

HIV protease inhibitor A-792611 is a novel functional inhibitor of human PXR

Christine Healan-Greenberg, Jeffrey F. Waring, Dale J. Kempf, Eric A.G. Blomme,
Rommel G. Tirona, Richard B. Kim

Abbott Laboratories: Global Pharmaceutical Research and Development, Abbott Park, Illinois,
USA (CHG; JFW; DJK; EAGB)

Division of Clinical Pharmacology, Department of Medicine, and Department of
Physiology & Pharmacology, The University of Western Ontario, Schulich School of
Medicine & Dentistry, London, Ontario, Canada (RGT; RBK)

Running title: A-792611 is a functional inhibitor of PXR

Corresponding Author:

Dr. Jeffrey F. Waring

Molecular and Cellular Toxicology

Abbott Laboratories BLDG. AP9A R463

100 Abbott Park Road,

Abbott Park, IL 60064-6104

Tel: (847) 935-4124

Fax: (847) 935-7845

e-mail: jeff.waring@abbott.com

Text Pages: 22

Number of Tables: 2

Number of Figures: 11

Number of References: 39

Abstract Words: 219

Introduction Words: 617

Discussion Words: 1003

Abbreviations: PXR - pregnane X receptor; CAR - constitutive androstane receptor;

CYP - cytochrome P450; FXR - farnesoid X receptor; VDR - vitamin D receptor;

PPAR α - peroxisome proliferator-activated receptor α

Abstract

Drug-drug interactions involving induction of cytochrome P450 enzymes (CYPs) can lead to loss of drug efficacy. Certain drugs, particularly those used to treat mycobacterial and human immunodeficiency virus (HIV) infections, are especially prone to induce CYPs. During studies to examine drug-interaction potential of compounds in cultured human hepatocytes, exposure with A-792611, a novel HIV protease inhibitor (PI) previously under investigation for the treatment of HIV infection, resulted in significant downregulation of constitutive CYP3A4 expression. Furthermore, co-administration of A-792611 was found to attenuate CYP3A4 induction mediated by known inducers rifampin and efavirenz. A-792611 also attenuated the rifampin and ritonavir-mediated activation of the human pregnane X receptor (PXR) in luciferase reporter assays. Microarray analysis on cultured human hepatocytes revealed that A-792611 treatment downregulated the expression of PXR target genes CYP3A4, CYP2B6, CYP2C8, and CYP2C9, whereas there was a lack of inductive effect observed in treated rat hepatocytes. A-792611 did not interact with other ligand-activated nuclear receptors that regulate CYP expression such as constitutive androstane receptor, farnesoid X receptor, vitamin D receptor and peroxisome proliferator-activated receptor α . These data suggest that A-792611 is a functional and effective human PXR inhibitor. Among the class of HIV-PIs, which are typically PXR activators, A-792611 appears to have a unique property for PXR antagonism and could be a useful tool for studying nuclear receptor pathway regulation.

Introduction

Hepatic gene induction occurs in response to exposure to various drugs and xenobiotics. It is characterized by an increase in levels of transporters and/or phase I or II metabolizing proteins. The cytochrome P450 (CYP) enzymes are phase I enzymes that are importantly involved in the metabolism of a variety of xenobiotics (Handschin and Meyer, 2003). Induction of one or more CYPs in response to drug administration is a common cause of drug-drug interactions, alterations in drug response or adverse drug reactions (Willson and Kliewer, 2002; Plant and Gibson, 2003).

CYP3A4 is an important drug metabolizing enzyme whose hepatic expression is regulated at the transcriptional level by nuclear receptors such as the pregnane X receptor (PXR, also called steroid and xenobiotic receptor, SXR) and the constitutive androstane receptor (CAR) (Handschin and Meyer, 2003; Martinez-Jimenez et al., 2007). In its inactive state, PXR is localized in the cytoplasm of cells. Upon compound binding, nuclear translocation and heterodimerization to the retinoid X receptor (RXR) occurs (Matic et al., 2007). Similarly, CAR is present in an inactive form in the cytoplasm and, during exposure to an inducing compound, translocates to the nucleus through a phosphorylation-dependent mechanism (Willson and Kliewer, 2002). Upon entry into the nucleus, PXR/RXR and CAR/RXR heterodimers bind to promoter elements of many genes involved in drug metabolism and transport to stimulate gene transcription (Stanley et al., 2006; Urquhart et al., 2007).

Certain drugs used to treat human immunodeficiency virus (HIV) infection are widely known to induce drug-metabolizing enzymes, especially CYP3A4. Nevirapine and efavirenz, two non-nucleoside HIV reverse transcriptase inhibitors, potently upregulate CYP3A4 (Fichtenbaum and Gerber, 2002; Bergshoeff et al., 2005), producing negative drug-drug interactions with a second drug class, the HIV protease inhibitors (HIV-PIs). The latter are almost exclusively metabolized by CYP3A4, and dose adjustments are often necessary when nevirapine or efavirenz is combined with a protease inhibitor (Bergshoeff et al., 2005).

The HIV-PI ritonavir is a well-known, potent CYP3A4 inhibitor, but this drug also causes upregulation of CYP3A4 expression by acting as a PXR agonist. However, inhibition of CYP3A4 by ritonavir supercedes induction, resulting in net lowering of

CYP3A4 activity (Luo et al., 2002). Because of this, co-administration with ritonavir is a widely used strategy to boost the plasma concentrations of other HIV-PIs, thereby increasing their potency (Zeldin and Petruschke, 2004).

Due to the potential of HIV drugs to cause induction of drug metabolizing enzymes and transporters, screening for induction early in the drug development process has become standard practice. Due to known species differences in the ligand-binding domains of PXR and CAR, induction studies are often performed using functional assays of the human receptors. Moreover, gene expression studies in cultured hepatocytes exposed to drug candidates are often performed to determine the potential for inductive drug interactions.

A-792611 {(S)-1-[(1S,3S,4S)-4-[(S)-2-(3-Benzyl-2-oxo-imidazolidin-1-yl)-3,3-dimethyl-butylamino]-3-hydroxy-5-phenyl-1-(4-pyridin-2-yl-benzyl)-pentylcarbamoyl]-2,2-dimethyl-propyl}-carbamic acid methyl ester} is a novel HIV protease inhibitor (Fig. 1) that was investigated preclinically. Metabolism studies showed that A-792611 is metabolized by and is a functional inhibitor of CYP3A4. In this study, we examined the effect of A-792611 on the regulation of drug metabolizing enzymes and transporters in cultured human and rat hepatocytes by microarray and quantitative gene expression analyses. Furthermore, receptor transactivation assays were performed to examine the interactions of A-792611 with various nuclear receptors. These studies showed that A-792611 caused a decrease in the levels of PXR-regulated genes, including CYP3A4, in human but not rat hepatocytes. Furthermore, the inductive effect of potent activators of PXR could be attenuated by co-administration with A-792611. While numerous PXR activators have been identified, to date there are few examples of potent inhibitors of human PXR. Here, we establish A-792611 as a novel functional antagonist of human PXR, which can be a useful chemical tool for modulating gene expression *in vitro* or *in vivo*.

Materials and Methods

Human hepatocyte culture. Plateable cryopreserved human hepatocytes from several donors were purchased from In Vitro Technologies (IVT, Baltimore, MD; Lots QKR, 130, NPV, and ZCA, Part # F00995-P), and Gentest (BD Disocover Labware, Bedford, MA; Lot 70, Catalog # 454551). Donor demographics are shown in Table 1. Vials of cryopreserved cells were gently shaken in a 37°C water bath until the ice was just melted. The contents of the vials were added to 37°C hepatocyte thawing medium (IVT, Part # Z90019) supplemented with 0.5 g/L D-glucose and 10% FBS. Cell suspension was centrifuged at 500 rpm for five minutes to gently pellet the hepatocytes. The supernatant was aspirated, and the cell pellet was resuspended in hepatocyte plating medium (IVT Part # Z90029). Cell count and viability was determined by Trypan blue exclusion, and hepatocyte suspensions were diluted to 700,000 viable cells/mL in plating medium. Suspended cells were added to BioCoat (BD Discovery Labware) 12-well plates (1.0 mL/well) and allowed to attach overnight at 37°C in 5% CO₂/95% air. After 24 hours, plating medium was aspirated and wells were refilled with 1.0 mL of 37°C hepatocyte incubation medium (IVT, Part #Z99009), then hepatocytes were incubated at 37°C in 5% CO₂/95% air for an additional 24 hours. Cells were then treated for 48 hours, with fresh dosing solutions added after 24 hours.

Cytotoxicity Assessment in Human Hepatocytes. Cytotoxicity for isolated human hepatocytes (donor KQG) treated with A-792611 by itself or in combination with rifampin or efavirenz was determined using both the lactate dehydrogenase assay (LDH) (CytoTox 96[®], Promega, Madison, WI; Cat. #9FB054), and the Cell Titer-Glo cell viability assay (Promega, Madison, WI; Cat. #G7571) according to the manufacturers instructions. All compounds were dosed at 10 µM.

Rat hepatocyte culture. Isolated rat hepatocytes for the microarray experiment were prepared as follows. Whole liver was removed from a naïve Sprague-Dawley rat, and the periportal vein was cannulated with an 18-gauge catheter. The liver was perfused for 4-5 minutes with perfusion buffer at pH 7.4 containing 148mM NaCl, 9.2mM Na-HEPES,

16.7mM D-fructose, 0.5mM EGTA, and 1000units/L heparin (Abbott Laboratories, Abbott Park, IL) at a flow rate of 25mL/minute with the Masterflex® L/S™ (Cole Parmer, Vernon Hills, IL) peristaltic pump, then perfused for 6-10 minutes with collagenase buffer at pH 7.4 containing 6.7mM KCl, 142mM NaCl, 9.2mM Na-HEPES, 5.8mM CaCl₂-H₂O, 16.7mM D-fructose, 30uM phenol red, 0.57% of fraction V bovine serum albumin, 170U/mL trypsin inhibitor (Sigma, St. Louis, MO), 100U/mL collagenase (Sigma, St. Louis, MO), and 93U/mL hyaluronidase (Sigma, St. Louis, MO). The liver was excised and transferred to a petri dish containing collagenase buffer. The capsule was mechanically broken up with a comb and hepatocytes were collected in a 50 mL conical tube and placed on ice. Cell suspension was drawn through a wide bore pipette to break up clumps, then passed through a 100 micron nylon mesh sleeve and collected in a 250 mL sterile centrifuge tube. IVT hepatocyte incubation medium was added to a total volume of 125 mL, and the suspension was centrifuged for 3 minutes at 50 x g. Supernatant was aspirated, and rinse was repeated with hepatocyte incubation medium. Cell pellet was resuspended in 40 mL of hepatocyte incubation medium and passed through a 106 micron sleeve. Cell count and viability was determined by Trypan blue exclusion, and cells were plated onto a 6-well plate at 1 million cells/well. Plated cells were incubated overnight in a 37°C, 5% CO₂ incubator. Rat hepatocyte dosing solutions were prepared so that they contained the desired concentration of compound and 0.1% DMSO. One well was treated for each treatment group. Cells were treated for 48 hours, with fresh dosing solutions added after 24 hours.

RNA Isolation. After 48 hours of treatment, dosing solutions were aspirated off of the cells, and cells were washed with phosphate buffered saline. 800 µL of TRIzol (Invitrogen, Carlsbad, CA) was added to each well, and cell suspensions were aliquoted into 1.5 mL microfuge tubes. 200 µL chloroform was added to each tube, and tubes were shaken by hand for 15 seconds. Samples were centrifuged at 12,200 x g at 4°C for 15 minutes. The upper aqueous phase of each sample was pipetted into a clean microfuge tube, and the lower organic phase was discarded. 2 µL glycogen and 500 µL isopropyl alcohol were added to each sample, and samples were allowed to incubate at room temperature for 10 minutes. Samples were centrifuged at 4°C for 10 minutes, and

supernatant was discarded. The RNA pellet was washed with 1 mL of 75% ethanol, and then resuspended in a small volume of RNase-free H₂O. Total RNA concentration was assessed by spectrophotometry, and RNA quality was assessed on the 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Quantitative real-time PCR analysis. Samples were diluted with RNase-free H₂O so that they contained 20 ng/μL total RNA. 5 uL of each sample was pipetted in duplicate into a 96-well optical reaction plate (Applied Biosystems, Foster City, CA). Reagent mix was made using the TaqMan EZ RT-PCR kit (Applied Biosystems). The following probe sets were used:

Human CYP3A4:

Forward: 5'- CTT CAT CCA ATG GAC TGC ATA AAT- 3'

Reverse: 5'- TCC CAA GTA TAA CAC TCT ACA CAG ACA A- 3'

TaqMan: 5'- /5-FAM/CCG GGG ATT CTG TAC ATG CAT TG/3-BHQ-1/- 3'

Human CYP2B6:

Forward: 5'- AAG CGG ATT TGT CTT GGT GAA- 3'

Reverse: 5'- TGG AGG ATG GTG GTG AAG AAG- 3'

TaqMan: 5'- /5-FAM/CGC CCG TGC CGA ATT GTT CC3-BHQ-1/- 3'

Human 28S (control to account for any variability in RNA levels):

Forward: 5'-TTC ACC AAG CGT TGG ATT GTT -3'

Reverse: 5'-TGT CTG AAC CTG CGG TTC CT -3'

TaqMan: 5'-/5-HEX/TCA CGA CGG TCT AAA CCC AGC TCA CG/3-BHQ-1/- 3'

RT-PCR was performed on the ABI Prism 7700. Results were analyzed by calculating the fold change of CYP induction caused by each compound compared to vehicle levels.

Microarray Analysis. Microarray analysis was performed using the standard protocol provided by Affymetrix (Santa Clara, CA). Briefly, approximately 5 μg of total RNA was reverse transcribed into cDNA using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions, with the exception that the primer used for the reverse transcription reaction was a modified T7 primer with 24 thymidines at the 5' end (Affymetrix). The sequence was

5'GGCCAGTGAATTGTAATACGAC-TCACTATAGGGAGGCGG-(dT)24-3'. cDNA

was purified via phenol/chloroform/isoamylalcohol (Invitrogen) extraction and ethanol precipitation. The purified cDNA was resuspended in molecular biology grade water. Following this procedure, biotin labeled cRNA was synthesized according to the manufacturer's instructions from the cDNA using the Enzo RNA Transcript Labeling Kit (ENZO Life Sciences, Farmingdale, NY). The labeled cRNA was then purified using RNeasy kits (Qiagen, Valencia, CA). Subsequently, cRNA concentration and integrity was evaluated. Approximately 20 µg of cRNA was then fragmented in a solution of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C for 35 minutes. Fragmented, labeled cRNA was hybridized to an Affymetrix rat genome RAE230A or human genome U133A array at 45°C overnight using an Affymetrix Hybridization Oven 640. The array was subsequently washed and stained twice with streptavidin-phycoerythrin (Invitrogen) using the Gene-Chip Fluidics Workstation 400 (Affymetrix). The array was then scanned using the Affymetrix Gene-Chip scanner (Part No. 900154). The microarray scanned image and intensity files (.cel files) were imported into Rosetta Resolver gene expression analysis software version 3.2 (Rosetta Inpharmatics), and ratios were built between the protease inhibitor-treated against the vehicle-treated controls. P-values for each gene expression change were calculated according to the Rosetta Resolver analysis software.

Reporter Constructs and Cell-Based Transactivation Assays. All assays involving reporter constructs were performed at Vanderbilt University in the laboratory of Dr. Richard Kim. A CYP3A4-XREM-Luc reporter plasmid driven by CYP3A4 regulatory elements (-362/+53) and (-7836/-7208) in pGL3 Basic vector (Promega, Madison, WI) was prepared as described previously (Zhang et al., 2001) based on the report of Goodwin and colleagues (Goodwin et al., 1999). The BSEP-luc reporter plasmid was prepared by cloning the -1.6 kb promoter of the bile salt export pump (BSEP) gene (-1540/+75) into pGL3 Basic (Marzolini et al., 2007). A PPAR responsive luciferase reporter plasmid and a PPAR α expression plasmid were kindly provided from Dr. Hong-Guang Xie (Vanderbilt University). PXR, CAR, VDR, and FXR cDNAs cloned in pEF6/V5-His expression vector (Invitrogen) were assembled as described previously (Tirona et al., 2003). Human hepatocarcinoma cells, HepG2, were seeded at 0.6×10^6

cells/well into 12-well plates. After 24 hours, cells were transfected with 250 ng/well of luciferase reporter plasmid, 250 ng/well of the nuclear receptor expression plasmid and 25 ng/well pCMV-TK (Promega) to control for transfection efficiency using Lipofectin (Invitrogen). Sixteen hours thereafter, cells were treated with compounds or vehicle (DMSO, 0.1%) for 24 hours. Luciferase activities were determined with the Dual Luciferase Assay Kit (Promega).

Results

Regulation of hepatocyte CYP3A4 and CYP2B6 expression by A-792611. Rifampin (20 μ M) caused a marked increase in CYP3A4 mRNA levels in hepatocytes from Donors 130 and QKR, and a moderate increase in CYP2B6 mRNA levels (Table 2). These observations are consistent with literature reports of rifampin as an inducer of CYP3A, and to a lesser extent CYP2B, in humans. Ritonavir (10 μ M) also induced CYP3A4 and CYP2B6 mRNA in both donors, consistent with literature reports of ritonavir as a transcriptional inducer (Table 2). Experimental compound A-792611 caused a decrease in CYP3A4 mRNA levels in both donors. At a concentration of 10 μ M, A-792611 caused CYP3A4 levels to drop to 6% of vehicle in Donor 130 and 3% of vehicle in Donor QKR. A-792611 also caused a slight decrease in CYP2B6 levels in both donors (Table 2).

The unusual downregulation of CYP3A4 and CYP2B6 mRNA by A-792611 caused us to investigate whether or not A-792611 could attenuate mRNA induction caused by known inducers. In donor QKR, rifampin and efavirenz caused dose-dependent upregulation of CYP3A4 mRNA levels. Co-dosing A-792611 with rifampin and efavirenz attenuated the CYP3A4 mRNA induction caused by the positive controls (Fig. 2AB and Table 2). Cytotoxicity assays performed on isolated hepatocytes treated with A-792611 alone or in combination with rifampin or efavirenz did not induce any detectable cell death (data not shown). Rifampin and efavirenz also caused dose-dependent upregulation of CYP2B6 mRNA in hepatocytes from donor QKR. As was the case with CYP3A4, co-dosing with A-792611 attenuated this induction. A-792611 (10 μ M) alone caused levels of CYP2B6 mRNA that were 35% of vehicle level (data not shown).

Interactions of A-792611 with human PXR. In order to determine whether the downregulation of CYP3A4 and CYP2B6 was PXR-mediated, activation of PXR in transfected HepG2 cells was measured by luciferase reporter assay. Treatment of transfected HepG2 cells with rifampin resulted in dose-dependent activation of PXR (Fig. 3A). When rifampin was co-dosed with A-792611, the activation of PXR decreased,

falling almost to vehicle levels, as the dose of A-792611 increased (Fig. 3A). The IC_{50} for A-792611 inhibition of rifampin-mediated PXR activation was approximately 2 μ M. Treatment of transfected HepG2 cells with ritonavir also resulted in dose-dependent activation of PXR. When ritonavir was co-dosed with A-792611, the activation of PXR again decreased as the dose of A-792611 increased (Fig. 3B). These results suggested that PXR antagonism plays a role in the regulation of CYP enzymes by A-792611.

Genomic analysis of A-792611-treated hepatocytes. To further investigate the possibility that inhibition of PXR was causing the downregulation of CYP3A4 and CYP2B6, microarray analysis was performed on human hepatocytes from donor 70. Microarray analysis confirmed that A-792611 caused the downregulation of CYP3A4, CYP2B6, CYP2C8, CYP2C9, and the liver transporters MDR1 and NTCP (Fig. 4A). Hepatocytes co-dosed with A-792611 and ritonavir also showed downregulation of CYP enzymes and transporters. By contrast, ritonavir, atazanavir, and amprenavir caused an induction of CYP enzymes and did not affect the expression of liver transporters, with the exception of MRP2, which was upregulated (Fig. 4A). A-792611 did not affect the expression of the nuclear receptors PXR, RXR, CAR, aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor α (PPAR α), farnesoid X receptor (FXR), vitamin D receptor (VDR), glucocorticoid receptor (GR), and estrogen receptor (ER) (data not shown). A lack of PPAR α agonist effects by A-792611 is also evidenced by unchanged CYP4A mRNA levels in human hepatocytes after treatment (data not shown). It is also unlikely that A-792611 is an AhR antagonist, as no decrease in expression levels were observed for CYP1A1 (Figure 4A). However, antagonism of the AhR cannot be entirely ruled out, since downregulation of the CYP1A2 gene was observed (Figure 4A).

In order to determine whether or not the CYP downregulation effect caused by A-792611 was species-specific, microarray analysis was also performed on primary rat hepatocytes treated with A-792611 and ritonavir. A-792611 and ritonavir both caused downregulation of Cyp2b3, Cyp2c6, and Ntcp, and upregulation of Cyp3a3 and Mdr1 (Fig. 4B). The expression of other Cyps and transporters was largely unchanged by treatment with these two compounds. The gene expression changes caused by dosing A-792611 together with ritonavir were very similar to those caused by each compound

alone (Fig. 3B). This is in sharp contrast to the primary human hepatocytes, where a distinct CYP and transporter gene expression pattern was observed in cells dosed with A-792611.

Lack of interaction between A-792611 and FXR, CAR, VDR and PPAR α Results from human hepatocytes and PXR activation assays supported the notion that A-792611 causes downregulation of CYP enzymes through functional inhibition of human PXR. Other nuclear receptors including FXR, CAR, VDR and PPAR α are known to regulate CYP expression (Tirona and Kim, 2005). Therefore, we investigated whether the effects of A-792611 on CYP expression were the result of functional interaction with nuclear receptors other than PXR. In cell-based, nuclear receptor transactivation assays, A-792611 did not activate human FXR, and a 20 μ M dose only slightly attenuated the FXR activation caused by positive control chenodeoxycholate (CDCA) (Fig. 5A). In a CAR reporter assay, CITCO (10 μ M), a CAR-specific agonist (19), caused a 2.5-fold activation of human CAR. A-792611 did not cause significant CAR activation at a concentration of 20 μ M, nor did it antagonize the effect of CITCO (Fig. 5B). Experiments in the reporter assays further showed that A-792611 was not an activator of VDR nor did it antagonize the inductive effects of 1 α ,25-dihydroxyvitamin D3 (Fig. 5C). Lastly, we examined the interaction of A-792611 with PPAR α and observed that it was not an agonist or antagonist of this receptor (Fig. 5D).

Discussion

Many peptidomimetic HIV-PIs including amprenavir, atazanavir, lopinavir, nelfinavir and ritonavir are known for their capacity to induce drug metabolizing enzymes via a PXR activation mechanism. In this report, we demonstrate that A-792611, an HIV-PI that was previously examined preclinically, downregulates constitutive expression of PXR-regulated genes CYP3A4, CYP2B6, CYP2C8, CYP2C9 and MDR1 in cultured human hepatocytes. These results are in contrast to the PXR agonists ritonavir, atazanavir, and amprenavir, which caused a general upregulation of CYP mRNA. These results suggest that constitutive CYP3A4 expression in hepatocytes may be controlled by endogenously synthesized PXR agonists. Moreover, the inductive responses caused by treatment of the xenobiotic PXR agonists rifampin and ritonavir could be attenuated by co-treatment of human hepatocytes with A-792611. Cell-based transactivation experiments confirmed that A-792611 inhibits the activity of human PXR but not other nuclear receptors that regulate CYP3A4. The effects of A-792611 appear to be largely species-specific, as we did not observe gene expression changes consistent with PXR antagonism in rat hepatocytes. While a luciferase reporter assay did show weak antagonism of PCN-induced rat PXR activation by A-792611, this response was not as robust as that observed in the human PXR assay (data not shown).

Since A-792611 is metabolized in hepatocytes, downregulation of CYP3A4 by parent compound *vs.* a metabolite has not been definitively established. However, the half-life of A-792611 in human hepatocytes is relatively long at 6 hours. Moreover, inhibition of PXR activation was demonstrated in HepG2 cells which have been shown to have little to no metabolic capability. Thus, it is likely that most, if not all, of the activity of A-792611 observed in this study reflects the action of the parent compound.

Results from cell-based, nuclear receptor transactivation assays indicate that A-792611 selectively antagonizes PXR and does not interact with other nuclear receptors tested, such as FXR, CAR, VDR and PPAR- α . Interestingly, microarray analysis in isolated human hepatocytes showed that treatment with A-792611 did cause a downregulation of CYP2B6, which is largely regulated by CAR (Faucette et al., 2006). However, extensive cross-talk exists between the nuclear receptors, and studies have shown that CYP2B6 is also regulated by PXR (Goodwin et al., 2001). Thus, quite

possibly, the downregulation of CYP2B6 mRNA by A-792611 may be due to antagonism of PXR.

An interesting finding that emerged from the microarray analysis of human hepatocytes is that treatment with A-792611 resulted in an upregulation of CYP1A1 and a downregulation of CYP1A2. This result is somewhat unusual since studies have shown that both of these genes can be regulated by the AhR (Ueda et al., 2006). It should be noted that the upregulation of CYP1A1 by A-792611 is relatively small (~4 fold). Treatment with classical activators of the AhR usually result in significantly higher levels of induction (see for instance (Zhang et al., 2006). Nonetheless, both the induction of CYP1A1 and repression of CYP1A2 were considered significant based on the Rosetta Resolver error model. Previous studies have shown that some differential response occurs between CYP1A1 and 1A2, and that CYP1A2 can be regulated by mechanisms independent of the AhR (Zaher et al., 1998). Thus, it may be that the response of CYP1A1 and 1A2 to A-792611 is due to AhR independent mechanisms.

Studies have shown that the antineoplastic agent ET-743 (Synold et al., 2001) and the antifungal agent ketoconazole (Takeshita et al., 2002) and the dietary isothiocyanate sulforaphane (Zhou et al., 2007) are also functional antagonists of PXR. While ET-743 is a potent inhibitor of PXR activation by SR12813 ($IC_{50} = 2$ nM) (Synold et al., 2001), its utility as an *in vitro* or *in vivo* tool may be hampered by its innate toxic actions as a DNA minor groove binder. Sulforaphane antagonizes rifampin-mediated PXR activation with an IC_{50} of 12 μ M and likely interacts with the receptor through covalent interactions from the chemically reactive isothiocyanate group (Zhou et al., 2007). Aside from PXR antagonist properties, sulforaphane is an inducer of several drug metabolizing and antioxidant enzymes acting via the Nrf2/Keap1 pathway (Morimitsu et al., 2002). Although ketoconazole inhibits PXR with an IC_{50} of 20 μ M (data not shown), it is also a well known antagonist of the glucocorticoid (Duret et al., 2006) and androgen receptors (Eil, 1992). Here, we describe a novel, effective PXR antagonist among the HIV protease inhibitor class of drugs that are largely known for their agonist effects towards this receptor. Aside from the anti-cancer compound ET-743, A-792611 is the most potent of the described PXR antagonists, with an IC_{50} of 2 μ M towards rifampin-mediated receptor activation.

Recently, molecular approaches that consider the three-dimensional structures of PXR have been employed to understand the molecular determinants of receptor-ligand interactions with the intent of modulating agonist effects or creating antagonists (Gao et al., 2007; Xue et al., 2007). This strategy has been somewhat ineffective in producing antagonists owing to the fact that the receptor appears to have conformational flexibility (Xue et al., 2007). Our results indicate that such antagonists can be identified. However, additional investigations will be required to understand the structure-activity relationships that determine the PXR agonist/antagonist behavior of HIV-PIs. Such information may have utility in the development of new HIV-PIs with minimal propensity for causing induction-type drug interactions, some of which have been recently described (Hogeland et al., 2007).

Aside from the important role in xenobiotic defense, PXR has been implicated in a number of physiological and pathophysiological processes. For example, PXR signaling contributes to bone (Tabb et al., 2003; Zhou et al., 2006a), cholesterol (Sporstol et al., 2005), lipid (Zhou et al., 2006c), mineralocorticoid (Zhai et al., 2007), glucocorticoid (Zhai et al., 2007), and thyroid hormone homeostasis (Wong et al., 2005), inflammatory responses (Gu et al., 2006; Zhou et al., 2006b) and uterine contractility (Mitchell et al., 2005). Hence, PXR antagonists such as A-792611 may have utility as chemical modulators to interrogate the physiological roles of PXR.

In conclusion, we report the identification of a novel, selective and functional inhibitor of human PXR. A-792611 should prove to be an important tool for understanding pharmacology and physiology of PXR regulated signaling pathways.

References

- Bergshoeff AS, Fraaij PL, Ndagijimana J, Verweel G, Hartwig NG, Niehues T, De Groot R and Burger DM (2005) Increased dose of lopinavir/ritonavir compensates for efavirenz-induced drug-drug interaction in HIV-1-infected children. *J Acquir Immune Defic Syndr* **39**:63-68.
- Duret C, Daujat-Chavanieu M, Pascussi JM, Pichard-Garcia L, Balaguer P, Fabre JM, Vilarem MJ, Maurel P and Gerbal-Chaloin S (2006) Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor. *Mol Pharmacol* **70**:329-339.
- Eil C (1992) Ketoconazole binds to the human androgen receptor. *Horm Metab Res* **24**:367-370.
- Faucette SR, Sueyoshi T, Smith CM, Negishi M, Lecluyse EL and Wang H (2006) Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *J Pharmacol Exp Ther* **317**:1200-1209.
- Fichtenbaum CJ and Gerber JG (2002) Interactions between antiretroviral drugs and drugs used for the therapy of the metabolic complications encountered during HIV infection. *Clin Pharmacokinet* **41**:1195-1211.
- Gao YD, Olson SH, Balkovec JM, Zhu Y, Royo I, Yabut J, Evers R, Tan EY, Tang W, Hartley DP and Mosley RT (2007) Attenuating pregnane X receptor (PXR) activation: a molecular modelling approach. *Xenobiotica* **37**:124-138.
- 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**:1329-1339.
- Goodwin B, Moore LB, Stoltz CM, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* **60**:427-431.
- Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, Gallo MA, Xie W and Tian Y (2006) Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem* **281**:17882-17889.
- Handschin C and Meyer UA (2003) Induction of drug metabolism: the role of nuclear receptors. *Pharmacol Rev* **55**:649-673.
- Hogeland GW, Swindells S, McNabb JC, Kashuba AD, Yee GC and Lindley CM (2007) Lopinavir/ritonavir reduces bupropion plasma concentrations in healthy subjects. *Clin Pharmacol Ther* **81**:69-75.
- Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E and Gan LS (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* **30**:795-804.

- 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.
- Marzolini C, Tirona RG, Gervasini G, Poonkuzhali B, Assem M, Lee W, Leake BF, Schuetz JD, Schuetz EG and Kim RB (2007) A Common Polymorphism in the Bile Acid Receptor FXR is Associated with Decreased Hepatic Target Gene Expression. *Mol Endocrinol*.
- Matic M, Mahns A, Tsoli M, Corradin A, Polly P and Robertson GR (2007) Pregnane X receptor: promiscuous regulator of detoxification pathways. *Int J Biochem Cell Biol* **39**:478-483.
- Mitchell BF, Mitchell JM, Chowdhury J, Tougas M, Engelen SM, Senff N, Heijnen I, Moore JT, Goodwin B, Wong S and Davidge ST (2005) Metabolites of progesterone and the pregnane X receptor: a novel pathway regulating uterine contractility in pregnancy? *Am J Obstet Gynecol* **192**:1304-1313; discussion 1313-1305.
- Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, Osawa T, Horio F, Itoh K, Iida K, Yamamoto M and Uchida K (2002) A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J Biol Chem* **277**:3456-3463.
- Plant NJ and Gibson GG (2003) Evaluation of the toxicological relevance of CYP3A4 induction. *Curr Opin Drug Discov Devel* **6**:50-56.
- Sporstol M, Tapia G, Malerod L, Mousavi SA and Berg T (2005) Pregnane X receptor-agonists down-regulate hepatic ATP-binding cassette transporter A1 and scavenger receptor class B type I. *Biochem Biophys Res Commun* **331**:1533-1541.
- Stanley LA, Horsburgh BC, Ross J, Scheer N and Wolf CR (2006) PXR and CAR: nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. *Drug Metab Rev* **38**:515-597.
- Synold TW, Dussault I and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* **7**:584-590.
- Tabb MM, Sun A, Zhou C, Grun F, Errandi J, Romero K, Pham H, Inoue S, Mallick S, Lin M, Forman BM and Blumberg B (2003) Vitamin K2 regulation of bone homeostasis is mediated by the steroid and xenobiotic receptor SXR. *J Biol Chem* **278**:43919-43927.
- Takeshita A, Taguchi M, Koibuchi N and Ozawa Y (2002) Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. *J Biol Chem* **277**:32453-32458.
- Tirona RG and Kim RB (2005) Nuclear receptors and drug disposition gene regulation. *J Pharm Sci* **94**:1169-1186.
- 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.
- Ueda R, Iketaki H, Nagata K, Kimura S, Gonzalez FJ, Kusano K, Yoshimura T and Yamazoe Y (2006) A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. *Mol Pharmacol* **69**:1924-1930.

- Urquhart BL, Tirona RG and Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol* **47**:566-578.
- Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. *Nat Rev Drug Discov* **1**:259-266.
- Wong H, Lehman-McKeeman LD, Grubb MF, Grossman SJ, Bhaskaran VM, Solon EG, Shen HS, Gerson RJ, Car BD, Zhao B and Gemzik B (2005) Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**:232-242.
- Xue Y, Chao E, Zuercher WJ, Willson TM, Collins JL and Redinbo MR (2007) Crystal structure of the PXR-T1317 complex provides a scaffold to examine the potential for receptor antagonism. *Bioorg Med Chem* **15**:2156-2166.
- Zaher H, Yang TJ, Gelboin HV, Fernandez-Salguero P and Gonzalez FJ (1998) Effect of phenobarbital on hepatic CYP1A1 and CYP1A2 in the Ahr-null mouse. *Biochem Pharmacol* **55**:235-238.
- Zeldin RK and Petruschke RA (2004) Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients. *J Antimicrob Chemother* **53**:4-9.
- Zhai Y, Pai HV, Zhou J, Amico JA, Vollmer RR and Xie W (2007) Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. *Mol Endocrinol* **21**:138-147.
- 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.
- Zhang ZY, Pelletier RD, Wong YN, Sugawara M, Zhao N and Littlefield BA (2006) Preferential inducibility of CYP1A1 and CYP1A2 by TCDD: differential regulation in primary human hepatocytes versus transformed human cells. *Biochem Biophys Res Commun* **341**:399-407.
- Zhou C, Assem M, Tay JC, Watkins PB, Blumberg B, Schuetz EG and Thummel KE (2006a) Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia. *J Clin Invest* **116**:1703-1712.
- Zhou C, Poulton EJ, Grun F, Bammler TK, Blumberg B, Thummel KE and Eaton DL (2007) The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Mol Pharmacol* **71**:220-229.
- Zhou C, Tabb MM, Nelson EL, Grun F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, Thummel KE and Blumberg B (2006b) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**:2280-2289.
- Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, Ren S, Evans RM and Xie W (2006c) A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* **281**:15013-15020.

Footnotes:

This work supported by U.S. Public Health Service Grants GM31304 (to R.B.K.)

Figure Legends

Figure 1. *Structure of A-792611.*

Figure 2. *CYP3A4 expression in cultured human hepatocytes treated with drugs for 48 hours.* Real-time PCR analysis of CYP3A4 mRNA in cultured human hepatocytes (Donor QKR) treated with the PXR agonists rifampin (A) or efavirenz (B) in combination with A-792611 (n=2). The error bars display standard deviation.

Figure 3. *Transactivation assays in HepG2 cells transiently transfected with CYP3A4-XREM-Luc reporter and PXR expression plasmids.* After 24 hours of drug treatment, luciferase activities were determined. (A) Concentration-dependent activation of human PXR by rifampin and attenuation of rifampin-mediated (10 μ M) PXR activation by escalating levels of A-792611 (n=4). (B) A-792611 attenuates PXR activation mediated by ritonavir treatment (n=3). Data are shown for a single experiment.

Figure 4. *Microarray analysis of cultured human hepatocyte gene expression after 48 hours treatment with drugs.* Drug treatment included ritonavir (RTV), atazanavir (ATV), amprenavir (AMP), A-792611 (611) alone or in combination. (A) Heat map showing CYP and transporter gene changes in human hepatocytes from Donor 70. (B) Heat map showing Cyp and transporter gene changes in isolated rat hepatocytes. Red, green and black colors represent genes that are upregulated, downregulated and unchanged, respectively, compared to the vehicle control. Genes were considered to be significantly regulated if the calculated p-value was less than or equal to 0.05. All compounds were dosed at 10 μ M.

Figure 5. *Lack of interaction between A-792611 and various nuclear receptors.* HepG2 cells were transfected with luciferase reporter constructs as well as expression plasmids for nuclear receptors and treated with A-792611, known inducers or vehicle (0.1% DMSO). Luciferase activities were determined after 24 hours of treatment. (A) FXR

reporter assay using BSEP-Luc reporter and the bile acid CDCA as positive agonist control (n = 6-12). (B) CAR assay using CYP3A4-XREM-Luc reporter and CITCO as agonist (n = 6). (C) VDR luciferase reporter assay with CYP3A4-XREM-Luc reporter and $1\alpha,25$ -dihydroxvitamin D₃ as positive agonist (n = 6). (D) PPAR α assay using PPRE-Luc reporter and Wy-14643 as positive control (n = 6-9). Data are shown for experiments performed on 2-3 occasions. * Significantly different from cells expressing FXR and treated with CDCA (Student's t-test).

Table 1. Demographics of human hepatocyte donors.

Lot	Age	Sex	Race	Cause of Death	Tobacco Use	Alcohol Use	Substance Use	CMV	Hepatitis B, C; HIV	Medical History
QKR	35	M	C	ICH	Yes	Yes	Yes	-	-	Seizure disorder, Subdural hematoma
130	2	F	C	Anoxia	No	No	No	+	-	None
70	1	M	C		No	No	No		-	
KQG	38	F	C	CVA	Yes	Yes	-	-	-	-

M, male; F, female; C, caucasian; ICH, intracerebral hemorrhage; CVA, cerebrovascular accident

Table 2. CYP3A4 and CYP2B6 expression in cultured human hepatocytes treated with rifampin, ritonavir and A-792611. Results are expressed as fold induction relative to vehicle treated control for human donors 130 and QKR.

Gene	Donor	Fold Induction		
		Rifampin (20 μ M)	Ritonavir (10 μ M)	A-792611 (10 μ M)
CYP3A4	130	64.01	18.78	0.06
	QKR	72.88	35.78	0.03
CYP2B6	130	4.62	1.72	0.57
	QKR	15.11	4.58	0.41

Figure 1.

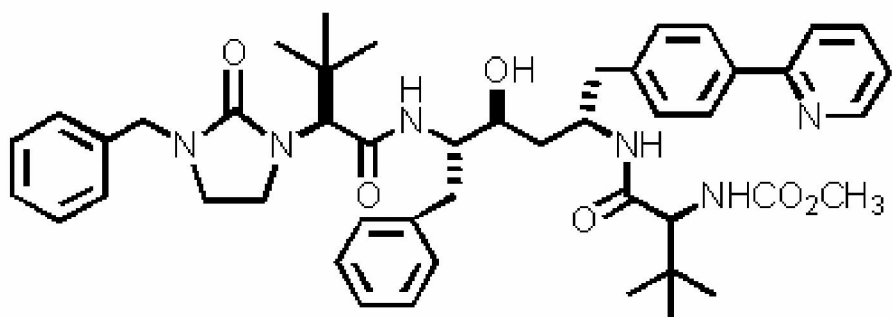


Figure 2A

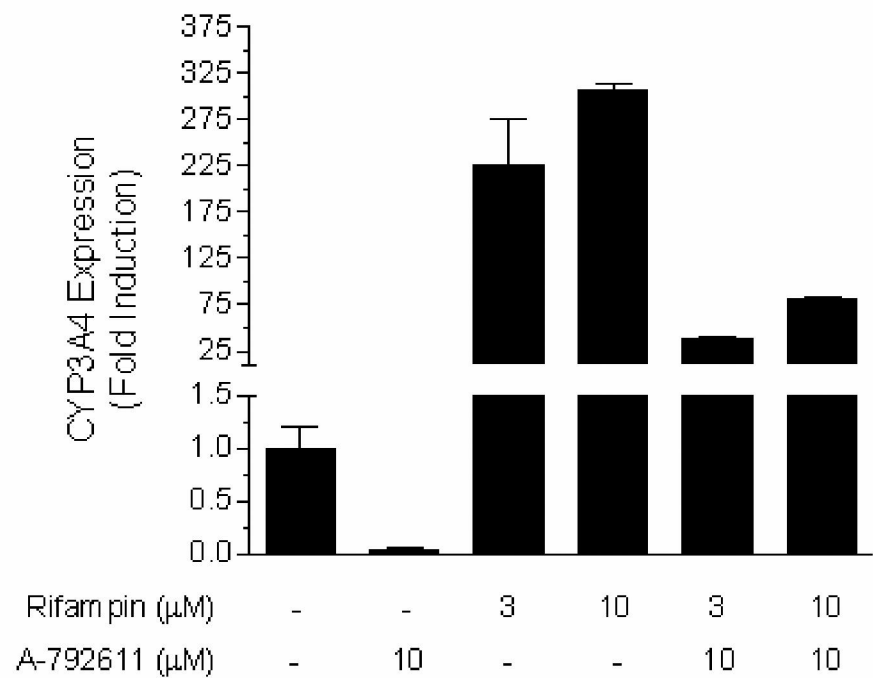


Figure 2B

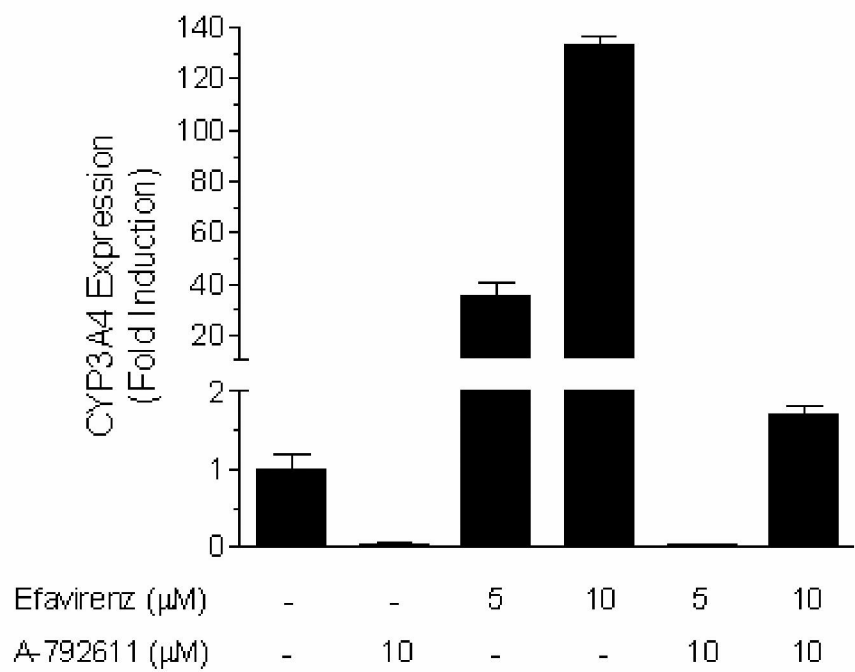


Figure 3A

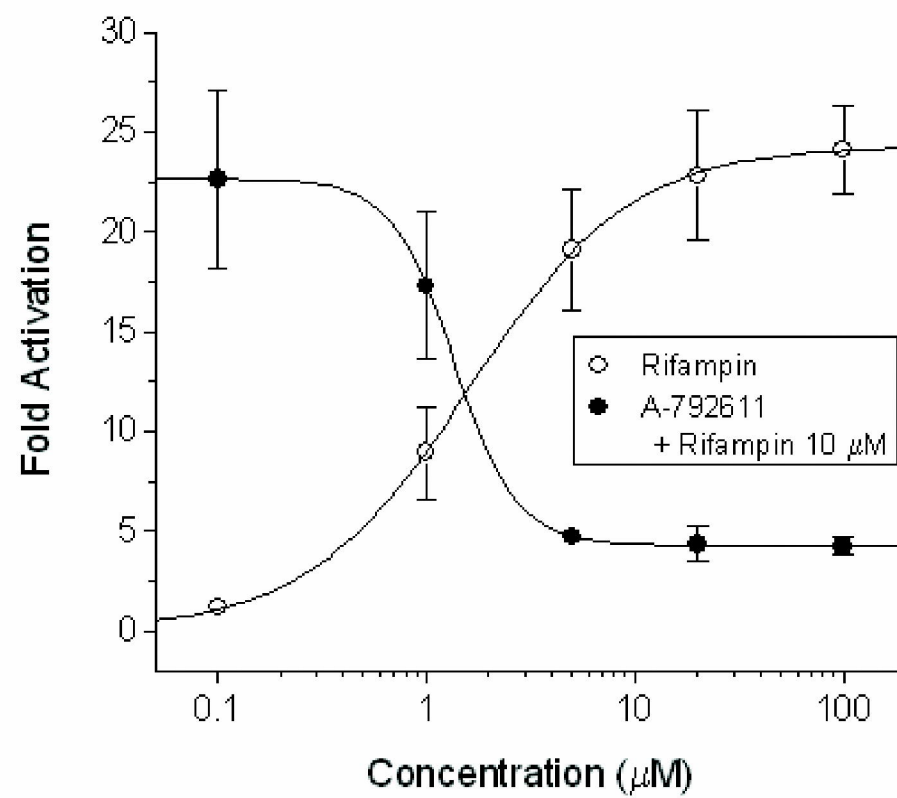


Figure 3B

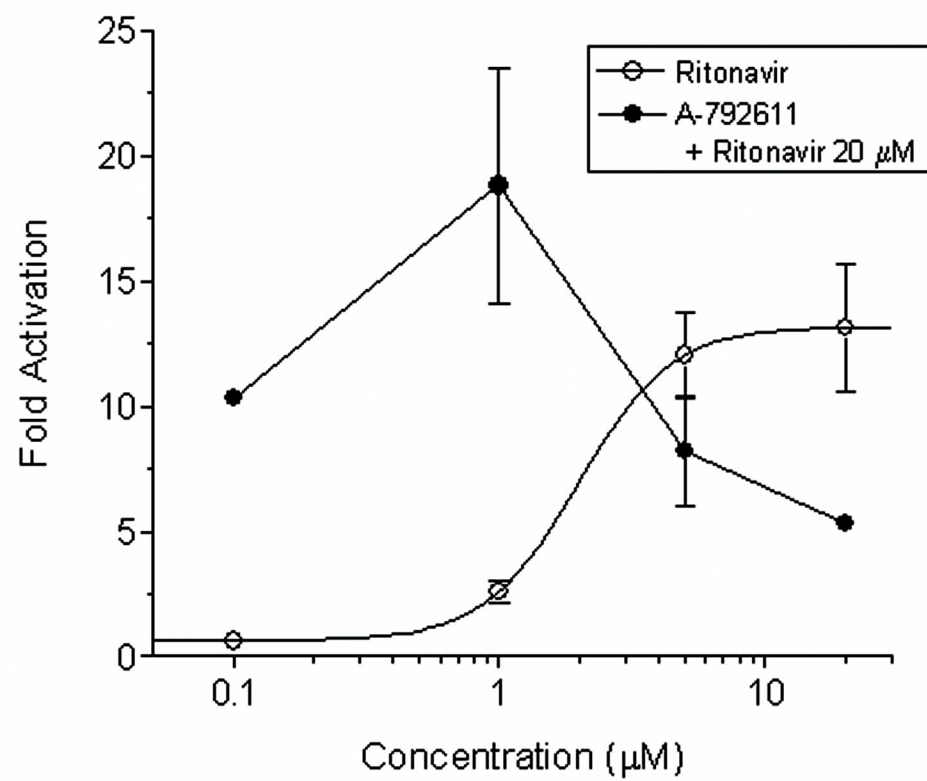


Figure 4A

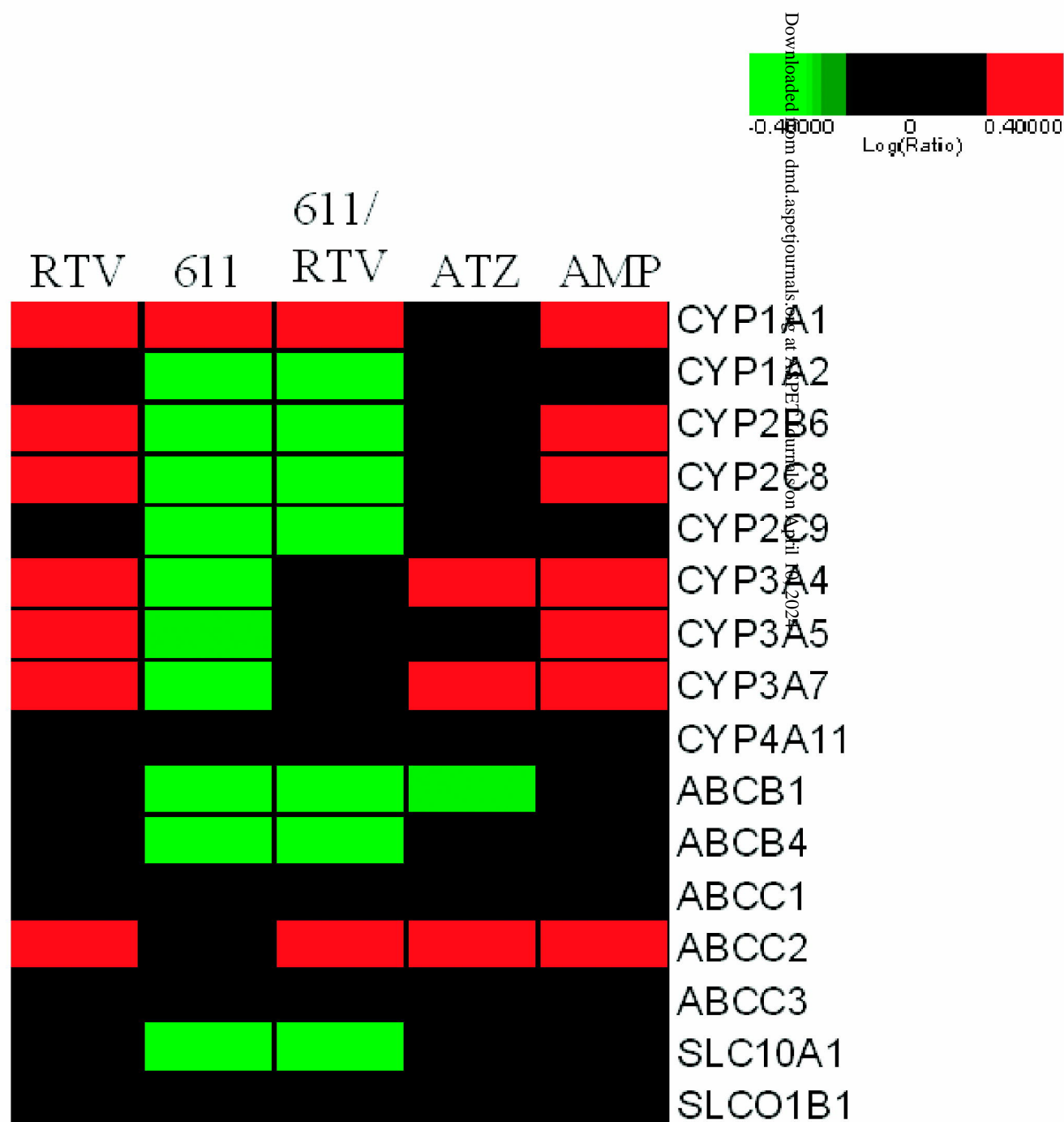
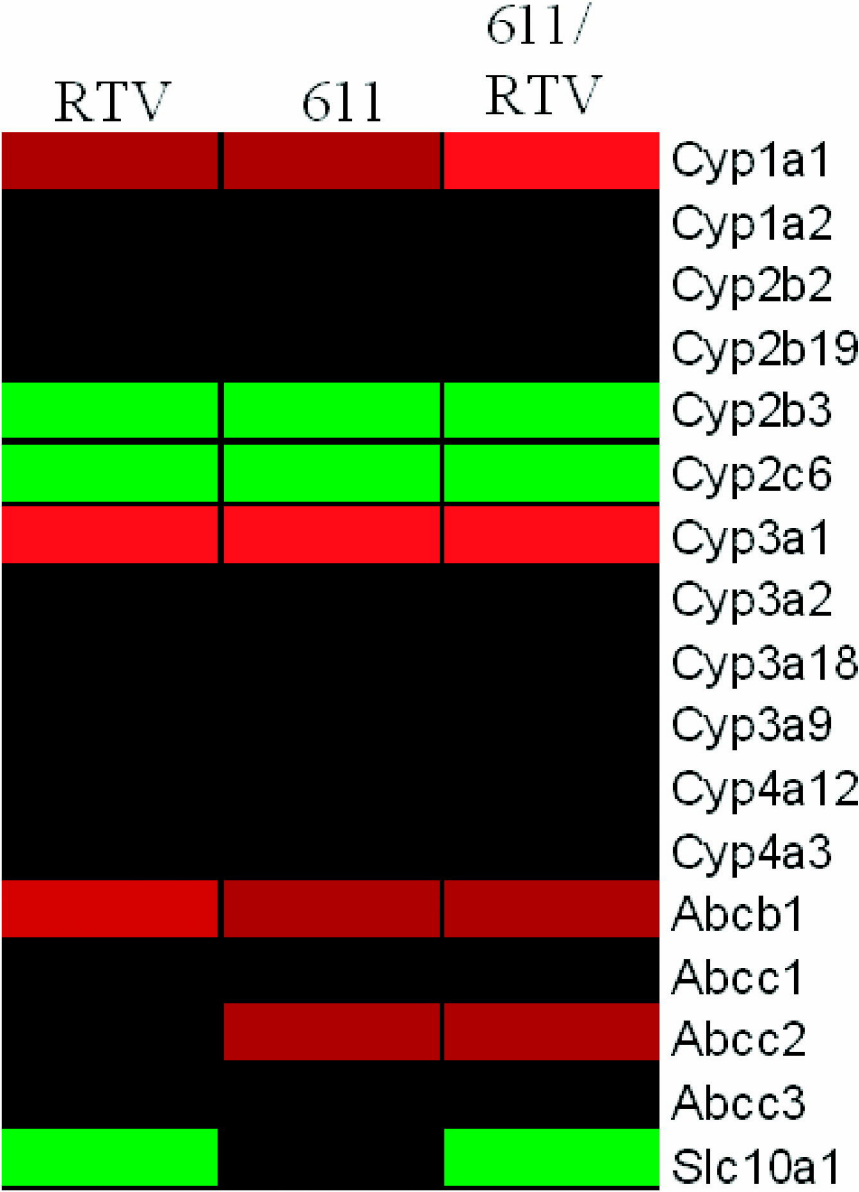


Figure 4B



Downloaded from dnd.aspetjournals.org at ASPET Journals on April 10, 2024

Log(Ratio)

-0.40000 0 0.40000

Figure 5A

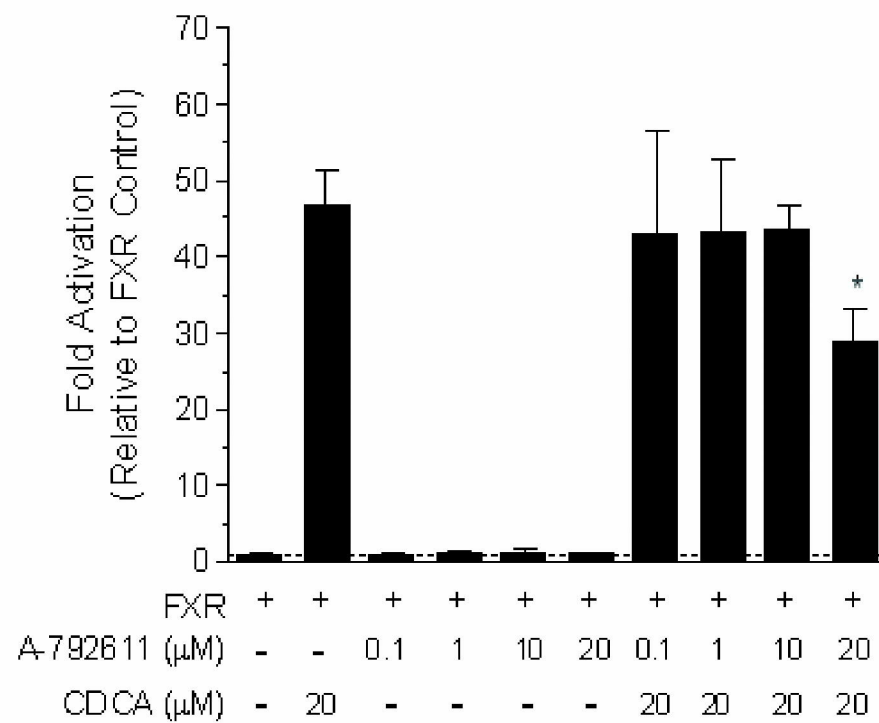


Figure 5B

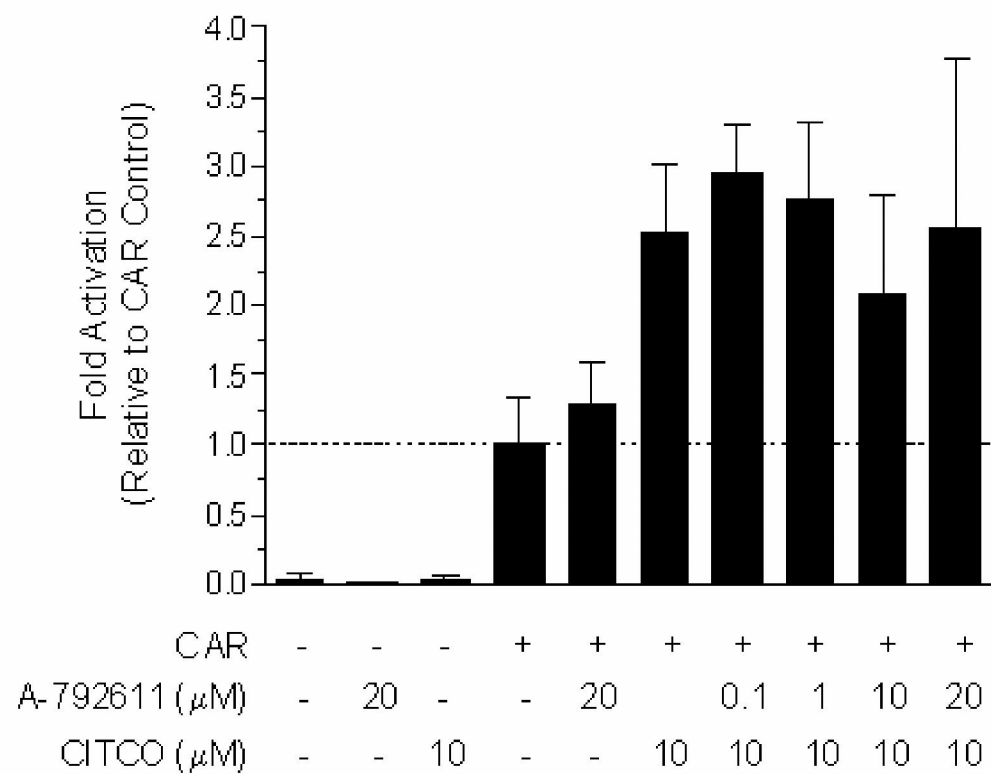


Figure 5C

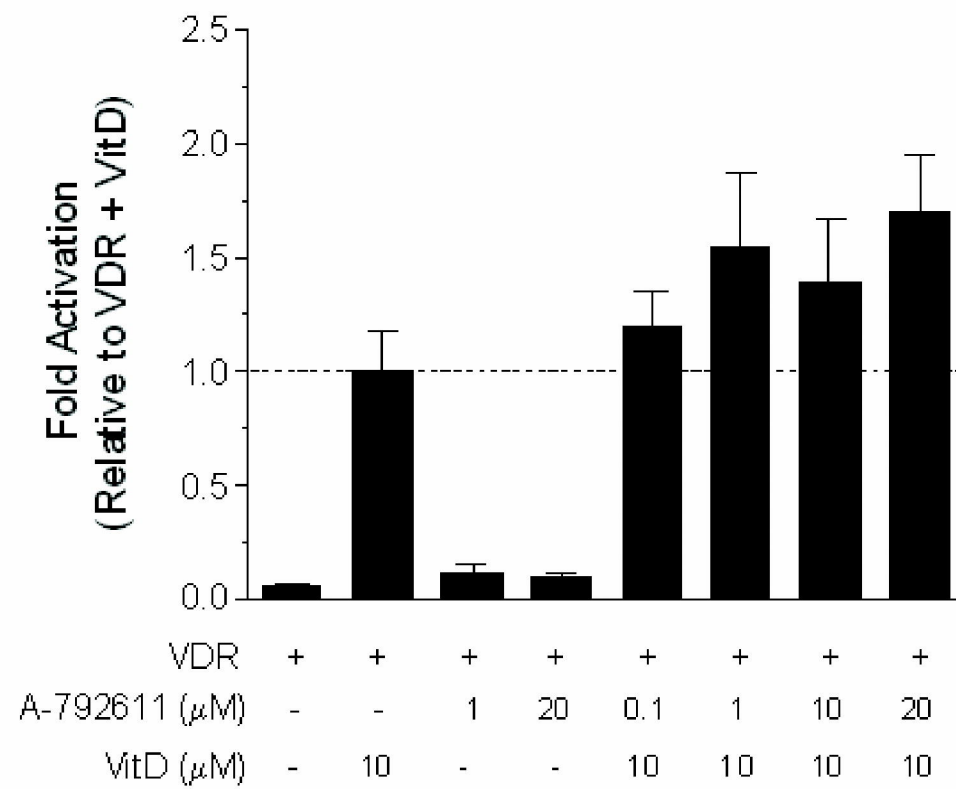


Figure 5D

