CYP3A5 Gene Variation Influences Cyclosporine A Metabolite Formation and Renal Cyclosporine Disposition

Songmao Zheng,1 Yasar Tasnif,2 Mary F. Hebert,2 Connie L. Davis,3 Yoshihisa Shitara,1 Justina C. Calamia,1 Yvonne S. Lin,1 Danny D. Shen,1,2 and Kenneth E. Thummel1,4

Background. Higher concentrations of AM19 and AM1c9, secondary metabolites of cyclosporine A (CsA), have been associated with nephrotoxicity in organ transplant patients. The risk of renal toxicity may depend on the accumulation of CsA and its metabolites in the renal tissue. We evaluated the hypothesis that CYP3A5 genotype, and inferred enzyme expression, affects systemic CsA metabolite exposure and intrarenal CsA accumulation.

Methods. An oral dose of CsA was administered to 24 healthy volunteers who were selected based on their CYP3A5 genotype. CsA and its six main metabolites in whole blood and urine were measured by liquid chromatography-mass spectrometry. In vitro incubations of CsA, AM1, AM9, and AM1c with recombinant CYP3A4 and CYP3A5 were performed to evaluate the formation pathways of AM19 and AM1c9.

Results. The mean CsA oral clearance was similar between CYP3A5 expressors and nonexpressors. However, compared with CYP3A5 nonexpressors, the average blood area under the concentration-time curve (AUC) for AM19 and AM1c9 was 47.4% and 51.3% higher in CYP3A5 expressors (P<0.040 and 0.011, respectively), corresponding to 30% higher \( \frac{\text{AUC}_{\text{metabolite}}}{\text{AUC}_{\text{CsA}}} \) ratios for AM19 and AM1c9 in CYP3A5 expressors. The mean apparent urinary CsA clearance based on a 48-hr collection was 20.4% lower in CYP3A5 expressors compared with CYP3A5 nonexpressors (4.2±1.0 and 5.3±1.3 mL/min, respectively; P=0.037), which is suggestive of CYP3A5-dependent intrarenal CsA metabolism.

Conclusions. At steady state, intrarenal accumulation of CsA and its secondary metabolites should depend on the CYP3A5 genotype of the liver and kidneys. This may contribute to interpatient variability in the risk of CsA-induced nephrotoxicity.

Keywords: Cyclosporine A, CYP3A5 genotype, Secondary metabolites, Chronic calcineurin inhibitor nephrotoxicity, Intrarenal metabolism.

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allele (inferred CYP3A5 expressor phenotype in individuals heterozygous or homozygous for CYP3A5*1) from the “inactive” CYP3A5*3, *6, or *7 allele (inferred CYP3A5 nonexpressor phenotype) (8–12). The CYP3A5 polymorphism contributes to interindividual differences in the metabolic clearance of a number of drugs, including CsA. However, in the case of CsA, the in vitro intrinsic metabolic clearance calculated from total metabolite formation is approximately 2.3-fold higher for CYP3A4 than for CYP3A5 (13). Thus, CYP3A4 plays a more dominant role than CYP3A5 in the metabolism of CsA and the influence of the CYP3A5 polymorphism on the bioavailability and total systemic clearance of CsA is limited (14).

Although the contribution of CYP3A5 to CsA oral clearance is modest, it might contribute more significantly to interindividually variation in CsA metabolite tissue exposure because of marked differences between the product selectivity of CYP3A4 and CYP3A5. The primary CsA metabolites AM1, AM9, and AM4N and several secondary and tertiary metabolites AM1c, AM19, and AM1c9 can be detected in the blood and urine (15). CYP3A4 catalyzes the formation of all three primary metabolites, whereas only AM9 is produced to a significant degree by CYP3A5 (13). Moreover, human liver microsomes from CYP3A5 expressors exhibit higher AM9 formation rates than liver microsomes from CYP3A5 nonexpressors (13). In the kidney, because CYP3A5, and not CYP3A4, is expressed in the tubular epithelium, the rate of AM9, AM19, and AM1c9 formation by human kidney microsomes is strongly associated with detection of CYP3A5 protein and presence of the CYP3A5*1 allele (13). Thus, interindividual variability in the systemic blood and renal concentration of CsA metabolites might be explained in part by differences in the expression and function of CYP3A5 in the major organs of drug elimination (16).

High blood and urinary concentrations of AM19 and AM1c9 have been associated with renal dysfunction in CsA-treated patients (17–19), although the causality has not been shown. It is unclear if greater than average metabolite exposure is the cause or the result of impaired kidney function. The primary and secondary metabolites of CsA are equivalent or less toxic than CsA in cultured renal epithelial cells (20, 21). In contrast, AM19 and AM1c9 (but not CsA or its primary metabolites) have been shown to alter renal mesangial cell function by increasing endothelin release (22). Accordingly, the presence of CYP3A5 in the small intestine, liver, and kidney may affect systemic and intrarenal concentrations of CsA and its putative nephrotoxic metabolites during drug therapy and, by inference, the risk of CNIT. To test this hypothesis, we measured and compared the concentrations of key CsA primary and secondary metabolites in blood and urine excretion among CYP3A5 expressors and nonexpressors. In addition, we evaluated the impact of CYP3A5 genotype on intrarenal CsA metabolism in vivo using the apparent urinary CsA clearance as a surrogate marker of intrarenal drug clearance.

**RESULTS**

**Demographic Characteristics of Healthy Volunteers**

The demographic characteristics of 24 healthy volunteers who participated in this study are shown in Table S1
higher in CYP3A5 expressors compared with nonexpressors (P = 0.040 and 0.011, respectively). In accordance, the AUC_{metabolite}/AUC_{CsA} ratio for AM19 and AM1c9 was 33.1% and 30.7% higher in CYP3A5 expressors compared with nonexpressors (P = 0.016 and 0.025), respectively (Table 2, Fig. 2C). Similarly, the AUC_{AM19}/AUC_{AM1} (Fig. 2D) and AUC_{AM1c9}/AUC_{AM1c} (not shown) ratios were 46.9% and 30.6% higher in CYP3A5 expressors compared with nonexpressors (P = 0.002 and 0.025), respectively.

**Renal Excretion of Cyclosporine A and Its Primary Metabolites**

The total amount of intact CsA excreted in urine over 48 hr after oral administration was comparable between CYP3A5 expressors and nonexpressors (1445.9±495.5 and 1677.0±450.2 ng, respectively). However, the mean apparent urinary CsA clearance based on the 48-hr collection was 20.4% lower in CYP3A5 expressors compared with CYP3A5 nonexpressors (4.2±1.0 and 5.3±1.3 mL/min, respectively; P = 0.037; Fig. 3A). Similarly, the eGFR-normalized apparent urinary CsA clearance based on the 48-hr collection was 28.5% lower in CYP3A5 expressors compared with CYP3A5 nonexpressors (0.03±0.01 and 0.05±0.02, respectively; P = 0.035; Fig. 3B). Although the interindividual variability was large, CYP3A5 expressors exhibited increased intrarenal CsA metabolism compared with nonexpressors as demonstrated by increased urinary CsA clearances over discrete urine collection time intervals (Fig. 3C).

The average cumulative amount of AM19 and AM1c9 excreted in urine was 48% and 50% higher in CYP3A5 expressors compared with nonexpressors (P = 0.077 and 0.069, respectively). This is in agreement with greater blood exposure for AM19 and AM1c9 in CYP3A5 expressors compared with nonexpressors. For the other CsA metabolites, the average amount excreted in urine in the two predicted phenotype groups was comparable. Interestingly, there was no CYP3A5-dependent difference in the apparent urinary clearance (amount excreted/AUC_{blood}) for all of the primary and secondary CsA metabolites measured.

**Formation of AM19 and AM1c9 by CYP3A4 and CYP3A5 In Vitro**

At a substrate concentration of 1 μM, CYP3A5 Supersomes converted AM1 to AM19 at a rate similar to that of CYP3A4 Supersomes (23.9±5.3 vs. 28.5±4.7 pmol/min/nmol, respectively).

AM9 was converted to AM19 much more efficiently by CYP3A4 (11.3±1.2 pmol/min/nmol) than by CYP3A5 (1.1±0.3 pmol/min/nmol). The formation of AM1c9 from AM1c by CYP3A4 and CYP3A5 was also comparable (20.5±5.5 vs. 13.0±0.1 pmol/min/nmol, respectively). Similar results were found when 200 nM AM1, AM9, and AM1c were incubated with CYP3A4 and CYP3A5 Supersomes for a shorter incubation of 30 min (data not shown).

**DISCUSSION**

Understanding the basis of interindividual differences in CsA clearance is an important step toward the goal of improving the safety and efficacy of immunotherapy. In the current study, we evaluated how CYP3A5 genetic variation (and the predicted enzyme expression phenotype) affected systemic and intrarenal CsA metabolism and exposure to its metabolites in blood.

Results showed that the mean oral CsA clearance for CYP3A5 expressors and nonexpressors was similar. This is in general agreement with some previous findings (23–26) but not with others (27, 28). The interindividual variability of CsA oral clearance was approximately 30% for both genotype groups. Thus, the interindividual variability of the CYP3A4 content may well have masked any effect of CYP3A5 expression. Because the AM9 pathway is only one of three primary CsA elimination routes and because CYP3A5 exhibits selective formation of only AM9 at an efficiency that is less than that of CYP3A4 (13), one would expect the total metabolic clearance to the primary metabolites to be influenced only

### TABLE 2. AUC_{0–∞} and AUC_{metabolite}/AUC_{CsA(0–∞)} of CsA and its metabolites for study participants stratified by predicted CYP3A5 phenotype

<table>
<thead>
<tr>
<th>CYP3A5 expressors</th>
<th>CYP3A5 nonexpressors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0–∞}</td>
<td>CsA</td>
<td>6098±1509</td>
</tr>
<tr>
<td>AM1</td>
<td>4711±1509</td>
<td>4900±2188</td>
</tr>
<tr>
<td>AM9</td>
<td>3186±766</td>
<td>2801±712</td>
</tr>
<tr>
<td>AM4N</td>
<td>418±118</td>
<td>456±94</td>
</tr>
<tr>
<td>AM1c</td>
<td>197±94</td>
<td>185±79</td>
</tr>
<tr>
<td>AM19</td>
<td>1360±602</td>
<td>923±343</td>
</tr>
<tr>
<td>AM1c9</td>
<td>162±62</td>
<td>107±29</td>
</tr>
<tr>
<td>AUC_{conjugate}/AUC_{CsA}</td>
<td>AM1</td>
<td>0.76±0.12</td>
</tr>
<tr>
<td>AM9</td>
<td>0.52±0.06</td>
<td>0.49±0.08</td>
</tr>
<tr>
<td>AM4N</td>
<td>0.07±0.02</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>AM1c</td>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>AM19</td>
<td>0.21±0.05</td>
<td>0.16±0.04</td>
</tr>
<tr>
<td>AM1c9</td>
<td>0.03±0.01</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. AUC, area under the concentration–time curve (ng hr/mL); CsA, cyclosporine A.
FIGURE 2. A, blood concentration-time profiles of AM19 and AM1c9 in CYP3A5 nonexpressors (n=12) and CYP3A5 expressors (n=12). B, blood concentration-time profiles of AM19 and AM1c9 displayed using a logarithmic Y-axis. Bars, SD. AUC ratios for (C) AUC_{AM19}/AUC_{CsA} and (D) AUC_{AM19}/AUC_{AM1} by predicted CYP3A5 phenotype. Solid line, mean ratios. *P<0.05; **P<0.005. AUC, area under the concentration-time curve.

modestly by the CYP3A5 genotype. In support of this prediction, both the AUCs and the AUC_{metabolite}/AUC_{CsA} ratios for AM1, AM9, AM4N, and AM1c were similar for the two CYP3A5 phenotype groups.

In contrast to what was seen for the primary CsA metabolites, the AUCs for both AM19 and AM1c9 were significantly higher in CYP3A5 expressors compared with nonexpressors. In addition, there were greater amounts of AM19 and AM1c9 excreted in the urine of CYP3A5 expressors compared with nonexpressors. Based on in vitro product formation rates and in vivo metabolite/parent AUC ratios, the predominant source of AM19 and AM1c9 appears to be through conversion of AM1 and AM1c to the secondary metabolites, reactions that can be catalyzed efficiently by both CYP3A4 and CYP3A5.

The above findings suggest that, at steady state, when CsA dose is adjusted to achieve a narrow therapeutic blood concentration range, there will be greater accumulation of AM19 and AM1c9 in the systemic blood of CYP3A5 expressors compared with nonexpressors. It has been previously suggested that the production and accumulation of the AM19 and AM1c9 secondary metabolites of CsA might contribute to drug-induced nephrotoxicity (17–19, 22). For example, Vollenbroeker et al. (17) reported that AM19 and AM1c9 were the only CsA metabolites to show a positive correlation with the concentration of C-reactive protein and interleukin-6 (biomarkers of organ inflammation) measured in 202 blood specimens from kidney transplant recipients. Christians et al. (18) found an inverse correlation between the steady-state blood concentration of AM1c9 and renal function in liver transplant patients during the early postoperative period. Likewise, Kempkes-Koch et al. (19) found elevated urine AM19 levels in patients with histologically confirmed CsA nephrotoxicity late after renal transplantation. Elevated secondary metabolites of CsA in patients with impaired renal function could be the result rather than the cause of CsA nephrotoxicity. Alternatively, individual variability in the formation and accumulation of secondary CsA metabolites in blood could contribute directly to differences in renal toxicity risk. With this in mind, formation of AM1c9 and AM19 may represent a toxification pathway.

Higher systemic levels of AM19 and AM1c9 in CYP3A5 expressors should enhance entry of these metabolites into the renal tubular cells either by secretion from the efferent arteriole or after reabsorption from the luminal side following glomerular filtration. This in turn could influence nephrotoxicity risk. Results from combination therapy with ketoconazole and CsA support this hypothesis. In a prospective, randomized study, when systemic levels of CsA were maintained at a similar level compared with the control arms, renal function was significantly better in the ketoconazole cotreatment group compared with CsA treatment alone (29). Interestingly, in human liver microsomal incubations with CsA, ketoconazole inhibited the formation of secondary metabolites more than the formation of primary CsA metabolites (30), further suggesting that the secondary metabolites of CsA are contributory to CsA nephrotoxicity.

The relationship between CYP3A5 genotype and CsA nephrotoxicity has been studied by several research groups. Some investigators report a significant inverse association between CYP3A5 expression and renal function, as measured by serum creatinine or eGFR or clinically evident CsA-related nephrotoxicity (31–33), whereas others found a positive association (34, 35). The impact of CYP3A5 expression on CsA nephrotoxicity is likely complicated by the dual role of CYP3A5 in CsA clearance within the kidneys and in the systemic formation of active secondary metabolites. Moreover, in studies of kidney transplant recipients, the relationship between genotype and nephrotoxicity is complicated by the fact that the phenotype of the donor kidney may differ from the recipient's intestinal and hepatic phenotype (36). The kidney transplant recipient's CYP3A5 genotype and hepatic and intestinal CYP3A5 activity should determine the levels of CsA and its metabolites to which the transplanted kidney is exposed. At the same time, the donor's renal CYP3A5 status would influence the amount of CsA and its metabolites formed locally in the renal tubular cells.

Results from the current study suggest that carriers of the CYP3A5*1 allele, and an inferred high CYP3A5 renal genotype and hepatic ABCC1 allele, and an inferred high CYP3A5 renal genotype and hepatic ABCC1 allele. Such a relationship between renal metabolism and the apparent urinary clearance of unchanged drug was first reported by Siriranni and Pang (37), who showed that the urinary clearance of enalapril was increased due to inhibition of its esterolysis by paraxxon in isolated perfused rat kidneys. In our study, the mean apparent urinary CsA clearance was 20.4% lower in CYP3A5 expressors compared with CYP3A5 nonexpressors, consistent with significant intrarenal CYP3A5-dependent CsA metabolism, presumably through AM9 formation (13). A semiphysiologic model was developed to evaluate the effect of CYP3A5 polymorphism on intrarenal metabolism and tubulointerstitial exposure to tacrolimus, another calcineurin inhibitor (38). In that case, the model fitting results supported the conclusion that reduced urinary tacrolimus clearance is due to increased intrarenal metabolism and decreased renal exposure to tacrolimus in metabolically competent cells, the tubular epithelia.

In individuals with significant renal CYP3A5 expression, one might expect higher intrarenal accumulation of AM19 and AM1c9, independent of an effect of intestinal and hepatic CYP3A5 genotype on systemic accumulation of the secondary metabolites. Such a difference might affect the risk of renal toxicity. However, the effect from a higher level of putatively nephrotoxic secondary metabolites might be counteracted by lower intrarenal levels of CsA. In addition, it is also important to consider the role of renal P-glycoprotein, which can transport CsA and in the renal tubular epithelium would act to reduce intracellular concentrations by active efflux activity. Polymorphisms in the ABCB1 gene, which putatively affect enzyme expression (14), have been associated with the risk of renal toxicity from CsA therapy (36, 39). High P-glycoprotein activity may independently influence intrarenal exposure to AM19 and AM1c9, if these metabolites are also substrates for active tubular efflux. This study was not designed to test the effect of ABCB1 gene variation on renal CsA clearance (would require a much larger number of subjects); however, we did conduct genotyping for the transporter and found that, as expected, there were no significant differences in key genotype or haplotype frequencies between CYP3A5 expressor and nonexpressor groups (see Table S1,
SDC, http://links.lww.com/TP/A768). Thus, the CYP3A5 expressor association that was observed should not have been influenced by the ABCB1 genotype status.

In summary, we found that individuals expressing CYP3A5 exhibited enhanced formation of AM19 and AM1c9, secondary metabolites of CsA that have been associated with an increased risk of CsA-induced nephrotoxicity. Moreover, the same phenotype influenced the apparent urinary clearance of CsA, suggesting the presence of significant intrarenal CsA metabolism for individuals that carry the functional CYP3A5*1 allele. These findings point toward the need for careful evaluation of the impact of both recipient and donor CYP3A5 genotypes on renal function in organ transplant patients receiving chronic CsA immunotherapy.

MATERIALS AND METHODS

Clinical Protocol

This protocol was approved by the University of Washington institutional review board. All study participants provided written informed consent and were selected based on their CYP3A5 genotype. Subjects (n=24) received a single oral dose of CsA (NEORAL Soft Gelatin Capsules, 5 mg/kg; Novartis, Basel, Switzerland). None of the subjects had a significant medical history or abnormal clinical laboratory test results, and none had taken a known inhibitor, inducer, or activator of CYP3A4/5 (other than oral contraceptives) for at least 1 month preceding the start of and during the pharmacokinetic investigation, and all abstained from grapefruit products and alcohol 1 week before the start until the end of the study. Sequential blood samples (5 mL) were collected in EDTA tubes predose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 22, 24, and 48 hr after oral drug administration of the CsA dose. Urine was collected in silanized glass containers over the following postdose intervals: 0–2, 2–4, 4–6, 6–12, 12–24, and 24–48 hr. All samples were stored at −80°C until analysis.

Genotyping

Buccal cell DNA was isolated using a DNeasy Blood & Tissue Kit or the Qiagen Gentra Puregene protocol (Qiagen, Valencia, CA). Single-nucleotide polymorphisms in the CYP3A5 gene (*5, *6, and *7 alleles; rs776746, rs10264272, and rs1303344, respectively) and the ABCB1 gene (C3435T, C1236T, and G2677T/A) were determined from a buccal swab tissue sample using previously published methods (9, 40) or a validated Taqman allelic discrimination assay from Applied Biosystems (Foster City, CA) (41).

Pharmacokinetic Analysis

Noncompartmental pharmacokinetic analysis was performed using WinNonLin version 5.2 (Pharsight, Mountain View, CA). Pharmacokinetic parameters were determined for CsA and metabolites. CL\textsubscript{unary} was calculated as the amount of drug or metabolite excreted in urine divided by AUCl\textsubscript{urine} for the drug or metabolite over the collection interval.

In Vitro Kinetic Protocol

To quantify rates of formation of secondary metabolites of CsA, the primary metabolites AM1, AM9, and AM1c (1 µM) were incubated in duplicate with CYP3A4 and CYP3A5 Supersomes (1000 pmol/mL coexpressed with cytochrome b\textsubscript{5}). The reactions were initiated by addition of NADPH or buffer after a 5-min preincubation period and were terminated after 1 hr. Metabolites were extracted and quantified as described (see Materials and Methods, SDC, http://links.lww.com/TP/A768).

Statistical Analysis

Descriptive statistics are presented as mean±SD. Normality of the data was confirmed before statistical analysis. Statistical comparisons were conducted using an unpaired two-sided Student’s t test by GraphPad Prism 5 (La Jolla, CA). P<0.05 was considered significant.

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REFERENCES


