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 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 Vmax and 1.8-fold higher Km for midazolam 1'-hydroxylation compared with the wild type. The mean values of Cmax and area under the concentration curve (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 < 0.07 in Cmax and P < 0.09 in AUC). In summary, the heterozygous CYP3A4*1/*18 does not appear to cause a significant change in midazolam disposition in vivo; however, the clinical relevance of CYP3A4*18/*18 remains to be evaluated.

CYP3A is the most abundantly expressed subfamily of cytochrome P450 (P450) 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 interindividual 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. Because approximately 85% of this variability is attributed to genetic factors (Ozdemir et al., 2000), genetic analysis is needed to understand interindividual 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 have been few data explaining their meaningful influences on its substrate clearance in humans. The CYP3A4*1B promoter SNP has been extensively studied because of its role in transcriptional regulation in vitro. However, no significant change associated with CYP3A4*1B was observed in midazolam (MDZ) clearance (Wandel et al., 2000; Garcia-Martín et al., 2002; Eap et al., 2004; He et al., 2005) and in cyclosporine clearance (Rivory et al., 2000), supporting the idea 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 interindividual variation in the enzyme activity; however, no key variant alleles of CYP3A4 have been found to support these variations (Lamba et al., 2002a).

CYP3A4*18 has been identified in 3 of the 118 Japanese subjects

ABBREVIATIONS: P450, cytochrome P450; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; MDZ, midazolam; HPLC, high performance liquid chromatography; Vmax, maximum reaction velocity; Km, the substrate concentration that corresponds to 50% of Vmax; Cmax, maximum plasma concentration; AUCinf, area under the concentration time curve from zero to infinity; OH, hydroxy; kb, kilobase(s); CPR, NADPH-CYP (P450) oxidoreductase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CO, carbon monoxide.
(Yamamoto et al., 2003), 22 of the 416 Japanese subjects and 6 of the 302 Chinese subjects (Hu et al., 2005), and 1 of 60 subjects in a Chinese population (Du et al., 2006). 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 CYP3A4*18 with respect to the disposition of MDZ in humans.

Materials and Methods

Chemicals and Materials. The QiAamp blood kit was obtained from QiAGEN (Valencia, CA). 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 (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 polymerase chain reaction-restriction fragment length polymorphism (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 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 their hands, new primers were designed as follows: for exon 3, forward primer 5'-CAGTGCGATCCCCCTCTGCTCTGTCTTTCTGAT-3' and reverse primer 5'-TCCCTATGGGTACCAACTCTTCA-3'; for exon 5 and 6, forward primer 5'-CATCAGCTAGACAGCTC-3' and reverse primer 5'-TTGGAATCCAAACACAAGCGGGA-3'. The distal enhancer region from 7 kb to 8 kb containing a xenobiotic-responsive enhancer module site was amplified by forward primer 5'-GTCCGTGGGACAACTCTTCA-3' and reverse primer 5'-GGGAATCCAAACACAAGCGGGA-3'. The proximal promoter region (from +71 to -1.1 kb) was amplified by forward primer 5'-TTGGGTCCATCTGCTGCC-3' and reverse primer 5'-TCTCTCCCTACAGCTCCTGTG-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 BsmBI instead of BsmAI (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'-GCAATCTCCATCTGATAGTCGAGACACCAGGAA-3' and 5'-ACGCGAGCTAGTCTTTCTGAC-3', and the PCR product was digested with HindIII. 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'-CATCAGCTAGACAGCTC-3' and 5'-AGGAATCCAAACACAAGCGGGA-3', and the PCR product was digested with MspI. The CYP3A4*18 allele was identified by the presence of the 283-bp and 167-bp DNA fragments, since the 7-C mutation in CYP3A4*18 introduces a MspI 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'-CAGCATAGGAAGATGCTCTGTCG-3' and 5'-Biotin-CATACTGAGATGCTCTGTCG-3'; and for CYP3A4*16, 5'-GATCTTTCTGAGATGCTCTGTCG-3' and 5'-Biotin-GATGAGCTACATCATACATC-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, MA), and the double-stranded DNAs were separated in denaturation solution (0.5 N NaOH). The immobilized template was washed with 10 mM Tris-acetate (pH 7.6), transferred to a PSQ 96 plate, and resuspended in 20 mM Tris-acetate (pH 7.6) annealing buffer that contained the following sequencing primers for CYP3A4*11, 5'-GACAGTGTTGTAATGAA-3'; and for CYP3A4*16, 5'-CTTACAGCATGAGGTGGTA-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 amplified with the specific primers 5'-CATGACTTATGAGCATGATGAG-3' and 5'-GGTCGGAAAAGAATA-3', and the PCR product was digested with SspI. 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 NAPDH-Cytochrome P450 (CYP) 450 oxidoreductase (CPR) genes were cloned by PCR using the cDNA prepared from total liver RNA. The primers used for CYP3A4 amplification were: 5'-GTCGACATGGTGCTCTCTATCCCA-3' and 5'-GTGGCATGATGGTGCTCTCTATCCCA-3'. The primers used for CPR amplification were: 5'-CTCGAGATGGGAGACTCCACCC-3' and 5'-GGATCCCTGAGTCATCACCATC-3'. The PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI), and the complete open reading frames 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'-CAAGATCTCGTCGTCGCAGCT-3' and 5'-GGTAAGGCAGTCATCACCATC-3'. The wild-type and mutant CYP3A4 cDNAs and CPR cDNA were subcloned into the pFastBac dual vector (Invitrogen, Carlsbad, CA). Sf9 cells were infected with the virus at a multiplicity of infection of 5 to 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 of cytochrome P450 in 50 mM potassium phosphate buffer (pH 7.4). The MDZ concentration range for the kinetic analysis was 0.5 to 25 µM. After a 5-min preincubation 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 β-NADPH, 3.3 mM MgCl2, 1 U/ml glucose-6-phosphate dehydrogenase), and the reactions were incubated at 37°C for 30 min. The reaction was stopped by placing the incubation tube on ice and adding 100 µl of ice-cold acetonitrile. The incubation mixtures were then centrifuged at 20,000g for 10 min 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, DE) and the API 3000 tandem mass spectrometer (Applied Biosystems). The liquid chromatograms were obtained by separation on a Luna C18 column (2 × 50 mm, 3 µm; Phenomenex, Torrance, CA) 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 for 1'-OH-MDZ were determined by fitting the unweighted kinetic data to the one-enzyme Michaelis-Menten equation (WinNonlin; 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/*1 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 before the commencement of the study. Medications, herbal drugs, alcohol, caffeine-containing beverages, and grapefruit products were prohibited for the 14-day period before 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 h and 10 h 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 h 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, and the study was conducted at the Clinical Pharmacology Center at Inje University Busan Paik Hospital. 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 guidelines.

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), alkalized with 0.3 ml of 2 N 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 (Agilent). Chromatographic separation was accomplished on a Luna C18 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 min, 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 interday assay precision for all concentrations of MDZ, 1'-OH-MDZ, and phenacetin were 1.8, 2.4, 2.7, and 4.3 min, respectively. The peak concentrations (C_{max}) of MDZ and its metabolites were obtained directly from the measured values. The peak concentrations were examined for the wild-type and mutant CYP3A4.18 proteins in HepG2 cells. The kinetic parameters of metabolic formation were examined for the wild-type and mutant CYP3A4.18 using MDZ as the probe substrate. The wild-type CYP3A4.1 and mutant CYP3A4.18 proteins were expressed together with 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 (Fig. 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 obtained for both the CYP3A4.1 and CYP3A4.18 proteins (Fig. 1B). The kinetics of metabolic 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 (Fig. 2). Relative to the wild-type enzyme, CYP3A4.18 produced lower levels of 1'-OH-MDZ at all the tested concentrations of MDZ (Fig. 2). The kinetic parameters for 1'-OH-MDZ were determined for the CYP3A4.1 and CYP3A4.18 enzymes. The K_{m} value of 1'-OH-MDZ in our reconstitution system was low compared with the values obtained from the Escherichia coli expression system.

### Table 1

<table>
<thead>
<tr>
<th>SNP</th>
<th>Number of Subjects</th>
<th>Location</th>
<th>Effect</th>
<th>Allele</th>
<th>Activity (in Vitro)</th>
<th>Number of Alleles</th>
<th>Observed Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>3857C&gt;T</td>
<td>50</td>
<td>Intron 2</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td>13880A&gt;G</td>
<td>298</td>
<td>Exon 5</td>
<td>I118V</td>
<td>*4</td>
<td>Decreased</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15711C&gt;G</td>
<td>298</td>
<td>Exon 7</td>
<td>P218R</td>
<td>*5</td>
<td>Decreased</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>17670Ains</td>
<td>298</td>
<td>Exon 9</td>
<td>Frameshift</td>
<td>*6</td>
<td>None</td>
<td>5</td>
<td>0.8*</td>
</tr>
<tr>
<td>21876C&gt;T</td>
<td>298</td>
<td>Exon 11</td>
<td>T363M</td>
<td>*11</td>
<td>Decreased</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>15612C&gt;G</td>
<td>298</td>
<td>Exon 7</td>
<td>T185S</td>
<td>*16</td>
<td>Decreased</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>20097T&gt;C</td>
<td>298</td>
<td>Exon 10</td>
<td>L293P</td>
<td>*18</td>
<td>?</td>
<td>10</td>
<td>1.7*</td>
</tr>
<tr>
<td>20239G&gt;A</td>
<td>50</td>
<td>Intron 10</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

* Nucleotides are numbered according to the reference sequence NC_000007.12.

* 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).

### 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 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 are g.3857C>T (found in four individuals as all heterozygous mutation in intron 2), g.17670Ains (CYP3A4*6) and g.20097T>C (CYP3A4*18) (found in one individual each as a heterozygous mutation), and g.20239G>A (found in 13 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 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 (Hsieh et al., 2001) and was expected to be observed in Koreans. However, we could not find the CYP3A4*4 allele in the present 298 Korean subjects. Except for the CYP3A4*4 allele, there were no significant differences in the allele frequency when compared with 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 alleles 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*3 allele.

The wild-type CYP3A4.1 and mutant CYP3A4.18 proteins were expressed together with CPR in Sf9 cells. The immunoblot of CYP3A4.1 and CYP3A4.18 proteins in our expression system (Fig. 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 obtained for both the CYP3A4.1 and CYP3A4.18 proteins (Fig. 1B). The kinetics of metabolic 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 (Fig. 2). Relative to the wild-type enzyme, CYP3A4.18 produced lower levels of 1'-OH-MDZ at all the tested concentrations of MDZ (Fig. 2). The kinetic parameters for 1'-OH-MDZ were determined for the CYP3A4.1 and CYP3A4.18 enzymes. The K_{m} value of 1'-OH-MDZ in our reconstitution system was low compared with the values obtained from the Escherichia coli expression system.
CYP3A5*3/*1 which resulted in a 5.2-fold decrease in
analyzed by LC-MS/MS. Each point represents the mean of duplicate determina-
in a final volume of 0.25 ml. The formation of 1
of wild type was minor for the comparison, resulting in a slight increase
2000). The extent of inhibition at a high concentration of MDZ in the
(Khan et al., 2002) and the human liver microsome (Wang et al.,
FIG. 2. Midazolam metabolism by the CYP3A4.1 and CYP3A4.18 proteins. Ki-
4.5
0.1 0.7
0.09). Although the
parameters for the metabolites revealed no significant differences be-
CYP3A4*1B
the discrimination, resulting in a slight increase of V
max (5.3 ± 0.5) and K
m (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 of MDZ was administered to the subjects, the concentrations of MDZ and its metabolites decreased exponentially (Fig. 3, A–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 Fig. 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 values 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 < 0.09). 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+4 enzyme shows large interindi-
vidual variability in activity and expression level. This polymorphic
activity of CYP3A4 may generate interindividual 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; Amirian et al., 2003; Min and Ellingrod, 2003; Matsumura
et al., 2004). In the present study, we identified for the first time, to our
knowledge, 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 frequen-
cies, 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 allele 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

![Fig. 1. Immunoblot and reduced CO-difference spectra of the recombiant wild-
type and mutant CYP3A4.18 proteins. A, Western blot analysis. Microsomal frac-
tions from Sf9 cells infected with wild-type or mutant CYP3A4 baculovirus were
separated by SDS-polyacrylamide gel electrophoresis. 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 bacu-
losome. 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. Ki-
etic 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 of P450 at 37°C for 30 min with MDZ (0.5, 2, 5, 10, and 25 μM)
in a final volume of 0.25 ml. The formation of 1
of wild type was minor for the comparison, resulting in a slight increase
2000). The extent of inhibition at a high concentration of MDZ in the
(Khan et al., 2002) and the human liver microsome (Wang et al.,

![Table 2. Comparisons of the kinetic parameters of midazolam 1'-hydroxylation
by the CYP3A4.1 and CYP3A4.18 proteins]

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vmax (pmol/min/pmol)</th>
<th>Km (μM)</th>
<th>Clint (μl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4.1</td>
<td>4.3 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>CYP3A4.18</td>
<td>1.4 ± 0.1</td>
<td>0.7 ± 0.4</td>
<td>2.1 ± 1</td>
</tr>
</tbody>
</table>
for nifedipine, but there is no effect on 6β-hydroxytestosterone activity (Sata et al., 2000). CYP3A4.12 shows greater activity for 15β- and 26β-hydroxytestosterone than does the wild type (Eiselt et al., 2001). CYP3A4.17 reduces testosterone 6β-hydroxylation, chlorpyrifos desulfuration, and nifedipine oxidation (Dai et al., 2001; Lee et al., 2005a), whereas CYP3A4.18 enhances the catalytic activities for testosterone and chlorpyrifos (Dai et al., 2001). The in vitro functionality of the CYP3A4.18 protein, when transiently 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 6β-hydroxytestosterone to an extent similar

with coding SNPs, nine nonsynonymous CYP3A4 variants have been shown to be associated with altered catalytic activities (http://www.cypalleles.ki.se/cyp3a4.htm). In brief, CYP3A4.2 has lower enzymatic activity for nifedipine, but there is no effect on 6β-hydroxytestosterone activity (Sata et al., 2000). CYP3A4.12 shows greater activity for 15β- and 26β-hydroxytestosterone than does the wild type (Eiselt et al., 2001). CYP3A4.17 reduces testosterone 6β-hydroxylation, chlorpyrifos desulfuration, and nifedipine oxidation (Dai et al., 2001; Lee et al., 2005a), whereas 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 6β-hydroxytestosterone to an extent similar

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CYP3A4*1/<em>1/CYP3A5</em>1/*3</th>
<th>CYP3A4*1/<em>1/CYP3A5</em>3/*3</th>
<th>CYP3A4*1/<em>1/CYP3A5</em>1/*3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.5 ± 0.6</td>
<td>2.5 ± 0.6</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.3 ± 0.8</td>
<td>0.6 ± 0.2</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>35.4 ± 11</td>
<td>56.6 ± 29</td>
<td>57.7 ± 30</td>
</tr>
<tr>
<td>CL/F (L/kg)</td>
<td>1.1 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>AUCinf (ng/ml)</td>
<td>112 ± 36</td>
<td>132 ± 63</td>
<td>148 ± 43</td>
</tr>
<tr>
<td>6β-OH-MDZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.3 ± 0.6</td>
<td>2.5 ± 0.5</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.2 ± 0.8</td>
<td>0.6 ± 0.2</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>18.2 ± 7.8</td>
<td>27.2 ± 14</td>
<td>27.7 ± 12</td>
</tr>
<tr>
<td>AUCinf (ng/ml)</td>
<td>46.9 ± 12</td>
<td>51.7 ± 17</td>
<td>61.9 ± 20</td>
</tr>
<tr>
<td>4-HO-MDZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.0 ± 0.5</td>
<td>2.8 ± 0.7*</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.5 ± 1.0</td>
<td>0.71 ± 0.3</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>2.1 ± 0.6</td>
<td>2.9 ± 0.8*</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>AUCinf (ng/ml)</td>
<td>6.5 ± 1.6</td>
<td>8.9 ± 3.1</td>
<td>9.2 ± 3.0*</td>
</tr>
</tbody>
</table>

**TABLE 3**
Pharmacokinetics of midazolam and its metabolites after oral administration of 7.5 mg of midazolam

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 ± S.D.

**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 of MDZ, plasma was serially collected and analyzed for MDZ content, as described under Materials and Methods. Closed circle, CYP3A4*1/*1 and CYP3A5*2/*1 subjects; closed square, CYP3A4*1/*1 and CYP3A4*1/*18 subjects; open square, CYP3A4*1/*18 and CYP3A5*3/*3 subjects. Each point represents the mean ± S.D.

**FIG. 4.** Scatter plots of Cmax (A), AUCinf (B), and oral clearance (CL/F) (C), as estimated after oral administration of a single dose of 7.5 mg of MDZ. A, CYP3A4*1/*1 and CYP3A5*1/*3 subjects; B, CYP3A4*1/*1 and CYP3A5*3/*3 subjects; C, CYP3A4*1/*18 and CYP3A5*1/*3 subjects. Each symbol with a bar indicates the mean ± S.E. of a group. The P values were determined by Student’s t test for differences between the CYP3A4 and CYP3A5 genotype groups.
to that of 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. (2001), the side-chain of the L293 residue is largely masked, being packed into the interior of the CYP3A4 protein. Owing to the nonconservative nature of the L293P amino acid substitution, this mutation may affect protein conformation, substrate access, and catalytic activity. Gottoh (1992) has proposed the presence of six substrate recognition sites in mammalian P450s. 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 laboratories, and 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 a rare allele in Asians and is 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, the clinical impact of CYP3A5*3 on the CYP3A substrates in vivo has also been controversial. The presence or absence of the CYP3A5*3 allele has affected tacrolimus disposition (Hesselink et al., 2003; Thertet et al., 2003; Haufroid et al., 2004), but not in 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/*3 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 of the role of the CYP3A4 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 polymorphisms have been shown to influence the MDZ clearance particularly in African Americans (He et al., 2006), suggesting that pregnane X receptor 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, compared with CYP3A4*/1 carriers with the same genetic 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 0.05 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 consent because of the low frequency of this allele in Asians, and its near-nonexistence 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. (2001) have also reported decreased MDZ clearance in CYP3A5*3/*3 carriers, compared with 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, 1 in approximately 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 and coworkers found an additional individual having homozygous mutation for CYP3A4*18 and two subjects were identified on the background of CYP3A5*1/*1 (Y. Saito, personal communication). Fukushima-Uesaka et al. (2004) reported a haplotype between CYP3A4*18 and CYP3A5*1E. This haplotype finding is also supported in the present results of our 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, when analyzed by SNP Alyze (version 4.1.1; Dynacom, Yokohama, Japan). All 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 the 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/*9 is not clinically significant. One of the reasons for this may be 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 allelic variant in the disposition of other CYP3A substrates.

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References


