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Pregnane X receptor activation potentiates ritonavir hepatotoxicity

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Introduction

With the advent of antiretroviral therapy (ART), the overall mortality and morbidity in HIV/AIDS patients has decreased substantially. However, the toxicity of ART is one of the major concerns of 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 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 for treating tuberculosis) or efavirenz (EFV) (a nonnucleoside reverse transcriptase inhibitor for treating HIV) followed by RTV-containing regimens (6–9). We first used 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 7 days followed by RTV (Figure 1, B and C). In addition, histological analysis revealed massive hepatocyte degeneration in hPXR/CYP3A4 mice pretreated with RIF followed by RTV (Figure 1, B and C). These data suggest interspecies 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 interspecies 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 7 days followed by RTV (Figure 1, E and F). 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 treat-
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AL 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 7 days potentiated RTV hepatotoxicity in hPXR/CYP3A4 mice (Supplemental Figure 5, B–F), which mimicked EFV- and RTV-induced liver injury observed in a previous clinical study (9). In addition, lead-in treatment with EFV for 7 days resulted in hepatocyte degeneration (Supplemental Figure 5E), exhibiting the same phenotype as RIF- and RTV-induced liver damage (Supplemental Figure 3).

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 7 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 7, A and B), and both metabolites are formed by CYP3A (20).

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 7 days significantly induced PXR target genes other than Cyp3a in the liver of hPXR/Cyp3a-null mice (Figure 2B and Supplemental Figure 4), indicating that PXR is functionally intact in these mice. Compared with hPXR/CYP3A4 mice, no liver injury was observed in hPXR/Cyp3a-null mice pretreated with RIF for 7 days followed by RTV (Figure 2, C–F), 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, such as constitutive androstane receptor (CAR), also upregulate CYP3A4 expression (25) and many CAR activators are found among 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 7 days potentiated RTV hepatotoxicity in hPXR/CYP3A4 mice (Supplemental Figure 5, B–F), which mimicked EFV- and RTV-induced liver injury observed in a previous clinical study (9). In addition, lead-in treatment with EFV for 7 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 1, E and F, and Supplemental Figure 5, B and C), which could be the result of enterohepatic circulation of RIF leading to long-term 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 5, B–F). 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. RIF had no effect on RTV metabolism and disposition (Supplemental Figure 6). However, pretreatment with RIF for 7 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 7, A and B), and both metabolites are formed by CYP3A (20). Compared with 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 7, C and D),
tase 3 (Cbr3), and solute carrier family 25 member 24 (Slc25a24) (Figure 3, E–G).

Similarly 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 pre-treatment 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 7 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, 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 7 days followed by RTV (Figure 4A).

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 7 days followed by RTV, but not in hPXR/Cyp3a-null mice with the same treatment (Figure 3, A–D). 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 (Chr3), and solute carrier family 25 member 24 (Slc25a24) (Figure 3, E–G). Similarly 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 pre-treatment 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 7 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 biomarker mRNAs, including C/EBP homologous protein (Chop), binding immunoglobulin protein (Bip), and cyclic AMP-dependent transcription factor 3 (Atf3) (Figure 4, B and C). ER stress also occurred in the livers of hPXR/CYP3A4 mice pretreated with EFV for 7 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 7 days followed by RTV, but not in hPXR/Cyp3a-null mice (Figure 4, D–F). These data suggest that lead-in treatment with PXR activators followed by RTV causes ER stress and hepatocellular injury and that it is CYP3A4 dependent.
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.

The ER is critical for protein maturation, including post-translational modification and proper folding (34). The activation of PXR, being a transcription factor, by ligands such as 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. Primers are listed in Supplemental Table 1.

Statistics. GraphPad Prism 7.0 (GraphPad Software) was used for statistical analysis. All data are shown as mean ± SEM. One-way or 2-way ANOVA was used with Tukey’s post hoc test to compare the differences among multiple groups. Two-tailed unpaired Student’s t test was performed for statistical analysis between 2 groups. 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
XM and AIS conceived the project and wrote the manuscript. AIS, JL, PW, JZ, YW, and XM performed the experiments. DY, WX, FJG, and DM contributed to the new reagents, analytic tools, and animal models. XM, WX, FJG, and DM contributed to the scientific discussion and experimental design.

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