Drug interaction of efavirenz and midazolam: Efavirenz activates the CYP3A-mediated midazolam 1'-hydroxylation in vitro

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Abbreviations: CYP, cytochrome P450; rCYP3A4, recombinant human cytochrome P450 3A4; rCYP3A5, recombinant human cytochrome P450 3A5; HLMs, human liver microsomes; NADPH-CYP reductase, NADPH-cytochrome P450 reductase; $K_m$, substrate concentration at half-maximal product formation rate; LC/MS/MS, liquid chromatography-tandem mass spectrometry; $V_{max}$, maximal product formation rate.
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Abstract

Cytochromes P450 (CYP) 3A4/3A5 are the most important drug metabolizing enzymes. For several drugs heteroactivation of CYP3A-mediated reactions has been demonstrated in vitro. Recent in vivo data suggested a possible acute activation of CYP3A4-catalyzed midazolam metabolism by efavirenz. We therefore 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 (HLMs) 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 (~1.5-fold increased at 5 μM efavirenz in HLMs and ~1.4-fold in rCYP3A4). The activation in rCYP3A4 was dependent on cytochrome b5, and the activating effect was also observed in rCYP3A5 supplemented with cytochrome b5 where V_max was ~1.3-fold enhanced. Concomitant inhibition of CYP3A activity with ketoconazole in HLMs abolished the increase in 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 and 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 cytochrome P450 (CYP) 3A subfamily contains the most abundant human CYP enzyme and plays a major role in phase I metabolism (Guengerich, 2006). Members of this enzyme subfamily catalyze the metabolism of approximately 40-50% of the currently marketed drugs (Thummel and Wilkinson, 1998). However, the wide substrate specificity facilitates major drug-drug interactions due to inhibition and induction of these enzymes (Lin and Lu, 1998). In addition to inhibition and induction, several CYP isoforms are stimulated in vitro. Activation (heterotropic positive cooperativity) occurs when CYP 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 (NNRTI) efavirenz was approved in combination with other antiretroviral agents for the treatment of HIV-1 infection (Adkins and Noble, 1998). Efavirenz is characterized by a high protein binding (>99.5%) and a long terminal half-life (52-76 h), which is reduced to 40-55 h due to 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.,
In different species, including humans, 8-hydroxyefavirenz, 7-hydroxyefavirenz, 8,14-dihydroxyefavirenz, and further secondary metabolites were also detected. Efavirenz acts both as a CYP3A4 inducer and inhibitor. 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 recently 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. We therefore aimed to investigate the effect of efavirenz on the in vitro metabolism of midazolam to clarify whether an 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 (HLMs) and recombinant human CYP3A4 and CYP3A5 (rCYP3A4 and rCYP3A5) confirmed the activating effect of efavirenz. Moreover, we also 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, d5-midazolam, and 13C3-1′-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 Na3, isocitric dehydrogenase, and β-nicotinamide-adenine dinucleotide phosphate disodium were purchased from Sigma-Aldrich (Steinheim, Germany), natriumdihydrogen-phosphate monohydrate and MgCl2·6H2O were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents were of HPLC grade and obtained from Sigma-Aldrich or Merck KGaA. 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).

HLMs and recombinant CYPs
HLMs (pooled, mixed gender, CMV-negative), rCYP3A4 Easy CYP Bactosomes and rCYP3A5 Easy CYP Bactosomes coexpressed with NADPH-cytochrome P450 reductase (NADPH-CYP reductase) in Escherichia coli, and supplemented with purified human cytochrome b5 were purchased from tebu-bio (Offenbach, Germany). P450 concentration, cytochrome b5 concentration, and NADPH-CYP reductase activity were 8.3 nmol/ml, 8.68 nmol/ml, and 194 nmol/min/mg protein for the HLMs. For both recombinant preparations, P450 concentration (1.0 nmol/ml) and cytochrome b5 concentration (5.0 nmol/ml) were identical while NADPH-CYP reductase activity was 568 nmol/min/mg protein for rCYP3A4 and 1460 nmol/min/mg protein for rCYP3A5. The in vitro preparations were stored at -80 °C until use and suspended in 0.1 M potassium phosphate buffer ahead of the experiments.

Incubation conditions
The incubation mixtures (final volume 250 μl) contained incubation buffer (0.1 M NaH₂PO₄ H₂O, 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 Na₃, 1.02 mg/ml MgCl₂·6H₂O), and either HLMs or rCYPs. The reaction was started by adding 25 μl of the NADPH-regenerating system and incubated for the respective time at 37 °C in a thermomixer. Incubations were terminated by addition of 225 μl acetonitrile. The samples were immediately centrifuged at 16,100 g for 3 min. Aliquots of the supernatants (50 μl) were diluted in LC mobile phase (600 μl) containing the internal standards d₅-midazolam and ¹³C₃-1´-hydroxymidazolam. An aliquot (20 μl) was injected into the liquid chromatography-tandem mass spectrometry (LC/MS/MS) system.

*Initial kinetic studies with HLMs and recombinant CYPs*

Initial kinetic studies were conducted to determine the optimal protein concentration for HLMs, rCYP3A4, and rCYP3A5 and the optimal incubation time. Midazolam concentrations above 15 μM were previously reported to decrease Vₘₐₓ in HLMs (Martínez et al., 2000). Therefore, HLMs (0, 0.005, 0.01, 0.05, 0.1, and 0.2 mg protein/ml) were incubated with 10 μM midazolam for 20 min. Optimum 1´-hydroxymidazolam formation rate was reached when 0.1 mg protein/ml pooled HLMs were used. For predefinition of optimal incubation time, HLMs (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. Due to higher metabolic rates in the presence of efavirenz, HLMs concentration was reduced to 0.05 mg protein/ml in all subsequent incubations with HLMs.

For rCYP3A4, 10 min incubation time and 0.1 mg protein/ml and for rCYP3A5, 10 min incubation time and 0.05 mg protein/ml were selected.

*Kinetic analyses in HLMs and recombinant CYPs*
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 HLMs (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 HLMs 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 HLMs (Gibbs et al., 1999). The CYP3A inhibition experiment was performed in HLMs under the incubation conditions described above and ketoconazole was added to the samples prior to addition of NADPH₂.

Quantification of midazolam and 1′-hydroxymidazolam

The formation of 1′-hydroxymidazolam was determined by LC/MS/MS analysis (Thermo Fisher Scientific Surveyor LC coupled to TSQ7000, Waltham, MA, USA) in the multiple reaction monitoring mode (MRM) as recently published (Hafner et al., 2010) with minor modifications. In brief, the compounds were separated at 40 °C on a Synergi Max-RP column (150 x 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, 13C₃-1′-hydroxymidazolam, midazolam, and d₅-midazolam was obtained with electrospray ionisation (ESI) in the positive mode. MS/MS transitions monitored in MRM 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 d₅-midazolam, and m/z 345→m/z 206 at 44 V for ¹³C₃-1′-hydroxymidazolam. ESI-in-source collision-induced dissociation value was set to 10V.

Calibration samples were prepared similarly to the incubation samples of HLMs, rCYP3A4, and rCYP3A5 to quantify midazolam concentrations between 0.11–21.5 µM (35-7000 ng/ml) and 1′-hydroxymidazolam concentrations between 0.03–5.85 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 weighing. The limit of quantification was 35 ng/ml for midazolam and 10 ng/ml for 1′-hydroxymidazolam.

Data Analysis

LC/MS/MS data was 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, USA). The formation of 1′-hydroxymidazolam (v) was described by the Michaelis-Menten model (equation 1).

\[ v = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \]  

(equation 1)

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

Kinetic analyses and heteroactivation of CYP3A-mediated midazolam metabolism in HLMs

The formation of 1′-hydroxymidazolam in HLMs displayed Michaelis-Menten kinetics and confirmed a decrease in metabolite formation for midazolam concentrations above 10 μM (data not shown). All data was fit to the Michaelis-Menten model and revealed that efavirenz effects \( V_{\text{max}} \) 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 maximal product formation rate (Fig. 1, Table 1). \( V_{\text{max}} \) in the presence of the highest efavirenz concentration (5 μM) was approximately 1.5-fold greater than 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 (\( V_{\text{max}}/K_m \) ratio) was 3-fold increased 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, HLMs were incubated with midazolam and increasing 8-hydroxyefavirenz concentrations (0.5, 1, and 5 μM). Apparent \( V_{\text{max}} \) 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 HLMs can be attributed to CYP3A, samples were also 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 HLMs 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 rCYP3A5 (Fig. 4, Table 1) and metabolite formation in the recombinant CYPs in the presence of efavirenz followed Michaelis-Menten kinetics. In rCYP3A4, V<sub>max</sub> increased from 1.3 to 1.8 pmol/min/pmol CYP and K<sub>m</sub> increased slightly compared to the control samples without efavirenz. Midazolam hydroxylation was also investigated in the absence of cytochrome b<sub>5</sub>. Without cytochrome b<sub>5</sub>, hydroxylation still occurred, albeit at a slower V<sub>max</sub> (1.0 pmol/min/pmol CYP), and was not altered by efavirenz. The extent of activation of midazolam hydroxylation (V<sub>max</sub>) in rCYP3A5 (28%) was comparable to the activation of rCYP3A4 (38%) and K<sub>m</sub> 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 ($\text{AUC}_{0-\infty}$) 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.

The formation of 1´-hydroxymidazolam in HLMs 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 $\mu$M, 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 HLMs, 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 HLMs, the increase in the formation of 1´-hydroxymidazolam correlated with efavirenz concentrations and the maximal activation was observed at a midazolam concentration of 1 $\mu$M i.e. close to the calculated $K_m$ value at 5 $\mu$M EFV. 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 force a repositioning towards more midazolam 1´-hydroxylation (Guengerich FP, personal communication). It was previously demonstrated that CYP2C9-mediated flurbiprofen 4´-hydroxylation was activated in the presence of dapsone due to repositioning of flurbiprofen more closely to the heme (Hummel et al., 2004). In vitro incubation with pooled HLMs 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 to 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 \( b_5 \). Noteworthy, 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 CYPs before. The first evidence that two different molecules (two substrates or substrate and activator) can simultaneously bind to the same CYP 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 alpha-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). More recently, midazolam 1´-hydroxylation was shown to be activated by sorafenib and sunitinib in CYP3A5 (Sugiyama et al., 2011) and ticagrelor in HLMs (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). Efavirenz, although preferentially metabolized by CYP2B6, 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 CYP. 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 μM 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 to 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 recent study investigating the effect of alpha-naphthoflavone on the sequential metabolism of nile red suggested a high-affinity binding site for alpha-naphthoflavone distal from the immediate heme environment (Woods et al., 2011). The identification of true allosterism, however, is hampered by CYP-CYP, CYP-NADPH-CYP reductase, and CYP-cytochrome $b_5$ interactions in which the behavior of an effector is dependent on substrate, effector-substrate concentration, and specific CYP isoform (Atkins, 2005). Cytochrome $b_5$ is mandatory and may stimulate or inhibit CYP enzymes depending on substrate and isoform. In CYP3A4 and CYP3A5, the presence of cytochrome $b_5$ increased $V_{\text{max}}$ 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 $b_5$ while 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). While the general role of an electron transfer component to
CYP is widely accepted, the exact mechanism of action remains ambiguous. One possible mechanism is the enhancement of coupling between CYP and NADPH-CYP reductase in the presence of cytochrome \( b_5 \) which in our case could have additionally be enhanced by efavirenz. Increased coupling was identified as a mechanism of CYP2C9 activation leading to increased product formation (Hutzler et al., 2003).

Recently, it was demonstrated that CYP3A5-mediated formation of 1'-hydroxymidazolam is highly sensitive to NADPH-CYP reductase activity (Christensen et al., 2011). Hence, the slightly varying level of activation in rCYP3A4 and rCYP3A5 may be explained by the difference in NADPH-CYP reductase activity whereas a low reductase activity promotes the impact of cytochrome \( b_5 \).

While the quantitative results of our study with recombinant CYP enzymes may not closely reflect the in vivo situation because of non-physiological ratios of cytochrome \( b_5 \) and NADPH-CYP 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 CYP induction via increased mRNA transcription as 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 before. Whether efavirenz can also activate the CYP3A-mediated metabolism of other substrates remains to be investigated.
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Authorship Contributions

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

Conducted experiments: Keubler, Burhenne

Contributed new reagents or analytical tools: -

Performed data analysis: Keubler, Weiss, Burhenne

Wrote or contributed to the writing of the manuscript: Keubler, Weiss, Haefeli, Mikus, Burhenne
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Christensen H, Hestad AL, Molden E, and Mathiesen L (2011) CYP3A5-mediated metabolism of midazolam in recombinant systems is highly sensitive to NADPH-cytochrome P450 reductase activity. Xenobiotica 41:1-5.


Figure legends:

**Fig. 1** Kinetic analysis of 1´-hydroxymidazolam formation in HLMs: HLMs were incubated with midazolam at 37 °C for 5 min in presence of efavirenz (0.5, 1, and 5 μM). Each data point represents the mean of the metabolite formation rate ± SD of triplicate samples. The lines represent best fit to the Michaelis-Menten model determined by nonlinear regression.

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

**Fig. 3** Inhibition of midazolam metabolism in HLMs 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 to the controls without inhibitor (closed symbols). Data represent the means ± SD 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 min and 5 min (rCYP3A5) in the presence of 5 μM efavirenz (EFV). Each data point represents the mean ± SD of triplicate samples. The lines represent the best fit to the Michaelis-Menten equation determined by nonlinear regression.
Table legends:

Table 1: Effect of efavirenz on the midazolam 1'-hydroxylation by human liver microsomes, rCYP3A4, and rCYP3A5

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<th>Efavirenz [µM]</th>
<th>$V_{\text{max}}^a$</th>
<th>$K_m$ [µM]</th>
<th>$V_{\text{max}}/K_m^b$</th>
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<td>Human liver microsomes</td>
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<td>5.0 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

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

$^a$ Units are nmol/min/mg protein for HLMs and pmol/min/pmol CYP for rCYP3A4 and rCYP3A5.

$^b$ For intrinsic clearance ($V_{\text{max}}/K_m$ ratio), units are µl/min/mg protein for HLMs and µl/min/pmol CYP for rCYP3A4 and rCYP3A5.
Figure 2

8-Hydroxyefavirenz

- ▼ 5 μM
- △ 1 μM
- □ 0.5 μM
- ● control

Midazolam 1'-hydroxylation [nmol/min/mg protein]

0.0 0.2 0.4 0.6 0.8

0 2 4 6 8 10

Midazolam [μM]
Figure 3

Midazolam 1'-hydroxylation [nmol/min/mg protein]

0.0 0.2 0.4 0.6 0.8
0 2 4 6 8 10

Midazolam [µM]

EFV
control
EFV + KTZ
control + KTZ
Figure 4

Midazolam 1'-hydroxylation
[pmol/min/pmol CYP3A] vs. Midazolam [μM]

- rCYP3A5
  - EFV
  - Control
- rCYP3A4
  - EFV
  - Control