**Drug Metabolism and Disposition**

# Supplemental material

## Relationship between in vivo CYP3A4 activity, CYP3A5 genotype and systemic tacrolimus metabolite/parent drug ratio.

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**Supplemental Table S1.** Characteristics of cohort A (n=50).

| Variable | Value | Range |
|---|---|---|
| Gender (male/female) | 35/15 | |
| CYP3A5 expressor (n) | 10 (20%) | |
| CYP3A4*22 carrier (n) | 1 (2%) | |
| Diabetes mellitus (n) | 16 (32%) | |
| Age (years) | 55.1 ± 13.8 | 25 – 80 |
| Time after transplantation | 3.5 ± 1.87 | 1.0 – 8.5 |
| Weight (kg) | 78.2 ± 15.6 | 44.3 – 107.0 |
| BMI ($kg/m^2$) | 26.0 ± 5.1 | 17.7 – 40.8 |
| Hematocrit (%) | 40.9 ± 4.5 | 30.0 – 55.0 |
| eGFR ($ml/min/1.73m^2$) | 50.4 ± 17.6 | 24.0 – 107 |
| Serum albumin (g/L) | 44.1 ± 3.1 | 39.4 – 54.3 |
| Methylprednisolone dose | 1.9 ± 1.9 | 0 – 4 |
| Midazolam CL/F (ml/min) | 722.5 ± 308.9 | 194.6 – 1497.5 |
| Midazolam CL/F/W | 9.5 ± 4.1 | 2.4 – 18.4 |
| Tacrolimus dose (mg/day) | 5.8 ± 3.3 | 1 – 14 |
| Tacrolimus CL/F (L/h) | 21.4 ± 10.5 | 5.0 – 49.1 |
| Tacrolimus CL/F/W (L/h/kg) | 0.28 ± 1.2 | 0.1 – 0.5 |

BMI, body mass index; CL/F, oral clearance; eGFR, estimated glomerular filtration rate

**Supplemental Table S2.** Correlation between midazolam oral clearance and selected tacrolimus pharmacokinetic parameters in cohort A.

| | **Non-expressors (n=40)** | | **Expressors (n=10)** | | **All patients (n=50)** | |
|---|---|---|---|---|---|---|
| | **Pearson R** | **P** | **Pearson R** | **P** | **Pearson R** | **P** |
| Tacrolimus CL/F | 0.67 | <0.001 | -0.13 | 0.718 | 0.45 | 0.001 |
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | -0.13 | 0.411 | -0.36 | 0.310 | -0.13 | 0.368 |
| $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ | -0.18 | 0.268 | -0.11 | 0.774 | -0.17 | 0.250 |
| $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ | -0.38 | 0.015 | -0.49 | 0.153 | -0.28 | 0.047 |
| $AUC_{metabolites}/AUC_{tacrolimus}$ | -0.19 | 0.248 | -0.40 | 0.259 | -0.17 | 0.243 |

AUC, area under the concentration-time curve; $AUC_{metabolites}$, sum of AUC for 13-DMT, 15-DMT and 31-DMT; CL/F, oral clearance; DMT, desmethyl tacrolimus

**Supplemental Table S3.** Independent predictors of tacrolimus metabolite/parent ratios in cohort A.

| Outcome parameter | Predictor variable(s) | Coefficient (B) | $R^2$ | P |
|---|---|---|---|---|
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | CYP3A5 expressor | 0.743 | 0.648 | <0.001 |
| | Hematocrit (%) | -0.027 | 0.075 | 0.003 |
| | Age (years) | 0.009 | 0.023 | 0.003 |
| $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ | Age (years) | 0.007 | 0.163 | 0.002 |
| $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ | CYP3A5 expressor | 0.797 | 0.595 | <0.001 |
| | MDZ CL/F | -0.258 | 0.056 | 0.002 |
| | Weight (kg) | 0.005 | 0.022 | 0.048 |

AUC, area under the concentration-time curve; CL/F, oral clearance; DMT, desmethyl tacrolimus; MDZ, midazolam

**Supplemental Table S4.** Characteristics of cohort B (healthy volunteers).

| | Healthy volunteers (n=16) | |
|---|---|---|
| **Variable** | **Value** | **Range** |
| Gender (male/female) | 16/0 | |
| Age (years) | 22.6 ± 2.6 | 18.2 – 28.1 |
| Weight (kg) | 77.7 ± 9.9 | 63.0 – 96.0 |
| BMI (kg/m$^2$) | 23.9 ± 3.0 | 18.8 – 29.4 |
| Hematocrit (%) | 45.9 ± 2.6 | 41.3 – 51.0 |
| eGFR (ml/min/1.73m$^2$) | 102.5 ± 13.6 | 74.0 – |
| Time after transplantation | - | |
| Methylprednisolone dose | - | |
| Diabetes mellitus (n) | 0 | |
| CYP3A5 expressor (n) | 4 (25%) | |
| CYP3A4*22 carrier (n) | 1 (6%) | |

BMI, body mass index; eGFR, estimated glomerular filtration rate.

Values refer to baseline conditions, before administration of the CYP3A4 inhibitor.

**Supplemental Table S5.** Tacrolimus metabolite/parent ratios in cohort B (healthy volunteers) before and after itraconazole, stratified by CYP3A5 genotype.

| Variable | Non-expressors (n=12) | Expressors (n=4) | Difference (%) | P |
|---|---|---|---|---|
| **Baseline** | | | | |
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | 0.055 ± 0.013 | 0.098 ± 0.038 | +78% | 0.007 |
| $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ | 0.024 ± 0.003 | 0.029 ± 0.011 | +20% | 0.359 |
| $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ | 0.003 ± 0.001 | 0.006 ± 0.003 | +50% | 0.119 |
| $AUC_{metabolites}/AUC_{tacrolimus}$ | 0.082 ± 0.015 | 0.132 ± 0.036 | +61% | 0.145 |
| **After itraconazole** | | | | |
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | 0.096 ± 0.024 | 0.141 ± 0.055 | +47% | 0.034 |
| $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ | 0.055 ± 0.012 | 0.047 ± 0.019 | -15% | 0.263 |
| $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ | 0.004 ± 0.001 | 0.008 ± 0.002 | +50% | 0.002 |
| $AUC_{metabolites}/AUC_{tacrolimus}$ | 0.155 ± 0.029 | 0.196 ± 0.058 | +26% | 0.145 |

AUC, area under the concentration-time curve; $AUC_{metabolites}$, sum of AUC for 13-DMT, 15-DMT and 31-DMT; DMT, desmethyl tacrolimus

**Brief LC-MS/MS methodology for tacrolimus and its metabolites**

The analytical method for the measurement of tacrolimus and its primary metabolites 13-DMT, 15-DMT and 31-DMT in human whole blood was newly developed. Tacrolimus was obtained from LC Laboratories (Woburn, MA, USA) and the internal standard tacrolimus-13C-d4 from Alsachim (Illkirch, France). 13-O-demethylated tacrolimus (M-I), 31-O-demethylated tacrolimus(M-II) and 15-O-demethylated tacrolimus(M-III) were generated, separated and purified with semi preparative chromatography after incubation of tacrolimus with rat liver microsomes. In short, the calibration curve covered a linear range from 0.035 to 72 ng/ml for tacrolimus, 0.07 to 10 ng/ml for the metabolite 13-DMT and 0.003 to 7 ng/ml for the metabolites, 15-DMT and 31-DMT. Calibration standards were prepared by spiking all compounds together in different concentrations in a final solution of Milli-Q water/ acetonitrile (50/50). Twelve calibration standards were made across the desired range and spiked into blank whole blood. Calibration curves were constructed using peak area ratios of analyte-to-internal standard using 1/X weighted linear regression. Tacrolimus-13C-d4 was used as internal standard for all compounds. For sample preparation, 20 µl Internal Standard (20 ng/ml tacrolimus-$^{13}$C-d4), 50 µl solution of Milli-Q water/acetonitrile (50/50), 50 µl zinc sulfate (0.1 M) were added to 50 µl whole blood. After vortex-mixing for 10 seconds, 200 µl acetonitrile was added. Subsequently the samples were vortex-mixed and centrifuged. The supernatant was then transferred into a 96-well 2ml collection plate, 100 µl Milli-Q water and 200µl of Milli-Q water/acetonitrile (50/50) were added to each sample and 50 µl was injected onto an UPLC (Acquity H Class, Waters, Zellik, Belgium). Chromatographic separation was performed on an Acquity BEHC18 column (2.1x50mm 1.7 µm particle size; Waters, Zellik, Belgium). The mobile phase, delivered at a flow rate of 0.5 ml/min at 55°C, was a gradient of Milli-Q water and acetonitrile. The detection was performed with a Xevo TQS tandem mass spectrometer (Waters, Zellik, Belgium). The most abundant, stable and sensitive adduct form was chosen for determination. Tacrolimus, the internal standard and the metabolites, 15-DMT and 31-DMT were determined as ammonium adduct, while 13-DMT was determined as sodium adduct. Ionization was achieved using electrospray positive ionization mode (ESI+). Nitrogen was used for nebulization and desolvation gas, while argon was used as collision gas. The multiple-reaction monitoring (MRM) transitions, cone voltage and collision energy were optimized for each individual compound and the optimal dwell time was experimentally determined for each component. The MRM

transitions were as follows: m/z 821.4 > 768.4, 826.6 > 773.4, 812.3 > 345.2 and 807.4 > 754.4 respectively for tacrolimus, the internal standard, metabolite 13-DMT and the metabolites, 15-DMT and 31-DMT with the same transition. The limit of detection and the lower limit of quantification were 0.002 and 0.035 ng/ml respectively for tacrolimus, 0.005 and 0.07 ng/ml respectively for 13-DMT and 0.003 and 0.007 ng/ml respectively for both 15-DMT and 31-DMT. The total, within-run, between-run and between-day precision (n=10) were all below 4 % and 10% coefficient of variation for tacrolimus and the metabolites respectively. The recovery of tacrolimus in whole blood was 104%, 105% for 13-DMT, 100% for 31-DMT and 97% for 15-DMT.

**Cohort C**

Because cohort B differs significantly from a clinical setting (particularly, 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 twice-daily tacrolimus (Prograft®, Astellas Pharma Europe, Staines, UK) and a moderate to potent CYP3A4 inhibitor (voriconazole, n=2; itraconazole, n=1; posaconazole, n=1; fluconazole, n=2; clarithromycin, n=1; diltiazem, n=2) for clinical reasons (cohort C). For these patients, $AUC_{0-8}$ of tacrolimus, MDZ and their metabolites were determined twice: once during co-therapy with the inhibitor and once without the inhibitor (usually several weeks later). Characteristics of cohort C are presented in Supplemental Table S6. As shown in Supplemental Table S7, CYP3A4 inhibition resulted in comparable increases in tacrolimus metabolite/parent ratios in cohorts B and C. Importantly, all the abovementioned CYP3A4 inhibitors (i.e. in spite of their differing inhibitory potency, potential effect on CYP3A5 and/or efflux pumps) resulted in an increase in tacrolimus metabolite/parent ratio.

**Supplemental Table S6.** Characteristics of cohort C.

| | Cohort C (n=9) | |
|---|---|---|
| **Variable** | **Value** | **Range** |
| Gender (male/female) | 6/3 | |
| Age (years) | 56.8 ± 13.7 | 30.0 – 73.0 |
| Weight (kg) | 67.3 ± 10.9 | 50.2 – 87.0 |
| BMI ($kg/m^2$) | 23.3 ± 2.3 | 20.2 – 27.5 |
| Hematocrit (%) | 37.7 ± 4.4 | 31.0 – 45.0 |
| eGFR ($ml/min/1.73m^2$) | 53.0 ± 21.9 | 17.6 – 85.0 |
| Time after transplantation | 1.4 ± 1.3 | 0.1 – 3.2 |
| Methylprednisolone dose | 4.7 ± 1.6 | 4.0 – 8.0 |
| Diabetes mellitus (n) | 0 | |
| CYP3A5 expressor (n) | 1 (11%) | |
| CYP3A4*22 carrier (n) | 2 (22%) | |

BMI, body mass index; eGFR, estimated glomerular filtration rate.

Values refer to baseline conditions, before administration of the CYP3A4 inhibitor.

**Supplemental Table S7.** Pharmacokinetic parameters of tacrolimus and its primary metabolites before and during CYP3A4 inhibition in cohorts B and C.

| | Baseline | CYP3A4 inhibition | % difference | P |
|---|---|---|---|---|
| **I. Cohort B (healthy volunteers, n=16)** | | | | |
| **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 metabolites** | | | | |
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | 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 |
| $AUC_{metabolites}/AUC_{tacrolimus}$ | 0.094 ± 0.030 | 0.166 ± 0.040 | +77% | <0.001 |
| **II. Cohort C (renal transplant recipients, n=9)** | | | | |
| **Tacrolimus** | | | | |
| $AUC_{0\text{-}24}$ (h.ng/ml) | 178.3 ± 41.5 | 203.1 ± 82.6 | +14% | 0.586 |
| CL/F (l/h) | 17.6 ± 8.3 | 10.6 ± 13.0 | -40% | 0.027 |
| **Midazolam** | | | | |
| CL/F (ml/min) | 704.0 ± 357.6 | 288.7 ± 312.9 | -59% | 0.001 |
| $AUC_{1'\text{-}HOMDZ}/AUC_{MDZ}$ | 0.338 ± 0.152 | 0.127 ± 0.079 | -62% | 0.002 |
| $AUC_{4\text{-}HOMDZ}/AUC_{MDZ}$ | 0.049 ± 0.013 | 0.038 ± 0.021 | -22% | 0.173 |
| **Tacrolimus metabolites** | | | | |
| $AUC_{13\text{-}DMT}/AUC_{tacrolimus}$ | 0.056 ± 0.023 | 0.076 ± 0.033 | +36% | 0.237 |
| $AUC_{15\text{-}DMT}/AUC_{tacrolimus}$ | 0.047 ± 0.017 | 0.087 ± 0.040 | +85% | 0.014 |
| $AUC_{31\text{-}DMT}/AUC_{tacrolimus}$ | 0.004 ± 0.001 | 0.005 ± 0.002 | +25% | 0.297 |
| $AUC_{metabolites}/AUC_{tacrolimus}$ | 0.107 ± 0.037 | 0.169 ± 0.064 | +58% | 0.017 |

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