Rifaximin Is a Gut-Specific Human Pregnane X Receptor Activator

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ABSTRACT

Rifaximin, a rifamycin analog approved for the treatment of travelers’ diarrhea, is also beneficial in the treatment of multiple chronic gastrointestinal disorders. However, the mechanisms contributing to the effects of rifaximin on chronic gastrointestinal disorders are not fully understood. In the current study, rifaximin was investigated for its role in activation of the pregnane X receptor (PXR), a nuclear receptor that regulates genes involved in xenobiotic and limited endobiotic deposition and detoxication. PXR-humanized (hPXR), Pxr-null, and wild-type mice were treated orally with rifaximin, and rifampicin, a well-characterized human PXR ligand. Rifaximin was highly concentrated in the intestinal tract compared with rifampicin. Rifaximin treatment resulted in significant induction of PXR target genes in the intestine of hPXR mice, but not in wild-type and Pxr-null mice. However, rifaximin treatment demonstrated no significant effect on hepatic PXR target genes in wild-type, Pxr-null, and hPXR mice. Consistent with the in vivo data, cell-based reporter gene assay revealed rifaximin-mediated activation of human PXR, but not the other xenobiotic nuclear receptors constitutive androstane receptor, peroxisome proliferator-activated receptor (PPAR)α, PPARγ, and farnesoid X receptor. Pretreatment with rifaximin did not affect the pharmacokinetics of the CYP3A substrate midazolam, but it increased the Cmax and decreased T1/2 of 1’’-hydroxymidazolam. Collectively, the current study identified rifaximin as a gut-specific human PXR ligand, and it provided further evidence for the utility of hPXR mice as a critical tool for the study of human PXR activators. Further human studies are suggested to assess the potential role of rifaximin-mediated gut PXR activation in therapeutics of chronic gastrointestinal disorders.

Rifaximin (RIFx; Xifaxan) was approved by the Food and Drug Administration in 2004 for the treatment of travelers’ diarrhea (Laustsen and Wimmett, 2005). RIFx was shown to be a general antibiotic that acts to inhibit bacterial RNA synthesis. Interestingly, accumulated data revealed the potential value of RIFx in the treatment of chronic gastrointestinal disorders. RIFx was effective in both acute and chronic hepatic encephalopathy (Loguercio et al., 2003; Mas et al., 2003). Several recent clinical trails have recommended the use of RIFx for prevention of the main complications in patients with diverticular disease (Latella et al., 2003; Pistoia et al., 2004; Papi et al., 2005). In patients with gas-related symptoms, the colonic production of H2 is increased, and RIFx significantly reduces this production and the excessive number of flatus episodes (Di Stefano et al., 2000). A combination of RIFx and ciprofloxacin was effective in patients with active chronic, treatment-resistant pouchitis (Gionchetti et al., 1999; Abdelrazeg et al., 2005). In addition, RIFx was beneficial in ulcerative colitis and Crohn’s disease (CD), two chronic inflammatory diseases generally referred to as inflammatory bowel disease; PXR, pregnane X receptor; DSS, dextran sulfate sodium; PCN, pregnenolone 16α-carbonitrile; RIF, rifampicin, 3-(4-methylpiperazinyl)-liminomethyl]rifamycin SV; hPXR, PXR-humanized; MDZ, midazolam; 1’’-OH-MDZ, 1’’-hydroxymidazolam; WT, wild-type; AUC, area under the serum concentration-time curve; LC-MS/MS, liquid chromatography-tandem mass spectrometry; qPCR, quantitative real-time polymerase chain reaction; GSTA, glutathione S-transferase α; MRP, multidrug resistance protein; OATP, organic anion transporting polypeptide; CAR, constitutive androstane receptor; PPAR, peroxisome proliferator-activated receptor; FXR, farnesoid X receptor; DMSO, dimethyl sulfoxide; Wy-14,643, pirinixic acid, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; WT, wild-type mice; Fwd, forward; Rev, reverse; S. intestine, small intestine; GW4064, 3-[2-[2-chloro-4-[[3-[2,6-dichlorophenyl]-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic.
to as inflammatory bowel diseases (IBDs). Despite the differences in dose and duration, RIFax was beneficial in active ulcerative colitis, mild-to-moderate CD as well as prevention of postoperative recurrence of CD (Shafran and Johnson, 2005; Gionchetti et al., 2006; Guslandi et al., 2006). The mechanism contributing to the beneficial effects of RIFax in chronic gastrointestinal disorders are not fully understood. Recently, it was revealed that the susceptibility to IBD was strongly associated with genetic variation in the pregnane X receptor gene (PXR; NR1I2), a member of the nuclear receptor family (Langmann et al., 2004; Dring et al., 2006). In a dextran sulfate sodium (DSS)-induced IBD mouse model, pregnenolone 16α-carbonitrile (PCN), a rodent-specific PXR ligand, demonstrated a protective role in DSS-induced colitis (Shah et al., 2007).

In the current study, RIFax was investigated for its role in activation of the PXR. PXR is an integral component of the body’s defense mechanism involved in endogenous and xenobiotic detoxication (Kliewer et al., 2002). PXR is activated by a broad spectrum of xenobiotics, including prescription drugs, herbal supplements, pesticides, endocrine disrupters, and other environmental contaminants (Carnahan and Redinbo, 2005). PXR activation regulates a number of genes involved in the metabolism and excretion of xenobiotics, including toxic chemicals (Kliewer, 2003; Rosenfeld et al., 2003; Sonoda et al., 2005). Several independent observations have led to the hypothesis of RIFax as a potential human gut PXR ligand: 1) the high degree of structural similarity between RIFax and rifampicin (RIF) (Fig. 1, A and B), a well known human PXR ligand (Bertilsson et al., 1998); 2) the high expression of PXR in human gut (Miki et al., 2005) and the high RIFax concentration in gut following oral treatment (Jiang et al., 2000); and 3) the induction of CYP3A4, a bona fide PXR target gene, following RIFax incubation in a human hepatocyte model (http://www.salix.com/). To test this hypothesis, a novel animal model was used, PXR-humanized (hPXR) mice, in which the entire human PXR gene was reintroduced into the Pxr-null background (Ma et al., 2007).

The present study identified RIFax as a human PXR ligand, and it provides further evidence for the utility of hPXR mice as a critical tool for human PXR study.

**Materials and Methods**

**Chemicals.** RIF, RIFax, and midazolam (MDZ) were obtained from Sigma-Aldrich (St. Louis, MO). 1′-Hydroxymidazolam (1′-OH-MDZ) was purchased from BD Gentest (Woburn, MA). All other chemicals were of the highest grade commercially available.

**Animals and Treatments.** hPXR, Pxr-null, and wild-type (WT) mice were maintained under a standard 12-h light/12-h dark cycle with water and chow provided ad libitum. Pxr-null and hPXR mice were described previously (Staudinger et al., 2001; Ma et al., 2007). To investigate the potential role of RIFax in PXR activation, 2- to 4-month-old male hPXR, Pxr-null, and WT mice were treated orally with 25 mg/kg/day RIFax for 3 days. RIF, a specific human PXR ligand, was used as positive control at 25 mg/kg/day (p.o.) for 3 days. Corn oil was used as vehicle for both RIF and RIFax treatment. All mice were killed by CO2 asphyxiation 24 h after the last dose. Liver and small intestine were collected and frozen at −80°C for further analysis. Handling was in accordance with animal study protocols approved by the National Cancer Institute Animal Care and Use Committee.

**RIFax Pharmacokinetics and Its Distribution in Intestinal Tract.** For pharmacokinetic analysis, WT, Pxr-null, and hPXR mice were treated with 10 mg/kg RIF or RIFax by oral gavage. Corn oil was used as vehicle for both RIF and RIFax treatment. Blood samples were collected from suborbital veins using heparinized tubes at predose and 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h after the administration. To compare the metabolic profiles of RIFax and RIF, 10 mg/kg RIFax and RIF were administered by i.v. and i.p. For i.p. injections, corn oil was used as vehicle for both RIF and RIFax, and blood samples were collected from suborbital veins at predose and 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h after the administration. For i.v. injections, 30% polyethylene glycol (wt 400) was used as vehicle for both RIF and RIFax, and blood samples were collected from suborbital veins at predose and 0.8833, 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h after the administration. Serum was separated by centrifugation at 8000g for 10 min. Fifty microliters of serum was mixed with 150 μl of methanol, vortexed twice for 20 s, and centrifuged at 14,000 rpm for 10 min at 4°C. The upper organic layer was then transferred to an autosampler vial for RIF or RIFax detection by LC-MS/MS. To detect the distribution in intestinal tract, mice were treated with 10 mg/kg RIFax or RIF (p.o.). At 1.5, 3, 6, 9, 12, 24, and 48 h after administration, the mice were killed, and the contents in different segments of the intestinal tract were collected. Intestinal contents were weighted and homogenized in 100 mg/ml methanol. The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The upper organic layer was then transferred to an autosampler vial for RIF or RIFax detection by LC-MS/MS.

**Analysis of RIFax and RIF by LC-MS/MS.** RIFax and RIF were determined by LC-MS/MS, carried out using a high-performance liquid chromatography system consisting of a PerkinElmer Series 200 quaternary pump, vacuum degasser, and autosampler with a 100-μl loop interface to LC-MS/MS as noted above. RIFax and RIF were separated on a Luna C18 (50 × 4.6 mm i.d.) column (Phenomenex, Torrance, CA). The flow rate through the column at ambient temperature was 0.25 ml/min with 88% methanol and 15% H2O.

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Fig. 1. LC-MS/MS analysis of RIF and RIFax. A, structure of RIF. B, structure of RIFax. C, typical chromatogram of RIF and RIFax. RIF and RIFax were detected by LC-MS/MS; m/z 823.5/791.5 for RIF (peak 1) and m/z 786.3/754.5 for RIFax (peak 2).
cells were plated on 24-well plates (5 × 10⁴ cells/well, cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum), and transfected with the various expression vectors using FuGENE transfection reagent (Roche Diagnostics, Indianapolis, IN). The mouse PPAR and CAR vectors were described in previous reports (Kliwer et al., 1992; Swales et al., 2005). The mouse FXR vector was provided by Dr. Christopher J. Sinal (Dalhousie University, Halifax, NS, Canada). After 24 h post-transfection, the cells were incubated with vehicle (DMSO) and 10 μM RIFax for 24 h. We used 250 nM 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, 10 μM Wy-14,643, 10 μM miglustat, and 25 μM GW4064 as positive controls for mouse CAR, PPARα, PPARγ, and FXR, respectively. A standard dual luciferase assay was used and normalized to a cotransfected control reporter (Promega, Madison, WI). Each in vitro assay was repeated at least three times.

Statistical Analysis. All values are expressed as the mean ± S.D., and data were analyzed by two-tailed Student’s t test. p < 0.05 was regarded as significantly different between groups.

Results

Metabolic Profiles and Intestinal Tract Distribution of RIFax in Mice. LC-MS/MS was used to develop assays to study the pharmacokinetics of RIF and RIFax. The retention time was 2.21 min for RIF, m/z 823.5/791.5 (Fig. 1C, peak 1), and 3.03 min for RIFax, m/z 786.3/754.5 (Fig. 1C, peak 2). The detection limit was 0.023 pmol for RIF and 0.012 pmol for RIFax. After a single oral dose of RIF or RIFax, mouse blood samples and intestinal contents were collected at different time points up to 48 h after treatment. In the pharmacokinetic study, the Cmax of serum RIFax was 0.04 μM, ~70-fold lower than that of 2.75 μM RIF. The AUC0–48 h of serum RIFax was ~300-fold lower than that of RIF (Fig. 2A). However, for intestinal tract distribution, the RIFax concentration was significantly higher than that of RIF. In the small intestine, the RIF concentration was below 20 μg/g at all time points measured (Fig. 2B). For RIFax, the concentration was ~160 μg/g, and it lasted up to 9 h after administration. The RIFax intestinal tract distribution in the cecum (Fig. 2C) and colon (Fig. 2D) was similar to that of the small intestine. No significant difference in RIFax metabolism was found among WT, Pxr-null, and hPXR mice after oral treatment. The Cmax values of RIFax (p.o. treatment) in WT, Pxr-null, and hPXR mice are shown in Fig. 2E. RIFax is well known as nonabsorbable rifamycin by oral treatment. By i.p. injection, RIFax was not well absorbed, and the bioavailability was significant lower than that of RIF (Fig. 2, F and G). Differences in metabolic profiles between RIFax and RIF were observed after i.v. treatment as ultrashort t1/2 and low AUC for RIFax compared with RIF (Fig. 2H).

PXR Activation by RIFax. PXR was detected in duodenum, jejunum, ileum, cecum, and colon, but not in stomach of WT and hPXR mice (Ma et al., 2007). Due to the high distribution of RIFax in the intestinal tract and expression of PXR in the gut, the effect of RIFax on gut PXR target genes was investigated by qPCR. In the small intestine of hPXR mice treated with RIFax, CYP3A11, GSTA1, MRP2, and OATP2 were all up-regulated (Fig. 3A). Intestinal CYP3A11 was increased ~4-fold compared with vehicle-treated hPXR mice, whereas expression was inhibited in WT mice, and no significant change was observed in Pxr-null mice (Fig. 3, B and C). Intestinal GSTA1 mRNA was up-regulated in all three mouse strains after RIFax treatment, with 87, 74, and 172% increases noted in WT, Pxr-null, and hPXR mice, suggesting

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<th>Primer Sequence</th>
<th>CYP3A11</th>
<th>Fwd: 5'-ACG AGG GAT GGA CCT GG-3'</th>
<th>Rev: 5'-GCG TAG AGG AAC ACC AA-3'</th>
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<tr>
<td>GSTA1</td>
<td>Fwd: 5'-CAC CCT GCC ACC AGC CAG A-3'</td>
<td>Rev: 5'-TCT GGT GCT CCA TCA ATG CA-3'</td>
<td></td>
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<tr>
<td>MRP2</td>
<td>Fwd: 5'-GCT GCC GCC TCT TTT TGG GCC AAT A-3'</td>
<td>Rev: 5'-TCA CAC CTT TTT TGG GCC ATT-3'</td>
<td></td>
</tr>
<tr>
<td>OATP2</td>
<td>Fwd: 5'-TCC GCA CTT CCA ACA AAA ATT CTG-3'</td>
<td>Rev: 5'-ACC TGA CAT GTA TAG ACC ATT GTC-3'</td>
<td></td>
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Pfd, forward; Rev, reverse.
that the Gsta1 gene may not be a direct PXR target but that it may be elevated by an indirect mechanism. One possible explanation for the effect of RIFax on GSTA1 is the antibiotic activity of RIFax. In the current study, RIFax was administered orally at 25 mg/kg for 3 days, which may change the gut bacterial composition and indirectly effect GSTA1 expression. A slight but significant up-regulation of intestinal MRP2 mRNA was noted in hPXR mice after RIFax treat-
ment, whereas its expression was significantly suppressed in WT mice, with no change observed in Pxr-null mice (Fig. 3, B and C). MRP2, which was reported to be activated by RIF and PCN in human and rat hepatocytes, respectively (Kast et al., 2002), was not markedly induced by RIF in liver and only modestly induced by RIF or RIFax in the gut in the current study. Indeed, others found that MRP2 is not significantly induced by mouse PXR ligands such as PCN (Maher et al., 2005). The finding that MRP2 is not induced by RIF in the hPXR mice suggests a possible species difference in the cis-elements controlling the Mrp2 gene between humans and mice. Intestinal OATP2 mRNA was increased 3.4-fold in hPXR mice after RIFax treatment, but no significant induction of this mRNA was noted in both WT and Pxr-null mice (Fig. 3, B and C). As expected, RIF also induced the four mRNAs in intestine but the extent of induction was less than that observed with RIFax (Fig. 3D). In contrast, RIF produced a significant induction of CYP3A11, GSTA1, and OATP2 in liver, whereas only GSTA1 mRNA was increased in the liver of RIFax-treated hPXR mice (Fig. 3, E and F). These data indicate that RIFax is a gut-specific human PXR ligand.

**Human PXR Activation by RIFax in a Cell-Based Reporter Assay.** A dose-dependent increase in luciferase activity was observed in a cell-based reporter assay for hPXR activation by RIFax. Incubation with 1, 10, and 100 μM RIFax in the hPXR reporter system produced a 2.1-, 6.7-, and 25.2-fold increase, respectively, versus DMSO control (Fig. 4A). RIFax at 100 nM had no significant effect on hPXR, whereas 10 μM RIFax produced no significant change in luciferase activity in the presence of PPARα, PPARγ, CAR, and FXR (Fig. 4B).

**Pharmacokinetic of MDZ in hPXR Mice Pretreated with RIFax.** After a single oral administration of 2.5 mg/kg MDZ, the serum concentration-time course of MDZ and 1'-OH-MDZ in hPXR mice was determined. Pharmacokinetic parameters were estimated by noncompartmental analysis. There were no significant changes (p > 0.05) for the Cmax, Tmax, and AUC0–90 min of MDZ in hPXR mice pretreated with or without RIFax. The RIFax pretreatment in hPXR mice had no significant effect on AUC0–90 min of 1'-OH-MDZ, the major metabolite of MDZ. However, the Cmax value of 1'-OH-MDZ was 50% higher (p < 0.05) in RIFax-pretreated hPXR mice, and the corresponding Tmax was 2-fold shorter than the control group (Table 2). These results suggested that the RIFax-mediated CYP3A11 up-regulation in hPXR mice intestine contributed to extrahepatic first-pass metabolism of MDZ.

**Discussion**

In the current study, the effect of RIFax on PXR was investigated. By using hPXR, Pxr-null, and WT mice, and a cell-based human PXR reporter gene assay, RIFax was identified as a gut-specific human PXR ligand. During the pharmaceutical development of RIFax, CYP3A4 induction by RIFax was noted in a human hepatocyte model (http://www.salix.com/). However, to our knowledge, there was no further study on the mechanism of RIFax-mediated CYP3A4 induction. The current study is the first report indicating RIFax as
a gut-specific human PXR ligand that up-regulates PXR target genes, including CYP3A. In the DPX2 cell line with stable recombinant human PXR expression, hPXR was significantly activated at RIFax concentrations over 1 μM, as indicated by at least a 2.1-fold increased luciferase activity versus vehicle. The EC₅₀ for activation of hPXR by RIFax in the DPX2 cell line was estimated to be around 20 μM. The RIFax concentration in intestine is much higher than 20 μM after RIFax treatments. In the current study, when mice were treated with 10 mg/kg RIFax (single dose p.o.), the RIFax concentration in the intestinal tract was up to 150 μg/g (∼200 μM) intestinal content. In humans, after 3 days of RIFax treatments, intestinal CYP3A induction and potential drug-drug interactions should be reassessed in future studies because of intestinal first-pass effects (Doherty and Charman, 2002; Granvil et al., 2003). Overall, RIFax-mediated intestinal CYP3A induction and potential drug-drug interactions should be reassessed in future studies because of intestinal first-pass effects (Doherty and Charman, 2002; Granvil et al., 2003).

The beneficial aspects of PXR activation is its role in detoxication by up-regulating the enzymes and transporters involved in elimination of the xenobiotics, including cytochromes P450, GST, OATP, and MRP (Kliewer, 2003; Saini et al., 2005; Wagner et al., 2005). PXR target genes are critical components in intestinal barrier function against xenobiotics and bacteria (Langmann et al., 2004). In the small intestine of hPXR mice treated with RIFax, several intestinal genes including transporters were up-regulated, such as the influx transporter OATP2, which may contribute to the increase of MDZ absorption. A bioavailability study on MDZ was not performed because of its poor bioavailability and large variation in mice (Granvil et al., 2003). Overall, RIFax-mediated intestinal CYP3A induction and potential drug-drug interactions should be reassessed in future studies because of intestinal first-pass effects (Doherty and Charman, 2002; Granvil et al., 2003).
Pelosini, 2005). The mechanisms contributing to the beneficial effects of Rifaxin in chronic gastrointestinal disorders are not fully understood, and they cannot be explained simply as Rifaxin antibiotic activity. Recent studies revealed that the susceptibility to IBD was strongly associated with the genetic variation in the PXR gene, and several PXR genes were dysregulated or downregulated in both ulcerative colitis and Crohn’s disease patients (Langmann et al., 2004; Dring et al., 2006). In a DSS-induced IBD mouse model, PCN-mediated PXR activation significantly prevented DSS-induced colitis (Shah et al., 2007), which indicates the potential value of PXR ligands as a therapeutic for IBD. Further human studies are suggested to assess the role of Rifaxin-mediated gut PXR activation in therapeutics of chronic gastrointestinal disorders.

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References


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