Mycophenolic acid (MPA) and its glucuronide metabolites interact with transport systems responsible for excretion of organic anions in the basolateral membrane of the human kidney

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Abstract

Background. Mycophenolic acid (MPA), the active moiety of the prodrug mycophenolate mofetil, is widely used in immunosuppressive regimens after kidney, liver or heart transplantation. MPA is metabolized predominantly to the inactive 7-O-glucuronide (MPAG). A minor fraction is converted to the pharmacologically active acyl glucuronide (AcMPAG). All compounds ultimately are eliminated via the kidneys. Due to their structures, MPA and its metabolites are candidate substrates for the human organic anion transporters 1 (OAT1) and 3 (OAT3) as well as for the Na+-dicarboxylate cotransporter 3 (NaDC3).

Methods. Human (h)OAT1, hOAT3 and hNaDC3 were expressed from in vitro synthesized cRNA in collagenase-defolliculated Xenopus laevis oocytes. On day 3 post-injection, measurements were made of (i) substrate-associated currents using MPA and MPAG (only in hNaDC3-expressing oocytes) and (ii) uptake of [3H]-p-aminohippurate (hOAT1) or [3H]estrone sulfate (hOAT3) in the absence or presence of either MPA, MPAG or AcMPAG.

Results. In hNaDC3-expressing oocytes at −60 mV, MPA (0.1 mM) as well as MPAG (0.1 mM) induced inward currents that were 17 and 25% of the currents evoked by succinate (1 mM). Vice versa, currents induced by succinate (1 mM) were partially inhibited by MPA and MPAG. hOAT1 and hOAT3 were potently inhibited by MPA (IC50 1.24 and 0.52 μM, respectively). Human OAT3, but not hOAT1, was additionally inhibited by both glucuronide metabolites of MPA in a concentration-dependent manner (IC50 15.2 μM for MPAG and 2.88 μM for AcMPAG), consistent with a preference of hOAT3 for more bulky substrates compared with hOAT1.

Conclusions. MPA and its metabolites potently interact with renal organic anion transporters hOAT1 and hOAT3, and thereby may interfere with the renal secretion of antiviral drugs, cortisol and other organic anions.

Keywords: glucuronide mycophenolate metabolites; hNaDC3; hOAT1/3; kidney; mycophenolic acid; transport

Introduction

Mycophenolic acid (MPA) is a potent immunosuppressant that is widely used in combination with calcineurin inhibitors, such as cyclosporin A (CsA), especially in kidney transplant recipients. It is administered as the prodrug mycophenolate mofetil (MMF), which is rapidly de-esterified in the body to MPA, or as an enteric-coated formulation. MPA is primarily metabolized by UDP-glucuronosyltransferases to the pharmacologically inactive phenolic glucuronide (MPAG) [1], and a minor pharmacologically active acyl glucuronide (AcMPAG) [2]. Pharmacokinetic studies [1,3] as well as in vitro metabolic investigations [4,5] using microsomes from liver, kidney and intestine have highlighted the importance of the liver for the systemic clearance of MPA. A significant amount of MPAG is excreted into the bile, and then undergoes bacterial deconjugation in the gut releasing MPA for reabsorption. This extensive entero-hepatic recirculation contributes substantially to the MPA exposure of MMF-treated patients [6], such that <5% of the total dose is eliminated via the faeces [1]. Recent studies [7,8] have demonstrated that MPAG secretion into the bile is mediated by the multi drug resistance-associated protein (Mrp) 2.
In addition to the liver, it has also been argued that glucuronidation of MPA can occur in renal tubule cells, which would allow direct tubular excretion of MPAG into urine. This was based on the comparison of the urinary clearance of MPAG after intravenous and oral administration of MMF to healthy subjects [3], and on a study into the effect of hepatic impairment on MPAG clearance [9]. Furthermore, human kidney microsomes were found to be much more efficient than either human liver or intestinal microsomes in transforming MPA to MPAG in vitro [5,10]. About 95% of the administered MPA is ultimately eliminated via the kidneys, almost exclusively as MPAG [1,3]. Less than 1% of the administered dose is excreted as MPA, or as the minor acylglucuronide. As ~82% of the MPAG in human plasma under MPA therapy is bound to albumin [1], transepithelial secretion rather than filtration is thought to be the primary mechanism of MPAG excretion [1]. MPA is even more strongly bound (97–99%) to plasma proteins, primarily albumin. With the exception of Mrp2, little is known about the transport mechanisms involved in the uptake and secretion of MPA and its metabolites through the kidneys. The purpose of the present study was to investigate whether the human organic anion transporters hNaDC3, hOAT1 and hOAT3 are potential candidates for the uptake of these compounds by renal proximal tubule cells.

Materials and methods

Chemicals

[3H]-Aminohippurate (PAH) (aminohippuric acid, p-[glycyl-2-3H], 1-5 Ci/mmol) and [3H]estrone sulfate (ES, ammonium salt, [6,7-3H(N)], 40–60 Ci/mmol) were obtained from NEN (Boston, MA, USA). MPA was obtained from Sigma (Deisenhofen, Germany). The glucuronide metabolites of MPA, MPAG and AcMPAG, were kind gifts of Roche Pharmaceuticals (Palo Alto, CA, USA). Collagenase was obtained from Biochrom (Berlin, Germany). 8500-[14C]glucuronide metabolites of MPA, MPAG and AcMPAG, were kind gifts of Roche Pharmaceuticals (Palo Alto, CA, USA). Borosilicate glass microelectrodes were filled with 3 M KCl and had resistances of ~1 MΩ. The resting membrane potential of the oocytes ranged between ~28 and ~46 mV and holding currents to achieve a potential of ~60 mV were in the range of ~10 to ~40 nA. Steady-state currents were obtained during 5 s voltage pulses from ~60 mV to potentials between ~90 and 0 mV in 10 mV steps. The current-voltage (I-V) relationships for substrate-induced currents, ΔI, were determined by subtraction of the steady-state currents in the absence of substrate from currents in the presence of substrate, respectively.

Uptake experiments

Uptake of [3H] PAH (5 μCi/ml) or [3H] ES (0.44 μCi/ml) was assayed at room temperature in ORI for 30 min or 1 h. After this time period, the uptake was terminated by aspiration of the incubation medium and 3 x 3 ml washes with ice-cold ORI. Each oocyte was then dissolved in 0.1 ml of 1 N NaOH and, after neutralization with 0.1 ml of 1 N HCl, the 3H content was assayed by liquid scintillation counting.

IC50 determinations

Substrate uptake of 0.2 μM [3H]PAH or 50 nM [3H]ES was assayed as described earlier, in the absence or presence of increasing concentrations of MPA or glucuronide metabolite for 30 min. Uptake was linear at least up to the 30 min time point at the PAH and ES concentrations used (data not shown) and should thus represent initial rate uptake. For determination of the concentration of MPA or one of its derivatives that blocked 50% of the respective substrate uptake (IC50), data were fitted to the equation $V = V_o[1 + (I/IC_{50})^h]$, where $V$ is the rate of substrate uptake in the presence of MPA or derivative, $V_o$ is the rate of substrate uptake in the absence of inhibitor, $I$ is the inhibitor concentration; and $h$ is the Hill coefficient. The SigmaPlot 8.0 program (SPSS Inc., Chicago, IL, USA) was used to fit the data by non-linear regression.

Kd determination

For hOAT1, 30 min PAH uptake was determined at 1 and 10 μM [3H]PAH in the presence of various concentrations of MPA. For hOAT3, 30 min ES uptake was determined at 50 and 200 nM [3H]ES, using again increasing concentrations
of MPA as inhibitor. The inverse of the uptake rates were plotted against the MPA concentration (Dixon plot).

Statistical analysis

All data presented are the means ± SE of the number of observations indicated in the text or figure legends. Unless otherwise stated, n is given as the number of experiments on oocytes from different donor animals, with the number of oocytes used per treatment in each individual experiment indicated. Statistical analysis using paired Student’s t-test was carried out with the GraphPad InStat program (version 3.00 for Windows 95; GraphPad Software, San Diego, CA, USA).

Results

Effect of MPA and MPAG on hNaDC3

In hNaDC3-expressing oocytes, addition of 0.1 mM MPA induced a small inward current compared with ORI-2 alone (Figure 1A). When sodium was replaced by N-methyl-D-glucamine, the MPA-associated inward current was abolished, indicating sodium-dependent translocation of MPA (Figure 1A). Figure 1 also shows the currents induced by 0.1 mM MPA (B, open circles) and 0.1 mM MPAG (C, open circles) in hNaDC3-oocytes as a function of membrane potential, each in comparison with those elicited by 1 mM succinate in the same oocytes (closed circles). At all potentials tested, the succinate-dependent inward currents were larger in magnitude as compared with MPA- or MPAG-induced currents. No inward currents were observed in H2O-injected oocytes when succinate, MPA, or MPAG was added (data not shown). When applied together with 1 mM succinate, MPA attenuated the inward currents by 16+/−10% and 28+/−8% at 0.1 and 1 mM, respectively. MPAG was somewhat more potent, eliciting a current decrease of 32+/−19% already at 0.1 mM (Figure 2). These data demonstrate that MPA and MPAG interact with, and are transported by human NaDC3.

MPA-Inhibition of substrate uptake via hOAT1 and hOAT3

We next investigated the effect of MPA on the basolateral organic anion transporters hOAT1 and hOAT3. In pilot experiments MPA significantly inhibited hOAT1-mediated PAH uptake at 10 μM (51.5+/−8.0%, P < 0.05) and 100 μM (91.7+/−2.1%, P < 0.001). Similarly, hOAT3-mediated PAH uptake was also significantly inhibited by the same MPA concentrations (87.6+/−3.6% at 10 μM, P < 0.001, and 99.8+/−3.7% at 100 μM, P < 0.01, respectively). When determining the inhibition constant of MPA for both transporters, PAH was again used as substrate for hOAT1, whereas hOAT3-mediated flux was measured with ES, which is a higher affinity substrate for this latter transporter. As shown in Figure 3A and 3B, MPA is a potent inhibitor for both transporters, with hOAT1 having a slightly lower sensitivity (IC50 1.24+/−0.06 μM) than hOAT3 (IC50 0.52+/−0.09 μM).

Kinetics of inhibition

To determine the type of inhibition of hOAT1 by MPA, we measured uptake at 1 and 10 μM [3H]PAH in the presence of various concentrations of MPA. The results are shown in Figure 4 as a Dixon plot. The intersection of the two straight lines above the X-axis demonstrates a competitive type of inhibition.
and reveals a $K_i$ of $1.52 \pm 0.9 \mu M$ (mean $\pm$ SE of five separate experiments). Similar experiments with hOAT3 using 50 and 200 nM $[^3H]$ES as substrate showed, at low concentrations of MPA, a stimulation rather than an inhibition of transport. It was, therefore, not possible to perform a meaningful Dixon analysis of the data (not shown).

**Effect of MPAG and AcMPAG on hOAT1- and hOAT3-mediated transport**

With respect to renal elimination, the glucuronide metabolites of MPA, especially MPAG, which are normally delivered to the kidney from the liver [1], are of particular importance. In contrast to MPA, the glucuronides MPAG and AcMPAG did not significantly attenuate PAH uptake by hOAT1-expressing oocytes at clinically relevant concentrations (Figure 5). However, ES uptake in hOAT3-oocytes was significantly inhibited by 100 $\mu M$ AcMPAG (80.9 $\pm$ 6.5%, $P < 0.01$), as well as by 10 $\mu M$ and 100 $\mu M$ MPAG (53.7 $\pm$ 5.2%, $P < 0.01$, and 69.2 $\pm$ 12.7%, $P < 0.05$, respectively) (Figure 5). In these experiments, inhibition by 10 $\mu M$ AcMPAG was slightly less than that by 10 $\mu M$ MPAG (46.8 $\pm$ 14.5%) and, due to its high variability, not statistically significant. However, the dose response curves indicate that AcMPAG is a more potent hOAT3 inhibitor (IC$_{50}$ 2.88 $\pm$ 0.42 $\mu M$) than MPAG (IC$_{50}$ 15.2 $\pm$ 18.5 $\mu M$) (Figure 6).

**Discussion**

Excretion of mycophenolate has been shown to occur mainly via the kidneys as its 7-O-glucuronide metabolite MPAG and is thought to involve tubular secretion [1]. Consistent with an almost exclusive renal elimination of MPAG, MPAG plasma clearance
strongly correlates with renal function, being decreased from \( \sim 1.81 \text{ l/h} \) in healthy subjects to \( \sim 0.261 \text{ l/h} \) in patients with severe renal impairment (GFR reduced to 1.5 l/h) [1]. In the present study, we investigated the interaction of MPA and its two glucuronide metabolites MPAG and AcMPAG with three human basolateral transport systems involved in renal secretion of organic anions, namely hNaDC3, hOAT1 and hOAT3.

Translocation of MPA by hNaDC3 was electrogenic as documented by the presence of an inward current, and this current was strictly sodium-dependent. Besides MPA, MPAG also produced potential-sensitive inward currents, which increased at hyperpolarizing potentials. This response to voltage is similar to that of other hNaDC3 substrates like \( \alpha \)-ketoglutarate or GA but was also observed for more bulky compounds such as folate and quinolinate [12]. Due to the low current amplitudes and problems of solubility of MPA and MPAG, it was not possible to determine kinetic parameters. Therefore, it is not clear at present to what extent MPA or MPAG interact with hNaDC3 \textit{in vivo}. However, both compounds attenuated succinate-induced currents suggesting a competition with succinate at the substrate binding site. The relatively small inward currents induced by MPA and MPAG even at concentrations well above plasma concentrations determined in MPA-treated patients [1] suggest that their transport rates via hNaDC3 are low and, thus, hNaDC3 probably does not contribute much to the secretion of mycophenolate and its derivatives \textit{in vivo}.

As for secretion of MPAG, the major MPA metabolite, hOAT1 is not likely to be involved, since even a 10-fold excess of MPAG over \( [\text{H}]\text{PAH} \) did not affect hOAT1-mediated PAH uptake. At similar concentrations, the acyl glucuronide AcMPAG also did not interact with hOAT1. hOAT3-mediated ES flux, on the other hand, was strongly decreased by MPAG at concentrations well within the range of unbound MPAG plasma levels reached under mycophenolate therapy in kidney transplant recipients (30 and 60 mg/l) [1]. hOAT3 was also inhibited by AcMPAG, indicating that both metabolites of MPA interact with human OAT3. Whereas the observed inhibition indicates that MPAG and AcMPAG bind to the transporter and inhibit translocation of ES, the data do not provide direct evidence for a translocation of these metabolites by hOAT3. It is, however, possible that MPAG and AcMPAG interfere with the renal excretion of other anionic drugs by competition for hOAT3. In this regard, it may be of importance that hOAT3 translocates cortisol [13]. Since cortisol
and in particular the synthetic analogues prednisone and prednisolone are often co-administered with MMF. MPA and its metabolites may decrease renal excretion of these steroids, potentially leading to higher plasma levels.

Interestingly, in patients with severe hepatic impairment, increased renal elimination of MPAG was observed [1]. This effect was attributed to an induction of renal glucuronidation of MPA, whereas normally MPA metabolism is thought to occur primarily in the liver [1]. Other studies report significant MPA glucuronidation rates in kidneys even under normal conditions [14]. Moreover, significant glucuronidation of MPA has recently been described in LLC-PK1 cells, a pig kidney cell line with some properties of proximal tubule cells [15]. MPA glucuronidation in the renal tubular epithelium requires cellular uptake, which might be expected to occur predominantly from the basolateral side due to the compound’s extensive protein binding. In the present study, we could demonstrate half-maximal inhibition of both hOAT1- and hOAT3-mediated transport by low to sub-micromolar MPA concentrations, which were in the range of free MPA plasma concentrations estimated from total MPA levels determined in MPA-treated patients [1,16]. For hOAT1 we found that MPA inhibited part of MPA levels determined in MPA-treated patients of free MPA plasma concentrations estimated from molar MPA concentrations, which were in the range of free MPA plasma concentrations estimated from total MPA levels determined in MPA-treated patients [1,16]. For hOAT1 we found that MPA inhibited PAH transport competitively with a Ki of 1.9 μM. A meaningful kinetic analysis was not possible for hOAT3 due to stimulation of ES uptake at low MPA concentrations. The reason for this cis-stimulation is not clear. The competitive inhibition seen with hOAT1 strongly suggests that MPA and PAH bind to the same site. As discussed earlier, the inhibition does not prove translocation of MPA. Until radioactively labelled MPA is available, we must leave the question open, whether hOAT1 and hOAT3 transport MPA into proximal tubule cells.

In this context, an interaction with hOAT1 of non-metabolized MPA could possibly explain the decreased elimination of antiviral drugs under mycophenolate therapy. Interaction studies conducted with acyclovir and ganciclovir in healthy subjects showed moderate decreases in the renal clearance of these antiviral drugs when co-administered with mycophenolate mofetil [1,17]. All antiviral drugs tested so far were transported by human OAT1 [18,19]. Since the affinity of hOAT1 for acyclovir and ganciclovir is low (Km for uptake 342 μM and 896 μM, respectively [19]), MPA (Kd 1.9 μM; this study) may potently interfere with hOAT1-mediated elimination of these compounds.

Although to be proven experimentally, human OAT1 and OAT3 could constitute the entry pathway for non-metabolized MPA into the renal epithelial cells for subsequent intrarenal glucuronidation. Candidate luminal efflux pathways would then include in particular the multi-drug resistance proteins MRP2 and MRP4, which are known to be expressed in the renal proximal tubule brush border membrane [20,21] and to transport glucuronidated substrates [22,23]. MRP2 has previously been reported to mediate MPAG transport across the bile canalicular membrane of rat liver [7,8], and efflux of MPAG from LLC-PK1 cells has been suggested to occur via Mrp homologues because of its sensitivity to an MRP inhibitor [15].

In summary, mycophenolate interacts with high affinity with both human OAT1 and OAT3, and inhibits substrate transport. The metabolites MPAG and AcmPAG were found to interact with hOAT3, but not with hOAT1. The potent inhibition with IC50 values well within the range of plasma concentrations encountered during mycophenolate therapy make hOAT1 and hOAT3 sensitive targets for interference with the renal elimination of co-administered hOAT1/ hOAT3 drug substrates, such as virustatics, corticosteroids or diuretics.

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References


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