Point-by-point responses

1. The editors recommend revising your title for clarity. We suggest: Antibiotic-mediated pregnane X receptor activation potentiates ritonavir hepatotoxicity. Ensure that your final title adheres to the JCI's strictly enforced 12-word limit.

   Response: We accept the editors' revision of the title, but we removed one word “Antibiotic-mediated” because (1) rifampicin is an antibiotic, but efavirenz is an antiviral, and both of them potentiate ritonavir hepatotoxicity through PXR activation (Figs 1 and 2; and Supplemental Fig 5); and (2) we discussed that PXR is a potential target for monitoring the safety of ritonavir, because many prescription drugs and herbal supplements are potent PXR activators that individuals may encounter in daily life [PMID:12822739; PMID: 23007437; PMID:11865669].

2. The published author list will appear as presented in your submitted Word document. Please confirm that all author names are correct and complete.

   Response: Confirmed.

3. If your funding agency requires a Creative Commons CC-BY license in order to support publication fees for this manuscript, please indicate this with a statement on the title page.

   Response: Not required.

4. Ensure that protein and gene symbols are formatted correctly and are consistent with Entrez Gene nomenclature. See Gene Nomenclature and Style for details: https://nam05.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.jci.org%2Fkiosk%2Fpubli sh%2Fgenestyle&data=02%7C01%7Cmxiaocha%40pitt.edu%7Cb3a5d8b0bf53418ed92608d6c2aa3616%7C9ef9f489e0a04eeb87cc3a526112fd0d%7C1%7C0%7C636910433653815655&a mp;sdata=HE%2FrNKtur7QDq7il0TMMJiGYYGyONPg4tb2BmLn%2Ft3S4%3D&amp;reserved=0. Please ensure the styling of mRNA is consistent between the text, figures, and legends with respect to italics.

   Response: Revised and ensured.

5. For all antibodies used, ensure that the source and catalog/clone number (in the case of commercial antibodies) or a description/reference to a description of the generation of custom antibodies is provided.

   Response: Ensured.

6. Ensure that the source of all cell lines/animals used is indicated.

   Response: Ensured.

7. Please ensure that all figures are prepared according to JCI style (https://nam05.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.jci.org%2Fkiosk %2Fpublish%2Ffigures&amp;data=02%7C01%7Cmxiaocha%40pitt.edu%7Cb3a5d8b0bf53418ed92608d6c2aa3616%7C9ef9f489e0a04eeb87cc3a526112fd0d%7C1%7C0%7C636910433653815655&amp;sd a=VwWrgq2ey5itqDjix%2FEcEYuR01%2F9X2XcmleJrhHTfo%3D&amp;reserved=0); in particular, labels should be in 8 pt Helvetica or Arial font, and no bold type should be used.

   Response: Revised and ensured.
8. JCI style dictates that different figure panels should show unique data. Any exceptions must be explicitly noted in the figure legend. Please include disclosures or provide independent replicates. Specifically, hPXR/CYP3A4, RIF+RTV image in 2E appears to be reshown in SF3. Response: We thank you for this comment. We have double checked all the Figs and found that SF3 was the only replicate. We have changed it using an independent replicate.
CONCISE COMMUNICATION

Role of Human Pregnane X Receptor Activation Potentiates in Modulating Ritonavir Hepatotoxicity

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Conflict of interest statement

The authors have declared that no conflict of interest exists.
Abstract

Ritonavir (RTV) is on the World Health Organization's List of Essential Medicines for antiretroviral therapy, but can cause hepatotoxicity by unknown mechanisms. Multiple clinical studies found that hepatotoxicity occurred in 100% of participants who were pretreated with rifampicin or efavirenz followed by RTV-containing regimens. Both rifampicin and efavirenz are activators of the pregnane X receptor (PXR), a transcription factor with significant inter-species differences in ligand-dependent activation. Using PXR-humanized mouse models, we recapitulated the RTV hepatotoxicity observed in the clinic. PXR was found to modulate RTV hepatotoxicity through CYP3A4-dependent pathways involved in RTV bioactivation, oxidative stress, and endoplasmic reticulum stress. In summary, the current work demonstrated the essential roles of human PXR and CYP3A4 in RTV hepatotoxicity, which can be applied to guide the safe use of RTV-containing regimens in the clinic.
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 (1), consequently leading to the emergence of drug resistance and/or virologic failure. Ritonavir (RTV) is the backbone of boosted protease inhibitor-based regimens in ART. In early clinical studies, treatment with a full dose of RTV frequently caused liver damage (2, 3). 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 (4, 5). 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 (6-9). We noted that both RIF and EFV are activators of the human pregnane X receptor (PXR), a ligand-dependent transcription factor that is highly expressed in the liver and upregulates drug metabolizing enzymes including CYP3A4 (10-14). In addition, CYP3A4 plays a critical role in RTV metabolism and bioactivation (15-20). These data led us to hypothesize that human PXR modulates RTV hepatotoxicity through CYP3A4-dependent pathways.

Genetically engineered PXR- and CYP3A4-humanized mouse models were developed and used to test our hypothesis.
Results and Discussion

This project was initiated from the clinical observations showing hepatotoxicity in subjects pretreated with RIF followed by RTV-containing regimens (Figure 1A) (6-8). We first used wild-type (WT) mice to mimic the hepatotoxicity that occurred in the clinical studies. However, no significant liver injury was found in WT mice pretreated with RIF for seven days followed by RTV (Figure 1B, 1C, and Supplemental Figure 1). 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 major hepatic drug-metabolizing enzyme CYP3A4 (11, 14). 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 both mouse Pxr and Cyp3a knockout (Figure 1D). As expected, treatment with RIF induced a panel of PXR target genes including CYP3A4 in the livers of hPXR/CYP3A4 mice (Supplemental Figure 2), indicating that human PXR is functional in these mice.

Using hPXR/CYP3A4 mice, we recapitulated the RTV hepatotoxicity observed in clinical studies (6-8), as the biomarkers of liver damage were significantly increased in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV (Figure 1E and 1F). In addition, histological analysis revealed massive hepatocyte degeneration in hPXR/CYP3A4 mice pretreated with RIF followed by RTV (Supplemental Figure 3). These data indicate that human PXR is the key mediator of 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 (21-24). Therefore, we suggest reviewing whether HIV/AIDs patients are under treatment with PXR activators before starting RTV-containing regimens.

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 (11, 13, 14), and CYP3A4 plays an important role in RTV metabolism and bioactivation (15-20). To test this hypothesis, we generated a humanized PXR mouse model deficient in Cyp3a (hPXR/Cyp3a-null) (Figure 2A). Treatment with RIF for seven days significantly induced PXR target genes other than Cyp3a in the liver of hPXR/Cyp3a-null mice (Figure 2B, Supplemental Figure 4), 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 2C-2F), 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 (25), and many CAR activators are found amongst prescription drugs (such as phenobarbital) and herbal supplements (26, 27). Hence, clinicians should be mindful of both PXR and CAR activators before starting RTV-containing regimens in HIV/AIDs patients, as they could induce CYP3A4 and predispose patients to the risk of liver injury.

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 (Supplemental Figure 5A). We found that lead-in treatment with EFV for seven days potentiated RTV hepatotoxicity in hPXR/CYP3A4 mice (Supplemental Figure 5B-5F), which mimicked EFV and RTV-induced liver injury observed in a previous clinical study (9). In addition, lead-in treatment with EFV for seven days followed by RTV resulted in hepatocyte degeneration (Supplemental Figure 5E), exhibiting the same phenotype as RIF and RTV-induced liver damage (Supplemental Figure 3). We also noted that RIF had a more significant impact than EFV on RTV hepatotoxicity as revealed by alanine transaminase (ALT) and aspartate transaminase (AST) values (Figure 1E and 1F, Supplemental Figure 5B and 5C), which could be the result of enterohepatic circulation of RIF leading to a long time exposure of liver to RIF (28). Furthermore, we found that the hepatotoxicity associated with lead-in treatment with EFV followed by RTV was CYP3A4-dependent (Supplemental Figure 5B-5F). These data further confirmed the roles of human PXR and CYP3A4 in RTV hepatotoxicity.

We next investigated the pharmacokinetic interactions between RIF and RTV in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice. RTV had no effect on RIF metabolism and disposition (Supplemental Figure 6). However, pretreatment with RIF for seven days significantly increased the metabolism and bioactivation pathways of RTV in the livers of hPXR/CYP3A4 mice, but not in hPXR/Cyp3a-null mice (Supplemental Figure 7). The major metabolism and bioactivation pathways of RTV were M1 followed by M13 (Supplemental Figure 7A and 7B), and both metabolites are formed by CYP3A (20). Compared to the control group, the production of M1 and M13 increased 19- and 7-fold, respectively, in liver microsomes of hPXR/CYP3A4 mice pretreated with RIF (Supplemental Figure 7C and 7D). Accompanied
with M1, 2-isopropylthiazole-4-carbaldehyde (M1-1) was produced, which can be further metabolized to form an adduct with glutathione (GSH) (20). 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 result in liver injury (29, 30). These data indicate that PXR-mediated CYP3A4 induction increases RTV bioactivation in the liver, which can potentially lead to liver damage.

CYP-mediated drug bioactivation can cause cellular stress (31, 32). Using a metabolomic approach, we found a dramatic increase in ophthalmic acid (OA), a biomarker of oxidative stress (33), in the livers 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 3A-3D). In addition, pretreatment with RIF followed by RTV in hPXR/CYP3A4 mice caused upregulation of mRNAs encoded by 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 3E-3G). Similar to the adverse drug interactions between RIF and RTV, lead-in treatment with the PXR activator EFV followed by RTV also caused oxidative stress in the livers of hPXR/CYP3A4 mice, but not in hPXR/Cyp3a-null mice (Supplemental Figure 8). These data indicate that pretreatment with PXR activators followed by RTV causes oxidative stress in the liver, which is dependent on CYP3A4.

Oxidative stress can lead to endoplasmic reticulum (ER) stress (34). 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 4A). In addition, lead-in treatment with RIF followed by RTV caused severe ER stress in the livers of hPXR/CYP3A4 mice, as indicated by increased expression of ER stress biomarkers mRNAs including C/EBP homologous protein (Chop), binding immunoglobulin protein (Bip), and cyclic AMP-dependent transcription factor 3 (Atf3) (Figure 4B and 4C). ER stress also occurred in the livers of hPXR/CYP3A4 mice pretreated with EFV for seven days followed by RTV, but not in hPXR/Cyp3a-null mice with the same treatment (Supplemental Figure 9). Persistent ER stress can lead to cell death (34). Concordantly, we observed a significant increase in the expression of mRNAs encoded by 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 4D-4F). These data suggest that lead-in treatment with PXR activators followed by RTV causes ER stress and hepatocellular injury, and it is CYP3A4-dependent.

The ER is critical for protein maturation, including posttranslational modification and proper folding (34). 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 leading to ER stress and hepatocellular injury (Figure 4G).

In summary, the current study demonstrated the essential roles of human PXR and CYP3A4 in RTV hepatotoxicity. These 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.
Methods

Details of experimental procedures are provided in the Supplemental Methods.

Statistics.  GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) was used for statistical analysis.  All data are shown as mean ± 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.

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

X.M., and A.I.S. conceived the project and wrote the manuscript. A.I.S., J.L., P.W., J.Z., Y.W., and X.M. performed the experiments. D.Y., W.X., F.J.G., and X.M. contributed to the new reagents, analytic tools, and animal models. X.M., W.X., F.J.G., and D.M. contributed to the scientific discussion and experimental design.
Acknowledgments

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References


30. Mizutani T, and Suzuki K. Relative hepatotoxicity of 2-(substituted phenyl)thiazoles and substituted thiobenzamides in mice: evidence for the involvement of thiobenzamides as


Figure 1. Role of human PXR in the hepatotoxicity cause 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 for seven days followed by RTV. (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 ± 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 2

A

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E (hPXR/CYP3A4, Control)

![Image](image7.png)

F (hPXR/Cyp3a-null, Control)

![Image](image8.png)
Figure 2. 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 seven days followed by RTV. (A) Genotyping results of hPXR/Cyp3a-null 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 seven days. Gapdh was used as a loading control. (C, D) Activities of ALT and AST in the serum. All data are expressed as means ± S.E.M. (*n* = 3-4). Statistical significance was determined by two-way ANOVA with Tukey’s post hoc test. ****P < 0.0001. (E, 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; original magnification 400X.
Figure 3. Metabolomics reveals oxidative stress in the liver of hPXR/CYP3A4 mice pretreated with RIF for seven 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 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. Ophthalmic acid (OA), a biomarker of oxidative stress, was identified as a top-ranking ion in the RIF+RTV group. (C) Structural illustration of OA by 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 qPCR. All data are expressed as means ± S.E.M. (n = 3-4). Statistical significance was determined by two-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 4

Figure 4. 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 followed by RTV. N, nucleus; (†) dilated ER; original magnification 4,000X. (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 ± 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.
Supplemental Material

Supplemental Methods

Supplemental Figure 1. Histological analysis of the liver in WT mice pretreated with RIF followed by RTV.

Supplemental Figure 2. Effects of RIF on the expression of PXR target genes in the liver of hPXR/CYP3A4 mice.

Supplemental Figure 3. Histological analysis of the liver in hPXR/CYP3A4 mice pretreated with RIF followed by RTV.

Supplemental Figure 4. Effects of RIF on the expression of PXR target genes in the liver of hPXR/Cyp3a-null mice.

Supplemental Figure 5. Roles of human PXR and CYP3A4 in the hepatotoxicity associated with lead-in treatment with EFV followed by RTV.

Supplemental Figure 6. Effects of RTV on RIF metabolism and disposition in hPXR/CYP3A4 mice.

Supplemental Figure 7. RIF-mediated PXR activation and CYP3A4 induction increase RTV metabolism and bioactivation.

Supplemental Figure 8. Oxidative stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV followed by RTV.

Supplemental Figure 9. ER stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV followed by RTV.

Supplemental Table 1. Primers for qPCR analysis.
Supplemental Methods

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.

Animals and treatment. The PXR- and CYP3A4-humanized mouse models were developed based on previously generated hPXR, TgCYP3A4/hPXR, and Cyp3a-null mouse models (1-3). In brief, CYP3A4 transgenic mice were generated by bacterial artificial chromosome (BAC) transgenesis, which contains the complete CYP3A4 and CYP3A7 genes including PXR response element (PXRE) (3). In Cyp3a-null mice, all eight Cyp3a genes were eliminated (1). TgCYP3A4/hPXR mice were crossed with Cyp3a-null mice to generate a mouse model expressing human PXR and CYP3A4, but deficient in the mouse Pxr and Cyp3a genes (hPXR/CYP3A4). In addition, Cyp3a-null mice were crossed with hPXR mice to generate a mouse model expressing human PXR, but deficient in the human CYP3A4 gene and the mouse Pxr and Cyp3a genes (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. 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 50 mg/kg of RTV (two doses, ip) or corn oil (vehicle). All mice were killed on the ninth day and blood and liver samples collected for evaluation of liver injury.

Assessment of liver injury. Alanine transaminase (ALT) and aspartate transaminase (AST) levels 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 µm sections were cut and stained with hematoxylin and eosin (H&E). For transmission electron microscopy (TEM), fresh liver samples (~ 1 mm³) 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.

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 by stable transfection of human PXR cDNA and a luciferase reporter gene. Briefly, DPX2 cells were cultured in a 96-well plate with a density of 2 × 10³ cells/ml. Ten µM RIF or EFV were incubated in DPX2 cells for 24 h followed by analysis of luciferase activity. The experiments were conducted in triplicate.
**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 μg of total RNA with a SuperScript II Reverse Transcriptase kit and random oligonucleotides (Invitrogen, Carlsbad, CA). PCR primers (Table S1) 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 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 μL. 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.

**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% 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.

**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 200 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).

**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 for seven days. Incubations were conducted in 1 × phosphate buffered saline (PBS, pH 7.4) containing 10 μM RTV and 0.2 mg MLM in a final volume of 95 μL. After 5 min of pre-incubation at 37 °C, the reaction was initiated by the addition of 5 μL of 20 mM NADPH and continued for 40 min with gentle shaking. Incubations were terminated by adding 100 μL 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-TOFMS.

**UPLC-TOFMS analysis.** Metabolite separation was performed on an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm; 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. TOFMS 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 eV was used for structural elucidations of metabolites.

**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 µL of water). Two hundred µL of acetonitrile: methanol (1:1, v/v) was added to 100 µL 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 using MarkerLynx software (Waters, Milford, MA). 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.

**References**


Supplemental Figure 1. Histological analysis of the liver in WT mice pretreated with RIF for seven days followed by RTV. H&E staining; CV, central vein; original magnification 400X.
Supplemental Figure 2. Effects of RIF on the expression of PXR target genes in the liver of hPXR/CYP3A4 mice. Mice were treated with RIF for seven days. CYP3A4 (A), Cyp2b10 (B), Gsta1 (C), Gstm1 (D), and Gstm2 (E) mRNAs were analyzed by qPCR. All data are expressed as means ± 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.
Supplemental Figure 3. Histological analysis of the liver in hPXR/CYP3A4 mice pretreated with RIF for seven days followed by RTV. H&E staining; CV, central vein; ψ indicates hepatocyte degeneration; original magnification 400X.
Supplemental Figure 4. Effects of RIF on the expression of PXR target genes in the liver of hPXR/Cyp3a-null mice. Mice were treated with RIF for seven days. 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.
Supplemental Figure 5. Roles of human PXR and CYP3A4 in the hepatotoxicity associated with lead-in treatment with EFV followed by RTV. (A) Effect of EFV (10 μM) on PXR activation in a cell-based reporter assay (n = 3). The data in control group were set as 1. RIF (10 μM) was used as a positive control. (B-F) Evaluation of liver damage in hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by RTV. (B, C) Activities of ALT and AST in the serum. All data are expressed as means ± 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; original magnification 400X.
Supplemental Figure 6. Effects of RTV on RIF metabolism and disposition in hPXR/CYP3A4 mice. Feces and liver samples were collected overnight after treatment with RIF or RIF plus RTV. 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 ± 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.
Supplemental Figure 7. RIF-mediated PXR activation and CYP3A4 induction increase 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 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 ± 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.
Supplemental Figure 8. Oxidative stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by RTV. *Gpx2* (A), *Cbr3* (B), and *Slc25a24* (C) mRNAs were analyzed by qPCR. All data are expressed as means ± 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.
Supplemental Figure 9. ER stress in the liver of hPXR/CYP3A4 and hPXR/Cyp3a-null mice pretreated with EFV for seven days followed by RTV. Chop (A), Bip (B), and Atf3 (C) mRNAs were analyzed by qPCR. All data are expressed as means ± 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.
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