Glucuronidation of Mycophenolic Acid by Wistar and Mrp2-Deficient TR\(^{-}\) Rat Liver Microsomes

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ABSTRACT:

In humans, mycophenolic acid (MPA) is metabolized 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 with control Wistar rats, suggesting a difference in the capacity of the TR\(^{-}\) rats to metabolize 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 and 0.65 nmol/min/mg, respectively (p < 0.33). Km values for control and TR\(^{-}\) microsomes were 0.47 and 0.50 mM, respectively (p = 0.81). The mean (S.E.M.) ratios of MPAGe formation by Wistar rat liver microsomes incubated with 50 \(\mu\)M MPA plus inhibitor versus 50 \(\mu\)M MPA alone were MPAGa 1.2 (0.1), CsA 0.7 (0.1) (p < 0.05), AM1 2.2 (0.3) (p < 0.05), AM1c 1.2 (0.2), and AM9 1.0 (0.2). Our results suggest that lower in situ glucuronidation of MPA in TR\(^{-}\) rats may be because of inhibition of glucuronidation by endogenous and exogenous compounds that accumulate in the transporter-deficient rat. Whereas CsA inhibits glucuronidation of MPA, its metabolite AM1 enhances MPAGe formation by rat liver microsomes.

The immunosuppressant mycophenolic acid (MPA) is metabolized primarily by glucuronidation in the liver (Bowalgaha 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 UDP glucuronosyltransferases (UGT) responsible for the formation of MPAGe and MPAGa are UGT1A9 (Bowalgaha and Miners, 2001) and UGT2B7 (Picard et al., 2005), respectively. Picard et al. (2005) identified a minor metabolite, 6-O-desmethyl-MPA, as a product of CYP3A4/5 and CYP2C8 (Picard et al., 2005). MPAGe undergoes extensive biliary secretion and in the gut is hydrolyzed by bacterial \(\beta\)-glucuronidase to reform MPA, which undergoes enterohepatic recirculation (Bullingham et al., 1996b). It has been estimated that between 10 and 61% of the MPA area under the plasma concentration versus time curve may be caused by enterohepatic recirculation, which results in a second peak in the concentration time profile of MPA at approximately 8 to 12 h following the administration of mycophenolate mofetil (the prodrug of MPA) (Bullingham et al., 1996a).

A drug interaction has been described in patients coadministered the immunosuppressants cyclosporine (CsA) and MPA that results in a decrease in plasma MPA concentrations (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-catalyzed metabolism of MPA (Zucker et al., 1999) and the hepatic basolateral transporters of the organic anion transporting polypeptide/Oatp family (Shitara et al., 2002, 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 of MPA were significantly lower compared with control Wistar rats (Westley et al., 2006). One possible mecha-
nism for this observation is that there was a difference in the capacity of the TR− rats to metabolize MPA compared with the control Wistar rats in situ. This was further shown by the significantly lower partial clearances of MPA to MPAGe and MPAGa and significantly greater amount of unmetabolized MPA recovered in the livers of TR− rats. Alternatively, in TR− rat livers there may be greater hydrolysis of the labelled MPAGa or MPAGe to MPA as a result of not being excreted into bile, thus decreasing net glucuronidation. However, this seemed unlikely because 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 with Wistar rats (Jager et al., 1998). However, there are few 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.

Materials and Methods

Materials. MPA, MPAGe, MPAGa, and carboxy butoxy ether mycophenolic acid were provided by Roche Pharmaceuticals (Palo Alto, CA). Alumethin, α-saccharic acid-1,4-lactone, and UDP-glucuronic acid (UDPGA)-trisodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Bovine Albumin Fraction V was purchased from Bayer Corporation (Kankanee, IL). CsA metabolites AM1, AM1c, and AM9 were supplied by Novartis Pharma AG (Basel, Switzerland). All the 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, Australia). Wistar rats (n = 6) were purchased from Adelaide University Animal House (Adelaide, SA, Australia). TR− rats (n = 6) were purchased from University of Queensland, Herston Medical Research Centre (Brisbane, QLD, Australia).

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

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

Microsomal Metabolism of MPA to MPAGe. Enzyme kinetics. Microsomal protein, 1.0 and 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 the incubations. Bovine serum albumin (10 mg/l) was also included containing MPA and microsomes without UDPGA, MPA and UDPGA with microsomes, or UDPGA and microsomes without MPA.

Inhibition studies. The formation of MPAGe from MPA (50 μM) 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 (200 μM). CsA and its metabolites were investigated because CsA is often coadministered with MPA and has been shown to alter the clinical pharmacokinetics of MPA. AM1 and AM9 were chosen because they are the major primary metabolites of CsA, whereas AM1c was chosen because 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 of 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 nonenzymatic MPAGe degradation. α-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 the 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 was added to 250 μl of acetonitrile containing the internal standard, carboxy butoxy ether mycophenolic acid (10 μg/ml), vortex-mixed, centrifuged for 5 min at 13,000g, and supernatant was analyzed by reverse-phase HPLC on a 250 × 4.6-mm Alltech Cyano 100A 5-μm 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:

\[ V = V_{\text{max}} \times \frac{S}{(K_m + S)} \]

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 (\( \text{Cl}_{\text{int}} \)) was calculated as \( \frac{V_{\text{max}}}{K_m} \).

Statistical Analyses. Results are given as mean values ± S.E.M. Differences in the kinetics of MPAGe formation (\( K_m \), \( V_{\text{max}} \), and \( \text{Cl}_{\text{int}} \), 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 were compared with 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) and 17.1 (0.6) g, respectively. Protein concentrations were 19.8 (2.0) 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) 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) 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 Fig. 1. \( \text{Cl}_{\text{int}} \) for the control and TR− rat liver microsomes was 1.17 (0.24) and 1.40 (0.21) μl/min/mg protein.
respectively. There were no statistically significant differences in the \( V_{\text{max}} \), \( K_m \), or \( \text{CL}_{\text{int}} \) of MPAGe formation kinetics between control and TR\(^{-}\) rat liver microsomes, as shown in Fig. 2.

**Inhibition of MPAGe Formation.** MPAGe formation in Wistar liver microsomes was investigated in the presence of MPAGa (200 \( \mu \)M), CsA (20 mM), or metabolites AM1 (100 \( \mu \)M), AM1c (100 \( \mu \)M), and AM9 (100 \( \mu \)M). The ratios of MPAGe formation in the presence versus absence of inhibitor are shown in Fig. 3. Mean (S.E.M.) MPAGe formation ratios in Wistar rat liver microsomes incubated with 50 \( \mu \)M 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 with control incubations \((p = 0.01)\), whereas CsA significantly reduced formation of MPAGe compared with control incubations with MPA alone \((p = 0.02)\).

**Hydrolysis of MPAGe.** The degradation of MPAGe to MPA by \( \beta \)-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, whereas \( \alpha \)-saccharic acid-1,4-lactone completely inhibited degradation of MPAGe by microsomal protein. The mean concentration of MPA (S.E.M.) resulting from the degradation of MPAGe (200 \( \mu \)M) by \( \beta \)-glucuronidase was 24.6 (2.7) and 19.8 (4.0) \( \mu \)M 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 shown a significantly lower in situ formation of MPAGe and MPAGa in TR\(^{-}\) compared with Wistar rat livers and proposed that this may have been because of a lower hepatic glucuronidation capacity in TR\(^{-}\) rats.

However, in the current study using Wistar and TR\(^{-}\) liver microsomes there was no difference in the in vitro formation of MPAGe. Webb et al. (2003) have also similarly reported that the in vivo formation of harmol glucuronide was significantly lower in TR\(^{-}\) rats compared with controls but that there was no significant difference in the in vitro formation of harmol glucuronide between TR\(^{-}\) and control Wistar rat liver microsomes. This suggests that intrahepatic accumulation of endogenous and/or exogenous compounds, as a result of the lack of the Mrp2 transporter, may affect in situ glucuronidation of MPA in the TR\(^{-}\) rat liver.

Johnson 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 rats compared with 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 rats may be isoform- and/or tissue-specific. In the same study, Mrp3 was also significantly up-regulated in the liver and kidney, respectively, of TR rats compared with control Wistar rats, whereas the expression of other transporters such as Oatp1a1, Bsep, and P-glycoprotein expression did not differ significantly between the TR rats and control Wistar rats. Our observation that there is no difference in the formation of MPAGe between TR rats and Wistar rat livers suggests that the UGT1A isozymes with increased expression in TR rats are not involved in the formation of MPAGe from MPA. Miles et al. (2005) showed that rat UGT1A1 and UGT1A7 are the likely enzymes involved in the metabolism of MPA to MPAGe in Sprague-Dawley rats. Assuming that the same enzymes catalyze the formation of MPAGe in Wistar and TR rats, our study suggests that up-regulation of the expression of UGT1A enzymes in TR rats may be isoform-selective and that hepatic UGT1A1 and UGT1A7 expression may not be up-regulated in TR rats. For example, similar observations have been reported for phase I metabolism. Jager et al. (1998) showed that differences in the total cytochrome P450 content may be caused by differential expression of individual isoenzymes in various mutant rat strains. Newton et al. (2005) showed that CYP2B1/2-mediated pentoxyresorufin O-demethylation activity and CYP2C11-mediated 2α- and 16α-hydroxylase activities were higher in TR rats, further showing 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 hepatic extraction ratio of MPA, and prolonged MPA area under the plasma concentration versus time curve and t₁/₂ previously reported in TR rat liver perfusions (Westley et al., 2006). Other glucuronidase conjugates have been reported to compete with parent aglycones for uptake transporters. For example, Sesink et al. (2005) showed that quercetin and quercetin glucuronides compete for intestinal absorption via Bcrp1, resulting in the inhibition of quercetin Bcrp1 efflux.

Miles et al. (2005) showed in HLM and Sprague-Dawley rat liver microsomes a significant difference in MPA metabolism between species with a mean Vₘₐₓ of 20.5 versus 6.7 nmol/mg protein/min, respectively, and mean Kₘ of 0.08 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) showed the affinity and capacity of the three UGT (UGT1A1, UGT1A6, and UGT1A7) responsible for the metabolism in Sprague-Dawley rats. In our study, Kₘ values were similar to those reported by Miles et al. (2005), but Vₘₐₓ 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 or methodological differences between laboratories. Shkipova et al. (2001) reported a mean Vₘₐₓ of 10.3 nmol/mg protein/min, mean Kₘ of 0.27 mM, and mean CLₑₓ of 34.3 μl/ (min/mg protein) for the formation of MPAGe in HLM. Similarly, Bowalgaha and Miners (2001) reported a mean Vₘₐₓ of 14.2 nmol/mg protein/min, mean Kₘ of 0.351 mM, and mean CLₑₓ of 46.6 μl/ (min/mg protein) in HLM.

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 suggest that CsA inhibits the UGT-catalyzed metabolism of MPA as previously shown 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 might inhibit the rat UGT isozymes catalyzing 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 are involved in the formation of MPAGe and MPAGa. CsA is a known substrate for UGT2B7 (Strassburg et al., 2001), the isoform catalyzing the formation of MPAGa in humans.

The significant increase in MPAGe formation in the presence of AM1 suggests positive cooperativity with the UGT 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 shown for cytochrome P450 enzymes in HLM, 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 UGT1A7 to further investigate this effect and identify the UGT involved, these data are the first of their kind to suggest positive cooperative activity in rat UGT. 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 the result of reduced uptake of the parent compound MPA into hepatocytes. This study is the first to suggest positive cooperation between the CsA metabolite, AM1, and MPA metabolism by rat UGT. However, further work is required to confirm and determine the true nature of this effect.

References

![Fig. 3. Ratios of MPAGe formation in the presence versus absence of potential inhibitors MPAGa, CsA, and CSA metabolites AM1, AM1c, and AM9.](image-url)
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