Glucuronidation of mycophenolic acid by Wistar and Mrp2 deficient TR⁻ rat liver microsomes.

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Abbreviations: cyclosporine, CsA; mycophenolic acid, MPA; mycophenolate ether glucuronide, MPAGE; mycophenolate acyl glucuronide, MPAGa; high performance liquid chromatography, HPLC; inosine monophosphate dehydrogenase, IMPDH; area under the plasma concentration versus time curve, AUC; human liver microsomes, HLM,
uridine diphosphate glucuronosyl transferase, UGT; clearance, CL; hepatic extraction ratio, $E_H$; half life, $t_{1/2}$; uridine diphosphate glucuronic acid, UDPGA; bovine serum albumin, BSA.
Abstract

In humans, mycophenolic acid (MPA) is metabolised primarily by glucuronidation in the liver to mycophenolate ether glucuronide (MPAGe) and mycophenolate acyl glucuronide (MPAGa). We have previously reported that in perfused livers of TR− rats (lacking the Mrp2 transporter), the clearance and hepatic extraction ratio of MPA were significantly lower compared to control Wistar rats, suggesting a difference in the capacity of the TR− rats to metabolise MPA in situ. There is very little information regarding the phase II metabolic capabilities of TR− rats, therefore the aim of this study was to investigate the in vitro glucuronidation of MPA in Wistar and TR− rat liver microsomal protein. A second aim was to determine whether MPAGa, cyclosporine (CsA) and/or its metabolites AM1, AM1c and AM9 inhibit the metabolism of MPA to MPAGe in rat liver microsomes. MPAGe formation rates by Wistar and TR− microsomes were 0.48 nmol/min/mg and 0.65 nmol/min/mg respectively (p=0.33). Km values for control and TR− microsomes were 0.47 mM and 0.50 mM respectively (p=0.81). The mean (SEM) ratios of MPAGe formation by Wistar rat liver microsomes incubated with 50 µM MPA + inhibitor versus 50 µM MPA alone were: MPAGa 1.2(0.1); CsA 0.7(0.1)*; AM1 2.2(0.3)*; AM1c 1.2(0.2); and AM9 1.0(0.2) (*p<0.05). Our results suggest that lower in situ glucuronidation of MPA in TR− rats maybe due to inhibition of glucuronidation by endogenous and exogenous compounds that accumulate in the transporter deficient rat. Whilst, CsA inhibits glucuronidation of MPA, its metabolite AM1 enhances MPAGe formation by rat liver microsomes.
Introduction

The immunosuppressant mycophenolic acid (MPA) is metabolised primarily by glucuronidation in the liver (Bowelgha and Miners, 2001) to form the inactive metabolite mycophenolate ether glucuronide (MPAGe) and the active metabolite mycophenolate acyl glucuronide (MPAGa) (Shipkova et al., 1999). In humans, the uridine diphosphate glucuronosyl transferases (UGT’s) responsible for the formation of MPAGe and MPAGa are UGT1A9 (Bowelgha and Miners, 2001) and UGT2B7 (Picard et al., 2005), respectively. Picard et al (2005) identified a minor metabolite, 6-O-desmethyl-MPA (DM-MPA) as a product of cytochrome P450 (CYP) 3A4/5 and CYP2C8 (Picard et al., 2005). MPAGe undergoes extensive biliary secretion and in the gut is hydrolysed by bacterial β-glucuronidase to reform MPA, which undergoes enterohepatic recirculation (Bullingham et al., 1996b). It has been estimated that between 10-61% of the MPA area under the plasma concentration versus time curve (AUC) may be due to enterohepatic recirculation which results in a second peak in the concentration time profile of MPA at approximately 8-12 hr following the administration of mycophenolate mofetil (the prodrug of MPA) (Bullingham et al., 1996a).

A drug interaction has been described in patients co-administered the immunosuppressants cyclosporine (CsA) and MPA that results in a decrease in plasma MPA concentrations (Smak Gregoor et al., 1999; Filler et al., 2001; Brown et al., 2002; Kuypers et al., 2003). Most pharmacokinetic interactions involving calcineurin inhibitors, such as CsA, involve substrates for intestinal and hepatic P-glycoprotein and the CYP3A enzyme family (Bohme et al., 1993; Lin and Lu, 1998). However, CsA has been shown to inhibit the UGT-catalysed metabolism of MPA (Zucker et al., 1999) as well as the...
hepatic basolateral transporters of the OATP/Oatp family (Shitara et al., 2002; Shitara et al., 2003).

Similar to humans, the metabolism of MPA in isolated perfused Wistar rat livers results primarily in the formation of MPAGe (84% of added MPA) and a much smaller fraction of MPAGa (3.9%). Using TR- rats, a strain of mutant Wistar rats lacking the Mrp2 transporter (Soroka et al., 2001), Mrp2 has been shown to be the key transporter involved in MPAGe biliary excretion in rats (Kobayashi et al., 2004; Hesselink et al., 2005; Westley et al., 2006). We have previously reported that in perfused TR- rat livers, the clearance (CL) and hepatic extraction ratio (E_h) of MPA were significantly lower compared to control Wistar rats (Westley et al., 2006). One possible mechanism for this observation is that there was a difference in the capacity of the TR- rats to metabolise MPA compared to the control Wistar rats in situ. This was further demonstrated by the significantly lower partial clearances of MPA to MPAGe and MPAGa and significantly greater amount of unmetabolised MPA recovered in the livers of TR- rats. Alternatively, in TR- rat livers there may be greater hydrolysis of the labile MPAGa or MPAGe to MPA as a result of not being excreted into bile, thus decreasing net glucuronidation. However, this seemed unlikely since MPAGa only accounted for 3.9% of the MPA dose recovered in control Wistar rat isolated liver perfusions (Westley et al., 2006).

A previous study has reported altered phase-I metabolic activity in TR- rats compared to Wistar rats (Jager et al., 1998). However, there is little data on the phase-II metabolic capacity of TR- rats. The aim of this study was to investigate the in vitro glucuronidation
of MPA in Wistar and TR-rat liver microsomal protein. A second aim was to determine whether MPAGa, CsA and/or its metabolites AM1, AM1c and AM9 inhibit the metabolism of MPA to MPAGe in rat liver microsomes.
METHODS

Materials

MPA, MPAGe, MPAGa and carboxy butoxy ether mycophenolic acid were provided by Hoffman La Roche, (Basel, Switzerland). Alamethicin, D-saccharic acid-1,4-lactone and uridine diphosphate glucuronic acid (UDPGA)-trisodium salt was purchased from Sigma Aldrich (St Louis, MO, USA). Bovine Albumin Fraction V, was purchased from Bayer Corporation (Kankanee, Il, USA). CsA metabolites AM1, AM1c and AM9 were supplied by Novartis Pharma AG (Basel, Switzerland). All other reagents were of analytical grade.

Animals

The experimental protocol was approved by the Animal Ethics committee of the Institute of Medical and Veterinary Science (Adelaide, SA, AUS). Wistar rats (n=6) were purchased from Adelaide University Animal House (Adelaide, SA, AUS). TR- rats (n=6) were purchased from University of Queensland, Herston Medical Research Centre (Brisbane, QLD, AUS).

Microsomal Preparation

Microsomes from rat livers were prepared by differential centrifugation as described by Zanger et al, (1988), resuspended in sucrose-tris buffer (0.25M sucrose, 0.05M Trisma) pH 7.4 and stored at -80ºC until use.

The microsomal protein concentration was determined by Bio-Rad protein estimation (Hercules, CA, USA). Bovine serum albumin (BSA) standards were prepared (0-1.0
mg/ml) and 50 µL of BSA standards or rat liver microsome suspension were added to 2.5 mL filtered dye reagent. BSA standards and microsomal sample absorbances were read at 595 nm on a Beckman DV-70 Spectrophotometer (Fullerton, CA, USA).

**Microsomal metabolism of MPA to MPAGe**

*Enzyme Kinetics*

Microsomal protein, 1.0 mg/ml or 0.5 mg/ml for control and TR− rats respectively, was incubated at 37°C with 0.5 mg/ml alamethicin, 5 µg/ml MgCl₂ and MPA (25 – 1000 µM). The reaction was activated by the addition of 5 mM UDPGA making a final reaction volume of 200 µL. Ethanol concentrations were maintained at 0.5% (v/v) for all incubations. Bowalgaha and Miners, (2001) have previously demonstrated the absence of non-specific binding of MPA to human liver microsomes by equilibrium dialysis. The reaction was ceased at 2 min by transferring 100 µL of the reaction mixture into a microcentrifuge tube containing 20 µL of 1.5 M perchloric acid. On addition of 250 µL of internal standard (carboxy butoxy ether mycophenolic acid, 10 mg/L) in acetonitrile, samples were placed on ice, and 30 µL of 1 M KOH was added to raise the pH. The sample was then vortex mixed and centrifuged at 13000g for 5 min and the formation of MPAGe was measured by high performance liquid chromatography (HPLC) (Westley et al., 2005). MPAGa formation was not determined due to insufficient compound available to prepare calibrator stocks.

Under the conditions described above MPAGe reaction rates were found to be linear with respect to incubation time and microsomal protein concentration. All incubations were
performed in duplicate. Control incubations were included containing either, MPA and microsomes without UDPGA, MPA and UDPGA without microsomes or UDPGA and microsomes without MPA.

**Inhibition Studies**

The formation of MPAGe from MPA (50µM) was also investigated using control Wistar liver microsomes (1 mg/ml protein) in the absence or presence of potential inhibitors of glucuronidation, CsA (20 mM), metabolites AM1 (100 µM), AM1c (100 µM), AM9 (100 µM) or MPAGa (200µM). CsA and its metabolites were investigated because CsA is often co-administered with MPA and has been shown to alter the clinical pharmacokinetics of MPA. AM1 and AM9 were chosen as they are the major primary metabolites of CsA, whilst AM1c was chosen as it is a substrate for glucuronidation (Christians and Sewing, 1995). Incubation and activation conditions were as described above. We previously determined that there was no significant hydrolysis of MPAGa to MPA during the incubation (data not shown).

**Hydrolysis of MPAGe**

The hydrolysis of MPAGe by microsomal β-glucuronidase was investigated by measuring MPA formation during incubation of MPAGe (200 µM) at 37°C in the presence of 1 mg microsomal protein from control Wistar and TR− rats. The incubation was terminated at 10 min by sampling 100 µL of the reaction mixture. On addition of acetonitrile containing internal standard, samples were placed on ice and MPA concentrations were determined by HPLC (Westley et al., 2005). Control samples
(lacking microsomal protein) were included with each experiment to determine whether there was non-enzymatic MPAGe degradation. D-saccharic acid-1,4-lactone was also included to determine whether MPAGe degradation was caused by enzymes other than β-glucuronidase. MPAGe degradation was linear with time under these conditions. Ethanol concentrations were maintained at 0.5% (v/v) for all incubations.

**HPLC analysis**

The concentrations of MPAGe or MPA were determined by HPLC analysis as previously described by Westley *et al.*, (2005). Briefly, 100 µL of each sample were added to 250 µL of acetonitrile containing the internal standard, carboxy butoxy ether mycophenolic acid (10 mg/L), vortex mixed, centrifuged for 5 min at 13000g and the supernatant was analysed by reverse phase HPLC on a 250 x 4.6 mm Alltech Cyano 100A 5µm column (Alltech Associates Inc, Deerfield, IL) with UV detection at a wavelength of 215 nm. The mobile phase consisted of 13/87% v/v acetonitrile / 0.1 M phosphoric acid pumped at a flow rate of 1 ml/min.

**Kinetic analyses**

Kinetic parameters for the formation of MPAGe were determined using the Michaelis Menten equation:

\[ V = \frac{V_{\text{max}} \cdot S}{K_m + S} \]

where \( V \) is reaction velocity, \( V_{\text{max}} \) is maximum reaction velocity, \( S \) is the concentration of substrate, MPA, and \( K_m \) is the Michaelis Menten constant. Intrinsic clearance (CL_{int}) was calculated as \( V_{\text{max}}/K_m \).
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Statistical Analyses

Results are given as mean values ± S.E.M. Differences in the kinetics of MPAGe formation (K_m, V_max, and CL_int), MPAGe hydrolysis, liver weights and protein concentrations between control Wistar and TR rats were compared using an unpaired t-test with Welch’s correction. The ratios of MPAGe formation in Wistar rats in the presence versus absence of inhibitors was compared to a theoretical mean of 1.0, using a one sample t test. A p-value <0.05 was considered significant.
RESULTS

Kinetic analysis of MPAGe Formation

Mean (S.E.M.) liver weights in control Wistar and TR^- rats were 13.4 (0.6) g and 17.1 (0.6) g, respectively. Protein concentrations were 19.8 (2.0) mg/ml and 24.8 (0.8) mg/ml, respectively. The mean liver weights of the TR^- rats were significantly greater than those of the Wistar rats, but there was no difference in microsomal protein concentrations. The significant difference in rat and liver weights identified in this study is consistent with those differences seen by Jager et al, (2003).

Mean (S.E.M.) $V_{\text{max}}$ values for the control and TR^- rats were 0.48 (0.10) nmol/ mg protein/ min and 0.65 (0.13) nmol/ mg protein/ min, respectively (p=0.33). Mean (S.E.M.) $K_m$ values for the control and TR^- rats were 0.47 (0.10) mM and 0.50 (0.11) mM, respectively (p=0.81). An example the kinetics of MPAGe formation in the control and TR^- rat liver microsomes is shown in Figure 1. Intrinsic clearance ($CL_{\text{int}}$) for the control and TR^- rat liver microsomes was 1.17 (0.24) µL/min/mg and 1.40 (0.21) µL/min/mg protein, respectively. There were no statistically significant differences in the $V_{\text{max}}$, $K_m$ or $CL_{\text{int}}$ of MPAGe formation kinetics between control and TR^- rat liver microsomes, as shown in Figure 2.

Inhibition of MPAGe Formation

MPAGe formation in Wistar liver microsomes was investigated in the presence of MPAGa (200 µM), CsA (20 mM) or metabolites AM1 (100 µM), AM1c (100 µM), AM9 (100 µM). The ratios of MPAGe formation in the presence versus absence of inhibitor are
shown in Figure 3. Mean (S.E.M.) MPAGe formation ratios in Wistar rat liver microsomes incubated with 50 µM MPA in the presence of MPAGa, CsA and metabolites AM1, AM1c and AM9 were 1.2 (0.1), 0.7 (0.1), 2.2 (0.3), 1.2 (0.2) and 1.0 (0.2), respectively.

AM1 significantly increased MPAGe formation compared to control incubations (p=0.01), whilst CsA significantly reduced formation of MPAGe compared to control incubations with MPA alone (p=0.02).

Hydrolysis of MPAGe

The degradation of MPAGe to MPA by β-glucuronidase was investigated during 10 min incubations with Wistar and TR⁻ liver microsomal protein (1.0 mg/mL). MPAGe was stable in the absence of microsomal protein whilst D-saccharic acid-1,4-lactone completely inhibited degradation of MPAGe by microsomal protein. The mean concentration of MPA (SEM) resulting from the degradation of MPAGe (200 µM) by β-glucuronidase was 24.6 (2.7) µM and 19.8 (4.0) µM in control and TR⁻ rat liver microsomes respectively, accounting for approximately 10% of the initial MPAGe concentration. There was no significant difference between the two groups (p=0.35). During the 2 min incubations of MPA with microsomal protein, hydrolysis of the formed MPAGe was negligible.
DISCUSSION

Using rat isolated perfused livers, we have previously demonstrated a significantly lower \textit{in situ} formation of MPAGe and MPAGa, in TR\textsuperscript{−} compared to Wistar rat livers, and proposed that this may have been due to a lower hepatic glucuronidation capacity in TR\textsuperscript{−} rats. However, in the current study using Wistar and TR\textsuperscript{−} liver microsomes there was no difference in the \textit{in vitro} formation of MPAGe. Webb \textit{et al.}, (2003) have also similarly reported that the \textit{in vivo} formation of harmol glucuronide was significantly lower in TR\textsuperscript{−} rats compared to controls but that there was no significant difference in the \textit{in vitro} formation of harmol glucuronide between TR\textsuperscript{−} and control Wistar rat liver microsomes. This suggests that intrahepatic accumulation of endogenous and/or exogenous compounds, due to the lack of the Mrp2 transporter, may affect \textit{in situ} glucuronidation of MPA in the TR\textsuperscript{−} rat liver.

Johnson \textit{et al} (2006) have recently reported approximately 3.5 and 5.5 fold higher expression of the UGT1A enzyme family by Western blotting in the liver and kidney, respectively, of TR\textsuperscript{−} rats compared to wild type Wistar rats. In addition they also showed there was no difference in UGT1A family expression in the duodenum, jejunum, ileum and colon suggesting that the altered expression of UGT1A enzymes in TR\textsuperscript{−} rats may be isoform and/or tissue-specific. In the same study Mrp3 was also significantly up-regulated in the liver and kidney in TR\textsuperscript{−} rats compared to control Wistar rats. Whilst the expression of other transporters such as Oatp1a1, Bsep and P-gp expression did not differ significantly between the TR\textsuperscript{−} rats and control Wistar rats. Our observation that there is no difference in the formation of MPAGe between TR\textsuperscript{−} and Wistar liver microsomes,
suggests that the UGT1A isoforms with increased expression in TR⁻ rats are not involved
in the formation of MPAGe from MPA. Miles et al. (2005) demonstrated that rat
UGT1A1 and 1A7 are the likely enzymes involved in the metabolism of MPA to MPAGe
in Sprague-Dawley rats. Assuming that the same enzymes catalyse the formation of
MPAGe in Wistar and TR⁻ rats, our study suggests that up regulation of the expression of
UGT1A enzymes in TR⁻ rat livers may be isoform selective, and that hepatic UGT1A1
and 1A7 expression may not be up regulated in TR⁻ rats.

For example, similar observations have been reported for phase-I metabolism. Jager et al
(1998) demonstrated that differences in the total CYP450 content may be due to
differential expression of individual isoenzymes in various mutant rat strains. Newton et al (2005) showed that CYP2B1/2 mediated pentoxyresorufin O-demethylation activity as
well as CYP2C11 mediated 2α- and 16α-hydroxylase activities were higher in TR⁻ rats
further demonstrating variation in mutant strains.

Another possible explanation for the lower in situ hepatic clearance of MPA in TR⁻ rats
may be decreased uptake into the hepatocytes. In addition, it is possible that MPAGe and
MPAGa compete with MPA for hepatic uptake and, in the Mrp2 deficient rats, increased
MPAGe and MPAGe basolateral efflux from hepatocytes results in increased inhibition
of MPA uptake into hepatocytes. This may result in the decreased CL and E_H of MPA,
and prolonged MPA AUC and t_{1/2} previously reported in TR⁻ rat liver perfusions
(Westley et al., 2006). Other glucuronide conjugates have been reported to compete with
parent aglycones for uptake transporters. For example, Sesink et al. (2005) demonstrated
that quercetin and quercetin glucuronides compete for intestinal absorption via Bcrp1 resulting in the inhibition of quercetin Bcrp1 efflux.

Miles et al (2005) demonstrated in HLM and Sprague-Dawley rat liver microsomes a significant difference in MPA metabolism between species with a mean \( V_{\text{max}} \) of 20.5 nmol/mg protein/min versus 6.7 nmol/mg protein/min, respectively, and mean \( K_m \) of 0.08 mM versus 0.20 mM respectively. The data from this study are consistent with the species differences in the metabolic capabilities between rat liver microsomes and HLM.

Furthermore, Miles et al (2005) demonstrated the affinity and capacity of the three UGT’s (UGT1A1, 1A6 and 1A7) responsible for the metabolism in Sprague-Dawley rats. In our study, \( K_m \) values were similar to those reported by Miles et al (2005), but \( V_{\text{max}} \) values were approximately 10-fold lower. This may be attributed to rat strain differences (Wistar versus Sprague-Dawley rats) in the expression of the respective UGT’s or methodological differences between laboratories. Shipkova et al (2001) reported a mean \( V_{\text{max}} \) of 10.3 nmol/mg protein/min, mean \( K_m \) of 0.27 mM and mean \( CL_{\text{int}} \) of 34.3 \( \mu \)L/(min/mg protein) for the formation of MPAGe in HLM. Similarly, Bowalgaha and Miners (2001) reported a mean \( V_{\text{max}} \) of 14.2 nmol/mg protein/min, mean \( K_m \) of 0.351 mM and mean \( CL_{\text{int}} \) of 46.6 \( \mu \)L/(min/mg protein) in HLM.

In order to determine whether accumulation of metabolites may affect the glucuronidation of MPA, we investigated MPAGa as a possible inhibitor of the formation of MPAGe. However, no significant inhibition was observed at an MPAGa concentration > 10-times the usual clinical range. We also investigated CsA and some of its metabolites.
as potential inhibitors of MPAGE formation. The data from these inhibition studies suggests that CsA inhibits the UGT-catalysed metabolism of MPA as previously demonstrated by Zucker et al (1999) with an apparent Ki of 2518 µg/L. In the present study the CsA concentrations used were approximately 170 µg/L, suggesting that, in Wistar rats, CsA may inhibit the rat UGT isoforms catalysing MPAGE formation at concentrations much lower than the Ki reported by Zucker et al (1999). Picard et al, (2005) have also reported that different human UGT’s are involved in the formation of MPAGE and MPAGa. CsA is a known substrate for UGT2B7 (Strassburg et al., 2001), the isoform catalysing the formation of MPAGa in humans.

The significant increase in MPAGE formation in the presence of AM1 suggests positive cooperativity with the UGT’s involved in MPA metabolism in Wistar rats. Homotropic positive cooperativity has been observed for the human UGT2B7 nonselective substrate 4-methylumbelliferone (Miners et al., 2004) and estradiol 3-glucuronidation by the addition of UGT1A1 substrates (Williams et al., 2002). A similar effect has also previously been demonstrated for CYP enzymes in human liver microsomes. For example, in the activation of CYP2C9-catalyzed tolbutamide hydroxylation by lansoprazole (Liu et al., 2005), and for the stimulation of CYP3A4 mediated metabolism of warfarin by quinidine (Ngui et al., 2001). Although, we were unable to obtain recombinant rat UGT1A1 and 1A7 to further investigate this effect and identify the UGT involved, this data is the first of its kind to suggest positive cooperativity in rat UGT’s. Further work is required to determine whether this may be clinically relevant.
In conclusion, the results from this study suggest that the *in situ* metabolism of MPA in TR- rats may be inhibited by endogenous compounds associated with the reduced biliary capabilities of the TR- rat or may be due to reduced uptake of the parent compound MPA into hepatocytes. This study is the first to suggest positive cooperativity between the CsA metabolite, AM1, and MPA metabolism by rat UGTs. However, further work is required to confirm and determine the true nature of this effect.
References


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Footnote

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Figure 1. Kinetics of MPAGe formation in liver microsomes from a control Wistar and a TR− rat liver.

Figure 2. Kinetic parameters for MPAGe formation by control Wistar and TR− rat liver microsomes.

Figure 3. Ratios of MPAGe formation in the presence versus absence of potential inhibitors MPAGa, CsA, and CSA metabolites AM1, AM1c and AM9.
Figure 1A

Graph showing the relationship between MPA concentration (mM) and enzyme activity (V nmol/min/mg).
Figure 2B

$K_m$ (mM)

Control  |  TR-