Glucuronide and glucoside conjugation of mycophenolic acid by human liver, kidney and intestinal microsomes

*¹M. Shipkova, ²C.P. Strassburg, ³F. Braun, ¹F. Streit, ⁴H.-J. Gröne, ¹V.W. Armstrong, ^{5,6}R.H. Tukey, ¹M. Oellerich & ¹E. Wieland

¹Abteilung für Klinische Chemie, Georg-August-Universität Göttingen, Germany; ²Abteilung für Gastroenterologie und Hepatologie, Medizinische Hochschule Hannover, Germany; ³Klinik für Transplantationschirurgie, Georg-August-Universität Göttingen, Germany; ⁴Deutsches Krebsforschungszentrum, Heidelberg, Germany; ⁵Department of Chemistry & Biochemistry, University of California, San Diego La Jolla, California, U.S.A. and ⁶Department of Pharmacology, University of California, San Diego La Jolla, California, U.S.A.

1 Mycophenolic acid (MPA) is primarily metabolized to a phenolic glucuronide (MPAG) as well as to two further minor metabolites: an acyl glucuronide (AcMPAG) and a phenolic glucoside (MPAGls). This study presents investigations of the formation of these metabolites by human liver (HLM), kidney (HKM), and intestinal (HIM) microsomes, as well as by recombinant UDP-glucuronosyltransferases.

2 HLM ($n=5$), HKM ($n=6$), HIM ($n=5$) and recombinant UGTs were incubated in the presence of either UDP-glucuronic acid or UDP-glucose and various concentrations of MPA. Metabolite formation was followed by h.p.l.c.

3 All microsomes investigated formed both MPAG and AcMPAG. Whereas the efficiency of MPAG formation was greater with HKM compared to HLM, AcMPAG formation was greater with HLM than HKM. HIM showed the lowest glucuronidation efficiency and the greatest interindividual variation. The capacity for MPAGls formation was highest in HKM, while no glucoside was detected with HIM.

4 HKM produced a second metabolite when incubated with MPA and UDP-glucose, which was labile to alkaline treatment. Mass spectrometry of this metabolite in the negative ion mode revealed a molecular ion of m/z 481 compatible with an acyl glucoside conjugate of MPA.

5 All recombinant UGTs investigated were able to glucuronidate MPA with K_M values ranging from 115.3 to 275.7 μM l^{-1} and V_{max} values between 29 and 106 $\text{pM min}^{-1} \text{mg protein}^{-1}$.

6 Even though the liver is the most important site of MPA glucuronidation, extrahepatic tissues particularly the kidney may play a significant role in the overall biotransformation of MPA in man. Only kidney microsomes formed a putative acyl glucoside of MPA.

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Abbreviations: AcMPAG, acyl glucuronide of mycophenolic acid; ESI, electrospray ionisation; HIM, human intestinal microsomes; HKM, human kidney microsomes; HLM, human liver microsomes; IMPDH, inosine monophosphate dehydrogenase; MMF, mycophenolate mofetil; MPA, mycophenolic acid; MPAC, mycophenolic acid carboxybutoxy ether; MPAG, mycophenolic acid 7-O-phenolic glucuronide; MPAGls, mycophenolic acid 7-O-phenolic glucoside; MRM, multiple reaction monitoring; MS, mass spectrometry; RP, reversed phase; UDP, uridine diphosphate; UDPGA, uridine diphosphate glucuronic acid; UDPGLs, uridine diphosphate glucose; UGT, uridine diphosphate glucuronosyltransferase

Introduction

The UDP-sugar-conjugating enzymes and their most important subfamily the UDP-glucuronosyltransferases (UGTs) play a major role in the biotransformation of exogenous and endogenous compounds. Occasionally, they also produce more biologically active and/or toxic compounds (Spahn-Langguth & Benet, 1992) as demonstrated for the analgesic properties of morphine-6-glucuronide (Portenoy *et al.*, 1992). Although the liver is generally accepted to be the major

contributor to overall drug biotransformation, several studies in the past 15 years have revealed that extrahepatic glucuronidation may significantly contribute to the elimination of xenobiotics in laboratory animals and man (Mulder, 1992; McGurk *et al.*, 1998; Hanninen *et al.*, 1987; Strassburg *et al.*, 1998). The major tissues of extrahepatic metabolism are those involved in absorption and excretion of xenobiotics such as the gastrointestinal mucosa and the kidney, respectively.

Mycophenolic acid (MPA), an uncompetitive, selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is used for immunosuppressive therapy

*Author for correspondence at: Georg-August-Universität, Abteilung Klinische Chemie, Robert-Koch-Strasse 40, D-37075 Goettingen; E-mail: maria.shipkova@med.uni-goettingen.de

after transplantation and several autoimmune diseases (Bullingham *et al.*, 1998; Behrend, 1998; Hood & Zaremski, 1997; Shaw *et al.*, 1998; Briggs *et al.*, 1998; Enk & Knop, 1997; Zimmer-Molsberger *et al.*, 1997; Nowack *et al.*, 1997), is the active compound of the prodrug mycophenolate mofetil (MMF). MMF is subject to rapid and complete first pass metabolism to MPA following oral administration. After absorption through the gastrointestinal wall, the primary metabolic pathway for MPA is formation of the phenolic glucuronide MPAG, which is pharmacologically inactive (Nowak & Shaw, 1997; Schütz *et al.*, 1999). It is assumed that hepatic glucuronosyltransferases (UGTs) are primarily responsible for the production of this metabolite, but the possible contribution of other tissues such as the kidney and the intestine has not been consequently evaluated. The kidney is the major organ involved in the excretion of MPA, and only about 5.5% of the recovered dose is eliminated with the faeces (Bullingham *et al.*, 1998). The glucuronide excreted into the bile can be deconjugated in the intestine and reabsorbed as MPA. This leads to a repeated encounter of MPA with the intestinal epithelium. We recently characterized two further metabolites of MPA, an acyl glucuronide (AcMPAG) and a phenolic 7-O-glucoside (MPAGIs) in the plasma of liver, kidney and heart transplant recipients receiving MMF (Shipkova *et al.*, 1999).

The acyl glucuronide is pharmacologically active and appears to have a potency similar to MPA in inhibiting inosine monophosphate dehydrogenase *in vitro* (Schütz *et al.*, 1999). Since acyl glucuronides are known to be reactive metabolites, which possess toxic potential (Spahn-Langguth & Benet, 1992), it is conceivable that the AcMPAG may also contribute to some of the adverse effects under MMF therapy, such as leukopenia and gastrointestinal disturbances. We found that it demonstrated a potential to induce cytokine release and cytokine mRNA expression in human mononuclear leukocytes (Wieland *et al.*, 2000), which may be a link to side effects under MMF treatment. To further elucidate this link to side effects, information by which tissues and enzymes the acyl glucuronide of MPA can be formed is needed.

Whereas N-linked glucoside drug metabolites have been described in man before (Tang, 1990), the MPAGIs is the first O-linked glucoside of a drug identified in human plasma. Glucosidation represents a relatively minor metabolic pathway in humans. In comparison with glucuronidation, there is only very limited information in the literature about this conjugation process. Radomska *et al.* (1993) characterized a UDP-Glucose (UDPGIs)-specific glucosyltransferase catalyzing the biosynthesis of 6-O-glucosides of the 6- α -hydroxylated bile acid hyodeoxycholic acid in human liver microsomes and provided evidence that two distinct enzyme systems are involved in 6-O-glucuronidation and 6-O-glucosidation of this acid (Little *et al.*, 1995). However, the contribution of extrahepatic tissues to the formation of such a glucoside has not yet been investigated.

The aim of the present investigation was to compare the glucuronide and glucoside conjugation of MPA by human liver, kidney, and intestinal microsomes. To better understand the contribution of these three organs to the glucuronidation of MPA we also studied the ability of different recombinant UDP-glucuronosyltransferases to convert MPA to MPAG.

Methods

Chemicals

UDP-glucose (UDPGIs), UDP-glucuronic (UDPGA) acid and saccharo-1,4-lactone were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sucrose, HClO₄, KCl, K₂HPO₄, KH₂PO₄, H₃PO₄, Na₂WO₄·2H₂O, MgCl₂, CH₃CONH₄, CH₃COOH, and NaOH were purchased from Merck (Darmstadt, Germany). 2[4-(2-hydroxyethyl)-1-piperazinol]-ethane sulphonic acid (HEPES) and tris-(hydroxymethyl)-aminomethane (TRIS) were from Aldrich, (Steinheim, Germany). Acetonitrile and methanol were obtained from J.T. Baker B.B. (Deventer, The Netherlands). All reagents were analytical grade. MPA, MPAG, and the internal standard carboxybutoxy ether of MPA (MPAC) were gifts from Hofmann-LaRoche (Basel, Switzerland).

Preparation of human liver, kidney and small intestine microsomes

Human liver, kidney and intestinal microsomes were prepared as previously described (Shipkova *et al.*, 1999; Strassburg *et al.*, 2000). Tissues samples were obtained from the Department of Transplant Surgery, Georg-August Universität, Göttingen, Germany (liver), the Department of Abdominal and Transplant Surgery, Hannover Medical School, Hannover, Germany (small intestine), and Deutsches Krebsforschungszentrum, Heidelberg, Germany (kidney). Kidney cortex was used for microsome preparation. For isolation of intestinal microsomes the intestinal mucosa was used once free of the muscularis and most of the submucosa. Human liver samples were obtained as either excess material removed during transplantation of livers from adult donors to pediatric recipients ($n=3$) or as material removed during surgery for hepatocellular carcinoma ($n=2$). Human kidney samples were obtained following nephrectomies due to renal tumors ($n=6$). Since the amount/activity of enzymes decreases along the intestine (Radomska-Pandya *et al.*, 1998; Strassburg *et al.*, 2000), segments from different parts of the small intestine (two duodenum, two jejunum, two ileum) were obtained. The samples were derived from patients undergoing surgery for cancer of the stomach, pancreas, small intestine, or colon. All tissues were microscopically examined to document normal histology. All tissue samples as well as microsomal preparations were stored at -80°C until use.

Preparation of recombinant UGT cell lysates

The cloning, characterization, and baculovirus-directed expression of the human recombinant UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15) were previously described in detail (Strassburg *et al.*, 1996; 1997; 1998; 1999a,b).

All enzymes were separately subcloned into the baculovirus transfer vector pBluebac4, and transfected into exponential-phase *Spodoptera Frugiperda* insect-cell (Sf9) monolayers as described previously (Strassburg *et al.*, 1996; Nguyen & Tukey, 1997; Guengerich *et al.*, 1997). UGT-recombinant virus supernatants with a titre of $1-2 \times 10^8$ p.f.u. ml⁻¹ were

generated and subsequently used to infect exponential-phase Sf9 cell monolayers at a multiplicity of infection of 10 (Strassburg *et al.*, 1996). Cells were collected at 60 h post-infection and lysed by three cycles of freeze/thawing in buffer containing 50 mM Tris/HCl (pH 7.4)/10 mM MgCl₂. Recombinant UGT cell lysates were stored at -80°C until analysis. Lysates of Sf9 cells containing the empty baculovirus expression vector did not possess glucuronidation capacity (Strassburg *et al.*, 1998).

Incubation of microsomes with MPA

Human liver (HLM), kidney (HKM) and intestinal (HIM) microsomes (0.25 ml, 1 mg protein ml⁻¹) were incubated in the presence of 0.01–1 mM MPA and 4 mM of either UDPGA or UDPGls for 15 min at 37°C in HEPES/KCl/MgCl₂ buffer (in mM): HEPES 50, KCl 150, MgCl₂ 5. The buffer was adjusted to pH 6.8 for the glucuronidation experiments, a pH value that is favourable for acyl glucuronide formation and stability. The incubations with UDPGls were generally performed at pH 7.4 with the exception of the metabolite identification experiments, in which the buffer pH was 6.8. Tissue samples from duodenum (*n*=1), jejunum (*n*=2), and ileum (*n*=2) were used in the glucuronidation experiments, whereas for the investigation of glucosidation, microsomes from two duodenum, two jejunum and one ileum tissue samples were prepared. The incubations were stopped using the sample preparation procedure described above. The low pH used during this procedure was favourable with respect to the stability of AcMPAG (Shipkova *et al.*, 2000).

Since the detergents that are usually used to stimulate glucuronidation activity of microsomes such as Triton X-100 and Brij 58 were found to be without effect on MPA metabolite formation, these additions were omitted in our experiments. Furthermore, to test whether the *in vitro* glucuronidation rate of MPA is affected by microsomal β -glucuronidase, incubations with MPA and UDPGA were performed with and without saccharo-1,4-lactone (1–15 mM) which however had no effect on the glucuronidation of MPA by HLM, HKM and HIM. In preliminary experiments we could also observe that the β -glucosidase inhibitor D-glucono-1,5-lactone did not enhance glucosidation of MPA (data not shown).

Reaction rates were found to be linear under the conditions described above. All incubations were done in duplicate. Control incubations contained either MPA and microsomes without any other additions or MPA plus co-substrates but no microsomes.

Michaelis constants (K_m) and maximal formation rates (V_{max}) were determined using linear regression analysis (Microsoft Excel software) through plotting the reciprocal initial formation rates (1/*V*) against the reciprocal substrate concentrations (1/*S*).

Catalytic activity of human recombinant UGTs

Recombinant UGT cell lysates (0.25 ml, 1 mg protein ml⁻¹) were incubated in the presence of 0.01–1 mM MPA and 4 mM UDPGA for 30 min at 37°C in HEPES/KCl/MgCl₂ buffer (in mM): HEPES 50, KCl 150, MgCl₂ 5, at pH 6.8. In analogy to the microsomal incubations, this pH value was

chosen to prevent degradation of the acyl glucuronides. Experiments were performed twice and Michaelis constants (K_m) and maximal formation rates (V_{max}) were determined as described above for incubations with microsomal preparations. The inter assay imprecision was below 10%.

H.p.l.c. analysis

MPA, MPAG as well as the recently identified metabolites MPAGls and AcMPAG were quantified by a modified h.p.l.c. procedure recently developed in our laboratory (Shipkova *et al.*, 2000). Briefly, 200 μ l microsomal preparation and 100 μ l acetonitrile containing the internal standard MPAC 0.036 mM (15 mg l⁻¹) were mixed by vortexing in a 1.5 ml polypropylene tube for 5 s. This was followed by the sequential addition of 20 μ l of 1.5 M perchloric acid and 20 μ l of 0.76 M sodium tungstate dihydrate. The tube was mixed by vortexing for 15 s after each addition. The sample was then centrifuged for 5 min at 10,000 $\times g$ and 80 μ l of the supernatant were injected on a 250 $\times$ 4.6 mm Hypersil C-18 reversed-phase column (MZ Analysentechnik, Mainz, Germany), maintained at 42°C as previously described (Shipkova *et al.*, 1999). The mobile phase consisted of solution A (250 ml acetonitrile, 750 ml phosphate buffer–pH 3.0 (20 mM final concentration) and solution B (450 ml acetonitrile and 550 ml phosphate buffer–pH 6.5, 40 mM final concentration) that formed the following gradient: 7%B (0.2 min); 7–35%B (2–17 min); 35%B (17–18 min); 35–100%B (18–18.5 min); 100%B (18.5–20 min); 100–7%B (20–20.5 min). The flow rate was 1.2 ml min⁻¹ and the compounds were detected at 215 nm. They were identified by their retention times and u.v. spectra. The u.v. spectra were recorded using a diode array detector. MPAGls and AcMPAG were quantified as MPAG-equivalents due to the lack of sufficient amounts of purified metabolites to obtain an accurate weight. AcMPAG (216, 250, and 306 nm) and MPAGls (215, 252, and 294 nm) have absorption spectra with characteristic absorption maxima, which are very similar to those of MPAG (214, 251, and 294 nm) (Shipkova *et al.*, 1999; 2000). This approach allows the comparison of the metabolic activity of the various tissues toward MPA. For calibration and control, microsomal preparations spiked with different MPAG concentrations (20–400 μ M) were used.

Electrospray ionisation mass spectrometry (ESI-MS)

ESI-MS was performed on a PE Applied Biosystems API 2000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) using an electrospray ion source (PE Sciex, Concord, Ontario, Canada) in the negative ionization mode. For LC-MS/MS analysis, the previously described chromatographic conditions (Shipkova *et al.*, 2000) were used with minor modifications. The phosphate buffers of the mobile phases were replaced by acetic acid (20 mM) in phase A and ammonium acetate (20 mM) in phase B. Furthermore, the sodium tungstate treatment was omitted during sample preparation. Samples were analysed after h.p.l.c. separation at a flow rate of 1.2 ml min⁻¹ and introduced into the ion source after 1:4 splitting (PE Sciex, Concord, Ontario, Canada) at a flow rate of 300 μ l min⁻¹. The MS/MS-experiments (multiple reaction monitoring (MRM)-transitions) were carried out at an ionisation voltage

of -5000 V, an orifice voltage of -36 V, and a ring electrode voltage of -350 V. The collision gas was argon and a collision energy of 28 eV were used. The temperature of the heater probe was set to 300°C . MRM-transitions $481 \rightarrow 319$ amu, $319 \rightarrow 275$ amu, and $319 \rightarrow 191$ amu, were monitored. The MS was operated with unit resolution for both Q1 and Q3 (e.g. 0.7 amu at 50% height).

Alkaline hydrolysis of MPA metabolites

Kidney microsomal preparations were incubated with MPA and UDPGls as described above ($n=2$) and pre-treated as described for h.p.l.c. analysis. To $100\ \mu\text{l}$ of the supernatant $10\ \mu\text{l}$ of $2\ \text{M}$ NaOH were added and the sample was then left at room temperature for $2\ \text{h}$. A control sample of the supernatant was stored at room temperature for $2\ \text{h}$ without addition of NaOH.

Protein measurement

Protein was determined using the BCA protein assay reagent kit supplied by Pierce (Rockford, IL, U.S.A.).

Statistical analyses

Catalytic activities of the different microsomal preparations were compared using the Mann-Whitney test and a P value below 0.05 was considered significant.

Results

Incubations with HLM, HKM and HIM

All microsomal preparations from the three different tissues investigated were capable of forming both MPAG and AcMPAG. The kinetic constants for these two metabolites are given in Table 1 and Table 2 respectively.

The $V_{\max}$ for MPAG formation was greatest with HKM followed by HLM and HIM ($P<0.05$). However, the K_M values were similar for the three microsomal preparations investigated ($P>0.05$) (Table 1). Whereas interindividual variation of the MPAG formation rate was low and only 2 fold in HLM (Table 1) or three fold with HKM, it was about

8 fold with HIM. The lowest formation rate in the HIM group was observed with duodenal microsomes ($V_{\max}=0.15\ \text{nM mg protein}^{-1}\ \text{min}^{-1}$). The jejunum- ($V_{\max}=1.04\ \text{nM mg protein}^{-1}\ \text{min}^{-1}$) and ileum ($V_{\max}=0.82\ \text{nM mg protein}^{-1}\ \text{min}^{-1}$ and $0.63\ \text{nM mg protein}^{-1}\ \text{min}^{-1}$) samples formed MPAG at a similar velocity. Liver microsomes turned out to be the most capable to form AcMPAG (Table 2), followed by the kidney, whereas the formation rate of AcMPAG by HIM was the lowest ($P<0.01$). The K_M values were not significantly different ($P>0.05$) between HKM, HLM, and HIM. However, as described above for MPAG formation, microsomes from the intestine showed the greatest inter-individual variability in AcMPAG formation, with the lowest formation rate found in the duodenal sample ($V_{\max}=10.4\ \text{pM mg protein}^{-1}\ \text{min}^{-1}$). While the microsomal preparations from the two jejunal tissue samples and one from the ileum samples formed AcMPAG at a similar rate ($V_{\max}=29.0\ \text{pM mg protein}^{-1}\ \text{min}^{-1}$, $11.4\ \text{pM mg protein}^{-1}\ \text{min}^{-1}$ and $13.3\ \text{pM mg protein}^{-1}\ \text{min}^{-1}$), the second ileum sample had a 5 – 10 times greater activity ($V_{\max}=112.4\ \text{pM mg protein}^{-1}\ \text{min}^{-1}$).

Both MPAG and AcMPAG were not found in control incubations in which either MPA or microsomal protein were omitted.

Glucosidation activity was detectable with both liver and kidney microsomes (Table 3) but not in intestinal mucosa. Interestingly, kidney microsomes were much more active toward MPAGs formation as compared to HLM. In contrast to the glucuronidation experiments, both $V_{\max}$ and K_M values for glucosidation of MPA (MPAGs) by HKM were significantly different ($P<0.01$) compared to those seen with HLM (Table 3). Furthermore, when HKM were incubated in the presence of MPA ($1\ \text{mM}$) and UDPGls ($4\ \text{mM}$) at pH 6.8 , a second peak with a very similar absorbance spectrum to MPAGs and a retention time of $8.2\ \text{min}$ was detected (Figure 1a) using h.p.l.c. This new peak had three absorption maxima at around 214 , 251 and $294\ \text{nm}$. We were not able to find this peak in incubations with HLM or HIM under the same conditions.

The MPAGs peak and the second newly detected peak were not present in both control incubations either without MPA or without microsomal protein.

Table 1 MPAG formation kinetic parameters with human liver, kidney and intestinal microsomes

Tissue	$V_{\max}$ (nM mg protein $^{-1}$ min $^{-1}$)	K_m (mM)	$V_{\max}/K_m$
HLM	10.3*	0.27	34.3
$n=5$	(8.38–14.3)	(0.23–0.58)	(18.7–45.3)
HKM	18.1*	0.31	75.8
$n=6$	(10.4–34.4)	(0.17–0.44)	(36.1–81.9)
HIM	0.64*	0.12	3.85
$n=5$	(0.15–1.04)	(0.06–0.38)	(2.49–6.83)

HKM, HLM and HIM were incubated ($0.25\ \text{ml}$, $1\ \text{mg protein ml}^{-1}$) in the presence of 0.01 – $1\ \text{mM}$ MPA and $4\ \text{mM}$ UDPGA for $15\ \text{min}$ at 37°C and pH 6.8 as mentioned in Methods. Pharmacokinetic parameters, Michaelis constants (K_m) and maximal formation rates ($V_{\max}$) were determined using linear regression analysis. Data are given as median with range. * $P<0.05$ vs all other microsomes.

Table 2 AcMPAG formation kinetic parameters with human liver, kidney and intestinal microsomes

Tissue	$V_{\max}$ (nM mg protein $^{-1}$ min $^{-1}$)	K_m (mM)	$V_{\max}/K_m$
HLM	2.73*	1.71	1.33
$n=5$	(1.00–3.64)	(0.83–3.86)	(0.58–1.83)
HKM	0.76*	0.99	0.82
$n=6$	(0.33–1.29)	(0.44–2.22)	(0.58–0.87)
HIM	0.013*	0.41	0.04
$n=5$	(0.01–0.11)	(0.31–2.22)	(0.01–0.09)

HKM, HLM and HIM were incubated ($0.25\ \text{ml}$, $1\ \text{mg protein ml}^{-1}$) in the presence of 0.01 – $1\ \text{mM}$ MPA and $4\ \text{mM}$ UDPGA for $15\ \text{min}$ at 37°C and pH 6.8 as mentioned in Methods. Pharmacokinetic parameters, Michaelis constants (K_m) and maximal formation rates ($V_{\max}$) were determined using linear regression analysis. Data are given as median with range. * $P<0.05$ vs all other microsomes.

Table 3 MPAGlc formation kinetic parameters with human liver, kidney and intestinal microsomes

Tissue	V_{max} (nm mg protein ⁻¹ min ⁻¹)	K_m (mM)	V_{max}/K_m
HLM <i>n</i> = 5	0.58* (0.34–0.69)	0.38* (0.32–0.79)	1.19 (0.87–1.86)
HKM <i>n</i> = 5	1.23* (1.19–1.40)	0.08* (0.06–0.12)	18.3 (10.0–20.7)
HIM <i>n</i> = 5	–	–	–

HKM, HLM and HIM were incubated (0.25 ml, 1 mg protein ml⁻¹) in the presence of 0.01–1 mM MPA and 4 mM UDPGLs for 15 min at 37°C and pH 7.4 as mentioned in Methods. Pharmacokinetic parameters, Michaelis constants (K_m) and maximal formation rates (V_{max}) were determined using linear regression analysis. Data are given as median with range. * P < 0.05 vs all other microsomes.

Mass spectrometric analysis of the putative MPA metabolite, generated by HKM

To further characterise the putative MPA metabolite found in the HKM incubations, it was analysed by tandem mass spectrometry (Figure 1b). In the negative ion mode this metabolite revealed a precursor ion peak of m/z 481, identical to that seen for MPAGls (Shipkova *et al.*, 1999). As with MPAGls, fragmentation of this precursor ion led to one major product ion with m/z 319, characteristic for MPA (Shipkova *et al.*, 1999). This fragmentation product can be explained by the loss of the dehydroglucose moiety. In addition, the MRM-transitions 319→275 amu and 319→191 amu, characteristic for MPA (Shipkova *et al.*, 1999) were also found for both MPAGls and the new metabolite. Since MPA has two potential sites for conjugation, one at the phenolic 7-hydroxyl group and one at the aliphatic carboxyl group of the acyl side chain (Figure 2), we inferred that the newly detected metabolite could be the acyl glucoside conjugate of MPA, which would have a molecular weight of 482 Da. Previously, two MPA glucuronates—MPAG (linked to the phenolic 7-hydroxyl group) and AcMPAG (linked to the aliphatic carboxyl group) (Shipkova *et al.*, 1999) had been identified.

Alkaline hydrolysis

In order to further investigate the structure of the metabolite, supernatant from the h.p.l.c. pre-treatment procedure was incubated in the presence and absence (control) of 0.18 M NaOH. After incubation, the concentrations of the metabolites in each of the samples were determined by h.p.l.c. In contrast to MPAGls, the putative new MPA-glucoside was found to be highly alkali-labile and was completely degraded under alkaline conditions.

MPA glucuronidation activity of human recombinant UGTs

To assess the individual contribution of different UGTs to the glucuronidation of MPA, human recombinant UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15, expressed

Figure 1 (a) Representative chromatogram of a kidney microsome preparation incubated in the presence of 1 mM MPA and 4 mM UDPGLs. The sample was pre-treated and chromatographed as described in Methods. Compounds eluting from the column were monitored continuously with a diode array detector. This chromatogram illustrates the u.v. absorption at 215 nm. The 7-O glucoside metabolite (MPAGls) eluted after 4.0 min. A second, as yet unknown metabolite was detected with a retention time of 8.2 min. This metabolite had a very similar u.v. absorption spectrum to that of MPAGls (shown in the inset). (b) Mass spectra of a kidney microsome preparation incubated in the presence of 1 mM MPA and 4 mM UDPGLs. The sample was pre-treated as described in Methods and introduced into the ion source after h.p.l.c. separation. An extracted ion chromatogram of multiple reaction monitoring (MRM)-transitions of 481/319 amu showed two peaks with retention times of 4.2 and 7.8 min respectively.

in baculovirus were tested. The presence of the recombinant proteins in Sf9 cells was documented by Western blot and their metabolic activity was confirmed by incubation with known substrates (data not shown). When cell lysates, containing individual UGTs were incubated with MPA and UDPGA, MPAG was generated by all UGTs (Table 4). Whereas V_{max} values varied about 4 fold (29.0–106.0 pM min⁻¹ mg protein⁻¹) within the UGTs investigated, the variation between the K_M values was only 2 fold (115.3–275.7 μ M⁻¹). All UGTs had similar capacities (V_{max}/K_M) for MPAG formation with UGT1A7 and UGT2B4 being most efficient. The differences in K_M values between microsomal preparations and recombinant UGTs can be explained by the absence or presence of activators and inhibitors in microsomes which are not found in the baculovirus system used. Even though the incubations were carried out at pH 6.8, which is favourable for the stability of acyl glucuronides, only traces of AcMPAG were found in all incubations precluding their quantification.

Figure 2 Proposed structural formulas of the phenolic and the acyl MPA glucosides.

Discussion

The liver is considered to be the most important site for the metabolism of many xenobiotics. The results of our *in vitro* investigation on the metabolism of the immunosuppressant MPA reveal that human kidney microsomes possess a significantly greater efficiency ($V_{\max}/K_M$) for its conversion to MPAG compared to HLM and HIM. In contrast, the highest AcMPAG formation rate was found with HLM. These data support and extend observations of Zucker *et al.* (1999) who reported that purified kidney extracts had higher amounts of enzyme capable of converting MPA to MPAG than liver. Significant extrahepatic glucuronidation, particularly in kidney tissue has been shown to occur in man for other drugs, such as morphine and propofol (Raoof *et al.*, 1996; Mazoit *et al.*, 1990). Direct renal metabolism of MPA in healthy subjects has been postulated (Bullingham *et al.*, 1996) based on the comparison of the urinary clearance of the metabolite MPAG after intravenous and oral administration of MMF. Further evidence for the participation of the kidney in the metabolism of MPA comes from a study into the pharmacokinetics of oral MMF in persons with varying degrees of hepatic oxidative impairment (Parker *et al.*, 1996). Since urine clearance of MPAG was 2 fold higher in patients with severe hepatic impairment than in mild or moderate impairment, despite similar creatinine clearances in all groups, the authors argued that impairment of hepatic glucuronidation of MPA must have led to induction of renal glucuronidation in patients with severe hepatic impairment. Taken together, the *in vitro* and *in vivo* findings show that in addition to the liver, the kidney can play a significant role in the metabolism of MPA, particularly when hepatic metabolism is impaired.

Kidney microsomes were also most active toward the formation of the phenolic glucoside MPAGs, when compared to other tissues. No glucosidation capacity was found

with HIM. These results suggest the presence of both a higher UDP-glucosyltransferase content (significantly greater $V_{\max}$ values) and of isoenzymes with a higher affinity for MPA (significantly greater K_M values) in HKM compared to other microsomes. Interestingly, kidney microsomes generated an as yet unknown MPA metabolite, namely the acyl glucoside conjugate of MPA. The evidence for the structural assignment is as follows: the metabolite was formed when microsomes were incubated with MPA and UDPGls; mass spectrometric analysis in the negative ion mode displayed a precursor ion of m/z 481 identical to that seen for the 7-O-MPAGs and a fragmentation product ion (m/z 319) characteristic for MPA; the metabolite was completely degraded to MPA on alkaline hydrolysis confirming that the glucose moiety was conjugated to the carboxyl group of MPA (alkali labile conjugate) and not to the 7-OH group of MPA (alkali stable conjugate). To our knowledge this is the first description of an acyl glucoside conjugate of a drug formed by human microsomes. Since the conjugation of drugs to glucose generally represents a minor metabolic pathway when glucuronidation is possible (Tang, 1990) and the newly detected metabolite was formed only by microsomes, and has not been generally observed in plasma samples of patients under MMF therapy (Shipkova *et al.*, 1999), this metabolic pathway may be of minor importance *in vivo*. However, we have recently observed this acyl glucoside of MPA in the urine as well as in trace amounts in the plasma of a kidney transplant recipient receiving MMF (unpublished observations). Our results suggest that the kidney may make a considerable contribution to O-glucosidation in humans.

When MMF is administered orally, the gastrointestinal tract is the initial site of direct contact with the drug. In contrast to HLM and HKM, HIM showed a 10–15 fold lower efficiency with respect to the conversion of MPA to MPAG (Tables 2, 3). This is in line with a recent report by Radomska-Pandya *et al.* (1998) who also reported that small phenols were glucuronidated with a much lower activity in intestinal mucosa than in liver. Furthermore, HIM showed a limited capacity to produce the acyl glucuronide and no MPAGs was detected, when these microsomes were incubated with MPA and UDPGls. However, the gut may nevertheless significantly contribute to extrahepatic MPA glucuronidation *in vivo* since it represents a large metabolically active external surface which extends to a length of 3–4 m in adults. The lowest efficiency for MPAG and AcMPAG formation was found with microsomal preparations from the duodenum whereas those from the jejunum and ileum formed glucuronidated MPA at a similar velocity. Even though we have only investigated microsomal preparations from a limited number of donors, our results support the observation of Radomska-Pandya *et al.* (1998) who found an increasing glucuronidation capacity in the intestine from proximal to distal with androstenedione and testosterone.

Another important impact on the MPA metabolism in small intestinal microsomes is the considerable interindividual variability of glucuronidation. In our experiments, HIM showed the greatest variability compared to HLM and HKM. The variation was 8 fold for MPAG formation and 10 fold for AcMPAG synthesis. Although variability in $V_{\max}$ values is to be expected as the UGT content in the tissues may differ between individuals, the observed variability in the

Table 4 MPAG formation kinetic parameters with human recombinant UDP-glucuronosyltransferases

Enzyme n = 2	V_{max} (pM mg protein ⁻¹ min ⁻¹)	K_m (μ M)	V_{max}/K_m
UGT1A1	39.9	197.0	0.20
UGT1A3	70.0	188.5	0.37
UGT1A4	46.0	266.3	0.17
UGT1A6	38.9	187.4	0.21
UGT1A7	85.2	158.6	0.54
UGT1A9	106.0	275.7	0.38
UGT1A10	69.2	250.8	0.28
UGT2B4	49.1	115.3	0.43
UGT2B7	39.0	122.7	0.32
UGT2B15	29.0	167.4	0.17

Recombinant UGT cell lysates (0.25 ml, 1 mg protein ml⁻¹) were incubated in the presence of 0.01–1 mM MPA and 4 mM UDPGA for 30 min at 37°C and pH 6.8. Pharmacokinetic parameters, Michaelis constants (K_m) and maximal formation rates (V_{max}) were determined using linear regression analysis. Data are shown as means of duplicate experiments.

K_M values implies that different UGTs with different affinities for MPA are present in the different HIM preparations. Furthermore, the higher variability found with HIM in comparison to recombinant UGTs suggests that in intestinal microsomes other yet unknown UGTs may be present which could not be investigated as recombinant enzymes.

Interindividual polymorphic regulation of the *UGT1* and *UGT2* genes has been already reported for the intestine, but not for the liver (Strassburg *et al.*, 2000). It is interesting to note, that the *UGT* genes subject to such regulation UGT1A1, UGT1A6, UGT2B4 and UGT2B7 contribute to MPA glucuronidation. As a consequence of the interindividual polymorphic *UGT* gene regulation, the rate of metabolism and the therapeutic efficacy or toxicity of drugs may be individually influenced by glucuronidation during or prior to resorption in the small intestine (Strassburg *et al.*, 2000). With respect to MPA metabolism, this fact is of importance, since the phenolic metabolites MPAG and MPAG1s are not pharmacologically active, while AcMPAG is both able to inhibit IMPDH *in vitro* and may also possess toxic potential.

To further elucidate the contribution of liver, kidney and small intestinal tissues to the glucuronidation of MPA, several recombinant human UDP-glucuronosyltransferases (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15) known to be present in these tissues were tested (Tukey & Strassburg, 2000; Radomska-Pandya *et al.*, 1999). In one recently published study using cDNA-expressed enzyme in COS-7 cells, Mackenzie reported, that three closely related UGTs (UGT1A8, UGT1A9 and, UGT1A10) have the capacity to glucuronidate MPA (Mackenzie, 2000). Of these,

UGT1A8 (Strassburg *et al.*, 1998) and UGT1A10 (Strassburg *et al.*, 1997) are present only in the gastrointestinal tract and hence can not be responsible for MPA glucuronidation in liver and kidney. In contrast, UGT1A9 gene products are found in the liver, but more abundantly in the kidney, and are therefore likely to play a role in MPA glucuronidation by this organ. However, in the study of Mackenzie the activity of UGT1A9 toward MPA was much lower than that of UGT1A8 and 1A10. The present investigation shows that all recombinant UGTs tested were capable of forming MPAG with similar efficiency (up to 3 fold variation). This is in parallel with reports from the literature which show that small phenolic molecules serve as substrates for most of the UGT proteins (Tukey & Strassburg, 2000). Furthermore, we found that MPAG was also formed by UGTs present in the liver such as UGT1A1, UGT 1A3, UGT1A6, UGT2B4, and UGTB7 which were previously considered by Mackenzie to lack the capability to glucuronidate MPA (Mackenzie, 2000). UGTB7 is also present in the kidney and may considerably contribute to MPA glucuronidation by HKM besides UGT1A9. In addition, it can be expected that other extrahepatic tissues not investigated in the present study also containing the UGT proteins mentioned above such as oesophagus, stomach, bile duct epithelium, colon, and pancreas etc. will also be able to glucuronidate MPA. The discrepancies to the studies of Mackenzie may be a result of differences in experimental conditions and the use of baculovirus expressed UGT proteins. However, when extrapolating from the *in vitro* system to the *in vivo* situation, the effect of other physiologic factors such as the amount of enzyme protein, the size of the organ, blood flow, and the absence or presence of activators as well as inhibitors must be considered.

Conclusion

This study presents the first detailed investigation on the *in vitro* metabolism of MPA by both hepatic and extrahepatic tissues. It demonstrates that: (i) even though the liver is quantitatively the most important site of glucuronidation, extrahepatic tissues, particularly the kidney, may play a significant role in the overall biotransformation of MPA in man, (ii) the kidney possesses the highest capacity for MPA glucosidation *in vitro* and is also able to form a newly described acyl glucoside of MPA, that is to our knowledge the first acyl glucoside of a drug, described in man, (iii) UGT enzymes investigated showed great redundancy toward MPA glucuronidation, indicating a highly conserved metabolism of compounds sharing chemical similarity with MPA by glucuronidation in humans.

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