Effect of Age on in Vitro Triazolam Biotransformation in Male Human Liver Microsomes

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Received August 29, 2003; accepted November 13, 2003

ABSTRACT

We studied age-related changes in enzyme kinetic parameters in human liver microsomes (HLMs) in vitro, using triazolam (TRZ), an index of CYP3A activity. HLMs were prepared from male livers from four age groups, n = 5 per group: A (14–20 years), B (21–40 years), C (41–60 years), and D (61–72 years). Mean $V_{\text{max}}$ values in groups B and C for both 1-hydroxy-triazolam (1-OH-TRZ) and 4-hydroxy-triazolam (4-OH-TRZ) formation were significantly greater as compared with groups A and D individually, as well as the net intrinsic clearance (sum of the two pathways). The mean net intrinsic clearance ($Cl_{\text{int}}$) values were 25.2, 89.8, 78, and 20.6 nl/min/mg protein in A, B, C, and D, respectively. TRZ $Cl_{\text{int}}$ correlated well with total CYP3A content ($r_s = 0.84; P < 0.0001$). Testosterone (TST) inhibited 1-OH TRZ formation and activated 4-OH TRZ formation in all age groups, with no significant differences among the groups; this suggests that the drug-drug interaction potential using TRZ and TST as index CYP3A substrates may not change with age. Reduced $V_{\text{max}}$ and $Cl_{\text{int}}$ for TRZ hydroxylation and CYP3A protein in livers from elderly men suggest reduced CYP3A gene expression in this group.

There is considerable pharmacokinetic evidence suggesting that age-related changes occur in drug disposition (Schmucker, 1985; Greenblatt et al., 1989; von Moltke et al., 1995a). However, in some clinical studies, no age-related changes were demonstrated in the biotransformation of CYP3A substrates (see reviews by Greenblatt et al., 1982; Cotreau et al., 2004). Factors that could influence drug clearance in the elderly include the expression, content, and function of catalytically active enzymes, as well as liver mass, hepatic blood flow, and renal function (Vestal, 1982; Greenblatt et al., 1986). It has been shown that triazolam (TRZ) clearance is reduced in the elderly (Greenblatt et al., 1983a, 1991).

In vitro studies could be helpful in examining the role of age on the metabolic activity of CYP3A and liver CYP3A content. However, the results of such studies have been inconsistent. Some studies using human liver microsomes (HLMs) have shown an age-related decline in CYP3A content (George et al., 1995; Sotaniemi et al., 1997) or total P450 activity (Sotaniemi et al., 1997), whereas others have found no change associated with age in content (Shimada et al., 1994; Transon et al., 1996) or activity of CYP3A (Schmucker et al., 1990; Hunt et al., 1992; Shimada et al., 1994; Transon et al., 1996).

Previous in vitro studies have evaluated the effect of age on metabolite formation rate via CYP3A-mediated biotransformation, but without consideration for enzyme affinity (Hunt et al., 1992; Transon et al., 1996). HLMs were classified into one or more classes over a wide age range, and these studies did not evaluate the possible contribution of CYP3A5 to age-related effects on CYP3A metabolism (Hunt et al., 1992; Shimada et al., 1994).

To address these issues, we examined the change of age on TRZ metabolism in HLMs from male donors ranging in age from 14 to 72 years. We also immunoquantified levels of both CYP3A5 and CYP3A4 to evaluate the possible contribution of CYP3A5 to CYP3A metabolism. To our knowledge, age-related changes in susceptibility to drug-drug interactions (DDIs) have not been evaluated in HLMs. Hence, we also evaluated the effect of varying concentrations of testosterone.
(TST) (0–100 μM) on a fixed concentration of TRZ (250 μM) in HLMs of varying ages to determine the age-related susceptibility to DDIs involving CYP3A, using TST and TRZ as prototypic CYP3A substrates.

Materials and Methods

Materials. 1-Hydroxy-triazolam (1-OH-TRZ), 4-OH-TRZ (4-hydroxy-triazolam), and TRZ were kindly provided by their pharmaceutical manufacturers or purchased from Ultrafine Chemicals (Manchester, UK). TST was purchased from Sigma-Aldrich (St. Louis, MO). Reaction cofactors (NADP+, di-isocitric acid, magnesium chloride, isocitric dehydrogenase, and potassium phosphate buffer solutions) were obtained from Sigma-Aldrich. Human CYP3A4 and CYP3A5 Western Blotting Kits (catalog numbers 458234 and 458235, respectively) were purchased from BD Gentest (Woburn, MA). Recombinant CYP3A4 and CYP3A5, which are expressed from human CYP3A4 and CYP3A5 cDNA using a baculovirus expression system, were purchased from BD Gentest. Sample protein concentration and P450 content were provided by the manufacturer.

Microsomal Preparation. Liver samples from male human donors were provided by the International Institute for the Advancement of Medicine (Exton, PA), the LIVER Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN), or the National Disease Research Interchange (Philadelphia, PA). These HLMs were classified into four age groups, the National Disease Research Interchange (Philadelphia, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN), or the National Disease Research Interchange (Philadelphia, PA). These HLMs were classified into four age groups, the National Disease Research Interchange (Philadelphia, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN), or the National Disease Research Interchange (Philadelphia, PA). These HLMs were classified into four age groups.

Incubation. Incubation mixtures contained 50 mM phosphate buffer, 5 mM MgCl₂, 0.5 mM NADP⁺, and an isocitrate/isocitric dehydrogenase regenerating system. Incubations were performed at 0 to 1500 μM TRZ to establish kinetic parameters for metabolite formation in HLMs (von Moltke et al., 1993). In brief, microsomes were prepared through ultracentrifugation; microsomal pellets were resuspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at –80°C until use. Total protein concentration was determined by a bicinchoninic acid protein assay (BCA assay; Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Antibodies and Quantitative Western Blotting. Antibodies and quantitative Western blotting were used to determine the age-related susceptibility to DDIs involving CYP3A, using TST and TRZ as prototypic CYP3A substrates. All postantibody washings were done three times (5 min each) in Tris-buffered saline (0.15 M NaCl, 0.04 M Tris-Cl, pH 7.7) containing 0.06% Tween 20. Blots were imaged (Kodak Image Station 440CF; Kodak, Rochester, NY) and bands were quantified using Kodak 1D Image Analysis Software (Kodak). A calibration curve of integrated band intensity (the product of band area and band intensity; Y) versus the quantity of P450 standard, in picomoles, as follows: Y = m (log x) + b for CYP3A5 and Y = mx + b for CYP3A4, where m and b are the slope and intercept terms, respectively. Integrated band densities of liver microsomal samples were used to determine the concentration of P450 per milligram of microsomal protein relative to the calibration curve. Antibodies used for CYP3A4 and CYP3A5 were specific, with no cross-reactivity, based on data from their manufacturers.

Effect of TST on TRZ Metabolism. For evaluating the effect of TST on TRZ metabolism, a fixed incubation time was used to determine the age-related susceptibility to DDIs involving CYP3A, using TST and TRZ as prototypic CYP3A substrates. All postantibody washings were done three times (5 min each) in Tris-buffered saline (0.15 M NaCl, 0.04 M Tris-Cl, pH 7.7) containing 0.06% Tween 20. Blots were imaged (Kodak Image Station 440CF; Kodak, Rochester, NY) and bands were quantified using Kodak 1D Image Analysis Software (Kodak). A calibration curve of integrated band intensity (the product of band area and band intensity; Y) versus the quantity of P450 standard, in picomoles, as follows: Y = m (log x) + b for CYP3A5 and Y = mx + b for CYP3A4, where m and b are the slope and intercept terms, respectively. Integrated band densities of liver microsomal samples were used to determine the concentration of P450 per milligram of microsomal protein relative to the calibration curve. Antibodies used for CYP3A4 and CYP3A5 were specific, with no cross-reactivity, based on data from their manufacturers.

Data Analysis. Kinetic parameters for TRZ biotransformation and TRZ metabolite formation with the addition of TST were determined through nonlinear least-squares regression analysis of untransformed data. Model selection was based on empiric goodness of fit criteria. 1-OH-TRZ and 4-OH-TRZ formation data for TRZ were fit to a Michaelis-Menten model. The parameters that were estimated are: Vₘₐₓ, the maximum reaction velocity (uninhibited); Kₘ, the substrate concentration corresponding to 50% of Vₘₐₓ; and Vₘₐₓ/Kₘ. 1000, the estimated in vitro intrinsic clearance. Since cell sizes were small and expected variance was large, values were rank-transformed and subjected to ANOVA. Pairwise comparisons were made on rank-transformed values using a Student-Newman-Keuls test. Nonparametric correlation analysis was performed using the Spearman correlation coefficient (Spearman's ρ) to assess the relationship between age and the primary end points.
man test \( (r_s) \). \( P \) value less than 0.05 was considered as statistically significant.

**Results**

**TRZ Biotransformation in Vitro.** Mean kinetic parameters for TRZ hydroxylation in the four age groups (Fig. 1) are shown in Table 1. TRZ 1-hydroxylation accounted for a greater proportion of estimated intrinsic clearance than did 4-hydroxylation in HLMs in all age groups (Table 1). Mean \( V_{\text{max}} \) values in Groups B and C for both 1-OH- and 4-OH-TRZ formation were significantly greater \( (P < 0.01) \) as compared with groups A and D individually, as well as the net intrinsic clearance (sum of the two pathways) (Figs. 1 and 2; Table 1). The mean net intrinsic clearance values were 25.2, 89.8, 78, and 20.6 nl/min/mg protein in Groups A, B, C, and D, respectively (Table 1).

**Western Blot Analysis.** CYP3A4 expression was variable, with levels ranging from 0.9 to 829 pmol/mg of microsomal protein. CYP3A5 was detected in 55\% (11 of 20) of the liver samples. CYP3A5 content varied from 0.01 to 9.6 pmol/mg protein. In our samples, the contribution of CYP3A5 was up to 18\% of CYP3A4 content. Multiple comparison analysis on rank-transformed values (Student-Newman-Keuls test) indicated that microsomal content of CYP3A4 differs significantly with age \( (P < 0.01, \text{Table 2}) \).

**Correlation of Net Intrinsic Clearance and Total CYP3A Content.** Net intrinsic clearance of TRZ was found to be correlated \( (r_s = 0.84; P < 0.0001) \) with total immunoreactive CYP3A (the sum of CYP3A4 and CYP3A5) (Fig. 3). Thus, a substantial proportion of the overall variability in TRZ hydroxylation activity can be explained by total CYP3A content, with elderly subjects generally showing the lower values.

**Effect of TST on TRZ Metabolite Formation.** TST inhibited 1-OH-TRZ formation (down to 31\% of control), whereas 4-OH-TRZ formation was significantly activated (up to 230\% of control) (Fig. 4) in all age groups. There were no significant differences (ANOVA, \( P > 0.05 \)) among the four groups with respect to maximum effect of TST on TRZ metabolite formation.

**Discussion**

The CYP3A subfamily is the most abundant class of cytochromes P-450 (P450s) in the liver responsible for the biotransformation of many drugs (von Moltke et al., 1995b; Transon et al., 1996). There is considerable pharmacokinetic evidence to indicate that age-related changes occur in drug disposition (Schmucker, 1985; Greenblatt et al., 1989; Schmucker, 2001). Old age has been shown to alter the metabolism of several CYP3A substrate drugs, including a number of benzodiazepine derivatives (Greenblatt et al., 1989; von Moltke et al., 1995a; Cotreau et al., 2004). According to some studies, the clearance of the CYP3A substrate midazolam is reduced in healthy elderly subjects (Greenblatt et al., 1984; Smith et al., 1984). Studies have also shown age-related changes in the metabolism of alprazolam and TRZ (Greenblatt et al., 1983a,b, 1991; Bertz et al., 1997). However, in some human in vivo studies, no age-related changes were demonstrated in the biotransformation of CYP3A substrates (see reviews by Greenblatt et al., 1982; Cotreau et al.,

![Fig. 1. Representative kinetic plots of in vitro biotransformation of TRZ in four age groups (A, B, C, and D). 1-OH-TRZ formation is indicated by filled circles and 4-OH-TRZ formation is indicated by open circles). Lines represent functions determined by nonlinear least-squares regression analysis.](image-url)
TABLE 1

In vitro biotransformation of TRZ in four age groups

Results are expressed as mean ± S.E. ANOVA was applied on rank-transformed values. $V_{\text{max}}$ values are expressed as nanomoles per minute per milligram of protein. $K_m$ values are expressed in micromoles per liter. $V_{\text{max}}/K_m$ (1000) values are expressed as nanoliters per minute per milligram of protein.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Group A (14–20 years)</th>
<th>Group B (21–40 years)</th>
<th>Group C (41–60 years)</th>
<th>Group D (61–72 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-OH TRZ pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>1.0 ± 0.2</td>
<td>3.3 ± 0.9**</td>
<td>2 ± 0.4**</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>$K_m$</td>
<td>67.7 ± 11</td>
<td>52.7 ± 9.2</td>
<td>36.1 ± 4.3</td>
<td>80.4 ± 22.8</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m$</td>
<td>19 ± 7.5</td>
<td>60.7 ± 14.1**</td>
<td>60.1 ± 13.5**</td>
<td>15 ± 4.8</td>
</tr>
<tr>
<td>4-OH TRZ pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>2.2 ± 0.4</td>
<td>7.1 ± 2.2**</td>
<td>4.7 ± 0.9**</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>$K_m$</td>
<td>381 ± 55</td>
<td>253.6 ± 31.1</td>
<td>317 ± 61</td>
<td>388 ± 53.6</td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m$</td>
<td>6.2 ± 1.5</td>
<td>29.2 ± 8.6**</td>
<td>17.9 ± 5.1**</td>
<td>5.7 ± 1.8</td>
</tr>
<tr>
<td>Net intrinsic clearance (sum of $V_{\text{max}}/K_m$ (1000))</td>
<td>25.2 ± 9</td>
<td>89.8 ± 22.1**</td>
<td>78 ± 17.5**</td>
<td>20.6 ± 6.4</td>
</tr>
</tbody>
</table>

** A Student-Newman-Keuls test on rank-transformed values indicated that groups A and D were different from groups B and C ($P < 0.01$).

TABLE 2

Expression of immunoreactive CYP3A4, CYP3A5, and total CYP3A in four age groups

Results are expressed as mean ± S.E. Immunoreactive P450 is expressed as picomoles per milligram of protein. ANOVA was applied on rank-transformed values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunoreactive CYP3A4</th>
<th>Immunoreactive CYP3A5</th>
<th>Total immunoreactive CYP3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15.2 ± 10.1</td>
<td>1.9 ± 1.9</td>
<td>17.1 ± 12</td>
</tr>
<tr>
<td>B</td>
<td>424.8 ± 149.7**</td>
<td>0.8 ± 0.5</td>
<td>425.6 ± 149.4**</td>
</tr>
<tr>
<td>C</td>
<td>126.2 ± 44.7**</td>
<td>1.0 ± 0.9</td>
<td>127.3 ± 44.5</td>
</tr>
<tr>
<td>D</td>
<td>43.4 ± 16.6</td>
<td>0.24 ± 0.2</td>
<td>43.7 ± 16.5</td>
</tr>
</tbody>
</table>

* A Student-Newman-Keuls test on rank-transformed values indicated that groups A and D were different from groups B and C ($P < 0.01$).

** A Student-Newman-Keuls test on rank-transformed values indicated that groups A and D were different from group B ($P < 0.01$).

Fig. 2. Total intrinsic clearance ($V_{\text{max}}/K_m$) of TRZ versus age (years) in HLMs.

Fig. 3. Correlation analysis of total intrinsic clearance ($V_{\text{max}}/K_m$) of TRZ versus quantity of immunoreactive CYP3A (the sum of CYP3A4 and CYP3A5) among all samples ($r_s = 0.84; P < 0.0001$).
activity in humans may be influenced by gender (Allen et al., 1980; Kremers et al., 1981; Tuenissen et al., 1987; Hunt et al., 1992; Shimada et al., 1994; Harris et al., 1995; Cotreau et al., 2004). The combined effect of age and gender in humans has been examined in vivo and in vitro with mixed results (Cotreau et al., 2004). Some in vitro studies have shown no change with either gender or age in P450 metabolic activity or content (Schmucker et al., 1990). Hence we used HLMs from males from four age groups to evaluate the effect of age on in vitro CYP3A activity and CYP3A protein content in the liver.

Mean $V_{\text{max}}$ values in groups B and C (ages 21–60 years) for both 1-OH- and 4-OH-TRZ formation were significantly greater as compared with groups A (14–20 years) and D (61–72 years) individually, as well as the net intrinsic clearance (sum of the two pathways) (Fig. 2, Table 1). This indicates reduced catalytic activity in groups A and D compared with groups B and C (Fig. 2). These findings are consistent with other studies (George et al., 1995; Stevens et al., 2003). As in previous reports (Wynne et al., 1988), $K_m$ values did not differ significantly among the four age groups (Table 1). These findings indicate that the reduced intrinsic clearance in groups A and D is attributable to decreased $V_{\text{max}}$ values (Table 2), whereas the qualitative nature of the enzyme (indicated by $K_m$ values) does not differ significantly with age. The reduced mean net intrinsic clearance in the elderly, therefore, is apparently due to the decreased protein expression, and is consistent with many in vivo studies in the elderly (Greenblatt et al., 1983a,b, 1989; von Moltke et al., 1995a; Burtz et al., 1997; Cotreau et al., 2004).

High clearance in adolescents compared with adults has been shown in in vivo studies (Murry et al., 1995; Reith et al., 2000; Kanamori et al., 2002). Our study has shown reduced in vitro clearance in adolescents (<21 years old) compared with adults (Table 1). The discrepancy in our in vitro results and the results of in vivo studies could possibly be due to larger livers normalized to body weight in children and adolescents than in adults, as opposed to higher CYP3A expression per unit of liver weight (Murry et al., 1995; Reith et al., 2000; Kanamori et al., 2002).

CYP3A5 is known to be catalytically equal or less active as compared with CYP3A4 (Williams et al., 2002; Patki et al., 2003). In people polymorphically expressing CYP3A5, the overall catalytic activity of CYP3A may be different from that in people only expressing CYP3A4. Previous studies did not evaluate the possible contribution of CYP3A5 to net CYP3A metabolism (Hunt et al., 1992; Shimada et al., 1994). We immunoquantified all liver samples for CYP3A4 as well as CYP3A5 content. Although 11 of 20 liver samples had detectable CYP3A5 expression, the amounts of CYP3A5 were small compared to CYP3A4 content. In view of the lower catalytic activity of CYP3A5 compared to CYP3A4 (Williams et al., 2002; Patki et al., 2003) and the minor amounts of CYP3A5 in livers expressing this protein in our study, the net contribution of CYP3A5 to the net intrinsic clearance may be minor. Our findings are consistent with a recent study (Westlund-Johnsson et al., 2003) indicating a minor role of CYP3A5 in drug metabolism. The correlation of net intrinsic clearance (a summation of the intrinsic clearance for both pathways) versus net immunoreactive CYP3A (the sum of CYP3A4 and CYP3A5) demonstrated a close relationship between TRZ biotransformation and quantity of CYP3A (Fig. 3). This relationship is essentially unchanged if CYP3A5 levels are excluded (data not shown). Thus, the reduced biotransformation observed in our study of TRZ in older as well as adolescent HLMs may be attributable to decreased quantities of CYP3A4. Stevens et al. (2003) also reported lower expression of CYP3A among individuals up to 15 years of age compared with adults. We did not evaluate the possible contribution of CYP3A4 polymorphisms to variability in catalytic activity or protein expression in this study.

Some previous studies evaluated age-associated changes in overall and/or isomorph-specific expression of P450s (George et al., 1995; Sotaniemi et al., 1997). Age-related changes were noted in overall P450 content (George et al., 1995; Sotaniemi et al., 1997), with a decline in P450 expression in the elderly. George et al. (1995) found a significant negative association between age and expression of CYP3A and CYP2E1, but not of CYP1A2 or CYP2C. Further studies are needed to evaluate the effect of age on the expression as well as the activity of other P450s.

To our knowledge, age-related changes in susceptibility to DDIs have not been evaluated in HLMs. Hence, in our study, we evaluated the effect of TST on TRZ metabolism in all four age groups. Consistent with the results of previous studies (Schrag and Wienkers, 2001; Patki et al., 2003), TST inhibited 1-OH-TRZ metabolite formation and activated 4-OH-TRZ metabolite formation, with no significant difference in the maximum change in the metabolite formation pattern among the age groups (Fig. 4), suggesting that the CYP3A-mediated DDI potential may not change with age.

In conclusion, reduced $V_{\text{max}}$ and intrinsic clearance for TRZ hydroxylation and CYP3A protein in liver samples from the elderly as well as adolescents suggests reduced CYP3A expression in these groups. These findings are consistent with many clinical studies showing reduced clearance of CYP3A substrates in elderly men (Cotreau et al., 2004). The results have implications with regard to appropriate adjustments in drug dosage for the elderly. Using TRZ and TST as index substrates of CYP3A, the effect of TST on TRZ metabolite formation does not differ significantly among any of the
age groups, suggesting that CYP3A-mediated DDI potential may not change with age.

References

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