DMD Fast Forward. Published on December 28, 2005 as doi:10.1124/dmd.105.006197

DMD# 6197

The role of PXR in 2-AAF-mediated induction of drug transport and metabolizing enzymes in mice.

Alexander Anapolsky, Shirley Teng, Santosh Dixit and Micheline Piquette-Miller

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada (A.A.; S.T.; M.P-M.)

Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee (S.D)

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Role of PXR in 2-AAF-mediated gene regulation.

Address correspondence to:

Dr. M. Piquette-Miller
Leslie Dan Faculty of Pharmacy
University of Toronto
19 Russell Street, Toronto, Ontario
Canada , M5S 2S2
Phone: 416-946-3057/ Fax: 416-978-8511
E-mail: m.piquette.miller@utoronto.ca

Number of Pages: 19

Number of Figures: 3

Number of References: 41

Word count:

Abstract – 225 words

Introduction – 370 words

Discussion – 1521 words

Abbreviations: PXR, pregnane X receptor; MRP, multidrug resistance-associated protein; RT-PCR, reverse transcription-polymerase chain reaction; OATP, organic anion transporting peptide; 2-AAF, 2-acetylaminofluorene; BCRP, breast cancer resistance protein; CYP, cytochrome p450; ABC, ATP-binding cassette transporter; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; 3-MC, 3-methylcholantrene; AhR, aryl hydrocarbon receptor.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Activation of the pregnane X receptor (PXR) mediates the induction of several drug transporters and metabolizing enzymes. *In vitro* studies have reported that several of these genes are induced after exposure to the hepatocarcinogen, 2-acetylaminofluorene (2-AAF). Thus, we hypothesized that PXR may play a role in the *in vivo* induction of gene expression by 2-AAF. We examined the expression of the drug metabolizing enzymes CYP1A2 and CYP3A11 and the drug transporters BCRP, MRP2 and OATP2. Wild-type ($PXR^{+/+}$) and PXR-null ($PXR^{-/-}$) C57BL/6 mice were injected daily for 7 days with 150 or 300 mg/kg of 2-AAF suspended in corn oil (i.p.), while control group received corn oil vehicle. Levels of mRNA isolated from liver were measured by RT-PCR and normalized to β-actin. Treatment of $PXR^{+/+}$ mice resulted in a dose-dependent 2- to 4-fold induction ($p<0.001$) of MRP2, OATP2, BCRP, CYP3A11 and CYP1A2, but no induction was observed in $PXR^{-/-}$ mice. Induction of PXR mRNA was observed in the 2-AAF-treated $PXR^{+/+}$ mice. Furthermore, a dose-dependent increase in CYP3A4 promoter construct activity was observed in HepG2 cells co-transfected with human or rat PXR, indicating that 2-AAF does indeed activate PXR. These results suggest that PXR is responsible for 2-AAF-mediated induction of drug efflux transporters and biotransformation enzymes in the liver. Moreover, novel findings demonstrate that PXR plays a role in regulation of the drug efflux transporter, BCRP, in mice.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

Short-term administration of the polycyclic aromatic amine, 2-acetylaminofluorene (2-AAF), in rodents is associated with pre-neoplastic changes in hepatocytes and the development of a drug-resistance phenotype (Gant et al., 1991). The hydroxylated metabolite of 2-AAF, produced predominantly by cytochrome p450 (CYP) 1A2, has been shown to account for genotoxicity (Schrenk et al., 1994; Russell et al., 1994). Several studies have suggested that exposure to 2-AAF causes induction of the multidrug resistant transporters MRP2, MRP3 (Kauffmann et al., 1997; Teng et al., 2003), MDR1 (Teeter et al., 1993; Schrenk et al., 1994), as well as the drug metabolizing enzyme CYP3A23 (Sparfel et al., 2003).

The overlap in genes induced by 2-AAF suggests a common molecular mechanism responsible for regulation of their expression. In particular it is believed that the pregnane X receptor (PXR) may be responsible for this induction (Kliewer et al., 1998). Genes shown to be regulated by PXR include the ABC drug transporters MDR1 (Geick et al., 2001), MRP2 (Kast et al., 2002) and MRP3 (Teng et al., 2003), the organic anion transporter OATP2 (Staudinger et al., 2001), as well as the CYP3A drug metabolizing enzyme. Recent *in vitro* studies have shown that administration of 2-AAF elicits a PXR-dependent induction of MRP2 and CYP3A23 (Kauffmann and Schrenk, 1998; Sparfel et al., 2003). *In vivo* studies elucidating the effects of 2-AAF on murine gene up-regulation have yet to be completed. Therefore, we examined the *in vivo* effects produced by 2-AAF administration on the expression of several murine hepatic genes which encode for active drug transporters and drug metabolizing enzymes. Novel findings from this study revealed a 2-AAF-mediated dose-dependent induction of the organic anion drug transporters MRP2 and OATP2, and the CYP3A11 and CYP1A2 drug metabolizing enzymes in wild-type ($PXR^{+/+}$), but not in PXR-null ($PXR^{-/-}$) mice, demonstrating involvement of murine

PXR (PXR) in the regulatory effects of 2-AAF. Activation of PXR by 2-AAF was further substantiated by CYP3A4 promoter construct studies which demonstrated an increase in luciferase reporter gene activity in HepG2 cells co-transfected with human or rat PXR. Moreover, the observed 2-AAF-mediated induction of the breast cancer resistance protein (BCRP) in $PXR^{+/+}$, but not in $PXR^{-/-}$ mice, is the first finding to demonstrate involvement of PXR in the regulation of this novel ABC-half-transporter.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

METHODS

In vivo Studies All animal studies were approved by the University of Toronto Animal Ethics Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Wild-type ($PXR^{+/+}$), eight-week old C57BL/6 mice (25-30g), were purchased from Charles River Canada (St. Constant, QC); PXR-null ($PXR^{-/-}$) mice colonies were originally obtained from Dr. Christopher Sinal (Dalhousie University, Halifax, NS). Animals were maintained in a temperature-controlled facility on a 12 h light/dark cycle and fed standard laboratory chow and water *ad libitum*.

Two $PXR^{+/+}$ and two $PXR^{-/-}$ groups of mice (n = 4 per group) were treated intraperitoneally (i.p.) for 7 days with 150 mg/kg or 300 mg/kg of 2-AAF (Sigma-Aldrich, Oakville, ON, Canada) suspended in corn oil. Each control group (n = 4-8 per group) was treated i.p. with corn oil vehicle using the same dosing schedule. On day eight all animals were sacrificed by cervical dislocation, their livers removed, snap-frozen in liquid nitrogen and stored at -80ºC until used for RNA isolation. Normal serum alanine aminotransferase (ALT) levels were found in all animals treated with these doses of 2-AAF, indicating the absence of liver necrosis.

Total RNA was extracted from control and 2-AAF-treated liver using the Amersham Quick-Prep™ RNA isolation kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's protocol. cDNA was synthesized from total RNA (0.5 μg) using the First Strand cDNA Synthesis Kit (MBI Fermentas, Flamborough, ON), according to the manufacturer's protocol. PCR standard curves for each gene product (β-actin, BCRP, CYP3A11, CYP1A2, MDR1a, MDR1b, MRP2, OATP2 and PXR) were generated as previously reported (Teng and

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

Piquette-Miller, 2004). PCR was performed in the presence of 3 mM $MgCl_2$, 200 μM dNTP and 50 pmol of each primer in a total volume of 50 μl using a GeneAmp ™ 2400 Thermocycler (Perkin-Elmer, Mississauga, ON). The reactions were initiated by the addition of 1.5 units of Taq polymerase (MBI Fermentas, Flamborough, ON) and amplification proceeded through 24 cycles for BCRP, 28 cycles for CYP3A11, 22 cycles for CYP1A2, 33 cycles for MDR1a and MDR1b, 26 cycles for MRP2, 19 cycles for OATP2 and 28 cycles for PXR. PCR products were run on a 2% agarose gel, stained with SYBR® Gold (Molecular Probes, Eugene, OR), and quantitated using Kodak Digital Science1D Image Analysis software. Sizes of DNA bands were confirmed using the Gene Ruler™ 100 bp DNA ladder (MBI Fermentas, Flamborough, ON). All mRNA levels were normalized to β-actin mRNA. Levels of MRP2, OATP2, BCRP, CYP3A11, CYP1A2 and PXR mRNA expression are reported as percentages of normalized values, as compared to controls. Data are presented as a mean value with standard error of the mean (SEM). Differences between $PXR^{+/+}$, $PXR^{-/-}$ treatment groups and controls were determined by one-way ANOVA, followed by Tukey's HSD test with a significance level of $*p<0.05$ or $**p<0.001$, using SPSS® Statistical Software (Version 11.0.0, SPSS Inc, Chicago, IL).

Luciferase Reporter Assay. A CYP3A4-XREM-Luc reporter plasmid driven by *CYP3A4* regulatory elements (-7836/-7208) (Goodwin et al., 1999) in pGL3 Basic vector (Promega, Madison, WI) was prepared as described previously (Tirona et al., 2003). Human PXR was cloned in pEF6/V5-His expression vector (Invitrogen, Calsbad, CA) as described previously (Zhang et al., 2001). A rat PXR expression plasmid was obtained by PCR from a rat liver cDNA library (BD Biosciences Clontech) and subsequent cloning into pEF6/V5-His, as described previously (Tirona et al., 2004).

Human hepatocarcinoma HepG2 cells (ATCC, Manassas, VA) were grown in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine system (FBS, Sigma) and 50 U/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured overnight at 37°C and then trypsinized and re-seeded at 3 x 10^5 cells/well in a 24-well plate (Corning Inc., Corning, NY) in DMEM containing 10% FBS. After 24 hours, cells were transfected using Lipofectin (Invitrogen) with 250 ng/well of CYP3A4-XREM-Luc, 250 ng/well of the human PXR (hPXR), rat PXR (rPXR) expression plasmids or pEF6 vector lacking cDNA insert, respectively. All wells were also co-transfected with a Renilla luciferase vector driven by a cytomegalovirus promoter (7.5 ng/well pRL-CMV) as an internal control for transfection efficiency. Sixteen hours thereafter, cells were treated with 2-AAF (1μM-100 μM) or vehicle (DMSO, 0.1%) for 48 hours. Luciferase activity was determined with the Dual Luciferase Assay Kit (Promega) as per manufacturer's instructions. Statistical differences between triplicate control and 2-AAF-treated cells were determined using Student's *t* test, with a *p* value of <0.05 was taken to be the minimum level of statistical significance.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

As shown in Figure 1A, several hepatic drug transporters were up-regulated in 2-AAF-treated mice. Daily doses of 150 and 300 mg/kg of 2-AAF resulted in 2.1- and 3-fold induction ($p<0.001$) of hepatic mRNA levels of MRP2 in $PXR^{+/+}$ mice, respectively. However, MRP2 levels in $PXR^{-/-}$ mice were not altered by 2-AAF. A dose-dependent induction of OATP2 mRNA levels was evident ($p<0.001$), when $PXR^{+/+}$ mice were treated with 150 mg/kg (3.4-fold induction) and 300 mg/kg (4.2-fold induction) of 2-AAF. $PXR^{-/-}$ mice treated with 150 mg/kg of 2-AAF showed a smaller but significant increase (1.9-fold) in levels of OATP2; however, doses of 300 mg/kg did not cause an induction. A 1.9- and 2.6-fold induction ($p<0.001$) of BCRP mRNA levels was present in both 2-AAF-treated groups of $PXR^{+/+}$ mice, respectively, whereas changes in BCRP levels were not observed in the 2-AAF-treated $PXR^{-/-}$ mice. On the other hand, levels of MDR1a and MDR1b were highly variable and not significantly altered in the 2-AAF treated mice (data not shown).

As shown in Figure 1B, the mRNA levels of CYP3A11 were up-regulated ($p<0.001$) 2.8- to 3.7-fold in $PXR^{+/+}$ mice treated with 150 and 300 mg/kg of 2-AAF, respectively. On the other hand, no induction was evident in $PXR^{-/-}$ mice. A 3.9-fold induction of CYP1A2 was seen in both 150 and 300 mg/kg 2-AAF-treated $PXR^{+/+}$ mice. The levels of CYP1A2 did not appear to be affected in 2-AAF-treated $PXR^{-/-}$ mice.

As shown in Figure 2, levels of PXR mRNA were increased in 2-AAF treated $PXR^{+/+}$ mice with a significant 1.8-fold induction of PXR mRNA observed in the 300 mg/kg 2-AAF $PXR^{+/+}$ mice ($p<0.05$). As compared with PXR +/+ mice, basal levels of MRP2 and, BCRP were significantly

lower (30-33% of wild-types) and levels of OATP2 significantly higher (2 fold) in $PXR^{-/-}$ mice. Basal levels of CYP1A2 and CYP3A11 were not significantly different between $PXR^{+/+}$ and $PXR^{-/-}$ vehicle-treated mice. No relationship was observed between basal mRNA expression to gene induction in the 2-AAF treated $PXR^{+/+}$ and $PXR^{-/-}$ mice. This finding was previously observed in mice treated with other known PXR inducers (Teng and Piquette-Miller, 2004).

Addition of 2-AAF (1 μM-100 μM) to HepG2 cells co-transfected with human or rat PXR resulted in a clear dose-dependent increase in luciferase activity (Figure 3). This was particularly evident for rat PXR, which displayed significant induction starting at 10 μM of 2-AAF, whereas human PXR was not activated at this concentration. Moreover, luciferase activity was induced approximately 10-fold greater with rat than human PXR treated with 100 μM 2-AAF. These findings clearly suggest 2-AAF is a ligand for both human and rat PXR, however, it appears to be a more potent agonist of rat PXR.

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Over the past two decades, it has been shown that drug efflux transporters and biotransformation enzymes are induced by an array of structurally diverse compounds. Interestingly, the same compounds that mediated changes in these genes were also shown to interact with steroid hormone receptors (Bertillson et al., 1998; Lehman et al., 1998). These findings were the first to suggest that common signal transduction pathways could be involved in the molecular regulation of both drug transport and metabolizing enzymes. Characterization of the nuclear receptor PXR in 1998 began to delineate the underlying regulatory molecular mechanisms involved (Kliewer et al., 1998). Since that time, various studies have shown that induction of CYP3A by a variety of xenobiotics is primarily mediated by PXR (Bertillson et al., 1998; Lehman et al., 1998; Guo et al., 2003). Studies performed with murine PXR showed that it is efficiently activated by the classic CYP3A inducer pregnenolone 16α-carbonitrile (PCN), as well as by glucocorticoids such as dexamethasone and spironolactone (Drocourt et al., 2001); the antiglucocorticoid RU486 (Staudinger et al., 2001; Xie et al., 2001); the HIV-1 protease inhibitor ritonavir (Dussault et al., 2001); and the anticancer drug paclitaxel (Synold et al., 2001). The 'promiscuity' of PXR towards ligands might contribute to the ability of various classes of xenobiotics to co-induce both CYP and drug efflux transporters (Goodwin et al., 2002).

Located on the canalicular membrane of hepatocytes, MRP2 is primarily responsible for biliary elimination of non-bile acids and organic anions into bile. Results from this study demonstrated a dose-dependent induction of MRP2 in 2-AAF-treated $PXR^{+/+}$, but not in $PXR^{-/-}$ mice. This implies both that 2-AAF may be a potent activator of PXR *in vivo* and that induction of MRP2

via 2-AAF likely occurs through a PXR-mediated pathway. Based on the ability of rifampicin to induce both murine and human CYP3A (Kliewer et al., 1998) and MRP2 (Fromm et al., 2000), it has been hypothesized that PXR could be the putative regulator of their transcription. Other investigators have shown a concentration-dependent up-regulation of MRP2 by 2-AAF in primary cultured rat hepatocytes and human HepG2 cells (Kauffmann et al., 1997; Schrenk et al., 2001). More importantly, studies conducted in knockout mice have demonstrated the absence of MRP2 induction in $PXR^{-/-}$ animals following the administration of various other PXR ligands (Kast et al., 2002; Teng and Piquette-Miller, 2004), demonstrating involvement of PXR in MRP2 induction.

Our results demonstrate a 2-AAF-mediated induction of OATP2 mRNA in $PXR^{+/+}$ mice, but not in $PXR^{-/-}$ mice, providing another line of evidence that 2-AAF exerts its effects on gene expression through PXR *in vivo*. OATP2, a basolateral transporter mediating hepatocellular uptake of bile acids and a wide range of xenobiotics, was initially isolated from rat brain (Noe et al., 1997), and, later, found to be abundantly expressed in rodent liver (Reichel et al., 1999). In addition to MRP2 and CYP3A11, OATP2 has been implicated in bile acid synthesis, transport, and metabolism (Noe et al., 1997; Reichel et al., 1999). Hence, it has been hypothesized that PXR might be directly involved in regulation of OATP2 gene transcription. Our findings are supported by earlier studies, which have reported a co-induction of CYP3A11 and OATP2 in $PXR^{+/+}$ mice, but not in $PXR^{-/-}$ model, after treatment with the known PXR activators RU486 and PCN (Staudinger et al., 2001; Teng and Piquette-Miller, 2004).

Interestingly, the observed induction of the breast cancer resistance protein, BCRP in 2-AAF-

treated $PXR^{+/+}$ mice, which was not seen in 2-AAF-treated $PXR^{-/-}$ mice, suggests that a PXR-dependent molecular mechanism may be involved in regulating BCRP expression. Transcriptional regulation of BCRP has not been previously established. BCRP, which is one of the most recently discovered ABC-drug efflux transporters, was first cloned from a doxorubicin-resistant MCF7 breast cancer cell line. BCRP is normally found on apical membranes of intestinal, hepatic and brain epithelia involved in drug disposition. Over-expression of BCRP, seen in various cancer cells and stem cells has been shown to cause multidrug resistance. Mouse and human cell lines expressed with BCRP have been demonstrated to extrude anticancer agents that overlap considerably with substrates for MRP2 (Schinkel and Jonker, 2003). Furthermore, BCRP has been shown to share structural similarity with MRP2 and other ABC transporters, but unlike them it is a half-transporter, and probably mediates its drug efflux functions either by homo- or hetero-dimerization (Kage et al., 2002; Schinkel and Jonker, 2003). Recent investigations reported an existence of putative estrogen response element (ERE) in the promoter region of BCRP in ER-positive cells (Ee et al., 2004). Expression of BCRP has also been shown to be up-regulated by hypoxia-inducible transcription factor complex HIF-1, suggesting the cyto-protective role of BCRP during hypoxia (Krishnamurthy et al., 2004). Recent studies indicate that BCRP, in addition to MRP1 and MRP3, may be relevant to hepatic cell survival during carcinogenesis (Zhou et al., 2002; Ros et al., 2003). Thus, in addition to structural resemblance, a dose-dependent induction of both BCRP and MRP2 in the present study suggests a similarity in function of these transporters during the administration of 2-AAF.

The cytochrome P450 enzymes (CYPs) are a superfamily of heme-thiolate proteins that play a central role in the oxidative, peroxidative, and reductive metabolism of a large spectrum of

endogenous compounds such as fatty acids, steroids, leukotrienes, prostaglandins, bile acids and fat-soluble vitamins. In addition, many of these enzymes are responsible for the detoxification of xenobiotics such as drugs, carcinogens, and environmental contaminants (Bertillson et al., 1998; Kliewer et al., 1998). As we observed induction of CYP3A11 and CYP1A2 in the 2-AAF-treated $PXR^{+/+}$, but not $PXR^{-/-}$ mice, our results indicate that PXR is involved in the *in vivo* induction of CYP3A11 and CYP1A2 imposed by 2-AAF. This supports previous *in vitro* findings of a 2-AAF-mediated up-regulation of CYP1A and CYP3A2/23 in primary rat hepatocytes (Tateishi et al., 1999; Sparfel et al., 2003). It has been generally accepted that polycyclic aromatic molecules such as 2-AAF are agonists of the aryl hydrocarbon receptor (AhR). Activation of the AhR has been linked to alterations in CYP1A1 and CYP1A2 mRNA levels during carcinogenesis (Cikryt et al., 1990; Gant et al., 1991). C57BL/6 mice, used in the present study, possess AhRs with a high affinity for aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholantrene (3-MC). Initial *in vivo* studies have shown that both chemicals induce CYP1A in mice (Gonzalez et al., 1984). Although affinity of 2-AAF for the AhR and an up-regulation of CYP1A has been shown in rats (Tateishi et al., 1999; Cikryt et al., 1990), the mechanistic role of AhRs in 2-AAF-induced CYP1A2 induction has not been established (Gant et al., 1991; Tateishi et al., 1999). Our results demonstrating an expected increased CYP1A2 expression in 2-AAF-treated $PXR^{+/+}$ mice, but a complete lack of CYP1A2 induction in 2-AAF-treated $PXR^{-/-}$ mice, indicates that PXR may play a more important role in CYP1A2 regulation than putative AhR *in vivo*. Induction of the multidrug resistance gene, MDR1, along with MRP2 and CYP1A1, has been reported during 2-AAF-induced carcinogenesis (Burt and Thorgeirrsson, 1988; Gant et al., 1991; Kauffmann et al., 1997; Tateishi et al., 1999). While we did not detect significant changes in mRNA levels of MDR1a or MDR1b in the present study, numerous

species- and strain-specific differences in 2-AAF-mediated induction of MDR1 have been reported (Lecureur et al., 1996). These differences are felt to primarily stem from the finding that the CYP1A2 metabolites of 2-AAF, N-hydroxylated-2-AAF and N-acetoxy-2-acetylaminofluorene, are responsible for MDR1 induction (Schrenk et al., 1994; Hill et al., 1996). Whether the 2-AAF-mediated induction of the drug transporters or drug metabolizing enzymes observed in this study occur due to 2-AAF or its metabolites remains to be determined.

In order to confirm whether observed PXR-dependent changes in transporter and CYP expression occurred as a result of direct activation of PXR by 2-AAF, *in vitro* reporter gene assays were performed in HepG2 cells co-transfected with a PXR-responsive CYP3A4-luciferase reporter and either the rat PXR or human PXR expression plasmids. Indeed, 2-AAF was found to be a highly efficacious activator of rat PXR. Interestingly, 2-AAF was also able to activate human PXR, but at higher concentrations. Of note, many compounds are able to activate PXR in different species; in particular, ligand specificity is almost entirely shared between rats and mice. Thus although these *in vitro* studies examined the activation of rat and human PXR whereas our *in vivo* studies were performed in mice, it is very likely that 2-AAF also serves as a PXR ligand in mice. Taken together, these findings suggest that 2-AAF is a ligand of PXR, and the observed induction of drug transporters and CYPs upon exposure to this compound is likely mediated through activation of PXR.

In conclusion, our findings demonstrated a dose-dependent induction in the hepatic expression of the drug transporters MRP2, OATP2 and BCRP and the CYP3A11 and CYP1A2 drug metabolizing enzymes in 2-AAF-treated $PXR^{+/+}$, but not PXR-null mice. Cell-based reporter

assays confirmed that 2-AAF serves as a ligand of PXR. Thus, induction of these genes occurs as a result of the activation of PXR by 2-AAF. Further studies elucidating the impact of 2-AAF on the activity of these genes and its impact on the drug disposition are warranted.

REFERENCES

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, and Berkenstam A. (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA* **95:**12208–12213.

Burt RK, and Thorgeirsson SS (1988) Coinduction of MDR-1 multidrug-resistance and cytochrome P-450 genes in rat liver by xenobiotics. *J Natl Cancer Inst* **80**:1383-1286.

Cikryt P, Gottlicher M, and Neumann HG (1990) Competitive binding affinity of carci-nogenic aromatic amines to the rat hepatic aromatic hydrocarbon (Ah) receptor in vitro and potency to induce monooxygenase activity in vivo. *Carcinogenesis* **11:**1359-1366.

Drocourt L, Pascussi JM, Assenat E, Fabre JM, Maurel P, and Vilarem MJ (2001) Calcium channel modulators of the dihydropyridine family are human pregnane X receptor activators and inducers of CYP3A, CYP2B, and CYP2C in human hepatocytes. *Drug Metab Dispos* **29:**1325-1331.

Dussault I, Lin M, Hollister K, Wang EH, Synold TW, and Forman BM (2001) Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. *J Biol Chem* **276:**33309-33312.

Ee PL, Kamalakaran S, Tonetti D, He X, Ross DD, and Beck WT (2004) Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res* **64**:1247-51.

Fromm MF, Kauffmann HM, Fritz P, Burk O, Kroemer HK, Warzok RW, Eichelbaum M, Siegmund W, and Schrenk D (2000) The effect of rifampin treatment on intestinal expression of human MRP transporters. *Am J Pathol* **157:**1575-1580.

Gant TW, Silverman JA, Bisgaard HC, Burt RK, Marino PA, and Trorgeirsson SS (1991) Regulation of 2-acetylaminofluorene-and 3-methylcholanthrene-mediated induction of multidrug resistance and cytochrome P450IA gene family expression in primary hepatocyte cultures and rat liver. *Mol Carcinog* **4:**499-509.

Geick A, Eichelbaum M, and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* **277:**2908-2915.

Gonzalez FJ, Tukey RH, and Nebert DW (1984) Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P1-450 and P3-450 mRNA levels by 3-methylcholanthrene. *Mol Pharmacol* **26:**117-121.

Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56**: 1329-39.

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.

Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB, Kliewer SA, Gonzalez FJ, and Sinal CJ (2003) Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* **278:**45062-45071.

Hill BA, Brown PC, Preisegger K-H, and Silverman J (1996) Regulation of mdr1b gene expression in Fischer, Wistar and Sprague-Dawley rats in vivo and in vitro. *Carcinogenesis* **17**:451-457.

Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, and Sugimoto Y (2002) Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int J Cancer* **97:**626-630.

Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, and Edwards PA (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* **277:**2908-2915.

Kauffmann HM and Schrenk, D (1998) Sequence analysis and functional characterization of the 5'-flanking region of the rat multidrug resistance protein 2 (MRP2) gene. *Biochem Biophys Res Commun* **245:**325-31.

Kauffmann HM, Keppler D, Kartenbeck J, and Schrenk D (1997) Induction of cMrp/cMoat gene expression by cisplatin, 2-acetylaminofluorene, or cycloheximide in rat hepatocytes. *Hepatology* **26:**980-985.

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

Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, Sarkadi B, Sorrentino BP, and Schuetz JD (2004) The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem* **279:**24218–24225.

Lecureur V, Guillouzo A, and Fardel O (1996) Differential regulation of mdr genes in response to 2-acetylaminofluorene treatment in cultured rat and human hepatocytes. *Carcinogenesis*, **17**:1157-1160.

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 Investig* **102:**1016–1023.

Noe B, Hagenbuch B, Stieger B, and Meier PJ (1997) Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc Natl Acad Sci U S A* **94:**10346-10350.

Reichel C, Gao B, Van Montfoort J, Cattori V, Rahner C, Hagenbuch B, Stieger B, Kamisako T, and Meier PJ (1999) Localization and function of the organic anion-transporting polypeptide OATP2 in rat liver. *Gastroenterology* **117:**688-695.

Ros JE, Roskams TA, Geuken M, Havinga R, Splinter PL, Petersen BE, LaRusso NF, van der Kolk DM, Kuipers F, Faber KN, Muller M, and Jansen PL (2003) ATP binding cassette transporter gene expression in rat liver progenitor cells. *Gut* **52:**1060-1067.

Russell AL, Henderson CJ, Smith G, and Wolf CR (1994) Suppression of multi-drug resistance gene expression in the mouse liver by 1,4-bis[2,(3,5-dichloro-pyridyloxy)]benzene.*Int J Cancer* **58:**550-4.

Schinkel AH and Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: anoverview. *Adv Drug Deliv Rev* **55:**3-29.

Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B, and Kauffmann HM (2001) Up-regulation of transporters of the MRP family by drugs and toxins. *Toxicol Lett* **120:**51-57.

Shrenk D, Gant TW, Michalke A, Orzechowski A, Silverman JA, Battula N, and Thorgeirsson SS (1994) Metabolic activation of 2-acetylaminfluorene is required for induction of multidrug resistance gene expression in rat liver cells. *Carcinogenesis* **15:**2541-2546.

Sparfel L, Payen L, Gilot D, Sidaway J, Morel F, Guillouzo A, and Fardel O (2003) Pregnane X receptor-dependent and -independent effects of 2-acetylaminofluorene on cytochrome P450 3A23 expression and liver cell proliferation. *Biochem Biophys Res Commun* **300:**278-284.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* **98:**3369–3374.

Synold TW, Dussault I, and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* **7:**584-590.

Tateishi T, Nakura H, Asoh M, Watanabe M, Tanaka M, Kumai T, and Koba-yashi S (1999) Multiple cytochrome P-450 subfamilies are co-induced with P-glyco-protein by both phenothiazine and 2-acetylaminofluorene in rats. *Cancer Lett* **138:**73-79.

Teeter LD, Estes M, Chan JY, Atassi H, Sell S, Becker FF, and Kuo MT (1993) Activation of distinct multidrug-resistance (P-glycoprotein) genes during rat liver regeneration and hepatocarcinogenesis. *Mol Carcinog* **8:**67-73.

Teng S and Piquette-Miller M (2004) The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. *J Pharmacol Exp Ther* **312:**841-848.

Teng S, Jekerle V, and Piquette-Miller M (2003) Induction of ABCC3 (MRP3) by pregnane X receptor activators. *Drug Metab Dispos* **31:**1296-1299.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG, and Kim RB (2003) The orphan nuclear receptor HNF4alpha

determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9:** 220-224.

Tirona RG, Leake BF, Podust LM, and Kim RB (2004) Identification of Amino Acids in Rat Pregnane X Receptor that Determine Species-Specific Activation. *Mol Pharmacol* **65:** 36-44.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98:**3375-3380.

Zhang J, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Wrighton SA, Hancock M, Kim RB, Strom S, Thummel K, Russell CG, Hudson JR Jr, Schuetz EG, and Boguski MS (2001) The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* **11:** 555-572.

Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, and Sorrentino BP (2002) BCRP1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc Natl Acad Sci U S A* **99:**12339-12344.

FOOTNOTES

Footnote to Title.

The authors would like to thank Dr. Richard B. Kim of the Vanderbilt University School of Medicine (Nashville, TN) for his generous collaboration on the reporter gene assays. Funding for this study was provided by a grant from the Canadian Institutes of Health Research (CIHR).

DMD Fast Forward. Published on December 28, 2005 as DOI: 10.1124/dmd.105.006197
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. The effect of 2-AAF on the hepatic mRNA expression of (A) Drug transporters and (B) Drug metabolizing enzymes in PXR$^{+/+}$ and PXR$^{-/-}$ mice. PXR$^{+/+}$ (WT) and PXR$^{-/-}$ (KO) C57BL/6 mice (n = 4-8) were injected i.p. daily for 7 days with corn oil vehicle (control), 150 mg/kg or 300 mg/kg of 2-AAF suspended in corn oil. Hepatic mRNA levels were determined by RT-PCR and results normalized to β-actin mRNA levels as described in *Materials and Methods*. **p*<0.05, ***p*<0.001.

Figure 2: The effect of 2-AAF on the hepatic mRNA expression of nuclear receptor PXR in PXR$^{+/+}$ mice. PXR$^{+/+}$ C57BL/6 mice (n = 4-8) were injected i.p. daily for 7 days with corn oil vehicle (control), 150 mg/kg or 300 mg/kg of 2-AAF suspended in corn oil. Hepatic mRNA levels were determined by RT-PCR and results normalized to β-actin mRNA levels as described in *Materials and Methods*. **p*<0.05, ***p*<0.001.

Figure 3: 2-AAF induces induces transactivation of the *CYP3A4* promoter through PXR in HepG2 cells. HepG2 cells (n=3) were co-transfected with human or rat PXR and the human *CYP3A4* promoter-driven luciferase construct as described in methods. Cells were incubated with increasing concentrations of 2-AAF or DMSO vehicle control for 48 hours followed by measurement of luciferase activity. **p*<0.05.

Figure 1A:

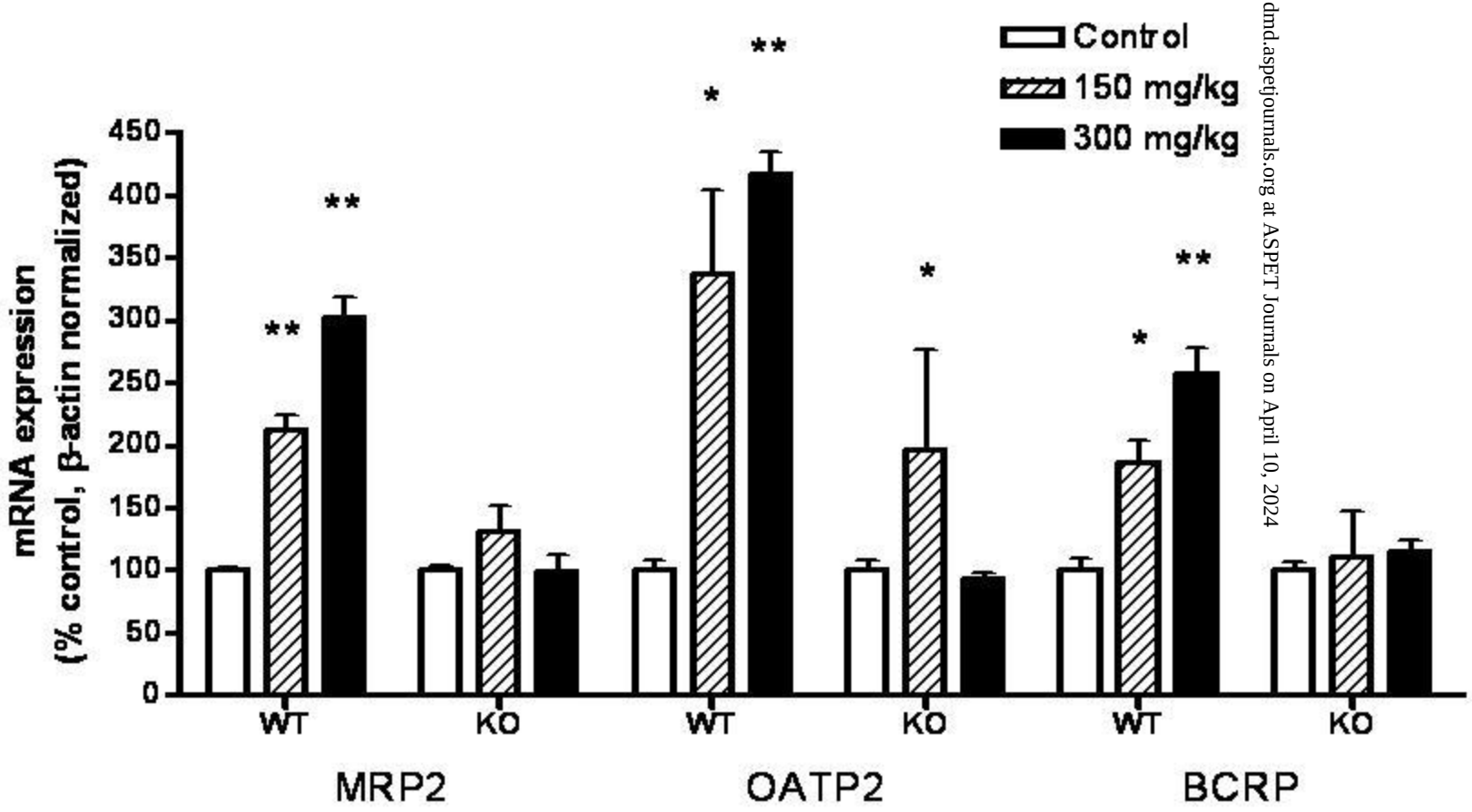


Figure 1B:

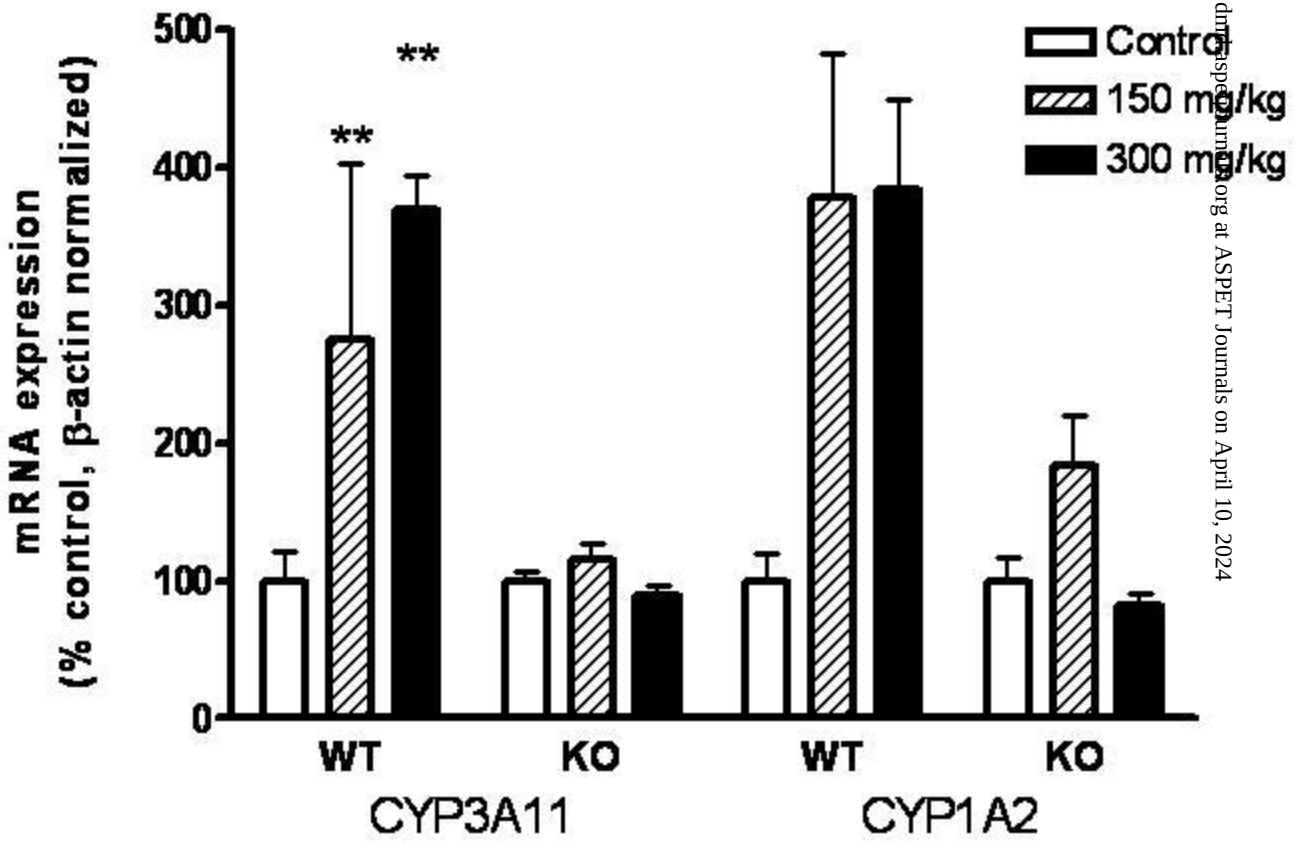
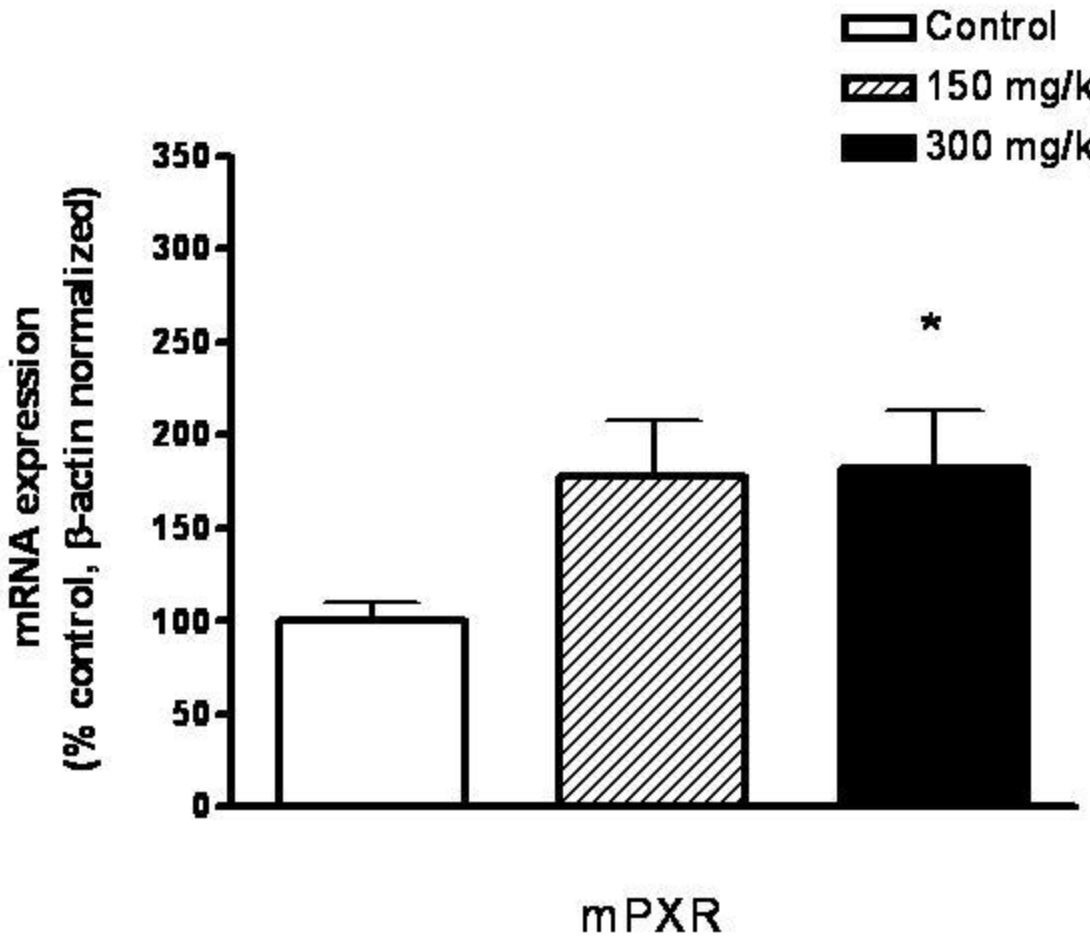


Figure 2:



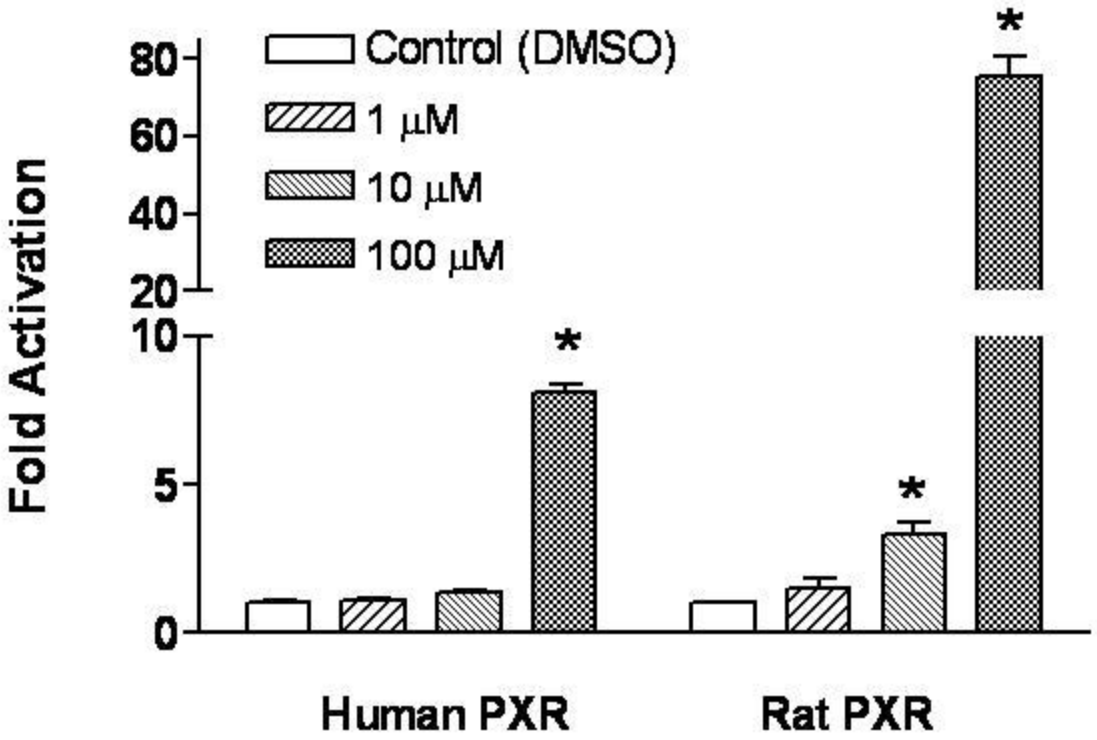


Figure 3