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AN INVESTIGATION OF HUMAN AND RAT LIVER MICROSOMAL MYCOPHENOLIC ACID GLUCURONIDATION: EVIDENCE FOR A PRINCIPAL ROLE OF UGT1A ENZYMES AND SPECIES DIFFERENCES IN UGT1A SPECIFICITY

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Running title: Human and rat liver microsomal MPA UGTs

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Abbreviations:

MPA	Mycophenolic acid
MPAG	Mycophenolic acid 7-O-glucuronide
UGT	UDP-glucuronosyltransferase
HEK	Human embryonic kidney 293 cells
BCA	Bicinchoninic acid
SDS	Sodium dodecyl sulfate
HLM	Human liver microsomes
HPLC	High performance liquid chromatography

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Abstract

Mycophenolic acid (MPA), the active metabolite of the immunosuppressant prodrug, mycophenolate mofetil, undergoes glucuronidation to its 7-O-glucuronide as a primary route of metabolism. Because differences in glucuronidation may influence the efficacy and/or toxicity of MPA, we investigated the MPA UDP-glucuronosyltransferase (UGT) activities of human (HLM) and rat liver microsomes with the goal of identifying UGTs responsible for MPA catalysis. HLM (n=23) exhibited higher average MPA glucuronidation rates (14.7 vs. 6.0 nmol/mg/min, respectively, $p<0.001$) and higher apparent affinity for MPA (K_m = 0.082 mM vs. 0.20 mM, $p< 0.001$) compared to rat liver microsomes. MPA UGT activities were reduced >80% in liver microsomes from Gunn rats. To identify the active enzymes, human and rat UGT1A enzymes were screened for MPA glucuronidating activity. UGT1A9 was the only human liver-expressed UGT1A enzyme with significant activity and exhibited both high affinity (K_m= 0.077 mM) and high activity (V_{max}= 28 nmol•min^{-1}•mg^{-1}). Spearman correlation analyses revealed a stronger relationship between HLM MPA UGT activities and 1A9-like content (r^2=0.79) relative to 1A1 (r^2=0.20), 1A4-like (r^2=0.22), and 1A6 (r^2=0.41) protein. A different profile was observed for rat with three active liver-expressed UGT1A enzymes: 1A1 (medium affinity/capacity), 1A6 (low affinity/medium capacity), and 1A7 (high affinity/capacity). Our data suggest that UGT1A enzymes are the major contributors to hepatic MPA metabolism in both species, but 1A9 is dominant in human while 1A1 and 1A7 are likely the principal mediators in control rat liver. This information should be useful for interpretation of MPA pharmacokinetic and toxicity data in clinical and animal studies.

Introduction

MPA (1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzylfuranyl)-4-methyl-4-hexenoate) is the active immunosuppressive metabolite of the 2-morpholinoethyl ester prodrug, mycophenolate mofetil (Cellcept®) currently used as an adjunct in anti-transplant rejection regimens. Although it was discovered over a century ago as a product of several *Penicillium* species, the clinical utility of MPA as an immunosuppressive was not realized until the mid-1990's when it was approved for use in prophylaxis against acute graft rejection in renal transplant patients (Mele and Halloran, 2000). Its use has since been extended to heart, liver, lung and other solid organ transplant patients. The mechanism of immunosuppressive activity of MPA is inhibition of inosine monophosphate dehydrogenase, the rate-limiting enzyme in the *de novo* pathway for synthesis of the nucleotide, guanosine (Sintchak and Nimmesgern, 2000). As a result, DNA synthesis and proliferation of T and B lymphocytes in response to cytokine or antigen stimulation is markedly inhibited in the presence of MPA.

After oral administration, mycophenolate mofetil undergoes rapid and complete absorption consequent with hydrolysis by serum carboxylesterases to release free MPA (Fig. 1) (for review, see (Bullingham *et al.*, 1998)). Most of the administered mycophenolate mofetil dose (87-94%) ultimately appears in the urine as the 7-O-glucuronide of MPA (MPAG) with small percentages reported to be metabolized to either the acyl glucuronide or glucoside conjugates. Although only 6% of administered MPA is excreted in feces, MPA exhibits prominent features of enterohepatic cycling with secondary peaks observed in the plasma MPA concentration-time profile. The latter is

considered to result from hepatic synthesis of MPAG, followed by biliary excretion, hydrolysis in the intestine to MPA, and subsequent reabsorption of MPA.

A common adverse effect of mycophenolate mofetil is gastrointestinal toxicity ranging in severity from gastrointestinal upset and constipation to more severe diarrhea and gastrointestinal perforation with hemorrhaging (Busca *et al.*, 2001). Mourad *et al*. reported an association between 30 min plasma MPA concentrations and occurrence of gastrointestinal side effects (Mourad *et al.*, 2001). The toxic effects are dose-dependent and can be controlled by dosage reduction. However, reduction of dose also adversely impacts the immunosuppressive efficacy of MPA, which correlates strongly with the area under the MPA plasma concentration-time curve (Shaw and Nowak, 1995). Few data are available regarding the mechanism of MPA-induced gastrointestinal toxicity, and its etiology remains poorly understood. Although further investigation is needed, a causal relationship between certain pharmacokinetic factors (e.g., the rate of MPA glucuronidation by liver or intestine itself) and occurrence of gastrointestinal toxicity seems plausible. An assessment of this relationship would be aided by knowledge of the enzymes that are responsible for catalysis of MPA glucuronidation, the UGTs.

A limited number of studies have characterized the glucuronidation of MPA by HLM and by recombinant expressed human UGTs. Human liver, kidney, and small intestine microsomes have all been demonstrated to be active in MPA glucuronidation (Bowalgaha and Miners, 2001; Shipkova *et al.*, 2001). Several studies have identified human UGTs with catalytic activity towards MPA. Collectively, these studies indicate potential roles of the human 1A7, 1A8, 1A9, and 1A10 in MPA glucuronidation (Mojarrabi and Mackenzie, 1997; Mackenzie, 2000; Bernard and Guillemette, 2004). Of

these forms, only the 1A9 enzyme is expressed at significant levels in human liver and has been proposed as the principal enzyme contributing to glucuronidation of MPA in human liver (Bernard and Guillemette, 2004).

Increasingly utilized as a model to investigate various aspects of MPA pharmacokinetics and disposition (Kobayashi *et al.*, 2004; Tian *et al.*, 2004), the laboratory rat will likely be valuable for investigating the relationship between glucuronidating activities of specific tissues (i.e., liver or gastrointestinal tract) and gastrointestinal toxicity of MPA. Limited data suggest that rats also metabolize MPA, principally to the 7-O phenolic glucuronide, which undergoes both biliary and renal excretion. However, no studies to our knowledge have characterized the MPAG forming activity of rat liver microsomes or recombinant expressed rat UGTs. In this study, we directly compared the MPA glucuronidating activities of human and rat liver microsomes and investigated the basis of these activities. Our data support some similarities as well as key differences in MPA glucuronidation by human and rat liver microsomes.

Materials and Methods

Chemicals and reagents. MPA (≥ 98% pure) was purchased from Sigma Chemical (St. Louis, MO). The synthesis and characterization of MPAG reference standard was described previously (Wiwattanawongsa *et al.*, 2001). Recombinant expressed human UGTs were generated in our laboratory by expression in human embryonic kidney cells (HEK) or were obtained from commercial sources (Panvera, San Diego, CA or Gentest Corp, Woburn, MA). Rat UGTs were expressed in human hepatoma HepG2 cells using cloned, recombinant, replication-defective adenoviruses coding for each of the 8 rat UGT1A as well as 3 UGT2B family enzymes. Preparation of adenoviral stock lysates was carried out in the Massey Cancer Center Adenovirus Core Facility of Virginia Commonwealth University. Solvents for high performance liquid chromatography were obtained from Fisher Scientific (Atlanta, GA). All other chemicals for this study were purchased from Sigma.

Animals. Heterozygous and homozygous jaundiced male Gunn rats and homozygous normal controls (6-8 weeks, 200-240 g body weight) were purchased from Harlan Sprague-Dawley (Fredrick, MD). Sprague-Dawley rats were purchased from the same supplier. All care and use of animals used in experiments followed current guidelines of the National Institutes of Health under protocols approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Upon arrival, the animals were housed two per cage and allowed at least one week to acclimate. The lighting was on a 12 hr light-dark cycle. Room temperature and humidity were maintained at 22°C and 21%, respectively. At all times, rats were allowed free access to

water and standard rat chow (Harlan Tekiad LM-485). Prior to preparation of microsomes, rats were anesthetized with isoflurane and exsanguinated after guillotining. Tissues were stored frozen at -80°C until the time of microsome preparation.

Human samples. Human liver samples for this study were a gift of the Anatomic Gift Foundation (Laurel, MD) to the Hepatocyte Isolation and Cryopreservation Core Facility at the Virginia Commonwealth Medical Center. Handling and use of these specimens was conducted according to an approved Virginia Commonwealth University Office of Protection of Research Subjects protocol. All samples were from viable livers prepared for hepatocyte isolation and cryopreservation by flushing with University of Wisconsin preservation solution. Samples of excess tissue (25-50 g) not used for the collagenase perfusion and hepatocyte isolation technique were transferred to 50 mL sterile Falcon tubes, frozen on liquid nitrogen, and stored at -80°C until the time of microsome preparation. All samples were from donors shown serologically to be negative for HIV, hepatitis B and hepatitis C virus exposure. For this study, HLM samples were obtained from 11 males and 10 females (no information was available for two). Donors ranged from 5-73 years of age with an average of 45.3 years. Ethnic groups represented were Caucasian (85%), Hispanic (10%) and African-Americans (5%). Information on alcohol, cigarette use, and drug medications was inconsistently reported and is omitted.

Microsome preparation. Liver microsomes were prepared as described (Kessler and Ritter, 1997). Briefly, liver samples were homogenized in 5 volumes of ice-cold 0.25 M sucrose using glass homogenizing tubes fitted with a drill-driven teflon pestle (three

passes). After removing the nuclear and mitochondrial fractions, the microsomes were collected by centrifuging at 105,000 x g for 45 min. After resuspending in 0.15 M KCl, the final microsome pellet was rehomogenized in 0.1M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 20% glycerol. Protein concentrations were determined using the bicinchoninic acid (BCA) method with a commercially available kit (Pierce Biochemical, Rockford, IL).

Recombinant UGT preparation. Clonal lines of HEK cells stably expressing human 1A1, 1A6, or 1A9 were established in our laboratory. The specific methods for generating, maintaining, and harvesting the cell lines for enzyme activity analyses followed our previous description for expression of the human 1A7 enzyme and variants (Guillemette *et al*., 2000). The HEK cell lines express the UGTs under the control of sequences from the pcDNA3 mammalian expression vector, i.e., cytomegalovirus promoter/enhancer. Briefly, the cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1000 μg/mL G-418 antibiotic for selection of the neo resistance factor in the pcDNA3 vector. The sources of human UGT cDNA sequences were 1A1 (Ritter *et al.*, 1992) and 1A6 and 1A9 (Ebner and Burchell, 1993). After culturing, cells were scraped and membrane fractions prepared by collecting the 5,000 x g supernatant fraction. Protein concentration was determined by the BCA method.

HepG2 cells were grown to 90-95% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, at which time individual plates were infected with adenovirus lysates encoding the UGTs indicated. Cells were harvested by gentle pipetting to release cells 72 hr after infection and centrifuging at 5000 rpm in a

clinical centrifuge at 4°C. Supernatants were discarded, and the resulting cell pellets were washed by resuspending in ice-cold 1X phosphate-buffered saline and recentrifuging. The washed cells were resuspended in 0.1 M potassium phosphate buffer, pH 7.5, 20% glycerol, and 1 mM EDTA. After three consecutive freeze/thaw cycles and a brief sonication to disperse aggregates, the cells were centrifuged at 5000 x g for 10 min. The supernatants were collected and transferred to microfuge tubes. Protein concentration was determined as described above. Membrane preparations were aliquotted and stored at -80°C until assay. Positive control reactions were performed to demonstrate the activities of the expressed UGT1A family enzymes were performed with the following substrates: bilirubin (UGT1A1), 7-hydroxybenzo[a]pyrene (UGT1A2 and UGT1A8), and estriol (UGT1A3 and UGT1A5).

MPA UGT assay. All reactions were performed in buffer containing 75 mM Tris-Cl (pH 7.45), 10 mM $MgCl_2$, and 6 mM saccharic acid 1,4-lactone (prepared fresh). Microsomes/membranes were preincubated on ice for 15 min with 50 μg/mg alamethicin, which was found to maximally activate microsomal MPA UGT activity. The protein concentration was 1 mg/mL. MPA (2 mM final concentration in our standard reaction) was added to the reaction in methanol (4% final methanol concentration, v/v). This concentration of methanol had no apparent inhibitory effect on MPA UGT activity of rat liver microsomes. Control reactions received methanol alone. For the kinetic studies, MPA concentration was varied between 0.0156 and 2 mM in serial 2-fold dilutions, and protein concentration was adjusted (0.1 to 1 mg/mL) as necessary to maintain linear product formation with respect to time. This was a particular concern at the low end of

the substrate concentration range (0.0156 to 0.125 mM). After equilibrating incubation tubes at 37°C for 3 min, reactions were initiated by the addition of UDP-glucuronic acid (3 mM final concentration) and were allowed to proceed at 37°C for 30 min. Reactions were stopped by the addition of concentrated perchloric acid (to 5.6% final concentration, v/v). Tubes were placed on ice for 5 min and then centrifuged at 16,000 x g for 5 min to remove the precipitate. A 20 µL aliquot was then analyzed by reversed-phase HPLC as described (Wiwattanawongsa *et al.*, 2001) using a Hewlett Packard 1050 HPLC with a Partisil 10 ODS-2 C_{18} column [4.6 mm x 25 cm] (Whatman Inc., NJ) and a Waters Resolve C_{18} Guard Pak precolumn cartridge (Millipore, MA). The mobile phase was 55% MeOH/45% aqueous trifluoroacetic acid (0.1% TFA). The flow rate was 1.5 ml/min. Absorbance was monitored with a Hewlett Packard 1050 diode array detector set at 250 nm. Under our conditions, the retention times for MPAG, the suprofen internal standard, and MPA were 3.3, 8.8, and 11.8 min, respectively. MPAG was quantitated using standard curves prepared using blank microsome reactions spiked with known amounts of authentic MPAG standard. Care was taken to perform all reactions under conditions when MPAG formation was linearly proportional to time and protein concentration. The limit of detectable activity of our assay was ~ 0.010 nmol/mg/min.

Data Analysis. The relationship between substrate concentration and rate of MPA glucuronide formation was evaluated by fitting the data to the hyperbolic Michaelis Menten function ($v = V_{max} \cdot [S]/(K_m + [S])$. For this purpose, both linear and non-linear regression methods were used and were found to yield similar results (<5%). For linear regression, Eadie-Hofstee plots were generated and data were analyzed in Microsoft

Excel using least squares linear regression to generate the best fit line. For non-linear regression, the data were fit to either Michaelis-Menten or a sigmoidal kinetic model (Hill equation $v = Vmax \cdot [S]^n/(S_{50} + [S]^n)$ using WinNonlin kinetic modeling software (Pharsight Corporation, Mountain View, CA). For the comparison of the kinetic parameters of rat and human microsomes, three liver microsome preparations were analyzed. Statistical analyses were conducted using Student's *t*-test, and differences between groups were considered statistically significant when the p value was <0.05.

Immunoblot analyses of recombinant UGTs and HLM. Relative UGT protein levels were determined using an immunoblotting procedure described previously with some modifications (Ritter et al., 1999; Guillemette et al., 2000). Recombinant and microsomal proteins were separated on a 7.5% SDS-PAGE gel and transferred onto nitrocellulose membranes. Both recombinant and liver microsomes were analyzed for UGT1A1, UGT1A4-like, UGT1A6, UGT1A9-like, and total UGT1A expression. Antisera were generated in mice immunized with bacterially-expressed fusion proteins raised against amino acid residues 29-159 of 1A1, 30-160 of 1A4, 77-183 of 1A6, and 31-191 of 1A9 from human as described (Ritter *et al.*, 1999). Antisera were tested for specificity by Western blot analysis of recombinant human UGT1A1, UGT1A4, UGT1A6, and UGT1A9 proteins. All antisera selectively detected their respective forms (see Fig. 5). Although we have not tested the reactivity of anti-hUGT1A4$_{30\text{-}160}$ towards UGT1A3/UGT1A5 or anti-hUGT1A9$_{31\text{-}191}$ towards UGT1A7, UGT1A8, and 1A10, cross-reactivity is likely due to the high homology of the amino terminal sequences within the 1A3-1A5 and 1A7-1A10 subfamilies. Therefore, the immunoreactivities of

the UGT1A4 and UGT1A9-antisera are referred to as UGT1A4-like and UGT1A9-like, respectively. Total UGT1A expression was determined using a commercially prepared antibody (Gentest Corp., Woburn, MA). Expressed rat UGTs were analyzed using an antiserum directed against the rat UGT1 common region (amino acid residues 444 of the rat UGT1A common region (Webb *et al*., 2005). UGT2B expression was evaluated using Ab1168. Proteins were detected using enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ), and the resulting images were captured on film and quantified using Image J™ software (NIH, Bethesda, MD).

MPA activity correlation plots of HLM. For semi-quantitative analysis of UGT content of HLM, dilutions of a high expressing sample were included on the membrane in order to generate a standard curve. Using Image J™, maximum pixel intensities in gray scale (0-256) were determined for the standards and adjacent samples. Relative amounts of UGT1A1, 1A4-like, 1A6, and 1A9-like protein were then estimated from the standard curve. Samples were normalized so that the highest expressing sample was assigned an arbitrary value of 1. In order to ensure that low activities were not due to poor condition of the human liver samples, microsomes were assayed towards two other substrates, morphine and acetaminophen. Samples possessing glucuronidation rates lower than 50% of the average for all three substrates were not included in the study. Correlation plots comparing 1A1, 1A4-like, 1A6, and 1A9-like protein expression versus MPA UGT activities were generated and analyzed by the Spearman rank order correlation test. The data were considered statistically significant when $p \leq 0.05$.

Results

To characterize and compare the MPA glucuronidating activities of rat and human liver microsomes, a collection of individual human liver microsome (n=23) and Sprague Dawley rat (n=9) liver microsome samples were assayed for MPA UGT activity under standardized conditions which included 2 mM MPA, 3 mM UDP-glucuronic acid, and 50 µg/mg alamethicin (Fig. 2). Liver microsomal MPA UGT activities were found to be higher for human compared to rat liver microsomes. The mean activities determined for HLM were 14.7 versus 6.0 nmol/mg/min for rat ($p<0.001$ using Student's t-test). The range of human liver microsomal MPA UGT activities was 6.5 to 30.4 nmol/mg/min, whereas the range for rat was 3.3 to 9.7 nmol/mg/min.

Human and rat liver microsomal MPA UGT activities were further compared to determine apparent substrate affinities and velocities of MPA glucuronidation. Initial rates of MPA glucuronidation were assessed in reactions containing MPA at concentrations ranging from 0.0156 to 2 mM. The observed kinetics were consistent with a standard Michaelis-Menten (hyperbolic) model. Eadie-Hofstee plots for all six samples of human and rat liver microsomes analyzed yielded linear functions (Fig. 3). Fitting the data to the sigmoidal cooperativity model yielded Hill coefficients near unity (data not shown). Estimates of kinetic parameters using WinNonlin modeling software (Table 1) were in close agreement with the values determined using standard linear regression techniques (Eadie Hofstee plots, Fig. 3). In addition to the considerably higher V_{max} value observed for two of the HLM samples (Table 1), the human microsomes were also distinctive in their higher apparent affinity for MPA. The average K_m value of the three human liver microsome preparations for MPA was 0.082 mM. This value is

significantly lower than the corresponding average rat K_m (0.200 mM) (statistically different at the $p<0.001$ level using Student's t-test).

An experiment was performed using Gunn rat liver microsomes to assess the relative contributions of UGT1 versus UGT2 family enzymes in catalysis of MPA glucuronidation by rat liver microsomes. The Gunn rat strain contains a frame-shift mutation in a UGT1A common exon that results in inactivation of all UGT1A-encoded activity. Any remaining activity in homozygous Gunn rats is considered to be attributable to UGT2 family members. The effect of genotype at the UGT1A locus on rat liver microsomal MPA UGT activity is shown in Fig. 4. Liver microsomes from heterozygote (j/+) rats showed a 27% reduction in MPA glucuronidating activity compared to control rats (i.e., homozygous UGT1A-normal controls, +/+). The effect was even more pronounced in the homozygous j/j rat liver microsomes, which showed a mean 83% reduction in activity. This finding suggests that UGT1A family enzymes contribute the bulk of liver microsomal glucuronidation of MPA in rats.

To investigate specific UGT1A enzymes responsible for MPA glucuronidation, preparations of recombinant enzymes representing nine human and eight rat UGT1A enzymes were tested for activity. Fig. 5A presents the results for three of the major forms found in human liver: 1A1, 1A6, and 1A9. Only the 1A9 enzyme exhibited significant capacity to catalyze MPA glucuronidation. All three preparations of 1A9 expressed in HEK cells exhibited the activity. The level of activity was consistent with the relative level of expressed enzyme determined by immunoblotting with a UGT1A common region antibody probe (Fig. 5A, bottom). In addition to the HEK cell expressed UGTs, we also assayed preparations of human UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9,

and 1A10 obtained from commercial sources. Consistent with the data for HEK cell expressed UGTs, 1A1 and 1A6 had low or undetectable activity, whereas the 1A9 preparation was highly active (16 nmol/mg/min). All three members of the 1A3-1A5 subfamily were found to be inactive, whereas the extrahepatic enzymes, 1A7, 1A8, and 1A10, exhibited significant activity (4.9, 4.3, and 0.6 nmol/mg/min, respectively).

A significantly different profile was observed for MPA glucuronidating activities of recombinant rat UGT1A enzymes (Fig. 5B, top). Of the seven enzymes shown, three yielded significant rates of MPA glucuronidating activity (>1.5 nmol/mg/min). The expressed 1A7 preparation had the highest apparent activity followed by 1A6 and 1A1 (21% and 15% of the 1A7-catalyzed rate, respectively). In contrast to the high activity observed for members of the human 1A7-1A10 subgroup, the other two members of the rat 1A7-1A10 family tested (1A8 and 1A10) were inactive (data for 1A10 not shown). The negative finding for MPA catalysis by 1A8 contrasts with the high activity observed for this form towards some other phenolic substrates (Webb *et al.*, 2005). Multiple preparations of recombinant rat UGTs were analyzed and produced similar results (data not shown). The results of an immunoblot analysis of the rat UGT1A enzymes to assess differences in enzyme content of the tested preparations are shown in Fig. 5B (bottom). The relative levels of expressed enzyme determined by densitometric analysis were as follows: 0.95 (1A1), 2.9 (1A2), 0.43 (1A3), 0.24 (1A5), 0.61 (1A6), 1.0 (1A7), and 0.61 (1A8). In this experiment, three members of the UGT2B family included in our analysis were each negative for formation of MPAG.

The human 1A9 and rat 1A1, 1A6 and 1A7 enzymes were further characterized in kinetic analyses (Table 1). As was observed for human and rat liver microsomes, the

data closely fit the Michaelis-Menten model. Estimated K_m and V_{max} parameters are shown in Table 1. The two independent preparations of HEK cell expressed-1A9 showed comparable apparent K_m values of 0.090 and 0.073 mM, which were similar in range to the apparent K_m values observed for three human liver microsome samples (average of 0.078 mM). V_{max} values for the two preparations differed, as expected, depending on the level of expressed 1A9 enzyme (2.7 - 28.7 nmol/mg/min). The rat UGT1A enzymes showed differing affinities and velocities for MPA glucuronidation. The 1A7 enzyme from rat was distinct in its high affinity-high velocity catalysis, compared to 1A1 and 1A6. The ratio of K_m/V_{max} (a measure of intrinsic clearance) was 380 for the 1A7 preparation versus 8 and 7 for the 1A1 and 1A6 forms, respectively.

Our findings showing high affinity/capacity of human 1A9 in MPA glucuronidation together with high affinity of HLM towards MPA led us to investigate whether the MPA glucuronidating activity of HLM (Fig. 6) might relate to their content of 1A9-like enzyme. The relative levels of 1A1, 1A4-like, 1A6, and 1A9-like proteins were determined by semi-quantitative immunoblotting using selective antisera raised in mice against these proteins. The correlation plot shown in Fig. 6 revealed a striking association between content of 1A9-like UGTs and the rate of MPA glucuronidation catalysis (assessed at 2 mM MPA) ($r^2 = 0.79$). In contrast, the MPA UGT activity showed weaker correlations between 1A1, 1A4-like, and 1A6 contents. Although we have found that the 1A9 antiserum does display cross-reactivity towards other members of the 1A7-1A10 subfamily, 1A9 is likely the principal source of signal observed with this antibody based on assessments of their relative expression in human liver. Our

observations are consistent with a principal contribution of 1A9 in human liver MPA glucuronidation.

Discussion

Bowalgaha and Miners (2001) proposed that the liver is likely the organ primarily responsible for the systemic clearance of MPA, basing their proposal on tissue microsomal intrinsic clearances for MPA formation and extrapolation to whole organ models. Understanding catalysis of MPAG formation by HLM may therefore provide important insights into interindividual differences in systemic MPA pharmacokinetics and in clinical responses to MPA, and several studies have reported characterizations of the MPA glucuronidating activities of HLM and of recombinant expressed UGTs. In the current study, we performed one of the most comprehensive surveys reported to date of human liver microsomal glucuronidation of MPA and compared it with corresponding data from rat, a species for which little information on MPA glucuronidation has heretofore been reported. In addition, extensive data were obtained for the MPA UGT activities of the complete families of expressed human and rat UGT1A family enzymes. A number of interesting findings were uncovered.

In our assessment of MPA UGT activities of 23 HLM samples, most activities were clustered in the range between 9 and 20 nmol/mg/min (19 of 23 samples). However, the overall range of variation was ~4.7-fold, which is consistent with reported variation in MPA pharmacokinetic parameters (Bullingham *et al.*, 1998). The basis for this variability has remained elusive but has been proposed to involve differences in hepatic glucuronidation. To investigate the basis of these differences, several studies have characterized human UGTs with MPA glucuronidating activity. Initial reports identified 1A8 and 1A10 as active enzymes (Mojarrabi and Mackenzie, 1997; Mackenzie, 2000), a finding confirmed by others (Bernard and Guillemette, 2004) as well

as in the current study. However, because these enzymes are not significantly expressed in liver, they are considered unlikely to contribute to hepatic MPA glucuronidation.

Bernard and Guillemette recently reported evidence for high MPA glucuronidating activity of the 1A9 enzyme and proposed that this enzyme may be a main contributor to hepatic glucuronidation of MPA (Bernard and Guillemette, 2004). While some differences are apparent, the data in our study confirm the high activity of the 1A9 enzyme and present additional evidence for a principal, if not exclusive, role of 1A9 in HLM MPA glucuronidation. The additional lines of evidence include: (1) the marked (>80%) reduction in MPA UGT activity of rat liver microsomes from Gunn rats, suggesting that functional UGT1A enzymes are required for high rates of MPA glucuronidation; (2) the lack of any significant overlapping activity towards MPA of other known hepatic-expressed human UGT1A enzymes (1A1, 1A3, 1A4, 1A5, and 1A6); (3) the high apparent affinity of the 1A9 enzyme for MPA, which is comparable to corresponding K_m values determined for HLM; and (4) the strong correlation observed between MPA UGT activity and the 1A9-like content of HLM (r^2=0.79, $p<0.05$).

The present study reports a lower value for apparent K_m of HLM and human 1A9 enzyme for MPA than has been previously reported. The average K_m determined for three HLM samples (0.082 mM) closely agrees with the average K_m determined for three different preparations of 1A9 enzyme (0.077 mM). Estimates of apparent K_m of HLM samples for MPA in other studies have generally ranged between 0.2 and 0.6 mM (Vietri *et al.*, 2000; Bowalgaha and Miners, 2001; Shipkova *et al.*, 2001; Vietri *et al.*, 2002). However, Bernard and Guillemette reported lower K_m values, closer to the estimates in our study (0.15 to 0.27 mM, with two sets of pooled human liver microsome samples

exhibiting values of 0.095 and 0.135 mM (Bernard and Guillemette, 2004). Similarly, estimates of the apparent K_m of 1A9 for MPA reported in two other studies (0.28, 0.29 mM) (Shipkova *et al.*, 2001; Bernard and Guillemette, 2004) were higher than the estimates obtained in our study. We are uncertain as to the reason for this discrepancy, but two potential explanations could underlie these differences. In our studies with the high affinity-high velocity enzymes (human 1A9 and rat 1A7), rapid substrate consumption, in some cases approaching 100%, was observed in reactions containing 1 mg/mL protein incubated for 30 min in the presence of the lower MPA test concentrations (0.0156 to 0.125 mM MPA). To accurately measure initial rates of MPAG formation, kinetic analyses of the high activity forms required use of lower protein concentrations and/or reduced incubation times. Underestimation of initial rates at low MPA concentration has the potential to result in the appearance of atypical kinetics with an upward shift in the apparent K_m estimate. Another possibility is that the observed K_m differences are attributable to differences in the incubation conditions. In some studies, Brij detergent was used (Vietri *et al.*, 2000; Bowalgaha and Miners, 2001), whereas other studies completely omitted detergents (Shipkova *et al.*, 2001; Bernard and Guillemette, 2004). In our study, the pore-forming agent, alamethicin, was used in place of detergents to maximally activate MPA UGT activity of HLM.

The current study provides, to our knowledge, the first characterization of MPA UGT activity of rat liver microsomes. In comparison to HLM, two notable differences were the lower overall rates of MPA glucuronidation (2.4-fold difference, $p<0.001$), as reflected in lower apparent V_{max} and higher K_m values. As already mentioned, a marked effect of UGT1A genotype on MPA UGT activity of rat liver microsomes was observed.

Characterization of the rat UGT1A family members with activity towards MPA uncovered some interesting differences between the species in the UGT1A isozymes capable of metabolizing MPA. In addition, the recombinant rat UGT kinetic characteristization data provides a possible explanation for the observed differences in kinetic parameters of human versus rat liver microsomes.

The screening of expressed rat UGTs yielded three UGT1A isoforms with activity towards MPA: UGT1A1, UGT1A6 and UGT1A7, a finding that contrasts with the observed very low or undetectable activity of the corresponding human 1A1 and 1A6 forms. Also in contrast to human, the 1A8 and 1A10 forms from rat showed very low or undetectable levels of activity towards MPA. The low activity of 1A8 is not due to lack of functionality, as this enzyme has been demonstrated to catalyze the glucuronidation of a number of phenolic and other compounds (Webb *et al.*, 2005). One question raised by these data is which of the three active MPA UGT1A enzymes contribute(s) to MPA glucuronidation in normal rat liver. All three are expressed or are potentially expressed in liver tissue. In microsomes prepared from adult Sprague Dawley rats fed normal diets without exposure to inducing agents, 1A1 content estimated by quantitative immunoblotting was found to be 4-fold higher than 1A6 and 20-fold higher than 1A7 (Miles, Stern, Smith, Kessler and Ritter, manuscript in preparation). Our data suggest that both UGT1A1 and UGT1A7 may contribute significantly to the MPA UGT activity in untreated control rats, and that the 1A7 form may be dominant at low MPA concentrations due to its high affinity.

It is also tempting to speculate that the lower and higher MPA glucuronidating activities of rat and human liver microsomes, respectively, reflect a combination of one or more of the following factors: (1) human liver normally expresses 1A9 at a high level, and 1A9 is a high affinity-high capacity enzyme, (2) liver microsomes from untreated, control rats normally exhibit low constitutive expression of the 1A7 enzyme, the only member of the rat bulky phenol UGT1A subfamily with high affinity-high activity towards MPA, and (3) the relatively low CL_{int} of rat 1A1 for MPA glucuronidation (i.e., 1A1 is a medium affinity/capacity enzyme in MPA glucuronidation).

Because the recombinant enzymes tested in our study were expressed using different systems (HepG2 for the rat enzymes versus HEK cells/ Sf9 insect cells for the human enzymes), it is worthwhile to consider the potential role of this factor in the observed differences in enzyme specificity of human and rat UGT enzymes. HepG2 cells were selected as the cell model because the rat UGTs are expressed at much higher levels in these cells following adenovirus infection. Some data in the literature suggest that UGT activities can be affected by certain posttranslational modifications, such as phosphorylation (Basu *et al.*, 2003), which may differ in the various host cells used for heterologous expression. To address this possibility, we performed MPA UGT activity assays using preparations of rat 1A1, 1A6, or 1A7 obtained from stably transfected HEK cell clones and observed similar results (significant catalytic rates for each of these forms, data not shown). These data therefore appear to exclude this possibility.

It is also noteworthy to consider that, although 1A1 may be the primary UGT1A enzyme underlying MPAG formation in control rat liver, the relative contribution of the phenol UGT1A enzymes, especially 1A7 (high affinity/capacity), may increase in states

of higher relative 1A6/1A7 expression. In this regard, the genes for both of these enzymes are known to be under the control of multiple induction mechanisms and/or specific inducing agents, including the arylhydrocarbon receptor (3-methylcholanthrene et. al.), phenolic antioxidants (*t*-butylhydroquinone and oltipraz), and barbiturates (phenobarbital). Our data predict that MPA UGT activity will be significantly increased in liver microsomes of induced rats.

The data in our study provide new information regarding the contributions of UGT1A versus UGT2 family enzymes in MPA glucuronidation by rat liver microsomes. Whether the metabolism of MPA in humans is also highly dependent on UGT1A enzymes remains less clear but is supported by the strong correlation observed in our study with the 1A9 enzyme. These data suggest that both Gunn rats as well as human patients with Crigler-Najjar Type 1 disease will likely exhibit drastically reduced rates of MPA glucuronidation. Given that the latter syndrome is commonly treated by orthotopic liver transplantation in conjunction with immunosuppression therapy, it would be interesting to examine whether these patients exhibit marked differences in the pharmacokinetics or toxicity of MPA in the pre- versus post-transplant state. To our knowledge no information is currently available regarding the use of MPA in Crigler-Najjar Type I patients who received a liver transplant.

During the preparation of this manuscript, Picard et al. (Picard *et al.*, 2005) reported the results of a study characterizing of human and rat liver microsomal MPA glucuronidation. In their work, chemical inhibition studies with a 1A9 inhibitor (propofol) supported a major contribution of the 1A9 isoform to MPAG formation which is consistent with the data of our immunoblot correlation study. The same study reported

induction of MPA UGT activity in rat liver microsomes by 3-methylcholanthrene, a known inducer of the phenol UGTs, UGT1A6 and UGT1A7. This finding is in agreement with the observation that both of these forms are active in MPAG formation catalysis, and particularly the high affinity, high capacity UGT1A7 form.

In summary, this study compared the MPA glucuronidating activities of the rat and human UGT systems. Our data for human UGTs support current evidence implicating members of the 1A7-1A10 subfamily in metabolism of MPA. While our data for rats indicate a similar reliance on UGT1A family enzymes for MPA UGT activity, species differences are apparent in the profile of UGT1A enzymes that underlie MPA glucuronidation. Continuing analysis and use of the rat as a model to investigate the relationship between MPA pharmacokinetics, rates of hepatic versus extrahepatic glucuronidation, and toxicity of MPA should be greatly facilitated by appreciation of these differences and will undoubtedly lead to new insights into the pharmacology and toxicology of this important immunosuppressive agent.

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Footnotes

Unnumbered footnote to the title

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Figure Legends

Figure 1. Schematic of metabolism of mycophenolate mofetil. MMF, mycophenolate mofetil. MPA, mycophenolic acid. MPAG, mycophenolate 7-O-glucuronide. The lower glucuronide shown is the acyl glucuronide of MPA and was not evaluated in this study.

Figure 2. Comparison of MPA UGT activities of human and rat liver microsomes. A bank of human (n=23) and Sprague-Dawley rat (n=9) liver microsomes were activated with 50 μg/mg alamethicin and assayed for MPA UGT activity as described under **Materials and Methods**. Reactions were performed in the presence of 2mM MPA and 3mM UDP-glucuronic acid. Values shown represent the mean ± SEM of the activities in units of nmol/mg/min. *$p<0.001$, Student's *t*-test.

Figure 3. Representative Eadie-Hofstee plots for catalysis of MPAG formation by human and rat liver microsomes. Liver microsomes from three individual human (A-C) and male Sprague Dawley rat (D-E) were analyzed for MPA UGT activity using different MPA concentrations (0.0156-2 mM). For each case, the concentration of microsomal protein (0.1-1 mg/mL) and/or time of incubation (10-30 min) was varied to ensure that not >10% of substrate was converted to product in the reactions. Indicated estimates of K_m and V_{max} (in units of mM and nmol/mg/min, respectively) were determined by linear regression (best fit line shown).

Figure 4. Influence of UGT1A genotype on liver microsomal MPA UGT activity. Liver microsomes from adult male homozygous normal (+/+), heterozygous (j/+), or homozygous UGT1A-defective rats (j/j) (n=3 per group) were assayed for MPA UGT activity as described under **Materials and Methods**. The data are expressed as the mean activity ± SD in units of nmol/mg/min. *$p<0.05$ compared to j/j group. **$p<0.05$ compared to +/+ group.

Figure 5. MPA glucuronidating activities of cloned, expressed human and rat UGT1A family enzymes. Preparations of the indicated (A) human UGT1A enzymes expressed in HEK cells or (B) rat UGT enzymes expressed in HepG2 cells were assayed for MPA UGT activity using 2 mM MPA and 3 mM UDP-glucuronic acid as described under **Materials and Methods**. Activities are expressed in units of nmol/mg/min. Bottoms of each panel show results of Western immunoblotting with the indicated antibodies to demonstrate specificity of antibodies and relative levels of UGT expression. Ab1168 is a polyclonal antibody generated against affinity-purified mouse liver UGT (Mackenzie *et al.*, 1984) and displays general reactivity towards UGTs from both the UGT1A and UGT2B families.

Figure 6. Correlation analyses between MPA UGT activity and content of human liver-expressed UGT1A family enzymes. Semi-quantitative immunoblot analyses were performed to determine relative human liver microsomal contents of UGT1A1, UGT1A4-like, UGT1A6, and UGT1A9-like (n=23 HLM samples). Lines represent the

best-fit estimate of least squares linear regression analyses. The Spearman coefficient of regression (r^2) is shown for each plot.

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Table 1

Comparison of kinetic parameters for MPA glucuronidation by human and rat liver microsomes and cloned UDP-glucuronosyltransferases

Liver microsomes or recombinant UGT preparations were incubated in the presence of 3 mM UDPGA and varying concentrations of MPA (0.0156- 2 mM) and the rate of MPA glucuronide formation determined. Kinetic parameters were estimated using Winnonlin software. For rat (RLM) and human liver microsomes (HLM), three different preparations were analyzed. For recombinant enzymes, single preparations were analyzed except for 1A9. Data are expressed as the mean +/- SD.

Sample	Apparent Km (mM)	Vmax (nmol/mg/min)	Clint (ul/min/ mg)
HLM (n=3)	0.082 +/- 0.025	20.5 +/- 12.4	250
h1A9 (n=3)			
Prep 1	0.108 +/- 0.010	2.7 +/- 0.07	25
Prep 2	0.048 +/- 0.005	15.2 +/- 0.4	317
Prep 3	0.076 +/- 0.012	28.7 +/- 1.2	378
1A9 Mean +/- SD	0.077 +/- 0.030	15.5 +/- 13.0	201
RLM (n=3)	0.200 +/- 0.016	6.7 +/- 1.1	34
r1A1	0.208 +/- 0.013	1.7 +/- 0.03	8.3
r1A6	1.28 +/- 0.02	4.8 +/- 0.1	3.7
r1A7	0.034 +/- 0.001	10.3 +/- 0.1	303

Fig. 1

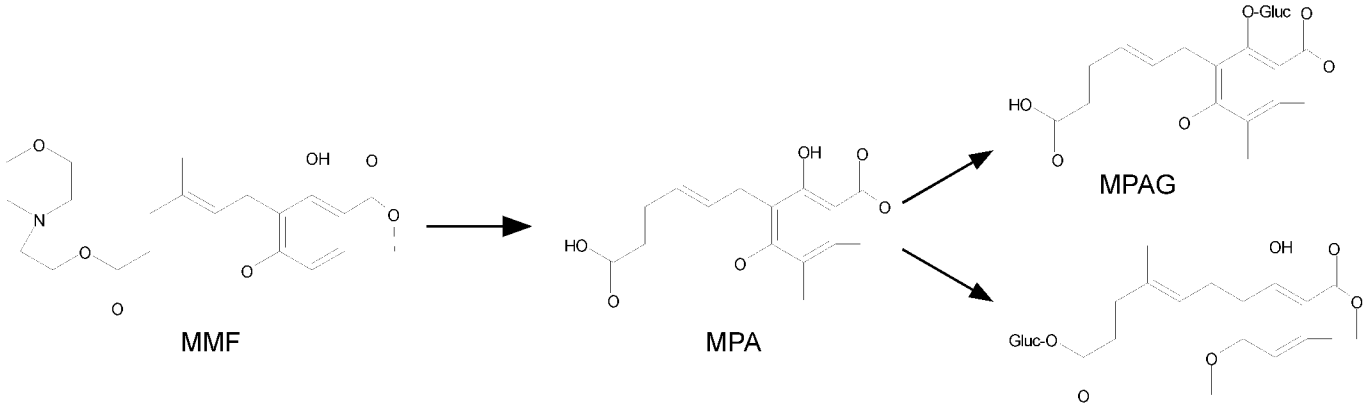


Fig. 2

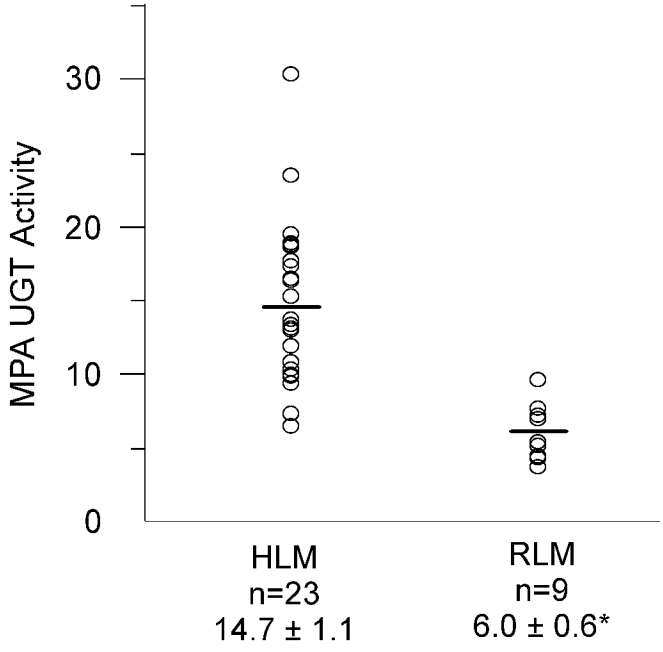
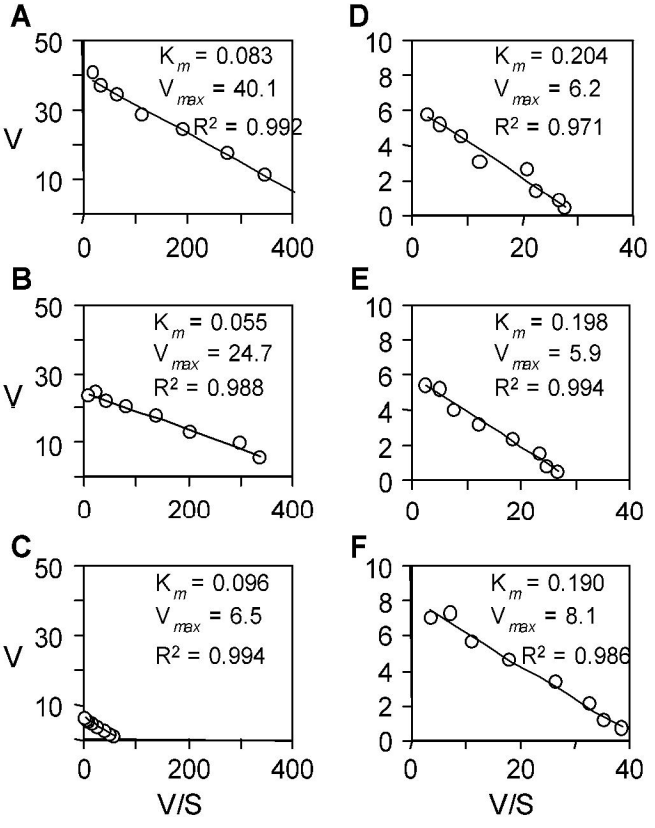


Fig. 3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V_{max} = 40.1
R^2 = 0.992
B
K_m = 0.055
V_{max} = 24.7
R^2 = 0.988
C
K_m = 0.096
V_{max} = 6.5
R^2 = 0.994
D
K_m = 0.204
V_{max} = 6.2
R^2 = 0.971
E
K_m = 0.198
V_{max} = 5.9
R^2 = 0.994
F
K_m = 0.190
V_{max} = 8.1
R^2 = 0.986
V
V/S

Fig. 4

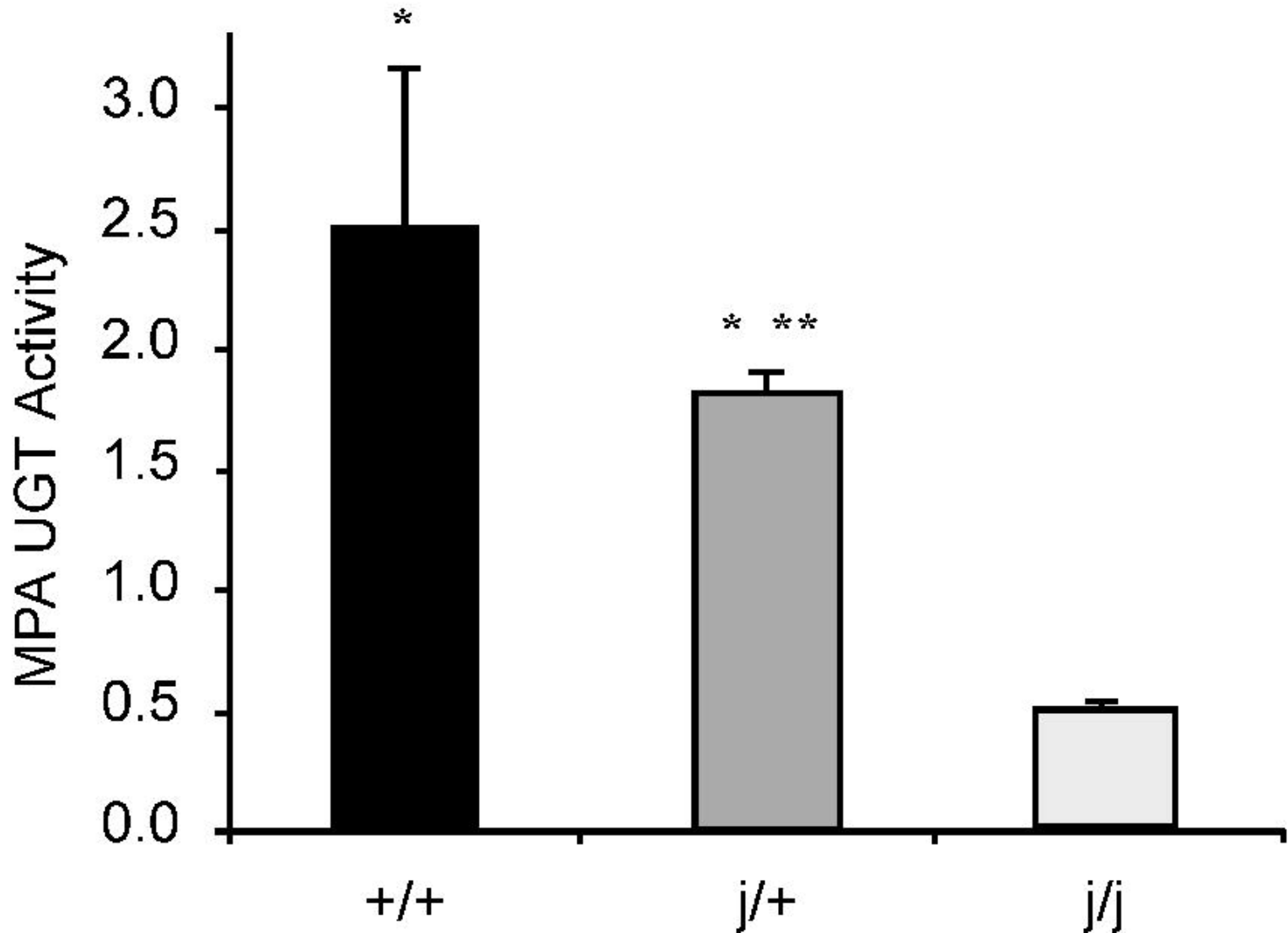
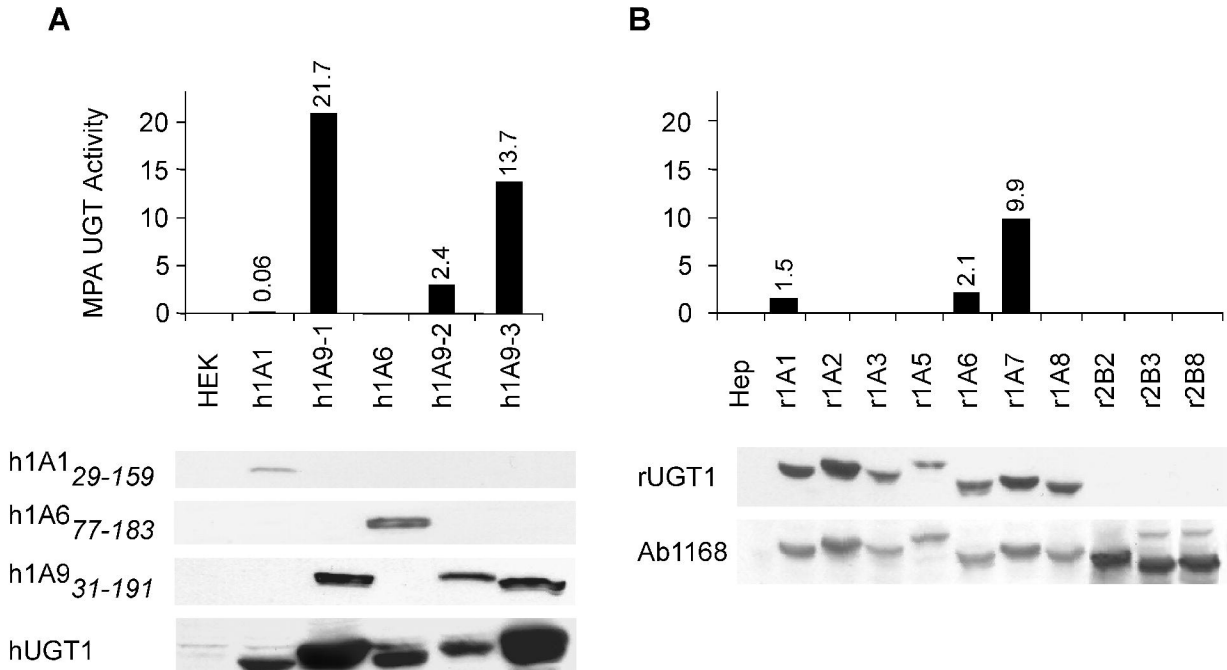


Fig. 5


A
MPA UGT Activity
20
15
10
5
0
0.06
21.7
2.4
13.7
HEK
h1A1
h1A9-1
h1A6
h1A9-2
h1A9-3
h1A1 29-159
h1A6 77-183
h1A9 31-191
hUGT1
B
20
15
10
5
0
1.5
2.1
9.9
Hep
r1A1
r1A2
r1A3
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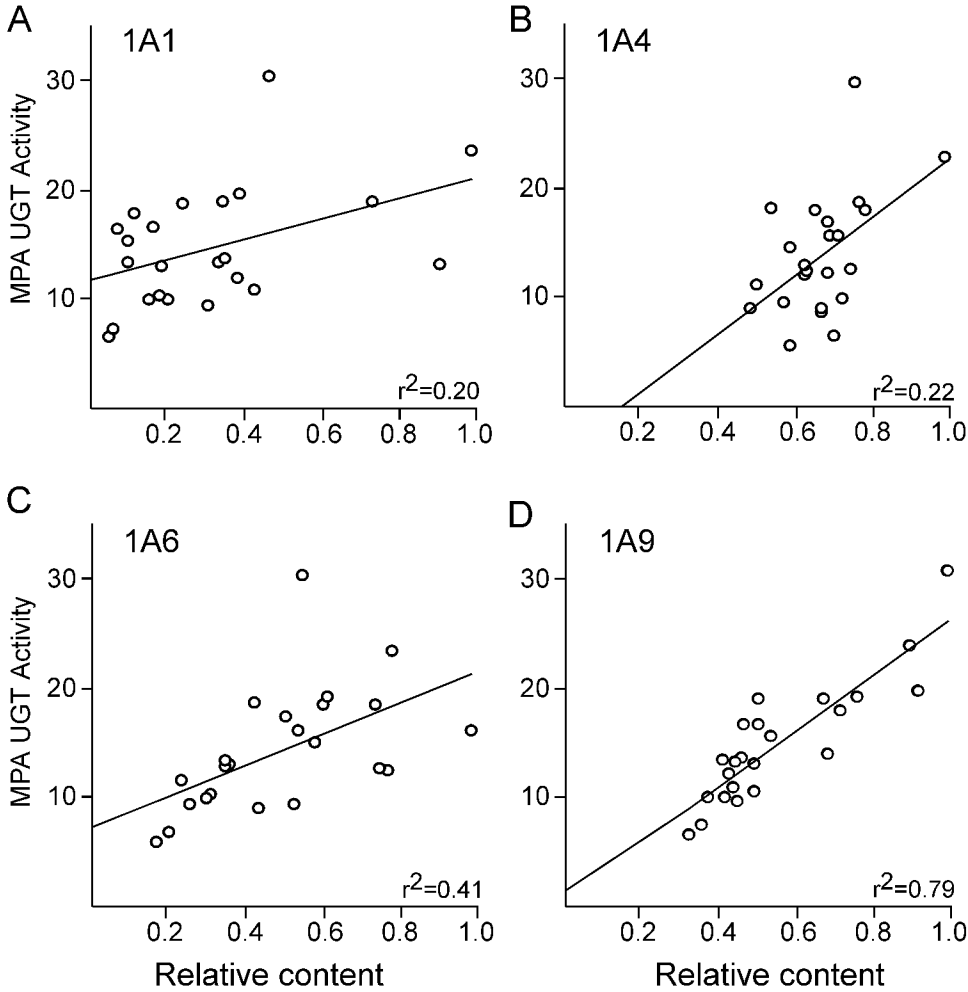
r2B8
rUGT1
Ab1168

Fig. 6


A
1A1
MPA UGT Activity
30
20
10
0.2
0.4
0.6
0.8
1.0
r2=0.20
B
1A4
r2=0.22
C
1A6
r2=0.41
D
1A9
r2=0.79
Relative content
Relative content