THE ROLE OF PREGNANE X RECEPTOR IN RITONAVIR-INDUCED LIVER INJURY

by

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Ritonavir (RTV) is a HIV protease inhibitor and an important component of anti-HIV therapy. Hepatotoxicity has been reported in ~10% of patients who receive RTV-containing regimens. However, the hepatotoxicity of RTV-containing regimens was significantly enhanced in subjects who were pretreated with rifampicin (RIF), a first-line anti-tuberculosis drug. RIF is also known as a ligand of human pregnane X receptor (PXR), a transcription factor that is highly expressed in the liver and regulates drug metabolism and many cellular functions. We hypothesize that RIF-mediated PXR activation potentiates RTV hepatotoxicity. We used a transgenic mouse model that expresses human PXR and human CYP3A4 (TgCYP3A4/hPXR). A CYP3A4 transgenic mouse model on the Pxr-null background (TgCYP3A4/Pxr-null) was used as the control. We found that pretreatment with RIF sensitized the TgCYP3A4/hPXR mice to RTV hepatotoxicity, and this sensitizing effect was abolished in the TgCYP3A4/Pxr-null mice. Furthermore, we found that PXR activation increased RTV bioactivation and unfolded protein response. In summary, PXR is a key modulator of RTV induced liver injury. The results from this study can be used to guide decisions on safety considerations regarding RTV-containing regimens in clinical practice.

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PREFACE

We would like to thank Mrs. Lucy Lu for all the technical help and Dr Feng Li for the metabolomic studies.

1.0 INTRODUCTION

HIV is a major public health issue. The introduction of highly active antiretroviral therapy (HAART) to HIV treatment has significantly improved the health outcomes of HIV patients Lipsky [1]. However, adverse drug reactions (ADRs) have emerged as an important problem and barrier to achieving the goals of HIV therapy [2]. An ADR is any damaging and unwanted effect of a drug at its normal dose in humans that results from its use for diagnosis, treatment or prophylaxis of a disease [3]. ADRs are relatively common in HIV patients due to side effects of the HIV drugs themselves, the presence of opportunistic infections like tuberculosis (TB) that need treatment alongside HIV, and the use of non-prescription medications such as herbs and dietary supplements together with HIV medications [4, 5]. ADRs have been reported to be the cause of non-adherence and discontinuation of HIV medications [6-10]. Hepatotoxicity is a common ADR that occurs with different combinations of HAART [11]. Therefore it is important to understand the mechanisms of ADRs associated with HIV medications.

TB is the second deadliest infectious disease after HIV accounting for about 1.5 million deaths worldwide in 2013. Out of the 1.5 million deaths, 400,000 deaths were attributed to HIV co-infection. (http://www.who.int/tb/publications/global_report/gtbr14_main_text.pdf?ua=1). This makes TB therapy in HIV positive patients of utmost importance. However, therapy is

limited by drug-drug interactions between HIV and TB drugs as well as an increased incidence of adverse effects due to the overlapping toxicities of HIV/TB drugs. Second paragraph.

1.1 PROTEASE INHIBITORS (PIS) IN HIV THERAPY

The HIV PIs have proved effective in treatment naïve as well as treatment experienced patients. They have been reported to show an increased threshold to development of resistance, which makes them an ideal choice of treatment in patients with adherence problems [12, 13] and those that have developed resistance to other classes of HIV drugs such as non-nucleoside reverse transcriptase inhibitors [12].

Ritonavir (RTV), a first generation PI, is the pillar of PI based regimen because of its ability to boost the therapeutic concentration, decrease dosing frequency and reduce the amount required for other PIs by inhibiting their CYP3A4 mediated metabolism [14]. Nonetheless, the use of PIs has been limited by an increased risk of side effects and drug-drug interactions with drugs like RIF used for the treatment of TB and herbal drugs like the St John's wort used for the treatment of depression [15-17].

RTV has been reported to be associated with an increased risk of liver injury and gastrointestinal side effects that has led to the discontinuation of therapy by patients [18]. In a study conducted in HIV patients on PI regimens in Italy, RTV treatment accounted for about 36% of treatment discontinuation after one year of therapy [6]. Further analysis identified RTV to account for the highest number of ADRs in HIV patients on PI-based treatment [6]. Another study in Uganda on HIV patients switched to second line RTV/lopinavir based regiment, there was a good virological response after 36 months of therapy but about 62% of patients

experienced at least one adverse effect. These study showed PI based regimens to be effective but limited by toxicities [19].

RTV metabolism has been studied in both humans and animals. The CYP3A4 is the principal enzyme responsible for RTV metabolism and to a lesser extent CYP2B6 [20, 21]. Major excretion route is hepatobiliary and about 30% of the drug is excreted in the feces of humans unchanged [22]. Studies have shown RTV to be a CYP3A4 mechanism based inhibitor. It is believed that RTV is metabolized by CYP3A4 to generate metabolites that bind and inhibit CYP3A4 activity thus suggesting the existence of RTV bioactivation pathways [23-25]. Our recent study profiled the bioactivation pathways of RTV and discovered 13 new metabolites, five of which were particularly interesting because of their chemical reactivity and propensity to react with glutathione [26]. Four of these five bioactivation pathways were found to be CYP3A dependent [26]. Results from this study further support the fact that RTV is a CYP3A substrate and undergoes bioactivation.

1.2 RIFAMPICIN (RIF) AND PREGNANE X RECEPTOR (PXR)

RIF is one of the first line drugs for TB treatment and belongs to the family of the rifamycins. It kills the mycobacteria TB by inhibiting the DNA dependent RNA polymerase thus terminating protein transcription [27]. RIF is a potent CYP3A4 enzyme inducer because of its ability to activate the PXR [28]. A significant number of the pharmacokinetic drug-drug interactions in HIV/TB therapy are mediated by RIF, because of its enzyme inducing effect. For example, RIF

was reported to reduce the therapeutic plasma concentration of indinavir and saquinavir both HIV protease inhibitors by about 92% and 80% respectively [15]. Therefore, RIF has been employed as a tool drug for studying PXR activation because of its potent ligand effect [29].

The PXR is a ligand dependent nuclear transcription factor that activates its target genes by forming a heterodimer with retinoid X receptor (RXR), which binds to the PXR DNA response element of target genes to induce their transcription [29]. It is highly expressed in the liver and small intestine [28-31].

The PXR regulates the expression of phase I enzymes like CYP3A4, CYP2B6, CYP2C9 and CYP24 [29, 32-35]; phase II enzymes namely glutathione S-transferase (GST), sulfotranferase 2A1 (SULT2A1), UDP-glucoronosyl transferase 1A1(UGT1A1) [36-40]; as well as transporters such as multidrug resistance protein (MDR1), multidrug resistance associated protein 2 (MRP2), and organic anion transporting polypeptide 2 (OATP2) [41-43]. Aside the regulation of drug metabolism, PXR also plays important physiological roles in glucose and lipid metabolism, inflammation, bile acid and bone homeostasis [36, 44-48].

A wide variety of PXR ligands exist from prescription drugs to herbal medicines, dietary food compounds, environmental chemicals/contaminants as well as endogenous compounds like steroid hormones and some essential vitamins [31, 49, 50]. The antibiotics RIF and clotrimazole are potent ligands of the human PXR [29]. The herbal medicine St John's wort was reported to be a more potent ligand than RIF [51]. Lignans such as enterolactone found in foods such as rye, berries and whole grains are low to moderate PXR ligands [52]. Organochlorine pesticides chlordane, dieldrin and endosulfan have also been reported to be PXR ligands [49]. Progestogens

like 5β Pregnan-3, 20-dione and estrogens like 17β estradiol are weak activators of the PXR [29, 52]. Dihydroxyvitamin D3, vitamin E and vitamin K are also PXR ligands [36, 50, 52].

Owing to the existence of huge number of PXR ligands and its effect on drug metabolizing enzymes and transporters, the PXR has been implicated in ADRs and pharmacokinetic drug-drug interactions in the clinic. Acetaminophen (APAP) a widely used over the counter analgesic causes liver injury with an overdose [53]. Induction of drug metabolizing enzymes especially CYP2E1 has been shown to enhance the conversion of APAP to the toxic metabolite *N-acetyl-p*-benzoquinone imine (NAPQI) that interacts with cellular macromolecules in the liver to cause injury [54-56]. However, activation of the PXR and induction of CYP3A4 by RIF was found to increase APAP induced liver injury in TgCYP3A4/hPXR mice co-treated with RIF and APAP compared to either treatment alone and their wild type, *hPXR* and *Pxr*-null counterparts [57]. Likewise, the human PXR was shown to modulate liver injury caused by RIF and isoniazid co-therapy through perturbation of the heme synthesis pathway, which causes the accumulation of the hepatotoxin protoporphrin IX [58].

Since the discovery of the Pxr in mouse, further research has revealed its activation to be specie specific [29, 31]. The PXR has a conserved DNA binding domain having an amino acid sequence similarity of >94% with other PXR orthologs in mice, rats, rabbits and rhesus monkey [59-61]. However, It was reported that the amino acids in the ligand-binding domain of the human PXR differs significantly from that of other species. For example, it shares a sequence similarity of only 77% with mice, which may account for the differences in its response to xenobiotic across species [61, 62]. In line with this example, pregnonelone 16α-carbonitrile

(PCN) is a potent mouse Pxr activator but a weak human PXR activator. Similarly, RIF is a strong human PXR activator but not a potent mouse Pxr ligand. Due to this specie differences, the *PXR*- humanized mouse model was generated to bridge the gap in studying the human PXR in vivo [61].

1.3 DRUG-INDUCED LIVER INJURY IN RIF AND RTV CONTAINING THERAPY

Reports from various pharmacokinetic trials optimizing doses of RTV boosted PIs when administered alongside potent enzyme inducers like RIF have shown an alarming rate of drug-induced liver injury in volunteers. In a study by Schmitt et al, volunteers who received RIF for two weeks before starting saquinavir/RTV (SQV/RTV-1000/100mg) presented with much severe adverse effects compared to those who started with SQV/RTV followed by RIF. The study had to be discontinued because all study participants had developed some level of hepatitis >2times the upper limit of normal [63]. Similar adverse effects were observed in HIV patients treated with a super boosted lopinavir /RTV (LPV/RTV-400/400mg) [64]. Despite these observations, the mechanism underlying liver injury during RIF pretreatment followed by an RTV containing regimen is not clearly understood. One may speculate that RIF pretreatment increases the expression of CYP3A4, which increases the bioactivation of RTV, possibly increasing the generation of reactive metabolites that can elicit liver injury. Therefore there is a need to understand the mechanisms behind RTV induced liver injury so that strategies can be designed to ameliorate or prevent such events in HIV patients exposed to PXR ligands.

1.4 SPECIFIC AIMS OF THE RESEARCH

The overall goal of this study was to investigate the mechanisms behind drug-induced liver injury during pretreatment with RIF followed by RTV regimen. The specific aims of the research were:

1. To determine if ligand activation of the human PXR modulates RTV induced liver injury. This was achieved by comparing TgCYP3A4/hPXR mice with the TgCYP3A4/Pxr-null mice pretreated with RIF followed by RTV.

2. To understand the processes that lead to liver injury associated with RIF pretreatment followed by RTV. This was accomplished by investigating the involvement of oxidative stress and endoplasmic reticulum (ER) stress in mediating RTV induced liver injury.

2.0 MATERIALS AND METHODS

2.1 ANIMAL MODELS

TgCYP3A4/hPXR and TgCYP3A4/*Pxr*-null mice were requested from Dr Frank Gonzalez lab in NIH. Animals were genotyped by polymerase chain reaction (PCR). Briefly, about 0.5 cm of tail was clipped from mice at the time of weaning and placed into a 1.5 ml eppendorf tube. About 500 μL of lysis buffer containing proteinase K was placed into the tube and incubated on a shaker at 37°C overnight. Next day, samples were centrifuged at 14,000 g for 10 min and supernatant was used for DNA extraction. DNA pellet obtained was air dried and re-suspended in 100 μL of distilled water.

The PCR reaction mix 25 μL containing 3 μL (100 ng) of DNA, 2.5 μL 10x PCR buffer minus Mg (Invitrogen, Carlsbad, CA), 0.5 μL 10 mM dNTPs (Invitrogen, Carlsbad, CA), 0.75 μL 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), and 0.125 μL iTaq DNA polymerase (Invitrogen, Carlsbad, CA) was heated for 5 min at 94°C, and then cycled 34 (29 for TgCYP3A4) times at 94°C for 30 sec (95°C for TgCYP3A4), 58°C for 30 sec (60°C for TgCYP3A4), 72°C for 45 sec and 72°C for 10 min (5 min for TgCYP3A4), then 37°C for 5 min (TgCYP3A4 only).The following primers were used to identify the mouse *PXR* WT and null alleles: PXR-Fwd1, 5'-CTGGTCATCACTGTTGCTGTACCA-3'; PXR-Rev2, 5' GCAGCATAGGACAAGTTATTCTAGAG-3'; and PXR-Rev3, 5'

CTAAAGCGCATGCTCCAGACTGC-3’ amplifying a PCR product of 348 bp for WT allele and 265 bp for *Pxr*-null allele [65]. Primers used for the human *CYP3A4* transgene were: CYP3A4-Fwd2, 5’-TGG AAT GAG GAC AGC CAT AGA GAC-3’; CYP3A4-REV2 5’-AGA AGA GGA GCC TGG ACA GTT ACT C-3’ amplifying a PCR product of 521 bp.

2.2 EXPERIMENTAL DESIGN

All male mice (two months old) were kept under a 12 h dark and 12 h light cycle with water and food ad libitum. Mice were fed with control diet and RIF 100 mg/kg diet for 6 days. RTV 25 mg/kg dissolved in 1% DMSO and corn oil was administered on the seventh day and continued for 5 more days. Handling was in accordance with University of Pittsburgh study protocols. Animals were sacrificed by cervical dislocation on the twelfth day. Blood and liver samples were collected and snap frozen and stored at -80°C till use.

2.3 MICROSOME PREPARATION AND WESTERN BLOT ANALYSIS

Liver tissue was homogenized in ice-cold buffer (containing 0.1 M phosphate buffer pH 7.5, sucrose 0.25 M and KCl 0.154 M). Microsomes were prepared by centrifuging liver homogenate at 12,100 rpm for 25 min at 4°C. The resulting supernatant was centrifuged at 37,100 rpm for 1h at 4°C. Microsomal pellets were re-suspended in same buffer used for homogenization. Protein quantification was carried out using the BCA method. For western blot analysis, 1μg protein from each sample was separated by SDS-polyacrylamide gel

electrophoresis, electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and probed using anti-mouse ubiquitin antibody (Santa Cruz). Goat anti-rabbit was used as secondary antibody. Immunoreactive proteins were detected by chemiluminescence blot detection kit (Thermo Scientific).

2.4 BIOCHEMICAL ASSAYS

Alanine amino transferase (ALT) and alkaline phosphatase (ALP) assays were carried out using serum. Briefly, serum was obtained by centrifuging blood samples collected in heparinized tubes at 10,000 g for 10 min at room temperature. Ten μL of serum samples was incubated with 200 μL of reagent mixture made according to the standard kit procedure (Pointe Scientific, INC). Absorbance was read using a spectrophotometer (Bio Rad) at 37°C and wavelength of 340 nm and 405 nm for ALT and ALP respectively.

2.5 RNA ANALYSIS

RNA was extracted from liver tissues using TRIzol reagent (Ambion, life technologies). Quantitative PCR (qPCR) was done using cDNA generated from 0.5 ug total RNA using the Invitrogen kit. qPCR reactions were carried out using the sybr green reagent in Quant studio 7600 (Applied Biosystems). qPCR values were quantified using the comparative cycle threshold (Ct) method and samples were normalized to cyclophilin D.

2.6 TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE DUTP NICK END LABELING (TUNEL) ASSAY

Liver slices about 5 mm thick were obtained from TgCYP3A4/hPXR and TgCYP3A4/*Pxr*-null mice and placed in tissue cassettes. The liver tissues were fixed in formaldehyde overnight. Tissue blocks were then dehydrated in varying concentrations of ethanol (70%, 95% and 100%) at one-hour intervals and then embedded in paraffin overnight. Blocks were then placed in xylene to remove fat and alcohol. The tissue was then infiltrated with paraffin by placing it in a beaker of melted paraffin overnight. Tissues were then removed from the cassettes and embedded in paraffin with the help of a mold.

The paraffin embedded tissue section was cut to a 4-5 um section. The section was deparafinized in an oven at 65°C for 30 min. Then xylene and various concentrations of alcohol were used to hydrate tissue sections. Sections were rinsed with PBS and excess water was removed using paper towels. Proteinase digestion was carried out by incubating slides with 20 μg/ml of proteinase K in TE buffer for 15 min at 21-37 °C. The TUNEL mixture was added to the sections according to the manufacturer's instructions. Slides were wrapped carefully in parafilm and incubated in a plastic chamber at 37°C for 1 h. Slides were then rinsed three times with PBS, mounted with DAPI and covered with glass slips and viewed under a fluorescent microscope.

2.7 METABOLOMICS

One hundred mg of liver was weighed from each sample and transferred to a small 12 x 75mm glass tube. About 500 μL of water (five times weight of liver) was added to each sample. Tissue was homogenized on ice using a tissue homogenizer (Thermo Scientific). One hundred μL of liver homogenate was transferred to an eppendorf tube and 100 μL of methanol was added and vortexed for 30 sec. The resulting mixture was centrifuged at 15,000 rpm for 10 min at room temperature. One hundred μL of supernatant was transferred to a new eppendorf tube and 100 μL of acetonitrile was added and vortexed well. Mixture was centrifuged again as described above and 100 μL of supernatant was transferred to UPLC sample vial for analysis on a qTOFMS.

2.8 STATISTICAL ANALYSIS

All tests were carried out using the student t-test comparing the treatment groups between the two genotypes. Significant P-values were set as <0.05.

3.0 RESULTS/DISCUSSION

3.1 GENOTYPING

We used the mouse models expressing human PXR and CYP3A4 to overcome the specie differences in the activation of PXR in mice and humans. A TgCYP3A4 on a Pxr-null background was used as the control to determine PXR dependent functions (Figure 1A). CYP3A4 was significantly induced by RIF treatment in the TgCYP3A4/hPXR but not by Pregnonelone-16α-carbonitrile (PCN), a mouse Pxr activator (Figure 1B).

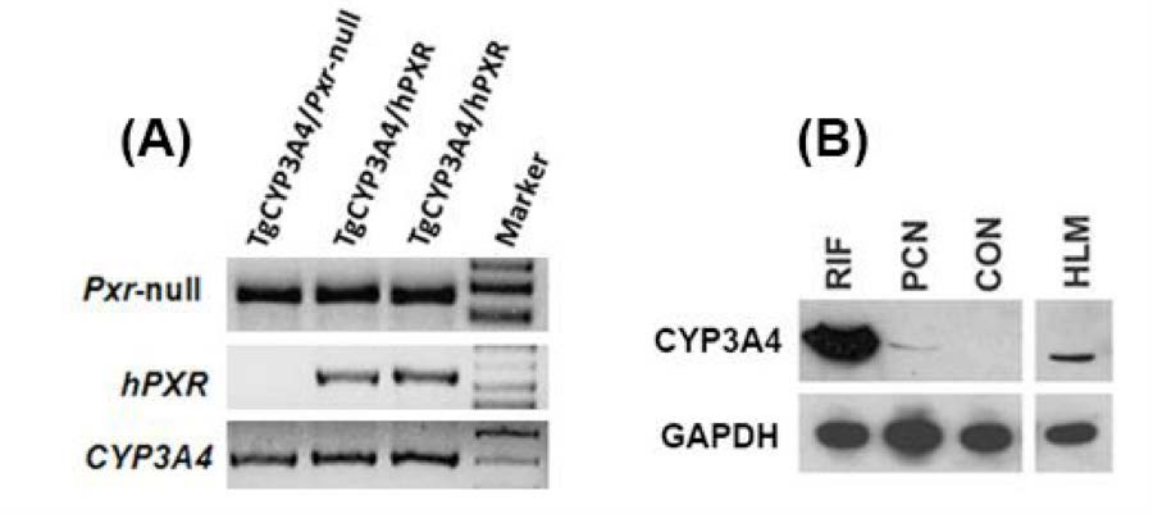

Figure 1. Mouse models

A) PCR genotyping results for TgCYP3A4/hPXR and TgCYP3A4/Pxr-null B) Regulation of CYP3A4 in TgCYP3A4/hPXR. Mice were treated with vehicle, RIF or PCN for 3 days. Liver microsomes were prepared and analyzed by Western blot. Human liver microsome (HLM) was used as the positive control. GAPDH was used as the loading control.

3.2 ACTIVATION OF HUMAN PXR MODULATES RTV-INDUCED LIVER INJURY

ALT activity has been reported to be a sensitive marker of liver injury [66]. In our study, there was a 7-fold increase in ALT activity in the transgenic mice expressing the human PXR pretreated with RIF followed by RTV compared to their *Pxr*-null counterparts (Figure 2A). Within the TgCYP3A4/hPXR mice group, there was approximately a 6-fold increase in ALT activity in RIF and RTV treatment group compared to vehicle control and treatment of either drug alone (Figure 2A). This indicates that liver injury is human PXR dependent and requires administration of both RIF and RTV. To further investigate if liver injury truly occurred, TUNEL assay, which is a technique used to detect nicked DNA in cells undergoing cell death, was carried out. There was a 6-fold increase in dead cells in transgenic mice expressing the hPXR compared to their *Pxr*-null controls further confirming liver injury (Figure 2B-D). ALP activity on the other hand showed no significant change between the treatment and control groups (data not shown) in both genotypes thus suggesting liver injury to be hepatocellular [67].

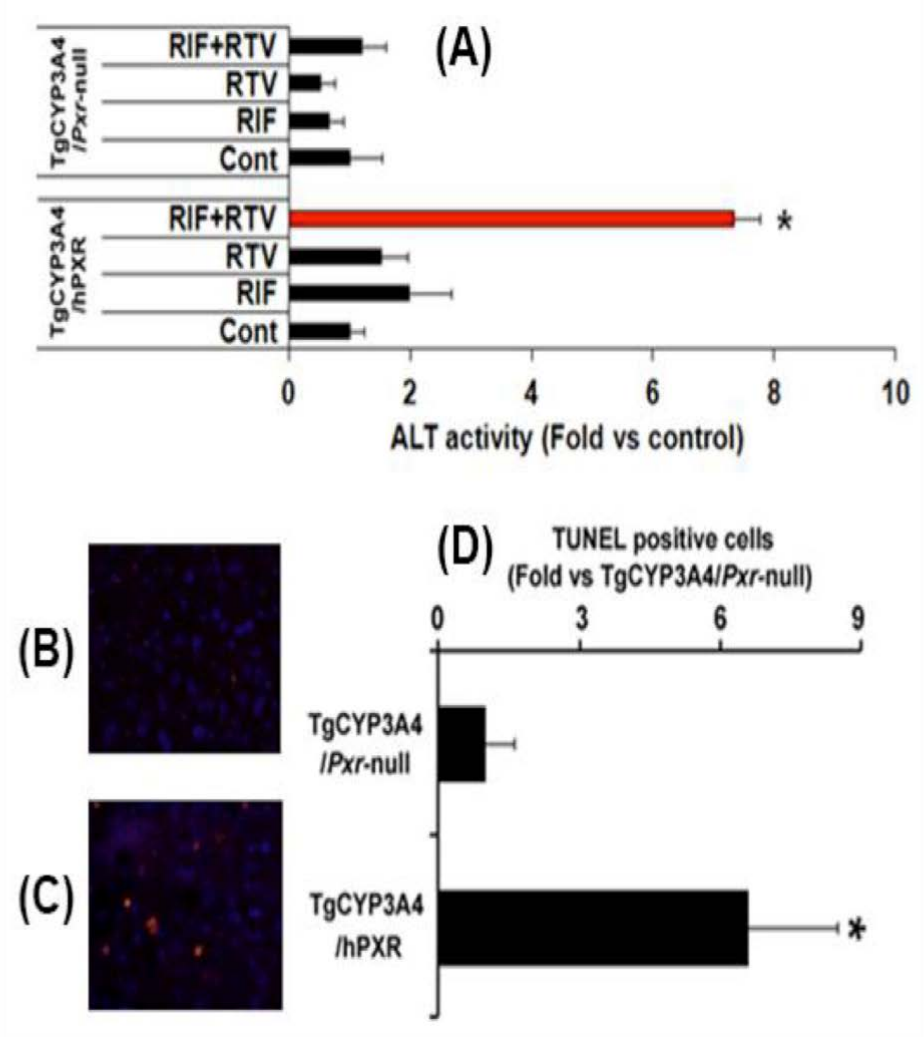

Figure 2 Human PXR modulates liver injury associated with RIF pretreatment followed by RTV

TgCYP3A4/hPXR and TgCYP3A4/Pxr-null mice were pretreated with vehicle (Cont) or RIF for 6 days. On day 7, RTV (25 mg/kg) was added and continued until day 11. On day 12, all mice were sacrificed and blood samples were collected for evaluation of liver injury. (A) Serum alanine aminotransferase (ALT) activity. The data are expressed as mean ± SD (n=3-5). *P<0.05 compared with control. ALT activity was set as 1 in the control group. (B-D) TUNEL assays in livers of TgCYP3A4/Pxr-null (B) and TgCYP3A4/hPXR (C) mice pretreated with RIF followed by RTV (200X). (D) Relative quantification of TUNEL positive cells. *P<0.05 compared with TgCYP3A4/Pxr-null mice (set as 1).

3.3 ENDOPLASMIC RETICULUM (ER) STRESS IN RTV-INDUCED LIVER INJURY

The ER is the domain in the cell where protein synthesis, folding and transport to other organelles like the golgi apparatus occurs. However, these processes can be disrupted by various physiological or noxious stimuli that lead to ER stress [68, 69]. Since, the ER is the focal organelle for drug biotransformation, toxic insults from the parent drug or metabolite or both may cause perturbation of ER functions leading to ER cellular stress [70]. The cell activates the ER stress response pathways to stop further protein synthesis, increase protein-folding capacity as well as inhibit the accumulation of misfolded proteins to promote cell survival. However, when the ER functions are severely damaged, the cells are targeted toward cell death [69].

In our studies, electron microscopy of ER revealed dilation and blebbing of the ER in the transgenic mice expressing the hPXR pretreated with RIF followed by RTV compared to their *Pxr*-null counterparts (Figure 3A and 3B). This provides evidence that the integrity of the ER is affected by RTV-induced liver injury. Furthermore, western blot analysis also revealed the accumulation of ubiquinated proteins in the liver of TgCYP3A4/hPXR mice pretreated with RIF followed by RTV (Figure 3C). This data is in agreement with a previous study on RTV showing proteasome inhibition as a possible mechanism of RTV-induced ER stress though we saw more accumulation of the bulk polyubiquinated protein [71]. This may be due to activation of hPXR by RIF, which leads to the increased transcription of proteins, but reactive metabolites of RTV target the ER proteins, which lead to the misfolding and accumulation of misfolded proteins or perturbation of proteasomal protein degradation.

C/EBP homologous protein (CHOP) is a transcription factor that is induced by ER stress

or some other hostile conditions in the cells. It is the main mediator of apoptotic cell death during ER stress [69]. We observed a 11-fold increase of CHOP in the TgCYP3A4/hPXR mice pretreated with RIF followed by RTV compared to their Pxr-null counterparts and individual drug treatment (Figure 3D). These data suggest that RIF pretreatment followed by RTV can induce ER stress. Based on our results, we propose that the ER stress is activated during RTV-induced liver injury. Since the ER stress cellular pathway has three major arms- IRE1-alpha, PERK and ATF-6 pathways [72], more studies will be carried out to determine which arm is particularly important in mediating RTV-induced liver injury.

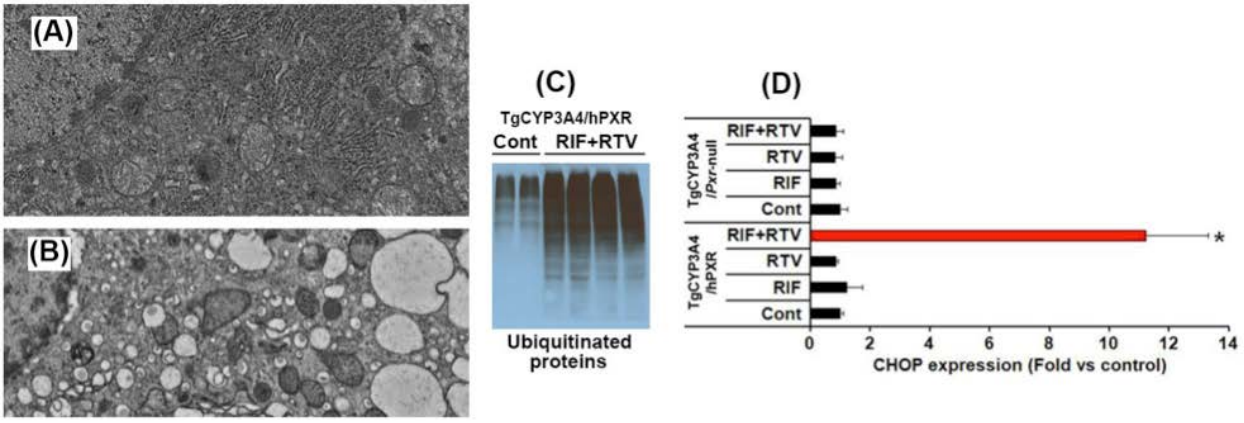

Figure 3: ER damage, accumulation of ubiquinated proteins and ER stress in the liver of TgCYP3A4/hPXR mice pretreated with RIF followed by RTV

(A and B) Liver images analyzed by transmission electron microscopy in TgCYP3A4/Pxr-null (A) and TgCYP3A4/hPXR (B) mice pretreated with RIF followed by RTV. The bubbles (B) are dilated ER. (C) Western blot analysis of ubiquitinated proteins in the liver of TgCYP3A4/hPXR mice. (D) CHOP expression in the liver. CHOP expression was quantified by qPCR. All data are expressed as mean ± SD (n=3-5). *P<0.05 compared with control.

3.4 RIF PRETREATMENT FOLLOWED BY RTV THERAPY INDUCED OXIDATIVE STRESS IN LIVER OF TGCYP3A4/HPXR MICE

Metabolomics has been used as an adept approach to study endobiotic homeostasis. The chemical profile of liver from TgCYP3A4/hPXR mice pretreated with RIF followed by RTV was significantly different from that of vehicle, RIF or RTV treatment groups (Figure 4A). Further analysis identified two ions GSSG and ophthalmic acid that are associated with oxidative stress. GSSG the oxidized form of glutathione (GSH) is a biomarker of oxidative stress [73] and was significantly increased in the liver of TgCYP3A4/hPXR (Figure 4B). Furthermore, ophthalmic acid, a GSH analog and sensitive indicator of oxidative stress [74] was also increased significantly (Figure 4B and 4C). These findings reveal considerable oxidative stress in the liver of TgCYP3A4/hPXR mice pretreated with RIF followed by RTV.

PXR activation has been reported to enhance oxidative stress as seen in transgenic mice with constitutively active PXR (VP-hPXR) showing increased sensitivity to paraquat toxicity, and treatment with mouse Pxr agonist, PCN, increased paraquat toxicity in wild type mice [75]. Moreover, increased sensitivity was accompanied by a down regulation of superoxide dismutase (SOD) and catalase, two enzymes important for the detoxification of reactive oxygen species from the body [75]. Thus activation of PXR by RIF in our studies may also enhance oxidative stress in addition to CYP3A4 induction that increases generation of RTV reactive metabolites. Further studies will be carried out to determine the role of superoxide dismutase (SOD) and catalase, in RTV-induced oxidative stress.

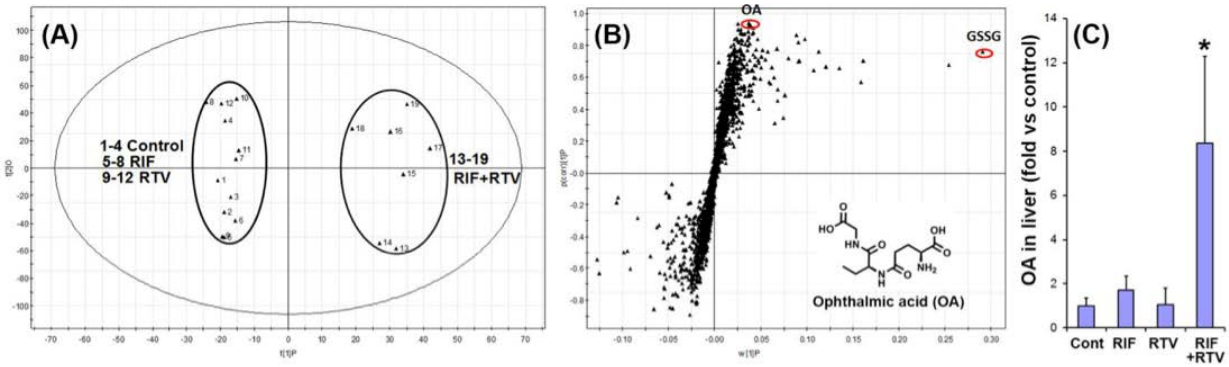

Figure 4 Metabolomic analysis of liver in TgCYP3A4/hPXR mice pretreated with RIF followed by RTV

(A) Group separation in an OPLS-DA score plot. The t [1] P and t[2]O values represent the score of each sample in principal component 1 and 2, respectively. (B) Loading S-plots generated by metabolomic analysis in mouse liver. 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. Two top ranking ions are labeled in S-plots, which are glutathione disulfide (GSSG) and ophthalmic acid (OA). (C) Relative quantifications of OA. All data are expressed as mean ± SD (n=4-7). *P<0.05 compared with control. The data in the control group was set as 1.

4.0 CONCLUSION

By using the PXR-humanized mouse model, we demonstrated that PXR potentiates RTV-induced liver injury (Figure 5). The presence of liver injury in our studies is consistent with findings from the clinical studies that reported drug induced liver injury in subjects pretreated with RIF followed by an RTV containing regimen [63, 64, 76]. Furthermore, we found that ER stress and oxidative stress contribute to liver injury associated with RTV. The results from this study can be used to guide clinical decisions on the use of PXR ligands before initiating RTV based therapy in patients. Further studies will be carried to provide a deeper insight into the mechanisms involved and pathways that can be targeted to ameliorate or prevent RTV induced liver injury.

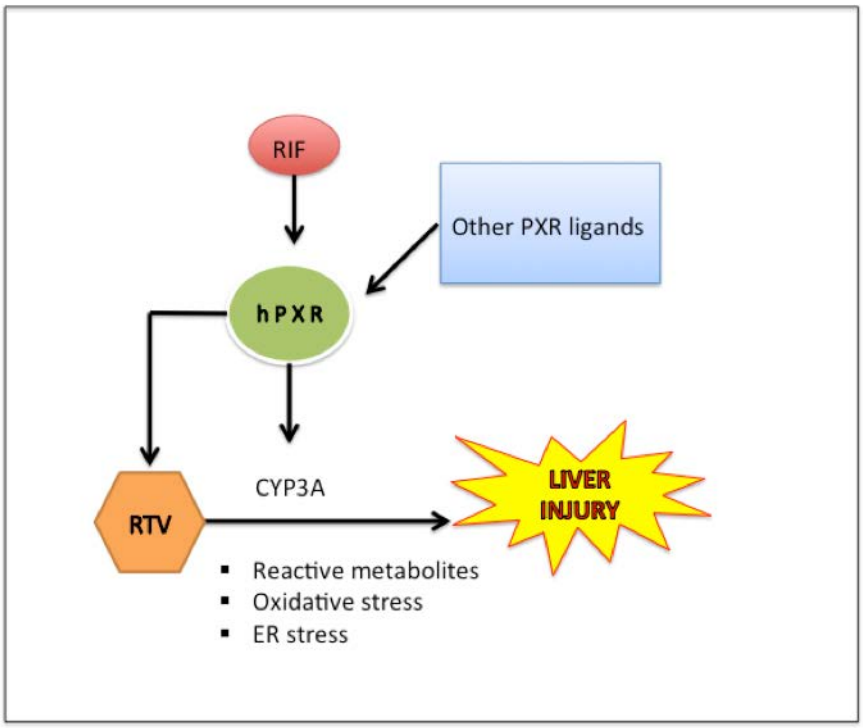

Figure 5: Project summary

RIF and other PXR ligands activate the hPXR, which induces CYP3A4 induction, thus exacerbating CYP3A4-mediated RTV bioactivation that potentiates oxidative stress and ER stress, leading to liver injury.

BIBLIOGRAPHY

1. Lipsky, J.J., Antiretroviral drugs for AIDS. The Lancet, 1996. 348(9030): p. 800-803.

2. Carr, A. and D.A. Cooper, Adverse effects of antiretroviral therapy. The Lancet, 2000. 356(9239): p. 1423-1430.

3. Karch, F.E. and L. Lasagna, Adverse drug reactions: a critical review. Jama, 1975. 234(12): p. 1236-1241.

4. Fellay, J., et al., Prevalence of adverse events associated with potent antiretroviral treatment: Swiss HIV Cohort Study. The Lancet, 2001. 358(9290): p. 1322-1327.

5. Ruschitzka, F., et al., Acute heart transplant rejection due to Saint John's wort. The Lancet, 2000. 355(9203): p. 548-549.

6. Bonfanti, P., et al., Incidence of adverse reactions in HIV patients treated with protease inhibitors: a cohort study. Coordinamento Italiano Studio Allergia e Infezione da HIV (CISAI) Group. Journal of acquired immune deficiency syndromes (1999), 2000. 23(3): p. 236-245.

7. Hänsel, A., et al., Reasons for discontinuation of first highly active antiretroviral therapy in a cohort of proteinase inhibitor-naive HIV-infected patients. JAIDS Journal of Acquired Immune Deficiency Syndromes, 2001. 26(2): p. 191-193.

8. Le Moing, V., et al., Impact of discontinuation of initial protease inhibitor therapy on further virological response in a cohort of human immunodeficiency virus–infected patients. Clinical infectious diseases, 2002. 34(2): p. 239-247.

9. Monforte, A.d.A., et al., Insights into the reasons for discontinuation of the first highly active antiretroviral therapy (HAART) regimen in a cohort of antiretroviral naive patients. Aids, 2000. 14(5): p. 499-507.

10. O'Brien, M.E., et al., Patterns and correlates of discontinuation of the initial HAART regimen in an urban outpatient cohort. JAIDS Journal of Acquired Immune Deficiency Syndromes, 2003. 34(4): p. 407-414.

11. Neuman, M.G., et al., HIV-antiretroviral therapy induced liver, gastrointestinal, and pancreatic injury. International journal of hepatology, 2012. 2012.

12. Naggie, S. and C. Hicks, Protease inhibitor-based antiretroviral therapy in treatment-naive HIV-1-infected patients: the evidence behind the options. Journal of Antimicrobial Chemotherapy, 2010. 65(6): p. 1094-1099.

13. Gupta, R., et al., Emergence of drug resistance in HIV type 1-infected patients after receipt of first-line highly active antiretroviral therapy: a systematic review of clinical trials. Clinical infectious diseases, 2008. 47(5): p. 712-722.

14. King, J.R., H. Wynn, and R. Brundage, Pharmacokinetic enhancement of protease inhibitor therapy. Clinical pharmacokinetics, 2004. 43(5): p. 291-310.

15. Burman, W.J., K. Gallicano, and C. Peloquin, Therapeutic implications of drug interactions in the treatment of human immunodeficiency virus-related tuberculosis. Clinical infectious diseases, 1999: p. 419-429.

16. Piscitelli, S.C., et al., Indinavir concentrations and St John's wort. The Lancet, 2000. 355(9203): p. 547-548.

17. Ernst, E., Second thoughts about safety of St John's wort. The Lancet, 1999. 354(9195): p. 2014-2016.

18. Sulkowski, M.S., et al., Hepatotoxicity associated with antiretroviral therapy in adults infected with human immunodeficiency virus and the role of hepatitis C or B virus infection. Jama, 2000. 283(1): p. 74-80.

19. Castelnuovo, B., et al., Three-year outcome data of second-line antiretroviral therapy in Ugandan adults: good virological response but high rate of toxicity. Journal of the International Association of Physicians in AIDS Care (JIAPAC), 2008.

20. Kumar, G.N., et al., Cytochrome P450-mediated metabolism of the HIV-1 protease inhibitor ritonavir (ABT-538) in human liver microsomes. Journal of Pharmacology and Experimental Therapeutics, 1996. 277(1): p. 423-431.

21. Kharasch, E.D., et al., Rapid clinical induction of hepatic cytochrome P4502B6 activity by ritonavir. Antimicrobial agents and chemotherapy, 2008. 52(5): p. 1663-1669.

22. Denissen, J.F., et al., Metabolism and disposition of the HIV-1 protease inhibitor ritonavir (ABT-538) in rats, dogs, and humans. Drug metabolism and disposition, 1997. 25(4): p. 489-489.

23. Ernest, C.S., S.D. Hall, and D.R. Jones, Mechanism-based inactivation of CYP3A by HIV protease inhibitors. Journal of Pharmacology and Experimental Therapeutics, 2005. 312(2): p. 583-591.

24. Von Moltke, L., et al., Potent mechanism-based inhibition of human CYP3A in vitro by amprenavir and ritonavir: comparison with ketoconazole. European journal of clinical pharmacology, 2000. 56(3): p. 259-261.

25. Koudriakova, T., et al., Metabolism of the human immunodeficiency virus protease inhibitors indinavir and ritonavir by human intestinal microsomes and expressed cytochrome

P4503A4/3A5: mechanism-based inactivation of cytochrome P4503A by ritonavir. Drug Metabolism and Disposition, 1998. 26(6): p. 552-561.

26. Li, F., J. Lu, and X. Ma, Metabolomic screening and identification of the bioactivation pathways of ritonavir. Chemical research in toxicology, 2011. 24(12): p. 2109-2114.

27. Wehrli, W., et al., Interaction of rifamycin with bacterial RNA polymerase. Proceedings of the National Academy of Sciences of the United States of America, 1968. 61(2): p. 667.

28. Bertilsson, G., et al., Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proceedings of the National Academy of Sciences, 1998. 95(21): p. 12208-12213.

29. Lehmann, J.M., et al., The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. Journal of Clinical Investigation, 1998. 102(5): p. 1016.

30. Blumberg, B., et al., SXR, a novel steroid and xenobioticsensing nuclear receptor. Genes & development, 1998. 12(20): p. 3195-3205.

31. Kliewer, S.A., et al., An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell, 1998. 92(1): p. 73-82.

32. Chen, Y., et al., Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. Journal of Pharmacology and Experimental Therapeutics, 2004. 308(2): p. 495-501.

33. Gerbal-Chaloin, S., et al., Induction of CYP2C genes in human hepatocytes in primary culture. Drug Metabolism and Disposition, 2001. 29(3): p. 242-251.

34. Goodwin, B., E. Hodgson, and C. Liddle, The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Molecular Pharmacology, 1999. 56(6): p. 1329-1339.

35. Pascussi, J.M., et al., Possible involvement of pregnane X receptor–enhanced CYP24 expression in drug-induced osteomalacia. Journal of Clinical Investigation, 2005. 115(1): p. 177-186.

36. Tabb, M.M., et al., Vitamin K2 regulation of bone homeostasis is mediated by the steroid and xenobiotic receptor SXR. Journal of Biological Chemistry, 2003. 278(45): p. 43919-43927.

37. Falkner, K., et al., Regulation of the rat glutathione S-transferase A2 gene by glucocorticoids: involvement of both the glucocorticoid and pregnane X receptors. Molecular pharmacology, 2001. 60(3): p. 611-619.

38. Runge-Morris, M., W. Wu, and T.A. Kocarek, Regulation of rat hepatic hydroxysteroid sulfotransferase (SULT2-40/41) gene expression by glucocorticoids: evidence for a dual mechanism of transcriptional control. Molecular pharmacology, 1999. 56(6): p. 1198-1206.

39. Sugatani, J., et al., Transcriptional regulation of human UGT1A1 gene expression: activated glucocorticoid receptor enhances constitutive androstane receptor/pregnane X receptor-mediated UDP-glucuronosyltransferase 1A1 regulation with glucocorticoid receptor-interacting protein 1. Molecular pharmacology, 2005. 67(3): p. 845-855.

40. Xie, W., et al., Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proceedings of the National Academy of Sciences, 2003. 100(7): p. 4150-4155.

41. Geick, A., M. Eichelbaum, and O. Burk, Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. Journal of Biological Chemistry, 2001. 276(18): p. 14581-14587.

42. Mills, J.B., et al., Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. Journal of Pharmacology and Experimental Therapeutics, 2004. 309(1): p. 303-309.

43. Synold, T.W., I. Dussault, and B.M. Forman, The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nature medicine, 2001. 7(5): p. 584-590.

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

45. Teng, S. and M. Piquette-Miller, The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. Journal of Pharmacology and Experimental Therapeutics, 2005. 312(2): p. 841-848.

46. Zhou, C., et al., Mutual repression between steroid and xenobiotic receptor and NF-κB signaling pathways links xenobiotic metabolism and inflammation. Journal of Clinical Investigation, 2006. 116(8): p. 2280-2289.

47. Kodama, S., et al., Human nuclear pregnane X receptor cross-talk with CREB to repress cAMP activation of the glucose-6-phosphatase gene. Biochem. J, 2007. 407: p. 373-381.

48. Nakamura, K., et al., Nuclear pregnane X receptor cross-talk with FoxA2 to mediate drug-induced regulation of lipid metabolism in fasting mouse liver. Journal of Biological Chemistry, 2007. 282(13): p. 9768-9776.

49. Coumoul, X., M. Diry, and R. Barouki, PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. Biochemical pharmacology, 2002. 64(10): p. 1513-1519.

50. Landes, N., et al., Vitamin E activates gene expression via the pregnane X receptor. Biochemical pharmacology, 2003. 65(2): p. 269-273.

51. Moore, L.B., et al., St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proceedings of the National Academy of Sciences, 2000. 97(13): p. 7500-7502.

52. Jacobs, M.N., G.T. Nolan, and S.R. Hood, Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicology and applied pharmacology, 2005. 209(2): p. 123-133.

53. Larson, A.M., et al., Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology, 2005. 42(6): p. 1364-1372.

54. Mitchell, J., et al., Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. Journal of Pharmacology and Experimental Therapeutics, 1973. 187(1): p. 185-194.

55. Nelson, S.D. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. in Seminars in liver disease. 1990.

56. Raucy, J.L., et al., Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. Archives of Biochemistry and Biophysics, 1989. 271(1): p. 270-283.

57. Cheng, J., et al., Rifampicin-activated human pregnane X receptor and CYP3A4 induction enhance acetaminophen-induced toxicity. Drug Metabolism and Disposition, 2009. 37(8): p. 1611-1621.

58. Li, F., et al., Human PXR modulates hepatotoxicity associated with rifampicin and isoniazid co-therapy. Nature medicine, 2013. 19(4): p. 418-420.

59. Iyer, M., E.J. Reschly, and M.D. Krasowski, Functional evolution of the pregnane X receptor. 2006.

60. Carnahan, V.E. and M.R. Redinbo, Structure and function of the human nuclear xenobiotic receptor PXR. Current drug metabolism, 2005. 6(4): p. 357-367.

61. Ma, X., J.R. Idle, and F.J. Gonzalez, The pregnane X receptor: from bench to bedside. 2008.

62. Watkins, R., S. Noble, and M. Redinbo, Structural insights into the promiscuity and function of the human pregnane X receptor. Current opinion in drug discovery & development, 2002. 5(1): p. 150-158.

63. Schmitt, C., et al., Unexpected hepatotoxicity of rifampin and saquinavir/ritonavir in healthy male volunteers. Archives of drug information, 2009. 2(1): p. 8-16.

64. Murphy, R.A., et al., Coadministration of lopinavir/ritonavir and rifampicin in HIV and tuberculosis co-infected adults in South Africa. PloS one, 2012. 7(9): p. e44793.

65. Ma, X., et al., The PREgnane X receptor gene-humanized mouse: a model for investigating drug-drug interactions mediated by cytochromes P450 3A. Drug metabolism and disposition, 2007. 35(2): p. 194-200.

66. Ozer, J., et al., The current state of serum biomarkers of hepatotoxicity. Toxicology, 2008. 245(3): p. 194-205.

67. Navarro, V.J. and J.R. Senior, Drug-related hepatotoxicity. New England Journal of Medicine, 2006. 354(7): p. 731-739.

68. Kaufman, R.J., Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes & development, 1999. 13(10): p. 1211-1233.

69. Oyadomari, S. and M. Mori, Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death & Differentiation, 2004. 11(4): p. 381-389.

70. Lafleur, M.A., J.L. Stevens, and J.W. Lawrence, Xenobiotic perturbation of ER stress and the unfolded protein response. Toxicologic pathology, 2013. 41(2): p. 235-262.

71. Gaedicke, S., et al., Antitumor effect of the human immunodeficiency virus protease inhibitor ritonavir induction of tumor-cell apoptosis associated with perturbation of proteasomal proteolysis. Cancer research, 2002. 62(23): p. 6901-6908.

72. Wu, J. and R. Kaufman, From acute ER stress to physiological roles of the unfolded protein response. Cell Death & Differentiation, 2006. 13(3): p. 374-384.

73. Carlberg, I. and B. Mannervik, [59] Glutathione reductase. Methods in enzymology, 1985. 113: p. 484-490.

74. Soga, T., et al., Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. Journal of Biological Chemistry, 2006. 281(24): p. 16768-16776.

75. Gong, H., et al., Orphan nuclear receptor pregnane X receptor sensitizes oxidative stress responses in transgenic mice and cancerous cells. Molecular endocrinology, 2006. 20(2): p. 279-290.

76. Rafaëlla, F., et al., Clinical experience with the combined use of lopinavir/ritonavir and rifampicin. Aids, 2009. 23(7): p. 863-865.