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The *CYP3A4*18* allele, the most frequent coding variant in Asian populations, does not significantly affect the midazolam disposition in heterozygous individuals

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ABSTRACT

The objective of this study was to identify *CYP3A4* variants in Koreans and to characterize their functional consequences *in vitro* and *in vivo.* Four single nucleotide polymorphisms (SNPs) were identified in 50 Koreans by direct DNA sequencing. In an additional genotyping using 248 subjects, *CYP3A4*18* was confirmed as the most frequent coding variant in Koreans at 1.7% and its frequency was similar to that of Asians, suggesting that *CYP3A4*18* would be the highest coding variant in Asians. The recombinant CYP3A4.18 protein prepared in baculovirus expression system showed 67.4% lower V_{max} and 1.8 fold higher K_m for midazolam 1′-hydroxylation compared to the wild-type. The mean values of C_{max} and AUC in the *CYP3A4*1/*18* and *CYP3A5*1/*3* subjects (N=8) were 63% and 32% higher than in *CYP3A4*1/*1* and *CYP3A5*1/*3* carriers (N=8), respectively. Although the *in vitro* assay exhibited a significant reduction of the enzyme activity for midazolam, the *in vivo* differences associated with the *CYP3A4*1/*18* tend to be low ($P < .07$ in C_{max} and $P < .09$ in AUC). In summary, the heterozygous *CYP3A4*1/*18* does not appear to cause significant change of midazolam disposition *in vivo*, however, the clinical relevance of *CYP3A4*18/*18* remains to be evaluated.

INTRODUCTION

Cytochrome P450 3A (CYP3A) is the most abundantly expressed subfamily of CYP enzymes in the human liver (Shimada et al., 1994). The CYP3A enzymes are responsible for the metabolism of more than 50% of clinically used drugs (Komori et al., 1990; Guengerich, 1999; Lamba et al., 2002a). Four human CYP3A enzymes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, have been identified. CYP3A4 is regarded as the most dominant CYP3A enzyme in the liver and small intestine of humans. It has been reported that CYP3A4 expression shows large inter-individual variation (Guengerich, 1999; Ozdemir et al., 2000; Lin et al., 2002). These variations can lead to different responses to human drugs that are substrates for CYP3A4. As approximately 85% of this variability is attributed to genetic factors (Ozdemir et al., 2000), genetic analysis is needed to understand inter-individual variability. To date, more than 39 allelic variants have been described (http://www.cypalleles.ki.se/cyp3a4.htm) (Dai et al., 2001; Eiselt et al., 2001; Kuehl et al., 2001; Lamba et al., 2002a; Fukushima-Uesaka et al., 2004). Among the *CYP3A4* variants, alleles with nonsynonymous single nucleotide polymorphisms (SNPs), i.e., *CYP3A4*2, *4, *5, *6, *17,* and **18,* have been shown to alter enzyme activity, compared with the wild-type (Lee and Goldstein,

2005). Although some *CYP3A SNPs* exhibited an altered intrinsic clearance of CYP3A substrates *in vitro*, there has been lack of actual clinical impact to exert their meaningful influences on its substrate clearance in humans. The *CYP3A4*1B* promoter SNP has been extensively studied due to its role in the transcriptional regulation *in vitro*. However, no significant change associated with the *CYP3A4*1B* was observed in midazolam (MDZ) clearance (Wandel et al., 2000; Garcia-Martin et al., 2002; Eap et al., 2004; He et al., 2005) and in the cyclosporine clearance (Rivory et al., 2000), supporting that this SNP may not have a significant role in the CYP3A4 expression (Westlind et al., 1999). Genetic polymorphisms in *CYP3A4* gene seem to be the reason for inter-individual variation in the enzyme activity, however no key variant alleles of *CYP3A4* have been found to support these variations (Lamba et al., 2002a).

It has been reported that *CYP3A4*18* is not rare allele in Asians showing three of the 118 Japanese subjects (Yamamoto et al., 2003), twenty-two of the 416 Japanese subjects, six of the 302 Chinese subjects (Hu et al., 2005) and one of 60 Chinese (Du et al., 2006) populations. However, there have been no discovery studies on *CYP3A4* genetic polymorphisms and the report of *CYP3A4*18* allele in the Korean population. Therefore, we sequenced

the *CYP3A4* gene in 50 subjects and evaluated the allele frequency in an additional 248 Korean subjects. Although the *CYP3A4*18* is a high frequency allele in Asians, there have been no *in vivo* clinical reports associated with the *CYP3A4*18* allele. Therefore, we evaluated for the first time the clinical relevance of the *CYP3A4*18* with respect to the disposition of midazolam (MDZ) in humans.

Materials and Methods

Chemicals and Materials. The QIAamp blood kit was obtained from Qiagen (Valencia, Calif). All of the restriction enzymes were purchased from TaKaRa Bio (Shiga, Japan), and the primers were synthesized by Bioneer (Daejon, Korea). MDZ and the two MDZ metabolites, 1′-OH-MDZ and 4-OH-MDZ, were purchased from Ultrafine Chemical (Manchester, UK). Phenacetin was obtained from Sigma-Aldrich Co. (St. Louis, Mo). All other reagents and chemicals were of analytical or HPLC grade.

Subjects. In all, 298 Korean subjects were recruited for the *CYP3A4* genotyping study. Genomic DNA samples prepared from 50 of the 298 subjects were sequenced directly to identify *CYP3A4* sequence variants and the DNA samples from the remaining subjects were used to screen for the identified functional *CYP3A4* SNPs by PCR-RFLP or pyrosequencing. All of the participants were healthy, according to medical history, physical examination, and routine laboratory tests. All subjects provided written informed consent before participating in the present study, which was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea).

Direct DNA Sequencing and PCR-RFLP. Genomic DNA was extracted from

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the peripheral whole blood of 298 subjects using the QIAamp blood kit (Qiagen). For direct DNA sequencing of the *CYP3A4* gene in 50 subjects, each pair of primers for the amplification of all exons was designed according to a previously published method (Eiselt et al., 2001) except for the exon 3, 5 and 6 regions. Since these three exons were not amplified properly in our hands, new primers were designed as follows: for the exon 3, forward primer 5'-CACTGTGCATTCTCTTCTGAT-3' and reverse primer 5'-TCCCATTGCGCAATACTCTA-3'; for the exon 5 and 6, forward primer 5'-CATCACCCAGTAGACAGTCAC-3' and reverse primer 5'-TGGAATAACCCAACAGCAGG-3'. The distal enhancer region from -7 kb to -8 kb containing a XEREM site was amplified by forward primer 5'-GTCCTGGAGATAACCATGTAAC-3' and reverse primer 5'-GAGGGCCATAACCACATGATC-3'. The proximal promoter region (from +71 to -1.1 kb) was amplified by forward primer 5'- TTGGGGTCCATCTGGCT-3' and reverse primer 5'- TCTTCCTTTCAGCTCTGTGT-3'. The PCR products were subjected to gel electrophoresis to ensure proper amplification and the DNA sequence of the PCR product was determined in both directions.

The presence of *CYP3A4*4*, *CYP3A4*5*, *CYP3A4*6*, and *CYP3A4*18*

was further analyzed in an additional 248 Korean subjects by PCR-RFLP. The presence of the *CYP3A4*4* allele was determined using a previously published method with a slight modification, in that we used *Bsm*BI instead of *Bsm*AI (Hsieh et al., 2001) The *CYP3A4*5* allele was detected as described previously (Hsieh et al., 2001). For the detection of *CYP3A4*6* and *CYP3A4*18*, new PCR-RFLP methods were developed. The 526-bp DNA fragment that contains the *CYP3A4*6* allele was amplified with the primer pair: 5′-GCCATCTCACATGATAGCCAGA-3′ and 5′-ACCGCAGACTGACTTTCTAGCA-3′, and the PCR product was digested with *Hinf*I. The *CYP3A4*6* allele was detected as fragments of 225-bp, 188-bp, and 28-bp, whereas the wild-type allele gave fragments of 225-bp, 165-bp, 82-bp, and 24-bp. The 450-bp DNA fragment that contains the *CYP3A4*18* allele was amplified with the specific primers 5′-CACATCAGAATGAAACCACC-3′ and 5′-AGAGCCTTCCTACATAGAGTCA-3′. The PCR product was digested with *Msp*I, and the *CYP3A4*18* allele was identified by the presence of the 283-bp and 167-bp DNA fragments, since the T>C mutation in *CYP3A4*18* introduces an *Msp*I site. The digested PCR products were analyzed on 3% agarose gels. The presence of *CYP3A4*11* and *CYP3A4*16* was determined by pyrosequencing.

The following specific primers were used: for *CYP3A4*11*, 5′-CAGTATGAGTTAGTCTCTGG-3′ and 5′-Biotin-CATAACTGATGACCTTCATCG-3′; and for *CYP3A4*16*, 5′-GTCTGTCTTGACTGGACATGTGG-3′ and 5′-Biotin-GATGATGGTCACACATATCTTC-3′. The biotinylated PCR product was immobilized onto streptavidin-coated beads (Streptavidin Sepharose High Performance; Amersham Biosciences, Uppsala, Sweden). The beads were transferred to a filter plate, then the liquid was removed by vacuum filtration (Multiscreen Resist Vacuum Manifold; Millipore, Billerica, Mass), and the double-stranded DNAs were separated in denaturation solution (0.5 M NaOH). The immobilized template was washed with 10 mM Tris-acetate (pH 7.6), transferred to a PSQ 96 plate, and re-suspended in 20 mM Tris-acetate (pH 7.6) annealing buffer that contained the following sequencing primers: for *CYP3A4*11*, 5′-GACATGGTGGTGAATGAAA-3′; and for *CYP3A4*16*, 5′-CCTACAGCATGGATGTGA-3′. The resulting mixture was analyzed on the PSQ 96MA Pyrosequencer (Pyrosequencing AB, Uppsala, Sweden). The *CYP3A5*3* allele was detected using a previously published method (Roy et al., 2005). The 293-bp DNA fragment that contains the *CYP3A5*3* allele was

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amplified with the specific primers 5′-CATGACTTAGTAGACAGATGA-3′ and 5′-GGTCCAAACAGGGAAGAAATA-3′, and the PCR product was digested with *Ssp*I. The presence of the *CYP3A5*3* allele was detected as fragments of 168-bp and 125-bp, whereas the wild-type allele gave fragments of 148-bp, 125-bp, and 20-bp.

Expression of Recombinant Wild-type and Mutant CYP3A4 Proteins. The *CYP3A4* and CPR genes were cloned by PCR using the cDNA prepared from total liver RNA. The primers used for *CYP3A4* amplification were: 5′-GTCGACATGGCTCTCATCCCA-3' and 5'-GTCGACATGGCTCTCATCCCA-3'. The primers used for CPR amplification were: 5'-CTCGAGATGGGAGACTCCCAC-3' and 5'-GGTACCCTAGCTCCACACGTC-3'. The PCR products were ligated into the pGEM T-Easy vector (Promega, Madison, Wisc), and the complete open reading frames (ORFs) for the cloned inserts were verified by sequencing. The full-length cDNA that encodes the CYP3A4.18 (L293P) protein was generated by an overlap extension procedure (Ho et al., 1989) using the specific primers 5'-CAAAGCTCTGTCCGATCCGGAGCTCGTGGCCCACTC-3' and 5'-GAGTGGGCCACGAGCTCCGGATCGGACAGAGCTTTG-3'. Introduction of

the mutation was verified by DNA sequencing. The wild-type and mutant *CYP3A4* cDNAs and CPR cDNA were sub-cloned into the pFastBac dual vector (Invitrogen, Carlsbad, Calif). Sf9 cells were infected with the virus at a multiplicity of infection of 5-6 in the presence of hemin chloride (2.5 µg/mL). CYP3A4 content was determined by difference spectroscopy, as described previously (Omura and Sato, 1964). The expression levels of the CYP3A4.1, CYP3A4.18, and CPR proteins were verified by Western blot analysis, as described previously (Lee et al., 2005b).

Enzyme Kinetics. The enzymatic activities of the wild-type and mutant CYP3A4 proteins were determined using MDZ as a prototype substrate. The incubation mixtures (0.25 mL) were composed of 5 pmol cytochrome P450 in 50 mM potassium phosphate buffer (pH 7.4). The MDZ concentration range for the kinetic analysis was 0.5-25 µM. Following a 5-minute pre-incubation step at 37°C, the reactions were initiated by the addition of the NADPH-generating system (3.3 mM glucose-6-phosphate, 1.3 mM ß-NADP+, 3.3 mM $MgCl_2$, 1 U/mL glucose-6-phosphate dehydrogenase), and the reactions were incubated at 37°C for 30 minutes. The reaction was stopped by placing the incubation tube on ice and adding 100 µL ice-cold acetonitrile. The incubation mixtures

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were then centrifuged at 20,000 × *g* for 10 minutes at 4°C. Aliquots (5 µL) of the supernatants were injected into the API 3000 LC/MS/MS system (Applied Biosystems), which comprises the Agilent 1100 series HPLC (Agilent, Wilmington, Del) and the API 3000 tandem mass spectrometer (Applied Biosystems). The LC chromatograms were obtained by separation on a Luna C_{18} column (2 × 50 mm, 3 µm; Phenomenex, Torrance, Calif) in an isocratic mobile phase of acetonitrile and water (3/7, v/v) at a flow rate of 0.2 mL/min. Detection of 1′-OH-MDZ was performed by monitoring the transitions of m/z 342 to 203. The apparent kinetic parameters of 1′-OH-MDZ were determined by fitting the unweighted kinetic data to the one-enzyme Michaelis-Menten equation (Pharsight, Mountain View, CA). The kinetics observed for 1′-OH-MDZ exhibited the best fit with the Michaelis-Menten equation, as reported previously (Williams et al., 2002). All of the data are presented as mean ± S.D.

Pharmacokinetics of MDZ. Eight subjects who carried the *CYP3A4 1/*1* and *CYP3A5 *1/*3* alleles, seven who carried the *CYP3A4*1/*1* and *CYP3A5*3/*3* alleles, and eight who carried the *CYP3A4*18/*1* and *CYP3A5*1/*3* alleles were used in the analysis of MDZ pharmacokinetics. All subjects were male and ranged in age from 21 to 29 years old and in body weight from 52 to 85 kg.

There was no statistical difference among genotype groups in demographic characteristics. Routine clinical laboratory tests were performed for all subjects 3 weeks prior to the commencement of the study. Medications, herbal drugs, alcohol, caffeine-containing beverages, and grapefruit products were prohibited for the 14-day period prior to the study and throughout the study period. All subjects gave written informed consent and fully understood the study protocol. After overnight fasting, 7.5 mg of MDZ was administered orally. A standardized meal was served 4 hours and 10 hours after drug administration. Blood samples (6 mL) were drawn through an intravenous catheter before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 hours after MDZ administration. Plasma was immediately separated and stored at -80°C. The study protocol was approved by the Institutional Review Board of Inje University Busan Paik Hospital (IUBPH), and the study was conducted at the Clinical Pharmacology Center at IUBPH. All procedures were performed in accordance with the recommendations of the Declaration of Helsinki on biomedical research involving human subjects and with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use-Good Clinical Practice (ICH-GCP) guidelines.

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Measurements of Midazolam and Its Metabolites. The plasma concentrations of MDZ, 1′-OH-MDZ, and 4-OH-MDZ were determined by LC-MS/MS as described previously, with some modifications (Kashuba et al., 1998). The samples (0.5 mL) were then spiked with an internal standard (25 µL of 0.5 µM phenacetin), alkalinized with 0.3 mL of 2 M NaOH, and extracted with 5 mL of diethyl ether: ethylene chloride (6:4). After centrifugation, the organic phase was evaporated to dryness at ambient temperature in a Speed-Vac (Savant, Holbrook, NY). The residue was reconstituted in 0.2 mL of the mobile phase, and 5 µL was injected into the API 3000 LC/MS/MS system, which was equipped with the Agilent 1100 series HPLC system (Agilent). Chromatographic separation was accomplished on a Luna C_{18} column (2.0 × 50 mm, 3 µm; Phenomenex) using a mobile phase that consisted of a water and acetonitrile mixture (8/2, v/v) and 0.1% formic acid, which was delivered at a flow rate of 0.2 mL/min. The approximate retention times for 4-OH-MDZ, MDZ, 1′-OH-MDZ, and phenacetin were 1.8, 2.4, 2.7, and 4.3 minutes, respectively. The detection limits for MDZ, 1′-OH-MDZ, and 4-OH-MDZ were 0.1, 0.25, and 0.025 ng/mL, respectively. The inter-day assay precision for all analysis was <13.2%.

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Pharmacokinetic Data Analysis. The peak concentrations (C_{max}) of MDZ and its metabolites were obtained directly from the measured values. Non-compartmental pharmacokinetic analysis was performed using the WinNonlin program. The area under the concentration-time curve (AUC_t) was calculated using a numerical integration method and was extrapolated to infinity for AUC_{inf}. The differences in various pharmacokinetic parameters between genotype pairs in relation to *CYP3A4*18* and *CYP3A5*3* were analyzed by the Student's *t*-test, and $P < .05$ was considered to be statistically significant.

Results

From the sequencing data of all the exon regions, proximal promoter (from +71-bp to -1.1 kb), and a distal region containing the xenobiotic-responsive enhancer module (XREM) at -7836 through -7607-bp of the *CYP3A4* gene, four *CYP3A4* variants were identified (Table 1). No novel variants were identified in the present study. The four variants include as follows: g.3857C>T (found in four individuals as all heterozygous mutation in intron 2), g.17670Ains (*CYP3A4*6*) and g.20079T>C (*CYP3A4*18*) (found in one individual each as a heterozygous mutation), and g.20239G>A (found in thirteen individuals as all heterozygous mutation in intron 10). To determine the allelic frequency of *CYP3A4* variants in a Korean population, the presence of *CYP3A4*4, *5, *6, *11, *16* or *18 was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or pyrosequencing in 248 additional subjects. We included *CYP3A4*4* for genotyping because this allele has been reported at a frequency of 1.5% in a Chinese population[12] and was expected to be observed in Koreans. However, we couldn't find the *CYP3A4*4* allele in the present 298 Korean subjects. Except the *CYP3A4*4* allele, there were no significant differences in the allele frequency when

compared to those of Asians. The overall allelic frequencies of the *CYP3A4* variants are summarized in Table 1. From the PCR-RFLP, we identified three *CYP3A4*6* carriers and eight *CYP3A4*18* carriers. The most frequent mutant allele was *CYP3A4*18*, which showed a frequency of 1.7% in 298 Korean subjects. All eight individuals who carried the *CYP3A4*18* allele were heterozygous for the *CYP3A5*3* allele. The frequencies of all the other variants were <1% and none of the coding variants were linked to other alleles as homozygote. Interestingly, all eight individuals who carried the *CYP3A4*18* allele were heterozygous for the *CYP3A5*1* allele.

The wild-type CYP3A4.1 and mutant CYP3A4.18 proteins were expressed together with NADPH-CYP oxidoreductase (CPR) in Sf9 cells. The immunoblot of CYP3A4 showed similar levels of expression for the CYP3A4.1 and CYP3A4.18 proteins in our expression system (Figure 1A). The expression levels of CPR in both the CYP3A4.1 and CYP3A4.18 expression systems were also similar (data not shown). A typical CO-difference spectrum with an absorbance maximum at 450 nm was observed for both the CYP3A4.1 and CYP3A4.18 proteins (Figure 1B). The kinetics of metabolite formation was examined for the wild-type and mutant CYP3A4.18 using MDZ as the probe

substrate. The wild-type CYP3A4.1 protein catalyzed the formation of substantial amounts of 1′-OH-MDZ (Figure 2). Relative to the wild-type enzyme, CYP3A4.18 produced lower levels of 1′-OH-MDZ at all the tested concentrations of MDZ (Figure 2). The kinetic parameters for 1′-OH-MDZ were determined for the CYP3A4.1 and CYP3A4.18 enzymes. The Km value of 1'-OH-MDZ in our reconstitution system was low compared to the values obtained from the *E. coli* expression system (Khan et al., 2002) and the human liver microsome (Wang et al., 2000). The extent of inhibition at high concentration of MDZ in the wild-type was minor for the comparison, resulting in slight increase of Vmax (5.3 ± 0.5) and Km (0.7 ± 0.2). CYP3A4.18 had a lower V_{max} (1.4 versus 4.3 pmol/min/pmol) and slightly higher K_m (0.74 versus 0.42 μM) than CYP3A4.1 with respect to 1′-OH-MDZ formation, which resulted in a 5.2-fold decrease in V_{max}/K_m (Table 2).

In our *in vitro* experiment, the CYP3A4.18 variant exhibited decreased metabolism of MDZ (67%). Thus, we evaluated the effect of this allele on MDZ pharmacokinetics in humans. After a single oral dose of 7.5 mg MDZ was administered to the subjects, the concentrations of MDZ and its metabolites were measured in the blood. Figure 3 shows the mean plasma concentration-

time profiles of MDZ in the subjects with *CYP3A4*1/*1-CYP3A5*3/*1*, *CYP3A4*1/*1 -CYP3A5*3/*3,* and *CYP3A4*18/*1-CYP3A5*3/*1*. After peaking at 30 minutes after oral administration, the MDZ concentration decreased exponentially in the *CYP3A4*1/*1-CYP3A5*3/*3* and *CYP3A4*18/*1-CYP3A5*3/*1* carriers. However, the *CYP3A4*1/*1-CYP3A5*3/*1* carriers required an additional 30 minutes for the MDZ concentration to peak after oral administration and, subsequently, the concentrations of MDZ and its metabolites decreased exponentially (Figure 3A-C). The pharmacokinetic parameters are summarized in Table 3. The C_{max} of MDZ in the *CYP3A4*18/*1-CYP3A5*3/*1* carriers was almost the same as that in the *CYP3A4*1/*1-CYP3A5*3/*3* carriers (57.7 ± 30 versus 56.6 ± 29), whereas *CYP3A4*1/*1-CYP3A5*1/*3* carriers exhibited a 37% decrease (35.4 ± 11) in each comparison. Detailed comparisons with the relevant statistics are shown in Figure 4. The mean value of C_{max} in the subject having *CYP3A4*1/*18* and *CYP3A5*1/*3* (n=8) were 63% higher than the subjects with *CYP3A4*1/*1* and *CYP3A5*1/*3* (n=8) ($P<0.07$). The AUC value for MDZ in the *CYP3A4*18/*1* carriers were 24% higher than those in the *CYP3A4*1/*1* carriers with the same background of the *CYP3A5* genotype (148 ± 43 versus 112 ± 36

ng.h/mL), however the difference was not statistically significant (P< .09). Although the parent MDZ showed slightly decreased kinetic parameters, the kinetic parameters for the metabolites revealed no significant differences between the *CYP3A4*1, CYP3A5*3*, and *CYP3A4*18* carriers. There was no significant difference in the metabolic ratios of 1′-OH-MDZ and 4-OH-MDZ in relation to the *in vivo* kinetic parameters.

Discussion

It is well known that the CYP3A4 enzyme shows large inter-individual variability in activity and expression level. This polymorphic activity of CYP3A4 may generate inter-individual variations in drug responses. One possible cause for the polymorphic activity is genetic variation of the *CYP3A4* gene (Sata et al., 2000; Murayama et al., 2002; Amirimani et al., 2003; Min and Ellingrod, 2003; Matsumura et al., 2004). In the present study, we identified for the first time genetic variants of *CYP3A4* in a Korean population. Four variants, two in the exon (*CYP3A4*6, *18)* and two in the intron, were identified in the direct DNA sequencing. The frequency of alleles with coding SNPs (from *CYP3A4*2* to *CYP3A4*20*) was <5% in all of the ethnic groups studied (Eiselt et al., 2001; Hsieh et al., 2001; Lamba et al., 2002b; Cavaco et al., 2003; Fukushima-Uesaka et al., 2004; Westlind-Johnsson et al., 2006). Despite their low frequencies, certain allelic variants have been observed exclusively in a specific subset of ethnic groups. For example, *CYP3A4*4, *5, *6,* and **16* have been found exclusively in Asians, including Chinese (Eiselt et al., 2001), Japanese (Lamba et al., 2002b), and Korean populations (except *4 in the present study), but not in Caucasians. On the other hand, *CYP3A4*7, *8, *9,*

and **10* have been observed exclusively in Caucasians (Eiselt et al., 2001). *CYP3A4*17* has been found in the Adygei ethnic group (Lee et al., 2005a). Ethnic differences in *CYP3A4* polymorphisms are highlighted by the fact that the allelic frequency of *CYP3A4*1B* in Blacks (66.7%) is much higher than in Caucasians (4-6.5%) and Asians (0%) (Sata et al., 2000; Lamba et al., 2002b; Cavaco et al., 2003). Of the 22 alleles with coding SNPs, nine nonsynonymous CYP3A4 variants have been shown to be associated with altered catalytic activities (http://www.cypalleles.ki.se/cyp3a4.htm). Briefly, CYP3A4.2 has lower enzymatic activity for nifedipine, but there is no effect on 6ß-hydroxytestosterone activity (Sata et al., 2000). CYP3A4.12 shows greater activity for 15 ß- and 2ß-hydroxytestosterone than does the wild-type (Eiselt et al., 2001). CYP3A4.17 reduces testosterone 6ß-hydroxylation, chlorpyrifos desulfuration, and nifedipine oxidation (Dai et al., 2001; Lee et al., 2005a), while CYP3A4.18 enhances the catalytic activities for testosterone and chlorpyrifos (Dai et al., 2001). The *in vitro* functionality of the CYP3A4.18 protein has proven to be controversial. One report has revealed that CYP3A4.18 expressed in *E. coli* shows a 2-fold increase in catalytic activity for testosterone. However, according to another report, the CYP3A4.18 protein,

when transiently expressed in HepG2 cells, catalyzes 6ß-hydroxytestosterone to an extent similar to wild-type. These contradictory *in vitro* results could be explained by the different expression systems used in the different laboratories. In the present study, CYP3A4.18 expressed in baculovirus-infected Sf9 cells showed a small decrease in MDZ metabolism. The different effects of CYP3A4.18 on MDZ metabolism may be explained by the presence of multiple substrate recognition sites on CYP3A4, resulting in the variable activities depending on different substrates. According to Dai et al., (Dai et al., 2001) the side-chain of the L293 residue is largely masked, being packed into the interior of the CYP3A4 protein. Owing to the non-conservative nature of the L293P amino acid substitution, this mutation may affect protein conformation, substrate access, and catalytic activity. Gotoh has proposed the presence of six substrate recognition sites in mammalian CYPs (Gotoh, 1992). According to this proposition, the L293P substitution is located within the fourth substrate recognition site, which is highly conserved in a variety of animal species and is thought to be associated with substrate specificity. Several confounding factors have caused the difficulty in the extrapolation of the *in vitro* result into the phenotype observation, although the variant is proven to have an altered

function *in vitro*. These factors may include unknown mutations in *CYP3A4* gene, unknown endogenous and exogenous compounds affecting its expression or activity, substrate overlapping with other enzymes, variation in nuclear receptors, different assay systems in different labs, and the complexity in the kinetics depending on the substrate. In the present study, we detected the *CYP3A4*18* variant at a frequency of 1.7% in 298 Korean subjects. The frequencies of the *CYP3A4*18* variant among Far East Asian populations show no significant differences. This variant has also been observed at a frequency of 2% in a Chinese population (Dai et al., 2001) and 1.3% in a Japanese population (Yamamoto et al., 2003), suggesting that it is not rare allele in Asians and an important allele to be tested *in vivo*. Therefore, we evaluated the clinical relevance of *CYP3A4*18* for MDZ disposition. To our knowledge, the present study is the first to evaluate the effect of *CYP3A4*18* on therapeutic drug disposition in humans. Recently, it has been reported that *CYP3A4*20* exhibits a clear genotype-phenotype correlation with MDZ pharmacokinetics (Westlind-Johnsson et al., 2006). MDZ has been reported to be extensively metabolized by CYP3A in the liver and intestine (Thummel and Wilkinson, 1998) but is not a substrate for P-glycoprotein (Kim et al., 1999). However, the

estimation of the relative contribution of CYP3A5 to total CYP3A activity has been a matter of debate, ranging from up to 50% of total CYP3A protein in the liver (Kuehl et al., 2001) to a minor contributor (Westlind-Johnsson et al., 2003). Therefore, clinical impact of *CYP3A5*3* on the CYP3A substrates *in vivo* has been also controversial. The presence or absence of the *CYP3A5*3* allele has affected tacrolimus disposition (Hesselink et al., 2003; Thervet et al., 2003; Haufroid et al., 2004), but not in the cyclosporine (Hesselink et al., 2003; Zhao et al., 2005). When it comes to MDZ, some reports showed a positive association with the existence of *CYP3A5*3* (Kuehl et al., 2001; Lin et al., 2002), but, no significant influence has been found in others (Shih and Huang, 2002; Floyd et al., 2003; Yu et al., 2004). The contribution of *CYP3A5*3* to the total metabolism of CYP3A substrates is still not clear *in vitro* as well as *in vivo* (Thummel, 2003; Wilkinson, 2004) and may differ depending on the substrates. In comparing the kinetic parameters of the MDZ metabolites between *CYP3A4*1/*1* and *CYP3A4*1/*18* carriers with the same background of *CYP3A5*3/*1* genotype, the heterozygous *CYP3A4*18* did not seem to affect the pharmacokinetics of 1′-OH-MDZ and 4-OH-MDZ. It could be difficult to link the role of the *CYP3A4*18* allele to a particular phenotype, especially with

respect to metabolite disposition, since the metabolite can be further metabolized to the conjugated products by phase II enzymes, thereby masking the primary effect of *CYP3A4*18* on MDZ metabolism. Genetic polymorphisms of phase II enzymes may define further confounding factors that will increase our understanding the role of the *CYP3A* genotype in phenotypic variations, especially with regard to multiple metabolite formation, as in the case of MDZ. In this context, *CYP3A4*18* may be associated with functionally altered SNPs of the phase II enzymes that are responsible for the conjugation of MDZ metabolites for plasma clearance. This may partly explain the lack of reduction of plasma MDZ metabolites in those *CYP3A4*18/*1* subjects who exhibited a slightly prolonged plasma concentration of the parent MDZ compound. Human pregnane X receptor (PXR) polymorphisms have been shown to influence the MDZ clearance particularly in African American (He et al., 2006), suggesting that PXR polymorphism would be another confounding factor in the MDZ clearance variation in the present study.

The *CYP3A4*18* carriers exhibited increases in C_{max} and AUC_{inf} for the parent MDZ compound, as compared to *CYP3A4*1* carriers with the same genotypic background of *CYP3A5*3/*1*. Although these results were not

statistically significant, *CYP3A4*18* seems to slightly affect MDZ clearance *in vivo*. From the post hoc power analysis for the AUC data obtained in the present study, at least 26 subjects per group are required to detect statistical significance in a *t* test comparison of mean value at the .05 significance level with 80% power. The evaluation of the *CYP3A4*18* on MDZ disposition using a larger number of subjects would be necessary for better assessment. However, it may be difficult to conduct the investigation using such a large number of subjects with the consent due to its low frequency in Asians, and even almost no existence of this allele in Caucasians.

The extent of the increases in C_{max} and AUC_{inf} for the parent MDZ compound in *CYP3A4*18/*1-CYP3A5*3/*1* carriers was similar to that in *CYP3A4*1/*1-CYP3A5*3/*3* carriers. Kuehl et al. (Kuehl et al., 2001) have also reported decreased MDZ clearance in *CYP3A5*3/*3* carriers, as compared to *CYP3A5*1/*3* carriers. All of the *CYP3A4*18* carriers examined in the present study were heterozygous for *CYP3A5*3*. Subjects who are homozygous for *CYP3A4*18* are relatively rare in the Korean population. According to the Hardy-Weinberg equation, one in about 3000 Koreans would be expected to have the homozygous *CYP3A4*18* genotype. Actually, an individual having

homozygous mutation for *CYP3A4*18* was identified in 418 Japanese subjects (Fukushima-Uesaka et al., 2004). Recently Fukushima-Uesaka *et al.* found an additional individual having homozygous mutation for *CYP3A4*18* and both of two subjects were identified on the background of *CYP3A5*1/*1* (Personal communication). Fukushima-Uesaka *et al.* reported a haplotype between *CYP3A4*18* and *CYP3A5*1E* (Fukushima-Uesaka et al., 2004). This haplotype finding is also supported in the present our result of the genotyping study. In the present study the expected haplotype frequency of *CYP3A4*18/CYP3A5*3* was 1.09×10^{-7}, whereas *CYP3A4*18/CYP3A5*1*, *CYP3A4*1/CYP3A5*1* and *CYP3A4*1/CYP3A5*3*, were 0.07, 0.42 and 0.51, respectively, analyzed by SNP Alyze Ver.4.1.1. All of eight individuals in the present study contained at least one *CYP3A5*1* allele. No individuals having *CYP3A4*18* have been reported to carry the homozygous mutation of *CYP3A5*3* allele so far (Yamamoto et al., 2003; Fukushima-Uesaka et al., 2004; Hu et al., 2005). The impact of the decreased activity against MDZ caused by *CYP3A4*18* allele may be compensated by the strong expresser genotype *CYP3A5*1* in terms of overall CYP3A activity, even in the case of the homozygous *CYP3A4*18/*18*.

In summary, the *CYP3A4*18* allele, which leads to decreased MDZ

metabolism *in vitro*, is often observed in Koreans as a heterozygous mutation. We suggest that the altered plasma concentration of MDZ *in vivo* caused by *CYP3A4*18/*1* is not clinically significant. One of these reasons may be from the linkage between *CYP3A4*18* and *CYP3A5*1* (Fukushima-Uesaka et al., 2004), compensating the lack of *CYP3A4* activity toward MDZ. Further clinical studies would be helpful to elucidate the role of the *CYP3A4*18* allele in the disposition of other CYP3A substrates.

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Footnotes

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Legends for figures

Fig. 1. Immunoblot and reduced CO-difference spectra of the recombinant wild-type and mutant CYP3A4.18 proteins. (A) Western blot analysis. Microsomal fractions from Sf9 cells infected with wild-type or mutant CYP3A4 baculovirus were separated by SDS-PAGE. The microsomal fraction infected with the baculovirus that lacks the CYP3A4 cDNA was analyzed as the negative control on the same membrane. Mock, empty baculosome; WT, CYP3A4.1 with CPR-containing baculosome; L293P, CYP3A4.18 with CPR-containing baculosome. (B) CO-difference spectra of microsomal fractions prepared from Sf9 cells that express the wild-type or mutant CYP3A4.18 protein.

Fig. 2. Midazolam metabolism by the CYP3A4.1 and CYP3A4.18 proteins. Kinetic parameter assessment using activity versus substrate concentration curves for 1′-OH-MDZ. All of the reactions were performed with a NADPH-regenerating system and 5 pmol P450 at 37°C for 30 minutes with MDZ (0.5, 2, 5, 10, and 25 μM) in a final volume of 0.25 mL. The formation of 1′-OH-MDZ metabolites was analyzed by LC/MS/MS. Each point represents the mean of duplicate determinations. Lines represent functions that were determined by

nonlinear least-squares regression analysis using the Michaelis-Menten equation (WinNonlin software).

Fig. 3. Time profiles of the MDZ plasma concentrations and hydroxy metabolites. (A) Plasma MDZ; (B) plasma 1′-OH-MDZ; (C) plasma 4-OH-MDZ. After oral administration of 7.5 mg MDZ, plasma was serially collected and analyzed for MDZ content, as described in the *Methods* section. The symbols in each panel are as follows: closed circle, *CYP3A4*1/*1* and *CYP3A5*3/*1* subjects; closed square, *CYP3A481/*1* and *CYP3A4*1/*18* subjects; open square, *CYP3A4*1/*1* and *CYP3A5*3/*3* subjects. Each point represents the mean ± SD.

Fig. 4. Scatter plots of (A) C_{max}, (B) AUC_{inf}, and (C) oral clearance (CL/F), as estimated following oral administration of a single dose of 7.5 mg MDZ. ●, *CYP3A4*1/*1* and *CYP3A5*1/*3* subjects; □, *CYP3A4*1/*1* and *CYP3A5*3/*3* subjects; ■, *CYP3A4*1/*18* and *CYP3A5*1/*3* subjects. Each symbol with a bar indicates the mean ± SE of a group. The *P* values were determined by the Student's *t*-test for differences between the *CYP3A4* and *CYP3A5* genotype

groups.

TABLE 1 *CYP3A4* genetic variants identified in a Korean population

SNP[a]	Number of subjects	Location	Effect	Allele	Activity (*in vitro*)	Number of alleles	Observed frequency (%)
3857C>T	50	Intron 2				4	4[b]
13880A>G	298	Exon 5	I118V	**4*	Decreased	0	0
15711C>G	298	Exon 7	P218R	**5*	Decreased	1	0.2
17670Ains	298	Exon 9	Frameshift	**6*	None	5	0.8[b]
21876C>T	298	Exon 11	T363M	**11*	Decreased	1	0.2
15612C>G	298	Exon 7	T185S	**16*	Decreased	1	0.2
20079T>C	298	Exon 10	L293P	**18*	?[c]	10	1.7[b]
20239G>A	50	Intron 10				13	13[b]

[a]Nucleotides are numbered according to the reference sequence NC_000007.12. [b]*CYP3A4* variants were identified by direct

DNA sequencing. *CYP3A4*6* and **18* alleles were identified in one individual each in direct DNA sequencing analysis. Further analysis of these SNPs in 248 samples revealed four individuals having **6* allele and nine for **18* allele. The combined allele frequencies (50+248 subjects) are presented in the table.

[c]The recombinant CYP3A4.18 enzyme expressed in *E. coli* showed enhanced metabolism of testosterone and chlorpyrifos (Dai et al., 2001). However, the same variant, when expressed in HepG2 cells does not affect testosterone hydroxylation (Murayama et al., 2002).

TABLE 2 Comparisons of the kinetic parameters of midazolam 1′-hydroxylation by the CYP3A4.1 and CYP3A4.18 proteins

P450s	Midazolam 1′-hydroxylation		
	V_{max} (pmol/min/pmol)	K_m (μM)	CL_{int} (μL/min/pmol)
CYP3A4.1	4.3 ± 0.3	0.4 ± 0.1	11 ± 3
CYP3A4.18	1.4 ± 0.1	0.7 ± 0.4	2.1 ± 1

The values are estimated from nonlinear least-squares regression analysis using the WinNonlin software. $V_{max,}$ the maximum reaction velocity; K_m, the substrate concentration that corresponds to 50% of V_{max}. The reactions include equal amounts of P450 (5 pmol) from the CO spectrum analysis, not P450 + P420. Detailed procedures are described in the *Methods* section.

TABLE 3 Pharmacokinetics of midazolam and its metabolites following oral administration of the 7.5 mg midazolam[a]

Genotype	*CYP3A4*1/*1 / CYP3A5*1/*3* N = 8	*CYP3A4*1/*1 / CYP3A5*3/*3* N = 7	*CYP3A4*18/*1 / CYP3A5*1/*3* N = 8
MDZ			
$t_{1/2}$ (h)	2.5 ± 0.6	2.5 ± 0.6	2.9 ± 0.8
T_{max} (h)	1.3 ± 0.8	0.6 ± 0.2	0.9 ± 0.9
C_{max} (ng/mL)	35.4 ± 11	56.6 ± 29	57.7 ± 30
CL/F (L/h/kg)	1.1 ± 0.4	1.0 ± 0.3	0.8 ± 0.1
AUC_{inf} (ng.h/mL)	112 ± 36	132 ± 63	148 ± 43
1′-OH-MDZ			
$t_{1/2}$ (h)	2.3 ± 0.6	2.5 ± 0.5	2.2 ± 0.5
T_{max} (h)	1.2 ± 0.8	0.6 ± 0.2	1.2 ± 1.1
C_{max} (ng/mL)	18.2 ± 7.8	27.2 ± 14	27.7 ± 12
AUC_{inf} (ng.h/mL)	46.9 ± 12	51.7 ± 17	61.9 ± 20

4-OH-MDZ

$t_{1/2}$ (h)	2.0 ± 0.5	2.8 ± 0.7*	2.5 ± 0.6
T_{max} (h)	1.5 ± 1.0	0.71 ± 0.3	1.3 ± 1.1
C_{max} (ng/mL))	2.1 ± 0.6	2.9 ± 0.8*	2.8 ± 1.2
AUC_{inf} (ng.h/mL)	6.5 ± 1.6	8.9 ± 3.1	9.2 ± 3.0^{+}

[a]The subjects were categorized into three groups according to the genotypes of *CYP3A4*1, CYP3A4*18,* and *CYP3A5*3*. The data are presented as the mean ± SD. T_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; $t_{1/2}$, terminal elimination half-life; CL/F, total oral clearance; AUC_{inf} and AUC_t, area under the concentration time curve from zero to infinity and last observation, respectively. *P < .05, Student's *t*-test for *3A4*1/*1* and *3A5*1/*3* versus *3A4*1/*1* and *3A5*3/*3* groups. ^{+}P < .05, Student's *t*-test for *3A4*1/*1* and *3A5*1/*3* versus *3A4*1/*18* and *3A5*1/*3* groups.

Figure 1

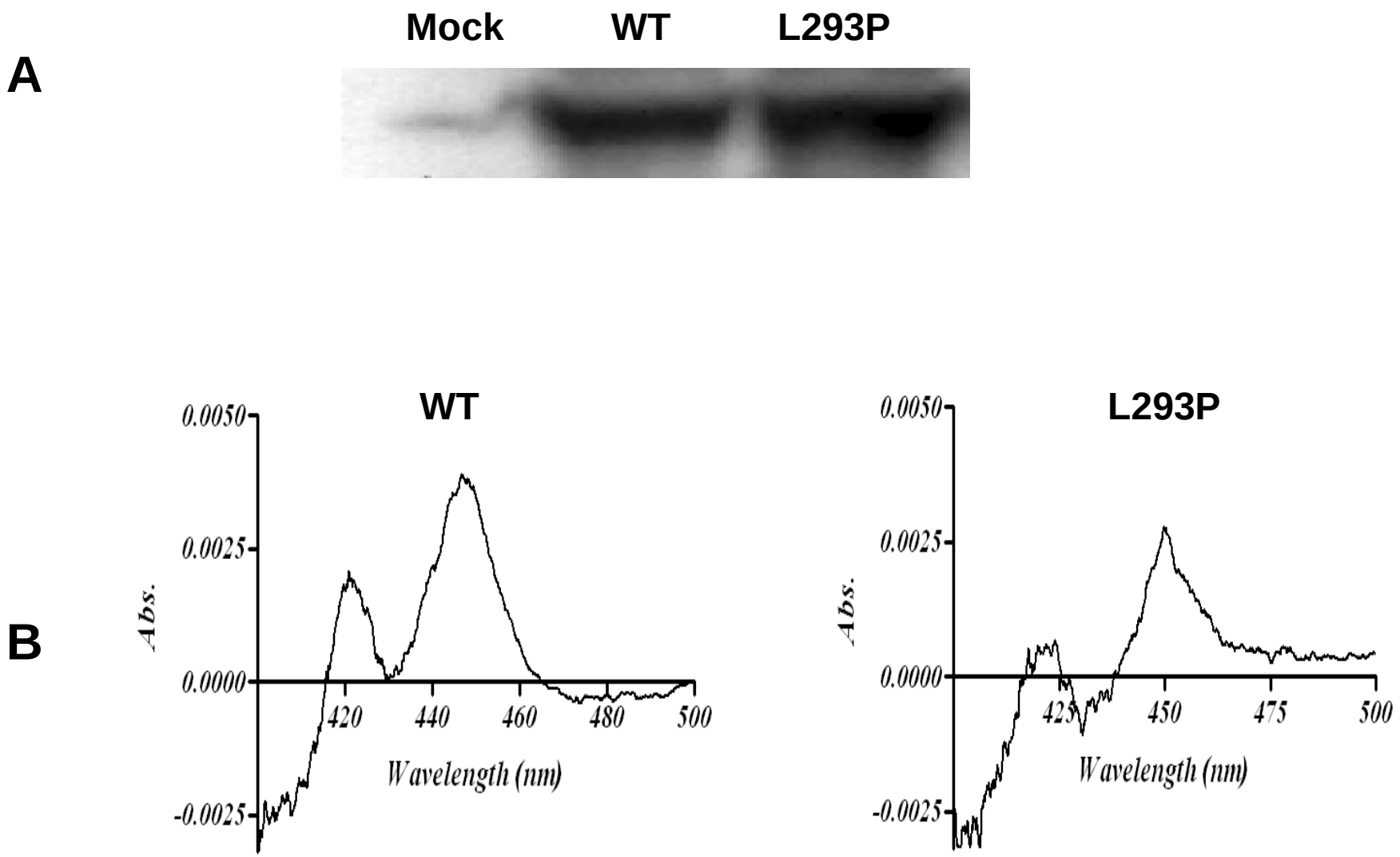

Figure 2

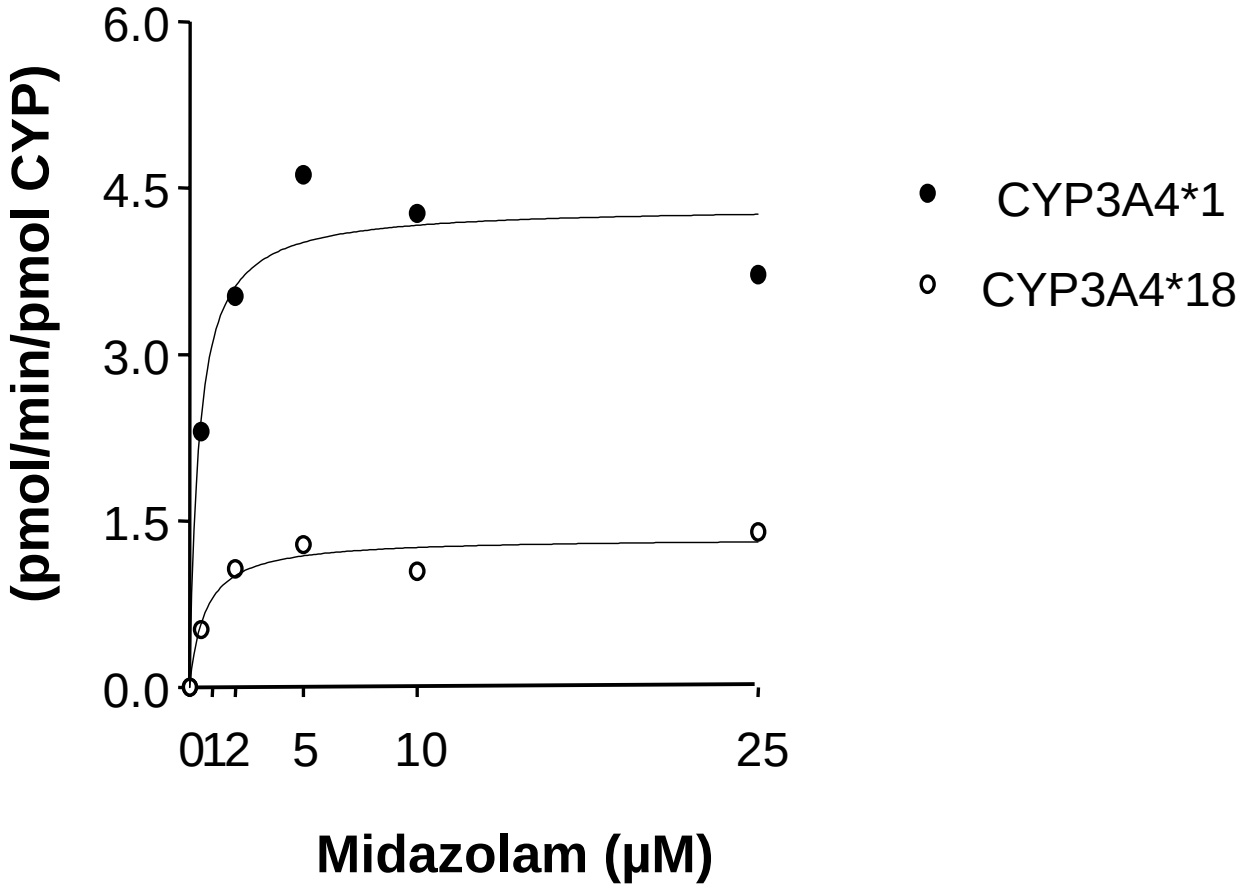

Figure 3

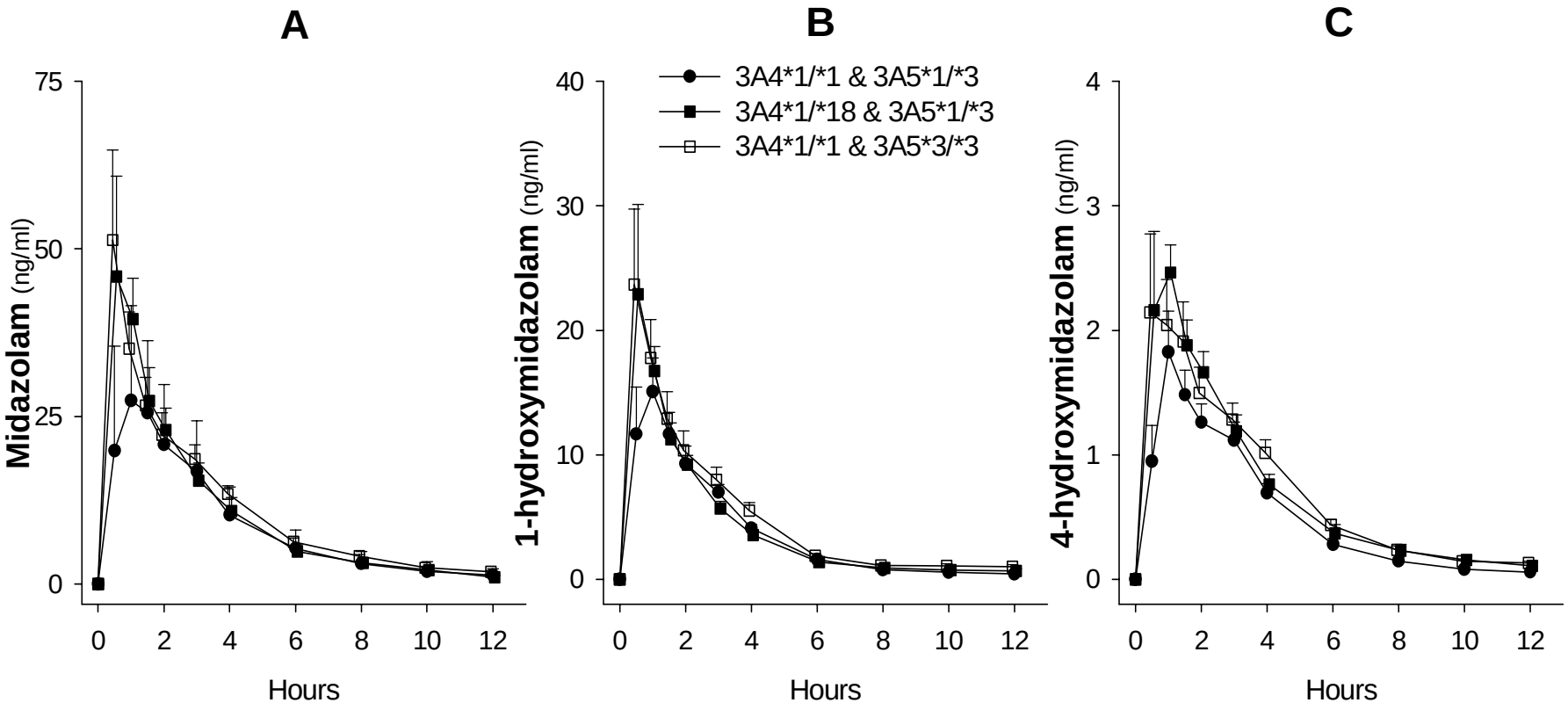

Figure 4