**In Vitro Analysis and Quantitative Prediction of Efavirenz Inhibition of Eight Cytochrome P450 (CYP) Enzymes: Major Effects on CYPs 2B6, 2C8, 2C9 and 2C19**

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**Summary:** In order to quantitatively predict drug interactions associated with efavirenz-based anti-HIV therapy, we evaluated reversible and time-dependent inhibitions of efavirenz on eight cytochrome P450 (CYP) enzymes in vitro. The present study showed that efavirenz was a potent competitive inhibitor of CYP2B6 (average $K_i = 1.68 \mu M$ in HLMs and $K_i = 1.38 \mu M$ in expressed CYP2B6) and CYP2C8 ($K_i = 4.78 \mu M$ in pooled HLMs and $K_i = 4.80 \mu M$ in HLMs with CYP2C8*3/*3 genotype). Efavirenz was a moderate inhibitor of CYP2C9 ($K_i = 19.46 \mu M$) and CYP2C19 ($K_i = 21.31 \mu M$); and a weak inhibitor of CYP3A ($K_i = 40.33 \mu M$). No appreciable inhibition was observed on CYP1A2, CYP2A6 or CYP2D6. No time-dependent inhibition of the CYPs by efavirenz was observed in this study. Quantitative predictions showed that single dose of efavirenz may substantially slow the elimination of drugs predominately cleared by CYP2B6, CYP2C19 or by both enzymes and may also lower the area under the plasma concentration time curve (AUC) of active metabolites of some pro-drugs (e.g., clopidogrel and proguanil) by up to 30%. Depending on substrates, chronic administration of efavirenz may increase the AUC of CYP2C8 and CYP2C9 substrates about 3.5–4.4-fold and 1.7–2.0-fold at steady state.

**Keywords:** antiretroviral agents; cytochrome P450; reversible inhibition; time-dependent inhibition; drug-drug interaction; quantitative prediction

**Introduction**

Efavirenz, a potent non-nucleoside reverse transcriptase inhibitor (NNRTI), remains a preferred component of highly active antiretroviral therapy (HAART) for treatment of naïve patients despite an emergence of second generation of NNRTIs and new classes of antiretroviral agents (Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services, March 27, 2012). It is the only antiretroviral agent currently on the market that has been combined with two other nucleoside/nucleotide reverse transcriptase inhibitors in a single tablet. Efavirenz is known to alter the pharmacokinetics of many 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 a substrate of cytochrome P450s (CYPs) and UDP-glucuronosyltransferases (UGTs). It is predominantly cleared by CYP2B6-mediated 8-hydroxylation, with a small contribution from other CYPs (e.g., CYP2A6, CYP3A and CYP1A2). Two minor pathways, efavirenz...
7-hydroxylation and N-glucuronidation, are predominantly catalyzed by CYP2A6\(^3\) and UGT2B7,\(^4\) 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.\(^5,6\) Therefore, many drug interactions associated with efavirenz at steady state including interactions associated with efavirenz at steady state includingitors\(^13\) can be primarily explained by the inductive effect of CYP2B6, CYP2C19 and CYP3A.\(^5,6\) Therefore, many drug interactions associated with efavirenz at steady state including

7) statins,\(^8\) omeprazole,\(^9\) interactions associated with efavirenz at steady state including

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CYP2B6, CYP2C19 and CYP3A.\(^5,6\) Therefore, many drug interactions associated with efavirenz at steady state including

and/or pregnane X receptor (PXR), enhances the expression of through activation of constitutive androstane receptor (CAR) (auto-induction) upon repeated administration compared to a single dose,\(^14\) probably through induction of CYP2B6 and other enzymes involved in its metabolism.\(^7,8\) Besides induction, there is \emph{in vitro} evidence that efavirenz may directly inhibit the activities of certain CYPs.\(^15\) Indeed, scattered clinical cases of adverse drug interactions, \emph{e.g.}, with amodiaquine,\(^19\) warfarin\(^20\) and phenytoin,\(^21\) suggest that efavirenz may alter the pharmacokinetics of co-administered drugs through inhibition of CYPs.

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 \emph{in vitro} studies describing inhibition of CYPs by efavirenz provide only qualitative information, without generating \emph{in vitro} inhibition parameters that will allow quantitative prediction of \emph{in vivo} conditions 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 isoforms. In order to better predict \emph{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 the present study was to systematically evaluate the \emph{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 \emph{in vivo} drug interactions was quantitatively predicted.

**Materials and Methods**

**Chemicals:** Efavirenz, 8-hydroxyefavirenz, 7-hydroxy-coumarin, bupropion, 4-hydroxybuproprion, desethylamodiaquine, S-mephenytoin, 4-hydroxymephenytoin, R-omeprazole, R-hydroxyomeprazole and ritonavir were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Acetaminophen, chloroquine, coumarin, dextromethorphan, dextrophan, desmethylazepam, 8-methoxypsoralen, phenacetin, tolbutamide, 4-hydroxyltolbutamide, testosterone, 6-β hydroxytestosterone, glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Amodiaquine and levallorphan were purchased from the United States Pharmacopeia (Rockville, MD). All the other chemicals were of high performance liquid chromatography (HPLC) grade.

**Microsomal preparations:** Pooled human liver microsomes (HLMs) from 24 individual donors, HLMs with the CYP2C8*3/*83 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 cytochrome (Cyt) b5 were obtained from BD Biosciences. All microsomal preparations were stored at ~80°C until analysis.

**General incubation conditions:** 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°C with HLMs (or with expressed CYP when required), 200 mM sodium phosphate reaction buffer (pH 7.4) and 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) 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 a speed vacuum before the addition of the incubation components. The following HLM 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).

**Enzyme activity assays:** 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-deethylolation (CYP1A2); coumarin 7-hydroxylation (CYP2A6); bupropion 4-hydroxylation (CYP2B6); amodiaquine N-deethylolation (CYP2C8); tolbutamide 4-hydroxylation (CYP2C9); S-mephenytoin 4-hydroxylation/R-omeprazole 5-hydroxylation (CYP2C19); dextromethorphan O-demethylation (CYP2D6); and testosterone 6-β hydroxylation (CYP3A). The methods to measure the activity of CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6 and CYP3A in the absence or presence of the test inhibitors were adopted from previous studies.\(^22,23\) LC/MS/MS assays were developed for quantification of bupropion 4-hydroxylation (CYP2B6) as described elsewhere.\(^24\) The MS/MS system was an API2000 MS/MS triple quadrupole system (Applied Biosystems, Foster City, CA) equipped with a turbo ion spray and was coupled with a Shimadzu (Columbia, MD) HPLC system consisting of an LC-20AB pump and SIL-20A HT autosampler (Applied Biosystems/MDS Sciex, Foster City, CA). In brief, bupropion, 4-hydroxybuproprion, and the internal standard (nevirapine) were separated using a Zorbax SB-C18 column (100 x 2.00 mm, 3-µm particle size), a Luna C18 guard column (30 x 4.6 mm, 5 µm), and an isocratic mobile phase that consisted of 75% formic acid (0.1% in H\(_2\)O) and 25% acetonitrile (flow rate, 0.3 ml/min). 4-Hydroxybuproprion 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. R-Omeprazole 5-hydroxylation (CYP2C19) was assayed as described previously.\(^24\) The MS/MS system was the same as that for bupropion hydroxylation assay above. R-Hydroxyomeprazole and the internal standard (R-lansoprazole) were separated by Chiral-AGP (150 x 4.60 mm; 5 µM; Phenomenex). A gradient elution profile was used: initial mobile phase, 95% of 20 mM ammonium acetate (adjusted to pH 6.5) and 5% of acetonitrile (v/v); and the secondary mobile phase consisted of 90% of acetonitrile and 10% of 20 mM ammonium acetate (adjusted to pH 6.5) (v/v). The secondary mobile phase was increased from 0% to 40% linearly between 0 and 8 min; the initial mobile phase conditions were resumed after 9 min and remained constant for an
additional 6 min, allowing the column to equilibrate. The selected reaction-monitoring transitions of the precursor ions to selected product ions were m/z 362.13/214.10 for R-5-hydroxymeprazole and m/z 370.25/252.30 for the internal standard (R-lansoprazole).

**Kinetic analysis:** 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°C in duplicate with HLMs (or expressed enzymes) and the NADPH-generating system. Phenacetin (5 to 1,000 µM), coumarin (0.1 to 50 µM), bupropion (1 to 1,000 µM), amodiaquine (0.1 to 100 µM), tolbutamide (5 to 500 µM), 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 substrate (without the inhibitor) at 37°C in duplicate with HLMs and cofactors. Kinetic analysis was performed for each substrate probe reaction before initiation of the inhibition experiments. 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 Vmax), 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.

**Assessment of time-dependent inhibition:** Efavirenz was reported to be a time-dependent inhibitor of expressed CYP2B6, with a Ki value of 30 µM. Therefore, time-dependent inhibition was tested on eight major CYPs other than 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°C. 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 Vmax), 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.

**Data analysis:** Apparent kinetic constants (Km, Vmax) were estimated by fitting formation rates of metabolites versus substrate concentrations to a simple single-site Michaelis-Menten equation by nonlinear regression analysis using SigmaPlot 11.0 (Systat Software Inc., Richmond, CA). To calculate Ki values, the inhibition data were fit to different models of enzyme inhibition (competitive, noncompetitive, and uncompetitive) by nonlinear least-squares regression analysis with Prism Version 5.0 software (GraphPad Software Inc., San Diego, CA). 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.

**Prediction of in vivo drug interactions:** Predictions of the in vivo drug interaction potential of efavirenz were made using the following equations, which have been described previously:

$$\frac{\text{AUC}_1}{\text{AUC}} = \frac{1}{\frac{f_m}{K_i} + (1 - f_m)}$$

where:

- $\frac{\text{AUC}_1}{\text{AUC}}$ is the ratio of the AUC of substrate after inhibition to the AUC of the uninhibited substrate, and 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 Cmax, free systemic Cmax, total hepatic inlet Cmax estimated after oral administration and free hepatic inlet Cmax in the prediction of drug interactions, have been compared before and estimation using free hepatic inlet Cmax yielded the most accurate predictions of the magnitude of drug interactions. But efavirenz is highly protein bound with the fraction unbound in plasma estimated to be 0.029. In order to avoid underestimation of
of potential risk for drug interaction, total hepatic inlet $C_{\text{max}}$ ($C_{\text{hep,inlet}}$) of efavirenz was used in the present study [Eq. (2)], which also showed a reasonably good prediction in the previous study.\(^{26}\)

$$C_{\text{hep,inlet}} = C_{\text{max}} + \frac{K_a F_a D}{Q_h} \quad (2)$$

where: $C_{\text{max}}$ is the maximum plasma concentration; $K_a$ is the absorption rate constant, which is estimated to be $0.3\text{h}^{-1}$; $F_a$ is the fraction of the inhibitor passing through the intestine unchanged; $D$ is the administered therapeutic dose ($600\text{mg/day}$); and $Q_h$ is hepatic blood flow ($87\text{L/h}$).\(^{29}\) Values of $F_a$ can be estimated from oral bioavailability and hepatic extraction. Since neither value is available for efavirenz, a value of unity for $F_a$ was assumed as the most cautious possibility.\(^{29,30}\) There is no evidence that efavirenz enhances the activities of CYP2C8 and CYP2C9 in vivo. Therefore, $C_{\text{max}}$ of efavirenz at steady state was used ($9.2–16.6\mu\text{M}$) to predict the AUC changes of CYP2C8 and CYP2C9 substrates [Product Information of Efavirenz (Sustiva), Bristol-Myers Squibb Company, June 2012]. However, efavirenz has been shown to enhance the activities of CYP2B6, CYP2C19 and CYP3A upon multiple dosing, suggesting its inhibition effect may be masked by the inductive effect of efavirenz in a time-dependent manner. Therefore, predicted AUC changes for CYP2B6, CYP2C19 and CYP3A substrates were estimated using $C_{\text{max}}$ ($4.6–8.4\mu\text{M}$) obtained after the administration of a single 600 mg oral dose of efavirenz to 20 healthy volunteers (unpublished data). Specifically, we focused on substrates that exhibit a 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 by pharmacogenetic method modified from a recent study for CYP2D6 substrate.\(^{36}\) The original method was based on the observation of the ratio of the AUC in a poor metabolizer (PM), AUC\(^{\text{PM}}\), to the AUC in an extensive metabolizer (EM), AUC\(^{\text{EM}}\). Assuming that genetic polymorphisms do not affect the elimination of the metabolite and have 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\(^{32}\) using the following equation:

$$\frac{\text{AUC}_m^{\text{EM}}}{\text{AUC}_m^{\text{PM}}} = \frac{1}{1 - f_m^{\text{EM}}} \quad (5)$$

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

**Results**

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 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-mephentoin) since a substrate-dependent effect on the CYP2C19 inhibition profile was observed previously.\(^{37}\) In the present study, efavirenz inhibited CYP2C19 activity by 35% and 70% at 10 and 50 µM, when S-mephentoin was used as a substrate (Fig. 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-mephentoin is more sensitive to CYP2C19 inhibi-

![Fig. 1. Inhibitory effects of efavirenz on human CYP activities in pooled HLMs](image-url)

A substrate probe of each specific CYP isoform 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 Materials and Methods. The specific concentrations of each probe used are illustrated in Materials and Methods. Each point represents the average of duplicate incubations. EFV, efavirenz.
tion than R-omeprazole. Therefore, S-mefloquine was used as a substrate of CYP2C19 in subsequent inhibition experiments.

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 the concentration ratio of paraxanthine/caffeine at 6 h (p < 0.0001). However, the present in vitro data derived from pooled HLMs shown in Figure 1 did not indicate that efavirenz inhibits CYP1A2 activity (Fig. 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 (Fig. 2), which suggests that 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) (Fig. 1).

Estimation of K_i values: In order to obtain quantitative prediction of the 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, the K_i value was determined in pooled HLMs because a previous study reported that the value of EC_{50} is around 20 µM using midazolam as a substrate.

Of all the CYPs tested, CYP2B6 was the most sensitive to efavirenz inhibition (Table 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. The 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 Figures 3A and 3B, respectively.

Inhibition of CYP2C8 by efavirenz was determined in two HLMs and expressed CYP2C8. As shown in Table 1, efavirenz showed potent competitive inhibition of CYP2C8 activity in pooled HLMs (K_i = 4.78 ± 2.24 µM). The second HLM 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 1). Efavirenz exhibited similar competitive inhibition in expressed CYP2C8 with an estimated K_i value of 6.05 ± 2.86 µM (Table 1). In Figure 4, Dixon plots for the inhibition of CYP2C8-catalyzed N-desethylamodiaquine by efavirenz in pooled HLMs (Fig. 4A), HLMs with the CYP2C8*3/*3 genotype (Fig. 4B) and expressed CYP2C8 (Fig. 4C) are shown.

Efavirenz was found to be a moderate inhibitor of CYP2C9 (K_i = 19.46 ± 2.78 µM; Table 1 and Fig. 5) and CYP2C19 (21.31 ± 2.57 µM; Table 1 and Fig. 6), and a weak inhibitor of CYP3A (K_i = 40.33 ± 0.33 µM; Table 1 and Fig. 7).

Time-dependent inactivation: As shown in Figure 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.

Quantitative prediction of in vivo drug interactions: The predicted ratio of AUC_i/AUC for each substrate co-administered with a single dose or multiple doses of efavirenz are listed in Table 2. Compared to control (without efavirenz), a single 600 mg oral dose of efavirenz was predicted to result in ~3-fold changes in the

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**Table 1.** K_i values of efavirenz for the inhibition of CYPs in HLMs and expressed CYPs

<table>
<thead>
<tr>
<th>CYP isoform</th>
<th>Substrate</th>
<th>Systems</th>
<th>K_i value (µM) (inhibition model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Pooled HLMs</td>
<td>2.96 ± 0.67 (competitive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CMV negative HLMs</td>
<td>0.39 ± 0.10 (competitive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed CYP2B6</td>
<td>1.38 ± 0.09 (competitive)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Pooled HLMs</td>
<td>4.78 ± 2.24 (competitive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLMs with CYP2C8*3/*3</td>
<td>4.80 ± 0.35 (competitive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed CYP2C8</td>
<td>6.05 ± 2.86 (competitive)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>Pooled HLMs</td>
<td>19.46 ± 2.78 (non-competitive)</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin</td>
<td>Pooled HLMs</td>
<td>21.31 ± 2.57 (competitive)</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Testosterone</td>
<td>Pooled HLMs</td>
<td>40.33 ± 0.33 (competitive)</td>
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</table>
exposure of methadone (CYP2B6 substrate). In addition, 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%

Fig. 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 the CYP2C8*3/*3 genotype (0.1 mg/ml) and cofactors at 37°C for 15 min with or without efavirenz (0–50 µM). Expressed CYP2C8 (26 pmol) was used in the inhibition study. Each point represents the average of duplicate incubations.

Fig. 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°C for 15 min with or without efavirenz (0–100 µM).

Fig. 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°C for 15 min with or without efavirenz (0–100 µM).

Fig. 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°C for 15 min with or without efavirenz (0–50 µM).

Fig. 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°C. 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.

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of CYP 2B6, 2C9 and 2C19 substrates in vivo by efavirenz

<table>
<thead>
<tr>
<th>Drug</th>
<th>t&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Predicted AUC ratio</th>
<th>Reported AUC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>0.75&lt;sup&gt;27&lt;/sup&gt;</td>
<td>2.9–3.1</td>
<td>N.A.</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>0.93&lt;sup&gt;26&lt;/sup&gt;</td>
<td>3.5–4.4</td>
<td>2.15–4.02&lt;sup&gt;19&lt;/sup&gt;</td>
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<tr>
<td>Phenytin</td>
<td>0.90&lt;sup&gt;27&lt;/sup&gt;</td>
<td>1.7–2.0</td>
<td>N.A.</td>
</tr>
<tr>
<td>S-Warfarin</td>
<td>0.91&lt;sup&gt;26&lt;/sup&gt;</td>
<td>1.7–2.0</td>
<td>N.A.</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>0.87&lt;sup&gt;26&lt;/sup&gt;</td>
<td>1.4–1.6</td>
<td>N.A.</td>
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<tr>
<td>Proguanil&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.84</td>
<td>29–33%</td>
<td>N.A.</td>
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<tr>
<td>Clopidogrel&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.56–0.64&lt;sup&gt;19&lt;/sup&gt;</td>
<td>17–29%</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Plasma concentrations of efavirenz after a single dose were used to predict its effect on the AUC change of methadone and omeprazole and the active metabolites of proguanil and clopidogrel. Plasma concentrations upon multiple doses were used for predicting the AUC change of amodiaquine, phenytin and S-warfarin.

The value of percentage change in the AUC of the active metabolite $(1 - \frac{AUC_{out}}{AUC_{in}})$ was predicted.

N.A.: not available.

Plasma concentrations of efavirenz in an extensive metabolizer of CYP2C19. However, when data generated using R-omeprazole 5-hydroxylation was 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: phenytin and S-warfarin) compared to controls (without efavirenz).

Discussion

The primary aim of this study is to characterize the inhibition constant (K<sub>i</sub>) of efavirenz in CYPs that showed inhibition and quantitatively predict its inhibition effect on the AUC of clinically important co-administered drugs. Previous in vitro studies only provided qualitative information (IC<sub>50</sub> values). In the present study, we have shown that efavirenz is a potent competitive inhibitor of CYP2B6 (average K<sub>i</sub> = 1.68 µM in HLMs and K<sub>i</sub> = 1.38 µM in expressed CYP2B6) and CYP2C8 (K<sub>i</sub> = 4.78 µM in pooled HLMs and K<sub>i</sub> = 4.80 µM in HLMs with the CYP2C8*3/*3 genotype). In pooled HLMs, efavirenz showed moderate inhibition of CYP2C9 (K<sub>i</sub> = 19.46 µM) CYP2C19 (K<sub>i</sub> = 21.31 µM), and weak inhibition of CYP3A (K<sub>i</sub> = 40.33 µM). Inhibition of CYP1A2, CYP2A6 and CYP2D6 by efavirenz was marginal. No time-dependent inactivation of the CYP isomers 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, and of omeprazole by 1.4- to 1.6-fold (CYP2C19 substrate), and may also lower the AUC of active metabolites of some prodrugs (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 phenytin 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 a K<sub>i</sub> 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, 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 on. Efavirenz has a higher binding affinity to CYP2B6 with K<sub>m</sub> values of 13–20 µM<sup>2,3</sup> than buyponprin with K<sub>m</sub> values of 90–130 µM.<sup>38,39</sup> Thus, it is plausible that the high inhibition potency of efavirenz on buyponprin hydroxylation could be due to the fact that efavirenz has higher binding affinity to CYP2B6 than buyponprin. A similar mechanism contributing to high inhibition potency has been reported for CYP2D6.<sup>40</sup> 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.<sup>41</sup> Efavirenz also enhances the metabolism of co-administered CYP2B6 substrates, including methadone<sup>42</sup> and buyponprin.<sup>43</sup> Considering the high inhibition potency of efavirenz, a substantial increase in the AUC of CYP2B6 substrates and potentially the risk of 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<sub>i</sub> values of 4.78 and 6.05 µM in pooled HLMs and expressed CYP2C8 respectively, which broadly concurs with an IC<sub>50</sub> of 4 µM reported in expressed CYPs.<sup>15</sup> The inhibition potency of efavirenz in HLMs with the CYP2C8*3/*3 genotype, the most frequent and functionally relevant variant in Caucasians,<sup>44</sup> was not different from that observed in pooled HLMs. The possibility of substrate-dependent interaction cannot be fully excluded,<sup>45</sup> but our data suggest that the CYP2C8*3 allele does not seem to alter susceptibility to efavirenz inhibition. We expect a ~3-fold higher AUC of amodiaquine and probably other substrates such as chloroquine, certain anti-diabetics, montelukast and rosiglitazone,<sup>46,47</sup> 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.<sup>39</sup> A 2.2- to 4-fold increase in the amodiaquine AUC was also noted<sup>40</sup> 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<sub>i</sub> = 19.46 µM) with a 1.7- to 2-fold predicted increase in the AUC of drugs mainly cleared by CYP2C9, consistent with an in vitro study reporting an IC<sub>50</sub> value of ~15 µM.<sup>16</sup> This enzyme is involved in the metabolism of more than 100 currently used drugs, including drugs with a narrow therapeutic range, e.g., oral anticoagulants, oral hypoglycemic agents and phenytin.<sup>48</sup> Therefore, co-administration of efavirenz may likely increase the risks of adverse effects of these drugs, which is supported by clinical cases of inhibition drug interactions of efavirenz with the CYP2C9 substrates phenytin<sup>21</sup> and warfarin.<sup>20</sup>

We found that the extent of CYP2C19 inhibition by efavirenz was substrate-dependent: modest inhibition of S-mephenytin 4-hydroxylation (K<sub>i</sub> = 21.31 µM) and marginal inhibition of R-
Efavirenz Inhibition of CYPs

Efavirenz-mediated in vivo inhibition of CYP3A3 seems unlikely given the high Kᵢ value (Kᵢ = ~40 µM) observed in the present study. Using triazolam as a substrate, another study reported lower IC₅₀ values (17–20 µM), but the significance of the in vivo inhibition of CYP3A, if any, is likely marginal. Efavirenz, through activation of PXR/CAR, induces CYP3A in vitro and in vivo. Hence, efavirenz enhances the elimination of many CYP3A substrates, including protease inhibitors, statins, 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. Efavirenz is not only a substrate but also a moderate inhibitor of UGT2B7, while no reversible inhibition or time-dependent inhibition by efavirenz was observed for CYP1A2 or CYP2A6 in the present study. Drug interaction with CYP2A6 substrates mediated by efavirenz inhibition seems very unlikely, but the possibility of efavirenz inhibiting the metabolism of CYP1A2 substrates cannot 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 inter-individual variability in its disposition and by often unpredictable and complex drug interactions. The extent of drug interactions with efavirenz varies greatly among individuals, and inter-patient 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; efavirenz activates CAR and PXR, induces CYP2B6 (and other drug disposition genes) and autoinduces its own metabolism upon repeated administration; and efavirenz is a potent inhibitor of CYP2B6 with no effect on CYP2A6 or CYP1A2 (present study) and a moderate inhibitor of UGT2B7 in vitro. 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 to be 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 inter-individual variability of efavirenz exposure and unpredictable drug interactions associated with it.

References


