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Factors influencing midazolam hydroxylation activity in human liver microsomes

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Abstract

The cytochrome P450 3A (CYP3A) subfamily (mainly CYP3A4 and CYP3A5) is responsible for metabolizing approximately half of currently marketed drugs, but with considerable inter-individual variability in expression and function. To investigate factors contributing to this variability, rates of midazolam (MDZ) 1'-hydroxylation, and CYP3A4 and CYP3A5 protein content were determined using a set of 54 human liver microsomes (HLMs). Genetic factors investigated included CYP3A4 and CYP3A5 single nucleotide polymorphisms (SNPs) and haplotypes, CYP3A4 mRNA alternative splicing, and CYP3A4 transcriptional start and poly-adenylation sites. Demographic and environmental factors investigated included age, gender, and histories of smoking and alcohol consumption. MDZ 1'-hydroxylation rate varied from 0.025 to 3.106 nmoles/min/mg protein, with significant correlation to CYP3A4 protein content (r_s=0.92, P<0.001) but less robust correlation to CYP3A5 protein content (r_s=0.60, P<0.001). We identified 8 CYP3A4 SNPs (5 novel), and 9 CYP3A5 SNPs (one novel), as well as 7 CYP3A4 and two CYP3A5 haplotypes (all novel). No influence of genotype or haplotype on MDZ 1'-hydroxylation rate was observed, although CYP3A5*3A (g.6986a>g; g.31611c>t) carriers had lower CYP3A5 protein content compared to non-carriers (P=0.004). No alternative splicing of CYP3A4 mRNA was found. Similarly, only a single transcriptional start site and poly-adenylation site for CYP3A4 mRNA were identified. Subjects with a history of alcohol consumption had 2.2-fold higher median MDZ 1'-hydroxylation (P=0.017), while no influence of age, gender or smoking was evident. In conclusion, the investigated genetic factors did not contribute substantially to

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the large inter-individual variability in midazolam hydroxylation, while alcohol consumption has a discernable but modest influence.

Introduction

The human cytochrome P450 (CYP) 3A subfamily is responsible for metabolizing approximately half of currently marketed drugs (Shimada et al., 1994; Thummel et al., 1996; Guengerich, 1999). CYP3A4 is the major isoform present in the liver and intestinal epithelium (Floyd et al., 2003). CYP3A5, a polymorphic form, is present in the livers of approximately 20% of Caucasians (Dai et al., 2001) and in more than 50% of African-Americans (Kuehl et al., 2001). CYP3A7 and CYP3A43 are considered to be minor forms in adults in that they appear to contribute minimally to drug metabolism in most individuals (Wrighton et al., 2000; Koch et al., 2002). Midazolam is considered to be a relatively specific CYP3A probe, and can be administered both orally and intravenously. It is widely used for both *in vivo* and *in vitro* studies (von Moltke et al., 1996; Greenblatt et al., 2003). *In vivo* studies have shown 5-fold variation (in 98% of study population) (Floyd et al., 2003) and 11-fold variation (in 90% of study population) (He et al., 2005) in the clearance of midazolam, similar to the 10-fold variation that have been observed for other CYP3A substrates (Wilkinson, 1996; Thummel and Wilkinson, 1998; Westlind et al., 2003). Variation in the rate of oxidation of CYP3A substrates by human liver samples *in vitro* is even larger (>30-fold) (Westlind et al., 1999). Estimates of the relative contribution of genetic variation to CYP3A function indicate that 70-90% of interindividual variability is attributable to genetic factors (Ozdemir et al., 2000). Environmental influences (Thummel and Wilkinson, 1998), including co-administered

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drugs (von Moltke et al., 1996; Greenblatt et al., 2000) as well as constituents of food such as grapefruit juice (Greenblatt et al., 2003), may also play a role.

Single nucleotide polymorphisms (SNPs) in the genes encoding CYP3A4 and CYP3A5 have been identified that produce defective proteins, either by changes in amino acid coding or by changes in transcriptional regulation. A database (http://www.imm.ki.se/CYPalleles/) lists identified SNPs and alleles of CYP3A4 and CYP3A5, and provides standardized nomenclature and references. However, there is still no consensus as to what effect, if any, the identified SNPs of the CYP3A4 and CYP3A5 genes have on overall CYP3A metabolic capacity. Many previous studies are confounded by inadequate CYP3A probe specificity, genetic linkage, and low statistical power indicating a need for future studies using specific CYP3A probes, haplotype analysis, and larger sample sizes.

After transcription of a gene, the resulting pre-mRNA is spliced before exiting the nucleus. Alternative splicing of a single pre-mRNA can give rise to different mRNA transcripts. Consequently, alternative splicing is also an important mechanism for generating protein diversity from a single gene. To our knowledge, no study has been reported that investigated the possibility of alternative splicing of CYP3A4 mRNA, although alternative splicing of the related CYP3A5 mRNA is common (http://www.imm.ki.se/CYPalleles/cyp3a5.htm).

The 5'-untranslated region (5'-UTR) of the mRNA generally extends to the transcriptional start site and can also be involved in regulation of gene transcription and translation. It can interact with important transcriptional factors, thereby influencing

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transcriptional efficiency (Stripecke et al., 1994), and can also form a stable mRNA secondary structure that can influence the translation efficiency (Brown and Schreiber, 1996). Hence, different 5'-UTR through use of different transcriptional start sites may lead to differential gene expression. The 3'-UTR formed by the use of poly-adenylation signals can also regulate aspects
of gene transcription and translation. It can incorporate with selenocysteine in mammalian proteins and control coding capacity (Berry et al., 1991), can control mRNA stability by promoting endonucleolytic cleavage (Wilson and Treisman, 1988) and can also regulate translational efficiency (Ostareck-Lederer et al., 1994). Three CYP3A4 mRNA sequences (NM_017460, BC069418 and AF182273) have been filed in NCBI GenBank with different lengths at the 3'-nontranslated region. Northern blotting has also been reported with two different sized hybridization bands identified in different human livers (Molowa et al., 1986). While the smaller of these mRNAs was predicted to be a major form of CYP3A4, the larger mRNA could represent CYP3A4 mRNA with an extended 3'-UTR formed by the use of alternate poly-adenylation signals (Molowa et al., 1986). Different ratios of these mRNAs could potentially account for the differential gene expression among individuals.

In this work, we investigated several aspects of the genetic control of hepatic midazolam hydroxylation activity including: (1) SNPs in genomic DNAs of CYP3A4 and CYP3A5; (2) alternative splicing of CYP3A4 mRNA; (3) transcriptional start site of CYP3A4 mRNA; (4) poly-adenylation termination site of CYP3A4 mRNA. We also investigated the relative contributions of CYP3A4 and CYP3A5 enzymes to hepatic midazolam hydroxylation, and the potential influence of such environmental factors as

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smoking and alcohol consumption on hepatic midazolam hydroxylation activity in human liver microsomes (HLMs).

Method

Reagents

MDZ (midazolam), 1'-OH-MDZ (1-hydroxy-midazolam) as well as 4-OH-MDZ were kindly provided by their pharmaceutical manufacturers or purchased from Ultrafine Chemicals (Oxford, UK). Reaction cofactors (NADP+, DL-isocitric acid, magnesium chloride, isocitric dehydrogenase, and potassium phosphate buffer solutions) were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile was purchased from Fisher (Fairlawn, NJ).

Human liver microsomes

Liver samples from human donors with no known liver disease were provided from either the National Disease Research Interchange (Philadelphia, PA) or the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis). All livers were either intended for transplantation but had failed to tissue match, were normal tissue adjacent to surgical biopsies, or were autopsy specimens. Donors were primarily of Caucasian ethnicity (n=48), but also included 4 African-Americans, and 2 Hispanics. Other available demographic information included gender (16 females, 38 males) and age (median 41 years; range 2 to 75 years). Smoking history was positive for 19 donors, negative for 29 donors and unknown for 6 donors, while moderate to heavy alcohol use (defined as 14 or more drinks per week) was positive for 13 donors, negative for 34

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donors and unknown for 8 donors. This information was provided by the liver tissue procurement service.

All microsomes were prepared using standard techniques previously described (von Moltke et al., 1993). In brief, microsomes were prepared through ultra-centrifugation;

microsomal pellets were re-suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at -80°C until use. The quality of the liver samples was ascertained by reference to at least 8 other CYP activities measured in this laboratory using the same set of livers. Livers that consistently showed low activity values (>2-fold lower for all measured activities) relative to the median activity value for the entire liver set were excluded from the study.

Midazolam hydroxylation assay

Midazolam hydroxylation activities were measured in duplicate using the entire set of HLMs as described in detail previously, with minor modifications (Patki et al., 2003). Briefly, incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg^{2+}, 0.5 mM β-$NADP^{+}$, and an isocitrate/isocitric dehydrogenase regenerating system. Incubations were performed at 50 μM MDZ and 32 μg of microsomal protein for 5 min. The volume of incubation reactions was 250 μl. Incubations were initiated by the addition of microsomal protein, and were stopped by cooling on ice and the addition of acetonitrile. Phenacetin was added as the internal standard. The incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis. Control incubations with no cofactor, no protein, and/or no substrate were

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performed concurrently to validate P450-dependent metabolism. The identity of metabolites was verified by comparing HPLC retention time with authenticated standards. Both intra- and inter- assay variabilities were less than 10%.

Western Blotting

Quantities of CYP3A4 and CYP3A5 proteins in 54 HLMs were determined by quantitative Western Blotting analysis as described previously (Patki et al., 2003). Protein was measured in duplicate and the mean value was expressed in units of pmoles per μg microsomal protein. Microsomal proteins (various amounts of recombinant CYP3A4 or CYP3A5 standards and an optimal amount of liver microsomal protein) were denatured for 5 min at 100 °C in 100 mM Tris buffer (pH = 6.8) containing 10% glycerol, 2% β-mercaptoethanol, 2% SDS and 5 mg/ml pyronin Y. Microsomal protein was separated by SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels (Bio-rad, Hercules, CA) in 25 mM Tris/0.192 M glycine/0.1% SDS running buffer (pH=8.3). Recombinant CYP3A4 and CYP3A5 protein (Gentest, Woburn, MA) were used to generate calibration standard. Samples were then transferred to Immobilon-P (PVDF membrane; Millipore, Bedford, MA) on the semi-dry transfer cell for 90 min at 15 V in 25 mM Tris/0.192 M glycine/20% methanol transfer buffer. Blots were blocked with 5% non-fat milk in TBS-Tween washing buffer for 1 hour while rocking, washed three times with TBS-Tween, and then incubated with primary anti-rabbit antibody (1:1000) (Gentest, Woburn, MA) containing 0.5% milk in TBS-Tween overnight at 4 °C cold room while rocking. Blots were then washed three times with TBS-Tween and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000) (PerkinElmer Life Sciences Inc, Boston,

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MA) containing 0.5% milk in TBS-Tween at room temperature for 1 hour while rocking. Blots were washed three times again with TBS-Tween, and incubated with West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 5 min at room temperature. Blots were analyzed by a Kodak Image Station 440 and quantification of CYP3A4 and CYP3A5 content was completed using KODAK 1D Image Analysis Software (Kodak, Rochester, NY). A standard curve in arbitrary units of net or mean intensity versus picomoles of CYP3A4 and CYP3A5 was created and fit to one of the following equations using non-linear least-squares regression: $y=m*x + B$, $y=m*\ln(x) + B$ or $\ln(y)=m*\ln(x) + B$. The amount of CYP3A4 protein in each sample was normalized to the quantity of protein loaded.

Genotyping

For this study, the reference sequences were the complete gene sequences contained in the NCBI GenBank file AF280107 for the CYP3A4 gene and file NG_00004.1 for the CYP3A5 gene. Nucleotide positions are given relative to the first nucleotide of the predicted translation start code ATG (nucleotide 253182 of the AF280107 CYP3A4 sequence and nucleotide 148999 of the NG_00004.1 CYP3A5 sequence).

Genomic DNA was isolated with DNAzol (Invitrogen, Carlsbad, CA) from the same human liver tissues used to prepare microsomes. Genetic polymorphisms were identified using polymerase chain reaction (PCR) amplification and direct sequence analysis of portions of the CYP3A4 and CYP3A5 genes. Primers used to screen for variants within selective regions of the CYP3A4 and CYP3A5 genes are listed in Table 1.

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PCR amplification was carried out in a 50 µl reaction mixture that contained PCR buffer, 2 mM $MgCl_2$, 0.2mM dNTP mix (Invitrogen, Carlsbad, CA), 5 µM of each primer, 2.5 U of Taq DNA polymerase (Platinum Taq, Invitrogen, Carlsbad, CA) and 100 ng of genomic DNA template. Denaturation was at 94 °C for 30 seconds, followed by annealing for 30 seconds at a temperature specific to primer pairs, and extending at 72 °C for 1 minute with 40 cycles. These PCR products were purified using QIAquick PCR purification Kit (Qiagen, Valencia, CA) and then subjected to direct sequencing with the same primers used for PCR amplification (ABI PRISM 3100, Applied Biosystems, Foster City, CA).

RT-PCR of CYP3A4 mRNA for evaluation of alternative splicing

Total RNAs were prepared using standard techniques previously described (Cotreau et al., 2000) using Trizol reagent (Sigma) according to the manufacturer's instructions. RNA pellets were stored in ethanol for no longer than 9 months at –80 °C. RNA concentrations were determined by UV absorbance spectrophotometry.

Primers for amplification of full-length CYP3A4 cDNA (Table 1) were designed and their homology and specificity were checked by use of the alignment and oligonucleotide analysis capability of the program Vector NTI Suite 7.0 (Invitrogen, Carlsbad, CA). All RNAs were treated with deoxyribonuclease (Invitrogen, Carlsbad, CA) to eliminate any DNA contamination before reverse transcription. To amplify CYP3A4 from human livers, first strand cDNA was synthesized from 1µg of total RNA from human liver samples according to the manufacturer's protocol (SuperScript II Reverse Transcriptase, Invitrogen, Carlsbad, CA). Using primer pair P11, the CYP3A4

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cDNA was amplified in a total volume of 25 μL consisting of PCR buffer, 2 mM $MgCl_2$, 0.2mM dNTP mix (Invitrogen, Carlsbad, CA), 5 μM of each primer, 2.5 U of Taq DNA polymerase (Platinum Taq, Invitrogen, Carlsbad, CA) and 1 μL of human liver cDNA. Denaturation was at 94 °C for 30 seconds, followed by annealing for 30 seconds at a temperature specific to primer pairs, and extending at 72 °C for 40 cycles. A second round nested amplification was performed using the primer pairs P12, P13 and P14 and appropriate dilutions
of the first round PCR products. The conditions used were the same as the first round amplification with minor modifications. After second round nested amplifications, the products were analyzed by agarose gel electrophoresis.

Rapid amplification of CYP3A4 3'-cDNA end

A nested 3'-RACE (rapid amplification of cDNA end) procedure was performed from the human liver CYP3A4 RNA common region as described previously with minor modifications (Court and Greenblatt, 2000). A total of 10 samples (5 samples with the highest, 5 with the lowest MDZ 1-hydroxylation activities) were used. Briefly, a first strand cDNA product was generated from 5 μg of liver total RNA with primer RACE-1 (SuperScript II Reverse Transcriptase, Invitrogen, Carlsbad, CA). A modified incubation protocol (37 °C for 1 hour; 42 °C for 30 minutes; 50 °C for 10 minutes; 70 °C for 15 minutes) for the reverse transcription reaction was also used to promote full-length transcription. The resultant first strand cDNA product was diluted to 400 μl in TE buffer, purified by using QIAquick PCR purification Kit (Qiagen, Valencia, CA) and re-suspended in 10 μl of TE buffer. The first round of the nested PCR procedure was then

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performed using 1 µl of the poly dA tailed cDNA, primers GSP-3 and RACE-2. The PCR product was diluted 20-fold in TE buffer and 1 µl of this used in a second round of amplification using primers GSP-4 and RACE-3. After a second round of nested amplifications, the products were analyzed by agarose gel electrophoresis. These PCR products were purified using QIAquick PCR purification Kit (Qiagen, Valencia, CA) and then subjected to direct sequencing with the same primers used for second round of PCR amplification (ABI

PRISM 3100, Applied Biosystems, Foster City, CA). The oligonucleotide sequences of the primers used are listed in Table 1.

Rapid amplification of CYP3A4 5'-cDNA end

Ten total RNA samples (5 samples with the highest, 5 with the lowest 1'-OH MDZ formation rates) were used to map the transcriptional start site of CYP3A4 with the 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 Kit (Invitrogen, Carlsbad, CA). Briefly, a first strand cDNA was generated from 1 µg of liver total RNA with GSP-5. The resultant first strand cDNA product was purified by a SNAP Column (Invitrogen, Carlsbad, CA) and used directly in the terminal deoxynucleotidyl transferase dC-tailing reaction. The first round of the nested PCR procedure was then performed using 5 µl of the poly dC tailed cDNA, primers GSP-6 and Abridge Anchor primer (Invitrogen, Carlsbad, CA). The PCR product was diluted 100-fold in TE buffer and 5 µl of this was used in a second round of amplification using primers GSP-2 and AUAP primer (Invitrogen, Carlsbad, CA). These PCR products were purified using QIAquick

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PCR purification Kit (Qiagen, Valencia, CA) and then subjected to direct sequencing with the same primers used for the second round of PCR amplification (ABI PRISM 3100, Applied Biosystems, Foster City, CA). The oligonucleotide sequences of the primers are listed in Table 1.

Quantification of CYP3A4 mRNA

CYP3A4 mRNA as well as 18S rRNA (control RNA) were quantified by a real-time PCR after reverse transcription (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA).

First strand cDNA was generated from total RNA (1 µg) with random hexamer (0.1 µg), diluted 10-fold with TE buffer and 10 µl of this used in real-time PCR amplification. Each 25µl reaction mixture also contained 12.5µl of SYBR Green 2X master mix (Applied Biosystems, Foster City, CA), 0.2 µM of forward and reverse primers, and 0.5 µl of distilled water. PCR conditions were as follows: initial denaturation was at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C (15 seconds), annealing and extension at 60°C (1 minute).

Two sets of CYP3A4 primers were used. The first set of CYP3A4 primers (P15) was designed to span from exon 4 to part of exon 5 (+219 bp to +347 bp relative to the first nucleotide of the start codon ATG of the GenBank reference sequence NM_017460), and the real-time PCR amplification product represents the total CYP3A4 mRNA. The second set of CYP3A4 primer pairs (P16) was designed to include the 5'-UTR of CYP3A4 mRNA according to the sequence from the 5'-RACE assay spanning the 5'-UTR (-100) to the first 23 bp of exon 1 (relative to the first nucleotide of the start codon

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ATG of the GenBank reference sequence NM_017460). The specificity of these PCR products were verified by a single peak in the dissociation curve and by direct sequencing with the same primers (ABI PRISM 3100, Applied Biosystems, Foster City, CA). The amount of CYP3A4 amplification product was described as $2^{(-C\tau)}$, in which $C\tau$ is the cycle threshold. The amount of CYP3A4 mRNA was normalized to 18S rRNA and expressed relative to the liver with the lowest value. Assays were performed in duplicate and results were averaged.

Data analysis

ARLEQUIN v2.001 was used to evaluate SNP genotype frequencies for consistency with the Hardy-Weinberg equilibrium by a modified Markov-chain random walk algorithm. Multiple haplotypes were constructed from the sequence genotype data using the program PHASE version 2.0.1. Sequence variants identified in the coding regions of each exon were evaluated for effects on the predicted amino acid sequence by use of the virtual translation capability of the program Vector NTI Suite 7.0 (Invitrogen, Carlsbad, CA). The GeneSplicer program (http://www.tigr.org/tdb/GeneSplicer) was used to predict the normal donor, acceptor and branch points in the intron region and the potential effect of the SNP at the post-transcriptional level. The ESEfinder program (http://exon.cshl/edu/ESE) was used to predict the binding sites of splicing regulatory proteins and the potential effect of SNPs in the intron region. The TESS program (http://www.cbil.upenn.edu/tess) was used to predict transcription factor binding sites in DNA sequences.

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Statistical analyses were conducted with the SigmaStat program (Version 3.0, SPSS Inc., Chicago, Illinois). Data were routinely evaluated for normality of distribution and equal variance, and in cases where these tests failed, nonparametric methods of statistical analysis were utilized. A value of $P<0.05$ was considered statistically significant. Correlations between age and midazolam hydroxylation activities, between the formation rates of MDZ 1'- and 4-hydroxylation, between CYP3A protein contents and 1'-OH MDZ formation rate, between CYP3A4 mRNA quantification value and 1'-OH MDZ formation rate, and between CYP3A4 mRNA quantification values using the 2 different primer sets were performed by Spearman's rank order correlation test. The influence of gender, histories of smoking and alcohol

consumption, the effect of an individual known or novel SNP and haplotype on MDZ 1'-hydroxylation activities and CYP3A4/CYP3A5 protein content, as well as the difference in CYP3A4 mRNA quantification between high and low CYP3A metabolism groups, was evaluated by ANOVA on rank transformed data (for 3 or more groups) or by Mann-Whitney rank sum test (for 2 groups). Possible interactions between these independent variables were evaluated by two-way ANOVA on rank-transformed data. Analyses were performed on the complete group of subjects, the chronic alcohol consumption subpopulation, the no-alcohol consumption subpopulation and the Caucasian subpopulation. In cases where the ANOVA on rank-transformed data test indicated a significant difference ($P<0.05$), multiple comparisons testing was performed using the Student-Newman-Keuls test. The influence of gender, ethnicity, histories of smoking and alcohol use, as well as haplotype was also evaluated using a Chi-square test (with Yates continuity correction for a 2×2 table) comparing groups defined by MDZ 1'-

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hydroxylation activities (upper third versus lower third of the population). A value of $P<0.05$ was considered statistically significant.

Results

Midazolam hydroxylation activities in the human liver bank

Formation rates of 1'-OH MDZ varied between 0.025 and 3.106 nmoles/min/mg protein, and 4-OH MDZ varied between 0.023 and 2.615 nmoles/min/mg protein. Formation rates of the two metabolites were essentially completely correlated ($r_s=1.0$, $P<0.0001$). Both 1'-OH MDZ and 4-OH MDZ formation rates were analyzed in this study. Since 1'-OH MDZ was the major metabolite observed, 1'-OH MDZ formation rates were used to represent hepatic midazolam hydroxylation activity. However, the results were not changed if 4-OH MDZ formation rates were used for analysis.

Factors associated with midazolam hydroxylation activity

Age and 1'-OH MDZ formation rate were not significantly correlated ($r_s=0.19$, $P>0.05$). There was no significant difference between female (n=16) and male (n=38) MDZ 1'-hydroxylation activities ($P>0.05$). No association of history of smoking with midazolam hydroxylation activities was evident ($P>0.05$). History of alcohol use was significantly associated with differences in activity, in that the group with a history of alcohol use had a 2.2-fold higher median level of 1'-OH MDZ formation rate compared with the group without alcohol exposure (Figure 1A, Mann-Whitney rank sum test, $P=0.017$; Figure 2, Chi-square test, $P<0.05$).

CYP3A4 and CYP3A5 genotypes and midazolam hydroxylation activity

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All of the DNA samples were genotyped in selected CYP3A4 and CYP3A5 gene regions as defined in Table 1 by direct sequencing. The frequency distributions of genotypes for most of the identified variants were consistent with Hardy-Weinberg equilibrium (P>0.05), indicating that the volunteer pool in this study was likely a representative sample of the population being studied. Eight CYP3A4 gene sequence variants, including 5 novel ones, were identified. The variants with relatively high frequencies included -392 a>g in the 5'-regulatory region (CYP3A4*1B), the novel SNP g.15753 t>g in intron 7, the novel SNP g.15977 c>t in intron 7, g.20230 g>a in intron 10 (CYP3A4*1G), and the novel SNP g.22545 g>a in intron 11. The variant frequencies in the entire population were 9.3%, 9.3%, 63.9%, 17.9% and 15.1% respectively. Other novel SNPs included g.15819-20 ca>tg in intron 7 and g.16161-2 ins t in intron 7. None of the novel CYP3A4 SNPs is located at the putatively important sites for post-transcriptional regulation process, such as splicing donor/acceptor sites, enhancer/suppressor binding sites or branch point.

Nine CYP3A5 gene sequence variants, including the novel g.14720 a>g variant, were identified. The variants with relatively high frequencies included g.6986 a>g (CYP3A5*3) in intron 3, g.27050 a>g in intron 10, g.27526 c>t in intron 11 and g.31611 c>t (CYP3A5*1D) in the 3'-UTR. The variant frequencies in the entire population were 87.0%, 9.3%, 8.3% and 86.7% respectively.

The association of each CYP3A4 and CYP3A5 genotype with 1'-OH midazolam formation rate was evaluated using the entire liver bank of 54 samples, as well as subsets stratified by alcohol use history. All data were also analyzed using the subset of Caucasians (n=48). Due to the small number of non-Caucasian samples, the effect of

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ethnicity could not be evaluated. Comparisons were made between individual genotype groups, as well as between reference genotype and variant carrier groups. No statistically significant relationship was evident between CYP3A4 or CYP3A5 genotype and MDZ 1'-hydroxylation activities, including -392 a>g (CYP3A4*1B) and g.6986 a>g (CYP3A5*3) (Figure 1B and Figure 1C), regardless of alcohol use history. Another previously reported functional variant (CYP3A5 g.14690 g>a (CYP3A5*6) (Kuehl et al., 2001)) was not present in any our 54 samples.

CYP3A4 and CYP3A5 haplotypes and midazolam hydroxylation activity

Seven CYP3A4 haplotypes and two CYP3A5 haplotypes (all of which were novel) could be inferred using the available genotype data. The nomenclature of the haplotypes we used is consistent with our previous publication (He et al., 2005). As previously observed by us (He et

al., 2005) and others (Lin et al., 2002; Floyd et al., 2003), the reference alleles for both CYP3A4 and CYP3A5 genes were not the highest frequency alleles, based on the NCBI GenBank reference gene file AF280107. This was also observed in our previous study with a mixed population.

The most common CYP3A4 haplotype CYP3A4*VI (g.15977 c>t) with a frequency of 53.7% in the entire population occurred almost exclusively in Caucasians, with none of the African-Americans and only one Hispanic DNA sample identified as carriers of this haplotype. Other novel haplotypes and their frequencies distributions are shown in Figure 2. None of the haplotypes identified were associated with differences in 1'-OH midazolam formation rates. The CYP3A4*VI haplotype (evaluated in detail

DMD Fast Forward. Published on April 25, 2006 as DOI: 10.1124/dmd.105.008904
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because of its relatively high frequency) was also not associated with midazolam hydroxylation activities, regardless of a history of alcohol use.

The most frequent haplotype in the CYP3A5 gene was CYP3A5*3A (g.6986 a>g, g.31611 c>t), with a frequency of 77.8% in the entire group, and 78.1% in 48 Caucasians. Two novel CYP3A5 haplotypes, including CYP3A5*1F (g.27526 c>t) and CYP3A5*3U (g.6986 a>g, g.27050 a>g), were identified with relatively low frequencies (Table 2, Figure 2). There was no haplotype frequency difference for individual CYP3A5 haplotypes between stratified MDZ 1'-hydroxylation phenotype groups analyzed by Chi-square test (P>0.05, Figure 2). The effect of CYP3A5*3A haplotype was evaluated in detail because of its relatively high frequency and

previous reports of functional significance (Floyd et al., 2003; Thummel, 2003). However, no association of this haplotype with differences in MDZ 1'-hydroxylation activities regardless of alcohol exposure history could be determined.

Seven distinct multi-gene haplotypes were also identified using the CYP3A4 and CYP3A5 gene variant data. The most common haplotype was CYP3A4*VI (g.15977 c>t) with CYP3A5*3A (g.6986 a>g, g.31611 c>t), which occurred exclusively in Caucasians, with an allele frequency of 51.0% (n = 48). Other multi-gene haplotypes occurred at much lower frequencies as shown in Figure 2. None of the multi-gene haplotypes were significantly associated with 1'-OH MDZ formation rate.

DMD Fast Forward. Published on April 25, 2006 as DOI: 10.1124/dmd.105.008904

Role of CYP3A4 and CYP3A5 in midazolam hydroxylation in human liver microsomes

CYP3A4 protein content and hepatic 1'-OH MDZ formation rate were strongly correlated (Figure 3A, Spearman correlation analysis, r_s=0.92, P<0.001). However, CYP3A5 protein content and hepatic midazolam hydroxylation activity were less robustly correlated (Figure 3A, Spearman correlation analysis, r_s=0.60, P<0.001).

The relationship between haplotype CYP3A5*3 (g.6986 a>g) and CYP3A5 protein content, and between CYP3A5*3 and hepatic CYP3A activity were also investigated (Figure 3B). The homozygous carriers of this haplotype had statistically lower CYP3A5 protein levels compared to non-carriers or heterozygous carriers of this haplotype (Kruskal-Wallis ANOVA on ranks test, P<0.001, followed by Student-Newman-Keuls test, P<0.05) (Figure 3B, left panel). There was no statistically significant difference in CYP3A5 protein content between non-carriers and heterozygous CYP3A5*3 carriers. Compared to defective CYP3A5 protein, hepatic CYP3A activity was not changed by the presence of haplotype CYP3A5*3. Similarly when haplotype CYP3A5*3A (g.6986 a>g, g.31611 c>t) was evaluated, the heterozygous and homozygous carriers of CYP3A5*3A had statistically lower CYP3A5 protein levels compared to non-carriers (ANOVA on ranks test, P<0.01, followed by Student-Newman-Keuls test, P<0.05, data not shown). Hepatic CYP3A activity was not influenced by the presence of haplotype CYP3A5*3A.

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CYP3A4 mRNA alternative splicing

CYP3A4 cDNA amplification was performed by nested PCR of reverse transcribed mRNA from10 livers with the extremes of high or low 1'-OH midazolam formation rates (5 each). As shown in Figure 4, only single band of the expected size was found in each of the samples.

CYP3A4 polyadenylation site

A 3'-RACE procedure was performed to map the polyadenylation sites of the CYP3A4 mRNA from 10 livers with the extremes of high or low 1'-OH midazolam formation rates (5 each). In each case, only a single band was detected on the agarose gel at around 600 bp in size. The sequence was identical to the reference CYP3A4 cDNA (NM_017460) with the polyadenylation consensus site AATAAA from +2051 to +2056 nucleotide position at the 3'-UTR.

CYP3A4 transcriptional start site and quantification

A 5'-RACE procedure was performed to map the transcriptional start sites of the CYP3A4 mRNA from 10 livers with the extremes of high or low 1'-OH midazolam formation rates (5 each). In each case, a single major band with a size of about 350 bp was detected on the agarose gel. Additional shorter bands were also detected. The sequence of the major band was

identical to the reference CYP3A4 cDNA (NM_017460) with the transcriptional start consensus site ATCACTGC from -105 to -98 nucleotide position at the 5'-UTR. No

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transcriptional start site consensus sequence could be identified in the sequences of minor bands.

Quantification of CYP3A4 mRNA with different length was applied with two different sets of primer pairs P15 and P16, with values normalized to the amount of 18S rRNA (as the control gene). There was a linear correlation in the amount of CYP3A4 mRNA between these two amplifications (Spearman correlation analysis, $r_s = 0.95$, $P<0.0001$).

Discussion

Single nucleotide polymorphisms that occur in the promoter, 5'-regulatory, 5'- and 3'-UTR, exonic and intronic regions of CYP3A4 and CYP3A5 genes could potentially influence the quantity and function of the respective expressed proteins. One variant in the 5'-regulatory region of the CYP3A4 gene and one in the intronic region of the CYP3A5 gene have been studied the most. It was reported that people with the A-392G mutation (CYP3A4*1B) within the putative nifedipine response element (NFSE) of the 5'-regulatory region of the CYP3A4 gene had a higher incidence of prostate cancer (Rebbeck et al., 1998) and secondary leukemia (Felix et al., 1998), possibly due to an altered CYP3A-mediated testosterone and xenobiotic
metabolism. However, both the current study and our previous *in vivo* study (He et al, 2005) failed to show any association of this polymorphism with altered CYP3A function as reflected by midazolam clearance. This is also consistent with findings of other studies (Floyd et al., 2003; Eap et al., 2004). It indicates that CYP3A4*1B may have linkage disequlibrium with other polymorphisms in a population specific manner.

DMD Fast Forward. Published on April 25, 2006 as DOI: 10.1124/dmd.105.008904
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The A6986G mutation (CYP3A5*3) in the intron region of CYP3A5 gene creates an alternative splice site in the pre-mRNA and production of aberrantly spliced mRNA that contains 131 bp of an intron 3 sequence (exon 3B) inserted between exons 3 and 4. This insertion results in a reading frame shift and predicted truncation of the CYP3A5 protein at amino acid position 102 (Kuehl et al., 2001). This variant has a high frequency among Caucasians, and in our study, around 90% of Caucasians were carriers. It was previously reported that CYP3A5 *3/*3 was strongly associated with very low or undetectable hepatic CYP3A5 protein content (Hustert et al., 2001), which was confirmed by the results of our study. However, in both the present HLM study and our previous *in vivo* study, there was no indication that this variant is associated with lower hepatic 1'-OH midazolam formation rates or oral midazolam clearance (He et al., 2005). This finding has also been reported by other groups (Shih and Huang, 2002; Floyd et al., 2003;Patki et al, 2003).

The contribution of CYP3A5 to hepatic drug metabolism *in vivo* is somewhat controversial (Kuehl et al., 2001; Floyd et al., 2003; Thummel, 2003). In addition to finding no effect of a known functional CYP3A5 polymorphism (CYP3A5*3) on midazolam hydroxylation *in vitro* and *in vivo* (He et al., 2005), we also found a relatively poor correlation between CYP3A5 protein content and midazolam hydroxylation activities, which contrasts with a highly

significant correlation between CYP3A4 protein content and midazolam hydroxylation activities. These results indicate that CYP3A5 has only a minor role in overall hepatic CYP3A mediated midazolam hydroxylation, and that CYP3A4 is likely the most

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important contributor. However, this conclusion may not necessarily generalize to all CYP3A substrates since differences in the
contribution of CYP3A4 and CYP3A5 to the metabolism of different substrates have been observed (Uesugi et al., 2006)

In this study, we identified 5 novel CYP3A4 variants. All of these SNPs are located in the intronic regions of CYP3A4 gene, but none is at the putatively important sites for post-transcriptional regulation process, such as splicing donor/acceptor sites, enhancer/suppressor binding sites or branch point. This is consistent with our finding that of a single CYP3A4 mRNA by RT-PCR without evidence for alternative splicing. However since we only studied liver, it is still possible that alternate splicing may occur in extrahepatic tissues. We also identified a novel variant g.15977 c>t in intron 7, which has a relatively high frequency in Caucasians. In our previous paper (He et al., 2005), we indicated that carriers with the multi-gene haplotype CYP3A4*VI – CYP3A5*3A (CYP3A4: g.15977 c>t; CYP3A5: g.6986 a>g, g.31611 c>t) had a significantly higher oral midazolam clearance. This was not confirmed in the present *in vitro* study, possibly because of differences in the race/ethnicity make-up of the 2 populations studied – primarily Caucasian in the present study, primarily African-American in the previous report (He et al., 2005).

Identical genes can have several 5'- transcriptional start sites. For example, there is an alternative splice variant incorporating exon 1B that has different nucleotide sequences from the usual exon 1 of PXR (pregnane X receptor) gene (Hustert et al., 2001). Although neither exon 1

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nor exon 1B is translated into amino acids, this difference could lead to different transcriptional and translational regulation of gene expression. In our study, we used the 5'-RACE technique to map the transcriptional start site and determine whether alternate 5'-UTRs of CYP3A4 mRNA exist. A similar strategy using 3'-RACE was also used to define the 3'-UTR and

polyadenylation site. Liver mRNA selected for analysis included those livers with extremes in 1'-OH midazolam formation rates in order to enhance our chances of finding variants that might be important for variability in MDZ hydroxylation. However in each instance the analyses showed only a single major mRNA species, indicating that there is essentially no variation in the 5'- and 3'-UTR region of the CYP3A4 mRNA that could explain MDZ hydroxylation variability.

Alcohol has been known to induce CYP2E1 and increase the risk of acetaminophen hepatotoxicity (Wrighton et al., 1987; Lieber et al., 1988). The ability of alcohol to induce CYP3A in humans is weaker. A recent study indicates that individuals consuming moderate amounts of alcohol have no alteration in hepatic CYP3A activity, but reduced midazolam oral bioavailability possibly as the result of intestinal CYP3A induction (Liangpunsakul et al., 2005). In our study, moderate to heavy chronic alcohol consumption (defined as 14 or more drinks per week) was associated with a small but discernible increase in hepatic CYP3A activity. The mechanism for this apparent effect of ethanol on CYP3A activity is as yet unknown, but could involve regulation via nuclear receptor transcription factors.

In conclusion, our results indicate that no single genetic, demographic or environmental factor is unlikely to completely explain the large inter-individual

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variability of CYP3A mediated drug metabolism in the population. Although alcohol consumption was associated with higher midazolam hydroxylation activity, the fold increase (2.2-fold between median levels) was far less than the overall variability in 1'-OH MDZ formation rates (>100-fold in 90% population samples). Furthermore, *in vivo* and *in vitro* studies are not fully consistent. CYPs are membrane bound proteins, and any change in reconstitution condition with NADPH reductase, cytochrome b5, or lipids may cause discrepancy in catalytic studies with CYPs *in vitro* (Lamba et al., 2002). In determining the contributions of genetic and environmental factors to the metabolic activity variability *in vivo*, monozygotic/dizygotic twin and adoption studies are necessary. These important tools have already been widely used in other other areas of research such as mental illness and alcoholism. The same methodology can be applied in pharmacogenomic studies.

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Footnotes:

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Legend for figures

Figure 1. Comparison of 1'-hydroxy midazolam (1'-OH MDZ) formation rates in groups sorted by the history of alcohol consumption, CYP3A4 variant –392 a>g (CYP3A4*1B) and CYP3A5 variant g.6986 a>g (CYP3A5*3). Indicated are the median, 75 percentile and 25 percentile values of 1'-OH MDZ formation rate. Mann-Whitney rank sum test or ANOVA on rank-transformed data was utilized in each case. (A). The median value of 1'-OH MDZ formation rate was 2.2-fold higher in the donors with the history of alcohol consumption (P = 0.017). (B). The CYP3A4 variant –392 a>g (CYP3A4*1B) and (C). The CYP3A5 variant g.6986 a>g (CYP3A5*3) had no significant effect on 1'-OH MDZ formation rate in either group sorted by the history of alcohol consumption (P>0.05).

Figure 2. Relationship between 1'-OH MDZ formation rate and CYP3A haplotypes determined in 54 human liver samples. Vertical bars shown in the graph at the top indicate 1'-OH MDZ formation rates (donor identifier shown on x-axis) and were ranked from lowest to highest. Below the graph is the gender (f, female; the remainder were male), known ethnicities (a, African-American; h, Hispanic; the remainder were Caucasians), smoking history and alcohol consumption history. Haplotypes of CYP3A4 and CYP3A5 genes, as well as multigene haplotypes (details are provided in Table 2 and the "Results" section), are also shown. Half-filled squares indicate one allele, and filled squares indicate the presence of both alleles of that haplotype. Also shown on the right side are P values for Chi-square test of demographics, history of smoking and alcohol use, and haplotype frequency differences between low (in the lower third) and high (in the upper third) 1'-OH MDZ formation rates. Analyses were conducted for all subjects,

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the Caucasians subpopulation, and subpopulations according to the history of alcohol consumption. na, not available.

Figure 3. The role of CYP3A4 and CYP3A5 in hepatic CYP3A mediated midazolam hydroxylation activity. (A). Correlation between rank orders of CYP3A4/CYP3A5 protein content and 1'-OH MDZ formation rate. Rank transformed data of CYP3A4 protein content is shown in filled dots, and that of CYP3A5 protein content is shown in open dots. (B). Left panel, the effect of haplotype CYP3A5*3 (g.6986 a>g) on CYP3A5 protein content. The homozygous carriers of haplotype CYP3A5*3 had significantly lower levels of CYP3A5 protein compared to non-carriers or heterozygous carriers (ANOVA on ranks test, **$P<0.001$, followed by Student-Newman-Kuels test, †$P<0.05$). Right panel, non-carriers, heterozygous and homozygous carriers of haplotype CYP3A5*3 did not have significantly different levels of midazolam hydroxylation activity. Indicated are the median, 75 percentile and 25 percentile values of CYP3A5 protein content or 1'-OH MDZ formation rate.

Figure 4. Nested PCR amplification of CYP3A4 cDNA. Ten total RNA samples (5 samples with the highest, 5 with the lowest 1'-OH MDZ formation rates) were isolated from human liver samples. The sketch on the top: the first round of the total cDNA amplification from exon 1 to exon 13 was performed using primer pair F1 and R1. Three nested PCR amplifications were performed using primer pair F2 and R2, primer pair F3 and R3 and primer pair F4 and R4. The amplification regions were partially overlapped.

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These three primer pairs were corresponding to primer pairs P12, P13 and P14 respectively in Table 1. Image on the bottom: gel electrophoresis of three nested PCR amplification products. High, 5 samples with the highest 1'-OH MDZ formation rates out of 54 samples; Low, 5 samples with the lowest 1'-OH MDZ formation rates out of 54 samples; pc, positive control; nc, negative control.

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Table 1. Polymerase chain reactions and the sequencing oligonucleotide primers used in study

Primer Pair	Location	Sequence (5'-3')
P1	CYP3A4 5'-region	CCAACAGAATCACAGAGGACCAGC
		CTCTGAGTCTTCCTTTCAGCTCTGTGT
P2	CYP3A4 Exon 6	GAATGAATCTGGTGGGGACAGGTAT
		CACCTCCTCATCGGAGCTGCC
P3	CYP3A4 Exon 7	GGAGTGTGATAGAAGGTGATCTAGTAGATC
		CACAATCTCATGGGATTTAGCAAAGG
P4	CYP3A4 Exon 10	TTAAGTTCCCTCAAACATATCTTCG
		ACACCACTATGGTGCCTGCTGC
P5	CYP3A4 Exon 11	CTTCCCGAATGCTTCCCACC
		CCTTCTCCTCCTTACCTTGTCTTCTCC
P6	CYP3A5 Exon 2	CCTGAGTAACTCACCAGCCCT
		CTCAAGCAACTCACCTGACGA
P7	CYP3A5 Intron 3	GAGTGGCATAGGAGATACCCAC
		TCTAGTTCATTAGGGTGTGACACACA
P8	CYP3A5 Exon 7	TACAGCATGGATGTGATTACTG
		AAAGAGAGAAAGAAATAATAGCC
P9	CYP3A5 Exon 11	AAATACTTCACGAATACTATGATCA
		CAGGGACATAATTGATTATCTTTG
P10	CYP3A5 Exon 13	TAAACGATGGATGGTGAGTGCTTTT
		GAAGCTGAGTCTACCTATCTGTCACTTACC
P11	CYP3A4 Exon 1	GGCTCTCATCCCAGACTTGGC
	CYP3A4 Exon 13	TTCTGGTTGAAGAAGTCCTCCTAAGCT
P12	CYP3A4 Exon 1	GGCTCTCATCCCAGACTTGGC
	CYP3A4 Exon 5	CTTTTCATAAATCCCACTGGACCA
P13	CYP3A4 Exon4	CTTTTATGATGGTCAACAGCCTGTG
	CYP3A4 Exon 9	GGGACTCAGTTTCTTTTGAATTCTGA
P14	CYP3A4 Exon 8	CAGTCTTTCCATTCCTCATCCCAA
	CYP3A4 Exon 13	TTCTGGTTGAAGAAGTCCTCCTAAGCT
P15	CYP3A4 Exon4	CTTTTATGATGGTCAACAGCCTGTG
	CYP3A4 Exon 5	CTTTTCATAAATCCCACTGGACCA
P16	CYP3A4 5'-UTR	GCTGTGCAGGGCAGGAAAGCTC
	CYP3A4 Exon 1	GCCAAGTCTGGGATGAGAGCCA
RACE-1		CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTT
RACE-2		CCAGTGAGCAGAGTGACG
RACE-3		GAGGACTCGAGCTCAAGC
GSP-1	CYP3A4 Exon 5	CTTTTCATAAATCCCACTGGACCA
GSP-2	CYP3A4 Exon 5	CACAGGCTGTTGACCATCATAAAAG
GSP-3		AGCTTAGGAGGACTTCTTCAACCAGAAA
GSP-4		TTGAGTCAAGGGATGGCACCG
GSP-5		TAAATCCCACTGGACCA
GSP-6		GTGATAGGCAGCACAGGCTGTTGACCA

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Table 2. Haplotypes inferred from CYP3A4 and CYP3A5 genotype data and their frequencies.

New	CYP3A allele	Nucleotide changes in gene	Frequency %	
			Entire group	Caucasian
	CYP3A4*1A	None	20.3	18.8
√	CYP3A4*I	-392 a>g, 15753 t>g, 15977 c>t, 20230 g>a	2.8	1.0
√	CYP3A4*V	15977 c>t, 20230 g>a, 22545 g>a	5.6	5.2
√	CYP3A4*VI	15977 c>t	53.7	59.4
√	CYP3A4*XI	-392 a>g, 15753 t>g, 20230 g>a, 22545 g>a	4.6	4.2
√	CYP3A4*XIV	20230 g>a, 22545 g>a	4.6	4.2
√	CYP3A4*XV	-392 a>g, 15753 t>g, 15977 c>t	1.9	2.1
	CYP3A5*1A	None	5.6	4.2
√	CYP3A5*1F	27526 c>t	7.4	5.2
	CYP3A5*3A	6986 a>g, 31611 c>t	77.8	78.1
	CYP3A5*3E	6986 a>g, 27050 a>g, 31611 c>t	8.3	9.4
√	CYP3A5*3U	6986 a>g, 27050 a>g	1.0	1.0

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Figure 1 (A)

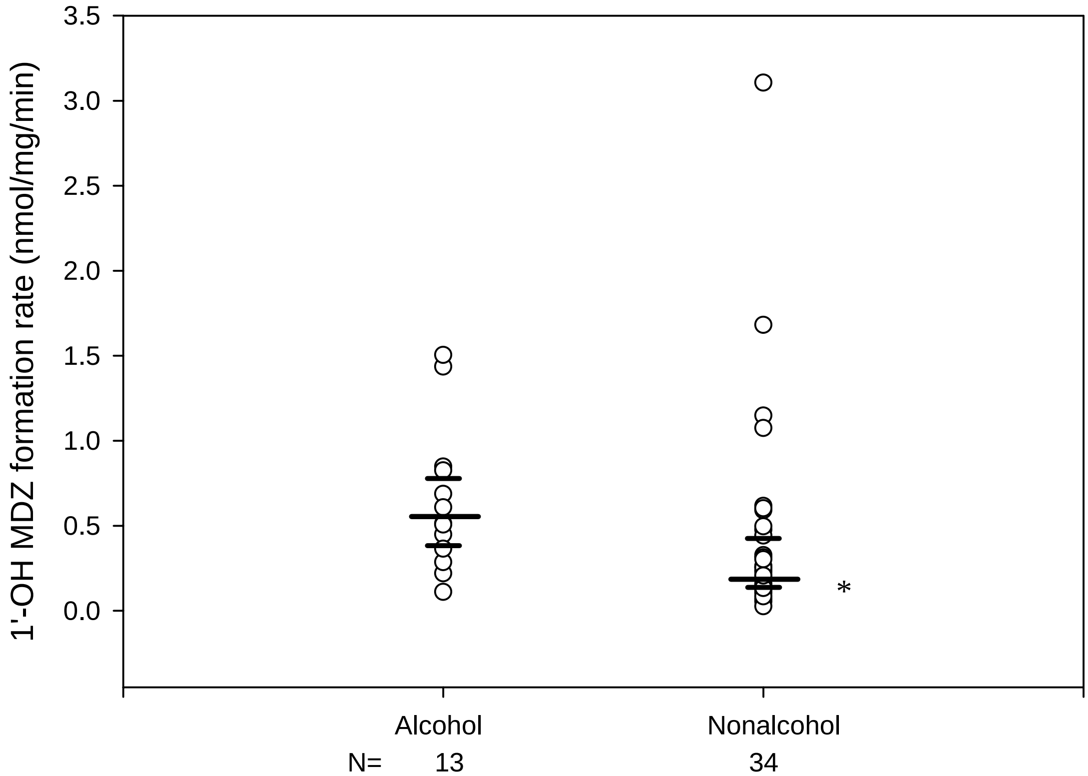

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Figure 1 (B)

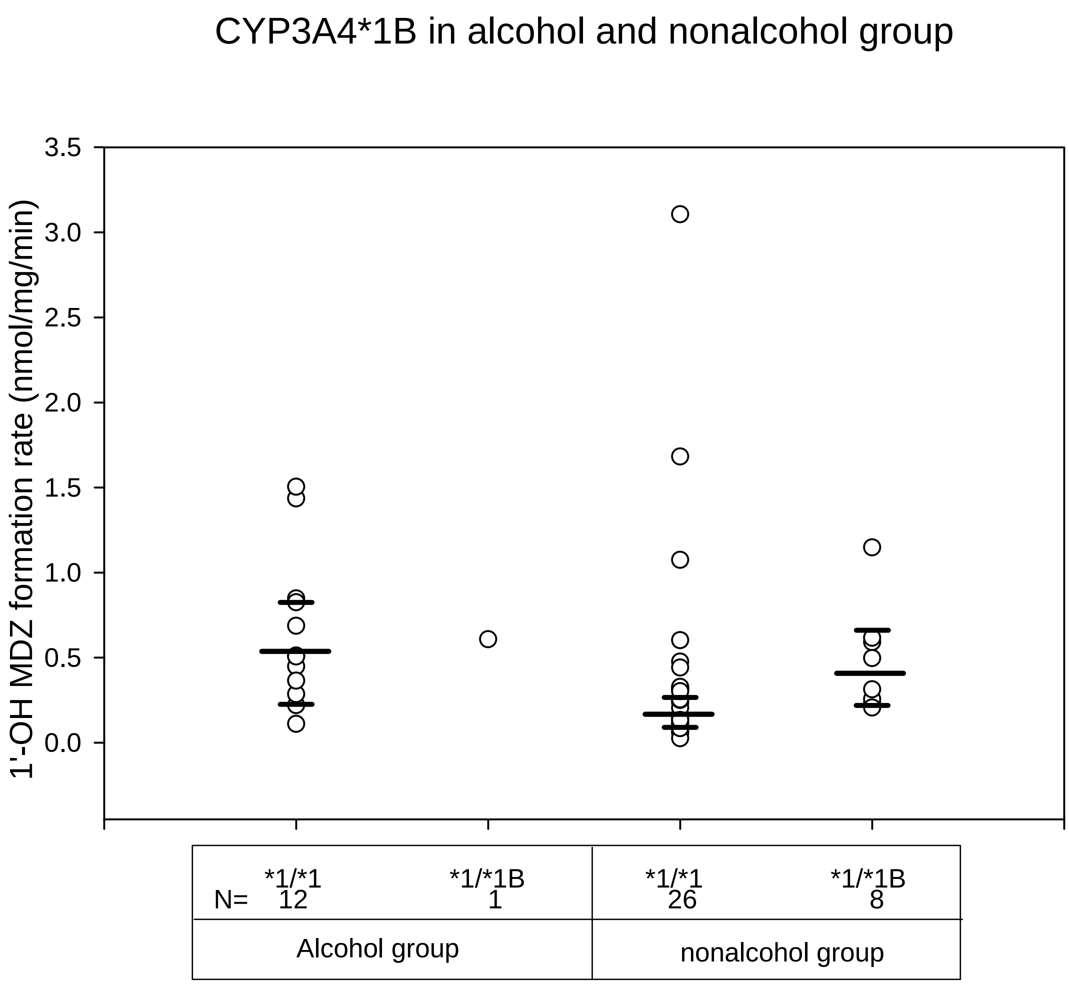

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Figure 1 (C)

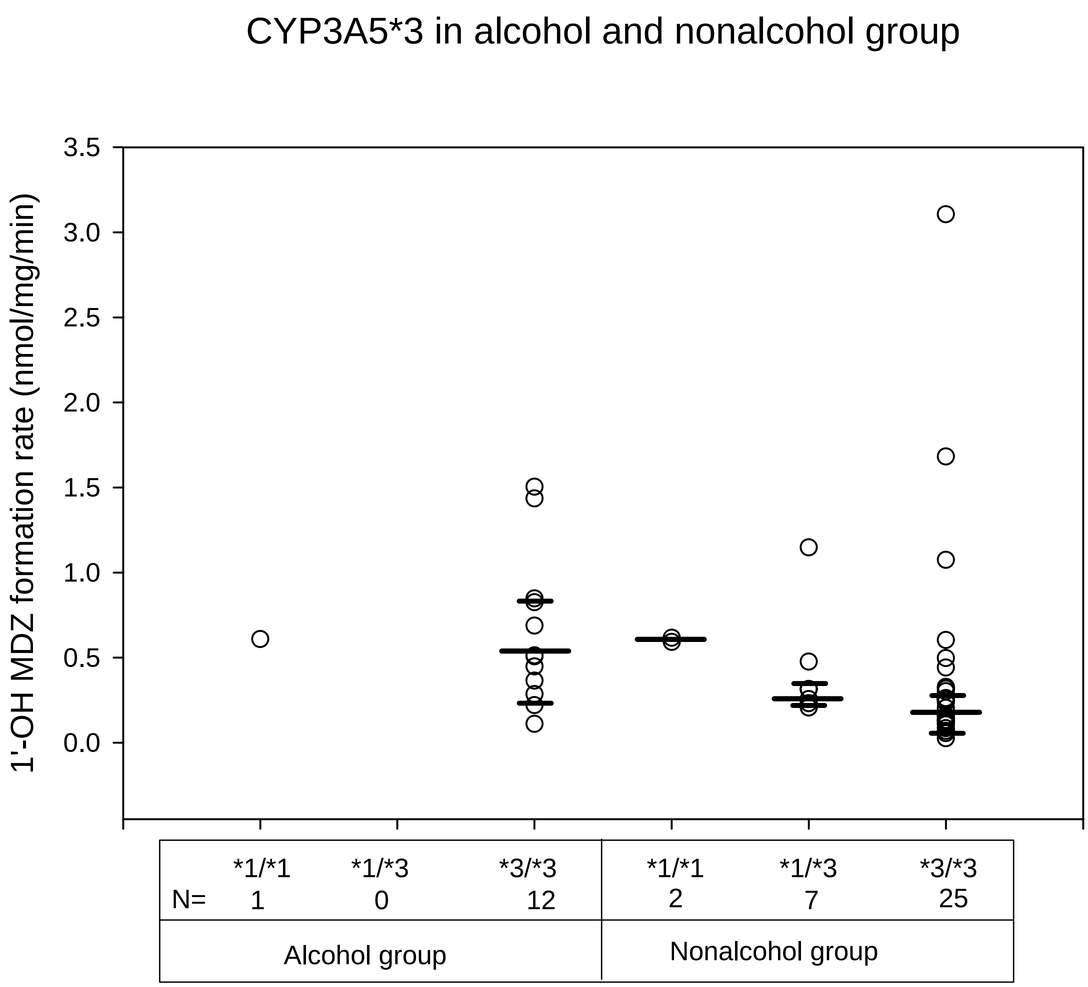

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Figure 2

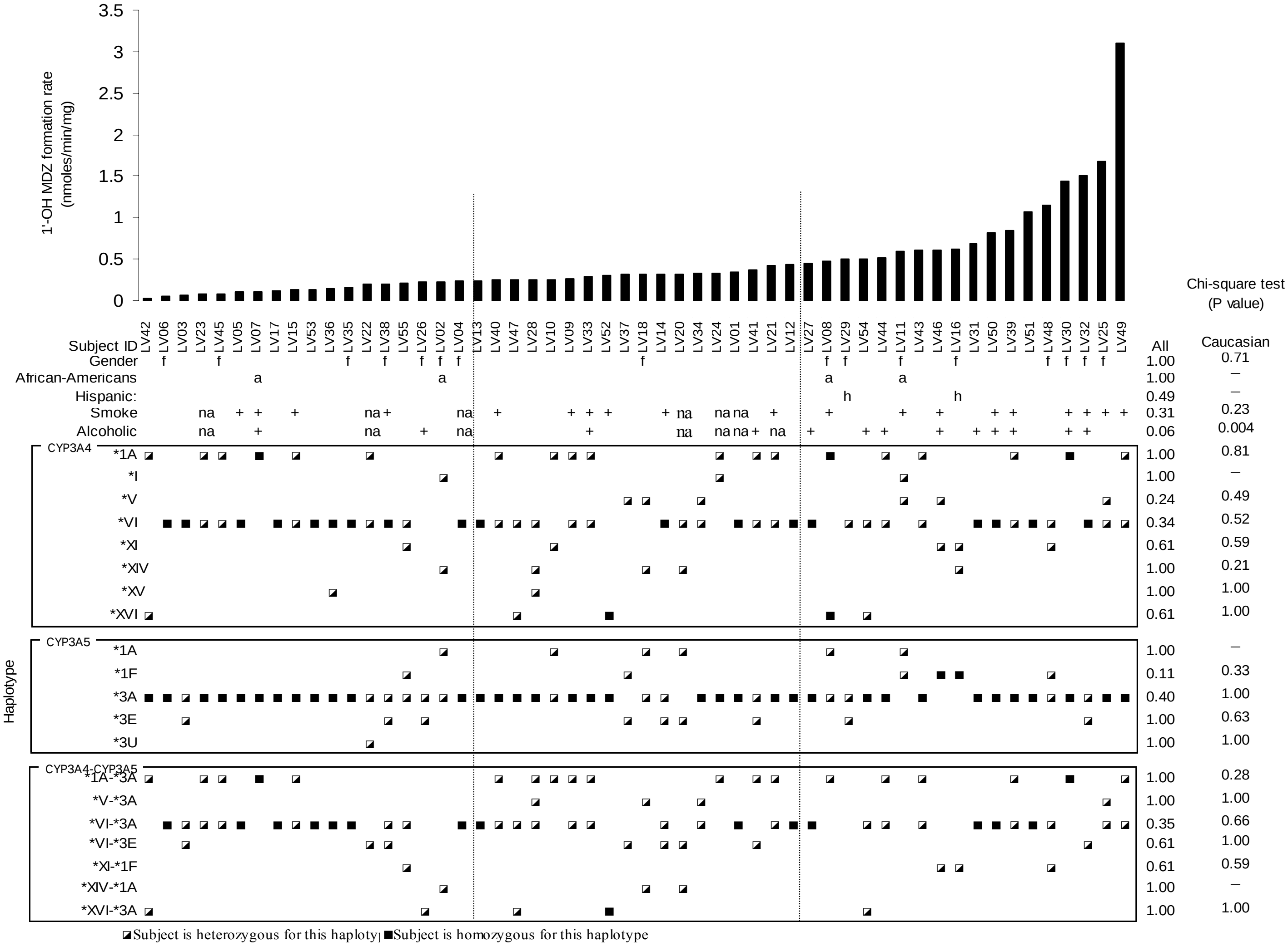

Figure 3 (A)

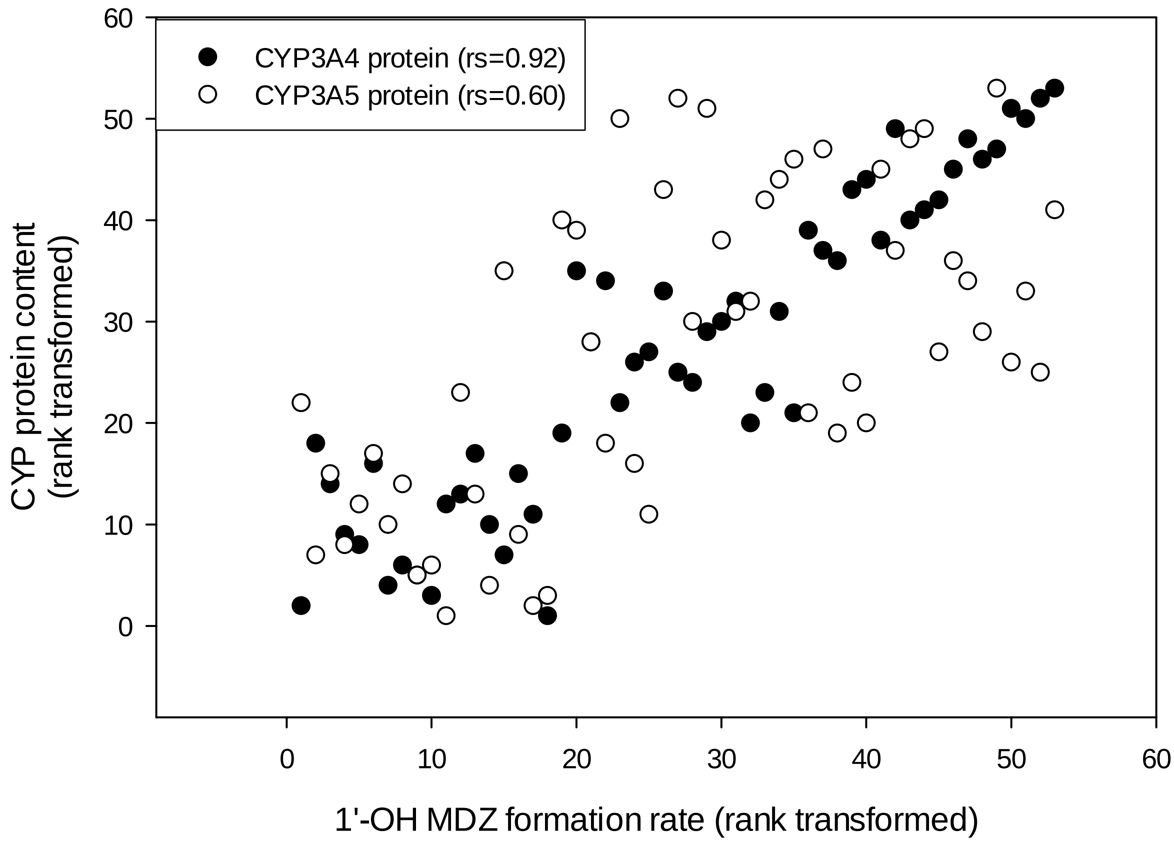
CYP3A4 protein (rs=0.92)
CYP3A5 protein (rs=0.60)
CYP protein content
(rank transformed)
1'-OH MDZ formation rate (rank transformed)
0
10
20
30
40
50
60

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Figure 3 (B)

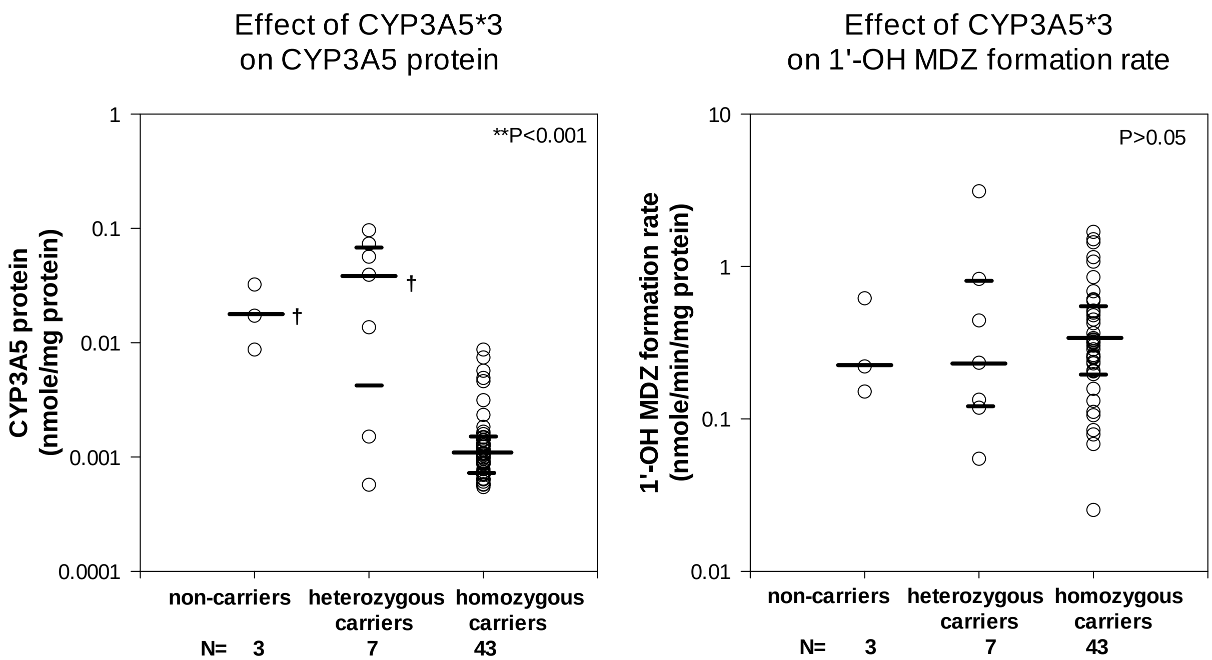

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Figure 4