Drug Interaction of Efavirenz and Midazolam: Efavirenz Activates the CYP3A-Mediated Midazolam 1′-Hydroxylation In Vitro

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ABSTRACT:

CYP3A4 and CYP3A5 are the most important drug-metabolizing enzymes. For several drugs, heteroactivation of CYP3A-mediated reactions has been demonstrated in vitro. In vivo data suggested a possible acute activation of CYP3A4-catalyzed midazolam metabolism by efavirenz. Therefore, we aimed to investigate the effect of efavirenz on the in vitro metabolism of midazolam. The formation of 1′-hydroxymidazolam was studied in pooled human liver microsomes (HLM) and recombinant human CYP3A4 and CYP3A5 (rCYP3A4 and rCYP3A5) in the presence of efavirenz (0.5, 1, and 5 μM). Product formation rates (V_{max}) increased with increasing efavirenz concentrations ($\sim$1.5-fold increase at 5 μM efavirenz in HLM and $\sim$1.4-fold in rCYP3A4). The activation in rCYP3A4 was depen-
dent on cytochrome b_5, and the activating effect was also observed in rCYP3A5 supplemented with cytochrome b_5, where V_{max} was $\sim$1.3-fold enhanced. Concomitant inhibition of CYP3A activity with ketoconazole in HLM abolished the increase in the 1′-hydroxymidazolam formation rate, further confirming involvement of CYP3A. The results of this study represent a distinct acute activation of midazolam metabolism and support the in vivo observations. Moreover, only efavirenz, but not its major metabolite 8-hydroxyefavirenz, was responsible for the activation. The increase in 1′-hydroxymidazolam formation may have been caused by binding of efavirenz to a peripheral site of the enzyme, leading to enhanced midazolam turnover due to changes at the active site.

Introduction

The CYP3A subfamily contains the most abundant human cytochrome P450 (P450) enzyme and plays a major role in phase I metabolism (Guengerich, 2006). Members of this enzyme subfamily catalyze the metabolism of approximately 40 to 50% of the currently marketed drugs (Thummel and Wilkinson, 1998). However, the wide substrate specificity facilitates major drug-drug interactions because of inhibition and induction of these enzymes (Lin and Lu, 1998). In addition to inhibition and induction, several P450 isoforms are stimulated in vitro. Activation (heterotropic positive cooperativity) occurs when P450 activity for a substrate is increased in the presence of another drug (Hutzler and Tracy, 2002) and may result in a change from hyperbolic Michaelis-Menten kinetics to nonhyperbolic kinetics (Atkins, 2005). Examples of CYP3A4 activators include 7,8-benzoflavone (Shou et al., 1994), quinidine (Ngui et al., 2001), and steroids (Henshall et al., 2008).

Midazolam is extensively metabolized by CYP3A. In vitro, two major metabolites, 1′-hydroxymidazolam and 4-hydroxymidazolam, are formed (Kronbach et al., 1989). The formation of 1′-hydroxymidazolam

is predominant at midazolam concentrations below 25 μM (Khan et al., 2002), and 4-hydroxymidazolam formation is minor at therapeutic doses of the drug. CYP3A4 and CYP3A5 catalyze the formation of both metabolites but exhibit different regioselectivity, and indeed the ratio of formed 1′-hydroxymidazolam to 4-hydroxymidazolam is significantly greater for CYP3A5 than CYP3A4 (Gorski et al., 1994). A minor catalytic role of CYP3A7 in midazolam metabolism was also suggested (Gorski et al., 1994).

The non-nucleoside reverse transcriptase inhibitor efavirenz was approved in combination with other antiretroviral agents for the treatment of HIV-1 infection (Adkins and Noble, 1998). Efavirenz is characterized by high protein binding ($>$99.5%) and a long terminal half-life (52–76 h), which is reduced to 40 to 55 h because of auto-induction of its own metabolism (Smith et al., 2001). The formation of the main metabolite 8-hydroxyefavirenz is preferentially catalyzed by CYP2B6 in vitro (Ward et al., 2003) and in vivo (Mutlib et al., 1999). In different species, including humans, 8-hydroxyefavirenz, 7-hydroxyefavirenz, 8,14-dihydroxyefavirenz, and other secondary metabolites were also detected. Efavirenz acts as both an inducer and an inhibitor of CYP3A4. In vivo, it induces hepatic but not intestinal CYP3A4 (Mouly et al., 2002), and in vitro studies demonstrated an induction of CYP3A4 transcription by efavirenz (Hariparsad et al., 2004; Weiss et al., 2009). In vitro, efavirenz also acts as a weak CYP3A inhibitor (von Moltke et al., 2001). In a clinical study, we found that efavirenz rapidly increased midazolam metabolism (Bayer et al., 2009), suggesting an acute activation of midazolam metabolism by efavirenz that occurred faster than expected for induction of the enzyme via mRNA transcription. Therefore, we aimed to

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ABBREVIATIONS: P450, cytochrome P450; HLM, human liver microsomes; r, recombinant; K_m, substrate concentration at half-maximal product formation rate; LC-MS/MS, liquid chromatography-tandem mass spectrometry; V_{max}, maximal product formation rate.

investigate the effect of efavirenz on the in vitro metabolism of midazolam to clarify whether activation of CYP3A might be the underlying mechanism. The kinetics of midazolam 1′-hydroxylation were investigated at increasing efavirenz concentrations. In vitro incubations of midazolam and efavirenz with human liver microsomes (HLM) and recombinant human CYP3A4 and CYP3A5 (rCYP3A4 and rCYP3A5, respectively) confirmed the activating effect of efavirenz. In addition, we studied the effect of 8-hydroxyefavirenz on midazolam metabolism and found that the observed effects can be attributed to efavirenz itself.

Materials and Methods

Chemicals. Efavirenz was obtained from Sequoia Research Products (Pangbourne, UK), 8-hydroxyefavirenz, midazolam, 1′-hydroxymidazolam, d_5-midazolam, and $[^{13}C_3]1′$-hydroxymidazolam were from Toronto Research Chemicals Inc. (North York, ON, Canada), and ketoconazole was obtained from Janssen Pharmaceutica N.V. (Olen, Belgium). DL-Isocitric acid Na$_3$, isocitric dehydrogenase, and β-nicotinamide-adenine dinucleotide phosphate disodium were purchased from Sigma-Aldrich (Steinheim, Germany), and natrium dihydrogen-phosphate monohydrate, and MgCl$_2$·6H$_2$O were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of high-performance liquid chromatography grade and were obtained from Sigma-Aldrich or Merck. Stock solutions of midazolam (100 μM) and ketoconazole (250 μM) were prepared in acetonitrile/water, 1:1 (v/v). Stock solutions of efavirenz (250 μM) and 8-hydroxyefavirenz (250 μM) were dissolved in methanol/water, 1:1 (v/v).

HLM and Recombinant P450s. HLM (pooled, mixed gender, cytomegalovirus-negative), rCYP3A4 EasyCYP Bactosomes and rCYP3A5 EasyCYP Bactosomes coexpressed with NADPH-P450 reductase in *Escherichia coli* and supplemented with purified human cytochrome b_5 were purchased from tebu-bio (Offenbach, Germany). P450 concentration, cytochrome b_5 concentration, and NADPH-P450 reductase activity were 8.3, 8.68 nmol · ml^{-1}, and 194 nmol · min^{-1} · mg protein^{-1} for the HLM, respectively. For both recombinant preparations, P450 concentration (1.0 nmol/ml) and cytochrome b_5 concentration (5.0 nmol/ml) were identical, whereas NADPH-P450 reductase activity was 568 nmol · min^{-1} · mg protein^{-1} for rCYP3A4 and 1460 nmol · min^{-1} · mg protein^{-1} for rCYP3A5. The in vitro preparations were stored at −80°C until use and were suspended in 0.1 M potassium phosphate buffer before the experiments.

Incubation Conditions. The incubation mixtures (final volume, 250 μl) contained incubation buffer (0.1 M NaH$_2$PO$_4$ H$_2$O, pH 7.4), a NADPH-regenerating system (10 μl/ml isocitric dehydrogenase (3–20 units/mg protein), 0.85 mg/ml β-nicotinamide-adenine dinucleotide phosphate disodium, 1.56 mg/ml DL-isocitric acid Na$_3$, 1.02 mg/ml MgCl$_2$·6H$_2$O), and either HLM or recombinant P450s (rP450s). The reaction was started by adding 25 μl of the NADPH-regenerating system and incubating for the respective time at 37°C in a thermomixer Eppendorf AG (Hamburg, Germany). Incubations were terminated by addition of 225 μl of acetonitrile. The samples were immediately centrifuged at 16,100g for 3 min. Aliquots of the supernatants (50 μl) were diluted in liquid chromatography mobile phase (600 μl) containing the internal standards d_5-midazolam and $[^{13}C_3]1′$-hydroxymidazolam. An aliquot (20 μl) was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Initial Kinetic Studies with HLM and Recombinant P450s. Initial kinetic studies were conducted to determine the optimal protein concentration for HLM, rCYP3A4, and rCYP3A5 and the optimal incubation time. Midazolam concentrations above 15 μM were previously reported to decrease V_{max} in HLM (Martínez et al., 2000). Therefore, HLM (0, 0.005, 0.01, 0.05, 0.1, and 0.2 mg protein/ml) were incubated with 10 μM midazolam for 20 min. An optimal 1′-hydroxymidazolam formation rate was reached when 0.1 mg protein/ml pooled HLM were used. For predefinition of optimal incubation time, HLM (0.1 mg protein/ml) were incubated with midazolam (10 μM) over periods of 0, 2.5, 5, 10, 15, 20, 30, and 40 min. At 5-min incubation time, a linear metabolic rate was maintained with not more than 10% midazolam being converted. Because of higher metabolic rates in the presence of efavirenz, HLM concentration was reduced to 0.05 mg protein/ml in all subsequent incubations with HLM. For rCYP3A4 and rCYP3A5, 10 min incubation time and 0.1 and 0.05 mg protein/ml were selected, respectively.

Kinetic Analyses in HLM and Recombinant P450s. To assess the kinetics of midazolam 1′-hydroxylation, midazolam (0.5–10 μM) was incubated in triplicates for 5 min at 37°C with pooled HLM (0.05 mg protein/ml) and a NADPH-generating system as described above in the presence of efavirenz (0.5–5 μM). Triplicate samples of rCYP3A4 (0.1 mg protein/ml) and rCYP3A5 (0.05 mg protein/ml) were incubated for 10 min at 37°C at the same midazolam concentrations. The influence of 8-hydroxyefavirenz on midazolam metabolism was studied in HLM by quantifying the formation of 1′-hydroxymidazolam in the presence of 8-hydroxyefavirenz (0.5, 1, and 5 μM).

Inhibition of CYP3A. CYP3A activity was inhibited by addition of ketoconazole (0.2 μM), a known inhibitor of CYP3A catalytic activity and potent inhibitor of 1′-hydroxymidazolam formation in HLM (Gibbs et al., 1999). The CYP3A inhibition experiment was performed in HLM under the incubation conditions described above, and ketoconazole was added to the samples before addition of NADPH$_2$.

Quantification of Midazolam and 1′-Hydroxymidazolam. The formation of 1′-hydroxymidazolam was determined by LC-MS/MS analysis (Surveyor LC coupled to TSQ7000; Thermo Fisher Scientific, Waltham, MA) in the multiple reaction monitoring mode as described previously (Hafner et al., 2010) with minor modifications. In brief, the compounds were separated at 40°C on a Synergi Max-RP column (150 × 2.0 mm i.d., 4-μm particle size; Phenomenex, Aschaffenburg, Germany). The flow rate was set to 0.4 ml/min, and the run time was 5 min. The isocratic mobile phase consisted of acetonitrile (46%) and 5 mM ammonium acetate containing 0.1% acetic acid and 5% acetonitrile (54%). Mass spectrometric data for 1′-hydroxymidazolam, $[^{13}C_3]1′$-hydroxymidazolam, midazolam, and d_5-midazolam were obtained with electrospray ionization in the positive mode. Tandem mass spectrometry transitions monitored in multiple reaction monitoring mode were m/z 326 → m/z 291 at 45-V collision energy for midazolam, m/z 342 → m/z 203 at 44 V for 1′-hydroxymidazolam, m/z 331 → m/z 296 at 45 V for d_5-midazolam, and m/z 345 → m/z 206 at 44 V for $[^{13}C_3]1′$-hydroxymidazolam. Electrospray ionization-in-source collision-induced dissociation value was set to 10 V.

Calibration samples were prepared similarly to the incubation samples of HLM, rCYP3A4, and rCYP3A5 to quantify midazolam concentrations between 0.11 and 21.5 μM (35–7000 ng/ml) and 1′-hydroxymidazolam concentrations between 0.03 and 5.85 μM (10–2000 ng/ml). Calibration was verified by quality-control samples at three concentration levels in the lower, middle, and upper calibration range. Calibration curves were established for both components using 1/X weighting. The limit of quantification was 35 for midazolam and 10 ng/ml for 1′-hydroxymidazolam.

Data Analysis. LC-MS/MS data were analyzed with Xcalibur and LCQuan software version 1.3 (Thermo Fisher Scientific). Apparent kinetic constants were analyzed by nonlinear regression analysis (curve fit) applying GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA). The formation of 1′-hydroxymidazolam (v) was described by the Michaelis-Menten model (eq. 1):

$$v = V_{max} \times [S]/(K_m + [S]) \tag{1}$$

In this model, V_{max} is the maximal product formation rate, and K_m is the substrate (S) concentrations at half-maximal product formation rate. The V_{max}/K_m ratio was calculated as intrinsic clearance.

Results

Kinetic Analyses and Heteroactivation of CYP3A-Mediated Midazolam Metabolism in HLM. The formation of 1′-hydroxymidazolam in HLM displayed Michaelis-Menten kinetics and confirmed a decrease in metabolite formation for midazolam concentrations above 10 μM (data not shown). All data were fit to the Michaelis-Menten model and revealed that efavirenz affects V_{max} rather than K_m. The presence of efavirenz altered the 1′-hydroxymidazolam formation rate in a concentration-dependent manner, and increasing efavirenz concentrations activated the V_{max} (Fig. 1; Table 1). V_{max} in the presence of the highest efavirenz concentration (5 μM) was approximately 1.5-fold greater than that of the control without efavirenz. At the same time, K_m results did not markedly change with increasing efavirenz concentrations. The K_m value was slightly decreased at 5 μM efavirenz, and the intrinsic clearance value (V_{max}/K_m ratio) was 3-fold increased
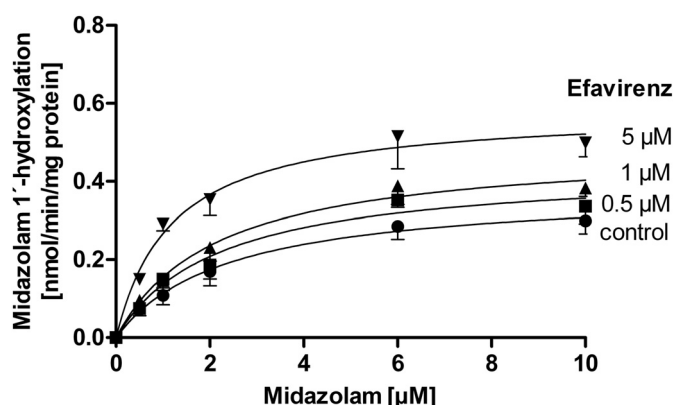
FIG. 1. Kinetic analysis of 1′-hydroxymidazolam formation in HLM: HLM were incubated with midazolam at 37°C for 5 min in the presence of efavirenz (0.5, 1, and 5 μM). Each data point represents the mean of the metabolite formation rate ± S.D. of triplicate samples. The lines represent best fit to the Michaelis-Menten model determined by nonlinear regression.

at the highest efavirenz concentration. The maximal heteroactivation (268% of control) was observed at 1 μM midazolam and an efavirenz/midazolam ratio of 5.

To ensure that the observed effects can be attributed to efavirenz and not to its main metabolite, HLM were incubated with midazolam and increasing 8-hydroxyefavirenz concentrations (0.5, 1, and 5 μM). Apparent V_{max} and K_m results were similar to the results of the control (Fig. 2), demonstrating that 8-hydroxyefavirenz did not alter 1′-hydroxymidazolam formation.

Inhibition of CYP3A. To verify that midazolam metabolism and its activation in HLM can be attributed to CYP3A, samples were incubated in the presence of the potent CYP3A inhibitor ketoconazole (0.2 μM), which significantly decreased the 1′-hydroxymidazolam formation rate (Fig. 3).

Heteroactivation in rCYP3A4 and rCYP3A5. The pronounced activation effect observed in HLM was further investigated in rCYP3A4 and rCYP3A5 supplemented with purified human cytochrome b_5 at midazolam concentrations up to 10 μM. The best fit for the formation of 1′-hydroxymidazolam in rCYP3A4 and rCYP3A5 was the Michaelis-Menten equation, and the metabolite formation rate in rCYP3A5 was more than 2-fold higher than the reaction catalyzed by rCYP3A4. Addition of 5 μM efavirenz activated 1′-hydroxylation of midazolam in both rCYP3A4 and
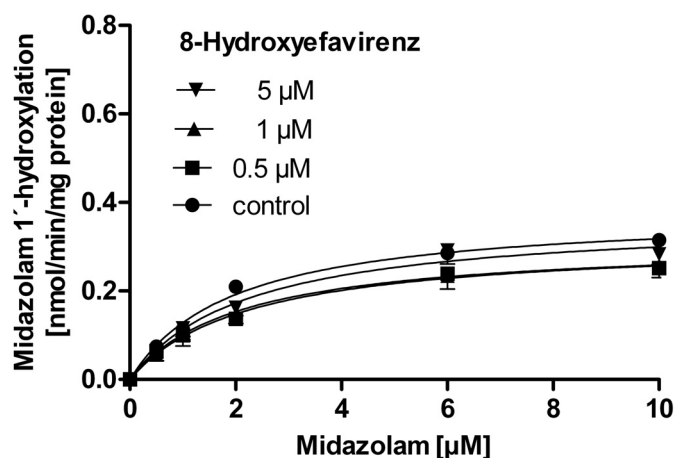

FIG. 2. Influence of 8-hydroxyefavirenz on midazolam metabolism in HLM: midazolam and HLM were incubated at 37°C for 5 min in the presence of 8-hydroxyefavirenz (0.5, 1, and 5 μM). Data are means ± S.D. of triplicate samples best fit to the Michaelis-Menten equation.

rCYP3A5 (Fig. 4; Table 1), and metabolite formation in the recombinant P450s in the presence of efavirenz followed Michaelis-Menten kinetics. In rCYP3A4, V_{max} increased from 1.3 to 1.8 pmol · min^{-1} · pmol P450^{-1}, and K_m increased slightly compared with the control samples without efavirenz. Midazolam hydroxylation was also investigated in the absence of cytochrome b_5. Without cytochrome b_5, hydroxylation still occurred, albeit at a slower V_{max} (1.0 pmol · min^{-1} · pmol P450^{-1}), and was not altered by efavirenz. The extent of activation of midazolam hydroxylation (V_{max}) in rCYP3A5 (28%) was comparable to the activation of rCYP3A4 (38%), and K_m did not change.

Discussion

Midazolam has become one of the paradigm markers of CYP3A activity in vitro and in vivo (Gorski et al., 1994; Thummel et al., 1994). Our in vitro study was a follow-up to an in vivo study in which a single dose of efavirenz (400 mg) significantly decreased midazolam exposure (area under the concentration-time curve from zero to infinity) likely by acute activation of midazolam metabolism (Bayer et al., 2009). Midazolam clearance significantly increased by 78%, and this effect occurred faster than induction of an enzyme via increase of mRNA transcription and could not be explained by reduced absorption. Therefore, the aim of this study was to clarify whether efavirenz may act as an activator of CYP3A.

TABLE 1

Effect of efavirenz on the midazolam 1′-hydroxylation by HLM, rCYP3A4, and rCYP3A5

All experiments were performed in triplicate. The kinetic parameters were calculated by nonlinear regression analysis (Michaelis-Menten, eq. 1). Data are presented as means ± S.D.

Efavirenz, μM	V_{max}[a]	K_m	V_{max}/K_m[b]
		μM	
Human liver microsomes			
0	0.38 ± 0.03	2.4 ± 0.7	158.3
0.5	0.44 ± 0.05	2.2 ± 0.9	200.0
1	0.49 ± 0.05	2.1 ± 0.5	233.3
5	0.58 ± 0.05	1.2 ± 0.3	483.3
rCYP3A4			
0	1.3 ± 0.2	0.8 ± 0.2	1.6
5	1.8 ± 0.2	1.6 ± 0.5	1.1
rCYP3A5			
0	3.9 ± 0.3	1.2 ± 0.3	3.3
5	5.0 ± 0.5	1.3 ± 0.5	3.8

[a] Units are nmol · min^{-1} · mg protein^{-1} for HLM and pmol · min^{-1} · pmol P450^{-1} for rCYP3A4 and rCYP3A5.

[b] For intrinsic clearance (V_{max}/K_m ratio), units are μl · min^{-1} · mg protein^{-1} for HLM and μl · min^{-1} · pmol P450^{-1} for rCYP3A4 and rCYP3A5.
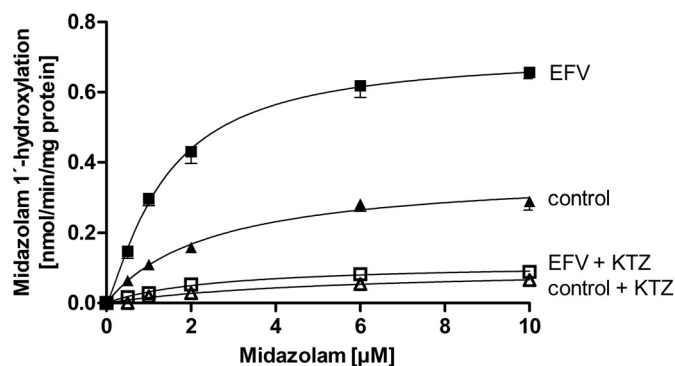

FIG. 3. Inhibition of midazolam metabolism in HLM with 0.2 μM ketoconazole (KTZ) in the presence of 5 μM efavirenz (EFV): inhibition of CYP3A decreased the formation of 1′-hydroxymidazolam significantly (open symbols) compared with the controls without inhibitor (closed symbols). Data represent the means ± S.D. of triplicate incubations.
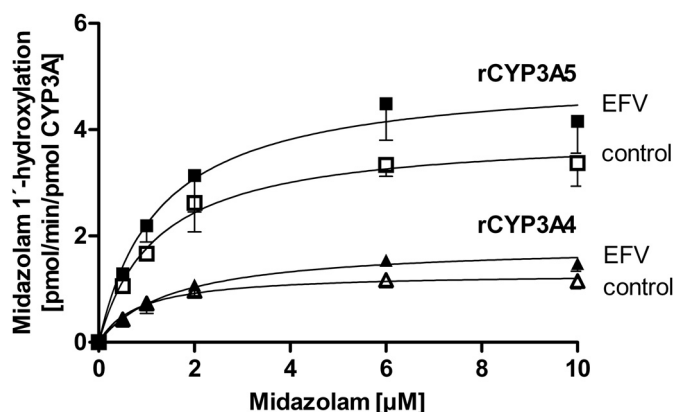
FIG. 4. Kinetic analyses of 1′-hydroxymidazolam formation in rCYP3A4 and rCYP3A5: midazolam and rCYP3A4 were incubated at 37°C for 10 and 5 min (rCYP3A5) in the presence of 5 μM efavirenz (EFV). Each data point represents the mean ± S.D. of triplicate samples. The lines represent the best fit to the Michaelis-Menten equation determined by nonlinear regression.

The formation of 1′-hydroxymidazolam in HLM displayed Michaelis-Menten kinetics in agreement with previous findings (Kronbach et al., 1989). We also confirmed a decrease in metabolite formation for midazolam concentrations above 10 μM, which is likely related to CYP3A4 inactivation by 1′-hydroxymidazolam (Martínez et al., 2000; Khan et al., 2002).

The formation of 1′-hydroxymidazolam was studied in HLM, rCYP3A4, and rCYP3A5, and the results of our study indeed provide evidence for a distinct acute activation of CYP3A in the presence of efavirenz. In HLM, the increase in the formation of 1′-hydroxymidazolam correlated with efavirenz concentrations, and the maximal activation was observed at a midazolam concentration of 1 μM, i.e., close to the calculated K_m value at 5 μM efavirenz. At the same time, K_m values did not substantially change, indicating that efavirenz does not increase the tightness of the binding of midazolam but somehow forces a repositioning toward more midazolam 1′-hydroxylation (F. P. Guengerich, personal communication). It was demonstrated previously that CYP2C9-mediated flurbiprofen 4′-hydroxylation was activated in the presence of dapsone as a result of repositioning of flurbiprofen more closely to the heme (Hummel et al., 2004). In vitro incubation with pooled HLM enables the formation of 8-hydroxyefavirenz, the main metabolite of efavirenz, by CYP2B6 (Ward et al., 2003). However, the efavirenz metabolite did not alter the 1′-hydroxymidazolam formation rate in our study, indicating that CYP3A activation was caused by the parent compound. This finding suggests that the steric configuration of the activator molecule influences its ability to activate the enzyme. Our data also indicate that activation rapidly reaches its maximum within minutes. Inhibition of the activation by ketoconazole demonstrates that activation occurs via CYP3A. The metabolite formation rate in rCYP3A5 was more than 2-fold greater compared with the rCYP3A4 catalyzed reaction, which is consistent with previous reports (Gorski et al., 1994). The activation was apparent in both rCYP3A4 and rCYP3A5 supplemented with cytochrome b_5. The activation in rCYP3A4 was strongly dependent on cytochrome b_5, and 1′-hydroxymidazolam formation was not activated in rCYP3A4 without cytochrome b_5.

Heterotropic positive cooperativity, activation of the metabolism of a substrate by another drug, has been observed with several P450s before. The first evidence that two different molecules (two substrates or substrate and activator) can simultaneously bind to the same P450 active site was provided when investigating phenanthrene and 7,8-benzoflavone CYP3A4-mediated metabolism (Shou et al., 1994). In an attempt to interpret the complex kinetics of CYP3A4, spectral titration studies provided evidence that at least two and probably three binding sites are relevant for CYP3A4 metabolism (Hosea et al., 2000). Moreover, two midazolam molecules may simultaneously bind to the CYP3A4 active site to form the two metabolites 1′- and 4-hydroxymidazolam, albeit only 1′-hydroxymidazolam is formed at clinically approved doses of the drug (Khan et al., 2002; Kapelyukh et al., 2008).

In adult and fetal liver microsomes and rCYP3A4, midazolam 1′-hydroxylation was activated by α-naphthoflavone (Mäenpää et al., 1998). Incubation of rCYP3A4 with midazolam and quinidine stimulated the formation of 1′-hydroxymidazolam at low quinidine concentrations (Galetin et al., 2002). Heterotropic positive cooperativity may also explain the interaction of thalidomide with midazolam in human CYP3A5 in which thalidomide increased midazolam 1′-hydroxylation and total midazolam oxidation (Okada et al., 2009). Midazolam 1′-hydroxylation was shown to be activated by sorafenib and sunitinib in CYP3A5 (Sugiyama et al., 2011) and ticagrelor in HLM (Zhou et al., 2011).

Two fundamental molecular mechanisms, multiple active site binding and binding at a peripheral allosteric site, have been proposed to describe the simultaneous binding of multiple ligands (Atkins, 2006). Although preferentially metabolized by CYP2B6, efavirenz is a substrate of CYP3A4 and CYP3A5 as well (Ward et al., 2003), indicating that it can bind to the active center of CYP3A4 (Mannu et al., 2011). Hence, the drug-drug interaction described here could occur via heterotropic positive cooperativity assuming that both midazolam and efavirenz bind simultaneously to the same P450. Multiple binding at the active site is expected to be minor, because the investigated efavirenz concentration (5 μM) is below the estimated K_m values of 19.1 in rCYP3A5 and 23.5 μM in rCYP3A4 (Ward et al., 2003), and the metabolic turnover of efavirenz at CYP3A4 and CYP3A5 is low compared with that of midazolam. Instead, efavirenz bound to a peripheral site could still alter the binding of midazolam within the active center of the enzyme and consequently increase midazolam 1′-hydroxylation. The high sequence homology between the two isoforms may explain why activation was observed in both rCYP3A4 and rCYP3A5. A study investigating the effect of α-naphthoflavone on the sequential metabolism of Nile red suggested a high-affinity binding site for α-naphthoflavone distal from the immediate heme environment (Woods et al., 2011). However, the identification of true allosterism is hampered by P450-P450, P450-NADPH-P450 reductase, and P450-cytochrome b_5 interactions in which the behavior of an effector is dependent on substrate, effector-substrate concentration, and specific P450 isoform (Atkins, 2005). Cytochrome b_5 is mandatory and may stimulate or inhibit P450 enzymes depending on substrate and isoform. In CYP3A4 and CYP3A5, the presence of cytochrome b_5 increased V_{max} values of midazolam 1′-hydroxylation (Yamaori et al., 2003). In our study, midazolam-1′-hydroxylation was activated in both rCYP3A4 and rCYP3A5 supplemented with cytochrome b_5, whereas the stimulation was not observed in rCYP3A4 without cytochrome b_5. Accordingly, baseline hydroxylation activity was slightly higher in rCYP3A4 with cytochrome b_5. Cytochrome b_5 plays various roles in P450 monooxygenase reactions (Schenkman and Jansson, 2003). Although the general role of an electron transfer component to P450 is widely accepted, the exact mechanism of action remains ambiguous. One possible mechanism is the enhancement of coupling between P450 and NADPH-P450 reductase in the presence of cytochrome b_5, which in our case could have additionally been enhanced by efavirenz. Increased coupling was identified as a mechanism of CYP2C9 activation, leading to increased product formation (Hutzler et al., 2003).

It was demonstrated that CYP3A5-mediated formation of 1′-hydroxymidazolam is highly sensitive to NADPH-P450 reductase activ-

ity (Christensen et al., 2011). Hence, the slightly varying level of activation in rCYP3A4 and rCYP3A5 may be explained by the difference in NADPH-P450 reductase activity, whereas a low reductase activity promotes the impact of cytochrome b_5.

Although the quantitative results of our study with recombinant P450 enzymes may not closely reflect the in vivo situation because of nonphysiological ratios of cytochrome b_5 and NADPH-P450 reductase, they may support qualitative conclusions. Indeed, examples of in vivo CYP3A stimulation are rare and include the enhancement of CYP3A-mediated hepatic clearance of diclofenac by quinidine in monkeys (Tang et al., 1999), the interaction between felbamate and carbamazepine caused by CYP3A4 heteroactivation (Egnell et al., 2003), and the alteration of the 1'-hydroxymidazolam/4-hydroxymidazolam ratio by fluconazole (Yang et al., 2012).

In conclusion, we confirmed a distinct acute activation of the metabolism of midazolam by efavirenz in vitro, supporting previously obtained data in a clinical study. A P450 induction via increased mRNA transcription as an underlying mechanism can be excluded, because the observed effect occurs immediately and in absence of the transcription machinery. Although our data do not elucidate the exact mechanism underlying the activation, they do suggest the involvement of multiple ligand binding at CYP3A. To our knowledge, in vitro heteroactivation of CYP3A by efavirenz has not been reported. Whether efavirenz can also activate the CYP3A-mediated metabolism of other substrates remains to be investigated.

Acknowledgments

Authorship Contributions

Participated in research design: Keubler, Weiss, Haefeli, Mikus, and Burhenne.

Conducted experiments: Keubler and Burhenne.

Performed data analysis: Keubler, Weiss, and Burhenne.

Wrote or contributed to the writing of the manuscript: Keubler, Weiss, Haefeli, Mikus, and Burhenne.

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