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PHARMACOKINETICS AND DYNAMICS OF MYCOPHENOLATE MOFETIL AFTER SINGLE DOSE ORAL ADMINISTRATION IN JUVENILE DACHSHUNDS

Megan Grobman, DVM¹, Dawn M. Boothe, DVM, MS, PhD, DACVIM, DACVCP², Hansjörg Rindt, PhD¹, Baye G. Williamson, DVM¹, Martin L. Katz, PhD³, Joan R. Coates, DVM, MS, DACVIM (Neurology)¹, and Carol R. Reinero, DVM, DACVIM, PhD^{1,*}

¹Department of Veterinary Medicine and Surgery, University of Missouri College of Veterinary Medicine

²Department of Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine Auburn University, Auburn Alabama

³Mason Eye Institute, University of Missouri School of Medicine, Columbia Missouri

Abstract

Mycophenolate mofetil (MMF) is recommended as an alternative/complementary immunosuppressant. Pharmacokinetic and dynamic effects of MMF are unknown in young-aged dogs. We investigated the pharmacokinetics and pharmacodynamics of single oral dose MMF metabolite, mycophenolic acid (MPA), in healthy juvenile dogs purpose-bred for the tripeptidyl peptidase 1 gene (*TPP1*) mutation. The dogs were heterozygous for the mutation (non-affected carriers). Six dogs received 13mg/kg oral MMF and 2 placebo. Pharmacokinetic parameters derived from plasma MPA were evaluated. Whole blood mitogen-stimulated T cell proliferation was determined using a flow cytometric assay. Plasma MPA C_{max} (mean $\pm$ Stdev, 9.33 ± 7.04 $\mu\text{g/mL}$) occurred at <1 hr. The $AUC_{0-\infty}$ (mean $\pm$ Stdev, 12.84 ± 6.62 h* $\mu\text{g/mL}$), MRT_{inf} (mean $\pm$ Stdev, 11.09 ± 9.63 min), $T_{1/2}$ (harmonic mean $\pm$ Pseudostdev 5.50 ± 3.80 min), and k/d (mean $\pm$ Stdev, 0.002 ± 0.001 1/min). Significant differences could not be detected between % inhibition of proliferating CD5+ T lymphocytes at any time point ($P=0.380$). No relationship was observed between MPA concentration and % inhibition of proliferating CD5+ T lymphocytes ($R=0.148$, $P=0.324$). Pharmacodynamics do not support the use of MMF in juvenile dogs at the administered dose based on existing therapeutic targets.

Keywords

Mycophenolic Acid; Flow Cytometry; $AUC_{0-\infty}$; Lymphocyte

*Corresponding author: reinero@missouri.edu, Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, 900 East Campus Drive, Columbia, MO 65211, USA.

^bWedgewood Compounding Pharmacy, Swedesboro NJ; 200 mg/mL

ⁿWinNonLin®, Pharsight Corporation, Mountain View, California (Recently purchased by Certara, St. Louis, MO)

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Mycophenolate mofetil (MMF), an immunosuppressant used in human medicine for solid organ transplantation, is being increasingly applied to treatment of immune-mediated disease.(Staatz & Tett, 2007; Kuypers, Le Meur et al., 2010; Abd Rahman, Tett et al., 2013) Mycophenolate has been popular in human medicine due to rapidity of onset and readily available intravenous and oral formulations.(Allison & Eugui, 2000) The use of MMF as an immunosuppressant in veterinary medicine is based on retrospective studies in dogs and cats.(Yuki, Sugimoto et al., 2007; Abelson, Shelton et al., 2009; Dewey, Cerda-Gonzalez et al., 2010; Bacek & Macintire, 2011) The active metabolite, mycophenolic acid (MPA), is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a critical enzyme in the *de novo* guanosine nucleotide synthesis pathway required for proliferation of activated lymphocytes.(Bullingham, Nicholls et al., 1998; Staatz & Tett, 2007) Inhibition of IMPDH results in predominantly lymphocyte-specific immunosuppression inhibiting cell-mediated and humoral immune responses.(Langman, Shapiro et al., 1996; Allison & Eugui, 2000; Sankatsing, Prins et al., 2008; Abd Rahman, Tett et al., 2013)

Selectivity for a specific immunosuppressive protocol depends upon clinician decision-making, patient's clinical status and financial considerations. In accordance with guidelines for specific diseases, a common protocol is daily administration of prednisone at an immunosuppressive dose. Adverse effects of high-dose corticosteroids include gastric ulceration, steroid-hepatopathy, muscle weakness, and iatrogenic hyperadrenocorticism. Additionally, monotherapy with glucocorticoids is frequently inadequate for disease control. Alternative immunosuppressive agents may be indicated and can limit the undesirable side-effects of corticosteroids. However, these drugs are not without risks including myelosuppression, hepatotoxicity, gastrointestinal disturbances and other drug-specific systemic effects; therefore regular monitoring of complete blood counts (CBC), serum biochemistry, and where possible, effective concentrations, are recommended.(Viviano, 2013) In pediatric animals immunosuppression poses additional challenges in part due to age related differences in drug metabolism, where existing safety, efficacy and toxicity data may lack accuracy when doses are extrapolated from studies in adult animals to younger immature dogs.(Adusumalli, Gilchrist et al., 1992; Tassinari, Benson et al., 2011)

There is a need for an effective immunosuppressant for juvenile/pediatric dogs in clinical and research settings. Non-infections, inflammatory brain diseases including steroid responsive meningitis-arteritis occur in juvenile/pediatric dogs and immunosuppression is required for successful treatment outcome.(Tipold & Schatzberg, 2010) Mycophenolate has been used successfully in human pediatric renal transplant patients, reflecting a possible therapeutic option in juvenile dogs requiring immunosuppression as part of their treatment regimen.(Downing, Pirmohamed et al., 2013; Dong, Fukuda et al., 2014) Administration of MMF has also enhanced the efficacy of gene therapy for Duchenne muscular dystrophy in adult dogs.(Shin, Yue et al., 2012) Based on these findings, MMF has been administered in combination with gene therapy in our *TPPI* null Dachshund model for neuronal ceroid lipofuscinosis (NCL). However, pharmacokinetic and dynamic information regarding MMF in juvenile/pediatric dogs is lacking.(Tipold & Schatzberg, 2010; Ellinwood, Ausseil et al., 2011; Shin, Yue et al., 2012)

Adverse effects of MMF in dogs include acute severe gastrointestinal toxicity.(Neerman & Boothe, 2003) This is similar to gastrointestinal signs in people receiving MMF.(Arns, 2007; Parfitt, Jayakumar et al., 2008) In humans, this is the most common cause for cessation of treatment, occurs in a non-dose dependent fashion, and has been reported long after commencing therapy.(Hood & Zarembski, 1997; Yu, Seidel et al., 1998; Staatz & Tett, 2014) In the authors' experience, gastrointestinal side-effects may be life-threatening in pediatric dogs.(Neerman & Boothe, 2003) Pharmacokinetic and pharmacodynamic evaluation is an important first step in establishing MMF as a viable immunosuppressant in this age cohort of dogs.

A pharmacodynamic assay used previously in the dog for MMF evaluated inhibition of IMPDH enzyme activity, with an IMPDH IC₅₀ of 200ug/mL in adult dogs(Langman, Shapiro et al., 1996). This assay measures an enzyme required for biosynthesis of purine nucleotides, with the assumption that that major mechanism for immunosuppression is inhibition of DNA synthesis of lymphocytes. Our laboratory has developed a highly sensitive, whole blood flow cytometric assay of lymphocyte proliferation which evaluates any mechanism by which MMF prevents lymphocyte proliferation.(Nafe, 2013; Bishop KA, 2016) In healthy dogs, the lymphocyte proliferation IC₅₀ for MPA was 106.3 ±157.7 nM (0.033 ± 0.05 ug/mL). (Bishop KA, 2016) Of note, this concentration is substantially lower than the previously published reports on the IMPDH IC₅₀ in adult dogs.(Langman, Shapiro et al., 1996) It is consistent with human studies and within a single dilution of the IC₅₀ for lymphocyte inhibition in a recent canine study.(de Lathouder, Gerards et al., 2002; Guzera, Szulc-Dabrowska et al., 2016) In a study of adult canine pharmacokinetics, a single dose of MMF led to blood concentrations of 10ug/mL, which is within the range of detection of our assay.(Lange, Mueller et al., 2008) The objectives of this study were to determine the pharmacokinetics of single oral dose MMF metabolite, MPA, in healthy juvenile miniature Dachshunds and to evaluate the pharmacodynamic effects of a single dose of MPA on T cell proliferation *ex vivo*.

Materials and methods

Animals

Eight (intact, 6 male, 2 female) age matched juvenile (120 ± 7 days) miniature longhaired Dachshunds (3.69 ± 0.84kg) from a purpose bred colony were studied.(Awano, Katz et al., 2006; Katz, Coates et al., 2014) The included dogs were unaffected heterozygotes for the tripeptidyl peptidase 1 gene (*TPPI*) mutation. The dogs were considered healthy based on physical examination, packed cell volume, serum total solids, blood smear, serum biochemical profile and urinalysis.(Faulks & Lane, 2003; von Dehn, 2014)

Dogs were sedated with intramuscular buprenorphine (0.01mg/kg) and dexmedetomidine (5–10 µg/kg) for placement of jugular catheters. Aseptic placement of 5 French 15 cm wire guided single lumen jugular catheters^a was performed in either the right or left jugular vein approximately 12 hours prior to the start of data collection. The catheters were secured and

^aCook medical, Bloomington, IN

heparin locked (100U/mL heparin sulfate). Sedation was reversed with atipamazole if clinically indicated.

Dogs received physical examinations prior to the start of sample collection, and at 12, and 24 hours post MMF administration. Dogs were housed in individual kennels. Catheters were heparin locked between the 12 hour and 24 hour collection times. The study was conducted with the approval of the Animal Care and Use Committee, University of Missouri, Columbia, Missouri, USA (protocol# 7848).

Mycophenolate Mofetil

Mycophenolate mofetil, was compounded from FDA approved 250mg capsules by Wedgewood Compounding Pharmacy^a in accordance with USP NF chapter <795>. The compounding MMF allowed specific dosing and the same formulation to be used amongst the study dogs. Mycophenolate mofetil was administered orally (13mg/kg) to 6 dogs after a 12 hour fast. Two dogs were untreated controls. The dogs were not fed for 2 hours after MMF administration to avoid influencing pharmacokinetic parameters [i.e., maximum MPA concentration (C_{max}) and time to maximum MPA concentration (T_{max})] associated with oral dosing. (Naesens, Verbeke et al., 2007) They were fed their commercial diet (Purina EN) after the 2 hour sample collection. Water was provided *ad libidum* at all times.

Blood Sampling

Blood was collected via the jugular catheters prior to dosing (0 min) and at 10, 20 and 50 min, and then 1, 1.5, 2, 3, 4, 6, 9, 12, and 24 hours after MMF administration. Collected blood at each time point was placed in tubes containing ethylene diamine tetra-acetate (EDTA; 1 ml) and/or lithium heparin (0.5 ml). Samples from EDTA tubes were used for pharmacokinetic analysis. Lithium heparin samples were used for pharmacodynamics analysis. Lithium heparin samples were not collected for the 10 min, 20 min, and 40 min time points. The EDTA samples were centrifuged within 1 hour and plasma was banked at -20°C prior to the mycophenolic acid pharmacokinetic assay.(Staatz & Tett, 2007) The jugular catheters were used and maintained as previously described.(Carnes, Axlund et al., 2011)

Mycophenolic Acid Assay

Samples were centrifuged for 10 minutes at 1900g within 2 hours of collection. Plasma was harvested and frozen at -20°C until analysis. Mycophenolic acid was detected and quantitated in canine plasma using a Food and Drug Administration human-approved immunoassay on a Siemens Dimension Xpand Plus integrated chemistry system.^c The assay exhibits very low (<1%) cross reactivity with the major (inactive) metabolite, mycophenolic acid glucuronide (MPAG) but does cross react with the active acyl glucuronide metabolite, resulting in MPA concentrations that are up to 40% higher compared to high performance liquid chromatography (HPLC) due to the detection of all active metabolites.(Shipkova, Schutz et al., 2000) The immunoassay was evaluated for matrix effects by testing pooled canine plasma samples spiked with mycophenolic acid at 2.5, 7.5, and 25.0 µg/mL. Mean

^cSiemens Healthcare, Erlangen, Germany

recoveries were 2.73 ± 0.058 , 8.13 ± 0.058 , and 25.67 ± 1.665 $\mu\text{g/mL}$, respectively. The assay was calibrated for canine plasma using the Siemens Dimension MPAT calibrator kit.^c A More Diagnostics® mycophenolic acid control kit was tested for quality assurance.^d The upper and lower limits of quantitation are 30.0 and 0.2 $\mu\text{g/mL}$, respectively. All controls predicted within $6.5 \pm 3.2\%$ (accuracy) whereas the coefficient of variation (precision) using the manufacturer's quality controls for the time period our samples were analyzed was less than 2 % for all 3 controls.

T Lymphocyte Proliferation Assay

T lymphocyte proliferation was evaluated using an assay previously validated in our laboratory against MPA. (Bishop KA, 2016) A 1:10 dilution of whole blood and cRPMI (RPMI 100 ml + FBS 10 mL + 100 μL diluted β -mercaptoethanol); with or without mitogens was plated (12 well flat bottom plate)^e in each of two wells at a final volume of 1250 μL per well. Mitogens added to one well included concanavalin A (ConA) (20 $\mu\text{g/mL}$) and LPS (15 $\mu\text{g/mL}$). (Nafe, 2013; Nafe, Dodam et al., 2014) Following a 4 day incubation at 37° C and 5% CO₂, red cells were lysed (ACK lysis buffer: 8.26 g NH₄Cl + 1.0g KHCO₃ + 0.037g Na₂EDTA in 1.0 liter deionized distilled H₂O) and washed twice with phosphate buffered saline (PBS). After resuspension in 1 mL PBS, leukocytes were stained with 1 μL of Fixable Viability Dye (eFluor 780)^f for 30 minutes at 4°C in the dark. Cells were washed twice in flow stain buffer (FSB; 950 mL PBS + 50 mL heat inactivated fetal bovine serum + 0.9 g Na Azide) and transferred to a 96 well round bottom plate^g in a final volume of 200 μL . The plate was centrifuged at 350 X g for 5 min and followed by a “flick” to remove supernatant. Cells were stained using 2.5 μL of an anti-canine pan T cell marker CD5 perCP-eFluor 710 antibody^h in 50 μL FSB for 15 min on ice in the dark. After two additional washes, 100 μL of an intracellular staining buffer (fixation/permeabilization concentrateⁱ and diluent^j) prepared according to manufacturer's instructions was added to each well and the plate was incubated for 30 minutes at 4° C in the dark. To study proliferation, washed cells were stained with anti-mouse/rat Ki-67 FITC antibody^k for 1 hour on ice in the dark. Washed cells were resuspended in 400 μL FSB for analysis. Controls included unstained cells and One Comp eBeads^l with a perCP eF710 antibody and with a FITC antibody as single positive controls to set compensation on the flow cytometer.

Analysis was performed using a CyAn ADP Flow Cytometer^m. A minimum of 20,000 gated events were collected with the gate set on lymphocytes on a plot of forward scatter (FSC) versus side scatter (SSC) (Figure 1a). Live cells were identified as cells staining negative for the fixed viability dye (i.e., excluding the dye) on a plot of eFluor780 versus SSC (Figure 1b). The combined gates of live cells and lymphocytes were then applied to a plot of Ki-67

^dMore Diagnostics, Los Osos, CA

^eFalcon, Corning, NY; 353043

^fE-bioscience, San Diego, CA

^gSigma-Aldrich, St. Louis, MO; Costar 3799

^hE-Bioscience, San Diego, CA; clone YKIX322.3

ⁱE-Bioscience, San Diego, CA; cat 00-5123

^jE-Bioscience, San Diego, CA; cat 00-5223

^kE-Bioscience, San Diego, CA; clone SolA15

^lE-Bioscience, San Diego, CA; cat 01-1111

^mBecton, Dickinson and Company, Franklin Lakes, New Jersey

FITC versus CD5 eFluor 710. Cells staining positive for both markers in the upper right quadrant were live, proliferating CD5+ lymphocytes in the G1, S, G2 and M phase of the cell cycle. (Figure 1c and 1d). Data were reported as percent proliferating cells after stimulation, calculated by subtracting the % CD5+ Ki-67+ cells in media alone (unstimulated) from the % CD5+ Ki-67+ cells stimulated with mitogens. Additionally, the percent inhibition was calculated for each time point after MMF administration:

$$\text{Percent inhibition} = [1 - (\text{treatment} / \text{pretreatment})] \times 100$$

“Pretreatment” refers to results obtained from stimulated blood without MPA at time=0 min and “treatment” refers to results from stimulated samples at various time points after administration of MPA. The same calculation was applied to control dogs to evaluate for intra-individual assay variation.

Pharmacokinetics, Pharmacodynamics, and Statistical Analysis

Pharmacokinetic analysis –Mycophenolic acid plasma concentration versus time curves were subjected to noncompartmental pharmacokinetic analysis using a linear log-trapezoidal method to determine area under the curve $AUC_{0-\infty}$), which was determined to infinity (Phoenix WinNonlin®; v.6.4, Certara USA, Inc. 100 Overlook Center, Suite 101, Princeton, NJ 08540 USA). In the absence of intravenous administration, the actual maximum concentration (C_{max}) was reported at the time to maximum concentration (T_{max}); the lowest concentration (C_{min}) and the time it occurred (T_{min}) also was reported. Further, disappearance rather than elimination rate constant (k_d) and half-life ($T_{1/2}$), which were determined by best-fit, were reported and clearance (CL) and volume of distribution (VD) were reported corrected for bioavailability (F; VD/F and CL/F). Other parameters reported included mean residence time (MRT). (Table 1).

Because of the small number of dogs the data were evaluated non-parametrically. Descriptive statistics were performed where appropriate. Statistical analysis of differences in T cell proliferation between time points was performed using a repeated measures ANOVA based on Ranks. Correlations between MPA concentrations and inhibition of CD5+ T cell proliferation were evaluated by Pearson product moment correlation coefficients.

Results

Adverse effects

All 6 dogs appeared to tolerate oral administration of a single dose of MMF with no visible indicators of adverse events. All dogs, including the 2 untreated control dogs, had normal vital parameters and physical examinations at the 0, 12 and 24 hr time points.

Pharmacokinetics

The pharmacokinetic data in 6 treated dogs including mean $\pm$ Stdev, CV, and the spread of the data around the mean are presented in Table 1. The percent of the area of the curve extrapolated from the last quantifiable time point was $30 \pm 21\%$. After oral administration of MMF, neither C_{max} (mean $\pm$ Stdev, $9.33 \pm 7.04 \mu\text{g/mL}$) nor $AUC_{0-\infty}$ (mean $\pm$ Stdev, 12.84

$\pm 6.60 \text{ h}^* \mu\text{g/mL}$) reached either the reported concentrations required to cause *in vitro* 50% IMPDH inhibition in dogs (200 $\mu\text{g/mL}$ or AUC of 30–60 $\text{h}^* \mu\text{g/mL}$) (Lange, Mueller et al., 2007) in any dog when administered at the current dose (Table 1, Fig 2, Fig 4). Cmax was consistent with previously reported concentrations in adult dogs (10 $\mu\text{g/mL}$) and exceeded the IC₅₀ for our lymphocyte proliferation assay tested in 6 adult dogs. (Lange, Mueller et al., 2008; Bishop KA, 2016) Inter-individual variability of all measured parameters was high (Table 1). The last time point where MPA could be quantitated (Tlast) was 11 ± 1 hours (95% CI, 4.7–17.29), although the majority of MPA was cleared by 90 min.

Pharmacodynamics: T Lymphocyte Proliferation

After oral administration of MMF, no statistically significant decrease in the percentage of proliferating CD5+ cells was observed at any time point ($P=0.529$) (Figure 3).

Administration of MMF did not result in inhibition of mitogen-stimulated lymphocyte proliferation, as there was no significant difference between % inhibition of CD5+ lymphocytes at any time point evaluated ($R=0.380$; Figure 4). No significant relationship was observed between the pharmacokinetic and pharmacodynamic parameters, specifically the MPA plasma concentrations and % inhibition of CD5+ lymphocytes ($R = 0.138$, $P=0.319$).

Discussion

Evidence that MMF can be used to treat juvenile diseases exists in human medicine. (Bacek & Macintire, 2011; West & Hart, 2014). Mycophenolate mofetil has been used in pediatric renal transplantation and represented a possible therapeutic option for juvenile patients with immune-mediated disease. (Downing, Pirmohamed et al., 2013; Dong, Fukuda et al., 2014) However, the results of this study suggest that oral MMF has unfavorable pharmacokinetics and pharmacodynamics at the administered dose, with the AUC_{0-∞} failing to reach therapeutic efficacy based on parameters in previous studies, concurrent with a lack of significant suppression of T cell proliferation (>50%) after MMF administration despite exceeding concentrations sufficient to suppress lymphocyte proliferation *in vitro*. (Langman, Shapiro et al., 1996; Creevy, Bauer et al., 2003; Premaud, Rousseau et al., 2011; Abd Rahman, Tett et al., 2013; Bishop KA, 2016) The use of pharmacokinetic and dynamic evaluations combines distributive and metabolic information with biologically relevant functional effects (e.g., immunosuppression) of a drug. To the authors' knowledge this is the first study to evaluate the pharmacokinetic and dynamics of a single orally administered dose of MMF in juvenile/pediatric dogs.

In people, onset of action of MPA is rapid, with pharmacodynamics assays indicating inhibition of IMPDH and T cell proliferation 1 hour after single dose administration. (Kamar, Glander et al., 2009) The therapeutic range (based on area under the curve) for plasma MMF in people is 30–60 $\mu\text{g}^* \text{h/mL}$. Concentrations below 30 $\mu\text{g}^* \text{h/mL}$ are associated with increased risk of graft rejection and is therefore an important, clinically established, therapeutic target. Human studies have also demonstrated that plasma MPA correlates with treatment efficacy, however across all studies MPA has demonstrated marked inter- and intra- subject variability. (Bullingham, Monroe et al., 1996; Staatz & Tett, 2014)

Additionally, human pediatric patients show different pharmacokinetic characteristics than adult patients.(Staatz & Tett, 2007; Abd Rahman, Tett et al., 2013; Downing, Pirmohamed et al., 2013; Staatz & Tett, 2014)

The concentration of MPA required to cause 50% IMPDH inhibition (I_{50}) in humans is 2.0–5.0 $\mu\text{g/mL}$.(Langman, LeGatt et al., 1995) However, there is marked variability between species. In dogs the IMPDH IC_{50} occurs at 200 $\mu\text{g/mL}$ of MPA.(Langman, LeGatt et al., 1995; Langman, Shapiro et al., 1996) However, our lymphocyte proliferation assay demonstrated an IC_{50} at $< 0.04 \mu\text{g/mL}$.(Bishop KA, 2016) This concentration is similar to a cytokine assay performed in people, and was within 1 dilution of a recently published canine study.(de Lathouder, Gerards et al., 2002; Guzera, Szulc-Dabrowska et al., 2016) Importantly, these concentrations were achieved using different pharmacodynamic assays, suggesting that MPA may inhibit lymphocyte proliferation by an IMPDH-independent mechanism. Both assays focus on *in vitro* effects without modification or alteration metabolite distribution of the immunosuppressant; thus, some differences could be expected if the assays were used *ex vivo*. This is in fact what happened in the current study as the concentrations of MPA determined by the PK portion of the study (i.e., $C_{\text{max}}=9\mu\text{g/mL}$) far exceeded what we have previously observed to be the IC_{50} for MPA *in vitro* ($<0.04\mu\text{g/mL}$). *Ex vivo* testing more closely resembles *in vivo* effects than *in vitro* testing because the drug undergoes absorption, distribution/tissue localization, biotransformation and elimination before removal of the cells for testing. The lack of an *ex-vivo* effect on lymphocyte proliferation in this study is yet undetermined, but underscores some of the challenges in PD testing.

After oral administration in people, MMF undergoes rapid absorption in the gastrointestinal tract and conversion by liver, peripheral tissue, and plasma esterases into MPA. Plasma proteins bind 97% of MPA. MPA is metabolized by the liver, with the major metabolite being inactive 7-O-MPA glucuronide (MPAG). However, the acyl glucuronide (AcMPAG) metabolite is active. Both MPAG and AcMPAG can undergo extensive enterohepatic recirculation, contributing substantively to bioactivity of MPA. One of the potential limitations of this study is the interaction of the immunoassay with both MPA and AcMPAG. In humans, the AcMPAG metabolite is equal to the parent in activity but only represented approximately 10% percent of the maximum drug concentration or total area under the curve of bioactivity in one study of humans simultaneously receiving cyclosporine.(Beckebaum, Armstrong et al., 2009) As such, the detection of AcMPAG is desirable.(Shipkova, Schutz et al., 2000) In human patients receiving mycophenolate, monitoring of MPAG based on the emit immunoassay consistently measured up to 40% higher maximum drug concentrations but similar area under the curve when compared to HPLC.(Weber, Shipkova et al., 2002) However, concentrations measured by Emit have been demonstrated to correlate to response as well as an HPLC assay.(Weber, Shipkova et al., 2002) The assay is well adapted for monitoring immunosuppressed patients.(Shaw, Figurski et al., 2007) Enterohepatic circulation of MPA (and its metabolites) impacts the pharmacokinetic profile of MPA in humans which classically is characterized by two peaks in the plasma drug concentrations, the first representing the initial rapid conversion and absorption of MPA and the second representing enterohepatic recirculation 6–12 hours later.(Bullingham, Nicholls et al., 1998; Allison & Eugui, 2000) While these juvenile dogs displayed rapid absorption, the second

peak concentration was not apparent in all dogs. This is in contrast to adult dogs, where rapid oral absorption and marked enterohepatic recirculation were observed.(Langman, Shapiro et al., 1996) Reported oral bioavailability in the canine species ranges from 54–87%.(Lupu, McCune et al.; Langman, Shapiro et al., 1996) In people MMF exhibits non-linear pharmacokinetics with decreasing bioavailability at higher doses. This is in contrast to the adult dog in which AUC was not significantly altered with increasing doses from 10–30 mg/kg.(Lange, Mueller et al., 2007; de Winter, Mathot et al., 2011) In the current study, 13 mg/kg MMF as single administered dose was selected based on lower doses avoiding dose related side effects without impacting overall drug exposure.(Langman, Shapiro et al., 1996; Lange, Mueller et al., 2007) This is the dose used in our gene therapy model for NCL and is considered the maximum tolerated dose for long term administration based on the development of severe gastrointestinal side effects.

Overall, the pharmacokinetics in these juvenile dogs are suggestive of rapid absorption and conversion of MMF to MPA as previously reported in adult dogs; however the $T_{1/2}$, terminal rate constant, C_{max} , and $AUC_{0-\infty}$ showed marked differences, compared to previously published reports in human pediatric transplant patients and healthy adults. As results obtained by immunoassay tend to be increased compared to HPLC, these decreases are unlikely to be due to the assay.(Weber, Shipkova et al., 2002) The data reported in this study were more comparable to studies in adult dogs, though in the $T_{1/2}$ was prolonged compared to previous published reports in adult dogs. (Table 2) (Lange, Mueller et al., 2007; Staatz & Tett, 2007; Downing, Pirmohamed et al., 2013)

The decreased AUC and C_{max} observed in dogs compared to humans may reflect differences in the intestinal microbiome, drug metabolism and clearance resulting in decreased AUC and C_{max} for individuals, species and age groups (Table 2).(Whitley & Day, 2011; Deng & Swanson, 2015) Enterohepatic recirculation accounts for 10–40% of the AUC in previous studies. (Staatz & Tett, 2007; Downing, Pirmohamed et al., 2013) Reduced contribution from impaired enterohepatic circulation has been shown to significantly decrease the AUC following oral MMF.(van Gelder, Klupp et al., 2001) Alterations in hepatic glucuronidation and metabolism of MPA to MAPG as well as age-related alterations in GFR may account for the differences in $T_{1/2}$ between adult and juvenile dogs.(Lu & Rosenbaum, 2014) A comparison of pharmacokinetic parameters from the current study and 3 previous studies evaluating different populations (adult dogs, pediatric renal transplant patients and healthy adult humans) are provided in Table 2.(Bullingham, Monroe et al., 1996; Langman, Shapiro et al., 1996; Staatz & Tett, 2007) Peak concentrations were comparable between people and dogs, however the AUC was markedly diminished compared to people, in both juvenile and adult dogs. Previous MPA studies demonstrated similar AUC when evaluated by HPLC and immunoassays.(Weber, Shipkova et al., 2002) The pharmacokinetic parameters in these juvenile dogs show significant inter-individual variability even under tightly controlled, experimental conditions. This is consistent with previous reports in people, which showed pronounced inter-individual variation with 32–60% variance for AUC_{0-12} .(Kuypers, Le Meur et al., 2010) Daily variability in enterohepatic recirculation, individual microbiota, or age dependent differences in P-glycoproteins may account for the differences observed here.(Tsai, Daood et al., 2002; Kearns, Abdel-Rahman et al., 2003; Fanta, Niemi et al., 2008; Deng & Swanson, 2015)

Previous *in vitro* and *in vivo* studies have demonstrated that MPA has high potency to inhibit T cell proliferation in a dose-dependent fashion.(Langman, Shapiro et al., 1996; Yu, Seidel et al., 1998; Gummert, Barten et al., 1999; Sankatsing, Prins et al., 2008) In rodents and people, MPA suppresses T cell proliferation.(Gummert, Barten et al., 1999; Staatz & Tett, 2007; Abd Rahman, Tett et al., 2013) Inhibition of lymphocyte proliferation was observed at low plasma MPA concentrations after a single dose in humans prior to renal transplantation. (Kamar, Glander et al., 2009) In adult dogs, *in vitro* incubation of MPA with canine lymphocytes and whole blood resulted in dose dependent inhibition of IMPDH with an [I₅₀] of 200 µg/mL, although this concentration was not achieved *in vivo*.(Lupu, McCune et al.; Langman, Shapiro et al., 1996; Lange, Mueller et al., 2007) Our laboratory determined the MPA IC₅₀ for T lymphocyte suppression to be 106.3 ± 157.7 nM (0.033 ± 0.05 µg/mL). In the current study, no statistically significant difference in the mean % inhibition of CD5+ T lymphocyte proliferation was observed at any time point in dogs following treatment with MMF despite exceeding the I₅₀ for MPA previously established. Non-significant decline was observed at 1 hour after MMF, however it failed to reach the target of 50% suppression. Further, no significant relationship was observed between plasma concentration and % inhibition of T cell proliferation (R=0.148, P=0.324). The pharmacokinetics and dynamics differences of MMF in pediatric as compared to adult human patients require higher doses to be administered.(Staatz & Tett, 2007; Downing, Pirmohamed et al., 2013) Though plasma concentrations exceeded those necessary to cause T cell suppression *in vitro*, it may be that higher concentrations are required for suppression *in vivo* or *ex-vivo*. However the benefit of administering high doses of MMF, given the severity of side effects observed in canine patients, requires additional investigation and should be done with caution.(Broadus, Tillson et al., 2006; West & Hart, 2014) Other possible weaknesses of this study design include a small number dogs increasing the probability of type 2 error. This study was also conducted in a single breed and results may not reflect the juvenile dogs population as a whole. However single breed pharmacokinetic studies have been used historically and are well represented in the veterinary literature.(Kilp, Ramirez et al., 2014; Norkus, Rankin et al., 2015) Additionally, the possibility that plasma concentrations do not truly reflect tissue concentrations should also be considered.

Marked species differences between dogs and people highlight the need for species-specific pharmacokinetic and dynamic evaluation of drugs. Failure to reach target therapeutic concentrations of MPA indicated by AUC_{0-∞}, as well as significant inter-individual variation and the lack of statistically significant effect on one pharmacodynamic assay does not support MMF as an effective immunosuppressant in juvenile dogs at the administered dose. This study investigated a single dose of MMF. High dose or repeated dosing may result in improved CD5+ T cell suppression over time however the C_{max} reached in our population was sufficient to cause T lymphocyte suppression *in vitro* and suppression was detectable after single oral dosing in humans.(Lange, Mueller et al., 2008) Plasma monitoring of MPA has been controversial in human medicine given the intra-individual variation in MPA concentrations related to enterohepatic recirculation. However more frequent administration may allow for adequate drug concentrations. Given the T_{1/2} of MMF in this population, a dosing interval of 8 hours may prove to be more optimal, though

the effect of this dosing interval on immune function and safety is unknown and warrants further investigation.

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Abbreviations

MMF	Mycophenolate mofetil
MPA	mycophenolic acid
TPP1	tripeptidyl peptidase 1
CLN2	ceroid lipofuscinosis 2
IMPDH	inosine monophosphate dehydrogenase
NCL	neuronal ceroid lipofuscinosis
EDTA	ethylene diamine tetra-acetate
C_{max}	maximum concentration
T_{max}	time to maximum concentration
MPAG	mycophenolic acid glucuronide
HPLC	high performance liquid chromatography
ConA	concanavalin A
LPS	lipopolysaccharide
AUC_{0-∞}	area under the curve from 0 to infinity
MRT	mean residence time
C_{min}	minimum plasma concentration
k/d	disappearance rate constant
T_{1/2}	half-life
T_{last}	time to last detectable concentration
I₅₀	inhibition concentration 50
MPAG	7-O-MPA glucuronide
AcMPAG	acyl glucuronide

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Figure 1.

(a–d). Flow cytometry was used to determine T cell proliferation in response to ex vivo stimulation with the mitogens conA and LPS in juvenile dogs treated with MMF and evaluated serially for 24 hours. Figure 1a and 1b show the gating strategy to include lymphocytes and exclude dead cells. Figure 1a is a plot of forward scatter (SSC) versus side scatter (FSC) for stimulated cells showing the gate encompassing resting and activated lymphocytes (representative sample). In figure 1b, a gate is placed around cells which do not stain with the fixed viability dye eFluor780 to allow inclusion of only live cells in subsequent analysis. Both gates are subsequently applied to a plot of Ki67 FITC versus CD5 perCP eF710 to show proliferating CD5+ T lymphocytes without (Fig 1c) and with (Fig 1d) the mitogens ConA and LPS. The percent proliferating T lymphocytes are provided in the upper right quadrants (1c and 1d).

Figure 2.

Figure 2a. Mean plasma MPA concentration $\pm$ Stdev across time. No MPA was detected after the 12 hour time point.

Figure 3.

A whole blood flow cytometric assay was used to determine the percentage of live, proliferating CD5+ T cells with mitogenic stimulation in 6 juvenile dogs before (time 0 hours) and serially for 24 hours after administration of oral MMF. Proliferating cells were those staining positively for CD5 and Ki-67 cells and negative for the fixed viability dye. The data represents the number of live proliferating cells after stimulation, minus the number of unstimulated proliferating cells at each time point. The lower box represents the 25th quartile, the upper box the 75th quartile, and the horizontal line, the median. The whiskers represent the range of the data. The black squares represent the mean. Treatment with oral MMF did not significantly dampen T cell proliferation over time ($P = 0.529$).

Figure 4.

The percent proliferating cells from untreated controls (dashed lines) are depicted against the mean $\pm$ Stdev of those treated with MMF (solid line). The dotted line marks the ideal target of immunosuppression with 50% lymphocyte inhibition. A decrease in lymphocyte inhibition is observed at 6 hours and likely reflects diurnal variability in lymphocyte activity. (Kronfol, Nair et al., 1997)

Table 1

Pharmacokinetic parameters (mean $\pm$ Stdev [95% confidence interval]) of mycophenolate after a single oral dose (13 mg/kg) to 6 healthy juvenile dogs. MRT_{INF} (h) = Mean residence time and represents the average (mean) amount of time the drug (molecule) resides in the body/plasma. T_{1/2} = elimination half-life, k/d (1/min) = Slope (disappearance rate constant), AUC_{0-∞} = area under the curve observed to infinity and reflects total drug exposure. T_{1/2} is reported as a harmonic mean and pseudostandard deviation.

Parameter	Mean $\pm$ Stdev (95% CI)	Coefficient of Variation %
C _{max} (μg/mL)	9.33 $\pm$ 7.04 (3.7–15.0)	77.44
C _{last} (μg/mL)	0.32 $\pm$ 0.15 (0.20–0.43)	47.28
C _{min} (μg/mL)	0.30 $\pm$ 0.12 (0.17–0.36)	39.14
C _{avg} (μg/mL)	0.46 $\pm$ 0.24 (0.27–0.66)	52.39
T _{max} (hour)	0.33 $\pm$ 0.18 (0.19–0.50)	54.77
T _{last} (hour)	11.00 $\pm$ 2.45 (9.04–13.00)	22.27
AUC _{0-∞} (h* μg/mL)	12.84 $\pm$ 6.62 (7.45–18.14)	51.53
AUC _{0-last} (h* μg/mL)	9.04 $\pm$ 5.06 (4.98–13.09)	56.04
AUC _{%ext-obs} (%)	30.87 $\pm$ 21.5 (13.67–48.07)	69.63
MRT _{INF} (h)	11.09 $\pm$ 9.63 (3.38–18.79)	86.88
T _{1/2} (h)	5.50 $\pm$ 3.80 (3.11–14.31)	80.31
kd (1/min)	0.002 $\pm$ 0.001 (0.001–0.003)	64.44
Clearance (mL/min/kg)	25.49 $\pm$ 14.60 (13.81–37.18)	57.27
Volume of distribution (mL/kg)	22879.69 $\pm$ 29359.72 (–612.98–46372.35)	128.32

Table 2

Pharmacokinetic parameters for comparison to juvenile dogs are listed from prior studies in adult dogs (Lange, Mueller et al., 2007), human pediatric (Staatz & Tett, 2007; Downing, Pirmohamed et al., 2013) renal transplant patients and healthy adult humans. (Bullingham, Monroe et al., 1996; Staatz & Tett, 2007) High performance liquid chromatography (HPLC)

Parameter	Present Study	Other Studies		
	Juvenile Dogs (13mg/kg) Mean± Stdev (CI 95%)	Adult Dogs (10mg/kg) Mean	Human Pediatric Pharmacokinetics (600mg/m2 PO) Mean	Healthy Adults (1g PO) Mean
Assay	Immunoassay	Immunoassay	HPLC	HPLC
MRTINF (h)	11.09 ± 9.63 (−13.67–35.84)	-	-	-
T1/2 (h)	5.50±3.80 (−9.28–26.71)	2.90	-	16.00
AUC0-∞ (h*µg/mL)	12.84±6.62 (−4.17–29.85)	15.50	55.80	63.90
Cmax (µg/mL)	9.33±7.04 (−8.78–27.43)	10.00	25.60	24.50
Tmax (h)	0.33±0.18 (−0.14–0.80)	0.70	0.60	0.80