Inhibition and stimulation of intestinal and hepatic CYP3A activity: studies in humanized CYP3A4 transgenic mice using triazolam


Running title: Inhibition and stimulation of intestinal and hepatic CYP3A

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Abbreviations: CYP, cytochrome P450; Cyp3a-/-, cytochrome P450 3A knockout mice; Cyp3a-/-;
Tg-3A4 hepat Cyp3a knockout mice with liver-specific transgenic expression of human CYP3A4;
Cyp3a-/-Tg-3A4 int, Cyp3a knockout mice with intestine-specific transgenic expression of human
CYP3A4; P-gp, P-glycoprotein, Tg, transgenic.
Abstract

CYP3A4 is an important determinant of drug-drug interactions. Here, we evaluated whether Cyp3a<sup>-/-</sup> and CYP3A4 transgenic (CYP3A4-Tg) mice can be utilized to study drug-drug interactions in the liver and intestine. Triazolam was used as a probe drug because it is a highly specific CYP3A substrate and not a P-glycoprotein substrate. Triazolam metabolism was profoundly reduced in Cyp3a<sup>-/-</sup> mice, both in vitro and in vivo. In vitro studies revealed clear species differences in human and mouse, but triazolam metabolism in microsomes derived from CYP3A4-Tg “humanized” mice closely resembled that in human microsomes. Interestingly, studies with tissue-specific CYP3A4-Tg mice revealed that intestinal CYP3A4 has a major impact on oral triazolam exposure, whereas the effect of hepatic CYP3A4 was limited. To mimic a drug-drug interaction, we co-administered triazolam with the prototypical CYP3A inhibitor ketoconazole, which increased triazolam exposure in all CYP3A-proficient mouse strains but not in Cyp3a<sup>-/-</sup> mice. We further found that the anti-cancer drug gefitinib is a potent stimulator of 1'-OH triazolam formation, in vitro. Importantly, also in vivo we could demonstrate stimulation of triazolam metabolism by gefitinib, resulting in a lower oral triazolam exposure. To our knowledge this is the first in vivo example of direct stimulation of CYP3A4 activity after oral drug administration. Overall, this study illustrates how Cyp3a<sup>-/-</sup> and CYP3A4-Tg mice can be utilized to study drug-drug interactions. The data clarify that also for drugs that are not P-glycoprotein substrates, intestinal metabolism can be more important than hepatic metabolism after oral administration.
Introduction

The cytochrome P450 3A (CYP3A) enzymes represent one of the most important drug metabolizing systems, affecting ~50% of currently prescribed drugs (Guengerich, 1999). Since so many drugs are substrates and/or inhibitors for CYP3A, the enzyme is also an important determinant of many drug-drug interactions (Thummel and Wilkinson, 1998; Dresser et al., 2000). The most common type of CYP3A-mediated drug-drug interaction is that one drug inhibits CYP3A activity, which leads to higher levels of other drugs metabolized by CYP3A, potentially leading to toxicity. This was for example the case for the antihistaminic terfenadine and the antihypertensive mibebradil, which were consequently both withdrawn from the market. While most drug-drug interactions are undesirable, in certain cases CYP3A can be inhibited on purpose to improve drug therapy. For instance, the CYP3A inhibitor ritonavir is given in combination with lopinavir, which improves the lopinavir oral bioavailability considerably (Kumar et al., 1999). Not only drugs but also food constituents can be potent inhibitors of CYP3A. For example, components of grapefruit juice potently inhibit CYP3A and several clinically relevant drug-grapefruit juice interactions have been described (Dresser and Bailey, 2003; Paine and Oberlies, 2007).

Another possible mechanism of a drug-drug interaction is that one drug directly increases the rate of CYP3A-mediated metabolism of another drug. This stimulation of the metabolism is also known as heterotropic positive cooperativity (Tang and Stearns 2001; Hutzler and Tracy, 2002; Houston and Galetin, 2005). Stimulation of metabolism could be of clinical relevance as it could result in sub-therapeutic drug levels. A classical example of a drug that is known to stimulate several CYP3A-mediated reactions is 7,8-benzoflavone, which for instance increases the metabolism of diazepam and aflatoxin B1 in vitro (Andersson et al., 1994; Ueng et al., 1997). It has to be noted that, although many in vitro examples of CYP3A stimulation have been published, there is only very limited in vivo evidence for this particular drug-drug interaction (Hutzler and Tracy, 2002; Wienkers and Heath, 2005).
Recently, we have generated *Cyp3a* knockout mice to study this important drug-handling system *in vivo* (van Herwaarden et al., 2007). In addition, we created *Cyp3a<sup>−/−</sup>* transgenic mice with expression of human CYP3A4 exclusively in the intestine or in the liver (van Herwaarden et al., 2007). We subsequently utilized these mice to determine the relative importance of intestinal versus hepatic CYP3A4 activity in first-pass drug metabolism. These studies revealed that intestinal CYP3A4 alone was sufficient to virtually abrogate net docetaxel entry from the gut, whereas hepatic CYP3A4 was more important in systemic docetaxel clearance (van Herwaarden et al., 2007). This study clarified the potential significance of intestinal metabolism, which has been a matter of debate for decades (Lin et al., 1999; Doherty and Charman, 2002; Thummel, 2007).

Besides being a good CYP3A4 substrate, however, docetaxel is also a very good substrate of the apical drug transporter P-glycoprotein (P-gp) (Bardelmeijer et al., 2002). It has been hypothesized that the function of P-gp could prevent saturation of intestinal CYP3A and give the enzyme repeated access to its substrates, resulting in highly efficient intestinal metabolism (Benet and Cummins, 2001). We considered that P-gp might have contributed greatly to the efficiency of intestinal CYP3A4-mediated docetaxel metabolism. Hence, it is not clear whether intestinal CYP3A can also dominate the metabolism of substrates that are not transported by P-gp, such as midazolam. Unfortunately, useful analysis of midazolam pharmacokinetics in our mouse models was precluded by the upregulation of (midazolam-metabolizing) mouse CYP2C in the *Cyp3a<sup>−/−</sup>* mice (van Waterschoot et al., 2008).

In contrast to midazolam, the closely related drug triazolam is a more specific substrate for CYP3A compared to other mouse CYP isoforms (Perloff et al., 2000). It might therefore be a better probe drug to characterize our *Cyp3a<sup>−/−</sup>* and CYP3A4 transgenic mouse lines. Similar to midazolam, triazolam is also not a P-gp substrate (von Moltke et al., 2004). In this study we therefore used triazolam to study the relative contribution of intestinal and hepatic CYP3A–mediated metabolism. Furthermore, we also assessed whether our mouse models could be utilized to study in vivo drug-drug interactions such as inhibition and stimulation of CYP3A activity.
Materials & Methods

Materials

Triazolam and 1’-OH triazolam were obtained from Sigma (St. Louis, MO, USA). D4-triazolam and D4-1’-hydroxy-triazolam were both obtained as 100 µg/ml solutions in methanol from Cerilliant (Round Rock, TX, USA). NADPH-generation system, pooled human liver and intestinal microsomes were obtained from BD Bioscience (Alphen aan den Rijn, The Netherlands). Gefitinib, imatinib, erlotinib, dasatinib, sorafenib and sunitinib were purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Pentobarbital (Nembutal) was obtained from Sanofi Sante BV (Maassluis, the Netherlands) and methoxyflurane (Metofane) was obtained from Medical Developments Australia Pty. Ltd. (Springvale, Australia). All other chemicals were of analytical grade and were obtained from commercial sources.

Animals

The mice used in this study were housed and handled according to institutional guidelines complying with Dutch legislation. The animals were kept in a temperature-controlled environment with a 12:12-hour light/dark cycle and permitted ad libitum consumption of acidified water and a standard (AM-II) diet (Hope Farms, the Netherlands), unless indicated otherwise. Wild-type, Cyp3a knockout (Cyp3a−/−), or Cyp3a−/− mice with specific expression of human CYP3A4 in either the liver (Cyp3a−/−Tg-3A4Hep (previously Cyp3a−/−A)), intestine (Cyp3a−/−Tg-3A4Int (previously Cyp3a−/−V)) or both (Cyp3a−/−Tg-3A4Hep/Int (previously Cyp3a−/−AV)) (van Herwaarden et al., 2007) were used. All mouse strains were of a homogeneous (>99%) FVB genetic background and all experiments were done using male mice between 8 and 12 weeks old.

Microsomal incubations

Mouse liver and intestinal microsomes were prepared using the whole tissues as previously described (van Waterschoot et al., 2008). The incubations were carried out in a total volume of
200 μl, containing 100 mM potassium phosphate (KPi) buffer pH 7.4. Protein concentrations were 0.5 mg/ml for liver microsomes and 1 mg/ml for intestinal microsomes, unless indicated otherwise. Formation of triazolam metabolites was linear with respect to incubation time and microsomal protein concentration. Control experiments without cofactor were performed to ascertain CYP-dependent metabolism. Final concentration of methanol was 0.5% in all incubations. After 5 min of preincubation at 37°C, the reactions were initiated by addition of a NADPH-regenerating system. After 20 minutes the reactions were stopped by adding 100 μl ice-cold acetonitrile and cooling on ice for 5 minutes. After centrifugation (10 min at 6,800 g), 50 μl of the sample was injected into the HPLC system.

The enzyme kinetics for both 1'-OH and 4-OH triazolam were investigated using GraphPad Prisms 4.0 non-linear regression analysis. Most of the kinetic data could be fitted using a standard Michaelis-Menten equation:

\[
V = \frac{V_{\text{max}} \cdot [s]}{K_m + [s]}
\]

Where indicated, a Michaelis-Menten kinetics model with non-competitive substrate inhibition (von Moltke et al., 1996) was used.

\[
V = \frac{V_{\text{max}} \cdot [s]}{K_m + [s] \cdot (1 + \frac{[s]}{K_s})}
\]

**Stimulation of 1'-OH triazolam formation in vitro**

Microsomes were pre-incubated for 4 minutes in the presence of gefitinib, imatinib, erlotinib, dasatinib, sorafenib and sunitinib (12.5 μM final concentration) leading to 0.2% DMSO in the final incubation. Subsequently, triazolam was added and pre-incubated for 1 minute after which the reaction was started by adding the NADPH-regenerating system. Other conditions were similar as described above.
Chemical and immunoinhibition

For the inhibition experiments two antibodies targeting rat CYP2C11 were used. Polyclonal anti-CYP2C11 goat antibodies were obtained from Daiichi Pure Chemical Co. (Tokyo, Japan) and monoclonal anti-CYP2C11 mouse antibodies were obtained from Invitrogen (Venlo, The Netherlands) referred to as Anti-CYP2C-A and Anti-CYP2C-B, respectively. After 10 min of pre-incubation with ketoconazole (2.5 μM final concentration) or one of two anti-CYP2C11 antibodies, the microsomal reaction mixture was incubated for 20 minutes. The final concentration of triazolam in the incubations was 50 μM. All other conditions were as described above.

HPLC

For determination of triazolam and its metabolites, the HPLC mobile phase consisted of 20% acetonitrile, 24% methanol and 56% 5 mM phosphate buffer (pH: 7.4, 0.15% triethylamine), with a flow rate of 0.4 ml/min. The analytical column was a reverse phase C18 XBridge; 3.0 x 150, 3.5 μm (Waters, Milford, MA, USA). Column effluent was monitored by UV absorption at 230 nm. Identity and quantity of 1’-OH and 4-OH triazolam metabolites were verified using the retention time and standard curve of an authentic 1’-OH triazolam standard. For determining triazolam in the presence of gefitinib, the mobile phase consisted of two solutions. Solution A consisted of 20% acetonitrile, 30% methanol and 50% 5 mM phosphate buffer (pH: 7.4, 0.15% triethylamine). Solution B consisted of 60% acetonitrile and 40% H2O. From 0 to 5 and from 8 to 20 minutes after injection, the mobile phase consisted of 100% A. Between 5 and 8 minutes after injection, the mobile phase consisted of a mix of A and B (10:90).

Triazolam pharmacokinetics in vivo

30 min before triazolam administration, fasted mice (2 hrs) received orally either ketoconazole (35 mg/kg) in PEG 400, gefitinib (25 mg/kg) in PEG 400 or vehicle (PEG 400). At t = 0, triazolam in ethanol and saline (1:99) was administered by oral gavage at 0.5 mg/kg, 10, 20, 40,
Blood samples were collected by tail sampling. Blood was centrifuged for 6 min at 6,800 g, after which 25 μl of serum was collected and stored at –20 ºC until further analysis.

**LC-MS/MS**

Mouse plasma samples were measured by LC-MS/MS. To a 20-μl plasma sample, 100 μl of 25% (v/v) methanol, 20 μl of 40 ng/ml D₄-triazolam and 10 ng/ml D₄-1′-hydroxy-triazolam in 25% (v/v) methanol and 200 μl of 5 mM sodium hydroxide were added. The analytes were extracted with 2 ml diethylether and the organic phase was evaporated under a stream of nitrogen at 30ºC. The residue was reconstituted in 100 μl of 50% (v/v) methanol before injection in the chromatographic system.

The LC-MS/MS equipment consisted of a DGU-14A degasser, a Sil-HTc autosampler, two LC10-ADvp-μ pumps and a CTO10-Avp column oven (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). Data were processed with the Finnigan Xcalibur software (version 1.4, Thermo Electron).

20 μl injections were made on a Polaris 3 C18-A column (50 x 2 mm, dₚ = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 x 2 mm, dₚ = 3 μm, Varian). The column temperature was maintained at 35ºC and the autosampler was maintained at 4ºC. The flow rate was 0.3 ml/min and the eluent comprised 35% (v/v) of 0.01% (v/v) formic acid in water and 65% (v/v) methanol.

Mass transitions (collision energies (V)) were 343→308(29), 239(43) and 315(28) for triazolam, 359→331(27), 239(44) and 250(38) for 1′-OH triazolam, 347→312(27) for D₄-triazolam and 365→337(29) for D₄-1′-OH triazolam (transition of ³⁷Cl-D₄-1′-hydroxy-triazolam was used because of isotopic interference, mainly by ³⁷Cl₂-1′-hydroxy-triazolam). The mass resolutions
were set at 0.2 full with at half height for the first quadrupole and at 0.7 full with at half height (unit resolution) for the third quadrupole for all compounds.
Results

Triazolam metabolism in liver and intestinal microsomes of Cyp3a−/− and CYP3A4-Tg mice

The biotransformation of triazolam by CYP3A enzymes yields 1′-OH and 4-OH triazolam as the principal metabolites (Kronbach et al., 1989; von Moltke et al., 1996). To evaluate the differences in metabolic activity between wild-type, Cyp3a−/− and CYP3A4 transgenic mice, we performed microsomal incubations with triazolam. In wild-type mouse liver microsomes triazolam was metabolized to its 1′-OH and 4-OH metabolites and both metabolite formation reactions obeyed normal Michaelis-Menten kinetics (Figure 1). Note that at lower triazolam concentrations 1′-OH triazolam is the major metabolite whereas 4-OH triazolam becomes more prominent at higher concentrations. These results are consistent with those observed in other mouse strains (Perloff et al., 1999; Perloff et al., 2000).

As expected, triazolam metabolism was profoundly reduced in liver microsomes from Cyp3a−/− mice, albeit not completely abolished, when compared to wild-type mice (Figure 1; Table 1). Accordingly, the intrinsic clearance (V_{max}/K_{m}) for the 1′-OH formation was reduced more than 40-fold and for 4-OH triazolam formation more than 14-fold (Table 1). The residual metabolic activity was NADPH-dependent, indicating that other CYPs in Cyp3a−/− mice can take over the triazolam metabolism to some extent. Notably, the kinetic profile for the 1′-OH triazolam formation in the Cyp3a−/− mouse liver microsomes was clearly different from that observed in wild-type and could be fitted into a substrate inhibition model (K_S = 507±129 µM) (Figure 1).

In liver microsomes of Cyp3a−/−Tg-3A4Hep mice, expressing human CYP3A4 exclusively in the liver, a different kinetic profile for the 1′-OH and 4-OH triazolam formation was observed when compared to wild-type (Figure 1). For 1′OH-triazolam formation, although the affinity of human CYP3A4 is 5-fold lower than the (combined) affinity of the mouse CYP3A enzymes, the higher V_{max} (3-fold) resulted in only a slightly lower intrinsic clearance (1.7-fold) when compared to wild-type (Table 1). For 4-OH triazolam formation the intrinsic clearance was increased 2.1-
fold. Importantly, the metabolic and kinetic profile observed in liver microsomes of CYP3A4 transgenic mice \((\text{Cyp3a}^{-/-}\text{Tg-3A4}_{\text{Hep}})\) closely resembled that of human liver microsomes (Figure 1), and the kinetic parameters were in the same range (Table 1).

We also tested liver microsomes from mice that have intestinal specific transgenic CYP3A4 expression \((\text{Cyp3a}^{-/-}\text{Tg-3A4}_{\text{Int}})\). Note that liver microsomes from these mice have no CYP3A activity and are in essence similar to those of Cyp3a\(^{-/-}\) mice (van Herwaarden et al., 2007). Indeed, the 1'-OH triazolam formation of these liver microsomes was roughly similar to that observed in liver microsomes of Cyp3a\(^{-/-}\) mice, including the substrate inhibition characteristics \((K_s = 478 \pm 43 \mu M)\) (Figure 1).

We also investigated triazolam metabolism in intestinal microsomes. In case of wild-type microsomes metabolite formation could only be detected at the highest triazolam incubation concentrations and kinetic parameters could not be deduced from these data. In addition, no metabolite formation at all could be detected in intestinal microsomes from Cyp3a\(^{-/-}\) and Cyp3a\(^{-/-}\) Tg-3A4\(_{\text{Hep}}\) mice. In contrast, 1'-OH and 4-OH metabolite formation could be readily detected in Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Int}}\) intestinal microsomes. Both 1'-OH triazolam and 4-OH triazolam formation obeyed normal Michaelis-Menten kinetics and closely resembled the kinetic profile of human intestinal microsomes (Figure 1, Table 1). Furthermore, the metabolic kinetic profile in Cyp3a\(^{-/-}\) Tg-3A4\(_{\text{Int}}\) intestinal microsomes was also very similar to that observed in liver microsomes from Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Hep}}\) mice (Figure 1). Note that we have used the whole small intestine for the isolation of mouse microsomes and that the inclusion of non-enterocyte tissue will result in lower apparent \(V_{\text{max}}\) values compared to liver. For the human intestinal microsomes only mature enterocytes, which are enriched in CYPs, were used, precluding a simple comparison between the mouse and human intestinal \(V_{\text{max}}\) values.

These data indicate that there are clear species differences between mouse and human in the in vitro metabolism of triazolam. Of importance, the data further show that mouse microsomes with transgenic CYP3A4 expression (in a Cyp3a\(^{-/-}\) background) closely resemble the triazolam metabolism in human hepatic and intestinal microsomes, both in terms of the metabolic
kinetic profile (Figure 1) as well as the intrinsic clearance (Table 1). Finally, they also confirm the tissue-specific activity of CYP3A4 in the transgenic strains.

**Ketoconazole inhibits triazolam metabolism in wild-type but not in Cyp3a<sup>-/-</sup> microsomes**

Co-incubation with the CYP3A inhibitor ketoconazole (2.5 μM), resulted in virtually complete inhibition of triazolam metabolite formation in wild-type but not in Cyp3a<sup>-/-</sup> mouse liver microsomes (Figure 2). To test whether CYP2C enzymes were responsible for the residual triazolam metabolism in Cyp3a<sup>-/-</sup> mouse liver microsomes, we co-incubated with one of two antibodies raised against rat-CYP2C11. Antibody A was able to lower the formation of both hydroxy metabolites to around 50% (Figure 2), indicating that CYP2C enzymes are, at least partly, responsible for the triazolam metabolism in Cyp3a<sup>-/-</sup> mouse liver microsomes. In addition, also antibody B efficiently inhibited the 1’-OH but not the 4-OH triazolam formation. This suggests specific and efficient inhibition of CYP2C enzymes that have a preference to produce 1’-OH triazolam over 4-OH triazolam by this antibody, in line with previous observations for midazolam (van Waterschoot et al., 2008). Taken together, these results indicate that the low-level, residual triazolam metabolism observed in Cyp3a<sup>-/-</sup> liver microsomes is for a large part mediated by CYP2C enzymes, even though in the wild-type situation the contribution of CYP2C is negligible.

**Gefitinib stimulates 1’-OH triazolam formation by human CYP3A4 but not by mouse CYP3A**

Recently, it was shown that the anti-cancer drug gefitinib stimulates the formation of 1’OH-midazolam in human liver microsomes (Li et al., 2007). Based on the structural similarity between midazolam and triazolam, we hypothesized that gefitinib could also stimulate triazolam metabolism. However, when we co-incubated gefitinib with triazolam we did not observe stimulation but rather inhibition of the 1’-OH triazolam formation in wild-type mouse liver microsomes. As expected, also no stimulation was observed in Cyp3a<sup>-/-</sup> microsomes. In contrast,
1’-OH triazolam formation was markedly stimulated in human liver microsomes (Figure 3). Consistent with these observations, we also found potent stimulation in Cyp3a−/−Tg-3A4Hep mouse liver microsomes (Figure 3). We note that the 4-OH triazolam formation was inhibited by gefitinib in both human and Cyp3a−/−Tg-3A4Hep liver microsomes (not shown). Co-incubation with imatinib, another tyrosine kinase inhibitor and known CYP3A4 substrate used as control, showed modest inhibition of 1’-OH triazolam formation in all preparations.

More detailed examination of the stimulation in Cyp3a−/−Tg-3A4Hep mouse microsomes revealed that gefitinib changes both $K_m$ and $V_{max}$ of 1’-OH triazolam formation (Figure 4; Table 2). The $K_m$ values for 1’-OH triazolam formation dropped 3.5- and 2.8-fold for liver and intestine, respectively (Table 2). In addition, the $V_{max}$ for the same metabolite showed a 2- and 2.2-fold increase, leading to a rise in intrinsic clearance of 6.7- and 6.3-fold for liver and intestine, respectively. In contrast, the formation of 4-OH triazolam was inhibited by gefitinib (Figure 4) and the intrinsic clearances were 2.9- and 4.1-fold decreased for liver and intestine, respectively (Table 2).

We subsequently tested whether more tyrosine kinase inhibitors could stimulate the 1’-OH triazolam formation. In addition to gefitinib, only erlotinib showed some stimulation of the 1’-OH formation in Cyp3a−/−Tg-3A4Hep microsomes, though this was clearly less pronounced compared to gefitinib (Figure 5). All other drugs inhibited the 1’-OH triazolam formation and dasatinib appeared to be the most potent CYP3A4 inhibitor of all tyrosine kinase inhibitors tested.

**Intestinal but not hepatic CYP3A4 determines oral triazolam exposure**

Next, we investigated the relative in vivo importance of intestinal and hepatic CYP3A in triazolam pharmacokinetics. Transgenic expression of CYP3A4 in the intestine (Cyp3a−/−Tg-3A4int) markedly reduced triazolam systemic exposure (~2.3-fold) after oral administration when compared to Cyp3a−/− mice (Figure 6; Table 3; Supplementary data 2). Surprisingly, no difference in AUC between the Cyp3a−/− Tg-3A4Hep and Cyp3a−/− mice was observed. In accordance, triazolam levels in the double-transgenic Cyp3a−/−Tg-3A4HepInt strain were similar to those in the
Cyp3a<sup>−/−</sup>Tg-3A4<sub>int</sub> strain. These results indicate that after oral administration of triazolam, intestinal metabolism by CYP3A4 is far more important than hepatic metabolism in determining triazolam systemic exposure.

We also measured the levels of 1'-OH triazolam but, due to technical limitations, not 4'-OH triazolam (Figure 6, Table 3). It should be noted that the interpretation of the in vivo behavior of metabolites is often complicated as both generation and further metabolism rates may be altered in the various strains. As expected, Cyp3a<sup>−/−</sup> mice had the lowest plasma levels of 1'-OH triazolam of all mouse strains investigated. Relatively high 1'-OH triazolam levels were observed in the strains with hepatic transgenic CYP3A4 expression (Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep</sub> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep/Int</sub>), whereas 1'-OH triazolam levels in the intestinal CYP3A4 transgenic strain (Cyp3a<sup>−/−</sup>Tg-3A4<sub>int</sub>) were not significantly different from the Cyp3a<sup>−/−</sup> mice. Apparently, the site where 1'-OH triazolam is formed has an important impact on its further disposition. Possible explanations for this could be that metabolites formed in the intestine could, in part, be directly excreted in the intestinal lumen and/or via the portal blood and liver - into the bile, thereby reducing their systemic exposure. In addition, 1'-OH triazolam formed in the intestine could be efficiently further metabolized by intestinal UDP glucuronosyltransferases (UGT) to its corresponding glucuronide-conjugates, thus also reducing the systemic exposure of 1'-OH triazolam.

**Inhibition and stimulation of intestinal and hepatic CYP3A activity**

To evaluate whether our mouse models are suitable for studying drug-drug interactions we co-administered ketoconazole or gefitinib with triazolam. Ketoconazole markedly increased the triazolam AUC in all mouse strains with transgenic CYP3A4 expression but, as expected, not in Cyp3a<sup>−/−</sup> mice (Figure 6 C,F; Table 3). Also the levels of 1'-OH triazolam were significantly reduced in all CYP3A-expressing strains, but not in Cyp3a<sup>−/−</sup> mice, consistent with specific inhibition of CYP3A. Interestingly, co-administration of gefitinib resulted in significantly lower triazolam exposure in Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep</sub> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep/Int</sub> but not in Cyp3a<sup>−/−</sup> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>int</sub> mice (Figure 6 A,B; Table 3). This indicates that stimulation of CYP3A4 activity by
gefitinib primarily takes place in the liver. These results illustrate how Cyp3a<sup>−/−</sup> and CYP3A4-Tg mice can be utilized to study CYP3A4-dependent drug-drug interactions <i>in vivo</i>. 


Discussion

In this study we demonstrate that the metabolism of triazolam is profoundly reduced in Cyp3a<sup>-/-</sup> mice, both in vitro and in vivo. Counter to our expectations, our studies with tissue-specific CYP3A4 transgenic mice revealed that intestinal CYP3A4 has a major impact on oral triazolam systemic exposure, whereas the effect of hepatic CYP3A4 was relatively modest. We further evaluated whether the CYP3A4 transgenic mouse strains could be utilized to study drug-drug interactions. This was exemplified by co-administration of the prototypical CYP3A inhibitor ketoconazole, which increases the triazolam exposure in all CYP3A proficient mouse strains but not in Cyp3a<sup>-/-</sup> mice. We found that the anti-cancer drug gefitinib is a potent stimulator of the 1'-OH triazolam formation by CYP3A4, in vitro. Interestingly, also in vivo we could demonstrate stimulation of triazolam metabolism by gefitinib, resulting in a lower triazolam plasma exposure. Whereas direct in vivo stimulation of CYP3A4 activity has been demonstrated previously after intraportal infusion of a drug (Tang et al., 1999), the observations of the present study represent to our knowledge the first example of such a drug-drug interaction after oral drug administration.

Previous efforts to use midazolam as a probe drug for the characterization of the Cyp3a<sup>-/-</sup> and CYP3A4 transgenic mouse strains were confounded by the upregulation of (midazolam-metabolizing) mouse CYP2C enzymes in Cyp3a<sup>-/-</sup> mice. When we gave midazolam at a dose of either 0.5 or 10 mg/kg i.v. there was no significant difference between wild-type and Cyp3a<sup>-/-</sup> in either the systemic exposure of midazolam or of its two major metabolites (van Waterschoot et al., 2008). Also after oral administration of 2 mg/kg there were no significant differences between wild-type and Cyp3a<sup>-/-</sup> mice (Supplementary data 1). In contrast to midazolam, triazolam is considered to be specific for the mouse CYP3A isoforms (Perloff et al., 2000). Nonetheless, here we demonstrated that also in case of triazolam, there is still some residual triazolam metabolism mediated by CYP2C, even though the contribution of CYP2C enzymes in the wild-type situation is negligible. However, compared to the effect on midazolam this compensatory triazolam metabolism is much lower.
Although the liver has long been considered as the most important organ where CYP3A-mediated metabolism takes place, evidence is accumulating that also intestinal CYP3A metabolism can have a pronounced impact on the oral bioavailability of drugs (van Herwaarden et al., 2009). Using Cyp3a<sup>-/-</sup>Tg<sub>hep</sub> and Cyp3a<sup>-/-</sup>Tg<sub>int</sub> mice, we previously demonstrated that for docetaxel intestinal CYP3A4 metabolism was much more significant than hepatic metabolism after oral administration, whereas after intravenous administration hepatic CYP3A4 metabolism was dominant and the impact of intestinal CYP3A4 was very minor (van Herwaarden et al., 2007). These experiments also illustrated that these transgenic mice have both very substantial hepatic and intestinal CYP3A4 activity. Yet whereas intestinal CYP3A4 is the major determinant of docetaxel oral bioavailability in mice, the question remained whether intestinal metabolism is also important for drugs that are, unlike docetaxel, not a substrate for intestinal drug efflux transporters like P-gp. We therefore tested triazolam. Interestingly, our mouse data revealed that also for oral triazolam exposure intestinal metabolism by CYP3A4 is more important than hepatic metabolism, qualitatively similar to what we found previously for docetaxel (van Herwaarden et al., 2007). Qualitatively similar results were also obtained with midazolam (Supplementary data 1). Overall, this demonstrates that, at least in mice, also for drugs that are not substrates for P-gp, intestinal CYP3A4 activity can be a more important determinant of oral bioavailability than hepatic CYP3A4 activity.

The fact that we did not observe a difference in triazolam AUCs between Cyp3a<sup>-/-</sup> and Cyp3a<sup>-/-</sup>Tg-3A4<sub>hep</sub> mice would suggest that hepatic metabolism does not play any role after oral administration. This, however, might be misleading as we know that CYP2C enzymes are upregulated in the livers of Cyp3a<sup>-/-</sup> mice but not in Cyp3a<sup>-/-</sup>Tg-3A4<sub>hep</sub> mice (van Waterschoot et al., 2009). Obviously, without the upregulated CYP2C enzymes there would have been less compensatory metabolism and triazolam plasma levels would consequently have been higher in Cyp3a<sup>-/-</sup> mice. So, although their AUCs appeared to be not different, in Cyp3a<sup>-/-</sup> mice the triazolam metabolism could be mainly attributed to the upregulated CYP2C enzymes, whereas in case of Cyp3a<sup>-/-</sup>Tg-3A4<sub>hep</sub> mice this can be primarily attributed to hepatic CYP3A4. This latter
interpretation is also supported by the fact that the CYP3A inhibitor ketoconazole markedly increased the triazolam systemic exposure in Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep</sub> mice but not in Cyp3a<sup>−/−</sup> mice (Table 3).

We found that the anti-cancer drug gefitinib is a potent stimulator of the 1’-OH triazolam formation, but inhibits the 4-OH triazolam formation, in vitro. Stimulation of CYP3A-mediated metabolism is thought to be attributable to presence of multiple binding sites within the enzyme’s active site (e.g., Tang and Stearns, 2001). Recent crystal structures have indeed verified the possible simultaneous binding of substrates to CYP3A4 and accompanying conformational changes (Ekroos and Sjögren, 2006). Most likely, binding of gefitinib to the active site of CYP3A4 results in a conformational change of the enzymes that favors the 1’-OH but inhibits the 4-OH triazolam formation. Such regio-selectivity where one metabolic route is stimulated and the other is inhibited has been observed previously. For example, testosterone could stimulate the 4-OH midazolam formation by CYP3A but it inhibited the 1’-OH metabolite formation (Wang et al., 2000). In contrast, 7,8-Benzoflavone stimulated 1’-OH, but inhibited 4-OH midazolam formation (Wang et al., 2000).

Interestingly, also in vivo co-administration of gefitinib with triazolam resulted in significantly lower triazolam exposure in Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep</sub> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>Hep/Int</sub> mice but not in Cyp3a<sup>−/−</sup> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>Int</sub> mice, indicating that stimulation of CYP3A4 primarily takes place in the liver. Although co-administration of gefitinib with triazolam resulted in lower triazolam plasma levels after oral administration, the effect was less pronounced than perhaps expected from the in vitro results. Clearly, as the in vivo situation is far more complex than an in vitro homogenate, it is not surprising that there is not a simple one-to-one quantitative relationship between in vitro and in vivo quantitative shifts. In the microsomal extract the only route for removal of triazolam is by metabolic conversion. In contrast, in vivo there are many alternative routes for clearance of triazolam, including clearance by transporters, diffusion, ultrafiltration, etc. It is thus more or less expected that the in vivo impact of CYP3A4 stimulation will be more limited than in vitro. Also the access of gefitinib to the metabolizing enzyme may be
much more limited in vivo than in a simple in vitro extract, as we are dealing with intact cells and organs. Also, as gefitinib itself is a substrate for CYP3A4 (McKillop et al., 2005), a substantial part of the gefitinib dose could already have been metabolized. Finally, administration of gefitinib, 30 min prior to triazolam administration, was used to obtain significant loading of the body with gefitinib, but the concentration of gefitinib in intestinal epithelial cells may already have started to decrease after 30 min, resulting in levels too low to exert a marked stimulatory effect in the intestine.

So far, there are no recognized examples known of clinically relevant drug-drug interactions that can be attributed to the direct stimulation of CYP3A-mediated metabolism (as opposed to induction of CYP3A expression, which is quite common). Clearly, as it typically concerns only a specific combination of drugs, stimulation of drug metabolism by CYP3A4 is much more difficult to predict than inhibition. We note that gefitinib does not only stimulate the CYP3A4-mediated metabolism of triazolam but also that of midazolam and the anti-cancer drug irinotecan in vitro (Fujita et al., 2005; Li et al., 2007). Also, a recent clinical study reported a drug-drug interaction between gefitinib and sorafenib (Adjei et al., 2007). When both drugs were given simultaneously, the AUC of gefitinib was reduced (38%) compared to when the drug was given alone, suggesting that sorafenib might stimulate gefitinib metabolism. In contrast, gefitinib had no effect on sorafenib pharmacokinetics. Clearly, as anti-cancer drugs in general have narrow therapeutic windows and are often combined, direct stimulation of CYP3A-mediated metabolism could be of clinical relevance.

CYP3A4 is undoubtedly one of the most important players in many drug-drug interactions. Unfortunately, the prediction of in vivo drug-drug interactions based on in vitro data is not always straightforward (Lin, 2000; Wienkers and Heath, 2005; Obach, 2009). In addition, as a result of species differences in CYP3A, animal studies are not always representative for the human situation. The fact that for some drugs the intestine and not the liver is the most important organ for CYP3A-mediated drug-drug interactions can further complicate predictions. Humanized mouse models for CYP3A4, as we characterized and used here, could therefore be of
great value to better understand and predict drug-drug interactions, especially at a relatively early stage in drug development. Nonetheless, one should always realize that humanized mice are still not humans and that there will always be limitations in utilizing these models for quantitative predictions of drug exposure in humans.
Acknowledgments

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References


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Footnotes

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Legends for figures

Figure 1. Plots of 1'-OH and 4-OH triazolam formation by pooled liver (A) and intestinal (B) microsomes of wild-type, Cyp3a<sup>−/−</sup>, Cyp3a<sup>−/−</sup>Tg-3A4<sub>int</sub>, Cyp3a<sup>−/−</sup>Tg-3A4<sub>hep</sub> mice and human. Note marked differences in y-axis scales between panels. Incubations were performed as described under Materials and Methods. Intestinal metabolic rates of wild-type, Cyp3a<sup>−/−</sup> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>hep</sub> mice were below the lower detection limit of our assay (< 2.5 pmol/min/mg protein). Note that mouse intestinal microsomes were isolated from the whole tissue whereas the human intestinal microsomes were isolated after enterocyte isolation and are enriched in CYP. Data are the means ± S.D. of triplicate incubations. Corresponding kinetic parameters are given in Table 1.

Figure 2. Inhibition of 1'-OH and 4-OH triazolam formation by ketoconazole (2.5 µM), or one of two different antibodies against rat CYP2C11 (anti-CYP2C-A or anti-CYP2C-B), in mouse liver microsomes. After a preincubation of 10 min at 37 °C with vehicle, ketoconazole (2.5 µM) or the anti-CYP2C11 antibodies, the reaction was started by adding a NADPH-regenerating system and the mixture was subsequently incubated for 20 minutes. The final concentration of triazolam in the incubations was 50 µM. All values are the means of n = 3 determinations. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to control values of the same strain. Note that the data are normalized for each mouse strain and that absolute metabolite formation rates are much lower for Cyp3a<sup>−/−</sup> mouse liver microsomes (see Figure 1).

Figure 3. Stimulation of 1’-OH triazolam formation in presence of gefitinib (12.5 µM) or imatinib (12.5 µM) in liver microsomes of wild-type, Cyp3a<sup>−/−</sup> and Cyp3a<sup>−/−</sup>Tg-3A4<sub>hep</sub> mice and of human. Incubations were performed as described under Materials and Methods. The final concentration of triazolam in the incubations was 25 µM and the protein concentration was 0.125 mg/ml. The incubation time was 10 min. Bars represent means ± S.D. of triplicate incubations. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to control values of the same strain.
Figure 4: Kinetics of triazolam metabolism in microsomes of Cyp3a\(^{-/-}\) Tg-3A4\(_{\text{Int}}\) and Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Hep}}\) mouse strains, in the presence or absence of gefitinib (12.5 µM). Incubations were performed as described under Materials and Methods. The protein concentration in the incubations was 0.125 mg/ml for liver microsomes and 0.25 mg/ml for intestinal microsomes. The incubation time was 10 min. Data are the means ± S.D. of triplicate incubations. Corresponding kinetic parameters are given in Table 2.

Figure 5: Stimulation or inhibition of 1’-OH triazolam formation by a panel of tyrosine kinase inhibitors in Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Hep}}\) mouse liver microsomes. Incubations were performed as described under Materials and Methods. The final concentration of tyrosine kinase inhibitor in the incubations was 12.5 µM and that of triazolam was 25 µM. The protein concentration in the incubations was 0.125 mg/ml. The incubation time was 10 min. Bars represent means ± S.D. of triplicate incubations. * \(P < 0.05\), ** \(P < 0.01\) and *** \(P < 0.001\) compared to control values.

Figure 6: Plasma concentration versus time curves of triazolam (A-C) and 1’-OH triazolam (D-F) after oral triazolam administration (0.5 mg/kg) and co-administration of gefitinib (25 mg/kg, B, E) or ketoconazole (35 mg/kg, C, F) are shown for Cyp3a\(^{-/-}\), Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Hep}}\), Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Int}}\) and Cyp3a\(^{-/-}\)Tg-3A4\(_{\text{Hep/Int}}\) mice. Note differences in scale for triazolam and 1’-OH triazolam panels. Data are shown as the mean concentrations, and error bars represent the S.D. (n = 4-5 per time point).
Table 1. Kinetic parameters for triazolam metabolism by liver and intestinal microsomes. All values are the means of three independent experiments ± S.D. Incubations were performed as described under Materials and Methods. Note that mouse intestinal microsomes were isolated from the whole tissue whereas the human intestinal microsomes were isolated after enterocyte isolation and are enriched in CYP.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>1’-OH triazolam</th>
<th>4-OH triazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m^a$</td>
<td>$V_{max}^b$</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>11.7 ± 1.96</td>
<td>742 ± 16.4</td>
</tr>
<tr>
<td>Cyp3a−/−</td>
<td>73.3 ± 18.8$^d$</td>
<td>115 ± 14.2$^d$</td>
</tr>
<tr>
<td>Cyp3a−/−Tg-3A4Hep</td>
<td>58.4 ± 8.46</td>
<td>2145 ± 124</td>
</tr>
<tr>
<td>Cyp3a−/−Tg-3A4Int</td>
<td>40.1 ± 2.73$^d$</td>
<td>56.5 ± 3.14$^d$</td>
</tr>
<tr>
<td>Human</td>
<td>37.2 ± 3.27</td>
<td>1156 ± 42.2</td>
</tr>
<tr>
<td><strong>Intestine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>$^e$</td>
<td>$^e$</td>
</tr>
<tr>
<td>Cyp3a−/−</td>
<td>$^f$</td>
<td>$^f$</td>
</tr>
<tr>
<td>Cyp3a−/−Tg-3A4Hep</td>
<td>$^f$</td>
<td>$^f$</td>
</tr>
<tr>
<td>Cyp3a−/−Tg-3A4Int</td>
<td>38.5 ± 4.04</td>
<td>194 ± 5.87</td>
</tr>
<tr>
<td>Human</td>
<td>53.7 ± 3.01</td>
<td>847 ± 19.6</td>
</tr>
</tbody>
</table>

$^a$ $K_m$ expressed in μM  
$^b$ $V_{max}$ expressed in pmol/min/mg protein  
$^c$ $V_{max}/K_m$ expressed in μL/min/mg protein  
$^d$ Fitted with substrate inhibition model as described under Materials and Methods  
$^e$ Metabolite observed but kinetics could not be assessed  
$^f$ No metabolite detected (< 2.5 pmol/min/mg protein)
Table 2. Kinetic parameters for triazolam metabolism with or without gefitinib (12.5 μM) by CYP3A4-transgenic mouse liver and intestinal microsomes. All values are the means of three independent experiments ± S.D. Incubations were performed as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Strain</th>
<th>1'-OH triazolam</th>
<th>4-OH triazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kam</td>
<td>Vmax</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Cyp3a-/-Tg-3A4Hep</td>
<td>77.2 ± 3.75</td>
<td>1320 ± 34.4</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Cyp3a-/-Tg-3A4Hep</td>
<td>22.5 ± 1.46</td>
<td>2602 ± 60.2</td>
</tr>
<tr>
<td>Intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Cyp3a-/-Tg-3A4Int</td>
<td>22.5 ± 1.72</td>
<td>349 ± 6.18</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Cyp3a-/-Tg-3A4Int</td>
<td>8.23 ± 0.74</td>
<td>812 ± 12.0</td>
</tr>
</tbody>
</table>

a Km expressed in μM
b Vmax expressed in pmol/min/mg protein
c Vmax/Km expressed in μl/min/mg protein
Table 3: Systemic exposure (AUC) of triazolam and 1’-OH triazolam after oral triazolam administration (0.5 mg/kg) and oral co-administration of gefitinib (25 mg/kg) or ketoconazole (35 mg/kg) to mice.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Gefitinib</th>
<th>Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triazolam</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>317 ± 62</td>
<td>294 ± 21</td>
<td>374 ± 50</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Hep&lt;/sub&gt;</td>
<td>370 ± 49</td>
<td>248 ± 14 *†</td>
<td>588 ± 68 **††</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Int&lt;/sub&gt;</td>
<td>140 ± 15 **</td>
<td>132 ± 23 **</td>
<td>458 ± 44 †††</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Hep/Int&lt;/sub&gt;</td>
<td>130 ± 19 **</td>
<td>77 ± 15 ***†</td>
<td>549 ± 38 †††</td>
</tr>
<tr>
<td>Wild-type</td>
<td>194 ± 23 *</td>
<td>n.d.</td>
<td>467 ± 43 *†††</td>
</tr>
<tr>
<td><strong>1’-OH Triazolam</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>165 ± 34</td>
<td>145 ±13</td>
<td>186 ± 19</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Hep&lt;/sub&gt;</td>
<td>304 ± 19 **</td>
<td>193 ± 56 †</td>
<td>97 ± 13 **†††</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Int&lt;/sub&gt;</td>
<td>211 ± 27</td>
<td>235 ± 35 *</td>
<td>144 ± 23 *†</td>
</tr>
<tr>
<td>Cyp3a&lt;sup&gt;−/−&lt;/sup&gt; Tg-3A4&lt;sub&gt;Hep/Int&lt;/sub&gt;</td>
<td>261 ± 40 *</td>
<td>202 ± 45</td>
<td>150 ± 22 †</td>
</tr>
<tr>
<td>Wild-type</td>
<td>237 ± 29</td>
<td>n.d.</td>
<td>144 ± 14 *††</td>
</tr>
</tbody>
</table>

AUC<sub>(0-320 min)</sub>, (hr.μg/l) area under plasma concentration-time curve up to 320 min. Data are presented as means ± SD, n = 4-5. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to Cyp3a<sup>−/−</sup> mice; † P < 0.05, †† P < 0.01 and ††† P < 0.001 compared to untreated strain. n.d. not determined.
Figure 1

A  Liver

1\text{OH}/4\text{OH} TRZ formation (pmol/min/mg protein)

Wild-Type

Cyp3a\textsuperscript{-/-}

Cyp3a\textsuperscript{-/-} Tg-3A\textsubscript{4int}

Cyp3a\textsuperscript{-/-} Tg-3A\textsubscript{4HeP}

Human

B  Intestine

1\text{OH}/4\text{OH} TRZ formation (pmol/min/mg protein)

\mu M triazolam

Cyp3a\textsuperscript{-/-} Tg-3A\textsubscript{4int}

Human
Figure 2

1'-OH triazolam

4-OH triazolam

% of control

Wild-type  Cyp3a−/−  Wild-type  Cyp3a−/−

- Keto  - Keto  - Keto  - Keto

Anti-CYP2C-A  Anti-CYP2C-B  Anti-CYP2C-A  Anti-CYP2C-B

***  ***  ***  ***  **  **
Figure 4

1'-OH triazolam

<table>
<thead>
<tr>
<th>Metabolism formation (pmol/min/mg protein)</th>
<th>Liver Cyp3a&lt;sup&gt;-/-&lt;/sup&gt;Tg-3A&lt;sub&gt;4&lt;/sub&gt;&lt;sub&gt;Hep&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>400</td>
<td>500</td>
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4-OH triazolam

<table>
<thead>
<tr>
<th>Metabolism formation (pmol/min/mg protein)</th>
<th>Intestine Cyp3a&lt;sup&gt;-/-&lt;/sup&gt;Tg-3A&lt;sub&gt;4&lt;/sub&gt;&lt;sub&gt;Int&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>400</td>
<td>500</td>
</tr>
</tbody>
</table>

μM triazolam