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Relationship between in vivo CYP3A4 activity, CYP3A5 genotype and systemic tacrolimus metabolite/parent drug ratio in renal transplant recipients and healthy volunteers.

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Running title: CYP3A4 activity, CYP3A5 genotype and tacrolimus metabolites

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Abbreviations

ALT	Alanine aminotransferase
AUC	Area under the curve
CL/F	Apparent oral clearance
Cmax	Maximum blood concentration
CYP3A	Cytochrome P450 3A
DMT	Desmethyl tacrolimus
eGFR	Estimated glomerular filtration rate
HOMDZ	Hydroxy midazolam
HT	Hydroxy tacrolimus
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MDZ	Midazolam

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P-gp	P-glycoprotein
SD	Standard deviation
SNP	Single nucleotide polymorphism
Tmax	Time to reach maximum blood concentration

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Abstract

CYP3A5 genotype is a major determinant of tacrolimus clearance, and has been shown to affect systemic tacrolimus metabolite/parent ratios in healthy volunteers, which may have implications for efficacy and toxicity. In a cohort of 50 renal transplant recipients who underwent quantification of CYP3A4 activity using the oral midazolam drug probe, we confirmed that CYP3A5 genotype is the single most important determinant of tacrolimus metabolite/parent ratio (CYP3A5 expressors displayed 2.7- and 2-fold higher relative exposure to 13-desmethyltacrolimus [DMT] and 31-DMT, respectively, P<0.001). There was, however, no relationship between CYP3A4 activity and tacrolimus metabolite/parent ratios. Additional analyses in 16 healthy volunteers showed that dual pharmacological inhibition of CYP3A4 and P-glycoprotein using itraconazole resulted in increased tacrolimus metabolite/parent ratios (+65%, +112% and 25% for 13-, 15- and 31-DMT, respectively, P<0.01). This finding was confirmed in a cohort of 9 renal transplant recipients who underwent tacrolimus pharmacokinetic assessments before and during CYP3A4 inhibition (58% increase in overall metabolite/tacrolimus ratio, P=0.017).

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1. Introduction

Tacrolimus, a calcineurin inhibitor used to prevent rejection in solid organ transplantation, is a dual substrate for the cytochrome P450 iso-enzymes 3A4 and -3A5 (collectively referred to as CYP3A) and the P-glycoprotein (P-gp) efflux pump (encoded by the ATP-binding cassette subfamily B member 1 [*ABCB1*] gene). CYP3A and P-gp are present in enterocytes (limiting intestinal uptake) and hepatocytes (contributing to first-pass metabolism and systemic clearance). The vast majority of systemically absorbed tacrolimus undergoes hepatic biotransformation to metabolites that are excreted in the faeces, while renal metabolism and excretion account for less than 5% of parent compound (Möller *et al.*, 1999). Between- and within-subject differences in the activity of CYP3A (and probably of P-gp) contribute significantly to tacrolimus' high pharmacokinetic and pharmacodynamic variability (Thomas Vanhove *et al.*, 2016). CYP3A5 expression and activity in intestine, liver, kidney and other organs is strongly reduced in carriers of two loss-of-function *CYP3A5* alleles *(CYP3A5*3*, **6* or **7*). In these 'CYP3A5 non-expressors', tacrolimus apparent oral clearance (CL/F) is about 2.5-fold lower compared with carriers of at least one active *CYP3A5*1* allele ('CYP3A5 expressors') (de Jonge *et al.*, 2013). The effect of *CYP3A5* genotype is not limited to an increased rate of metabolic clearance. As Zheng et al. have demonstrated (Zheng *et al.*, 2012), CYP3A5 genotype also strongly influences relative systemic exposure to tacrolimus metabolites and likely influences local tissue concentrations of tacrolimus (and its metabolites). In a study in 24 healthy volunteers, systemic metabolite/parent area under the curve (AUC) ratios for the primary metabolites 13-*O*-desmethyl tacrolimus (13-DMT, M1), 31-*O*-desmethyl tacrolimus (31-DMT, M2) and 12-hydroxy tacrolimus (12-HT, M4) were 2.0 to 2.7-fold higher in CYP3A5 expressors (Zheng *et al.*, 2012). Furthermore, a semiphysiological model predicted 47% lower tacrolimus exposure in renal epithelium

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(normalized to blood AUC) in CYP3A5 expressors compared with CYP3A5 non-expressors. The fact that systemic and intrarenal exposure to tacrolimus and its primary metabolites are related to CYP3A5 genotype could have pharmacodynamic (i.e. efficacy) and toxicodynamic implications. It has, for example, been shown that the immunosuppressive activity of 31-DMT is comparable to that of tacrolimus (Iwasaki *et al.*, 1995). Whether tacrolimus metabolites are nephrotoxic has not been studied, although the nephrotoxicity of several metabolites of the related calcineurin inhibitor ciclosporin is well established *in vitro* and in animal models; some evidence suggests it may also be relevant in humans (Copeland *et al.*, 1990; Radeke *et al.*, 1991; Stephens *et al.*, 2006).

It is unclear to what extent tacrolimus metabolite/parent ratio varies in a relevant population of renal transplant recipients and how much *CYP3A5* genotype contributes to interindividual variability in metabolite exposure in such a population. Particularly since the allelic frequency of *CYP3A5*1* is only 5-15% in Caucasians (International HapMap Project, n.d.), variability in other determinants of tacrolimus metabolism, namely the activity of CYP3A4, may also contribute to differences in relative metabolite exposure. We have previously shown that *in vivo* CYP3A4 activity, assessed using the oral midazolam (MDZ) probe, explains up to 30% of interindividual variability in steady-state tacrolimus clearance (de Jonge *et al.*, 2012). The goals of this study, therefore, were (1) to determine the degree of interindivual variability in tacrolimus metabolite/parent ratio in a population of renal transplant recipients, (2) to analyze whether this variability can be related to differences in CYP3A4 activity, and (3) to assess whether combined pharmacological inhibition of CYP3A4 and P-gp can be used to influence tacrolimus metabolite/parent ratio.

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2. Materials and methods

2.1 Study populations

Two cohorts were analyzed. The principal cohort (A) comprised 50 renal allograft recipients in tacrolimus steady state, in which AUC_{0-8} profiles of midazolam, tacrolimus and their primary metabolites were determined. The goal of this analysis was to determine whether interindividual differences in CYP3A4 activity are related to interindividual differences in tacrolimus metabolite/parent ratio. Cohort B addressed the question of whether tacrolimus metabolite/parent ratio can be influenced by pharmacological inhibition of CYP3A4 activity. In this study, 16 male healthy volunteers underwent AUC_{0-24} profiles for MDZ and (single dose) tacrolimus before and immediately after administration of the potent CYP3A4/P-gp inhibitor itraconazole. Methodological details for both cohorts are provided below.

Cohort A was a subgroup of patients from a previously reported prospective single center study (Vanhove *et al.*, 2017). Stable renal allograft recipients ≥ 1 year posttransplant treated with once-daily tacrolimus (Advagraf®, Astellas Pharma Europe, Staines, UK) were asked to participate. Exclusion criteria were age less than 18 years, combined organ transplants (except kidney-pancreas), women of childbearing potential not using acceptable contraceptive measures, pregnant women, hemoglobin < 8 g/dl, albumin < 25 g/l, intestinal malabsorption, liver cirrhosis, alanine aminotransferas (ALT) or bilirubin > 2 x upper limit of normal, estimated glomerular filtration rate < 15 ml/min (eGFR, calculated from serum creatinine using the modified Modification of Diet in Renal Disease (MDRD) equation) (Levey *et al.*, 1993), change in tacrolimus dose in the 3 days prior to the study, documented noncompliance, addiction to any known drug or alcohol, known allergy or intolerance to MDZ or fexofenadine and use of a moderate or potent CYP3A4

inhibitor or inducer. Patients were asked to abstain from alcohol use 1 week prior to the study and from any fruit juice, grapefruit or pomelo 3 days prior to the study. The details of the pharmacokinetic study protocol have been previously described (Vanhove *et al.*, 2017). Briefly, patients presented at the outpatient clinic after an overnight fast. At 8:00 AM, patients were orally administered 2 mg of MDZ (2 ml of a 1 mg/ml MDZ solution, Dormicum, Roche, Basel, Switzerland) in 30 ml of a 5% glucose solution, their usual dose of tacrolimus and other immunosuppressive medication, followed by 100ml of water to rinse the glass. All other medication was ingested at 10:00 AM, followed by a standard breakfast. Two 4-ml EDTA blood tubes were collected immediately before and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 8h after probe drug administration.

Cohort B was a prospective single-arm open-label study. Sixteen healthy adult non-smoking males with no significant medical history were recruited. None of the subjects had taken any CYP3A4/5 interfering medication for at least 1 month prior to the start of and during the study. All subjects abstained from alcohol, grapefruit or pomelo from 7 days prior to the start of the study until study conclusion. On days 1 and 6, subjects were administered a single oral dose of 3mg tacrolimus (Advagraf®, Astellas Pharma Europe, Staines, UK) and 2mg of MDZ (2ml of a 1 mg/ml solution of Dormicum®; Roche, Basel, Switzerland) with 250 ml of water in the morning after an overnight fast. Two 4-ml blood samples were collected in ethylenediaminetetraacetic acid-containing tubes before and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24h after drug administration. One whole blood sample was stored at -80°C pending tacrolimus metabolite quantification. The other sample was centrifuged for 10 minutes at 1,860g and 4°C, after wich plasma was stored at -80°C pending MDZ quantification. On days 2 through 5, two hundred mg of itraconazole (2 tablets of 100mg Sporanox®; Janssen-Cilag, Beerse, Belgium) was administered twice daily with 250ml of water, 2 hours before breakfast/dinner.

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All parts of this study were performed according to the latest version Declaration of Helsinki and were approved by the ethics committee of the University Hospitals Leuven (ML5159; S51157; S58603) and the Belgian Federal Agency for Medicines and Health Products (EudraCT 2008-004158-33 and 2015-004518-74, https://eudract.ema.europa.eu). All participants provided written informed consent.

2.2 Analytical methods

Plasma concentrations of MDZ and its major metabolites 1'-hydroxyMDZ and 4-hydroxyMDZ were measured using a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, as previously described (de Loor *et al.*, 2011). Whole blood concentrations of tacrolimus and its major metabolites 13-DMT, 15-DMT and 31-DMT were measured using an ultraperformance LC-MS/MS method, as described in the online Supplemental Material. Tacrolimus and its metabolites form ionic adducts with eiter ammonium or sodium. Only the adduct which was most abundant and most stable across measurements was used for analysis. For tacrolimus, 15-DMT and 31-DMT, this was the ammonium adduct. For 13-DMT, this was the sodium adduct.

2.3 Genotyping

Genomic DNA was isolated from whole blood samples using a salting out procedure (Miller *et al.*, 1988). The quantity and quality of genomic DNA were verified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) before being assayed on an OpenArray platform (Life Technologies, Carlsbad, California, USA). Participants were genotyped for the the following single nucleotide polymorphisms (SNPs), among others: *CYP3A5*1* (rs776746); *CYP3A4*22* (rs35599367); *ABCB1* 3435C>T

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(rs1045642), 2677G>T/A (rs2032582) and 1236C>T (rs1128503). Hardy weinberg equilibrium and linkage disequilibrium between SNPs were assessed using Haploview (Barrett *et al.*, 2005). Haplotypes were inferred using the program PHASE version 2.1 (Stephens *et al.*, 2001). *ABCB1* SNPs were grouped into 4 diplotype categories based on the presence of wild type CGC and triple mutated (presumed loss-of-function) TTT haplotypes: CGC-CGC, CGC-TTT, TTT-TTT and 'other' (containing 1 or more other haplotypes) (T Vanhove *et al.*, 2016).

2.4 Pharmacokinetic and statistical analysis

Data are presented as mean ± standard deviation (SD) except when stated otherwise. Normality was tested using the Shapiro-Wilk test. AUC, Cl/F, Cmax and $AUC_{metabolite}/AUC_{tacrolimus}$ values were not normally distributed and Ln-transformed for analysis. Differences between means were assessed using independent samples t-test for normally distributed continuous data (ANOVA in case of more than 2 categories of the predictor variable) and using Mann-Whitney U test for ordinal data. Since steady-state exposure to tacrolimus and its primary metabolites was the outcome measure of interest, no compartmental analysis was performed. Independent predictors of tacrolimus metabolite/parent AUC ratio were determined using multivariable linear regression. The following variables were entered as possible predictors of metabolite/parent ratio: age, time after transplantation, hematocrit, actual body weight, presence of diabetes mellitus, *CYP3A5* genotype, *ABCB1* functional diplotype group, MDZ CL/F, $AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$ ratio and tacrolimus CL/F (the latter not included in the same model as MDZ CL/F), followed by stepwise removal of non-significant predictor variables. A two-sided p-value <0.05 was considered statisically significant. All reported R^2 values are semipartial. Statistical analyses were performed using IBM SPSS Statistics version 22 (IBM, New York City, NY,

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USA). Calculation of NCA pharmacokinetic parameters and figure generation was performed using Graphpad Prism version 6 (Graphpad Prism, La Jolla, CA, USA).

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3. Results

3.1 Tacrolimus metabolite/parent ratio is related to CYP3A5 genotype, but not to baseline CYP3A4 activity

First, the relationship between CYP3A5 genotype, CYP3A4 activity and systemic tacrolimus metabolite/parent ratio was examined in a cohort of 50 tacrolimus-treated renal recipients under steady-state conditions (cohort A). Characteristics of cohort A are summarized in Supplemental Table S1. The *CYP3A5*1* allele was present in 10 renal recipients (i.e. 20% expressors). One CYP3A5 non-expressor carried the *CYP3A4*22* allele. Non-compartmental analysis (NCA) pharmacokinetic parameters of MDZ, tacrolimus and their primary metabolites are presented in Table 1. Mean concentration-time profiles are shown in Figure 1.

MDZ CL/F did not differ between CYP3A5 expressors and non-expressors, in agreement with previous reports (Kharasch *et al.*, 2007; de Jonge *et al.*, 2013). As expected, mean tacrolimus CL/F was 2-fold higher in CYP3A5 expressors, whose tacrolimus AUC was similar to that of non-expressors despite a daily tacrolimus dose that was 2.2-fold higher on average. Independent predictors of tacrolimus CL/F in multivariable regression were CYP3A5 genotype (B=0.67, R^2=0.349, P<0.001), MDZ CL/F (B=0.55, R^2=0.223, P<0.001), hematocrit (B=-3.40, R^2=0.061, P=0.001) and weight (B=0.01, R^2=0.036, P=0.019; overall R^2=0.669). When limiting the analysis to CYP3A5 non-expressors, predictors of tacrolimus CL/F were MDZ CL/F (B=0.65, R^2=0.433, P<0.001) and hematocrit (B=-3.14, R^2=0.100, P=0.005; overal R^2=0.533). In CYP3A5 expressors, the only predictor of tacrolimus CL/F was weight (B=0.009, R^2=0.435, P=0.023).

Relative systemic exposure to 13-DMT and 31-DMT, measured using $AUC_{metabolite}/AUC_{tacrolimus}$, was 178% and 100% higher in CYP3A5 expressors compared with CYP3A5 non-expressors (P<0.001); $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ did not differ between

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CYP3A5 expressors and non-expressors. The overall result was a 94% higher $AUC_{metabolites}/AUC_{tacrolimus}$ ratio in CYP3A5 expressors (P<0.001). Within CYP3A5 non-expressors, the range of observed metabolite/parent ratios was relatively narrow (range 1.9 – 6.7% for $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ and 0.1 – 0.5% for $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$, although it was 0.2 – 5.0% for $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$). Tacrolimus metabolite/parent ratios did not differ between *ABCB1* functional diplotype categories (P ≥ 0.4 for all analyses).

There was a consistently negative correlation between MDZ CL/F and all tacrolimus metabolite/parent ratios, but this only reached statistical significance for $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ in the subgroup of CYP3A5 non-expressors (R=-0.38, P=0.015). No significant correlation was observed between MDZ CL/F and $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ or $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ in either CYPA5 non-expressors or expressors, as shown in Figure 2 and Supplemental Table S2. Similarly, there was no significant correlation between MDZ metabolite/parent ratios ($AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$ or $AUC_{4\text{-}HOMDZ}/AUC_{MDZ}$) and the different $AUC_{metabolite}/AUC_{tacrolimus}$ ratios (data not shown). Tacrolimus CL/F was moderately correlated with $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ (r=0.627, P<0.001) and $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ (r=0.386, P=0.006), indicating that relative exposure to 13-DMT and 31-DMT increased in parallel with higher tacrolimus clearance (Figure 2). This effect was largely driven by high tacrolimus CL/F values in the CYP3A5 expressors. In the subgroup of CYP3A5 non-expressors, the association between $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ and tacrolimus CL/F was weaker (r=0.397, P=0.011) and that between $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ was reversed (r=-0.384, P=0.014). In multivariable analysis, CYP3A5 genotype was the dominant determinant of $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ and $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$, explaining 64.8% and 59.5% of their interindividual variability, respectively (see Supplemental Table S3).

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3.2 Itraconazole increases the tacrolimus metabolite/parent ratio

Next, we studied whether pharmacological CYP3A4/P-gp inhibition alters the tacrolimus metabolite/parent ratio in a cohort of 16 healthy volunteers treated with itraconazole (cohort B). Characteristics of cohort B are presented in Supplemental Table S4. Pharmacokinetic parameters of tacrolimus and its 3 primary metabolites before and during CYP3A4 inhibition are shown in Table 2.I; mean blood concentration-time profiles are presented in Figure 3. Metabolite/parent ratios for 13-DMT and 31-DMT were higher in CYP3A5-expressing healthy volunteers (see Supplemental Table S5), analogous to the renal transplant recipients in cohort A. Mean tacrolimus $AUC_{0\text{-}24}$ increased 3.3-fold as a result of itraconazole treatment. Metabolite/parent ratios for all 3 metabolites were significantly increased during CYP3A4/P-gp inhibition (plotted in Figure 4). This increase in relative exposure averaged 1.7-fold, 2.1-fold, 1.3-fold and 1.8-fold for 13-DMT, 15-DMT, 31-DMT and all 3 metabolites combined, respectively ($P<0.01$ for all). There was a numerically smaller increase in $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ resulting from itraconazole treatment in CYP3A5 expressors vs. CYP3A5 non-expressors but this difference was not statistically significant (68±30% vs. 132±66%, $P=0.077$). Similarly, there was no difference between CYP3A5 expressors and non-expressors with regard to changes in tacrolimus AUC ($P=0.944$), $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ ($P=0.353$) or $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ ($P=0.878$). Baseline $AUC_{metabolites}/AUC_{tacrolimus}$ was strongly correlated to the degree of change (delta) in $AUC_{metabolites}/AUC_{tacrolimus}$ resulting from itraconazole inhibition ($R=0.67$, $P=0.005$; see Figure 5).

Contrary to tacrolimus, relative exposure to MDZ metabolites decreased as a result of CYP3A4 inhibition. $AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$ was 91% lower during CYP3A4 inhibition (see Table 2). No adverse events occurred in any of the study cohorts; no subjects withdrew from the study. Because cohort B differs significantly from a clinical setting (particularly,

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lack of steady state resulting from administration of single doses of tacrolimus), these analyses were repeated on a cohort of 9 renal transplant recipients co-treated with tacrolimus and a variety of moderate to potent CYP3A4 inhibitors for clinical reasons (cohort C), detailed in the online supplemental material.

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4. Discussion

In an analysis of 50 tacrolimus-treated renal transplant recipients in steady state (cohort A), *CYP3A5* genotype was the single most important determinant of systemic tacrolimus metabolite/parent ratio. Compared with CYP3A5 non-expressors, CYP3A5 expressors displayed 2.7- and 2-fold higher relative exposure to 13-DMT and 31-DMT, respectively, while relative exposure to 15-DMT was identical. These findings are in close agreement with the observations made by Zheng et al. in 24 healthy volunteers administered a single dose of tacrolimus (Zheng *et al.*, 2012). In the current population of renal transplant recipients in steady state, interindividual variability in tacrolimus metabolite/parent ratios was relatively limited. Furthermore, around 60% of interindividual variability in the metabolite/parent ratio of 13-DMT and 31-DMT was explained by CYP3A5 genotype, with only minimal overlap between CYP3A5 expressors and non-expressors (see Figure 2).

To our knowledge, this is the first study to investigate the relationship between *in vivo* combined intestinal and hepatic CYP3A4 activity, assessed using the oral MDZ probe, and relative systemic exposure to the metabolites of a CYP3A4 substrate such as tacrolimus. MDZ CL/F varied considerably (more than 6-fold) between renal transplant recipients and explained 43% of interindividual variability in tacrolimus CL/F in CYP3A5 non-expressors, in line with previous observations (de Jonge *et al.*, 2012) and confirming that variability in CYP3A4 activity is a major determinant of variability in tacrolimus CL/F. However, this 'baseline' CYP3A4 activity bore little relationship to tacrolimus metabolite/parent ratios. MDZ CL/F was negatively correlated with metabolite/parent ratios, but this was only statistically significant in CYP3A5 non-expressors for 31-DMT, a quantitatively minor metabolite with concentrations 10- to 20-fold lower than those of 13-DMT and 15-DMT. To further elaborate on this relationship, analyses were performed

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in healthy volunteers (cohort B) which demonstrated that administration of a dual CYP3A4/P-gp inhibitor significantly increased metabolite/parent ratios of all 3 primary tacrolimus metabolites. This observation suggests that itraconazole has a more pronounced impact on the clearance of the metabolites than on the clearance of tacrolimus.

The underlying mechanisms for the increased metabolite/parent ratios, both in CYP3A5 expressors and after CYP3A4/P-gp inhibition, remain speculative. In CYP3A5 expressors the significantly higher metabolite/parent ratios for 13-DMT and 31-DMT indicate that enhanced formation of these metabolites is not compensated for by a proportional increase in their elimination rates. This implies that 13-DMT and 31-DMT display non-linear pharmacokinetic behavior, or a proportional decrease in clearance (i.e. elimination capacity) with increasing metabolite levels, possibly related to different affinities of tacrolimus and its metabolites for the catalytic site of CYP3A5. This may be especially relevant for CYP3A5, as it is a much less abundant enzyme compared to CYP3A4 and therefore more susceptible to enzymatic saturation, particularly when parent and metabolites are competing. It is also possible that partial saturation of biliary excretion of metabolites occurs when metabolite generation rate is increased (i.e. in CYP3A5 expressors). Indeed, tacrolimus is almost exclusively eliminated through biliary excretion of its metabolites (rather than the unchanged parent molecule) (Möller *et al.*, 1999). The observations that biliary concentrations of primary tacrolimus metabolites are significantly higher than those of tacrolimus (Shimomura *et al.*, 2008) and that cholestasis results in accumulation of second-order metabolites (Gonschior *et al.*, 1996) suggest that (P-gp mediated) biliary excretion of primary metabolites may contribute more to their clearance than further metabolism to second- and third-order metabolites. If P-gp mediated biliary excretion were to be the rate-limiting step in tacrolimus metabolite

elimination and a source of non-linear pharmacokinetic behavior, this would also imply that the increase in metabolite/parent ratio resulting from itraconazole administration may primarily be related to the P-gp inhibiting properties of itraconazole and its metabolites (Wang *et al.*, 2002; Vermeer *et al.*, 2016), rather than its CYP3A4 inhibiting effects. Indeed, the fact that itraconazole reduced $AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$ indicates that, for a drug that is no P-gp (or hepatic transporter) substrate, CYP3A4 inhibition results in less metabolite formation, as is to be expected. It is clear, however, that these are only hypotheses, the verification of which would require additional (*in vitro*) experiments.

Some limitations must be noted. Firstly, urine collections were not available, so apparent urinary tacrolimus clearances could not be calculated. Secondly, only 8-hour AUCs were available for renal transplant recipients (cohort A). However, because these were performed under steady-state pharmacokinetic conditions, it is reasonable to assume that tacrolimus $C_0 = C_{24}$. Thirdly, 12-hydroxy tacrolimus was not measured, although this is a quantitatively minor metabolite (Zheng *et al.*, 2012).

In conclusion, CYP3A5 was the single most important determinant of tacrolimus metabolite/parent ratios in a cohort of stable renal transplant recipients, in agreement with previous data. We demonstrated that CYP3A4 activity, assessed using the MDZ probe, bore little to no relationship with tacrolimus metabolite/parent ratios. In addition, these results suggest that 13-DMT and 31-DMT may display non-linear elimination behavior during exposure to the combined CYP3A4/P-gp inhibitor itraconazole, possibly due to saturation of P-gp dependent biliary efflux of metabolites and/or competition with tacrolimus for the catalytic site of CYP3A.

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Authorship contributions

Participated in research design: Vanhove, Annaert and Kuypers

Conducted experiments: Vanhove, de Jonge, de Loor and Oorts

Contributed new reagents or analytic tools: de Loor and Pohanka

Performed data analysis: Vanhove

Wrote or contributed to the writing of the manuscript: Vanhove, de Jonge, de Hoon, Annaert and Kuypers

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Figure legends

Figure 1. Mean log concentration-time profiles of midazolam, tacrolimus and their primary metabolites in cohort A. The left-hand panel displays CYP3A5 non-expressors and the right-hand panel displays CYP3A5 expressors. Error bars represent the standard deviations.
DMT, desmethyl tacrolimus.

Figure 2. Correlation between parent/metabolite AUC ratio for tacrolimus' three primary metabolites, oral midazolam clearance (MDZ CL/F; scatterplots A, C and E) and tacrolimus CL/F (plots B, D and F) in cohort A.

Figure 3. Mean log concentration-time profiles of tacrolimus and its primary metabolites in cohort B (healthy volunteers), before and immediately after inhibition of CYP3A4 using itraconazole. Error bars represent the standard deviations.

Figure 4. Tacrolimus metabolite/parent ratios during baseline and CYP3A4 inhibited conditions in cohort B (healthy volunteers). **, $P<0.01$

Figure 5. Correlation between baseline $AUC_{metabolites}/AUC_{tacrolimus}$ and fold change (delta) $AUC_{metabolites}/AUC_{tacrolimus}$ resulting from itraconazole inhibition in cohort B (healthy volunteers).

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Table 1. Pharmacokinetic parameters of midazolam, tacrolimus and their primary metabolites in cohort A, stratified by CYP3A5 genotype.

Variable	Non-expressors (n=40)	Expressors (n=10)	Difference (%)	P
Midazolam				
AUC_{0-8} (h.ng/ml)	3373 ± 1764	3355 ± 1219		0.770
T_{max} (h)[a]	0.5 [0.3-0.5]	0.5 [0.5-1.0]		0.519
C_{max} (ng/ml)	16.8 ± 8.1	15.9 ± 7.2	-5.4%	0.811
CL/F (ml/h)	735 ± 324	673 ± 249	-8.4%	0.770
Midazolam				
$AUC_{1'\text{-HOMDZ}}/AUC_{MDZ}$	0.43 ± 0.22	0.39 ± 0.17	-9.3%	0.544
$AUC_{4\text{-HOMDZ}}/AUC_{MDZ}$	0.05 ± 0.01	0.04 ± 0.01	-20.0%	0.108
Tacrolimus				
AUC_{0-24} (h.ng/ml)	266.0 ± 66.3	291.8 ± 59.2	+9.7%	0.257
Dose (mg/day)	4.7 ± 2.2	10.5 ± 3.1	+123.4%	<0.001
T_{max} (h)[a]	2.0 [1.6-3.0]	1.75 [1.0-3.0]		1.000
C_{max} (ng/ml)	17.9 ± 5.0	25.6 ± 7.3	+43.0%	0.002
CL/F (L/h)	17.8 ± 7.6	35.6 ± 8.2	+100.0%	<0.001
Tacrolimus				
$AUC_{13\text{-DMT}}/AUC_{tacrolimus}$	0.036 ± 0.012	0.100 ± 0.024	+177.8%	<0.001
$AUC_{15\text{-DMT}}/AUC_{tacrolimus}$	0.031 ± 0.007	0.031 ± 0.007		0.814
$AUC_{31\text{-DMT}}/AUC_{tacrolimus}$	0.003 ± 0.001	0.006 ± 0.001	+100.0%	<0.001
	0.070 ± 0.017	0.136 ± 0.028	+94.3%	<0.001

AUC, area under the concentration-time curve; $AUC_{metabolites}$, sum of AUC for 13-DMT, 15-DMT and 31-DMT; CL/F, oral clearance; C_{max}, maximum blood concentration; MDZ, midazolam; DMT, desmethyl tacrolimus; HOMDZ, hydroxy midazolam; T_{max}, time to reach maximum blood concentration
[a]median [interquartile range]

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Table 2. Pharmacokinetic parameters of tacrolimus and its primary metabolites before and during CYP3A4 inhibition in 16 healthy volunteers (cohort B).

	Baseline	CYP3A4 inhibition	% difference	P
Tacrolimus				
$AUC_{0\text{-}24}$ (h.ng/ml)	50.6 ± 21.6	167.9 ± 73.2	+232%	<0.001
CL/F (l/h)	69.7 ± 28.9	21.4 ± 9.3	-69%	<0.001
Midazolam				
CL/F (ml/min)	762.7 ± 327.7	83.4 ± 24.9	-89.1%	<0.001
$AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$	0.434 ± 0.133	0.041 ± 0.013	-90.6%	<0.001
$AUC_{4\text{-}HOMDZ}/AUC_{MDZ}$	0.081 ± 0.017	0.030 ± 0.009	-63.0%	<0.001
Tacrolimus				
$AUC_{13\text{-}DMT}/AUC_{tacrolimus}$	0.065 ± 0.028	0.107 ± 0.038	+65%	<0.001
$AUC_{15\text{-}DMT}/AUC_{tacrolimus}$	0.025 ± 0.006	0.053 ± 0.014	+112%	<0.001
$AUC_{31\text{-}DMT}/AUC_{tacrolimus}$	0.004 ± 0.002	0.005 ± 0.002	+25%	0.007
	0.094 ± 0.030	0.166 ± 0.040	+77%	<0.001

AUC, area under the concentration-time curve; $AUC_{metabolites}$, sum of AUC for 13-DMT, 15-DMT and 31-DMT; CL/F, oral clearance; C_{max}, maximum blood concentration; DMT, desmethyl tacrolimus; HOMDZ, hydroxy midazolam; T_{max}, time to reach maximum blood concentration

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Figure 1

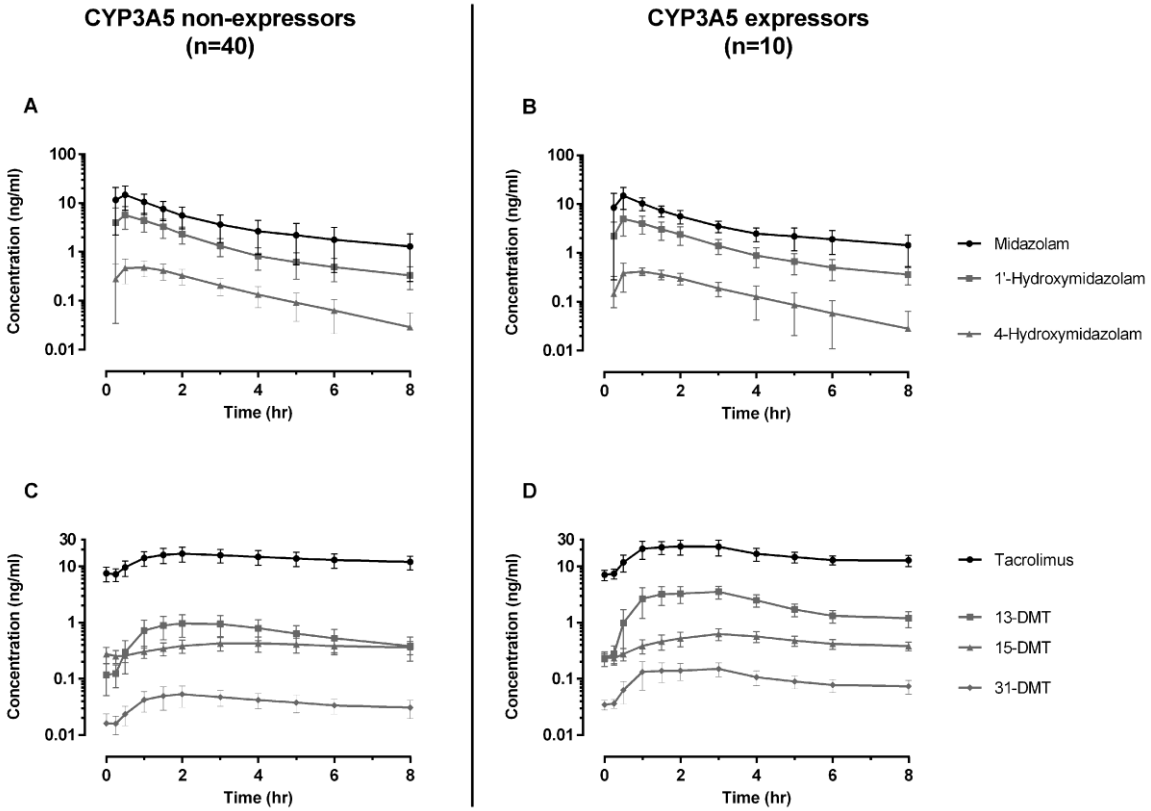

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Figure 2

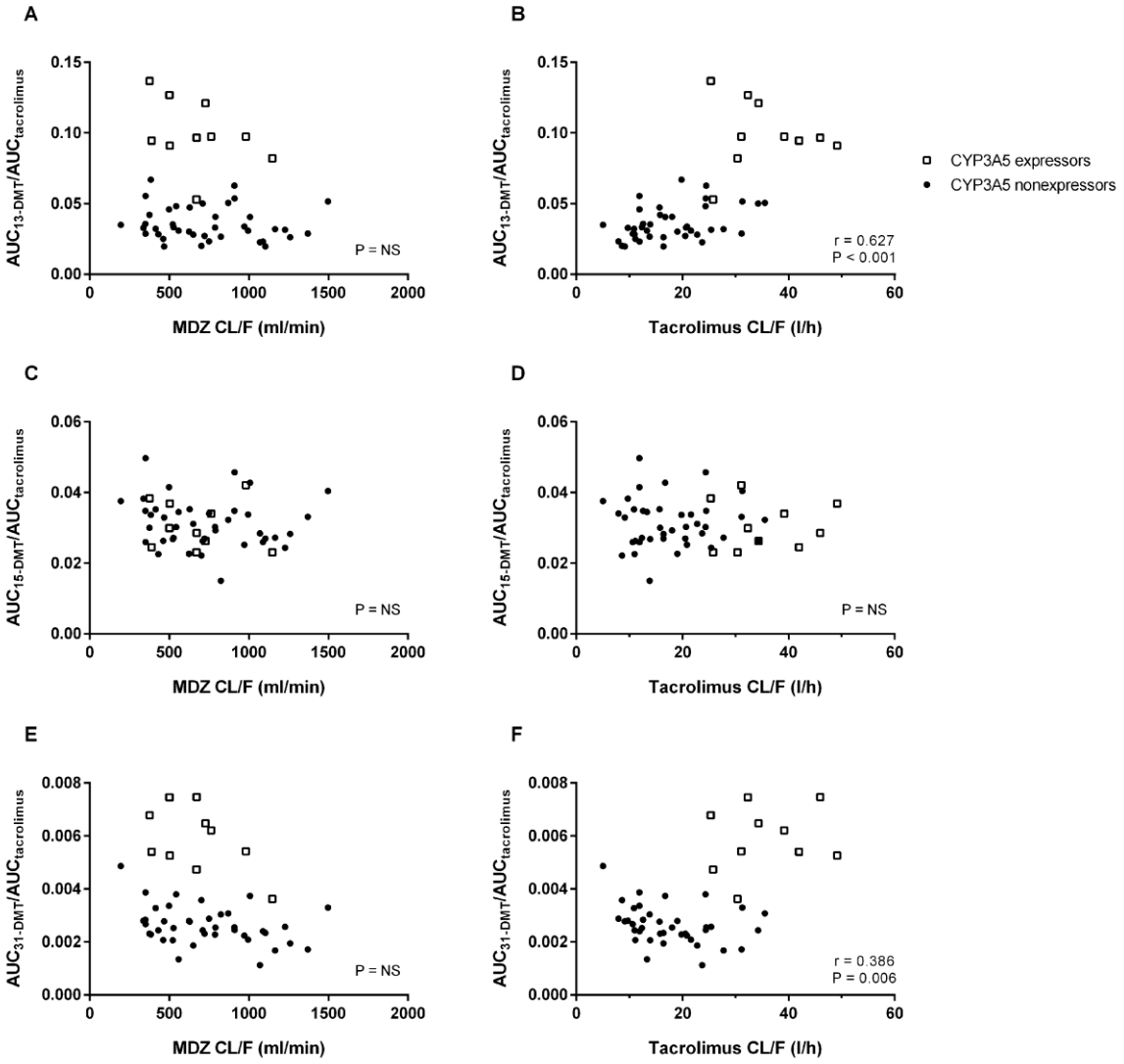

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Figure 3

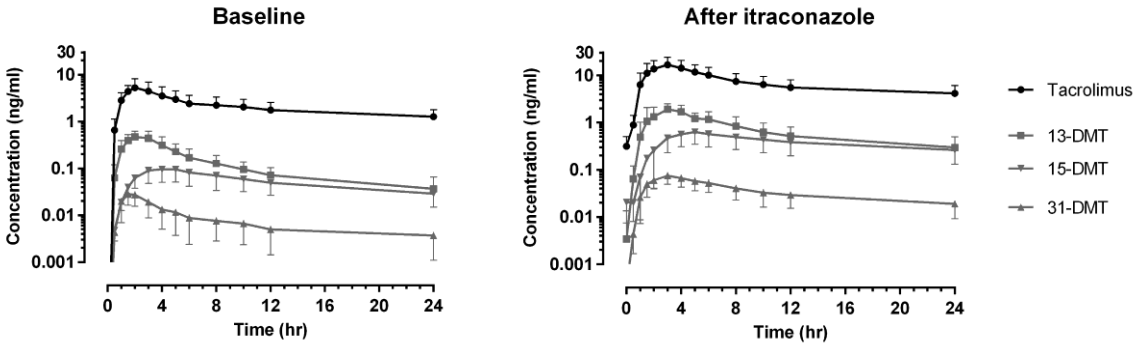

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Figure 4

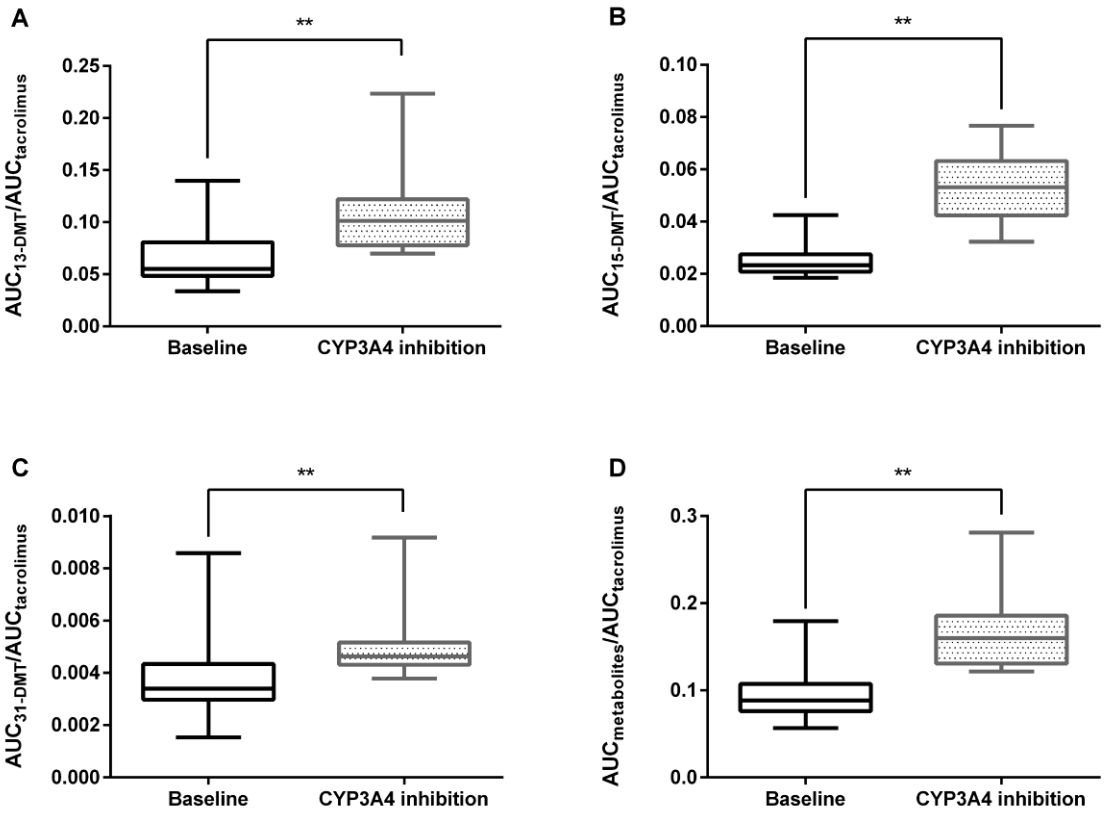

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Figure 5