

The role of organic anion-transporting polypeptides and their common genetic variants in mycophenolic acid pharmacokinetics

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Abstract

The goal of this study was to determine the role of the Organic Anion Transporting Polypeptides, OATP1A2, OATP1B1 and OATP1B3 and their genetic variants in the pharmacokinetics of the immunosuppressive drug mycophenolate mofetil (MMF). Using OATP-transfected HEK cells, we measured the uptake of mycophenolic acid (MPA) and its glucuronide (MPAG). MPAG, but not MPA, significantly accumulated in cells expressing OATP1B3 or OATP1B1 ($p < 0.05$). MPA and MPAG pharmacokinetics were significantly influenced by the OATP1B3 polymorphism, 334T>G/699G>A, in 70 renal transplant patients co-treated with tacrolimus or sirolimus but not in 115 patients co-treated with cyclosporine. The decrease in MPA dose-normalized exposure and concomitant increase in the MPAG/MPA metabolic ratio are consistent with reduced enterohepatic cycling in patients carrying the OATP1B3 334G-699A haplotype. Further studies demonstrated that this variant of OATP1B3 exhibited a reduced V_{max} in transfected HEK cells, providing functional support of our clinical findings.

MESH Keywords Cell Line ; Genetic Variation ; genetics ; Humans ; Mycophenolic Acid ; pharmacokinetics ; Organic Anion Transporters ; genetics ; physiology ; Peptides ; genetics ; physiology ; Polymorphism, Genetic ; genetics

Author Keywords immunosuppressive agents ; transplantation ; transporters ; pharmacogenetics ; mycophenolic acid.

Introduction

Mycophenolic acid (MPA), the active moiety of mycophenolate mofetil (MMF), is a non-nephrotoxic, cornerstone immunosuppressant in kidney transplantation. MPA undergoes extensive entero-hepatic recirculation initiated by almost complete hepatic glucuronidation into the inactive MPA-7-O-glucuronide (MPAG) (1) and active biliary excretion of this metabolite by the Multidrug Resistance-associated Protein-2 (MRP2) (2). It is thought that, after MPAG hydrolysis by intestinal flora, the amount of MPA reabsorbed could represent 10 to 60% of total MPA exposure (1). Consequently, inhibitors of MPA entero-hepatic cycling (including cyclosporine) affect MPA levels (3, 4). Although significant associations between polymorphisms in enzymes involved in MPA glucuronidation and MMF pharmacokinetics were described, results differed depending on the post-transplantation period and comedications and could not always be replicated (see supplementary Table 1). The hepatic uptake of MPA and/or MPAG being a prerequisite for MPA entero-hepatic recirculation, it could contribute to the pharmacokinetic variability and drug-drug interactions of MPA-based formulations. There has been only one indirect hint about the implication of the Organic Anion Transporting Polypeptides (OATPs) localized to the sinusoidal membrane of the hepatocytes in the disposition of MMF. Specifically, Miura et al. reported an association between OATP1B3 and OATP1B1 polymorphisms and MPAG and MPA pharmacokinetics in Japanese renal transplants (5, 6).

This study aimed to determine whether MPA and MPAG are substrates of the main OATP expressed in hepatocytes (OATP1B1 and 1B3) or enterocytes (OATP1A2) and the effect of genetic variations in competent transporters on MPA pharmacokinetics in renal transplant recipients receiving MMF combined with different other immunosuppressants, taking into account other SNPs previously reported to affect MPA pharmacokinetics.

Results

Cellular uptake of MPA and MPAG in OATP-expressing cells

After 5 min incubation in cells expressing OATP1A2, 1B1 or 1B3, accumulation of MPA was not affected as compared to that observed in cells transfected with an empty vector (Figure 1a). In contrast, MPAG uptake was significantly and substantially enhanced by OATP1B3 and to a lesser extent by OATP1B1, but not by OATP1A2 (Figure 1b).

Experiments with radiolabeled model substrates confirmed the activity of each transporter (see supplementary data).

Identification and frequency distribution of patient genotypes

Our clinical pharmacogenetic investigation was primarily based on our aforementioned in-vitro results and thus aimed to assess the effect of SLCO1B3 and SLCO1B1 polymorphisms on MMF pharmacokinetics. In addition, patients were genotyped for polymorphisms in UGT1A9, UGT1A8 and ABCC2, previously reported to significantly affect MPA pharmacokinetics.

SNPs to be investigated in SLCO genes were selected from the literature and from the SOPHIE (Study of Pharmacogenetics in Ethnically Diverse Populations) database (<http://pharmacogenetics.ucsf.edu/index.html>). Two frequent coding, non-synonymous SNPs in complete linkage disequilibrium were reported in SLCO1B3 (T334G, G699A) (7). In the Caucasian group of the SOPHIE cohort one additional common non-synonymous SNP was found in SLCO1B3 (G767C) with allele frequency of 0.18, and three in SLCO1B1 (A388G, C463A and T521C) with allelic frequencies of 0.44, 0.16 and 0.17, respectively.

No linkage disequilibrium was observed between the SLCO1B3 T334G and G767C SNPs. In contrast, for SLCO1B1, the A388G SNP showed a strong linkage disequilibrium with the C463A ($D'=1$; $r^2=0.21$) and the T521C ($D'=0.79$; $r^2=0.17$) SNPs, while there was no linkage between the C463A and the T521C SNPs. This led to six SLCO1B1 haplotypes: A-C-T (0.53), G-C-C (known as SLCO1B1*15; 0.15), G-C-T (0.14), G-A-T (0.13), A-C-C (0.021), and G-A-C (0.004).

All genotypes were in Hardy-Weinberg equilibrium. Their distributions were compared to those observed in the HapMap Caucasian panel (www.hapmap.org; Release 27, Feb 09), except for UGT1A9 C-440T, T98C and SLCO1B3 C767G, not reported in HapMap. No significant difference was found, except for ABCC2 C-24T ($p=0.01592$) whose allelic distribution was however not significantly different from that reported in the literature for Caucasians (8, 9).

Influence of comedications on MPA pharmacokinetics

As the nature of the immunosuppressant coadministered with MMF influences MPA pharmacokinetics, this effect was evaluated before the pharmacogenetic association study. Since no difference in MMF dose (1416 ± 528 vs 1304 ± 478 mg; $p=0.3633$), MPA dose-normalized (dn) AUC_{0-12h} (64.6 ± 34.7 vs 66.0 ± 29.7 mg.h/l/g, $p=0.6401$), $dn-C_0$ (4.4 ± 3.5 vs 3.9 ± 2.5 mg/l/g, $p=0.6958$) and $dn-C_{max}$ (20.1 ± 9.4 vs 27.2 ± 21 mg/l/g, $p=0.2674$) or MPAG $dn-AUC_{0-9h}$ (1011 ± 595 vs 1183 ± 611 mg.h/l/g; $p=0.1220$), $dn-C_0$ (99 ± 75 vs 108 ± 59 mg/l/g, $p=0.3436$) and $dn-C_{max}$ (152 ± 84 vs 177 ± 95 mg/l/g, $p=0.2059$) was observed between patients receiving the drug in combination with sirolimus or tacrolimus, the two sub-groups of patients were combined for the pharmacogenetic study. In contrast, patients co-treated with cyclosporine were studied independently as they received significantly higher doses of MMF (1890 ± 679 mg) and had lower MPA $dn-AUC_{0-12h}$ (44.3 ± 20.8 mg.h/l/g) as compared to patients on sirolimus or tacrolimus ($p<0.0001$).

SLCO genotypes and MMF pharmacokinetics

In patients receiving MMF in combination with cyclosporine ($n=115$), no significant association between SLCO1B3 or 1B1 genotypes or haplotypes and MPA $dn-AUC_{0-12h}$ was found (Table 1).

In 70 patients co-treated with sirolimus or tacrolimus, MPAG $dn-AUC_{0-9h}$ showed a trend to a gradual decrease with the number of SLCO1B3 T334 alleles (Table 2). Although not significant, important differences in MPAG dose-normalized levels were observed at all the sampling time points (Figure 2b). The metabolic ratio of MPAG to MPA showed a very significant decrease in carriers of the SLCO1B3 T334 allele ($p=0.0001$) (Figure 2c).

In these patients, MPA dose-normalized exposure was significantly different depending on SLCO1B3 T334G genotype (Table 2): the SLCO1B3 T334 allele resulted in significantly higher MPA dose-normalized levels at every concentration-time points, except T_{20min} ($p=0.0636$) and T_{120min} ($p=0.0975$) (Figure 2a). Consequently, carriers of a least one SLCO1B3 T334 allele ($n=22$) had 1.42-fold higher (IC95%: 1.39–1.45; $p=0.0003$) MPA $dn-AUC_{0-12h}$ and a 1.38-fold higher (IC95%: 1.32–1.49; $p=0.0010$) $dn-C_{max}$ as compared to carriers of the 334GG genotype ($n=48$). There was no difference in T_{max} (Table 2). MPA AUC_{4-9h} was studied as a surrogate for enterohepatic cycling: patients heterozygous or homozygous for the SLCO1B3 T334 allele ($n=22$) had significantly higher MPA $dn-AUC_{4-9h}$ than other patients ($n=48$; $p=0.0027$). However, they also had higher $dn-AUC_{0-4h}$ ($p=0.0008$), resulting in unchanged AUC_{4-9h}/AUC_{0-9h} ratio ($p=0.4945$).

The SLCO1B3 T334 allele was associated with a similar increase in MPA $dn-AUC_{0-12h}$ ($p=0.0074$ and $p=0.0651$, respectively) and decrease in MPAG/MPA metabolic ratio ($p=0.0517$ and 0.0002 , respectively) in the subgroups of patients co-treated with sirolimus ($n=42$) and tacrolimus ($n=28$).

There was no association between the SLCO1B3 T334G genotype and MMF daily dose, categorized as low (≤ 1 g), intermediate ($= 1.5$ g) or standard/high (≥ 2 g) ($p=0.1080$). However, a significant association was observed between this genotype and MPA AUC_{0-12h} categorized as below, in, or above the generally admitted therapeutic range (30–60 mg.h/l; $p=0.03548$). One third of the patients with the 334 TT or TG genotype ($n=7/22$) had MPA AUC_{0-12h} higher than the admitted therapeutic range, as compared to only 8% of patients of the

334GG genotype (n=4/48). Conversely, in carriers of the 334GG genotype, 40% (n=19/48) had MPA AUC_{0-12h} below the therapeutic range as compared to 23% (n=5/22) patients of the TT or TG genotype. There was no association of the second SLCO1B3 SNP tested (G767C) with any of the exposure indices.

Despite a trend to higher MPA $dn-AUC_{0-12h}$ in heterozygous carriers of the SLCO1B1 T521C SNP as compared to homozygous TT521 carriers (p=0.0534), this SNP was not associated with MPAG exposure (Table 2). The other SLCO1B1 SNP tested (A388G and C463A) did not show any association with MPA or MPAG pharmacokinetics (Table 2).

Thesias® haplotype analysis revealed an association between the SLCO1B1*15 allele and MPA $dn-AUC_{0-12h}$ (p=0.0246) or MPAG/MPA ratio (p=0.0267), but not with MPAG $dn-AUC_{0-9h}$.

No statistical interaction between the effect of SLCO1B3 T334G genotype and SLCO1B1*15 allele was found using ANOVA, but the effect of the SLCO1B1*15 allele did not remain significant regarding MPA $dn-AUC_{0-12h}$ (p=0.0553) and only showed a trend regarding MPAG/MPA ratio (p=0.0302, NS after Bonferroni correction). In contrast, the effect of SLCO1B3 T334G genotype remained highly significant (p values of 0.0008 and 0.0043 for MPA $dn-AUC_{0-12h}$ and MPAG/MPA ratio, respectively).

UGT and ABCC2 genotypes and MMF pharmacokinetics

In patients co-treated with cyclosporine, no influence of UGT1A8 and 1A9 polymorphisms was observed on MPA $dn-AUC_{0-12h}$ (Table 1). There was a trend towards an association between the ABCC2 C-24T SNP and MPA $dn-AUC_{0-12h}$, however, without apparent additivity: heterozygotes for this SNP showed higher MPA $dn-AUC_{0-12h}$ than homozygotes for the reference or the variant genotype.

No significant association of ABCC2, UGT1A8 or UGT1A9 genotypes with MPAG exposure was found in patients co-treated with tacrolimus or sirolimus (Table 3). Regarding MPA pharmacokinetics, a trend to lower MPA $dn-C_{max}$ was found in two patients heterozygous for the UGT1A9 T98C genotype as compared to patients of the reference genotype (p=0.0344; NS after Bonferroni correction). However, no association between this polymorphism and other MPA (or MPAG) exposure indices or with MPAG/MPA ratio was found (Table 3).

The UGT1A8 C518G SNP tended to be associated with MPA $dn-C_0$ (p=0.0130), C_{max} (p=0.0368) and $dn-AUC_{0-12h}$ (p=0.0171; Table 3) but not with MPAG exposure indices (Table 3) or MPAG/MPA ratio. Finally, patients homozygous for the UGT1A9 C-440T variant genotype (n=4) had significantly lower MPA T_{max} than patients heterozygous or homozygous for the reference allele, but similar MPA or MPAG exposure indices.

Transport activity of OATP1B3 reference and variant proteins

The effect of the SLCO1B3 variant found to be associated with MPA clinical pharmacokinetics was investigated in-vitro.

The uptake of MPAG into cells expressing the OATP1B3 variant protein (i.e., 334G-699A haplotype) was markedly lower than in cells expressing the reference sequence. OATP1B3 variant protein showed a similar K_m (122±109 vs 114±69.8 μM) with the reference OATP1B3 but a reduced V_{max} (313±188 vs 508±205 pmol/min/mg protein, respectively) (Figure 3).

Discussion

This study provides direct evidence that MPAG is a substrate of the liver-specific uptake transporter OATP1B3 and that the pharmacokinetics of MPA is significantly influenced by the common SLCO1B3 T334G polymorphism of this gene in renal transplant patients receiving MMF in a cyclosporine-free immunosuppressive regimen. Reduced transport of MPAG was observed in cells expressing this particular OATP1B3 variant, which is consistent with the trend towards a gene-dose decrease in MPAG exposure with the number of variant alleles observed in these patients. We found a significant decrease in MPA $dn-C_{max}$ and $dn-AUC_{0-12h}$ in carriers of the 334G allele. Based on our in-vitro findings and on the change in MPAG exposure in transplant patients, we suggest that this is secondary to reduced MPAG hepatic uptake, hence reduced re-absorption of MPA via the entero-hepatic cycling.

Entero-hepatic cycling is indeed a major feature of MPA pharmacokinetics and its contribution to MPA exposure is well-known. MPAG is extensively produced in the liver, partly excreted into the bile and substantially hydrolysed to MPA in the small intestine, leading to MPA re-absorption estimated to contribute 10 to 60% to total MPA exposure (1). It is generally thought that MPAG biliary excretion occurs just after its production in the liver. Here we suggest that, additionally, the active transport of circulating MPAG into hepatocytes substantially contributes to MPA enterohepatic cycling and then in MPA systemic exposure. Indeed, MPAG plasma concentrations are by far higher than those of MPA and this study strongly suggests that MPAG is actively taken up by the hepatocyte. The trend towards higher MPAG plasma levels in patients carrying the SLCO1B3 G-allele is consistent with our in-vitro findings and

supports the hypothesis that reduced OATP1B3 activity would decrease MPAG hepatic uptake. Nonetheless, the change in MPAG levels was not significant, maybe because renal elimination is the main disposition pathway for this metabolite. Thus, despite reducing MPA re-circulation, the decrease in MPAG biliary excretion might have a limited effect on MPAG overall elimination.

We did not observe any association between this SNP and MPA exposure in 115 patients co-treated with cyclosporine, which might be due to the inhibitory effect of cyclosporine on MPA biliary excretion. Inhibition of MRP2 by cyclosporine will indeed reduce MPA recirculation, whatever the activity of OATP1B3 is. Looking at the means \pm SD in the three genotype groups (Table 1) as well as to the p value obtained, it seems that the number of patients in this comedication subgroup was sufficient to exclude any clinically relevant influence of this particular SNP on MPA exposure in patients receiving cyclosporine. In contrast, increasing the number of patients cotreated with tacrolimus or sirolimus might have allowed us to investigate a potential additive gene-dose effect.

Human OATP1B3 is mainly expressed in hepatocytes where it mediates, together with OATP1B1, the uptake of a number of drugs or endogenous compounds from blood to hepatocytes (10). In this study, we showed that cell expression of either transporter resulted in the facilitated uptake of the main metabolite of MPA (MPAG). However, OATP1B1 activity with respect to MPAG was relatively modest as compared to that of OATP1B3, suggesting limited implication of OATP1B1 in MPAG uptake in-vivo as compared to OATP1B3. The finding that none of the OATP1B1 polymorphisms or haplotypes tested in this study was significantly and markedly associated with MPA or MPAG pharmacokinetics supports this hypothesis.

We did not observe any difference between HEK293T native and OATP-expressing cells regarding the uptake of the parent drug (MPA). We cannot totally exclude that MPA is a substrate of OATP. Indeed, passive diffusion (or transport via some unknown transporter in HEK) apparently plays a much higher role in MPA than MPAG cellular transport, with a net uptake rate approximately 3-fold higher for MPA than MPAG in HEK293T native cells (0.021 vs 0.008 pmol/mg/min). This may mask the active transport of the drug (if any) in our cellular model.

OATP1A2 is highly expressed in the intestine, the kidney, in cholangiocytes and at the blood-brain barrier (10 , 11) where it is supposed to play an important role in drug absorption and drug-drug interactions (11). We observed no difference between native and OATP1A2-expressing HEK293T cells regarding MPA or MPAG uptake, suggesting that these compounds are not substrates of OATP1A2.

The first study investigating the functional consequences of SLCO1B3 polymorphisms showed in stably transfected MDCKII and HEK293 cells that variant OATP1B3 proteins resulting from either of the two coding non-synonymous SNPs T334G (S122A) and G699A (M233I) had similar basolateral membrane localization with the reference protein (12). They did not find differences in uptake activities between these proteins using several model substrates (Estrone-3-sulfate, bromosulphophthalein and 17- β -glucuronosyl estradiol). However, the authors did not investigate the consequence of the combined amino acid substitutions resulting from these two SNPs (in complete linkage disequilibrium). Hamada et al. found that COS-7 cells transfected with the variant SLCO1B3 haplotype (334G-699A) had a significantly reduced ability to uptake testosterone as compared to cells transfected with the reference SLCO1B3 gene or a sequence containing either the 334G or the 699A SNP (7). In the present study, our SLCO1B3 plasmid integrated simultaneously the two SNPs and we obtained results in line with those of Hamada et al, with a decreased uptake of MPAG in cells transfected with the 334G-699A variant as compared to the reference sequence. Our clinical results are compatible with decreased activity of the OATP1B3 variant protein but surprisingly different from those recently published for the same drug in Japanese patients. Miura et al. described higher $dn-AUC_{6-12}$ (regarded a marker of MPA recirculation) in patients carrying the 334GG (or 699AA) genotype as compared to carriers of the 334TT genotype (6). Based on this pharmacokinetic observation, the authors proposed that MPA uptake into hepatocytes and excretion into bile might be increased in patients with SLCO1B3 334GG genotype. This is in contradiction with previous (12) and the present in-vitro evidence that the 334G allele reduces OATP1B3 activity. Also, the present results do not support this hypothesis as MPA is not a substrate of OATP1B3 in-vitro, and yet we found a lower dose-normalized MPA exposure in carriers of the 334GG genotype. It should be noted that Miura et al. recently reported, in accordance with our hypothesis, that patients homozygous for the SLCO1B3 334G-699A allele had significantly higher MPAG $dn-AUC_{0-12}$ than patients heterozygous or homozygous for the reference allele (5).

We observed an important decrease of MPA exposure in patients receiving MMF combined with cyclosporine as compared to tacrolimus or sirolimus. This difference is well-known and is thought to result from the inhibitory effect of cyclosporine on MPAG biliary excretion. The mechanisms presumably involved in this drug-drug interaction and in the pharmacogenetic association we observed are very close. It is even tempting to hypothesize that the inhibitory effect of cyclosporine might not only concern MRP2 (as generally admitted) but also OATP1B3. Indeed, in-vitro studies showed that cyclosporine is a potent inhibitor of OATP1B1 and OATP1B3 (13). This inhibitory effect was associated with drug-drug interactions of clinical relevance, in particular with statins (14 , 15).

Most of the pharmacogenetic studies performed regarding MMF focused on UGT enzymes or MRP2 but weak associations were mainly reported (see supplementary Table 1). In the present study, we tested the effect of SNPs previously associated with MPA pharmacokinetics using stringent criteria for statistical analysis (i.e., Bonferroni correction): none of the SNPs evaluated significantly

influenced MPA pharmacokinetics, whatever the comedication. It is possible that this absence of effect, in particular for UGT1A9 SNPs previously associated in-vitro with a decreased enzyme activity or expression (16, 17) is related to differences in the post-transplantation periods at which patients were studied. Association between the UGT1A9 C-2152T, T98C or UGT1A8 C518G SNPs were indeed observed in patients studied at early post-transplantation periods (<30 days) (18–20), when MPA apparent clearance (CL/F) is known to be higher than at later time-points (21), presumably owing to several time-dependent factors such as the renal function, albumin and haemoglobin levels, decreasing cyclosporine predose levels (22) and inductive effect of corticosteroids on UGT (23). However, this cannot explain the discrepancy with the significant influence of UGT1A9 C-440T/T-331C on MPA $dn-AUC_{0-12h}$ found by Baldelli et al. in stable renal transplants receiving a steroid-free regimen (9).

Our study certainly has some limitations: in addition to those mentioned above, potential covariates such as serum albumin, previously associated with MPAG disposition in renal transplants (9), could not be taken in account in multivariate analyses to ponder the associations (or the absence of associations) found here, contrary to what van Shaik et al. recently did (20), because they were not available in our databases.

Apart from the association of SLCO1B3 genotype with MPA pharmacokinetics (of which the relevance for MMF efficacy or tolerance remains to be investigated in a larger population), our study demonstrates for the first time that OATP proteins are involved in the pharmacokinetics of MPA. It gives new hints regarding the mechanism of drug-drug interactions, in particular with cyclosporine whose known effects on MPA and MPAG plasma levels are similar to those of the mutant SLCO1B3 allele herein.

In conclusion, the pharmacokinetics of MPA in stable renal transplant patients receiving a cyclosporine-free regimen is influenced by genetic polymorphisms of the hepatic uptake transporter OATP1B3, of which MPAG is a substrate. Cyclosporine might inhibit the transporter function and thus attenuate the effect of its polymorphism.

Methods

Cellular assays

SLCO1B3 -pcDNA5/FRT plasmids containing the reference sequence (NM_019844) or a sequence with two nucleotide exchanges corresponding to the SLCO1B3 T334G (rs4149117) and SLCO1B3 G699A (rs7311358) polymorphisms were constructed as described in supplementary data. SLCO1A2 -PSG5 and SLCO 757-PCMV6-XL4 plasmids were a generous gift from Theo J. Visser, Department of Internal Medicine, Erasmus University Medical Center.

Human embryonic kidney (HEK) 293 T/17 cell lines (American Type Culture Collection, ATCC) were routinely cultured at 37°C with 5% CO₂ and 95% humidity in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin. Cells were seeded in 10-cm dishes and transfected with 10µg of SLCOs cDNA (or empty vector), 20µl of lipofectamine LTX (Invitrogen, Carlsbad, CA) and 2ml of Optimem (Invitrogen). 24h after transfection, cells were trypsinized and seeded in 24-well poly-d-lysine-coated plates (BD Discovery Labware, Bedford, MA) at a density of 1.5×10⁵ cells/well in order to reach confluence within 24h for uptake assays.

Transport activities were analyzed through measuring intra-cellular accumulation of MPA and MPAG. (20µM). Uptake was initiated by adding the compounds in Hank's Buffer Salt Solution (HBSS, Invitrogen) after cells had been washed twice and preincubated with HBSS at 37°C for 15 min. After 5 to 10 minutes, the cells were washed twice with ice-cold uptake buffer, lysed in 250µl of NaCl 0.2N (1 hour at 4°C) and lysates neutralized by adding 25µl of 2N HCl. MPA and MPAG were determined using a previously described validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay (3). Results were normalized to per-well protein content as measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

OATP1B3-mediated net uptake kinetics was studied after 10 min of incubation with MPAG, after subtracting the uptake in empty vector-transfected cells. K_m and V_{max} were obtained by fitting the Michaelis–Menten equation (GraphPad Software, San Diego, CA).

Clinical pharmacogenetic analysis

A first group of 70 kidney transplant patients enrolled in one of three phase 4 clinical trials gave their informed consent for providing blood samples for pharmacogenetic investigations related to the pharmacokinetics of MMF. 42 patients received MMF in combination with sirolimus and 28 with tacrolimus. MMF was administered at an initial fixed dose of 1g twice daily, which was reduced if necessary based on clinical criteria. Tacrolimus was administered twice daily and sirolimus once daily, targeting a whole blood trough concentration between 0.05 to 0.015 mg/l and 0.010 to 0.012 mg/l, respectively. For MMF pharmacokinetics, blood samples were collected in each patient between 3 and 6 months post-grafting at 0, 20, 40, 60, 90, 120, 180, 240, 360 and 540 min after the morning dose. MPA and MPAG plasma concentrations were determined by use of a validated LC-MS/MS method (24). Area under the concentration-time curve (AUC_{0-12h}) were calculated using validated Bayesian models. In the absence of an adequate model for MPAG, AUC_{0-9h} were estimated using the linear trapezoidal rule. The plasma metabolic ratio of MPAG to MPA was estimated as the AUC_{0-9h} ratio. There was no

difference in renal function between patients receiving tacrolimus or sirolimus as assessed by comparing serum creatinine levels at the time of blood sampling ($p=0.7659$).

A second group of 115 kidney transplants out of 137 patients enrolled in the multicenter, open-label, randomized trial Apomygre (Clinical Trial Registry No. NCT0019967) provided written informed consent for pharmacogenetic investigations related to MMF pharmacokinetics. These patients received the drug in combination with cyclosporine. Study aim and design, patients population and primary results were recently published (25). Blood samples were collected at the same times as in the first group at 3 or 6 months post-transplantation. MPA AUC_{0-12h} were estimated using concentration at three sampling time (20, 60 and 180min) using previously published, validated Bayesian models (21, 26).

All these trials were approved by ethic committees.

There was no statistical difference in corticosteroid dose between patients co-treated with cyclosporine and tacrolimus or sirolimus at the time of blood sampling ($p>0.05$).

Patients' DNA was isolated from EDTA-treated blood as previously described in details (27) and was characterized for SNPs in *SLCO1B3*, *SLCO1B1*, *UGT1A8*, *UGT1A9* and *ABCC2* genes using validated TaqMan allelic discrimination assays on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France).

Statistical analysis

Pairwise linkage disequilibrium between polymorphisms was evaluated by means of D' and r^2 (calculated using Thesias® software (28)) and patients' most probable haplotypes inferred using the PHASE program (29). Categorical variables were compared using the Fisher's exact test and continuous variables using the Kruskal–Wallis or Mann-Whitney tests, or ANOVA when studying the interaction between variables. A Bonferroni correction was applied leading to a p threshold for significance of 0.005 (analysis of 10 SNPs). All statistical comparisons were performed using the Stat View program (SAS Institute, Cary, NC, version 5.0).

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Footnotes:

Conflict of Interest/Disclosure: The authors declared no conflict of interest.

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Figure 1

Mycophenolic acid (MPA; a) and mycophenolic acid phenyl-glucuronide (MPAG; b) (20 μ M) uptake into HEK293T cells transiently transfected with OATP1B1, 1B3 and 1A2. Empty vector (EV) transfected cells served as control. Data are mean \pm SD of three independent experiments performed in triplicate. Uptake time was 5 min. (*p<0.05 for comparison with control cells).

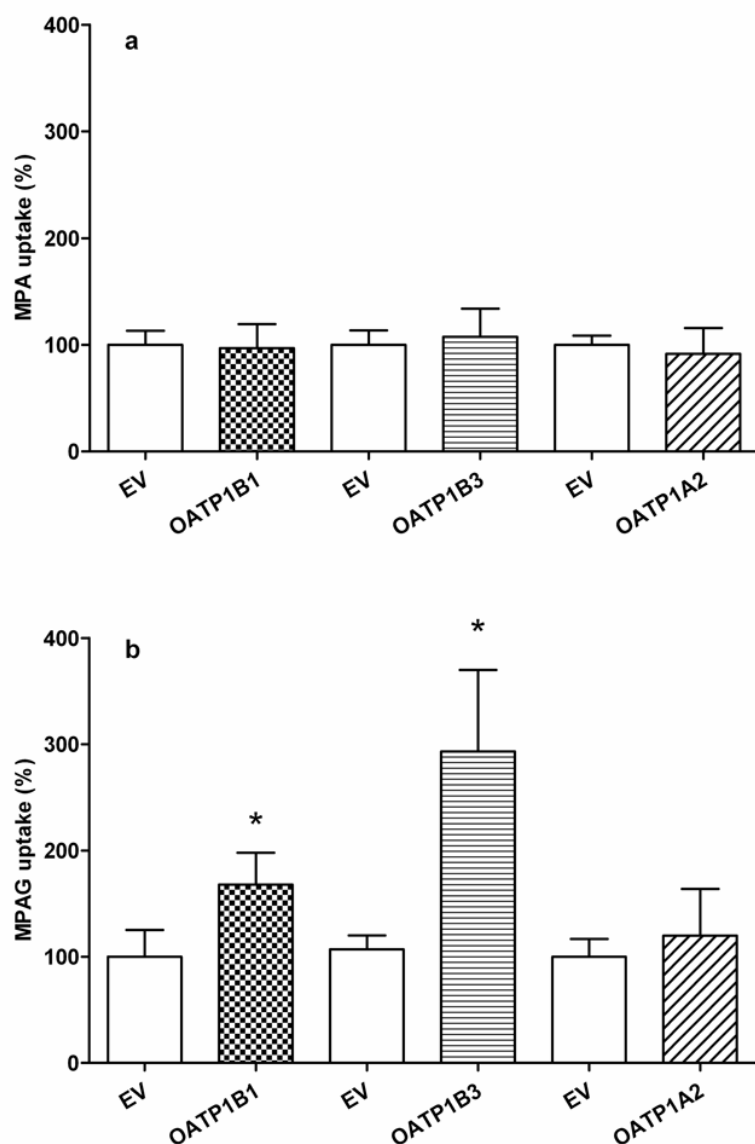


Figure 2

Mean \pm SEM of dose normalized concentration-time profiles of mycophenolic acid (MPA; a), MPA-phenyl glucuronide (MPAG; b) and distribution of MPA to MPAG metabolic ratio (MPAG/MPA AUC_{0-9h} ratio; c) as a function SLCO1B3 T334G genotype in patients co-treated with tacrolimus or sirolimus. In panel c, the lines represent the mean values. * $p < 0.05$; ** $p < 0.005$

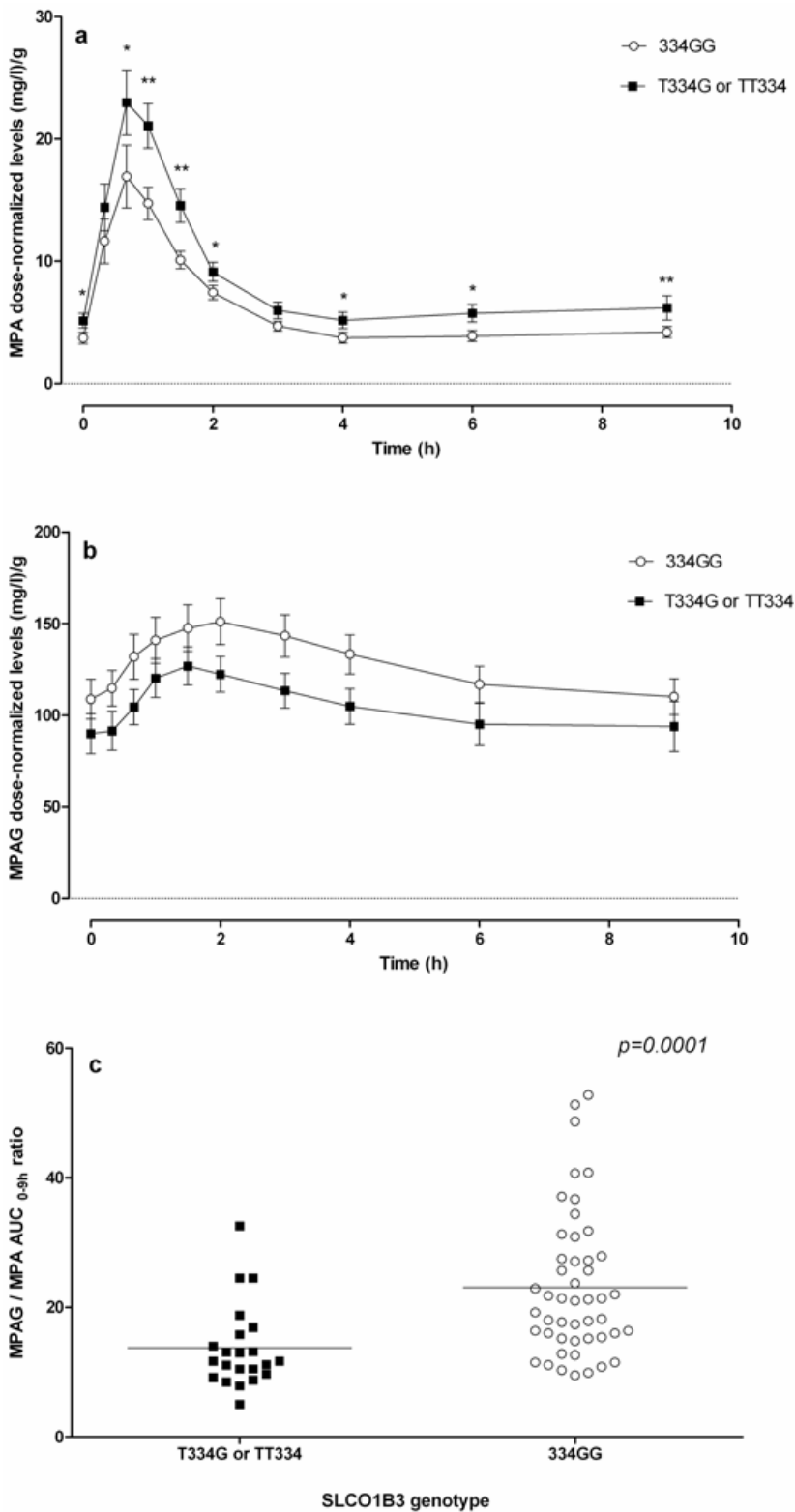


Figure 3

Kinetics of mycophenolic acid phenyl-glucuronide (MPAG) transport by reference (T334-G699) and variant (334G-699A OATP1B3). HEK293 cells transiently expressing either the OATP-reference or the OATP-variant proteins were incubated in the presence of increasing concentrations of MPAG for 10 min. Data were fitted to the Michaelis-Menten equation after subtracting background uptake. Data points represent the mean \pm SD from n=3 experiments.

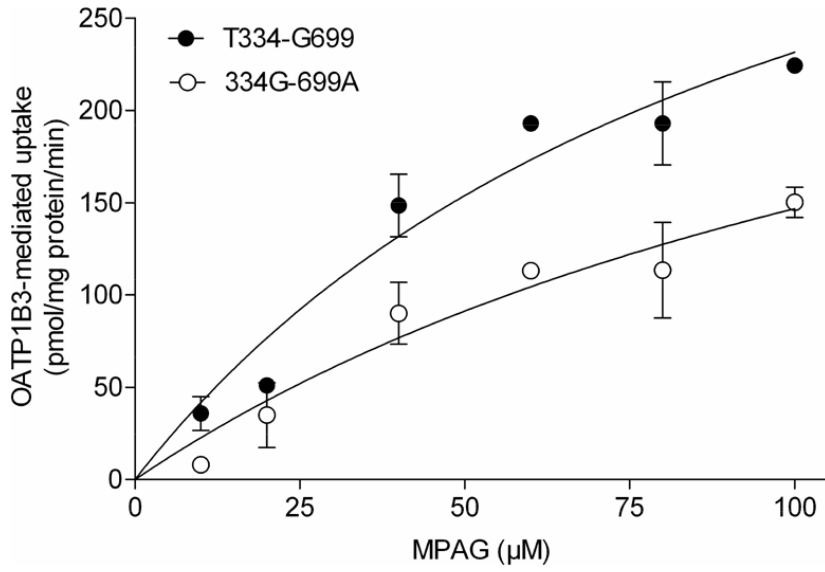


Table 1Mycophenolic acid (MPA) dose-normalized (dn) AUC_{0-12h} according to SLCO , UGT or ABCC2 Single Nucleotide Polymorphisms (SNPs) in patients co-treated with cyclosporine

Gene	SNP	Patients genotypes (n)	MPA dn-AUC _{0-12h} (mg.h/l)/g	P values
SLCO1B3	T334G	TT (n=6)	42.7±18.4	0.4747
		TG (n=32)	48.6±22.9	
		GG (n=76)	42.7±20.2	
	G767C	GG (n= 79)	47.1±23.2	0.0469
		GC (n= 36)	38.1±12.4	
	T521C	TT (n=75)	44.8±20.7	0.7507
		TC (n=36)	43.7±22.1	
		CC (n=4)	38.6±10.9	
SLCO1B1	A388G	AA (n=37)	45.5±17.1	0.2800
		AG (n=52)	44.1±22.2	
		GG (n=26)	42.8±23.3	
	C463A	CC (n=83)	43.6±18.3	0.9717
		CA (n=30)	46.3±27.0	
		AA (n=2)	42.8±25.0	
UGT1A8	C518G	CC (n=59)	43.7±18.9	0.9066
		CG (n=50)	44.1±22.1	
		GG (n=6)	51.4±29.3	
	C-440T	CC (n=69)	40.8±15.3	0.2721
		CT (n=39)	49.2±27.1	
		TT (n=7)	50.7±24.0	
UGT1A9	T-275A and/or C-2152T*	TTandCC(n=101)	44.1±21.2	0.4498
		TAandCT or CC(n=12)	47.8±19.1	
		AA and TT (n=2)	31.8±7.9	
	T98C	TT (n=105)	43.7±21.1	0.0634
		TC (n=10)	50.7±17.1	
ABCC2	C-24T	CC (n=68)	40.2±14.5	0.0087
		CT (n=35)	53.6±26.0	
		TT (n=12)	39.9±26.7	

* SNPs in strong linkage disequilibrium: $D'=1$; $r^2=0.87$

Table 2

Pharmacokinetic parameters of mycophenolic acid (MPA) and its glucuronide (MPAG) according to SLCO Single Nucleotide Polymorphisms (SNPs) in patients co-treated with tacrolimus or sirolimus

Gene	SNP	Genotypes (n)	MPA dn-C ₀ (mg/l)/g	MPA dn-C _{max} (mg/l)/g	MPA T _{max} (min)	MPA dn-AUC _{0-12h} (mg.h/l)/g	MPAG dn-C ₀ (mg/l)	MPAG dn-C _{max} (mg/l)	MPAG T _{max} (min)	MPAG dn-AUC _{0-9h} (mg.h/l)
T334G		TT (n= 2)	3.4±0.6	21.4±10.5	65.0±35.3	73.9±30.2	79.6±42.7	111.6±32.8	90.0±42.4	621.7±235.3
		TG (n=20)	5.3±2.8	28.6±10.7	71.5±111.6	82.6±32.6	91.0±51.0	137.4±54.0	114.5±112.1	964.8±457.6
		GG (n=48)	3.7±3.1	20.6±16.7	69.6±86.7	57.5±30.2	108.8±74.7	173.7±98.7	123.3±75.6	1146.2±644.2
		p values	0.0413	0.0012	0.8113	0.0040	0.6296	0.3664	0.3297	0.2477
SLCO1B3		p values (TT+TG vs GG)	0.0150	0.0004	0.8233	0.0009	0.3774	0.1947	0.1364	0.2394
G767C		GG (n=49)	4.4±3.2	23.5±13.2	63.5±74.5	68.5±33.4	103.9±72.1	157.2±82.7	117.9±88.1	1042.6±524.5
		GC (n=21)	3.7±2.7	21.5±19.8	85.2±126.0	57.4±30.0	100.9±60.2	171.7±101.7	124.3±83.5	1165.1±741.9
		p values	0.3153	0.1644	0.9183	0.2234	0.9365	0.5010	0.6570	0.4769
T521C		TT (n=47)	3.6±2.3	22.1±17.0	64.0±75.7	60.0±30.0	112.3±76.3	170.0±96.7	116.6±84.1	1137.9±639.8
		TC (n=23)	5.3±4.1	24.6±11.6	82.2±121.0	75.8±35.6	84.8±44.7	144.3±66.6	126.5±91.8	959.7±482.2
		p values	0.0942	0.0902	0.6753	0.0534	0.1168	0.2685	0.9695	0.2578
SLCO1B1 A388G		AA (n=19)	4.0±2.5	18.5±9.0	80.0±115.2	58.2±30.4	123.9±93.7	167.8±95.9	117.4±114.9	1079.4±545.2
		AG (n=43)	4.0±3.3	23.7±16.3	71.6±89.9	65.9±33.0	91.0±55.7	156.6±87.1	127.4±75.5	1054.0±634.9
		GG (n=8)	5.2±3.1	29.4±20.5	37.5±16.7	77.9±34.8	118.9±52.3	173.2±85.5	85.0±58.1	1164.6±542.6
		p values	0.4849	0.1966	0.1858	0.2692	0.0883	0.6920	0.2154	0.5720
C463A		CC (n=52)	3.9±2.3	23.3±15.1	74.8±106.4	65.7±33.0	96.7±70.2	154.2±86.1	119.2±90.5	1015.1±562.9
		CA (n=17)	4.6±4.6	22.1±16.9	58.2±21.9	63.5±33.2	119.5±62.6	185.2±96.2	127.6±72.3	1291.0±672.6
		AA (n=1)	8.6	18.3	20.0	62.9	137.0	145.6	20.0	824.9
		p values	0.2799	0.7794	0.1598	0.9049	0.1393	0.3723	0.1249	0.1680

dn: dose-normalized

Table 3

Pharmacokinetic parameters of mycophenolic acid (MPA) and its glucuronide (MPAG) according to UGT or ABCC2 Single Nucleotide Polymorphisms (SNPs) in patients co-treated with tacrolimus or sirolimus

Gene	SNP	Patients genotypes (n)	MPA dn-C ₀ (mg/l)/g	MPA dn-C _{max} (mg/l)/g	MPA T _{max} (min)	MPA dn-AUC _{0-12h} (mg.h/l)/g	MPAG dn-C ₀ (mg/l)	MPAG dn- C _{max} (mg/l)	MPAG T _{max} (min)	MPAG dn-AUC _{0-9h} (mg.h/l)
	C-440T (T-331C)	CC (n=39)	4.1±3.7	22.0±18.3	75.6±93.8	63.1±35.0	108.5±88.3	171.9±104.2	130.0±95.1	1143.5±692.2
		TC (n=27)	4.1±2.3	23.3±11.3	68.5±97.4	68.7±30.1	95.0±50.6	144.7±59.0	111.5±76.5	974.3±429.7
		TT (n=4)	4.4±2.1	29.0±6.7	25.0±10.0	61.5±17.7	102.2±46.6	174.7±86.4	77.5±35.0	1163.1±559.7
		p values	0.5049	0.0753	0.0330	0.5080	0.7807	0.7867	0.3018	0.6777
UGT1A9	T-275A or C-2152T*	TT(CC) (n=63)	4.3±3.1	23.3±15.8	71.9±97.2	65.7±30.4	105.8±69.8	163.4±90.4	118.4±88.9	1090.6±605.2
		TA (CT) (n=7)	2.8±2.8	19.3±11.7	52.8±22.1	60.3±51.2	79.7±50.6	144.9±69.9	132.8±59.6	978.2±523.4
		p values	0.0912	0.3325	0.8469	0.1799	0.5119	0.9143	0.2858	0.9143
		T98C	TT (n=68)	4.1±3.0	23.3±15.4	70.1±93.8	66.2±32.5	103.7±68.9	163.4.2±88.7	119.0±87.2
TC (n=2)	6.1±6.5		8.9±3.1	65.0±35.3	30.9±8.7	78.9±38.3	98.0±51.0	150.0±42.4	745.6±371.3	
p values	0.5671		0.0344	0.5957	0.0570	0.6764	0.2172	0.2358	0.4175	
UGT1A8	C518G	CC (n=40)	4.2±3.2	25.4±18.1	69.0±94.5	66.8±29.6	105.1±75.5	166.8±95.4	109.7±73.8	1066.9±587.5
		CG (n=26)	4.5±3.0	21.0±9.8	73.5±97.8	67.9±36.8	105.1±60.9	160.7±41.9	139.6±105.6	1040.4±643.9
		GG (n=4)	1.1±0.4	10.1±6.0	57.5±28.7	30.9±8.3	68.4±30.5	114.2±41.8	92.5±37.7	807.7±263.5
		p values	0.0130	0.0368	0.4694	0.0171	0.5331	0.5350	0.5786	0.6701
ABCC2	C-24T	CC (n=48)	4.1±3.2	22.7±17.6	60.4±51.6	64.1±33.0	98.4±72.5	158.1±93.7	127.1±78.0	683.1±299.7
		CT (n=19)	4.7±3.0	23.0±9.8	96.8±157.5	67.4±33.6	112.8±60.1	165.1±75.9	103.15±110.05	1107.0±648.1
		TT (n=3)	2.6±1.2	26.2±4.7	53.3±11.5	68.0±27.7	116.2±49.4	193.9±95.4	110.0±17.3	1341.8±577.8
		p values	0.5066	0.1804	0.7746	0.8393	0.3411	0.2371	0.0913	0.3835

* SNPs in complete linkage disequilibrium