

Title Page

***In vitro* investigation of human UDP-glucuronosyltransferase isoforms responsible for tacrolimus glucuronidation: predominant contribution of UGT1A4.**

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Running Title Page

Running title: Tacrolimus glucuronidation by UGT1A4

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Pages: 15

Table: 1

Figures: 3

References: 13

Words: Abstract: 208

Introduction: 288

Results/Discussion: 741

Abbreviations: 3'-azido-3'-deoxythymidine (AZT), glucuronide (-G), inhibition constant (K_i), lamotrigine (LTG), tacrolimus (Tacro), trifluoperazine (TFP), UDP-glucuronosyltransferase (UGT).

Abstract

Tacrolimus (Tacro) is a potent immunosuppressant and a central agent in prevention of post-transplantation rejection. Tacro is characterized by a narrow therapeutic index and wide interindividual pharmacokinetic fluctuation. The contribution of human UDP-glucuronosyltransferase in its metabolism has not been extensively studied. *In vitro* metabolism studies support that the liver produced Tacro-glucuronide (Tacro-G) while its formation was minimal or undetectable in the presence of intestine and kidney microsomes. Among 17 human UGTs tested, UGT1A4 was the sole enzyme involved in Tacro-G formation. This conclusion is supported by the finding of inhibition with a specific substrate of UGT1A4 lamotrigine with K_i values highly similar for both human liver and UGT1A4 microsomes and the correlation with trifluoperazine-glucuronide formation by liver microsomes ($r_s=0.551$; $p=0.02$). Formation of Tacro-G by liver samples was variable between individuals (6.4-fold variation; $n=16$) and common non-synonymous variants may contribute to this variability. In the HEK293 cellular model, no significant differences in enzyme kinetics could be revealed for UGT1A4*2 (P²⁴T) and *3 (L⁴⁸V) while the allozyme *4 (R¹¹W) displayed a 2-fold higher velocity ($p<0.01$) compared to the UGT1A4*1 enzyme preparation. In human liver samples, carriers of the UGT1A4 variants did not display statistically different efficiency in Tacro-G formation compared to homozygote for the reference genotype UGT1A4*1/*1. We conclude that UGT1A4 is the major isoform involved in Tacro glucuronidation while additional studies are required to assess the contribution of UGT1A4 genetic factors in tacrolimus glucuronidation variability.

Introduction

Among factors involved in patient and graft survival following transplantation, immunosuppressant exposure and efficiency is critical in order to optimize clinical outcome. Immunosuppressive regimens in post-transplantation typically include a calcineurin inhibitor, cyclosporine or tacrolimus (Tacro), as well as other steroid and immune modulators such as mycophenolate mofetil and azathioprine, to name only a few. Tacro suppresses lymphocyte proliferation and interleukin synthesis, likely through a calcineurin-dependent inhibition of nuclear translocation and subsequent activation of nuclear factor of activated T cells transcription factors (Halloran, 2004).

Because of the complex pharmacokinetics, pharmacodynamics, and pharmacogenetics of Tacro, the potential exists for adverse reactions and a high incidence of drug interactions. Tacro is characterized by a narrow therapeutic index and a large interindividual variation that would be explained, at least in part, by its metabolism. Tacro is metabolized by cytochromes P450 (CYP3A4 and CYP3A5) in both liver and small intestine and transported by P-glycoprotein (ABCB1) efflux transporter (Wallemacq et al., 2009). Drugs that are substrates of these pathways, as well as inhibitors and inducers, can cause significant interactions with Tacro (Mertz et al., 2009). In addition, genetic variations in genes encoding for these proteins have been shown to interfere with Tacro pharmacokinetics, efficacy and toxicity (Kuypers et al., 2010; Staatz et al., 2010). Tacro is also conjugated to glucuronide (-G) by the UDP-glucuronosyltransferase enzymes (UGTs) (Firdaous et al., 1997; Strassburg et al., 2001). In support, Tacro-G is measured in human bile (Firdaous et al., 1997). However, limited data are available with regards to the involvement of specific enzymes involved. In this study, we compared the glucuronidation activity for Tacro of major human metabolizing tissues; identified main UGT isozymes involved in the formation of Tacro-G and tested common genetic variants in UGT1A4 coding regions.

Materials and Methods

Chemical and Reagents. Tacro was obtained from New England Biolabs Ltd. (Pickering, ON, Canada). Lamotrigine (LTG), 3'-azido-3'-deoxythymidine (AZT) and trifluoperazine (TFP) were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada), deuterated cyclosporine from Toronto Research Chemical (Downsview, ON, Canada) and anti-calnexin antibody from Stressgen Biotechnologies (Victoria, BC, Canada). All chemicals and reagents were of the highest grade commercially available. Liver tissues from sixteen individuals were kindly provided by Dr. Ted T. Inhaba (Trottier et al., 2010). Human pooled colon and kidney microsomes were purchased from BD Gentest (Woburn, USA) and Celsis in vitro technologies (Chicago, IL, USA), respectively.

Glucuronidation assays. Microsomal protein extracts from UGT1A- and UGT2B-overexpressing HEK cells, and tissues were prepared by differential centrifugation, quantified by western blot and assays conducted as described previously (Benoit-Biancamano et al., 2009). UGT1A4 first exon was amplified by PCR from each liver samples, and sequenced as described earlier (Benoit-Biancamano et al., 2009). The initial screening was performed for 16 hours with 200 μ M Tacro at 37°C for liver, kidney, intestine microsomes and nine human UGT1A and seven UGT2B functionally active enzymes characterized to date (**Fig.1**). Enzyme kinetics were then assessed in liver samples from individual donors and in one human intestine pool, as well as positive UGT, namely UGT1A4 reference protein *1 (R¹¹P²⁴L⁴⁸) as well as for its common variant alloenzymes *2 (T²⁴), *3 (V⁴⁸) and *4 (W¹¹). Assays were performed with Tacro ranging from 25 to 750 μ M for 1 hour, while TFP assays were done according to the procedure described earlier (Court, 2005; Benoit-Biancamano et al., 2009). Inhibition constant (K_i) values were determined using Tacro (25–250 μ M), LTG (0–3mM) and AZT (0.1-2.5mM). The enzyme kinetic model was selected as described previously (Benoit-

Biancamano et al., 2009), using Sigma Plot 11.2 assisted by Enzyme Kinetics 1.3 (SSI, San Jose, CA, USA). Values are expressed with mean \pm SD of triplicate determinations of three independent experiments. Enzymatic activities using UGT overexpressing cell lines were considered statistically significant for p value below 0.05 according to the Student's *t*-test variance analysis. The relationship between UGT1A4 genotype and UGT activity in liver samples was evaluated by performing an analysis of variance (ANOVA); assuming an unequal variance between groups we used Kruskal-Wallis non-parametric ANOVA on ranks. Correlations were evaluated by Spearman rank correlation coefficient.

Tacro-G detection by mass spectrometry. Detection of Tacro-G, expressed as peak area ratio of Tacro-G over cyclosporine d4, was performed by liquid chromatography coupled to a triple-quadrupole mass spectrometer (API3200 Applied Biosystems, ON, Canada) operating with a turbo ion-spray source in single ion monitoring mode. The HPLC system consisted of the Agilent 1200 LC (Agilent Technology, Qc, Canada), coupled to a Luna C8 column (50 X 4.6 mm, 5 μ m) from Phenomenex (Torrance, CA, USA). The mobile phase is composed of binary solution, 1 mM ammonium acetate and 0.01% acetic acid (solvent A), and methanol with 1 mM ammonium acetate and 0.01% acetic acid (solvent B). The analytes were eluted at 0.9 ml/min flow rate, using the following protocol: initial condition, 50%B; 0-0.5 min, linear gradient 50-97% B; 0.5-3 min, isocratic 97% B; 3-3.1 min, linear gradient 97-50% B; 3.1-6 min, isocratic 50% B. Electro-spray was set to positive-ion mode with an ionization and declustering potential energy of 5000 and 100V respectively, and ion source temperature was set to 500°C. The following mass ions (*m/z*) was used for detection: 997.8 [M+NH₄]⁺ for Tacro-G and 1224 [M+NH₄]⁺ for cyclosporine d4 (internal standard).

Results and Discussion

Glucuronidation of Tacro is exclusively performed by UGT1A4. Data reveals that Tacro glucuronidation is formed by liver microsomes and to a lesser extent by intestine proteins, consistent with a previous report (Strassburg et al., 2001). In the presence of recombinant human UGT proteins, Tacro-G detected by mass spectrometry, was found exclusively for UGT1A4 (**Fig.1C**). This contrasts with a previous study that identified UGT2B7 as the major UGT enzyme involved in Tacro-G formation based on enzymatic assays using microsomal protein extracts from Sf9 cells infected with UGT-recombinant baculovirus and detection of the glucuronide product by thin layer chromatography (Strassburg et al., 2001).

Evidence against a role of UGT2B7 in Tacro-G formation is the absence of its glucuronidation by UGT2B7-HEK293 microsomes and by a commercial preparation of UGT2B7 recombinant proteins, both positive for the glucuronidation of a UGT2B7 substrate, AZT (data not shown). In addition, Tacro-G remained undetectable in the presence of human kidney microsomes that express abundantly UGT2B7 but not UGT1A4 (**Fig.1C**) (Ohno and Nakajin, 2009). A role for UGT2B7 in Tacro-G formation is likely not significant and this reaction would be performed exclusively by UGT1A4. Data from competitive assays performed with liver and UGT1A4-HEK293 microsomes using specific substrates LTG for UGT1A4 and AZT for UGT2B7 (Court, 2005), also sustain this conclusion. Tacro-G formation was influenced in a concentration-dependent manner by LTG for both liver and UGT1A4 -derived proteins but not in the presence of AZT. Furthermore, the apparent K_i values estimated for inhibition of Tacro glucuronidation by LTG were almost identical in liver and UGT1A4 (0.43 ± 0.26 and 0.47 ± 0.28 mM, respectively) in support of the involvement of this sole enzyme (**Fig.2**). Another observation is the affinity of UGT1A4 that appears higher for Tacro compared to

LTG, as indicated by the $K_i > K_m$ ($K_i = 0.43 \pm 0.26$ and 0.47 ± 0.28 mM for liver and UGT1A4 microsomes, respectively). Lastly, a significant correlation was observed between formation of Tacro-G and TFP-G by liver microsomes ($r_s = 0.551$; $p = 0.02$). We therefore conclude that UGT1A4 is responsible for Tacro-G formation and that UGT2B7 would not be involved.

Tacro-G formation may be altered by common UGT1A4 variant allozymes. Experimental evidence demonstrated that genetic polymorphisms in the *UGT1A4* gene significantly influence glucuronidation activity *in vitro* (Ehmer et al., 2004; Sun et al., 2006; Benoit-Biancamano et al., 2009). We then sought to examine whether common polymorphisms in the coding region of *UGT1A4* (frequency $\geq 1\%$) influence Tacro-G formation. Kinetics for Tacro revealed that variant allozymes UGT1A4*1 (R¹¹P²⁴L⁴⁸), UGT1A4*2 (Thr²⁴), UGT1A4*3 (Val⁴⁸) and UGT1A4*4 (Trp¹¹) have similar apparent K_m values (**Table 1**). UGT1A4*4 (Trp¹¹) displayed a significant alteration of Tacro-G formation rates compared to the reference UGT1A4*1 protein (3.14 ± 0.51 for *4 vs. 1.65 ± 0.34 for *1; $p = 0.007$), resulting in a 67% increase in the ratio V_{max}/K_m for UGT1A4*4. It remains to be determined if UGT1A4*4 could possibly result in a lower tacrolimus exposure *in vivo*. No significant changes of Tacro-G formation could be revealed for UGT1A4*2 and UGT1A4*3 allozymes whereas significant alteration of enzyme kinetics for other substrates has been reported previously, suggesting a substrate specific effect of these coding variations (Ehmer et al., 2004; Benoit-Biancamano et al., 2009). Formation of Tacro-G by 16 liver samples further supports variability between individuals (0.16 – 1.03 area/min/mg; 6.4-fold variation). Kinetic parameters of homozygote carriers of UGT1A4*1 (n=2), and heterozygotes UGT1A4*1/*2 (n=2) or UGT1A4*1/*3 (n=2) was also assessed, while no individual with the UGT1A4*4 allele could be tested because of its low frequency (**Table 1**). Rates of Tacro-G formation between *1/*1 carriers was highly similar

($V_{\max}=0.32\pm 0.03$ area/min/mg). Compared to livers with the $*1/*1$ genotype, mean values of Tacro-G formation (V_{\max}) for $UGT1A4*1/*2$ and $UGT1A4*1/*3$ carriers were not statistically different. However, these observations are based on a very limited number of samples and should be confirmed in a larger liver bank. We could not exclude either the potential effect of common variations in regulatory or non coding regions of the *UGT1A4* gene previously shown to be in strong linkage with these cSNP (Benoit-Biancamano et al., 2009).

In conclusion, data indicate that Tacro-G is mainly produced by the liver and is exclusively dependent on the *UGT1A4* pathway. Structural information on the position of glucuronidation of Tacro requires additional investigations. Besides, common genetic variations in the *UGT1A4* gene are associated with an altered in vitro formation of Tacro-G and may have a potential effect *in vivo*.

Authorship Contributions

Participated in research design: C. Guillemette, E. Lévesque

Conducted experiments: I. Laverdière, P. Caron

Performed data analysis: I. Laverdière, P. Caron, C. Guillemette

Wrote or contributed to the writing and revision of the manuscript: I. Laverdière, P. Caron, M. Harvey, É. Lévesque and C. Guillemette.

Other: E. Lévesque and C. Guillemette acquired funding for this research.

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Footnotes

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Legends for Figures

Figure 1. Detection of Tacro-G by LC-MS/MS in human tissues and HEK293-overexpressing UGTs. **A)** Representative chromatogram of Tacro-G from incubated sample. Retention time was 2.3 min for Tacro-G and 2.9 min for CsA d₄. **B)** MS/MS scan confirms the glucuronide nature of the observed Tacro-G. **C)** Tacrolimus glucuronidation by microsomes prepared from human tissues and HEK293 cell lines that overexpressed UGT1A or UGT2B enzymes.

Figure 2. Lineweaver-Burk plots for the inhibition of Tacro-G formation by LTG in A) human liver samples and **B)** in UGT1A4-overexpressing HEK293 cell lines. In liver samples, addition of 3 mM LTG to 250 μ M Tacro resulted in a reduced V_{\max} and increased K_m by 36% and 50% respectively. Similar results were observed with UGT1A4 microsomes.

Figure 3. Enzyme kinetics data. Formation of glucuronides in microsomal proteins from **A)** liver and **B)** UGT1A4-overexpressing HEK293 cell lines. Eadie-Hofstee plots were conclusive of Michaelis Menten profile, in **C)** liver, **D)** intestine, as well as in **E)** UGT1A4 recombinant.

Tables

Table 1. Kinetic parameters for the glucuronidation of Tacro by human tissues and UGT1A4 variant allozymes.

	Apparent K_m (μ M)	V_{max} (area/min/mg) ¹	V_{max}/K_m (μ l/min/mg)
UGT1A4 Allozymes			
1A4*1 R ¹¹ P ²⁴ L ⁴⁸	91 ± 28	1.65 ± 0.34	0.018
1A4*2 T ²⁴	114 ± 29	1.65 ± 0.31	0.014
1A4*3 V ⁴⁸	126 ± 51	2.23 ± 0.37	0.018
1A4*4 W ¹¹	101±48	3.14 ± 0.51*	0.030
Individual liver samples (UGT1A4 status)			
K28 (*1/*1)	41.2 ± 1.5	0.32 ± 0.03	0.008
K29 (*1/*1)	33.5 ± 8.6	0.33 ± 0.02	0.010
K21 (*1/*2)	52.8 ± 6.6	0.28 ± 0.02	0.005
K22 (*1/*2)	39.4 ± 5.1	0.29 ± 0.02	0.007
K14 (*1/*3)	36.8 ± 8.4	1.03 ± 0.10	0.028
K23 (*1/*3)	38.7 ± 7.2	0.36 ± 0.03	0.009
Intestine pool	51.8 ± 5.7	0.04 ± 0.01	0.001

Results are expressed with mean ± SD of triplicate determinations of three independents experiments.

¹The relative Vmax values were adjusted for UGT1A4 protein content: UGT1A4*1 (arbitrary set at 1); UGT1A4*2= 0.09; UGT1A4*3= 0.56; UGT1A4*4= 0.86.

*P value <0.01 vs *1

Figure 1

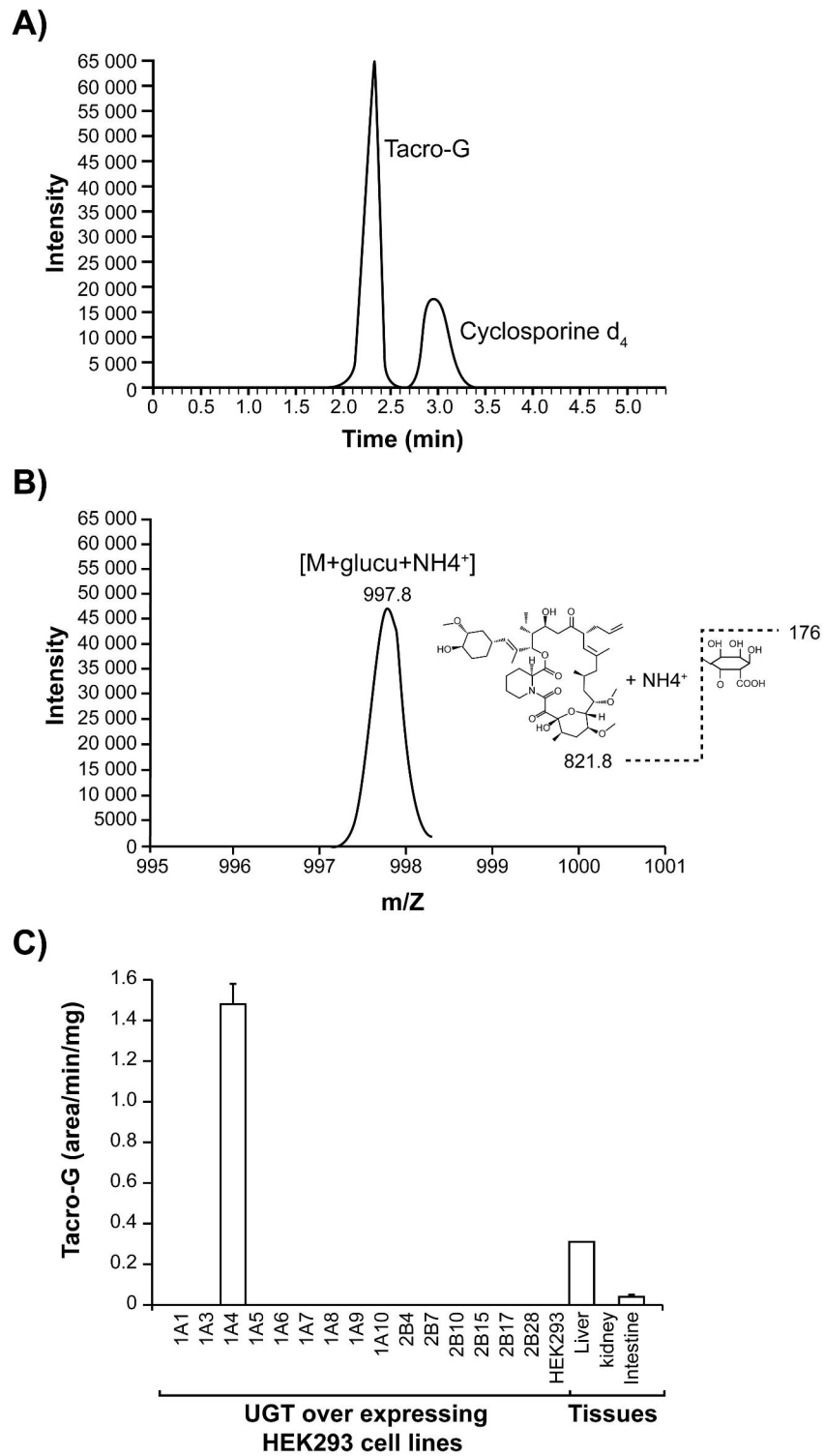
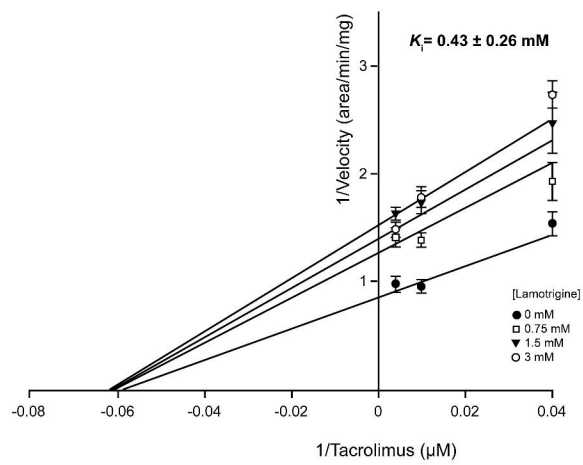


Figure 2

A) Human liver



B) UGT1A4

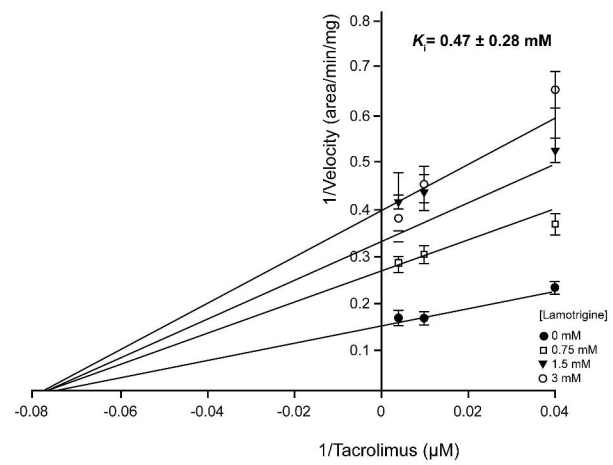


Figure 3

