Effect of Common CYP3A4 and CYP3A5 Variants on the Pharmacokinetics of the Cytochrome P450 3A Phenotyping Probe Midazolam in Cancer Patients


Abstract

Purpose: To evaluate the effect of naturally occurring variants in genes encoding the cytochrome P450 (CYP) isoforms CYP3A4 and CYP3A5 in patients with cancer receiving midazolam as a phenotyping probe.

Experimental Design: Five variants in CYP3A4 and CYP3A5 were evaluated in 58 patients (21 women and 37 men) receiving a short i.v. bolus of midazolam (dose, 0.0145 or 0.025 mg/kg). Midazolam concentrations in plasma were determined using liquid chromatography-mass spectrometry, and pharmacokinetic variables were calculated using noncompartmental analysis. Genomic DNA was characterized for the variants by PCR-RFLP, and all genotypes were confirmed by direct nucleotide sequencing.

Results: The mean clearance of midazolam was 24.4 ± 9.12 L/h, and phenotypic CYP3A activity varied about 4-fold in this population (range, 10.8-44.3 L/h). There were six carriers of the CYP3A4*1B allele (allele frequency, 0.061). No variant alleles for CYP3A4*17, CYP3A4*18A, or CYP3A5*6 were identified. Forty-eight of the 58 patients were homozygous variant for CYP3A5*3C; eight were heterozygous, and two were homozygous wild type (allele frequency, 0.897). No associations were noted between any of the studied genotypes and the phenotypic measures (P ≥ 0.16). Likewise, a common variant in exon 26 in the gene encoding P-glycoprotein [i.e., ABCB1 (MDR1) 3435C>T] that was previously reported to be linked to CYP3A4 mRNA levels was unrelated to any of the studied phenotypic measures (P ≥ 0.49).

Conclusions: The studied genetic variants in CYP3A4 and CYP3A5 are unlikely to have an important functional significance to phenotypic CYP3A activity in patients with cancer.
contributor to the variability of both the severity of side effects and therapeutic response (8). Furthermore, the metabolism of 37% of all currently approved cytotoxic and/or cytotoxic anticancer agents is known to be mediated, at least in part, by the CYP3A isomers. Hence, identification of factors affecting the clearance of CYP3A substrates could aid in predicting or adapting appropriate, individualized doses of anticancer drugs. Here, we evaluated the effect of common naturally occurring variants in genes encoding CYP3A4 and CYP3A5 in cancer patients receiving midazolam as a phenotyping probe.

Materials and Methods

Patient selection. This study was conducted under two Institutional Review Board–approved protocols and all patients signed an informed consent form before treatment. Patients with a histologically or cytologically confirmed diagnosis of malignant solid tumor were eligible for two clinical trials in which patients underwent CYP3A phenotyping with i.v. given midazolam (9, 10). Eligibility criteria included age >18 years; performance status ≤1; and adequate hematopoietic (leukocytes, ≥4.0 × 10^9/L; neutrophils, ≥1.5 × 10^9/L; and platelets, ≥100 × 10^9/L), hepatic (bilirubin within normal limits; transaminases, ≤2 times the upper limit of normal), and renal function (creatinine clearance, ≥50 mL/min). All patients were required to have an estimated life expectancy of >12 weeks and no previous chemotherapy was allowed for about half of the patients, 1, 2, 3, 4, and 5 hours following bolus i.v. administration of midazolam 3 weeks after the administration of midazolam.

CYP3A Phenotype/Genotype Relations

In about half of CYP3A5*3C allele, an initial PCR was done using the sample DNA and 20 pmol of each primer, 5'-AGGTGTGGCTTGTTGGGATG-3' (CYP3A5*3C R2) in a 50-μL reaction that contained 1× PCR buffer (Perkin-Elmer), 1.5 mmol/L MgCl₂, 2 mmol/L deoxynucleotide triphosphates, and 2 units of Platinum Taq polymerase (Invitrogen). The cycle conditions were: one cycle at 94°C for 5 minutes followed by 20 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds, with a final 7-minute cycle at 72°C. Sequencing was done using the following primers: (CYP3A4*1B F5) 5'-AGGTGTGGCTTGTTGGGATG-3' for the forward strand and (CYP3A4*1B R5) 5'-TCGAAATTCAATGAGGAAGGC-3' for the reverse strand.

For the CYP3A5*3C allele, primers were designed and chosen as follows: 5'-TCTCTCAGAATCCACACCGTG-3' (CYP3A5*3C F1) and 5'-TTATTGTCTGAGAAGAGGAGC-3' (CYP3A5*3C R1). These primers were used in a 20-μL reaction that contained 1× PCR buffer (Perkin-Elmer), 1.5 mmol/L MgCl₂, 2 mmol/L deoxynucleotide triphosphates, and 2 units of Platinum Taq polymerase (Invitrogen). The cycle conditions were: one cycle at 94°C for 5 minutes followed by 20 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds, ending with a final 7-minute cycle at 72°C. After initial amplification, 3 μL of amplified product were removed and an additional PCR amplification (as described above in a total of 50 μL final volume) was done using 40 pmol of primers 5'-AGCAGTGATGATCCACCCG-3' (CYP3A5*3C F2) and 5'-CCAGAGAACACGGTGATTGATG-3' (CYP3A5*3C R2). PCR conditions for this reaction were 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, with a final 7-minute cycle at 72°C. After initial amplification, 3 μL of amplified product were removed and an additional PCR amplification using the above reaction ingredients was done using 40 pmol of the designed primers 5'-TCTCGTCAATGATGGAGGAC-3' and 5'-TCAGAAATTCAATGAGGAAGGC-3' on September 22, 2017. © 2005 American Association for Cancer Research.

Table 1. Demographic characteristics

<table>
<thead>
<tr>
<th>Patient demographics</th>
<th>Mean</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>56.7</td>
<td>58</td>
<td>29</td>
<td>73</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.73</td>
<td>1.74</td>
<td>1.55</td>
<td>1.91</td>
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<tr>
<td>Weight (kg)</td>
<td>80.27</td>
<td>77.85</td>
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<td>137</td>
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<td>BSA (m²)</td>
<td>1.94</td>
<td>1.91</td>
<td>1.46</td>
<td>2.46</td>
</tr>
<tr>
<td>Bilirubin (μg/dl)</td>
<td>0.50</td>
<td>0.47</td>
<td>0.23</td>
<td>1.23</td>
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</table>
was observed in all but one sample (for Concordance in results between RFLP and direct sequence analysis ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). dRhodamine Terminator Cycle Sequencing Ready Reaction kit on an each variant was determined by direct nucleotide sequencing using the TGTGTGAGGGCTCTAGATTGAC-3

V

were 5'-30 seconds, with a final 7-minute cycle at 72°C. The sequencing primers were 5'-TTGGGCGCTACAGCATGGATG-3' (CYP3A5*6 F3) and 5'-TTGTGAGGGCTCTAGATTGAC-3' (CYP3A5*6 R3). The existence of each variant was determined by direct nucleotide sequencing using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). Concordance in results between RFLP and direct sequence analysis was observed in all but one sample (for CYP3A5*5C only); results from sequence analysis were used for correlation with midazolam pharmacokinetic variables.

**ABC1 (MDR1) genotype analysis.** The following primers were designed for the ABC1 3435C>T variant in exon 26: 5'-AGCAACCTTACATCTTACTAC-3' (ABC1 3435C>T F) and 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T R) and nested primers 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T F) and 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T R; Invitrogen). The outside primers and −100 ng of genomic DNA were added to a 15-μL reaction mixture consisting of 1 mmol/L deoxynucleotide triphosphates, 1× PCR buffer, 2.5 mmol/L MgCl2, and 0.6 units of

**Table 2. Summary of midazolam pharmacokinetics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>Sample size</th>
<th>Population (route)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (ng × h/mL)</td>
<td>90.2</td>
<td>80.6</td>
<td>35.8</td>
<td>39.0</td>
<td>204</td>
<td>58</td>
<td>Cancer patients (i.v.)</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>79.2</td>
<td>71.2</td>
<td>31.9</td>
<td>37.5</td>
<td>194</td>
<td>31</td>
<td>Cancer patients (i.v.)</td>
</tr>
<tr>
<td>Vss (L)</td>
<td>78.0</td>
<td>70.1</td>
<td>40.1</td>
<td>24.9</td>
<td>228</td>
<td>22</td>
<td>CYP3A5*1A/*3C volunteers (orally)</td>
</tr>
<tr>
<td>T1/2, z (h)</td>
<td>3.40</td>
<td>2.8</td>
<td>2.37</td>
<td>0.62</td>
<td>17.5</td>
<td>24</td>
<td>Volunteers (i.v.)</td>
</tr>
</tbody>
</table>

Abbreviation: Cmax, dose-normalized peak plasma concentration.

* Data not provided.

† Calculated based on AUC and dose published.

(CYP3A5*6 F2) and 5'-TTGTGAGGGCTCTAGATTGAC-3' (CYP3A5*6 R2). PCR conditions for this reaction were 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 30 seconds, with a final 7-minute cycle at 72°C. The sequencing primers were 5'-TTGGGCGCTACAGCATGGATG-3' (CYP3A5*6 F3) and 5'-TTGTGAGGGCTCTAGATTGAC-3' (CYP3A5*6 R3). The existence of each variant was determined by direct nucleotide sequencing using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). Concordance in results between RFLP and direct sequence analysis was observed in all but one sample (for CYP3A5*5C only); results from sequence analysis were used for correlation with midazolam pharmacokinetic variables.

**ABC1 (MDR1) genotype analysis.** The following primers were designed for the ABC1 3435C>T variant in exon 26: 5'-AGCAACCTTACATCTTACTAC-3' (ABC1 3435C>T F) and 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T R) and nested primers 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T F) and 5'-GGAGAGAAGTGGAGTTTCA-3' (ABC1 3435C>T R; Invitrogen). The outside primers and −100 ng of genomic DNA were added to a 15-μL reaction mixture consisting of 1 mmol/L deoxynucleotide triphosphates, 1× PCR buffer, 2.5 mmol/L MgCl2, and 0.6 units of

**Table 3. Genotype and allele frequencies for the studied variants**

<table>
<thead>
<tr>
<th>Polymorphism*</th>
<th>Nomenclature</th>
<th>Effect†</th>
<th>Genotype frequencies‡</th>
<th>Allele frequencies§</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Wt</td>
<td>Het</td>
</tr>
<tr>
<td>CYP3A4*1A/*3C</td>
<td>CYP3A4*1B</td>
<td>Promoter</td>
<td>51</td>
<td>89.5</td>
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<tr>
<td>CYP3A4 1561T/C</td>
<td>CYP3A4*17</td>
<td>F189S</td>
<td>58</td>
<td>100</td>
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<tr>
<td>CYP3A4 20070T/C</td>
<td>CYP3A4*18A</td>
<td>L293P</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>CYP3A5 6986A/G</td>
<td>CYP3A5*3C</td>
<td>Splicing defect</td>
<td>2</td>
<td>3.45</td>
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<tr>
<td>CYP3A5 14690G/A</td>
<td>CYP3A5*6</td>
<td>Splicing defect</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>ABC1 3435CT</td>
<td>Not available</td>
<td>I1145I</td>
<td>12</td>
<td>20.69</td>
</tr>
</tbody>
</table>

Abbreviations: Wt, homozygous wild-type patient; Het, heterozygous variant patient; Var, homozygous variant patient.

* Number represents position in nucleotide sequence based on gene AF280107 (for CYP3A4*1B, CYP3A4*17, and CYP3A4*18A). There is still no genomic CYP3A5 sequence available corresponding to the CYP3A5*1A allele initially described on the cDNA level and encoding a functional CYP3A5 enzyme. The reference sequence for the CYP3A5*1A allele has been obtained by using the CYP3A5 sequence found in accession no NG_000004.2 (corresponding to the CYP3A5*3A allele) and replacing base 6986 with an A and base 3161 with a C (numbering based on translation start as +1). See http://www.imm.ki.se/CYPalleles/cyp3a5.htm and http://www.imm.ki.se/CYPalleles/cyp3a5f.htm.

† Number represents amino acid codon.

‡ Number represents number of patients with percentage in parenthesis; the difference in total number of patients for CYP3A4*1B is due to the fact that one sample did not show PCR amplification.

§ Hardy-Weinberg notation for allele frequencies (p, frequency for wild-type allele and q, frequency for variant allele).
Amplitaq Gold (Applied Biosystems). For the nested PCR reaction, 2 µL of the first PCR product were used as template for amplification. The thermocycler conditions were an initial cycle at 95°C for 12 minutes followed by 10 cycles 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute, 20 cycles of 89°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, and ending with a final 10-minute cycle at 72°C. The restriction enzyme Mbol (New England Biolabs, Beverly, MA) was used for the determination of the variants. Enzyme (0.5 µL) and 2 µL of enzyme buffer were added to 20 µL of PCR product and incubated at 37°C for 1 hour followed by an incubation at 65°C for 20 minutes. Blue juice (1 µL of 5× solution) was added to 10 µL of restricted PCR product and run on a 2.0% agarose gel with ethidium bromide as a fluorescence detector. A “T” was determined by bands of 408, 263, 158, 103, and 59 bp; “C” was determined by bands of 263, 236, 172, 158, and 59 bp; heterozygous samples contained all bands. The existence of the variant was also determined by direct nucleotide sequencing after a cleanup step with MinElute PCR purification kit (Qiagen) in a total volume of 12 µL, which included 6 µL of DNA at 3 ng per 100 bp of amplified products and 6 µL of either the forward or reverse primer. Sequencing in the forward and reverse direction was done using an ABI 3730XL Sequencer (Applied Biosystems). Concordance in results between RFLP and direct sequence analysis was observed in all but six samples; results from sequence analysis were used for correlation with midazolam pharmacokinetic variables.

**Statistical considerations.** All data are presented as mean values ± SD, unless stated otherwise. Genotype-frequency analysis of Hardy-Weinberg equilibrium was carried out using Clump version 1.9. Linkage disequilibrium was calculated using EMLD (Qi Qing Huang; see http://linkage.rocketefeller.edu/soft/). The linkage between each pair of SNPs was determined in terms of the classic statistic D’. The absolute value for D’ (|D’|) of 1 denotes complete linkage disequilibrium, whereas a value of 0 denotes complete linkage equilibrium.

To relate pharmacokinetic variables with the variant genotypes, the Kruskal-Wallis test, a nonparametric one-way ANOVA, was used for each polymorphism examined. (NCSS v2001; J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT). Although this study was mainly exploratory in intent, a Bonferroni adjustment was used to evaluate the significance of the multiple comparisons. Two-sided Ps < 0.017 (0.05 divided by the three observed variant genotypes) were regarded as statistically significant and Ps < 0.05 were considered a trend. These levels were chosen to reduce the risk of finding purely coincidental associations in view of the number of variant genotypes analyzed concurrently.

**Results**

**Patients.** A total of 58 adult patients (21 women and 37 men) were evaluated during this study. Patients were of Caucasian (n = 55), African American (n = 2), or Hispanic (n = 1) descent and were between 29 and 73 years old (median age, 57 years). Additional patient demographics are shown in Table 1.

**Midazolam disposition.** Complete midazolam pharmacokinetic data following i.v. administration were available in all 58 patients. The mean dose-normalized AUC was 90.2 ± 35.8 ng h/ml, whereas the mean clearance was 24.4 ± 9.12 L/h, which is consistent with earlier findings obtained in cancer patients following i.v. administration of midazolam (15, 16). Table 2 provides a comparison of the clearance values determined in previously conducted trials in cancer patients and healthy volunteers, which involved genotyping for CYP3A5*3C.

**Variant genotypes.** Genotyping results were available for all patients except for CYP3A4*1B, which had available data for 57 of the 58 patients (Table 3). Of these 57 patients, there were six carriers of the CYP3A4*1B allele (five heterozygous CYP3A4*1A/*1B and one homozygous CYP3A4*1B/*1B). Both of the subjects of African American descent were wild type for CYP3A4*1B (homozygous CYP3A4*1A/*1A). No patients were identified carrying variant alleles for CYP3A4*17, CYP3A4*18A, or CYP3A5*6. Forty-eight of the 58 patients were homozygous variants for CYP3A5*3C and there were eight heterozygotes.

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**Fig. 1.** Midazolam clearance as a function of the CYP3A4*1B, CYP3A5*3C, and ABCB1 3435C>T genotypes. Horizontal lines indicate mean values in each group. Abbreviations: Wt, homozygous wild-type patient; Het, heterozygous patient; Var, homozygous variant patient. Two-sided Ps are given above the panels.
(CYP3A5*1A/*3C). The ABCB1 3435C>T genotypes were distributed as follows: 12 wild type (C/C), 35 heterozygous (C/T), and 11 variant (T/T).

ABCB1 3435C>T was in linkage equilibrium with both CYP3A4*1B and CYP3A5*3C (\(D' = 0.183\) and 0.0933, respectively). Linkage disequilibrium was apparent between CYP3A4*1B and CYP3A5*3C (\(D' = 0.656\)), as described previously (17).

**Genotype-phenotype relations.** There were no significant correlations between any of the variant genotypes and clearance, volume of distribution at steady state, or AUC (Fig. 1; Table 4). Although the mean midazolam clearance was 1.2 times higher in patients with the CYP3A5*1A/*1A genotype compared with patients that were homozygous variant (CYP3A5*3C/*3C), this difference was not statistically significant (\(P = 0.50\)). Furthermore, there was no correlation between CYP3A5*3C genotype status and clearance, when patients with the homozygous variant genotype (CYP3A5*3C/*3C) were compared with patients that had at least one wild-type allele (CYP3A5*1A/*3C and CYP3A5*1A/*3C; \(P = 0.24\)).

**Comparison of predicted and observed area under the plasma concentration-time curve.** The AUC of midazolam was also calculated with a limited-sampling model based on the 4-hour sampling time point (4-h conc.) using the limited-sampling model equation: AUC = 9.91 + 12.2 × [4-h conc.], as described previously (12). The dotted line indicates a linear regression fit, whereas the solid line represents the line of identity.

It has been proposed that genotyping for CYP3A4 and CYP3A5 variants may be useful for prediction of total CYP3A activity because of the genetic diversity in the genes encoding these proteins (2, 3). Over 30 SNPs in CYP3A4 have been published; however, most are unlikely to contribute substantially to the interindividual variability of CYP3A4 activity in vivo, based on their limited functional significance and/or low allele frequency (3, 19, 20). Similar to earlier findings (21), the very low allele frequency of the functional CYP3A4*17 and CYP3A4*18A variants evaluated in the present study suggests that these SNPs most likely have no broad relevance to CYP3A4 activity and function in predominantly Caucasian populations. The one additional SNP that was studied here, CYP3A4*14B, is a promoter variant in the so-called nifedipine-specific element. The allele frequency of this variant is known to depend on racial ancestry, being absent in Japanese and Chinese subjects, and present in 2% to 10% in Caucasians and 35% to 67% in African Americans, respectively (3). The currently observed frequency of 6.1% is consistent with previous estimates for predominantly Caucasian populations. Although the functional significance of this SNP presently remains unclear, the preponderance of the evidence suggests that CYP3A4*1B does not affect the metabolism and clearance of CYP3A4 substrate drugs (22–26). Likewise, in the current study, the pharmacokinetic profile of midazolam was not significantly different between patients with or without the CYP3A4*1B variant allele.

**Discussion**

Interindividual variability in drug efficacy and toxicity, resulting in unpredictable patient responses, is commonly observed in all therapeutic areas (18). However, these differences are particularly important in the field of cancer therapy because many anticancer agents have a narrow therapeutic index. It is now well recognized that in addition to environmental and physiologic factors, such interpatient variation is commonly associated with polymorphisms in genes encoding drug-metabolizing enzymes, drug transporters, and/or drug targets. In this study, we tested the hypothesis that the disposition of the prototypical CYP3A substrate drug midazolam in cancer patients is dependent on genetic variability in the CYP3A4 and CYP3A5 genes.

**Table 4. Genotype-phenotype relationships**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>CL (L/h)</th>
<th></th>
<th></th>
<th>(P)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt</td>
<td>Het</td>
<td>Var</td>
<td></td>
<td>Wt</td>
</tr>
<tr>
<td>CYP3A4*1B</td>
<td>22.5(10.8-44.3,51)</td>
<td>32.1(22.4-42.2,5)</td>
<td>22.0(1)</td>
<td>0.16</td>
<td>77.6(24.9-228,51)</td>
</tr>
<tr>
<td>CYP3A5*3C</td>
<td>28.5(16.8-40.2,2)</td>
<td>26.4(16.5-42.2,8)</td>
<td>22.3(10.8-44.3,48)</td>
<td>0.50</td>
<td>81.0(42.5-119,2)</td>
</tr>
<tr>
<td>ABCB1 3435C&gt;T</td>
<td>22.8(10.8-43.3,12)</td>
<td>23.6(11.4-44.3,35)</td>
<td>18.3(11.4-40.8,11)</td>
<td>0.56</td>
<td>88.8(32.8-130,12)</td>
</tr>
</tbody>
</table>

NOTE: Numbers represent median (range, number of subjects with each genotype) or two-sided \(P\) values. Abbreviations: Wt, homozygous wild-type patient; Het, heterozygous variant type patient; Var, homozygous variant type patient.
As mentioned previously and in contrast to CYP3A4, the CYP3A5 protein isoform is known to be expressed in only a small percentage of Caucasian individuals and this has been linked to a common transition in intron 3 of the CYP3A5 gene (CYP3A5*3C), which introduces a frameshift during translation and results in a truncated, nonfunctional protein (4, 5). Approximately 70% to 90% of Caucasian subjects are homozygous variant for CYP3A5*3C and thus are deficient in functionally active CYP3A5 (27), which is consistent with the currently observed genotype frequency of 89.7%. In the present study, no change in midazolam pharmacokinetic variables was noted between 10 patients with at least one wild-type (CYP3A5*1A) allele and 48 patients carrying two variant (CYP3A5*3C) alleles. This is similar to findings obtained in healthy subjects using midazolam (24, 25, 28, 29), or various other CYP3A phenotyping probes, including erythromycin (23, 25) and nifedipine (30). Interestingly, in a small cohort of Australian cancer patients, Wong et al. recently observed ~ 1.4-fold higher systemic clearance of midazolam in four patients with the CYP3A5*1A/*3C genotype compared with 39 patients with the CYP3A5*3C/*3C genotype (P = 0.01; ref. 16). In an effort to explain this discrepant finding, it is noteworthy that the interindividual variability in CYP3A phenotypic activity was substantially greater in the study reported by Wong et al. than that found in the various other studies [8-fold for systemic clearance and 24-fold for apparent oral clearance (16) versus 3.3- to 5.2-fold (24, 25, 28, 29)], suggesting the possibility of altered expression of either CYP3A4, CYP3A5, or both as a result of differences in advancement of the disease state of patients in the studied cohort (31).

Wong et al. estimated the systemic midazolam clearance from a previously developed limited-sampling model based on the collection of one plasma sample obtained 4 hours after drug administration (16). Although a retrospective validation of this model on our own data set indicated a good correlation between the predicted AUC and the observed AUC (R² = 0.749), the model had a statistically significant tendency for systematic negative error and showed poor precision. To illustrate this problem, two different patients in our cohort that had very similar observed AUC values of 117.0 and 117.2 ng h/mL, respectively, had a predicted AUC based on the previously developed limited-sampling model of 37.3 and 165 ng h/mL, respectively. This raises the possibility that some of the proposed genotype-phenotype associations described previously that were based on variable estimates from a limited-sampling model might be particularly prone to the risk of false-positive error due to inaccurate phenotypic assessments. In addition, some error may also be attributed to the smaller sample size used in the previous study (16). Collectively, current evidence does not suggest that CYP3A5*3C-associated differences in the extent of drug metabolism of CYP3A substrates are clinically important.

The extent to which the CYP3A isoforms metabolize midazolam is likely to be determined, at least in part, by intracellular drug concentrations in the liver and, to a lesser extent, the intestine. This process, in turn, is partially dependent on the ATP-binding cassette transporters like ABCB1 (P-glycoprotein) that are localized in the apical membrane of hepatocytes and enterocytes. Although it has been suggested that midazolam is not a substrate for ABCB1 (32), recent data have indicated that midazolam exhibits characteristics of a highly permeable ABCB1 substrate (33). The clearance of midazolam has also been shown to be correlated (r = 0.6; P = 0.0005) with the clearance of docetaxel (15), a known dual substrate of CYP3A and ABCB1 (34), suggesting that varying expression of ABCB1 may alter midazolam clearance. In recent years, various genetic variants in the ABCB1 gene have been described that may effect transporter expression or function (35). The most extensively studied ABCB1 variant to date is a common synonymous C-to-T transition at nucleotide position 3435 at a wobble position in exon 26 (36). Although this transition does not change its encoded amino acid, recent findings have indicated that this variant is associated with altered protein expression in different human tissues (37), and this may result in decreased hepatobiliary and/or intestinal secretion of substrate drugs. Furthermore, a reduced expression of intestinal CYP3A4 mRNA has been observed in subjects carrying the homozygous variant genotype of the ABCB1 3435C>T polymorphism in a Japanese population (38), further pointing to the possibility of ABCB1 genotype affecting midazolam clearance. However, in this study, midazolam pharmacokinetic variables were not significantly influenced by the ABCB1 3435C>T genotype, which is consistent with prior observations in healthy volunteers (25, 39). It should be noted that the lack of relationships with this SNP is, as expected, consistent with preclinical observations in Abcb1a-deficient mice that metabolism rather than transport is the prominent elimination pathway for midazolam (40). This further suggests that the involvement of ABCB1 in the disposition of midazolam may be relatively unimportant regardless of ABCB1 genotype status.

The lack of statistically significant relations in this study does not necessarily mean that there are none, especially in light of the few individuals studied in our population with a homozygous variant genotype. However, the variant genotype effects are minor and/or the genotype heterogeneity is

### Table 4. Genotype-phenotype relationships (Cont’d)

<table>
<thead>
<tr>
<th>Het</th>
<th>Var</th>
<th>P</th>
<th>Wt</th>
<th>AUC (ng h/mL)</th>
<th>Var</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.9 (36.8-189, 5)</td>
<td>69.9 (1)</td>
<td>0.97</td>
<td>80.7 (39.0-204, 51)</td>
<td>69.0 (49.5-108, 5)</td>
<td>99.9 (1)</td>
<td>0.46</td>
</tr>
<tr>
<td>45.6 (24.9-189, 8)</td>
<td>73.8 (32.8-228, 48)</td>
<td>0.77</td>
<td>86.4 (60.0-113, 2)</td>
<td>73.2 (49.5-117, 8)</td>
<td>86.8 (39.0-204, 48)</td>
<td>0.56</td>
</tr>
<tr>
<td>69.9 (31.9-228, 35)</td>
<td>69.7 (24.9-145, 11)</td>
<td>0.87</td>
<td>77.2 (47.8-204, 12)</td>
<td>89.0 (39.0-168, 35)</td>
<td>79.3 (53.4-166, 11)</td>
<td>0.85</td>
</tr>
</tbody>
</table>
sufficiently small to conclude that the presently studied variants in the CYP3A4 and CYP3A5 genes do not cause a substantial interindividual difference in midazolam clearance and thus are unlikely to have an important functional significance in cancer patients treated with CYP3A substrate drugs. For example, even in a population of this size, if CYP3A5 genotype accounted for the large degree of variability in midazolam pharmacokinetics, detection of this effect would have been expected.

There may be a number of other factors contributing to the variability in midazolam pharmacokinetics, because the tested genetic variants do not seem to explain this finding. A recent study has shown an inverse correlation between liver dysfunction, particularly total bilirubin levels, and CYP3A activity (23). Although all participants in the current study had total bilirubin levels within 1.5 times the upper limit of normal, there was still a considerable range observed (Table 1). Inflammatory response in patients with cancer, resulting in increased α-1 acid glycoprotein serum concentrations, has also been suggested to influence CYP3A activity. Furthermore, many drugs and herbal medications are known to interact with CYP3A. Although patients were asked to abstain from any drugs known to affect the function and/or expression of either CYP3A or ABCB1, this possibility cannot be entirely excluded. Finally, it is possible that additional genes, genetic variants and/or haplotypes of importance to the pharmacokinetics of CYP3A substrate drugs like midazolam may yet be discovered and/or evaluated. Further investigation is needed to determine the relative role of genetic variation and environmental variables (e.g., concomitant drugs or herbs) on the pharmacokinetics of anticancer drugs that are substrates of CYP3A4 and CYP3A5.

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