Glucuronidation and Subsequent Biliary Excretion of Mycophenolic Acid in Rat Sandwich-cultured Hepatocytes

Kazuhiro TETSUKA*, Nicolas GERST, Kouichi TAMURA and Jeffrey N. MASTERS

Astellas Research Institute of America LLC, Skokie, Illinois, USA

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Summary: Rat sandwich-cultured hepatocytes (SCH) were used to correlate the in vitro hepatic disposition of mycophenolic acid (MPA) with published in vivo data, as well as mechanistic studies on drug-drug interaction. The major metabolite of MPA in SCH was 7-O-glucuronide (MPAG) followed by acyl-glucuronide (AcMPAG). MPAG and AcMPAG, but not MPA, showed significant in vitro biliary excretion with biliary excretion indexes (BEI) of 40% for MPAG and 45% for AcMPAG. While these BEIs were similar, the biliary excretion amount (BEA) of MPAG (120 pmol/mg protein) was orders of magnitude higher than that of AcMPAG (0.34 pmol/mg protein). Since MPAG is the major metabolite in in vivo bile, we propose that BEA is a better qualifier of biliary excretion. Quercetin inhibited MPAG and AcMPAG production, while chrysin inhibited only MPAG production, showing that chrysin is not a pan-glucuronidation inhibitor. Cyclosporin A (CysA) reduced the BEI of MPAG and increased intracellular MPA accumulation without changing MPAG amounts. These results suggest that CysA causes inhibition of biliary excretion of MPAG, as well as a mixed inhibition of glucuronidation of MPA and sinusoidal efflux of MPA/MPAG. In conclusion, the present study demonstrates a good agreement of hepatic MPA disposition between SCH and in vivo rats.

Keywords: biliary excretion; glucuronidation; sandwich-cultured hepatocytes; mycophenolic acid; drug-drug interaction

Introduction

Drugs are typically distributed into the liver by passive diffusion and/or transporters, where they are then metabolized and/or excreted to the bile or the blood. Drugs cleared by biliary excretion have the potential to be absorbed back into the gastrointestinal (GI) tract where they can reenter the blood (enterohepatic circulation: EHC). Pharmacokinetic (PK) parameters of drugs exhibiting EHC can be difficult to determine because EHC can cause multiple-peaks and uncertain t-half estimations in plasma. Another source of EHC is from glucuronidated metabolites since these metabolites excreted in the bile often undergo deconjugation and re-absorption in the GI tract.

The ability of sandwich-cultured hepatocytes (SCH) to form in vitro bile pockets makes them a viable tool for evaluating biliary excretion of compounds, transporter based drug-drug interaction (DDI), and hepatotoxic potential concerning transporter inhibition. Parameters obtained by SCH have also been incorporated into hepatic clearance prediction, as well as physiologically based PK modeling. Moreover, there are some reports investigating in vitro biliary excretion of metabolites in addition to the parent compound in SCH.

Mycophenolic acid (MPA), an inosine 5'-monophosphate dehydrogenase inhibitor, is an immunosuppressive agent to prevent acute rejection in organ transplantation. MPA is frequently administered as a pro-drug (mycophenolic acid mofetil: MMF) that is rapidly converted to MPA in vivo. The major metabolic pathway of MPA is glucuronidation, where 7-O-glucuronide (MPAG) is the major metabolite and acyl-glucuronide (AcMPAG) is the minor metabolite. These metabolites are known to be excreted into urine in humans. In humans and rats, MMF is also known to exhibit EHC of MPA because its metabolite MPAG undergoes biliary excretion followed by deconjugation to MPA in the GI tract and absorption to the blood. Since MMF is commonly administered in combination with other agents such as calcineurin inhibitors and steroids, many DDIs are reported. For example, co-delivery of cyclosporin A (CysA) can reduce plasma MPA concentration because CysA is believed to inhibit biliary excretion of MPAG, resulting in reduced EHC of MPA. In addition, CysA is also reported to inhibit various metabolic enzymes including UDP-glucuronosyltransferase (UGT). Therefore, a comprehensive understanding DDI events is important because an unexpected PK profile of MPA due to DDI often causes insufficient efficacy or unfavorable side effects. These unique characteristics
including DDI by CysA are also observed in rats.24,25)

The present study investigates the in vitro hepatic disposition of MPA in terms of glucuronidation and biliary excretion, as well as explores the DDI profiles between MPA and CysA. For these studies, the extent of cellular accumulation and in vitro biliary excretion of MPAG, AcMPAG, and MPA were determined using rat SCH. We also evaluated potential pan-glucuronidation inhibitors on in vitro hepatic MPA disposition.

**Methods**

**Chemicals:** Alamethicin, chrysin, CysA, MPA, and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma (St. Louis, MO). AcMPAG and MPAG were obtained from Toronto Research Chemicals (North York, Canada). Quercetin was from Tocris Bioscience (Bristol, United Kingdom). All other chemicals were of analytical grade.

**SCH studies:** Male Sprague-Dawley rat SCH in 24-well plates were purchased from Qualyst Transporter Solutions (Durham, NC). All experiments with SCH were done using 4-day cultures and performed according to manufacturer’s protocol and supplied reagents. Briefly, SCH were washed twice by either Plus (+) buffer or Minus (−) buffer and incubated at 37°C for 10 min following a third addition of these buffers; Plus (+) buffer containing calcium maintains tight junctions formed in SCH while Minus (−) buffer disrupts the tight junctions by removing divalent ions from SCH. After removal of these buffers, MPA in Plus (+) buffer was added onto SCH and maintained in a CO2 incubator at 37°C for up to 20 min. Incubations were terminated by buffer removal and washing SCH three times with cold Plus (+) buffer. For determining MPA, MPAG, and AcMPAG concentration, SCH was frozen, then treated with 0.1% formic acid for 10 min prior to MPA incubation, in addition to the 10 min incubation with CysA.

**Liver microsomes studies:** Male Sprague-Dawley rat liver microsomes (RLM; Xenotech, Lenexa, KS) were diluted to 0.1 mg protein/mL in assay buffer (50 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 50 µg/mg microsome alamethicin) and 1 µM MPA. After pre-incubation for 30 min at 37°C, glucuronidation reactions were initiated by addition of UDPGA to a 2 mM final concentration with or without CysA up to 30 µM. Aliquots were periodically taken and added into stop solution (0.1% formic acid, 90% acetonitrile, and 1,000 nM dichlofenac in water), followed by centrifugation (1,600 × g for 10 min). The supernatants were transferred to fresh 96-well plates and dried under nitrogen, then reconstituted with 0.1% formic acid containing 10% acetonitrile in water.

**Liver microsomes experiments:** Male Sprague-Dawley rat liver microsomes (RLM; Xenotech, Lenexa, KS) were diluted to 0.1 mg protein/mL in assay buffer (50 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 50 µg/mg microsome alamethicin) and 1 µM MPA. After pre-incubation for 30 min at 37°C, glucuronidation reactions were initiated by addition of UDPGA to a 2 mM final concentration with or without CysA up to 30 µM. Aliquots were periodically taken and added into stop solution (0.1% formic acid, 90% acetonitrile, and 1,000 nM dichlofenac in water), followed by centrifugation (1,600 × g for 10 min). The supernatants were transferred to fresh 96-well plates for determining MPA, MPAG, and AcMPAG concentrations.

**Measurement for MPA, MPAG, and AcMPAG concentration:** A combination of a Prominence 20 series liquid chromatography system (LC; Shimadzu, Kyoto, Japan) and an API-5000 mass spectrometer (MS/MS; AB Sciex, Foster City, CA) was used as the LC-MS/MS. Samples were fractionated using an AQUASIL C18 column (100 × 2.1 mm, 3 µm; Thermo Scientific, Waltham, MA) at 50°C with a 0.2 mL/min flow rate and a gradient mobile phase condition, where mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Mobile phase B was retained 30% from 0 min to 0.5 min post injection, then gradually increased to 90% from 0.5 min through 4 min. After 6 min post injection, mobile phase B was set to 30% for the next injection. Negative electrospray ionization mode with transitions of 319–190 m/z was used for MPA detection, while MPAG and AcMPAG detection used transitions of 495–319 m/z. MPAG was detected at an earlier retention time than AcMPAG, typically 2.7 min for MPAG and 4.3 min for AcMPAG. MPAG and diclofenac (294–250 m/z transition) were detected at 5.4 min and 6.1 min, respectively. Standard curve dilutions for determining intracellular contents were prepared by adding stock solution mixture including known concentrations of MPA, MPAG, and AcMPAG to untreated SCH (blank cell plates). The stock solutions were prepared with acidified buffer for stability. All cellular concentrations were calculated using Analyst 1.4.2 software (AB Sciex).

**Parameter calculation for data obtained by SCH studies:** AccumulationPlus (+) (Acccells+bile) and AccumulationMinus (−) (Acccells) were obtained according to vendor’s protocol. Briefly, these parameters indicate the mass amounts in SCH (pmol per mg protein) after correction for nonspecific binding. Using these parameters, the biliary excretion amount (BEA) and the biliary excretion index (BEI) were calculated by the following equations:

\[
\text{BEA (pmol/mg protein)} = \frac{\text{Acccells+bile} - \text{Acccells}}{\text{Acccells}}
\]

\[
\text{BEI} (%) = \frac{\text{Acccells+bile} - \text{Acccells}}{\text{Acccells+bile}} \times 100
\]

**Data analysis:** Student’s t-test was used for the evaluation of difference between two groups while analysis of variance (ANOVA) was conducted for comparison of differences among more than two groups. Unless otherwise indicated, statistical probability (p) was expressed as * for p < 0.05, ** for p < 0.01, and *** for p < 0.001.

**Results**

**MPA metabolism and biliary excretion in vitro:** The Acccells+bile of MPA, MPAG, and AcMPAG in SCH is shown in Figure 1A. Both glucuronides MPAG and AcMPAG were detected in SCH from the earliest sampling time. The amount of MPAG at 10 min was over 100-fold higher than that of AcMPAG (Fig. 1A). Given the plateau of Acccells+bile for all compounds, 10 min sampling times were selected for our analysis. The Acccells+bile and Acccells of MPA in 4 different SCH preparations are summarized in Figure 2. Due to the lack of significant difference between Acccells+bile and Acccells, the in vitro biliary excretion is deemed negligible. A dominant MPAG production compared with AcMPAG was also observed in RLM in the presence of UDPGA (Fig. 1B).

**Biliary excretion of MPAG and AcMPAG after applying MPA onto SCH:** The Acccells+bile of both MPAG and AcMPAG was significantly higher than the respective Acccells (Fig. 3), indicating obvious biliary excretion of these metabolites. The BEI of MPAG and AcMPAG was 49% and 74%, respectively (Fig. 3). The in vitro biliary excretion of these metabolites was also observed in 3 additional preparations of SCH (Table 1). The average BEI of MPAG and AcMPAG with these 4 preparations was 40 ± 6% and 45 ± 24%, respectively. While this difference of BEI was not significant, the average BEA of MPAG (120 ± 94

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pmol/mg protein) was significantly higher than that of AcMPAG (0.340 ± 0.322 pmol/mg protein).

**Effects of quercetin, chrysin, and CysA on MPA disposition in SCH:** The effects of quercetin and chrysin on MPA disposition in rat SCH are shown in Figure 4. Quercetin reduced total production of MPAG and AcMPAG, with almost complete reduction by 100 µM (Figs. 4A and 4B). Chrysin, on the other hand, only partially reduced MPAG content to 52% of control (Fig. 4A). In addition, chrysin increased total production of AcMPAG by 750% at 100 µM (Fig. 4B). Both quercetin and chrysin increased MPA accumulation in SCH up to 280% and 230%, respectively (Fig. 4C), most likely due to inhibition of MPAG production (MPA's major glucuronidated metabolite).

Treatment of SCH by CysA did not change BEI, Acccells + bile, or Acccells for AcMPAG (Table 2). However, CysA reduced MPAG BEI; the BEI in the presence and absence of 10 µM CysA was 15% and 46% respectively (Table 2). In addition, 10 µM CysA increased the BEI of MPAG by 160%, while having no effect on the Acccells + bile of MPAG. This CysA effect of increased MPAG without a concomitant decrease in MPAG contrasts with the quercetin effect of glucuronidation inhibition, where MPA is increased with a concomitant decrease of MPAG (Fig. 4). These results suggest a different mechanism of action of CysA compared to quercetin.

**Effect of CysA on MPA metabolism in RLM:** The % of control of MPA and MPAG levels in RLM with increasing CysA concentration are summarized in Figure 5. Addition of 10 µM CysA reduced the relative MPAG level to 69% of control while increasing the relative MPA level to 115% of control, confirming a previous report showing CysA inhibits the glucuronidation of MPA in Wistar rat microsomes.22) These results contrast with the SCH data showing that CysA does not change Acccells + bile of MPAG and increases MPA by 160% at 10 µM (Table 2).

**Discussion**

In the present study, we investigated the hepatic disposition of MPA in terms of metabolism and biliary excretion using rat SCH. To the best of our knowledge, this is the first report of MPA disposition in SCH. In addition, the mechanisms of quercetin, chrysin, and CysA effects on hepatic disposition of MPA and its metabolites were investigated.

In the rat SCH as well as RLM, MPAG is the predominant metabolite of MPA followed by AcMPAG (Fig. 1). Westley
in vitro we detected SCH can exhibit an agreement with after applying MPA onto SCH (than that of AcMPAG (and AcMPAG were similar, the BEA of MPAG was much higher than that of CysA).<ref>Fig. 4. Effects of quercetin and chrysin on MPAG (A), AcMPAG (B), and MPA (C) amounts in SCH after applying MPA</ref> Data are processed for showing % of control of Accells and expressed as mean ± S.D. (n = 9 in the control, n = 3 in the presence of CysA). * and ** represent statistically significant difference from control with p < 0.05 and p < 0.001, respectively.

Table 2. Effect of CysA on MPAG, AcMPAG, and MPA disposition in SCH

<table>
<thead>
<tr>
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<th>MPAG</th>
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<tr>
<td></td>
<td>Acccells+bile</td>
<td>Acccells</td>
<td>BEI</td>
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<tr>
<td>Control</td>
<td>218 ± 27</td>
<td>117 ± 22</td>
<td>46</td>
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<tr>
<td>CysA 1µM</td>
<td>237 ± 16</td>
<td>150 ± 10</td>
<td>37</td>
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<tr>
<td>CysA 3µM</td>
<td>247 ± 12</td>
<td>188 ± 21***</td>
<td>24</td>
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<tr>
<td>CysA 10µM</td>
<td>234 ± 11</td>
<td>199 ± 21***</td>
<td>15</td>
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All data are from 10 min incubation after applying 2.5 µM MPA. Acccells+bile and Acccells are expressed as mean ± S.D. (n = 9 in the control, n = 3 in the presence of CysA).<ref>Fig. 5. Effects of CysA on MPA glucuronidation in rat liver microsomes</ref> Serial concentrations of CysA (0, 0.3, 1, 3, 10 and 30 µM) were co-incubated with 1 µM MPA for 60 min in rat liver microsomes in the presence of UDPGA. The % of controls of MPA (C) and MPAG (B) levels (mean of duplicates) were plotted vs. CysA concentration, where MPA and MPAG levels in the absence of CysA represent the control (100%).

metabolite in in vitro bile pockets. Gao et al.<ref>Fig. 2</ref> investigated the SD rat PK profile of MMF, which is rapidly converted to MPA, and reported 33% biliary recovery of MPAG and 1% biliary recovery of AcMPAG at 9 h post MMF dosing. A liver MPA perfusion study also showed biliary MPAG recovery was much greater than AcMPAG recovery.<ref>Comparing our SCH results to these in vivo reports shows that in vitro SCH reflects the in vivo hepatic disposition of MPA in terms of glucuronidation and biliary excretion. The SCH cultures did not show significant biliary excretion of MPA (Fig. 2), which also reasonably reflects the in vivo situation as only 0.08% recovery of MPA was found in the bile after dosing MMF in SD rats.</ref>

Pan-inhibitors of cytochrome P450, such as 1-aminobenzotriazole, have been used for mechanistic PK studies to distinguish absorption processes from metabolic processes when the chemicals exhibit poor plasma exposure after oral dosing<ref>in addition to raising plasma concentrations of compounds when assessing toxicological effects of metabolites and parent compounds.</ref> Therefore, a pan-UGT inhibitors would also be beneficial for drug discovery. Nakamori et al. have reported that quercetin was metabolized by 12 different subtypes of UGT-overexpressed supersomes including the UGT1A and 1B family<ref>postulating quercetin as a potential pan-inhibitor to UGT metabolism. The present study revealed that quercetin can reduce both MPAG and AcMPAG accumulation in SCH with a concomitant increase in MPA accumulation (Fig. 4). This increase in MPA is likely due to inhibition of MPA glucuronidation to both MPAG and AcMPAG. Thus these data support the hypothesis that quercetin may be a pan-UGT inhibitor that can be used similarly to 1-aminobenzotriazole for UGT-specific questions. In contrast, our results show that chrysin is not a pan-UGT inhibitor as it only partially inhibits MPAG production and enhances AcMPAG production (Figs. 4A</ref>
MPA and/or MPAG, where MPA is increased and MPAG is unchanged.

The enhanced AcMPAG production is most likely due to the selective inhibition of the major MPA-MPAG metabolic pathway by chrysin, allowing a change in metabolic route due to the preserved MPA-AcMPAG metabolic pathway (metabolite switching). Walsky et al. have reported that chrysin inhibits human UGT1A1-mediated metabolism effectively, but does not inhibit human UGT1A6-mediated metabolism very well, supporting the supposition that chrysin does not inhibit all UGT subtypes. An alternative explanation could be metabolic stimulation by chrysin for AcMPAG production.

The successful detection of MPAG in both cells and bile pockets of SCH allows investigation of DDI events. CysA is often co-administered with MMF/MPA and is known to show DDI in the clinic. The major DDI event is reduction of plasma MPA concentration due to an inhibition of MPAG biliary excretion, resulting in lower EHC. This DDI is also observed in rats as Kobayashi et al. reported that CysA inhibits biliary excretion of MPAG through the multidrug resistance-associated protein 2 (Mrp2). Westley et al. also reported potential CysA inhibition of MPA glucuronidation in addition to Mrp2-mediated MPAG transport. The inhibition of glucuronidation by CysA is also observed in Wistar rat microsomes and human UGT-overexpressed supernatants. Our study showed a reduction of MPAG BEI by CysA (Table 2), clearly implicating an inhibition of its biliary excretion as previously shown in vivo. In addition, CysA also increased $\text{Acc}_{\text{cells+bile}}$ of MPA up to 160% relative to control without a concomitant decrease in $\text{Acc}_{\text{cells+bile}}$ of MPAG (Table 2). We hypothesize this MPA accumulation without MPAG reduction is due to a mixed inhibition of glucuronidation and sinusoidal efflux of MPA and/or MPAG (Fig. 6). This hypothesis is explained by considering the mass balance of MPA and MPAG; we discount any AcMPAG effects on the mass balance in this argument because if it is orders of magnitude less abundant, then its contribution to MPA and/or MPAG is also affected by CysA in order to account for the unchanged MPAG $\text{Acc}_{\text{cells+bile}}$ and increased MPA $\text{Acc}_{\text{cells+bile}}$ in SCH. For further understanding of this comprehensive DDI mechanism, an identification of the responsible transporter(s) for this sinusoidal efflux of MPA and MPAG as well as a full elucidation of CysA’s effect on sinusoidal efflux will be required in the future. In this regard, we have measured sinusoidal efflux of both MPA and MPAG in SCH.

Contrary to findings with MPAG, CysA did not change the AcMPAG BEI (Table 2). This suggests that unique or additional transporter(s) are involved in biliary excretion of AcMPAG. There is a report of Mrp2 and another transporter’s contribution to biliary excretion of AcMPAG in Wistar rats while Mrp2 seems to be the major transporter of MPAG. Future study is needed to specify the responsible AcMPAG transporter(s) in the canalicular membrane of SD rats. In addition, CysA did not change the $\text{Acc}_{\text{cells+bile}}$ of MPAG while CysA is reported to inhibit MPA glucuronidation to AcMPAG in Wistar rats. Therefore, CysA may also inhibit the sinusoidal efflux of AcMPAG for maintaining its abundance in SCH.

In conclusion, we found that SCH shows good agreement with in vivo hepatic disposition of MPA in terms of metabolism and biliary excretion. The major metabolite of MPA in SCH is MPAG followed by orders of magnitude reduced AcMPAG, and both are excreted into in vitro bile with similar BEI. The BEA may be better than BEI when reporting in vitro biliary excretion results because it identifies the major metabolite in the bile. Finally, SCH is useful in mechanistic DDI studies. While CysA is known to inhibit MPAG biliary excretion and MPAG glucuronidation, we implicate additional DDI effects on inhibition of MPA/MPAG sinusoidal efflux as well. Thus, SCH is a powerful tool to obtain insights into hepatic disposition of MPA and hopefully other drugs causing EHC.

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References


