receptor 4, initiating a signaling cascade of kinases to inhibit CYP7A1 action (39). The net effect results in a decrease of newly synthesized bile acids.

As a feedback mechanism in response to endogenous changes in bile acid levels, regulation of bile acids can be used to purposefully reduce serum cholesterol amounts by forcing a decrease in hepatic bile acids. The drug cholestyramine interferes with the reabsorption of bile acids into the liver, by sequestering them in the intestinal lumen, therefore the liver senses a need for bile acids, and initiates the catabolism of cholesterol, which can be pulled from the sera (48). Bile acids diverted from the liver are excreted from the body, allowing bile acid homeostasis to remain intact.

1.4.2 Role of bile acids in the body

One of the most notable functions of bile acids is the emulsification of dietary fats and fat-soluble vitamins from the intestine (44). Their amphipathic quality allows bile acids to form micelles, which allows their hydrophilic ends to interact with the intracellular fluid, forming an outer shell, and their hydrophobic tails to stay protected inside of the micelle (49-51). The inner hydrophobic core of the micelle interacts with dietary fats and vitamins, in addition to toxic endo- and xenobiotics which need to be removed from the body, and sequester them inside, away from unfavorable interactions with the cytoplasm (51). Once the lipids or toxic constituents are sequestered inside of the bile acid micelles, they can easily be transported through the intestine and liver, for further processing or removal from the body, respectively. The sequestration of dietary lipids and their delivery to the liver provides an additional mechanism for bile acids in the regulation of lipids. Outside of their direct interaction with lipids, as mentioned above, bile acids also serve as signaling molecules, binding to nuclear receptors and g-protein coupled receptors to initiate downstream effects (52).

1.4.3 Enterohepatic circulation

The most abundant bile acids in humans are CA, CDCA, and DCA, with the other species contributing minimally to the bile acid pool (53). Bile acid levels in the body are maintained via two mechanisms: de novo synthesis, as discussed above, or enterohepatic circulation. The majority of the bile acid pool, which is ~2-3 g (53, 54), is recycled from the intestines in a process known as enterohepatic circulation, limiting the amount of bile acids synthesized de novo each day to between 200-600 mg (53). This recycling of bile acids is vital to ensuring bile acid homeostasis, which is necessary for its variety of endogenous functions.

Enterohepatic circulation maintains flow of bile acids from the liver where they are synthesized, to the gallbladder for storage, and eventually the small intestines where they aid in the absorption of dietary fats and vitamins (55, 56). Once biliary micelles contain these lipids, they are shuttled back to the liver where they can deposit their contents and start the process again (56). As the bile acids individually, and in the form of micelles, can inflict toxic effects, this flow is essential not only to function, but also to the protection of biliary tissue from toxic accumulation of bile acids.

1.4.4 Biliary transporters

In order to facilitate flow of bile through their respective tissues, several biliary transporters are expressed. Bile acids are taken up into the liver by the sodium-taurocholate cotransporter polypeptide (NTCP), located on the basolateral membrane, and to a lesser extent, by the organic anion transporter proteins (OATPs) (57). The primary route of elimination for bile acids from the liver once synthesized, is through the

bile salt export pump (BSEP), which is expressed on the apical membrane of hepatocytes. Another transporter found at the apical membrane is the multi-drug resistance-associated protein 2 (MRP2). In addition to transporting sulfated bile acids, MRP2 is important in excreting bilirubin and glutathione into the bile canaliculi, both essential components of bile (58, 59). Similarly, multi-drug resistance protein 3 (MDR3) and the p-type ATPase ATP8B1 flippases contribute to bile flow by ensuring phospholipids are present in the bile canaliculi (60). Additional anions are needed for bile formation, and are transported by the multi-drug resistance protein (MDR1) (61). Mixed micelles are formed from the bile constituents once they have been transported to the bile canaliculi.

From the liver, bile travels through the bile ducts to the gallbladder for storage. The gallbladder releases bile acids into the small intestine upon consumption of a meal to initiate absorption of dietary lipids (49). The major transporters in the small intestines and colon responsible for biliary transport are the apical sodium-dependent bile-salt transporter and organic solute transporter α and β heterodimer (OSTα/β) (53). Once recycled through the small intestine, bile acids travel back to the liver through the portal vein.

1.4.5 Hepatic transporters

Enterohepatic circulation of bile acids starts and ends in the liver. There are several transporters expressed to ensure successful flow of bile acids and all other constituents that make up bile (61) (Figure 1.1). In addition to primary transporters for uptake and efflux, there are secondary transporters, as well as compensatory transporters

that flow in the opposite direction of traditional bile flow. This ensures a proper balance of bile acids and maintains toxicity that may associate with bile acids.

1.4.5.1 Basolateral uptake transporters

The sodium-dependent taurocholate cotransporter polypeptide, NTCP (SLC10A1) was first identified in 1978 and is the first member of its family of transporters (62). As the name suggests, NTCP requires the selective transport of sodium ions in order to transport bile acids (57). Specifically, two sodium ions must be present for biliary transport, and transport occurs in the same direction. While NTCP is selective for sodium, it can co-transport a range of substrates, including hormones and drugs. The affinity of NTCP for drugs allows competitive inhibition of bile acid transport, in addition to potential inhibition on away from the active site (57, 63). NTCP is responsible for transporting 80% of conjugated bile acids, therefore disruption of NTCP activity can have significant effects on bile acid homeostasis (63).

Serving as a supportive biliary uptake transporter, the organic anion transporting polypeptide family of transporters (SLCO; formerly SLC21), transport unconjugated bile acids in a sodium-independent manner (64). OATP typically transports molecules with molecular weights $\geq$ 350 daltons, including drugs bound to albumin (65). Many features of OATP oppose those of NTCP, including the co-transport of one or two negatively charged molecules with the transport of bile acids (65). While there are other minor uptake transporters available, NTCP and OATP maintain the majority of bile acid transport into hepatocytes.

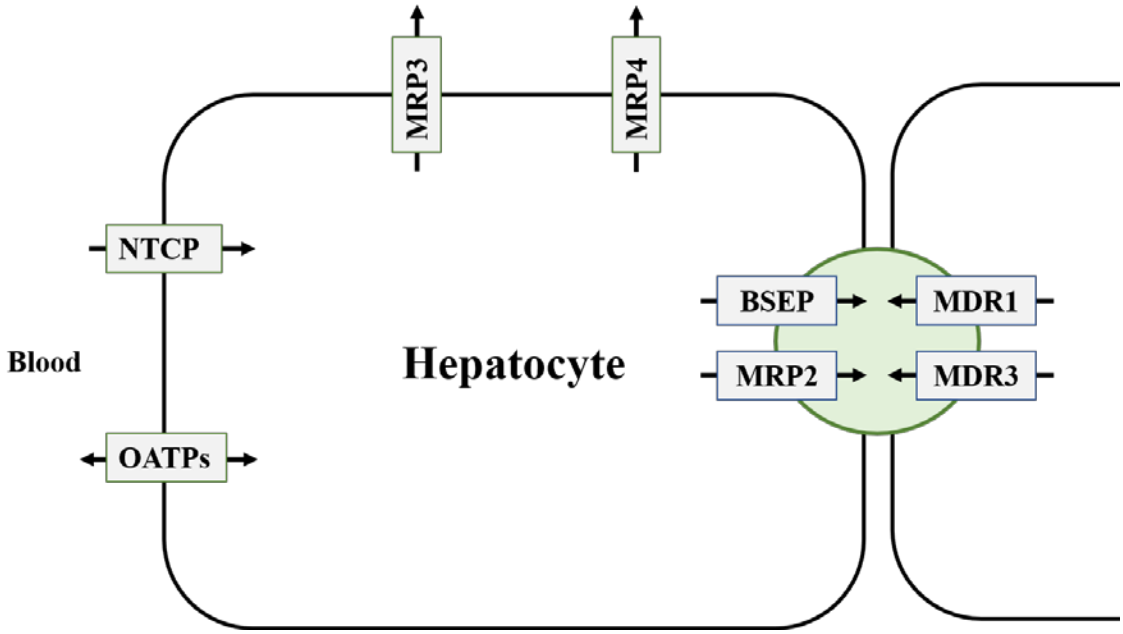


Figure 1.1 Hepatobiliary transporters. The hepatobiliary transporters discussed in this chapter are shown, in relation to their position in hepatocytes. The basolateral membrane contains the uptake transporters, NTCP and OATPs, as well as the efflux transporters MRP3 and MRP4. The bile canaliculi (in green), depicted between two hepatocytes, contains the canalicular efflux transporters BSEP, MRP2, MDR1, and MDR3. Arrows indicate direction of transport.

1.4.5.2 Canalicular efflux transporters

Known as the rate-limiting step of bile acid excretion from the liver, the bile salt export pump (ABCB11) was first cloned in rats in 1998, however it was first identified as the "sister of p-glycoprotein," as it shared 88% sequence homology with p-glycoprotein when the sequence was characterized in 1995 (66, 67). BSEP is located on the canalicular membrane and transports conjugated bile acids from the liver in an ATP-dependent manner (64). Substrate preference for BSEP is highly preferential for bile acids, however numerous inhibitors have been identified, with some reported as competitive inhibitors (55).

Unusual bile acids not transported by BSEP can be excreted into the bile canaliculi by either the multi-drug resistance-associated protein 2 (ABCC2) or the multi-drug resistance protein 1 (ABCB1) (68). Divalent conjugates of bile acids, bilirubin, glucuronides, and glutathione conjugates are a few examples of substrates for MRP2. MDR1 is involved in transporting bulky amphipathic anionic conjugates (68). Both transporters also belong to the ATP-binding cassette (ABC) superfamily of transporters, and require ATP to function (61).

Though not involved in bile acid transport, the mult-drug resistance protein 3 (ABCB4) is essential in its role for translocating phospholipids to bile (61). Also expressed at the canalicular membrane, MDR3 is an ABC transporter dependent on ATP for function.

1.4.5.3 Basolateral efflux transporters

The major route of elimination for bile acids is through the canalicular membrane, however, in the event that transport in that direction is not possible, compensatory

mechanisms are available on the basolateral membrane (68). Multi-drug resistance-associated protein 3 and 4 (ABCC3/4) specifically transport divalent conjugated bile acids and are dependent on ATP. During normal conditions, the basal expression of MRP3 and MRP4 are quite low, however under conditions with excess bile acids in hepatocytes, these transporters are upregulated (68).

1.4.6 Transcriptional regulation of biliary network by FXR

Regulation of bile homeostasis primarily relies on the farnesoid x receptor (FXR; NR1H4) (69). FXR is a member of the nuclear receptor family of transcriptional regulators, and is predominantly expressed in tissues related to bile acids: liver, intestines, and kidney. As a regulator of bile acids, FXR is directly bound and activated by primary, and to a lesser extent, secondary bile acids (both conjugated and unconjugated), before dimerizing with the retinoic x receptor (NR2B1) (52). FXR binds to a specific DNA-binding motif, called the inverted repeat 1, with the following sequence: AGGTCA, separated by one base pair.

Also referred to as the bile acid receptor, FXR is involved in the transcriptional regulation of bile acid metabolizing enzymes, binding proteins, and transporters (40). In response to low bile acid levels in the liver, FXR transactivates CYP7A1, the enzyme responsible for the catabolism of cholesterol to bile acids. Additionally, the non-classical pathway of bile acid synthesis, initiated by CYP8B1 is also regulated by FXR (70). As mentioned above, intestinal FXR induces FGF19, which inhibits CYP7A1 in the liver in response to excess bile acids in the small intestine (52). The ileal bile acid binding protein, which facilitates transport of bile acids through enterocytes to be taken up into the portal blood system, is also induced by FXR activation in the intestines (71). These

mechanisms ensure adequate levels of bile acids, needed to maintain enterohepatic circulation. Transporters play a pivotal role in facilitating bile flow, many of which are also regulated by FXR.

Biliary uptake and efflux transporters are also regulated by FXR, including: NTCP, OATPs, BSEP, MDR3, and MRPs (52, 72). Uptake transporters NTCP and OATPs are upregulated when hepatic bile acid levels are low, allowing an influx of bile acids to enter the liver, and down-regulated when there are excess bile acids. Conversely, BSEP is upregulated when bile acid levels exceed normal levels. Notably, MRP3 and MRP4 have been shown to be upregulated in excessive bile acid conditions, again, allowing toxic bile acids to escape the liver, however this regulation is through the pregnane x receptor (PXR; NR1I2) (52). A schematic indicating the hepatic proteins regulated by FXR can be found in Figure 1.2 (68). The downregulation of NTCP occurs through FXR induction of the small heterodimer partner, (SHP).

1.5 Cholestatic liver injury

Disruption of bile flow, whether metabolic or mechanical, can result in the accumulation of bile acids, predominantly in the liver. Due to their amphipathic nature, bile acids are inherently toxic and cause damage to the hepatocytes if they remain in the liver for an extended period (73). Clinically DILI is characterized by an elevation in alkaline phosphatase and bilirubin levels in the blood, compared to hepatocellular injury, which typically experiences elevations in alanine aminotransferase levels (73). The detergent-like nature of bile acids produces damage to the plasma membrane, even leading to holes. In addition to perturbations to the plasma membrane, the mitochondrial membrane can also be targeted by excess bile acids, eventually inducing apoptosis and

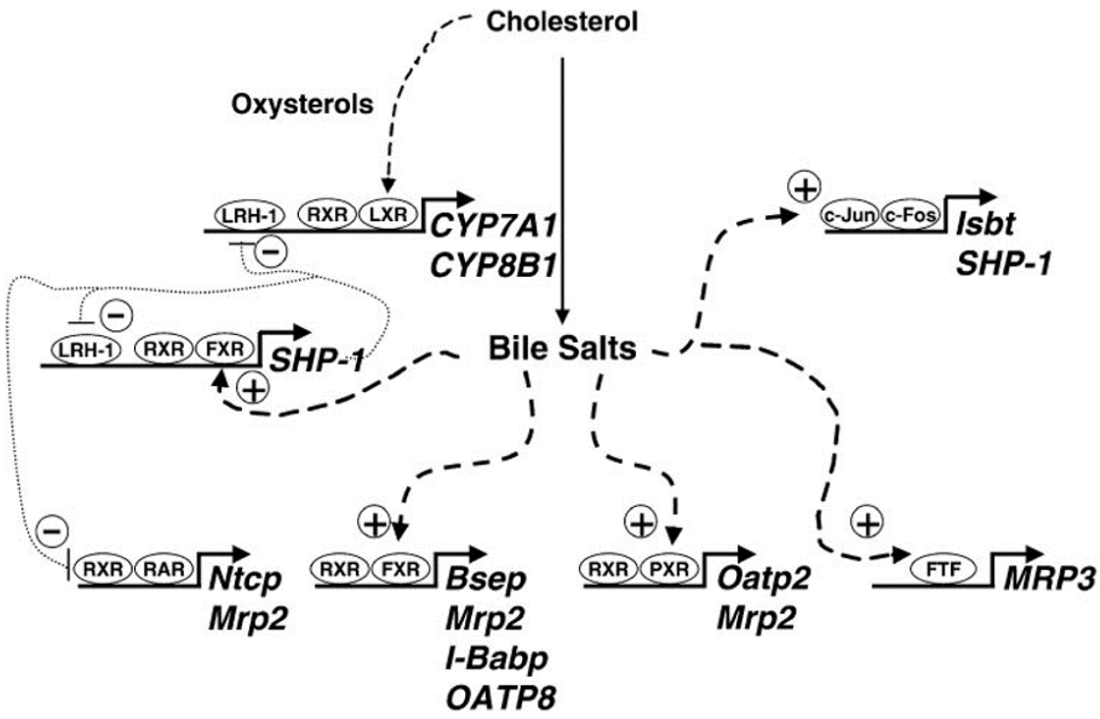


Figure 1.2: Role of ligand-activated transcription factors in the transcriptional regulation of bile salt synthesis and transport. Conversion of cholesterol to bile salts by CYP7A1 and 8B1 is stimulated by oxysterol-activated liver X receptor (LXR). Bile salts inhibit their own synthesis by farnesoid X receptor (FXR)dependent activation of SHP-1, which in turn suppresses CYP7A1 and 8B1 transcription. Bile salts also suppress retinoic acid receptor (RAR)-dependent genes such as Ntcp via the same pathway. Activation of FXR by bile salts stimulates transcription of Bsep, Mrp2, I-Babp, and OATP8. Activation of pregnane X receptor (PXR) by bile salts induces transription of Oatp2 and Mrp2. Finally, liver receptor homolog-1 (LRH-1) may mediate induction of MRP3 by bile salts in human enterocytes. RXR, retinoid X receptor; FTF, fetoprotein transcription factor. *Modified from Trauner and Boyer, 2002 (68).

cell death (43). Cholestasis can be acquired or inherited, with several subclasses of cholestasis possible.

1.5.1 Inherited forms of cholestasis

Several inherited forms of cholestasis arise from mutations in hepatobiliary genes. Generally genetic mutations in the hepatic transporters results in non-functional and severely diminished expression of these transporters, with some forms abolishing expression altogether (74). The prognosis of inherited cholestasis is quite bleak, as there is limited therapy available and typically liver surgery or transplantation becomes the only remedy (75).

The familial intrahepatic cholestasis type can present with mutations in three different hepatobiliary transporters, and ranges from benign recurrent intrahepatic cholestasis (BRIC) to progressive familial intrahepatic cholestatsis (PFIC) (75). There are three types of PFIC, annotated PFIC1/2/3, each with different causative mutations: PFIC1 derives from a mutation in ATP8B1, PFIC2 derives from an ABCB11 mutation, and PFIC 3 derives from a mutation in ABCB4. While PFIC1 and 2 tend to progress to a more severe injury, they do not typically associate with elevated gamma-glutamyl transferase (GGT) levels in serum (76).

Similar to PFIC1 and 2, BRIC 1 and 2 are also derived from mutations in the ATP81 and ABCB11 genes, however benign recurrent intrahepatic cholestasis has less toxicity overall (75). Typically, individuals with BRIC experience spontaneous, benign attacks of intrahepatic cholestasis which resolve spontaneously without lasting liver damage. The result of ATP81 deficiency is a reduction in biliary phospholipids, enabling toxic bile acids to remain free and interact with membranes. Conversely, ABCB11

deficiency would prevent bile acids from entering the bile canaliculi, however they would accumulate in hepatocytes, imposing their toxicity (77).

1.5.2 Acquired forms of cholestasis

The bile salt export pump is also implicated in acquired forms of cholestasis, specifically in intrahepatic cholestasis of pregnancy (ICP) and drug-induced cholestasis. The predominant cause of BSEP-related acquired development of ICP requires the presence of MRP2, in addition to BSEP (55). The metabolites of estrogen and progesterone produced during pregnancy cause an interaction with BSEP, resulting in the its inhibition, however the dependence on MRP2 suggests the interaction occurs from inside the bile canaliculi (55, 78).

The most commonly identified etiology for drug-induced cholestasis is deficient BSEP function, often due to direct inhibition of BSEP function (whether competitive or allosteric) (55, 77). Drug-induced cholestasis represents the most common and most severe form of drug-induced liver injury, speculated to be under-reported (79).

1.6 Bile Salt Export Pump

The bile salt export pump (ABCB11), a member of the ABC superfamily of transporters, requires ATP as an energy source for active transport. Similar to other ATP-binding cassette family members, the BSEP protein contains twelve transmembrane domains, which form two motifs referred to as the Walker A and B motifs (66, 80). Transport of bile acids (and to a small degree xenobiotics) occurs through the tunnel-like structure created from the two six-transmembrane domain motifs. Substrates preferred by

the bile salt export pump include the conjugated monovalent bile acids with high specificity. While a handful of drugs have been reported as substrates or competitive inhibitors for BSEP, it is generally very selective for bile acid transport (68).

As the primary method of biliary excretion from hepatocytes, expression of the bile salt export pump is tightly regulated. Transcription factors FXR, nuclear-factor-erythroid-2-like 2 (NRF2), and liver receptor homolog 1 (LRH1) have previously been reported to regulate BSEP; regulation through FXR has been the most characterized (81-83). Activation of FXR by endogenous bile acids creates a feed-forward mechanism of BSEP regulation, as excess levels of bile acids induce BSEP expression, which allows it to facilitate their removal (80).

1.6.1 Inhibition of BSEP in drug-induced cholestasis

Inhibition of BSEP function is the hallmark mechanism investigated in the study of drug-induced liver injury. Disruption of bile flow from the liver can result in the accumulation of bile acids, which left untreated, can result in toxicity, including cell death and liver failure. Drug inhibition of BSEP is the most straightforward approach which can be studied in drug development and toxicity assays, therefore significant efforts have been dedicated to understanding this strategy.

Insect membrane vesicles provide a simplified system to study transport; transporters of interest can be over-expressed and their function be monitored specifically, without other interfering factors (84). Although this system is selective for a transporter of interest, the physiologically relevant interactions of the transporter with its natural environment cannot be investigated. This limitation has prompted the use of human primary hepatocytes for the study of BSEP inhibition, however there are

drawbacks to this system as well. Hepatocytes are more difficult to obtain, requiring procuremet from liver resection or donations, and there exists a natural variability in the expression and function of proteins from one individual to another (85). Both systems are still widely used, however there must be careful consideration of the endpoints.

1.6.2 Modeling of BSEP in drug-induced cholestasis

Computational modeling approaches to predict BSEP inhibition in drug-development have been gaining interest in recent years, as a means to screen and eliminate potentially hazardous drugs from drug studies. Several approaches have been utilized to develop these models, including structure-activity pharmacophore generation, Bayesians modeling of physiochemical drug characteristics, and in silico mechanistic modeling of drug-induced liver injury (86-89). With increasing information regarding the class of drugs associated with DILI, patterns of injury experienced, and other cellular mechanisms which influence BSEP function, modeling has provided a strong platform to narrow-down lead compounds, which can be further characterized in vitro for potential hepatotoxicity.

1.7 Rationale and hypothesis

Drug-induced liver injury persists as the leading cause for drug attrition from the pharmaceutical market, with drug-induced cholestasis being the most severe form reported in recent years. Without sufficient methods to predict and study the cholestatic potential of drugs in the drug development process, avoidance of cholestasis in future drug candidates is unlikely. The most commonly employed strategy for studying the potential development of drug-induced cholestasis is through BSEP inhibitory studies.

The bile salt export pump (BSEP) represents the limiting step in excretion of bile acids from the liver. As such, disruption of BSEP function will likely interrupt bile flow. While inhibition assays study the direct competition or disruption of BSEP function, downregulation or loss of BSEP expression observed in hereditary forms of cholestasis suggest drug-mediated repression of BSEP could also provide an additional pathway for the development of drug-induced cholestasis.

Investigating the role of repression in conjunction with inhibition, as well as on its own, will provide valuable insight into mechanisms for the development of cholestasis, and offer a novel approach to identifying cholestatic drugs in development. To this end, our hypothesis for this work is that ***drugs determined to repress BSEP expression will significantly reduce its functional output, potentially resulting in clinical consequences upon treatment; additionally, modeling of repressors will help predict additional BSEP repressors from currently approved drugs, while providing structural insight for future drug development.*** To address this hypothesis and determine the role of BSEP repression in drug-induced cholestasis, a number of BSEP inhibitors, as well as cholestatic BSEP non-inhibitors, will be screened for their effects on BSEP expression in human primary hepatocytes (HPH). Further characterization of the effects BSEP repressors have on function will also be investigated using a biliary excretion assay, also performed in HPH.

Upon successful identification of drugs capable of repressing BSEP expression and function, computational modeling will be used to develop a prediction method for additional BSEP repressors. Lead compounds generated from such studies will be characterized in gene expression assays in HPH.

Chapter 2: The Role of Bile Salt Export Pump Gene Repression in Drug-Induced Cholestatic Liver Toxicity[1]

2.1 Introduction

The primary function of the ATP-binding cassette transporter bile salt export pump (BSEP, ABCB11) is to facilitate enterohepatic circulation by expelling bile salts from hepatocytes to the bile (67). Bile salts are synthesized in the liver via the catabolism of cholesterol; however, the majority of bile salts is recycled from the small intestine where they assist in the absorption of dietary fat (90). BSEP represents one of the rate-limiting mechanisms involved in the enterohepatic circulation (91). Disruption of BSEP function has been linked to severe forms of cholestasis, characterized by accumulation of bile salts in the liver, jaundice caused by hyperbilirubinemia, and intestinal malabsorption of dietary fat (92). Cholestasis can occur either through inherited gene mutation or acquired via environmental factor-induced impairment of bile flow (93, 94). The bile salts accumulated in the liver are polar molecules and, at high levels, can cause inflammation, apoptosis, and lead to various liver diseases (95).

Although a close correlation between hereditary defects in BSEP gene and the progressive familial intrahepatic cholestasis type 2 has been firmly established, hereditary forms of cholestasis are clinically rare. In contrast, many xenobiotics including clinically used drugs are frequently associated with acquired cholestasis, becoming an increasingly recognized cause of liver disease (96). However, the mechanism(s) underlying the

[1]Garzel B, Yang H, Zhang L, Huang SM, Polli JE, Wang H. The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity. Drug Metab Dispos. 2014;42(3):318-322.

involvement of BSEP in the development of drug-induced cholestasis remains unclear. Previous reports have focused primarily on the ability of drugs to inhibit BSEP function, without adequately considering the potential drug-induced perturbation of BSEP expression (97, 98). Endpoints for inhibition studies often measure direct efflux competition between bile salts and drugs using plasma-membrane vesicles overexpressing BSEP instead of whole viable cells (99). In some other reports that used rodent or human primary hepatocyte cultures, which provide a physiologically more relevant in vitro hepatic environment, transporter inhibition was evaluated over a short period of time (10–60 minutes) after drug exposure (97, 100). Thus, in these studies, potential contribution of BSEP expression in drug-induced cholestasis was largely unexplored.

Functioning as the major determinant of bile acid secretion and bile formation, BSEP gene expression is tightly controlled at the transcriptional level by a number of liver enriched transcription factors. Farnesoid X-receptor (FXR), a ligand-activated nuclear receptor, plays a pivotal role in the inductive expression of BSEP (81). Several bile acids, such as chenodeoxycholic acid (CDCA) and lithocholic acid, are endogenous ligands for FXR, and when accumulated in the liver, these bile acids bind to FXR and trigger the expression of the BSEP gene (101). This feedback mechanism ensures the removal of excess bile salts from the hepatocytes. Notably, BSEP expression is partially retained in the liver of FXR-/- mice, suggesting the existence of additional regulators of BSEP expression (102). Recent evidence reveals that expression of BSEP is also regulated by the nuclear factor erythroid-derived 2-like 2 (NRF2) and the liver receptor homolog-1 (LRH-1). Knockdown of NRF2 or knockout of LRH-1 was associated with

decreased expression of BSEP, whereas activation of NRF2 by oltipraz increased BSEP mRNA (82, 83). Clearly, BSEP expression can be influenced by both endogenous and exogenous chemicals through their interaction with a number of transcription factors. Therefore, it is reasonable to speculate that drug-induced perturbation of BSEP expression in human liver can contribute to acquired cholestasis.

In this study, we use sandwich-cultured human primary hepatocytes to investigate the role of BSEP repression in drug-induced cholestatic liver injury. Expression of BSEP, FXR, NRF2, and LRH-1 was determined in human hepatocytes upon treatment with an array of clinically used drugs that were reported BSEP inhibitors. Luciferase activation assay for FXR was carried out in HepG2 cells. Correlation of BSEP repression, inhibition, and clinical reported cholestatic injury for these drugs was analyzed. Collectively, our results emphasize that in addition to inhibition, drug-induced BSEP repression is another critical factor that contributes to drug-induced cholestatic liver injury.

2.2 Materials and methods

2.2.1 Chemicals

Troglitazone, erythromycin estolate, cinnarizine, 17-alpha ethynyl estradiol, simvastatin, benzbromarone, and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). Bosentan was acquired from Waterstone Technology (Carmel, IN). Glyburide and telmisartan were purchased from AK Scientific Inc. (Union City, CA). Pioglitazone, cyclosporine-A, gefitinb, lapatinib, sorafenib, and imatinib were purchased from Selleck Chemicals (Houston, TX). Glimepiride, lopinavir, paclitaxel, pazopanib,

indinavir sulfate, fusidic acid sodium salt, and ketoconazole were obtained from Axxora (Farmingdale, NY). Nefazodone, nicardipine hydrochloride, fenofibrate, nelfinavir, saquinavir, itraconazole, ritonavir, and CDCA were generously provided by Dr. James Polli. The Dual-Luciferase reagent was obtained through Promega (Madison, WI). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Matrigel was received from BD Biosciences (Bedford, MA). All other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Sigma-Aldrich.

2.2.2 Plasmids

The FXR expression plasmid and BSEP luciferase reporter vector were kindly provided by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX) and Dr. Ruitang Deng (University of Rhode Island School of Pharmacy, Princeton, RI), respectively, as previously described (2, 103). The pRL-TK Renilla luciferase plasmid used to normalize firefly luciferase activities were from Promega.

2.2.3 Human Primary Hepatocytes

Human liver tissues were obtained after surgical resection by qualified pathology staff with donor consent and prior approval from the Institutional Review Board at the University of Maryland School of Medicine. Hepatocytes were isolated from human liver specimens as described previously (104) or obtained from BioReclamationIVT reclaimation (Baltimore, MD). Hepatocytes were seeded at 7.5×10^5 cells/ well in 12-well collagen-coated plates and cultured in the sandwich format as described previously (105). Forty-eight hr after seeding, hepatocytes were treated with 0.1% DMSO or

specified compounds for 24 and 72 hr for detection of mRNA and protein expression, respectively. Culture medium was replaced on a daily basis.

2.2.4 Real-Time Polymerase Chain Reaction

Total RNA was isolated from hepatocytes using the TRIzol reagent and reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instruction. Primer sequences for real-time polymerase chain reaction (PCR) can be found in Table 2.1. Target gene mRNA expressions were normalized against that of GAPDH. PCR assays were performed in 96-well optical plates on an Applied Biosystems StepOnePlus Real-Time PCR System with SYBR Green PCR Master Mix. Fold induction values were calculated according to the equation: fold over control = $2\Delta\Delta Ct$, where ΔCt represents the differences in cycle threshold numbers between the target gene and GAPDH, and $\Delta\Delta Ct$ represents the relative change in these differences between control and treatment groups.

2.2.5 Western Blotting

Homogenate proteins (40 mg) from hepatocytes were resolved on SDS-polyacrylamide gels and transferred onto polyvinylidenefluoride membranes. Subsequently, membranes were incubated with specific antibodies against BSEP (Kamiya, Seattle, WA) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed with horseradish peroxidase-labeled IgG antibodies. Membranes were developed with West Femto chemiluminescent substrate (Thermo, Rockford, IL).

Table 2.1: Primers used for RT-PCR gene expression assays

Species	Gene	Sequence (5' to 3')
Human	GAPDH	CCCATCACCATCTTCCAGGAG GTTGTCATGGATGACCTTGGC
Human	BSEP	ACATGCTTGCGAGGACCTTTA GGAGGTTCGTGCACCAGGTA
Human	FXR	ATGGGAATGTTGGCTGAATG CCTGCATGACTTTGTTGTCG
Human	NRF2	TCCAGTCAGAAACCAGTGGAT GAATGTCTGCGCCAAAAGCTG
Human	LRH1	TGATGGGATTGAAGTTCATGGC GGCAGAAGAGCACTGATCGTA

2.2.6 Transfection in HepG2 Cells

HepG2 cells cultured in 24-well plates at 1 x 10^5 cells/well were transfected with the XtremeGene 9 DNA transfection reagent (Roche Applied Science, Indianapolis, IN) 24 hour after seeding. The transfection mixes contained the BSEP luciferase reporter plasmid, FXR expression plasmid, and the pRL-TK construct as internal control. Sixteen hr after transfection, cells were treated with vehicle control (DMSO 0.1%) or CDCA (50 mM) in the presence and absence of selected compounds for 24 hr before harvesting. Cell lysates were assayed for firefly activities and normalized against the activities of Renilla luciferase using the Dual-Luciferase Kit (Promega). Data are represented as mean ± S.D. of three individual transfections.

2.2.7 MTT Assay

Human primary hepatocytes seeded 7.5 x 10^4 cells/well in 96- well plates were cultured for 48 hr before treatment with BSEP inhibitors at previously indicated concentrations. A typical 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was carried out as described previously (106). Cell viability was expressed as percent of vehicle control (0.1% DMSO).

2.2.8 Data Analysis

Results from real-time PCR and reporter gene assays are expressed as mean ± S.D. of triplicate determinations unless otherwise indicated. Statistical analyses were made where appropriate using one-way analysis of variance and $\chi 2$ tests.

2.3 Results

2.3.1 Repression of BSEP gene expression by inhibitors

Thirty known BSEP inhibitors, with IC_{50} of 25 μM, were screened in multiple donors of human primary hepatocytes (HPH) to determine their repressive potential. Upon treatment with inhibitors, BSEP gene expression changes were established and shown in Figure 2.1 as a percentage of negative control (0.1% DMSO) treatment. These inhibitors can be further classified into three categories: potent repression, moderate repression, or no repression. Half of the drugs had no significant effect on BSEP gene expression, while ten only had moderate effects. Interestingly, five BSEP inhibitors were found to potently repress BSEP greater than 60% of DMSO treatment (Figure 2.1A). Repression was determined not to be a side effect of toxicity, by performing an MTT assay (Figure 2.1B), confirming a handful of BSEP inhibitors were also capable of repression.

2.3.2 Potent repression of BSEP is concentration dependent

Further investigation of the five inhibitors which repressed BSEP expression in Figure 2.1 revealed a concentration-dependent reduction in gene expression when human primary hepatocytes were treated with multiple concentrations (Figure 2.2). At the highest concentration for each inhibitor, which match previously used concentrations in the screening of 30 inhibitors, the same order of potency (lopinavir-greatest; glimepiride-5^{th} most potent) was observed. Additionally, when treated with the highest chosen concentration for each inhibitor, BSEP protein expression was also potently repressed.

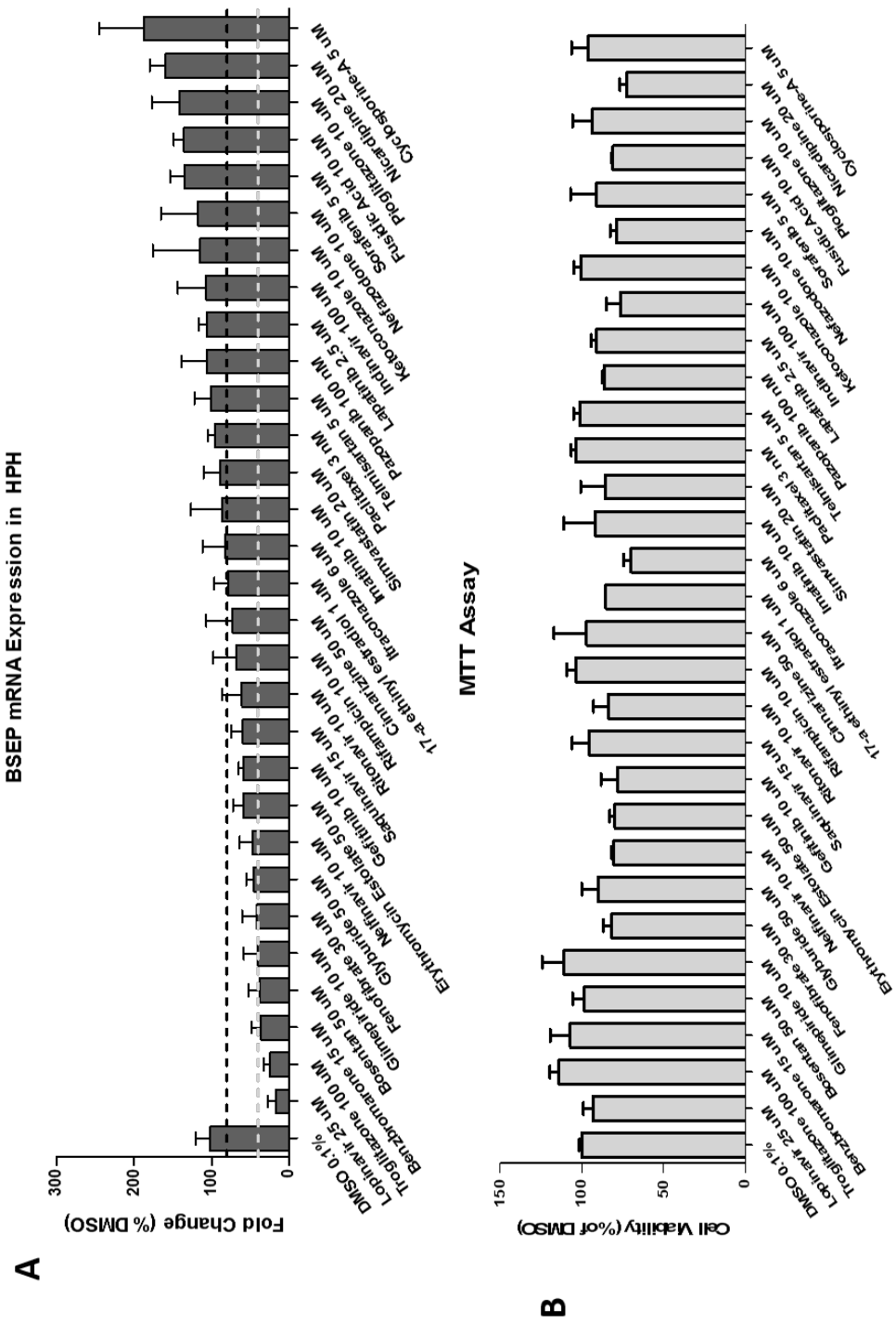


Figure 2.1 Repression of BSEP gene expression upon treatment with reported BSEP inhibitors in human primary hepatocytes. BSEP mRNA expression was measured using real-time PCR in hepatocytes from at least three donors upon treatment with BSEP inhibitors as described in Materials and Methods (A). Dotted lines indicate 20% (dark) and 60% (light) BSEP repression compared with vehicle control (0.1% DMSO). A standard MTT assay as described in Materials and Methods was performed in human primary hepatocytes to monitor cytotoxicities associated with BSEP inhibitors at selected concentrations (B). All the data are expressed as mean ± S.D. (n = 3). Modified from Garzel et al. 2014 (107).

2.3.3 FXR is partially involved in BSEP repression

A brief investigation into the mechanism of repression revealed FXR expression was not altered by BSEP inhibitors (Figure 2.3A), however two of the five potent repressors (lopinavir and troglitazone) disrupted FXR activity at their highest concentrations, as demonstrated with a luciferase reporter assay (Figure 2.3B). A known endogenous activator of FXR, chenodeoxycholic acid (CDCA), pre-induced BSEP expression, before co-treatment with repressors. After co-treatment, only lopinavir and troglitazone were able to attenuate FXR-mediated induction of BSEP through CDCA-activation (Figure 2.3B). This data provides preliminary information regarding the regulation of BSEP expression by BSEP inhibitors.

2.3.4 Expression of NRF2 and LRH1 are unchanged by BSEP repressors

In addition to studying the involvement of the prototypical BSEP regulator, FXR, in inhibitor-mediated repression of BSEP, the transcription factors NRF2 and LRH1 were also investigated. As demonstrated in Figure 2.4, most compounds had no effect on NRF2 or LRH1, even at multiple concentrations. There was a moderate decrease in NRF2 expression at the highest concentration of glimepiride (Figure 2.4A), and similarly a small decrease in LRH1 expression with treatment of the highest concentration of bosentan (Figure 2.4B). Overall transcription factor expression was not altered by BSEP inhibitors.

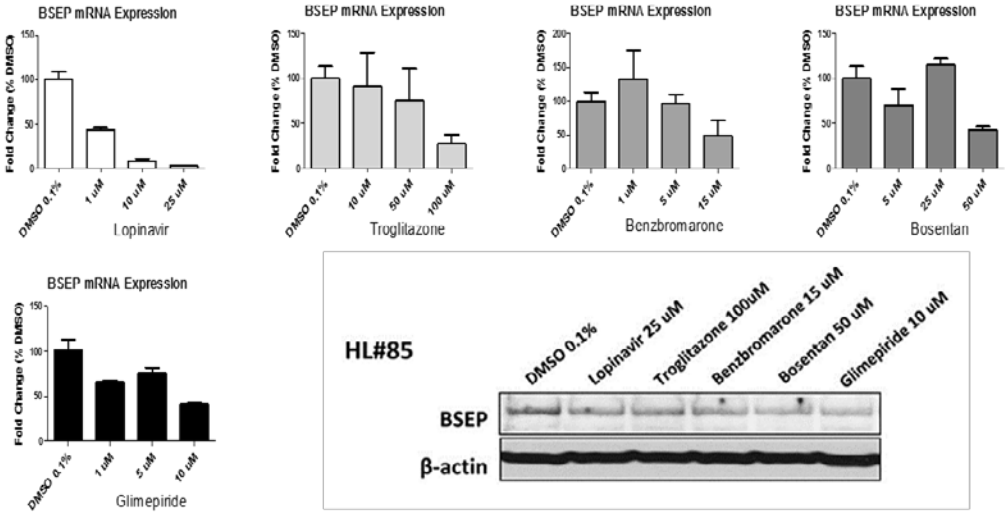


Figure 2.2 Concentration-dependent repression of BSEP. In human primary hepatocytes, five potent repressors of BSEP (≥60% BSEP repression, lopinavir, troglitazone, benzbromarone, bosentan, and glimepiride) were investigated in a concentration-dependent manner to determine their effects on BSEP mRNA expression as described in Materials and Methods. Repression of the BSEP protein was demonstrated in hepatocytes from liver donor #85 (HL#85). Modified from Garzel et al. 2014 (107).

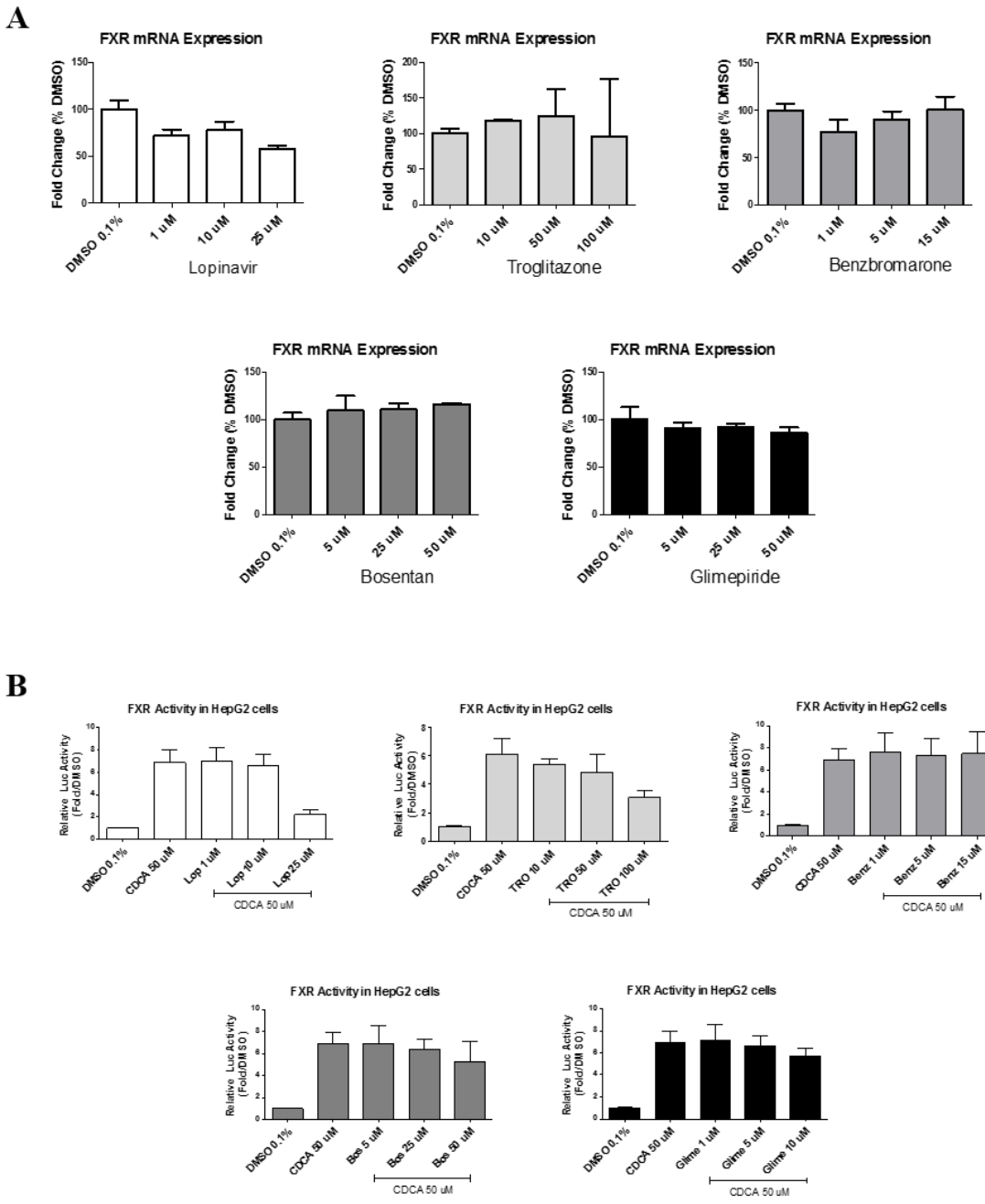


Figure 2.3 Effect of BSEP repressors on FXR expression and function. (A) In human primary hepatocytes, five potent repressors of BSEP (≥60% BSEP repression, lopinavir, troglitazone, benzbromarone, bosentan, and glimepiride) were investigated in a concentration-dependent manner to determine their effects on FXR mRNA expression as described in Materials and Methods. (B) A cell-based luciferase assay was performed in HepG2 cells by cotransfection of the FXR expression and BSEP reporter constructs as detailed in Materials and Methods. The known FXR activator CDCA (50 µM) was used as a positive control. Influence of drugs on FXR activity was determined by comparison between CDCA alone and CDCA + drugs. All the data are expressed as a mean ± S.D. (n = 3). ***P , 0.001; **P , 0.01; *P , 0.05. Modified from Garzel et al. 2014 (107).

2.3.5 Repression of BSEP by inhibitors positively correlates with DILI

BSEP inhibitors were categorized by drug-induced liver injury (DILI) potential, as described by Chen et al (108), and when compared to their repressive capabilities, there was a greater association with DILI for drugs which potently repressed BSEP (Figure 2.5A). A chi-squared test revealed a positive correlation between inhibitors which also repress and drugs with "most DILI concern (Figure 2.5B), with four out of five inhibitors having sever clinical manifestations. Similarly, average BSEP expression changes differed significantly between the three classes of repressors, compared to average IC_{50}, where there was no difference (Figure 2.5C). While all thirty drugs chosen were potent inhibitors, the additional repressive characteristic contributed to their potential to cause clinical manifestations of liver injury.

2.4 Discussion

Preclinical prediction of drug-induced liver injury (DILI), the leading cause of attrition in drug development, has proven to be challenging. Oftentimes endogenous and/or exogenous chemicals were accumulated in the hepatocytes and led to inflammation, cholestasis, apoptosis, or necrosis during DILI (84, 109). Currently, mounting evidence revealed that inhibition of BSEP function is correlated with a large number of drug-induced cholestatic liver liabilities (92). Many BSEP inhibitors have been identified. Nevertheless, whether transcriptional repression of BSEP expression could contribute to such correlation remains unclear. Utilizing membrane vesicles from transfected Sf9 insect cells, Morgan et al. (2010) evaluated more than 200 compounds including Food and Drug Administration-approved drugs and suggested a relatively strong association

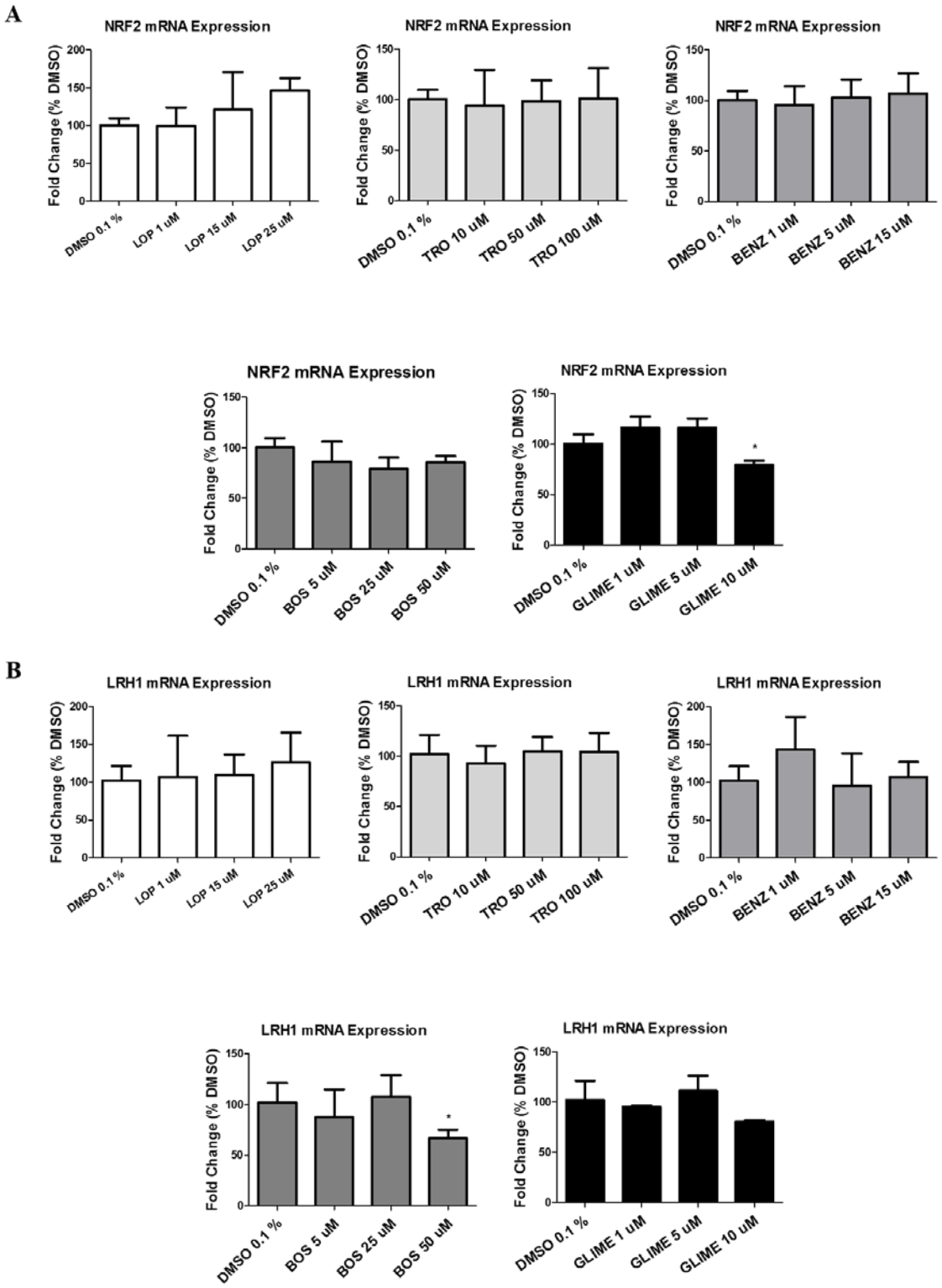
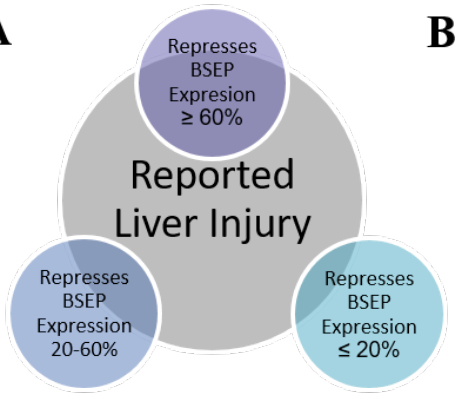


Figure 2.4 Effect of BSEP repressors on NRF2 and LRH-1 mRNA expression. Human primary hepatocytes from two donors were treated with vehicle control (0.1% DMSO) or multiple concentrations of lopinavir, troglitazone, benzbromarone, bosentan, and glimepiride as detailed in "Materials and Methods". NRF2 (A) and LRH-1 (B) mRNA expression was measured using real-time PCR.

A



B

BSEP Repression	DILI Drug (+)	DILI Drug (-)
Potent, ≥ 60%; (+)	a=4	b=1
Moderate or Non-Repressors, ≤ 60% (-)	c=8	d=17

$\chi^2 = 4$; $p = 0.045$
Consistent rate = (a+d)/(a+b+c+d)*100% = 70%

C

	Average Expression Change (% DMSO)	Average Human BSEP IC_{50} (μM)
Represses BSEP Expression ≥ 60%	30.5 ± 15.5	15.7 ± 5.9
Represses BSEP Expression 20-60%	59.3 ± 23.4	11.9 ± 6.6
Represses BSEP Expression ≤ 20%	122 ± 45.2	11.6 ± 8.1

Figure 2.5 Correlation between BSEP repression and clinically reported DILI. A, Number of DILI drugs in each group of BSEP repressors. B, The correlation between potent BSEP repression and DILI was analyzed by χ2 test. DILI was determined using criteria set forward in Chen et al., (2011). C, Average BSEP expression and reported IC_{50} for potent (5), moderate (10), and non-repressors (15). One-way analysis of variance with post-hoc test Tukey analysis was performed. ***P , 0.001. Modified from Garzel et al. 2014 (107).

between the pharmacological interference with BSEP function and human liver toxicity. In the current study, 30 drugs with a BSEP IC_{50} value of 25 µM or less from Morgan's report (98) were analyzed for the BSEP mRNA expression in HPH. At non-toxic concentrations, BSEP mRNA and protein expression was repressed by five of the thirty BSEP inhibitors, underlining an under-explored mechanism for toxicity which can work in addition to inhibition.

Unlike immediate functional inhibition, alteration of BSEP transcription is regulated by a number of transcription factors and can only be reliably measured in physiologically relevant viable cells or in vivo. Among others, FXR has been recognized as one of the key nuclear receptors that mediates drug-induced as well as basal expression of BSEP (81). Expression of FXR was largely unchanged upon treatment with potent BSEP repressors. It is noteworthy to point out that although the expression level of some nuclear receptors correlates well with their target gene expression, prototypical agonists or antagonists often do not affect the expression of their target receptors (110, 111). FXR activity was disrupted by lopinavir and troglitazone treatment, however benzbromarone, bosentan, and glimepiride had no effect. Intriguingly, a previous report revealed that in Huh7 cells, although troglitazone (10 µM) moderately induces the basal expression of BSEP, it dose-dependently represses CDCA-induced BSEP mRNA in a FXR-dependent manner (112). It appears that some of these potent repressors, however, may suppress BSEP expression via FXR-independent mechanisms. Notably, the expression of NRF2 and LRH-1, two transcription factors that have recently been linked with BSEP expression (82, 83), was not significantly affected by the same panel of potent repressors

in human hepatocytes. Further activity assays are warranted for elucidating the role of NRF2 and LRH-1 in drug-induced BSEP repression.

Correlation of the drug-induced BSEP expression profile with clinically reported DILI further reveals that among the five potent repressors, troglitazone and benzbromarone were withdrawn from the market because of severe liver toxicity, and the clinical use of bosentan, an endothelin receptor antagonist, requires monthly monitoring of liver function partly because of its well-known role in intracellular accumulation of cytotoxic bile salts (Table 2.2) (113). Although it is difficult to quantify these clinically observed DILI, our findings imply that among the 30 selected BSEP inhibitors, the five potent repressors are generally associated with more severe clinical DILI (108). Interestingly, the average BSEP IC_{50} values among the three groups of drugs classified based on BSEP repression were not significantly different, further supporting the contribution of BSEP expression in DILI. Meanwhile, in the current study, we do recognize that a number of known BSEP inhibitors exhibited moderate induction of BSEP mRNA. Although it is extremely challenging to dissect the net effects of inhibition versus induction on the same target molecule, moderate induction of BSEP is unlikely to be a risk factor that enhances BSEP inhibition-mediated liver injury.

In conclusion, the present study in human primary hepatocytes identifies that a number of known BSEP inhibitors are also potent repressors of this gene. Dual inhibitors and repressors of BSEP are often associated with severe clinically reported DILI. Although further systematic and intensive investigations with a larger number of drugs are clearly needed, our current findings indicate that in addition to the well-studied BSEP

inhibition, altered expression of this gene should be taken into consideration in predicting drug-induced liver toxicities.

Table 2.2: BSEP repressive and inhibitory values for BSEP inhibitors

	Compounds	Average BSEP Expression (% DMSO)	Human BSEP IC_{50} (µM)*	DILI Severity Score**
Represses BSEP ≥ 60%	Lopinavir	21.9 ± 9.5	17.3	8; WP, MDC
	Troglitazone	24.2 ± 8.9	5.9	-2; W, MDC
	Benzbromarone	32.4 ± 4.2	17.5	-2; W, MDC
	Bosentan	35.8 ± 5.9	22.0	7; BW, MDC
	Glimepiride	39.2 ± 9.0	15.7	7; AR, LDC
Represses BSEP 20-60%	Fenofibrate	42.0 ± 7.6	15.3	3; WP, LDC
	Glyburide	45.2 ± 3.5	6.1	--
	Nelfinavir	47.4 ± 6.5	11.8	--
	Erythromycin estolate	58.8 ± 5.6	13.0	5; WP, MDC
	Gefitinib	59.2 ± 2.2	10.9	--
	Saquinavir	59.6 ± 6.0	4.9	3; WP, LDC
	Ritonavir	60.8 ± 8.5	2.2	5; WP, MDC
	Rifampicin	68.1 ± 10.4	25.3	8; WP, MDC
	Cinnarizine	72.8 ± 12.1	15.7	--
	17-alpha ethinyl estradiol	79.1 ± 7.0	14.0	2; AR, LDC
Represses BSEP ≤ 20%	Itraconazole	81.9 ± 20.9	18.0	--
	Imatinib	86.2 ± 14.3	25.1	--
	Simvastatin	89.4 ± 8.5	24.7	3; WP, LDC
	Paclitaxel	95.9 ± 3.5	15.0	--
	Telmisartan	100.9 ± 15.1	16.2	--

*BSEP IC_{50} values reported by Morgan et al. (2010)
**DILI severity score determined by Chen et al. (2011) and the associated LTKB benchmark dataset (FDA)

Table 2.2 BSEP expression changes as a percentage of DMSO, as depicted in Figure 2.1 are summarized, along with BSEP IC50 values and reported DILI severity score. Drugs that did not meet the criteria set forth in Chen et al. (2011) and were determined not to have DILI concern are indicated by (--). Abbreviations used for DILI represent withdrawn drugs (W); DILI implicated in the box warning (BW), warnings and precautions (WP), or adverse reactions (AR) sections of label; and most DILI concern (MDC) or less DILI concern (LDC) classifications. Modified from Garzel et al. 2014 (107).

Chapter 3: Metformin-mediated AMPK activation disrupts bile acid efflux through repression of BSEP

3.1 Introduction:

Drug-induced liver injury (DILI) is one of the most common causes of drug attrition, from both drug development and post-marketing - a major concern for pharmaceutical industries, regulatory agencies, and clinical practice (114, 115). DILI can manifest in a variety of ways, mimicking a wide range of disorders associated with both acute and chronic liver diseases (116). One of the most prevalent manifestations of DILI is drug-induced cholestasis (114), characterized by interrupted bile flow from the liver to the intestines and toxic levels of bile acids accumulate in the liver, particularly within hepatocytes (117). Physiologically, bile acids play an important role in lipid and fat-soluble vitamin emulsification and absorption from the small intestine. To facilitate their function, bile acids have distinct polar and non-polar regions that allow them to form micelles (118, 119). These detergent-like micelles are necessary for lipid-trapping; however, this property also contributes to toxicity.

Typically, drug-induced cholestasis occurs soon after initiating a new drug, and is confirmed when discontinuing the drug alleviates toxicity (120, 121). While, often not life-threatening when diagnosed early, prolonged cholestasis can result in severe liver injury, liver transplantation, and death (115, 117). With an increasing number of drugs reported to cause cholestasis after FDA approval, drug-induced cholestasis has become a major focus of investigation (86, 122).

The bile salt export pump (BSEP, ABCB11) is the rate-limiting step of bile flow from the liver to the intestine (66, 123). As the key determinant of hepatic bile acid elimination, BSEP is a major focus of drug-induced cholestasis research. Most current studies focus on disruption of BSEP function through direct inhibition of bile acid transport (124, 125). Indeed, the recently issued FDA guidance for industry on transporter-based drug interactions focuses on transporter inhibition (126, 127). Nevertheless, BSEP inhibition alone cannot fully explain BSEP-associated cholestasis. Genetic mutations in *BSEP* are linked to substantial decreases and even absent BSEP expression (e.g. progressive familial intrahepatic cholestasis) (123). A group of compounds with cholestatic liability did not inhibit BSEP activity in BSEP functional assays (128). Notably, our previous study showed that several agents classified as BSEP inhibitors were also capable of repressing BSEP expression (107). Of importance, dual inhibitors and repressors of BSEP appear to be associated with severe DILI. Based on these observations, we pursued the current study wherein drug-mediated repression of BSEP expression is shown to play an important role in drug-induced cholestasis.

Metformin is one of the most commonly prescribed drugs used to treat type 2 diabetes mellitus. While generally considered a safe medication, there have been several reports of cholestasis with metformin use, and discontinuation of the drug alleviated cholestatic toxicity (121, 129, 130). Notably, metformin does not directly inhibit BSEP (128). Accordingly, we hypothesized that instead of directly inhibiting BSEP activity, metformin represses BSEP expression, thus inducing cholestatic liver injury. Given that activation of the cellular energy sensor, AMP-activated protein kinase (AMPK), is an important mechanism through which metformin exerts its pharmacological actions (131),

we also hypothesized that metformin represses expression of BSEP in HPH by activating AMPK.

In this report, we provide experimental evidence to show that metformin exhibits potent repression of BSEP expression in HPH, which is contradictory to its effect in mouse primary hepatocytes. Utilizing combined pharmacologic and genetic approaches, we showed that a number of known AMPK activators other than metformin also repress BSEP expression, and adenovirus-driven genetic activation of AMPK alone is sufficient to repress BSEP expression in HPH. Collectively, our results indicate that a class of metformin-like compounds has the potential to repress expression of BSEP, an underappreciated mechanism that may contribute to drug-induced cholestasis.

3.2 Materials and methods:

3.2.1 Reagents:

Metformin, GW-4064, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), chenodeoxycholic acid (CDCA), rapamycin, (S)-(+)-4-[1-(4-*tert*-Butylphenyl)-2-oxo-pyrrolidin-4-yl]methoxybenzoic acid (S-2E), fatostatin, and U0126 were purchased from Sigma Aldrich (St. Louis, MO). [^{3}H]-taurocholic acid was purchased from Perkin Elmer (Waltham, MA). Erlotinib, VTX-11e, and SB-203580 were purchased from Selleck Chemicals (Houston, TX). PCR Primers were synthesized by Integrated DNA Technologies (Coralville, IA). High Capacity cDNA Archive Kit was obtained from Applied Biosystems (Foster City, CA). The Dual-Luciferase assay kit was obtained through Promega (Madison, WI). Matrigel was received from BD Biosciences (Bedford,

MA). All other cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

3.2.2 Culture and treatment of HPH:

Human liver tissues were obtained after surgical resection by qualified pathology staff, with donor consent and prior approval from the Institutional Review Board at the University of Maryland School of Medicine. Hepatocytes were isolated from human liver specimens as described previously (104) or obtained from BioreclamationIVT (Baltimore, MD). The demographic information of liver donors is listed in Table 3.1. Hepatocytes (viability > 85%) were seeded at 1.5 x 10^6 cells/well and 7.5 × 10^5 cells/well in 6-well and 12-well collagen-coated plates, respectively, and cultured in the sandwich format as described previously (105). Hepatocytes were treated 48 h after seeding with 0.1% DMSO or specified compounds for 24 h for mRNA analysis or 72 h for western blotting and functional assays. Culture medium was replaced on a daily basis.

3.2.3 Isolation of mouse primary hepatocytes

Mouse primary hepatocytes were isolated from 6-8 week-old C57BL/6J male mice (Charles River Laboratories, Inc.; Germantown, MD) using a two-step collagenase perfusion method as described previously (100). Mice were kept in 12:12 h dark/light cycle with free access to water and food prior to surgery. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of Maryland Baltimore, MD). Isolated hepatocytes were seeded at 7.5 x 10^5 cells/well in 12-well collagen coated plates and cultured in the sandwich format

. After 24 hr of culture, hepatocytes were were treated with vehicle control (0.1% DMSO), CDCA (10, 50, and 100 µM), AICAR (0.1, 0.25, and 0.5 µM), or metformin (0.1, 0.5, and 1 mM) for 24 h.

3.2.4 RT-PCR:

Total RNA was isolated from HPH using the TRIzol® reagent and reverse transcribed using a High Capacity cDNA Archive Kit following the manufacturers' instructions. Primer sequences used for amplification of GAPDH, BSEP, AMPK, FAS, FXR, Nrf2, and NQO1 are shown in Table 3.2. Target gene mRNA expression was normalized against that of GAPDH. Real-time PCR assays were performed in 96-well optical plates on an StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix as described previously (132). Data are represented as mean ± SD of three individual experiments.

3.2.5 Western blotting:

Antibodies against BSEP (F-6) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphor-AMPKα (Thr172) (40H9) and AMPKα were purchased from Cell Signaling Technology (Danvers, MA). Crude protein lysates from treated HPH were harvested using RIPA buffer, and protein concentrations were determined using a bicinchoninic acid (BCA) based assay from Thermo Fisher Scientific. SDS-polyacrylamide gels (4-12% bis-tris) were loaded with 50

Table 3.1: Demographic information for human primary hepatocytes

HL Donor #	Sex	Age (years)	Race
66	Male	61	Caucasian
69	Female	78	Caucasian
70	Female	57	Black/Hispanic
75	Male	53	Caucasian
90	Male	41	Caucasian
91	Male	44	Caucasian
93	Male	76	Caucasian
106	Female	48	Caucasian
107	Male	47	Caucasian
109	Male	51	Hawaiian
111	Male	60	Caucasian
113		Data Not Available	
118	Male	45	Caucasian
119	Male	36	Caucasian
120	Male	45	Caucasian
123	Female	30	Hispanic
126	Female	47	Caucasian
127	Male	39	Caucasian

Table 3.2: Primers used for RT-PCR gene expression assays

Species	Gene	Sequence (5' to 3')
Human	GAPDH	GTGACCAGGCGCCCAATACGA TTCCTTTTGCGTCGCCAGCCGA
Human	BSEP	ACATGCTTGCGAGGACCTTTA GGAGGTTCGTGCACCAGGTA
Rat	AMPK	TAGAACACACCCCCTCGGAT ACCTGAAGCCAGAGAACGTG
Human	FAS	TGTGGACATGGTCACGGAC GGCATCAAACCTAGACAGGTC
Human	FXR	ATGGGAATGTTGGCTGAATG CCTGCATGACTTTGTTGTCG
Human	Nrf2	TCCAGTCAGAAACCAGTGGAT GAATGTCTGCGCCAAAAGCTG
Human	NQO1	TGATGGGATTGAAGTTCATGGC GGCAGAAGAGCACTGATCGTA

μg of protein lysate and used to separate proteins before transferring to polyvinylidene difluoride membranes. Primary antibodies against BSEP (1:250 dilution), phosphor-AMPK (1:1000 dilution), AMPK (1:1000 dilution), and β-actin (1:30,000 dilution) probed membranes overnight at 4ºC, followed by horseradish peroxidase-conjugated secondary antibodies. β-actin was used as loading control. Membranes were developed with chemiluminescent reagents and the relative protein expression was estimated by densitometry analysis using NIH ImageJ software (Bethesda, MD).

3.2.6 Biliary Efflux Assay:

Human primary hepatocytes were seeded on 12-well plates as described above, and cultured for 96 h after Matrigel overlay for BSEP functional assays. In a modification to the transport assay described previously (133), transport of the tritiated BSEP substrate taurocholic acid ([^{3}H]-TCA) was monitored to assess BSEP function in the presence of selected drugs. Briefly, cells were incubated with standard or calcium(Ca^{2+})-free Hank's balanced salt solution (HBSS) for 10 min, before HBSS (standard and Ca^{2+}-free) containing [^{3}H]-TCA was applied to cells for a 10-min incubation. During the inhibition assay, biliary efflux was monitored in the presence of metformin at different concentrations for 10 min. Conversely, the effect of BSEP repression on its function was monitored by pretreating HPH for 72 h with metformin and followed by monitoring [^{3}H]-TCA biliary efflux for 10 min in HBSS (standard or Ca^{2+}-free) free of drug. The assay was terminated by aspirating the [^{3}H]-TCA containing HBSS, and adding ice-cold standard HBSS. Cell lysates harvested in 0.5% Triton X-100 were analyzed by scintillation counting and normalized to protein concentration. Data are represented as a

mean ± SD of treatments in duplicate. The amount of bile acids excreted from hepatocytes, as a measurement of [^{3}H]-TCA, was calculated into a percentage and referred to as the biliary excretion index (BEI) as described previously (133).
BEI = (([^{3}H]-TCA in ST HBSS – [^{3}H]-TCA in CF HBSS) / [^{3}H]-TCA in ST HBSS) x 100%.

3.2.7 Adenoviral Overexpression of CA-AMPK in HPH:

HPH cultured on 12-well plates were infected with adenoviral CA-AMPK-GFP (generously provided by Dr. Benoit Viollet; INSERM, Paris, France) (134) in increasing volumes (2, 6, 10, and 40 µL) for 48 h. Successful infection was monitored through the GFP on a Nikon Eclipse TI fluorescence microscope (Nikon, Melville, NY). Cells were infected for 48 h or treated with select compounds for an additional 24 h before being harvested for RNA extraction. Subsequent gene expression assays were conducted as described above for CA-AMPK, BSEP, and FAS expression.

3.2.8 Luciferase Reporter Assay:

The FXR expression plasmid and BSEP luciferase reporter vector were kindly provided by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX) and Dr. Ruitang Deng (University of Rhode Island School of Pharmacy, Princeton, RI), respectively (2, 103). HepG2 cells were obtained from ATCC (Manassas, VA) and authenticated by using the short tandem repeat DNA profile every 6 months (Biopolymer/Genomics Core Facility, University of Maryland School of Medicine), and were used for no more than 35 passages. Cells were cultured in 24-well plates at 1 ×

10^5 cells/well for 24 h, and were transfected with the BSEP luciferase reporter plasmid (60 ng/well), the FXR expression plasmid (30 ng/well), and the pRL-TK construct (10 ng/well) using XtremeGene 9 DNA transfection reagent (Sigma-Aldrich). Sixteen hr after transfection, cells were treated with vehicle control (DMSO 0.1%), CDCA (50 μM), or increasing concentrations of metformin for 24 h before harvesting. Cell lysates were assayed for firefly activities and normalized against the activities of Renilla luciferase using the Dual-Luciferase Kit (Promega). Data are represented as mean ± S.D. of three individual transfections.

3.2.9 Statistical Analysis:

Results from real-time PCR, functional efflux assays, and reporter gene assays are expressed as mean ± S.D. of triplicate determinations unless otherwise indicated. Statistical analyses used one-way analysis of variance (ANOVA) and Dunnett's posttest.

3.3 Results:

3.3.1 Metformin represses BSEP expression and increases AMPK phosphorylation in HPH.

The effects of metformin on BSEP expression were evaluated in sandwich-cultured HPH prepared from multiple liver donors. As demonstrated in Figure 3.1A and 3.1B, expression of *BSEP* mRNA was markedly repressed by metformin treatment in a concentration-dependent manner. Maximal repression (approximately 75%) of *BSEP* mRNA expression was achieved with 1 mM metformin, a concentration that is not cytotoxic in HPH (135, 136). As expected, the positive control, GW-4064, robustly

induced *BSEP* expression. Western blotting analysis confirmed metformin repressed BSEP protein expression in a pattern consistent with the changes in *BSEP* mRNA (Figure 3.1C and 3.1D). Metformin is a well-known activator of AMPK (131, 137); as expected, metformin markedly increased AMPK phosphorylation without altering expression of total AMPK (Figure 3.1E and 3.1F). These results reveal metformin as a potent repressor of *BSEP* expression in HPH at concentrations that activate AMPK.

3.3.2 Metformin alters BSEP function by repressing its expression.

To investigate whether metformin alters BSEP function, two transport assays were performed in HPH. The effects of metformin on hepatic bile acid efflux were tested in a prototypical inhibition assay with 10 min drug exposure (Figure 3.2A) or in a modified inhibition assay condition where a 72-h drug pre-treatment precedes the bile acid efflux measurement (Figure 3.2B). Our results demonstrated that under typical inhibitory conditions, metformin did not alter bile acid efflux compared to the vehicle control (0.1% DMSO) (Figure 3.2C and 3.2E). As a positive control, a known cholestatic BSEP-inhibitor, troglitazone, potently inhibited bile acid transport through BSEP. Interestingly, when HPH were pre-treated for 72 h with metformin (0.1, 0.5, and 1 mM), [^{3}H]-TCA efflux was significantly decreased (Figure 3.2D and 3.2F). Metformin (1 mM) did not alter clearance of [^{3}H]-TCA (82.45% for metformin vs. 81.72% for vehicle control) under typical

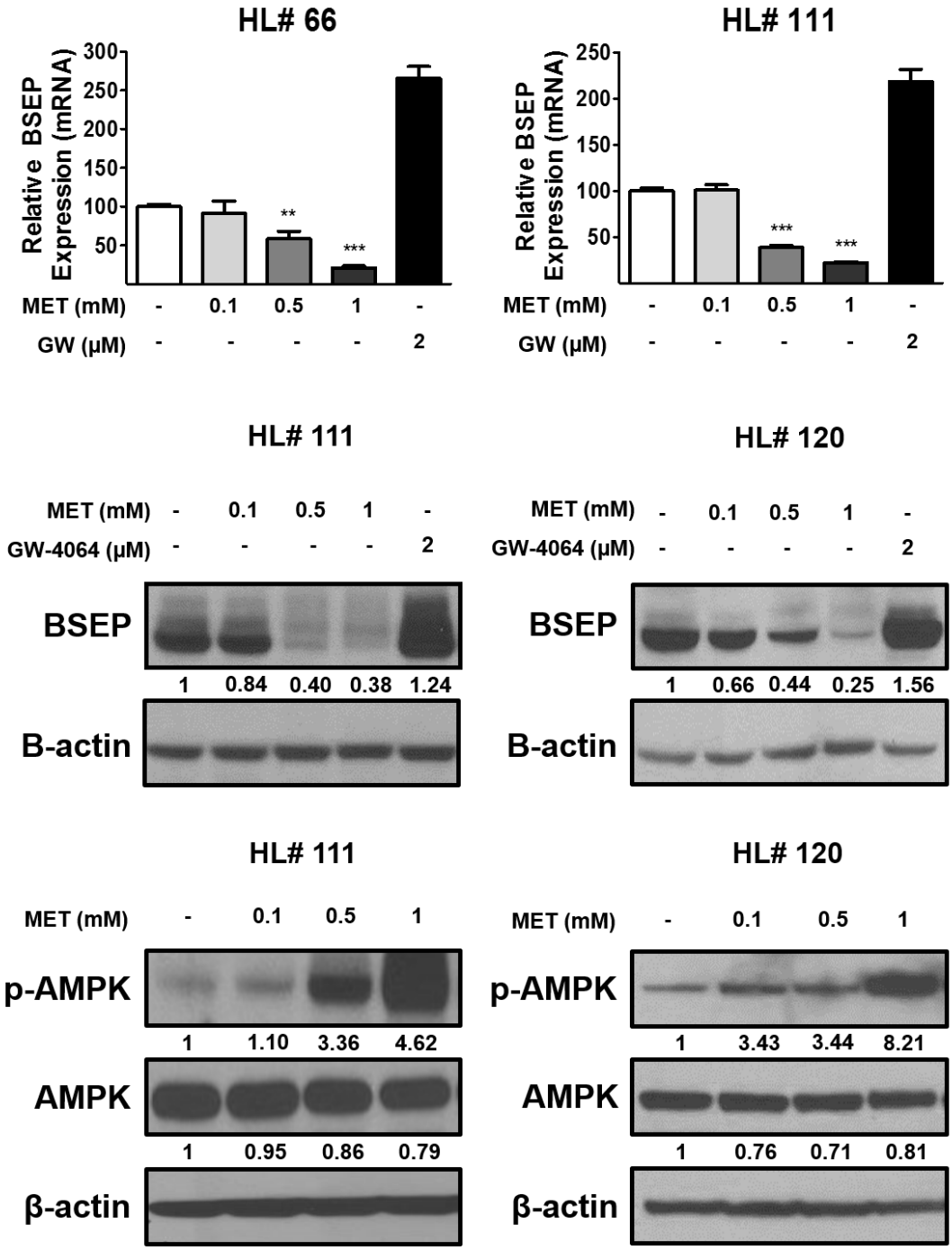
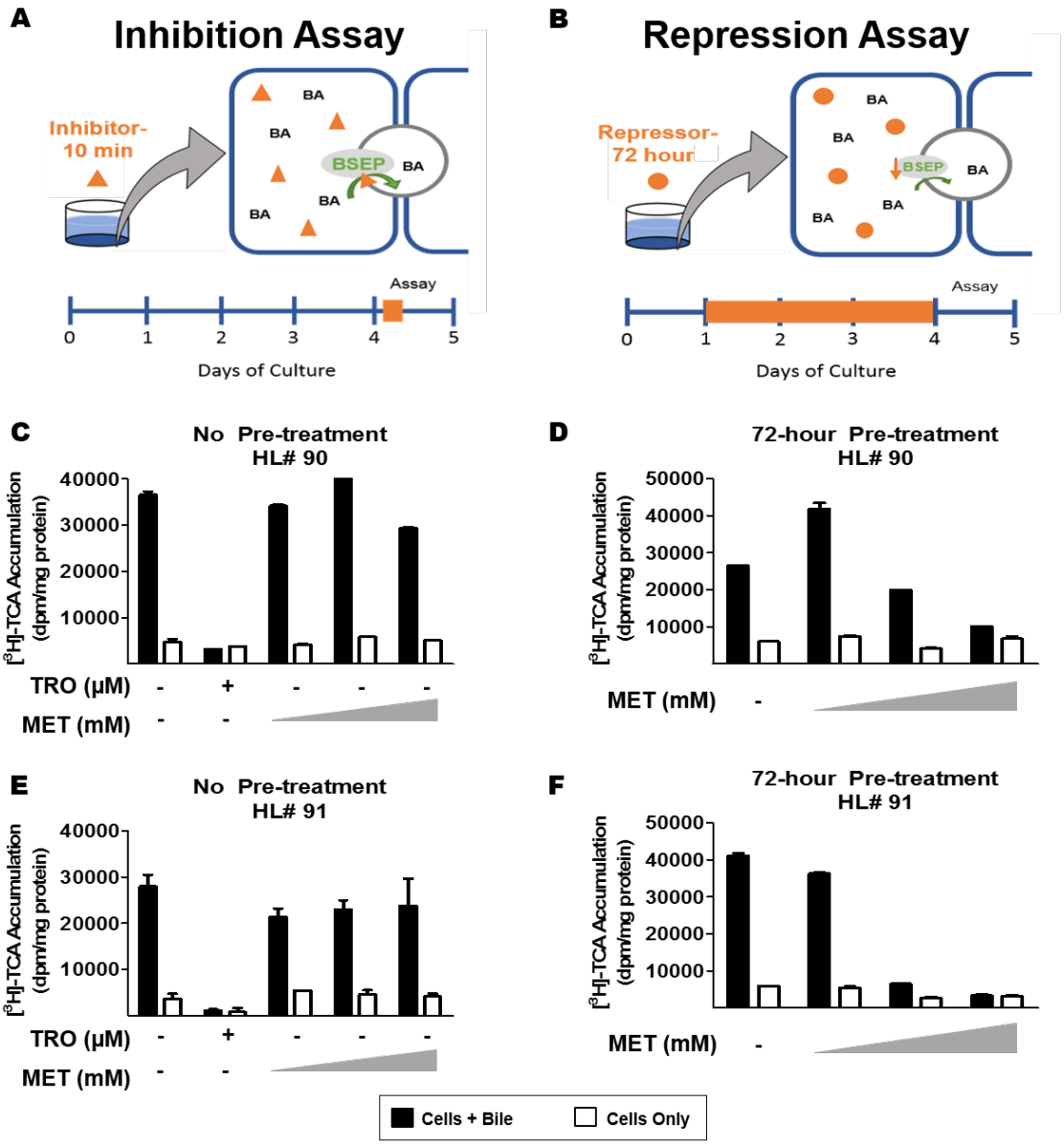


Figure 3.1 Metformin represses BSEP expression in human primary hepatocytes. (A & B) mRNA expression of *BSEP* was measured using RT-PCR in two human primary hepatocyte donors, HL #66 (A) & #111 (B), treated with vehicle control (0.1% DMSO), metformin (0.1, 0.5, and 1 mM), or positive control *BSEP* inducer, GW-4064 (2 μM). Data are expressed as mean ± S.D. (n=3). BSEP (C & D), AMPK phosphorylated at Thr 172 (p-AMPK) (E & F), total AMPK (AMPK) (E & F), β-actin (C-F) protein expression was measured using Western blotting in two donors, HL #111 (C & E) & #120 (D & F). Human primary hepatocytes were treated for 72 h with vehicle control (0.1% DMSO), metformin (0.1, 0.5, and 1 mM), or GW-4064 (2 μM).



G

	No Pre-treatment (Inhibition)	72-hour Pre-treatment (Repression)
DMSO 0.1%	81.72 ± 6.75	81.52 ± 4.83
MET 0.1 mM	82.71 ± 8.53	83.50 ± 1.75
MET 0.5 mM	84.18 ± 2.14	68.95 ± 11.77
MET 1 mM	82.45 ± 1.08	19.22 ± 16.22 ***

Figure 3.2 Metformin represses BSEP function in human primary hepatocytes. (A) Schematic of inhibition assay – 10-min incubation with metformin. (B) Schematic of repression assay – 72-h incubation with metformin. (C & E) BSEP inhibition assay measured via bile flow of [^{3}H]-TCA in two human primary hepatocytes donors, HL #90 (C) & #91 (E), upon 10-min exposure to vehicle control (0.1% DMSO), positive control BSEP inhibitor troglitazone 100 µM, or metformin (0.1, 0.5, and 1 mM). Solid bars represent dpm/mg protein of [^{3}H]-TCA that accumulated in the cells + bile canaliculi (standard buffer used), while open bars represent dpm/mg protein [^{3}H]-TCA in the cells only (calcium-free buffer used). Data are expressed as mean ± S.D. (n =2). (D & F) BSEP repression assay measured via bile flow of [^{3}H]-TCA in two human primary hepatocytes donors, HL #90 (D) & 91(F), upon 72-h pre-treatment with vehicle control (0.1% DMSO) or metformin (0.1, 0.5, and 1 mM). Solid bars represent dpm/mg protein of [^{3}H]-TCA that accumulated in the cells + bile canaliculi (standard buffer used), while open bars represent dpm/mg protein [^{3}H]-TCA in the cells only (calcium-free buffer used). Data are expressed as mean ± S.D. (n =2). (G) Tabular representation of biliary excretion index (BEI; equation in methods) of DMSO or metformin treatments during inhibition and repression assays. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

inhibition assay conditions (10-min incubation), whereas bile acid clearance in the presence of metformin was reduced to 19.22% compared to the control value of 81.52% in the 72-h repression assay (Figure 3.2G). These results indicate that metformin does not inhibit BSEP through direct substrate competition; instead, longer exposure to metformin repressed BSEP expression, thereby decreasing bile acid clearance capacity at 72 h.

3.3.3 Both chemical and genetic activation of AMPK repress BSEP expression in HPH.

AMPK activation is a key mediator of metformin actions. The role of AMPK activation in *BSEP* repression was investigated using a known AMPK activator, AICAR. Treatment of HPH (HL #69, #70, #75) with 0.1, 0.25, and 0.5 mM AICAR significantly repressed *BSEP* mRNA levels (Figure 3.3A). Similar repressive effects on the expression of BSEP protein by AICAR were also observed in HPH prepared from HL#119 and HL#126 (Figure 3.3B and 3.3C). In addition to small molecular modulation, the role of AMPK in BSEP expression was investigated further using the constitutively activated (CA)-AMPK (134). Infection of HPH with increasing amounts of the CA-AMPK-GFP-expressing virus was monitored using fluorescence microscopy (Figure 3.3D) and AMPK over-expression confirmed by qPCR (Figure 3.3E). As shown in Figure 3.3F, overexpression of CA-AMPK reduced *BSEP* mRNA expression. As a positive control, we examined the action of CA-AMPK on expression of fatty acid synthase (FAS), which is known to be repressed by AMPK activation; as expected, increasing amounts of CA-AMPK

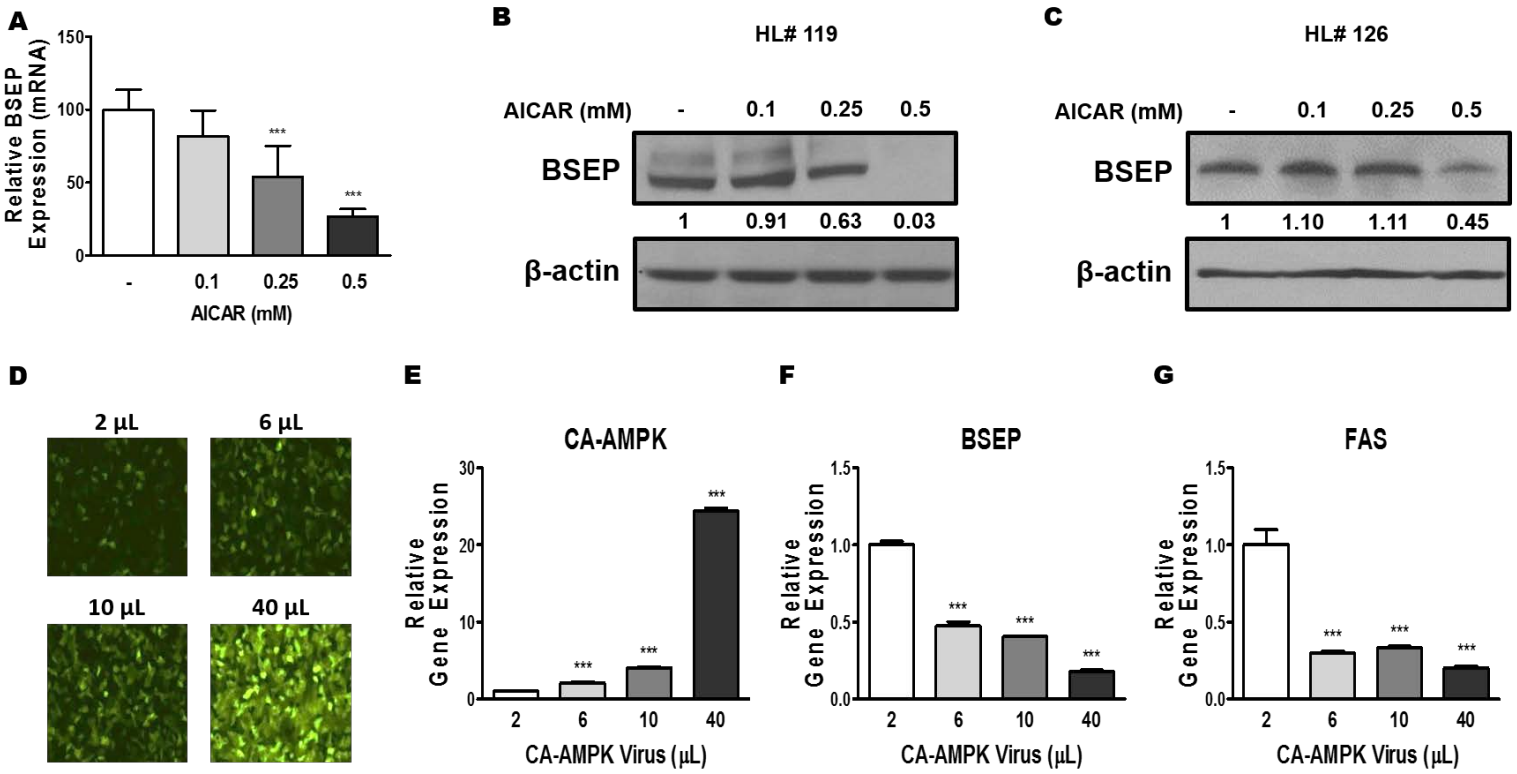

A
Relative BSEP Expression (mRNA)
150
100
50
0
***
***
-
0.1
0.25
0.5
AICAR (mM)
B
HL# 119
AICAR (mM) - 0.1 0.25 0.5
BSEP
1 0.91 0.63 0.03
β-actin
C
HL# 126
AICAR (mM) - 0.1 0.25 0.5
BSEP
1 1.10 1.11 0.45
β-actin
D
2 μL
6 μL
10 μL
40 μL
E
CA-AMPK
Relative Gene Expression
30
20
10
0
***
***
***
2
6
10
40
CA-AMPK Virus (μL)
F
BSEP
Relative Gene Expression
1.5
1.0
0.5
0.0
***
***
***
2
6
10
40
CA-AMPK Virus (μL)
G
FAS
Relative Gene Expression
1.5
1.0
0.5
0.0
***
***
***
2
6
10
40
CA-AMPK Virus (μL)

Figure 3.3 AMPK activation represses BSEP expression. (A) mRNA expression of *BSEP* was measured using RT-PCR in three human primary hepatocyte donors (HL #69, #70, and #75) treated with vehicle control (0.1% DMSO) or AICAR (0.1, 0.25, and 0.5 mM). Data are expressed as mean ± S.D. (n=3). (B & C) BSEP and β-actin protein expression in two donors, HL #118 (B) & #126(C), was measured using Western blotting. Human primary hepatocytes were treated for 72 h with vehicle control (0.1% DMSO) or AICAR (0.1, 0.25, and 0.5 mM). (D) Fluorescence microscopy images of GFP in human primary hepatocyte donor HL #105 upon infection with 2, 6, 10, and 40 µL GFP-tagged CA-AMPK containing adenovirus; 48 h post-infection. mRNA expression of *CA-AMPK* (E), *BSEP* (F), and *fatty acid synthase* (*FAS*) (G) was measured using RT-PCR in one representative human primary hepatocyte donor, HL #105, infected with CA-AMPK (2, 6, 10, and 40 µL). Data are expressed as mean ± S.D. (n=3). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

progressively reduced FAS expression (Figure 3.3G). Together, these results support the hypothesis that metformin-induced AMPK activation in represses BSEP expression.

3.3.4 Downstream targets of AMPK activation, Hmg-CoA reductase and ACC, potently repress BSEP expression

To determine their effect on BSEP expression, down-regulated targets of AMPK activation—mechanistic target of rapamycin (mTOR), 3-hydroxy-3-methyl-glutaryl-coenzyme A (Hmg-CoA) reductase, acetyl coenzyme A carboxylase (ACC), and sterol regulatory element binding protein 1 (SREBP1)—were investigated using chemical inhibitors. The dual inhibitor S-2E (Hmg-CoA reductase and ACC) repressed BSEP expression in HPH potently, while the selective inhibitors rapamycin (mTOR) and fatostatin (SREBP1) had no effect (Figure 2.4A). When exploring the inhibitor S-2E in three consecutive concentrations, it was found to repress BSEP in a concentration-dependent manner (Figure 2.4B). These results establish an additional link between AMPK activation and BSEP repression.

In addition to exploring targets of the AMPK pathway, downstream targets of the mitogen-activated protein kinase (MAPK) pathway were investigated to explore the role of other kinases in the repression of BSEP. The extracellular signal-regulated kinases 1/2 (ERK 1/2), p38 MAP kinases, MAPK and ERK kinases 1/2 (MEK 1/2), and epidermal growth factor receptor (EGFR) were targeted with selective inhibitors in HPH (VX-11e, SB-203580, U0126, and erlotinib respectively), and BSEP expression was assessed. Interestingly, none of the MAPK inhibitors chosen for this study repressed BSEP expression (Figure 2.4A).

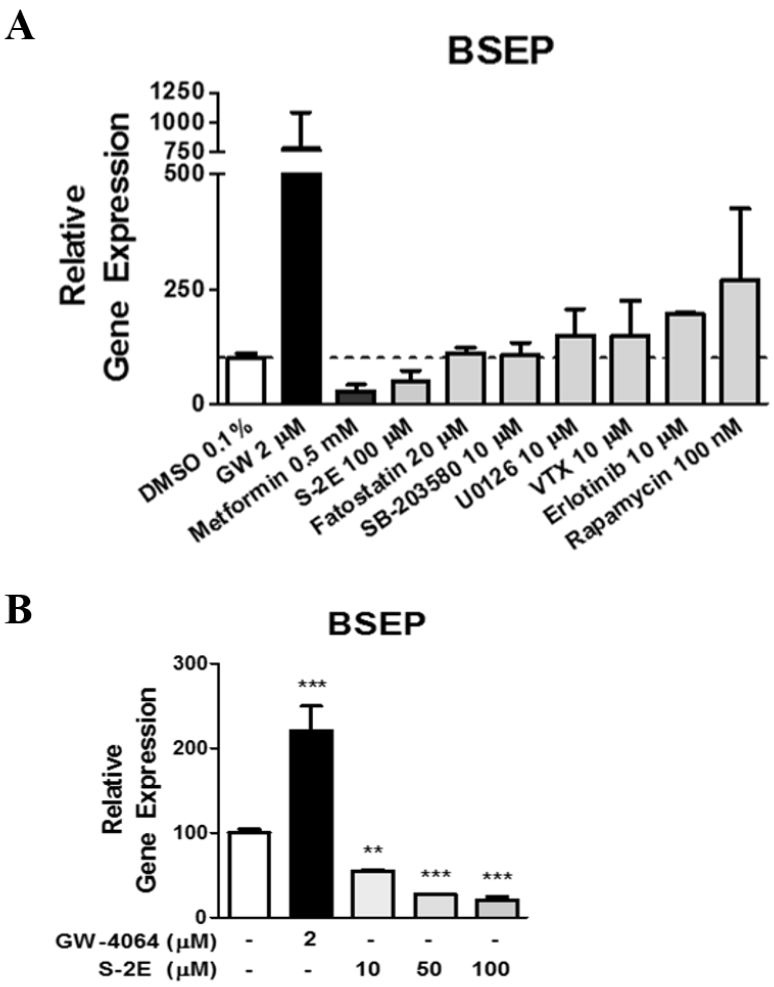


Figure 3.4 Downstream targets of AMPK, Hmg-CoA reductase and ACC, potently repress BSEP expression. mRNA expression of BSEP was measured using RT-PCR in two human primary hepatocyte donors (HL #113 and #114) treated with (A) vehicle control (0.1% DMSO), GW-4064 (2 µM), metformin (0.5 mM), S-2E (100 µM), fatostatin (20 µM), SB-203580 (10 µM), U0126 (10 µM), VX-11e (10µM), erlotinib (10µM), or rapamycin (100 nM). (B) BSEP mRNA expression was measured using RT-PCR in one representative donor (HL #114) treated with vehicle control (0.1% DMSO), GW-4064 (2µM), or S-2E (10, 50, and 100 µM). Data are expressed as mean ± S.D. (n=3). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

3.3.5 AMPK activation induces BSEP expression in mouse primary hepatocytes

To explore the effects of AMPK activation in another species, mouse human primary hepatocytes were treated with metformin and AICAR before changes in BSEP expression were assessed. All three concentrations of AICAR induced mBSEP expression (~ 5 fold induction at 250 μM), to levels ≥ a positive control, CDCA (~3 fold induction at 50 μM) (Figure 3.5). Conversely, up to 1 mM metformin had no effect on mBSEP expression.

3.3.6 FXR and Nrf2 do not contribute to metformin-mediated repression of BSEP in HPH.

The transcription factors FXR and Nrf2 play key roles in regulating BSEP expression in hepatocytes (83, 138). Both xenobiotic and endobiotic chemicals, including bile acids such as CDCA, can induce BSEP expression by activating FXR (81, 139). We explored whether FXR and Nrf2 were involved in metformin-induced repression of BSEP expression. In HPH, CDCA, a potent activator of FXR, induced BSEP expression, an action that was efficiently repressed by co-treatment with metformin (Figure 3.4A) or over-expression of CA-AMPK (Figure 3.4B). In contrast, metformin neither altered expression of *FXR* in HPH (Figure 3.4C), nor affected FXR activity in luciferase reporter assays (Figure 3.4D). Additionally, metformin did not alter *Nrf2* expression and only exhibited moderate induction of *NQO1*, a Nrf2 target gene (Figure 3.4E). Collectively, these results confirm metformin attenuates FXR activator-mediated BSEP expression, while neither FXR nor Nrf2 contribute to metformin-mediated repression of basal BSEP expression.

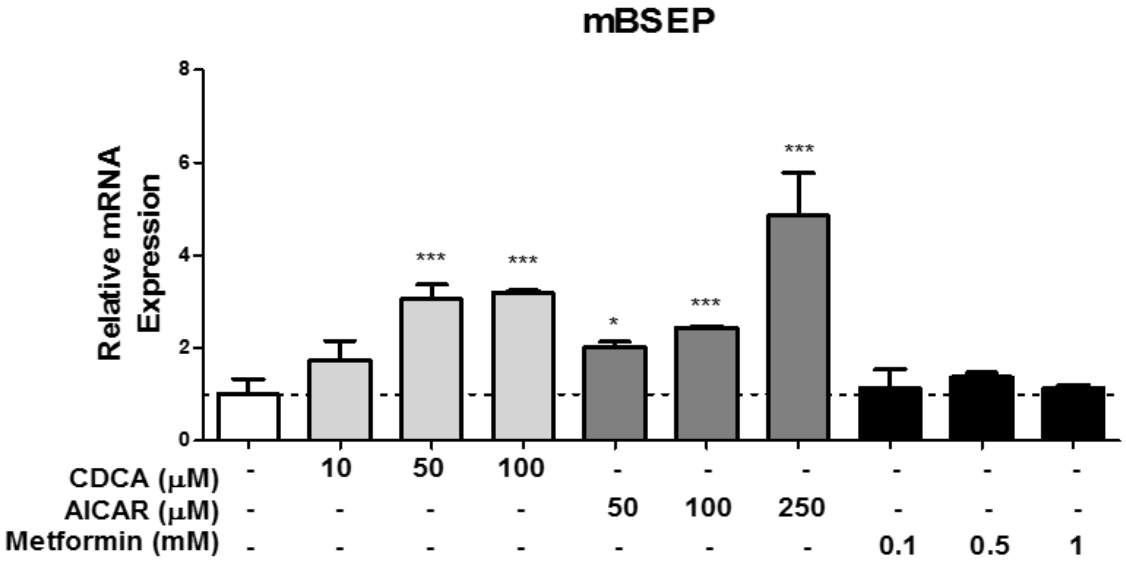
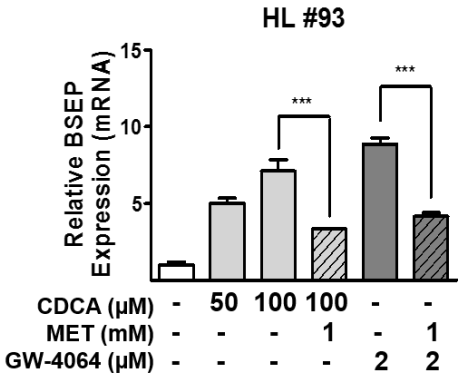
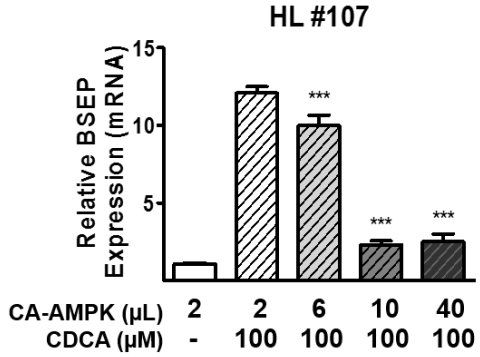
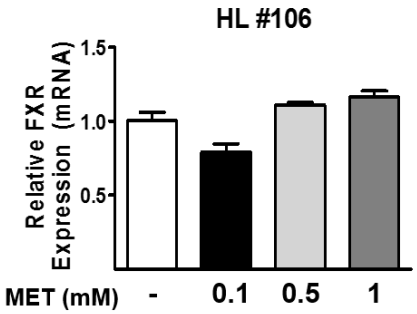
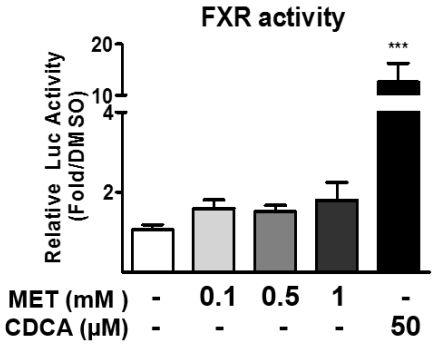


Figure 3.5 AICAR-mediated activation of AMPK induces BSEP expression in mouse primary hepatocytes. BSEP mRNA expression was measured using RT-PCR in three mouse primary hepatocyte donors treated with vehicle control (0.1% DMSO), CDCA (10, 50, and 100 μM), AICAR (0.05, 0.1, and 0.25 mM), and metformin (0.1, 0.5, and 1 mM). Data are expressed as mean ± S.D. (n=3) in three separate donors. ****P* < 0.001; ***P* < 0.01; **P* < 0.05.


HL #93
Relative BSEP Expression (mRNA)
15
10
5
***
***
CDCA (μM) - 50 100 100 - -
MET (mM) - - - 1 - 1
GW-4064 (μM) - - - - 2 2


HL #107
Relative BSEP Expression (mRNA)
15
10
5
***
***
***
CA-AMPK (μL) 2 2 6 10 40
CDCA (μM) - 100 100 100 100


HL #106
Relative FXR Expression (mRNA)
1.5
1.0
0.5
MET (mM) - 0.1 0.5 1


FXR activity
Relative Luc Activity (Fold/DMSO)
20
10
4
2
***
MET (mM) - 0.1 0.5 1 -
CDCA (μM) - - - - 50

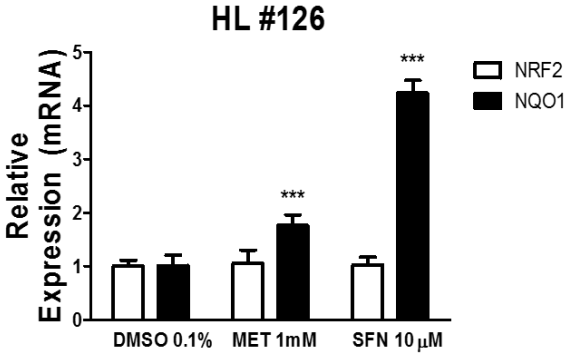

HL #126
Relative Expression (mRNA)
5
4
3
2
1
0
***
***
NRF2
NQO1
DMSO 0.1%
MET 1mM
SFN 10 μM

Figure 3.6 Transcriptional regulators FXR and Nrf2 are not involved in basal BSEP gene repression. (A) mRNA expression of *BSEP* was measured using RT-PCR in one representative human primary hepatocyte donor (HL #93) treated with vehicle control DMSO (0.1%), CDCA (50 and 100 µM), GW-4064 (2 µM), or in combination with metformin (1 mM). Data are expressed as mean ± S.D. (n=3). (B) mRNA expression of *BSEP* was measured using RT-PCR in one representative human primary hepatocyte donor (HL #107) infected with CA-AMPK (2, 6, 10, and 40 µL) containing adenovirus, in the presence of CDCA 100 µM, or with 2 µL CA-AMPK alone containing adenovirus. Data are expressed as mean ± S.D. (n=3). (C) mRNA expression of FXR was measured using RT-PCR in one representative human primary hepatocyte donor (HL #106) treated with vehicle control DMSO (0.1%) or metformin (0.1, 0.5 and 1 mM). Data are expressed as mean ± S.D. (n=3). (D) A dual-luciferase reporter assay was performed in HepG2 cells by co-transfection with FXR expression, BSEP reporter, and pRL-TK background reporter plasmids. Transfected cells were treated with vehicle control (0.1% DMSO), metformin (0.1, 0.5, and 1 mM), or BSEP inducer positive control CDCA (50 µM). Data are expressed as a mean ± S.D. (n = 3). (E) mRNA expression of *NQO1* and *Nrf2* were measured using RT-PCR in one representative human primary hepatocyte donor (HL #126) treated with vehicle control DMSO (0.1%), metformin (1 mM), or NQO1 inducer, SFN (10 µM). Data are expressed as mean ± S.D. (n=3) ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

3.4 Discussion:

Drug-induced cholestasis is a leading cause of drug attrition, prompting an expanding research effort to understand the underlying mechanisms (87, 140). Until recent years, the primary focus has centered on investigating the ability of drugs to inhibit bile acid efflux from the liver by interaction with the bile salt export pump BSEP (124, 125). Although this strategy has elucidated the mechanism of action for many cholestatic drugs, some chemicals with cholestatic liability do not directly inhibit BSEP activity (128), suggesting alternate mechanisms may contribute to drug-induced cholestasis. Our previous screen of 30 known BSEP inhibitors identified five potent (> 60% repression) and 10 moderate (20-60% repression) repressors of BSEP expression (107). Importantly, drugs exhibiting both potent repression of BSEP expression and inhibition of its activity were associated with the greatest DILI concern, i.e. black box warnings and withdrawal from the market (108). Using metformin as a model compound, the present study unequivocally shows that drug-induced BSEP repression alone is sufficient to affect bile acid clearance from HPH and, thus, may represent an overlooked mechanism leading to drug-induced cholestatic liver injury.

As the first-line medication for the treatment of type 2 diabetes, metformin exhibits several metabolic benefits and is generally well-tolerated with few side effects. Nonetheless, several case reports revealed the association of metformin with intrahepatic cholestasis and liver injury in type 2 diabetic patients (121, 129, 130). In mice, metformin treatment increased bile acid accumulation in both the liver and the blood (141). The underlying mechanisms of these observations however remained elusive. Using membrane vesicles from Sf9 cells overexpressing human BSEP, Pedersen et al., screened

250 compounds for BSEP inhibition and identified metformin as a non-inhibitor of BSEP (142). In the current study, we found that metformin markedly repressed BSEP expression in sandwich-cultured HPH at pharmacologically-relevant concentrations. These results suggest metformin may represent a pure repressor of human BSEP that could influence bile acid clearance without direct substrate competition.

Transport activity of membrane proteins has often been evaluated using insect membrane vesicles, immortalized cell lines ectopically expressing particular transporters, or sandwich-cultured hepatocytes that maintain functional endogenous transporters (125, 133, 143, 144). Typical inhibition assays involve a short-term (10- to 30-min) incubation of test compounds with substrate probes in membrane vesicles or cultured cells (125, 145). Utilizing this traditional approach, we showed that metformin does not directly inhibit BSEP-mediated efflux of taurocholic acid in HPH, which is consistent with earlier observations in inverted insect membrane vesicles (142). Nevertheless, this methodology fails to evaluate a drug's effects on BSEP after an extended period of exposure. In our modified experiments depicted in Figure 3.2B, the 72-h pre-treatment prior to bile acid efflux measurements in HPH allowed a transport assay to coordinate effects of gene expression on transporter function, and confirmed that repression of BSEP expression alone can have a significant effect on BSEP-mediated bile acid transport.

It is well established that activation of the cellular energy regulator, AMPK, is a key mechanism by which metformin exerts its pharmacological effects (131, 137). AICAR, an analog of adenosine monophosphate, has often been cited as another AMPK activator (146). We found that similar to metformin, AICAR robustly repressed BSEP

expression, and the role of AMPK was further confirmed when a constitutively active AMPK adenoviral construct was overexpressed in HPH.

In contrast, previous studies in mice demonstrated that activation of AMPK by metformin and/or AICAR, in addition to genetic alterations with adenoviral-mediated overexpression, enhanced BSEP expression (134, 141). While results varied depending on the use of primary hepatocytes or *in vivo* treatment with AMPK activators, the overall trend was induction in BSEP expression, or at the very least no change. Interestingly under these conditions, both serum and liver bile acid levels observed an increase, suggesting a cholestatic phenotype (141). These results seem paradoxical, as the increased expression of BSEP should alleviate liver accumulation of bile acids, however the order of these events (bile acid accumulation and overexpression of BSEP) may be indicative of a compensatory mechanism, rather than direct effects of metformin treatment. Our own experiments in mice mirrored these mRNA changes in mouse primary hepatocytes, with AMPK activation either inducing BSEP expression or having no effect. Although mechanisms for these contradictory observations are largely unknown, as increasingly recognized, species differences between mice and humans contribute to the relatively low predictability of preclinical models for toxicological evaluation during drug development (97, 147, 148).

Exploring the downstream mechanism(s) following AMPK activation involved in BSEP repression revealed two potential targets, Hmg-CoA reductase and ACC. The drug S-2E is a dual-inhibitor of both enzymes, therefore it is not clear which, if not both, are involved in BSEP repression. Notably, a recent study showed that atorvastatin, a selective Hmg-CoA reductase inhibitor, repressed BSEP expression in mice (149). While no

current reports link ACC inhibition to BSEP, the role of Hmg-CoA reductase as a downstream participant in AMPK-mediated repression BSEP is likely. Further studies to investigate the role of these enzymes alone or in combination in human primary hepatocytes is necessary to clarify this pathway.

The most highly studied transcription factors associated with BSEP expression are FXR and Nrf2 (81, 83, 138, 139, 148). In accordance with a previous report, we found that CDCA-mediated induction of BSEP through FXR activation can be disrupted in HPH by both metformin treatment and genetic activation of AMPK (150). However, metformin did not alter the expression and the basal transcriptional activity of FXR. We also found that metformin does not repress the expression or activity of Nrf2 in HPH. Together these findings suggest metformin-mediated repression of basal BSEP expression is independent of either FXR or Nrf2.

In conclusion, BSEP inhibition cannot fully explain drug-induced cholestasis; hence, we have directed our work at identifying alternate mechanisms. In the current study, we found that metformin alters BSEP function through repression of *BSEP* expression without directly inhibiting its activity. AMPK activation was found, at least in part, to mediate metformin-induced BSEP repression. Although AMPK activation also disrupts FXR activation by CDCA, which interferes with BSEP induction by FXR, neither FXR nor Nrf2 is associated with metformin-induced repression of basal BSEP expression. Regardless of the exact mechanisms, repression of BSEP decreases bile acid efflux from the liver. Our results suggest transcriptional BSEP repression represents an overlooked mechanism underlying drug-induced cholestasis.

Chapter 4: Predicting Cholestatic BSEP Repressors

4.1 Introduction

Drug-induced cholestasis is most often associated with direct inhibition of the bile salt export pump (BSEP; ABCB11). While the prevailing view is that the primary mechanism of BSEP-related cholestasis studied is direct inhibition, emerging evidence suggests alternatives that are both dependent and independent of BSEP function. After BSEP inhibition is excluded, secondary bile acid transporters are often evaluated for their potential contribution to drug-induced cholestasis. Compensatory biliary efflux transporters, multi-drug resistance-associated protein 3 and 4 (MRP3; ABCC3, MRP4; ABCC4), as well as bile transporters, multi-drug resistance protein 3 (MDR3; ABCB4) and multi-drug resistance-associated protein 2 (MRP2; ABCC2) are most commonly studied alternatives, as they play important roles in enterohepatic circulation, especially when BSEP function has been disrupted. In addition to the above bile transporters, breast cancer resistance protein (BCRP; ABCG2) and multi-drug resistance protein (MDR1; ABCB1) may also be considered, as they regulate the ion concentration of bile. Generally, transport inhibition remains the primary mechanism considered regarding these transporters, similar to BSEP studies.

As a consequence of BSEP inhibition or dysfunction, bile acid transporters MRP3 and MRP4 are upregulated and responsible for excreting excess bile acids from the liver, into the circulation. Recent studies by the Swann and Brouwer research groups have investigated the role of these transporters, both when BSEP is inhibited but also as primary mechanisms. In their 2015 publication, Welch et al. reported inhibition of MRP4 often correlated with BSEP inhibition, and this dual inhibition shut down two of the three

hepatic elimination routes for bile acids. Drugs associated with this additional inhibition of MRP4, were more likely to cause cholestasis, as the compensatory pathway for efflux was disrupted. A conclusion drawn from that work suggests MRP3 would be the sole efflux mechanism available for bile acid elimination, and cannot accommodate the need. Interestingly, a more recent study by the same group uncovered that MRP3 can also be subject to drug inhibition, underlining the importance of biliary transporter inhibition studies. With all routes of elimination potential targets of drug inhibition, biliary transport can be significantly disrupted, inevitably leading to drug-induced cholestasis.

The aforementioned studies represent one example of current research regarding MRP3 and MRP4 involvement in drug-induced cholestasis; specifically, using computational modeling as a tool to predict and characterize transporter inhibition. Modeling has become a valuable tool in drug discovery, allowing studies to eliminate poor performers or toxic compounds from *in vitro* testing. Providing a more successful pool of compounds in efficacy studies, while limiting potential toxicants, saves time, money, and disappointment. In the following studies, a combination of Bayesian and pharamacophore modeling, as well as literature evidence, will be employed to better predict BSEP repressors, with or without inhibitory capabilities.

As described in the previous chapters, our studies have provided solid evidence to suggest repression of the BSEP gene, and thus protein, can result in deficient bile salt excretion. If allowed to persist, this deficiency could lead to cholestasis, and eventually liver failure. Using the information obtained from the previous repression studies, in addition to literature reports of cholestasis, a model for BSEP repression will be determined. Our strategy will generate a Bayesian model from drugs associated with

cholestasis but not with BSEP inhibition—referred to as cholestatic BSEP non-inhibitors. A pharmacophore model of the BSEP repressor identified previously by our group, metformin, will be coupled with the Bayesian model, to predict additional metformin-like repressors. Lead compounds will be screened in human primary hepatocytes (HPH) for their effect on BSEP gene expression, with any successful hits being characterized further.

Establishing a model for BSEP repression can advance the development of safer drugs, especially when combined with the current BSEP inhibition screens. As BSEP is the primary mechanism for removing bile acids from the liver, it is important that disruption of BSEP function, whether by inhibition or repression, be easily determined, preferably early in the drug development process.

4.2 Materials and Methods

4.2.1 Materials

Carbamazepine and cimetidine were purchased from Perkin Elmer (Waltham, MA); GW-4064, d-penicillamine, nitrofurantoin, and nortriptyline were purchased from Sigma Aldrich (St. Louis, MO). Ibuprofen, tamoxifen, trimethoprim, and verapamil were purchased from AK Scientific (Union City, CA). PCR Primers were synthesized by Integrated DNA Technologies (Coralville, IA). High Capacity cDNA Archive Kit was obtained from Applied Biosystems (Foster City, CA). Matrigel was received from BD Biosciences (Bedford, MA). All other cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

4.2.2 Culture and treatment of HPH:

Human liver tissues were obtained after surgical resection by qualified pathology staff, with donor consent and prior approval from the Institutional Review Board at the University of Maryland School of Medicine. Hepatocytes were isolated from human liver specimens as described previously (104) or obtained from BioreclamationIVT (Baltimore, MD). The demographic information of liver donors is listed in Table 4.1. Hepatocytes (viability > 85%) were seeded at 1.5 x 10^6 cells/well and 7.5 × 10^5 cells/well in 6-well and 12-well collagen-coated plates, respectively, and cultured in the sandwich format as described previously (105). Hepatocytes were treated 48 h after seeding with 0.1% DMSO or specified compounds for 24 h for mRNA analysis or 72 h for western blotting. Culture medium was replaced daily.

4.2.3 Computational model to determine BSEP repressors

A combination of computational approaches was used to identify a preliminary screen of potential BSEP repressors. Previous data from Chapter 2, which identified BSEP inhibitors with a range of repressive capabilities, were used to create a pharmacophore model of BSEP repressors. Additionally, a Bayesian model was created from the BSEP inhibitors which potently repressed BSEP; this model uses even more descriptors than structure to predict BSEP repressors (as compared to pharmacophore modeling). Finally, a pharmacophore was developed from the structure of metformin, as it was found in Chapter 3 to potently repress BSEP without a potentially confounding inhibitory signature. These approaches combined with literature reports of cholestasis, identified the 10 compounds examined in this study.

4.2.3.1 Generation of a BSEP repressor pharmacophore

Chemical structures for BSEP inhibitors which potently repressed BSEP—lopinavir, troglitazone, benzbromarone, bosentan, and glimepiride—were retrieved from PubChem and imported into the LigandScout software with default settings (version 3.12, build 20130912; Inte:Ligand, Vienna, Austria) for pharmacophore generation. Common structural features were determined among the five drugs and a shared-feature pharmacophore was generated. For the five drugs modeled, 10 pharmacophores were generated and the pharmacophore with the highest score, and aligned best with the five drugs was selected for further testing. The pharmacophore model was then used to screen 814 commonly prescribed drugs, derived from the SCUT database.

4.2.3.2 Bayesian model of BSEP repressors

Of the 30 BSEP inhibitors screened in Chapter 2, 28 were used to create a Bayesian model of BSEP repressors (cyclosporin A and paclitaxel were excluded due to structural complexity that could not be modeled). Specifically, the dataset was split in half, with drugs which repressed BSEP $\geq$ 20% of vehicle control deemed repressors, and the remaining 14 drugs labeled non-repressors. Discovery Studio 4.0 (Accelrys, Inc., San Diego, CA) was used to generate the model, applying the "Create Bayesian Model" protocol, with the number of bins set to 10. Sixteen molecular descriptors (Table 4.1), as well as functional-class fingerprints maximum diameter 6 (FCFP_6) (151), were calculated for all compounds**.** The models were validated with a leave-one-out cross-validation statistics receiver operating characteristic area under the curver (LOO ROC

Table 4.1: Molecular descriptors used in Bayesian model

ALogP
Formal charge
LogD
Molecular weight
Number of aromatic rings
Number of hydrogen-bond acceptors
Number of hydrogen-bond donors
Number of rings
Number of rotatable bonds
Molecular fractional polar solvent accessible surface area
Molecular fractional surface area
Molecular polar solvent accessible surface area
Molecular polar surface area
Molecular solvent accessible surface area
Molecular solvent accessible volume
Molecular surface area

AUC) method. A dataset of 1510 U.S. Food and Drug Administration-approved drugs, retrieved from DrugBank (http://www.drugbank.ca) (152), excluding ionic salts and large polymeric drugs and proteins (leaving ~1488 drugs), was screened using the Bayesian model.

4.2.3.3 Metformin pharmacophore

Using the PubChem structure for metformin, all pharmacophore features of metformin were assigned using LigandScout software with default settings (version 3.12, build 20130912). The metformin pharmacophore was then compared to 25 drugs (structures also retrieved from PubChem) which have been reported as "cholestatic BSEP non-inhibitors" (128). Each drug sampled up to 255 conformations, and those which shared at least four of the pharmacophore's eight features were ranked according to the best fit with the model.

3.2.4 RT-PCR:

Total RNA was isolated from HPH using the TRIzol® reagent and reverse transcribed using a High Capacity cDNA Archive Kit following the manufacturers' instructions. Primer sequences used for amplification of GAPDH and BSEP are as follows: GAPDH 5'-GTGACCAGGCGCCCAATACGA-3' (forward), 5'-TTCCTTTTGCGTCGCCAGCCGA-3' (reverse); BSEP 5'-ACATGCTTGCGAGGACCTTTA-3' (forward), 5'-GGAGGTTCGTGCACCAGGTA-3' (reverse). Target gene mRNA expression was normalized against that of GAPDH. Real-time PCR assays were performed in 96-well optical plates on an StepOnePlus Real-

Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix as described previously (132). Data are represented as mean ± SD of three individual experiments.

3.2.5 Western blotting:

Antibodies against BSEP (F-6) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Crude protein lysates from treated HPH were harvested using RIPA buffer, and protein concentrations were determined using a bicinchoninic acid (BCA)-based assay from Thermo Fisher Scientific. SDS-polyacrylamide gels (4-12% bis-tris) were loaded with 50 µg of protein lysate and used to separate proteins before transferring to polyvinylidene difluoride membranes. Primary antibodies against BSEP (1:250 dilution) and β-actin (1:30,000 dilution) probed membranes overnight at 4ºC, followed by horseradish peroxidase-conjugated secondary antibodies. β-actin was used as loading control. Membranes were developed with chemiluminescent reagents and the relative protein expression was estimated by densitometry analysis using NIH ImageJ (Bethesda, MD).

4.3 Results

4.3.1 Pharmacophore model of BSEP repressors identifies 21 lead drugs

In order to predict additional BSEP repressors, a number of computational models were developed. The first, a shared-feature pharmacophore model, was created using the five potent repressors from Chapter 2. Ten pharmacophores were generated and the BS1-1 model with the highest-fit score was chosen for screening of 814 drugs from the SCUT

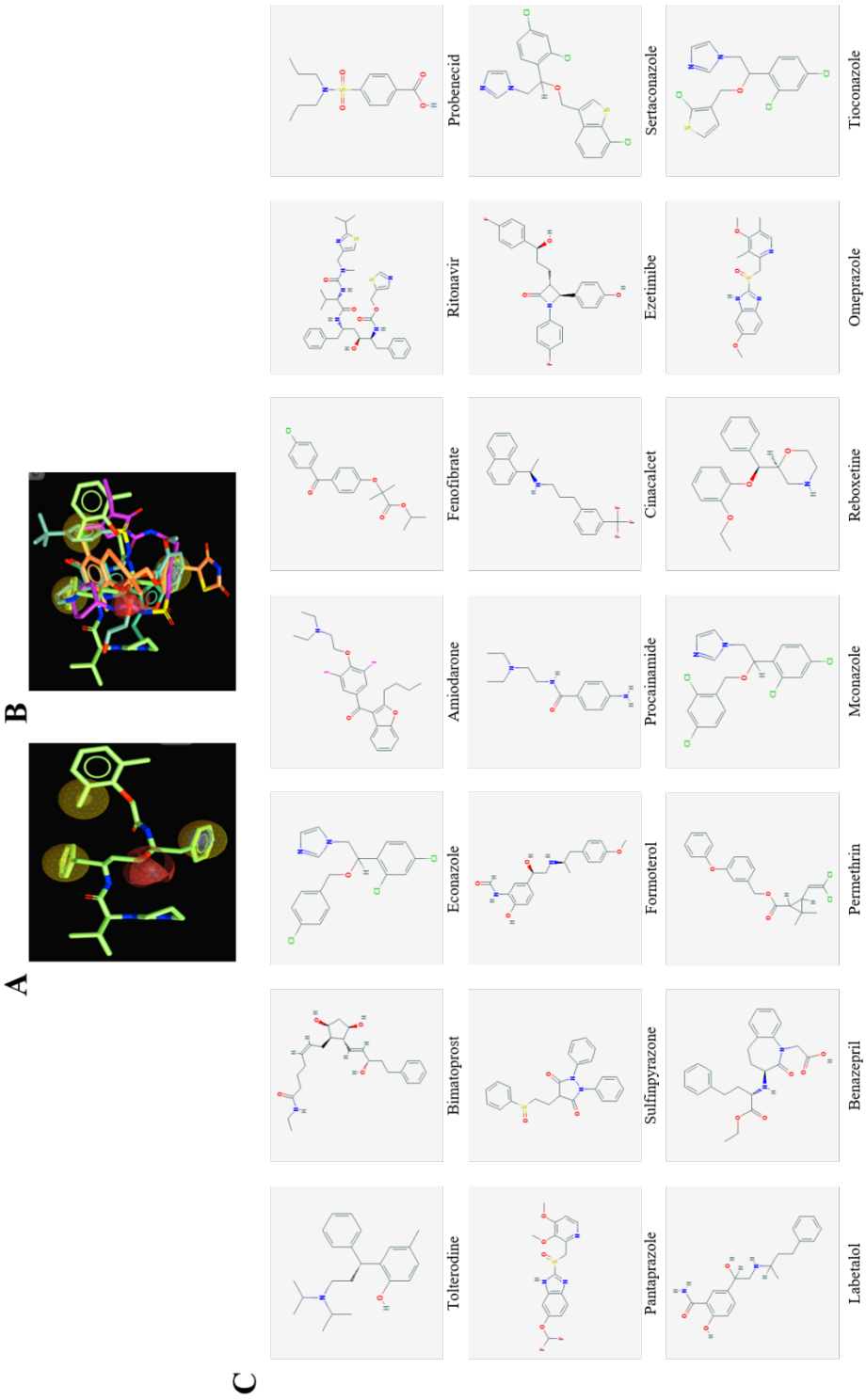


Figure 4.1 Pharmacophore model of BSEP repressors generates 21 lead drugs when screened against the SCUT database. BS1-1 pharmacophore model generated from LigandScout software, with lopinavir (A) or all five repressors (lopinavir, troglitazone, benzbromarone, bosentan, and glimepiride) (B) overlaid. Yellow spheres = hydrophobic groups (3), red sphere = hydrogen-bond acceptor (1), and purple (within bottom yellow sphere) = aromatic group (1). Chemical structures of 21 drugs (C) which shared all five features of the pharmacophore, listed in order of highest-fit score.

database (Figure 4.1A and B). Figure 4.1 A shows the pharmacophore with lopinavir overlaid, while Figure 4.1B includes all five potent repressors overlaid. The pharmacophore is color-coordinated, with yellow = hydrophobic group, red = hydrogen-bond acceptor, and purple = aromatic ring. Twenty-one drugs were identified that shared all features with the pharmacophore and their structures are provided in Figure 4.1C.

4.3.2 Bayesian modeling of BSEP repressors

A Bayesian model was also created for the BSEP repressors in Chapter 2, however drugs were split into two groups: those with ≥ 20% BSEP repression and those that did not repress. This equal division of drugs (14 in each group) allowed a stronger model to be developed, compared to only five repressors (data not shown). The leave-one-out cross validation receiver operating characteristic area under the curve for the BSEP repressor Bayesian model was calculated to be 0.832, with a confusion matrix (true positive/false negative/false positive/true negative) of 8/6/0/14; correctly identifying all but six drugs (which were false negatives). Applying the Bayesian model to the DrugBank set of 1510 compounds generated 119 predicted BSEP repressors (Table 4.2).

4.3.3. Cholestatic BSEP non-inhibitors screened against metformin pharmacphore

The key feature of metformin discovered from Chapter 3 was the ability to repress BSEP expression and function, without directly inhibiting BSEP. These characteristics are promising features for a cholestatic BSEP repressor, therefore additional drugs which have been linked to reports of cholestasis, however do not inhibit BSEP, were identified and utilized in the third modeling strategy (128). Pharmacophore features of metformin

Table 4.2: Positive hits generated from Bayesian model of BSEP repressors

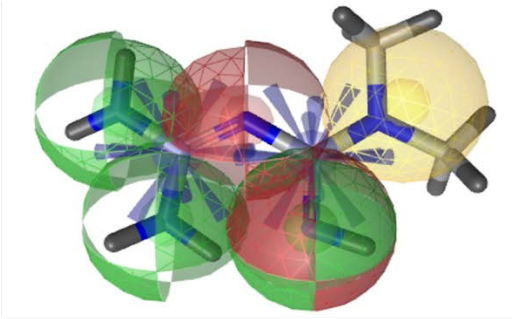
Rifampicin	Estrone	Amprenavir	Labetalol	Atovaquone	Chlorpropamide
Rifapentine	Terlipressin	Oxytetracycline	Gonadorelin	Flavoxate	Venlafaxine
Saquinavir	Gefitinib	Diethylstilbestrol	Amiodarone	Molindone	Norethindrone
Ritonavir	Butorphanol	Lisuride	Tetracycline	Ergonovine	Phenylephrine
Nelfinavir	Desmopressin	Doxycycline	Epirubicin	Lymecycline	Vincristine
Lopinavir	Trabectedin	Vindesine	Doxorubicin	Dezocine	Hesperetin
Rifaximin	Gliquidone	Estramustine	Nabilone	Dopamine	Pergolide
Glyburide	Pentagastrin	Glisoxepide	Vinblastine	Pasireotide	Phentolamine
Glimepiride	Fenofibrate	Arbutamine	Guaifenesin	Metaraminol	Fenproporex
Rifabutin	Fulvestrant	Methacycline	Phenelzine	Azlocillin	Methylphenobarbital
Ethinyl Estradiol	Octreotide	Carbetocin	Bevantolol	Lisdexamfetamine	Salicylamide
Bosentan	Caspofungin	Carfilzomib	Zolmitriptan	Melatonin	Idarubicin
Glipizide	Novobiocin	Valrubicin	Amoxicillin	Cefadroxil	Tolterodine
Mestranol	Vitamin E	Levorphanol	Agomelatine	Butenafine	Colistin
Quinestrol	Felypressin	Propafenone	Equilin	Betaxolol	Tofisopam
Estradiol	Conjugated Estrogens	Ceruletide	Cabergoline	Methylergometrine	Primidone
Vapreotide	Plicamycin	Levallorphan	Nalbuphine	Sulpiride	Penbutolol
Estriol	Fosamprenavir	Desvenlafaxine	Dobutamine	Torasemide	Oxybenzone
Cinnarizine	Polymyxin B Sulfate	Dienestrol	Fidaxomicin	Ecabet	
Estradiol valerate/Dienogest	Dronabinol	Cinchocaine	Tolbutamide	Pentazocine	

were first assigned, before a group of 25 cholestatic BSEP non-inhibitors were compared to the model. The metformin pharmacophore is shown in Figure 4.2A, with hydrogen bond donors indicated with green spheres and hydrogen bond acceptors indicated with red spheres; yellow spheres indicate hydrophobic groups and blue starbursts indicate positively ionizable groups. Figure 4.2B lists the drugs which shared at least four of the eight pharmacophore features with metformin, along with the features that matched, and their pharmacophore-fit scores. The 10 with pharmacophore-fit scores ≥ 50 were considered the most promising. All 25 drugs screened are listed in Table 4.3.

4.3.4. Drug-induced repression of BSEP expression is not unique to metformin.

In addition to metformin, a prototype drug used to study BSEP repression in Chapter 3, other drugs are reported to cause cholestasis without inhibiting BSEP (128). After three computational models were developed, 10 drugs which were reported as cholestatic, and had successful prediction in one or more BSEP repressor models were evaluated for their effects on BSEP expression. As shown in Figure 4.3A, most of these compounds caused modest repression of BSEP expression. However, tamoxifen, an estrogen antagonist widely used for breast cancer treatment, potently repressed BSEP expression, confirmed in hepatocytes from additional liver donors at both the mRNA (Figure 4.3B and 4.3C) and protein (Figure 4.3D and 4.3E) levels. While only tamoxifen showed potent repression, it is notable to mention some of level of repression measured for each of the drugs screened may indicate successful prediction power of the models generated.

A



B

Drug	Matching features	Pharmacophore-fit score
Metformin	H|HBA|HBA|HBD|HBD|HBD|PI|PI	88.47
Famotidine	H|HBA|HBA|HBD|HBD|HBD|PI|	76.01
Cimetidine	H|HBA|HBA|HBD|HBD|HBD|PI|PI	74.87
D-penicillamine	H|HBA|HBA|HBD|HBD|HBD|PI|PI	66.53
Tolbutamide	H|HBA|HBA|HBD|HBD|HBD|PI|PI|	66.53
Chloramphenicol	H|HBA|HBA|HBD|HBD|HBD|PI|	65.41
Fluoxetine	H|HBA|HBA|HBD|HBD|HBD|PI|	56.68
Chlorpropamide	H|HBA|HBA|HBD|HBD|HBD|PI|	56.42
Ranitidine	H|HBA|HBA|HBD|HBD|HBD|PI|	55.25
Quinine	H|HBA|HBA|HBD|HBD|HBD|PI|	55.13
Trimethoprim	H|HBA|HBA|HBD|HBD|HBD|PI|	55.05
Furosemide	H|HBA|HBA|HBD|HBD|HBD|PI|	47.68
Haloperidol	H|HBA|HBA|HBD|HBD|HBD|PI|	47.3
Sulfasalazine	H|HBA|HBA|HBD|HBD|HBD|PI|	46.41

Figure 4.2 Pharmacophore model of metformin predicts ten compounds to repress BSEP. Pharmacophore features were assigned to metformin, with hydrophobic groups shown in yellow, hydrogen-bond acceptors in green, hydrogen-bond donors in red, and positively ionizable points in blue (A). Fourteen cholestatic BSEP non-inhibitors shared at least four pharmacophore features with metformin and are listed in order of pharmacophore-fit score (B). The features which were shared with metformin are also indicated, and color-coordinate with the metformin pharmacophore.

Table 4.3: Cholestatic BSEP non-inhibitors

Bezafibrate
Carbamazepine*
Chloramphenicol
Chlorpromazine
Chlorpropamide
Cimetidine*
Desipramine
D-Penicillamine*
Famotidine
Fluoxetine
Furosemide
Haloperidol
Ibuprofen*
Maprotiline
Metformin
Nitrofurantoin*
Nortriptyline*
Promethazine
Quinine
Ranitidine
Sulfasalazine
Sulindac*
Tamoxifen*
Tolbutamide
Trimethoprim*
Verapamil*

* Drugs selected for use in *in vitro* screening

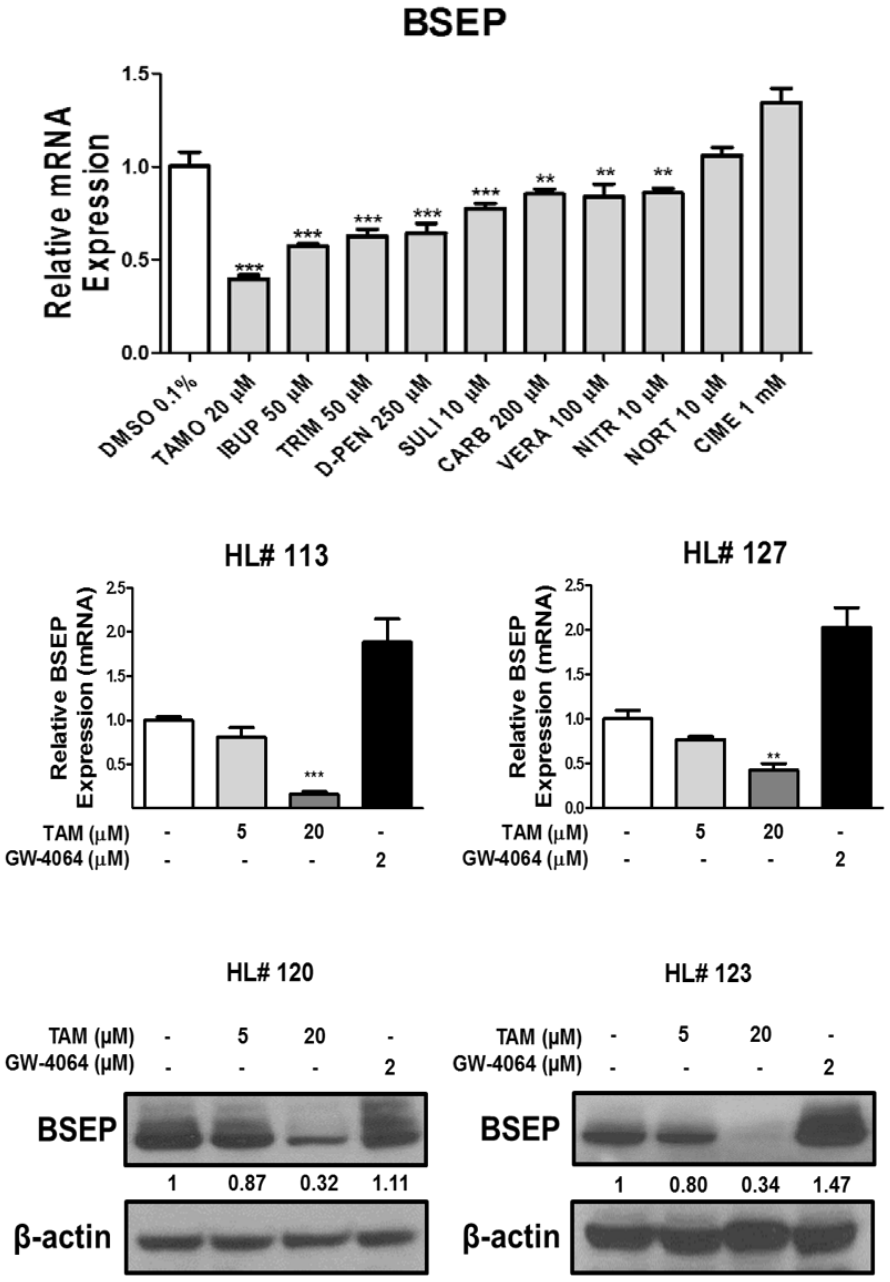


Figure 4.3 Cholestatic non-BSEP- inhibitor tamoxifen represses BSEP expression. (A) mRNA expression of BSEP was measured using RT-PCR in a human primary hepatocyte donor (HL #109). Cells were treated with vehicle control DMSO (0.1%), tamoxifen (20 µM, TAM), ibuprofen (50 µM, IBUP), trimethoprim (50 µM, TRIM), D-penicillamine (250 µM, D-PEN), sulindac (10 µM, SULI), carbamazepine (200 µM, CARB), verapamil (100 µM, VERA), nitrofurantoin (10 µM, NITR), nortriptyline (10 µM, NORT), and cimetidine (1 mM, CIME). Data are expressed as mean ± S.D. (n=3). (B & C) mRNA expression of BSEP was measured using RT-PCR in two human primary hepatocyte donors, HL #113 (B) & #127 (C), treated with vehicle control DMSO (0.1%), GW-4064 (2 µM, GW), or tamoxifen (5, 20, 40 µM). Data are expressed as mean ± S.D. (n=3). (D & E) Protein expression of BSEP was measured using Western blotting in two human primary hepatocyte donors, HL #120 (D) & #123 (E), treated with vehicle control DMSO 0.1% (DMSO), tamoxifen 5 or 20 µM for 72 h, and positive control BSEP inducer, GW-4064 (2 µM). ***P, 0.001; **P, 0.01; *P, 0.05.

4.4 Discussion

Drug-induced cholestasis remains a major concern in the development and prescribing of drugs. In an effort to better understand the causes for drug-induced cholestasis, and develop models for prediction, inhibition of hepatic transporters, including the bile salt export pump, is heavily investigated (86, 88, 153). However, few studies consider the repression of hepatic transporters, let alone develop models to predict this. While it is inherently more difficult to model repression, as there could be multiple transcription factors and pathways involved, our current study developed pharmacophore and Bayesian models for drugs which inhibit and repress BSEP, as identified from our previous work (107).

Three models were developed, including two pharmacophore and one Bayesian, based on previous work by our group investigating BSEP repressors. Interestingly, when compared to the pharmacophore model of BSEP repressors, four drugs were also predicted by the Bayesian model: ritonavir, labetalol, amiodarone, and tolterodine. These drugs warrant further investigation as likely BSEP repressors. The first pharmacophore and Bayesian models used a group of drugs which repressed BSEP, however they were also potent inhibitors. While still important, as it was determined that drugs that both inhibit and repress BSEP are associated with more severe drug-induced liver injury (107), the focus of the current study was to identify BSEP repressors and establish their role in drug-induced liver injury. For this reason, the third model was developed.

Recently, a list of drugs labeled “cholestatic BSEP non-inhibitors” were reported to induce cholestatic toxicity without inhibiting BSEP activity (128). We speculated a plausible explanation for the cholestatic effects of these drugs might be repression of

BSEP gene expression. Included in this list was metformin, which we established in Chapter 3 to potently repress BSEP expression and function. Using metformin as a model, pharmacophore features were assigned, and the 24 remaining drugs in the list of cholestatic BSEP non-inhibitors were screened for BSEP repression.

Of the drugs screened against the metformin pharmacophore model, 10 were investigated in human primary hepatocytes for BSEP repression. Tamoxifen potently repressed BSEP expression, while the rest had moderate or no effects. Notably, tamoxifen has been reported to activate AMPK (154), and is classified as a selective estrogen receptor modulator (SERM) that can act as either an agonist or antagonist in a tissue-specific manner (155). Interestingly, previous reports have demonstrated that estrogen receptor alpha (ERα) exhibits transrepressive effects on BSEP expression, and estradiol-activated ERα contributes to intrahepatic cholestasis of pregnancy (156, 157). As an ERα agonist in the liver (158), it is possible that, like estradiol, tamoxifen induces ERα-mediated transrepressive effects on BSEP; this merits further investigation (156). Our results support BSEP repression as a mechanism of drug-induced cholestasis, and suggest other cholestatic drugs may exert their toxicity by this mechanism.

While the strategy for modeling repressors of drug transporters has not been attempted previously, our studies suggest combining multiple approaches may produce promising leads. Only a small subset of potential repressors were investigated in the current study, with most having some repressive effects on BSEP. Additional mRNA profiling of the remaining drugs is warranted, as few drugs overlap in more than one model. Successful identification of BSEP repressors from these modeling strategies can

be used to revise and strengthen the current models, until one effective model of BSEP repression is established.

Chapter 5: Conclusions and Future Directions

With persistent reports of hepatotoxicity, understanding and predicting drug-induced liver injury (DILI) remains a major focus of the pharmaceutical field. As the leading cause for post-marketing withdrawal and/or regulatory action, DILI impacts all aspects of drug development, including the drug industry, regulators, clinicians, and patients (159). There are many forms of DILI, making it unpredictable by nature, and difficult to distinguish from other types of liver disease. This may lead to under-reporting and/or misdiagnosis (12). Advances in the development of biomarkers would help diagnosis of DILI, while better predictive models would help avoid new drugs that may cause DILI. Though idiosyncratic DILI is considered unpredictable, recent findings have begun to make connections and common associations between drugs linked to DILI (8, 32). Continuing to investigate mechanisms involved in the development of DILI, and determining the pathways targeted will provide the understanding needed to optimize biomarkers and models.

One of the most prevalent forms of DILI is cholestasis (27, 79). The primary determinant of hepatobiliary excretion, the bile salt export pump (BSEP) is most commonly investigated mechanism in the development of drug-induced cholestasis (55). Current strategies focus on inhibition of BSEP function, through functional assays with an endogenous substrate. While important, numerous drugs associated with drug-induced cholestasis have been shown not to inhibit BSEP (128). To this end, work described in this dissertation ***confirmed drug-mediated repression of the bile salt export pump could reduce the transport of bile acids in human primary hepatocytes (HPH), with clinical implications regarding drug-induced liver injury. Additionally, combined***

computational modeling and literature-based strategies successfully identified a novel, potent BSEP repressor, tamoxifen.

Completion of the above studies provided novel insights into the contribution of BSEP repression to the development of cholestasis. A screening of 30 BSEP inhibitors revealed five potent repressors of BSEP mRNA and protein expression. When considering the clinical implications inhibition and repression have on DILI, drugs capable of both inhibiting and repressing BSEP were associated with the greatest concern. A deeper, mechanistic study identified metformin as a potent BSEP repressor, however, consistent with literature, metformin did not directly inhibit BSEP function. Additionally, our studies established the effects of BSEP repression in a modified functional assay, demonstrating that without drug-inhibition BSEP repression can impair the excretion of bile acids from HPH.

Computational modeling of the gene repression studies provided the foundation for identifying additional BSEP repressors. When combined with literature-based identification of potential BSEP repressors, the metformin pharmacophore model predicted 10 drugs would repress BSEP. Screening of these drugs in HPH revealed one potent repressor, tamoxifen, with moderate repression observed for most drugs. Considering the role of tamoxifen and metformin as activators of AMPK, further investigation into the role of AMPK revealed it contributed at least partially to BSEP gene repression.

Future directions for this work include further validation of the computational models, and additional optimization, as more information is elucidated. Several promising candidates were predicted from the pharmacophore and Bayesian models of

BSEP repression, including labetalol, amiodarone, and tolterodine. Investigating these drugs in HPH for their effect on BSEP expression should be considered.

Characterizing the clinical impact of in vitro BSEP repression is very difficult, therefore developing a method which would determine the effect on BSEP expression in vivo (human clinical data) would be useful. Disruption of bile flow from the liver, should result in increased serum bile acids, which have been diverted from the liver or effluxed from the basolateral membrane. Measuring bile acid levels in patients before use of a suspected cholestatic drug (such as metformin), and during the first 24-48 hr after dosing, could provide information about the bile homeostasis response to the drug.

It has been suggested that the incidence of drug-induced cholestasis is under-reported, with some individuals showing only elevated liver enzymes in serum and no symptoms; therefore, it would be beneficial to conduct a retrospective study to measure liver enzymes in asymptomatic patients taking drugs highly associated with cholestasis.

Better understanding of drug-induced cholestasis, including its causes, susceptibility factors and biomarkers is urgently needed, therefore any information or strategy to advance knowledge in this field is warranted.

References

1. Grant DM. Detoxification pathways in the liver. J Inherit Metab Dis. 1991;14(4):421-430.
2. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell. 2000;6(3):507-515.
3. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci. 2002;65(2):166-176.
4. Chalasani NP, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ, Practice Parameters Committee of the American College of G. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. Am J Gastroenterol. 2014;109(7):950-966; quiz 967.
5. Onakpoya IJ, Heneghan CJ, Aronson JK. Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: a systematic review of the world literature. BMC Med. 2016;14:10.
6. Watkins PB. Drug safety sciences and the bottleneck in drug development. Clin Pharmacol Ther. 2011;89(6):788-790.
7. Alempijevic T, Zec S, Milosavljevic T. Drug-induced liver injury: Do we know everything? World J Hepatol. 2017;9(10):491-502.
8. Weiler S, Merz M, Kullak-Ublick GA. Drug-induced liver injury: the dawn of biomarkers? F1000Prime Rep. 2015;7:34.

9. Zimmerman HJ. Iatrogenic hepatic injury. In. Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver. Philadelphia, PA: Lippincott Williams & Wilkins; 1999. p. 427-456.

10. Hayashi PH, Fontana RJ. Clinical features, diagnosis, and natural history of drug-induced liver injury. Semin Liver Dis. 2014;34(2):134-144.

11. Lu RJ, Zhang Y, Tang FL, Zheng ZW, Fan ZD, Zhu SM, Qian XF, Liu NN. Clinical characteristics of drug-induced liver injury and related risk factors. Exp Ther Med. 2016;12(4):2606-2616.

12. Kaplowitz N. Idiosyncratic drug hepatotoxicity. Nat Rev Drug Discov. 2005;4(6):489-499.

13. Lewis JH. 'Hy's law,' the 'Rezulin Rule,' and other predictors of severe drug-induced hepatotoxicity: putting risk-benefit into perspective. Pharmacoepidemiol Drug Saf. 2006;15(4):221-229.

14. Reuben A. Hy's law. Hepatology. 2004;39(2):574-578.

15. Temple R. Hy's law: predicting serious hepatotoxicity. Pharmacoepidemiol Drug Saf. 2006;15(4):241-243.

16. Benichou C, Danan G, Flahault A. Causality assessment of adverse reactions to drugs--II. An original model for validation of drug causality assessment methods: case reports with positive rechallenge. J Clin Epidemiol. 1993;46(11):1331-1336.

17. Danan G, Benichou C. Causality assessment of adverse reactions to drugs--I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. J Clin Epidemiol. 1993;46(11):1323-1330.

18. Rochon J, Protiva P, Seeff LB, Fontana RJ, Liangpunsakul S, Watkins PB, Davern T, McHutchison JG, Drug-Induced Liver Injury N. Reliability of the Roussel Uclaf Causality Assessment Method for assessing causality in drug-induced liver injury. Hepatology. 2008;48(4):1175-1183.

19. Kaplowitz NaD, L.D. Histopathological evaluation of drug-induced liver disease. In. Drug-induced liver disease: Elsevier Science; 2013. p. 241-264.

20. Raschi E, De Ponti F. Drug-induced liver injury: Towards early prediction and risk stratification. World J Hepatol. 2017;9(1):30-37.

21. Suk KT, Kim DJ. Drug-induced liver injury: present and future. Clin Mol Hepatol. 2012;18(3):249-257.

22. Zhu XW, Li SJ. In silico Prediction of Drug-induced Liver Injury Based on Adverse Drug Reaction Reports. Toxicol Sci. 2017.

23. Iorga A, Dara L, Kaplowitz N. Drug-Induced Liver Injury: Cascade of Events Leading to Cell Death, Apoptosis or Necrosis. Int J Mol Sci. 2017;18(5).

24. Roth RA, Ganey PE. Intrinsic versus idiosyncratic drug-induced hepatotoxicity--two villains or one? J Pharmacol Exp Ther. 2010;332(3):692-697.

25. Roth AD, Lee MY. Idiosyncratic Drug-Induced Liver Injury (IDILI): Potential Mechanisms and Predictive Assays. Biomed Res Int. 2017;2017:9176937.

26. Ju HY, Jang JY, Jeong SW, Woo SA, Kong MG, Jang HY, Lee SH, Kim SG, Cha SW, Kim YS, Cho YD, Jin SY, Kim HS, Kim BS. The clinical features of drug-induced liver injury observed through liver biopsy: focus on relevancy to autoimmune hepatitis. Clin Mol Hepatol. 2012;18(2):213-218.

27. Ghabril M, Chalasani N, Bjornsson E. Drug-induced liver injury: a clinical update. Curr Opin Gastroenterol. 2010;26(3):222-226.

28. Holt MP, Ju C. Mechanisms of drug-induced liver injury. AAPS J. 2006;8(1):E48-54.

29. Gunawan B, Kaplowitz N. Clinical perspectives on xenobiotic-induced hepatotoxicity. Drug Metab Rev. 2004;36(2):301-312.

30. Russmann S, Jetter A, Kullak-Ublick GA. Pharmacogenetics of drug-induced liver injury. Hepatology. 2010;52(2):748-761.

31. Yu K, Zhang J, Chen M, Xu X, Suzuki A, Ilic K, Tong W. Mining hidden knowledge for drug safety assessment: topic modeling of LiverTox as a case study. BMC Bioinformatics. 2014;15 Suppl 17:S6.

32. Liu Z, Shi Q, Ding D, Kelly R, Fang H, Tong W. Translating clinical findings into knowledge in drug safety evaluation--drug induced liver injury prediction system (DILIps). PLoS Comput Biol. 2011;7(12):e1002310.

33. Zhu X, Kruhlak NL. Construction and analysis of a human hepatotoxicity database suitable for QSAR modeling using post-market safety data. Toxicology. 2014;321:62-72.

34. Kleiner DE, Chalasani NP, Lee WM, Fontana RJ, Bonkovsky HL, Watkins PB, Hayashi PH, Davern TJ, Navarro V, Reddy R, Talwalkar JA, Stolz A, Gu J, Barnhart H, Hoofnagle JH, Drug-Induced Liver Injury N. Hepatic histological findings in suspected drug-induced liver injury: systematic evaluation and clinical associations. Hepatology. 2014;59(2):661-670.

35. Ramachandran R, Kakar S. Histological patterns in drug-induced liver disease. J Clin Pathol. 2009;62(6):481-492.

36. Padda MS, Sanchez M, Akhtar AJ, Boyer JL. Drug-induced cholestasis. Hepatology. 2011;53(4):1377-1387.

37. Myant NB, Mitropoulos KA. Cholesterol 7 alpha-hydroxylase. J Lipid Res. 1977;18(2):135-153.

38. Boyer JL. Bile formation and secretion. Compr Physiol. 2013;3(3):1035-1078.

39. Keely SJ, Walters JR. The Farnesoid X Receptor: Good for BAD. Cell Mol Gastroenterol Hepatol. 2016;2(6):725-732.

40. Chiang JY. Bile acid regulation of hepatic physiology: III. Bile acids and nuclear receptors. Am J Physiol Gastrointest Liver Physiol. 2003;284(3):G349-356.

41. St-Pierre MV, Kullak-Ublick GA, Hagenbuch B, Meier PJ. Transport of bile acids in hepatic and non-hepatic tissues. J Exp Biol. 2001;204(Pt 10):1673-1686.

42. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol. 2009;15(14):1677-1689.

43. Maillette de Buy Wenniger L, Beuers U. Bile salts and cholestasis. Dig Liver Dis. 2010;42(6):409-418.

44. Zwicker BL, Agellon LB. Transport and biological activities of bile acids. Int J Biochem Cell Biol. 2013;45(7):1389-1398.

45. Vitek L, Haluzik M. The role of bile acids in metabolic regulation. J Endocrinol. 2016;228(3):R85-96.

46. Taoka H, Yokoyama Y, Morimoto K, Kitamura N, Tanigaki T, Takashina Y, Tsubota K, Watanabe M. Role of bile acids in the regulation of the metabolic pathways. World J Diabetes. 2016;7(13):260-270.

47. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell. 2000;6(3):517-526.

48. Chiang JY, Miller WF, Lin GM. Regulation of cholesterol 7 alpha-hydroxylase in the liver. Purification of cholesterol 7 alpha-hydroxylase and the immunochemical evidence for the induction of cholesterol 7 alpha-hydroxylase by cholestyramine and circadian rhythm. J Biol Chem. 1990;265(7):3889-3897.

49. Hofmann AF. Bile Acids: The Good, the Bad, and the Ugly. News Physiol Sci. 1999;14:24-29.

50. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev. 2009;89(1):147-191.

51. Chiang JY. Bile acid metabolism and signaling. Compr Physiol. 2013;3(3):1191-1212.

52. Copple BL, Li T. Pharmacology of bile acid receptors: Evolution of bile acids from simple detergents to complex signaling molecules. Pharmacol Res. 2016;104:9-21.

53. Chiang JY. Bile acids: regulation of synthesis. J Lipid Res. 2009;50(10):1955-1966.

54. Stellaard F, Sackmann M, Sauerbruch T, Paumgartner G. Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using 13C-labeled bile acids. J Lipid Res. 1984;25(12):1313-1319.

55. Stieger B. Role of the bile salt export pump, BSEP, in acquired forms of cholestasis. Drug Metab Rev. 2010;42(3):437-445.

56. Jansen PL, Ghallab A, Vartak N, Reif R, Schaap FG, Hampe J, Hengstler JG. The ascending pathophysiology of cholestatic liver disease. Hepatology. 2017;65(2):722-738.

57. Anwer MS, Stieger B. Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters. Pflugers Arch. 2014;466(1):77-89.

58. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol. 1999;55(5):929-937.

59. Konig J, Nies AT, Cui Y, Leier I, Keppler D. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim Biophys Acta. 1999;1461(2):377-394.

60. Linton KJ. Lipid flopping in the liver. Biochem Soc Trans. 2015;43(5):1003-1010.

61. Muller M, Jansen PL. Molecular aspects of hepatobiliary transport. Am J Physiol. 1997;272(6 Pt 1):G1285-1303.

62. Anwer MS, Hegner D. Effect of Na on bile acid uptake by isolated rat hepatocytes. Evidence for a heterogeneous system. Hoppe Seylers Z Physiol Chem. 1978;359(2):181-192.

63. Dong Z, Ekins S, Polli JE. Structure-activity relationship for FDA approved drugs as inhibitors of the human sodium taurocholate cotransporting polypeptide (NTCP). Mol Pharm. 2013;10(3):1008-1019.

64. Dawson PA, Lan T, Rao A. Bile acid transporters. J Lipid Res. 2009;50(12):2340-2357.

65. Pastor CM, Mullhaupt B, Stieger B. The role of organic anion transporters in diagnosing liver diseases by magnetic resonance imaging. Drug Metab Dispos. 2014;42(4):675-684.

66. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, Meier PJ. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem. 1998;273(16):10046-10050.

67. Childs S, Yeh RL, Georges E, Ling V. Identification of a sister gene to P-glycoprotein. Cancer Res. 1995;55(10):2029-2034.

68. Trauner M, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev. 2003;83(2):633-671.

69. Eloranta JJ, Kullak-Ublick GA. The role of FXR in disorders of bile acid homeostasis. Physiology (Bethesda). 2008;23:286-295.

70. Kim I, Ahn SH, Inagaki T, Choi M, Ito S, Guo GL, Kliewer SA, Gonzalez FJ. Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. J Lipid Res. 2007;48(12):2664-2672.

71. Ferrebee CB, Dawson PA. Metabolic effects of intestinal absorption and enterohepatic cycling of bile acids. Acta Pharm Sin B. 2015;5(2):129-134.

72. Matsubara T, Li F, Gonzalez FJ. FXR signaling in the enterohepatic system. Mol Cell Endocrinol. 2013;368(1-2):17-29.

73. de Lima Toccafondo Vieira M, Tagliati CA. Hepatobiliary transporters in drug-induced cholestasis: a perspective on the current identifying tools. Expert Opin Drug Metab Toxicol. 2014;10(4):581-597.

74. Pauli-Magnus C, Meier PJ. Pharmacogenetics of hepatocellular transporters. Pharmacogenetics. 2003;13(4):189-198.

75. van der Woerd WL, Houwen RH, van de Graaf SF. Current and future therapies for inherited cholestatic liver diseases. World J Gastroenterol. 2017;23(5):763-775.

76. Gaur K, Sakhuja P. Progressive familial intrahepatic cholestasis: A comprehensive review of a challenging liver disease. Indian J Pathol Microbiol. 2017;60(1):2-7.

77. van der Woerd WL, van Mil SW, Stapelbroek JM, Klomp LW, van de Graaf SF, Houwen RH. Familial cholestasis: progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis and intrahepatic cholestasis of pregnancy. Best Pract Res Clin Gastroenterol. 2010;24(5):541-553.

78. Stieger B, Geier A. Genetic variations of bile salt transporters as predisposing factors for drug-induced cholestasis, intrahepatic cholestasis of pregnancy and therapeutic response of viral hepatitis. Expert Opin Drug Metab Toxicol. 2011;7(4):411-425.

79. Yang K, Kock K, Sedykh A, Tropsha A, Brouwer KL. An updated review on drug-induced cholestasis: mechanisms and investigation of physicochemical properties and pharmacokinetic parameters. J Pharm Sci. 2013;102(9):3037-3057.

80. Alrefai WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res. 2007;24(10):1803-1823.

81. Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 2001;276(31):28857-28865.

82. Song X, Kaimal R, Yan B, Deng R. Liver receptor homolog 1 transcriptionally regulates human bile salt export pump expression. J Lipid Res. 2008;49(5):973-984.

83. Weerachayaphorn J, Cai SY, Soroka CJ, Boyer JL. Nuclear factor erythroid 2-related factor 2 is a positive regulator of human bile salt export pump expression. Hepatology. 2009;50(5):1588-1596.

84. Dawson S, Stahl S, Paul N, Barber J, Kenna JG. In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. Drug Metab Dispos. 2012;40(1):130-138.

85. Cheng Y, Woolf TF, Gan J, He K. In vitro model systems to investigate bile salt export pump (BSEP) activity and drug interactions: A review. Chem Biol Interact. 2016;255:23-30.

86. Kotsampasakou E, Ecker GF. Predicting Drug-Induced Cholestasis with the Help of Hepatic Transporters-An in Silico Modeling Approach. J Chem Inf Model. 2017;57(3):608-615.

87. Ritschel T, Hermans SM, Schreurs M, van den Heuvel JJ, Koenderink JB, Greupink R, Russel FG. In silico identification and in vitro validation of potential cholestatic compounds through 3D ligand-based pharmacophore modeling of BSEP inhibitors. Chem Res Toxicol. 2014;27(5):873-881.

88. Welch MA, Kock K, Urban TJ, Brouwer KL, Swaan PW. Toward predicting drug-induced liver injury: parallel computational approaches to identify multidrug resistance protein 4 and bile salt export pump inhibitors. Drug Metab Dispos. 2015;43(5):725-734.

89. Woodhead JL, Yang K, Siler SQ, Watkins PB, Brouwer KL, Barton HA, Howell BA. Exploring BSEP inhibition-mediated toxicity with a mechanistic model of drug-induced liver injury. Front Pharmacol. 2014;5:240.

90. Esteller A. Physiology of bile secretion. World J Gastroenterol. 2008;14(37):5641-5649.

91. Reichen J, Paumgartner G. Uptake of bile acids by perfused rat liver. Am J Physiol. 1976;231(3):734-742.

92. Ogimura E, Sekine S, Horie T. Bile salt export pump inhibitors are associated with bile acid-dependent drug-induced toxicity in sandwich-cultured hepatocytes. Biochem Biophys Res Commun. 2011;416(3-4):313-317.

93. Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LW, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RH, Freimer NB. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat Genet. 1998;18(3):219-224.

94. Maddrey WC. Drug-induced hepatotoxicity: 2005. J Clin Gastroenterol. 2005;39(4 Suppl 2):S83-89.

95. Stieger B. Recent insights into the function and regulation of the bile salt export pump (ABCB11). Curr Opin Lipidol. 2009;20(3):176-181.

96. Bjornsson E, Olsson R. Outcome and prognostic markers in severe drug-induced liver disease. Hepatology. 2005;42(2):481-489.

97. Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharski P, Cai H, Sinclair JF, Sahi J. Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. Toxicol Sci. 2003;76(1):220-228.

98. Morgan RE, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, Afshari CA, Qualls CW, Jr., Lightfoot-Dunn R, Hamadeh HK. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. Toxicol Sci. 2010;118(2):485-500.

99. van Staden CJ, Morgan RE, Ramachandran B, Chen Y, Lee PH, Hamadeh HK. Membrane vesicle ABC transporter assays for drug safety assessment. Curr Protoc Toxicol. 2012;Chapter 23:Unit 23 25.

100. Swift B, Brouwer KL. Influence of seeding density and extracellular matrix on bile Acid transport and mrp4 expression in sandwich-cultured mouse hepatocytes. Mol Pharm. 2010;7(2):491-500.

101. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. Science. 1999;284(5418):1362-1365.

102. Kubitz R, Droge C, Stindt J, Weissenberger K, Haussinger D. The bile salt export pump (BSEP) in health and disease. Clinics and research in hepatology and gastroenterology. 2012;36(6):536-553.

103. Deng R, Yang D, Yang J, Yan B. Oxysterol 22(R)-Hydroxycholesterol Induces the Expression of the Bile Salt Export Pump through Nuclear Receptor Farsenoid X Receptor but Not Liver X Receptor. Journal of Pharmacology and Experimental Therapeutics. 2006;317(1):317-325.

104. LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, Richert L. Isolation and culture of primary human hepatocytes. Methods Mol Biol. 2005;290:207-229.

105. Faucette SR, Sueyoshi T, Smith CM, Negishi M, Lecluyse EL, Wang H. Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. J Pharmacol Exp Ther. 2006;317(3):1200-1209.

106. Wang D, Li L, Yang H, Ferguson SS, Baer MR, Gartenhaus RB, Wang H. The constitutive androstane receptor is a novel therapeutic target facilitating cyclophosphamide-based treatment of hematopoietic malignancies. Blood. 2013;121(2):329-338.

107. Garzel B, Yang H, Zhang L, Huang SM, Polli JE, Wang H. The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity. Drug Metab Dispos. 2014;42(3):318-322.

108. Chen M, Vijay V, Shi Q, Liu Z, Fang H, Tong W. FDA-approved drug labeling for the study of drug-induced liver injury. Drug Discov Today. 2011;16(15-16):697-703.

109. Yamazaki M, Miyake M, Sato H, Masutomi N, Tsutsui N, Adam KP, Alexander DC, Lawton KA, Milburn MV, Ryals JA, Wulff JE, Guo L. Perturbation of bile acid homeostasis is an early pathogenesis event of drug induced liver injury in rats. Toxicol Appl Pharmacol. 2013;268(1):79-89.

110. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. Physiol Rev. 2001;81(3):1269-1304.

111. Li L, Sinz MW, Zimmermann K, Wang H. An insulin-like growth factor 1 receptor inhibitor induces CYP3A4 expression through a pregnane X receptor-independent, noncanonical constitutive androstane receptor-related mechanism. J Pharmacol Exp Ther. 2012;340(3):688-697.

112. Kaimal R, Song X, Yan B, King R, Deng R. Differential modulation of farnesoid X receptor signaling pathway by the thiazolidinediones. J Pharmacol Exp Ther. 2009;330(1):125-134.

113. Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B, Meier PJ. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. Clin Pharmacol Ther. 2001;69(4):223-231.

114. Oorts M, Baze A, Bachellier P, Heyd B, Zacharias T, Annaert P, Richert L. Drug-induced cholestasis risk assessment in sandwich-cultured human hepatocytes. Toxicol In Vitro. 2016.

115. Vatakuti S, Pennings JL, Gore E, Olinga P, Groothuis GM. Classification of Cholestatic and Necrotic Hepatotoxicants Using Transcriptomics on Human Precision-Cut Liver Slices. Chem Res Toxicol. 2016;29(3):342-351.

116. Schadt HS, Wolf A, Pognan F, Chibout SD, Merz M, Kullak-Ublick GA. Bile acids in drug induced liver injury: Key players and surrogate markers. Clinics and research in hepatology and gastroenterology. 2016.

117. Noor F. A shift in paradigm towards human biology-based systems for cholestatic-liver diseases. The Journal of physiology. 2015;593(23):5043-5055.

118. Qiu X, Zhang Y, Liu T, Shen H, Xiao Y, Bourner MJ, Pratt JR, Thompson DC, Marathe P, Humphreys WG, Lai Y. Disruption of BSEP Function in HepaRG Cells Alters Bile Acid Disposition and Is a Susceptive Factor to Drug-Induced Cholestatic Injury. Mol Pharm. 2016;13(4):1206-1216.

119. Chatterjee S, Richert L, Augustijns P, Annaert P. Hepatocyte-based in vitro model for assessment of drug-induced cholestasis. Toxicol Appl Pharmacol. 2014;274(1):124-136.

120. NAMAS R, MARQUARDT A. Case Report and Literature Review: Quinacrine-induced Cholestatic Hepatitis in Undifferentiated Connective Tissue Disease. The Journal of Rheumatology. 2015;42(7):1354-1355.

121. Saadi T, Waterman M, Yassin H, Baruch Y. Metformin-induced mixed hepatocellular and cholestatic hepatic injury: case report and literature review. International journal of general medicine. 2013;6:703-706.

122. Parmentier C, Couttet P, Wolf A, Zaccharias T, Heyd B, Bachellier P, Uteng M, Richert L. Evaluation of transcriptomic signature as a valuable tool to study drug-induced cholestasis in primary human hepatocytes. Arch Toxicol. 2017.

123. Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. Nat Genet. 1998;20(3):233-238.

124. Marroquin LD, Bonin PD, Keefer J, Schroeter T. Assessment of Bile Salt Export Pump (BSEP) Inhibition in Membrane Vesicles Using Radioactive and LC/MS-Based Detection Methods. Curr Protoc Toxicol. 2017;71:14 14 11-14 14 20.

125. Montanari F, Pinto M, Khunweeraphong N, Wlcek K, Sohail MI, Noeske T, Boyer S, Chiba P, Stieger B, Kuchler K, Ecker GF. Flagging Drugs That Inhibit the Bile Salt Export Pump. Mol Pharm. 2016;13(1):163-171.

126. Giacomini KM, Huang SM. Transporters in drug development and clinical pharmacology. Clin Pharmacol Ther. 2013;94(1):3-9.

127. Tweedie D, Polli JW, Berglund EG, Huang SM, Zhang L, Poirier A, Chu X, Feng B, International Transporter C. Transporter studies in drug development: experience to date and follow-up on decision trees from the International Transporter Consortium. Clin Pharmacol Ther. 2013;94(1):113-125.

128. Kock K, Ferslew BC, Netterberg I, Yang K, Urban TJ, Swaan PW, Stewart PW, Brouwer KL. Risk factors for development of cholestatic drug-induced liver

injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. Drug Metab Dispos. 2014;42(4):665-674.

129. Desilets DJ, Shorr AF, Moran KA, Holtzmuller KC. Cholestatic jaundice associated with the use of metformin. Am J Gastroenterol. 2001;96(7):2257-2258.

130. Nammour FE, Fayad NF, Peikin SR. Metformin-induced cholestatic hepatitis. Endocr Pract. 2003;9(4):307-309.

131. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108(8):1167-1174.

132. Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M, Wang H. The Peripheral Benzodiazepine Receptor Ligand 1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide Is a Novel Antagonist of Human Constitutive Androstane Receptor. Molecular Pharmacology. 2008;74(2):443-453.

133. Swift* B, Pfeifer* ND, Brouwer KLR. Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. Drug Metabolism Reviews. 2010;42(3):446-471.

134. Chopra AR, Kommagani R, Saha P, Louet JF, Salazar C, Song J, Jeong J, Finegold M, Viollet B, DeMayo F, Chan L, Moore DD, O'Malley BW. Cellular energy depletion resets whole-body energy by promoting coactivator-mediated dietary fuel absorption. Cell Metab. 2011;13(1):35-43.

135. Nygaard EB, Vienberg SG, Orskov C, Hansen HS, Andersen B. Metformin stimulates FGF21 expression in primary hepatocytes. Exp Diabetes Res. 2012;2012:465282.

136. Wanninger J, Neumeier M, Weigert J, Liebisch G, Weiss TS, Schaffler A, Aslanidis C, Schmitz G, Scholmerich J, Buechler C. Metformin reduces cellular lysophosphatidylcholine and thereby may lower apolipoprotein B secretion in primary human hepatocytes. Biochim Biophys Acta. 2008;1781(6-7):321-325.

137. Fryer LG, Parbu-Patel A, Carling D. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem. 2002;277(28):25226-25232.

138. Weerachayaphorn J, Mennone A, Soroka CJ, Harry K, Hagey LR, Kensler TW, Boyer JL. Nuclear factor-E2-related factor 2 is a major determinant of bile acid homeostasis in the liver and intestine. Am J Physiol Gastrointest Liver Physiol. 2012;302(9):G925-936.

139. Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL, Muller M. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology. 2002;35(3):589-596.

140. Chen M, Hong H, Fang H, Kelly R, Zhou G, Borlak J, Tong W. Quantitative structure-activity relationship models for predicting drug-induced liver injury based on FDA-approved drug labeling annotation and using a large collection of drugs. Toxicol Sci. 2013;136(1):242-249.

141. Chen Q, Yang X, Zhang H, Kong X, Yao L, Cui X, Zou Y, Fang F, Yang J, Chang Y. Metformin impairs systemic bile acid homeostasis through regulating SIRT1 protein levels. Biochim Biophys Acta. 2017;1864(1):101-112.

142. Pedersen JM, Matsson P, Bergstrom CA, Hoogstraate J, Noren A, LeCluyse EL, Artursson P. Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABCB11). Toxicol Sci. 2013;136(2):328-343.

143. Kis E, Ioja E, Nagy T, Szente L, Heredi-Szabo K, Krajcsi P. Effect of membrane cholesterol on BSEP/Bsep activity: species specificity studies for substrates and inhibitors. Drug Metab Dispos. 2009;37(9):1878-1886.

144. Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF, Sugiyama Y. Vectorial transport of unconjugated and conjugated bile salts by monolayers of LLC-PK1 cells doubly transfected with human NTCP and BSEP or with rat Ntcp and Bsep. Am J Physiol Gastrointest Liver Physiol. 2006;290(3):G550-556.

145. Wolf KK, Vora S, Webster LO, Generaux GT, Polli JW, Brouwer KL. Use of cassette dosing in sandwich-cultured rat and human hepatocytes to identify drugs that inhibit bile acid transport. Toxicol In Vitro. 2010;24(1):297-309.

146. Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, Beri RK. Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. FEBS Lett. 1994;353(1):33-36.

147. Yang K, Pfeifer ND, Kock K, Brouwer KL. Species differences in hepatobiliary disposition of taurocholic acid in human and rat sandwich-cultured hepatocytes:

implications for drug-induced liver injury. J Pharmacol Exp Ther. 2015;353(2):415-423.

148. Song X, Chen Y, Valanejad L, Kaimal R, Yan B, Stoner M, Deng R. Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor. J Lipid Res. 2013;54(11):3030-3044.

149. Li WK, Li H, Lu YF, Li YY, Fu ZD, Liu J. Atorvastatin alters the expression of genes related to bile acid metabolism and circadian clock in livers of mice. PeerJ. 2017;5:e3348.

150. Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, Dehondt H, Ploton M, Colin S, Lucas A, Patrice A, Pattou F, Diemer H, Van Dorsselaer A, Rachez C, Kamilic J, Groen AK, Staels B, Lefebvre P. Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. J Clin Invest. 2014;124(3):1037-1051.

151. Rogers D, Brown RD, Hahn M. Using extended-connectivity fingerprints with Laplacian-modified Bayesian analysis in high-throughput screening follow-up. J Biomol Screen. 2005;10(7):682-686.

152. Law V, Knox C, Djoumbou Y, Jewison T, Guo AC, Liu Y, Maciejewski A, Arndt D, Wilson M, Neveu V, Tang A, Gabriel G, Ly C, Adamjee S, Dame ZT, Han B, Zhou Y, Wishart DS. DrugBank 4.0: shedding new light on drug metabolism. Nucleic Acids Res. 2014;42(Database issue):D1091-1097.

153. Jain S, Grandits M, Richter L, Ecker GF. Structure based classification for bile salt export pump (BSEP) inhibitors using comparative structural modeling of human BSEP. J Comput Aided Mol Des. 2017.

154. Daurio NA, Tuttle SW, Worth AJ, Song EY, Davis JM, Snyder NW, Blair IA, Koumenis C. AMPK Activation and Metabolic Reprogramming by Tamoxifen through Estrogen Receptor-Independent Mechanisms Suggests New Uses for This Therapeutic Modality in Cancer Treatment. Cancer Res. 2016;76(11):3295-3306.

155. Goodsell DS. The molecular perspective: tamoxifen and the estrogen receptor. Oncologist. 2002;7(2):163-164.

156. Chen Y, Vasilenko A, Song X, Valanejad L, Verma R, You S, Yan B, Shiffka S, Hargreaves L, Nadolny C, Deng R. Estrogen and Estrogen Receptor-alpha-Mediated Transrepression of Bile Salt Export Pump. Mol Endocrinol. 2015;29(4):613-626.

157. Song X, Vasilenko A, Chen Y, Valanejad L, Verma R, Yan B, Deng R. Transcriptional dynamics of bile salt export pump during pregnancy: mechanisms and implications in intrahepatic cholestasis of pregnancy. Hepatology. 2014;60(6):1993-2007.

158. Yoshikawa Y, Miyashita T, Higuchi S, Tsuneyama K, Endo S, Tsukui T, Toyoda Y, Fukami T, Nakajima M, Yokoi T. Mechanisms of the hepatoprotective effects of tamoxifen against drug-induced and chemical-induced acute liver injuries. Toxicol Appl Pharmacol. 2012;264(1):42-50.

159. Navarro VJ, Senior JR. Drug-related hepatotoxicity. N Engl J Med. 2006;354(7):731-739.