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

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Running title: Effect of age on TRZ metabolism

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Abbreviations: CYP, cytochrome P-450; TRZ, triazolam; TST, testosterone; rCYP3A4, recombinant CYP3A4; rCYP3A5, recombinant CYP3A5; S.E., standard error; HPLC, high-performance liquid chromatography

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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_{max} values in groups B and C for both 1-OH and 4-OH-TRZ formation were significantly greater as compared to A and D individually, as well as the net intrinsic clearance (sum of the two pathways). The mean net intrinsic clearance (Cl_{int}) values were: 25.2, 89.8,78 and 20.6 nL/min/mg protein in A, B, C and D respectively. TRZ Cl_{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_{max} and Cl_{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 (Greenblatt et al., 1989; Schmucker., 1985; 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., 2003). 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 CYP activity (Sotaniemi et al., 1997), whereas others have found no change associated with age in content (Transon et al., 1996; Shimada et al., 1994) 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 (Transon et al., 1996; Hunt et al., 1992), 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 (Shimada et al., 1994; Hunt et al., 1992).

To address these issues, we examined the effect 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 the 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 effect of varying concentrations of TST (0-100μM) 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.

Methods

Materials:

1-OH-TRZ (1-hydroxy-triazolam), 4-OH-TRZ (4-hydroxy-triazolam), and TRZ were kindly provided by their pharmaceutical manufacturers or purchased from Ultrafine Chemicals (Manchester, England). TST (testosterone) was purchased from Sigma Chemical Co. (St. Louis, MO). Reaction cofactors (NADP+, DL-isocitric acid, magnesium chloride, isocitric dehydrogenase, and potassium phosphate buffer solutions) were obtained from Sigma Chemical Co. (St. Louis, MO). Human CYP3A4 and CYP3A5 Western Blotting Kits (Catalog No. 458234 and 458235, respectively) were purchased from Gentest (Woburn, MA). Recombinant CYP3A4 and CYP3A5, which are expressed from human CYP3A4 and CYP3A5 cDNA using a baculovirus expression system, were purchased from Gentest (Woburn, MA). Sample protein concentration and CYP 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), or the National Disease Research Interchange (Philadelphia, PA). These HLMs were classified in four age groups, n = 5 per group: A (14-20 years), B (21-40 years), C (41-60 years), and D (61-72 years). All microsomes were prepared using standard techniques previously described (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 BSA as a standard.

Incubation:

Incubation mixtures contained 50 mM phosphate buffer, 5 mM $MgCl_2$, 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., 1996a,b; Perloff et al., 2000). Reaction mixtures with HLMs were incubated at 0.25 mg/ml microsomal protein. The volume of incubation reactions was 250 µl. Formation of metabolites with HLMs was linear with respect to incubation time and microsomal protein concentration over ranges relevant to this study. Incubations were initiated by the addition of microsomal protein. After 20 min, at 37°C, reactions were stopped by cooling on ice and the addition of acetonitrile. Phenacetin was added as the internal standard for TRZ, and for the effect of TST on TRZ. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. All samples were processed in duplicate. The formation rate of individual metabolites in reaction mixtures was determined based on calibration curves constructed from a series of standards containing varying known amounts of metabolite standards together with internal standard. Reaction velocities were calculated in units of nanomoles of product formed per minute per milligram of microsomal protein. Control incubations with no cofactor, no protein, and/or no

substrate were performed concurrently to validate CYP-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards. All samples were processed in duplicate. The analytical assays used gave a coefficient of variation (n=6) below 10%. Calibration curves were linear and passed through the origin.

HPLC analysis:

For TRZ and its metabolites and for the effect of TST on TRZ metabolism, the HPLC mobile phase consisted of 70%/20% /10 % 50mM phosphate buffer/acetonitrile/methanol, with a flow rate of 1.5 ml/min. The analytical column (3.9 x150 mm) was a stainless steel reverse phase C-18 Nova-Pak (Waters Associates, Milford, MA). Column effluent was monitored by ultraviolet absorbance at 220 nm for TRZ and for the effect of TST on TRZ metabolism.

Antibodies and Quantitative Western Blotting:

Immunoreactive quantities of CYP3A4 and CYP3A5 in HLMs from twenty livers were determined by quantitative Western blotting as described previously (Perloff et al., 2000; Patki et al., 2003). Microsomal protein (varying amounts of recombinant CYP standards and an optimal amount of liver microsomal protein) was denatured for 5 min at 100°C in 100 mM Tris buffer (pH 6.8) containing 10% glycerol, 2% beta-mercaptoethanol, 2% SDS, and 5 mg/ml pyronin Y. Recombinant CYP3A4 and CYP3A5 (Gentest) were used to generate calibration standards in concentrations ranging from 0.007 to 0.25 pmol/well and 0.039 to 5 pmol/well respectively. Protein

was separated by SDS-polyacrylamide gel electrophoresis in precast 7.5% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) in 25 mM. Tris/0.192 M glycine/0.1% SDS running buffer (pH 8.3) and transferred to Immobilon-P paper (0.45-mm pore size; Millipore, Bedford, MA) by electroblotting at 100 V for 1 h in 25 mMTris/0.192 M glycine/20% methanol transfer buffer. Blots were blocked, incubated with primary antibody for 1 h, washed, incubated with HRP-labeled secondary antibody for CYP3A4 and peroxidase-conjugated anti-rabbit secondary antibody for CYP3A5 for 1 h, washed again, and the bound HRP signal was activated by enhanced chemiluminescence (ECL) using the Super Signal Cl-HRP substrate system (Pierce, Rockford, IL). All postantibody washings were done three times (5 min each) in TBS (0.15 M NaCl, 0.04 M Tris Cl, pH 7.7) containing 0.06% Tween 20 (TBS-Tween). Blots were imaged (Kodak image station 440CF; Kodak, Rochester, NY) and bands 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 CYP standard in picomoles as follows: $Y= m (logx) +b$ for CYP3A5 and $Y= mx +b$ for CYP3A4; where m and b are slope and intercept terms, respectively. Integrated band densities of liver microsomal samples were used to determine the concentration of CYP 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 concentration of TRZ (250 μM) was incubated with a range of TST concentrations (0-100 μM). Maximum change in TRZ metabolite formation produced by TST was expressed as a percentage of control with no TST, based on metabolite to internal standard peak height ratio. Briefly, reaction mixtures were incubated with human microsomes (0.25 mg/ml). After 20 min at 37°C, reactions were stopped by cooling on ice and by the addition of acetonitrile. Phenacetin was added as the internal standard for both TST and TRZ. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. All samples were processed in duplicate. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards.

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: V_{max}, the maximum reaction velocity (uninhibited); K_m, the substrate concentration corresponding to 50% of $V_{max;}$ V_{max}/ K_m*1000, the estimated *in vitro* intrinsic clearance. As 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 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 (Figure 1) are shown in Table 1. TRZ 1-hydroxylation accounted for a greater proportion of estimated intrinsic clearance than 4-hydroxylation in HLMs in all age groups (Table 1). Mean V_{max} values in Groups B and C for both 1-OH and 4-OH-TRZ formation were significantly greater ($P<0.01$) as compared to A and D individually, as well as the net intrinsic clearance (sum of the two pathways) (Figure 1, 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 out 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 ($P<0.01$, 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). 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) (Figure 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 (CYP) 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 (Greenblatt et al., 1989; Schmucker, 1985; Schmucker et al., 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., 2003). 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 (ALP) 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., 2003). 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. (Vestal, 1982; Greenblatt et al., 1986). *In vitro* biotransformation studies using HLMs provide a valuable tool in research to examine age-related changes in CYP mediated biotransformation. These studies have the advantage of being uncomplicated by other factors that may influence drug disposition in vivo, such as changes in absorption, distribution, blood flow, and renal function. Some of the limitations of *in vitro* studies include variable causes of death among age groups (cancer as a common cause of death in elderly vs. vehicle

accident in young and adolescents), and the possible effect of these causes on CYP mediated biotransformation *in vitro*. Other potential sources of bias include the consumption by patients of multiple medications including enzyme inducers, and time elapsed between death and collection of liver tissue. In our study, none of the subjects were on known enzyme inducers, except one subject in group C. However, this did not seem to affect our results as this subject showed one of the lowest expression levels of CYP3A protein as well as catalytic activity in group C. Most of the livers in our study were collected within one hour of subjects' death.

Many of the previous studies evaluating the effect of age on drug metabolism *in vitro* have considered only the metabolite formation rate at a single substrate concentration, but without consideration of enzyme affinity and/or the estimated intrinsic clearance (Shimada et al., 1994; Schmucker et al., 1990; Beaune et al., 1986). For drugs metabolized by first order kinetics, the affinity of the enzyme for substrate is an important determinant of reaction rate at low substrate concentrations. In a previous study (Wynne et al., 1988), no age related changes were found in enzyme affinity. In our study we evaluated effect of age on the metabolite formation rate, on the affinity, as well as on the estimated intrinsic clearance (V_{max}/K_m). Some studies also suggest that CYP3A 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., 2003). The combined effect of age and gender in humans has been examined *in vivo* and *in vitro* with mixed results (Cotreau et al., 2003). Some *in vitro* studies have shown no change with either gender or age in CYP 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_{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 to Groups A (14-20 years) and D (61-72 years) individually, as well as the net intrinsic clearance (sum of the two pathways) (Table 1). This indicates reduced catalytic activity in Groups A and D compared to Groups B and C. 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_{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 (von Moltke et al., 1995a; Greenblatt et al., 1983a,b, 1989; Bertz et al., 1997; Cotreau et al., 2003).

High clearance in adolescents compared to adults has been shown in *in vivo* studies (Murry et al., 1995; Kanamori et al., 2002; Reith et al., 2000). Our study has shown reduced *in vitro* clearance in adolescents (< 21 years old) compared to 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 liver weight (Murry et al., 1995; Kanamori et al., 2002; Reith et al., 2000).

CYP3A5 is known to be catalytically equal or less active as compared to CYP3A4 (Williams et al., 2002, Patki et al., 2003). In people polymorphically expressing CYP3A5, the overall catalytic activity of CYP3A may be different than in people only expressing CYP3A4. Previous studies did not evaluate the possible contribution of CYP3A5 to net CYP3A metabolism (Shimada et al., 1994; Hunt et al., 1992). We immunoquantified all liver samples for CYP3A4 as well as CYP3A5 content. Although 11 out of 20 liver samples had detectable CYP3A5 expressions, 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 (Westlind-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 (Figure 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 to 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 isoform-specific expression of CYPs (George et al., 1995; Sotaniemi et al., 1997). Age-related changes were noted in overall CYP content (George et al., Sotaniemi et al., 1997), with a decline in CYP 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 effect of age on the expression as well as the activity of other CYPs.

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 the four age groups. Consistent with the results of previous studies (Patki et al., 2003; Schrag et al., 2001), 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 (Figure 4), suggesting that the CYP3A mediated DDI potential may not change with age.

In conclusion, reduced V_{max} and intrinsic clearance for TRZ hydroxylation and CYP3A protein in liver samples from elderly as well as adolescents suggest reduced CYP3A gene expression in these groups. These findings are consistent with many clinical studies showing reduced clearance of CYP3A substrates in elderly men (Cotreau et al., 2003). 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.

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Footnotes

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Figure Legends

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.

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

Fig. 3. Correlation analysis of total intrinsic clearance (V_{max}/K_m) of TRZ versus quantity of immunoreactive CYP3A (the sum of CYP3A4 and CYP3A5) among all samples ($r_s = 0.84$; $P<0.0001$).

Fig. 4. Effect of TST on in vitro TRZ biotransformation (250 μM) in four age groups (A, B, C and D). Each bar in the bar graph represents maximum change in TRZ metabolite formation (mean ± S.E.) produced by TST, expressed as percent of the control reaction velocity (without TST) for 4 different liver preparations.

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Table 1. In vitro biotransformation of TRZ in four age groups.

Results are expressed as Mean ± SE. ANOVA was applied on rank transformed values. V_{max} values are expressed as nanomoles per minute per milligram protein. K_m values are expressed in micromoles per liter. V_{max}/K_m *1000 are expressed as nanoliters per minute per milligram protein.

	A (14-20 yrs)	B (21-40 yrs)	C (41-60 yrs)	D (61-72 yrs)
1-OH TRZ pathway				
V_{max}	**1.0 ± 0.2**	**3.3 ± 0.9****	**2 ± 0.4****	**0.9 ± 0.3**
K_m	**67.7 ± 11**	**52.7 ± 9.2**	**36.1 ± 4.3**	**80.4 ± 22.8**
V_{max}/K_m*1000	**19 ± 7.5**	**60.7 ± 14.1****	**60.1 ± 13****	**15 ± 4.8**
4-OH TRZ pathway				
V_{max}	**2.2 ± 0.4**	**7.1 ± 2.2****	**4.7± 0.9****	**2.1± 0.8**
K_m	**381 ± 55**	**253.6 ± 31.1**	**317 ± 61**	**388 ± 53.6**
V_{max}/K_m*1000	**6.2 ± 1.5**	**29.2 ± 8.6****	**17.9 ± 5.1****	**5.7 ± 1.8**
Net intrinsic clearance	**25.2 ±9**	**89.8 ± 22.1****	**78 ± 17.5****	**20.6 ± 6.4**
(Sum of V_{max} / K_m*1000)				

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

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Table 2. Expression of immunoreactive CYP3A4, CYP3A5 and total CYP3A in four age groups.

Results are expressed as Mean ± SE. Immunoreactive CYP is expressed as pmol/mg protein. ANOVA was applied on rank transformed values.

	A (14-20 yrs)	B (21-40 yrs)	C (41-60 yrs)	D (61-72 yrs)
Immunoreactive CYP3A4	**15.2±10.1**	**424.8±149.7***	**126.2±44.7***	**43.4±16.6**
Immunoreactive CYP3A5	**1.9±1.9**	**0.8±0.5**	**1.0±0.9**	**0.24±0.2**
Total immunoreactive CYP3A	**17.1±12**	**425.6±149.4****	**127.3±44.5**	**43.7±16.5**

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

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

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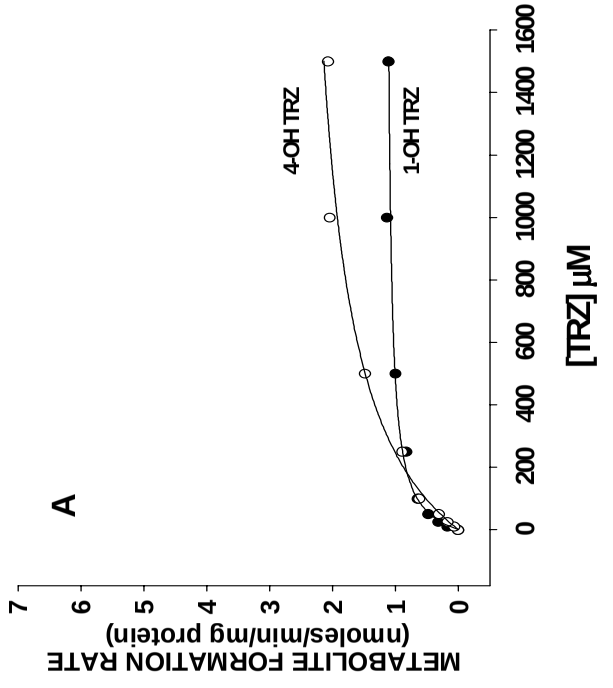

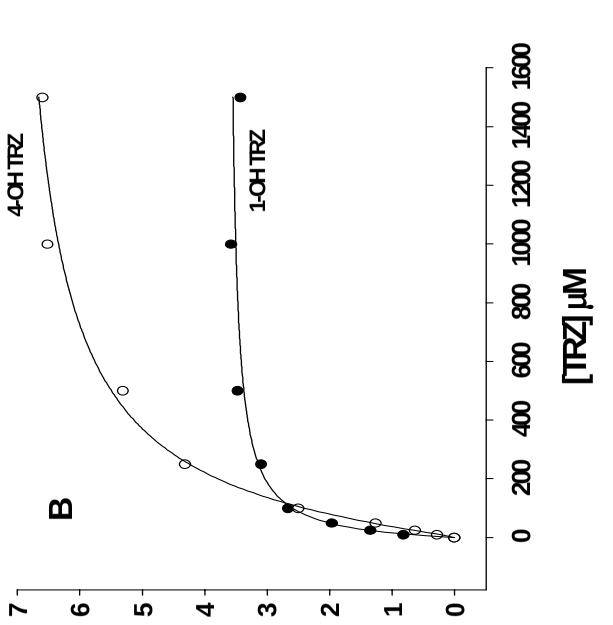

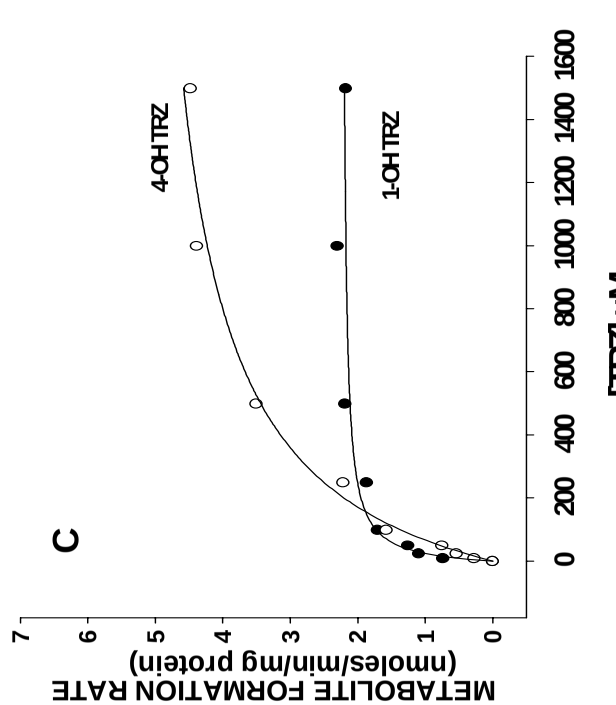

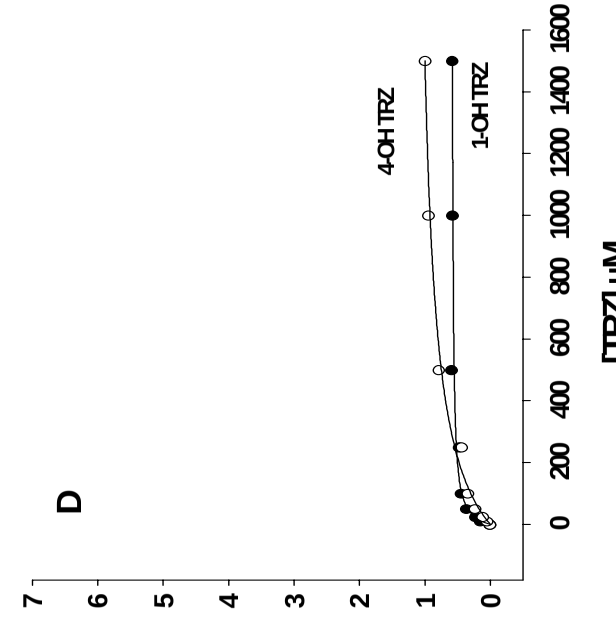

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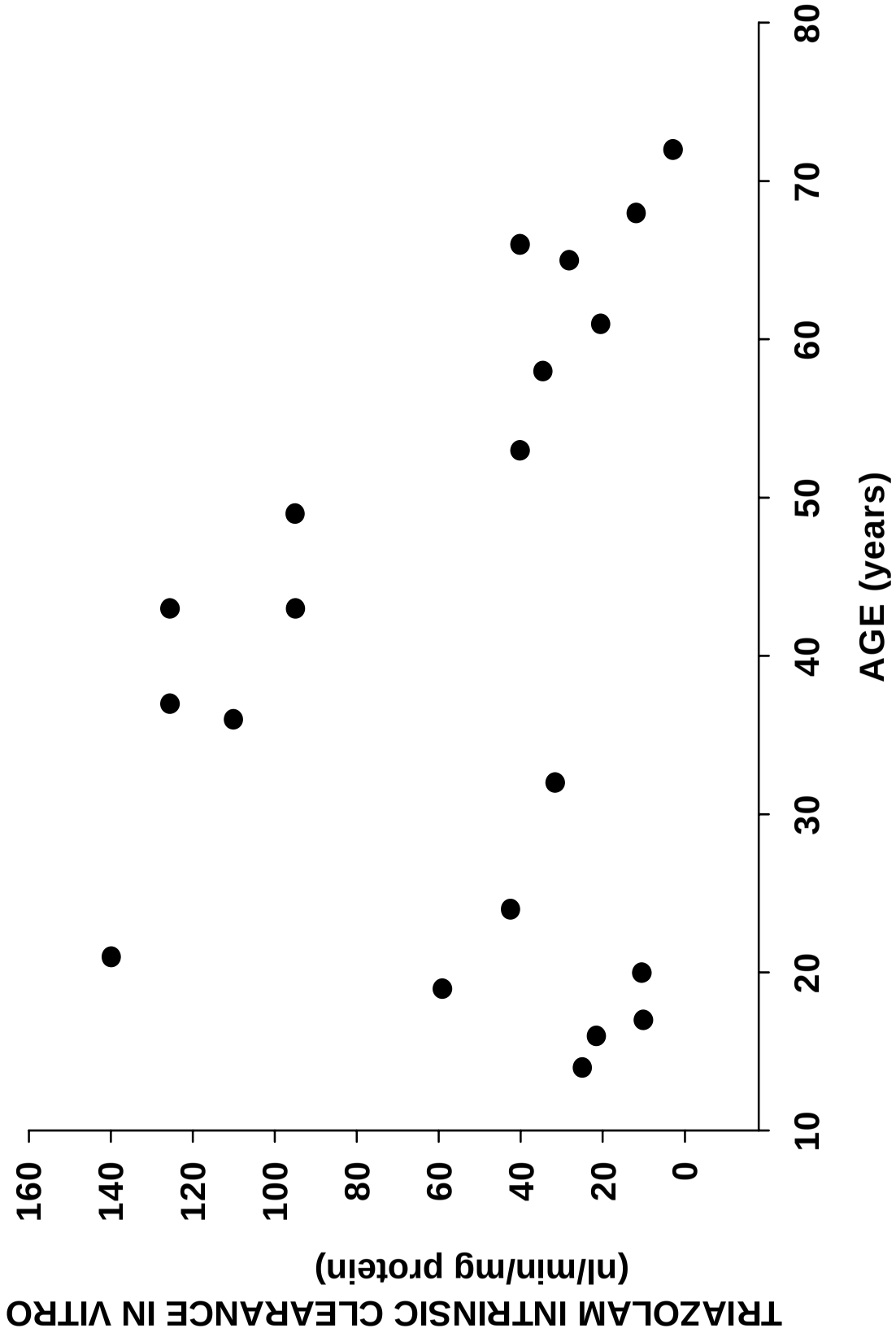

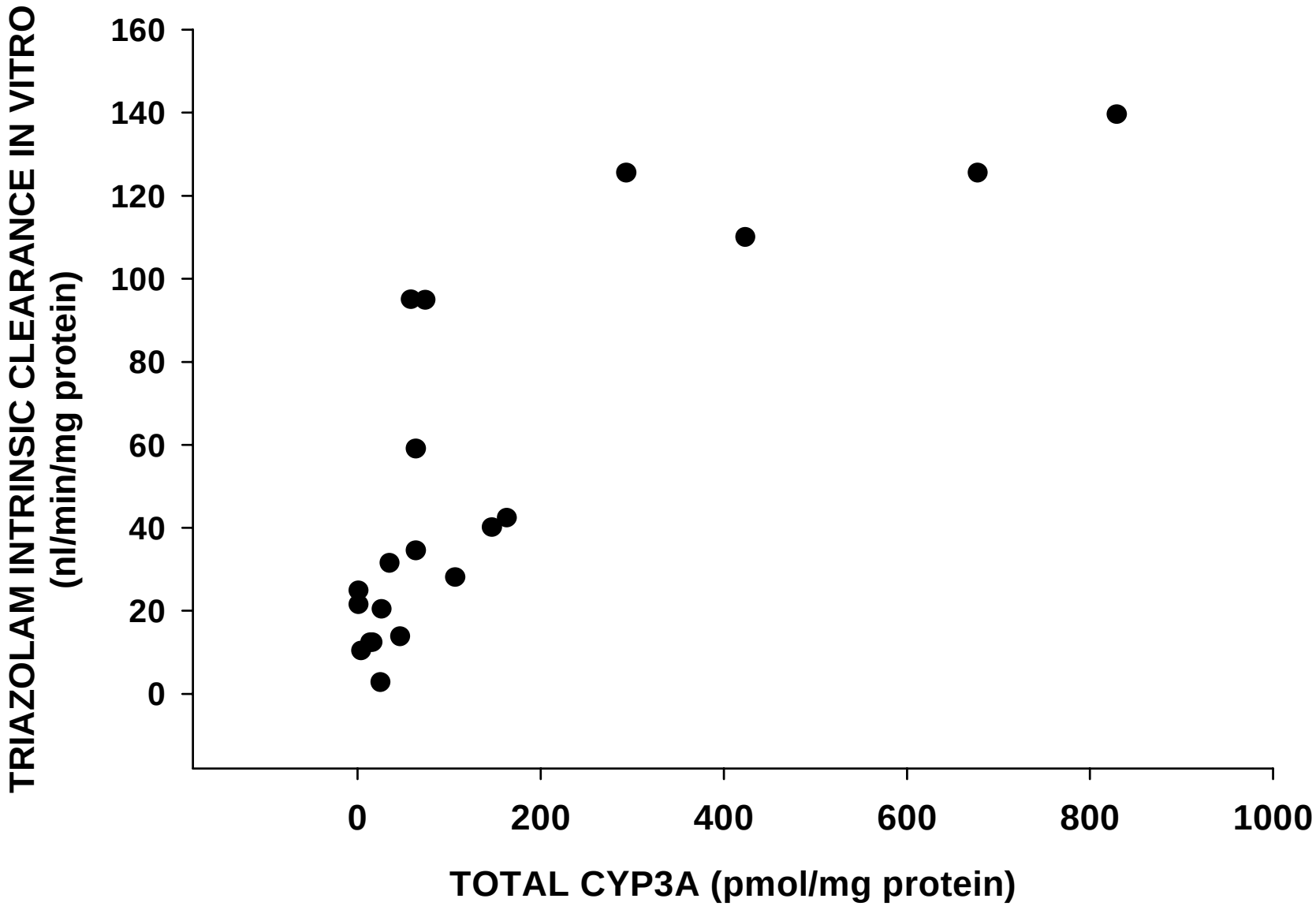

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