DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 79160

Role of c-Jun-N-terminal kinase in pregnane X receptor-mediated induction of human cytochrome P4503A4 in vitro

Guncha Taneja, Chun Chu, Paramahamsa Maturu, Bhagavatula Moorthy, and Romi Ghose

G.T & R.G - Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX. 1441 Moursund Street, Houston TX 77030, USA.

C.C, P.M & B.M –Department of Pediatrics, Baylor College of Medicine, 1102 Bates Avenue, Suite 530, Houston, TX 77030, USA

Current affiliation for G.T- DILIsym Services, A Simulations Plus Company, Research Triangle Park, North Carolina, 27709, USA

Primary Laboratory of Origin: RG

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 79160

Running Title: JNK: Novel regulator of PXR-mediated CYP3A4 induction *in vitro*

Corresponding author: Romi Ghose, Ph.D.

College of Pharmacy

Department of Pharmacological & Pharmaceutical Sciences

University of Houston

1441 Moursund Street

Houston, TX 77030, USA

Tel: 832-842-8343

Fax: 832-842-8305

E-mail: rghose@central.uh.edu

Co-Corresponding author: Bhagavatula Moorthy, Ph.D.

Number of text pages: 18

Number of tables: 0

Number of figures: 7

Number of references: 68

Number of words- a) Abstract: 231

b) Introduction: 812

c) Discussion: 1333

Section Assignment: Metabolism, Transport, and Pharmacogenomics

Abbreviations: CYP, Cytochrome P450; NR, nuclear receptor; RIF, rifampicin; PXR, pregnane X-receptor; VDR, vitamin D receptor; AUC, area under the curve; JNK, c-JUN N-terminal kinase; CRPs, cytoplasmic retention proteins.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Cytochrome P450 (CYP) 3A4 is the most abundant drug metabolizing enzyme and is responsible for the metabolism of ~50% of clinically available drugs. Induction of CYP3A4 impacts the disposition of its substrates and leads to harmful clinical consequences such as failure of therapy. In order to prevent such undesirable consequences, molecular mechanisms of regulation of CYP3A4 need to be fully understood. *CYP3A4* induction is primarily regulated by the xenobiotic nuclear receptor, pregnane-X-receptor (PXR). After ligand binding, PXR is translocated to the nucleus, where it binds to the *CYP3A4* promoter and induces its gene expression. It is known that PXR function is modulated by phosphorylation(s) by multiple kinases. In this study, we determined the role of the c-Jun-N-terminal kinase (JNK) in PXR-mediated induction of CYP3A4 enzyme *in vitro*. HepG2 cells were transfected with CYP3A4 luciferase and PXR plasmids, followed by treatment with JNK inhibitor (SP600125; SP) and PXR activators, rifampicin (RIF) or hyperforin. Our results indicate that SP treatment significantly attenuated PXR-mediated induction of *CYP3A4* reporter activity, as well as gene expression and enzyme activity. JNK knockdown by siRNA (targeting both JNK 1 and 2) also attenuated CYP3A4 induction by RIF. Interestingly, SP treatment attenuated JNK activation by RIF. Furthermore, treatment with RIF increased PXR nuclear levels and binding to the *CYP3A4* promoter; SP attenuated these effects. This study shows that JNK is a novel mechanistic regulator of CYP3A4 induction by PXR.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cytochrome P450 (CYP) 3A4 enzyme is the most important contributor of hepatic and intestinal metabolism in adults (Guengerich, 1995; Leeder & Okey, 1996; Krishna & Klotz, 1994; Wilkinson, 2005). CYP3A4 is responsible for the metabolism of ~50% of drugs currently used therapeutically for a wide spectrum of disorders such as cancer, fungal/bacterial infections, neurological disorders, hepatitis, AIDS etc. (Guengerich, 1999; Veith et al, 2009; Zanger et al, 2013). CYP3A4 also plays a crucial endogenous role in bile acid detoxification and metabolism of steroid hormones (Waxman et al, 1991; Araya et al, 1999).

Induction of CYP3A4 enzyme expression/activity is known to alter the absorption, disposition, metabolism and/or elimination of co-administered drugs (Thummel & Wilkinson, 1998). For example, St. John wort's supplementation significantly increased the clearance and decreased the mean area under the curve (AUC) of the chemotherapeutic drug, docetaxel (Goey et al, 2014). Similarly, a phase 1 trial showed that CYP3A4 induction by the chemotherapeutic compound, bexarotene caused 50% reduction of AUC of atorvastatin, a lipid-lowering agent and CYP3A4 substrate (Wakelee et al, 2012). CYP3A4 induction is known to cause drug-drug interactions (DDIs), failure of therapy or drug toxicities in patients.

CYP3A4 gene is both constitutively expressed by a number of liver-specific transcription factors as well as nuclear receptors and transcriptionally induced (Martínez-Jiménez et al, 2007). CYP3A4 induction is mediated by nuclear receptors (NRs) such as pregnane-X-receptor (PXR), constitutive androstane receptor, glucocorticoid receptor and vitamin D-receptor (VDR) (Pascussi et al, 2003; Luo et al, 2004). Among these NRs, PXR is primarily responsible for CYP3A4 induction (Goodwin et al, 2002, Xie et al, 2004). PXR can be activated by structurally diverse moieties including rifampicin, ritonavir, clotrimazole etc (Harmsen et al, 2007; Lehmann et al,

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

1998). PXR is localized in the cytosol and upon ligand binding, it is activated and translocated into the nucleus where it heterodimerizes with retinoid-X–receptor to bind to the proximal PXR response element and distal xenobiotic responsive enhancer module on the *CYP3A4* promoter (Kliewer et al, 1998). Since *CYP3A4* gene activation by PXR is a critical determinant of metabolism, transport and elimination of potentially toxic chemicals, the mechanism of PXR-mediated *CYP3A4* regulation needs to be fully elucidated in order to improve therapeutic outcome in patients.

There is a growing body of evidence that phosphorylation of PXR provides an important mechanism for *CYP3A4* regulation (Staudinger et al, 2011). So far, PXR phosphorylation has been shown to have a repressive effect on the transcription of its target genes. PXR phosphorylation by cyclic AMP-dependent protein kinase A (PKA) represses *CYP3A4* gene expression in a species-specific manner (Ding et al, 2005a; Lichti-Kaiser et al, 2009a). Similarly, cyclin-dependent kinase (Cdk2 & 5) phosphorylates PXR to inhibit *CYP3A4* expression. PXR phosphorylation by Cdk2 at Ser350 inhibits *CYP3A4* gene expression in HepG2 cells (Lin et al, 2008). Activation of protein kinase C (PKC) by pro-inflammatory cytokines inhibits PXR transcriptional activity in hepatocytes (Ding et al, 2005b). Furthermore, PXR phosphorylation at Thr57 by p70 S6K inhibited PXR activity (Pondugula et al, 2009). Although direct phosphorylation of PXR is not involved, recent studies showed that casein kinase 2 (CK2)-mediated phosphorylation of heat shock protein 90 (HSP90) stabilizes PXR and induces its downstream genes (Kim et al, 2015). HSP90 is a cytoplasmic retention protein which binds to and sequesters PXR in the cytosol (Squires et al, 2004). Interestingly, the mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) was shown to be required for optimal activation of *CYP3A4* gene by 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which is a known ligand for the NR, VDR (Yasunami et al, 2004; Rochel et

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

al, 2000; Vanhooke et al, 2004). MAPKs consist of two additional members, extracellular signal-regulated kinase (ERK 1/2) and p38 kinase (Zhou et al., 2009). These serine/threonine kinases transduce extracellular signals from activated receptors on cell surface to cellular responses by phosphorylating transcriptional factors or downstream kinases (Houliston et al., 2001).

The goal of this investigation was to determine the mechanistic role of JNK in CYP3A4 induction by PXR *in vitro*. We observed that inhibition of JNK significantly attenuated PXR-mediated induction of CYP3A4 reporter activity, gene expression and enzyme activity in HepG2 cells. We also confirmed that CYP3A4 enzyme activity was attenuated by treatment of HepaRG cells with a JNK inhibitor. Furthermore, activation of JNK was observed upon treatment of HepG2 cells with the PXR ligand, rifampicin. Interestingly, JNK inhibitors also attenuated ligand-induced PXR nuclear translocation and binding to the *CYP3A4* promoter.

To date, PXR phosphorylation at multiple sites have been shown to inhibit PXR activity, leading to attenuation of CYP3A4 induction by PXR. To our knowledge, this is the first study showing that JNK is required for PXR-mediated CYP3A4 induction. This indicates that JNK may have a novel mechanistic role in CYP3A4 regulation by modulation of PXR function. These findings may provide insight into understanding functional interactions between cell signaling pathways and drug metabolism and their consequences in drug efficacy and/or toxicity.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Reagents and Materials. HepG2 cells were purchased from ATCC (Manassas, VA). HepaRG cells were purchased from Thermo Fisher Scientific (Waltham, MA, # HPRGC10). Rifampicin (3-4-Methylpiperazinyliminomethyl) rifamycin) (#R3501), Hyperforin (dicyclohexylammonium) salt ($C_{35}H_{52}O_4 \cdot C_{12}H_{23}N$) (#H1792), Curcumin (Diferuloylmethane) (#C1386) and Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). SP600125 (1, 9-Pyrazoloanthrone) (#tlrl-sp60), SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl] pyridine) (#tlrl-sb20) and PD098059 (2-(2-amino-3-methoxyphenyl) chromen-4-one) (#tlrl-pd98) were purchased from InvivoGen (San Diego, CA). A custom siRNA sequence (AGAAUGUCCUACCUUCUUUUU) that simultaneously targets JNK1 and JNK2 and a control siRNA targeting luciferase were both synthesized by Dharmacon (Lafayette, CO). DharmaFECT Duo Transfection reagent was also purchased from Dharmacon. SuperFect Transfection Reagent was purchased from Qiagen (Valencia, CA, #301305). p-CYP3A4-pGL3B luciferase plasmid was obtained as a kind gift from Dr. Rommel G. Tirona, The University of Western Ontario, London, Ontario, Canada. h-PXR-pSG5 plasmid was obtained as a kind gift from Dr. Steven Kliewer, UT Southwestern Medical Center, Dallas, Texas, United States. pRL-TK vector (#E2241), Dual-Glo® luciferase reporter assay kit (#E1910) and P450-Glo™ CYP3A4 Assay (Luciferin-IPA) (#V9002) was purchased from Promega (Madison, WI). Antibodies against phospho-JNK (#9251), JNK (#9252), phospho-c-Jun (#9164), c-Jun (#9165) were purchased from Cell Signaling (Beverly, MA), PXR (#PA5-19080) was purchased from Thermo Scientific (Waltham, MA) and Lamin A/C (#sc-20681) was purchased from Santa Cruz Biotechnology (Dallas, TX). Goat Anti-Rabbit IgG HRP Conjugate antibody (#1706515) was purchased from Bio-Rad (Hercules, CA). Donkey Anti-goat IgG-HRP (#sc-2020) was purchased from Santa Cruz Biotechnology (Dallas, TX). Chromatin

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Immunoprecipitation assay was performed using the Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit (#17-10460) purchased from EMD-Millipore (Billerica, MA). Anti-PXR ChIP grade antibody (SC-25381X) was purchased from Santa Cruz Biotechnology (Dallas, TX).

Cell culture and Transfection. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA). Briefly, cells were seeded in 96 well (2.0 X 10^4 cells/ well), 24 well (8.0 X 10^4 cells/ well) or 6 well format (4.0 X 10^5 cells/well) depending on the type of assay at 80% confluency. After 24 hours, cells achieved 95% viability, and were transfected with CYP3A4 luciferase (luc) promoter construct (contains the proximal promoter (-362/+53) and distal XREM (-7836/-7208; inserted in pGL3 Basic vector) and h-PXR-pSG5 plasmid together with the pRL-TK plasmid (that expresses *Renilla* luciferase) using SuperFect transfection reagent. For siRNA experiments, co-transfection of plasmids and siRNA was carried out with DharmaFect Duo reagent. A custom siRNA sequence targeting JNK 1 & JNK2 simultaneously and a control non-targeting siRNA were used for these experiments. HepG2 cells were transfected with JNK1/2 siRNA along with CYP3A4 luciferase and PXR plasmid using DharmaFect Duo reagent for 24 hours before treatment. HepaRG™ cells were cultured according to the manufacturer's protocol. Cryopreserved cells were thawed with Williams E Medium containing HepaRG™ Thawing/ Plating medium supplement. Cells were seeded in 96 well plates (100 µl/ well) at a density of 0.1 million cells/ ml. The cells were allowed to recover at 37°C with a 5% CO_2 for 72 hours to achieve ~90% viability.

Measurement of Luciferase Reporter Gene Expression. After 24 hours of transfection, HepG2 cells were pretreated with 30 µM SP600125 (SP; specific JNK 1/2/3 inhibitor) or 25 µM curcumin (JNK pathway inhibitor) or 10 µM SB203580 (p-38 inhibitor) or 25 µM PD098059 (ERK

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

inhibitor) or DMSO (0.1%) as control for 30 minutes followed by treatment with 1 μM hyperforin or 10 μM rifampicin (RIF) or DMSO (0.1%) as control for 24 hours. Following treatment, cells were lysed and Dual-Glo® luciferase assay was carried out, according to manufacturer's instructions (Promega, #E1910). Briefly, Dual-Glo® Luciferase buffer containing Dual-Glo® Luciferase substrate was added to the lysed cells and luciferase activity was recorded. *Firefly* luciferase activity was normalized to *Renilla* luciferase activity and compared among different treatments.

Quantitative Real-Time PCR. HepG2 cells were seeded in 6 well plate at 80% confluency. After 24 hours, at ~95% viability, cells were pre-treated with vehicle (0.1% DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 0, 2, 4, 6, 8 & 12 h. At each time point, total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Sigma Aldrich; St Louis, MO). cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems; Foster City, CA) as described previously (Shah et al., 2014; Ghose et al, 2004, 2007). In short, each 25 μl reaction mixture contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 μl of TaqMan Universal PCR Master Mix. PCR thermocycling parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. We extrapolated the quantitative expression values from standard curves and these values were normalized to GAPDH. Data are represented as relative gene expression (normalized to vehicle control).

CYP450-Glo Activity Assay. HepG2 and HepaRG cells were cultured on white-walled, collagen-coated 96 well culture plates at a density of 1.0 $X10^4$ cells/ well. HepG2 and HepaRG cells were incubated for 24 hours and 72 hours respectively followed by pre-treatment with DMSO (0.1%)

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

or 30 μM SP for 30 minutes and treatment with 10 μM RIF or DMSO (0.1%). After treatment, cells were lysed and luciferase enzymatic activity was measured using a P450-Glo™ commercial kit as per the manufacturer's protocol (Promega, # V9002). Specific CYP3A4 luminogenic substrate, luciferin-IPA, was added to the wells at a concentration of 3 μM and incubated at 37^{o}C for 60 minutes. Light emission from the samples was detected by SpectraMax Microplate Reader/Luminometer and net signal was calculated by subtracting background luminescence values.

Immunoblotting. HepG2 cells were seeded in 6 well plate at 80% confluency. After 24 hours, at ~95% viability, cells were pre-treated with vehicle (0.1% DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF. Whole cell, nuclear and cytosolic extracts were prepared as described previously (Ghose et al., 2007, 2011) and the protein concentrations were determined using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A). Immunoblotting was performed as described in previous publications (Ghose et al., 2007, 2011; Moorthy et al, 2000; Jiang et al, 2004). Briefly, equal amounts of protein (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were incubated with phospho-JNK, JNK, phospho-c-Jun, c-Jun, PXR and Lamin A/C primary antibodies overnight at 4^{o} C followed by incubation with goat anti-rabbit IgG-HRP secondary antibody or donkey anti-goat IgG secondary antibody for 60 minutes at room temperature. The membranes were then washed and incubated with HyGlo HRP Antibody detection reagent (Denville Scientific, #E2500) as per the manufacturers' instructions. The immunoreactive bands were detected by chemiluminescence method and the band density was analyzed by Image J software (National Institutes of Health, Bethesda, Maryland). Band density of phospho-JNK 1 & 2 was measured and normalized to JNK 1 & 2 respectively. Phospho-cJun

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

was normalized to c-jun bands. Total lamin A/C was used as a loading control or as a housekeeping gene for nuclear extracts.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed to assess specific binding of the PXR complex to the PXR response element on the *CYP3A4* promoter as described in the manufacturer's protocol (EMD Millipore Inc., #17-10460). HepG2 cells were seeded ~80 to 90% confluency (1.0 X 10^7 cells/well) in a 150 mm culture dish containing 20 mL of growth media. Next day, HepG2 cells were pre-treated with SP (30 μM) or DMSO (0.1%) for 30 minutes, followed by RIF (10 μM) for 24 hours. The cells were then cross-linked with 37% formaldehyde at room temperature for 10 minutes, washed twice with ice-cold phosphate-buffered saline (PBS) and collected in 1 ml of ice-cold PBS. Cells were pelleted at 800*g* and digested by sonication (15 pulses, 20 sec each, 30 sec. rest in between). The protein-DNA complexes were immunoprecipitated by using ChIP-grade anti-PXR antibody (Santa Cruz #SC-25381X). As a negative control, the beads were incubated with lysates without anti-PXR antibody. The chromatin was reverse cross-linked and eluted in 50 μl elution buffer. DNA recovered from this assay was analyzed by end point PCR using primers for PXR binding site in the *CYP3A4* promoter region (forward 5'-AGAACCCAGAACCCTTGGAC-3' and reverse 5'-CTGCCTGCAGTTGGAAGAG-3'). PCR products were analyzed by agarose gel electrophoresis. 10% of the total cell lysate was used as "input". Eluted DNA was further analyzed by real time PCR using same primer mix as mentioned above. ChIP DNA Ct values were normalized to input Ct values by subtracting the Ct value of the input (and dilution factor) from each sample according to the method II of the manufacturer's protocol. Estimated fold enrichment of the positive locus sequence in ChIP DNA over negative control is expressed as fold enrichment or fold induction.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Statistical Analysis. All data are presented as the mean ± standard deviations (SDs) form at least three independent experiments (n = 3), unless otherwise specified. Differences between the groups were compared using Student's unpaired two-tailed t test. One-way analysis of variance (ANOVA) with Dunnett's post hoc test was applied to the data if more than two groups were analyzed. P-value <0.05 was considered to be statistically significant.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Induction of *CYP3A4* luciferase activity by PXR was mediated by JNK. In order to determine the role of JNK in induction of *CYP3A4* reporter gene expression, we co-transfected HepG2 cells with plasmids expressing *CYP3A4* reporter gene and PXR, followed by treatment with the PXR ligand, RIF in the presence of JNK inhibitors. *CYP3A4* reporter activity increased ~8 folds in the presence of 10 μM RIF as compared to control (Fig. 1A). This *CYP3A4* reporter induction by RIF was significantly attenuated by treatment with SP, indicating that JNK may be involved in regulating CYP3A4 induction by PXR. We observed that PD098059 (ERK inhibitor) co-treatment also inhibited *CYP3A4* luciferase activity but not SB203580 (p38 inhibitor) (data not shown). In addition, we also studied the effect of curcumin, a JNK pathway inhibitor on *CYP3A4* reporter expression. As shown in Fig. 1A, 15 μM curcumin significantly attenuated RIF-mediated *CYP3A4* induction by almost 50%. Significant difference was not observed between curcumin treatment alone and curcumin with RIF treatment. We further confirmed these findings by treating HepG2 cells with another PXR ligand, hyperforin in the presence of the SP (Fig. 1B). Hyperforin is an active component of St. John's wort and a potent activator of human PXR (Moore et al., 2000; Chen et al., 2004). *CYP3A4* luciferase activity was induced ~4 fold by 2 μM hyperforin at 24 hours, and this induction was significantly attenuated by SP treatment. SP had no effect on *CYP3A4* luciferase activity in the absence of RIF or hyperforin (Figs. 1A and 1B), indicating that JNK likely does not affect the basal expression of the *CYP3A4* gene.

To further confirm our results, we investigated the effect of simultaneous knockdown of JNK1 and JNK2 (using a siRNA that targets a homologous region present in both kinases) on *CYP3A4* reporter gene induction (Fig 2A). Knockdown of JNK1/2 expression reduced the ability of RIF to induce *CYP3A4* luciferase activity in HepG2 cells, validating our previous data with

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

pharmacological inhibitors. There were no significant differences between control and JNK siRNA-treated cells in the absence of RIF. The validity of the siRNAs was confirmed by the lack of JNK and phospho-JNK expression in JNK siRNA-treated cells (Fig. 2B). The fact that inhibition of JNK by pharmacological inhibitors or siRNA attenuated CYP3A4 induction by two different PXR ligands (RIF and hyperforin), strongly indicate that PXR is likely regulated by JNK-dependent signaling mechanism. Furthermore, while *CYP3A4* reporter expression in HepG2 cells without PXR plasmid was below the level of detection (data not shown), the same signal was significantly enhanced in PXR-transfected cells, suggesting a PXR-dependent effect.

Induction of *CYP3A4* gene expression by PXR was in part mediated by JNK. To determine the role of JNK in regulating endogenous *CYP3A4* gene expression, HepG2 cells were treated with SP and RIF (Fig. 3). The cells were lysed to prepare mRNA and *CYP3A4* gene expression was analyzed. RIF treatment significantly induced *CYP3A4* gene expression from 2 to 8 hours (~3-4.5 fold); the expression was lower at the 12 hour time-point (~2-fold), although it was significantly induced compared to the control. We also observed ~2-fold CYP3A4 mRNA induction by RIF at 24h (data not shown). SP treatment significantly attenuated this induction. About 2-fold CYP3A4 mRNA induction by RIF was observed in HepG2 cells by other groups at longer time-points (Novotna et. al., 2014). These results were in agreement with our *CYP3A4* luciferase activity data in transfected HepG2 cells. Since significant attenuation of *CYP3A4* reporter gene activity was observed with ERK inhibitor, we also carried out gene expression studies using the ERK inhibitor, PD098059; we found that PD098059 had no effect on RIF-induced *CYP3A4* mRNA levels (data not shown).

Induction of CYP3A4 enzyme activity by PXR was mediated by JNK. We performed P450-Glo™ assay to determine the role of JNK in PXR-mediated induction of CYP3A4 enzyme activity.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

As shown in Fig.4A, treatment of HepG2 cells with RIF significantly induced CYP3A4 enzyme activity (~2.5 fold) as compared to control, and SP attenuated this induction of CYP3A4 enzyme activity (as measured by the metabolism of the CYP3A4-specific substrate, luciferin IPA). In order to confirm our findings, we also conducted CYP3A4 activity assays in HepaRG™ cells, which are terminally differentiated hepatic cells derived from a human hepatic progenitor cell line that retains many characteristics of primary human hepatocytes (Parent et al, 2004). We observed a similar trend in HepaRG™ cells; RIF treatment significantly induced CYP3A4 enzyme activity from 4-12 h; this induction was attenuated by the JNK inhibitor, SP (Fig. 4B). In these cell lines we did not see any changes in DMSO treated cells over time, and SP by itself did not have any effect on CYP3A4 activity (data not shown). Consistent with our gene expression results, SP does not affect CYP3A4 enzyme activity in the absence of RIF, indicating that JNK is likely not involved in regulating basal CYP3A4 activity.

RIF treatment activated JNK *in vitro.* Since treatment with JNK inhibitors resulted in attenuation of PXR-mediated *CYP3A4* reporter activity, gene expression as well as enzyme activity, we determined the role of RIF in activation of JNK. Whole cells extracts were prepared from treated HepG2 cells and immunoblot analysis was carried out to determine phospho-JNK 1 and 2 protein expression. JNK exists in 3 distinct isoforms (JNK1-3). While JNKl and JNK2 genes are ubiquitously expressed including liver, the JNK3 gene is selectively expressed in the brain, heart, and testis (Ip and Davis, 1998). We found that both phospho-JNK1 (P-JNK1) and phospho-JNK2 (P-JNK2) levels were increased by RIF treatment starting at 60 minutes; while this increase was attenuated in the presence of the JNK inhibitor, SP (Fig. 5). At 2 and 4 hours, p-JNK protein levels increased as compared to 0 hour time point, but this effect was not significant. In agreement

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

with these findings, we found that the JNK substrate, c-Jun was phosphorylated after RIF treatment for 4 h, and this phosphorylation was attenuated in the presence of SP (Fig. 5C and D).

PXR nuclear levels were regulated by JNK. Our results indicate that JNK likely affects PXR function, therefore we determined the role of JNK in regulating PXR nuclear levels in RIF-treated HepG2 cells. As expected, PXR nuclear protein levels significantly increased from 4 to 24 hours in the presence of RIF (Fig. 6). Importantly, SP treatment attenuated RIF-mediated nuclear accumulation of PXR, indicating that PXR protein levels in the nucleus is regulated by JNK.

PXR binding to the *CYP3A4* promoter was mediated by JNK. Lastly, we performed *in vitro* ChIP assays to analyze whether decreased accumulation of PXR in the nucleus, due to JNK inhibition impacts the binding of PXR to the *CYP3A4* promoter. A commercially available ChIP-grade anti-PXR antibody was used to precipitate DNA-protein complexes. RIF treatment alone significantly increased the association of PXR with the regulatory regions of the *CYP3A4* gene as compared to control. Both our end point and qRT-PCR data showed decreased binding of PXR to *CYP3A4* promoter when JNK is inhibited (Fig. 7A & B), supporting the hypothesis that JNK was required for optimum binding of PXR to its response elements on *CYP3A4* gene. We saw very faint bands in cell lysates precipitated without anti-PXR antibody, which might be due to non-specific binding. Input DNA from total cell lysate confirmed amplification of the *CYP3A4* regulatory region targeted by specific primers.

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Induction of CYP3A4 enzyme expression/activity can have adverse effects on therapeutic outcome in patients. Therefore, the mechanism of CYP3A4 induction needs to be fully elucidated. This *in vitro* study demonstrates that CYP3A4 induction by PXR was mediated in part by a JNK-dependent mechanism. Research carried out in the last decade has shown that PXR-mediated gene expression is regulated not only by xenobiotics and endobiotics (Kliewer et al., 1998; Lehmann et al., 1998; Staudinger et al., 2007; Pondugula et al., 2009), but also by cellular signaling pathways. Post-translational modifications of PXR, especially direct phosphorylation primarily led to an inhibition of its transcriptional activity (Wang YM, 2012); thereby downregulating *Cyp3a* expression. Our *in vitro* data, on the other hand, shows that JNK is required for optimum induction of CYP3A4 via PXR. We provide additional evidence showing that PXR nuclear translocation as well as binding of PXR to its response elements on *CYP3A4* gene is JNK-dependent.

To comprehensively understand the role of MAPKs in PXR-mediated CYP3A4 signaling, we investigated the role of all MAPKs by treating HepG2 cells with specific ERK 1/2 pathway inhibitor (PD098059), JNK 1/2/3 inhibitor (SP600125) and p38 inhibitor (SB203580). Specifically, SP600125, an anthrapyrazolone compound, is a potent inhibitor of all isoforms of JNK with an IC_{50} of 0.04 µM but exhibits greater than 300 fold selectivity against other MAPKs-ERK and p38 (IC_{50} > 10 µM) (Bennett et al, 2001). In our studies, while JNK and ERK 1/2 inhibition significantly attenuated PXR-mediated CYP3A4 luciferase activity, p-38 inhibition showed no significant change. Curcumin, which can inhibit both JNK (IC_{50}: 5 µM) and ERK (IC_{50}: 20 µM) (Chen and Tan, 1998), also attenuated PXR-mediated CYP3A4 luciferase activity. MAPKs phosphorylate downstream kinases and nuclear factors such as c-Jun, c-Fos, c-Myc, SP1, Elk1 etc. Since different MAPKs phosphorylate varying nuclear factors, it could be possible that

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

only nuclear factors activated by JNK and ERK might be involved in CYP3A4 regulation. However, the possibility of ERK being involved was eliminated from the results that the ERK inhibitor, PD098059 had no effect on RIF-induced *CYP3A4* mRNA expression in HepG2 cells (data not shown). A similar study by Yasunami *et. al.* reported that the induction of CYP3A4 mRNA and promoter activity by the VDR ligand, $1,25(OH)_2D_3$ was attenuated in the presence of a JNK inhibitor, while ERK or p-38 inhibition had no effect (Yasunami et al, 2004). SP is one of the most widely used and extensively studied ATP-competitive JNK inhibitors (Zhang et al, 2012; Davies et al, 2012; Bogoyevitch et al, 2011; Feng et al, 2013; Koch et al, 2014), and curcumin has been shown to inhibit JNK signaling pathways (Suh et al, 2007; Chen et al, 1998); however, these compounds can target additional pathways to affect CYP3A4 expression. For example, both SP and curcumin can activate aryl hydrocarbon receptor (AHR) (Dvorak et al, 2008; Bachelda et al, 2008; Ciolino et al, 1998; Mohammadi-Bardbori et al, 2012); recent studies have shown that AHR activation decreases basal and RIF-induced CYP3A4 expression in HepaRG cells (Rasmussen et al, 2017). So it is possible that SP or curcumin can activate AHR to reduce CYP3A4 expression; however, these compounds did not affect basal CYP3A4 expression in our experiments. In order to address the potential issues associated with non-specific effects of the pharmacological inhibitors, we confirmed our results by siRNA experiments. Knock down of JNK1/2 expression by siRNA also attenuated *CYP3A4* induction by RIF in HepG2 cells. These data confirmed that JNK is indeed required for optimal activation of CYP3A4 as pharmacological inhibition as well as genetic knock-down of JNK1 & JNK2 attenuated its promoter activity significantly at 24 hours. However, the role of individual isoforms of JNK needs to be further investigated.

Additionally, we studied the effect of SP on RIF-mediated CYP3A4 mRNA and activity in HepG2 cells. HepG2 cells, human liver carcinoma cells, are frequently used for *in vitro* biotransformation

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

assays (Westerink & Schoonen, 2007). Although HepG2 is a cheap and easy to use cell line, endogenous activity and expression of enzymes and NRs is relatively low in HepG2 cells as compared to primary human hepatocytes (Xu et al, 2004). Despite this limitation, HepG2 cells are routinely used for induction studies, and previous reports have shown significant induction of *CYP3A4* gene expression (~3-6 folds) as well as enzyme activity by RIF in these cells (Westerink & Schoonen, 2007; Usui et al, 2003; Pascussi et al, 2001). As an additional approach, we performed enzyme activity studies in HepaRG cells which is a well-established model for biotransformation applications (Gripon et al. 2002; Lambert et al. 2009; Anthérieu et al. 2010). In our data, attenuation of PXR-mediated induction of CYP3A4 enzyme activity in the presence of JNK inhibitor, irrespective of cell line, confirmed that JNK is required for induction of CYP3A4 and this is not a cell-line specific effect.

To understand the role of JNK in PXR regulation, we demonstrated that RIF activated JNK in HepG2 cells. Activation of PXR by RIF has previously been shown to increase phosphorylation of p38 MAPK; which was attributed to induction of *GADD45β* gene (Kodama et al, 2010). *GADD45β*, is an immediate-early response gene induced by various physiological and environmental stressors, including cytokines and genotoxic stresses (Liebermann and Hoffman, 2008). Our data showing activation of JNK by RIF in HepG2 cells could potentially be mediated via similar targets of PXR. Alternatively, PXR may increase JNK expression, however, we did not detect any increase in total JNK protein levels in our experiments. Further studies to determine the mechanism of JNK activation by PXR are ongoing.

To determine the mechanism involved in JNK-mediated regulation of CYP3A4, we studied the role of JNK in regulating PXR nuclear translocation or it's binding to *CYP3A4* gene. PXR exists as a phospho-protein in cells (Lichti-Kaiser et al, 2009b) and previous studies have shown that

kinases primarily decrease PXR transcription by either strengthening PXR-co-repressor interaction or weakening PXR-co-activator interactions. Recent studies have shown that activation of AMP-activated protein kinase (AMPK) and JNK by metformin lead to down-regulation of PXR expression, and reduced expression of carboxylesterases (Shan et al, 2017). In contrast, our studies show that JNK positively regulated PXR function, without affecting its expression (data not shown). JNK may positively regulate PXR function by multiple mechanisms (Fig. 8), including: 1) JNK directly phosphorylates PXR and increases its nuclear translocation/binding to the *CYP3A4* gene; 2) JNK phosphorylates cytoplasmic retention proteins (CRPs) associated with PXR, and impacts its translocation; 3)) JNK decreases PXR-co-repressor binding, or 4) JNK increases PXR-co-activator binding. Although we did not study whether PXR is directly phosphorylated by JNK, we found that JNK is required for PXR nuclear translocation and binding to the *CYP3A4* promoter, ultimately affecting PXR function. Using *in silico* computer-based analysis using PhosphoSitePlus®, we found 9 serine phosphorylation sites and 7 threonine phosphorylation sites on PXR and further studies to reveal their association with JNK are ongoing. Similar to our study, 7 serine/threonine residues were also identified in human PXR protein which are good potential substrates for an array of kinases, including MAPKs (Lichti-Kaiser et al, 2009b). Using mass spectrometry analysis, some of these serine-threonine residues such as S114, T133/135, S167, and S200 in PXR were also found to be phosphorylated by cyclin-dependent kinase 2 (Elias et al, 2014).

In summary, our *in vitro* data indicate that JNK, but not ERK or p38, is required for *CYP3A4* gene activation by PXR. Further studies exploring the role of JNK on CYP3A4 induction *in vivo* will be clinically relevant in studying PXR-mediated target gene expression. JNK is activated by various extracellular stimuli and thus regulates gene expression through phosphorylation of

transcription/nuclear factors. Hence, elucidation of the contribution of JNK in the xenobiotic-induced expression of P450 genes may be instrumental in understanding the mechanism of induction of P450s which can impact therapeutic outcome in patients undergoing treatment with multiple medications. Ultimately, understanding the role of JNK in induction of CYP3A4 could provide novel strategies to address concerns of loss of drug safety and/or efficacy due to alteration of expression and activity of the CYP3A4 enzyme.

Authorship Contributions

Participated in research design: Taneja, Moorthy and Ghose

Conducted experiments: Taneja, Maturu and Chu

Contributed new reagents or analytic tools: Moorthy and Ghose

Performed data analysis: Taneja

Wrote or contributed to the writing of the manuscript: Taneja, Moorthy and Ghose

References

Anthérieu S, Chesné C, Li R, Camus S, Lahoz A, Picazo L, Turpeinen M, Tolonen A, Uusitalo J, Guguen-Guillouzo C, and Guillouzo A (2010) Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metab Dispos* **38**:516–525.

Araya Z, and Wikvall K (1999) 6α-Hydroxylation of taurochenodeoxycholic acid and lithocholic acid by CYP3A4 in human liver microsomes. *Biochim Biophys Acta - Mol Cell Biol Lipids* **1438**:47–54.

Bachleda P, & Dvorak Z (2008) Pharmacological inhibitors of JNK and ERK kinases SP600125 and U0126 are not appropriate tools for studies of drug metabolism because they activate aryl hydrocarbon receptor. *Gen Physiol Biophys* **27:** 143-145.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, and Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci* **98**:13681–13686.

Bogoyevitch MA, and Arthur PG (2008) Inhibitors of c-Jun N-terminal kinases: JuNK no more? *Biochim Biophys Acta* **1784:** 76-93.

Ciolino HP, Daschner PJ, Wang TT, & Yeh GC (1998) Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem Pharmacol* **56:** 197-206.

Chen Y, Ferguson SS, Negishi M, and Goldstein J a (2004) Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* **308**:495–501.

Chen YR, and Tan TH (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* **17**:173–178.

Davies C, and Tournier C (2012) Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. *Biochem Soc Trans* **40:** 85-89.

Ding X, and Staudinger JL (2005a) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J Pharmacol Exp Ther* **312**:849–856.

Ding X, and Staudinger JL (2005b) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochem Pharmacol* **69**:867–873.

Dvorak Z, Vrzal R, Henklova P, Jancova P, Anzenbacherova E, Maurel P, Svecova L, Pavek P, Ehrmann J, Havlik R, Bednar P, Lemr K, Ulrichova J (2008) JNK inhibitor SP600125 is a partial agonist of human aryl hydrocarbon receptor and induces CYP1A1 and CYP1A2 genes in primary human hepatocytes. *Biochem. Pharmacol*. **75** (2), 580-588.

Elias A, High AA, Mishra A, Ong SS, Wu J, Peng J, and Chen T (2014) Identification and characterization of phosphorylation sites within the pregnane X receptor protein. *Biochem Pharmacol* **87**:360–370.

Feng Y, Chambers JW, Iqbal S, Koenig M, Park H, Cherry L (2013) A small molecule bidentate-binding dual inhibitor probe of the LRRK2 and JNK kinases. *ACS Chem Biol* **8:** 1747-1754.

Ghose R, Guo T, Vallejo JG, and Gandhi A (2011) Differential role of toll-interleukin 1 receptor domain-containing adaptor protein in toll-like receptor 2-mediated regulation of gene expression of hepatic cytokines and drug-metabolizing enzymes. *Drug Metab Dispos* **39**:874–881.

Ghose R, Mulder J, von Furstenberg RJ, Thevananther S, Kuipers F, and Karpen SJ (2007)

Rosiglitazone attenuates suppression of RXRα-dependent gene expression in inflamed liver. *J Hepatol* **46**:115–123.

Ghose R, Zimmerman TL, Thevananther S, and Karpen SJ (2004) Endotoxin leads to rapid subcellular re-localization of hepatic RXRα: A novel mechanism for reduced hepatic gene expression in inflammation. *Nucl Recept* **2**:4.

Goey AKL, Meijerman I, Rosing H, Marchetti S, Mergui-Roelvink M, Keessen M, Burgers JA, Beijnen JH, and Schellens JHM (2014) The effect of St John's Wort on the pharmacokinetics of docetaxel. *Clin Pharmacokinet* **53**:103–110.

Goodwin B, Redinbo MR, and Kliewer SA (2002) Regulation of cyp3a gene transcription by the pregnane x receptor. *Annu Rev Pharmacol Toxicol* **42**:1–23.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, and Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* **99**:15655–15660.

Guengerich FP (1995) Human cytochrome P-450 enzymes. "*Cytochrome P450*" *(*P.R. Ortiz de Montellano ed.), pp. 473–535. New York: Plenum. 2nd ed.

Guengerich FP (1999) CYTOCHROME P-450 3A4: Regulation and Role in Drug Metabolism. *Annu Rev Pharmacol Toxicol* **39**:1–17.

Harmsen S, Meijerman I, Beijnen JH, and Schellens JHM (2007) The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. *Cancer Treat Rev* **33**:369–380.

Houliston R a, Pearson JD, and Wheeler-Jones CP (2001) Agonist-specific cross talk between ERKs and p38(mapk) regulates PGI(2) synthesis in endothelium. *Am J Physiol Cell Physiol* **281**:C1266-76.

Ip YT, and Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)- from

inflammation to development. *Curr Opin Cell Biol* **10**:205–19.

Jiang W, Welty SE, Couroucli XI, Barrios R, Kondraganti SR, Muthiah K, Yu L, Avery SE and Moorthy B (2004) Disruption of the Ah receptor gene alters the susceptibility of mice to oxygen-mediated regulation of pulmonary and hepatic cytochromes P4501A expression and exacerbates hyperoxic lung injury. *J Pharmacol Exp Ther* **310:** 512-519.

Kim SW, Hasanuzzaman M, Cho M, Heo YR, Ryu MJ, Ha NY, Park HJ, Park HY, and Shin JG (2015) Casein kinase 2 (CK2)-mediated phosphorylation of Hsp90β as a novel mechanism of rifampin-induced MDR1 expression. *J Biol Chem* **290**:17029–17040.

Kliewer S a, Moore JT, Wade L, Staudinger JL, Watson M a, Jones S a, McKee DD, Oliver BB, Willson TM, Zetterström RH, Perlmann T, and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73–82.

Koch P, Gehringer M, & Laufer SA (2015) Inhibitors of c-Jun N-terminal kinases: an update. *J Med Chem* **58:** 72-95.

Kodama S and Negishi M (2010) Pregnane X Receptor PXR Activates the GADD45β Gene, Eliciting the p38 MAPK Signal and Cell Migration. *J Biol Chem* **286**, 3570-3578.

Krishna DR, and Klotz U (1994) Extrahepatic Metabolism of Drugs in Humans. *Clin Pharmacokinet.* **26(2)**:144-60.

Lambert CB, Spire C, Claude N, and Guillouzo A (2009) Dose- and time-dependent effects of phenobarbital on gene expression profiling in human hepatoma HepaRG cells. *Toxicol Appl Pharmacol* **234**:345–360.

Leeder JS, and Okey AB (1996) Cytochromes P-450 and liver injury. *In Drug Induced Hepatotoxicity pp. 119–53.*

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, and Kliewer SA (1998) The

human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016–1023.

Lichti-Kaiser K, Xu C, and Staudinger JL (2009a) Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner. *J Biol Chem* **284**:6639–49.

Lichti-Kaiser K, Brobst D, Xu C, and Staudinger JL (2009b) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* **331**:65–76.

Liebermann DA and Hoffman B (2008) Gadd45 in stress signaling. *J Mol Signal* **3**: 15.

Lin W, Wu J, Dong H, Bouck D, Zeng FY, and Chen T (2008) Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *J Biol Chem* **283**:30650–30657.

Luo G, Guenthner T, Gan L-S, and Humphreys WG (2004) CYP3A4 induction by xenobiotics: biochemistry, experimental methods and impact on drug discovery and development. *Curr Drug Metab* **5**:483–505.

Martinez-Jimenez CP, Jover R, Donato MT, Castell JV, and Gomez-Lechon MJ (2007) Transcriptional regulation and expression of CYP3A4 in hepatocytes. *Curr Drug Metab* **8:** 185-194.

Mohammadi-Bardbori A, Bengtsson J, Rannug U, Rannug A, and Wincent E (2012) Quercetin, resveratrol, and curcumin are indirect activators of the aryl hydrocarbon receptor (AHR). *Chem Res Toxicol* **25:** 1878-1884.

Moore LB, Goodwin B, Jones S a, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, and Kliewer S a (2000) St. John's wort induces hepatic drug metabolism through activation of

the pregnane X receptor. *Proc Natl Acad Sci U S A* **97**:7500–7502.

Moorthy B, Parker KM, Smith CV, Bend JR, and Welty SE (2000) Potentiation of oxygen-induced lung injury in rats by the mechanism-based cytochrome P-450 inhibitor, 1-aminobenzotriazole. *J Pharmacol Exp Ther* **292:** 553-560.

Novotna A, Dvorak Z. Omeprazole and lansoprazole enantiomers induce CYP3A4 in human hepatocytes and cell lines via glucocorticoid receptor and pregnane X receptor axis. *PLoS One. 2014*; **9**:e105580.

Parent R, Marion MJ, Furio L, Trépo C, and Petit MA (2004) Origin and Characterization of a Human Bipotent Liver Progenitor Cell Line. *Gastroenterology* **126**:1147–1156.

Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P, and Vilarem MJ (2001) Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur. J. Biochem.* **268(24)**:6346-58

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P, and Vilarem MJ (2003) The expression of CYP2B6, CYP2C9 and CYP3A4 genes: A tangle of networks of nuclear and steroid receptors, in *Biochimica et Biophysica Acta - General Subjects* pp 243–253.

Pondugula SR, Brimer-Cline C, Wu J, Schuetz EG, Tyagi RK, and Chen T (2009a) A phosphomimetic mutation at threonine-57 abolishes transactivation activity and alters nuclear localization pattern of human pregnane X receptor. *Drug Metab Dispos* **37**:719–730.

Pondugula SR, Dong H, and Chen T (2009b) Phosphorylation and protein–protein interactions in PXR-mediated CYP3A repression. *Expert Opin Drug Metab Toxicol* **5**:861–873.

Rasmussen MK, Daujat-Chavanieu M, and Gerbal-Chaloin S (2017) Activation of the aryl

hydrocarbon receptor decreases rifampicin-induced CYP3A4 expression in primary human hepatocytes and HepaRG. *Toxicol Lett* **277:** 1-8.

Rochel N, Wurtz JM, Mitschler A, Klaholz B, and Moras D (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol Cell* **5:** 173-179.

Shah P, Guo T, Moore DD, and Ghose R (2014) Role of constitutive androstane receptor in toll-like receptor-mediated regulation of gene expression of hepatic drug-metabolizing enzymes and transporters. *Drug Metab Dispos* **42**:172–181.

Shan E, Zhu Z, He S, Chu D, Ge D, and Zhan Y (2017) Involvement of pregnane X receptor in the suppression of carboxylesterases by metformin in vivo and in vitro, mediated by the activation of AMPK and JNK signaling pathway. *Eur J Pharm Sci* **102:** 14-23.

Squires EJ, Sueyoshi T, and Negishi M (2004) Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *J. Biol. Chem.* 279, 49307–49314

Staudinger JL, and Lichti K (2007) Cell Signaling and Nuclear Receptors : New Opportunities for Molecular Pharmaceuticals in Liver Disease. *Mol Pharm* **5**:17–34.

Staudinger JL, Xu C, Biswas A, and Mani S (2011) Post-translational modification of pregnane x receptor. *Pharmacol Res* **64**:4–10.

Suh HW, Kang S, and Kwon KS (2007) Curcumin attenuates glutamate-induced HT22 cell death by suppressing MAP kinase signaling. *Mol Cell Biochem* **298:** 187-194.

Thummel KE, and Wilkinson GR (1998) In vitro and in vivo drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* **38**:389–430.

Usui T, Saitoh Y, and Komada F (2003) Induction of CYP3As in HepG2 Cells by Several Drugs. Association between Induction of CYP3A4 and Expression of Glucocorticoid Receptor.

Biol Pharm Bull **26(4)**:510-7.

Vanhooke JL, Benning MM, Bauer CB, Pike JW, and DeLuca HF (2004) Molecular structure of the rat vitamin D receptor ligand binding domain complexed with 2-carbon-substituted vitamin D3 hormone analogues and a LXXLL-containing coactivator peptide. *Biochemistry* **43:** 4101-4110.

Veith H, Southall N, Huang R, James T, Fayne D, Artemenko N, Shen M, Inglese J, Austin CP, Lloyd DG, and Auld DS (2009) Comprehensive characterization of cytochrome P450 isozyme selectivity across chemical libraries. *Nat Biotechnol* **27**:1050–1055.

Wakelee HA, Takimoto CH, Lopez-Anaya A, Chu Q, Middleton G, Dunlop D, Ramlau R, Leighl N, Rowinsky EK, Hao D, Zatloukal P, Jacobs CD, and Rodon J (2012) The effect of bexarotene on atorvastatin pharmacokinetics: Results from a phase i trial of bexarotene plus chemotherapy in patients with advanced non-small cell lung cancer. *Cancer Chemother Pharmacol* **69**:563–571.

Wang Y-M, Ong SS, Chai SC, and Chen T (2012) Role of CAR and PXR in xenobiotic sensing and metabolism. *Expert Opin Drug Metab Toxicol* **8**:803–817.

Waxman DJ, Lapenson DP, Aoyama T, Gelboin H V., Gonzalez FJ, and Korzekwa K (1991) Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Arch Biochem Biophys* **290**:160–166.

Westerink WMA, and Schoonen WGEJ (2007) Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicol Vitr* **21**:1592–1602.

Wilkinson GR (2005) Drug metabolism and variability among patients in drug response. *N Engl J Med* **352**:2211–2221.

Xie W, Uppal H, Saini SPS, Mu Y, Little JM, Radominska-Pandya A, and Zemaitis MA (2004) Orphan nuclear receptor-mediated xenobiotic regulation in drug metabolism. *Drug Discov Today* 15;9(10):442-9.

Xu JJ, Diaz D, and O'Brien PJ (2004) Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chem Biol Interact.* 150(1):115-28.

Yasunami Y, Hara H, Iwamura T, Kataoka T, and Adachi T (2004) C-jun N-terminal kinase modulates 1,25-dihydroxyvitamin D3-induced cytochrome P450 3A4 gene expression. *Drug Metab Dispos* **32**:685–8.

Zanger UM, and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol. Ther.* **138**, 103–141

Zhang T, Inesta-Vaquera F, Niepel M, Zhang J, Ficarro SB, Machleidt T, Xie T, Marto JA, Kim N, Sim T, Laughlin J, Park H, LoGrasso PV, Patricelli M, Nomanbhoy TK, Sorger P, Alessi DR, and Gray NS (2012) Discovery of potent and selective covalent inhibitors of JNK. *Chem Biol* **19:** 140-154.

Zhou H, Lu N, Chen ZQ, Song QL, Yu HM, and Li XJ (2009) Osteopontin mediates dense culture-induced proliferation and adhesion of prostate tumour cells: Role of protein kinase C, p38 mitogen-activated protein kinase and calcium. *Basic Clin Pharmacol Toxicol* **104**:164–170.

Footnotes

a) This work was supported by National Institutes of Health [Grant R21DA035751-01A1 to R.G.], and [Grant R01 ES-019689, ES-001932, HL-129794, and HL-112516 to B.M.]

b) Citation of Meeting Abstracts:

Taneja, G; Chu, C; Moorthy, B; Ghose, R. C-Jun-N-terminal kinase (JNK): Novel regulator of PXR-mediated CYP3A4 induction; ASPET-Experimental Biology; San Diego, 2016

Taneja, G; Chu, C; Moorthy, B; Ghose, R. Regulation of human hepatic CYP3A4 expression: Role of cytokines and MAP-Kinases at 2015 AAPS Annual Meeting and Exposition; Orlando, 2015

c) Reprints requested: Romi Ghose

1441 Moursund Street

Houston, TX 77030, USA

Tel: 832-842-8343

E-mail: rghose@central.uh.edu

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Fig. 1. Induction of *CYP3A4*–luciferase activity by RIF was attenuated by JNK inhibitors. *CYP3A4* luciferase reporter was co-transfected with PXR plasmid into HepG2 cells. Cells were pre-treated with vehicle (DMSO) or **A)** 30 μM SP/25 μM curcumin for 30 min., followed by 10 μM RIF for 24 h, or **B)** 30 μM SP for 30 min., followed by 2 μM hyperforin for 48 h and harvested for luciferase activity assays as described under Materials and Methods. The data are expressed as *Firefly* luciferase/*Renilla* luciferase activity. The data are presented as the mean ± SD from three independent experiments (n = 3) performed in triplicates. * $p<0.05$ as compared to control treatment. # $p<0.05$ as compared to DMSO + RIF treatment.

Fig. 2. Induction of *CYP3A4* luciferase activity by RIF was attenuated by JNK siRNA. Custom-made JNK1/2 siRNA and control siRNA were co-transfected with *CYP3A4* luciferase plasmid and PXR plasmid in HepG2 cells. Following transfection, cells were treated with the vehicle (DMSO) or 10 μM RIF. **A)** Cells were lysed after 24h, and the data are expressed as *Firefly* luciferase/*Renilla* luciferase activity. The data are presented as the mean ± SD from three independent experiments (n = 3) performed in triplicates. * $p<0.05$ as compared to vehicle control. # $p<0.05$ as compared to RIF-treated control siRNA transfected cells. **B)** Cells were lysed after 1h, and phospho (P)-JNK and JNK protein levels in whole cell extracts were determined by immunoblotting.

Fig. 3. Induction of *CYP3A4* gene expression by RIF was attenuated by SP. HepG2 cells were pre-treated with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 0, 2, 4, 6, 8 & 12 h. Relative CYP3A4 mRNA expression (0 h samples are set as 1) levels are plotted at different times. The data are presented as the mean ± SD from three independent experiments (n = 3) performed in triplicates and are expressed as the relative change normalized to vehicle treated

cells. * p<0.05 as compared to DMSO + RIF 0 h group. # p<0.05 as compared to DMSO + RIF for each time point.

Fig. 4. Induction of RIF-mediated CYP3A4 activity was attenuated by SP. **A)** HepG2 cells were pre-treated with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 24 hours. Following treatment, cells were incubated with specific CYP3A4 substrate, 3 μM Luciferin IPA for 60 min and luminescence was detected. The data are presented as the mean ± SD from three independent experiments (*n* = 3) performed in triplicates. *p<0.05 as compared to DMSO + RIF 0 h group. # p<0.05 as compared to DMSO + RIF 24 h. **B)** HepaRG cells were pre-treated with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 2, 4, 6, 8 & 12 h. Following treatment, cells were incubated with specific CYP3A4 substrate, 3 μM Luciferin IPA for 60 min and luminescence was detected. The data are presented as the mean ± SD from three independent experiments (*n* = 3) performed in triplicates. *p<0.05 as compared to DMSO + RIF 2 h group. # p<0.05 as compared to DMSO + RIF for each time point.

Fig. 5. RIF activates JNK in whole cell extracts prepared from HepG2 cells. **A)** Immunoblot of phospho (P)-JNK and JNK in whole cell extracts after treatment of HepG2 cells with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 0, 0.5, 1, 2 & 4 h. **B)** Representation of quantification of total p-JNK (sum of p-JNK normalized levels) by densitometry after normalizing the P-JNK 1 & 2 levels over JNK 1 & 2 respectively. **C)** Immunoblot of phospho-c-Jun in whole cell extracts after treatment of HepG2 cells with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 4 h. **D)** Representation of quantification of total phospho-c-Jun levels by densitometry after normalizing over total c-Jun. Replicates from three experiments performed in duplicates were quantified by densitometry. * p<0.05 as compared to untreated

control (DMSO). #p<0.05 as compared to DMSO + RIF. The contrast was adjusted to improve legibility.

Fig. 6. Attenuation of RIF-induced PXR nuclear levels by SP. **A)** Immunoblot of PXR in nuclear extracts after treatment of HepG2 cells with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 0, 4, 12 & 24 h. **B)** Quantification of blots by densitometry after normalizing PXR levels over total Lamin A/C nuclear housekeeping protein. Replicates from three experiments performed in duplicates were quantified by densitometry. * p<0.05 as compared to DMSO + RIF 0 h group. # p<0.05 as compared to DMSO + RIF for each time point.

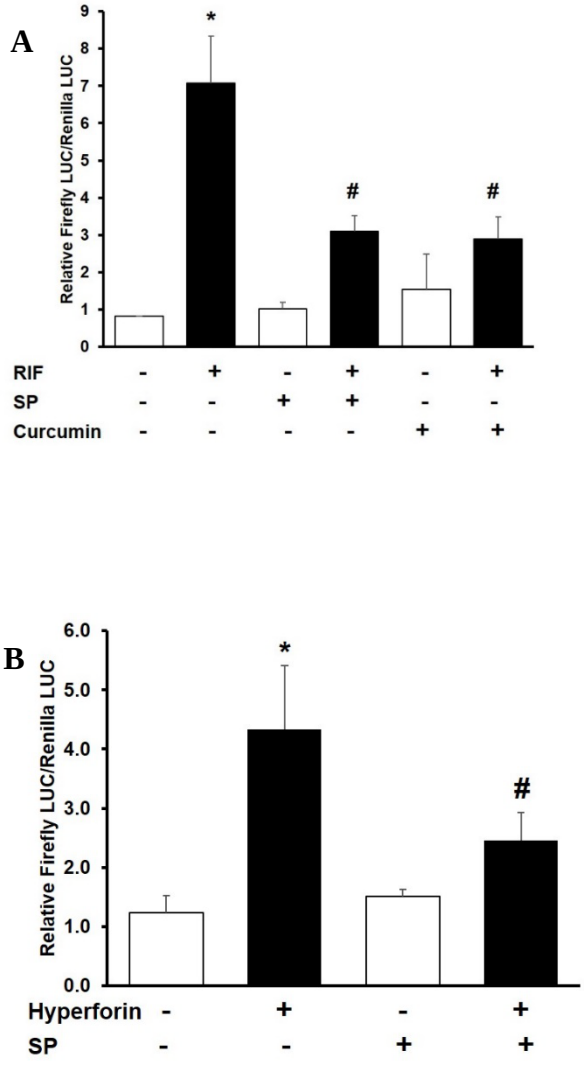
Fig. 7. Attenuation of binding of PXR to *CYP3A4* promoter region by SP. HepG2 cells were pre-treated with vehicle (DMSO) or 30 μM SP for 30 min, followed by 10 μM RIF for 24 hours. An antibody against PXR was used to immunoprecipitate DNA-protein complexes. As a negative control, the beads were incubated with lysates without anti-PXR antibody. ChIP assays were performed as described under Material and Methods. **A)** End point PCR was performed using forward and reverse primers designed in the promoter region of CYP3A4 and analyzed on a 2% agarose gel. 10% of the total cell lysate was used as "input". **B)** Quantitative real time-PCR was performed using forward and reverse primers designed in the promoter region of CYP3A4. Estimated fold enrichment of the positive locus sequence in ChIP DNA over negative control is expressed as fold enrichment or fold induction. Data represents mean of triplicates ± SD from two independent experiments. * p<0.05 as compared to control. # p<0.05 as compared to RIF treatment alone.

Fig. 8. Potential roles of JNK in PXR-mediated induction of CYP3A4

DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

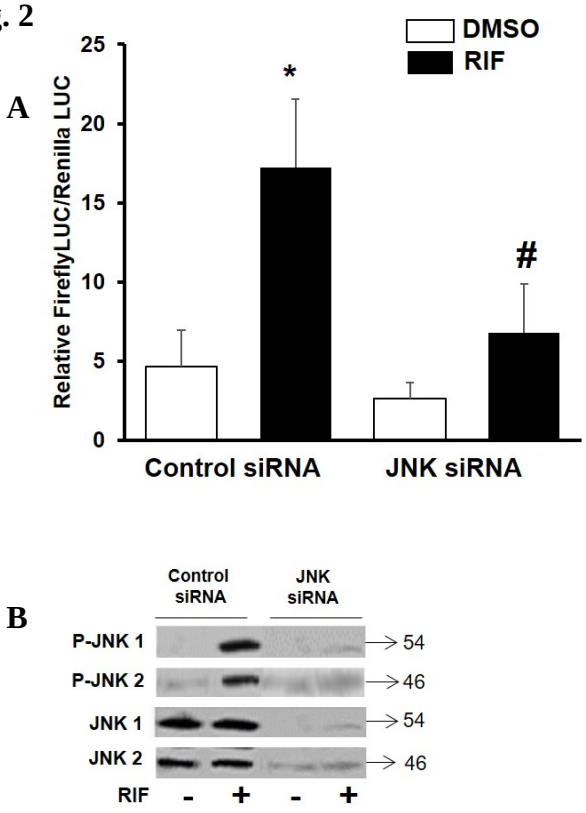
Figures

Fig. 1



DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2



DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3

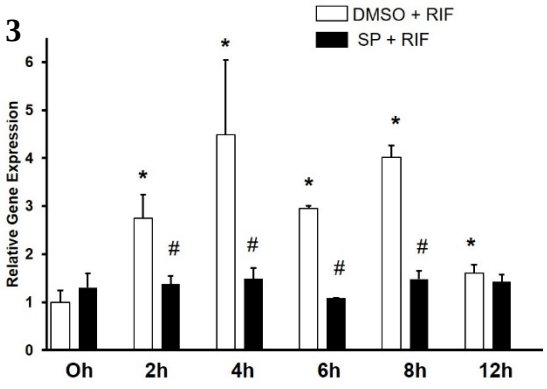
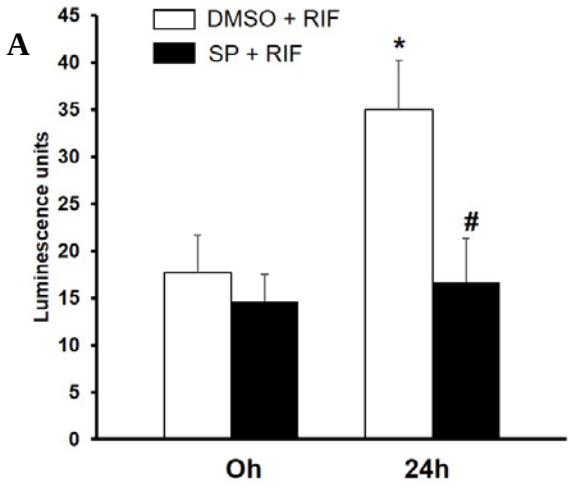
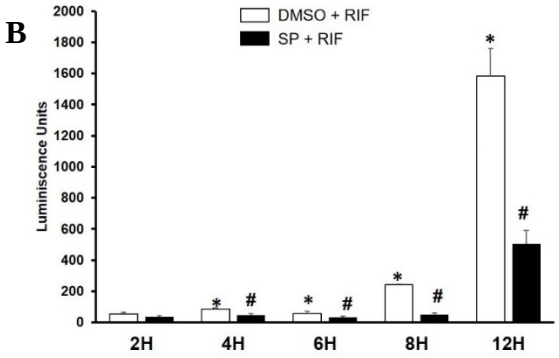


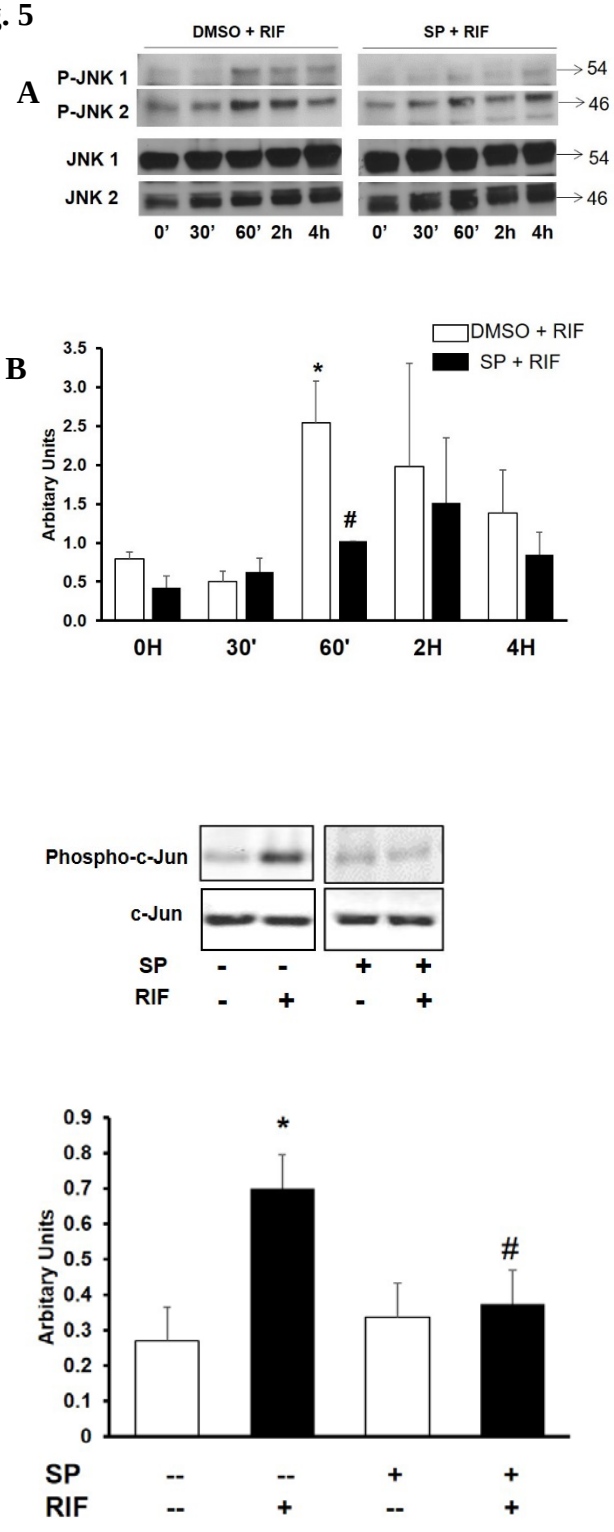
Fig. 4





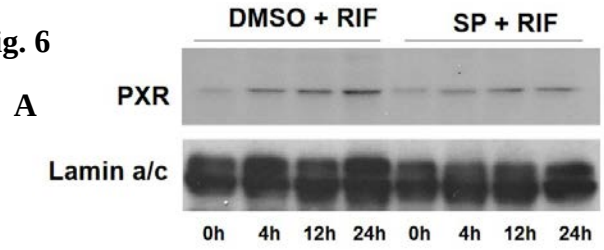
DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

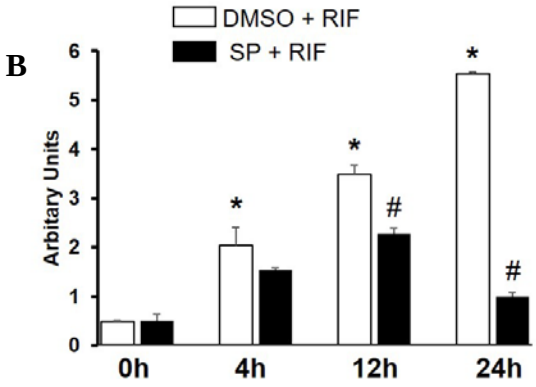
Fig. 5



DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

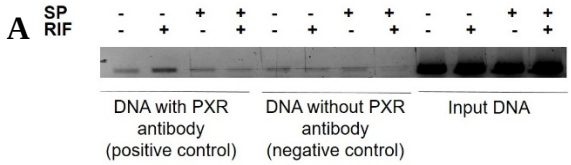
Fig. 6

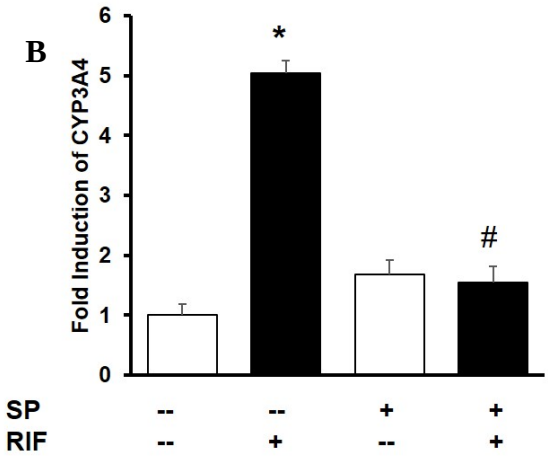




DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 7





DMD Fast Forward. Published on February 12, 2018 as DOI: 10.1124/dmd.117.079160
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8