Effects of Age on In Vitro Midazolam Biotransformation in Male CD-1 Mouse Liver Microsomes

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ABSTRACT

To study age-related changes in drug metabolism, we examined the in vitro biotransformation of midazolam (MDZ), a human cytochrome P-450 (CYP) 3A substrate, using liver microsomes from three age groups of male CD-1 mice ranging from 6 weeks to 2 years old. MDZ was metabolized to two major products, α-OH- and 4-OH-MDZ, which were quantified by HPLC. For both metabolites, V_max values were reduced in old livers (P < .05), while K_m values did not change with age. The net intrinsic clearance (the sum of V_max/K_m for both pathways) also was reduced in the old animals (P < .05). The capacity of ketoconazole, a CYP3A inhibitor in humans, to inhibit the biotransformation of MDZ and of alprazolam, another human CYP3A substrate, did not differ significantly with age. At 100 μM alprazolam, 0.5μM ketoconazole inhibited metabolite formation by >80%. At 30 μM MDZ, 2.5 μM ketoconazole impaired 4-OH-MDZ formation by 88%, whereas it reduced α-OH-MDZ formation by only 46%. Immunoinhibition studies with polyclonal anti-rat CYP3A1/2 and CYP2C11 antibodies confirmed that 4-OH-MDZ formation was largely CYP3A-dependent, while α-OH-MDZ formation was mediated by CYP3A and -2C isoforms. Western blot analysis revealed decreased microsomal content of CYP3A in old livers. Net intrinsic clearance of MDZ was correlated with total CYP3A content (P < .001). These results demonstrate a reduction in MDZ biotransformation in old male mice, which may be attributable, in part, to decreased CYP3A content in old livers. Changes in expression and activity of CYP2C isoforms also may contribute to age-related changes in MDZ biotransformation, but this requires more investigation.

As the most abundant class of cytochromes P-450 (CYP) in the liver, CYP3A isoforms are responsible for the biotransformation of many drugs (von Moltke et al., 1995b; Transon et al., 1996) and have been linked to age-related changes in drug metabolism (Greenblatt et al., 1988; Horbach et al., 1992). In particular, old age alters the metabolism of several benzodiazepines that have been shown to be CYP3A substrates (Greenblatt et al., 1987; von Moltke et al., 1995a). For example, some studies demonstrate that clearance of midazolam, an index reaction for activity of CYP3A isoforms in humans (Gorski et al., 1994), is reduced in healthy elderly subjects (Greenblatt et al., 1984; Smith et al., 1984). Studies also have demonstrated age-related changes (Greenblatt et al., 1983a; Bertz et al., 1997) and the role of CYP3A (von Moltke et al., 1995a,b; Venkatakrishnan et al., 1998) in the metabolism of alprazolam (ALP). Nonetheless, some human in vivo studies have demonstrated that the biotransformation of CYP3A substrates does not change with age (Hunt et al., 1992b).

Factors that could influence drug clearance with age include the expression, content, and function of catalytically active enzymes, as well as liver mass and hepatic blood flow. In vitro studies can be used to examine the role of age in CYP3A metabolic activity and liver content more directly. The results of such studies have proven inconsistent. Whereas some studies with human liver microsomes have shown an apparent age-related reduction in CYP3A content (George et al., 1995) or total CYP activity (Sotaniemi et al., 1997), others have found no change with age in content (Transon et al., 1996) or in CYP3A activity (Schmucker et al., 1990, 1992a; Shimada et al., 1994, 1995). In vivo and in vitro human studies may be complicated by confounding factors such as disease, medications, diet, tobacco or alcohol use, and genetic composition (von Moltke et al., 1993, 1995b; George et al., 1995; Transon et al., 1996). In vitro studies using autopsy, transplant or surgical tissue.

ABBREVIATIONS: CYP, cytochrome P-450; MDZ, midazolam; ALP, alprazolam.

reflect additional variability arising from the time between death and tissue removal and preservation. Animal models can attenuate some of this variability.

Few studies have examined the role of age on CYP3A activity in mice. Barnhill et al. (1990) demonstrated a diminished clearance of clonazepam, a putative mouse CYP3A substrate, in aging male CD-1 mice in vivo. Charpentier et al. (1997) did not detect significant age-related differences in the in vitro clearance of ALP, a probable CYP3A substrate, in CD-1 mice; in this study, however, the oldest animals did not exceed 12 months old.

Using mouse liver microsomes, we investigated the role of age in the in vitro biotransformation of MDZ, a CYP3A substrate in humans (Gorski et al., 1994; von Moltke et al., 1996). We examined the effect of age on MDZ metabolism in three age groups, and also determined the susceptibility of MDZ biotransformation to inhibition by ketoconazole and by polyclonal antibodies raised against CYP3A1/2 and CYP2C11. To examine further the relationship between CYP3A activity and age and to determine whether age-related changes are specific to MDZ, we inhibited the biotransformation of ALP, another putative CYP3A substrate in mice, with ketoconazole. Finally, by Western blot immunonchantment, we compared CYP3A microsomal content in the three age groups.

Experimental Procedures

Materials. Eighteen male CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). We studied six livers from each of the three age groups (Table 1: young (6–8 weeks), intermediate (12 months), and old (23–24 months). Hepatic microsomal fractions were prepared via differential centrifugation, as described previously (von Moltke et al., 1993; Charpentier et al., 1997).

MDZ, ALP, and their metabolites were kindly provided by their manufacturers or purchased from commercial sources. Solutions of the two major metabolites, a-OH-MDZ and 4-OH-MDZ, were prepared in methanol and stored at −20°C. Other chemicals and reagents were purchased from commercial sources.

Incubations. To determine Vmax and Km, we used varying amounts of MDZ at final concentrations ranging from 1 to 250 μM. All reactions were performed in duplicate. Prior to incubations, the medium was evaporated from each sample. A cofactor preparation was added to each reaction to yield final concentrations of 6.3 mM MgCl2, 0.5 U of isocitric dehydrogenase, 3.8 mM isocitric acid, and 0.5 mM NADP+ in 0.05 M phosphate buffer at pH 7.4. Incubations were initiated with the addition of microsomal protein (0.5 mg/ml) and maintained at 37°C for 5 min. The final reaction volume was 250 μl. Reactions were terminated with 100 μl of acetonitrile and placed on ice. Phenacetin (0.6 μg) was added as an internal standard. Samples were centrifuged at 15,000 rpm for 10 min and supernatants were analyzed by HPLC.

Concentrations of the two major metabolites, a-OH-MDZ and 4-OH-MDZ, were measured at a wavelength of 220 nm, as described previously (von Moltke et al., 1996). The formation rates for both metabolites were linear with respect to both time and protein concentration.

Reaction velocities were calculated based on standard curves of varying known quantities of metabolites, subjected to the same incubation conditions. Because the 4-OH-MDZ metabolite was not available during the kinetic studies, we used the slope of the a-OH-MDZ calibration curve for both metabolites. Since then, we have determined that the 4-OH-MDZ slope is within 15% of the a-OH-MDZ slope.

Ketoconazole Inhibition. Inhibition of MDZ metabolism was conducted in duplicate at 30 μM MDZ with varying concentrations of ketoconazole (0–2.5 μM). Samples were incubated as described above. A more simplified approach was applied to inhibition of ALP biotransformation, which was used to compare MDZ to another CYP3A substrate. Using two concentrations of ketoconazole (0.25 or 0.5 μM), the biotransformation of ALP was evaluated at four fixed concentrations of ALP (100, 250, 500, and 1000 μM). ALP incubations and HPLC quantitation of 4-OH-ALP were conducted as described previously (von Moltke et al., 1993, 1994; Charpentier et al., 1997). Samples were incubated for 20 min in duplicate. To identify metabolites, retention times of MDZ and ALP metabolites were compared with known standard metabolites of MDZ (Hoffmann-La Roche Inc., Nutley, NJ) and ALP (Pharmacia and UpJohn, Kalamazoo, MI).

Antibody Inhibition. Immunoinhibition reactions were performed as described previously (Schmider et al., 1996). MDZ (30 μM) was preincubated with microsomes (at 0.5 μg/μl) and antibody (at 10 μg/μl) for 30 min at 37°C. The antibodies that were used were either polyclonal rabbit anti-rat CYP3A1/2 (Xenotech, Kansas City, KS) or polyclonal goat anti-rat CYP2C11 (Gentest, Woburn, MA). Reactions were initiated with the cofactor mixture described above. Control samples with either rabbit or goat serum or 0.1 M potassium phosphate buffer in place of antibody were run alongside samples with antibodies. Metabolite formation rates in samples containing antibody or serum were expressed as a percentage of the reaction velocities in samples containing potassium phosphate buffer.

Data Analysis: Kinetics of a-OH- and 4-OH-MDZ Formation. As previously reported in humans (von Moltke et al., 1996), formation of 4-OH-MDZ from MDZ was consistent with single-enzyme Michaelis-Menten kinetics (Fig. 1). Accordingly, the standard Michaelis-Menten equation was fitted to 4-OH-MDZ data points:

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

in which V is the rate of 4-OH-MDZ formation, and S is the concentration of the substrate, midazolam. Vmax (the maximum velocity) and Km (the substrate concentration corresponding to 50% Vmax) were calculated by nonlinear regression.

The pattern of a-OH-MDZ formation from MDZ was consistent with Michaelis-Menten kinetics with competitive substrate inhibition (von Moltke et al., 1996) (Fig. 1). The following equation was fitted to a-OH-MDZ data points:

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

### TABLE 1

Animal and liver weights for the three age groups

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Mean ± S.D. Values for Age Group</th>
<th>Value of F from ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (6–8 weeks)</td>
<td>36.0 ± 1.90</td>
<td>44.4, P &lt; .001</td>
</tr>
<tr>
<td>Intermediate (12 months)</td>
<td>54.2 ± 4.07</td>
<td>44.0, P &lt; .001</td>
</tr>
<tr>
<td>Old (23–24 months)</td>
<td>38.8 ± 4.31</td>
<td>44.4, P &lt; .001</td>
</tr>
</tbody>
</table>

* A Student-Newman-Keuls test indicated that the intermediate group differs significantly (P < .001) from young and old, but young and old do not differ from each other.

** A Student-Newman-Keuls test indicated that all three groups differ from each other (P < .05).
in which $V$, $V_{\text{max}}$, $S$, and $K_{m}$ are as described previously, and $K_{s}$ represents the substrate inhibition constant.

For both MDZ metabolites, the $V_{\text{max}}/K_{m}$ ratio, termed intrinsic clearance, was calculated. This quantity represents the proportionality constant between $S$ and $V$ at low values of $S$ and is commonly used as an index of intrinsic enzyme activity. Ketoconazole inhibition data were analyzed by nonlinear regression (von Moltke et al., 1998) to determine the estimated maximum degree of inhibition ($E_{\text{max}}$) and, when possible, the ketoconazole concentrations producing a reduction in reaction velocity to 50% of the control value without inhibitor ($IC_{50}$).

**Fig. 1.** In vitro MDZ biotransformation in representative young, intermediate, and old livers. Aggregate $V_{\text{max}}$ and $K_{m}$ values are shown in Table 2.

**Fig. 2.** Ketoconazole inhibition of MDZ biotransformation in young, intermediate, and old liver microsomes. Increasing concentrations of ketoconazole (0, 0.1, 0.25, 0.5, 1.0, 2.5 $\mu$M) were used to inhibit biotransformation of 30 $\mu$M MDZ. Each point is the mean $\pm$ S.E. for six liver samples.

**Statistical Analyses.** One-way ANOVA and Student-Newman-Keuls multiple comparison tests were used to determine the statistical significance of kinetic parameters among the three age groups.

**Western Analysis.** Western blot analysis was used to quantify CYP3A content, as described previously (Perloff et al., 1999). Using precast 7.5% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA), microsomes (0.75 or 1 $\mu$g) were loaded alongside known concentrations (0.5–3.5 pmol) of lymphoblast cDNA-expressed CYP3A4 protein (Gentest, Woburn, MA) and a molecular weight marker (Bio-Rad).

Samples were separated and then transferred to polyvinylidene
In vitro studies on biotransformation of MDZ in three age groups

$V_{\text{max}}$ values are expressed as nanomoles per minute per milligram protein. $K_m$ values are expressed in micromoles per liter. The relatively large S.D. of the mean $K_m$ value for the 4-OH-MDZ pathway in the old group is attributable to a single high value (66.4 μM). Six livers were used for each group.

**TABLE 2**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Young (Mean ± S.D.)</th>
<th>Intermediate (Mean ± S.D.)</th>
<th>Old (Mean ± S.D.)</th>
<th>$P$ from ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-OH-MDZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$V_{\text{max}}$</td>
<td>1.35 ± 0.22</td>
<td>1.74 ± 0.35</td>
<td>0.87 ± 0.20</td>
<td>16.4, $P &lt; .001$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>5.60 ± 0.95</td>
<td>6.00 ± 1.49</td>
<td>4.49 ± 0.58</td>
<td>3.15, NS</td>
</tr>
<tr>
<td>$K_m^{**}$</td>
<td>411.5 ± 147.7</td>
<td>278.7 ± 107.9</td>
<td>773.9 ± 499.2</td>
<td>4.2, $P &lt; .05$</td>
</tr>
<tr>
<td>1000$V_{\text{max}}/K_m$</td>
<td>240.9 ± 20.7</td>
<td>294.6 ± 46.0</td>
<td>191.8 ± 29.2</td>
<td>14.0, $P &lt; .001$</td>
</tr>
<tr>
<td>4-OH-MDZ pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.70 ± 0.17</td>
<td>0.88 ± 0.14</td>
<td>0.45 ± 0.09</td>
<td>15.2, $P &lt; .001$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>31.2 ± 4.4</td>
<td>28.7 ± 4.2</td>
<td>31.0 ± 17.5</td>
<td>0.10, NS</td>
</tr>
<tr>
<td>1000$V_{\text{max}}/K_m^{**}$</td>
<td>22.8 ± 6.7</td>
<td>31.2 ± 7.0</td>
<td>16.4 ± 5.7</td>
<td>7.88, $P &lt; .05$</td>
</tr>
<tr>
<td>Sum of both pathways</td>
<td>263.7 ± 23.0</td>
<td>325.8 ± 48.2</td>
<td>208.2 ± 32.8</td>
<td>16.1, $P &lt; .001$</td>
</tr>
</tbody>
</table>

* A Student-Newman-Keuls test indicated that each age group was different from the others ($P < .05$).
** A Student-Newman-Keuls test detected a difference of the intermediate group from the young and the old ($P < .05$).
*** A Student-Newman-Keuls test detected a difference between the intermediate and the old groups ($P < .05$).

Animals and Kinetics of MDZ Biotransformation. The intermediate group of animals had the highest mean body weights and liver weights (Table 1). The mean ± S.D. values for $V_{\text{max}}$, $K_m$, and intrinsic clearance in each of the three age groups are listed in Table 2. On average, intrinsic clearance via the $\alpha$-OH-MDZ pathway accounted for >90% of the total intrinsic clearance.

$V_{\text{max}}$, for both the $\alpha$-OH and 4-OH pathways, was significantly different among the three age groups ($P < .001$). The intermediate age group demonstrated the greatest average $V_{\text{max}}$, while the old group had the lowest average $V_{\text{max}}$ values (Fig. 1). The $K_m$ values for the two pathways did not significantly differ among the three groups.

Largely due to changes in $V_{\text{max}}$ values, the intrinsic clearance for $\alpha$-OH-MDZ and 4-OH-MDZ and the net intrinsic clearance were significantly different among the three groups to varying degrees (Table 2). In all cases, the intermediate group of animals had significantly higher intrinsic clearance values than the other two groups ($P < .05$), whereas the lowest values occurred in the older group.

**Ketoconazole Inhibition of MDZ Biotransformation.**

Using 2.5 μM ketoconazole at 30 μM MDZ, the degree of inhibition was 88% for the 4-OH pathway in all three age groups; however, the degree of inhibition was only 46% for the $\alpha$-OH-MDZ pathway among the three groups (Fig. 2). Table 3 reflects the projected $E_{\text{max}}$ values for both pathways and the $IC_{50}$ values for the 4-OH-MDZ pathway. This data suggests that 4-OH-MDZ production is largely mediated by CYP3A. Since ketoconazole only partially inhibited $\alpha$-OH-MDZ production, it is likely that some other enzyme also contributes to the $\alpha$-OH-MDZ pathway. Comparison of $E_{\text{max}}$ values or reaction velocities at 2.5 μM ketoconazole did not reveal a significant difference among the three age groups (Table 3). Although it did not reach statistical significance, the extent to which ketoconazole inhibited MDZ metabolite formation tended to be less in the old group for the $\alpha$-OH-MDZ pathway.

**Ketoconazole Inhibition of ALP Biotransformation.**

Using 0.5 μM ketoconazole at 100 μM ALP, an average of 90% of 4-OH-ALP production and 83% of $\alpha$-OH-ALP production was inhibited (Fig. 3). Consistent with competitive inhibition, the degree of inhibition could be overcome by increasing the amount of substrate. There was no statistically significant difference in ketoconazole inhibition of ALP biotransformation among the three age groups.

**Antibody Inhibition of MDZ Biotransformation.**

Perloff et al. (2000) have demonstrated that isoforms from the CYP2C subfamily also contribute to $\alpha$-OH-MDZ production. The relative contributions of CYP2C and CYP3A isoforms with age in MDZ biotransformation were evaluated by immunoinhibition (Fig. 4). As expected, the CYP2C11 antibody did not inhibit the 4-OH-MDZ pathway. Due to limited quantities of...
microsomal tissue and antibodies, only one representative sample in each age group was tested. However, since only one liver was evaluated from each age group, the results could not be evaluated statistically and extrapolations from this data are limited.

**Western Blot Analysis.** As shown in Fig. 5, the polyclonal rabbit anti-rat CYP3A1/2 antibody identified two bands in the mouse liver microsomes. This Western blot analysis revealed that microsomal content of CYP3A differs with age. Age-related differences were observed in both the upper band \( (P < .001) \) and the lower band \( (P < .05) \). These differences mainly reflected reductions in the old group. The upper band was almost absent in the old livers.

**Correlation of Intrinsic Clearance and Total CYP3A Content.** Net intrinsic clearance of MDZ was found to be correlated \( (r = 0.85, P < .001) \) with total immunoreactive CYP3A (the summation of the top and bottom bands), as shown in Fig. 6. Thus, a substantial proportion of the overall variability in MDZ hydroxylation activity can be explained by total CYP3A content, with older animals showing the lowest values of both.

**Discussion**

We used an aging mouse model to examine the effect of age on in vitro CYP3A activity and liver content. In the old animals, we demonstrated reduced intrinsic clearance \( (V_{\text{max}}/K_m) \) for the two pathways of MDZ hydroxylation. This reduction in the old group is attributable to a reduced \( V_{\text{max}} \), which is proportional to the quantity of metabolically active enzyme.

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*Fig. 3.* Ketoconazole inhibition of ALP biotransformation in young, intermediate, and old liver microsomes. Two concentrations of ALP (100 and 1000 \( \mu \)M) were exposed to three concentrations of ketoconazole (0, 0.25, 0.5 \( \mu \)M). Each point is the mean \( \pm \) S.E. for six liver samples. Production of both metabolites displayed a similar profile, although only the \( \alpha \)-OH-ALP pathway is shown.

*Fig. 4.* Immunoinhibition of the biotransformation of MDZ (30 \( \mu \)M) in a young, intermediate, and old liver. Inhibition of 4-OH-MDZ production (A) and \( \alpha \)-OH-MDZ production (B) by polyclonal rabbit anti-rat P450–3A1/2 and goat anti-rat P450–2C11 antibodies. ■, samples incubated with control sera (rabbit serum for CYP3A controls and goat serum for CYP2C controls); □, samples incubated with antibodies.
per milligram of microsomal protein. The $K_m$ values, which reflect the affinity of the enzyme for the substrate MDZ, did not change with age. These findings are consistent with the Western blot data. Using a polyclonal anti-rat CYP3A1/2 antibody, we demonstrated a significant decline in immunoreactive CYP3A in the old group. The top band, which may represent CYP3A11 (mol. wt. 57,853 Da; Yanagimoto et al., 1992), was affected by age to a greater degree than the bottom band, which may be CYP3A13 (mol. wt. 57,491 Da; Yanagimoto et al., 1994). The correlation of total intrinsic clearance (a summation of the intrinsic clearance for both pathways) versus net immunoreactive CYP3A (a summation of the top and bottom bands on the Western blot) demonstrates a close relationship between MDZ biotransformation and quantity of CYP3A. The reduced biotransformation of MDZ in older male mice appears to be attributable to decreased quantities of CYP3A and not to a difference in affinity.

We addressed the role of CYP3A in the biotransformation of MDZ more directly with chemical and antibody inhibition. In humans, 2.5 $\mu$M ketoconazole appears to be relatively specific for CYP3A (Newton et al., 1995). Both methods demonstrated that CYP3A is largely responsible for 4-OH-MDZ formation in mice, although some other enzyme may be responsible for the remaining 10% of 4-OH-MDZ production seen in both these and previous ketoconazole inhibition studies (Perloff et al., 2000). Chemical and antibody inhibition studies demonstrated that CYP3A is only partly responsible for $\alpha$-OH-MDZ production. The present results together with previous work (Perloff et al., 2000) suggest that isoforms from the CYP2C subfamily are responsible for an important component of $\alpha$-OH-MDZ production. This conclusion is further supported by regression analysis of total MDZ clearance versus immunoreactive CYP3A, in which the nonzero y-intercept may represent the component of clearance not contributed by CYP3A. Ketoconazole inhibition studies with ALP as the substrate demonstrated that, in contrast to MDZ, both pathways of ALP hydroxylation appear to be largely mediated by CYP3A.

The ketoconazole inhibition of both MDZ and ALP did not reveal significant changes in mean $E_{\text{max}}$ or IC$_{50}$ values with

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**Fig. 5.** Immunoquantification of CYP3A in young, intermediate, and old livers. A, representative Western blot comparing quantities of young (lanes 7 and 10), intermediate (lanes 8 and 11), and old (lanes 9 and 12) age microsomal protein (1 $\mu$g) probed with polyclonal rabbit anti-rat CYP3A1/2 antibody. Lanes 2 to 5 consist of a standard curve of known quantities of human CYP3A4 (0.5, 0.75, 1.5, 3.5 pmol), which were probed with the same antibody. A molecular weight marker was loaded in lane 1 and no samples were loaded into lane 6. B, quantitative analysis of the Western blot demonstrating age-related changes in the bottom and top bands. Differences among the three age groups reached statistical significance for both bands, as evaluated by ANOVA. Six livers were evaluated for each age group.

**Fig. 6.** Correlation analysis of total intrinsic clearance ($V_{\text{max}}/K_m$) of MDZ versus quantity of immunoreactive CYP3A (the summation of the quantified bottom and top bands) comparing young, intermediate, and old age groups.
age. The old group may have a slightly reduced susceptibility to ketoconazole inhibition of formation of $\alpha$-OH-MDZ from MDZ, but this was not supported statistically.

The majority of animal experimentation on the role of age in CYP-mediated metabolism has been conducted in rats. With notable exceptions, most studies have reported an increase in body and liver weights in aging rats (van Be zooinen et al., 1986; Rikans, 1989; Horbach et al., 1992) with a concomitant decline in total microsomal protein (Horbach et al., 1992) and total CYP content (Schmucker and Wang, 1980; Rikans and Notley, 1982; Rikans, 1989). The impact of age varies depending on the specific CYP. Previous studies have demonstrated an age-related decline in CYP2C11 activity, as measured by aldrin epoxidation (Horbach et al., 1992), and in the CYP3A content (Imaoka et al., 1991) of rat liver microsomes.

The pattern of MDZ biotransformation in rats only partly resembles that seen in mice (Ghosal et al., 1996). 4-OH-MDZ and $\alpha$-OH-MDZ are the major metabolites of MDZ biotransformation in both species. However, while the $\alpha$-OH-MDZ pathway is the major route of clearance in mice and humans, 4-OH-MDZ is the predominant pathway in the rat. Also, unlike mouse MDZ biotransformation, both metabolites in the rat are 90 to 100% susceptible to ketoconazole inhibition at 5 $\mu$M ketoconazole (Ghosal et al., 1996). To our knowledge, however, MDZ biotransformation in aging rats has not been examined.

Similar to the pattern seen in mouse liver microsomes, $\alpha$-OH-MDZ production is the predominant metabolic pathway of MDZ biotransformation in human liver microsomes, while 4-OH-MDZ formation accounts for a relatively small fraction of clearance (von Molteke et al., 1996). In both species, the $K_m$ values for the respective pathways are similar. In the human, the mean $K_m$ values for the $\alpha$-OH- and 4-OH-MDZ pathways are 3.3 and 57 $\mu$M, respectively (von Molteke et al., 1996). Nonetheless, both MDZ oxidative pathways in humans appear to be exclusively mediated by CYP3A.

The inconsistent findings of human studies may be attributable to a number of factors, including diet, medications, disease, and genetic composition. Importantly, some studies suggest that CYP3A4 activity in humans may be influenced by gender (Harris et al., 1995). The combined effect of age and gender in humans has been examined in vivo and in vitro with mixed results. Some in vitro studies have shown no change with either sex or age in metabolic activity or content (Schmucker et al., 1990; Shimada et al., 1994). Yet, Sotaniemi et al. (1997) have demonstrated a reduction in total CYP content in both sexes to an equal degree with age, whereas Hunt et al. (1992a) have shown a change associated with sex, but not age, in CYP3A4 activity. In contrast, George et al. (1995) demonstrated a change with age, but not with sex, in total CYP content.

In vivo studies examining changes in CYP-mediated metabolism with age and sex have been more conclusive, although some variability remains. Two studies on the effects of age and gender on MDZ metabolism have indicated reduced clearance in elderly males versus young male controls, but have demonstrated no significant change with age in females (Greenblatt et al., 1984; Holazo et al., 1988). Reduced clearance in elderly men has also been reported for ALP (Greenblatt et al., 1983a). Age-dependent decrements in triazolam clearance have been reported for both men and women (Greenblatt et al., 1983b, 1991). Any variability in results may depend on the substrates and enzymes involved as well as on how these changes were measured. Studies using oral dosing are further complicated by possible gender differences in bioavailability and oral clearance. Although sex can theoretically influence drug absorption through changes in gastric emptying time and intestinal transit time, it is not yet clear whether differences in intestinal CYP3A contribute to sex-dependent changes in drug metabolism (Harris et al., 1995). Nonetheless, given the possible role of sex in altering drug metabolism with age in humans and in rats (Rikans, 1989), the present work should be extended to female CD-1 mice.

In contrast to previous studies on aging in which 2-year-old mice weighed more than younger controls (Webster et al., 1976; Barnhill et al., 1990), we found a reduction in total weight in the old age group. We also found a reduction in liver weight in the old group. These findings may reflect possible limitations of this study. Although there was no evidence of illness, the weight reduction may reflect general debility rather than a specific effect of age on CYP3A. Due to the limited amount of hepatic tissue available, we were not able to clarify this further by determining the metabolic activity and immunologic content of other CYP enzymes. We adjusted for total protein amount by using equal amounts of microsomal protein in both our kinetic experiments and our Western blots for all animals. In any case, these findings suggest that this model may be further explored to assess the mechanism of the apparently reduced expression of CYP3A in the aging animals. This model also may be useful in examining reduced MDZ biotransformation in humans.

References


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