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ADENOVIRUS-MEDIATED GENE THERAPY TO RESTORE EXPRESSION AND FUNCTIONALITY OF MULTIPLE UDP-GLUCURONOSYLTRANSFERASE 1A ENZYMES IN GUNN RAT LIVER

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Abbreviations:

AUC – area under the plasma concentration-time curve

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C_{max} – peak plasma concentration

HD – high adenovirus dose

HPLC – high performance liquid chromatography

ID- intermediate adenovirus dose

LD- low adenovirus dose

MMF – mycophenolate mofetil

MPAG – 7-O-mycophenolate glucuronide

OD – optical density units

RLM – rat liver microsomes

T_{max} – time at peak plasma concentration

UGT – Uridine diphosphate-glucuronosyltransferases

$T_{1/2\alpha}$ – Plasma elimination half life, early phase

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 MPA glucuronide concentration-time curves after mycophenolic acid administration (80 mg/kg, i.p.). Functional reconstitution was also apparent in the correction of the mycophenolic acid $T_{1/2\alpha}$ and the $AUC_{MPA,0\text{-}8hr}$:$AUC_{MPAG,0\text{-}8hr}$ ratio. Twenty-four hours post-mycophenolic acid, severe signs of toxicity were noted in the naïve Gunn group including reduced food consumption. The effect on food consumption was reduced but not completely prevented in the UGT adenovirus-treated Gunns. *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.

Introduction

Mycophenolate mofetil (MMF, Cellcept®) is now considered one of the first line drugs for maintenance immunosuppression therapy in transplant patients (Sintchak and Nimmesgern, 2000). Use of this drug, however, be limited by one of its common side effects, the development of gastrointestinal toxicity, which usually manifests as diarrhea presenting many weeks or months following the start of therapy. The occurrence of diarrhea is of clinical significance, because it necessitates dosage reductions for MMF which can negatively impact 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; Miles *et al.*, 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 man (Bullingham *et al.*, 1998) is the 7-O-glucuronide (MPAG), which is inactive as a DNA synthesis inhibitor. UGTs expressed at high levels in intestinal epithelial cells could be protective by keeping intracellular mycophenolic acid concentrations low.

Glucuronidation represents one of the major phase II biotransformation pathways in the detoxification of drugs, carcinogens, and dietary components. UGTs 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; Picard *et al.*, 2005; Miles *et al.*, 2005). Of the three rat UGT1A enzymes identified as having mycophenolic acid glucuronidating activity (UGT1A1, 1A6, and 1A7), the 1A1 and 1A7 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; Miles *et al.*, 2006), a strain that is deficient in UGT1A enzymes due to 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 UGTs as modulators of drug toxicity is limited. To circumvent this limitation, we considered the possibility of using replication-defective adenoviruses to correct hepatic UGT1As, leaving extrahepatic UGT1As deficient. Adenoviruses are currently being extensively investigated as potential gene therapy vectors and have been demonstrated 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; Amalfitano, 2004; Herrmann *et al.*, 2004; Toietta *et al.*, 2005). Although the available data for adenovirus-mediated UGT gene therapy demonstrate the correction of a single enzyme (UGT1A1), it remains unclear if 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 UGTs expressed in rat liver, UGT1A1, 1A6, and 1A7, could reconstitute a normalized profile for each of these enzymes in Gunn rat liver. The specific objectives were to obtain evidence for functional reconstitution using both *in vivo* and *in vitro* measures. For the *in vivo* assessment, the plasma kinetics of mycophenolic acid and MPAG were determined following a single injection of mycophenolic acid (80 mg/kg, i.p.). For the *in vitro* measures, liver microsomes were prepared and analyzed for UGT activities towards bilirubin, 4-nitrophenol, and mycophenolic acid, as well as for relative levels of expressed UGT1A1, 1A6, and 1A7 proteins.

Methods

Chemicals and reagents.

All chemicals used in this study were of the highest grade available. Organic solvents were purchased from Fisher Scientific (New Lawn, NJ). Mycophenolic acid, UDP-glucuronic acid, and other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. The preparation and characterization of MPAG was described previously (Wiwattanawongsa *et al.*, 2001). Suprofen was from the Pharmaceutical Research Institute (Springhouse, PA).

Adenovirus amplification and purification.

The adenoviruses used in this study were obtained from the Massey Cancer Center viral production core facility at Virginia Commonwealth University. All are based on the replication-defective strain Ad5*dl*324 (Pandak *et al.*, 2001), which carries deletions of the E1 and E3 early genes. Complete coding regions for rat UGT1A1, 1A6, 1A7, and 1A10 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 Ad5*dl*324. The resulting pZeroTG/CMV-UGT plasmids were co-transformed with ClaI-linearized pTG/CMV (containing the entire Ad5*dl*324 genome) into *E. coli*. Resulting recombinant plasmids were screened for inserts prior to transfection into human embryonic kidney 293B cells (American Type Culture Collection, Manassas, VA) to propagate and amplify the virus preparations as previously described (Pandak *et al.*, 2001). Briefly, 293B cells were grown to confluence and infected with the individual adenoviruses described above. Cells were harvested 72 hr post-infection and spun at 2,700 x g for 10 min at 4°C. The

cell pellet was washed by resuspension in phosphate-buffered saline and recentrifuged. After a final resuspenson, the cells were submitted to five consecutive freeze-thaw cycles and were centrifuged at 9,400 x g for 5 min at 4°C. The recovered supernatant was layered onto a CsCl gradient and centrifuged for 2 hr at 155,000 x g 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 $MgCl_2$; and 10% glycerol). The number of virions per mL 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% sodium dodecyl sulfate, and 1 mM EDTA), where 1 OD_{260} unit = 1 x 10^{12} virions per mL (Herrmann *et al.*, 2004). adenovirus stocks were aliquotted and stored at –80°C until use.

Animal care and housing.

All 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 body weight) were obtained from a Gunn rat colony established on-site and maintained two per cage using a standard 12 hour light-dark cycle. Room temperature and humidity were maintained at 22°C and 21%, respectively. Animals were given free access to rodent chow (Harlan Tekiad Lab Diet LM-485) and water.

Animal treatments.

For this study, three groups of homozygous male Gunn rats (j/j genotype, 3 per group) were infected at different dose levels with a fixed ratio mixture of adenoviruses encoding rat UGT1A1, 1A6, and 1A7. 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 Gunns) received 0.26 $OD_{260\ units}$ total consisting of 0.2 $OD_{260\ units}$ adenovirus-CMV-r1A1, 0.05 $OD_{260\ units}$ adenovirus-CMV-r1A6, and 0.01 $OD_{260\ units}$ adenovirus-CMV-r1A7 (or a total of 2.6 x 10^{11} virions). The intermediate and low dose groups (ID and LD Gunns) received 0.13 and 0.0625 $OD_{260\ units}$ total of the fixed ratio mixture, respectively. Two control groups were used in this study: uninfected homozygous (j/j) Gunn rats and heterozygous Gunns infected with a high dose (0.26 $OD_{260\ units}$) of adenovirus coding for a negative control UGT (i.e., rat UGT1A10, which shows no detectable activity towards mycophenolic acid) (Miles *et al.*, 2005). Five days post-infection, when adenovirus-expressed UGT has reached maximum (data not shown), animals received a single dose of mycophenolic acid (80 mg/kg) delivered intraperitoneally. Serial blood samples (100 μL each) were collected from the tail vein at 15, 30, 60, 90, 120, 240, 360, and 480 min post mycophenolic acid administration. Blood was collected in Microtainer® EDTA tubes and spun at 16,000 x g for 5 min at 4°C. The resulting plasma was transferred to a microfuge tube and stored at –20°C until analysis. After dosing the animals were housed individually and observed over the next 48 hr. 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 HPLC procedure with some modification (Wiwattanawongsa *et al.*, 2001). Briefly, suprofen internal standard (5 µL of a 0.5 µg/µL solution) was mixed with 20 µL of plasma followed by 4µL of concentrated perchloric acid to precipitate proteins. The samples were then placed on ice for 5 min and centrifuged at 16,000 x g 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 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 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 µg/mL 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 non-compartmental model using

WinNonlin™ pharmacokinetic software v. 2.0 (Pharsight Corporation, Palo Alto, CA). Statistical analyses were performed using one-way analysis of variance (ANOVA) 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). Briefly, livers were homogenized in five volumes of ice-cold 0.25M sucrose using glass Potter-Elvehjem tubes fitted with Teflon pestles. After removing nuclear and mitochondrial fractions, samples were spun at 155,000 x g for 45 min at 4°C. Microsomes were then resuspended in 0.15M KCl and subjected to a second ultracentrifugation spin as described above. Final microsomal resuspensions were made in 0.1M 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 Biochemical, Rockford, IL).

Mycophenolic acid UGT activities were determined as previously described (Miles *et al.*, 2005). Briefly, microsomal reactions contained: 75 mM Tris-Cl (pH 7.45), 10 mM $MgCl_2$, 6 mM saccharic acid 1,4-lactone (prepared fresh), and 1 mM mycophenolic acid. Microsomes were activated with 50μg alamethicin/mg protein for 15 min on ice, prior to 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 UGTs.

The antibodies for Western immunoblot analysis were prepared in our laboratory. The generation of the rat UGT1A1 (anti-r1A1$_{29\text{-}162}$) and 1A7 (anti-r1A7$_{21\text{-}149}$) 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-r1A6$_{77\text{-}183}$) was prepared using a similar strategy. Antisera were tested for specificity against recombinant rat UGT1A (1A1-1A10 excluding 1A4 and 1A9) and UGT2B (2B2, 2B3, and 2B8) proteins by Western blot analysis. Briefly, twenty micrograms of crude cell membrane protein from recombinant rat UGT-expressing HepG2 cells were 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 (ECL) (Amersham Biosciences, Piscataway, NJ). Resulting images were captured on film and analyzed.

Western immunoblot analyses of naïve and infected Gunn RLMs were performed as previously described with minor modifications (Ritter *et al.*, 1999; Guillemette *et al.*, 2000). Microsomal proteins were subjected to 7.5% SDS-PAGE in parallel to serial

dilutions of recombinant rat UGT1A1, 1A6, and 1A7 and transferred to a nitrocellulose membrane. UGTs 1A4 and 1A9 were not monitored because these UGTs are pseudogenes in rats. Blots were blocked in 5% milk suspension then probed for rat UGT1A1, 1A6, and 1A7 using lab-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 ANOVA 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 μL) was added to 0.65 mL of reconstituted ALT assay reagent at room temperature, and the change in absorbance at 340 nm due to NADH consumption was monitored using a Shimadzu UV160U spectrophotometer. The activity was calculated using 6.22 as the millimolar absorptivity of NADH. A commercially available reference sample (Catatrol™I, 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 Fig. 1. For mycophenolic acid, two phases were apparent: an early (alpha) phase from 0 to 2 hours and a late phase from 2 to 8 hours. In at least three of the groups (HD and ID Gunns and the control infected heterozygotes), a distinct secondary rise in plasma mycophenolic acid concentrations was observed. These data are consistent with the late phase being due to enterohepatic cycling of mycophenolic acid. Differences in both the early and late phases of mycophenolic acid kinetics were evident between the naïve Gunn rat and treated Gunn rat groups, which are consistent with dose-dependent correction of the deficient mycophenolic acid glucuronidation phenotype by UGT adenovirus.

In the control Gunn rat group, a higher peak concentration of mycophenolic acid (Table 1, $p<0.001$ vs. all groups) and a slower rate of decline in early plasma mycophenolic acid concentrations ($T_{1/2,\alpha}$=1.6 hrs, $p<0.05$ vs 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 $AUC_{MPAG,\ 0\text{-}8\ hr}$ of all groups (22.2 μg/mL•h, $p < 0.001$ compared to all groups).

Among the UGT adenovirus-treated Gunn rat groups, an inverse relationship between UGT adenovirus dose and $AUC_{MPA,\ 0\text{-}8\ hrs}$ 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 vs. 442 μg/mL·hr group), suggesting an essentially complete correction of the mycophenolic acid glucuronidation deficiency in the HD group. Interestingly, the HD Gunns exhibited significantly greater $AUC_{MPAG,\ 0\text{-}8\ hr}$ compared to 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 $AUC_{MPA,\ 0\text{-}8\ hr}$, C_{max}, and $T_{1/2,\alpha}$ and decreased $AUC_{MPAG,\ 0\text{-}8hr}$. Restoration of the imbalance between mycophenolic acid and MPAG exposures in Gunn rats relative to heterozygotes (AUC_{MPA} : AUC_{MPAG} 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 AUC_{MPA} : AUC_{MPAG} 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 to the HD group (over corrected) and LD groups (significantly under corrected).

Toxicological observations.

For the two day period following 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 naïve Gunn rats by 24 hours post-dose exhibited severe signs of toxicity (ruffled fur, hunched posture, and lack of any locomotor response to stimulation) and were therefore euthanized after 24 hours. In the groups of UGT adenovirus-treated Gunns, 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 hour period prior to the mycophenolic acid treatment, all groups exhibited comparable rates of food consumption (~60-70 g/kg body weight, 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 groups compared to heterozygotes). Differences across groups in the magnitude of this effect were evident. The effect was most pronounced in the naïve Gunn and LD Gunn groups and was significantly attenuated but not completely reversed in the HD Gunn. 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 naïve Gunns (data not shown), consistent with the decreased food consumption. During the second 24 hour post-dose period, signs of recovery from the acute mycophenolic acid-induced toxicity were apparent in the UGT adenovirus Gunn groups.

In vitro measures of adenovirus reconstitution of hepatic UGT1As

Liver and colon microsomes prepared from the naïve and adenovirus-infected Gunns and heterozygotes (2 days post-mycophenolic acid dose) were assayed for their glucuronidating activities towards mycophenolic acid. In addition, the liver microsomes were assayed for bilirubin UGT (a 1A1-dependent marker), and 4-nitrophenol (an overlapping but primarily UGT1A6 substrate). As previously reported (Miles *et al.*, 2005), homozygous Gunn RLMs showed a deficiency in mycophenolic acid UGT activity ($p<0.001$ versus control j/+) (Table 2). Treatment of rats with increasing doses

of UGT-encoding adenovirus resulted in dose-dependent increases in activity towards all three substrates. The average mycophenolic acid glucuronidation rate for the HD Gunn RLMs 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, while LD group microsomes did not show an increased rate of mycophenolic acid glucuronidation when compared to naïve controls.

Increases in the relative UGT1A1, 1A6, and 1A7 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) ($p<0.05$). As compared to infected heterozygote controls, RLM prepared from this group contained 2.5-, 2.0-, and 2.0-fold more 1A1, 1A6, and 1A7, respectively. The ID Gunn samples had roughly similar amounts of 1A1, 1A6, and 1A7 compared to 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 1A1, 1A6, and 1A7 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).

Mycophenolic acid 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 acid UGT

activity. These data demonstrate that, in contrast to liver, the adenovirus virus does not effectively transduce intestinal or kidney cells following 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 +/- standard error (in units of U/L) were 74±45, 16±3, 33±15, 33±19, and 46±36 for the HD Gunn, ID Gunn, LD Gunn, naïve 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 demonstrate that combination gene therapy can be applied to restore or normalize the expression of multiple UGTs 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 to, or higher than, that of controls (i.e., heterozygote j/+ 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 UGTs 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 UGTs in hepatotoxicity and thyroid hypertrophy 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 due to the adenovirus being selectively taken up and expressed in hepatocytes compared to extrahepatic tissues such as intestine.

Of specific interest to our laboratory is the role of hepatic versus intestinally-expressed UGTs 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, and 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 $AUC_{MPA,0\text{-}8hr}$ 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 $AUC_{MPAG,0\text{-}8hr}$ or the $AUC_{MPA,0\text{-}8hr}$:$AUC_{MPAG,0\text{-}8hr}$ 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 demonstrate for the first time the effect of expressing specific UGTs 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 co-expression 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-Dawleys) leads to the development of diarrhea with an onset of 3-5 days depending on the gender (Stern *et al.*, 2006). 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 UGTs 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 demonstrate 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-hr period following the mycophenolic acid dose, and (2) that even at the low adenovirus dose, plasma mycophenolic acid glucuronide concentrations are restored to levels comparable to 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 MPAG excretors, and increased biliary excretion of MPAG would be expected to increase exposure of the gastrointestinal 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 demonstrates that a combination of rat UGT-encoding adenoviruses could be administered to Gunn rats in order to reconstitute a range of 1A1, 1A6, and 1A7 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 appears to have considerable potential for mechanistic studies to understand the relationship of hepatic and extrahepatic 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 UGTs 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 in order to assess specific characteristics of human UGT1A enzymes in drug metabolism and toxicity.

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Footnotes

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Legends for Figures

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 naïve (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 minutes. Plasma was analyzed for mycophenolic acid and MPAG by HPLC. Each value represents the mean ± SE of 3 rats.

Fig. 2. The effect of mycophenolic acid on food consumption by adenovirus-infected and naive Gunn rats. Food consumption for the pre-dose period and the post-dose periods corresponding to 0-24 hrs and 24-48 hrs was determined by weighing. The data represent the average +/- SE of food consumption expressed in units of g per kg body weight. *** $p<0.001$ as compared to j/j, ‡ $p<0.001$ as compared to j/+.

Fig. 3. Relative contents of UGT1A1, 1A6, and 1A7 in liver microsomes from adenovirus-infected Gunn and control-infected heterozygote rats. Liver microsomes prepared from the different groups of adenovirus-infected Gunn and heterozygote rats were analyzed for UGT1A1, 1A6, and 1A7 protein by Western blotting as described under Materials and Methods. Each bar represents the average relative level of UGT

detected ± SE in three different samples. Recombinant UGT proteins were used as standards. The most intense signal detected was assigned a value of 1.0 and was used to normalize the remaining signals. Note that the relative levels shown do not reflect the natural abundance of the UGTs (UGT1A1 > UGT1A6 > UGT1A7). * $p<0.05$ as compared to j/+, † $p<0.05$ as compared to HD.

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Table 1

Plasma Mycophenolic acid and MPAG Kinetic Parameters for Naive and Adenovirus-Infected Gunn Rats After a Single Mycophenolic acid Administration

Mycophenolic acid (80 mg/kg) was administered intraperitoneally, and blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 hours post-dosing. Plasma mycophenolic acid and MPAG concentrations were determined and the various kinetic parameters shown were calculated using WinNonLin (non-compartmental model). AUC_{MPA}, mycophenolic acid area under the plasma concentration-time curve; C_{max}, peak plasma concentration; $T_{1/2,\alpha}$, plasma elimination half-life-alpha; T_{max}, mean peak time; MPAG area under the plasma concentration-time curve; *** $p<0.001$ as compared to j/j; † $p<0.05$ as compared to j/j; ‡ $p<0.001$ as compared to j/+. Each value representative of 3 rats ± SE.

Pharmacokinetic Parameters	HD Gunn ($0.26\ OD_{260\ units}$)	ID Gunn ($0.13\ OD_{260\ units}$)	LD Gunn ($0.065\ OD_{260\ unit}$)	Control *j/+* Gunn ($0.26\ OD_{260\ units}$)	(uninfected)
$AUC_{MPA,\ 0\text{-}8\ hr}$ (μg/mL•h)	477 ± 60 ***	568 ± 156 ***	808 ± 16	442 ± 59 ***	1041 ± 210
$AUC_{MPA,\ 4\text{-}8\ hr}$ (μg/mL•h)	216 ± 18	240 ± 62	325 ± 16	165 ± 19	374 ± 106
C_{max} (μg/mL)	213 ± 30***	205 ± 32***	254 ± 18 ***	231 ± 34 ***	359 ± 10
T_{max} (μg/mL)	15 ± 0	15 ± 0	15 ± 0	15 ± 0	20 ± 8
$T_{1/2,\alpha}$ (h)	0.71 ± 0.11†	1.06 ± 0.13‡	1.31 ± 0.15	0.53 ± 0.01	1.55 ± 0.18 ‡
CL/F	0.41 ± 0.07***	0.30 ± 0.06***	0.16 ± 0.01	0.35 ± 0.05***	0.11 ± 0.02
V/F	0.40 ± 0.04	0.46 ± 0.11	0.30 ± 0.02	0.26 ± 0.05	0.26 ± 0.05
$AUC_{MPAG,\ 0\text{-}8\ hr}$ (μg/mL•h)	323 ± 24*** ‡	171 ± 11***	94 ± 11*** ‡	183 ± 10***	22 ± 3
C_{max} (μg/mL)	199 ± 41*** ‡	100 ± 8	65 ± 8	46 ± 7	18 ± 3
T_{max} (μg/mL)	30 ± 0	40 ± 10	60 ± 0	110 ± 66	60 ± 0
AUC_{MPA} : AUC_{MPAG}	1.4	3.2	8.2	2.7	44.9

Table 2

***In vitro* UGT activities of adenovirus-infected and naive Gunn rats.**

UGT activities were determined toward the indicated substrates in reactions containing 1 mg/mL of RLMs and 50 μg alamethicin/mg protein. Aglycone concentrations were mycophenolic acid (1 mM), bilirubin (0.33 mM), and 4-nitrophenol (0.1 mM). Values expressed represent the average rate (nmol/mg/min) ± SE of n=3 rats. ** $p<0.01$ as compared to j/j, * $p<0.05$ as compared to j/j ‡ $p<0.001$ as compared to j/+. n.d., not detectable.

	UGT activities (nmol/mg/min)		
Group	MPA	Bilirubin	4-Nitrophenol
HD Gunn	4.04 +/- 0.64**‡	1.14 +/- 0.07**‡	0.22 +/- 0.07*
ID Gunn	1.62 +/- 0.80	0.46 +/- 0.09**	0.08 +/- 0.06
LD Gunn	0.68 +/- 0.08	0.06 +/- 0.04	0.02 +/- 0.01
Control-infected j/+	2.72 +/- 0.16**	0.37 +/- 0.06**	0.22 +/- 0.04**
Naïve j/j	1.04 +/- 0.13	n.d.	0.02 +/- 0.01

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Fig. 1

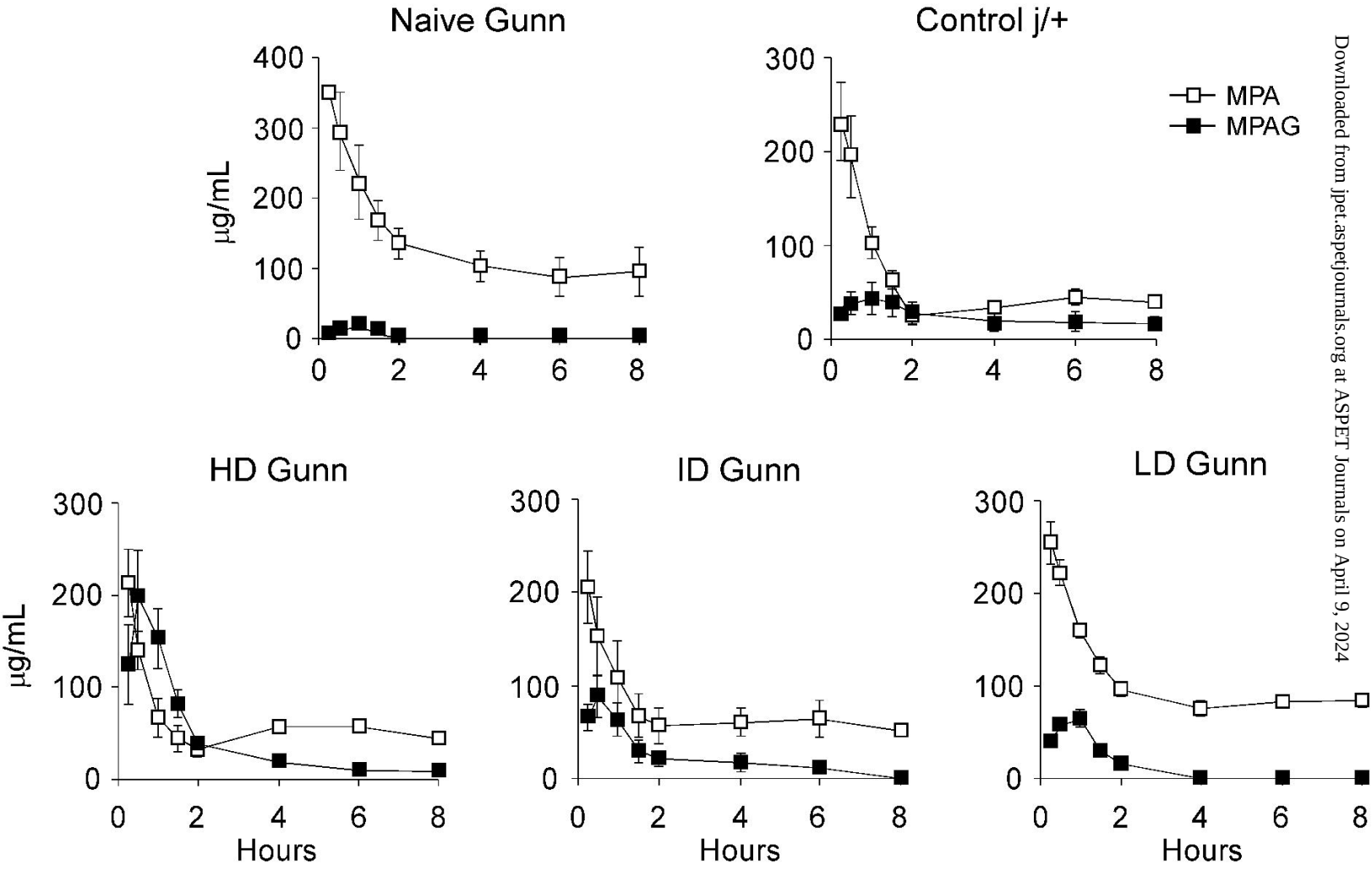

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Fig. 2

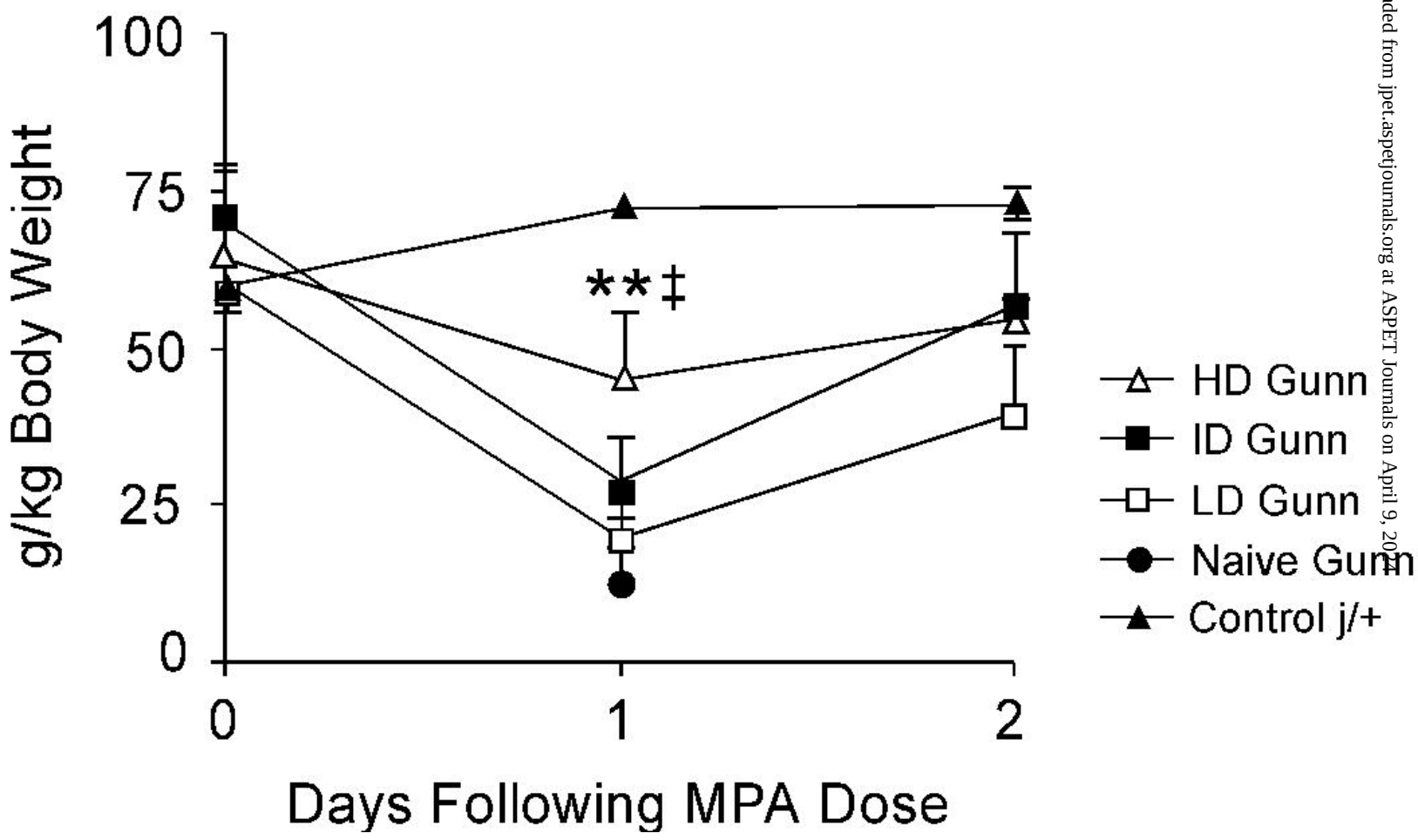

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Fig. 3