Roles of Toll-Like Receptors, Nuclear Receptors and Obesity in Drug Metabolism

A Dissertation Presented to the

Faculty of the Department of Pharmacological and Pharmaceutical Sciences

College of Pharmacy, University of Houston

In Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

By

Pranav Shah

May, 2014

Dedicated to my grandfather, Rasiklal C Shah

ACKNOWLEDGEMENTS

"There is no such thing as a self-made man. You will reach your goals only with the help of others"-George Shinn. I could not be who I am today without the help and support of hundreds of people who have touched my life. Although I will not be able to thank them individually, I sincerely express my deepest gratitude to all of them who help me reached where I am today.

First and foremost, I would like to thank my primary advisor, Dr. Romi Ghose. She was gracious enough to accept me under her wings even when she was fully aware that I had no prior research experience. I have learned practically everything about research during my time in her lab. I have enjoyed all my interactions with her and she has helped shape me up not only scientifically but on a personal level as well. She has helped inculcate a strong sense of professionalism in me which is going to be helpful to me throughout my career. Thank you Dr. Ghose for bearing me for 5 years. I will always cherish my time with you and I will always remember the time I spent here with great fondness.

I have been lucky to have been trained by Dr Diana Chow and Dr Ming Hu during my time here. Their lectures and course materials helped build a strong foundation which will be helpful to me during my entire career. I would also like to sincerely thank Dr Bhagavatula Moorthy and Dr Sundararajah Thevananther for being a part of my thesis committee. Their inputs during my interactions with

them and during my proposal defense helped shape up and optimize my study designs. Thank you all for always supporting me during my time here.

I would like to especially thank Dr Adarsh Gandhi, Dr Ozozoma Omoluabi and Tao Guo. I have learned all laboratory techniques from them and they were a constant source of support in the lab. The friendly and helpful environment they created in the lab was instrumental in my research. I would like to thank my current lab members Pankajini Mallick and Guncha Taneja for their help during my research projects. I would also like to thank my friends in Houston; Sumit, Zhen Lili, Kastubh, Nounou, Zhen, Fady, Renu, Sheetanshu, Nikesh, Ridham, Sushant, Sonal, Santosh, Sahil, Manas, Prajakta & Sameer for their support system which made me feel at home away from home. Thank you Tristan and Doris for their help in my time here.

Speaking of home, a special thanks to my beloved family, who although being thousands of miles away physically, was always present emotionally. I am and will always be really grateful to my late grandmother Bhanumati R Shah, who unfortunately will not see me graduate, grandfather (Rasisklal Shah), mother (Mrs. Charu Shah), father (Dr. Ashwin Shah) and my brother (Harit Shah) for their unwavering and unending support. Their blinding beliefs in me gave me the strength and courage to carry on when things seemed tough. I would also like to

thank my extended family; the families of Dr Pradip Shah, Vijay Shah and Dr Devendra Shah for their support.

Special thanks to my wife, Nikitha Nair. Although we have been married only for a few months, we have been together since the last 9 years. Her contribution in my success has been immense. She has been a pillar of support during my time in Houston and I am lucky to have her as my life partner and I look forward to my life with her. I would also like to thank my in-laws, Vinod Nair, Usha Nair and Nivisha Nair for their support.

The great Ralph Waldo Emerson once said “The glory of friendship is not the outstretched hand, nor the kindly smile, nor the joy of companionship; it's the spiritual inspiration that comes to one when he discovers that someone else believes in him and is willing to trust him with his friend.”. And last but definitely not the least special mention to Tanay Samant, whom I met here at the University of Houston. Thank you Kunal Desai and Prathamesh Malvankar for 20 years of your friendship and support. I will cherish your friendships forever.

Pranav Shah

University of Houston

TABLE OF CONTENTS

Acknowledgements.....iii

Abstract xvi

List of Tables xiv

List of figures.....xv

List of Abbreviations xxi

Chapter 1: General Introduction 1

1.1: Drug metabolism in the liver 2

1.2: Regulation of drug metabolism during infections and inflammation 4

1.2.1 Toll-like receptors (TLRs) 5

1.2.2 TLR-signaling in the liver.....8

1.2.3 Drug metabolism: Role of cytokines 12

1.2.4 Drug metabolism: Role of NF-κB.....15

1.2.5 Drug metabolism: Role of MAP kinases 17

1.2.6 Drug metabolism: Role of oxidative stress.....19

1.2.7 DMET down-regulation: Molecular mechanisms 21

1.2.7.1 Transcriptional regulation.....22

1.2.7.2 RNA degradation.....23

1.2.7.3 Protein synthesis and degradation.....24

1.2.7.4 Enzyme inhibition.....24

1.3 Nuclear receptors.....25

1.3.1 Location and activation of CAR & PXR.....27

1.3.2 PXR.....29

1.3.3 CAR.....30

1.3.4 Role of CAR AND PXR during inflammation.....31

1.3.5 Role of CAR and PXR in drug metabolism and toxicity.....33

1.4 DMET alterations during obesity.....35

Chapter 2: Hypotheses and Specific Aims 38

2.1. A Specific Aim 1A.....40

2.1. B Specific Aim 1B.....41

2.2: Specific Aim 2.....42

Chapter 3: Experimental Methods 45

3.1 Reagents used throughout the dissertation.....45

3.2 Animals.....45

3.3 Treatments.....46

3.4 RNA isolation and real-time PCR analysis.....47

3.5 Isolation of primary mouse hepatocytes 54

3.6 Hepatocyte culture 56

3.7 Hepatocyte treatments.....58

3.8 Alanine aminotransferase assay.....58

3.9 Perfusion of liver for activity assays.....59

3.10 Preparation of microsome and S9 fractions.....60

3.11 BCA assay for quantification of protein 62

3.12 Cyp3a11 activity assay 63

3.13 Preparation of nuclear and cytosolic extracts 64

3.14 Immunoblotting for protein analysis.....65

3.15 Collection of blood and plasma 68

3.16 Drug extraction from plasma samples 69

3.17 Pharmacokinetic studies, LC-MS/MS sample preparation and Analysis

.....69

3.18 Pharmacokinetic analysis.....71

Chapter 4: Role of TRIF-dependent signaling in regulation of gene expression of DMEs and transporters.....72

4.1 Abstract.....73

4.2 Introduction.....75

4.3 Materials and methods.....79

4.3.1 Materials 79

4.3.2 Animals.....79

4.3.3 Treatments.....80

4.3.4 real-time PCR.....80

4.3.5 Primary hepatocyte isolation and treatment.....81

4.3.6 Immunoblotting.....82

4.3.7 Statistical analysis.....82

4.4 Results.....83

4.4.1 Role of TRIF in down-regulation of gene expression of DME/transporters in LPS-treated mice.....83

4.4.2 Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following LPS-administration.....85

4.4.3 Regulation of gene expression of DME/transporters in poly I:C-treated $TRIF^{+/+}$ mice.....87

4.4.4 Activation of cytokines, nuclear receptors (NRs) and cell-signaling pathways by LPS and poly I:C.....90

4.4.5 Role of TRIF in down-regulation of gene expression of DMETs in poly I:C-treated $TRIF^{+/+}$and $TRIF^{-/-}$ mice.....94

4.4.6 Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice 96

4.4.7 Role of MAP kinases; JNK, ERK and p38 in TLR4 & TLR3 mediated DMET down-regulation.....98

4.5 Discussion.....101

Chapter 5: Role of constitutive androstane receptor in Toll-like receptor 2 & 4-mediated regulation of gene expression of hepatic drug metabolizing enzymes and transporters 106

5.1 Abstract 107

5.2 Introduction 109

5.3 Materials and methods 113

5.3.1 Materials 113

5.3.2 Animals and treatments 114

5.3.2 real-time PCR 114

5.3.3 Primary hepatocyte culture and treatments 115

5.3.4 Immunoblotting 116

5.3.5 Cyp3a11 enzyme activity 116

5.3.6 Liquid chromatography tandem mass spectroscopy analysis (LC-MS/MS) 117

5.3.7 Statistical analysis 118

5.4 Results 119

5.4.1 Effect of LTA treatment on DME/transporter gene expression in $CAR^{+/+}$ and $CAR^{-/-}$ mice 119

5.4.2: Effect of LPS treatment on DME/transporter gene expression in $CAR^{+/+}$ and $CAR^{-/-}$ mice 123

5.4.3 Effect of LTA or LPS treatment on DME/transporter gene expression in TCPOBOP-treated Mice 126

5.4.4 Effect of LTA or LPS treatment on DME/transporter gene expression in PB-treated hCAR mice.....132

5.4.5 Effect of LTA or LPS treatment on DME/transporter gene expression in primary hepatocytes from $CAR^{+/+}$ and $CAR^{-/-}$ mice.....135

5.4.6 Effect of LTA or LPS treatment on DME/Transporter gene expression in primary hepatocytes pre-treated with TCPOBOP.....138

5.4.7 Effect of LTA administration on TLR2 mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$).....141

5.4.9 Effect of LTA or LPS on Cyp3a11 activity in $CAR^{+/+}$ and $CAR^{-/-}$ mice

.....146

5.5 Discussion.....149

Chapter 6: Role of obesity in UGT-mediated glucuronidation of irinotecan metabolite.....156

6.1 Abstract.....157

6.2 Introduction.....159

6.3 Materials and methods.....163

6.3.1 Materials.....163

6.3.2 Animals.....163

6.3.3 Treatments.....164

6.3.4 Preparation of S9 fractions and Ugt1a1 activity assay.....164

6.3.5 Pharmacokinetic studies, LC-MC/MS sample preparation and analysis.....165

6.3.6 β-Glucuronidase assay.....166

6.3.6 real-time PCR.....167

6.3.7 Serum TNF-α assay.....167

6.3.8 Statistical analysis.....168

6.4 Results.....169

6.4.1 Ugt1a1 mediated glucuronidation of SN-38 169

6.4.2 Pharmacokinetic analysis of irinotecan and its metabolites 171

6.4.3 Effect of obesity on hepatic TNF-α mRNA expression and TNF-α concentration in plasma 174

6.4.4 β-glucuronidase activity assay 177

6.5 Discussion 179

BIBLIOGRAPHY 184

Abstract

Drug metabolism is affected in various diseases and altered physiological states such as cancer, cardiovascular diseases, diabetes, obesity, liver disorders, etc. The common factor underlying all these disease states is inflammation. Accumulating evidence has shown that inflammation can alter the gene expression and activity of several drug metabolizing enzymes and transporters (DMETs) however, the exact mechanism is unknown. Although well established studies have attributed the down-regulation of DMET activities and protein expression to the reduction in their gene expression levels, further research is needed to establish the clinical relevance of this down-regulation. Furthermore, clinical relevance of down-regulation of DMETs during inflammation or pathophysiological conditions needs to be ascertained. Our ***overall aim*** is to determine molecular mechanisms that regulate gene expression of DMETs during inflammation and ascertain the clinical relevance of this down-regulation in a mouse model of obesity. Inflammatory responses in the liver are primarily mediated by specific trans-membrane receptors known as Toll-like receptors (TLRs). These receptors are key components of the innate immune response and are present on immune cells as well as hepatocytes in the liver. Out of the 13 known TLRs, we selected TLR2, TLR3 and TLR4 (activated by gram-positive bacterial components, viral components and gram-negative bacterial components, respectively) as they play major roles in immune reactions elicited during various pathophysiological

conditions. TLR signaling is mediated by adaptor proteins Toll/interleukin (IL)1 receptor (TIR) domain containing adaptor protein (TIRAP) and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF). We have shown that activation of TLR2 by lipoteichoic acid (LTA) and TLR4 by lipopolysaccharide (LPS) caused down-regulation of gene expression and activity of key hepatic DMETs. This down-regulation was associated with reduced expression of the nuclear receptors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR), which heterodimerize with the central nuclear receptor, retinoid x receptor alpha (RXRα). Our central hypothesis is that drug metabolism is altered by activation of innate immune components and in obesity. We propose to achieve our goals by pursuing the following Specific Aims: **Aim 1:** To determine the role of the adaptor protein, TRIF down-regulating the gene expression of DMEs and transporters. We hypothesize that the adaptor protein TRIF mediates the down-regulation of DMETs through TLR4 and TLR3. We observed that down-regulation of DMETs on activation of TLR4 and TLR3 is independent of the adaptor protein TRIF. We also found that the MAP kinase, c-Jun-N-terminal kinase (JNK) is involved in mediating the down-regulation of DMET through TLR3 & TLR4. **Aim 2:** To determine the role of nuclear receptor CAR in TLR2- & 4-mediated down-regulation of DMEs and transporters. We hypothesize that CAR mediates the down-regulation of DMEs and transporters through TLR2 and TLR4. CAR was shown to play a differential role in regulating gene expression

of DMETs in TLR2 and TLR4 induced inflammation in mice. We found that CAR is involved in down-regulation of hepatic DMET genes through TLR2, but not through TLR4.

Aim 3: We have shown that gene expression of uridine glucuronosyl transferase (Ugt) 1a1 enzyme is reduced during obesity. Our goal is to determine the role of obesity in Ugt-mediated glucuronidation of irinotecan metabolite. We hypothesize that obesity induced-alteration in Ugt enzymes leads to accumulation of the active and toxic metabolite of irinotecan i.e. SN-38. We found that the rate of formation of SN-38 Glucuronide (SN-38G) was 2-fold lower in the DIO mice compared to the lean controls. Plasma exposure of SN-38 increased by 2-folds and that of SN-38G decreased significantly in the DIO mice compared to the lean controls. Thus, reduction in Ugt1a expression and activity in DIO mice contributes to accumulation of SN-38 which could be the potential reason for irinotecan-induced liver toxicity during obesity.

Since TLRs are activated in a variety of pathophysiological conditions, this study will have a significant impact on drug development. The outcomes of this proposed study will provide important information regarding the signaling pathways that govern drug metabolism and hepatotoxicity of clinically relevant drugs at therapeutic doses during pathophysiological conditions.

LIST OF TABLES

Table 1.1 Human nuclear receptor superfamily.....27

Table 2.1: Probe and primer sequences of DMET/cytokine/NR genes.....51

Table 5.4.1 Enzyme kinetic parameters of MDZ.....148

Table 6.4.1: Pharmacokinetic parameters of irinotecan and its metabolites..173

LIST OF FIGURES

Fig 1.1: TLRs and their ligands 6

Fig 1.2: TLR signaling pathways 11

Fig 1.3: Activation of Nuclear Receptors 28

Fig 2.1.A: Regulation of gene expression of DMET by TLR signaling 40

Fig 2.1.B: Role of nuclear receptors in regulating drug metabolism during inflammation 41

Fig 2.2: Regulation of CPT-11 toxicity in diet-induced obese mice 43

Fig 4.4.1: Regulation of DME mRNA levels in $TRIF^{+/+}$ and $TRIF^{-/-}$ mice following LPS administration 84

Fig 4.4.2: Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following LPS administration 86

Fig 4.4.3: Regulation of DMET mRNA levels in C57BL/6 mice on poly I:C administration 88

Fig 4.4.4.1: Regulation of cytokines on activation of TLR3 91

Fig 4.4.4.2: Regulation of nuclear receptors by poly I:C.....92

Fig 4.4.2: Regulation of cell-signaling pathways by poly I:C 93

Fig 4.4.5: Regulation of DMET mRNA levels in $TRIF^{+/+}$ and $TRIF^{-/-}$ mice following poly I:C administration.....95

Fig 4.4.6: Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following poly I:C administration.....97

Fig 4.4.7.1 & 4.4.7.2: Regulation of DMEs by MAP kinases on LPS or poly I:C treatments 99

Fig 5.4.1: Regulation of DME and transporter mRNA levels in $CAR^{+/+}$ and $CAR^{-/-}$ mice following LTA administration. 121

Fig 5.4.2: Regulation of DME and transporter mRNA levels in $CAR^{+/+}$ and $CAR^{-/-}$ mice following LPS administration. 124

Fig 5.4.3.1: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LTA administration. 128

Fig 5.4.3.2: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LPS administration. 130

5.4.4: Effect of LTA or LPS treatment on DME/transporter gene expression in PB-treated hCAR mice 133

Fig 5.4.5: Regulation of DME and transporter gene expression by LTA or LPS in primary hepatocytes from $CAR^{+/+}$ and $CAR^{-/-}$ mice.....136

Fig 5.4.6: Regulation of DME and transporter gene expression by LTA in TCPOBOP pre-treated primary hepatocytes. 139

Fig 5.4.7: Regulation of TLR2 mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$) following LTA administration. 142

Fig 5.4.8: Regulation of cytokine mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$) following LTA administration. 144

Figure 5.4.9: Regulation of Cyp3a11 activity in $CAR^{+/+}$ and $CAR^{-/-}$ mice ...147

Fig 6.1: Metabolic pathway of irinotecan 160

Fig 6.4.1: Ugt1a1 mediated glucuronidation of SN-38 in DIO and lean mice

.....170

Fig 6.4.2: PK profiles of irinotecan, SN-38 and SN-38G 172

Fig 6.4.3.1: Regulation of TNF-α mRNA levels in Lean and DIO mice following irinotecan administration 175

6.4.3.2: Regulation of TNF-α plasma levels in Lean and DIO mice following irinotecan administration 176

Fig 6.4.4: β-glucuronidase activity in lea**n** and DIO mice.....178

List of Abbreviations

ALT	Alanine Aminotransferase
APR	Acute phase response
AUC	Area under curve
BCA	Bicinchoninic acid
BCG	Bacillus Calmette–Guérin
BSA	Bovine serum albumin
Bsep	Bile salt exporter pump
CAR	Constitutive androstane receptor
CARDs	Contain caspase-recruiting domains
CCRP	CAR Cytoplasmic Retention Protein
CDKs	Cyclin-dependent kinases
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4 dichlorobenzyl)oxime

CL	Cleared from body
Cmax	Maximum plasma concentration
CPT	Camptothecin
CPT11	Irinotecan
CPT11-HCl	Irinotecan hydrochloride
CYP450	Cytochrome P450
DBD	DNA binding domain
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DIO	Diet induced mice
DMETs	Drug metabolizing enzymes and transporters
ds	Double-stranded
DTT	Dithioerythritol
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase

ERK	Extracellular related protein kinase
FBS	Fetal bovine serum
GR	Glucocorticoid receptor
HCV	Hepatitis C virus
HSP90	Heat Shock Protein 90
IKK	IκB kinase
IL	Interleukin
IP	Intra peritoneal
IPS-1	IFN-β promoter stimulator
IRFs	IFN regulatory factors
IS	Internal standard
ITS	Insulin-transferrin-sodium selenite
IVC	Inferior vena cava
JNK	c-Jun-N-terminal kinase
K_e	Elimination rate constant

LBD	Ligand binding domain
LBP	LPS-binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MDZ	Midazolam
Mrp	Multi drug resistance-associated protein
MyD88	Myeloid differentiation factor 88
NO	Nitric oxide
Ntcp	Sodium taurocholate co-transporting polypeptide
PAMPs	Pathogen-associated molecular
PB	Phenobarbital
PBREM	Phenobarbital responsive enhancer modules
P-JNK	Phosphorylated JNK
PK	Pharmacokinetic

poly I:C	Polyinosinic:polycytidylic acid
PV	Portal vein
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RXR	Retinoid-X-receptor\
RXRα	Retinoid X receptor alpha
SN-38	7-ethyl-10-hydroxycamptotheci
SN38G	SN-38 Glucuronide
ss	Single-stranded
Stat	Signal transduction and activation of transcription
$t_{1/2}$	Half- life
TCPOBOP	1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene
TCR	T-cell receptor
TIRAP	Toll-interleukin receptor domain containing adaptor protein
TLRs	Toll-like receptors

TNF	Tumour-necrosis factor
TRAM	TRIF-related adaptor molecule
Ugt	Uridine glucuronosyl transferase

CHAPTER 1

General Introduction

1.1: Drug metabolism in the liver:

The liver is the biggest internal organ in the body. It is reddish brown in color and has four lobes of unequal size and shape which weighs around 3-3.5 lbs (Cotran et al., 2005). It is connected to the hepatic artery and the portal vein. The hepatic artery carries blood from the heart, whereas the portal vein carries blood containing digested nutrients from the gastrointestinal tract as well as the spleen and pancreas. These blood vessels subdivide into capillaries, which then lead to a lobule. Each lobule is made up of millions of hepatic cells which are the basic metabolic cells. Lobules are the functional units of the liver (Cotran et al., 2005). More than 500 functions have been identified and some of the important functions of the liver include metabolism of a wide variety of endogenous and exogenous compounds, production of bile, cholesterol and plasma proteins, regulation of blood clotting, protection of the body against infection by clearing bacteria from the blood stream, conversion of glucose to glycogen for storage, etc (Robbins and Cotran., 2005). The liver also plays an important role in illiciting an inflammatory response to infection, trauma, foreign invasions or other possible causes of tissue damage. Of all these, one of the most important functions of the liver is the metabolism of xenobiotics and endogenous compounds by the help of enzymatic systems. More often than not, metabolism converts highly lipophilic compounds into readily excreted polar compounds. Drug metabolism or detoxification is divided into two phases. Phase I metabolism can lead to either activation or

deactivation of a drug where as Phase II metabolism mainly leads to detoxification of drugs (Guengerich FP., 1999; Jakoby et al., 1990).

The major enzymatic system responsible for metabolism of xenobiotics is the cytochrome P450 (CYP450) isoenzyme family (Nebert et al., 2002). CYP450s are responsible for more than 75% of drug metabolism and the major isoform amongst these enzymes is CYP3A4 which is responsible for metabolism of ~50% of known xenobiotics in humans (Guengerich FP, 1999; 2008). Inflammation has been long associated with suppression of expression and activity of CYP450s. Since CYP450s are the major enzymes responsible for drug metabolism, changes in expression and activity will have a major impact on drug metabolism and clearance. In order to study the roles of different drug metabolizing enzymes, microsomes or S9 fractions are used. Microsomes are vesicle-like artifacts re-formed from pieces of the endoplasmic reticulum (ER) when cells are broken up (Voet et al., 2004). S9 fractions contain the cytosol, mitochondria and ER fractions. On higher centrifugation, only the ER fractions will sediment out as pellets. While CYP450 and UGT family of enzymes are almost exclusively localized in the ER (Wheeler et al., 2001; Pandya et al., 2003), others are located in cytosol e.g., alcohol dehydrogenase (Crabb et al., 2004), smooth ER e.g ketone-reductase (Bohren et al., 1987), mitochondria e.g. monoamine oxidase (Slotkin et al., 1999). Sulfotransferase enzymes are found only in cytosol (Negishi et al., 2001).

1.2: Regulation of drug metabolism during infections and inflammation:

Infection, Inflammation or trauma induces a pathophysiological condition called as the acute phase response (APR) (Christou et al., 1989). Induction of the APR by LPS or pro-inflammatory cytokine administration is associated with the suppression of CYP450 enzyme expression and activity in the liver and dramatic alterations in lipid and lipoprotein metabolism (Hardardóttir et al., 1992).

One of the earliest incidences of reduced clearance of drugs during infections was reported by Gray et al., 1983 who showed that theophylline clearance was reduced during Bacillus Calmette–Guérin (BCG) infections. Antipytine clearance was found to be reduced during pneumonia infections (Sonne et al., 1985). Induction of inflammation by administration of endotoxin to healthy human volunteers reduced the clearance of theophylline, antipyrine and hexobarbitone (Shedlofsky et al., 1994; 1997). Turpentine-induced inflammation is another well known model of inflammation. Plasma levels of gimatecan, an anti-cancer drug were 2-fold higher in mice pre-treated with turpentine (Frapolli et al., 2010).

PK of levofloxacin and fluconazole was found to be altered in patients with HIV infections (Goodwin et al., 1994; Tett et al., 1995). Decreased exposure of amprenavir, an anti-retroviral drug was found in patients with hepatitis C virus (HCV)-infected patients (Gatti et al., 2009). There have been several cases of

altered drug metabolism during bacterial and viral infections however; the exact mechanism remains to be elucidated.

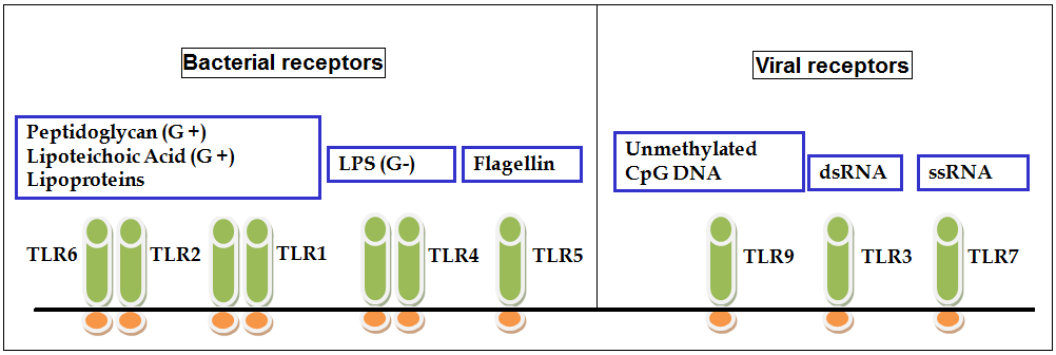
1.2.1: Toll-like receptors (TLRs):

Toll like receptors (TLRs) are the major mediators of inflammatory responses in the liver. TLRs facilitate innate immune responses for the initial host defense against microorganisms. TLRs are widely expressed on immune cells and recognize distinct microorganism products as pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006).

The TLR family is among the best-characterized pattern recognition receptor families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (Medzhitov et al., 1997; Akira et al., 2003). TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1Receptor (TIR) domain. Ten different TLRs have been identified in humans and 13 in mice. Different TLRs recognize different molecular patterns of microorganisms and endogenous components (Fig 1.1).

TLR2 can sense various components from bacteria, mycoplasma, fungi, and viruses. Lipoteichoic acid (LTA), a component of the cell wall of gram-positive

Fig 1.1: TLRs and their ligands (Adapted from Lee et al., 2012)



bacteria activates TLR2 (Takeuchi et al., 1999; Aliprantis et al., 1999). TLR2 recognizes its ligands by forming heterodimers with either TLR1 or TLR6. The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize distinct ligands (triacyl and diacyl lipoproteins, respectively). TLR4 recognizes lipopolysaccharide (LPS) on the cell surface (Tapping et al., 2000) with myeloid differentiation factor 2 (MD2). TLR4 requires the association with LPS-binding protein (LBP) & CD14 in addition to MD2 to recognize LPS. LPS is a component derived from the outer membrane of gram-negative bacteria and is known to elicit strong immune responses in animals. TLR5 is highly expressed by dendritic cells (DCs) of the lamina propria in the small intestine and it recognizes flagellin from flagellated bacteria (Means et al., 2003). TLR11 is present in mice but not in humans and shows close homology to TLR5. TLR11 recognizes uropathogenic bacteria and a profilin-like molecule derived from the intracellular protozoan *Toxoplasma gondii* (Yarovinsky et al., 2005).

A set of TLRs, comprising TLR3, TLR7, TLR8, and TLR9, recognize nucleic acids derived from viruses and bacteria, as well as endogenous nucleic acids. TLR3 detects viral double-stranded (ds) RNA in the endolysosome. TLR3 is involved in the recognition of polyinosinic polycytidylic acid (poly I:C), a synthetic dsRNA analog (Alexopoulou et al, 2001). Mouse TLR7 and human TLR7/8 recognize single-stranded (ss) RNAs from RNA viruses, as well as small purine analog compounds (imidazoquinolines) (Hattermann et al., 2007). TLR7 also detects RNAs from bacteria such as Group B Streptococcus in endolysosomes in conventional DCs (Mancuso et al., 2009).

TLR9 senses unmethylated DNA with CpG motifs derived from bacteria and viruses. In addition to DNA, TLR9 also recognizes hemozoin, a crystalline metabolite of hemoglobin produced by the malaria parasite. TLR9 directly binds to hemozoin, and a crude extract of the malaria parasite elicits parasite-antigen-specific immune responses via TLR9 (Coban et al., 2005, 2010). This report though is controversial.

A recent report showed that, immune complexes consisting of Immunoglobulin G bound to mammalian chromatin have been shown to activate TLR9 (Leadbetter et al., 2002). Given that endogenous ligands are potent TLR ligands and may facilitate autoimmunity (Barrat et al., 2008), TLRs that recognize endogenous ligands are compartmentalized to avoid unwanted activation. Although TLR-

mediated microbial recognition is very important for host defense against pathogens, excess responses to TLR ligands may induce lethal septic shock syndrome. Thus, appropriate activation of TLRs is vital for eradicating invading pathogens without causing any harmful damage to the host.

1.2.2: TLR-signaling in the liver:

In the liver, TLRs are present on the cell surface of various immune cells such as Kupffer cells, hepatic stellate cells, biliary epithelial cells, liver sinusoidal endothelial cells and hepatic dendritic cells as well as on the hepatocytes (Scott et al., 2009; Seki and Brenner, 2008). Upon the recognition of PAMPs, TLRs recruit adaptor proteins such as myeloid differentiation factor 88 (MyD88), Toll/interleukin (IL)1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing interferon (IFN)-β (TRIF), and TRIF-related adaptor molecule (TRAM). In spite of chronic exposure to a high load of bacterial products, the normal liver shows no activation of TLR-signaling pathways. However, under pathologic conditions, TLRs promote pro-inflammatory signaling such as induction of nuclear factor-κB, c-Jun-N-terminal kinase (JNK), p38 and induction of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, TNF-α and IFNs in the immune cells in the liver and regulate antiviral/antibacterial responses, hepatic injury, and wound healing (Fig 1.2).

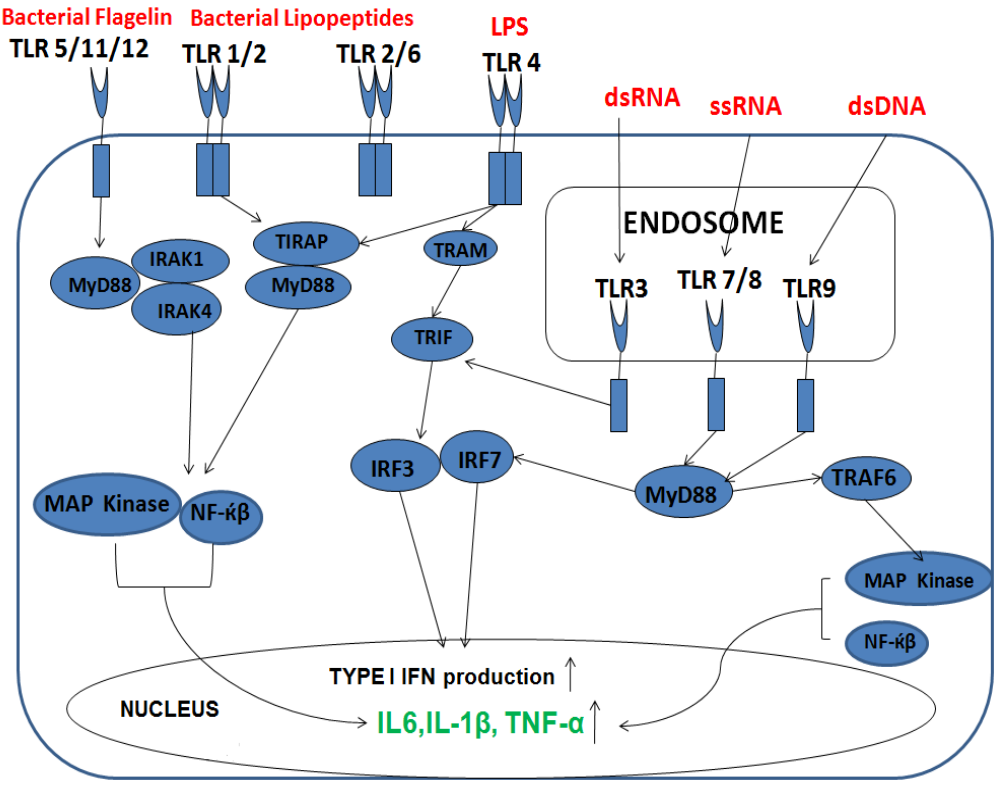
The TLR-signaling pathway shows remarkable similarity to the IL-1 receptor signaling pathway with which it shares many components including highly conserved cytoplasmic TIR domains and several intracellular adapter molecules (Schwabe et al., 2006). Despite divergent PAMP ligands, most TLRs including TLR1, 2, 4, 5, 6, 7 and 9, share a common signaling pathway via the adaptor molecule MyD88. Reports have shown MyD88 is a crucial adapter in all TLR receptor signaling pathways except for TLR3. This has led to the classifying of downstream signaling pathways as MyD88-dependent and MyD88-independent. Apart from the above mentioned TLRs, MyD88 interacts with IL-1 and IL-18 receptors as well. MyD88 has an N-terminal death domain and a C-terminal TIR domain through which it interacts with the TIR domains of TLRs or IL-1 and IL-18 receptors (Ohnishi et al., 2009).

The adapter molecule TIRAP also called Mal (MyD88 adapter like) associates with MyD88 and is required for MyD88-dependent TLR2 and TLR4 signaling. TIRAP has a C-terminal TIR domain but lacks an N-terminal death domain. $TIRAP^{-/-}$ mice have impaired responses upon activation of TLR4 and TLR2 (Akira et al., 2003; Horng et al., 2002). The adapter molecule TRIF mediates TLR3 and TLR4 signaling. TRIF is important for induction of interferon-regulating factor 3, production of INF-β and also for activation and maturation of dendritic cells (Yamamato et al., 2003). TRIF is involved in mediating NF-κB activation in response to TLR3 ligands and is involved in the MyD88-independent

prolonged NF-κB activation in response to TLR4 ligands (Yamamato et al., 2003). There is direct interaction between TLR3 and TRIF but the interaction between TRIF and TLR4 is mediated by TRAM (Fig 1.2). TRAM-deficient mice show impaired responses on activation of TLR4 but show normal responses on activation of TLR2, 3, 7 and 9 (Yamamato et al., 2003).

We have shown that activation of TLR2 and TLR4 by bacterial components leads to down-regulation of expression and activity of DMETs (Ghose et al, 2008, 2009; Gandhi et al., 2012). We have recently shown that regulation of DMEs through TLR4 is independent of TIRAP (Ghose et al., 2008). While the role of bacterial TLRs has been studied, there is little information about the role of viral TLRs in regulating expression and activities of DMETs. It is becoming increasingly evident that the down-regulation of DMEs during inflammation is mediated by the TLRs and hence attaining knowledge about different TLR signaling pathways is crucial.

Fig 1.2: TLR signaling pathways



1.2.3: Drug metabolism: Role of cytokines:

Kupffer cells are specialized macrophages located in the liver, lining the walls of the sinusoids. They are the principal liver cells for phagocytosis, antigen presentation and the production of pro-inflammatory mediators like cytokines, nitric oxide, prostanoids etc. The anatomical link between the liver and the intestine make kupffer cells the primary cells that are exposed to gut-derived toxins (Mathison et al., 1979; Schwabe et al., 2006). Kupffer cells express TLR4 and respond to LPS stimulation (H. Van Bossuyt et al., 1988). On stimulation by LPS, Kupffer cells produce TNF-α, IL-1β, IL-6, IL-12, IL-18 and several chemokines. IL-12 and IL-18 then activate hepatic NK cells to produce a cytokine IFN-γ, which has an important role in hepatic wound healing and microbial eradication (Wysocka et al., 1995). Since Kupffer cells are constantly exposed to LPS, they do not respond to low levels of LPS i.e. they have acquired tolerance for LPS. Kupffer cells isolated from fresh human liver also secrete IL-10, an anti-inflammatory cytokine which negatively regulates the pro-inflammatory cytokines like IL-6 and TNF-α after LPS stimulation (Knolle et al., 1995).

We have shown that activation of TLR2 and TLR4 by bacterial components leads to down-regulation of expression and activity of DMETs (Ghose et al, 2008, 2009; Gandhi et al., 2012). It is generally thought that this down-regulation during inflammation is because of the induction of pro-inflammatory cytokines because

cytokines administered *in vivo* or *in vitro* have mimicked selective effects on P450 expression (Muntane-Relat et al., 1995; Morgan, 1997). TNF-α , IL-1β & IL-6-treatment in primary human hepatocytes led to down-regulation of mRNA and activities of CYP1A2, CYP2C, CYP2E1, CYP3A where as treatment with IFN-γ led to down-regulation of expression and activities of CYP1A2 and CYP2E1 (Abdel-Razzak et al., 1993). IFN-α treatment led to down-regulation of gene expression of CYP2C11 in rat hepatocytes (Chen et al., 1995). IL-1β-treatment was also shown to decrease the mRNA expression of glutathione-S-transferases in a primary rat hepatocyte culture (Maheo et al., 1997). Additionally, sodium taurocholate co-transporting polypeptide (Ntcp) and Multi drug resistant protein (Mrp2) were also down-regulated in primary rat hepatocytes exposed to IL-1β (Denson et al., 2000).

IL-6-treatment in mice led to significant decreases in the mRNA expression and protein expression of bile salt exporter pump (bsep), Mrp2, and Cyp3a11 in mice (Teng-Piquette Miller et al., 2005). Studies have also investigated the role of individual cytokines in altering expression of DMETs by using gene knockout mice or receptor knockout mice. LPS-administration led to down-regulation of Cyp1a, 2b, 3a protein and activities in wild-type as well as TNF-α-receptor deficient mice (Warren et al., 1999). LPS-treatment led to suppression of Cyp1a2, Cyp2e1 and Cyp3a11 mRNA expression in wild-type and IL-6 gene knockout

mice (Siewert et al., 2000). This could point towards functional redundancy of the cytokines in altering Cyp450s.

We and others have shown that TLRs are present on the hepatocytes themselves (Ferrari et al., 2001; Ghose et al., 2011). We have also shown that LPS or LTA treatment of primary mouse hepatocytes can directly affect the DMEs via TLRs present on the hepatocytes themselves indicating that cytokines may not be the only players involved in down-regulation of DMEs during bacterial infections (Ghose et al., 2011). Hepatocytes make up bulk of the liver's cytoplasmic mass. These cells are involved in protein synthesis, protein storage, synthesis of cholesterol, bile salts and phospholipids, detoxification and excretion of exogenous and endogenous compounds and formation and secretion of bile. They are important mediators of the acute phase response. The hepatocytes express mRNA for all TLRs but they have very low expression of TLR2, TLR3, TLR4 and TLR5. TLR2 and TLR4 weakly respond to their ligands in the hepatocytes. LPS, TNF-α, IL-1, IL-6 induce the expression of TLR2 which insinuates that hepatocytes are more responsive to TLR2 ligands during inflammation. TLR4 expression is induced by LPS, but not induced by the above mentioned pro-inflammatory mediators (Matsamura et al., 2000).

While LPS-induced inflammation leads to release of several cytokines like IL-6, IL-1β and TNF-α, turpentine-induced inflammation leads to induction of only IL-

6 and IL-6 gene deficiency blocks the suppression of Cyp1a2, 2a5 and 3a11 mRNA expression (Siewert et al., 2000). This indicates that the effects of inflammation on drug metabolism are stimulus dependant.

1.2.4: Drug metabolism: Role of NF-κB:

NF-κB regulates the expression of cytokines, chemokines, growth factors, immunoregulatory molecules, cell adhesion molecules, acute-phase response proteins, stress response genes, cell surface receptors, regulators of apoptosis, viruses, enzymes and others (Kumar et al., 2004). Thus NF-κB has broad physiological functions apart from regulating innate as well as adaptive immune systems. Aberrant activation of the NF-κB pathway is involved in the pathogenesis of a number of human diseases including those related to inflammation, enhanced cellular proliferation, viral infection, and genetic diseases (Kumar et al., 2004).

NF-κB activity can be regulated by the direct modification of NF-κB proteins through phosphorylation and acetylation. Generally, the regulatory proteins IκB retain NF-κB proteins in the cytoplasm as an inactive form. IκBα, IκBβ and IκBε are the most common IκB forms (Hayden et al., 2004; Viatour et al., 2005). The degradation of IκB is mediated by phosphorylation at its specific serine residues

at N-terminus by IκB kinase (IKK) complex. IκBα is degraded rapidly in response to stimuli and quickly resynthesized because of NF-κB activation (Hayden et al., 2004). NF-κB is activated rapidly in response to a wide range of stimuli, including pathogens, stress signals and pro-inflammatory cytokines, such as tumour-necrosis factor (TNF) and IL-1. NF-κB activity is stimulated by many pathways, including lipopolysaccharide (LPS), tumour-necrosis factor (TNF) and T-cell receptor (TCR) signalling.

NF-κB activation has been shown in conditions like oxidative stress and inflammatory conditions both of which lead to the down-regulation of DMETs (Kumar et al., 2004; Morgan et al., 2002). Thus it is likely that NF-κB may be involved in regulating the expression and activity of DMETs during inflammation. It was recently seen that NF-κB signaling pathways cross-talk with nuclear receptors which regulate important DMETs (De Bosscher et al., 2006). NF-κB binding sites have been identified in the promoter regions of some DMEs (Morgan et al., 2002). It has been shown that CAR expression was reduced after stimulation with IL-6 or LPS both are known activators of NF-κB (Pascussi et al., 2000; Beigneux et al., 2002). It has been shown that NF-κB inhibits the binding of the PXR-RXRα heterodimer to the binding site of CYP3A4 promoter region (Gu et al., 2006). There is mutual repression between NF-κB and PXR signaling pathways; however, the exact mechanism is unknown. NF-κB inhibits the

expression of RXRα which is a common heterodimerizing partner for CAR and PXR (Na et al., 1999).

NF-κB binding to the NF-κB binding response element on the promoter region of CYP2D5 led to down-regulation of CYP2D5 expression (Abdulla et al., 2005). NF-κB is also thought to be involved directly in regulating the expression of CYP2E1 and CYP3A7 (Peng et al., 2000; Nakamura et al., 2001). More research is needed to understand the full picture about the role of NF-κB in regulation of DMET levels.

1.2.5: Drug metabolism: Role of MAP kinases:

Mitogen-activated protein kinases also known as MAP kinases are serine/threonine/tyrosine-specific protein kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) kinase group (Han et al., 2002). The closest relatives of MAPKs are the cyclin-dependent kinases (CDKs). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis among many others (Houliston et al., 2001). MAP kinases are found in eukaryotes only, but they are fairly diverse and encountered in all animals, fungi and plants, and even in an array of unicellular eukaryotes.

The MAPK signaling pathways generally refers to a family of signaling cascades, which consist of the extracellular signal regulated kinase (ERK 1/2), Jun N-terminal kinase (JNK) and p38 kinase pathways (Zhou et al., 2009). These cell-signaling components are involved in regulation of DMEs and transporters by modulating the activity of some of nuclear receptors (Ghose et al, 2004, 2008; D Li, 2002). JNK and p38 on activation, lead to activation of transcription factor AP-1 (Davis et al., 2000). IL-1 is known to regulate the activity of a variety of target genes and transcription factors through several signal transduction cascades including JNK, ERK, and p38 MAPK (Saklatvala et al., 1999; Schoemaker et al., 1997).

A key component in the TLR-signaling pathway is JNK. JNK exists in 3 distinct isoforms (JNK1-3) and is expressed in various tissues such as liver, heart & brain (Ip and Davis, 1998). JNK is known to be activated by TNF-α or UV radiation (Whitmarsh and Davis, 1996). JNK displays two distinct activation profiles: early/transient and late/sustained (Guo et al., 1998; Roulston et al., 1998). While transient activation of JNK was shown to be involved in cell survival, sustained activation leads to apoptosis. We have also shown that sustained activation of JNK could be responsible for the idiosyncratic toxicity of chlorpromazine (Gandhi et al., 2013). It has been shown that IL-1β regulates the activity of Ntcp through a JNK-dependant mechanism (Li et al., 2002). IL-1β was also shown to

reduce RXRα levels in the nucleus through a JNK-dependant mechanism (Zimmermann et al., 2006).

One of the mechanisms through which NF-κB promotes survival signals in TNF-α -induced cell death is the inhibition of the prolonged activation of JNK (Schwabe et al., 2006). JNK signaling is an important mediator of apoptosis in the liver during embryogenesis and suppression of this signaling is a key mechanism by which NF-κB promotes survival during liver development (Chang et al., 2006). Like NF-κB, p38 also suppresses JNK activation (Sakurai et al., 2008; Heinrichhsdorff et al., 2008).

All these studies indicate that the role of JNK may be central in mediating responses during inflammation. JNK1 and JNK2 are abundantly present in the liver however, JNK1/JNK2 double knockout mice are lethal and JNK1 and JNK 2 show redundancy in mediating signaling during inflammation. There are no well known *in vivo* inhibitors of JNK and hence, it is difficult to elucidate the role of JNK in signaling *in vivo.*

1.2.6: Drug metabolism: Role of oxidative stress:

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. ROS are formed as a natural byproduct of the normal metabolism of

oxygen and have important roles in cell signaling and homeostasis (Devasagayam et al., 2004). However, during times of environmental stress (e.g., heat exposure, U.V radiation or ionizing radiation), ROS levels can increase dramatically and this may result in significant damage to cell structures. This phenomenon is called as oxidative stress (Devasagayam et al., 2004).

ROS are known to modulate the expression of several genes including pro-inflammatory cytokines. ROS have also been recently implicated in activating JNK and p38 pathways as well (Ichijo et al., 1997; Allen et al., 1997). It has been shown recently that activation of TLR4 by LPS leads to induction of proinflammatory cytokines as well as ROS such as O^2 and nitric oxide (NO) (Tran-Thi et al., 1995; Hida et al., 2003). Elevated levels of O^2 and nitric oxide are known to activate NF-κB and pro-inflammatory cytokines (Bautista et al., 1990). As mentioned earlier, individual cytokine receptor knockout mice did not have any attenuation on the effects of LPS. This led to suggest that ROS may have an important role in inflammation-mediated effects.

It has been shown by Xu et al., 2005, that the effect of LPS on down-regulation of Cyp3a gene expression is partly mediated by ROS. In another study, LPS-treatment was shown to down-regulate the gene expression of Cyp3a11 in placenta. Administration of an anti-oxidant before treatment with LPS led to attenuation of this down-regulation suggesting that ROS are involved in LPS-

mediated down-regulation of DMETs (Chen et al., 2005). Oxidative stress has also been shown to play an important role in structural, functional and inflammatory responses in an acute lung injury model (Trajano et al., 2011).

Thus, targeting ROS by use of anti-oxidants might have benefial effects during inflammation-mediated down-regulation of DMETs.

1.2.7: DMET down-regulation: Molecular mechanisms

The effects of inflammation on CYP450 levels in liver and cultured primary hepatocytes can be attributed to decreases in the levels of specific CYP450 mRNAs (Morgan, 1997). As mentioned earlier, different cytokines down-regulate different DMETs both *in vivo* and *in vitro* which implies that different mechanisms exist for different cytokines. The decreases in mRNA due to cytokines have been thought to be due to decreased transcription because decrease in the protein levels is significantly slower. Hence, it is thought that the down-regulation of mRNA is responsible for the reduction in protein levels of DMETs. However, one cannot rule out the possibility of reduced DMET translation and/or mRNA/protein stability.

As described above, many studies have shown the reduction of protein levels of specific forms of DMEs both *in vivo* and *in vitro*, and in most cases, suppression

of the mRNAs encoding these enzymes precedes the protein loss. The fact that the magnitudes of the observed decreases in transcription are usually sufficient to account for the decreases in protein and that the mRNA effects can often be shown to precede those on the protein, suggests that the primary mechanism of regulation is transcriptional. However, there are several cases which show that protein turnover and mRNA degradation contribute to this down-regulation.

1.2.7.1: Transcriptional regulation

Using a nuclear run-on assay, Wright et al., 1990 showed that the down-regulation of CYP2C11 mRNA at 24 h after LPS or turpentine treatments in male rats is transcriptional. However, in female rats, the transcription of CYP2C11 was suppressed much lesser than the actual mRNA levels indicating that posttranscriptional mechanisms may be involved (Wright et al., 1990).

Control of negative or positive transcription of genes is achieved by interaction of regulatory proteins with specific DNA sequences on the regulated genes. It has been shown that IL-1 and TNF-α stimulate transcription of several genes by activation of NF-κB (L. A. J. O'Neill., 1995). It has also been shown that IL-6, IFNγ and IFNα regulate transcription of genes through activators of proteins of the signal transduction and activation of transcription (stat) family. When a ligand

binds to IL-6 or IFN receptor, it activated tyrosine kinases of the Janus family and subsequently leads to phosphorylation of stat protein subunits. These stat protein subunits homodimerize or heterodimerize to become active transcription factors (Schindler et al., 1995). AP-1 or IFN regulatory factors (IRFs) are other cytokine activated factors that could modulate the expression of DMETs (L. A. J. O'Neill., 1995; Schindler et al., 1995).

1.2.7.2: RNA degradation

In matrigel cultured rat hepatocytes, the mRNA for CYP2C12 was found to have a half-life of ~10 h (Tollet et al., 1990). Morgan et al., 1989 found that there was ~95% suppression of CYP2C12 mRNA on treatment of rat hepatocytes with LPS indicating the presence of posttranscriptional mechanisms. Tollet et al., 1990 also showed that there is maximal reduction of CYP2E1, CYP2C11 and CYP3A2 mRNAs within 6 h in LPS-treated rats further indicating the presence of posttranscriptional mechanisms. However, it should be clearly understood that degradation of mRNA is not a universal effect during inflammation. It was shown that turpentine treatment in rats, mRNA degradation is inhibited and poly I:C treatment in mice leads to increased mRNA content (Saadane et al., 1996).

1.2.7.3: Protein synthesis and degradation

The first incidence of reduction of hepatic microsomal CYP450 due to increased degradation was seen on treatment with IFN inducers. Another study by Renton et al., 1997 showed that treatment of mice with Poly I:C led to decrease in protein synthesis of CYP450s. It was later explained that part of the reduction in protein was due to reduction of mRNA levels (Anari et al., 1994; Sakai et al., 1992). Rats that were treated with LPS had parallel reductions in mRNA and protein levels of CYP2C12 (Morgan et al., 1989). This rapid change in protein cannot be accounted only by mRNA changes and it indicates degradation of the protein itself as a possible mechanism (Morgan et al., 1989). In another study, it was found that poly I:C treatment led to degradation of microsomal protein for the first 10 h and thereafter, it was inhibited (Gooderham et al., 1990).

1.2.7.4: Enzyme inhibition

In most cases, when the protein expression of a CYP450 is measured, its corresponding enzyme activity is also measured and in most cases, the reduction in activity of the enzyme can be explained on the basis of the reduced amount of the enzyme. However, there have been cases when this is not true. Rat hepatocytes cultured on matrigel showed ~70% down-regulation of protein levels

as compared to control but showed ~97% reduction in enzyme activity (Tapner et al., 1996). In another study where rats were treated with IFN-α, the activities of Cyp1a1 and Cyp2b were down-regulated without any changes in the corresponding protein levels (Stanley et al., 1991). Similar discord in Cyp1a and Cyp2a activity and protein levels were found when arthritic rats were treated with IL-1 (Ferrari et al., 1993).

The mechanisms of how inflammatory stimulants mediate these effects are not clear however, they could involve oxidative stress, inhibition by nitric oxide or other mechanisms.

1.3: Nuclear receptors

Since 1985, when the cloning of the first nuclear receptor cDNA encoding the human glucocorticoid receptor (GR) was discovered (Weinberger et al., 1985), there has been great progress in this field. To date a total of 49 members of nuclear receptor superfamily have been characterized in mouse and 48 members have been characterized in humans. Nuclear receptors are one of the largest known families of transcription factors and they function as regulators of gene expression. They have three major protein domains called the AF-1 or the activation function 1 domain, DNA binding domain (DBD) and a ligand binding

domain (LBD). The nuclear receptors share high structure similarity. Nuclear receptors regulate many diverse functions, like homeostasis, reproduction, development and metabolism. The function of nuclear receptors was investigated by relating the expression profile of nuclear receptors in multiple tissues with the function of the tissues in mice. The results obtained showed that the nuclear receptors form a hierarchy network governing two physiologic parameters (i) reproduction, growth and development & (ii) uptake of nutrients, excretion and metabolism (Gronemeyer et al., 1999; McEwan, 2004).

Xenobiotic nuclear receptors, CAR (Constitutive androstane receptor), PXR (Pregnane-X-receptor) as well as the central nuclear receptor RXR (Retinoid-X-receptor) α are the most important nuclear receptors implicated in drug metabolism changes and toxicities in patho-physiological conditions (Moore et al., 2002; Wei et al., 2002; Wagner et al., 2005). They are extensively expressed in the metabolic tissues, the liver and intestines. As xenosensors, PXR and CAR generally respond to different xenobiotics, and regulate different genes. However, there is also some overlap in the response and regulation of these receptors. There are three different RXR isotypes encoded by three distinct genes: RXRα, RXRβ and RXRγ. Each isotype exists in several isoforms with specific tissue distributions (Philippe et al., 2010).

Table 1.1 Human nuclear receptor superfamily

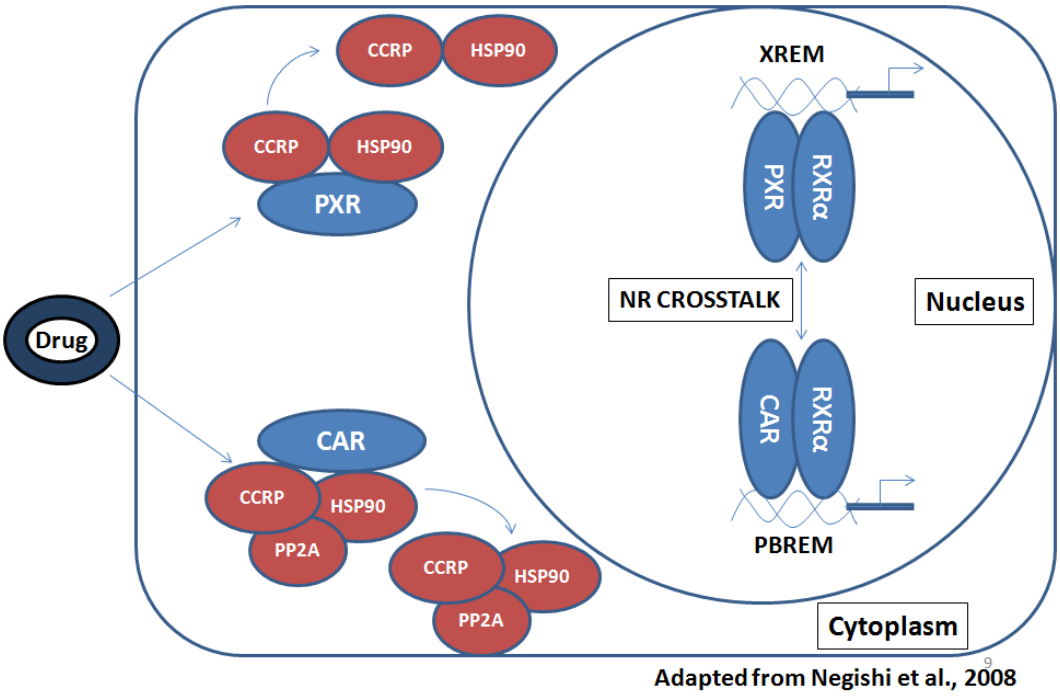
Name	Abbreviation	Nomenclature	Ligands
Retnoid X receptor	RXRα	NR2B1	9-cis-retinoic acid
Retnoid X receptor	RXRβ	NR2B2	9-cis-retinoic acid
Retnoid X receptor	RXRγ	NR2B3	9-cis-retinoic acid
Pregnane X receptor	PXR	NR1I2	Xenobiotics, steroids, bile acids
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, PB, steroids

1.3.1: Location and activation of CAR & PXR:

CAR is present in high amounts in the cytoplasm. It is bound in the cytoplasm by CAR Cytoplasmic Retention Protein (CCRP), Heat Shock Protein 90 (HSP90). CAR is unique because it can be activated by direct binding as well as indirect binding. On activation of CAR, CAR is released from the co-repressors and based on the activity of Protein Phosphatase PP2A, CAR translocates into the nucleus and binds to its partner RXR after which the heterodimer binds to the transcription site on the nucleus. Inside the nucleus, CAR-RXR dimer binds to Phenobarbital (PB) responsive enhancer module or PBREM. Activators of CAR include PB, TCPOBOP in mice, CITCO in humans etc. PXR is not active on its own unlike CAR. Also it can be activated only by direct activation unlike CAR.

PXR is also present in the cytoplasm where it is bound by CCRP, HSP90. On activation of PXR, it translocates into the nucleus and binds to RXR and forms a heterodimer and binds to the Xenobiotic responsive enhancer module or XREM.

Fig. 1.3: Activation of Nuclear Receptors (Adapted from Negishi et al., 2008)



1.3.2: PXR

PXR is a sensor for endogenous and xenobiotic compounds and a trans-regulator for the expression of many drug metabolism–related genes. The rodent PXR and its human homolog i.e. SXR can be activated by certain xenobiotic and endogenous compounds (Kliewer et al., 1998; Lehmann et al., 1998; Blumberg et al., 1998). The most important drug metabolism gene regulated by PXR is the Cyp3a subfamily of CYP450s both in mouse and humans. Other genes that are regulated by PXR include multiple drug resistant genes such as Mdr1, Mrp2 & Mrp3 (Synold et al., 2001; Kast et al., 2002) as well as genes involved in metabolism and transport of endogenous molecules. A number of UGTs, including UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9, have been identified as PXR targets (Xie et al., 2003; Chen et al., 2003; Yueh et al., 2003). PXR/RXR heterodimer can also interact with other nuclear receptor pathways (like CAR/RXR) by mutually binding to the regulatory DNA sequences leading to redundancy in regulating the metabolism and clearance of various xenobiotics and endogenous compounds (Xie et al., 2000)

Dexamethasone and lithocholic acid have been shown to induce PXR gene expression in primary human hepatocytes Clofibrate, perfluorodecanoic acid, isoniazid, and troleandomycin have been shown to alter PXR gene expression in rat livers (Zhang et al., 1999). PXR gene expression can be regulated by many

different stimuli including xenobiotics and metabolites (Aouabdi et al., 2006). PXR gene expression can also activated by farnesoid X receptor (FXR) in response to bile acids (Jung et al., 2006). Three alternatively spliced transcripts of PXR that encode different isoforms have been described, one of which encodes two products through the use of alternative translation initiation codons. Additional transcript variants have been shown to exist, although these variants have not been fully described (Bertilsson et al., 1998; Dotzlaw et al., 1999; Gardner-Stephen et al., 2004).

1.3.3: CAR

CAR is expressed primarily in the liver and kidneys with some expression in the heart, brain and intestines (Timsit et al., 2007). The primary target drug metabolism genes of CAR are in the cyp2b subfamily, uridine 5'-diphospho-glucuronosyltransferase (Ugt)1a1 and Mrp2. As the name suggests, CAR does not require ligand binding to become activated. Instead, it readily forms heterodimers with RXR and targets Phenobarbital responsive enhancer modules (PBREM) in target gene promoters. Much like PXR, CAR functions as a chemical sensor and regulates a broad range of hepatic and intestinal phase I DMEs (CYP3A4, CYP2Bs and CYP2Cs), phase II DMEs (UGTs and GSTs) (Sugatani et al., 2001),

and drug transporters (MDR1, MRPs and OATP2) (Aseem et al., 2004; Kast et al., 2002).

Androstanol and androstenol are inverse agonists of CAR i.e. they repress the constitutive activity of CAR by recruitment of co-repressors instead of co-activators (Forman et al., 1998). Even though mouse CAR and human CAR share many similarities, like nuclear translocation on PB treatment and binding on PBREM in the nucleus, clear species-specific effects exist. For example, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4 dichlorobenzyl)oxime (CITCO) is a potent human, but not mouse, CAR agonist. Phenobarbital-like inducer, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the most potent mouse CAR ligand known, does not activate human or rat CAR (Moore et al., 2000; Tzameli et al., 2000). Thus data generated in mouse models of CAR may not accurately describe human CAR functions.

1.3.4: Role of CAR AND PXR during inflammation

Several studies have shown that down-regulation of PXR, CAR and RXRα was associated with decreased DME and transporter gene expression during inflammation (Beigneux et al., 2000, 2002; Ghose et al., 2004, 2008, 2009). Thus,

it is likely that nuclear receptors play an important role in inflammation-mediated down-regulation of gene expression of DMEs and transporters.

It has been shown recently that mutual inhibition between PXR and NF-κB exists which may prove to be the molecular link between drug metabolism and imflammation (Xie et al., 2006; Zhou et al., 2006). NF-κB controls a lot of genes involved during inflammation and it is found to be activated in almost all types of inflammation. It was shown that PXR and NF-κB mutually inhibit each other. This study was taken further by another group. They conducted experiments in $PXR^{-/-}$ mice and found that the levels of NF-κB were higher than in mice with PXR. It was later found that PXR actually controls the levels of NF-κB (Langmann et al., 2004). Recently there was co-relation found between PXR and inflammatory bowel diseases like Crohn's disease. It was also found that $PXR^{-/-}$ mice have higher levels of inflammation in the small intestine (Dring et al., 2006). As of now, not much is known or studied about the role of CAR in inflammation or inflammatory bowel disease. But these studies indicate that PXR and potentially CAR have an important role to play during inflammation.

1.3.5: Role of CAR and PXR in drug metabolism and toxicity

CAR and PXR together regulate a wide variety of Phase I enzymes, Phase II enzymes and transporters. CYP3A4 which is the most important Phase I enzyme is regulated by CAR and PXR but primarily by PXR which is also responsible for its basal activity. CAR and PXR have overlapping substrate specificity for activating CYP3A4. PXR and CAR are responsible for the regulation of CYP2C19, CYP2C9 and CYP2D6 as well. These 4 enzymes are responsible for Phase I metabolism of more than 90% known drugs. Hence CAR and PXR play an important role in the detoxification of most drugs. However, they are not only involved in regulating Phase I DME genes, they also regulate a variety of Phase II genes like UGTs, sulfotransferases, glutathione-S-transferases etc (Xie et al. 2003; Xu et al. 2005; Zhou et al. 2005). These Phase II enzymes make the drug hydrophilic and help in its excretion from the body through kidneys and bile. In addition to these, CAR and PXR can regulate expression of organic anion transporters like Ntcp, bsep, Mrp3 & Oatps (Staudinger et al. 2003; Geier et al. 2005). These transporters help in the transport of drugs and their conjugated metabolites through the body. Through the regulation of these enzymes and transporters, CAR and PXR can also regulate the metabolism and clearance of endogenous molecules. An example of this is bilirubin clearance. Bilirubin is a byproduct of heamoglobin breakdown. If its levels are high in blood, PXR and CAR will activate the uptake transporters so that the cells take up the bilirubin

from blood. CAR and PXR will also activate Ugt1a1 so as to conjugate bilirubin and they will also induce efflux transporters which will cause excretion of the harmful substances (Guo et al. 2003; Geier et al. 2005). Thus CAR and PXR are extremely important in regulating drug metabolism and act as a defense mechanism to prevent any adverse effects.

As mentioned earlier, CAR and PXR induce the expression of DMEs and transporters so that bilirubin is immediately conjugated and thrown out of the body (Guo et al., 2003). If bilirubin levels in blood remain high, they cause neurological damage and jaundice. Thus by enhancing its removal from the body, PXR and CAR prevented toxicity of bilirubin. Similarly, PXR and CAR prevent toxicities from xenobiotics as well. Patients undergoing hepatectomy (removal of 1/3rd or 2/3rd of the liver), have high circulating levels of bile acids and bilirubin. Treatment with CAR activator Phenobarbital causes normal levels in blood (Tien & Negishi., 2008). CAR and PXR have been implicated in cholestatic liver disease as well. CAR activation protects mice from lithocholic acid-induced toxicity (Beilke et al., 2009). All these are examples of how CAR and PXR ameliorate toxicities. However there are instances when activation of CAR and PXR cause toxicity. Recently, CAR activation was linked with hepatocellular carcinoma (Yamamoto et al., 2004). In the rodent model of Non-Alcoholic steatohepatitis, CAR activation causes liver fibrosis and other complications (Yamazaki et al., 2007). Activation of PXR using rifampicin caused liver issues in

TB patients (Morere et al., 1975). CAR activators are also known to cause hepatomegaly. Although much is known about these nuclear receptors, a lot is yet to be discovered.

Considering their roles in drug metabolism, toxicities, inflammation etc, nuclear receptors CAR and PXR will remain attractive therapeutic targets for various pathological/patho-physiological conditions.

1.4: DMET alterations during obesity:

Obesity is a condition characterized by a low grade chronic inflammation (Shoelson et al., 2007). Generally, obesity results from excessive energy storage over a prolonged period of time. Studies in animal models have demonstrated two distinct types of obesity; (i) genetic, as seen in zucker rats or leptin deficient mice (Phillips et al., 1996; Zhang et al., 1994) and (ii) combination of genetic and environmental factors. Among environmental factors, long-term high-fat intake has been most extensively studied because of its contribution to the development of both obesity and diabetes in humans and rodents (Olefsky et al., 1975). The C57BL/6J mouse strain has been widely used as a human model of obesity because this strain develops obesity when fed a high-fat and high-sucrose diet; however, remains lean if the fat content of the diet is restricted (Lin et al., 2000).

Cyp3a11 and Cyp4a10 gene expression was induced in mice fed on a 36% Kcal-fed diet (Kim et al., 2004). In the same study, the gene expression of GST, an important phase II enzyme was also found to be induced. There have been many contrasting reports on regulation of DMETs during different models of obesity. Cyp3a activity was reported to be reduced or induced in diabetic fa/fa Zucker rats (Irizar et al., 1995; Wang et al., 2007). Cyp2b levels were found to be induced in an obese zucker rat model (Blouin et al., 1993). Mice fed a high fat diet (36% Kcal diet) have significant reductions in Cyp3a11 at the protein and RNA levels in ICR mice (Yoshinari et al., 2006). We have also seen that mice fed on a high fat diet (60% Kcal diet) had significant down-regulation in the expression of several DMETs like Cy3a11, Cyp2b10 and Ugt1a1 at the gene as well as the protein levels (Ghose et al., 2011). This is an understudied area and more studies are required to understand the mechanisms behind alterations in DMETs as well as to understand the clinical consequences of down-regulation of DMETs during obesity.

It has been recently found that TLR1, 2, 3, 4, 5, 7 and 9 are expressed in human and mouse adipocytes and all of them are active (Batra et al., 2006). It was shown that fatty acids that are circulating in high concentrations in obese patients can activate TLR signaling (Suganami et al., 2007). In humans TLR2 and TLR4 were shown to be functional in the adipocytes (Bes-Houtmann et al., 2007). It was also shown that TLR4 deficient mice are resistant to diet-induced obesity (Davis et al.,

2008; Tsukumo et al., 2007). It has been reported that there is down-regulation of expression of phase I DMEs, phase II DMEs and transporters during obesity (Omoluabi et al., 2011; Osabe et al., 2008). It is fairly well known that changes in expression and activities of DMEs and transporters leads to altered pharmacokinetics and pharmacodynamics of various drugs (Wilkinson et al., 1997; Gandhi et al., 2012). Powis et al., 1987 found that the clearance of the anti-cancer drug cyclophosphamide was reduced in obese breast cancer patients. Rodvold et al., 1988 found that the clearance of doxorubicin was reduced in severely obese individuals. Obese patients have increased sensitivity for psychomotor response to a benzodiazepine derivative drug, triazolam (Smith et al., 1995). Schmid et al., 2009 reported that, in patients with BMI >30 kg/m^2, there was an increased dose requirement for insulin to reach target glucose levels.

We have seen that treatment of mice with a high-fat diet leads to down-regulation of gene expression of several phase I & phase II genes (Ghose et al., 2011). It is very important we try and understand the clinical consequences of this down-regulation because this could lead to altered PK and PD of drugs and altered PK and PD may lead to toxicity or inefficacy depending on whether the parent drug or the metabolite is the active component.

CHAPTER 2

Hypotheses and Specific Aims

Specific Aims:

Aim 1A: We will test the hypothesis that the down-regulation of gene expression of DMETs through TLR4 and TLR3 is mediated through TRIF.

Aim 1B: We will test the hypothesis that the down-regulation of DMEs and transporters in LTA or LPS-induced inflammation is dependent on the nuclear receptor CAR.

Aim 2: We will test the hypothesis that obesity induced accumulation of the active metabolite SN-38 is most likely responsible for irinotecan-induced liver toxicity. We will also test the hypothesis that repeated insult with irinotecan will lead to development of steatohepatitis in a mouse model.

2.1. A: Specific Aim 1A:

TLR3 and TLR4 mediate responses by viral and gram -ve bacterial components respectively, through adaptor molecules TIRAP and TRIF. While TLR4 signaling can recruit TIRAP and TRIF, TRIF is the only adaptor protein involved in the TLR3 pathway. We showed that TIRAP is involved in TLR2-mediated but not TLR4-mediated down-regulation of DMETs. Thus, we hypothesize that the down-regulation of DMETs through TLR4 and TLR3 is mediated by TRIF.

Aim 1A: (i) We will test the hypothesis that the down-regulation of gene expression of DMETs through TLR4 and TLR3 is mediated through TRIF.

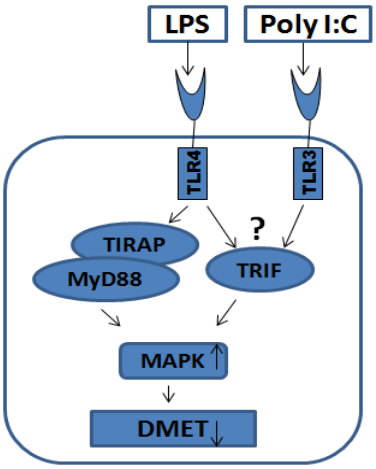


Fig 2.1.A: Regulation of gene expression of DMETs through TLR signaling.

2.1.B: Specific Aim 1B:

Down-regulation of DMETs during TLR-induced inflammatory responses involves the down-regulation of gene expression of PXR and CAR. Studies in $PXR^{-/-}$ mice revealed no role of PXR during LPS-induced inflammation. We have shown that activation of TLR2 and TLR4 by LTA and LPS respectively leads to suppression of CAR and its target genes like Cyp3a11, Cyp2b10, Amine-N-Sulfotransferase (Sultn), Cyp2a4, Ugt1a1 & Mrp2. Thus, we hypothesize that CAR is the main nuclear receptor involved in DMET down-regulation during LTA and LPS-induced inflammation and that the absence of CAR will attenuate this down-regulation.

Aim 1B: We will test the hypothesis that the down-regulation of DMEs and transporters in LTA or LPS-induced inflammation is dependent on the nuclear receptor CAR.

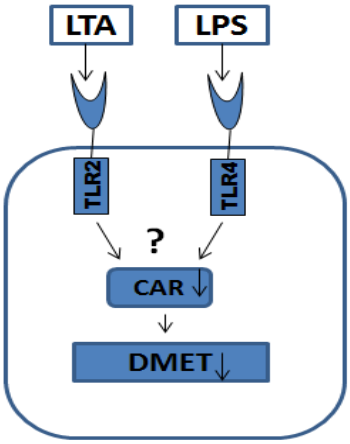


Fig 2.1.B: Role of nuclear receptors in regulating drug metabolism during inflammation

2.2: Specific Aim 2:

Apart from bacterial or viral components, TLRs are also activated by pathological conditions such as obesity (Suganami et al., 2007). Thus, one of our goals is to determine the role of obesity in altered drug metabolism. Irinotecan (CPT-11) is a topoisomerase I inhibitor which is highly effective in treatment of variety of cancers (Trifan et al., 2002). Clinical studies revealed that patients with a BMI>25 were twice as much susceptible to developing irinotecan-induced liver toxicity than patients with BMI<25 (Pilgrim et al., 2011). CPT-11 is metabolized to SN-38, which then undergoes glucuronidation by Ugt1a1 to form SN-38 glucuronide (SN-38G). Excess accumulation of the toxic metabolite SN-38 is known to cause fatal diarrhea in cancer patients. We have seen that mice fed on a high fat diet have significant down-regulation in gene expression of Ugt1a1. We hypothesize that reduction in Ugt1a1-mediated SN-38 glucuronidation will lead to increased accumulation of the toxic metabolite of irinotecan, SN-38.

Aim 2: We will test the hypothesis that obesity causes reduction of Ugt1a1-mediated SN-38 glucuronidation leading to increased accumulation of SN-38 *in vivo*. We will also test the hypothesis that repeated insult with irinotecan will lead to development of steatohepatitis in a mouse model.

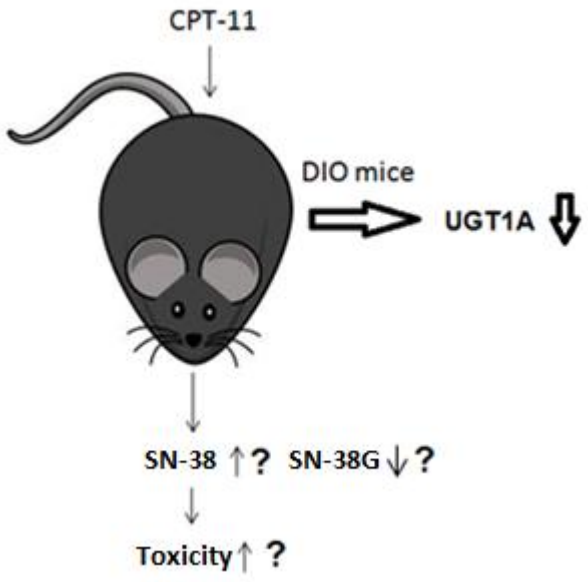


Fig 2.2: Regulation of CPT-11 toxicity in diet-induced obese mice

CHAPTER 3

Experimental Methods

3.1: Reagents used throughout the dissertation:

Lipopolysaccharide (E. coli, Cat # tlrl-pslta), lipoteichoic acid (S. aureus, Cat # tlrl-pelps) and Poly I:C (HMW; Cat # 31852-29-6) were purchased from InvivoGen (San Diego, CA) and dissolved in endotoxin free sterile water to get a stock solution with a concentration of 5 mg/mL of LPS, 5 mg/mL of LTA and 5 mg/mL of Poly I:C and stored at -20°C. Irinotecan hydrochloride (CPT-11-HCl) solution for injections was purchased from Martin Surgical Supplies (Houston, TX, USA, Stock# 4434-11) at a concentration of 20 mg/ml stock solution. Irinotecan (CPT-11) and Camptothecin (CPT; internal standard (IS)) were purchased from Sigma-Aldrich, St. Louis, MO. SN-38 and SN-38G were a kind gift from Dr Ming Hu's lab at the University of Houston, Houston, TX (Santa Cruz Biotechnology; sc-212931).

3.2: Animals:

All animals used in this study followed care of the animals and experimental procedures complied strictly with the Institutional Animal Care and Use Committee guidelines of the University of Houston. Adult, male C57BL/6 mice, aged 5-6 weeks with approximate weight of 20-22 g were purchased from The Harlan Laboratory (Houston, Texas, USA). $TRIF^{-/-}$ (Stock #5037) (~6-8 weeks weighing 20-25 g) were obtained from Jackson laboratories (Bar Harbor, Maine). The hCAR and $CAR^{-/-}$ mice (8–10 weeks weighing 25-30 g) on a C57BL/6

genetic background were a kind gift from Dr. David Moore (Baylor College of Medicine, Houston, TX, USA). Male, 12 weeks old diet-induced obese (DIO) mice (60% kcal fed) (Cat #382250) and Lean mice (10% kcal fed) (Cat #382256) on a C57BL/6 background were purchased from Jackson Labs (Bar Harbor, Maine). All the animals were maintained in a temperature and humidity controlled environment and 12 h light/dark cycle with free access to water and rodent chow ad libitum.

3.3: Treatments:

For injections with LPS, 50 µL of the 5 mg/ml stock was freshly reconstituted with 1200 µL of sterile deionized water. For injections with LTA, 150 µL of 5 mg/ml stock was freshly reconstituted with 1100 µL of sterile deionized water. For injections with Poly I:C, 100 µL of the 5mg/mL stock was freshly reconstituted with 900 µL of sterile deionized water. A 10 µL of each reconstituted solution was injected by the intra peritoneal (i.p.) route for every 1 g of mouse weight.

For *in vivo* studies, the animals were injected with saline, 2 mg/kg of LPS, 6 mg/kg of LTA or 5mg/kg of Poly I:C. The animals were returned to their cages and sacrificed after 1, 4, 8 & 16 h to harvest livers in order to extract mRNA or microsome/S9 fractions, which is described in the subsequent sections. For pharmacokinetic (PK) studies, lean and DIO mice were treated with saline or

irinotecan hydrochloride and returned to their cages as one animal per cage for easy identification of animals during PK studies.

For injections at a dose of 10 mg/kg, CPT-11-HCl was diluted 10X to get a concentration of 2 mg/ml. Then 100 μL of 2 mg/ml of CPT-11-HCl solution was administered via oral gavage for every 20 g of mouse weight.

3.4: RNA isolation and real-time PCR analysis:

Reagents:

TRIzol reagent (Cat # T9424), chloroform (Cat # C2432) and iso-propyl alcohol (I9516) were bought from Sigma-Aldrich, St. Louis. Plastic cuvettes were purchased from VWR (Z330388). The cDNA synthesis was performed using a High Capacity Reverse Transcription Kit from Applied Biosystems (Foster City, CA, Cat # 4368813). The sequences for all probes and primers are described in table 2.1 and unless specified, probes and primers were bought from Sigma-Genosys, Houston TX.

Method:

Total RNA was isolated from mouse liver tissues using TRIzol reagent according to the manufacturer's instructions. The details are: approximately 0.1 g of liver tissues stored at -80°C, were collected in 2 ml of microcentrifuge tubes. Then 0.75 ml of cold TRIzol reagent was added. The tubes were placed on ice and

homogenized with a hand-held Beckman Polytron homogenizer (highest setting) for ~30 sec. After each tube, the homogenizer was washed in the sequence with RNA Zap reagent (Life technologies; AM9780), 70% ethanol, MilliQ water and TRIzol reagent. The samples were incubated at room temperature for 5 min. Then 0.2 ml of chloroform was added. The tubes were tightly capped and vigorously shaken for 15 sec and incubated for an additional 15 min at room temperature. The tubes were centrifuged at 12,000 rpm for 15 min at 4°C. After this step, the upper colorless phase was transferred (~70% of the volume of TRIzol) to another clean Eppendorf tube. Then 0.5 ml of isopropyl alcohol was added and tubes were mixed vigorously and incubated for 10 min at room temperature. The tubes were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet (often invisible formed at the bottom) was mixed with 1 ml of 75% ethanol, vortex mixed and centrifuged at 7600 rpm for 5 min at 4°C. All the ethanol was removed and the RNA pellet was allowed to dry in the hood for 2-3 min. The pellet was then dissolved in 50-70 μL of RNAase-free water or diethylpyrocarbonate (DEPC, 0.1% v/v solution) (Sigma-aldrich; D5758) water by passing the solution several times with a pipette tip. If necessary, the tubes were warmed for 5-10 min in a water bath preset at 55°C.

Total RNA was measured by diluting 1 μL of sample in 500 μL of TE buffer (pH 8.8) in 1.5 ml clear disposable plastic cuvettes on a UV-vis spectrophotometer (Beckman Coulter, DU800) at 260 and 280 nm wavelengths. The ratio of 260/280

was used to quantitatively determine the RNA concentration. RNA preparations with a ratio of > 2 were used in the subsequent experiments.

The following reagents per reaction were used as follows: 4 μL of 10X RT buffer, 1.6 μL of 25X dNTP mix (100 nm), 4 μL of 10X random primers, 2 μL of multiscribe reverse transcriptase, 2 μL of RNAase inhibitor, 6.4 μL of DEPC water. So a total of 20 μL of these reagents were added to 20 μL of RNA samples in PCR tubes (0.2 ml capacity) to get the final reaction volume of 40 μL. The tubes were gently tapped and placed in the PCR cycler to construct the cDNA. The conditions for PCR were: 25°C for 0-10 min, 37° C for 11-70 min, held constant at 37° C from 71-130 min, 85° C for 5 sec, then cooled down to 4° C for 90 min.

Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA). For each 10 μL of sample, 15 μL of PCR mix reagent was added to each well of a 96-well PCR plate. The contents of the 15 μL of PCR mix reagent are as follows: 10 μL cDNA (50-100ng), 0.075 μL forward primer (100 μM stock), 0.075 μL reverse primer (100 μM stock), 0.05 μL TaqMan probe (100 μM stock), 11.25 μL of Roche PCR Master Mix (Roche Diagnostics, Indianapolis, IN, Cat # 04914058001) and 3.55 μL of dd water in a PCR plate. In the case of 20X master-mix assays bought from Applied-Biosystems, instead of 0.075 μL forward primer,

0.075 μL of reverse primer and 0.05 μL probe, 0.2 μL of this 20X master mix was added per reaction. The reaction volume was 25 μL per well. The plate was tightly sealed and centrifuged for 10-15 sec to allow proper mixing of the reagents. The reaction conditions were 50°C for 2 min (stage 1), denaturing at 95°C for 10 min (stage 2), denaturing at 95°C for 15 sec and annealing at 60°C for 1 min (stage 3, 50 cycles). Quantitative expression values were normalized to cyclophilin.

Table 2.1: Probe and primer sequences of DMET/cytokine/NR genes

Gene	Forward Primer	Reverse Primer	Probe sequence
Cyp1a2	TGGAGCTGGCTTTGACACAG	CGTTAGGCCATGTCACAAGTAGC	CACCACAGCCATCACCTGGAGCATTT
Cyp2e1	AAGCGCTTCGGGCCAG	TAGCCATGCAGGACCACGA	TCACACTGCACCTGGGTCAGAGGC
Cyp2a4	CAGCCAACGTTATGGTCCTGTA	GTCCGCACAGCACCACAA	TCACCATCTATCTGGGATCTCGCCGA
Cyp3a11	GGATGAGATCGATGAGGCTCTG	CAGGTATTCCATCTCCATCACAGT	CCAACAAGGCACCTCCCACGTATGA
Cyp2b10	CAATGGGGAACGTTGGAAGA	TGATGCACTGGAAGAGGAAC	TTCGTAGATTCTCTCTGGCCACCATGAGA
Sult1a1	GACAATGGAGCAACTGCAGAAC	CCAGACTTTGGGTACGTGCTG	CAGCCTGGCCTGATGATGTGCTCA
Ugt1a1	TCTGAGCCCTGCATCTATCTG	CCCCAGAGGCGTTGACATA	TGGTATAAATTGCCTTCAGAAAAAGCCCCTATC

Sult2a1	GGAAGGACCACGACTCATAAC	GATTCTTCACAAGGTTTGTGTTACC	CCATCCATCTCTTCTCCAAGTCTTTCTTCAG
Sultn	TTTGGAGCATTGCTGAGCAT	GGGATATGTGGAGATCAAAATGTCA	TCCCAAGTAGAGTCATTTGAAGCCCGG
Mrp2	GCTGGGAGAAATGGAGAATGTC	GACTGCTGAGGGACGTAGGCTA	TGGGCATATCACCATCAAGGGCTCC
Mdr1b	GCTGGACAAGCTGTGCATGA	TGGCAGAATACTGGCTTCTGCT	CTTCCCCTCTTGATGCTGGTGTTTGGAAAC
Mrp3	CCACTTTTCGGAGACAGTAAC	ACTGAGGACCTTGAAGTCTTGGA	CACCAGTGTCATTCGGGCCTATGGC
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	CTGTGTAATGAAAGACGGCACACCCACC
IFN-β	Mm00439552_s1 purchased from AB sciex as a 20X mix		
IL-6	Mm00446190_m1 purchased from AB sciex as a 20X mix		

TLR2	CCTACATTGGCCATGGTGAC	CCTCTATTGTATTGATTCTGCTGGA	CGAGCGTGTGCGAACCTCCA
CAR	CAGGGTTCCAGTACGAGTTTTG	AGGCTCCTGGAGATGCAGTC	AGTCGATCCTCCACTTCCATAAAAACCTGAA
PXR	TCCAGCGCAGCGTGGTA	GCAGGATATGGCCGACTACAC	TTTGCCCTCACCCTGAAGGCCTACA

3.5: Isolation of primary mouse hepatocytes:

Reagents:

Perfusion buffer 1 was purchased from VWR Chemicals (Cat # 45000-446) and consisted of EGTA, 95 mg; PBS (without Ca/Mg), 500 ml and 1 M HEPES solution, 5 ml. 1 M HEPES was prepared by adding 131 g of HEPES (H3375; Sigma-Aldrich) powder to 1L of deionized water with the pH adjusted to 7.4 and stored at 4°C. Perfusion buffer 2 (VWR, Cat # 45000-430) consisted of PBS with Ca/Mg. The perfusion buffer 2 was prepared by adding 20 mg of collagenase Type IV (Sigma-Aldrich, C-5138).

Method:

Prior the experiment, water bath was warmed at 37°C, the microcentrifuge was fast cooled at 4°C and perfusion buffers 1 and 2 were warmed in the water bath. A total of 50 ml. of buffer 1 and 62.5 ml of buffer 2 were pre-warmed in the water bath for one mouse. For isolation of primary mouse hepatocytes, the mouse was lightly anesthetized in a jar containing a cotton ball wet in Isoflurane. The mouse was allowed to remain in the jar for less than a min after which the mouse was placed on its back on a sterile Styrofoam lid covered with aluminum foil. The head of the mouse was gently placed in a conical tube containing a cotton ball lightly wet with the Isoflurane. The fore and hind legs of the mouse were fixed by

piercing pins so that a tight and flat abdominal surface is available for the experiment. The procedure was conducted inside the hood and light was turned on to keep the mouse warm and conscious during the entire procedure. An incision was cut on holding the abdominal skin of the mouse tightly with forceps. Then the abdomen was cut wide open by moving the scissors so as to avoid damage to any other internal organs. The intestine was gently pushed away with cotton tips to expose the inferior vena cava (IVC) and portal vein (PV). The muscles next to the IVC were gently ruptured with a curved forcep so that the hemostat can pass. A silk thread was curved in the hemostat at the tip and put under through the IVC. The thread was pulled from the other end and a loose knot was tied with 2 throws. At an angle parallel to the IVC, a catheter was inserted in the IVC till the needle went ¾ inside and the knot was tightened. Then the needle was carefully removed and after checking the blood flow from the white end of the needle and the tubing was attached. The PV was quickly cut and the pump was turned on to perfuse the liver first with perfusion buffer 1. The liver was perfused for 4-5 min. at a flow rate of 6-8 ml/min (~ 30-40 ml. was perfused). Then the tube was removed from buffer 1 and immersed in buffer 2 which was perfused for an additional 3-4 min. until the liver begins dissociating under glisson's capsule. We check the liver by poking holes with a curved forcep. The liver is ready when a soft touch by the forcep creates a lasting indentation in the liver. The pump was then stopped; the liver was then removed and immersed in cold plating cell culture medium.

3.6: Hepatocyte culture:

Reagents:

The compositions of the cell culture medium are listed below:

Plating medium: Williams medium E, 500 ml. (Invitrogen; 12551-032); penicillin/streptomycin, 5 ml. of stock (Invitrogen; 15140-122); glutamine/gentamycin, 5 ml. of stock (Sigma-Aldrich; G9654); Insulin-transferrin-sodium selenite (ITS) (25 mg/10ml) (Sigma-Aldrich; I1884), 1 ml of stock to get final conc. of 5 μg/ml; glucagon, 20 μL of stick (2 μg/500 ml) (Sigma-Aldrich; G3157) and fetal bovine serum (FBS) (Invitrogen; 10082-14), 50 ml.

Treatment medium: Williams medium E, 500 ml.; penicillin/streptomycin, 5 ml. of stock and glutamine/gentamycin, 5 ml. of stock.

Method:

Both the mediums were filter sterilized in the cell culture hood and stored at 4°C for further use. The liver, which was placed in 40 ml. of cold plating medium was shaken vigorously enough to break the liver. The suspension was then poured through a 70 micron cell strainer (Fisher; 087712) and collected in another tube. Some debris is left and do not worry about breaking that tissue that as it may damage the hepatocytes. The 40 ml. crude suspension was centrifuged at 50 rcf

for 2 min. at 4°C. The supernatant was discarded as it contained the non-parenchymal cells. Then, 25 ml. of fresh medium was added with 12 ml. of 100% Percoll solution (Sigma-Aldrich; P4937). Percoll is used to establish a density gradient which allows efficient removal of dead cells. The tube was inverted 4-5 times gently to mix the cells and then spun down at 50 rcf for 10 min at 4°C. The supernatant was aspirated and the cells were again washed 2 times with 30 ml. of ice cold plating medium and spun at 50 rcf for 2 min. each. The supernatant was aspirated and the cells were resuspended in 10 ml. of ice cold plating medium. The cells were gently mixed and then counted on a hemocytometer under a light microscope set at 20X. A good preparation yields around 2 million cells/ml. Only preparations with a viability >90% was used in further experiments. A seeding density of 500,000 cells/2 ml/well in 6-well plates (BD PharMingen, Cat # 353847) was used.

The plates were incubated at 37°C in a humidified incubator supplied with 95% relative humidity and 5%CO_2. The cells were allowed to attach for 3-4 h after which the medium was changed to treatment medium and the cells were returned to the incubator for overnight. The cells were allowed to stabilize for 48 h and only then treatments were carried out.

3.7: Hepatocyte treatments:

Reagents:

JNK inhibitor (SP600125); cat# 420119, ERK inhibitor (PD98059); cat# 513000, and p38 inhibitor (SB203580); cat# 559389 were purchased from Calbiochem, USA.

Methods:

For *in vitro* studies, the hepatocytes were treated with saline, 1 μg/mL of LPS, 50 ng/mL of LTA or 50 μg/mL of Poly I:C for 8 or 16 h.

To assess the roles of MAPK, primary hepatocytes were isolated from C57BL/6 mice and treated with and without the following inhibitors: JNK (SP00125, 10μM), ERK (PD98059, 20μM) and p38 (SB203580, 25μM) for 60 min followed by treatment with Poly I:C (50μg/ml) or LPS (1μg/ml) for 8 h and 16 h respectively. RNA was extracted from cells and gene expression was analyzed by qPCR analysis.

3.8: Alanine aminotransferase assay:

Alanine Aminotransferase (ALT) is routinely used as a marker to assess liver toxicity due to pathophysiological conditions or chemical insults. The reagent was reconstituted with the volume of distilled or deionized water as stated on the vial

label (ALT-GPT Infinity, Thermo Scientific, Middletown, VA, USA, Cat # TR18503) and stored at 4°C for future use. For determination of ALT activity in samples, 5 µL of serum specimen or 20 µL of cell culture supernatant was added in a half-area UV absorbance 96-well plate. Then 50 µL or 200 µL of ALT reagent was added into the plate. The plate was mixed well and after 30 seconds the absorbance was recorded at 340 nm for 3 min at 1 min intervals. The change in absorbance per min (ΔA/min) was calculated from using the mean from the 3 readings. To get the final ALT activity in U/L, the ΔA/min was multiplied by a predefined factor based on the manufacturer's instructions.

3.9: Perfusion of the liver for activity assays:

Reagents:

Perfusion buffer A comprised of 8 mM KH_2PO_4 (P0662), 5.6 mM Na_2HPO_4 (S7907), 1.5 mM KCl (P9333) and 96 mM NaCl (S7653). Perfusion buffer B comprised of 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, and 1.5 mM EDTA dehydrate (03664). Both the buffers were stored at 4^0 C and prepared the day before the experiments. On the day of the experiments, 10 µL of 2 mM dithioerythritol (DTT, D9779) and 10 µL of PMSF solution (P7626) 1000X prepared by adding 24 mg in 0.6 ml of methanol) were added to 100 ml of perfusion buffer. Perfusion buffer A contained only PMSF. All these reagents were purchased from Sigma-Aldrich, St. Louis, USA.

Method:

Microsomal fractions are widely used to study drug metabolizing enzyme activity and drug-drug interaction studies. But in order to obtain the purest form of microsomal fractions, the livers need to be perfused to remove any remaining blood which may interfere in the assays. For preparation of liver microsomes, the livers were perfused with perfusion buffers A and B. An incision was cut in the abdomen of the mice and the livers were perfused with buffer A for 2-3 min, followed by buffer B for an additional 2-3 min until there was no blood visible in the liver. The livers were then excised and put in a beaker containing cold sterile saline solution. The livers were placed on ice all the time after harvesting.

3.10: Preparation of microsome and S9 fractions:

Reagents:

Homogenization buffer comprised of 10 mM potassium phosphate solution (pH 7.4), 250 mM sucrose (S7903), 1 mM EDTA (EDS). All reagents were purchased from Sigma-Aldrich.

Method:

Microsomes were prepared to study CYP3A-mediated reaction to study the metabolism of Midazolam (MDZ) in $CAR^{+/+}$ and $CAR^{-/-}$ mice. S9 fractions were prepared to study Ugt1a-mediated glucuronidation of SN-38, an active and toxic

metabolite of CPT-11. The perfused livers were minced on a glass plate placed on ice with a sharp and clean blade to make the liver ready for homogenization. The minced livers were then transferred into 10 ml cylindrical homogenization glass tubes (Wheaton, Millville, NJ, Cat # 358007) placed on ice. The livers were then homogenized using a motorized homogenization gun fitted with a teflon pestle in ice-cold homogenization buffer at a ratio of 1:3 i.e. 3 ml of homogenization buffer for every 1 g of liver. The mixture was then transferred into clean polycarbonate centrifuge tubes (Beckman Coulter, Palo Alto, CA, Cat # 357000) and centrifuged at 15,400 rpm for 15 min at 4°C. The pellet which contained cell debris and unwanted waste was discarded and the supernatant (this is the actual S9 fraction) was collected into clean polycarbonate ultracentrifuge tubes (Beckman Coulter, Palo Alto, CA, Cat # 355618) and centrifuged again at 35,000 rpm for 60 min at 4°C. The fat layer on the top was carefully aspirated using rubber droppers to yield the microsomal pellets. The microsomal pellet was then washed twice with 500 μL of 250 mM sucrose solution. The pellet was then resuspended in approximately 500-700 μL of 250 mM sucrose and mixed with a teflon pestle. The mixture was then pipetted with a 1000 μL pipetted into a clean 2 ml cylindrical homogenization glass tube (Wheaton, Cat # 358003) which was placed on ice. The mixture was manually homogenized by grinding the microsomal pellet with 10-12 strokes of the teflon pestle. The microsomes were then aliquoted

(~50 µL per tube) and stored at -80°C. Protein concentration was determined in one tube from each mouse using a BCA protein assay kit on the same day.

3.11: BCA assay for quantification of protein:

To perform this experiment, microsomes placed on ice all the time. 3 µL of sample was added to 57 µL or 117 µL of dd water to get 20 or 40 folds dilution, respectively. The Pierce® BCA protein assay kit was purchased from Thermo Scientific, Rockford, IL, Cat # 23225). The standards were prepared from the stock of 2 mg/ml to get final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml respectively. Water served as a blank or 0 mg/ml concentration. All the standards prepared were at least 30 µL as 20 µL was to be used in the calibration curve. Then 10 µL of microsome samples or standards were added in duplicates in a clear bottom 96 well plate. The working reagent was prepared by mixing 50 parts of solution A with 1 part of solution B as per the manufacturer's recommendation. Then 200 µL of working reagent was added to each well containing the samples or standards. The plate was then covered with aluminum foil and incubated for 30-35 min at 35 – 37^{o}C in an incubator. Once this time elapsed, the plate was cooled to room temperature for 2-3 min and the absorbance was read on a Biotek plate reader (Biotek, Winooski, VT, USA). The plate reader used to read the absorbance was set to quick read, single settling, 96 well plate, and 570 nm wavelength.

3.12: Cyp3a11 activity assay:

Reagents:

Midazolam (MDZ) (Cat # 451028) was purchased from BD Biosciences (San Diego, CA, USA). The Glucose-6-phosphate (Cat # G6378-500UN), $MgCl_2$ (Cat # 208337), glucose-6-phosphate dehydrogenase (Cat # G6378-2KU) and NADP+ (Cat # N0505) were purchased from Sigma-Aldrich, St. Louis, MO. Phenacetin (Cat # 77440) and 1'-hydroxymidazolam (Cat # UC430) were purchased from Sigma Aldrich (St. Louis, MO, USA). Regenerating system solution contained: 3.3 mM of Glucose-6-phosphate, 3.3 mM of $MgCl_2$ and 1.3 mM of NADP+.

Method:

Typical Phase I reactions were carried out in liver microsomes using MDZ as a Cyp3a specific probe substrate. For determining Cyp3a11 activity, 0.05 mg/ml of microsomal protein was incubated with various concentrations of MDZ (0-16 μM). The reaction mixture consisted of 50 mM KPi solution (pH 7.4), 25 μL of regenerating system solution, 10 μL of MDZ stock solutions (25 fold concentrated) and 0.05 mg/ml of final microsomal protein concentration. The reactions were carried out in duplicate and the reaction mixtures were placed in an ice water bath all the time. The reactions were initiated by adding 25 μL of 100 units/ml of glucose-6-phosphate dehydrogenase solution to get a final

concentration of 1 unit/ml in the reaction mixture. The volume of KPi solution was adjusted according to the microsome concentration for which the volume was fixed at 25 μL. The tubes were tightly capped and reversed upside-down to mix the solution properly. The tubes were then incubated for 5 min at 37°C in a shaking water bath (35-40 horizontal oscillations per min). After 5 min, the reactions were stopped by the adding 100 μL of 100% acetonitrile containing phenacetin which was the internal standard (IS, 1 μg/ml).

3.13: Preparation of nuclear and cytosolic extracts:

Reagents:

Hypotonic buffer consisted of 10mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCL, 0.5 mM DTT and 10 μL/mL of protease inhibitor cocktail (P8340; Sigma-Aldrich). Lysis buffer contained 140 mM NaCl, 2 mM EDTA, 1 % NP-40, 50 mM Tris-HCL, pH 7.2 and 10 μL/mL protease inhibitor.

Method:

To prepare nclear and cytosolic extracts from liver cells, a 6-well plate was placed on top of packed ice. The media was quickly aspirated. Then, the plate was washed two times with 1 mL of cold PBS. Ice-cold PBS was added once and swirled gently. PBS was aspirated off and the washing was repeated. 350 μL of hypotonic buffer was added to each well. The cells were then scraped with a cell

scraper and transferred to glass homogenizers and then were dounce homogenized (10 strokes of dounce A followed by 10 strokes of dounce B). The solutions were transferred to Eppendorf tubes and spun for 5 minutes at 5000 rpm and 4°C. Pellet was stored and the supernatant was transferred and respun in the same conditions. This time, a smaller pellet will be seen. Collect the supernatant and store in -80°C as cytosolic extracts. 100 μL of lysis buffer was added to the eppendorf tube with the smaller pellet. The contents of that tube were mixed and transfered to the tube containing the bigger pellet. Lysis was done by pipetting up and down ~15 times using P-1000 pipet. After incubating on ice for 30 min and centrifuging at maximum speed for 5 min the supernatents were collected and stored as nuclear fractions. Protein concentration was determined using the BCA assay.

3.14: Immunoblotting for protein analysis:

Reagents:

Anti-Lamin A/C (sc-20681), Anti-lactate dehydrogenase (LDH; sc-33781), β-actin (sc-47778) and Anti-CAR antibody (#sc-8538) were purchased from Santa Cruz Biotechnology. Anti-JNK (#9252) and anti-phospho-JNK (#9251) were purchased from Cell-Signaling (Beverly, MA, USA). Homogenization buffer comprising each of 50 mM Tris HCl, 0.5 M NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 (X-100; Sigma-Aldrich) and 0.25% deoxycholate, 1 mM sodium fluoride (NaF) (S7920; Sigma-Aldrich), 1 mM sodium vanadate

(Na_3VO_4) (S6508; Sigma-Aldrich), 2 mM of DTT and 100 µL of protease inhibitor cocktail solution per 10 ml of homogenization buffer.

Method:

Immunoblotting analysis was used to determine the protein expression in liver whole cell extracts or nuclear and cytosolic extracts from the liver. Lamin A/C was used as a loading control or as a housekeeping gene for all the nuclear extracts and LDH was used as a control for cytosolic extracts. Beta actin was used as a loading control or as a housekeeping gene for whole cell extracts. After determination of protein concentration by BCA assay, the samples were diluted with homogenization buffer. The samples were diluted so as to load 10 µg of protein per well in the gel. After dilution of the samples with the buffer, the tubes were tightly capped and gently vortexed. Then an equal amount of 1X loading dye was added to all the wells. 1X loading dye was prepared by mixing 950 µL of Laemmeli sample buffer with 50 µL of 2-mercaptoethanol. The protein samples were then briefly vortexed and spun down and then heated at 90-95°C in a heating block. By this time, the gels, prepared the previous day, were loaded into the chambers to check any leakage of the buffer. After 10 min, the samples were cooled on ice for 10 more min and then spun down for 10 seconds again. The first well of the gel was loaded with 5 µL of protein Fisher-EZ run Pre stained Rec protein ladder (Fisher Scientific, Cat # BP 3603-500) which consists of a mixture

of different proteins with varying molecular weights. Then 10 μL of samples were loaded into the respective wells and the gel was run at 200 V electrophoretically on a electrophoresis chamber (Bio-Rad, Hercules, CA, USA) for about 35-40 min. After the run, the gels were transferred onto the nitrocellulose membranes as a sandwich consisting of this sequence: 1 wet sponge, 1 sheet of 3 mm wet paper, 1 pre-wet nitrocellulose membrane, gel, 1 sheet of 3 mm wet paper and then the wet sponge again. Care was taken to avoid any air bubbles in this step. The gel was set up for transfer in the transferring buffer with continuous stirring at 250 mAmp for 90 min at 4°C. After this the membranes were removed and blocked in a 5% non-fat dry milk (NFDM) solution overnight at 4°C on a horizontal shaker to avoid any non-specific binding. Then the membranes were washed 2 times in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 5 min each. The membranes were then incubated for 1 h at room temperature with the anti-mouse CAR, Anti-LDH or Anti-Lamin A/C antibodies in 50 ml conical tubes consisting of 5 ml of 5% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO, Cat # A3059) solution prepared in TBST. The final dilution of the antibody was determined to be 1:500.

The membranes were washed thrice with TBST followed by incubation with the goat anti-rabbit IgG-alkaline phosphatase secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, Cat # sc-2007) at a dilution of 1:2000 in 5% NFDM for 1 h at room temperature with continuous shaking. Then the

membranes were washed for the final 3 times with TBST and incubated with an electro chemiluminescence reagent (~ 1 ml of reagent was added per membrane) for 10 min covered in dark (I use a box cover and turn off the lights in that lab to protect my membranes from exposure to light). The bands were then analyzed on a FluorChem FC2 Imaging System with chemiluminescence filter initially for 2 min. Depending on the intensity of the bands, exposure time was determined accordingly.

For JNK and P-JNK, the conditions were slightly different. Blocking with NFDM was done for 1 h at room temperature. Membranes were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. The remaining procedure remained constant.

3.15: Collection of blood and plasma:

Reagents:

Heparin sodium salt (2106-10VL) was purchased from Sigma-Aldrich.

Method:

Approximately 10-15 μL of blood samples were collected at predetermined time points (0, 5, 15, 30, 60, 120, 240, 360 and 480 min) from the tail vein in heparinized Eppendorf tubes. The heparinized tubes were prepared on the day before the experiment by coating the tubes with Qtips immersed in a solution of

1000 units heparin prepared in deionized water. Plasma was immediately isolated from the blood by centrifuging the tubes at 8000 rpm for 4 min at room temperature.

3.16: Drug extraction from plasma samples:

CPT-11, SN-38, SN-38G and CPT stock solutions (1mM) were prepared in 50% methanol solution, aliquoted and stored in -80°C. 5 µl of plasma sample from the mouse was diluted in 40 µl blank plasma and 5 µl of IS (CPT, 1 µg/ml) and vortexed briefly. Standard samples were prepared by serial dilutions of 45 µl of the highest standard (1000 ng/ml of CPT-11, SN-38 and SN-38G) with 45 µl blank plasma. Then 5 µl of 1 µg/ml of IS was added and the tubes were briefly vortexed. A mixture of 450 µl acetonitrile (ACN) and 450 µl methanol was added to the samples and standards and the tubes were vortexed for 30 sec and centrifuged at 13,200 rpm for 15 min. The supernatant organic solvent (~825 µl) was transferred to clean eppendorf tubes and evaporated in a gentle stream of air at room temperature. The dried extracts were reconstituted in a solution of 50%ACN/50% methanol/1% glacial acetic acid.

3.17: Pharmacokinetic studies, LC-MS/MS sample preparation and analysis:

For quantification of analytes in plasma, LC-MS/MS method was utilized. In order to quantitate CPT-11, SN-38, SN-38G and CPT in plasma an API 5500

Qtrap triple quadrapole mass spectrophotometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with a Turbospray ^{TM} source was used by multiple reaction monitoring (MRM) method operated in a positive ion mode. The optimization of the LC-MS/MS conditions for analysis of irinotecan and its metabolites was performed as follows. The m/z transition for irinotecan was 587.1/124.1, m/z transition for SN-38 was 393.1/349.1, m/z transition for SN-38G was 569.5/393.1, m/z for CPT was 349.0/305.1 in positive ion mode. The instrument dependent and compound dependent parameters for all the compounds were optimized as described. The following compound dependent parameters were optimized: declustering potential (DP), entrance potential (EP), cell exit potential (CXP) and the collision energy (CE). The following instrument dependent parameters were optimized for the compounds: ion spray voltage, ion source temperature, nebulizer gas (gas 1), turbo gas (gas 2) and the curtain gas. The flow rate of the sample during Mass method optimization was set between 7 - 20 μL/min. The UPLC conditions for the compounds were: system, Waters Acquity™ (Milford, MA, USA) with DAD detector; column, Acquity UPLC BEH C18 column (50×2.1mm I.D., 1.7μm, Waters); mobile phase A, 0.1% formic acid; mobile phase B, 100%, acetonitrile; gradient, 0-0.5 min, 10% B, 0.5-1 min, 25% B, 1-2 min, 40% B, 2-2.5 min, held constant at 40% B, 2.5-3 min, 10% B, respectively. Flow rate was 0.4 ml/min, column temperature, 45 degree; injection volume, 10 μL.

3.18: Pharmacokinetic analysis:

The PK data was analyzed using the WinNonlin 3.3 PK modeling software from Pharsight Corporation (Mountain View, California). The data was fitted to a non-compartmental model for pharmacokinetic analysis of CPT-11 and its metabolites. Pharmacokinetic parameters, including maximum plasma concentration (Cmax), time to reach Cmax (Tmax), elimination rate constant (ke), half-life ($t_{1/2}$), rate at which drug is cleared from the body (CL), volume of distribution (V_d) and plasma exposure or area under the curve (AUC) were directly derived from WinNonlin.

CHAPTER 4

Role of TRIF-dependent signaling in regulation of gene expression of DMEs and transporters

4.1 Abstract

The expression and activity of hepatic drug metabolizing enzymes and transporters (DMETs) is altered on activation of an inflammatory response by bacterial or viral components. Inflammatory responses in the liver are primarily mediated by Toll-like receptor (TLR)-signaling which involves adaptor molecules Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP) and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF). Lipopolysaccharide (LPS) is the ligand involved for gram-negative bacterial receptor, TLR4 and polyinosinic:polycytidylic acid (poly I:C) activates the viral receptor, TLR3. We have shown previously that TIRAP is not involved in LPS-mediated down-regulation of DMETs. To determine the role of TRIF, we treated $TRIF^{+/+}$ and $TRIF^{-/-}$ mice with saline, LPS (2mg/kg) or poly I:C (5mg/kg). LPS treatment led to down-regulation of RNA levels of Cyp3a11 (~80%) and Ugt1a1 (~40%) in $TRIF^{+/+}$ as well as $TRIF^{-/-}$ mice. Poly I:C treatment led to down-regulation of RNA levels of Cyp3a11 (~60%), Cyp2a4 (~40%), Cyp1a2 (~40%), Cyp2b10 (~35%), Ugt1a1 (~45%), Mrp2 (~30%) and Mrp3 (~30%). Surprisingly, this down-regulation was not attenuated in $TRIF^{-/-}$ mice. TLRs promote inflammatory response by activation of several signaling pathways like induction of pro-inflammatory cytokines, IL-6, IL-1β and TNF-α., activation of mitogen-activated protein (MAP) kinases c-Jun-N-terminal kinase (JNK), p38 and extracellular related protein kinase (ERK). Induction of hepatic pro-inflammatory

cytokines by LPS was observed in both $TRIF^{+/+}$ and $TRIF^{-/-}$. The induction of hepatic cytokines is delayed in poly I:C-treated $TRIF^{-/-}$ mice indicating that other mechanisms of recognition of dsRNA may exist. To assess the roles of MAPK, primary hepatocytes were treated with specific inhibitors of JNK, ERK & p38 for 1 h followed by treatment with LPS or poly I:C. We find that the JNK inhibitor completely attenuated the down-regulation of DMETs, while p38 and ERK inhibitors had no effect on down-regulation of DMETs. These results show that down-regulation of DMETs through TLR4 & 3 is independent of TRIF, but likely involves JNK dependent signaling pathways.

4.2: Introduction

Inflammation and infection have been known to decrease the activity of drug metabolizing enzymes and cause changes in clearance of drugs however; the mechanism is still not exactly known (Morgan ET, 1997; Renton KW 2001, 2004). This change in activity of enzymes has been attributed to the alterations in their gene expression (Sewer et el., 1997). Toll like receptors (TLRs) are the major mediators of inflammatory responses in the liver. TLRs promote initial host defense against microorganisms by recognizing distinct microbial/viral components as as pathogen-associated molecular patterns (PAMPs) (Rock et al., 1998).

Upon the recognition of PAMPs, TLR signaling induces potent innate immune responses that signal through adaptor molecules including myeloid differentiation factor 88 (MyD88), Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP), and TIR domain containing adaptor inducing interferon (IFN)-β (TRIF). TLRs promote pro-inflammatory signaling by activating down-stream signaling pathways such as the nuclear factor (NF)-κB, mitogen-activated protein (MAP) kinases, c-Jun-N-terminal kinase (JNK), p38 and extracellular related protein kinase (ERK) pathways in the liver (Abdulla et al., 2005). These cell-signaling components are also involved in regulation of DMEs and transporters directly or by modulating the activity of some of their regulatory

nuclear receptors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Pascussi et al., 2003; Adam-Stitah et al., 1999; Yu et al., 1999; Ghose et al, 2004, 2008; Li et al., 2002). This suggests a role of the nuclear receptors CAR and PXR in mediating expression and activities of DMEs during inflammation.

TLRs are present on non-parenchymal cells including Kupffer cells as well as on hepatocytes in the liver (Akira et al., 2001; Liu et al., 2002; Matsumura et al., 2003). We have shown that activation of TLR4 by lipopolysaccharide (LPS), the gram-negative bacterial components leads to down-regulation of gene expression and activity of DMETs (Ghose et al., 2008, 2009; Gandhi et al., 2012). LPS-treated rodents are well-established models of inflammation, and LPS treatment induces pro-inflammatory cytokines, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α. These cytokines act on hepatocytes to reduce the expression of DME/transporter genes. (Renton KW., 2004; Aitken et al., 2006). TLR3 detects viral double-stranded (ds) RNA in the endo-lysosome. TLR3 is involved in the recognition of polyinosinic polycytidylic acid (poly I: C), a synthetic dsRNA analog (Medzhitov et al, 2002). However, the role of TLR3 regulation of DMETs has not yet been studied.

While TRIF is the only adaptor protein involved in mediating TLR3-signaling pathway, TLR4 signaling can be mediated by TRIF or TIRAP. TRIF is important

for induction of interferon (IFN)-regulating factor 3, production of INF-β and also for activation and maturation of dendritic cells (Yamamato et al., 2003). TRIF is involved in mediating NF-κB activation in response to TLR3 ligands and is involved in the MyD88-independent prolonged NF-κB activation in response to TLR4 ligands (Yamamato et al., 2003). There is direct interaction between TLR3 and TRIF but the interaction between TRIF and TLR4 is mediated by TRAM (Yamamato et al., 2003). We found no role of TIRAP in TLR4 mediated DMETs down-regulation and cytokine induction in the liver however; the plasma cytokine induction by LPS seems to be mediated by TIRAP (Ghose et al., 2011).

The goal of this paper is to determine the role of TRIF in TLR3 and TLR4-mediated down-regulation of DMETs. We find that LPS administration led to significant down-regulation of gene expression of key DMEs including Cyp3a11 and Ugt1a1 in $TRIF^{+/+}$ as well as $TRIF^{-/-}$ mice. Induction of pro-inflammatory cytokines, IL-1β, IL-6 & TNF-α was also not attenuated in LPS-treated $TRIF^{-/-}$ mice indicating no role for TRIF in LPS signaling. Upon treatment of $TRIF^{+/+}$ mice with poly I:C, we see significant down-regulation of gene expression of Cpy3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sult1a1 and Mrp2. Surprisingly, the down-regulation of DMETs by poly I:C was not attenuated in $TRIF^{-/-}$ mice. Poly I:C administration also leads to induction of gene expression of pro-inflammatory cytokines IL-1β, IL-6 & TNF-α however, this induction is delayed in $TRIF^{-/-}$ mice indicating that alternate pathways may be involved in recognizing poly I:C-

mediated responses. In order to study the role of MAP kinases in regulating gene expression of DMETs, primary hepatocytes were treated with specific inhibitors of MAP kinases before treatment with LPS or poly I:C. We find that inhibition of JNK led to complete attenuation of LPS and poly I:C-mediated down-regulation of gene expression of DMETs

Our results indicate that TRIF is not involved in either TLR4 or TLR3-mediated down-regulation of DMETs. We had previously seen that TLR4 activation caused down-regulation of DMETs in a TIRAP–independent manner. Recent studies have shown that dsRNA including poly I:C can also activate cytoplasmic receptors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Thus it is possible that in the absence of TRIF, poly I:C activates the alternate pathways to reduce DMET genes. Our results indicate that the down-regulation of DMETs during TLR4 and TLR3-induced inflammation could most likely involve JNK-dependant signaling pathways. Overall these studies show that there is redundancy in signaling pathways and targeting multiple signaling components may be important to counter deleterious consequences of inflammation in DMET genes.

4.3: Materials and methods:

4.3.1: Materials

Highly purified and LPS (*Escherichia coli*) and Poly I:C (high molecular weight) was purchased from InvivoGen (San Diego, U.S.A) and freshly diluted to the required concentration in 0.9% saline. The sequences of the primers and probes were obtained from the literature as reported previously (Shah et al., 2013). All oligonucleotides were purchased from Sigma Genosys (Houston, U.S.A) and all real-time PCR reagents were purchased from Applied Biosystems, (Foster city, CA, U.S.A.). Anti-JNK (#9252) and anti-phospho-JNK (#9251) were purchased from Cell-Signaling (Beverly, MA, USA). Anti-IκB-α antibody (sc-371) was purchased from Santa Cruz biotechnology, San Diego, USA. JNK inhibitor (SP600125); cat# 420119, ERK inhibitor (PD98059); cat# 513000, and p38 inhibitor (SB203580); cat# 559389 were purchased from Calbiochem, USA. Cell culture media and media supplements were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Unless specified, all other materials were purchased from Sigma-Aldrich (St Louis, MO, USA.).

4.3.2: Animals

C57BL/6 mice (~6-8 weeks) weighing 20–25 g were obtained from Harlan Laboratories (Houston, Texas, USA). $TRIF^{+/+}$ and $TRIF^{-/-}$ mice (~6-8 weeks)

were obtained from Jackson laboratories (Bar Harbor, Maine). The animals were maintained in a temperature and humidity-controlled environment and were provided with water and rodent chow ad libitum. All animal experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

4.3.3: Treatments

$TRIF^{+/+}$ and $TRIF^{-/-}$ mice were intraperitoneally (i.p.)-injected with saline, poly I:C (5 mg/kg body wt.) or LPS (2 mg/kg body wt.) for 1, 4, 8 & 16 h. Livers were harvested and cryopreserved in liquid nitrogen and stored at -80°C for further studies. All experiments were performed in triplicate and repeated three to four times.

4.3.4: real-time PCR

Total RNA was isolated from mouse liver using TRIzol reagent (Sigma-Aldrich, St Louis, MO, U.S.A) according to the manufacturer's protocol. cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems) as described previously (Shah et al., 2013). In short, each reaction mixture (total of 25µl) contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer,

200 nM fluorogenic probe, and 15 μl of TaqMan Universal PCR Master Mix. We extrapolated the quantitative expression values from standard curves and these values were normalized to cyclophilin.

4.3.5: Primary hepatocyte isolation and treatment

Primary mouse hepatocytes were isolated from C57BL/6 mice using the two step collagenase perfusion technique as described previously (Li et al., 2002; Ghose et al., 2011; Shah et al., 2013). In short, after digestion, the liver was excised and then the hepatocytes were purified using a percoll gradient (33%), washed and screened for viability using trypan blue exclusion technique. Only isolations with viability of more than 90% were used for these studies. Cells were plated at a density of 500,000 cells per well in six-well Primaria plates (BD biosciences, San Diego, CA, U.S.A) and allowed to attach for 4 h. Cells were maintained for 48 h with daily change of medium.

The cells were incubated with serum-free Williams medium E two hours prior to treatment with 50 μg/ml poly I:C (8 h) or 1 μg/ml LPS (16 h). In order to identify the role of MAP kinases, the cells were pre-treated with specific inhibitors 1 h prior to treatment with poly I:C or LPS. RNA was then isolated for real-time PCR analysis as described above.

4.3.6: Immunoblotting

Whole cell extracts were prepared as described previously (Ghose et al., 2004) and the protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A). Equal amounts of protein (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were probed with anti-JNK (1:500), anti-P-JNK (1:500) or IκB-α (1:1000) antibody followed by probing with a goat anti-rabbit IgG-AP secondary antibody (1:2000). The membranes were then washed and incubated with Tropix® CDP star® nitroblock II ^{TM} ECL reagent as per the manufacturers' instructions (Applied Biosystems, CA, U.S.A.). The membranes were analyzed using FlourChem FC imaging system (Alpha Innotech). The images were quantified by densitometer using AlphaEase software.

4.3.7: Statistical analysis

Treatment groups were compared using Students t-test with $p < 0.05$. The results were presented as mean $\pm$ SD.

4.4: Results:

4.4.1: Role of TRIF in down-regulation of gene expression of DME/transporters in LPS-treated mice

We have previously shown that administration of LPS leads to down-regulation of gene expression of Cyp3a11 and Ugt1a1 and this down-regulation is independent of TIRAP (Ghose et al., 2008). In order to investigate the role of TRIF in LPS-mediated down-regulation of DMEs, we treated $TRIF^{+/+}$ and $TRIF^{-/-}$ mice with LPS. RNA was isolated from the livers harvested at the indicated time points and analyzed by real-time PCR. RNA levels of Cyp3a11 (~80%) and Ugt1a1 (~50-55%) were significantly down-regulated in LPS-treated $TRIF^{+/+}$ mice. These results are in agreement with our previous results. However, this down-regulation was not attenuated in $TRIF^{-/-}$ mice indicating redundancy in TLR4 signaling pathways (Fig 4.4.1.).

Fig 4.4.1.

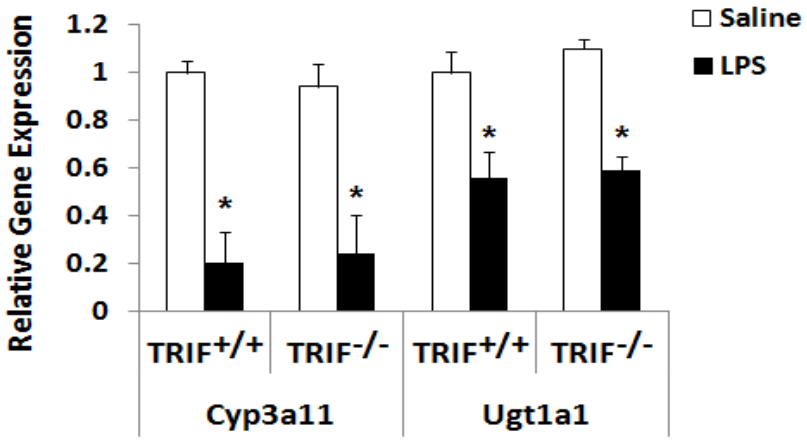


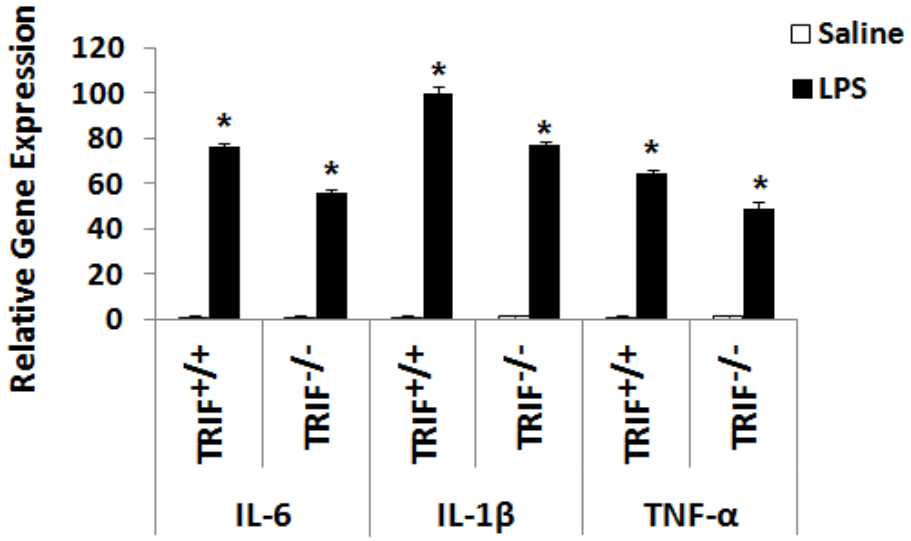
Fig: 4.4.1.: Regulation of DME mRNA levels in TRIF$^{+/+}$ and TRIF$^{-/-}$ mice following LPS administration

TRIF$^{+/+}$ and TRIF$^{-/-}$ were i.p. injected with saline or LPS (2 mg/kg) and livers were harvested at 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of Cyp3a11 & Ugt1a1 were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LPS-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and LPS groups. The experiments were repeated at least thrice.

4.4.2: Regulation of cytokine mRNA levels in $TRIF^{+/+}$ & $TRIF^{-/-}$ mice following LPS-administration

We have also shown that the induction of cytokines in the liver on activation of TLR4 is independent of the adaptor protein TIRAP (Ghose et al., 2008). Our goal is to identify the role of TRIF in TLR4-mediated induction of cytokines. As seen earlier, on activation of TLR4, there is significant induction of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in $TRIF^{+/+}$ mice and there is no attenuation of this induction in LPS-treated $TRIF^{-/-}$ mice (Fig 4.4.2). Our results indicate that there is redundancy in TLR4 signaling in that signaling can proceed through TIRAP or TRIF. Inhibition of both TIRAP and TRIF in $TRIF^{-/-}$/$TIRAP^{-/-}$ double knockout mice most likely block the down-regulation of DMETs by LPS.

Fig 4.4.2



4.4.2: Regulation of cytokine mRNA levels in TRIF$^{+/+}$ & TRIF$^{-/-}$ mice following LPS administration

TRIF$^{+/+}$ and TRIF$^{-/-}$ mice were i.p. injected with saline or LPS (2 mg/kg) and livers were harvested at 1 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNF-α were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LPS-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and LPS groups. The experiments were repeated at least thrice.

4.4.3: Regulation of gene expression of DME/transporters in poly I:C-treated C57BL/6 mice

Our next goal was to determine the role of the TLR3 ligand, poly I:C in regulating gene expression of DMETs. This will provide the basis for further investigation into the role of TRIF. Thus we treated C57BL/6 mice with 5 mg/kg of Poly I:C for 8 & 16 h. We show for the first time that activation of TLR3 by poly I:C leads to down-regulation of several DMET genes. RNA levels of the key phase I enzyme, Cyp3a11 were significantly down-regulated at 8 h (~35%) and 16 h (~60%) in C57BL/6 mice. RNA levels of Cyp1a2 and Cyp2a4 (~30-40%) were significantly down-regulated at 8 and 16 h. Cyp2b10 RNA levels were down-regulated (~35%) at 8 h. The RNA levels of key phase II genes, Ugt1a1 and Sult1a1 were down-regulated significantly (~40% and ~60% respectively) at 8 h. RNA levels of key transporters, Mrp2 and Mrp3 were significantly (~30-35%) down-regulated at 8 and 16 h respectively (Fig 4.4.3).

Fig 4.4.3

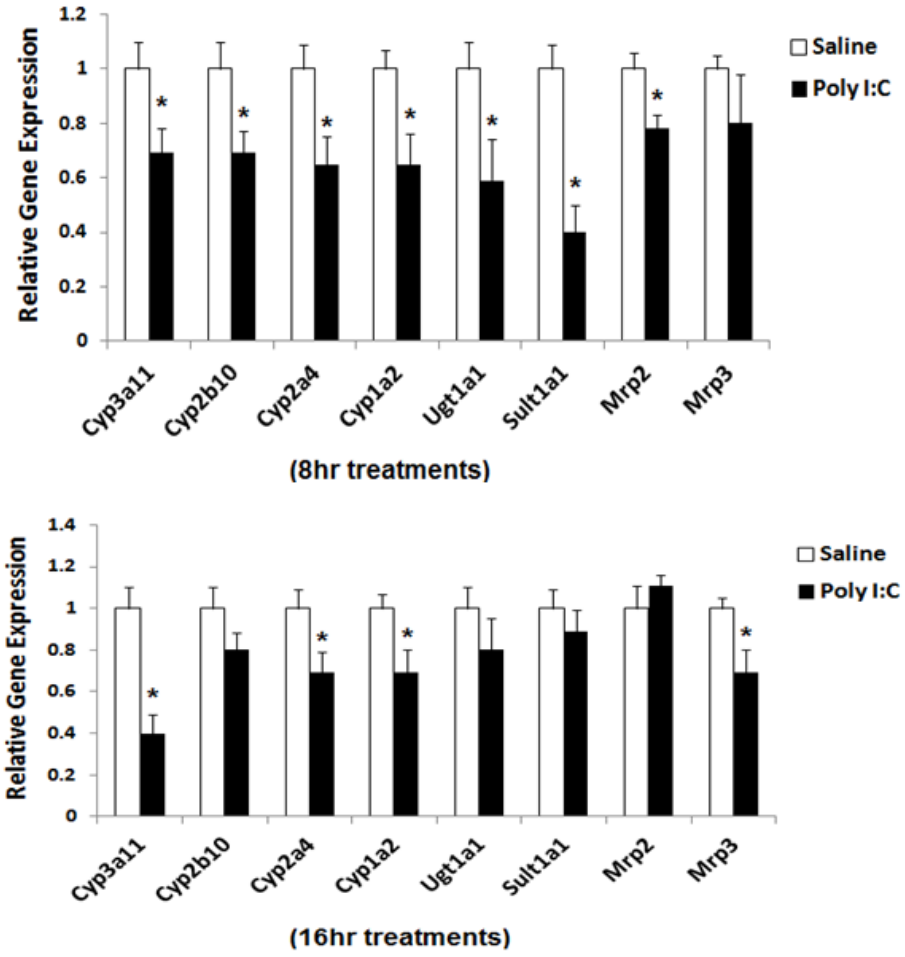


Fig 4.4.3: Regulation of DMET mRNA levels in C57BL/6 by poly I:C

C57BL/6 mice were i.p. injected with saline or poly I:C (5 mg/kg) and livers were harvested at 8 & 16 h (*n* = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I, phase II and transporter genes were determined by real-time PCR

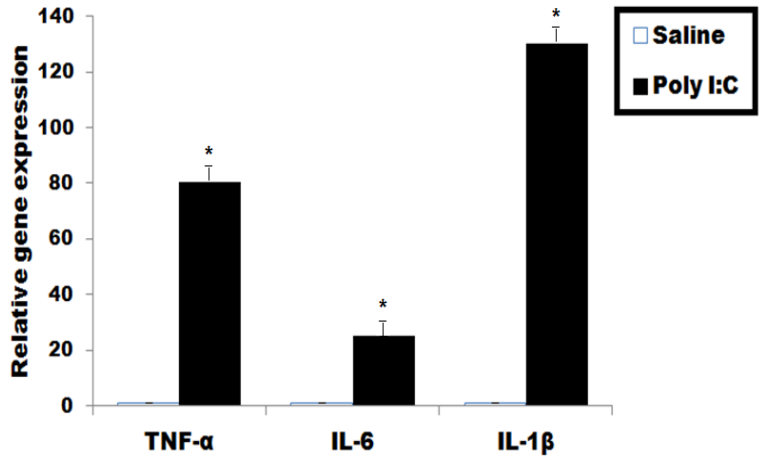
analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after poly I:C-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and poly I:C groups. The experiments were repeated at least thrice.

4.4.4: Activation of cytokines, nuclear receptors (NRs) and cell-signaling pathways by poly I:C

It is known that cytokines play an important role in altering the gene expression of DMETs in hepatocytes. We have previously shown that activation of TLR2 and TLR4 leads to the induction of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α (Ghose et al., 2008; 2009). JNK and NF-κB are known to be critical components of the TLR signaling pathway (Kawai et al., 2005) and the alterations in hepatic DMETs in LPS-induced inflammation involves cross-talk between cell-signaling and NRs. We have also shown that activation of TLR4 and TLR2 leads to the activation of JNK and NF-κB and alterations in expression of nuclear receptors (Ghose et., 2008; 2009). However, it is not clear if activation of poly I:C will lead to activation of these signaling components and whether they play a role in mediating the effects on DMETs. We show that on treatment of poly I:C there is significant induction of mRNA levels of pro-inflammatory cytokines, IL-1β (~120-folds), IL-6 (~25-folds) and TNF-α (~80-folds) (Fig 4.4.4.1). There is down-regulation of the gene expression of CAR and RXRα at 8 and 16 h respectively while there is a slight up-regulation of gene expression of PXR at 16 h (Fig 4.4.4.2). We also see activation of phosphorylated JNK (P-JNK) in whole cell extracts (Fig 4.4.4.3). Activation of NF-κB was determined by degradation of the inhibitory subunit, IκBα after poly I:C treatment (Fig. 4.4.4.3). We see

activation of NF-κB at 1 h indicating that these signaling components are involved in mediating TLR3-signaling pathways as well.

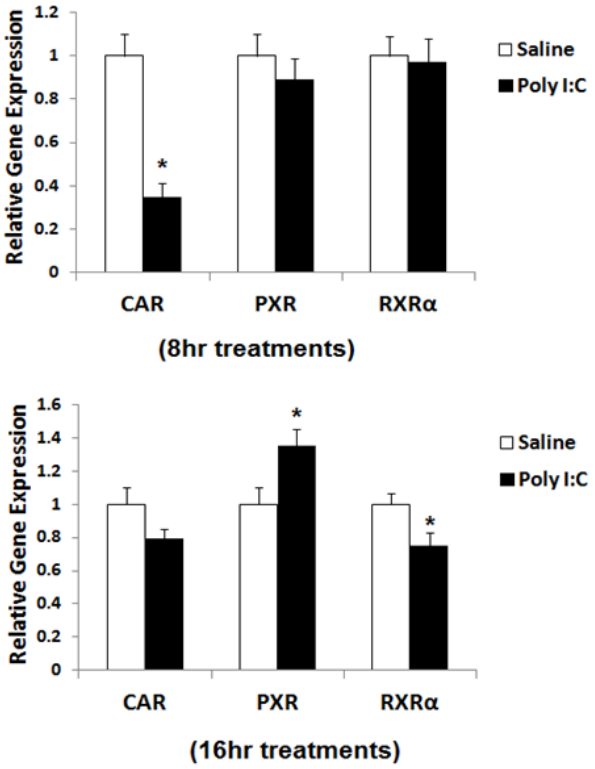
Fig 4.4.4.1



4.4.4.1: Regulation of cytokines on activation of TLR3

C57BL/6 mice were i.p. injected with saline or poly I:C (5 mg/kg) and livers were harvested at 1 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNF-α were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after poly I:C-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and poly I:C groups. The experiments were repeated at least thrice.

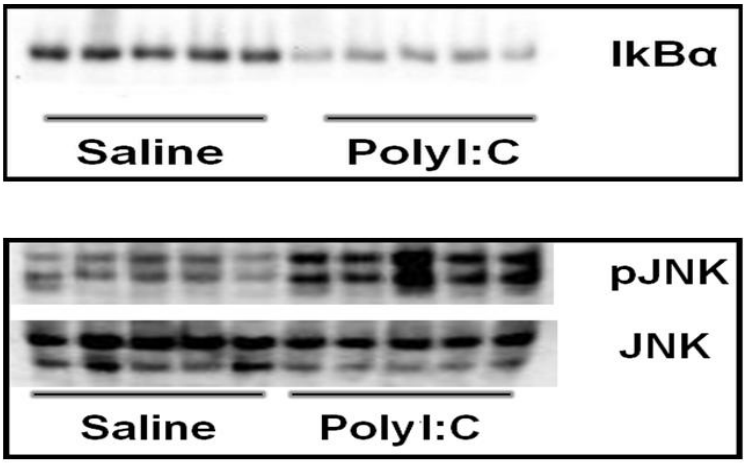
Fig 4.4.4.2



4.4.4.2: Regulation of nuclear receptors by poly I:C

C57BL/6 mice were i.p. injected with saline or poly I:C (5 mg/kg) and livers were harvested at 8 and 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of CAR, PXR and RXRα were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after poly I:C-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and poly I:C groups. The experiments were repeated at least thrice.

Fig 4.4.4.3



4.4.4.3: Regulation of cell-signaling pathways on by poly I:C

Whole cell extracts were prepared from the livers of saline and poly I:C-injected mice at 1 h, and the samples were analyzed by immunoblotting. The phosphorylated forms of JNK (P-JNK) and degradation of IkBα were measured as markers of JNK and NF-κB activation, respectively. The experiments were repeated at least thrice.

4.4.5: Role of TRIF in down-regulation of gene expression of DMETs in poly I:C-treated TRIF$^{+/+}$and TRIF$^{-/-}$ mice

Since we have shown that the TLR3 ligand poly I:C down-regulates DMET genes, our next goal was to determine the role of TRIF in poly I:C-mediated regulation of DMET genes. Since we saw maximal down-regulation of DMETs at 8 h on treatment with poly I:C, we chose the 8 h time-point for studies in TRIF$^{-/-}$ mice. We find that the RNA levels of Cyp3a11 (~40%), Cyp2a4 (~40%), Cyp2b10 (~45%), Ugt1a1 (~50%), Sult1a1 (~50%) and Mrp2 (~30%) were significantly down-regulated at in TRIF$^{-/-}$ mice at 8 h and the extent of down-regulation was similar to that in TRIF$^{+/+}$ mice (Fig 4.4.5).

These results show that the down-regulation of DMETs through TLR3 is independent of TRIF.

Fig 4.4.5

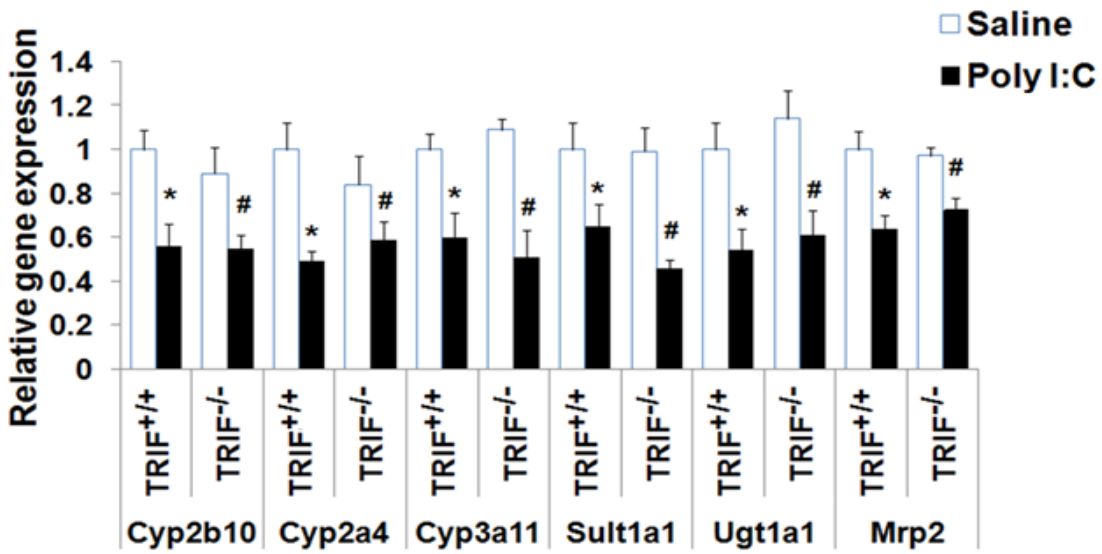


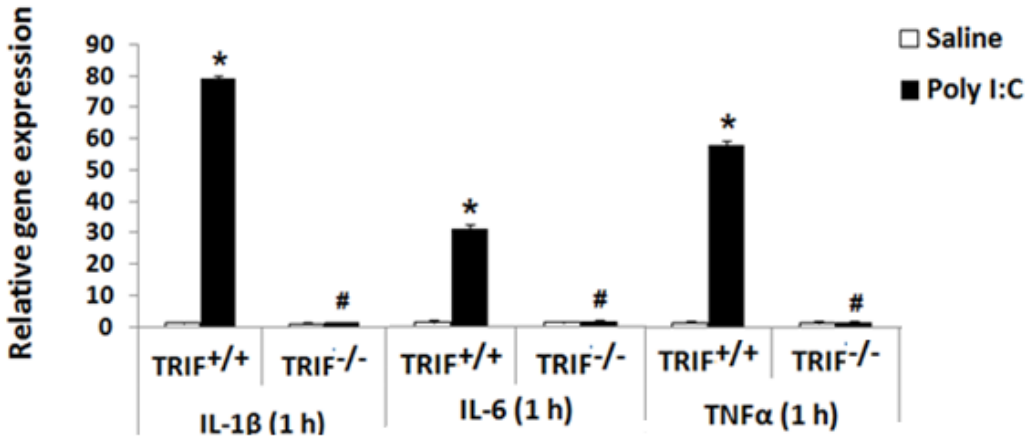
Fig 4.4.5 Regulation of DMET mRNA levels in TRIF$^{+/+}$ and TRIF$^{-/-}$ mice following poly I:C administration

TRIF$^{+/+}$ and TRIF$^{-/-}$ were i.p. injected with saline or poly I:C (5 mg/kg) and livers were harvested at 8 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I, phase II and transporter genes were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after poly I:C-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and poly I:C group in TRIF$^{+/+}$ mice and # indicates significant difference ($p < 0.05$) between saline and poly I:C group in TRIF$^{-/-}$ mice. The experiments were repeated at least thrice.

4.4.6: Regulation of cytokine mRNA levels in TRIF$^{+/+}$ & TRIF$^{-/-}$ mice

Induction of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in TRIF$^{+/+}$ mice is completely attenuated in poly I:C-treated TRIF$^{-/-}$ mice at 1 h (4.4.6). Since cytokines have been implicated in altering the gene expression of DMETs, it is surprising that there is no induction of cytokine gene expression in TRIF$^{-/-}$ mice treated with poly I:C. Hence, we studied the induction of cytokines at 4 h and we find that there is significant induction of IL-1β (~60-folds), IL-6 (~35-folds) and TNF-α (~40-folds) in poly I:C-treated TRIF$^{-/-}$ mice (Data not shown). There is a delayed response in the induction of cytokines in TRIF$^{-/-}$ mice treated with poly I:C suggesting that poly I:C can potentially mediate viral responses through multiple mechanisms. We did not find any differences in the plasma levels of IL-1β in poly I:C-treated TRIF$^{+/+}$ and TRIF$^{-/-}$ mice at 1 h.

Fig 4.4.6



4.4.6: Regulation of cytokine mRNA levels in TRIF$^{+/+}$ & TRIF$^{-/-}$ mice following poly I:C administration

TRIF$^{+/+}$ and TRIF$^{-/-}$ were i.p. injected with saline or poly I:C (5 mg/kg) and livers were harvested at 1 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of IL-1β, IL-6 and TNF-α were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after poly I:C-treatment was compared to the saline-treated controls. ***** indicates significant difference ($p < 0.05$) between saline and poly I:C groups. # indicates significant difference in poly I:C-treated TRIF$^{+/+}$ and TRIF$^{-/-}$ mice. The experiments were repeated at least thrice.

4.4.7: Role of MAP kinases; JNK, ERK and p38 in TLR4 & TLR3 mediated DMET down-regulation

MAP kinases have been implicated in altering the gene expression of DMET during inflammation due to their cross-talk with NRs. In order to determine their roles in TLR4 and TLR3-mediated down-regulation of DMETs, we isolated primary hepatocytes from C57BL/6 mice and treated them with chemical inhibitors of JNK, ERK and p38, 1 h before treatments with LPS (1 μg/ml; 16 h) or poly I:C (50 μg/ml; 8 h). We see that on treatments of primary hepatocytes with LPS, there is down-regulation of gene expression of Cyp3a11 and Ugt1a1 (Fig 4.4.7.1). Poly I:C treatment leads to down-regulation of gene expression of Cyp3a11, Cyp2a4 and Sultn (Fig 4.4.7.2). This down-regulation of DMEs is completely attenuated on treatment of specific JNK inhibitor where as ERK and p38 inhibitor have no effects in attenuating the LPS or poly I:C-mediated down-regulation of DMEs. Thus our results indicate that the down-regulation of DMEs through TLR4 and TLR3 is JNK-mediated.

Fig 4.4.7.1

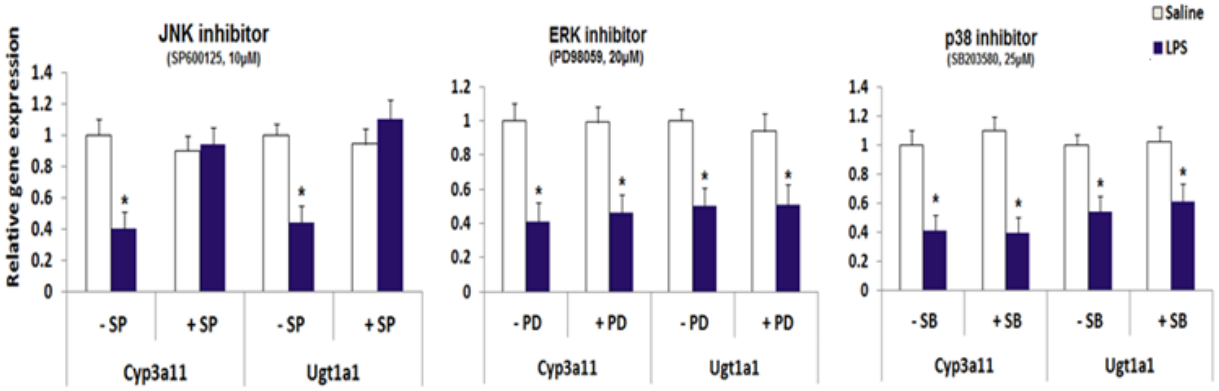
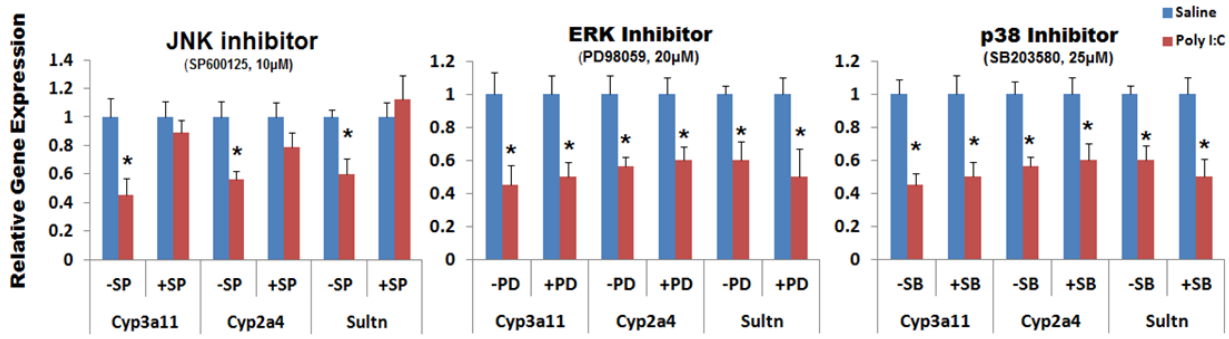


Fig 4.4.7.2



4.4.7: Regulation of DMEs by MAP kinases on LPS or poly I:C treatments

Primary hepatocytes from C57BL/6 mice were treated with DMSO or JNK inhibitor (SP600125, 10 μM), ERK inhibitor (PD98059, 20 μM) or p38 inhibitor (SB203580, 25

μM) for 1 h, before treatment with saline or LPS (1 μg/ml) (upper panel) or poly I:C (50 μg/ml) (lower panel) for 8 h. RNA was isolated, and real-time PCR was performed as described earlier. n = 5-6 per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. ***** indicate significant difference ($p < 0.05$) between saline and LPS or poly I:C groups. The experiments were repeated at least thrice.

4.5: Discussion:

Infection and inflammation can alter the expression and activities of several DMETs (Hartmann et al., 2001). This can have potentially harmful consequences in patients and it is extremely important to understand the mechanisms behind alterations of DMET during inflammation. We have shown that expression and activities of hepatic DMETs are altered in a gram-negative and gram-positive bacterial-inflammatory model however, the role of viral infections in altering DMETs has not been studied (Ghose et al., 2009; Gandhi et al., 2012). We show for the first time that activation of TLR3 by poly I:C leads to down-regulation of gene expression of DMET, induction of pro-inflammatory cytokines, alterations in nuclear receptor levels and activation of cell signaling components. We also show that the down-regulation of DMETs on activation of TLR4 and TLR3 is independent of TRIF however *in vitro* results show that it is dependent on the MAP kinase, JNK.

Activation of TLR4 can recruit TIRAP or TRIF. Initial studies with $TIRAP^{-/-}$ mice had shown that TIRAP is important for the induction of cytokines by LPS in dendritic cells and peritoneally derived macrophages (Horng et al., 2002; Yamamoto et al., 2002). However, our studies with $TIRAP^{-/-}$ mice indicated that TIRAP is not essential for induction of hepatic cytokines or down-regulation of hepatic DMETs through TLR4 signaling (Ghose et al., 2008). In this study, we

show that LPS administration leads to induction of pro-inflammatory cytokines and down-regulation of DMETs in $TRIF^{+/+}$ as well as $TRIF^{-/-}$ mice. Our results indicate that LPS-mediated signaling can proceed through TIRAP or TRIF and experiments in TIRAP/TRIF double-knockout mice will most likely block the effects of LPS on DMET genes. If double knockout mice are not able to block the responses of LPS on DMET, it could be because TIRAP is not essential for recruitment of Myd88 and TLR4 signaling. If that is the case, LPS mediated down-regulation of DMET would be blocked in Myd88/TRIF double-knockout mice.

Poly I:C administration led to down-regulation of several DMETs such as Cyp3a11, Cyp2a4, Cyp2b10, Cyp1a2, Ugt1a1 Sult1a1, Mrp2 and Mrp3 genes in $TRIF^{+/+}$ mice at 8 and 16 h. We show for the first time that TLR3 activation by viral components can down-regulate gene expression of hepatic DMET genes. We also show that activation of TLR3 by poly I:C leads to (a) induction of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, (b) alterations of nuclear receptor levels of CAR, PXR and RXRα and (c) activation of signaling components like JNK and NF-κB. Since TRIF is the only adaptor protein involved in TLR3 signaling, we expected attenuation of down-regulation of DMET genes in $TRIF^{-/-}$ mice. Surprisingly, the down-regulation of DMETs on poly I:C treatment is not attenuated in $TRIF^{-/-}$ mice. Since cytokines have been implicated in altering gene expression of DMETs during inflammation, we

expected no changes in the induction pattern of pro-inflammatory cytokines in poly I:C-treated TRIF$^{-/-}$ mice. Surprisingly, the induction of IL-1β, IL-6 and TNF-α was completely abolished in poly I:C-treated TRIF$^{-/-}$ mice at 1 h. Further investigation showed that this induction is delayed and there is significant induction of these pro-inflammatory cytokines at 4 h (data not shown). This indicates that in the absence of TRIF, poly I:C is likely activating other signaling pathways to induce the cytokine expression in the liver.

RIG-I and MDA5 are cytoplasmic DEx (D/H) box helicases that contain caspase-recruiting domains (CARDs) and can detect intracellular viral products such as genomic RNA to signal IRF3, IRF7 & NF-κB (Onoguchi et al., 2007; Yoneyama et al., 2004). These receptors are important in sensing dsRNA because dendritic cells or fibroblast cells lacking in TLR3 secrete type I IFNs on exposure to dsRNA (Hemmi et al., 2004). RIG-I and MDA5 have been implicated in mediating responses to dsRNA infections (Yoneyama et al., 2005; Kovacsovics et al., 2002). IFN-β promoter stimulator (IPS-1) is an adaptor protein that mediates RIG-I- and MDA5-dependent antiviral responses (Akira et al., 2005). IPS-1 is also called as CARDIF. It has been shown that induction of plasma IFN-α/β and IL-6 is not attenuated in TRIF$^{-/-}$ mice however, the induction of gene expression of IFN-α/β in the spleen is attenuated and delayed in TRIF$^{-/-}$ mice treated with poly I:C (Kumar et al., 2008). Cytokine production is totally inhibited only in

IPS-1 and TRIF-double knockout mice indicating that both TRIF and IPS-1 play important roles in mediating anti-viral responses.

It is possible that in our study that the delayed induction of cytokines in $TRIF^{-/-}$ mice is due to RIG-I and MDA5-mediated signaling. Thus, these cytokines and the redundancy of down-stream pathways (IPS-1 and TRIF) is likely responsible for DMET down-regulation by poly I:C in $TRIF^{-/-}$ mice. Studies in poly I:C-treated IPS-1/TRIF double knockout mice will be able to prevent the down-regulation of DMETs during viral infections.

Inflammation and infection leads to alterations in the activity of a variety of target genes and transcription factors through several signal transduction cascades, including the three main MAP kinases, JNK, ERK and p38. In order to investigate the roles of JNK, ERK and p38 in regulating DMET genes during gram negative and viral infections, we treated primary hepatocytes with specific inhibitors 1 h prior to treatment with LPS or poly I:C. We find that the TLR4 and TLR3-mediated down-regulation of DMETs is completely attenuated by the JNK inhibitor where as ERK and p38 inhibitors have no effects on attenuating the down-regulation of DMETs. JNK is found in multiple isoforms. JNK1 and JNK2 are present in most tissues and JNK3 is found almost exclusively in the brain. JNK1/JNK2 double knockout mice are embryonically lethal and JNK1 knockout and JNK2 knockout mice show redundancy in mediating the effects of LPS in the

liver (Karpen et al., 2009). Our in vitro studies indicate that JNK could be a potential target to reverse or attenuate inflammation-mediated effects on drug metabolism.

Our study is the first of its kind to show that activation of TLR3 will lead to down-regulation of gene expression of several DMETs. The down-regulation of DMETs and transporters during inflammation and infection is a complex process, and the molecular mechanisms are not fully understood. We show that the adaptor protein TRIF is not involved in the TLR4 or TLR3-mediated down-regulation of gene expression of DMETs however; the MAP kinase JNK is a major player in regulating DMET genes during infections caused by gram-negative and viral components. Redundancy in signaling pathways indicate that multiple pathways may be needed to be targeted to counter the deleterious effects of bacterial or viral infections on hepatic detoxification process.

CHAPTER 5

Role of constitutive androstane receptor in Toll-like receptor 2 & 4-mediated regulation of gene expression of hepatic drug metabolizing enzymes and transporters

5.1: Abstract:

Impairment of drug disposition in the liver during inflammation has been attributed to down-regulation of gene expression of drug metabolizing enzymes (DMEs) and drug transporters. Inflammatory responses in the liver are primarily mediated by toll-like receptors (TLRs). We have recently shown that activation of TLR2 or TLR4 by lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively, leads to the down-regulation of gene expression of DMEs/transporters. However, the molecular mechanism underlying this down-regulation is not fully understood. The xenobiotic nuclear receptors, pregnane-X-receptor (PXR) and constitutive androstane receptor (CAR) regulate the expression of DMEs/transporter genes. Down-regulation of DMEs/transporters by LTA or LPS was associated with reduced expression of PXR and CAR genes. To determine the role of CAR, we injected, $CAR^{+/+}$ and $CAR^{-/-}$ mice with LTA or LPS, which significantly down-regulated (~40-60%) RNA levels of the DMEs, Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and the transporter, Mrp2 in $CAR^{+/+}$ mice. Suppression of most of these genes was attenuated in LTA-treated $CAR^{-/-}$ mice. In contrast, LPS-mediated down-regulation of these genes was not attenuated in $CAR^{-/-}$ mice. Induction of these genes by mouse CAR activator 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4 bis(pyridyloxy)benzene (TCPOBOP) was sustained in LTA but not in LPS-treated mice. Similar observations were obtained in humanized CAR mice. We

have replicated these results in primary hepatocytes as well. Thus LPS can down-regulate DME/transporter genes in the absence of CAR whereas, the effect of LTA on these genes is attenuated in the absence of CAR, indicating the potential involvement of CAR in LTA-mediated down-regulation of DME/transporter genes.

5.2: Introduction:

Impairment of drug disposition during inflammation is attributed to down-regulation of gene expression of phase I and phase II drug metabolizing enzymes (DMEs) and transporters (Sewer et el., 1997; Renton & Nicholson, 2000). The modulation of DMEs and transporters at the transcriptional level is regulated by basal transcription factors as well as the xenobiotic nuclear receptors (NRs), pregnane-X-receptor (PXR) and constitutive androstane receptor (CAR). The NRs heterodimerize with retinoid-X-receptor (RXR)α to bind to the promoter regions of the target genes (Shen & Kong, 2009; Zordoky & El-Kadi, 2009; Mangelsdorf et al., 1995; Kakizaki et al., 2008; Tien & Negishi, 2006). The molecular mechanism by which DMEs and transporters are down-regulated during inflammation is not fully understood.

Toll-like receptors (TLRs) are the major mediators of inflammatory responses in the liver, and recognize microbial components as well as endogenous ligands from damaged or stressed cells (Takeda & Akira, 2001; Ishii & Akira, 2004; Schwabe et al., 2006). TLRs are present on non-parenchymal cells including Kupffer cells as well as on hepatocytes in the liver (Liu et al., 2002; Matsumura et al., 2003). We have shown that activation of TLR4 by LPS (gram-negative bacterial component) down-regulates the gene expression of select DMEs and transporters (Ghose et al., 2008). LPS-treated rodents are well-established models

of inflammation, and LPS treatment induces pro-inflammatory cytokines, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)α. These cytokines act on hepatocytes to reduce the expression of DME/transporter genes. (Renton KW., 2004; Aitken et al., 2006). We have also shown that activation of TLR2 by LTA (gram-positive bacterial component) down-regulates the expression of select DME and transporter genes (Ghose et al., 2009, 2011).

Several studies have shown that down-regulation of PXR and CAR genes was associated with decreased DME and transporter gene expression (Beigneux et al., 2000, 2002; Ghose et al., 2004, 2008, 2009). We have also shown that there is preferential suppression of CAR and its target hepatic genes on administration of LTA (Ghose et al., 2009). Thus, it is likely that nuclear receptors play an important role in inflammation-mediated down-regulation of gene expression of DMEs and transporters.

Studies in $PXR^{-/-}$ mice showed that PXR is not responsible for the regulation of cytochrome P450 (Cyp) genes during LPS-mediated inflammation (Richardson & Morgan, 2005; Hartmann et al., 2001). On the other hand, PXR was shown to be involved in down-regulation of Cyp3a11, multi drug resistance-associated protein (Mrp)2 and bile salt exporter pump in IL-6 treated mice (Teng & Piquette-Miller, 2005).

The goal of this paper is to determine the role of CAR in down-regulation of DMEs and transporters by comparing the effects of LTA or LPS in $CAR^{+/+}$, $CAR^{-/-}$ and CAR-activated mice (treated with mouse CAR activator, TCPOBOP). We find that LTA administration led to significant down-regulation of gene expression of key DMEs, Cyp3a11, Cyp2a4, Cyp2b10, uridine diphosphate glucuronosyltransferase (Ugt)1a1, amine N-sulfotransferase (Sultn), and the transporter, Mrp2 in $CAR^{+/+}$ mice. Down-regulation of Cyp3a11, Cyp2a4, Cyp2b10 and Ugt1a1 was completely attenuated whereas the down-regulation of Sultn and Mrp2 was attenuated at certain time-points in LTA-treated $CAR^{-/-}$ mice. On the other hand, LPS administration led to significant down-regulation of Cyp3a11, Cyp2a4, Ugt1a1 and Mrp2 in $CAR^{+/+}$ as well as $CAR^{-/-}$ mice. Activation of mouse CAR with TCPOBOP increased nuclear CAR protein levels and induced the expression of all the above-mentioned genes as expected. Surprisingly, the induction of all these genes, and increased CAR protein levels in the nucleus was still detected in the presence of LTA. On the other hand, the induction of these genes by TCPOBOP was attenuated in the presence of LPS, along with attenuation of CAR protein levels in the nucleus. We have replicated the above results in primary hepatocytes as well. These results were also confirmed in humanized CAR (hCAR) mice treated with the universal CAR activator, PB.

The outcome of these studies demonstrates that CAR is required for down-regulation of hepatic DME/transporter genes by LTA, but LPS can down-regulate these genes in the absence of CAR. Surprisingly, induction of CAR-mediated genes in TCPOBOP-treated mice was maintained in LTA, but not in LPS-injected mice. This was likely due to the inability of LTA, in the presence of TCPOBOP to down-regulate gene, and consequently protein expression of CAR. These results indicate that differential CAR expression may contribute to the regulation of hepatic DMEs and transporters upon infections by gram-positive and gram-negative bacterial components.

5.3: Materials and methods

5.3.1: Materials:

Highly purified lipoteichoic acid (*Staphylococcus aureus*) and lipopolysaccharide (*Escherichia coli*) were purchased from InvivoGen (San Diego, U.S.A) and freshly diluted to the required concentration in 0.9% saline. The sequences of the primers and probes were obtained from the literature as reported previously (Ghose et al., 2004, 2009, 2011). All oligonucleotides were purchased from Sigma Genosys (Houston, U.S.A) and all real-time PCR reagents were purchased from Applied Biosystems, (Foster city, CA, U.S.A.). Rabbit anti-CAR antibody (#sc-8538), lactate dehydrogenase (LDH) (#sc-33781) and lamin A/C (#sc-20681) (Santa Cruz biotechnology CA, U.S.A) were used as per the manufacturer's instructions. Midazolam (Cat # 451028) was purchased from BD Biosciences (San Diego, CA, USA). Phenacetin (Cat # 77440) and 1'-hydroxymidazolam (Cat # UC430) were purchased from Sigma Aldrich (St. Louis, MO, USA). 1'-hydroxymidazolam glucuronide was a kind donation from Dr. Gérard Fabre, (Sanofi-Aventis, France). Midazolam hydrochloride solution for injections was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA).Cell culture media and media supplements were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Unless specified, all other materials were purchased from Sigma-Aldrich (St Louis, MO, USA.).

5.3.2: Animals and treatments:

Adult, male, (~8 weeks old, 20-25g) $CAR^{-/-}$ mice (Wei et al., 2000) and the corresponding wild-type mice on a C57BL/6 background were maintained in a 12 h dark/light cycle and in a temperature-and-humidity-controlled environment. The mice had access to regular rodent chow and water *ad libitum.* The mice were intraperitoneally (i.p.)-injected with LTA (6 mg/kg b.w) or LPS (2 mg/kg b.w) and the vehicle, saline. Livers were harvested at various time-points for RNA and protein analysis. In order to activate CAR, mice were i.p.-injected with 3 mg/kg b.w of TCPOBOP i.p. in corn oil for 3 days (Baskin-Bey et al., 2006) or PB (80mg/kg/day, i.p. in saline) for 3 days prior to LTA or LPS treatment. The hCAR mice used were generated using the knock-in strategy as described previously (Zhang et al., 2002). All the animal care and use protocols were approved by the Institutional Animal Care and Use Committee guidelines. All experiments were performed in triplicate and repeated three to four times.

5.3.2: real-time PCR:

Total RNA was isolated from mouse liver using TRIzol reagent (Sigma-Aldrich, St Louis, MO, U.S.A) according to the manufacturer's protocol. cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems) as described

previously (Ghose et al., 2004). In short, each reaction mixture (total of 25µl) contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 µl of TaqMan Universal PCR Master Mix. We extrapolated the quantitative expression values from standard curves and these values were normalized to cyclophilin.

5.3.3: Primary hepatocyte culture and treatments:

Primary mouse hepatocytes were isolated from $CAR^{+/+}$ and $CAR^{-/-}$ mice using the two step collagenase perfusion technique as described previously (Li et al., 2002; Ghose et al., 2011). In short, after digestion, the liver was excised and then the hepatocytes were purified using a percoll gradient (33%), washed and screened for viability using trypan blue exclusion technique. Only isolations with viability of more than 90% were used for these studies. Cells were plated at a density of 500,000 cells per well in six-well Primaria plates (BD biosciences, San Diego, CA, U.S.A) and allowed to attach for 4 h. Cells were maintained for 48 h with daily change of medium. The cells were incubated with serum-free Williams medium E two hours prior to treatment with 50 ng/mL LTA (8 h) or 1 µg/mL LPS (16 h). In order to activate CAR, the cells were treated with 250 nM of TCPOBOP 24 h or DMSO (0.025%v/v) prior to treatment with LTA or LPS. RNA was then isolated for real-time PCR analysis as described above.

5.3.4: Immunoblotting:

Nuclear extracts were prepared as described previously (Ghose et al., 2004) and the protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A). Equal amounts of protein (10 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were then probed with rabbit anti-CAR (1:500), anti Lamin A/C (1:500) or anti-LDH (1:500) antibody followed by probing with a goat anti-rabbit IgG-AP secondary antibody (1:2000). The membranes were then washed and incubated with Tropix® CDP star® nitroblock II ^{TM} ECL reagent as per the manufacturers' instructions (Applied Biosystems, CA, U.S.A.). The membranes were analyzed using FlourChem FC imaging system (Alpha Innotech). The images were quantified by densitometer using AlphaEase software.

5.3.5: Cyp3a11 enzyme activity:

MDZ hydroxylation was measured using mouse liver microsomes as described in detail by He et al., 2006, with minor modifications. The formation of 1'-OHMDZ was used as a specific indicator for mouse Cyp3a11 activity. In short, incubation mixtures (performed in triplicates) contained 0.05 mg of total microsomal protein, MDZ (0-16 μM), 1.3 mM NADPH and reaction cofactors in 50 mM potassium phosphate buffer (pH 7.4). The reaction mixtures were incubated at 37°C.

Initiated was done by addition of glucose-6-phosphate dehydrogenase (1 unit mL-1). After 5 min, the reactions were stopped by the addition of 100 μL of acetonitrile containing phenacetin (I.S). The incubation mixture was centrifuged and the supernatant was analyzed by LC-MS/MS analysis. The identity of 1'OHMDZ and I.S was verified by comparing with authenticated standards. The data were fit to the substrate inhibition model and analyzed by GraphPad Prism 4.0 software (GraphPad Inc., La Jolla, CA).

5.3.6: Liquid chromatography tandem mass spectroscopy analysis (LC-MS/MS):

To determine the concentrations of MDZ and its metabolites in microsomal samples, an API 3200 Q-trap triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) was used by MRM (Multiple Reaction Monitoring) method in the positive ion mode. A UPLC system, Waters Acquity™ with diode-arrayed detector was used. The UPLC conditions for analyzing MDZ, 1'-OHMDZ, 1'-OHMDZ-gluc and phenacetin (IS) were: column, Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm, Waters, Milford, MA, USA); mobile phase A, 0.1% formic acid; mobile phase B, 100% acetonitrile performed in a gradient from 0 to 3 min as follows: 0-0.5 min: 90% A; 0.5-1.0 min: 75% A; 1.0-2.0 min: 60% A and 2.0-2.5 min: held constant at 60% A, 2.5-3.0 min: 90% A, respectively. The quantification was performed

using MRM method with the transitions of m/z 326.1 → m/z 291.1 for MDZ, 342.1 → 324.1 for 1'-OHMDZ, 518.1 → 324.1 for 1'-OHMDZ-gluc and 180.0 → 110.0 for phenacetin. The retention times were: MDZ, 1.97 min; 1'-OHMDZ, 1.92 min; 1'-OHMDZ-gluc, 1.70 min and phenacetin, 1.76 min.

5.3.7: Statistical analysis:

Treatment groups were compared using 2 way ANOVA followed by a post-hoc test (tukey's post hoc test) with $p < 0.05$. The results were presented as mean $\pm$ SD.

5.4: Results

5.4.1: Effect of LTA treatment on DME/transporter gene expression in $CAR^{+/+}$ and $CAR^{-/-}$ mice

We have shown previously that LTA causes preferential suppression of CAR and its target genes (Ghose et al., 2009). To investigate the role of CAR in LTA-mediated down-regulation of DMEs and transporters, $CAR^{+/+}$ and $CAR^{-/-}$ mice were injected with LTA. RNA was isolated from the livers harvested at the indicated time points and analyzed by real-time PCR. RNA levels of the key phase I enzyme, Cyp3a11 were down-regulated at 4-16 h (~30-40%) in the $CAR^{+/+}$ mice after LTA administration, whereas this down-regulation was absent in $CAR^{-/-}$ mice. Cyp3a11 is the human analog of CYP3A4, which is responsible for metabolism of more than ~50% of marketed drugs. The basal expression of Cyp3a11 is significantly lower (~50-60%) in the $CAR^{-/-}$ mice. This observation is in agreement with other reports published previously (Assem et al., 2004). Cyp2a4 RNA levels were significantly down-regulated in $CAR^{+/+}$ mice at 4 and 8 h (~60-80%), whereas this down-regulation was attenuated in the $CAR^{-/-}$ mice. Cyp2a4 is the human analog of CPY2A13, which is responsible for metabolism of several drugs such as nicotine, phenacetin etc. As seen previously (Ghose et al., 2009), the RNA levels of Cyp2b10 in $CAR^{+/+}$ mice were down-regulated at 4 h (~75-80%) and remained reduced (~40%) until 16 h after LTA administration (Fig. 5.4.1). Cyp2b10 is the human analog of CYP2B6, which is responsible for

the metabolism of drugs such as propofol, efavirenz, cyclophosphamide etc. The basal expression of Cyp2b10 in the $CAR^{-/-}$ mice is too low to make any conclusive inference.

In the case of phase II enzymes, Ugt1a1 RNA levels were significantly (~40%) reduced at 16 h in $CAR^{+/+}$ mice treated with LTA, while this reduction was attenuated in the $CAR^{-/-}$ mice. Ugt1a1 RNA levels remained unchanged at 4 h and 8 h in LTA-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice. Ugt1a1 is the human analog of UGT1A1, which is responsible glucuronidation of several known marketed drugs. Sultn RNA levels were down-regulated in $CAR^{+/+}$ mice at 4 and 8 h (~40-55%), while no reduction was seen at 16 h. The down-regulation of Sultn in $CAR^{-/-}$ mice was not detected at 4 h and 8 h of LTA treatment, though there was down-regulation of Sultn at 16 h (~60%). This suggests delayed action of LTA on Sultn in the absence of CAR, and/or the existence of alternate mechanisms.

RNA levels of the transporter, Mrp2 were down-regulated (~40%) by LTA at 4 h in both $CAR^{+/+}$ and $CAR^{-/-}$ mice, whereas at 8 h, Mrp2 down-regulation (~35%) was detected only in the $CAR^{+/+}$ mice. Mrp2 gene expression was unaffected at 16 h in both $CAR^{+/+}$ and $CAR^{-/-}$ mice treated with LTA. We also examined RNA levels of two other transporter genes namely Mrp4 (unchanged in $CAR^{+/+}$ and $CAR^{-/-}$ mice) and Mdr1b (up-regulated in both $CAR^{+/+}$ and $CAR^{-/-}$ mice) (data not

shown). This finding indicates that basal expression of CAR is required for LTA to reduce DME and transporter genes.

Fig 5.4.1

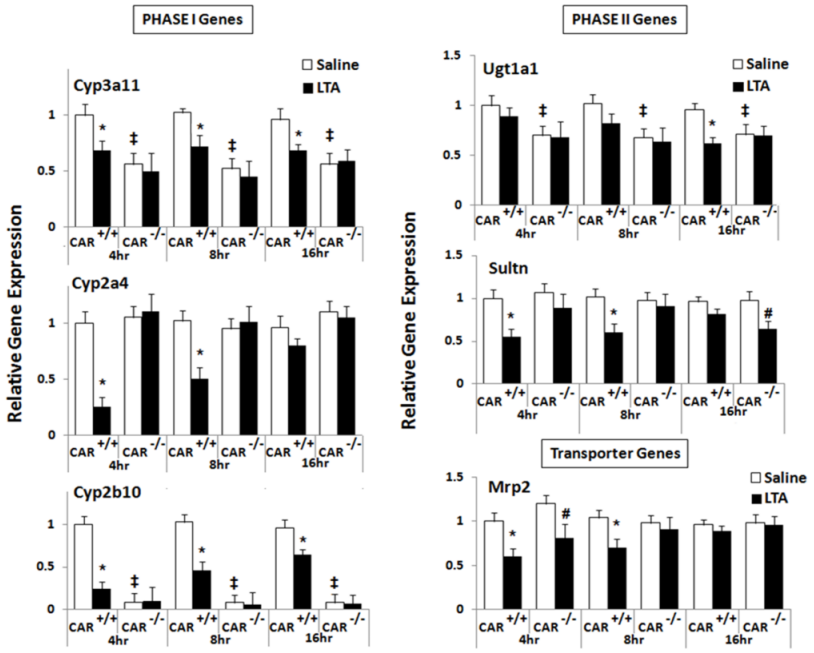


Fig 5.4.1: Regulation of DME and transporter mRNA levels in CAR$^{+/+}$ and CAR$^{-/-}$ mice following LTA administration.

CAR$^{+/+}$ and CAR$^{-/-}$ were i.p. injected with saline or LTA (6 mg/kg) and livers were harvested at 4, 8 and 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I enzymes, phase II enzymes and transporters were determined by

real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls. ***** and # indicate significant difference ($p < 0.05$) between saline and LTA groups in $CAR^{+/+}$ mice and $CAR^{-/-}$ mice respectively and ‡ indicates basal level differences between $CAR^{+/+}$ and $CAR^{-/-}$ mice. The experiments were repeated at least thrice.

5.4.2: Effect of LPS treatment on DME/transporter gene expression in $CAR^{+/+}$ and $CAR^{-/-}$ mice

LPS administration significantly down-regulated RNA levels of the phase I enzymes (Cyp3a11, Cyp2a4), the phase II enzyme, Ugt1a1 and the transporter, Mrp2 in both $CAR^{+/+}$ and $CAR^{-/-}$ mice at 16 h (Fig. 5.4.2). Amongst all the genes studied, Cyp3a11 was the most affected (~80% reduction) followed by Cyp2a4 (~65% reduction), Ugt1a1 (~50% reduction) and Mrp2 (~40% reduction). Since, LPS down-regulates DME/transporter genes in the absence of CAR, these results indicate that CAR is not required for LPS-mediated down-regulation of DME and transporter genes. Cyp2b10 and Sultn mRNA levels remained unchanged in both $CAR^{+/+}$ and $CAR^{-/-}$ mice. We selected the 16 h time point because we saw optimal down-regulation of these DMEs and transporters at this time-point (Ghose et al., 2004).

Fig 5.4.2

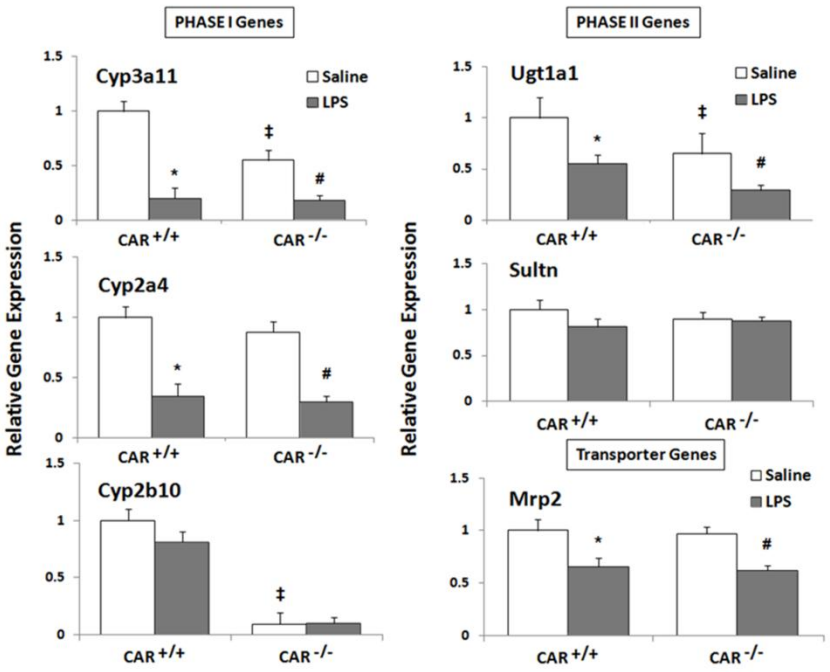


Fig 5.4.2: Regulation of DME and transporter mRNA levels in $CAR^{+/+}$ and $CAR^{-/-}$ mice following LPS administration.

$CAR^{+/+}$ and $CAR^{-/-}$ mice were i.p. injected with saline or 2 mg/kg LPS and livers were harvested at 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I enzymes, phase II enzymes and transporters were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change

after LPS treatment was compared to the saline-treated controls. * and # indicate significant difference ($p < 0.05$) between saline and LPS groups in $CAR^{+/+}$ mice and $CAR^{-/-}$ mice respectively and ‡ indicates basal level differences between $CAR^{+/+}$ and $CAR^{-/-}$ mice. The experiments were repeated at least thrice.

5.4.3: Effect of LTA or LPS treatment on DME/transporter gene expression in TCPOBOP-treated Mice:

To further confirm the role of CAR in LTA-mediated down-regulation of DME/transporter genes, we activated CAR using specific mouse CAR activator TCPOBOP (3 mg/kg/day in corn oil i.p.) 3 days prior to administration of LTA (Fig. 5.4.3.1). TCPOBOP treatment caused a significant induction of Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and Mrp2 genes (Fig. 5.4.3.1A; B). Since we saw maximum down-regulation of DMEs and transporters by LTA at 4 h, we treated these TCPOBOP treated $CAR^{+/+}$ mice with LTA for 4 h. Surprisingly, this induction was still detected in the presence of LTA. LTA treatment at longer time-points (8-16 h) did not affect the induction of these DMEs/transporters by TCPOBOP (data not shown). Since LTA down-regulated Ugt1a1 at 16 h in $CAR^{+/+}$ mice (Fig. 5.4.1), we analyzed mRNA levels of Ugt1a1 after 16 h treatment with LTA in the TCPOBOP-treated mice (Fig. 5.4.3.1B). As seen with other DMEs, induction of Ugt1a1 RNA levels by TCPOBOP was not affected by LTA treatment. Similar results were observed by treatment of mice with the universal CAR activator, PB (data not shown).

In order to understand the mechanism underlying the lack of effect of LTA on TCPOBOP-mediated induction of DME/transporter genes, we measured expression of CAR at the gene and protein levels in the presence of TCPOBOP.

As observed in our previous publications (Ghose et al., 2009), we found that CAR gene expression and cytosolic protein levels were reduced by LTA in the absence of TCPOBOP (data not shown); nuclear levels of CAR were too low to obtain a conclusive inference.

TCPOBOP, by itself caused increased accumulation of CAR in the nucleus with no effect on CAR gene expression as expected. Interestingly, we did not observe any changes in CAR gene expression and nuclear/cytosolic levels by LTA in the presence of TCPOBOP (Fig. 5.4.3.1 C, D & E). Presence of high amount of CAR in TCPOBOP/LTA-treated mice may account for increased expression of CAR-target genes in these mice compared to corn oil/LTA-treated controls.

We find that LPS down-regulated the DME and transporter genes to the same extent in mice pre-treated with TCPOBOP or the vehicle control, corn oil (Fig. 5.4.3.2A). We also found that CAR gene expression as well as nuclear/cytosolic protein levels were down-regulated by LPS to the same extent in $CAR^{+/+}$ and TCPOBOP-treated mice (Fig. 5.4.3.2 B, C & D). As shown in our previous publications (Ghose et al., 2004), LPS down-regulated CAR expression in the absence of TCPOBOP (data not shown).

Fig 5.4.3.1

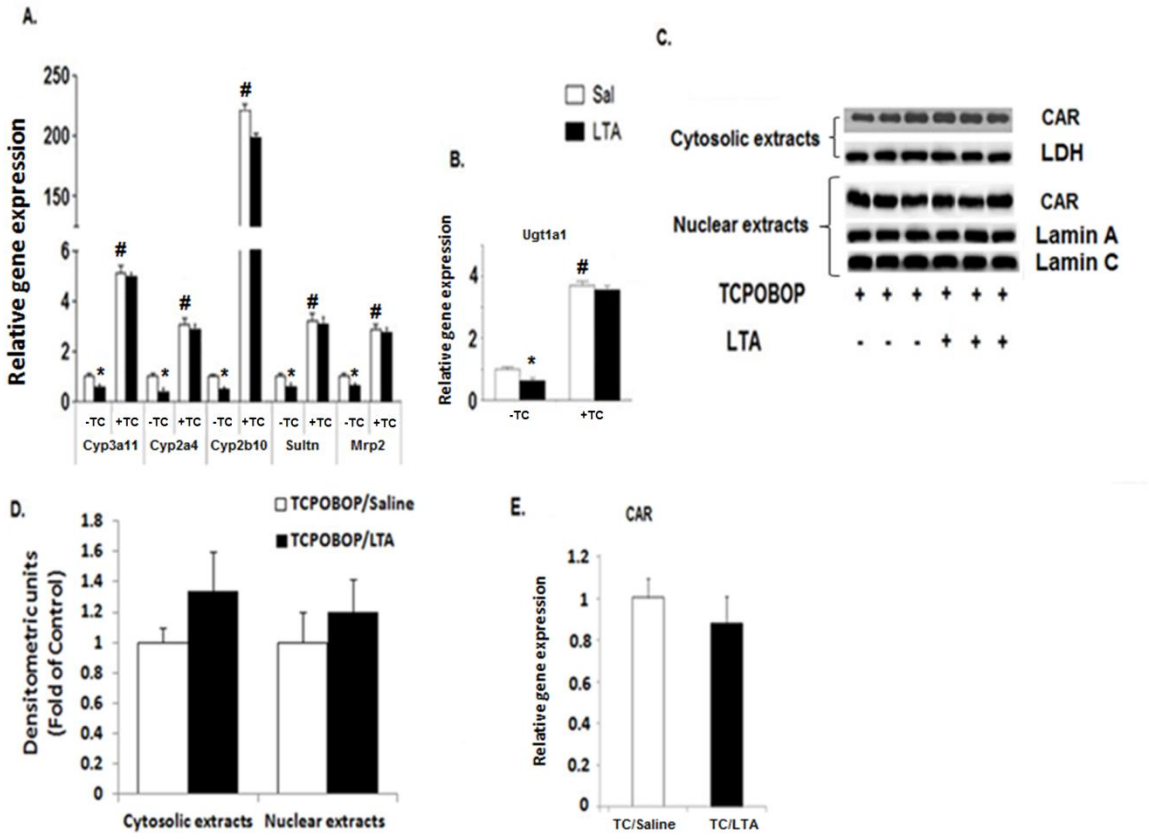


Fig 5.4.3.1: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LTA administration.

$CAR^{+/+}$ mice were treated with the specific CAR activator, TCPOBOP (3 mg/kg/day) for 3 days prior to i.p. administration of saline or LTA (6 mg/kg). Livers were harvested after: (A) 4 h and (B) 16 h and mRNA levels were analyzed by real-time PCR as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA treatment

was compared to the saline-treated controls. ***** indicate significant difference ($p < 0.05$) between saline and LTA groups and # indicates significant differences between saline samples of -TCPOBOP (-TC) and +TC groups. The experiments were repeated at least thrice. (C) Cytosolic and nuclear extracts were prepared from livers from $CAR^{+/+}$ mice treated with TCPOBOP (3 mg/kg/day) for 3 days prior to saline and LTA (6 mg/kg) i.p. injections (4 h) and CAR protein levels were measured by western blotting. (D) The images were quantified by densitometer using AlphaEase software. The normalized values of fold difference, relative to the expression of LDH for cytosolic extracts and Lamin A/C for nuclear extracts, which was set to 1, are presented as mean ±SD values. (E) Regulation of CAR mRNA levels by LTA in TCPOBOP pre-treated mice**.** C57BL/6 mice were pre-treated for 3 days with 3 mg/kg TCPOBOP (i.p.) in corn oil prior to treatment with saline or LTA (6 mg/kg) and livers were harvested at 2 h *(n* = 5 per group). All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in TCPOBOP/saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls.

Fig 5.4.3.2

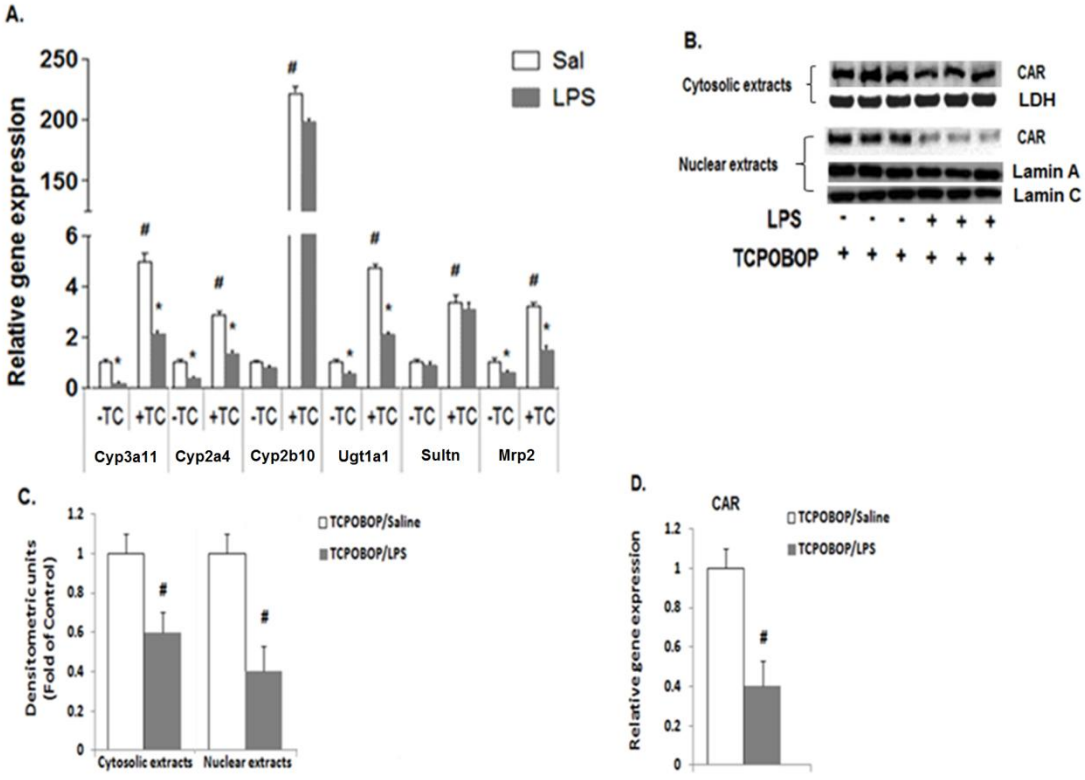


Fig 5.4.3.2: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LPS administration.

(A) $CAR^{+/+}$ mice were treated with the specific CAR activator TCPOBOP 3 mg/kg/day for 3 days prior to LPS (2 mg/kg) i.p. administration. Livers were harvested after 16 h and mRNA levels were analyzed by real-time PCR as described earlier. All data are

presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LPS treatment was compared to the saline-treated controls. ***** indicate significant difference ($p < 0.05$) between saline and LPS groups and # indicates significant difference ($p < 0.05$) between saline samples of –TCPOBOP (TC) and +TC groups. The experiments were repeated at least thrice. (B) Cytosolic and nuclear extracts were prepared from livers of $CAR^{+/+}$ mice treated with TCPOBOP 3 mg/kg/day for 3 days prior to saline and LPS (2 mg/kg) i.p. injections (16 h) and CAR protein levels were measured by Western blotting. (C) The images were quantified by densitometer using AlphaEase software. The normalized values of fold difference, relative to the expression of LDH for cytosolic extracts and Lamin A/C for nuclear extracts, which was set to 1, are presented as mean ±SD values. $^{\#}$ indicates significant difference ($p < 0.05$) between saline and LPS treatment groups. (D) Regulation of CAR mRNA levels by LPS in TCPOBOP pre-treated mice**.** C57BL/6 mice were pre-treated for 3 days with 3 mg/kg TCPOBOP (i.p.) in corn oil prior to treatment with saline or LPS (2 mg/kg) and livers were harvested at 16 h *(n* = 5 per group). All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in TCPOBOP/saline-treated mice was set to 1, fold change after LPS treatment was compared to the saline-treated controls. $^{\#}$ indicates significant difference ($p < 0.05$) between saline and LPS groups.

5.4.4: Effect of LTA or LPS treatment on DME/transporter gene expression in PB-treated hCAR mice

In order to test the relevance of these findings on human CAR, we injected hCAR mice with PB (80 mg/kg i.p.) for 3 days, prior to administration of LTA or LPS. LTA treatment led to down-regulation of gene expression of Cyp3a11 (~45%), Cyp2a4 (~65%), Cyp2b10 (~60%), Ugt1a1 (~50%), Sultn (~55%) and Mrp2 (~50%) (Fig. 5.4.4 A and B). PB treatment significantly induced the expression of these genes, while LTA treatment did not attenuate this induction (Fig. 5.4.4 A and B). On the other hand, LPS administration in hCAR mice led to down-regulation of gene expression of Cyp3a11 (~80%), Cyp2a4 (~60%), Ugt1a1 (~55%) and Mrp2 (~45%) (Fig. 5.4.4 C). Interestingly, LPS down-regulated the gene expression of these genes to the same extent in mice pre-treated with or without PB (Fig. 5.4.4 C). This data is in agreement with the previous findings in LTA or LPS-injected $CAR^{+/+}$ mice pre-treated with TCPOBOP.

Fig 5.4.4

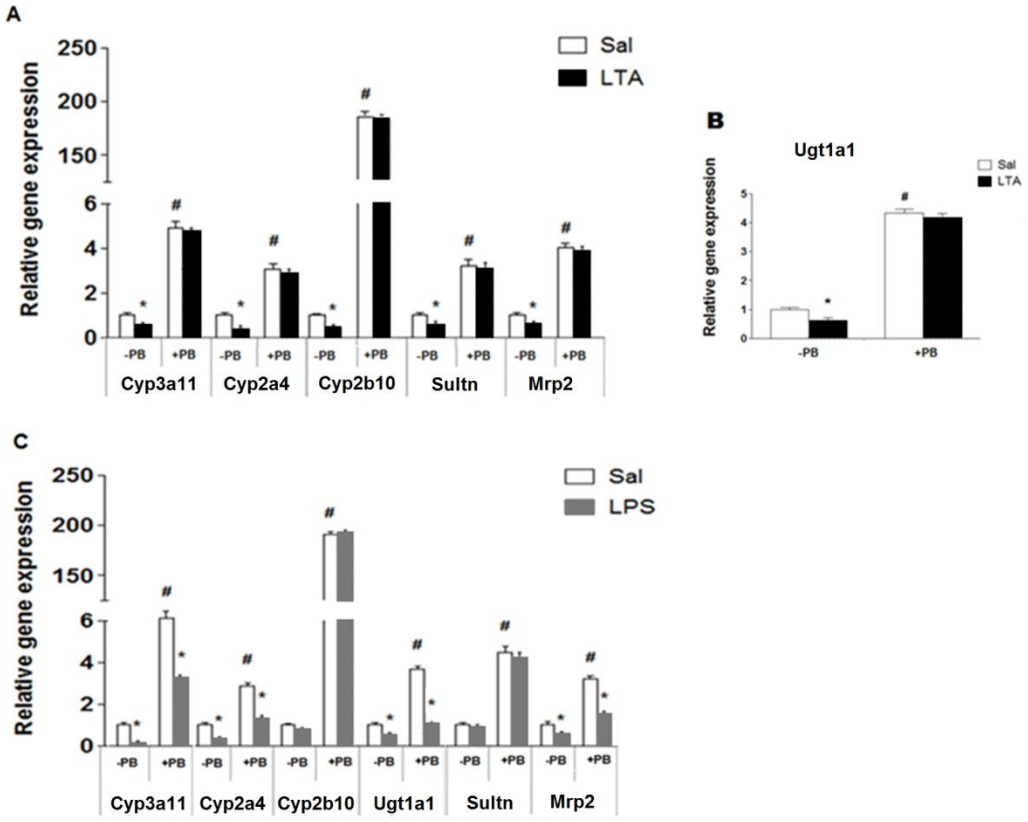

A
Relative gene expression
Sal
LTA
-PB
+PB
Cyp3a11
Cyp2a4
Cyp2b10
Sultn
Mrp2
B
Ugt1a1
C
LPS
Ugt1a1

Fig 5.4.4: Regulation of DME and transporter mRNA levels by LTA or LPS in hCAR mice pre-treated with PB.

hCAR mice were treated with the universal CAR activator PB (80 mg/kg/day) for 3 days prior to i.p. administration of: (A) saline or LTA (6 mg/kg) for 4 h and (B) 16 h. (C) hCAR mice were treated with PB 80 mg/kg/day for 3 days prior to saline or LPS (2 mg/kg) treatment for 16 h. Livers were harvested and mRNA levels were analyzed by real-time PCR as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA or LPS treatment was compared to the saline-treated controls. ***** indicate significant difference ($p < 0.05$) between saline and LTA or LPS groups and # indicates significant differences between saline samples of –PB and +PB groups. The experiments were repeated at least thrice.

5.4.5: Effect of LTA or LPS treatment on DME/transporter gene expression in primary hepatocytes from $CAR^{+/+}$ and $CAR^{-/-}$ mice.

The role of CAR was further examined in primary hepatocytes treated with saline or LTA (50 ng/ml) for 8 h. RNA levels of Cyp3a11, Cyp2a4, Cyp2b10 and Ugt1a1 were significantly down-regulated (~40-45%) in LTA-treated $CAR^{+/+}$ hepatocytes, and this down-regulation was attenuated in $CAR^{-/-}$ hepatocytes (Fig. 5.4.5 A). Sultn gene expression was significantly down-regulated (~40%) in the $CAR^{+/+}$ as well as in the $CAR^{-/-}$ hepatocytes, however, the extent of down-regulation was lower in the $CAR^{-/-}$ hepatocytes (~20%). We did not see any change in the Mrp2 expression either in the $CAR^{+/+}$ or $CAR^{-/-}$ hepatocytes. As seen *in vivo*, there were significant differences in the basal levels of Cyp2b10, Cyp3a11 and Ugt1a1 expression between hepatocytes isolated from $CAR^{+/+}$ and $CAR^{-/-}$ mice. The difference in the trends of Sultn gene expression and Mrp2 gene expression on treatment with LTA is most likely because of the difference in *in vitro* and *in vivo* conditions.

$CAR^{+/+}$ and $CAR^{-/-}$ hepatocytes were treated with saline or LPS (1 μg/ml) for 16 h. Cyp3a11 (~70%), Cyp2a4 (~50%), Ugt1a1 (~60%) and Mrp2 (~50%) were significantly down-regulated in LPS-treated $CAR^{+/+}$ as well as in $CAR^{-/-}$ hepatocytes (Fig. 5.4.5 B).

Fig 5.4.5

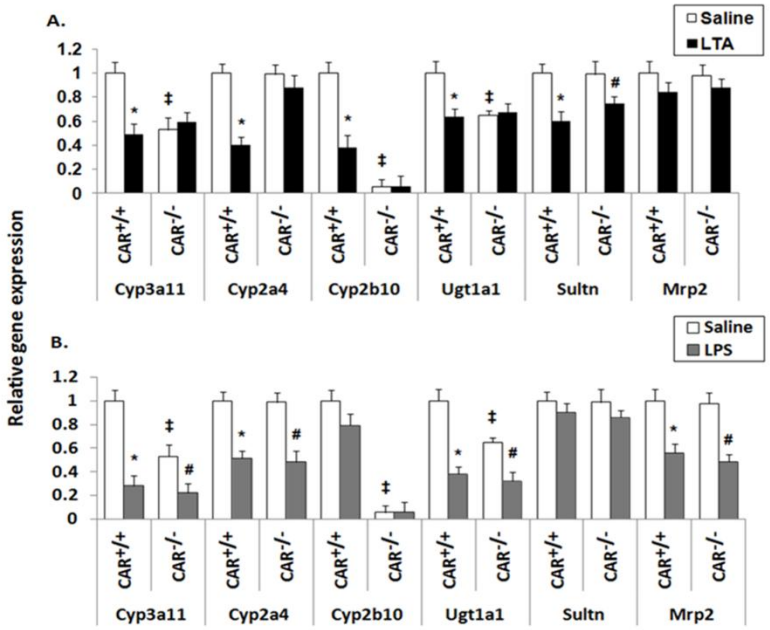


Fig 5.4.5: Regulation of DME and transporter gene expression by LTA or LPS in primary hepatocytes from $CAR^{+/+}$ and $CAR^{-/-}$ mice.

Primary hepatocytes from $CAR^{+/+}$ and $CAR^{-/-}$ mice were treated with: (A) saline or LTA (50 ng/ml) for 8 h, or (B) saline or LPS (1 μg/ml) for 16 h. RNA was isolated, and real-time PCR was performed as described earlier. *n* = 5-6 per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. ***** indicate significant difference

($p < 0.05$) between saline and LTA/LPS of $CAR^{+/+}$ groups and ‡ indicates significant differences ($p < 0.05$) between saline samples of $CAR^{+/+}$ and $CAR^{-/-}$ groups and # indicates significant difference ($p < 0.05$) between saline and LTA/LPS of $CAR^{-/-}$ group. The experiments were repeated at least thrice.

5.4.6: Effect of LTA or LPS treatment on DME/Transporter gene expression in primary hepatocytes pre-treated with TCPOBOP

To activate CAR *in vitro*, $CAR^{+/+}$ hepatocytes were treated with 250 nM TCPOBOP 24 h prior to treatment with saline or LTA (50 ng/ml). TCPOBOP induced the expression of all the DMEs and transporters significantly. As seen *in vivo*, the down-regulation of DMEs and transporters by LTA was attenuated in hepatocytes treated with TCPOBOP (Fig. 5.4.6 A).

$CAR^{+/+}$ hepatocytes were pre-treated with 250nM TCPOBOP 24 h prior to treatment with LPS (1 μg/ml). LPS administration led to down-regulation of Cyp3a11 (~75%), Cyp2a4 (~50%), Ugt1a1 (~50%) and Mrp2 (~50%). As expected, LPS caused no change in the gene expression of Cyp2b10 or Sultn in hepatocytes treated with TCPOBOP (Fig. 5.4.6 B). Our *in vitro* findings concur with our *in vivo* results suggesting that down-regulation of DME/transporter genes by LPS is associated with down-regulation of gene expression of CAR however; the DME/transporter genes are down-regulated by LPS even in the absence of CAR suggesting the presence of alternative mechanisms. We also performed these experiments in PB-treated primary mouse hepatocytes from hCAR mice and found same results (data not shown).

Fig 5.4.6

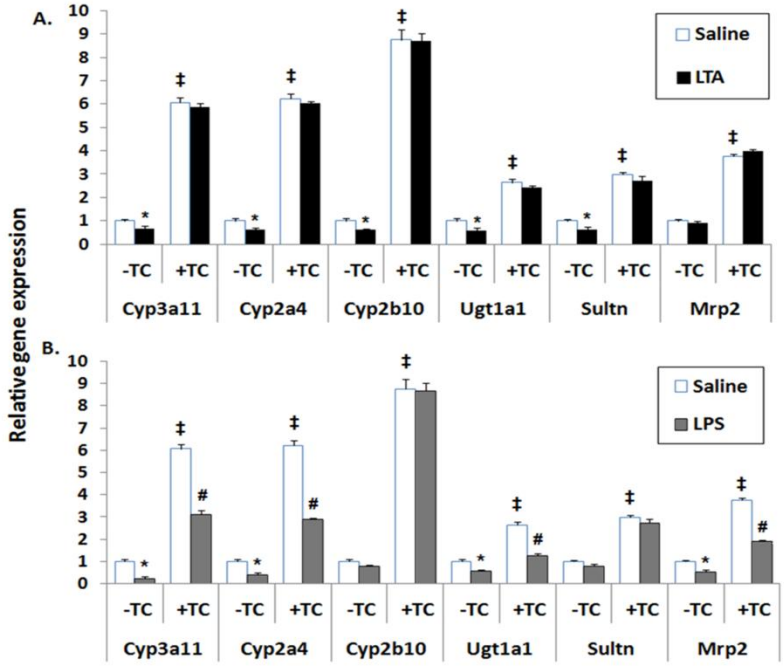


Fig 5.4.6: Regulation of DME and transporter gene expression by LTA in TCPOBOP pre-treated primary hepatocytes.

Primary hepatocytes from $CAR^{+/+}$ were isolated and treated with specific CAR activator TCPOBOP (250 nM) for 24h prior to treatment with: (A) saline or LTA (50 ng/ml) for 8 h, or (B) saline or LPS (1 μg/ml) for 16 h. RNA was isolated, and real-time PCR was

performed as described earlier. $n = 5$ per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. ***** indicate significant difference ($p < 0.05$) between saline and LTA/LPS of –TCPOBOP (TC) groups and ‡ indicates significant differences ($p < 0.05$) between saline samples of –TC and +TC groups and # indicates significant difference ($p < 0.05$) between saline and LTA/LPS of + TC group. The experiments were repeated at least thrice.

5.4.7: Effect of LTA administration on TLR2 mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$):

We have previously seen that on activation of TLR2 by LTA leads to the induction of TLR2 levels by ~50 folds (Ghose et al., 2009). In order to determine the effects of LTA-administration on the expression of TLR2, we treated $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice (mice pre-treated with TCPOBOP for 3 days) with LTA (6mg/kg) for 4 h. Our results show that the levels of TLR2 were induced significantly in $CAR^{+/+}$ and $CAR^{-/-}$ mice (~45-50 folds) however, there was severe attenuation of the induction of TLR2 (~5 folds) in CAR-activated mice (Fig 5.4.7). Even though the levels of TLR2 are induced in $CAR^{-/-}$ mice, we do not see a reduction in the levels of DMEs probably because of the absence of CAR.

Fig 5.4.7

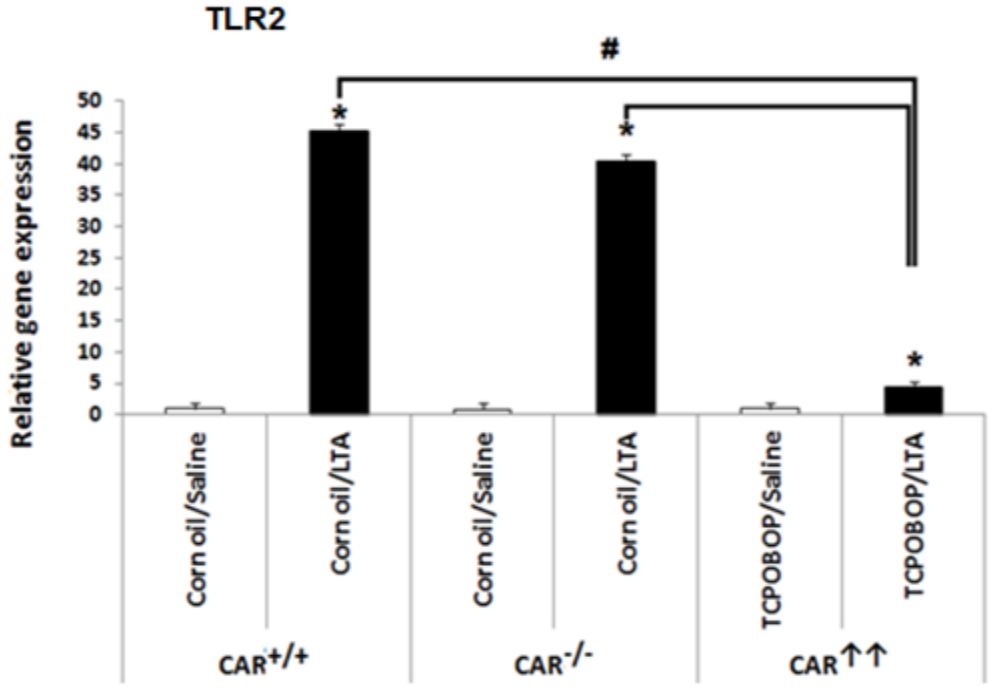


Fig 5.4.7: Regulation of TLR2 mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$) following LTA administration.

The mice were pre-treated for 3 days with 3 mg/kg TCPOBOP (i.p.) in corn oil or corn oil prior to treatment with saline or LTA (6 mg/kg) and livers were harvested at 2 h (n = 5 per group). RNA was isolated from the livers and mRNA levels of TLR2 were determined by real-time PCR analysis as described earlier. All data are presented as ± SD and standardized for cyclophilin mRNA levels. Expression in corn oil/saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated

controls. * indicates significant difference ($p < 0.05$) between saline and LTA groups and # indicates significant differences between LTA-treated groups. The experiments were repeated at least thrice.

5.4.8: Effect of LTA administration on cytokine mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$):

We have previously seen that on activation of TLR2 by LTA leads to the induction of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α (Ghose et al., 2009). In order to determine the effects of LTA-administration on the expression of pro-inflammatory cytokines, we treated $CAR^{+/+}$, $CAR^{-/-}$ and CAR-activated mice (mice pre-treated with TCPOBOP for 3 days) with LTA (6mg/kg) for 1 h. We see similar induction of IL-1β (~35-40 folds) and IL-6 (~60-80 folds) in $CAR^{+/+}$, $CAR^{-/-}$ and CAR-activated mice on treatment with LTA. There is similar induction of the levels of TNF-α in $CAR^{+/+}$ (~60 folds) and $CAR^{-/-}$ (~65 folds) mice, however the level of induction of TNF-α is attenuated in CAR-activated mice (~10 folds) (Fig 5.4.8).

Fig 5.4.8:

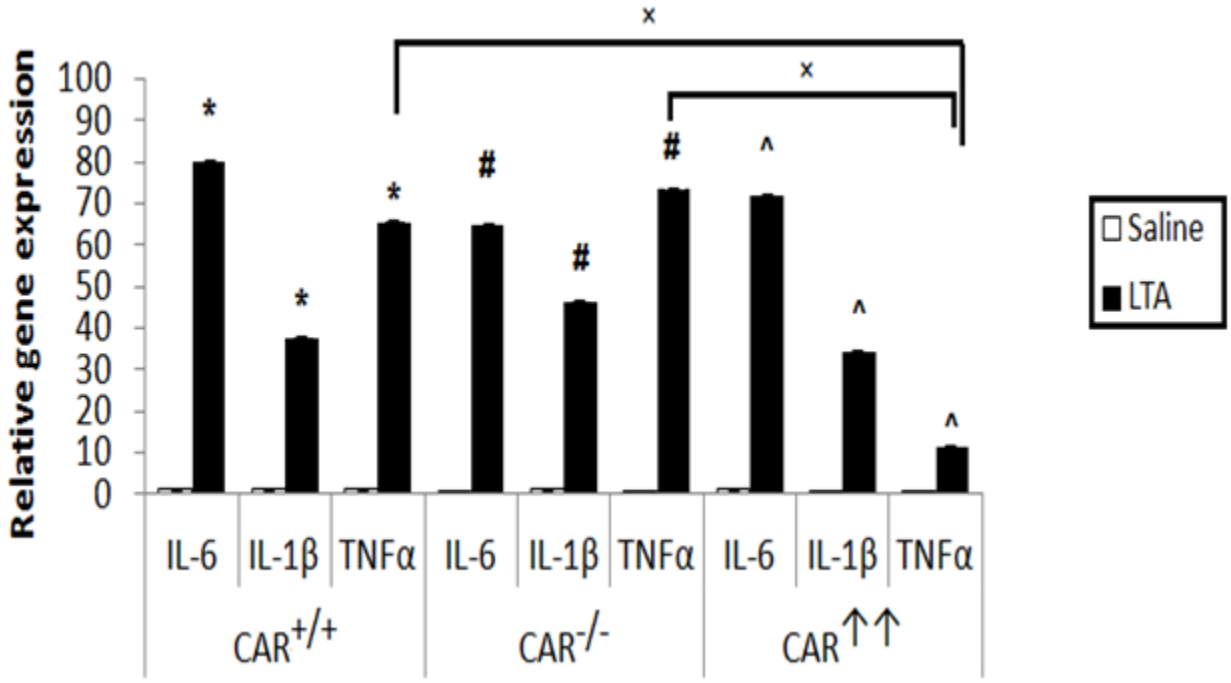


Fig 5.4.8: Regulation of cytokine mRNA levels in $CAR^{+/+}$, $CAR^{-/-}$ and CAR activated mice ($CAR^{\uparrow\uparrow}$) following LTA administration.

$CAR^{+/+}$ and $CAR^{-/-}$ mice were treated with saline or LTA (6 mg/kg) (i.p.) and livers were harvested at 2 h (*n* = 5 per group). $CAR^{\uparrow\uparrow}$ were pre-treated with 3 mg/kg/day i.p. dose of TCPOBOP for 3 days before treatment with saline or LTA (6 mg/kg) for 2 h. RNA was isolated from the livers and mRNA levels of IL-6, IL-1β and TNF-α were determined by real-time PCR analysis as described earlier. All data are presented as ± SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls. * indicates

significant difference ($p < 0.05$) between saline and LTA groups in $CAR^{+/+}$ mice, # indicates significant differences between saline and LTA-treated groups in $CAR^{-/-}$ mice, ^ indicates significant difference between saline and LTA groups in $CAR^{\uparrow\uparrow}$ mice and x indicates significant difference between TNF-α groups of $CAR^{+/+}$, $CAR^{-/-}$ and $CAR^{\uparrow\uparrow}$ mice. The experiments were repeated at least thrice.

5.4.9: Effect of LTA or LPS on Cyp3a11 activity in $CAR^{+/+}$ and $CAR^{-/-}$ mice

We have seen that gene expression of DMETs is attenuated almost completely in LTA-treated in $CAR^{-/-}$ mice where as there is no attenuation of down-regulation of DMETs in LPS-treated $CAR^{-/-}$ mice. In order to determine whether the mRNA expression correlates to the activity of the enzymes, we performed MDZ-hydroxylation assay for determining the activity of Cyp3a11 enzyme in $CAR^{+/+}$ and $CAR^{-/-}$ mice. $CAR^{+/+}$ and $CAR^{-/-}$ mice were treated with LTA or LPS for 16 h and microsomes were prepared from freshly harvested livers as described earlier. We find that the enzyme activity of Cyp3a11 is down-regulated on treatment with LPS in $CAR^{+/+}$ mice as well as $CAR^{-/-}$ mice (Fig. 5.4.9) as seen by the K_m and V_{max} values in Table 5.4.1. The activity of Cyp3a11 is down-regulated only in LTA-treated $CAR^{+/+}$ mice and not in the LTA-treated $CAR^{-/-}$ mice which is similar to our mRNA data. This suggests that targeting of CAR will have clinical benefits in patients with gram positive infections.

Fig 5.4.9

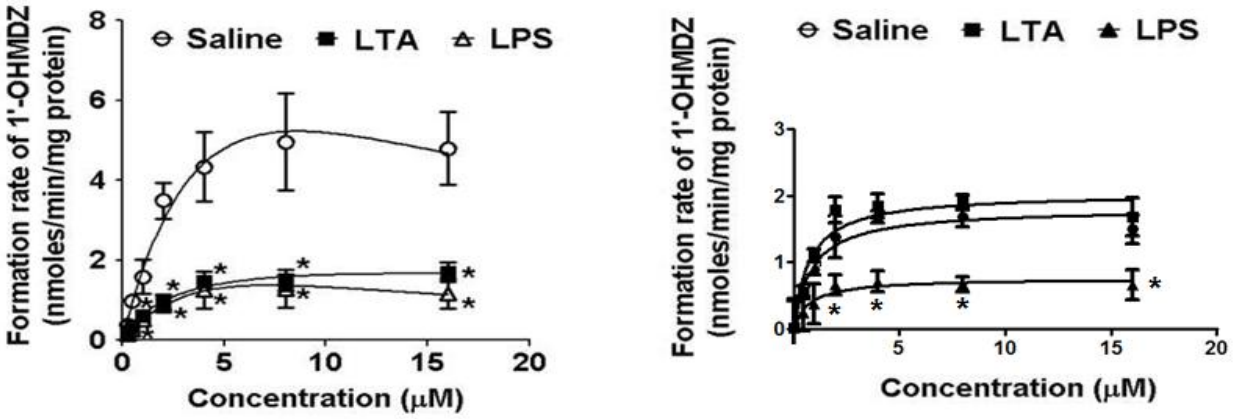


Figure 5.4.9: Regulation of Cyp3a11 activity in $CAR^{+/+}$ and $CAR^{-/-}$ mice

Mice were i.p-injected with saline, LPS (2 mg/kg) or LTA (6 mg/kg). Livers were harvested after 16 h (n=4 per group). Microsomes were prepared as described previously in Materials and Methods. Hydroxylation of MDZ was used as a marker of Cyp3a11 activity. Data are shown as mean ± S.D. * indicates significant difference at $p<0.05$.

Table 5.4.1

	CAR$^{+/+}$			CAR$^{-/-}$		
	Saline	LPS	LTA	Saline	LPS	LTA
K_m (μM)	6.58± 1.83	3.24±1.32*	4.85± 1.11*	0.77±0.33#	0.73±0.54	0.78±0.39
V_{max} (nm/min*mg)	14.14±3.1	2.88±1.65*	3.42±2.1*	1.77±0.29#	0.78±0.21*	1.86± 0.23

Table 5.4.1 Enzyme kinetic parameters of MDZ

*Significant difference at $p<0.05$ when LPS or LTA-treatments compared to saline of its own group and # indicates significant difference in saline groups of CAR$^{+/+}$ and CAR$^{-/-}$ mice.

5.5: Discussion

Infection and inflammation can alter the expression and activities of several of phase I and phase II DMEs and transporters (Cheng & Morgan, 2001; Hartmann et al., 2001). Down-regulation of hepatic DME/transporter genes during inflammation and infection is associated with reduced gene expression of NRs, PXR and CAR. Although, studies have shown that PXR has no role in LPS-mediated regulation of hepatic genes, the role of CAR is not known. In this study, we found that basal levels of CAR are required for LTA to down-regulate the expression of DME/transporter genes. On the other hand, LPS can down-regulate these genes even in the absence of CAR in $CAR^{-/-}$ mice.

Our results indicate that both LTA and LPS down-regulate CAR expression, however, they differentially regulate DME and transporter genes. In addition, the time-line of down-regulation of CAR gene expression is different for LTA and LPS, which can partially account for the differential effects on CAR-target genes. LTA down-regulates CAR gene expression (~65%) at 2-4 h, whereas LPS down-regulates CAR gene expression (~70%) at 16 h (Ghose et al., 2008; 2009). LTA administration led to down-regulation of Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and Mrp2 genes in $CAR^{+/+}$ mice at time points ranging from 4-16 h as we have seen previously (Ghose et al., 2009). The reason for discord in the time course of suppression could be the mechanism of down-regulation is different for

different genes. Furthermore, LTA may affect corepressor expression and recruitment differently which can lead to differences in the time course of down-regulation of DMEs and transporters. We have also seen attenuation of the down-regulation of Cyp3a11 activity in LTA-treated $CAR^{-/-}$ mice (Fig 5.9). LTA-mediated down-regulation of most of these DME and transporter genes was attenuated in the $CAR^{-/-}$ mice suggesting that CAR plays an important role in LTA-mediated down-regulation of these genes.

LPS administration led to down-regulation of Cyp3a11, Cyp2a4, Ugt1a1 and Mrp2 genes in both $CAR^{+/+}$ and $CAR^{-/-}$ mice, suggesting that LPS can down-regulate DME/transporter genes in the absence of CAR. It is possible that this LPS-mediated down-regulation may occur through PXR in the absence of CAR. However, we did not find any difference in the expression of PXR between $CAR^{+/+}$ and $CAR^{-/-}$ mice treated with saline or LPS (data not shown). We also see that the down-regulation of Cyp3a11 activity is similar in LPS-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice (Fig 5.9). This indicates that down-regulation of DME/transporter genes by LPS in $CAR^{-/-}$ mice are not due to over-expression of PXR. Experiments in PXR/CAR double knockout mice will further explain the roles of PXR and CAR in LTA- or LPS-mediated DME and transporter down-regulation.

Treatment of $CAR^{+/+}$ mice with specific mouse CAR activator TCPOBOP, results in induction of gene expression of all the DME/transporters mentioned above.

Immunoblotting of nuclear extracts from TCPOBOP pre-treated mice revealed that induction of CAR protein levels in the nucleus remained unchanged by LTA treatment (Fig. 5.3.1 C and D). Furthermore, CAR gene expression in TCPOBOP/LTA-treated mice was significantly higher than corn oil/LTA-injected mice (Fig. 5.3.1 E).

Since LTA administration in TCPOBOP treated mice did not change gene expression of DMEs and transporters, we administered 12 mg/kg dose of LTA in TCPOBOP-pre-treated mice (double the original dose). However, gene expression of DMEs and transporters still remained unchanged (data not shown). We did not find any down-regulation of DME/transporter genes at different time points (2, 4, 8, 16, 24 and 48 h) in TCPOBOP/LTA-treated mice (data not shown). In order to rule out a ligand dependent effect, we tried another CAR activator PB (80 mg/kg/day i.p. for 3 days) and observed the same results (data not shown).

The mechanism of how LTA down-regulates CAR and its target genes is unclear. We find that activation of CAR with TCPOBOP leads to accumulation of CAR in the nucleus, and LTA treatment does not affect the concentration of nuclear CAR protein levels. Cytosolic CAR protein and CAR gene expression were also unaffected by LTA in the presence of TCPOBOP. Since LTA treatment of $CAR^{-/-}$ mice did not cause down-regulation of DME/transporter genes, we anticipated that induction of CAR by TCPOBOP would facilitate the down-regulation of

these genes by LTA. However, in TCPOBOP-treated mice, LTA did not affect DME and transporter gene expression likely due to its lack of effect on CAR gene and protein expression. It is possible that the induction of DME and transporter genes on administration of TCPOBOP led to enhanced clearance of LTA. Previous studies have shown that LTA is cleared mainly by the liver and kidneys (Hyzy et al., 1992). Thus, the induction of DME/transporter genes may lead to faster clearance of LTA in TCPOBOP pre-treated $CAR^{+/+}$ mice, leading to diminished effects of LTA on DME/transporter genes.

Activation of TLR2 by its ligands leads to up-regulation of its expression. LTA induced TLR2 expression by ~50 fold in the liver (Ghose et al., 2009), and ~5 fold in odontoblasts (Durand et al., 2006). Another TLR2 ligand, i.e. porin of *Shigella dysenteriae* also induced the expression of TLR2 in hemopoietic cells (Ray et al., 2008). It is possible that induction of TLR2 expression is required for mediating the effects of TLR2 ligands. We find that TLR2 RNA levels were significantly induced by LTA treatment of $CAR^{+/+}$ and $CAR^{-/-}$ mice (~40-45 fold; Fig. 5.7). Interestingly, TCPOBOP pre-treatment attenuated the induction of TLR2 by LTA. We did not find any induction of TLR2 RNA levels in TCPOBOP/saline-treated mice (Fig. 5.7). Lower TLR2 expression can cause reduced binding of LTA, which can contribute towards lower *in vivo* concentration of LTA, thus leading to lack of its effect on DME/transporter genes.

LPS administration in $CAR^{-/-}$ mice led to down-regulation of DME and transporter genes (Fig. 5.2), hence, we expected that LPS-mediated down-regulation of the genes would be independent of CAR. As expected, LPS treatment led to down-regulation of DMEs and transporters even in mice pre-treated with TCPOBOP (Fig. 5.3.2 A). Interestingly, TCPOBOP-induced CAR accumulation in the nucleus was significantly attenuated by LPS. Cytosolic CAR protein was also reduced by LPS in TCPOBOP-treated mice. This is likely due to the reduction in CAR gene expression by LPS in the presence of TCPOBOP (Fig. 5.3.2 B, C & D). The effect of LPS on CAR nuclear protein levels is contrary to the effects of LTA in TCPOBOP-pre-treated mice, and this is reflected in the different effects of LPS and LTA on DME/transporter genes.

Pro-inflammatory cytokines have been implicated in the down-regulation of hepatic genes during inflammation (Barker et al., 1992; Muntane-Relat et al., 1995). Our results show that the induction of cytokines in the liver by LPS and LTA occurred to different extents. LTA administration led to ~80, 35 and 65 fold induction in IL-6, IL-1β and TNF-α respectively, whereas LPS administration led to ~15, 210 and 160 fold inductions (Ghose et al., 2008; 2009). The differential regulation of DME/transporter genes by LTA and LPS may be caused by the differences in induction levels of the cytokines.

LTA treatment induces the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α at 2 h, which coincides with the down-regulation of CAR and its target genes (Ghose et al., 2009). In $CAR^{-/-}$ mice, these cytokines are induced at comparable levels by LTA at 2 h (Fig. 5.8), but DME/transporter genes are not down-regulated by LTA, probably because of the absence of CAR. IL-6 and IL-1β RNA levels are induced similarly in TCPOBOP pre-treated mice on treatment with LTA, however, the extent of TNF-α induction is significantly lower in comparison to $CAR^{+/+}$ mice (Fig. 5.8). It is possible that lower expression of TNFα in TCPOBOP/LTA-treated mice accounts for lack of effect of LTA on DME/transporter genes in these mice. Circadian cycle had no effect on our experiments as we treated the mice at the same time of the day during each experiment and each experiment had their respective control animals which were treated at the same time.

Our study is the first of its kind to determine the role of CAR in regulating DMEs and transporters during inflammation. The down-regulation of DMEs and transporters during inflammation is a complex process, and the molecular mechanism is not fully understood. The effect on hepatic DME/transporter genes depends on the type of inflammatory stimuli, and our results show that there are distinct mechanistic differences between TLR2 and TLR4-induced bacterial inflammation. This indicates that patients exposed to gram-positive and gram-

negative infections may have differences in drug disposition due to mechanistic differences in the regulation of DME/transporter genes. Targeting CAR may have therapeutic implications in countering the deleterious effects of gram-positive bacterial infections on hepatic detoxification processes.

CHAPTER 6

Role of obesity in UGT-mediated glucuronidation of irinotecan metabolite

6.1: Abstract:

Colorectal cancer is the second most common cause of cancer related deaths in the United States. Out of 1 million clinical cases of colorectal cancer, which are clinically diagnosed every year, a significant number of colorectal cancer patients develop colorectal liver metastasis (CLM). Due to its potent anticancer activity, irinotecan (CPT-11), a topoisomerase I inhibitor, is used for CLM treatment by itself or in combination with other drugs. It has been shown recently that CPT-11 administration is associated with liver toxicity and this effect is compounded by baseline obesity. It was found that patients with a BMI index of >25 were twice as much susceptible to developing liver toxicity than patients with BMI index of <25. CPT-11 metabolizes to SN-38, which then undergoes glucuronidation by uridine glucuronosyl transferase (Ugt)1a1 to form SN-38 glucuronide (SN-38G). Excess accumulation of the toxic metabolite SN-38 is known to cause fatal diarrhea in cancer patients. We have previously shown that mice fed on a high-fat diet had reduced gene expression of Ugt1a1. Thus, we hypothesize that the reduction in Ugt1a1 expression in DIO mice will lead to accumulation of SN-38 which could be responsible for irinotecan-induced liver toxicity during obesity. Our studies show that the rate of formation of SN-38G was 2-fold lower in the diet-induced obese (DIO) mice compared to the lean controls. We did not observe significant changes in the area under the curve (AUC) or clearance (CL) of CPT-11 between DIO and lean mice. However, plasma and fecal exposure of SN-38

increased by 2-folds and plasma levels of SN-38G decreased ~2-fold in the DIO mice compared to the lean controls. We also observed significantly higher mRNA and prolonged induction of plasma TNF-α in the DIO mice as compared to the lean mice. Higher TNF-α levels are known to be associated with liver toxicity. Thus, CPT-11 dosage should be closely monitored for effective and safe chemotherapy in obese patients.

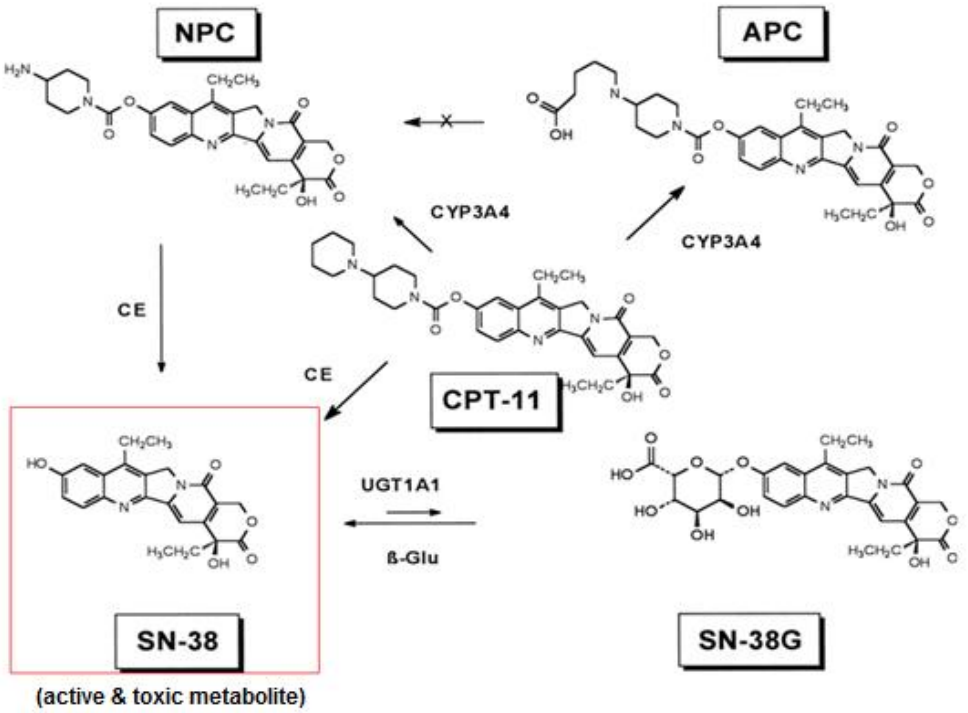
6.2: Introduction:

Colorectal cancer (CRC) is the second most leading cause of cancer related deaths in the United States. It is estimated that worldwide, around 1 million new cases of colorectal cancer are clinically diagnosed; approximately half of the colorectal cancer patients develop liver metastases (Ferlay et al., 2010; Abdalla et al., 2004). The survival of patients with untreated colorectal liver metastases (CLM) is as low as 4 months. Secondary liver resection is considered to be an effective treatment for CLM (Saif et al., 2009). However, only 15-20% of patients with CLM are candidates for surgical resection at the time of diagnosis (Scheele et al., 1995).

Among the various chemotherapeutic agents, the topoisomerase I inhibitor, irinotecan (CPT-11, Camptosar) has been highly effective to treat malignant colorectal cancers (Czejka et al., 2011). Irinotecan has been shown to be highly effective in treatment of colon, stomach, pancreas, and non-small cell lung cancers (Trifan et al., 2002). Irinotecan is a prodrug which is metabolized by carboxylesterases (CES; two isoforms 1 & 2, present in plasma, intestine and high concentrations in the liver) to form the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) (Weekes et al., 2009). SN-38 is a inhibitor of topoisomerase-I, which is an enzyme essential for DNA transcription, replication and repair.

Secondary metabolism of SN-38 is catalyzed Ugt1a1 and 1a9, forming SN-38 glucuronide (SN-38G) which is inactive. The second metabolic pathway of irinotecan is its oxidation by cytochrome P450 (Cyp) 3A4 resulting in the formation of two inactive metabolites, APC ({7-ethyl-10[4-N-(5-aminopentanoicacid)-1-piperidino] carbonyloxycamptothecin}) and NPC ({7-ethyl-10[4-amino- 1 piperidino] carbonyloxycamptothecin}). NPC can be converted to SN-38 by the CES enzymes. The inactive SN-38G metabolite, is excreted via bile duct into small intestine where bacterial β-glucuronidases convert it back to SN-38 (Takasuna et al., 1996).

Fig 6.1: Metabolic pathway of irinotecan



One of the major side effects of irinotecan is life-threatening diarrhea which accounts for the dose limiting toxicity of irinotecan. Irinotecan–induced diarrhea is of two kinds; (i) early-onset, which is due to its inhibitory activity on acetylcholinesterase, or (ii) delayed-onset, which is due to accumulation of SN-38 in the bowel (Hecht, 1998). It has recently been shown that irinotecan administration is associated with steatohepatitis and this effect is compounded by baseline obesity. It was found that patients with a BMI index of >25 were twice as much susceptible to developing steatohepatitis than patients with BMI index of <25 and that irinotecan is associated with steatohepatitis (Pilgrim et al., 2011).

Recently, irinotecan treatment along with etoposide in patients with Non-Hodgkin's lymphoma developed hepatotoxicities which were possibly linked with higher plasma levels of the SN-38 (Ohtsu et al., 1998). However, the mechanism was not investigated. We have seen previously that administration of high-fat diet leads to the reductions in gene expression and protein levels of Ugt1a1 which is primarily responsible for the detoxification of SN-38 (Ghose et al., 2011). Based on these observations, we hypothesize that accumulation of SN-38 is associated with increased liver toxicity of CPT-11 in obesity. Our goal was to determine the role of obesity in reducing SN-38 glucuronidation. We chose the high fat diet model for obesity because this model is clinically relevant. It mimics the human phenotype which includes higher body weight and increased free fatty acids in the systemic circulation (Collins et al., 2004). We find that SN-38 glucuronidation

was significantly reduced in DIO mice as compared to lean controls. Plasma PK profiles of CPT-11, SN-38 and SN-38G were significantly altered in irinotecan-treated DIO mice as demonstrated by alterations in AUC and C_{max} values. Hepatic gene expression of TNF-α and plasma TNF-α levels were significantly induced in irinotecan-treated DIO mice upto 8 h.

This is the first study of its kind attempting to isolate the key factors that may be involved in the higher incidences of inrinotecan-induced liver toxicity during obesity. Activity of Ugt1a1 enzyme should be monitored in obese patients on irinotecan therapy and patients with Ugt1a1 polymorphisms in should be identified and treated accordingly. The outcome of our studies demonstrate that Ugt1a1 mediated glucuronidation of SN-38 maybe the key factor in predicting irinotecan-induced liver toxicity during obesity.

6.3: Materials and methods:

6.3.1: Materials:

Standard CPT-11 and Camptothecin (CPT; internal standard (IS)) were purchased from Sigma-Aldrich; St. Louis, MO. Irinotecan hydrochloride for injections was purchased from APP Pharmaceuticals, Schaumburg, IL (Cat# NDC-63323-19305). SN-38 and SN-38G were a kind gift from Dr Ming Hu's lab at the University of Houston, Houston, TX (SantaCruz Biotechnology; sc-212931). TNF-α Elisa Max ^{TM} Deluxe assay kit (Cat # 430904) was purchased from Biolegend, San Diego, CA. Solvents purchased for chromatography were LC-MS grade and were purchased from VWR international, LLC (Suwanee, GA, USA). Unless specified, all other materials were purchased from Sigma-Aldrich (St Louis, MO, USA.).

6.3.2: Animals

Male, 12 weeks old diet-induced obese (DIO) mice (60% kcal fed) (Cat #382250) and Lean mice (10% kcal fed) (Cat #382256) on a C57BL/6 background were purchased from Jackson Labs, Bar Harbor, ME. The animals were maintained in a 12 h dark/light cycle and in a temperature-and-humidity-controlled environment. The mice had access their respective chow and water *ad libitum.* All the animal care and use protocols were approved by the Institutional Animal Care and Use

Committee guidelines. All experiments were performed in triplicate and repeated at least three times.

6.3.3: Treatments

Irinotecan hydrochloride solution (20 mg/ml) was diluted in sterile water to get a concentration of 2 mg/ml for injections. Mice (n= 5-6) were treated with vehicle or irinotecan hydrochloride solution (10 mg/kg). Blood samples (20-25 µl) were obtained at various time points ranging from 0-8 h. Livers were harvested at 8 h, cryopreserved and stored in -80°C until further use. For serum TNF-α analysis, approximately 100 µl of blood was collected at 1 h and at the end of the study i.e. 8 h. At the end of the study, feces samples were collected from 0-8 h, combined and stored at -80°C to prevent loss of enzyme activity.

6.3.4: Preparation of S9 fractions and Ugt1a1 activity assay

Mouse liver S9 fractions were prepared using a procedure adopted from the literature with minor modifications as described below (Chen et al., 2003, Gandhi et al., 2012). Livers from untreated DIO and lean mice were perfused with sodium phosphate buffer (pH 7.4) and homogenized in ice-cold homogenization buffer (~3 ml) (50 mM potassium phosphate buffer (pH 7.4), 250 mM sucrose, 1 mM EDTA). This homogenate was centrifuged at 15,400 rpm for 15 min at 4°C. The supernatant fractions were collected after discarding the fat layer and stored at -

80°C. Protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A).

For Ugt1a1 activity assay, each reaction contained 2 mg/ml hepatic S9 fractions, 15 μM of SN-38 (1.7 μl of 15 mM stock solution) along with 24 μl of solution A and 30 μl of solution B in 0.05 M Potassium phosphate (KPi, pH 7.4) buffer (total volume 170 μl) for 90 min. Solution A contained 25 mM UDPGA triammonium salt and solution B contained 25 mM saccharolactone, 5 mM $MgCl_2$ and 0.125 mg/ml alamethacin. The reaction was quenched by adding 50 μl of 94% ACN and 6% glacial acetic acid containing 1 μg/ml of internal standard (IS; CPT). Standards were prepared using S9 and different concentrations of SN-38 ranging from 0-15 μM without the cofactors. The samples were then analyzed for SN-38 and SN-38G using LC-MS/MS as describer earlier.

6.3.5: Pharmacokinetic studies, LC-MC/MS sample preparation and analysis

Lean and DIO mice were treated with irinotecan hydrochloride at a dose of 10mg/kg by oral gavage. Blood samples (20-25μl) were collected from the tail vein in heparinized tubes at 0, 5, 15, 30, 60, 120, 240 & 480 min respectively. For quantification of analytes in plasma, LC-MS/MS method was utilized. In order to quantitate CPT-11, SN-38, SN-38G and CPT in plasma an API 5500 Qtrap triple quadrapole mass spectrophotometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with a Turbospray ^{TM} source was used by multiple

reaction monitoring (MRM) method operated in a positive ion mode. Plasma was isolated from blood samples by centrifuging at 9,000 rpm for 3 min. at room temperature. Then, 5 µl of plasma sample was diluted in 40 µl blank plasma and 5 µl of IS (1 µg/ml) and vortexed briefly. Standard samples were prepared by serial dilutions of 45 µl of the highest standard (1000 ng/ml of CPT-11, SN-38 and SN-38G) with 45 µl blank plasma. Then 5 µl of 1 µg/ml of IS was added and the tubes were briefly vortexed. A mixture of 450 µl ACN and 450 µl methanol was added to the samples and standards and the tubes were vortexed for 30 sec and centrifuged at 13,200 rpm for 15 min. The supernatant organic solvent (~825 µl) was transferred to clean eppendorf tubes and evaporated in a gentle stream of air at room temperature. The dried extracts were reconstituted in a solution of 50%ACN/50% methanol/1% glacial acetic acid.

6.3.6: β-Glucuronidase assay

The β-glucuronidase activity in feces samples was determined using an absorbance spectrophotometer (DU800; Beckman Coulter Inc., Indianapolis, IN). Roughly, 1 ml of solvent mixture (50% methanol: 50% water) per g of feces was taken in an eppeodorf tube and homogenized. This fecal extract was stored in -80°C to prevent loss of enzyme activity. In brief, the reaction mixture contained 0.2 ml of 1mM p-nitrophenyl-β-D-glucuronide (Sigma-Aldrich, Cat# N1627), 0.25 ml of 0.1 M phosphate buffer, pH 7.0 and 0.05 ml of the fecal suspension.

The mixture was incubated at 37°C water bath for 30 min. The reaction was quenched by adding 0.5 ml of 0.5 N NaOH. The mixture was then centrifuged at 3000 rpm for 10 min and the supernatant (700 µl) was transferred to clear plastic disposable UV cuvettes and UV absorbance was measured at 540 nm.

6.3.6: real-time PCR

Total RNA was isolated from mouse liver using TRIzol reagent (Sigma-Aldrich, St Louis, MO, U.S.A) according to the manufacturer's protocol. cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems) as described previously (Ghose et al., 2004). In short, each reaction mixture (total of 25µl) contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 µl of TaqMan Universal PCR Master Mix. We extrapolated the quantitative expression values from standard curves and these values were normalized to cyclophilin.

6.3.7: Serum TNF-α assay:

Serum TNF-α was quantified by ELISA kit according to the manufacturer's instructions. Standard curve was performed in duplicates and concentration of TNF-α was calculated using the standard curve as reference. The optical density

at 450 nm and background at 570 nm were measured on a Synergy II microplate reader (BioTek, USA). Calculation of validation parameters of the ELISA showed that the limit of detection for the assay was 0.19 ng/ml. The intra-assay variability was less than 10% and the inter-assay variability was less than 9% between 3 separate days.

6.3.8: Statistical analysis

All data presented as mean ± S.D from 4-5 mice per group. The data were analyzed by un-paired student's t-test using GraphPad Prism 5.2 software (GraphPad Inc., La Jolla, CA) and *$p < 0.05$ was considered to be statistically significant.

6.4: Results:

6.4.1: Ugt1a1 mediated glucuronidation of SN-38

We determined the role of Ugt1a1 in glucuronidation of SN-38 in S9 fractions. As compared to lean mice which had an SN-38G concentration of 16.71 nM, the SN-38G concentration in DIO mice was 10.02 nM ($p < 0.05$) (Fig 6.4.1). This down-regulation of Ugt1a1 activity is in agreement with our earlier study which demonstrated the down-regulation of gene expression of Ugt1a1 in DIO mice (Ghose et al., 2011).

Fig 6.4.1

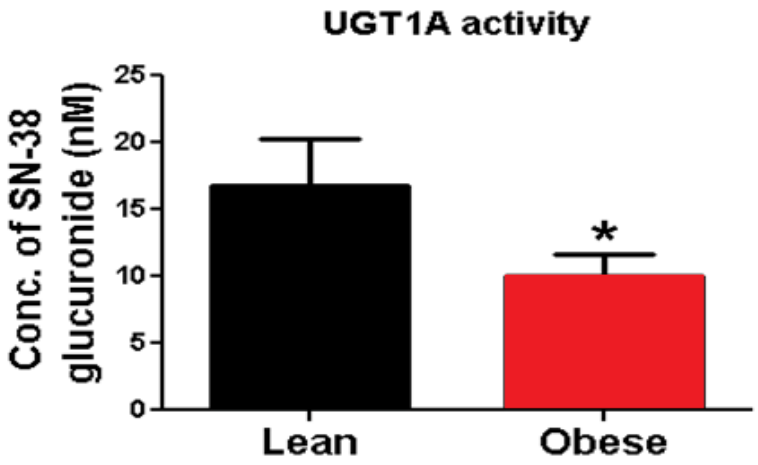


Fig 6.4.1: Ugt1a1 mediated glucuronidation of SN-38 in DIO and lean mice

Livers were harvested from untreated lean and DIO mice (n=6). S9 fractions were prepared as described in materials and methods. Glucuronidation of SN-38 was used as a marker of Ugt1a1 activity. Data are shown as mean ± S.D. * indicates statistical significance at $p<0.05$ when compared to the lean group.

6.4.2: Pharmacokinetic analysis of irinotecan and its metabolites

In order to measure changes in irinotecan and its metaboliets, we performed pharmacokinetic (PK) studies in lean and DIO mice. The data was analyzed by non-compartmental analysis using WinNonLin software 3.3 (Pharsight Corporation Mountain View, California). The plasma PK data revealed no significant differences in AUC and CL of irinotecan in DIO mice compared to the lean controls (Table 6.4.1). Similarly, the concentration of irinotecan in liver tissue and fecal extracts did not vary significantly in DIO mice compared to the lean controls. However, concentrations of SN-38 in plasma, liver tissues and fecal extracts were significantly higher in DIO mice as compared to the lean controls. The concentration of SN-38G in plasma was significantly lower in DIO mice as compared to the lean controls (Fig. 6.4.2). This is in agreement with our earlier experiment where we showed that the activity of Ugt1a1 decreases in DIO mice. We found no significant difference in the levels of SN-38 in liver and fecal extracts in both the groups (data not shown).

Fig 6.4.2

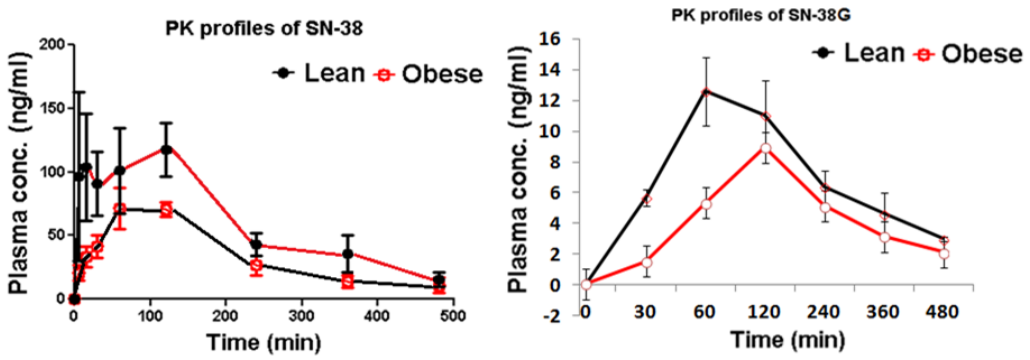


Fig 6.4.2: PK profiles of irinotecan, SN-38 and SN-38G

Plasma concentrations versus time profiles from 0 to 8 h for irinotecan (A), SN-38 (B) and SN-38G (C) in DIO and lean mice upon oral injection of irinotecan (10 mg/kg) are shown. Plasma samples were processed as described earlier. n = 5-6. Data are shown as mean ± S.D.

Table 6.4.1

Parameter	CPT-11		SN-38		SN-38G	
	Lean	Obese	Lean	Obese	Lean	Obese
AUC $_{0\text{-}8h}$ (ng.min/ml)	4448.92 ± 2283.46	4652.27 ± 2993.3	18654.1 ± 4486.83	29599.5 ± 6917.83 *	5987.12 ± 1055.36	3698.88± 994.63 *
T_{max} (min)	8.33 ± 5.77	51.67 ± 60.48	80.0 ± 34.64	64.0 ± 55.16	68.69± 15.36	127.36± 21.33 *
C_{max} (ng/ml)	8.97 ± 2.78	12.87 ± 1.06	108.2 ± 36.30	224.4 ± 78.63 *	12.60± 2.24	8.25± 3.72 *

Table 6.4.1: Pharmacokinetic parameters of irinotecan and its metabolites

Pharmacokinetics parameters of irinotecan, SN-38 and SN-38G were calculated using WinNonlin 5.2 software. Data are shown as mean ± S.D. * indicates statistical significance at $p<0.05$ when compared to the lean group.

6.4.3: Effect of obesity on hepatic TNF-α mRNA expression and TNF-α concentration in plasma

TNF-α is known to cause apoptosis in cells. It was shown that CPT-11 when injected together with TNF-α in mice, was shown to cause significant liver damage (Hentze et al., 2004). We found significant induction in the mRNA expression of TNF-α in DIO mice on treatment with irinotecan (~2-folds) in DIO mice as compared to lean controls at 8 h (Fig 6.4.3.1). Similarly, we observed a moderate, though significant and increase in plasma concentration of TNF-α in both groups at 1 h (Fig. 6.4.3.2). However, the extent of induction was significantly higher and it sustained till 8 h longer in the DIO mice as compared to lean controls.

Fig 6.4.3.1

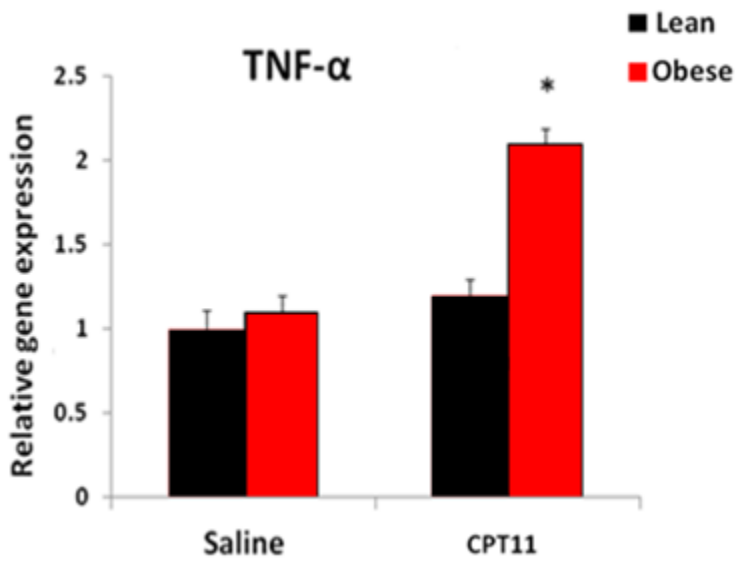
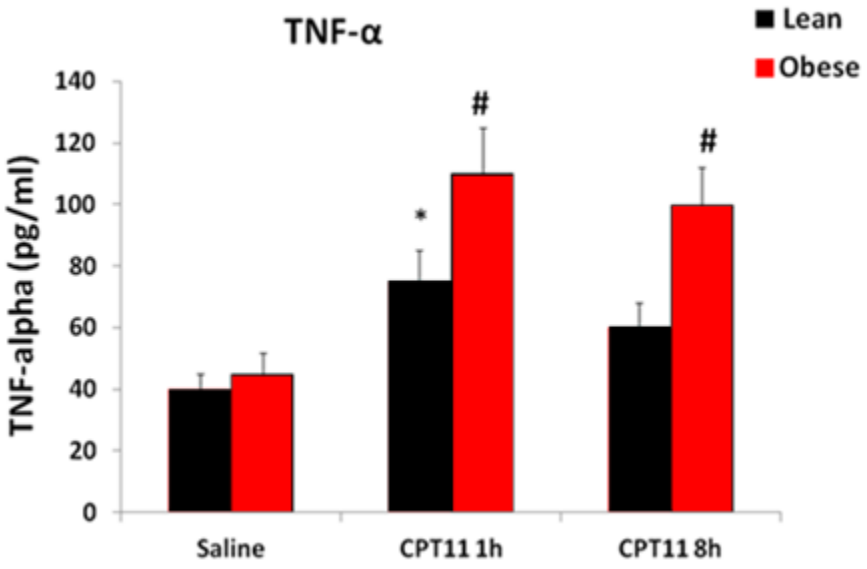


Fig 6.4.3.1: Regulation of TNF-α mRNA levels in Lean and DIO mice following irinotecan administration

Lean and DIO mice were injected with saline or irinotecan (10 mg/kg) via oral gavage and livers were harvested at 8 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of TNF-α were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after irinotecan treatment was compared to the saline-treated controls. * indicates significant difference ($p < 0.05$) between saline and irinotecan groups in lean and DIO mice. The experiments were repeated at least thrice.

Fig 6.4.3.2



6.4.3.2: Regulation of TNF-α plasma levels in Lean and DIO mice following irinotecan administration

Plasma levels of TNF-α were determined at 1 and 8 h after irinotecan administration by ELISA. All the data represented are mean ± S.D. from 5-6 mice per group. * and # indicates statistical significance at $p<0.05$ when irinotecan-treated group compared to the respective saline groups.

6.4.4: β-glucuronidase activity assay

Studies have shown that the intestinal β-glucuronidase enzyme may play in important role in increasing toxicity of irinotecan by increasing circulating SN-38 concentration (Takasuna et al., 1996). In our study, we observed ~2 fold induction in fecal β-glucuronidase enzyme activity in irinotecan-treated DIO mice compared to the lean controls (Fig. 6.4.4).

Fig 6.4.4

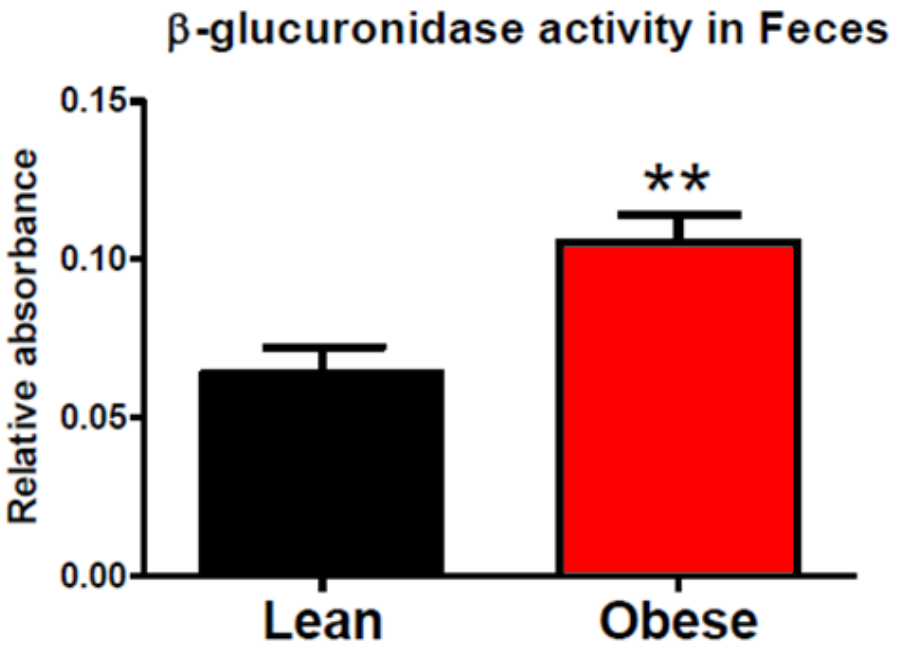


Fig 6.4.4: β-glucuronidase activity in lean and DIO mice

Feces were collected from untreated lean and DIO mice over a period of 24 h. β-glucuronidase enzyme activity was determined as described earlier. All the data represented are mean ± S.D. from 4-5 mice per group. ** indicates statistical significance at $p<0.05$ when compared to the lean group.

6.5: Discussion:

Several studies have reported changes in hepatic Ugt1a1 activity in obese animal models. Although, our previous study showed that Ugt1a1 enzyme activity is decreased in DIO mice (Ghose et al., 2011), the expression and activity of hepatic Ugt1a1 was shown to be increased in genetically obese mice deficient in leptin signaling (ob/ob) (Xu *et al.*, 2012). Thus, these two studies provide contrasting evidences on regulation of hepatic Ugt1a1 activity in obese animal models. In this study, we observed significant down-regulation of hepatic Ugt1a1 gene expression and activity in DIO mice compared to the lean controls. The plasma concentrations of SN-38 were significantly higher and SN-38G concentrations in plasma were significantly lower in DIO mice as compared to the lean controls. The activity of β-glucuronidase enzyme in fecal extracts of DIO mice was ~ 2-folds higher as compared to the lean controls. In addition, hepatic mRNA and plasma levels of TNF-α were induced significantly in irinotecan-treated DIO mice.

In the present study, we observed significant down-regulation of SN-38G formation in DIO mice due to decreased hepatic Ugt1a1 activity. High levels of SN-38 have been associated with life-threatening diarrhea. Irinotecan-induced toxicity (even at low doses) such as neutropenia or diarrhea in humans were correlated with alterations in UGT1A1 activities due to genetic polymorphisms in

the UGT1A1*28/*28 allele (Hu *et al.*, 2010). The authors also concluded that the dose-dependent manner of SN-38 glucuronidation could possible explain the link between UGT1A1*28 and neutropenia. Thus, the results from our study hold clinical significance in justifying that UGT1A1 activity in obese human patients on irinotecan therapy should be monitored.

It has been reported that there is down-regulation of expression of phase I DMEs, phase II DMEs and transporters during obesity (Omoluabi et al., 2011; Osabe et al., 2008). It is well known that changes in expression and activities of DMEs and transporters leads to altered pharmacokinetics and pharmacodynamics of various drugs (Wilkinson et al., 2005; Gandhi et al., 2012). Powis et al., 1987 found that the clearance of the anti-cancer drug cyclophosphamide was reduced in obese breast cancer patients. Rodvold et al., 1988 found that the clearance of doxorubicin was reduced in severely obese individuals. Obese patients have increased sensitivity for psychomotor response to a benzodiazepine derivative drug, triazolam (Derry et al., 1995). Schmid et al., 2009 reported that, in patients with BMI >30 kg/m^2, there was an increased dose requirement for insulin to reach target glucose levels. This altered pharmacokinetics and pharmacodynamics may lead to toxicity or inefficacy depending on whether the parent drug or the metabolite is the active component. Clinical PK of irinotecan is widely reported however, the PK of irinotecan shows a high degree of interpatient variability. Some reports suggest that this variability may be due to genetic variations or drug

interactions (Sasaki et al., 1994). Our previous results show that obesity may be a contributing factor to this wide inter-patient variability (Ghose et al., 2011). However, PK of irinotecan in context of obesity has not been studied before. Obesity is associated with serious comorbidities with a steep global increase in its prevalence. Increased BMI is considered as a risk factor in causing recurrence of CRC (Murphy *et al.*, 2000; Meza *et al.*, 2008). Irinotecan treatment led to significant development of steatohepatitis in CRC patients with a BMI > 25 kg/m2 (Vauthey *et al.*, 2006), however the cause was not explored. Our results show twice the AUC levels of SN-38 with no significant changes in the parent compound in DIO mice compared to lean mice. It is probable that increased plasma levels of SN-38 play a role in liver injury in obese patients.

Several factors, individually or collectively, can play a role in increasing SN-38 concentration *in vivo*. Down-regulation of Ugt1a1 and subsequently lower conversion to SN-38G can account for increased plasma and liver concentration of SN-38 in DIO mice. However, obesity induces CES1 expression in human adipose tissues (Jernas *et al.*, 2009). Thus, increased CES levels in DIO mice could also play a role in increased production of SN-38 from irinotecan. Also, cancer is known to induce CES2 expression (Xu *et al.*, 2002) in humans. Graewin et al., 2013 showed that there is reduced activity of CES2 in leptin-deficient mice. Therefore, further studies exploring the role of obesity on CES will be of clinical relevance in studying irinotecan-induced steatohepatitis in obese populations.

Intestinal bacterial β-glucuronidases have been shown to increase circulating levels of SN-38 in animals (Kaneda *et al.*, 1990; Takasuna *et al.*, 1996) as well as humans (Sparreboom *et al.*, 1998). This observation is commonly known as enterohepatic recirculation and is accounted for by a second peak of SN-38. To assess the importance of SN-38G deconjugation with respect to potential increase in concentration of SN-38, we evaluated β-glucuronidase activity in fecal extracts of DIO and lean mice. We find in our study that the fecal β-glucuronidase activity is ~2-fold higher in DIO mice as compared to the lean controls. Thus, this could be another potential factor in the accumulation of circulating SN-38 levels. However, since SN-38 is not a highly permeable drug, the contribution of β-glucuronidases in accumulation of SN-38 in plasma might be low.

We also explored the role of obesity in irinotecan-induced increase in hepatic and plasma TNF-α. Steatohepatitis encompasses steatosis (fatty liver) with infiltration of inflammatory cells and mediators including TNF-α (Kleiner *et al.*, 2005). Our gene expression studies showed significant increase (~2-fold) in TNF-α mRNA levels in irinotecan treated DIO mice as compared to their lean controls at 8 h. Plasma levels of TNF-α were induced in irinotecan-treated lean as well as treated DIO mice at 1 h however the fold induction was significantly higher in the treated DIO mice. Plasma TNF-α levels remained high in the treated DIO mice whereas they were back to basal levels in the treated lean mice at 8 h. We did not see any basal level differences in the gene levels of TNF-α in lean and DIO mice and this

is in contrast to our previously published results (Ghose *et al*., 2011). This difference could be attributed to the strain difference i.e. CD-1 mice then and C57BL/6 mice now.

An irinotecan-induced steatohepatitis mouse model would be ideal to study the exact mechanism and it will reveal new potential targets to prevent toxicity of irinotecan. As obese patients are at a higher risk for developing irinotecan-induced steatohepatitis, our study provides novel findings on the potential role of reduced Ugt1a1-mediated glucuronidation leading to increased concentration of SN-38 in plasma. Monitoring and altering hepatic Ugt1a1 activity during obesity should be considered as potential therapeutic biomarker/target for preventing adverse reactions of irinotecan.

BIBLIOGRAPHY:

Aitken AE, Richardson TA, and Morgan ET (2006) Regulation of drug-metabolizing enzymes and transporters in inflammation. Annu Rev Pharmacol Toxicol (46):123–149.

Abdalla, E. K., Vauthey, J. N., Ellis, L. M., Ellis, V., Pollock, R., Broglio, K. R., Hess, K., and Curley, S. A. (2004) Recurrence and outcomes following hepatic resection, radiofrequency ablation, and combined resection/ablation for colorectal liver metastases. Ann Surg (239):818-25

Abdel-Razzak, Z., Loyer, P., Fautrel, A., Gautier, J. C., Corcos, L., Turlin, B., Beaune, P., and Guillouzo, A. (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. Molecular pharmacology (44):707-15.

Abdulla, D., Goralski, K.B., Garcia Del Busto Cano, E. and Renton, K.W. (2005) The signal transduction pathways involved in hepatic cytochrome P450 regulation in the rat during an LPS-induced model of CNS inflammation. Drug Metab Dispos. 33(10):1521-31.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity (9):143-50.

Adam-Stitah, S., Penna, L., Chambon, P., and Rochette-Egly, C. (1999) Hyperphosphorylation of the retinoid X receptor alpha by activated c-Jun NH2-terminal kinases. J Biol Chem (274):18932-41.

Akira S, Sato S. (2001) Toll-like receptors and their signaling mechanisms. Scand J Infect Dis. 35(9):555-62.

Akira, S., Takeda, K., and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nature immunology (2):675-80.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature (6857):732-8.

Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P., and Zychlinsky, A. (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science (285):736-9.

Allen, R. G (1998) Oxidative stress and superoxide dismutase in development, aging and gene regulation. Age 21: 47– 76.

Anari, M. R., A. E. Cribb (1995) The duration of induction and species influences the downregulation of cytochrome P450 by the interferon inducer polyinosinic acid-polycytidylic acid. Drug Metab Dispos 23(5): 536-541.

Aouabdi, S., Gibson, G. and Plant, N. (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. Drug Metab Dispos (34):138-44.

Assem M, Schuetz EG, Leggas M, Sun D, Yasuda K, Reid G, Zelcer N, Adachi M, Strom S, Evans RM, Moore DD, Borst P, Schuetz JD (2004) Interactions between hepatic Mrp4 and Sult2a as revealed by the constitutive androstane receptor and Mrp4 knockout mice. J Biol Chem 279(21):22250-22257.

Barker C.W, Fagan J.B, Pasco D.S (1992) Interleukin-1 beta suppresses the induction of 4501A1 and P4501A2 mRNAs in isolated hepatocytes. J Biol Chem (267): 8050-8055.

Barrat, F. J. and R. L. Coffman (2008) Development of TLR inhibitors for the treatment of autoimmune diseases. Immunol Rev (223): 271-283.

Baskin-Bey ES, Huang W, Ishimura N, Isomoto H, Bronk SF, Braley K, Craig RW, Moore DD, Gores GJ (2006) Constitutive androstane receptor (CAR) ligand, TCPOBOP, attenuates Fas-induced murine liver injury by altering Bcl-2 proteins. Hepatology 44(1):252-262

Batra A, Pietsch J, Fedke I, Glauben R, Okur B, Stroh T, Zeitz M, Siegmund B. (2007) Leptin-dependent toll-like receptor expression and responsiveness in preadipocytes and adipocytes. Am J Pathol. (6):1931-41.

Bautista, A. P.; Meszaros, K.; Bojta, J.; Spitzer, J. J. (1990) Superoxide anion generation in the liver during the early stage of endotoxemia in rats. J. Leukoc. Biol. 48: 123– 128.

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR (2000) The acute phase response is associated with retinoid X receptor repression in rodent liver. J Biol Chem 275(21):16390-16399.

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR (2002) Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. Biochem Biophys Res Commun 293(1):145-149.

Beilke LD, Aleksunes LM, Holland RD, Besselsen DG, Beger RD, Klaassen CD, Cherrington NJ. (2009) Constitutive androstane receptor-mediated changes in bile acid composition contributes to hepatoprotection from lithocholic acid-induced liver injury in mice. Drug Metab Dispos 37(5):1035-45.

Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P. and Berkenstam, A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci U S A (95):12208-13.

Bès-Houtmann S, Roche R, Hoareau L, Gonthier MP, Festy F, Caillens H, Gasque P, Lefebvre d'Hellencourt C, Cesari M. (2007) Presence of functional TLR2 and TLR4 on human adipocytes. Histochem Cell Biol. 127(2):131-7.

Blouin RA, Warren GW. (1999) Pharmacokinetic considerations in obesity.J Pharm Sci 88(1):1–7.

Blumberg, B., Sabbagh, W., Jr., Juguilon, H., Bolado, J., Jr., van Meter, C.M., Ong, E.S. and Evans, R.M. (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev (12): 3195-205.

Bohren KM, von Wartburg JP, Wermuth B. (1987) Kinetics of carbonyl reductase from human brain. Biochem J.244(1):165-71.

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, Liu YC, Karin M. (2006)The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell (124):601–613.

Chen JQ, Ström A, Gustafsson JA, Morgan ET. (1995) Suppression of the constitutive expression of cytochrome P-450 2C11 by cytokines and interferons in primary cultures of rat hepatocytes: comparison with induction of acute-phase genes and demonstration that CYP2C11 promoter sequences are involved in the suppressive response to interleukins 1 and 6. Mol Pharmacol 47(5): 940-947.

Chen YH, Wang JP, Sun MF, Wang H, Wei LZ, Wei W (2005). Lipopolysaccharide treatment downregulates the expression of the pregnane X receptor, cyp3a11 and mdr1a genes in mouse placenta. Toxicology 211 (3): 242–252

Chen, C., J. L. Staudinger, et al. (2003) Nuclear receptor, pregname X receptor, is required for induction of UDP-glucuronosyltranferases in mouse liver by pregnenolone-16 alpha-carbonitrile. Drug Metab Dispos 31(7): 908-915.

Cheng PY, Morgan ET (2001) Hepatic cytochrome P450 regulation in disease states. Curr Drug Metab 2(2):165-183.

Cherrington, N. J., A. L. Slitt (2004) Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. Drug Metab Dispos 32(7): 734-741.

Choti, M. A., Sitzmann, J. V., Tiburi, M. F., Sumetchotimetha, W., Rangsin, R., Schulick, R. D., Lillemoe, K. D., Yeo, C. J., and Cameron, J. L. (2002) Trends in long-term survival following liver resection for hepatic colorectal metastases. Ann Surg (235): 759-66.

Christou NV, Tellado-Rodriguez J, Chartrand L, Giannas B, Kapadia B, Meakins J, Rode H, Gordon J. (1989) Estimating mortality risk in preoperative patients using immunologic, nutritional, and acute-phase response variables. Ann Surg. 210(1):69-77.

Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T, Ohata K, Tsukui T, Takeshita F, Sakurai K, Ikegami T, Nakagawa A, Horii T, Nuñez G, Ishii KJ, Akira S. (2010) Immunogenicity of whole-parasite vaccines against Plasmodium

falciparum involves malarial hemozoin and host TLR9. Cell Host Microbe 7(1):50-61.

Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N, Horii T, Akira S. (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med. 201(1):19-25.

Coban, C., K. J. Ishii, et al. (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med 201(1): 19-25.

Coban, C., T. Horii, et al. (2010) TLR9 and endogenous adjuvants of the whole blood-stage malaria vaccine. Expert Rev Vaccines 9(7): 775-784.

Collins, S., Martin, T. L., Surwit, R. S., and Robidoux, J. (2004) Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiol Behav (81): 243-8.

Cotran, Ramzi S.; Kumar, Vinay; Fausto, Nelson; Nelso Fausto; Robbins, Stanley L.; Abbas, Abul K. (2005) Robbins and Cotran pathologic basis of disease (7th ed.).

Crabb DW, Matsumoto M, Chang D, You M. (2004) Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. Proc Nutr Soc.63(1):49-63

Czejka, M., J. Schueller (2005) Pharmacokinetics and metabolism of irinotecan combined with capecitabine in patients with advanced colorectal cancer. Anticancer Res 25(4): 2985-2990.

Dasu MR, Devaraj S, Park S, Jialal I (2010) Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care 33(4):861-8.

Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. (2008) Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. Obesity 16(6):1248-55.

Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. Cell (103): 239-52.

De Bosscher, K., W. Vanden Berghe, et al. (2006). Cross-talk between nuclear receptors and nuclear factor kappaB. Oncogene 25(51): 6868-6886.

Denson LA, Auld KL, Schiek DS, McClure MH, Mangelsdorf DJ, Karpen SJ. (2000) Interleukin-1beta suppresses retinoid transactivation of two hepatic transporter genes involved in bile formation. J Biol Chem. 275(12):8835-43.

Denson, L. A., K. L. Auld, et al. (2000) Interleukin-1beta suppresses retinoid transactivation of two hepatic transporter genes involved in bile formation. J Biol Chem 275(12): 8835-8843.

Derry CL, Kroboth PD, Pittenger AL, Kroboth FJ, Corey SE, Smith RB. (1995) Pharmacokinetics and pharmacodynamics of triazolam after two intermittent doses in obese and normal-weight men. J Clin Psychopharmacol.15(3):197-205.

Devasagayam, TPA; Tilak JC, Boloor KK, Sane Ketaki S, Ghaskadbi Saroj S, Lele RD (2004) Free Radicals and Antioxidants in Human Health: Current Status and Future Prospects. Journal of Association of Physicians of India 52: 796.

Dotzlaw, H., Leygue, E., Watson, P. and Murphy, L.C. (1999) The human orphan receptor PXR messenger RNA is expressed in both normal and neoplastic breast tissue. Clin Cancer Res (5):2103-7.

Dring, M. M., C. A. Goulding (2006) The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. Gastroenterology 130(2): 341-348.

Durand SH, Flacher V, Romeas A, Carrouel F, Colomb E, Vincent C, Magloire H, Couble ML, Bleicher F, Staquet MJ, Lebecque S, Farges JC (2006) Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts. J Immunol (176): 2880–2887.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127(12):2893-917.

Fernandez, F. G., Drebin, J. A., Linehan, D. C., Dehdashti, F., Siegel, B. A., and Strasberg, S. M. (2004) Five-year survival after resection of hepatic metastases

from colorectal cancer in patients screened by positron emission tomography with F-18 fluorodeoxyglucose (FDG-PET). Ann Surg (240):438-4

Fernandez, F. G., Ritter, J., Goodwin, J. W., Linehan, D. C., Hawkins, W. G., and Strasberg, S. M. (2005) Effect of steatohepatitis associated with irinotecan or oxaliplatin pretreatment on resectability of hepatic colorectal metastases. J Am Coll Surg (200): 845-53.

Ferrari, L., J. Y. Jouzeau (1993) Interleukin-1 beta differentially represses drug-metabolizing enzymes in arthritic female rats. J Pharmacol Exp Ther 264(2): 1012-1020.

Ferrari, L., N. Peng, Morgan ET. (2001) Role of nitric oxide in down-regulation of CYP2B1 protein, but not RNA, in primary cultures of rat hepatocytes. Mol Pharmacol 60(1): 209-216.

Forman, B. M., I. Tzameli (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. Nature 395(6702): 612-615.

Frapolli R, Zucchetti M, Sessa C, Marsoni S, Vigano L, Locatelli A, Rulli E, Compagnoni A, Bello E, Pisano C, Carminati P, D'Incalci M (2010) Clinical pharmacokinetics of the new oral camptothecin gimatecan: the inter-patient variability is related to alpha1-acid glycoprotein plasma levels. Eur J Cancer. 46(3):505–516.

Gandhi A, Guo T, Shah P, Moorthy B, Ghose R. (2013) Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice. Toxicol Appl Pharmacol 266(3):430-8.

Gandhi, A. S., Guo, T., Shah, P., Moorthy, B., Chow, D. S., Hu, M., and Ghose, R. (2012) CYP3A-dependent drug metabolism is reduced in bacterial inflammation in mice. Br J Pharmacol. 166(7):2176-87.

Gardner-Stephen, D., Heydel, J.M., Goyal, A., Lu, Y., Xie, W., Lindblom, T., Mackenzie, P. and Radominska-Pandya, A. (2004) Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. Drug Metab Dispos (32):340-7.

Gatti F, Nasta P, Loregian A, Puoti M, Matti A, Pagni S, de Requena DG, Prestini K, Parisi SG, Bonora S, Palù G, Carosi G. (2009) Unboosted fosamprenavir is associated with low drug exposure in HIV-infected patients with mild-moderate liver impairment resulting from HCV-related cirrhosis. J Antimicrob Chemother. 63(3):575-8

Geier, A., M. Wagner (2007) Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. Biochim Biophys Acta 1773(3): 283-308.

Ghose R, Guo T, Haque N (2009) Regulation of gene expression of hepatic drug metabolizing enzymes and transporters by the Toll-like receptor 2 ligand, lipoteichoic acid. Arch Biochem Biophys 481(1):123-130.

Ghose R, Guo T, Vallejo JG, Gandhi A (2011) Differential role of Toll-interleukin 1 receptor domain-containing adaptor protein in Toll-like receptor 2-mediated regulation of gene expression of hepatic cytokines and drug-metabolizing enzymes. Drug Metab Dispos 39(5):874-881.

Ghose R, White D, Guo T, Vallejo J, Karpen SJ (2008) Regulation of hepatic drug-metabolizing enzyme genes by Toll-like receptor 4 signaling is independent of Toll-interleukin 1 receptor domain-containing adaptor protein. Drug Metab Dispos 36(1):95-101.

Ghose R, Zimmerman TL, Thevananther S, Karpen SJ (2004) Endotoxin leads to rapid subcellular re-localization of hepatic RXRalpha: A novel mechanism for reduced hepatic gene expression in inflammation. Nucl Recept 2(1):4.

Ghose, R., Guo, T., Vallejo, J. G., and Gandhi, A. (2011) Differential role of Toll-interleukin 1 receptor domain-containing adaptor protein in Toll-like receptor 2-mediated regulation of gene expression of hepatic cytokines and drug-metabolizing enzymes. Drug Metab Dispos (39): 874-81.

Ghose, R., Omoluabi, O., Gandhi, A., Shah, P., Strohacker, K., Carpenter, K. C., McFarlin, B., and Guo, T. (2011) Role of high-fat diet in regulation of gene expression of drug metabolizing enzymes and transporters. Life Sci (89):57-64.

Ghose, R., White, D., Guo, T., Vallejo, J., and Karpen, S. J. (2008) Regulation of hepatic drug-metabolizing enzyme genes by Toll-like receptor 4 signaling is

independent of Toll-interleukin 1 receptor domain-containing adaptor protein. Drug metabolism and disposition: the biological fate of chemicals (36):95-101.

Gooderham, N. J. and G. J. Mannering (1990) In vitro translational activity of messenger-RNA isolated from mice treated with the interferon inducer, polyriboinosinic acid.polyribocytidylic acid. Biochem Pharmacol 39(5): 865-871.

Goodwin, S. D., Gallis, H. A., Chow, A. T., Wong, F. A., Flor, S. C., and Bartlett, J. A. (1994) Pharmacokinetics and safety of levofloxacin in patients with human immunodeficiency virus infection. Antimicrobial agents and chemotherapy (38):799-804.

Graewin SJ, Kiely JM, Lu D, Svatek CL, Al-Azzawi HH, Swartz-Basile DA, Pitt HA (2008) Leptin regulates gallbladder genes related to gallstone pathogenesis in leptin-deficient mice. J Am Coll Surg. 206(3):503-10.

Grimm M, Kim M, Rosenwald A, Heemann U, Germer CT, Waaga-Gasser AM, Gasser M (2010) Toll-like receptor (TLR) 7 and TLR8 expression on CD133+ cells in colorectal cancer points to a specific role for inflammation-induced TLRs in tumourigenesis and tumour progression. Eur J Cancer 46(15):2849-57.

Gronemeyer, H. (1999) Structure and function of nuclear receptors. EMBO workshop. IDrugs 2(8): 760-762.

Gu, X., S. Ke (2006) Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. J Biol Chem 281(26): 17882-17889.

Guengerich, F. P. (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. Annu Rev Pharmacol Toxicol (39): 1-17.

Guengerich, F. P. (2008) Cytochrome p450 and chemical toxicology. Chem Res Toxicol 21(1): 70-83.

Guo, G. L., G. Lambert (2003) Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. J Biol Chem 278(46): 45062-45071.

Guo, Y. L., Baysal, K., Kang, B., Yang, L. J., and Williamson, J. R. (1998) Correlation between sustained c-Jun N-terminal protein kinase activation and

apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. The Journal of biological chemistry (273): 4027-34.

H. Ichijo, E. Nishida, K. Irie (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275 (5296): 90–94.

Han, D., Shinohara, M., Ybanez, M. D., Saberi, B., and Kaplowitz, N. (2010) Signal transduction pathways involved in drug-induced liver injury. Handb Exp Pharmacol, 267-310.

Han, Z., D. L. Boyle (2001) c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J Clin Invest 108(1): 73-81.

Hardardóttir I, Doerrler W, Feingold KR, Grünfeld C. (1992) Cytokines stimulate lipolysis and decrease lipoprotein lipase activity in cultured fat cells by a prostaglandin independent mechanism. Biochem Biophys Res Commun 186(1):237-43.

Hartmann G, Kim H, Piquette-Miller M (2001) Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. Int Immunopharmacol 1(2):189-199.

Hartmann, G., Vassileva, V., and Piquette-Miller, M. (2005) Impact of endotoxin-induced changes in P-glycoprotein expression on disposition of doxorubicin in mice. Drug Metab Dispos (33): 820-8.

Hattermann K, Picard S, Borgeat M, Leclerc P, Pouliot M, Borgeat P. (2007) The Toll-like receptor 7/8-ligand resiquimod (R-848) primes human neutrophils for leukotriene B4, prostaglandin E2 and platelet-activating factor biosynthesis. FASEB J. 21(7):1575-85

Hayden, M.S., West, A.P. and Ghosh, S. (2006) NF-kappaB and the immune response. Oncogene (25):6758-80.

Hecht, J. R. (1998) Gastrointestinal toxicity or irinotecan. Oncology (12): 72-8.

Heinrichsdorff J, Luedde T, Perdiguero E, Nebreda AR, Pasparakis M. (2008) p38alpha MAPK inhibits JNK activation and collaborates with IκB kinase 2 to prevent endotoxin-induced liver failure. EMBO Rep. (9):1048–1054.

Hentze, H., Latta, M., Kunstle, G., Dhakshinamoorthy, S., Ng, P. Y., Porter, A. G., and Wendel, A. (2004) Topoisomerase inhibitor camptothecin sensitizes mouse hepatocytes in vitro and in vivo to TNF-mediated apoptosis. Hepatology (39):1311-20.

Hida, A. I.; Kawabata, T.; Minamiyama, Y.; Mizote, A.; Okada, S (2003) Saccharated colloidal iron enhances lipopolysaccharide-induced nitric oxide production in vivo. Free Radic. Biol. Med. 34: 1426– 1434.

Horng T, Barton GM, Flavell RA, Medzhitov R. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature (21); 420(6913):329-33.

Houliston, R. A., J. D. Pearson (2001) Agonist-specific cross talk between ERKs and p38(mapk) regulates PGI(2) synthesis in endothelium. Am J Physiol Cell Physiol 281(4): C1266-1276.

Hu, Z. Y., Q. Yu (2010) Dose-dependent association between UGT1A1*28 genotype and irinotecan-induced neutropenia: low doses also increase risk. Clin Cancer Res 16(15): 3832-3842.

Hyzy J, Sciotti V, Albini B, Stinson M (1992) Deposition of circulating streptococcal lipoteichoic acid in mouse tissues. Microb Pathog 13(2):123-132.

Ip, Y. T., and Davis, R. J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Current opinion in cell biology (10): 205-19.

Irizar A, Barnett CR, Flatt PR, Ioannides C. (1995) Defective expression of cytochrome P450 proteins in the liver of the genetically obese Zucker rat. Eur J Pharmacol 293(4):385–93.

Ishii KJ, Akira S (2004) Toll-like Receptors and Sepsis. Curr Infect Dis Rep 6(5):361-366.

J.C. Mathison, R.J. Ulevitch (1979) The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J Immunol, (123): 2133–2143

Jakoby WB, Ziegler DM (1990) The enzymes of detoxication. J. Biol. Chem. 265 (34): 20715

Janssens S, Burns K, Tschopp J, Beyaert R. (2003) MyD88S, a splice variant of MyD88, differentially modulates NF-kappaB- and AP-1-dependent gene expression. FEBS Lett. 548(1-3):103-7.

Jernas, M., Olsson, B., Arner, P., Jacobson, P., Sjostrom, L., Walley, A., Froguel, P., McTernan, P. G., Hoffstedt, J., and Carlsson, L. M. (2009) Regulation of carboxylesterase 1 (CES1) in human adipose tissue. Biochem Biophys Res Commun (383): 63-7.

Jeyaseelan S, Young SK, Fessler MB, Liu Y, Malcolm KC, Yamamoto M, Akira S, Worthen GS (2007) Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-mediated signaling contributes to innate immune responses in the lung during Escherichia coli pneumonia. J Immunol. 178(5):3153-60.

Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. (2001) Endotoxin-induced maturation of MyD88-deficient dendritic cells. J Immunol (166): 5688-94.

Kakizaki S, Yamazaki Y, Takizawa D, Negishi M (2008) New insights on the xenobiotic-sensing nuclear receptors in liver diseases CAR and PXR. Curr Drug Metab 9(7):614-621.

Kaneda, N., Nagata, H., Furuta, T., and Yokokura, T. (1990) Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. Cancer Res (50):1715-20.

Kast, H.R., Goodwin, B., Tarr, P.T., Jones, S.A., Anisfeld, A.M., Stoltz, C.M., Tontonoz, P., Kliewer, S., Willson, T.M. and Edwards, P.A (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem (277): 2908-15.

Kawai T, Akira S. (2005) Toll-like receptor downstream signaling. Arthritis Res Ther. 7(1):12-9.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity (11):115-22.

Kawai, T., K. Takahashi, S. Akira (2005) IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 6(10): 981-988.

Kim S, Sohn I, Ahn JI, Lee KH, Lee YS, Lee YS. (2004) Hepatic gene expression profiles in a long term high-fat diet-induced obesity mouse model. Gene 340(1):99-109.

Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., and Sanyal, A. J. (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology (41): 1313-21.

Kliewer, S. A. and T. M. Willson (2002) Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. J Lipid Res 43(3): 359-364.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., McKee, D.D., Oliver, B.B., Willson, T.M., Zetterstrom, R.H., Perlmann, T. and Lehmann, J.M. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell (92):73-82.

Knolle, P., J. Schlaak (1995) Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. J Hepatol 22(2): 226-229.

Kovacsovics, M., F. Martinon (2002) Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. Curr Biol 12(10): 838-843.

Kraemer, M. J., Furukawa, C. T., Koup, J. R., Shapiro, G. G., Pierson, W. E., and Bierman, C. W. (1982) Altered theophylline clearance during an influenza B outbreak. Pediatrics (69): 476-80.

Kumar, A., Takada, Y., Boriek, A.M. and Aggarwal, B.B. (2004) Nuclear factor-kappaB: its role in health and disease. J Mol Med (82): 434-48.

Kumar, H., S. Koyama (2008) Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 180(2): 683-687.

Langmann, T., C. Moehle (2004) Loss of detoxification in inflammatory bowel disease: dysregulation of pregnane X receptor target genes Gastroenterology 127(1): 26-40.

Lee, C. C., Avalos, A. M., and Ploegh, H. L. (2012) Accessory molecules for Toll-like receptors and their function. Nat Rev Immunol (12):168-79.

Lehmann, J.M., McKee, D.D., Watson, M.A., Willson, T.M., Moore, J.T. and Kliewer, S.A.: The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. J Clin Invest 102 (1998) 1016-23.

Li D, Zimmerman TL, Thevananther S, Lee HY, Kurie JM, Karpen SJ (2002) Interleukin-1 beta-mediated suppression of RXR: RAR transactivation of the Ntcp promoter is JNK-dependent. J Biol Chem 277(35):31416-31422.

Li D, Zimmerman TL, Thevananther S, Lee HY, Kurie JM, Karpen SJ (2002) Interleukin-1 beta-mediated suppression of RXR: RAR transactivation of the Ntcp promoter is JNK-dependent. J Biol Chem 277(35):31416-31422.

Lima Trajano ET, Sternberg C, Caetano M, Santos Silva MA, Porto LC, Santos JC, Ribeiro ML, Magalhães CB, Zin WA, Benjamim CF, Valença SS (2011) Endotoxin-induced acute lung injury is dependent upon oxidative response. Inhal

Liu S, Gallo DJ, Green AM, Williams DL, Gong X, Shapiro RA, Gambotto AA, Humphris EL, Vodovotz Y and Billiar TR (2002) Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infect Immun (70): 3433-3442.

Liu, S., Gallo, D. J., Green, A. M., Williams, D. L., Gong, X., Shapiro, R. A., Gambotto, A. A., Humphris, E. L., Vodovotz, Y., and Billiar, T. R. (2002) Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infect Immun (70): 3433-42.

Maheo, K., J. Antras-Ferry (1997) Modulation of glutathione S-transferase subunits A2, M1, and P1 expression by interleukin-1beta in rat hepatocytes in primary culture. J Biol Chem 272(26): 16125-16132.

Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, Teti G, Beninati C. (2009) Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol. 10(6):587-94.

Mancuso, G., M. Gambuzza (2009) Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol 10(6): 587-594.

Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. Cell 83 (6):841-850.

Matsumura T, Degawa T, Takii T, Hayashi H, Okamoto T, Inoue J, Onozaki K (2003) TRAF6-NF-kappaB pathway is essential for interleukin-1-induced TLR2 expression and its functional response to TLR2 ligand in murine hepatocytes. Immunology 109(1):127-136.

Matsumura T, Ito A, Takii T, Hayashi H, Onozaki K. (2000) Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. J Interferon Cytokine Res. 20(10):915-21.

McEwan, I. J. (2004) Sex, drugs and gene expression: signalling by members of the nuclear receptor superfamily. Essays Biochem (40): 1-10.

Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. (2003) The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol. 170(10):5165-75.

Medzhitov R, Preston-Hurlburt P, Janeway CA (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388 (6640): 394–7.

Meza, R., J. Jeon (2010) Colorectal cancer incidence trends in the United States and United kingdom: evidence of right- to left-sided biological gradients with implications for screening. Cancer Res 70(13): 5419-5429.

Moore, L. B., D. J. Parks (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J Biol Chem 275(20): 15122-15127.

Moore, L.B., Maglich, J.M., McKee, D.D., Wisely, B., Willson, T.M., Kliewer, S.A., Lambert, M.H. and Moore, J.T. (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. Mol Endocrinol (16):977-86.

Morere, P., G. Nouvet (1975) Information obtained by liver biopsy in 100 tuberculous patients. Sem Hop 51(31-34): 2095-2102.

Morgan, E. T. (1989) Suppression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin.Mol Pharmacol 36(5): 699-707.

Morgan, E. T. (1997) Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev (29), 1129-88.

Morgan, E. T., T. Li-Masters (2002) Mechanisms of cytochrome P450 regulation by inflammatory mediators. Toxicology)181-182): 207-210.

Muntane-Relat J, Ourlin J.C, Domergue J, Maurel P (1995) Differential effects of cytokines on the inducible expression of CYP1A1, CYP1A2, and CYP3A4 in human hepatocytes in primary culture. Hepatology (22) 1143-1153.

Muntane-Relat, J., Ourlin, J. C., Domergue, J., and Maurel, P. (1995) Differential effects of cytokines on the inducible expression of CYP1A1, CYP1A2, and CYP3A4 in human hepatocytes in primary culture. Hepatology (22): 1143-53.

Murphy TK, Calle EE, Rodriguez C, Kahn HS, Thun MJ. (2000) Body mass index and colon cancer mortality in a large prospective study. Am J Epidemiol. 152(9):847-54.

Na, S.Y., Kang, B.Y., Chung, S.W., Han, S.J., Ma, X., Trinchieri, G., Im, S.Y., Lee, J.W. and Kim, T.S (1999) Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFkappaB. J Biol Chem (274):7674-80.

Nakamura, T., Y. Saito (2001) Apparent low frequency of sequence variability within the proximal promoter region of the cytochrome P450 (CYP) 3A5 gene in established cell lines from Japanese individuals. Biol Pharm Bull 24(8): 954-957

Nebert, D. W., and Russell, D. W. (2002) Clinical importance of the cytochromes P450. Lancet (360): 1155-62.

Negishi M, Pedersen LG, Petrotchenko E, Shevtsov S, Gorokhov A, Kakuta Y, Pedersen LC. (2001) Structure and function of sulfotransferases. Arch Biochem Biophys. 390(2):149-57

Ohnishi H, Tochio H, Kato Z, Orii KE, Li A, Kimura T, Hiroaki H, Kondo N, Shirakawa M. (2009) Structural basis for the multiple interactions of the MyD88 TIR domain in TLR4 signaling. Proc Natl Acad Sci U S A. 106(25):10260-5

Ohtsu, T., Sasaki, Y., Igarashi, T., Murayama, T., Kobayashi, Y., and Tobinai, K. (1998) Unexpected hepatotoxicities in patients with non-Hodgkin's lymphoma treated with irinotecan (CPT-11) and etoposide. Jpn J Clin Oncol (28):502-6.

Olefsky JM, Reaven GM. (1975) Effects of age and obesity on insulin binding to isolated adipocytes. Endocrinology 96(6):1486-98.

O'Neill, L. A. (1995) Interleukin-1 signal transduction. Int J Clin Lab Res 25(4): 169-177.

Onoguchi, K., M. Yoneyama (2007) Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 282(10): 7576-7581.

Osabe, M., J. Sugatani (2008) Expression of hepatic UDP-glucuronosyltransferase 1A1 and 1A6 correlated with increased expression of the nuclear constitutive androstane receptor and peroxisome proliferator-activated receptor alpha in male rats fed a high-fat and high-sucrose diet. Drug Metab Dispos 36(2): 294-302.

P Shah, T Guo, D Moore & R Ghose. Role of constitutive androstane receptor in Toll-like receptor -mediated down-regulation of drug metabolizing enzymes and transporters. Drug Metabolism and Disposition 2014 42(1):172-81.

Pascussi JM, Busson-Le Coniat M, Maurel P, Vilarem MJ. (2003) Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. Mol Endocrinol. 17(1):42-55.

Pascussi, J. M., Dvorak, Z., Gerbal-Chaloin, S., Assenat, E., Maurel, P., and Vilarem, M. J. (2003) Pathophysiological factors affecting CAR gene expression. Drug Metab Rev (35): 255-68.

Pascussi, J. M., S. Gerbal-Chaloin (2000) Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. Biochem Biophys Res Commun 274(3): 707-713.

Peng, H. M. and M. J. Coon (2000) Promoter function and the role of cytokines in the transcriptional regulation of rabbit CYP2E1 and CYP2E2. Arch Biochem Biophys 382(1): 129-137.

Philippe Lefebvre, Yacir Benomar and Bart Staels. (2010) Retinoid X receptors: common heterodimerization partners with distinct functions. Trends Endocrinol. Metab. (21): 676–683

Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, Hess JF. (1996) Leptin receptor missense mutation in the fatty Zucker rat. Nat Genet. (1):18-9.

Pilgrim, C. H., Thomson, B. N., Banting, S., Phillips, W. A., and Michael, M. (2012) The developing clinical problem of chemotherapy-induced hepatic injury. ANZ J Surg (82): 23-9.

Powis G, Reece P, Ahmann DL, Ingle JN. (1987) Effect of body weight on the pharmacokinetics of cyclophosphamide in breast cancer patients. Cancer Chemother Pharmacol. 20(3):219-22.

R. A. Blouin, A. M. Bandyopadhyay, I. Chaudhary, L. W. Robertson, B. Gemzik, and A. (1993) Parkinson. Cytochrome P450 2B enzyme (CYP2B) induction defect following Phenobarbital treatment in the fa/fa Zucker rat: molecular characterization. Arch. Biochem. Biophys. (303):313Y 320

Radominska-Pandya A, Ouzzine M, Fournel-Gigleux S, Magdalou J. (2005) Structure of UDP-glucuronosyltransferases in membranes. Methods Enzymol. (400):116-47.

Ray A, Chatterjee NS, Bhattacharya SK, Biswas T (2003) Porin of Shigella dysenteriae enhances mRNA levels for Toll-like receptor 2 and MyD88, up-regulates CD80 of murine macrophage, and induces the release of interleukin-12. FEMS Immunol Med Microbiol 5; 39(3):213-219.

Renton KW (2004) Cytochrome P450 regulation and drug biotransformation during inflammation and infection. Curr Drug Metab (5):235–243.

Renton KW, Nicholson TE (2000) Hepatic and central nervous system cytochrome P450 are down-regulated during lipopolysaccharide-evoked localized inflammation in brain. J Pharmacol Exp Ther 294(2):524-530.

Renton, K. W., and Nicholson, T. E. (2001) Hepatic and central nervous system cytochrome P450 are down-regulated during lipopolysaccharide-evoked localized inflammation in brain. J Pharmacol Exp Ther (294):524-30.

Richardson TA, Morgan ET (2005) Hepatic cytochrome P450 gene regulation during endotoxin-induced inflammation in nuclear receptor knockout mice. J Pharmacol Exp Ther 314(2):703-709.

Robbins and Cotran pathologic basis of disease (7th ed.). St. Louis, MO: Elsevier Saunders. p. 878.

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. (1998) A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A. 95(2):588-93.

Rodvold KA, Rushing DA (1988) Tewksbury DA. Doxorubicin clearance in the obese. J Clin Oncol. 6(8):1321-7.

Roulston, A., Reinhard, C., Amiri, P., and Williams, L. T. (1998) Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. The Journal of biological chemistry (273): 10232-9.

Saadane, A., N. Neveux (1996) Inhibition of liver RNA breakdown during acute inflammation in the rat. Biochem J (317): 907-912.

Sabbah A, Bose S. (2009) Retinoic acid inducible gene I activates innate antiviral response against human parainfluenza virus type 3. Virol J. (17) 6: 200.

Saif, M. W. (2009) Secondary hepatic resection as a therapeutic goal in advanced colorectal cancer. World J Gastroenterol (15): 3855-64.

Sakai, H., T. Okamoto (1992) Suppression of hepatic drug metabolism by the interferon inducer, polyriboinosinic acid:polyribocitidylic acid. J Pharmacol Exp Ther 263(1): 381-386.

Saklatvala, J., J. Dean (1999) Protein kinase cascades in intracellular signalling by interleukin-I and tumour necrosis factor. Biochem Soc Symp (64): 63-77.

Sakurai T, Maeda S, Chang L, Karin M. (2006) Loss of hepatic NF-κB activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. Proc Natl Acad Sci USA (103):10544–10551.

Scheele, J., Stang, R., Altendorf-Hofmann, A., and Paul, M. (1995) Resection of colorectal liver metastases. World J Surg (19): 59-71.

Schindler, C. and J. E. Darnell, Jr. (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem (64): 621-651.

Schmid C, Krayenbühl P, Wiesli P. (2009) Increased insulin dose requirement of long-acting insulin analogues in obese patients with type 2 diabetes. Diabetologia 52(12):2668-9.

Schnare, M., Holt, A. C., Takeda, K., Akira, S., and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr Biol (10): 1139-42.

Schoemaker, M. H., L. Conde de la Rosa, et al. (2004) Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. Hepatology 39(6): 1563-1573.

Schwabe RF, Seki E, Brenner DA (2006) Toll-like receptor signaling in the liver. Gastroenterology 130(6):1886-1900.

Scott, M. J., Liu, S., Shapiro, R. A., Vodovotz, Y., and Billiar, T. R. (2009) Endotoxin uptake in mouse liver is blocked by endotoxin pretreatment through a

suppressor of cytokine signaling-1-dependent mechanism. Hepatology (49):1695-708.

Seki, E., and Brenner, D. A. (2008) Toll-like receptors and adaptor molecules in liver disease: update. Hepatology (48):322-35.

Sewer MB, Koop DR, Morgan ET (1997) Differential inductive and suppressive effects of endotoxin and particulate irritants on hepatic and renal cytochrome P-450 expression. J Pharmacol Exp Ther 280(3):1445-1454.

Sewer, M. B., Koop, D. R., and Morgan, E. T. (1996) Endotoxemia in rats is associated with induction of the P4504A subfamily and suppression of several other forms of cytochrome P450. Drug Metab Dispos (24):401-7.

Shedlofsky, S. I., B. C. Israel. (1997) Endotoxin depresses hepatic cytochrome P450-mediated drug metabolism in women. Br J Clin Pharmacol 43(6): 627-632.

Shedlofsky, S. I., Israel, B. C., McClain, C. J., Hill, D. B., and Blouin, R. A. (1994) Endotoxin administration to humans inhibits hepatic cytochrome P450-mediated drug metabolism. J Clin Invest (94): 2209-14.

Shen G, Kong AN (2009) Nrf2 plays an important role in coordinated regulation of Phase II drug metabolism enzymes and Phase III drug transporters. Biopharm Drug Dispos 30(7):345-355.

Shoelson SE, Goldfine AB. (2009) Getting away from glucose: fanning the flames of obesity-induced inflammation. Nat Med. 15(4):373-4.

Siewert, E., Bort, R., Kluge, R., Heinrich, P. C., Castell, J., and Jover, R. (2000) Hepatic cytochrome P450 down-regulation during aseptic inflammation in the mouse is interleukin 6 dependent. Hepatology (32):49-55.

Siewert, E., R. Bort, et al. (2000) Hepatic cytochrome P450 down-regulation during aseptic inflammation in the mouse is interleukin 6 dependent. Hepatology 32(1): 49-55.

Slotkin TA (1999) Mary Bernheim and the discovery of monoamine oxidase. Brain Res Bull. (5-6):373.

Sonne, J., Dossing, M., Loft, S., and Andreasen, P. B. (1985) Antipyrine clearance in pneumonia. Clin Pharmacol Ther (37): 701-4.

Sparreboom, A., de Jonge, M. J., de Bruijn, P., Brouwer, E., Nooter, K., Loos, W. J., van Alphen, R. J., Mathijssen, R. H., Stoter, G., and Verweij, J. (1998) Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res (4) 2747-54.

Stanley, L. A., D. J. Adams (1991) Differential effects of recombinant interferon alpha on constitutive and inducible cytochrome P450 isozymes in mouse liver. Biochem Pharmacol 42(2): 311-320.

Staudinger, J. L., A. Madan, et al. (2003) Regulation of drug transporter gene expression by nuclear receptors. Drug Metab Dispos 31(5): 523-527.

Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, Ogawa Y. (2007) Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol. 27(1):84-91.

Sugatani J, Kojima H, Ueda A, Kakizaki S, Yoshinari K, Gong QH, Owens IS, Negishi M, Sueyoshi T. (2001) The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. Hepatology. 33(5):1232-8.

Synold, T.W., Dussault, I. and Forman, B.M (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nat Med (7):584-90.

Takagi K, Matsumura S, Okuda-Ashitaka E, Okuda K, Watanabe J, Takahashi H, Iwakura Y, Ito S. (2007) Interleukin-1 is not essential for expression of inducible NOS in hepatocytes induced by lipopolysaccharide in vivo. Nitric Oxide. 16(4):433-41.

Takasuna, K., Hagiwara, T., Hirohashi, M., Kato, M., Nomura, M., Nagai, E., Yokoi, T., and Kamataki, T. (1996) Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res (56): 3752-7.

Takasuna, K., Hagiwara, T., Hirohashi, M., Kato, M., Nomura, M., Nagai, E., Yokoi, T., and Kamataki, T. (1996) Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res (56):3752-7.

Takeda K, Akira S. (2010) Regulation of innate immune responses by Toll-like receptors. Jpn J Infect Dis. 54(6):209-19.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity (11):443-51.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Muhlradt, P. F., and Akira, S. (2000) Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol (164):554-7.

Tapner, M., Liddle, C., Goodwin, B., George, J., and Farrell, G. C. (1996) Interferon gamma down-regulates cytochrome P450 3A genes in primary cultures of well-differentiated rat hepatocytes. Hepatology (24): 367-73.

Tapping, R. I., S. Akashi (2000) Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. J Immunol 165(10): 5780-5787.

Teng S, Piquette-Miller M (2005) The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. J Pharmacol Exp Ther 312(2):841-848

Tett, S., Moore, S., and Ray, J. (1995) Pharmacokinetics and bioavailability of fluconazole in two groups of males with human immunodeficiency virus (HIV) infection compared with those in a group of males without HIV infection. Antimicrobial agents and chemotherapy (39):1835-41.

Tien ES, Negishi M (2006) Nuclear receptors CAR and PXR in the regulation of hepatic metabolism. Xenobiotica 36(10-11):1152-1163.

Timsit, Y. E. and M. Negishi (2007) CAR and PXR: the xenobiotic-sensing receptors.Steroids 72(3): 231-246.

Tollet, P., B. Enberg (1990) Growth hormone (GH) regulation of cytochrome P-450IIC12, insulin-like growth factor-I (IGF-I), and GH receptor messenger RNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I, and thyroid hormone. Mol Endocrinol 4(12): 1934-1942.

Tran-Thi, T. A.; Decker, K.; Baeuerle, P. A (1995) Differential activation of transcription factors NF-kappa B and AP-1 in rat liver macrophages. Hepatology 22: 613– 619.

Trifan OC, Durham WF, Salazar VS, Horton J, Levine BD, Zweifel BS, Davis TW, Masferrer JL. (2002) Cyclooxygenase-2 inhibition with celecoxib enhances antitumor efficacy and reduces diarrhea side effect of CPT-11. Cancer Res 62(20):5778-84.

Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araújo EP, Vassallo J, Curi R, Velloso LA, Saad MJ. (2007) Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 56(8):1986-98.

Tzameli, I., P. Pissios, Moore DD. (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. Mol Cell Biol 20(9): 2951-2958.

Van Bossuyt H, Desmaretz C, Rombaut B, Wisse E. (1988) Response of cultured rat Kupffer cells to lipopolysaccharide. Arch Toxicol. 62(4):316-24.

Van Bossuyt, H., C. Desmaretz (1988) Response of cultured rat Kupffer cells to lipopolysaccharide. Arch Toxicol 62(4): 316-324.

Vauthey, J. N., Pawlik, T. M., Ribero, D., Wu, T. T., Zorzi, D., Hoff, P. M., Xiong, H. Q., Eng, C., Lauwers, G. Y., Mino-Kenudson, M., Risio, M., Muratore, A., Capussotti, L., Curley, S. A., and Abdalla, E. K. (2006) Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. J Clin Oncol (24): 2065-72.

Viatour, P., M. P. Merville (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci 30(1): 43-52.

Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, Quax PH, Lim SK, Borst C, Pasterkamp G, de Kleijn DP (2002) In vivo evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation 106(15):1985-90.

Voet Donald; Voet, Judith G. (2004). Biochemistry (3rd ed.). Wiley. p. 1309

Wagner, M., Halilbasic, E., Marschall, H.U., Zollner, G., Fickert, P., Langner, C., Zatloukal, K., Denk, H. and Trauner, M. (2005) CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. Hepatology (42):420-30.

Wang T, Shankar K, Ronis MJ, Mehendale HM. (2007) Mechanisms and outcomes of drug and toxicant-induced liver toxicity in diabetes. Crit Rev Toxicol 37(5):413–59.

Warren, G. W., Poloyac, S. M., Gary, D. S., Mattson, M. P., and Blouin, R. A. (1999) Hepatic cytochrome P-450 expression in tumor necrosis factor-alpha receptor (p55/p75) knockout mice after endotoxin administration. J Pharmacol Exp Ther (288): 945-50.

Weekes, J., Lam, A. K., Sebesan, S., and Ho, Y. H. (2009) Irinotecan therapy and molecular targets in colorectal cancer: a systemic review. World J Gastroenterol (15): 3597-602.

Wei P, J Zhang, M Egan-Hafley, S Liang, D.D Moore (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. Nature (407) 920–923

Wei, P., Zhang, J., Dowhan, D.H., Han, Y. and Moore, D.D (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. Pharmacogenomics (J 2):117-26.

Weinberger, C., S. M. Hollenberg, et al. (1985) Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. Nature 318(6047): 670-672.

Wheeler JB, Stourman NV, Thier R, Dommermuth A, Vuilleumier S, Rose JA, Armstrong RN, Guengerich FP. (2001) Conjugation of haloalkanes by bacterial and mammalian glutathione transferases: mono- and dihalomethanes. Chem Res Toxicol 14(8):1118-27.

Whitmarsh, A. J., and Davis, R. J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. Journal of molecular medicine (Berlin, Germany) (74):589-607.

Wilkinson, G. R. (1997) The effects of diet, aging and disease-states on presystemic elimination and oral drug bioavailability in humans. Adv Drug Deliv Rev (27): 129-159.

Wright, K., and Morgan, E. T. (1990) Transcriptional and post-transcriptional suppression of P450IIC11 and P450IIC12 by inflammation. FEBS letters (271):59-61.

Wysocka M, Kubin M, Vieira LQ, Ozmen L, Garotta G, Scott P, Trinchieri G. (1995) Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. Eur J Immunol. 25(3):672-6.

Xie, W. and Y. Tian (2006) Xenobiotic receptor meets NF-kappaB, a collision in the small bowel. Cell Metab 4(3): 177-178.

Xie, W., Barwick, J.L., Simon, C.M., Pierce, A.M., Safe, S., Blumberg, B., Guzelian, P.S. and Evans, R.M (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev (14):3014-23.

Xie, W., M. F. Yeuh (2003) Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proc Natl Acad Sci U S A 100(7): 4150-4155.

Xu, C., Li, C.Y. and Kong, A.N. (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res (28):249-68.

Xu, G., Zhang, W., Ma, M. K., and McLeod, H. L. (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. Clin Cancer Res (8): 2605-11.

Xu, J., Kulkarni, S. R., Li, L., and Slitt, A. L. (2012) UDP-glucuronosyltransferase expression in mouse liver is increased in obesity- and fasting-induced steatosis. Drug Metab Dispos (40): 259-66.

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301(5633):640-3.

Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 169(12):6668-72.

Yamamoto Y, Moore R, Goldsworthy TL, Negishi M, Maronpot RR (2004) The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice. Cancer Res. 64(20):7197-200.

Yamamoto, M., S. Sato (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420(6913): 324-329.

Yamazaki, Y., S. Kakizaki (2007) The role of the nuclear receptor constitutive androstane receptor in the pathogenesis of non-alcoholic steatohepatitis. Gut 56(4): 565-574.

Yarovinsky, F., D. Zhang (2005) TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 308(5728): 1626-1629.

Yoneyama, M., M. Kikuchi (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5(7): 730-737.

Yoneyama, M., M. Kikuchi (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 175(5): 2851-2858.

Yoshinari K, Takagi S, Yoshimasa T, Sugatani J, Miwa M. (2006) Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. Pharm Res 23(6):1188–200.

Yu, R., Lei, W., Mandlekar, S., Weber, M. J., Der, C. J., Wu, J., and Kong, A. N. (1999) Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. J Biol Chem (274): 27545-52.

Yueh, M. F., Y. H. Huang (2003) Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. J Biol Chem 278(17): 15001-15006.

Zhang J, Huang W, Chua SS, Wei P, Moore DD (2002) Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. Science (298):422–424.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. (1996) Positional cloning of the mouse obese gene and its human homologue. Nature 372(6505):425-32.

Zhang, H., E. LeCulyse (1999) Rat pregnane X receptor: molecular cloning, tissue distribution, and xenobiotic regulation.Arch Biochem Biophys 368(1): 14-22.

Zhang, J., W. Huang. (2004). The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. J Biol Chem 279(47): 49517-49522.

Zhou, C., M. M. Tabb (2006) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. J Clin Invest 116(8): 2280-2289.

Zhou, H., N. Lu. (2009) Osteopontin mediates dense culture-induced proliferation and adhesion of prostate tumour cells: role of protein kinase C, p38 mitogen-activated protein kinase and calcium. Basic Clin Pharmacol Toxicol 104(2): 164-170.

Zhou, J., J. Zhang, et al. (2005) Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyl-transferases. Curr Drug Metab 6(4): 289-298.

Zimmerman TL, Thevananther S, Ghose R, Burns AR, Karpen SJ (2006) Nuclear export of retinoid X receptor alpha in response to interleukin-1beta-mediated cell signaling: roles for JNK and SER260. J Biol Chem 281(22): 15434-15440.

Zordoky BN, El-Kadi AO (2009) Role of NF-kappaB in the regulation of cytochrome P450 enzymes. Curr Drug Metab 10(2):164-178.