ORIGINAL ARTICLE

# Species differences in sinusoidal and canalicular efflux transport of mycophenolic acid 7-*O*-glucuronide in sandwich-cultured hepatocytes

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## Abstract

Metabolism and sinusoidal/canalicular efflux of mycophenolic acid (MPA) was investigated using sandwich-cultured hepatocytes (SCHs). After applying MPA to SCHs from humans, wild-type rats, and multidrug resistance-associated protein (Mrp) 2-deficient rats, the MPA metabolites 7-*O*-glucuronide (MPAG) and acyl glucuronide (AcMPAG) were detected in the intracellular compartment of the SCHs. Sinusoidal efflux of MPAG was detected in all SCH preparations including Mrp2-deficient rat SCHs, whereas canalicular efflux of MPAG was observed in wild-type rat and human SCHs but not in Mrp2-deficient rat SCHs. The ratio of canalicular efflux to net (canalicular plus sinusoidal) efflux was $37 \pm 8\%$ in wild-type rat SCHs, while the ratio in human SCHs was significantly lower ($20 \pm 2\%$, $P < 0.05$), indicating species differences in the direction of hepatic MPAG transport. This 20% ratio in human SCHs corresponds to a high sinusoidal MPAG efflux (80%) that can in part account for the urine-dominated recovery of MPAG in humans. Both sinusoidal and canalicular MPAG efflux in rat SCHs shows a good correspondence to urinary and biliary recovery of MPAG after MPA dosing. The sinusoidal efflux of AcMPAG in human SCHs was detected from one out of three donors, suggesting donor-to-donor variation. In conclusion, this study demonstrates the predictive value of SCHs for elucidating the interplay of metabolism and efflux transport, in addition to demonstrating a species difference between rat and human in sinusoidal and canalicular efflux of MPAG.

### Abbreviations

AcMPAG, acyl glucuronide of mycophenolic acid; EHC, enterohepatic circulation; KO, knockout; MMF, mycophenolic acid mofetil; MPA, mycophenolic acid; MPAG, 7-*O*-glucuronide of mycophenolic acid; MRP or Mrp, multidrug resistance-associated protein; SCH, sandwich-cultured hepatocyte; SD, Sprague–Dawley; UGT, UDP-glucuronosyltransferase.

## Introduction

Mycophenolic acid (MPA), an inosine 5′-monophosphate dehydrogenase inhibitor, is an immunosuppressive agent to prevent acute rejection in organ transplantation. It is frequently administered as a pro-drug form (mycophenolic acid mofetil; MMF) that is rapidly and completely converted to MPA by esterases in the gut wall, blood, liver, and tissues. MPA is metabolized in the liver, small intestine, and kidney, with 7-*O*-glucuronide (MPAG) as the major metabolite and acyl glucuronide (AcMPAG) as the minor metabolite (Staatz and Tett 2007). Thus, while the metabolism of MPA in the body has been well characterized, its excretion and that of its metabolites from the body have not been fully investigated.

The few studies conducted thus far on the transport of MPA and its metabolites have demonstrated that enterohepatic circulation (EHC) is a well-known pharmacokinetic profile of MPA in human, as shown by the multiple peaks in the time-plasma concentration profile (Bullingham et al.

1996). The detailed mechanisms of EHC of MPA have been investigated using rats. It is believed that the biliary excretion of MPAG plays a crucial role because MPAG excreted into the bile can undergo deconjugation to MPA at the gastrointestinal-tract and re-absorption into the blood (Staatz and Tett 2007). In addition, the multidrug resistance-associated protein (Mrp) 2 is reported to be responsible for biliary excretion of MPAG (Kobayashi et al. 2004; Westley et al. 2006). However, rat and human may show species differences in pharmacokinetic profiles of MMF/MPA in terms of urinary and fecal/biliary recovery of MPAG. In humans, ~87% of orally administered radiolabeled MMF is retrieved as MPAG in urine while only 6% of the radioactivity is recovered in feces (the reference is available from Drugs@FDA in U.S. Food and Drug Administration). In contrast to humans, urinary recovery of MPAG is reported to be only 55% in normal adult Wistar rats (Ishizaki et al. 2012) following intravenous injection of MPA. Furthermore, oral MMF administration to Sprague–Dawley (SD) rats yields a recovery of 33% of MPAG, 0.08% of MPA, and 1.06% of AcMPAG in the bile (Gao et al. 2011). It is also reported that intravenous dosing of MPA to Wistar rats yields 66% of MPAG biliary recovery (Ishizaki et al. 2012). Therefore, we may reasonably hypothesize species differences in urinary and biliary MPAG recovery. However, the lack of comparative and comprehensive in vitro studies has limited the better understanding of the species differences.

Sinusoidal efflux is a route for secretion of molecules from the liver to the blood prior to urinary excretion or re-entry to the liver. Zelcer et al. (2005) have reported that mice deficient in Mrp3 (a transporter located at the sinusoidal membrane) can reduce plasma levels of morphine-3-glucuronide after morphine administration, as well as change the excretion route from urine to feces. This report indicates the importance of sinusoidal efflux for plasma metabolite concentration in addition to urinary or fecal (biliary) recovery. However, few attempts have been made to investigate sinusoidal and canalicular efflux for correlation with urinary and biliary excretion.

Sandwich-cultured hepatocytes (SCHs) forming in vitro bile pockets are a well-known tool for investigating biliary excretion (Ghibellini et al. 2006). We recently reported in vitro biliary excretion of MPAG and AcMPAG as metabolites of MPA in rat SCHs (Tetsuka et al. 2014). The tight junction formation in SCHs can divide the culture into three different components (i.e., culture media component, intracellular component, and bile pocket component), thereby facilitating elucidation of sinusoidal (basolateral) efflux transport in addition to canalicular efflux transport (Swift et al. 2010; Pfeifer et al. 2013).

In this study, we investigated a series of events in SCHs — including metabolism, sinusoidal efflux, and canalicular efflux of MPA — to determine species differences in MPA disposition between rats and humans. In addition, SCHs prepared from Mrp2 knockout (KO) rats were also used for further characterization of the hepatic disposition.

## Materials and Methods

### Experimental materials

AcMPAG and MPAG were purchased from Toronto Research Chemicals (North York, ON, Canada), and MPA was obtained from Sigma (St. Louis, MO). SCHs in 24-well plate format (B-CLEAR®) were purchased from Qualyst Transporter Solutions (Durham, NC), where SCHs from male SD rats and male Mrp2 SAGE® KO rats (SD strain) had been cultured for 4 days at the start of the experiment while human SCHs had been cultured for 7 days. The human SCHs were prepared from female Caucasian donors with age ranges of 33–66 years. Plus (+) and Minus (−) buffer were also purchased from Qualyst Transporter Solutions; Plus (+) buffer containing calcium can maintain tight junctions in SCHs while Minus (−) buffer can disrupt the tight junctions by removing divalent ions from SCHs. All other chemicals were of analytical grade.

### Sinusoidal and canalicular efflux study

SCHs were washed twice with Plus (+) buffer then treated with 2.5 µmol/L MPA in Plus (+) buffer, initiating uptake and metabolism in SCH. After incubation at 37°C for 20 min, the SCHs were washed twice, and buffer was replaced using either warm Plus (+) or Minus (−) buffer. These buffers were collected after predetermined time points and stored at −20°C until measurement of MPA, MPAG, and AcMPAG, as described previously (Tetsuka et al. 2014). Intracellular amounts of MPA, MPAG, and AcMPAG after 20-min incubation were also measured and expressed as $Acc_{cells+bile}$.

### Data analysis

Amounts of MPAG and AcMPAG in Plus (+) buffer (Mass (+)) and Minus (−) buffer (Mass (−)) were calculated from their concentration and buffer volume. These amounts may be used to calculate Sinusoidal Efflux and Canalicular Efflux via the following equations (Swift et al. 2010).

$$\text{Sinusoidal Efflux (pmol/mg protein)} = \text{Mass (+)}$$

$$\text{Canalicular Efflux (pmol/mg protein)}$$
$$= \text{Mass (−)} - \text{Mass (+)}$$

In addition, relative canalicular efflux (%) was calculated using the following equation: [Mass (−) − Mass

(+)]/Mass (−) × 100, which can represent the extent of canalicular efflux to total efflux, and relative sinusoidal efflux (%) was calculated from 100 − relative canalicular efflux. Student's *t*-test was used to evaluate differences between two groups. Statistical probability (*P*) was expressed as * for *P* < 0.05, ** for *P* < 0.01, and *** for *P* < 0.001, respectively.

## Results

### Sinusoidal and canalicular efflux of MPA and its metabolites

Typical time courses of MPAG and AcMPAG efflux after applying MPA to SCHs from SD rats, Mrp2 KO rats, and humans are shown in Figure 1A–F. An increase in Mass (+) of MPAG and AcMPAG was observed over time in all SCH preparations, indicating sinusoidal efflux of MPAG and AcMPAG after metabolism from MPA. In addition,

the canalicular efflux of MPAG was demonstrated by the MPAG Mass (−) being higher than Mass (+) in rat and human SCHs (Fig. 1A and C). In contrast, no differences were noted between Mass (+) and Mass (−) of MPAG in Mrp2 KO rat SCHs (Fig. 1B). Regarding the canalicular efflux of AcMPAG, we were able to detect a significant difference between AcMPAG Mass (+) and Mass (−) in human SCHs after 5 min (Fig. 1F). However, a conclusive statement on the canalicular efflux of AcMPAG in human SCHs cannot be made due to the lack of any statistically significant difference in rat SCHs (Fig. 1D) in this study compared to that with successful detection of in vitro biliary excretion of AcMPAG in rat SCHs in the previous study (Tetsuka et al. 2014). The inability to detect canalicular efflux in rat SCHs in this study may be due to the markedly low amounts of AcMPAG in buffer components during intricate experiments measuring the sinusoidal and canalicular efflux of the minor metabolite.
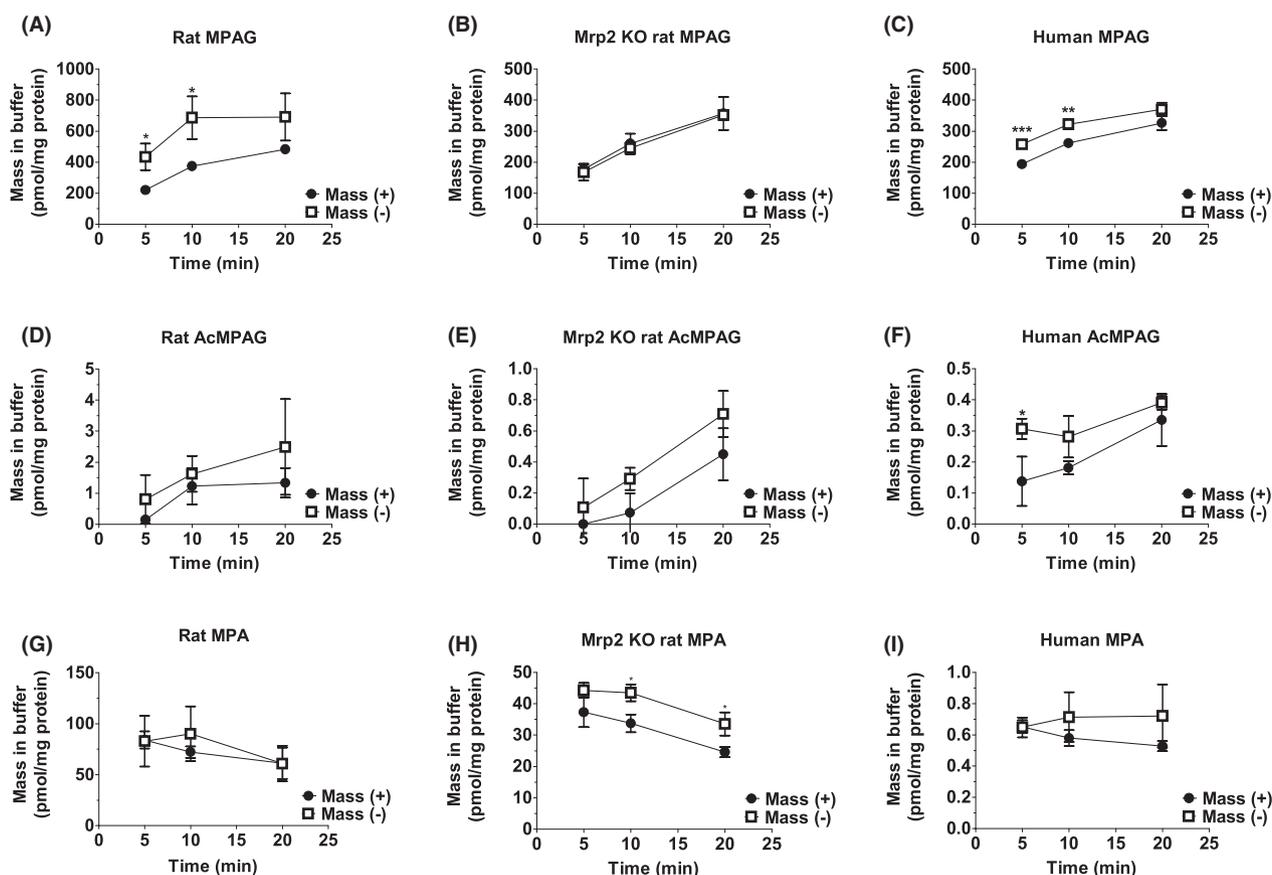


**Figure 1.** Typical time course of MPAG, AcMPAG, and MPA efflux to buffer after applying MPA to rat, Mrp2 KO rat, and human SCH. Closed circles (●) and open squares (□) represent mass in Plus (+) buffer (Mass (+)) and mass in Minus (−) buffer (Mass (−)), respectively. Mass (+) can represent sinusoidal efflux while Mass (−) can represent net efflux (sinusoidal plus canalicular efflux). Data are expressed as mean ± SD (*n* = 3). *P* < 0.05, **P* < 0.01, and ***P* < 0.001, respectively.

Although we also monitored the efflux of MPA (Fig. 1G–I), we did not observe a time-dependent increase in Mass (+) or Mass (−), due to the rapid efflux of MPA or un-washable MPA in the pre-incubation process. Buffer sampling within a shorter time period would clarify this point. However, such a study would prevent the detection of AcMPAG in these buffers in terms of detection limit. Despite the time course of MPA flux not being optimal, significant differences between Mass (+) and Mass (−) were detected in Mrp2 KO rat SCHs (Fig. 1H).

## Species differences in accumulation and sinusoidal/canalicular efflux

As we were able to detect the sinusoidal and canalicular efflux of MPAG in addition to sinusoidal efflux of AcM-PAG, the donor number was increased in further analysis (Table 1; Efflux from SCHs). We also determined the amount of MPA, MPAG, and AcMPAG in SCHs before initiating the efflux study (Table 1; Accumulation in SCHs before efflux). The intracellular amount of MPAG was 2–3 orders of magnitude greater than that of AcMPAG in all SCH preparations, showing that MPAG is the major metabolite in humans and rats, even when deficient for Mrp2. The amount of MPA in rat SCHs ranging from 44–241 pmol/mg protein (mean, 146 pmol/mg protein) was 9.5-fold higher than that in human SCHs, which ranged from 11–23 pmol/mg protein (mean, 15 pmol/mg protein), but the difference was not significant ($P = 0.084$). Canalicular efflux in addition to sinusoidal efflux of MPAG was observed in all preparations of rat and human SCHs, while canalicular efflux of MPAG

in Mrp2 KO rat SCHs was not significant. Regarding AcMPAG, sinusoidal efflux in rat SCHs was observed in all preparations, whereas that in human SCHs was observed in only one donor out of three. In contrast, intracellular AcMPAG in human SCHs before initiating the efflux study and intracellular MPA that can be a source of AcMPAG production during the efflux study were detected in all donors. The direction of MPAG efflux from SCHs is highlighted in Figure 2. The relative canalicular efflux in rat SCHs ranged from 30–45%, which is significantly higher than that in human SCHs (19–22%, $P = 0.019$), or 78–81% of MPAG oriented to sinusoidal efflux in humans.

## Discussion

Given that species differences in the biliary excretion of drugs have been reported between rats, dogs, and humans (Grime and Paine 2013), species differences may also exist in drug metabolites with respect to their levels and proportions in the bile in addition to their final destination that is feces or urine. MPA is one such drug with reported species differences, as its major metabolite MPAG is predominantly recovered in urine of humans and in both urine and bile of rats. Prior to urinary excretion, hepatic metabolites must undergo sinusoidal efflux. Despite the importance of sinusoidal efflux for pharmacokinetics, investigation concerning this pathway has been limited, restricting a comprehensive understanding of the hepatic disposition of drugs and their metabolites. In this study, sinusoidal and canalicular efflux of MPA metabolites were investigated. We clearly demonstrated a sinusoidal efflux pathway of MPAG and AcMPAG following the

**Table 1.** Accumulation and efflux parameters of MPA and its metabolites in rat, Mrp2 KO rat, and human SCHs.

| | Accumulation in SCHs before efflux | | | Efflux from SCHs | | | |
|---|---|---|---|---|---|---|---|
| | MPA | MPAG | AcMPAG | MPAG | | | AcMPAG |
| | $Acc_{cells+bile}$ (pmol/mg protein) | | | Sinusoidal Efflux (pmol/mg protein) | Canalicular Efflux (pmol/mg protein) | | Sinusoidal Efflux (pmol/mg protein) |
| Rat | 154 ± 35 | 354 ± 31 | 0.67 ± 0.11 | 375 ± 15 | 312* | | 1.23 ± 0.59 |
| Rat | 241 ± 8 | 429 ± 18 | 0.39 ± 0.06 | 431 ± 104 | 242* | | 0.67 ± 1.16 |
| Rat | 44 ± 1 | 477 ± 21 | 1.23 ± 0.58 | 302 ± 38 | 129** | | 1.94 ± 0.54 |
| Mrp2 KO rat | 190 ± 33 | 180 ± 11 | 0.40 ± 0.10 | 305 ± 77 | 65 | | 0.82 ± 0.22 |
| Mrp2 KO rat | 113 ± 3 | 277 ± 21 | 0.43 ± 0.05 | 260 ± 32 | (−15) | | 0.07 ± 0.13 |
| Human | 12 ± 1 | 258 ± 27 | 0.16 ± 0.03 | 214 ± 5 | 49* | | ND |
| Human | 23 ± 8 | 229 ± 26 | 0.89 ± 0.18 | 264 ± 13 | 60** | | 0.18 ± 0.02 |
| Human | 11 ± 2 | 180 ± 11 | 0.40 ± 0.06 | 117 ± 7 | 34* | | ND |

After 20-min incubation of MPA with SCHs, intracellular MPA, MPAG, and AcMPAG were measured and expressed as $Acc_{cells+bile}$. An additional 10-min incubation after replacing dosing solution with either Plus (+) or Minus (−) buffer was conducted to obtain efflux parameters. $Acc_{cells+bile}$ and Sinusoidal Efflux are expressed as mean ± SD ($n = 3$) whereas Canalicular Efflux is expressed as mean value as a result of data subtraction. ND, not detected. *$P < 0.05$ and **$P < 0.01$ between Mass (+) and Mass (−), respectively.
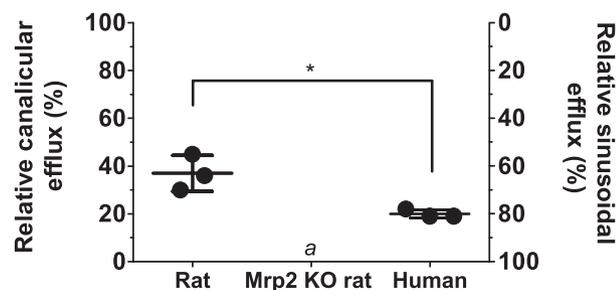
**Figure 2.** Relative sinusoidal/canalicular efflux of MPAG among rat, Mrp2 KO rat, and human SCH. Given observations of statistical significance in rat and human SCHs between Mass (+) and Mass (−) (n = 3), analysis to determine relative sinusoidal/canalicular efflux was conducted. The results of three independent SCH preparations are expressed as closed circles (●). Mean ± SD lines are also plotted. [a]In Mrp2 KO rat SCHs, statistic significance was not observed between Mass (+) and Mass (−) (n = 3) in two independent SCH preparations. *P < 0.05.

metabolism from MPA in rats and humans (Fig. 1). Recently, Pfeifer et al. (2013) reported a novel protocol for a sinusoidal (basolateral) efflux assay with SCHs using rosuvastatin as a model compound. The present study is a successful expansion of this assay for elucidating sequential glucuronidation followed by sinusoidal efflux.

For both rats and humans, EHC is a well-known pharmacokinetic profile of MPA. The mechanism of EHC is postulated to be biliary excretion of MPAG followed by de-conjugation to MPA at the gastrointestinal-tract and subsequent absorption into the blood (Staatz and Tett 2007). We previously reported that rat SCHs can model the biliary excretion of MPAG (Tetsuka et al. 2014). This study, therefore, involved the canalicular efflux of MPAG after metabolism from MPA in human SCHs. In addition, no canalicular efflux of MPAG was observed in Mrp-2 KO rat SCHs in this study suggesting that Mrp2 is the transporter responsible for the biliary excretion of MPAG in rats, findings in agreement with those of previous reports (Kobayashi et al. 2004; Westley et al. 2006).

Both canalicular and sinusoidal efflux are pathways of hepatic disposition that eliminate drugs and their metabolites from hepatocytes. Of these pathways, canalicular efflux has been emphasized as a route to the bile side that can cause EHC or fecal excretion, suggesting the similar importance of sinusoidal efflux as a route to the blood stream capable of causing the presence of metabolites in the blood and potentially urine as well. Understanding this direction of efflux is important for the pharmacokinetics of drugs and their metabolites. Here, we demonstrated species differences between rats and humans regarding hepatic MPAG efflux, with the relative sinusoidal efflux of MPAG in human SCHs (80%) being significantly higher than that in rat SCHs (Fig. 2). This

substantial contribution of sinusoidal MPAG efflux in human SCHs is supported by recently reported mathematical modeling of MPA disposition in human SCHs (Matsunaga et al. 2014). In addition, efflux in the opposite direction is one potential reason for the different MPAG recovery between humans and rats, where urine-dominated MPAG recovery is observed in humans while both urinary and biliary recovery is observed in rats. Further investigation on renal disposition of MPA and its metabolites will be required to improve understanding of this species difference. Regarding the mechanism behind the species differences in hepatic MPAG efflux between rats and humans, one explanation could be the different affinity between human MRP2 and rat Mrp2 as reported by Takekuma et al. (2007). In addition, other MRP transporters on the sinusoidal side, such as MRP3, MRP4, and MRP6 (Giacomini et al. 2010), might also contribute to species differences. Recently, MPAG was reported to be a substrate of human MRP3 and MRP4 (Matsunaga et al. 2014). Further investigation regarding protein expression level of these transporters in addition MPAG affinity to rat Mrp transporters will be required to provide a conclusive explanation for these species differences in hepatic disposition.

Among MRP transporters, MRP3 may play a key role in sinusoidal efflux given reports citing its role in transporting acetaminophen glucuronide (Ghanem et al. 2005; Allegaert and Tibboel 2006) and morphine glucuronide (Zelcer et al. 2005) to the urine and blood stream. Furthermore, increases in Mrp3, in addition to UDP-glucuronosyltransferase (UGT) enzymes, have been reported in Mrp2-deficient rats (TR-rat; Johnson et al. 2006). However, of note, the source of Mrp2 deficiency in that previous study differs from the one used in this study. Regarding the hepatic disposition of MPA and its metabolites, the canalicular efflux of MPAG in SCHs was diminished due to Mrp2 deficiency (Table 1), suggesting that Mrp2 is the transporter responsible for the efflux of MPAG to the bile side regardless of Mrp3 recognition. We were unable to statistically compare the events occurring in rat SCHs and Mrp2 KO rat SCHs due to the limited number of SCH preparations from KO rats. However, a change in the degree of protein expression of un-identified MPA transporter(s) to the bile side might be occurring in Mrp2 KO SCHs given that the canalicular efflux of MPA was detected in the culture (Fig. 1H) but not in that of normal rat SCHs (Fig. 1G).

Sinusoidal efflux of AcMPAG in human SCHs was observed in only one of the three donors but in all rat samples (Table 1). However, intracellular AcMPAG was detected in human SCHs from all three donors showing metabolic capacity (Table 1). Although these results suggest potential donor-to-donor variation of AcMPAG

transport function, the case numbers are limited. This interpretation is consistent with the known patient-to-patient variation of AcMPAG concentration in human plasma after MMF administration (Shipkova et al. 2002). Future identification of sinusoidal efflux transporters of AcMPAG could be useful for pharmacogenomic elucidation in MMF therapy with recent reports incorporating MRP2 and UGT polymorphism (Lloberas et al. 2011; Dupuis et al. 2012; Fukuda et al. 2012). In addition, future direct comparison between MPA disposition and genotyping in the same human SCH sample will prove informative, as SCHs enable mechanistic studies to be conducted to elucidate the interplay between metabolism and transport.

In conclusion, this study explored sinusoidal and canalicular efflux of MPAG and AcMPAG after metabolism from MPA in SCHs. We successfully detected a series of events regarding metabolism and sinusoidal/canalicular efflux. In addition, the study revealed species differences in the canalicular efflux of MPAG between rats and humans, as well as demonstrating sinusoidal-oriented MPAG efflux in humans. These findings can, in part, account for the species differences in MPAG recovery, where urine-dominated recovery is found in humans while biliary and urinary recovery is found in rats. Donor-to-donor differences in the sinusoidal efflux of AcMPAG from human SCHs were also observed. Given that sinusoidal efflux indicates the secretion of molecules from the liver to the blood, the utilization of SCHs in the evaluation of the interplay between metabolism and sinusoidal efflux will help facilitate the investigation of plasma metabolite levels and their urinary excretion.

## Acknowledgement

## Disclosure

None declared.

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