ABSTRACT:

The CYP3A subfamily represents the most abundant cytochrome P450 in the human liver and gastrointestinal tract and plays very important role in xenobiotic metabolism. CYP3A5 is expressed in a relatively small population of whites and Orientals. We recruited 42 Chinese volunteers to determine the genotypes of CYP3A5 by polymerase chain reaction-restriction fragment length polymorphism. Genotype analyses revealed that CYP3A5*3 allele existed in 39 of 42 volunteers, CYP3A5*4 and CYP3A5*5 alleles were found in one volunteer each; and CYP3A5*2 and CYP3A5*6 alleles were not found. The most frequent CYP3A5*3 allele is known not to express CYP3A5. We excluded other genotypes of CYP3A5 to study the significance of CYP3A5*3 in midazolam pharmacokinetics. In this study, each volunteer was given a midazolam tablet (7.5 mg) orally. Blood samples were collected to analyze the time-dependent concentrations of midazolam and 1'-hydroxymidazolam by high-performance liquid chromatography. The average area under plasma concentration curve (AUC, 0–8 h) of midazolam was 9237 ± 1050 ng-min/ml (mean ± S.E.M.) in homozygous CYP3A5*3 (n = 14) subjects and 7934 ± 768 ng-min/ml in heterozygous CYP3A5*1*3 (n = 12) subjects, respectively. The average AUC (0–8 h) of 1'-hydroxymidazolam was 3748 ± 427 ng-min/ml in homozygous CYP3A5*3 subjects and 3920 ± 402 ng-min/ml in heterozygous CYP3A5*1*3 subjects. The results indicated that the pharmacokinetics of midazolam and 1'-hydroxymidazolam was independent of CYP3A5 expression. Although the genetic polymorphism of CYP3A5 is well known, the results of this study suggested that the clinical consequence might be insignificant.

Cytochrome P450s (P450)1 are heme-containing monoxygenases and play an essential role in the oxidative biotransformation of endogenous compounds such as steroids, fatty acids, prostaglandins, and exogenous chemicals, including drugs, natural plant products, carcinogens, and environmental pollutants. CYP3A enzymes are the most abundantly expressed cytochrome P450 enzymes in liver (Hashimoto et al., 1993) and are responsible for the metabolism of over 50% of all clinically used drugs, including substances as diverse as steroids, antidepressants, benzodiazepines, immunosuppressive agents, midazolam, imidazole antimicrotics, and macrolide antibiotics (Li et al., 1995; Paulussen et al., 2000).

In human, four members of the CYP3A subfamily, CYP3A4, CYP3A5, CYP3A7 (Nelson et al., 1996), and CYP3A43 (Domanski et al., 2001; Westlind et al., 2001), have been identified. The four CYP3A genes are localized in a cluster on chromosome 7q21-q22.1 (Spurr et al., 1989; Finta and Zaphiropoulos, 2000) and are characterized by a high structure similarity and protein sequence identity. The encoding genes have a well conserved exon-intron structure, all consisting of 13 exons (Hashimoto et al., 1993).

The predominant P450 form, CYP3A4, is a major P450 in the liver and intestine and has been reported to be involved in the metabolism of more than 60% of medically relevant drugs (Li et al., 1995). The expression of CYP3A5 is highly polymorphic and is estimated to comprise on average 7 to 8% of the total P450 content in human adult liver (Wrighton et al., 1989). It is also a major P450 in the kidney and intestine (Schuetz et al., 1992; Lin et al., 2002). CYP3A7 accounted for 30 to 50% of total P450 in fetal liver (Schuetz et al., 1994). CYP3A43 was identified recently (Domanski et al., 2001; Westlind et al., 2001). Overlapping substrate specificities between CYP3A4 and CYP3A5 have made it difficult to differentiate the two enzymes using probe drugs. The delineation of CYP3A4 and CYP3A5 metabolism has been shown to be possible only by using midazolam (Gorski et al., 1994; Hachner et al., 1996) or cyclosporine as a probe drug. Two metabolites of midazolam are formed: 1'-OH-MDZ and 4'-hydroxymidazolam. Wandel et al. (1994) quantified the hydroxylated metabolites by HPLC after incubation with P450 enzymes. They reported that CYP3A5 converted more MDZ to 1'-OH-MDZ (127.4 ng/10 min/0.5 mg protein) than CYP3A4 did (26.8 ng/10 min/0.5 mg protein).

Immunoblotting and Northern blot analyses have detected CYP3A5 expression in only 10 to 30% of human livers (Aoyama et al., 1989; Wrighton et al., 1990; Schuetz et al., 1994). Another study showed that only 3% of human liver had detectable CYP3A5 by immunoblotting (Boobis et al., 1996). These findings suggested that CYP3A5 polymorphism might play an important role in functional regulation. The single nucleotide polymorphisms in CYP3A5*3 and CYP3A5*6
resulted in the absence of CYP3A5 in tissues (Kuehle et al., 2001). Among the CYP3A5*3 subjects, CYP3A5 expression comprises only 4.2% of total CYP3A in the liver and 2.7% of total CYP3A in the jejunum (Lin et al., 2002). However, among the heterozygous CYP3A5*1/*3 subjects, CYP3A5 expression is appreciable, with 50% of total CYP3A in the liver and 61% of CYP3A in the jejunum. We report the results of CYP3A5 genotypes in 42 Chinese and of the pharmacokinetics of MDZ and 1'-OH-MDZ in some Chinese subjects with different CYP3A5*3 genotypes after oral ingestion of MDZ (Wandel et al., 1998).

Materials and Methods

Human Subjects and DNA Isolation. We recruited 42 healthy male subjects from the Chinese (Han) population living in Taiwan. Whole blood (10 ml) was obtained from each subject, and genomic DNA was isolated from peripheral leukocytes using the DNA isolation kit (Puregene; Gentra Systems, Inc., Minneapolis, MN).

PCR-RFLP for CYP3A5*2 in Intron 11. A PCR-based test of CYP3A5*2 (T3098N) was developed. To detect the C1492SHIH AND HUANG

TABLE 1
Primers used to identify CYP3A5 variants

<table>
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<tr>
<th>Variant</th>
<th>Location</th>
<th>Name</th>
<th>Primers Seq.(5'→3')</th>
<th>Position on Reference Sequence (bp)</th>
<th>PCR Fragment (bp)</th>
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<tr>
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<tr>
<td></td>
<td></td>
<td>*2(R)</td>
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<td>43317</td>
<td></td>
</tr>
<tr>
<td>CYP3A5*3</td>
<td>Intron 3</td>
<td>pri-IN3(S)</td>
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<td></td>
</tr>
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<td></td>
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<td>1542</td>
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</table>

PCR-RFLP for CYP3A5*3 in Intron 3. A PCR-based test of CYP3A5*3 was developed. To detect the A1492SHIH AND HUANG

In Vivo MDZ Hydroxylation Assay. Twenty-eight healthy male subjects were selected from the genotyping test for the pharmacokinetic study. The written consent of volunteers was obtained from each volunteer. The study was approved by the Ethics Committee of the Medical Center, National Cheng Kung University (Tainan, Taiwan). Each volunteer was given an MDZ tablet (7.5 mg, Dormicum; F. Hoffmann-La Roche, Basel, Switzerland) orally. Blood samples, obtained at 10 different time points (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, and 8 h), were collected to analyze the time-dependent concentrations of MDZ and 1'-OH-MDZ concentrations by HPLC. Plasma samples were stored at −20°C until assay.

The plasma extraction procedure was carried out as described previously (Eckhoudt et al., 1998). Midazolam and its metabolites were extracted with 3 ml of cyclohexane/diethyl ether (3:7) after the addition of 10 µl of 2% sodium hydroxide. The organic phase was removed and evaporated to dryness under nitrogen, and the residue was reconstituted in 200 µl of the mobile phase (methanol/10 mM phosphate buffer, pH 7.4/acetonitrile, 35:45:20). Ninety microliters of the mixture was injected for chromatographic analysis.

HPLC Analysis. The HPLC system consisted of a 2690 separation module (Waters, Milford, MA), a 759A absorbance detector (Applied Biosystem, Ramsey, NJ), and an HP3395 integrator (Hewlett Packard, Avondale, PA). A Hypersil ODS column (5 µm particles, 4.6 mm i.d. × 250 mm; Thermo Hypersil Ltd., Cheshire, UK) was used. The separation of MDZ and the metabolites was achieved isocratically using the mobile phase (methanol/10 mM phosphate buffer, pH 7.4/acetonitrile, 35:45:20). The flow rate was 1.0 ml/min and the UV absorbance was monitored at 254 nm. All chromatographic separations were performed at room temperature.

Data Analysis. The AUC (0–8 h) was calculated by the trapezoidal rule. The half-lives of MDZ and 1'-OH-MDZ were determined by linear regression of data determined from samples obtained in the last three or four time points. The extrapolation of AUC was based on the last plasma concentration and the terminal slope (Cpntanh). The ratio of 1'-OH-MDZ/MDZ was also calculated to assess the CYP3A activity. The pharmacokinetic parameters were compared between two groups of subjects by two-tailed T test.

Results

We recruited 42 Chinese volunteers in Taiwan to determine the genotypes of CYP3A5 by PCR-RFLP. Genotype analyses revealed that the CYP3A5*3 allele was detected in 39 of 42 Chinese volunteers. CYP3A5*4 and CYP3A5*5 were found in one volunteer each; CYP3A5*2 and CYP3A5*6 were not found (Table 4). For CYP3A5*3, the homozygous wild-type allele (*1/*1) was 7.1%
(3 of 42), the heterozygous allele (*1/*3) was 40.5% (17 of 42), and the homozygous mutant-type allele (*3/*3) was 52.4% (22 of 42).

To avoid the additive effect of other genotypes, we excluded subjects with genotypes of CYP3A5*4 and CYP4A5*5 to study the significance of CYP3A5*5 in midazolam pharmacokinetics. The average AUC (0–8 h) of MDZ was 9237 ± 1050 ng-min/ml in heterozygous CYP3A5*3 subjects and 3920 ± 402 ng-min/ml in heterozygous CYP3A5*1/*3 subjects (Fig. 1). The two values are not significantly different (t = 0.97, P = 0.34). The average AUC (0–8 h) of 1′OH-MDZ was 3748 ± 427 ng-min/ml in homozygous CYP3A5*3 subjects and 3920 ± 402 ng-min/ml in heterozygous CYP3A5*1/*3 subjects (Fig. 1). The two values are also not significantly different (t = 0.29, P = 0.77). The AUC values were extrapolated and normalized by body weight. The AUC values of MDZ and 1′OH-MDZ in CYP3A5*3 subjects are 235 ± 37.8 and 81.6 ± 10.1 ng-min/ml-kg, respectively; AUC values of MDZ and 1′OH-MDZ in CYP3A5*1/*3 subjects are 215 ± 41.9 and 81.4 ± 13.6 ng-min/ml-kg, respectively. The values are not statistically different (t =...
0.35, \( P = 0.72 \) for MDZ; \( t = 0.01, P = 0.99 \) for 1'OH-MDZ). The ratios of 1'OH-MDZ/MDZ are similar (0.54 ± 0.11 in homozygous CYP3A5*3 subjects and 0.48 ± 0.075 in heterozygous CYP3A5*1*/3 subjects; \( t = 0.22, P = 0.83 \)). The two subjects of homozygous CYP3A5*1 allele (16 and 20) also showed similar pharmacokinetics of MDZ and 1'OH-MDZ (Fig. 1). The results showed that pharmacokinetics of MDZ and 1'OH-MDZ is of no difference in subjects with or without CYP3A5 expression.

### Discussion

CYP3A5 is polymorphically expressed in 10 to 30% of human liver (Aoyama et al., 1989; Wrighton et al., 1990; Schuetz et al., 1994). It becomes clear that CYP3A5*3 is the most important allele responsible for the genetic polymorphism (Kuehl et al., 2001). The splicing variant, CYP3A5*3, forms the premature stop codon at amino acid 102, and therefore loses the enzyme activity.
Sporine is the same in homozygous disposition of cyclosporine. They found that the clearance of cyclosporine is important in the pharmacokinetics of MDZ or 1′-OH-MDZ. Recently, Yates et al. (2002) studied the effect of CYP3A5 and subject no.20 with CYP3A5*1/*1 allele (H11032) are shown. Each point represents the mean ± S.E.M. Subject no.16 with wild-type CYP3A5*1/*1 allele (H11032) and subject no.20 with CYP3A5*1/*1 allele (H11032) are shown.

In this study, the CYP3A5*3 allele with an a22893→g point mutation in the 3′ of the CYP3A5 gene was examined by PCR-RFLP. We conclude that the CYP3A5*3 variant allele is abundantly present in the Japanese population, displaying an allelic frequency of 72.6%. For comparison, Hustert et al. (2001) reported that the CYP3A5*3 allele was detected in 73% of Chinese population, 71% of Japanese, 70% of Korean, and 27% of African American individuals. Fukuen et al. (2002) reported that the CYP3A5*3 allele was detected in 76.8% of Japanese population, 85.2% of Caucasian, and 47.5% of African American.

CYP3A5*4 and CYP3A5*5 (Chou et al., 2001) were found in 1 of 42 volunteers with an allelic frequency of 1.2% each, and CYP3A5*2 (Jounaidi et al., 1996) and CYP3A5*6 (Kuehl et al., 2001) were not found in our study population (Table 4). In repeated experiments, we did not detect any CYP3A5*2 variant in Chinese population (Chou et al., 2001). This is in contrast to the finding that there was 1.9% of CYP3A5*2 in Caucasian (Hustert et al., 2001). With respect to the absence of CYP3A5*6 variant, the results of this study are identical to that in Japanese population (Fukuen et al., 2002). It seems that the CYP3A5*6 allelic frequency is also very low in the Oriental. Presently, the CYP3A5*3 allele and CYP3A5*5 allelic variants have only been found in the Chinese (Chou et al., 2001).

Kuehl et al. (2001) reported that individuals with a CYP3A5*1 allele had higher CYP3A5 protein expression and the metabolic activity than those with homozygous CYP3A5*3 allele as detected by the probe drug MDZ metabolism in vitro. In this study, the average AUC value of MDZ in homozygous CYP3A5*3 subjects was close to the value in heterozygous CYP3A5*1/*3 subjects. The average AUC value of 1′-OH-MDZ was also very similar in the two groups. There were only two homozygous CYP3A5*1 subjects. Their MDZ and 1′-OH-MDZ concentrations were also very similar to those of other subjects (Fig. 1). CYP3A5 expression level does not seem to be important in the pharmacokinetics of MDZ or 1′-OH-MDZ. Recently, Yates et al. (2002) studied the effect of CYP3A5 genotypes in the disposition of cyclosporine. They found that the clearance of cyclosporine is the same in homozygous CYP3A5*3 and heterozygous CYP3A5*1/*3 subjects. The result was in agreement with that in the present study.

The ratio of 1′-OH MDZ/MDZ AUCs was found to correlate with CYP3A activity well (Thummel et al., 1994a). In theory, the ratio reflects the formation clearance divided by the total elimination clearance of 1′-OH-MDZ. We found average values of 0.51 for homozygous CYP3A5*3 subjects and 0.48 for heterozygous CYP3A5*1/*3 subjects. The values are not statistically different. Again, the results indicated that the rates of formation of 1′-OH-MDZ are similar in the two genotypes of subjects.

It is interesting to find that the CYP3A5*3 genotype affects CYP3A5 level but does not affect MDZ pharmacokinetics in vivo. It is difficult to interpret the results. One possibility is that CYP3A5 is not the major enzyme in MDZ metabolism. There may be other enzymes responsible for converting MDZ into 1′-OH-MDZ.

A recent report of tissue donors from Caucasians revealed that CYP3A5 protein was 70.7 pmol/mg in the liver and 3.6 pmol/mg in the jejunum for CYP3A5*1/*3 subjects, whereas CYP3A4 protein was detected in 76.8% of Chinese population, displaying an allelic frequency of 72.6%. For CYP3A5*3 subjects, CYP3A4 protein was detected in 73% of Chinese population, 71% of Japanese, 70% of Korean, and 27% of African American individuals. CYP3A5*2 (Jounaidi et al., 1996) and CYP3A5*6 (Kuehl et al., 2001) were not found in our study population (Table 4). In repeated experiments, we found in our study population (Table 4). In repeated experiments, we found average values of 0.51 for homozygous CYP3A5*3 subjects and 0.48 for heterozygous CYP3A5*1/*3 subjects. The values are not statistically different. Again, the results indicated that the rates of formation of 1′-OH-MDZ are similar in the two genotypes of subjects.

In previous studies, midazolam clearance after intravenous administration correlated better with hepatic CYP3A content (Thummel et al., 1994b) than the apparent oral midazolam clearance (Wandel et al., 1998). There is a possibility that the route of administration affects the phenotype difference in subjects of different genotypes. Lin et al. (2002) also showed that jejunal CYP3A4 up-regulated in homozygous CYP3A5*3 subjects. An intravenous midazolam study may give a larger metabolic activity difference between subjects of different CYP3A5 genotypes. The possibility remains to be elucidated.

The alternative hypothesis is that CYP3A genes are not regulated as expected. There is a possibility that CYP3A5*3 in Chinese subjects may have an increased expression of other functional proteins by unknown mechanism. Finta and Zaphiropoulos (2002) found that the intergenic mRNA molecules resulting from trans-splicing in CYP3A4 cluster. Even though only CYP3A4-CYP3A43-CYP3A5 chimeric protein were detected in very low percentage, CYP3A5*3 premRNA might have an alternative intergenic splicing to create an alternative functional protein in Chinese. The complicated chimeric CYP3A mRNA levels and structures in the liver and the intestine need to be searched in the future to verify the hypothesis.

Furthermore, it is interesting to find that G/A→a polymorphism of CYP3AP1 promoter has strong association with a22893→g polymorphism in intron 3 of CYP3A5 in Chinese (Table 4). Other associated mutations might also exist to cause the compensation of enzymatic activity. Unidentified polymorphism in CYP3A4, CYP3A7, and CYP3A43 may exist in the population. The polymorphic sites may segregate with G/A→a or a22893→g site. The metabolic activity or expression of either members of CYP3A subfamily may have altered in CYP3A5*3 subjects.

MDZ and 1′-OH-MDZ are both pharmacologically active. Even though the mechanism is unknown, the results suggest that CYP3A5 genotypes may not be an important factor in using MDZ. Other than
Several studies have highlighted the importance of CYP3A5 in the metabolism of various drugs. Eeckhoudt SL, Desager JP, Horsmans Y, De Winne AJ, and Verbeeck RK (1998) Sensitive assay for midazolam and cyclosporine, importance of CYP3A5 in the metabolism of other CYP3A substrates needs to be investigated. Although CYP3A5 polymorphism is very interesting and widely observed, its clinical significance remains to be established.

**References**


Finta C and Zaphiropoulos PG (2000) The human cytochrome P450 3A locus. **Gene evolution polymorphism is very interesting and widely observed, its clinical significance remains to be established.**


