Influence of ABCB1 Genotype in Collies on the Pharmacokinetics and Pharmacodynamics of Loperamide in a Dose-Escalation Study

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ABSTRACT

Thirty-three Collies (14 male and 19 female) were used in a dose-escalation study to determine the impact of ABCB1 genotype on loperamide pharmacokinetics (PK) and pharmacodynamics (PD). Loperamide was orally administered in four ascending doses (0.01, 0.05, 0.1, or 0.2 mg/kg) over a 4-wk period to fasted Collies. Comparisons were made within each dose to genotype, phenotype, and whether Collies received three (3D) or four (4D) loperamide doses. The 3D and 4D groupings had statistically significant differences in systemic drug exposure (defined by the area under the concentration-versus-time profile estimated from time zero to the last quantifiable drug concentration, AUC_{0-last}). In contrast, statistical differences in AUC_{0-last} only occurred in the comparison between wild-type (WT) Collies versus homozygous mutant (Mut) Collies administered 0.1 mg/kg. Statistical differences in the proportionality relationship were observed when comparing 3D to 4D Collies, and the WT to Mut Collies. Intersubject variability in drug exposure tended to be twice as high between Mut and WT Collies. Associations were observed between systemic drug exposure and ataxia or depression but not between systemic drug exposure and mydriasis or salivation. Thus, Collies expressing the greatest sensitivity to CNS-associated effects of loperamide (Mut) tended to have higher drug exposure compared with those less sensitive to the adverse effects of loperamide. Genotype and phenotype only partially explained differences in loperamide PK and PD, suggesting this relationship may not be straightforward and that other factors need to be considered. Accordingly, the PD and PK of one P-glycoprotein substrate only partially predicted the likelihood of adverse responses to unrelated substrates.

Introduction

The ABCB1 gene encodes for the efflux transporter P-glycoprotein (P-gp). P-gp is a major efflux pump for a wide variety of human and veterinary drugs (Sadique et al., 2000; Elkiweri et al., 2009).

In Collies possessing a four-nucleotide deletion, the ABCB1-1Δ genotype results in a frame shift mutation that yields a truncated, nonfunctional P-gp molecule (Mealey et al., 2001). Collies homozygous for this genotype (Mut) have an increased risk of toxic responses to P-gp substrates compared with the wild-type (WT) counterpart (Neff et al., 2004; Geyer et al., 2005a, 2005b, 2007; Mealey et al., 2005; Graham et al., 2011). Drugs reported to have canine genotype–associated toxicities include ivermectin (IVM), milbemycin, moxidectin, digoixin, mexiletine, loperamide, and vincristine (Tranquilli et al., 1991; Staley and Staley, 1994; Hugnet et al., 1996; Sartor et al., 2004; Henik et al., 2006; Mealey et al., 2008). The response of heterozygous Collies to these various compounds is not fully understood.

In humans possessing the 2677TT- and 3435TT-associated P-gp polymorphisms, both sedation and elevated plasma loperamide concentrations were observed at pharmacologically relevant doses (Skarke et al., 2003). However, conflicting reports have been published on the impact of this ABCB1-1Δ polymorphism on canine plasma loperamide concentrations. For example, Kitamura et al. (2008) observed that a pharmacological dose of loperamide (0.01 mg/kg) was associated with elevated plasma loperamide levels in Collies homozygous for the ABCB1-1Δ mutation. Conversely, no corresponding differences in loperamide PK were reported at a suprapharmacological dose of 0.2 mg/kg (Mealey et al., 2010). Although it has been suggested that this discrepancy may reflect a dose-associated saturation of intestinal P-gp activity (Dufek et al., 2013), confirmation of the underlying cause for this difference in observations remains undetermined. Accordingly, further evaluation of this question is warranted.

To explore this issue, a dose-escalation study was conducted to determine if P-gp saturation could be responsible for the lack of effect on loperamide pharmacokinetics (PK) as observed by Mealey et al. (2010). Furthermore, we matched these PK responses to clinical toxic outcomes to determine if any PK/pharmacodynamic (PD) correlations could be identified. Lastly, the questions remained as to whether dogs...
heterozygous for this defect behaved in a manner similar to the WT Collies or to the Mut Collies and if prior adverse reactions to IVM are predictive of loperamide toxicity.

Materials and Methods

Animals

Thirty-three intact Collies (14 males and 19 females) were enrolled in this study; all Collies were research animals obtained from a research colony, and returned at the end of the study. The ABCB1 genotype and sensitivity to IVM (0.1 mg/kg) of each Collie had been determined by the research colony prior to enrollment. All homozygous mutant Collies were sensitive to IVM. The Collies were individually housed and were kept in a facility in which the temperature was maintained between 68 and 72°F. There was a 3-week acclimation period prior to study initiation. The Collies were fed once daily (Pride 22/16; The Hyland Co., Ashland, KY); this was the identical feed offered to the Collies at the research colony. Water was provided ad libitum using nipple waterers and individual bowls of water. The Collies were provided at least once daily with the ability to socialize and exercise with other Collies. Each Collie was also provided various forms of enrichment. All animal activities were approved by the FDA Center for Veterinary Medicine Institutional Animal Care and Use Committee. The average age, weight, and gender composition are listed in Table 1. The table also lists the numbers of Collies in each group that received just the three lowest doses of loperamide, or received all four doses (see below also).

Dose Groups

The Collies were arranged into four dosing groups on the basis of the genotype and sensitivity to CNS toxicities induced by IVM. The four groups (and number per group) were wild-type (7), heterozygous mutant, non-IVM sensitive (9), heterozygous mutant-IVM sensitive (7), and homozygous mutant (10). Collies were grouped according to their genotype of WT, Mut, or heterozygous. The heterozygotes were further categorized into IVM-sensitive (HS) or IVM nonsensitive (HNS), for a total of four treatment groups. As genotypic/phenotypic information was known prior to study enrollment, the Collies were randomly ordered within a given study group. This ranking served as the basis for dosing group assignment. Prior to dosing group assignment, all Collies were assigned a random number using a random number table for purposes of clinical observations to ensure that making clinical observations were masked to genotype/phenotype.

Loperamide Administration

All Collies were weighed prior to initiation of the dose-escalation study. An over-the-counter generic loperamide solution (1 mg/5 ml) was administered using an oral dosing syringe. The same product lot was used throughout the study. Collies scheduled to receive loperamide were fasted overnight, and fed approximately 10 hours after loperamide administration. Dosing of Collies within each group occurred in 5-minute intervals. Loperamide was administered at doses of 0.01, 0.05, 0.10, and 0.20 mg/kg, starting at the lowest dose. All Collies were administered one dose per week, with a 7-day wash-out separating administration of loperamide dose.

Plasma Collection and Observation for Clinical Signs of Toxicity. All Collies had an indwelling catheter placed into the cephalic vein immediately prior to loperamide administration. The catheter was secured in place using surgical tape and Vetwrap, with an injection cap sealing the end of the catheter. The catheters remained in place through the entire blood sampling period and...
were removed after the 24-hour sample collection. Catheter patency between sampling was maintained with heparinized saline (10 IU/ml). Blood samples (approx. 5 ml per collection period) were collected at 0 hour (prior to loperamide administration) and at 1, 2, 5, 10, and 24 hours after oral administration of loperamide. Prior to the 0-hour sample collection, the dogs were observed for clinical signs of central nervous system (CNS) toxicity (depression, ataxia, mydriasis, and/or salivation). The individuals evaluating each Collie were masked to treatment, genotype, and phenotype.

A predefined four-level scoring system for depression, ataxia, mydriasis, and salivation was employed to assess the clinical impact of loperamide administration (Fassler et al., 1991; Paul et al., 2000). The scoring criteria for each parameter ranged from normal (0) through severe (3), with narrative descriptions included for each scoring category and parameter. A single standardized scoring sheet was used for each Collie at each observation time. These observation sheets were collected by the study director at the end of each observation period to prevent observer bias during subsequent observation periods. Once a Collie exhibited any signs of CNS toxicity, loperamide administration was discontinued and the dog did not receive higher doses of loperamide. Although excessive, life-threatening adverse events were not anticipated, naloxone and other supportive therapies were available if needed.

Loperamide Analysis. The method, slightly modified from a published procedure (Ganssmann et al., 2001), comprises sample extraction and instrumental analysis. First, 0.5 ml of internal standard solution (D,L-methadone, 1 ng/ml in borate buffer, pH 8.5) was added to an aliquot of 0.5 ml plasma sample, followed by addition of 1 ml ethyl acetate for extraction. After centrifugation, 700 μl of the supernatant was evaporated to dryness, and the residue was reconstituted with 50 μl of mobile phase (4 mM ammonium acetate buffer + acetonitrile + methanol, 14:13:13, v/v/v). A Shimadzu (Columbia, MD) Prominance system, composed of two LC20AD pumps, a DGU20A5 degasser, a SIL-20AC auto-sampler, and a CTO-20A column oven, was used for liquid chromatography, with a Zorbax Eclipse XDB-C8 column (150 × 2.1 mm, 5 μm; Agilent Technologies, Santa Clara, CA). Mobile phase was run in isocratic mode at 0.25 ml/min for 5 minutes, and the column oven temperature was kept at 30°C. For signal detection, an Sciex (Framingham, MA) 4000 QTRAP mass spectrometer equipped with TurboIonSpray source (run in positive mode) was used. The ionization source was set at 550°C, 5000 V, and optimized interface gas supply rates. Two transitions were monitored for loperamide (“Lop1”, 477.3 → 266.1 m/z, and “Lop2”, 477.3 → 210.1 m/z), and one transition for desmethyl loperamide (DML; 463.3 → 252.1 m/z), and methadone (310.2 → 265.1 m/z), respectively.

The sum of the two transitions (Lop1 + Lop2) was used for loperamide quantitation, and a matrix-matched standard curve (with methadone as internal standard) was constructed for each batch. Retention time for loperamide was around 3.3 minutes, and DML eluted about 0.6 minutes earlier. Lacking

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**Fig. 1.** Box-plot comparisons of natural log-transformed AUC for Collies grouped by genotype and phenotype for all four doses used in this study. The four doses of loperamide used in this study were 0.01, 0.05, 0.10, and 0.20 mg/kg. See Table 1 for information on the sample size of each group.
reference standards for DML, estimates of their respective concentration were made on the basis of the calibration curve of the Lop1 transition (loperamide) with internal standard correction. In addition, the ratio of Lop1 to Lop2 was used to verify identity of loperamide in positive samples, along with retention time.

The method was validated over a linear quantitation range of 0.01 to 10 ng/ml (after correction with concentration factor) for loperamide in canine plasma, at four spiking levels (5, 0.5, 0.05, and 0.01 ng/ml, respectively), on multiple days. Linear regression with $r^2$ higher than 0.99 was achieved in all batches. The average recoveries at these levels were between 98% and 104%, with interday precision (described as relative standard deviation) ranging from 5% to 15%. No significant interference or carryover was found in blank control samples. An estimate of detection limit was made on the basis of instrument response in blank controls (0.0023 ng/ml), which was well below the lower limit of quantitation. For verification of analyte identity, all positive controls ($N = 19$) met preset confirmation criteria, as none of the five blank controls did. Loperamide was also found to be stable in extract (refrigeration) for at least 24 hours, and in plasma ($<-70{^\circ C}$) over the period of analysis. Lastly, performance of the method was retrospectively reviewed with the spiked predosing plasma samples from different Collies that were analyzed in various days. The average accuracy was 104.6% ($N = 111$) with an 11.6% relative standard deviation.

**PK Analyses.** The data were evaluated on the basis of Collie genotype/phenotype groupings, whether Collies previously exhibited IVM sensitivity, and if the Collies received all four doses of loperamide (4D) or only received the three lowest doses of loperamide (3D) owing to manifestation of CNS toxicity at one of the lowest three doses of loperamide.

A noncompartmental model approach using the observed total plasma concentrations (free plus bound-to-plasma proteins) were evaluated on the basis of the area under the concentration/time profile (AUC), and terminal elimination half-life ($T_{1/2}$). AUC values were obtained using the linear trapezoidal rule (slopes defined using no less than three consecutive points and a uniform weighting procedure. Several cases were encountered where $T_{1/2}$ could not be defined). These noncompartmental estimates were obtained using the Phoenix 64 WinNonlin 6.3 software program (Build 6.3.0.395). In addition to estimating the AUC from time zero to the last quantifiable concentration ($AUC_{0-\text{last}}$), the area was also estimated from hours 0–2 ($AUC_2$) or hours 0–5 ($AUC_5$). Because our primary concern was extent of exposure, $C_{\text{max}}$ was indirectly evaluated by a comparison of the variability in the individual concentration-versus-time profiles.

**Dose Proportionality.** The individual AUC values for all Collies at each loperamide dose were used to assess dose proportionality using the slope function in Excel. Statistical comparisons for dose proportionality were conducted three different ways: 1) using the original phenotype/genotype groupings; 2) IVM-sensitivity classification (sensitive = IVMS; nonsensitive = IVMNS); and 3) whether the Collies were classified as 3D or 4D dogs.

**Statistical Analyses**

Comparisons were generated using an analysis of variance (ANOVA) model (Proc GLM, SAS 9.3; SAS, Cary, NC) by dose levels. The between-ani-
classes comparisons focused either on genotype/phenotype, IVM sensitivity, or whether the Collie could tolerate only three or all four loperamide dose levels. Each ANOVA model included one of the three categorical covariates: genotype/phenotype, IVM sensitivity, and 3D/4D. The Sidak multiplicative inequality was used to control maximum experiment-wise error rate under the set of null hypotheses related to the four categories of genotype/phenotype,

Fig. 3. Time-concentration curves for the Collies when grouped by Collies that received the three lowest doses of loperamide (3D) versus Collies that received all four doses of loperamide. The results are grouped by treatment dose. The results shown are the mean values ± S.D.
assuming positive dependence among the test statistics of interest. Statistical tests were two-sided with significance defined as $P < 0.05$.

Tabulated means and standard deviations provided in the tables reflect the simple arithmetic (untransformed) calculations.

An integral part of this study was the observation of clinical signs in the Collies after each dose of loperamide. The PK/PD comparative analyses were performed three different ways: 1) using the original phenotype/genotype groupings; 2) IVM-sensitivity classification (sensitive = IVMS; nonsensitive =

**Fig. 4.** Time-concentration curves for the Collies when grouped by sensitivity to exhibit ivermectin-induced CNS toxicities. The Collies are grouped into ivermectin-sensitive Collies versus ivermectin-nonsensitive Collies, and further grouped by treatment dose. The results shown are the mean values ± S.D.
IVMNS); and 3) whether the Collies were classified as 3D or 4D dogs. As the multinomial clinical scores were ordinal in nature, they were analyzed using a cumulative logit model with exposure parameters (AUC2, AUC5 and AUC0–last) as covariates and the group variables defined above as fixed effects. Note that we do not necessarily have to take the ordering into account. However, ordinality in the response is vital information; ignoring it almost always will lead to suboptimal models. Taking the natural ordering into account can lead a simpler, more parsimonious model and increased power to detect

Fig. 5. Time-concentration curves for the Collies when grouped by genotype and phenotype for the four doses of loperamide used in this study. The four doses of loperamide used in this study were 0.01, 0.05, 0.10, and 0.20 mg/kg. The results shown are the mean values ± S.D.
relationships with other variables. The cumulative logit model was implemented using the Proc Genmod (SAS 9.3) with generalized estimating equation (GEE) methods. Probability of a clinical score \( y \) less than or equal to category \( j \) \((j=0, 1, 2, \text{and } 3)\), \( P(y \leq j) \) is of interest rather than \( P(y=j) \).

**Results**

The variability in \( \text{AUC}_{0\text{-last}} \) values, expressed as the coefficient of variation (\%CV), were nearly two times greater in the Mut Collies compared with the WT dogs (Table 2, Fig. 1). Variability of the heterozygotes (HNS and HS) tended to be greater than that seen with the WT but less than that associated with the Mut dogs. Similar differences in variability were seen in the IVMNS compared with the IVMS dogs (Table 2). No corresponding trends could be identified solely on the basis of dose. This genotypic/phenotypic difference in variability of \( \text{AUC}_{0\text{-last}} \) values was one of the most outstanding features of this dataset.

Because there was an observed relationship between the magnitude of drug exposure and the ability of the dogs to continue in the study through the final dose level, we further evaluated the exposure-response relationship by segregating the animals into 3D (Collies that could not be dosed \( > 0.1 \text{ mg/kg} \)) and 4D groups (Collies that could tolerate all 4 doses). When divided in this manner, the 3D group consisted of Mut, HNS, and HS dogs and the 4D group contained all four genotypes/phenotypes. When evaluating the time-concentration values for the 3D and 4D groups over the first three doses of loperamide, the 4D Collies exhibited a lower intersubject variability (Fig. 2). The 3D Collies exhibited a wider spread in their plasma loperamide concentrations at the 0.01, 0.05, and 0.10 mg/kg dose groups (Figs. 2 and 3). Interestingly, although the IVMNS dogs tended to have lower loperamide exposure compared with the IVMS dogs, the magnitude of this difference was substantially less than that seen between the 3D and 4D animals (Table 2). Furthermore, a pairwise comparison of \( \text{AUC}_{0\text{-last}} \) values in HS-versus-HNS dogs revealed significant differences with respect to whether they displayed IVM sensitivity (Table 2).

In the absence of intravenous data, the impact of genotype and phenotype on loperamide pre- and postabsorptive processes cannot be differentiated. Accordingly, data were evaluated from the perspective of total PK drug characteristics using the \( \text{AUC}_{0\text{-last}} \) values (Tables 2). Statistically significant differences in WT-versus-Mut \( \text{AUC}_{0\text{-last}} \) values were achieved after the 0.1 mg/kg dose. However, when

**Table 3**

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<th>Significant Diff*</th>
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<td>350.21</td>
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*P values are shown only for group comparisons with statistically significant differences. All group comparisons were calculated.

**Fig. 6.** Loperamide \( \text{AUC}_{0\text{-last}} \) (expressed as ng*hr/ml) versus dose as a function of genotype/phenotype. The dashed line reflects the regression line derived on the basis of the observed values. The solid black line represents the expected relationship between dose and \( \text{AUC}_{0\text{-last}} \) in the presence of perfect dose proportionality. Note that in HS dogs, the lighter dashed line reflects dose proportionality estimated on the basis of the 0.05 mg/kg rather than on the 0.01 mg/kg dose. In all cases, the average exposure was proportional to dose, even though there were differences in the magnitude of the exposure across the various dog groups.
examining the mean concentration-versus-time profiles across all genotypes/phenotypes, a definite trend is seen, with higher loperamide plasma concentrations being in the Mut-versus-other classifications (Fig. 5). The 3Ds had a statistically significantly higher total exposure compared with that observed in the 4D dogs at all but the 0.05 mg/kg dose level (Table 2 and Figs. 2 and 3), suggesting either a lower systemic clearance in the 3D group or a higher fraction of drug absorbed.

The dose proportionality of AUC$_{0-last}$ value, expressed as the slope of the regression of AUC$_{0-last}$ versus dose, was evaluated as a function of genotype/phenotype, IVM sensitivity, and 3D/4D classification (Table 3). Statistically significant differences in slope were observed between the 3D and 4D dogs (4D Collies exhibited lower values). Differences in slope were also observed between the WT and Mut dogs. No differences in dose proportionality were observed on the basis of phenotype when the IVMS-versus-IVMNS dogs were compared (Table 3). The results of these evaluations are further illustrated by the graph of the dose-versus-loperamide exposure relationship as a function of genotype/phenotype (Fig. 6) or 3D-versus-4D dogs (Fig. 7). In these graphs, the solid black line reflects the predicted AUC$_{0-last}$ loperamide value on the basis of linear increases in exposure (estimated on the basis of the average concentration observed following a 0.01 mg/kg dose or, for the HNS dose group, the 0.01 mg/kg and 0.5 mg/kg dose groups). The hatched lines represent the linear regression on the basis of the observed mean of the AUC$_{0-last}$ values at each dose. Note that up through the 0.1 mg/kg dose, the concentrations of loperamide increased in a dose-proportional manner for all groupings. Nevertheless, the concentrations in the 3D dogs are substantially greater than those seen in the 4D animals.

The observed differences in loperamide concentrations could be attributable to either drug enterocyte permeability or to differences in presystemic drug loss. To further explore the potential contribution of drug metabolism, we considered the potential contribution of first pass drug loss. We measured the exposure (expressed as AUC$_{0-last}$ values) for the primary metabolite, desmethyl loperamide. If the reason for the higher loperamide exposure in the 3D-versus-4D dogs was attributable to a reduction in its metabolic conversion, we would expect that the ratio of loperamide to DML AUC$_{0-last}$ values would differ between these two groups. If such a difference is not observed, we would conclude that the higher levels of loperamide are associated with a higher permeability (greater intestinal absorption). As seen in Fig. 8 (dose range 0.01 to 0.1 mg/kg), the vast majority of the 3D dogs were indistinguishable from their 4D counterparts. We did see two dogs that appeared to have a higher loperamide to DML ratio. Therefore, we further segregating the dogs into genotype/phenotype classification.

Of note: When graphed as a function of genotype/phenotype, all groups exhibited a small negative trend in the loperamide to DML AUC$_{0-last}$ ratios, indicating that as dose increased, the metabolite exposure tended to increase relative to that of the loperamide. A negative slope was observed in all but 5 dogs (3 Mut, 1 HNS, 1 HS; 4 = 4D; 1 = 3D). Since the loperamide and DML $T_{max}$ values did not differ across doses (averaged within dose across all groups: loperamide $T_{max}$ = 3.0, 2.6, 2.7, and 2.4; DML $T_{max}$ values = 13.5,
14.6, 12.3, and 13 hours after the 0.01, 0.05, 0.1, and 0.2 mg/kg doses, respectively), we cannot attribute this negative slope to a delay in the absorption as a function of dose. Moreover, since this trend was observed across all groups, we conclude that this observation was not associated with P-gp saturation. The lack of consistent differences in T_{1/2} values, regardless of grouping, along with the marked variability in these estimates (Table 4) further supports the contention that the differences in exposure are not attributable to mutation-associated effects on drug clearance or volume of distribution. Thus, the higher plasma drug in 3D-versus-4D dogs appears to be largely a function of the drug absorption process, with no apparent difference in the proportion of loperamide that undergoes first pass metabolism.

To ascertain whether there is a correlation between exposure and clinical response, AUC_{0–last}, AUC_{2}, and AUC_{5} were evaluated. A lower correlation was observed between the clinical signs and AUC_{0–last} compared with that associated with either AUC_{2} or AUC_{5} (data not shown). Because most clinical signs were observed by hour 5 postdose, all the analyses were performed using AUC_{5} as the exposure variable. Furthermore, upon examining all four clinical signs observed during this study (depression, ataxia, mydriasis, and salvation), only ataxia and depression exhibited a phenotypic/genotypic distinction in exposure-response relationships. Therefore, subsequent assessments focused solely on ataxia and depression versus AUC_{5}.

The cumulative probability of the less than—or-equal to category j (j=0, 1, 2, and 3) of the clinical score for ataxia and depression were evaluated and plotted with regard to the various categorical variables (Figs. 10–12). The Mut Collies had a much greater cumulative probability of exhibiting ataxia and depression compared with the wild-type, HNS, or HS dogs. The exposures at which these responses occurred tended to be lower than that seen in any of the other groups, indicating a greater sensitivity to circulating loperamide. In addition, the Mut dogs had a greater propensity toward a higher severity of response (graded level 0–3) in ataxia and depression when an adverse event was seen (Figs. 10–11). Ataxia was associated with a greater risk at level 2 or 3 scores compared with that seen for depression.

HNS Collies had no incidents of depression, although several dogs exhibited level 1 ataxia, with the probability of an event linked to drug exposure. Within the HS group, a similar AUC/response relationship for depression and ataxia was observed. Both of these signs occurred with a greater likelihood in HS compared with HNS dogs. In all cases, WT Collies had less than a 10% probability of exhibiting any sign of depression or ataxia but did exhibit mydriasis and salvation. Depression and ataxia were observed in both 3D and 4D groups. However, the level of severity was less in the 4D Collies compared with the 3D Collies. Furthermore, the 4D dogs were associated with a distinct right-shift of the exposure-response relationship both for ataxia and depression (Fig. 12). Therefore, more drug exposures (ln AUC_{0–5}) were needed to achieve an adverse response in the 4D Collies compared with 3D dogs. Accordingly, evaluation of the effects from both the PK and the PKPD relationships showed that the 3D dogs were associated at higher drug exposure with a greater overall risk of an adverse event and at lower drug exposures with a greater likelihood of ataxia and depression.

Five Mut Collies (two in the 3D group, three in the 4D group) exhibited a score of 1 for only one of the clinical signs. In four of these five dogs, ataxia was observed. The fifth dog had a clinical sign of depression. This underscores the range of sensitivity to loperamide, even among the Mut genotype.
To further explore the sensitivity differences, we restricted our assessments to the Mut Collies in the 3D-versus-4D groups. Within this group, for any AUC5 value (i.e., across all dose levels), the likelihood of ataxia and depression was greater for Collies in the 3D group compared with Collies in the 4D group. When these clinical signs were manifested, the 3D dogs trended toward a greater level of severity. Thus, irrespective of drug exposure, it would appear that the physiologic effects of loperamide within the CNS of the Mut 3D dogs differed from that of the Mut 4D dogs. Because all of these Collies had nonfunctional P-gp, it is highly probable that this difference simply reflected typical population variability in drug response.

Lastly, all IVMS Collies were compared with all IVMNS Collies to determine the prognostic implications of this phenotypic classification. The results demonstrated that if a Collie was IVMNS, there was a corresponding low probability that it would also exhibit ataxia (and nearly zero probability of depression) in response to loperamide (Table 5). However, the converse could not be assumed, and IVMS Collies (with the exception of the Mut dogs) could not be predicted to be sensitive to loperamide.

**Discussion**

P-gp recognizes and effluxes a multitude of structurally and biochemically unrelated substrates (cyclic, linear, basic, uncharged, zwitterionic, negatively charged, hydrophobic, aromatic, nonaromatic, amphipathic) that range from a molecular weight of 250 to 4000 (Hodges et al., 2011). The impact of the P-gp mutation on human loperamide PK has been a subject of debate (Benet et al., 2004). The current investigation in dogs was unique in that it not only provided an opportunity to examine WT and Mut Collies, but it also evaluated heterozygous Collies, and distinguished this group of Collies on the basis of genotype and phenotype (response to IVM). This study also examined the influence of the ABCB1-1Δ gene on PK and PD as a function of dose, thereby elucidating potential factors that may be responsible for observed disparities in the literature.

Although the presence of a P-gp mutation affected the magnitude of loperamide exposure, the PKs remained dose-proportional, irrespective of whether this was evaluated on the basis of genotype/phenotype or 3D versus 4D. Therefore, the higher slope associated with the P-gp Mut dogs reflect the higher drug concentration at a given dose. To determine if differences in concentrations were a function of loperamide permeability or metabolism, we examined the ratio of loperamide to DML AUC0–last values. To avoid bias attributable to differences in amount of loperamide absorbed, this issue was evaluated from the perspective of the ratio of loperamide to DML AUC0–last values. No differences in these ratios as a function of dog classification, it was concluded that differences in drug absorption rather than clearance or first pass drug loss was affected by the functionality of canine P-gp. This was further confirmed by the lack of difference in T_{1/2} values.

It is important to note that loperamide conversion to DML (on the basis of human hepatic microsomes) has been associated not only with CYP3A4 but also with CYP2C8, CYP2B6, and CYP2D6 (Kaligutkar and Nguyen 2004; Kim et al., 2004). Accordingly, several metabolic
pathways may be involved. Although loperamide undergoes first pass and hepatic metabolism (Baker, 2007), the substantial delay in DML $T_{\text{max}}$ relative to that of loperamide is consistent with the majority of DML formation occurring after its first pass through the enterocyte or the liver. Accordingly, the observed differences between WT and Mut dogs are reflective of the influence of the P-gp mutation on drug absorption rather than on loperamide metabolism. In this regard, when and if functional P-gp enhances or reduces intestinal drug absorption remains a topic of debate (Tam et al., 2003; Benet et al., 2004, Dufek et al., 2013).

Another interesting observation was that for all but five dogs (all either homozygous or heterozygous for the ABCB1Δ gene), the loperamide to DML $AUC_0$–last ratio tended to decrease as a function of dose. The similarity of loperamide and DML $T_{\text{max}}$ values across all doses indicates that this change in ratio was probably not a consequence of loperamide effects on gastrointestinal transit time. The late DML $T_{\text{max}}$ observed in all dogs prohibited an evaluation of the elimination portion of the DML concentration-time profile. Nevertheless, considering the dose proportionality of loperamide systemic concentrations across all genotypes/phenotypes (and 3D-versus–4D dogs), the dose-associated decrease in the $AUC_0$–last ratio is probably attributable to a reduction in DML elimination. On the basis of data generated in human liver microsomes and rats, loperamide is characterized by a complex set of metabolic pathways, with DML being its primary metabolite. (Kalghatkar and Nguyen, 2004). Saturation of any of these pathways could lead to increased DML concentrations. However, with respect to the relevance of this observation to the current investigation, we observed that this decrease in ratio was not influenced by the competency of dog P-gp.

Of particular note was not necessarily the impact of the mutation on mean systemic drug exposure but rather on the variability of drug exposure that occurred as a function of Collie classification. Loperamide exposure (expressed as $AUC_0$–last values) and the corresponding variability in that exposure in the Mut dog typically exceeded that of their WT counterparts. With the small number of subjects included in the investigation by Mealey et al. (2010), and differences with the results seen by Kitamura et al. (2008), the apparent interstudy disparities may simply be a function of “chance.” Moreover, we observed that those Collies trending toward a lower adverse response to loperamide (4D) also tended to exhibit lower plasma level variability compared with Mut or 3D Collies. Reasons for this genotype/phenotype-associated higher variability (which we concluded is most probably attributable to the absorption phase of the PK profile) is a question worthy of further investigation.

With regard to the heterozygotes, the variability in $AUC_0$–last values ranged between that of the WT and Mut dogs. No significant differences could be detected between the $AUC_0$–last values of heterozygotes that were classified as HS versus HNS. With regard to their clinical response to loperamide, some of the dogs classified as IVMNS were found to be sensitive to loperamide-induced CNS toxicities, and some dogs classified as IVMS were nonsensitive to loperamide. As with the Mut dogs, differences in response to loperamide compared with IVM is probably a function of the integrity of the blood brain barrier as well as the interindividual differences in drug response if the drug enters the brain. Thus, when comparing exposure-response toxicities across all four genotypes/phenotypes, it is important to consider: 1) the ability of loperamide to cross the blood-brain barrier (i.e., if loperamide does not get across, it will not cause a toxic response); and 2) the pharmacology of loperamide within the CNS of the individual animal.

Considering the range of PK/PD characteristics seen in the heterozygotes, it would appear that the presence of the ABCB1-1Δ mutation leads to a unique biology that needs to be explored further. From a therapeutic perspective, this variability can have tremendous implications with regard to the possible range of heterozygosity on disease expression (Bonneau et al., 2014). In other words, even if the trait is recessive, there may be other downstream consequences associated with being a carrier of just one recessive gene (e.g., Stribl et al., 2014). Potentially, this variability may also be associated with tissue-specific gene expression (Lo et al., 2003; Loeuillet et al., 2007; Lo et al., 2003; Loeuillet et al., 2007; Zhang et al., 2009.).

The complexity of the PK/PD responses to ABCB1-1Δ mutation raises the question of the mechanism for the magnitude of variability within a given genotype. For example, does the ABCB1-1Δ mutation influence the translation of genes other than those that encode P-gp? The rationale for this hypothesis relates to the intersubject variability observed in dogs expressing this mutation (e.g., Mut dogs). Could the remnant of this one gene influence the expression of other genes, or elicit some downstream epigenetic modifications that might normally occur if the gene did not possess a premature stop codon (e.g., Ingelman-Sundberg et al., 2013; Kim et al., 2014)? If this is the case, then much of the work with knockout mice (where the gene is totally removed) could lead to a biased study result, whereby there is an inappropriate loss of ability to identify these “innocent victim” effects of the mutation.

For heterozygote dogs, it may also be worthwhile to consider whether there is differential tissue-specific expression of the functioning

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**TABLE 4**

Mean $T_{\text{max}}$ estimates for each loperamide dose analyzed by 3D versus 4D, IVM sensitivity/nonsensitivity, or genotype/phenotype grouping

P values are shown only for group comparisons with statistically significant differences. All group comparisons were calculated. No significant differences exist between any of the groups in this table.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Group</th>
<th>Mean</th>
<th>n</th>
<th>% CV</th>
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<td>48.3</td>
</tr>
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<tr>
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<td>54.3</td>
</tr>
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</table>
and/or nonfunctioning gene. Individuals heterozygous for the ABCB1 3435C>T polymorphism show differential expression of one or the other alleles at a single cell level (Loeuillet et al., 2007). Differences in allelic expression are also a common phenomenon elsewhere within the human genome (Lo et al., 2003). MRP2 knockout mice exhibit an upregulation in P-gp (Hoffmann and Löscher, 2007). Thus, it is conceivable that Collies heterozygous for the ABCB1 Δ gene may have differential tissue expression of the mutant gene.

Further supporting this hypothesis are the multiple pathways controlling production and persistence of P-gp. P-glycoprotein gene expression and protein production are controlled at several discrete points in the regulatory pathways leading from DNA to expression of functional protein, with final protein levels a function of translational and post-translational/epigenetic control. P-gp expression is under the control of transcription factors (Chen and Sikic, 2012; Henrique et al., 2013; Kobori et al., 2014) that can be activated by a wide variety of agents (Cascorbi, 2011). Expression is also altered epigenetically through changes in DNA methylation or acetylation (Reed et al., 2010; Chen and Sikic, 2012; Tomiyasu et al., 2014) or changes in ubiquitination (Zhang et al., 2004; Nawa et al., 2012). Changes in P-gp expression can also be influenced by changes in the expression of other transporter genes and prior drug exposure (Xia et al., 2009; Kobori et al., 2014). This epigenetic regulation may be of particular importance to dogs that are heterozygous for this trait.

When considering factors influencing gene expression, it is important to recognize the dynamic nature of cellular biology and the potential for alterations in transcription and translational processes over time. For example, with regard to P-gp, age-associated decreases in P-gp function appear to occur in elderly humans (Toornvliet et al., 2006). This decrease may not be uniformly expressed but localized in specific tissues. Using positive emission tomography, this decrease in P-gp activity observed in older human subjects appeared to preferentially influence the white matter of the brain (Bartels et al., 2010). Likewise, age-associated decreases in P-gp activity has been observed in the brains of rats (Silverberg et al., 2010), and dogs (Pekcec et al., 2011). In canine postmortem tissue from 23 nonlaboratory dogs, the dentate hilus and dentate gyrus indicated 77% and 80% reductions, respectively, in dogs aged 37–99 months in comparison with younger individuals. In contrast, P-gp expression rates in the parahippocampal cortex increased with further aging in dogs with plaque formation (Pekcec et al., 2011). Given the results of our current investigation, it is interesting to consider whether decreased P-gp expression in the aged canine brain may be more apparent in heterozygous dogs compared with their WT counterparts.

Ultimately, the results of this study underscore the complexity of effects that can be associated with genetic mutations and the need to consider this complexity when we try to extrapolate effects from one drug to another or from one animal model to the target species. As seen in this investigation, prior expression of sensitivity to P-gp substrates is suggestive, but not perfectly predictive, of a higher risk of sensitivity to other P-gp substrates. Whereas P-gp variants can be expected to have a range of potential effects across therapeutic compounds, the ABCB1-1Δ mutation is unique in that the resulting effects are complex and depend on multiple factors. The potential for alterations in transcription and translational processes over time is also a significant consideration in the interpretation of these findings.
P-gp molecule is truncated and nonfunctional. Accordingly, one might suspect that all P-gp substrates would be comparably affected by this mutation. In other words, because the ABCB1-1D mutation results in a truncated (nonfunctional) transporter, we anticipated that any drug whose entry into the CNS is restricted by P-gp would be likewise affected by the absence of this efflux transporter. However, given that sensitivity to IVM did not necessarily translate into a corresponding sensitivity to loperamide in Mut, HS, and HNS dogs, it is probable that the therapeutic impact of this transporter defect will be drug-specific, reflecting the inherent range of physiologic effects that can occur within the CNS. Therefore, when P-gp substrates are administered to dogs that are potential carriers of this defect, animals should be carefully monitored for potential adverse effects, even if animals are heterozygous for the mutation. Importantly, we now recognize that there are genetic mutations other than the ABCB1-1D mutation that can lead to defective P-gp activity (Mizukami et al., 2013). It is incorrect to assume that the PK consequences of such defects will be uniform across all drugs.

Clearly, with respect to loperamide, with the exception of the WT dogs, sensitivity to IVM does not necessarily correctly identify Collies that would express an adverse reaction to loperamide. In part, across genotype and phenotype, differences were observed both in terms of dose-exposure relationships and in terms of exposure-response relationships. With respect to dose-exposure relationships, the high variability observed in dogs carrying the genetic mutation suggest that other factors downstream from the P-gp itself are influenced by the presence of the ABCB1-1Δ mutation. The mechanism for this additional effect cannot be discerned from this investigation but appears to influence loperamide presystemic PK characteristics.

In some ways, this research raised more questions than it answered. Thus, one of the important contributions of this work is the identification of questions that need to be considered whenever exploring potential implications of any mutation and genotype on the PK and exposure-response relationships.

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Authorship Contributions
Participated in research design: Myers, Martinez, Troutman, Sharkey, Yancy.
Conducted experiments: Myers, Li, Troutman, Sharkey, Yancy.
Performed data analysis: Myers, Martinez, Qiu, Yancy.
Wrote or contributed to the writing of the manuscript: Myers, Martinez, Li, Qiu, Troutman, Sharkey, Yancy.

References
Predicted Cumulative Probabilities for time5
With 95% Confidence Limits

Fit computed at Phenon=Mut

Fig. 12. The probability that a given loperamide InAUC value will result in a CNS-induced ataxic score of 0, (black), 1 (blue), 2, (red), or 3 (green). Clinical scores range from 0 (no response) to 3 (maximum response). Results are depicted by 3D-versus-4D Collies. HNS, heterozygous, nonivermectin-sensitive Collies (one normal ABCB1 gene and one ABCB1-1Δ gene), HS, heterozygous; ivermectin-sensitive Collies (one normal ABCB1 gene and one ABCB1-1Δ gene); Mut, homozygous mutant Collies (two ABCB1-1Δ genes); WT, wild-type Collies (two normal A BCB1 genes).

TABLE 5
Clinical signs of loperamide CNS toxicity observed in IVM-nonsensitive and IVM-sensitive Collies

<table>
<thead>
<tr>
<th>Group</th>
<th>Clinical Sign</th>
<th>No. of Collies</th>
</tr>
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<td>IVM-nonsensitive Collies</td>
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<tr>
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<tr>
<td></td>
<td>Depression</td>
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<tr>
<td></td>
<td>Mydriasis</td>
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<tr>
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</tr>
<tr>
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<td>Salivation</td>
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</tr>
<tr>
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<td>Depression</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Mydriasis</td>
<td>11</td>
</tr>
</tbody>
</table>


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