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Canine Albumin Polymorphisms and Their Impact on Drug Plasma Protein Binding [S]

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ABSTRACT

Drug binding to plasma proteins is routinely determined during drug development. Albumin polymorphisms c.1075G>T (p.Ala359Ser) and c.1422A>T (p.Glu474Asp) were previously shown to alter plasma protein binding of a drug candidate (D01-4582, 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid) in a colony of Beagles. Our study investigated the hypothesis that drug-protein binding in plasma from dogs with the albumin H1 (reference) allele would be greater than in plasma from dogs with the albumin H2 allele (c.1075G>T and c.1422A>T) (*n* = 6 per group). The plasma protein binding extent of four drugs (D01-4582, celecoxib, mycophenolic acid, and meloxicam) was evaluated using ultracentrifugation or equilibrium dialysis. Free and total drug concentrations were analyzed by liquid chromatography–mass spectrometry. The albumin gene coding region was sequenced in 100 dogs to detect novel gene variants, and H1/H2 allele frequency was determined in a large and varied population (*n* = 1446 from 61 breeds and mixed-breed dogs). For meloxicam, H1 allele plasma had statistically significant higher free drug fractions (*P* = 0.041) than H2 allele plasma. No significant difference was identified for plasma protein binding of D01-4582, celecoxib, or mycophenolic acid. c.1075G>T and c.1422A>T were the most common single nucleotide polymorphisms in canine albumin, present concurrently in most study dogs and occasionally identified independently. Our findings suggest a potential influence of c.1075G>T and c.1422A>T on plasma protein binding. This influence should be confirmed in vivo and for additional drugs. Based on our results, albumin genotyping should be considered for canine research subjects to improve interpretation of pharmacokinetic data generated during the drug development process for humans and dogs.

Introduction

For some drugs, the risk of adverse effects (toxicity or lack of efficacy) depends on the concentration of free drug achieved in plasma. In some cases, free drug concentrations and the extent of drug binding to plasma proteins can be affected by differences in the albumin genotype. Canine albumin polymorphisms c.1075G>T (p.Ala359Ser; rs852211303) and c.1422A>T (p.Glu474Asp; rs851238996) have been identified as a cause of pharmacokinetic variability of a drug candidate (D01-4582, 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl) ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid) in Beagle dogs (Ito et al., 2009). [To note, the description of protein sequence variants used by the authors is based on recommendations by the Human Genome Variation Society, with the position of the amino acid substituted calculated based on the precursor peptide (proalbumin) and not the mature peptide as in the article by Ito et al. (2009).] Specifically, dogs homozygous for both single nucleotide polymorphisms (SNPs) had an up to 6-fold greater unbound drug fraction compared with wild-type dogs, resulting in significantly greater drug clearance. Although the report by Ito et al. (2009) identified an association between canine albumin genotypes and high interindividual variability in drug disposition, the study was limited to only one experimental drug in a colony of research Beagles. The impact of canine albumin polymorphisms on plasma protein binding of other drugs and in a more heterogeneous dog population (outside of research colonies) remains to be investigated.

The primary objective of our study was to investigate the hypothesis that plasma samples collected from dogs with the albumin H1 allele (reference) have a higher extent of drug-protein binding than that of plasma collected from dogs with the albumin H2 allele (c.1075G>T and c.1422A>T). To test our hypothesis, we compared the extent of plasma protein binding of four highly albumin-bound drugs (D01-4582, meloxicam, celecoxib, and mycophenolic acid) in plasma obtained from dogs with H1 and H2 alleles. Secondary objectives of our study were to screen for other nonsynonymous variants in the coding region of the canine albumin gene that could influence protein tertiary structure and to describe c.1075G>T and c.1422A>T genotype frequencies among a large number of different dog breeds.

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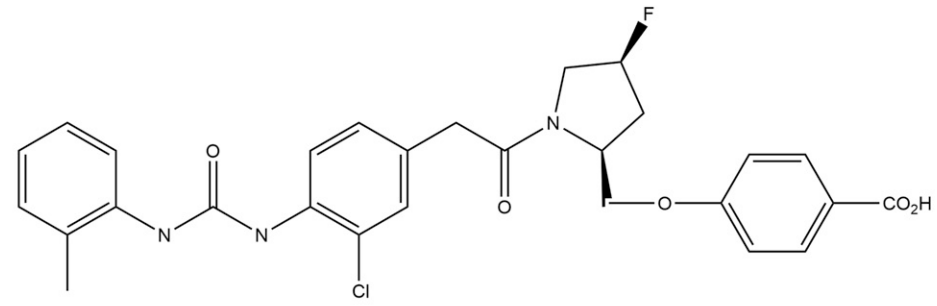
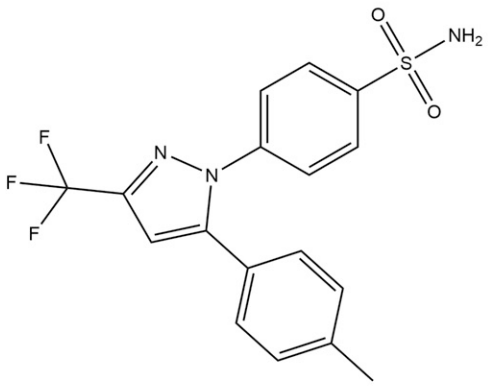
ABBREVIATIONS: ACN, acetonitrile; CV, coefficient of variation; D01-4582, 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl) ureido]phenylacetyl]-(4S)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; PCR, polymerase chain reaction; QC, quality control; SNP, single nucleotide polymorphism; WSU, Washington State University.



D01-4582



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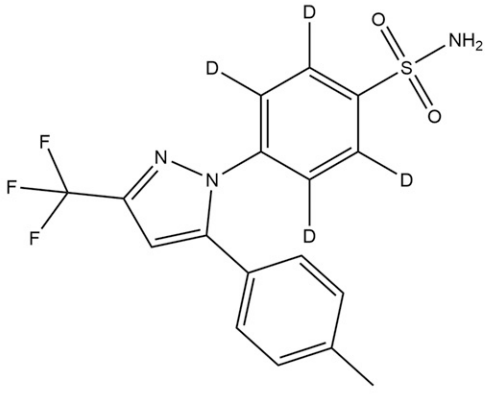
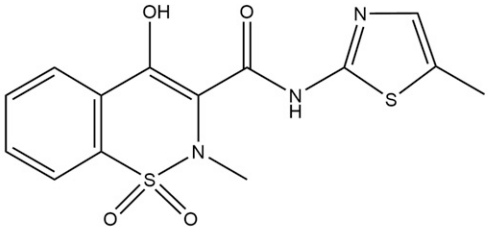


Celecoxib-d4



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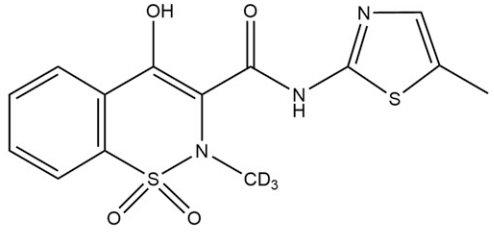

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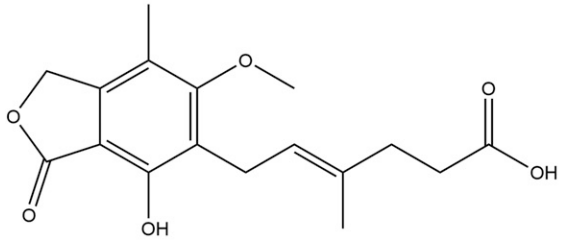
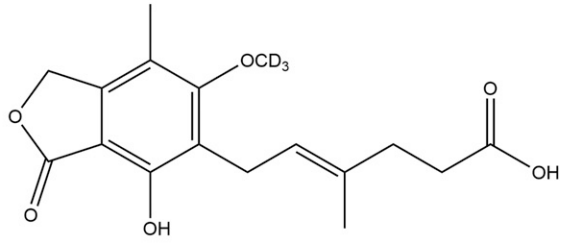


Mycophenolic Acid



Mycophenolic Acid-d3

Fig. 1. Chemical structure for D01-4582, meloxicam, celecoxib, and mycophenolic acid.

Materials and Methods

Reagents and Chemicals

Mycophenolic acid, meloxicam, and celecoxib, as well as their deuterated forms (mycophenolic acid-d_3, meloxicam-d_3, and celecoxib-d_4), were purchased from Toronto Research Chemicals (Toronto, ON, Canada). D01-4582 was synthesized and generously provided by the Daiichi Sankyo Medicinal Chemistry Research Laboratory (Tokyo, Japan). Chemical structures of each analyte and its deuterated form are included in Fig. 1. High-performance liquid chromatography (HPLC)–grade methanol and acetonitrile (ACN) were from Fisher Scientific (Pittsburgh, PA). Milli-Q water of at least 18 MΩ resistance was used to prepare the mobile phase. Potassium phosphate monobasic (99%) was from Fisher Chemical (Fair Lawn, NJ). Sodium chloride (99%) and potassium chloride (99%) were from Fisher Bioreagents (Fair Lawn, NJ). Sodium phosphate dibasic and formic acid (88%) were from J.T. Baker (Phillipsburg, PA).

TABLE 1

Relevance of the drugs selected for our protein binding experiments in dogs

Drug	Clinical Relevance in Dogs	Drug Class Clinically Relevant in Dogs	Presumed Binding to Albumin Site I	Presumed Binding to Albumin Site II	Previously Studied in Dogs
D01-4582		N/A[a]		X	x[b]
Meloxicam	X	Nonsteroidal anti-inflammatory drug	X		
Celecoxib		Nonsteroidal anti-inflammatory drug		X	
Mycophenolic acid	X	Immunomodulatory		X	

N/A: not applicable.
[a]Previously reported.
[b]Ito et al. (2009).

Canine Plasma Samples

Blood samples were collected using procedures approved by the Washington State University (WSU) Institutional Animal Care and Use Committee (ASAF numbers 04662-004 and 04797-001). Owners provided written consent for their dogs' participation in the study. Pooled canine plasma collected into sodium citrate tubes from clinically healthy dogs ($n = 6$) was used for initial analytical assay validation (methods described below). The sample size requirement to evaluate the effect of albumin genotype (H1/H1 vs. H2/H2) on drug binding in plasma was determined based on mean ± S.D. results from a previous report (Ito et al., 2009). This analysis indicated that a minimum of five dogs per group was needed to achieve an α value of 0.05 with a power value of 0.80 for a 6-fold difference in mean free drug fraction. Therefore, we proposed to study six dogs per genotype group.

Privately owned dogs were recruited from the WSU student, faculty, and employee population and genotyped for the c.1075G>T and c.1422A>T variants (method described below). After initial screening, six dogs with the H1/H1 genotype (c.1075G and c.1422A) and six dogs with the H2/H2 genotype (c.1075T and c.1422T) were selected for our study. Breeds included in the H1/H1 genotype group were as follows: Labrador Retriever ($n = 3$), Afghan Hound ($n = 1$), English Pointer ($n = 1$), and mixed breed ($n = 1$). Breeds included in the H2/H2 genotype group were as follows: Golden Retriever ($n = 2$), Borzoi ($n = 1$), Pitbull ($n = 1$), German Shorthaired Pointer ($n = 1$), and mixed breed ($n = 1$). All study dogs (five castrated males and seven spayed females) were judged to be clinically healthy based on history and physical examination, with no medications received for at least 14 days before blood collection. Owners were asked to fast the dogs on the morning of blood sample collection. Blood samples were collected by venipuncture into tubes with sodium citrate as an anticoagulant. Plasma was obtained by centrifugation (1800*g* for 8 minutes) of blood samples, transferred to cryotubes, and stored at −80°C until analysis.

Determination of the Extent of Plasma Protein Binding of D01-4582, Celecoxib, Mycophenolic Acid, and Meloxicam

D01-4582 was included in our study because of its reported binding differences associated with canine albumin genotype. The remaining compounds were selected based on their reported high plasma protein binding (>95%), site-specific binding to human albumin (Er et al., 2013), and previous clinical use in dogs (Table 1). The method (ultracentrifugation or equilibrium dialysis) used to determine the extent of each drug plasma binding was primarily selected based on methods previously reported for that drug Jolliet et al., 1997; Lapicque et al., 2000; Ito et al., 2009; Cooper et al., 2014; Morassi et al., 2018).

To control the potential effect of pH differences on drug-protein binding, individual plasma samples were adjusted with 1 N HCl to achieve a mean pH of 7.34 ± 0.04 (Mettler Toledo, Columbus, OH). The volume of acid used in these adjustments was <0.5% of the total mixture. The plasma albumin concentration for each genotyped plasma sample was determined using a standard bromocresol green method, and absorbance was monitored at 630 nm (SEKURE chemistry albumin assay; Sekisui Diagnostics, Lexington, MA).

Ultracentrifugation (for D01-4582). Initially, 10 μl D01-4582 standard solution (2500 μg/ml) was added to 490 μl plasma for a final concentration of 50 ppm (92.6 μM). Samples were then incubated for 30 minutes at 37°C and 210 μl of each plasma sample was centrifuged in a 0.5-ml thick-wall polycarbonate tube on a fixed-angle rotor Thermo Sorvall MX-150 Micro-Ultracentrifuge (Sorvall Instruments, Newton, CT) at 200,000*g* for 16 hours at 4°C. The ultracentrifugation resulted in visually distinct layers: superficial (lipid), middle (clear), and deep (protein pellet). To minimize disruption of the lipid superficial layer, the supernatant sample was collected from the middle layer immediately after centrifugation, as recommended (Dr. Takashi Ito, personal communication). In addition, the rotor was kept in ice during collection to maintain sample temperature. A 50-μl aliquot of the supernatant was collected into a 0.6-ml Eppendorf tube and prepared for HPLC–tandem mass spectrometry (MS/MS) analysis, as described below. The unbound fraction of D01-4582 was estimated by dividing the unbound concentration by the nominal incubation concentration. Plasma from a nongenotyped dog was used to confirm acceptable inter- and intrarun precision [coefficient of variation (CV) < 15%]. The experiments for each dog were conducted in duplicate and repeated on 3 separate days.

High-Throughput Equilibrium Dialysis (for Celecoxib, Mycophenolic Acid, and Meloxicam). A high-throughput equilibrium dialysis device (96-well, 75-μl half-cell capacity) and cellulose membranes (12–14 kDa molecular mass cutoff) (HT-Dialysis, Gales Ferry, CT) were used to determine the extent of protein binding for celecoxib, mycophenolic acid, and meloxicam. The dialysis membranes and device were conditioned and assembled as recommended by the manufacturer. Plasma samples were spiked with celecoxib (4 μM), mycophenolic acid (12.5 μM), and meloxicam (1.6 μM).

Compound spiked plasma samples were transferred to the plasma (donor) chambers of the dialysis device (75 μl per half-well), and an equal volume of phosphate buffer (15 mM, pH 7.3 ± 0.1) was placed in the buffer (receiver) chambers. The dialysis device was sealed using the kit adhesive (HT-Dialysis), and dialysis was conducted in an orbital shaker (100 rpm) maintained at 37°C. Complete equilibrium was achieved in the HT-Dialysis apparatus within 3.5 hours (data not shown) and samples were collected at the end of 4 hours. Samples were then processed for quantification by HPLC-MS/MS analysis as described below.

The extent of plasma protein binding of each analyte in all of the plasma samples was estimated using the following mathematical expressions:

$$\% \text{ Bound} = (\text{Cb}/\text{Cp}) \times 100\%$$
$$\% \text{ Unbound} = 100 - \% \text{ Bound}$$

where Cb and Cp represent the measured concentration of the compound from the buffer and plasma chambers of the dialysis device, respectively.

Quality controls (QCs) using mycophenolic acid (12.5 μM) (previously validated binding evaluation by equilibrium dialysis in canine plasma) were included in each row of the dialysis apparatus to monitor dialysis membrane integrity and assay performance. Experiments for each dog and compound were conducted in triplicate and repeated on 3 separate days.

Determination of Drug Concentrations

Chromatographic Conditions. The concentration of each analyte was determined by liquid chromatography with mass spectrometry detection using an HPLC apparatus (Agilent 1100; Agilent Technologies, Santa Clara, CA) connected to a triple quadrupole mass spectrometry detector (AB Sciex API4000; Applied Biosystems/Life Technologies, Framingham, MA) operated in positive ion mode. The mobile phase consisted of 95% (v/v) water [containing 0.1% (v/v)

formic acid] and 5% (v/v) ACN at 1 ml per minute through a Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm, 5 μm; Phenomenex, Torrance, CA). Mass transitions monitored included *m/z* 540.3→402.2 (D01-4582), *m/z* 382.2→362 (celecoxib), *m/z* 386.2→366 (celecoxib-d_4), *m/z* 321.2→207.1 (mycophenolic acid), *m/z* 324.2→210.1 (mycophenolic acid-d_3), *m/z* 352.1→115.1 (meloxicam), and *m/z* 355.1→115.1 (meloxicam-d_3). Retention times for D01-4582, celecoxib, mycophenolic acid, and meloxicam were 2.20, 2.41, 2.11, and 2.14 minutes, respectively.

Calibration Standards. Calibration standards and QC samples (n = 3 for each concentration) were prepared in canine plasma before protein precipitation (for celecoxib, mycophenolic acid, and meloxicam) or in the supernatant (D01-4582). The concentration ranges used for calibration curves and QCs for each analyte are listed in Table 2.

Sample Preparation for HPLC-MS/MS Analysis

For D01-4582 samples, methanol (50 μl) containing the internal standard (meloxicam-d_3) was added to the supernatant sample (50 μl). The mixture was vortexed and centrifuged at 10,000*g* for 15 minutes at 4°C, with the supernatant (20 μl) introduced into the HPLC-MS/MS.

For the celecoxib, mycophenolic acid, and meloxicam samples, each 50-μl aliquot of plasma (calibration standard or QC samples) was transferred into a 2-ml Eppendorf tube with 50 μl phosphate buffer, 1000 μl precipitating solution [ACN for celecoxib and meloxicam; methanol/ACN 1:1 (v/v) for mycophenolic acid], and the respective internal standard (4 μl for celecoxib-d_4, 3 μl for mycophenolic acid-d_3, and 5 μl for meloxicam-d_3). For dialysis samples, the matrix was equilibrated before protein precipitation by adding 50 μl plasma or phosphate buffer to the corresponding sample (e.g., plasma to dialysis buffer samples, and buffer to dialysis plasma samples) before protein precipitation and the addition of internal standard. Each extract was then vortexed for 30 seconds and centrifuged at 17,000*g* for 10 minutes. A 1050-μl aliquot of supernatant was evaporated to dryness using a Speed-Vac concentrator (Savant Instruments Inc., Farmingdale, NY). The residue was resuspended in 100 μl 95% (v/v) water [containing 0.1% (v/v) formic acid] and 5% (v/v) ACN, mixed, and centrifuged at 17,000*g* for 10 minutes for further sample cleanup. The second supernatant was injected into the chromatographic system.

Method Validation

Methods used for determination of D01-4582, celecoxib, mycophenolic acid, and meloxicam were validated according to the Guidelines for Bioanalytical Method Validation published by the U.S. Food and Drug Administration in May 2001. Quantification of all analytes was performed using internal standard calibration. All QC samples and calibration standards were prepared in triplicate in canine plasma (for celecoxib, mycophenolic acid, and meloxicam) or in the supernatant (for D01-4582). The lower limit of quantitation, the limit of detection, and the concentration ranges used for calibration curves and QCs for each analyte are listed in Table 2. Calibration curves were obtained with weighting of 1/x.

For all four analytes evaluated, the procedure produced a linear curve ($r^2 > 0.998$) over the concentration ranges evaluated with a high degree of repeatability. Both intra- and interday precision CVs were <15%, with accuracy between −11% and 14%. All analytes were evaluated with run times of <5 minutes. All analytes were stable under the experimental conditions used in this study, with reproducible results (93%–100% of the nominal concentration) after 24 hours at room temperature. Experimentally determined concentration values in plasma samples were considered acceptable with precision and accuracy higher than 85%.

TABLE 2

Concentrations used to validate the methods in the quantification of D01-4582, meloxicam, mycophenolic acid, and celecoxib in canine plasma

Drug	LOD	LLOQ	QC	Concentration Range
			μM	
D01-4582	0.002	0.01	0.014, 0.045, 0.11	0.01–0.12
Meloxicam	0.02	0.03	0.09, 1.2, 2	0.045–2.2
Mycophenolic acid	0.01	0.025	0.078, 7, 16	0.03–14
Celecoxib	0.03	0.05	0.15, 0.8, 4.4	0.06–4.5

LLOQ, lower limit of quantitation; LOD, limit of detection.

Canine Albumin Gene Sequencing

The canine albumin cDNA sequence has been reported for 26 Beagle dogs, identifying two linked SNPs at an allele frequency of 40% (Ito et al., 2009). Since that initial report included only one dog breed, we sequenced all 14 exons of the canine albumin gene using genomic DNA from 100 dogs consisting of 37 breeds. DNA samples were obtained from the WSU College of Veterinary Medicine DNA Bank. By sequencing a wider variety of breeds, we intended to identify previously unreported polymorphisms in the canine albumin gene.

Primers were designed to amplify all 14 canine albumin exons from genomic DNA. National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) genomic DNA reference sequence NW_003726083 was used to design oligonucleotide primers and develop sequencing strategies for this study. The mRNA reference sequence (AB090854) was used for comparison. Primers were designed to anneal in the flanking intron DNA sequences to amplify each exon using the PrimerQuest online design tool (http://www.idtdna.com/Primerquest). Standard polymerase chain reaction (PCR) amplifications were carried out using 20 ng genomic DNA.

PCR products were visualized on a 1.2% agarose gel before the products were treated with ExoSAP-IT reagent (Affymetrix, Cleveland, OH) according to the manufacturer's directions. Treated PCR products were Sanger dye terminator sequenced with the same primers used for amplification using Big Dye 3.1 sequencing mix as directed (Applied Biosystems/Life Technologies, Grand Island, NY). Sequence data were analyzed using Sequencher 5.2 software (Gene Codes Corp., Ann Arbor, MI). Nucleotide sequences were evaluated for differences from the canine reference genome (CanFam3.1).

Canine Albumin c.1075G>T and c.1422A>T Genotyping

Canine albumin genotypes at two previously reported polymorphic sites (c.1075G>T and c.1422A>T) were determined using genomic DNA obtained from the WSU College of Veterinary Medicine DNA Bank. DNA samples were randomly selected to include 1446 dogs representing 61 different breeds (minimum of 10 dogs per breed) and 136 mixed-breed dogs. The breed of dog was based on owner designation at the time of admission to the WSU Veterinary Teaching Hospital. Genotyping was conducted using TaqMan allele discrimination assays (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA) according to the manufacturer's directions. The primers and probes for c.1075G>T were 5′-CAA GTA AAA CTA TTT CAT TTT CAT CTG AAT CAG GT-3′ (forward primer), 5′-TGG CGA GTC TCA AAA GCA ATG A-3′ (reverse primer), 5′-TTG TAT GAA TAC GCA AGA AG-3′ (G-allele probe labeled with Vic), and 5′-TTT TGT ATG AAT ACT CAA GAA G-3′ (T-allele probe labeled with Fam). The primers and probes for c.1422A>T were 5′-GGC ACC AAA TGT TGT AAG AAA CCT-3′ (forward primer), 5′-GTT AGC TTT GTC CAT TTC TAA AGG CAA A-3′ (reverse primer), 5′-TGT GCT GAA GAC TTT-3′ (A-allele probe labeled with Vic), and 5′-CTG TGC TGA TGA CTT T-3′ (T-allele probe labeled with Fam). Genotype results for the dogs included in the study were confirmed with two separate runs.

Statistical Analysis

All experimental measurements regarding the extent of plasma protein binding are reported as means ± S.D. (n = 12) unless otherwise noted. In vitro experiments were performed with two (D01-4582) or three (celecoxib, mycophenolic acid, and meloxicam) replicates per sample, randomly distributed in each run, on 3 separate days. Descriptive and comparative analysis was performed using Microsoft Office Professional Plus Excel 2013 (Microsoft Corporation, Redmond, WA) and SigmaPlot 12 software (Systat, San Jose, CA) to analyze and report study results. ChemDraw 18.1 software (Cambridge, MA) was used to construct Fig. 1. GraphPad Prism 3.03 software (La Jolla, CA) was used to construct Fig. 3. Normality of the unbound concentrations for each analyte was assessed with the Shapiro–Wilk test. Statistical power analysis was performed for sample size estimation, based on data from Ito et al. (2009), comparing dogs with and dogs without c.1075G>T and c.1422A>T variants. Median unbound fractions for all drugs were compared using the Mann–Whitney U test to determine differences in the extent of plasma protein binding between dogs with

haplotypes H1 and H2. Statistical differences between the mean serum albumin concentration of each group were evaluated with the unpaired t test. For all comparisons, statistical significance was set at ≤ 0.05.

Results

Canine Albumin Exon Sequencing

All 14 exons in the canine albumin gene were sequenced using DNA samples from 100 dogs representing 37 different breeds, and the results were compared with the reference sequence (NW_003726083). Six exons (2, 6, 7, 10, 12, and 14) showed no variation from the reference sequence, whereas eight exons (1, 3, 4, 5, 8, 9, 11, and 13) contained SNPs. Of the SNPs identified, 13 were synonymous (data not shown) and 3 were nonsynonymous (Table 3). Among the three nonsynonymous SNPs, c.268C>T was relatively uncommon. On the other hand, c.1075G>T and c.1422A>T were present in 90 of 100 dogs sequenced. Because of the low frequency of the uncommon nonsynonymous SNPs, they were not investigated further.

TABLE 3

Canine albumin gene nonsynonymous SNPs identified by sequencing all exons in 100 dogs representing 37 different breeds

Exon	cDNA	Amino Acid	Number of Dogs		
			REF	HET	VAR
3	c.268C>T	p.Leu91Phe	98	2	0
9	c.1075G>T	p.Ala359Ser	24	32	44
11	c.1422A>T	p.Glu474Asp	24	30	46

The position of each variant is given in reference to the first nucleotide of the coding sequence (for cDNA) or first amino acid of the predicted protein. The number of dogs with the reference (REF), heterozygous (HET), or homozygous variant (VAR) sequence for each SNP is listed.

Effect of H1 and H2 Allelic Variation on Drug Binding to Plasma

Drug plasma protein binding was evaluated in plasma samples collected from dogs homozygous for the H1 allele ($n = 6$) and the H2 allele ($n = 6$). Plasma albumin concentrations in all dogs tested ($n = 12$) were considered within normal limits (range, 2.5–3.3 mg/dl), with no statistically significant difference identified between the H1 and H2 allele groups ($P = 0.41$). The extent of drug binding (expressed as the mean ± S.D. percent bound) for all 12 dogs was 99.93 ± 0.04 for D01-4582, 98.22 ± 0.3 for celecoxib, 95.36 ± 1.1 for mycophenolic acid, and 94.90 ± 1.72 for meloxicam.

Considering the high extent of protein binding of the compounds analyzed as well as the pharmacological importance of unbound (free) drug, free drug fractions for each genotype are reported (Fig. 2). Unbound fraction values calculated for each plasma-drug combination had a CV < 20% (data not shown). Median unbound drug fractions with D01-4582 and celecoxib were considerably smaller (<2%) than those determined for mycophenolic acid and meloxicam. Free drug fractions in plasma from dogs with the albumin H1 allele ranged from 0.02% to 0.13% (mean 0.06% ± 0.04%) for D01-4582, 1.72% to 2.43% (1.86% ± 0.34%) for celecoxib, 4.03% to 7.80% (6.10% ± 1.29%) for meloxicam, and 3.92% to 6.51% (5.17% ± 0.83%) for mycophenolic acid. Plasma from dogs with albumin H2 allele had free drug fractions between 0.04% and 0.18% (0.09% ± 0.05%) for D01-4582, 1.41% and 2.00% (1.69% ± 0.19%) for celecoxib, 2.38% and 5.93% (4.10% ± 1.55%) for meloxicam, and 2.75% and 5.35% (4.10% ± 1.11%) for mycophenolic acid. The difference in free drug fraction between albumin genotypes reached statistical significance for meloxicam only ($P = 0.0411$).

Canine Albumin c.1075G>T and c.1422A>T Haplotypes and Breed Distribution

c.1075G>T and c.1422A>T genotypes were determined in 1260 dogs from 61 different breeds and 186 mixed-breed dogs. Haplotypes that were resolved by inference are shown in Table 4 and haplotype frequencies for each breed are given in Supplemental Table 1. As shown in Fig. 3, the frequency distribution of albumin haplotypes varied greatly across dog breeds. H1 (reference) and H2 (variant for both SNPs) were the predominant haplotypes in all dog breeds examined. Haplotype H1 was most frequently detected in Cocker Spaniels (95%), Miniature Dachshunds (89.5%), and Italian Greyhounds (75.6%), whereas haplotype H2 was most common in Great Danes (100%), Basenjis (98%), Rottweilers (97.5%), Golden Retrievers (97.1%), Boxers (95%), and Yorkshire Terriers (95%). Median and interquartile range (IQR) frequencies for haplotypes H1 and H2 across all breeds were 39% (25%–50%) and 60% (50%–71%), respectively. By comparison, H1 and H2 haplotype frequencies in mixed-breed dogs were 37% and 63%, respectively. Haplotype H3 (c.1075G>T) was found in nine breeds, including Cirneco dell'Etna, Pomeranian, Portuguese Podengo, Weimaraner, Pembroke Welsh Corgi, Australian Shepherd, Rhodesian Ridgeback, Peruvian Inca Orchid, and Afghan Hound, with a median breed frequency of 5.9% (IQR, 2.8%–14%). Haplotype H4 (c.1422A>T) was found in 11 breeds, including Afghan Hound, Border Collie, Siberian Husky, Borzoi, Pharaoh Hound, Greyhound, Longhaired Whippet, Whippet, Italian Greyhound, Silken Windhound, and Scottish Deerhound, with a median breed frequency of 1.8% (IQR, 1.1%–4.0%). Afghan Hound was the only breed with all four haplotypes detected. Haplotypes H3 and H4 were not found in any of the 186 mixed-breed dogs tested.

Discussion

In our study, canine albumin genotype affected plasma protein binding of meloxicam, with no significant difference detected between dogs with mean free fractions of the albumin H1 allele (reference) and the H2 allele (c.1075G>T and c.1422A>T) for the other three drugs assessed (D01-4582, celecoxib, and mycophenolic acid). Drugs selected for our study had high preferential binding to albumin sites I or II and a narrow therapeutic index (Er et al., 2013). Meloxicam and mycophenolic acid were used in our experiments due to their preferential binding in humans to albumin site I and site II, respectively (Nowak and Shaw, 1995; Er et al., 2013). In addition, celecoxib and D01-4582 were used because of their reported pharmacokinetic variability in dogs that could be associated with differences in protein binding (Paulson et al., 1999a; Ito et al., 2009).

Our results were consistent with the mean extent of plasma protein binding previously reported for dogs (99.9% for D01-4582, 97% for meloxicam, 98.5% for celecoxib, and 95%–97% for mycophenolic acid) (Busch et al., 1998; Paulson et al., 1999b; Ito et al., 2009; Morassi et al., 2018). For D01-4582, our findings differ from a previous report (Ito et al., 2009) in which free drug fractions were greater in plasma from Beagles with the albumin H2 allele than in plasma from Beagles with the H1 allele. Free fractions of D01-4582 in Beagles were reported to be 0.029% ± 0.017% (CV = 59%, $n = 35$), with dogs with the albumin H1 allele having statistically significant lower values (0.015% ± 0.003%) compared with dogs with the albumin H2 allele (0.059% ± 0.016%) (Ito et al., 2009). In our study population, D01-4582 free fractions were somewhat higher (mean 0.07% ± 0.04%), with no statistically significant difference between dogs with the albumin H1 allele and those with the albumin H2 allele. There are some possible explanations for the contrasting results between the two studies. First, there are differences in the canine populations assessed. Whereas a single research Beagle colony was evaluated in the previously published study, our experiments

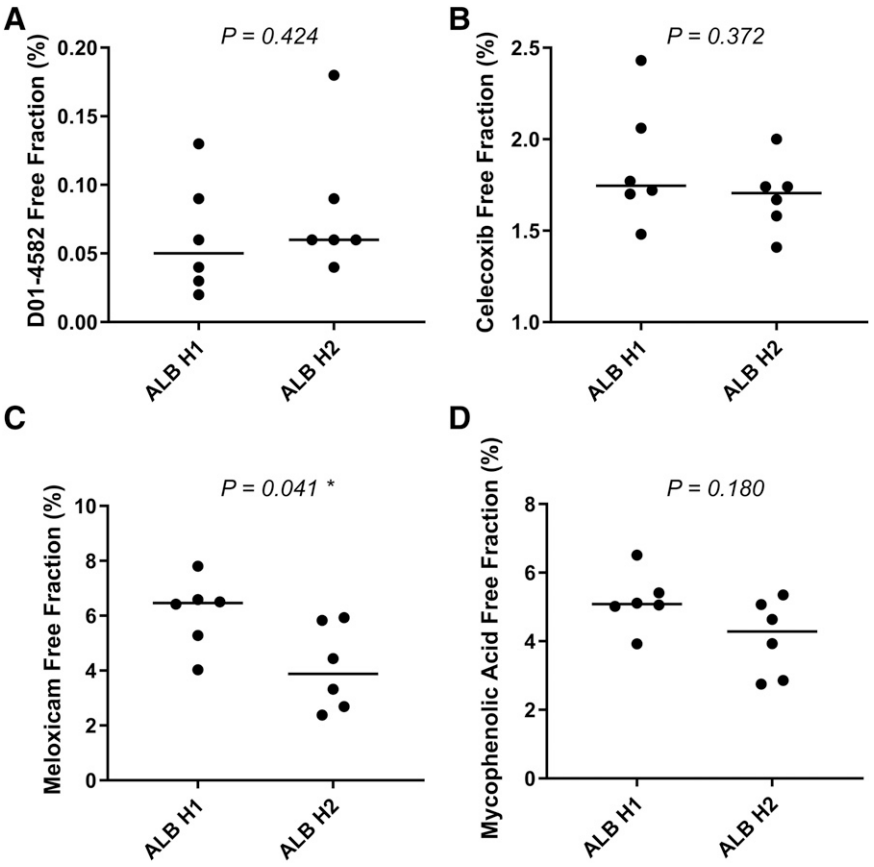


Fig. 2. Scatter dot plots of unbound fractions of D01-4582 (A), celecoxib (B), meloxicam (C), and mycophenolic acid (D), in relation to the albumin haplotype (H1 and H2) for each dog evaluated ($n = 6$ per group; average of three replicates from three separate runs). Lines represent the median unbound fraction for each compound. The difference between the median unbound fraction for both haplotypes was statistically significant for meloxicam ($P = 0.041$) but not the other compounds analyzed (Mann–Whitney U test, significance set at $^*P < 0.05$). ALB, albumin.

included plasma from multiple dog breeds. Lower free fractions of D01-4582 may be common in Beagles; however, the inclusion of different breeds, representing greater genetic heterogeneity, likely increased the group biologic variability and resulted in higher free drug fractions. Evaluating plasma protein binding in dogs from multiple breeds likely provides a more comprehensive and representative assessment of the overall canine population; however, the higher variability in free drug fractions may have contributed to the lack of statistical differences between groups. A second explanation is associated with the potential variability of lipid fraction in the plasma from our study population. D01-4582 is a highly lipophilic drug with plasma protein binding >99%, which makes it likely to bind to lipoproteins in the plasma. Ultracentrifugation leads to the formation of a superficial chylomicron layer that can make the collection of supernatant difficult. In plasma samples, the presence and size of this chylomicron layer can lead to overestimation of free drug fraction. Even though plasma samples were collected while fasting, they were obtained from dogs from a variety of breeds, food regimen and body condition scores that likely contributed to a lipid profile more diverse than the one obtained from the research Beagle colony used by Ito et al. (2009). Breed, as well as diet and body condition score, has been identified as a source of difference in plasma lipids in dogs and could have contributed to the higher biologic variability in plasma lipid content in our samples compared with colony research animals (Downs et al., 1993; Jeusette et al., 2005). For celecoxib, our findings suggest that albumin genotype is unlikely to have caused the pharmacokinetic variability reported by Paulson et al. (1999a).

TABLE 4

Canine albumin c.1075G>T and c.1422A>T haplotypes

Haplotype	c.1075G>T	c.1422A>T
H1	G	A
H2	T	T
H3	T	A
H4	G	T

Multiple factors other than genotype can affect binding to albumin in plasma and may have contributed to the differences noted between dogs. Endogenous compounds in plasma (e.g., globulins, cells, or metabolites) can influence free drug fractions by competitively displacing drugs from albumin binding sites and/or inducing conformational changes in the protein (Otagiri, 2005). For example, bilirubin and free fatty acids can competitively bind to human albumin and increase free drug fractions of the antibiotic cefazolin in vitro (Decroix et al., 1988). Although the content of other plasma components in study samples was not controlled, we estimate their impact to be small for celecoxib, meloxicam, and celecoxib considering their high binding preference for albumin at the drug concentrations used in our experiments (below the reported C_{max} in dogs). Lower albumin concentrations can also increase drug free fractions, in particular for drugs highly bound to albumin (Ikenoue et al., 2000). Plasma albumin concentrations in our study were considered normal and unlikely to affect our results, with no statistical difference in albumin concentrations between allele groups. Albumin can also undergo post-translational modifications (e.g., glycation, cysteinylation, and carbamoylation) that can cause structural changes to the molecule and affect albumin's ability to interact with some drugs (Lee and Wu, 2015). These were not assessed in our study population. Sex-related differences in our plasma protein binding experiments were not statistically significant ($P > 0.05$).

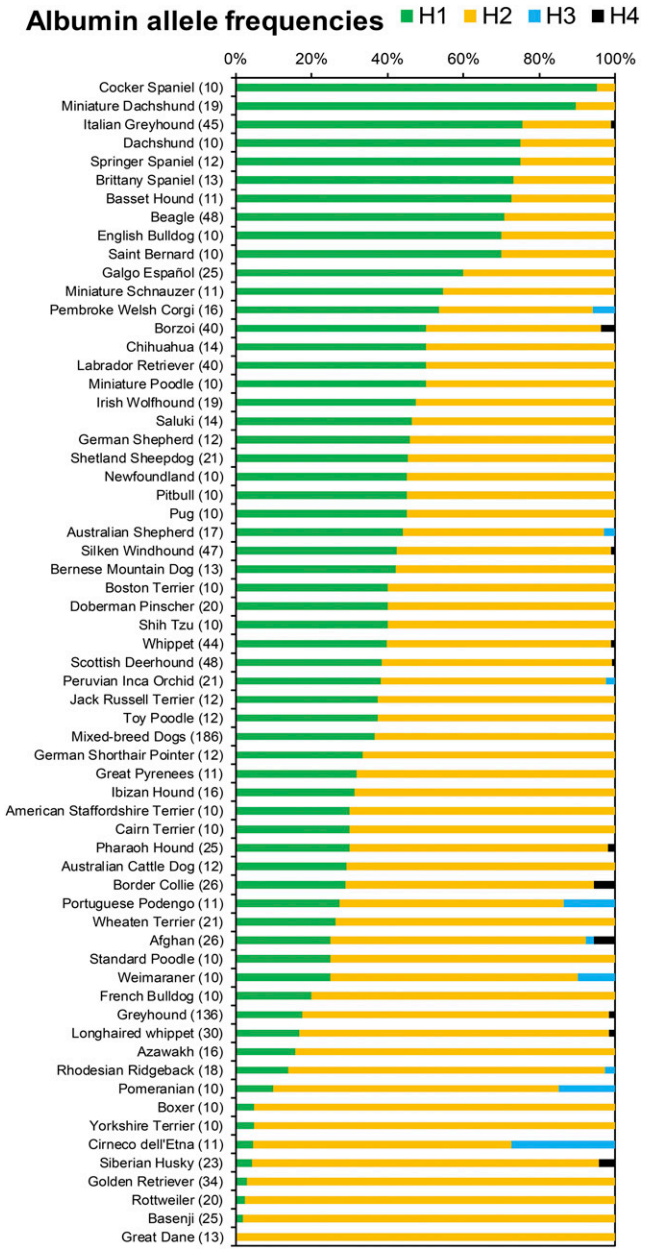


Fig. 3. Albumin H1, H2, H3, and H4 allele frequencies determined for 61 dog breeds and mixed-breed dogs. The number of dogs sampled per breed is given in parentheses.

Albumin genotype seemed to impact the plasma protein binding of meloxicam in our ex vivo study, as dogs with the albumin H1 allele had a significantly higher free drug fraction than dogs with the albumin H2 allele ($P = 0.041$). This contrasts the initial hypothesis that the albumin H1 allele would be associated with lower free drug fractions (and therefore higher binding). Unlike the other three drugs tested in our study, meloxicam is the only one that binds to albumin site I in humans (Er et al., 2013). Although the preferential binding site for these drugs has not been extensively evaluated in canine albumin, it is possible that the role of albumin genotype in plasma protein binding is drug dependent. While the amino acid changes associated with the albumin H2 allele (c.1075G>T and c.1422A>T) may decrease binding of drugs commonly bound to site II, they may have the opposite effect on drugs bound to a different region of the albumin molecule in dogs. The difference in the mean free drug fraction of meloxicam between albumin genotypes noted ex vivo may be relevant and should be confirmed in vivo. Although changes in free drug fraction can affect total drug concentrations in vivo, the impact on free drug concentrations and pharmacologic effect is usually minor.

Another new finding of our study is that c.1075G>T and c.1422A>T polymorphisms are independent, occurring separately in a small percentage of dogs. Although median allele frequencies in Beagles in our study population were similar to those in a previous report (Ito et al., 2009), they varied considerably between dog breeds. This difference in allele frequencies may be particularly important for veterinary drug development programs since single-breed studies may introduce bias in preclinical results.

Interestingly, the presence of more than one form of canine albumin was first reported in 1985, with two albumin phenotypes ("slow" and "fast") identified based on their electrophoretic mobility in a large population of dogs (Christensen et al., 1985). The H2 allele breed frequencies from our study had a strong correlation ($Rs = 0.86$; $P < 0.0001$) with the corresponding frequencies for the slow form reported by Christensen et al. (1985) (Fig. 4). This indirectly suggests that the slow migration pattern of the S variant could result from the two predicted amino acid changes associated with the presence of the H2 allele. Unfortunately, we were unable to reproduce the starch gel electrophoresis technique used in the previous report to determine the electrophoretic mobility (phenotype) of the dogs used in our study.

The findings of our study should be interpreted considering its limitations. Comparing binding properties between genetic variants was previously done using a small number of marker ligands for different binding regions of human serum albumin (Kragh-Hansen et al., 1990; Vestberg et al., 1992). Although binding properties of human albumin have been extensively investigated, similar information regarding canine albumin is scarce. Canine albumin is expected to follow the same tertiary molecular structure as human albumin; however, it may differ in the number and specificity of binding sites (Kosa et al., 1997). Therefore, it is possible that the drugs we intended to interrogate sites I and II may actually bind to different sites on canine albumin. Our findings cannot predict the effect of c.1075G>T and c.1422A>T polymorphisms on

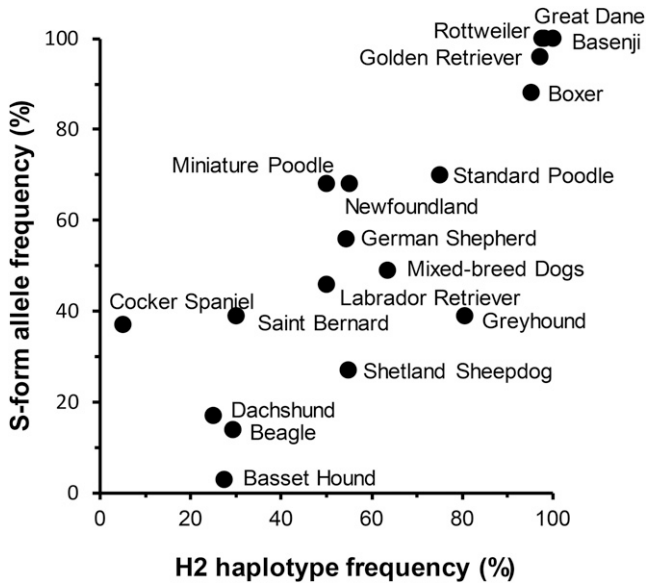


Fig. 4. Comparison of H2 haplotype frequencies determined in this study with frequencies of the albumin electrophoretic mobility slow (S) form allele reported previously by Christensen et al. (1985) for the same dog breeds. Also shown is the Spearman correlation coefficient and associated P value (significance set at $P < 0.05$).

specific binding sites and/or on plasma protein binding of other compounds. For that reason, additional studies evaluating the impact of albumin genotype on the extent of plasma protein binding of a large variety of drugs are recommended. Although the difference in plasma protein binding of meloxicam seen in our ex vivo study suggests a significant impact of albumin genotype on free drug fraction, this should be confirmed with in vivo studies. The lack of significant differences in free drug fractions of D01-4582, celecoxib, and mycophenolic acid between albumin genotypes in our study is likely unrelated to the number of dogs evaluated in each group ($n = 6$), since the previous report (Ito et al., 2009) included only five dogs with the H2 allele that showed statistically higher free drug fractions of D01-4582 compared with dogs with the H1 allele.

In conclusion, this study provides novel information on the frequency and pharmacokinetic impact of the most common nonsynonymous canine albumin polymorphisms (c.1075G>T and c.1422A>T) across multiple breeds. In our study, dogs with the albumin H1 allele had significantly higher free drug fractions (and consequently lower plasma protein binding) of meloxicam than dogs with the albumin H2 allele. Albumin alleles H1 and H2 seem to be the most frequent across breeds and c.1075G>T and c.1422A>T are not completely linked, a finding that has not been previously reported. Our findings suggest that albumin genotype may influence free drug fractions at physiologically relevant concentrations of select drugs in dogs. Because changes in plasma protein binding commonly do not affect free drug concentrations, the significance of the influence of albumin genotype in vivo will depend on the drug studied and its intrinsic clearance. However, albumin genotyping may improve the interpretation of pharmacokinetic data generated in canine research populations during the developmental process of human and veterinary drugs.

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Authorship Contributions

Participated in research design: Costa, Court, Mealey, Villarino.

Conducted experiments: Costa, Burke, Zhu.

Contributed new reagents or analytic tools: Court, Mealey, Villarino.

Performed data analysis: Costa, Court, Mealey, Villarino.

Wrote or contributed to the writing of the manuscript: Costa, Court, Mealey, Villarino.

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