UNIVERSITY OF CINCINNATI

Date: JUNE 1, 2006

I, NIRESH HARIPARSAD,

hereby submit this work as part of the requirements for the degree of:

Doctor of Philosophy (Ph.D.)

in:

Pharmaceutical Sciences

It is entitled:

Hepatic and Extra-Hepatic Induction of Drug Metabolizing Enzymes and Drug Transporters by Antiretrovirals, in the Presence and Absence of Viral Infection

This work and its defense approved by:

Chair: Pankaj Desai

Hepatic and Extra-Hepatic Induction of Drug Metabolizing Enzymes and Drug Transporters by Antiretrovirals, in the Presence and Absence of Viral Infection

A dissertation submitted to the

Division of Research and Advanced Studies

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in the Department of Pharmaceutical Sciences

of the College of Pharmacy

2006

by

Niresh Hariparsad

B. Pharm., University of Durban-Westville, Durban, South Africa, 1995

M.S. (Clin. Pharm.) University of Durban-Westville, Durban, South Africa, 1999

Committee Chair: Pankaj B. Desai, Ph.D.

ABSTRACT

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) are important antiretroviral drugs (ARVs) included in the currently used highly active antiretroviral therapy (HAART) regimen. While these agents have significantly improved the morbidity and mortality associated with HIV/AIDS, their clinical use remains fraught with numerous drug-drug interactions (DDIs) including induction of drug clearance pathways. This study represents an attempt to better understand the impact of these agents on key drug-metabolizing enzymes and drug transporters (DMTs) in hepatic and extra-hepatic tissues. In preliminary studies we observed that NNRTI efavirenz, and PIs, nelfinavir and ritonavir, activate the pregnane-X receptor (PXR), a key transcriptional regulator of DMTs. Based on this observation and other published reports, we hypothesized that these agents induce hepatic and extra-hepatic (intestinal and $CD4^+$ T-cells) DMTs, and that the magnitude of induction is modulated by disease state. In Aim 1, employing primary human hepatocytes and LS174T colon carcinoma cell line, a clinically relevant model for intestinal drug metabolism studies, we observed that these drugs have a profound effect on several DMTs. Our studies revealed that efavirenz is potent inducer of CYP3A4, whereas the PIs increase CYP3A4 mRNA levels without a correlative increase in CYP3A4 activity. The three drugs also induced CYP2B6, UGT1A1 and UGT1A6, but only nelfinavir induced UGT2B7. Both PIs markedly induced P-gp. In Aim 2, we evaluated the effect of these drugs on expression and activity of DMTs in $CD4^+$ T-cells. While all three ARVs produced significant increases in the P-gp function in $CD4^+$ T-cells, nelfinavir was the most potent P-gp inducer. In the third Specific Aim, we observed that exposure of nelfinavir-treated $CD4^+$ T-cells to infectious-HIV decreased P-gp activity which corresponded with a reduction in hPXR expression levels. A comparison of the effects on exposed-infected and exposed-uninfected cells revealed a significant decrease of P-gp activity in nelfinavir-treated

exposed-infected cells, but an increase in the exposed-uninfected cells. The expression of hPXR was lower in nelfinavir-treated exposed-infected cells than exposed-uninfected cells. Taken together, our studies have provided novel insights into the factors that may increase the potential for drug-drug interactions and alter the intracellular pharmacology of important ARVs.

ACKNOWLEDGEMENTS

I would like to start my acknowledgements by thanking **Dr Pankaj B. Desai**. Dr Desai has supported my research interests, in HIV/AIDS, from the get-go and has made every attempt to ensure that I obtained all of the knowledge and experience that I needed in getting my degree. He is an excellent mentor who has tirelessly worked to ensure that my experience in his laboratory was joyous and fruitful. None of my achievements over the past 5 years would have been possible without him.

I would also like to extend a sincere thank you to **Dr Julie AE. Nelson**. Dr Nelson was extremely meticulous in her criticism of my work which helped to significantly improve the quality of my dissertation. None of the work that I pursued in Specific Aims 2 and 3 would have been possible without her guidance. I appreciate the fact that she treated my research like her own. I learnt a tremendous amount from her, not only from her guidance of my research but also her professional and friendly mannerisms and attitude.

I wish also to thank my other committee members; **Dr Arthur R. Buckley**, **Dr William Cacini** and **Dr Judith E. Feinberg** whose classes I enjoyed and whose criticisms contributed to the improved quality of my work. I am extremely grateful to Dr Buckley and Donna Buckley for allowing me the use of their lab space.

I would like to thank my current lab mates; Fang Li and Ganesh Mugundu. I would especially like to thank Dr Srikanth Nallani and Dr Rucha Sane who taught me important laboratory techniques which helped me in the pursuit of my degree. I would like to thank some of my other past lab mates and friends, Sandya, Abhijeeth, Murad, Andy, John and Philip.

Finally, I would like to thank the College of Pharmacy and all of the administrators who facilitated the completion of my degree.

I would like to dedicate this work to my supportive, loving, caring and patient wife

Monica Marie Hariparsad

None of my accomplishments would have been possible without the kindness, love and support I obtained from my mother

Saraswathie Hariparsad

I would also like to thank my uncles; Nilraj Hariparsad, Bissondaw Hariparsad and Ganagaram Hariparsad who helped make me the person I am today.

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ACTG	AIDS clinical trial group
ABC	ATP-binding cassette
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of Variance
APV	Amprenavir
AUC	Area under the plasma concentration versus time curve
AZT	Zidovudine
BCRP	Breast cancer resistance protein
BFC	7-benzyloxy-4-trifluoromethylcoumarin
Con	Control (DMSO/solvent/vehicle treated group)
CAR	Constitutive androstane receptor
cDNA	Deoxyribonucleic acid sequence complementary to RNA
Cl	Total systemic clearance
C_{max}	Maximum measured plasma concentration over the time span
C_{min}	Minimum measured plasma concentration over the time span
C_{ss}	Plasma concentration at steady state
CYP	Cytochrome P450 enzymes
DBD	Deoxyribonucleic acid binding domain
DDI	Drug-drug interaction
DLV	Delavirdine
DME	Drug metabolizing enzyme
DMT	Drug metabolizing enzymes and transporters

DMSO	Dimethylsulfoxide
DR-3	Direct repeat with a three nucleotide spacer
EFV/EFZ	Efavirenz
EFC	7-ethoxy-4-trifluoromethylcoumarin
ER-6	Everted repeat with a six nucleotide spacer
GR	Glucocorticoid receptor
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HMM	Hepatocyte maintenance medium
HNF	Hepatocyte nuclear factor
HPLC	High pressure liquid chromatography
HRP	Horse-radish peroxidase
IDV	Indinavir
LBD	Ligand binding domain
LS174T	Colon carcinoma cell line
MDR	Multidrug resistance protein
MEM	Minimum essential medium
mRNA	messenger Ribonucleic acid
MRP	Multidrug resistance protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NEL/NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor

NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PB	Phenobarbital
PBMC	Peripheral blood mononuclear cells
PBREM	Phenobarbital response element module
PBS	Phosphate buffered saline (pH 7.4)
PBS-T	Phosphate buffered saline with 0.1% Tween 20 (pH 7.4)
PCN	Pregnenolone 16α-carbonitrile
PHA	Phytohemaglutinin
P-gp	P-glycoprotein
PI	Protease inhibitor
pSG5	Commercial expression vector employed to carry plasmid of interest
PXR	Pregnane-X receptor
Rh-123	Rhodamine-123
RTV	Ritonavir
RXR	Retinoid-X receptor
RIF	Rifampin
RNA	Ribonucleic acid
SQV	Saquinavir
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t½	Elimination half-life of the drug in plasma
UGT	Uridilyl diphosphate glucuronosyltransferase
XREM	Xenobiotic response element module

1. INTRODUCTION

The pandemic of HIV/AIDS has had a catastrophic impact on the world population and has been identified as the "defining medical and public health issue of our generation and ranks among the greatest infectious disease scourges in history" (Fauci, 1999). The world first became aware of this disease in a small population of homosexuals in 1981 in San Francisco (Table 1.1). However, since this discovery, the disease has spread in successive waves affecting every corner of the globe. By 2005, HIV had infected a cumulative total of 40.3 million people worldwide, up from 37.5 million in 2003 (Zarocostas, 2005). Unfortunately, the disease continues to wreak havoc in less developed nations which highlights the fact that the full impact of the AIDS pandemic has not been fully realized. This is evidenced by the continued spread of the virus, notably in sub-Saharan Africa, which has been hardest hit, China, India and parts of Eastern Europe and central Asia (von Overbeck, 2005). HIV and AIDS continue to exact an enormous toll even in more developed countries, such as the United States (~1.5 million HIV-positive individuals, with about 40 000 new cases every year) where their incidence is accelerating among minorities and the 25-44 year old age-group (Anonymous 2001), (Anonymous 2001).

Initially, most patients who were diagnosed with HIV/AIDS died within 2 years (Rothenberg *et al.*, 1987). However, major breakthroughs were made with the isolation of HIV-1 and the development of blood-screening antibody tests indicating the presence or absence of the virus (Table 1.1) (Barre-Sinoussi *et al.*, 1983), (Gallo *et al.*, 1983), (Silberner, 1985). These breakthroughs lead to the recognition of **CD4$^{\pm}$ T-cells as the as the primary receptor for the virus on a subset of T cells** and monocytes (Dalgleish *et al.*, 1984), (Gottlieb *et al.*, 1981), (Klatzmann *et al.*, 1984), (Masur *et al.*, 1981). With these findings, clinicians began using viral load as an indication of disease progression in a patient. In addition, having identified the

primary site of attack by HIV-1, scientists were also able to begin identifying potential drug target sites against the virus.

Table 1.1: HIV-1 therapy time-line (Adapted from Pomerantz et al. 2003)

1981	Acquired Immunodeficiency Syndrome/AIDS was identified in San Francisco (Anonymous 1981).
1983	Disease management was challenging prior to the isolation of the HIV-1 in 1983 and the development of antibodies to HIV-1 (Barre-Sinoussi *et al.*, 1983), (Gallo *et al.*, 1983)
1987	The identification of potential drug target sites resulted in the development of the first nucleoside reverse transcriptase inhibitor (NRTIs), zidovudine (AZT), which was approved by the FDA in 1987 (Ezzell, 1987),
1993	Even with the NRTIs, HIV/AIDS continued to have a major socioeconomic impact and became the leading cause of death in young adults in the United States (Anonymous 1996)
1994	A major drawback with the currently available antiretroviral regimen was the rapid resistance to monotherapy (Anonymous 1994), (Volberding *et al.*, 1995). However, AZT was identified to limit mother to child transmission of HIV.
1995/96	AIDS Clinical Trials Group (ACTG) 320 Study Team, undertook a study of the first protease inhibitors in triple combination therapy (Hammer *et al.*, 1997), (Hirsch *et al.*, 1999)
1996	Nevirapine, first non-nucleoside reverse transcriptase inhibitor (NNRTI) was approved by the FDA. Combinations from all 3 major antiretroviral classes referred to as highly active antiretroviral therapy (HAART) (Anonymous 2002) causes a significant reduction in deaths related to HIV/AIDS in developed nations. However, clinicians note significant side effects (lipodystrophy and metabolic complications) associated with HAART (Carr and Cooper, 2000), (Carr *et al.*, 1998), (Carr *et al.*, 2000)
1999	Further, drug disposition studies identify antiretroviral agents as modulators and substrates of drug metabolizing enzymes and transporters. This makes the eradication of HIV-1 from reservoir sites such as the brain, currently unachievable (Kulkosky and Pomerantz, 2002), (Kulkosky and Pomerantz, 2002), (Pomerantz, 2002)
2003	Introduction of fusion inhibitors, such as Enfuvirtide which mimic the sequence of the HR2 domain in gp41. It inhibits virus–cell fusion by binding to the HR1 domain of gp41, preventing formation of the 6-helix bundle and thereby arresting the HIV entry process.
2003	Therapeutic vaccination (Hardy *et al.*, 2002)
2004	The use of growth factors and cytokines was used to treat opportunistic infections in HIV-1 disease (Jaworowski *et al.*, 2004)
2005	Genetic influences on HIV infection have major implications for vaccine development (Smith and Kent, 2005)

1.1 Antiretroviral Drugs

The first major site of attack identified by scientists was the reverse transcriptase involved in the conversion of HIV-1 single-stranded viral RNA to double-stranded DNA. This lead to the development of zidovudine (AZT), a nucleoside analog that inhibits HIV-1 reverse transcriptase, which was approved by the FDA in 1987 (Table 1.1) (Ezzell, 1987). AZT, interferes with HIV-1 replication by competitively inhibiting reverse transcriptase, thus leading to chain termination of HIV-1 proviral DNA (Figure 1.1) (Furman and Barry, 1988), (Richman, 2001).

However, despite the original excitement surrounding AZT and the wide release of another NRTI, didanosine, in 1991 (Gershon, 1991) scientists began to recognize that the effect of monotherapy was brief and limited in activity against HIV-1 (Table 1.1) (Anonymous 1994), (Volberding *et al.*, 1995). With greater understanding of the virus, drug discovery targets additional to reverse transcriptase were recognized which included viral protease, the enzyme responsible for the maturation of viral particles to infectious virions. Protease inhibitors bind to the active site of the enzyme where protein cleavage occurs (Figure 1.1) and new viral particles cannot mature and become infectious (Richman, 2001), (Venaud *et al.*, 1992).

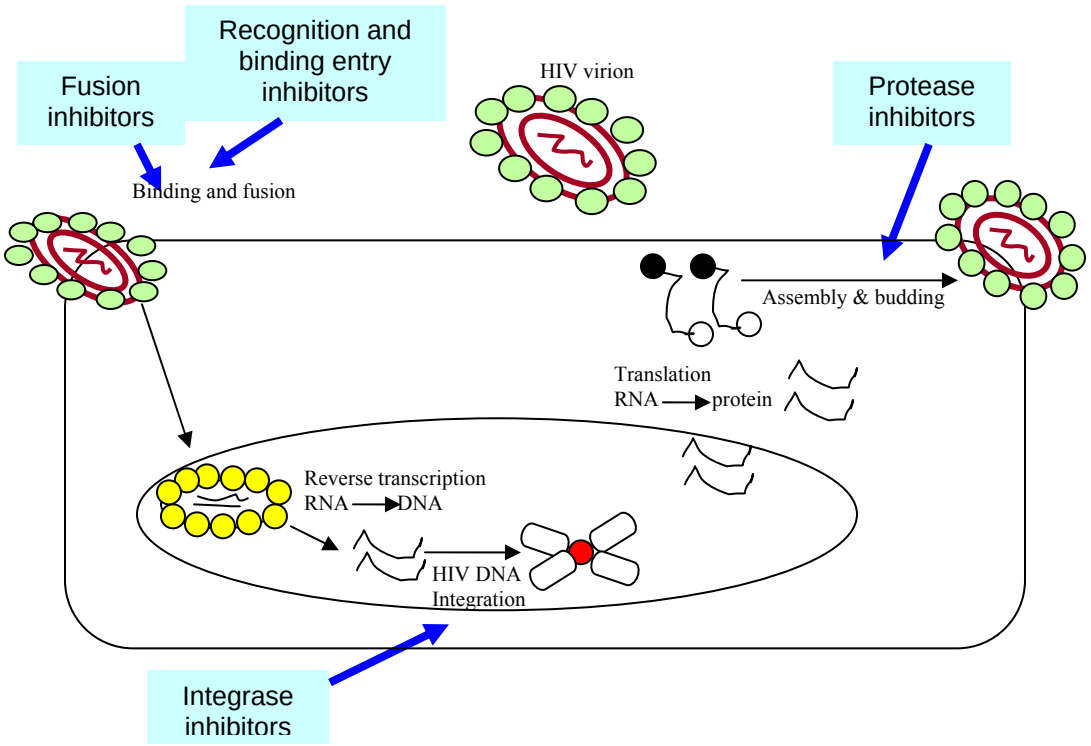

Figure 1.1: HIV-1 viral life-cycle with sites of antiretroviral drug activity

(Adapted from the Bristol Myers Squibb Website, accessed April 5, 2005)

1.2 Highly Active Antiretroviral Therapy

The clinical study, AIDS Clinical Trial Group (ACTG) 320 showed the therapeutic benefits of combining PIs with NRTIs. These combinations became more efficacious with the approval of more potent PIs, such as ritonavir and nelfinavir. Subsequent landmark studies proved the efficacy of triple combination therapy, or Highly Active Antiretroviral Therapy (HAART), in markedly reducing mortality (Hammer *et al.*, 1997) and inducing viral load suppression (Hirsch *et al.*, 1999). The use of HAART effectively decreased the plasma viral load below the detection limits of reverse transcription-polymerase chain reaction (RT-PCR) for plasma HIV-1 RNA. Scientists realized that sustained suppression of HIV-1 titers could

minimize viral replication, which would, in turn, deter the development of resistance to the new agents and allow for immune reconstitution (Gulick *et al.*, 2000), (Sterling *et al.*, 2003). The development of non-nucleoside reverse transcriptase inhibitors (NNRTIs) made additional HAART combinations possible which were further able to suppress HIV-1 replication (Staszewski *et al.*, 1999). NNRTIs, such as efavirenz, are a group of structurally diverse agents with a reasonable toxicity profile that bind at a region distant from the active site of reverse transcriptase, which results in conformational changes of the active site and, thereby inhibiting its activity (Figure 1.1). As with the other antiretrovirals, when these agents are used as monotherapy, there is a rapid emergence of resistant viral strains associated with single point mutations in the reverse transcriptase gene (Saag *et al.*, 1993).

1.3 Limitations of Highly Active Antiretroviral Therapy

In spite of the undeniable clinical benefits obtained with HAART, between 20% and 50% of patients who initiate HAART present with virological failure during the first year of treatment and the incidence of failure increases rapidly during successive treatments (Casado *et al.*, 1998), (Deeks *et al.*, 1999), (Mocroft *et al.*, 2001), (Paredes *et al.*, 2000), (Smit *et al.*, 2002). Treatment failure may occur due to a multitude of reasons, which may be patient-, virus-, or drug-related (Fletcher, 1999). A major factor contributing to drug related reasons for treatment failure is related to the complexities in the drug disposition (Figure 1.2).

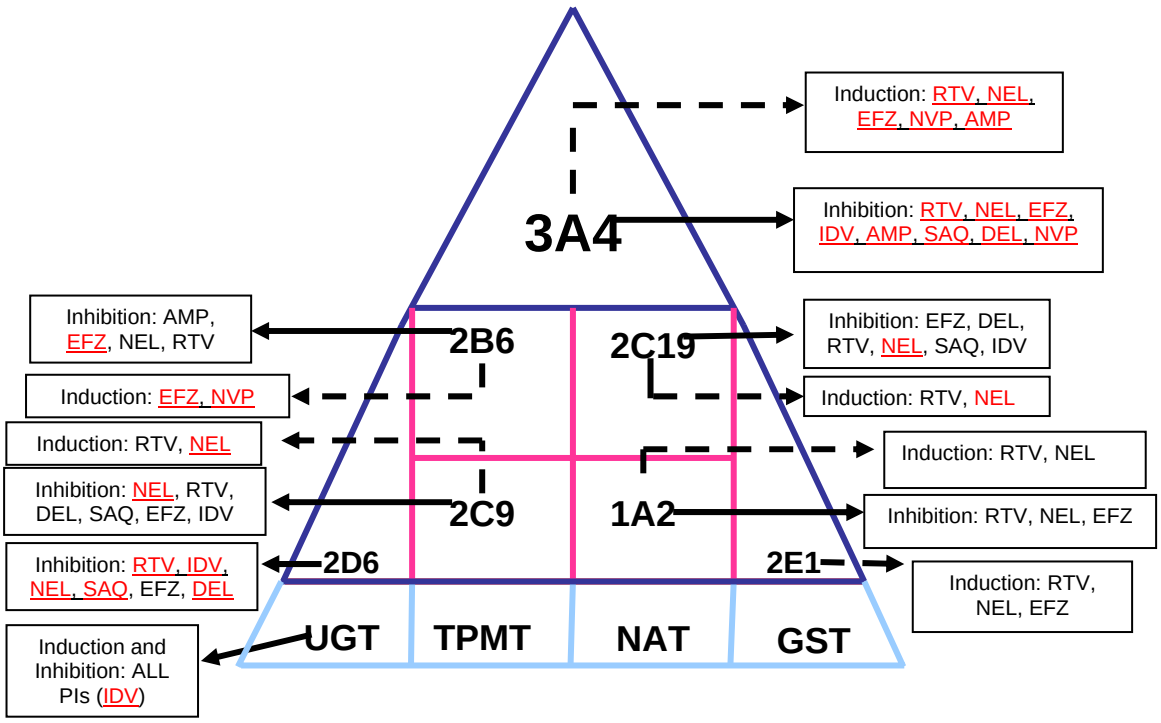

Figure 1.2: Effect of antiretrovirals on various phase I/II enzymes. The pyramid pattern shows the relative importance of the various enzymes in the metabolism of clinically used drugs. Drugs that are underlined are also substrates of these enzymes. **APV** = amprenavir; **DLV** = delavirdine; **EFV** = efavirenz; **IDV** = indinavir; **NFV** = nelfinavir; **NVP** = nevirapine **RTV** = ritonavir; **SQV** = saquinavir. (Adapted from Fichtenbaum *et al*. (Fichtenbaum and Gerber, 2002)

The pharmacokinetics of most antiretroviral agents is complex and is associated with significant inter-subject variability, which is reflected in corresponding variability in the outcome of drug therapy, and serious drug-drug interactions. Further complications are the changes in physiological function, due to the HIV infections and or other co-morbidities. The principal pathway of systemic elimination of most antiretroviral agents entails biotransformation at hepatic and extra-hepatic sites. Additionally, many of these agents are substrates for transporters that restrict their systemic absorption or participate in their clearance. A majority of the

aforementioned inter-patient variability in drug efficacy or toxicity and drug-drug interactions is related to the expression and activity of DMTs. Furthermore, as will be discussed in subsequent sections, antiretrovirals are known to cause marked effects on the activity and regulation of these enzymes and transporters (Figure 1.3). Such effects may affect their own clearance as well as that of other co-administered drugs and ultimately impact the overall safety and efficacy of the HAART regimens and other co-medications (Baede-van Dijk *et al.*, 2001), (Barry *et al.*, 1998), (Burger *et al.*, 2003), (Csajka *et al.*, 2003), (Marzolini *et al.*, 2001a), (Marzolini *et al.*, 2001b) , (Regazzi *et al.*, 1999), (Smith *et al.*, 2001), (Veldkamp *et al.*, 2001) .

1.4 Drug Metabolizing Enzymes and Transporters

As indicated in the previous section, the principal pathway of systemic elimination of most antiretroviral agents entails biotransformation. Biotransformation involves the conversion of highly lipophilic compounds, such as antiretrovirals, to more hydrophilic metabolites by oxidative (Phase I) and conjugating (Phase II) enzymes. This is a cellular defense mechanism against chemicals to which our body is frequently exposed (Conney, 1982).

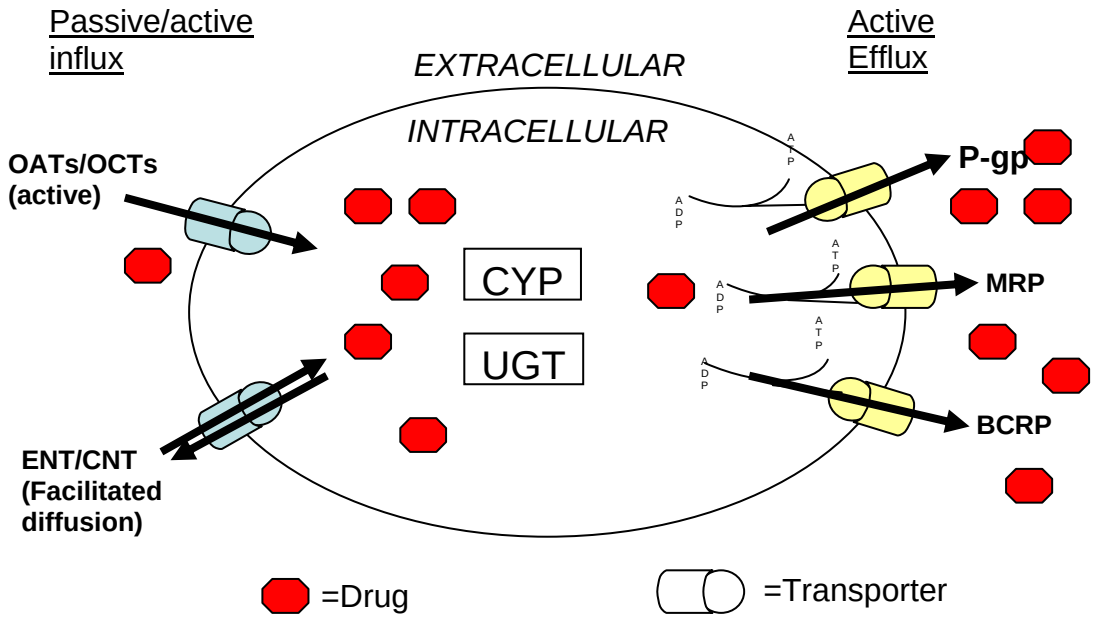

Figure 1.3: Expression of drug metabolizing enzymes and drug transport systems in a typical cell. OAT – organic anion transporter, OCT – organic cation transporter, ENT – equilibrative nucleoside transporter, CNT – constitutive nucleoside transporter, P-gp – p-glycoprotein, MRP – multi resistance protein, BCRP – breast cancer resistance protein. (Adapted from Ford *et al*. (Ford *et al*., 2004)

These enzymes and transporters, which are located in both the gut and liver represent the first barrier for orally administered drugs to reach the systemic circulation. Drug absorption is known to involve both passive and active pathways, with passive processes depending mainly on the physicochemical characteristics of the drug (Table 1.2). It is now well established that efflux drug transporters may also reduce drug absorption (Fromm, 2000), (Lin and Yamazaki, 2003). The dynamic interplay between DMTs to limit the oral bioavailability of drugs is referred to as first pass metabolism. Phase I biotransformation is usually the first step in which primarily CYP family of enzymes (e.g. CYP3A4, CYP2B6) metabolizes the drug to either a more hydrophilic or a conjugable form.

Table 1.2: Physicochemical properties of antiretrovirals (adapted from Ford *et al.*, 2004)

	Nelfinavir	Ritonavir	Efavirenz
Accumulation ratio	5.3 (2.3-16.2)	1.25 (0.20-4.19)	1.3 (0.7-3.3)
pKa	6.0	2.8	10.2
Lipophilicity	2.9	1.2	2.07
Protein binding	>98%	98-99%	>99%

This includes biotransformation reactions such as oxidation, hydroxylation, reduction and hydrolysis. Phase II biotransformation usually follows phase I biotransformation in which a bulky hydrophilic group such as sulfate or glucuronic acid is conjugated to either the drug or its phase I metabolite. Also known as the synthetic phase of drug metabolism, it is catalyzed by conjugating phase II enzymes such as UDP-glucuronosyltransferases (UGTs). Usually, this biotransformation makes the drug pharmacologically less active and chemically more hydrophilic thereby facilitating its rapid elimination. Finally, the drug or its metabolites and conjugates may be effluxed out of the enterocytes along the GI lumen or hepatobiliary canal by transporter proteins such as P-gp or multi drug resistance protein (MRP) family members which constitute the so called phase III of drug disposition. Figure 1.4 shows the predominant phase I and phase II enzymes expressed in liver tissues.

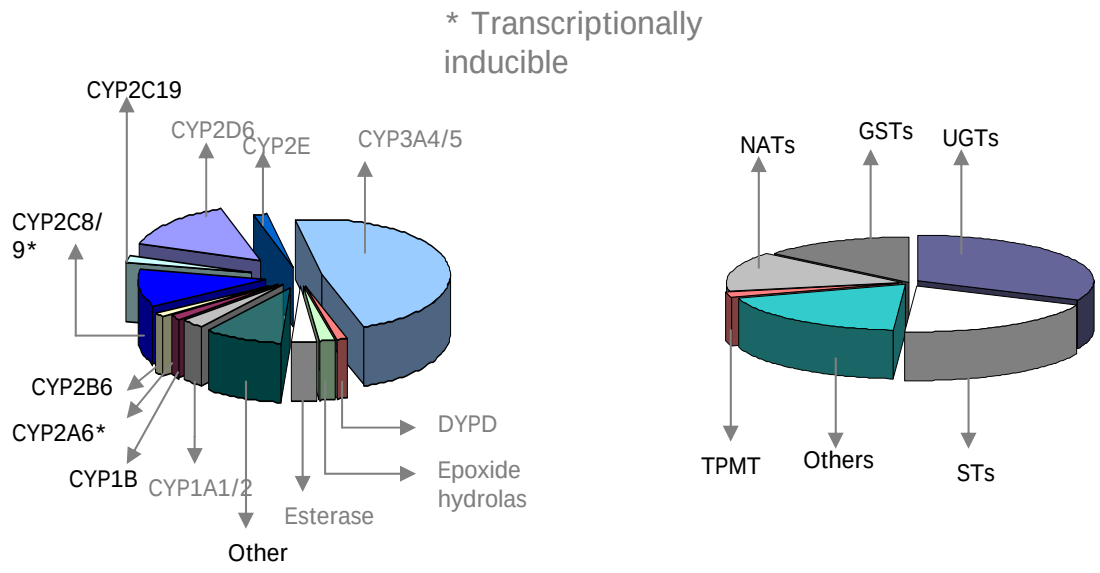

Figure 1.4: Pie chart of various phase I/II drug metabolizing enyzmes present in the human liver

1.4.1 Drug Metabolizing Enzymes

Physiological Role of Drug Metabolizing Enzymes

The existence of CYP enzymes has been approximated to 3.5 billion years. These isoenzymes are present in several different organisms including animals, plants, fungi and bacteria. They are responsible for the biosynthesis and/or the degradation of endogenous compounds such as fatty acids, steroid hormones and cholesterol, in addition to other major functions (Coon *et al*., 1992). Such functions include; involvement in signal transduction in the brain (Warner *et al*., 1993), and adrenal and gonadal steroidgenesis (Morhashi *et al*., 1996). In addition, researchers have recently found that the metabolites of CYP enzymes may alter ion permeability of membranes and the enzyme activity and turnover of membranes (Capdevila *et al*., 1992). These metabolites have been shown to have vasodilatory and vasoconstrictive effects

which suggest that CYPs play an important role in the integration of body fluid volume and thus influence blood pressure (Wrighton *et al*., 1992).

1.4.1.1 Cytochrome P450 3A

The CYP 3A forms are among the most important of the xenobiotic-metabolizing CYP enzymes. These isoforms mediate the metabolism of numerous xenobiotics, including pollutants, pesticides, tobacco smoke constituents, food contaminants and pharmaceuticals. The human *CYP3A* locus, on chromosome 7, consists of four functional genes: *CYP3A4*, *CYP3A5*, *CYP3A7* and *CYP3A43* (Burk *et al.*, 2002). CYP3A4 enzyme is the predominant form in adult liver and intestine. It also catalyzes the metabolism of more than half of all drugs, and represents the primary route of elimination for many drugs (Schuetz, 2004). The average fraction of the total P450 in liver accounted for by CYP3A4 is ~25-35% (Shimada *et al.*, 1994); in the small intestine, the fraction attributed to CYP3A4 is even higher. A study with the selective inhibitor gestodene, which destroys CYP3A4, indicates that CYP3A4 can constitute 60% of the total hepatic CYP content (Guengerich, 1990).

CYP3A4 is also expressed in some extrahepatic tissues, including lung (Ding and Kaminsky, 2003), (Kelly *et al.*, 1997), stomach, colon (Ding and Kaminsky, 2003) and adrenal glands (weak) (Koch *et al.*, 2002). The literature is mixed on whether expression occurs in PBMCs. Nakamoto *et al*., (2000) found that the CYP3A4 mRNA level was increased by treatment with rifampin in PBMCs. Another study by Krovat *et al*. (2000), in which the character and variability of human CYP gene expression in human blood cells was assessed, found that CYP2D6 (9.1×10^5 molecules/µg total RNA) and CYP2E1 (2.6×10^5 molecules/µg total RNA) were typically expressed at the highest levels, while CYP3A levels (1.3×10^4 molecules/µg total RNA) were near the detection limit of the assay.

1.4.1.2 Cytochrome P450 2B6

CYP2B6, plays a major role in the biotransformation of several therapeutically important drugs, including the cytostatic prodrug, cyclophosphamide (Chang *et al.*, 1993); the antidepressant, bupropion (Faucette *et al.*, 2000), (Hesse *et al.*, 2000); and efavirenz (Ward *et al.*, 2003). CYP2B6 is expressed primarily in the liver. The expression and function of CYP2B6 are highly variable between and within individuals, with estimates ranging from 28 to 80 pmol CYP2B6 per milligram of protein (Code *et al.*, 1997), (Ekins *et al.*, 1998), (Stresser and Kupfer, 1999). Results by Hanna *et al*. (Hanna *et al.*, 2000) was in line with the lower estimates of expression levels (mean ~ 1% of total CYP), with values rarely exceeding 5% even in samples from individuals administered inducers.

The *CYP2B6* gene is located within a *CYP2* gene cluster on chromosome 19 (Hoffman *et al.*, 2001). Approximately 40 allelic variants of CYP2B6 have been discovered (http://www.imm.ki.se/CYPalleles/criteria.htm) (Lang *et al.*, 2001). Some of the aforementioned allelic variants have been associated with decreased expression of CYP2B6 protein in human liver (Lang *et al.*, 2001) and/or to be deficient in heterologous expression systems. Evidence for the clinical relevance of *CYP2B6* polymorphisms is accumulating subsequent to the discovery that efavirenz, is a specific drug substrate of this enzyme (Ward *et al.*, 2003). The sequential oxidative metabolism of efavirenz results in the production of 8-hydroxy- and 8,14-dihdroxyefavirenz *in vitro* and it was shown that CYP3A plays only a minor role (Ward *et al.*, 2003). Pronounced interindividual differences in efavirenz bioavailability and an inverse correlation between average drug exposure and viral load, as well as a trend with central nervous system toxicity, were found in a recent population pharmacokinetic analysis (Csajka *et al.*, 2003).

1.4.1.3 UDP-glucuronosyltransferase enzymes

While phase I enzymes have garnered the most interest in the biotransformation of antiretroviral compounds, attention is also being focused on phase II enzymes (e.g. uridine diphosphoglucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), glutathione *S*-transferase (GST), thiopurine methyltransferase (TPMT) and sulfotransferase (ST) (Burchell *et al.*, 2000). Of the aforementioned phase II enzymes UGT is regarded as a major biotransformation pathway for several antiretroviral compounds.

The human UGTs are a superfamily of enzymes that conjugate a variety of endogenous substances and exogenous compounds. UGT-catalyzed glucuronidation reactions are responsible for approximately 35% of all drugs metabolized by phase II enzymes, and glucuronidation is a listed clearance mechanism for 1 in 10 of the top 200 prescribed drugs (Kiang *et al.*, 2005), (King *et al.*, 2000).

UGT enzymes are bound to the internal membrane and face the luminal side of the endoplasmic reticulum, a location which confers both an advantage and a disadvantage (Tukey and Strassburg, 2000). The advantage is that these enzymes have direct access to metabolites produced by phase I biotransformation reactions. The disadvantage is that this restricts the access of drug substrates, cofactors, and glucuronidated products to and from the active sites of UGT enzymes. This access restriction by the endoplasmic reticulum is one of the factors that causes a recognized decrease in UGT enzyme activity in isolated microsomes and is the phenomenon commonly referred to as "latency." This contributes to one of the difficulties in predicting *in vivo* effects of UGT enzymes based on data obtained from *in vitro* experiments, such as those with tissue microsomes (Fisher *et al.*, 2001), (Lin and Wong, 2002).

To date, 15 functionally active human UGTs have been characterized and classified into two separate families (UGT1 and UGT2) based on amino acid sequence similarity (Mackenzie *et al.*, 1997). Liver is the major organ for glucuronidation in the body as it is directly exposed to the influx of drugs from the hepatic portal vein during oral absorption (Fisher *et al.*, 2000), (Fisher *et al.*, 2001), (Tukey and Strassburg, 2000). The major extrahepatic tissues of glucuronidation include those that are responsible for the absorption and/or excretion of drugs such as the intestine, lungs, and kidneys (Fisher *et al.*, 2001). In addition to the aforementioned extra-hepatic sites, Hu *et al*. (Hu and Wells, 2004) observed UGT expression in PBMCs from healthy volunteers, albeit with significant interindividual variability (~200-fold).

1.4.2 Transporters

Transporters that mediate uptake and efflux of endogenous nutrients and exogenous toxins including drugs across biological membranes are necessary for the survival of mammalian species. These transporters are classified into the solute carrier superfamily, for which gene symbols are prefixed with *SLC*, and ATP binding cassette transporters, for which gene symbols are prefixed with *ABC* (http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml).

Uptake transporters

Organic anion and cation transporters are two major classes of transport proteins expressed in tissues such as the liver, intestine, placenta and etc. However, unlike the ATP-binding cassette (ABC)-transporters these transporters are involved in the trafficking of drugs into cells. Organic anion transporters (OAT) have been cloned and belong to one of the following gene families: OAT, OATP or OAT-K (Dresser *et al.*, 2001) whilst organic cation transporters (OCT) belong to either the OCT or OCTN family (Lee and Kim, 2004). Few studies have investigated the role of organic anion and cation transport proteins in the transport of

antiretrovirals. However, the PIs saquinavir, ritonavir, indinavir and nelfinavir have been shown to inhibit OCT1 *in vitro*, which has the potential to cause drug interactions (Zhang *et al.*, 2000). A more recent study identified that OATP-A transports saquinavir *in vitro* (Su *et al.*, 2004) and the clinical relevance of this finding remains to be determined.

Efflux transporters

1.4.2.1 P-glycoprotein

The *ABCB1* [multidrug resistance 1 (*MDR1*)] gene, which is located on chromosome 7 of humans and consists of 28 exons, encodes the 1280 amino acid P-gp. This protein belongs to the ATP-binding cassette (ABC) family and functions as a transmembrane efflux pump that translocates its substrates from its intracellular domain to its extracellular domain. It consists of two homologous halves, each containing six transmembrane domains and an ATP-binding site. P-gp is one of the most important ABC transporters for drug disposition in humans. It was first described in certain tumor cells that were protected against anticancer agents as a result of P-gp overexpression (Juliano and Ling, 1976). It is now established that P-gp is expressed constitutively in many normal, non-tumorous tissues (Cordon-Cardo *et al.*, 1990), (Thiebaut *et al.*, 1987). Together with xenobiotic-metabolizing enzymes, constitutive P-gp expression is believed to be an important protective mechanism against potentially toxic xenobiotics.

P-gp transports a broad variety of structurally diverse compounds that are usually hydrophilic and amphiphatic. In addition to various anticancer agents, P-gp translocates cardiac drugs, Ca^{2+} channel blockers, β-adrenoceptor antagonists, HIV protease inhibitors, antibiotics, immunosuppressants and histamine H_1 receptor antagonists. Most of these P-gp substrates are also substrates of the major drug-metabolizing enzyme CYP3A4.

As a result of its anatomical localization, P-gp functions in three main ways (Figure 1.5); (i) P-gp limits drug entry into the body after oral drug administration as a result of its expression in the luminal (apical) membrane of enterocytes. (ii) Once the drug has reached the circulation, P-gp promotes drug elimination into bile and urine as a result of its expression in the canalicular membrane of hepatocytes and in the luminal membrane of proximal tubule cells in the kidneys, respectively. (iii) In addition, once a xenobiotic has reached the systemic blood circulation, P-gp limits drug penetration into sensitive tissues (e.g. into the brain, testis, **lymphocytes** and fetal circulation). The latter function of P-gp is likely to be a determinant of the success of drug therapy because adequate local intracellular drug concentrations (e.g. in $CD4^{\pm}$ lymphocytes) are required for therapeutic effectiveness (e.g. during HIV therapy) (Kim, 2003).

Intestinal P-glycoprotein and bioavailability

Enterocytes, like hepatocytes, simultaneously express the major drug-metabolizing enzyme CYP3A4 and the efflux transporter P-gp (Watkins, 1997). This leads to a drug efflux–metabolism alliance, which increases the access of drugs to metabolism by CYP3A4 through repeated cycles of absorption and efflux (Benet and Cummins, 2001), (Suzuki and Sugiyama, 2000), (Wacher *et al.*, 1995), (Watkins, 1997). The level of CYP3A4 expression decreases from proximal to distal small intestine, whereas the level of P-gp expression increases from proximal to distal regions (Mouly and Paine, 2003).

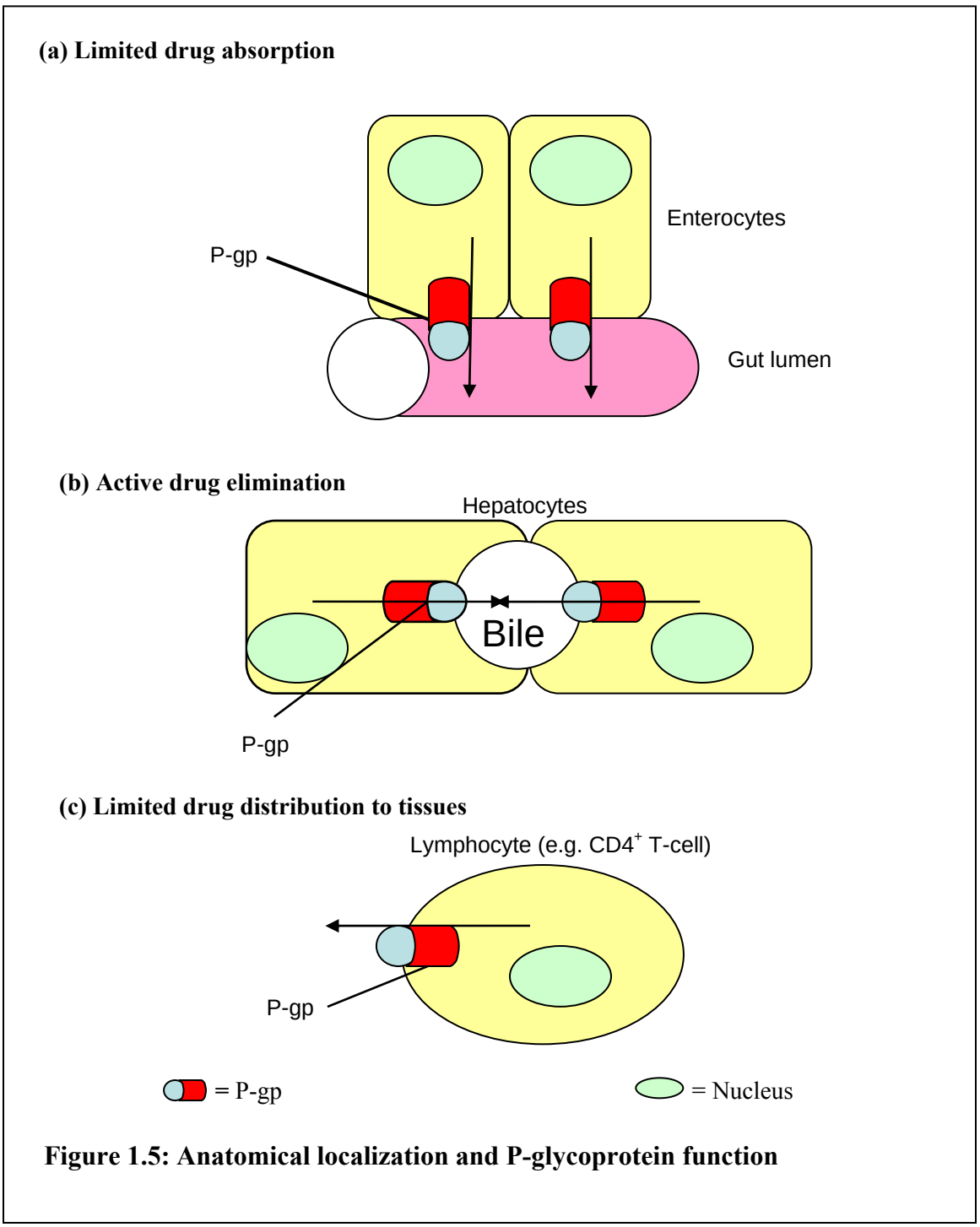

Figure 1.5: Anatomical localization and P-glycoprotein function

DMTs in the gut wall mucosa are now recognized as major determinants of the bioavailability of orally administered drugs (Suzuki and Sugiyama, 2000), (Watkins, 1997). *In vitro* experiments using the P-gp-expressing intestinal cell line Caco-2 and animal studies indicate polarized, basal to apical transport of P-gp substrates (i.e. transport from the enterocyte

into the gut lumen) (Fromm *et al.*, 1999), (Mayer *et al.*, 1996), (Mayer *et al.*, 1997). Accordingly, the bioavailability of several drugs including various HIV protease inhibitors or anti-cancer drugs such as paclitaxel is considerably higher in P-gp (-/-) mice compared with P-gp-expressing control animals (Kim *et al.*, 1998), (Sparreboom *et al.*, 1997). Moreover, gut wall mucosa appear to function as an organ with direct excretory function. After intravenous administration of digoxin to bile duct-ligated, P-gp-expressing mice, 16% of the dose was directly secreted into the gut lumen within 90 min (Mayer *et al.*, 1996), (Mayer *et al.*, 1997). Virtually no direct digoxin secretion into the gut lumen was observed in P-gp knockout mice and in P-gp-expressing mice after oral administration of the P-gp inhibitor valspodar (Mayer *et al.*, 1996), (Mayer *et al.*, 1997). Corresponding observations were made recently in humans using a multi-lumen perfusion catheter (Drescher *et al.*, 2003). In this study, 0.45% of an intravenous dose of digoxin was eliminated directly into a 20-cm isolated segment of proximal small intestine (proximal jejunum). The total amount of drug secreted into the small intestine *in vivo* would be expected to be considerably higher than that secreted into the 20-cm segment because the overall length of the small intestine is 3–5 m and P-gp expression increases from proximal to distal small intestine (Mouly and Paine, 2003). In accordance with the involvement of an active transport process, direct digoxin elimination into the gut lumen was significantly reduced (to 0.23% of the dose; $p<0.03$) by luminal administration of a P-gp inhibitor (quinidine) (Drescher *et al.*, 2003).

Lymphocyte P-glycoprotein

The expression of P-gp in PBMCs has been observed in many studies. Such studies have also attempted to establish a link between the expression of P-gp on PBMCs and intracellular concentrations of drugs. An i*n vitro* study undertaken by Jones *et al*. (2001) revealed marked

differences in the intracellular accumulation of antiretroviral PIs in CEM_{VBL} Leukemic T-Cell line overexpressing P-gp. In these experiments CEM_{VBL} cells expressing high levels of P-gp had reduced intracellular concentrations of PI's compared with control CEM cells. These changes corresponded to falls of between 37-65%. Similar results were obtained by Nascimbeni *et al.* (1999) who observed that differences in the antiviral kinetics of the PI could be a result of differences in the rates of cellular clearance (Nascimbeni *et al.*, 1999).

1.4.2.2 Multidrug Resistance Protein 1

Multidrug Resistance Protein (MRP1) (*ABCC1*) is also a drug effluxing member of the ABC family that together with P-gp actively pumps multiple chemically unrelated substrates across the plasma membrane. MRP1 shares approximately 15% amino acid sequence identity with P-gp (Huai-Yun *et al.*, 1998) and to date there are nine members of the MRP family (Chan *et al.*, 2004), (Kruh and Belinsky, 2003) that all transport organic anions or neutral drugs conjugated to glutathione, glucuronide or sulphate. The increased expression of MRP1 has been reported in a variety of hematological and solid tumors, suggesting a significant role for this transport protein in clinical drug resistance (Cole *et al.*, 1992), (Hipfner *et al.*, 1999).

MRP1 is expressed in most tissues with relatively high levels found in the lung, testis, kidneys, skeletal muscle, and PBMCs while relatively low levels are found in the liver (Cole *et al.*, 1992), (Flens *et al.*, 1996), (Stride *et al.*, 1996) . In most tissues (with the possible exception of brain endothelial cells), MRP1 is localized to the basolateral cellular surface, which in certain tissues results in the efflux of its substrates into the blood.

1.4.2.3 Breast Cancer Resistance Protein

The human breast cancer resistance protein (BCRP) (*ABCG2*) contains 16 exons (ranging from 60 to 532 bp), 15 introns and spans over 66 kb, is located on chromosome 4q22. Sequence

analysis revealed that BCRP is a 655 amino acid ABC protein (72 kDa). Structurally, BCRP belongs to the ABCG gene subfamily. Unlike most of the other ABC transporters, BCRP contains only one NBD. Based on transfection studies, BCRP has been proposed to be a half-transporter that may function as a homodimer or recently suggested tetramer bridged by disulphide bonds (Xu *et al.*, 2004).

Like P-gp, BCRP confers high levels of resistance to anthracyclines, mitoxantrone, and the camptothecins by enhancing drug efflux from the cell (Bates *et al.*, 2001), (Ejendal and Hrycyna, 2002), (Litman *et al.*, 2000). Indeed, in acute leukemia, BCRP may play an important role in resistance to flavopiridol (Nakanishi *et al.*, 2003), (Robey *et al.*, 2001). In addition to its role in resistance to chemotherapeutic agents, BCRP actively transports structurally diverse organic drugs, conjugated or unconjugated, such as estrone-3-sulfate, 17β-estradiol 17-(β-D-glucuronide), and methotrexate (Imai *et al.*, 2003), (Volk and Schneider, 2003), (Volk *et al.*, 2002). BCRP is prominently expressed in placental syncytiotrophoblasts, in the epithelium of the small intestine, and in the liver canalicular membrane (Doyle and Ross, 2003), (Maliepaard *et al.*, 2001). Studies by Wang *et al*. (2003b) and Taipalensuu *et al*. (2001) have found that BCRP transcript is expressed in PBMCs and the intestine in greater amounts than P-gp. This strategic and substantial tissue localization implies that BCRP also functions as a protective drug efflux pump in the placenta, intestine and PBMCs.

1.5 Nuclear Receptors

General Properties

Nuclear receptors are ligand-activated, DNA-binding regulators of gene expression that are best exemplified by receptors for the steroid and thyroid hormones and retinoids (Whitfield *et al.*, 1999). Humans have 48 members in the nuclear receptor gene family (Maglich *et al.*, 2001),

and they share a common modular structure that consists of a central DNA-binding domain (DBD) and a carboxyterminal ligand binding domain (LBD). The DBD governs recognition of the DNA, and thus the specificity for target genes. The LBD region includes amino acid sequences that are important not only for ligand recognition but also for receptor dimer formation, localization to the cell nucleus, and binding of co-regulator proteins (Bourguet *et al.*, 2000a), (Bourguet *et al.*, 2000b). Functionally, binding of an agonist will induce a chemical conformational change in the LBD. This conformational switch leads to recruitment and interaction with nuclear receptor co-activators, followed by a complex cascade that culminates in activation of the nuclear receptor target genes.

1.5.1 Regulation of Target Genes by Transcription Factors

The past decade has witnessed remarkable progress in the understanding of the regulation of key DMTs. CYPs, UGTs and transporters are coexpressed in hepatic and extra-hepatic tissues (Bellamy and Weinstein, 1994). There is also a significant overlap between compounds that are substrates, inhibitors and inducers of the aforementioned DMTs (Figure 1.2) (Schuetz *et al.*, 1996). These observations have led to the hypothesis that these genes acted in concert as a defense mechanism against xenobiotics, which may otherwise persist in our body for extended periods. Not only do these DMTs share ligands, their expression also appears to be coregulated by some of these ligands.

1.5.2 Pregnane X Receptor and Constitutive Androstane Receptor

In 1998, two members of the gene superfamily of nuclear receptors (Mangelsdorf *et al.*, 1995), (Schuetz *et al.*, 1993), namely the pregnane X receptor (PXR) (NR1I2) and the constitutive androstane receptor (CAR) (NR1I3), were identified in rodents and humans as key

transcription factors in hepatic CYP induction by xenobiotics (Kliewer *et al.*, 1999), (Waxman, 1999). In addition to being expressed in hepatic tissue, PXR is expressed in the intestine, kidney, breast tissue, osteoclasts, lung and PBMCs (Albermann *et al.*, 2005), (Lamba *et al.*, 2004b), (Moore and Kliewer, 2000), (Owen *et al.*, 2004). CAR is mainly expressed in the liver with lower expression levels in the intestine, heart, muscle, testis, adrenal, kidney and lung (Baes *et al.*, 1994), (Lamba *et al.*, 2004a). PXR, which was first shown to mediate ligand-dependent induction of CYP3A4, has now come to be regarded as a master regulator of DMTs, since it is thought to mediate the ligand dependent transcription of multiple genes including; *CYP3A4, CYP2B6*, *UGT1A1*, *UGT1A6* and *MDR1*.

PXR and CAR function as ligand-dependent transcription factors by binding to specific DNA sequences called response elements within the promoter region of genes such as CYP3A4 (Figure 1.6). CAR seems to prefer a direct repeat (DR) of hexamer AGTTCA motifs that are separated by 4 bp (DR4), but motif repeats, such as DR2, DR3, and DR5, and everted repeats (ER) with 6 or 8 bp spacing can be recognized (Sueyoshi and Negishi, 2001), (Tzameli *et al.*, 2000). PXR also binds to a variety of elements including DR3, DR4, DR5, ER6, ER8, and inverted repeat (IR0) motifs (Goodwin *et al.*, 2002), (Sonoda *et al.*, 2002), (Sueyoshi and Negishi, 2001). Such response elements may be configured as a single element or as two tandem elements in a direct, everted, or inverted repeat, which permits binding of nuclear receptors as monomers, homodimers, or heterodimers. For instance, the proximal promoter of the CYP3A4 gene (bases – 172 to – 149) contains two copies of an AG(G/T)TCA hexamer recognized by PXR/CAR. As shown by Barwick *et al.* (1996) these half-sites are organized as an ER-6 (everted repeat sequences separated by six nucleotides). Goodwin *et al.* (1999) discovered an important 230-bp xenobiotic-responsive enhancer module (XREM) at the -7836 to -7606 in the

CYP3A4 5' flanking region which harbors multiple copies of the ER6 sequences. They further demonstrated cooperativity between the response elements in the proximal promoter and the distal XREM regions of CYP3A4, which is essential for maximal induction of CYP3A4.

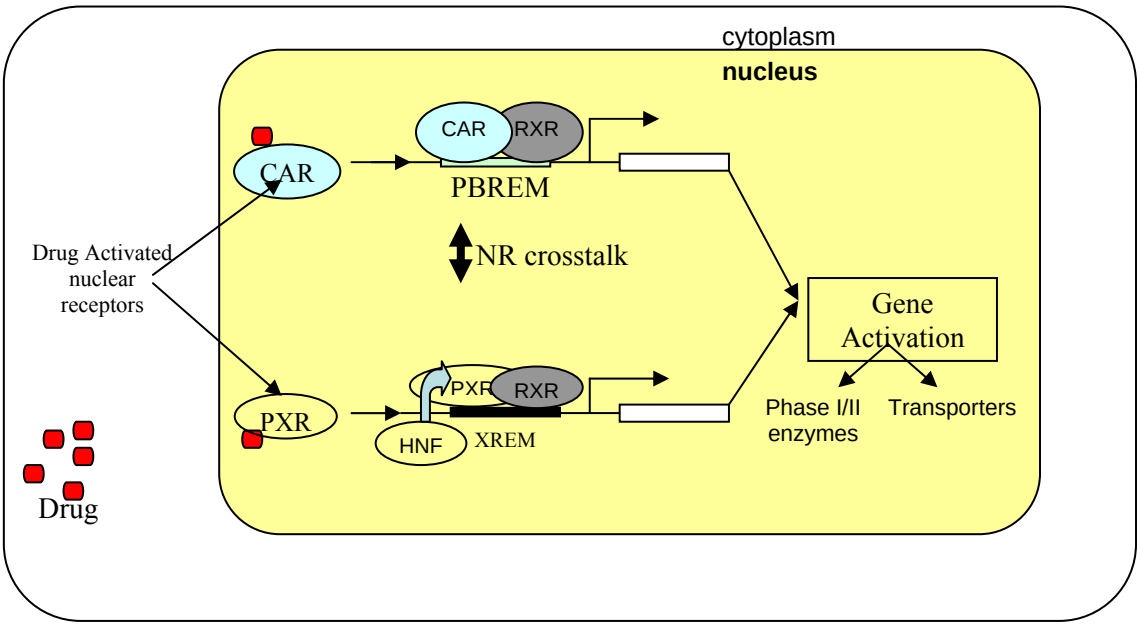

Figure 1.6: Activation of the nuclear receptors CAR and PXR by xenobiotics which result in increased transcription of target genes. PXR – pregnane-X receptor, CAR – constitutive androstane receptor, PB/XREM – xenobiotic-responsive enhancer module, NR – nuclear receptor, HNF – hepatocyte nuclear factor, RXR – retinoid X receptor. (Adapted from Handschin *et al*. (2003) and Hariparsad *et al*. (2006).

PXR and CAR heterodimerize with retinoid X receptor (RXR) and bind to the above response elements and activate target gene expression (Mangelsdorf and Evans, 1995), (Mangelsdorf *et al.*, 1990). While PXR is predominantly activated by dexamethasone/rifampin-type inducers and affects preferentially CYP3A transcription, CAR activity is more influenced by the phenobarbital-type class of compounds and increases transcription of CYP2B and CYP2C

genes (Akiyama and Gonzalez, 2003), (Karpen, 2002), (Pascussi *et al.*, 2003). However, recent studies revealed a considerable overlap of these two receptors both in terms of the spectrum of genes regulated and their affinity to DNA-response elements (Wei *et al.*, 2002).

The LBD sequences of CAR and PXR between rodents and humans are only about 70-75% conserved, raising the possibility of species dependent ligand recognition. The species specificity of some inducers such as PCN, which is a Cyp3A inducer in rats but not humans, is attributed to these differences in LBDs. Another characteristic of the LBD of PXR is that it is fairly promiscuous. Ligand binding studies have shown that the LBDs accommodate structurally diverse compounds, which include small molecular weight compounds such as phenobarbital (molecular weight 232 Da) to larger molecular weight compounds such as paclitaxel (molecular weight 856 Da). This flexibility accommodates a vast array of compounds which is consistent with the known spectrum of structurally diverse CYP inducers.

Since CYP3A4 induction is species specific, studies on CYP3A4 mediated drug interactions by investigational agents have to rely primarily upon use of human hepatocytes. Given the numerous complexities in the availability of human hepatocytes and challenges of working with primary cultures from freshly isolated and cryopreserved cells, the use of high throughput tools that facilitate screening large libraries of investigational agents for their propensity to induce CYP3A is highly desirable. With the role of hPXR in transcriptional regulation of CYP3A4 induction well established, hPXR expression plasmid and CYP3A4 promoter plasmid can be used in cell based reporter assays to determine if a compound is a hPXR activator, which may be indicative of its ability to induce CYP3A4 transcription via hPXR (Moore *et al.*, 2000b).

1.5.3 Beyond Pregnane X Receptor and Constitutive Androstane Receptor

In addition to CAR and PXR, many orphan and adopted orphan NRs have recently been identified as key regulators of DMT genes. Indeed, nuclear receptors including farnesoid X receptor, peroxisome proliferator-activated receptors, and hepatocyte nuclear factors (1alpha, 3 and 4alpha) exhibit overlapping ligand specificities and regulate multiple gene targets, resulting in tissue- and organ-specific expression of drug disposition genes (Dixit *et al.*, 2005a). Intense research has also revealed permissive roles of other receptors such as glucocorticoid receptor (GR) and vitamin D receptor (VDR) in ligand dependent DMT induction.

More recently, attention is being focused on the Y-box-binding protein (YB-1). YB-1 is a member of the DNA-binding protein family. It binds to the Y-box, an inverted CCAAT box, in the promoter region of the human multidrug resistance 1 gene, which encodes P-gp. Nuclear localization of YB-1 protein has been reported to be associated with the intrinsic expression, i.e. basal expression, of P-gp in human breast cancer (Oda *et al.*, 1998). Giminez-Bonafe *et al*. (Gimenez-Bonafe *et al.*, 2004) observed that early in prostate cancer progression, increased expression of YB-1 may increase P-gp activity which may, in turn, lower androgen levels in the prostate tumor cells.

1.6 Clinical Implications of Induction and Inhibition of Drug Metabolizing Enzymes and Transporters

The preceding sections highlighted some of the key phase I/II enzymes and drug transporters involved in the metabolism of drugs. In addition to being metabolized by DMTs certain drugs have the ability to modulate these DMTs and may therefore alter the metabolism of co-administered drugs. This has a direct impact on the safety and efficacy of co-administered

drugs as well as their metabolites which are dependent upon these enzymes and transporters. As such these enzymes and transporters are highly regulated by their substrates and other drugs which could lead to induction or inhibition of these genes and subsequent drug interactions resulting from the changes in systemic clearance of drugs.

The first phenomenon that we shall discuss is induction. Induction results in increased clearance of the substrates. Most often, the metabolic end products of drug metabolizing enzymes do not retain the therapeutic activity of the parent compound and therefore, induction results in markedly reduced drug activity. Alternatively, induction can lead to increased adverse events if the metabolites have systemic toxicity. Compounds such as rifampin and phenobarbital transcriptionally induce CYPs and UGTs resulting in serious drug-drug interactions (Moore *et al.*, 2000b). For instance, rifampin significantly increases the rate of metabolism of the CYP2B6 substrate, efavirenz, resulting in ineffective blood levels and causing drug resistance in HIV-positive patients (Lopez-Cortes *et al.*, 2002). In another example, women taking St. John's Wort as a dietary supplement had decreased exposure to nevirapine due to increased levels of CYP3A4 (de Maat *et al.*, 2001).

In addition to increasing the clearance of co-administered drugs, an inducing agent can also enhance its own metabolism, a process known as auto-induction. Several compounds including efavirenz and rifampin exhibit autoinduction (Bristol Myers Squibb, 2002), (Strolin Benedetti and Dostert, 1994). For such compounds, there is a significant increase in drug clearance resulting in reduced plasma drug levels and drug efficacy upon multiple-dosing.

CYP and UGT inhibition is usually at the protein level and relatively instantaneous, whereas induction usually occurs at the level of transcriptional regulation. Antiretroviral drugs such as PIs and NNRTIs are involved in a significant number of drug-drug interactions related to

the modulation of DMTs, which will be further discussed later. Regardless of the overall outcome of the induction of DMTs, it is important to understand the mechanisms that underlie these interactions so that potentially adverse outcomes can be anticipated and alterations in the dosing regimen (dose, dosing interval, sequence of drug administration) can be made in order to avert these outcomes.

1.7 Biotransformation of Antiretroviral compounds

All currently approved PIs and NNRTIs are known to undergo CYP-mediated phase I metabolism (Erickson *et al.*, 1999), (Flexner, 1998), (Smith *et al.*, 2001) (Figure 1.2). These lipophilic drugs are either metabolized by one or more of the CYP isozymes to make them more water soluble for eventual elimination, or simply inhibit or induce these enzymes without being a substrate for metabolism. Again, CYP3A4 is the principal enzyme involved in their metabolism. For instance PIs such as saquinavir, indinavir, amprenavir, lopinavir, ritonavir and nelfinavir and NNRTIs such as efavirenz and nevirapine are metabolized by CYP3A4 to some extent (Chiba *et al.*, 1997), (Decker *et al.*, 1998), (Fitzsimmons and Collins, 1997), (Kumar *et al.*, 1999).

Following metabolism by phase I enzymes antiretroviral drugs are often subjected to phase II reactions such as glucuronidation. Given that phase I reactions are the slower of the two processes, they represent the rate-limiting step in the overall metabolic drug clearance of antiretroviral drugs. As discussed later, we focused our research on three important antiretroviral agents, efavirenz, ritonavir and nelfinavir. Therefore the metabolism of these drugs is discussed below.

1.7.1 Nelfinavir

Nelfinavir is metabolized by multiple CYP enzymes, including CYP3A and CYP2C19, resulting in the formation of one major and several minor oxidative metabolites (Agouron Pharmaceuticals Inc.). M8, the major metabolite formed by CYP2C19 mediated oxidation of the parent drug, has an *in vitro* antiretroviral activity similar to that of nelfinavir (Zhang *et al.*, 2001). The majority (82-86%) of radioactivity in plasma of healthy volunteers receiving a single oral dose of ^{14}C-nelfinavir 750mg was due to the parent drug (Agouron Pharmaceuticals Inc.), (Zhang *et al.*, 2001), the remainder comprised nelfinavir metabolites (Zhang *et al.*, 2001). Nelfinavir terminal t½ in plasma is <6 hours (Agouron Pharmaceuticals Inc.), (Kaeser *et al.*, 2005), (Regazzi *et al.*, 2001). After administration of a single oral 750 mg dose, most (87%) of ^{14}C-labelled nelfinavir is excreted in feces, with unchanged nelfinavir and its oxidative metabolites accounting for 22% and 78% of fecal radioactivity (Agouron Pharmaceuticals Inc.). A small proportion of the dose (1-2%) was recovered in urine, mainly as unchanged nelfinavir (Agouron Pharmaceuticals Inc.).

1.7.1.1 Nelfinavir Drug-drug interactions

Nelfinavir has been reported to be an inducer, an inhibitor and a substrate of DMTs (Figure 1.2). Since it is metabolized primarily by CYP3A and by CYP2C19, drugs that induce or inhibit these pathways raise the chances of potential interactions with nelfinavir (Agouron Pharmaceuticals Inc). Conversely, nelfinavir is an inhibitor of CYP3A and can therefore alter the pharmacokinetics of drugs metabolized by this isozyme (Agouron Pharmaceuticals Inc).

A recent study by Kurowski *et al*. (Kurowski *et al.*, April 2005) observed an increase in nelfinavir (57.4%) and M8 (124%) C_{min} when co-administered with atazanavir. This treatment regimen did not contain ritonavir. Similar results were observed when nelfinavir was co-

administered with delavirdine. Delavirdine increased the AUC (107%), C_{max} (88%) and C_{min} (136%) of nelfinavir (750 mg q8h) after 14 days. However, nelfinavir reduced the AUC (31%), C_{max} (27%) and C_{min} (33%) of delavirdine following 7 days of patients receiving this regimen. The appropriate dosage regimen for this combination has not yet been established.

The inhibitory potency of nelfinavir is highlighted by the significant increases in saquinavir pharmacokinetics when co-administered with nelfinavir. The AUC and C_{max} of saquinavir increased 392 and 179%, respectively, however, saquinavir did not alter the pharmacokinetics of nelfinavir (Perry *et al.*, 2005).

In addition to other antiretrovirals agents, nelfinavir could cause significant drug-drug interactions when combined with amiodarone, quinidine, pimozide, midazolam, ergot derivatives, terfenadine, astemizole and cisapride (Agouron Pharmaceuticals Inc.). These drugs are highly dependent on CYP3A4 for clearance and their elevated plasma concentrations are associated with serious and/or life-threatening events. Concomitant use of nelfinavir with St John's wort (*Hypericum perforatum*) is not recommended as it may substantially reduce plasma nelfinavir concentrations (Agouron Pharmaceuticals Inc.). Administration with nelfinavir may increase the plasma concentrations of some other drugs that are substrates for CYP3A e.g. bepiridil, tacrolimus, sirolimus, cyclosporine, and erectile dysfunction agents. Nelfinavir is also known to interact with medications for the treatment of cardiovascular disease (Table 1.3) (Hare *et al.*, 2002), (Hsyu *et al.*, 2001), (Izzedine *et al.*, 2004).

Table 1.3: Drug-Drug Interactions Associated with Efavirenz, Nelfinavir and Ritonavir

	Co-administered Drug	Class of co-administered drug	Effect on co-administered drug
Efavirenz	Simvastatin	Cardiovascular	58% decrease in AUC
	Pravastatin	Cardiovascular	40% decrease in AUC
	Atovarstatin	Cardiovascular	43% decrease in AUC
	Rifampin	Opportunistic infection	20% Decrease AUC, 26% decrease in Cmax
	Clarithromycin	Opportunistic infection	40% decrease in AUC
	Methadone	Withdrawal from drugs of abuse	60% decrease in AUC
	Buprenorphine	Withdrawal from drugs of abuse	Decrease AUC over 24 hours, 55% for Cmax, 49% for Cmin, and 72% for t ½
	Cyclosporine A	Immunosuppressant	75% Decrease in AUC
Nelfinavir	Felodipine	Cardiovascular	60% increase in felodipine
	Simvastatin	Cardiovascular	505% increase in AUC, 517% increase in Cmax
	Atovarstatin	Cardiovascular	74% increase in AUC, 122% increase in Cmax
	Ethinyl Estradiol	Anti-infective	47% decrease in AUC, 28% decrease in Cmax, +62% decrease in Cmin
	Azithromycin	Anti-Infective	>100% increase in AUC
Ritonavir	Corticosteroids	Immunosuppressant	>30% increase in AUC

1.7.2 Ritonavir

Drug metabolic studies to discern the metabolic pathways of ritonavir in human microsomes have shown that 3 major metabolites – the des-thiazoylyl carbamate product (M1), the isopropylthiazolyl oxidation product (M2) and the des-isopropylthiazolyl product (M11) –

are formed primarily by CYP3A (Denissen *et al.*, 1997), (Kumar *et al.*, 1996). CYP2D6 was also found to produce the metabolite M2 (Kumar *et al.*, 1996). Based on *in vitro* data, and the low fraction of CYP2D6 present in human liver relative to CYP3A (Shimada *et al.*, 1994), it was projected that CYP3A is the dominant isoform in humans. This was confirmed by the lack of consistent correlation between CYP2D6 genotype and ritonavir clearance (Abbott Laboratories, 1997), (Bertz *et al.*, 1996) and by the relatively small effect of fluoxetine (a potent CYP2D6 inhibitor, with lesser effects on CYP3A) on the pharmacokinetics of ritonavir (Abbott Laboratories, 1997).

1.7.2.1 Ritonavir Drug-drug interactions

Experiments undertaken *in vitro* indicate ritonavir to be a potent inhibitor of CYP3A (IC_{50} = 1.4mg/L) and CYP2D6 (IC_{50} = 1.8mg/L). In addition to these CYP enzymes, ritonavir also less potently inhibits CYP2C8, CYP2C9, CYP2C10, CYP2C19 and CYP1A2 *in vitro* with IC_{50} ranging from 5.8 to 36 mg/L (Kumar *et al.*, 1996). Based on these *in vitro* experiments it is evident that significant drug interactions are possible when ritonavir is prescribed in combination with any drugs that are extensively metabolized by CYP3A and had high intrinsic clearances and significant first pass metabolism (Hsu *et al.*, 1998a), (Lal *et al.*, 1997), (Lal *et al.*, 1998), (Merry *et al.*, 1997).

1.7.2.2 Ritonavir-boosted Protease Inhibitor Regimen

The potent inhibition of CYP3A by ritonavir is being utilized to improve clinical outcomes in the management of HIV. Ritonavir is now a commonly used booster-agent that is used in combination with low orally bioavailable antiretrovirals (Cooper *et al.*, 2003). This combination, with other PIs in a therapeutic dose, proved to be safe and effective over 5 years.

There are however, several limitations to this combination which could affect patient compliance, the most significant of which is gastrointestinal symptoms. Because ritonavir-associated toxicity is related to its level of exposure, lower doses of ritonavir have further improved the tolerability. Doses of 100 mg sufficiently enhance the pharmacokinetics of co-administered PIs to allow for twice- or once-daily dosing of the boosted PI, which may improve patient compliance. Simultaneous ingestion of ritonavir and the boosted PI is required to realize the beneficial interaction, as illustrated by a study in which subjects received saquinavir 4 hrs before ritonavir. The AUC of saquinavir was 37% of the AUC observed after simultaneous ingestion of these PIs.

When fixed doses of either saquinavir or indinavir are administered to patients, the addition of ritonavir resulted in a significant increase in the AUC and C_{min} values of saquinavir and indinavir (Hsu *et al.*, 1996), (Hsu *et al.*, 1998b), and significantly reduce the interindividual variability in the bioavailability of saquinavir from 84% to approximately 50% (Hsu *et al.*, 1998a), (Merry *et al.*, 1997). Also, compared to standard regimen of indinavir alone, the addition of ritonavir increased the steady-state C_{min} value of indinavir 3-fold at steady-state (Hsu *et al.*, 1998). While saquinavir, a weak CYP3A inhibitor, had no effect on ritonavir metabolism (Hsu *et al.*, 1998a), (Merry *et al.*, 1997) indinavir slightly increased the steady-state plasma concentration of ritonavir (Hsu *et al.*, 1998).

The interaction between ritonavir and nelfinavir is an example of complex interactions between drugs that are metabolized by multiple enzymes, which are known to be both inhibited and induced by various xenobiotics. In single dose studies, ritonavir increased the AUC of nelfinavir by 152%, while nelfinavir had no effect on the pharmacokinetics of ritonavir (Agouron Pharmaceuticals Inc.), (Kerr *et al.*, 1997). The results of a recent Phase II study in 2 groups of

patients with HIV receiving the same dosage of ritonavir (400mg twice daily) revealed that the group receiving nelfinavir 750mg twice daily had lower ritonavir C_{min} values compared with those receiving nelfinavir 500mg twice daily; this suggests a dose-dependence in the degree of enzyme induction (Flexner *et al.*, 1998). Ritonavir significantly increased the plasma concentrations of nelfinavir, however, the $AUC_{0\text{-}24\ hrs}$ values were similar between doses of nelfinavir 500mg and 750mg. Ritonavir also significantly increased the plasma concentrations of nelfinavir metabolite, M8, for both groups possibly indicating that ritonavir induced the activity of CYP2C isozymes, which ostensibly produce the metabolite (Flexner *et al.*, 1998), (Wu *et al.*, 1996), (Zhang *et al.*, 1998).

Ritonavir when administered with AZT decreased the AZT C_{max} and $AUC_{0\text{-}24\ hrs}$ by 27% and 26%, respectively. These changes in the pharmacokinetic profile of AZT are probably due to the induction of UGT by ritonavir (Figure 1.2) (Cato *et al.*, 1998).

Delavirdine is primarily metabolized by CYP3A and possibly also by CYP2D6 (Pharmacia & Upjohn, 1998). During co-administration with ritonavir delavirdine significantly increased the AUC of ritonavir by 60% in patients maintained on ritonavir 600mg twice daily (Morse *et al.*, 1998). Surprisingly ritonavir had no effect on the pharmacokinetics of delavirdine (Shelton *et al.*, 1997).

When co-administered with ritonavir, efavirenz increased the concentration of ritonavir by 22% at steady-state (Fiske *et al.*, 1998) while ritonavir had the same effect on the AUC of efavirenz (18% increase) (Cato *et al.*, 1998).

1.7.3 Efavirenz

Efavirenz is converted to inactive metabolites; 8-hydroxyefavirenz (major) and 7-hydroxyefavirenz (minor) via the CYP system, primarily CYP2B6 (90%) (Ward *et al.*, 2003).

Ward *et al*. (Ward *et al.*, 2003) noted that recombinant human CY1A2, CYP3A5, and CYP3A4 formed 8-hydroxyefavirenz from efavirenz, but the contributions of these isoforms to efavirenz metabolism appear minor. The intrinsic clearance (CL_{int}) for 8-hydroxyefavirenz formation by CYP1A2, CYP3A5, and CYP3A4 was 5.8-, 13.5-, and 60-fold lower, respectively than was obtained in recombinant human CYP2B6 (Ward *et al.*, 2003). Glucuronide and sulfate conjugates of 7-hydroxyefavirenz and 8,14-dihydroxyefavirenz had been identified in human urine (Mutlib *et al.*, 1999).

1.7.3.1 Efavirenz Drug-Drug Interactions

Combining a protease inhibitor with efavirenz may result in suboptimal protease inhibitor trough concentrations that may result from induction of drug clearance. The magnitude of these drug interactions tends to be variable and unpredictable between patients, providing clinicians with significant challenges when deciding on the need for dose adjustments or evaluating potential drug interactions involving efavirenz and a specific protease inhibitor.

HIV-positive patients receiving efavirenz in combination with indinavir (800 mg three times daily) have a 31% and 16% reduction in the observed indinavir AUC and $C_{max,}$ respectively (Staszewski *et al.*, 1999). Efavirenz was also shown to significantly reduce the AUC and C_{max} of saquinavir by 62% and 50% when administered as a soft gel capsule to 12 healthy volunteers (Bristol Myers Squibb, 2002). Conversely, neither indinavir nor saquinavir had any effect on efavirenz pharmacokinetics (Bristol Myers Squibb, 2002), (Staszewski *et al.*, 1999). However, when efavirenz was administered together with ritonavir both drugs produced an approximately 20% increase in their respective AUCs (Fiske *et al.*, 1998). The pharmacokinetics of efavirenz was also determined when administered with nelfinavir in healthy volunteers and at steady-state in adults and children with HIV-infection (Starr *et al.*, 1999), (Villani *et al.*, 1999). None of

these studies found any significant effect on either drug with efavirenz producing a 20% increase in the AUC of nelfinavir.

NNRTIs are also used in combination with 2 protease inhibitors which makes discerning the complex drug-drug interactions more difficult given that all of the drugs could either be inducers, inhibitors or substrates of DMTs (Figure 1.2). A study undertaken by Aarnoutse *et al*. (Aarnoutse *et al.*, 2002) in which 18 healthy volunteers were administered indinavir and ritonavir 800/100 mg daily for 14 days after which efavirenz 600 mg daily was added to the regimen. In this study efavirenz reduced the AUC and C_{min} of indinavir by 19% and 48%, respectively. However, the C_{min} value remained above the IC_{50} concentration (0.10 mg/L) of wild-type virus negating the need for dose adjustment (Aarnoutse *et al.*, 2002).

Previous clinical studies have shown that efavirenz decreases the AUC and C_{min} of amprenavir (Barry *et al.*, 1999), (Falloon *et al.*, 2000), (Wintergerst *et al.*, 2000). Thus it was hypothesized that when the aforementioned combination is administered with ritonavir, a known inhibitor of amprenavir metabolism, the inductive effects of efavirenz will be offset. A clinical study was undertaken in 19 HIV-positive patients in which, each patient received amprenavir 200mg twice a day, ritonavir 200 or 500mg twice daily, and efavirenz 600mg daily in a stepwise fashion. The findings from this study revealed that ritonavir significantly increased the AUC of amprenavir without a reduction by efavirenz. When ritonavir was replaced with nelfinavir similar findings were observed (Piscetelli *et al.*, 2000). These results were confirmed in the clinical studies undertaken by Fellay *et al*. (Fellay *et al.*, 2005) in which the inhibition of CYP3A by ritonavir or nelfinavir offset the inductive effects of efavirenz administered concomitantly. They concluded that no induction of CYP3A activity, by efavirenz, was

noticeable after long-term administration of ritonavir at low dosages (200 mg/day b.i.d.) or of nelfinavir at standard dosages (2,500 mg/day b.i.d.).

Among the newer PIs, efavirenz decreased atazanavir AUC by 74% (Preston *et al.*, 2002). In an attempt to negate this reduction in AUC, investigators increased the dose of atazanavir from 400 mg to 600 mg, however, the concentrations remained lower than with atazanavir 400mg alone. Due to these results the combination is not recommended. When ritonavir 100mg was added to this dual combination, atazanavir 300 mg and efavirenz 600 mg, ritonavir increased the atazanavir AUC by 39% compared with atazanavir 400 mg alone (O'Mara *et al.*, 2002). Subsequently, in efavirenz-containing regimen, atazanavir must be boosted with ritonavir.

In addition to drug-drug interactions with other antiretrovirals, efavirenz is also known to interact with drugs used for the treatment of drug-abuse (Clarke and Mulcahy, 2000), (Clarke and Mulcahy, 2000), (Clarke *et al.*, 2001), (Marzolini *et al.*, 2000), immunosuppressant drugs (Tseng and Foisy, 1997), (Tseng *et al.*, 2002), cardiovascular medications (Fontas *et al.*, 2004), (Gerber *et al.*, 2005), (Simon *et al.*, 2002), (Tashima *et al.*, 2003) and with medications for opportunistic infections (Benedek, 1998), (McCance-Katz *et al.*, 2005) (Table 1.3).

1.8 Pharmacokinetics of Antiretrovirals

From literature search we determined that all 3 antiretroviral agents are well absorbed after oral administration with efavirenz, nelfinavir and ritonavir having bioavailability (F) values of 60, 88 and 98%, respectively. Efavirenz after oral administration achieved C_{max} of 1.6 to 9.1 μM, 5 hours following single oral doses ranging from 100-1600 mg (Bristol Myers Squibb, 2002). In HIV-positive patients, oral administration of 600 mg daily had been reported to produce steady-state C_{max} of 12.9 ± 3.7 μM and steady-state trough drug concentrations (C_{min}) of

5.6 ± 3.2 μM (Bristol Myers Squibb, 2002), (Staszewski *et al.*, 1999). The observed C_{max} and C_{min} values following oral nelfinavir (1250 mg twice/thrice daily) to healthy volunteers were ~7.0 μM and 2.1 μM (range 0.12 - 3.9 μM) (Maserati *et al.*, 1999), (Perry *et al.*, 2005). The therapeutic dosage of ritonavir is 600 mg twice daily, or 400 mg twice daily. However, nowadays ritonavir is mainly used in combination with other PIs as a booster of these compounds. A low dose of ritonavir (100 or 200 mg), the so-called 'baby dose', results in nontherapeutic plasma concentrations of ritonavir. For therapeutic doses of ritonavir, significant correlations between antiviral activity and plasma drug concentrations have been demonstrated in various clinical studies in HIV-1-infected patients. A ritonavir C_{min} of 2.9 μM was extracted as a target for adequate viral suppression. After a dose of 600 mg twice daily the C_{max} obtained was 14.9 μM and the C_{min} was 4.9 μM (Abbott Laboratories, 1997), (Gatti *et al.*, 1999).

Efavirenz has a long half-life, ranging from 52 to 76 hours following single oral doses, and 40 to 55 hours following long-term administration as a result of auto-induction of efavirenz metabolism. In comparison the t½'s of nelfinavir and ritonavir are ~6 hrs. Furthermore, all 3 antiretrovirals are highly bound to α_1-acid glycoproteins (>90%) (Table 1.4.2) (Abbott Laboratories, 1997), (Agouron Pharmaceuticals Inc.), (Bristol Myers Squibb, 2002), (Kempf *et al.*, 1995), (Zhang *et al.*, 2001). The apparent volumes of distribution of efavirenz, nelfinavir and ritonavir are 252 L (Csajka *et al.*, 2003), 525L (Agouron Pharmaceuticals Inc.), and 40L (Abbott Laboratories, 1997), respectively, which indicates that most of the administered dose is present in peripheral compartments.

1.9 Induction of Drug Metabolizing Enzymes and Transporters by Antiretrovirals

Drugs such as efavirenz, nelfinavir and ritonavir can cause a clinically significant increase in the metabolism of other antiretrovirals which are substrates and therefore metabolized via the same drug metabolizing enzyme (saquinavir, delavirdine and AZT) (Figure 1.2), because they stimulate the CYP/UGT system. This can significantly reduce the concentrations of co-administered drugs to subtherapeutic levels (Dybul *et al.*, 2002). Even a short exposure to suboptimal levels of antiretroviral agents can lead to irreversible viral resistance and loss of clinical benefit (especially among drugs with a low barrier to resistance, such as lamivudine and NNRTIs).

A study by Berruet *et al*. (Berruet *et al.*, 2005) investigated the effects of efavirenz on intestinal P-gp and hepatic CYP function to predict drug interactions. They studied the effect of orally administered efavirenz (150 mg/kg) on rat intestinal P-gp function (using everted gut sacs and in situ intestinal perfusion). In parallel, they investigated the effect of efavirenz on rat hepatic P450 metabolism using hepatic microsomes, prepared from rats with or without pretreatment with efavirenz. They observed that in everted gut sacs, P-gp function was not modified and in the in situ intestinal perfusion, rhodamine 123 (P-gp probe substrate) clearance was not affected by efavirenz. They also observed that concentrations of the metabolites of midazolam (CYP3A probe substrate), 1-OH midazolam and 4-OH midazolam were higher in efavirenz pretreated rats than those in the control group. Based on these findings Berruet *et al*. (Berruet *et al.*, 2005) concluded that efavirenz may not alter intestinal absorption of co-administered P-gp substrates, but may decrease plasma concentrations of co-administered drugs metabolized by P450.

Similarly Kageyama *et al*. (Kageyama *et al.*, 2005) attempted to assess the functional changes of CYP3A or Pgp during chronic administration of ritonavir in rats. Intravenous administration of erythromycin (CYP3A substrate) to ritonavir-treated rats, resulted in increased erythromycin $AUC_{(0\text{-infinity})}$ on Day 3 and Day 5 compared to the control untreated rats. This increase is likely due to initial inhibition of DMTs with ritonavir. However, in rats that received a 7-day treatment of ritonavir and in rifampin-treated rats, the $AUC_{(0\text{-infinity})}$ of erythromycin decreased significantly by 82% and 42%, respectively, as compared to the control. Also, the excretion clearance of Rh123, a marker of P-gp activity, from blood circulation to the intestinal lumen and the biliary excretion clearances increased by 2.2-fold and 2.6-fold over controls, in rats that received ritonavir treatment for 14 days. The authors concluded that both CYP3A and P-gp are induced by ritonavir.

All of the studies and preceding discussions provide evidence that antiretrovirals such as efavirenz, nelfinavir and ritonavir induce various DMTs. However, several issues regarding antiretroviral effect on DMTs still remain unexplored.

A vast majority of the studies employed animal models. It is well recognized that CYP enzymes exhibit marked inter-species variability in their expression and regulation. This is a major factor limiting the extrapolation of results obtained in animal studies to humans. In order to improve the *in vitro- in vivo* correlations of antiretrovirals on human enzymes, it is vital to employ clinically relevant models. Of the various experimental systems currently used in the assessment of the regulation and functional changes in various hepatic DMTs, the best approach entails the use of primary cultures of human hepatocytes. As with liver cells it would be ideal to utilize primary human enterocytes for investigating issues pertaining to intestinal drug metabolism. However bacterial contamination is a major factor limiting its use. A widely used

alternative is the intestinal colon carcinoma cell lines, such as LS174T (a clinically relevant model for intestinal drug metabolism studies). There is also limited data on the induction of CYP2B6 which is now known to metabolize 8% of drugs marketed in the United States (specifically efavirenz). Given the complex metabolic pathways involved in antiretroviral metabolism, it would also be prudent to assess the modulation of phase II enzymes such as UGT.

There is also a paucity of information regarding the induction of DMTs in $CD4^+$ T-cells. While modulation of DMTs in the liver and intestine may alter the plasma concentrations of the active drug, the clinical efficacy of these agents is dependent upon their intracellular concentration given that their site of action is inside the infected $CD4^+$ T-cell. Therefore, of critical importance to the efficacy of these antiretrovirals is the modulation of these DMTs in $CD4^+$ T cells (Asghar *et al.*, 2002), (Hu and Wells, 2004), (Krovat *et al.*, 2000), (Lucia *et al.*, 2002), (Nakamoto *et al.*, 2000). In HIV-1-infected peripheral blood mononuclear cells (PBMCs) and cell lines, the antiviral activity of antiretrovirals has been highly correlated with the intracellular concentration of drugs (Bilello and Drusano, 1996), (Nascimbeni *et al.*, 1999) which has formed the rationale for further studies on intracellular antiretrovirals.

Related to the paucity of information regarding the modulation of DMTs in $CD4^+$ T-cells is the impact of HIV on the induction potential of DMTs. The attack by HIV on $CD4^+$ T-cells occurs in phases which suggest that while some cells are infected other $CD4^+$ cells may be exposed to HIV but remain uninfected. Both scenarios could have a significant impact on the efficacy of antiretrovirals.

1.10 Limitations of the In Vitro Models

Primary cultures of human hepatocytes have overcome some of the drawbacks of other *in vitro* models for induction studies. For example, the influence of the processes of drug diffusion

and transport and metabolites on the overall cellular disposition is accounted for in this model system. Also, regulatory pathways remain functional for several days after isolating and plating cells which makes it feasible to assess upregulation/downregulation of drug metabolizing enzymes.

However, it has been widely recognized that cultured hepatocytes are subject to a gradual loss of liver-specific functions, with special reference to a decreased CYP expression. This loss is different for the specific CYP isoforms; for some isoforms it becomes evident after a few days of culture (CYP2E1 and CYP3A4), while others remain nearly unaffected by the isolation and culturing processes (CYP1A2 and CYP2C9) (George *et al*. 1997). Various culturing methods have been explored in an effort to maintain the liver-specific characteristics of hepatocytes during prolonged culture. This includes the use of cell double-layer collagen gel sandwich, which can be used to study not only biotransformation, but also transporter-mediated biliary excretion (De Smet *et al.*, 2000).

Unlike the use of primary culture of human hepatocytes, which is considered to be the best *in vitro* model for evaluating drug effect on hepatic DMTs, *in vitro* models for intestinal drug metabolism are limited and not established. Previous studies have used primary cultures of enterocytes in short term cultures to undertake enzyme inhibition studies. However, enzyme induction studies require drug treatment for ~72 hours, which necessitates that cells remain in culture for ~72-96 hours. A major concern with the maintenance of primary enterocytes is the potential contamination from brush border bacteria. This restriction requires the use of transformed cell lines and careful consideration of the experimental aims is needed for selecting the cell line. Several cell lines that are widely utilized in the study of polarized intestinal transport of orally administered drugs lack key pathways involved in the regulation of drug

metabolism. For example, Caco-2 cells do not express PXR and in some instances CYP3A4 itself (Cummins *et al.*, 2004), (Pfrunder *et al.*, 2003). With the above considerations of PXR expression and it's previously reported use in studying the regulation of drug metabolism and transport, we preferred to use LS174T cells in our studies compared to other available colon carcinoma cell lines (Pfrunder *et al.*, 2003), (Schuetz *et al.*, 1996). Furthermore, compounds that induce hepatic CYP3A4, such as rifampin, have shown marked induction of CYP3A4 in LS174T cells, which indicates that this model has potential to be clinically relevant.

2. HYPOTHESIS AND SPECIFIC AIMS

AIDS remains a major public health issue and has been described as the "defining medical and public health issue of our generation and ranks among the greatest infectious disease scourges in history". In 1995, the clinical efficacy of using a protease inhibitor in combination with two nucleoside reverse transcriptase inhibitors (NRTIs) was recognized. The subsequent approvals of more potent protease inhibitors, such as ritonavir and nelfinavir, rendered feasible the prolonged survival of patients with AIDS and HIV-1 infection. Subsequent landmark studies proved the efficacy of triple combination therapy, or Highly Active Antiretroviral Therapy (HAART), in markedly reducing mortality and inducing viral load suppression. Other triple combination therapies became possible with the introduction of non-nucleoside reverse transcriptase inhibitors (NNRTIs), which when combined with two NRTIs, further enhanced the efficacy, in suppressing HIV-1 replication. In spite of the undeniable clinical benefits obtained with HAART, between 20% and 50% of patients who initiate HAART present with virological failure during the first year of treatment and the incidence of failure increases rapidly during successive treatments. Treatment failure may occur due to a multitude of reasons, which may be patient-, virus-, or drug-related.

It is known that antiretroviral agents such as protease inhibitors activate the human pregnane X receptor (hPXR) and the human constitutive androstane receptor (hCAR), nuclear receptors that are known to upregulate the expression of several key drug metabolizing enzymes and transporters (DMTs). These agents are also known to induce CYP3A4 expression, resulting in serious clinically relevant drug-drug interactions. However, several mechanistically and clinically important aspects of such inductive effects have remained unresolved. Firstly, the inductive effects of some of the novel agents such as the NNRTI, efavirenz, were not known at the time of this undertaking. Secondly, a clear understanding of the comparative risk for drug-

drug interactions (DDIs) between efavirenz and other PIs was not known. Thirdly, the effects of these agents on the other phase I and II enzymes and drug transporters in not known. Fourthly, the inductive effects of antiretroviral agents on the extra-hepatic expression of DMTs, especially in the $CD4^+$ T-cells has not been investigated. While hepatic and intestinal metabolism determines the overall systemic exposure to the antiretrovirals, the target-specific intracellular drug concentration (in this case, in $CD4^+$ T-cells) may be the most clinically relevant drug levels. Finally, the influence of the HIV infection on the activity and expression of DMTs in $CD4^+$ T-cells is not known. These gaps in our knowledge represent an important barrier to the safe and efficacious use of antiretroviral agents. Our research represents an important step in bridging this gap. The overarching hypothesis of our research was that **antiretroviral compounds, efavirenz and PIs, induce hepatic and extra-hepatic drug metabolizing enzymes and drug transporters, and the magnitude of induction is modulated by disease state, in human *in vitro* models.**

To investigate this hypothesis we pursued the following specific aims:

Specific Aim 1: *To assess the extent to which efavirenz and PIs (nelfinavir and ritonavir) induce phase I (CYP3A4 and CYP2B6) and phase II (UGT1A1, UGT1A6 and UGT2B7) enzymes and drug transporters (P-gp) in primary cultures of human hepatocytes and a colon carcinoma cell line, LS174T.*

Specific Aim 2: *To investigate whether efavirenz and PIs induce DMTs in primary cultures of $CD4^+$ T-cells.*

Specific Aim 3: *To examine the influence of HIV on the induction of DMTs in primary cultures of $CD4^+$ T-cells.*

Anticipated Outcomes

It was projected that completion of the aforementioned specific aims will provide a comparative evaluation of efavirenz and the PIs to induce DMTs in human hepatocytes which may indicate the potential for alteration in hepatic metabolism, transport and clearance of drugs. Secondly, it would also be possible to evaluate potential differences in the inductive effects of these agents in hepatocytes versus intestinal tissue. Our studies also assessed whether DMTs are expressed and induced in $CD4^+$ T-cells. This together with our observations of the impact of HIV on the expression of key transcription factors and the induction of P-gp will add to the current paucity of information in this regard. The knowledge acquired from this study will ultimately aid in designing newer antiretrovirals that lower the potential for drug-drug interactions and the development of antiretroviral resistance.

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

Chemicals

Rifampin, phenobarbital, dexamethasone, testosterone, 6ß-hydroxytestosterone, 11α-hydroxyprogesterone, midazolam, ß-glucuronidase/arylsulfatase, 7-ethoxy-4-trifluoromethylcoumarin (EFC), 7-benzyloxy-4-trifluoromethylcoumarin, 7-hydroxycoumarin, 7-hydroxy-4-trifluoromethylcoumarin (HFC), 4-hydroxycoumarin, Rhodamine 123, estradiol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phytohemaglutinin (PHA) were obtained from Sigma Chemical Co. (St. Louis, MO). ECL chemiluminescence detection system was obtained from GE Healthcare (Amersham Biotech, Piscataway, NJ). All PCR-reagents, lymphocyte separation medium, mitoxantrone, calcein-AM, RPMI-1640 medium, fetal bovine serum and dimethylsulfoxide (DMSO) were obtained from Fisher Scientific (Hampton, NH). $CD4^+$ isolation kit (MACS cell separation) and columns (MACS column) were obtained from Miltenyi Biotech (Auburn, CA). MK571 was obtained from Cayman Chemical (Ann Arbor, MI). Fumitremorgin–C (FTC) was purchased from Alexis Biochemicals (San Diego, CA). Efavirenz, nelfinavir and ritonavir were kindly provided by the NIH AIDS Reagent Procurement Program.

Biological Reagents

All primary ($CD4^+$, P-gp, MRP1 and BCRP) and rat anti-mouse IgG_{2a+b} secondary protein specific antibodies for flow cytometry were purchased from BD Biosciences (San Jose, CA) and Chemicon International (Temecula, CA). Primers for real-time PCR were custom made by Integrated DNA technologies (Coralville, IA) and the University of Cincinnati DNA Core (Cincinnati, OH). HIV-1 gp41 monoclonal antibody was kindly provided by the NIH AIDS Reagent Procurement Program. Interleukin-2 (IL-2) was donated by Julie E. Nelson, Ph.D.

(Department of Molecular Genetics, University of Cincinnati). The MACS separation magnet and reagents for flow cytometry were kindly provided by Rodney P. DeKoter Ph.D. (Department of Molecular Genetics, University of Cincinnati). The hPXR expression plasmid, pSG5-hPXR-ΔATG, the CYP3A4 promoter plasmid pGL3-CYP3A4 XREM-tk-luc and the CAR expression plasmid pSG5-hCAR were kindly donated by Bryan Goodwin, Ph.D. (Glaxosmithkline Inc.) (Goodwin *et al.*, 1999) . The 780-basepair CYP3A4 cDNA probe was obtained from Oxford Biomedical Research Inc. (Oxford, MI). Enzyme specific antibodies (CYP3A4, CYP2B6, P-gp, UGT1A1, UGT1A6 and UGT2B7) for immunoblotting were obtained from BD Biosciences (Gentest Corp, Woburn, MA).

Cell lines, Cell Culture Media and Supplements

Human hepatocellular carcinoma (HepG2) and colon carcinoma (LS174T) cell lines were obtained from American Type Culture Collection (Manassas, VA). Hepatocyte Maintenance Medium (HMM, Clonetics, Walkerville, MD) and media supplements were obtained from BioWhittaker (San Diego, CA). William's E medium was obtained from Caisson Labs Inc. (Rexburg, ID).

3.2 Methods Employed to Assess the Induction of Drug Metabolizing Enzymes and Transporters

3.2.1 Human Pregnane-X Receptor Activation Assays

Transient transfection assays in hepatoma (HepG2) and LS174T cells were performed as described previously (Goodwin *et al.*, 1999) . Briefly, cells were plated in 24-well plates in antibiotic free DME medium supplemented with 10 % delipidated fetal calf serum at a density of 2.4×10^5 cells per well. Following 24-hour of plating the cells, overnight transfections were performed employing Lipofectamine and Plus reagent (Invitrogen), exactly as suggested by the manufacturer. Transfection mixes contained 100 ng human PXR expression vector (pSG5-humanPXR), 400 ng luciferase reporter gene construct (XREM-CYP3A4 -tk-luc), 400 ng pCH110 (an expression vector containing β-galactosidase cDNA under T7 promoter, Amersham). Control transfections with 100 ng blank vector pSG5, 400 ng luciferase reporter gene construct, 400 ng pCH110 were also maintained and treated with drugs. Following transfection, plasmid- containing media were replaced with drug-containing media (replaced after 24 hours) and incubated for 48 hours. The cell layers were washed twice with ice cold PBS (pH 7.4) and scraped and collected in 250 µl reporter lysis buffer provided with the Luciferase Assay System (Promega). Protein assay was performed on an aliquot of the cell lysate and 10 µl of the cell lysate was used for determining the luciferase activity employing a Luciferase Assay System (Promega). An aliquot of cell lysate (50 µl) was used for determining the β-galactosidase activity exactly as described by the manufacturer's protocol and reagents were obtained from Promega. The luciferase activity levels were normalized to the β-galactosidase

activity and expressed as fold-activation with respect to the solvent (0.1 % DMSO) treated controls.

3.2.2 Cell Culture and Drug Treatment of Cells

Primary human hepatocytes, isolated from lobes of liver from separate donors (Table 3.2.1), were provided by the Liver Tissue Procurement and Distribution System, which was funded by the National Institutes of Health (NIH) contract N01-DK-9-2310.

Table 3.2.1: Human Liver Donor Information

Batch Number	Age (yr)	Sex	Race	Cause of Death	Drugs used in ER/OR
HH940	28	M	C	MVA	Dopamine, DDAVP, NaHCO3, insulin, clindamycin, ceftazadime, mannitol, KCl,Ca Gluconate, Kphos, Ancef, Erythromycin, neomycin, nystatin, solumedrol
HH946	8	F		Brain dead	
HH993	46	F	C	CVA/Stroke	
HH1087	57	M	C	CVA	Flomax, atenolol, divertic, aspirin
HH1097	35	F	C	Brain dead	Dopamine
HH1105	15	F	C	Anoxia	
HH1117	68	F	C	Stroke	Solumedrol, insulin, levothyroxine
HH1139	3	M	C	MVA	Dopamine, atropine, etomidate, vecuronium, versed, propofol, benedryl, lasix, vasopressin, thyroxine
HH1174	75	F	C	CVA	Neosynephrine
HH1182	66	F	C	CVA	
HH1183	54	M	C	Fall, brain dead	Dopamine, neosynephrine, vasopressin, vencuronium, Propofol, thyroxine
HH1196	56	F	C	Asystole	Propofol, neosynephrine, vasopressin, levophed, milirone, fentanyl, dobutamine, insulin, naficillin, caspofungin
HH1200	53	F	C	CVA	Dopamine

HH1201	69	M	C	CVA/Anoxia	Epinephrine, insulin, ampicillin, zosyn, genomyson, levoquin
HH1205	45	M	H	CVA	Solumedrol, vecuronium
HH1210	35	F	C	Head injury	Dopamine, neosynephrine, vasopressin, synthroid, hydrocortisone, levaquin, metronidazole
HH1220	47	F	C	CVA	
HH1221	45	F			
HH1223	58	F	C	Anoxia/CVA	
HH1247	3	M			
HH1249	59	M	H	CVA	Lipitor, labetalol, lopressor, insulin, vancomycin, dilantin, pertobarbital, nipride
HH1177	60	M	C	Anoxia	Dopamine, levophed

HH = human hepatocyte, M = male, F = female, C = Caucasian, H = Hispanic, CVA = cardiovascular accident, MVA = motor vehicle accident

Hepatocytes were plated in collagen-coated T25 cm^2 flasks (4×10^6 cells/well) for the determination of CYP/UGT activity and immunoreactive protein levels. In parallel, cells were plated in 6-well plates (1×10^6 cells/well) for Northern Blot or mRNA analysis of CYP3A4-specific mRNA. Collagen-coated 24-well plates ($1.25 \text{ X } 10^5$) were used for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Figure 3.1).

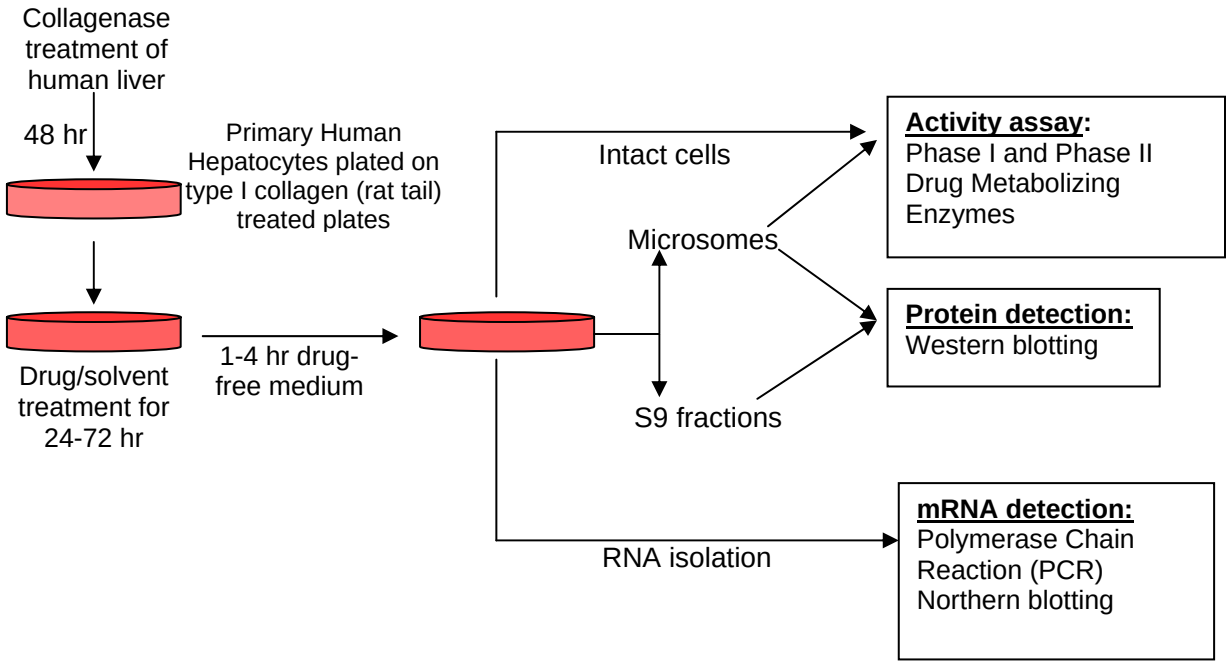

Figure 3.1: Scheme of hepatocyte treatment for induction studies

Hepatocytes were maintained in Williams' E medium (BioWhittaker). LS174T (colon carcinoma) cell line was obtained from The American Type Culture Collection (ATCC). Cells were maintained in minimum essential medium (MEM) supplemented with non-essential amino acids, 7.5% sodium bicarbonate and 10% fetal bovine serum (Fisher). LS174T cells utilized for CYP and UGT studies were from passage 5 to passage 15.

Stock drug solutions (1000X) were prepared in DMSO and diluted prior to use. Forty-eight hours after plating, hepatocytes/LS174T cells were treated with vehicle, which contained the same amount of DMSO (0.1%) (Desai *et al.*, 2002), efavirenz (0.5-10 μM), nelfinavir (0.5-10 μM), ritonavir (0.5-10 μM), rifampin (10 μM) or Pb (2 mM) for 72 hrs. Previously reported studies from our laboratory (Desai *et al.*, 2002), (Nallani *et al.*, 2001) and those of others (Kostrubsky *et al.*, 1998), (Kostrubsky *et al.*, 1999) have shown that DMSO at levels ranging from those in our experiments to levels twice as high do not alter the expression of CYP

enzymes. For each donor 2 replicates were done for each treatment (i.e. for control, phenobarbital, rifampin or each different concentration of test compound). Drug-containing medium was replaced every 24 hrs for the 72 hr period. Cell viability was assessed daily during the course of drug exposure by using the MTT assay as described earlier (Carmichael *et al.*, 1987). No significant difference between the viability of cells treated with the vehicle and cells treated with rifampin, phenobarbital, ritonavir or efavirenz was observed. Toxicity was however, observed at higher concentrations (≥ 5 μM) of nelfinavir in LS174T cells.

3.2.3 Measurement of Cytochrome P450 3A4 Activity in Intact Cells

At the end of the 72 hr drug treatment period the drug-containing media from the T25 cm^2 flasks were removed, and the cells were incubated in drug-free medium for 4 hours. Cells were then incubated with testosterone (250 μM) in William's E medium (1 ml/well) or MEM (1 ml/well) for 30 min. This medium was then collected. The rate of testosterone conversion to 6β-hydroxytestosterone, a reaction catalyzed by CYP3A4, in intact hepatocytes was employed to assess the enzyme activity, as described earlier (Kostrubsky *et al.*, 1999). Twenty five microliters of 11α-hydroxyprogesterone (1 μg/10 μL) used as an internal standard for High Performance Liquid Chromatography (HPLC) quantitation, was added to the medium. An aliquot of the medium was extracted with 2.5 mL of dichloromethane, and the solvent was evaporated under nitrogen. The residue was then reconstituted in 60:40 vol/vol methanol/water and analyzed for 6β-hydroxytestosterone levels employing a published HPLC method routinely employed in our laboratory (Desai *et al.*, 2002) , (Nallani *et al.*, 2001), (Waxman *et al.*, 1983). Briefly, Waters 510 pumps were used to elute the mobile phase comprising 60:40 methanol: water mobile phase at 1 ml/min through a C_{18} μ-bondapak column (3.9 mm X 30 mm). A Waters 486 UV/VIS detector at a wavelength of 242 nm was employed for the detection of 6β-

hydroxytestosterone. The total run lasted for 30 minutes, with 6β-hydroxytestosterone, 11α-hydroxyprogesterone (internal standard) and testosterone eluting at 9.2, 14.9, and 23.2 minutes, respectively. The interday and intraday variability in the HPLC analysis was <5%, and the detection limit was 100 pmol for 6β-hydroxytestosterone.

3.2.4 Cytochrome P450 2B6 Activity Assay

The conversion of bupropion to hydroxybupropion was used as a marker of CYP2B6 activity. Solutions of bupropion hydrochloride, hydroxybupropion and trazodone were prepared in methanol. The reaction was carried out using microsomes (0.25 mg/ml) in 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM $NADP^+$, an isocitrate/isocitric dehydrogenase regenerating system, and 5 mM $MgCl_2$. Reactions were initiated with the addition of microsomes, and the final volume was 0.25 ml. The incubation tubes were placed in a shaking water bath (37^0C) for 20 min and terminated by the addition of 1N hydrochloric acid (HCl) (50 µl). The internal standard, trazodone 125 µM (25 µl) was added to the incubation test tube, vortexed and spun at 16 000g for 10 min.

The metabolite concentrations (hydroxybupropion) were determined by HPLC using a method adapted from Cooper *et al*. (Cooper *et al.*, 1984). Briefly, a 300 X 3.9-mm µBondapak C_{18} column (Water Inc.) was used for separation with a flow rate of 2 ml/min and ultraviolet detection at 214 nm. The mobile phase consisted of 79% 50 mM KH_2PO_4 (adjusted to pH 3 with 1N HCL) and 21% acetonitrile. Standard curves were prepared by adding known amounts of hydroxybupropion (50-2000 ng) to an incubation tube and evaporating off the solvent. Chromatograms were analyzed by measuring peak height using the internal standard method. The interday and intraday variability in the HPLC analysis was <4%, and the detection limit was 50 ng for hydroxybupropion.

3.2.5 UDP-glucuronosyltransferase Activity Assay

3.2.5.1 UDP-glucuronosyltransferase 1A

The conversion of 7-hydroxy-coumarin to its glucuronide was employed as a marker for UGT1A activity. The reaction was carried out employing microsomes (80 µg) in a Tris Buffer (pH = 7.4) containing $MgCl_2$ (10mM), alamethicin (50 µg/mg microsomal protein), sacchralactone (5 mM) and the substrate (7-hydroxy-coumarin; 0.77 mM). The final volume was 400 µl. The reaction was started with the addition of uridine diphosphate glucuronic acid (UDPGA) 3 mM. The test tubes containing the reaction mixture were shaken at 37^0C in a water bath for 20 min. The reaction was then terminated with the addition of tricholoro acetic acid. The substrate and the glucuronide metabolite were assayed employing a validated HPLC system. The current procedure in our laboratory employs a Waters C-18 µBondapak column and a gradient mobile phase. 4-hydroxy-coumarin served as the internal standard. The eluent was monitored at 320 nm. The solvent system comprised of Methanol (solvent A) and Water/Acetic Acid (1000:2; solvent B) pH = 3. The gradient employed was as follows: 0-14 min: 20:80 A:B; 14 to 20 min 50:50 A:B; 20 to 35 min 50:50 A:B; 35 to 41 20:80 A:B; 41 to 51 min 20:80 A:B. The retention times were: 7-hydroxy-coumarin glucuronide, 6.3 min; 7-hydroxy-coumarin at 15.1 min and 4-hydroxy-coumarin, 24.8 min.

3.2.5.2 UDP-glucuronosyltransferase 2B7

AZT, 3-Acetamidophenol and AZT-glucuronide solutions were prepared by dissolving in methanol. AZT 500 µM was added to each incubation tube and dried down using nitrogen gas. Tubes were then placed on ice, and microsomes (0.5 mg/ml), alamethicin (50 µg alamethicin/mg of microsomal protein), and aqueous buffer (50 mM potassium phosphate) were added to a

volume of 50 µl. A UDPGA cofactor solution was also prepared on ice containing UDPGA (5 mM final concentration) and $MgCl_2$ (5 mM final concentration) in buffer.

Microsomal fractions were then pre-incubated for 5 min in a water bath set at 37^0C, and reactions were initiated by the addition of 50 µl of the UDPGA cofactor solution to each tube, vortexing briefly, and returning to the water bath. Reactions were terminated by the addition of 100 µl of ice-cold methanol containing 3-Acetamidophenol (internal standard). Tubes were then centrifuged at 14 000g for 10 min, and the supernatant transferred to glass test-tubes, dried down using nitrogen gas and reconstituted with 100 µl of water. AZT and AZT-glucuronide were assayed employing a validated HPLC system (Court *et al.*, 2003). The current procedure in our laboratory employed an Alltech C-18 column (100A 5µ) and a gradient mobile phase. The eluent was monitored at 260 nm. The solvent system consisted of acetonitrile (solvent A) and 20 mM potassium phosphate (solvent B) pH = 2.2. The gradient employed was as follows: 10% A for min, 15-50% A over the next 10 min, and 100% of B for 20 min. The retention times were: 3-Acetamidophenol (internal standard), 10 min; AZT-glucuronide, 12 min; and AZT at 14 min.

3.2.6 P-glycoprotein Activity Assay

At the end of the 72 hr drug treatment period, the medium was discarded and cells were incubated for 2 hrs in serum- and drug-free medium to facilitate drug elimination. The Pgp index substrate rhodamine-123 (Rh123), and the Pgp inhibitor cyclosporine A as positive control, have been used previously in induction studies in adenocarcinoma cells (Perloff *et al.*, 2001b). One set of plates was incubated for 1 hour with drug- and serum-free medium containing cyclosporine A 25 µM (Pgp inhibitor). Plates were then incubated for 30 min with Rh123 (final concentration 20 µM) with 1 set incubated with both cyclosporine A and Rh123.

After 30 min plates were removed from the incubator and washed thrice with ice-cold phosphate buffered saline (PBS). Plates were then lysed with 1X Reporter Lysis buffer (Promega) and shaken at room temperature for 30 min. Rh123 lysates were quantified by fluorescence determined at 500/550 (excitation/emission). The data was then normalized to protein concentration in each well. The fold increase in Rh123 retention in the presence of cyclosporine A was calculated using the following formula:

$$\text{Ratio} = \frac{(\text{sample/control})^{+\text{Inhibitor}}}{(\text{sample/control})^{-\text{Inhibitor}}}$$

3.2.7 Immunodetection of Cytochrome P450/ UDP-glucuronosyltransferase Protein

After the 72 hr drug treatment period cells were harvested in phosphate buffered saline (PBS) and spun at 4000 rpm for 10 minutes. The supernatant was discarded and cells were homogenized in homogenization buffer (10 mM HEPES, 1 mM EDTA, 20% glycerol pH 7.4) using a Tissue-Tearor (Biospec Products). Cells were then spun at 9000 rpm for 20 minutes. The supernatant was transferred to polycarbonate tubes and ultra-centrifuged at 100 000g for 1 hour. Pellets were then resuspended in Tris-Resuspension buffer (50 mM Tris base, 1 mM EDTA, 20% glycerol, pH 7.4). Protein quantitation of the microsomes was undertaken using the Lowry method.

Protein fractions (3-25 μg) were resolved employing SDS-PAGE (8-12% acrylamide) and transferred to nitrocellulose membranes. The membranes were then blocked with 3% bovine serum albumin in phosphate buffer saline supplemented with Tween 20 (0.1 M, pH 7.4, 0.1% Tween 20) for 45 min and then treated with primary anti-CYP/UGT antibody (Gentest) followed

by horseradish peroxidase-conjugated anti-mouse secondary antibody. The protein bands were visualized using enhanced chemiluminescence detection (Amersham) and quantitated by photodensitometry. To quantitate photodensitometric readings we first constructed a plot of known CYP amounts and intensity/area of the blot. The plot was observed to be linear over the range of 5 – 50 fmoles (r = 0.984).

3.2.8 Northern Blot Analysis of Cytochrome P450 3A4 mRNA

Total cellular RNA from control and drug-treated hepatocytes was isolated using TRIzol (GIBCO BRL). A 10 μg aliquot was fractionated by electrophoresis in 1% agarose gels containing formaldehyde (2.2 M) and transferred on to a nylon membrane (Millipore). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S rRNA, which was visualized and photographed under UV illumination. The membranes were hybridized as described previously (Church and Gilbert, 1984), (Desai *et al.*, 2002) with CYP3A4 cDNA probe (780 base pairs, Oxford Biomed. Res., Inc.) labeled with [α^{32} P]-dCTP (NEN) using the random primer method.

3.2.9 Real time-Polymerase Chain Reaction for Detecting mRNA Levels

Although, we initially utilized Northern blot analysis for detecting mRNA expression levels of CYP3A4, we subsequently used RT-PCR which is more sensitive. Following the 72 hr treatment period cells were lysed using Trizol reagent. The total cellular RNA was isolated using the phenol-chloroform RNA isolation technique. cDNA was derived from 2 μg of total RNA using AMV-Reverse Transcriptase (Fisher). The PCR mix consisted of; SYBR® Green PCR Master Mix (Applied Biosystems), 2 μl of cDNA, and each primer at 200 nM per reaction. Real time (RT)-PCR was carried out employing ABI-7000 (Applied Biosystems) thermocycler

with each cycle of PCR including 30 sec of denaturation at 94°C, 1 min of primer annealing at 55°to 60°C and 2 min of extension at 72°C. CYP/UGT and P-gp sequences were amplified using previously published primers (Table 3.2.2). RT-PCR efficiencies were calculated from the given slopes in the Millenium software. The corresponding RT-PCR efficiency (*E*) of one cycle in the exponential phase was calculated using the formula: $E = 10^{[-1/\text{-slope}]}$

The mathematical model used for determining relative quantification of the target genes was (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP \text{ target (control – sample)}}}{(E_{\text{ref}})^{\Delta CP \text{ ref (control – sample)}}}$$

All necessary precautions against contamination of PCR reactions were observed. Results were normalized to GAPDH. Standard curve construction for absolute quantification of genes was undertaken with 10-fold serial dilutions of plasmid containing the sequence of the target. The curve range was from 10^6 to 10 copies. Dilutions were freshly prepared for each experiment from one aliquot of plasmid stock solution stored at -20°C.

Table 3.2.2: List of Primers utilized for RT-PCR

Gene	Forward Primer	Reverse Primer	Reference
hPXR (NM 003889)	5'-GCA TCATCA GCT TTG CCA AAG – 3'	5' – CCG CGT TGA ACA CTG TGT TG – 3'	(Wang *et al.*, 2003a)
HNF4α (NM 178849)	5' –GGT GTC CAT ACG CAT CCT TGA - 3'	5'- TGG CTT TGA GGT AGG CAT ACT CA-3'	(Iwahori *et al.*, 2003)
RXRα (NM 002957)	5' – GAG GCC TAC TGC AAG CAC AAG – 3'	5' – CAG GCG GAG CAA GAG CTT AG – 3'	(Haugen *et al.*, 2004)
GAPDH (NM 002046)	5' – GAA GGT GAA GGT CGG AGT C - 3'	5' – GAA GAT GGT GAT GGG ATT TC - 3'	(Hurteau and Spivack, 2002)
CYP3A4 (DR11131)	5' – CTT CAT CCA ATG GAC TGC ATA AAT – 3'	5' – TCC CAA GTA TAA CAC TCT ACA CAG ACA A – 3'	(Bowen *et al.*, 2000)
UGT1A1 (NM 000463)	5'-GGT GAC TGT CCA GGA CCT ATT GA-3'	5'-TAG TGG ATT TTG GTG AAG GCA GTT-3'	(Basten *et al.*, 2002)
UGT1A6 (NM 001072)	5'-GAA AAG CAG TGG TTT GTT TA-3'	5'-TCT TAA ATA TAT TTA AAT AA-3'	(Nishimura *et al.*, 2003)
UGT2B7 (NM 001074)	5'-AGT TGG AGA ATT TCA TCA TGC AAC AGA-3'	5'-TCA GCC AGC AGC TCA CAA CAG GGG-3'	(Congiu *et al.*, 2002)
CYP2B6 (NM 000767)	5'-CCC CAA GGA CAC AGA AGT ATT TC-3'	5'-GAT TGA AGG CGT GTT TTT C-3'	(Chang *et al.*, 2003)
MDR1 (NM 000927)	5'-GCA GAC AGC AGG AAA TGA AGT TGA-3'	5'-CAT AAT CCT CCA AAA GGA AAC TGG A-3'	(Noonan *et al.*, 1990)
MRP1 (NM 004996)	5'-ATG TCA CGT GGA ATA CCA GC-3'	5'-GAA GAC TGA ACT CCC TTC CT	(Gutmann *et al.*, 1999)
BCRP (NM 004827)	5'-AGA TGG GTT TCC AAG CGT TCA T-3'	5'-CCA GTC CCA GTA CGA CTG TGA CA-3'	(Sauerbrey *et al.*, 2002)

3.2.10 Microarray Analysis

Microarray analysis of solvent control (0.1% DMSO treated cells) versus efavirenz (10 μM treated cells) was undertaken for 4 different batches of hepatocytes at the University of Cincinnati Microarray Core facility.

The microarray experiments were carried out essentially as described in published reports and references therein (Guo *et al.*, 2004), (Karyala *et al.*, 2004), (Sartor *et al.*, 2004). The human 70-mer oligonucleotide library version 2 (22,291 optimized oligos) (Qiagen) was suspended in 3X SSC at 30 μM and printed at 22°C and 65% relative humidity on aminosilane-coated slides

(Cel Associates) using a high-speed robotic Omnigrid machine (GeneMachines) with Stealth SMP3 pins (Telechem). The complete gene list can be viewed at http://microarray.uc.edu. Spot volumes were 0.5 nl and spot diameters are 75-85 µm. The oligonucleotides were crosslinked to the slide substrate by exposure to 600 mJ of ultraviolet light.

Fluorescence-labeled cDNAs were synthesized from total RNA using an indirect amino allyl labeling method via an oligo (dT)-primed, reverse transcriptase reaction. The cDNA was decorated with monofunctional reactive Cyanine-3 and Cyanine-5 dyes (Cy3 and Cy5; Amersham). Details can be found at http://microarray.uc.edu.

The microarrayed DNA probes were incubated in Prehybridization Buffer (5X SSC, 0.1% SDS, and 1% bovine serum albumin) at 42^{o}C for 90 min. For hybridization, the microarray slides are spotted with 42 µl Hybridization Buffer (25% formamide, 5X SSC, 0.1% SDS, 5 µg/µl COT1-DNA, 5 µg/µl poly(A)-DNA, and 2µg/µl yeast tRNA) and the fluorescence-labeled target cDNA; covered with glass coverslips (Fisher Scientific), and placed in humidified hybridization chambers (Corning). The hybridization chambers were placed in a water bath at 48°C for 66 hr. The slides were placed in a slide rack set in a staining dish and washed in 1X SSC with 0.2% SDS for 4 min at 48°C with agitation and transferred to a staining dish with 0.1X SSC and 0.2% SDS and washed with agitation for 4 min at room temperature and two times for 4 min each in 0.1X SSC at room temperature. The slides were spun-dried immediately after washing. More details of slide preparation can be found at http://microarray.uc.edu.

Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments) and associated software from Axon Instruments, Inc. The microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 and Cy5 fluorescence emittance. Images were captured in JPEG and TIFF files, and DNA spots captured

by the adaptive circle segmentation method. Information extraction for a given spot is based on the median value for the signal pixels minus the median value for the background pixels to produce a gene set data file for all the DNA spots. The Cy3 and Cy5 fluorescence signal intensities were normalized.

3.3. Methods Employed to Assess the Induction of Drug Metabolizing Enzymes/Transporters in CD4$^+$ (T-lymphocytes)

3.3.1. Isolation of Peripheral Blood Mononuclear Cells

Whole blood was obtained from healthy volunteers (100 mL) or from the Hoxworth Blood Center (<500 mL) with approval from the University of Cincinnati Institutional Review Board. PBMCs were isolated using Lymphocyte Separation Medium (Fisher) and resuspended in PBMC growth medium (RPMI-1640 with L-glutamine containing with 20% fetal bovine serum, penicillin streptomycin and interleukin-2) at 2 x 10^6 cells/ml. Phytohemagglutinin (5 μg/ml) was added to stimulate the cells for 3 days, after which the cells were pelleted and resuspended in fresh PBMC growth medium. CD4$^+$ T-cells were isolated from the PBMCs using a CD4$^+$ T-cell Negative Selection Kit (Miltenyi Biotech) following the manufacturers instructions (Figure 3.2). Isolated CD4$^+$ T-cells were used for all RNA analyses while PBMCs were used for all other experiments.

Cells were placed in 6-well plates at 1 × 10^6 cells/well. Stock drug solutions (1000X) were prepared in DMSO and diluted prior to use in PBMC growth medium. Cells were treated for 72 hrs with efavirenz 10 μM, nelfinavir 10 μM, ritonavir 10 μM or rifampin 10 μM which contained the same amount of DMSO (0.1%) as the drug solutions (Desai *et al.*, 2002) . For each donor 2 replicates were treated with each compound. Cell viability was assessed daily

during the course of drug exposure by using the MTT assay as described earlier (Carmichael *et al.*, 1987). No significant difference between the viability of cells treated with the vehicle and cells treated with rifampin, efavirenz, ritonavir or nelfinavir was observed.

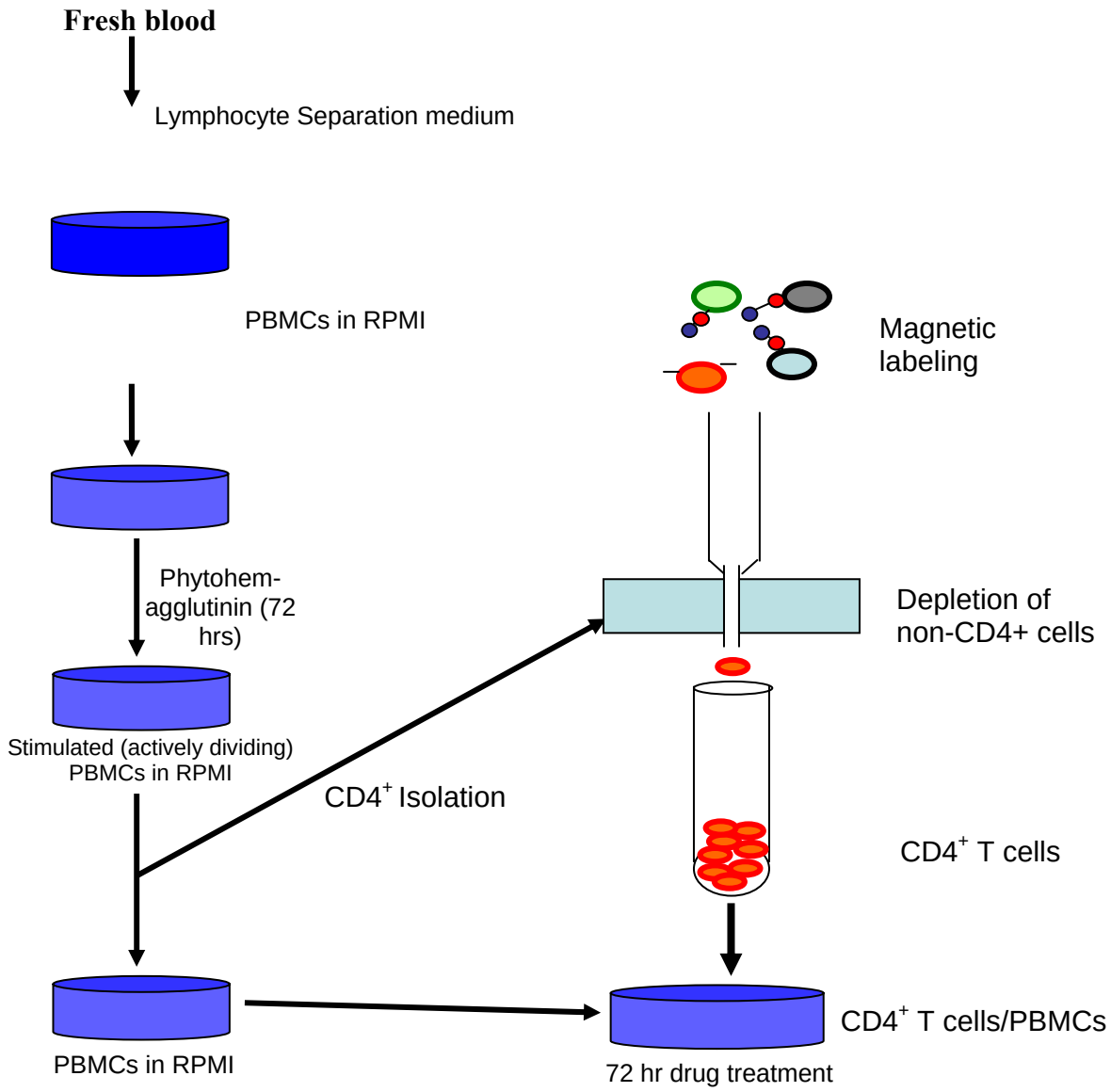

Figure 3.2: Scheme of peripheral blood mononuclear cell treatment for induction studies.

(Adapted from the Miltenyi Biotech website, accessed May 15, 2006)

3.3.2 Fluorimetric Probes for Cytochrome P450 Activities in Intact $CD4^+$ T-Cells.

Fluorescence-based P450 assays were performed by direct incubation of cells cultured in 6-well plates with selected substrates (7-ethoxy-4-trifluoromethylcoumarin [EFC] for CYP2B6 and 7-benzyloxy-4-trifluoromethylcoumarin [BFC] for CYP3A4). The method employed was the same as Donato et al. (Donato *et al.*, 2004). The fluorescent metabolite formation was quantified fluorimetrically (410/510 nm) by means of a Spectra Max Gemini XS fluorescence microplate reader (Molecular Devices) (Donato *et al.*, 2004). Results were expressed as picomoles of metabolite [7-hydroxy-4-trifluoromethylcoumarin (HFC)] formed per minute and per milligram of total cell protein.

3.3.3 Efflux Assays and Flow Cytometry

3.3.3.1 Transporter Functional Activity Assays

The cells were resuspended in complete growth medium containing the P-gp substrate Rh123 (0.5 μM for 1 hour) (Chaudhary and Roninson, 1991), the MRP1 substrate calcein-AM (50nM for 15 min) (Dogan *et al.*, 2004) or the BCRP substrate mitoxantrone (3 μM for 30 min) (Minderman *et al.*, 2002) at 37^0C, 5% CO_2. The cells were washed twice with ice-cold PBS, resuspended in ice-cold dye-free medium, and 0.5 x 10^5 cells were transferred to each tube. Two tubes of cells were centrifuged (300g for 10 minutes) and fixed with formaldehyde (8%) to serve as loading controls. The P-gp inhibitor cyclosporine A (2.5 μM) (Ejendal and Hrycyna, 2005), (Leier *et al.*, 1994), the MRP1 inhibitor MK571 (10 μM) (Dogan *et al.*, 2004), or the BCRP inhibitor fumitremorgin C (10 μM) (Minderman *et al.*, 2002) was added to 2 tubes of the appropriate cells to inhibit efflux.

Cells were placed in a shaking (200rpm) water bath (37^0C) or rotator in a CO_2 incubator for 1 to 3 hours to allow for substrate efflux. Following efflux, the tubes were immediately placed on ice and centrifuged (300g for 10 minutes, 4^0C). Thereafter the cells were fixed on ice for 1 hour in 8% formaldehyde.

3.3.3.2 Antibody Staining and FACSCalibur Flow Cytometer Analysis

Formaldehyde-fixed PBMCs were incubated with 1 µg/ml of anti-P-gp UIC2 (Chemicon International), or 5 µg/ml of anti-BCRP (BD Biosciences) or 10 µg/ml of anti-MRP1 (BD Biosciences) mouse anti-human antibodies for 30 min. In addition, PBMCs were also exposed to biotinylated anti-$CD4^+$ antibody for 30 min. The cells were washed with PBS and incubated with rat anti-mouse IgG_{2a+b}-PerCP (BD Biosciences) secondary antibody (specific for anti-Pgp, anti-BCRP, or anti-MRP1) and streptavidin APC specific for the anti-$CD4^+$ antibody. Cells were then washed, resuspended in MACS buffer, and immediately analyzed by flow cytometry.

Cells were analyzed using a BD Biosciences FACSCalibur system by gating on forward and side scatter characteristics for optimal size and shape of cells. Secondary gating involved the selection of $CD4^+$ T-cells. Ten thousand gated events were recorded for each sample.

The mixture for detecting P-gp expression and function contained; Rh123, anti-$CD4^+$ antibody, streptavidin-APC, anti-P-gp antibody and anti-mousePerCP. The mixture for detecting MRP1 expression and function contained; Calcein-AM, anti-$CD4^+$ antibody, streptavidin-APC, anti-MRP1 antibody and anti-mousePerCP. Finally, the mixture to detect BCRP expression and function consisted of; Mitoxantrone (MTX), anti-$CD4^+$ antibody, streptavidin-APC, anti-BCRP antibody and anti-mousePerCP. FACScan compensation was set to reduce as much as possible any bleed-over effect into other channels. CELLQuest software (BD Biosciences) was used to develop diagrams/graphs and analyze the statistics

3.3.4 Isolation of RNA, cDNA Synthesis and Quantification of mRNA Expression

RNA was isolated from $CD4^+$ cells using the RNeasy Mini Kit (Qiagen). Quality and purity of RNA was verified by gel electrophoresis. cDNA was derived from 2 µg of total RNA using AMV-Reverse Transcriptase (Fisher). The PCR mix consisted of; SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl of cDNA, and each primer at 200 nM per reaction. Real time (RT)-PCR was carried out employing ABI-7000 (Applied Biosystems) thermocycler with each cycle of PCR including 30 sec of denaturation at 94°C, 1 min of primer annealing at 55°to 60°C and 2 min of extension at 72°C.

3.4. Methods Employed to Assess the Induction of P-glycoprotein in HIV-Exposed and -Unexposed $CD4^+$ T-Cells

3.4.1 HIV infection of Peripheral Blood Mononuclear Cells/$CD4^+$ T-Cells and Drug Treatment of Infected Cells

Stimulated PBMCs were divided into unexposed cells and cells to be exposed to HIV-1. The cells to be infected with HIV were centrifuged at 300 g for 10 minutes at room temperature. For each infection, 2.5 x 10^6 cells were resuspended in 200µl medium containing 2250 infectious units of NL_{4-3} strain of HIV-1. These cells were incubated at 37^0C, 5% CO_2 for 1 hour. The cells were transferred into a 12-well plate and 2300 µl of the complete PBMC growth medium was added to each well. Thereafter, both the unexposed and HIV-exposed cells were treated with 2.5 µL of DMSO control or stock solutions of nelfinavir (10 mM) for 72 hours.

3.4.2 Separation of Exposed-Uninfected and Exposed-Infected $CD4^+$ T-Cells

After drug treatment, $CD4^+$ T-cells which were exposed to HIV-1 were separated into exposed-uninfected cells and exposed-infected cells using magnetic sorting with indirect microbeads. Briefly, HIV-1 exposed cells were incubated with HIV-1 gp41 monoclonal antibody (NIH AIDS Research & Reference Reagent Program) for 10 minutes. Thereafter, cells were washed with ice-cold PBS and exposed to mouse anti-human IgG microbeads (Miltenyi Biotech). After washing, the cells were passed through a LS column in a VarioMACS separator magnet and the exposed-uninfected cells were collected. The column was then removed from the magnet and the HIV-1 infected cells were flushed into a collection tube using MACS buffer. The RNA from these cells was isolated using the RNeasy Mini Kit (Qiagen). mRNA expression of P-gp was ascertained using real-time PCR as described above.

3.4.3 Assessment of P-glycoprotein Functional Activity in HIV-1 Exposed Cells

P-gp function was determined in HIV-exposed cells (with Rh-123) using the flow cytometry method described earlier. Fixed PBMCs were stained in order to separate HIV-1 exposed-infected and exposed-uninfected cells. Briefly, cells were incubated with anti-HIV-1 gp41 monoclonal antibody for 30 minutes on ice. The cells were then washed with ice-cold PBS and exposed to anti-human IgG PE-CY5 secondary antibody (BD Biosciences) for 30 minutes. This was followed by another wash with PBS. The cells were then resuspended in ice-cold MACS buffer and immediately subjected to flow cytometry. Exposed-infected and exposed-uninfected cells were separated by gating on the highest and the lowest PE-Cy5 expression.

3.5 Statistical and Data Analysis

The difference in the fold increase in CYP, UGT, P-gp, BCRP and MRP1 activity, immunoreactive protein content, mRNA levels, fold activation of transcription factors (hPXR) and cell viability using the MTT assay between untreated control vs. treated groups was analyzed using one way analysis of variance (ANOVA), followed by the Student-Newman-Keuls Test for the comparison of mean values at an $\alpha = 0.05$ (Sigmastat version 3.1).

The data representing background subtracted spot intensities generated by GenePix® Pro version 5.0 software were analyzed to identify differentially expressed genes. Data normalization was performed in two steps for each microarray separately (Guo *et al.*, 2004), (Sartor *et al.*, 2004). First, background adjusted intensities were log-transformed and the differences (M) and averages (A) of log-transformed values were calculated as $M = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy5 and Cy3 intensities, respectively. Second, normalization was performed by fitting the array-specific local regression model of M as a function of A. Normalized log-intensities for the two channels were then calculated by adding half of the normalized ratio to A for the Cy5 channel and subtracting half of the normalized ratio from A for the Cy3 channel. The statistical analysis was performed for each gene separately by fitting the following Analysis of Variance model (Dudoit et al., 2002): $Y_{ijk} = \mu + A_i + S_j + C_k + \Omega_{ijk}$, where Y_{ijk} corresponds to the normalized log-intensity on the i^{th} array, with the j^{th} treatment, and labeled with the k^{th} dye (k = 1 for Cy5, and 2 for Cy3). μ is the overall mean log-intensity, A_i is the effect of the i^{th} array, S_j is the effect of the j^{th} treatment and C_k is the gene-specific effect of the k^{th} dye. Resulting t-statistics from each contrast were modified using an empirical Bayesian moderated-T method (Smyth, G. K., 2004). This method uses variance estimates from all genes to improve the variance estimates of each individual gene. Estimates of

fold-change were calculated, and genes with p-value < 0.1 and average spot intensity > 50 were considered for followup. Data analysis was performed using the statistical software R and the Bioconductor platform.

The gene list was analyzed to determine which gene categories were enriched with differentially expressed genes. This was performed using Expression Analysis Systematic Explorer (EASE), and the gene sets tested were the three branches of the Gene Ontology database. Gene Ontology is a multi-organism, controlled vocabulary database containing three separate ontologies: biological process, molecular function, and cellular component, and it is commonly used for assessing the results of microarray analyses (Ashburner *et al.*, 2000). Fisher's Exact probability, using the Benjamini FDR multiple testing adjustment, was calculated for each gene category (Hosack *et al.*, 2003), and an FDR cutoff of 0.05 was used for significance.

4. RESULTS

As is evident from our earlier discussions, induction of DMTs by the antiretrovirals may underlie some of the clinical problems associated with the use of efavirenz, ritonavir and nelfinavir including drug-drug interactions. To that effect, we first examined the potential of the aforementioned antiretrovirals to induce CYP3A, CYP2B6, UGT1A, UGT2B7 and P-gp employing primary human hepatocytes and LS174T cells as *in vitro* models for hepatic and intestinal drug metabolism, respectively. We further assessed the induction of the major efflux transporters (P-gp, MRP1 and BCRP) by antiretrovirals in $CD4^+$ T-cells, which is the primary site of attack of all antiretrovirals. We finally, determined whether the presence of HIV influences the induction potential of P-gp and the expression of key transcription factors.

4.1 Specific Aim 1

4.1.1 Activation of Transcription factors

Pregnane-X Receptor

The activation of hPXR by efavirenz, ritonavir and nelfinavir were examined by transient transfection of a CYP3A4 reporter gene construct harboring hPXR-responsive regions of the *CYP3A* gene in HepG2 hepatoma cells and in LS174T, colon carcinoma cells. We compared the activation of hPXR by efavirenz (0.5 - 10 μM), ritonavir (0.5 – 10 μM), nelfinavir (0.5 – 10 μM), rifampin (10 μM) and phenobarbital (PB) (2 mM) (Figure 4.1.1). In HepG2 cells we observed a ~22-fold activation of hPXR following treatment with both rifampin 10 μM and PB 2 mM (p=0.01). Efavirenz at concentrations of 5 and 10 μM efficaciously increased hPXR activation by ~14- and 21-fold, respectively (Figure 4.1.1). These results compared well with those obtained with ritonavir at 10 μM. However, ritonavir 5 μM produced a higher increase, in PXR activation, than was observed with efavirenz at 5 μM.

hPXR activation in LS174T cells was lower than that observed in HepG2 cells. The positive controls rifampin 10 μM and PB 2 mM produced ~12-and 13-fold increase in hPXR activation relative to control. Efavirenz (0.5-10 μM) was observed to result in a dose-dependent increase, ranging from ~2-to-9-fold, in hPXR activation relative to vehicle treated control. We did not conduct studies to assess hPXR activation in LS174T cells for the PIs as these drugs were not observed to cause differential induction of CYP3A4 in liver versus intestinal cells.

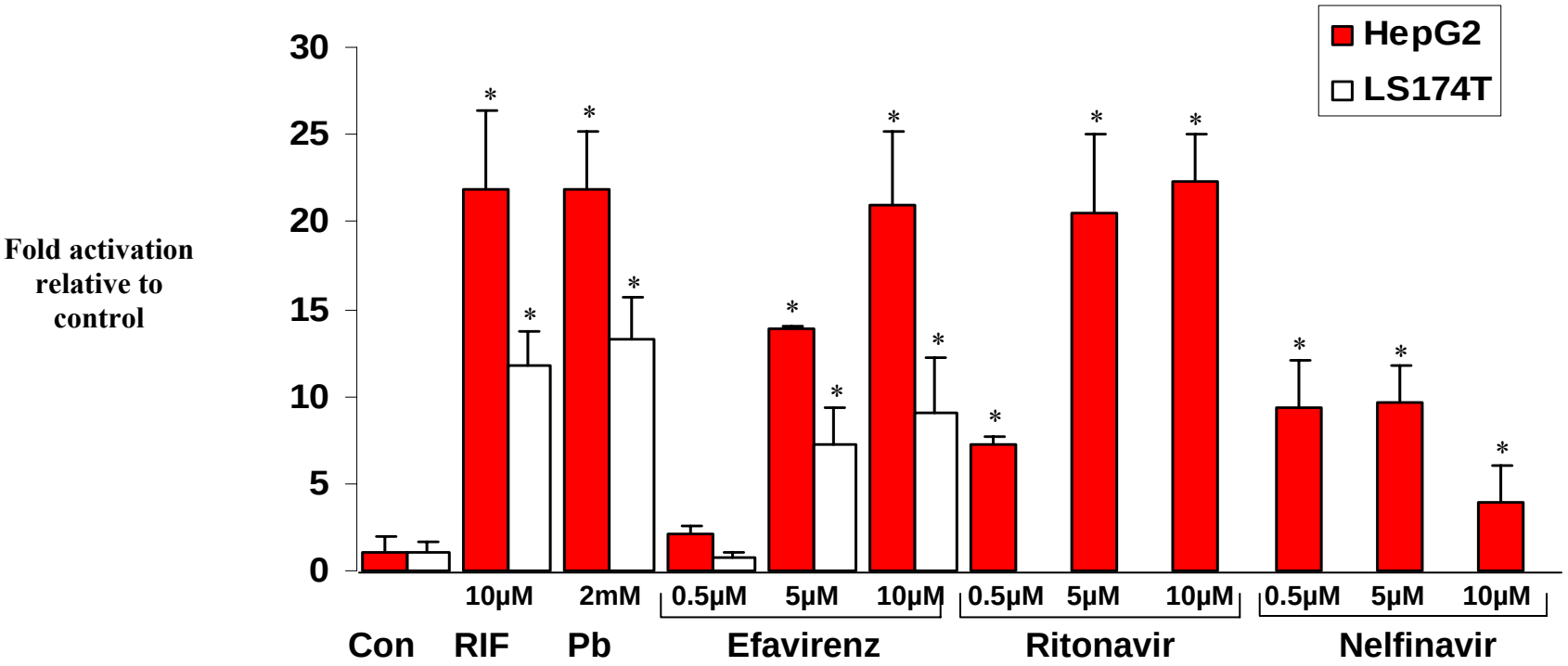

Figure 4.1.1: Fold Activation of hPXR in HepG2 and LS174T cells (n=6) following treatment with efavirenz, ritonavir and nelfinavir, rifampin (RIF 10 μM) and phenobarbital (2 mM). * $p < 0.05$ compared to untreated controls.

4.1.2 Comparative Expression of Transcription Factors

Pregnane-X Receptor (PXR) and constitutive androstane receptor (CAR) heterodimerize with retinoid X receptor (RXR) and bind to response elements and activate target gene expression (Mangelsdorf and Evans, 1995), (Mangelsdorf *et al.*, 1990). Quantification of PXR and CAR expression levels in hepatocytes, LS174T intestinal cells, and $CD4^+$ cells revealed hepatocytes to have the highest levels, followed by LS174T cells and finally $CD4^+$ T-cells (Table 4.1.1). This was evaluated in five batches in each of the three different cells types. Significant variability was observed among PXR expression levels in all three cell types. In comparison, CAR expression levels were ~40-fold lower than PXR expression levels in all cell types. Among five individuals, CAR levels in $CD4^+$ cells were barely detectable from two individuals.

RXR expression levels showed much less variability than was observed with the PXR/CAR. Surprisingly, the highest expression was observed in $CD4^+$ T-cells, which was ~2-fold higher than was observed in hepatocytes and LS174T cells.

Table 4.1.1: Quantification of Pregnane-X Receptor (PXR) and constitutive androstane receptor (CAR) expression in hepatocytes, LS174T cells and CD4+ T-cells

	Transcription Factor	
	PXR (transcript molecules/ng of total RNA)	CAR (transcript molecules/ng of total RNA)
Hepatocytes	987-86683	202-2901
Intestinal cells (LS174T)	474 – 373183	35-320
$CD4^+$ T-Cells	98-1152	11-20 (n=3)

Range, n=5

4.1.3 Induction of Hepatic and Intestinal Cytochrome P450 3A4

We first examined the influence of efavirenz, ritonavir and nelfinavir employed at clinically relevant concentrations (0.5-10 μM) on hepatic and intestinal cells (LS174T). Fold change in CYP3A4 activity as well as CYP3A4 immunoreactive protein and mRNA levels in drug-treated hepatocytes and LS174T cells, relative to the vehicle control (0.1% DMSO), were examined as described in the Material and Methods section. Briefly, following the drug treatment, CYP3A4 activity in intact hepatocytes and LS174T was determined by incubating the cells with testosterone-containing culture medium. The conversion of testosterone to 6β-hydroxytestostrone, a CYP3A4 catalyzed reaction, was monitored in the intact cells by HPLC and the rate of 6β-hydroxytestostrone was used as a marker of CYP3A4 activity. Figure 4.1.2 shows a typical HPLC chromatogram depicting the CYP3A4 generated metabolite 6β-hydroxytestostrone, the parent substrate testosterone and the internal standard 11α-hyrdoxyprogesterone. Increases in 6β-hydroxytestostrone formation as seen with rifampin and efavirenz denotes induction of CYP3A4 activity.

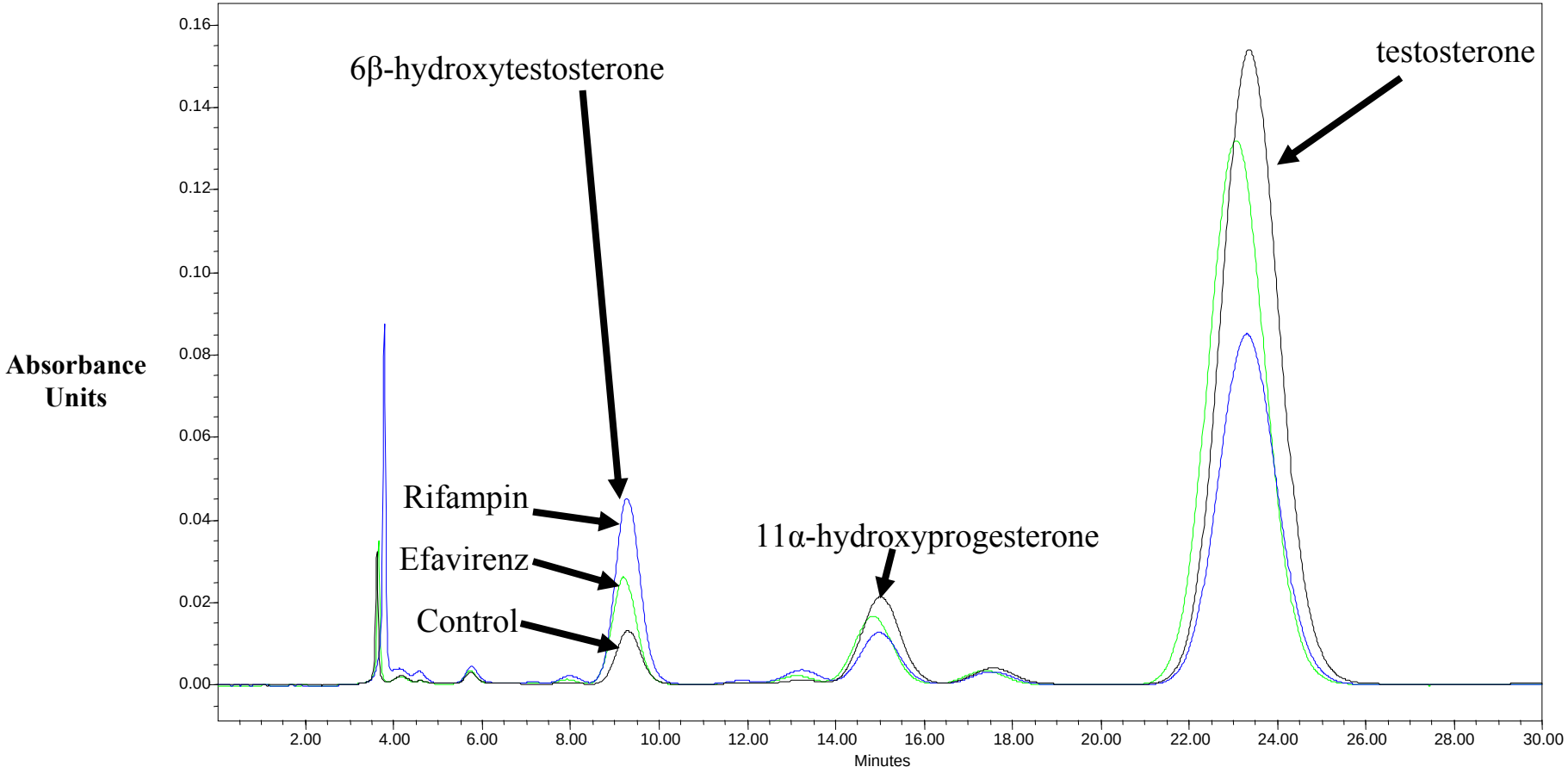

Figure 4.1.2: HPLC chromatogram of 6β-hydroxytestosterone, 11α hydroxyprogesterone and testosterone. HPLC chromatogram depicting 6β-hydroxytestosterone formation in control (black), efavirenz (green) and rifampin (blue) treated human hepatocytes following 72 hours drug treatment period

Fold increases in CYP3A4 activity by the antiretrovirals in drug treated compared to solvent treated cells are shown in Figure 4.1.3. Efavirenz caused a concentration-dependent increase in CYP3A4 activity in primary human hepatocytes (Hariparsad *et al.*, 2004). While the increase in the 6β-hydroxylation of testosterone was not statistically significant at efavirenz 0.1 μM ($p = 0.741$), there was a statistically significant increase in CYP3A4 activity ($p = 0.005$) at drug concentrations ≥ 5 μM. Accordingly, we observed a ~1.3-to 3.6-fold increase in testosterone 6β-hydroxylation in cells treated with 0.5, 5, and 10 μM efavirenz. In comparison, rifampin 10 μM caused a ~8.3-fold increase in the enzymatic activity, respectively. The consistent dose-dependent increase in CYP3A4 activity observed with efavirenz was not evident with the protease inhibitors nelfinavir and ritonavir in hepatocytes. In LS174T cells, efavirenz again produced a dose-dependent increase in CYP3A4 activity with the most significant increase observed at 10 μM ($p = 0.001$), while rifampin 10 μM produced a ~2.5-fold increase in the enzymatic activity. It is important to note that variability that was observed in CYP3A4 enzymatic activity between the hepatocytes from the 4 different livers used in our studies. This is not uncommon and has been evident in the ~40 livers that we have worked with in our laboratory. One of the factors that may contribute to variability in CYP3A4 enzymatic activity is hPXR expression. While the effect of hPXR expression on CYP3A4 inducibility is poorly understood, it is generally believed that the inducibility of CYP3A4 is inversely proportional to the basal level of CYP3A4 expression (Kostrubsky *et al.*, 1999). Thus, the use of a prototypical inducer such as rifampin allows us to make comparisons and therefore rank the test compounds against it.

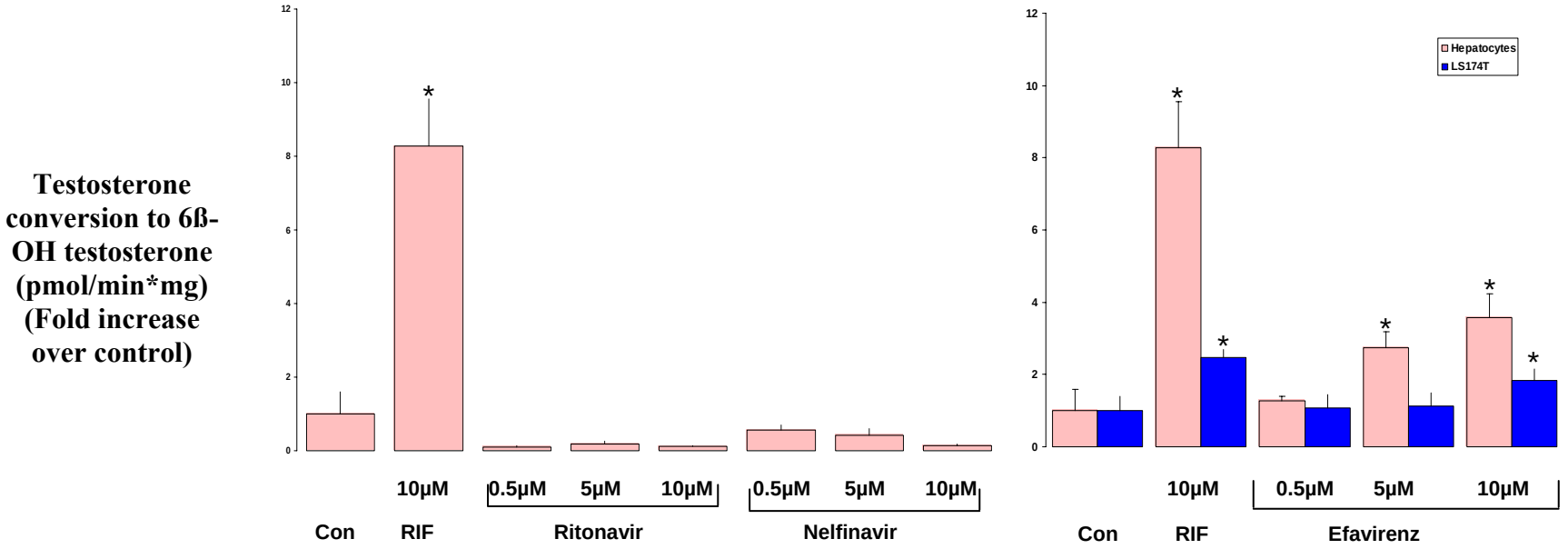

Figure 4.1.3: Effect of efavirenz, nelfinavir and ritonavir on CYP3A4 activity in primary human hepatocytes and LS174T cells. Human hepatocytes and LS174T cells were treated, in duplicate, with: vehicle; rifampin 10 µM; or the test drugs; efavirenz (0.5, 5 and 10 µM), ritonavir (0.5, 5 and 10 µM), nelfinavir (0.5, 5 and 10 µM) concentrations. n=4, *p<0.05. Hariparsad et al. (2004)

A representative western blot analysis of CYP3A4 immunoreactive protein in microsomes prepared from untreated and treated hepatocytes is shown in Figure 4.1.4.A. The mean ± SD of the fold increases in the expression of CYP3A4 immunoreactive protein, quantitated employing photodensitometric analysis, are shown in Figure 4.1.4.B. Similar to the observed increases in CYP3A4 activity, efavirenz at concentrations ≥ 5 μM augmented CYP3A4 levels. At 0.5, 5 and 10 μM concentration, efavirenz caused a ~1.1-, 3.3-and 3.4-fold increase in CYP3A4 immunoreactive protein levels. A ~3-and 2-fold increase was observed in cells treated with rifampin 10 μM and phenobarbital 2 mM consistent with the increased activity. Conversely, neither nelfinavir nor ritonavir produced a consistent increase in immunoreactive protein levels, again consistent with the activity levels. In fact, at 0.5 and 5 μM ritonavir produced a statistically significant ($p = 0.035$) decrease in CYP3A4 immunoreactive protein levels compared to control, with a slight increase in protein levels at ritonavir 10 μM.

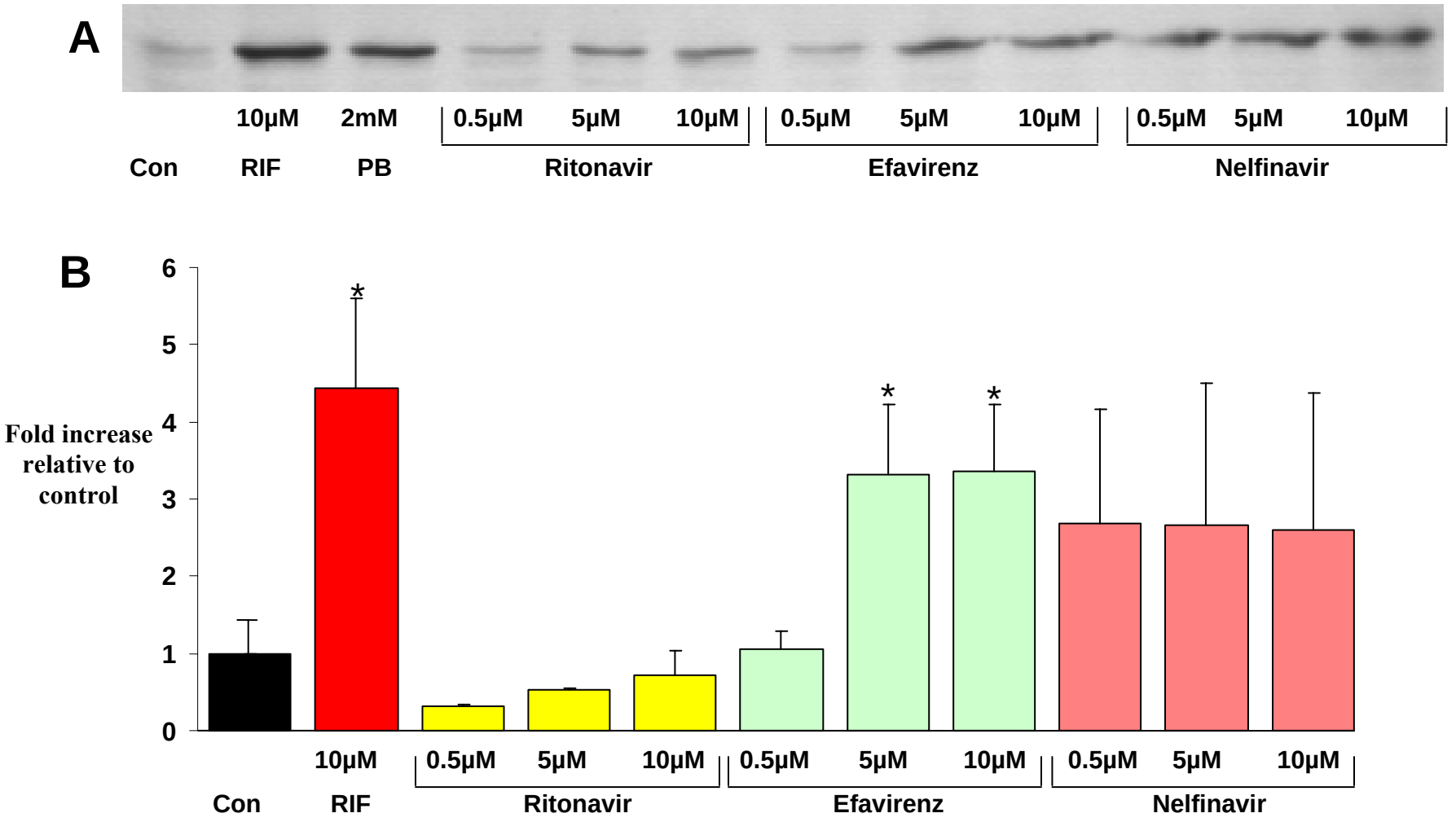

Figure 4.1.4: Western blot analysis of immunoreactive CYP3A4 levels in human hepatocytes (n=4) treated, in duplicate, with efavirenz, ritonavir, nelfinavir and rifampin (RIF 10 μM). Immunoreactive CYP3A4 protein bands from one batch (HH1040) are shown in A. The bands were quantitated and the mean ± SD are shown in B (n=4). *$p<0.05$

As shown in the representative northern blot of CYP3A4-specific mRNA levels following treatment with efavirenz, rifampin and phenobarbital in Figure 4.1.5, efavirenz caused a concentration-dependent induction of CYP3A4. At efavirenz concentrations ranging from 1 – 10 μM, there was a 1.2- to 4.1-fold increase in CYP3A4 mRNA levels. Increased enzymatic activity or expression may result from either increased enzyme synthesis or decreased enzyme degradation. Our observations indicate that elevated CYP3A4 activity and immunoreactive protein levels are a result of increased transcription. Using real-time PCR, we observed an increase in the CYP3A4 mRNA levels in hepatocytes and LS174T cells treated with nelfinavir and ritonavir. Hepatocytes and LS174 T cells treated with 0.5, 5 and 10 μM ritonavir increased mRNA levels by 1.9, 3.4 and 7.3-fold over control. Similarly, hepatocytes and LS174T cells treated with nelfinavir (0.5 μM-10 μM) produced between 1.3 to 14.9-fold increases in CYP3A4 mRNA levels.

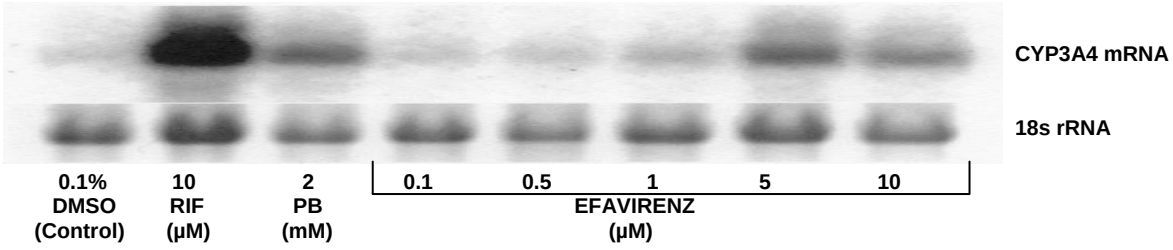

Figure 4.1.5: Northern blot analysis depicting CYP3A4 mRNA levels in human hepatocytes (n=4) treated, in duplicate, with efavirenz, rifampin (RIF 10 μM) and phenobarbital (2 mM).

Thus, the transcriptional increase in CYP3A4 levels for all 3 drugs did not consistently translate into an increase in protein and functional activity. Functional activity data revealed ritonavir and nelfinavir to be inhibitors of CYP3A4. This occurs even in the absence of ritonavir and nelfinavir given that a wash-out period of 4 hours was done prior to measuring testosterone

conversion to 6ß-OH testosterone. Efavirenz was the only drug which consistently resulted in an increase in CYP3A4 transcription, translation and functional activity. When compared to rifampin, efavirenz produced ~50% induction of CYP3A4 functional activity.

4.1.4 Induction of Cytochrome P450 2B6

Utilizing clinically relevant concentrations (0.5-10 μM) of efavirenz, ritonavir and nelfinavir we also examined the influence of antiretrovirals on another phase I enzyme, CYP2B6. CYP2B6 plays a major role in the biotransformation of several therapeutically important drugs (Chang *et al.*, 1993), (Faucette *et al.*, 2000), (Hesse *et al.*, 2000), (Ward *et al.*, 2003) and is expressed primarily in the liver. Thus, our studies were only conducted in primary human hepatocytes, currently considered the most clinically relevant *in vitro* model. Fold change in CYP2B6 activity as well as CYP2B6 immunoreactive protein and mRNA levels in drug-treated hepatocytes, relative to the vehicle control (0.1% DMSO), were examined as described in the Material and Methods section. Briefly, following drug treatment, hepatocytes were harvested and microsomes were prepared. The microsomal protein was incubated in a mixture containing bupropion. The conversion of bupropion to hydroxybupropion, a CYP2B6 catalyzed reaction, was ascertained in incubation mixtures containing microsomal protein by HPLC and the rate of hydroxybupropion conversion was used as a marker of CYP2B6 activity. Figure 4.1.6 represents a typical HPLC chromatogram depicting the CYP2B6 generated metabolite hydroxybupropion, the parent substrate bupropion and the internal standard, trazodone. Increases in hydroxybupropion formation as seen with nelfinavir denotes induction of CYP2B6 activity. Hepatocytes treated with nelfinavir 10 μM produced the most significant increase in hydroxybupropion levels (Table 4.1.2). In comparison efavirenz, ritonavir and rifampin at 10 μM produced ~0.9-1.4-fold increase in hydroxybupropion levels.

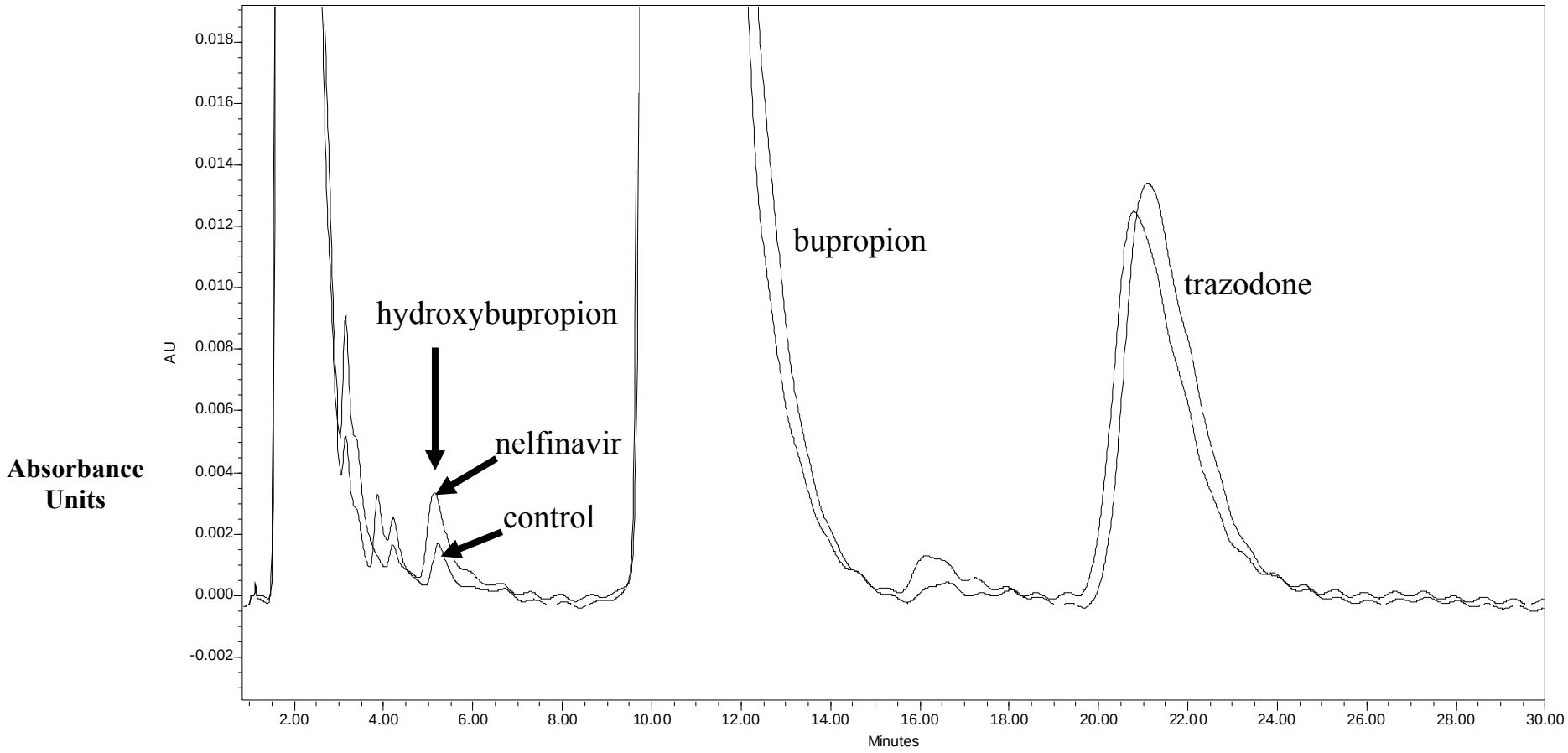

Figure 4.1.6: HPLC chromatogram of hydroxybupropion, trazodone and bupropion. HPLC chromatogram depicting hydroxybupropion formation in control, and nelfinavir treated human hepatocytes following 72 hours drug treatment period

A dose-dependent increase was also evident with the increase in CYP2B6 immunoreactive protein levels. At 10 µM concentration all 3 antiretrovirals produced a comparative increase in protein levels when compared to the prototypical inducer, rifampin (Table 4.1.2).

Similar to CYP3A4, CYP2B6 mRNA levels for all 3 antiretroviral compounds increased in hepatocytes compared to DMSO-treated control (Table 4.1.2). Efavirenz, nelfinavir and ritonavir at 10 µM concentration produced almost equivalent CYP2B6 mRNA increases around 3-fold which was less than the induction by the prototypical inducer rifampin 10 µM resulted in a 5.0±1.4-fold increase in CYP2B6 mRNA levels.

Table 4.1.2: Effect of antiretrovirals on hepatic CYP2B6 activity, protein and mRNA levels. Summary table showing fold increase in CYP2B6 activity, immunoreactive protein and mRNA levels compared to DMSO (0.1%) control in primary human hepatocytes following 72 hours with efavirenz, ritonavir, nelfinavir and rifampin.

	RIF	RTV			EFZ			NEL		
	10 µM	0.5µM	5µM	10µM	0.5µM	5µM	10µM	0.5µM	5µM	10µM
CYP2B6 mRNA	5.0 ± 1.4*	1.2 ±.0.1	3.1 ± 2.8	3.3 ±2.4	1.2 ± 0.2	1.7 ± 0.2	3.2 ± 0.5*	1.7 ± 0.3	2.5 ± 1.5	2.9 ± 1.1*
CYP2B6 Protein	1.9 ± 0.3*	0.7 ± 0.5	1.3 ± 0.8	1.6 ± 0.5	1.0 ± 0.8	1.2 ± 0.7	1.7 ± 0.5	1.2 ± 0.2	1.5 ± 0.6	1.8 ± 0.3*
CYP2B6 Activity	1.5 ± 0.2*	1.1 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	0.7 ± 0.3	1.2 ± 0.2	2.2 ± 0.5*

Mean ± SD

n=3, *$p < 0.05$,

4.1.5 Induction of UDP-glucuronosyl transferase

Our next step involved assessing the potential of efavirenz, nelfinavir and ritonavir to induce phase II enzymes such as UGT1A1, UGT1A6 and UGT2B7.

4.1.5.1 UDP-glucuronosyl transferase 1A

Microsomes obtained from drug treated hepatocytes were employed in UGT1A activity. Conversion of 7-hydroxycoumarin to its glucuronide was used as a UGT1A activity marker. 7-hydroxycoumarin is not a UGT-specific substrate and its glucuronidation can be carried out by numerous members in the UGT1A family. The formation of 7-hydroxycoumarin was detected and quantified using HPLC. Figure 4.1.7 shows a typical chromatograph depicting 7-hydroxycoumarin glucuronide, the substrate 7-hydroxycoumarin and the internal standard 4-hydroxycoumarin.

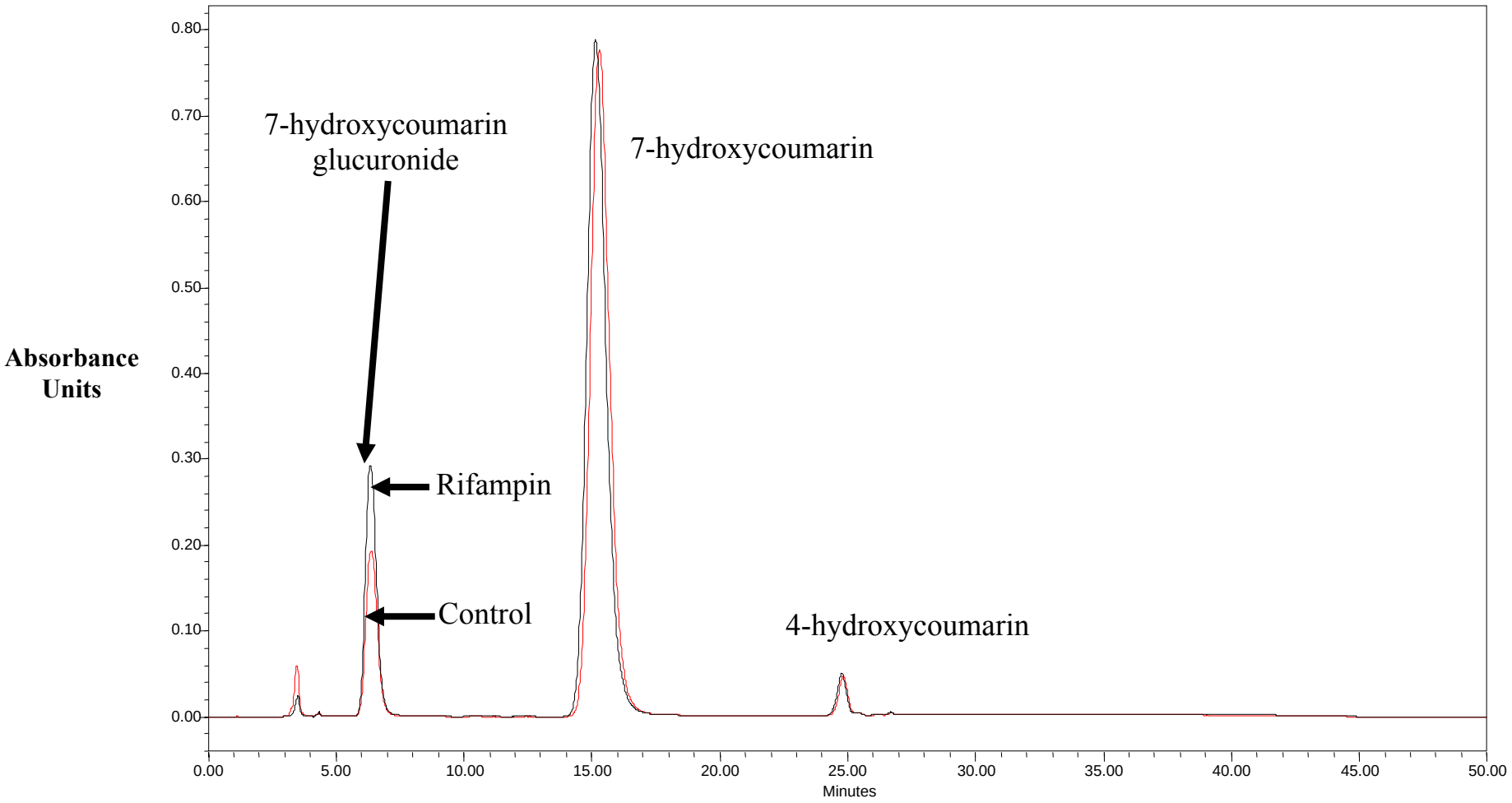

Figure 4.1.7: HPLC chromatogram of 7-hydroxycoumarin glucuronide, 7-hydroxycoumarin and 4-hydroxycoumarin. HPLC chromatogram depicting 7-hydroxycoumarin glucuronide formation in control (red) and rifampin (black) treated human hepatocytes following 72 hours drug treatment period

When compared to CYP enzymes, UGTs are usually only moderately elevated even by the prototypical inducer rifampin (Soars *et al.*, 2004), which was included in our experiments. Thus, the overall extent of induction of UGTs compared to that of CYP3A4 is low, which is consistent with previous findings (Soars *et al.*, 2004). As shown in Figure 4.1.8, all three test compounds (efavirenz, ritonavir and nelfinavir) caused a modest concentration-dependent increase in UGT1 activity of the same magnitude as rifampin 10 μM. There was high variability in UGT1 induction levels present between the hepatocytes from three different livers, and could be due to differences in PXR expression levels and basal levels of UGT.

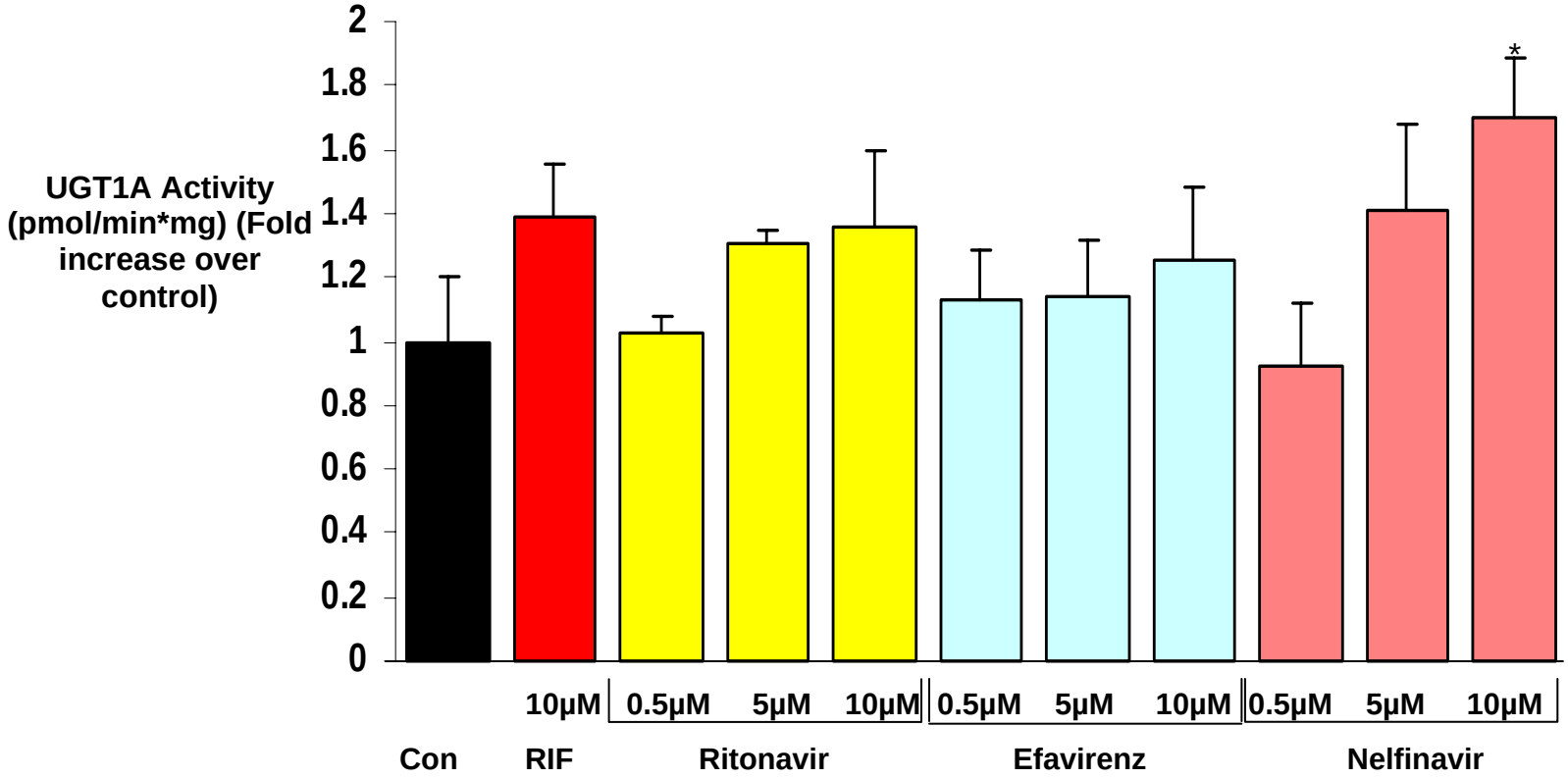

Figure 4.1.8: Effect of efavirenz, ritonavir and nelfinavir on the UGT1 activity in primary human hepatocytes.

Human hepatocytes obtained from three different donors (n=3) were treated, in duplicate, with: vehicle (DMSO Control, filled column); 10 μM rifampin (RIF, red column); efavirenz 0.5, 5 and 10 μM (green column); ritonavir 0.5, 5 and 10 μM (yellow columns) and nelfinavir 0.5, 5 and 10 μM concentrations (red columns). n=3,* $p < 0.05$

We also observed a corresponding increase in the immunoreactive UGT1A1 and UGT1A6 levels following efavirenz, ritonavir and nelfinavir treatments. Representative western blot analyses of UGT1A1 and UGT1A6 immunoreactive protein in microsomal fractions prepared from untreated and treated hepatocytes are shown in Figures 4.1.9.A and 4.1.9.B, respectively. The means ± SD of the fold increases in the expression of UGT1A1 and UGT1A6 protein, quantitated employing photodensitometric analysis, are shown in Table 4.1.2. Similar to the observed increases in UGT1 activity, all three compounds produced increases in UGT1A1 and UGT1A6 immunoreactive protein levels. Both ritonavir 10 μM and nelfinavir 10 μM caused higher immunoreactive protein levels than the prototypical inducer rifampin at the same concentration. The results suggest the following hierarchy of UGT1A1 immunoreactive protein expression; nelfinavir>ritonavir>efavirenz. In contrast, UGT1A6 immunoreactive protein expression levels were more comparable between all 3 drugs at 5-10 μM concentration (±3-fold increase).

As shown in Table 4.1.3, efavirenz, ritonavir and nelfinavir caused concentration-dependent induction of both UGT1A1 and UGT1A6. With immunoreactive protein levels nelfinavir produced higher increases in both UGT1A1 and UGT1A6 at lower concentrations than both ritonavir and nelfinavir. Increased enzymatic activity or expression may result from either increased enzyme synthesis or decreased enzyme degradation. Our observations indicate that elevated UGT activity and immunoreactive protein levels are a result of increased transcription.

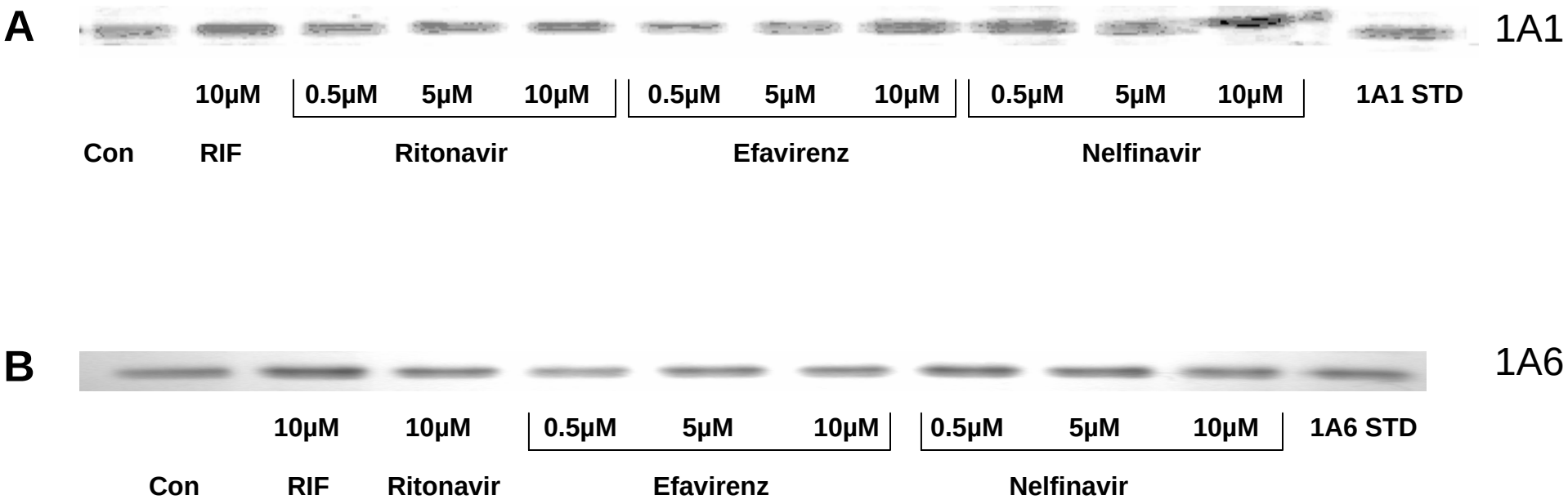

Figure 4.1.9: Western blot analysis of immunoreactive A) UGT1A1 and B) UGT1A6 in primary human hepatocytes treated with efavirenz, ritonavir, nelfinavir and rifampin (RIF 10 μM).

Table 4.1.3: UGT1A1 and UGT1A6 mRNA and immunoreactive protein levels in human hepatocytes treated, in duplicate, with efavirenz, ritonavir and nelfinavir and rifampin (RIF 10 µM).

		Rifa-mpin	Efavirenz			Ritonavir			Nelfinavir		
		10 µM	**0.5 µM**	**5 µM**	**10 µM**	**0.5 µM**	**5 µM**	**10 µM**	**0.5 µM**	**5 µM**	**10 µM**
UGT 1A1	mRNA	13.0± 9.6	1.4± 0.2	3.9± 2.0	4.4± 2.1	2.1± 1.9	1.9± 0.5	4.2± 0.2	1.2± 1.5	2.3± 1.6	3.9± 1.8
	Immunoreactive protein	1.6± 0.2*	1.2± 0.2	1.3± 0.2	1.4± 0.3	0.9± 0.4	1.2± 0.1	1.8± 0.3*	1.6± 0.7	1.7± 0.7	2.1± 0.7*
UGT 1A6	mRNA	4.7± 1.4*	2.6± 2.2	1.7± 0.4	2.4± 0.5*	3.1± 2.2	4.0± 1.1*	4.7± 4.3	3.0± 1.2*	2.4± 1.0	4.2± 0.6*
	Immunoreactive protein	2.3± 1.2	1.2± 0.9	1.4± 0.3	3± 2.7	1.9± 2.2	2.4± 1.8	3± 0.5*	2.9± 1.4	3.3± 0.4*	2.4± 1.8

n=3, *$p < 0.05$

4.1.5.2 UDP-glucuronosyl transferase 2B7

Microsomes obtained from drug treated hepatocytes were employed in UGT2B7 activity assays. Conversion of AZT to its glucuronide was used as a UGT2B7 activity marker. AZT is a more specific marker for UGT2B7 than morphine, but it has been shown to be glucuronidated by UGT2B4 to a limited extent (Court *et al.*, 2003). The formation of AZT-glucuronide was detected and quantified using HPLC. Figure 4.1.10 shows a typical chromatograph depicting AZT-glucuronide, the substrate AZT and the internal standard 3-Acetamidophenol.

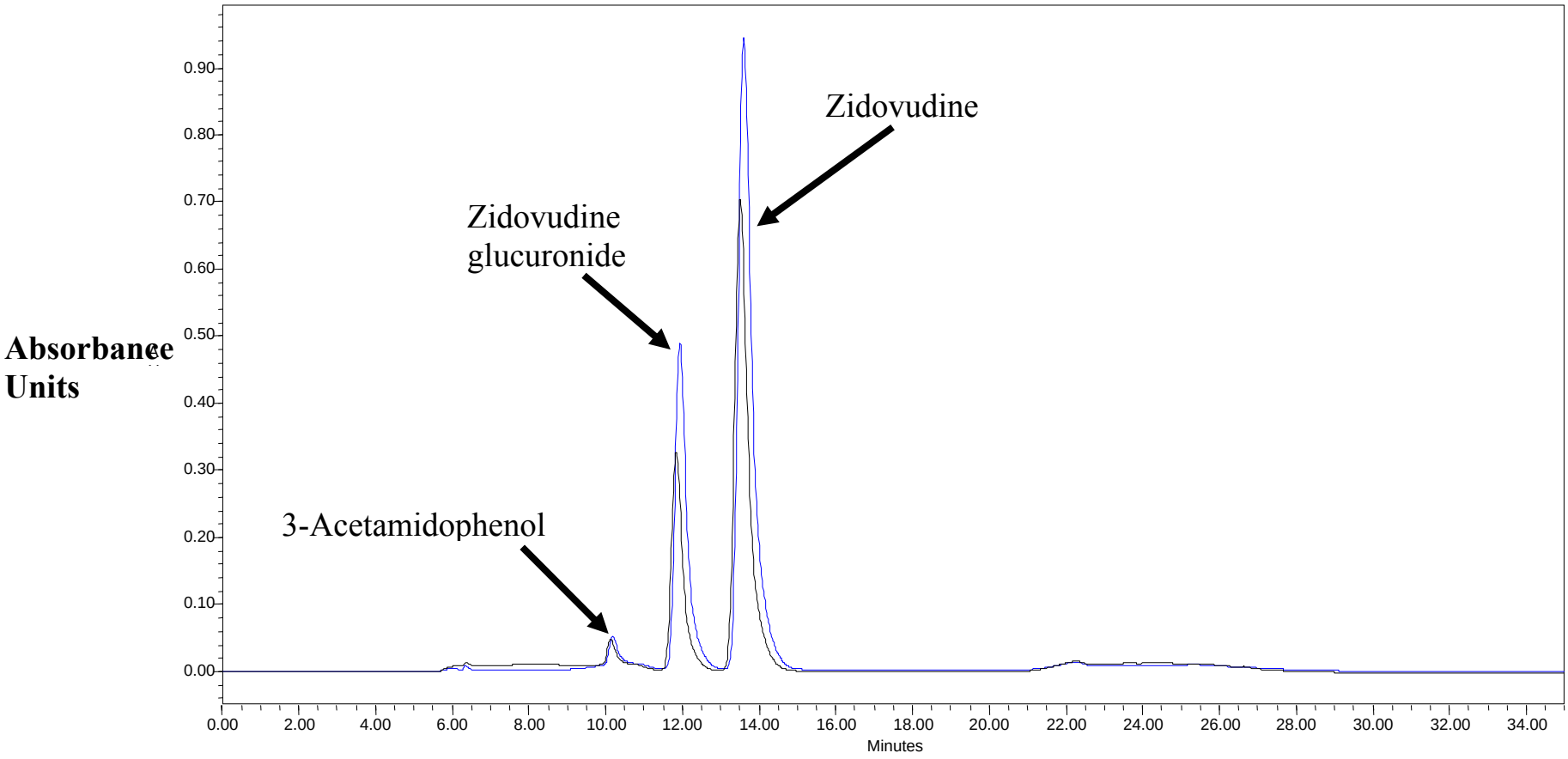

Figure 4.1.10: HPLC chromatogram of 3-Acetamidophenol, zidovudine glucuronide and zidovudine. HPLC chromatogram depicting zidovudine glucuronide formation in control (black) and rifampin (blue) treated human hepatocytes following 72 hours drug treatment period

All 3 drugs robustly induced UGT2B7 mRNA levels (Table 4.1.4), with much greater variability than was observed for UGT1A1 and UGT1A6. Only nelfinavir 10 μM increased immunoreactive protein levels to the same amount as rifampin. The high levels of mRNA expression did not lead to an increase in immunoreactive protein levels with either ritonavir or efavirenz.

Table 4.1.4: Effect of antiretrovirals on hepatic UGT2B7 activity, protein and mRNA levels. Summary table showing fold increase in UGT2B7 activity, immunoreactive protein and mRNA levels compared to DMSO (0.1%) control in primary human hepatocytes following 72 hours with efavirenz, ritonavir, nelfinavir and rifampin.

	RIF	RTV			EFZ			NEL		
	10 μM	**0.5μM**	**5μM**	**10μM**	**0.5μM**	**5μM**	**10μM**	**0.5μM**	**5μM**	**10μM**
mRNA	7.7 ± 4.9	1.4 ± 0.7	1.6 ± 0.9	3.9 ± 2.5	4.6 ± 3.2	3.1 ± 2.1	15.8 ± 5.5	2.7 ± 1.1	9.4 ± 3.8	18.9 ± 7.8
Protein	2.3 ± 0.4*	0.4 ± 0.3	0.9 ± 0.8	1.5 ± 0.7	1.1 ± 0.9	1.2 ± 0.7	1.3 ± 0.5	1.2 ± 0.5	1.6 ± 0.8	2.4 ± 0.8*

Mean ± SD

n=3, *$p < 0.05$

4.1.6 Induction of P-glycoprotein

To evaluate whether the antiretrovirals have the potential to induce P-gp in hepatocytes and LS174T cells, we employed a fluorescent P-gp substrate, Rhodamine 123 (RH123), which is a widely used probe substrate to determine P-gp activity. Rh123 is effluxed out of the cells

primarily by P-gp and hence can be employed in fluorescence- based assays to estimate the amount of Rh123 retained within the cells. Induction of P-gp in hepatocytes results in enhanced efflux of Rh123, leading to a reduction in the overall Rh123 retained inside the cells. Extended exposure (72 hrs) to efavirenz (0.5-10 μM) caused only a slight reduction in Rh123 accumulation in hepatocytes and LS174T cells, which was statistically insignificant. In contrast in primary human hepatocytes, ritonavir and nelfinavir produced robust efflux of Rh123 (Figure 4.1.11). A similar trend was observed in LS174T cells in which ritonavir again produced robust efflux of Rh123. However, interestingly nelfinavir did not show the induction that was observed in hepatocytes. Rifampin 10 μM treated LS174T cells produced a statistically significant increase in Rh123 efflux, unlike hepatocytes treated with rifampin.

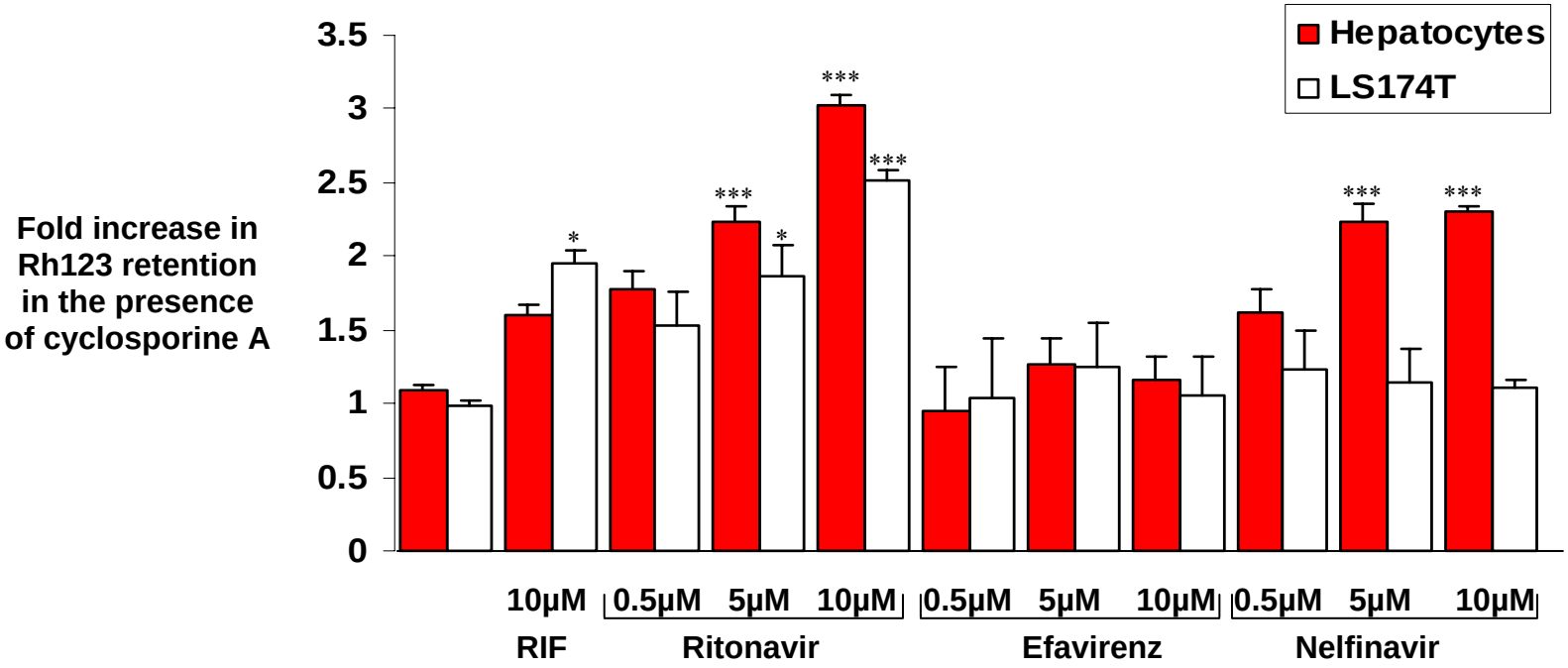

Figure 4.1.11: Effect of antiretrovirals on hepatic and intestinal P-gp activity in primary human hepatocytes and LS174T cells. Fold increase in the percentage of Rh123 remaining in the presence of cyclosporine A (25μM) after efflux, as a function of P-gp in cells treated for 72 hours with test drug rifampin, ritonavir and nelfinavir. N=4, treatments in duplicate. ***$p < 0.001$, *$p < 0.05$ compared to untreated controls.

Relative quantification of P-gp mRNA in hepatocytes and LS174T cells showed a similar trend to that observed with functional activity. Ritonavir again significantly increased P-gp expression in both hepatocytes and LS174T cells. These results compared well to that observed in LS174T cells. LS174T cells treated with 0.5, 5 and 10 μM of ritonavir produced ~1-to 13-fold increase in mRNA levels, while hepatocytes produced ~2 to 7- fold increase in P-gp mRNA levels. As was evident in P-gp functional activity studies, no increase in P-gp mRNA was observed with nelfinavir in LS174T cells. Rifampin, the prototypical inducer produced comparative results in hepatocytes and in LS174T cells (Table 4.1.5).

Table 4.1.5: Relative quantification of hepatic and intestinal P-gp mRNA levels in primary human hepatocytes and LS174T cells. Fold increase in P-gp mRNA detected using real - time PCR in primary human hepatocytes and LS174T cells treated with test drugs for 72 hours.

Treatment	Fold increase in hepatocytes	Fold increase in LS174T cells
Rifampin	2.5 ± 1.4	3.7 ± 1.1*
Efz 0.5 μM	0.5 ± 0.3	0.9 ± 0.3
Efz 5 μM	1.5 ± 0.5	1.4 ± 0.5
Efz 10 μM	2.1 ± 0.7	2.0 ± 1.2
Ritonavir 0.5 μM	1.2 ± 0.2	1.0 ± 0.5
Ritonavir 5 μM	8.5 ± 2.1*	9.2 ± 3.2*
Ritonavir 10 μM	10.5 ± 2.8*	12.7 ± 4.1*
Nelfinavir 0.5 μM	2.2 ± 1.2	1.3 ± 0.5
Nelfinavir 5 μM	2.9 ± 2.8	0.4 ± 0.2
Nelfinavir 10 μM	7.0 ± 2.4*	0.5 ±0.3

Mean ± standard deviation, *$p < 0.05$

4.1.7 Microarray Analysis

The technology of cDNA microarray is a conceptually simple and cost-effective method for monitoring the relative expression of thousands of genes simultaneously (Schena *et al.*, 1995). PCR-amplified cDNA fragments are spotted at high density (10-50 spots/mm^2) onto a microscope slide and probed against fluorescently labeled cDNA. The intensity of signal observed is proportional to the amount of transcript present in the RNA population being studied. Differences in intensity reflect differences in transcript level between treatments. Statistical and bioinformatics analyses on the microarray data are used to generate that may be tested with established molecular biological approaches (Brown and Botstein, 1999).

Microarray analysis was performed on 4 batches of primary human hepatocytes treated with efavirenz 10 μM or vehicle. The Expression Analysis Systematic Explorer (EASE) results obtained indicated that the cytochrome P450 activity gene ontology was most significantly changed when comparing cells that were treated with efavirenz to those that were untreated (p = 0.0012). In contrast, the ATP-binding cassette transporter family was not significantly different (p = 0.37) between efavirenz-treated versus control cells.

The microarray data showed that cytochrome P450 was highly expressed in the liver (Table 4.1.6). CYP3A made up the highest proportion of all the CYPs present in the liver with high fluorescence intensities observed for CYP3A4, CYP3A5, CYP3A7 and CYP3A43. The expression of CYP3A4 (p = 0.0093), CYP3A5 (p = 0.038) and CYP3A7 (p = 0.003) were significantly higher in primary human hepatocytes treated with efavirenz 10 μM compared to untreated cells. In addition, the expression of other phase I enzymes was also increased, including CYP2C8 (1.69-fold, p = 0.038) and CYP2D6 (1.74-fold, p = 0.0048).

The expression of phase II drug metabolizing enzymes was also increased. UGT1A1, UGT1A6 and UGT2B7 produced 1.5-, 1.6 and- 1.2-fold increases in expression. Of these phase II enzymes, UGT2B7 was the most highly expressed in the liver (Table 4.1.6).

The presence of all of the major efflux transporters was confirmed by our microarray data. MRP2 levels were highest and produced a significant increase in expression when treated with efavirenz 10 μM as compared to vehicle (DMSO 0.1%) treated control. None of the other efflux transporters; P-gp, MRP1 or BCRP produced a statistically significant increase in expression. The influx transporters OATP-A and OCT were expressed in the primary human hepatocytes but neither showed a change in expression between efavirenz treated and untreated cells.

As expected, all of the major transcription factors in the regulation of DMTs were expressed in the liver, with PXR expressed at the highest level. CAR, VDR, RXRα and HNF4α were all expressed, but showed no change with efavirenz treatment.

Table 4.1.6: Microarray results of key drug metabolizing enzymes and transporters in cells treated with efavirenz 10 µM and DMSO (0.1%) control

Accession ID	Name	Symbol	Intensity	Fold change (Efz/Con)	*p*-value	Molecular function
NM 000927	ATP-binding cassette, sub-family B (MDR/TAP), member 1	***ABCB1***	546	1.41	0.0996	Drug efflux transporter
NM 004996	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	***ABCC1***	296	-1.33	0.196	Drug efflux transporter
NM 000392	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	***ABCC2***	1969	1.47	0.032	Drug efflux transporter
NM 004827	ATP-binding cassette, sub-family G (WHITE), member 2	***ABCG2***	64	1.19	0.4662	Drug efflux transporter
NM 000761	cytochrome P450, family 1, subfamily A, polypeptide 2	***CYP1A2***	1014	1.07	0.7002	Phase 1 Drug metabolizing enzymes
NM 000771	cytochrome P450, family 2, subfamily C, polypeptide 9	***CYP2C9***	3562	1	0.9999	Phase 1 Drug metabolizing enzymes
NM 000773	cytochrome P450, family 2, subfamily E, polypeptide 1	***CYP2E1***	10006	-4.06	0.0014	Phase 1 Drug metabolizing enzymes
NM 000767	cytochrome P450, family 2, subfamily B, polypeptide 6	***CYP2B6***	1831	1.07	0.728	Phase 1 Drug metabolizing enzymes
NM 030878	cytochrome P450, family 2, subfamily C, polypeptide 8	***CYP2C8***	6019	1.69	0.0375	Phase 1 Drug metabolizing enzymes
NM 000106	cytochrome P450, family 2, subfamily D, polypeptide 6	***CYP2D6***	2677	1.74	0.0048	Phase 1 Drug metabolizing enzymes
NM 017460	cytochrome P450, family 3, subfamily A, polypeptide 4	***CYP3A4***	7607	2.3	0.0093	Phase 1 Drug metabolizing enzymes
NM 000765	cytochrome P450, family 3, subfamily A, polypeptide 7	***CYP3A7***	5363	2.05	0.003	Phase 1 Drug metabolizing enzymes

Table 4.1.6 continued

NM 000463	UDP glycosyltransferase 1 family, polypeptide A1	UGT1A1	829	1.47	0.1	Phase 2 Drug metabolizing enzymes
NM 001072	UDP glycosyltransferase 1 family, polypeptide A6	UGT1A6	2378	1.6	0.1086	Phase 2 Drug metabolizing enzymes
NM 001074	UDP glycosyltransferase 2 family, polypeptide B7	UGT2B7	24672	1.15	0.3257	Phase 2 Drug metabolizing enzymes
NM 000457	hepatocyte nuclear factor 4, alpha	HNF4α	108	-1.04	0.8107	Transcription co-activator
NM 002957	retinoid X receptor, alpha	RXR α	1330	-1.71	0.0061	Transcription factor
NM 000376	vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	354	-1.04	0.7973	Transcription factor
AK056001	Homo sapiens cDNA FLJ31439 fis, clone NT2NE2000707.	CAR	811	1.02	0.8981	Transcription factor
NM 003889	nuclear receptor subfamily 1, group I, member 2	PXR	2259	1.53	0.0944	Transcription factor
NM 021094	solute carrier family 21 (organic anion transporter), member 3	OATP-A	174	1.16	0.3548	Influx transporter
NM 002555	solute carrier family 22 (organic cation transporter), member 1-like	OCT	3779	-1.34	0.073	Influx transporter

4.2 Specific Aim 2

Our results obtained in Specific Aim 1 suggested that induction of phase I/II drug metabolizing enzymes and drug transporters (DMTs), namely P-glycoprotein, or P-gp, occurs in hepatic and intestinal tissue. In clinical practice it is known that modulation of DMTs in the liver and intestine will alter the plasma concentrations of the active drug. However, the clinical efficacy of these antiretroviral agents is dependent upon their intracellular concentration given that their site of action is inside the $CD4^+$ T-cell. Thus, of critical importance to the efficacy of these antiretroviral drugs is the presence and modulation of DMTs in $CD4^+$ T-cells (Asghar *et al.*, 2002), (Hu and Wells, 2004), (Krovat *et al.*, 2000), (Lucia *et al.*, 2002), (Nakamoto *et al.*, 2000). In HIV-1-infected PBMCs and cell lines, the antiviral activity of antiretrovirals has been highly correlated with the intracellular concentration of drugs (Bilello and Drusano, 1996), (Nascimbeni *et al.*, 1999) which has formed the rationale for *in vitro* studies on intracellular antiretroviral drugs.

4.2.1 Induction of Phase I and II Drug Metabolizing Enzymes

Given the results obtained in Specific Aim 1, our next aim was to confirm the presence and induction of drug metabolizing enzymes in $CD4^+$ T-cells. We evaluated the phase I enzymes CYP3A4 and CYP2B6, and the phase II enzymes UGT1A1 and UGT1A6. Gene expression of these four DMTs in all cells is regulated by pregnane-X receptor (PXR), which heterodimerizes to retinoid-X receptor (RXR) to increase the expression of DMTs. To determine whether the expression of these four DMTs are affected by antiretroviral drugs in $CD4^+$ T cells, we treated PBMCs with rifampin 10 μM, ritonavir 10 μM, efavirenz 10 μM or nelfinavir 10 μM, and compared them with control PBMCs treated with DMSO (0.1%). PBMCs were isolated from

whole blood obtained from 5 healthy volunteers, with the experiment being repeated in 1 individual (n=5). $CD4^+$ T-cells were isolated from the PBMCs prior to drug treatment for 72 hours.

First, we determined the mRNA levels for each of the four DMTs. Following drug treatment, total cellular RNA was isolated from the $CD4^+$ T-cells and real-time PCR was carried out using cDNA derived from 2 µg of total RNA. CYP and UGT sequences were amplified using previously published primers (Table 3.2.).

RNA levels were higher for all of the treated cells compared with the control cells (Table 4.2.1). We found significant differences between the treated cells and the control cells for CYP3A4 ($p = 0.009$), CYP2B6 ($p = 0.02$), UGT1A1 ($p = 0.01$), and UGT1A6 ($p = 0.017$). Cells treated with nelfinavir 10 µM produced the most robust induction of CYP3A4 and UGT1A1 compared to control ($p = 0.008$ and $p = 0.01$, respectively). Cells treated with efavirenz 10 µM had the highest increase in CYP2B6 and UGT1A6 mRNA, at least among the antiretroviral treatments; CYP2B6 induction was the same for efavirenz and rifampin, while rifampin induction of UGT1A6 was higher than with antiretroviral treatment.

Table 4.2.1: Relative quantification of PXR-regulated gene mRNA in Primary human $CD4^+$ T-Cells (Fold increase relative to control).

mRNA	Rifampin 10 µM	Efavirenz 10 µM	Ritonavir 10 µM	Nelfinavir 10 µM
CYP3A	7.6±3.2*	13.9±2.3***	7.5±3.6 *	30.0±12.6 *
CY2B6	22.3±8.1*	22.6±12.9 *	5.0±4.1	6.1±2.5 *
UGT1A1	10.5±5.0*	12.4±10.9	7.7±2.2*	14.7±10.1
UGT1A6	18.1±3.5**	9.3±3.1**	7.2±4.3*	7.9±4.1*

n=5

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,

To determine the level of CYP3A4 functional activity and compare to mRNA levels, the same assay that we used on hepatocytes and LS174T cells was also used with PBMCs. Briefly, following 72 hr of drug treatment, the PBMCs were incubated with testosterone. The level of 6β-hydroxytestostrone was monitored in intact cells by HPLC as a marker of CYP3A4 activity (Figure 4.1.2). However, no measurable levels of 6β-hydroxytestostrone were observed using this method in PBMCs. Therefore this assay is apparently not sensitive enough to measure changes in CYP3A4 at the translational level in these cells. We also used fluorochromes, which are purported to be 100 times more sensitive than testosterone conversion to 6β-hydroxytestosterone (Donato *et al.*, 2004), to detect CYP3A4 activity in PBMCs, but again, we detected no conversion by CYP3A4. The levels of CYP3A4 activity in PBMC is likely very low and will require new and more sensitive methods of detection.

4.2.2 Induction of Efflux Transporters

Several ATP-binding cassette efflux transporters are known to be expressed in PBMCs. Previous studies revealed that an increase/decrease in the expression levels of these transporters on PBMCs alters the intracellular concentration of antiretroviral drugs such as saquinavir and nelfinavir. Therefore, in addition to P-gp that we examined in hepatocytes in Specific Aim 1, we also determined the induction of MRP1 and BCRP by antiretrovirals.

4.2.2.1 Induction of P-glycoprotein Transcription and Functional Activity

P-gp is one of the most important ABC transporters for drug disposition in humans. It transports a broad variety of structurally diverse compounds that are usually hydrophilic and amphipathic. As a result of its anatomical localization, one of the ways that P-gp functions is by limiting drug penetration into sensitive tissues (e.g. lymphocytes) once the drug has reached the

systemic circulation (Figure 1.5). This function of P-gp is likely to be a determinant of the success of HIV drug therapy because adequate local intracellular drug concentrations in $CD4^{\pm}$ lymphocytes are required for therapeutic effectiveness (Kim, 2003).

Using primers listed in Table 3.2.2, P-gp mRNA levels were measured in $CD4^{+}$ T-cells treated with rifampin 10 μM, ritonavir 10 μM, efavirenz 10 μM, or nelfinavir 10 μM (Table 4.2.2). Treatment with all of the drugs led to higher P-gp mRNA levels than control cells. Treatment with nelfinavir resulted in the highest increase, followed by ritonavir and efavirenz.

Table 4.2.2: Relative quantification of PXR-regulated gene mRNA in Primary human CD4+ T-Cells (Fold increase relative to control).

mRNA	Rifampin 10 μM	Efavirenz 10 μM	Ritonavir 10 μM	Nelfinavir 10 μM
P-gp	7.8±1.2***	10.2±1.5***	9.6±2.1***	14.9±3.0*
MRP1	7.6±3.2*	2.8±1.3	3.8±3.2	9.0±5.2*
BCRP	1.2±0.4	0.3±0.2	0.4±0.3	0.3±0.1

n=5

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,

To evaluate whether antiretroviral treatment induced P-gp activity in $CD4^{+}$ T-cells, we employed the fluorescent P-gp substrate Rhodamine 123 (Rh123) and measured the levels of this substrate in individual cells using flow cytometry. Rh123 is effluxed out of the cells primarily by P-gp and hence can be employed in fluorescence-based assays to estimate the amount of Rh123 retained within the cells over time. Induction of P-gp in $CD4^{+}$ T-cells results in enhanced efflux of Rh123 within 1 hr, leading to a reduction in the overall Rh123 retained inside the cells. To confirm the efflux of Rh123 via P-gp, we utilized the P-gp inhibitor cyclosporine A.

PBMCs were treated with the antiretroviral drugs for 72 hr, then loaded with Rh123 for 60 min, allowed to efflux Rh123 for 60 min, and fixed. Sixty minute efflux represents a time point during the linear phase of Rh123 efflux. The fixed cells were stained with anti-human CD4-biotin/streptavidin-APC that allowed gating on only the $CD4^+$ cells (Figure 4.2.1). Rh123 was measured in $CD4^+$ cells that had been fixed immediately after the loading period (time = 0 min) to assess loading efficiency, and in $CD4^+$ cells that had been fixed immediately after the efflux period (time = 60 min). Rh123-loaded cells incubated with cyclosporine A were also measured at time = 60 min to assess transporter specificity.

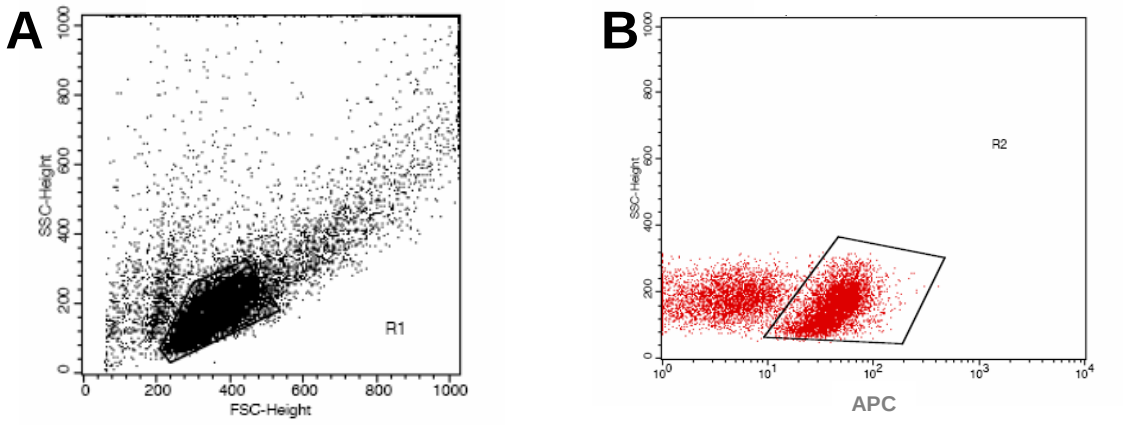

Figure 4.2.1: Flow cytometry of fixed and stained PBMCs

A) The correct size for T-cells were selected by gating on forward and side scatter characteristics for optimal size and shape of cells. B) Secondary gating to select $CD4^+$ T-cells by intensity of APC staining

Initial experiments were done with day-old blood obtained from Hoxworth Blood Center (n=6). However, significant variability in the loading of Rh123 was observed (Figure 4.2.2).

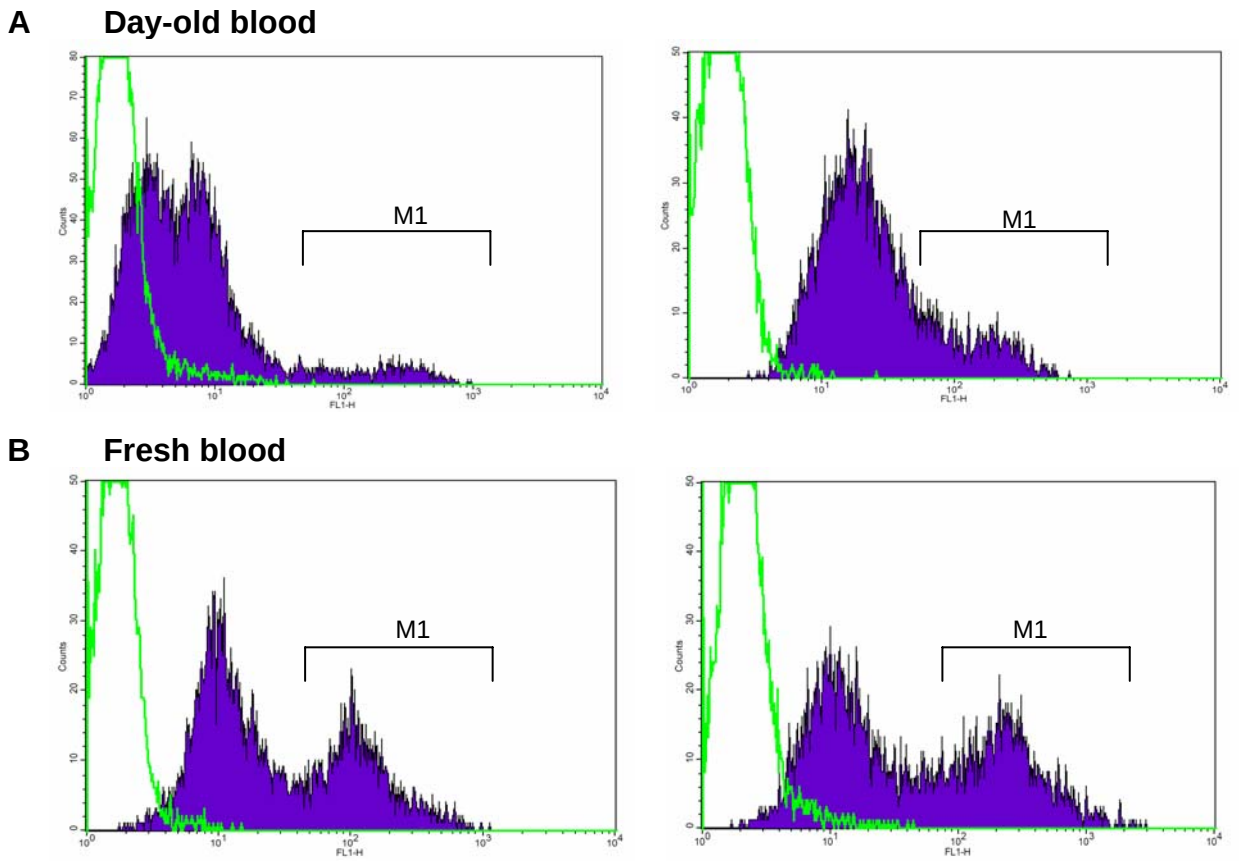

Figure 4.2. 2: Histogram of the Rh123 loading of control treated CD4+ T-cells. Figures A represents day old Rh123 loaded cells from 2 different individuals to show loading. Figures B represents Rh123 loaded cells from fresh blood of 2 different individuals. M1 represents fully loaded cells.

We attempted to reduce this variability by adding cyclosporine A during Rh123 loading, but the presence of cyclosporine A adversely affected the loading and subsequent efflux of Rh123 even after a 2 hour washout period (Figure 4.2.3).

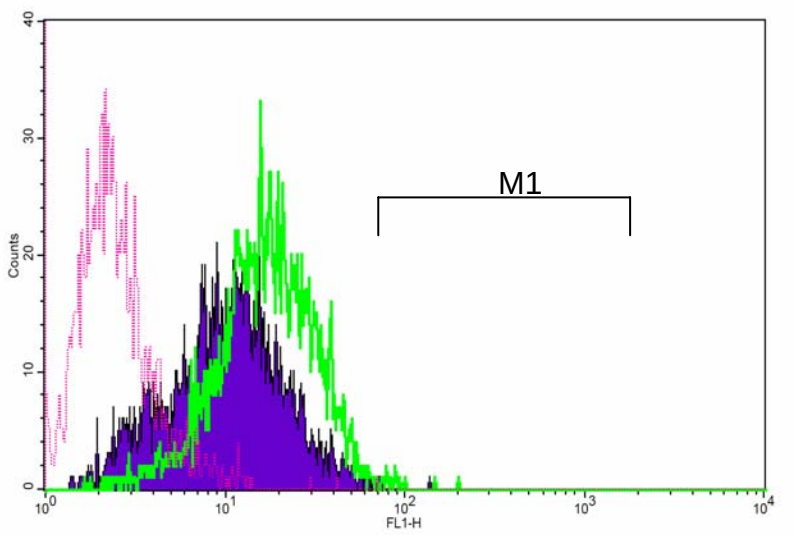

Figure 4.2.3: Histogram of nelfinavir 10 µM treated CD4$^+$ T-cells from day-old blood in the presence (purple) and in the absence (green) of cyclosporine A during Rh123 loading. PBMCs not loaded with Rh123 (pink) were also included for comparison of loading efficiency.

Following these tests, we decided to use fresh blood obtained from healthy volunteers in which more uniform loading of Rh123 was observed (Figure 4.2.2). The following results were obtained using PBMCs from 5 healthy volunteers. In order to confirm the consistency of the results, we repeated the experiment with PBMCs from one of the individuals and found that the loading and efflux of Rh123 were consistent (Figure 4.2.4).

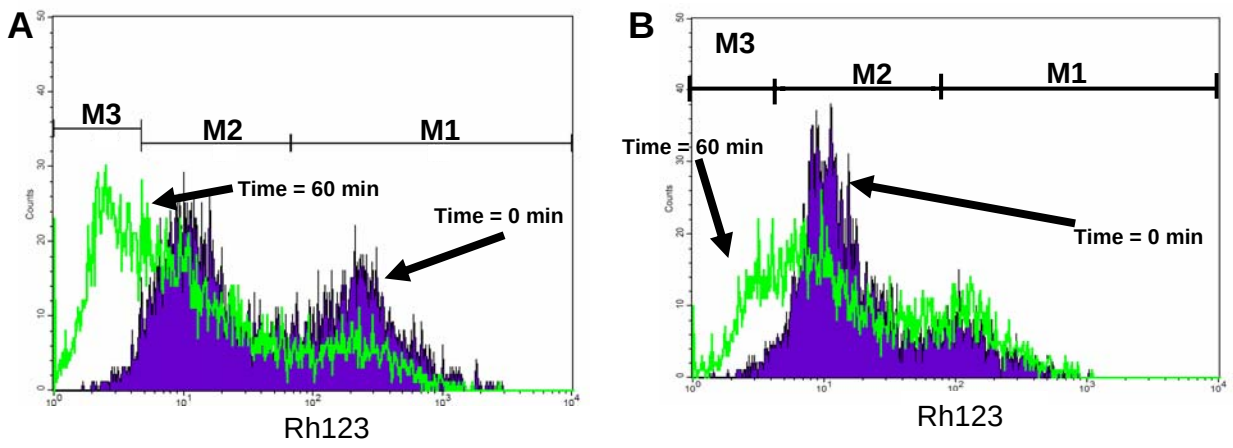

Figure 4.2.4. Repeat of analysis on one individual to evaluate consistency of results. A: Histogram of nelfinavir 10 µM treated CD4+ T-cells before efflux at time = 0 min (purple) and after efflux at time = 60 min (green). B: Histogram of nelfinavir 10 µM treated CD4+ T-cells from blood obtained from the same individual 1 month later.

The cells loaded consistently (observed at time = 0) with mean fluorescence intensity (MFI) values ranging between 61.9 and 74.7. MFI represents the total number of events divided by the total number of gated cells. Overall means obtained after 60 minutes of efflux revealed that nelfinavir-treated cells effluxed Rh123 most efficiently ($p = 0.01$, Figure 4.2.5). The MFI values for cells treated with ritonavir and efavirenz were lower than nelfinavir-treated cells, but were still higher than for control cells ($p < 0.05$). The results observed with rifampin were not significantly different from control cells. The observed MFI values were essentially the same in cells co-incubated with the P-gp inhibitor cyclosporine A (2.5 µM) at time = 60 min compared with time = 0 min cells (between 95-120% of time = 0 min values).

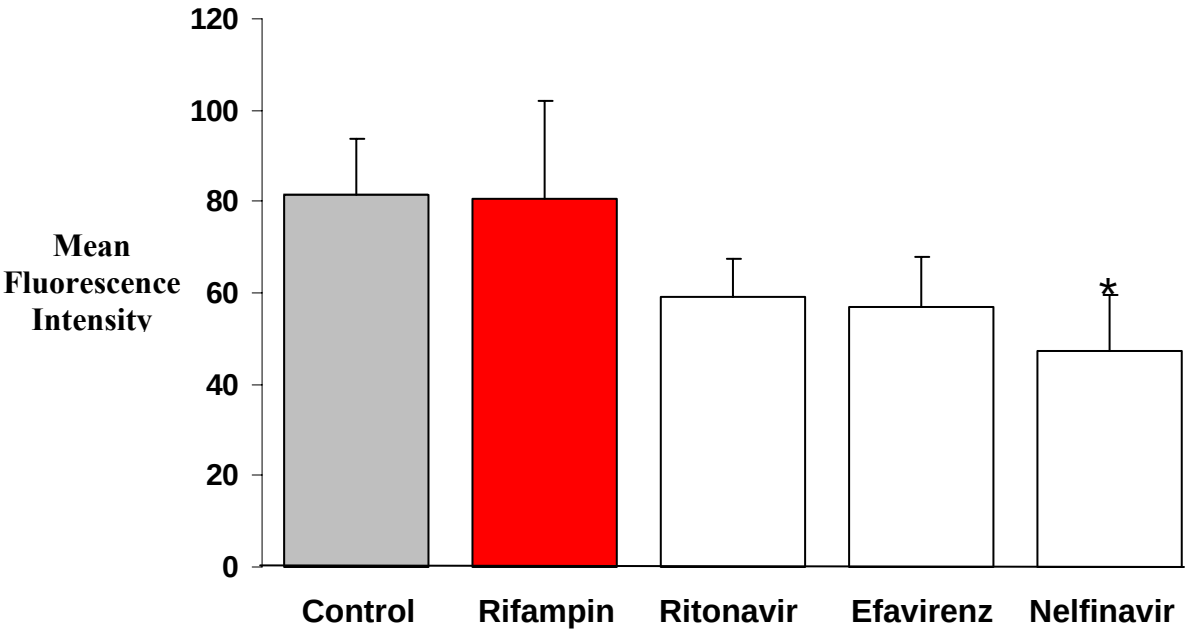

Figure 4.2.5: P-gp functional activity assay assessing the overall Rh123 mean fluorescence intensity (MFI) following 60 min efflux in drug-treated CD4+ T-cells. Drug treated cells were compared to untreated control cells. n=5, treatments in duplicate. *p<0.05

The data were also analyzed by separating the populations based on Rh123 fluorescence. As shown in Figure 4.2.6, marker M1 bounded the fully-loaded $CD4^+$ T-cells, M2 bounded the cells with intermediate levels of Rh123 (either not fully loaded or partially effluxed), and M3 bounded the cells that had fully effluxed the Rh123. M3 was set using PBMCs which had not been loaded with Rh123. Thus, a higher percentage of $CD4^+$ T-cells in M3, following 60 minutes of efflux time, represents more efficient Rh123 efflux, which translates into increased P-gp expression. In the example in Figure 4.2.6, it appears that the fully-loaded cells at time 0 (in M1) moved to M3 as they fully effluxed, while there was little change in the cell population within M2.

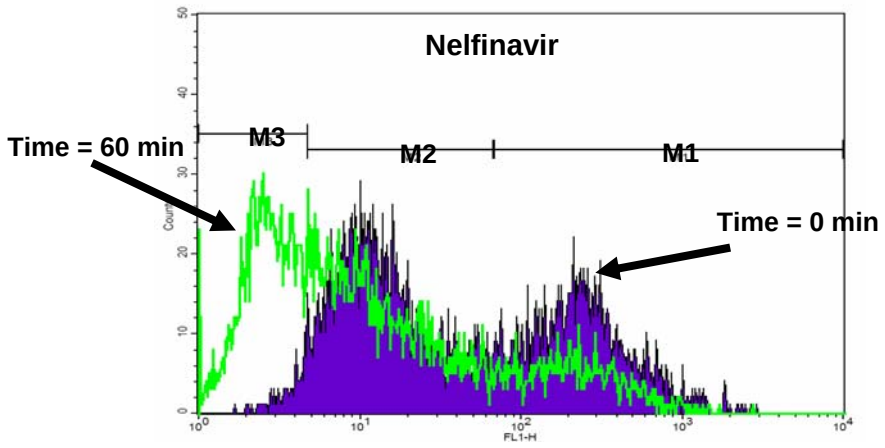

Figure 4.2.6: Histogram of nelfinavir 10 µM treated CD4+ T-cells before efflux at time = 0 min (purple) and after efflux at time = 60 min (green). M1 = fully loaded cells, M2 = partially loaded cells and/or partially effluxed cells, and M3 = cells that have completely effluxed Rh123

Table 4.2.3 is a representation of the CELLQuest statistics obtained at time zero and time 60. At time zero the largest percentage of cells was present in M1 and M2, suggesting that the cells loaded well. In contrast, the lowest percentage of cells was in M3, which indicates that the cells had not effluxed Rh123. However, at time 60 the opposite was observed, a large percentage of cells moved away from M1, while a large increase was observed in the percentage of cells in M3.

Table 4.2.3: CELLQuest statistics of nelfinavir 10 µM treated CD4+ T-cells before Rh123 efflux (time = 0 min) and after efflux (time = 60 min).

	Time = 0 min	Time = 60 min
Marker	**% Cells**	**% Cells**
All	100.00	100.00
M1	37.83	15.02
M2	57.59	45.96
M3	6.49	39.20

M1 = fully loaded cells, M2 = partially loaded cells and/or partially effluxed cells, and M3 = cells that have completely effluxed Rh123. % cells represents the percentage of $CD4^+$ T-cells that are in the given marker region of the histogram.

As was evident with observed changes in the overall MFI values, $CD4^+$ T-cells treated with nelfinavir had the greatest percentage of cells in M3 (30.4±4.3%) compared to control (14.0±3.6%) ($p = 0.003$) (Figure 4.2.7). Cells treated with ritonavir, rifampin and efavirenz also had more cells in M3 following 60 minutes of efflux. At time = 0, the percentage of cells present in the M3 region was consistent between the different treatments and ranged between 4.6% and 5.7% (red bars in Figure 4.2.7).

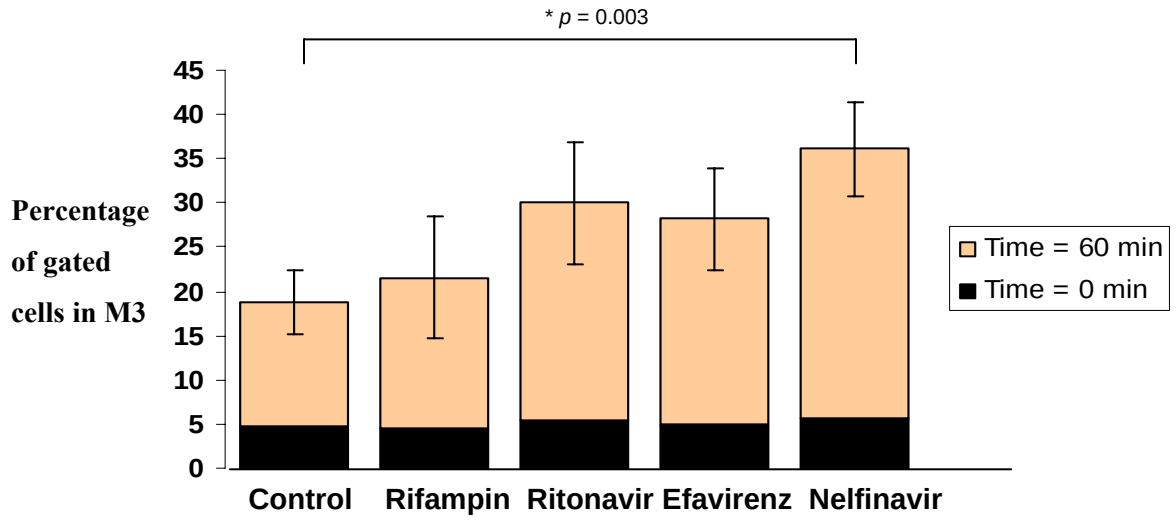

Figure 4.2.7: Percentage of Drug Treated Cells in M3 (cells that have completely effluxed Rh123) following 60 minutes of Rh123 efflux. Time = 0 min represents the percentage of cells in M3 prior to efflux. n=5, treatments in duplicate.

4.2.2.2 Induction of Multidrug Resistance Protein 1 Transcription and Functional Activity

MRP1 is expressed in most tissues throughout the body with relatively high levels found in PBMCs (Cole *et al.*, 1992), (Flens *et al.*, 1996), (Stride *et al.*, 1996). Meaden *et al.* (2002) found patients with lower MRP1 expression had a significantly higher accumulation of both ritonavir and saquinavir than those with higher MRP1 expression. They concluded that increased expression of MRP1 on lymphocytes is associated with lower intracellular accumulation of saquinavir and ritonavir. These results were reproduced by Janneh *et al.* (2005) in which the transport and accumulation of ^{3}[H]-saquinavir was measured in PBMC in the absence or

presence of specific and non-specific inhibitors of MRP1. They also found that the expression of MRP1 mRNA is important in the intracellular accumulation of saquinavir in PBMCs.

Relative quantification of MRP1 mRNA revealed all test compounds to increase the transcription of MRP1. Rifampin (7.6±3.2) ($p < 0.05$) and nelfinavir (9.0±5.2) ($p < 0.05$) led to the greatest increases, while efavirenz (2.8±1.3) and ritonavir (3.8±3.2) treatments showed lesser induction (Table 4.2.2).

To evaluate whether the antiretrovirals could induce MRP1 function/activity in $CD4^+$ T-cells, we employed the MRP1 substrate, calcein-AM, to determine MRP1 activity. Calcein was measured by flow cytometry in PBMCs at time = 0 min (loading efficiency) and time = 180 min (efflux). Cells incubated in the presence of MK571 were also measured at time =180 min (transporter specificity). In contrast to the Rh123 loading, all of the $CD4^+$ T-cells were in a single population after loading and after efflux (Figure 4.2.8). We therefore utilized the overall MFI of the $CD4^+$ T-cells to ascertain the ability of the cells to efflux calcein. As represented in Figure 4.2.8, the cells were efficiently loaded with calcein. After 180 min, the prototypical inducer rifampin and all 3 antiretrovirals were able to efflux calcein slightly more efficiently compared to vehicle-treated control cells (Figure 4.2.9). This efflux, however, was not inhibited by MK571, suggesting that efflux may be occurring via another transporter.

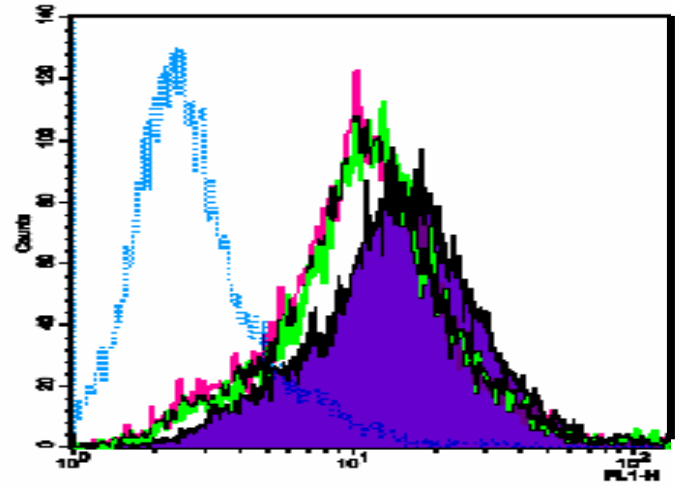

Figure 4.2.8: Histogram of nelfinavir 10 µM treated CD4$^+$ T-cells. Cells not loaded with Calcein-AM (blue). Cells loaded with Calcein-AM before efflux at time = 0 min (purple), after efflux at time = 180 min (green), and after 180 min of efflux in the presence of the MRP1 inhibitor MK571 (pink).

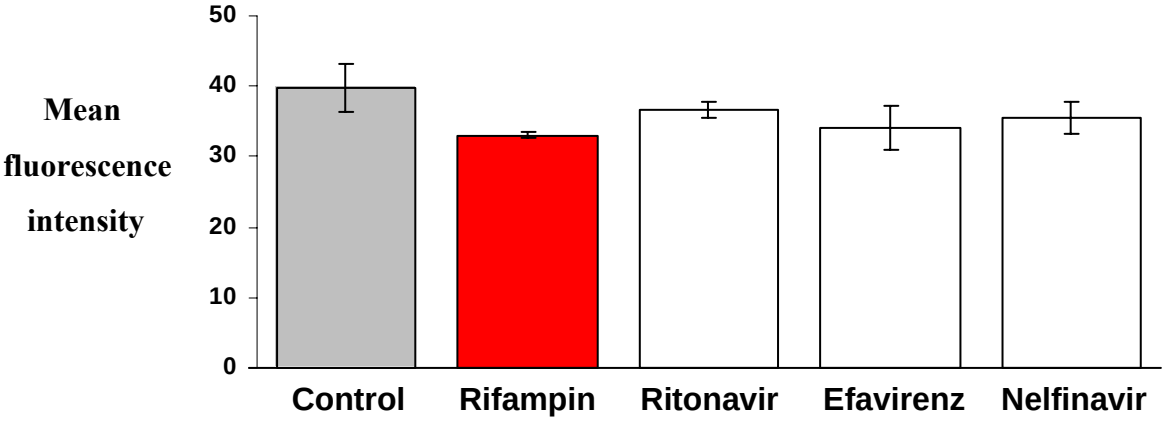

Figure 4.2.9: MRP1 functional activity assay assessing the overall calcein mean fluorescence intensity (MFI) following 180 min efflux in drug-treated CD4+ T-cells. n=5

4.2.2.3 Induction Breast Cancer Resistance Protein Transcription and Functional Activity

Studies by Wang e*t al.* (2003b) and Taipalensuu *et al.* (2001) have found that BCRP transcript is expressed in PBMCs and the intestine in greater amounts than P-gp. Wang *et al.* (2003b) found that a high level of BCRP expression in $CD4^+$ T cells conferred cellular resistance to AZT. Therefore, we measured mRNA levels and efflux by this protein in $CD4^+$ T-cells.

Surprisingly, efavirenz, ritonavir, and nelfinavir treatments dropped BCRP mRNA levels in $CD4^+$ T-cells to one-third of the control cell levels (Table 4.2.2). In contrast, rifampin showed no changed in BCRP mRNA levels. Therefore, even though BCRP may be regulated by PXR, it was not induced by these antiretroviral drugs or rifampin.

To evaluate whether the antiretrovirals had the potential to induce BCRP function in $CD4^+$ T-cells, we employed the fluorescent BCRP substrate, mitoxantrone (MTX), which loaded well into $CD4^+$ T-cells (Figure 4.2.10). MTX was measured by flow cytometry in PBMCs after 0 (to check loading) and 90 min of efflux. Cells incubated in the presence of the BCRP inhibitor Fumitremorgin–C were also measured at 90 min to check transporter specificity (Diah *et al.*, 2001). As shown in Figure 4.2.10, no MTX efflux was recorded in the $CD4^+$ T-cells with and without treatment with antiretroviral drugs, or with incubation with Fumitremorgin–C. Therefore, BCRP was not active in these cells.

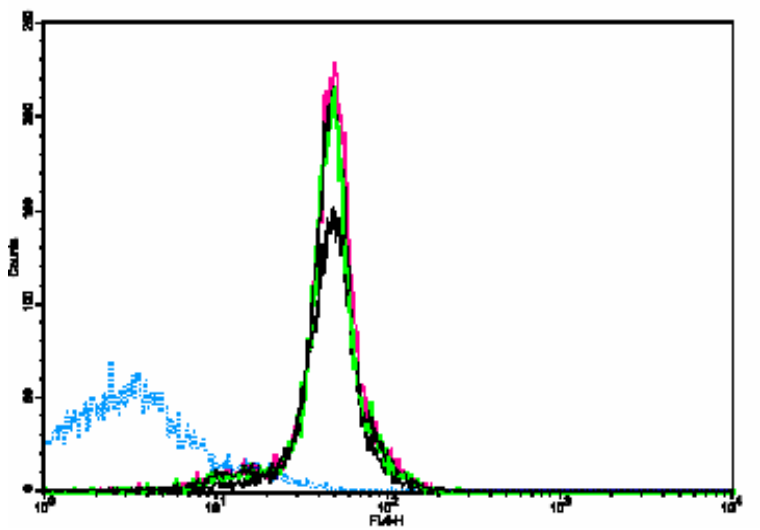

Figure 4.2.10: Histogram of nelfinavir 10 µM treated CD4+ T-cells A: Cells not loaded with Mitoxantrone (blue). Cells loaded with Mitoxantrone before efflux at time = 0 min (black), after efflux at time = 90 min (green), and after 90 min in the presence of the BCRP inhibitor Fumitremorgin (pink).

4.3 Specific Aim 3

There have been numerous reports of infectious diseases causing impaired metabolism of drugs in humans, with important clinical consequences. The presence of HIV proteins, such as Tat, have been associated with increased expression of efflux transporters such as P-gp and MRP1 (Hayashi *et al.*, 2005a), (Hayashi *et al.*, 2005b). The potential influence of HIV on the expression of hPXR and inducibility of DMT genes has not been investigated and was addressed in this specific aim. In addition, it is important to assess the amount of drug efflux from the target cells for antiretroviral drugs, $CD4^+$ T-cells, within the context of HIV infection. Based upon the results we obtained in aim 2, our studies here are focused on the induction of P-gp in nelfinavir-treated cells, since P-gp (among the DMTs examined) showed the most induction with

antiretroviral treatment, and nelfinavir (among the antiretrovirals examined) had the greatest impact on P-gp.

4.3.1 Induction of P-gp in HIV-Exposed and -Unexposed $CD4^+$ T-Cells

$CD4^+$ T-cells were incubated with HIV_{NL4-3} for one hour and then treated either with vehicle (DMSO 0.1%) or with nelfinavir 10 μM for 72 hours. Cellular RNA was harvested and subjected to RT-PCR using primers listed in Table 3.2.2 for P-gp and PXR. As shown in Figure 4.2.1, cells incubated with HIV showed a slight reduction in P-gp mRNA induction with nelfinavir treatment compared with cells that were not incubated with HIV (~10 vs. ~13-fold). However, hPXR transcript levels were reduced 26-fold in cells incubated with HIV. The large change in hPXR expression could be due to unusual hPXR expression in this individual or it could be related to an alteration in the ability of infected $CD4^+$ T-cells to express hPXR. It should be emphasized here that hPXR expression should correspond with the induction of DMTs, such as P-gp; although both P-gp and hPXR showed reductions in expression, the effect of HIV exposure on hPXR was substantially greater.

The effect of HIV on nelfinavir induction of P-gp function in $CD4^+$ T-cells was next determined using the same flow cytometry methodology utilized in Specific Aim 2. PBMCs were incubated with HIV_{NL4-3} and treated with nelfinavir or DMSO for 72 hrs as described above. The cells were loaded with Rh123, allowed to efflux for 1 hr, and fixed. The fixed PBMCs were then stained with antibodies against CD4 followed by a secondary antibody conjugated to a fluorochrome. Rh123 levels were measured after 0 and 60 min of efflux for the total $CD4^+$ cell population. Rh123-loaded cells incubated with cyclosporine A were also measured after 60 min efflux to check transporter specificity.

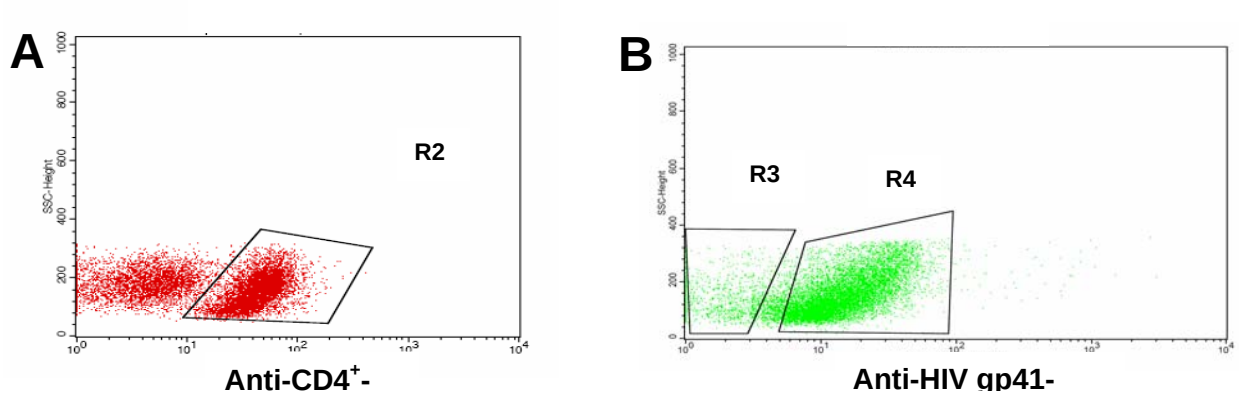

Figure 4.3.2: Gating of HIV_{NL4-3}-Infected CD4+ T-Cells.

A: CD4+ T-cells were gated by high fluorescence of APC. B: Cells with the lowest fluorescence with anti-gp41 were gated as HIV^- and cells with the highest fluorescence with anti-gp41 as HIV^+ cells.

We used the M1 marker to determine the percentage of $CD4^+$ cells that contained a high level of Rh123 in our comparison of the HIV-exposed and -unexposed cells, since the cells in M1 undergo the most efflux (Figure 4.2.6). As shown in Figure 4.3.3, there was no difference in Rh123 efflux between the HIV-exposed and -unexposed cells when the entire $CD4^+$ cell populations were examined in the control cells. In comparison, nelfinavir treated cells showed a significantly ($p < 0.05$) lower ability to efflux Rh123 after 60 minutes of efflux in HIV-exposed cells. These results support the RNA data in which a reduction in P-gp mRNA expression was observed.

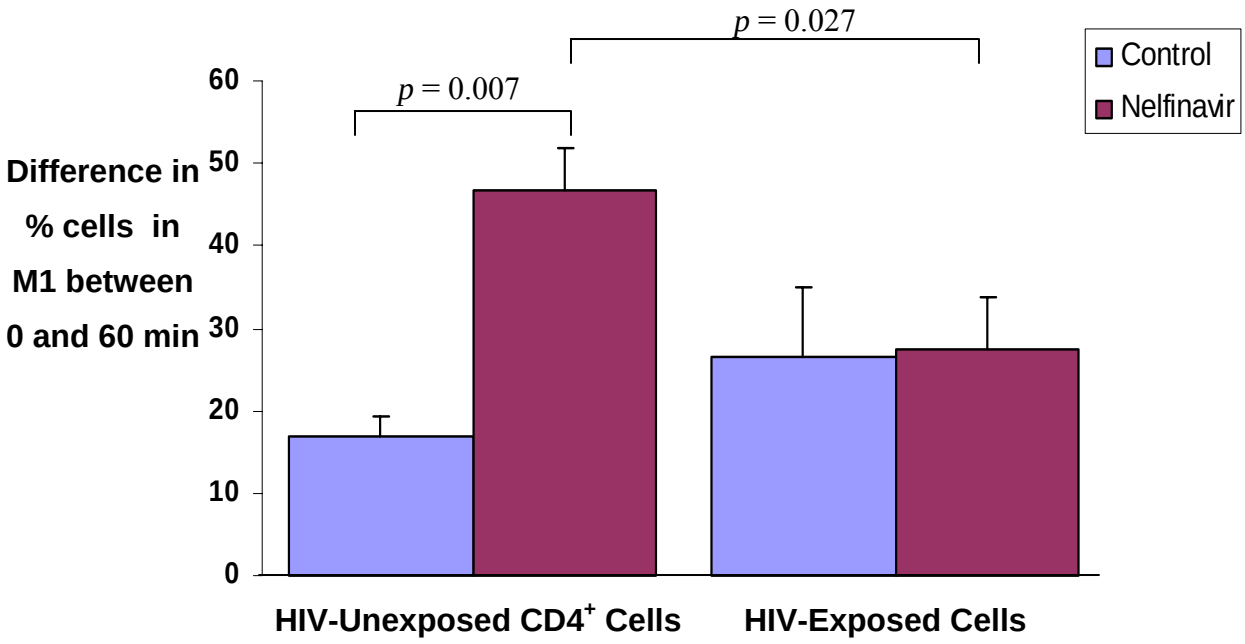

Figure 4.3.3: Difference in the percentage of cells before and after efflux (Time = 60 min) of Rh123 loaded cells in the M1 region (fully loaded population). n=3, treatments in duplicate.

4.3.2 Induction of P-glycoprotein in HIV-Exposed-Infected and- HIV-Exposed-Uninfected CD4$^+$ T-Cells

Although the cells with reduced P-gp and hPXR mRNA described above had been exposed to HIV, there was insufficient virus to infect all of the cells, and the length of incubation of the cells post-infection was not long enough for the virus to spread to all of the cells. However, the virus stock contained high amounts of noninfectious viral particles (J. Nelson Ph.D, personal communication), which could bind to cells without infecting them. Interaction with HIV proteins could lead to a significant impact on the efficacy of antiretroviral drugs in

those cells. Previous studies have shown that the presence of viral proteins has an effect on the expression of efflux transporters such as MRP1 and P-gp (Hayashi *et al.*, 2005a), (Hayashi *et al.*, 2005b). Hayashi et al. (Hayashi *et al.*, 2005b) observed an increase in the expression and functional activity of P-gp in the presence of a viral regulatory protein, Tat. Thus, to determine the effects of HIV on cells that were infected and those that were only exposed and not infected, we used a monoclonal antibody against the HIV envelope protein gp41 that is expressed on the surface of infected cells along with magnetic beads to separate the infected and uninfected $CD4^+$ T-cells after nelfinavir treatment. Among the control cells, the infected cells had the lowest expression of P-gp mRNA and the unexposed cells had the highest expression. Among the nelfinavir-treated cells, the infected cells showed very low induction of P-gp mRNA while the exposed-uninfected cells had greater induction than did the unexposed cells (Figure 4.3.4). Similar results were seen with PXR, with the nelfinavir-treated infected cells having the lowest expression of hPXR mRNA and nelfinavir-treated exposed-uninfected cells showing the highest expression. However, there were differences between the control cells between P-gp and hPXR. hPXR levels were the same with HIV infection in the absence of nelfinavir, while HIV exposure without infection in the absence of nelfinavir led to substantially higher levels of hPXR mRNA (Figure 4.3.5). Comparison of our results with and without separation of infected/uninfected cells suggests that a majority of the cells were infected by HIV, leading to an overall drop in P-gp and hPXR mRNA among unsorted cells.

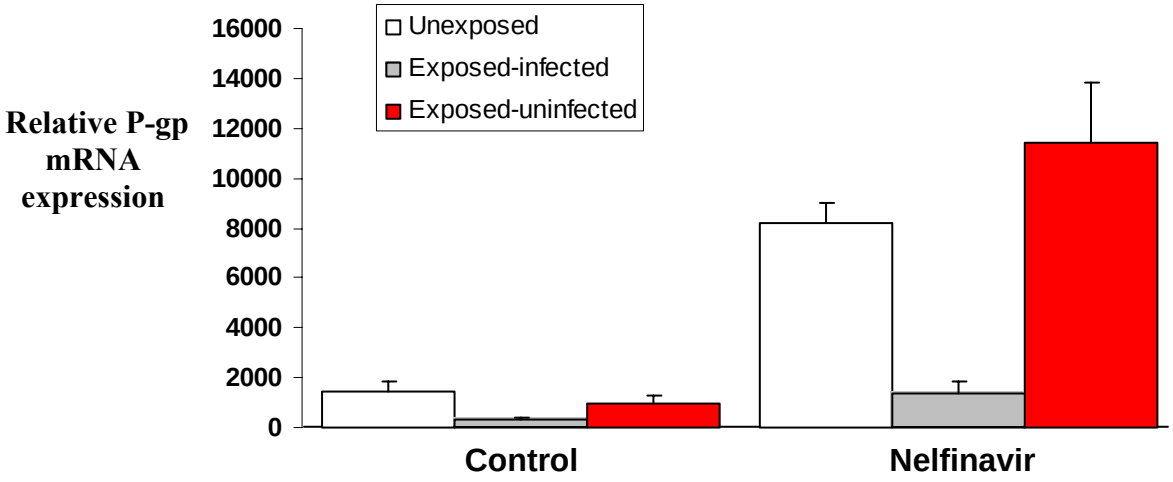

Figure 4.3.4: Relative quantification of P-gp expression in control and nelfinavir treated cells. HIV-exposed cells were divided into infected and uninfected cells prior to RNA extraction. n=2, treatments in duplicate. The standard curve for PXR expression was used for relative quantification of P-gp.

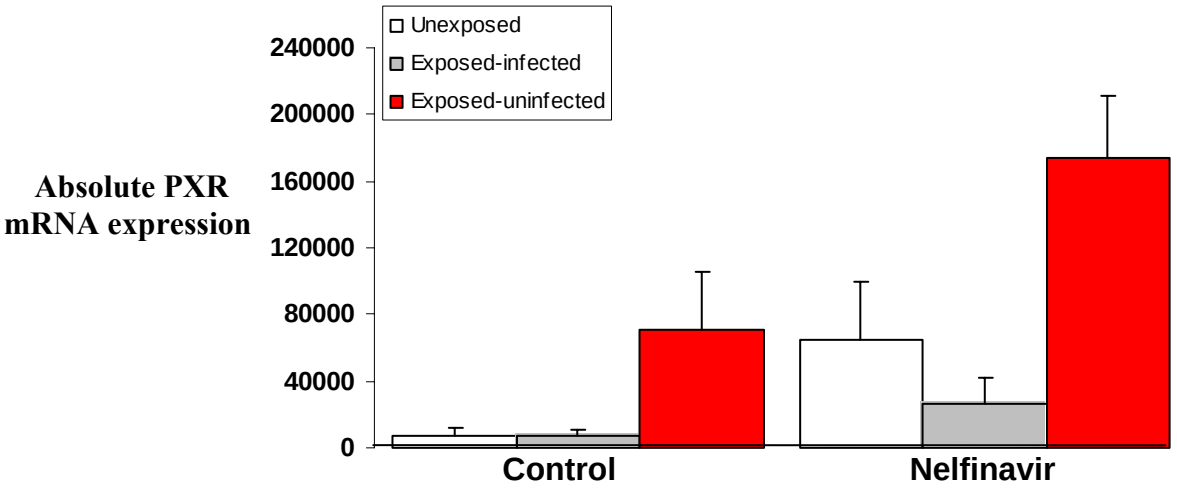

Figure 4.3.5: Absolute quantification of PXR mRNA expression in control and nelfinavir treated cells. HIV-exposed cells were divided into infected and uninfected cells prior to RNA extraction. n=2, treatments in duplicate.

Again, P-gp function in $CD4^+$ T-cells was assessed employing flow cytometry. In addition to the CD4 antibody, an antibody specific for the HIV envelope protein gp41 that is expressed on infected cells was used to stain the cells. $CD4^+$ cells were gated (Figure 4.3.2A), and to analyze the infected cells in the HIV-exposed samples, a gate was set for the highest gp41 expression (Figure 4.3.2B). The lowest gp41 expressing cells were gated as the HIV-exposed uninfected cells. These separate analyses of the HIV-exposed-infected and HIV-exposed-uninfected cells indicated real differences between these cell populations, just as was seen with the P-gp and PXR mRNA levels. As shown in Figure 4.3.6A, exposed-uninfected and infected cells both had significantly lower M1 values immediately after Rh123 loading than unexposed cells, but there was no difference with nelfinavir treatment at this time point (Figure 4.3.6 A).

After 60 min efflux, the level of efflux, as measured by the difference in the percentage of cells in M1 between 0 and 60 min, indicated that the combination of nelfinavir treatment and HIV exposure led to significant changes in efflux (Figure 4.3.6B). Unexposed and exposed-uninfected cells had similar levels of efflux with DMSO and nelfinavir treatment, with the expected boost in efflux with nelfinavir treatment. These similarities were seen despite the differences in loading between the HIV exposed cells and the unexposed cells seen in Figure 4.3.6A. In contrast, the infected cells showed the opposite effect. The control infected cells showed increased efflux on the level of nelfinavir-treated unexposed cells, while the nelfinavir-treated infected cells had a significantly lower ability to efflux Rh123 from M1 as compared to control ($p = 0.013$).

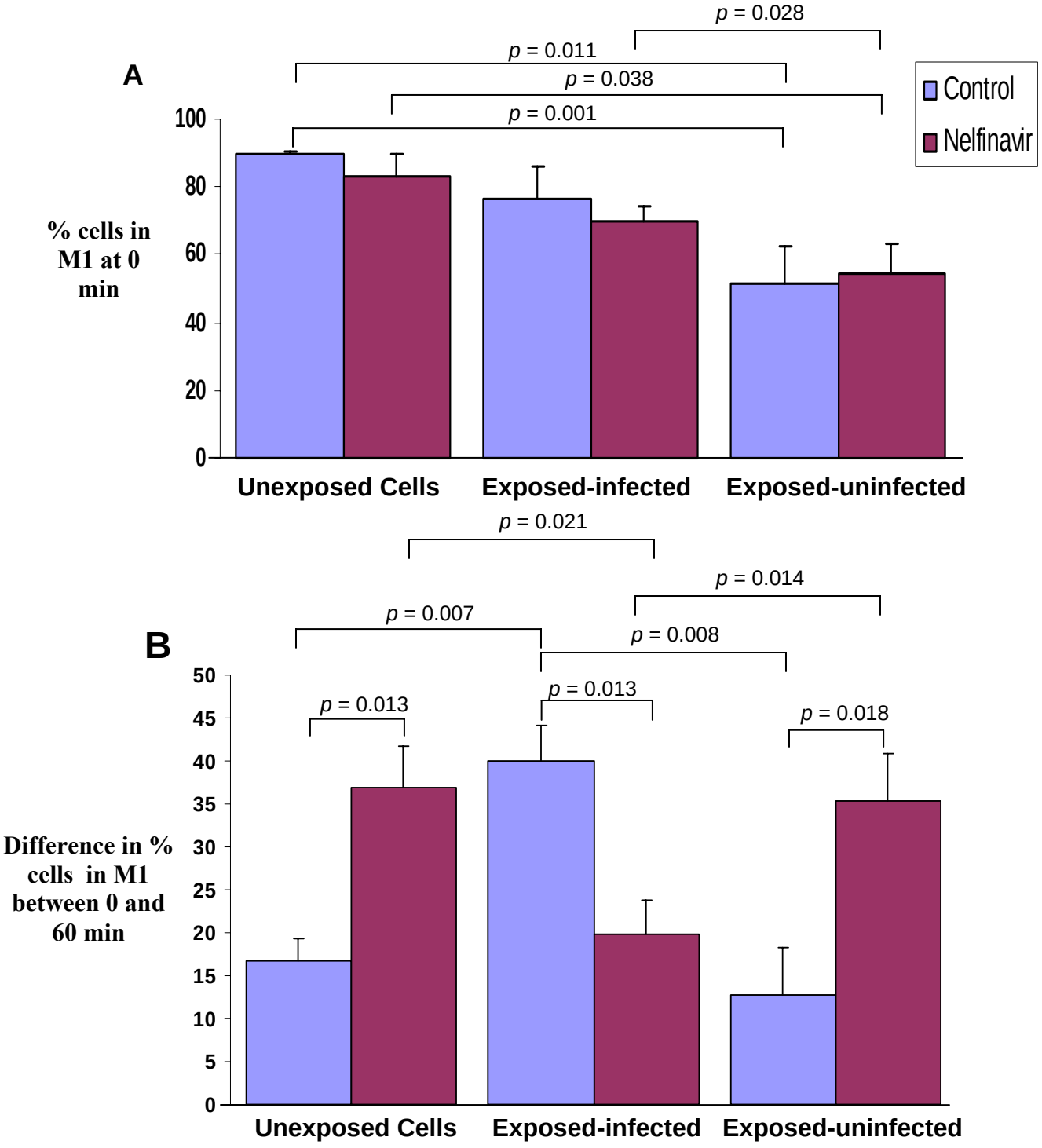

Figure 4.3.6: Rh123 loaded cells in the M1 region (fully loaded population) A: before efflux (Time = 0 min) and B: difference in the percentage of cells before and after efflux (Time = 60 min). n=3, treatments in duplicate.

We also analyzed the percentages of cells moving into M3, i.e. cells that had completely effluxed Rh123 (Figure 4.3.7). These results corresponded well with those obtained with M1 in Figure 4.3.6B, indicating that the loaded cells in M1 were moving into M3 due to effluxing all of the Rh123. There was, however, more variability present in the M3 comparisons, so some of the comparisons had nearly significant differences of just slightly above $p = 0.05$.

P-gp mRNA levels and P-gp functional activity were correlated (r = 0.931), indicating the induction of the transcription led to the increase in activity. In addition, an excellent correlation was observed between P-gp functional activity and PXR expression levels (r = 0.9870) (Figure 4.3.8), suggesting that PXR expression drives the induction of P-gp functional activity.

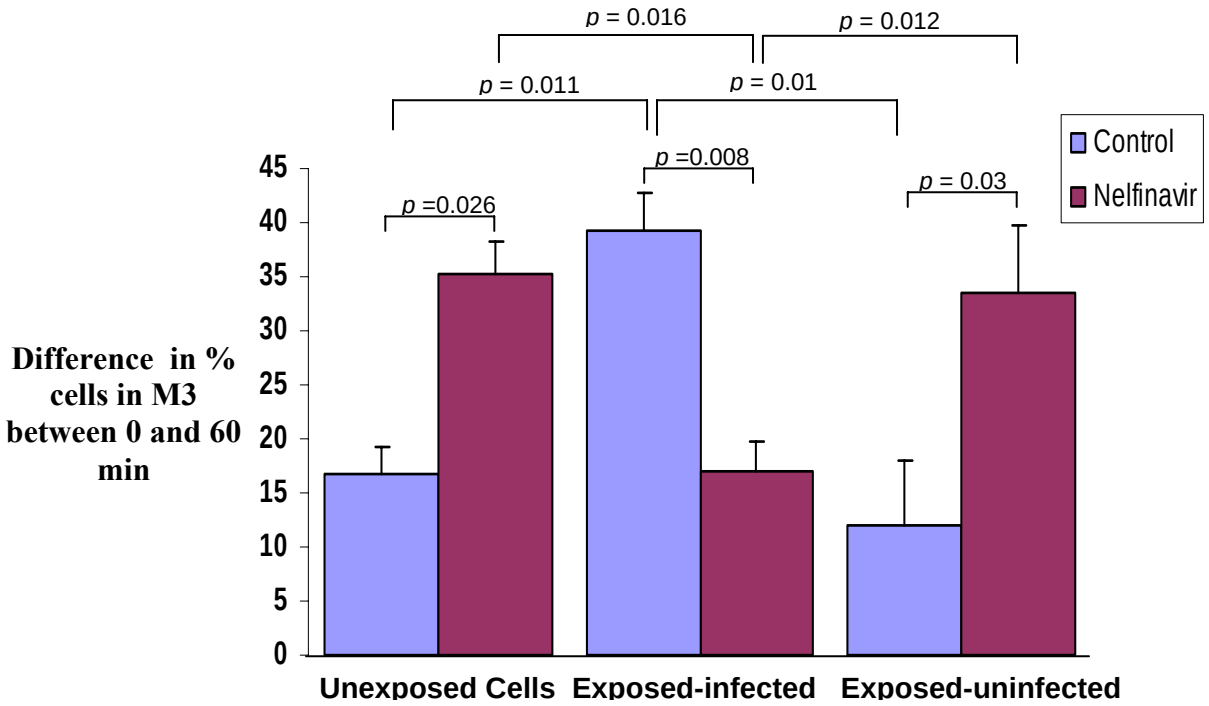

Figure 4.3.7: Difference in the percentage of $CD4^+$ T-cells in M3 at time = 0 min and time = 60 min in control and nelfinavir-treated cells. n=3, treatments in duplicate.

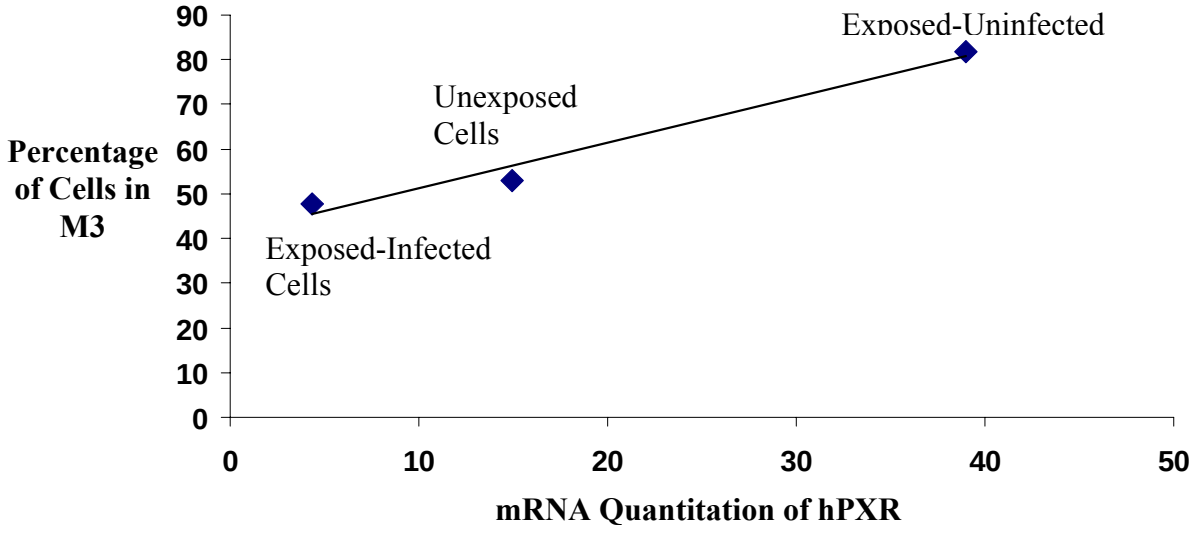

Figure 4.3.8: Correlation between P-gp percent of cells in M3 versus PXR expression treated with nelfinavir. n=3, treatments in duplicate. r=0.9870

5. DISCUSSION

Highly active antiretroviral therapy (HAART) consists of a combination of potent antiretroviral agents, two classes of which are protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). In spite of the undeniable clinical benefits obtained with HAART, between 20% and 50% of patients who initiate HAART present with virological failure during the first year of treatment and the incidence of failure increases rapidly during successive treatments (Casado *et al.*, 1998), (Deeks *et al.*, 1999), (Mocroft *et al.*, 2001), (Paredes *et al.*, 2000), (Smit *et al.*, 2002). Treatment failure may occur due to a multitude of reasons that may be patient-, virus-, or drug-related (Fletcher, 1999).

Preliminary studies in our laboratory and previous studies by others have shown that antiretroviral agents such as PIs and NNRTIs activate the hPXR and the hCAR, nuclear receptors that are known to upregulate the expression of several key DMTs. These agents are also known to induce CYP3A4 expression, resulting in serious clinically relevant drug-drug interactions (DDIs). CYP3A4 enzyme is the predominant form in adult liver and intestine. It also catalyzes the metabolism of more than half of all drugs, and represents the primary route of elimination for many drugs (Schuetz, 2004), which increases the potential for DDIs for drugs cleared via this pathway. However, several mechanistically and clinically important aspects of such inductive effects had remained unresolved. Firstly, the inductive effects of some of the agents such as the NNRTI efavirenz had not been previously studied. Secondly, a clear understanding of the comparative risk for DDIs between efavirenz and other PIs was not known. Thirdly, the effects of these agents on the other phase I and II metabolizing enzymes and drug transporters was not known. Fourthly, the inductive effects of antiretroviral agents on the extra-hepatic expression of DMTs, especially in the $CD4^+$ T-cells, had not been investigated. While hepatic and intestinal metabolism determines the overall systemic exposure to the antiretrovirals, the target-specific

intracellular drug concentration (in this case, in $CD4^+$ T-cells) may be the most clinically relevant drug levels. Finally, the influence of HIV infection on the activity and expression of DMTs in $CD4^+$ T-cells was not known. These gaps in our knowledge represent an important barrier to the safe and efficacious use of antiretroviral agents. This led us to the central hypothesis of our research that antiretroviral compounds, including efavirenz and PIs, induce hepatic and extra-hepatic drug metabolizing enzymes and drug transporters, and that the magnitude of induction is modulated by disease state.

In order to test our hypothesis, we employed primary cultures of human hepatocytes and human colon carcinoma cells LS174T, as clinically relevant models of hepatic and intestinal drug metabolism and transport. We observed that efavirenz and the PIs nelfinavir and ritonavir considerably influenced multiple clearance pathways. First, we observed efavirenz to be a potent inducer of CYP3A4 but a moderate inducer of P-gp. The PIs increased CYP3A4 mRNA levels without a correlative increase in CYP3A4 immunoreactive protein or activity. Both PIs were potent inducers of P-gp, with significant increases observed at concentrations as low as 0.5 μM. This concentration is lower than that observed with standard doses of the PIs and NNRTIs and probably represents a C_{min} plasma concentration. We noted that the three drugs also induced CYP2B6, UGT1A1, and UGT1A6 RNA, protein and functional activity. In comparison, nelfinavir was the only drug to increase UGT2B7 RNA and protein expression. This would suggest that drugs that are cleared primarily via these metabolic pathways are subject to potential DDIs when combined with the aforementioned PIs and efavirenz.

To discern the modulation of DMTs at the antiretroviral site of action we also conducted studies in primary $CD4^+$ T-cells. We observed transcriptional upregulation of all the key DMTs such as P-gp and multidrug resistance protein (MRP1) in $CD4^+$ T-cells in the presence of

efavirenz, ritonavir, and nelfinavir. All 3 of these drugs produced significant increases in P-gp transcription and function, while only nelfinavir and the control inducer rifampin increased MRP1 transcription. In contrast to the data obtained in primary human hepatocytes and LS174T cells, nelfinavir was observed to be the most potent P-gp inducer in $CD4^+$ T-cells, when compared to efavirenz, ritonavir and rifampin. In addition, we noted marked differences in the pattern of expression of hPXR in $CD4^+$ T-cells compared to primary hepatocytes.

Finally, we assessed the impact of HIV on the induction of P-gp in $CD4^+$ T-cells. Separation of exposed cells into exposed-infected and exposed-uninfected $CD4^+$ T-cells revealed a significant decrease in the P-gp activity of nelfinavir-treated exposed-infected cells. However, the control cells (DMSO 0.1%) had a higher P-gp activity in exposed-infected cells than the unexposed or exposed-uninfected cells.

A critical component of *in vitro* studies is to ensure that the results can be extrapolated to the *in vivo* scenario. Having determined which *in vitro* models to use (primary human hepatocytes, LS174T cells and primary lymphocytes), our next undertaking was to determine the most clinically relevant drug concentrations to use. From literature search we determined that all 3 antiretroviral agents are well absorbed after oral administration with efavirenz, nelfinavir and ritonavir having bioavailability (F) values of 60, 88 and 98%, respectively. Efavirenz after oral administration achieved C_{max} of 1.6 to 9.1 μM, 5 hours following single oral doses ranging from 100-1600 mg (Bristol Myers Squibb, 2002). In HIV-positive patients, oral administration of 600 mg daily had been reported to produce steady-state C_{max} of 12.9 ± 3.7 μM and steady-state trough drug concentrations (C_{min}) of 5.6 ± 3.2 μM (Bristol Myers Squibb, 2002), (Staszewski *et al.*, 1999). The observed C_{max} and C_{min} values following oral nelfinavir (1250 mg twice/thrice

daily) to healthy volunteers were ~7.0 μM and 2.1 μM (range 0.12 - 3.9 μM) (Maserati *et al.*, 1999), (Perry *et al.*, 2005). The therapeutic dosage of ritonavir is 600 mg twice daily or 400 mg twice daily. However, nowadays ritonavir is mainly used in combination with other PIs as a booster of these compounds. A low dose of ritonavir (100 or 200 mg), the so-called 'baby dose', results in nontherapeutic plasma concentrations of ritonavir. For therapeutic doses of ritonavir, significant correlations between antiviral activity and plasma drug concentrations have been demonstrated in various clinical studies in HIV-1-infected patients. A ritonavir C_{min} of 2.9 μM was extracted as a target for adequate viral suppression. After a dose of 600 mg twice daily the C_{max} obtained was 14.9 μM and the C_{min} was 4.9 μM (Abbott Laboratories, 1997), (Gatti *et al.*, 1999).

In addition to the plasma concentrations obtained following oral administration, it is also important to highlight the t½'s of these drugs and the distribution of these agents into peripheral compartments, which is dependent upon their volumes of distribution and their plasma protein binding potential. Efavirenz has a long half-life, ranging from 52 to 76 hours following single oral doses, and 40 to 55 hours following long-term administration as a result of auto-induction of efavirenz metabolism. In comparison the t½'s of nelfinavir and ritonavir are ~6 hrs. Furthermore, all 3 antiretrovirals are highly bound to α_1-acid glycoproteins (>90%) (Table 1.4.2) (Abbott Laboratories, 1997), (Agouron Pharmaceuticals Inc.), (Bristol Myers Squibb, 2002), (Kempf *et al.*, 1995), (Zhang *et al.*, 2001). The apparent volumes of distribution of efavirenz, nelfinavir and ritonavir are 252 L (Csajka *et al.*, 2003), 525L (Agouron Pharmaceuticals Inc.), and 40L (Abbott Laboratories, 1997), respectively, which indicates that most of the administered dose is present in peripheral compartments. Of particular importance to our studies in hepatocytes, is the high liver uptake of these agents, with the hepatic uptake ranging between ~6-

100-fold higher than that in serum (Dupin *et al.*, 2002), (Maserati *et al.*, 1999), (Salama *et al.*, 2005). Thus, given the high partitioning of the drugs into hepatocytes, despite high protein binding, it is reasonable to conclude that the concentration of drugs in human liver cells may in fact be greater than that used in our study. This further underscores the clinical relevance of our findings.

Prior to developing our hypothesis, we undertook studies to assess whether the antiretrovirals efavirenz, ritonavir and nelfinavir activate hPXR, which is involved in the regulation of several key DMTs. In addition, we also determined the expression of key transcription factors (hPXR, CAR and RXR) in hepatic and extrahepatic cells. hPXR ligands are known to activate key DMTs including; CYP3A4, CYP2B6, UGT1A1, UGT1A6, P-gp and MRP1. Limited data were available regarding the activation of PXR by antiretrovirals. Studies by Luo *et al.* (2002) conducted in HepG2 cells observed ritonavir to efficiently activate PXR as was observed in our studies. A study by Dussault *et al.* (2001) confirmed the results obtained by Luo *et al.* (2002), however their studies showed that nelfinavir does not activate PXR. A limitation of the study by Dussault *et al.* (2001) was that mouse PXR was used, which as we indicated earlier is only 76% homologous with hPXR. In addition, neither study utilized the PXR construct that contains the xenobiotic responsive enhancer module (XREM) present in our PXR construct. The XREM is a critical component of the PXR construct necessary for gene activation (Goodwin *et al.*, 1999), (Goodwin *et al.*, 2002).

In addition to the results we observed with ritonavir and nelfinavir we observed efavirenz to be a potent activator of hPXR both in HepG2 cells and in LS174T cells. The hPXR activation observed in LS174T cells was lower than that observed with HepG2 cells. This may be related

to a lack of or lower expression of transcription factors and co-activators present in these cells. Our results confirmed the significantly lower expression of hPXR and hCAR in LS174T cells.

Given that antiretroviral agents target intracellular sites in $CD4^+$ T-cells, we thought it prudent to also assess the expression of hPXR and hCAR in these cells. A previous study by Albermann *et al.* (2005) identified the expression of hPXR in PBMCs, although, no studies assessed hPXR or hCAR expression in $CD4^+$ T-cells specifically. We found that hPXR expression levels were significantly lower in $CD4^+$ T-cells than in hepatocytes or LS174T cells, which is possibly responsible for the lower expression levels of DMTs in $CD4^+$ T-cells as opposed to hepatic and intestinal expression and activity.

Following studies in which we assessed hPXR activation, we initially evaluated the potential of efavirenz, ritonavir and nelfinavir to induce CYP3A4 in primary cultures of human hepatocytes at clinically relevant concentrations (1-10 μM). It can be concluded from our results that efavirenz markedly induce, *CYP3A4*, resulting in a significant increase in the enzymatic activity. In contrast, ritonavir and nelfinavir significantly inhibit CYP3A4 enzymatic activity. Here, several observations are noteworthy. First, the CYP3A4 induction in human hepatocytes by efavirenz is comparable with other prototypical inducers such as hyperforin and paclitaxel (Moore *et al.*, 2000a), (Nallani *et al.*, 2004). Second, the observed magnitude of CYP3A4 induction with efavirenz was comparable to that of rifampin, one of the most potent CYP3A4 inducers in hepatocytes reported in the literature thus far. Thirdly, the CYP3A4 catalytic activity correlated well with both the protein and mRNA expression levels, suggesting that the antiretroviral drug-mediated regulation occurs at the transcriptional level. Fourthly, the correlative increase in mRNA, protein and catalytic activity was not observed in hepatocytes treated with ritonavir and nelfinavir. In fact, inhibition of CYP3A4 enzymatic activity observed

with ritonavir and nelfinavir was observed in the absence of these drugs, even though a 4-hour washout period was conducted following drug treatment. This appears to be consistent with the reported mechanism-based inhibition in which ritonavir covalently binds CYP3A4 protein, thus preventing an increase in CYP3A4 expression and activity without affecting the increase in mRNA expression (Koudriakova *et al.*, 1998).

To our knowledge, the influence of efavirenz and nelfinavir on human CYP3A4 expression is an original and novel contribution of our research and had not been reported until our undertaking. As reported in the introduction section, while there were reports on efavirenz-, ritonavir- and nelfinavir-mediated induction of DMTs, these studies were conducted employing only animal models. Induction of drug-metabolizing enzymes is known to exhibit interspecies variability. For example, rifampin is a potent inducer of CYP3A in rabbit and human liver, but is a poor inducer in rats (Lu and Li, 2001). Conversely, antiglucocortioid pregnenolone 16α-carbonitrile and glucocorticoid dexamethasone are potent inducers of CYP3A in rats and mice but not in human liver (Kocarek, 1995). The species-specific induction of CYP3A has been attributed in part to the differences in amino acid sequence in the ligand binding domain of PXR in different species. Across various species such as mice, rats, rabbits and humans, the (DNA-binding domain) DBD of PXR is well conserved with >96% amino acid sequence homology. However, the ligand binding domains of rat and human PXR exhibit 24% divergence, which results in species-specific differences in binding and activation of PXR. The inter-species variability in CYP3A induction poses a major limitation on the use of animals and animal tissues for the assessment of clinically relevant issues pertaining to CYP3A4 induction. Indeed, nelfinavir has been previously reported to be an inducer of CYP3A in rats but not in mice, suggesting that nelfinavir exhibits interspecies variability (Huang *et al.*, 2001), (Matheny *et al.*,

2004). Therefore, the observations made in these animal studies regarding nelfinavir-mediated CYP3A may not extrapolate to humans. In fact, the lack of such information has been indicated as a significant deficiency in publications that documented efavirenz, ritonavir and nelfinavir interactions with other antiretroviral drugs.

Primary human hepatocytes express the complete complement of drug-metabolizing enzymes and regulatory proteins present in the liver and provide reliable estimates of intrinsic hepatic clearance for drugs. Consequently, in recognition of such interspecies differences, the FDA recommends use of cultures of primary human hepatocytes as the most clinically relevant model to assess induction of hepatic drug metabolizing enzymes and transporters *in vitro*. Simultaneous use of a prototypical inducer such as rifampin, which is known to cause many CYP3A4 induction related drug interactions, can provide further insights into the potential of a test compound to cause clinically relevant interactions.

Similar to the results obtained in human hepatocytes, efavirenz induced CYP3A4 activity in LS174T cells in culture. Since LS174T is a transformed cell line derived from colon carcinoma, interpretation of the data must be done with caution. Unlike the use of primary human hepatocytes, *in vitro* models for intestinal drug metabolism are not established. Primary cultures of enterocytes may be used in short term cultures to study enzyme inhibition, but maintenance of their long term cultures is difficult due to potential contamination from brush border bacteria. This restriction requires the use of transformed cell lines and careful consideration of the experimental aims is needed for selecting the cell line. Several cell lines, such as Caco-2 and MDCKII that are widely utilized in the study of polarized intestinal transport of orally administered drugs, lack key pathways involved in the regulation of drug metabolism. For example, Caco-2 cells do not express PXR and, in some instances, CYP3A4 (Cummins *et*

al., 2004), (Pfrunder *et al.*, 2003) and thus these cells may not be a relevant model for studying hPXR-dependent enzyme induction.

With the above considerations of PXR expression and its previous use to study the regulation of drug metabolism and transport, we preferred to use LS174T cells for our studies (Pfrunder *et al.*, 2003), (Schuetz *et al.*, 1996). Furthermore, compounds that induce hepatic CYP3A4, such as rifampin, have shown marked induction of CYP3A4 in LS174T cells, which indicates that this model has potential to be clinically relevant. Accordingly, it appears that efavirenz does not exhibit tissue-dependent CYP3A4 induction as observed by Mouly *et al.* (2002). Several factors may have resulted in a lack of intestinal CYP3A4 induction following the administration of efavirenz 400mg daily in healthy volunteers. Firstly, the relatively short duration of exposure of the intestinal cells to efavirenz following oral administration. Given that efavirenz is fairly rapidly absorbed (reaching t_{max} of 2 to 3 hours) and that it is administered only once daily, and that no enterohepatic recirculation of the parent drug occurs, the total duration of exposure of the enterocyte to parent efavirenz during treatment may be insufficient to cause a significant increase in gene transcription. Secondly, the mechanism for induction of hepatic CYP3A4 in liver may involve novel mechanisms not present in the intestine. Thirdly, the techniques used to measure CYP activity and protein in the intestine may not be sensitive enough to measure any changes that may be present.

CYP2B6 is gaining more notoriety as significant polymorphic and ethnic differences have been observed in the expression of this enzyme and more drugs are recognized to be cleared via this pathway. These factors have had a major impact on the clearance of drugs. Efavirenz has been observed to be metabolized primarily (>90%) by CYP2B6 (Ward *et al.*, 2003). All 3 drugs, nelfinavir, efavirenz and ritonavir were observed to be inhibitors of CYP2B6 (Hesse *et al.*,

2001). There existed however, a paucity of information regarding the induction of CYP2B6 in humans by antiretroviral agents.

Mouly *et al.* (2002) attempted to assess the induction of CYP2B6 in intestinal tissue following the administration of 400 mg efavirenz daily to healthy volunteers. They did not find a change in CYP2B6 protein expression levels in enterocytes. This is probably due to the extremely low expression of CYP2B6 in the intestine given that in the liver where CYP expression is much higher, CYP2B6 makes up <1% of the total CYP expression. At the time they were not able to probe CYP2B6 activity in the liver due to the lack of a specific probe. In studies undertaken by Faucette *et al.* (2004) ritonavir 10 μM was observed to cause a 6-fold increase in CYP2B6 activity relative to control.

In the view that antiretrovirals such as AZT are metabolized by both CYP and UGTs, induction of UGTs by efavirenz, ritonavir and/or nelfinavir may also contribute to the observed reduced plasma levels in patients simultaneously treated with these antiretroviral drugs. There is, however, a paucity of data on the induction of UGT by HAART. Clinical studies do indicate that nelfinavir and ritonavir reduce the area under the plasma-concentration–time curve of AZT (a known UGT substrate) by 35 percent and 25 percent, respectively, presumably because of the induction of glucuronyl transferases (Abbott Laboratories, 1997), (Abbott Laboratories, 1997), (Flexner, 1998). In addition, a study undertaken by Rotger *et al.* (2005) showed a reduction in bilirubin levels in HIV-positive patients treated with efavirenz. This observation is consistent with the known activation of PXR which leads to the induction of UGT1A1. Since all three antiretroviral drugs upregulate hPXR, which appears to regulate a large number of DMTs including UGT1A1 and UGT1A6, we examined the potential induction of these genes by the antiretroviral drugs. In our studies, efavirenz, ritonavir and nelfinavir produced a comparable

dose-dependent increase in UGT1A activity with the following grade-scale; nelfinavir>rifampin>ritonavir>efavirenz. Our findings on rifampin are consistent with those obtained in other laboratories with the prototypical inducer, rifampin. For instance, Soars *et al.* (2004) observed ~1.5-fold increase in UGT1A activity in primary human hepatocytes treated with rifampin.

Our studies have built on the very limited data currently available on human UGT induction by examining the response of antiretrovirals at concentrations that have been historically used to investigate the regulation of expression of the P450s. This has allowed an initial induction profile for each UGT form to be determined at the enzyme activity level and has yielded results that suggest that the UGTs appear to respond to a lesser degree and with variability to inducers than do the P450s. Although few in number, several important drug interactions have been attributed to induction of UGT isoforms (Kiang *et al.*, 2005). Due to the abundance of these phase II enzymes, even moderate increases in UGT levels in fact will substantially increase the levels of these enzymes in the body. Thus, it is reasonable to assume that the observed increase in UGT activity may lead to clinically observed interactions.

Several manuscripts have highlighted the dynamic interplay between P-gp and CYP3A (Benet and Cummins, 2001), (Benet *et al.*, 1999), (Cummins *et al.*, 2002), (Wacher *et al.*, 1995). Previous studies have assessed the induction of P-gp by nelfinavir in different animal models. Studies undertaken by Huang *et al.* (2001) revealed nelfinavir to be an inducer of P-gp in rat intestine however Matheny *et al.* (2004) observed no change in the intestinal expression of P-gp. Interestingly, while we observed robust induction of P-gp by nelfinavir in hepatocytes, no induction of P-gp was observed in LS174T cells. The differential induction we observed between hepatocytes and LS174T cells was in contrast to the results observed by Huang *et al.*

(2001) in rats in which intestinal but not hepatic induction of P-gp was observed. These studies again highlight the interspecies differences observed when assessing the induction of DMTs which could be related.

Our observations with the induction of P-gp by efavirenz and ritonavir concur with those of other research groups, in which efavirenz was observed to be a modest inducer of P-gp. Ritonavir was observed to be a potent inducer in cell culture (Perloff *et al.*, 2000), (Stormer *et al.*, 2002). Interestingly, we observed robust induction of P-gp treated with ritonavir and nelfinavir at concentrations as low as 0.5 μM with only limited increase with an increase in the dose. This is extremely important in the case of ritonavir given that the current trend is the use of ritonavir at much lower doses to boost other PIs. It could therefore be argued that even though the dose may be subtherapeutic, induction of P-gp by ritonavir may occur which could limit the absorption of substrates of P-gp.

Following our observations in hepatocytes and LS174T cells which impact the plasma concentration of drugs, we determined whether exposure of antiretroviral drugs could alter DMT expression in $CD4^+$ T-cells. Induction of DMTs would likely alter the intracellular concentration of antiretroviral compounds that are known to be substrates of several DMTs. Information regarding the presence of phase I and phase II drug metabolizing enzymes and drug transporters in extrahepatic tissue (such as $CD4^+$ T-cells) beyond our study here is limited. Our observations concur with those of Nakamoto *et al.* (2000) who found that CYP3A4 mRNA level was increased by treatment with rifampin in PBMCs. In addition to the induction of phase I enzymes, we also observed a robust increase in phase II enzyme UGT mRNA expression which was similar to that observed by Hu and Wells (2004). As was the case in previous studies, we

were unable to measure a change in phase I or phase II protein expression and functional activity in $CD4^+$ T-cells. This is most likely due to the low expression of these enzymes below the limit of detection of the currently available assays.

We focused our attention on transporters because several reports had indicated the expression of these transporters on PBMCs (Chandler *et al.*, 2003), (Ford *et al.*, 2004), (Ford *et al.*, 2004), (Janneh *et al.*, 2005), (Owen *et al.*, 2004). A correlation between the expression of transporters and the intracellular concentration of substrate drugs had also been reported (Chandler *et al.*, 2003), (Meaden *et al.*, 2002). Further analysis of the literature revealed that efavirenz, ritonavir, and nelfinavir are lipophilic drugs that primarily pass into cells by passive diffusion and are therefore not significantly influenced by the expression of influx transporters (Table 1.2) (Ford *et al.*, 2004). This was confirmed in studies by Almond *et al.* (2005) in which the intracellular concentration of efavirenz was highly associated with the percentage of protein bound. Studies by Maffeo *et al.* (2004) observed a decrease in the intracellular concentration of saquinavir which correlated well with an increase in P-gp expression. Similarly, Jones *et al.* (2001) observed a decrease in intracellular PI concentration with increased MRP1 expression. Reduced intracellular drug concentrations have a major impact on viral replication, and may lead to the development of sanctuary sites and accelerated viral resistance. Both of these studies were conducted in CEM cells ($CD4^+$ T-cell line) overexpressing either MRP1 or P-gp. As indicated previously, the use of primary cells is preferred over transformed cell lines since the primary cells are more likely to show effects that would be seen *in vivo*.

BCRP may play an important role in the transport of some antiretroviral drugs, since it appears to be expressed on lymphocytes to a similar extent as P-gp, and confers cellular resistance to AZT (Taipalensuu *et al.*, 2001), (Wang *et al.*, 2003b). However, relationships

between antiretroviral drug accumulation and BCRP expression remain to be determined *in vitro*. Our studies indicated that all 3 antiretroviral drugs tested led to lower levels of BCRP mRNA. Previous studies by Gupta *et al.* (2004) revealed PIs to be inhibitors but not substrates of BCRP. Because BCRP participates in cellular detoxification, it may have been thought to be regulated by nuclear receptors that upregulate genes involved in cellular detoxification. However, BCRP expression is only minimally affected by hPXR or hCAR activators such as rifampin, as was observed in our studies. Therefore, although BCRP is localized to the plasma membrane and apical surfaces of polarized cells, it appears unlikely to be activated by hPXR and hCAR as part of a cellular detoxification cascade (Krishnamurthy and Schuetz, 2006).

Meaden *et al.* (2002) found patients with lower MRP1 protein expression in PBMCs, had a significantly higher accumulation of both ritonavir and saquinavir than those with higher MRP1 expression. They concluded that increased expression of MRP1 protein on lymphocytes was associated with lower intracellular accumulation of saquinavir and ritonavir. These results were confirmed by Janneh *et al.* (2005) in experiments where the transport and accumulation of ^{3}[H]-saquinavir were measured in PBMC in the absence or presence of specific and non-specific inhibitors of MRP1. They also found that the expression of MRP1 mRNA was important in the intracellular accumulation of saquinavir in PBMCs. These studies confirm the importance of efflux transporters in the clinical outcome following the administration of antiretroviral agents. As mentioned above, subtherapeutic intracellular concentrations of these agents could result in the development of sanctuary sites which could lead to drug resistance. Limited data existed regarding the induction of MRP1 by antiretroviral agents, specifically in $CD4^{+}$ T-cells. A study by Perloff *et al.* (2001a) found that ritonavir induced MRP1 expression in LS180 cells (colon carcinoma cell line). These studies are the first to assess the induction of MRP1 by antiretroviral

drugs in $CD4^+$ T-cells. While we observed an increase in MRP1 mRNA expression in $CD4^+$ T-cells treated with rifampin and nelfinavir, there was no corresponding increase in functional activity, which may be related to a lack of change in the normal expression levels of MRP1.

As mentioned above, P-gp is likely to be a determinant of the success of antiretroviral therapy because adequate local intracellular drug concentrations (e.g. in $CD4^+$ T-cells) are required for therapeutic effectiveness (Kim, 2003). Previous studies have confirmed the expression of P-gp in PBMCs (Albermann *et al.*, 2005), (Lucia *et al.*, 2002), (Meaden *et al.*, 2002) with a limited number of studies assessing the induction of P-gp by antiretroviral agents in $CD4^+$ T-cells. Chandler *et al.* (2003) studied the induction of P-gp in CEM $CD4^+$ T-cells with PI and NNRTI treatment. They observed that nelfinavir was the only PI and efavirenz was the only NNRTI to increase P-gp expression. Similarly, Ford *et al.* (2003) undertook experiments to assess the effect of PI-containing regimens on P-gp expression in primary PBMCs from healthy volunteers. Their studies, which did not include efavirenz, also found an increase in P-gp expression of cells treated with nelfinavir. A limitation of both of these studies was that they did not conduct functional activity assays. In our studies, we focused on $CD4^+$ T-cells within the PBMCs, and treated the cells with efavirenz, ritonavir, and nelfinavir; in addition, we were able to assess the rate of efflux by P-gp using Rh123. We found that nelfinavir and efavirenz, as well as ritonavir, treatment led to the high P-gp mRNA levels and to efficient efflux of Rh123 out of the cells. Through our functional assay, we were able to identify the population of $CD4^+$ T-cells which completely effluxed Rh123 out of the cells suggesting an increase in expression of P-gp in those cells. Our studies identified nelfinavir as the most potent inducer of P-gp of the three antiretroviral drugs tested. This suggests that a drug that was co-administered with nelfinavir,

such as AZT, would be effluxed out of a population of $CD4^+$ T-cells prior to its' activation and inhibitory effect on reverse transcriptase.

Based on the results obtained in Aim 2, our studies to assess the impact of HIV infection on DMTs focused specifically on P-gp and only on $CD4^+$ T-cells treated with nelfinavir. We observed a very slight reduction in P-gp mRNA levels in HIV-exposed cells as opposed to HIV-unexposed cells, and no significant differences in P-gp functional activity with or without nelfinavir treatment. A study by Owen et al. (2004) observed a significant correlation between hPXR expression and P-gp expression in PBMCs. Accordingly, we expected to observe only a slight change in hPXR expression levels between the exposed and unexposed $CD4^+$ T-cells. However, hPXR transcript levels were substantially lower in the cells incubated with HIV. This large change in hPXR expression could have been due to unusual hPXR expression in this individual or it could have been related to an alteration in the ability of infected $CD4^+$ T-cells to express hPXR. Alternatively, there could be differences in hPXR expression within different $CD4^+$ T-cells in the population (based on HIV infection or CD4 cell subset), such that the total expression of P-gp and hPXR do not reflect differences in the individual cells. For this reason, we also evaluated P-gp and hPXR in cells separated by whether they were HIV infected or not. Differences between subsets of $CD4^+$ T-cells were not evaluated due to a lack of knowledge of the markers that could separate the different cell populations.

As mentioned in the results, the amount of HIV that was used in the infections was not sufficient for all of the cells to become infected with the 3 days of incubation. Therefore, there were cells in the sample that were exposed to HIV proteins but remained uninfected, while other cells were infected with HIV and had begun producing HIV proteins. Previous studies have

shown that the presence of viral proteins has an effect on the expression of efflux transporters such as MRP1 and P-gp (Hayashi *et al.*, 2005a), (Hayashi *et al.*, 2005b). Hayashi et al. (Hayashi *et al.*, 2005b) observed an increase in the expression and functional activity of P-gp in the presence of a viral regulatory protein, Tat. To separate the infected and uninfected cells within the HIV-exposed cells, we used a monoclonal antibody against one of the HIV envelope proteins that is expressed on the surface of infected cells. We observed that cells infected by HIV in the absence of nelfinavir had higher P-gp functional activity than unexposed cells, which could be due to increased oxidative stress that may have resulted in activation of the nuclear factor κB (NF- κB) and a subsequent increase in P-gp expression (Dixit *et al.*, 2005b), (Schreck *et al.*, 1991). When comparisons were made between cells treated with nelfinavir and untreated cells, we found a reduction in P-gp functional activity in HIV-exposed-infected cells with nelfinavir treatment (Figure 4.3.4). The exposed but uninfected cells were more similar to the unexposed cells, in that nelfinavir increased P-gp functional activity. The reduction in efflux with HIV infection plus nelfinavir was surprising given our observation that HIV infection alone led to higher efflux. Three possible explanations for the reduction in efflux in HIV-infected, nelfinavir-treated cells are (i) these cells had lower expression of PXR, (ii) the additional stress of drug treatment led to reduced P-gp functional activity, and (iii) an HIV protein could be influencing the effect of nelfinavir in the cells. Nonetheless, reduced P-gp function in HIV-infected cells within an individual treated with nelfinavir would result in higher intracellular concentrations of the antiretroviral drugs that are substrates of P-gp, which would help limit further HIV replication.

In comparison, the cells that were exposed to HIV but remained uninfected had essentially the same levels of P-gp functional activity as unexposed cells, with a boost in activity

with nelfinavir treatment. These results suggest that in patients treated with nelfinavir, the $CD4^+$ T-cells that remain uninfected will efficiently efflux the antiretroviral drugs that are substrates of P-gp, including AZT and the protease inhibitors. Since the protease inhibitors are needed after infection occurs, increased P-gp function in uninfected cells is not likely to be harmful. However, AZT functions immediately after virus entry and is needed at higher concentrations prior to infection, so lower intracellular concentrations of AZT and other reverse transcriptase inhibitors would increase the possibility that these as yet uninfected cells could become infected. Our results also confirmed the regulatory influence PXR-expression has on P-gp expression and function, which was observed by other research groups (Albermann *et al.*, 2005). The observed changes in PXR expression may be related to disruption of the DNA structure during the incorporation of the viral DNA into the host genome. This process may be occurring at a site which may disrupt the expression of PXR in infected cells.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Significant advances have been made in assessing the induction of DMTs as a causative factor in drug-drug interactions. This includes the availability and the techniques for the use of human tissues which most closely simulate the *in vivo* scenario. Given the "life-saving" status afforded to antiretroviral drugs, timely development and approval of these agents is critical. Although highly active antiretroviral therapy (HAART) has had a major impact in the management of HIV/AIDS, the induction of DMTs remains largely unexplored. The doctoral dissertation work presented here has made several major contributions in this regard. Firstly, prior to the development of our hypothesis, we assessed the activation of pregnane-X receptor (PXR), the "master" regulator of several key DMTs, by antiretroviral drugs. We also determined the expression of this transcription factor and constitutive androstane receptor in both hepatic and extra-hepatic tissues. We observed an increase in hPXR activation by all 3 drugs and expression of hPXR in hepatic and extra-hepatic tissues, albeit at significantly different levels.

Next, employing primary human hepatocytes and human-specific methodologies, we showed efavirenz to be a potent inducer of CYP3A4 and a mild/moderate inducer of P-gp. This was converse to the results observed with the protease inhibitors (PIs). Even at low doses ritonavir and nelfinavir were observed to potently induce P-gp with ritonavir being more potent a P-gp inducer than nelfinavir. Interestingly, our findings in $CD4^+$ T-cells revealed nelfinavir and efavirenz to be more potent inducers of P-gp than ritonavir or rifampin. When we separated the cells into different populations based upon Rh123 efflux potential we observed nelfinavir to most efficiently efflux all of the Rh123 out, suggesting a significant induction of P-gp. Our studies were conducted in PBMCs obtained from the blood of healthy volunteers. The trends observed from our results in $CD4^+$ T-cells suggest that a similar phenomenon could be occurring in HIV-positive patients being maintained with drug regimens consisting of nelfinavir and efavirenz.

Thus, the next step from the results obtained in our studies would be to isolate PBMCs from HIV-positive patients being administered the aforementioned agents and to utilize the techniques used in our studies to assess the induction of P-gp in their blood. In addition, given that antiretrovirals are administered as combination therapy, it may be prudent to treat PBMCs with multiple drugs *in vitro* (which we have not yet undertaken in our laboratory) to observe any changes that may occur when a combination of drugs are used. Another important study will be to relate the intracellular concentration of a P-gp substrate drug (such AZT) to the therapeutic outcome when combined with a drug like nelfinavir. It is also important to note that nelfinavir itself is a substrate of P-gp and may therefore limit its own intracellular concentration.

The last part of our study in which we assessed the impact of HIV exposure on the induction of DMTs revealed interesting new findings that require further exploration. It recently became evident that the presence of viral proteins resulted in the increased expression of efflux transporters such as P-gp (Hayashi *et al.*, 2005b) . Thus, even if cells are not infected with HIV, the presence of the virus may modulate P-gp. Using an HIV-specific monoclonal antibody we were able to separate cells into exposed-infected and exposed-uninfected cells. We were therefore able to compare the results between: (i) HIV-exposed and unexposed cells, (ii) exposed-infected and exposed-uninfected cells, and (iii) nelfinavir-treated and vehicle-treated control cells. Of particular interest to us was the finding that nelfinavir-treated exposed-uninfected cells had a significant increase in P-gp mRNA expression. This could have dire consequences in the clinical setting, increasing the potential infection of uninfected cells. The experiments should be repeated using PBMCs from patients who are managed with a regimen that includes nelfinavir. The PBMCs obtained from these individuals should be separated using the techniques that we utilized in our studies and P-gp mRNA expression and functional activity

should be assessed in these cells. In order to make comparisons to unexposed PBMCs, blood should be obtained from healthy volunteers.

Converse to the results obtained with exposed-uninfected cells we observed a decrease in P-gp functional activity in nelfinavir-treated exposed-infected cells, and an increase in the DMSO-control cells. Further studies need to be undertaken in this group of cells to determine which viral protein is the likely cause of the aforementioned effects.

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