Adenovirus-Mediated Gene Therapy to Restore Expression and Functionality of Multiple UDP-Glucuronosyltransferase 1A Enzymes in Gunn Rat Liver


Department of Pharmacology and Toxicology, Virginia Commonwealth University Medical Center, Richmond, Virginia (K.K.M., F.K.K., L.J.W., J.K.R.); and Department of Drug Delivery and Disposition, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (P.C.S.)

Received March 17, 2006; accepted June 7, 2006

ABSTRACT

The Gunn rat has been a valuable model for investigating the effect of UDP-glucuronosyltransferase 1A (UGT1A) deficiencies on drug metabolism and toxicity, but it is limited in some aspects. For example, the native Gunn rat model cannot distinguish between hepatic and extrahepatic UGT1A deficiencies in toxicological mechanisms. To extend the model’s utility, we investigated the use of replication-defective recombinant UGT1A adenoviruses for the purpose of selectively restoring hepatic UGT1A function. Mycophenolic acid, the active metabolite of the anti-transplant rejection drug mycophenolate mofetil and suspected gastrointestinal toxicant, was used as a model UGT1A-dependent substrate. Treatment with UGT1A adenoviruses normalized the plasma mycophenolic acid and 7-O-mycophenolate glucuronide (MPAG) (concentration-time curves after mycophenolic acid administration (80 mg/kg intraperitoneally). Functional reconstitution was also apparent in the correction of the mycophenolic acid $t_{1/2}$ and the area under the curve (AUC)$_{MPAG,0–8\ h}/$AUC$_{MPAG,0–8\ h}$ ratio. Twenty-four hours after administration of mycophenolic acid, severe signs of toxicity were noted in the naive Gunn group, including reduced food consumption. The effect on food consumption was reduced but not completely prevented in the UGT adenovirus-treated Gunn rats. In vitro analyses indicated adenovirus dose-dependent reconstitution of mycophenolic acid UGT activities and UGT1A contents in liver but not intestinal microsomes. In the highest adenovirus dose group, the liver microsomal UGT1A markers exceeded those of the heterozygote controls. The ability to selectively manipulate multiple hepatic UGT1A enzymes in Gunn rats should provide a novel way to assess the importance of intestinal or other extrahepatic UGT1A enzymes in toxicities induced by mycophenolic acid and other cytotoxic drugs and dietary agents.

Mycophenolate mofetil (MMF, Cellcept) is now considered one of the first-line drugs for maintenance immunosuppression therapy in transplant patients (Sinthchak and Nimmesgern, 2000). Use of this drug, however, may be limited by one of its common side effects, the development of gastrointestinal toxicity, which usually manifests as diarrhea presenting many weeks or months after the start of therapy. The occurrence of diarrhea is of clinical significance because it necessitates dosage reductions for MMF, which can negatively affect its immunosuppressive efficacy. Neither the origin of the effect nor factors that determine the variable clinical presentation are understood but have been proposed to involve its active metabolite, mycophenolic acid. Mycophenolic acid is a cytotoxic drug that interferes with DNA synthesis by inhibition of guanosine-inosine monophosphate dehydrogenase, the rate-limiting enzyme in the de novo purine nucleotide synthesis pathway (Neerman and Boothe, 2003). Our laboratories have proposed that deficient glucuronidation of mycophenolic acid in the intestinal mucosa itself (Miles et al., 2005, 2006) could be an important contributing factor in the onset of MMF-induced diarrhea. The primary metabolite of mycophenolic acid in both rodents (Hesselink et al., 2005) and humans (Bullingham et al., 1998) is the 7-O-mycophenolate glucuronide (MPAG), which is inactive as a DNA synthesis inhibitor. UDP-glucuronosyltransferase (UGT) expressed at high levels in intestinal epithelial cells could be protective by keeping intracellular mycophenolic acid concentrations low.

This work was supported by National Institutes of General Medical Sciences and Environmental Health Science Research Grants R01GM61188 and R01ES07672 and an institutional A. D. Williams Trust grant.

ABBREVIATIONS: MMF, mycophenolate mofetil; MPAG, 7-O-mycophenolate glucuronide; i.p., intraperitoneally; UGT, UDP-glucuronosyltransferase(s); OD, optical density; RLM, rat liver microsome(s); ALT, alanine aminotransferase; HPLC, high-performance liquid chromatography; AUC, area under the plasma concentration-time curve; $t_{1/2}$, plasma elimination half-life, early phase.
Glucuronidation represents one of the major phase II biotransformation pathways in the detoxification of drugs, carcinogens, and dietary components. UGT that are active in mycophenolic acid catalysis have been characterized using various approaches. In human liver, UGT1A9 is proposed to be a dominant enzyme (Bernard and Guillemette, 2004; Miles et al., 2005; Picard et al., 2005). Of the three rat UGT1A enzymes identified as having mycophenolic acid glucuronidating activity (UGT1A1, UGT1A6, and UGT1A7), the UGT1A1 and UGT1A7 forms were suggested to be the principal mediators in liver (Miles et al., 2005) and intestine (Miles et al., 2006), respectively, based in part on the high abundances of these enzymes in rat liver and intestine. The important contribution of UGT1A enzymes to hepatic and intestinal mycophenolic acid glucuronidation in rats is supported by the marked reduction of mycophenolic acid UGT activity in homozygous Gunn rats (Miles et al., 2005, 2006), a strain that is deficient in UGT1A enzymes because of a frameshift mutation in common exon 4 of the UGT1A gene locus (Iyanagi, 1991).

Although the Gunn rat has been used to study the relationship between glucuronidation and xenobiotic toxicity (de Morais and Wells, 1989; Kim and Wells, 1996; Kato et al., 2005) and as a gene therapy test model (Takahashi et al., 1996; Danko et al., 2004; Jia and Danko, 2005), its utility for mechanistic studies to distinguish the roles of hepatic versus extrahepatic UGT as modulators of drug toxicity is limited. To circumvent this limitation, we considered the possibility of using replication-defective adenoviruses to correct hepatic UGT1A, leaving extrahepatic UGT1A deficient. Adenoviruses are currently being extensively investigated as potential gene therapy vectors and have been shown to be effective for delivering functional UGT1A1 to the livers of Gunn rats and correcting the hyperbilirubinemic phenotype (Takahashi et al., 1996; Ilan et al., 1997; Li et al., 1998; Toietta et al., 2005). A major advantage of adenoviruses over other available gene therapy approaches is the highly selective uptake and expression in the liver, the high adenovirus transduction efficiency for hepatocytes, and the chronic nature of adenovirus transgene expression (Amalfitano et al., 1998, 2004; Herrmann et al., 2004; Toietta et al., 2005). Although the available data for adenovirus-mediated UGT gene therapy show the correction of a single enzyme (UGT1A1), it remains unclear whether the approach is useful for the correction of the multiple UGT1A deficiencies known to exist in Gunn rats.

The purpose of the present study was to determine whether gene therapy with a mixture of adenoviruses encoding the three major mycophenolic acid UGT expressed in rat liver—UGT1A1, UGT1A6, and UGT1A7—could reconstitute the hyperbilirubinemic phenotype (Pandak et al., 2001). UGT1A1 and UGT1A7 forms were cloned in the correct orientation into the multiple cloning site of pZero Tg-CMV, immediately downstream of the cytomegalovirus promoter sequence, with flanking 5' and 3' genomic DNA sequence from Ad5dI324. The resulting pZeroTg-CMV-UGT plasmids were cotransformed with Clal-linearized pTG/CMV (containing the entire Ad5dI324 genome) into Escherichia coli. Resulting recombinant plasmids were screened for inserts before transfection into human embryonic kidney 293B cells (American Type Culture Collection, Manassas, VA) to propagate and amplify the virus preparations as described previously (Pandak et al., 2001). In brief, 293B cells were grown to confluence and infected with the appropriate adenoviruses described above. Cells were harvested 72 h postinfection and spun at 2700g for 10 min at 4°C. The cell pellet was washed by resuspension in phosphate-buffered saline and recenterfuged. After a final resuspension, the cells were submitted to five consecutive freeze-thaw cycles and were centrifuged at 9400g for 5 min at 4°C. The recovered supernatant was layered onto a CsCl gradient and centrifuged for 2 h at 155,000g at 20°C. Infectious adenovirus was removed and subjected to an overnight CsCl equilibrium centrifugation. The twice-purified virus was dialyzed overnight with three changes of dialysis buffer (10 mM Tris-Cl, pH 7.4, 1 mM MgCl2, and 10% glycerol). The number of virions per milliliter was established by measurement of the optical density (OD) at 260 nm of viral dilutions prepared in lysis buffer (10 mM Tris-Cl, pH 7.4, 0.1% SDS, and 1 mM EDTA), where 1 OD260 u = 1 x 1012 virions/ml (Herrmann et al., 2004). Adenovirus stocks were aliquoted and stored at −80°C until use.

Animal Care and Housing. All of the animal experiments were conducted according to the National Institutes of Health Guidelines for the Care and Use of Animals using a protocol approved by both the Biohazard Safety and Institutional Animal Care and Use Committees of Virginia Commonwealth University. Heterozygous (j/+ and homozygous mutant (j/j) male Gunn rats (225–275 g b.wt.) were obtained from a Gunn rat colony established on-site and maintained two per cage using a standard 12-h light/dark cycle. Room temperature and humidity were maintained at 22°C and 50%, respectively. Animals were given free access to rodent chow (Harlan Teklad Lab Diet LM-485) and water.

Animal Treatments. For this study, three groups of homozygous male Gunn rats (j/j) genotype, three per group) were infected at different dose levels with a fixed ratio mixture of adenoviruses encoding rat UGT1A1, UGT1A6, and UGT1A7. The fixed ratio was 20 virions adenovirus-CMV-r1A1/5 virions adenovirus-CMV-r1A6/1 virion adenovirus-CMV-r1A7, which was chosen to reflect the natural abundance ratios of these enzymes in control rat liver microsomes (RLM) (Miles et al., 2006). The high-dose group (HD Gunn rats) received 0.26 OD260 u total, consisting of 0.02 OD260 u adenovirus-CMV-r1A1, 0.05 OD260 u adenovirus-CMV-r1A6, and 0.01 OD260 u adenovirus-CMV-r1A7 for a total of 2.6 x 1011 virions. The intermediate- and low-dose groups received 0.13 and 0.0625 OD260 u total of the fixed ratio mixture, respectively. Two control groups were used in this study: uninfected homozygous (j/j)
Gunn rats and heterozygous Gunn rats infected with a high dose (0.26 OD 260 U) of adenosine coding for a negative control UGT (i.e., rat UGT1A10, which shows no detectable activity toward mycophenolic acid) (Miles et al., 2005). Five days postinfection, when adenovirus-expressed UGT has reached maximum (data not shown), animals received a single dose of mycophenolic acid (80 mg/kg) delivered i.p. Serial blood samples (100 μl each) were collected from the tail vein at 15, 30, 60, 90, 120, 240, 360, and 480 min after mycophenolic acid administration. Blood was collected in Microtainer EDTA tubes and spun at 16,000g for 5 min at 4°C. The resulting plasma was transferred to microfuge tube and stored at −20°C until analysis. After dosing, the animals were housed individually and observed over the next 48 h. Animals were sacrificed by isoflurane overdose, followed by guillotining. A final blood sample was collected for analysis of serum alanine aminotransferase (ALT) activity. Livers were collected, frozen on dry ice, and stored at −80°C until needed for microsome preparation.

Plasma Mycophenolic Acid and MPAG Kinetic Analysis. Plasma samples were analyzed for mycophenolic acid and MPAG using a published reverse-phase high-performance liquid chromatography (HPLC) procedure with some modification (Wiwattanawongsa et al., 2001). In brief, suprofen internal standard (5 μl of a 0.5 μg/μl solution) was mixed with 20 μl of plasma, followed by 4 μl of concentrated perchloric acid to precipitate proteins. The samples were then placed on ice for 5 min and centrifuged at 16,000g for 5 min at 4°C. Ten microliters of cleared supernatant was injected onto a Hewlett Packard 1050 HPLC containing a Partisil 10 ODS-2 C18 column (4.6 mm × 55 mm) (Whatman Inc., Clifton, NJ) and a Waters Resolve C8 Guard Pak precolumn cartridge (Millipore, Billerica, MA). The mobile phase consisted of 55% methanol/45% aqueous trifluoroacetic acid (0.1% trifluoroacetic acid). The flow rate and column temperature were 1.5 ml/min and 40°C, respectively. Absorbance was monitored using a Hewlett Packard 1050 diode array detector set at 250 nm. Under these conditions, the retention times for MPAG, suprofen, and mycophenolic acid were 3.3, 8.8, and 11.8 min, respectively. Plasma mycophenolic acid and MPAG concentrations were determined from standard curves prepared from plasma samples containing known concentrations of added mycophenolic acid and MPAG. The limit of detection in plasma was ~2 and ~3 μg/ml for mycophenolic acid and MPAG, respectively.

The area under the plasma concentration-time curve (AUC), peak plasma concentration (C_{max}), and the time of peak plasma concentration (T_{max}) for mycophenolic acid and MPAG were calculated based on a noncompartmental model using WinNonlin pharmacokinetic software v. 2.0 (Pharsight Corporation, Palo Alto, CA). Statistical analyses were performed using one-way analysis of variance testing. When the test indicated that a statistically significant difference existed between one or more groups, a Student-Newman-Keuls post hoc test was performed. Differences were considered statistically significant when p < 0.05.

Microsome Preparation and Mycophenolic Acid UGT Assays. Liver microsomes were prepared based on a previously described method (Kessler and Ritter, 1997). In brief, livers were homogenized in five volumes of ice-cold 0.25 M sucrose using glass Potter-Elvehjem tubes fitted with Teflon pestles. After removing nucleic acid and mitochondrial fractions, samples were spun at 155,000g for 45 min at 4°C. Microsomes were then resuspended in 0.15 M KCl and subjected to a second ultracentrifugation spin as described above. Final microsomal resuspensions were made in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 20% glycerol. Protein concentration was determined by the bicinchoninic acid method using a commercially available kit (Pierce Biotechnology, Rockford, IL).

Mycophenolic acid UGT activities were determined as described previously (Miles et al., 2005). In brief, microsomal reactions contained 75 mM Tris-Cl, pH 7.45, 10 mM MgCl2, 6 mM saccharic acid 1,4-lactone (prepared fresh), and 1 mM mycophenolic acid. Microsomes were activated with 50 mg alamethicin/mg protein for 15 min on ice before the initiation of enzymatic reactions. After pre-equilibration at 37°C, reactions were initiated by the addition of UDP-glucuronic acid (3 mM final concentration) and allowed to proceed for 30 min. Reactions were stopped by addition of concentrated perchloric acid (to 5.6% final concentration, v/v). Five microliters of suprofen internal standard was added, and the tubes were placed on ice and centrifuged as described above. Control reactions prepared without mycophenolic acid were also included. An aliquot of supernatant was analyzed for MPAG by HPLC as described above. The MPAG concentration was estimated from an MPAG standard curve prepared in a standard mycophenolic acid UGT reaction matrix. The limit of detection expressed as rate of MPAG formation was ~0.01 nmol/mg/min.

Western Blot Analysis of Liver Microsomal UGT. The antibodies for Western immunoblot analysis were prepared in our laboratory. The generation of the rat UGT1A1 (anti-r1A129–162) and UGT1A7 (anti-r1A721–149) antisera has been previously reported (Kessler et al., 2002; Webb et al., 2005). An antisera recognizing rat amino acid residues 77 to 183 of rat UGT1A6 (anti-r1A677–183) was prepared using a similar strategy. Antisera were tested for specificity against recombinant rat UGT1A (UGT1A1-1A10 excluding UGT1A4 and UGT1A9) and UGT2B (UGT2B2, UGT2B3, and UGT2B8) proteins by Western blot analysis. In brief, 20 μg of crude cell membrane protein from recombinant rat UGT-expressing HepG2 cells was electrophoresed in parallel. Membranes were incubated in diluted antisera, then in horseradish peroxidase-conjugated sheep-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and detected with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Resulting images were captured on film and analyzed.

Western immunoblots analyses of naive and infected Gunn RLM were performed as described previously with minor modifications (Ritter et al., 1999; Guillemette et al., 2000). Microsomal proteins were subjected to 7.5% SDS-polyacrylamide gel electrophoresis in parallel to serial dilutions of recombinant rat UGT1A1, UGT1A6, and UGT1A7 and transferred to a nitrocellulose membrane. UGT1A4 and UGT1A9 were not monitored because these UGT are pseudogenes in rats. Blots were blocked in 5% milk suspension and then probed for rat UGT1A1, UGT1A6, and UGT1A7 using liver-generated antisera. Membranes were then incubated in horseradish peroxidase-conjugated sheep-anti-mouse IgG. Proteins were detected using enhanced chemiluminescence, and images were captured on film. Quantification of UGT bands was performed using Image J software (NIH, Bethesda, MD). Standard curves generated from the serially diluted recombinant proteins were used to estimate UGT content. The highest expressing sample was assigned an arbitrary value of 1 and was used to normalize the remaining samples. Data were statistically analyzed using analysis of variance with the Student-Newman-Keuls post hoc test. Values were considered statistically significant when p < 0.05.

Serum ALT Determinations. Serum ALT activities were determined using a commercially available ALT assay kit (Sigma Diagnostics). Serum (10 ml) was added to 0.65 ml of reconstituted ALT assay reagent at room temperature, and the change in absorbance at 340 nm resulting from NADH consumption was monitored using a Shimadzu UV1600 spectrophotometer. The activity was calculated using 6.22 as the millimolar absorbivity of NADH. A commercially available reference sample (Catatrol, Catachem, Bridgeport, CT) stated to contain 38 U/l yielded an ALT activity of 28 U/l under the conditions of our assay.

Results

Pharmacokinetic Analysis after Dosing with Mycophenolic Acid (80 mg/kg). The kinetic profiles for plasma mycophenolic acid and MPAG in the two control groups and three UGT adenovirus-treated Gunn groups are shown in
and t1/2α = 1.6 h, p < 0.05 versus the HD Gunn and the control infected heterozygotes) was observed. This effect is consistent with lower mycophenolic acid glucuronidation in the control Gunn rats, which exhibited the lowest AUCMPAG,0–8 h of all of the groups (22.2 μg/ml/h, p < 0.001 compared with all of the groups).

Among the UGT adenovirus-treated Gunn rat groups, an inverse relationship between UGT adenovirus dose and AUCMPAG,0–8 h was evident, although the differences between groups and the control-infected heterozygotes was not statistically significant. This finding supports the hypothesis that rats with lower hepatic mycophenolic acid glucuronidating activity have higher systemic exposure to mycophenolic acid. The statistical means of the HD Gunn and control infected heterozygote groups were almost identical (477 versus 442 mg/ml/h group), suggesting an essentially complete correction of the mycophenolic acid glucuronidation deficiency in the HD group. Interestingly, the HD Gunn rats exhibited significantly greater AUCMPAG,0–8 h compared with the heterozygotes, suggesting that the deficiency in the HD group may be overcorrected.

Differences in the plasma mycophenolic acid and MPAG kinetic profiles of the ID and LD Gunn rat groups were more subtle, with trends apparent toward increased AUCMPA,0–8 h, Cmax, and t1/2α and decreased AUCMPAG,0–8 h. Restoration of the imbalance between mycophenolic acid and MPAG exposures in Gunn rats relative to heterozygotes (AUCMPA/AUCMPAG Ratios of 44.9 and 2.7, respectively) is apparent for each of the adenovirus-treated Gunn rat groups, again with evidence of adenovirus dose dependence. Using the AUCMPA/AUCMPAG ratio as an index of correction relative to the control heterozygote group, the data suggest that the ID dose group is almost fully corrected compared with the HD group (overcorrected) and LD groups (significantly undercorrected).

**Toxicological Observations.** For the 2-day period after mycophenolic acid dosing, the rats were monitored for signs of general toxicity (appearance of fur coat, posture, and response to stimulation, food consumption, and body weight). Heterozygote animals appeared normal throughout the monitoring period, whereas the naive Gunn rats by 24 h postdose exhibited severe signs of toxicity (ruffled fur, hunched posture, and lack of locomotor response to stimulation) and were therefore euthanized after 24 h. In the groups of UGT adenovirus-treated Gunn rats, this toxicity appeared to be attenuated as a function of increasing UGT adenovirus dose. The data for food consumption during the monitoring period are shown in Fig. 2. For the 24-h period before the mycophenolic acid treatment, all of the groups exhibited comparable rates of food consumption (ca. 60–70 g/kg b.wt., not statistically significant). After mycophenolic acid treatment, the heterozygote group exhibited a slight increase in food consumption, whereas food consumption was significantly reduced in all of the Gunn groups (p < 0.001 for all of the groups compared with heterozygotes). Differences across groups in the magnitude of this effect were evident. The effect was most pronounced in the naive Gunn and LD Gunn groups and was significantly attenuated but not completely reversed in the HD Gunn group. No groups exhibited any evidence of loose stools or diarrhea. However, a striking reduction in feces production was apparent in the cages of the Gunn groups, especially the naive Gunn rats (data not shown), consistent with the decreased food consumption. During the second 24-h postdose period, signs of recovery from the acute mycophenolic acid-induced toxicity were apparent in the UGT adenovirus Gunn groups.

**Fig. 1.** Plasma mycophenolic acid and MPAG kinetic profiles of adenovirus-infected and naive Gunn rats. Groups of Gunn rats were infected with the indicated doses of UGT adenoviruses as described under Materials and Methods. HD, high dose; ID, intermediate dose; LD, low dose. The two control groups, control virus (adenovirus-CMV-rUGT1A10)-infected heterozygote (J/+ ) rats and naive (uninfected) Gunn rats, are included for comparison. Five days after the adenovirus treatments, animals received a single dose of mycophenolic acid (80 mg/kg i.p.), and blood samples were collected at 15, 30, 60, 90, 120, 240, 360, and 480 min. Plasma was analyzed for mycophenolic acid and MPAG by HPLC. Each value represents the mean ± S.E. of three rats.
TABLE 1
Plasma mycophenolic acid and MPAG kinetic parameters for naive and adenovirus-infected Gunn rats after a single mycophenolic acid administration

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>HD Gunn</th>
<th>ID Gunn</th>
<th>LD Gunn</th>
<th>Control j/j</th>
<th>Gunn</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCMPA,μg/mL</td>
<td>0.06 OD_{280} U</td>
<td>0.13 OD_{280} U</td>
<td>0.065 OD_{280} U</td>
<td>0.26 OD_{280} U</td>
<td>Uninfected</td>
</tr>
<tr>
<td>AUCMPAG,μg/mL/h</td>
<td>477 ± 60**</td>
<td>568 ± 156***</td>
<td>808 ± 16</td>
<td>442 ± 59***</td>
<td>1041 ± 210</td>
</tr>
<tr>
<td>C_{max} (μg/mL)</td>
<td>216 ± 18</td>
<td>240 ± 62</td>
<td>325 ± 16</td>
<td>165 ± 19</td>
<td>374 ± 10</td>
</tr>
<tr>
<td>T_{1/2a} (h)</td>
<td>15 ± 0</td>
<td>15 ± 0</td>
<td>15 ± 0</td>
<td>15 ± 0</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>CL/F (mL/min/kg)</td>
<td>0.71 ± 0.11</td>
<td>1.06 ± 0.13</td>
<td>1.31 ± 0.15</td>
<td>0.53 ± 0.01</td>
<td>1.55 ± 0.18</td>
</tr>
<tr>
<td>V/F (mL/kg)</td>
<td>0.41 ± 0.07***</td>
<td>0.30 ± 0.06**</td>
<td>0.18 ± 0.01</td>
<td>0.35 ± 0.05***</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>AUC_{MPA-MPA} (μg/mL)</td>
<td>323 ± 24***</td>
<td>171 ± 11***</td>
<td>94 ± 11***</td>
<td>183 ± 10***</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>C_{max} (μg/mL)</td>
<td>199 ± 41***</td>
<td>100 ± 8</td>
<td>65 ± 8</td>
<td>46 ± 7</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>T_{max} (μg/mL)</td>
<td>30 ± 0</td>
<td>40 ± 10</td>
<td>60 ± 0</td>
<td>110 ± 66</td>
<td>60 ± 0</td>
</tr>
<tr>
<td>AUC_{MPA}/AUC_{MPAG}</td>
<td>1.4</td>
<td>3.2</td>
<td>8.2</td>
<td>2.7</td>
<td>44.9</td>
</tr>
</tbody>
</table>

AUCMPA, mycophenolic acid area under the plasma concentration-time curve; C_{max}, peak plasma concentration; t_{1/2a}, plasma elimination half-life-alpha; T_{max}, mean peak time; MPAG area under the plasma concentration-time curve.

***p ≤ 0.001 as compared with j/j.
† p < 0.05 as compared with j/j.
‡ p < 0.001 as compared with j/j*.

In Vitro Measures of Adenovirus Reconstitution of Hepatic UGT1A.

Liver and colon microsomes prepared from naive and adenovirus-infected Gunn and heterozygote rats were assayed for their glucuronidating activities toward mycophenolic acid. In addition, the liver microsomes were assayed for bilirubin (a UGT1A1-dependent marker) and 4-nitrophenol (an overlapping but primarily UGT1A6 substrate). As previously reported (Miles et al., 2005), homozygous Gunn RLM showed a deficiency in mycophenolic acid UGT activity (p < 0.001 versus control j/j) (Table 2). Treatment of rats with increasing doses of UGT-encoding adenovirus resulted in dose-dependent increases in activity toward all three substrates. The average mycophenolic acid glucuronidation rate for the HD Gunn RLM was 2.2- and 7.0-fold higher than groups ID and LD, respectively (p < 0.05). Comparable mycophenolic acid UGT activities were observed for ID samples and infected controls, whereas LD group microsomes did not show an increased rate of mycophenolic acid glucuronidation compared with naive controls.

Increases in the relative UGT1A1, UGT1A6, and UGT1A7 microsomal protein content that were dependent on the UGT adenovirus dose were also observed (Fig. 3). The highest relative contents were observed in the HD Gunn group (Fig. 3).
3) (p < 0.05). Compared with infected heterozygote controls, RLM prepared from this group contained 2.5-, 2.0-, and 2.0-fold more UGT1A1, UGT1A6, and UGT1A7, respectively. The ID Gunn samples had roughly similar amounts of UGT1A1, UGT1A6, and UGT1A7 compared with the heterozygote controls, whereas the LD Gunn microsomes had the lowest levels of the enzymes, which were 7.0-, 5.5-, and 10.5-fold lower on average than the UGT1A1, UGT1A6, and UGT1A7 enzyme levels of the heterozygote control (p < 0.05). As expected, based on the frameshift mutation located in exon 4 of the UGT1A common region, none of the forms analyzed were detected in naive Gunn RLM (data not shown).

Mucosal acidic UGT activities of colon microsomes remained below detectable limits (<0.01 nmol/mg/min) in all of the Gunn rat groups analyzed (data not shown). The activity could only be measured in the heterozygote control samples (range from 0.85 and 2.2 nmol/mg/min, p < 0.001). In addition, we have been unable to detect any effect of UGT adenovirus treatments on kidney microsomal mycophenolic acidic UGT activity. These data show that, in contrast to liver, the adenovirus virus does not effectively transduce intestinal or kidney cells after intravenous administration.

ALT Activities. The ALT activities of the serum samples obtained at sacrifice were determined, and there was no significant effect of either adenovirus virus or mycophenolic acid treatment on serum ALT activity. The mean ALT activities ± S.E. (in U/l) were 74 ± 45, 16 ± 3, 33 ± 15, 33 ± 15, and 46 ± 36 for the HD Gunn, ID Gunn, LD Gunn, naive Gunn, and control virus-treated heterozygote groups, respectively. These data support minimal hepatotoxicity of the adenoviruses used in this study.

Discussion
To our knowledge, this is the first report to show that combination gene therapy can be applied to restore or normalize the expression of multiple UGT in livers of homozygous Gunn rats. Depending on the dose of adenovirus, UGT1A1, UGT1A6, and UGT1A7 can be expressed and manipulated at levels below, comparable with, or higher than that of controls (i.e., heterozygote y+/− rats in the current study). The development of this model is significant for at least two reasons. First, it provides a way to manipulate and thereby assess the roles of specific hepatic-expressed UGT in toxicological mechanisms of specific drugs. For example, manipulation of UGT1A enzymes important for acetaminophen or thyroid hormone glucuronidation will be useful for studies to explore the roles of specific-expressed liver UGT in hepatotoxicity and thyroid hyperthyroidism induced by these agents, respectively. The ability to manipulate hepatic glucuronidation by the adenovirus-based gene therapy approach should also provide a way to distinguish between hepatic- and extrahepatic-expressed UGT1A enzymes in toxicological manifestations of drugs administered to Gunn rats. This is because of the adenovirus being selectively taken up and expressed in hepatocytes compared with extrahepatic tissues such as intestine.

Of specific interest to our laboratory is the role of hepatic versus intestinally expressed UGT in the mechanism of mycophenolic acid-induced gastrointestinal toxicity. Intestinal mucosa is replete with UGT1A enzymes in contrast to the UGT2B family members (Shelby et al., 2003). The evidence indicates that the major expressed UGT1A enzyme in the rat gastrointestinal tract is the UGT1A7 form, which not only exhibits vastly superior to UGT1A1 and UGT1A6 in its 100-fold higher $V_{max}/K_m$ ratio in the turnover of mycophenolic acid to MPAG but also quantitatively dominates the total UGT1A content of rat intestinal microsomes (estimated to be more than 50% of the total) (Shelby et al., 2003; Miles et al., 2006). Although the UGT1A expression profile in human intestine differs from that of rats, the human intestine appears similar to rat in the characteristic that UGT1A enzymes with high activity toward mycophenolic acid are expressed at high levels in intestine (i.e., UGT1A8 and UGT1A10) (Mackenzie, 2000; Bowalgaha and Miners, 2001). The role of these enzymes in protection of intestinal mucosa against mycophenolic acid, as well as dietary phenols and other phytochemicals in general, has been proposed, but to date direct evidence has been lacking.

The current study provides clear support for the hypothesis that hepatic mycophenolic acid glucuronidation can be restored to varying degrees depending on the dose of UGT-encoding adenovirus. These include in vivo functional evidence (mycophenolic acid and MPAG kinetics), as well as conventional in vitro measures (determination of hepatic microsomal UGT activities and UGT content). Interestingly, using mycophenolic acid as a substrate, the degree of reconstitution seemed to depend on the specific endpoint examined. For example, in the case of the AUCMPAG,0–8 h data, the high adenovirus dose group resulted in systemic mycophenolic acid exposure most closely approximating that of the heterozygote controls. In contrast, the data for AUCMPAG,0–8 h or the AUCMPAG,0–8 h /AUCMPAG,0–8 h ratio suggest that the intermediate adenovirus dose results in complete or nearly complete reconstitution. The conflict also is apparent for the in vitro measurements. Estimations of the UGT1A content suggest that the ID dose conditions are closest, whereas the mycophenolic acid UGT activity data suggest that the HD dose condition is closest. Overall, the data suggest that the ID and HD doses result in proportional degrees of functional hepatic UGT reconstitution that bracket the controls in our study. In addition, the ID and HD UGT adenovirus dose groups may be useful in and of themselves to assess the importance of different degrees of hepatic glucuronidating activity on a pharmacological or toxicological effect of interest, such as mycophenolic acid-induced gastrointestinal toxicity.

Using our model, it was possible to directly show for the first time the effect of expressing specific UGT in liver on the glucuronidation of drugs in vivo or in vitro. Our data suggest that it could be applied for expression of any UGT1A or likely UGT2B enzyme as well (although the latter are not deficient in Gunn rats). One advantage of the multi-UGT gene therapy approach is that coexpression of UGT1A1 with other UGT1A enzymes allows the effects of UGT1A expression on drug metabolism and toxicity to be assessed under conditions when serum bilirubin levels are normalized. Use of the native Gunn rat model is complicated by the severe hyperbilirubinemia present in this mutant strain. Although serum bilirubin levels were not measured in our study, the correction of the jaundiced phenotype of the Gunn rats at the HD and ID adenovirus dose levels was apparent by several outward indicators, most notably the normalization of the serum.
color, normally a straw yellow in homozygous Gunn rats. At the low adenovirus dose level, the yellow phenotype was not fully corrected but was reduced. The latter findings are consistent with the observed bilirubin UGT activity data shown in Table 2.

The present study reports for the first time the marked sensitivity of the Gunn rat to toxicity from a single dose of mycophenolic acid. This was an unexpected finding, and the experimental design was not set up to address whether the origin of the toxicity was gastrointestinal. Although no diarrhea was observed in our study, signs point to the toxicity being gastrointestinal in origin based on visual inspection of the lower intestine of the naïve Gunn rat group during our necropsy procedure. The 80-mg/kg dose of mycophenolic acid used in this study was selected because daily exposure to this level in rats with normal UGT1A gene function (Sprague-Dawley) leads to the development of diarrhea with an onset of 3 to 5 days depending on the gender (S. Stern, M. Tallman, K. Miles, J. Ritter, R. Dupuis, and P. Smith, unpublished observations). In terms of human clinical relevance, the 80-mg/kg dose is equivalent to approximately one-third of a human clinical dose (1 g) expressed per unit body surface area. Further work will be needed to establish an appropriate dose range and frequency to study the diarrhea-inducing effect of mycophenolic acid in the genetically modified Gunn rats and their heterozygote counterparts.

A diminishment in the intensity of the toxic response to the single mycophenolic acid treatment was observed in the adenovirus-treated Gunn rats, and this effect was adenovirus dose-dependent. This observation provides evidence that hepatic UGT1A6 themselves are fundamental determinants of mycophenolic acid-induced toxicity. Furthermore, the impact of the adenovirus therapy on serum mycophenolic acid concentration, systemic exposure to mycophenolic acid, and other parameters of mycophenolic acid metabolism show the importance of hepatic mycophenolic acid glucuronidation for the overall pharmacokinetics of mycophenolic acid. Two surprising observations are (1) that the intermediate and high adenovirus-dose Gunn rats still exhibited demonstrable evidence of toxicity from mycophenolic acid, as indicated by diminished food consumption during the first 24-h period after the mycophenolic acid dose; and (2) that even at the low adenovirus dose, plasma mycophenolic acid glucuronide concentrations are restored to levels comparable with or even exceeding the heterozygote controls. The first observation occurred despite the pharmacokinetic data indicating full or near-full correction of the hepatic mycophenolic acid glucuronidation deficiency and the microsomal analysis data, which indicated near-complete or more than complete reconstitution of enzyme levels. Gunn rats may be higher biliary excretors and increased biliary excretion of MPAG would be expected to increase exposure of the gastrointesti- nal tract to mycophenolic acid, subsequent to its hydrolysis. Future studies performing biliary excretion studies could address this possibility. Alternatively, the absence of intestinal mucosal UGT expression in the adenovirus-treated Gunn rats may predispose these animals to the development of toxicity. The second observation is surprising, given that UGT levels in the LD dose group remain much below the level of the heterozygote (Fig. 3). The reasons for this remain unexplained but could involve alterations in the balance of sinusoidal versus canalicular glucuronide efflux transporters. Ogawa et al. (2000) have shown that Gunn rats exhibit elevated levels of Mrp3, a glucuronide transporter expressed on the apical surface of hepatocytes.

In summary, the present study shows that a combination of rat UGT-encoding adenoviruses could be administered to Gunn rats to reconstitute a range of UGT1A1, UGT1A6, and UGT1A7 hepatic expression profiles. UGT expression using this system was found to be dose-dependent and resulted in functional enzymes allowing for the pharmacokinetic analysis of mycophenolic acid in Gunn rats. This model system seems to have considerable potential for mechanistic studies to understand the relationship of hepatic and extrareplicative UGT expression levels to the onset and progression of toxicities from cytotoxic drugs, such as mycophenolic acid or genotoxic drugs. The technique may also be useful to model disease or physiologic states associated with altered rates of hepatic glucuronidation, as well as to investigate the activities of recombinant UGT expressed in their normal microsomal environments in vivo. Finally, it may also be easily applied for the purpose of humanizing the UGT1A system of rats to assess specific characteristics of human UGT1A enzymes in drug metabolism and toxicity.

Acknowledgments

We thank Melanie Tallman for valuable input during these studies.

References

Li Q, Murphree SS, Willer SS, Bolli R, and French BA (1998) Gene therapy with...


Address correspondence to: Joseph K. Ritter, Virginia Commonwealth University Medical Center, Department of Pharmacology and Toxicology, Medical Sciences Building, 1217 East Marshall Street, Room 531, Richmond, VA 23298. E-mail: jkriter@vcu.edu