

Pregnane X receptor activation potentiates the hepatotoxicity of pharmacoenhancers

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Ritonavir (RTV) is a first generation pharmacoenhancer used in anti-HIV therapy. Hepatotoxicity is one of the major safety concerns for HIV/AIDS patients receiving ritonavir (RTV)-containing antiretroviral regimens. In a recent series of clinical trials, the hepatotoxicity associated with RTV-containing regimens occurred in 100% of subjects who were pretreated with rifampicin (RIF) or efavirenz (EFV). Both RIF and EFV are potent ligands of human pregnane X receptor (PXR), a ligand-dependent transcription factor that upregulates Cytochrome P450 3A4 (CYP3A4) expression. Therefore, we hypothesize that drug mediated PXR activation potentiates RTV hepatotoxicity. Due to specie differences in PXR activation, humanized PXR and CYP3A4 mouse models were utilized. Pretreatment with both RIF and EFV potentiated RTV hepatotoxicity. Further studies showed that PXR mediated CYP3A4 induction increased RTV bioactivation in a humanized PXR/CYP3A4 mouse model which resulted in oxidative stress, endoplasmic reticulum stress and cellular injury.

The second part of this dissertation focused on cobicistat (COBI), the new second generation pharmacoenhancer and structural analog of RTV. Clinical trials comparing the safety of COBI to RTV showed a similar rate of liver injury adverse events between the COBI and RTV treatment arms suggesting a similarity in their adverse effect profile. Based on our initial findings

that the PXR/CYP3A4 axis plays an essential role in RTV hepatotoxicity, we explored the role of PXR/CYP3A4 axis in COBI hepatotoxicity. Using our humanized mouse models, hepatocellular injury was observed in PXR/CYP3A4-humanized mice pretreated with RIF followed by COBI which was similar to the phenotype observed with RTV. In addition, pretreatment with a constitutive androstane receptor (CAR) activator (another nuclear receptor), significantly up-regulated CYP3A4 expression and potentiated COBI hepatotoxicity in a CYP3A4-transgenic mouse model deficient in PXR. Further studies illustrated that induction of CYP3A4 increased COBI metabolism and bioactivation resulting in oxidative stress, endoplasmic reticulum stress, and hepatocellular injury as observed with RTV.

Collectively, this work established the essential roles of hPXR and CAR ligands/activators that can induce CYP3A4 expression as risk factors for RTV/COBI hepatotoxicity. Our results can be used to develop novel strategies based upon PXR, CYP3A4, and their downstream pathways to ensure the safe use of RTV/COBI-containing regimens in the clinic.

Table of Contents

PREFACE.....	xiv
1.0 INTRODUCTION TO DRUG HEPATOTOXICITY.....	1
1.1 Mechanisms of DIH.....	6
1.1.1 Cell Death (Apoptosis and Necrosis)	6
1.1.2 Reactive metabolites formation (Bioactivation)	7
1.1.3 Drug transporter mediated DIH.....	8
1.1.4 Immune-mediated response	8
1.1.5 Mitochondrial dysfunction	9
1.1.6 Activation of cellular stress signaling pathways.....	10
1.2 Risk factors for DIH.....	11
1.2.1 Age	11
1.2.2 Gender.....	12
1.2.3 Genetic factors.....	13
1.2.4 Immune response	14
1.2.5 Daily dose of drug and Metabolism.....	14
1.3 The role of Pregnane X receptor in DIH.....	15
1.3.1 PXR in drug metabolism and its role in DIH	17
1.3.1.1 Regulation of cytochrome P450 (CYP) enzymes by PXR and its role in DIH.....	17
1.3.1.2 Regulation of phase II conjugating enzymes by PXR and its role in DIH.....	19

1.3.1.3 Regulation of transporters by PXR and its role in DIH.....	20
1.3.2 PXR in endobiotic metabolism and its roles in DIH	21
1.3.2.1 Regulation of glucose metabolism by PXR and its role in DIH.....	21
1.3.2.2 Regulation of lipid metabolism by PXR and its role in DIH	22
1.3.2.3 Regulation of heme biosynthesis by PXR and its role in DIH	23
1.3.3 PXR in oxidative stress and its role in DIH	25
1.3.4 PXR in inflammation and its role in DIH	25
1.3.5 PXR in apoptosis and its role in DIH	27
1.3.6 PXR in cell proliferation and regeneration and its role in DIH	28
1.3.7 PXR polymorphisms and its role in DIH	29
2.0 PREGNANE X RECEPTOR ACTIVATION MODULATES RITONAVIR	
HEPATOTOXICITY	35
2.1 INTRODUCTION	35
2.2 METHODS.....	36
2.2.1 Animal models	36
2.2.2 Experimental design	37
2.2.3 Chemicals and reagents	37
2.2.4 Study approval	38
2.2.5 Assessment of liver injury	38
2.2.6 Cell-based reporter assay of human PXR activation.....	38
2.2.7 qPCR analysis.....	39
2.2.8 Western blotting.....	40
2.2.9 Effects of RTV on RIF metabolism	41

2.2.10 Effects of RIF on RTV metabolism	42
2.2.11 UPLC-QTOFMS analysis.....	42
2.2.12 Liver metabolomics.....	43
2.2.13 Statistics	43
2.3 RESULTS AND DISCUSSION.....	44
2.4 CONCLUSION	65
3.0 LEAD-IN TREATMENT WITH CYP3A4 INDUCERS POTENTIATES COBICISTAT HEPATOTOXICITY	66
3.1 INTRODUCTION	66
3.2 METHODS.....	68
3.2.1 Animals models	68
3.2.2 Experimental design	68
3.2.3 Chemicals and reagents	69
3.2.4 Assessment of liver injury	69
3.2.5 qPCR analysis.....	69
3.2.6 Western blotting.....	70
3.2.7 Effects of RIF on COBI metabolism.	70
3.2.8 UPLC-QTOFMS analysis.....	71
3.2.9 Statistical analysis.	71
3.3 RESULTS	72
3.4 DISCUSSION.....	95
3.5 CONCLUSION	96
4.0 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES.....	97

Bibliography	104
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List of Tables

Table 1. Drugs withdrawn from the market due to hepatotoxicity (United States, European Union, United kingdom and France).....	2
Table 2. Examples of drugs currently used in the clinic that can cause hepatotoxicity	3
Table 3. Summary of PXR polymorphisms associated with DIH.	31
Table 4. Primers for qPCR analysis.....	40

List of Figures

Figure 1. General mechanisms of DIH and cell death.....	5
Figure 2. Risk factors for developing DIH.....	12
Figure 3. The roles of PXR in drug-drug interactions and their contributions to DIH.....	17
Figure 4. The role of PXR in heme biosynthesis and its contribution to RIF+INH induced liver injury.	24
Figure 5. Summary of pathways regulated by PXR and their contribution to DIH.	34
Figure 6. Role of human PXR in the hepatotoxicity caused by pretreatment with RIF followed by RTV.	46
Figure 7. Histological analysis of the liver in WT mice pretreated with RIF diet for seven days followed by two doses of RTV.....	47
Figure 8. Effects of RIF on the expression of PXR target genes in the liver of hPXR/CYP3A4 mice.....	48
Figure 9. Histological analysis of the liver in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by two doses of RTV.....	49
Figure 10. Role of CYP3A4 in the hepatotoxicity caused by pretreatment with RIF followed by RTV	51
Figure 11. Effects of RIF on the expression of PXR target genes in the liver of hPXR/Cyp3a-null mice.....	53
Figure 12. Roles of human PXR and CYP3A4 in the hepatotoxicity associated with lead-in treatment with EFV followed by RTV.....	55
Figure 13. Effects of RTV on RIF metabolism and disposition in hPXR/CYP3A4 mice.....	57

Figure 14. RIF-mediated PXR activation and CYP3A4 induction increases RTV metabolism and bioactivation.....	58
Figure 15. Metabolomics reveals oxidative stress in the liver of hPXR/CYP3A4 mice pretreated with RIF for 7 days followed by RTV.....	60
Figure 16. Oxidative stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by two doses of RTV.....	61
Figure 17. Endoplasmic reticulum (ER) is a target organelle in the hepatotoxicity associated with RIF pretreatment for seven days followed by RTV.....	63
Figure 18. ER stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by two doses of RTV.....	64
Figure 19. Lead-in treatment with RIF followed by COBI causes hepatotoxicity in hPXR/CYP3A4 mice, but not in WT mice.....	73
Figure 20. Histological analysis of livers from WT mice.....	75
Figure 21. Lead-in treatment with RFB followed by COBI does not cause hepatotoxicity in hPXR/CYP3A4 mice.....	77
Figure 22. Role of PXR in the hepatotoxicity caused by pretreatment with RIF followed by COBI.	79
Figure 23. Histological analysis of livers from Pxr-null/CYP3A4 and hPXR/Cyp3a-null mice.	80
Figure 24. Role of CYP3A4 in the hepatotoxicity caused by pretreatment with RIF followed by COBI.	83
Figure 25. PXR-mediated CYP3A4 induction increases COBI metabolism and bioactivation.	86

Figure 26. CYP3A4-dependent oxidative stress and ER stress in the hepatotoxicity caused by pretreatment with RIF followed by COBI.	88
Figure 27. The expressions of genes related to oxidative stress in the liver of hPXR/CYP3A4, Pxr-null/CYP3A4, and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI.	90
Figure 28. The effects of antioxidant CDDO-Im on the liver injury in hPXR/CYP3A4 mice pretreated with RIF followed by COBI.	91
Figure 29. The expressions of genes related to cell death in the liver of hPXR/CYP3A4, Pxr-null/CYP3A4, and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI.	93
Figure 30. The expressions of genes related to ER stress and cell death in the liver of Pxr-null/CYP3A4 mice pretreated with TCPOBOP followed by COBI.	94

Preface

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1.0 INTRODUCTION TO DRUG HEPATOTOXICITY

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Amina I. Shehu, and Xiaochao Ma. "Pregnane X receptor in drug-induced liver injury: Friend or foe?" *Liver Research* (2018) (2).

Drug-induced hepatotoxicity (DIH) is an important clinical problem in the United States and around the world. It is one of the primary reasons for failure of drug candidates during preclinical drug development, early-phase clinical trials, and Food and Drug Administration drug withdrawal from the market after drug approval (examples in Tables 1 and 2) (3). Previous reports have shown that drug-mediated hepatotoxicity is responsible for more than 50% of reported cases of acute liver failure in the United States (4). Although acetaminophen (APAP) accounts for a majority of cases of DIH, other drugs also account for acute liver failure more frequently than viral hepatitis and other causes(4). In a population-based study in Iceland, the incidence of DIH was reported to be as high as 19 cases per 100,000 people (5).

Table 1. Drugs withdrawn from the market due to hepatotoxicity (United States, European Union, United kingdom and France). Nonsteroidal antiinflammatory agent (NSAID)

Drug	Class	Mechanism of Toxicity
Troglitazone	Antidiabetic/anti-inflammatory	Reactive metabolites
Benaxaprofen	NSAID	Reactive metabolites
Bromfenac	NSAID	Reactive metabolites
Ibufenac	NSAID	Reactive metabolites
Temafloxacin	Fluoroquinolone antibiotic	Not clear
Alatrofloxacin	Fluoroquinolone antibiotic	Not clear
Trovaflaxacin	Fluoroquinolone antibiotic	Mitochondrial dysfunction, inflammatory stress
Benzarone	Thrombolytic	Reactive metabolites
Ximelagatran	Anticoagulant	Immune mediated
Clomacron	Psychotropic drug	Not clear
Nefazodone	Antidepressant	Reactive metabolites
Cyclofenil	Antiestrogen	Not clear
Dilevalol	Antihypertensive	Immune mediated
Sitaxentan	Antihypertensive	Mitochondrial dysfunction Covalent binding to liver proteins
Tienilic acid	Antihypertensive	Reactive metabolites, immune response
Pemoline	CNS stimulant	Partly immune mediated, not completely clear

Table 2. Examples of drugs currently used in the clinic that can cause hepatotoxicity

Drug/Class	Pattern of injury	Mechanism of toxicity
Amoxicillin-clavulanate (antibiotic)	Cholestasis, mixed or hepatocellular	Immunoallergic
Isoniazid (antibiotic)	Hepatocellular	Reactive metabolites, immunoallergic
Ketoconazole(anti-fungal)	Hepatocellular, cholestatic	Reactive metabolites?
Ibuprofen (analgesic)	Mixed or cholestatic	immunoallergic
Nitrofurantoin(antibiotic)	Hepatocellular	Reactive metabolites, autoimmune mediated
**Propylthiouracil (antithyroid)	Hepatocellular, cholestatic or mixed	Immunoallergic
Carbamazepine(anti-epileptic)	Cholestatic, mixed or hepatocellular	Reactive metabolites, immunoallergic
**Valproate(anti-epileptic)	Mixed or hepatocellular	Mitochondrial dysfunction
Asparaginase (anticancer)	Cholestatic	Mitochondrial dysfunction ?
Azathioprine(immunosuppressant)	Cholestatic, mixed	Reactive metabolites, immunoallergic
Infliximab	Hepatocellular, cholestatic	Autoimmune mediated
diclofenac	Hepatocellular	Immunoallergic
Flutamide	Hepatocellular	Reactive metabolites?

DIH can present as acute liver failure or chronic liver failure, which makes it difficult to distinguish DIH from other liver diseases. DIH usually appears as elevations of serum liver enzymes with or without an increase in bilirubin. DIH is defined as an increase in alanine aminotransferase (ALT) 5 times above the upper limit of normal or baseline value; alkaline phosphatase (ALP) 2 times above the upper limit of normal; or a combination of ALT 3 times above the upper limit of normal and bilirubin 2 times above the upper limit of normal (6). The pattern of liver enzyme increase is further classified into 3 subtypes based on the R value, which is defined as the ratio of ALT to ALP expressed in multiples of the upper limit of normal. An R value greater than 5 denotes hepatocellular injury; R value of 2 to 5 is mixed; and R value less than 2 is cholestatic type of injury. Hepatocellular pattern of liver injury is characterized by cellular necrosis and inflammation with little or no elevation of bilirubin. ALT and aspartate aminotransferase (AST) levels are usually high whereas ALP levels are mildly increased. Patients usually present with malaise and exhaustion. The cholestatic pattern of injury is typified by accumulation of bile in the hepatocytes due an insult to the bile ducts, increased levels of bilirubin and ALP, and jaundice with itching on the skin. The mixed pattern of injury is often encountered in DIH and combines the features of hepatocellular and cholestatic pattern of liver injury. Patients may present with both exhaustion and itching, elevated levels of ALT and ALP, and bile accumulation. DIH can also appear in the form of other liver diseases, like acute or cholestatic hepatitis, steatosis, acute necrosis, chronic hepatitis, and nonalcoholic fatty liver disease (http://livertox.nih.gov/Phenotypes_enzy.html).

DIH can be dose dependent and predictable or it can be idiosyncratic, which occurs only in specific individuals and is not strictly drug dose dependent. Many efforts have been made in understanding the mechanisms that drive DIH. General mechanisms involved in DIH include cell

death, metabolism-mediated reactive metabolite formation, immune-mediated reaction, and mitochondrial dysfunction. Multiple mechanisms together seem to contribute to clinically observed DIH (Figure 1).

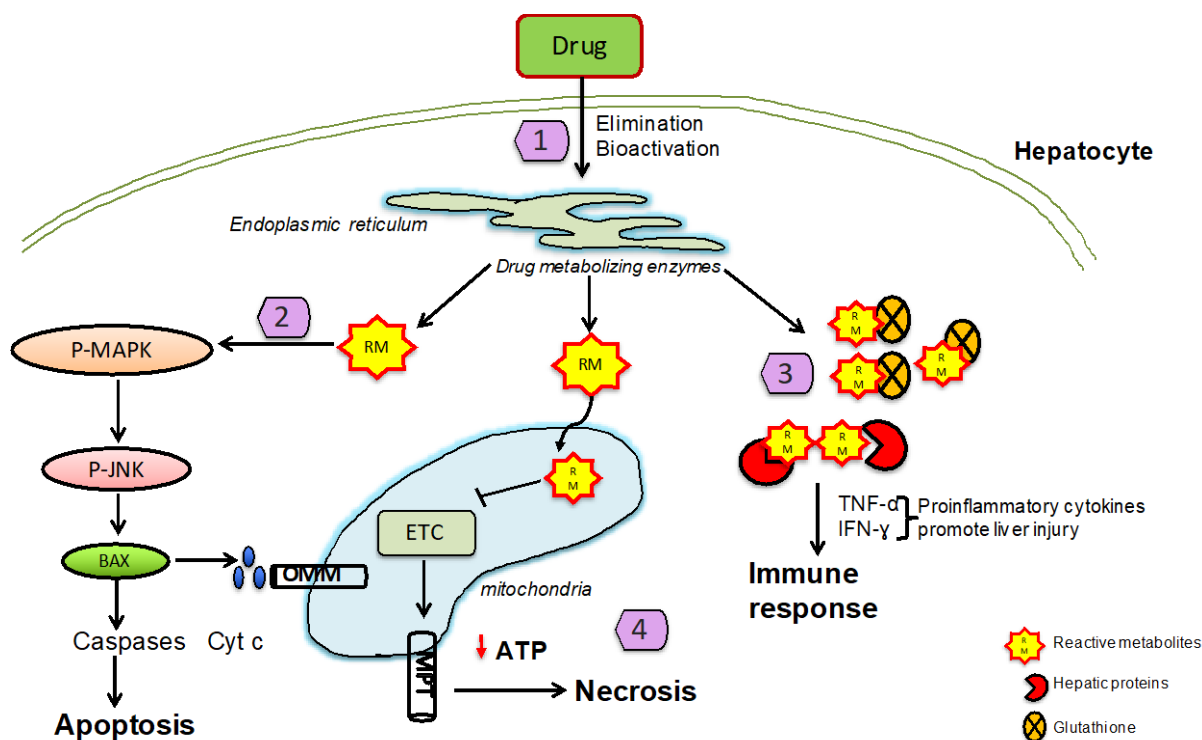


Figure 1. General mechanisms of DIH and cell death.

(1) Drug is metabolized in the endoplasmic reticulum by cytochrome P450s or other enzymes to reactive metabolites or polar metabolites for elimination. (2) RMs can lead to the activation of stress signaling pathways like the JNK pathway to induce apoptosis through the recruitment of Bax to the OMM. Insertion of Bax into the OMM leads to the permeability of the OMM and the release of mitochondrial proteins like Cyt c. Cyt c release can lead to the activation of caspases and the induction of apoptosis. (3) RMs are usually detoxified by cellular antioxidants like glutathione. However, depletion of glutathione can allow RM to covalently bind to hepatic proteins and trigger an immune response through the activation of cytotoxic T cells. (4) RMs can lead to the production of ROS in the mitochondria leading to the inhibition of ETC chain and opening of the MPT pore. This can lead to the collapse of mitochondrial respiration, inhibition of ATP synthesis and cell death via necrosis. Bax, Bcl-2-associated X protein; Cyt c, cytochrome c; ETC, electron transport chain; MPT, mitochondrial permeability transition; OMM, outer mitochondrial membrane; RMs, reactive metabolites.

1.1 Mechanisms of DIH

1.1.1 Cell Death (Apoptosis and Necrosis)

The hallmark of DIH is death of hepatocytes or sometimes cholangiocytes and endothelial cells. Different modes of cell death are encountered with different drugs. The 2 most common forms of cell death in DIH are apoptosis and necrosis. Apoptosis, also known as programmed cell death, is an ATP-dependent process characterized by shrinking of the cell, condensation of chromatin, and extensive distortion of the extracellular membrane but without the release of cellular contents to the extracellular medium (7). Surrounding phagocytes immediately clear the dying cells. In this mode of cell death, inflammation is limited because cellular contents are not released into the surrounding cells (7). The proteolytic enzymes caspases are responsible for the controlled degradation of cells during apoptosis. Apoptosis can occur through intrinsic or extrinsic pathways. The intrinsic pathway is activated by intracellular stimuli, such as toxins, radiation, or depletion of growth factors in the cell. This results in the activation of proapoptotic mitochondrial proteins, like Bax, Bid (BH3 Interacting Domain Death Agonist), and Bim (BCL2 like 11), that guide the opening of the outer mitochondrial membrane and the release of cytochrome c, endoribonuclease G, and other mitochondrial proteins. The release of cytochrome c leads to the activation of initiator caspase 9 and eventually caspase 3 that executes apoptosis (7). On the other hand, the extrinsic pathway of apoptosis is induced by ligand binding to death receptors of the tumor necrosis factor (TNF)- α family present on the transmembrane surface of a cell. Some

examples of death receptors and their ligands that transmit lethal stimuli to intracellular signaling pathways include FAS receptor and ligand and the TNF receptor 1 and TNF- α . Activation of death receptors leads to the subsequent activation of caspase 8 and the executioner caspase 3, which leads to cell death (7). Diclofenac is a drug that induces apoptosis in hepatocytes (8).

Necrotic cell death, on the other hand, involves swelling of the endoplasmic reticulum (ER) and mitochondria, complete dissolution of the nuclear fragment, and rupture of the cell membrane to release cellular contents (9). Unlike apoptosis, necrosis is usually accompanied by inflammation due to the recruitment of chemotactic signals resulting from the release of cellular contents to the neighboring cells (7). Also, there is loss of mitochondrial membrane potential due ion gradient collapse and ATP depletion (10). Necrosis used to be considered an unregulated process until recent years, when studies have shown its regulation by activation of receptor-interacting protein kinases 1 and 3 and involves the mitochondria (11). APAP is a classic example of a drug that induces necrosis in hepatocytes.

1.1.2 Reactive metabolites formation (Bioactivation)

The metabolism of drugs normally involves the breakdown of lipophilic compounds to more water-soluble substances that can be readily excreted out of the body. Drug biotransformation, however, can sometimes lead to the formation of reactive chemical metabolites that can bind to nucleic acids, cellular proteins, and lipids, thus leading to DNA damage, loss of protein function, and lipid peroxidation (12). Reactive metabolite generation can also activate the adaptive immune response and induce stress in the ER and mitochondria, which all together

contribute to liver damage (13). The formation of reactive metabolites as a mechanism for hepatotoxicity is reported for drugs like APAP, halothane, and tienilic acid.

1.1.3 Drug transporter mediated DIH

Hepatic drug transporters are involved in the uptake and efflux of endogenous compounds and certain drugs into and out of hepatocytes, respectively. Altered expression (genetic polymorphism) or inhibition of these transporters can predispose patients to DIH by allowing toxic drugs, their metabolites or bile acids to accumulate in the hepatocytes and cause injury (14). Polymorphism in the uptake transporter, organic anionic transporting polypeptide (OATP1B1), predisposes patients to RIF-mediated liver injury (15). Also, polymorphism in the canalicular transporter multidrug resistance-associated protein (MRP) 2 is associated with elevated levels of bilirubin and jaundice (16). Certain drugs, like cyclosporine, estradiol, and bosentan, can alter the activity of the biliary transporter bile salt excretory pump (BSEP) and lead to cholestatic or mixed type of liver injury (16). Transporters, like MRP2 and MRP3, are involved in the secretion of drugs and metabolites into bile. Altered expression of these transporters have been reported in patients with drug-induced liver injury (17).

1.1.4 Immune-mediated response

Injury to hepatocytes can trigger the release of chemicals that can activate cells of the innate immune system that are resident in the liver. The Kupffer cells, natural killer cells, and natural killer T cells exist in large numbers in the liver to protect it from harm by viral or bacterial toxins and xenobiotics. Activation of these cells, however, promotes DIH through the recruitment of

proinflammatory cytokines, such as TNF- α , interferon (IFN)- γ , and interleukin (IL)-1 β , that potentiate the inflammatory response resulting in further tissue damage (13). Genome-wide association studies have shown a connection between various HLA haplotypes and DIH. For example, abacavir is generally well tolerated, but hypersensitivity reactions take place in 5% to 8% of patients due to the activation of HLA-B*5701 (18, 19). DIH associated with the adaptive immune response presents with allergic reaction-related symptoms, like skin rash, fever, eosinophilia, and detection of antibodies directed against modified or native hepatic proteins indicative of an immune-mediated response. In addition, re-challenge with the drug causes toxicity. Examples of drugs that show such reactions are halothane and phenytoin (20).

1.1.5 Mitochondrial dysfunction

The mitochondria are the powerhouse of the cell where energy is produced for normal cellular function. Many drugs used clinically target this organelle to cause toxicity by interfering with different functions of the mitochondria, such as fatty acid β -oxidation, mitochondrial permeability transition pore formation (MPTP), oxidative phosphorylation, and mitochondrial DNA replication. The MPTP is a protein pore that is located in the inner membrane of the mitochondria. MPTP pore induction increases mitochondrial permeability to molecules greater than 1.5 kDa, which in turn allows the inflow of water and ions like calcium into mitochondria and the escape of protons. As a result, mitochondria can undergo swelling and rupture of the outer mitochondrial membrane (21). This leads to disruption of the electrochemical gradient, loss of membrane potential, generation of reactive oxygen species (ROS), and ATP depletion due to collapse of the electron transport chain (22). IFN- α and nucleoside analog drugs can affect the replication of mitochondrial DNA and ibuprofen can inhibit fatty acid beta oxidation (23, 24).

Mitochondrial toxicity can lead to cell death via necrosis (due to ATP depletion) and apoptosis (through the release of mitochondrial proteins like cytochrome c (25). Mitochondrial dysfunction leads to the activation of other cellular stress pathways that are discussed in the following section.

1.1.6 Activation of cellular stress signaling pathways

The c-jun N-terminal kinase (JNK) serves diverse functions in hepatocytes, including cell death, regeneration, and differentiation (26). JNK is activated by a wide variety of stress signals, like ROS, drugs, radiation, cytokines, and pathogens. JNK is regulated upstream by 2 successive mitogen-activated protein kinases (MAPKs). The MAP kinase kinase kinases (MAP3Ks [e.g. ASK1 and MLK]) activates MAP kinase kinases (MAP2Ks [e.g. MKK4 and MKK7]), which then activate JNK (MAPK) at the threonine tyrosine residue (27). Once JNK is activated, it induces its protein substrates downstream to execute its functions. JNK activates mitochondrial proteins, like antiapoptotic Bcl2 and Bcl-XL; proapoptotic Bad, Bim, and Bid; and other non-mitochondrial proteins, like Bax, c-jun, p53, and c-Myc (26). TNF- α is one of the common cytokines induced during hepatocyte injury that serves as a stimulus for JNK activation. Sustained JNK activation and its interaction with diverse signaling pathways have been implicated in the pathology of many liver diseases, including but not limited to ischemia and reperfusion liver injury, nonalcoholic liver disease, hepatocellular carcinoma, APAP-induced hepatotoxicity, and liver fibrosis (26). TNF-related apoptosis-inducing ligand potentiates Fas-induced hepatocyte apoptosis through JNK activation that leads to the phosphorylation of Bim and subsequent mitochondrial dysfunction (28).

ER stress is another stress pathway that can lead to the induction of JNK to mediate cell death via apoptosis (26). The ER is the organelle where the CYP metabolizing enzymes are located. The ER is also responsible for proper protein folding, secretion, and transportation to other target organs (29). Hepatocytes are rich in ER. Misfolding or accumulation of proteins in the ER due to stress signals like ROS, drugs, calcium depletion, and toxins, can trigger the unfolded protein response (UPR) to help restore proper ER function. Three signal transducers that are located on the ER membrane control the UPR. This includes inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor ATF-6 (29). They act by inhibiting global protein synthesis, induction of chaperones to increase protein folding, and activation of degradation pathways to clear accumulated proteins. The IRE1 pathway can activate JNK through its interaction with TRAF-2 to induce apoptosis when the UPR is not able to restore normal ER homeostasis (29).

1.2 Risk factors for DIH

1.2.1 Age

Age has an important effect on DIH (Figure 2). People greater than 40 years are normally more susceptible to liver injury due to altered drug disposition, excretion, and the intake of multiple drugs at the same time. On the contrary, children are at more risk of DIH with drugs like valproate and aspirin. Also, a study conducted using cases of DIH in a Spanish registry reported that older

patients experienced more cholestatic type of liver injury compared with a younger population who experienced hepatocellular type of injury (30) .

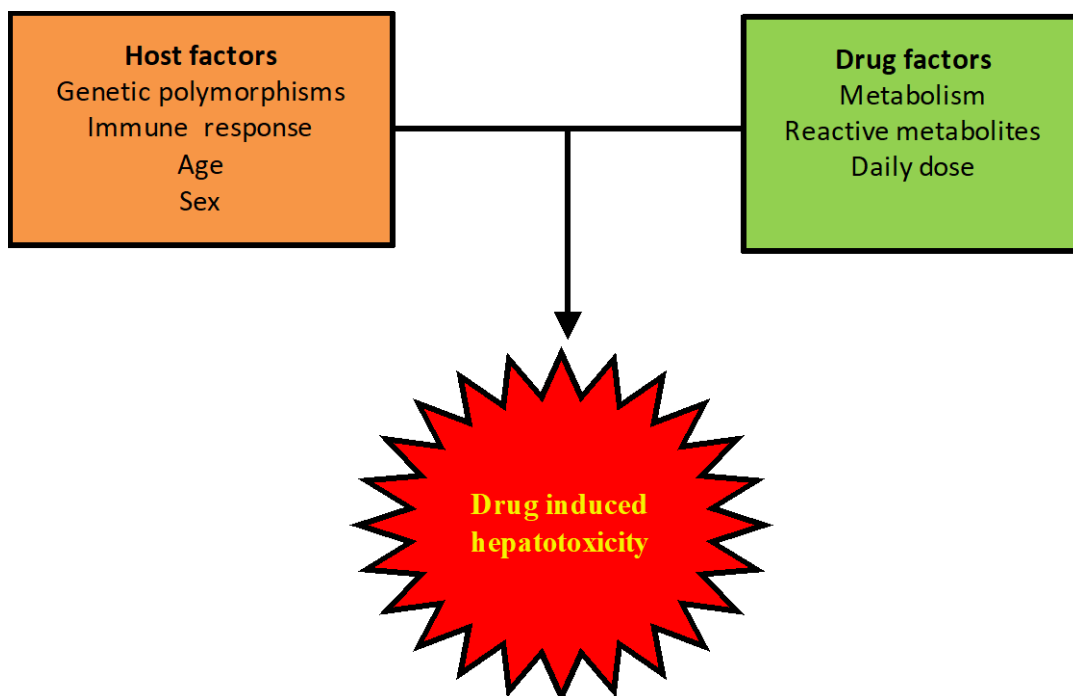


Figure 2. Risk factors for developing DIH

Most DIH is idiosyncratic, thus the genetic make-up of individuals as well as their health condition and the interplay of other factors increase the susceptibility to liver injury.

1.2.2 Gender

Although the exact mechanism is not known, sex might influence differences in susceptibility to DIH in men and women. Some reasons proposed are factors that affect the pharmacokinetics and pharmacodynamics of drugs- examples include differences in the expression of drug metabolizing enzymes and transporters, sex hormones, plasma protein binding, body mass index, body weight, and organ blood flow (31). In one study, women were shown to be more susceptible to autoimmune-mediated DIH and to liver injury caused by drugs like isoniazid

(INH), halothane, and erythromycin (32). In addition, women with DIH were also reported to have a worse outcome that results in fulminant liver injury and transplantation (30). Men on the other hand have been shown to be more susceptible to the cholestatic type of injury which correlated with older age of >60 years. Furthermore, men have been reported to be more susceptible to liver injury caused by azathioprine and amoxicillin-clavunate (30, 33, 34).

1.2.3 Genetic factors

Genetic factors, such as mutations in CYPs or phase II enzyme genes, mitochondrial DNA, and antioxidant genes, are believed to contribute significantly to DIH, especially idiosyncratic drug reactions. For example, mutations in CYP2E1 (CYP2E1 c1/c1) and N-acetyltransferase (NAT [NAT *5, *6, *7]) both enzymes associated with isoniazid (INH) metabolism, increase susceptibility to INH hepatotoxicity (35). In patients with NAT slow acetylator phenotype, the conversion of INH to acetyl-INH and acetyl hydrazine to diacetyl hydrazine is decreased. This process is required for the breakdown of INH to non-toxic metabolites. This results in formation of toxic byproducts that are believed to mediate INH hepatotoxicity.³² Polymorphisms associated with detoxification and antioxidant enzymes like glutathione S transferase (GST), and mitochondrial superoxide dismutase are reported to be associated with increased risk of DIH. Genetic variation in the expression of the bile salt export pump (BSEP) transporter has been associated with increased susceptibility to cholestatic DIH caused by drugs like troglitazone (36). In addition to genes regulating drug metabolism and transport, genes regulating human leukocyte antigens, cytokines and oxidative stress may also impact DIH.

1.2.4 Immune response

Immune response in individuals is a major determinant of idiosyncratic DIH. The presence of preexisting inflammatory response, polymorphisms in cytokine-encoding genes, and HLA class II antigens contributes to how the body responds to drugs and the occurrence of immune-mediated DIH. For example, genetic variants of the anti-inflammatory cytokine IL-10 were found more in individuals who had diclofenac-induced hepatotoxicity compared with those who were on diclofenac but did not develop DIH or their healthy controls (37). Also, a genome-wide association study showed that flucloxacillin DIH was associated with the HLA-B*5701 genotype (38). Reactive metabolites covalently bind to hepatic proteins and may serve as antigens that falsely trigger an immune response and that leads to the formation of antibodies against the modified or native proteins and the induction of cytotoxic response to clear antigens (37). This may contribute to the pathogenesis of DIH although a direct causal relationship has not been established.

1.2.5 Daily dose of drug and Metabolism

In two studies conducted using a database on oral prescription drugs most commonly prescribed in the United States, drugs administered at a dose of greater than 50 mg a day and are extensively metabolized by the liver were more likely to cause DIH compared to those administered at a daily dose of <10mg or have less hepatic metabolism (39, 40). Also, many drugs that have been withdrawn from the market or those that have the black box warning due to hepatotoxicity are given at daily doses of >50-100 mg and were associated with formation of reactive metabolites (41, 42). In a large population study carried out in Iceland, 88% of patients that developed liver injury on a single prescription drug were given daily doses of >50 mg, 10%

were given 11-49 mg and 2% were given <10 mg (5) . Although, daily dose alone cannot be used to predict DIH, several studies have shown a strong correlation between drugs that are given at a high daily dose and hepatotoxicity.

Other factors, such as preexisting diseases, like viral hepatitis and diabetes, increase the risk of DIH. The consumption of alcohol and concomitant use of other hepatotoxic drugs is also associated with increased susceptibility to DIH. Furthermore, the duration of exposure to the drug may determine the severity of hepatotoxicity (43). A combination of these factors predisposes individuals to DIH (Figure 2).

1.3 The role of Pregnane X receptor in DIH

Because drug metabolism is one of the key factors that can lead to DIH, we decided to focus on the PXR which is a nuclear receptor that regulates drug metabolizing enzymes, transporters and other important gene networks that contribute to the pathogenesis of DIH. One risk factor for the development of DIH is metabolism-mediated drug-drug interactions arising from the combination of multiple drugs (44, 45) . For example, the rate of hepatotoxicity is increased in patients when isoniazid (INH) (anti-tuberculosis) or acetaminophen (painkiller) is taken together with RIF compared to taking either drug alone (46, 47).

PXR is a nuclear hormone receptor that belongs to the nuclear receptor subfamily 1, group I, member 2 (NR1I2) (48, 49). PXR serves as a sensor of foreign toxic compounds and in response acts as a transcription factor that upregulates the expression of enzymes that help to clear these compounds out of the body. In addition to xenobiotic metabolism, PXR also regulates endobiotic

metabolism(48, 49). The mouse PXR was first discovered and cloned in 1997 by screening the University of Washington expressed sequence tag mouse liver library database based on the similarity of its ligand binding domain to other nuclear receptors (48). Subsequently the human PXR was identified as the steroid and xenobiotic sensing receptor (SXR) and the pregnane activated receptor (PAR) with similar structure and activation profile as the mouse PXR (49-51). PXR has a large and promiscuous ligand binding pocket which allows it to bind and be activated by a wide variety of structurally diverse compounds, including steroids, glucocorticoids, bile acids, prescription drugs, herbal medicines, dietary supplements and environmental toxicants (48-53). Upon ligand binding, PXR regulates gene expression by forming a heterodimer with another nuclear receptor-retinoid X receptor in the cytoplasm. The heterodimer then translocates into the nucleus and binds to the DNA response element of the target gene (54, 55). PXR can bind to different response elements containing the half site consensus sequence AGTTCA organized mostly as direct repeats (DR) with 3,4, or 5 base pair spacing and less likely everted repeats (ER) with 6 and 8 base pair spacing (56). In addition, the transcriptional activity of PXR is enhanced by the recruitment of co-activators like proteins from the p160 family (e.g. steroid receptor coactivator, glucocorticoid receptor interacting protein) (57). The PXR like other nuclear receptors structurally contains an NH₂-terminal ligand independent activation function domain (AF-1,A/B region), a highly conserved DNA binding domain (C region) , followed by a less conserved hinge domain (D region), a promiscuous C-terminal ligand binding domain (E region) and an activation function 2 domain (58).

Activation of PXR regulates the expression of phase I and II drug-metabolizing enzymes and transporters thus plays a major role in drug absorption, metabolism and elimination (50, 51). In addition, activation of PXR modulates many cellular processes beyond drug metabolism, such

as endobiotic metabolism, oxidative stress response, apoptosis, inflammation, cell proliferation and regeneration (59-62). Furthermore, the activation of PXR is specie specific; in that mouse PXR ligands like pregnenolone 16 α -carbonitrile (PCN) do not activate the human PXR (hPXR) (63). Therefore, humanized mouse models have been engineered to study the effects of PXR activation by human ligands like RIF in mice (64). PXR can modulate drug-drug interactions and potentially contribute to DIH (Figure 3).

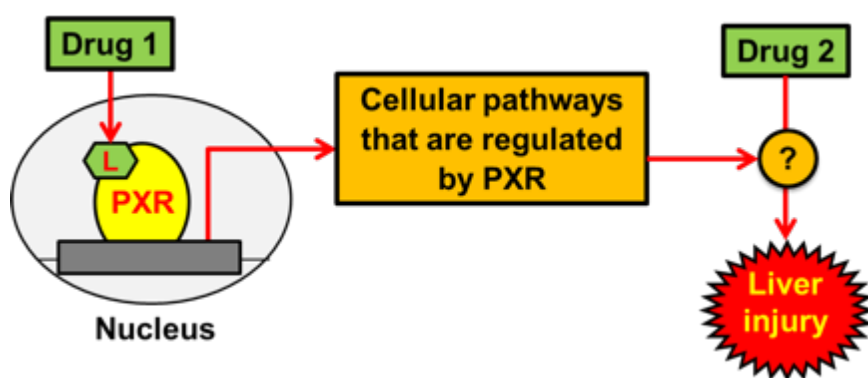


Figure 3. The roles of PXR in drug-drug interactions and their contributions to DIH.

Ligand (L)-dependent PXR activation can regulate multiple cellular pathways, alter cellular functions, and impact the DIH of co-administered drug(s).

1.3.1 PXR in drug metabolism and its role in DIH

1.3.1.1 Regulation of cytochrome P450 (CYP) enzymes by PXR and its role in DIH

PXR is expressed in the liver and the intestine, two organs that play a significant role in drug metabolism (48). The CYPs referred to as phase I enzymes are responsible for catalyzing oxidation reactions in drug metabolism (65). PXR regulates the expression of CYP3A, 2A, 2B, and 2C family of enzymes in the liver (66).

CYP3A4 is the most abundant CYP in the liver, and is responsible for the metabolism of many clinically used drugs (67). CYP3A4 can be transcriptionally induced by drugs that activate PXR or CAR (68). Upon ligand activation, PXR regulates the expression of CYP3A4 by forming a heterodimer with retinoid X receptor (RXR), which together translocate into the nucleus and bind to the xenobiotic response element in the proximal promoter region located close to the transcriptional start site of CYP3A4 (67, 69). CAR is also a nuclear hormone receptor that belongs to the same family as the PXR. Studies have shown that PXR and CAR share similarities in amino acid sequence of their DNA binding domain which makes it possible to bind similar DNA binding motifs and regulate an overlapping set of genes (70). Ligand mediated activation of CAR regulates CYP3A4 expression by forming a heterodimer with RXR, translocating into the nucleus where they bind to the xenobiotic response element located in the distal and proximal promoter of the CYP3A4 gene (71). Furthermore, CYP3A4 can be regulated by other nuclear receptors such as farnesoid X receptor (FXR), vitamin D receptor (VDR), hepatic nuclear factor 4 α (HNF4- α) and other factors which are beyond the scope of this review.

Many PXR ligands can lead to drug-drug interactions through CYP3A4 induction (49). The products of metabolism are usually non-toxic, more polar and ready for elimination. However, some drugs are metabolized to more toxic electrophiles that can bind to cellular components and lead to liver injury (65). This makes PXR an important target to study in drug metabolism and drug-drug interactions that lead to DIH.

Acetaminophen is a widely used over the counter (OTC) pain relieve medication that has been associated with hepatotoxicity. CYPs are critical in the formation of acetaminophen's reactive metabolite, N-acetyl benzoquinone imine (NAPQI) that binds to liver proteins to cause mitochondrial dysfunction and hepatocyte necrosis (72, 73). Studies have shown that PXR

activation can upregulate CYP3A and potentiate acetaminophen hepatotoxicity through CYP3A-mediated bioactivation of acetaminophen (74-76).

Flucloxacillin is an antimicrobial agent that is used for the treatment of staphylococcal infections. However, its use has been limited by its propensity to cause cholestatic liver injury (77). In vitro studies have found that CYP3A mediates the formation of 5'-hydroxymethyl metabolite of flucloxacillin, which is toxic to biliary epithelial cells (78). Further studies using primary human hepatocytes revealed that flucloxacillin is a PXR agonist and can induce the expression of CYP3A4 thus establishing PXR as an important factor in flucloxacillin-induced liver injury (79).

1.3.1.2 Regulation of phase II conjugating enzymes by PXR and its role in DIH

The phase II enzymes uridine 5'-diphospho-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) have been found to be PXR target genes (80). These enzymes catalyze the conjugation reactions of drugs to increase their water solubility (81). UGT is a cytosolic enzyme that catalyzes the transfer of glucuronic acid to hydrophobic molecules to form more water-soluble glucuronide metabolites that are usually unreactive and easily excreted from the body (82). However, UGTs can metabolize carboxylic acid containing drugs to form acyl glucuronides some of which are reactive and can bind to deoxyribonucleic acid (DNA) or proteins and contribute to drug toxicity (83). For example, uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) is the major enzyme responsible for the biotransformation of trovafloxacin, an antibiotic that has been withdrawn from the market due to its ability to cause idiosyncratic hepatotoxicity (84). UGT1A1 can metabolize trovafloxacin to form acyl glucuronide (85, 86). Since UGT1A1 is a PXR target gene, induction of this enzyme by PXR ligands may increase the metabolism of trovafloxacin to form reactive acyl glucuronides thus potentiating liver injury. GSTs

are usually associated with detoxifying drug metabolites by conjugating them to glutathione (GSH). Clozapine is an antipsychotic drug that has been reported to cause DIH (87, 88). Although the exact mechanism is unknown, it has been proposed that the formation of reactive metabolites might be involved (89). Glutathione S-transferase A1 (GSTA1) and glutathione S-transferase M1 (GSTM1) catalyze the inactivation of reactive metabolites produced from clozapine metabolism (90). GSTA1 and GSTM1 are PXR target genes (91). Therefore, co-administration of PXR ligands with clozapine might be beneficial if the upregulation of GSTA1 and GSTM1 are sufficient to protect from DIH in susceptible patients on clozapine therapy.

1.3.1.3 Regulation of transporters by PXR and its role in DIH

Drug transporters play an imminent role in the transport of drugs and endogenous compounds in and out of cells. PXR regulates multidrug resistance gene (MDR) 1, multidrug resistance associated protein (MRP) 1 and 2, and organic anion-transporting polypeptides (OATPs) transporters (92). Preclinical and clinical studies have shown that PXR activation may have a therapeutic value in the treatment of cholestasis (93-97). PXR activation detoxifies the toxic bile acid lithocholic acid (LCA) (96, 97). Treatment of wild type mice with PXR ligand PCN led to an increased expression of Cyp3a11 and the uptake transporter Oatp2 (96). Upregulation of Oatp2 increases the uptake of bile acids from the blood into hepatocytes for metabolism by Cyp3a11 (96). Therefore, PXR ligands have been proposed for use in the treatment of cholestatic liver injury.

1.3.2 PXR in endobiotic metabolism and its roles in DIH

1.3.2.1 Regulation of glucose metabolism by PXR and its role in DIH

Several studies have shown an association between the use of PXR ligands and glucose intolerance, impaired insulin sensitivity and type II diabetes (98-102). In addition, diabetes mellitus has been considered as a risk factor for developing liver disease and DIH (103, 104). PXR activation by PCN in mice has been shown to suppress hepatic gluconeogenesis through binding to forkhead box protein O1 (FoxO1) thereby inhibiting FoxO1 from binding to insulin response sequence in its target genes glucose-6-phosphatase enzyme (G6Pase) and phosphoenolpyruvate carboxykinase (Pepck) (105). In addition, PXR can form a complex with cyclic adenine monophosphate (cAMP) response element-binding protein (Creb) and repress Creb binding to G6Pase promoter thus leading to decreased expression of G6Pase and Pepck (106). Both FoxO1 and Creb are transcription factors that activate the expression of genes like G6Pase and Pepck to promote gluconeogenesis. Thus, the overall effect of PXR activation in mice is a decrease in glucose production. In contrast to mice, PXR activation in humans may cause an increase in glucose production. Treatment with human PXR activator RIF in primary human hepatocytes and Shp51 cells induced the expression of G6Pase, PEPCCK and serum- and glucocorticoid regulated kinase 2 (SGK2) (107). PXR-mediated induction of G6Pase was found to be dependent on SGK2 (107). Interestingly, it was found that treatment with high doses of RIF (which can activate the mouse PXR) in mouse liver cells led to the repression of G6Pase and Sgk2 indicating a difference in the regulation of SGK2 in humans and mice (107). The finding that PXR activation can lead to an increase in glucose production and hyperglycemia in humans is supported by a clinical study in which healthy volunteers were treated with RIF and blood glucose levels were increased during the oral glucose tolerance test (99). In addition, the statins atorvastatin and simvastatin used for

the treatment of high cholesterol are PXR agonists and have been reported to cause hyperglycemia and increased risk of type II diabetes (108).

A high level of glucose in the body is associated with an increase in the production of free radicals because glucose can undergo auto-oxidation and lead to the formation of superoxides or ketoaldehydes (109). In addition, glucose can bind to amino groups on proteins to produce an amadori product and advanced glycation end products (AGEs). These AGEs can bind to proteins to form adducts or protein crosslinks leading to the loss of protein function (109). Furthermore, AGEs can bind to their receptors and stimulate the activation of many signaling cascades including nuclear factor kappa B (NF- κ B) that eventually elicit inflammation and apoptosis in various cell types (109). In the context of DIH, PXR activation in humans is linked with an increased risk of hyperglycemia which is associated with an increase in oxidative stress and inflammation (110). Caution should be exercised when administering PXR ligands with other drugs that can cause hyperglycemia or in patients with disease conditions like obesity and diabetes as this can increase the susceptibility of individuals to DIH (111-113).

1.3.2.2 Regulation of lipid metabolism by PXR and its role in DIH

In vitro studies showed that both PXR activation and knockout cause hepatic steatosis. PXR knockdown led to an increase in the expression of aldo-keto reductase (AKR) 1B10, which enhanced the levels of acetyl-CoA carboxylase (ACC)-the key enzyme in fatty acid synthesis that catalyzes the conversion of acetyl-CoA to malonyl-CoA (114). PXR activation in HepG2 cells and primary human hepatocytes led to an increase in sterol regulatory element-binding protein (SREBP) 1a, a transcription factor that positively regulates lipogenesis (115).

In transgenic mice carrying constitutively activated hPXR transgene and hPXR mice treated with RIF, both showed that PXR activation promotes lipid synthesis and fat accumulation

in the liver. There was an increase in the levels of the fatty acid uptake transporter CD36, the adipogenesis transcription factor- peroxisome proliferator-activated receptor (PPAR) γ , the lipogenic genes stearoyl-CoA desaturase-1 (Scd1) and fatty acid elongase (Fae) thus leading to hepatic steatosis (116). In addition, activation of PXR by PCN in mice led to the repression of fatty acid beta-oxidation (FAO) and ketogenesis by inhibiting the binding of Foxa2 to the promoter of carnitine palmitoyltransferase 1a (Cpt1a) and mitochondrial 3-hydroxy-3-methylglutarate-CoA (HMG-CoA) synthase 2 respectively (117). A decrease in FAO leads to an increase in fatty acids and triglycerides accumulation in hepatocytes resulting in steatosis. Therefore, PXR activation promotes hepatic steatosis by increasing de novo lipogenesis and decreasing FAO and co-administration of PXR ligands with drugs that promote hepatic steatosis can lead to an increased risk of DIH (118).

1.3.2.3 Regulation of heme biosynthesis by PXR and its role in DIH

Heme is an important molecule comprised of iron attached to a porphyrin ring (119). Heme is required for various cellular functions such as oxygen transport in hemoglobin and electron transport in hemoproteins like the CYPs (119, 120). Heme is synthesized in the liver and bone marrow by a series of well-controlled enzymatic processes that starts with the condensation of succinyl coA and glycine by the first and rate limiting enzyme alanine synthase 1 (ALAS1); and ends with the insertion of ferrous iron into the protoporphyrin IX (PPIX) ring to form heme by ferrochelatase (FECH) (119). Both mPXR and hPXR have been reported to upregulate the expression of ALAS1 by binding to the ALAS1 drug-responsive enhancer sequences in the 5' flanking region of ALAS1 (120, 121) .

RIF and INH are two first line drugs used in tuberculosis therapy. The combination of these two drugs has been linked with an increased risk of developing hepatotoxicity in tuberculosis

patients (122). A study using PXR-humanized mice has shown that PXR-mediated perturbation of the heme biosynthesis pathway facilitates RIF and INH-induced hepatotoxicity through the accumulation of the hepatotoxic compound PPIX, an intermediate in the heme biosynthesis pathway (46). PXR activation by RIF increases the expression of ALAS1 and INH also induces ALAS1 and on the other hand inhibits the expression of the enzyme that converts PPIX to heme (123). Thus, the combination of RIF and INH leads to the accumulation of PPIX in the liver thereby promoting liver injury (Figure 4).

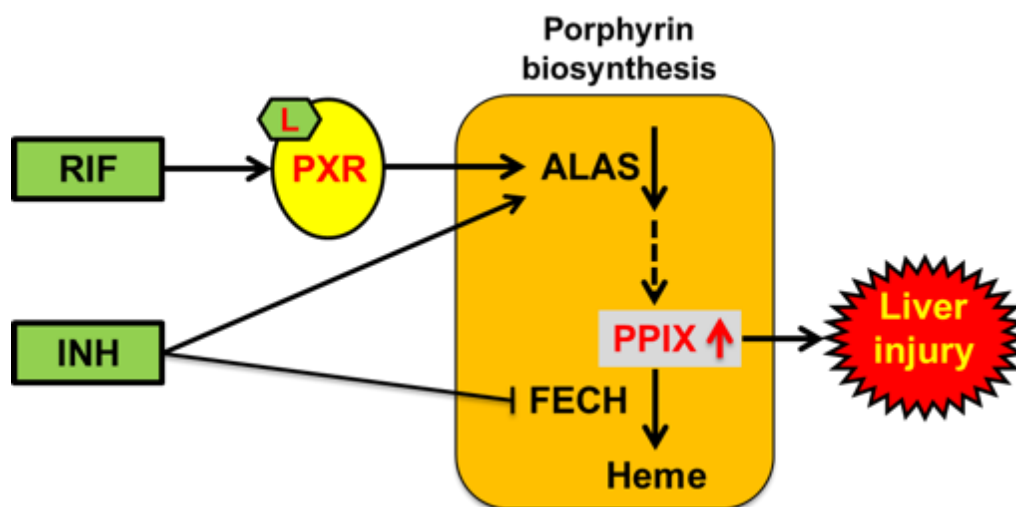


Figure 4. The role of PXR in heme biosynthesis and its contribution to RIF+INH induced liver injury.

PXR activation by RIF can up regulate the expression of ALAS1, the rate-limiting enzyme in heme biosynthesis while isoniazid can inhibit Ferrochelatase, the last enzyme that catalyzes the insertion of Ferrous ion into protoporphyrin IX to form heme. The net effect is an increase in the formation of protoporphyrin IX – a hepatotoxin, which leads to hepatotoxicity.

1.3.3 PXR in oxidative stress and its role in DIH

The liver is the major organ that expresses enzymes that catalyze chemical reactions to eliminate xenobiotics or other environmental pollutants. These chemical reactions can generate free radicals that can lead to oxidative stress. Cells have established antioxidant mechanisms like the use of antioxidant enzymes (e.g. superoxide dismutase, catalase, and glutathione peroxidase) to maintain low levels of free radicals and defend themselves against oxidative stress (*124*). However, in pathological conditions, these defense systems are either overwhelmed or their function is compromised leading to injury and cell death by either apoptotic or necrotic mechanisms (*125*). PXR activation in transgenic and wild type mice has been shown to lower the activity of superoxide dismutase and catalase, two enzymes important in redox cycling (*126*). The reduction in antioxidant enzyme activities was associated with increased sensitivity to the hepatotoxicant herbicide, paraquat (*126*). The mechanism by which PXR activation modulates the activities of superoxide dismutase and catalase activities is not understood but it seems to be an indirect mechanism of regulation. Thus, PXR activation may lower the body's defense against reactive oxygen species produced by some drugs and increase the risk of developing DIH. The role of PXR in response to oxidative stress in humans is unknown and this will be an interesting area of future research.

1.3.4 PXR in inflammation and its role in DIH

It has long been shown that inflammation and proinflammatory cytokines suppress the expression of drug metabolizing enzymes and transporters in inflammatory disease conditions like

inflammatory bowel disease, sepsis, and inflammation induced cholestasis (127-130). However, the molecular mechanism behind the dysregulation of drug metabolizing enzymes and transporters was discovered less than two decades ago. Treatment of wild type mice with endotoxin or interleukin 6 (IL-6) led to a decrease in the expression of PXR messenger ribonucleic acid (mRNA) and protein as well as down regulation of PXR target genes Cyp3a11, Mrp2, and Bsep (59). On the other hand, PXR activation has been shown to negatively regulate NF- κ B, a key regulatory molecule in inflammation and immune defense (131, 132). Using primary human hepatocytes and HepG2 cells transfected with NF- κ B or PXR, Zhou et al. showed that human PXR agonists like phenytoin and RIF can inhibit the expression of multiple NF- κ B target genes [IL-2, tumor necrosis factor alpha (TNF- α), IL-6, and cyclooxygenase (Cox)-2] and reduce the transcriptional activation of NF- κ B (131, 132). In addition, PXR activation in wild type mice inhibited NF- κ B signaling (131). In another study, primary cultures of hepatocytes from human, wild-type mice, and transgenic human PXR mice were treated with lipopolysaccharide (LPS) to induce the acute phase response. The results showed that PXR represses the expression of LPS inducible cytokines notably IL-1b and the mechanism proposed is that PXR can increase the expression of secreted IL-1 receptor antagonist, which is known to decrease the secretion of cytokines (133). Thus, PXR plays an anti-inflammatory role in the liver. LPS/D-galactosamine (GalN) treatment in mice is a classic model of acute inflammatory liver injury. LPS is a bacterial endotoxin that elicits strong immune response in animals while GalN is a transcriptional inhibitor that mitigates NF- κ B function and increases hepatocyte sensitivity to LPS (134). TNF- α is the major modulator of apoptosis and liver injury in this model (135). Wang et al. found that Pxr-null mice treated with LPS/GalN were more susceptible to liver injury compared to wild type mice and the mechanism of injury was due to an increase in the phosphorylation of c-Jun N-terminal kinase (JNK), delayed

activation of hepatic Stat 3, increased activation of NF- κ B and a down regulation of antiapoptotic and autophagy pathways (136). Thus, PXR plays a hepatoprotective role in LPS/GaIN-induced liver injury. Despite the aforementioned studies on the anti-inflammatory role of PXR in mice, the exact clinical relevance in humans is not clear and needs further research.

1.3.5 PXR in apoptosis and its role in DIH

Apoptosis is a programmed mode of cell death in which a cell undergoes shrinkage and chromatin fragmentation. Apoptosis is executed by caspases, which are activated through an intrinsic or extrinsic pathway depending on the stimuli (137). Apoptosis is one of the common pathways involved in DIH and studies have shown that PXR activation predominantly has anti-apoptotic effects (60, 138).

Constitutively activated PXR or pharmacological activation of PXR by RIF protected cells from deoxycholic acid-induced apoptosis as well as adriamycin-induced cell death (139). A similar anti-apoptotic effect was observed in transgenic mice expressing constitutively active PXR treated with lithocholic acid (a toxic bile acid that leads to apoptotic cell death) (139). The mechanism by which PXR activation inhibits apoptosis appears to be through the upregulation of antiapoptotic proteins like BIRC2, B cell leukemia 2 (Bcl-2) associated athanogene 3 (BAG3) and myeloid cell leukemia-1(MCL-1) while down regulating the pro-apoptotic genes like brassinosteroid insensitive1-associated receptor kinase1 (BAK1) and p53 (139). In addition, PXR activation by dexamethasone was found to prevent apoptosis by upregulating the expression of the anti-apoptotic proteins Bcl and Bcl-XL respectively (60). Furthermore, a recent study found that PXR could bind and sequester the p53 gene thereby preventing it from binding to its apoptotic target

genes and suppressing its transcriptional activity (*140*). Since apoptosis is a key mechanism of cell death in DIH, PXR activation in the context of DIH may have a protective effect.

1.3.6 PXR in cell proliferation and regeneration and its role in DIH

Liver regeneration is the ability of the liver to recover to its original mass after massive hepatocyte loss or injury. The regeneration process involves a complex interplay of cytokines, growth factors and metabolic pathways (*141*). Liver regeneration involves cell proliferation and DNA synthesis that all promote tissue repair (*141*). Early toxicological studies using model hepatotoxicants like acetaminophen have shown that the activation of tissue repair processes influences the outcome of liver injury (*142*). An increase in tissue repair mechanisms can slow down or reverse the progression of a toxic insult to the liver.

Studies have shown that PXR activation leads to an increase in the size of the liver as well as an upsurge in cell proliferation (*62, 143*). Further studies in mice found the PXR to be an important factor in driving liver regeneration. Dai et al. showed that loss of function of PXR led to a decline in the overall size of the liver, decreased deposition of triglycerides and cholesterol as well as reduced expression of some lipogenic genes. They also found a decrease in cell proliferation indicated by a decline in the number of Kiel antibody 67 (Ki-67) positive hepatocytes, and inhibition of Stat 3 expression, a transcription factor that promotes liver regeneration (*61*). Triglycerides and cholesterol have been reported to be an important source of energy for hepatocytes during regeneration (*144*). PXR promotes the early phase of regeneration by activation of genes involved in triglyceride and cholesterol synthesis. In addition, PXR activation was shown to promote liver enlargement and regeneration by increasing (i) the population of hybrid hepatocytes that are involved in regeneration after liver injury, (ii) cell size, and (iii) promoting the

proliferation of hepatocytes and hybrid hepatocytes (145). The mechanism by which PXR mediates this regeneration is dependent on its interaction with the yes-associated protein (YAP). PXR activation triggers the nuclear translocation of YAP, which leads to the activation of the YAP signaling pathway (145). Another study in mice showed that PXR activation by PCN alone was not sufficient to drive hepatocyte proliferation. However, PCN was found to heighten chimeric antigen receptor (CAR)-mediated and peroxisome proliferator activated receptor alpha (PPARα)-mediated hepatocyte proliferation by their ligands (146). The authors further found that PCN treatment increased the ribonucleic acid (RNA) content of quiescent hepatocytes and decreased the mRNA levels of p27 and p130, which are negative regulators of the cell cycle (146). In another study, PXR activation was found to stimulate growth factor-mediated cell proliferation in immortalized mouse hepatocytes (147). Also, PCN treatment in carbon tetrachloride-treated mice (a chemical model for liver regeneration) increased the number of proliferating cells and *Ccna2* and *Ccnb1* mRNA levels (cell cycle promoting genes) in the liver of wild-type mice but not in *Pxr*-null mice (147). Furthermore, PCN treatment decreased the mRNA levels of cell cycle suppressor genes *Rbl2*, *Cdkn1a* and *Cdkn1b* by inhibiting *foxo3*-mediated transcription of these genes (147). Therefore, PXR activation can promote liver regeneration and possibly attenuate DIH while PXR inhibition can slow down liver regeneration, leading to a negative effect on tissue repair after a toxic insult to the liver.

1.3.7 PXR polymorphisms and its role in DIH

Individual differences in the expression of PXR have been implicated in DIH and liver diseases (Table 3) (79, 148-151). In a clinical study, the frequency of PXR polymorphism rs3814055 with the CC genotype was found to be significantly increased in patients who developed

DIH after flucloxacillin therapy (79). PXR polymorphism in the -25385 promoter region and with the CC genotype is associated with a decrease in PXR and CYP3A4 expression (79, 148). A decrease in PXR expression can have a negative effect on the downstream gene network controlled by PXR. In the case of flucloxacillin, a PXR agonist, a decrease in PXR and CYP3A4 expression may slow down flucloxacillin metabolism thus leading to its accumulation in the hepatocytes and causing DIH (79). (Table 3)

Table 3. Summary of PXR polymorphisms associated with DIH.

Reference SNP	SNP genotypes	Impact on PXR functions	Drugs/diseases affected	Outcomes	References
rs3814055	CC	Decreased PXR basal expression and CYP3A4 expression	Flucloxacillin	Promotes liver injury	(79)
rs3814055	TT	Increased CYP3A4 expression	Anti-tuberculosis therapy	Increases susceptibility to DIH	(151),(148)
rs2461823	AA	Unknown	Anti-tuberculosis therapy	Increases risk of DIH	(150)
rs7643645	GG	Regulates CYP3A4 expression, Disrupts the binding site of HNF4 α	Anti-tuberculosis therapy	Decreases risk of DIH	(150)
rs7643645	GG	Regulates CYP3A4 expression, disrupts the binding site of HNF4 α	Associated with liver injury in Nonalcoholic liver disease	Increases susceptibility to liver injury	(149)
rs2461823	AA	Unknown	Associated with liver injury in Nonalcoholic liver disease	Increases susceptibility to liver injury	(149)

In Indonesian patients who had tuberculosis and were placed on standard tuberculosis therapy, genotyping results revealed that patients with the same PXR polymorphism rs3814055 but with the TT genotype were more susceptible to tuberculosis drug-induced hepatotoxicity compared to those with the wild type CC genotype or heterozygous CT genotype (151). The rs3814055 polymorphism with the TT genotype is associated with an increase in CYP3A4 expression (148). (Table 3) Another clinical study in tuberculosis patients in Taiwan revealed that female patients carrying the PXR variant rs2461823 with the AA genotype showed an increased frequency of anti-tuberculosis (TB) drug induced hepatitis compared to those carrying other genotypes AG and GG (150). On the contrary, female patients with the AA genotype at rs7643645 were protected from anti-tuberculosis drug induced hepatitis compared to those carrying the AG or GG genotypes (150). The number of G alleles at the rs7643645 was associated with an increased risk of anti-tuberculosis drug induced hepatitis in female patients and patients with the GG genotype showed a higher incidence of drug induced hepatitis (150). The PXR polymorphism rs2461823 has no transcription factor binding sequence identified at that site and no functional effect on PXR has been reported (149). However, rs7643645 is positioned at the binding site of HNF-4 α and the G allele leads to the disruption of the HNF4- α binding site on PXR (152). HNF4- α is a nuclear receptor that regulates the expression of PXR, PXR mediated transcription of CYP3A4 and controls other gene networks in the liver (153). Therefore disruption of its binding to PXR will affect both the expression of PXR and CYP3A4 (153). Interestingly, the frequencies of PXR variants were similar between male patients who did and did not develop hepatitis (150).

Diseases affecting the liver like non-alcoholic steatosis increase the susceptibility of individuals and sensitivity of the liver to injury. Two of the PXR single nucleotide polymorphisms already discussed above rs7643645 with the GG genotype and rs2461823 with the AA genotype were found to be associated with disease severity in patients with non-alcoholic liver steatosis (149). The rs7643645/G and rs2461823/A haplotype were also associated with alanine transaminase (ALT) levels increase (149). This makes PXR an interesting target for understanding susceptibility to DIH.

The role of PXR in DIH is a two-edged sword (Figure 5). PXR activation can promote DIH through its ability to increase the expression of phase I and phase II drug metabolizing enzymes that can catalyze the bioactivation of drugs to form reactive metabolites; decrease utilization of glucose in cells or promote the production of glucose to cause hyperglycemia; increase lipid synthesis and fatty acid uptake into the liver while decreasing fatty acid beta oxidation, which leads to lipid accumulation and steatosis; increase porphyrin synthesis, and down regulation of enzymes involved in antioxidant defense. On the contrary, PXR activation can decrease the risk of DIH through its regulation of detoxification, its anti-inflammatory and anti-apoptotic effects, liver regeneration and proliferation promoting properties. Future studies should be focused on understanding the implication of these PXR functions in humans and PXR as a target in mitigating DIH.

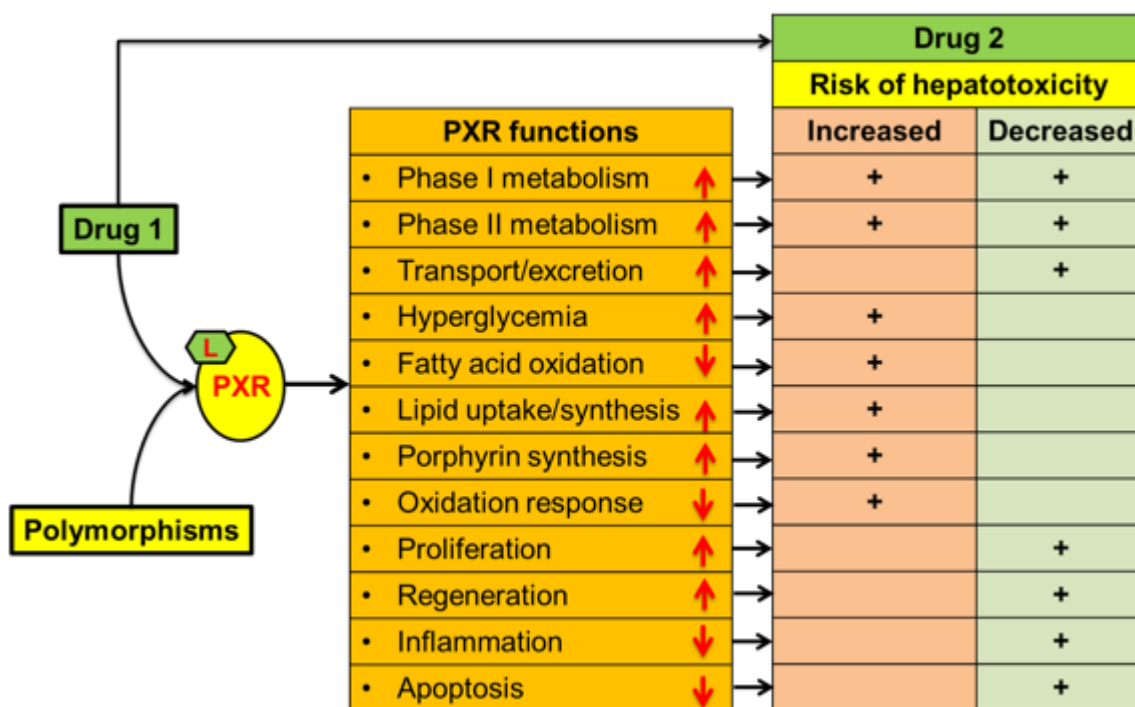


Figure 5. Summary of pathways regulated by PXR and their contribution to DIH.

Ligand (L)-dependent PXR activation together with PXR polymorphisms impact the DIH of co-administered drug(s) through the cellular pathways that are regulated by PXR

2.0 PREGNANE X RECEPTOR ACTIVATION MODULATES RITONAVIR HEPATOTOXICITY

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2.1 INTRODUCTION

With the advent of antiretroviral therapy (ART), the overall mortality and morbidity in HIV/AIDS patients has decreased significantly. However, the toxicity of ART is one of the major concerns for the HIV/AIDS community. A considerable number of patients have poor adherence to ART in part due to drug toxicity (155), consequently leading to the emergence of drug resistance and/or virologic failure. RTV is the backbone of boosted protease inhibitor-based regimens in ART. In early clinical studies of RTV, treatment with a full dose of RTV frequently caused liver damage (156, 157). The use of a low dose RTV as a pharmacoenhancer for RTV-containing regimens decreased the overall rate of liver injury. However, RTV is still considered the cause of liver damage during treatment with RTV-containing regimens (158, 159). Because the mechanisms of RTV hepatotoxicity remain elusive, no approach is currently available to predict and prevent such toxicity.

Remarkably, multiple clinical studies found that hepatotoxicity occurred in 100% of participants who were pretreated with rifampicin (RIF, an antibiotic to treat tuberculosis) or efavirenz (EFV, a non-nucleoside reverse transcriptase inhibitor to treat HIV) followed by RTV-containing regimens (160-163). We noted that both RIF and EFV are activators of human PXR, a ligand-dependent transcription factor that is highly expressed in the liver and upregulates drug metabolizing enzymes including Cytochrome P450 3A4 (CYP3A4) (48-51, 164). In addition, CYP3A4 plays a critical role in RTV metabolism and bioactivation (165-170). These data led us to hypothesize that human PXR modulates RTV hepatotoxicity through CYP3A4-dependent pathways. Genetically engineered PXR and CYP3A4 mouse models were developed and used to test our hypothesis.

2.2 METHODS

2.2.1 Animal models

The *PXR*- and *CYP3A4*-humanized mouse models were developed based on previously generated hPXR, TgCYP3A4/hPXR, and Cyp3a-null mouse models (171-173). In brief, the *CYP3A4* transgenic mice were generated by bacterial artificial chromosome (BAC) transgenesis, which contains the complete *CYP3A4* and *CYP3A7* genes including PXR response element (PXRE) (173). In Cyp3a-null mice, all eight *Cyp3a* genes were eliminated (171). TgCYP3A4/hPXR mice were crossed with Cyp3a-null mice to generate a mouse model expressing human *PXR* and *CYP3A4*, but deficient in mouse *Pxr* and *Cyp3a* (hPXR/CYP3A4). In addition, Cyp3a-null mice were crossed with hPXR mice to generate a mouse model expressing human

PXR, but deficient in human *CYP3A4*, and mouse *Pxr* and *Cyp3a* (hPXR/Cyp3a-null). hPXR/CYP3A4 and hPXR/Cyp3a-null mice (3 weeks old) were verified by PCR genotyping of human *PXR* and *CYP3A4*, and mouse *Pxr* and *Cyp3a*. In addition, quantitative PCR (qPCR) and/or Western blotting were used to determine the expression of PXR target genes including CYP3A4.

2.2.2 Experimental design

To determine the roles of PXR and CYP3A4 in RTV hepatotoxicity, WT, hPXR/CYP3A4, and hPXR/Cyp3a-null mice (male, 2-4 months old) were treated with RIF (50 mg in 1 kg diet) or EFV (500 mg in 1 kg diet) for seven days. On the eighth day, the mice were administered with 50 mg/kg of RTV (two doses, ip) or corn oil (vehicle). All mice were sacrificed on the ninth day. Blood and liver samples were collected for evaluation of liver injury. All drug doses for animal experiments were calculated according to the food and drug administration (FDA) draft guidance for calculating human equivalent dose (HED).

2.2.3 Chemicals and reagents

Ritonavir (RTV) and rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO). Efavirenz (EFV) was purchased from TCI Chemical (Portland, OR). The solvents for metabolite analysis were of the highest grade commercially available.

2.2.4 Study approval

All mice were handled in accordance with study protocols approved by the University of Pittsburgh Animal Care and Use Committee.

2.2.5 Assessment of liver injury

Alanine transaminase (ALT) and aspartate transaminase (AST) in serum were analyzed by standard assays according to the kit procedures (Point Scientific Inc, Canton, MI). Sections of liver tissues were fixed in 4% formaldehyde phosphate solution overnight for histological analysis. Dehydration of fixed liver tissues was achieved by passing them through an increasing gradient of alcohol and then xylene solutions. Liver tissues were then embedded in paraffin and 4 μ m sections were cut and stained with hematoxylin and eosin (H&E). For transmission electron microscopy (TEM), fresh liver samples ($\sim 1 \text{ mm}^3$) were placed into Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde). The liver samples were further processed using 1% osmium tetroxide, phosphate buffered saline and gradients of ethanol (30-100%) solution. Afterward, the liver tissues were kept overnight in Epon/propylene oxide solution (1:1), and then embedded and sectioned for TEM imaging.

2.2.6 Cell-based reporter assay of human PXR activation

The effects of RIF and EFV on human PXR activation were examined in the DPX2 cell line (Puracyp Inc., Carlsbad, CA), which was derived from HepG2 cells with the stable

transfection of human *PXR* and a luciferase reporter gene. Briefly, DPX2 cells were cultured in a 96-well plate with a density of 2×10^5 cells/ml. Ten μ M RIF or EFV were incubated in DPX2 cells for 24 h followed by analysis of luciferase activity. The experiments were conducted in triplicate.

2.2.7 qPCR analysis

Total mRNA was extracted from 50 mg of liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was made from 1 μ g of total RNA with a SuperScript II Reverse Transcriptase kit and random oligonucleotides (Invitrogen, Carlsbad, CA). PCR primers (Table 4) were obtained from the Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>) or designed by the Primer blast-NCBI-NIH (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). qPCR was carried out using 25 ng cDNA, 150 nM of each primer, and 5 μ L of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 μ L. The qPCR plate was analyzed on an ABI-Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Cyclophilin was used as the housekeeping gene and the values were calculated using the comparative CT method.

Table 4. Primers for qPCR analysis

Gene	Forward 5' -3'	Reverse 3'-5'
Atf3	GAGGATTTTGCTAACCTGACACC	TTGACGGTAACTGACTCCAGC
Bax	TCCCCCGAGAGGTCTTTT	CGGCCCCAGTTGAAGTTG
Bip	AGTGGTGGCCACTAATGGAG	CAATCCTTGCTTGATGCTGA
Cbr3	GGCACTCCGGTTAAGTAACTC	TGTCACTTGGTCGAATTTGTTCA
Chop	CTGCCTTTCACCTTGGGAC	CGTTTCCTGGGGATGAGATA
Cyp2b10	GGGAAAGCGCATTTGTCTTG	GATGGACGTGAAGAAAAGGAACA
Dr5	ATAAAAAGAGGCTGTGAACGGG	GGTCCAAGAGAGACGA
Gpx2	GCCTCAAGTATGTCCGACCTG	GGAGAACGGGTCATCATAAGGG
Gsta1	GCAGGGGTGGAGTTTGAAGA	CAGGGCTCTCTCCTTCATGTC
Gstm1	GAGGATCCGTGCAGACATT	ACTCTGGCTTCTGCTTCTCA
Gstm2	TGACTACATGAAGAGCAGCCG	CTTTGGGTTCCTAAAAGGCCA
Mcp1	CAACTCTCACTGAAGCCAGCTCT	CAGGCCCAGAAGCATGACA
Slc25a24	AGGCTTTCGGCAGATGGTAAA	CCTTCCTCGGTAAGCAACTTCT

2.2.8 Western blotting

Microsomal or total liver protein was used for blotting. Microsomal protein was prepared by homogenizing liver tissues in ice-cold buffer A (0.1 M phosphate buffer pH 7.5, sucrose 0.25 M, KCL 0.154 M). Liver homogenates were centrifuged at 12,100 g for 25 min at 4 °C and the

resulting supernatant was spun at 38,100 g for 1 h. Microsomal pellets were re-suspended in buffer B (0.1 M phosphate buffer saline and 20% glycerol). For total protein, liver tissue was homogenized in ice-cold Ripa buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). The resulting homogenates were centrifuged at 10,000 g for 10 min and the supernatants were collected. Protein concentration was measured using the Bio-Rad protein assay (Hercules, CA). Twenty µg of protein from each sample was resolved on a 10 or 12 % SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to PVDF membranes and probed using primary antibodies against Chop (Cell Signaling Technology, #2895S), Bip (Cell Signaling Technology, #3177S), CYP3A4 (Laboratory of Metabolism, NCI, #275-1-2), and Gapdh (EMD Millipore, #MAB374). Immunoreactive proteins were identified by a chemiluminescence detection method.

2.2.9 Effects of RTV on RIF metabolism

hPXR/CYP3A4 mice were treated with RIF (10 mg/kg, po) or RIF plus RTV (50 mg/kg, ip). Eighteen h after the treatment, the liver and feces were collected for analysis of RIF and its metabolite. In brief, liver and feces were weighed and homogenized in water (100 mg of liver in 400 µL of water and 100 mg of feces in 1000 µL of water). One hundred µL of the liver homogenate was mixed with 600 µL of acetonitrile to methanol (1:1 v/v) followed by vortexing. For feces, 100 µL of the homogenate was mixed with 200 µL of acetonitrile followed by vortexing. The samples were then centrifuged twice at 15,000 g for 10 min and the supernatants were transferred to the autosampler vials for metabolite analysis by ultra-performance liquid chromatography (UPLC) coupled with quadrupole time-of-flight mass spectrometry (QTOFMS) system (Waters Corporation, Milford, MA).

2.2.10 Effects of RIF on RTV metabolism

The effects of RIF on RTV metabolism were determined using mouse liver microsomes from hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with or without RIF diet 50 mg/kg for seven days. Incubations were conducted in $1 \times$ phosphate buffered saline (PBS, pH 7.4) containing 10 μ M RTV and 0.2 mg MLM in a final volume of 95 μ L. After 5 min of pre-incubation at 37 °C, the reaction was initiated by the addition of 5 μ L of 20 mM NADPH and continued for 40 min with gentle shaking. Incubations were terminated by adding 100 μ L of acetonitrile followed by vortexing for 30 s and centrifuging at 15,000 g for 10 min. Each supernatant was transferred to an autosampler vial for metabolite analysis by UPLC-QTOFMS.

2.2.11 UPLC-QTOFMS analysis

Metabolite separation was performed on an Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 μ m; Waters Corporation, Milford, MA). The flow rate of the mobile phase was 0.5 ml/min using a gradient ranging from 2% to 98% acetonitrile/water containing 0.1% formic acid. The column temperature was maintained at 50 °C. QTOFMS was operated in positive mode with electrospray ionization. MS data were acquired over a range of 50-1000 Da in centroid format. Tandem mass fragmentation with collision energy ramping from 15 to 45 V was used for structural elucidations of metabolites.

2.2.12 Liver metabolomics

Liver metabolome was analyzed in hPXR/CYP3A4 mice treated with vehicle, RIF, RTV, or RIF plus RTV. Briefly, liver samples were weighed and homogenized in water (100 mg of tissues in 500 μ L of water). Two hundred μ L of acetonitrile: methanol (1:1, v/v) was added to 100 μ L of each homogenate, followed by vortexing and centrifugation at 15,000 g for 10 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation at 15,000 g for 10 min, and then transferred to an autosampler vial for metabolite analysis by UPLC-QTOFMS. A data matrix, including retention time and m/z, was generated through alignment of all the samples. The data matrix was further exported into SIMCA-P+ software (Version 13, Umetrics, Kinnelon, NJ). Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were conducted to maximize the class discrimination. The metabolites that significantly contributed to the discrimination between groups were subjected to structure identification.

2.2.13 Statistics

GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. All data are shown as mean \pm standard error of the mean (S.E.M.). One-way or two-way analysis of variance (ANOVA) was used with Tukey's post hoc test to compare the difference among multiple groups. A two-tailed unpaired Student's t-test was performed for statistical analysis between two groups. A *P* value < 0.05 was considered statistically significant.

2.3 RESULTS AND DISCUSSION

WT mice are not sensitive to lead-in treatment with RIF followed by RTV

This project was initiated from the clinical observations showing hepatotoxicity in subjects pretreated with RIF followed by RTV-containing regimens (Figure 6A) (160, 161, 163). We first used wild-type (WT) mice to mimic the hepatotoxicity that occurred in clinical studies. However, no significant liver injury was found in WT mice pretreated with RIF for seven days followed by RTV (Figure 6B, C, and Figure 7). These data suggest inter-species differences between mice and humans in response to RIF and/or RTV. RIF is a human-specific activator of PXR, a ligand-dependent transcription factor highly expressed in the liver, which regulates a gene network including the hepatic drug-metabolizing enzyme CYP3A4 (49, 50). To overcome the inter-species differences in RIF-mediated PXR activation, we generated a double transgenic mouse model expressing human PXR and CYP3A4 (hPXR/CYP3A4) on the background of mouse *Pxr* and *Cyp3a* deficiency (Figure 6D). As expected, treatment with RIF induced a panel of PXR target genes including CYP3A4 in the liver of hPXR/CYP3A4 mice (Figure 8), indicating that human PXR is functional in these mice.

Lead-in treatment with RIF potentiates RTV hepatotoxicity in hPXR/CYP3A4 mice

Using hPXR/CYP3A4 mice, we recapitulated the RTV hepatotoxicity observed in clinical studies, as the biomarkers of liver damage were significantly increased in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV (Figure 6E and 6F). In addition, histological analysis revealed massive hepatocyte degeneration in hPXR/CYP3A4 mice pretreated with RIF followed by RTV (Figure 9). These data indicate that human PXR is the key mediator in the hepatotoxicity caused by lead-in treatment with RIF followed by RTV. This information is

extremely important for the HIV/AIDS community because many prescription drugs and herbal supplements are potent PXR activators that individuals may encounter in daily life (70, 174-176). Therefore, we suggest reviewing whether HIV/AIDS patients are under treatment with PXR activators before starting RTV-containing regimens.

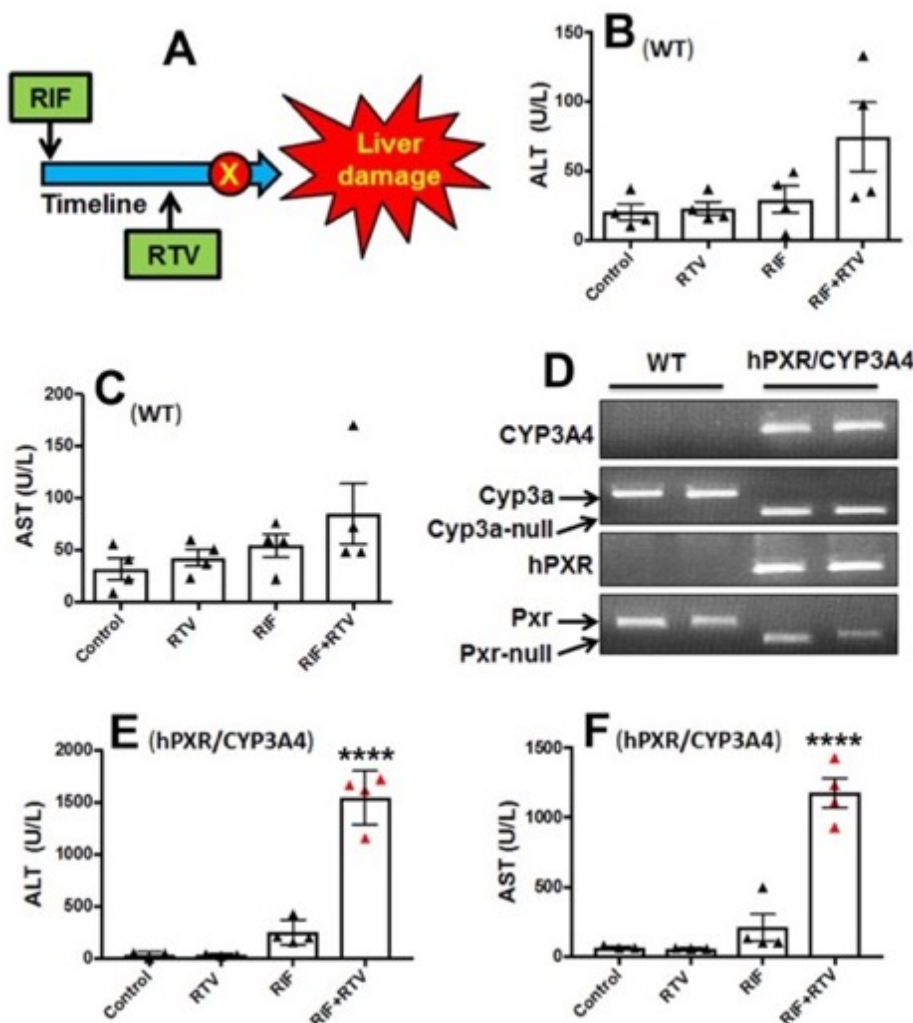


Figure 6. Role of human PXR in the hepatotoxicity caused by pretreatment with RIF followed by RTV.

(A) A scheme showing the adverse drug interactions between RIF and RTV in humans that led to the early termination of clinical studies. (B-F) Evaluation of liver damage in WT and hPXR/CYP3A4 mice pretreated with RIF diet 50 mg/kg for seven days followed by two doses of RTV (50 mg/kg, ip). (B, C) Activities of alanine transaminase (ALT) and aspartate transaminase (AST) in the serum of WT mice. (D) Genotyping results of hPXR/CYP3A4 mice, which are positive for human PXR and CYP3A4, but negative for mouse Pxr and Cyp3a. (E, F) Activities of ALT and AST in the serum of hPXR/CYP3A4 mice. All data are expressed as means \pm S.E.M. (n = 3-4). Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. ****P < 0.0001 for RIF+RTV group vs control, RTV, and RIF groups.

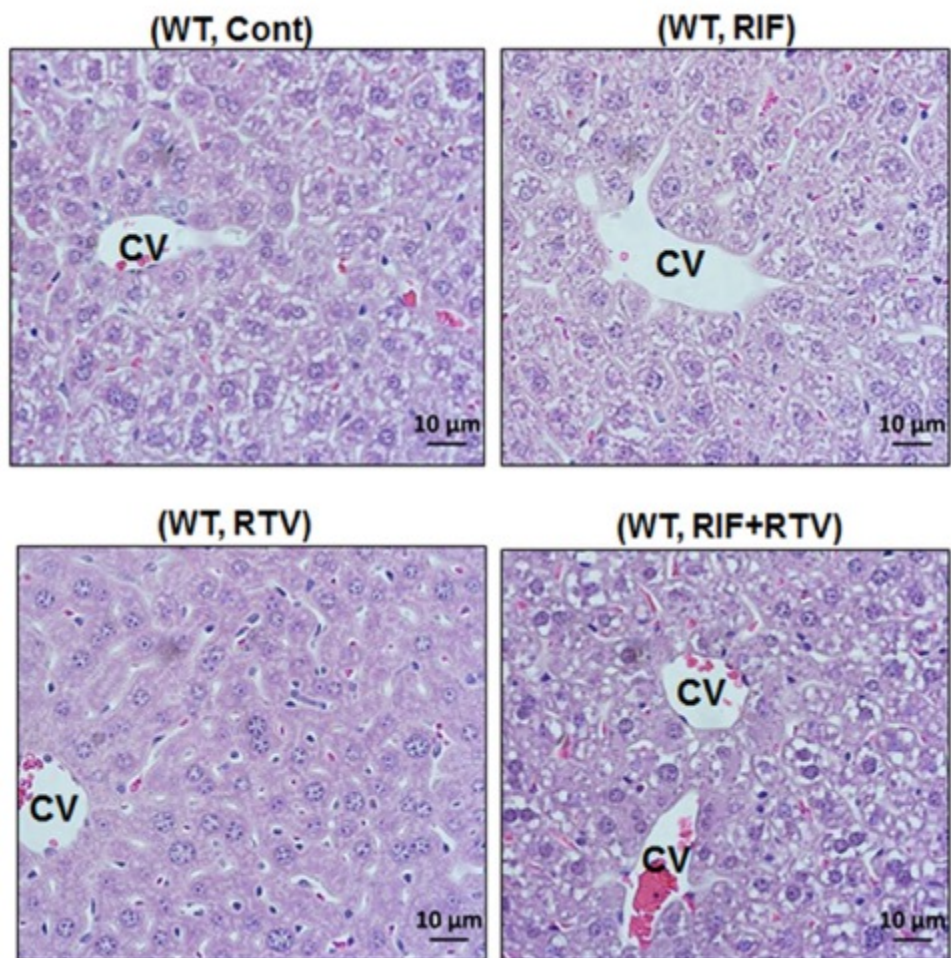


Figure 7. Histological analysis of the liver in WT mice pretreated with RIF diet for seven days followed by two doses of RTV.

H&E staining; CV, central vein. Scale bar: 10 μm.

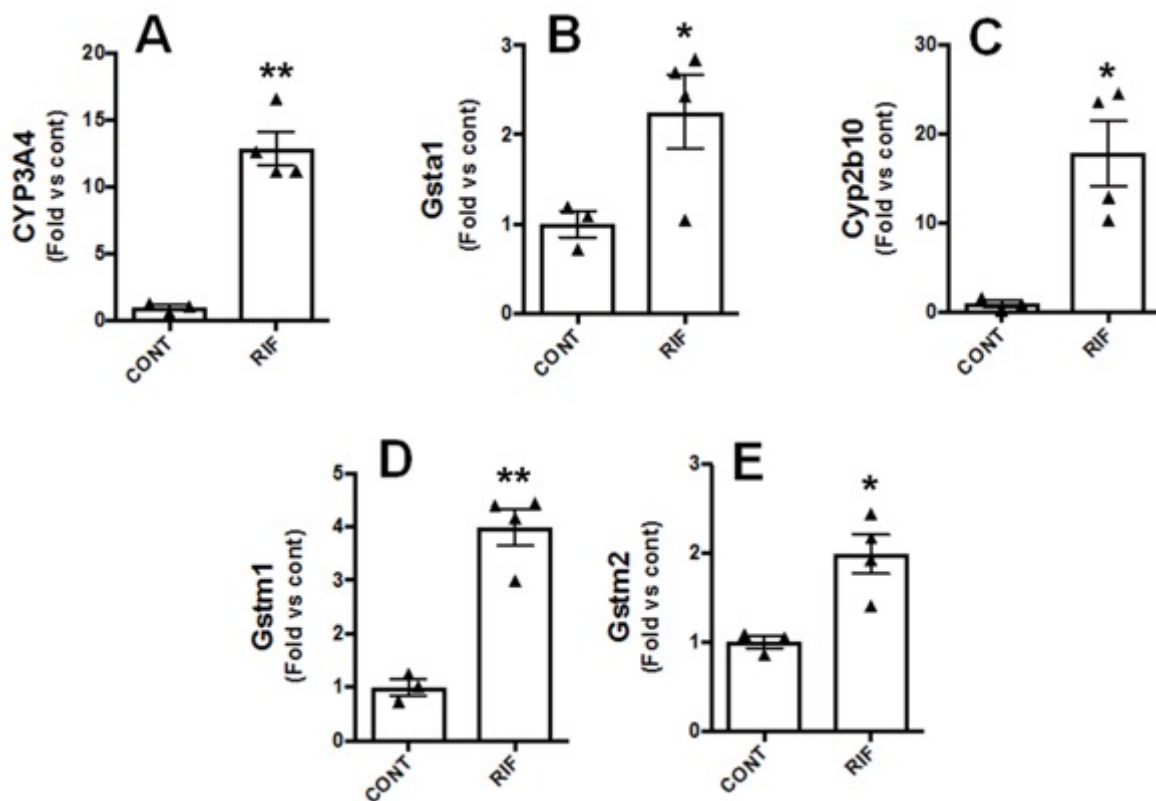


Figure 8. Effects of RIF on the expression of PXR target genes in the liver of hPXR/CYP3A4 mice.

Mice were treated with RIF diet (50 mg in 1kg diet) for seven days and liver samples were analyzed for expression of PXR target genes. *CYP3A4* (A), *Cyp2b10* (B), *Gsta1* (C), *Gstm1* (D), and *Gstm2* (E) mRNAs were analyzed by qPCR. All data are expressed as means \pm S.E.M. ($n = 3-4$). The data in control groups were set as 1. Statistical significance was determined by two tailed unpaired t-test. * $P < 0.05$, ** $P < 0.01$ vs control.

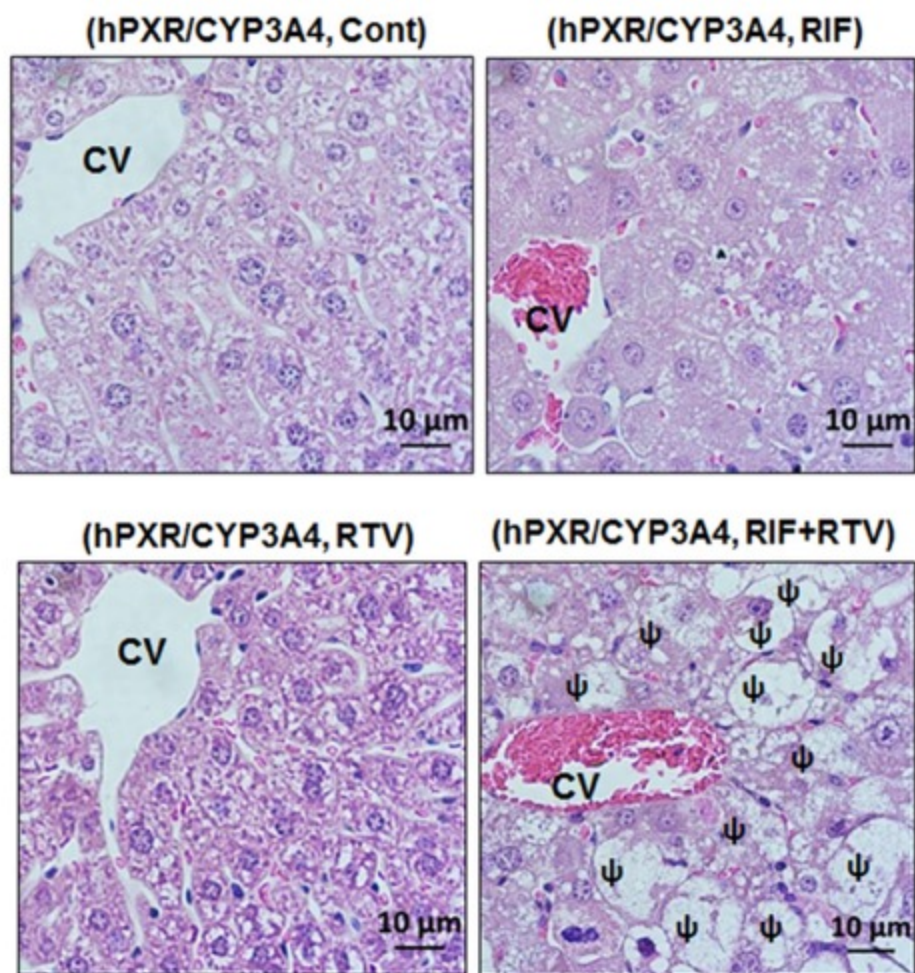


Figure 9. Histological analysis of the liver in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by two doses of RTV.

H&E staining; CV, central vein; ψ indicates hepatocyte degeneration. Scale bar: 10 μm.

CYP3A4 loss of function mitigates hepatotoxicity caused by lead-in treatment with RIF followed by RTV

We next explored the pathways downstream of PXR that lead to RTV hepatotoxicity. We hypothesized that PXR modulates RTV hepatotoxicity through CYP3A4-dependent pathways because CYP3A4 is a primary PXR target gene (*49-51*), and CYP3A4 plays an important role in RTV metabolism and bioactivation (*165-170*). To test this hypothesis, we generated a human PXR mouse model deficient in Cyp3a (hPXR/Cyp3a-null) (Figure 10A). Treatment with RIF for seven days significantly induced PXR target genes other than Cyp3a in the liver of hPXR/Cyp3a-null mice (Figure 10B, Figure 11), indicating that PXR is functionally intact in these mice. Compared to hPXR/CYP3A4 mice, no liver injury was observed in hPXR/Cyp3a-null mice pretreated with RIF for seven days followed by RTV (Figure 10C-10F), indicating that PXR modulates RTV hepatotoxicity through CYP3A4-dependent pathways. These data suggest that CYP3A4 induction should be considered as a risk factor for RTV hepatotoxicity. Apart from PXR, other nuclear receptors like constitutive androstane receptor (CAR) also upregulate CYP3A4 expression (*177*), and many CAR activators are found amongst prescription drugs (such as phenobarbital) and herbal supplements (*178, 179*). Hence, clinicians should be careful with both PXR and CAR activators before starting RTV-containing regimens in HIV/AIDS patients, as this can induce CYP3A4 and predispose patients to the risk of liver injury.

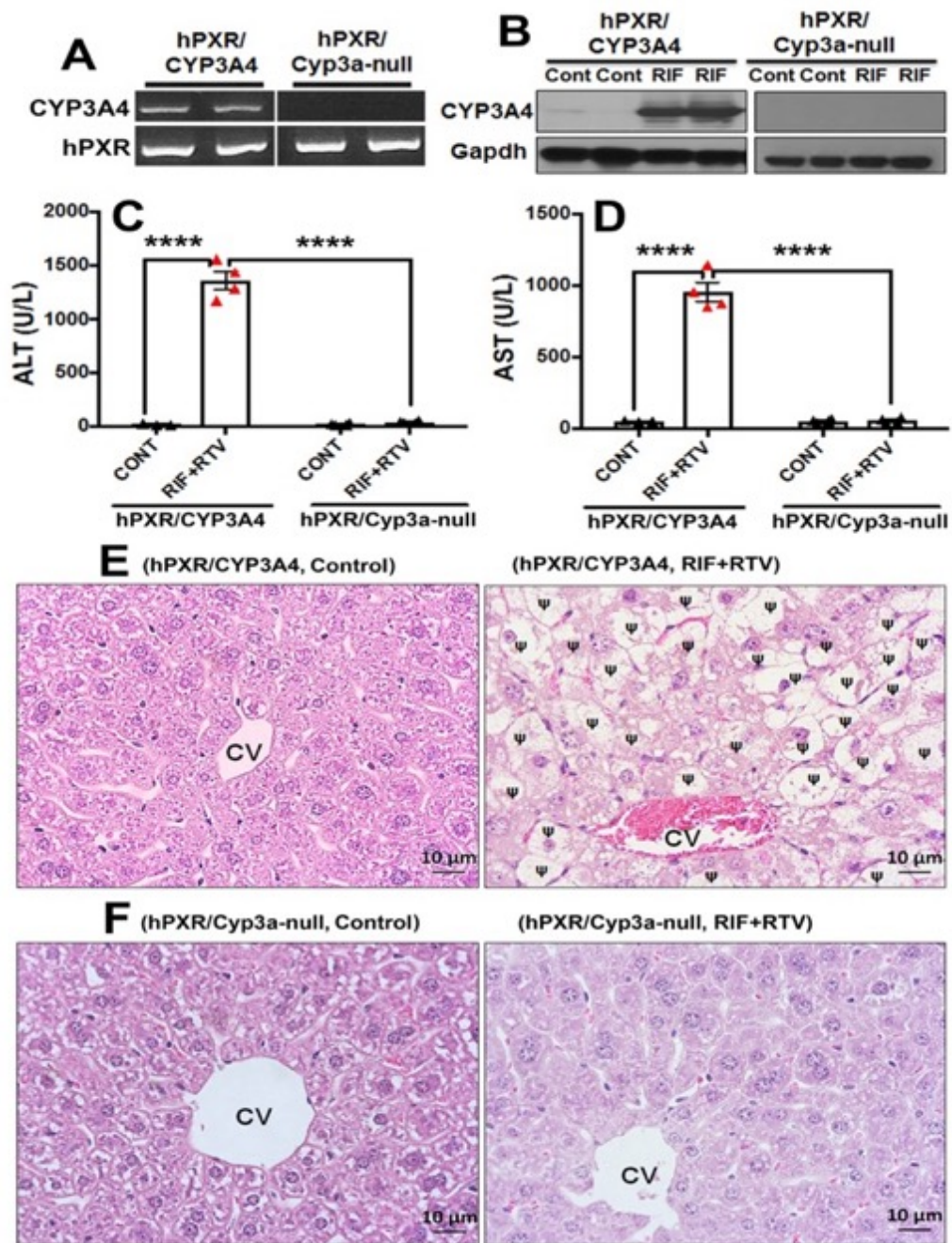


Figure 10. Role of CYP3A4 in the hepatotoxicity caused by pretreatment with RIF followed by RTV
hPXR/CYP3A4 and hPXR/Cyp3a-null mice were pretreated with RIF for 7 days followed by RTV. (A) Genotyping results of hPXR/Cyp3anull mice, which are positive for human PXR, but negative for human CYP3A4. (B) Expression of CYP3A4 in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with or without PXR

ligand RIF for 7 days. Gapdh was used as a loading control. (C and D) Activities of ALT and AST in the serum. All data are shown as mean \pm SEM. (n = 3-4). Statistical significance was determined by 2-way ANOVA with Tukey's post hoc test. ****P < 0.0001. (E and F) Histological analysis of liver samples from control and RIF+RTV groups of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. H&E staining. CV, central vein; ψ indicates hepatocyte degeneration. Scale bar: 10 μ m.

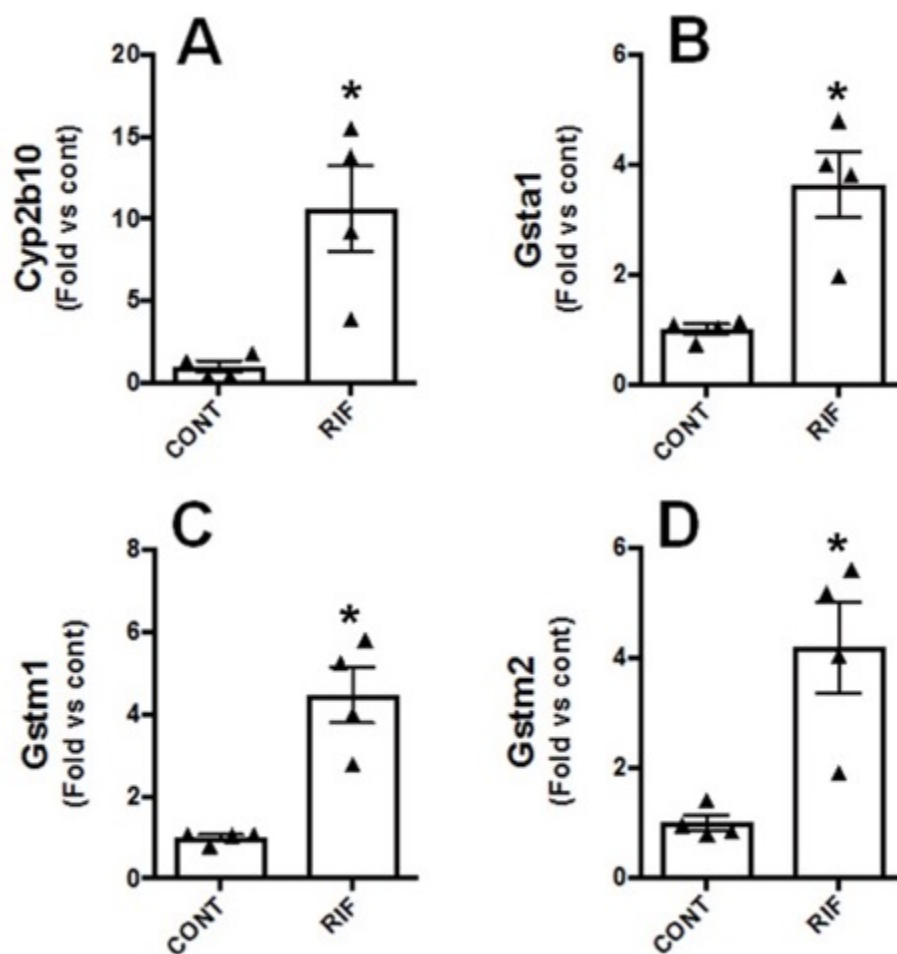


Figure 11. Effects of RIF on the expression of PXR target genes in the liver of hPXR/Cyp3a-null mice.

Mice were treated with RIF (50 mg in 1kg diet) for seven days and liver samples were analyzed for expression of PXR target genes. *Cyp2b10* (A), *Gsta1* (B), *Gstm1* (C), and *Gstm2* (D) mRNAs were analyzed by qPCR. All data are expressed as means ± S.E.M. ($n = 4$). The data in control groups were set as 1. Statistical significance was determined by two tailed unpaired t-test. * $P < 0.05$ vs control.

Lead-in treatment with EFV followed by RTV caused hepatotoxicity in hPXR/CYP3A4 mice

To further verify the role of human PXR and CYP3A4 in RTV hepatotoxicity, the adverse interactions between EFV and RTV were investigated in hPXR/CYP3A4 and hPXR/Cyp3a-null mice. EFV is also a PXR activator (Figure 12A). We found that lead-in treatment with EFV for seven days potentiated RTV hepatotoxicity in hPXR/CYP3A4 mice (Figure 12B-12F), which mimicked EFV and RTV-induced liver injury observed in a previous clinical study (*162*). In addition, lead-in treatment with EFV for seven days followed by RTV resulted in hepatocyte degeneration (Figure 12E), exhibiting the same phenotype as RIF and RTV-induced liver damage (Figure 9). We also noted that RIF had a more significant impact than EFV on RTV hepatotoxicity as seen by comparing the ALT and AST values in Figure 6E and 6F, Figure 12B and 12C. This increased effect of RIF can be the result of enterohepatic circulation of RIF leading to consistent distribution of RIF in the liver (*180*). Furthermore, we found that the hepatotoxicity associated with lead-in treatment with EFV followed by RTV was CYP3A4-dependent (Figure 12B-12F). These data further confirmed the roles of human PXR and CYP3A4 in RTV hepatotoxicity.

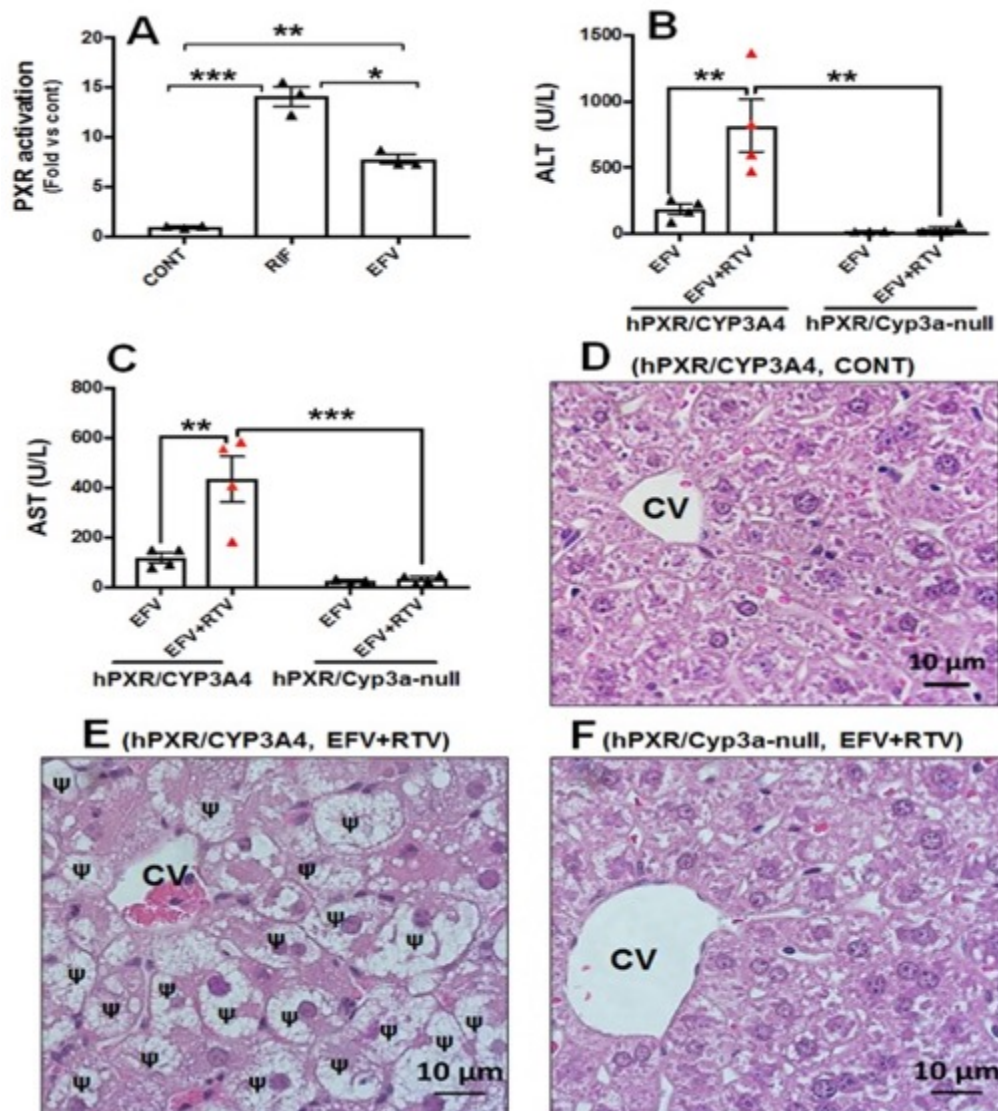


Figure 12. Roles of human PXR and CYP3A4 in the hepatotoxicity associated with lead-in treatment with EFV followed by RTV.

(A) Effect of EFV (10 μ M) on PXR activation in a cell-based reporter assay ($n = 3$). The data in control group were set as 1. RIF (10 μ M) was used as a positive control. (B-F) Evaluation of liver damage in hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV diet (500 mg/kg) for seven days followed by two doses of RTV(50 mg/kg,ip). (B, C) Activities of ALT and AST in the serum. All data are expressed as means \pm S.E.M. ($n = 4$). Statistical significance was determined by One or two-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D-F) Histological analysis of liver. H&E staining; CV, central vein; ψ indicates hepatocyte degeneration. Scale bar: 10 μ m

Lead-in treatment with RIF increases RTV metabolism and bioactivation in hPXR/CYP3A4 mice

We next investigated the pharmacokinetic interactions between RIF and RTV in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. We found that RTV had no effect on RIF metabolism and disposition (Figure 13). However, pretreatment with RIF for seven days significantly increased the metabolism and bioactivation pathways of RTV in the liver of hPXR/CYP3A4 mice, but not in hPXR/Cyp3a-null mice (Figure 14). The major metabolism and bioactivation pathways of RTV were M1 followed by M13 (Figure 14A and 14B), and both of them are mediated by CYP3A (20). We found that the production of M1 and M13 increased 19- and 7-fold, respectively, in the liver microsomes of hPXR/CYP3A4 mice pretreated with RIF for seven days (Figure 14C and 14D). Accompanied with M1, 2-isopropylthiazole-4-carbaldehyde (M1-1) was produced, which can be further metabolized to form an adduct with glutathione (GSH) (170). In addition, M13 and 2-methylpropanethioamide (M13-1) were isopropylthiazole ring-open metabolites of RTV. The ring-open metabolites of thiazole derivatives can be further oxidized and cause liver injury (181, 182). These data indicate that PXR-mediated CYP3A4 induction increases RTV bioactivation in the liver, which can potentially lead to liver damage.

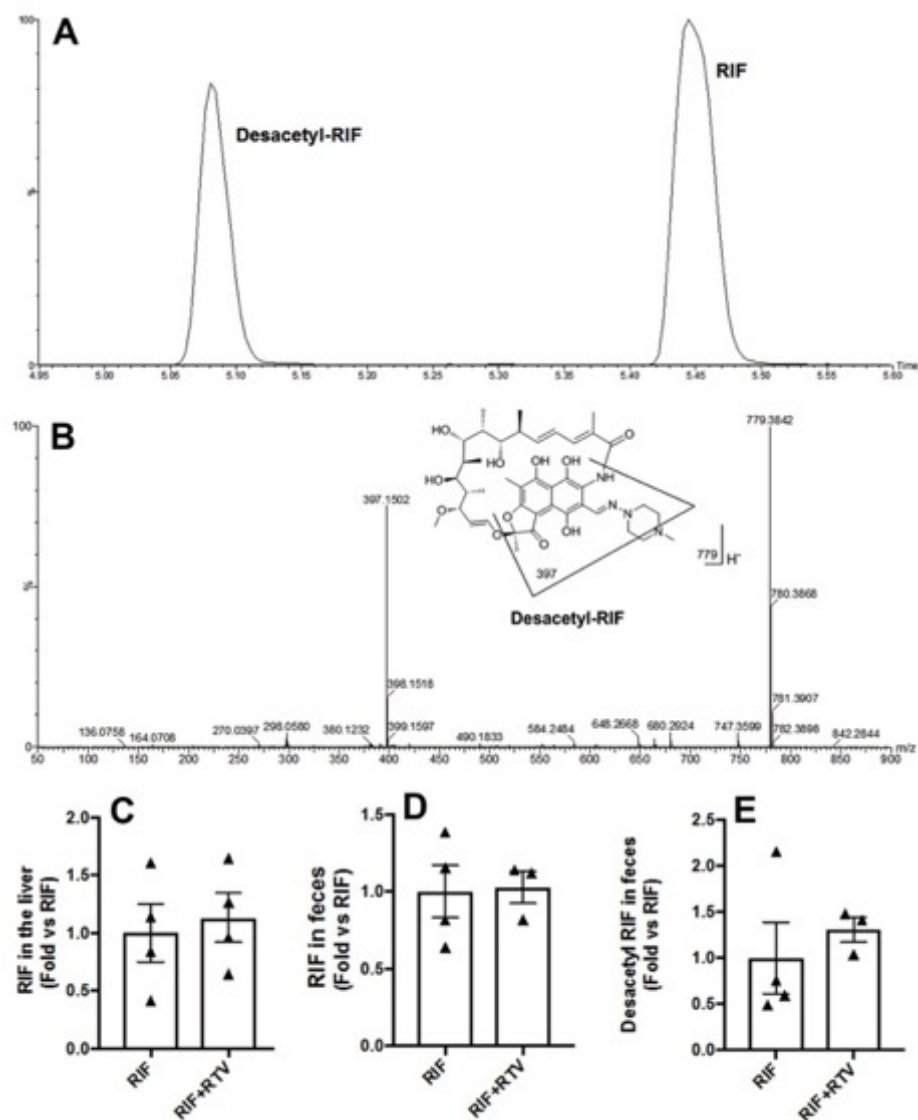


Figure 13. Effects of RTV on RIF metabolism and disposition in hPXR/CYP3A4 mice.

Feces and liver samples were collected overnight after treatment with RIF (10 mg/kg, po) or RIF plus RTV (50 mg/kg, ip). RIF and its metabolite desacetyl-RIF were analyzed by UPLC-QTOFMS. (A) Extracted chromatograms of RIF and desacetyl-RIF from feces. (B) MS/MS spectrum of desacetyl-RIF. The structure of desacetyl-RIF with fragmental patterns is inlaid in the spectra. (C) The relative abundance of RIF in the liver. (D) The relative abundance of RIF in feces. (E) The relative abundance of desacetyl-RIF in feces. All data are expressed as means \pm S.E.M. ($n = 3-4$). The data in RIF group were set as 1. Statistical significance was determined by two tailed unpaired t-test.

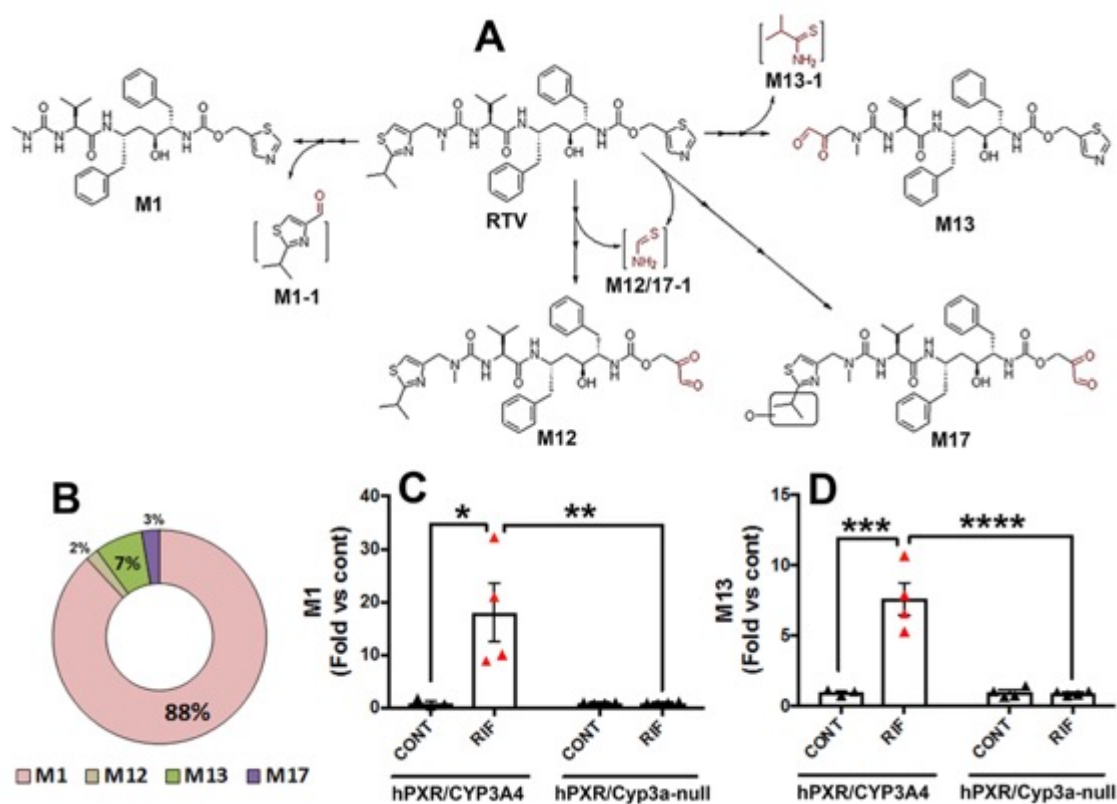


Figure 14. RIF-mediated PXR activation and CYP3A4 induction increases RTV metabolism and bioactivation.

(A) A scheme showing the bioactivation pathways of RTV. (B) Relative abundances of M1, M12, M13, and M17 produced in the liver microsomes of hPXR/CYP3A4 mice pretreated with RIF diet (50 mg/kg) for seven days.

These metabolites were analyzed by UPLC-QTOFMS and the total peak areas of these 4 metabolites were set as

100%. M1 was identified as the most abundant metabolite followed by M13. (C, D) Production of M1 and M13 in the liver microsomes of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with or without RIF for seven days.

The data are expressed as means \pm S.E.M. ($n = 3-4$). The data in control groups of hPXR/CYP3A4 mice were set as

1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. $*P < 0.05$, $**P < 0.01$,

$***P < 0.001$, $****P < 0.0001$.

Lead-in treatment with RIF followed by RTV caused oxidative stress in the liver of hPXR/CYP3A4 mice

CYP-mediated drug bioactivation can cause cellular stress (41, 183). Using a metabolomic approach, we found a dramatic increase in ophthalmic acid (OA), a biomarker of oxidative stress (184), in the liver of hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV, but not in hPXR/Cyp3a-null mice with the same treatment (Figure 15A-15D). In addition, we found that pretreatment with RIF followed by RTV in hPXR/CYP3A4 mice caused an upregulation of genes that are involved in cellular responses to oxidative stress in the liver, including glutathione peroxidase 2 (Gpx2), carbonyl reductase 3 (Cbr3), and solute carrier family 25 member 24 (Slc25a24) (Figure 15E-15G). Similar to the adverse drug interactions between RIF and RTV, lead-in treatment with PXR activator EFV followed by RTV also caused oxidative stress in the liver of hPXR/CYP3A4 mice, but not in hPXR/Cyp3a-null mice (Figure 16). These data indicate that pretreatment with PXR activators followed by RTV causes oxidative stress in the liver, which is dependent on CYP3A4.

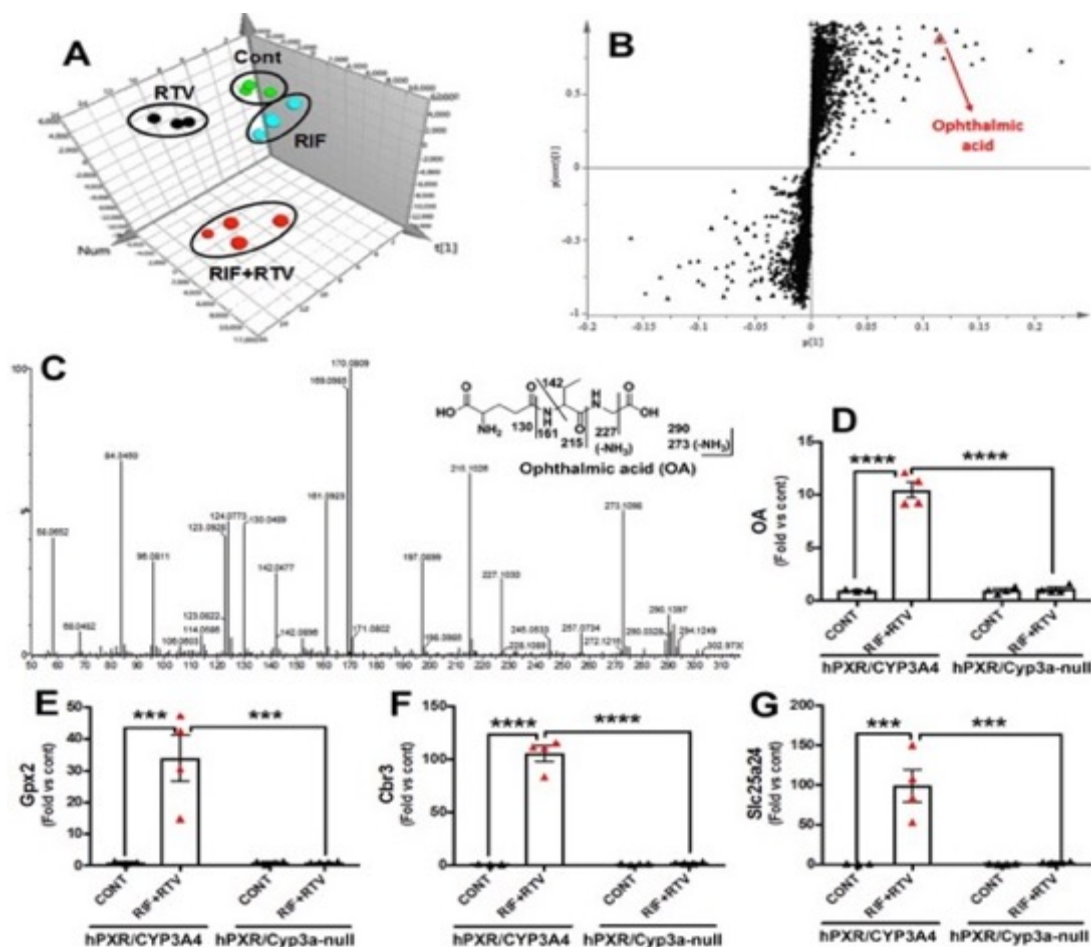


Figure 15. Metabolomics reveals oxidative stress in the liver of hPXR/CYP3A4 mice pretreated with RIF for 7 days followed by RTV.

Liver samples were analyzed by UPLC-QTOFMS. (A) Principal component analysis (PCA) of liver samples from control, RIF, RTV, and RIF/RTV groups of hPXR/CYP3A4 mice. (B) Loading S plots generated by orthogonal projections to latent structures discriminant analysis (OPLS-DA) analysis of liver samples. The x axis is a measure of the relative abundance of ions, and the y axis is a measure of the correlation of each ion to the model. OA, a biomarker of oxidative stress, was identified as a top-ranking ion in the RIF+RTV group. (C) Structural illustration of OA by tandem mass spectrometry (MS/MS) fragmental analysis. (D) Relative quantification of OA in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. (E–G) The expressions of genes related to oxidative stress. *Gpx2* (E), *Cbr3* (F), and *Slc25a24* (G) mRNAs were analyzed by quantitative PCR (qPCR). All data are shown as mean \pm SEM. ($n = 3$ –4). Statistical significance was determined by 2-way ANOVA with Tukey's post hoc test. The data in the control group of hPXR/CYP3A4 mice were set as 1. *** $P < 0.001$; **** $P < 0.0001$.

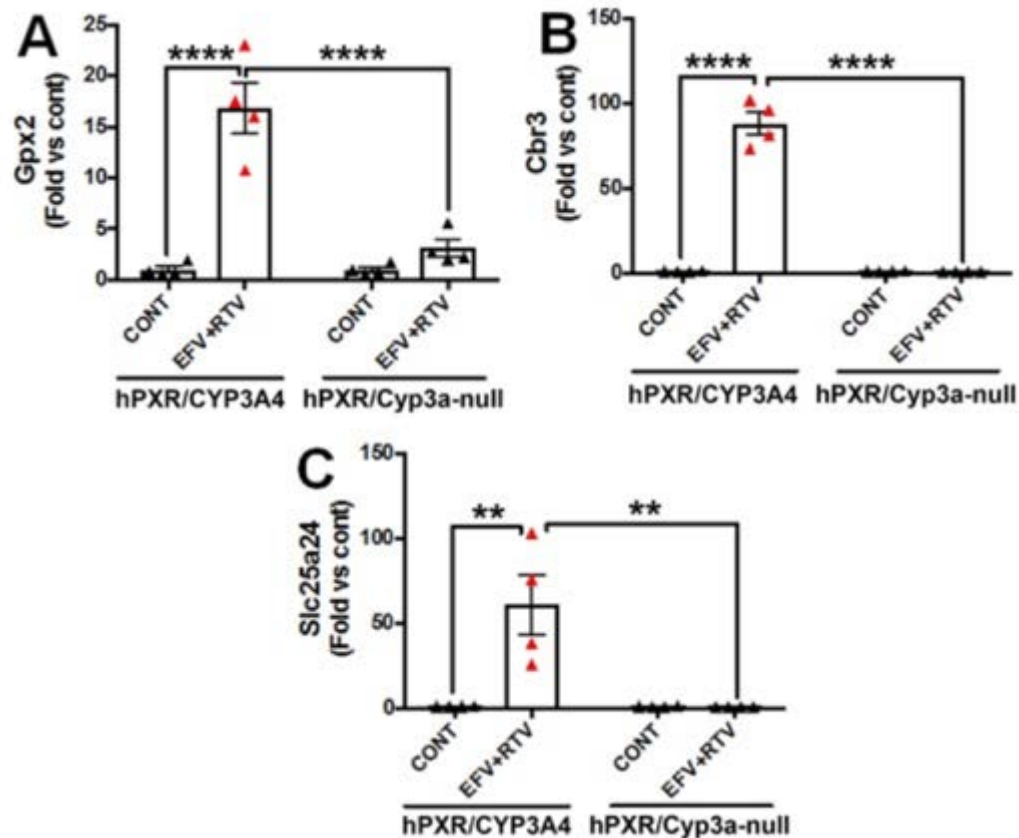


Figure 16. Oxidative stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by two doses of RTV.

Gpx2 (A), Cbr3 (B), and Slc25a24 (C) mRNAs were analyzed by qPCR. All data are expressed as means \pm S.E.M. (n = 4). The data in the control group of hPXR/CYP3A4 mice were set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. **P < 0.01, ****P < 0.0001.

Lead-in treatment with RIF followed by RTV caused ER stress in the liver of hPXR/CYP3A4 mice

Oxidative stress can lead to endoplasmic reticulum (ER) stress (*185*). Indeed, we found that the ER is a target organelle of RTV hepatotoxicity, as electron-microscopic analysis revealed massive ER dilation in hepatocytes of hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV (Figure 17A). In addition, we found that lead-in treatment with RIF followed by RTV caused severe ER stress in the liver of hPXR/CYP3A4 mice, as indicated by the increased expression of ER stress biomarkers including C/EBP homologous protein (Chop), binding immunoglobulin protein (Bip), and cyclic AMP-dependent transcription factor 3 (Atf3) (Figure 17B and 17C). ER stress also occurred in the liver of hPXR/CYP3A4 mice pretreated with EFV for seven days followed by RTV, but not in hPXR/Cyp3a-null mice with the same treatment (Figure 18). Persistent ER stress can lead to cell death (*185*). Concordantly, we observed a significant increase in the expression of genes associated with cell death and tissue injury, including death receptor 5 (Dr5), BCL2-associated X (Bax), and monocyte chemoattractant protein 1 (Mcp1) in the liver of hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV, but not in hPXR/Cyp3a-null mice (Figure 17D-17F). These data suggest that lead-in treatment with PXR activators followed by RTV causes ER stress and hepatocellular injury, and it is CYP3A4-dependent.

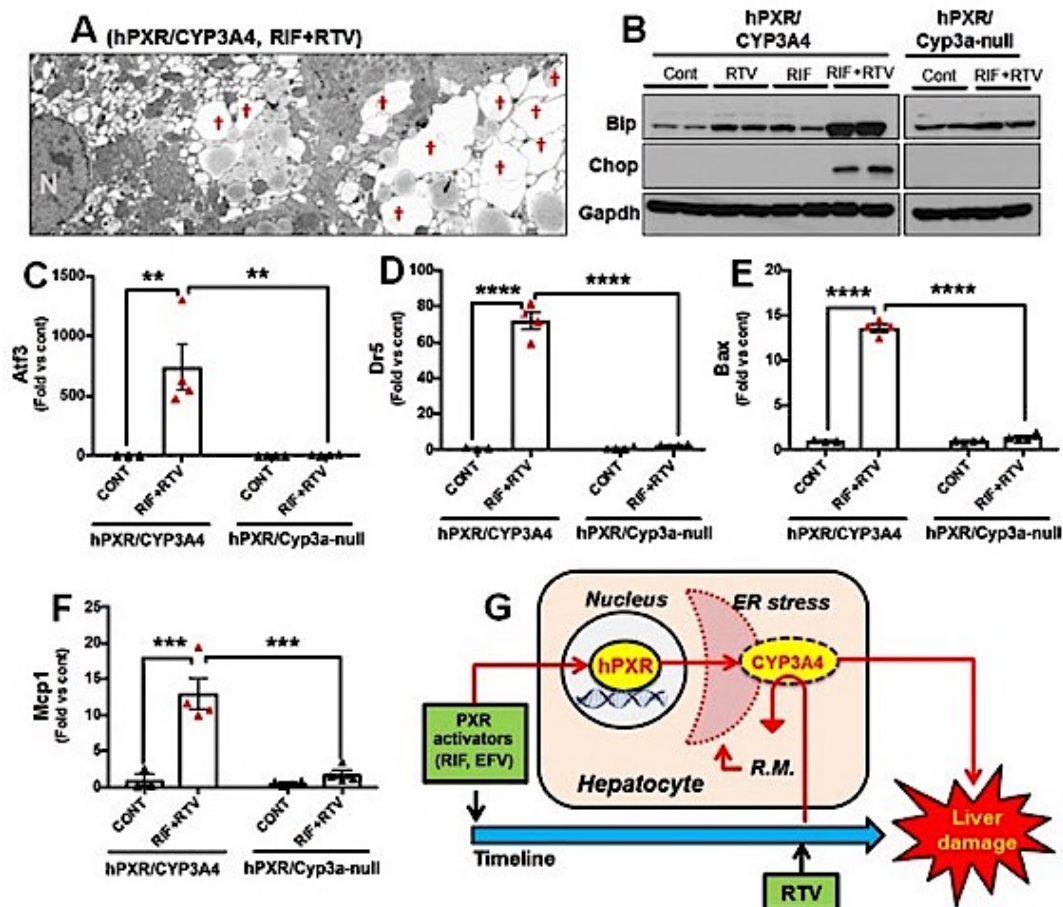


Figure 17. Endoplasmic reticulum (ER) is a target organelle in the hepatotoxicity associated with RIF pretreatment for seven days followed by RTV.

(A) Electron-microscopic examination of the liver in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by two doses of RTV. N, nucleus; (†) dilated ER. (B, C) ER stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. Bip, Chop, and Atf3 were used as biomarkers of ER stress. Bip and Chop were analyzed by Western blotting. Gapdh was used as a loading control. Atf3 mRNA was analyzed by qPCR. (D-F) The expressions of genes related to cell death and tissue injury in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. Dr5 (D), Bax (E) and Mcp1 (F) mRNAs were analyzed by qPCR. All data are expressed as means \pm S.E.M. ($n = 3-4$). The data in control groups of hPXR/CYP3A4 mice were set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (G) Schematic representation for the roles of human PXR and CYP3A4 in modulating RTV hepatotoxicity: (i) Ligand-dependent activation of PXR upregulates CYP3A4 expression; (ii) Overexpressed CYP3A4 is located in the ER; (iii) CYP3A4

catalyzes RTV to produce reactive metabolites (R.M.); and (iv) The ER is exposed to a high level of R.M. of RTV, which leads to ER stress and hepatocellular injury.

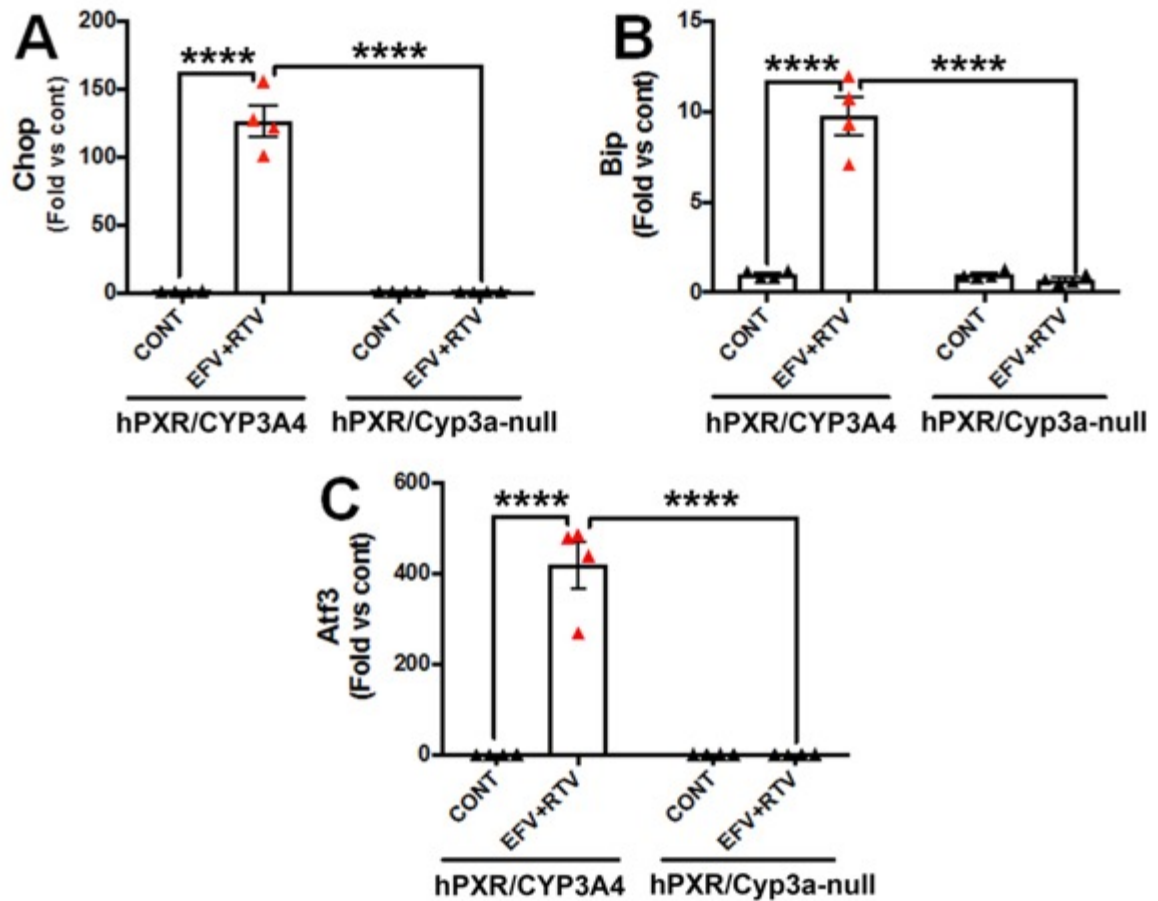


Figure 18. ER stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by two doses of RTV.

Chop (A), *Bip* (B), and *Atf3* (C) mRNAs were analyzed by qPCR. All data are expressed as means \pm S.E.M. ($n = 4$). The data in the control group of hPXR/CYP3A4 mice were set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. **** $P < 0.0001$.

The ER is critical for protein maturation, including posttranslational modification and proper folding (185). The activation of PXR, being a transcription factor, by ligands like RIF and EFV upregulates a network of genes including CYP3A4 and thus increases the workload of the ER for protein maturation and processing. On the other hand, CYP3A4 is located in the ER, and PXR-mediated CYP3A4 induction increases the production of RTV reactive metabolites that can directly target the ER and undermine its functions leading to ER stress and hepatocellular injury (Figure 17G).

2.4 CONCLUSION

In summary, the current study demonstrated the essential roles of human PXR and CYP3A4 in RTV hepatotoxicity. Our results can be used to develop novel strategies based upon PXR, CYP3A4, and their downstream pathways to improve the safety profile of RTV-containing regimens in the clinic.

3.0 LEAD-IN TREATMENT WITH CYP3A4 INDUCERS POTENTIATES COBICISTAT HEPATOTOXICITY

This chapter is adapted from the following manuscript in preparation:

Amina I. Shehu, Jie Lu, Junjie Zhu, Deborah McMahon, Wen Xie, Frank J. Gonzalez, Xiaochao Ma. Lead-in treatment with CYP3A4 inducers potentiates cobicistat hepatotoxicity.

3.1 INTRODUCTION

Cobicistat (COBI) is a second-generation pharmacokinetic enhancer in HIV therapy, which has a similar structure and the same mechanism-of-action as ritonavir (RTV), the first-generation pharmacoenhancer (*186-188*). However, COBI has no inducing effects on CYPs or uridine 5'-diphospho-glucuronosyltransferase (UGT) when compared to RTV, which potentially reduces the risk of drug-drug interactions (*189, 190*). In addition, COBI has no anti-HIV activity and has fewer effects than RTV on lipid metabolism (*188, 191, 192*). Furthermore, COBI has more favorable physicochemical properties than RTV, which allows for co-formulation with other drugs (*193*). These beneficial factors make COBI an emerging pharmacokinetic enhancer in clinical practice.

In spite of the aforementioned advantages of COBI, adverse effects of COBI have been observed in clinical studies (*194-196*). In addition, a similar rate of treatment discontinuation was observed with RTV- or COBI-containing regimens in clinical studies suggesting a similarity of COBI to RTV in safety profiles (*194-196*). Clinical studies have reported that lead-in treatment

with rifampicin (RIF), a human specific activator of pregnane X receptor (PXR)(48-51, 63), followed by RTV-containing regimens led to hepatotoxicity(160, 161, 163). Using PXR- and CYP3A4-humanized mouse models, our work revealed that RIF-mediated PXR activation potentiates RTV hepatotoxicity through CYP3A4-dependent pathways (154). RIF belongs to the rifamycin family used in tuberculosis (TB) therapy and many rifamycins are human PXR activators (197). These data raise concern on the safety of initiating treatment with rifamycins followed by COBI-containing regimens because the World Health Organization (WHO) recommends lead-in treatment of TB followed by HIV therapy in the subjects co-infected with HIV and TB (198).

The current work explored the risk factors of COBI hepatotoxicity with a focus on the PXR/CYP3A4 axis. Genetically engineered PXR and CYP3A4 mouse models were utilized. In addition to RIF, we investigated the effects of rifabutin (RFB) on COBI hepatotoxicity, because RFB is a member of the rifamycin family and typically used in subjects co-infected with HIV and TB (198). We also investigated the roles of the constitutive androstane receptor (CAR)/CYP3A4 axis in COBI hepatotoxicity because CAR plays a similar role to PXR in CYP3A4 regulation (199). The findings from this work can be used to guide the safe use of COBI-containing regimens in clinical practice.

3.2 METHODS

3.2.1 Animals models

PXR- and CYP3A4-humanized (hPXR/CYP3A4) and PXR-humanized mice deficient in Cyp3a (hPXR/Cyp3a-null) mice were developed in our previous work (154). CYP3A4-humanized mice deficient in Pxr (Pxr-null/CYP3A4) mice were developed in the current study. In brief, Pxr-null/CYP3A4 mice were generated by crossing hPXR/CYP3A4 with Pxr-null mice. All mouse models were verified by PCR genotyping of human PXR and CYP3A4, and mouse Pxr and Cyp3a. CYP3A4 expression in the liver was also verified using Western blotting. These mice (male, 2-4 months old) were treated with PXR or CAR activators to determine the roles of PXR, CAR, CYP3A4, and their downstream pathways in COBI hepatotoxicity. All mice were handled in accordance with study protocols approved by the University of Pittsburgh Animal Care and Use Committee.

3.2.2 Experimental design

To determine the roles of PXR and CYP3A4 in COBI hepatotoxicity, hPXR/CYP3A4, Pxr-null/CYP3A4 and hPXR/Cyp3a-null mice were pretreated with either RIF (50 mg/kg in diet) or RFB (50 mg/kg in diet) for seven days. To determine the roles of CAR-mediated CYP3A4 induction in COBI hepatotoxicity, Pxr-null/CYP3A4 mice were pretreated with TCPOBOP (3 mg/kg, i.p.) for 3 days. On the following day, 50 mg/kg of COBI (two doses, i.p.) were administered. All mice were euthanized one day after COBI treatment. To determine the roles of

oxidative stress in COBI hepatotoxicity, hPXR/CYP3A4 mice were treated with CDDO-Im (2 mg/kg, i.p.) on day six and seven during RIF treatment followed by COBI on day eight. Blood and liver samples were collected for evaluation of liver injury.

3.2.3 Chemicals and reagents

Rifampicin (RIF), rifabutin (RFB), and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Cobicistat (COBI) was provided by Medchem Express (South Brunswick, NJ). TCPOBOP and CDDO-Im were purchased from Tocris Bioscience (Bristol, UK).

3.2.4 Assessment of liver injury

Biochemical and histological analyses were conducted to evaluate liver damage. In brief, the biomarkers of liver injury alanine transaminase (ALT) and aspartate transaminase (AST) in serum were analyzed using the standard kit (Point Scientific Inc, Canton, MI). Liver histology was carried out using paraffin embedded liver tissues. Four μ M sections of liver tissues were cut and stained with hematoxylin and eosin (H&E). In addition, transmission electron microscopy (TEM) was used to examine the target organelle of COBI hepatotoxicity.

3.2.5 qPCR analysis.

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract RNA from liver tissues. One μ g of total RNA was used to make Complementary DNA (cDNA) using a SuperScript II

Reverse Transcriptase kit and random oligonucleotides (Invitrogen, Carlsbad, CA). The qPCR reaction contained 25 ng of cDNA, 150 nM of each primer, and 5 μ L of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 μ L. ABI-Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for plate analysis, and the values were calculated using the comparative CT method. Cyclophilin was used as the internal control.

3.2.6 Western blotting.

Twenty μ g of liver proteins were resolved on a 10% SDS-polyacrylamide gel electrophoresis. PVDF membranes were used for protein transfer. Proteins were then probed using primary antibody against CYP3A4 (Laboratory of Metabolism, NCI, #275-1-2) and Gapdh (EMD Millipore, #MAB374). A chemiluminescence detection method was used to identify the target protein. Gapdh was used as the internal control.

3.2.7 Effects of RIF on COBI metabolism.

Liver microsomes were used to determine the effects of RIF-mediated PXR activation and CYP3A4 induction on COBI metabolism. In brief, the incubation was carried out in 1x phosphate buffer saline (PBS, pH 7.4) containing 30 μ M of COBI and 1 mg of liver microsomes. The mixture was pre-incubated for 5 min and then started by adding NADPH, a co-factor of CYP-mediated reactions. The incubation was continued with gentle shaking for 30 min and terminated by adding acetonitrile: methanol (1:1). The resulting mixture was vortexed for 1 min and centrifuged at

15,000 g for 10 mins. The supernatant was transferred to an autosampler vial for analysis by UPLC-qTOFMS (Waters Corporation, Milford, MA).

3.2.8 UPLC-QTOFMS analysis.

UPLC-qTOFMS analysis was conducted to quantify COBI metabolites and ophthalmic acid (OA), a biomarker of oxidative stress. In brief, the Acquity UPLC BEH C18 column (2.1×100 mm, $1.7 \mu\text{m}$) was used for metabolite separation. Acetonitrile/water containing 0.1% formic acid was used as mobile phase. qTOFMS (50-1000 Da) was operated in positive mode.

3.2.9 Statistical analysis.

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). One-way or two-way analysis of variance (ANOVA) was used to compare the differences among multiple groups while the two-tailed Student's t-test was used for statistical analysis between two groups. All data are shown as mean \pm standard error of the mean (SEM). Statistical significance was established when the P value was < 0.05 .

3.3 RESULTS

Lead-in treatment with RIF sensitizes hPXR/CYP3A4 mice to COBI hepatotoxicity

Because of species differences in RIF-mediated PXR activation (48-51, 63), we utilized the hPXR/CYP3A4 mouse model. Pretreatment with RIF followed by COBI resulted in hepatotoxicity in hPXR/CYP3A4 mice as shown by a marked elevation of liver injury biomarkers including serum alanine transaminase (ALT) and aspartate transaminase (AST) (Figure 19A and 19B). Cell death was observed in the liver of hPXR/CYP3A4 mice treated with RIF+COBI, but not in the control, COBI or RIF groups (Figure 19C-F). Histological analysis also revealed massive hepatocyte degeneration with cell enlargement and the formation of large vacuoles in hPXR/CYP3A4 mice treated with RIF+COBI (Figure 19F). In addition, a similar study was conducted in WT mice pretreated with RIF followed by COBI, but no notable liver injury was observed (Figure 19A, B, and Figure 20). These results suggest that RIF potentiates COBI hepatotoxicity through human PXR.

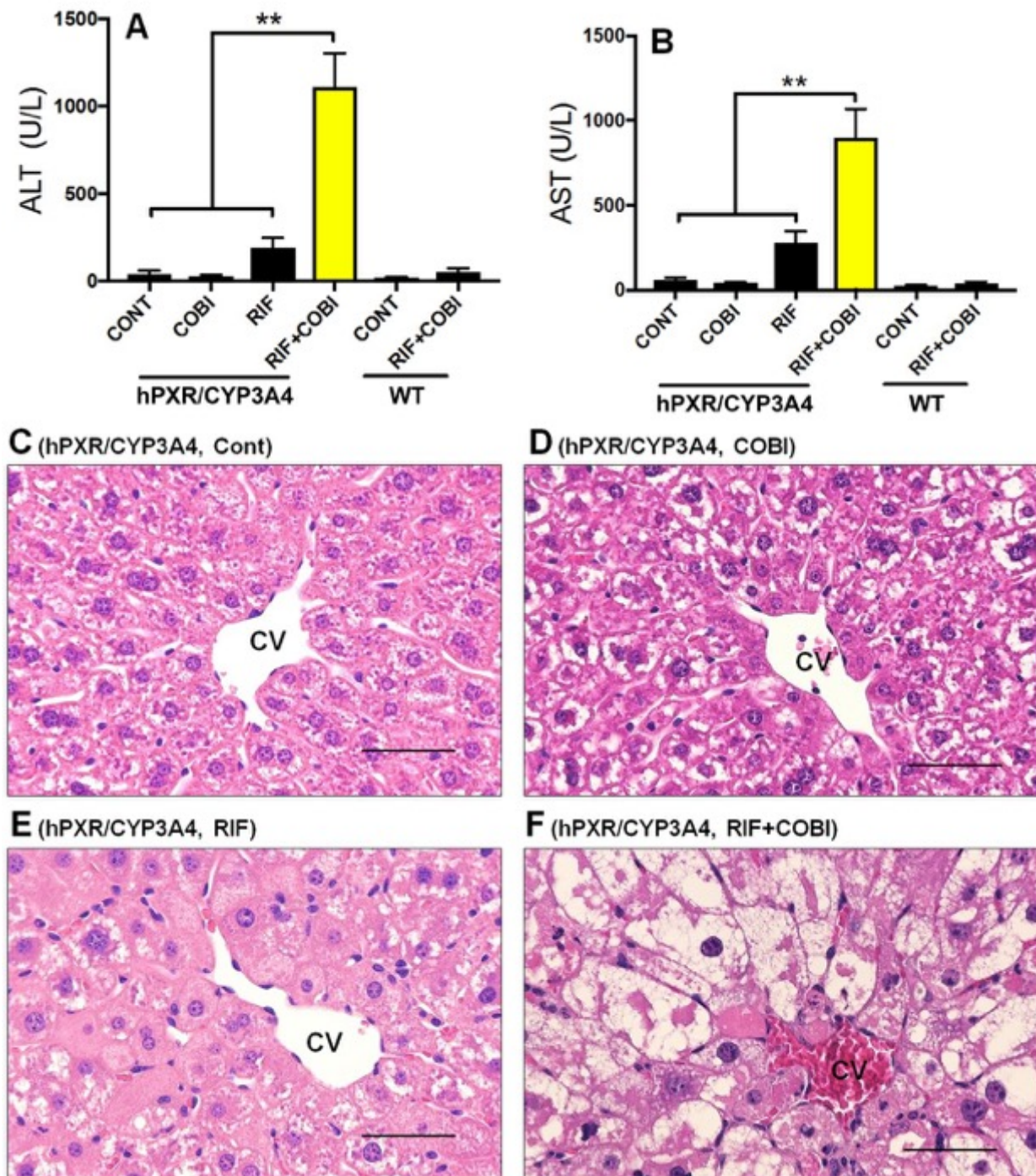


Figure 19. Lead-in treatment with RIF followed by COBI causes hepatotoxicity in hPXR/CYP3A4 mice, but not in WT mice.

WT and hPXR/CYP3A4 mice were pretreated with RIF for seven days followed by COBI for one day. (A, B) Serum activities of alanine transaminase (ALT) and aspartate transaminase (AST) in hPXR/CYP3A4 and WT mice. All data are expressed as means \pm SEM (n = 3-7). Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. $**P < 0.01$ for RIF+COBI group vs control, COBI and RIF groups in hPXR/CYP3A4 mice. (C-F) Histological analysis of liver samples from control, COBI, RIF and RIF+COBI groups of hPXR/CYP3A4 mice. Hepatocyte degeneration (*) and cell death (^) were observed in RIF+COBI group of hPXR/CYP3A4 mice. H&E staining. CV, central vein. Scale bars: 50 μ m.

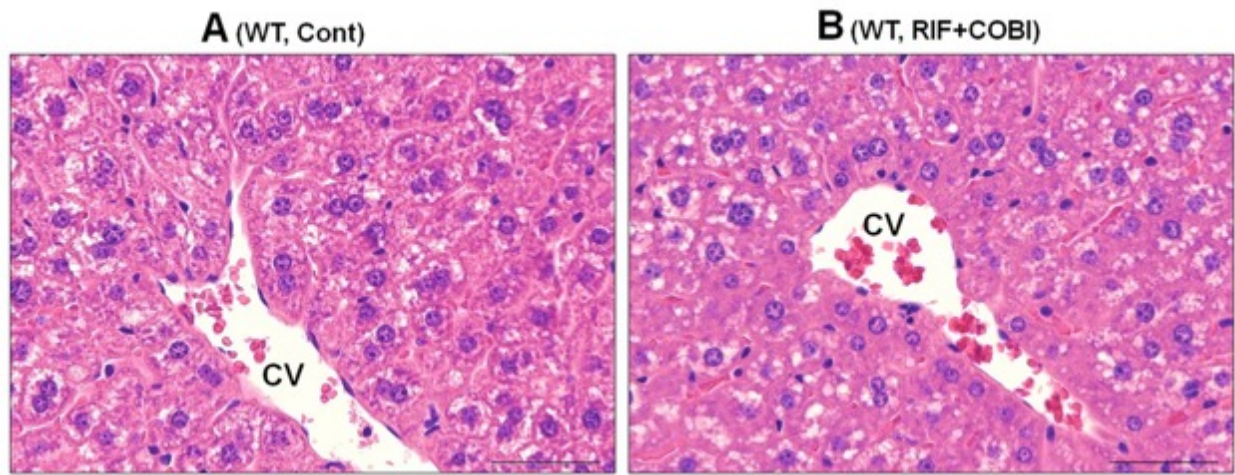


Figure 20. Histological analysis of livers from WT mice.

control (A) and RIF+COBI (B) groups. The mice were pretreated with RIF for seven days followed by COBI for one day. H&E staining. CV, central vein. Scale bars: 50 μ m.

Lead-in treatment with RFB does not potentiate COBI hepatotoxicity in hPXR/CYP3A4 mice

According to the WHO guidelines for treatment of HIV and TB co-infection, it is recommended that TB therapy should be started 2-8 weeks before starting anti-retroviral therapy (198) (Figure 21A). RFB belongs to the same class of drugs as RIF and typically used in subjects co-infected with HIV and TB (198, 200). We therefore asked whether lead-in treatment with RFB potentiates COBI hepatotoxicity (Figure 21A). The effect of RFB on PXR activation was compared to RIF in hPXR/CYP3A4 mice using CYP3A4 as a marker because it is the primary target gene of PXR (48-51). As expected, RIF-mediated PXR activation strongly induced CYP3A4 expression in the liver of hPXR/CYP3A4 mice, but RFB had a very weak effect on CYP3A4 induction (Figure 21B), indicating that RFB is a weak PXR activator, which agreed with previous reports (201, 202). In hPXR/CYP3A4 mice pretreated with RFB followed by COBI, no liver injury was observed (Figure 21C and D), indicating that RFB is safe for lead-in treatment in HIV and TB co-infected subjects receiving COBI-containing regimens. These data also suggest that the extent of PXR activation plays an important role in modulating COBI hepatotoxicity.

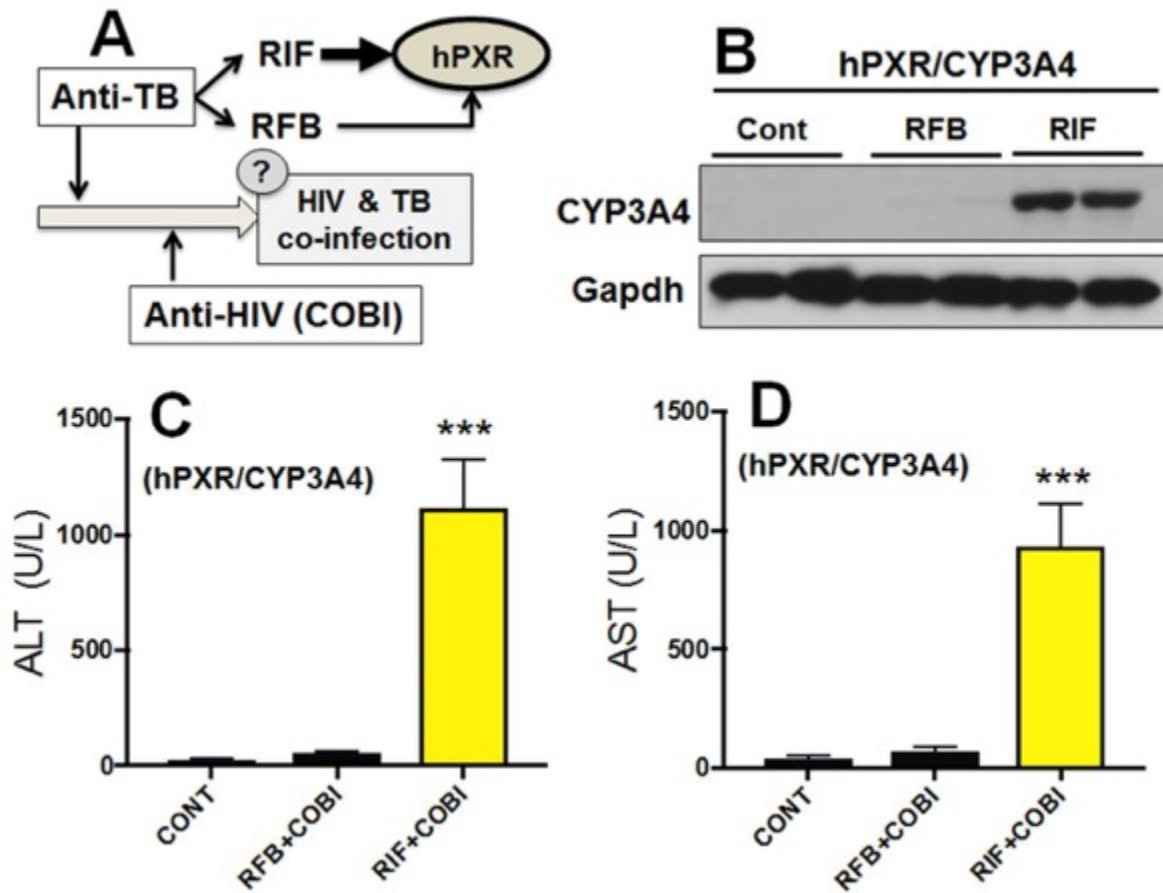


Figure 21. Lead-in treatment with RFB followed by COBI does not cause hepatotoxicity in hPXR/CYP3A4 mice.

(A) A scheme showing the WHO guideline on the treatment of HIV and tuberculosis (TB) co-infections, in which lead-in treatment with anti-TB drugs is suggested. Both RIF and RFB are commonly used anti-TB drugs, but RFB has less effect on PXR activation. We asked whether lead-in treatment with RFB will potentiate COBI hepatotoxicity. (B) Effects of RIF and RFB on PXR-mediated CYP3A4 induction in the liver of hPXR/CYP3A4 mice. The mice were pretreated with RIF or RFB for 7 days. Expression of CYP3A4 was analyzed by Western blotting. Gapdh was used as a loading control. (C, D) Serum activities of ALT and AST in hPXR/CYP3A4 mice pretreated with RIF or RFB followed by COBI. All data are shown as mean \pm SEM (n = 3-7). Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. *** $P < 0.001$.

Loss-of-function of PXR abolishes the hepatotoxicity caused by lead-in treatment with RIF followed by COBI

To further determine the role of PXR in the hepatotoxicity caused by lead-in treatment with RIF followed by COBI, we developed a mouse model that expresses CYP3A4 but is deficient in Pxr (Pxr-null/CYP3A4) (Figure 22A). As expected, treatment with RIF in Pxr-null/CYP3A4 mice did not induce CYP3A4 expression indicating that PXR is not functional in these mice (Figure 22B). Compared to hPXR/CYP3A4 mice, lead-in treatment with RIF followed by COBI did not cause liver injury in Pxr-null/CYP3A4 mice (Figure 22 C, D, and Figure 23). These data confirm that human PXR is essential in the hepatotoxicity caused by lead-in treatment with RIF followed by COBI. These data also suggest that lead-in treatment with PXR activators is a risk factor for COBI hepatotoxicity.

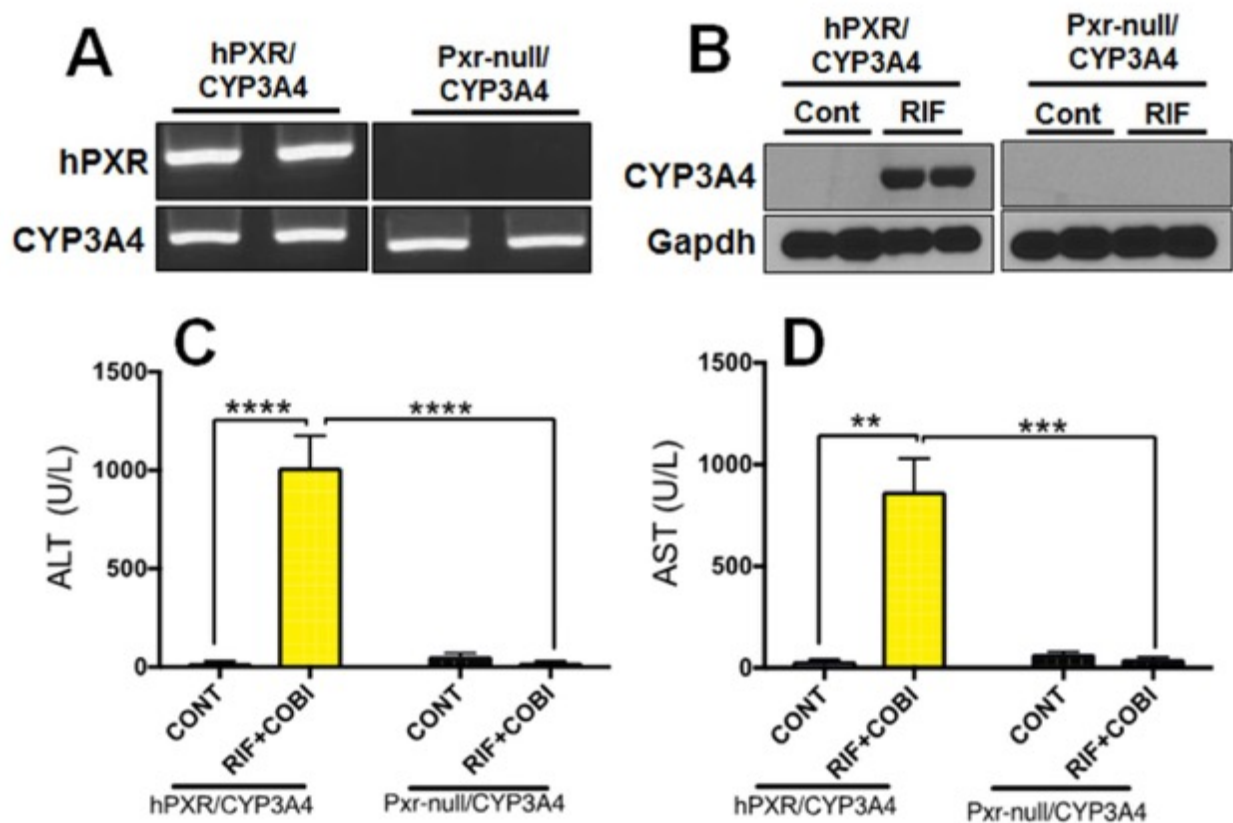


Figure 22. Role of PXR in the hepatotoxicity caused by pretreatment with RIF followed by COBI.

(A) Genotyping results of hPXR/CYP3A4 and Pxr-null/CYP3A4 mice. Pxr-null/CYP3A4 mice are positive for human CYP3A4 but negative for PXR. (B) Expression of CYP3A4 in the liver of hPXR/CYP3A4 and Pxr-null/CYP3A4 mice pretreated with or without RIF for 7 days. Gapdh was used as a loading control. (C, D) Serum activities of ALT and AST in hPXR/CYP3A4 and Pxr-null/CYP3A4 mice pretreated with RIF followed by COBI. All data are shown as mean \pm SEM (n = 3-7). Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

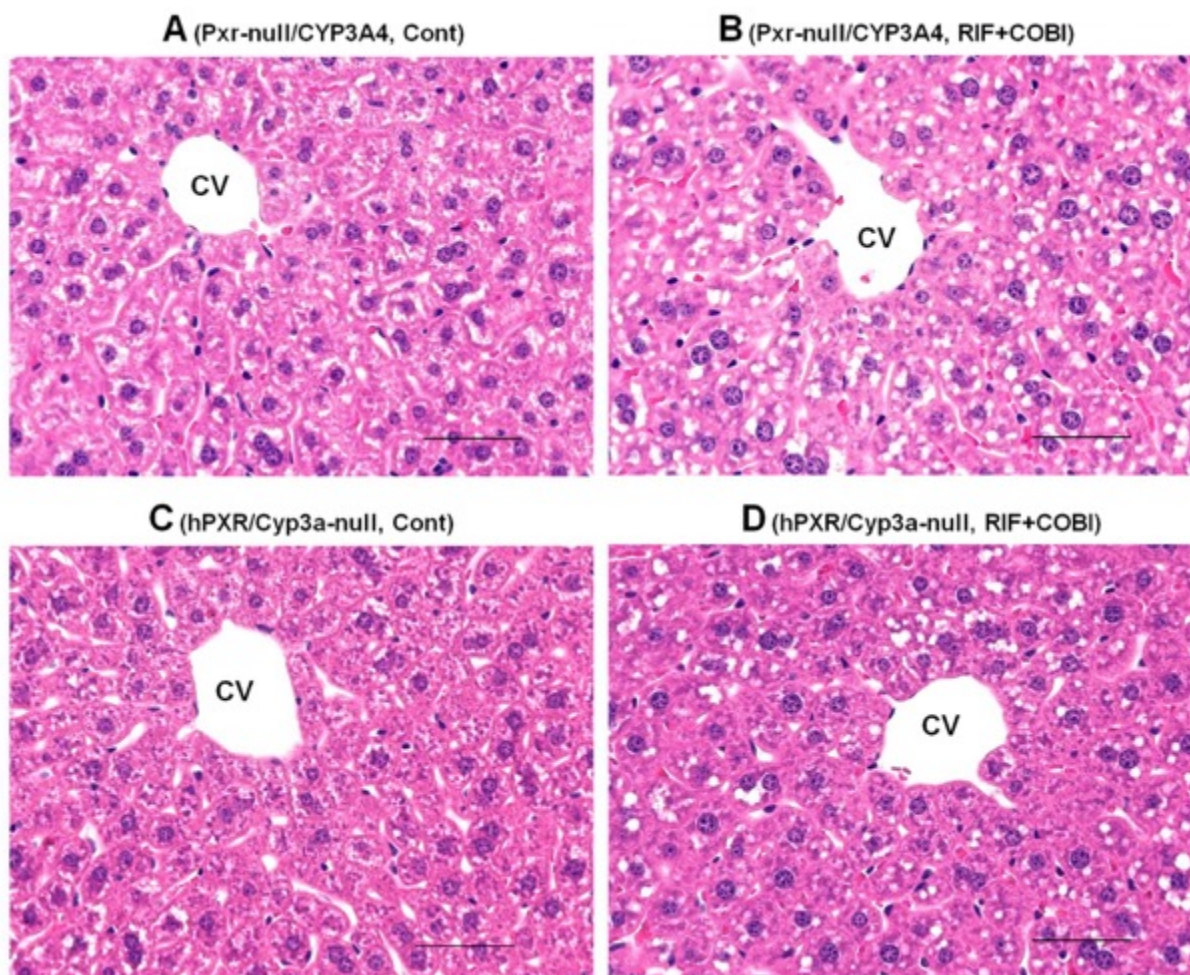


Figure 23. Histological analysis of livers from Pxr-null/CYP3A4 and hPXR/Cyp3a-null mice.

Control (A, C) and RIF+COBI (B, D) groups. The mice were pretreated with RIF for seven days followed by COBI for one day. H&E staining. CV, central vein. Scale bars: 50 μ m.

Loss-of-function of CYP3A4 abolishes the hepatotoxicity caused by lead-in treatment with RIF followed by COBI

CYP3A4 is a PXR target gene and it plays an important role in COBI metabolism and bioactivation (48-51, 203). Therefore, we hypothesized that RIF as a human PXR activator modulates COBI hepatotoxicity through CYP3A4-dependent pathways. We utilized a PXR-humanized mouse model with Cyp3a deficiency (hPXR/Cyp3a-null) to test this hypothesis. Compared to hPXR/CYP3A4 mice, liver injury was not observed in hPXR/Cyp3a-null mice pretreated with RIF followed by COBI (Figure 23 and Figure 24A, B). These results suggest that CYP3A4 is critical in the liver injury caused by lead-in treatment with RIF followed by COBI.

Activation of CAR re-sensitizes Pxr-null/CYP3A4 mice to COBI hepatotoxicity

No liver injury was observed in Pxr-null/CYP3A4 mice pretreated with RIF followed by COBI because RIF-mediated PXR activation and CYP3A4 induction were abrogated (Figure 22B-D). To further determine the role of CYP3A4 in COBI hepatotoxicity, Pxr-null/CYP3A4 mice were pretreated with 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), a potent activator of CAR that up-regulates CYP3A4 expression (204, 205). As expected, TCBOPOB significantly induced CYP3A4 expression in the liver of Pxr-null/CYP3A4 mice (Figure 24C). In addition, lead-in treatment with TCPOBOP followed by COBI caused a significant elevation of serum ALT and AST in Pxr-null/CYP3A4 mice (Figure 24D and E). Furthermore, severe hepatocyte degeneration and cell death were observed in the livers of Pxr-null/CYP3A4 mice pretreated with TCPOBOP followed by COBI (Figure 24F and G), which are similar to the phenotypes observed in the livers of hPXR/CYP3A4 mice pretreated with RIF followed by COBI (Figure 19F). These

results further confirmed the importance of CYP3A4 in COBI hepatotoxicity. These data also indicate that lead-in treatment with CAR activators is a risk factor for COBI hepatotoxicity.

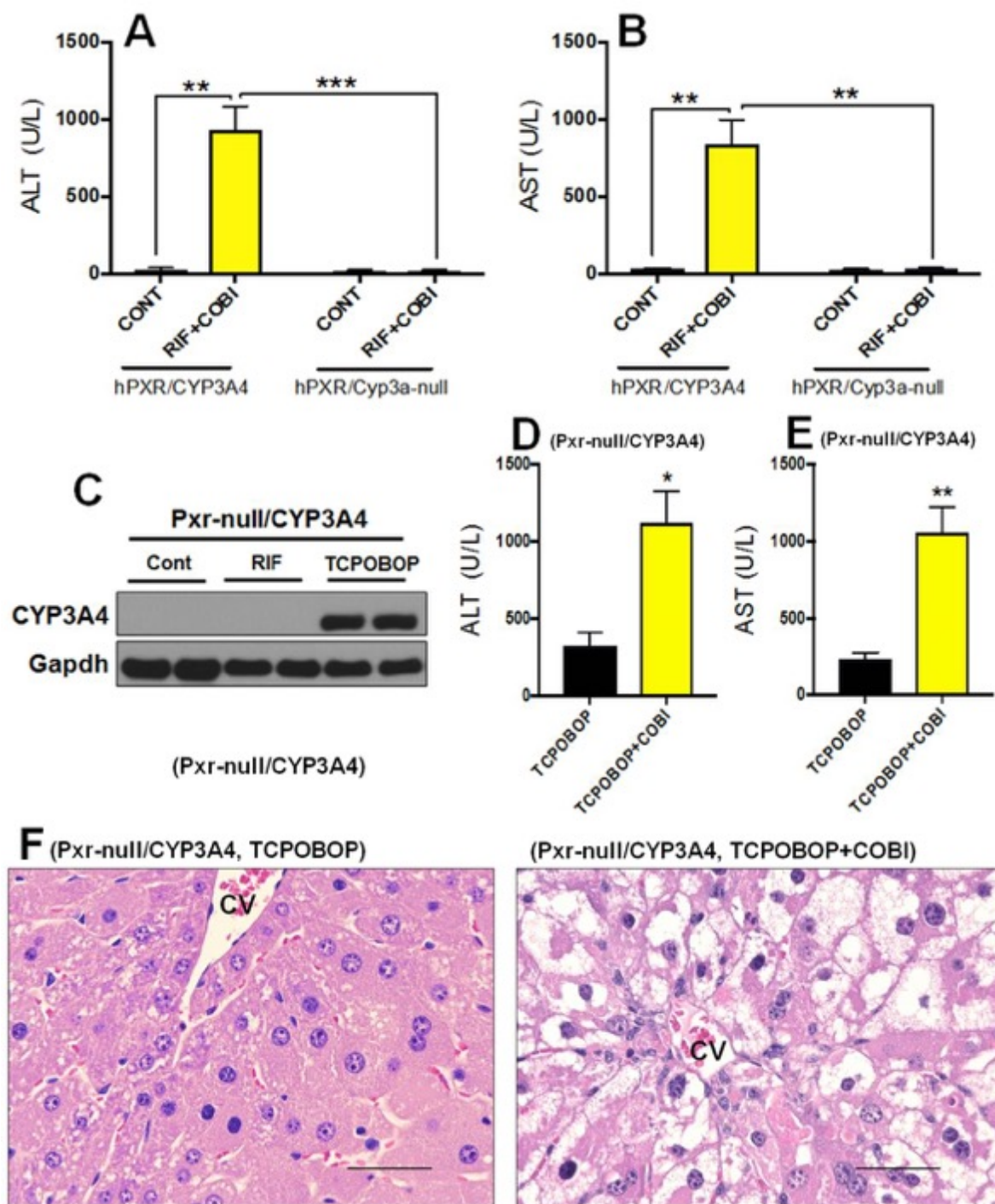


Figure 24. Role of CYP3A4 in the hepatotoxicity caused by pretreatment with RIF followed by COBI.

(A, B) Serum activities of ALT and AST in hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI. (C) CYP3A4 expression in the liver of Pxr-null/CYP3A4 mice pretreated with RIF or

TCPOBOP. TCPOBOP is a CYP3A4 inducer through the activation of the nuclear receptor CAR, but not PXR.

Gapdh was used as a loading control. (*D, E*) Serum activities of ALT and AST in Pxr-null/CYP3A4 mice pretreated with TCPOBOP for three days followed by COBI for one day. All data are shown as mean \pm SEM (n = 3-7).

Statistical significance was determined by two tailed unpaired t-test or two-way ANOVA with Tukey's post hoc test.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (*F, G*) Histological analysis of liver samples from Pxr-null/CYP3A4 mice treated with TCPOBOP and TCPOBOP+COBI. Hepatocyte degeneration (*) and cell death (^) were observed in TCPOBOP+COBI group of Pxr-null/CYP3A4 mice. CV: central vein. Scale bars: 50 μ m.

Lead-in treatment with RIF increases COBI metabolism and bioactivation in the liver of hPXR/CYP3A4 mice

COBI undergoes metabolism and bioactivation through CYP3A4-dependent pathways (203). M2 and M9 are the major metabolites of COBI (Figure 25A). The byproduct through the M2 pathway of COBI is an N-acetyl cysteine conjugated metabolite, suggesting the formation of a reactive intermediate that interacts with glutathione, an important antioxidant in cells (203). In addition, the M9 pathway of COBI produces an unstable metabolite 2-isopropylthiazole-4-carbaldehyde (Figure 25A). Furthermore, M18 and M19 are thiazole ring-open metabolites of COBI (Figure 25A), which can undergo oxidation and cause oxidative stress (181, 203). We observed dramatic increases in productions of M2, M9, M18, and M19 in the liver microsomes of hPXR/CYP3A4 mice pretreated with RIF, but not in Pxr-null/CYP3A4 or hPXR/Cyp3a-null mice (Figure 25B-E), indicating that lead-in treatment with RIF increases COBI metabolism and bioactivation through PXR- and CYP3A4-dependent pathways.

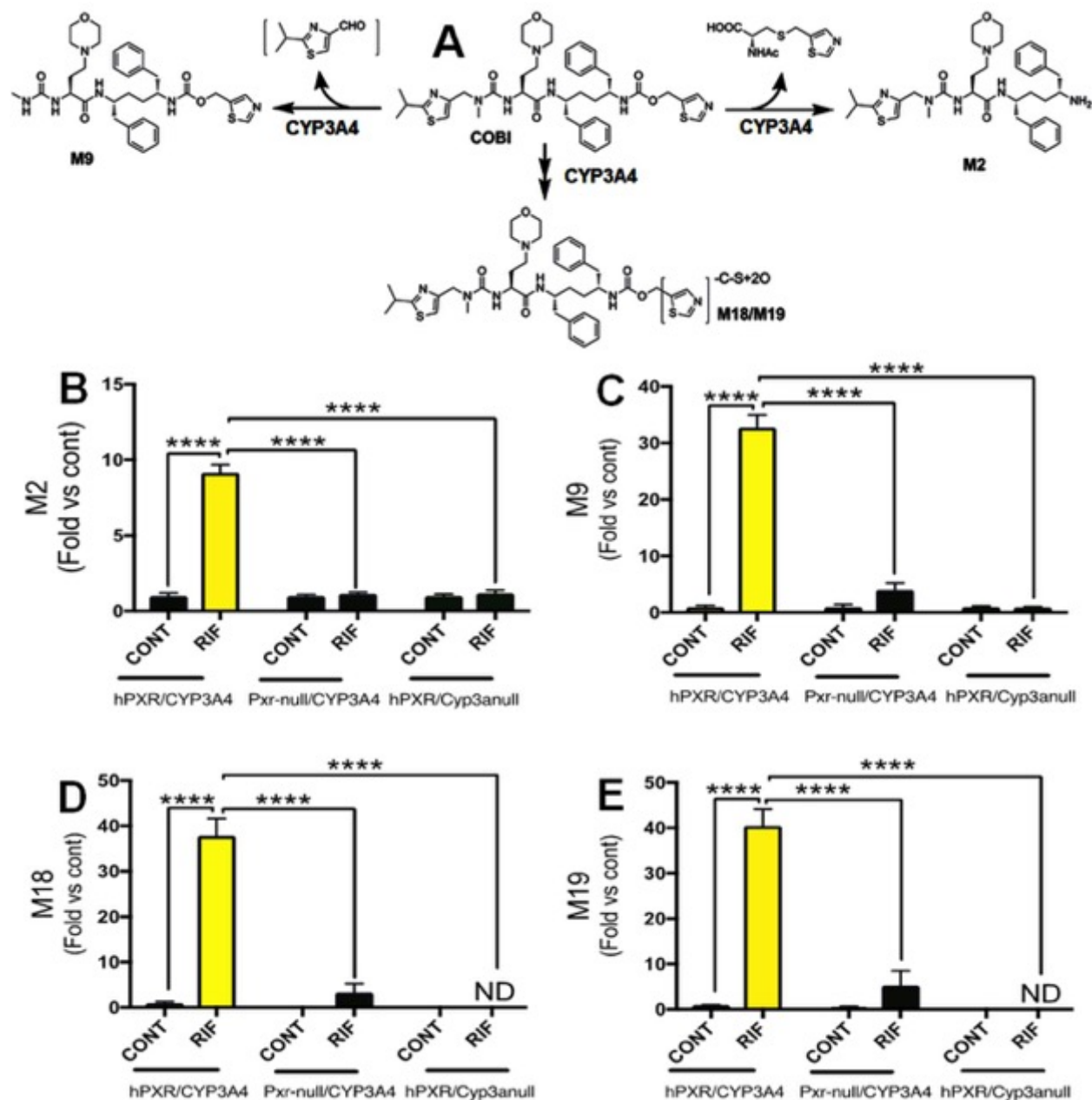


Figure 25. PXR-mediated CYP3A4 induction increases COBI metabolism and bioactivation.

(A) A scheme showing the roles of CYP3A4 in the metabolic pathways M2, M9, M18 and M19 of COBI. (B-E) The abundance of M2, M9, M18 and M19 produced in liver microsomes of hPXR/CYP3A4, Pxr-null/CYP3A4 and hPXR/Cyp3a-null mice pretreated with or without RIF. COBI metabolites were analyzed by UPLC-qTOFMS. All data are expressed as means \pm SEM (n = 3-4). Data in control groups are set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. **** $P < 0.0001$. ND, not detected.

Lead-in treatment with RIF followed by COBI caused oxidative stress in the liver of hPXR/CYP3A4 mice

Metabolic activation of drugs by CYPs can lead to oxidative stress (206). Indeed, we observed a substantial up-regulation of antioxidant responsive genes including glutathione peroxidase (Gpx), carbonyl reductase 3 (Cbr3), and solute carrier family 7 member 11 (Slc7a11) and family 25 member 24 (Slc25a24) in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI, but not in Pxr-null/CYP3A4 or hPXR/Cyp3a-null mice with the same treatment (Figure 26A-C, and Figure 27). In addition, we observed a high level of ophthalmic acid (OA), a biomarker of oxidative stress (184), in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI (Figure 28A). These results indicate that lead-in treatment with RIF followed by COBI causes oxidative stress in the liver of hPXR/CYP3A4 mice. To further determine the role of oxidative stress in COBI hepatotoxicity, hPXR/CYP3A4 mice were pretreated with an antioxidant, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic imidazolid (CDDO-Im), which significantly decreased oxidative stress in the liver and protected against the hepatotoxicity in hPXR/CYP3A4 mice pretreated with RIF followed by COBI (Figure 28B-D). These results indicate that oxidative stress plays an important role in the hepatotoxicity caused by lead-in treatment with RIF followed by COBI.

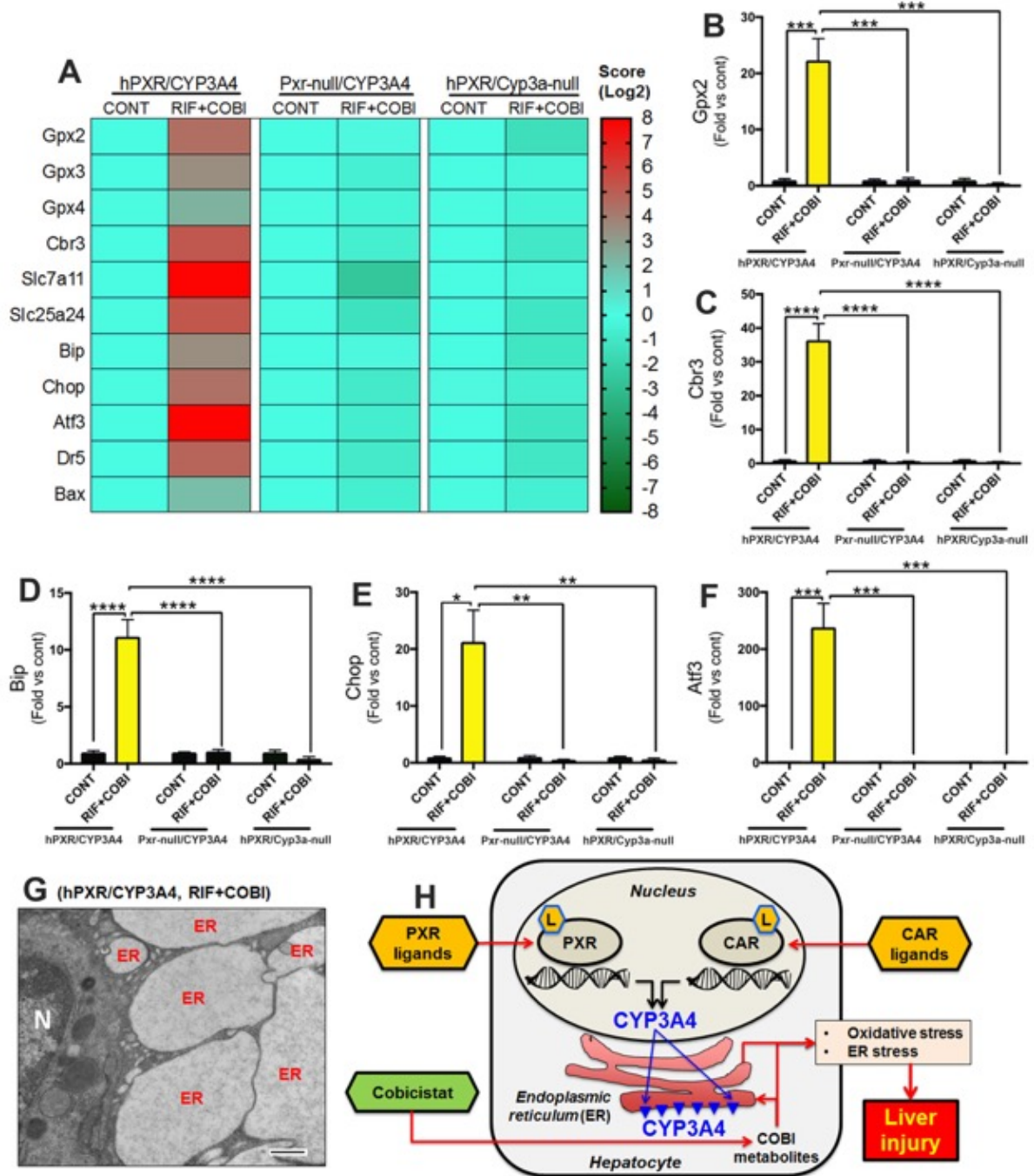


Figure 26. CYP3A4-dependent oxidative stress and ER stress in the hepatotoxicity caused by pretreatment with RIF followed by COBI.

(A) A heatmap of genes related to oxidative stress, ER stress, and cell death in the liver of hPXR/CYP3A4, Pxr-null/CYP3A4, and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI. Fold change values were log 2 transformed. (B, C) Expression of genes associated with oxidative stress. (D-F) Expression of genes associated with

ER stress. mRNAs were analyzed by qPCR. All data are expressed as means \pm SEM (n = 3-7). Data in control group are set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (G) A TEM image showing ER dilation in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI. N, nucleus. (H) A scheme showing the role of CYP3A4 in COBI hepatotoxicity. Ligand-dependent activation of PXR and CAR leads to CYP3A4 induction in hepatocytes located in the ER. Overexpressed CYP3A4 in the ER increases the metabolism and bioactivation of COBI to form reactive metabolites, which can directly target ER leading to oxidative stress, ER stress, and hepatocellular injury.

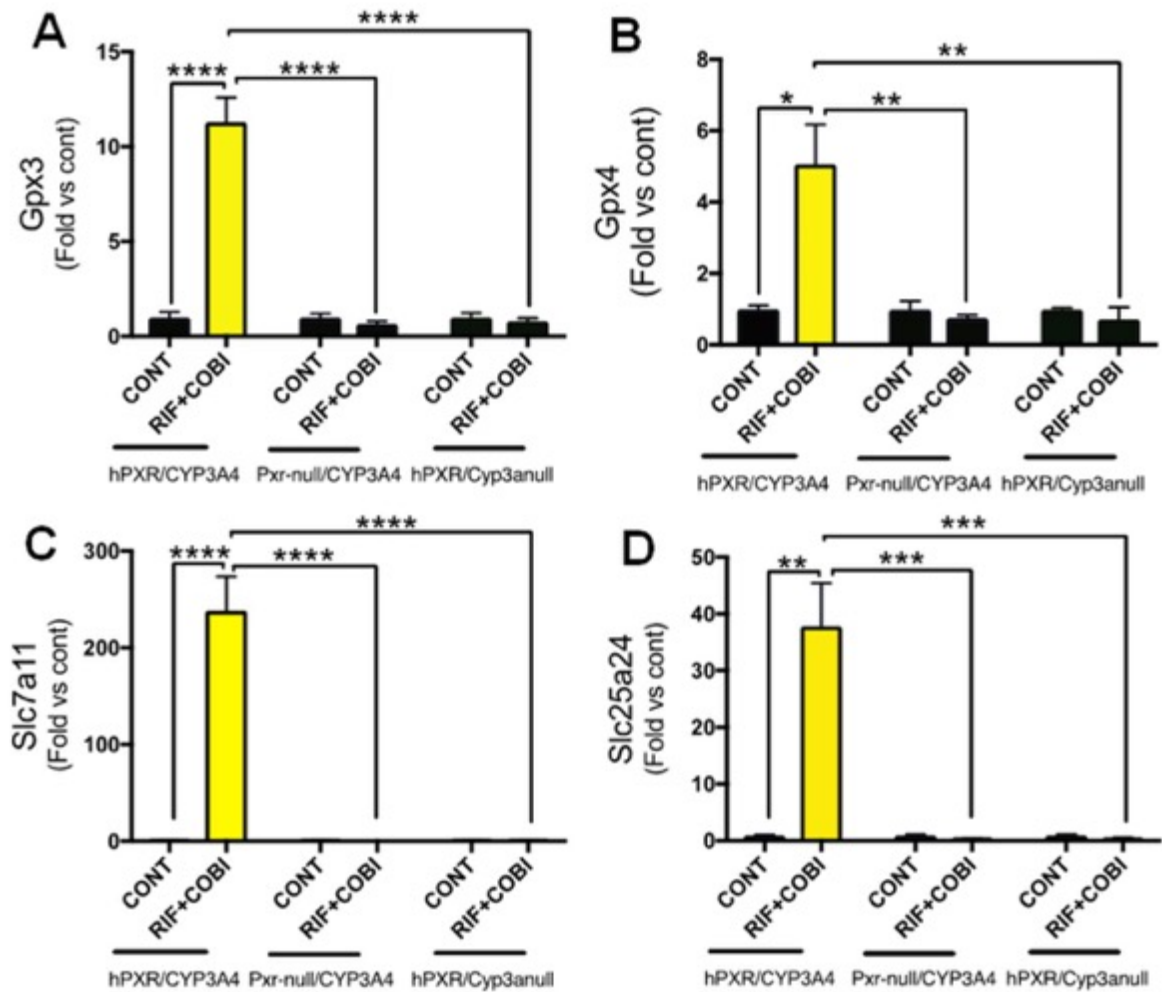


Figure 27. The expressions of genes related to oxidative stress in the liver of hPXR/CYP3A4, Pxr-null/CYP3A4, and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI.

Gpx3 (A), Gpx4 (B), Slc7a11 (C), and Slc25a24 (D) mRNAs were analyzed by qPCR. All data are expressed as means \pm SEM (n = 3-7). Data in control group are set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

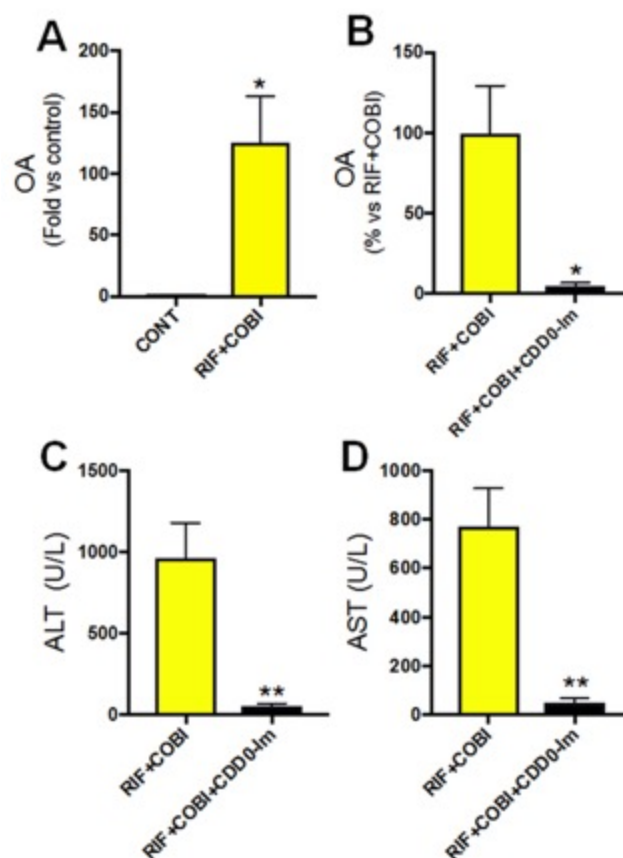


Figure 28. The effects of antioxidant CDDO-Im on the liver injury in hPXR/CYP3A4 mice pretreated with RIF followed by COBI.

(A) The levels of ophthalmic acid (OA), a biomarker of oxidative stress, in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI. (B) The effects of CDDO-Im on OA levels in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI. OA in the liver was extracted and analyzed by UPLC-qTOFMS. (C, D) The effects of CDDO-Im on the liver injury in hPXR/CYP3A4 mice pretreated with RIF followed by COBI. Serum activities of ALT (C) and AST (D) were analyzed by a biochemical approach. All data are expressed as means \pm SEM (n = 3-7). Statistical significance was determined by the two-tailed Student's t-test. * P < 0.05, ** P < 0.01.

Lead-in treatment with RIF followed by COBI caused endoplasmic reticulum (ER) stress in the liver of hPXR/CYP3A4 mice

Severe oxidative stress can lead to ER stress and cell death (185). We observed a notable increase in ER stress biomarkers including C/EBP homologous protein (Chop), binding immunoglobulin protein (Bip), and cyclic AMP-dependent transcription factor 3 (Atf3) in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI, but not in the Pxr-null/CYP3A4 and hPXR/Cyp3a-null mice with the same treatment (Figure 26A, D-F). Further analysis using transmission electron microscopy (TEM) provided additional evidence of ER stress, as remarkable ER dilation was observed in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI (Figure 26G). Prolonged ER stress causes cell death 35. Indeed, the expressions of genes associated with cell death including death receptor 5 (Dr5) and BCL2-associated X (Bax) were significantly increased in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by COBI, but not the liver of Pxr-null/CYP3A4 or the hPXR/Cyp3a-null mice (Figure S5). In addition, lead-in treatment with CAR activator TCPOBOP followed by COBI also led to an upregulation of genes associated with ER stress and cell death (Figure S6). These data suggest that the ER is a target organelle in the hepatotoxicity caused by pretreatment with PXR and CAR activators followed by COBI (Figure 26H).

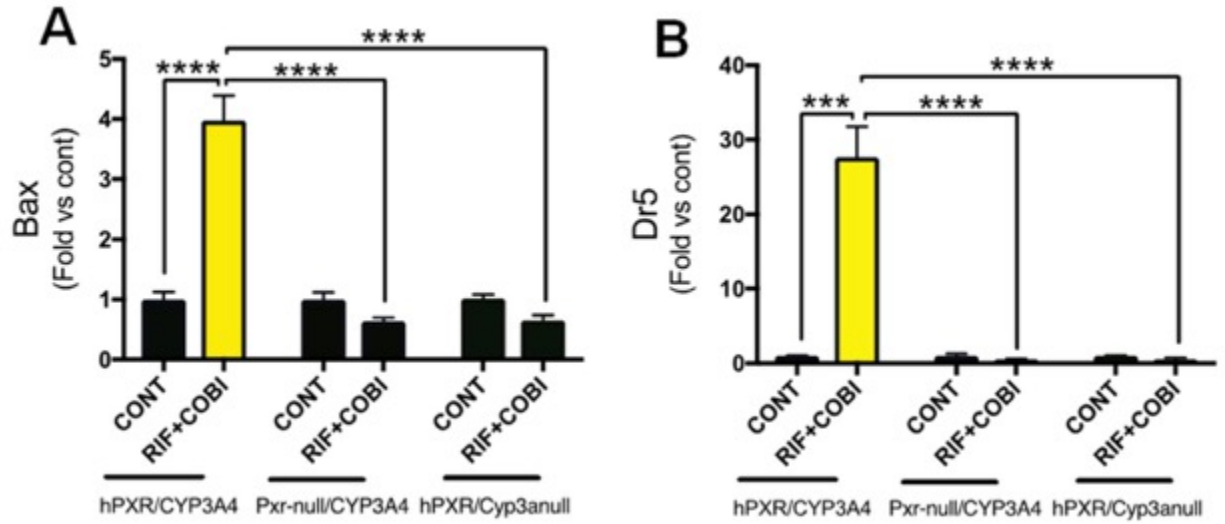


Figure 29. The expressions of genes related to cell death in the liver of hPXR/CYP3A4, Pxr-null/CYP3A4, and hPXR/Cyp3a-null mice pretreated with RIF followed by COBI.

Bax (A) and *Dr5* (B) mRNAs were analyzed by qPCR. All data are expressed as means \pm SEM (n = 3-7). Data in control group are set as 1. Statistical significance was determined by two-way ANOVA with Tukey's post hoc test.

*** $P < 0.001$, **** $P < 0.0001$.

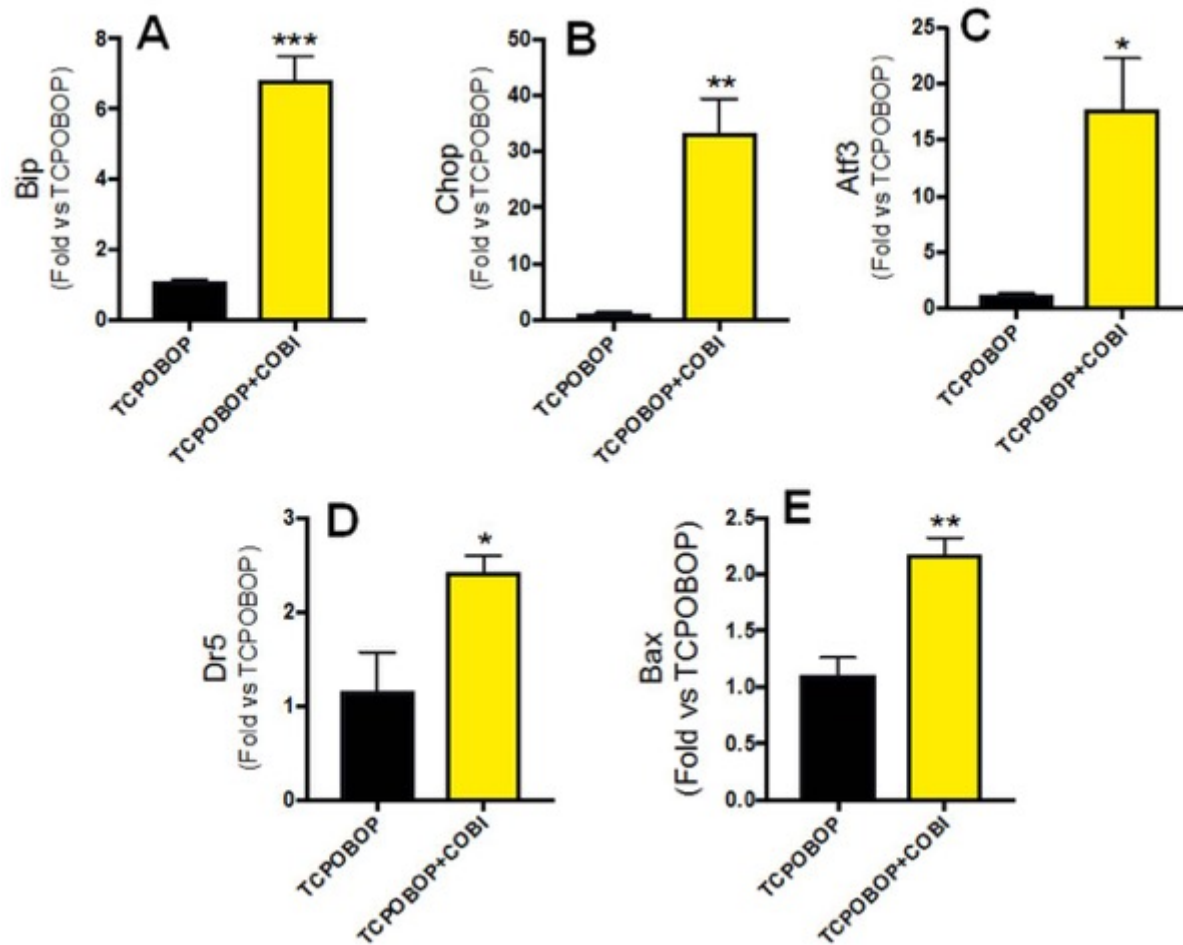


Figure 30. The expressions of genes related to ER stress and cell death in the liver of Pxr-null/CYP3A4 mice pretreated with TCPOBOP followed by COBI.

mRNA expressions of *Bip* (A), *Chop* (B), *Atf3* (C), *Bax* (D), and *Dr5* (E) were analyzed by qPCR. All data are expressed as means \pm SEM (n = 4-6). Data in TCPOBOP group are set as 1. Statistical significance was determined by the two-tailed Student's t-test. * P < 0.05, ** P < 0.01, *** P < 0.001.

3.4 DISCUSSION

Adverse drug interactions are concerns in the HIV/AIDS field because of the use of multiple drug classes in HIV therapy and the use of drugs for the treatment of pre-existing diseases and/or opportunistic infections like TB (207, 208). Therefore, understanding the mechanisms of drug-drug interactions associated with HIV drugs will help to reduce the risk of side effects and increase medication adherence in the clinic. Using genetically engineered mouse models, we demonstrated that lead-in treatment with CYP3A4 inducers potentiates COBI hepatotoxicity. Specifically, ligand-dependent activation of PXR and CAR up-regulates CYP3A4 expression in the ER, which increases the production of reactive metabolites of COBI and in turn causes oxidative stress, ER stress, and hepatocellular injury (Figure 6H).

We found that lead-in treatment with RIF potentiates COBI hepatotoxicity in PXR-humanized mice, but not in WT mice, suggesting that human PXR is a key modulator of COBI hepatotoxicity. In addition, we developed and used a PXR deficient mouse model to prove that the hepatotoxicity caused by lead-in treatment with RIF followed by COBI is dependent on PXR. Consistent with these data, we found that lead-in treatment with RFB, a weak PXR ligand, does not cause COBI hepatotoxicity. Both RIF and RFB are commonly used drugs in TB therapy (197, 198). As WHO guideline recommends lead-in treatment with anti-TB drugs followed by anti-HIV therapy in subjects co-infected with TB and HIV, our work suggests that RFB is safer than RIF in combination with COBI-containing regimens.

We next demonstrated that RIF-mediated PXR activation potentiates COBI hepatotoxicity through CYP3A4-dependent pathways, as COBI hepatotoxicity was abolished in hPXR/Cyp3a-null mice.

CYP3A4 is one of the most important enzymes in drug metabolism (67). Ligand-dependent PXR activation induces CYP3A4 and therefore leads to many drug-drug interactions and adverse drug reactions (49, 154). In addition to PXR, the nuclear receptor CAR also up-regulates CYP3A4 expression (209). Here we showed that CAR activation induces CYP3A4 and potentiates COBI hepatotoxicity in a CYP3A4 transgenic mouse model with Pxr deficiency, indicating the vital role of CYP3A4 in COBI hepatotoxicity. These findings are important for the safe use of COBI in the clinic because many prescription drugs, herbs, and dietary supplements in daily life can activate PXR and/or CAR and induce CYP3A4 (210).

3.5 CONCLUSION

In conclusion, our work illustrated the crucial role of CYP3A4 in modulating COBI hepatotoxicity. Our findings can be applied in the clinic to guide the safe use of COBI-containing regimens by avoiding lead-in treatment with PXR and CAR activators that up-regulates CYP3A4 expression.

4.0 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

HIV protease inhibitors (PIs) are an important class of anti-HIV drugs that interrupt the lifecycle of the virus by inhibiting its protease enzyme. However, this class of drugs were plagued by poor bioavailability and high pill burden (211). PIs are CYP3A4 substrates, therefore intentional inhibition of CYP3A4 was seen as a strategy to improve their bioavailability, boost systemic exposure, reduce the frequency of dosing and improve adherence to therapy. This strategy led to the basis of using pharmacoenhancers RTV and COBI in HIV therapy (211). RTV/COBI are mechanism-based inhibitors of CYP3A4 that impede the metabolism and clearance of PIs (167, 187, 188). The concept of mechanism-based inhibition (MBI) (also known as suicide inhibition) is when a substrate drug is metabolized by CYP3A4 to produce reactive metabolites that come back to inhibit the enzyme by either (i) forming a covalent bond with the protein (ii) quasi-irreversibly bind to the prosthetic heme of the enzyme or (iii) alkylation of the heme in the active site of the enzyme (212). Some common features of CYP3A4 MBIs include time and concentration dependent inhibition, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (co-factor required for CYP450 reactions)-dependent inhibition and irreversible inhibition. Because CYP3A4 enzyme is rendered non-functional by MBIs, a new CYP3A4 protein has to be synthesized (213). CYP3A4 is the most abundant CYP isoenzyme responsible for the metabolism of >60% of clinically used drugs, therefore MBI of CYP3A4 can lead to drug-drug interactions and sometimes adverse drug reactions (67). Therefore, my research was focused on understanding adverse drug interactions associated with RTV/COBI that can lead to hepatotoxicity.

The basis of my research were clinical studies in humans that reported that lead in treatment with RIF (a first-line anti-TB drug and potent PXR activator) followed by RTV led to hepatotoxicity (160-163). My research had the unique advantage of using humanized PXR and CYP3A4 mouse models to recapture the phenotype observed in human studies. This is important because the use of wild type mice was not able to recapture the phenotype due to the species differences in PXR activation in response to RIF (48, 50, 51, 63). In addition, our results highlight the importance of human CYP3A4 in modulating RTV and COBI hepatotoxicity over mouse Cyp3a11. Taken together, our findings show the importance of using humanized mouse models over wildtype mice for drug-drug interaction studies at the preclinical stage. Therefore, the animal models generated in this research hPXR/CYP3A4, hPXR/Cyp3a-null and Pxr-null/CYP3A4 can be used in preclinical studies to screen drugs that are PXR ligands and CYP3A4 inducers as well as study drug-drug interactions.

The findings from my research provided novel insights into the critical role of PXR in modulating RTV and COBI hepatotoxicity. The results show that the timeline and sequence of dosing PXR ligands and CYP3A4 inducers matters before starting RTV/COBI containing antiretroviral regimens. One clinical implication of this finding is in the treatment of TB and HIV co-infection where TB treatment requires the use of a rifamycin like RIF or RFB (197). The WHO guidelines recommend initiating TB therapy first for 2-8 weeks before starting HIV therapy in HIV treatment naïve patients (198). Based on this recommendation, our results support the clinical recommendation of not using RIF with RTV/COBI containing regimens. In addition, in situations where a PI boosted regimen is considered after starting TB therapy, our work provides evidence that RFB, a weak PXR ligand is a safer alternative to RIF, which is line with the current guidelines.

For future studies, the development and use of PXR/CAR antagonist can be used as a strategy to prevent RTV/COBI hepatotoxicity in circumstances where the use of PXR/CAR ligands that induce CYP3A4 is inevitable (214).

The use of the hPXR/Cyp3a-null mouse model established the vital role of CYP3A4 in modulating RTV/COBI hepatotoxicity. Our results showed that increased CYP3A4 induction by RIF led to an increase in reactive metabolites that were produced as a result of increased RTV/COBI bioactivation. However, we were not able to specifically identify which of the metabolites (RTV- (M1, M12, M13, and M17); COBI (M2, M9, M18 and M19) and how the metabolites contributed to RTV/COBI hepatotoxicity. In addition, since RTV/COBI are both CYP3A4 MBIs, it not clear which reactive metabolite(s) is responsible for the inhibition of CYP3A4 and whether that/those metabolite(s) are associated with toxicity (215). Therefore, future studies should investigate which metabolite(s) are responsible for contributing to RTV/COBI hepatotoxicity.

To further understand the pathways that are involved in lead-in treatment with RIF followed by RTV/COBI induced hepatotoxicity, we conducted metabolomic studies to understand changes at the functional level and gene expression studies to understand how changes in gene expression might be affecting function. In the metabolomics study, ophthalmic acid (OA) was identified as one of the metabolites that was highly upregulated in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by RTV/COBI. OA is a tripeptide analog of glutathione and reported to be a biomarker of oxidative stress, as it indirectly correlates with glutathione utilization in the liver (184). Consistent with this finding, gene expression studies showed an upregulation of

genes that are involved in glutathione synthesis (*Slc7a11*) and glutathione utilization (*Gpx2*, *Gpx3*, *Gpx4*). Hence, there was a correlation between pathway changes observed at the mRNA level and metabolites observed at the functional level. However, we did not conduct an in-depth analysis of the liver metabolome in hPXR/CYP3A4 mice pretreated with RIF followed by RTV/COBI. Future studies should explore in-depth pathway analysis using metabolomics as this may provide more information about other pathways that might be involved in RTV/COBI hepatotoxicity.

Consistent with the metabolomics findings above, oxidative stress and ER stress were observed as potential mechanisms that are involved in RTV/COBI hepatotoxicity. Indeed, oxidative stress and ER stress have been reported to promote the pathophysiology of HIV infection as well as the pathogenesis of coinfections like HCV (216, 217). This suggests that HIV patients with HCV coinfections might be at a higher risk of liver damage associated with lead-in treatment with PXR/CAR ligands followed by RTV/COBI. In addition, my research showed a marked increase in several isoforms of glutathione peroxidases (*Gpx2*, *Gpx3*, *Gpx4*) which are enzymes that catalyze the detoxification of hydrogen peroxides to water using glutathione as a cofactor (218), indicating the activation of cellular defense against oxidative stress in the liver of hPXR/CYP3A4 mice. Interestingly, these panel of genes are regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (*Nrf2*) that is a known master regulator of cellular antioxidant response (219). Furthermore, pretreatment with the antioxidant CDDO-lm (a synthetic oleanane triterpenoid that activates *Nrf2* before COBI treatment protected hPXR/CYP3A4 mice against hepatotoxicity caused by lead-in treatment with RIF followed by COBI. This finding is in line with previous studies in which CDDO-lm was shown to protect against acetaminophen and ischemia-reperfusion-induced liver injury in mice through the *Nrf2*-keap1 dependent pathway

(220-222). Currently, only one *Nrf2* activator dimethyl fumarate (DMF) has been approved by the FDA for the treatment of multiple sclerosis in 2013. However, several *Nrf2* activators including the synthetic oleanane triterpenoids are under clinical development by various pharmaceutical companies for use in disease conditions like pulmonary hypertension and kidney disease (219). Therefore, pretreatment with antioxidants like the synthetic oleanane (if approved by the FDA) can be repurposed and used as a strategy to mitigate COBI/RTV hepatotoxicity when using CYP3A4 inducers.

The biomarkers of ER stress (*Chop*, *Bip*, *Atf3*) were also significantly upregulated in the liver of hPXR/CYP3A4 mice pretreated with RIF followed by RTV/COBI. Also, a marked dilation of the ER was observed in liver TEM images of hPXR/CYP3A4 mice pretreated with RIF followed by RTV/COBI, further supporting the activation of ER stress. We speculate that the generation of RTV/COBI reactive metabolites by CYP3A4 in the ER undermines protein folding and other functions of the ER. This in turn activates ER stress to restore cellular homeostasis. However, prolonged ER stress can lead to cell death and we observed a marked increase in *Dr5* and *Bax* which are biomarkers of cell death. Since ER stress seems to be secondary to the formation of reactive metabolites and oxidative stress, the use of ER stress inhibitors might not protect against hepatotoxicity caused by lead-in treatment with RIF followed by RTV.

My research focused specifically on the role of PXR and CYP3A4 in modulating liver injury. However, RTV/COBI have high rates of gastrointestinal side effects associated with their use. Also, previous studies have reported that ER stress is an important cellular mechanism by which HIV protease inhibitors like RTV and lopinavir cause liver injury and gastrointestinal

disturbance (223, 224). PXR and CYP3A4 are both expressed in the intestine and they contribute to the metabolism of RTV in the intestine. Thus, future studies should evaluate the effect of PXR activation and CYP3A4 induction on the metabolism of RTV/COBI in the intestine and whether it is important in mediating their gastrointestinal side-effects.

A major limitation of our study includes the use of *in vivo* mouse models which are complex systems and raise the possibility of off-target effects. Also, we used healthy animals in our studies just like the clinical studies in healthy adult volunteers that showed a high incidence of hepatotoxicity. However, studies in HIV patients co-infected with tuberculosis show a variability in the incidence of hepatic adverse events but still exist. The reason for this discrepancy might be the following points; Differences in study design; Differences in the absorption and bioavailability of tuberculosis and HIV drugs in healthy volunteer's vs HIV patients; Attenuated immune response in HIV patients; small sample sizes and variability in individual responses to drugs. So, future studies using the humanized PXR and CYP3A4 animal models infected with HIV might provide more information as HIV itself might affect individual responses to drugs.

Finally, It has been reported that liver disease is one of the leading causes of death in HIV patients other than AIDs (225). In addition, hepatitis B and C (HBV/HCV) coinfection increases the severity and mortality of liver disease in HIV patients. The presence of such comorbidities and treatment of opportunistic infections like TB all increases the risk of DIH in HIV (226). In this context, our findings suggest that physicians/or pharmacist should review their patients medications carefully before starting them on RTV/COBI containing therapy especially given the wide number of clinically used drugs, dietary supplements and herbs that are PXR/CAR ligands

that can induce CYP3A4 (227). Furthermore, information gathered in the literature over the last two decades indicate that HIV-virus itself can promote liver inflammation and fibrosis through mechanisms similar to that of DIH (228). Thus, drug-drug interactions that can lead to further liver damage should try to be avoided at all cost.

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