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Impact of Interindividual Differences in Plasma Fraction Unbound on the Pharmacokinetics of a Novel Syk Kinase Inhibitor in Beagle Dogs[S]

Andy Pike, Barry Jones,[1] Roshini Markandu,[2] and Daniel O'Neill

DMPK (A.P., R.M., B.J.) and Discovery Biology, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Cambridge, United Kingdom

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ABSTRACT

Inconsistencies in pharmacokinetic parameters between individual animals in preclinical studies are a common occurrence. Often such differences between animals are simply accepted as experimental variability rather than as indications of specific differences in animal phenotype that could lead to a different interpretation of the data. The fraction unbound in plasma is one factor influencing pharmacokinetic parameters and is typically determined using pooled plasma from multiple animals, making the assumption that there is limited population variance. However, this assumption is not often tested and may not hold true if there are polymorphisms affecting binding or variation in the concentrations of individual plasma proteins that could give rise to different fraction unbound phenotypes in individual animals. During profiling of a novel Syk inhibitor, AZ8399, striking interindividual differences in total plasma clearance and volume of distribution were observed between dogs consistent with differences in fraction unbound between animals. Determination of the fraction unbound showed a ~5-fold difference in fraction unbound between the animals in the study. Broader analysis of individual dogs across a colony demonstrated a correlation between individual animal fraction unbound with total plasma clearance and volume of distribution. The concentrations of the common drug-binding proteins albumin and α1-acid glycoprotein in plasma were determined, and α1-acid glycoprotein levels were found to correlate with fraction unbound. Finally, single-nucleotide polymorphisms were identified at c.502 and c.522 of exon 5 of the dog α1-acid glycoprotein gene that may be correlated to the α1-acid glycoprotein concentration phenotype observed.

SIGNIFICANCE STATEMENT

The current work demonstrates the potential for significant interindividual differences in plasma fraction unbound in beagle dogs and goes on to examine the underlying cause for the compound described. The findings suggest that the application of a population mean value of fraction unbound generated from a pooled sample may not always be appropriate and could introduce significant errors in scaling of in vitro clearance values, PBPK understanding, and interpretation of PKPD or toxicokinetic data in the context of unbound concentrations.

Introduction

Observing inconsistent pharmacokinetic parameters between individual animals in preclinical pharmacokinetic (PK) studies is a common occurrence, especially after oral dosing in which absorption is an additional variable. However, it can be challenging to explain such observations after the fact, and it is often tempting to accept differences between animals as simply reflecting experimental variability rather than as evidence of specific differences in animal phenotype that could lead to a different interpretation of the data.

In drug discovery, the fraction unbound (f_u) in plasma is often determined in preclinical species for purposes of in vitro to in vivo scaling of clearance, understanding of PBPK relationships and interpretation of PKPD, or toxicokinetic exposure data in the context of unbound concentrations. Typically, it is determined using pooled plasma from multiple individuals and, concentration dependence aside, the resulting value is usually assumed to represent a broadly applicable mean with the potential for variability within the population rarely considered.

However, there are two reasons why this assumption may not hold in practice. Firstly, concentrations of plasma proteins can change in response to physiological factors, such as inflammatory state. For example, it has been reported that inflammatory disease in dogs can give rise to a ~6-fold increase in α1-acid glycoprotein (AGP) concentration with a corresponding increase in drug binding (Dello et al., 1988). It can be hypothesized that such an effect would be likely to result in a continuum of values for protein concentration and, therefore, drug f_u proportional to the severity of inflammation in cases in which binding is principally to AGP. Secondly, genetic variation within the population may affect protein expression or produce proteins with altered binding affinity for drugs. For example, Nagase rats do not express serum albumin because of a seven base pair deletion in the gene that prevents mRNA splicing (Nagase et al., 1979). Similarly, some extremely rare cases of analbuminemia have been reported in humans arising from various mutations of the albumin gene (Watkins et al., 1994). In beagle dogs, a pair of linked single-nucleotide polymorphisms (SNPs),

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[1]Current affiliation: Pharmaron, Rushden, United Kingdom.
[2]Current affiliation: Regulatory Safety, Clinical Pharmacology & Safety Sciences, R&D, AstraZeneca, Cambridge, United Kingdom.
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ABBREVIATIONS: AGP, α1-acid glycoprotein; CL_p, plasma clearance; f_u, fraction unbound; HPLC, high-performance LC; LC, liquid chromatography; MeCN, acetonitrile; PBPK, physiologically based PK; PK, pharmacokinetic; PKPD, PK pharmacodynamic; SNP, single-nucleotide polymorphism; V_{ss}, volume of distribution steady state.

Ala335Ser and Glu450Asp, in the albumin gene has been reported to result in the expression of albumin with altered binding affinity (Ito et al., 2009). The compound reported in this study (D01-4582) was an acidic $\alpha4\beta1$ integrin antagonist and showed an approximately 4-fold greater f_u than those for "wild-type" dogs.

AZ8399 was synthesized as a novel, highly selective Syk kinase inhibitor for the treatment of diffuse large B-cell lymphoma and related conditions (Fig. 1). The origin of this compound has previously been described, but for the purposes of this manuscript it should be noted that the compound is a moderately lipophilic ($logD_{7.4}$ = 2.4) weak base (pK_a = 7.3) (Grimster et al., 2020).

Unusual data were observed in a dog PK study of AZ8399 with both plasma clearance (CL_p) and volume of distribution steady state (V_{ss}) showing a significant and comparable fold difference between animals. The current work examines interindividual differences in AZ8399 dog pharmacokinetics within a dog colony resulting from differences in plasma f_u. The underlying changes in AGP concentration and a potentially causative genetic mutation are also discussed.

Materials and Methods

Compound. AZ8399 was obtained from AstraZeneca compound management (synthesis previously reported as compound 30 in Grimster et al., 2020) (Fig. 1).

Reagents. Rapid Equilibrium Dialysis Device Single-Use Plate with Inserts, 8K MWCO, and PBS pH 7.4 were obtained from Thermo Fisher Scientific (Loughborough, UK). Pooled male control beagle plasma was obtained from BioReclamationIVT (Frankfurt, Germany). Albumin and α1-acid glycoprotein (AGP) ELISA kits and dog albumin protein were obtained from Abcam (Cambridge, UK). Acetonitrile (MeCN) and methanol (HPLC grade) were obtained from HiPerSolv CHROMANORM. Formic acid (analytical grade >98%) was obtained from Merck KGaA (Darmstadt, Germany).

Animals. Pharmacokinetic studies were conducted in male beagle dogs at Charles River Laboratories (Tranent, UK). Dogs 001M–004M, 007M, and 008M were transferred to Charles River Laboratories from AstraZeneca (Alderley Park, UK). All other animals were obtained from Marshall BioResources, UK. Animals were in the range 3–7 years of age and weighed 12–17 kg at time of use. All in vivo experiments were performed according to the UK Home Office Animals Scientific Procedures Act 1986 under project license number PF6EFEB3E and approved by Charles River Laboratories Animal Welfare and Ethical Review Body. As standard, the animals were offered food each day based on body weight. Food was withdrawn on the afternoon preceding dose administration, and the dogs were then fed approximately 3 hours after end of intravenous infusion administration of the test compound AZ8399. Tap water was available ad libitum. Animals were kept to 12-hour light/dark cycle. ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were used for reporting the procedures (Kilkenny et al., 2010).

Dog Pharmacokinetic Studies. AZ8399 was formulated as a solution in 5% DMSO:95% cyclodextrin solution (30% w/v) at 0.5 mg/ml. Dogs were singly housed for the duration of the sampling period to enable urine collection (unpublished data). The compound was administered by intravenous infusion over 15 minutes (dose volume of 1 ml/kg) (n = 8, of which two animals, 003M and 004M, were coadministered ("cassette-dosed") with three other compounds (see Supplemental Material). Blood samples (approximately 1 ml) were collected via the jugular vein into K_2EDTA tubes predose and 0, 0.0833, 0.25, 0.283, 0.333, 0.417, 0.75, 1.25, 3.25, 6.25, 12.25, and 24.25 hours after intravenous administration; plasma was prepared by centrifugation and stored at −20°C prior to analysis.

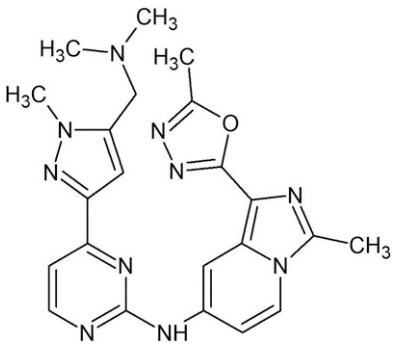

Fig. 1. Structure of AZ8399.

Bioanalysis of AZ8399 in Dog Pharmacokinetic Experiments. Plasma, 100 µl (plasma control matrix, bulk standard/quality control, or sample), was added to a 96 round well bottom plate, and protein was precipitated with internal standard in acetonitrile (300 µl) and pelleted by centrifugation. Then 100 µl of the supernatant was transferred to a clean plate, 100 µl of water/formic acid (100/0.2, v/v) was added, and the samples were vortex-mixed prior to analysis. A calibration curve was prepared, and these were injected at the beginning and end of each set of plasma and dose aliquot samples.

Quantitative data were acquired on a Sciex API4000 mass spectrometer connected to a Perkin Elmer Series 200 LC pump using a CTC Analytics HTS Pal autosampler. Separation was achieved by reversed-phase liquid chromatography using a Poroshell EC-C18 column (2.1 × 50 mm, 2.7 µm) in a column oven set to 60°C. Mobile phase A consisted of MeCN/formic acid (100/0.2, v/v), and mobile phase B consisted of H_2O/formic acid (100/0.2, v/v). The flow rate was 0.75 ml/min with the following elution gradient: linear gradient 2% A to 100% A, 0.00 to 1.2 minutes; isocratic hold, 100% A 1.20 to 1.8 minutes; gradients from 100% A to 100% B in 0.2-minute cycles until reaching 100% at 3.0 minutes: and 100% A to 2% A, 3.0 to 3.5 minutes.

The mass spectrometer was equipped with an electrospray ionization source, which was operated in positive ion mode, and the settings were as follows: ionspray voltage = 5500 V; source temperature was set to 600°C; curtain gas = 20; collision gas = 6; gas 1 setting = 50; and gas 2 setting = 50. Detection of the ions was performed in the multiple reaction monitoring mode 445.2 > 372.3, with a dwell time of 75 milliseconds, declustering potential of 75 eV, entrance potential of 11 eV, collision energy of 36 eV, and cell exit potential of 11 eV. Peak integration was performed using Analyst (Version 1.6.2) software.

The determined concentration for calibration standards was within 100% ± 25% of the nominal concentration. Quality control sample accuracy and precision was within 100% ± 25% and ≤25%, respectively. The limit of quantification was 1 nM.

Determination of Unbound Fraction in Dog Plasma. Fraction unbound was determined by equilibrium dialysis using Rapid Equilibrium Dialysis single-use plates. After the initial cassette PK study in dogs 003M and 004M, plasma protein binding was determined in the pooled 0.083- and 0.25-hour plasma samples giving a 0.8–2 µM incubation concentration. Subsequently, f_u was determined in blank samples from individual colony animals and pooled male beagle control plasma at 5 µM to ensure adequate analytical sensitivity, for potentially lower f_u samples, and for comparison with historical data at that concentration. Values for dogs 003M and 004M were comparable between the pharmacokinetic study samples and spiked control plasma (see Table 1).

To determine the fraction unbound in dog plasma, 400 µl of PBS pH 7.4 was added to the buffer chamber as well as 200 µl of plasma containing compound to the sample chamber. The unit was sealed and incubated for 18 hours in an incubator shaker at 37°C and 100 rpm. At the end of the incubation, samples (25 µl) from both buffer and plasma chambers were removed for analysis. Samples and standards were matrix-matched, and protein was precipitated with 200 µl MeCN containing internal standard. The samples were then centrifuged at 3000 rpm for 10 minutes, and 50 µl of supernatant was diluted with 300 µl water before LC–tandem mass spectrometry analysis. The f_u in plasma was calculated as follows:

$$f_u = \text{Conc.}_{\text{buffer chamber}}/\text{Conc.}_{\text{plasma chamber}}$$

AZ8399 calibration curves were prepared by sequential dilution of a 2 mM stock in 100% DMSO to give working solutions of 0.02 to 200 µM. Then 2.5 µl of the DMSO stocks were spiked into 47.5 µl control dog plasma to generate a 1–10000 nM calibration curve (a DMSO control was also included). The calibration standards were then processed as stated for samples.

Bioanalysis of AZ8399 in Protein Binding Experiments. Quantitative data for the protein binding experiments was acquired on a Waters Xevo TQ-XS mass spectrometer connected to a Waters Acquity Ultra HPLC. The Waters Acquity system consisted of an autosampler, binary Ultra HPLC pump, column oven, autoinjector, and a photodiode array detector. Analytes (AZ8399 and internal standard) were separated by reversed-phase liquid chromatography

TABLE 1

Summary of f_u, albumin, and AGP conc. and pharmacokinetic parameters in individual colony dogs

Data shown in italics indicate animals received AZ8399 as part of a cassette-dosing study with three other compounds (see Supplemental Material).

Dog	f_u	Albumin	AGP	CL_p	$CL_{p,u}$[c]	V_{ss}	$V_{ss,u}$[c]
		mg/ml	*μg/ml*	*ml/min/kg*	*ml/min/kg*	*l/kg*	*l/kg*
001M	0.051	36.6	207	6.4	125	1.5	29
002M	0.027	33.1	300	7.2	268	1.4	52
003M	*0.091 (0.10)*[a]	*27.7*	*76.0*	*16*	*173*	*5.0*	*55*
004M	*0.021 (0.020* [a]	*25.5*	*465*	*4.1*	*202*	*1.7*	*80*
005M	0.019 (0.016)[b]	27.0	421	3.4	180	1.1	58
006M	0.025 (0.035)[b]	26.5	292	5.8	236	1.9	77
007M	0.090	23.9	90.4	14	158	5.0	55
008M	0.082	27.8	86.6	12	143	4.7	57
009M	0.10	23.3	109	No data generated			
010M	0.12	23.7	105				
011M	0.037	16.3	595				
012M	0.034	16.9	543				
013M	0.034	11.9	588				
014M	0.027	26.7	833				
Control plasma[d]	0.089						
Mean				9	186	2.8	58
S.D.				4.7	48	1.8	16
CoV[e] (%)				54	26	64	27

[a]Values shown in () were determined from pooled pharmacokinetic plasma samples after an initial cassette PK study.
[b]Values shown in () are from a second set of samples taken from dogs 005M and 006M at the same time as those from dogs 009M—014M.
[c]Subscript "u" denotes value corrected for fraction unbound. $CL_{p,u}$ = unbound plasma clearance; $V_{ss,u}$ = unbound volume of distribution steady state.
[d]Pooled male control plasma (see *Materials and Methods*).
[e]Coefficient of variation.

using a Kinetex C18 column (50 × 2.1 mm, 2.6 μm) preceded by a Krud-Katcher Ultra in-line filter (Phenomenex, Macclesfield, UK) in a column oven at 60°C. The mobile phase consisted of HPLC grade water with 0.1% formic acid (eluent A) and methanol with 0.1% formic acid (eluent B). The elution profile was: linear gradient 95% A to 5% A, 0.00 to 1.5 minutes; isocratic hold, 5% A 1.50 to 2.6 minutes; and re-equilibration 95% A, 2.61 to 2.8 minutes. The flow rate was 0.6 ml/min; eluent was introduced to the mass spectrometer via the divert valve at 0.5 minutes.

The Xevo TQ-XS was equipped with an electrospray ionization source, which was operated in positive ion mode. The mass spectrometer source settings were as following: the capillary voltage was 0.6 kV. The desolvation temperature was set to 600°C. Nitrogen was used as the desolvation gas (1000 l/h) and cone gas (150 l/h). Detection of the ions was performed in the multiple reaction monitoring mode, 445.2472 > 371.9968 (parent > daughter m/z), dwell time of 0.163 seconds, cone voltage 34 V, and collision energy of 26 V. Peak integration and calibrations were performed using TargetLynx software (Version 4.1, Waters, Milford, MA). AZ8399 concentrations were quantified using a linear regression calibration model with a 1/x weighting factor. The percentage deviation for individual standards differed by no more than ±30% from the nominal concentration, and >70% of all calibration standards passed this acceptance criteria. The limit of quantitation for AZ8399 was 5 nM.

Quantification of Dog Albumin and α1-Acid Glycoprotein. Sandwich ELISA was used to quantify the concentration of albumin and AGP in the individual and pooled dog plasma samples. Albumin and AGP amounts were quantified using the canine albumin and canine AGP ELISA kits, respectively. The ELISA procedure was carried out as stated in the manufacturer's protocol. All samples were run in duplicate. The ELISA absorbance at 450 nm was obtained using a Spectramax i3 (Molecular Devices, San Jose, CA). Data interpretation was performed in GraphPad Prism version 8.0.1 using a four-parameter algorithm to fit the standard curve. To estimate the protein levels in the plasma, the unknown sample concentrations were then interpolated against the standard curve.

DNA Sequencing of α1-Acid Glycoprotein Gene. Blood samples were collected from all dogs using the PAXgene Blood DNA Tubes according to manufacturer's instructions and stored at −20°C pending processing (QIAGEN, UK). DNA was subsequently extracted using the PAXgene Blood DNA Kit according to manufacturer's instructions (QIAGSEN, UK) and eluted in nuclease-free water (Ambion). Each exon of canine α1 acid glycoprotein was fully Sanger-sequenced by Eurofins Genomics (Ebersberg, Germany); a full report is available (see Supplemental Material).

Statistical Analysis. Dog pharmacokinetic data for AZ8399 were collected on animals 001M–008M inclusive (n = 8), as these animals comprised the colony at the start of the experimental period. The data set was compiled from three separate pharmacokinetic studies in which the following animals were included: 1) October 2017: 003M and 004M, 2) May 2018: 006M and 007M, 3) September 2018: 001M, 002M, 005M, and 008M. Data from all animals are included in subsequent analysis.

No pharmacokinetic data were collected on animals subsequently introduced to the colony (009M–014M). As the objective of the study is to compare the plasma concentration versus time profiles and calculated pharmacokinetic parameters, no control or reference groups (e.g., nondosed animals) were used during this study.

Correlation of CL_p, V_{ss}, and f_u of dogs 001M–008M to concentrations of albumin and AGP were assessed using two-sided Spearman's rank correlation coefficient (GraphPad Prism, version 8.4.2), assuming a monotonic relationship. P value < 0.05 was considered statistically significant.

For comparison of AGP concentrations between genotypes, a combination of Mann-Whitney and Kruskal-Wallis tests were used (GraphPad Prism, version 8.4.2). Data from dogs 001M–014M were included in the analysis. See *Results* section for description of specific tests applied to individual exons.

Results

Dog Pharmacokinetics. As part of an initial dog cassette PK study, AZ8399 was dosed to dogs 003M and 004M and showed large interindividual differences in CL_p (16 vs. 4.1 ml/min/kg) and V_{ss} (5.0 vs. 1.7 l/kg). Other compounds in the cassette (AZ-Y and AZ-Z) study showed a similar trend, with interindividual differences being apparently inversely correlated with f_u (see Supplemental Fig. 2; Supplemental Table 1). However, one compound (AZ-X), with a high f_u of 0.67, which showed no significant difference between animals, suggesting an inadvertent misdose was unlikely to be the explanation for the interanimal differences observed for the other compounds. Although a difference in intrinsic clearance between the animals could not be ruled out, the apparent difference in unbound V_{ss} was unusual, and the comparable magnitude of the shift in CL_p and V_{ss} suggested the two observations might be related.

After confirmation that the f_u did in fact vary considerably between individuals (see next section), the remaining dogs in the colony (at the time)

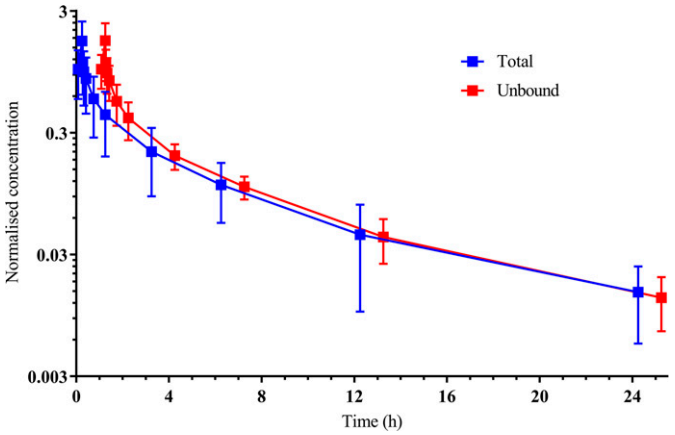

Fig. 2. Normalized total and unbound concentration versus time profiles of dogs 001M–008M (mean ± S.D., n = 8). For purposes of comparison, total and unbound concentrations have been normalized to the first concentration measured (value = 1). Unbound concentrations are offset by +1 hour.

were subsequently administered with AZ8399 at the same dose in single-compound studies. These studies confirmed the hypothesis that the phenotypes observed were in large part driven by interindividual differences in f_u.

The plasma concentration profiles and pharmacokinetic parameters of AZ8399 are summarized in Table 1 and Fig. 2, respectively.

Plasma Protein Binding in Individual Colony Animals. Following the initial cassette study the plasma samples were obtained from and following pooling of samples from each individual dog, the f_u was measured (see Supplemental Table 1). This experiment showed f_u values of 0.10 and 0.021 for dogs 003M and 004M respectively, which appeared broadly consistent with the differences in PK parameters with a 3.9-fold lower total CL_p and 2.9-fold lower V_{ss} in dog 004M.

To confirm this finding in a more controlled experiment and investigate the prevalence of the different phenotypes within the dog colony, control plasma samples were obtained from all animals in the colony (at the time 001M–008M) and the f_u of AZ8399 was determined. This showed an f_u range of 0.019–0.09 within the group and demonstrated that the contrasting phenotype was present in other dogs (see Table 1). This observation of different f_u phenotypes was subsequently followed up with dog PK studies to confirm relevance to the measured CL_p and V_{ss}.

After replacement of several animals in the colony, control samples were acquired from new dogs (009M–014M) as well as repeat samples from dogs 005M and 006M, which had been retained. For the new dogs, the data showed the same phenotypic differences between animals with a similar f_u range of 0.027–0.12, whereas resampling of retained dogs showed comparable values to previous data.

Concentrations of Albumin and α1-Acid Glycoprotein in Plasma. After the observation that individual dogs showed a large range of f_u values, it was hypothesized that this was most likely to be driven by variability in the concentration of albumin and AGP, which are common binding partners for drugs in plasma, or else a change in the K_d value. Therefore, the albumin and AGP concentrations were determined in control plasma samples (the same samples that were used for f_u determinations) from animals with pharmacokinetic data.

Albumin concentrations in animals with PK data were found to vary within a relatively narrow range from approximately 20 to 35 mg/ml (Fig. 3; Table 1). No significant correlation was observed with total CL_p and V_{ss} (Fig. 4, A and B). Likewise, in the expanded animal data set (including animals 009M–014M) no significant correlation to individual f_u values was observed (Fig. 4C).

In contrast, concentrations of AGP varied over a >10× range (~76–833 μg/ml) (Fig. 3; Table 1). Furthermore, a significant, strong inverse correlation was observed, with total CL_p (P = 0.005, r = −0.905) and V_{ss} (P = 0.032, r = −0.767) suggesting AGP concentrations could explain the variability in PK observed (Fig. 4, D and E). AGP concentrations also showed a significant, strong inverse correlation to individual f_u (P = 0.019, r = −0.625) values in the expanded animal data set supporting the PK effect being driven through impact on f_u (Fig. 4F).

DNA Sequencing of α1-Acid Glycoprotein. After sequencing of the α1-acid glycoprotein gene in dogs 1–14, the following SNPs were noted. In exon 1: dogs 6, 8, and 9 share the same heterozygous missense mutation at position c.70 G > A, which results in an amino acid change from alanine to threonine. In exon 5, dogs 1, 2, 5, 6, 11, 12, 13, and 14 share synonymous mutations at position c.502 C > T and position c.522 G > A, of which dogs 1, 2, 6, 11, and 12 are heterozygous and 5, 13, and 14 are homozygous. Finally, in exon 6 only dog 2 was found to have a synonymous mutation at position c.582 G > A. All the mutations identified are known variants in ensemble CanFam3.1 dog reference genome. As only exon 1 and 5 showed mutations with high frequency in the data set, further analysis was restricted to these exons (Fig. 5).

For exon 1: a Mann-Whitney test found no significant differences in AGP concentrations between the c.70GG and c.70GA animals (P = 0.1167). For exon 5: A Kruskal-Wallis test was used to compare AGP levels between the c.502CC/c.522GG, c.502CT/c.522GA, and c.502TT/c.522AA animals. Post hoc testing was conducted with a Dunnett's test to adjust the P values for multiple testing. Statistically significant differences were seen between the c.502CC/c.522GG and

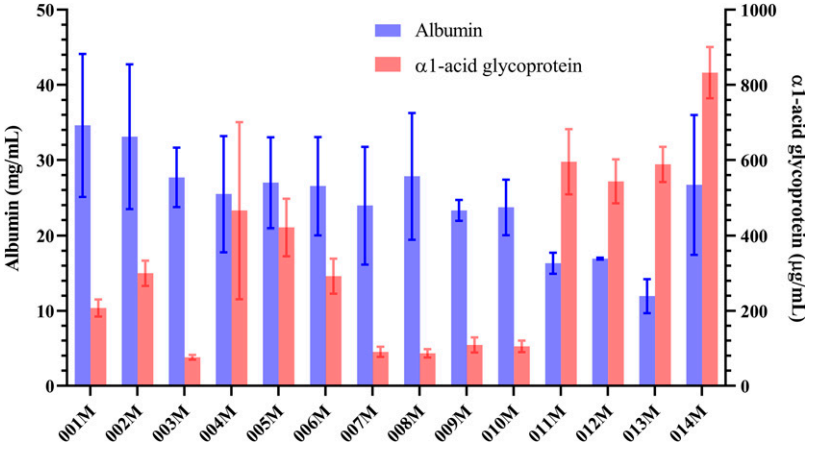

Fig. 3. Concentrations of albumin and AGP from individual colony animals (n = 2–6, error bars = S.D.).

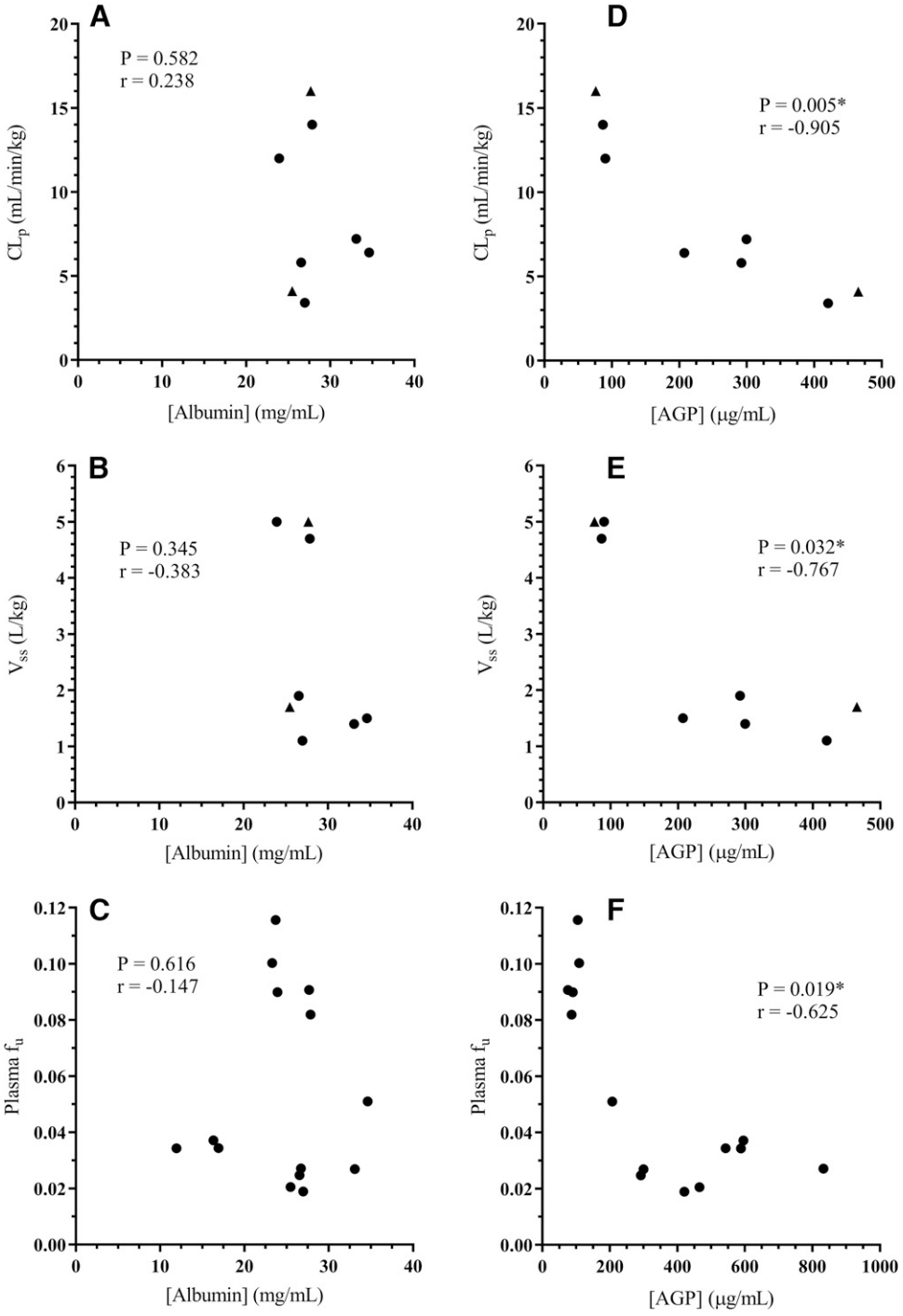

Fig. 4. Relationship of concentrations of albumin (A–C) and AGP (D–F) to CL_p, V_{ss}, and fraction unbound in individual dogs. The significance (P) and strength (r) of relationships have been assessed using Spearman's rank correlation coefficient. For *P* value * denotes significance ($\alpha = 0.05$); r limits of 1, −1, and 0 indicate maximum positive, maximum negative, and no correlation, respectively. Triangular symbols denote cassette-dosed animals.

the c.522CT/c.522GA ($p' = 0.0350$, mean rank differences = −5.1) and the c.502TT/c.522AA ($p' = 0.0065$, mean rank differences = −7.5). No statistically significant difference was seen between the c.522CT/c.522GA and the c.502TT/c.522AA genotypes ($P = 0.3621$, mean rank differences = −2.4).

Visual inspection indicates a trend between AGP level and SNP status suggesting higher AGP concentrations (c.502TT/c.522AA > c.502CT/c.522GA > c.502CC/c.522GG). However, a Shirley Williams test, which assesses for a relationship between dose levels of a treatment and outcome, returned a borderline *P* value of 0.0764. Further data would be needed to understand whether a true dose-response relationship exists, as the data has a low and high variance.

Discussion

During the initial preclinical evaluation of a novel Syk inhibitor AZ8399 in dogs, pharmacokinetic data were produced showing an apparent sizable difference in unbound CL_p and V_{ss} between the two animals when a pooled control plasma f_u was applied. The direction and comparable magnitude of the fold difference between total CL_p and V_{ss} was consistent with a difference in plasma f_u value between individual dogs.

To investigate this hypothesis, we obtained the pharmacokinetic samples from the study and measured the f_u from the individual animals, revealing an approximate 5-fold difference consistent with that seen in CL_p and V_{ss}. Subsequent evaluation of AZ8399 f_u in other colony animals showed this phenotypic difference was not limited to the two

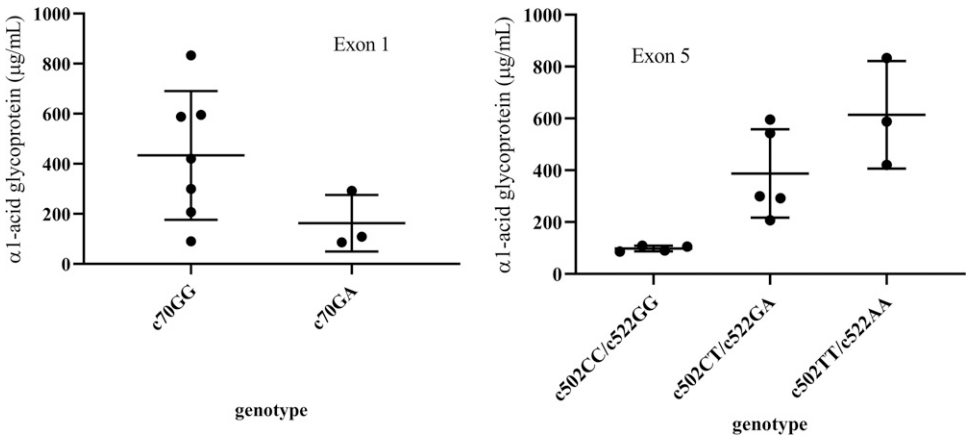

Fig. 5. The relationship between α1-acid glycoprotein mutational status in exons 1 and 5 (as inferred by Sanger sequencing) and individual dog AGP concentration (individual values indicated by dots. Bars show mean ± S.D., n = 3–7).

animals previously tested. Therefore, the intravenous PK parameters of animals not previously dosed with AZ8399 were determined to confirm the correlation to the experimental f_u. This confirmed statistically significant, strong negative correlations between f_u and total CL_p and V_{ss}. Determination of AZ8399 f_u in control samples from a cohort of newly acquired animals (009-014M) showed the same distinct phenotypes with four "high" and two "low" binding animals, suggesting the phenotype exists in the broader population.

Based on these data, we hypothesized that the observed phenotypes were most likely to arise from differences in binding to either albumin or AGP driven by either variation in protein concentration or else differing K_d values due to polymorphisms in the protein(s). Therefore, the albumin and AGP concentrations were determined in control plasma samples (the same samples that were used for f_u determinations) from animals with pharmacokinetic data. Albumin levels in animals with PK data varied within a relatively narrow range from approximately 20 to 35 mg/ml, and no significant correlations were observed with measured PK parameters or individual f_u values. By contrast, AGP concentrations were found to vary more than 10-fold from ∼76 to 833 μg/ml and demonstrate significant, strong inverse correlations with the individual animal total CL_p, V_{ss} and f_u (Fig. 4, D–F). It should be noted that although supporting protein concentration as a factor, this data did not rule out a difference in K_d.

The phenotypic data could be loosely grouped into "high" f_u animals (002M, 004M, 005M, 006M, 011M, 012M, 013M, 014M) and "low" f_u animals (003M, 007M, 008M, 009M, 010M) possibly consistent with a genetic polymorphism affecting compound K_d, expression levels, or AGP K_{deg}. We therefore decided to sequence the exons to determine whether any SNPs were present consistent with the two groups. SNPs were identified in exons 1, 5, and 6, which interestingly have recently been reported in a wider population of dogs (Costa et al., 2021). The frequency of the exon 6 SNP was too low to allow any correlation to AGP to be examined. The data suggest a lack of any robust correlation to AGP levels for the exon 1 c.70G > A variant, which is consistent with a lack of effect on f_u for four AGP-binding drugs reported by Costa et al. (2021). In contrast, the SNPs in exon 5 were found to be positively correlated with increased AGP concentrations. Given that the exon 5 SNPs are synonymous, there is no structural rationale to suggest that an altered K_d could explain the observed f_u changes; however, we hypothesize that these SNPs may alter mRNA stability and gene expression, ultimately leading to increased levels.

It is also interesting to note that the mean albumin concentrations determined (24.6 mg/ml) were close to the value (26.3 mg/ml) in the commonly quoted publication "Physiological parameters in laboratory animals and humans" (Davies and Morris, 1993). In contrast, the mean AGP levels measured in the current study (0.337 mg/ml) are approximately 10-fold lower than those quoted in the same reference (3.7 mg/ml). Even animals at the high end of the range in the current experiment are approximately 4-fold lower than this reference value.

This left the question of the underlying factor driving differing concentration of AGP in plasma, and this remains an area for future investigation.

The current work has demonstrated that within a specific colony population of beagle dogs, different phenotypes exist with respect to the plasma f_u of a novel Syk inhibitor, AZ8399. To date, the phenotypic difference reported here has been confirmed only with compounds from the chemical series from which AZ8399 was derived. Therefore, it is difficult to generalize on how often the phenotypes observed will significantly impact measured PK parameters, although it seems that a low f_u driven to a large extent by AGP is likely a requirement. Likewise, it is not possible based on the analysis of such a limited population to predict with any accuracy how prevalent this phenotype is within the broader population, albeit the observation that newly introduced animals from an external source showed the same phenotypes at least shows it is not limited to an isolated colony. However, it was interesting to note that pooled control plasma gave a value that was comparable with our highest f_u animals rather than midrange.

In terms of pharmacokinetics, understanding the plasma f_u is important for in vitro to in vivo scaling of metabolic clearance, interpretation of renal clearance properties (defining passive filtration as glomerular filtration rate × f_u), and PBPK-based modeling. Likewise, it is required for interpretation of PKPD or toxicokinetic exposure data in terms of unbound concentrations. However, these data suggest that the possibility exists for individual differences in f_u within the dog population and that the application of an assumed population mean value to data interpretation may not always be appropriate. Therefore, when individual pharmacokinetic data are consistent with differences in f_u it may be appropriate to conduct further investigations to understand if this is the case. We have not to date studied the impact of individual f_u on circulating metabolite levels insofar as total clearance could be impacted. However, it could be argued that interpretation of metabolites in safety testing data, the total clearance of a given metabolite being f_u dependent, could be changed dependent on the binding phenotype of individual animals, and this may be an area for further investigation. In summary, this work demonstrates that interindividual variability in f_u between dogs driven by differences in AGP concentration can contribute significantly to pharmacokinetic behavior of test compounds. Prior evaluation of AGP concentrations or genotype in colony dogs used for PK studies could be beneficial for prediction and interpretation of PK parameters.

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Authorship Contributions

Participated in research design: Pike, Jones, Markandu, O'Neill.

Conducted experiments: Markandu.

Performed data analysis: Pike, Markandu, O'Neill.

Wrote or contributed to the writing of the manuscript: Pike, Jones, Markandu, O'Neill.

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Address correspondence to: Andy Pike, E-mail: andrew.pike@astrazeneca.com