Midazolam Metabolism in Cytochrome P450 3A Knockout Mice Can Be Attributed to Up-Regulated CYP2C Enzymes

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ABSTRACT

The cytochrome P450 3A (CYP3A) enzymes represent one of the most important drug-metabolizing systems in humans. Recently, our group has generated cytochrome P450 3A knockout mice to study this drug-handling system in vivo. In the present study, we have characterized the Cyp3a knockout mice by studying the metabolism of midazolam, one of the most widely used probes to assess CYP3A activity. We expected that the midazolam metabolism would be severely reduced in the absence of CYP3A enzymes. We used hepatic and intestinal microsomal preparations from Cyp3a knockout and wild-type mice to assess the midazolam metabolism in vitro. In addition, in vivo metabolite formation was determined after intravenous administration of midazolam. We were surprised to find that our results demonstrated that there is still marked midazolam metabolism in hepatic (but not intestinal) microsomes from Cyp3a knockout mice. Accordingly, we found comparable amounts of midazolam as well as its major metabolites in plasma after intravenous administration in Cyp3a knockout mice compared with wild-type mice. These data suggested that other hepatic cytochrome P450 450 enzymes could take over the midazolam metabolism in Cyp3a knockout mice. We provide evidence that CYP2C enzymes, which were found to be up-regulated in Cyp3a knockout mice, are primarily responsible for this metabolism and that several but not all murine CYP2C enzymes are capable of metabolizing midazolam to its 1'-OH and/or 4-OH derivatives. These data illustrate interesting compensatory changes that may occur in Cyp3a knockout mice. Such flexible compensatory interplay between functionally related detoxifying systems is probably essential to their biological role in xenobiotic protection.

The cytochrome P450 enzymes (P450s) play a pivotal role in the phase I metabolism of drugs and other xenobiotics. In addition, P450s are involved in the synthesis and metabolism of a broad range of endogenous substrates, including steroids, bile acids, and arachidonic acids. Members of the cytochrome P450 3A (CYP3A) subfamily are of particular interest because of their broad substrate specificity and their high inter- and intraindividual variation in expression and activity levels. In humans, four CYP3A enzymes have been identified, but only CYP3A4 and CYP3A5 are considered to be relevant for drug metabolism in adults. In general, CYP3A4 and CYP3A5 have similar substrate specificities, although they may have distinct affinities and turnovers for some substrates (Williams et al., 2002).

It is estimated that CYP3A enzymes contribute to the metabolism of approximately half of currently marketed drugs (Guengerich, 1999). Because the CYP3A enzymes are strategically located in the liver and intestinal wall, they have a strong effect on the first-pass metabolism, oral bioavailability, and elimination of administered drugs. Furthermore, the induction and inhibition of CYP3A enzymes are considered important determinants in the therapeutic efficacy and toxicity of numerous drugs (Dresser et al., 2000; Lamba et al., 2002). Accordingly, interactions at the CYP3A

ABBREVIATIONS: P450, cytochrome P450; RT-PCR, real-time polymerase chain reaction; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CAR, constitutive androstane receptor.
level are often the cause of pronounced drug-drug interactions (Thummel and Wilkinson, 1998).

Given the importance of CYP3A, the in vitro screening of novel drug candidates as potential substrates of CYP3A has become routine in the preclinical drug development stage. However, these in vitro studies are not always indicative for the in vivo situation. To allow a more systematic in vivo evaluation of CYP3A-mediated metabolism, we have recently generated mice lacking all Cyp3a genes (Cyp3a/−/*−) as well as CYP3A4 transgenic mice in a Cyp3a knockout background (van Herwaarden et al., 2007). It is noteworthy that Cyp3a/−/*− mice are viable, fertile, and do not show obvious physiological abnormalities. These observations suggest that the CYP3A enzymes do not have an essential endogenous physiological function and could be primarily dedicated to the detoxification of xenobiotics.

A classic probe for CYP3A activity in humans is midazolam. This drug is considered highly specific because no other human P450s contribute significantly to its metabolism. The biotransformation of midazolam by CYP3A enzymes yields 1′-OH and 4-OH midazolam as the principal metabolites (Kronbach et al., 1989). In case of the recombinant human CYP2C and CYP3A enzymes, 20 pmol of enzyme was used, and the reactions were allowed to proceed preincubation of 5 min, the mixture was incubated for 15 min. All other conditions were as described for the microsomal incubations.

Preparation and Reconstitution of Recombinant Murine CYP2C Enzymes. The heterologous expression of the recombinant murine CYP2C enzymes in Escherichia coli as well as their partial purification and reconstitution has been reported previously (Luo et al., 1998; Tsao et al., 2001; DeLozier et al., 2004; Wang et al., 2004). CYP2C65, CYP2C65, and CYP2C70 were prepared in *E. coli* using similar methods (J. A. Bradbury and D. C. Zeldin, personal communication). The final concentration of midazolam in the incubations was 25 μM, and 25 pmol of CYP2C enzyme was used. After a preincubation of 5 min, the mixture was incubated for 15 min. All other conditions were as described for the microsomal incubations. In case of the recombinant human CYP2C and CYP3A enzymes, 20 pmol of enzyme was used, and the reactions were allowed to proceed for 10 min.

Plasma Pharmacokinetics. Midazolam was dissolved in 0.9% NaCl and was injected i.v. into the tail vein of mice at 0.5 or 10 mg/kg body weight. At *t* = 7.5, 15, 30, 60, and 90 min, blood samples were taken by cardiac puncture under anesthesia with methoxyflurane, after which the mice were sacrificed by cervical dislocation (n = 3–5 for each time point). Blood samples were centrifuged at 2100g for 10 min at 4°C, and the plasma fraction was collected and stored at −20°C until analysis. Samples were processed and measured by LC-MS/MS as described below.

HPLC. HPLC analyses were performed using a Symmetry C18 column; 3.0 × 150 mm, 3.5 μm (Waters, Ettan-Leer, The Netherlands). Isocratic analyses were carried out at a flow rate of 0.4
ml/min. The mobile phase consisted of 33% acetonitrile/23% methanol/44% 10 mM phosphate buffer, pH 7.4, and 0.2% triethylamine. The identities of 1'-OH and 4-OH midazolam were verified by comparing the retention times with authentic standards. Metabolites were detected at 230 nm and quantitated by using standard curves for 1'-OH and 4-OH midazolam.

**LC-MS/MS.** Mouse plasma samples were measured by LC-MS/MS. To a 20-μl plasma sample, 100 μl of water, 20 μl of 0.5 μg/ml clonazepam (internal standard) in 50% (v/v) methanol and 200 μl of 5 mM sodium hydroxide were added. The analytes were extracted with 2 ml of diethyl ether and the organic phase was evaporated under a stream of nitrogen. The residue was reconstituted in 100 μl of 0.1% (v/v) acetic acid in 5% (v/v) acetonitrile before injection in the chromatographic system.

The LC-MS/MS equipment consisted of a DDU-14A degasser, a Sli-HTC autosampler, two LC10-ADvp 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 Fisher Scientific, Waltham, MA). Data were processed with the Finnigan Xcalibur software (version 1.4; Thermo Fisher Scientific).

**RNA Isolation and cDNA Synthesis.** Mouse livers were excised and immediately placed in an appropriate volume of RNAlater (QIAGEN, Valencia, CA). They were stored at 4°C for several days until RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer’s protocol for the purification of total RNA from animal tissues. Subsequently, cDNA was generated using 5 μg of total RNA in a synthesis reaction using random hexamers (Applied Biosystems, Foster City, CA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the supplier’s protocols. The reverse transcription reaction was performed for 60 min at 42°C with a deactivation step of 15 min at 70°C. cDNA was stored at -20°C until use.

**RT-PCR Analysis.** Real-time PCR (RT-PCR) was performed using specific primers (QIAGEN) for the individual mouse CYP2C subfamily members on an Applied Biosystems 7500 real-time cycler system according to the manufacturers protocol. In brief, in a MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems), 25-μl reaction mixtures containing 5 μl of cDNA (0.1 ng/μl), 12.5 μl of SyBr Green PCR master mix, 2.5 μl of sample primer mix (QuantiTect Primer Assays; QIAGEN) and 5 μl of aqua Braun were pipetted. After sealing the plates with optical adhesive film (Applied Biosystems), the plates were briefly centrifuged. The cycling conditions were initiated at 50°C for 2 min with an enzyme activation step of 95°C for 10 min, followed by 45 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Analysis of the results was done by the comparative C, method as described previously (Livak and Schmittgen, 2001). In brief, quantitation of the target cDNAs in all samples was normalized to glyceroldehyde-3-phosphate dehydrogenase cDNA (\(C_{T\text{CY}} - C_{T\text{GAPDH}} = \Delta C_t\)) and the difference in expression for each target cDNA in the Cyp3a-/- mice was expressed relative to the amount in the wild-type mice (\(\Delta C_{T\text{wild-type}} - \Delta C_{T\text{cyp3a-/-}} = \Delta \Delta C_t\)). Subsequently, fold changes in target gene expression were determined by taking 2 to the power of this number (2^\(-\Delta \Delta C_t\)) Statistic analysis was performed on ΔCT values (Yuan et al., 2006).

**Western Blot Analysis.** Immunohistochemical staining of CYP2C55 in mouse liver tissues was performed as described previously (Wang et al., 2004; van Herwaarden et al., 2005). Blots were probed with rabbit anti-CYP2C55pep1 IgG antibody (1:1000). After extensive washing, the secondary horseradish peroxidase-labeled antibody (goat anti-rabbit IgG horseradish peroxidase (1:10,000)) was added. Bands were visualized by enhanced chemiluminescence. Equal loading across the lanes was confirmed with total protein staining (ponceau S and India ink).

**Results**

**Midazolam Metabolism in Wild-Type and Cyp3a-/- Mouse Liver Microsomes.** In wild-type mouse liver microsomes, midazolam was metabolized to its 1'-OH and 4-OH metabolites with kinetic parameters as reported in Table 1. Whereas normal Michaelis-Menten kinetics was observed for the 4-hydroxylation, substrate inhibition was seen for the 1'-hydroxylation reaction (K_m = 204 ± 27 μM) (Fig. 1). These results are consistent with those observed in other mouse strains (Perloff et al., 2000; Granvil et al., 2003). We were surprised to find that when using liver microsomes from Cyp3a-/- mice, we still found substantial 1'-OH- and 4-OH midazolam formation in an NADPH-dependent manner, indicating that other P450 enzymes can take over these reactions from CYP3A (Table 1). The K_m for the midazolam 1'-hydroxylation was increased approximately 7-fold with a
concomitant modest increase in $V_{\text{max}}$, resulting in an intrinsic clearance that was roughly 6-fold lower compared with wild-type. It is noteworthy that, analogous to wild-type microsomes, data for the 1'-hydroxylation by Cyp3a$^{-/-}$ microsomes could also be fitted in a substrate inhibition model ($K_s = 354 \pm 56 \mu M$) (Fig. 1). Analysis of the 4-hydroxylation reaction in Cyp3a$^{-/-}$ liver microsomes revealed that the $K_m$ was increased 2-fold with a concomitant 4.5-fold decrease in $V_{\text{max}}$ compared with wild-type microsomes (Table 1). Overall, these data indicate that there is still significant midazolam metabolism in liver microsomes from Cyp3a$^{-/-}$ mice.

Midazolam Metabolism in Cyp3a$^{+/-}$ and Cyp3a$^{-/-}$ Mouse Intestinal Microsomes. In addition to liver, we also investigated the midazolam metabolism in small intestinal microsomes from wild-type and Cyp3a$^{-/-}$ mice. In intestinal microsomes from wild-type mice, significant 1'-OH and 4-OH midazolam formation was observed (Table 1). Consistent with the kinetic profiles in mouse liver microsomes, substrate inhibition was observed for the 1'-OH midazolam formation and normal Michaelis-Menten kinetics was seen for the 4-OH midazolam formation (data not shown).

In contrast to Cyp3a$^{-/-}$ mouse liver microsomes, no midazolam metabolism was observed in Cyp3a$^{-/-}$ intestinal microsomes, indicating that, in wild-type intestine, the CYP3A enzymes were primarily responsible for the midazolam metabolism. In agreement with this, coincubation with the CYP3A inhibitor ketoconazole (2.5 $\mu M$) showed a virtually complete inhibition of both 1'-OH and 4-OH midazolam formation in intestinal microsomes from wild-type mice (data not shown).

Chemical And Immunochemical Inhibition. Inhibition experiments with ketoconazole in liver microsomes from wild-type mice supported that both midazolam 1'- and 4-hydroxylation reactions are mostly CYP3A mediated. However, ketoconazole (2.5 $\mu M$) was not able to completely inhibit the 1'-OH midazolam formation in wild-type mice (Fig. 2A). In contrast, complete inhibition of the 4-OH midazolam formation with ketoconazole was observed, suggesting that in wild-type liver microsomes, this reaction is entirely CYP3A dependent (Fig. 2B). As expected, ketoconazole had no inhibitory effect on the 1'- and 4-OH metabolite formation in liver microsomes from Cyp3a$^{-/-}$ mice.

Previous studies have indicated that murine CYP2C enzymes may contribute to the 1'-OH midazolam formation in the mouse (Perloff et al., 2000, 2003). In the absence of established inhibitors for the murine CYP2C enzymes, we used 2 different antibodies raised against rat CYP2C11 to evaluate the involvement of CYP2C enzymes. Both antibodies were able to partially inhibit the 1'-OH midazolam formation in liver microsomes from wild-type mice (Fig. 2A). The combined use of either of the two antibodies with ketoconazole resulted in a complete inhibition of the 1'-OH midazolam formation in wild-type microsomes, indicating that CYP3A and CYP2C enzymes were primarily responsible for this reaction, with no significant contribution of other P450s. Consistent with these observations, we found that in Cyp3a$^{-/-}$ mouse liver microsomes, the anti-CYP2C11 antibodies were able to significantly inhibit the 1'-OH metabolite formation (down to ~5% of control values), although clear differences in the degree of inhibition were observed between the two antibodies (Fig. 2A). The differences in inhibition potential between these two anti-CYP2C11 antibodies were even more pronounced for the 4-OH midazolam formation and, interestingly, reversed, compared with the inhibition of 1'-OH midazolam formation. Whereas antibody A inhibited the 4-OH midazolam formation efficiently, antibody B was not at all able to inhibit this reaction in Cyp3a$^{-/-}$ microsomes (Fig. 2B). It should be noted that these antibodies have not been evaluated against the murine CYP2C enzymes, but our results strongly suggest that they differ markedly in their capability to inhibit different mouse CYP2C enzymes. These data do suggest, however, that in Cyp3a$^{-/-}$ liver microsomes, distinct CYP2C enzymes are responsible for 1'-OH and 4-OH midazolam formation, respectively.

Expression Levels of CYP2C Enzymes in Cyp3a$^{-/-}$. Based on the findings above, we hypothesized that one or more CYP2C enzymes would be up-regulated in the Cyp3a$^{-/-}$ mice. We performed RT-PCR analyses for a set of Cyp2c genes to determine whether alterations in mRNA expression levels in the liver of Cyp3a$^{-/-}$ compared with wild-type mice could be detected. Indeed, we found that CYP2C29, CYP2C38, CYP2C39, CYP2C50, and CYP2C66 were significantly up-regulated with roughly 1.5- to 3-fold differences (Fig. 3). None of the Cyp2c genes tested was down-regulated in livers from Cyp3a$^{-/-}$ mice. Most notable was the more than 30-fold up-regulation of CYP2C55, although its RNA levels remained

![Fig. 1. Representative plots of 1'-OH midazolam (A) and 4-OH midazolam (B) formation by liver microsomes of Cyp3a$^{+/-}$ and Cyp3a$^{-/-}$ mice. Incubations were performed as described under Materials and Methods. The corresponding kinetic parameters are summarized in Table 1.](http://molpharm.aspetjournals.org/issue)
low even after induction (Supplementary Data 1). In addition, at the protein level, CYP2C55 seemed to be highly up-regulated in the livers of Cyp3a<sup>-/-</sup> mice compared with wild-type mice (Fig. 4).

**Midazolam Metabolism by Recombinant Mouse CYP2C Enzymes.** The murine CYP2C subfamily consists of many closely related enzymes that can—unlike CYP3A enzymes—differ strikingly in substrate specificity (Goldstein and de Morais, 1994). We screened a panel of currently available recombinant expressed mouse CYP2C enzymes to identify which enzymes were capable of metabolizing midazolam (Table 2). Among these enzymes, it seemed that CYP2C29 and CYP2C65 were capable of hydroxylating midazolam at both its 1<sup>1</sup> and 4 positions, whereas CYP2C39 and CYP2C55 solely catalyzed the 1<sup>1</sup>-OH midazolam reaction and CYP2C70 primarily did the 4-hydroxylation. For several CYP2C enzymes, however, we detected no 1<sup>1</sup>- or 4-OH metabolites. Unfortunately, it is not possible to compare these activities with the

![Fig. 2. Inhibition of 1<sup>1</sup>OH midazolam (A) and 4-OH (B) midazolam formation by ketocnazole and/or two different antibodies against rat CYP2C11 from Invitrogen (monoclonal, anti-CYP2C-A) or Daiichi (polyclonal, anti-CYP2C-B), respectively, in mouse liver microsomes. After a preincubation of 15 min at 37°C with vehicle, ketoconazole (2.5 μM), and/or the anti-CYP2C11 antibodies, the reaction was started by adding a NADPH-regenerating system, and the mixture was subsequently incubated for 6 min. The final concentration of midazolam in the incubations was 50 μM. All values are the means of n = 2 to 5 determinations.](#)

![Fig. 3. CYP2C mRNA expression measured by RT-PCR. Results are expressed as the fold change in liver of Cyp3a<sup>-/-</sup> mice (n = 4) compared with wild-type mice (n = 4). Data were normalized against the endogenous control glyceraldehyde-3-phosphate dehydrogenase. Each sample was assayed in duplicate in at least two independent experiments. *, P < 0.05; **, P < 0.01; and ***, P < 0.001, indicate that the ΔC<sub>T</sub> value for Cyp3a<sup>-/-</sup> mice is significantly different from that for wild-type mice. ΔC<sub>T</sub> values are given in Supplementary Data 1.](#)

![Fig. 4. Expression of mouse CYP2C55 in liver of Cyp3a<sup>+/+</sup> and Cyp3a<sup>-/-</sup> mice as detected by Western blotting. Microsomal protein (30 μg) was loaded per lane. Recombinant CYP2C55 enzyme, migrating at 50 kDa (arrow), was used as positive control (0.5 pmol/lane). Total protein staining (Ponceau S and India Ink) confirmed equal loading across the lanes (not shown).](#)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>1&lt;sup&gt;1&lt;/sup&gt;-OH MDZ</th>
<th>4-OH MDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C29</td>
<td>0.91 ± 0.04</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>CYP2C37</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CYP2C38</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CYP2C39</td>
<td>0.10 ± 0.1</td>
<td>—</td>
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<tr>
<td>CYP2C44</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CYP2C54</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CYP2C55</td>
<td>0.016 ± 0.002</td>
<td>0.008 ± 0.003</td>
</tr>
<tr>
<td>CYP2C65</td>
<td>0.021 ± 0.006</td>
<td>—</td>
</tr>
<tr>
<td>CYP2C66</td>
<td>—</td>
<td>0.28 ± 0.06</td>
</tr>
</tbody>
</table>

—, no metabolite detected (detection limit < 0.005 min<sup>-1</sup>)
individual murine CYP3A enzymes, because these enzymes are not readily available.

We also tested whether human CYP2C enzymes are capable of metabolizing midazolam and compared this with the activities of CYP3A4 and CYP3A5 (Fig. 5). For all the known human CYP2C enzymes, we found modest amounts of the 1'-OH metabolite formed but could not detect any 4-OH midazolam. However, compared with CYP3A4 and CYP3A5, the formation of 1'-OH midazolam by human CYP2C enzymes was very low.

**In Vivo Midazolam Metabolism.** Most of the in vivo metabolism and clearance of midazolam in wild-type mice occurs through CYP3A, as previously demonstrated by ketoconazole inhibition experiments (e.g., Granvil et al., 2003). Given the up-regulation of CYP2C enzymes and their marked contribution to midazolam metabolism in Cyp3a<sup>−/−</sup> microsomes, we wanted to know the consequences of the Cyp3a knockout for the in vivo metabolism of midazolam. We therefore administered 0.5 mg/kg midazolam intravenously and subsequently determined the plasma levels of midazolam and its 1'- and 4-OH metabolites at several time points. It is noteworthy that, as indicated in Fig. 6A and Table 3, the plasma concentration curves and areas under the curve of midazolam were not significantly different between wild-type and Cyp3a<sup>−/−</sup> mice. Accordingly, the clearance did not differ between the two strains. Also the plasma levels of the 1'- and 4-OH metabolites were comparable (Fig. 6, B and C). Similar results were obtained when administering a much higher dose of 10 mg/kg midazolam (Supplementary Data 2 and Table 3). Taken together, these data clearly demonstrate that in vivo there is still marked midazolam metabolite formation in Cyp3a<sup>−/−</sup> mice and hence that midazolam is very efficiently cleared despite the absence of CYP3A.

**Discussion**

Midazolam is one of the probes used most widely to assess CYP3A activity in vitro and in vivo, and in this study we have used this drug to further characterize our recently generated Cyp3a knockout mouse model (van Herwaarden et al., 2007). We here report that CYP2C enzymes able to metabolize midazolam are up-regulated in Cyp3a<sup>−/−</sup> mice and that, consequently, midazolam is still very efficiently metabolized and cleared in Cyp3a<sup>−/−</sup> mice.

The biotransformation of midazolam in both human and mouse is comparable, yielding 1'-OH midazolam as the major metabolite and 4-OH midazolam as a minor metabolite. Consistent with previous in vitro studies (Perloff et al., 2000, 2003), our data suggest that in wild-type mice, 1'-OH midazolam formation is not only dependent on CYP3A but also

![Fig. 5. 1'-OH midazolam formation by human CYP2C and CYP3A enzymes. The final concentration of midazolam in the incubations was 25 μM, and 20 pmol of enzyme was used. After a preincubation of 5 min, the reaction was started by adding a NADPH-regenerating system, and the mixture was subsequently incubated for 10 min. All values are the means of duplicate determinations.](image)

![Fig. 6. Plasma concentration versus time curves of midazolam (A), 1'-OH midazolam (B) and 4-OH midazolam (C) after intravenous midazolam administration (0.5 mg/kg) are shown for Cyp3a<sup>+/+</sup> and Cyp3a<sup>−/−</sup> mice. Note the different axis scale for 4-OH midazolam. n = 3 ± S.D. for each time point.](image)
has a significant CYP2C component. Accordingly, we had anticipated observation of some 1'-OH midazolam formation in Cyp3a−/− mice as a result of CYP2C activity, but we were surprised by the extent of this formation because it was only marginally reduced compared with that in wild-type mice. Even more surprising was that we also observed significant 4-OH midazolam formation in Cyp3a−/− mouse liver microsomes, a reaction that was also considered to be CYP3A-specific in mice (Perloff et al., 2000). Consistent with this latter report, our own ketoconazole studies demonstrated complete inhibition of the 4-OH metabolite formation in wild-type microsomes. Because we could not detect midazolam metabolism in small intestinal microsomes from Cyp3a−/− mice, it is likely that the small intestinal expression of CYP2C enzymes involved in midazolam metabolism is too low to significantly contribute to the intestinal metabolism of midazolam. This is consistent with studies that were not able to detect small intestinal CYP2C expression at the protein level (Emoto et al., 2000).

We demonstrated that several but not all CYP2C enzymes are up-regulated in Cyp3a−/− mice. It is noteworthy that almost all CYP2C enzymes that were able to metabolize midazolam also had up-regulated mRNA levels. Most prominent was the ~35-fold up-regulation of CYP2C55, which was also capable of performing the 1'-OH midazolam formation. This RNA up-regulation was also reflected in a large increase in CYP2C55 protein levels, as demonstrated by immunoblotting. However, the up-regulated RNA expression level of CYP2C55, as judged from RT-PCR, would still be lower than for most other CYP2C genes (e.g., CYP2C29). The contribution of CYP2C55 to the total 1'-OH midazolam formation in Cyp3a−/− mice might therefore still be limited.

Given the high up-regulation of CYP2C55, it might be interesting to investigate whether this enzyme is partly under a different regulatory mechanism from the other CYP2c genes. It is noteworthy that in mice with a liver-specific deletion of the NADPH-P450 reductase gene, a 17-fold induction in CYP2C55 mRNA levels was observed (Weng et al., 2005). The mechanism of regulation of the different mouse CYP2C enzymes is still under investigation. The mouse Cyp2c locus is complex and contains 15 genes, whereas in humans, only four CYP2C genes have been identified (Nelson et al., 2004). It has been shown that the induction of CYP2C29 and CYP2C37 can be mediated by the constitutive androstane receptor (CAR) (Jackson et al., 2004, 2006). However, CYP2C44 could not be up-regulated by either CAR or pregnane X receptor activators (DeLozier et al., 2004). Although little is yet known about the regulation of the other CYP2C enzymes, there clearly is accumulating evidence that various members of the CYP2C subfamily are differently regulated.

The CAR-mediated induction of various CYP2C family members indicates that these genes can be regulated by a diverse range of xenobiotic inducers. This could provide a clue to the possible mechanism behind the CYP2C up-regulation in the Cyp3a knockout mouse. It is likely that CYP3A normally metabolizes one or more inducers of the various CYP2c genes, and that levels of these inducers are much higher in Cyp3a−/− mice. These inducers might in part be endogenous (e.g., steroids, bile acids), but an obvious source of these inducers would be xenobiotics (phytoestrogens, etc.) that occur in the normal food. Indeed, we have recently tested the effect of replacing normal food with a semisynthetic diet, and found much lower induction levels of CYP2C55 RNA in the livers of Cyp3a−/− mice compared with wild-type (5-fold instead of ~35-fold on normal food). This suggests that a major factor in the CYP2C up-regulation in the Cyp3a−/− mice is induction by food-derived xenobiotics that are normally metabolized by CYP3A.

During the preparation of our manuscript, Emoto and Iwasaki (2007) reported that, in addition to CYP3A enzymes, human CYP2C19 is also capable of catalyzing the midazolam 1'-hydroxylation. We found that not only CYP2C19 but also the three other human CYP2C enzymes could catalyze this reaction, although with much lower turnover rates than CYP3A enzymes. Based on the turnover rates as well as the lower expression levels of CYP2C versus CYP3A enzymes, the contribution of CYP2C enzymes to the total midazolam metabolism in humans will most likely be negligible.

At first, we used docetaxel for the in vitro and in vivo pharmacokinetic characterization of our Cyp3a−/− mice (van Herwaarden et al., 2007). Docetaxel is a widely used anticancer drug and is known to be primarily metabolized by members of the CYP3A subfamily (Shou et al., 1998). It turned out that the formation of docetaxel metabolites was completely absent in hepatic and intestinal microsomes from Cyp3a−/− mice, indicating that no other mouse P450s could take over the docetaxel metabolism. In vivo, the lack of CYP3A mediated metabolism resulted in 18- and 7-fold higher areas under the curve for docetaxel in Cyp3a−/− mice after oral and i.v. administration, respectively (van Herwaarden et al., 2007).

Consistent with the in vitro data from the present study, but in strong contrast to the earlier docetaxel data, our in vivo experiments demonstrated that after i.v. administration of midazolam, there were only marginal differences between Cyp3a+/+ and wild-type mice in plasma levels of midazolam and of its 1'- and 4-OH metabolites.

### TABLE 3

Plasma pharmacokinetic parameters after i.v. administration of 0.5 or 10 mg/kg midazolam to Cyp3a+/+ and Cyp3a−/− mice

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<th>Cyp3a+/+</th>
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<th>Cyp3a+/+</th>
<th>Cyp3a−/−</th>
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<tbody>
<tr>
<td><strong>Midazolam</strong></td>
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<tr>
<td>CYP2C55</td>
<td>29.7 ± 1.62</td>
<td>26.2 ± 3.25</td>
<td>0.95 ± 0.08</td>
<td>1.14 ± 0.09</td>
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<tr>
<td>1'-OH Midazolam</td>
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<tr>
<td>CYP2C55</td>
<td>1849 ± 234</td>
<td>1147 ± 199*</td>
<td>23.6 ± 5.1</td>
<td>15.8 ± 3.4</td>
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<td>AUC_{0-1.5h} μg/l</td>
<td>842 ± 168</td>
<td>833 ± 111</td>
<td>0.012 ± 0.002</td>
<td>0.012 ± 0.002</td>
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<tr>
<td>Cl, l/h</td>
<td>0.012 ± 0.002</td>
<td>0.012 ± 0.002</td>
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</table>
Combined with our recently generated CYP3A4 transgenic mouse models (van Herwaarden et al., 2007), we consider the Cyp3a<sup>−/−</sup> mouse model as an appropriate tool to study the impact of CYP3A on drug levels in an in vivo situation. However, other up-regulated P450 enzymes can cause some drugs to affect the results obtained. For example, CYP3A and CYP2C enzymes have overlapping substrate specificities and this overlap may be different between species. Clearly, proper in vitro evaluation of the background metabolism of drugs of interest in Cyp3a<sup>−/−</sup> mice would be recommended to optimize application of this novel mouse model.

In summary, we investigated the metabolism of midazolam in Cyp3a<sup>−/−</sup> mice. Both our in vitro and in vivo data showed that in the absence of CYP3A, the metabolism of midazolam was only marginally reduced. We provided evidence that CYP2C enzymes were primarily responsible for this compensatory metabolism, and we demonstrated that several but not all CYP2C enzymes were capable of catalyzing the 1’- and/or 4-hydroxylation reactions. Moreover, the Cyp3a knockout apparently resulted in a significant up-regulation of some of the CYP2C enzymes. From a biological point of view, this study demonstrated that in the absence of an important xenobiotic metabolizing enzyme subfamily, organisms can still deal with some xenobiotics as a result of the overlapping substrate specificity of P450s and the potential up-regulation of these enzymes. Such flexibility is probably essential to the important biological function of detoxification of these enzyme systems.

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**References**


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