PREGNANE X RECEPTOR AND CONSTITUTIVE ANDROSTANE RECEPTOR
ACTIVATION BY NON-NUCLEOSIDE HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

by

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Abstract

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are important anti-retroviral drugs indicated in combination therapy of human immunodeficiency virus-1 (HIV-1) infection. NNRTI therapy is associated with pharmacokinetic drug interactions, the underlying mechanisms of which are poorly understood. The present study investigated the effects of NNRTIs on the activity of pregnane X receptor (PXR) and constitutive androstane receptor (CAR), key transcriptional factors regulating the expression of various drug-metabolizing enzymes and transporters. The experimental approaches included cell-based luciferase reporter gene assays, *in vitro* competitive ligand binding assay, nuclear translocation analysis by confocal imaging, coactivator recruitment assays, and target gene expression determination in primary human hepatocytes. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, were identified as agonists of PXR and inducers of cytochrome P450 3A4 (CYP3A4), a target gene of PXR. By comparison, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, were indirect activators of the wild-type isoform of CAR and inducers of cytochrome P450 2B6, a target gene of CAR. Among the NNRTIs investigated, only efavirenz activated the SV23 and SV24 splice variants of CAR, indicating that NNRTIs activated CAR in a drug-specific and isoform-selective manner. To further understand PXR regulation by rilpivirine, the role of microRNA in rilpivirine activation of PXR was investigated. MicroRNAs are small non-coding RNAs that are post-transcriptional regulators causing mRNA degradation or translational repression by binding to complementary regions in target mRNA. Bioinformatic analysis (www.microRNA.org) predicted sequence complementarity between hsa-miR-18a-5p and PXR. Reporter gene assays revealed a functional hsa-miR-18a-5p microRNA recognition
element in the 3’-untranslated region of PXR. In cell-based assays, over-expression of hsa-miR-18a-5p by transfecting LS180 human colon adenocarcinoma cells with a mimic of hsa-miR-18a-5p decreased PXR mRNA and protein expression. Rilpivirine and rifampin did not affect PXR expression, but it decreased endogenous expression of hsa-miR-18a-5p in LS180 cells. In contrast, over-expression of hsa-miR-18a-5p decreased PXR mRNA expression and CYP3A4 inducibility by rilpivirine and rifampin. These data suggest that hsa-miR-18a-5p regulates PXR and contributes to drug activation of PXR. Overall, the present study shows activation of PXR and CAR by select NNRTIs and provides mechanistic understanding of NNRTI-mediated drug-drug interactions.
Preface

Chapter 2. A version of Chapter 2 has been published (Sharma D, Lau AJ, Sherman MA, Chang, TKH (2013) Agonism of human pregnane X receptor by rilpivirine and etravirine: Comparison with first generation non-nucleoside reverse transcriptase inhibitors. Biochem Pharmacol 85: 1700-1711). With inputs from all authors, I designed the research question and experimental plan. I, along with Dr. Lau conducted the experiments and performed data analysis. Dr. Chang and I wrote the paper, with input and discussion from all of the co-authors.

Chapter 3. A version of Chapter 3 has been published (Sharma D, Lau AJ, Sherman MA, Chang, TKH (2015) Differential activation of human constitutive androstane receptor and its SV23 and SV24 splice variants by rilpivirine and etravirine. Br J Pharmacol 172: 1263-1276). With inputs from all authors, I designed the research question and experimental plan. I, along with Dr. Lau conducted the experiments and performed data analysis. Dr. Chang and I wrote the paper, with input and discussion from all of the co-authors.

Chapter 4. With guidance from Dr. Chang and Dr. Xu, I was responsible for the research design. I, along with Mr. Abdullah Turkistani and Ms. Wenjun Chang, conducted experiments, collected, analyzed, and interpreted data. I was responsible for writing this chapter. This chapter will be revised further and submitted for publication.
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<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
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<td>C-C chemokine receptor type 5</td>
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<tr>
<td>NNRTI</td>
<td>non-nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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I express my sincere gratitude to my supervisor Dr. Thomas Chang for being a great mentor. In addition to being a profound source of knowledge and inspiration, he has always been a role model of modesty and simplicity. His intellectual offerings and scientific temperament laid the foundation that helped me accomplish this scientific endeavor.

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Dedicated to My Loved Ones
Chapter 1: Introduction

1.1 Human Immunodeficiency Virus (HIV)

HIV, a retrovirus belonging to the *Retroviridae* family, is the causal organism for acquired immunodeficiency syndrome (AIDS) (Sharp and Hahn, 2011). Ever since its discovery in the early 1980s (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983), AIDS has become one of the greatest human disease pandemics (Piot and Quinn, 2013). HIV targets cell-mediated immune system in humans and infects helper T cells expressing cluster of differentiation 4 (CD4), macrophages, and antigen-presenting dendritic cells (Maartens *et al.*, 2014). Loss of cell-mediated immunity post-HIV infection occurs via several mechanisms including apoptosis of infected CD4 T cells, CD8 cell-mediated destruction of bystander cells uninfected CD4 T cells, and limited recovery of new CD4 T cells due to inhibition of regeneration process in thymus gland (McCune, 2001; Cohen *et al.*, 2008). The net effect of destruction and impaired regeneration of CD4 T cells leads to a gradual deterioration in cell-mediated immune response, thereby increasing host susceptibility to various opportunistic infections (McCune, 2001; Cohen *et al.*, 2008).

Structurally, a mature HIV virion particle, which is about 120 nm in diameter, consists of a cone shaped nucleocapsid containing two single stranded genomic ribonucleic acid (RNA) molecules, viral protease, integrase, reverse transcriptase, and accessory proteins such as viral infectivity factor, viral protein R, viral protein U, and negative regulatory factor (Turner and Summers, 1999). The genomic viral RNA is stabilized by forming a ribonucleoprotein complex with nucleocapsid protein p7. A matrix membrane, composed of matrix protein p17, encapsulates viral nucleocapsid. The outermost covering of HIV is composed of a host-cell
derived lipid bilayer that expresses envelope glycoprotein gp120 and gp41, along with other host cell-derived membrane proteins such as major histocompatibility antigens, actin, and ubiquitin. The membrane glycoproteins gp120 and gp41 play an important role in attachment of HIV to the host cell (Turner and Summers, 1999; Sierra et al., 2005). Fig. 1.1 shows a schematic diagram of HIV virion structure.

Figure 1.1 Diagram of HIV Virion (reprinted with permission from (Sierra et al., 2005)). CA: capsid protein; gp41: glycoprotein 41; gp120: glycoprotein 120; MC: matrix protein; NC: nucleocapsid protein; RT: reverse transcriptase
HIV mainly targets activated T cells expressing CD4 and C-C chemokine co-receptor type 5 (CCR5) or C-X-C chemokine co-receptor type 4 (CXCR4) (Maartens et al., 2014). Other cell types infected by HIV include resting CD4 T cells, macrophages, and dendritic cells that express CD4 and chemokine co-receptor (Maartens et al., 2014). At the beginning of infection, viral glycoprotein gp120 interacts with CD4 receptor on host cell surface to initiate attachment (Sierra et al., 2005). This is followed by conformational changes in gp120 to facilitate its interaction with chemokine co-receptors (CCR5 or CXCR4) (Sierra et al., 2005). Binding with chemokine co-receptors induces conformational changes in another viral envelope protein, gp41, allowing for membrane fusion and release of viral capsid into host cell cytoplasm (Sierra et al., 2005). After uncoating, viral RNA is reverse transcribed into double-stranded deoxyribonucleic acid (DNA) with the help of viral reverse transcriptase, which after entering the nucleus, gets integrated into host genome as proviral DNA using viral integrase enzyme (Sierra et al., 2005). The integrated proviral DNA may remain latent or start replication. In multiple steps and as a result of different splicing events, proviral DNA transcribes for viral genome RNA and viral messenger RNA (mRNA) that gets processed into genomic RNA and various viral polyproteins, respectively (Sierra et al., 2005). The viral genomic RNA and various viral polyproteins move to the cellular membrane for assembly of immature virions and budding. Budding activates viral protease that catalyzes conversion of viral polyproteins into various structural proteins and enzymes (Sierra et al., 2005). Fig. 1.2 shows various steps in HIV replication.
Figure 1.2 HIV-1 Replication Cycle (reprinted with permission from (Sierra et al., 2005)). IN: viral integrase; PR: viral protease; RT: viral reverse transcriptase.
1.1.1 Epidemiology

Two sub-types of HIV, namely HIV-1 and HIV-2, have been characterized (Cohen et al., 2008; Sharp and Hahn, 2011). These sub-types differ in virulence, pathogenicity, prevalence, and origin. HIV-1 has greater virulence, pathogenicity, and prevalence when compared to HIV-2 (Cohen et al., 2008). HIV-1 originated from apes (chimpanzee and gorilla), whereas HIV-2 is originated from Sooty mangabey (Sharp and Hahn, 2011). HIV-1 constitutes the majority of infections globally, while infections due to HIV-2 are mostly confined to West Africa (Cohen et al., 2008; Maartens et al., 2014). HIV-1 and HIV-2 are further divided into groups and subtypes based on genome differences (Cohen et al., 2008).

Since its discovery in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1983), HIV-1 has infected over 60 million people and has caused over 25 million AIDS-related deaths (Cohen et al., 2008). According to the latest statistics, an estimated 35.3 million people are infected with HIV globally, with approximately 2.3 million new cases reported in 2012 alone (Maartens et al., 2014). Geographically, sub-Saharan Africa has the highest global HIV/AIDS burden, constituting approximately 70.8% of the total HIV/AIDS infections. Although better access to antiretroviral therapy has affected HIV in positive ways, this disease of human immune system still remains to be one of the leading causes of mortality in humans claiming an estimated 1.6 million lives in 2012 (Maartens et al., 2014). In high-income countries, the majority of HIV/AIDS-related deaths are due to cancers, liver infections, and cardiovascular diseases, whereas tuberculosis is the major cause of mortality in middle- and low-income countries (Maartens et al., 2014).
1.1.2 Transmission and Infection

HIV has been reported to be present in various body fluids including blood, semen, cervicovaginal secretions, rectal secretion, saliva, cerebrospinal fluid, and breast milk (Shepard et al., 2000; Pilcher et al., 2001). HIV infection occurs when these fluids are exchanged between carrier and the naive host via the bloodstream or the mucosal membrane. Any sexual contact with an infected person and blood-to-blood contact, either during transfusion or exchange of infected needles, remains the major routes of HIV transmission (Maartens et al., 2014).

Transmission of HIV is a complex interplay of a wide variety of biological and social risk factors. The major factors associated with transmission of HIV through any route include concentration of the virus in various body fluids of the carrier, susceptibility of the naive host, and virus-specific determinants (Cohen et al., 2008). Viral load in various body fluids has been closely associated with transmission of HIV and a higher viral load during acute infection poses a greater risk for HIV transmission when compared with chronic infection (Pilcher et al., 2007). Host-specific factors, such as concomitant sexually-transmitted diseases and pregnancy, may increase risk for HIV transmission (Maartens et al., 2014). Chemokine co-receptors (CCR5 or CXCR4) and viral tropic determinants, that governs whether macrophages or activated T cells would be infected, have been reported to affect HIV transmission (Berger et al., 1999; Cohen et al., 2008).

Various social and behavioral factors have been identified as risk factors for HIV transmission. Multiple sexual partners, serodiscordant partners, and homosexual partners increase risk of HIV transmission (Maartens et al., 2014). Sharing of injection needles and unprotected sex may increase risk for HIV transmission (Maartens et al., 2014).
1.1.3 Drug Classes

To date, over 25 drugs have been approved for the treatment HIV/AIDS (De Clercq, 2009) that target various steps in infection and replication cycle of HIV. These drugs include:

1. Co-receptor inhibitor: These drugs inhibit interactions between virus and host cell co-receptors (CCR5).

2. Fusion inhibitor: These drugs inhibit fusion of the virus particle with the host cell membrane.

3. Reverse transcriptase inhibitors: These drugs inhibit synthesis of double-stranded proviral DNA from single-stranded genomic RNA of HIV. This class includes nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors. The research focus for this thesis was kept on non-nucleoside reverse transcriptase inhibitors (NNRTIs).

4. Integrase inhibitors: These drugs inhibit integration of double-stranded proviral DNA into the host genomic DNA.

5. Protease inhibitors: These drugs inhibit proteolytic processing of viral polyproteins into structural and functional proteins.

The currently used anti-HIV drugs and fixed dose drug combinations approved by the United States Food and Drug Administration are listed in Table 1.1 (United States Food and Drug Administration, 2015).
<table>
<thead>
<tr>
<th>Drugs Class</th>
<th>Approved Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-receptor inhibitor</td>
<td>Maraviroc</td>
</tr>
<tr>
<td>Fusion inhibitor</td>
<td>Enfuvirtide</td>
</tr>
<tr>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
<td>Delavirdine, Efavirenz, Etravirine, Nevirapine, Rilpivirine</td>
</tr>
<tr>
<td>Nucleoside reverse transcriptase inhibitors</td>
<td>Abacavir, Didanosine, Emtricitabine, Lamivudine, Stavudine, Zidovudine</td>
</tr>
<tr>
<td>Nucleotide reverse transcriptase inhibitor</td>
<td>Tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>Integrase inhibitors</td>
<td>Dolutegravir, Elvitegravir, Raltegravir</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Atazanavir, Darunavir, Fosamprenavir, Indinavir, Nelfinavir, Ritonavir, Saquinavir, Tipranavir</td>
</tr>
<tr>
<td>Fixed dose combinations</td>
<td>Abacavir and Lamivudine</td>
</tr>
<tr>
<td></td>
<td>Abacavir, Dolutegravir, and Lamivudine</td>
</tr>
<tr>
<td></td>
<td>Abacavir, Lamivudine, and Zidovudine</td>
</tr>
<tr>
<td></td>
<td>Efavirenz, Emtricitabine, and Tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td></td>
<td>Abacavir and Lamivudine</td>
</tr>
<tr>
<td>Drugs Class</td>
<td>Drugs</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Fixed dose combinations</td>
<td>Abacavir, Dolutegravir, and Lamivudine</td>
</tr>
<tr>
<td></td>
<td>Abacavir, Lamivudine, and Zidovudine</td>
</tr>
<tr>
<td></td>
<td>Efavirenz, Emtricitabine, and Tenofovir disoproxil fumarate</td>
</tr>
</tbody>
</table>
1.1.3.1 Highly Active Anti-retroviral Therapy (HAART) Regimen

Therapeutic benefits of combining antiretroviral drugs in the treatment of HIV-1 infection were first reported in 1997 (Hammer et al., 1997; Gulick et al., 1997) and has since been a widely accepted treatment strategy. Antiretroviral therapy is initiated with specific aims of: (a) maximal and durable HIV viral load suppression, (b) immune function restoration, (c) reduction of morbidity and mortality associated with HIV; and (d) prevention of transmission of HIV (Department of Health and Human Services, 2014). According to the current treatment guideline from the United States Department of Health and Human Services (Department of Health and Human Services, 2014), it is recommended to prescribe HAART regimen to all patients infected with HIV in order to minimize HIV-associated morbidity and mortality, and prevent transmission of HIV. At the same time, patients are encouraged to commit to HAART regimen and understand fully the benefits and risks associated with the treatment (Department of Health and Human Services, 2014). The World Health Organization recommends initiation of HAART regimen in all patients with CD4 counts less than 500 cells per cubic millimeter (World Health Organization, 2013). Other factors such as pregnancy or lactation may require modification in the treatment regimen (World Health Organization, 2013). In a population-based association study performed in British Columbia, Canada, a positive correlation was reported between usage of HAART regimen and decrease in viral load (Montaner et al., 2010). The same study reported a 547% increase in the number of HIV patients receiving HAART regimen and a 52% decrease in new HIV diagnosis over the period of 1996 and 2009 (Montaner et al., 2010). Overall, HAART regimen has been reported to be effective in reducing HIV transmission.
1.2 Non-nucleoside Reverse Transcriptase Inhibitors

NNRTIs represent an important class of drugs that are routinely used, along with other anti-retrovirals, for the treatment of HIV-1 infection (Gazzard et al., 2008; World Health Organization, 2013; Department of Health and Human Services, 2014). These drugs are inhibitors of HIV-1 reverse transcriptase enzyme and differ from nucleoside reverse transcriptase in characteristics listed in Table 1.2 (adapted from (de Bethune, 2010)).

Table 1.2 Comparison between Nucleoside and Non-nucleoside Reverse Transcriptase Inhibitors

<table>
<thead>
<tr>
<th>Features</th>
<th>Nucleoside reverse transcriptase inhibitors</th>
<th>Non-nucleoside reverse transcriptase inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotype</td>
<td>Nucleoside analogues</td>
<td>Structurally diverse</td>
</tr>
<tr>
<td>Metabolic activation</td>
<td>Require metabolic conversion to active 5′-triphosphates</td>
<td>Not required</td>
</tr>
<tr>
<td>Inhibition mechanism</td>
<td>Competitive</td>
<td>Mixed (non- and un-competitive)</td>
</tr>
<tr>
<td>Binding site</td>
<td>Catalytic</td>
<td>Allosteric</td>
</tr>
<tr>
<td>Activity</td>
<td>Broad-spectrum anti-retrovirals</td>
<td>Specific inhibitors of HIV-1 reverse transcriptase enzyme</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Low to moderate</td>
<td>High</td>
</tr>
</tbody>
</table>
Clinically, NNRTI mono-therapy has not been successful in treatment of HIV-1 infections as the virus rapidly develops resistance against these drugs by mutating amino acid sequences in the NNRTI binding pocket (Balzarini *et al.*, 1998; Menendez-Arias *et al.*, 2011). However, due to their effectiveness when given in combination with nucleoside reverse transcriptase inhibitors and/or protease inhibitors, these drugs have become an integral part of the HAART regimen (Gazzard *et al.*, 2008; De Clercq, 2009; World Health Organization, 2013; Department of Health and Human Services, 2014). In terms of activity spectrum, NNRTIs specifically inhibit reverse transcriptase of HIV-1 (except strains in group O), but are inactive against HIV-2 and animal retroviruses (Usach *et al.*, 2013).

In the present thesis, NNRTIs have been arbitrarily classified into two categories, i.e., first generation NNRTIs (including nevirapine, delavirdine, and efavirenz) and second generation NNRTIs (including etravirine and rilpivirine). This classification was used to compare effects of these drugs on pregnane X receptor and constitutive androstane receptor.

### 1.2.1 First Generation NNRTIs

Discovery of inhibitory properties of 1-(2-2-hydroxyethoxymethyl)-6-(phenylthio)thymine (Baba *et al.*, 1991) and tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one and -thione (Pauwels *et al.*, 1994) for HIV-1 reverse transcriptase initiated development of NNRTIs. Nevirapine was the first drug to receive approval from the United States Food and Drug Administration (1996) for the treatment of HIV-1 infection. In subsequent years, delavirdine (1997) and efavirenz (1998) received regulatory approval to be used as anti-HIV drugs (De Clercq, 2009; Usach *et al.*, 2013). Chemical structures of nevirapine, delavirdine, and efavirenz are shown in Fig. 1.3.
Figure 1.3 Chemical Structures of First Generation Non-nucleoside Reverse Transcriptase Inhibitors: Nevirapine, Delavirdine, and Efavirenz.
First generation NNRTIs, nevirapine, delavirdine, and efavirenz, are generally safe and well tolerated in the clinic and due to their long plasma half-life (except delavirdine), can be used as a once-daily regimen (Usach et al., 2013). However, their clinical use is often restricted due to low genetic barrier to development of resistance by HIV-1 and potential for cross-resistance (Usach et al., 2013). Also, nevirapine has been associated with hepatotoxicity (Sulkowski et al., 2002) and Stevens-Johnson syndrome (Warren et al., 1998). Similarly, delavirdine is associated with severe skin rashes that in rare cases may lead to erythema multiforme or Stevens-Johnson syndrome (Gangar et al., 2000; Usach et al., 2013). Efavirenz, on the other hand, has been associated with neuropsychiatric adverse effects (Gutierrez et al., 2005).

1.2.2 Second Generation NNRTIs

The second generation NNRTIs belong to the di-aryl-pyrimidine chemotype (de Bethune, 2010; Usach et al., 2013). Etravirine was the first drug in this class to receive regulatory approval (2008) (De Clercq, 2009), followed by rilpivirine (2011) (Usach et al., 2013). Both etravirine and rilpivirine (Fig. 1.4) have shown activity against treatment-naive and treatment-resistant strains of HIV-1 (Goebel et al., 2006; Arasteh et al., 2009; Santos et al., 2011; Gazzard et al., 2011), although rilpivirine has been approved for clinical use in only treatment-naive HIV-1 infection (De Clercq, 2012). Greater potency, smaller dosage, and requirement of less frequent dosing has enabled development of rilpivirine-based triple drug combination branded as Complera® (containing 25 mg rilpivirine, 300 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the United States of America) and Eviplera® (containing 25 mg rilpivirine, 245 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the European Union) for treatment-naive HIV-1 infection (De Clercq, 2012). In clinical trials, both etravirine
and rilpivirine have been found to be generally safe and well tolerated. Compared to efavirenz, fewer neuropsychiatric adverse effects have been reported for etravirine (Nelson et al., 2011). Similar results were obtained in clinical trials wherein rilpivirine was found to be better tolerated than efavirenz (Cohen et al., 2012). Table 1.3 enlists various pharmacokinetic parameters for rilpivirine, etravirine, nevirapine, delavirdine, and efavirenz.

Figure 1.4 Chemical Structures of Second Generation Non-nucleoside Reverse Transcriptase Inhibitors: Etravirine and Rilpivirine.
**Table 1.3 Pharmacokinetic Parameters of First and Second Generation NNRTIs** (adapted from (Usach et al., 2013))

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Nevirapine</th>
<th>Delavirdine</th>
<th>Efavirenz</th>
<th>Etravirine</th>
<th>Rilpivirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosage (mg)</td>
<td>200, bid</td>
<td>400, tid</td>
<td>600, od</td>
<td>200, bid</td>
<td>25, od</td>
</tr>
<tr>
<td>(t_{\text{max}}) (h)</td>
<td>4</td>
<td>1.2</td>
<td>3-5</td>
<td>4 (200 mg/12h)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (400 mg/12 h)</td>
<td></td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>2</td>
<td>3.3</td>
<td>4.1</td>
<td>0.4 (200 mg/12h)</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7 (400 mg/12 h)</td>
<td></td>
</tr>
<tr>
<td>Cl (L/h)</td>
<td>1.5, SD</td>
<td>60.3, SD</td>
<td>9.4</td>
<td>43.7</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>3.3, MD</td>
<td>7.79, MD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>1.2</td>
<td>0.8-1</td>
<td>3.8</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>&gt;90%</td>
<td>85%</td>
<td>40-45%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(t_{1/2}) (h)</td>
<td>45, SD</td>
<td>2.4</td>
<td>52-76, SD</td>
<td>30-40</td>
<td>47.7</td>
</tr>
<tr>
<td></td>
<td>25-30, MD</td>
<td>40-55, MD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>62%</td>
<td>98%</td>
<td>&gt;99%</td>
<td>99.9%</td>
<td>99.7%</td>
</tr>
</tbody>
</table>

bid: twice-daily; od: once-daily; tid: thrice-daily; MD: multiple dose; SD: single dose
Cl: apparent plasma clearance; F: bioavailability; \(t_{\text{max}}\): time required to reach maximum concentration in plasma (C\(_\text{max}\)); \(t_{1/2}\): half-life in plasma; Vd: apparent volume of distribution
1.2.3 Mechanism of Action

Replication of HIV-1 requires four different viral enzymes, reverse transcriptase, ribonuclease H, integrase, and protease. All of these are encoded by viral polymerase (Pol) gene (de Bethune, 2010). Out of these enzymes, drugs have been developed to target reverse transcriptase, integrase, and protease (De Clercq, 2009). NNRTIs specifically inhibit HIV-1 reverse transcriptase by a mixed (non- and un-competitive) mechanism (Sluis-Cremer and Tachedjian, 2008). This asymmetrical heterodimeric enzyme is composed of two subunits, p66 (66 kDa; 560 amino acids) and p51 (51 kDa; 440 amino acids). A three dimensional domain structure of p66 subunit shares anatomical resemblance with the right hand, and contains a finger (amino acids 1-85 and 118-155), a palm (amino acids 86-117 and 156-237), and a thumb (amino acids 238-318) region. The palm region (amino acids 86-117 and 156-237) of the p66 subunit of HIV-1 reverse transcriptase contains the active site, along with a hydrophobic pocket at a distance of ~10 angstroms (Kohlstaedt et al., 1992). This hydrophobic pocket serves as an allosteric binding site for NNRTIs and contains aromatic (Tyr181, Tyr188, Phe227, Trp229, and Tyr232), hydrophobic (Pro59, Leu100, Val106, Val179, Leu234, and Pro236), and hydrophilic (Lys101, Lys103, Ser105, Asp132, and Glu224) amino acids of the p66 subunit, along with two amino acids belonging to the p51 subunit (Ile135 and Glu138) (Kohlstaedt et al., 1992). Binding of NNRTIs to this hydrophobic pocket results in loss of conformational flexibility of the enzyme, which in turn leads to inhibition of the enzyme. Changes or a loss of amino acid in the hydrophobic pocket due to mutations have resulted in resistance development against NNRTIs (Sarafianos et al., 2009). These mutations are common in HIV-1 due to high replication rate (Perelson et al., 1996) and lack of exonucleolytic proof-reading functionality that lets replication
error go undetected (Roberts et al., 1988). A schematic diagram of three-dimensional structure of HIV-1 reverse transcriptase enzyme illustrating NNRTI binding pocket is shown in Fig. 1.5.

![Figure 1.5 Three-dimensional Structure of HIV-1 Reverse Transcriptase](image)

**Figure 1.5 Three-dimensional Structure of HIV-1 Reverse Transcriptase** (reprinted with permission from (De Clercq, 2009)).

NRTI: nucleoside reverse transcriptase inhibitors; NtRTIs: nucleotide reverse transcriptase inhibitors; NNRTIs: non-nucleoside reverse transcriptase inhibitors

The amino acid sequence of HIV-1 and HIV-2 reverse transcriptase are very similar, however, NNRTIs lack any activity against HIV-2 (De Clercq, 1998). Structural analysis revealed differences in residues 181 and 188. While HIV-1 has a tyrosine residue at both 181 and 188 position, the same are replaced by isoleucine and leucine at position 181 and 188, respectively, in HIV-2 (Condra et al., 1992). This prevents binding of NNRTIs to the hydrophobic pocket of HIV-2 reverse transcriptase rendering inactivity to NNRTIs against HIV-2 infections.
1.3 Nuclear Receptors

Nuclear receptors belong to a large superfamily of conserved ligand-activated, DNA-binding transcription factors (Gronemeyer et al., 2004; Germain et al., 2006; Sladek, 2011). The analysis of the human genome has identified 48 members in the nuclear receptor superfamily (Schulman and Heyman, 2004; Sladek, 2011) that play key regulatory roles in normal physiological processes, such as cellular homeostasis, growth, development, and differentiation, and various pathophysiological conditions, such as cancer (Sherman et al., 2012; Safe et al., 2014), metabolic syndrome (Sonoda et al., 2008), and various other diseases in humans (Anbalagan et al., 2012; Dasgupta et al., 2014).

In 1995, nuclear receptors were grouped in four broad categories based on their dimerization and DNA-binding properties (Mangelsdorf et al., 1995), as shown in Fig. 1.6. “Class I receptors include the known steroid hormone receptors, which function as ligand induced homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors heterodimerize with RXR and characteristically bind to direct repeats (although some bind to symmetrical repeats as well). Exclusive of the steroid hormones, this group includes all other known ligand dependent receptors. Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories” (Mangelsdorf et al., 1995).
Figure 1.6 Nuclear Receptor Classification Based on Their Dimerization and DNA-binding Properties (reprinted with permission from (Olefsky, 2001)).

GR: glucocorticoid receptor, MR: mineralocorticoid receptor; PR: progesterone receptor; AR: androgen receptor; ER: estrogen receptor; T3R: thyroid receptor; RAR: retinoic acid receptor; VDR: vitamin D receptor; PPAR: Peroxisome proliferator-activated receptors; EcR: ecdysone receptor; FXR: fornesoid X receptor; CAR: constitutive androstane receptor; LXR: liver X receptor; PXR: pregnane X receptor; RXR: retinoic X receptor; COUP: COUP-TF-like receptors; HNF-4: hepatocyte nuclear factor-4; TR2: testicular receptors; TLX: Tailless-like receptors; GCNF: Germ cell nuclear factor receptor; NGF1-B: nerve growth factor-induced clone B; SF-1: steroidogenic factor 1; Rev-erb: Rev-Erb receptors; ROR: Retinoic acid-related orphans; ERR: Estrogen-related receptors
A more systematic classification system categorized the members of nuclear receptor superfamily into different groups based on the phylogenetic analysis and sequence homology (Nuclear Receptors Nomenclature Committee, 1999; Germain et al., 2006; Alexander et al., 2013). According to the Nuclear Receptor Nomenclature Committee recommendations, gene subfamilies are designated by Arabic numbers, groups by capital letters, and individual genes by Arabic numerals, for example, NR2 (subfamily) E (group) 3 (individual gene). Functional but structurally distinct variants of the same gene are designated by a lower-case letter added at the end of the name, for example, NR2E3a or NR2E3b (Nuclear Receptors Nomenclature Committee, 1999). Various members of the nuclear receptor subfamily are listed in Table 1.4.
### Table 1.4 Nuclear Receptor Classification (Alexander et al., 2013)

<table>
<thead>
<tr>
<th>Class</th>
<th>Name</th>
<th>Receptor Sub-types</th>
<th>Gene Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Thyroid hormone receptors</td>
<td>Thyroid hormone receptor-α</td>
<td>NR1A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thyroid hormone receptor-β</td>
<td>NR1A2</td>
</tr>
<tr>
<td>1B</td>
<td>Retinoic acid receptors</td>
<td>Retinoic acid receptor-α</td>
<td>NR1B1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retinoic acid receptor-β</td>
<td>NR1B2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retinoic acid receptor-γ</td>
<td>NR1B3</td>
</tr>
<tr>
<td>1C</td>
<td>Peroxisome proliferator-activated receptors</td>
<td>Peroxisome proliferator-activated receptor-α</td>
<td>NR1C1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peroxisome proliferator-activated receptor-β/δ</td>
<td>NR1C2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peroxisome proliferator-activated receptor-γ</td>
<td>NR1C3</td>
</tr>
<tr>
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<td>Rev-Erb receptors</td>
<td>Rev-Erb receptor-α</td>
<td>NR1D1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev-Erb receptor-β</td>
<td>NR1D2</td>
</tr>
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<td>Small heterodimer partner</td>
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Figure 1.7 Domain Structure of Nuclear Receptors (Adapted from (Pawlak et al., 2012)).

AF-1: activation function domain-1; DBD: DNA-binding domain; LBD: ligand-binding domain, AF-2: activation function domain-2

The commonalities in structural organization of nuclear receptors are shown in Fig. 1.7 (Pawlak et al., 2012). The N-terminal region of nuclear receptor contains the Activation Function 1 (AF-1) domain that can be transactivated in a ligand-independent manner and is highly variable depending on the receptor (Kumar and Thompson, 1999; Pawlak et al., 2012). The DNA-binding domain (DBD) is a compact globular domain that has the most conserved amino acid sequence within the nuclear receptor family. This domain consists of two zinc-finger motifs (P-box and D-box) that coordinate binding with zinc atoms and formation of tertiary helical structures. These tertiary helices play a role in recognition of and binding to specific DNA sequences called response elements. The DBD of nuclear receptors binds to DNA in a sequence-specific manner either as a monomer, homodimer, or heterodimer with retinoid X receptor α (RXRα) (Kumar and Thompson, 1999; Pawlak et al., 2012). The hinge region is a flexible linker between the DBD and the ligand-binding domain (LBD) and contains specific nuclear localization sequences regulating distribution of nuclear receptors within the cells. The LBD at the C-terminal region contains ligand-dependent activation function 2 (AF-2) domain. The LBD shares low sequence homology between nuclear receptors. This domain plays
multifunctional role by providing an interface for receptor homo- and hetero-dimerization and co-modulator binding region, along with serving as a ligand-binding site. Ligand-binding to this domain causes conformational changes, resulting in transcriptional activation or repression (Kumar and Thompson, 1999; Pawlak et al., 2012). Despite having structural similarities, nuclear receptors in different classes, as listed in Table 1.4, vary greatly in their dimerization and DNA binding properties, and these differences are have been compared in previously published reports (Moore et al., 2006; Benoit et al., 2006). The studies in the present thesis will focus on pregnane X receptor (PXR, NR1I2), and constitutive androstane receptor (CAR, NR1I3), two transcriptional factors/nuclear receptors involved in regulation of various physiological, pathophysiological, and detoxification processes by controlling the expression of their target genes (Kretschmer and Baldwin, 2005; Timsit and Negishi, 2007; di Masi et al., 2009; Wang et al., 2012; Chai et al., 2013). Differences in dimerization, DNA binding properties, and cellular localization of PXR and CAR are discussed in subsequent sections.

1.3.1 Pregnane X Receptor (PXR)

PXR belongs to the superfamily of ligand-activated, DNA-binding transcription factor that is encoded by the NR1I2 gene (Kliewer et al., 2002). It was first discovered and cloned from the mouse liver cDNA library (Kliewer et al., 1998). In the same study, various synthetic pregnanes were identified as ligands for this nuclear receptor, hence it was named as pregnane X receptor (Kliewer et al., 1998). Shortly after the discovery of mouse PXR, its human ortholog was discovered by three independent research groups (Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998). Different research groups referred to human PXR by terms such as steroid and xenobiotic receptor (Blumberg et al., 1998) or pregnane activated receptor (PAR)
In subsequent years, PXR orthologs were discovered in other animal species such as rat (Zhang et al., 1999), rabbit (Savas et al., 2000), and rhesus monkey (Moore et al., 2002). Analysis of expressed sequence tag database revealed that alternatively spliced variants exist for approximately 40% of human genes (Brett et al., 2000). As a result, multiple proteins, with different structural and functional properties, can be coded from a single gene. This information suggest differences in expression levels, DNA-binding, sub-cellular localization, co-modulator protein interactions, target genes, and biological function for nuclear receptors (Keightley, 1998). Nucleotide sequencing analysis of the mouse liver cDNA revealed the presence of two clones corresponding to mouse PXR, designated as PXR.1 (wild type) and PXR.2 (splice variant), with a loss of 41 amino acids in the LBD of PXR.2 (Kliewer et al., 1998). Later, similar splice variants were discovered for the human ortholog of PXR. Two variants of hPXR, formed as a result of alternative splicing, were first reported in 1998 as human PAR-1 (hPXR wild type, hPXR-WT) (T1) and hPAR-2 (T2) (Bertilsson et al., 1998). Amino acid sequence comparison revealed differences in the N-terminal regions of T2 that contained extra 39 amino acids in the open reading frame when compared to T1 (Bertilsson et al., 1998). Subsequently, another variant of hPXR (hPXR.2 or T3), with a deletion of 37 amino acids in the LBD (amino acid 174 to 210), was identified using reverse-transcription polymerase chain reaction (RT-PCR) (Dotzlaw et al., 1999). Fig. 1.8 illustrates the structure of human PXR transcript (T1) in comparison with its T2 and T3 splice variants. Among other animal species, porcine orthologs of PXR and its splice variants have also been characterized (Pollock et al., 2007). By comparison, the porcine PXR protein shares 85% amino acid sequence homology with human PXR, and is more similar to human PXR when compared to other animal species.
(Pollock et al., 2007). Similar to the human PXR, splice variants of porcine PXR (porcine PXR.2 - porcine PXR.7) with modifications in the LBD have been identified (Pollock et al., 2007). Northern blot analysis showed presence of porcine PXR in various tissues including liver, small intestine, kidney, and colon, with detectable expression in heart, adrenal gland, thymus, ovary, uterus, trachea, thymus, bladder, spleen and lymph nodes (Pollock et al., 2007). Tissues with high porcine PXR mRNA levels (liver, small intestine, kidney, and colon) showed presence of splice variants (porcine PXR.2 - porcine PXR.6) (Pollock et al., 2007), indicating intensive alternative splicing of porcine PXR.

![Comparison of Wild-type Human PXR Transcript Structure with its Splice Variants](adapted from (Gardner-Stephen et al., 2004)).

DBD: DNA-binding domain; LBD: ligand-binding domain; T1: Wild-type hPXR; T2: hPXR splice variant T2 (extra 39 amino acids in the N-terminal region); T3: hPXR splice variant T3 (37 amino acids deletion in the LBD; deleted amino acids: 174-210)
1.3.1.1 Tissue Distribution and Ontogeny

As assessed by RT-PCR analysis of total RNA from single and pooled organ specimen, PXR mRNA expression is detectable in several human organs, with very high levels in organs such as liver, small intestine, and colon (Nishimura et al., 2004). This finding corroborates with earlier studies where Northern blot analysis revealed hPXR expression in human liver, small intestine, and colon (Lehmann et al., 1998; Bertilsson et al., 1998). Although low, PXR mRNA is still detectable in organs such as kidneys, adrenal gland, lungs, stomach, heart, brain, cerebellum, skeletal muscles, spleen, thymus, testes, uterus, placenta, bone marrow, trachea, and salivary glands (Nishimura et al., 2004). RT-PCR analyses have indicated mRNA expression of all three variants of human PXR i.e., T1, T2, and T3, in normal human intestine, liver and liver-derived hepatocellular carcinoma cells (HepG2) (Gardner-Stephen et al., 2004). Only the T1 variant is expressed in the human Caco-2 colorectal adenocarcinoma cell line (Gardner-Stephen et al., 2004).

PXR mRNA expression levels in humans show developmental changes suggesting ontogenetic regulation (Miki et al., 2005; Vyhlidal et al., 2006). The expression level of PXR is very low and highly variable (345-fold) in pre- and neonatal livers. PXR is detectable in the liver and small intestine of infants less than 6 months of age, reaches its highest levels in the 15-38 year old age group, and then decreases in the 45-65 year old age group before dropping to fetal levels in the elderly (67-85 years) (Miki et al., 2005; Vyhlidal et al., 2006). Similar changes have been reported in PXR protein levels (Miki et al., 2005). In other tissues such as kidneys and large intestine, PXR mRNA expression were found to be high in fetal groups and low in rest of the age groups (Miki et al., 2005). Lungs, on the other hand, showed low and variable PXR mRNA expression in all the age groups (Miki et al., 2005). Similar age-dependent
effect on PXR expression have been observed in both male and female rats, where levels are low in embryonic stages, increases during postnatal development and reaches the peak expression levels at adulthood (Balasubramaniyan et al., 2005).

1.3.1.2 Factors Affecting PXR Expression or Function

Expression and function of PXR may be affected by factors such as infection, chemical exposure, and pregnancy. Lipopolysaccharide, a component of gram-negative bacterial cell wall (Jacob et al., 1977), down-regulates PXR expression in mouse liver (Beigneux et al., 2002), intestine (Xu et al., 2006), and placenta (Chen et al., 2005). Similarly, interleukin-6, an inflammation marker (Rincon, 2012), down-regulates PXR in primary cultures of human hepatocytes (Pascussi et al., 2000c). Similarly, chronic exposure to alcohol down-regulates hepatic expression of PXR in mice (Wang et al., 2005). Elevated levels of steroidal hormones may increase the activity of PXR and its target genes (Reschly and Krasowski, 2006).

1.3.1.3 Mechanism of Activation

Activation of PXR is multi-step process that is initiated by ligand-binding. In the absence of a ligand, PXR remain inactive and resides mainly in the cytoplasmic compartment of the cells (Kawana et al., 2003), complexed with chaperone proteins such as heat shock protein 90 and cytoplasmic constitutive androstane receptor retention protein (Squires et al., 2004). Binding of a ligand to the LBD of PXR causes dissociation of the receptor from chaperone proteins and translocation of the receptor into the nucleus. The xenochemical response sequence, nuclear localization sequence, and activation function domain-2 regions of PXR plays a crucial role in its translocation from cytoplasm into the nucleus (Squires et al., 2004). Once
inside the nucleus, PXR forms a heterodimer with RXRα (Mangelsdorf and Evans, 1995) and this complex binds to DNA response element (DRE) in the promoter or enhancer region of the target genes of PXR. Depending on the target gene, PXR-RXRα complex is capable of binding to a wide range of response element motifs oriented either as direct repeat (DR-3 or DR-4) or as everted repeat (ER-6 or ER-8) (Kliewer et al., 2002). This causes conformational changes in PXR-RXRα heterodimer complex leading to dissociation of corepressor proteins and association of coactivator proteins. Corepressors of PXR include proteins such as small heterodimer partner (Ourlin et al., 2003), nuclear receptor corepressor protein (Takeshita et al., 2002), and silencing mediator for retinoid and thyroid receptors (Johnson et al., 2006). Some of the coactivators of PXR include receptor-associated coactivator-3 (Johnson et al., 2006), glucocorticoid receptor-interacting protein 1 (Takeshita et al., 2002), steroid receptor coactivator 1 (SRC1) (Kliewer et al., 1998; Mani et al., 2005), and peroxisome proliferator-activated receptor-γ coactivator 1-α (Bhalla et al., 2004). Ligand-dependent activation of PXR eventually leads to an increase in target gene expression. Micro-RNA-mediated post-transcriptional and post-translational modifications in the form of ubiquitylation, phosphorylation, SUMOylation, and acetylation may also affect PXR and its target genes (Smutny et al., 2013). Fig. 1.9 shows a schematic diagram of PXR activation mechanism.
Figure 1.9 Schematic Representation of PXR Activation Mechanism.
CCRPI: cytoplasmic constitutive androstane receptor retention protein; DRE: DNA response element; HSP90: heat shock protein 90
1.3.1.4 Target Genes

PXR regulates many genes that play important roles in maintaining normal physiological processes (Sonoda et al., 2008; di Masi et al., 2009; Ihunnah et al., 2011; Kakizaki et al., 2011; Gao and Xie, 2012; Dasgupta et al., 2014). Activated PXR represses expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase by interfering with transcription factors like forkhead transcription factor 1 (Kodama et al., 2004) and cAMP-response element-binding protein (Kodama et al., 2007), thereby affecting pancreatic hormone (insulin and glucagon) signaling (Kodama and Negishi, 2013). Cross-talk between PXR and Akt-regulated forkhead transcription factor represses β-oxidation and ketogenesis, and increases lipogenesis by activating carnitine palmitoyltransferase 1A, 3-hydroxy-3-methylglutarate-CoA synthase 2, and stearoyl-CoA desaturase 1 (Nakamura et al., 2007; Kodama and Negishi, 2013). Activated PXR also competes with hepatic nuclear factor 4 α to form a complex with peroxisome proliferator activating receptor-γ coactivator-1 that leads to repression of CYP7A1 and CYP8B1 protein levels affecting physiological processes such as glucose, cholesterol, and bile acid metabolism (Bhalla et al., 2004; Kodama and Negishi, 2013). Studies suggest that activated PXR may also interfere with nuclear factor κB and growth arrest and DNA damage-inducible protein β signaling, thereby affecting immune and cellular responses to physiological and environmental stresses, respectively (Zhou et al., 2006; Kodama and Negishi, 2011; Kodama and Negishi, 2013).

PXR is a master regulator of genes encoding various proteins involved in metabolism and transport of xenobiotics (Kliewer, 2003; Handschin and Meyer, 2003; Timsit and Negishi, 2007; Li and Wang, 2010). Among phase I drug-metabolizing enzymes, PXR has been shown to be involved in regulation of CYP2B6 (Goodwin et al., 2001), CYP2C8 (Gerbal-Chaloin et al.,
CYP2C9 (Gerbal-Chaloin et al., 2001), CYP2C19 (Gerbal-Chaloin et al., 2001), CYP3A4 (Lehmann et al., 1998), CYP3A5 (Burk et al., 2004), and CYP3A7 (Pascussi et al., 1999). PXR also regulates expression of phase II conjugating enzymes such as glutathione S-transferase A2 (GSTA2) (Falkner et al., 2001), sulfotransferases (SULT) (SULT1A1, SULT2A1, and SULT2A3) (Runge-Morris and Kocarek, 2005), and uridine 5′-diphosphoglucuronosyltransferases (UGT) (UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9) (Zhou et al., 2005). Drug transporters regulated by PXR include P-glycoprotein (Geick et al., 2001), multidrug resistance-associated protein 2 (Kast et al., 2002), and organic anion-transporting polypeptide 1A2 (Miki et al., 2006).

1.3.1.5 Modulators of PXR Activity

The activity of PXR can be modulated by a wide variety of structurally diverse chemicals, including prescription drugs, natural products, and endogenous substances (Chang and Waxman, 2006). A large LBD cavity volume (1150 cubic angstroms) makes human PXR capable of binding with a wide range of compounds by allowing hydrogen bond formation or van der Waals interactions with a ligand (Watkins et al., 2001). For instance, crystallographic studies between SR12813 [Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate], a ligand of human PXR (Jones et al., 2000), and LBD of PXR revealed three distinct orientations in which SR12813 bind to the receptor (Watkins et al., 2001).

Modulators of PXR can be broadly classified into two groups, agonists and antagonists. Based on in vitro assays, prescriptions drugs such as rifampin (Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998), clotrimazole (Lehmann et al., 1998), phenobarbital (Jones et al., 2000), ritonavir (Dussault et al., 2001), dexamethasone (Pascussi et al., 2001), paclitaxel
(Synold et al., 2001), and tamoxifen (Sane et al., 2008) have been reported to be human PXR agonists. Among natural products, artemisinin (Burk et al., 2005b), forskolin (Ding and Staudinger, 2005), hyperforin (Wentworth et al., 2000), Ginkgo biloba extract (Lau et al., 2010), ginkgolide A and ginkgolide B (Lau et al., 2012) are PXR agonists. Other prescription drugs such as bosentan (van Giersbergen et al., 2002), carbamazepine (El Sankary et al., 2001), and lovastatin (Lehmann et al., 1998), and natural products such as Kava extract (Raucy, 2003) and St. John’s wort (Wentworth et al., 2000) have been reported to activate hPXR. Among the antagonists that bind to hPXR, but do not alter its activity include compounds such as L-sulforaphane (Zhou et al., 2007), trabectedin (ecteinascidin-743 or ET-743) (Synold et al., 2001), and ketoconazole (Huang et al., 2007). The fact that hPXR activity can be modulated by a wide variety of structurally diverse compounds signify the importance of this receptor in regulation of genes involved in various biological functions, including drug disposition. Table 1.5 enlists various human PXR activators, agonists, and antagonists.
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<td>Spironolactone, Tamoxifen, Tpiramate, Topotecan</td>
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<td>Nifedipine, Phenobarbital, Schisandrol B; Schisandrins A, Schisandrins B,</td>
</tr>
<tr>
<td></td>
<td>Solomonsterol A, Solomonsterol B, Rifampin, Rifaximin, Ritonavir</td>
</tr>
<tr>
<td>Antagonist</td>
<td>Coumestrol, Ketoconazole, L-sulfuraphane, Trabectedin (ecteinascidin-743 or</td>
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<td>ET-743), Seamin</td>
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1.3.1.6 Species-dependent PXR Activation

Receptor cloning experiments have found marked species-dependent differences in the LBD of PXR. It has been shown that the amino acid sequence of the LBD of mouse, rat, rabbit, and human isoforms of PXR have only ~80% homology (Jones et al., 2000). This translates to inter-species differences in PXR activation profiles. For example, rifampin, a macrolide antibiotic, activate human and rabbit isoforms of PXR, but has no effect on mouse or rat receptor isoforms (Jones et al., 2000). The same study reported similar effects for troglitazone and SR12813 (Jones et al., 2000). Pregnenolone 16α-carbonitrile (PCN) is a potent activator of mouse and rat PXR, but has no effect on the activity of rabbit or human isoforms (Jones et al., 2000). Site-directed mutagenesis studies have shown that Leu-308 (Tirona et al., 2004) and Gln-285 (Ostberg et al., 2002) in the LBD of hPXR, Phe-305 and Asp-318 (Song et al., 2005) in the LBD of rat PXR, and Ile-282 (Ostberg et al., 2002) in the LBD of mouse PXR as the amino acids that impart inter-species specificity for ligand-dependent activation of PXR. By comparison, cell-based reporter gene assays revealed common ligands for porcine and human PXR, including rifampin and several endogenous steroids (Gray et al., 2010).

1.3.2 Constitutive Androstane Receptor (CAR)

CAR, designated as NR113 (Giguere, 1999), is another member of the superfamily of nuclear receptors (Germain et al., 2006). It was first discovered in 1994 while sequencing the conserved DBD regions of nuclear receptors in the human liver cDNA, and was named MB67 (Baes et al., 1994). Shortly after, using cloning techniques, another research group isolated the mouse ortholog of MB67 (Choi et al., 1997). Owing to the ability of these mouse and human receptors to constitutively transactivate a group of retinoic acid receptor elements, they were
termed as mouse CAR (Choi et al., 1997) and human CAR (Baes et al., 1994), respectively. Subsequently, two androstane metabolites, androstenol (5α-androst-16-en-3α-ol) and androstanol (5α-androstan-3α-ol) were identified as naturally occurring inverse agonists of mouse CAR (Forman et al., 1998).

Like PXR, splice variants of CAR have also been identified in various animal species. As a result of alternative splicing, mouse CAR encodes for two different proteins, named mouse CAR1 and mouse CAR2 (Choi et al., 1997). mCAR1 shares 88%, 71%, and 72% sequence homology with the corresponding DBD, hinge, and LBD of hCAR (Choi et al., 1997). Sequence analysis of mouse CAR2 revealed similarities with mouse CAR1, except for a 107-base pair deletion at the C-terminal LBD (Choi et al., 1997). Loss of ligand-binding/dimerization motif rendered mouse CAR2 incapable to binding with retinoic acid receptor elements and LBD transactivation (Choi et al., 1997). RT-PCR analysis of cDNA samples from rat liver identified three splice variants, namely rat CAR.1, rat CAR.2, and rat CAR.3 (Kanno et al., 2003; Lamba et al., 2005). Among other animal species, porcine orthologs of CAR and its splice variants have also been identified (Gray et al., 2009). By comparison, the porcine CAR shares 86% sequence homology with human CAR, with 83% homology in the DBD and 84% homology in the LBD (Gray et al., 2009). RT-PCR analysis of cDNA samples from porcine liver identified five splice variants for porcine CAR (splice variant 1 - splice variant 5) (Gray et al., 2009).

For the human CAR gene, 26 splicing variants (SV-1 through SV-26) encoding a variety of alternate mRNAs have been identified (Lamba et al., 2005). The most common splice variant of human CAR is hCAR-SV23, which has an in-frame insertion of four amino acids (SPTV) between helices 6 and 7 and is capable of transactivating target genes (Auerbach et al., 2003; Lamba et al., 2005). Insertion of four amino acids in hCAR-SV23 has been suggested to alter
receptor conformation (DeKeyser et al., 2011). Another important splice variant of human CAR is the SV24 variant (hCAR-SV24) with an in-frame insertion of five amino acids (APYLT) between helices 8 and 9 (Jinno et al., 2004; Arnold et al., 2004; Lamba et al., 2005). Insertion of five amino acids has been reported to compromise receptor heterodimerization and transactivation of target genes (Lamba et al., 2005; Omiecinski et al., 2011). By comparison, hCAR-SV25 has both four amino acid (SPTV, identical to hCAR-SV23) inserts and five amino acids (APYLT, identical to hCAR-SV25) inserts in the sequence (Auerbach et al., 2003; Savkur et al., 2003). Other variants of the wild-type form of human CAR (hCAR-WT) include hCAR-SV5 that lacks α-helices 7 and 8 rendering the receptor incapable of binding to the DBD and target gene activation. hCAR-SV26 has an out-frame deletion of the first 76-base pair in exon 9 that causes loss of α-helices H10, H11, and H12 of LBD, and AF2 domain compromising receptor heterodimerization and target gene transactivation (Arnold et al., 2004; Lamba et al., 2005). With complete loss of exon 2, hCAR-SV22, expressed only in prostate tissue, has compromised DNA-binding ability due to lost zinc finger in the DBD (Lamba et al., 2004). All other splice variants of CAR have been reported to result in truncated proteins with no LBD (Lamba et al., 2004; Lamba et al., 2005). The presence of alternate splice variants may explain variability in CAR-regulated functions.

1.3.2.1 Tissue Distribution and Ontogeny

RT-PCR analysis of total RNA from single and pooled human organ specimen indicated CAR mRNA expression predominantly in liver (Baes et al., 1994; Nishimura et al., 2004; Lamba et al., 2004). Other tissues such as kidney, small intestine, testis, skeletal muscle, stomach, lung, adrenal gland, brain, placenta, and trachea has low, yet detectable expression of CAR mRNA
(Nishimura et al., 2004). Another research group investigated expression of hCAR-WT and its variants in various human tissues and found only hCAR-WT expression in intestine while spleen, heart, and prostate expressed only splice variants (Lamba et al., 2004). This tissue-specific expression of CAR suggests differential transcriptional regulation of diverse biological processes by hCAR-WT and its splice variants (Lamba et al., 2004).

Like PXR, CAR mRNA expression seems to be regulated by age-dependent factors (Vyhlidal et al., 2006). Quantitative RT-PCR analysis indicated that the expression level of CAR is very low during gestational age and prenatal livers in neonates and infants (age < 6 months), but increase over time in older children during postnatal development (Vyhlidal et al., 2006). However, the expression levels are variable (Vyhlidal et al., 2006).

1.3.2.2 Factors Affecting CAR Expression or Function

Factors that may affect CAR expression include chemical exposure, infections or inflammation, and circadian rhythm. Dexamethasone, a glucocorticoid receptor agonist (LaLone et al., 2012), has been reported to induce CAR expression in human hepatocytes (Pascussi et al., 2000b; Pascussi et al., 2003b). This observation was defined mechanistically when a glucocorticoid response element was discovered in the distal region of the CAR gene promoter (Pascussi et al., 2003a). While drugs like dexamethasone can induce CAR expression, others like colchicine, a microtubule disrupting agent (Skoufias and Wilson, 1992), down-regulates expression of CAR by inhibiting glucocorticoid receptor transcriptional activity (Dvorak et al., 2003; Pascussi et al., 2003b). Similarly, lipopolysaccharide and interleukin-1b have been shown to down-regulate hepatic mRNA expression of CAR (Beigneux et al., 2002; Pascussi et al., 2003b). This effect is believed to be a downstream effect of nuclear factor κB-mediated
inhibition of the glucocorticoid response element in the distal region of CAR gene promoter (Pascussi et al., 2003b). Further, hepatic levels of mCAR have been shown to follow a circadian cycle where the expression levels are at highest in the early night suggesting temporal changes in receptor expression (Yang et al., 2006; Yang et al., 2007).

1.3.2.3 Mechanism of Activation

As other nuclear receptors, the domain structure of CAR includes a highly variable AF1 region at the N-terminal, a DBD, a hinge region, a LBD, and AF2 region at the C-terminal (Baes et al., 1994; Lamba et al., 2005; Yang and Wang, 2014). However, an interesting fact about CAR is that it can be activated by both ligand-independent (indirect) (Kawamoto et al., 1999) and ligand-dependent (direct) (Maglich et al., 2003) mechanisms. CAR, in its inactive state, resides inside the cytoplasm in a complexed state with chaperone proteins such as cytoplasmic constitutive androstane receptor retention protein, heat shock protein 90, and protein phosphatase1 regulatory subunit 16A (Kobayashi et al., 2003; Timsit and Negishi, 2007; Sueyoshi et al., 2008). Experimental evidence suggests that nuclear translocation is the rate limiting step in CAR activation and a balancing act between cytosolic retention and nuclear translocation controls the effect of CAR on its target genes (Timsit and Negishi, 2007). Once inside the nucleus, CAR forms a heterodimer with RXRα, causing conformational changes promoting coactivator recruitment and corepressor dissociation (Timsit and Negishi, 2007; Chang, 2009; Yang and Wang, 2014). Some of the coactivator proteins of CAR include SRC1 (Forman et al., 1998), SRC2 (Yan et al., 2015), SRC3 (Chen et al., 2012a), peroxisome proliferator-activated receptor-γ coactivator 1-α (Shiraki et al., 2003), and peroxisome proliferator-activated receptor-binding protein/mediator 1 (Jia et al., 2005; Guo et al., 2006),
whereas nuclear receptor corepressor protein has been identified as CAR corepressor (Lempainen et al., 2005). After heterodimerization and coactivator recruitment, the highly conserved DBD of CAR binds to specific binding site upstream of the CYP2B6 gene (Yang and Wang, 2014). These enhancer modules have been identified as phenobarbital-responsive enhancer module (Honkakoski et al., 1998) and xenobiotic-responsive enhancer module (Wang et al., 2003). CAR-binding response elements usually contains hexameric sequence of AG(G/T)TCA with motifs oriented either as DR-3 or DR-4 (Yang and Wang, 2014).

Indirect activators of CAR include a wide range of chemicals including prescription drugs, environmental chemicals, and endogenous steroid metabolites (Chang and Waxman, 2006; Yang and Wang, 2014). Indirect activators of CAR, for example phenobarbital, stimulate nuclear translocation of CAR without directly binding to the receptor (Kawamoto et al., 1999; Moore et al., 2000). In a previous study, dephosphorylation of conserved Thr-38 residue of CAR appeared to be essential for nuclear translocation of the receptor suggesting an important role of protein kinase-mediated phosphorylation/dephosphorylation in indirect activation of CAR (Mutoh et al., 2009). Various studies have implicated enzymes such as protein phosphatase 2A, extracellular signal-regulated kinase and p38 mitogen-activated protein kinase, and adenosine monophosphate activated protein kinase in indirect activation of CAR (Mutoh et al., 2009; Yang and Wang, 2014). A recent study associated phenobarbital-mediated inhibition of epidermal growth factor receptor signaling as an indirect CAR mechanism in primary cultures of mouse hepatocytes (Mutoh et al., 2013). However, it remains to be investigated if indirect activation of hCAR follows the same mechanism. Fig. 1.10 shows a schematic diagram of CAR activation mechanisms.
Figure 1.10 Schematic Representation of CAR Activation Mechanism.
CCRP: cytoplasmic constitutive androstane receptor retention protein; HSP90: heat shock protein 90; PBREM: phenobarbital-responsive enhancer module; XREM: xenobiotic-responsive enhancer module.
1.3.2.4 Target Genes

Like its closest relative PXR, CAR plays a key role in regulating a wide range of target genes involved in various physiological and pathophysiological functions along with regulating genes involved in absorption, distribution, metabolism, and excretion of drugs and endogenous chemicals (Willson and Kliewer, 2002; Kretschmer and Baldwin, 2005; Qatanani and Moore, 2005; Timsit and Negishi, 2007; Kakizaki et al., 2011; Kachaylo et al., 2011; Gao and Xie, 2012; Chai et al., 2013).

Phenobarbital, a known CAR activator (Sueyoshi et al., 1999), has been shown to improve insulin sensitivity and decrease glucose levels in diabetic patients suggesting CAR-dependent regulation of glucose metabolism (Lahtela et al., 1985). Later, phenobarbital and TCPOBOP treatment were shown to repress phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase in mouse liver (Manenti et al., 1987) and rat hepatocytes (Argaud et al., 1991). The suggested mechanism behind this observation is that activated CAR prevents binding of FoxO1 to insulin response sequence element thereby repressing gluconeogenesis via down-regulation of phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase (Kodama et al., 2004; Konno et al., 2008). Activation of CAR has been implicated in inhibition of hepatic nuclear factor 4α by competing for DNA binding and coactivator (glucocorticoid receptor interacting protein 1 and peroxisome proliferator-activated receptor-gamma coactivator 1α) thereby down-regulating CYP7A1, CYP8B1, and phosphoenolpyruvate carboxykinase 1 and suppressing bile acid production, β-oxidation of fatty acids, and gluconeogenesis in the liver (Miao et al., 2006).

CAR regulates the expression of various target genes encoding drug transporters along with phase I and phase II drug-metabolizing enzymes (Willson and Kliewer, 2002). Phase I
drug-metabolizing enzymes regulated by hCAR include CYP2B6 (Sueyoshi et al., 1999), CYP2C8 (Gerbal-Chaloin et al., 2001), CYP2C9 (Gerbal-Chaloin et al., 2001), and CYP2C19 (Gerbal-Chaloin et al., 2001). Owing to its weaker binding to promoter regions of CYP3A4, the effect of CAR on CYP3A4 regulation is minimal (Faucette et al., 2006). Among phase II conjugating enzymes, CAR is involved in the regulation of UGT1A1 (Sugatani et al., 2001; Zhou et al., 2005). CAR has also been implicated in the regulation of ABC transporter expression including P-glycoprotein (Burk et al., 2005a; Burk et al., 2005b), multidrug resistance-associated protein 2 (Kast et al., 2002), multidrug resistance-associated protein 3 (Huang et al., 2006), and multidrug resistance-associated protein 4 (Assem et al., 2004).

1.3.2.5 Modulators of CAR Activity

CAR activity can be modulated by a wide range of structurally diverse chemicals including synthetic drugs and herbal products (Chang and Waxman, 2006; Chang, 2009). Based on in vitro activity assays, prescription drugs like phenobarbital (Sueyoshi et al., 1999), phenytoin (Wang et al., 2004), statins (fluvastatin, atorvastatin, cerivastatin, and simvastatin) (Kobayashi et al., 2005) have been identified as hCAR activators. Among natural products, artemisinin (Burk et al., 2005b) and Ginkgo biloba extract (Lau et al., 2011a) activate human CAR. An interesting fact about human CAR is that certain modulators may selectively activate one isoform of human CAR, while having no effect on others. For example, the flavonol analogues (galangin, datiscetin, kaempferol, quercetin, isorhamnetin, and tamarixetin) (Lau and Chang, 2013) and phenobarbital (Lau et al., 2011a) activate hCAR-WT while having no effect on hCAR-SV23 or hCAR-SV24. On the other hand, chemicals like di-isononyl phthalate (DeKeyser et al., 2009) and pheniramine (Dring et al., 2010) activate hCAR-SV23 and hCAR-
SV24, respectively, and none of them activate hCAR-WT. Further, CAR has also been reported to show species-dependent chemical modulation. For example, TCPOBOP, clotrimazole, chlorpromazine, and meclizine act as agonists of mouse CAR while having no effect on human CAR (Chang and Waxman, 2006). By comparison, CITCO is an agonist of human CAR (Chang, 2009) and porcine CAR (Gray et al., 2009), but not mouse CAR (Chang, 2009). Chemicals that act as inverse agonists of mouse CAR include androstenol and androstanol (Forman et al., 1998), whereas 1-(2-chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide (PK11195) has been identified as a human CAR antagonist (Li et al., 2008). Table 1.6 enlists various human CAR activators, agonists, and antagonists.
**Table 1.6 Modulators of Human CAR** (complied from (Chang and Waxman, 2006; Chang, 2009; Gao and Xie, 2012; Lau and Chang, 2013; Kanno et al., 2013; Kanno et al., 2014))

<table>
<thead>
<tr>
<th>Type</th>
<th>Modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activators</td>
<td>Acetaminophen, Atorvastatin, Cerivastatin, Datiscetin, Fluvastatin, Galangin,</td>
</tr>
<tr>
<td></td>
<td>3-hydroxyflavone, Isorhamnetin, Kaempferol, Phenobarbital, Phenytoin,</td>
</tr>
<tr>
<td></td>
<td>Quercetin, Simvastatin, Tamarixetin</td>
</tr>
<tr>
<td>Agonists</td>
<td>CITCO, 6,7-Dimethylesculetin</td>
</tr>
<tr>
<td>Inverse Agonist</td>
<td>Andtrostanol, Clotrimazole, S07662, TO901317, Nigramide J</td>
</tr>
<tr>
<td>Antagonist</td>
<td>PK11195</td>
</tr>
</tbody>
</table>
1.3.3 PXR-CAR Cross-talk

PXR and CAR are two closely related nuclear receptors that not only share structural similarities but also ligands, target genes, and capabilities of binding to each other’s DNA response elements (Moore et al., 2003; Pascussi et al., 2003c; Pascussi et al., 2008). The cross-talk between CAR and PXR, and their interactions with other transcription factors such as farnesoid X receptor, thyroid receptor, liver X receptor, vitamin D receptor, hepatic nuclear factor 4α, forkhead transcription factor 1, glucocorticoid receptor, and small heterodimer partner have been shown previously (Pascussi et al., 2008). Various studies have indicated potential protective role of PXR and CAR in bile acid toxicity due to their interaction with farnesoid X receptor (Staudinger et al., 2001; Handschin et al., 2002; Guo et al., 2003). In mice model, PXR and CAR not only influence metabolism of cholesterol and synthesis of bile acid, but also affect bile acid elimination pathways making PXR and CAR as a potential target for the treatment of cholestatic liver injury (Stedman et al., 2005). Another transcription factor VDR has been reported to regulate expression of CAR and PXR target genes such as CYP2B6, CYP2C9, and CYP3A4 suggesting potential cross-talks between vitamin D receptor, PXR, and CAR (Drocourt et al., 2002). As described earlier, inhibitory interaction of PXR and CAR with forkhead transcription factor 1 and hepatic nuclear factor 4α is responsible for regulating homeostasis of carbohydrate and lipid metabolism. As described in earlier sections, both PXR and CAR act as important xenosensors and can be modulated by various xeno- and endo-chemicals. For example, dexamethasone, a glucocorticoid receptor agonist (LaLone et al., 2012), has been reported to induce expression of PXR (Pascussi et al., 2000a) and CAR (Pascussi et al., 2000b) in human hepatocytes, suggesting a functional cross-talk between these nuclear receptors.
1.4 MicroRNA

Approximately 98.5% of genomic DNA in humans does not code for proteins, despite transcribing various types of RNAs (Mattick, 2004). Non-coding RNAs, including ribosomal RNA, transfer RNA, small nuclear RNA, small nucleolar RNA, and micro RNA, do not translate into proteins. Instead, they play an important role in the regulation of genes involved in various biological processes, both physiological and pathophysiological (Yu and Pan, 2012). Among these, microRNAs have been extensively studied for their pivotal role in post-transcriptional regulation of mRNAs. The seminal work of Lewis et al. predicted more than 5300 human genes (~30% of human gene set) as targets for microRNAs (Lewis et al., 2005). A complex network connects microRNAs with their targets mRNAs, which is evident from the fact that a microRNA can regulate several mRNAs, whereas a single mRNA can be a target of several microRNAs (Kim, 2005).

The first microRNA to be discovered was lin-4 from Caenorhabditis elegans in 1993 (Lee et al., 1993). Since then, more than 2588 mature microRNAs have been discovered in humans (www.mirbase.org), that have been predicted to regulate > 60% of genes encoding proteins (Friedman et al., 2009). MicroRNAs are have been shown to play a key function in diverse biological functions, ranging from growth and development, differentiation, apoptosis, proliferation, metabolism, and various human diseases (Bartel, 2004; Kim, 2005). MicroRNAs also regulate the expression of genes involved in metabolism and transport of drugs and other chemicals (Yu, 2009; Yu and Pan, 2012; Ikemura et al., 2014; He et al., 2015). Owing to their diverse regulatory roles, in both normal and disease conditions, microRNAs expression levels have been reported to show temporal changes in a tissue/organ-specific manner (He et al., 2015).
According to the current standard nomenclature system (Ambros et al., 2003; Griffiths-Jones et al., 2006; Ha and Kim, 2014), a microRNA is named following conventions listed below:

- MicroRNA name initiates with a prefix “miR” followed by a dash and a number (an indicator the order of naming). For example, miR-124.
- Mature microRNAs are denoted by capital “R” in “miR” prefix, precursor or primary microRNAs are represented by lowercase “r”.
- Genes encoding microRNAs are denoted as “MIR” (Wright and Bruford, 2011).
- Species of origin for microRNA is identified by a three-letter prefix before “miR”. For example, human miR-124 is named hsa-miR-124 while mouse miR-124 is named mmu-miR-124.
- MicroRNAs with virtually identical sequences, with differences in 1–2 nucleotides, are represented with an additional lowercase letter. For example, hsa-miR-124a and hsa-miR-124b.
- When double-stranded precursor microRNA produces two mature microRNAs in nearly similar amounts, a -3p or -5p suffix is added to denote anti-sense and sense microRNA, respectively. For example, hsa-miR-124a-3p and hsa-miR-124b-5p.

1.4.1 Biogenesis

A mature microRNA is a single-stranded, small, non-coding RNA that usually contains ~22 (18-25) nucleotides (Bartel, 2004; Tetreault and De Guire, 2013). The majority of microRNAs are synthesized via the canonical biogenesis pathway, a multi-step process starting with transcription of primary microRNA transcript from microRNA gene with the help of RNA
polymerase II in the nucleus. The primary microRNA transcript contains stem-loop structures and can be several thousand nucleotides long. Subsequently, primary microRNA is cleaved by Drosha microprocessor complex to form precursor microRNA which is about 70 nucleotides long. The Drosha microprocessor complex contains nuclear ribonuclease III protein Drosha and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8). Next, precursor microRNA is transported out of the nuclear compartment to the cytoplasm with the help of Exportin-5 transporter. Once in the cytoplasmic compartment, precursor microRNA is acted upon by ribonuclease III protein Dicer that cleaves the hairpin of the precursors generating a double-stranded RNA duplex (approximately 22 nucleotides long) that contains mature microRNA and its complimentary sequence strand. The microRNA duplex then unwinds and interacts with Argonaute proteins that retains mature microRNA and incorporates it into RNA-induced silencing complexes that regulates mRNA expression (Bartel, 2004; Kim, 2005; Tetreault and De Guire, 2013).

MicroRNAs may bypass the canonical biogenesis and follow Drosha-independent or Dicer-independent pathways (Miyoshi et al., 2010). Four Drosha-independent microRNA biogenesis pathways have been identified in mammals. Mirtron pathway is the most common non-canonical microRNA biogenesis pathway in which precursor microRNA gets generated from mirtrons (short introns) as a result of splicing, bypassing the Drosha-mediated cleavage. Debranched mirtrons are then exported out of the nucleus and processed following the canonical pathway, as discussed above. Other Drosha-independent pathways include small nucleolar RNA-derived pathway, transfer RNA-derived pathway, and short hairpin RNA-derived pathway (Miyoshi et al., 2010). Although rare, but mature microRNA can also be generated via Argonaute 2 protein-dependent or transfer ribonuclease Z-dependent pathways thereby skipping
Dicer-dependent precursor microRNA cleavage (Miyoshi et al., 2010; Goodall et al., 2013). Fig. 1.11 illustrates various canonical and non-canonical pathways for microRNA biogenesis in various animal species, including mammals, and are described in greater details in a previous report (Miyoshi et al., 2010).
Figure 1.11 Various Canonical and Non-canonical Pathways for MicroRNA Biogenesis (Reprinted with permission from Miyoshi et al., 2010).
1.4.2 Mechanism of Action of MicroRNA

As mentioned above, subsequent to processing by Dicer, microRNA duplex is loaded onto RNA-induced silencing complex. Argonaute protein containing the PAZ and the PIWI structural domains forms the core of RNA-induced silencing complex. The PAZ domain anchors 3′-end of the guide strand of mature microRNA, whereas the PIWI domain holds 5′-end of the guide microRNA strand (Pillai, 2005). Mature microRNA in the RNA-induced silencing complex recognize and usually bind to complimentary sequence in the 3′-untranslated region (3′-UTR) of its target mRNA causing mRNA cleavage or translational repression (Djuranovic et al., 2011). MicroRNA-mediated target mRNA cleavage or translational repression depends on sequence complementarity. Binding of microRNA to its target mRNA does not require absolute sequence complementarity. As few as 6-7 complementary nucleotides between the seed region of microRNA (nucleotide position 2-8) and microRNA recognition element (MRE) in target mRNA is sufficient for RNA-induced silencing complex activity (Tetreault and De Guire, 2013). While sufficient sequence complementarity causes mRNA cleavage, partial complementary base paring between microRNA and target mRNA leads to translational repression (Bartel, 2004). Requirement for partial complementarity for translational repression explains why a microRNA can regulate several mRNAs and a single mRNA can be a target of several microRNAs (Kim, 2005).

1.4.3 Gene Regulation by MicroRNA

To date, the sequences of 2588 mature microRNA have been identified in humans, as listed in the miRBase online database (www.mirbase.org). Various bioinformatics tools such as TargetScan, miRanda, miRIAD, and MicroCosm use this information for predicting interactions
between microRNA and mRNA which is based on sequence complementarity (partial or complete) and binding stability (He et al., 2015). MicroRNAs have been investigated for their potential regulatory role in genes involved in absorption, distribution, metabolism, and excretion of drugs. A recent review associated 120 microRNAs in the regulation of 261 genes involved in drug absorption, distribution, metabolism, and excretion, including various phase I and II drug-metabolizing enzymes, drug transporters, and various nuclear receptors (He et al., 2015).

1.5 Rationale

Rilpivirine and etravirine are second generation NNRTIs that are indicated as part of the HAART regimen for the treatment of HIV-1 infection (De Clercq, 2009; De Clercq, 2012). Combination therapies with both rilpivirine and etravirine, and the first generation NNRTIs (nevirapine, delavirdine and efavirenz) have been reported to cause pharmacokinetic drug interactions (Ma et al., 2005; Brown et al., 2009). Induction of various drug-metabolizing enzymes is a potential reason for NNRTI-mediated drug interactions. Among the first generation NNRTIs, efavirenz has been shown to increase transcriptional expression of CYP3A4 (Hariparsad et al., 2004) and CYP2B6 (Faucette et al., 2007). Similarly, nevirapine has been shown to induce CYP2B6 mRNA in primary human hepatocytes (Faucette et al., 2007). Further, in vitro studies have indicated an inducing effect of NNRTIs on various drug transporters. Efavirenz is reported to induce expression of P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 3, and breast cancer resistance protein; nevirapine and delavirdine have been reported to induce P-glycoprotein; etravirine is reported to induce P-glycoprotein, multidrug resistance-associated protein 3, and breast cancer resistance protein (Weiss et al., 2007; Weiss et al., 2008; Weiss et al., 2009; Weiss and Haefeli, 2010).
The molecular mechanisms responsible for NNRTI-mediated induction of various drug-metabolizing enzymes and transporters have not been elucidated. Transcriptional expression of these enzymes and transporters is regulated by nuclear receptors such as PXR and CAR (Willson and Kliwer, 2002; Klaassen and Slitt, 2005; Timsit and Negishi, 2007; di Masi et al., 2009; Wang et al., 2012). In various in vitro studies, NNRTIs have been studied for their activity on PXR and CAR (Hariparsad et al., 2004; Faucette et al., 2006; Faucette et al., 2007; Yanakakis and Bumpus, 2012). These literature reports indicate potential association of activation of PXR and CAR in NNRTI-mediated effects on drug-metabolizing enzymes. However, these results are either conflicting or inconclusive, and the underlying mechanisms are not known. Furthermore, the scientific literature lack reports on whether rilpivirine and etravirine affect transcriptional activity of PXR or CAR.

As described in earlier sections, PXR is an important nuclear receptor that is involved in diverse biological roles (Willson and Kliwer, 2002; Gao and Xie, 2010; Kodama and Negishi, 2013; Dasgupta et al., 2014). A previous study demonstrated a lack of a correlation between PXR mRNA and PXR protein expression in a panel of 25 human livers, indicating post-transcriptional regulatory mechanism(s) (Takagi et al., 2008). In recent years, microRNAs have gained significant importance in post-transcriptional regulation of various genes involved in drug metabolism and transport processes (Yu, 2009; Yu and Pan, 2012; Ikemura et al., 2014; He et al., 2015). PXR has been shown to be regulated by microRNA-148a (Takagi et al., 2008). Another microRNA that has been suggested to indirectly regulate PXR include microRNA-34a which regulates expression and function of RXRα, a heterodimeric partner of PXR (Oda et al., 2014). Correlation analysis done by Lamba et al. also suggest that microRNA-34a may be involved in PXR (Lamba et al., 2014). Using the online bioinformatics database
www.microRNA.org (Betel et al., 2008), we identified microRNA-18a as another microRNA that has sequence complementarity in the 3’-UTR region of PXR. Previously, microRNA-18a has been reported to regulate glucocorticoid receptor (Vreugdenhil et al., 2009), a nuclear hormone receptor that has been reported to functionally interact with PXR and induce CYP3A4 (Pascussi et al., 2000a). It is unknown whether microRNA-18a is involved in regulation of PXR expression and activation by PXR agonists.

1.6 Hypothesis and Research Objectives

The research hypothesis is that NNRTIs activate PXR and CAR and induce specific drug-metabolizing enzymes by a mechanism involving microRNA.

The research objectives are as follows:

- To characterize the effect of NNRTIs on human PXR (Chapter 2) and human CAR and its splice variants hCAR-SV23 and hCAR-SV24 (Chapter 3)
- To investigate the molecular mechanism of human PXR and CAR activation by NNRTI (Chapter 2 and Chapter 3)
- To characterize the role of microRNA-18a in regulation of PXR expression and function (Chapter 4)
- To investigate the effect of microRNA-18a on activation of human PXR by rilpivirine and rifampin (Chapter 4)
Chapter 2: Agonism of Human Pregnane X Receptor by Rilpivirine and Etravirine: Comparison with First Generation Non-nucleoside Reverse Transcriptase Inhibitors

2.1 Introduction

Human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) still remains one of the leading causes of mortality in humans, claiming an estimated 1.8 million lives annually (Flexner, 2007). Unfortunately, a cure for this disease has not been found yet; however, the current therapeutic options are able to suppress viral load and increase Cluster of Differentiation 4 (CD4) counts in HIV/AIDS patients (Arts and Hazuda, 2012; Deeks et al., 2013). An important class of drugs that is routinely used for the treatment of HIV-1 infection, along with other anti-retroviral drugs, is the non-nucleoside reverse transcriptase inhibitors (Jayaweera and Dilanchian, 2012). Efavirenz, nevirapine, and delavirdine (Fig. 1.1) are first generation non-nucleoside reverse transcriptase inhibitors approved for clinical use by the United States Food and Drug Administration. The most recent additions to this drug class are rilpivirine and etravirine (Fig. 1.1), which were approved for clinical use by the United States Food and Drug Administration in May 2011 (De Clercq, 2012) and January 2008 (De Clercq, 2009), respectively. Rilpivirine and etravirine, like other non-nucleoside reverse transcriptase inhibitors, inhibit HIV-1 reverse transcriptase enzyme allosterically (De Clercq, 2009). They are potent second generation non-nucleoside reverse transcriptase inhibitors used in combination with other anti-retrovirals. Recently, the United States Food and Drug Administration approved
a rilpivirine-based triple drug combination with the brand names of Complera® (containing 25 mg rilpivirine, 300 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the U.S.A.) and Eviplera® (containing 25 mg rilpivirine, 245 mg tenofovir disoproxil, and 200 mg emtricitabine; available in the European Union) for treatment-naive HIV-1 infection (De Clercq, 2012). On the other hand, etravirine is reported to be effective in both non-nucleoside reverse transcriptase inhibitor-naive and non-nucleoside reverse transcriptase inhibitor-resistant strains of HIV-1, and is used in combination with other anti-retrovirals (Croxtall, 2012).

Nuclear receptors represent a broad group of ligand-activated transcriptional factors and are categorized as endocrine receptors, adopted orphan receptors, and orphan receptors (Sonoda et al., 2008). They play a key regulatory role in various biological processes, ranging from development and differentiation to metabolic homeostasis. Pregnane X receptor (PXR; designated as NR1I2 according to the nomenclature of nuclear receptors) is a member of the superfamily of nuclear receptors (Germain et al., 2006). It acts as xenobiotic sensors and regulates expression of genes involved in drug transport, such as \(ABCB1\) (P-glycoprotein) (Geick et al., 2001) and \(ABCC2\) (MRP2) (Kast et al., 2002), and those involved in drug metabolism, such as \(CYP3A4\) (Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998). Other than playing a major role in many of the pharmacokinetic drug interactions (Wang et al., 2012), PXR has been linked to the development of bile acid toxicity (Staudinger et al., 2001), drug-induced hepatotoxicity (Guo et al., 2004), and anticancer drug resistance (Chen et al., 2012b). It has also been proposed as a therapeutic target for various disease states, including cholestatic liver disease (Kakizaki et al., 2011), inflammatory bowel disease (Shah et al., 2007), and dyslipidemia (Gao and Xie, 2012).
Among the first generation non-nucleoside reverse transcriptase inhibitors, efavirenz has been reported to activate human PXR (hPXR) (Hariparsad et al., 2004; Faucette et al., 2007; Svard et al., 2010). By comparison, nevirapine only weakly activates hPXR, and this occurs not at a therapeutic concentration (7.5 µM) (Svard et al., 2010), but at a suprapharmacological concentration (50 µM) (Faucette et al., 2007). However, the mechanism on how these drugs activate hPXR is still unknown. In a recent study, treatment of primary cultures of human hepatocytes with etravirine (10 µM) increased CYP3A4 mRNA expression (Yanakakis and Bumpus, 2012). This increase in CYP3A4 mRNA expression by etravirine was attenuated by pretreatment with sulforaphane (Yanakakis and Bumpus, 2012), which is an in vitro antagonist of hPXR (Zhou et al., 2007). However, there is no direct experimental evidence as to whether the second generation non-nucleoside reverse transcriptase inhibitors (i.e. rilpivirine and etravirine) affect the transcriptional activity of PXR or how they may activate hPXR.

In the present study, we systematically evaluated the effects of rilpivirine and etravirine on the activity of hPXR, including their mode of activation, and compared them to those of first generation non-nucleoside reverse transcriptase inhibitors; i.e., efavirenz, nevirapine, and delavirdine. Given that PXR is known to show pronounced species-dependent activation by drugs and other chemicals (Jones et al., 2000), we determined whether rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors activate mouse PXR (mPXR) and rat PXR (rPXR). The experimental approaches involved cell-based luciferase reporter gene assays, competitive ligand binding assay by time-resolved fluorescence resonance energy transfer (TR-FRET), in vitro nuclear translocation analysis by confocal microscopy, mammalian two-hybrid assay, and hPXR target gene (CYP3A4) expression analysis in human hepatocytes in culture.
The results are discussed in the context of drug-dependent activation of human and rodent PXR by select non-nucleoside reverse transcriptase inhibitors and their mode of activation.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Dextran, Triton X-100 (Union Carbide Corporation, Houston, TX, U.S.A.), rifampin, pregnenolone 16α-carbonitrile (PCN), 4-pregnane-20β-ol-3-one, sodium phenobarbital, and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, MO, U.S.A.). Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl) ethenyl-1,1-bisphosphonate (SR12813) was obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, U.S.A.). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids (Newport, RI, U.S.A.). Cytotoxicity Detection Kit (LDH) and FuGENE 6 transfection reagent were purchased from Roche Diagnostics (Laval, QC, Canada). Matrigel basement membrane matrix was obtained from BD Biosciences (Mississauga, ON, Canada). Hanks’ balanced salt solution (HBSS), PureLink RNA Mini Kit, PicoGreen Double-Stranded DNA Quantification Kit, LanthaScreen TR-FRET PXR Competitive Binding Assay, and ProLong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were procured from Life Technologies (Burlington, ON, Canada), and the Dual-Luciferase Reporter Assay System was from Promega (Madison, WI, U.S.A.). Charcoal-stripped fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.) was bought from Thermo Fisher Scientific (Nepean, ON, Canada), and all other reagents to culture HepG2 cells were obtained
from Life Technologies (Burlington, ON, Canada). Cryopreserved hepatocyte thawing medium, hepatocyte plating medium, hepatocyte maintenance medium, and various medium supplements were provided by Triangle Research Labs, LLC (Research Triangle Park, NC, U.S.A.).

2.2.2 Plasmids

pCMV6-XL4-hPXR, pCMV6-entry-mPXR, pCMV6-AC-rPXR, pCMV6-AC-GFP-hPXR, pCMV6-XL4, pCMV6-AC, and pCMV6-entry were obtained from OriGene Technologies (Rockville, MD, U.S.A.). pFR-luc reporter were purchased from Agilent Technologies (Santa Clara, CA, U.S.A.). The internal control Renilla reniformis luciferase pGL4.74 [hRluc/TK] plasmid was procured from Promega (Madison, WI, U.S.A.). The pGL3-basic-CYP3A4-XREM-luc reporter construct was prepared according to a published method (Goodwin et al., 1999). pVP16-hPXR-LBD, pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID were constructed as detailed previously (Lau et al., 2010; Lau et al., 2011a). The pVP16 and pM empty vectors were purchased from Clontech (Mountain View, CA, U.S.A.). The plasmid constructs were sequenced by the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC, Canada) and their sequence identity was confirmed by comparing with published sequence.

2.2.3 HepG2 Cells

HepG2 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in T-75 culture flasks in minimum essential medium supplemented with heat-inactivated fetal bovine serum (10% v/v), penicillin G (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2
mM. Cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂. Culture medium was changed every 3 days, and cells were sub-cultured weekly.

2.2.4 Determination of Cytotoxicity of Non-nucleoside Reverse Transcriptase Inhibitors

Cultured HepG2 cells were plated in 24-well plates at a density of 100,000 cells/well. At 48 h post-plating, the cells were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 0.1-25 µM), dextran (1% w/v; negative control), or Triton X-100 (0.1% v/v; positive control) in a volume of 0.5 ml culture medium. At the end of the 24 h treatment period, culture supernatant was collected and cells were lysed in 0.5 ml lysis buffer containing 2% v/v Triton X-100 and 20 mM EDTA in phosphate-buffered saline (pH 7.4). LDH levels were quantified in the culture supernatant and cell lysate using the Roche Cytotoxicity Detection Kit (LDH), as described earlier (Lau et al., 2010). Results are expressed as the amount of LDH in the culture supernatant as a percentage of the total cellular LDH; i.e., sum of LDH in the culture medium and cell lysate. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

2.2.5 Transient Transfection and Reporter Gene Assays

To investigate the effect of rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors on the activity of hPXR, mPXR, and rPXR, HepG2 cells were plated onto 24-well microplates at a density of 100,000 cells/well in a volume of 0.5 ml of culture medium. At 24 h post-plating, cultured cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), serum-free Opti-MEM (20 µl/well), pGL4.74 [hRluc/TK]
internal control vector (5 ng/well), a reporter construct (50 ng/well), and either a receptor expression plasmid or the corresponding empty vector (50 ng/well) for 24 h, as detailed in the appropriate figure legend. Transfected cells were treated with 0.5 ml of supplemented culture medium containing DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), a negative control, or a positive control at the concentrations indicated in the appropriate figure legend. At the end of the 24 h treatment period, HepG2 cells were lysed. Firefly luciferase and Renilla reniformis luciferase activities were quantified using a Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured in a GloMax 96 microplate luminometer (Promega). Normalized luciferase activity was determined by calculating the ratio of Firefly luciferase activity and R. reniformis luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

2.2.6 hPXR-LBD Transactivation Assay

The hPXR-LBD transactivation assay was performed as described previously (Lau et al., 2012). Briefly, cultured HepG2 cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), serum-free Opti-MEM (20 µl/well), pGL4.74 [hRluc/TK] internal control vector (5 ng/well), pFR-luc reporter (100 ng/well), and either the pM-hPXR-LBD (Met-107 to Ser-434) expression plasmid or the pM empty vector (100 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented culture medium containing DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine,
etavirine, efavirenz, nevirapine, or delavirdine; 5 µM), PCN (10 µM; negative control for hPXR (Jones et al., 2000)), rifampin (10 µM; positive control for hPXR (Jones et al., 2000)), or SR12813 (10 µM; positive control for hPXR (Jones et al., 2000)) for 24 h. Firefly luciferase and *Renilla reniformis* luciferase activities were quantified as described above. Each experiment was performed in triplicate and a total of five independent experiments were conducted.

### 2.2.7 Competitive Ligand-binding Assay

A LanthaScreen hPXR competitive binding assay based on the principle of time-resolved fluorescence resonance energy transfer (TR-FRET) was conducted as described previously (Lau et al., 2012). A non-nucleoside reverse transcriptase inhibitor (rilpivirine, etavirine, efavirenz, nevirapine, or delavirdine; 5 µM), PCN (10 µM; negative control), phenobarbital (1000 µM; positive control), SR12813 (10 µM; positive control), or DMSO (1% v/v; vehicle control) was incubated with hPXR ligand-binding domain (5 nM), Fluormone PXR Green (40 nM), terbium-labeled anti-glutathione transferase (10 nM), and dithiothreitol (0.05 mM) at ambient temperature for 1 h in the dark. TR-FRET was quantified using a PHERAstar FS microplate reader (BMG Labtech GmbH, Allmendgruen, Germany) with the following settings: excitation wavelength, 337 nm; emission wavelengths 490 nm (terbium emission) and 520 nm (fluorescein emission); delay time, 100 µs; and integration time, 200 µs. TR-FRET ratio, background TR-FRET ratio, and net TR-FRET ratio were determined as detailed previously (Lau et al., 2012). Data are expressed as percentage of the net TR-FRET ratio in the DMSO-treated control groups. Each experiment was performed in triplicate and a total of three independent experiments were conducted.
2.2.8 *In Vitro* Nuclear Translocation of Green Fluorescence Protein Tagged hPXR

HepG2 cells were cultured on poly-d-lysine-coated glass cover slips in 24-well plate at a density of 25,000 cells/well. At 24 h post-plating, cultured HepG2 cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), serum-free Opti-MEM (20 µl/well), and pCMV6-AC-GFP-hPXR (green fluorescence protein-tagged hPXR; 50 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented medium containing DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 µM), PCN (10 µM; negative control for hPXR) (Jones *et al.*, 2000), or rifampin (10 µM; positive control for hPXR (Jones *et al.*, 2000)) for 24 h. Subsequently, the cells were fixed with *p*-formaldehyde (4% v/v) for 20 min on ice. After three washes in phosphate-buffered saline (pH 7.4), the cover slips containing cells were mounted on glass slides using ProLong® Gold Antifade Reagent with DAPI. The cells were visualized under an Olympus FV10i confocal microscope interfaced with the FluoView 10i software (FV10i version 1.2c) and analyzed using the NIH, ImageJ software (National Institutes of Health, U.S.A.).

2.2.9 Mammalian Two-hybrid Assay

Recruitment of steroid receptor coactivator 1 (SRC 1), SRC2, and SRC3 to hPXR was assessed by a mammalian two-hybrid assay (Lau *et al.*, 2011a). Briefly, at 24 h post-plating, cultured HepG2 cells were transfected with pGL4.74 [hRluc/TK] internal control vector (10 ng/well), pFR-luc reporter plasmid (100 ng/well), a coactivator expression plasmid (10 ng/well), and either pVP16-hPXR-LBD expression plasmid or the corresponding pVP16 empty vector (40 ng/well). The coactivator expression plasmids were pM-hSRC1-RID, pM-hSRC2-RID, and pM-
hSRC3-RID. At 24 h post-transfection, cells were treated with supplemented culture medium containing DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 µM), PCN (10 µM; negative control for hPXR), rifampin (10 µM; positive control for hPXR), or SR12813 (10 µM; positive control for hPXR) for 24 h. Luciferase activity was measured and normalized as described under Section 2.2.5. Each experiment was performed in triplicate and a total of five independent experiments were conducted.

2.2.10 Culture and Treatment of Human Hepatocytes

The demographics of the donors (GC4008 and HUM4021) are listed in Table 2.1. Cryopreserved human hepatocytes (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) were thawed and plated according to protocols listed at [http://triangleresearchlabs.net/products/cryopreserved-hepatocytes/](http://triangleresearchlabs.net/products/cryopreserved-hepatocytes/). Cell viability was 88% and 90% for hepatocyte samples GC4008 and HUM4021, respectively, as assessed by trypan blue exclusion (Jauregui et al., 1981). Hepatocytes were plated and cultured at a density of $3.5 \times 10^5$ cells per well in a BioCoat 24-well plate at 37°C in a humidified incubator with 95% air and 5% CO$_2$. They were allowed to attach for 4 to 6 h. Subsequently, the medium was aspirated and 0.5 ml of fresh hepatocyte maintenance medium (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) and Matrigel (0.25 mg/ml) were added to each well. Plates were placed in the incubator, and this was followed by a change in the culture medium on Day 2 and drug treatment was initiated on Day 3. Cultured hepatocytes were treated with hepatocyte maintenance medium containing DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor
<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Race</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Reported BMI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Smoking Status</th>
<th>Alcohol Use</th>
<th>Drug Use</th>
<th>Serological Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC4008</td>
<td>Caucasian</td>
<td>Male</td>
<td>69</td>
<td>24.7</td>
<td>Stopped 24 years ago</td>
<td>No</td>
<td>No</td>
<td>All Negative</td>
</tr>
<tr>
<td>HUM4021</td>
<td>Caucasian</td>
<td>Male</td>
<td>13</td>
<td>18.0</td>
<td>Non-smoker</td>
<td>No</td>
<td>No</td>
<td>All negative</td>
</tr>
</tbody>
</table>

<sup>a</sup>Body Mass Index
(rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; 5 µM), or rifampin (10 µM; positive control for CYP3A4 expression (Pichard et al., 1990)), as described in the figure legend. The drug-containing culture medium was replaced every 24 h and the treatment was continued for 48 h for hepatocyte sample GC4008 and 72 h for hepatocyte sample HUM4021.

2.2.11 Isolation of Total RNA, Reverse Transcription, and Real-time PCR Analysis

RNA isolation and reverse transcription were conducted as described previously (Lau et al., 2010). Briefly, cultured human hepatocytes were lysed and total cellular RNA was isolated using PureLink RNA Mini Kit (Life Technologies, Burlington, ON, Canada). Total RNA was reverse-transcribed using Superscript II reverse transcriptase, and total cDNA was quantified using PicoGreen Double-Stranded DNA Quantitation Kit (Life Technologies, Burlington, ON, Canada).

The sequences of the primers used to amplify CYP3A4 cDNA (Schuetz et al., 1996) and HPRT cDNA (Qiu et al., 2007) were specified in a previous publication (Lau et al., 2010). The primers were synthesized and their specificity was verified by sequencing the purified amplicons (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.).

CYP3A4 cDNA and HPRT1 cDNA were amplified by real-time PCR in a LightCycler (Roche Diagnostics, Laval, QC, Canada) according to conditions described previously (Lau et al., 2010). To construct calibration curves (cross point versus log cDNA copies), CYP3A4, and HPRT amplicons were generated from human liver QUICK-Clone cDNA (Clontech, Mountain View, CA, U.S.A.) and then purified and quantified as described previously (Chang et al., 2006). CYP3A4 mRNA expression was normalized to that of a housekeeping gene (hypoxanthine-guanine phosphoribosyltransferase, HPRT).
2.2.12 Testosterone 6β-Hydroxylation Assay

Testosterone 6β-hydroxylation assay was conducted in cultured human hepatocytes as described previously (Lau et al., 2010). Briefly, cultured human hepatocytes were first washed twice with HBSS and subsequently incubated for 15 min at 37°C with testosterone (200 µM final concentration), a concentration that has been previously shown to be closer to maximum rate of 6β-hydroxytestosterone metabolite formation (Wang et al., 1997). Supernatant from the incubation samples were collected and transferred to a 24-well plate for storage at -80°C until analysis. High-performance liquid chromatographic (HPLC) analyses of 6β-hydroxytestosterone was performed as described previously (Chang et al., 2006), but with several modifications. In the present study, 4-pregnene-20β-ol-3-one (in acetonitrile) was selected as the internal standard based on its structural similarity to the analyte (6β-hydroxytestosterone), and 10 µl of the internal standard (2000 pmol) was added to 150 µl of the hepatocytes incubation sample. A 50 µl aliquot of that sample was injected onto the HPLC. The detector response of the internal standard was used to normalize the detector response of 6β-hydroxytestosterone. The HPLC system (Waters, Milford, MA, U.S.A.) was equipped with a Waters model 1525 binary pump, Waters model 717 plus autosampler, and Waters model 2487 dual wavelength absorbance detector. The chromatographic separation was carried out on a Waters SymmetryShield C18 column (150 × 4.6 mm; i.d., 5 µm) linked to a Phenomenex (Torrance, CA, U.S.A.) SecurityGuard cartridge (4.0 x 3.0 mm; i.d., 5 µm). The mobile phases consisted of water containing 0.5% v/v acetic acid (A) and methanol containing 0.5% v/v acetic acid (B), and the optimized flow rate was 0.8 ml/min. A gradient method was used to separate 6β-hydroxytestosterone from the internal standard and the matrix components with the following solvent conditions: linear gradient from
55 to 90% B (0-12 min), isocratic at 90% B (12-15 min), linear gradient from 90 to 55% B (15-16 min), and isocratic at 55% B (16-25 min). The detector response for 6β-hydroxytestosterone and internal standard was recorded at a wavelength of 242 nm. Data acquisition and processing were conducted using the Waters Breeze software (version 3.20). A calibration curve was constructed with the authentic 6β-hydroxytestosterone standard (100-12,500 pmol; diluted in HBSS) for each experiment.

2.2.13 Data Analyses

The half-maximal effective concentration (EC_{50}) and maximal response (E_{max}) for hPXR activation were calculated using the following equation in GraphPad Prism® 5.00 (GraphPad Software, Inc., La Jolla, CA, U.S.A.): 

\[ E = E_0 + \frac{(E_{max} - E_0)}{1 + 10^{\frac{\log EC_{50} - C}{10}}} \]

where \( E \) is effect (i.e. fold increase over vehicle-treated control group), \( E_0 \) is basal effect, and \( C \) is drug concentration. Data analyses were performed by one-way or two-way analysis of variance. Where appropriate, two-way analysis of variance was followed by the Student Newman-Keuls multiple comparison test (SigmaPlot 11.0; Systat Software, Inc., San Jose, CA, U.S.A.). The level of statistical significance was set a priori at \( P < 0.05 \).

2.3 Results

2.3.1 Cytotoxicity Assessment of Rilpivirine, Etravirine, and Other Non-nucleoside Reverse Transcriptase Inhibitors

To determine non-cytotoxic concentrations of the selected non-nucleoside reverse transcriptase inhibitors for investigation in the subsequent \textit{in vitro} cell-based reporter gene
assays, release of intracellular LDH into the culture medium was employed as a cytotoxicity marker (Jauregui et al., 1981). LDH release was quantified in cultured HepG2 cells treated with increasing concentrations (0.1-25 µM) of rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine. LDH release did not increase at drug concentrations of up to 10 µM ($P > 0.05$) for rilpivirine (Fig. 2.1A), etravirine (Fig. 2.1B), efavirenz (Fig. 2.1C), nevirapine (Fig. 2.1D), or delavirdine (Fig. 2.1E). Expected results were obtained for the positive control (0.1% v/v Triton X-100; Fig. 2.1A-E) ($P < 0.001$) and the negative control (1% w/v dextran; Fig. 2.1A-E) ($P > 0.05$). In all subsequent experiments, the concentration of the non-nucleoside reverse transcriptase inhibitors did not exceed 10 µM.

2.3.2 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Activate hPXR

The effect of rilpivirine and etravirine on the activity of hPXR was assessed in cell-based reporter gene assays and compared with an equimolar concentration (5 µM) of efavirenz, nevirapine, and delavirdine in hPXR-transfected HepG2 cells. As shown in Fig. 2.2A, rilpivirine and etravirine increased hPXR activity by 11- ($P < 0.001$) and 17-fold ($P < 0.001$), respectively, over the vehicle-treated control group. Among the other non-nucleoside reverse transcriptase inhibitors tested, efavirenz (7-fold) ($P < 0.001$), but not nevirapine ($P < 0.771$) or delavirdine ($P < 0.148$), activated hPXR. Rifampin (10 µM), a positive control for hPXR activation (Lehmann et al., 1998), produced a 20-fold increase over the vehicle-treated control group ($P < 0.001$), whereas PCN (10 µM), a negative control (Jones et al., 2000), did not have an effect ($P < 0.804$).
Concentration-response Relationship in the Effect of Non-nucleoside Reverse Transcriptase Inhibitors on hPXR Activity

Concentration-response experiments were performed to further characterize the activation of hPXR by rilpivirine, etravirine, and efavirenz. As shown in Fig. 2.2B, rilpivirine, at concentrations of 0.05 and 0.1 µM, had no effect on the activity of hPXR, whereas it activated hPXR at greater concentrations (0.5-10 µM) \( (P < 0.001) \), with a maximal response of 11-fold produced at 5 µM \( (P < 0.001) \). Etravirine had a profile similar to rilpivirine as 0.05 and 0.1 µM had no effect on hPXR activity, whereas greater concentrations (0.5-10 µM) of this drug activated hPXR \( (P < 0.001) \), with maximal response (17-fold) produced at 5 µM \( (P < 0.001) \). Efavirenz at a concentration of 0.5 or 1 µM had no effect on hPXR activity, whereas at 5 and 10 µM, it increased the activity by 7- \( (P < 0.001) \) and 10-fold \( (P < 0.001) \), respectively. In contrast to rilpivirine, etravirine and efavirenz, neither nevirapine \( (P < 0.872) \) nor delavirdine \( (P < 0.651) \) activated hPXR at concentrations up to and including 10 µM (Fig. 3B). Given that rilpivirine and etravirine produced maximal hPXR activation at 5 µM, all subsequent experiments were conducted at this concentration. Also, efavirenz, nevirapine, and delavirdine were used at the same concentration to have an equimolar comparison.

The calculated \( EC_{50} \) and \( E_{\text{max}} \) values (mean ± S.E.M.) for rilpivirine-mediated hPXR activation were 0.4 ± 0.2 µM and 11 ± 1 fold, respectively, whereas those for hPXR activation by etravirine were 0.6 ± 0.1 µM and 17 ± 1 fold, respectively. These values are comparable with the literature \( EC_{50} \) and \( E_{\text{max}} \) values for hPXR activation by rifampin (Chang, 2009), which is a prototypic hPXR agonist (Lehmann et al., 1998). It was not possible to calculate the \( EC_{50} \) and \( E_{\text{max}} \) values for efavirenz because maximal effect was not achieved in the dose-response experiment (Fig. 2.2B).
Figure 2.1 Effect of Non-nucleoside Reverse Transcriptase Inhibitors on LDH Release in HepG2 Cells.
Cultured cells were treated with varying concentrations (0.1–25 μM) of (A) rilpivirine, (B) etravirine, (C) efavirenz, (D) nevirapine, or (E) delavirdine for 24 h. The control treatments were DMSO (0.1%, v/v; vehicle for the non-nucleoside reverse transcriptase inhibitors), culture medium (vehicle for dextran and Triton-X), dextran (1%, w/v; negative control), and Triton X-100 (0.1%, v/v; positive control). LDH levels were measured in the culture medium and cell lysates, and the amount of LDH in the culture medium is expressed as a percentage of the total cellular LDH. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M percentage of total LDH content for three independent experiments. *, significantly different from the control group treated with DMSO ($P < 0.05$).
2.3.4 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Activate mPXR and rPXR

Pronounced species-dependent chemical activation of PXR has been reported (Jones et al., 2000). Therefore, we determined the effect of the non-nucleoside reverse transcriptase inhibitors on the activity of mPXR and rPXR. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated mPXR (Fig. 2.3A) and rPXR (Fig. 2.3B) \((P < 0.001)\). This pattern of response is similar to the profile shown for hPXR (Fig. 2.2A). PCN (10 µM), which is a positive control for ligand activation of mPXR and rPXR (Moore et al., 2000), activated both mPXR \((P < 0.001)\) and rPXR \((P < 0.001)\), whereas rifampin (10 µM) \((P < 0.080)\), which is a negative control (Moore et al., 2000), had no effect (Fig. 2.3A and 2.3B).

2.3.5 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Transactivate the Ligand-binding Domain of hPXR

To investigate whether rilpivirine, etravirine, and efavirenz act as agonists of hPXR, a series of mechanistic experiments were performed, starting with a cell-based reporter gene assay whereby HepG2 cells were transfected with a plasmid (i.e. pM-hPXR-LBD; Met-107 to Ser-434) expressing the ligand-binding domain of hPXR. As shown in Fig. 2.4A, rilpivirine, etravirine, and efavirenz produced a 4.8- \((P < 0.001)\), 6.4- \((P < 0.001)\), and 3.5-fold \((P < 0.001)\) increase in the luciferase activity, respectively. As expected, nevirapine \((P < 0.961)\) and delavirdine \((P < 0.737)\) had no effect on the transactivation of hPXR-LBD, consistent with data shown in a reporter gene assay using the full-length hPXR (Fig. 2.2A and 2.2B). The positive controls (10 µM rifampin and 10 µM SR12813) \((P < 0.001)\) and the negative control (10 µM) \((P < 0.859)\) produced the expected results (Fig. 2.4A).
Figure 2.2 Comparative Effect of Rilpivirine, Etravirine, Efavirenz, Nevirapine, and Delavirdine on the Activity of hPXR.

Cultured HepG2 cells were transfected with pGL3-basic-CYP3A4-XREM-luc, pGL4.74\([hRluc/TK]\), and either pCMV6-XL4-hPXR or pCMV6-XL4 (empty vector) for 24 h. (A) For the single concentration experiment, transfected cells were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), PCN (10 µM; negative control), or rifampin (10 µM; positive control) for 24 h. (B) Concentration-response curves for hPXR activation by rilpivirine, etravirine, and other non-nucleoside reverse transcriptase inhibitors were generated by treating transfected cells with DMSO (0.1% v/v; vehicle control) or a non-nucleoside reverse transcriptase inhibitor at 0.05-10 µM for 24 h. Firefly luciferase and \(R.\ reniformis\) luciferase activities were measured and normalized by calculating the ratio of Firefly luciferase activity and \(R.\ reniformis\) luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for three independent experiments. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the hPXR expression plasmid \(P < 0.05\).
Figure 2.3 Activation of mPXR and rPXR by Rilpivirine, Etravirine, and Efavirenz.

Cultured HepG2 cells were transfected with (A) pGL3-basic-CYP3A4-XREM-luc, pGL4.74 [hRLuc/TK], and either pCMV6-entry-mPXR, or pCMV6-entry (empty vector); or (B) pGL3-basic-CYP3A4-XREM-luc, pGL4.74 [hRLuc/TK], and either pCMV6-AC-rPXR or pCMV6-AC (empty vector) for 24 h. Transfected cells were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), rifampin (10 µM; negative control), or PCN (10 µM; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of Firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for three independent experiments. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the mPXR or rPXR expression plasmid ($P < 0.05$).
Figure 2.4 (A) Effect of Rilpivirine, Etravirine, Efavirenz, Nevirapine, and Delavirdine on Transactivation of the Ligand-binding Domain of hPXR. (B) TR-FRET Analysis of the Binding of Rilpivirine, Etravirine, and Other Non-nucleoside Reverse Transcriptase Inhibitors to the Ligand-binding Domain of hPXR.

(A) Cultured HepG2 cells were transfected with pGL4.74 [hRluc/TK], pFR-luc, and either pM-hPXR-LBD or the pM empty vector for 24 h. Transfected cells were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), PCN (10 µM; negative control), rifampin (10 µM; positive control), or SR12813 (10 µM; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of Firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for five independent experiments. *, significantly different from the same treatment group transfected with the pM empty vector and from the vehicle-treated control group transfected with pM-hPXR-LBD (P < 0.05). (B) A non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), PCN (10 µM; negative control), phenobarbital (1000 µM; positive control), SR12813 (10 µM; positive control), or DMSO (0.1% v/v; vehicle control) was incubated with human PXR ligand-binding domain (5 nM), Fluormone PXR Green (40 nM), terbium-labeled anti-glutathione transferase (10 nM), and dithiothreitol (0.05 mM). Net TR-FRET ratio was determined by subtracting the background TR-FRET ratio from the TR-FRET ratio. Results are expressed as a percentage of net TR-FRET ratio in the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for five independent experiments. *, significantly different from the vehicle-treated control group (P < 0.05).
2.3.6 TR-FRET Analyses Indicate Binding of Rilpivirine, Etravirine, and Efavirenz to the Ligand-binding Domain of hPXR

To corroborate the hPXR-LBD transactivation data (Fig. 2.4A), a TR-FRET competitive ligand-binding assay was conducted. As shown in Fig. 2.4B, rilpivirine, etravirine, and efavirenz (each at 5 µM) decreased the net TR-FRET emission ratio to 48% ($P < 0.001$), 58% ($P < 0.001$), and 72% ($P = 0.006$) of the control level, respectively, whereas nevirapine ($P = 0.877$) and delavirdine ($P = 0.747$) had no effect. Analyses with positive controls indicate that phenobarbital (1000 µM) produced an effect (61% of the control level) ($P < 0.001$) comparable to that of rilpivirine, etravirine, and efavirenz, whereas SR12813 (10 µM) was the most effective (< 1% of the control level) ($P < 0.001$). The negative control (10 µM PCN) yielded the expected result ($P < 0.114$).

2.3.7 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Stimulate Nuclear Translocation of GFP-tagged hPXR

In its inactive form, hPXR resides mainly in the cytoplasmic compartment of the cells, and upon activation by an agonist, the agonist-activated receptor complex translocates from the cytoplasm into the nucleus (Kawana et al., 2003). According to confocal microscopy (Fig. 2.5), the GFP-tagged hPXR translocated to the nucleus in HepG2 cells treated with rilpivirine, etravirine, or efavirenz, as evident by the predominant localization of green fluorescence in the nucleus. A similar finding was obtained in cells treated with the positive control rifampin, in agreement with published data (Lichti-Kaiser et al., 2009). In contrast, the fluorescence was predominantly in the cytoplasm in GFP-hPXR-transfected cells treated with nevirapine, delavirdine, or PCN (negative control).
Figure 2.5 Localization of GFP-tagged hPXR in HepG2 Cells Treated with Rilpivirine, Etravirine, or a First Generation Non-nucleoside Reverse Transcriptase Inhibitor. Cultured HepG2 cells were transfected with pCMV6-AC-GFP-hPXR for 24 h and treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), PCN (10 µM; negative control), or rifampin (10 µM; positive control) for another 24 h. Subsequently, the cells were fixed with p-formaldehyde (4% v/v) and mounted on glass slides using ProLong® Gold Antifade Reagent with DAPI for confocal microscopy. Shown are representative photomicrographs illustrating the localization of GFP-tagged hPXR, DAPI-stained nuclei, and merged images for each treatment group.
Cultured HepG2 cells were transfected with pGL4.74 [hRluc/TK], pFR-luc, a coactivator expression plasmid, and either pVP16-hPXR-LBD or pVP16 (empty vector) for 24 h. The coactivator plasmids were (A) pM-hSRC1-RID, (B) pM-hSRC2-RID, and (C) pM-hSRC3-RID. Transfected cells were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), PCN (10 µM; negative control), rifampin (10 µM; positive control), or SR12813 (10 µM; positive control) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized by calculating the ratio of Firefly luciferase activity and *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with the corresponding empty vector. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for five independent experiments. *, significantly different from the same treatment group transfected with corresponding empty vector and from the vehicle-treated control cells transfected with pVP16-hPXR-LBD expression plasmid (P < 0.05).
2.3.8 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Promote Recruitment of Coactivators to hPXR

To assess whether rilpivirine, etravirine, and efavirenz recruit coactivators to hPXR, mammalian two-hybrid assays were conducted. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, increased the luciferase reporter activity in HepG2 cells transfected with pVP16-hPXR-LBD and a coactivator expression plasmid for SRC1 (Fig. 2.6A) \( (P < 0.001) \), SRC2 (Fig. 2.6B) \( (P < 0.001) \), or SRC3 (Fig. 2.6C) \( (P < 0.001) \). Experiments with the positive controls (10 µM rifampin and 10 µM SR12813) \( (P < 0.001) \) and negative control (10 µM PCN) yielded the expected results (Fig. 2.6A-7C).

2.3.9 Rilpivirine, Etravirine, and Efavirenz, but not Nevirapine or Delavirdine, Increase the Expression of a hPXR Target Gene (CYP3A4) in Cultured Human Hepatocytes

Primary cultures of human hepatocytes were used to compare the effect of first and second generation non-nucleoside reverse transcriptase inhibitors on the induction of CYP3A4, which is a known target gene of hPXR (Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998). In sample GC4008 (Fig. 2.7A), rilpivirine, etravirine, and efavirenz increased CYP3A4 mRNA expression by 5.7-, 4.3-, and 1.8-fold, respectively, whereas nevirapine and delavirdine had no effect. A similar trend was observed in sample HUM4021 where rilpivirine, etravirine, and efavirenz increased CYP3A4 mRNA expression by 3.1-, 6.3-, and 1.7-fold, respectively, and nevirapine and delavirdine had no effect (Fig. 2.7B). By comparison, rifampin (10 µM; positive control) increased the expression of CYP3A4 mRNA by 3.8-fold in sample
Figure 2.7 Induction of CYP3A4 in Human Hepatocytes Treated with Rilpivirine, Etravirine, or a First Generation Non-nucleoside Reverse Transcriptase Inhibitor.
Cultured hepatocytes were treated with DMSO (0.1% v/v; vehicle control), a non-nucleoside reverse transcriptase inhibitor (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine; each at 5 µM), or rifampin (10 µM; positive control) for (A and C) 48 or (B and D) 72 h. Hepatocytes were lysed, total RNA was isolated from pooled cell lysates (three wells per treatment group), and CYP3A4 and HPRT mRNA levels were analyzed by real-time PCR. CYP3A4 mRNA level was normalized to HPRT mRNA level. Data are shown as mean ± S.D. of triplicate PCR analyses for hepatocyte samples (A) GC4008 and (B) HUM4021. For determining the CYP3A4 catalytic activity, testosterone 6β-hydroxylation assay was performed using HPLC analyses. Data are expressed as mean ± S.D. of three wells for hepatocyte samples (C) GC4008 and (D) HUM4021. Due lack of enough biological replicates, statistical analysis on this data could not be performed.
GC4008 and 4.8-fold in sample HUM4021. Drug treatment did not alter endogenous expression of HPRT and therefore, it was appropriate to use it as the reference gene to normalize the CYP3A4 mRNA data. CYP3A catalytic activity, as determined by the metabolic conversion of testosterone to 6β-hydroxytestosterone (Yuan et al., 2002), was also measured. In sample GC4008, etravirine and efavirenz increased CYP3A catalytic activity by 2.5- and 2.8-fold, respectively (Fig. 2.7C), whereas rilpivirine and delavirdine decreased it and nevirapine had no effect. By comparison, in sample HUM4021, rilpivirine, etravirine, and efavirenz increased CYP3A catalytic activity by 3.1-, 3.9-, and 3.2-fold, respectively (Fig. 2.7D), whereas delavirdine decreased it and nevirapine had no effect. In the same experiment, rifampin increased CYP3A activity by 8.7- and 4.1-fold in samples GC4008 and HUM4021, respectively. Due to limited access to primary human hepatocytes, experiments were conducted in only two biological replicates, and as a result, statistical analysis of the experimental data could not be performed.

2.4 Discussion

PXR is a ligand-activated transcriptional factor belonging to the superfamily of nuclear receptors (Germain et al., 2006) and it is known to regulate the expression of biologically important genes (Gao and Xie, 2012). We report for the first time that the second generation non-nucleoside reverse transcriptase inhibitors rilpivirine and etravirine act as orthosteric agonists of hPXR. This conclusion is based on the experimental evidence indicating that both rilpivirine and etravirine 1) bound to the ligand-binding domain of hPXR, as demonstrated by hPXR-LBD transactivation and TR-FRET competitive ligand-binding assay; 2) stimulated
nuclear translocation of GFP-tagged hPXR into the nuclear compartment, as shown by confocal microscopy; and 3) recruited steroid receptor coactivators SRC1, SRC2, and SRC3 to hPXR, as indicated by mammalian two-hybrid assays. Concentration-response data indicated that rilpivirine and etravirine activated hPXR at a concentration range of 0.5-10 µM, with maximal effect observed at 5 µM. As a comparison, the steady-state maximum plasma concentrations of rilpivirine, etravirine, and efavirenz have been reported to be 0.30 ± 0.08 µM (mean ± S.D.) (Goebel et al., 2006), 1.81 ± 0.66 µM (mean ± S.D.) (DeJesus et al., 2010), and 12.98 µM (95% confidence interval, 7.95-18.27 µM) (Nanzigu et al., 2012), respectively, in specific studies conducted in HIV patients. Further, data from TR-FRET competitive ligand-binding assay revealed that rilpivirine and etravirine were competitive ligands of hPXR. Whether these drugs compete with other PXR ligands, especially the ones with low binding affinity, and if that has any physiological relevance needs further investigations. Overall, these data show that rilpivirine and etravirine activate hPXR at low micromolar concentrations and by a mechanism, which involves orthosteric agonism of the receptor.

Comparison of rilpivirine and etravirine with the first generation non-nucleoside reverse transcriptase inhibitors, such as efavirenz, nevirapine and delavirdine, revealed drug-specific agonism of hPXR by this class of anti-HIV drugs, as demonstrated by the mechanistic experiments in the present study. Among the five drugs investigated, only rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, were identified as agonists of hPXR. At an equimolar concentration (5 µM), the rank order in the activation of hPXR was etravirine > rilpivirine > efavirenz, based on data from the cell-based reporter gene assay. By comparison, as shown in the present study, nevirapine did not activate hPXR at ≤ 10 µM, in agreement with a previous study reporting a lack of an effect at 7.5 µM (Svard et al., 2010). This drug weakly
activates hPXR at a concentration of 50 µM (Faucette et al., 2007). Overall, it is interesting that despite the chemical diversity within the first and second generation non-nucleoside reverse transcriptase inhibitors (De Clercq, 2009), three out of the five drugs investigated are capable of activating hPXR. This not only points to the well-known broad substrate specificity of hPXR (Chang and Waxman, 2006), but it also provides an opportunity to utilize this information to deduce the structure-activity relationship for newer non-nucleoside reverse transcriptase inhibitors that may be valuable in designing newer drugs in this class.

PXR has been reported to show remarkable species-dependent differences in the ligand-binding domain (Jones et al., 2000). Illustrating this fact are the examples of rifampin and PCN. Rifampin is a well-known activator of hPXR, but it has no activity on mPXR or rPXR (Moore et al., 2000). In contrast, PCN activates mPXR and rPXR, but not hPXR (Moore et al., 2000). As assessed at an equimolar concentration (5 µM) in the present study, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated mPXR and rPXR, a profile that is qualitatively similar to that obtained in the hPXR activation experiment. According to site-directed mutagenesis studies, Leu-308 (Tirona et al., 2004) and Gln-285 (Ostberg et al., 2002) in the ligand-binding domain of hPXR, Ile-282 in the ligand-binding domain of mPXR (Ostberg et al., 2002), and Phe-305 and Asp-318 in the ligand-binding domain of rPXR (Song et al., 2005) were identified as amino acids imparting ligand-dependent species-specific activation of PXR. Future in silico docking studies, including those that target these specific amino acid residues, may provide insights into the binding pattern of non-nucleoside reverse transcriptase inhibitors in the ligand-binding domain of hPXR, mPXR, and rPXR, and the lack of species-dependent PXR activation by this class of anti-HIV drugs. Overall, these data suggest that it may be appropriate
to utilize mouse and rat models to assess the biological interactions between hPXR and the non-nucleoside reverse transcriptase inhibitors rilpivirine, etravirine, and efavirenz.

As shown in the experiment with human hepatocytes in culture, agonism of hPXR by rilpivirine was associated with an increase in the mRNA expression of \textit{CYP3A4}, which is a target gene of hPXR (Lehmann \textit{et al.}, 1998; Bertilsson \textit{et al.}, 1998; Blumberg \textit{et al.}, 1998). The increase was accompanied by an elevation in CYP3A-mediated testosterone 6β-hydroxylation activity in hepatocyte sample HUM4021 (Fig. 2.7D), but a decrease in sample GC4008 (Fig. 2.7C). The reason for this differential finding in testosterone 6β-hydroxylation activity is not clear, but perhaps rilpivirine elicits a non-genomic effect (e.g. mechanism-based inactivation) that dominates over the genomic effect (i.e. transcriptional increase in \textit{CYP3A4} gene expression) and accounts for the net decrease in testosterone 6β-hydroxylation that occurs after 2 days of drug treatment (Fig. 2.7C). Further, human hepatocytes used in the present study were from two male donors. Given the known sexually dimorphic expression of CYP3A4 (Thangavel \textit{et al.}, 2011), whether rilpivirine and etravirine exert similar effect on CYP3A4 expression and function in human hepatocytes from female donors require further investigations.

The reported induction of CYP3A enzymes by etravirine ((Yanakakis and Bumpus, 2012 and present study) and efavirenz (Hariparsad \textit{et al.}, 2004; Faucette \textit{et al.}, 2007; Svard \textit{et al.}, 2010; Mugundu \textit{et al.}, 2010; Kamiguchi \textit{et al.}, 2010) in cultured human hepatocytes also occurs \textit{in vivo} in humans, as inferred from pharmacokinetic studies. The administration of etravirine decreases the area under the plasma concentration-time profile for maraviroc (Kakuda \textit{et al.}, 2011), which is metabolized almost exclusively by CYP3A enzymes (Lu \textit{et al.}, 2012). Similarly, human pharmacokinetic studies have shown that efavirenz increases the elimination of CYP3A substrates, as assessed by the erythromycin breath test (Mouly \textit{et al.}, 2002), the metabolic
formation of omeprazole sulfone from omeprazole (Michaud et al., 2012), and the metabolic formation of 4β-hydroxycholesterol from cholesterol (Josephson et al., 2008). The lack of CYP3A4 induction by nevirapine at ≤ 10 µM in cultured human hepatocytes (Svard et al., 2010 and present study) is consistent with a lack of an effect of therapeutic doses of this drug on CYP3A activity in vivo, as determined by the erythromycin breath test (Mouly et al., 2002). In the case of delavirdine at a concentration of ≤ 10 µM, this drug did not activate hPXR or increase CYP3A4 gene expression; rather, in the cultured human hepatocyte experiment, it decreased CYP3A-mediated testosterone 6β-hydroxylation, consistent with the identification of delavirdine as a CYP3A inhibitor in vivo in human subjects (Cheng et al., 1997) and a CYP3A mechanism-based inactivator in vitro in microsomal incubations (Voorman et al., 1998). Overall, given that PXR plays a major role in regulating the expression of genes involved not only in drug transport and metabolism, but also in other biological processes (Gao and Xie, 2012), it will be important to assess the effects of rilpivirine and etravirine on the expression of hPXR target genes other than CYP3A4. Studies are currently underway to investigate in detail the functional consequences of hPXR activation by the second generation non-nucleoside reverse transcriptase inhibitors.

In summary, among the five non-nucleoside reverse transcriptase inhibitors investigated in the present study, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, are activators of hPXR, mPXR, and rPXR. Agonism of hPXR by rilpivirine, etravirine, and efavirenz was demonstrated by the ability of these drugs to bind to the ligand binding domain of hPXR, promote nuclear translocation of GFP-tagged hPXR, and recruit receptor coactivators (SRC1, SRC2, and SRC3). hPXR activation by rilpivirine, etravirine, and efavirenz was associated with induction of a hPXR target gene (CYP3A4). Overall, these novel findings
indicate ligand-dependent regulation of PXR function by select non-nucleoside reverse transcriptase inhibitors and provide insight into the molecular mechanism of hPXR activation by rilpivirine, etravirine, and efavirenz. As activation of PXR and induction of target gene expression form the molecular basis of drug-drug interactions (Wang et al., 2012) and other biological actions (Gao and Xie, 2012), the results from the present study provide a rational basis for investigating novel actions of the second generation non-nucleoside reverse transcriptase inhibitors rilpivirine and etravirine.
Chapter 3: Differential Activation of Human Constitutive Androstane Receptor and its SV23 and SV24 Splice Variants by Rilpivirine and Etravirine

3.1 Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are allosteric inhibitors of human immunodeficiency virus (HIV) reverse transcriptase (De Clercq, 2009). When used as part of the highly active antiretroviral therapy (HAART) regimen with other anti-HIV drugs, NNRTIs have been reported to decrease HIV load and increase Cluster of Differentiation 4 (CD4) counts in HIV/acquired immune deficiency syndrome (AIDS) patients (Camacho and Teofilo, 2011). Rilpivirine and etravirine (Fig. 1.1) belong to the second generation NNRTIs that are clinically approved for the treatment of HIV/AIDS infection (Usach et al., 2013). In various studies, rilpivirine and etravirine have been reported to be active against both treatment-naive and treatment-resistant strains of HIV-1 (Goebel et al., 2006; Arasteh et al., 2009; Santos et al., 2011; Gazzard et al., 2011), although rilpivirine has been approved for clinical use in only treatment-naive HIV-1 infection (De Clercq, 2012). Due to greater potency, smaller dosage and less frequent dosing, two fixed-dose combinations have been developed and approved for rilpivirine (De Clercq, 2012).

 Constitutive androstane receptor (CAR) is a member of the superfamily of nuclear receptor (subfamily 1, group I, member 3; NR1I3). It plays an important role in absorption, distribution, metabolism, and excretion of xenobiotics and endogenous chemicals by regulating the expression of genes involved in transport and biotransformation (Yang and Wang, 2014). CAR has also been implicated in energy metabolism, endocrine regulation, liver diseases, and
other physiological processes and pathophysiological conditions (Jiang and Xie, 2013). Human CAR (hCAR) target genes include the prototypic cytochrome P450 2B6 (CYP2B6) (Sueyoshi et al., 1999). Multiple isoforms of human CAR (hCAR) have been identified, including the wild-type (hCAR-WT) and the naturally occurring splice variants (Lamba et al., 2005; Choi et al., 2013), such as hCAR-SV23 (insertion of amino acids SPTV) (Auerbach et al., 2003) and hCAR-SV24 (insertion of amino acids APYLT) (Jinno et al., 2004; Arnold et al., 2004).

hCAR is known to be activated by a diverse range of chemicals, including endogenous chemicals, prescription drugs, and herbal medicines (Chang and Waxman, 2006). Like hCAR, human pregnane X receptor (hPXR) is another important nuclear receptor that is involved in diverse biological roles and is activated by various endobiotics and xenobiotics (Kodama and Negish, 2013). Whereas some modulators, such as phenobarbital, activate both hCAR (Sueyoshi et al., 1999) and hPXR (Moore et al., 2000), others exhibit receptor selectivity. For example, carbamazepine preferentially activates hCAR (Faucette et al., 2007), whereas meclizine activates hPXR but not hCAR (Lau et al., 2011b). Previous studies showed rilpivirine [(Sharma et al., 2013; Weiss and Haefeli, 2013), also see Chapter 2] and etravirine [(Sharma et al., 2013), also see Chapter 2] are activators of hPXR. These drugs appear to activate hPXR by a mechanism that involves binding to the LBD of the receptor and recruitment of steroid receptor coactivator 1 (SRC1), SRC2, and SRC3 [(Sharma et al., 2013), also see Chapter 2]. However, it remains to be investigated whether and how rilpivirine and etravirine activate hCAR.

The present study systematically investigated the effects of rilpivirine and etravirine on the activity of hCAR-WT and its hCAR-SV23 and hCAR-SV24 splice variants. As a comparison, we also determined the effects of the first generation NNRTIs (efavirenz, nevirapine, and delavirdine, Fig. 1.1). The experimental approaches involved the use of cell-
based luciferase reporter gene assays to assess receptor activation, mammalian two-hybrid assay to examine steroid receptor co-activator recruitment, and primary cultures of human hepatocytes to characterize nuclear translocation of hCAR-WT and hCAR target gene (CYP2B6) expression. The results are discussed from the perspective of drug-dependent and isoform-selective activation of hCAR by NNRTIs.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Etravirine, efavirenz, and nevirapine were obtained from the National Institutes of Health AIDS Reagent Program (Bethesda, MD, U.S.A.). Rilpivirine, delavirdine, and hydroxybupropion were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). FuGENE 6 and Effectene® transfection reagent were purchased from Roche Diagnostics (Laval, QC, Canada), and QIAGEN Inc. (Mississauga, ON, Canada), respectively. Charcoal-stripped fetal bovine serum (FBS), minimum essential medium/Earle’s Balanced Salts Solution (MEM/EBSS), minimum essential medium-reduced serum (MEM-RS), and minimum essential medium/non-essential amino acid solution (100×) (HyClone Laboratories, Logan, UT, U.S.A.) were purchased from Thermo Fisher Scientific (Nepean, ON, Canada). All other chemicals, reagents, and assay kits were purchased from sources listed previously [(Lau et al., 2011a; Sharma et al., 2013), also see Chapter 2]. Cryopreserved hepatocyte thawing medium, hepatocyte plating medium, hepatocyte maintenance medium, and various medium supplements were provided by Triangle Research Labs, LLC (Research Triangle Park, NC, U.S.A.).
3.2.2 Culture and Treatment of Human Hepatocytes

The demographics of the donors (GC4008, HUM4021, HUM4034, and HUM4038) are listed in Table 3.1. Cryopreserved human hepatocytes (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) were thawed and plated according to protocols listed at http://triangleresearchlabs.net/products/cryopreserved-hepatocytes/. Cell viability was 88%, 90%, 94%, and 93% for hepatocyte samples GC4008, HUM4021, HUM4034, and HUM4038, respectively, as assessed by trypan blue exclusion (Jauregui et al., 1981). Hepatocytes were plated and cultured as described previously [(Sharma et al., 2013), also see Chapter 2]. Cultured hepatocytes were treated with DMSO (vehicle control), rilpivirine, etravirine, efavirenz, nevirapine, delavirdine, or a known CYP2B6 inducer such as rifampin (Chang et al., 1997), CITCO (Maglich et al., 2003), or phenobarbital (Chang et al., 1997), as described in the appropriate figure legends. Drug-containing culture medium was replaced every 24 h.

3.2.3 Isolation of Total RNA, Reverse Transcription and Real-time PCR Analysis

Isolation of total RNA and reverse transcription were conducted as described previously (Lau et al., 2011b). The sequences of the primers used to amplify human CYP2B6 cDNA, HPRT1 cDNA, 18s rRNA cDNA, and cyclophilin cDNA are listed in Table 3.2. The primers were synthesized and their specificity was verified by sequencing the purified amplicons (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.). CYP2B6 cDNA and HPRT cDNA were amplified by real-time PCR in a LightCycler (Roche Diagnostics, Laval, QC, Canada), according to conditions described in Table 3.2. Each PCR reaction contained 1 ng total cDNA, 1 U Platinum Taq DNA polymerase in 1× PCR buffer (2 mM Tris-HCl, pH 8.4 and 50 mM KCl), 4 mM MgCl2, 0.2 mM deoxynucleoside triphosphate, 0.25 mg/ml bovine serum albumin, 0.2 μM
forward and reverse primers (0.5 µM for HPRT), and 1:30,000 SYBR Green I. To construct the calibration curves (cross point versus log cDNA copies) for CYP2B6 and HPRT1 amplicons were generated using human liver QUICK-Clone cDNA (Clontech, Mountain View, CA, U.S.A.) and then purified and quantified as described previously (Lau et al., 2011b). CYP2B6 mRNA expression was normalized to that of HPRT.
<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Race</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Reported BMI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Smoker</th>
<th>Alcohol Use</th>
<th>Medicine Use</th>
<th>Serological Data</th>
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<tr>
<td>GC4008</td>
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<td>69</td>
<td>24.7</td>
<td>Stopped 24 years ago</td>
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<td>No</td>
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<td>No</td>
<td>No</td>
<td>All negative</td>
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<tr>
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<td>Yes</td>
<td>Marijuana</td>
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</tr>
<tr>
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<td>26.0</td>
<td>No</td>
<td>Rare</td>
<td>No</td>
<td>CMV+ *</td>
</tr>
</tbody>
</table>

<sup>a</sup> Body Mass Index; * CMV+ (cytomegalovirus positive)
### Table 3.2 Primer Sequences and RT-PCR Cycling Conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6</td>
<td>GCG-TGT-GGT-TCA-TTC-ACA-AA (forward)</td>
<td>95°C, 5 s</td>
<td>65°C, 5 s</td>
<td>72°C, 15 s</td>
<td>(Chang et al., 2003a)</td>
</tr>
<tr>
<td></td>
<td>AAT-TTA-GCC-AGG-CGT-GGT-G (reverse)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>GAA-GAG-CTA-TTG-TAA-TGA-CC (forward)</td>
<td>95°C, 5 s</td>
<td>60°C, 10 s</td>
<td>72°C, 15 s</td>
<td>(Qiu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>GCG-ACC-TTG-ACC-ATC-TTT-G (reverse)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.4 Bupropion Hydroxylation Assay

The CYP2B6-catalyzed bupropion hydroxylation assay was performed as described previously (Lau et al., 2011b). Briefly, at 72 h post-treatment, cultured human hepatocytes were first washed twice with HBSS and subsequently incubated with 0.5 ml of HBSS containing bupropion (final concentration 500 μM) for 20 min at 37°C. Final concentration for bupropion was selected based on a CYP2B6-catalyzed bupropion hydroxylation kinetics published previously (Faucette et al., 2000). At the end of incubation period, sample supernatant for each treatment groups were collected and transferred to a 24-well plate for storage at -80°C until analysis. Formation of hydroxybupropion was quantified using an optimized ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method (Lau and Chang, 2009). Hepatocyte incubation samples (75 μl) were spiked with 75 μl of triprolidine (internal standard; 1 μM final concentration) and transferred to a 96-well plate, and 2 μl sample aliquot was injected onto the UHPLC-MS/MS. The UHPLC-MS/MS system comprised of an AB SCIEX QTrap 5500 hybrid linear ion-trap triple quadrupole mass spectrometer (AB SCIEX, Concord, ON, Canada) with an Agilent 1290 (Agilent, Mississauga, ON, Canada) as the frontend UHPLC system. Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C_{18} column (100×2.1 mm i.d., 1.7 μm) using mobile phases containing 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at a flow rate optimized at 0.2 ml/min. A gradient method was used to separate hydroxybupropion from the internal standard and the matrix components with the following solvent compositions: isocratic at 98% A (0.0 –1.5 min), linear gradient from 98% to 2% A (1.5–1.6 min), isocratic at 2% A (1.6 – 4.0 min), linear gradient from 2% to 98% A (4.0 – 4.1 min), and isocratic at 98% A (4.1– 6.0 min). A multiple reaction monitoring mode was used to detect specific mass-to-charge transitions for
hydroxybupropion (257.3 → 239.1) and triprolidine (280.5 → 208.8) in positive ion mode. Various mass spectrometer parameters were optimized as follows: declustering potential: 81 volts; collision energy: 17 electronvolt (hydroxybupropion) and 23 electronvolt (triprolidine); and collision cell exit potential: 10 volts (hydroxybupropion) and 12 volts (triprolidine). Mass spectrometric data were acquired using Analyst® software, version 1.5.2 (AB SCIEX, Concord, ON, Canada). The peak area of hydroxybupropion was normalized by triprolidine (internal standard) peak area. A calibration curve was constructed using an authentic hydroxybupropion standard (0.025 - 1 µM) and the metabolite amount in hepatocyte incubation samples were determined using weighted (1/x²) linear least-squares regression analysis.

3.2.5 Cell Culture

HepG2 human hepatocellular carcinoma cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in T-75 culture flasks in minimum essential medium/EBSS supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc., Burlington, ON, Canada), penicillin G (100 U/ml), streptomycin (100 µg/ml), MEM/non-essential amino acid (1×), and L-glutamine (2 mM). Cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO2. Culture medium was changed every 3 days, and cells were sub-cultured weekly.

3.2.6 Plasmids

pCMV6-XL4-hCAR-WT, pCMV6-neo-hCAR-SV23, pCMV6-XL4-hCAR-SV24, pCMV6-XL4-hRXRα, pCMV6-AC-GFP-hCAR-WT, pCMV6-neo, and pCMV6-XL4 were purchased from OriGene Technologies, Inc. (Rockville, MD, U.S.A.). The pFR-luc reporter was
purchased from Agilent Technologies (Santa Clara, CA, U.S.A.). The internal control *Renilla reniformis* luciferase pGL4.74 [hRluc/TK] plasmid was procured from Promega (Madison, WI, U.S.A.). The pVP16 and pM empty vectors were purchased from Clontech (Mountain View, CA, U.S.A.). pM-hCAR-WT-LBD, pVP16-hCAR-WT-LBD, pM-hCAR-SV23-LBD, pVP16-hCAR-SV23-LBD, pM-hCAR-SV24-LBD, pVP16-hCAR-SV24-LBD, pGL3-basic-CYP2B6-PBREM/XREM-luc, pM-hSRC1-RID, pM-hSRC2-RID, pM-hSRC3-RID, pVP16-hSRC1-RID, pVP16-hSRC2-RID, and pVP16-hSRC3-RID were constructed as mentioned previously (Lau *et al.*, 2011a; Lau and Chang, 2013). The constructs were sequenced (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.) and their sequence identity was confirmed by comparing with published sequence.

### 3.2.7 Transient Transfection and Reporter Gene Assays

HepG2 cells were cultured in minimum essential medium-RS supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 1% v/v charcoal-stripped fetal bovine serum. This smaller concentration of serum was used to decrease the normally high basal activity associated with hCAR-WT as a result of receptor activation by contaminants (Lau and Chang, 2014), such as DEHP, which is present in fetal bovine serum (DeKeyser *et al.*, 2009). Cells were seeded at a density of 100,000 cells/well in a volume of 0.5 ml of supplemented culture medium. At 24 h post-plating, transfection of cultured HepG2 cells was carried out as described previously (Lau and Chang, 2014), except that minimum essential medium-RS was used instead of serum-free Opti-MEM. The transfection master mix contained FuGENE 6 transfection reagent (3 μl/μg of DNA), minimum essential medium-RS (20 μl/well), and various plasmids. In the hCAR-WT-dependent reporter gene assays, the transfection master
mix contained pGL4.74 [hRluc/TK] internal control vector (5 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc reporter construct (50 ng/well), and either a receptor expression plasmid or the corresponding empty vector (50 ng/well) for 24 h, as detailed in the appropriate figure legend. In the hCAR-SV23- and hCAR-SV24-dependent reporter gene assays, the transfection master mix also contained pCMV6-XL4-hRXRα (10 ng/well) (Auerbach et al., 2005). Transfected cells were treated with 0.5 ml of supplemented culture medium (without charcoal-stripped fetal bovine serum) containing DMSO (vehicle control), a NNRTI (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), TCPOBOP (negative control) (Lau et al., 2011a), CITCO (positive control for hCAR-WT and hCAR-SV24) (Maglich et al., 2003; Auerbach et al., 2005), or DEHP (positive control for hCAR-SV23) (DeKeyser et al., 2009), at the concentrations indicated in appropriate figure legends. In hCAR-WT-dependent reporter gene assays, androstanol (10 µM), which is an inverse agonist of the receptor (Moore et al., 2000), was added to decrease the constitutive activity (Burk et al., 2005b). At 24 h post-treatment, HepG2 cells were lysed. Firefly luciferase and \textit{Renilla reniformis} (internal control) luciferase activities were quantified and normalized as described previously [(Sharma et al., 2013), also see Chapter 2]. Results are expressed as fold increase over the vehicle-treated control group. Each experiment was performed in triplicate and a total of four or five independent experiments were conducted.

3.2.8 LBD-transactivation Assay

The hCAR-WT-LBD transactivation assays were performed as described previously (Lau and Chang, 2014), except that minimum essential medium-RS was used instead of serum-free Opti-MEM. At 24 h post-plating, cultured HepG2 cells were transfected with a master mix
containing FuGENE 6 transfection reagent (3 µl/µg of DNA), minimum essential medium-RS (20 µl/well), pFR-luc reporter plasmid (100 ng/well), pGL4.74 [hRluc/TK] internal control plasmid (1 ng/well), and either the pM-hCAR-WT-LBD (Gln-105 to Ser-348) receptor expression plasmid or the pM empty vector (40 ng/well) for 24 h. The hCAR-SV23-LBD- and hCAR-SV24-LBD-dependent transactivation assays were carried out in HepG2 cells transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), minimum essential medium-RS (20 µl/well), pCMV6-XL4-hRXRα (10 ng/well), pFR-luc reporter plasmid (100 ng/well), pGL4.74 [hRluc/TK] internal control plasmid (1 ng/well), and either a receptor expression plasmid or the pM empty vector (40 ng/well) for 24 h. The receptor expression plasmids were pM-hCAR-SV23-LBD (Gln-105 to Ser-352) and pM-hCAR-SV24-LBD (Gln-105 to Ser-353). Transfected cells were treated with 0.5 ml of supplemented culture medium (without charcoal-stripped fetal bovine serum) containing DMSO (vehicle control), a NNRTI (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), TCPOBOP (negative control) (Lau et al., 2011a), CITCO (positive control for hCAR-WT and hCAR-SV24) (Maglich et al., 2003; Auerbach et al., 2005), or DEHP (positive control for hCAR-SV23) (DeKeyser et al., 2009) for 24 h, at the concentrations detailed in appropriate figure legends. In the hCAR-WT-dependent LBD-transactivation assay, cells were co-treated with a hCAR-WT inverse agonist, androstanol (10 µM) (Moore et al., 2000). Luciferase activity was measured and normalized as described under Transient transfection and reporter gene assays. Each experiment was performed in triplicate and a total of three independent experiments were conducted.
3.2.9 In Vitro Nuclear Translocation of GFP-tagged hCAR-WT in Primary Cultures of Human Hepatocytes

Human hepatocytes (HUM4038) were cultured on collagen-coated glass cover slips in 24-well plates at a density of 150,000 cells/well. After cell attachment (at 6 h post-plating), the medium was aspirated and 0.5 ml of fresh hepatocyte maintenance medium (Triangle Research Labs, LLC, Research Triangle Park, NC, U.S.A.) was added to each well. At 24 h post-plating, human hepatocytes were transfected with a pCMV6-AC-GFP-hCAR-WT (100 ng/well) for 24 h using Effectene® Transfection Reagent (25 µl/µg of DNA) following manufacturer’s protocol. Transfected hepatocytes were treated with 0.5 ml of supplemented hepatocyte maintenance medium containing DMSO (vehicle control), a NNRTI (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), or a positive control (CITCO or PB) (Sueyoshi et al., 1999; Maglich et al., 2003) for 24 h, at concentrations described in the figure legend. At 24 h post-treatment, samples were prepared for confocal imaging as described previously [(Sharma et al., 2013), also see Chapter 2]. The images were collected using a Carl Zeiss LSM 700 confocal microscope interfaced with the Zen 2011 SP2 software (version 8.0) (Carl Zeiss Microscopy GmbH, Jena, Germany). Post-imaging adjustments in brightness and contrast of the entire image were made in compliance with the Clinical and Laboratory Images in Publications (CLIP) principles (Lang et al., 2012) using Adobe® Photoshop® CS6, version 13.0 x64 (Adobe Systems Corporation, San Jose, CA, U.S.A.).

3.2.10 Mammalian Two-hybrid Assay

Recruitment of steroid receptor coactivator 1 (SRC1), SRC2, and SRC3 to hCAR-WT-LBD, hCAR-SV23-LBD, and hCAR-SV24-LBD was assessed by a mammalian two-hybrid
assay as described previously (Lau and Chang, 2014), except that minimum essential medium-RS was used instead of serum-free Opti-MEM. At 24 h post-plating, cultured HepG2 cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), minimum essential medium-RS (20 µl/well), pFR-luc reporter plasmid (100 ng/well), pGL4.74 [hRluc/TK] internal control plasmid (10 ng/well), a receptor expression plasmid (or the pVP16 empty vector; 40 ng/well), and a coactivators expression plasmid (10 ng/well) for 24 h. The receptor expression plasmids were pVP16-hCAR-WT-LBD, pVP16-hCAR-SV23-LBD, and pVP16-hCAR-SV24-LBD, whereas the coactivator expression plasmids were pM-hSRC1-RID, pM-hSRC2-RID, and pM-hSRC3-RID. In another set of experiments, the mammalian two-hybrid assay was performed as described above, except that a different vector (pM) was used in constructing the receptor expression plasmids (pM-hCAR-WT-LBD, pM-hCAR-SV23-LBD, pM-hCAR-SV24-LBD) and a different vector (pVP16) was used in constructing the coactivator expression plasmids (pVP16-hSRC1-RID, pVP16-hSRC2-RID, pVP16-hSRC3-RID). In the hCAR-SV23-LBD- and hCAR-SV24-LBD-dependent mammalian two-hybrid assay, cells were co-transfected with pCMV6-XL4-hRXRα (10 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented culture medium (without charcoal-stripped fetal bovine serum) containing DMSO (vehicle control), a NNRTI (rilpivirine, etravirine, efavirenz, nevirapine, or delavirdine), TCPOBOP (negative control) (Lau et al., 2011a), CITCO (positive control for hCAR-WT and hCAR-SV24) (Maglich et al., 2003; Auerbach et al., 2005), or DEHP (positive control for hCAR-SV23) (DeKeyser et al., 2009) for 24 h, as detailed in the respective figure legends. In the hCAR-WT-dependent co-activator recruitment assay, cells were co-treated with a hCAR-WT inverse agonist, androstanol (10 µM) (Moore et al., 2000). Luciferase activity was measured and normalized as described under Transient transfection and reporter gene assays.
Each experiment was performed in triplicate and a total of three independent experiments were conducted.

3.2.11 Data Analyses

Data were analyzed by two-way analysis of variance. Where appropriate, it was followed by the Student Newman-Keuls multiple comparison test (SigmaPlot 11.0; Systat Software, Inc., San Jose, CA, U.S.A.). The level of statistical significance was set \textit{a priori} at $P < 0.05$.

3.3 Results

3.3.1 Rilpivirine and Etravirine Induce Hepatic Expression of a hCAR Target Gene (CYP2B6)

Sandwich-cultured human hepatocytes were used as an experimental model to compare the effects of rilpivirine and etravirine with first generation NNRTIs (efavirenz, nevirapine, and delavirdine) on the induction of \textit{CYP2B6}, a target gene of hCAR (Sueyoshi \textit{et al.}, 1999). As shown in Fig. 3.1A for hepatocyte sample GC4008, rilpivirine, etravirine, and efavirenz (5 µM) increased CYP2B6 mRNA expression by 3.4-, 2.6-, and 3.4-fold, respectively, whereas nevirapine and delavirdine had no effect. Therefore, subsequent gene expression experiments assessed the effects of only rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine. Similar effects were observed for sample HUM4021 (Fig. 3.1B), wherein rilpivirine, etravirine, and efavirenz (5 µM) increased CYP2B6 mRNA level by 3.6-, 3.3-, and 4.3-fold, respectively. In human hepatocytes from two other donors (HUM4034 and HUM4038), rilpivirine (0.5 and 5 µM), etravirine (2.5 and 5 µM), and efavirenz (5 and 10 µM)
increased CYP2B6 mRNA expression at all the concentrations investigated (Fig. 3.1C and 3.1D). The positive controls CITCO (1 µM) and rifampin (10 µM) (Maglich et al., 2003) yielded the expected result (Fig. 3.1A-D). The same pattern of response was obtained regardless of whether HPRT1 or 18s rRNA was used as the reference gene to normalize the CYP2B6 mRNA data. As shown in Fig. 3.1E-F, the CYP2B6-catalyzed bupropion hydroxylation data were consistent with the CYP2B6 mRNA results (Fig. 3.1A-D). CYP2B6 mRNA expression levels were also measured in cDNA samples from treated human hepatocyte samples (GC4008 and HUM4021) from the previous study [(Sharma et al., 2013); see Chapter 2]. However, due to limited access to primary human hepatocytes, CYP2B6-catalyzed bupropion hydroxylation assay was conducted in only two biological replicates (HUM4034 and HUM4038), and as a result, statistical analysis of the experimental data could not be performed.
Figure 3.1 Induction of CYP2B6 in Primary Cultures of Human Hepatocytes by Rilpivirine and Etravirine.

Cultured hepatocytes were treated with culture medium (vehicle control for phenobarbital; PB), DMSO (0.1% v/v; vehicle control for the other drugs), rilpivirine (RPV), etravirine (ETV), efavirenz (EFV), nevirapine (NVP), delavirdine (DLV), rifampin (RIF; positive control), CITCO (positive control), or PB (positive control) for 48 h (samples GC4008, HUM4034, and HUM4038) or 72 h (sample HUM4021) at the concentrations indicated in panels A to D. The hepatocytes were lysed and total RNA was isolated from pooled cell lysates (four wells), and CYP2B6 mRNA and HPRT1 mRNA levels were analyzed by real-time PCR, as described under Section 3.2.3. CYP2B6 mRNA level was normalized to HPRT1 mRNA level. Data are shown as mean ± S.D. of triplicate PCR analyses for hepatocyte samples GC4008 (A), HUM4021 (B), HUM4034 (C), and HUM4038 (D). CYP2B6 catalytic activity was measured by bupropion hydroxylation using an optimized LC-MS/MS method, as detailed under Section 3.2.4. Data are expressed as mean ± S.D. of three wells for hepatocyte samples HUM4034 (E) and HUM4038 (F). Due lack of enough biological replicates, statistical analysis on this data could not be performed (see Section 3.3.1 for further explanation).
3.3.2 Rilpivirine and Etravirine Activate hCAR-WT without Involving the LBD

Data from the previous study indicated that rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine at concentrations ≤ 10 µM were not cytotoxic to HepG2 cells [(Sharma et al., 2013), also see Chapter 2]. Therefore, in the present study, the concentration of each of these drugs did not exceed 10 µM in the cell-based reporter gene assays. At an equimolar concentration (5 µM), rilpivirine (P < 0.001), etravirine (P < 0.001), and efavirenz (P < 0.001) increased, whereas nevirapine (P < 0.932) and delavirdine (P < 0.508) had no effect on the transcriptional activity of hCAR-WT (Fig. 3.2A). By comparison, the positive control (CITCO) (P < 0.001) (Maglich et al., 2003) and the negative control (TCPOBOP) (P < 0.893) (Moore et al., 2000) produced the expected results. Dose-response experiments indicated the range of concentrations of rilpivirine (1-10 µM, Fig. 3.2B) (P < 0.001 - 0.003), etravirine (2.5-10 µM, Fig. 3.2C) (P < 0.001), and efavirenz (2.5-10 µM, Fig. 3.2D) (P < 0.001 - 0.013) that increased hCAR-WT activity.

To determine whether the NNRTIs transactivate the LBD of hCAR-WT, cultured HepG2 cells were transfected with a hCAR-WT-LBD expression plasmid (i.e., Gln-105 to Ser-348) and treated with 5 µM rilpivirine, etravirine, efavirenz, nevirapine or delavirdine. As shown in Fig. 3.3, none of the NNRTIs increased the luciferase activity in cells transfected with hCAR-WT-LBD (P > 0.05). As expected, CITCO (positive control) (Maglich et al., 2003), but not TCPOBOP (negative control) (Lau et al., 2011a), transactivated hCAR-WT-LBD.
Figure 3.2 Ligand-specific Activation of Wild-type hCAR (hCAR-WT) by NNRTIs. Cultured HepG2 cells were transfected with pGL3-basic-CYP2B6-PBREM/XREM-luc reporter plasmid, pGL4.74 [hRluc/TK] internal control plasmid, and either the pCMV6-XL4-hCAR-WT receptor expression plasmid or the pCMV6-XL4 empty vector for 24 h. (A) Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), or CITCO (10 µM; positive control) for 24 h. (B, C, D) In the concentration-response experiment, transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 0.1-10 µM), etravirine (ETV; 0.1-10 µM), or efavirenz (EFV; 0.1-10 µM) for 24 h. In all cases, cells were co-treated with androstanol (10 µM; inverse agonist for hCAR-WT) to decrease the constitutive activity of this receptor. Firefly luciferase and R. reniformis luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± S.E.M. for four or five independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the receptor expression plasmid ($P < 0.05$). Androstanol reduced the constitutive activity of hCAR-WT by 32 ± 3%.
Figure 3.3 Effect of NNRTIs on Transactivation of the LBD of hCAR-WT.
Cultured HepG2 cells were transfected with pFR-luc reporter plasmid, pGL4.74 [hRluc/TK] internal control plasmid, and either the pM-hCAR-WT-LBD receptor expression plasmid or the pM empty vector for 24 h. Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), or CITCO (10 µM; positive control) for 24 h. In all cases, cells were co-treated with androstanol (10 µM; inverse agonist for hCAR-WT) to decrease the constitutive activity of this receptor. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector and from the vehicle-treated control cells transfected with receptor expression plasmid (*P* < 0.05). Androstanol reduced the constitutive activity of hCAR-WT by 30 ± 19%. 

![Graph showing the effect of NNRTIs on transactivation of the LBD of hCAR-WT](image-url)
3.3.3 Rilpivirine and Etravirine Trigger Nuclear Translocation of GFP-tagged hCAR-WT

To determine whether the NNRTIs triggered nuclear translocation of hCAR-WT, primary cultures of human hepatocytes were transfected with a GFP-tagged hCAR-WT expression plasmid. As shown in Fig. 3.4, confocal image analyses and comparison with vehicle control (DMSO) group revealed that at a concentration of 5 µM, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, triggered nuclear translocation of GFP-tagged hCAR-WT in primary cultures of human hepatocytes. Control analysis showed that CITCO and phenobarbital triggered nuclear translocation of hCAR-WT, in agreement with previous findings (Maglich et al., 2003; Li et al., 2009).

3.3.4 In Contrast to CITCO, Rilpivirine and Etravirine do not Recruit SRC1, SRC2, or SRC3 to hCAR-WT-LBD

Mammalian two-hybrid assay was performed to determine whether the NNRTIs recruit steroid receptor coactivator to the LBD of hCAR-WT. Rilpivirine, etravirine, efavirenz, nevirapine, and delavirdine at 5 µM did not increase the luciferase activity in HepG2 cells co-transfected with a pVP16-hCAR-WT-LBD expression plasmid and a pM-SRC1 (Fig. 3.5A) ($P > 0.05$), pM-SRC2 (Fig. 3.5B) ($P > 0.05$), or pM-SRC3 coactivator expression plasmid (Fig. 3.5C) ($P > 0.05$). The same results were obtained in a pM-containing receptor expression plasmid and a pVP16-containing coactivator expression plasmid (data shown in Appendix A.1). By comparison, CITCO (positive control) (Maglich et al., 2003) and TCPOBOP (negative control) (Lau et al., 2011a) produced the expected results (Figs. 3.5A, 3.5B, and 3.5C).
Figure 3.4 Localization of GFP-tagged hCAR-WT in Primary Cultures of Human Hepatocytes (HUM4038) Treated with a NNRTI.

Cultured hepatocytes were transfected with pCMV6-AC-GFP-hCAR-WT for 24 h and subsequently treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), CITCO (10 µM; positive control), or phenobarbital (PB; 1 mM; positive control) for 24 h. Cells were fixed with 4% p-formaldehyde and mounted on glass slides using ProLong® Gold Antifade Reagent with DAPI for confocal microscopy. Shown are representative photomicrographs illustrating the localization of GFP-tagged hCAR-WT, DAPI-stained nuclei, and merged images for each treatment group.
Figure 3.5 Mammalian Two-hybrid Assay to Evaluate Recruitment of Steroid Receptor Coactivators to the LBD of hCAR-WT by Specific NNRTIs.

Cultured HepG2 cells were co-transfected with a pVP-16-hCAR-WT-LBD receptor expression plasmid (or the pVP-16 empty vector), a coactivator expression plasmid, pFR-luc reporter plasmid, and pGL4.74 [hRLuc/TK] internal control plasmid. The coactivator expression plasmids
were pM-hSRC1-RID (A), pM-hSRC2-RID (B), and pM-hSRC3-RID (C). Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), or CITCO (10 µM; positive control) for 24 h. In all cases, cells were co-treated with androstanol (10 µM; inverse agonist for hCAR-WT) to decrease the constitutive activity of this receptor. Firefly luciferase and R. reniformis luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with the empty vector and from the vehicle-treated control cells transfected with receptor expression plasmid (P < 0.05). Androstanol reduced the constitutive activity of hCAR-WT by 49 ± 8%, 36 ± 20%, and 45 ± 9% in cells co-transfected with pVP16-hCAR-WT-LBD and pM-hSRC1-RID, pM-hSRC2-RID, or pM-hSRC3-RID, respectively.
Figure 3.6 Ligand-specific Activation of Functional Splice Variants hCAR-SV23 and hCAR-SV24 by NNRTIs.

Cultured HepG2 cells were transfected with pCMV6-XL4-hRXRα, pGL3-basic-CYP2B6-PBREM/XREM-luc reporter plasmid, pGL4.74 [hRluc/TK] internal control plasmid, and either a receptor expression plasmid [pCMV6-neo-hCAR-SV23 (A, C) or pCMV6-XL4-hCAR-SV24 (B, D)] or an empty vector (pCMV6-neo or pCMV6-XL4) for 24 h. Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM or 0.1-10 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), DEHP (10 µM; positive control for hCAR-SV23), or CITCO (10 µM; positive control for hCAR-SV24) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized as described under *Section 3.2.7*. Data are expressed as mean ± S.E.M. for four or five independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the receptor expression plasmid (*P* < 0.05).
Figure 3.7 Effect of Rilpivirine, Etravirine, Efavirenz, Nevirapine, and Delavirdine on Transactivation of the LBD of hCAR-SV23 and hCAR-SV24, and Recruitment of Steroid Receptor Coactivator SRC1 to the Ligand Binding Domain of hCAR-SV23 and hCAR-SV24.

LBD-transactivation assays were performed in HepG2 cells were transfected with pCMV6-XL4-hRXRα, pFR-luc reporter plasmid, pGL4.74 [hRLuc/TK] internal control plasmid, and either a receptor expression plasmid [pM-hCAR-SV23-LBD (A) or pM-hCAR-SV24-LBD (B)] or an empty vector (pM) for 24 h. In the mammalian two-hybrid assays, HepG2 cells were co-transfected with a pM-hSRC1-RID coactivator expression plasmid, pCMV6-XL4-hRXRα, pFR-luc reporter plasmid, pGL4.74 [hRLuc/TK] internal control plasmid, and either a receptor expression plasmid [pVP16-hCAR-SV23-LBD (C) or pVP16-hCAR-SV24-LBD (D)] or an empty vector (pVP16) for 24 h. Transfected cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), or delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), DEHP (10 µM; positive control for hCAR-SV23), or CITCO (10 µM; positive control for hCAR-SV24) for 24 h. Firefly luciferase and R. reniformis luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with corresponding empty vector and from the vehicle-treated control cells transfected with respective receptor expression plasmid (P < 0.05).
3.3.5 **Selective Activation of hCAR Isoforms by Rilpivirine and Etravirine**

As shown in Fig. 3.2A-C, rilpivirine and etravirine activated hCAR-WT. Therefore, the next experiment was to investigate whether NNRTIs influence the activity of other hCAR isoforms. At an equimolar concentration (5 µM), only efavirenz increased, whereas rilpivirine, etravirine, nevirapine, and delavirdine had no effect on the activity of hCAR-SV23 (Fig. 3.6A) ($P < 0.001$) or hCAR-SV24 (Fig. 3.6B) ($P = 0.037$). Concentration-response data revealed that 0.5-10 µM efavirenz increased hCAR-SV23 activity (Fig. 3.6C) ($P < 0.001$), whereas ≥ 5 µM efavirenz increased hCAR-SV24 activity (Fig. 3.6D) ($P < 0.001$). The effect of an equimolar concentration (10 µM) of efavirenz on hCAR-SV24 (Figure 7D) was smaller than the response elicited by CITCO (Fig. 3.6B).

None of the drugs at 5 µM transactivated the LBD of hCAR-SV23 (Gln-105 to Ser-352) (Fig. 3.7A) ($P > 0.05$) or hCAR-SV24 (Gln-105 to Ser-353) (Fig. 3.7B) ($P > 0.05$). Among the NNRTIs investigated in the mammalian two-hybrid assay for each combination of the LBD of a hCAR splice variant (pVP16-hCAR-SV23-LBD or pVP16-hCAR-SV24-LBD) and a steroid receptor coactivator (pM-hSRC1-RID, pM-hSRC2-RID, or pM-hSRC3-LBD) (Fig. 3.7C, 3.7D, and Appendix A.2), only efavirenz minimally recruited SRC1 to the LBD of hCAR-SV23 (Fig. 3.7C) ($P < 0.001$). No recruitment was obtained in cells transfected with a pM-containing receptor expression plasmid and a pVP16-containing coactivator expression plasmid (data shown in Appendix A.3). In each of the above assays, positive control analysis with DEHP and CITCO and negative control analysis with TCPOBOP (Lau et al., 2011a) produced the expected results (Fig. 3.7C, 3.7D, and Appendix A.2, and A.3).
3.4 Discussion

CAR is a transcriptional factor of practical importance as changes in its activity influence a variety of physiological functions and pathological conditions (Jiang and Xie, 2013). Moreover, owing to its effect on genes involved in transport and biotransformation of various endogenous substances and xenobiotics, alteration in CAR activity represents a molecular basis for pharmacokinetic drug interactions and chemical toxicity (Yang and Wang, 2014). This is the first study to demonstrate that rilpivirine and etravirine are activators of hCAR-WT. This conclusion is based on various experimental evidences indicating that rilpivirine and etravirine: 1) activated hCAR-WT, as assessed in a cell-based reporter gene assay in which cultured HepG2 cells were transfected with a plasmid expressing the full-length hCAR-WT; 2) triggered nuclear translocation of GFP-tagged hCAR-WT in primary cultures of human hepatocytes; and 3) induced hCAR target gene (*CYP2B6*) expression in primary cultures of human hepatocytes. The activation of hCAR-WT by rilpivirine and etravirine did not appear to involve direct interaction between each of these drugs and the LBD of hCAR-WT, as suggested by the lack of an effect in a hCAR-WT-LBD transactivation assay and the lack of coactivator (SRC1, SRC2, and SRC3) recruitment in a mammalian two-hybrid assay. The molecular mechanism of hCAR-WT activation is still not well-understood. Previous studies have shown orthosteric agonism as a mechanism of hCAR-WT activation by CITCO (Maglich *et al.*, 2003) and 3-hydroxyflavone (Lau and Chang, 2013). However, other ligands, such as phenobarbital (Moore *et al.*, 2000), phenytoin (Wang *et al.*, 2004), and *Ginkgo biloba* (Lau *et al.*, 2011a), do not activate hCAR-WT by binding directly to the LBD of the receptor and recruiting coactivators to the ligand-receptor complex. A recent study indicated inhibition of epidermal growth factor receptor (EGFR)
signaling as a mechanism of indirect activation of mouse CAR by phenobarbital (Mutoh et al., 2013). Given the pronounced species-dependency in chemical activation of CAR (Chang and Waxman, 2006; Omiecinski et al., 2011), it remains to be investigated whether the EGFR signaling pathway also contributes to the activation of hCAR by rilpivirine, etravirine, and other indirect activators of hCAR. Overall, these data provide the novel identification of rilpivirine and etravirine as activators of hCAR-WT and show that the activation occurs in a manner distinct from the orthosteric agonism of hCAR-WT by CITCO (Maglich et al., 2003) and 3-hydroxyflavone (Lau and Chang, 2013).

Another novel finding of the present study is that rilpivirine and etravirine activate hCAR in an isoform-dependent manner. Rilpivirine and etravirine activated hCAR-WT, but not hCAR-SV23 or hCAR-SV24. This finding corroborates with the notion that these hCAR isoforms do not have identical ligand activation profiles (Lau et al., 2011a). This may be explained by the proposed alteration in receptor conformation of hCAR-SV23 as a result of the amino acid (SPTV) insertion between helices 6 and 7 (DeKeyser et al., 2011) and the proposed compromise in receptor heterodimerization of hCAR-SV24 as a result of the amino acid (APYLT) insertion between helices 8 and 9 (Omiecinski et al., 2011). As reported previously, phenobarbital (Lau et al., 2011a) and various flavonol analogues (galangin, datiscetin, kaempferol, quercetin, isorhamnetin, and tamarixetin) (Lau and Chang, 2013) activate hCAR-WT but not hCAR-SV23 or hCAR-SV24. In contrast, di-isononyl phthalate (DeKeyser et al., 2009) and pheniramine (Dring et al., 2010) activate hCAR-SV23 and hCAR-SV24, respectively, whereas neither affects the activity of hCAR-WT. The finding that rilpivirine and etravirine activate hCAR-WT but not hCAR-SV23 or hCAR-SV24 leads to the possibility of inter-individual differences in hCAR-
mediated effects, given the inter-individual differences reported for hepatic gene expression of hCAR-WT (Chang et al., 2003a).

Among the first generation NNRTIs investigated in the present study, efavirenz at concentrations of 2.5-10 µM, 0.5-10 µM, and 5-10 µM activated hCAR-WT, hCAR-SV23, and hCAR-SV24, respectively. This occurred by a mechanism that did not appear to involve binding to the LBD of the respective receptor or recruitment of the SRC1, SRC2, and SRC3 coactivators. In a previous report (Svard et al., 2010), 10 µM efavirenz was shown not to activate hCAR-WT in a cell-based reporter gene assay. However, any effect of efavirenz on hCAR-WT activity may have been masked in that study (Svard et al., 2010) because their HepG2 cells were not treated with an inverse agonist (e.g. androstanol) to attenuate the high basal activity normally associated with hCAR-WT in cultured HepG2 cells (Burk et al., 2005b). The results on activation of hCAR-SV24 by efavirenz are consistent with those reported in a previous study (Faucette et al., 2007). Among the other first generation NNRTIs investigated in the present study, nevirapine and delavirdine at concentrations up to 10 µM did not influence the activity of hCAR-WT, hCAR-SV23, or hCAR-SV24. In the case of nevirapine, it appears that a greater concentration is needed to activate hCAR-SV24, as shown in a previous finding that suprapharmacological concentrations (50 and 100 µM) of this drug activate hCAR-SV24 (Faucette et al., 2007; Chen et al., 2010). Overall, the first generation NNRTIs activated hCAR in an isoform-selective and ligand-specific manner. Furthermore, efavirenz activated hCAR-WT, hCAR-SV23 and hCAR-SV24 by a mechanism that is distinct from that of hCAR-WT and hCAR-SV24 activation by CITCO and of hCAR-SV23 activation by DEHP (Lau and Chang, 2013).

hCAR regulates the expression of CYP2B6, which is its prototypical target gene (Sueyoshi et al., 1999). The CYP2B6 enzyme metabolizes a diverse group of drugs, including
bupropion (Hesse et al., 2000), efavirenz (Ward et al., 2003), and nevirapine (Erickson et al., 1999). The CYP2B6-inducing concentrations of rilpivirine (0.5-5 µM), etravirine (2.5-5 µM), and efavirenz (5-10 µM) evident in the sandwich-cultured human hepatocyte experiment are comparable to the steady-state maximum plasma concentrations reported for rilpivirine (0.30 ± 0.08 µM; mean ± S.D.) (Goebel et al., 2006), etravirine (up to 5 µM) (Gagliardini et al., 2014), and efavirenz (12.98 µM; 95% confidence interval, 7.95-18.27 µM) (Nanzigu et al., 2012). Whether rilpivirine and etravirine influence the elimination pharmacokinetics of CYP2B6-metabolized drugs is not known, but efavirenz has been reported to decrease by approximately one-half the systemic exposure to bupropion (i.e. the area under the plasma bupropion concentration-time curve) in a study of healthy human subjects (Robertson et al., 2008). Interestingly, *in vitro* experiments have identified rilpivirine (IC$_{50}$ = 4.2 ± 1.6 µM) (Weiss and Haefeli, 2013) and efavirenz (at 10 and 100 µM concentrations) (Hesse et al., 2001) as inhibitors of CYP2B6 catalytic activity. Therefore, in the human hepatocyte samples treated with one of these drugs, the magnitude of bupropion hydroxylation reflects perhaps not only an inductive effect on *CYP2B6* gene expression, but also an inhibitory effect on CYP2B6 catalytic activity by rilpivirine and efavirenz.

In conclusion, NNRTIs activate hCAR in an isoform-selective and drug-specific manner. Rilpivirine and etravirine activate hCAR-WT, whereas efavirenz activates hCAR-WT and its SV23 and SV24 splice variants. The activation of these hCAR isoforms occurs by a mechanism that does not appear to involve binding to the LBD of the respective receptor or recruitment of SRC1, SRC2, or SRC3 coactivators. Finally, rilpivirine, etravirine, and efavirenz are not a CITCO-type of hCAR-WT and hCAR-SV24 activator nor are they DEHP-type of hCAR-SV23 activator.
Chapter 4: Role of MicroRNA-18a in Activation of Pregnane X Receptor by Rilpivirine

4.1 Introduction

Pregnane X receptor (PXR) is a ligand-activated, DNA-binding nuclear receptor that belongs to the NR1I superfamily (Tolson and Wang, 2010). Since its discovery in 1998 (Lehmann et al., 1998; Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998), PXR has been shown to regulate diverse biological processes, ranging from energy metabolism (Gao and Xie, 2010) to pathophysiological states, such as bile acid toxicity (Staudinger et al., 2001), dyslipidemia (Gao and Xie, 2012), inflammatory bowel disease (Shah et al., 2007). PXR also regulates several genes encoding drug-metabolizing enzymes and transporters involved in the disposition of endobiotics and xenobiotics (Chai et al., 2013). The expression of this receptor is regulated transcriptionally by a complex network involving several other nuclear receptors, including glucocorticoid receptor (Pascussi et al., 2000a; Pascussi et al., 2001), peroxisome proliferator-activated receptor-α (Aouabdi et al., 2006), and hepatic nuclear factor-4α (Iwazaki et al., 2008). However, post-transcriptional mechanisms may also be involved (Smutny et al., 2013).

MicroRNAs belong to a family of small non-coding RNAs, consisting of approximately 18-25 nucleotides (Bartel, 2004). They are involved in post-transcriptional regulation of genes controlling various physiological and pathophysiological processes, such as development and differentiation, metabolism, cancer, and other infections (Huang et al., 2011). The advent of
bioinformatics tool has facilitated prediction of specific microRNAs targets with potential regulatory association with downstream targets (Lagana, 2015). Based on *in silico* predictions and *in vitro* experiments to date, over 100 microRNAs have been associated with regulation of more than 260 genes involved in drug absorption, distribution, metabolism, and excretion, including those encoding various drug-metabolizing enzymes, drug transporters, and nuclear receptors (He *et al.*, 2015). Based on the sequence complementarity, microRNA-mediated post-transcriptional regulation leads to either target mRNA degradation or translational repression (Bartel, 2004). It has been demonstrated in a panel of 25 human livers that PXR mRNA expression does not correlate with PXR protein expression, suggesting involvement of post-transcriptional regulatory mechanism(s) (Takagi *et al.*, 2008). In the same study, microRNA-148a (miR-148a) (Takagi *et al.*, 2008) was shown to directly regulate PXR by binding to microRNA-specific recognition elements in the 3′-untranslated region (3′-UTR) of PXR. However, other studies (Rieger *et al.*, 2013; Wei *et al.*, 2013) reported conflicting findings, disputing the claims made earlier (Takagi *et al.*, 2008).

Previously, we identified rilpivirine as an agonist of PXR and an inducer of a PXR target gene [(Sharma *et al.*, 2013), also see Chapter 2]. The overall purpose of the present study was to investigate whether there is an association between a select microRNA and drug activation of PXR. The specific aims were to: 1) perform bioinformatic analysis to identify microRNAs with sequence complementary to the 3′-UTR of PXR; 2) conduct cell-based assays to determine the effect of a select microRNA on the expression and functionality of PXR; and 3) investigate whether a select microRNA plays a role in PXR activation by rilpivirine. The experimental approaches involved *in silico* prediction, cell-based reporter gene assays, overexpression of
microRNA, and target gene and protein expression analysis. The results provide the first demonstration that microRNA-18a (miR-18a) is a regulator of PXR.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Rilpivirine was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), whereas rifampin and pregnenolone 16α-carbonitrile (PCN) were bought from and Sigma-Aldrich (St. Louis, MO, U.S.A.). Charcoal-stripped fetal bovine serum (FBS), minimum essential medium/Earle’s Balanced Salts Solution (MEM/EBSS), and minimum essential medium/non-essential amino acid solution (100×) (HyClone Laboratories, Logan, UT, U.S.A.) were purchased from Thermo Fisher Scientific (Nepean, ON, Canada). All other chemicals and reagents related to cell culture were purchased from sources listed previously [(Sharma et al., 2013; Sharma et al., 2015), also see Chapter 2 and Chapter 3].

TaqMan® microRNA Assay Kit for hsa-miR-18a-5p (miR-18a) (catalog number: 4427975, assay id: 002422) and hsa-miR-16 (miR-16) (catalog number: 4427975, assay id: 000391), mirVana microRNA mimic negative control #1 (mimic control) (catalog number: 4464058), hsa-miR-18a-5p mirVana microRNA mimic (miR-18a mimic) (catalog number: 4464066, assay id: MC12973), hsa-miR-16 mirVana microRNA mimic (miR-16 mimic) (catalog number: 4464066, assay id: MC10339), TaqMan® MicroRNA Reverse Transcription Kit (catalog number: 4366596), Qubit® RNA HS Assay Kit (catalog number: Q32855), TRIZol® Reagent, SuperSignal™ West Femto Maximum Sensitivity Substrate, PicoGreen Double-Stranded DNA Quantification Kit, Lipofectamine® RNAiMAX, and Lipofectamine® 2000 were
purchased from Life Technologies (Thermo Fisher Scientific, Burlington, ON, Canada). EvaGreen 2× qPCR MasterMix-ROX and TaqProbe 2× qPCR MasterMix-ROX were bought from Applied Biological Materials (Richmond, BC, Canada). The Dual-Luciferase Reporter Assay System was from Promega (Madison, WI, U.S.A.). Recombinant human PXR (hPXR), human constitutive androstane receptor (hCAR), and human vitamin D receptor (hVDR) protein were purchased from OriGene Technologies (Rockville, MD, U.S.A.). Primary antibodies (anti-PXR H-11 mAb and anti-GAPDH 6C5 mAb), horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (sc-2005), and radioimmunoprecipitation assay (RIPA) lysis buffer system were purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). DC™ Protein Assay Kit was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada).

4.2.2 Plasmids

pCMV6-XL4-hPXR, pCMV6-XL4, pGL3-basic-CYP3A4-XREM-luc reporter, and the internal control *Renilla reniformis* luciferase pGL4.74 [hRluc/TK] plasmid were procured from sources as described previously [(Sharma et al., 2013), also see Chapter 2]. The pGL3-promoter was purchased from Promega (Madison, WI, U.S.A.). The portion of human PXR mRNA from +1041 to +1064 upstream to the coding sequence was identified as the miR-18a microRNA recognition element (MRE) using www.microRNA.org, an online bioinformatics tool (Betel et al., 2008). Three copies of the predicted MRE of miR-18a (5′-GAA-GAA-CCA-TTT-ACA-TGC-ACC-TTA-3′) in the 3′-UTR of human PXR were amplified and cloned into the pGL3-promoter vector using *KpnI* and *XhoI* restriction sites downstream of the *luciferase* gene. This construct was named pGL3-promoter-3×PXR-MRE18a. Similarly, complementary sequence of 3 copies of miR-18a MRE was amplified and cloned into the pGL3-promoter vector. This
construct was named pGL3-promoter-3×PXR-MRE18a-REV. A portion containing perfect matching sequence (5′-CTA-TCT-GCA-CTA-GAT-GCA-CCT-TA-3′) of the mature miR-18a was amplified and cloned into the pGL3-promoter vector. This construct was named pGL3-promoter-complementary-18a. Each of these constructs was synthesized at GenScript (Piscataway, NJ, U.S.A.).

4.2.3 Cell Culture

LS180 human colon adenocarcinoma cells have been previously reported to endogenously express PXR and CYP3A4 (Gupta et al., 2008), but lack endogenous expression of constitutive androstane receptor (Gupta et al., 2008) or glucocorticoid receptor (Maier et al., 2007). Hence, LS180 cells were utilized as an experimental model in the present study. LS180 cells (CL-187) (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in T-75 culture flasks in minimum essential medium (MEM)/EBSS supplemented with 10% v/v heat-inactivated fetal bovine serum (Life Technologies, Inc., Burlington, ON, Canada), penicillin G (100 U/ml), streptomycin (100 µg/ml), MEM/non-essential amino acid (1×), and L-glutamine (2 mM). Cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂. Culture medium was changed once every 3 days, and cells were sub-cultured weekly. All experiments were conducted in MEM-EBSS medium supplemented with 10% v/v charcoal-stripped fetal bovine serum as described previously [(Sharma et al., 2015), also see Chapter 3].

4.2.4 Transient Transfection and Luciferase Reporter Gene Assays

The effect of miR-18a mimic on 3′-UTR of PXR was investigated in transiently transfected LS180 cells using luciferase reporter gene assay. LS180 cells were seeded in 24-well
plates at a density of 100,000 cells/well in a volume of 0.5 ml of supplemented culture medium. At 24 h post-plating, cells were transfected with pGL3-promoter (empty vector control), pGL3-promoter-3×PXR-MRE18a, pGL3-promoter-3×PXR-MRE18a-REV, or pGL3-promoter-complementary-18a (each at 200 ng/well)] and 50 nM of mirVana microRNA mimic negative control #1 (mimic control) or hsa-miR-18a-5p mirVana microRNA mimic (miR-18a mimic) for 48 h using Lipofectamine® 2000, as described in the manufacturer’s protocol. An internal control pGL4.74 [hRluc/TK] vector (10 ng/well) was added to each well. Firefly luciferase and *Renilla reniformis* (internal control) luciferase activities were quantified and normalized as described previously [(Sharma *et al.*, 2013), also see Chapter 2]. Results are expressed as ratio of Firefly luciferase and *Renilla reniformis* luciferase activities. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

To determine the effect of rilpivirine and rifampin on hPXR-dependent reporter activity in LS180 cells, luciferase reporter gene assay was performed as described previously [(Sharma *et al.*, 2013), also see Chapter 2]. Cells were transfected with a master mix containing FuGENE 6 transfection reagent (3 µl/µg of DNA), serum-free Opti-MEM (20 µl/well), pGL4.74 [hRluc/TK] internal control vector (5 ng/well), a pGL3-basic-CYP3A4-XREM-luc reporter construct (50 ng/well), and either a pCMV6-XL4-hPXR receptor expression plasmid or the corresponding pCMV6-XL4 empty vector (50 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of supplemented medium containing DMSO (0.1 % v/v, vehicle control), rifampin (10 µM), or rilpivirine (5 µM) for 24 h. Firefly luciferase and *Renilla reniformis* (internal control) luciferase activities were quantified as described above. Each experiment was performed in triplicate and a total of three independent experiments were conducted.
4.2.5 Transfection of LS180 Cells with a Mimic of miR-18a

An experiment was performed to demonstrate that transfection reagent or the mimic control does not alter endogenous expression of miR-18a. Briefly, LS180 cells were seeded in 10 cm petri dishes at a density of $1 \times 10^6$ cells/plate. At 24 h post-plating, cells were transfected with either Lipfectamine® RNAiMAX or Lipfectamine® RNAiMAX containing 25 or 50 nM mimic control for 48 h. At the end of transfection period, total RNA or whole cell lysates were isolated as described in Sections 4.2.7 and 4.2.10, respectively. Reverse transcription was performed, and miR-18a and miR16 (to normalize miR-18a data) expression were measured as described under Section 4.2.8. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

In another experiment, the appropriate duration of transfection was determined. LS180 cells were transfected with 25 nM mimic control or a miR-18a mimic for 24, 48, or 72 h using Lipfectamine® RNAiMAX. To determine an appropriate concentration of a miR-18a mimic, LS180 cells were transfected with either 25 or 50 nM mimic control or miR-18a mimic for 48 h using Lipfectamine® RNAiMAX transfection reagent. At the end of transfection period, total RNA or whole cell lysates were isolated from the cells and target gene and protein expression was measured as described under Sections 4.2.7, 4.2.8, and 4.2.10. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

To determine the effect of miR-18a over-expression on PXR expression and PXR target gene ($CYP3A4$) expression, LS180 cells were seeded in 10 cm petri dishes at a density of $1 \times 10^6$ cells per plate. At 24 h post-plating, cells were transfected with either 50 nM mimic control or a miR-18a mimic using Lipfectamine® RNAiMAX transfection reagent. At 48 h post-transfection, culture medium was replaced with MEM-EBSS containing rilpivirine (5 µM),
rifampin (10 µM), or DMSO (0.1 % v/v; control) for 24 h. At the end of drug treatment, total RNA and whole cell lysates were isolated. mRNA (PXR, CYP3A4, or HPRT) and protein (PXR) levels were determined as described under Sections 4.2.7, 4.2.9, and 4.2.10. Each experiment was performed in triplicate and three independent experiments were conducted.

4.2.6 Temporal Profiling of Endogenous Expression of miR-18a, PXR mRNA and CYP3A4 mRNA Expression in LS180 Cells Treated with Rilpivirine or Rifampin

The temporal profile for the effect of drug treatment on endogenous miR-18a expression was characterized in LS180 cells treated with rilpivirine or rifampin. LS180 cells were plated in 10 cm petri dishes at a density of $1 \times 10^6$ cells per dish. At 48 h post-plating, the culture medium was replaced with MEM-EBSS containing rilpivirine (5 µM), rifampin (10 µM), or DMSO (0.1 % v/v; control) for 0, 3, 6, 12, or 24 h. At the end of treatment period, total RNA was isolated and reverse transcribed. miR-18a, miR-16 (to normalize miR-18a data), PXR, CYP3A4, and HPRT (to normalize PXR and CYP3A4 mRNA data) expression levels were determined as described under Sections 4.2.7, 4.2.8, and 4.2.9. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

4.2.7 Isolation and Quantitation of Total RNA

Total RNA was isolated using TRIzol® (Chang et al., 2003b) and quantified using the Qubit® RNA HS Assay Kit (Thermo Fisher Scientific, Burlington, ON, Canada). A working solution of Qubit® RNA HS Reagent (1:200 dilution) was prepared in Qubit® RNA HS Buffer. Samples were prepared by adding 199 µl of working solution of Qubit® RNA HS Reagent and 1 µl total RNA. After brief mixing, samples were incubated at room temperature for 2 min. Total
RNA was quantified in a Qubit® 2.0 Fluorometer at an excitation maximum wavelength of 644 nm and an emission maximum wavelength of 673 nm.

4.2.8 Determination of miR-18a and miR-16 Expression

Total RNA was reverse transcribed using miR-18a or miR-16 specific primers (TaqMan® microRNA assay, Thermo Fisher Scientific, Burlington, ON, Canada) and reagents supplied in the TaqMan® microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Burlington, ON, Canada). Briefly, each 15 µl volume of the reverse transcription reaction contained 5 µl diluted RNA (20 ng/µl), 3 µl of 5× reverse transcription primers specific for miR-18a or miR-16, and 7 µl reverse transcription master mix. Each 7 µl of the reverse transcription master mix contained 1 µl Multiscribe® RTase, 1.5 µl of 10× reverse transcription buffer, 0.19 µl of RNAse inhibitor, 0.15 µl of dNTP, and 4.16 µl of PCR water. Reverse transcription was performed at 16°C for 30 min followed by 42°C 30 min, 85°C for 5 min, and 4°C for 10 min. Subsequently, miR-18a cDNA or miR-16 cDNA was amplified by real-time PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems, Burlington, ON, Canada). Each 20 µl volume of the PCR reaction contained 10 µl of TaqProbe 2× qPCR MasterMix-ROX, 1 µl of 20× PCR primers specific for miR-18a or miR-16 diluted in 8 µl of PCR water, and 1 µl of the amplicon. PCR was initiated at 95°C for 10 min (activation of Taq enzyme) followed by 40 cycles of denaturation (95 °C for 15 sec) and annealing/extension (60°C for 60 sec). miR-16 expression was used to normalize miR-18a expression. Calibration curves (cross point versus log copy number, 1 pM - 100 nM) for miR-18a and miR-16 were constructed using miR-18a mimic and miR-16 mimic, respectively. Results are expressed as ratio of miR-18a and miR-16.
Each experiment was performed in triplicate and a total of three independent experiments were conducted.

4.2.9 Determination of PXR and CYP3A4 mRNA Expression

Total RNA was isolated and reverse transcribed [(Sharma et al., 2013), also see Chapter 2]. cDNA concentrations were quantified using the PicoGreen Double-Stranded DNA Quantitation Kit (Life Technologies, Burlington, ON, Canada) (Singer et al., 1997). PXR cDNA, CYP3A4 cDNA, and HPRT cDNA were amplified by real-time PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems, Burlington, ON, Canada). Each 20 µl volume of the PCR reaction contained 10 µl of EvaGreen 2× qPCR MasterMix-ROX, 5 µl of gene-specific primers, and 5 µl of the amplicon. PXR, CYP3A4, and HPRT primer sequences have been shown previously (Lau et al., 2011b). The PCR cycling conditions were 95°C for 10 min (activation of Taq enzyme), followed by 40 cycles of 95°C for 15 s (denaturation), 55°C for 10 s (annealing), and 60°C for 50 s (elongation). Calibration standards for specific gene were generated using method described previously (Chang et al., 2006). PXR and CYP3A4 mRNA expression was normalized to HPRT mRNA expression. Each experiment was performed in triplicate and a total of three independent experiments were conducted.

4.2.10 Immunoblot Analysis

Whole cell lysates were prepared using the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Dallas, TX, U.S.A.). Total protein concentrations in cell lysates were quantified using the DC™ Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Whole cell lysates (75 µg) were separated on 12% sodium-dodecyl sulfate - polyacrylamide gel (SDS-
PAGE) and transferred on to Immobilon®-P polyvinylidene fluoride membranes (EMD Millipore, Etobicoke, ON, Canada). After blocking in 5% milk, membranes were incubated over-night with anti-PXR H-11 mAb (1: 500) (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) at 4°C. Subsequently, after washing, the membranes were incubated with HRP-conjugated anti-mouse secondary antibody (1:5000) (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) at room temperature. Proteins were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (600 µl) (Thermo Fisher Scientific, Burlington, ON, Canada) and images of the protein bands were taken using the Alpha Innotech FluorChem 8800 Gel Box Imager. Densitometric image analysis was performed using the NIH ImageJ software (National Institutes of Health, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.

4.2.11 Data Analyses

Data analyses were performed by one-way or two-way analysis of variance. Where appropriate, it was followed by the Student Newman-Keuls multiple comparison test (SigmaPlot 11.0; Systat Software, Inc., San Jose, CA, U.S.A.). The level of statistical significance was set a priori at $P < 0.05$.

4.3 Results

4.3.1 Prediction of miR-18a MRE in the 3′-UTR of PXR

To predict the identity of microRNA target(s) with potential involvement in regulating the expression of PXR, an online bioinformatics database, [www.microRNA.org](http://www.microRNA.org), was utilized that
predicts potential targets based on sequence complementarity between and thermodynamic stability of mRNA-microRNA duplex (Betel et al., 2008). Based on the selection criteria (target site of conserved microRNAs with good mirSVR scores), a total of 29 miR targets were predicted (listed in Table 4.1). miR-18a was selected for further investigation based on preliminary data and a previous report where miR-18a was shown to regulate the expression of glucocorticoid receptor (Vreugdenhil et al., 2009), a nuclear receptor known to regulate the expression of PXR (Pascussi et al., 2000b; Pascussi et al., 2001). As shown in Fig. 4.1, the predicted MRE of miR-18a in the 3′-UTR of PXR stretches from position 1041 to 1064 upstream of the coding sequence. This information about MRE was utilized in designing various reporter plasmids to investigate the potential involvement of miR-18a in regulation of 3′-UTR of human PXR.

### Table 4.1 List of Predicted MicroRNA with Sequence Complementarity in the 3′-UTR of Human PXR

<table>
<thead>
<tr>
<th>microRNA</th>
<th>mirSVR Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-18a</td>
<td>-1.1567</td>
</tr>
<tr>
<td>hsa-miR-18b</td>
<td>-1.1567</td>
</tr>
<tr>
<td>hsa-miR-34a</td>
<td>-0.4060</td>
</tr>
<tr>
<td>hsa-miR-34c-5p</td>
<td>-0.4122</td>
</tr>
<tr>
<td>hsa-miR-98</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
<td>-0.1535</td>
</tr>
<tr>
<td>hsa-miR-148b</td>
<td>-0.1535</td>
</tr>
<tr>
<td>hsa-miR-152</td>
<td>-0.1535</td>
</tr>
<tr>
<td>microRNA</td>
<td>mirSVR Score</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>hsa-miR-219-5p</td>
<td>-0.4185</td>
</tr>
<tr>
<td>hsa-miR-214</td>
<td>-0.2183</td>
</tr>
<tr>
<td>hsa-miR-222</td>
<td>-0.3377</td>
</tr>
<tr>
<td>hsa-miR-299-3p</td>
<td>-0.1632</td>
</tr>
<tr>
<td>hsa-miR-221</td>
<td>-0.3377</td>
</tr>
<tr>
<td>hsa-miR-338-3p</td>
<td>-0.1242</td>
</tr>
<tr>
<td>hsa-miR-340</td>
<td>-1.0855</td>
</tr>
<tr>
<td>hsa-miR-374a</td>
<td>-0.1135</td>
</tr>
<tr>
<td>hsa-miR-374b</td>
<td>-0.1158</td>
</tr>
<tr>
<td>hsa-miR-377</td>
<td>-0.1937</td>
</tr>
<tr>
<td>hsa-miR-449a</td>
<td>-0.4185</td>
</tr>
<tr>
<td>hsa-miR-449b</td>
<td>-0.4185</td>
</tr>
<tr>
<td>hsa-miR-758</td>
<td>-0.2116</td>
</tr>
<tr>
<td>hsa-let-7a</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-let-7b</td>
<td>-0.5639</td>
</tr>
<tr>
<td>hsa-let-7c</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-let-7d</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-let-7e</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-let-7f</td>
<td>-0.5567</td>
</tr>
<tr>
<td>hsa-let-7g</td>
<td>-0.5603</td>
</tr>
<tr>
<td>hsa-let-7i</td>
<td>-0.5639</td>
</tr>
</tbody>
</table>
4.3.2 miR-18a Targets 3'-UTR of PXR in LS180 Cells

Various plasmids were cloned into the pGL3-promoter vector downstream of the luciferase gene (Fig. 4.2A). LS180 cells were transfected in the presence of a miR-18a mimic or a mimic control. As shown in Fig. 4.2B, the luciferase activity in cells transfected with pGL3-promoter-3×PXR-MRE-18a (construct containing 3 copies of miR-18a MRE) or pGL3-promoter-complementary-18a (construct with perfect matching complement of miR-18a) and miR-18a mimic was decreased when compared to the group transfected with the corresponding reporter plasmid and mimic control, and the group transfected with pGL3-promoter (empty vector control) and a miR-18a mimic ($P < 0.001$). As expected, the luciferase activity with pGL3-promoter-3×PXR-MRE-18a-REV (construct containing 3 copies complementary sequence of miR-18a MRE) was not affected in the presence of a miR-18a mimic ($P = 0.313$). These results indicate that miR-18a regulates PXR by targeting its recognition site in the 3'-UTR of PXR.
Figure 4.1 Schematic Representation of the Sequence Complementarity between miR-18a and the Predicted MRE on Human PXR.

At the 5' end, number 1840 refers to the start of the coding sequence that continues to position 3141, with a stop codon at 3142-3144. The predicted MRE for miR-18a (23 nucleotide sequence) is located at position 1041 to 1064 upstream to the coding sequence in the 3'-UTR of human PXR mRNA.
Figure 4.2 Effect of miR-18a on 3′-UTR of PXR in LS180 Cells.

(A) Diagrammatic representation of pGL3-promoter (empty vector control), pGL3-promoter-3×PXR-MRE18a (reporter construct containing 3 copies of miR-18a MRE), pGL3-promoter-3×PXR-MRE18a-REV (reporter construct containing 3 copies complementary sequence of miR-18a MRE), and pGL3-promoter-complementary-18a (reporter construct with perfect matching complement of miR-18a). All plasmid sequences were inserted downstream of the Firefly luciferase gene using Kpn1 and Xho1 restriction sites. (b) LS180 cells were transfected with either a mimic control or miR-18a mimic (50 nM) and one of the following constructs: 1) pGL3-promoter (empty vector control), 2) pGL3-promoter-3×PXR-MRE18a, 3) pGL3-promoter-3×PXR-MRE18a-REV, or 4) pGL3-promoter-complementary-18a (each at 200 ng/well) for 48 h. Cells in each well were also transfected with pGL4.74 [hRluc/TK] (10 ng/well; internal control). Firefly luciferase and Renilla reniformis luciferase activities were measured and normalized as described in Section 4.2.4. Data are expressed as mean ± SEM for three independent experiments performed in triplicate. *, significantly different from the group cotransfected with the corresponding reporter plasmid and mimic control, and the group cotransfected with pGL3-promoter (empty vector control) and a miR-18a mimic (P < 0.05).
4.3.3 miR-16 is an Appropriate microRNA for Normalizing miR-18a Expression

miR-16 is a microRNA appropriate for normalizing microRNA expression (Davoren et al., 2008; McDermott et al., 2013; Xiang et al., 2014). As confirmed in an initial experiment, transfection reagent (Fig. 4.3A), mimic control (Fig. 4.3A), and either rilpivirine or rifampin (Fig. 4.3B) did not alter miR-16 expression. Therefore, miR-16 was used to normalize to miR-18a expression.

4.3.4 Endogenous miR-18a Expression is not Affected by Transfection Reagent or Mimic Control

To rule out the possibility that the transfection reagent or mimic control affects the endogenous expression of miR-18a, cultured LS180 cells were transfected with transfection reagent (Lipofectamine® RNAiMAX) or transfection reagent containing a mimic control (25 or 50 nM). Endogenous levels of miR-18a were quantified by RT-PCR and compared with that of control LS180 cells (untransfected cells). As shown in Fig. 4.4, miR-18a expression was comparable in untransfected LS180 cells or in LS180 cells transfected with transfection reagent or a mimic control.
Figure 4.3 Effect of Transfection or Drug Treatment on Endogenous Expression of miR-16 in LS180 Cells.

(A) Cultured LS180 cells were untransfected or transfected with Lipofectamine® RNAiMAX (transfection reagent control), or Lipofectamine® RNAiMAX containing a mimic control (25 nM) for 48 h. (B) Cultured LS180 cells were treated with DMSO (0.1% v/v; control), rilpivirine (RPV; 5 µM), or rifampin (RIF; 10 µM) for 24 h. At the end of transfection or drug treatment period, cells were lysed and total RNA was isolated. Absolute miR-16 copy number data were quantified by qPCR, as described in Section 4.2.8. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate.
Figure 4.4 Endogenous miR-18a Expression in LS180 Cells Transfected with Transfection Reagent or a Mimic Control.

Cultured LS180 cells were untransfected or transfected with Lipofectamine® RNAiMAX (transfection reagent), or Lipofectamine® RNAiMAX containing a mimic control (25 or 50 nM). At 48 h post-transfection, cells were lysed and total RNA was isolated. miR-18a and miR-16 expression levels were measured by qPCR, as described in Section 4.2.8. miR-18a expression was normalized to miR-16 expression. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate.
### 4.3.5 Expression Profile of miR-18a in LS180 Cells Transfected with a Mimic of miR-18a

Following transfection with 25 nM mimic of miR-18a, the increase in miR-18a expression was greatest at 24 h post-transfection \((P < 0.001)\), and started declining at 48 h \((P < 0.001)\) and 72 h \((P < 0.001)\), as shown in Fig. 4.5A. The increases in miR-18a expression with 25 or 50 nM mimic were comparable \((P = 0.913)\), but significantly different from corresponding mimic control groups \((P < 0.001)\), as shown in Fig. 4.5B. Therefore, 48 h transfection period and 50 nM mimic were used in subsequent experiments.
Figure 4.5 Expression Profile of miR-18a in LS180 Cells Transfected with a Mimic of miR-18a.

(A) Cultured LS180 cells were transfected with 25 nM mimic control or miR-18a mimic for 24, 48, or 72 h. (B) LS180 cells were transfected with 25 or 50 nM of mimic control or miR-18a mimic for 48 h. At the end of each transfection period, total RNA was isolated and miR-18a and miR-16 expression was measured, as described under Section 4.2.8. miR-18a expression was normalized to miR-16 expression. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding mimic control group ($P < 0.05$).
4.3.6 Transfection of LS180 Cells with a Mimic of miR-18a Decreases PXR mRNA and Protein Expression

The effect of transfecting LS180 cells with a miR-18a mimic on PXR expression was investigated. Transfecting LS180 cells with a miR-18a mimic at a concentration (25 or 50 nM) that increased the level of miR-18a (Fig. 4.5A and 4.5B) was associated with a decrease in PXR mRNA (Fig. 4.6A) \( (P < 0.001) \) and protein (Fig. 4.6B) expression. Control immunoblot analysis indicated that the anti-PXR antibody reacted with human PXR, but it did not cross-react with human CAR or VDR (Fig. 4.6B). These data suggest that miR-18a regulate PXR at both the mRNA and protein levels.
Figure 4.6 PXR mRNA and Protein Expression in LS180 Cells Transfected with a Mimic of miR-18a.
LS180 cells were transfected with a miR-18a mimic or mimic control (each at 25 or 50 nM) for 48 h. (A) PXR mRNA expression was measured using qPCR, as described in Section 4.2.8. PXR mRNA expression was normalized to HPRT mRNA expression. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding mimic control group ($P < 0.05$). (B) Lysates from transfected LS180 cells, human recombinant PXR protein (positive control), human recombinant constitutive androstane receptor protein (CAR; negative control), and human recombinant vitamin D receptor protein (VDR; negative control) were subjected to SDS-PAGE. Immunoblots were probed with the anti-PXR H-11 mAb antibody, as described in Section 4.2.9. PXR protein expression were normalized to GAPDH protein expression. Data are expressed as mean ± S.E.M. of three independent experiments performed in triplicate. *, significantly different from the corresponding mimic control group ($P < 0.05$).
4.3.7 Human PXR Agonists, Rilpivirine and Rifampin, Decreased Endogenous Expression of miR-18a

In cultured LS180 cells, both rifampin and rilpivirine activated PXR in a cell-based reporter gene assay ($P < 0.001$) (Fig. 4.7). To investigate whether treatment with a known agonist of human PXR modulates endogenous expression of miR-18a, a time course experiment was performed in cultured LS180 cells. Preliminary data showed no drug-dependent changes in endogenous expression of miR-18a at time points > 24 h. Hence, subsequent experiment looked at changes in endogenous expression of miR-18a in LS180 cells treated with rilpivirine (5 µM), rifampin (10 µM), or DMSO (0.1% v/v; vehicle control) for 3, 6, 12, and 24 h. As shown in Fig. 4.8A, rilpivirine started to decrease miR-18a expression at 3 h post-treatment ($P < 0.001$), with a maximal decrease observed at 6 h ($P < 0.001$) and 12 h ($P < 0.001$) post-treatment and returned back to basal levels by 24 h ($P = 0.071$). By comparison, rifampin treatment decreased miR-18a expression as early as 3 h ($P < 0.001$) and continued this effect until 6 h ($P < 0.001$) post-treatment. The expression of miR-18a returned to basal levels at 12 h ($P = 0.066$) and 24 h ($P = 0.073$) post-treatment. As expected, LS180 cells treated with the vehicle control (DMSO, 0.1% v/v) did not change miR-18a expression over the 24 h period ($P > 0.05$). These data indicate that the decrease in miR-18a expression by rilpivirine and rifampin have different temporal profiles and that it is necessary to include early time points for capturing any changes in endogenous expression of microRNA expression.

In LS180 cells treated with rilpivirine or rifampin, drug treatment did not modulate PXR mRNA expression over the course of 24 h, as shown in Fig. 4.8B ($P = 0.062 - 0.742$). By comparison, rilpivirine and rifampin increased CYP3A4 mRNA expression, although the time profiles were different (Fig.4.8C). The increase in CYP3A4 mRNA in LS180 cells treated with
rilpivirine did not occur until 24 h after drug treatment ($P < 0.001$), whereas rifampin induced CYP3A4 expression at 12h ($P < 0.001$) and 24 h ($P < 0.001$) after drug treatment. In a previous experiment, both rilpivirine and rifampin induced CYP3A4 expression at 24 h after treatment of primary cultures of human hepatocytes [(Sharma et al., 2013), also see Chapter 2].

Figure 4.7 Effect of Rifampin and Rilpivirine on PXR Activation in LS180 Cells Transfected with a PXR Expression Plasmid.

Cultured LS180 cells were transfected with pGL3-basic-CYP3A4-XREM-luc (50 ng/well), pGL4.74 [hRluc/TK] (10 ng/well), and either pCMV6-XL4-hPXR or pCMV6-XL4 (empty vector) (50 ng/well). At 24 h post-transfection, cells were treated with DMSO (0.1% v/v; control), PCN (10 µM), rilpivirine (RPV; 5 µM), or rifampin (RIF; 10 µM) for 24 h. Firefly luciferase and Renilla reniformis luciferase activities were measured and normalized as described under Section 4.2.4. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with empty vector and the vehicle-treated control group transfected with the hPXR expression plasmid ($P < 0.05$).
Figure 4.8 Effect of Rifampin and Rilpivirine on the Endogenous Expression of miR-18a, PXR mRNA, and CYP3A4 mRNA in LS180 Cells.

Cultured LS180 cells were either untreated or treated with DMSO (0.1% v/v; control), rilpivirine (RPV; 5 µM), or rifampin (RIF; 10 µM) for 3, 6, 12, or 24 h. At the end of treatment period, cells were lysed and total RNA was isolated. Expression levels of (A) miR-18a, (B) PXR mRNA, and (C) CYP3A4 mRNA were measured.
mRNA, and (C) CYP3A4 mRNA were quantified by qPCR, as described under Sections 4.2.8 and 4.2.9. miR-18a expression was normalized to miR-16 expression, while PXR and CYP3A4 mRNA expression were normalized to HPRT mRNA expression. Data are compared with corresponding 0 h sample generated from untreated LS180 cells and expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding DMSO treated control group and the 0 h time point within the same drug treated samples ($P < 0.05$).
4.3.8 Transfection of miR-18a Mimic Decreases PXR and CYP3A4 Expression in LS180 Cells Treated with Rilpivirine or Rifampin

The effects of miR-18a over-expression on PXR and PXR target gene (CYP3A4) expression were investigated in LS180 cells treated with rilpivirine or rifampin. As shown in Fig. 4.9A, transfection of LS180 cells with a mimic of miR-18a decreased PXR mRNA expression. The extent of decrease was similar in cells treated with rilpivirine ($P < 0.001$), rifampin ($P < 0.001$), or vehicle ($P < 0.001$) (Fig. 4.9A). Immunoblot analysis (Fig. 4.9B) showed a similar profile at PXR protein levels [rilpivirine ($P = 0.006$), rifampin ($P = 0.005$), or vehicle ($P = 0.032$)]. As shown in Fig. 4.10, over-expression of miR-18a decreased induction of CYP3A4 expression in cells treated with rilpivirine ($P < 0.001$) or rifampin ($P < 0.001$).
Figure 4.9 Effect of Transfecting with a Mimic of miR-18a on PXR Expression in Cultured LS180 Cells Treated with Rifampin or Rilpivirine.
Cultured LS180 cells were transfected with 50 nM of miR-18a or a mimic control for 48 h, and then treated with DMSO (0.1% v/v), rifampin (RIF; 10 µM), or rilpivirine (RPV; 5 µM) for 24 h. (A) PXR mRNA and HPRT mRNA expression was measured using qPCR, as described in Section 4.2.8. PXR mRNA expression was normalized to HPRT mRNA expression. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding mimic control group (P < 0.05). (B) PXR protein expression was measured by immunoblot analysis and normalized to the GAPDH protein levels, as described in Section 4.2.9. Results are represented as fold change over the vehicle-treated control group and are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the corresponding mimic control group (P < 0.05).
Figure 4.10 Effect of Transfecting with a Mimic of miR-18a on CYP3A4 Expression in Cultured LS180 Cells Treated with Rifampin or Rilpivirine.

Cultured LS180 cells were transfected with 50 nM of miR-18a or mimic control for 48 h, following by treatment with DMSO (0.1% v/v), rifampin (RIF; 10 µM), or rilpivirine (RPV; 5 µM) for 24 h. CYP3A4 mRNA and HPRT mRNA levels was measured in these samples using qPCR, as described in Section 4.2.9. CYP3A4 mRNA expression was normalized to HPRT mRNA expression. Data are expressed as mean ± S.E.M. for three independent experiments done in triplicate. *, significantly different from the corresponding mimic control group ($P < 0.05$).
4.4 Discussion

PXR is a member of the superfamily of nuclear receptors (Germain et al., 2006). It regulates the expression and function of diverse sets of biologically significant genes (Tolson and Wang, 2010), including those that function in drug metabolism and transport (Chai et al., 2013). A novel finding of the present study is that miR-18a is a post-transcriptional regulator of human PXR. This conclusion is supported by the findings that: 1) bioinformatics analysis of the 3′-UTR of human PXR revealed a MRE for miR-18a; 2) miR-18a MRE in the 3′-UTR of human PXR was functional, as demonstrated by a cell-based reporter gene assay; and 3) over-expression of miR-18a decreased PXR mRNA and protein expression in LS180 cells, suggesting that miR-18a degrades PXR mRNA, thereby leads to decreased protein synthesis. Previously, miR-18a was shown to regulate the expression of glucocorticoid receptor (Vreugdenhil et al., 2009), a nuclear receptor known to regulate the expression of PXR (Pascussi et al., 2000b; Pascussi et al., 2001). It is possible miR-18a cross-talks with glucocorticoid receptor in mediating its modulatory effect on PXR. However, LS180 cells, the experimental model used in the present study, lack endogenous expression of glucocorticoid receptor (Maier et al., 2007), discounting any contribution of glucocorticoid receptor towards PXR regulation by miR-18a. Overall, these data indicate that miR-18a regulates PXR expression by targeting its MRE in the 3′-UTR of PXR.

Another novel finding of the present study is that over-expression of miR-18a affects PXR activation and CYP3A4 inducibility by rilpivirine and rifampin, which are known PXR agonists and CYP3A4 inducers [(Lehmann et al., 1998; Bertilsson et al., 1998; Sharma et al., 2013), see
This conclusion is supported by the finding that over-expression of miR-18a attenuated induction of CYP3A4 by rilpivirine and rifampin in LS180 cells (Fig. 4.10). By comparison, over-expression of miR-18a did not alter CYP3A4 expression in DMSO treated LS180 cells, suggesting a lack of an effect of miR-18a on CYP3A4 basal expression. Consistent with these results, miR-18a has sequence complementarity in the 3′-UTR of PXR but not CYP3A4. Overall, miR-18a modulates activation of PXR and induction of CYP3A4 by rilpivirine and rifampin in LS180 cells. It remains to be determined whether such an association occurs in vivo. In a previous study, another microRNA, miR-148a, was demonstrated to regulate PXR post-transcriptionally (Takagi et al., 2008). However, in livers from Chinese Han population, a lack of correlation exists between liver miR-148a and PXR protein/PXR mRNA ratio (Wei et al., 2013). Similar results were reported in livers from Caucasians, wherein miR-148a levels did not correlate with PXR or CYP3A4 levels (Rieger et al., 2013). These findings indicate the complex nature of regulatory pathways in vivo.

As shown in the present study, rilpivirine and rifampin decreased endogenous expression of miR-18a in LS180 cells in a time-dependent manner. It appears that drug-mediated modulation of miR-18a is an early event in PXR activation and induction of PXR target genes. Further, in a previous experiment, psychoactive drugs, such as dimethyly tryptamine, 5-methoxy dimethyly tryptamine, harmaline, methylphenidate, fluoxetine, and methadone, decreased miR-18a expression in human neuroblastoma cell line SH-SY5Y (Rodrigues et al., 2011). On the other hand, only harmaline decreased miR-18a expression in human neuroblastoma cell line BE(2)-M-17 cells, indicating that in different human cell lines microRNA expression is modulated in a drug- and cell line-dependent manner (Rodrigues et al., 2011). Interestingly, over-expression of miR-18a has been implicated in various forms of malignancies, including
lung cancer (Shen et al., 2015) and esophageal squamous cell carcinoma (Hirajima et al., 2013). Whether drug-dependent downregulation of miR-18a has any therapeutic implications in such cases require future investigations.

Overall, the conclusions from the present study performed in LS180 cells are: (A) miR-18a is a post-transcriptional regulator of PXR, (B) over-expression of miR-18a attenuates PXR activation and induction of CYP3A4 by rilpivirine and rifampin, and (C) rilpivirine and rifampin decrease miR-18a endogenous expression. Given that PXR is a master regulator of various genes important in drug metabolism and transport (Urquhart et al., 2007), it would be of interest to investigate whether miR-18a compromises the expression of other PXR target genes, such as \textit{ABCB1}, which encodes P-glycoprotein (Geick et al., 2001). The over-expression of this efflux transporter results in development of anti-cancer drug resistance (Fedoruk et al., 2004; Bao et al., 2011). Whether miR-18a decreases P-glycoprotein expression and whether that leads to enhanced efficacy of anti-cancer drugs remain to be investigated.
Chapter 5: Concluding Remarks

5.1 Summary of Major Findings and Conclusions

1. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated human, mouse, and rat PXR, as determined in cell-based luciferase reporter gene assays (Chapter 2). Rilpivirine and etravirine activated human PXR (hPXR) at concentrations 0.5-10 μM, with maximal effect at 5 μM. Efavirenz increased hPXR activity at concentration ≥ 5 μM. The EC$_{50}$ and Emax values for rilpivirine and etravirine were comparable with the literature EC$_{50}$ and Emax values for rifampin-mediated hPXR activation. hPXR-LBD transactivation and time-resolved fluorescence resonance energy transfer (TR-FRET) assays indicated that rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, bound to the ligand-binding domain of hPXR. Rilpivirine, etravirine, and efavirenz treatment triggered nuclear translocation of a green fluorescence protein-tagged hPXR, as visualized by confocal imaging. Mammalian two-hybrid assay revealed recruitment of steroid receptor coactivator1 (SRC1), SRC2, and SRC3 to hPXR, upon rilpivirine-, etravirine-, and efavirenz mediated hPXR activation. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, increased hPXR target gene (CYP3A4) expression in primary cultures of human hepatocytes. Overall, rilpivirine and etravirine are agonists of hPXR. Among the first generation NNRTIs, only efavirenz, but not nevirapine or delavirdine, is an agonist of hPXR.

2. As shown in Chapter 3, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, induced expression of hCAR target gene (CYP2B6) in primary cultures of
human hepatocytes. Rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, activated hCAR-WT, as assessed in cell-based reporter gene assays. Rilpivirine, etravirine, and efavirenz activated hCAR-WT by a mechanism that does not involve the receptor LBD (Gln-105 to Ser-348) transactivation. In primary human hepatocytes, rilpivirine, etravirine, and efavirenz, but not nevirapine or delavirdine, triggered nuclear translocation of GFP-tagged hCAR-WT. Mammalian two-hybrid assay revealed that hCAR-WT activation by rilpivirine, etravirine, and efavirenz did not involve recruitment of SRC1, SRC2, and SRC3 to hCAR-WT-LBD. Among the hCAR splice variants investigated in the study (i.e., hCAR-SV23 and hCAR-SV24), only efavirenz increased, whereas rilpivirine, etravirine, nevirapine, and delavirdine had no effect on the activity of hCAR-SV23 or hCAR-SV24. None of the drugs transactivated the LBD of hCAR-SV23 (Gln-105 to Ser-352) or hCAR-SV24 (Gln-105 to Ser-353). Among the non-nucleoside reverse transcriptase inhibitors investigated in the mammalian two-hybrid assay for each combination of a hCAR splice variant (hCAR-SV23 or hCAR-SV24) and a steroid receptor coactivator (SRC1, SRC2, or SRC3), only efavirenz recruited SRC-1 to the LBD of hCAR-SV23. Overall, rilpivirine and etravirine are indirect activators of hCAR-WT. Among the first generation NNRTIs, only efavirenz, but not nevirapine or delavirdine, activated hCAR-WT and its SV23 and SV24 splice variants.

3. As described in Chapter 4, bioinformatics analysis predicted a microRNA-18a recognition element in the 3′-UTR of PXR mRNA that stretched from position 1041 to 1064 upstream to the coding sequence. The predicted microRNA-18a recognition
element in the 3′-UTR of PXR mRNA was functional as transfection of LS180 human colon adenocarcinoma cells with a microRNA-18a mimic and pGL3-promoter-3xPXR-MRE18a or pGL3-promoter-complementary-18a decreased luciferase reporter gene activity. Over-expression of microRNA-18a in LS180 cells decreased PXR mRNA and PXR protein, suggesting that microRNA-18a caused PXR mRNA degradation that resulted in decreased PXR protein expression. Treatment of LS180 cells with a PXR agonist, rilpivirine or rifampin, did not affect PXR expression, but decreased endogenous expression of hsa-miR-18a-5p. Over-expression of microRNA-18a decreased PXR mRNA expression and attenuated rilpivirine- and rifampin-mediated PXR target gene (CYP3A4) expression in LS180 cells. Overall, microRNA-18a regulates PXR and contributes to rilpivirine activation of PXR.

5.2 Strengths and Limitations of the Present Work

5.2.1 Strengths

In the various in vitro and cell-based assays performed in the present study, rilpivirine (0.5-10 µM), etravirine (0.5-10 µM), or efavirenz (5-10 µM) activated PXR (Chapter 2, Fig. 2.2). Similarly, rilpivirine (1-10 µM), etravirine (2.5-10 µM), or efavirenz (2.5-10 µM) activated CAR-WT (Chapter 3, Fig. 3.2). These concentrations are comparable to plasma concentrations achieved following drug administration in HIV patients (Goebel et al., 2006; Nanzigu et al., 2012; Gagliardini et al., 2014). Further, primary cultures of human hepatocytes have been used as a “gold standard” in cell culture studies of the functionality of nuclear receptors (Thomas et al., 2014). In the present study, data from mechanistic studies performed in HepG2 cells were
corroborated by gene expression data in primary cultures of human hepatocytes (see Chapter 2 and Chapter 3). Finally, the conclusions in Chapter 2 and Chapter 3 were drawn based on multiple *in vitro* or cell-based assays; i.e., cell-based luciferase reporter gene assays, LBD transactivation assay, competitive ligand binding assay, *in vitro* nuclear translocation analysis, mammalian two-hybrid assay, and target gene expression assays. The conclusions in Chapter 4 were drawn based on *in silico* prediction and experimental data obtained from cell-based luciferase reporter gene assays, target gene expression, and immunoblot analysis.

### 5.2.2 Limitations

In the present study, it is unknown whether the amount of receptor expression plasmid used to investigate the effects of NNRTIs on PXR (Chapter 2) and CAR (Chapter 3) in HepG2 cells expresses these transcription factors at levels that is physiologically relevant. Further, primary cultures of human hepatocytes remain an *in vitro* experimental model that lacks the dynamics of an *in vivo* system. However, compared to some of the other cell-based models, for example human hepatocellular carcinoma HepG2 cells, hepatocytes have greater physiological relevance (Wilkening *et al.*, 2003). Lastly, primary cultures of human hepatocytes were used as an experimental model to investigate the effects of NNRTIs on PXR target gene, CYP3A4 (Chapter 2), and CAR target gene, CYP2B6 (Chapter 3). However, due to limited availability of human hepatocytes, there were only two biological samples in each experiment. As a result, statistical analysis for the experimental data could not be performed.
5.3 Future Studies

1. Although rilpivirine, etravirine, and efavirenz increased CYP3A4 expression levels (Chapter 2) in primary cultures of human hepatocytes, there were some discrepancies in mRNA expression and enzyme activity data. Rilpivirine induced CYP3A4 mRNA expression over a period of 48-72 h (Chapter 2, Fig. 2.7A and 2.7B). However, rilpivirine treatment showed a decrease and an increase in CYP3A-mediated testosterone 6β-hydroxylation in hepatocytes treated for 48 or 72 h, respectively (Chapter 2, Fig. 2.7C and 2.7D). Perhaps rilpivirine is a mechanism-based inactivator of CYP3A4 and the inhibitory effect dominates over transcriptional activation by rilpivirine after 2 days of drug treatment. This hypothesis requires further investigation.

2. Rilpivirine, etravirine, and efavirenz activated CAR-WT by a mechanism that did not involve binding to the LBD of CAR-WT (Chapter 3, Fig. 3.3) or recruitment of SRC1, SRC2, or SRC3 (Chapter 3, Fig. 3.5). However, the mechanism(s) contributing to activation of CAR-WT by these drugs remains to be determined. Inhibition of epidermal growth factor receptor signaling has been proposed as an indirect mechanism for activation of mouse CAR (Mutoh et al., 2013). Similarly, nuclear translocation triggered by dephosphorylation of threonine-38 has been proposed to be another indirect CAR activation mechanism (Mutoh et al., 2009). Whether these signaling pathways contribute to activation of human CAR-WT by rilpivirine, etravirine, or efavirenz warrants further investigation.
3. The effect of miR-18a was investigated on rilpivirine-mediated PXR activation (Chapter 4). However, among the other NNRTIs, etravirine and efavirenz were also identified as PXR activators and inducers of CYP3A4 (Chapter 2, Fig. 2.2). It remains to be verified whether miR-18a modulates effects of etravirine or efavirenz on PXR. Further, this study demonstrated that rilpivirine decreased endogenous expression of miR-18a without altering PXR mRNA expression in LS180 cells. It is not known whether etravirine or efavirenz exert a similar effect on miR-18a.

4. miR-18a has been demonstrated to attenuate rilpivirine- or rifampin-mediated induction of CYP3A4 (Chapter 4), a known target gene of PXR (Lehmann et al., 1998; Bertilsson et al., 1998). Given that PXR is a key regulator of various genes involved in drug disposition (Urquhart et al., 2007), including P-glycoprotein (Geick et al., 2001) and multidrug resistance-associated protein 2 (Kast et al., 2002), it is possible miR-18a modulate expression or function of P-glycoprotein and multidrug resistance-associated protein 2. Whether miR-18a decreases expression of these transporters and whether that leads to enhanced efficacy of drug therapy remain to be investigated.

5. As shown in Chapter 4, miR-18a is associated with post-transcriptional regulation expression and function of PXR in LS180 cells. In a previous report, miR-18a was demonstrated to regulate expression of the glucocorticoid receptor (Vreugdenhil et al., 2009), a known transcriptional regulator of PXR (Pascussi et al., 2000b; Pascussi et al., 2001). Other transcription factors that are known to regulate PXR include peroxisome proliferator-activated receptor-α (Aouabdi et al., 2006) and hepatic nuclear factor-4α
(Iwazaki et al., 2008). It is not known whether there is functional cross-talk between miR-18a and other transcriptional regulators of PXR.

6. As shown in Chapter 4, miR-18a regulates PXR post-transcriptionally in LS180 cells. Previously, a lack of correlation between PXR mRNA and PXR protein in a panel of 25 human liver samples from a Japanese population suggested role of microRNA in regulation of PXR (Takagi et al., 2008). In the same study, miR-148a was discovered as a post-transcriptional regulator of PXR (Takagi et al., 2008). However, these results were disputed as miR-148a did not correlate with PXR in human livers from Chinese Han (Wei et al., 2013) or Caucasians (Rieger et al., 2013). These differences have been attributed to ethnic differences in the populations under study and the complexity of gene regulatory pathways in vivo. Whether the association between miR-18a and PXR occurs in vivo, it still needs to be determined.

7. MicroRNAs, such as miR-34a (Oda et al., 2014), miR-128-2 (Adlakha et al., 2013), and miR-27a/b (Ji et al., 2009) have been shown to regulate expression of known regulator of RXRα, which is a known heterodimeric partner of PXR (Mangelsdorf and Evans, 1995) and is required for PXR activation (Kliewer et al., 2002). Due to their effects on RXRα, these microRNAs may indirectly regulate PXR and affect induction of PXR target genes by rilpivirine, etravirine, or efavirenz.

8. Human CAR has been demonstrated to be regulated at post-transcriptional level by miR-137 (Takwi et al., 2014; Chen et al., 2014). Similarly, miR-122 has been implicated in
phenobarbital-dependent indirect activation of mouse CAR (Shizu et al., 2012). Given that rilpivirine, etravirine, and efavirenz indirectly activate CAR (Chapter 2), a mechanism similar to phenobarbital, it remains to be investigated whether these drugs modulate expression or function of miR-122 or miR-137.

5.4 Overall Conclusions

In summary, following are the conclusions from the present study:

1. Rilpivirine and etravirine activate human PXR and CAR, but this occurs by distinct mechanisms.
2. NNRTIs activate hCAR in a drug-specific and isoform-selective manner.
3. Not all NNRTIs activate human PXR or CAR.
4. MicroRNA-18a post-transcriptionally regulates hPXR and contributes to rilpivirine- and rifampin-mediated activation of hPXR.
5. Rilpivirine and rifampin are novel modulators of miR-18a expression.

The present study provides mechanistic understanding of how rilpivirine, etravirine, and efavirenz activate nuclear receptor PXR and CAR. Further, findings of this study also provide evidence for a link between miRNA-18a and PXR, and the role of miR-18a in rilpivirine and rifampin activation of PXR. Overall, these results have provided answers for some of the underlying molecular mechanisms contributing to NNRTI-mediated pharmacokinetic drug interactions and may provide rational basis for selection of anti-HIV therapies.
References


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Figure A.1 Mammalian Two-hybrid Assay to Evaluate the Effect of Alternate Combination of Expression Vectors for Receptor LBD and Coactivator on Recruitment of Steroid Receptor Coactivator to the LBD of hCAR-WT by Specific NNRTIs.

Cultured HepG2 cells were co-transfected with a pM-hCAR-WT-LBD receptor expression plasmid (or the pM empty vector), pVP-16-hSRC1-RID coactivator expression plasmid, pFR-luc reporter plasmid, and pGL4.74 [hRLuc/TK] internal control plasmid. Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), or CITCO (10 µM; positive control) for 24 h. In all cases, cells were co-treated with androstanol (10 µM; inverse agonist for hCAR-WT) to decrease the constitutive activity of this receptor. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± SD for an experiment performed in triplicate.
Figure A.2 Mammalian Two-hybrid Assay to Evaluate Recruitment of Steroid Receptor Coactivators to the LBD of hCAR-SV23 and hCAR-SV24 by Specific NNRTIs.

In the mammalian two-hybrid assays, HepG2 cells were co-transfected with a coactivator expression plasmid, pCMV6-XL4-hRXRα, pFR-luc reporter plasmid, pGL4.74 [hRluc/TK] internal control plasmid, and either a receptor expression plasmid [pVP16-hCAR-SV23-LBD (A and C) or pVP16-hCAR-SV24-LBD (B and D)] or an empty vector (pVP16) for 24 h. The coactivator expression plasmids were pM-hSRC2-RID (A and B) and pM-hSRC3-RID (C and D), and pM-hSRC3-RID (C). Transfected cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), or delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), DEHP (10 µM; positive control for hCAR-SV23), or CITCO (10 µM; positive control for hCAR-SV24) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± S.E.M. for three independent experiments performed in triplicate. *, significantly different from the same treatment group transfected with corresponding empty vector and from the vehicle-treated control cells transfected with respective receptor expression plasmid ($P < 0.05$).
Figure A.3 Mammalian Two-hybrid Assay to Evaluate the Effect of Alternate Combination of Expression Vectors for Receptor LBD and Coactivator on Vector Swapping on Recruitment of Steroid Receptor Coactivator to the LBD of hCAR-SV23 and hCAR-SV24 by Specific NNRTIs.

Cultured HepG2 cells were co-transfected with a receptor expression plasmid (or the pM empty vector), pVP-16-hSRC1-RID coactivator expression plasmid, pFR-luc reporter plasmid, and pGL4.74 [hRLuc/TK] internal control plasmid. The receptor expression plasmids were pM-hCAR-SV23-LBD (A) and pM-hCAR-SV24-LBD (B). Transfected HepG2 cells were treated with DMSO (0.1% v/v; vehicle control), rilpivirine (RPV; 5 µM), etravirine (ETV; 5 µM), efavirenz (EFV; 5 µM), nevirapine (NVP; 5 µM), delavirdine (DLV; 5 µM), TCPOBOP (0.25 µM; negative control), or CITCO (10 µM; positive control) for 24 h. In all cases, cells were co-treated with androstanol (10 µM; inverse agonist for hCAR-WT) to decrease the constitutive activity of this receptor. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized as described under Section 3.2.7. Data are expressed as mean ± SD for experiment performed in triplicate.