

**Pregnane X Receptor Mediates Dyslipidemia Induced by the HIV Protease Inhibitor
Amprenavir in Mice**

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Running title: Amprenavir activates PXR and induces hyperlipidemia in mice

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37 Text pages

0 Tables

7 Figures

56 References

Abstract: 212 words

Introduction: 750 words

Discussion: 1,402 words

Abbreviations: HAART, highly active anti-retroviral therapy; PI, protease inhibitor; PXR, Pregnane X receptor; SXR, steroid and xenobiotic receptor; FXR, farnesoid X receptor; LXR, liver X receptor; ER, estrogen receptor. CAR, constitutive androstane receptor; VDR, vitamin D receptor; RAR, retinoid acid receptor; RXR, retinoid X receptor, PPAR, peroxisome proliferator-activated receptor; SRC-1, steroid receptor coactivator-1; PBP, PPAR binding protein; NCoR, nuclear receptor co-repressor; SMRT, silencing mediator of retinoid and thyroid hormone; LBD, ligand-binding domain; DBD, DNA-binding domain; CYP, cytochrome P450; GST, glutathione transferase; MDR1, multidrug resistance 1; ApoE, apolipoprotein E; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; WT, wild-type; LDLR, LDL receptor; RIF, rifampicin; PCN, pregnenolone 16 α -carbonitrile; QPCR, quantitative real-time PCR; LipA, lysosomal lipase; LipF, gastric lipase.

Abstract

HIV protease inhibitors (PIs) have been used successfully in extending the lifespan of people infected with HIV. The use of PIs has also been associated with dyslipidemia and an increased risk of cardiovascular disease but the underlying mechanisms remain elusive. Several PIs have been implicated to activate the nuclear receptor pregnane X receptor (PXR), which acts as a xenobiotic sensor to regulate xenobiotic metabolism in the liver and intestine. Recent studies indicate that PXR may also play an important role in the regulation of lipid homeostasis. In the present study, we identified amprenavir, a widely used HIV PI, as a potent PXR-selective agonist. Computational docking studies combined with site-direct mutagenesis identified several key residues within the ligand binding pocket of PXR that constitute points of interaction with amprenavir. Amprenavir efficiently activated PXR and induced PXR target gene expression *in vitro* and *in vivo*. Short-term exposure to amprenavir significantly increased plasma total cholesterol and atherogenic low-density lipoprotein cholesterol levels in wild-type mice, but not in PXR-deficient mice. Amprenavir-mediated PXR activation stimulated the expression of several key intestinal genes involved in lipid homeostasis. These findings provide critical mechanistic insight for understanding the impact of PIs on cardiovascular disease and demonstrate a potential role of PXR in mediating adverse effects of HIV PIs in humans.

Introduction

The introduction of highly active anti-retroviral therapy (HAART) has led to a marked increase in survival for people infected with HIV (Bhaskaran et al., 2008). However, HAART has also been associated with dyslipidemia and an increased risk of cardiovascular disease (Carr et al., 1998; Distler et al., 2001; Friis-Moller et al., 2007; Friis-Moller et al., 2003; Lang et al., 2010; Periard et al., 1999; Stein et al., 2001). For example, findings from the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study suggested that the incidence of cardiovascular disease increased with longer exposure to HAART (Friis-Moller et al., 2003). Another large-scale study including more than 23,000 HIV patients analyzed the combination of drugs used in HAART and concluded that protease inhibitors (PIs) are associated with an increased risk of cardiovascular disease, which is partly explained by dyslipidemia (Friis-Moller et al., 2007). A Swiss HIV Cohort Study found that PIs are the cause of dyslipidemia in patients (Periard et al., 1999) and several other studies have reached the same conclusion (Lang et al., 2010; Stein et al., 2001). Despite the strong evidence linking PIs with dyslipidemia and cardiovascular disease, the underlying mechanisms responsible for the adverse effects of PIs remain elusive.

Several PIs such as ritonavir have been implicated to activate the pregnane X receptor (PXR, also known as steroid and xenobiotic receptor, or SXR) (Dussault et al., 2001; Zhou et al., 2009b). PXR is a nuclear receptor activated by numerous endogenous hormones, dietary steroids, pharmaceutical agents, and xenobiotic chemicals (Blumberg et al., 1998; Kliewer et al., 1998; Zhou et al., 2009b). PXR functions as a xenobiotic sensor that induces expression of genes required for xenobiotic metabolism in the liver and intestine, including cytochrome P450s (CYP), conjugating enzymes (e.g., glutathione transferase (GST)), and ABC family transporters

(e.g., multidrug resistance 1 (MDR1)) (Kliewer et al., 2002; Zhou et al., 2009b). In the past decade, the role of PXR as a xenobiotic sensor has been well established (Zhou et al., 2009b).

Recent evidence indicates that PXR may also play an important role in the regulation of lipid homeostasis (de Haan et al., 2009; Gao and Xie, 2012; Zhou et al., 2009a; Zhou et al., 2006b). It is well known that many clinically relevant PXR ligands can elevate plasma lipid levels in patients and may increase their risk of cardiovascular disease (Eiris et al., 1995; Khogali et al., 1974; Lutjohann et al., 2004; Shafran et al., 2005; Zhou et al., 2009a). A recent meta-analysis of seven genome-wide association studies indicated that common genetic variants in PXR can affect plasma low-density lipoprotein (LDL) cholesterol levels in humans (Lu et al., 2010). Modulation of PXR activity has been found to alter lipid homeostasis and affect plasma lipid levels in several animal models. We recently reported that chronic activation of PXR led to increased levels of plasma total cholesterol and atherogenic LDL and very-low-density lipoprotein (VLDL) in wild-type (WT) mice, but not in PXR-deficient (PXR^{-/-}) mice (Zhou et al., 2009a). Activation of PXR also increased plasma total cholesterol and VLDL levels in apolipoprotein E (ApoE)*3-Leiden mice which exhibit a human-like lipoprotein distribution on a cholesterol-rich diet (de Haan et al., 2009). Another report showed that acute activation of PXR increased plasma triglyceride levels in both LDL receptor-deficient (LDLR^{-/-}) and ApoE-deficient (ApoE^{-/-}) mice but decreased LDL cholesterol levels in LDLR^{-/-} mice (Hoekstra et al., 2009). Activation of PXR can regulate many genes involved in lipid homeostasis including CD36, Stearoyl-CoA desaturase-1, 7-dehydrocholesterol reductase, S14, and lipin-1 in the liver, intestine, or macrophages of several animal models (Cheng et al., 2012; de Haan et al., 2009; He et al., 2013; Hoekstra et al., 2009; Moreau et al., 2009; Zhou et al., 2009b; Zhou et al., 2006b). These studies indicate that PXR can mediate cholesterol and lipid homeostasis at multiple levels.

Here we report that amprenavir, a widely used HIV PI, is a potent PXR-selective agonist. Computational docking studies together with site-directed mutagenesis identified several key residues within PXR's ligand binding pocket that constitute points of interaction with amprenavir. Amprenavir efficiently activated PXR and induced PXR target gene expression *in vitro* and *in vivo*. Short-term exposure to amprenavir significantly increased plasma total cholesterol and atherogenic lipoprotein LDL cholesterol levels in WT mice, but not in PXR^{-/-} mice. Amprenavir-mediated PXR activation significantly regulated several key intestinal genes involved in lipid homeostasis. These findings provide critical mechanistic insight for understanding the impact of PIs on cardiovascular disease and demonstrate a potential role of PXR in mediating adverse effects of PIs in humans.

Materials and Methods

Reagents and Plasmids

Pregnenolone 16 α -carbonitrile (PCN) and rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO). Amprenavir, ritonavir, nelfinavir, saquinavir, and indinavir sulfate were obtained from NIH AIDS Research and Reference Reagent Program. All chemicals were dissolved in dimethyl sulfoxide (DMSO). Human (h) and mouse (m) PXR expression vectors; GAL4 DNA-binding domain (DBD)-linked nuclear receptor ligand-binding domain (LBD) vectors (GAL4-hPXR, GAL4-mPXR, GAL4-rPXR, GAL4-RAR α , GAL4-RXR, GAL4-FXR, GAL4-LXR, GAL4-PPAR α , GAL4-PPAR γ and GAL4-VDR); and CMX- β -gal expression vectors have been described before (Sui et al., 2012; Zhou et al., 2007; Zhou et al., 2006a). VP16-PXR, GAL4-NCOR, GAL4-SMRT, GAL4-SRC1, GAL4-PBP, PXR-dependent CYP3A4 promoter reporter (CYP3A4XREM-Luciferase); CYP3A2 promoter reporter ((CYP3A2)₃-luciferase); and GAL4 reporter (MH100-Luciferase) have also been described previously (Sui et al., 2012; Zhou et al., 2007; Zhou et al., 2006a; Zhou et al., 2004).

Cell culture and transfections

The human hepatic cell line HepG2 and intestine epithelial cell line LS180 were obtained from the American Type Culture Collection. The human hepatoma HepaRG cells were purchased from Life Technologies (Carlsbad, CA). Transfection assays were performed as described previously (Sui et al., 2012; Zhou et al., 2007). The cells were transfected with various expression plasmids or hPXR mutants as well as the corresponding luciferase reporter plasmids, together with CMX- β -galactosidase control plasmids using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). The cells were then incubated with the corresponding ligands as

indicated in the figure legends for 24 h, and β -gal and luciferase assays were performed as described (Sui et al., 2012; Zhou et al., 2007). Fold activation was calculated relative to solvent controls. Each data point represents the average of triplicate experiments \pm SD and was replicated in 3-5 independent experiments. EC₅₀ values were calculated by curve fitting of data, using Prism software (GraphPad Software, San Diego, CA). For the mammalian two-hybrid assays, HepG2 cells were transfected with GAL4 reporter, VP16-hPXR, and GAL-SRC1, GAL-PBP, GAL-NCOR, and GAL-SMRT (Sui et al., 2012; Zhou et al., 2007). The cells were then treated with compounds at the indicated concentrations.

Computational docking studies

Preparation of the protein structure for subsequent ligand-receptor docking studies was conducted as previously described (Sui et al., 2012). Briefly, the structural coordinates of the tethered hPXR, linker, steroid receptor coactivator 1 (PXR/SRC-1) were retrieved from the RSCB Protein Data Bank entry 3HVL. The larger PXR fragment of chain A, Gly142-Glu458, was extracted for molecular modeling using MOE 2010 software (Chemical Computing Group, Montreal, Quebec, Canada), and for ligand-receptor docking studies using GOLD software (version 5.0) (Payne and Glen, 1993). Water molecules, salt ions, ligand (SR12813), and co-receptor fragments were deleted. After the addition of hydrogen atoms and assigning of the AMBER99 force-field charges to the protein, the hydrogen atomic positions were allowed to relax (Wang et al., 2008). The resulting protein structural coordinates were saved in Tripos mol2 format and used later for GOLD docking.

The ligands were docked to the 3HVL chain A using semiflexible docking whereby the ligand has full conformational flexibility and the hydroxyl groups of designated protein side chains in the binding pocket can rotate to optimize hydrogen bond contacts. Each ligand was

docked 50 independent times. The binding pocket was defined as all atoms within an 8-Å radius around the bound ligand, SR12813.

Site-directed mutagenesis

The hPXR full-length plasmid was used as a wild-type template to generate a series of mutant plasmids using the QuikChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) according to the manufacturer-supplied protocol as described before (Sui et al., 2012). The primers used for mutant generation are listed in Supplemental Table 1.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from mouse tissues, intestinal LS180, and HepaRG cells using TRIzol Reagent (Life Technologies, Carlsbad, CA) per the manufacturer-supplied protocol. Quantitative real-time PCR (QPCR) was performed using gene-specific primers and the SYBR green PCR kit (Life Technologies, Carlsbad, CA) as described previously (Sui et al., 2012; Zhou et al., 2007). The primer sets used in this study are listed in Supplemental Table 2.

Animals

C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory. PXR-deficient mice (PXR^{-/-}) on C57BL/6 background were employed as described previously (Zhou et al., 2009a). All the animals were housed in the Division of Laboratory Animal Resources, University of Kentucky, and approved by the Institutional Animal Care and Use Committee in the specific pathogen-free environment with a light-dark cycle. 8-week-old male wild-type or PXR^{-/-} mice were fed a semisynthetic low-fat AIN76 diet containing 0.02% cholesterol (Research Diet; New Brunswick, NJ) (Sui et al., 2011; Teupser et al., 2003; Zhou et al., 2009a) and treated by oral gavage with vehicle (corn oil) or 10 mg/kg body weight (BW) amprenavir daily for seven days. On the day of euthanization, mice were anesthetized by intraperitoneal injection with

Ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa). Mice were exsanguinated by left ventricular puncture and blood was collected into EDTA-containing syringes. Plasma was prepared by spinning at 16,000 g for 10 min. The circulation was flushed with PBS, intestinal and liver tissues were collected and stored in RNAlater solution (Life Technologies, Carlsbad, CA).

Plasma analysis

Plasma total cholesterol and triglyceride concentrations were determined enzymatically by colorimetric methods as described previously (Roche; Indianapolis, IN) (Park et al., 2012; Zhou et al., 2009a). Plasma from multiple mice (n=4-7) was pooled and plasma lipoprotein cholesterol distributions were determined by fast-performance liquid chromatography (FPLC) (Sui et al., 2011). Lipoproteins fractions were isolated by spinning 60 μ l of plasma in a TL-100 ultracentrifuge (Beckman Coulter, Brea, CA) at its own density (1.006 g/ml) at 70,000 RPM for 3 hours to harvest the supernatant, and then after adjusting the infranatant with solid KBr to a density of 1.063 g/ml spinning it for 70,000 RPM for 18 hours to harvest the supernatant (Zhou et al., 2011). The cholesterol content of each supernatant and the final infranatant were measured and taken to be very-low-density lipoprotein (VLDL) (<1.006 g/ml), low-density lipoprotein (LDL) ($1.006 \leq d \leq 1.063$ g/ml), and high-density lipoprotein (HDL) ($d > 1.063$ g/ml) cholesterol, respectively. Cholesterol concentrations in all three fractions were then determined enzymatically by a colorimetric method (Roche, Indianapolis, IN). Plasma triglyceride levels were determined enzymatically in the original plasma sample.

Statistical Analysis

All data are expressed as means \pm SD unless otherwise noted. Statistical analysis was performed using a two-sample, two-tailed Student t-test unless otherwise noted, with

$P < 0.05$ regarded as significant. One-way analysis of variance was used when multiple comparisons were made, followed by Dunnett's *t* test for multiple comparisons to a control.

Results

Amprenavir is a potent PXR-selective agonist

We tested several widely used HIV PIs including amprenavir, ritonavir, nelfinavir, saquinavir, and indinavir sulfate for PXR activation by use of transfection assays. Since PXR exhibits considerable differences in its pharmacology across species (viz., mouse vs. human) (Blumberg et al., 1998; Zhou et al., 2009b), the potent PXR ligands rifampicin (RIF) and pregnenolone 16 α -carbonitrile (PCN) were used as the positive control for human (h) and mouse (m) PXR, respectively. Consistent with a previous report (Dussault et al., 2001), ritonavir activated PXR and induced PXR-mediated reporter activity (Fig. 1A,B). Interestingly, amprenavir was a more potent PXR agonist than any of the other tested PIs (Fig. 1A,B). Amprenavir can activate both human and mouse PXR and induced reporter gene activity in a dose dependent manner (Fig. 1A,B). Dose-response analysis indicated that amprenavir was able to activate PXR at concentrations as low as 1 μ M, reaching peak activation at 30 μ M (Figure 1C). The EC₅₀ for amprenavir activation of PXR-mediated CYP3A4 promoter activity was 8.6 μ M (Fig. 1C). We next tested the ability of amprenavir to activate a panel of other nuclear receptors, including human retinoid acid receptor (RAR) α , retinoid X receptor (RXR), farnesoid X receptor (FXR), liver X receptor (LXR) α , peroxisome proliferator-activated receptor (PPAR) α , PPAR γ , vitamin D receptor (VDR), estrogen receptor (ER) α , and ER β (Fig. 1D). Amprenavir was unable to activate any of these other nuclear receptors. Results from this panel emphasize the importance of studying the effects of amprenavir-mediated PXR agonism.

Amprenavir promotes PXR co-activator recruitment and co-repressor disassociation

Nuclear receptor co-regulators play critical roles in nuclear receptor signaling (Kliewer et al., 2002). We used a mammalian two-hybrid assay (Sui et al., 2012; Zhou et al., 2007) to evaluate the effects of amprenavir on PXR co-regulator interaction. Similar to the potent PXR ligand RIF, amprenavir promoted the specific interactions between PXR and the coactivators SRC-1 (steroid receptor coactivator-1) and PBP (PPAR binding protein) (Fig. 2A). Consistent with our previous reports (Sui et al., 2012; Zhou et al., 2007), unliganded PXR interacted with co-repressors, nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone (SMRT). Amprenavir disrupted this interaction as did the RIF (Fig. 2B). Binding of amprenavir to PXR inhibits PXR/co-repressor interaction and promotes PXR/co-activator recruitment, thereby inducing PXR transcriptional activation in a concentration-dependent manner.

Computational docking and modeling studies

To investigate the potential interaction pattern between amprenavir and PXR, a structure-based approach was employed. Amprenavir was docked into the high-resolution crystal structure of human PXR in complex with SR12813 (Wang et al., 2008). The results from GOLD docking are shown in Figure 3A. The binding pose of amprenavir in the PXR ligand-binding domain (LBD) is similar to SR12813. The PXR LBD appears to possess four hydrophobic subpockets and a connection region in the middle. Amprenavir occupies all four subpockets, and its hydroxyl group forms a hydrogen bond with Ser247 which is located in the connection region of PXR to help to position the drug in the optimal orientation inside the receptor. Another two hydrogen bonds were formed between amprenavir and PXR (Gln285 and His407) that provide additional electrostatic interactions. Nonpolar contacts also play a key role in stabilizing

amprenavir within the ligand binding pocket of PXR. As displayed in Figure 3B, the interaction map of amprenavir and PXR revealed an important π - π stacking between ring A of amprenavir and Phe288 in subpocket 1 of PXR. Tyr306 forms a 3.5-Å edge-to-face contact to the same phenol ring of the drug that also engages in hydrophobic interactions with Trp299, the other hydrophobic residue in subpocket 1. In subpocket 2, amprenavir forms direct contacts with one residue on α AF of the PXR AF-2 surface, Phe429, which may stabilize the active AF-2 conformation of the receptor and contribute to the agonist activity of amprenavir on PXR. Subpockets 3 and 4 of PXR are located on the bottom of the cavity, which corresponds to the most structurally flexible region of the PXR LBD. This region is dominated by hydrophobic residues (e.g., Met323 and Leu209) that form van der Waals contacts with amprenavir.

Key LBD residues of PXR are required for amprenavir's agonistic activity

To validate the results of the docking analysis, we mutated the key amino acids responsible for amprenavir's agonist activity including Ser247, Gln285, Phe288, and Tyr306. We also included Thr248, a key amino acid known to be important for PXR/co-activator interaction (Sui et al., 2012; Ueda et al., 2005), and Leu411, an amino acid predicted by the docking analysis not to interact with amprenavir, as the putative positive and negative control for our mutagenesis study, respectively. As shown in Figure 3C, Ser247Leu and Tyr306Leu mutations completely blocked amprenavir's agonist activity at all tested drug concentrations. Gln285Leu and His407Ala exerted little or no effects on the drug at low concentration (5 μ M). At higher concentrations (10 and 20 μ M), these mutations showed inhibitory effects on amprenavir's activity. Phe288Ala showed some inhibitory effects on amprenavir's activity. As expected, Thr248Leu mutation abolished the activity of amprenavir; and Leu411Phe did not

inhibit but rather slightly promoted amprenavir as an agonist. In summary, our site-directed mutagenesis analysis confirmed the docking model and revealed the key residues within PXR's binding pocket responsible for amprenavir's agonist effects.

Amprenavir induces PXR target gene expression in human cells.

We next used human hepatoma HepaRG cells (Kanebratt and Andersson, 2008) and intestinal LS180 cells (Zhou et al., 2007; Zhou et al., 2006a) to test the effects of amprenavir exposure on PXR activity and target gene expression. Similar to the known human PXR ligand RIF, amprenavir induced the expression of bona fide PXR target genes involved in phase I (CYP3A4), phase II (UGT1A1) and phase III (MDR1) metabolism in both HepaRG cells (Fig. 4A) and LS180 cells (Fig. 4B). These results suggest that amprenavir can activate PXR and induce PXR target gene expression in human cells.

Amprenavir elevates plasma lipid levels in WT mice, but not in PXR^{-/-} mice

To further investigate the effects of amprenavir on PXR activity *in vivo*, WT and PXR^{-/-} mice were treated with vehicle (corn oil) or 10 mg/kg body weight amprenavir daily by oral gavage for one week. Amprenavir-treated WT mice had significantly increased total cholesterol levels compared with control WT mice (Fig. 5A). By contrast, amprenavir did not affect plasma cholesterol levels in PXR^{-/-} mice (Fig. 5B). Fast-performance liquid chromatography (FPLC) analysis of cholesterol distribution pattern revealed that amprenavir increased atherogenic LDL cholesterol fractions in WT mice, but not in PXR^{-/-} mice (Fig. 5C,D). Lipoprotein fractions (VLDL, LDL, and HDL) were isolated by ultracentrifugation (Zhou et al., 2011) and cholesterol concentrations in all three fractions were then measured. Consistent with FPLC results (Fig.

5C,D), amprenavir treatment did not affect HDL or VLDL cholesterol levels but significantly increased LDL cholesterol levels in WT mice (Fig. 6A). By contrast, amprenavir did not affect any of the lipoprotein levels in PXR^{-/-} mice (Fig. 6B). These results suggest that the adverse effects of amprenavir on plasma cholesterol levels are mediated, at least in part, through PXR in mice.

Amprenavir affects genes involved in lipid homeostasis

To determine whether amprenavir can activate PXR in these mice, expression levels of known PXR target genes were measured in the intestine and liver. Amprenavir stimulated expression of known PXR target genes including CYP3A11, GSTA1, and MDR1a in the intestine (Fig. 7A) of WT mice, but not in PXR^{-/-} mice. Interestingly, amprenavir did not affect the expression of hepatic PXR target genes (Fig. 7B), which might be due to the insufficient accumulation of amprenavir in the liver. To elucidate possible molecular mechanisms through which amprenavir-mediated intestinal PXR activation might induce dyslipidemia, expression levels of genes involved in lipid homeostasis were measured (Fig. 7C). Of the genes surveyed, CD36 showed significant change, with its expression levels increased more than 10-fold. CD36, a class B scavenger receptor, plays an important role in intestinal lipid absorption (Abumrad and Davidson, 2012). Consistent with a recent report (Cheng et al., 2012), the expression levels of diacylglycerol acyltransferase 1 and 2 (DGAT1 and 2), two enzymes involved in intestinal lipid transportation and chylomicron secretion (Ables et al., 2012), were also induced by amprenavir-mediated PXR activation in WT mice. It was recently reported that a *Drosophila* PXR ortholog DHR96 regulates expression of the intestine lipase Magro (CG5932) which mediates cholesterol and triglyceride homeostasis in *Drosophila*. Magro protein is most similar to mammalian gastric

lipase (LipF) (56% similarity) and lysosomal lipase (LipA) (50% similarity) (Sieber and Thummel, 2012). Interestingly, amprenavir-mediated PXR activation stimulated the expression of both LipF and LipA in the intestine of WT mice, but not in PXR^{-/-} mice (Fig. 7C), indicating a possible role of intestinal PXR in mediating dietary lipid breakdown and absorption in mammals. Taken together, upregulation of those genes by PXR activation contribute to amprenavir-elicited hyperlipidemia.

Discussion

PIs have been associated with dyslipidemia and an increased risk of cardiovascular disease (Friis-Moller et al., 2007; Lang et al., 2010; Periard et al., 1999), but the underlying mechanisms are still unknown. Several PIs have been shown to activate PXR which functions as a xenobiotic sensor to regulate genes required for xenobiotic metabolism in the liver and intestine (Kliewer et al., 2002; Zhou et al., 2009b). In the current study, we identified a widely used PI amprenavir as a potent and selective PXR agonist. Amprenavir is usually taken by patients with or without ritonavir. Interestingly, a case-control study suggested that the risk of cardiovascular disease was increased by exposure to all PIs and, in particular, to amprenavir with or without ritonavir (Lang et al., 2010). We found that amprenavir activated both human and mouse PXR but did not affect the activity of other nuclear receptors. Short-term exposure to amprenavir significantly increased plasma total cholesterol and LDL cholesterol levels in WT mice, but not in PXR^{-/-} mice. Amprenavir-mediated PXR activation regulated several key intestinal genes involved in lipid homeostasis. These findings provide critical mechanistic insight for understanding the impact of PIs on cardiovascular disease and demonstrate a potential role of PXR in mediating adverse effects of HIV PIs in humans.

Using *in silico* ligand-PXR docking studies combined with site-directed mutagenesis, we identified Tyr306, Ser247, His407, and Phe288 to participate in the amprenavir-PXR interaction (Fig. 3). A critical polar interaction with Ser247 helps to orient amprenavir in the ligand binding cavity that enables formation of nonpolar interactions with a highly hydrophobic region of PXR's ligand binding pocket lined by Phe288, Trp299 and Tyr306. These three residues are highly conserved among different species and interact with structurally diverse PXR agonists as shown in multiple published PXR X-ray crystal structures. This Ser247 directed orientation of

amprenavir also guided the selection of the most plausible binding alignment among the multiple poses generated by our docking simulations. Based on our previous modeling results on diverse ligands with PXR, we conclude that two essential features need to be satisfied for ligand binding to PXR, viz., (1) one key hydrogen bond with Ser247 or His407 and (2) van der Waals contacts with subpocket 1. Besides the interactions formed by these two essential features, numerous hydrophobic contacts between amprenavir and PXR further stabilize the ligand binding and indirectly promote contact with the AF-2 region, therefore forming the optimal orientation of α AF helix to interact with coactivators. Our observations regarding the key pharmacophoric features for PXR binding will hopefully provide guidance useful for PXR ligand screening in the future.

The role of PXR in xenobiotic metabolism has been well established; however, recent studies have revealed the role of PXR in dyslipidemia and atherosclerosis. We recently demonstrated the pro-atherogenic effects of PXR in animals (Sui et al., 2011; Zhou et al., 2009a). We previously reported that chronic activation of PXR elicited by feeding mice the mouse PXR ligand PCN led to increased levels of plasma total cholesterol and VLDL and LDL in WT mice, but not in PXR^{-/-} mice (Zhou et al., 2009a). In the present study, mice were treated with 10 mg/kg/day amprenavir to investigate its potential effects on lipid levels. Patients usually take a daily dose of 1,400 mg or 2,800 mg of the amprenavir prodrug, fosamprenavir, which is rapidly and extensively converted to amprenavir after oral administration (Wire et al., 2006). Pharmacokinetic studies have demonstrated that the mean peak plasma concentration of amprenavir is approximately 18 μ M after a 1,200 mg single-dose (Sadler et al., 1999; Sadler and Stein, 2002) which is sufficient to activate PXR based on our results (Fig. 1). Therefore, we believe that amprenavir can activate PXR and regulate PXR target genes in patients taking this

drug routinely. The dose of 10 mg/kg/day amprenavir used in the present study to treat animals is considered low or modest. Nevertheless, after one week treatment, amprenavir significantly increased plasma total cholesterol and LDL cholesterol levels in WT mice, but not in PXR^{-/-} mice, indicating the significant role of PXR in mediating the adverse effects of amprenavir (Figs. 5,6). Interestingly, amprenavir regulated PXR target genes in the intestine but not in the liver (Fig. 7). This could be explained by the relative low dose of amprenavir and short-term treatment in this study. In addition, amprenavir is metabolized by CYP3A4 enzyme in the liver (Barry et al., 1999). Therefore, the relative low dose of amprenavir used in the present study may be insufficient for amprenavir to reach the high concentrations required to activate PXR in the liver.

Intestinal lipid transportation plays a central role in lipid homeostasis. In addition to prototypic PXR target genes (e.g., CYP3A11, MDR1a), amprenavir significantly stimulated expression of several key genes involved in intestinal lipid homeostasis in WT mice including CD36, DGAT1, and DGAT2. We and others previously reported that activation of PXR induces CD36 expression and increases lipid accumulation in the liver (Zhou et al., 2006b), intestine (Cheng et al., 2012), and macrophages (Zhou et al., 2009a). The role of CD36 in mediating intestinal fatty acid uptake has been well established (Abumrad and Davidson, 2012; Nassir et al., 2007; Nauli et al., 2006); and several studies have also indicated that CD36 mediates cholesterol uptake in the intestine (Nassir et al., 2007; Nauli et al., 2006). For example, cholesterol uptake was significantly decreased in the enterocytes isolated from CD36-deficient (CD36^{-/-}) mice (Nassir et al., 2007). In a lipid infusion study, CD36^{-/-} mice exhibited accumulation of dietary cholesterol in the intestinal lumen and reduction of cholesterol transport into the lymph (Nauli et al., 2006). Therefore, the PXR-mediated CD36 upregulation may

contribute to amprenavir-stimulated elevation of cholesterol levels. In addition, Liang et al. (Liang et al., 2001) previously reported that PIs such as ritonavir inhibited degradation and secretion of apolipoprotein B (ApoB), the primary apolipoprotein of chylomicrons and LDL. Interestingly, the inhibitory effects of PI on ApoB secretion were reversed by oleic acid, a fatty acid that stimulates neutral-lipid biosynthesis (Liang et al., 2001). In the presence of oleic acid, PI treatment even increased ApoB secretion above controls (Liang et al., 2001). Since CD36 plays an important role in the absorption of oleic acid (Nassir et al., 2007), it is plausible that PXR-mediated CD36 expression increases oleic acid absorption and contributes to PI-induced ApoB secretion and hyperlipidemia.

The precise mechanisms through which PXR modulates lipid homeostasis and cholesterol levels, in various animal models as well as in humans, remain to be determined. By contrast, the *Drosophila* PXR ortholog DHR96 has been established to be a key regulator in mediating cholesterol and triglyceride homeostasis (Horner et al., 2009; Sieber and Thummel, 2009; Sieber and Thummel, 2012). Recent studies showed that DHR96 regulates expression of the intestine lipase Magro which is most similar to mammalian LipA and LipF (Sieber and Thummel, 2012). Both LipA and LipF were upregulated by amprenavir-mediated PXR activation in the intestine of WT mice but not in PXR^{-/-} mice (Fig. 7C). LipA is an enzyme responsible for hydrolysis of cholesterol esters and triglycerides within lipoprotein particles internalized by receptor-mediated endocytosis (Anderson and Sando, 1991; Sheriff et al., 1995). Previous studies have demonstrated an important role of LipA in maintaining cholesterol levels in mice (Du et al., 2001) and mutations in LipA result in cholesterol ester storage disease (CESD) associated with hyperlipidemia in humans (Pisciotta et al., 2009). LipF also contributes to lipid catabolism by hydrolysis of dietary triglycerides in the stomach and intestine sequentially producing free fatty

acids and diacylglycerol (Carriere et al., 1993; Gargouri et al., 1986). It would be interesting to further investigate the detailed mechanisms by which PXR transcriptionally regulates LipA and LipF expression and mediates intestinal lipid homeostasis. Upregulation of those key genes by PXR activation contribute to amprenavir-elicited hyperlipidemia.

In summary, we demonstrated that amprenavir as a selectively potent PXR agonist. Amprenavir efficiently activated PXR and stimulated PXR target gene expression *in vitro* and *in vivo*. Computational docking studies and site-directed mutagenesis analysis identified key residues within PXR's ligand binding pocket that interact with amprenavir. Our study provides critical insight for understanding the mechanism by which clinically relevant PXR ligands interact and activate PXR. We also showed, for the first time, that amprenavir-mediated activation of PXR elevated lipid levels in an animal model, which has direct clinical consequence for patients under long-term treatment with amprenavir and other PIs. Activation of PXR may present a key mechanism for adverse effects of PIs in humans.

Acknowledgments:

We thank B. Blumberg (UC, Irvine) and B.M. Forman (City of Hope National Medical Center) for plasmids; R. M. Evans (Salk Institute) and B. Blumberg for PXR^{-/-} mice; A. Daugherty and D. Howatt for FPLC analysis; and R. Greenberg for valuable discussions.

Authorship Contributions:

Participated in research design: Helsley, Sui, Welsh, and Zhou

Conducted experiments: Helsley, Sui, Ai, Park, and Zhou

Contributed new reagents or analytical tools: Helsley, Sui, Ai, and Zhou

Perform data analysis: Helsley, Sui, Ai, Park, and Zhou

Wrote or contributed to the writing of the manuscript: Helsley, Ai, Welsh, and Zhou

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Footnotes:

This work was supported in part by the National Institutes of Health [P20GM103527; P30HL101300]. R.N.H. was supported by a training grant from the National Institutes of Health [T32HL072743]. C.Z. was supported by the American Heart Association [09SDG2150176].

Robert N. Helsley and Yipeng Sui contributed equally to this work.

Figure Legends

Figure 1. Amprenavir is a potent PXR-selective agonist.

(A and B) HepG2 cells were transfected with (A) full-length hPXR together with a hPXR reporter (CYP3A4-luc) or (B) full-length mPXR together with a mPXR reporter ((CYP3A2)₃-luc) and CMX- β -galactosidase control plasmid. Cells were then treated with DMSO control, amprenavir, ritonavir, nelfinavir, saquinavir, indinavir sulfate, RIF (a hPXR ligand) or PCN (a mPXR ligand) at the indicated concentrations for 24 h. (C) HepG2 cells were co-transfected with hPXR together with CYP3A4-luc reporter and CMX- β -galactosidase plasmid. Cells were then treated with amprenavir or RIF at the indicated concentrations for 24 h. (D) HepG2 cells were co-transfected with a GAL4 reporter and a series of GAL4 constructs in which the GAL4 DBD is linked to the indicated nuclear receptor LBD. Cells were treated with DMSO control or 10 μ M amprenavir for 24 h. Data are shown as fold induction of normalized luciferase activity compared with DMSO treatment and represent the mean of triplicate experiments.

Figure 2. Amprenavir promotes PXR co-activator recruitment and co-repressor disassociation.

HepG2 cells were transfected with a GAL4 reporter and VP16-hPXR as well as expression vector for GAL4 DBD or GAL4 DBD linked to the receptor interaction domains of the indicated PXR co-activators (GAL-SRC1 and GAL-PBP) (A) or co-repressor (GAL-SMRT and GAL-NCoR) (B). Cells were then treated with DMSO control, amprenavir, or RIF at the indicated concentrations for 24 h.

Figure 3. Key residues of PXR LBD are required for amprenavir's agonistic activity.

(A) Overlay of docked amprenavir and SR12813 (green) inside LBP of PXR. Atoms in amprenavir were colored by atom type (oxygen in red, phosphor in yellow, nitrogen in blue and carbon in grey), while all the atoms in SR12813 were colored in green for clear comparison. Four subpockets were presented by black circles. (B) Interaction map between amprenavir and PXR. Amprenavir was computationally docked in the ligand binding pocket of the hPXR X-ray crystal structure (3hvl.pdb). The amprenavir-PXR complex was stabilized by three hydrogen bonds with residues Ser247, Gln285 and His407, respectively. The noncovalent contacts with the aromatic side chains Phe288, Trp299, and Tyr306 lining in the subpocket 1 contributed significantly to amprenavir binding. Phe288 was well positioned to engage in π -stacking interactions with the phenyl ring of amprenavir. The interaction diagram reveals that amprenavir occupied the major portion of the large, flexible PXR ligand binding pocket. (C) HepG2 cells were co-transfected with a full-length PXR WT plasmid or mutant PXR plasmids as indicated, together with CYP3A4-luciferase reporter and CMX- β -galactosidase plasmid. After transfection, cells were treated with control medium or medium containing amprenavir at indicated concentrations for 24 h.

Figure 4. Amprenavir induces PXR target gene expression in human cells.

Human HepaRG hepatoma cells (A) and LS180 intestinal cells (B) were treated with control medium or medium containing 10 μ M amprenavir for 24 h. Total RNA was isolated and gene expression levels were analyzed by QPCR with primers for human CYP3A4, UGT1A1 or MDR1 (n=3, **P<0.01, ***P<0.001).

Figure 5. Amprenavir elicits hyperlipidemia in WT, but not in PXR^{-/-} mice.

(A and B) 8-week-old WT (A) and PXR^{-/-}(B) mice were treated with vehicle (corn oil) or 10 mg/kg of amprenavir by oral gavage for 1 week. Plasma total cholesterol levels were measured by a standard method (n= 4-7; *P<0.05 compared to control group). (C and D) Plasma cholesterol distribution of WT (C) and PXR^{-/-} mice (D) was analyzed by FPLC.

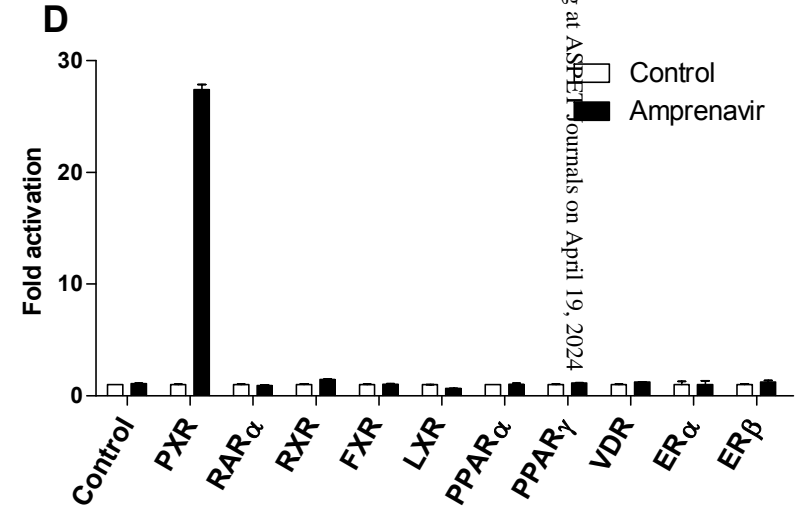
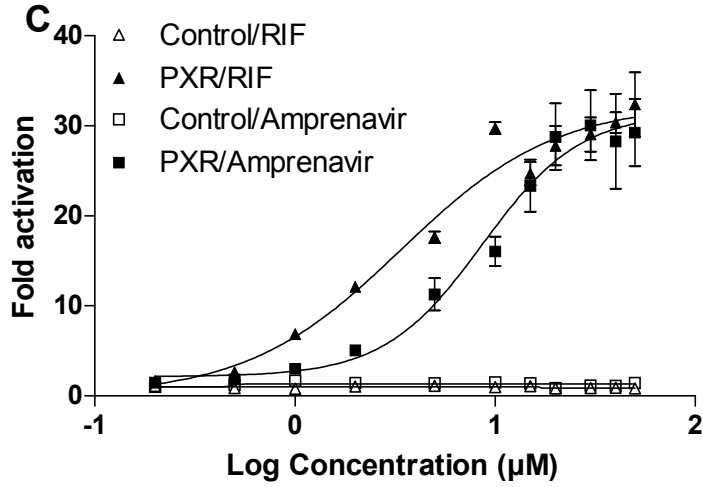
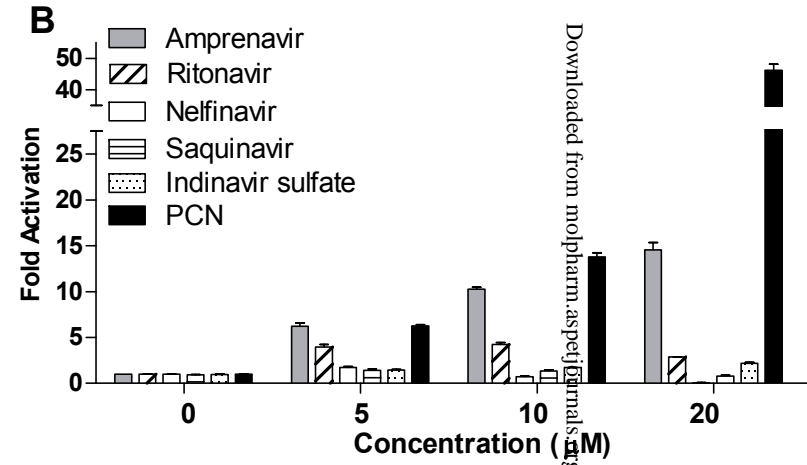
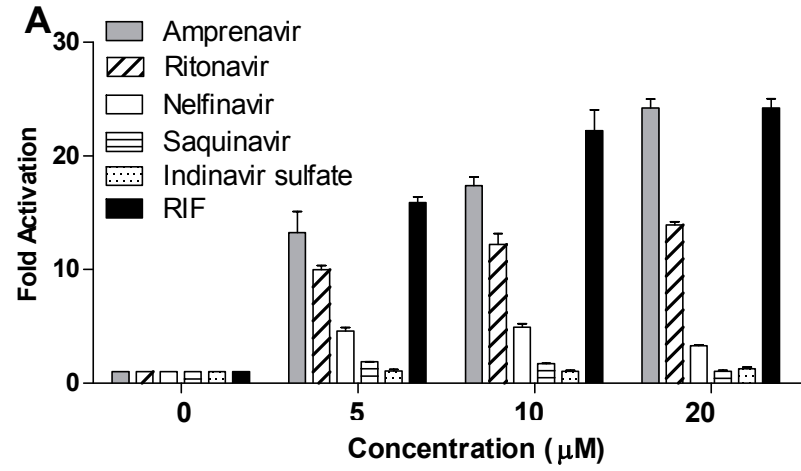
Figure 6. Amprenavir elevates atherogenic LDL cholesterol levels in WT mice.

8-week-old WT (A) and PXR^{-/-}(B) mice were treated with vehicle or 10 mg/kg of amprenavir by oral gavage for 1 week. Lipoprotein fractions (VLDL, LDL, and HDL) were isolated and the cholesterol levels of each fraction were measured by a standard method (n= 4-7 per group; *P<0.05 compared to control group).

Figure 7. Activation of PXR by amprenavir stimulates the expression of intestinal genes involved in lipid homeostasis.

8-week-old WT and PXR^{-/-} mice were treated with vehicle (corn oil) or 10 mg/kg of amprenavir by oral gavage for 1 week. Total RNA was isolated from the liver and small intestine (duodenum). (A and B) The mRNA levels of known PXR target genes, CYP3A11, GSTA1, and MDR1a, in the intestine (A) and liver (B) were measured by QPCR. (C) Expression levels of intestinal genes involved in lipid homeostasis were measured by QPCR. (n=4-5 per group, *P<0.05 and **P<0.01).

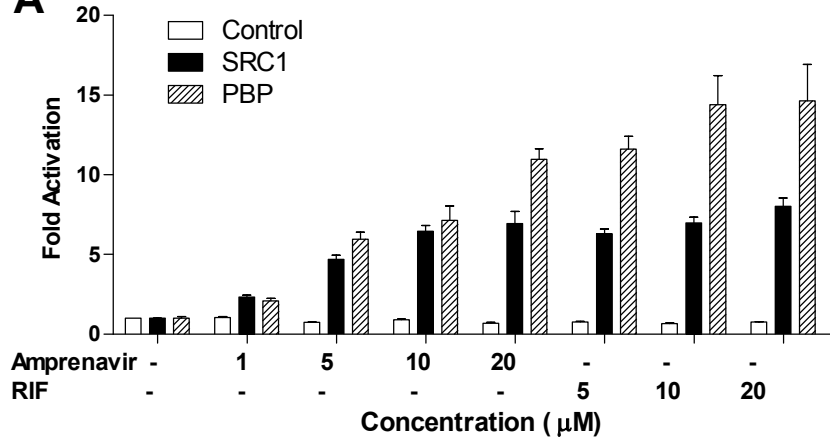
Figure 1



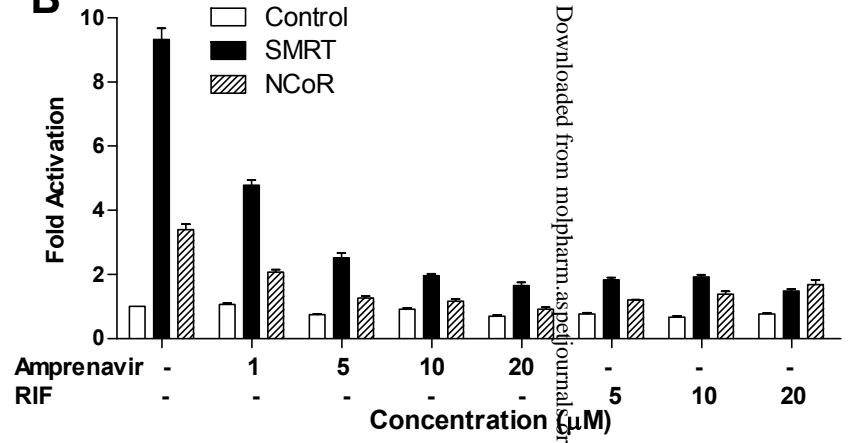
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Figure 2

A



B



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Figure 3

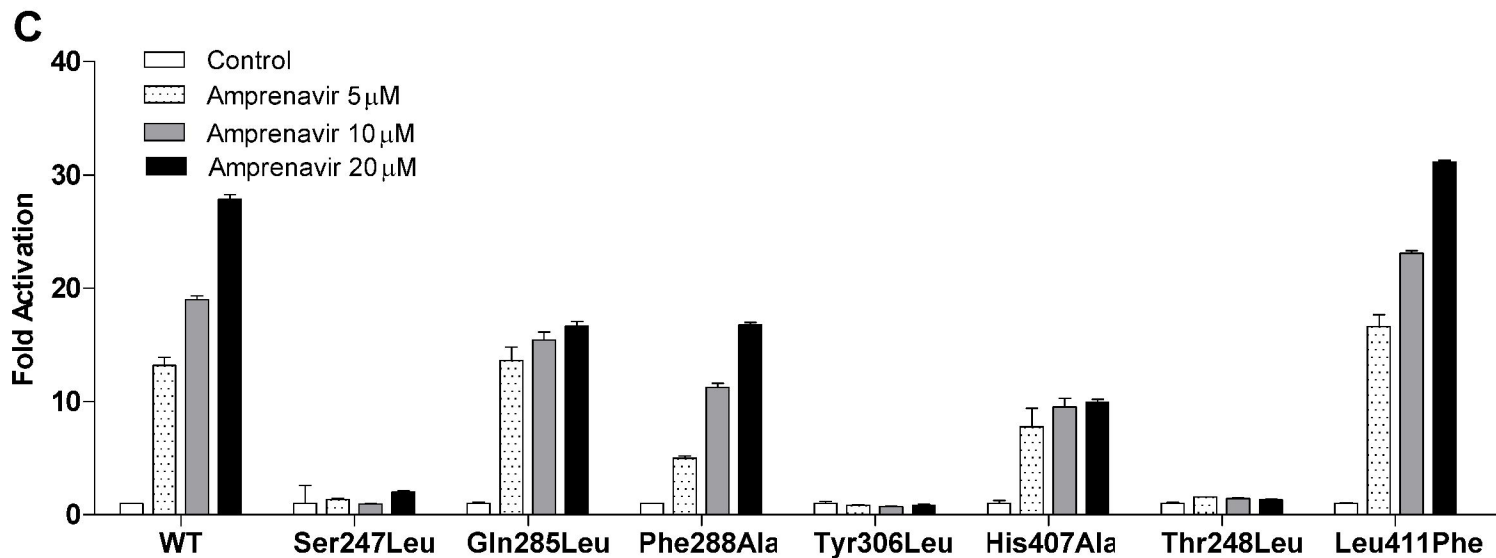
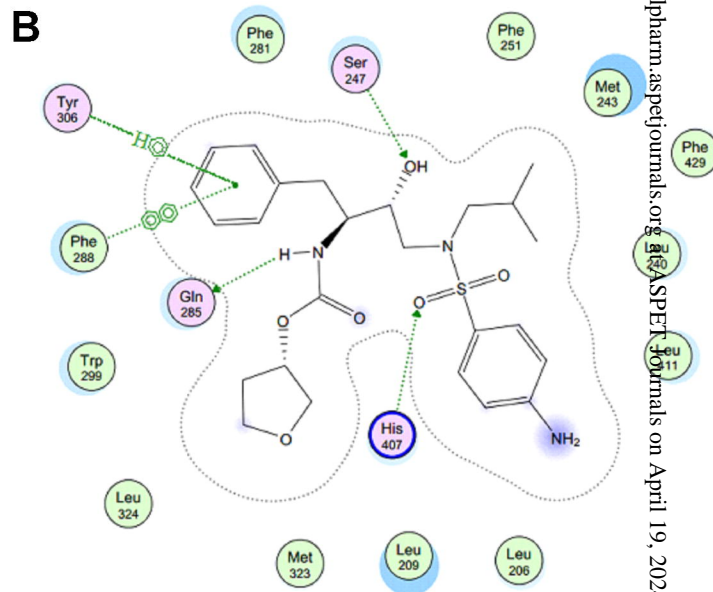
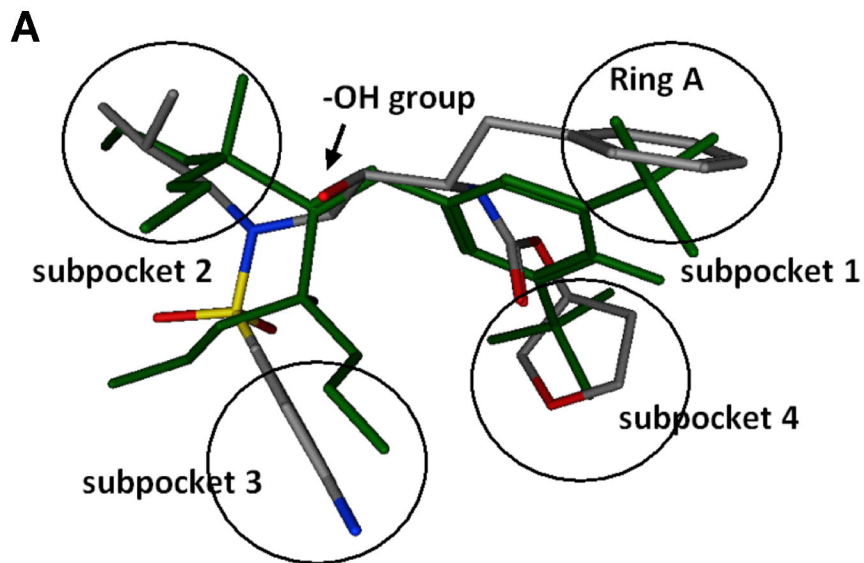


Figure 4

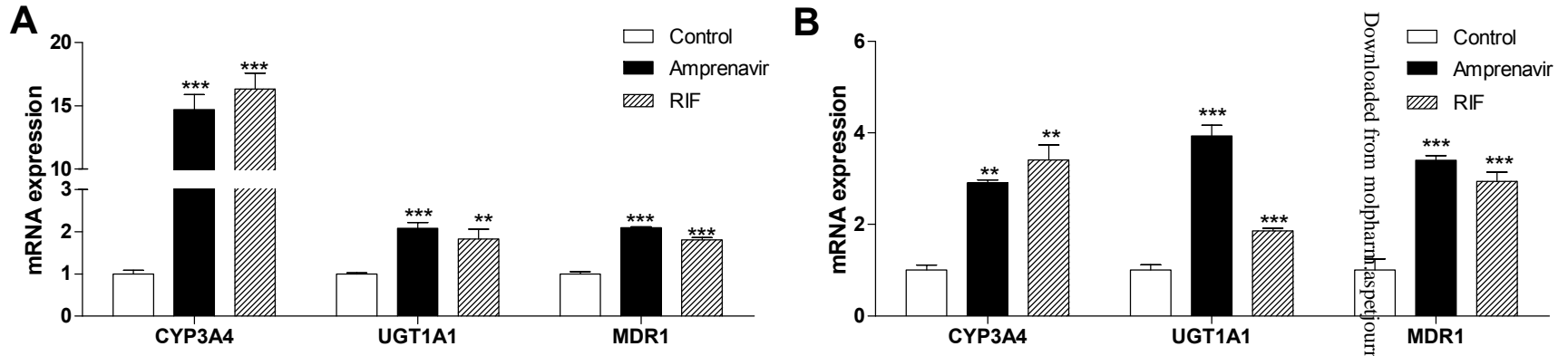


Figure 5

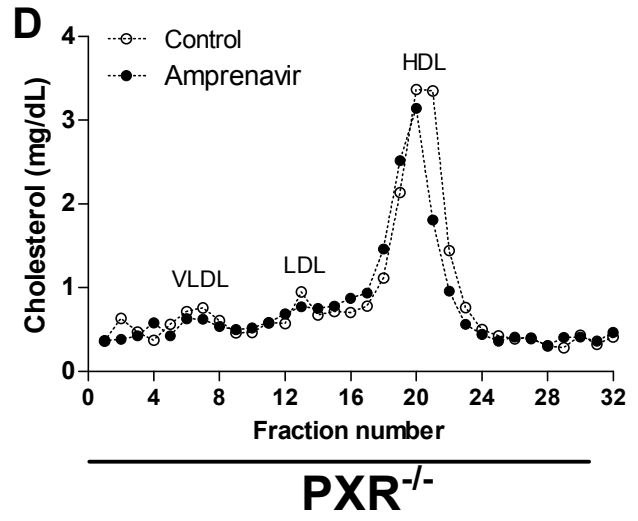
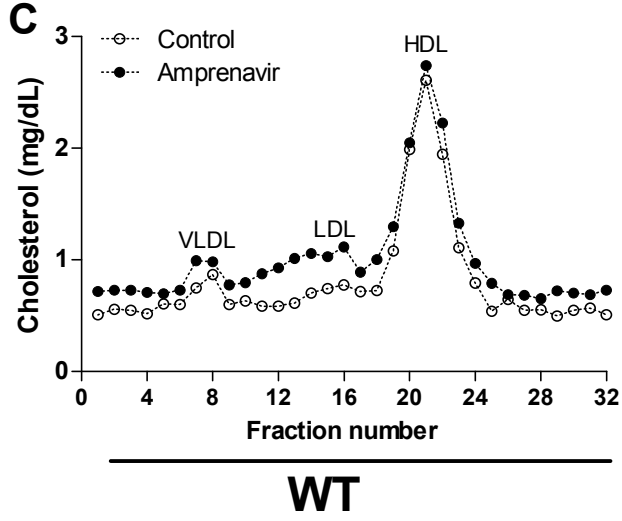
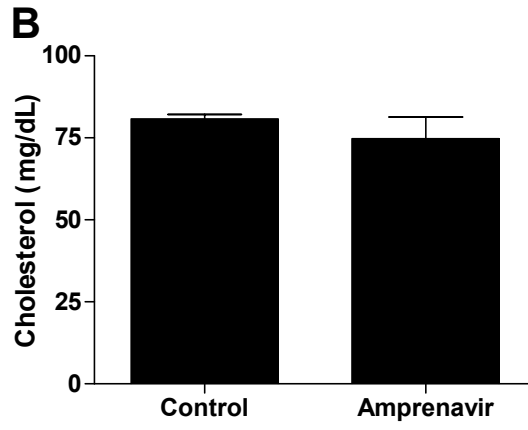
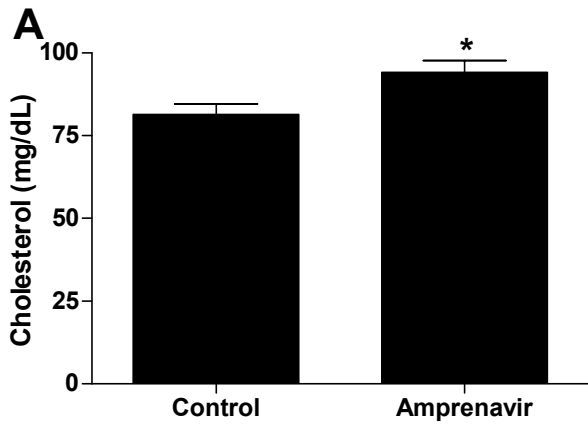


Figure 6

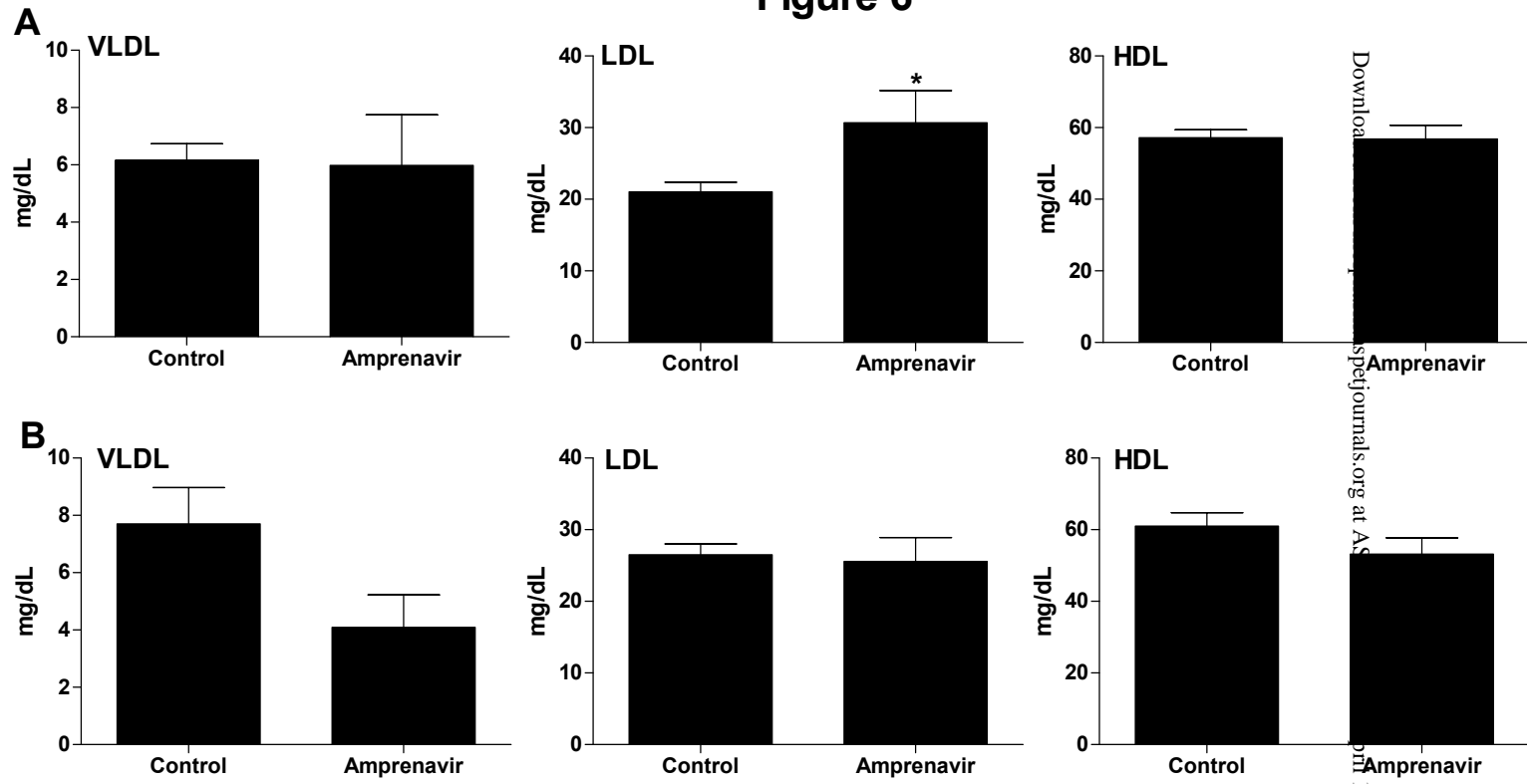


Figure 7

