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INFLUENCE OF NONSYNONYMOUS POLYMORPHISMS OF UGT1A8 AND UGT2B7 METABOLIZING ENZYMES ON THE FORMATION OF PHENOLIC AND ACYL GLUCURONIDES OF MYCOPHENOLIC ACID.

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Abbreviations used are: MPA, mycophenolic acid; MMF, mycophenolate mofetil; MPAG, mycophenolic acid phenolic glucuronide; AcMPAG, mycophenolic acid acyl glucuronide; UGT; UDP-glucuronosyltransferase, UDPGA; UDP-glucuronic acid, LC/MS; high performance liquid chromatography coupled with mass spectrometry.

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ABSTRACT

Mycophenolic acid (MPA) is the active metabolite of mycophenolate mofetil (MMF), a standard immunosuppressive drug approved for clinical use in the prevention of acute allograft rejection following organ transplantation. This study examines the role of the genetic variants of UDP-glucuronosyltransferase (UGT) 1A8 and 2B7 enzymes involved in the formation of the primary metabolite of MPA, the inactive phenolic glucuronide (MPAG), and the reactive acyl glucuronide (AcMPAG). The first exon of *UGT1A8* was first resequenced in the region encoding for the substrate binding domain in 254 Caucasians and 41 African-Americans. Eight nonsynonymous changes were observed and led to the following amino acid substitutions : $S^{43}L$, $H^{53}N$, $S^{126}G$, $A^{144}V$, $A^{173}G$, $A^{231}T$, $T^{240}A$ and $C^{277}Y$. Thirteen haplotypes were inferred, comprising only two previously described alleles, namely UGT1A8*2 ($A^{173}G$) and UGT1A8*3 ($C^{277}Y$). Upon stable expression in HEK-293 cells, the UGT1A8*3 ($C^{277}Y$), *5 ($G^{173}A^{240}$), *7 ($A^{231}T$), *8 ($S^{43}L$) and *9 ($N^{53}G$) proteins were associated with the most profound decreases in the formation of MPAG and AcMPAG, indicating that these amino acids are critical for substrate binding and enzyme function. Altogether the low activity UGT1A8 enzymes are carried by 2.8-4.8% of the population. The variant of the UGT2B7 protein (UGT2B7*2 Y^{268}), the main enzyme involved in the formation of AcMPAG, demonstrated a catalytic efficiency comparable to that of UGT2B7*1 (H^{268}). In conclusion, while the common UGT2B7*2 variant is predicted to have limited impact, several UGT1A8 variants identified may potentially account for the large inter-individual variance in MMF pharmacokinetics and deserve further clinical investigations.

INTRODUCTION

Mycophenolate mofetil (MMF, Cellcept), an immunosuppressive drug, is approved for clinical use in the prevention of acute allograft rejection following organ transplantation, as well as haematopoietic stem cell transplantation (Sollinger, 1995; Bullingham et al., 1998; Cohn et al., 1999). Mycophenolic acid (MPA), its active metabolite, is a selective inhibitor of inosine monophosphate dehydrogenase (IMPDH). The metabolism of MPA involves mainly its conjugation by UDP-glucuronosyltransferase (UGT) enzymes, yielding two glucuronide conjugates, namely the major derivative MPAG and the minor metabolite AcMPAG (Bullingham et al., 1998; Shipkova et al., 2001b). MPAG has no inhibitory effects on IMPDH and is the major urinary excretion product of MPA (Bullingham et al., 1996; Schutz et al., 1999). In contrast, AcMPAG may be biologically active by inhibiting IMPDH, leukocyte proliferation, and inducing cytokine release (Schutz et al., 1999; Wieland et al., 2000; Shipkova et al., 2001b). A relationship between plasma levels of MPA and clinical outcomes in transplant patients has been demonstrated (Hale et al., 1998; van Gelder et al., 1999; Oellerich et al., 2000; Weber et al., 2002). Besides, AcMPAG has been suggested to be involved in some of the toxicities experienced by patients receiving MMF, including neutropenia and gastro-intestinal disorders (Wieland et al., 2000; Maes et al., 2002). Therefore, factors affecting the extent of MPA glucuronidation are likely to be clinically significant.

Recently, UGT1A9 has been identified as the main enzyme involved in the hepatic formation of MPAG (Bernard and Guillemette, 2004). This enzyme was predicted to be the key determinant of MPAG formation *in vivo* since the metabolism of MPA takes place mainly in the liver (Bowalgaha and Miners, 2001; Shipkova et al., 2001a; Bernard and Guillemette,

2004). Functional genetic variants within the *UGT1A9* gene have been uncovered recently by our group (Villeneuve et al., 2003; Girard et al., 2004). In human liver microsomes, the presence of the variants *-275A>T* and *-2152T>C* of the *UGT1A9* promoter region were associated with a 2.3-fold higher hepatic expression of *UGT1A9* and a 2.1-fold increased glucuronidation activity to generate MPAG (Girard et al., 2004). These *in vitro* observations were confirmed recently in a clinical setting by the group of Kuypers (Kuypers et al., 2005). In renal transplant recipients carrying these polymorphisms, a reduced MPA exposure and an increased MPA clearance were observed, demonstrating the clinical importance of genetic variability in the *UGT* genes involved in the *in vivo* metabolism of MPA.

MPAG is also produced by UGT1A8, which is expressed in the gastrointestinal tract and not in the liver (Cheng et al., 1998; Tukey and Strassburg, 2000; Zheng et al., 2002; Bernard and Guillemette, 2004). UGT1A8 has demonstrated the highest catalytic efficiency for MPAG formation *in vitro* (Bernard and Guillemette, 2004). Based on these metabolic studies, UGT1A8 could also play a role in the formation of AcMPAG, along with UGT2B7 that appears as the predominant enzyme responsible for its formation (Picard et al., 2005). Other extrahepatic UGTs, namely UGT1A7 and UGT1A10, demonstrated a lower reactivity towards MPA glucuronidation and are predicted to play a minor role compared to UGT1A8, UGT1A9 and UGT2B7 (Basu et al., 2004; Bernard and Guillemette, 2004; Picard et al., 2005).

To this day, two coding region polymorphisms have been reported in the *UGT1A8* gene, namely the variants $A^{173}G$ (UGT1A8*2) and $C^{277}Y$ (UGT1A8*3) (Huang et al., 2002). *In vitro* metabolic studies with heterologous expression of these variant allozymes revealed that

the polymorphism at codon 277 induces a drastic reduction in the formation of MPAG while no significant effect was observed for the codon 173 variation (Bernard and Guillemette, 2004). The effect of these variations on the formation of the AcMPAG remains to be determined. As for the *UGT2B7* gene, a frequent polymorphism (*UGT2B7*2*; $H^{268}Y$) has been reported in more than 50% of Caucasian individuals (Jin et al., 1993; Lampe et al., 2000). The functional impact of this polymorphism on the formation of AcMPAG has never been assessed.

The aim of this study was to further investigate genetic variations in the *UGT1A8* gene by resequencing the first exon and assessing the functional impact of newly found and known variants on the formation of both MPAG and AcMPAG. As a secondary aim, we explored the role of known UGT2B7*1 (H^{268}) and UGT2B7*2 (Y^{268}) variant enzymes in the formation of AcMPAG. Together, results of this study identify new genetic factors resulting in structural changes in the UGT1A8 protein that could potentially alter MPA metabolism in extrahepatic tissues. In contrast, the UGT2B7*2 common variant allozyme is predicted to have a modest influence on drug metabolism *in vivo*.

MATERIALS AND METHODS

Reagents and chemicals

MPA was obtained from Sigma Diagnostics Canada (Mississauga, ON, Canada). MPAG and AcMPAG were generous gifts from Hoffmann-La Roche (Mississauga, ON, CA). All other chemicals and reagents were of the highest grade and commercially available.

Genomic DNA samples

DNA samples from 254 healthy unrelated Caucasian subjects were obtained from the Quebec Family Study (QFS) for *UGT1A8* single-nucleotide polymorphism (SNP) genotyping (Simonen et al., 2002). Additional random DNA samples from African-American subjects (n=41) used in a previous study were sequenced (Butler et al., 2005). Subject identifiers for these samples had been removed prior to their reception in our laboratory. All subjects provided written consent for experimental purposes and the present study was reviewed and approved by the Institutional Review Boards (CHUL Research Center and Laval University).

Resequencing of the *UGT1A8* gene and genotyping

The first exon of *UGT1A8* (-34/+935) was amplified using a previously described strategy (Thibaudeau et al., 2006). Briefly, three pairs of primers were designed to amplify overlapping fragments covering the coding region of the first exon, a small portion of the 5'-flanking region and the intron-exon junction. PCR conditions for the amplification primers were 3 min at 95º for denaturation, followed by 35 cycles at 95º for 30 s, a 30 s annealing period and 72º for 30 s, with a final extension at 72º for 7 min. PCR products were sequenced using an ABI 3700 automated sequencer. Samples with ambiguous sequencing chromatograms and samples with SNPs were subjected to a second, independent amplification, followed by DNA sequencing. Sequences were analyzed with the Staden preGap4 and Gap4 programs. Allelic and genotype frequencies were calculated for all

alleles. Haplotypes and their respective frequencies were inferred using the Phase 1.0.1 software (Stephens et al., 2001).

UGT-HEK293 microsomal preparations

The *UGT1A8*1*, *UGT2B7*1* and *UGT2B7*2* constructions were kindly provided by Dr. Thomas Tephly (Cheng et al., 1998). The *UGT1A8*2* and *UGT1A8*3* variants were prepared as described previously (Bernard and Guillemette, 2004). All other *UGT1A8* variants were generated by PCR site-directed mutagenesis using the primers presented in Table 1 and inserted in the pcDNA3 vector. Prior to enzymatic assays, Western blot analyses and catalytic activities on known substrates were performed for each preparation. Stable HEK293 cells transfection with variant pcDNA3-UGT expression plasmids, preparation of microsomes by differential centrifugation and determination of UGT protein levels by Western blot has been previously described (Villeneuve et al., 2003). Protein expression levels for *UGT1A8* alleles were determined by Western blot using a polyclonal anti-UGT1A antibody (in-house, #RC-71) and were *1 (1.0), *2 (0.7), *3 (0.5), *4 (0.4), *5 (0.5), *6 (0.5), *7 (0.2), *8 (1.4), *9 (1.5), $H^{53}N$ (1.1), $A^{144}V$ (0.4) and $T^{240}A$ (0.4). Protein expression levels for UGT2B7 allozymes were determined previously (Thibaudeau et al., 2006).

Analytical procedures for MPA, MPAG and AcMPAG detection

Detection of MPA, MPAG and AcMPAG was supported by a previously published high performance liquid chromatography coupled with mass spectrometry (LC/MS) protocol used with slight modifications (Bernard and Guillemette, 2004). Briefly, the analysis system consisted of a HPLC module (Alliance model 2690, Waters Corporation, Milford, MA, USA) and a triple quadrupole mass spectrometer API 3000. Acidified assays were centrifuged for 6 min at 14000 x g and 250 uL of supernatant were collected. Ten uL samples, maintained at 4°C, were injected on a 100 mm x 4.6 mm (4.0 um diameter)

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Synergic RP-Hydro® C-18 reversed-phase column (Phenomenex, Torrance, CA). The mobile phase consisted of solution A (MeOH + 3 mM ammonium formate) and solution B (H_2O+ 3 mM ammonium formate) using the following gradient: 62% A (0-4.5 min), 95% A (4.5-6.5 min), 62% A (6.5-9.5 min). The flow rate was 0.9 ml/min. MS detection of MPA was followed in the multiple reactions monitoring (MRM) positive ion mode with mass fragmentation of 321.1>207.2 (MPA) and 514.3>321.1 (MPAG and AcMPAG). Under these conditions, retention times for MPAG, AcMPAG and MPA were 1.66, 2.47 and 4.41 min, respectively. The signals were found to be linear from 10 to 5000 ng/mL for MPAG, AcMPAG and MPA. The limit of quantification was 10 ng/mL using a signal-to-noise ratio of 3. The within-day precision was <5.0% and the between-day precision was <10%.

Enzymatic assays and kinetic parameters determination

The procedure for enzymatic assays was described previously (Bernard and Guillemette, 2004). Briefly, incubations were performed for 1h at 37°C with 50 µg of UGT protein, 50 mM Tris-HCl (pH 6.8), 10mM $MgCl_2$, 2 mM UDPGA, pepstatin and phosphatidylcholine. Determination of V_{max} and K_m was performed for all *UGT1A8* and *UGT2B7* variants with MPA ranging from 25 to 1250 µM. Absolute velocity values were adjusted according to protein expression levels relative to the corresponding *UGT*1* allele determined by Western blot. Visual inspection of fitted functions (V as a function of [S]) and Eadie-Hofstee plots (V as a function of V/[S]) was used to select the best fit enzyme kinetic model (Venkatakrishnan et al., 2001). Kinetic parameters calculations were performed with the SigmaPlot 8.0 software assisted by the Enzyme Kinetics 1.1 software (SPSS, Chicago, IL). Values were expressed as the mean of two to five experiments performed in duplicate. Means comparisons were performed with the JMP 4.0.2 software (SAS Institute, NC, USA) using Student's t test with a statistical significance threshold of $p < 0.05$.

RESULTS

Identification of novel missense mutations in the *UGT1A8* first exon

The resequencing of the first exon of *UGT1A8* led to the discovery of four novel missense mutations in the Caucasian population (n=254) at nucleotides 376 (A>G), 431 (C>T), 691 (G>A) and 718 (A>G) relative to the start codon and at nucleotides 128 (C>T) and 157 (C>A) in the African-American population (n=41). These nonsynonymous changes led to the following amino acid substitutions: $S^{43}L$, $H^{53}N$, $S^{126}G$, $A^{144}V$, $A^{231}T$ and $T^{240}A$ (Figure 1). The allelic frequencies of these variants were 0.2 to 1.4% (Table 2). The two previously reported single-nucleotide polymorphisms (SNP) of *UGT1A8* at codons 173 and 277 were confirmed with genotypic frequencies of 0.238 and 0.012, respectively. In addition to these, four synonymous variations were found at nucleotides 90 (G>A; $V^{30}V$), 441 (T>G; $L^{147}L$), 765 (A>G; $T^{255}T$) and 804 (T>C; $N^{268}N$) and one intronic missense mutation was found at nucleotide 883 relative to the start codon, that is 27 bp downstream of the end of exon 1 (IVS1+27). All SNP frequencies were found to follow the Hardy-Weinberg equilibrium (data not shown).

Eleven haplotypes were inferred in the Caucasian population (n=508 chromosomes). In contrast, five haplotypes, of which two were not encountered in the Caucasians, were observed in the African Americans (n=82 chromosomes) (Table 3). These haplotypes generated ten different diplotypes (Table 5). The *UGT1A8*1a*, *UGT1A8*1b* (T255T) and *UGT1A8*2a* ($A^{173}G$) alleles were found to be the most frequent, at 58.1, 13.4 and 22%, respectively. The other haplotypes with nonsynonymous variations were found at frequencies of 0.2-1.4%. The subjects with the $A^{144}V$ variant also presented the $A^{173}G$ variant, resulting in the *UGT1A8*4* allele. The $T^{240}A$ variant was found only once and in the presence of the $A^{173}G$ variant, representing the *UGT1A8*5* allele. The $S^{126}G$ and $A^{231}T$ variants were found

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alone generating the *UGT1A8*6* and *UGT1A8*7* alleles, respectively. The *UGT1A8*1*, *2 and *4 alleles were also found in the African-American population at frequencies of 92.7, 3.7 and 1.2%, respectively. The $S^{43}L$ polymorphism (*UGT1A8*8)* was found only in one individual, while the $H^{53}N$ polymorphism was found in combination with the $A^{173}G$ variant, generating the *UGT1A8*9* allele.

Kinetic analyses of UGT1A8 variant allozymes on MPAG and AcMPAG formation

In order to assess the impact of nonsynonymous *UGT1A8* polymorphisms on the glucuronidation of MPA, kinetic analyses were performed on all variants for the determination of K_m, V_{max} and Cl_{int} values. Kinetic estimates are presented in Table 5. A novel finding of this study is the observation that UGT1A8 has the capability to generate both MPAG and AcMPAG. The UGT1A8*3, *7, *8 and *9 enzymes were associated with the most profound effects on the level of MPAG formation with 3.3 to 82.5-fold reduced Cl_{int} values. This decrease was mostly explained by an altered velocity of the enzyme, except for the codon 277 variation (*3) that affects both the affinity and the velocity of the protein, consistent with our previous observations (Bernard and Guillemette, 2004). In contrast, the UGT1A8*4 protein appears as a high activity enzyme for the formation of MPAG with a 1.8-fold higher Cl_{int} value, explained by both a significantly better affinity and an increased velocity caused by the $V^{144}G^{173}$ mutations compared to the reference *1 protein. In the case of the UGT1A8*5 protein, despite an enhanced affinity for the formation of MPAG, the velocity of the enzyme was drastically reduced, leading to a clearance value similar to the *1 protein. In turns, the UGT1A8*2 and *6 proteins demonstrated modest modifications of their kinetic parameters compared to the reference *1 protein.

With regards to AcMPAG, its formation was undetectable for the *3 and *7 proteins and severely reduced for the *5 protein (20-fold reduction) due to an altered velocity. Velocities

were also significantly reduced for the *2, *4, *6 and *8 enzymes, by 2- to 4-fold compared to the UGT1A8*1 protein. The affinity of variant proteins *2, *4, *5, *6, *8 and *9 was not significantly altered compared to UGT1A8*1. In the individuals tested, 2.8% of Caucasians and 4.8% of African-Americans carry at least one of the low activity alleles (*3, *7, *8 and *9).

In order to gain insight into the nucleic acid positions responsible for critical changes in the kinetic parameters of the UGT1A8 protein, few mutations were tested alone. Results indicate that the replacement of a threonine by an alanine at codon 240 abolishes the ability of the UGT1A8 protein to form the acyl glucuronide and compromised drastically the formation of the phenolic glucuronide. In turns, the $H^{53}N$ change acts only on the velocity of the protein while the $A^{144}V$ mutation alters specifically the formation of the MPAG (K_m and V_{max}) with no significant detectable effect on the kinetic parameters for the formation of the acyl.

Kinetic analyses of the UGT2B7*1 and *2 alleles AcMPAG formation

The UGT2B7 enzyme was found to generate high levels of AcMPAG with no detectable formation of MPAG. Both the affinity and the capacity of the UGT2B7*1 protein were higher for the formation of the acyl glucuronide compared to the UGT1A8*1 protein, with a 31-fold higher Cl_{int} value. No significant changes in the kinetic parameters were associated with the UGT2B7*2 protein, with K_m and V_{max} values similar to those observed for the UGT2B7*1 protein.

DISCUSSION

In a recent study, we revealed the existence of common variations in the upstream region of the *UGT1A9* gene (-275T>A and -2152C>T) associated with higher protein expression and higher glucuronidation activity in human liver samples (Girard et al., 2004). These genetic variants were further shown to influence the pharmacokinetics of MMF in transplant recipients (Kuypers et al., 2005). Based on *in vitro* data, two additional UGTs, UGT1A8 and UGT2B7, are proposed to play a critical role in the metabolism of MPA (Basu et al., 2004; Bernard and Guillemette, 2004; Picard et al., 2005). In this study, polymorphisms conferring a low activity phenotype were identified in UGT1A8, the extra-hepatic MPA-metabolizing enzyme that demonstrates the highest catalytic efficiency for MPAG formation. It is thus predicted that specific UGT1A8 variants identified here may have an impact on the pharmacokinetics of MMF and potentially on the metabolism of other UGT1A8 substrates.

Six novel nonsynonymous variations in the coding region of *UGT1A8* ($S^{43}L$, $H^{53}N$ $S^{126}G$, $A^{144}V$, $A^{231}T$ and $T^{240}A$) were identified and the two previously described variants *2 ($A^{173}G$) and *3 ($C^{277}Y$) were also observed. The $A^{173}G$ and $C^{277}Y$ variants were initially reported with frequencies of 14.5 and 2.2%, respectively (Huang et al., 2002). While the $C^{277}Y$ variant is reported in this study with a similar frequency, a 2-fold higher frequency was observed for the $A^{173}G$ and could be attributed to several differences in the population studied (254 healthy subjects of French-Canadian origin *vs* 69 individuals with lung cancer patients, their family members and other volunteers).

Among the variants found, UGT1A8*3 ($C^{277}Y$), *5 ($G^{173}A^{240}$), *7 ($A^{231}T$), *8 ($S^{43}L$) and *9 ($N^{531}G$) were associated with the most profound decreases in the formation of MPA

glucuronides *in vitro*. The formation of MPAG in the GI tract is predicted to be reduced in the presence of those alleles, occurring in 2.8% of Caucasians and 4.8% of African-Americans. As for AcMPAG, while UGT2B7 is the most active UGT compared to UGT1A8 (Clint = 12 uL/min/mg *vs* 0.39 uL/min/mg), it is predicted that UGT1A8 variants would have a limited impact on the formation of the acyl glucuronide *in vivo*. Prior to this study, UGT1A8*3 had been identified as a low activity protein on various substrates, including a dramatic reduction in MPAG formation, but its impact on AcMPAG formation was not assessed (Huang et al., 2002; Bernard and Guillemette, 2004). Our study further shows that a similar effect can be observed for AcMPAG. Such an impact could be explained by the fact that this amino acid variation involves the substitution of a highly conserved cysteine for a tyrosine. Similarly, the $T^{240}A$ variant (not encountered alone in the population studied) appears to be responsible for the reduced capacity observed for the UGT1A8*5 protein ($G^{173}A^{240}$) to generate MPA glucuronides, as the activity of the *5 ($G^{173}A^{240}$) and $T^{240}A$ variants are similar. This also suggests a negligible role of the $A^{173}G$ variation (*2 allele) on UGT1A8 protein activity, as seen previously (Huang et al., 2002; Bernard and Guillemette, 2004). The dramatic activity reduction associated with the UGT1A8*7 ($A^{231}T$) protein is somehow surprising since the alanine-threonine substitution represents a fairly conservative change. Nevertheless, the velocity of the UGT1A8*7 ($A^{231}T$) protein was reduced by 281-fold for MPAG compared to UGT1A8*1, indicating a critical role of this amino acid for enzyme function. As for the UGT1A8*8 ($S^{43}L$) and *9 ($N^{53}G^{173}$) proteins, they were both associated with similar decreases in velocity, with 2.8 and 3.3-fold reduced V_{max} values, respectively. The $H^{53}N$ variation involves the substitution of a highly conserved histidine for an asparagine, which could explain the reduced activity observed with the variant protein.

One of the UGT1A8 variant allozymes, UGT1A8*4 ($V^{144}G^{173}$) demonstrated an effect specific to the glucuronide product formed. The combination of variations at codons 144 and 173 led to an enhanced activity of the protein specifically for the formation of MPAG, while the formation of AcMPAG was lowered. According to the kinetic properties of the V^{144} alone and the G^{173} alone (UGT1A8*2), the effect on AcMPAG formation would be a consequence of the amino acid substitution at codon 173, whereas the increased capacity of MPAG formation would mostly be caused by the change at codon 144.

UGT2B7 has been identified as the main enzyme involved in the formation of AcMPAG (Picard et al., 2005) and is expressed in the liver and the intestine (Jin et al., 1993; Radominska-Pandya et al., 1998). The common *UGT2B7*2* (Y^{268}) allele, carried by 27% of Asians and up to 54% of Caucasians (Jin et al., 1993; Guillemette et al., 2000; Lampe et al., 2000), demonstrated a catalytic efficiency comparable to that of *UGT2B7*1* in most studies for various substrates, including opioids, androgens, AZT, and morphine (Coffman et al., 1998; Barbier et al., 2000; Bhasker et al., 2000; Innocenti et al., 2001). Thus, it is predicted that the *in vivo* formation of AcMPAG in the liver and the GI tract, is likely not significantly modulated by UGT2B7*2. Other variants of UGT2B7, namely SNPs of the 5'-regulatory region, could affect the levels of expression of the gene and would deserve further consideration with regards to AcMPAG formation. One example is the -79 G>A polymorphism in the *UGT2B7* gene, in complete linkage disequilibrium with UGT2B7*2, that results in a reduction of 2.5- to 7-fold lower promoter activity and found in approximately 5% of the population (Duguay et al., 2004).

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The pharmacokinetics of MPA and its metabolites have been shown to be highly variable in various transplant subpopulations (Bullingham et al., 1998; Ensom et al., 2002; Jacobson et al., 2005; Srinivas et al., 2005). One of the factors likely involved is the genetic diversity of *UGT* genes, such as the *UGT1A9* -275/-2152 variants recently associated with significantly lower MPA exposure in renal transplant patients (Kuypers et al., 2005). As proposed by the authors, the altered pharmacokinetics related to the presence of these common polymorphisms is believed to be at least partially caused by a reduction of enterohepatic recirculation, a process accounting for up to 40% of the plasma MPA area under the concentration-time curve (Seifeldin, 1995; van Gelder et al., 2001). Given the important role of this process in the pharmacokinetics of MMF, the intestinal conjugation of MPA certainly deserves further attention (Bullingham et al., 1998). UGT1A8 is one of few UGT enzymes to be specifically expressed in the gastro-intestinal tract (Cheng et al., 1998; Tukey and Strassburg, 2000; Zheng et al., 2002), along with UGT1A9 and 2B7 (Tukey and Strassburg, 2000; Turgeon et al., 2001). This isoform could significantly contribute to the intestinal formation of the inactive glucuronide MPAG, but also the reactive and potentially toxic metabolite AcMPAG, yet to a much lesser degree than UGT2B7 (31-fold lower Cl_{int} value).

In conclusion, while the common variant of UGT2B7 at codon 268 is predicted to have limited impact *in vivo*, several UGT1A8 variants were identified and could contribute to the inter-individual variability in MMF pharmacokinetics and deserve further clinical investigation.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 *UGT1A8* genetic variants. (A) Schematic representation of polymorphisms located in *UGT1A8*. Amino acid position is relative to UGT1A8 according to the reference sequence (AF297093). (B) Amino acid alignment of UGT1A proteins at UGT1A8 polymorphic sites. ref, reference sequence; var, variant sequence.

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Table 1 Site-directed mutagenesis primers sequences for *UGT1A8* variants.

UGT1A8 variants	Primer Sequence[a]
$S^{43}L$	(F) 5'-GGTTCACCATGCAGT**T**GGTGGTGGAGAAACT
	(R) 5'-AGTTTCTCCACCACC**A**ACTGCATGGTGAACC
$H^{53}N$	(F) 5'-CTTATCCTCAGGGGG**A**ATGAGGTGGTTGTAG
	(R) 5'-CTACAACCACCTCAT**T**CCCCCTGAGGATAAG
$S^{126}G$	(F) 5'-TTCGCATTGCAGG**G**GTTTGTTTAATGACCG
	(R) 5'-CGGTCATTAAACAAAC**C**CCTGCAATGCGAA
$A^{144}V$	(F) 5'-GGAGAGTTCTTTTGATG**T**GGTGTTTCTTGATCC
	(R) 5'-GGATCAAGAAACACC**A**CATCAAAAGAACTCTCC
$A^{231}T$	(F) 5'-GCCCTAGAAATA**A**CCTCTGAAATTCTCC
	(R) 5'-GGAGAATTTCAGAGG**T**TATTTCTAGGGC
$T^{240}A$	(F) 5'-CTCCAAACACCTGTC**G**CAGCATATGATCTC
	(R) 5'-GAGATCATATGCTG**C**GACAGGTGTTTGGAG

F, Forward; R, Reverse;

[a] Accession number for the *UGT1A8* reference sequence : AF297093.

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Table 2 Frequency of *UGT1A8* variants in the Caucasian and African-American populations.

Caucasians (n=254 subjects)

Nucleic acid changes	Amino acid changes	Genotype frequency (%)			SNP frequency (%)
		Homozygous	Heterozygous	Homozygous variant	
90 A>G	$V^{30}V$	99.6	0.4	0.0	0.2
376 A>G	**$S^{126}G$**	99.6	0.4	0.0	0.2
431 C>T	**$A^{144}V$**	97.2	2.8	0.0	1.4
441 C>T	$L^{147}L$	94.9	4.3	0.8	3.0
518 C>G	**$A^{173}G$**	55.9	40.6	3.5	23.8
691 G>A	**$A^{231}T$**	99.6	0.4	0.0	0.2
718 A>G	**$T^{240}A$**	99.6	0.4	0.0	0.2
765 A>G	$T^{255}T$	74.8	23.6	1.6	13.4
803 T>C	$N^{268}N$	99.6	0.4	0.0	0.2
830 G>A	**$C^{277}Y$**	97.6	2.4	0.0	1.2
883 G>A	IVS1+27[a]	99.6	0.4	0.0	0.2

African-Americans (n=41 subjects)

Nucleic acid changes	Amino acid changes	Genotype frequency (%)			SNP frequency (%)
		Homozygous	Heterozygous	Homozygous variant	
128 C>T	**$S^{43}L$**	97.6	2.4	0.0	1.2
157 C>A	**$H^{53}N$**	97.6	2.4	0.0	1.2
431 C>T	**$A^{144}V$**	97.6	2.4	0.0	1.2
518 C>G	**$A^{173}G$**	87.8	12.2	0.0	6.1
765 A>G	$T^{255}T$	75.6	19.5	4.9	14.6

[a] IVS1+27 : intronic variation 27 bp downstream of exon 1. SNP, single-nucleotide polymorphism.

Table 3 *UGT1A8* haplotypes in Caucasian and African-American subjects.

H	$V^{30}V$ A>G	$S^{43}L$ C>T	$H^{53}N$ C>A	$S^{126}G$ A>G	$A^{144}V$ C>T	$L^{147}L$ T>G	$A^{173}G$ C>G	$A^{231}T$ G>A	$T^{240}A$ A>G	$T^{255}T$ A>G	$N^{268}N$ T>C	$C^{277}Y$ G>A	IVS1+27 G>A	Caucasians (n=508) n	Caucasians (n=508) Frequency	African-Americans (n=82) n	African-Americans (n=82) Frequency
***1a**	A	C	C	A	C	T	C	G	A	A	T	G	G	297	0.585	76	0.927
***1b** [a]	A	C	C	A	C	T	C	G	A	G	T	G	G	66	0.130	0	
***1c**	A	C	C	A	C	G	C	G	A	A	T	G	G	15	0.030	0	
***1d**	A	C	C	A	C	T	C	G	A	A	C	G	G	1	0.002	0	
***2a**	A	C	C	A	C	T	G	G	A	A	T	G	G	112	0.220	3	0.037
***2b**	A	C	C	A	C	T	G	G	A	A	T	G	A	1	0.002	0	
***3**	A	C	C	A	C	T	C	G	A	A	T	A	G	6	0.012	0	
***4**	A	C	C	A	T	T	G	G	A	A	T	G	G	7	0.014	1	0.012
***5** [a]	G	C	C	A	C	T	G	G	G	A	T	G	G	1	0.002	0	
***6**	A	C	C	G	C	T	C	G	A	A	T	G	G	1	0.002	0	
***7**	A	C	C	A	C	T	C	A	A	A	T	G	G	1	0.002	0	
***8**	A	T	C	A	C	T	C	G	A	A	T	G	G	0		1	0.012
***9** [a]	A	C	A	A	C	T	G	G	A	A	T	G	G	0		1	0.012

Haplotypes (H) and their respective frequencies were inferred with the Phase 1.0.1. n, number of chromosomes analyzed. [a] Haplotypes *2b, *5 and *9 could not be confirmed in more than one individual and therefore are considered as hypothetical alleles.

Table 4 *UGT1A8* diplotypes in Caucasian and African-American subjects.

Diplotypes	Caucasians (n=254)		African-Americans (n=41)	
	n	Frequency	n	Frequency
1/1	134	0.528	35	0.854
1/2	95	0.374	3	0.073
2/2	9	0.035		
1/3	6	0.024		
1/4	7	0.028	1	0.024
1/5	1	0.004		
1/6	1	0.004		
1/7	1	0.004		
1/8			1	0.024
1/9			1	0.024

Table 5 Kinetic estimates for MPAG and AcMPAG formation by UGT1A8 and UGT2B7 variant allozymes.

UGT1A8 allozymes	MPAG			AcMPAG		
	K_m (μM)	Relative V_{max} (pmol/min/mg)	Cl_{int} (μL/min/mg)	K_m (μM)	Relative V_{max} (pmol/min/mg)	Cl_{int} (μL/min/mg)
*1[a]	411 ± 65	112826 ± 5434	165	703 ± 144	532 ± 39	0.39
*2 (G^{173})	296 ± 31	87992 ± 4263	211	566 ± 9	242 ± 5 **	0.22
*3 (Y^{277})	89 ± 11 *	3296 ± 359 **	38	Not detectable		
*4 ($V^{144}G^{173}$)	105 ± 13 *	30470 ± 1102 **	300	468 ± 40	120 ± 13 *	0.26
*5 ($G^{173}A^{240}$)	75 ± 10 *	9273 ± 400 **	132	278 ± 36	26 ± 5 *	0.09
*6 (G^{126})	333 ± 120	48880 ± 8735 *	164	483 ± 95	128 ± 20 *	0.29
*7 (T^{231})	288 ± 136	402 ± 1 **	2	Not detectable		
*8 (L^{43})	531 ± 18	40451 ± 416 *	50	1125 ± 226	164 ± 12 *	0.11
*9 ($N^{53}G^{173}$)	683 ± 262	33773 ± 3705 *	39	672 ± 125	166 ± 8 *	0.13
$H^{53}N$[b]	441 ± 74	44809 ± 1387 *	63	773 ± 188	204 ± 23 *	0.15
$A^{144}V$[b]	130 ± 25 *	66479 ± 9498 *	652	553 ± 43	403 ± 101	0.71
$T^{240}A$[b]	75 ± 18 *	7261 ± 1386 **	101	Not detectable		
UGT2B7						
*1 (H^{268})	Not detectable			183 ± 99	1993 ± 193	12
*2 (Y^{268})	Not detectable			146 ± 61	2610 ± 805	18

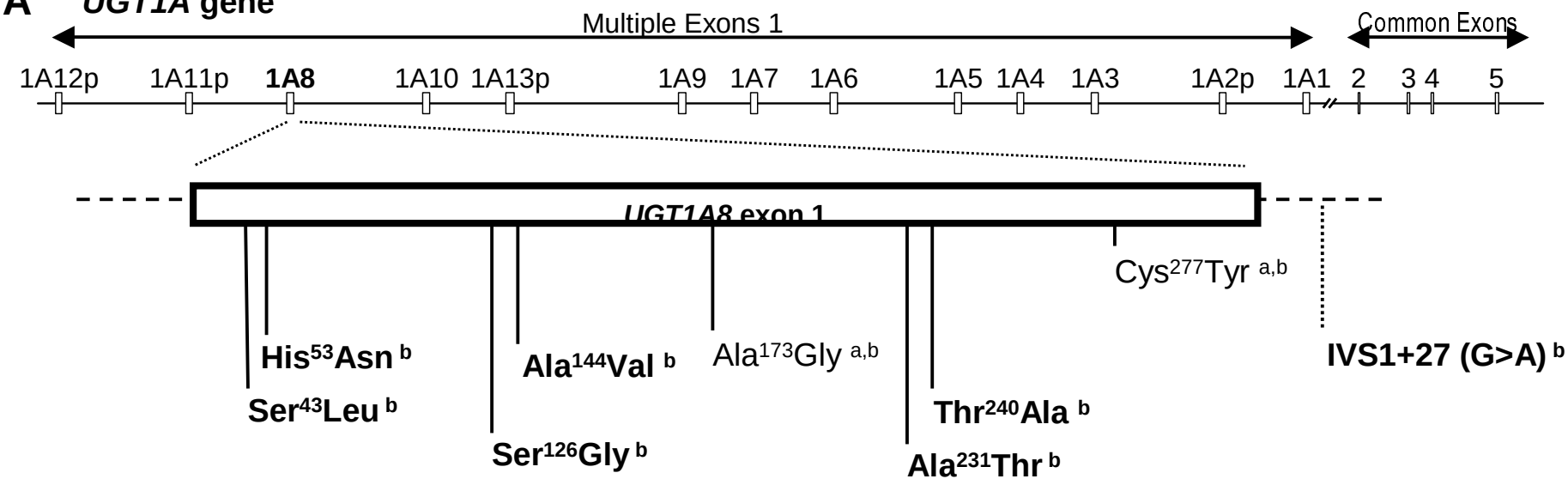
Kinetic parameters determination was performed by incubating MPA (25 to 1250 μM) with HEK-293 cells stably expressing UGT1A8 and UGT2B7 variants. Velocity values were adjusted according to protein expression levels relative to the *1 allele determined by Western blot. All values are expressed as the mean ± SEM of two to five experiments performed in duplicate. Significant differences from the *1 allele; * $p < 0.05$, ** $p < 0.001$.

[a] UGT1A8*1 ($S^{43}H^{53}S^{126}A^{144}A^{173}A^{231}T^{240}C^{277}$).

[b] Not encountered alone in the population studied.

Figure 1

A ***UGT1A* gene**



Legend :

[a] Huang *et al.* Pharmacogenetics 2002

[b] **Present work**

B

UGT	*UGT1A8* amino acid changes							
	$S^{43}L$	$H^{53}N$	$S^{126}G$	$A^{144}V$	$A^{173}G$	$A^{231}T$	$T^{240}A$	$C^{277}Y$
1A8 (ref)	**S**	**H**	**S**	**A**	**A**	**A**	**T**	**C**
***1A8* (var)**	***L***	***N***	***G***	***V***	***G***	***T***	***A***	***Y***
1A10	S	H	S	A	F	F	T	C
1A7	S	H	S	A	F	F	T	C
1A9	S	H	S	A	L	L	T	C
1A6	D	H	S	A	P	P	D	C
1A3	E	H	E	V	P	P	S	C
1A5	E	H	E	V	P	P	S	C
1A4	E	H	E	V	P	P	S	C
1A1	G	H	H	V	P	P	T	C