CYP2B6 genotype-dependent inhibition of CYP1A2 and induction of CYP2A6 by the antiretroviral drug efavirenz in healthy volunteers

Running title: Efavirenz alters *in vivo* CYP1A2 and CYP2A6

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Abstract

We investigated the effect of efavirenz on the activities of CYP1A2, CYP2A6, xanthine oxidase (XO) and N-acetyltransferase 2 (NAT2), using caffeine as a probe. A single 150 mg oral dose of caffeine was administered to healthy volunteers (n = 58) on two separate occasions; with a single 600 mg oral dose of efavirenz; and after treatment with 600 mg/day efavirenz for 17 days. Caffeine and its metabolites in plasma and urine were quantified using liquid chromatography/tandem-mass spectrometry. DNA was genotyped for *CYP2B6*4 (785A>G), CYP2B6*9 (516G>T)*, and *CYP2B6*18 (983T>C)* alleles using TaqMan assays. Relative to single-dose efavirenz treatment, multiple-doses of efavirenz decreased CYP1A2 (by 38%) and increased CYP2A6 (by 85%) activities ($P < 0.05$); XO and NAT2 activities were unaffected. *CYP2B6*6*6* genotype was associated with lower CYP1A2 activity following both single and multiple doses of efavirenz. No similar association was noted for CYP2A6 activity. This is the first report showing that efavirenz reduces

hepatic CYP1A2 and suggesting chronic efavirenz exposure likely enhances the elimination of CYP2A6 substrates. This is also the first to report the extent of efavirenz-CYP1A2 interaction may be efavirenz exposure- and *CYP2B6* genotype-dependent.

Introduction

The non-nucleoside HIV-1 reverse transcriptase inhibitor efavirenz, in combination with two nucleoside/nucleotide reverse transcriptase inhibitors, has been the preferred first-line regimen for over 15 years for treatment naive HIV infected adults (1) and children (2). Although regimens containing efavirenz are considered alternative therapy since 2015 due to the increased effectiveness of newer agents such as integrase inhibitors (3), the World Health Organization continues to recommend efavirenz-based therapies as cost-effective and convenient first-line therapy in resource-limited countries with pandemic HIV (4). However, the use of efavirenz is associated with numerous and often unpredictable pharmacokinetic drug-drug interactions (DDIs) (5). These DDIs are of major clinical concerns as they potentially increase the risk for loss of efficacy or adverse effects of those drugs co-administered with efavirenz. Thus, understanding the mechanisms of these DDIs is crucial to predict and manage them.

Most efavirenz mediated DDIs can be explained by efavirenz's ability to induce drug disposition genes via activation of the constitutive androstane receptor (CAR) (6) and pregnane X receptor (PXR) (7), nuclear receptors that regulate the transcription of drug-metabolizing enzymes and transporters (8). As a result, chronic administration of efavirenz increases its own elimination ("autoinduction" of metabolism) (9),(6) and those of others through induction of drug metabolizing

enzymes including CYP2B6, CYP2C19 and CYP3A (10),(11),(12). However, not all DDIs with efavirenz are due to induction of drug disposition genes. Our *in vitro* data show that efavirenz directly inhibits certain enzymes at therapeutically relevant concentrations (e.g., CYP2B6, CYP2C8, CYP2C19 and CYP2C9) (13), with reduction in the elimination of CYP2C8 and CYP2C9 substrates *in vivo* (14),(15) and efavirenz being a mixed inhibitor/inducer of CYP2B6(16) and CYP2C19(11). The potential *in vivo* effect of efavirenz on the activity of other drug metabolizing enzymes, such as CYP1A2, CYP2A6, xanthine oxidase (XO) and N-acetyltransferase 2 (NAT 2), is not fully investigated. In HIV/TB-co-infected patients, model predicted exposure of isoniazid exposure was marginally reduced by efavirenz (by 29%) in those who are fast acetylators, but no effect was observed in slow acetylators (17). However, these patients were receiving multiple medications, making it difficult to attribute the observed effect to efavirenz alone. Using human liver microsomal (HLMs) preparations and/or expressed enzymes, we have shown that efavirenz does not inhibit/inactivate the activities of CYP1A2, CYP2A6 (13). While HLMs are important *in vitro* experimental tools to estimate the estimation and prediction of inhibition DDI potential of a drug candidate, this approach would not be appropriate to test the effect of efavirenz on gene expression (e.g., due to enzyme induction). Appropriate *in vitro* models (e.g., cell lines or human hepatocytes) or *in vivo* studies using probe substrates are required to exclude the potential effect of efavirenz on CYP1A2 and CYP2A6.

Efavirenz is mainly metabolized by hepatic CYP2B6 (18). Two gene variants (*CYP2B6*6* and *CYP2B6*18*) produce higher efavirenz exposure after standard dosing in HIV patients (19). The extent of efavirenz autoinduction of metabolism is influenced by *CYP2B6* genetic variants (20). Recent evidence indicate that the

extent of efavirenz-mediated DDIs with such CYP3A substrates as lumefantrine (21) and levonogestrel (22) also depends on the variability in *CYP2B6* gene expression, suggesting that variable efavirenz exposure to functionally relevant *CYP2B6* genetic variants and other factors may be important determinant of the magnitude of DDIs.

The main objectives of this study were to test whether (1) efavirenz alters the activity of CYP1A2 in healthy volunteers and (2) the magnitude of the alteration correlated with genetic variation in *CYP2B6* expression. Caffeine is extensively metabolized in humans with at least 17 metabolites identified in the urine following caffeine administration (23),(24),(25). Approximately 80 to 90% of the total caffeine elimination in humans is mediated via hepatic CYP1A2-mediated demethylation to paraxanthine (26),(25) and caffeine is a well-established and validated probe drug for CYP1A2. The secondary objective of this study is to explore the effect of efavirenz on the activities of CYP2A6, xanthine oxidase (XO) and N-acetyltransferase 2 (NAT2). CYP2A6, XO and NAT2 are involved in the formation of caffeine metabolites (24),(25). Although these enzymes catalyze minor metabolic pathways which make difficult to validate against oral clearance of caffeine, urinary metabolic ratios of caffeine have been widely used as convenient markers of CYP1A2, CYP2A6, XO and NAT2 activities in population, epidemiological and drug-drug interactions (27),(24),(25). Activities of hepatic CYP1A2, CYP2A6, XO and NAT2 were profiled in healthy volunteers using plasma and urinary caffeine and metabolites as an *in vivo* probes following the administration of caffeine on two occasions: with a single 600 mg oral dose of efavirenz and after chronic efavirenz (600 mg/day) treatment for 17 days.

MATERIALS AND METHODS

Study subjects. Healthy male and female volunteers (age, 18 to 49 years old; and BMI, 20-32 kg/m^2) were recruited to participate in this study. Potential subjects underwent a pre-enrollment screening examination (within a maximum of six weeks prior to enrollment into the study) for any medical abnormalities, which included physical examination, medical history and standard clinical laboratory tests. Details of inclusion and exclusion criteria are described in our previous publications (11),(28),(29),(30). During the screening, a blood sample (~10 ml) was obtained from each subject to extract genomic DNA for genotyping purposes. The Indiana University (IU) Institutional Review Board approved the protocol and the study was conducted at the IU Clinical Research Center. All participants gave written informed consent. The study was registered at http://www.clinicaltrials.gov (ClinicalTrials.gov number NCT00668395).

Study design. This study used a two-session sequential design to characterize efavirenz autoinduction, pharmacogenetics and drug interactions (Figure S1). To determine efavirenz-mediated changes in hepatic drug metabolizing enzyme activity, volunteers received a cocktail probe drugs orally with water on two occasions: concomitantly with a single 600 mg oral dose of efavirenz and again at the end of 17-day treatment with 600 mg efavirenz daily. On day 1, the participants were admitted to the hospital ~7 AM, after an overnight fast. Following a predose blood sample, each participant received a single 600-mg oral dose of efavirenz (Sustiva®, Bristol-Myers Squibb, Princeton, NJ) with 240 mL water on an empty stomach. One hour later, a cocktail probe drugs was administered orally with water to assess potential drug interactions: 150 mg caffeine, 250 mg tolbutamide, 20 mg omeprazole, and 1

mg of midazolam syrup. Although high doses of omeprazole can induce CYP1A2 after chronic administration, the small single dose (20 mg) used in this study is not expected to alter CYP1A2 activity. In fact, there is no mutual pharmacokinetic interaction of caffeine and omeprazole (or among the other components of the cocktail used here) and these probe drugs (including caffeine and omeprazole) have been validated for simultaneous use without fear of interaction and thus widely used in several CYP phenotyping cocktails. Venous blood samples (10 mL) were collected from an indwelling catheter 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16 and 24 hours following efavirenz administration while participants were in the hospital and at 48, 72 and 144 hours as outpatients. All urine voided during the 24 hour after efavirenz administration was collected in fractions. Plasma was separated by centrifugation for 20 min at 3,000 rpm within an hour of collection and two 10-ml urine aliquots were saved from each time point after the total urine volume is recorded. Plasma and urine samples were immediately stored at –80^{o}C until analysis. The volunteers began chronic oral efavirenz (600 mg/day) in the evening, from day 7 to day 23. On the morning of day 24, participants were again admitted to the hospital. After pre-dose blood collection, participants received their final dose of oral efavirenz (600 mg) and one hour later, a cocktail probe drugs was administered as in day 1. All other procedures and sample processing were identical to day 1. We present here the data that relate to efavirenz interaction with caffeine metabolism as marker of CYP1A2, CYP2A6, NAT2 and XO.

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DNA genotyping.

The *CYP2B6* gene is highly polymorphic, with over 38 alleles reported to date (https://www.pharmvar.org/gene/CYP2B6). The *CY2B6*6* allele, which consists of the *CYP2B6*4* and *CYP2B6*9* variants on the same haplotype, has functional relevance and occurs at high frequency across ethnically and geographically diverse populations (19). In addition, the *CYP2B6*18* allele which does not express functional protein is frequent in black population (4-12%) (19). Briefly, genomic DNA was extracted from whole blood (QIAamp DNA Mini Kit, Qiagen, Valencia, CA). Polymerase chain reaction (PCR) was performed on the Quant Studio 12K Flex real-time PCR instruments blocks for 96-well plates. CYP2B6*9, rs3745274 (516G>T, Q172H); CYP2B6*4, rs2279343 (785A>G, K262R); and CYP2B6*18, rs28399499 (983T>C, I328T) were genotyped using TaqMan Assay-Reagent Allelic Discrimination Kits (Applied Biosystems, Foster City, CA) according to the supplier's instructions. Genotype groups considered for the analysis were normal metabolizer (**1/*1* genotype, n=36), intermediate metabolizer (**1/*6* genotype, n=15) and slow metabolizer (**6/*6** genotype, n=5 and **1/*18* genotype, n=1). The additional variants in the *CYP2B6* gene and other *CYP* variants that were genotyped in these samples have been previously published (28),(30).

Measurement of drugs and metabolites in plasma and urine. “Plasma samples from single and multiple doses were assayed in the same run. Similarly, urine samples from single and multiple doses were run together. Plasma and urine samples were run separately as the number of analytes tested and the methods used were slightly different”.

Chemicals. Caffeine (1,3,7 TMX), 1-methylxanthine (1 MX), 1-methyluric acid (1 MU), 1,7-dimethyluric acid (1,7 DMU), 5-acetylamino-6-formylamino-3-methyluracil (AFMU), and 1,7-dimethylxanthine (1,7 DMX) or paraxanthine and theobromine and theophylline were purchased from Sigma-Aldrich (St. Louis, MO). Efavirenz, 8-hydroxyefavirenz, 8,14-dihydroxyefavirenz, 7-hydroxyefavirenz, and β-glucuronidase were purchased from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals and solvents were HPLC grade or the highest quality commercially available.

Plasma extraction and quantification of efavirenz, caffeine and their respective metabolites. Plasma (250 µL) was mixed with 250 µl of 0.2M sodium acetate (pH=5), 12.5µL of 600mM sodium azide and 25µL of β-glucuronidase (1000U/mL) and incubated at 37°C for 18 hours to hydrolyze circulating glucuronide conjugates of efavirenz. Efavirenz-d4 (30µL of 1µg/mL) and 1 µg/ml acetaminophen were added as internal standards and the sample vortex mixed. Hydrochloric acid (250µL of 0.1M) and 6 mL of ethyl acetate/hexane (50/50, v/v) was added and the sample mixture shaken for 10 min and then centrifuged at 3600 rpm for 10min at 0°C (Beckman Coulter, Schaumburg, IL). The organic layer was evaporated to dryness and reconstituted in 120 µL of 50% mobile phase A and 50% mobile phase B. Aliquot (25 µL) was injected onto the high performance liquid chromatography/tandem-mass spectrometry (HPLC-MS/MS) system.

Caffeine and its metabolites were quantified on an API 3200 triple-quadrupole mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA) equipped with a turbo ion spray source. The HPLC system consist of two LC-20AD pumps, SIL-20AHT UFLC auto-sampler, DGU-20A3 degasser and a CBM-20A controller (Shimadzu, Columbia, MD). The chromatographic separation was achieved using a Restek C8

(250X4.6mm, 5µm particle size) column (Bellefonte, PA). Before and after each injection, the sampling needle was washed with acetonitrile/water (75%/25%, v/v). Mobile phase A [methanol : formic acid (0.1% in water; 1/99, v/v)] and mobile phase B [methanol : formic acid (0.1% in water; 99/1, v/v)] was pumped at 0.8 ml/min using a gradient elution described elsewhere (30). MS optimization was achieved via adjustment of both the compound- and instrument-dependent parameters for caffeine, theophylline, paraxanthine and acetaminophen (internal standard) in positive mode. The analytes were optimized at a source temperature of 400°C in positive mode under unit resolution for quadrupole 1 and 3, and were given a dwell time of 60 ms and a setting time of 700 ms. Gas pressures for all analytes, including the internal standards were optimized as described previously (30). Multiple Reaction Monitoring (MRM) at *m/z* of 195.12/138.12, 181.13/124.20, 181.13/124.2, and 152.13/110.20 for caffeine, theophylline, paraxanthine, and acetaminophen (internal standard), respectively, in positive mode was used. Theophylline and paraxanthine were chromatographically separated. Data acquisition and processing were performed using Analyst® software. The lower limit of quantification was 1 ng/ml for all analytes and was linear over a wide range of therapeutically relevant concentrations. The intra-day and inter-day variabilities were less than 20%.

Efavirenz, 8-hydroxyefavirenz, 7-hydroxyefavirenz and 8,14-dihydroxyefavirenz were quantified by LC/MS/MS in negative mode using efavirenz-d4 as an internal standard as detailed in our previous publication (30). MRMat *m/z* of 313.97/244.01, 329.98/210.0, 329.98/257.89, 345.91/262.0, and 318.01/247.95 was used to measure first and third quadrupole (Q1/Q3) transitions for efavirenz, 8-hydroxyefavirenz, 7-hydroxyefavirenz, 8,14-dihydroxyefavirenz, and efavirenz-d4

(internal standard), respectively, in negative mode. Metabolites (8-and 7-hydroxyefavirenz) were separated chromatographically.

Urine extraction and quantification. Urine (250 µL) was spiked with 30µL of internal standard (nevirapine, 500ng/mL). Each sample was extracted by adding 300 µL of acetic acid (0.1% in water) and 6mL of dichloromethane: isopropyl alcohol (85:15), vortex-mix for 20s, centrifuged at 3600 rpm for 5 minutes at 10°C. The aqueous layer was decanted by suction and the organic layer evaporated using speed vacuum. Residue was reconstituted in 100 µL of 99% ammonium acetate (20mM, pH = 4.8)/1% methanol and vortex-mix for 15 seconds. An aliquot (50µL) was injected onto LC/MS/MS system described above for plasma. Analytes were separated on a Zorbax Eclipse XDB-C8 column (4.6 x 150mm, 3.5µM, Agilent Technologies, Santa Clara, CA). The gradient mobile phase was pumped at a flow rate of 0.5 mL/min and consist of phase A [99% ammonium acetate (20mM, pH=3.5)/1% methanol (v/v)] and phase B [10% ammonium acetate (20mM, pH=3.5)/90% methanol (v/v)]. MRM at *m/z* 195.12/138.12 for caffeine, 181.13/124.2 for theophylline, 181.13/124.2 for paraxanthine, 181/138 for theobromine, 166/149 for 1-methylxanthine, 183/105 1-methyluric acid, 195/180 for 1,7-dimethyluric acid, 225/197 for 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 152.1/110.2 for acetaminophen (internal standard) was used to measure first and third quadrupole (Q1/Q3) transitions, in positive mode. Theophylline, paraxanthine and theobromine peaks were separated chromatographically.

Pharmacokinetic analyses:

Pharmacokinetic parameters were estimated using WinNonlin software (Version 5.01; Pharsight, Mountain View, CA). Area under the plasma concentration vs. time curve (AUC) from time zero to the time of the last quantifiable concentration (AUC_{0-t}) was estimated using the linear and logarithmic trapezoidal rule for the respective up and down portions of the curve. The elimination rate constant (λ_z) was determined by linear regression analysis of the terminal portion of the log concentration *vs*. time curve. The AUC ($AUC_{0-\infty}$) from zero to infinity was calculated as the sum of C_t/ λ_z and AUC_{0-t}, where C_t was the last measured concentration. The terminal elimination half-life ($t_{1/2}$) was calculated as the quotient of 0.693 and λ_z. The maximum plasma concentration (C_{max}) and the time to C_{max} (t_{max}) were determined by visual inspection of the respective individual concentration-time curves. Total clearance (CL/F) was calculated as the quotient of the administered dose and $AUC_{0-\infty}$.

Calculation of metabolic ratios:

Systemic clearance, caffeine AUC and/or plasma paraxanthine to caffeine metabolic ratios (MRs) 5–7 h after administration of caffeine are preferred (or accepted) and validated *in vivo* metrics of CYP1A2 activity (26). In addition, urinary metabolic ratios are useful markers of CYP1A2, CYP2A6, XO and NAT2 activities. Therefore, the following plasma and urinary caffeine MRs were calculated as indices of the respective enzyme activity besides estimation of pharmacokinetic parameters of caffeine.

Plasma MRs:

CYP1A2 activity = Paraxanthine concentrations (**C**5 hour + **C**7 hour)

Caffeine concentrations (**C**5 hour + **C**7 hour)

Where **C** is plasma concentrations at 5 and 7 hours post caffeine dosing.

Urinary MRs:

CYP1A2 Activity = (AFMU+ 1 MU + 1 MX)/ 1,7 DMU

CYP2A6 Activity = 1,7 DMU/1,7 DMX

XO Activity = (1 MU)/ (1 MX+1 MU)

NAT2 Activity = AFMU/ (AFMU+1 MU+1 MX).

Abbreviations: *1 MX = 1-methylxanthine; 1 MU = 1-methyluric acid; 1,7 DMU = 1,7-dimethyluric acid; AFMU = 5-acetylamino-6-formylamino-3-methyluracil; and 1,7 DMX = 1,7-dimethylxanthine (paraxanthine).*

Statistical analyses:

Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA, USA). Comparison of pharmacokinetic parameters or enzyme activity between single and multiple doses of efavirenz was performed using the Wilcoxon-matched paired test. For testing the association between *CYP2B6* genotype and the activities of CYP1A2 and CYP2A6 following single and multiple doses of efavirenz were evaluated by nonparametric analysis of variance (Kruskall-Wallis test) using Dunn's post-test for multiple comparison correction. Correlation analyses between efavirenz exposure and enzyme activities

(CYP1A2 and CYP2A6) were determined from the Spearman's Rank-Order Correlation (rs). Differences were judged to be due to chronic efavirenz treatment rather than single dose efavirenz administration when $P < 0.05$.

A sample size of at least 52 subjects was calculated as sufficient to provide a 95% power to detect a 40% difference in CYP1A2 activity between a single efavirenz dose versus multiple doses of efavirenz at a two-sided alpha level of 5%. Power calculation for efavirenz's effect on CYP2A6, NAT2 and XO was not performed *apriori*. However, data from a relatively large sample size were analyzed in this study as compared to conventional drug-drug interactions reported in the literature, which often involves sample size less than 15 subjects.

RESULTS

Human Volunteers

Sixty healthy normal volunteers were recruited and completed the entire study protocol. Two participants were excluded due to missing samples from one of the efavirenz treatment phases. Caffeine metabolic ratios were calculated from 58 individuals. Plasma pharmacokinetic parameters were available from only 52 volunteers after documentation of exogenous caffeine intake by six individuals during the study; this was an exclusion criteria.

The demographics of the 58 participants completing the study were: 36 male and 22 female; median age of 28.5 years (range 18 to 50 years); body weight of 74.6 $\pm$ 13.7 kg; and BMI of 24.6 $\pm$ 3.8 kg/m^2. Based on self-identification, 74 % were white/Caucasian and 19% were black/African-American. The remaining ~7% belonged to other ethnic/racial groups.

The pharmacokinetics of caffeine and metabolite data are shown in Table 1. The caffeine half-life ($t_{1/2}$) was prolonged ($P < 0.0001$) and $AUC_{0-\infty}$ increased ($P < 0.01$) following chronic efavirenz compared to a single dose of efavirenz. Accordingly, chronic administration of efavirenz decreased the C_{max} of paraxanthine ($P < 0.0001$), the major metabolite of caffeine. Relative to the single dose phase, chronic efavirenz treatment decreased CYP1A2 activity (plasma MRs) by 38 percent (Table 1 and Figure 1A, $P < 0.0001$) with marked interindividual variability (range: -78.2% to 31.7%) (Figure 1B). Similarly, CYP1A2 activity measured by urinary MRs decreased 28.9% ($P < 0.0001$) from 5.7 to 3.6 after chronic treatment with efavirenz (Table 1; Figure 1C). The percent change in urine MR also varied widely among individuals (ranging from -65.1% to 43.2%) (Figure 1D). CYP1A2 activity measured by the plasma MRs correlated with CYP1A2 activity measured by urinary MRs in the single dose efavirenz phase (Spearman rs = 0.57; P < 0.0001) and after chronic efavirenz (r = 0.33, $P = 0.013$) and combination of both phases (single dose and after multiple doses of efavirenz) (rs = 0.57; P < 0.0001) [Figure S2 (A-C)]. Plasma caffeine MRs are the preferred phenotype marker for CYP1A2 rather than urinary MRs (27). Thus, only results from plasma MRs are presented as markers of CYP1A2 subsequently.

CYP2A6, XO and NAT-2 activities were estimated from respective caffeine MRs as described in the Methods section. Chronic efavirenz treatment significantly increased CYP2A6 activity (Table 1; Figure 2A) with marked inter-subject variability (range: 45.4 to 281.4%) (Figure 2B). Urinary metabolic ratios reflecting XO and NAT2 activities were not significantly altered by chronic efavirenz exposure (Table 1).

Approximately 7-fold between subject differences in efavirenz exposure was observed following a single dose and multiple doses of efavirenz. To test whether changes in CYP1A2 and CYP2A6 activities were dependent on the exposure of efavirenz and its metabolites, correlations between efavirenz exposure and indices of CYP1A2 and CYP2A6 were examined. Greater reduction in CYP1A2 activity was observed with higher efavirenz exposure as shown by the negatively significant correlation between: CYP1A2 activity and efavirenz $AUC_{0-\infty}$ at single dose (Pearson r_s = - 0.32; P < 0.01) as well as efavirenz AUC_{0-24h} after multiple doses (r_s = -0.61, P < 0.001). The percent change in CYP1A2 was negatively correlated with efavirenz AUC_{0-24h} after multiple doses (r_s = -0.41, P < 0.01) (Figure S3). Efavirenz exposure at single dose or at steady state did not significantly correlate with urine marker of CYP2A6 activity (Figure S4). Exposure of efavirenz metabolites did not correlate with CYP1A2 or CYP2A6 activity.

The correlation analysis suggest that the extent of reduction of CYP1A2 activity dependent on efavirenz exposure (the higher the efavirenz exposure the greater reduction in CYP1A2). Genotype-phenotype data from the same subjects analyzed in this study and published elsewhere (30),(29) show that efavirenz exposure following: a single dose of efavirenz ($AUC_{0-\infty}$) was 2.1- and 1.8-fold higher; and multiple doses of efavirenz (AUC_{0-24}) was 2.5- and 2-fold higher in *CYP2B6*6/*6* genotypes compared to *CYP2B6*1/*1* and *CYP2B6*1/*6* genotypes, respectively. Difference in efavirenz exposure between *1/*1 and *1/*6, although statistically different, was small. To test whether the extent of efavirenz-CYP1A2 interaction is dependent on *CYP2B6* genotypes, data analyses was performed after stratifying by *CYP2B6*1/*1, CYP2B6*1/*6* and *CYP2B6*6/*6 genotypes.* One subject with *1/*18 genotype was grouped with *6/*6 as the genotype-predicted phenotype was close to

slow metabolizer. The demographics are comparable among the three genotypes (Table S1). CYP1A2 activity was lower for the CYP2B6*6/*6 genotypes compared to *1/*1 or *1/*6 genotypes following single dose (Kruskal-Wallis test, $P < 0.05$; Figure 3A) and multiple doses of efavirenz ($P < 0.01$; Figure 3B). Compared to the single dose phase, chronic efavirenz inhibited CYP1A2 activity within each genotype ($P < 0.05$, (Figure 3C). The percent change in CYP1A2 activity was greater in *6/*6 genotypes when compared with *1/*1 and *1/*6 genotypes (Kruskal-Wallis test, $P < 0.05$) (Figure 3D). Similarly, caffeine $AUC_{0-\infty}$ was significantly higher for *6/*6 genotype in both efavirenz treatment groups compared to *1/*1 genotype [Figure S5 (A-B), while caffeine $AUC_{0-\infty}$ and CYP1A2 for the *1/*6 genotype were not different from the wild type *1/*1 genotype in either treatment group.

The impact of *CYP2B6* genotypes on the extent of efavirenz-mediated induction of CYP2A6 was also tested. CYP2A6 activity was not statistically different among the *CYP2B6* genotypes either at single dose of efavirenz (Figure 4A) or after multiple doses of efavirenz (Figure 4B). Compared to the single dose phase, chronic efavirenz increased the urinary caffeine metabolic ratio for CYP2A6 in *1/*1 and *1/*6 genotypes, but not in **6/*6* genotype (Figure 4C). Although percent change in CYP2A6 activity was lower in **6/*6* genotypes when compared with *1/*1 and *1/*6 genotypes, this did not reach a statistically significant level (Figure 4D).

DISCUSSION

In the present study, chronic administration of efavirenz significantly reduced and increased the *in vivo* activity of hepatic CYP1A2 and CYP2A6, respectively. The effect of efavirenz on CYP1A2 activity was more pronounced in individuals with higher efavirenz exposure and those expressing *CYP2B6*6*6* genotype, while the . increase in CYP2A6 activity was neither efavirenz exposure- nor *CYP2B6* genotype-dependent. Efavirenz did not significantly altered the activity XO or NAT-2. These data suggest that there is an increased risk for drug-drug interactions in individuals receiving efavirenz-based HIV therapy who are co-medicated with CYP1A2 or CYP2A6 substrates. Moreover, *CYP2B6* genotypes associated with reduce efavirenz clearance may particularly alter the risk for adverse drug interactions involving CYP1A2.

Efavirenz activates two nuclear receptors that regulate a range of genes involved in drug disposition, namely the constitutive androstane receptor (CAR, *NR1I3*) and pregnane X receptor (PXR, *NR1I2*) (6), (7). Accordingly, efavirenz treatment enhances the elimination of numerous drugs metabolized by the cytochromes P450 [e.g., CYP3A, CYP2B6 and CYP2C19 (10),(11),(12)]. This together with the fact that CYP2A6 is also transcriptionally regulated via PXR (31) and CAR (32) may explain our observed increase in CYP2A6 activity following efavirenz treatment. CYP2A6 catalyzes fully or partially the metabolism of several clinically used drugs (e.g., letrozole, metronidazole, efavirenz and tegafur)) (33) and it is anticipated that efavirenz would alter the disposition of these drugs. CYP2A6 is also the main metabolic pathway for nicotine elimination and contributes to the biotransformation of several nicotine-derived carcinogens (N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)(33)). Hepatic CYP2A6

activity has been associated with number of cigarettes smoked per day, smoking topography (puff volume, duration, and velocity) and smoking cessation (33). Thus, it is conceivable that cigarette smokers would be at increased risk for tobacco-related illnesses when co-prescribed efavirenz-based HIV therapy. Our data show that neither efavirenz exposure nor the presence of the CYP2B6 polymorphisms altered the magnitude of the increase in CYP2A6 activity suggesting that maximum nuclear receptor activation occurs at relatively low efavirenz concentrations.

In contrast to the induction effects of efavirenz on gene expression, drug interactions also occur as a result of direct enzymatic inhibition by efavirenz (15),(34). Our findings clearly indicate that chronic doses of efavirenz significantly reduced CYP1A2 activity *in vivo* compared to CYP1A2 activity determined when caffeine was administered with a single dose of efavirenz. The mechanism by which efavirenz reduces CYP1A2 activity remains unknown. *In vitro* studies indicate that efavirenz is neither a reversible inhibitor nor a mechanism-based (i.e., time-dependent) inactivator of of CYP1A2 activity(13). It is unlikely that efavirenz metabolites produce CYP1A2 inhibition because the major oxidative metabolite, 8-hydroxyefavirenz, had no effect on CYP1A2 activity *in vitro*(13). Efavirenz hydroxylated metabolites undergo extensive conjugation mainly by glucuronosyltransferases. With few exceptions [e.g., glucuronides of gemfibrozil(35) and clopidogrel)(36)], glucuronide conjugates are relatively hydrophilic and do not appear to produce clinically significant drug interactions. The possibility that efavirenz reduces CYP1A2 activity *in vivo* via mechanisms other than direct enzymatic inhibition/inactivation cannot be excluded. However, a limitation of these findings is that the study design in which probe substrates including caffeine were administered on two occasions (with single dose of efavirenz and after multiple

doses of efavirenz) without inclusion of caffeine alone arm (absence of efavirenz) to determine baseline CYP1A2 did not allow accurate determination of the true magnitude of efavirenz-CYP1A2 interaction.. Initially, the study was designed to address potential induction of drug metabolism by efavirenz.

Of note, the CYP1A2 probe, caffeine, has low intrinsic clearance with no significant pre-systemic metabolism. The magnitude of the efavirenz-CYP1A2 interaction reported here is likely an underestimate for any CYP1A2 drugs known to exhibit significant pre-systemic (i.e., first-pass) metabolism and low bioavailability (e.g., tizanidine) (37). Thus, efavirenz co-administration with CYP1A2 substrates possessing narrow therapeutic ranges (e.g., clozapine, olanzapine, duloxetine, remolten and tizanidine), would likely produce clinically significant drug interactions. For example, ciprofloxacin and fluvoxamine (potent inhibitors of CYP1A2) increase the plasma exposure of tizanidine up to 30-fold, concomitant increased toxicity (37), (38). Tizanidine is a α_2-adrenergic agonist approved for therapy of muscle spasm and other conditions. It is frequently prescribed, off-label, for suppression of the symptoms associated with opiate withdrawal during detoxification (39),(40). Because some individuals with HIV infection may also suffer from and be treated for opioid abuse, the likelihood that tizanidine and efavirenz may be co-administered is high.

Drug interactions with efavirenz are difficult to predict because of the marked interindividual variability in its plasma exposure (41). Efavirenz is mainly eliminated by metabolism via CYP2B6, with minor contributions from CYP2A6, CYP3A, CYP1A2, and UGT2B7 (18),(42),(43). Patients with variants in CYP2B6 gene (e.g., CYP2B6*6 and CYP2B6*18) demonstrate higher efavirenz plasma exposure after administration of single (30) and multiple doses(19) of efavirenz. Consistent with the literature, genotype-phenotype data from the same subjects analyzed in this study

and published elsewhere (30),(29) show that efavirenz exposure is over 2-fold higher in *CYP2B6*6/*6* genotypes compared to *CYP2B6*1/*1 genotype.* Evidence exist that genetic variants in the *CYP2B6* gene that contributes to variable efavirenz exposure may dictates the magnitude of certain induction drug interactions caused by efavirenz. For example, efavirenz reduced lumefantrine concentrations in patients co-infected with HIV and malaria, resulting in poor malaria treatment response, and the decrease in lumefantrine concentrations was greater in those with higher efavirenz exposure and in CYP2B6 poor metabolizers (21). Efavirenz-based HIV therapy has been associated with lower levonorgestrel concentrations in women with the contraceptive subdermal implant (22), resulting in a risk for unintended pregnancy (44) particularly in CYP2B6 poor metabolizers. *CYP2B6* genotypes are also associated with differential efavirenz autoinduction of metabolism (20). However, this is the first report to suggest greater reduction of CYP1A2 in carriers of the *CYP2B6*6/*6* genotype compared to *1/*1 and *1/*6 genotypes. These data along with the correlation analysis showing efavirenz exposure-dependent reduction in CYP1A2 activity suggest that factors that increase efavirenz exposure would increase the risk for efavirenz-CYP1A2 interaction. The genotype-dependent effect may have particularly important clinical consequences in certain populations, e.g., this likely pose unique risks in African or African American populations as the allele frequencies of relevant CYP2B6 genotypes (e.g., *6 and *18) are higher in these populations compared to other ethnic and racial groups (45). Taken together, while the association of *CYP2B6*6/*6* genotype on the extent of efavirenz-CYP1A2 interaction appears plausible and anticipated, our sample size for the CYP2B6*6/*6 was small. Thus, a well designed (including caffeine alone arm) and more detailed

study in adequate sample size of *CYP2B6* genotype subgroups may be warranted to validate our findings and to assess the true extent of efavirenz-CYP1A2 interaction.

In summary, we have demonstrated that efavirenz is an inhibitor of CYP1A2 activity and an inducer of CYP2A6 activity and is likely to alter the disposition and efficacy of drugs metabolized by these enzymes. The results further suggest that genetic and non-genetic factors will increase the risk for efavirenz mediated-drug interactions. Finally, specific populations expressing higher frequencies of *CYP2B6* variants (e.g., Black and African American) may be at increased risk for efavirenz drug interactions. Concomitant use of CYP1A2 and CYP2A6 substrate drugs with efavirenz-based HIV therapy will require careful monitoring to minimize the risk of adverse drug effects.

STUDY HIGHLIGHTS

What Is the Current Knowledge on the Topic?

Efavirenz-based HIV therapy is associated with complex and often unpredictable drug-drug interactions, potentially compromising efficacy and toxicity of coadministered drugs. While efavirenz cause in vivo induction and/or inhibition of selected drug metabolizing enzymes (e.g., CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A), its effect on other enzymes such as CYP1A2 and CYP2A6 is unknown.

What Question Did This Study Address?

This work tested the effect of efavirenz on the in vivo hepatic activities of four enzymes: CYP1A2, CYP2A6, xanthine oxidase (XO) and N-acetyltransferase 2 (NAT2). The impact of efavirenz exposure and genetic variation in *CYP2B6* expression on the magnitude of interaction was also tested. The plasma and urinary

disposition of a single 150 mg oral dose of caffeine was profiled in healthy volunteers (N = 58) as *in vivo* probes following a single 600 mg oral dose of efavirenz and after chronic efavirenz treatment for 17 days.

What Does This Study Add To Our Knowledge?

This is the first clinical study showing that efavirenz reduces hepatic CYP1A2 and suggesting chronic efavirenz exposure likely enhances the elimination of CYP2A6 substrates. This is also the first to report the extent of efavirenz-CYP1A2 interaction is CYP2B6 genotype-dependent.

How Might This Change Clinical Pharmacology or Translational Science?

Concomitant use of CYP1A2 and CYP2A6 substrate drugs with efavirenz-based HIV therapy will require careful monitoring to minimize the risk of adverse drug effects. Moreover, CYP2B6 genotypes associated with increased efavirenz exposure may be particularly at increased risk for adverse drug interactions involving CYP1A2.

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Author Contributions:

I.F.M., N.D., R.E.G., and Z.D. wrote the article; Z.D. designed the research; I.F.M., Y.K., and J.B.L.L. performed the research; I.F.M., N.D., R.E.G., and Z.D. analyzed the data.

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Figure legends

Figure 1. Relative CYP1A2 activity following single dose (SD, open circles) and after multiple doses of efavirenz (MD, closed circles) in healthy volunteers (N=58). Plasma **(A)** and urine **(C)** caffeine metabolic ratios (MRs) were calculated as the surrogate for relative changes in CYP1A2 activity. Ranked percent change in CYP1A2 activity using MRs in plasma **(B)** and urine **(D)** of 58 subjects (multiple doses versus a single dose of efavirenz) are shown. CYP1A2 activity following single dose and after multiple doses of efavirenz was compared using Wilcoxon signed-rank test. ****P < 0.0001.

Figure 2. Relative CYP2A6 activity following single dose (SD, open circles) and after multiple doses of efavirenz (MD, closed circles) in healthy volunteers (N=58). (A), CYP2A6 activity was calculated from urinary caffeine metabolic ratios as described and **(B),** ranked percent change in CYP2A6 activity of 58 individuals (multiple doses versus a single dose of efavirenz). CYP2A6 activity following single dose and after multiple doses of efavirenz was compared using Wilcoxon signed-

rank test. ****P < 0.0001 (between CYP1A2 activity following SD and after MD of efavirenz).

Figure 3. Relative CYP1A2 activity stratified by *CYP2B6* genotypes following a single dose and multiple doses of efavirenz in health volunteers (N=58). Plasma metabolic ratio of paraxanthine to caffeine represents relative CYP1A2 activity. **(A)** CYP1A2 activity following a single dose of efavirenz. **(B)** CYP1A2 activity after chronic administration of efavirenz. **(C)** Comparison between single and multiple efavirenz dosing within each genotype group. **(D)** Represents percent change in CYP1A2 activity. CYP1A2 activity was compared between a SD and MDs of efavirenz in each genotype using Wilcoxon matched pairs test. Comparison among genotypes was tested using nonparametric ANOVA (Kruskal-Wallis test) with post-hoc Dunn's multiple comparison test. ****P<0.0001, ***P<0.001, **P<0.01, * P<0.05. NS, not statistically significant (P>0.05).

Figure 4. Relative CYP2A6 activity stratified by *CYP2B6* genotypes (1/*1,* n=37; **1/*6,* n=15; and **6/*6,* n=6) following a single dose (SD) and multiple doses of efavirenz (MD) in health volunteers (N=58). (A)** Relative CYP2A6 activity following a single dose of efavirenz. **(B)** Relative CYP2A6 activity after multiple doses of efavirenz. **(C)** Compares CYP2A6 activity between single and multiple dosing within each genotype group. **(D)** Represents percent change in CYP2A6 activity (MDs versus SD efavirenz). CYP2A6 activity was compared between a SD and MDs of efavirenz in each genotype using Wilcoxon matched pairs test. Comparison among genotypes was tested using nonparametric ANOVA (Kruskal-Wallis test) with post-hoc Dunn's multiple comparison test. **** $P < 0.0001$, *** $P < 0.001$. NS, $P > 0.05$.

Supplementary Materials

(Supplemental Tables and Figures)

Supplemental Tables and Figures

Table 1. Pharmacokinetic parameters of caffeine and its main metabolite paraxanthine, and activities of select enzymes calculated from plasma and urinary metabolic ratios (MRs), following co-administration of caffeine with a single dose and after multiple doses of efavirenz.

	Single Dose	Multiple Dose	Mean Percent Change
Caffeine (1,3,7 TMX)			
T_{max} (h)	1 (0.5 – 3)	1 (0.5 – 5)	
C_{max} (ng/mL)	1,825 ± 972	1,897 ± 1,050	17.4
$AUC_{0-\infty}$ (mcg/mL*h)	17.3 ± 11.6	22.7 ± 18.4**	46.2
$T_{1/2}$ (hr)	7.61 ± 8.29	8.87 ± 4.98***	32.5
Paraxanthine (1,7 MX)			
T_{max} (h)	7 (1.5 – 15)	7 (1 – 15)	
C_{max} (ng/mL)	504 ± 236	358 ± 208***	-24.5
$AUC_{0-\infty}$ (mcg/mL*h)	10.8 ± 6.1	8.6 ± 5.8*	-8.6
$T_{1/2}$ (hr)	10.85 ± 9.21	12.22 ± 7.88**	26.3
Relative Change in Enzyme Activity			
Plasma MR‡			
CYP1A2	0.52 ± 0.28	0.31 ± 0.18***	-38.1
Urinary MR‡			
CYP1A2	5.2 ± 2.4	3.5 ± 1.3***	-28.9
CYP2A6	0.58 ± 0.38	0.94 ± 0.59***	85.5
XO‡	0.37 ± 0.16	0.37 ± 0.16	1.1
NAT-2‡	0.31 ± 0.21	0.32 ± 0.22	4.5

Data are expressed as mean ± SD except T_{max} which was presented as median (minimum – maximum). ‡MR, metabolic ratio; XO, xanthine oxidase; and NAT-2, N-acetyltransferase 2

*$P < 0.05$, **P<0.01; ***P<0.0001, Wilcoxon-matched pairs test (single dose versus multiple doses of efavirenz).

T_{max}, time to maximum concentration; C_{max}, Maximum concentration; AUC, area under the plasma concentration-time curve; and $T_{1/2}$, terminal elimination half-life