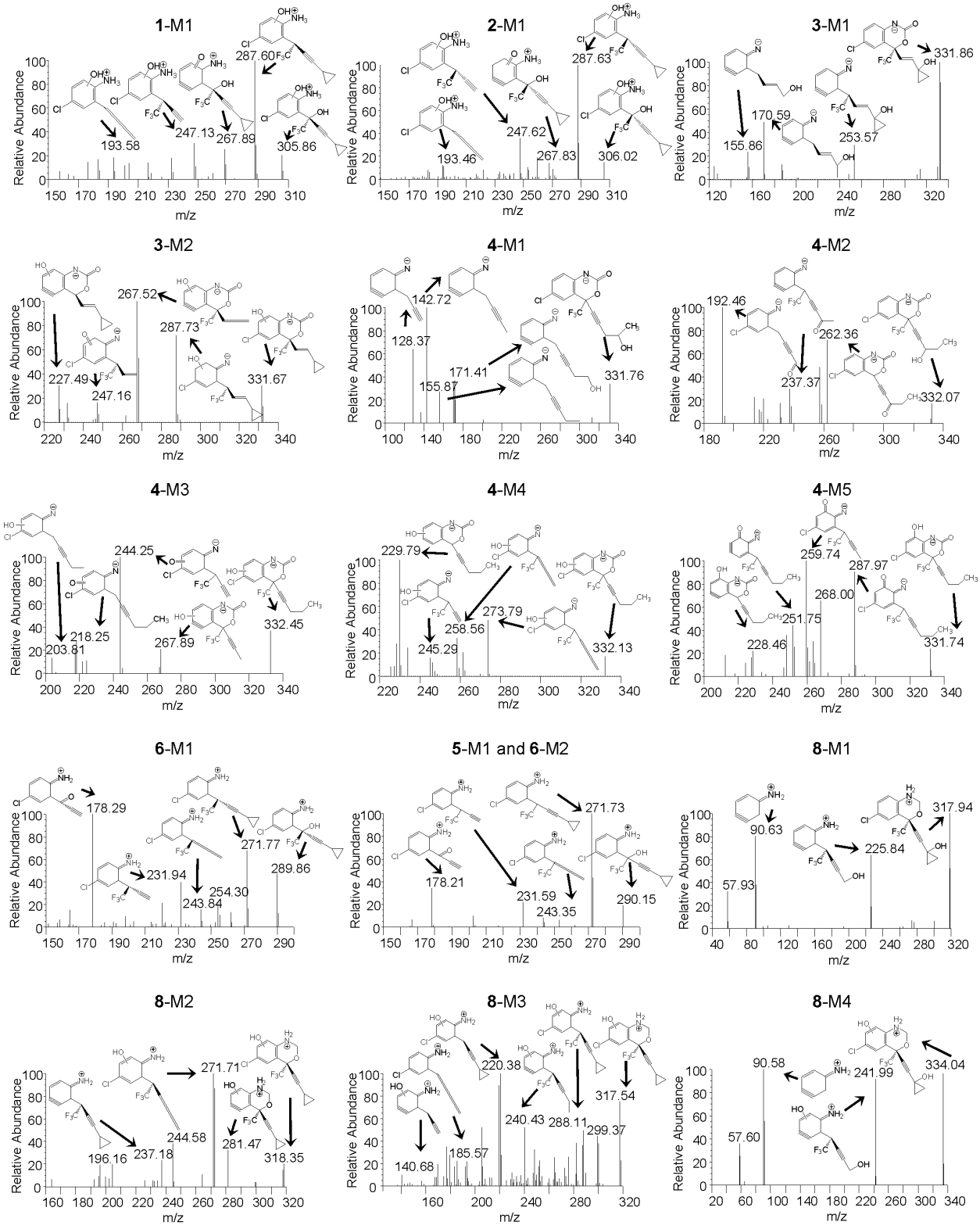
SUPPORTING INFORMATION

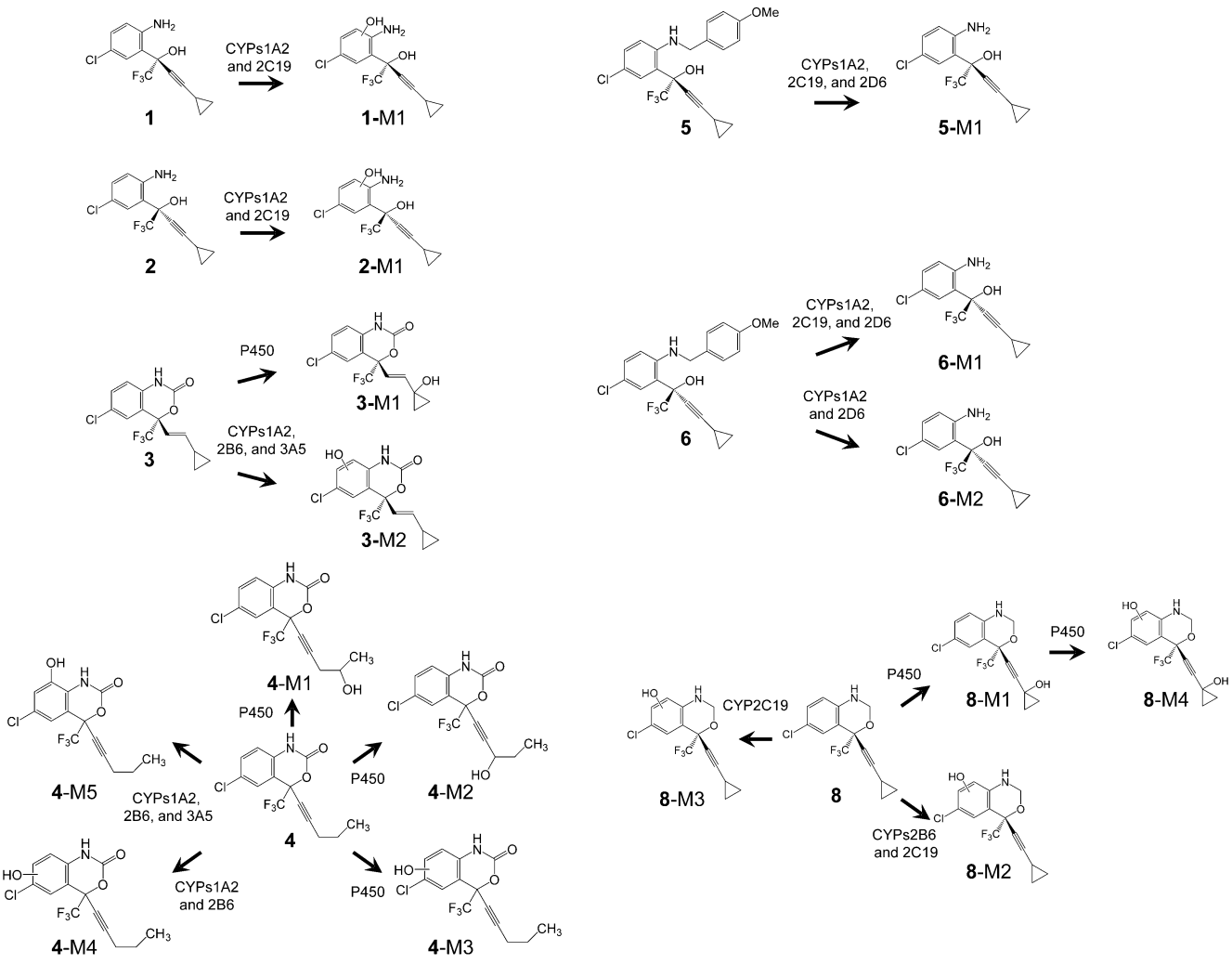
Structure–Activity Studies Reveal the Oxazinone Ring Is a Determinant of Cytochrome P450 2B6 Activity Toward Efavirenz.

Philip M. Cox and Namandjé N. Bumpus

Table of contents:



Supplemental Figure 1. Mass spectra for EFV analogue metabolites identified using human liver microsomes. Each EFV analogue (10 μM) was incubated individually with human liver microsomes (2 mg/mL) for 60 minutes at 37 ˚C in potassium phosphate buffer in the presence of an NADPH regenerating system. Fragmentation was conducted in product ion mode scanning for the parent mass plus 16 or 32 m/z for mono- and dihydroxylated metabolites, and for m/z 290 for N-dealkylated metabolites. (N=3)



Supplemental Figure 2. Cytochrome P450-catalyzed metabolism schemes for EFV analogues **1**-**6** and **8**. Each EFV analogue (10 µM) was incubated with individual cDNA-expressed P450s (10 nM) for 60 minutes at 37 ˚C in 100 mM potassium phosphate buffer in the presence of an NADPH regenerating system. Metabolite detection was performed using uHPLC-MS/MS as described under materials and methods. In each scheme, individual P450s found to form each metabolite are indicated or "P450" is used to demarcate those metabolites that were not found to be formed by any of the individuals P450 enzymes tested. We did not detect metabolites from EFV analogue **7**. (N=3)

EFV analogues.
EFV, all EFV analogues, and bupropion hydrochloride were obtained from Toronto Research Chemicals (Toronto, Canada) and were ≥ 97% pure, according to the manufacturer. The analogues used in this study were **1** – (αS)-2-Amino-5-chloro-α-(2-cyclopropylethynyl)-α-trifluoromethyl)benzenemethanol, **2** – (αR)-2-Amino-5-chloro-α-(2-cyclopropylethynyl)-α-(trifluoromethyl)benzenemethanol, **3** – (E)-Dihydroefavirenz, **4** – rac 6-Chloro-1,4-dihydro-4-(1-pentynyl)-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one, **5** – (R)-5-Chloro-α-(cyclopropylethynyl)-2-[[(4-methoxyphenyl)methyl]amino]-α-(trifluoromethyl)benzenemethanol, **6** – (S)-5-Chloro-α-(cyclopropylethynyl)-2-[[(4-methoxyphenyl)methyl]amino]-α-(trifluoromethyl)benzenemethanol, **7 –** rac N-[4-Chloro-2-[3-cyclopropyl-1-hydroxy-1-(trifluoromethyl)-2-propynyl]phenyl]-4-methoxybenzamide, and **8** - (4S)-6-Chloro-4-(2-cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazine.

EFV analogue metabolism by human liver microsomes and cDNA-expressed P450s.
Metabolism assays with human liver microsomes (Xenotech LLC, Lenexa, KS) or cDNA-expressed P450s (Supersomes, BD Biosciences) were performed as described previously[1, 2] using 10 μM EFV analogue and 2 mg/mL HLM or 10 nM cDNA-expressed P450 for 60 minutes. To aid in fragment assignment, metabolism reactions with human liver microsomes were also performed in the presence of $H_2{}^{18}O$ (Cambridge Isotope Laboratories, Andover, MA). Reactions were performed in polypropylene tubes except those for analogue **8**, which were performed in silanized borosilicate glass tubes. All samples were analyzed using uHPLC-MS/MS.

Substrate depletion kinetics.
K_m and V_{max} values for EFV and EFV analogues **3**, **4** and **8** were obtained using a substrate depletion approach essentially as described previously.[3] Briefly, 10 nM CYP2B6 was pre-incubated with six concentrations of EFV or EFV analogues ranging over four to six orders of magnitude for 5 minutes at 37 ˚C in 100 mM potassium phosphate buffer. At 0, 2, 5, 10, 20, and 30 minutes after addition of an NADPH-regenerating system (BD Biosciences), 100 μL aliquots were taken and diluted into an equal volume of acetonitrile containing the internal standard fluorinated efavirenz.[4] The height of the analyte peak and internal standard peak were determined by uHPLC-MS/MS.

Inhibition using bupropion as a probe.
Each analogue (10 μM) was incubated with bupropion (40 μM) and CYP2B6 (50 μM) in 100 mM potassium phosphate buffer for 10 minutes in the presence of an NADPH regenerating system at 37 ˚C. Hydroxybupropion formation was linear under these conditions. Reactions were terminated as described previously.[1, 2] Data were collected using uHPLC-MS/MS.

Data Analysis.
Non-linear regression, curve fitting, and statistical analyses were performed using GraphPad Prism (version 6; GraphPad Software Inc., San Diego, CA). Statistical comparisons were made using unpaired parametric *t*-tests. Substrate depletion data analysis was performed essentially as previously described.[3] Briefly, the natural logarithm of the ratio of analyte to internal standard for each substrate concentration was calculated and normalized to the ratio at time 0. The normalized ratio was then plotted versus time and the resulting curves fit to equation (1) to obtain the depletion rate constant (k_{dep}).

$$Y=Y_0\times e^{-k_{dep}t} \quad (1)$$

Only the linear portion of the depletion curve was used to calculate k_{dep}. Any points not contributing to log-linearity were excluded. To calculate K_m, the k_{dep} values were plotted versus the logarithm of the substrate concentration and fit to equation (2), where $k_{dep\ max}$ is the maximum depletion rate constant.

$$k_{dep} = k_{dep\ max} \times \left(1 - \frac{[S]}{[S] + K_m}\right) \quad (2)$$

V_{max} values were determined as described previously.[5] Briefly, the $k_{dep\ max}$ values obtained from fitting the data to equation 2, were divided by the enzyme concentration yielding the Cl_{int}. The Cl_{int} values were then divided by the calculated K_m to obtain the V_{max}.

uHPLC-MS/MS analysis of EFV, EFV analogues, and hydroxybupropion.
For metabolism assays, samples were injected onto a Dionex UltiMate 3000 uHPLC system coupled to a TSQ Vantage Triple Stage Quadrupole mass spectrometer (Thermo Scientific, Pittsburgh, PA) and resolved with an Xterra C18 column (2.5 µm, 2.1 by 50 mm; Waters, Milford, MA). For EFV and EFV analogue assays, a linear gradient of mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid) consisting of 5% B from 0-0.5 min, 5-95% B from 0.5-9.5 min, 95% B from 9.5-11.5 min, and re-equilibration at 5% B from 11.6-12.6 minutes at a flow rate of 0.4mL/min was used. For separation and detection of hydroxybupropion, a linear gradient of the same mobile phases consisting of 20% B from 0-0.5 min, 20-50% B from 0.5-3 min, 50-95% B from 3.0-3.1 min, 95% B from 3.1-4.0 min, and re-equilibration at 20% B from 4.1-5.0 min was used. All reagents used for mobile phases were of the highest grade commercially available. For hydroxybupropion and EFV analogues **1**, **2**, and **5-8** resolution was achieved in positive ion mode, while EFV, EFV analogues **3** and **4**, and fluorinated efavirenz were resolved in negative ion mode. EFV analogue metabolite identification and determination of relative abundance was accomplished in product ion mode. Hydroxybupropion relative abundance was determined using selected reaction monitoring with a transition of m/z 256.1 > 139.06. For substrate depletion experiments, selected reaction monitoring was used to monitor the parent peak height over time. All samples were injected onto a Polaris C18-A column (5 µM, 100 by 2 mm) at a flow rate of 0.6 mL/min. A linear gradient of mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid) consisting of 10% B from 0-0.5 min, 10-95% B from 0.5-5 min, 95% B from 5-6 min, and re-equilibration at 10% B from 6.1-7 min. For analogue **8**, a baseline of 20% B was used in order that the parent peak elute during the gradient phase. Selected reaction monitoring transitions used were as follows: EFV: m/z 314 > 243.87; fluorinated efavirenz: m/z 297.97 > 227.93; analogue **3** m/z 315.9 > 245.9; analogue **4**: m/z 316 > 218; and analogue **8**: m/z 302.1 > 203.97.

References

(1) Lade, J. M.; Avery, L. B.; Bumpus, N. N. Human biotransformation of the nonnucleoside reverse transcriptase inhibitor rilpivirine and a cross-species metabolism comparison. *Antimicrob. Agents Chemother.* **2013**, 57, 5067-79.

(2) Lu, Y.; Hendrix, C. W.; Bumpus, N. N. Cytochrome P450 3A5 plays a prominent role in the oxidative metabolism of the anti-human immunodeficiency virus drug maraviroc. *Drug Metab. Dispos.* **2012**, 40, 2221-30.

(3) Obach, R. S.; Reed-Hagen, A. E. Measurement of Michaelis constants for cytochrome P450-mediated biotransformation reactions using a substrate depletion approach. *Drug Metab. Dispos.* **2002**, 30, 831-7.

(4) Avery, L. B.; Parsons, T. L.; Meyers, D. J.; Hubbard, W. C. A highly sensitive ultra performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) technique for quantitation of protein free and bound efavirenz (EFV) in human seminal and blood plasma. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci*. **2010**, 878, 3217-24.

(5) Lutz, J. D.; Dixit, V.; Yeung, C. K.; Dickmann, L. J.; Zelter, A.; Thatcher, J. E.; Nelson, W. L.; Isoherranen, N. Expression and functional characterization of cytochrome P450 26A1, a retinoic acid hydroxylase. *Biochem. Pharmacol.* **2009**, 77, 258-68.