MECHANISMS AND QUANTITATIVE PREDICTION OF EFAVIRENZ METABOLISM, PHARMACOGENETICS AND DRUG INTERACTIONS

Cong Xu

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Pharmacology and Toxicology,
Indiana University

August 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

Doctoral Committee

_____

Sherry F. Queener, Ph.D., Chair

_____

Zeruesenay Desta, Ph.D.

_____

Lang Li, Ph.D

_____

David R. Jones, Ph.D.

March 28, 2013

_____

Jian-Ting Zhang, Ph.D.

DEDICATION

This work is dedicated to my thesis mentor, Dr. Zeruesenay Desta and my friends and family, especially my parents, Zhangsun Xu and Wanling Chai.

ACKNOWLEDGEMENTS

I would not have completed my Ph.D. dissertation without the tremendous support and guidance of many people who have influenced my life and shaped my experience both personally and professionally.

First and foremost, I would like to sincerely thank my family. To my mother, Wanling, and father, Zhangsun, who have always supported me to pursue my career interests and encouraged me during periods of struggles.

I especially would like to thank my mentor, Dr. Zeruesenay Desta. I sincerely appreciate that Dr. Desta granted me the opportunity to grow into a mature professional. Dr. Desta introduced me into the arena of clinical pharmacology by his favorite efavirenz project. He provided expert assistance during periods of trouble shooting, gave freedom to develop an independent research project, allowed me to cultivate collaboration project and helped improving my scientific writing and presentation skills as an international student. I would like to particularly thank for his support to my internship in Pfizer Inc. during the summer of 2012. I feel extremely fortunate to have Dr. Desta as my Ph.D. mentor, who helped me to grow not only scientifically but also mentally during my graduate training.

I would also like to thank the members of my thesis committee, Dr. Sherry Queener, Dr. Jianting Zhang, Dr. David Jones and Dr. Lang Li for their guidance, suggestions, and constructive critiques.

Besides my committee, I would like to thank the faculty and staff of the Clinical Pharmacology division and department of Pharmacology and Toxicology for all their guidance and help. I would like to acknowledge Dr. David Flockhart for his generous

support for me to take additional scientific trainings, e.g. online classes and scientific conferences. I would like to acknowledge Dr. David Jones who generously shared his resources and experiences of microsomal incubation and mass spectrometry quantification. I would like to acknowledge Dr. Lang Li and Dr. Sara Quinney for their guidance and support to learn Simcyp and apply it in my dissertation project. I would like to acknowledge the significant help from Evan Ogburn, a former lab technician of my laboratory. My experimental part of dissertation would not have been made possible without him. I would also like to acknowledge Jessica Lu and Yvonne Kreutz for their support as lab colleagues and friends.

ABSTRACT

Cong Xu

Mechanisms and quantitative prediction of efavirenz metabolism, pharmacogenetics and drug interactions

The antiretroviral drug efavirenz remains a cornerstone for treatment-naïve HIV patients. Subsequent to the demonstration that efavirenz is a substrate of cytochrome P450 (CYP) 2B6, a number of clinical studies found that the *CYP2B6*6* allele is significantly associated with higher efavirenz exposure and/or adverse reactions. However, the mechanism of reduced efavirenz metabolism by this genetic variant is not fully understood and whether this variant exhibits differential susceptibility to metabolic inhibition is also unknown. Ths use of efavirenz is further complicated by the drug interactions associated with it. Therefore, I hypothezised that 1) the *CYP2B6*6* allele reduces efavirenz metabolism by altering catalytic properties of CYP2B6; 2) efavirenz alters the pharmacokinetics of co-administered drugs by inhibiting drug metabolizing enzymes. A series of studies was carried out in hepatic microsomal preparations to determine the functional consequences of the *CYP2B6*6* allele and to assess inhibition potency of efavirenz on 8 CYPs. The major findings for these studies include: 1) the *CYP2B6*6* allele reduces efavirenz metabolism by decreasing substrate binding and catalytic efficiency; 2) functional consequences of the *CYP2B6*6* allele appear to be substrate- and cytochrome b5-dependent; 3) the *CYP2B6*6* allele confers increased susceptibility to metabolic inhibition; and 4) efavirenz inhibits the activities of CYP2B6, 2C8, 2C9 and 2C19 at therapeutically relevant concentrations. In addition, I explored the hypothesis that the incorporation of *in vitro* mechanism by which the *CYP2B6*6* allele

reduced efavirenz metabolism predicts the genetic effect of this allele on efavirenz clearance after a single oral dose by modeling approach. A pharmacogenetics-based *in vitro-in vivo* extrapolation (IVIVE) model was developed to predict human efavirenz clearance. Taken together, results from this dissertation provide new mechanistic information on how the *CYP2B6*6* allale alters substrate metabolism and drug interactions; demonstrate new mechanisms of efavirenz-mediated inhibition interactions; and demonstrate the utility of a pharmacogenetics-based predictive model that can serve as a basis for future studies with efavirenz and other CYP2B6 substrates. Overall these data provide improved understanding of genetic and non-genetic determinant of efavirenz disposition and drug interactions associated with it.

Sherry F. Queener, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HIV	human immunodeficiency virus
AIDS	acquired immunodeficiency syndrome
HTLV	human T-lymphotropic virus
LAV	lymphadenopathy-associated virus
RTI	reverse transcriptase inhibitor
InSTI	integrase inhibitor
PI	protease inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse-transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
HAART	highly active antiretroviral therapy
ACTG	AIDS Clinical Trial Group
FDA	Food and Drug Administration
CYP	cytochrome P450
GI	Gastrointestinal
hCAR	human constitutive androstane receptor
hPXR	human pregnane X receptor
PBREM	phenobarbital-responsive enhancer module
RXR	retinoid X receptor
XREM	xenobiotic-responsive enhancer module
PBMCs	peripheral blood mononuclear cells
ABC	ATP-binding cassette

POR	P450 oxidoreductase
HLMs	human liver microsomes
Cyt b5	cytochrome b5
HPLC	high performance liquid chromatography
LC/MS/MS	liquid chromatography/tandem mass spectrometry
EFV	Efavirenz
7-OHEFV	7-hydroxyefavirenz
8-OHEFV	8-hydroxyefavirenz
BUP	Bupropion
SNP	single nucleotide polymorphism
K_m	Michaelis-Menten constant
V_{max}	maximum enzyme velocity
PBPK	physiologically-based pharmacokinetic model
IVIVE	*in vitro-in vitro* extrapolation
AUC	area under the plasma concentration-time curve
EM	extensive metabolizer
PM	poor metabolizer
ISEF	inter-system extrapolation factor

Chapter I: *Introduction*

1. HIV epidemic and anti-HIV therapy

The world first became aware of acquired immunodeficiency syndrome (AIDS), a disease of the human immune system, in 1981. A growing number of homosexual patients and injection drug users were developing rare types of opportunistic infections, Kaposi's sarcoma, and pneumonia in New York and California (Weiss, 2008). In 1983, scientists have established the causative organism from AIDS patients when two separate research groups independently isolated a novel retrovirus from AIDS patients named human T-lymphotropic viruses-III (HTLVs-III) (Gallo et al., 1983) and lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983), respectively. These two retroviruses were the same (Ratner et al., 1985) and were renamed human immunodeficiency virus infection (HIV) by the International Committee on Taxonomy of Viruses in 1986. Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is the virus that causes the majority of HIV infections globally (De Cock et al., 1993; Marlink et al., 1994).

Following its recognition in 1981, the HIV epidemic has evolved to become a great challenge in global health. In 2011, more than 34.2 million people were infected with HIV, and more than 25 million lives lost over the last 30 years (World Health Organization, July 2012). The most affected region of the worldis sub-Saharan African, where over 60% of HIV patients are living (World Health Organization, July 2012).

HIV primarily infects $CD4^+$ T cells, macrophages, and dendritic cells (Wu and KewalRamani, 2006; Carter and Ehrlich, 2008). HIV begins its life cycle when it binds to a CD4 receptor and one of two co-receptors (CCR5 and CXCR4) on the surface of target

cells (Alkhatib et al., 1996; Feng et al., 1996) (Figure 1.1). Then the virus fuses with the host cell and viral RNA and proteins are injected into cell. An HIV enzyme called reverse transcriptase converts the single-stranded HIV RNA to double-stranded HIV DNA. The newly formed HIV DNA enters the host cell's nucleus, and is integrated into the host cell's own DNA by integrase to use the host cells' genetic machinery to produce new virus. The integrated HIV DNA is called provirus and may remain inactive for several years. When the host cell receives a signal to become active, the provirus uses a host enzyme called RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins. The newly assembled virus pushes out ("buds") from the host cell. During budding, the new virus steals part of the cell's outer envelope. This envelope, which acts as a covering, is studded with protein/sugar combinations called HIV glycoproteins. These HIV glycoproteins are necessary for the virus to bind CD4 and co- receptors. The final step of HIV life cycle is maturation of virion, where the polyprotein is cut into proper size by protease, and the new copies of HIV can now move on to infect other cells (Debouck et al., 1987; Kohl et al., 1988).

Zidovudine, nucleoside reverse transcriptase inhibitors (NRTIs), was the first anti-HIV agent approved by the Food and Drug Administration (FDA) in 1987. This drug prevents HIV-1 replication by competitively inhibiting RT, leading to chain termination of the viral DNA. Despite the initial high hopes of zidovudine therapy, physicians have quickly realized that its use as a single agent (monotherapy) resulted in rapid emergence of viral drug resistance and treatment failure. In 1991, didonasine, another NRTI, was developed and dual NRTI therapy was tested, no therapeutic breakthrough could be

achieved and HIV treatment remained unsuccessful. By 1993, HIV-1 became the number one killer of the USA young population. Thus, developing new drugs and refining combination regimens that are safe and effective continues to this date. However, the demonstration, in 1994, that zidovudine monotherapy during pregnancy prevents HIV-1 transmission to the neonates (decrease from 25.5 to 8.3%) remained a landmark therapeutics approach (Connor et al., 1994). As improved understanding of the molecular biology of the virus is achieved, additional drug targets, including viral protease (the enzyme responsible for the maturation of viral particles to infectious virons ready to infect new host cells), were identified. Through advanced drug discovery processes, potent protease inhibitors that bind to the active site of the enzyme where protein cleavage occurs were synthesized and tested for efficacy. By 1995, there was preliminary understanding that combination of PIs with NRTIs shows clinical efficacy, and this has paved the way for the start of the era of a three-drug combination in 1996, also known as the highly active antiretroviral therapy (HAART). By 1997, there were convincing clinical data regarding the effectiveness of HAART (Gulick et al., 1997; Hammer et al., 1997). HAART was quickly incorporated into clinical practice and rapidly showed significant decrease in the incidence, death and hospitalization rate caused by AIDS (Palella et al., 1998). Despite certain intial limitations of HAART in terms of adverse effects, pill burden or development of resistant virus, HAART represented landmark treatment approach for this otherwise deadly disease because these drug combinations significantly decreased incidence of HIV-1 and mortality from AIDS.

Currently, 26 antiretroviral drugs are approved by the FDA that targets different steps of the HIV life cycle: fusion inhibitors, co-receptor inhibitors, reverse transcriptase

inhibitors (RTIs), integrase inhibitors (InSTI) and protease inhibitors (PIs). There are three subgroups under RTIs: nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse-transcriptase inhibitors (NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs compose the first class of antiretroviral drugs developed. They are analogues of the naturally occurring deoxynucleotides and compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. In order to be incorporated into the viral DNA, NRTIs must be activated in the cell by the addition of three phosphate groups to their deoxyribose moiety, to form NRTI triphosphates. Since NRTIs lack a 3'-OH moiety in the ribose ring, this incorporation results in the termination of transcription. The mode of action of NtRTIs is essentially the same to NRTIs. All NRTIs and NtRTIs are classified as competitive inhibitors. In contrast, NNRTIs inhibit reverse transcriptase by a completely different mechanism, which bind to a specific pocket binding site within the HIV-1 reverse transcriptase distinct from the catalytic site (Broder, 2010). The availability of these drugs has significantly contributed to refine drug combinations of HAART in terms of improved efficacy, safety and adheremce. As a result, HIV-1 infection has now become a chronic illness in patients as long as continued treatment access and long-term adherence is ensured.

All adults with HIV infection should be offered antiretroviral therapy (ART) regardless of CD4 cell count, based on recent observational cohort data that all patients may benefit from ART and data from a randomized controlled trial showing that ART reduces the likelihood of HIV transmission while providing clinical benefit to treated individuals (Cohen et al., 2011b). Recommended initial regimens include 2 NRTIs

(tenofovir/emtricitabine or abacavir/lamivudine) plus a NNRTI (efavirenz), a ritonavir-boosted PIs (atazanavir or darunavir), or an integrase strand transfer inhibitor (raltegravir) or rarely, an agent that blocks the CCR5 (Thompson et al., 2012). Suppression of plasma HIV-1 RNA to less than 50 copies/ml by 24 weeks should occur with effective therapy, regardless of prior treatment experience (Thompson et al., 2012).

The introduction of HAART in clinics has dramatically decreased morbidities and mortalities of AIDS (Palella et al., 1998; Porter et al., 2003; Broder, 2010). However, the effective and safe use of HAART is compromised by the wide intra and interindividual variability observed both in responses to therapy and in the adverse effects of certain antiretroviral drugs. This variability can be attributed to factors that regulate the availability of drugs (pharmacokinetics), effects on the host (pharmacodynamics), and the activity of the virus itself (viral pharmacodynamics), including adherence, drug resistance, tolerability, co-morbidity and concomitant treatment (Michaud et al., 2012a). Adherence is a major predictor of the success of HAART, with higher adherence rates leading to a lower risk of viral rebound and resistance development (Maggiolo et al., 2007). The complexity of the treatment regimen is an important barrier to good adherence and patients generally prefer the simplicity of once-daily regimen (Maggiolo, 2009). The effectiveness of therapy is also affected by potency of viral suppression and more importantly the viral sensitivity to a drug. HIV has very high genetic variability coupled with a high mutation rate, thereby enabling the virus to easily adapt. The consequences of drug resistance include treatment failure, the need to start more costly second-line treatment for patients, and the spread of resistant strains of HIV. Tolerability is another important factor that affects adherence. Different categories of anti-retroviral agents are

associated with different toxicity profiles. NRTIs are more often associated with lactic acidosis, hepatic steatosis and lipodystropy, while NNRTIs are more likely associated with rash, neuropsychiatric symptoms and hepatotoxicity. All PIs are associated with metabolic abnormalities, dyslipidemia, hyperglycemia, insulin resistance, and lipodystrophy (Thompson et al., 2012). Co-morbid conditions such as metabolic disorders (diabetes mellitus), malignancies and renal, hepatic or cardiac dysfunctions may also interfere with the drug metabolism and/or require treatments that may interact with antiretroviral drugs. As a consequence, management of HAART should take into account together with all the aforementioned factors.

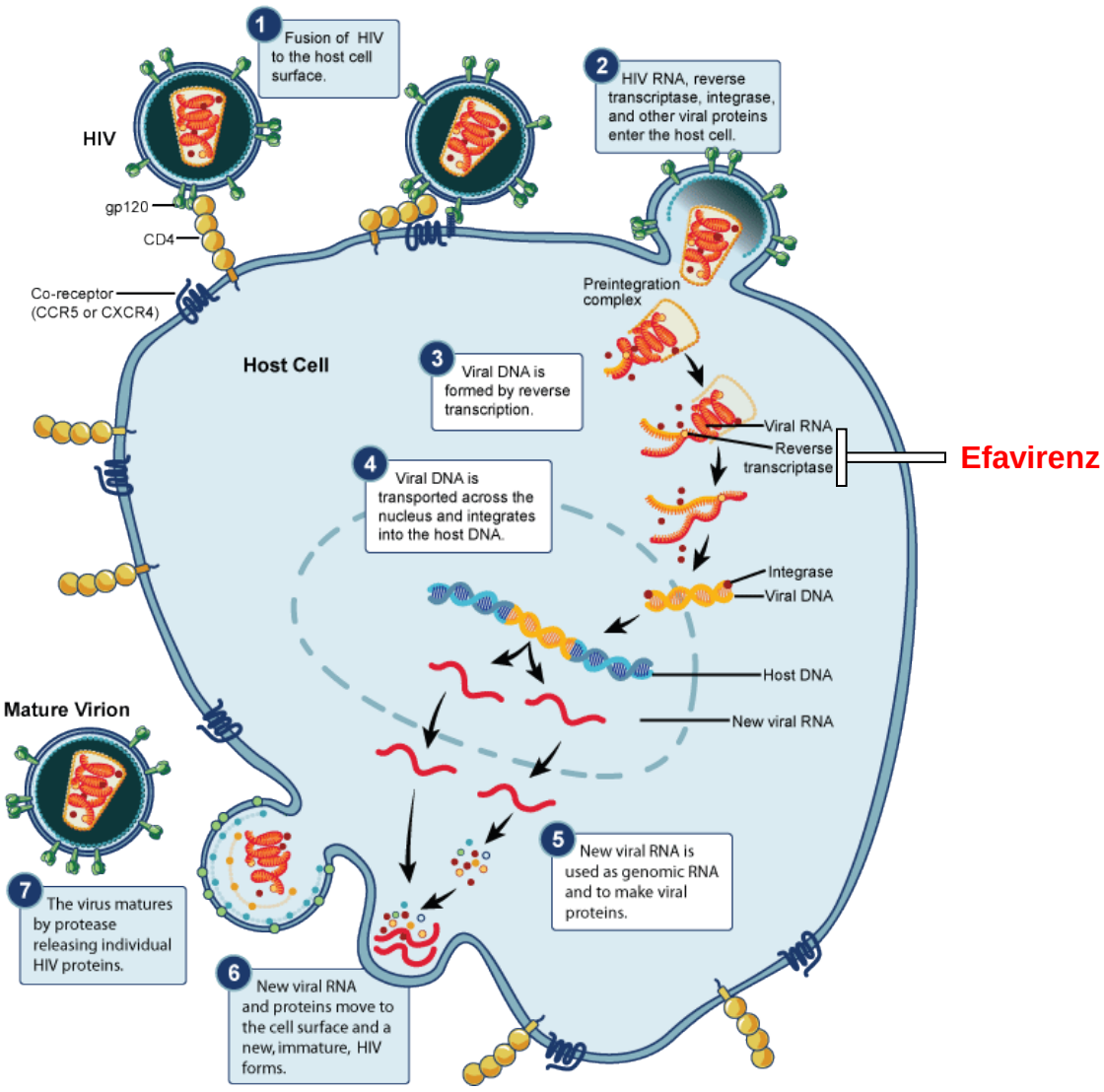

Figure 1.1 HIV life cycle and antiretroviral drug targets. The virus first attaches to the cell membrane of the target cell by interacting with the receptors. Then the virus fuses with the target cell and viral DNA and proteins are injected into cell. The viral RNA is transcribed to DNA by reverse transcriptase. Efavirenz is an inhibitor targeting at this enzyme. Viral DNA is integrated into the DNA of host cell by integrase. The final step is maturation of virion, where the polyprotein is cut into proper size by protease. Targeting at these key steps, entry inhibitors, fusion inhibitors, co-receptor inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors were developed.

(This figure was modified from HIV Replication Cycle, National Institue of Allergy and Infectious Diseases)

2. Efavirenz-based anti-HIV regimen

Efavirenz (Sustiva®) [(S)-6-chloro-4-(cyclopropylethynyl)-1, 4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazin-2-one] is a NNRTI approved for the treatment of HIV-1 infection by FDA in 1998 (Maggiolo, 2009). Subsequent study established the role of efavirenz with 2 NRTIs in the management of HIV-1 infections (Staszewski et al., 1999). The chemical structure of efavirenz is shown in Figure 1.2. Efavirenz is the most commonly prescribed NNRTI in treatment naïve patients, in combination with 2 NRTIs and is recommended regimen for initial therapy in the current US and UK guidelines of antiretroviral treatment for adult HIV infection (Gazzard et al., 2008; Thompson et al., 2012). The efficacy of efavirenz has been established in numerous randomized clinical trials and observational studies in HAART-naïve patients, including those with advanced infection. Efavirenz-based regimen was superior to or as effective as unboosted PIs (Maggiolo et al., 2003; Squires et al., 2004; Yeni et al., 2006). The AIDS Clinical Trial Group (ACTG) performed a landmark comparison study of efavirenz versus ritonavir-boosted lopinavir, where efavirenz showed greater virological efficacy than the boosted PI (Riddler et al., 2008). Efavirenz is also more effective as a third agent than the nucleoside analogue abacavir (Gulick et al., 2004).

There are another four NNRTIs currently in the market, i.e. delavirdine, nevirapine, etravirine and rilpivirine. Nevirapine and efavirenz, both as first generation NNRTIs, are cornerstones of first line HAART. Delavirdine is less used due to common and serious adverse drug interactions. The first generation NNRTIs are characterized by a

lower genetic barrier to the development of drug resistance compared to second generation NNRTIs, i.e. rilpivirine and etravirine. Some, but not all, studies have suggested that efavirenz is more effective than nevirapine (Keiser et al., 2002; van Leth et al., 2004). Patients also tolerated efavirenz better than nevirapine, since nevirapine is associated with more adverse events than efavirenz. Rash is more common and usually more severe with nevirapine than with efavirenz (van Leth et al., 2004). Severe hepatotoxicity is also more common among the patients prescribed nevirapine (Sulkowski et al., 2002; van Leth et al., 2004). The clinical development of etravirine was conducted exclusively in treatment experienced patients. Therefore, extravirine is only recommended to be used as as sequential therapy in patients with NNRTI resistant HIV-1. Rilpivirine was non-inferior to efavirenz, although rates of virologic failure were higher with rilpivirine, while rates of adverse events were higher with efavirenz (Cohen et al., 2011a; Molina et al., 2011).

Efavirenz is generally well tolerated: rash and neuropsychiatric disturbances are the most notable adverse events. Neuropsychiatric disturbances (such as dizziness, insomnia, nightmares, lack of concentration and drowsiness) generally develop early in treatment among more than 50% of patients and they tend to resolve with continued administration, but they are persistent in about 5 ~ 9 % of patients [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012]. However, severe events such as depression, psychosis, mania, and paranoid have also been reported in some patients. Efavirenz has less effect on plasma lipid profiles than some boosted PIs. Lipodystrophy can occur under treatment with efavirenz, but it may be reduced if the concurrent use of thymidine analogues is avoided. However, there is concern regarding

the use of efavirenz during the first trimester of pregnancy because of animal data and rare case reports in humans indicating a potential association with neural tube defects (De Santis et al., 2002; Saitoh et al., 2005). Thus efavirenz has been assigned to pregnancy category D by the FDA. Recently, efavirenz was co-formulated with tenofovir disoproxil fumarate and emtricitabine in one tablet marketed as Atripla ™, providing the most convenient HAART regimen in one pill once a day. In light of these features, efavirenz-based therapy remains the preferred intial therapy for treatment naïve HIV-1 patients (Thompson et al., 2012).

Efavirenz exhibits narrow therapeutic range. The proposed therapeutic range of efavirenz is 1-4 mg/L. Clinical studies have suggested that patients with plasma concentrations below 1 mg/L are at increased risk for failure of antiretroviral therapy (Marzolini et al., 2001; Csajka et al., 2003), and those with concentrations higher than 4 mg/L experience more frequent CNS side effects (Marzolini et al., 2001; Haas et al., 2004; Rotger et al., 2005; Motsinger et al., 2006). Therefore, the goal of efavirenz-based therapy is to maintain maximum virally suppressive efavirenz concentrations that will prevent the emergence of resistance and avoid treatment failure, while also ensuring an adverse event profile. However, the achievement of this goal is made difficult by the large interindividual variability associated with efavirenz pharmacokinetics (Csajka et al., 2003; Rotger et al., 2007). Wide interindividual variations in efavirenz pharmacokinetics may occur during absorption and distribution, but the most frequent and dramatic effects are due to changes in drug metabolism and excretion, which is probably driven by the genetic and nongenetic (e.g., drug interactions) factors. The current knowledge of the contribution of genetic polymorphisms in drug metabolizing enzymes and drug

transporters to wide variability in efavirenz pharmacokinetics is discussed in details in **Chapter I-4**.

Besides the large interindividual variability in efavirenz pharmacokinetics, complex and unpredictable drug interactions compromise rational use of efavirenz-bsaed regimen. The likelihood of these pharmacokinetic drug interactions in HIV patients is particularly high, because efavirenz is always used in combination therapy, frequently in the presence of herbal and nutritional supplements or in concert with drugs directed at the treatment of opportunistic infections and other HIV-related disorders. Co-administered drugs could influence efavirenz disposition, while efavirenz is known to alter the pharmacokinetics of several drugs through not only induction but also inhibition of drug metabolizing enzymes and drug transporters (discussed in **Chapter I-3**). The drug interactions with efavirenz mediated by the inhibition/induction of the drug metabolizing enzymes involved in efavirenz metabolism are discussed in **Chapter I-5**. It is worthy to notice the bidirectional drug interaction phenomenon in efavirenz-based anti-HIV therapy. For example, voriconazole is used to treat the opportunistic fungal infections in HIV patients. At steady state, efavirenz substantially decreased voriconazole exposure, while voriconazole increased efavirenz exposure by about 40% (Liu et al., 2008). Therefore, with voriconazole, it is suggested to decrease efavirenz dose to 300 mg once daily and increase voriconazole maintenance dose to 400 mg every 12 hours [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012].

3. Clinical pharmacology of efavirenz 0

3.1 Efavirenz absorption

Efavirenz is a crystalline lipophilic solid (log octanol water partition coefficient of 4.6) with a molecular mass of 315.68 and an aqueous solubility of 9.0 μg/ml (Maurin et al., 2002). It is a class II drug (low solubility, high permeability) according to the biopharmaceutical classification system guidance by the FDA (Kasim et al., 2004). Highly permeable, poorly soluble drugs often demonstrate poor gastrointestinal (GI) absorption due to inadequate drug solubility in GI fluids. Furthermore, efavirenz has a considerably low intrinsic dissolution rate of 0.037 mg/cm^2/min (Sathigari et al., 2009), which suggests dissolution rate-limited absorption problems for this drug. Peak efavirenz plasma concentrations of 1.6-9.1 μM were attained by 5 hours following single oral dose of 100 mg to 1600 mg administered to uninfected volunteers [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012]. Dose-related increases in C_{max} and AUC were seen for doses up to 1600 mg; the increases were less than proportional suggesting diminished absorption in higher doses. Since there is no intravenous formulation of efavirenz available, the bioavailability of efavirenz is not known. A one-month mass balance/excretion study using 400 mg efavirenz once daily with a 14C-labeled dose administered on Day 8 may provide some hints about the bioavailability. Approximately 14~ 34% of the radiolabel was recovered in the urine and 16-61% was recovered in the feces. Nearly all of the urinary excretion of the radio-labeled drug was in the form of metabolites. Efavirenz accounted for the majority of the total radioactivity measured in feces [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012]. However, it is unclear that the excretion of

efavirenz in feces is from biliary excretion after it was first absorbed or it was not absorbed from the GI tract in the first place. The food intake was associated with a mean increase of about 20% in efavirenz AUC and a mean increase of 50% in efavirenz C_{max}, respectively (Lamorde et al., 2012). Therefore, it is recommended that efavirenz be taken on an empty stomach.

3.2 Efavirenz distribution

Efavirenz is highly bound (approximately 99.5-99.75%) to human plasma proteins, predominantly albumin and distributed well to sanctuary sites including cerebrospinal fluid and testicles, reaching therapeutic levels in just a few days after initiating treatment (Avery et al., 2013). In HIV-1 infected patients (n=9) who received efavirenz 200 to 600 mg once daily for at least one month, cerebrospinal fluid concentrations ranged from 0.26 to 1.19% (mean 0.69%) of the corresponding plasma concentration. This proportion is approximately 3-fold higher than the non-protein bound (free) fraction of efavirenz in plasma. Efavirenz has been reported to accumulate in cells, reaching intracellular concentrations well above its 90% inhibitory concentration (Almond et al., 2005).

3.3 Efavirenz metabolism and excretion

The scheme of human efavirenz metabolism pathways is depicted in Figure 1.2. Efavirenz is mainly cleared by oxidation via cytochrome P450s (CYPs) (Mutlib et al., 1999). The metabolites identified in human plasma and urine (almost exclusively as glucuronide or sulfate conjugates) were 7- and 8-hydroxyefavirenz (primary metabolites) and 8, 14-dihydroxyefavirenz (secondary metabolite). Direct N-glucuronidation of efavirenz was also observed (Mutlib et al., 1999). Less than 1% of efavirenz is excreted

unchanged in the urine. The metabolites undergo further conjugation by UDP-glucuronosyltransferase (UGT) (Mutlib et al., 1999). *In vitro* studies have shown that 8-hydroxylation catalyzed predominantly by CYP2B6, with minor contributions from CYP1A2, CYP2A6 and CYP3A4/5, represents the main clearance pathway of efavirenz (Ward et al., 2003; Desta et al., 2007). Our laboratory provide direct evidence that CYP2A6 is the sole enzyme responsible for 7-hydroxylation that accounts for ~23% of total efavirenz metabolism *in vitro* (Ogburn et al., 2010). Recent clinical studies in HIV patients implicate that CYP2A6 may play a role in efavirenz 7-hydroxylation (Arab-Alameddine et al., 2009; di Iulio et al., 2009; Kwara et al., 2009a; Kwara et al., 2009b). We also found that efavirenz is metabolized sequentially to novel dihydroxylated metabolite (s), via CYP2B6-mediated 7- and 8-hydroxyefavirenz hydroxylation as intermediary; and 8, 14-dihydroxyefavirenz is formed *in vivo* but not *in vitro*, suggesting novel metabolic reactions and challenging previous notion that it is formed through direct 14-hydroxylation of 8-hydroxyefavirenz (Mutlib et al., 1999; Ward et al., 2003; Ogburn et al., 2010). UGT2B7-mediated N-glucuronidation represents an additional minor clearance route of efavirenz (Belanger et al., 2009). However, its contribution to overall efavirenz clearance appears to be minimal *in vivo* (Cho et al., 2011).

The gender effect on the pharmacokinetics of efavirenz is controvercial. While no significant gender effect was observed in previous study (Csajka et al., 2003), some more recent studies suggest that females have higher efavirenz exposure compared to males (Burger et al., 2006; Floridia et al., 2008). The pharmacokinetics of efavirenz has not been studied in patients with renal insufficiency; however, less than 1% of efavirenz is

excreted unchanged in the urine, so the impact of renal impairment on efavirenz elimination should be minimal.

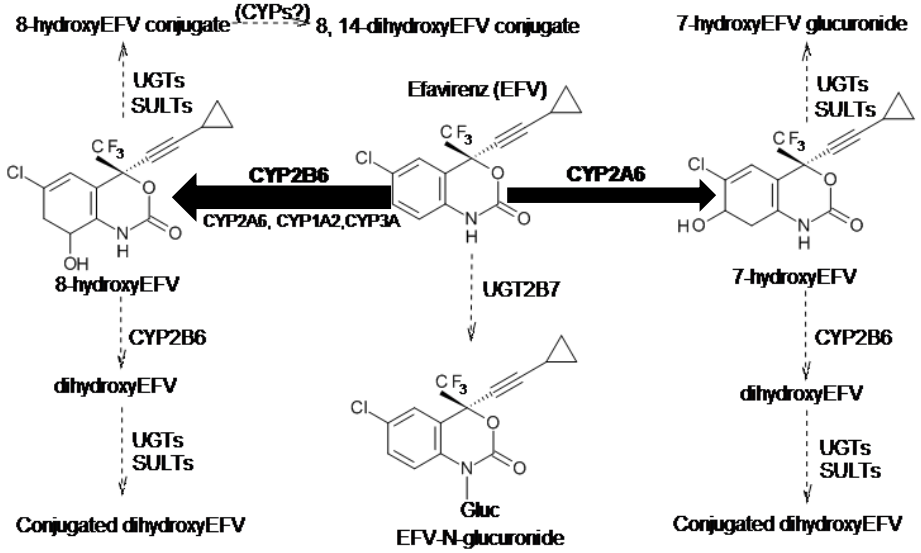

Figure 1.2 Human metabolism of efavirenz. Efavirenz is predominantly metabolized to 8-hydroxyefavirenz via CYP2B6 (Mutlib et al., 1999; Ward et al., 2003; Ogburn et al., 2010), while CYP2A6-catalyzed 7-hydroxylation to 7-hydroxyefavirenz represents the alternative pathway (Ogburn et al., 2010). Once primary metabolites form, they will undergo further hydroxylation and conjugation. All these pharmacologically inactive metabolites or their conjugates are excreted in the urine. Besides the oxidation pathways, efavirenz can undergo direct conjugation by UGTs (Belanger et al., 2009), although the contribution of this route to the overall clearance remains to be marginal (Cho et al., 2011). In sum, CYP2B6 is the main enzyme responsible for the major clearance pathway of efavirenz.

3.4 Efavirenz induction and inhibition on drug metabolizing enzymes and drug transporters

Efavirenz has a terminal elimination half-life of 52-76 hours after a single dose and 40-55 hours after multiple doses, mainly due to its ability to induce CYP2B6 expression and thus accelerate its own metabolism (auto-induction) (Zhu et al., 2009). Examples of auto-induction for other CYP2B6 substrates include the widely used anticonvulsant carbamazepine (Kudriakova et al., 1992), anticancer drug cyclophosphamide (Hassan et al., 1999) , anti-HIV agent nevirapine (Faucette et al., 2007) and antimalarial artemisinin (Burk et al., 2005). Previous study demonstrates that human constitutive androstane receptor (hCAR), alone or in cross-talk to human pregnane X receptor (hPXR), mediates the auto-inductive effects of efavirenz, as well as its ability to alter the pharmacokinetics and efficacy of other drugs (Faucette et al., 2007). Efavirenz auto-induction has important clinical implications because enhanced drug metabolism results in lower drug exposure at the site of action and may lead to a suboptimal therapeutic response or the development of drug resistance (Ngaimisi et al., 2011). The inductive effect of efavirenz on the metabolism of co-administered drugs has also been reported. Efavirenz has been associated with decreased exposure of methadone (Kharasch et al., 2012), statins (Gerber et al., 2005), omeprazole (Michaud et al., 2012b), voriconazole (Liu et al., 2008), proguanil (van Luin et al., 2010), etravirine (Boffito et al., 2009), protease inhibitors (Staszewski et al., 1999), and bupropion (Robertson et al., 2008), which can be primarily explained by its induction of CYP3A, CYP2B6 and CYP2C19 activities (Hariparsad et al., 2004; Faucette et al., 2007).

Induction of most drug metabolizing gene expression is predominantly regulated at the transcriptional level by PXR and CAR. Both PXR and CAR are orphan (or 'X') receptors, grouped into the nuclear receptor subfamily 1, group I (NR1I) play an important role in detecting xenobiotics and stimulating genes involved in drug disposition, including phase I and phase II enzymes such as CYPs, UGTs, sulfotransferases as well as drug transporters. PXR and CAR are predominately expressed in the liver, but also expressed in the intestine, lung and other tissues (Willson and Kliewer, 2002). Several studies demonstrate that CAR and PXR can mutually bind to and activate response elements in the promoter regions of several P450 genes, suggesting that cross-talk occurs between these receptors in the regulation of these genes (Xie et al., 2000; Goodwin et al., 2001; Goodwin et al., 2002). The role of CAR as a xenobiotic sensor was first suggested by the work of Negishi and colleagues, who characterized the transcription factor that regulate the expression of *CYP2B* (Zelko and Negishi, 2000; Sueyoshi and Negishi, 2001). In primary hepatocytes and intact liver, hCAR is predominately localized in cytoplasm and translocates to the nucleus upon activation by either direct ligand binding or indirect mechanisms (Kawamoto et al., 1999). Most findings to date suggest that CAR binds to a phenobarbital-responsive enhancer module (PBREM) located approximately 2 kb upstream from the *CYP2B* gene transcriptional start site as a heterodimer with retinoid X receptor (RXR) (Honkakoski et al., 1998; Zelko and Negishi, 2000). CAR response elements have been mapped in a number of human genes, including *CYP2B6*, *CYP3A4*, members of the CYP2C family, *UGT1A1* and *ABCB1* (Swales and Negishi, 2004). Efavirenz induction of CYP2B6 is mediated preferentially through CAR and it induced nuclear translocation of CAR at levels

comparable with or greater than well known hCAR activators, phenobarbital and imidazole derivative 6-(4-chlorophenyl)imidazo[2,1-β][1,3]thiazole-5-carbaldehyde-O-(3,4 dichlorobenzyl)oxime (CITCO), in cell-based reporter gene assay (Faucette et al., 2007). CAR also binds to a xenobiotic-responsive enhancer module (XREM) in the distal region of the *CYP2B6* promoter together with the PBREM mediates optimal drug-induced expression of CYP2B6 (Wang et al., 2003).

Like CAR, PXR regulates the expression of target genes by binding to xenobiotic response elements as a heterodiner with RXR (Kliewer et al., 1998). The target genes of PXR broadly overlap with those of CAR hPXR is capable of binding to the same response elements in the promoter regions of the *CYP3A4* and *CYP2B6* genes (Goodwin et al., 1999; Wang et al., 2003).

Besides drug metabolizing enzymes, efavirenz also has been shown to induce the expressions of multiple drug transporters in Jurkat cells (Weiss et al., 2009). The results from a recent collaborative study from our laboratory also provide strong evidence that exposure to efavirenz significantly increases markers of hepatic drug metabolism and target gene expression in peripheral blood mononuclear cells (PBMCs) but not the CAR target genes in the intestine *in vivo* (Meyer zu Schwabedissen et al., 2012). However, these findings are in contrast to those recently reported by Burhenne *et al.*, who found no significant induction of ABCB1 and ABCC2 expression in PBMCs after 14 days of efavirenz treatment (Burhenne et al., 2010).

Although many drug-drug interactions associated with efavirenz can be explained by its induction potential, some of them may be attributed to direct enzymatic inhibition effect. *In vitro* evidence shows that efavirenz may directly inhibit the activities of certain

CYPs, including CYP2B6 (Hesse et al., 2001), CYP2C8 (Parikh et al., 2007), CYP2C9 and CYP2C19 (von Moltke et al., 2001). Indeed, scattered clinical cases of adverse drug interactions, e.g., with CYP2C8 substrate amodiaquine (German et al., 2007; Soyinka et al., 2013), CYP2C9 substrates warfarin (Bonora et al., 2008) and phenytoin (Robertson et al., 2005) suggest that efavirenz may alter the pharmacokinetics of co-administered drugs through inhibition of CYPs. The clinical significance of efavirenz inhibition effect on drug transporters is unclear currently. Efavirenz is known to inhibit breast cancer resistant protein (BCRP) coded by *ABCG2* gene *in vitro* (Weiss et al., 2007), but the chronic treatment with efavirenz induces the expression of *ABCG2* in a leukemia cell line without modification in intracellular accumulation of the drug (Weiss et al., 2009). The same founding was reported in rats (Peroni et al., 2011).

4. *Pharmacogenetics of efavirenz*

Emerging evidence suggest that the large interindividual variability in efavirenz exposure and clinical responses may be primarily attributed to the large variability in the expression and activity of the drug metabolizing enzymes and/or drug transporters involved in its disposition mainly due to genetic polymorphisms.

4.1 CYP2B6

As described above, CYP2B6 is the main enzyme responsible for catalyzing efavirenz 8-hydroxylation, the principal metabolic pathway of the drug. Studies have shown that there is a large interindividual variation in CYP2B6 protein expression (20- to 280-fold) and activity (25- to 100-fold) among human livers, in part due to extensive genetic polymorphisms in the *CYP2B6* gene and non-genetic factors, e.g., induction and inhibition drug interactions (Code et al., 1997; Stresser and Kupfer, 1999; Lang et al.,

2001; Zanger et al., 2007). The *CYP2B6* gene is highly polymorphic. To date, 37 alleles of *CYP2B6* (**1A* [wild type to **37*) and multiple sub-alleles have been reported in the *CYP2B6* upstream sequence, introns and 9 exons, which have extensive linkage disequilibrium causing complex haplotype structures (http://www.cypalleles.ki.se/cyp2b6.htm).

The first systematic analysis of genetic polymorphisms in *CYP2B6* gene identified nine SNPs of *CYP2B6* gene in a Caucasian population, resulting in six different alleles designated as *CYP2B6*2* (c.64C>T), *CYP2B6*3* (c.777C>A), *CYP2B6*4* (c.785A>G), *CYP2B6*5* (c.1459C>T), *CYP2B6*6* (c.516G>T and c.785A>G) and *CYP2B6*7* (c.516G>T, c.785A>G and c.1459C>T) (Lang et al., 2001). Some of these variants have been shown to change CYP2B6 hepatic expression and activity (Lang et al., 2001). Subsequent studies have confirmed that genetic polymorphisms in *CYP2B6* gene are associated with enzyme expression and activity *in vitro* (Lamba et al., 2003; Hesse et al., 2004; Lang et al., 2004; Desta et al., 2007), efavirenz exposure in HIV patients (Haas et al., 2004; Tsuchiya et al., 2004; Rotger et al., 2005; Wang et al., 2006; Rotger et al., 2007; Arab-Alameddine et al., 2009; Holzinger et al., 2012) and CNS adverse effect (Haas et al., 2004). Most of these studies focused on the *CYP2B6*6* allele, which is defined by two amino acid alterations, Q172H (516G>T) and K262R (785A>G). This variant occurs at high frequencies in all ethnic populations studied to date, ranging from about 15% in Asian, 25% in Caucasians, to almost 50% among black individuals (Zanger et al., 2007; Li et al., 2012) (Figure 1.3). The *CYP2B6*6* allele is consistently associated with efavirenz clearance (Tsuchiya et al., 2004; Rotger et al., 2007), CNS side effects (Haas et al., 2004), hepatic toxicity (Yimer et al., 2011), treatment discontinuation (Wyen

et al., 2011), virological failure (Motsinger et al., 2006), and drug interactions (Ngaimisi et al., 2011). More *CYP2B6* novel variants (e.g. **11, *15, *16, *18, *27* and **28*) with diminished or loss of CYP2B6 functions have been reported recently via candidate gene approach (Wang et al., 2006; Rotger et al., 2007). A recent genome-wide association analyses replicates the association of 516G>T (**9*) and 983T>C (**4*) with efavirenz plasma concentrations and also identifies a new intron variant associated with efavirenz pharmacokinetics (Holzinger et al., 2012). The 983T>C SNP is found mainly in subjects of African descent, but rare in other ethnic groups. All these findings together demonstrate the significant contribution of the *CYP2B6* genetic polymorphism to the variable pharmacokinetics and clinical responses of efavirenz and lay the foundation of CYP2B6 genotype-based efavirenz dose adjustment.

The second most frequent candidate SNP 1459C>T in allele **5*, which has been previously linked to reduced expression in human liver microsomes (Lang et al., 2001), did not affect efavirenz kinetics (Rotger et al., 2007). This is in agreement with another report that could not find an effect of this allele on bupropion kinetics in health populations (Kirchheiner et al., 2003). The apparently unaffected *in vivo* activity of **5* may indicate a compensation of reduced expression by enhanced metabolism through the variant enzyme (Zanger et al., 2007; Zhang et al., 2011b).

The *CYP2B6*4* allele occurs more frequently in Caucasian and Asian population (approximately 2-6%), whereas this allele appears to be virtually absent in African-Americans and Africans (Zanger et al., 2007) (Figure 1.3). The observed trend for lower efavirenz AUC in **1/*4* heterozygotes compared to **1/*1* carriers (Rotger et al., 2007) is in agreement with increased specific activity of the recombinant K262R variant towards

efavirenz compared to the wild type protein (Ariyoshi et al., 2011). However, inconsistent effect of this allele was observed on bupropion kinetics between *in vitro* and *in vivo*. Higher bupropion clearance after single-dose administration is observed in the individuals with *CYP2B6*4* allele (Kirchheiner et al., 2003), while lower catalytic efficiency of bupropion was characterized in the CYP2B6.4 (Zhang et al., 2011b). This inconsistence may be due to that the N-terminally truncated CYP2B6 protein was used in Zheng et al.'s study and its has been shown that the modified protein had significantly reduced K_m for bupropion compared with that of human liver microsomes (Zanger et al., 2007).

The *CYP2B6*9* allele (516G>T) occurs at very low frequency (below 1%) in most populations (Zanger et al., 2007). The substrate-dependent effect of this allele on enzyme function was noticed. While the expressed N-terminally modified enzyme displayed increased turnover of 7-ethoxycoumarin O-deethylase activity (Ariyoshi et al., 2001), the decreased catalytic efficiency of this mutant was observed in the metabolism of several other substrates (Watanabe et al., 2010).

The *CYP2B6*18* allele (983T>C) (Klein et al., 2005) and related *CYP2B6*16* allele (785A>G and 983T>C) (Wang et al., 2006) were not found in Caucasians, but were found in various African populations with frequencies of about 7% and in African-Americans with frequencies of approximately 4% (Zanger et al., 2007). The SNP resulted in undetectable expression and activity in COS-1 cells (Klein et al., 2005) and COS-7 cells (Watanabe et al., 2010), but a protein with reduced activity could be expressed in insect cells (Klein et al., 2005). The 983T>C polymorphism is reported to be significantly

associated with higher efavirenz plasma concentrations and toxicity (Rotger et al., 2007; Wyen et al., 2008; Ribaudo et al., 2010).

The *CYP2B6*2/*2* genotype is very rare but the association between this genotype and a case of efavirenz-induced neurological symptoms has been reported (Usami et al., 2007). Mean expression and activity levels in liver heterozygous *CYP2B6*2* carriers were similar to wild type. In expressed enzymes, no significant difference in the expression and activity was observed for this variant protein either (Watanabe et al., 2010).

The -82T>C promoter variant, characterizing the **22* allele, with frequency of about 3% in Caucasion (Zukunft et al., 2005; Rotger et al., 2007) and 7% in black population (Zukunft et al., 2005; Rotger et al., 2007). This genetic variant allele has previously been reported to be associated with enhanced transcription, and to result in increased expression/activity of the protein at baseline (Zukunft et al., 2005).It also appears to enhance induction of CYP2B6 by rifampin.But no significant difference in efavirenz plasma exposure was found in the *CYP2B6*22* carrier *in vivo*, probably due to the small sample size (Rotger et al., 2007).

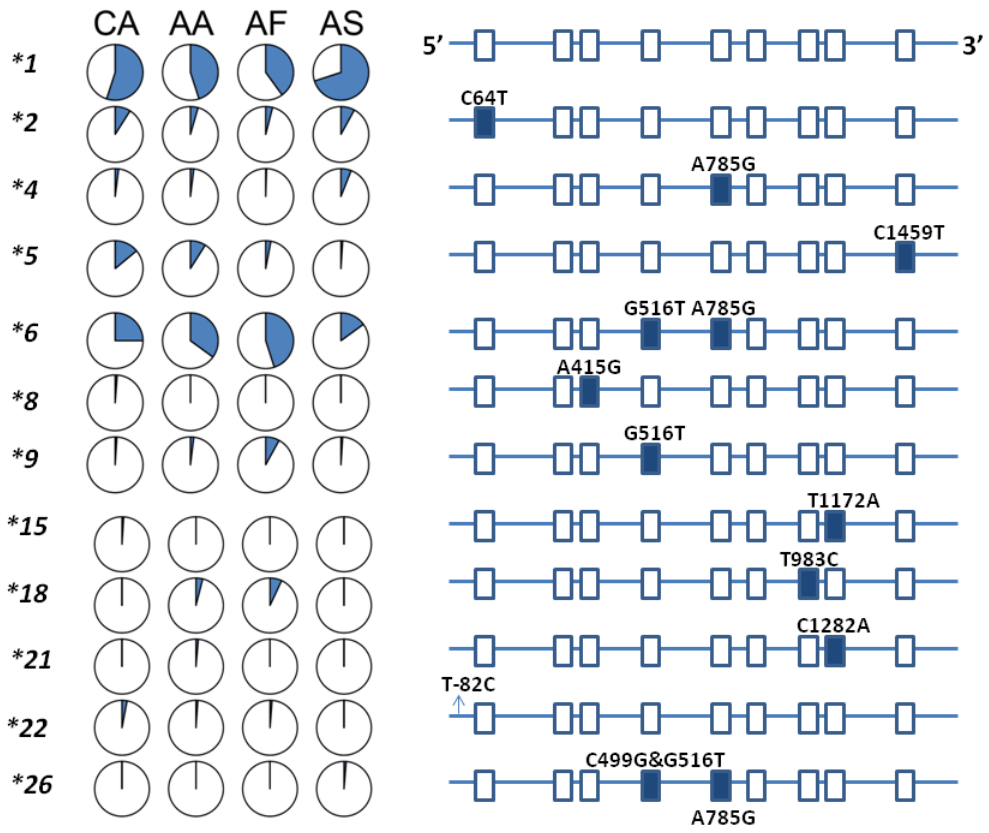

Figure 1.3 Frequency and structure of the most common *CYP2B6* alleles (modified from Zanger, et al. 2007 and Li, et al. 2012). On the left, allele frequencies across four major ethnic groups are indicated as filled fractions of the respective circles (completely filled circle representing a frequency of 100%). On the right, corresponding amino acid variants are shown, which are above the exons represented by black rectangles. CA: White European or North American of Caucasian origin; AA: African-American; AF: African (Ghanaian and other West African, Tanzanian); AS: Asians (Chinese, Korean and Japanese).

4.2 CYP2A6, CYP3A4/5, CYP1A2 and UGT2B7

Most previous pharmacogenetic studies related to efavirenz pharmacokinetics focused on CYP2B6, which is the main catalyst of efavirenz metabolism and responsible for the major metabolic pathway (8-hydroxylation). However, not all the efavirenz pharmacokinetic variability can be explained by the CYP2B6 or 8-hydroxylation pathway alone. The identified *CYP2B6* genetic polymorphisms can explain up to about 30% of the interindividual variability in efavirenz pharmacokinetics and a large inter-subject variability in efavirenz plasma concentrations remains after accounting for known *CYP2B6* genetic variations (Rotger et al., 2007; Arab-Alameddine et al., 2009; Holzinger et al., 2012). This unexplained variability suggests the contribution of genetic polymorphisms in other genes involved in efavirenz metabolism, such as CYP2A6, CYP3A4/5, CYP1A2 and UGT2B7 (Ward et al., 2003; Ogburn et al., 2010). Indeed, the predictive value of patient genotype of efavirenz plasma concentrations can be enhanced by taking into account of the genetic status of *CYP2A6*9B* and/or **17* polymorphism (Kwara et al., 2009b). Also, Kwara et al. reported that *UGT2B7* polymorphisms along with *CYP2A6* genetic variations demonstrate independent effects on efavirenz disposition after accounting for the *CYPB6* genetic polymorphisms (Kwara et al., 2009a). The effect of *CYP2A6* and *CYP3A4* genetic polymorphisms on efavirenz metabolism, especially in CYP2B6 slow metabolizers, also has been replicated by other studies (Arab-Alameddine et al., 2009; di Iulio et al., 2009). However, other investigators were unable to replicate the role of polymorphisms in these candidate genes in efavirenz clearance (Cabrera et al., 2009; Elens et al., 2010; Maimbo et al., 2012) or their effects are small that genotype-phenotype associations are susceptible for confounding factors, particularly in HIV

patients taking multiple drugs. As described above, in a recent GWAS study, only variants in the *CYP2B6* locus predicted efavirenz concentrations (Holzinger et al., 2012). Other variants in other genes did not reach a statistically significant level.

4.3 Drug transporters

Drug transporters fall into two groups: the ABC superfamily of transporters (ATP-binding cassette proteins) and the SLC superfamily of transporters (solute carrier proteins). These pumps located in the intestinal epithelium actively remove drugs in the basolateral-apical direction, against a concentration gradient. The influence of genetic variations of *ABCB1* gene on efavirenz pharmacokinetics and pharmacodynamics is not clearly defined since contradictory results were reported. Most of the studies focused on the most common SNP, i.e. 3435C>T. Clinical studies showed that 3435C>T polymorphism in *ABCB1* gene may predict plasma concentrations (Fellay et al., 2002; Mukonzo et al., 2009), responses (Haas et al., 2005; Elens et al., 2010) and toxicities (Yimer et al., 2011) in HIV patients. However, conflicting results also exist (Nasi et al., 2003; Haas et al., 2004; Tsuchiya et al., 2004). Recently, a new variant allele in *ABCB1* gene (c.4036A>G) was identified as a major predictor of efavirenz pharmacokinetics in healthy subjects (Mukonzo et al., 2009), which indicates that further investigation of the influence of *ABCB1* polymorphism on efavirenz pharmacokinetics may be warranted. A recent study showed that efavirenz is a substrate of BCRP (Peroni et al., 2011), but no effect of genetic variants of BCRP on efavirenz pharmacokinetics was identified in HIV patients (Sanchez et al., 2011). Instead, a SNP of *ABCC4* (1497C>T) encoding multidrug resistance protein 4 (MRP4) was identified as a major factor influencing efavirenz oral

clearance, suggesting the potential importance of MRP4 in the distribution of efavirenz (Sanchez et al., 2011).

4.4 Nuclear receptors

Efavirenz is known to induce its own metabolism and the metabolism of other co-administered drugs through activation of CAR and/or PXR. At least CYP3A4 (Hariparsad et al., 2004), CYP2B6 (Faucette et al., 2007), CYP2C19 (Liu et al., 2008; Michaud et al., 2012b) and multiple drug transporters (Weiss et al., 2009) can be induced by efavirenz. Most of the pharmacogenetic studies so far have been focused on genetic variations in oxidation/conjugation drug metabolizing enzymes and drug transporters, but genetic variations in nuclear receptor can also contribute to the observed variability in the expression of genes involved in drug disposition and drug interactions, particularly for efavirenz which is known for its induction potential (Zhu et al., 2009). It has been shown that hPXR (Lamba et al., 2010) and hCAR (Ikeda et al., 2005) variants affect the transactivation of CYP3A4 and thus contribute to the high variability of inducibility. A recent study also has shown that the genetic polymorphism in CAR contributes to early treatment discontinuation for efavirenz-based regimens (Wyen et al., 2011), which indicates that genetic polymorphisms of nuclear receptors may play an important role in the large interindividual variability of efavirenz pharmacokinetics and clinical response. In a recent study, CAR variants have been shown to alter efavirenz exposure, but the effect seems modest (Cortes et al., 2013).

5. Human CYP2B6

The CYP gene superfamily currently consists of 57 functional genes and 58 pseudogenes (Nelson et al., 2004). The members of families *CYP1, CYP2*, and *CYP3* are

localized in the endoplasmic reticulum of both liver and extrahepatic tissues, where they catalyze a variety of biotransformations of numerous endogenous and exogenous substrates, including many drugs currently in use. The family member *CYP2B6* was first described in 1989 (Yamano et al., 1989) as the human ortholog to the phenobarbital-inducible *CYP2B* genes in rodents. Located on the long arm of chromosome 19, *CYP2B6* is mapped with closely related pseudogene *CYP2B7* and several other members of the *CYP2* gene family (Yamano et al., 1989). The *CYP2B6* gene is composed of 9 exons, coding for a protein of 48 KDa with 491 amino acids (Yamano et al., 1989). Like most other CYP isoforms, CYP2B6 is primarily expressed in the liver and involved in the metabolism of ingested drugs, but has also been detected in several extrahepatic tissues including brain, kidney, intestine, endometrium, bronchoalveolar macrophages, peripheral blood lymphocytes and skin (Gervot et al., 1999; Janmohamed et al., 2001; Ding and Kaminsky, 2003). Initially underestimated, recent studies using more sensitive and specific immunochemical detection methods demonstrate that the average expression of CYP2B6 ranges from 2% to 10% of the total hepatic CYP content (Code et al., 1997; Ekins et al., 1998; Stresser and Kupfer, 1999; Hesse et al., 2000).

5.1 CYP2B6 substrates

The number of drugs recognized as CYP2B6 substrates has been constantly increasing, and several clinically important drugs are now known to be metabolized by this enzyme. These include the anticancer prodrug cyclophosphamide (Chang et al., 1993; Roy et al., 1999); the ansthetics ketamine (Yanagihara et al., 2001; Desta et al., 2012) and propofol (Court et al., 2001); the antidepressant bupropion (Faucette et al., 2000; Hesse et al., 2000); the antimalarial drug artemisinin (Svensson and Ashton, 1999);

and anti-HIV drugs efavirenz (Ward et al., 2003) and nevirapine (Erickson et al., 1999); synthetic opioids methadone (Kharasch et al., 2004) and meperidine (Ramirez et al., 2004). Other substrates are listed in the following reviews (Wang and Tompkins, 2008; Mo et al., 2009).

Until recently, progress on the clinical relevance of CYP2B6 was hampered by the lack of selective and safe *in vivo* probes of its activity. Bupropion is generally accepted as the traditional CYP2B6 probe. However, the contribution of CYP2B6-mediated pathway to the overall clearance is small and non-CYP2B6 mediated metabolism contributes significantly to the overall clearance of this drug (Turpeinen et al., 2005; Loboz et al., 2006). The metabolism of bupropion by CYP2B6 has also been shown to be stereoselective. Bupropion is used clinically as a racemic mixture of R- and S-enantiomers and CYP2B6 selectively catalyzes the hydroxylation of (S)-bupropion over that of (R)-bupropion. Given the documented limitations of bupropion as an *in vivo* probe for CYP2B6 activity, the stereo-specific catalysis allowed researchers to identify the (S, S)-hydroxybupropion metabolite as a more specific *in vivo* probe (Kharasch et al., 2008). The stereo-selective behavior of CYP2B6 suggests that other chiral substrates of CYP2B6 catalysis could exhibit similar stereo-specific outcomes. Besides bupropion, efavirenz is the other sensitive *in vivo* CYP2B6 substrate recommended by the FDA (Guidance for Industry, Drug Interaction Studies- Study Design, Data Analysis, Implications for Dosing and Labeling Recommendations, February, 2012). Efavirenz is not only an *in vitro* and *in vivo* activity probe, but may also serve as a prototype model drug for evaluating the clinical relevance of *CYP2B6* genetic polymorphisms, because of the association between the *CYP2B6* genetic polymorphisms and efavirenz

pharmacokinetics and pharmacodynamics (Haas et al., 2004; Tsuchiya et al., 2004; Rotger et al., 2007).

In addition to drugs, CYP2B6 is able to both detoxify and bioactivate a number of procarcinogens and environmental agents including pesticides and herbicides (Mo et al., 2009). CYP2B6 also catalyzes the metabolism of several endogenous compounds (Rendic, 2002). These include arachidonic acid, lauric acid, 17ß-estradiol, estrone, and testosterone. The role of CYP2B6 in the metabolism of these endogenous compounds is minor compared to other CYPs such as CYP3A members play a more important role in the oxidative metabolism of these substances.

5.2 CYP2B6 inhibitors and inducers

In addition to genetic polymorphisms, drug-drug interactions mediated by inhibition and induction of CYP2B6 are also major factors that may contribute to the large inter-individual variability in efavirenz pharmacokinetics. [2-phenyl-2-(1-piperidinyl) propane (PPP) (Chun et al., 2000), cytostatic triethylenethiophosphoramide (thioTEPA) (Rae et al., 2002; Richter et al., 2005), antiplatelet agents clopidogrel (Richter et al., 2004), ticlopidine (Richter et al., 2004; Walsky et al., 2006) and prasugrel (Nishiya et al., 2009) have been shown to inhibit CYP2B6 activity. All of them are mechanism-based inhibitors of CYP2B6, while ThioTEPA is not only a mechanism-based inhibitor but also a non-competitive inhibitor of CYP2B6 (Rae et al., 2002). Under a reversible inhibition experimental protocol, clopidogrel seems to possess adequate selectivity for CYP2B6 (Walsky and Obach, 2007). Our recent study showed that ticlopidine seems to possess greater inhibition potency of CYP2B6 than ThioTEPA and less inhibits CYP2A6-mediated 7-hydroxylation of efavirenz (Ogburn et al., 2010), but it

potently inhibits CYP2C19 activity (Walsky and Obach, 2007). Besides CYP2B6, thioTEPA also inhibits CYP3A and CYP2A6 (Walsky and Obach, 2007; Ogburn et al., 2010). Under an irreversible inactivation condition, PPP, clopidogrel and ticlopidine displayed significant selectivity, while thioTEPA demonstrated a minimal selectivity for CYP2B6. PPP served as a selective probe for CYP2B6 inhibition, but restricted its use to *in vitro* studies only, as PPP is not approved for human administration. The next selective inhibitors, clopidogrel and ticlopidine, already approved as clinical agents, could be used in clinical studies under circumstances that promote selectivity (Walsky and Obach, 2007). To date, all the *in vivo* CYP2B6 inhibitors (clopidogrel, ticlopidine and prasugrel) are classified as weak inhibitors, since less than 2-fold increase in AUC was observed (Guidance for Industry, Drug Interaction Studies- Study Design, Data Analysis, Implications for Dosing and Labeling Recommendations, February, 2012). Recently, our laboratory showed that voriconazole is a highly potent competitive inhibitor of CYP2B6 *in vitro* (Jeong et al., 2009). The investigation of its inhibition potency of CYP2B6 activity in humans is ongoing in our laboratory.

A number of drugs have been shown to induce CYP2B6 *in vitro* and *in vivo*. The mechanism of CYP2B6 induction has been discussed in 3.4 of this chapter. The most prototypical CYP2B6 inducer is phenobarbital (Mo et al., 2009). Other known CYP2B6 inducers include cyclophosphamide (Gervot et al., 1999), rifampicin (Goodwin et al., 2001), phenytoin (Wang et al., 2004), artemisinin (Burk et al., 2005), carbamazepine (Oscarson et al., 2006), nevirapine (Faucette et al., 2007), ritonavir and efavirenz (Faucette et al., 2004).

6. The *CYP2B6*6* allele

As described above, the *CYP2B6*6* allele is the most frequent allele across different ethnic populations among all the *CYP2B6* variant alleles identified so far (Zanger et al., 2007; Li et al., 2012). It is established that this allele is associated with increased risk for higher efavirenz exposure (Haas et al., 2004; Tsuchiya et al., 2004), CNS side effects (Haas et al., 2004), hepatic toxicity (Yimer et al., 2011), treatment discontinuation (Wyen et al., 2011) and virological failure (Motsinger et al., 2006). Recently, it also has been shown that this allele plays a significant role in determining the extent of CYP2B6 induction (Ngaimisi et al., 2011).

Consistent to the association with the reduced efavirenz metabolism *in vivo*, the *CYP2B6*6* allele also markedly reduces efavirenz metabolism in expressed CYP2B6 (Ariyoshi et al., 2011; Zhang et al., 2011b) and human liver micrsomes (HLMs) (Desta et al., 2007). In HLMs, the *CYP2B6*6* allele is associated with reduced CYP2B6 protein expression (Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007), probably due to the aberrant splicing of pre-mRNA of CYP2B6 (Lamba et al., 2003; Hofmann et al., 2008). However, mounting evidence indicate that reduced protein expression alone may not explain the functional consequences of this allele. For substrates that include cyclophosphamide, this allele is associated with enhanced metabolism despite reduced protein expression (Xie et al., 2003), which appears due to substantially lower K_m in the variant versus wild type protein (Ariyoshi et al., 2011). Other *in vitro* studies, mostly in expression systems, have also reported that the *CYP2B6*6* allele or the amino acids harbored in it influence catalytic properties, although the extent and direction of effect appears to depend on the substrate and the enzyme sources used (Ariyoshi et al., 2001;

Jinno et al., 2003; Bumpus and Hollenberg, 2008; Watanabe et al., 2010; Zhang et al., 2011b). Therefore, in addition to reduced protein expression, altered enzyme function by the *CYP2B6*6* allele may contribute to altered substrate metabolism.

Previous studies show that the two amino acid mutations at residues of 172 and 262 harbored by the *CYP2B6*6* allele are not within the active site of the enzyme depicted in Figure 1.4 (Gay et al., 2010; Shah et al., 2011). Therefore, it is very likely that they are involved in the ligand binding and substrate catalysis indirectly. Indeed, a recent publication characterizing the crystal structure of CYP2B6 genetic variant (Y226H, K262R) indicates that the side chain of residue 172 may interact with the residues at active site and thus could affect the orientation of active site residues on the I-helix and substrate binding affinity (Gay et al., 2010; Shah et al., 2011). It is noteworthy that the other mutated amino acid carried by *CYP2B6*6* allele, K262R, is located at the G/H loop and is part of a small hydrogen-bonding network (Gay et al., 2010). The concerted movement of the G and H helices, which could be affected by this hydrogen-bonding network, may influence the orientation of active site residues on the G helix (Gay et al., 2010). In addition, part region of G helix forms substrate recognition sequence and substrate egress channels, which may both affect substrate specificity (Cojocaru et al., 2007). Indeed, substrate-dependent effects of the *CYP2B6*6* allele on pharmacokinetics have been observed *in vivo*. *In vivo*, the *CYP2B6*6/*6* genotype is associated with 3-fold increase in efavirenz exposure compared with *CYP2B6*1/*1* genotype (Rotger et al., 2007), but its effect on plasma exposure of bupropion or 4-hydroxybupropion was marginal (Kirchheiner et al., 2003) and its effect on exposure of cyclophosphamide was opposite (Nakajima et al., 2007). K262R may also involve in the interaction between the

enzyme and its redox partner, CYP reductase (Bumpus and Hollenberg, 2008; Gay et al., 2010). The oxidation reaction catalyzed by CYPs requires transferring of two electrons from NADPH. The first electron is generally thought to be transferred by CYP reductase, while the second can be transferred by either CYP reductase or cytochrome b5. That altered electron transfer from CYP reductase to CYP2B6 variant proteins may influence substrate metabolism was suggested by a recent study (Zhang et al., 2011b). It is, therefore, reasonable to suggest that amino acid changes by the *CYP2B6*6* allele may influence the interaction between the CYPs and electron transfer proteins and thus alter the catalysis of substrates in cytochrome b5- and substrate-dependent manner.

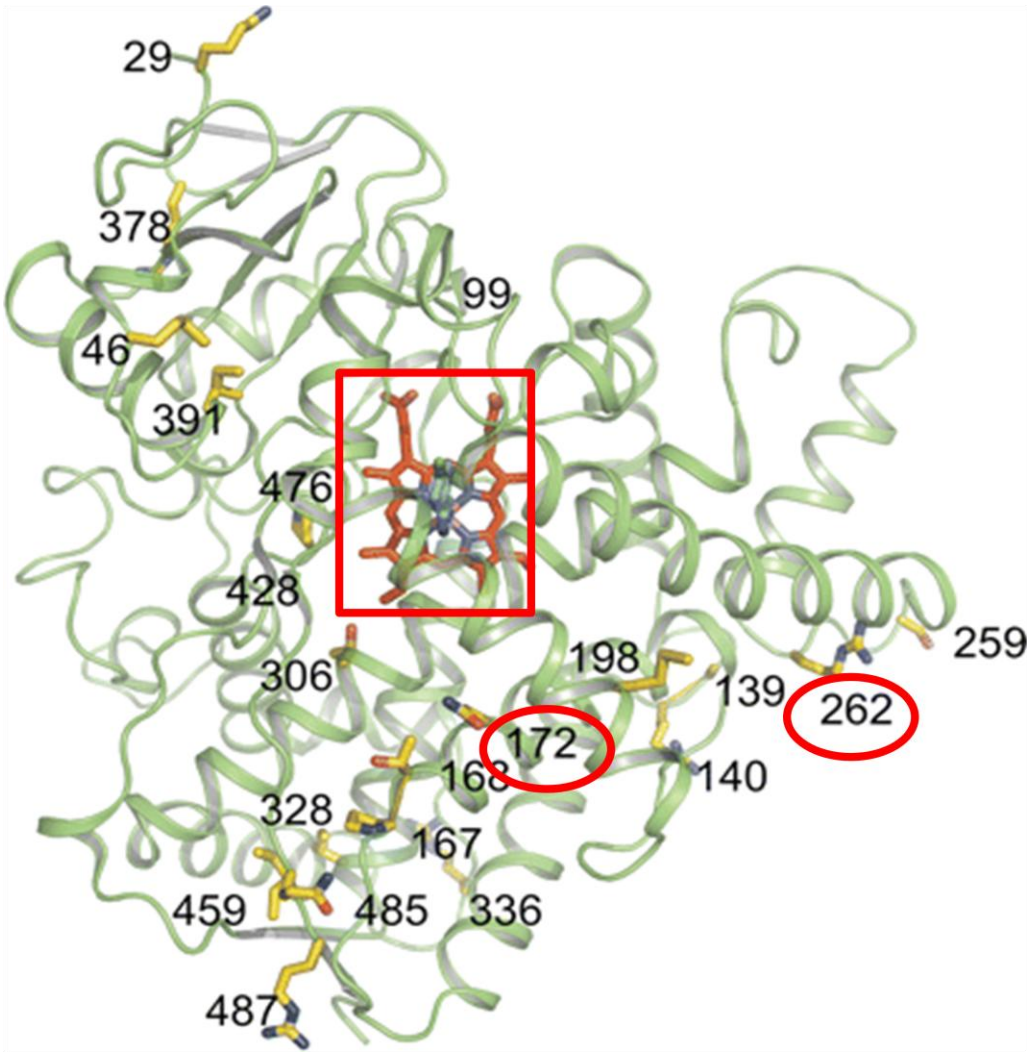

Figure 1.4 Ribbon diagram showing the location of amino acid residues with known mutations (yellow sticks) in CYP2B6. The heme is shown as red sticks; 4-CPI, cyan sticks. The majority of the known 2B6 coding sequence variants contain substitutions that occur relatively far away from the active site, and none of them actually lies within the active site, and none of them actually lies within the active site The two amino acid residues harbored by the variant protein coded by the *CYP2B6*6* allele are highlighted in red circles. (This figure is modified from Gay et al., 2010).

7. Hypothesis and specific aims:

Efavirenz, a potent non-nucleoside reverse transcriptase inhibitor (NNRTI), remains a preferred component of highly active antiretroviral therapy (HAART) for treatment naïve patients despite an emergence of second generation of NNRTIs and new classes of antiretroviral agents. However, the optimal and safe use of efavirenz is impaired by its large interindividual variability in pharmacokinetics, and by the unpredictable drug interactions associated with it. Thus, better understanding of mechanisms controlling systemic exposure of efavirenz and its interactions with other medications used in HIV/AIDS therapy is critical to avoid adverse reactions and optimizing beneficial effects.

Efavirenz is primarily metabolized by cytochrome P450 (CYP) 2B6 and to some extent CYP2A6 and UDP-glucuronosyl transferase (UGT) 2B7. *In vitro* and population pharmacokinetic studies in HIV patients have shown that *CYP2B6* genetic polymorphisms, particularly the *CYP2B6*6* allele, which is the most frequent variant across different populations, significantly alters efavirenz plasma concentrations and/or adverse effects. However, the mechanism of reduced efavirenz metabolism by the *CYP2B6*6* allele is not fully understood and whether this variant exhibits differential susceptibility to metabolic inhibition is also unknown. In addition, some *in vitro* and *in vivo* evidences also indicate that the functional consequences of the *CYP2B6*6* allele appear to be substrate- and cytochrome (Cyt) b5-dependent.

In addition, efavirenz alters the pharmacokinetics of many drugs co-administered with it and are often associated with reduced efficacy or increased toxicity of the affected drugs. It has been well documented that some of the drug interactions are mediated

through efavirenz induction activity of certain drug metabolizing enzymes and transporters via the constitutive androstane receptor (CAR) and pregnane X receptor (PXR). However, not all pharmacokinetic drug interactions involving efavirenz can be explained by its known inductive effect. Scatter clinical cases of adverse drug interactions were reported, suggesting that efavirenz may also directly inhibit the activities of certain CYPs. The *in vitro* studies describing inhibition of CYPs by efavirenz provide only qualitative information, without generating *in vitro* inhibition parameters that will allow quantitative prediction of *in vivo* condition and without taking the contribution of time-dependent inactivation into account.

The main objectives of this proposal are: 1) to identify mechanisms that contribute to the reduced efavirenz metabolism by the *CYP2B6*6* allele, and 2) to determine mechanisms by which efavirenz alters the pharmacokinetics of co-administered drugs. This work explores the hypothesis that the *CYP2B6*6* allele reduces efavirenz metabolism by altering catalytic properties of CYP2B6 and efavirenz alters the pharmacokinetics of co-administered drugs by inhibiting drug metabolizing enzymes. To test this hypothesis, we will pursue the following specific aims:

1. Determine whether the *CYP2B6*6* allele alters catalytic properties and susceptibility to metabolic inhibition of CYP2B6.
2. Determine whether the functional consequences of the *CYP2B6*6* allele are substrate- and Cyt b5-dependent.
3. Explore whether the incorporation of *in vitro* mechanism of reduced efavirenz metabolism by the *CYP2B6*6* allele can predict genetic effect on efavirenz pharmacokinetics after a single dose by modeling approach.

4. Determine whether efavirenz inhibits the activities of eight major human CYP isoforms *in vitro* and determine the mechanisms involved. For those isoforms that are inhibited in pilot experiments, inhibition constants (K_i values) will be estimated with which the extent of *in vivo* drug interactions is quantitatively predicted.

Together, this proposal will improve the safe and effective use of HIV medications through better understanding of genetic and non-genetic determinants of efavirenz disposition and the drug interactions associated with it. The accomplishment of this proposal will also allow the prevention of deleterious drug interactions that currently appear idiosyncratic and the optimization of dosing for a growing number of important HIV medications.

Chapter II: *Materials and methods*

This section describes common methods and specific materials utilized in experimental research sections that follow.

1. Chemicals

Efavirenz, 7, 8-hydroxyefavirenz, bupropion, 4-hydroxybupropion, nevirapine, ritonavir, voriconazole, clopidogrel, 7-hydroxycoumarin, desethylamodiaquine, S-mephenytoin, 4-hydroxy-S-mephenytoin, R-omeprazole, R-hydroxyomeprazole, R-lansoprazole and ritonavir were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). Acetaminophen, chloroquine, coumarin, dextromethorphan, dextrorphan, desmethyldiazepam, 8-methoxypsolaren, phenacetin, tolbutamide, 4-hydroxytolbutamide, chlorpropamide, testosterone, 6β-hydroxytestosterone, glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase and glycine were purchased from Sigma-Aldrich (St. Louis, MO). Amodiaquine and levallorphan were purchased from the United States Pharmacopeia (Rockville, MD). Monobasic and dibasic sodium phosphate, monobasic potassium phosphate, magnesium chloride, acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). All drug solutions were prepared by dissolving each compound in methanol or acetonitrile, and were stored at -20 ℃. All the other chemicals were of high performance liquid chromatography (HPLC) grade.

2. Microsomal preparations

2.1 Expressed CYP2B6.1 and CYP2B6.6 proteins

CYP2B6.1 and CYP2B6.6 proteins without and with co-expression of cytochrome b5 (Cyt b5) and plasmid-transfected negative controls were produced by BD

Biosciences (Woburn, MA) and kindly provided by Dr. Guo (Eli Lilly and Company, Indianapolis, IN). Briefly, CYP2B6 is expressed from human CYP2B6 cDNA using a baculovirus expression system. Baculovirus infected insect cells were used to prepare these microsomes. These microsomes also contain cDNA-expressed human P450 oxidoreductase (POR). In those proteins expressed without Cyt b5, the protein content, CYP 450 content using spectral assay and Cyt c reductase activity were 26.6 mg/ml, 1451 pmol/ml and 1739 nmol/(min*mg protein) for CYP2B6.1, and 33.5 mg/ml, 1582 pmol/ml and 1489 nmol/(min*mg protein) for CYP2B6.6. Assuming that a specific activity of 3.0 micromoles of Cyt c reduced per minute per nanomole of reductase (Parikh et al., 1997), the molar ratios of CYP 450 : reductase for CYP2B6.1 and CYP2B6.6 were 1 : 10.6 and 1:10.5, respectively. In those proteins co-expressed with Cyt b5, the protein content, CYP 450 content, Cyt c reductase activity and Cyt b5 content were 9.0 mg/ml, 1000 pmol/ml, 1900 nmol/(min*mg protein) and 220 pmol/mg protein for CYP2B6.1, while they were 2.7 mg/ml, 1000 pmol/ml and 851 nmol/(min*mg protein) and 370 pmol/mg protein for CYP2B6.6. The molar ratio of CYP 450 : reductase : Cyt b5 of CYP2B6.1 was 1 : 5.4 : 2 and that of CYP2B6.6 was 1: 0.73 : 1. All microsomal preparations were stored at -80 ℃ until analysis.

2.2 Human liver microsomes (HLMs) genotyped for the *CYP2B6*6* allele

HLMs obtained from liver tissues with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes were used for the metabolism and inhibition studies. Two HLMs sources were used. HLMs that were obtained from Medical College of Wisconsin (Milwaukee, WI), Medical College of Virginia (Richmond, VA), Indiana University School of Medicine (Indianapolis, IN) and University of Pittsburgh (Pittsburgh, PA) under protocols

approved by the appropriate committees for the conduct of human research were prepared by Eli Lilly and Company (Indianapolis, IN) and kindly provided by Dr. Guo. Liver microsomes were prepared by differential centrifugation (van der Hoeven and Coon, 1974). Additional HLMs for inhibition study to evaluate the effect of the *CYP2B6*6* allele on the inhibition susceptibility of CYP2B6 were obtained from in-house human liver tissues, which were medically unsuitable for transplantation and were prospectively collected in the Division of Clinical Pharmacology by Dr. Hall through the liver transplantation units of Indiana University hospitals. HLMs were prepared from these liver tissues by ultracentrifugation and protein concentrations were determined using standard protocols (Desta et al., 1998). Genotyping for the *CYP2B6*6* allele was performed in those human liver tissues from which HLMs were prepared. Liver samples were homogenized and genomic DNA was isolated using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The concentration of DNA was determined using the PicoGreen assay and the quality of DNA was checked by Agarose gel and PCR. The DNA samples were stored at -80˚C until analysis. The two SNPs tagging *CYP2B6*6* allele, 516G>T and 785A>G, were genotyped using either the Affymetrix DMET Premier Pack (Santa Clara, CA) or TaqMan® SNP genotyping assays (Foster City, CA) according to the respective manufacturer's protocols. *CYP2B6*1* was designated as the allele without these two tagging SNPs. All microsomal preparations were stored at -80 ℃ until analysis.

2.3 HLMs for efavirenz inhibition study

Pooled HLMs from 24 individual donors, HLMs with *CYP2C8*3/*3* genotype, and other HLMs were obtained from BD Biosciences (Woburn, MA). Human CYP2B6

and CYP2C8 expressed in baculovirus infected insect cells with oxidoreductase and without co-expression of Cyt b5 were obtained from BD Biosciences. All microsomal preparations were stored at -80 ℃ until analysis.

3. General incubation conditions

3.1 General incubation conditions of efavirenz and bupropion kinetics study

Efavirenz 8-hydroxylation and bupropion 4-hydroxylation (Figure 2.1) have been shown to be mainly catalyzed by CYP2B6 (Faucette et al., 2000; Ward et al., 2003). Therefore, we used these two probe reactions to determine CYP2B6 activity in expressed CYP2B6 proteins (CYP2B6.1 and CYP2B6.6) and HLMs obtained from human liver tissues genotyped for the *CYP2B6*6* allele. Efavirenz 7-hydroxylation is a minor pathway of its metabolism and solely catalyzed by CYP2A6 (Ogburn et al., 2010). Therefore, the formation of 7- and 8-hydroxyefavirenz from efavirenz incubations in HLMs obtained from liver tissues genotyped for the *CYP2B6*6* allele were simultaneously quantified by LC/MS/MS. All incubations were carried out using incubation times and protein concentrations that were within the linear range for reaction velocity. The key steps of the enzyme incubation are depicted in the Figure 2.2. Efavirenz and bupropion were dissolved and diluted in methanol to the required concentrations (1 to 200 μM efavirenz and 10 to 1000 μM bupropion), and methanol was removed by drying in speed vacuum before the addition of the incubation components. The reaction components contain 200 mM sodium phosphate buffer (pH 7.4), expressed CYP2B6 (10 to 15 pmol) or 25 μl of HLMs (2.5 mg/ml) and a substrate (efavirenz or bupropion) (total incubation volume of 250 μl). The incubation mixture was pre-warmed for 5 min at 37 ℃. The reaction was initiated by adding a NADPH-regenerating system (1.3 mM

NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 1 µl/ml glucose-6-phosphate dehydrogenase). Reaction was allowed to proceed for 15 min and then was terminated by placing tubes on ice and immediate addition of 500 µl acetonitrile. After an internal standard was added, the sample was vortex mixed and centrifuged at 14,000 rpm for 5 min. Ritonavir (50 µl of 0.01 mg/ml) and nevirapine (50 µl of 500 ng/ml) were used as an internal standard for 7, 8-hydroxyefavirenz assay by HPLC/UV and LC/MS/MS methods, respectively. For 4-hydroxybupropion assay, nevirapine (25 µl of 50 µM) was used as an internal standard for the HPLC/UV assay and 25 µl of 5 µM of nevirapine for the LC/MS/MS assay. The supernatant layer was moved to a clean tube and was extracted with 500 µl of 0.5 ml glycine/ NaOH buffer (pH 11.3) and 6 ml of ethyl acetate and then centrifuged at 36,000 rpm for 15 min. The organic layer was removed and evaporated to dryness. The residue was reconstituted with 100 µl of mobile phase, and an aliquot was injected into an HPLC or LC/MS/MS. The stock solutions of 7, 8-hydroxyefavirenz and 4-hydroxybupropion were prepared at a concentration of 1mg/ml in methanol. For HPLC assays, dilutions prepared in methanol at concentrations of 0.1 to 5 µM were used to prepare standard curves of 7, 8-hydroxyefavirenz, while concentrations of 0.5 to 20 µM were used for 4-hydroxybupropion. For LC/MS/MS assays, dilutions prepared in methanol at concentrations of 0.01 to 2.5 µM were used to prepare standard curves of 7, 8-hydroxyefavirenz, while concentrations of 0.1 to 10 µM were used for 4-hydroxybupropion. Efavirenz and bupropion metabolites were quantified by using the ratio of peak area of the metabolite to peak area of internal standard and standard curves that were constructed using known metabolite concentrations. Negative control incubations were run in parallel that included exclusion of efavirenz and bupropion, a

NADPH-generating system, or microsomes (bovine serum albumin was used instead) from the incubation mixture.

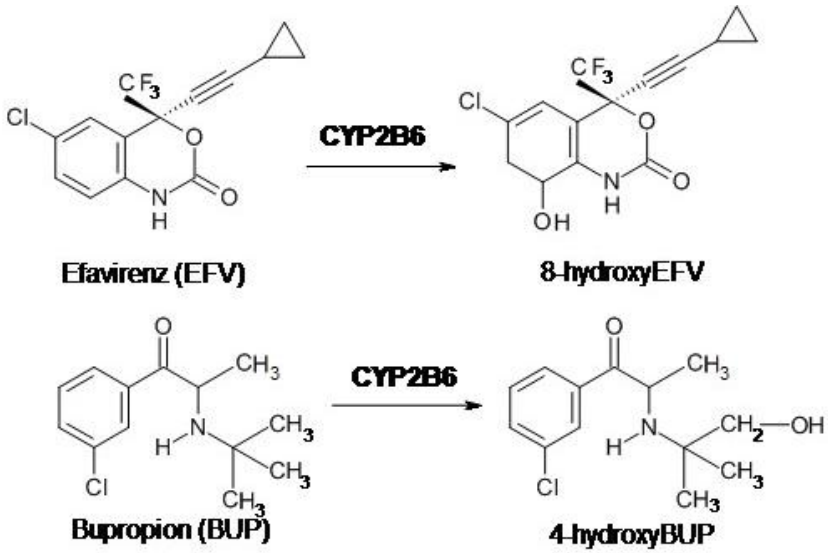

Figure 2.1 Structure of probe substrate reactions: CYP2B6-mediated 8-hydroxylation of efavirenz and CYP2B6-mediated 4-hydroxylation of bupropion.

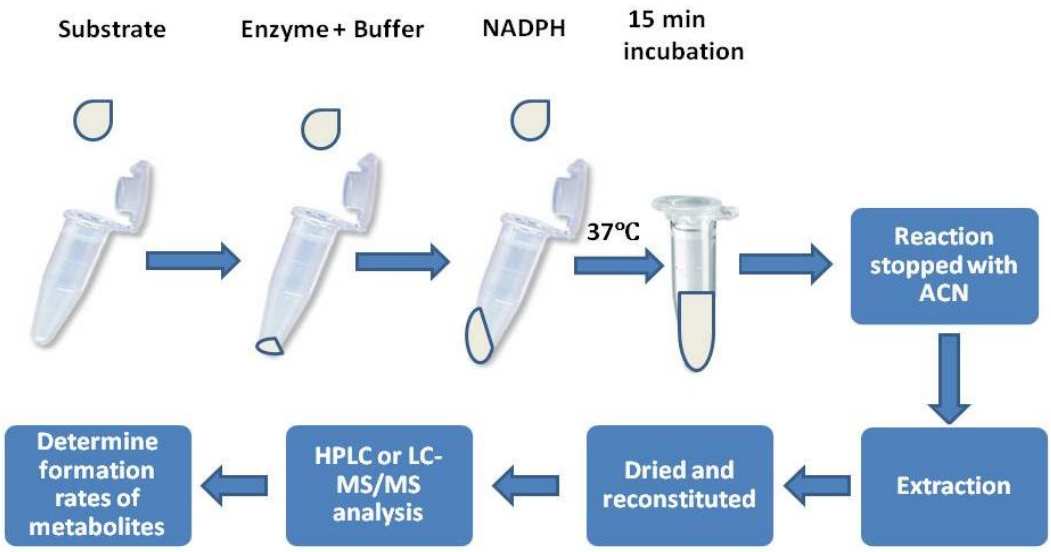

Figure 2.2 Key steps of enzyme incubation method. Substrates were dissolved and diluted in methanol to the required concentrations, and methanol was removed by drying in speed vacuum before the addition of the incubation components. The reaction components contain 200 mM sodium phosphate buffer (pH 7.4), microsomal enzymes and a substrate (total incubation volume of 250 µl). The incubation mixture was pre-warmed for 5 min at 37 ℃. The reaction was initiated by adding a NADPH-regenerating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 1 µl/ml glucose-6-phosphate dehydrogenase). Reaction was allowed to proceed for 15 min and then was terminated by placing tubes on ice and immediate addition of 500 µl acetonitrile. After an internal standard was added, the sample was vortex mixed and centrifuged at 14,000 rpm for 5 min. The supernatant layer was moved to a clean tube and was extracted and then centrifuged at 36,000 rpm for 15 min. The organic layer was removed and evaporated to dryness. The residue was reconstituted with mobile phase, and an aliquot was injected into an HPLC or LC/MS/MS. The amount of metabolites were quantified by using the ratio of peak area of the metabolite to peak area of internal

standard and standard curves that were constructed using known metabolite concentrations.

3.2 General incubation conditions of efavirenz inhibition study

Using incubation conditions specific to each isoform that were linear for time, substrate and protein concentrations, isoform selective substrate probes were incubated in duplicate at 37 ℃ with HLMs (or with expressed CYP when required), 200 mM sodium phosphate reaction buffer (pH 7.4) and NADPH-regenerating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 1 μl/ml glucose-6-phosphate dehydrogenase) in the absence or presence of varying concentrations of efavirenz (or 8-hydroxyefavirenz). The test inhibitors were dissolved and diluted in methanol to required concentrations and methanol was removed by drying in speed vacuum before the addition of the incubation components. The following HLMs concentrations were used: 1 mg protein/ml for CYP1A2, CYP2C9, CYP2C19 and CYP2D6; 0.5 mg protein/ml for CYP2A6, 0.25 mg protein/ml for CYP2B6 and CYP3A; and 0.1 mg/ml for CYP2C8 incubations. Inhibition constants (K_i values) were determined in expressed CYP2C8 (26 pmol) and CYP2B6 (5 pmol).

The inhibitory effects of efavirenz on the activities of different CYP isoforms were studied in HLMs, expressed CYP2B6 and CYP2C8 using the following selective reaction probes: phenacetin O-deethylation (CYP1A2); coumarin 7-hydroxylation (CYP2A6); bupropion 4-hydroxylation (CYP2B6); amodiaquine N-desethylation (CYP2C8); tolbutamide 4-hydroxylation (CYP2C9); S-mephenytoin 4-hydroxylation/R-omeprazole 5-hydroxylation (CYP2C19); dextromethorphan O-demethylation (CYP2D6); and testosterone 6β-hydroxylation (CYP3A). Kinetic analysis was performed for each substrate probe reaction before initiation of the inhibition experiments, and the data generated were used as a guide for selection of the appropriate concentrations of the

substrate probes in the subsequent inhibition experiments. Thus, the kinetic parameters for the metabolism of each probe substrate were determined by incubating a range of different concentrations of the substrate (without the inhibitor) at 37 ℃ in duplicate with HLMs (or expressed enzymes) and the NADPH-generating system. Phenacetin (5 to 1000 μM), coumarin (0.1 to 50 μM), bupropion (1 to 1000 μM), amodiaquine (0.1 to 100 μM), tolbutamide (5 to 500M), S-mephenytoin (5 to 100 μM), R-omeprazole (1 to 200 μM), dextromethorphan (1 to 200 μM), and testosterone (1 to 200 μM) were used. Formation rates of metabolite versus the substrate concentrations were fit to appropriate enzyme kinetic equations to estimate the apparent kinetic parameters.

4. Specific enzyme assays

The substrate reactions to determine the activities of different CYP isoforms and specific inhibitors for each CYP isoform are summarized in Table 2.1. The specific enzyme assays of 8 major human CYP isoforms were described below.

4.1 CYP1A2 activity (phenacetin O-deethylation to acetaminophen)

HLMs (1 mg/ml), different concentrations of phenacetin, the NADPH-generating system, and phosphate reaction buffer (pH 7.4) were incubated at 37 ℃ for 30 min. Acetonitrile (500 μl) was used to terminate the reaction. 25 μl of 50 μM nevirapine was used as an internal standard. After centrifugation at 14,000 rpm for 5 min, the supernatant layer was moved to a clean tube and was extracted with 500 μl of 0.5 ml glycine/NaOH buffer (pH 11.3) and 6 ml of ethyl acetate and then centrifuged at 36,000 rpm for 15 min. The organic layer was removed and evaporated to dryness. The residue was reconstituted with 150 μl of mobile phase, and an aliquot of 50 μl was injected into an HPLC. The HPLC system consisted of a Shimadzu LC-10AT pump, SIL-10AD auto-sampler, SCL-

10A system controller and SPD-10A UV-VIS detector. The HPLC eluates (acetaminophen and nevirapine) were separated with a Zorbax SB-C_{18} column (150 × 4.6 mm, 3.5-μm particle size; Phenomenex, Torrance, CA), a Luna C_{18} Guard column (30 × 4.6 mm, 5 μm; Phenomenex), and a mobile phase composed of 85% 50 mM KH_2PO_4 (pH 4.5) and 15% (v/v) acetonitrile (flow rate, 0.7 ml/min). Detection was performed with UV detection at 245 nm. The retention time of phenacetin, acetaminophen and nevirapine (IS) was 21.6, 4.5 and 13.0 min.

4.2 CYP2A6 activity (coumarin 7-hydroxylation to 7-hydroxycoumarin)

Coumarin was dissolved in methanol (stock, 100 mM) and was serially diluted in distilled water to the required concentration (final methanol concentration, ≤ 0.1%). Coumarin, 0.5 mg/ml HLMs, the NADPH-generating system, and phosphate reaction buffer (pH 7.4) were incubated at 37 ℃ for 15 min. The reaction was terminated by adding 100 μl of acetonitrile. The solution of internal standard, 8-methoxypsolaren, should be made fresh before the experiment, because it may degrade over time. After the internal standard (50 μl of 20 μg/ml) was added, the mixture was vortex mixed and centrifuged and an aliquot (150 μl) of the supernatant was transferred to HPLC vials; 100 μl of this aliquot was injected onto the HPLC system. The HPLC and separation column was the same as that used for the CYP1A2 assay described above. The mobile phase composed of 70% 10 mM KH_2PO_4 (pH 3.0) and 30% (v/v) acetonitrile (flow rate, 0.8 ml/min). The column eluate was monitored by UV detection at 280 nm (internal standard) or with a fluorescence detector at an excitation wavelength of 370 nm and an emission wavelength of 450 nm (7-hydroxycoumarin). The retention time of coumarine,

7-hydroxycoumarine and 8-methoxypsolaren (IS) was approximately 9.1, 4.6 and 17.7 min.

4.3 CYP2B6 activity (efavirenz 8-hydroxylation to 8-hydroxyefavirenz and bupropion 4-hydroxylation to 4-hydroxybupropion)

8-Hydroxyefavirenz formed from efavirenz incubations in expressed CYP2B6 was quantified by HPLC/UV system with slight modification as described previously (Ward et al., 2003). Briefly, the HPLC system consisted of a Shimadzu LC-10AT pump, SIL-10AD auto-sampler, SCL-10A system controller and SPD-10A UV-VIS detector. The separation system consisted of a Zorbax SB-C_{18} column (150 ×4.6 mm, 3.5-μm particle size; Phenomenex, Torrance, CA), a Luna C_{18} Guard column (30 ×4.6 mm, 5 μm; Phenomenex), and a mobile phase composed of 55% 10 mM KH_2PO_4 (adjusted to pH 2.7 with 1% phosphoric acid) and 45% (v/v) acetonitrile (flow rate, 1 ml/min). The column eluate was monitored by UV detection at 245 nm. The retention time of efavirenz, 8-hydroxyefavirenz and ritonavir (IS) was 21.6, 12.0 and 16.7 min. 8-Hydroxyefavirenz was quantified by using the ratio of peak area of the metabolite to peak area of internal standard and standard curves that were constructed using known 8-hydroxyefavirenz concentrations.

Due to the slow formation rates of 8-hydroxyefavirenz in HLMs samples, particularly in those with *CYP2B6*6/*6* genotype, a sensitive and selective LC/MS/MS method was developed to assay 8-hydroxyefavirenz from HLMs incubation and implemented as described in our previous publication (Ogburn et al., 2010). 7-Hydroxyefavirenz, a minor metabolite catalyzed solely by CYP2A6 in HLM incubations (Ogburn et al., 2010), was also simultaneously quantified by the same LC/MS/MS

method. The MS/MS system was an API 2000 MS/MS triple quadruple system (Applied Biosystems, Foster City, CA) equipped with a turbo ion spray and was coupled with a Shimadzu HPLC system (Columbia, MD) consisting of an LC-20AB pump and SIL-20A HT autosampler, all controlled by Analyst 1.4.2 software (Applied Biosystems/MDS Sciex, Foster City, CA) in conjunction with Windows 2000 (Microsoft, Redmond, WA). 7, 8-Hydroxyefavirenz and nevirapine were detected using multiple reactions monitoring (MRM) at a m/z of 332.2/248.3 and 267.1/226.4 in positive ion mode, respectively. The separation of 7, 8-hydroxyefavirenz are based on their different retention times. A representative MRM trace chromatogram of 7, 8-hydroxyefavirenz and nevirapine (IS) are depicted in Figure 2.3.

An HPLC assay method with UV detection was developed for the quantification of 4-hydroxybupropion from bupropion incubation in expressed enzymes. The HPLC and separation column were the same as those used for quantification of 8-hydroxyefavirenz. The mobile phase composed of 85% 10 mM KH_2PO_4 (adjusted to pH 3 with 85% phosphoric acid) and 15% (v/v) acetonitrile (flow rate, 1 ml/min). The column eluate was monitored by UV detection at 214 nm for 4-hydroxybupropion and 282 nm for internal standard (nevirapine). A LC/MS/MS assay was developed for the quantification of bupropion metabolite in HLMs incubation samples. The MS/MS system was the same as for efavirenz described above. In brief, bupropion, 4-hydroxybupropion and the internal standard (nevirapine) were separated using a Zorbax SB-C_{18} column (100 × 2.00 mm, 3 μm particle size; Phenomenex, Torrance, CA), a Luna C_{18} guard column (30 × 4.6 mm, 5 μm; Phenomenex), and an isocratic mobile phase that consisted of 75% formic acid (0.1% in H_2O) and 25% acetonitrile (flow rate, 0.3 ml/min). 4-Hydroxybupropion and

nevirapine were detected using multiple reactions monitoring at a m/z of 256.1/238.0 and 267.2/224.4 in positive ion mode, respectively. A representative MRM trace chromatogram of 4-hydroxybupropion and nevirapine (IS) are depicted in Figure 2.4.

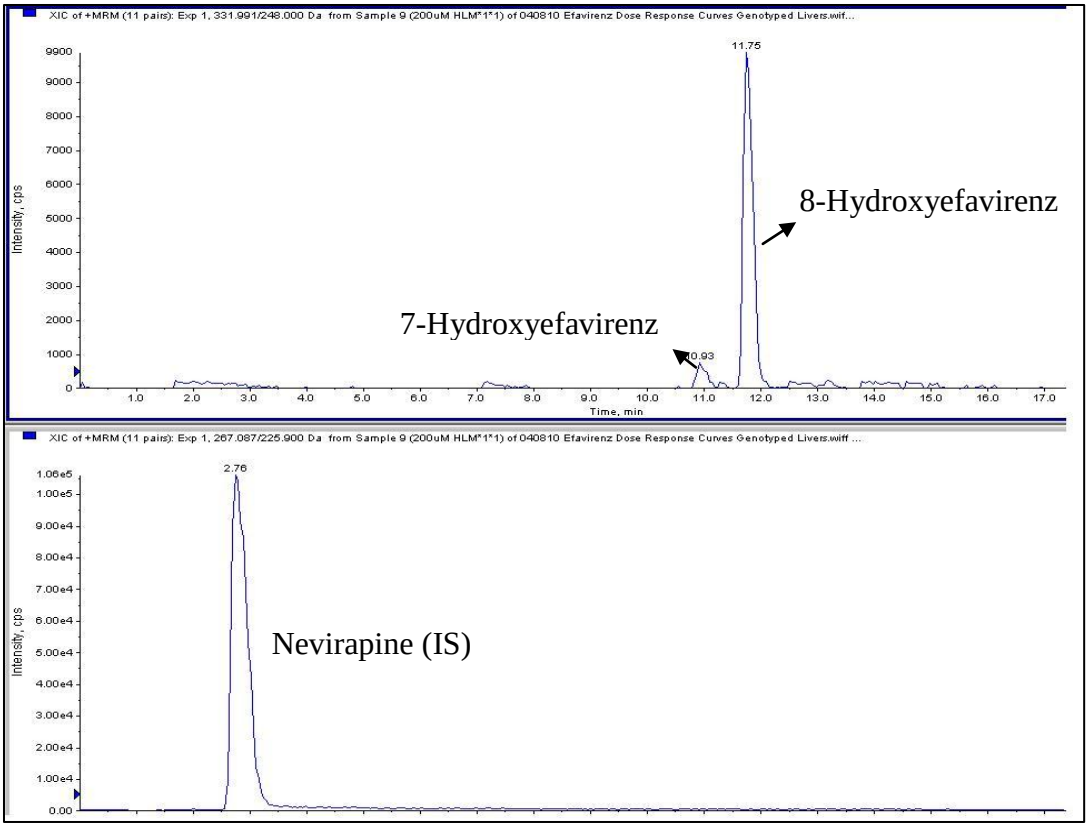

Figure 2.3 Multiple reactions monitoring (MRM) trace chromatograms of 7, 8-hydroxyefavirenz and nevirapine (IS) in microsomal incubates. Efavirenz was incubated in HLM samples and cofactors for 15 min at 37 ℃. Subsequent sample processing and LC/MS/MS conditions were described under *Materials and Methods*. 7, 8-Hydroxyefavirenz and nevirapine were detected using multiple reactions monitoring (MRM) at a m/z of 332.2/248.3 and 267.1/226.4 in positive ion mode, respectively. The separation of 7, 8-hydroxyefavirenz are based on their different retention times.

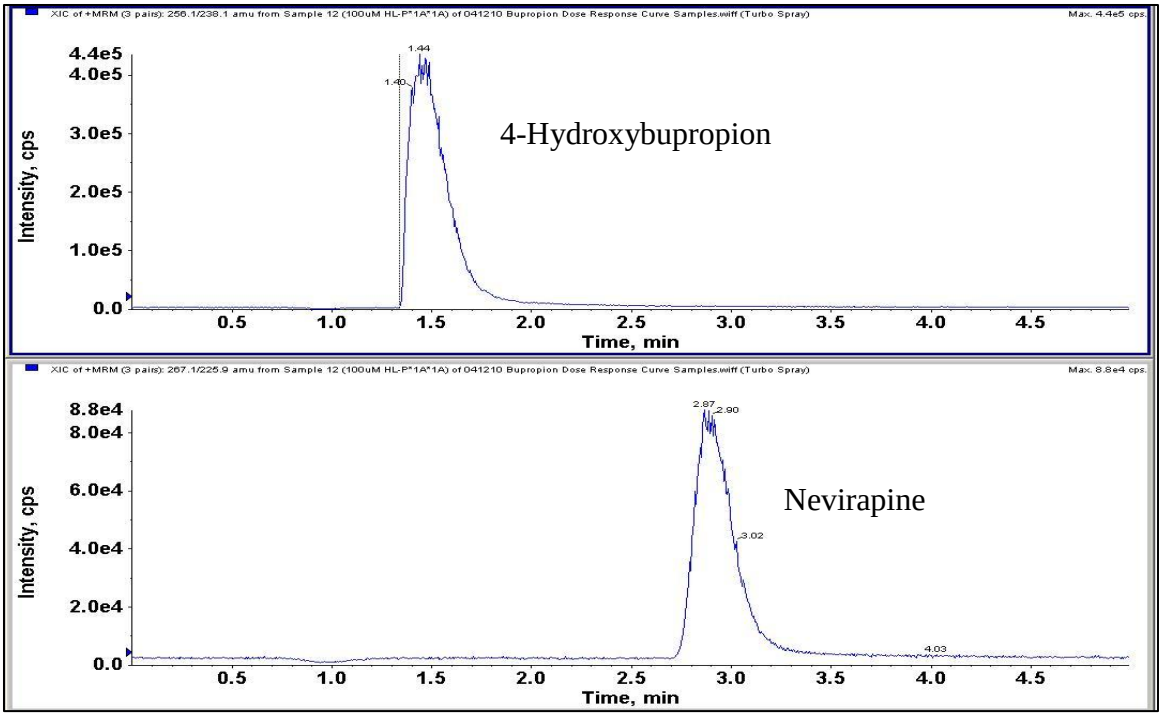

Figure 2.4 Multiple reactions monitoring (MRM) trace chromatograms of 4-hydroxybupropion and nevirapine (IS) in microsomal incubates. Bupropion was incubated in HLM samples and cofactors for 15 min at 37 ℃. Subsequent sample processing and LC/MS/MS conditions were described under *Materials and Methods*. 4-Hydroxyefavirenz and nevirapine were detected at a m/z of 256.1/238.0 and 267.2/224.4 in positive ion mode, respectively.

4.4 CYP2C8 activity (amodiaquine desethylation to desethylamodiaquine)

A mixture of amodiaquine, HLMs (0.1 mg/ml) or expressed CYP2C8 (26 pmol/ml), and the NADPH-generating system was incubated at 37 ℃ for 15 min. The reaction was terminated by addition of 100 µl acetonitrile, and chloroquine (50 µM) was added as an internal standard. The methods for further processing of the sample and the separation column were the same as those used in the CYP2A6 assay. We used a mobile phase that consisted of water, 16% (v/v) methanol, and 0.1% (v/v) triethylamine (pH 2.2); a flow rate of 1 ml/min; and UV detection at 340 nm. The retention time of amodiaquine, desethylamodiaquine and chloroquine (IS) was 15.4, 12.4 and 9.4 min.

4.5 CYP2C9 activity (tolbutamide 4-hydroxylation to 4-hydroxytolbutamide)

Tolbutamide, HLMs (1 mg/ml), and the NADPH-generating system were incubated for 60 min at 37 ℃. The reaction was terminated by addition of 100 µl 10% perchloric acid ($HClO_4$) and 50 µl of the internal standard, chlorpropamide (10 µg/ml), was added. After centrifugation at 14,000 rpm for 5 min, the supernatant layer was moved to a clean tube and was extracted with 500 µl of H_2O and 3 ml of ethyl acetate and then centrifuged at 36,000 rpm for 15 min. The organic layer was removed and evaporated to dryness. The residue was reconstituted with 150 µl of mobile phase, and an aliquot of 50 µl was injected into an HPLC. The separation column was the same as that used for the CYP1A2 assay. A mobile phase that consisted of 70% 10 mM ammonium acetate (pH 5.4) and 30% (v/v) acetonitrile (flow rate, 0.7 ml/min) and UV detection at 230 nm were used. The retention time of tolbutamide, 4-hydroxytolbutamide and chlorpropamide (IS) was 19.0, 4.6 and 8.0 min.

4.6 CYP2C19 activity (S-mephenytoin 4-hydroxylation to 4-hydroxy-S-mephenytoin and R-omeprazole 5-hydroxylation to R-hydroxyomeprazole)

S-Mephenytoin, HLMs (0.5 mg/ml), and the NADPH-generating system were incubated for 60 min at 37 ℃. The reaction was terminated by addition of 100 µl acetonitrile. The sample was centrifuged after addition of phenytoin (5 µg/ml) as an internal standard, and the supernatant was extracted with dichloromethane (2 ml). The organic layer was removed and evaporated to dryness with a speed vacuum. The residue was reconstituted in mobile phase (75% 50 mM KH_2PO_4 [pH 4.0], 25% [v/v] acetonitrile), and 100 µl was injected into an HPLC system. The separation columns were the same as those used for the CYP1A2 assay. The mobile phase was delivered at 0.7 ml/min, and the column eluate was monitored by UV detection at 211 nm. The retention time of S-mephenytoin, 4-hydroxy-S-mephenytoin and phenytoin (IS) was 21.0, 6.6 and 33.5 min.

A LC/MS/MS assay was developed for the quantification of the formation of R-hydroxyomeprazole. First, 25 µl of 1µg/ml R-lansoprazole was added to each sample as an internal standard. The incubation mixture was then extracted by the addition of 500 µl of 0.025 M NaCl (pH 7.5) and 6 ml of ethyl acetate. After centrifugation at 36,000 rpm for 15 min, the organic layer was evaporated to dryness and then reconstituted in 100 µl of mobile phase. The separation column used was Chiral-AGP (150 × 4.60 mm; 5 µM; Phenomenex). A gradient elution profile was used: initial mobile phase: 95% (v/v) 20 mM ammonium acetate (pH 6.5) and 5% acetonitrile; secondary mobile phase: 10% 20 mM ammonium acetate (pH 6.5) and 90% acetonitrile. The secondary mobile phase was increased from 0% to 40% linearly between 0 and 8 min; the initial mobile phase was

resumed after 9 min and remained constant for an additional 6 min, allowing the column to equilibrate. The eluate was introduced, without splitting, at 0.5 ml/min to the turbo ion source. R-hydroxyomeprazole and R-lansoprazole were detected using multiple reactions monitoring at m/z values of 362.13/214.10 and 370.25/252.30, respectively.

4.7 CYP2D6 activity (dextromethorphan O-demethylation to dextrorphan)

Dextromethorphan, HLMs (1 mg/ml), and the NADPH-generating system were incubated at 37 ℃ for 30 min. The reaction was terminated by addition of 20 µl 60% HClO4. Levallorphan (40 µl of 16 µM) was added as an internal standard and was processed further as described above for the CYP2A6 assay. An aliquot of the supernatant (100 µl) was injected into the HPLC system, which consisted of the separation column described above for the CYP2A6 assay; the mobile phase was 10 mM KH_2PO_4 with 0.114% triethylamine (pH 4.0), 23% (v/v) acetonitrile, and 20% (v/v) methanol (flow rate, 0.5 ml); and fluorescence detection was performed at an excitation wavelength of 200 nm and an emission wavelength of 304 nm. The retention time of dextromethorphan, dextrorphan and levallorphan was 13.3, 5.3 and 7.1 min.

4.8 CYP3A activity (testosterone hydroxylation to 6β-hydroxytestosterone)

Testosterone, HLMs (1 mg/ml), and the NADPH-generating system were incubated at 37 ℃ for 30 min. The reaction was terminated by addition of 20 µl 60% HClO4. Levallorphan (40 µl of 16 µM) was added as an internal standard and was processed further as described above for the CYP2A6 assay. An aliquot of the supernatant (100 µl) was injected into the HPLC system, which consisted of the separation column described above for the CYP2A6 assay; the mobile phase was 40% 30 mM ammonium acetate (pH 6.3) and 60% (v/v) methanol (flow rate, 1 ml); and UV

detection was set at 254 nm. The retention time of testosterone, 6β-hydroxytestosterone and desmethyldiazepam was 10.0, 5.0 and 10.4 min.

Table 2.1 Summary of substrate reactions and inhibitors for 8 major human cytochrome P450 enzymes

Cytochrome P450s	Substrate reactions	Inhibitors
CYP1A2	phenacetin O-deethylation	furafylline
CYP2A6	coumarin 7-hydroxylation	pilocarpine
CYP2B6	bupropion 4-hydroxylation	thioTEPA, clopidogrel,
	efavirenz 8-hydroxylation	voriconazole
CYP2C8	amodiaquine N-desethylation	quercetin
CYP2C9	tolbutamide 4-hydroxylation	sulfaphenazole
CYP2C19	S-mephenytoin 4-hydroxylation	ticlopidine
	R-omeprazole 5-hydroxylation	
CYP2D6	dextromethorphan O-demethylation	quinidine
CYP3A	testosterone 6β-hydroxylation	ketoconazole

5. Inhibition of CYP2B6 by voriconazole and clopidogrel in HLMs

To test the impact of the *CYP2B6*6* allele on metabolic inhibition of CYP2B6, inhibition experiments were performed in HLMs obtained from human liver tissues genotyped for the *CYP2B6*6* allele. IC_{50} values for the inhibition of CYP2B6 by voriconazole and clopidogrel were determined by incubating efavirenz (10 μM) with a NADPH-generating system and 25 μl of HLMs (2.5 mg/ml) at 37 ℃ for 15 min in the absence or presence of voriconazole (0.01 to 4 μM) and clopidogrel (0.003 to 2.5 μM) (total incubation volume of 250 μl). Dixon plots for the inhibition of CYP2B6 by voriconazole were determined by incubating efavirenz (10 to 100 μM) with a NADPH-generating system and 25 μl of HLMs (2.5 mg/ml) at 37 ℃ for 15 min in the absence or presence of voriconazole (0.1 to 10 μM) (total incubation volume of 250 μl). The samples were processed and 8-hydroxyefavirenz formed was quantified by LC/MS/MS as described above.

6. Determination of efavirenz in vitro Cl_{int} in HLMs and expressed CYP2B6

Efavirenz is primarily cleared by CYP2B6-mediated 8-hydroxylation and partially by CYP2A6-mediated 7-hydroxylation (Ward et al., 2003; Ogburn et al., 2010). Therefore, the formation rates of 7- and 8-hydroxyefavirenz from efavirenz incubations in HLMs obtained from liver tissues genotyped for the *CYP2B6*6* allele (n=5 for each genotype) as well as CYP2B6.1 and CYP2B6.6 without co-expression of Cyt b5 were determined by a LC/MS/MS method described above. Metabolism data from expressed CYP2B6 without co-expression of Cyt b5 was selected for the present study. Because Cyt b5 affects catalytic properties of CYP2B6 in a genotype- and substrate-dependent manner (Xu et al., 2012). The details of microsomal preparations, incubation conditions and assay

method have been described above. Briefly, 1-200 μM efavirenz were incubated with 200 mM potassium phosphate buffer (pH 7.4), expressed CYP2B6 (10-15pmol) or 25 μl of HLMs (2.5 mg/ml) and a NADPH-generating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, and l μl/ml glucose-6-phosphate dehydrogenase) for 15 min at 37 ℃. The formation rates of 7- and 8-hydroxyefavirenz were simultaneously measured with the quantifier MRM and confirmed with the qualifier MRM transition. The parent and daughter ions were the same for 7- and 8-hydroxyefavirenz and therefore these two metabolites were quantified based on chromatographic separation (Figure 2.1). Apparent kinetic constants (K_m and V_{max}) were estimated by fitting formation rates of metabolites versus efavirenz concentrations to simple single-site Michaelis-Menten equation by nonlinear regression analysis using Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). *In vitro* intrinsic clearance (Cl_{int}) was given as V_{max}/K_m.

7. Prediction of efavirenz clearance by Simcyp

7.1 Simcyp population physiologically-based pharmacokinetic (PBPK) modeling

The *in vitro-in vivo* extrapolation (IVIVE) of efavirenz single-dose pharmacokinetics was performed using the Simcyp® Population-based ADME Simulator (version 11.00; Simcyp Ltd., Sheffield, UK). The algorithm, physiological basis, and differential equations used by the software have been described in previous publications (Rostami-Hodjegan and Tucker, 2007; Jamei et al., 2009). Input parameters including the physicochemical properties of efavirenz, *in vitro* Cl_{int} in HLMs and expressed enzymes are listed in Table 2.2. All other parameters used for simulations were set to default Simcyp values. Although UGT 2B7 has been suggested to be also involved in efavirenz metabolism *in vitro* (Belanger et al., 2009) and *in vivo* (Kwara et al., 2009a), the

contribution of N-glucuronidation pathway to efavirenz overall metabolism seems minimal (Cho et al., 2011). Therefore, the present study did not take UGT2B7-mediated pathway into account. Simulations were performed using a virtual population consisted of the built-in Sim-healthy volunteers population in 20 trials, and in each trial the number of subjects was matched to that in the clinical trial (*CYP2B6*1/*1*: n=8; *CYP2B6*1/*6*: n=9, *CYP2B6*6/*6*: n=3). The total number of 160 subjects were simulated for the *CYP2B6*1/*1* genotype; the total number of 180 subjects were simulated for the *CYP2B6*1/*6* genotype; the total number of 60 subjects were simulated for the *CYP2B6*6/*6* genotype. Mean, median and standard deviation of key pharmacokinetic parameters were determined. Simulations were done on an exclusive extensive metabolizer (EM) CYP2B6 population and an exclusive slow metabolizer (SM) CYP2B6 population. Initially, all combinations of the three models for absorption and two models for distribution, i.e. minimal and full PBPK models embedded within Simcyp®, were tested and simulated efavirenz time-concentration profiles were compared with the observed plasma concentrations in the clinical trial. The final model was selected based on visual predictive checks and comparison between the mean (5^{th} and 95^{th} percentile) of simulated and observed key pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0-\infty}$ and CL_{po}).

7.2 Recalculation of intersystem extrapolation factors (ISEFs)

Intersystem extrapolation factors (ISEFs) built in the Simcyp were calculated on the basis of V_{max} for each individual CYP. The default values of ISEFs for BD CYP2B6 and CYP2A6 supersomes are 0.43, and were initially tested in our model. Then, the ISEFs for CYP2B6.1 and CYP2B6.6 were recalculated on the basis of V_{max} and Cl_{int} of efavirenz 8-hydroxylation determined in HLMs with *CYP2B6*1/*1 and *6/*6* genotypes

and expressed CYP2B6 proteins, respectively. The CYP2B6 abundance was assumed to be 17 pmol/mg and 6 pmol/mg in the wild type and homozygote according to Simcyp. In addition, ISEF for CYP2A6 was also recalculated using V_{max} and Cl_{int} of efavirenz 7-hydroxylation determined in HLMs and expressed CYP2A6. Since no significant difference in V_{max} and Cl_{int} of efavirenz 7-hydroxylation determined in the HLMs with different *CYP2B6*6* genotypes was observed and CYP2A6 was suggested to be the sole enzyme responsible for 7-hydroxylation *in vitro* (Ogburn et al., 2010), mean value calculated from V_{max} and Cl_{int} determined in individual HLM was used in the recalculations of ISEF for CYP2A6.

Table 2.2 *In vitro* input parameters of efavirenz in Simcyp

PhysicoChem	Value	Reference	CL_{int} from HLMs (Pathway) µl/min/mg protein		CL_{int} from rhCYP (Pathway) µl/min/pmol P450	
MW	315.67		CYP2B6*1/*1 (8-OH)[a]	14.5	CYP2B6.1 (8-OH)[a]	0.39
LogP	4.6	(Drug Bank)	CYP2B6*1/*6 (8-OH)[a]	6.4	CYP2B6.6 (8-OH) [a]	0.19
B/P ratio	0.74	(Balani et al., 1999)	CYP2B6*6/*6 (8-OH)[a]	2.4	CYP2A6 (7-OH)[c]	0.05
$f_{u,plasma}$	0.029	(Shou et al., 2008)	CYP2B6*1/*1 (7-OH)[b]	1.1	CYP2A6 (8-OH) [c]	0.08
f_{umic}	0.30	(Rekic et al., 2011)	CYP2B6*1/*6 (7-OH) [b]	1.5	CYP3A4 (8-OH)[d]	0.007
Plasma binding protein	albumin		CYP2B6*6/*6 (7-OH)[b]	1.3	CYP3A5 (8-OH) [d]	0.03
Caco-2 permeability (10^{-5} cm/s)	8.92	(Takano et al., 2006)			CYP1A2 (8-OH) [d]	0.07

[a] Data from Xu, et al. (2012)

[b] Data from the present study

[c] Data from Ogburn, et al. (2011)

[d] Data from Ward, et al. (2003)

8. Prediction of efavirenz clearance by Well-stirred liver model

Prediction of mean efavirenz CL_{po} extrapolated from *in vitro* Cl_{int} determined in HLMs and expressed CYP2B6 was also performed by Well-stirred liver model.

8.1 Extrapolation of expressed CYP metabolism data

ISEFs were applied to account for the differences in turnover number between expressed proteins and HLMs (Proctor et al., 2004). ISEFs were defined with respect o either the V_{max} of metabolite formation of a probe substrate or Cl_{int} using equations as described below:

$$\text{VISEF} = \frac{V_{max}\,(\text{HLM})}{V_{max}\,(\text{rhCYP}) \times \text{CYP abundance (HLM)}} \quad (1)$$

$$\text{CLISEF} = \frac{Cl_{int}\,(\text{HLM})}{Cl_{int}\,(\text{rhCYP}) \times \text{CYP abundance (HLM)}} \quad (2)$$

The *in vivo* Cl_{int} was scaled on the basis of measured *in vitro* Cl_{int} in expressed CYPs and ISEFs using equation 3 modified from Proctor et al.:

$$Cl_{int} = \left[\sum_{j=1}^{n} \frac{Cl_{intj}\,(\text{rhCYP}) \times CYP_j \text{ abundance} \times \text{ISEF}(CYP_j)}{f_{umic}} \right] \times \text{MPPGL} \times \text{liver weight} \quad (3)$$

where the values of milligrams of microsomal protein per gram of liver (MPPGL) and total liver weight are both set to the same as Simcyp for the purpose of comparison. The mean of MPPGL value built in Simcyp is 39.8 mg/g and the average liver weight is 1717 g, which are similar to published values (Obach, 1999; Wilson et al., 2003). The importance of correcting for nonspecific protein binding in IVIVE has been shown (Obach, 1996; Ito and Houston, 2005). Therefore, the prediction of Cl_{int} was corrected for nonspecific protein binding in equation 3.

8.2 Extrapolation of HLM metabolism data

Assuming that 7- and 8-hydroxylation are the main clearance mechanisms of efavirenz and that other elimination pathways are negligible and that sequential metabolism is minimal in the incubation conditions used in this study. The total *in vitro* Cl_{int} of efavirenz in HLMs was calculated as the summation of Cl_{int} estimated from these two pathways:

$$Cl_{int,\ HLM} = Cl_{int,\ 8\text{-}OHEFV} + Cl_{int,\ 7\text{-}OHEFV} \qquad (4)$$

The overall *in vivo* Cl_{int} of efaivrenz was scaled from *in vitro* Cl_{int} generated from HLMs using equation 5:

$$Cl_{int} = \frac{Cl_{int,HLM}}{f_{umic}} \times MPPGL \times \text{liver weight} \qquad (5)$$

where the values of scaling factors are the same as described above.

8.3 Prediction of efavirenz clearance by Well-stirred liver model

When $f_u \times Cl_{int} << Q_h$, the hepatic clearance can be estimated from *in vivo* Cl_{int} using the well-stirred liver model by a simplified equation defined in equation 6:

$$CL_h = \frac{Q_h \times f_u \times Cl_{int}}{Q_h + f_u \times Cl_{int}} \qquad (6)$$

where f_u is the fraction unbound of efavirenz in the plasma (~0.029) (Shou et al., 2008), CL_h is the hepatic blood clearance, and Q_h is the hepatic blood flow (~86 L/h) (Obach, 1999). Since IV formulation of efavirenz is not available, CL_h of the drug is unknown. However, efavirenz is mainly cleared by hepatic metabolism and no major first pass metabolism appears to occur, we assumed that the value of oral clearance (CL_{po}) is similar to that of CL_h. Thus, efavirenz CL_h predicted from *in vitro* Cl_{int} was compared to

observed CL_{po} after administration of a single 600 mg oral dose to healthy volunteers in the clinical trial.

9. Clinical study: efavirenz single-dose pharmacokinetics

Efavirenz clinical data was obtained from a pharmacokinetic study that was conducted as part of a drug interaction trial. In a randomized, placebo controlled crossover clinical trial, healthy subjects (n=20) genotyped for the *CYP2B6*6* allele received a single 600 mg oral dose of efavirenz after 10 day treatment with placebo or rifampin. The study was approved by the Institutional Review Board (IRB) of Indiana University School of Medicine. Signed and dated written informed-consent forms were obtained from each subject. Eligible subjects were randomized to take either a daily 600-mg oral dose of rifampin or placebo from day 1 through day 10. On day 11, after predose blood collection, subjects were given a single 600-mg oral dose of efavirenz on an empty stomach. Blood samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48, and 72 h after efavirenz dosing for pharmacokinetic analysis. After a washout period of 11 days, subjects started taking rifampin or placebo in a crossover fashion for 10 consecutive days and underwent the same procedure as in the first phase of the study. Plasma samples were separated by centrifugation at 3,000 rpm for 20 min within an hour of blood collection. Plasma samples were immediately stored at -80 ℃ until analysis. Plasma concentrations of efavirenz and its metabolites were determined by a validated LC/MS/MS method described previously (Ogburn et al., 2010). Data from the placebo treated arm were used in the present study. All pharmacokinetic parameters (C_{max}, T_{max}, AUC $_{0-\infty}$ and CL_{po}) were calculated by noncompartmental analysis using WinNonlin professional software (version 5.01; Pharsight, Mountain View, CA).

10. *In vitro* analysis of efavirenz inhibition of eight human cytochrome P450s

10.1 Screening of inhibition on multiple CYPs

Kinetic analysis was performed for each substrate probe reaction before initiation of the inhibition experiments, and the data generated were used as a guide for selection of the appropriate concentrations of the substrate probes in the subsequent inhibition experiments. Thus, the kinetic parameters for the metabolism of each probe substrate were determined by incubating a range of different concentrations of the substrate (without the inhibitor) at 37 ℃ in duplicate with HLMs (or expressed enzymes) and the NADPH-generating system. Phenacetin (5 to 1000 μM), coumarin (0.1 to 50 μM), bupropion (1 to 1000 μM), amodiaquine (0.1 to 100 μM), tolbutamide (5 to 500M), S-mephenytoin (5 to 100 μM), R-omeprazole (1 to 200 μM), dextromethorphan (1 to 200 μM), and testosterone (1 to 200 μM) were used. Formation rates of metabolite versus the substrate concentrations were fit to appropriate enzyme kinetic equations to estimate the apparent kinetic parameters. Apparent kinetic constants (K_m, V_{max}) were estimated by fitting formation rates of metabolites versus substrate concentrations to simple single-site Michealis-Menten equation by nonlinear regression analysis using SigmaPlot 11.0 (Systat Software Inc., Richmond, CA).

A single isoform-specific substrate concentration at about the respective K_m value (50 μM phenacetin, 10 μM coumarin, 50 μM bupropion, 25 μM amodiaquine, 150 μM tolbutamide, 50 μM S-mephenytoin, 25 μM R-omeprazole, 10 μM dextromethorphan, and 10 μM testosterone) was incubated at 37 ℃ in duplicate with HLMs and the NADPH-generating system in the absence or the presence of efavirenz concentrations at 10 and 50 μM, which covered the range of average steady-state C_{max} receiving 600mg

once daily (Bristol-Myers Squibb Company, June 2012). Processing of the incubation mixture and HPLC analysis of the metabolites formed were performed as described above.

Positive control experiments were run in parallel by incubating each probe substrate at 37 ℃ in duplicate with HLMs and the NADPH-generating system in the absence (control) and the presence of the following isoform-specific inhibitors: furafylline (20 μM; specific for CYP1A2), pilocarpine (50 μM; specific for CYP2A6), thioTEPA (50 μM; specific for CYP2B6), ticlopidine (5 μM; specific for CYP2B6), quercetin (10 μM; specific for CYP2C8), sulfaphenazole (25 μM; specific for CYP2C9), ticlopidine (5 μM; specific for CYP2C19), quinidine (1 μM; specific for CYP2D6), and ketoconazole (1 μM; specific for CYP3A). The substrate probes and concentrations that were used in the screening experiments (see above) were used for these positive control experiments. Formation rates of the metabolites in the presence of the isoform-specific inhibitor were compared with that for controls in which the inhibitor was replaced with vehicle.

10.2 Determination of inhibition constants (K_i values)

In pilot experiments, efavirenz showed inhibition on the activities of CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A (by >20% at 50 μM); inhibition on the other CYPs (CYP1A2, 2A6, and 2D6) was minimal. Therefore, Dixon plots for the inhibition of CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A were determined by incubating multiple concentrations of the respective substrate probe in the presence and absence of multiple concentrations of efavirenz with HLMs and cofactors. K_i values in expressed enzymes were determined for two CYP isoforms that showed potent inhibition in pooled

HLMs with K_i < 10 μM, i.e. CYP2B6 and CYP2C8. The following isoform-specific probe substrate concentrations were used: 25 to 75 μM bupropion for CYP2B6; 10 to 100 μM amodiaquine for CYP2C8; 50 to 250 μM tolbutamide for CYP2C9; 15 to 75 μM S-mephenytoin for CYP2C19 and 5 to 50 μM testosterone for CYP3A. The concentrations of efavirenz used were 0 to 100 μM. The inhibition data obtained from the pilot experiments were used as a guide to generate appropriate probe substrate and test inhibitor concentrations for the determination of the K_i values for each isoform. To calculate K_i values, the inhibition data were fit to different models of enzyme inhibition (competitive, noncompetitive, and uncompetitive) by nonlinear least-squares regression analysis with the Prism Version 5.0 software (GraphPad software Inc., San Diego, CA, USA). The final model for each data set was selected on the basis of visual inspection of Lineweaver-Burk, Dixon, and Eadie-Hofstee plots, as well as the size of the sum of squares of residuals, the Akaike information criterion, and Schwartz criterion values.

10.3 Assessment of time-dependent inhibition

Efavirenz was reported to be a time-dependent inhibitor of expressed CYP2B6, with a K_I value of 30 μM (Bumpus et al., 2006). Therefore, time-dependent inhibition was tested on eight major CYPs except for CYP2B6 using pooled HLMs. Efavirenz (50 μM) was pre-incubated in duplicate with HLMs and 200 mM sodium phosphate reaction buffer (pH 7.4) (without or with the NADPH-generating system) in the absence of a substrate probe for 0, 5, 10, 15 and 30 min at 37 ℃. The preincubation reaction was started by adding the NADPH-generating system. Controls were pre-incubated for 0 min without efavirenz and without the NADPH generating system. The total volume of the preincubation mixture was 600 μl. After 0, 5, 10, 15 and 30 min of preincubation, 50 μl

of preincubation mixture was added to a glass tube containing 950 µl mixture that consisted of a substrate (final concentration corresponding to the V_{max}), phosphate reaction buffer and NADPH-generating system. The mixture was further incubated for the time specific for each assay. The reaction was stopped and processed as described above for the co-incubation experiments.

11. Quantitative prediction of in vivo drug interactions

Predictions of *in vivo* drug interaction potential of efavirenz were made using the following equations, which has been described previously (Obach et al., 2006):

$$\frac{AUC_I}{AUC} = \frac{1}{\frac{f_m}{1+\frac{[I]}{K_i}} + (1-f_m)} \quad (7)$$

where $\frac{AUC_I}{AUC}$ is the ratio of the AUC of substrate after inhibition to the AUC of the uninhibited substrate; f_m is the fraction of substrate metabolized by the inhibited CYP pathway. The utilities of four different values for *in vivo* inhibitor concentrations, which are the total systemic C_{max}, free systemic C_{max}, total hepatic inlet C_{max} estimated after oral administration and free hepatic inlet C_{max} in the prediction of drug interactions have been compared before and estimation using free hepatic inlet C_{max} yielded the most accurate predictions of the magnitude of drug interactions (Obach et al., 2006). But efavirenz is highly protein bound with fraction unbound in plasma estimated to be 0.029 (Shou et al., 2008). In order to avoid underestimation of potential risk for drug interaction, total hepatic inlet C_{max} ($C_{hep,inlet}$) of efavirenz were used in present study (equation 8), which also showed a reasonably good prediction in the previous study (Obach et al., 2006).

$$C_{hep,inlet} = C_{max} + \frac{K_a F_a D}{Q_h} \quad (8)$$

Where C_{max} is maximum plasma concentration; K_a is the absorption rate constant, which is estimated to be 0.3 h^{-1} (Csajka et al., 2003); F_a is the fraction of the inhibitor passing through the intestine unchanged; D is the administered therapeutic dose (600 mg/day); and Q_h is hepatic blood flow (87 L/h) (Walsky et al., 2006). Values of F_a can be estimated from oral bioavailability and hepatic extraction. Since both values are not available for efavirenz, a value of unity for F_a was assumed as the most cautious possibility (Obach et al., 2005; Walsky et al., 2006). There is no evidence that efavirenz enhances the activities of CYP2C8 and CYP2C9 *in vivo*. Therefore, C_{max} of efavirenz at steady state was used (9.2 -16.6 μM) to predict the AUC changes of CYP2C8 and CYP2C9 substrates (Bristol-Myers Squibb Company, June 2012). However, efavirenz has been shown to enhance the activities of CYP2B6, CYP2C19 and CYP3A upon multiple dosing, suggesting inhibition effect may masked by inductive effect of efavirenz in a time-dependent manner. Therefore, predicting AUC changes CYP2B6, CYP2C19 and CYP3A substrates was estimated using C_{max} (4.6-8.4 μM) obtained after the administration of a single 600 mg oral dose of efavirenz to 20 healthy volunteers (Xu, et al. 2012). Specifically, we focused on substrates that exhibit narrow therapeutic range and thus initiation of efavirenz to patients stabilized on these drugs may increase the risk of adverse effects (methadone) or failure of therapy (clopidogrel and proguanil). Of note, clopidogrel and proguanil are pro-drugs that require conversion to pharmacologically active metabolites primarily by CYP2C19 (Carrington et al., 1951; Jeppesen et al., 1997; Hulot et al., 2006). For these prodrugs that require conversion by CYPC19 to active metabolites, the following equation published previously (Lutz and Isoherranen, 2012) was slightly modified to predict the extent of inhibition of bioactivation:

$$\frac{AUC_m}{AUC_p} = \frac{f_{u,p} \times Cl_f}{f_{u,m} \times Cl_m} \quad (9)$$

where $\frac{AUC_m}{AUC_p}$ is the ratio of the AUC of metabolite to the AUC of parent compound. The $f_{u,p}$ and $f_{u,m}$ are the plasma fraction unbound of the parent and metabolite. Cl_f and Cl_m are the formation and elimination clearance of metabolite.

The majority of clopidogrel is metabolized by an esterase (Lins et al., 1999) and relative importance of CYP2C19 for the overall elimination of proguanil is much lower than it is for the formation of cycloguanil (Jeppesen et al., 1997). Assuming that efavirenz does not affect the elimination of metabolite and the change in the AUC of parent is negligible, the equation 10 can be derived from equation 7 and 9 as:

$$\frac{AUC_m}{AUC_{m,I}} = \frac{Cl_f}{Cl_{f,I}} = \frac{1}{\frac{f_m}{1+\frac{[I]}{K_i}} + (1-f_m)} \quad (10)$$

where $\frac{AUC_m}{AUC_{m,I}}$ is the ratio of the uninhibited AUC of metabolite to the AUC of metabolite with inhibition. $\frac{Cl_f}{Cl_{f,I}}$ is the ratio of the uninhibited formation clearance to the formation clearance after inhibition. f_m for clopidogrel and proguanil is defined as the fraction of active metabolites formed by CYP2C19. Since f_m for proguanil is not available in the literature, it was estimated using a pharmacogenetic method modified from a recent study for CYP2D6 substrate (Tod et al., 2011). The original method was based on the observation that the ratio of AUC in poor metabolizer (PM), AUC^{SM}, to the AUC in extensive metabolizer (EM), AUC^{EM}. Assuming that genetic polymorphisms do not affect the elimination of metabolite and has limited effects on the AUC of the parent compound, the value of f_m for proguanil metabolized to cycloguanil by CYP2C19 was estimated from a clinical study (Jeppesen et al., 1997) using the following equation:

$$\frac{AUC_m^{EM}}{AUC_m^{PM}} = \frac{1}{1-f_m^{EM}} \quad (11)$$

where $\frac{AUC_m^{EM}}{AUC_m^{PM}}$ is the ratio of metabolite AUC in poor metabolizer (PM) to the metabolite AUC in extensive metabolizer (EM).

12. Statistical analysis

Statistical comparisons of metabolism and inhibition kinetic parameters among genotypes were performed using one-way ANOVA with Dunn's post-hoc test for multiple comparison correction. Independent t-test was used to compare parametric data from two groups. Mann-Whitney test or Wilcoxon test was performed for nonparametric data. Correlation analysis was performed by a nonparametric test (Spearman's rank correlation test). All statistical tests were performed using GraphPad. $p < 0.05$ was considered statistically significant.

The variability and accuracy of extrapolated efavirenz clearance using different values of ISEFs by Simcyp and well-stirred liver model were evaluated. Root mean square error (RMSE) and mean residual sum (MRS) were calculated based on equation 12 and 13. A smaller RMSE indicates less variability between the observed and extrapolated data. The MRS was sued to measure the bias of different methods. A negative value indicates underestimation and a positive value indicates overestimation.

$$RMSE = \sqrt{\frac{\sum_1^n (\text{predicted CL-observd CL})^2}{n}} \quad (12)$$

$$MRS = \frac{\sum_1^n (\text{predicted CL-observed CL})}{n} \quad (13)$$

The overall accuracies of the static prediction using different ISEFs were determined by equation 14 (Obach, 1999):

$$\text{Average fold error} = 10^{\left|\frac{\sum \log\left(\frac{\text{predicted}}{\text{observed}}\right)}{n}\right|} \qquad (14)$$

CHAPTER III: *Altered catalytic properties by the CYP2B6*6 allele may contribute to reduced efavirenz metabolism*

1. *Introduction*

Efavirenz, a potent non-nucleoside reverse transcriptase inhibitor (NNRTI), remains preferred component of antiretroviral therapy (ART) for treatment naïve patients despite an emergence of second generation of NNRTIs and new classes of antiretroviral agents (Thompson et al., 2012). However, its effective and safe use is compromised by the large inter-individual variability in its pharmacokinetics and clinical responses. Subsequent to the demonstration that CYP2B6 is the principal clearance mechanism of efavirenz *in vitro* (Ward et al., 2003), a number of clinical studies have documented that the genetic polymorphism of *CYP2B6* are significantly associated with higher efavirenz plasma concentrations (Haas et al., 2004; Tsuchiya et al., 2004; Wang et al., 2006; Rotger et al., 2007; Holzinger et al., 2012), intracellular concentrations (Rotger et al., 2005; Elens et al., 2010), and/or increased risk to adverse events (Haas et al., 2004; Gounden et al., 2010; Yimer et al., 2011; Mugusi et al., 2012).

The *CYP2B6* gene is highly polymorphic (Zanger et al., 2007) as reflected by 37 associated alleles, many sub-alleles and SNPs (http://www.imm.ki.se/CYPalleles/cyp2b6.htm). Of the variants so far identified, the *CYP2B6*6* haplotype defined by two non-synonymous single nucleotide polymorphisms (SNPs), 516G>T (Q172H) and 785A>G (K262R), is clinically important because this allele or the SNP tagging it (G516T) occurs at high frequency in all ethnic populations [14-62% (Zanger et al., 2007; Li et al., 2012)] and has been consistently found to be associated with increased risk for higher efavirenz exposure and/or adverse effects (Haas

et al., 2004; Tsuchiya et al., 2004; Zanger et al., 2007; Gounden et al., 2010; Yimer et al., 2011; Mugusi et al., 2012). In addition, this variant has also been associated with the elimination and/or response of other CYP2B6 substrate drugs including nevirapine (Rotger et al., 2005), cyclophosphamide (Nakajima et al., 2007) and methadone (Eap et al., 2007).

Four possible mechanisms have been proposed to contribute to the altered drug metabolism by the *CYP2B6*6* allele: (1) the *CYP2B6*6* allele is associated with a lower transcription rate due to linked promoter variants, (2) this variant allele leads to aberrant splicing of CYP2B6 pre-mRNA and results in reduced protein expression, (3) this variant allele may be associated with altered post-transcriptional modification, e.g. degradation of the enzyme, and (4) this variant allele may alter function of the enzyme due to the altered protein structure by the amino acid changes.

Previous study reported that this variant results in aberrant splicing of pre-mRNA and thereby reduces CYP2B6 expression and activity, while no promoter variant linked with reduced transcription rate has been identified yet (Hofmann et al., 2008). Nitric oxide has been shown to play a significant role in the down-regulation of CYP2B6 protein by a post-transcriptional manner (Aitken et al., 2008), but no evidence has been shown that the *CYP2B6*6* allele alters this regulation.

Our hypothesis of that the *CYP2B6*6* allele may alter function of the enzyme due to the altered protein structure by the amino acid changes is based on the evidence of structure-function relationship of CYP2B6. The *CYP2B6*6* allele has been associated with functional consequences in expressed systems (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus and Hollenberg, 2008; Watanabe et al., 2010; Ariyoshi et al., 2011; Zhang

et al., 2011b) and human liver microsomes (HLMs) (Lang et al., 2001; Lamba et al., 2003; Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007). In HLMs, the *CYP2B6*6* allele is associated with reduced total amount of CYP2B6 protein (Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007). However, mounting evidence indicate that reduced protein expression alone may not explain the functional consequences of this allele. For substrates that include cyclophosphamide, this allele is associated with enhanced metabolism despite reduced protein expression (Xie et al., 2003), which appears due to substantially lower K_m in the variant versus wild type protein (Ariyoshi et al., 2011). Other *in vitro* studies, mostly in expression systems, have also reported that the *CYP2B6*6* allele or the amino acids harbored in it influence catalytic properties, although the extent and direction of effect appears to depend on the substrate and the enzyme sources used (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus and Hollenberg, 2008; Watanabe et al., 2010; Zhang et al., 2011b). Therefore, in addition to reduced protein expression, altered enzyme function by the *CYP2B6*6* allele may contribute to altered substrate metabolism.

In this dissertation I explored the mechanism by which the *CYP2B6*6* allele alters drug metabolism *in vitro* and tested whether the incorporation of *in vitro* mechanism of reduced efavirenz metabolism by this genetic variant can predict genetic effect on efavirenz pharmacokinetics. In this section I tested the hypothesis that the *CYP2B6*6* allele changes catalytic properties, i.e. binding affinity and/or catalytic efficiency and this may contribute to reduced efavirenz metabolism. I determined the kinetics of efavirenz metabolism to 8-hydroxyefavirenz in expressed CYP2B6.1 and CYP2B6.6 as well as HLMs obtained from liver tissues genotyped for the *CYP2B6*6* allele. Since the ability of

cytochrome b5 (Cyt b5) to influence P450-mediated drug oxidation (increase, inhibit or no effect) has been described for multiple P450s (Schenkman and Jansson, 2003), I utilized the expressed CYP2B6 without coexpression of Cyt b5 first to exclude its confounding effects. The results presented here demonstrated that the amino acid changes harbored in the *CYP2B6*6* allele may reduce efavirenz metabolism by decreasing binding affinity and catalytic efficiency.

2. *Results*

2.1 Efavirenz 8-hydroxylation by CYP2B6.1 and CYP2B6.6 proteins without coexpression of Cyt b5

To determine whether the *CYP2B6*6* allele alters binding affinity and/or catalytic efficiency, both expressed CYP2B6.1 and CYP2B6.6 proteins were included in the present study to characterize catalytic properties of the enzymes using efavirenz as a probe (see **Chapter II-2.1 and II-3.1** for Methods). The kinetic profiles for formation of 8-hydroxyefavirenz from efavirenz in these proteins are depicted in Figure 3.1. The kinetic parameters estimated are summarized in Table 3.1. The K_m and V_{max} values for the formation of 8-hydroxyefavirenz were significantly higher in the CYP2B6.6 than in the CYP2B6.1 protein (Table 3.1). The *in vitro* intrinsic clearance (V_{max}/K_m or Cl_{int}) in the CYP2B6.6 protein was significantly lower than in the CYP2B6.1 protein (Table 3.1).

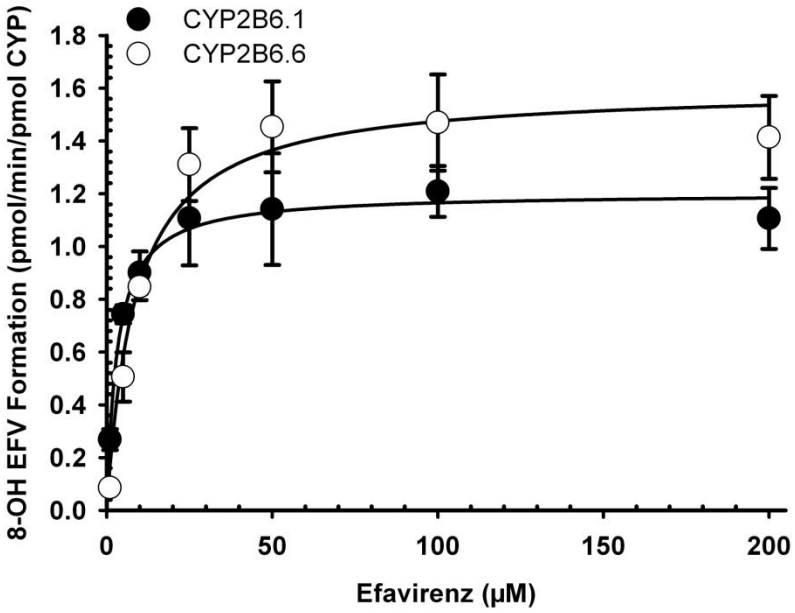

Figure 3.1 Efavirenz concentrations versus formation rate of 8-hydroxyefavirenz in microsomes containing cDNA-expressed CYP2B6.1 and CYP2B6.6 without coexpression of Cyt b5. Efavirenz (1-200 μM) was incubated with reconstituted systems containing either CYP2B6.1 or CYP2B6.6 (15 pmol) and a NADPH-generating system for 15 min at 37 ℃. Formation rate of 8-hydroxyefavirenz (pmol/min/pmol CYP) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represents mean ± S.D. of three replicates. The curve represents the line of best fit.

Table 3.1 Kinetic parameters for the formation of 8-hydroxyefavirenz from efavirenz in expressed CYP2B6.1 and CYP2B6.6 without coexpression of Cyt b5.

	CYP2B6.1(-b5)	CYP2B6.6 (-b5)
Efavirenz 8-Hydroxylation		
V_{max} (pmol/min/pmol)	1.21 ± 0.15	1.61 ± 0.11**
K_m (μM)	3.2 ± 1.0	8.8 ± 1.6***
Cl_{int} (μl/min/pmol)	0.39 ± 0.08	0.19 ± 0.04**

Kinetic data are presented as mean ± S.D. (n=3 incubation were performed in duplicate). *In vitro* Cl_{int} was calculated as V_{max}/K_m. Kinetic parameters for the formation of 8-hydroxyefavirenz were estimated by fitting the velocity versus substrate concentrations to the simple single-site Michaelis-Menten equation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared CYP2B6.6 to CYP2B6.1.

2.2 Efavirenz 8-hydroxylation in HLMs genotyped for the *CYP2B6*6* allele

To further evaluate the effect of the *CYP2B6*6* allele on efavirenz metabolism *in vitro*, the kinetics of efavirenz 8-hydroxylation were characterized in 15 HLM samples with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotypes (n=5 for each genotype) (see **Chapter II-2.2 and II- 3.1** for Methods).

In Figure 3.2, efavirenz concentrations versus formation rate of 8-hydroxyefavirenz in the different genotypes are shown. Formation rates of 8-hydroxyefavirenz versus efavirenz concentrations were fit into a Michaelis-Menten equation to estimate kinetic parameters. The mean $\pm$ S.D. of these parameters for each genotype are listed in Table 3.2. The kinetic parameters for individual HLM are summarized in Table 3.3. None of the kinetic parameters were statistically different among the three genotypes, probably due to the high inter-HLMs variability in V_{max} and Cl_{int} for the formation of 8-hydroxyefavirenz, particularly in HLMs with *CYP2B6*1/*1* and **1/*6* (CV of more than 100%); this variability was smaller in HLMs with **6/*6* genotype (CV of about 30% for V_{max} and 65% for Cl_{int}). Despite this lack of statistical significance, it is noteworthy that the average V_{max} in HLMs with *CYP2B6*6/*6* genotype were lower by 71 and 75% compared to values in HLMs with *CYP2B6*1/*1* and **1/*6* genotypes, respectively. The K_m values in *CYP2B6*6/*6* genotype were higher on average by 114% and 58% than that in *CYP2B6*1/*1* and **1/*6* genotypes, respectively. Accordingly, the Cl_{int} values in HLMs with *CYP2B6*6/*6* were 83% lower compared to that of HLMs with *CYP2B6*1/*1* and 62% lower compared to that of HLMs with **1/*6* genotype.

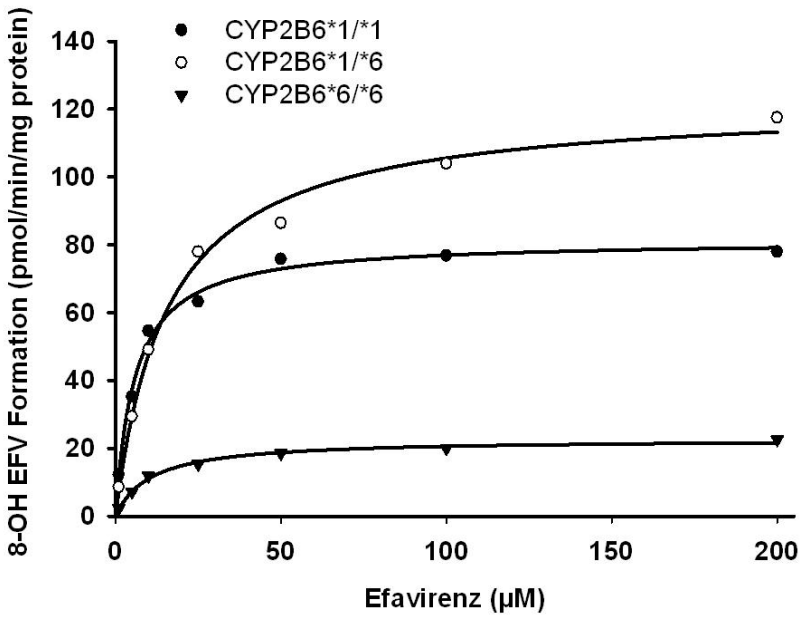

Figure 3.2 Efavirenz concentrations versus formation rate of 8-hydroxyefavirenz in 15 human liver microsomal samples with CYP2B6*1/*1, *1/*6 and *6/*6 genotypes (n=5 HLMs for each genotype). Efavirenz (1-200 μM) were incubated with human liver microsomal samples (0.25mg/ml) with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system for 15 min at 37 ℃ in duplicate. The formation rate of 8-hydroxyefavirenz (pmol/min/mg protein) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represented as the average of five individual incubations in human liver microsomal samples with the same *CYP2B6* genotype. The curve represents the line of best fit.

Table 3.2 Kinetic parameters (mean ± S.D.) for the formation of 8-hydroxyefavirenz from efavirenz in 15 human liver microsomal samples with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype).

HLMs	8-Hydroxyefavirenz		
	V_{max} (pmol/min/mg protein)	K_m (μM)	Cl_{int} (μl/min/mg protein)
*CYP2B6*1/*1*	87.1 ± 87.4	11.2 ± 6.7	14.5 ± 22.6
*CYP2B6*1/*6*	100.6 ± 143.7	15.2 ± 8.0	6.4 ± 7.8
*CYP2B6*6/*6*	25.0 ± 6.7	24.0 ± 31.3	2.4 ± 1.6

Efavirenz (1-200 μM) were incubated with human liver microsomal samples (0.25mg/ml) with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLM for each genotype) and a NADPH-generating system at 37 ℃ for 15min in duplicate. Kinetic parameters (V_{max} and K_m) for the formation of 8-hydroxyefavirenz were estimated by fitting the velocity versus efavirenz concentrations to the simple single-site Michaelis-Menten equation. *In vitro* Cl_{int} was calculated as V_{max}/K_m. The kinetic parameters (V_{max}, K_m and Cl_{int}) for each genotype group are listed in the Table 3.3. The data presented here are mean ±S.D. calculated from 5 individual HLMs values for each genotype.

Table 3.3 Individual kinetic parameters for the formation of 8-hydroxyefavirenz from efavirenz in 15 HLM samples with *CYP2B6*1/*1, *1/*6 and *6/*6* genotypes (n=5 HLMs for each genotype).

HLMs	8-Hydroxyefavirenz		
	V_{max} (pmol/min/mg protein)	K_m (μM)	Cl_{int} (μl/min/mg protein)
*CYP2B6*1/*1*			
HL-U	62.9	20.6	3.1
HL-M	24.8	15.5	1.6
HL-P	110.5	9.2	12.0
HH-689	10.2	6.6	1.5
MCV-65	227.1	4.2	54.1
Mean ± S.D.	87.1 ± 87.4	11.2 ± 6.7	14.5 ± 22.6
*CYP2B6*1/*6*			
HL-S	125.5	11.7	10.7
HL-C	12.9	11.0	1.2
HL-O	342.0	19.0	18.0
MCV-49	11.9	7.1	1.7
HH-488	10.4	27.3	0.4
Mean ± S.D.	100.6 ± 143.7	15.2 ± 8.0	6.4 ± 7.8
*CYP2B6*6/*6*			
HL-K	32.4	21.5	1.5
HH-1180	31.1	7.0	4.5

IIAM-091698-1	16.6	5.8	2.9
HH-525	21.0	7.0	3.0
HH-478	23.6	78.9	0.3
Mean ± S.D.	24.9 ± 6.7	24.0 ± 31.3	2.4 ± 1.6

Efavirenz (1-200 μM) were incubated with HLM samples (0.25mg/ml) with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system at 37 ℃ for 15min in duplicate. Kinetic parameters for the formation of 8-hydroxyefavirenz were estimated by fitting the velocity versus efavirenz concentrations to the simple single-site Michaelis-Menten equation. Kinetic parameters in the individual HLM samples are presented. *In vitro* Cl_{int} was calculated as V_{max}/K_m. Kinetic parameters (V_{max}, K_m and Cl_{int}) are presented as mean ± S.D. calculated from values of individual HLMs carrying the same genotype.

3. *Discussion*

In this section, I explored the hypothesis that altered substrate binding and/or catalytic properties by the *CYP2B6*6* allele may contribute to reduced efavirenz metabolism. Data presented in this chapter have shown that the *CYP2B6*6* allele is associated with increased efavirenz K_m and decreased Cl_{int} in expressed CYP2B6 proteins and similar trend is also observed in HLMs. These data suggest that the mechanism by which the *CYP2B6*6* allele is associated with reduced efavirenz metabolism may be in part due to amino acid changes that modify catalytic properties of the variant versus wild type protein.

Consistent with previous reports in various expressed systems (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus et al., 2006; Raccor et al., 2012), we noted that the V_{max} values for the formation of 8-hydroxyefavirenz significantly higher in CYP2B6.6 than in CYP2B6.1 proteins expressed without Cyt b5. However, V_{max} values for the formation of 8-hydroxyefavirenz were substantially decreased (by ~70%) in HLMs with *CYP2B6*6/*6* genotype versus HLMs with *CYP2B6*1/*6* and *CYP2B6*1/*1* genotypes. Our interpretation is that the expressed variant protein inherently increases catalytic activity of efavirenz, whereas the decreased V_{max} value in HLMs is probably mainly due to reduced protein expression by the *CYP2B6*6/*6* genotype (Hesse et al., 2004; Desta et al., 2007; Hofmann et al., 2008). The average K_m value for the formation of 8-hydroxyefavirenz in CYP2B6.6 protein expressed without Cyt b5 was increased by 175% and in HLMs with *CYP2B6*6/*6* genotype was also increased by 58% and 114% than in HLMs with *CYP2B6*1/*6* and *CYP2B6*1/*1* genotypes, respectively. These data concur with a recent report (Zhang et al., 2011b). However, since some K_m values derived from

HLMs with **1/*1* and **1/*6* were outliers and may have skewed the average data, these data should be interpreted with caution.

A series of endeavors have been made by Dr. Halpert's group to elucidate how the two SNPs (K262R and Q172H) harbored in the *CYP2B6*6* allele alter binding affinity and/or catalytic efficiency of the enzyme (Gay et al., 2010; Shah et al., 2011). Considering that the two amino acid mutations are not within the active site of the enzyme, it is very likely that they involve in the ligand binding and substrate catalysis indirectly. Indeed, a recent publication characterizing the crystal structure of CYP2B6 genetic variant (Y226H, K262R) indicates that the side chain of residue 172 may interact with the residues at active site and thus could affect the orientation of active site residues on the I-helix and substrate binding affinity (Gay et al., 2010; Shah et al., 2011). It is noteworthy that the other mutated amino acid carried by *CYP2B6*6* allele, K262R, is located at the G/H loop and is part of a small hydrogen-bonding network (Gay et al., 2010). The concerted movement of the G and H helices, which could be affected by this hydrogen-bonding network, may influence the orientation of active site residues on the G helix (Gay et al., 2010). In addition, part region of G helix forms substrate recognition sequence and substrate egress channels, which may both affect substrate specificity (Cojocaru et al., 2007). Indeed, substrate-dependent effects of the *CYP2B6*6* allele on pharmacokinetics have been observed *in vivo*. *In vivo*, the *CYP2B6*6/*6* genotype is associated with 3-fold increase in efavirenz exposure compared with *CYP2B6*1/*1* genotype (Rotger et al., 2007), but its effect on plasma exposure of bupropion or 4-hydroxybupropion was marginal (Kirchheiner et al., 2003) and its effect on exposure of cyclophosphamide was opposite (Nakajima et al., 2007). Hence, I hypothesize that the

functional consequences of the *CYP2B6*6* allele may be substrate-dependent. I next tested this hypothesis in **Chapter IV** by determining the catalytic properties of CYP2B6 using another traditional *in vitro* substrate, bupropion, in expressed CYP2B6 and HLMs genotyped for the *CYP2B6*6* allele.

K262R may also involve in the interaction between the enzyme and its redox partner, CYP reductase (Bumpus and Hollenberg, 2008; Gay et al., 2010). The oxidation reaction catalyzed by CYPs requires transferring of two electrons from NADPH. The first electron is generally thought to be transferred by CYP reductase, while the second can be transferred by either CYP reductase or Cyt b5. That altered electron transfer from CYP reductase to CYP2B6 variant proteins may influence substrate metabolism was suggested by a recent study (Zhang et al., 2011b). It is, therefore, reasonable to suggest that amino acid changes may influence the interaction between the CYPs and electron transfer proteins and thus alter the catalysis of substrates in Cyt b5- and substrate-dependent manner. I tested this hypothesis in **Chapter V** by determining the catalytic properties of CYP2B6 using efavirenz and bupropion in expressed CYP2B6 with coexpression of Cyt b5.

In summary, the results presented in this section of my dissertation provide *in vitro* evidence that amino acid changes harbored in the *CYP2B6*6* allele alter efavirenz binding and catalytic efficiency, and therefore may contribute to efavirenz reduced metabolism in individuals with the *CYP2B6*6/*6* genotype. Specifically, I showed the significantly higher V_{max} and K_m values for 8-hydroxyefavirenz formation and ~2-fold lower Cl_{int} in expressed CYP2B6.6 protein compared with that of CYP2B6.1 protein. In HLMs, *CYP2B6*6/*6* genotype was associated with markedly lower V_{max} (and moderate

increase in K_m) and thus lower Cl_{int} values for efavirenz metabolism, but no difference in catalytic properties was noted between *CYP2B6*1/*1* and *CYP2B6*1/*6* genotypes. Having determined the functional consequences of the *CYP2B6*6* allele using efavirenz as substrate, I further explore whether the functional consequences of this genetic variant is substrate- and Cyt b5-dependent.

CHAPTER IV: *Substrate-dependent effects on the functional consequences of the CYP2B6*6 allele*

1. *Introduction*

A number of *in vitro* studies, mostly in expressed systems, have reported that the *CYP2B6*6* allele or the amino acids harbored in it influence catalytic properties. However, the extent and direction of effect appears to depend on the substrate and the enzyme sources used (Table 4.1). It is noteworthy that two recent papers reported the altered catalytic properties of CYP2B6 using bupropion and efavirenz by the *CYP2B6*6* allele (Ariyoshi et al., 2011; Zhang et al., 2011b). However, the interesting findings by Dr. Zhang's group should be interpreted with caution, since the N-terminally truncated CYP2B6 protein used in their studies had marked reduced K_m for bupropion compared with that of HLMs (Zanger et al., 2007). In addition, the effects of enzyme source on the kinetics also been shown for other CYP isoforms previously (Kumar et al., 2006a; Kaspera et al., 2011). Therefore, further study in native CYP2B6 enzyme using different expression sources is warranted.

In this section I tested the hypothesis that the functional consequences of the *CYP2B6*6* allele may be substrate-dependent. I determined the kinetics of bupropion metabolism to 4-hydroxybupropion in expressed CYP2B6.1 and CYP2B6.6 as well as HLMs obtained from liver tissues genotyped for the *CYP2B6*6* allele. The changes of catalytic properties of CYP2B6 using bupropion are compared to that obtained using efavirenz as a substrate (presented in the **Chapter III**).

Table 4.1 Substrate-specific changes in function consequences of the *CYP2B6*6* allele

Substrate	% change of V_{max}	% change of K_m	% change of Cl_{int}	Expressed System	Reference
7-EFC	162.1% ↑	40.2% ↑	88.7% ↑	COS-1 cells	(Jinno et al., 2003)
7-EFC	41.7% ↑	91.7% ↑	30.8% ↓	E. coli cells	(Zhang et al., 2011b)
Cyclophosphamide	1.4% ↓	39.6% ↓	64.3% ↑	Sf9 insect cells	(Ariyoshi et al., 2011)
Cyclophosphamide	55.2% ↑	11.1% ↑	40.1% ↑	E. coli cells	(Raccor et al., 2012)
Bupropion	75.0% ↑	300% ↑	56.9% ↓	E. coli cells	(Zhang et al., 2011b)
Efavirenz	19.1% ↓	61.0% ↑	50% ↓	Sf9 insect cells	(Ariyoshi et al., 2011)
Efavirenz	462.5% ↑	2712.3% ↑	80.0% ↓	E. coli cells	(Zhang et al., 2011a)

Artemether	316.7% ↑	116.8% ↑	92.0% ↑	COS-7 cells	(Honda et al., 2011)

7-EFC, 7-hydroxy-4-trifluoromethylcoumarin
Wild type V_{max}, K_m, Cl_{int} = 100%

2. *Results*

2.1 Bupropion 4-hydroxylation by CYP2B6.1 and CYP2B6.6 proteins without coexpression of Cyt b5

To determine whether the *CYP2B6*6* allele alters binding affinity and/or catalytic efficiency in a substrate-dependent manner, I characterized catalytic properties of expressed CYP2B6.1 and CYP2B6.6 using bupropion as a probe (see **Chapter II-2.1 and II-3.1** for Methods). The kinetic profiles for the formation of 4-hydroxybupropion from bupropion in these proteins are depicted in Figure 4.1. The kinetic parameters estimated are summarized in Table 4.2. As shown in Figure 4.1 and Table 4.2, V_{max} value for the formation of 4-hydroxybupropion in the CYP2B6.6 protein was significantly higher than that estimated from CYP2B6.1 protein, whereas there was no statistically significant difference in the K_m values between the variant and the wild type proteins. Accordingly, the Cl_{int} for the formation of 4-hydroxybupropion was significantly increased in the CYP2B6.6 protein compared to that of the CYP2B6.1 protein (Table 4.2).

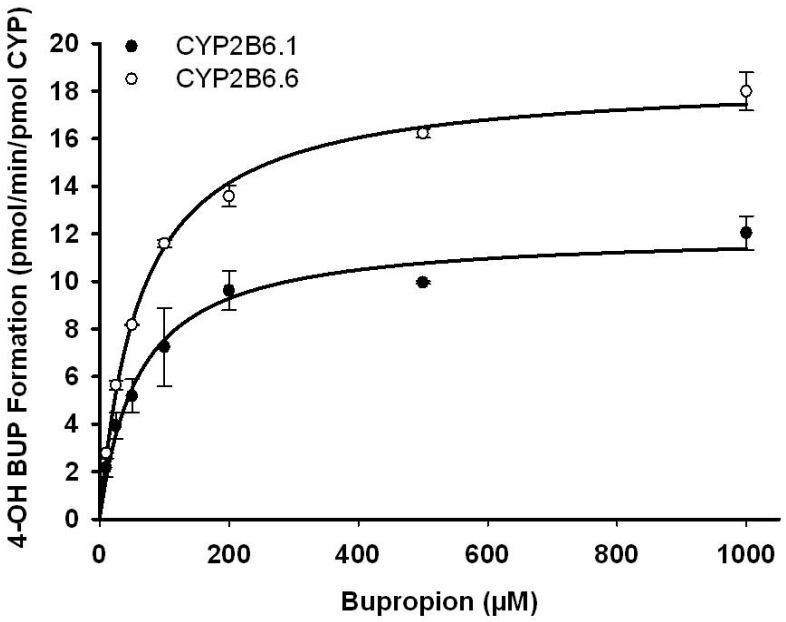

Figure 4.1 Bupropion concentrations versus formation rate of 4-hydroxybupropion in microsomes containing cDNA-expressed CYP2B6.1 and CYP2B6.6 without coexpression of Cyt b5. Bupropion (10 - 1000 μM) was incubated with reconstituted systems containing either CYP2B6.1 or CYP2B6.6 (15 pmol) and a NADPH-generating system for 15 min at 37 ℃. Formation rate of 4-hydroxybupropion (pmol/min/pmol CYP) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represents mean ± S.D. of three replicates. The curve represents the line of best fit.

Table 4.2 Kinetic parameters for the formation of 4-hydroxybupropion from bupropion in expressed CYP2B6.1 and CYP2B6.6 without coexpression of Cyt b5.

	CYP2B6.1(-b5)	CYP2B6.6 (-b5)
Bupropion 4-Hydroxylation		
V_{max} (pmol/min/pmol)	13.37 ± 0.97	18.55 ± 0.82**
K_m (μM)	64.2 ± 13.4	62.6 ± 7.3
Cl_{int} (μl/min/pmol)	0.21 ± 0.03	0.30 ± 0.03**

Kinetic data are presented as mean ± S.D. (n=3 incubation were performed in duplicate). *In vitro* Cl_{int} was calculated as V_{max}/K_m. Kinetic parameters for the formation of 4-hydroxybupropion were estimated by fitting the velocity versus substrate concentrations to the simple single-site Michaelis-Menten equation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared CYP2B6.6 to CYP2B6.1.

2.2 Bupropion 4-hydroxylation in HLMs genotyped for *the CYP2B6*6* allele

Kinetic analyses for the formation of 4-hydroxybupropion were also performed in the same 15 HLM samples that were used for the characterization of efavirenz metabolism. In Figure 4.2, bupropion concentrations versus formation rate of 4-hydroxybupropion in the different genotypes are shown. Formation rates of 4-hydroxybupropion versus bupropion concentrations were fit into a Michaelis-Menten equation to estimate kinetic parameters. The mean $\pm$ S.D. of these parameters for each genotype are listed in Table 4.3. The kinetic parameters for individual HLM are summarized in the Table 4.4. Similar to that observed for the kinetics of 8-hydroxyefavirenz, the values of V_{max} and Cl_{int} for the formation of 4-hydroxybupropion in HLMs also exhibited a large variability. HLMs with *CYP2B6*1/*1* and **1/*6* genotypes showed higher variability (CV of more than 100%) than that in HLMs with **6/*6* (CV of about 60% and 65%, respectively). The average V_{max} values for the formation of 4-hydroxybupropion were also much lower in HLMs with *CYP2B6*6/*6* genotype than that in wild type and heterozygotes. The HLMs with *CYP2B6*1/*1* exhibited lower average K_m values compared with HLMs with *CYP2B6*1/*6* and **6/*6*, although this difference didn't reach statistical significance (Table 4.3). The Cl_{int} in *CYP2B6*6/*6* genotype was decreased by over 95% when compared with *CYP2B6*1/*1* and *CYP2B6*1/*6* (Table 4.3).

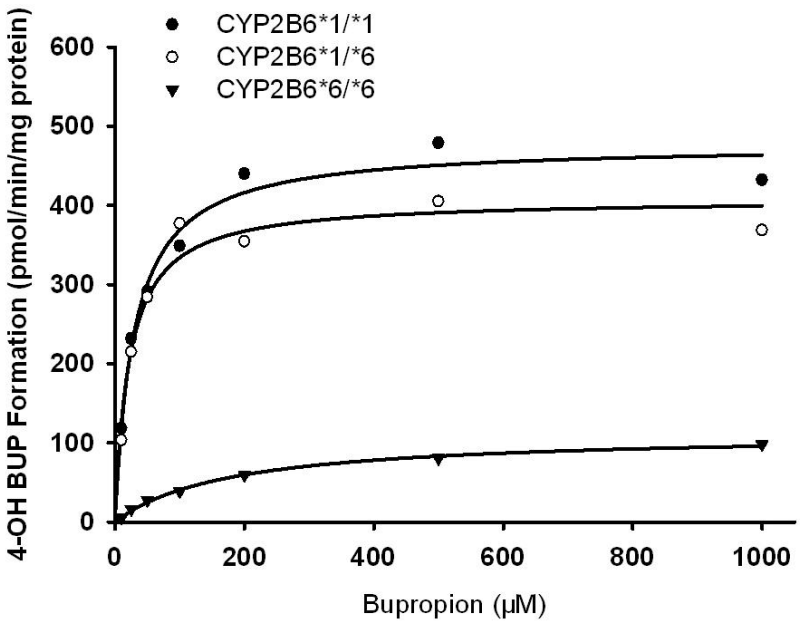

Figure 4.2 Bupropion concentrations versus formation rate of 4-hydroxybupropion in 15 human liver microsomal samples with CYP2B6*1/*1, *1/*6 and *6/*6 genotypes (n=5 HLMs for each genotype). Bupropion (10-1000 μM) were incubated with human liver microsomal samples (0.25mg/ml) with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system for 15 min at 37 ℃ in duplicate. The formation rate of 4-hydroxybupropion (pmol/min/mg protein) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represented as the average of five individual incubations in human liver microsomal samples with the same *CYP2B6* genotype. The curve represents the line of best fit.

Table 4.3 Kinetic parameters (mean ± S.D.) for the formation of 4-hydroxybupropion from bupropion in 15 human liver microsomal samples with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype).

HLMs	4-Hydroxybupropion		
	V_{max} (pmol/min/mg protein)	K_m (μM)	Cl_{int} (μl/min/mg protein)
*CYP2B6*1/*1*	492.8 ± 427.9	86.0 ± 75.7	18.8 ± 26.3
*CYP2B6*1/*6*	441.6 ± 583.0	212.1 ± 221.1	21.8 ± 44.2
*CYP2B6*6/*6*	112.9 ± 66.7	204.2 ± 66.1	0.6 ± 0.5

Bupropion (10-1000 μM) were incubated with HLM samples (0.25mg/ml) with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system at 37 ℃ for 15min in duplicate. Kinetic parameters (V_{max} and K_m) for the formation of 4-hydroxybupropion were estimated by fitting the velocity versus bupropion concentrations to the simple single-site Michaelis-Menten. *In vitro* Cl_{int} was calculated as V_{max}/K_m. The kinetic parameters (V_{max}, K_m and Cl_{int}) for each genotype group are listed in the Supplemental Table 2. The data presented here are mean ±S.D. calculated from 5 individual HLMs values for each genotype.

Table 4.4 Individual kinetic parameters for the formation of 4-hydroxybupropion from bupropion in 15 HLM samples with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype).

HLMs	4-Hydroxybupropion		
	V_{max} (pmol/min/mg protein)	K_m (μM)	Cl_{int} (μl/min/mg protein)
*CYP2B6*1/*1*			
HL-U	342.3	92.3	3.7
HL-M	207.0	90.0	2.3
HL-P	564.4	22.5	25.1
HH-689	147.7	205.9	0.7
MCV-65	1202.7	19.3	62.4
Mean ± S.D.	492.8 ± 427.9	86.0 ± 75.7	18.8 ± 26.3
*CYP2B6*1/*6*			
HL-S	329.9	48.2	6.9
HL-C	201.9	264.0	0.8
HL-O	1471.0	14.6	100.6
MCV-49	88.7	168.0	0.5
HH-488	116.6	565.7	0.2
Mean ± S.D.	441.6 ± 583.0	212.9 ± 221.1	21.8 ± 44.2
*CYP2B6*6/*6*			
HL-K	81.5	211.6	0.4

HH-1180	187.4	188.5	1.0
IIAM-091698-1	85.4	161.5	0.5
HH-525	177.3	145.7	1.2
HH-478	33.1	313.5	0.1
Mean ± S.D.	112.9 ± 66.7	204.2 ± 66.1	0.6 ± 0.5

Bupropion (10 - 1000 μM) were incubated with HLM samples (0.25mg/ml) with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system at 37 ℃ for 15min in duplicate. Kinetic parameters for the formation of 4-hydroxybupropion were estimated by fitting the velocity versus bupropion concentrations to the simple single-site Michaelis-Menten. *In vitro* Cl_{int} was calculated as V_{max}/K_m. Kinetic parameters (V_{max}, K_m and Cl_{int}) are presented as mean ± S.D. calculated from values of individual HLMs carrying the same genotype.

3. *Discussion*

In this section, I explored the hypothesis that functional consequences of the *CYP2B6*6* allele may be substrate-dependent. Data presented in this chapter have shown that the V_{max} and Cl_{int} values for 4-hydroxybupropion formation were significantly higher in CYP2B6.6 than in CYP2B6.1 protein, with no difference in K_m. These changes are different from that using efavirenz as a substrate, which is associated with significantly higher V_{max} and K_m values and thus lower Cl_{int} in CYP2B6.6 protein. In HLMs, *CYP2B6*6/*6* genotype was associated with markedly lower V_{max} (and moderate increase in K_m) and thus lower Cl_{int} values for 4-hydroxybupropion formation similar to that observed in the formation kinetics of 8-hydroxyefavirenz. In addition, different effects of the *CYP2B6*6* allele on the K_m values for 4-hydroxybupropion formation were observed between the expressed system and HLMs. These data suggest that the *CYP2B6*6* allele influences metabolic activity by altering substrate binding and catalytic activity in a substrate-dependent manner. The effects of the *CYP2B6*6* allele on catalytic properties of CYP2B6 seem to be different in expressed protein compared to that in HLMs using bupropion.

The average K_m value for the formation of 4-hydroxybupropion in CYP2B6.6 protein did not differ from CYP2B6.1, while that for the formation of 8-hydroxyefavirenz in CYP2B6.6 protein was markedly increased by 175%. While the average K_m value for the formation of 8-hydroxyefavirenz in HLMs with *CYP2B6*6/*6* genotype was also increased by 58% and 114% than in HLMs with *CYP2B6*1/*6* and *CYP2B6*1/*1* genotypes, respectively, only a slight increase in K_m for bupropion 4-hydroxylation was noted in HLMs with **1/*6* or **6/*6* genotypes compared to HLMs with **1/*1* genotype.

These results suggest that the amino acid changes harbored in *CYP2B6*6* allele may influence substrate binding with pronounced effect on efavirenz than bupropion. However, since some K_m values derived from HLMs with **1/*1* and **1/*6* were outliers and may have skewed the average data, these data should be interpreted with caution.

Our *in vitro* data concurs with clinical observations. *In vivo*, the *CYP2B6*6/*6* genotype is associated with >3-fold increase in efavirenz exposure compared to *CYP2B6*1/*1* genotype (Rotger et al., 2007), but its effect on plasma exposure of bupropion or 4-hydroxybupropion was marginal (Kirchheiner et al., 2003). It is well recognized that variants in other CYPs, e.g. CYP2C8 and CYP2C9, that change amino acids affect metabolic activity in a substrate-dependent manner (Maekawa et al., 2009; Kaspera et al., 2011). However, the *CYP2B6*6* allele seems unique in that its effect on catalytic activity is not only substrate-dependent but also results in opposite effects. This variant has been associated with enhanced cyclophosphamide metabolism *in vitro* (Xie et al., 2003; Ariyoshi et al., 2011; Raccor et al., 2012) and *in vivo* (Nakajima et al., 2007), which appears to be primarily driven by the significantly lower K_m for cyclophosphamide 4-hydroxylation in CYP2B6.6 than in CYP2B6.1 proteins (Ariyoshi et al., 2011). The *CYP2B6*6* allele appears to alter substrate metabolism in two ways: by decreasing (e.g., cyclophosphamide) or increasing (e.g., efavirenz) substrate binding (present data; Zhang et al., 2011; Ariyoshi et al., 2011) probably due to changes in the three dimensional structures of the protein; and by reducing catalytic efficiency secondary to reduced protein expression (Desta et al., 2007; Hofmann et al., 2008). Overall, altered substrate binding and/or catalytic activity as a result of amino acid changes seem to play a critical

role in determining the substrate-dependent functional consequences of the *CYP2B6*6* allele.

How the two SNPs (K262R and Q172H) harbored in the *CYP2B6*6* allele that are not within the active site of the enzyme regulate the substrate specificity? It is noteworthy that one amino acid carried by *CYP2B6*6* allele, K262R, is located at the G/H loop and is part of a small hydrogen-bonding network (Gay et al., 2010). The concerted movement of the G and H helices, which could be affected by this hydrogen-bonding network, could influence the orientation of active site residues on the G helix (Gay et al., 2010). In addition, part region of G helix forms substrate recognition sequence and substrate egress channels, which may both affect substrate specificity (Cojocaru et al., 2007).

Data from the present study also implicate that the effects of the *CYP2B6*6* allele on catalytic properties of CYP2B6 seem to be different in expressed protein compared to that in HLMs using bupropion. In HLMs with *CYP2B6*6/*6* and *CYP2B6*1/*6* genotype, the average K_m values for the formation of 4-hydroxybupropion was increased by 137% compared to that in HLMs with *CYP2B6*1/*1* genotype, while there is no significant difference was noticed between the wild type and variant expressed proteins. The disconnect between the metabolism data generated from HLMs and expressed enzymes were observed in many other CYPs, e.g. CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Venkatakrishnan et al., 2000; Kumar et al., 2006a; Chen et al., 2011), but this is the first study that showed the differential magnitude of changes in catalytic properties by the genetic variant utilizing the two systems, i.e. expressed proteins and genotyped HLMs. This discrepancy can be attributed to intrinsic differences between the two *in vitro* systems including variability in intrinsic activity or

turnover number (activity per unit amount of P450 enzyme), differences in the expression levels of accessory proteins (primarily CYP reductase and Cyt b5), membrane lipid compositions, ionic strength of the *in vitro* incubation matrix and most importantly the abundance of the respective P450 isoforms (Crespi and Miller, 1999; Venkatakrishnan et al., 2000). When evaluating the kinetic differences between polymorphic variants, use of an expressed enzyme system is often a necessity because of the scarcity of donor material available from individual having the genetic variation of interest. Therefore, it is critical to determine how directly comparable an expressed enzyme system or a HLM system is to the human situation.

In summary, data presented in this section of my dissertation provide *in vitro* evidence that functional consequences of the *CYP2B6*6* allele may be substrate-dependent. In addition, these data also show that the effects of the *CYP2B6*6* allele on catalytic properties of CYP2B6 seem to be different in expressed protein compared to that in HLMs using bupropion.

CHAPTER V: *Cytochrome b5-dependent effects on the functional consequences of the CYP2B6*6 allele*

1. *Introduction*

Cytochrome (Cyt) b5 is a 16.7-kDa hemoprotein found in the endoplasmic reticulum and mitochondrial membrane of hepatocytes. The membrane-bound form of Cyt b5 found in the endoplasmic reticulum is also referred to as microsomal Cyt b5 and can transfer electrons for a wide array of oxidative reactions catalyzed by CYPs. CYP-mediated metabolism occurs via a catalytic cycle that involves several steps: 1) substrate binding; 2) one-electron addition to the substrate-P450 complex by CYP reductase; 3) oxygen binding to the ferrous P450; 4) transfer of a second electron by either CYP reductase or Cyt b5 and protonation of the resulting iron-peroxo anion intermediate; 5) cleavage of the O–O bond to generate H_2O; 6) oxidation of the substrate; and 7) and release of product with subsequent regeneration of ferric P450 (Schlichting et al., 2000; Ortiz de Montellano and De Voss, 2002; Groves, 2003).

Cyt b5 anchored by a C-terminal hydrophobic helix, interacts directly with the membrane-bound P450s and CYP reductase to alter the rate of P450 catalysis. The addition of Cyt b5 to mixture of P450s and CYP reductase results in varied effects on the activity of P450-mediated drug oxidation. It appears that Cyt b5 has a rather complex interaction with CYP, which is greatly influenced by the form of CYPs, as well as its substrate. As has been shown for CYP2C8 (Kaspera et al., 2011), CYP2C9 (Kumar et al., 2006a) and probably many other CYPs (Venkatakrishnan et al., 2000), several factors inherent to specific enzyme sources that include differences in Cyt b5 contents may influence *in vitro* kinetic parameters and inhibition constants in a substrate-dependent

manner. Cyt b5 has been reported to activate several CYPs including CYP2B6 (Reed and Hollenberg, 2003; Jushchyshyn et al., 2005), but its influence on the catalytic properties of CYP2B6.6 protein has not been studied. Therefore, the purpose of the present study was to determine the influence of Cyt b5 on metabolic activities of expressed CYP2B6.1 and CYP2B6.6 proteins. I tested the hypothesis that the functional consequence of the *CYP2B6*6* allele may be Cyt b5-dependent. I determined the kinetics of efavirenz metabolism to 8-hydroxyefavirenz and bupropion metabolism to 4-hydroxybupropion in expressed CYP2B6.1 and CYP2B6 with the coexpression of Cyt b5. And the results are compared to that obtained from expressed CYP2B6 without coexpression of Cyt b5 (presented in the **Chapter III and Chapter IV**).

2. *Results*

To evaluate the potential effect of Cyt b5 on catalytic properties of the variant versus wild type protein, kinetic parameters for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion were determined in CYP2B6.1 and CYP2B6.6 proteins that were co-expressed with Cyt b5 and the results were compared with those data obtained from CYP2B6.1 and CYP2B6.6 proteins without coexpression of Cyt b5 (see **Chapter II-2.1 and II-3.1** for Methods). Kinetic profiles for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion in CYP2B6.1 and CYP2B6.6 proteins are shown in Figure 5.1. The corresponding kinetic parameters are summarized in Table 5.1. In contrast to the significant changes by the *CYP2B6*6* allele observed in expressed system without co-expression of Cyt b5, the V_{max} value for the formation of 8-hydroxyefavirenz was not significantly different (p = 0.20) between CYP2B6.1 and CYP2B6.6 proteins co-expressed with Cyt b5 (Figure 5.1A; Table 5.1). Although the K_m value in CYP2B6.6 protein was higher by 81% than that in the CYP2B6.1 protein and the Cl_{int} value was decreased by 43% consistent with the data obtained from system without Cyt b5 (Table 5.1), the differences did not reach a statistically significant level (Figure 5.1A; Table 5.1) (p=0.059). When bupropion 4-hydroxylation was used as a reaction probe, a significantly lower V_{max} value was observed in CYP2B6.6 protein compared to the value obtained from the CYP2B6.1 protein (Figure 5.2B; Table 5.1). The K_m value was increased from 90.6 μM in the CYP2B6.1 protein to 110.0 μM in the variant protein, but this did not reach a statistically significant difference (p = 0.27). A significant decrease in Cl_{int} was observed in the CYP2B6.6 protein compared with CYP2B6.1 protein.

As described above, the catalytic properties of CYP2B6 appeared to be genotype- and Cyt b5-dependent. To gain further insight regarding the differential effect of Cyt b5 on CYP2B6.1 versus CYP2B6.6 protein, kinetic parameters for efavirenz 8-hydroxylation and bupropion 4-hydroxylation obtained in the presence of Cyt b5 were compared with those values obtained in CYP proteins expressed without Cyt b5 (Table 5.1). In the CYP2B6.1 protein, none of the kinetic parameters of efavirenz 8-hydroxylation were significantly different compared to values obtained from CYP2B6.1 protein without co-expression of Cyt b5. Whereas, co-expression of Cyt b5 with the CYP2B6.6 protein significantly decreased the V_{max} value for the formation of 8-hydroxyefavirenz compared to the value obtained from CYP2B6.6 protein without co-expression of Cyt b5. However, since the K_m value was also tended towards decrease in the CYP2B6.6 protein, the Cl_{int} value in the CYP2B6.6 protein co-expressed with Cyt b5 was not significantly different than that obtained from the CYP2B6.6 protein without Cyt b5 (Table 5.1). Similar to the findings with efavirenz 8-hydroxylation, differential effects of Cyt b5 on the catalytic properties of CYP2B6.1 and CYP2B6.6 were observed with bupropion 4-hydroxylation (Table 5.1). Compared to the CYP2B6.1 protein without Cyt b5, the CYP2B6.1 protein co-expressed with Cyt b5 exhibited modest increase in the V_{max} and K_m values for the formation of 4-hydroxybupropion, while the Cl_{int} tended towards decrease (Table 5.1). The presence of Cyt b5 with CYP2B6.6 protein decreased the V_{max} value to 11.77 pmol/min/pmol P450 from 18.55 pmol/min/pmol P450 in the CYP2B6.6 protein without Cyt b5 (p=0.0002) A significant increase was observed in K_m value of CYP2B6.6 protein co-expressed with Cyt b5 compared to that of CYP2B6.6 protein co-expressed without Cyt b5. As a result, Cl_{int} for the formation of 4-

hydroxybupropion was significantly decreased in CYP2B6.6 co-expressed with Cyt b5 (Table 5.1).

We recognized that recombinant protein systems have limitations, including differences in cofactor expression between variants and wild type or even between batches of the same protein. In the expressed enzymes used in this experiment, the amounts of Cyt b5 expressed in CYP2B6.1 and CYP2B6.6 proteins were slightly different (220 and 370 pmol/mg protein, respectively). However, we have observed substrate-dependent effects for the *CYP2B6*6* allele in the presence of Cyt b5, i.e. no significant differences were found in V_{max} and K_m comparing CYP2B6.1 to CYP2B6.6 using efavirenz as substrate, while V_{max} was significantly lower in CYP2B6.6 using bupropion. These data suggest that expression differences in Cyt b5 do not seem to significantly contribute to the differences in kinetic parameters observed.

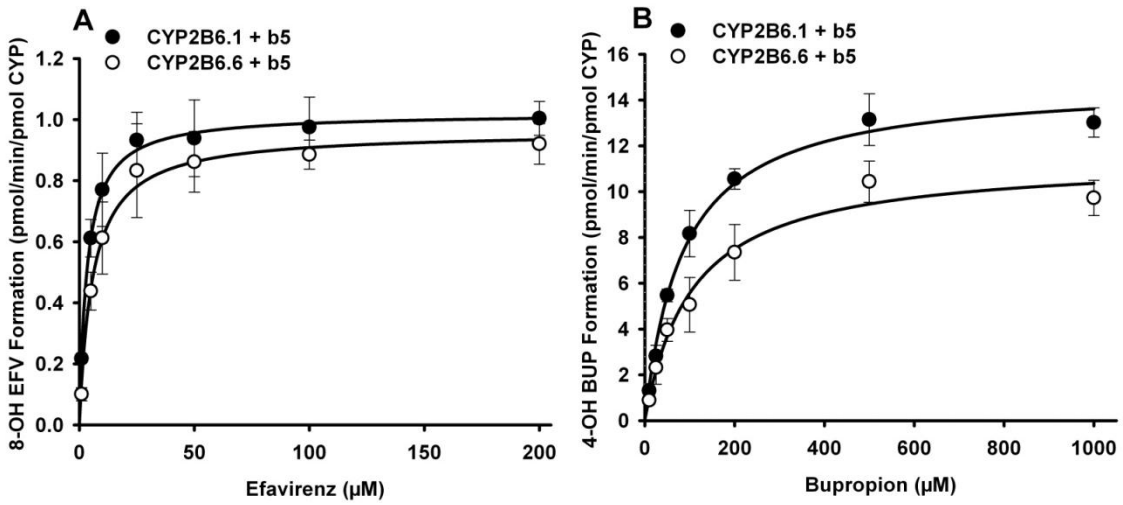

Figure 5.1 Efavirenz concentrations versus formation rate of 8-hydroxyefavirenz (A) and bupropion concentrations versus formation rate of 4-hydroxybupropion (B) in microsomes containing cDNA-expressed CYP2B6.1 and CYP2B6.6 with coexpression of Cyt b5. Efavirenz (1 - 200 μM) or bupropion (10 - 1000 μM) was incubated with reconstituted systems containing either CYP2B6.1 or CYP2B6.6 with coexpression of Cyt b5 (15 pmol) and a NADPH-generating system for 15 min at 37 ℃. The formation rate of 8-hydroxyefavirenz and 4-hydroxybupropion (pmol/min/pmol CYP) versus substrate concentrations were fit to the simple single-site Michaelis-Menten equation. Each point represents mean ± S.D. of three replicates. The curve represents the line of best fit.

Table 5.1 Kinetic parameters for the formation of 8-hydroxyefavirenz from efavirenz and 4-hydroxybupropion from bupropion in expressed CYP2B6.1 and CYP2B6.6 without and with co-expression of Cyt b5.

	Without Cyt b5		With Cyt b5		*p* value (comparing with versus without b5) §	
	CYP2B6.1	CYP2B6.6	CYP2B6.1	CYP2B6.6	CYP2B6.1	CYP2B6.6
Efavirenz 8-Hydroxylation						
V_{max} (pmol/min/pmol)	1.21 ± 0.15	1.61 ± 0.11**	1.02 ± 0.11	0.95 ± 0.02	0.14	0.00002
K_m (μM)	3.2 ± 1.0	8.8 ± 1.6***	3.4 ± 0.6	6.2 ± 2.6	0.74	0.14
Cl_{int} (μl/min/pmol)	0.39 ± 0.08	0.19 ± 0.04**	0.30 ± 0.06	0.17 ± 0.07	0.18	0.69
Bupropion 4-Hydroxylation						
V_{max} (pmol/min/pmol)	13.37 ± 0.97	18.55 ± 0.82**	15.18 ± 0.99	11.77 ± 1.46**	0.04	0.0002
K_m (μM)	64.2 ± 13.4	62.6 ± 7.3	90.6 ± 10.3	110.0 ± 29.8	0.02	0.02
Cl_{int} (μl/min/pmol)	0.21 ± 0.03	0.30 ± 0.03**	0.17 ± 0.02	0.11 ± 0.04*	0.07	0.0002

Kinetic data are presented as mean ± S.D. (n=3 incubation were performed in duplicate). *In vitro* Cl_{int} was calculated as V_{max}/K_m. Kinetic parameters for the formation of 8-hydroxyefavirenz and 4-hydroxybupropion were estimated by fitting the velocity versus substrate concentrations to the simple single-site Michaelis-Menten equation.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared CYP2B6.6 to CYP2B6.1 with co-expression of Cyt b5, respectively. §Kinetic parameters of CYP2B6.1 with and without Cyt b5 as well as CYP2B6.6 with and without Cyt b5 were also compared.

3. *Discussion*

In this section, I explored the hypothesis that functional consequences of the *CYP2B6*6* allele may be Cyt b5-dependent. Data presented in this chapter have shown that Cyt b5 abolished the significant changes of kinetic parameters for the formation of 8-hydroxyefavirenz by CYP2B6.6 compared to CYP2B6.1, while it reversed the genetic effect on the kinetic parameters for the formation of 4-hydroxybupropion. These data suggest that Cyt b5 affects catalytic properties in a genotype- and substrate-dependent manner and highlight the fact that interpretation of *in vitro* studies performed with expressed proteins may vary depending on the presence or absence of Cyt b5, substrate used and underlying genotype.

The ability of Cyt b5 to influence CYP-mediated drug oxidation (increase, inhibit, or no effect) has been described for multiple CYPs (Schenkman and Jansson, 2003). The mechanisms by which Cyt b5 might alter substrate metabolism include: providing the second electron during the catalytic cycle of CYPs; interacting physically with CYPs and thus modifying conformation of the protein, which, in turn, influences interaction with the substrate or reductase; or by competing for same binding site with CYP reductase, thereby preventing reduction of ferric CYP and initiation of the catalytic cycle (Zhang et al., 2008). In this study, Cyt b5 had no impact on CYP2B6.1-catalyzed efavirenz 8-hydroxylation. However, in sharp contrast to the results obtained from CYP2B6.6 without Cyt b5, V_{max} for efavirenz 8-hydroxylation was significantly reduced by Cyt b5 (with no effect on K_m) in CYP2B6.6 protein, effectively abolishing the genotype-dependent effect observed in CYP2B6.6 protein expressed without Cyt b5. The kinetic properties were different for bupropion 4-hydroxylation. Cyt b5 significantly increased

the V_{max} and K_m values for bupropion 4-hydroxylation in CYP2B6.1 protein compared to CYP2B6.1 without Cyt b5. In CYP2B6.6, the K_m for bupropion 4-hydroxylation was significantly increased and V_{max} was significantly reduced by Cyt b5, leading to marked reduction in Cl_{int} in the CYP2B6.6 protein (Table 5.1). For both substrates, CYP2B6.1 exhibited similar or increased catalytic activities with coexpression of Cyt b5 compared to that without Cyt b5, whereas Cyt b5 significantly decreased V_{max} values in CYP2B6.6. These data suggest an overlapping binding site between CYP reductase and Cyt b5 in CYP2B6.6 but probably not in CYP2B6.1. The possibility that the observed effect of Cyt b5 could be due to difference in the expression of Cyt b5 or POR among the genotypes cannot be excluded. In our study, the POR level was relatively lower in CYP2B6.6 with coexpression of Cyt b5 than that without coexpression of Cyt b5. Thus, the possibility that the lower expression of POR in the CYP2B6.6 protein may influence the magnitude of effect of Cyt b5 among the genotypes and substrates cannot be fully excluded. However, variation in kinetic parameters were observed even when the POR level was balanced between the variant and wild type protein (Ariyoshi et al., 2011; Zhang et al., 2011b). However, POR is much less functionally variable in general population than hepatic drug-oxidation CYPs (Venkatakrishnan et al., 2000; Huang et al., 2004). Although some POR SNPs have been found to affect activities of CYP1A2, CYP2C8, CYP2C19 and CYP3A4, no POR SNP has been identified to significantly influence CYP2B6 activity up to date (Gomes et al., 2009). Therefore, we believe that the differences in kinetics we observed are most likely due to the effect of Cyt b5. Further studies are warranted to identify the mechanism underlying the substrate-dependent effect of Cyt b5 and to provide insight into the topology of the variant.

CHAPTER VI: *Altered susceptibility to metabolic inhibition by the CYP2B6*6 allele*

1. *Introduction*

I have shown that the *CYP2B6*6* allele alters the catalytic properties of CYP2B6 in a substrate- and Cyt b5-dependent manner in previous chapters (**Chapter III**, **Chapter IV** and **Chapter V**). It is likely that the same property of the variant that influences substrate metabolism also affects the susceptibility to inhibition. Indeed, it has been shown that amino acid substitutions, such as those found in the variant of *CYP2B6*6* allele, may alter the degree of susceptibility to competing metabolic inhibitors for certain CYP2B6 variants (Bumpus et al., 2006; Bumpus and Hollenberg, 2008; Talakad et al., 2009). In addition, differential genotype-dependent inhibition by a battery of inhibitors has been reported for the *CYP2C9*3* allele (Kumar et al., 2006b), as inhibition profile may be dependent on a specific inhibitor-substrate interaction with the CYP proteins.

Therefore, I tested the hypothesis that the *CYP2B6*6* allele is associated with altered susceptibility to metabolic inhibition. To test my hypothesis, I initially conducted a pilot study where I determined IC_{50} values for two CYP2B6 inhibitors, clopidogrel (Richter et al., 2004) and voriconazole (Jeong et al., 2009) towards efavirenz 8-hydroxylation in HLMs with *CYP2B6*1/*1* and *CYP2B6*6/*6*. Next I determined inhibition constant (K_i) values for the formation of 8-hydroxyefavirenz by voriconazole in the HLMs with *CYP2B6*1/*1*, *CYP2B6*1/*6* and *CYP2B6*6/*6* genotype.

2. *Results*

2.1 Determination of IC_{50} values for the inhibition of CYP2B6 by voriconazole and clopidogrel in HLMs with *CYP2B6*1/*1* and *CYP2B6*6/*6* genotype

To test whether susceptibility to metabolic inhibitors differs between HLMs that carry the *CYP2B6*6* allele and the wild type, inhibition potency of voriconazole and clopidogrel towards efavirenz 8-hydroxylation was determined in HLMs obtained from tissues genotyped for the *CYP2B6*6* allele (see **Chapter II-2.3 and II-5** for Methods). Inhibition of CYP2B6 by voriconazole and clopidogrel in HLMs with *CYP2B6*1/*1* and *CYP2B6*6/*6* is shown in Figure 6.1 A and B, respectively. IC_{50} values for voriconazole inhibition in HLMs with *CYP2B6*1/*1* and *CYP2B6*6/*6* were 0.40 and 0.16 μM, respectively. IC_{50} value for clopidogrel inhibition of efavirenz 8-hydroxylation in HLMs with *CYP2B6*1/*1* (IC_{50}= 0.14 μM) was also higher than that in HLMs with *CYP2B6*6/*6* (IC_{50}= 0.05 μM).

2.2 Determination of K_i values for the inhibition of CYP2B6 by voriconazole in HLMs with *CYP2B6*1/*1, CYP2B6*1/*6* and *CYP2B6*6/*6* genotype

I next determined K_i values for the inhibition on 8-hydroxylation of efavirenz by voriconazole in HLMs with *CYP2B6*1/*1* (n=5), *CYP2B6*1/*6* (n=6) and *CYP2B6*6/*6* (n=5) genotype (see **Chapter II-2.3 and II-5** for Methods). The K_i values were determined here because the IC_{50} value could be influenced by the substrate concentration and other experimental conditions used. Representative Dixon plots for the inhibition of efavirenz 8-hydroxylation in the HLMs with **1/*1, *1/*6* and **6/*6* genotypes are shown in Figure 6.2. The individual K_i values for the formation of 8-hydroxyefavirenz by voriconazole in the 16 HLM samples are listed in the Table 6.1. As

shown in Figure 6.3, there was a statistically significant difference among the K_i values estimated from the three genotypes ($p = 0.04$). There was no significant difference between the K_i values estimated from *CYP2B6*1/*6* (average K_i value = 1.55 μM) and *CYP2B6*6/*6* (average K_i value = 1.64 μM) ($p = 0.85$). But the K_i values estimated from HLMs with *CYP2B6*6/*6* ($p = 0.04$) and *CYP2B6*1/*6* ($p = 0.04$) genotypes were both significantly lower than that estimated from *CYP2B6*1/*1* genotype (average K_i value = 3.03 μM) (Figure 6.3). When the data from HLMs with *CYP2B6*6/*6* and *CYP2B6*1/*6* genotypes were combined and compared against HLMs with the *CYP2B6*1/*1* genotype, the K_i values for the inhibition of efavirenz 8-hydroxylation by voriconazole in the HLMs with *CYP2B6*1/*6 + *6/*6* genotypes was significantly lower ($p = 0.009$) than those observed in HLMs with *CYP2B6*1/*1* genotype.

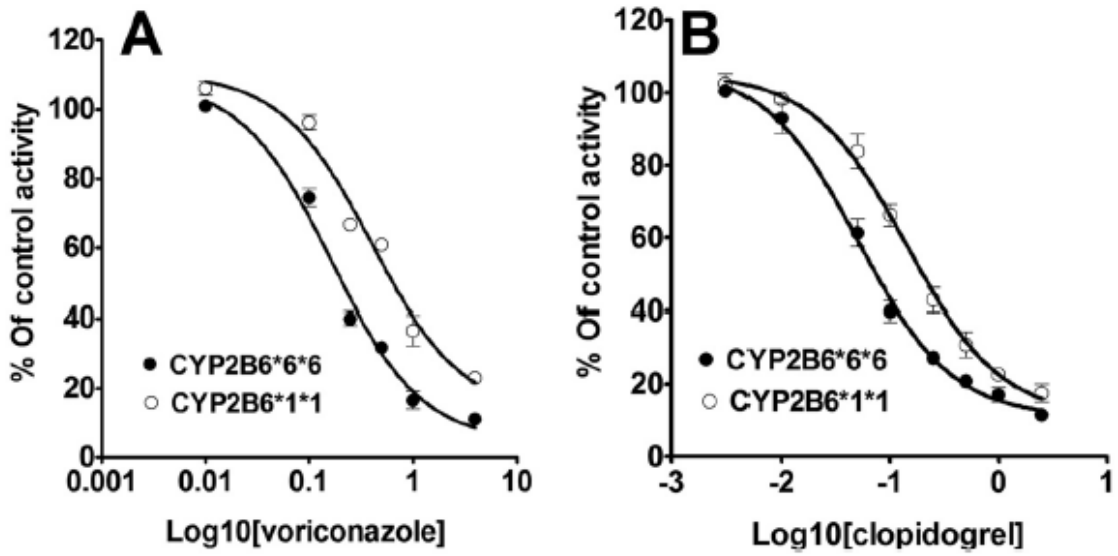

Figure 6.1 Inhibition of CYP2B6 by voriconazole (A) and clopidogrel (B) in HLMs with CYP2B6*1/*1 and CYP2B6*6/*6. Efavirenz (10 μM) was incubated with HLMs (0.25 mg/ml) and the NADPH-generating system for 15 min without or with voriconazole (0 to 4 μM) and clopidogrel (0 to 2.5 μM). Each point represents the mean of duplicate.

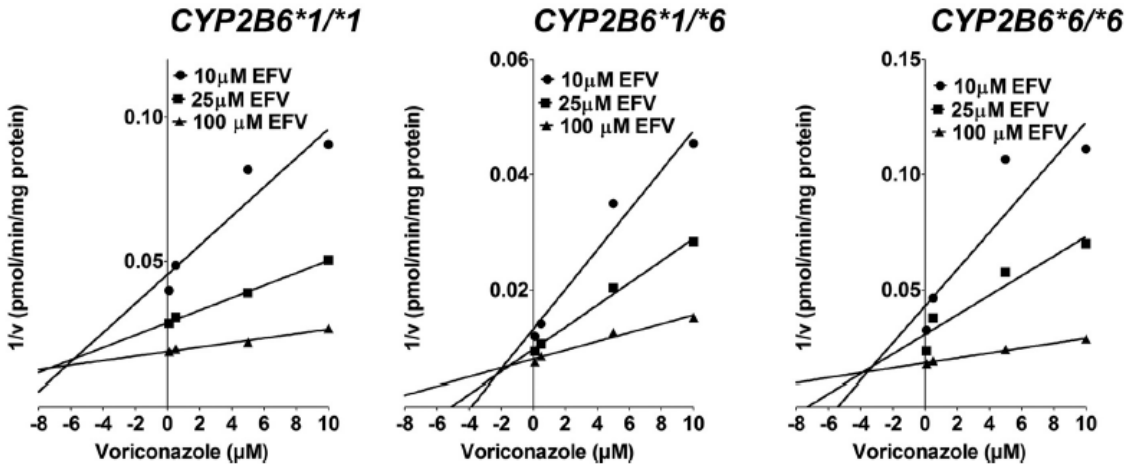

Figure 6.2 Representative Dixon plots for the inhibition on 8-hydroxylation of efavirenz by voriconazole in HLMs with CYP2B6*1/*1 (A), CYP2B6*1/*6 (B) and CYP2B6*6/*6 (C). Efavirenz (10 to 100 μM) was incubated with HLMs (0.25 mg/ml; IU 5, IU 73 and HL-G) and the NADPH-generating system at 37℃ for 15 min without or with voriconazole (0.1 to 10 μM). Each point represents the mean of duplicate. The line represents the best fit for the data.

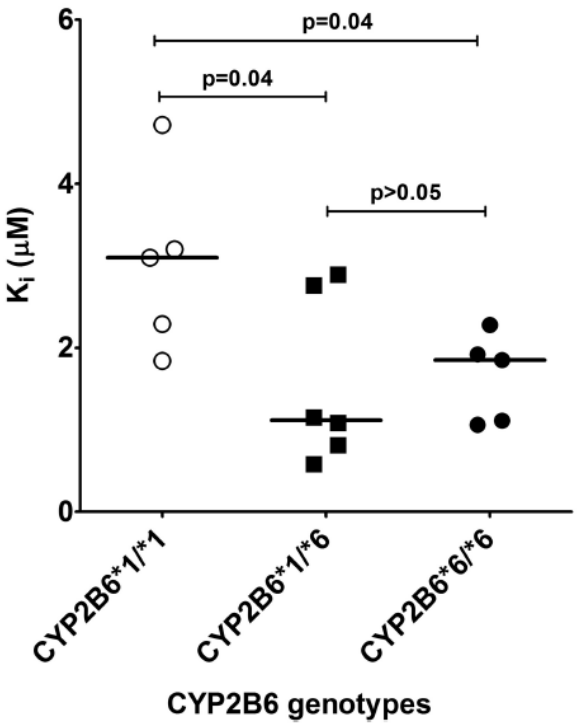

Figure 6.3 The K_i values for the formation of 8-hydroxyefavirenz by voriconazole in the HLMs with CYP2B6*1/*1, CYP2B6 *1/*6 and *6/*6 genotype. The horizontal line indicates the median K_i value and the box covers the 25^{th} to 75^{th} percentiles. Dots represent the K_i values generated using each individual human liver microsomal samples.

Table 6.1 Individual K_i values for the formation of 8-hydroxyefavirenz by voriconazole in the 16 HLM samples with *CYP2B6*1/*1, *1/*6* and **6/*6* genotypes.

HLMs	Genotype	K_i (μM)
IU-5	*CYP2B6*1/*1*	3.20
IU-58	*CYP2B6*1/*1*	4.72
IU-59	*CYP2B6*1/*1*	1.84
IU-61	*CYP2B6*1/*1*	3.10
HL-F	*CYP2B6*1/*1*	2.29
Mean ± S.D.		3.03 ± 1.10
IU-31	*CYP2B6*1/*6*	1.15
IU-33	*CYP2B6*1/*6*	0.81
IU-65	*CYP2B6*1/*6*	2.89
IU-73	*CYP2B6*1/*6*	0.58
HL-E	*CYP2B6*1/*6*	1.08
HL-S	*CYP2B6*1/*6*	2.76
Mean ± S.D.		1.55 ± 1.01
IU-6	*CYP2B6*6/*6*	1.92
IU-42	*CYP2B6*6/*6*	2.28
IU-84	*CYP2B6*6/*6*	1.06
HL-K	*CYP2B6*6/*6*	1.11
HL-G	*CYP2B6*6/*6*	1.85
Mean ± S.D.		1.64 ± 0.54

3. *Discussion*

In this section I explored the hypothesis that the *CYP2B6*6* allele is associated with altered susceptibility to metabolic inhibition. Data presented in this chapter have shown that inhibition of efavirenz 8-hydroxylation by voriconazole was greater in HLMs with *CYP2B6*6* allele (K_i = 1.6 ± 0.8 μM) than HLMs with *CYP2B6*1/*1* genotype (K_i = 3.0 ± 1.1 μM). These data suggest that the CYP2B6 variant protein is more susceptible to metabolic inhibition than the wild type. This suggestion is further supported by our data using clopidogrel as an inhibitor (2.8-fold lower IC_{50}).

I have shown that the *CYP2B6*6* allele alters the catalytic property of CYP2B6 in a substrate- and Cyt b5-dependent manner in previous chapters (**Chapter III**, **Chapter IV** and **Chapter V**). The same property of the variant that influences substrate metabolism seems also to confer susceptibility to inhibition. However, our data are in contrast to a previous study reporting decreased susceptibility of CYP2B6.6 protein to metabolic inhibition (Talakad et al., 2009). I speculate that several factors could contribute to this discrepancy, including the different type and composition of the proteins, substrates and inhibitors used in our study versus the other study. It has been shown that K262R, one of the amino acid mutations carried by the *CYP2B6*6* allele, loses the mechanism-based inactivation by different CYP2B6 inhibitors (Bumpus et al., 2006; Shebley and Hollenberg, 2007; Bumpus and Hollenberg, 2008). This alteration of inhibition profile by the variant allele has been suggested be related to the altered electron transfer and the addition of Cyt b5 can facilitate inactivation of the enzyme (Bumpus and Hollenberg, 2008). Previous studies have revealed the differences in the expression levels of Cyt b5 between the expressed CYP proteins and HLMs (Venkatakrishnan et al., 2000;

Huang et al., 2004). Therefore, it is very likely that the different amount of Cyt b5 in the two systems, i.e. expressed CYP2B6 protein in Talakad's versus HLMs in ours, may result in the opposite changes observed for the inhibition susceptibility by the *CYP2B6*6* allele. The fact that the presence of Cyt b5 influences the alteration of catalytic properties by the *CYP2B6*6* allele (see **Chapter V**) also lends more support to this notion. It is also worthwhile to note that the results generated in Talakad's study using N-terminal modified CYP2B6 protein should be interpreted with caution, since altered catalytic properties of such kind of modified CYP2B6 protein have been reported before (Zanger et al., 2007). Different substrate probe reactions used in two studies, i.e. O-deethylation of 7-methoxy-4-(trifluoromethyl)coumarin, versus 8-hydroxylation of efavirenz, could also contribute to the discrepancy, because the evidence of substrate-dependent inhibition has been shown for a number of CYP isoforms, e.g. CYP3A4 (Kenworthy et al., 1999), CYP2C8 (VandenBrink et al., 2011a), CYP2C9 (Kumar et al., 2006b), CYP2C19 (Foti and Wahlstrom, 2008) and CYP2D6 (Vandenbrink et al., 2011b). We included clopidogrel as a CYP2B6 inhibitor in the present study, since it was used in Talakad's study. In contrast to 6-fold increase in K_i with clopidogrel (Talakad et al., 2009), 2.8-fold lower IC_{50} was observed by the variant protein in the present study. However, clopidogrel is not a selective CYP2B6 inhibitor. It has been found to inhibit the activity of CYP2A6 (Walsky and Obach, 2007) that appears to contribute to the overall clearance of efavirenz by being the sole catalyst of efavirenz 7-hydroxylation, a pathway that accounts on average for ~23% of efavirenz metabolism, and by participating (~20-30%) in efavirenz 8-hydroxylation (Ogburn et al., 2010). Therefore, we further confirmed the greater

inhibition susceptibility by the variant protein using voriconazole as a CYP2B6 inhibitor, because it has marginal inhibition effect on CYP2A6 (Jeong et al., 2009).

The phenomenon of genotype-dependent inhibition has also been reported for other polymorphic CYPs, e.g. CYP2C9, CYP2C19 and CYP2D6. In contrast to the increased susceptibility to inhibition by the CYP2B6 variant observed in the present study, a greater degree of inhibition susceptibility of wild type CYP2C19 and CYP2D6 enzyme compared with variant forms have been consistently observed in humans (Hamelin et al., 2000; Lessard et al., 2001; Lindh et al., 2003; Uno et al., 2006). The variant alleles of CYP2C19, as well as those of CYP2D6, in the above studies cause either a splicing defect or a frame shift resulting in either premature termination of translation or a truncated protein. Thus, the genotype-dependent inhibition results for CYP2C19 and CYP2D6 polymorphisms identified in those studies would be expected for those CYP isoforms, whose polymorphisms result in the expression of inactive proteins, such that no residual activity is present to be inhibited. But for CYPs whose variant is associated with reduced activity, e.g. CYP2B6.6 and CYP2C9.3, it is important to take both genotype and fraction metabolized by a given pathway when predicting genotype-dependent drug-drug interaction (Kumar et al., 2008). 8-hydroxylation of efavirenz which is mainly mediated by CYP2B6 has been estimated to account for about 80% of total clearance of efavirenz independent of *CYP2B6*6* genotype from *in vitro* metabolism data (estimation based on the formation kinetics of 7, 8-hydroxyefavirenz in HLMs with *CYP2B6*1/*1*, *CYP2B6*1/*6* and *CYP2B6*6/*6* genotype; formation kinetics of 8-hydroxyefavirenz presented in **Chapter III** and formation kinetics of 7-hydroxyefavirenz presented in **Chapter VII**). Thus, the inhibition of CYP2B6 activity by other co-

administered medications in the anti-HIV regimen may result in the disproportionately marked reduction of efavirenz metabolism in slow metabolizers but probably not in the extensive metabolizers. However, the prediction of the impact of genotype-dependent inhibition on efavirenz exposure is further complicated by its ability to induce its own metabolism (autoinduction) at steady state (Zhu et al., 2009). A clinical study is ongoing to evaluate the genotype-dependent inhibition by voriconazole on efavirenz pharmacokinetics in our laboratory. This knowledge is potentially clinically important because differential dosage adjustments may be needed in individuals with the *CYP2B6*6* genotype compared with the more prevalent wild type-expressing individuals, when a known interacting drug is co-administered.

CHAPTER VII: *CYP2B6 pharmacogenetics-based in vitro-in vivo extrapolation (IVIVE) of efavirenz clearance: comparison between models and enzyme preparations*

1. *Introduction*

CYP2B6 plays a more important role than previously estimated in the metabolism of a growing list of drugs. It fully or partially metabolizes 8-13% of clinically important drugs (e.g., efavirenz, nevirapine, methadone, cyclophosphamide, ketamine, bupropion and propofol) as well as a long list of other xenobiotics of toxicological significance (Mo et al., 2009). Until recently, progress on the clinical relevance of this enzyme was hampered by the lack of selective and safe *in vivo* probes of its activity. Research on the clinical link between CYP2B6 metabolic status and drug disposition was accelerated subsequent to our demonstration of CYP2B6 as the main clearance mechanism of efavirenz (Ward et al., 2003). 8-Hydroxylation catalyzed predominantly by CYP2B6, with minor contributions from CYP1A2, CYP2A6 and CYP3A4/5, represents the main clearance pathway of efavirenz (Ward et al., 2003). CYP2A6-catalyzed 7-hydroxylation and UGT 2B7-mediated N-glucuronidation are minor metabolic pathways of the drug (Belanger et al., 2009; Ogburn et al., 2010; Cho et al., 2011). Emerging evidence suggest that the large interindividual variability in efavirenz exposure and clinical responses may be primarily attributed to the large variability in CYP2B6 protein expression (20- to 280-fold) and activity (25- to 100-fold) among human livers, in part due to extensive genetic polymorphisms in the *CYP2B6* gene and non-genetic factors, e.g., induction and inhibition drug interactions (Zanger et al., 2007). As described in **Chapter III**, the *CYP2B6* gene is highly polymorphic and the *CYP2B6*6* allele is by far the most frequent allele across populations and functionally relevant among all the variants (Zanger et al.,

2007). It is established that variants in the *CYP2B6* gene, particularly the *CYP2B6*6* allele, are associated with efavirenz clearance (Tsuchiya et al., 2004; Rotger et al., 2007), CNS side effects (Haas et al., 2004), hepatic toxicity (Yimer et al., 2011), treatment discontinuation (Wyen et al., 2011), virological failure (Motsinger et al., 2006), and drug interactions (Ngaimisi et al., 2011). Thus, efavirenz is not only an *in vitro* and *in vivo* activity probe, but can also serve as a prototype model drug for evaluating the clinical relevance of *CYP2B6* genetic polymorphisms. Other substrates for which CYP2B6 metabolic status is a major determinant of the clearance, drug interaction and response and/or toxicity include nevirapine, methadone, cyclophosphamide, bupropion, ketamine, propofol and sibutramine (Zanger et al., 2007).

Our previous study has shown an association between the *CYP2B6*6* allele with reduced CYP2B6 amount in HLMs (Desta et al., 2007), probably due to the aberrant splicing of pre-mRNA of CYP2B6 (Lamba et al., 2003; Hofmann et al., 2008). We have shown this allele also influences metabolic activity by altering substrate binding and catalytic activity *in vitro*, an effect that was highly dependent on the enzyme source used (expressed versus HLMs) in **Chapter III**. Taking all these findings together, the reduced efavirenz metabolism in individuals with the *CYP2B6*6/*6* genotype may be due to both reduced protein expression and altered binding affinity and catalytic efficiency. In order to test whether the mechanism that identified *in vitro* also applies to *in vivo*, I explored the hypothesis that the incorporation of *in vitro* efavirenz metabolism data predicts genetic effect of the *CYP2B6*6* allele on efavirenz pharmacokinetics by *in vitro-in vivo* extrapolation (IVIVE) in the present chapter.

Often, *in vitro-in vivo* extrapolation (IVIVE) is a well accepted and cost effective approach to quantitatively predict human drug metabolism *in vivo*, potential for metabolism-mediated drug interactions and genotype-phenotype associations in both industry and academic research. However, its success depends on the use of relevant *in vitro* systems that accurately and reliably generate high quality kinetic parameters as well as the selection of appropriate models for prediction. Several conventional modeling approaches have been used in the IVIVE. The well-stirred liver model (Obach, 1999; Ito and Houston, 2005) is probably the most widely employed liver model to predict average metabolic clearance with some success (Rowland et al., 2011). However, this model has limitations, particularly when attempts to predict inter-subject variability in drug exposure are made. In contrast to the well-stirred liver model, physiologically-based pharmacokinetic (PBPK) modeling can be utilized to predict the full time-concentration profile, the complexities of oral drug absorption, and likely the inter-individual variability in pharmacokinetics (Rowland et al., 2011). PBPK models divide the body into anatomically and physiologically meaningful compartments connected by the circulatory system, using system-specific parameters to describe the absorption, distribution, metabolism, and elimination (ADME) processes. These physiological parameters are coupled with drug-specific data such as physicochemical parameters to predict the plasma and tissue concentration versus time profiles of a compound.

The enzyme sources and their composition used may influence precise estimation of kinetic parameters. Enzyme sources that include hepatocytes, human liver fractions such as HLMs, and expressed enzymes are standard *in vitro* systems for IVIVE of drug clearance and potential drug interactions. Due to the limited availability of well-

characterized HLMs and hepatocytes with specific genotype information, expressed CYP2B6 variant and wild type proteins are often employed to determine the influence of pharmacogenetics on substrate metabolism and interaction. However, our previous study and data from other investigators indicate that kinetic profiles of CYP2B6 substrates are highly affected by the enzyme sources used (Ariyoshi et al., 2011; Zhang et al., 2011a; Xu et al., 2012). The same is true, when the impact of genetics on the activity of other enzymes (e.g., CYP2C9 and CYP2C8) is evaluated (Kumar et al., 2006a; Kaspera et al., 2011).

Pharmacogenetics-based IVIVE of efavirenz pharmacokinetics may be further complicated not only by its large inter-individual variability (Csajka et al., 2003; Rotger et al., 2007) but also its ability to enhance its own metabolism (autoinduction) upon multiple doses (Zhu et al., 2009), probably through induction of CYP2B6 (Faucette et al., 2007). The effect of efavirenz autoinduction on its exposure also appears to be CYP2B6 genotype-dependent (Ngaimisi et al., 2011). To exclude these confounding variables at steady state, prediction of efavirenz pharmacokinetics following a standard 600 mg oral dose should first be established.

In the present study, we have used rich *in vitro* efavirenz kinetic data set generated under the same experimental conditions from expressed CYP2B6.1 and CYP2B6.6 proteins as well as HLMs derived from liver tissues genotyped for the *CYP2B6*6* allele to: 1) predict efavirenz exposure after a single 600 mg oral dose of efavirenz in healthy volunteers; 2) systematically evaluate which enzyme sources (HLMs versus expressed CYP2B6) may better predict efavirenz exposure; and 3) compare the

performance of PBPK and well-stirred liver model in the prediction of mean efavirenz clearance for individuals with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotype.

2. *Results*

2.1 *In vitro* metabolism data of efavirenz

In two HLMs (HH-488, a **1/*6* carrier; and HH-478, a **6/*6* carrier), incubation of efavirenz did not produce quantifiable 7-hydroxyefavirenz (Table 7.1). Kinetics for the formation of 7-hydroxyefavirenz were determined in the rest of HLMs. Formation rates of 7-hydroxyefavirenz versus efavirenz concentrations were fit into a simple single-site Michaelis-Menten equation to estimate kinetic parameters (V_{max} and K_m) for individual HLM. The mean Cl_{int} of 7-hydroxylation calculated from individual HLM with the same genotype are shown in Table 2.2 (Materials and Method). The Cl_{int} values for the formation of 8-hydroxyefavirenz in expressed CYP2B6 and HLMs were reported in our recent publication and used in the present simulation (Xu et al., 2012).

Table 7.1 Kinetic parameters for the formation of 7-hydroxyefavirenz from efavirenz in 15 HLM samples with *CYP2B6*1/*1, *1/*6* and **6/*6 genotypes* (n=5 HLMs for each genotype).

HLMs	7-Hydroxyefavirenz		
	V_{max}	K_m	Cl_{int}
*CYP2B6*1*1*			
HL-U	27.67	43.32	0.64
HL-M	14.89	19.32	0.77
HL-P	11.85	12.39	0.96
HH-689	6.18	11.66	0.53
MCV-65	20.15	7.30	2.76
Mean	16.15	18.80	1.13
S.D.	8.19	14.39	0.92
*CYP2B6*1/*6*			
HL-S	79.21	19.24	4.12
HL-C	4.13	16.01	0.26
HL-O	49.36	66.94	0.74
MCV-49	9.52	10.92	0.87
HH-488	ND	ND	ND
Mean	35.56	28.28	1.50
S.D.	35.41	26.00	1.77
*CYP2B6*6/*6*			
HL-K	38.13	41.84	0.91

HH-1180	27.06	11.00	2.46
IIAM-091698-1	8.09	22.17	0.36
HH-525	8.99	7.22	1.25
HH-478	ND	ND	ND
Mean	20.57	20.56	1.25
S.D.	14.61	15.54	0.89

Increasing concentrations of efavirenz (1 - 200 μM) were incubated with HLM samples (0.25mg/ml) with *CYP2B6*1/*1*, **1/*6* and **6/*6* genotypes (n=5 HLMs for each genotype) and a NADPH-generating system at 37 ℃ for 15min in duplicate. Kinetic parameters for the formation of 7-hydroxyefavirenz were estimated by fitting the velocity versus efavirenz concentrations to the simple single-site Michaelis-Menten equation. Kinetic parameters in the individual HLM samples are presented. *In vitro* Cl_{int} was calculated as V_{max}/K_m. V_{max}, pmol/min/mg protein; K_m, μM; Cl_{int}, μl/min/mg protein. ND: not detected.

2.2 Efavirenz pharmacokinetics

The pharmacokinetic parameters following a single 600 mg oral dose of efavirenz are summarized in Table 7.3 and are consistent with a previous study (Haas et al., 2009). The values for efavirenz CL_{po} of individuals with CYP2B6 wild type (n=8) and heterozygotes (n=9) were 8.5 (5^{th} to 95^{th} percentile: 5.4- 13.4) L/h and 8.3 (4.7-11.5) L/h, respectively. Correspondingly, the $AUC_{0-\infty}$ were 80.0 (45.3- 110.6) mg/L•h and 81.6 (52.3- 132.8) mg/L•h, respectively. The efavirenz CL_{po} for individuals with *CYP2B6*6/*6* (n=3) was 5.9 (5.6-6.4) L/h and the $AUC_{0-\infty}$ was 101.7 (93.9- 106.4) mg/L•h. Although the difference in the values of CL_{po} between individuals with *CYP2B6*1/*1* & **1/*6* and *CYP2B6*6/*6* did not reach statistical significance (p= 0.23) probably mainly due to the small size of the study, it was associated with 30% reduction in CL_{po} and 27% increase in $AUC_{0-\infty}$ of homozygtes compared with wild type.

2.3 Prediction of efavirenz single oral dose pharmacokinetics using HLM Cl_{int} by PBPK Modeling

Compartmental absorption transit (CAT) model with a full PBPK model fitted efavirenz time-concentration profile best based on visual predictive checks and therefore were selected to perform simulations in the present study. The simulated versus observed efavirenz pharmacokinetics of individuals with *CYP2B6*1/*1, *1/*6* and **6/*6* using *in vitro* Cl_{int} determined in HLMs with corresponding genotypes are shown in Figure 7.1. The corresponding observed and Simcyp-simulated pharmacokinetic parameters are summarized in Table 7.2. For individuals with *CYP2B6*1/*1* genotype, Simcyp-predicted values of C_{max}, T_{max}, $AUC_{0-\infty}$ and CL_{po} were in good agreement with observed values (Table 7.2). For individuals with *CYP2B6*1/*1* genotype, predicted mean values

of C_{max}, T_{max}, $AUC_{0-\infty}$ and CL_{po} were in good agreement with observed values (Table 7.2). However, the PBPK model under-predicted efavirenz CL_{po} for individuals with *CYP2B6*1/*6* genotype [simulated: 4.7 ± 3.2 L/h versus observed: 8.3 ± 2.8 L/h] and **6/*6* [simulated: 2.2 ± 1.4 L/h versus observed: 5.9 ± 0.5 L/h] using HLM data. The predicted mean of CL_{po} for individuals with *CYP2B6*1/*6* and *CYP2B6*6/*6* was 43% and 63% lower than that obtained from the clinical trial, respectively. Accordingly, the predicted mean $AUC_{0-\infty}$ for individuals with *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes was 103.2% and 200.0% higher than the observed values. Individuals with *CYP2B6*6/*6* genotype were predicted to have a 74% reduction in mean CL_{po} compared to individuals with *CYP2B6*1/*1* genotype, while a smaller extent of reduction (~30%) was observed in the clinical trial.

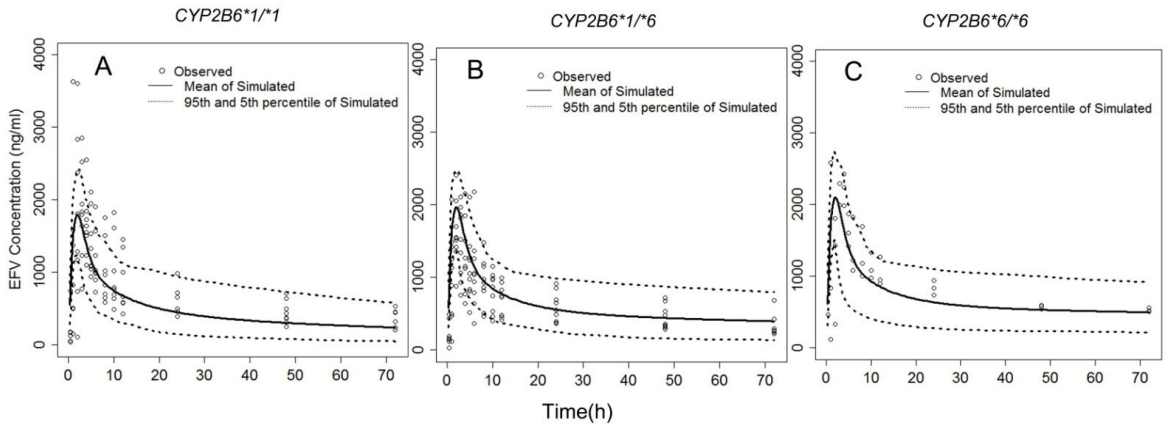

Figure 7.1 Observed and PBPK-predicted efavirenz (EFV) concentration-time profile after a single 600 mg oral dose of efavirenz in individuals with *CYP2B6*1/*1* (A), *CYP2B6*1/*6* (B) and *CYP2B6*6/*6* (C) genotypes using *in vitro* Cl_{int} determined in HLMs obtained from liver tissues with corresponding *CYP2B6*6* genotypes. Symbols represent the observed efavirenz plasma concentrations (0-72 h) from healthy volunteers after a single 600 mg oral dose of efavirenz with *CYP2B6*1/*1* (n=8), *CYP2B6*1/*6* (n=9) and *CYP2B6*6/*6* (n=3) genotypes. The solid line represent the mean of simulated efavirenz plasma concentrations, while the dashed lines represent the 5^{th} and 95^{th} percentile of simulated efavirenz plasma concentrations. 20 trials were simulated for each genotype.

Table 7.2 Observed and simulated pharmacokinetic parameters for a single 600 mg oral dose of efavirenz in 20 healthy subjects by PBPK model (All the values are presented as mean ±S.D.).

		Pharmacokinetic Parameters			
		T_{max} (h)	C_{max} (mg/L)	$AUC_{0-\infty}$ (mg•h/L)	CL_{po} (L/h)
Observed	*CYP2B6*1/*1* (n=8)	2.3 ± 1.0	2.3 ± 0.7	79.8 ± 28.4	8.5 ± 3.4
Predicted	*CYP2B6*1/*1* HLM	2.0 ± 0.4	1.8 ± 0.4	75.6 ± 43.1	8.5 ± 6.9
Predicted	CYP2B6.1(Simcyp)	2.0 ± 0.4	1.8 ± 0.4	175.1 ± 83.5	4.1 ± 2.2
Predicted	CYP2B6.1 (VISEF)	2.0 ± 0.4	1.8 ± 0.4	84.3 ± 57.2	9.8 ± 6.9
Predicted	CYP2B6.1 (CLISEF)	1.9 ±0.3	1.6 ± 0.4	39.0 ± 35.2	24.3 ± 20.8
Observed	*CYP2B6*1/*6* (n=9)	2.6 ± 1.7	1.7 ± 0.5	81.6 ± 33.7	8.3 ± 2.8
Predicted	*CYP2B6*1/*6* HLM	2.1 ± 0.4	2.0 ± 0.3	165.8 ± 94.1	4.7 ± 3.2
Observed	*CYP2B6*6/*6* (n=3)	2.7 ± 1.5	2.4 ± 0.2	101.7 ± 7.9	5.9 ± 0.5
Predicted	*CYP2B6*6/*6* HLM	2.1 ± 0.4	2.2 ± 0.3	305.1 ± 135.8	2.2 ± 1.4
Predicted	CYP2B6.6 (Simcyp)	2.1 ± 0.4	1.9 ± 0.3	235.9 ± 98.8	2.8 ± 1.6
Predicted	CYP2B6.6 (VISEF)	2.0 ± 0.4	2.0 ± 0.4	136.0 ± 83.8	6.6 ± 6.4
Predicted	CYP2B6.6 (CLISEF)	2.0 ± 0.4	2.1 ± 0.4	183.7 ± 93.3	4.2 ±3.1

2.3 Predicted efavirenz single oral dose pharmacokinetics using expressed CYP2B6 Cl_{int} by PBPK Modeling

The simulated versus observed efavirenz pharmacokinetics of individuals with *CYP2B6*1/*1*and *CYP2B6*6/*6* using *in vitro* Cl_{int} determined in expressed CYP2B6.1 and CYP2B6.6 are shown in Figure 7.2. The corresponding observed and Simcyp-simulated pharmacokinetic parameters are summarized in Table 7.2. PBPK model predicted the mean values of C_{max} and T_{max} for individuals with *CYP2B6*1/*1* and *CYP2B6*6/*6* reasonably well. However, it under-predicted efavirenz mean CL_{po} for individuals with *CYP2B6*1/*1* [simulated: 4.1 ± 2.2 versus observed: 8.5 ± 3.4 L/h and *CYP2B6*6/*6* [simulated: 2.8 ± 1.6 L/h versus observed: 5.9 ± 0.5 L/h] (Table 7.2). The predicted mean of CL_{po} for individuals with *CYP2B6*1/*1* and *CYP2B6*6/*6* genotypes was 52% and 53% lower than that observed in the clinical trial, respectively. Accordingly, the predicted mean of $AUC_{0-\infty}$ for individuals with *CYP2B6*1/*1* and *CYP2B6*6/*6* genotypes was 119.4% and 132.0% higher than that observed in the clinical trial, respectively. Individuals with *CYP2B6*6/*6* genotype were predicted to have a 32% reduction in mean CL_{po} compared to individuals with *CYP2B6*1/*1*.

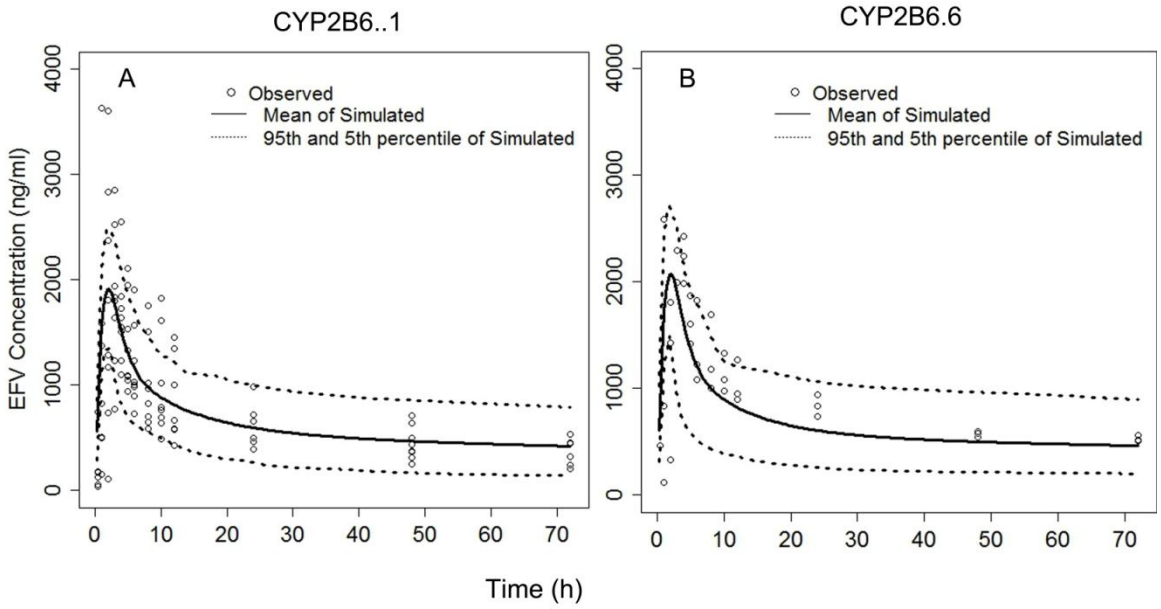

Figure 7.2 Observed and PBPK-predicted efavirenz (EFV) concentration-time profile after a single 600 mg oral dose of efavirenz in individuals with *CYP2B6*1/*1* (A) and *CYP2B6*6/*6* (B) genotypes using *in vitro* Cl_{int} generated from expressed CYP2B6.1 and CYP2B6.6 proteins. Symbols represent the observed efavirenz plasma concentrations (0-72 h) from healthy volunteers after a single 600 mg oral dose of efavirenz with *CYP2B6*1/*1* (n=8) and *CYP2B6*6/*6* (n=3) genotypes. The solid line represent the mean of simulated efavirenz plasma concentrations, while the dashed lines represent the 5^{th} and 95^{th} percentile of simulated efavirenz plasma concentrations. 20 trials were simulated for each genotype.

2.4 Recalculation of ISEFs

Previous study indicates that ISEF values may vary widely depending on CYP expression system, HLM preparations, probe substrate selected, and/or assay conditions used in each laboratory (Proctor et al., 2004). Thus, the under-prediction of CL_{po} using *in vitro* Cl_{int} in expressed CYP2B6 may indicate that the default settings of ISEFs for CYP2B6 in Simcyp may not apply to our system. Therefore, ISEFs of expressed CYP2B6.1 and CYP2B6.6 were calculated on the basis of V_{max} and Cl_{int} estimated from the kinetics of bupropion 4-hydroxylation determined in the HLMs and expressed CYPs from our previous publication (Xu et al., 2012). The ISEF values determined on the basis of V_{max} (VISEF) and Cl_{int} (CLISEF) for CY2B6 are shown in Table 7.3. For CYP2B6.1, the VISEF value was 1.9 and CLISEF value was 6.5. For CYP2B6.6, the VISEF value was 1.6 and CLISEF value was 0.9. The same simulations were performed as described above to evaluate the effect of different ISEF values on the prediction of efavirenz CL_{po}. The simulated key pharmacokinetic parameters are summarized in Table 7.2. The predicted population median, 5^{th} and 95^{th} percentile (using Cl_{int} determined in expressed CYP2B6.1 and CYP2B6.6 correcting for Simcyp ISEF, recalculated VISEF and CLISEF) versus observed CL_{po} are shown in Figure 7.3. For expressed CYP2B6.1, the prediction using VISEF reasonably captured the observed values of efavirenz CL_{po}, while under-prediction using Simcyp ISEFs and over-prediction using CLISEF were observed. For expressed CYP2B6.6, simulations using recalculated VISEF and CLISEF both improved the prediction of efavirenz CL_{po} for individuals with the *CYP2B6*6/*6* genotype.

Table 7.3 Recalculation of ISEFs.

	VISEF	CLISEF	Simcyp
CYP2B6.1	1.9	6.5	0.43
CYP2B6.6	1.6	0.9	NA

VISEF: calculated on the basis of V_{max}.

CLISEF: calculated on the basis of Cl_{int}.

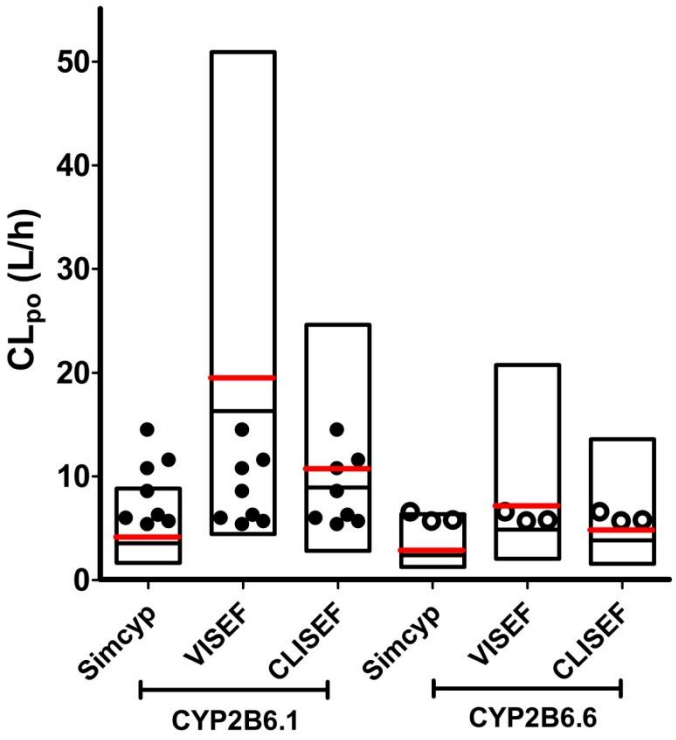

Figure 7.3 Observed and PBPK-predicted efavirenz oral clearance (CL_{po}) after a single 600 mg oral dose of efavirenz in individuals with *CYP2B6*1/*1* and *CYP2B6*6/*6* genotypes using *in vitro* Cl_{int} determined in expressed CYP2B6.1 and CYP2B6.6 correcting for different values of ISEFs. The simulations of CL_{po} were performed incorporating default values of ISEFs for expressed CYP2B6 and CYP2A6 built in the Simcyp (Simcyp), ISEF determined on the basis of V_{max} (VISEF) and Cl_{int} (CLISEF). Horizontal black lines represent the 95th percentile, median and 5th percentile of simulations. Horizontal red line represents the mean of simulations. Symbols represent CL_{po} estimated from pharmacokinetics observed in the individuals with *CYP2B6*1/*1* (n=8) and *CYP2B6*6/*6* (n=3) in the clinical trial. 20 trials were simulated for each genotype.

2.5 Prediction of efavirenz CL_{po} by Well-stirred liver model

The predicted efavirenz CL_h of individuals with *CYP2B6*1/*1, *1/*6* and **6/*6* extrapolated from *in vitro* Cl_{int} determined in the HLMs with corresponding genotypes by well-stirred liver model are shown in Table 7.4. The predicted efavirenz CL_h of individuals with *CYP2B6*1/*1* and **6/*6* extrapolated from *in vitro* Cl_{int} determined in expressed CYP2B6.1 and CYP2B6.6 correcting for different ISEF values are also shown in Table 7.4. The predicted CL_h extrapolated from Cl_{int} in HLMs and expressed enzymes with different ISEFs were compared with the observed mean CL_{po} of individuals carrying the same *CYP2B6*6* genotype in clinical trial (Table 7.4). Predictions using expressed CYP2B6 corrected for CLISEFs by well-stirred liver model showed best prediction in terms of accuracy and variability. Predictions using Simcyp ISEFs were associated with largest variability and average fold error. Predictions using HLMs, Simcyp ISEFs and VISEFs all tended to underestimate efavirenz CL_{po}.

2.6 Comparison of predictions by PBPK and Well-stirred liver model

The performances of efavirenz CL_{po} and CL_h prediction by PBPK and well-stirred liver model using HLM and expressed systems were evaluated by comparing predicted with observed mean efavirenz clearance (Table 7.4). Since IV formulation of efavirenz is not available, bioavailability of the drug is unknown. Thus, efavirenz CL_h was predicted by well-stirred liver model and used for comparison in Table 7.4. Predictions using expressed CYP2B6 corrected for CLISEFs by PBPK model showed best prediction in terms of accuracy and variability. Predictions using Simcyp ISEFs were associated with largest variability and average fold error by well-stirred liver model. In general, PBPK model had better prediction of mean CL_{po} than well-stirred liver model with the

exception of that when Simcyp ISEFs were used. Well-stirred liver model had comparable prediction to Simcyp model when CLISEF was corrected for the expressed system. The tendency of over-prediction and/or under-prediction was the same by PBPK and well-stirred liver model except that when VISEFs were used. IVIVE using HLM data tended to underestimate efavirenz CL_{po} using both models.

Table 7.4 Prediction of efavirenz oral clearance (CL_{po}) and hepatic clearance (CL_h) from *in vitro* data generated in expressed CYP2B6 and genotyped HLMs by PBPK and well-stirred liver model.

		PBPK model				Well-stirred liver model			
		Predicted Mean CL_{po}	RMSE	MRS	Average fold error	Predicted Mean CL_h	RMSE	MRS	Average fold error
HLMs			3.0	-2.5	2.3		4.3	-4.2	4.1
	*CYP2B6*1/*1*	8.5				5.8			
	*CYP2B6*1/*6*	4.7				3.0			
	*CYP2B6*6/*6*	2.1				1.4			
rhCYPs (Simcyp ISEF)			3.8	-3.8	3.2		5.5	-5.4	6.1
	CYP2B6.1	4.1				2.2			
	CYP2B6.6	2.8				1.3			
rhCYPs (New VISEF)			7.8	6.1	2.2		2.8	-2.7	2.5

	CYP2B6.1	16.3				6.7			
	CYP2B6.6	4.9				2.4			
rhCYPs			1.7	0.6	0.1		2.9	0.2	0.7
(New CLISEF)									
	CYP2B6.1	9.0				11.5			
	CYP2B6.6	4.8				3.2			

Simcyp ISEF: Simcyp-default ISEF values. New VISEF: ISEF values determined on the basis of V_{max} obtained from our study. New CLISEF: ISEF values determined on the basis of CL_{int} obtained from our study. RMSE, MRS and average fold error were calculated by comparing the predicted efavirenz mean clearance using PBPK and well-stirred liver model to observed mean values. Since IV formulation of efavirenz is not available, bioavailability of the drug is unknown. Thus, efavirenz CL_h was predicted by well-stirred liver model and used for comparison.

3. *Discussion*

In this section I explored the hypothesis that the incorporation of *in vitro* efavirenz metabolism data predicts genetic effect of the *CYP2B6*6* allele on efavirenz pharmacokinetics by IVIVE. We have shown that 1) expressed CYP2B6 system has provided improved prediction of efavirenz clearance following a single dose compared to genotyped HLMs with more accuracy achieved by PBPK model than well-stirred liver model; 2) the most reliable predictions were achieved by recalculating ISEFs for CYP2B6.1 and CYP2B6.6 using metabolism data generated from expressed CYP2B6. These findings suggest that: 1) expressed CYP2B6 system seems superior to HLMs in pharmacogenetics-based IVIVE of drug clearance; 2) it may be critical to establish ISEFs for wild type and variant proteins when expressed enzymes are used for IVIVE; and 3) expressed CYP2B6 may be a reliable system to investigate the effect of *CYP2B6*6* allele on metabolism of CYP2B6 substrates.

HLMs and expressed CYPs are the most commonly utilized *in vitro* systems in predicting drug clearance during drug discovery and development. Generally, HLMs are considered to be more physiologically relevant than expressed system. However, its use is limited by the availability, variable quality of tissues and variability between donors (Hallifax et al., 2010). Indeed, there is a large viability in Cl_{int} and catalytic activity of efavirenz 8-hydroxylation determined in HLMs even within the same genotype (Desta et al., 2007; Xu et al., 2012). In our study, for the first time, we compared the utility of these two *in vitro* systems in pharmacogenetics-based IVIVE under the same experimental condition. Expressed CYP2B6 system using recalculated CLISEFs captured the mean values of efavirenz CL_{po} with better accuracy and less variability than HLMs (Table 7.4).

Taking a closer look into the results, the wild type HLMs actually provided comparable prediction of efavirenz clearance for individuals with the *CYP2B6*1/*1* genotype. The inferiority of HLMs in pharmacogenetics-based IVIVE is primarily due to its under-prediction of efavirenz clearance for individuals with *CYP2B6*1/*6* and **6/*6*, which can be attributed to several factors. Firstly, altered binding affinity and catalytic efficiency secondary to reduced protein expression may both contribute to reduced efavirenz metabolism in HLMs with the *CYP2B6*6/*6* genotype (Xu et al., 2012). The default unit of HLM Cl_{int} in Simcyp is normalized to mg microsomal protein. Simcyp default setting of CYP2B6 expression amount is 17 pmol/mg protein in CYP2B6 extensive metabolizer (EM) versus 6 pmol/mg in CYP2B6 poor metabolizer (PM). Thus, additive effect of reduced protein expression by the *CYP2B6*6* allele on catalytic efficiency and overall extrapolated Cl_{int} may lead to the under-prediction of efavirenz clearance in homozygote. Secondly, the mechanism by which the *CYP2B6*6* allele influences the function of heterozygote is not fully understood. The under-prediction of efavirenz clearance in heterozygote is just expected regarding the discrepancy between *in vitro* and *in vivo* observed for several CYP2B6 substrates in heterozygote. Both CYP2B6 expression and efavirenz metabolism are found to be reduced in HLMs with *CYP2B6*1/*6* compared to wild type (Desta et al., 2007; Xu et al., 2012). However, the phenotypes of heterozygote and wild type are overlapping for a number of CYP2B6 substrates including efavirenz, methadone and cyclophosphamide *in vivo* (Tsuchiya et al., 2004; Crettol et al., 2005; Nakajima et al., 2007). Thirdly, only the *CYP2B6*6* allele was genotyped in those human liver tissues where HLMs were prepared. Therefore, the

possibility that those liver samples also contain other unknown *CYP2B6* genetic variants with reduced functions cannot be excluded.

While genotyped HLMs have the aforementioned limitations and confounding factors, expressed system has been used for pharmacogenetics-based IVIVE of pharmacokinetics and/or pharmacodynamics for several polymorphic CYPs, e.g. CYP2C9 (Dickinson et al., 2007a; Kusama et al., 2009), CYP2D6 (Dickinson et al., 2007b) and CYP2B6 (Siccardi et al., 2012). Despite the success in predicting phenotypes for wild type, very limited success was obtained for homozygote (Dickinson et al., 2007b; Siccardi et al., 2012). We speculate that the over-prediction of efavirenz clearance for individuals carrying the *CYP2B6*6/*6* genotype in the recent study by Siccardi et al. could be due to that the reduced efavirenz binding affinity and catalytic efficiency by the *CYP2B6*6* allele was not taken into account in their model (Siccardi et al., 2012). The importance of considering functional changes by the CYP2B6 variants in pharmacogenetics-based IVIVE is further supported by the fact that not only reduced protein expression but also the altered catalytic properties may contribute to the altered substrate metabolism by the *CYP2B6*6* allele *in vitro* (Jinno et al., 2003; Watanabe et al., 2010; Ariyoshi et al., 2011; Zhang et al., 2011a; Xu et al., 2012). Furthermore, the present study that incorporated altered catalytic efficiency of CYP2B6.6 protein showed significantly improved prediction of efavirenz clearance for individuals with *CYP2B6*6/*6* genotype. Therefore, it is essential to obtain a comprehensive understanding of the mechanisms by which the genetic variants alter substrate metabolisms and incorporate them into the modeling exercise for successes in

pharmacogenetics-based IVIVE of pharmacokinetics and/or pharmacodynamics for drugs metabolized by polymorphic CYPs.

The initial under-prediction of efavirenz clearance using metabolism data generated from expressed CYP2B6 propels us to explore the possible *in vitro* factors that could contribute to the under-prediction, including unbound fraction in expressed CYP2B6 (f_{umic}) and ISEFs, etc. Several predictive equations for f_{umic} determined by drug lipophilicity and protein concentrations have been developed (Austin et al., 2002; Hallifax and Houston, 2006; Turner et al., 2007). The protein concentrations used in HLM and CYP2B6 incubations were 0.25 mg/ml ~1 mg/ml and thus f_{umic} of efavirenz was estimated to be 0.2~0.4. A published f_{umic} value of 0.3 with reasonably good prediction of efavirenz clearance (Rekic et al., 2011) was contained in that range and therefore was used in the present study. However, the uncertainties on f_{umic} determinations contribute to the under-prediction of clearance cannot be excluded. ISEF is the other important factor with great impact on the extrapolation of clearance from expressed CYPs. It has been employed to account for the difference in turnover number (activity per unit amount of P450) between expressed CYPs and HLMs (Proctor et al., 2004) and successfully used for IVIVE to predict *in vivo* clearance in a number of studies (Howgate et al., 2006; Dickinson et al., 2007a; Dickinson et al., 2007b; Siccardi et al., 2012). ISEFs have been shown to depend on the expressed systems, HLM preparations, substrates used and assay conditions (Proctor et al., 2004). Especially considering the substrate-dependent effects on the alteration of substrate binding and catalytic activity by the *CYP2B6*6* allele (Jinno et al., 2003; Watanabe et al., 2010; Ariyoshi et al., 2011; Zhang et al., 2011a; Xu et al., 2012), it is very likely that the ISEFs determined using

other CYP2B6 substrate, e.g. bupropion and cyclophosphamide, may not be applied to efavirenz. Furthermore, the ISEF values are determined on the basis of enzyme abundance, K_m and V_{max} (Proctor et al., 2004), which all have been shown to be altered by the *CYP2B6*6* allele (Hofmann et al., 2008; Zhang et al., 2011a; Xu et al., 2012). Therefore, it is important to test the effect of this allele on ISEFs of CYP2B6. Indeed, incorporation of recalculated CLISEF greatly improved the prediction of efavirenz clearance by PBPK and well-stirred liver model. PBPK model using CLISEF showed the best prediction in terms of accuracy and bias, probably due to that CLISEF accounts for the differences in efavirenz K_m from expressed and HLM systems. This result highlights the importance of establishing ISEFs for individual CYP isoform and its variant proteins using specific substrate when general values of ISEFs did not work.

In the present study, PBPK model performed generally better prediction for mean values of efavirenz CL_{po} compared to well-stirred liver model, when the same metabolic data and extrapolation factors were used. But it is worthy of notice that well-stirred liver model had very comparable prediction to PBPK model when CLISEF was used, supporting its utility in IVIVE when only limited resources are available. A comprehensive knowledge of drug properties, physiological and anatomical compositions of human body is a necessity to achieve good prediction by PBPK model, which may not be available in early drug development. Therefore, well-stirred liver model may still retain its important role in IVIVE because of its simplicity.

Although PBPK modeling requires much more information about the drug and *in vivo* system than well-stirred liver model as mentioned above, a particular strength of PBPK modeling is the capability, with the use of Monte Carlo methods, to predict not

only the mean values of pharmacokinetics but also the variability in it beyond observed limits and to anticipate the subpopulation who are likely to be associated with extreme pharmacokinetics (Rowland et al., 2011). The prediction of the variability is particularly important for drugs that are associated with a large interindividual variability in pharmacokinetics and thus pharmacodynamics like efavirenz (Csajka et al., 2003; Haas et al., 2004). It appears that PBPK model captured the variability of efavirenz CL_{po} in wild type comparing the simulated 5^{th} and 95^{th} percentile with observed values in the present and previous studies (Haas et al., 2009). But it seems to overestimate the variability associated with efavirenz CL_{po} of individuals carrying *CYP2B6*6/*6* genotype. Whether this overestimation is due to the overestimated variability associated with CYP2B6 expression or other physiological parameters in homozygotes warrant further investigation.

In summary, we systematically investigated the predictability of expressed CYP2B6 and HLMs genotyped for the *CYP2B6*6* allele in pharmacogenetics-based IVIVE of efavirenz clearance under the same experimental conditions. PBPK and well-stirred liver models are able to predict efaivirenz single-dose pharmacokinetics incorporating the *in vitro* mechanisms underlying the reduced efavirenz metabolism by the *CYP2B6*6* allele in a genotype-based manner. We provided evidence that expressed CYPs seems to be a better system than HLMs for pharmacogenetics-based IVIVE of CYP2B6 substrates using efavirenz as an example. Expressed CYP2B6 may be a useful tool to study the effect of *CYP2B6* genetic polymorphism on the substrate metabolism in the early drug development. We also showed the importance of establishing ISEFs for specific substrate and variant proteins in the prediction of drug clearance using expressed

CYP2B6. PBPK model developed in the present study can be a base model for the simulation of efavirenz steady-state pharmacokinetics, and drug-drug interactions.

CHAPTER VIII: *In vitro analysis and quantitative prediction of efavirenz inhibition of eight Cytochrome P450s (CYPs)*

1. Introduction

The propensity for clinically important drug-drug interactions of efavirenz is high, because it is always used in combination therapy and in concert with drugs directed at the treatment of opportunistic infections, cancers and other HIV-related co-morbidities. The alterations in pharmacokinetics of co-administered drugs by efavirenz lead to either lack of efficiency or adverse drug reactions of the victim drug (Deeks and Perry, 2010). In order to predict and avoid adverse drug interactions with efavirenz-based therapy, it is important to identify mechanisms underlying those drug interactions.

Efavirenz is known to alter the pharmacokinetics of a long list of co-administered drugs [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012], probably by modulating the activities of multiple drug metabolizing enzymes and/or drug transporters. Efavirenz is predominantly cleared mainly by CYP2B6-mediated 8-hydroxylation, with small contribution from other CYPs (e.g., CYP2A6, CYP3A and CYP1A2) (Ward et al., 2003; Ogburn et al., 2010). Two minor pathways, efavirenz 7-hydroxylation and N-glucuronidation, are predominantly catalyzed by CYP2A6 (Ogburn et al., 2010) and UGT2B7 (Belanger et al., 2009), respectively. Efavirenz, through activation of constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR), enhances the expression of multiple enzymes regulated by these nuclear receptors, including CYP2B6, CYP2C19 and CYP3A (Hariparsad et al., 2004; Faucette et al., 2007). Therefore, many drug interactions associated with efavirenz at steady state including decreased exposures of methadone (Clarke et al., 2001), statins

(Gerber et al., 2005), omeprazole (Michaud et al., 2012b), voriconazole (Liu et al., 2008), proguanil (van Luin et al., 2010) and etravirine (Boffito et al., 2009), and protease inhibitors (Staszewski et al., 1999) can be primarily explained by the inductive effect of efavirenz. Furthermore, efavirenz enhances its own metabolism (auto-induction) upon repeated administration compared to a single dose (Zhu et al., 2009), probably through induction of CYP2B6 and other enzymes involved in its metabolism (Ward et al., 2003; Belanger et al., 2009; Ogburn et al., 2010). Besides induction, there is *in vitro* evidence that efavirenz may directly inhibit the activities of certain CYPs (Hesse et al., 2001; von Moltke et al., 2001; Bumpus et al., 2006; Parikh et al., 2007). Indeed, scattered clinical cases of adverse drug interactions, e.g., with amodiaquine (German et al., 2007), warfarin (Bonora et al., 2008) and phenytoin (Robertson et al., 2005) suggest that efavirenz may alter the pharmacokinetics of co-administered drugs through inhibition of CYPs.

A comprehensive inhibitory analyses that encompass all major drug-metabolizing CYPs are important because: a) not all pharmacokinetic drug interactions involving efavirenz can be explained by the known inductive effect of efavirenz and by the CYPs studied so far; and b) the *in vitro* studies describing inhibition of CYPs by efavirenz provide only qualitative information, without generating *in vitro* inhibition parameters that will allow quantitative prediction of *in vivo* condition and without taking the contribution of time-dependent inactivation into account. In addition, the net effect on the pharmacokinetics of co-administered drugs seems to depend on its varied potencies of inhibition and induction on individual CYP isoform. In order to better predict *in vivo* drug-drug interactions associated with such mixed mechanisms, it is necessary to

simultaneously take reversible inhibition, time-dependent inhibition, and induction into account.

The purpose of present study was to systematically evaluate the *in vitro* inhibitory potency of efavirenz on eight major human CYP isoforms and determine the mechanisms involved. For those isoforms that were inhibited in pilot experiments, inhibition constants (K_i values) were estimated with which the extent of *in vivo* drug interactions was quantitatively predicted.

2. Results

2.1 Screening for inhibition of multiple CYPs by efavirenz

The inhibitory effect of efavirenz at 10 and 50 μM on the activities of eight CYP isoforms in pooled HLMs is shown in Figure 8.1. Efavirenz was a potent inhibitor of CYP2B6 (by 90% at 10 and 50 μM). It also showed inhibition of CYP2C8, CYP2C9 and CYP2C19 by >20% at 10 μM and by >50% at 50 μM. Efavirenz only showed weak inhibition on the activity of CYP3A (testosterone β-hydroxylation) by 10% at 10 μM and by 40% at 50 μM.

The activity of CYP2C19 was assessed using two substrates (R-omeprazole and S-mephenytoin) since substrate-dependent effect on CYP2C19 inhibition profile was observed previously (Foti and Wahlstrom, 2008). In the present study, efavirenz inhibited CYP2C19 activity by 35% and 70% at 10 and 50 μM, when S-mephenytoin was used as a substrate (Figure 8.1), but its effect on CYP2C19-mediated R-omeprazole 5-hydroxylation was marginal (by 10% at 50 μM efavirenz) (data not shown). This result is consistent with a previous report that S-mephenytoin is more sensitive to CYP2C19

inhibition than R-omeprazole (Foti and Wahlstrom, 2008). Therefore, S-mphenytoin was used as a substrate of CYP2C19 in subsequent inhibition experiment.

We have *in vivo* evidence that efavirenz reduces CYP1A2 activity, as measured by caffeine metabolism: compared to a single efavirenz dose (600 mg orally), pretreatment with efavirenz (600 mg/day for 17 days) significantly decreased concentration ratio of paraxanthine/caffeine at 6 hour ($P<0.0001$) (Metzger et al., 2012). However, the present *in vitro* data derived from pooled HLMs did not indicate that efavirenz inhibits CYP1A2 activity (Figure 8.1). Therefore, we tested whether the major metabolite of efavirenz, 8-hydroxyefavirenz, contributes to inhibition of CYP1A2 and showed that 8-hydroxyefavirenz inhibited CYP1A2 by ~20% up to 10 μM (Figure 8.2), which suggests that other alternative mechanisms should account for the reduced CYP1A2 activity that we observed *in vivo*.

The inhibitory effect of efavirenz on the activity of CYP2A6 and CYP2D6 was negligible (less than 10% at both efavirenz concentrations) (Figure 8.1).

2.2 Estimation of K_i values

In order to obtain quantitative prediction of magnitude of drug interaction *in vivo*, further experiments were performed to determine the K_i values for the inhibition of CYP2B6, 2C8, 2C9 and 2C19 by efavirenz. Although relatively weak inhibition of CYP3A was observed by efavirenz, K_i value was determined in pooled HLMs because a previous study reported that the value of IC_{50} is around 20 μM using midazolam as a substrate (von Moltke et al., 2001).

Of all the CYPs tested, CYP2B6 was the most sensitive to efavirenz inhibition (Table 8.1). Visual inspection of the Dixon plot and further analysis of the parameters of

the enzyme inhibition models suggested that the inhibition data fit well to a competitive type of inhibition. The K_i values estimated by using a nonlinear regression model for competitive enzyme inhibition of CYP2B6-catalyzed bupropion 4-hydroxylation in pooled HLMs and CMV negative HLMs were 2.96 ± 0.67 μM and 0.39 ± 0.10 μM, respectively. K_i value determined in expressed CYP2B6 was 1.38 ± 0.09 μM. Representative Dixon plots for the inhibition of CYP2B6 in CMV negative HLMs and expressed CYP2B6 are shown in Figure 8.3A and 3B, respectively.

Inhibition of CYP2C8 by efavirenz was determined in two HLMs and expressed CYP2C8. As shown in Table 8.1, efavirenz showed potent competitive inhibition of CYP2C8 activity in pooled HLMs ($K_i = 4.78 \pm 2.24$ μM). The second HLMs was obtained from human liver tissues with the *CYP2C8*3/*3* genotype and the K_i value (4.80 ± 0.35 μM) derived from this HLM was not different from that derived from pooled HLMs (Table 8.1). Efavirenz exhibited similar competitive inhibition in expressed CYP2C8 with an estimated K_i value of 6.05 ± 2.86 μM (Table 8.1). In Figure 8.4, Dixon plots for the inhibition of CYP2C8-catalyzed N-desethylation of amodiaquine by efavirenz in pooled HLMs (Figure 8.4A), HLMs with *CYP2C8*3/*3* genotype (Figure 8.4B) and expressed CYP2C8 (Figure 8.4C) are shown.

Efavirenz was found to be a moderate inhibitor of CYP2C9 ($K_i = 19.46 \pm 2.78$ μM; Table 8.1 and Figure 8.5) and CYP2C19 (21.31 ± 2.57 μM; Table 8.1 and Figure 8.6), and a weak inhibitor of CYP3A ($K_i = 40.33 \pm 0.33$ μM; Table 8.1 and Figure 8.7).

2.3 Time-dependent inactivation

As shown in Figure 8.8, efavirenz preincubation for 30 min only marginally inhibited the activity of those CYPs tested. Shorter preincubation times (5-15 min) were also tested, but did not show any indication of time-dependent inactivation.

2.4 Quantitative prediction of *in vivo* drug interactions

The predicted ratios of $\frac{AUC_I}{AUC}$ for each substrate co-administered with a single dose or multiple doses of efavirenz are listed in Table 8.2. Compared to control (without efavirenz), a single 600 mg oral dose of efavirenz was predicted to result in ~3-fold changes in the exposure of methadone (CYP2B6 substrate). Also, based on the inhibition data generated using S-mephenytoin hydroxylation as a marker of CYP2C19, we predicted lower AUCs of active metabolites of clopidogrel and proguanil (by 17%-29% and 29%-33% respectively), and higher omeprazole AUC (by 1.4- to 1.6-fold) in extensive metabolizer of CYP2C19. However, when data generated using R-omeprazole 5-hydroxylation is used, no inhibition could be predicted *in vivo*. Based on our *in vitro* data, a single dose of efavirenz is unlikely to alter the pharmacokinetics of CYP3A substrates.

After multiple doses of efavirenz, the AUC of CYP2C8 and CYP2C9 substrates was predicted to be ~3.5- to 4.4-fold higher (CYP2C8 substrate: amodiaquine) and 1.7- to 2.0-fold higher (CYP2C9 substrates: phenytoin and S-warfarin) compared to controls (without efavirenz).

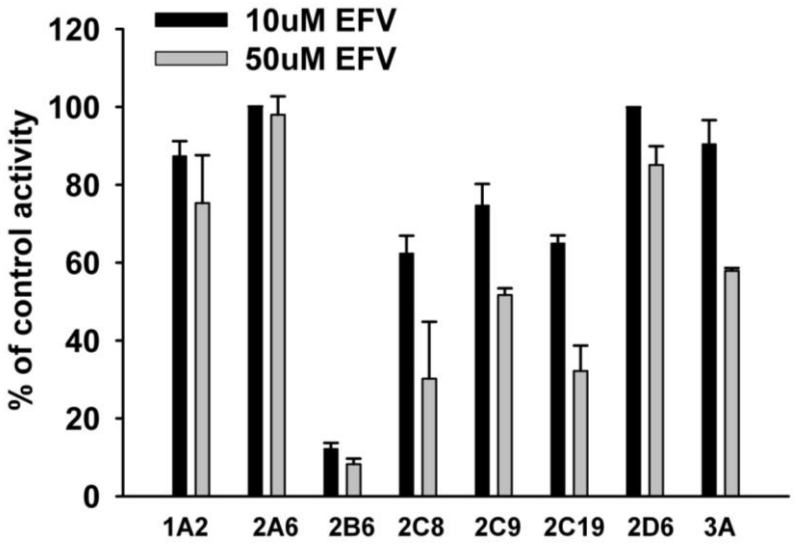

Figure 8.1 Inhibition of CYP isoforms by efavirenz in pooled HLMs. A substrate probe at a single concentration was incubated with pooled HLMs and cofactors in the absence (control) or the presence of efavirenz (10 and 50 μM) for times and with protein concentrations that were linear for the respective reaction described in detail in Material and Methods. The specific concentrations of each probe used are illustrated in Materials and Methods. Each point represented the average of duplicate incubations. EFV, efavirenz.

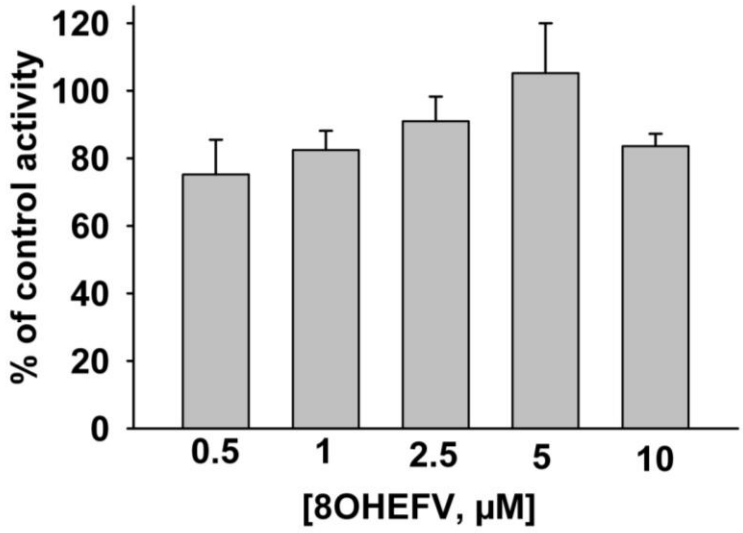

Figure 8.2 Inhibition of CYP1A2 by 8-hydroxyefavirenz in pooled HLMs. Phenacetin (50 µM) was incubated with pooled HLMs (1 mg/ml) and cofactors in the absence (control) or the presence of 8-hydroxyefavirenz (0.5 to 10 µM) at 37 ℃ for 30 min. Each point represented the average of duplicate incubations. 8OHEFV, 8-hydroxyefavirenz.

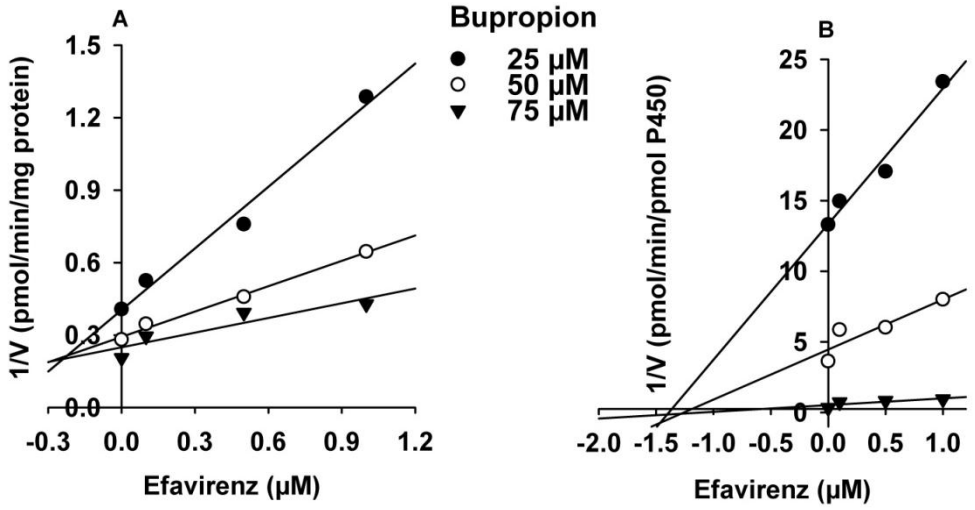

Figure 8.3 Dixon plots for the inhibition of bupropion 4-hydroxylation by efavirenz in CMV Negative HLMs (A) and expressed CYP2B6 (B). Bupropion (25 to 75 µM) was incubated with CMV negative HLMs (0.25 mg/ml) or expressed CYP2B6 (5 pmol) and cofactors at 37℃ for 15 min with or without efavirenz (0-1 µM). Each point represented the average of duplicate incubations. The line represents the best fit for the data.

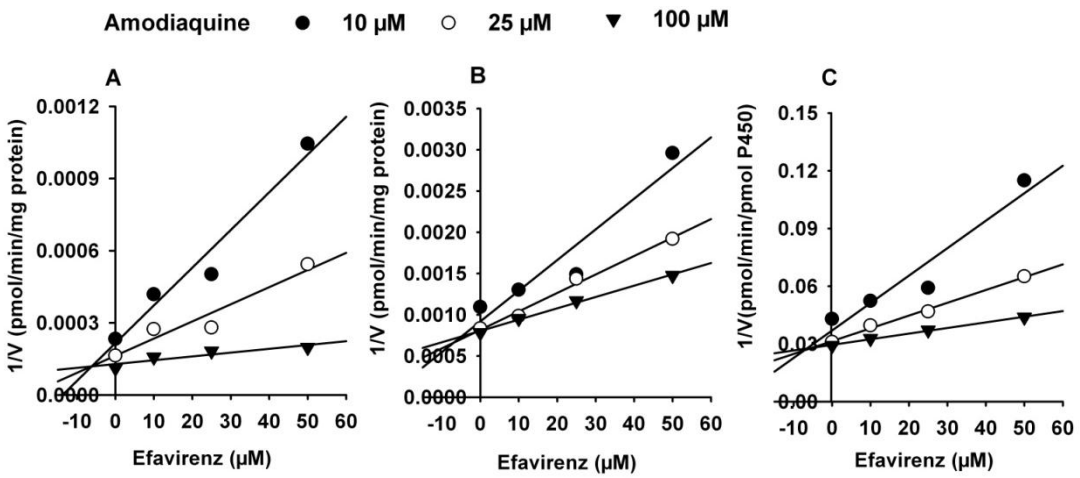

Figure 8.4 Dixon plots for the inhibition of amodiaquine desethylation by efavirenz in pooled HLMs (A), HLMs with CYP2C8*3/*3 genotype (B) and expressed CYP2C8 (C). Amodiaquine (10 to 100 µM) was incubated with pooled HLMs (0.1 mg/ml) or HLMs with *CYP2C8*3/*3* genotype (0.1 mg/ml) and cofactors at 37 ℃ for 15 min with or without efavirenz (0-50 µM). Expressed CYP2C8 (26 pmol) was used in the inhibition study. Each point represented the average of duplicate incubations. The line represents the best fit for the data.

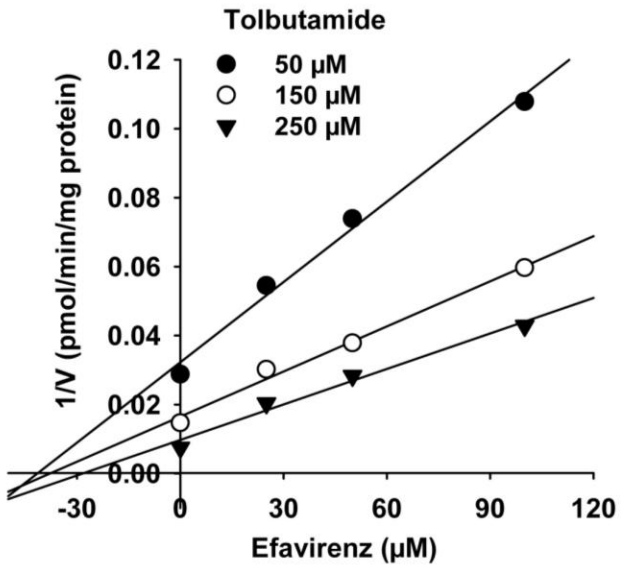

Figure 8.5 Dixon plot for the inhibition of tolbutamide 4-hydroxylation by efavirenz in pooled HLMs. Tolbutamide (50 to 250 µM) was incubated with pooled HLMs (1 mg/ml) and cofactors at 37 ℃ for 15 min with or without efavirenz (0-100 µM). The line represents the best fit for the data.

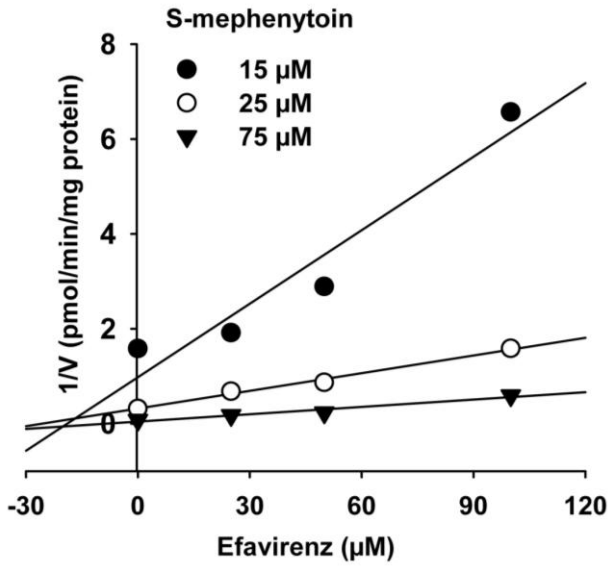

Figure 8.6 Dixon plot for the inhibition of S-mephenytoin 4-hydroxylation by efavirenz in pooled HLMs. S-mephenytoin (15 to 75 µM) was incubated with pooled HLMs (1 mg/ml) and cofactors at 37 ℃ for 15 min with or without efavirenz (0-100 µM). The line represents the best fit for the data.

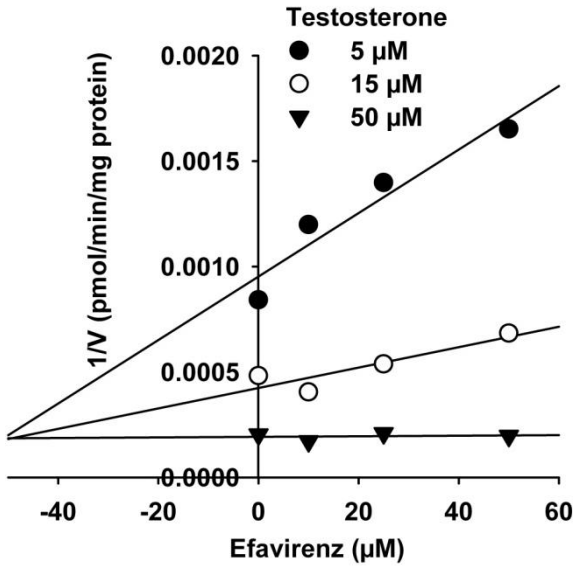

Figure 8.7 Dixon plot for the inhibition of testosterone β-hydroxylation by efavirenz in pooled HLMs. Testosterone (5 to 50 µM) was incubated with pooled HLMs (0.25 mg/ml) and cofactors at 37 ℃ for 15 min with or without efavirenz (0-50 µM). The line represents the best fit for the data.

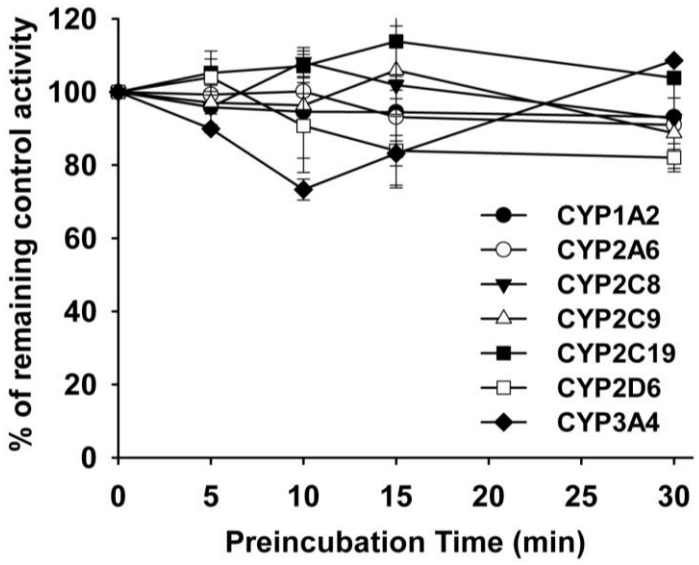

Figure 8.8 Time-dependent inhibition of CYP isoforms by efavirenz in pooled HLMs. Efavirenz (50 μM) was preincubated in duplicate with HLMs and phosphate reaction buffer (pH 7.4) (without or with the NADPH-generating system) in the absence of a substrate probe for 0, 5, 10, 15 and 30 min at 37 ℃. Controls were preincubated for 0 min without efavirenz and without the NADPH generating system. Protein concentrations and the specific concentrations of each probe used are illustrated in Materials and Methods. Each point represents the average of duplicate incubations.

Table 8.1 K_i values of efavirenz for the inhibition of CYPs in HLMs and expressed CYPs.

CYP Isoform	Substrate	Systems	K_i value (μM) (inhibition model)
CYP2B6	Bupropion	pooled HLMs	2.96 ± 0.67 (competitive)
		CMV negative HLMs	0.39 ± 0.10 (competitive)
		expressed CYP2B6	1.38 ± 0.09 (competitive)
CYP2C8	Amodiaquine	pooled HLMs	4.78 ± 2.24 (competitive)
		HLMs with *CYP2C8*3/*3*	4.80 ± 0.35 (competitive)
		expressed CYP2C8	6.05 ± 2.86 (competitive)
CYP2C9	Tolbutamide	pooled HLMs	19.46 ± 2.78 (non-competitive)
CYP2C19	S-mephenytoin	pooled HLMs	21.31 ±2.57 (competitive)
CYP3A	Testosterone	pooled HLMs	40.33 ± 0.33 (competitive)

Table 8.2 Prediction of changes in AUC of CYP2B6, 2C9 and 2C19 substrates *in vivo* by efavirenz.

	f_m	Predicted AUC Ratio	Reported AUC Ratio
Methadone	0.75 (Shou et al., 2008)	2.9 - 3.1	N.A.
Amodiaquine	0.93 (Li et al., 2002)	3.5 - 4.4	2.15 – 4.02 (German et al., 2007)
Phenytoin	0.90 (Giancarlo et al., 2001)	1.7 - 2.0	N.A.
S-Warfarin	0.91(Obach et al., 2006)	1.7 - 2.0	N.A.
Omeprazole	0.87 (Obach et al., 2006)	1.4 -1.6	N.A.
Proguanil*	0.84	29% ~33%	
Clopidogrel*	0.56-0.64 (Boulenc et al., 2011)	17% ~29%	N.A.

Plasma concentrations of efavirenz after a single dose were used to predict its effect on AUC change of methadone, omeprazole and the active metabolites of proguanil and clopidogrel. Plasma concentrations upon multiple doses were used for predicting AUC change of amodiaquine, phenytoin and S-warfarin. *: The value of percentage change in the AUC of active metabolite (1- $\frac{AUC_{m,I}}{AUC_{m,}}$) was predicted. N.A.: not available

3. Discussion

In this section I explored the hypothesis that efavirenz alters pharmacokinetics of co-administered drugs by inhibiting CYPs activities. We have shown that efavirenz is a potent competitive inhibitor of CYP2B6 (average K_i= 1.68 μM in HLMs and K_i= 1.38 μM in expressed CYP2B6) and CYP2C8 (K_i = 4.78 μM in pooled HLMs and K_i = 4.80 μM in HLMs with *CYP2C8*3/*3* genotype). In pooled HLMs, efavirenz showed moderate inhibition of CYP2C9 (K_i= 19.46 μM) CYP2C19 (K_i= 21.31 μM), and a weak inhibitor of CYP3A (K_i= 40.33 μM). Inhibition of CYP1A2, CYP2A6 and CYP2D6 by efavirenz was marginal. No time-dependent inactivation of the CYP isoforms tested was observed. Based on the *in vitro* to *in vivo* quantitative prediction, efavirenz is expected to : a) increase the AUC of methadone (CYP2B6 substrate) by 2.9- to 3.1-fold, omeprazole by 1.4- to 1.6-fold (CYP2C19 substrate),and may also lower the AUC of active metabolites of some pro-drugs (e.g. clopidogrel and proguanil) by up to 30% during initiation of efavirenz-based anti-HIV therapy; and b) increase the AUC of amodiaquine by ~3-fold (CYP2C8 substrate) and phenytoin and warfarin by 1.7- to 2.0-fold (CYP2C9 substrates) during a single dose or multiple doses of efavirenz. Our data suggest that efavirenz may increase the risk for adverse effects by increasing the exposure of the parent drug or reduce efficacy by diminishing the formation of pharmacologically active metabolites from prodrugs.

Of the CYPs tested, CYP2B6 was most sensitive to efavirenz inhibition with K_i value of ~1.7 μM in HLMs and ~1.38 μM in expressed CYP2B6. Although the ability of efavirenz to inhibit CYP2B6 was previously reported (Hesse et al., 2001; Bumpus et al., 2006), the present data provide key information that allowed *in vivo* quantitative

prediction of the magnitude of interaction. The high inhibition potency of efavirenz in our study is worth commenting. Efavirenz has a higher binding affinity to CYP2B6 with K_m values of 13 ~ 20 μM (Ward et al., 2003; Ogburn et al., 2010) than bupropion with K_m values of 90 ~ 130 μM (Faucette et al., 2000; Hesse et al., 2000). Thus, it is plausible that the high inhibition potency of efavirenz on bupropion hydroxylation could be due to the fact that efavirenz has higher binding affinity to CYP2B6 than bupropion. Similar mechanism contributing to high inhibition potency has been reported for CYP2D6 (Vandenbrink et al., 2011b). To put the *in vitro* inhibition data on CYP2B6 into perspective, it is important to point out that efavirenz enhances its own metabolism upon multiple doses preferentially through CAR-mediated induction of CYP2B6 (Oswald et al., 2012). Efavirenz also enhances the metabolism of co-administered CYP2B6 substrates, including methadone (Clarke et al., 2001; Kharasch et al., 2012) and bupropion (Robertson et al., 2008). Considering the high inhibition potency of efavirenz, a substantial increase in AUC of CYP2B6 substrates and potentially the risk to adverse effects may be expected, when efavirenz-based therapy is initiated in patients who are stabilized on CYP2B6 substrates. We predicted approximately 2.9- to 3.1-fold increase in methadone AUC, when a single 600 mg oral dose of efavirenz is co-administered. However, during chronic administration, inhibition of CYP2B6 by efavirenz appears to be masked by its marked induction and the net effect becomes induction.

Our study demonstrates that efavirenz inhibits CYP2C8-mediated amodiaquine desethylation with K_i values of 4.78 and 6.05 μM in pooled HLMs and expressed CYP2C8 respectively, which broadly concurs with an IC_{50} of 4μM reported in expressed CYPs (Parikh et al., 2007). The inhibition potency of efavirenz in HLMs with the

*CYP2C8*3/*3* genotype, the most frequent and functionally relevant variant in Caucasians (Daily and Aquilante, 2009), was not different from that observed in pooled HLMs. The possibility of substrate-dependent interaction cannot be fully excluded (Kaspera et al., 2011), but our data suggest that the *CYP2C8*3* allele does not seem to alter susceptibility to efavirenz inhibition. We expect ~3-fold higher AUC of amodiaquine and probably other substrates such as chloroquine, certain anti-diabetics, montelukast and rosiglitazone (Totah and Rettie, 2005; Lai et al., 2009), when co-administered with efavirenz. A clinical study that was designed to evaluate drug interactions between anti-malarials and efavirenz-based anti-HIV therapy was prematurely discontinued after the first two subjects developed hepatotoxicity (German et al., 2007). A 2.2- to 4-fold increase in amodiaquine AUC was also noted (German et al., 2007) and it is highly likely that this interaction occurred through inhibition of CYP2C8, as predicted from our *in vitro* data.

Our data showed that efavirenz inhibits CYP2C9 activity (K_i= 19.46 μM) with 1.7- to 2-fold predicted increase in AUC of drugs mainly cleared by CYP2C9, consistent with an *in vitro* study reporting an IC_{50} value of ~15μM (von Moltke et al., 2001). This enzyme is involved in the metabolism of more than 100 currently used drugs, including drugs with narrow therapeutic range, e.g., oral anticoagulants, oral hypoglycemic agents and phenytoin (Rettie and Jones, 2005). Therefore, co-administration of efavirenz may likely increase the risks to adverse effects of these drugs, which is supported by clinical cases of inhibition drug interactions of efavirenz with the CYP2C9 substrate phenytoin (Robertson et al., 2005) and warfarin (Bonora et al., 2008).

We found that the extent of CYP2C19 inhibition by efavirenz was substrate-dependent: modest inhibition of S-mephenytoin 4-hydroxylation (K_i= 21.31 μM) and marginal inhibition of R-omeprazole 5-hydroxylation consistent with a previous study reporting that R-omeprazole is less sensitive to CYP2C19 inhibitors than S-mephenytoin (Foti and Wahlstrom, 2008). The clinical relevance of efavirenz inhibition on CYP2C19 and the mechanism of substrate-dependent inhibition remain unclear, but this interaction may be important for prodrugs that require bioactivation by CYP2C19. For example, at a single dose or acute dosing, efavirenz may inhibit the formation of active metabolite of clopidogrel by CYP2C19 and to some extent by CYP2B6 and CYP2C9 (Kazui et al., 2010). Provided that efavirenz is a potent inhibitor of CYP2B6 and a moderate inhibitor of CYP2C19 and CYP2C9 (present data), it is likely that administration of efavirenz to patients stabilized on clopidogrel may result in substantially reduced formation of active metabolite and lack of efficacy. At steady state, efavirenz may be a mixed inhibitor and inducer of CYP2C19 (Michaud et al., 2012b), although induction appears to predominate as shown with the enhanced metabolism of CYP2C19 substrates including voriconazole (Liu et al., 2008), omeprazole (Michaud et al., 2012b), etravirine (Boffito et al., 2009). Contradictory effects were observed in another CYP2C19 substrate proguanil, with decreased proguanil exposure co-administered with 600 mg/day efavirenz for 1 month (van Luin et al., 2010) and increased proguanil exposure co-administered with 400 mg/day efavirenz for 9 days (Soyinka and Onyeji, 2010). This discrepancy may be due to differences in dose regimens, duration of treatment and study populations.

Efavirenz-mediated *in vivo* inhibition of CYP3A seems unlikely given the high K_i value (K_i= ~40μM) observed in the present study. Using triazolam as a substrate, another

study reported lower IC_{50} values (17-20μM) (von Moltke et al., 2001), but the significance of *in vivo* inhibition of CYP3A, if any, is likely marginal. Efavirenz, through activation of PXR/CAR, induces CYP3A *in vitro* (Hariparsad et al., 2004; Faucette et al., 2007) and *in vivo* (Mouly et al., 2002; Michaud et al., 2012b). Hence, efavirenz enhances the elimination of many CYP3A substrates, including protease inhibitors, statins (Gerber et al., 2005), calcium channel blockers and anti-fungals [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012]. Therefore, induction drug interactions between efavirenz and CYP3A substrates appear to predominate at steady-state.

CYP1A2, CYP2A6 and UGT2B7 all have been shown to be involved in efavirenz metabolism (Ward et al., 2003; Belanger et al., 2009). Efavirenz is not only a substrate but also a moderate inhibitor of UGT2B7 (Belanger et al., 2009), while no reversible inhibition or time-dependent inhibition by efavirenz was observed for CYP1A2 and CYP2A6 in the present study. The drug interaction with CYP2A6 substrates mediated by efavirenz inhibition seems very unlikely, but the possibility of efavirenz inhibiting the metabolism of CYP1A2 substrates can't be excluded, because the preliminary results from our laboratory showed that efavirenz reduces CYP1A2 activity as measured by caffeine metabolism *in vivo*.

The use of efavirenz is made difficult by high interindividual variability in its disposition and by often unpredictable and complex drug interactions. The extent of drug interactions with efavirenz varies greatly among individuals, and interpatient differences in efavirenz exposure contribute to this variability. Efavirenz exposure is governed by complex factors: efavirenz is mainly cleared by CYP2B6, with some contribution from

accessory pathways catalyzed by enzymes that include CYP2A6, CYP1A2, CYP3A and UGT2B7 (Ward et al., 2003; Belanger et al., 2009; di Iulio et al., 2009; Kwara et al., 2009a; Ogburn et al., 2010; Cho et al., 2011); efavirenz activates CAR and PXR, induces CYP2B6 (and other drug disposition genes) and autoinduces its own metabolism (Oswald et al., 2012) upon repeated administration; and efavirenz is a potent inhibitor of CYP2B6 with no effect on CYP2A6 and CYP1A2 (present study) and a moderate inhibitor of UGT2B7 *in vitro* (Belanger et al., 2009). These complex inhibition/induction processes and genetic variations of CYP2B6 would likely contribute to variable efavirenz exposure and drug interactions. The net effect of efavirenz on drug interactions (induction versus inhibition) *in vivo* is likely to be dependent on: duration of efavirenz administration (acute versus chronic); genetic and nongenetic factors; and the potency with which efavirenz induces or inhibits drug metabolizing enzymes. Although predicting the extent and direction of drug interactions with efavirenz *in vivo* might be difficult for the individual patient, general comments could be made based on our data and the literature. During initiation of efavirenz-based therapy, it is expected that efavirenz reduces the elimination of CYP2B6, CYP2C8, CYP2C9 and CYP2C19 substrates. Mixed induction/inhibition occurs upon repeated administration of efavirenz, but the net effect of efavirenz appears to be induction for CYP2B6 and CYP2C19, while inhibition appears to dominate for CYP2C8 and CYP2C9. Induction appears the main mechanism for efavirenz interactions involving CYP3A. Together, efavirenz's complex interaction with enzymes involved in its own metabolism and the metabolism of co-administered drugs may contribute to the large interindividual variability of efavirenz exposure and unpredictable drug interactions associated with it.

CHAPTER IX: *Conclusions and future directions*

1. Conclusions

The major focuses of this dissertation were to determine the mechanisms: 1) by which the *CYP2B6*6* allele reduced efavirenz metabolism *in vitro,* and 2) by which efavirenz alters the pharmacokinetics of co-administered drugs. Results from this dissertation provided evidence that amino acid changes harbored in the *CYP2B6*6* allele may reduce efavirenz metabolism by decreasing binding affinity and catalytic efficiency. The functional consequences of the *CYP2B6*6* allele appears to be substrate- and Cyt b5-dependent. Additionally, this dissertation found that CYP2B6 variant protein is more susceptible to metabolic inhibition than the wild type. Moreover, data from this dissertation also showed that models incorporating *in vitro* mechanisms of reduced efavirenz metabolism by the *CYP2B6*6* allele can predict efavirenz clearance in pharmacogenetics-based manner. Finally, results from this dissertation demonstrated that efavirenz may alter pharmacokinetics of co-administered drugs by inhibiting the activities of CYP2B6, 2C8, 2C9 and 2C19. Based on the major findings outlined above, four overarching conclusions can be drawn concerning the body of work presented in this dissertation: 1) the *CYP2B6*6* allele alters catalytic properties, i.e. binding affinity and/or catalytic efficiency, and susceptibility to metabolic inhibition that may contribute to reduced efavirenz metabolism by this variant allele, 2) the functional consequences of the *CYP2B6*6* allele is substrate- and Cyt b5-dependent, 3) incorporation of *in vitro* mechanism of reduced efavirenz metabolism by the *CYP2B6*6* allele can predict efavirenz clearance after a single oral dose in pharmacogenetics-based manner, and 4) efavirenz may alter the pharmacokinetics of co-administered medications by inhibiting

the activities of CYP2B6, 2C8, 2C9 and 2C19. Each of these overarching conclusions is discussed in the section which follows.

*A. The CYP2B6*6 allele alters catalytic properties, i.e. binding affinity and/or catalytic efficiency, and susceptibility to metabolic inhibition that may contribute to reduced efavirenz metabolism by this variant allele*

The *CYP2B6*6* allele is clinically important because this allele or the SNP tagging it (G516T) occurs at high frequency in all ethnic populations [14-62% (Zanger et al., 2007)] and has been associated with functional consequences in expressed systems (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus and Hollenberg, 2008; Watanabe et al., 2010; Ariyoshi et al., 2011; Zhang et al., 2011b) and human liver microsomes (HLMs) (Lang et al., 2001; Lamba et al., 2003; Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007). In HLMs, the *CYP2B6*6* allele is associated with reduced total amount of CYP2B6 protein (Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007). The G516T SNP was predicted to disrupt an exonic splicing enhancer *in silico* (Lamba et al., 2003). Subsequently, Hofmann et al. provided evidence that this variant indeed affects splicing and thereby reduces CYP2B6 expression and activity (Hofmann et al., 2008). However, mounting evidence indicate that reduced protein expression alone may not explain the functional consequences of this allele. For substrates that include cyclophosphamide, this allele is associated with enhanced metabolism despite reduced protein expression (Xie et al., 2003), which appears due to substantially lower K_m in the variant versus wild type protein (Ariyoshi et al., 2011).

As part of this dissertation I examined the influence of the *CYP2B6*6* allele on catalytic properties measured by efavirenz 8-hydroxylation (Ward et al., 2003) as a probe

of activity using expressed enzymes and HLMs obtained from liver tissue samples genotyped for the *CYP2B6*6* allele (**Chapter III**). The results presented in this thesis demonstrated that that the *CYP2B6*6* allele is associated with decreasing binding affinity and/or catalytic activity using efavirenz as a substrate. Consistent with previous reports in various expressed systems (Ariyoshi et al., 2001; Jinno et al., 2003; Bumpus et al., 2006; Raccor et al., 2012), we noted that the V_{max} values for the formation of 8-hydroxyefavirenz significantly higher in CYP2B6.6 than in CYP2B6.1 proteins expressed without Cyt b5. However, V_{max} values for the formation of 8-hydroxyefavirenz were substantially decreased (by ~70%) in HLMs with *CYP2B6*6/*6* genotype versus HLMs with *CYP2B6*1/*6* and *CYP2B6*1/*1* genotypes. Our interpretation is that the expressed variant protein inherently increases catalytic activity of efavirenz, whereas the decreased V_{max} value in HLMs is probably mainly due to reduced protein expression by the *CYP2B6*6/*6* genotype (Hesse et al., 2004; Desta et al., 2007; Hofmann et al., 2008). Consequently, additional experiments are needed to quantify the amount of CYP2B6 protein in human liver samples genotyped for the *CYP2B6*6* allele and determine whether the decreased V_{max} value in HLMs is due to reduced protein expression and/or reduced catalytic activity of the variant protein.

In addition, it has been shown that amino acid substitutions, such as those found in the variant of *CYP2B6*6* allele, may also alter the degree of susceptibility to competing metabolic inhibitors for certain CYP2B6 variants (Bumpus et al., 2006; Bumpus and Hollenberg, 2008; Talakad et al., 2009). As part of the dissertation, I explored whether the *CYP2B6*6* allele is associated with altered susceptibility to metabolic inhibition. The fact that the K_i values for CYP2B6 inhibition by voriconazole

was significantly lower in HLMs with *CYP2B6*6* allele than in those with the *CYP2B6*1/*1* genotype suggests that the variant protein is more susceptible to metabolic inhibition than the wild type (**Chapter VI**).

The greater inhibition susceptibility by the variant protein may contribute to marked reduction of efavirenz metabolism and thus elevated efavirenz exposure in individuals with *CYP2B6*6/*6* genotype, when a known interacting drug is co-administered. Taking the data presented in this dissertation together, the *CYP2B6*6* allele alters catalytic properties, i.e. binding affinity and/or catalytic efficiency, and susceptibility to metabolic inhibition that may contribute to reduced efavirenz metabolism by this variant allele.

The suggestion of genotype-based efavirenz dose reduction in individuals with the *CYP2B6*6/*6* is primarily based on pharmacogenetic association study in humans. The *in vitro* mechanisms by which the *CYP2B6*6* allele reduced efavirenz metabolism identified in this dissertation indicates that amino acid changes harbored in this allele variant may alter the protein structure and thus lead to altered catalytic properties of the enzyme. The causal relationship between the genetic variation and reduced efavirenz metabolism shown in this dissertation lends support to the implement of dose adjustment based on the *CYP2B6*6* genotype in anti-HIV treatment.

Efavirenz exposure is governed by complex factors: efavirenz is mainly cleared by polymorphic CYP2B6, with some contribution from accessory pathways catalyzed by enzymes that include CYP2A6, CYP1A2, CYP3A and UGT2B7 (Ward et al., 2003; Belanger et al., 2009; di Iulio et al., 2009; Kwara et al., 2009a; Ogburn et al., 2010; Cho et al., 2011); efavirenz activates CAR and PXR, induces CYP2B6 (and other drug

disposition genes) and autoinduces its own metabolism (Oswald et al., 2012) upon repeated administration in a genotype-dependent manner (Ngaimisi et al., 2011); efavirenz is a potent inhibitor of CYP2B6 with no effect on CYP2A6 and CYP1A2 (present study) and a moderate inhibitor of UGT2B7 *in vitro* (Belanger et al., 2009); and the inhibition susceptibility of CYP2B6 is also dependent on the *CYP2B6*6* genotype. These complex inhibition/induction processes and genetic variations of CYP2B6 would likely contribute to variable efavirenz exposure and drug interactions.

Efavirenz may serve as an effective probe of CYP2B6 and CYP2A6 activity *in vitro* and *in vivo* due to their primary contribution to efavirenz 8-hydroxylation and 7-hydroxylation, respectively (Ward et al., 2003; Ogburn et al., 2010). Clinical studies are ongoing in our laboratory to clarify the precise contribution of CYP2A6 and CYP2B6 in efavirenz metabolism using genetic and drug interactions as markers.

*B. The functional consequences of the CYP2B6*6 allele is substrate- and Cyt b5-dependent*

A number of *in vitro* studies, mostly in expressed systems, have reported that the *CYP2B6*6* allele or the amino acids harbored in it influence catalytic properties. However, the extent and direction of effect appears to depend on the substrate and the enzyme sources used. Therefore, I hypothesized that the functional consequences of the *CYP2B6*6* allele may be substrate-dependent. I determined the kinetics of bupropion metabolism to 4-hydroxybupropion in the same expressed CYP2B6 and HLMs used for the characterization of efavirenz kinetics in **Chapter IV**. I found that the *CYP2B6*6* allele may influence substrate binding with pronounced effect on efavirenz than bupropion. The data presented in **Chapter III** and **Chapter IV** in conjunction with the

previous studies suggest that the *CYP2B6*6* allele alters catalytic properties in a substrate-dependent manner.

Several factors inherent to specific enzyme sources that include differences in Cyt b5 contents may influence *in vitro* kinetic parameters and inhibition constants in substrate-dependent manner. Cyt b5 has been reported to activate several CYPs including CYP2B6 (Reed and Hollenberg, 2003; Jushchyshyn et al., 2005), but its influence on the catalytic properties of CYP2B6.6 protein has not been studied. I examined the influence of Cyt b5 on the catalytic properties of expressed CYP2B6.1 and CYP2B6.6 by determining the kinetics of efavirenz and bupropion in CYP2B6 enzymes coexpressed with Cyt b5 (**Chapter V**). Our data show that Cyt b5 affects catalytic properties of CYP2B6 in genotype- and substrate-dependent manner and highlight the fact that interpretation of *in vitro* studies performed with expressed proteins may vary depending on the presence or absence of Cyt b5, substrate used and underlying genotype.

In addition, I also found that for both substrates, CYP2B6.1 exhibited similar or increased catalytic activities with co-expression of Cyt b5 compared to that without Cyt b5, whereas Cyt b5 significantly decreased V_{max} values in CYP2B6.6. These data suggest an overlapping binding site between CYP reductase and Cyt b5 in CYP2B6.6 but probably not in CYP2B6.1.

A recent study characterizing the crystal structure of CYP2B6 genetic variant (Y226H, K262R) also supports our hypothesis that the amino acid changes harbored in the *CYP2B6*6* allele may influence the interaction between the CYPs and electron transfer proteins and thus alter the catalysis of substrates in Cyt b5- and substrate-dependent manner. The two SNPs (K262R and Q172H) harbored in the *CYP2B6*6* allele

are not within the active site of the enzyme. The two amino acid mutations harbored by the *CYP2B6*6* allele may indirectly involve in the ligand binding and substrate catalysis. The side chain of residue 172 may interact with the residues at active site and thus could affect binding affinity (Gay et al., 2010). It is noteworthy that the other mutated amino acid carried by *CYP2B6*6* allele, K262R, is located at the G/H loop, which may involve in the interaction between the enzyme and its redox partner, CYP reductase (Bumpus and Hollenberg, 2008; Gay et al., 2010). The oxidation reaction catalyzed by CYPs requires transferring of two electrons from NADPH. The first electron is generally thought to be transferred by CYP reductase, while the second can be transferred by either CYP reductase or Cyt b5. That altered electron transfer from CYP reductase to CYP2B6 variant proteins may influence substrate metabolism was suggested by a recent study (Zhang et al., 2011b). Therefore, the functional consequences of the *CYP2B6*6* allele may be substrate- and Cyt b5-dependent.

*C. Incorporation of in vitro mechanism of reduced efavirenz metabolism by the CYP2B6*6 allele predicts efavirenz clearance after a single oral dose in pharmacogenetics-based manner*

I showed that the reduced efavirenz metabolism in individuals with the *CYP2B6*6/*6* genotype may be due to decreasing altered binding affinity and catalytic efficiency (**Chapter III**). I tested that whether the incorporation of *in vitro* efavirenz metabolism data predicts genetic effect of the *CYP2B6*6* allele on efavirenz clearance by *in vitro-in vivo* extrapolation (IVIVE) in this dissertation. I found that PBPK and well-stirred liver models are able to predict efaivirenz clearance after a single oral dose by incorporating the *in vitro* mechanisms underlying the reduced efavirenz metabolism by

the *CYP2B6*6* allele in a pharmacogenetics-based manner. In addition, I povided evidence that expressed CYPs seems to be a better system than HLMs for pharmacogenetics-based IVIVE of CYP2B6 substrates using efavirenz as an example. These data suggest that expressed CYP2B6 may be a useful tool to study the effect of *CYP2B6* genetic polymorphism on the substrate metabolism in the early drug development.

D. Efavirenz may alter the pharmacokinetics of co-administered medications by inhibiting the activities of CYP2B6, 2C8, 2C9 and 2C19

Efavirenz is known to alter the pharmacokinetics of a long list of co-administered drugs, probably by modulating the activities of multiple drug metabolizing enzymes and/or drug transporters. However, not all pharmacokinetic drug interactions involving efavirenz can be explained by the known inductive effect of efavirenz and by the CYPs studied so far. Therefore, I systematically evaluated the *in vitro* inhibitory potency of efavirenz on eight major human CYP isoforms and determine the mechanisms involved (**Chapter VIII**). I found that efavirenz is a potent competitive inhibitor of CYP2B6 and CYP2C8, a moderate inhibitor of CYP2C9 and CYP2C19, and a weak inhibitor CYP3A. Inhibition of CYP1A2, CYP2A6 and CYP2D6 by efavirenz was marginal. No time-dependent inactivation of the CYP isoforms tested was observed. Based on the *in vitro* to *in vivo* quantitative prediction, it is expected that efavirenz reduces the elimination of CYP2B6, CYP2C8, CYP2C9 and CYP2C19 substrates during initiation of efavirenz-based therapy. Mixed induction/inhibition occurs upon repeated administration of efavirenz, but the net effect of efavirenz appears to be induction for CYP2B6 and CYP2C19, while inhibition appears to dominate for CYP2C8 and CYP2C9. Induction

appears the main mechanism for efavirenz interactions involving CYP3A. Our data suggest that efavirenz may increase the risk for adverse effects by increasing the exposure of the parent drug or reduce efficacy by diminishing the formation of pharmacologically active metabolites from prodrugs. Together, efavirenz's complex interaction with enzymes involved in its own metabolism and the metabolism of co-administered drugs may contribute to the large interindividual variability of efavirenz exposure and unpredictable drug interactions associated with it.

2. Future directions

Based on the findings of my work, it would be valuable to further evaluate the following directions in the future:

1. To quantify CYP2B6 protein amount in human liver samples genotyped for the *CYP2B6*6* allele.
2. To identify the mechanism underlying the substrate-dependent effect of Cyt b5 and to provide insight into the topology of the variant.
3. To study the genotype-dependent inhibition of CYP2B6 inhibition using other CYP2B6 substrates and inhibitors.

APPENDIX

Author permissions

9650 Rockville Pike
Bethesda, MD 20814-3995

Phone: (301) 634-7060
Fax: (301) 634-7061

info@aspet.org
www.aspet.org

January 11, 2013

Xu Cong
Pharmacology
Indiana University
West 10th St.
Indianapolis, IN 46202

Email: smartxucong@gmail.com

Dear Xu Cong:

This is to grant you permission to reproduce the following figure in your thesis titled "Mechanisms and quantitative prediction of efavirenz metabolism, pharmacogenetics and drug interactions" for the Indiana University School of Medicine:

> Figure 5 from Sean C. Gay, Manish B. Shah, Jyothi C. Talakad, Keiko Maekawa, Arthur G. Roberts, P. Ross Wilderman, Ling Sun, Jane Y. Yang, Stephanie C. Huelga, Wen-Xu Hong, Qinghai Zhang, C. David Stout, and James R. Halpert, Crystal Structure of a Cytochrome P450 2B6 Genetic Variant in Complex with the Inhibitor 4-(4-Chlorophenyl)imidazole at 2.0-Å Resolution, *Mol Pharmacol* April 2010 77:529-538

Permission to reproduce the figure is granted for worldwide use in all languages, translations, and editions, and in any format or medium including print and electronic. The authors and the source of the materials must be cited in full, including the article title, journal title, volume, year, and page numbers.

Sincerely yours,

Richard Dodenhoff
Journals Director

American Society for Pharmacology and Experimental Therapeutics

REFERENCES

Aitken AE, Lee CM, and Morgan ET (2008) Roles of nitric oxide in inflammatory downregulation of human cytochromes P450. Free Radic Biol Med **44:**1161-1168.

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, and Berger EA (1996) CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-1958.

Almond LM, Hoggard PG, Edirisinghe D, Khoo SH, and Back DJ (2005) Intracellular and plasma pharmacokinetics of efavirenz in HIV-infected individuals. J Antimicrob Chemother **56:**738-744.

Arab-Alameddine M, Di Iulio J, Buclin T, Rotger M, Lubomirov R, Cavassini M, Fayet A, Decosterd LA, Eap CB, Biollaz J, Telenti A, and Csajka C (2009) Pharmacogenetics-based population pharmacokinetic analysis of efavirenz in HIV-1-infected individuals. Clin Pharmacol Ther **85:**485-494.

Ariyoshi N, Miyazaki M, Toide K, Sawamura Y, and Kamataki T (2001) A single nucleotide polymorphism of CYP2b6 found in Japanese enhances catalytic activity by autoactivation. Biochem Biophys Res Commun **281:**1256-1260.

Ariyoshi N, Ohara M, Kaneko M, Afuso S, Kumamoto T, Nakamura H, Ishii I, Ishikawa T, and Kitada M (2011) Gln172His Replacement Overcomes Effects on the Metabolism of Cyclophosfamide and Efavirenz Caused by CYP2B6 Variant with Arg262. Drug Metab Dispos **39:** 2045-2048.

Avery LB, Sacktor N, McArthur JC, and Hendrix CW (2013) Protein-free efavirenz concentrations in cerebrospinal fluid and blood plasma are equivalent: applying the law of mass action to predict protein-free drug concentration. Antimicrob Agents Chemother **57:**1409-1414.

Balani SK, Kauffman LR, deLuna FA, and Lin JH (1999) Nonlinear pharmacokinetics of efavirenz (DMP-266), a potent HIV-1 reverse transcriptase inhibitor, in rats and monkeys. Drug Metab Dispos **27:**41-45.

Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, and Montagnier L (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-871.

Belanger AS, Caron P, Harvey M, Zimmerman PA, Mehlotra RK, and Guillemette C (2009) Glucuronidation of the antiretroviral drug efavirenz by UGT2B7 and an in vitro investigation of drug-drug interaction with zidovudine. Drug Metab Dispos **37:**1793-1796.

Boffito M, Jackson A, Lamorde M, Back D, Watson V, Taylor J, Waters L, Asboe D, Gazzard B, and Pozniak A (2009) Pharmacokinetics and safety of etravirine administered once or twice daily after 2 weeks treatment with efavirenz in healthy volunteers. J Acquir Immune Defic Syndr **52:**222-227.

Bonora S, Lanzafame M, D'Avolio A, Trentini L, Lattuada E, Concia E, and Di Perri G (2008) Drug interactions between warfarin and efavirenz or lopinavir-ritonavir in clinical treatment. Clin Infect Dis **46:**146-147.

Boulenc X, Djebli N, Shi J, Perrin L, Brian W, Van Horn R, and Hurbin F (2011) Effects of Omeprazole and Genetic Polymorphism of CYP2C19 on the Clopidogrel Active Metabolite. Drug Metab Dispos.

Broder S (2010) The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. Antiviral Res **85:**1-18.

Bumpus NN and Hollenberg PF (2008) Investigation of the mechanisms underlying the differential effects of the K262R mutation of P450 2B6 on catalytic activity. Mol Pharmacol **74:**990-999.

Bumpus NN, Kent UM, and Hollenberg PF (2006) Metabolism of efavirenz and 8-hydroxyefavirenz by P450 2B6 leads to inactivation by two distinct mechanisms. J Pharmacol Exp Ther **318:**345-351.

Burger D, van der Heiden I, la Porte C, van der Ende M, Groeneveld P, Richter C, Koopmans P, Kroon F, Sprenger H, Lindemans J, Schenk P, and van Schaik R (2006) Interpatient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. Br J Clin Pharmacol **61:**148-154.

Burhenne J, Matthee AK, Pasakova I, Roder C, Heinrich T, Haefeli WE, Mikus G, and Weiss J (2010) No evidence for induction of ABC transporters in peripheral blood mononuclear cells in humans after 14 days of efavirenz treatment. Antimicrob Agents Chemother **54:**4185-4191.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF, and Eichelbaum M (2005) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. Mol Pharmacol **67:**1954-1965.

Cabrera SE, Santos D, Valverde MP, Dominguez-Gil A, Gonzalez F, Luna G, and Garcia MJ (2009) Influence of the cytochrome P450 2B6 genotype on population pharmacokinetics of efavirenz in human immunodeficiency virus patients. Antimicrob Agents Chemother **53:**2791-2798.

Carrington HC, Crowther AF, Davey DG, Levi AA, and Rose FL (1951) A metabolite of paludrine with high antimalarial activity. Nature **168:**1080.

Carter CA and Ehrlich LS (2008) Cell biology of HIV-1 infection of macrophages. Annu Rev Microbiol **62:**425-443.

Chang TK, Weber GF, Crespi CL, and Waxman DJ (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. Cancer Res **53:**5629-5637.

Chen Y, Liu L, Nguyen K, and Fretland AJ (2011) Utility of intersystem extrapolation factors in early reaction phenotyping and the quantitative extrapolation of human liver microsomal intrinsic clearance using recombinant cytochromes P450. Drug Metab Dispos **39:**373-382.

Cho DY, Ogburn ET, Jones D, and Desta Z (2011) Contribution of N-glucuronidation to efavirenz elimination in vivo in the basal and rifampin-induced metabolism of efavirenz. Antimicrob Agents Chemother **55:**1504-1509.

Chun J, Kent UM, Moss RM, Sayre LM, and Hollenberg PF (2000) Mechanism-based inactivation of cytochromes P450 2B1 and P450 2B6 by 2-phenyl-2-(1-piperidinyl)propane. Drug Metab Dispos **28:**905-911.

Clarke SM, Mulcahy FM, Tjia J, Reynolds HE, Gibbons SE, Barry MG, and Back DJ (2001) The pharmacokinetics of methadone in HIV-positive patients receiving the non-nucleoside reverse transcriptase inhibitor efavirenz. Br J Clin Pharmacol **51:**213-217.

Code EL, Crespi CL, Penman BW, Gonzalez FJ, Chang TK, and Waxman DJ (1997) Human cytochrome P4502B6: interindividual hepatic expression, substrate specificity, and role in procarcinogen activation. Drug Metab Dispos **25:**985-993.

Cohen CJ, Andrade-Villanueva J, Clotet B, Fourie J, Johnson MA, Ruxrungtham K, Wu H, Zorrilla C, Crauwels H, Rimsky LT, Vanveggel S, and Boven K (2011a) Rilpivirine versus efavirenz with two background nucleoside or nucleotide reverse transcriptase inhibitors in treatment-naive adults infected with HIV-1 (THRIVE): a phase 3, randomised, non-inferiority trial. Lancet **378:**229-237.

Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, Hakim JG, Kumwenda J, Grinsztejn B, Pilotto JH, Godbole SV, Mehendale S, Chariyalertsak S, Santos BR, Mayer KH, Hoffman IF, Eshleman SH, Piwowar-Manning E, Wang L, Makhema J, Mills LA, de Bruyn G, Sanne I, Eron J, Gallant J, Havlir D, Swindells S, Ribaudo H, Elharrar V, Burns D, Taha TE, Nielsen-Saines K, Celentano D, Essex M, and Fleming TR (2011b) Prevention of HIV-1 infection with early antiretroviral therapy. N Engl J Med **365:**493-505.

Cojocaru V, Winn PJ, and Wade RC (2007) The ins and outs of cytochrome P450s. Biochim Biophys Acta **1770:**390-401.

Connor EM, Sperling RS, Gelber R, Kiselev P, Scott G, O'Sullivan MJ, VanDyke R, Bey M, Shearer W, Jacobson RL, and et al. (1994) Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N Engl J Med **331:**1173-1180.

Cortes CP, Siccardi M, Chaikan A, Owen A, Zhang G, and la Porte CJ (2013) Correlates of efavirenz exposure in Chilean patients affected with human immunodeficiency virus reveals a novel association with a polymorphism in the constitutive androstane receptor. Ther Drug Monit **35:**78-83.

Court MH, Duan SX, Hesse LM, Venkatakrishnan K, and Greenblatt DJ (2001) Cytochrome P-450 2B6 is responsible for interindividual variability of propofol hydroxylation by human liver microsomes. Anesthesiology **94:**110-119.

Crespi CL and Miller VP (1999) The use of heterologously expressed drug metabolizing enzymes--state of the art and prospects for the future. Pharmacol Ther **84:**121-131.

Crettol S, Deglon JJ, Besson J, Croquette-Krokkar M, Gothuey I, Hammig R, Monnat M, Huttemann H, Baumann P, and Eap CB (2005) Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. Clin Pharmacol Ther **78:**593-604.

Csajka C, Marzolini C, Fattinger K, Decosterd LA, Fellay J, Telenti A, Biollaz J, and Buclin T (2003) Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. Clin Pharmacol Ther **73:**20-30.

Daily EB and Aquilante CL (2009) Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies. Pharmacogenomics **10:**1489-1510.

De Cock KM, Adjorlolo G, Ekpini E, Sibailly T, Kouadio J, Maran M, Brattegaard K, Vetter KM, Doorly R, and Gayle HD (1993) Epidemiology and transmission of HIV-2. Why there is no HIV-2 pandemic. JAMA **270:**2083-2086.

De Santis M, Carducci B, De Santis L, Cavaliere AF, and Straface G (2002) Periconceptional exposure to efavirenz and neural tube defects. Arch Intern Med **162:**355.

Debouck C, Gorniak JG, Strickler JE, Meek TD, Metcalf BW, and Rosenberg M (1987) Human immunodeficiency virus protease expressed in Escherichia coli exhibits autoprocessing and specific maturation of the gag precursor. Proc Natl Acad Sci U S A **84:**8903-8906.

Deeks ED and Perry CM (2010) Efavirenz/emtricitabine/tenofovir disoproxil fumarate single-tablet regimen (Atripla(R)): a review of its use in the management of HIV infection. Drugs **70:**2315-2338.

Desta Z, Kerbusch T, Soukhova N, Richard E, Ko JW, and Flockhart DA (1998) Identification and characterization of human cytochrome P450 isoforms interacting with pimozide. J Pharmacol Exp Ther **285:**428-437.

Desta Z, Moaddel R, Ogburn ET, Xu C, Ramamoorthy A, Venkata SL, Sanghvi M, Goldberg ME, Torjman MC, and Wainer IW (2012) Stereoselective and regiospecific hydroxylation of ketamine and norketamine. Xenobiotica **42:**1076-1087.

Desta Z, Saussele T, Ward B, Blievernicht J, Li L, Klein K, Flockhart DA, and Zanger UM (2007) Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro. Pharmacogenomics **8:**547-558.

di Iulio J, Fayet A, Arab-Alameddine M, Rotger M, Lubomirov R, Cavassini M, Furrer H, Gunthard HF, Colombo S, Csajka C, Eap CB, Decosterd LA, and Telenti A (2009) In vivo analysis of efavirenz metabolism in individuals with impaired CYP2A6 function. Pharmacogenet Genomics **19:**300-309.

Dickinson GL, Lennard MS, Tucker GT, and Rostami-Hodjegan A (2007a) The use of mechanistic DM-PK-PD modelling to assess the power of pharmacogenetic studies - CYP2C9 and warfarin as an example. Br J Clin Pharmacol **64:**14-26.

Dickinson GL, Rezaee S, Proctor NJ, Lennard MS, Tucker GT, and Rostami-Hodjegan A (2007b) Incorporating in vitro information on drug metabolism into clinical trial simulations to assess the effect of CYP2D6 polymorphism on pharmacokinetics and pharmacodynamics: dextromethorphan as a model application. J Clin Pharmacol **47:**175-186.

Ding X and Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annu Rev Pharmacol Toxicol **43:**149-173.

Eap CB, Crettol S, Rougier JS, Schlapfer J, Sintra Grilo L, Deglon JJ, Besson J, Croquette-Krokar M, Carrupt PA, and Abriel H (2007) Stereoselective block of hERG channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. Clin Pharmacol Ther **81:**719-728.

Ekins S, Vandenbranden M, Ring BJ, Gillespie JS, Yang TJ, Gelboin HV, and Wrighton SA (1998) Further characterization of the expression in liver and catalytic activity of CYP2B6. J Pharmacol Exp Ther **286:**1253-1259.

Elens L, Vandercam B, Yombi JC, Lison D, Wallemacq P, and Haufroid V (2010) Influence of host genetic factors on efavirenz plasma and intracellular pharmacokinetics in HIV-1-infected patients. Pharmacogenomics **11:**1223-1234.

Erickson DA, Mather G, Trager WF, Levy RH, and Keirns JJ (1999) Characterization of the in vitro biotransformation of the HIV-1 reverse transcriptase inhibitor nevirapine by human hepatic cytochromes P-450. Drug Metab Dispos **27:**1488-1495.

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM, and Lindley CM (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. Drug Metab Dispos **28:**1222-1230.

Faucette SR, Wang H, Hamilton GA, Jolley SL, Gilbert D, Lindley C, Yan B, Negishi M, and LeCluyse EL (2004) Regulation of CYP2B6 in primary human hepatocytes by prototypical inducers. Drug Metab Dispos **32:**348-358.

Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, Negishi M, and Wang H (2007) Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. J Pharmacol Exp Ther **320:**72-80.

Fellay J, Marzolini C, Meaden ER, Back DJ, Buclin T, Chave JP, Decosterd LA, Furrer H, Opravil M, Pantaleo G, Retelska D, Ruiz L, Schinkel AH, Vernazza P, Eap CB, and Telenti A (2002) Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet **359:**30-36.

Feng Y, Broder CC, Kennedy PE, and Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-877.

Floridia M, Giuliano M, Palmisano L, and Vella S (2008) Gender differences in the treatment of HIV infection. Pharmacol Res **58:**173-182.

Foti RS and Wahlstrom JL (2008) CYP2C19 inhibition: the impact of substrate probe selection on in vitro inhibition profiles. Drug Metab Dispos **36:**523-528.

Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, and Popovic M (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220:**865-867.

Gay SC, Shah MB, Talakad JC, Maekawa K, Roberts AG, Wilderman PR, Sun L, Yang JY, Huelga SC, Hong WX, Zhang Q, Stout CD, and Halpert JR (2010) Crystal structure of a cytochrome P450 2B6 genetic variant in complex with the inhibitor 4-(4-chlorophenyl)imidazole at 2.0-A resolution. Mol Pharmacol **77:**529-538.

Gazzard BG, Anderson J, Babiker A, Boffito M, Brook G, Brough G, Churchill D, Cromarty B, Das S, Fisher M, Freedman A, Geretti AM, Johnson M, Khoo S, Leen C, Nair D, Peters B, Phillips A, Pillay D, Pozniak A, Walsh J, Wilkins E, Williams I, Williams M, and Youle M (2008) British HIV Association Guidelines for the treatment of HIV-1-infected adults with antiretroviral therapy 2008. HIV Med **9:**563-608.

Gerber JG, Rosenkranz SL, Fichtenbaum CJ, Vega JM, Yang A, Alston BL, Brobst SW, Segal Y, and Aberg JA (2005) Effect of efavirenz on the pharmacokinetics of simvastatin, atorvastatin, and pravastatin: results of AIDS Clinical Trials Group 5108 Study. J Acquir Immune Defic Syndr **39:**307-312.

German P, Greenhouse B, Coates C, Dorsey G, Rosenthal PJ, Charlebois E, Lindegardh N, Havlir D, and Aweeka FT (2007) Hepatotoxicity due to a drug interaction between amodiaquine plus artesunate and efavirenz. Clin Infect Dis **44:**889-891.

Gervot L, Rochat B, Gautier JC, Bohnenstengel F, Kroemer H, de Berardinis V, Martin H, Beaune P, and de Waziers I (1999) Human CYP2B6: expression, inducibility and catalytic activities. Pharmacogenetics **9:**295-306.

Giancarlo GM, Venkatakrishnan K, Granda BW, von Moltke LL, and Greenblatt DJ (2001) Relative contributions of CYP2C9 and 2C19 to phenytoin 4-hydroxylation in vitro: inhibition by sulfaphenazole, omeprazole, and ticlopidine. Eur J Clin Pharmacol **57:**31-36.

Gomes AM, Winter S, Klein K, Turpeinen M, Schaeffeler E, Schwab M, and Zanger UM (2009) Pharmacogenomics of human liver cytochrome P450 oxidoreductase: multifactorial analysis and impact on microsomal drug oxidation. Pharmacogenomics **10:**579-599.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. Mol Pharmacol **62:**359-365.

Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol **56:**1329-1339.

Goodwin B, Moore LB, Stoltz CM, McKee DD, and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. Mol Pharmacol **60:**427-431.

Gounden V, van Niekerk C, Snyman T, and George JA (2010) Presence of the CYP2B6 516G> T polymorphism, increased plasma Efavirenz concentrations and early neuropsychiatric side effects in South African HIV-infected patients. AIDS Res Ther **7:**32.

Groves JT (2003) The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies. Proc Natl Acad Sci U S A **100:**3569-3574.

Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, Richman DD, Valentine FT, Jonas L, Meibohm A, Emini EA, and Chodakewitz JA (1997) Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med **337:**734-739.

Gulick RM, Ribaudo HJ, Shikuma CM, Lustgarten S, Squires KE, Meyer WA, 3rd, Acosta EP, Schackman BR, Pilcher CD, Murphy RL, Maher WE, Witt MD, Reichman RC, Snyder S, Klingman KL, and Kuritzkes DR (2004) Triple-nucleoside regimens versus efavirenz-containing regimens for the initial treatment of HIV-1 infection. N Engl J Med **350:**1850-1861.

Haas DW, Gebretsadik T, Mayo G, Menon UN, Acosta EP, Shintani A, Floyd M, Stein CM, and Wilkinson GR (2009) Associations between CYP2B6 polymorphisms and pharmacokinetics after a single dose of nevirapine or efavirenz in African americans. J Infect Dis **199:**872-880.

Haas DW, Ribaudo HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, Clifford DB, Hulgan T, Marzolini C, and Acosta EP (2004) Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS **18:**2391-2400.

Haas DW, Smeaton LM, Shafer RW, Robbins GK, Morse GD, Labbe L, Wilkinson GR, Clifford DB, D'Aquila RT, De Gruttola V, Pollard RB, Merigan TC, Hirsch MS, George AL, Jr., Donahue JP, and Kim RB (2005) Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: an Adult Aids Clinical Trials Group Study. J Infect Dis **192:**1931-1942.

Hallifax D, Foster JA, and Houston JB (2010) Prediction of human metabolic clearance from in vitro systems: retrospective analysis and prospective view. Pharm Res **27:**2150-2161.

Hamelin BA, Bouayad A, Methot J, Jobin J, Desgagnes P, Poirier P, Allaire J, Dumesnil J, and Turgeon J (2000) Significant interaction between the nonprescription antihistamine diphenhydramine and the CYP2D6 substrate metoprolol in healthy men with high or low CYP2D6 activity. Clin Pharmacol Ther **67:**466-477.

Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Jr., Feinberg JE, Balfour HH, Jr., Deyton LR, Chodakewitz JA, and Fischl MA (1997) A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N Engl J Med **337:**725-733.

Hariparsad N, Nallani SC, Sane RS, Buckley DJ, Buckley AR, and Desai PB (2004) Induction of CYP3A4 by efavirenz in primary human hepatocytes: comparison with rifampin and phenobarbital. J Clin Pharmacol **44:**1273-1281.

Hassan M, Svensson US, Ljungman P, Bjorkstrand B, Olsson H, Bielenstein M, Abdel-Rehim M, Nilsson C, Johansson M, and Karlsson MO (1999) A mechanism-based pharmacokinetic-enzyme model for cyclophosphamide autoinduction in breast cancer patients. Br J Clin Pharmacol **48:**669-677.

Hesse LM, He P, Krishnaswamy S, Hao Q, Hogan K, von Moltke LL, Greenblatt DJ, and Court MH (2004) Pharmacogenetic determinants of interindividual variability in bupropion hydroxylation by cytochrome P450 2B6 in human liver microsomes. Pharmacogenetics **14:**225-238.

Hesse LM, Venkatakrishnan K, Court MH, von Moltke LL, Duan SX, Shader RI, and Greenblatt DJ (2000) CYP2B6 mediates the in vitro hydroxylation of bupropion: potential drug interactions with other antidepressants. Drug Metab Dispos **28:**1176-1183.

Hesse LM, von Moltke LL, Shader RI, and Greenblatt DJ (2001) Ritonavir, efavirenz, and nelfinavir inhibit CYP2B6 activity in vitro: potential drug interactions with bupropion. Drug Metab Dispos **29:**100-102.

Hofmann MH, Blievernicht JK, Klein K, Saussele T, Schaeffeler E, Schwab M, and Zanger UM (2008) Aberrant splicing caused by single nucleotide polymorphism c.516G>T [Q172H], a marker of CYP2B6*6, is responsible for decreased expression and activity of CYP2B6 in liver. J Pharmacol Exp Ther **325:**284-292.

Holzinger ER, Grady B, Ritchie MD, Ribaudo HJ, Acosta EP, Morse GD, Gulick RM, Robbins GK, Clifford DB, Daar ES, McLaren P, and Haas DW (2012) Genome-wide association study of plasma efavirenz pharmacokinetics in AIDS Clinical Trials Group protocols implicates several CYP2B6 variants. Pharmacogenet Genomics **22:** 858-867.

Honda M, Muroi Y, Tamaki Y, Saigusa D, Suzuki N, Tomioka Y, Matsubara Y, Oda A, Hirasawa N, and Hiratsuka M (2011) Functional characterization of CYP2B6 allelic variants in demethylation of antimalarial artemether. Drug Metab Dispos **39:**1860-1865.

Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. Mol Cell Biol **18:**5652-5658.

Howgate EM, Rowland Yeo K, Proctor NJ, Tucker GT, and Rostami-Hodjegan A (2006) Prediction of in vivo drug clearance from in vitro data. I: impact of inter-individual variability. Xenobiotica **36:**473-497.

Huang W, Lin YS, McConn DJ, 2nd, Calamia JC, Totah RA, Isoherranen N, Glodowski M, and Thummel KE (2004) Evidence of significant contribution from CYP3A5 to hepatic drug metabolism. Drug Metab Dispos **32:**1434-1445.

Hulot JS, Bura A, Villard E, Azizi M, Remones V, Goyenvalle C, Aiach M, Lechat P, and Gaussem P (2006) Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. Blood **108:**2244-2247.

Ikeda S, Kurose K, Jinno H, Sai K, Ozawa S, Hasegawa R, Komamura K, Kotake T, Morishita H, Kamakura S, Kitakaze M, Tomoike H, Tamura T, Yamamoto N, Kunitoh H, Yamada Y, Ohe Y, Shimada Y, Shirao K, Kubota K, Minami H, Ohtsu A, Yoshida T, Saijo N, Saito Y, and Sawada J (2005) Functional analysis of four naturally occurring variants of human constitutive androstane receptor. Mol Genet Metab **86:**314-319.

Ito K and Houston JB (2005) Prediction of human drug clearance from in vitro and preclinical data using physiologically based and empirical approaches. Pharm Res **22:**103-112.

Jamei M, Dickinson GL, and Rostami-Hodjegan A (2009) A framework for assessing inter-individual variability in pharmacokinetics using virtual human populations and integrating general knowledge of physical chemistry, biology, anatomy, physiology and genetics: A tale of 'bottom-up' vs 'top-down' recognition of covariates. Drug Metab Pharmacokinet **24:**53-75.

Janmohamed A, Dolphin CT, Phillips IR, and Shephard EA (2001) Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. Biochem Pharmacol **62:**777-786.

Jeong S, Nguyen PD, and Desta Z (2009) Comprehensive in vitro analysis of voriconazole inhibition of eight cytochrome P450 (CYP) enzymes: major effect on CYPs 2B6, 2C9, 2C19, and 3A. Antimicrob Agents Chemother **53:**541-551.

Jeppesen U, Rasmussen BB, and Brosen K (1997) Fluvoxamine inhibits the CYP2C19-catalyzed bioactivation of chloroguanide. Clin Pharmacol Ther **62:**279-286.

Jinno H, Tanaka-Kagawa T, Ohno A, Makino Y, Matsushima E, Hanioka N, and Ando M (2003) Functional characterization of cytochrome P450 2B6 allelic variants. Drug Metab Dispos **31:**398-403.

Jushchyshyn MI, Hutzler JM, Schrag ML, and Wienkers LC (2005) Catalytic turnover of pyrene by CYP3A4: evidence that cytochrome b5 directly induces positive cooperativity. Arch Biochem Biophys **438:**21-28.

Kasim NA, Whitehouse M, Ramachandran C, Bermejo M, Lennernas H, Hussain AS, Junginger HE, Stavchansky SA, Midha KK, Shah VP, and Amidon GL (2004) Molecular properties of WHO essential drugs and provisional biopharmaceutical classification. Mol Pharm **1:**85-96.

Kaspera R, Naraharisetti SB, Evangelista EA, Marciante KD, Psaty BM, and Totah RA (2011) Drug metabolism by CYP2C8.3 is determined by substrate dependent interactions with cytochrome P450 reductase and cytochrome b5. Biochem Pharmacol **82:**681-691.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol **19:**6318-6322.

Kazui M, Nishiya Y, Ishizuka T, Hagihara K, Farid NA, Okazaki O, Ikeda T, and Kurihara A (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. Drug Metab Dispos **38:**92-99.

Keiser P, Nassar N, White C, Koen G, and Moreno S (2002) Comparison of nevirapine- and efavirenz-containing antiretroviral regimens in antiretroviral-naive patients: a cohort study. HIV Clin Trials **3:**296-303.

Kenworthy KE, Bloomer JC, Clarke SE, and Houston JB (1999) CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. Br J Clin Pharmacol **48:**716-727.

Kharasch ED, Hoffer C, Whittington D, and Sheffels P (2004) Role of hepatic and intestinal cytochrome P450 3A and 2B6 in the metabolism, disposition, and miotic effects of methadone. Clin Pharmacol Ther **76:**250-269.

Kharasch ED, Mitchell D, and Coles R (2008) Stereoselective bupropion hydroxylation as an in vivo phenotypic probe for cytochrome P4502B6 (CYP2B6) activity. J Clin Pharmacol **48:**464-474.

Kharasch ED, Whittington D, Ensign D, Hoffer C, Bedynek PS, Campbell S, Stubbert K, Crafford A, London A, and Kim T (2012) Mechanism of efavirenz influence on methadone pharmacokinetics and pharmacodynamics. Clin Pharmacol Ther **91:**673-684.

Kirchheiner J, Klein C, Meineke I, Sasse J, Zanger UM, Murdter TE, Roots I, and Brockmoller J (2003) Bupropion and 4-OH-bupropion pharmacokinetics in relation to genetic polymorphisms in CYP2B6. Pharmacogenetics **13:**619-626.

Klein K, Lang T, Saussele T, Barbosa-Sicard E, Schunck WH, Eichelbaum M, Schwab M, and Zanger UM (2005) Genetic variability of CYP2B6 in populations of African and Asian origin: allele frequencies, novel functional variants, and possible implications for anti-HIV therapy with efavirenz. Pharmacogenet Genomics **15:**861-873.

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

Kohl NE, Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA, Scolnick EM, and Sigal IS (1988) Active human immunodeficiency virus protease is required for viral infectivity. Proc Natl Acad Sci U S A **85:**4686-4690.

Kudriakova TB, Sirota LA, Rozova GI, and Gorkov VA (1992) Autoinduction and steady-state pharmacokinetics of carbamazepine and its major metabolites. Br J Clin Pharmacol **33:**611-615.

Kumar V, Brundage RC, Oetting WS, Leppik IE, and Tracy TS (2008) Differential genotype dependent inhibition of CYP2C9 in humans. Drug Metab Dispos **36:**1242-1248.

Kumar V, Rock DA, Warren CJ, Tracy TS, and Wahlstrom JL (2006a) Enzyme source effects on CYP2C9 kinetics and inhibition. Drug Metab Dispos **34:**1903-1908.

Kumar V, Wahlstrom JL, Rock DA, Warren CJ, Gorman LA, and Tracy TS (2006b) CYP2C9 inhibition: impact of probe selection and pharmacogenetics on in vitro inhibition profiles. Drug Metab Dispos **34:**1966-1975.

Kusama M, Maeda K, Chiba K, Aoyama A, and Sugiyama Y (2009) Prediction of the effects of genetic polymorphism on the pharmacokinetics of CYP2C9 substrates from in vitro data. Pharm Res **26:**822-835.

Kwara A, Lartey M, Sagoe KW, Kenu E, and Court MH (2009a) CYP2B6, CYP2A6 and UGT2B7 genetic polymorphisms are predictors of efavirenz mid-dose concentration in HIV-infected patients. AIDS **23:**2101-2106.

Kwara A, Lartey M, Sagoe KW, Rzek NL, and Court MH (2009b) CYP2B6 (c.516G-->T) and CYP2A6 (*9B and/or *17) polymorphisms are independent predictors of efavirenz plasma concentrations in HIV-infected patients. Br J Clin Pharmacol **67:**427-436.

Lai XS, Yang LP, Li XT, Liu JP, Zhou ZW, and Zhou SF (2009) Human CYP2C8: structure, substrate specificity, inhibitor selectivity, inducers and polymorphisms. Curr Drug Metab **10:**1009-1047.

Lamba V, Lamba J, Yasuda K, Strom S, Davila J, Hancock ML, Fackenthal JD, Rogan PK, Ring B, Wrighton SA, and Schuetz EG (2003) Hepatic CYP2B6 expression: gender and ethnic differences and relationship to CYP2B6 genotype and CAR (constitutive androstane receptor) expression. J Pharmacol Exp Ther **307:**906-922.

Lamba V, Panetta JC, Strom S, and Schuetz EG (2010) Genetic predictors of interindividual variability in hepatic CYP3A4 expression. J Pharmacol Exp Ther **332:**1088-1099.

Lamorde M, Byakika-Kibwika P, Tamale WS, Kiweewa F, Ryan M, Amara A, Tjia J, Back D, Khoo S, Boffito M, Kityo C, and Merry C (2012) Effect of Food on the Steady-State Pharmacokinetics of Tenofovir and Emtricitabine plus Efavirenz in Ugandan Adults. AIDS Res Treat **2012:**105980.

Lang T, Klein K, Fischer J, Nussler AK, Neuhaus P, Hofmann U, Eichelbaum M, Schwab M, and Zanger UM (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. Pharmacogenetics **11:**399-415.

Lang T, Klein K, Richter T, Zibat A, Kerb R, Eichelbaum M, Schwab M, and Zanger UM (2004) Multiple novel nonsynonymous CYP2B6 gene polymorphisms in Caucasians: demonstration of phenotypic null alleles. J Pharmacol Exp Ther **311:**34-43.

Lessard E, Yessine MA, Hamelin BA, Gauvin C, Labbe L, O'Hara G, LeBlanc J, and Turgeon J (2001) Diphenhydramine alters the disposition of venlafaxine through inhibition of CYP2D6 activity in humans. J Clin Psychopharmacol **21:**175-184.

Li J, Menard V, Benish RL, Jurevic RJ, Guillemette C, Stoneking M, Zimmerman PA, and Mehlotra RK (2012) Worldwide variation in human drug-metabolism enzyme genes CYP2B6 and UGT2B7: implications for HIV/AIDS treatment. Pharmacogenomics **13:**555-570.

Li XQ, Bjorkman A, Andersson TB, Ridderstrom M, and Masimirembwa CM (2002) Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. J Pharmacol Exp Ther **300:**399-407.

Lindh JD, Annas A, Meurling L, Dahl ML, and A AL-S (2003) Effect of ketoconazole on venlafaxine plasma concentrations in extensive and poor metabolisers of debrisoquine. Eur J Clin Pharmacol **59:**401-406.

Lins R, Broekhuysen J, Necciari J, and Deroubaix X (1999) Pharmacokinetic profile of 14C-labeled clopidogrel. Semin Thromb Hemost **25 Suppl 2:**29-33.

Liu P, Foster G, LaBadie RR, Gutierrez MJ, and Sharma A (2008) Pharmacokinetic interaction between voriconazole and efavirenz at steady state in healthy male subjects. J Clin Pharmacol **48:**73-84.

Loboz KK, Gross AS, Williams KM, Liauw WS, Day RO, Blievernicht JK, Zanger UM, and McLachlan AJ (2006) Cytochrome P450 2B6 activity as measured by bupropion hydroxylation: effect of induction by rifampin and ethnicity. Clin Pharmacol Ther **80:**75-84.

Lutz JD and Isoherranen N (2012) Prediction of relative in vivo metabolite exposure from in vitro data using two model drugs: dextromethorphan and omeprazole. Drug Metab Dispos **40:**159-168.

Maekawa K, Harakawa N, Sugiyama E, Tohkin M, Kim SR, Kaniwa N, Katori N, Hasegawa R, Yasuda K, Kamide K, Miyata T, Saito Y, and Sawada J (2009) Substrate-dependent functional alterations of seven CYP2C9 variants found in Japanese subjects. Drug Metab Dispos **37:**1895-1903.

Maggiolo F (2009) Efavirenz: a decade of clinical experience in the treatment of HIV. J Antimicrob Chemother **64:**910-928.

Maggiolo F, Airoldi M, Kleinloog HD, Callegaro A, Ravasio V, Arici C, Bombana E, and Suter F (2007) Effect of adherence to HAART on virologic outcome and on the selection of resistance-conferring mutations in NNRTI- or PI-treated patients. HIV Clin Trials **8:**282-292.

Maggiolo F, Ripamonti D, Gregis G, Quinzan G, Callegaro A, Arici C, Ravasio L, and Suter F (2003) Once-a-day therapy for HIV infection: a controlled, randomized study in antiretroviral-naive HIV-1-infected patients. Antivir Ther **8:**339-346.

Maimbo M, Kiyotani K, Mushiroda T, Masimirembwa C, and Nakamura Y (2012) CYP2B6 genotype is a strong predictor of systemic exposure to efavirenz in HIV-infected Zimbabweans. Eur J Clin Pharmacol **68:**267-271.

Marlink R, Kanki P, Thior I, Travers K, Eisen G, Siby T, Traore I, Hsieh CC, Dia MC, Gueye EH, and et al. (1994) Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science **265:**1587-1590.

Marzolini C, Telenti A, Decosterd LA, Greub G, Biollaz J, and Buclin T (2001) Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. AIDS **15:**71-75.

Maurin MB, Rowe SM, Blom K, and Pierce ME (2002) Kinetics and mechanism of hydrolysis of efavirenz. Pharm Res **19:**517-521.

Meyer zu Schwabedissen HE, Oswald S, Bresser C, Nassif A, Modess C, Desta Z, Ogburn ET, Marinova M, Lutjohann D, Spielhagen C, Nauck M, Kroemer HK, and Siegmund W (2012) Compartment-specific gene regulation of the CAR inducer efavirenz in vivo. Clin Pharmacol Ther **92:**103-111.

Michaud V, Bar-Magen T, Turgeon J, Flockhart D, Desta Z, and Wainberg MA (2012a) The dual role of pharmacogenetics in HIV treatment: mutations and polymorphisms regulating antiretroviral drug resistance and disposition. Pharmacol Rev **64:**803-833.

Michaud V, Ogburn E, Thong N, Aregbe AO, Quigg TC, Flockhart DA, and Desta Z (2012b) Induction of CYP2C19 and CYP3A Activity Following Repeated Administration of Efavirenz in Healthy Volunteers. Clin Pharmacol Ther **91:** 475-482.

Mo SL, Liu YH, Duan W, Wei MQ, Kanwar JR, and Zhou SF (2009) Substrate specificity, regulation, and polymorphism of human cytochrome P450 2B6. Curr Drug Metab **10:**730-753.

Molina JM, Cahn P, Grinsztejn B, Lazzarin A, Mills A, Saag M, Supparatpinyo K, Walmsley S, Crauwels H, Rimsky LT, Vanveggel S, and Boven K (2011) Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naive adults infected with HIV-1 (ECHO): a phase 3 randomised double-blind active-controlled trial. Lancet **378:**238-246.

Motsinger AA, Ritchie MD, Shafer RW, Robbins GK, Morse GD, Labbe L, Wilkinson GR, Clifford DB, D'Aquila RT, Johnson VA, Pollard RB, Merigan TC, Hirsch MS, Donahue JP, Kim RB, and Haas DW (2006) Multilocus genetic interactions and response to efavirenz-containing regimens: an adult AIDS clinical trials group study. Pharmacogenet Genomics **16:**837-845.

Mouly S, Lown KS, Kornhauser D, Joseph JL, Fiske WD, Benedek IH, and Watkins PB (2002) Hepatic but not intestinal CYP3A4 displays dose-dependent induction by efavirenz in humans. Clin Pharmacol Ther **72:**1-9.

Mugusi S, Ngaimisi E, Janabi M, Minzi O, Bakari M, Riedel KD, Burhenne J, Lindquist L, Mugusi F, Sandstrom E, and Aklillu E (2012) Liver enzyme abnormalities and associated risk factors in HIV patients on efavirenz-based HAART with or without tuberculosis co-infection in Tanzania. PLoS One **7:**e40180.

Mukonzo JK, Roshammar D, Waako P, Andersson M, Fukasawa T, Milani L, Svensson JO, Ogwal-Okeng J, Gustafsson LL, and Aklillu E (2009) A novel polymorphism in ABCB1 gene, CYP2B6*6 and sex predict single-dose efavirenz population pharmacokinetics in Ugandans. Br J Clin Pharmacol **68:**690-699.

Mutlib AE, Chen H, Nemeth GA, Markwalder JA, Seitz SP, Gan LS, and Christ DD (1999) Identification and characterization of efavirenz metabolites by liquid chromatography/mass spectrometry and high field NMR: species differences in the metabolism of efavirenz. Drug Metab Dispos **27:**1319-1333.

Nakajima M, Komagata S, Fujiki Y, Kanada Y, Ebi H, Itoh K, Mukai H, Yokoi T, and Minami H (2007) Genetic polymorphisms of CYP2B6 affect the pharmacokinetics/pharmacodynamics of cyclophosphamide in Japanese cancer patients. Pharmacogenet Genomics **17:**431-445.

Nasi M, Borghi V, Pinti M, Bellodi C, Lugli E, Maffei S, Troiano L, Richeldi L, Mussini C, Esposito R, and Cossarizza A (2003) MDR1 C3435T genetic polymorphism does not influence the response to antiretroviral therapy in drug-naive HIV-positive patients. AIDS **17:**1696-1698.

Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics **14:**1-18.

Ngaimisi E, Mugusi S, Minzi O, Sasi P, Riedel KD, Suda A, Ueda N, Janabi M, Mugusi F, Haefeli WE, Bertilsson L, Burhenne J, and Aklillu E (2011) Effect of rifampicin and CYP2B6 genotype on long-term efavirenz autoinduction and plasma exposure in HIV patients with or without tuberculosis. Clin Pharmacol Ther **90:**406-413.

Nishiya Y, Hagihara K, Ito T, Tajima M, Miura S, Kurihara A, Farid NA, and Ikeda T (2009) Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. Drug Metab Dispos **37:**589-593.

Obach RS (1996) The importance of nonspecific binding in in vitro matrices, its impact on enzyme kinetic studies of drug metabolism reactions, and implications for in vitro-in vivo correlations. Drug Metab Dispos **24:**1047-1049.

Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab Dispos **27:**1350-1359.

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB, and Tremaine LM (2006) The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. J Pharmacol Exp Ther **316:**336-348.

Obach RS, Walsky RL, Venkatakrishnan K, Houston JB, and Tremaine LM (2005) In vitro cytochrome P450 inhibition data and the prediction of drug-drug interactions: qualitative relationships, quantitative predictions, and the rank-order approach. Clin Pharmacol Ther **78:**582-592.

Ogburn ET, Jones DR, Masters AR, Xu C, Guo Y, and Desta Z (2010) Efavirenz primary and secondary metabolism in vitro and in vivo: identification of novel metabolic pathways and cytochrome P450 2A6 as the principal catalyst of efavirenz 7-hydroxylation. Drug Metab Dispos **38:**1218-1229.

Ortiz de Montellano PR and De Voss JJ (2002) Oxidizing species in the mechanism of cytochrome P450. Nat Prod Rep **19:**477-493.
Oscarson M, Zanger UM, Rifki OF, Klein K, Eichelbaum M, and Meyer UA (2006) Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. Clin Pharmacol Ther **80:**440-456.

Oswald S, Meyer zu Schwabedissen HE, Nassif A, Modess C, Desta Z, Ogburn ET, Mostertz J, Keiser M, Jia J, Hubeny A, Ulrich A, Runge D, Marinova M, Lutjohann D, Kroemer HK, and Siegmund W (2012) Impact of efavirenz on intestinal metabolism and transport: insights from an interaction study with ezetimibe in healthy volunteers. Clin Pharmacol Ther **91:**506-513.

Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, and Holmberg S.D. (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med **338:**853-860.

Parikh A, Gillam EM, and Guengerich FP (1997) Drug metabolism by Escherichia coli expressing human cytochromes P450. Nat Biotechnol **15:**784-788.

Parikh S, Ouedraogo JB, Goldstein JA, Rosenthal PJ, and Kroetz DL (2007) Amodiaquine metabolism is impaired by common polymorphisms in CYP2C8: implications for malaria treatment in Africa. Clin Pharmacol Ther **82:**197-203.

Peroni RN, Di Gennaro SS, Hocht C, Chiappetta DA, Rubio MC, Sosnik A, and Bramuglia GF (2011) Efavirenz is a substrate and in turn modulates the expression of the efflux transporter ABCG2/BCRP in the gastrointestinal tract of the rat. Biochem Pharmacol **82:**1227-1233.

Porter K, Babiker A, Bhaskaran K, Darbyshire J, Pezzotti P, and Walker AS (2003) Determinants of survival following HIV-1 seroconversion after the introduction of HAART. Lancet **362:**1267-1274.

Proctor NJ, Tucker GT, and Rostami-Hodjegan A (2004) Predicting drug clearance from recombinantly expressed CYPs: intersystem extrapolation factors. Xenobiotica **34:**151-178.

Raccor BS, Claessens AJ, Dinh JC, Park JR, Hawkins DS, Thomas SS, Makar KW, McCune JS, and Totah RA (2012) Potential contribution of cytochrome P450 2B6 to hepatic 4-hydroxycyclophosphamide formation in vitro and in vivo. Drug Metab Dispos **40:**54-63.

Rae JM, Soukhova NV, Flockhart DA, and Desta Z (2002) Triethylenethiophosphoramide is a specific inhibitor of cytochrome P450 2B6: implications for cyclophosphamide metabolism. Drug Metab Dispos **30:**525-530.

Ramirez J, Innocenti F, Schuetz EG, Flockhart DA, Relling MV, Santucci R, and Ratain MJ (2004) CYP2B6, CYP3A4, and CYP2C19 are responsible for the in vitro N-demethylation of meperidine in human liver microsomes. Drug Metab Dispos **32:**930-936.

Ratner L, Gallo RC, and Wong-Staal F (1985) HTLV-III, LAV, ARV are variants of same AIDS virus. Nature **313:**636-637.

Reed JR and Hollenberg PF (2003) Comparison of substrate metabolism by cytochromes P450 2B1, 2B4, and 2B6: relationship of heme spin state, catalysis, and the effects of cytochrome b5. J Inorg Biochem **93:**152-160.

Rekic D, Roshammar D, Mukonzo J, and Ashton M (2011) In silico prediction of efavirenz and rifampicin drug-drug interaction considering weight and CYP2B6 phenotype. Br J Clin Pharmacol **71:**536-543.

Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev **34:**83-448.

Rettie AE and Jones JP (2005) Clinical and toxicological relevance of CYP2C9: drug-drug interactions and pharmacogenetics. Annu Rev Pharmacol Toxicol **45:**477-494.

Ribaudo HJ, Liu H, Schwab M, Schaeffeler E, Eichelbaum M, Motsinger-Reif AA, Ritchie MD, Zanger UM, Acosta EP, Morse GD, Gulick RM, Robbins GK, Clifford D, and Haas DW (2010) Effect of CYP2B6, ABCB1, and CYP3A5 polymorphisms on efavirenz pharmacokinetics and treatment response: an AIDS Clinical Trials Group study. J Infect Dis **202:**717-722.

Richter T, Murdter TE, Heinkele G, Pleiss J, Tatzel S, Schwab M, Eichelbaum M, and Zanger UM (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. J Pharmacol Exp Ther **308:**189-197.

Richter T, Schwab M, Eichelbaum M, and Zanger UM (2005) Inhibition of human CYP2B6 by N,N',N''-triethylenethiophosphoramide is irreversible and mechanism-based. Biochem Pharmacol **69:**517-524.

Riddler SA, Haubrich R, DiRienzo AG, Peeples L, Powderly WG, Klingman KL, Garren KW, George T, Rooney JF, Brizz B, Lalloo UG, Murphy RL, Swindells S, Havlir D, and Mellors JW (2008) Class-sparing regimens for initial treatment of HIV-1 infection. N Engl J Med **358:**2095-2106.

Robertson SM, Maldarelli F, Natarajan V, Formentini E, Alfaro RM, and Penzak SR (2008) Efavirenz induces CYP2B6-mediated hydroxylation of bupropion in healthy subjects. J Acquir Immune Defic Syndr **49:**513-519.

Robertson SM, Penzak SR, Lane J, Pau AK, and Mican JM (2005) A potentially significant interaction between efavirenz and phenytoin: a case report and review of the literature. Clin Infect Dis **41:**e15-18.

Rostami-Hodjegan A and Tucker GT (2007) Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. Nat Rev Drug Discov **6:**140-148.

Rotger M, Colombo S, Furrer H, Bleiber G, Buclin T, Lee BL, Keiser O, Biollaz J, Decosterd L, and Telenti A (2005) Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. Pharmacogenet Genomics **15:**1-5.

Rotger M, Tegude H, Colombo S, Cavassini M, Furrer H, Decosterd L, Blievernicht J, Saussele T, Gunthard HF, Schwab M, Eichelbaum M, Telenti A, and Zanger UM (2007) Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. Clin Pharmacol Ther **81:**557-566.

Rowland M, Peck C, and Tucker G (2011) Physiologically-based pharmacokinetics in drug development and regulatory science. Annu Rev Pharmacol Toxicol **51:**45-73.

Roy P, Yu LJ, Crespi CL, and Waxman DJ (1999) Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. Drug Metab Dispos **27:**655-666.

Saitoh A, Hull AD, Franklin P, and Spector SA (2005) Myelomeningocele in an infant with intrauterine exposure to efavirenz. J Perinatol **25:**555-556.

Sanchez A, Cabrera S, Santos D, Valverde MP, Fuertes A, Dominguez-Gil A, and Garcia MJ (2011) Population pharmacokinetic/pharmacogenetic model for optimization of efavirenz therapy in Caucasian HIV-infected patients. Antimicrob Agents Chemother **55:**5314-5324.

Sathigari S, Chadha G, Lee YH, Wright N, Parsons DL, Rangari VK, Fasina O, and Babu RJ (2009) Physicochemical characterization of efavirenz-cyclodextrin inclusion complexes. AAPS PharmSciTech **10:**81-87.

Schenkman JB and Jansson I (2003) The many roles of cytochrome b5. Pharmacol Ther **97:**139-152.

Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, and Sligar SG (2000) The catalytic pathway of cytochrome p450cam at atomic resolution. Science **287:**1615-1622.

Shah MB, Pascual J, Zhang Q, Stout CD, and Halpert JR (2011) Structures of cytochrome P450 2B6 bound to 4-benzylpyridine and 4-(4-nitrobenzyl)pyridine: insight into inhibitor binding and rearrangement of active site side chains. Mol Pharmacol **80:**1047-1055.

Shebley M and Hollenberg PF (2007) Mutation of a single residue (K262R) in P450 2B6 leads to loss of mechanism-based inactivation by phencyclidine. Drug Metab Dispos **35:**1365-1371.

Shou M, Hayashi M, Pan Y, Xu Y, Morrissey K, Xu L, and Skiles GL (2008) Modeling, prediction, and in vitro in vivo correlation of CYP3A4 induction. Drug Metab Dispos **36:**2355-2370.

Siccardi M, Almond L, Schipani A, Csajka C, Marzolini C, Wyen C, Brockmeyer NH, Boffito M, Owen A, and Back D (2012) Pharmacokinetic and pharmacodynamic analysis of efavirenz dose reduction using an in vitro-in vivo extrapolation model. Clin Pharmacol Ther **92:**494-502.

Soyinka JO and Onyeji CO (2010) Alteration of pharmacokinetics of proguanil in healthy volunteers following concurrent administration of efavirenz. Eur J Pharm Sci **39:**213-218.

Squires K, Lazzarin A, Gatell JM, Powderly WG, Pokrovskiy V, Delfraissy JF, Jemsek J, Rivero A, Rozenbaum W, Schrader S, Sension M, Vibhagool A, Thiry A, and Giordano M (2004) Comparison of once-daily atazanavir with efavirenz, each in combination with fixed-dose zidovudine and lamivudine, as initial therapy for patients infected with HIV. J Acquir Immune Defic Syndr **36:**1011-1019.

Staszewski S, Morales-Ramirez J, Tashima KT, Rachlis A, Skiest D, Stanford J, Stryker R, Johnson P, Labriola DF, Farina D, Manion DJ, and Ruiz NM (1999) Efavirenz plus zidovudine and lamivudine, efavirenz plus indinavir, and indinavir plus zidovudine and lamivudine in the treatment of HIV-1 infection in adults. Study 006 Team. N Engl J Med **341:**1865-1873.

Stresser DM and Kupfer D (1999) Monospecific antipeptide antibody to cytochrome P-450 2B6. Drug Metab Dispos **27:**517-525.

Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. Annu Rev Pharmacol Toxicol **41:**123-143.

Sulkowski MS, Thomas DL, Mehta SH, Chaisson RE, and Moore RD (2002) Hepatotoxicity associated with nevirapine or efavirenz-containing antiretroviral therapy: role of hepatitis C and B infections. Hepatology **35:**182-189.

Svensson US and Ashton M (1999) Identification of the human cytochrome P450 enzymes involved in the in vitro metabolism of artemisinin. Br J Clin Pharmacol **48:**528-535.

Swales K and Negishi M (2004) CAR, driving into the future. Mol Endocrinol **18:**1589-1598.

Takano R, Sugano K, Higashida A, Hayashi Y, Machida M, Aso Y, and Yamashita S (2006) Oral absorption of poorly water-soluble drugs: computer simulation of fraction absorbed in humans from a miniscale dissolution test. Pharm Res **23:**1144-1156.

Talakad JC, Kumar S, and Halpert JR (2009) Decreased susceptibility of the cytochrome P450 2B6 variant K262R to inhibition by several clinically important drugs. Drug Metab Dispos **37:**644-650.

Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, Eron JJ, Gunthard HF, Hammer SM, Reiss P, Richman DD, Rizzardini G, Thomas DL, Jacobsen DM, and Volberding PA (2012) Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. JAMA **308:**387-402.

Tod M, Goutelle S, and Gagnieu MC (2011) Genotype-Based Quantitative Prediction of Drug Exposure for Drugs Metabolized by CYP2D6. Clin Pharmacol Ther **90:**582-587.

Totah RA and Rettie AE (2005) Cytochrome P450 2C8: substrates, inhibitors, pharmacogenetics, and clinical relevance. Clin Pharmacol Ther **77:**341-352.

Tsuchiya K, Gatanaga H, Tachikawa N, Teruya K, Kikuchi Y, Yoshino M, Kuwahara T, Shirasaka T, Kimura S, and Oka S (2004) Homozygous CYP2B6 *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens. Biochem Biophys Res Commun **319:**1322-1326.

Turpeinen M, Uusitalo J, Jalonen J, and Pelkonen O (2005) Multiple P450 substrates in a single run: rapid and comprehensive in vitro interaction assay. Eur J Pharm Sci **24:**123-132.

Uno T, Shimizu M, Yasui-Furukori N, Sugawara K, and Tateishi T (2006) Different effects of fluvoxamine on rabeprazole pharmacokinetics in relation to CYP2C19 genotype status. Br J Clin Pharmacol **61:**309-314.

Usami O, Ashino Y, Komaki Y, Tomaki M, Irokawa T, Tamada T, Hayashida T, Teruya K, and Hattori T (2007) Efavirenz-induced neurological symptoms in rare homozygote CYP2B6 *2/*2 (C64T). Int J STD AIDS **18:**575-576.

van der Hoeven TA and Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. J Biol Chem **249:**6302-6310.

van Leth F, Phanuphak P, Ruxrungtham K, Baraldi E, Miller S, Gazzard B, Cahn P, Lalloo UG, van der Westhuizen IP, Malan DR, Johnson MA, Santos BR, Mulcahy F, Wood R, Levi GC, Reboredo G, Squires K, Cassetti I, Petit D, Raffi F, Katlama C, Murphy RL, Horban A, Dam JP, Hassink E, van Leeuwen R, Robinson P, Wit FW, and Lange JM (2004) Comparison of first-line antiretroviral therapy with regimens including nevirapine, efavirenz, or both drugs, plus stavudine and lamivudine: a randomised open-label trial, the 2NN Study. Lancet **363:**1253-1263.

van Luin M, Van der Ende ME, Richter C, Visser M, Faraj D, Van der Ven A, Gelinck L, Kroon F, Wit FW, Van Schaik RH, Kuks PF, and Burger DM (2010) Lower atovaquone/proguanil concentrations in patients taking efavirenz, lopinavir/ritonavir or atazanavir/ritonavir. AIDS **24:**1223-1226.

VandenBrink BM, Foti RS, Rock DA, Wienkers LC, and Wahlstrom JL (2011a) Evaluation of CYP2C8 inhibition in vitro: utility of montelukast as a selective CYP2C8 probe substrate. Drug Metab Dispos **39:**1546-1554.

Vandenbrink BM, Foti RS, Rock DA, Wienkers LC, and Wahlstrom JL (2011b) Prediction of CYP2D6 Drug Interactions from In Vitro Data: Evidence for Substrate-Dependent Inhibition. Drug Metab Dispos **40:** 47-53.

Venkatakrishnan K, von Moltke LL, Court MH, Harmatz JS, Crespi CL, and Greenblatt DJ (2000) Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. Drug Metab Dispos **28:**1493-1504.

von Moltke LL, Greenblatt DJ, Granda BW, Giancarlo GM, Duan SX, Daily JP, Harmatz JS, and Shader RI (2001) Inhibition of human cytochrome P450 isoforms by nonnucleoside reverse transcriptase inhibitors. J Clin Pharmacol **41:**85-91.

Walsky RL, Astuccio AV, and Obach RS (2006) Evaluation of 227 drugs for in vitro inhibition of cytochrome P450 2B6. J Clin Pharmacol **46:**1426-1438.

Walsky RL and Obach RS (2007) A comparison of 2-phenyl-2-(1-piperidinyl)propane (ppp), 1,1',1"-phosphinothioylidynetrisaziridine (thioTEPA), clopidogrel, and ticlopidine as selective inactivators of human cytochrome P450 2B6. Drug Metab Dispos **35:**2053-2059.

Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M, and LeCluyse E (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. J Biol Chem **279:**29295-29301.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M, and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. J Biol Chem **278:**14146-14152.

Wang H and Tompkins LM (2008) CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme. Curr Drug Metab **9:**598-610.

Wang J, Sonnerborg A, Rane A, Josephson F, Lundgren S, Stahle L, and Ingelman-Sundberg M (2006) Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. Pharmacogenet Genomics **16:**191-198.

Ward BA, Gorski JC, Jones DR, Hall S.D., Flockhart DA, and Desta Z (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. J Pharmacol Exp Ther **306:**287-300.

Watanabe T, Sakuyama K, Sasaki T, Ishii Y, Ishikawa M, Hirasawa N, and Hiratsuka M (2010) Functional characterization of 26 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.28, except CYP2B6.22). Pharmacogenet Genomics **20:**459-462.

Weiss J, Herzog M, Konig S, Storch CH, Ketabi-Kiyanvash N, and Haefeli WE (2009) Induction of multiple drug transporters by efavirenz. J Pharmacol Sci **109:**242-250.

Weiss J, Rose J, Storch CH, Ketabi-Kiyanvash N, Sauer A, Haefeli WE, and Efferth T (2007) Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. J Antimicrob Chemother **59:**238-245.

Weiss RA (2008) Special anniversary review: twenty-five years of human immunodeficiency virus research: successes and challenges. Clin Exp Immunol **152:**201-210.

Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. Nat Rev Drug Discov **1:**259-266.

Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW, and Tucker GT (2003) Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. Br J Clin Pharmacol **56:**433-440.

Wu L and KewalRamani VN (2006) Dendritic-cell interactions with HIV: infection and viral dissemination. Nat Rev Immunol **6:**859-868.

Wyen C, Hendra H, Siccardi M, Platten M, Jaeger H, Harrer T, Esser S, Bogner JR, Brockmeyer NH, Bieniek B, Rockstroh J, Hoffmann C, Stoehr A, Michalik C, Dlugay V, Jetter A, Knechten H, Klinker H, Skaletz-Rorowski A, Fatkenheuer G, Egan D, Back DJ, and Owen A (2011) Cytochrome P450 2B6 (CYP2B6) and constitutive androstane receptor (CAR) polymorphisms are associated with early discontinuation of efavirenz-containing regimens. J Antimicrob Chemother **66:**2092-2098.

Wyen C, Hendra H, Vogel M, Hoffmann C, Knechten H, Brockmeyer NH, Bogner JR, Rockstroh J, Esser S, Jaeger H, Harrer T, Mauss S, van Lunzen J, Skoetz N, Jetter A, Groneuer C, Fatkenheuer G, Khoo SH, Egan D, Back DJ, and Owen A (2008) Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. J Antimicrob Chemother **61:**914-918.

Xie HJ, Yasar U, Lundgren S, Griskevicius L, Terelius Y, Hassan M, and Rane A (2003) Role of polymorphic human CYP2B6 in cyclophosphamide bioactivation. Pharmacogenomics J **3:**53-61.

Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, and Evans RM (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev **14:**3014-3023.

Xu C, Ogburn ET, Guo Y, and Desta Z (2012) Effects of the CYP2B6*6 Allele on Catalytic Properties and Inhibition of CYP2B6 in vitro: Implication for the Mechanism of Reduced Efavirenz Metabolism and Other CYP2B6 Substrates in vivo. Drug Metab Dispos **40:** 717-725.

Yamano S, Nhamburo PT, Aoyama T, Meyer UA, Inaba T, Kalow W, Gelboin HV, McBride OW, and Gonzalez FJ (1989) cDNA cloning and sequence and cDNA-directed expression of human P450 IIB1: identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. Biochemistry **28:**7340-7348.

Yanagihara Y, Kariya S, Ohtani M, Uchino K, Aoyama T, Yamamura Y, and Iga T (2001) Involvement of CYP2B6 in n-demethylation of ketamine in human liver microsomes. Drug Metab Dispos **29:**887-890.

Yeni P, Cooper DA, Aboulker JP, Babiker AG, Carey D, Darbyshire JH, Floridia M, Girard PM, Goodall RL, Hooker MH, Mijch A, Meiffredy V, and Salzberger B (2006) Virological and immunological outcomes at 3 years after starting antiretroviral therapy with regimens containing non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or both in INITIO: open-label randomised trial. Lancet **368:**287-298.

Yimer G, Amogne W, Habtewold A, Makonnen E, Ueda N, Suda A, Worku A, Haefeli WE, Burhenne J, Aderaye G, Lindquist L, and Aklillu E (2011) High plasma efavirenz level and CYP2B6*6 are associated with efavirenz-based HAART-induced liver injury in the treatment of naive HIV patients from Ethiopia: a prospective cohort study. Pharmacogenomics J **12:** 499-506.

Zanger UM, Klein K, Saussele T, Blievernicht J, Hofmann MH, and Schwab M (2007) Polymorphic CYP2B6: molecular mechanisms and emerging clinical significance. Pharmacogenomics **8:**743-759.

Zelko I and Negishi M (2000) Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes. Biochem Biophys Res Commun **277:**1-6.
Zhang H, Hamdane D, Im SC, and Waskell L (2008) Cytochrome b5 inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. J Biol Chem **283:**5217-5225.

Zhang H, Sridar C, Kenaan C, Amunugama H, Ballou DP, and Hollenberg PF (2011a) Polymorphic variants of cytochrome P450 2B6 (CYP2B6.4-CYP2B6.9) exhibit altered rates of metabolism for bupropion and efavirenz: a charge-reversal mutation in the K139E variant (CYP2B6.8) impairs formation of a functional cytochrome p450-reductase complex. J Pharmacol Exp Ther **338:**803-809.

Zhu M, Kaul S, Nandy P, Grasela DM, and Pfister M (2009) Model-based approach to characterize efavirenz autoinduction and concurrent enzyme induction with carbamazepine. Antimicrob Agents Chemother **53:**2346-2353.

Zukunft J, Lang T, Richter T, Hirsch-Ernst KI, Nussler AK, Klein K, Schwab M, Eichelbaum M, and Zanger UM (2005) A natural CYP2B6 TATA box polymorphism (-82T--> C) leading to enhanced transcription and relocation of the transcriptional start site. Mol Pharmacol **67:**1772-1782.

CURRICULUM VITAE

Cong Xu

EDUCATION

- **Ph.D. Clinical Pharmacology** 08/2013
  Division of Clinical Pharmacology, Indiana University, Indianapolis, IN
  Dissertation: Mechanisms and Quantitative Prediction of Efavirenz Metabolism, Pharmacogenetics and Drug Interactions
  Mentor: Zeruesenay Desta, Ph.D.

- **M.D.** 08/2003-06/2008
  Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

SKILLS AND TECHNIQUES

- Extensive experience in various disciplines of preclinical and clinical research, including drug metabolism, enzymology, drug-drug interactions, pharmacokinetics (PK), pharmacodynamics (PD), pharmacometrics and pharmacogenetics
- Clinical study: Phase I PK/PD and drug-drug interaction study design, execution, data management and data analysis
- Preclinical study: *in vitro* drug metabolism and drug-drug interaction studies
- Modeling & simulation software: expert in Simcyp; proficient with NONMEM and R; hands-on experience with WinNonlin, SigmaPlot and GraphPad Prism
- Therapeutic areas: infectious disease, oncology and CNS
- Bioanalytical methods: HPLC and LC/MS/MS

WORK EXPERIENCE

Pfizer Inc., Clinical Pharmacology, Primary Care Business Unit, New York, NY
Summer intern with Dr. Ellen Q. Wang and Dr. Piet Van Der Graaf, 06/2012-08/2012

- Developed a population pharmacokinetic model using NONMEM and R
- Performed covariate modeling using full model estimation approach

Indiana University School of Medicine, Division of Clinical Pharmacology, Indianapolis, IN **Research assistant** with Dr. Zeruesenay Desta, 2009-present

- Initiated a collaborative project with Drug Disposition Laboratories of Eli Lilly & Co., developed and validated a CYP2B6 pharmacogenetics-based PBPK model of efavirenz using Simcyp
- Developed a genotype-based pharmacokinetic model for efavirenz autoinduction using NONMEM and R

- Conducted non-compartmental analysis of efavirenz single-dose pharmacokinetics from a phase I trial (n=20) using WinNonlin
- Determined the effects of the *CYP2B6*6* allele on efavirenz-induced central nervous system (CNS) side effects in healthy subjects (n=58)
- Designed and conducted experiments to determine the effects of the *CYP2B6*6* allele on catalytic properties and inhibition susceptibility of CYP2B6 *in vitro*; and to study inhibition drug interactions of several small molecules *in vitro*
- Analyzed enzyme kinetics and inhibition drug-drug interaction data using SigmaPlot and GraphPad Prism

CLINICAL EXPERIENCE

The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China
Clerkship, 05/2007-06/2008

- Rotated through the departments of internal medicine, surgery, obstetrics & gynecology, pediatrics and infectious disease

PUBLICATIONS

- **Xu C**, et al. CYP2B6 Pharmacogenetics-based in vitro-in vivo Extrapolation (IVIVE) of Efavirenz Clearance by PBPK Modeling. Drug Metab Dispos. (*Accepted*)

- **Xu C**, Desta Z. *In vitro* Analysis and Quantitative Prediction of Efavirenz Inhibition of Eight Cytochrome P450 (CYP) Enzymes: Major Effects on CYPs 2B6, 2C8, 2C9 and 2C19. Drug Metab and Pharmacokinet. 2013, Feb 5 [Epub ahead of print].

- **Xu C**, et al. Effects of the *CYP2B6*6* Allele on Catalytic Properties and Inhibition of CYP2B6 in vitro: Implication for the Mechanism of Reduced Efavirenz Metabolism and Other CYP2B6 Substrates *in vivo*. Drug Metab and Dispos. 2012, 40(4): 717-725.

- Desta Z, Moaddel R, Ogburn ET, **Xu C**, et al. Stereoselective and Regiospecific Hydroxylation of Ketamine and Norketamine. Xenobiotica. 2012, 42(11): 1076-1087.

- Lu WJ, **Xu C**, et al. The Tamoxifen Metabolite Norendoxifen is a Potent and Selective Inhibitor of Aromatase (CYP19) and a Potential Lead Compound of Novel Therapeutic Agents. Breast Cancer Res Treat. 2012, 133(1): 99-109.

- Lu WJ, Ferlito V, **Xu C**, et al. Enantiomers of Naringenin as Pleiotropic, Stereoselective Inhibitors of Cytochrome P450 Isoforms. Chirality. 2011, 23(10): 891-896.

- Ogburn ET, Jones DR, Masters AR, **Xu C**, et al. Efavirenz Primary and Secondary Metabolism *in vitro* and *in vivo*: Identification of Novel Metabolic Pathways and Cytochrome P450 2A6 as the Principal Catalyst of Efavirenz 7-Hydroxylation. Drug Metab Dispos. 2010, 38(7): 1218-1229.

MEETING ABSTRACTS

- Mechanism of Reduced Efavirenz Metabolism by the *CYP2B6*6* Allele *in vitro* and Genotype-based Prediction of Efavirenz Single-dose Pharmacokinetics by Physiologically-based Pharmacokinetic Modeling. 2012 AAPS Annual Meeting and Exposition. Chicago, IL.

- Genotype-Based *in vitro-in vivo* Extrapolation (IVIVE) of Efavirenz Pharmacokinetics Using a Physiologically-Based Pharmacokinetic Model. 2012 Annual Meeting of ASCPT. National Harbor, MD.

- Inhibitory Effect of Efavirenz on CYP2C8-Catalyzed Amodiaquine N-desethylation *in vitro*. 2011 Annual Meeting of ASCPT. Dallas, TX.

HONORS AND AWARDS

- Global Health Focus Group Travel Award of AAPS Annual Meeting — 2012
- Indiana University Biomedical Gateway Fellowship — 2008
- Sun Yat-sen University Outstanding Graduate Award (top 5%) — 2006
- First Prize of Sun Yat-sen University Scholarship — 2006
- Second Prize of Sun Yat-sen University Scholarship — 2004, 2005, 2007