Phenacetin Pharmacokinetics in CYP1A2 Deficient Beagle Dogs

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Running Title: **Phenacetin Pharmacokinetics in CYP1A2 Deficient Dogs**

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Abbreviations: CYP, cytochrome P450; ADME, absorption, distribution, metabolism, excretion; IV, intravenous; PO, oral administration; CC, wild type; CT, heterozygous; TT, homozygous mutant; PEG 400, polyethylene glycol 400, AUC, area under the curve; $C_{\max}$, maximum concentration; MRT, mean resonance time; V_{ss} volume at steady state; CL, clearance; LC/MS/MS, liquid chromatography/tandem mass spectrometry; UPLC, ultra high pressure liquid chromatography; ESI, electrospray ionization

Abstract

Phenacetin is widely used as an *in vitro* probe to measure CYP1A2 activity across species. To investigate whether phenacetin can be used as an *in vivo* probe substrate to phenotype CYP1A2 activity in dogs, beagle dogs previously genotyped for a single nucleotide polymorphism that yields an inactive CYP1A2 protein were selected and placed into one of three groups: CC (wild type), CT (heterozygous), or TT (homozygous mutants). The dogs were dosed with phenacetin orally at 5 and 15 mg/kg and intravenously (IV) at 15 mg/kg. Plasma samples were analyzed by LC/MS/MS and phenacetin and its primary metabolite, acetaminophen, were monitored. After IV dosing, all groups showed similar exposures to phenacetin irrespective of genotype. After oral dosing at 15 mg/kg, the exposure of phenacetin in CC & CT dogs were similar, but phenacetin exposure was 2-fold greater in TT dogs. Exposure of the metabolite, acetaminophen was similar in all groups, however the mean acetaminophen:phenacetin ratio in TT dogs was 1.7 times less than that observed in CC dogs. Similar trends between the groups of dogs with respect to phenacetin exposure were also observed following a lower 5 mg/kg oral dose of phenacetin, however, a proportionally greater amount of acetaminophen was generated. Although oral exposure of phenacetin was 2-fold higher and acetaminophen exposure was 2-fold lower in CYP1A2 deficient (TT) dogs, these results were considered modest and suggest that phenacetin is not a selective or robust *in vivo* probe to measure CYP1A2 enzyme activity in the dog.

Introduction

Cytochrome P450 (CYP) enzymes play a significant role in the oxidative metabolism and elimination of xenobiotics and endogenous substances. CYP enzymes have been extensively studied in humans and rodents, however dogs are commonly used for pharmacological and ADME/toxicology studies of new drug candidates and studies of their CYP enzymes and polymorphisms are few (Tenmizu et al., 2006; Zhou et al., 2010). Although genetic polymorphisms have been reported in the dog, our understanding of their contribution to the inter-individual variability in drug pharmacokinetics related to pharmaceutical drug development and veterinary practice is limited (Mise et al., 2004a).

The CYP1A2 enzyme is expressed in all mammals with few species differences in function or regulation and all have very similar amino acid sequences (Parkinson et al., 2008). In humans, CYP1A2 polymorphisms in the coding region are rare and only occasionally appear to decrease enzyme activity (Faber et al., 2005). A CYP1A2 polymorphism in beagle dogs has been reported (Tenmizu et al., 2004). The mutation is a single nucleotide polymorphism, CYP1A2 1117 C>T, which results in an amino acid change from arginine to an early stop codon at position 373 (truncated inactive protein). Pharmacokinetic variability was observed in dogs after administration of AC-3933 and YM-64227, both metabolized primarily by CYP1A2 (Mise et al., 2004b; Tenmizu et al., 2006). In CYP1A2 deficient dogs (TT), AC-3933 and YM-64227 exhibited 5.2 and 24.7-fold higher mean oral exposures (AUC) of parent drug, respectively, compared to wild type or heterozygous dogs.

Enzyme-selective probe substrates are widely used in the field of drug metabolism and pharmacokinetics for the screening and characterization of individual CYP enzymes and for phenotyping various CYP activities. Subpopulations can be identified and categorized by their inherent capacity to metabolize such probe drugs (Frye, 2004). Phenacetin is a substrate widely used as an *in vitro* and *in vivo* probe to measure the activity of CYP1A2 because the metabolism of phenacetin to acetaminophen is thought to be a selective CYP1A2-mediated reaction. [Fig. 1]. However, recent *in vitro* studies by Zhou et al., have found that the dog CYP2A13 enzyme has a similar *in vitro* intrinsic clearance to catalyze phenacetin to acetaminophen as compared to CYP1A2, suggesting that phenacetin may not be a selective

probe for dog CYP1A2 enzyme activity *in vitro* (Zhou et al., 2010). To investigate the effects of the CYP1A2 genotypic deficiency and the potential contribution of CYP2A13 on the *in vivo* metabolism of phenacetin; previously genotyped dogs (Whiterock et al, 2007) were dosed orally (PO) and intravenously (IV) with phenacetin. The exposures and pharmacokinetic parameters of phenacetin and its metabolite (acetaminophen) were compared between groups of dogs to assess the utility of phenacetin as an *in vivo* probe substrate of CYP1A2 enzyme activity in the dog.

Materials and Methods

Materials: Phenacetin, acetaminophen, alprazolam, sodium carbonate, ethyl acetate and PEG 400 were obtained from Sigma-Aldrich (St. Louis, MO). Formic acid (98%) was from EMD Chemicals Inc. (Gibbstown, NJ) and HPLC grade acetonitrile and water were from JT Baker (Phillipsburg, NJ).

Experimental Animals: Male beagle dogs were obtained from Marshall Farms (North Rose, NY). The studies described were approved by the IACUC and adhered to the 'Principles of Laboratory Animal Care' (NIH publication 85-23, revised in 1985). The animals were housed on a 12-h light cycle with free access to food and water when not on study.

Study Design: Adult male beagle dogs were genotyped for the single nucleotide polymorphism, CYP1A2 1117 C>T (Whiterock et al., 2007). Based on genotype, dogs were selected and placed into three groups (n=3/group): CC (wild type), CT (heterozygous), and TT (homozygous mutants). Dosing solutions were prepared in 75% PEG 400:25% water (IV) or 100% PEG 400 (PO). Each group of dogs was dosed with 15 mg/kg IV (5 min infusion, 1 mL/kg) of phenacetin and blood samples were taken at pre-dose and through 8 h. For oral studies, all dogs were fasted overnight and then administered 15 mg/kg phenacetin PO by capsule. Blood samples were taken pre-dose and through 8 h. All dogs were given access to food 4 h post dose. In a cross-over experiment, the CC and TT groups of dogs were fasted overnight and dosed phenacetin PO at 5 mg/kg (0.5 mL/kg). Blood samples were obtained through 6 h. Blood samples were centrifuged to obtain plasma and stored at -20° C until analysis.

Quantitation of Phenacetin and Acetaminophen in Dog Plasma: Liquid-liquid extraction was performed for each plasma sample by adding 100 μ L of a 0.1 M sodium carbonate solution containing internal standard (alprazolam) followed by addition of 1 mL of ethyl acetate. The samples were vortexed for 2 min followed by centrifugation at 3000 rpm for 15 min. Eight hundred microliters of the ethyl acetate layer was evaporated under nitrogen and reconstituted with 200 μ L of 1:1 acetonitrile/water with 0.1% formic acid. Quantitation of phenacetin and acetaminophen was performed using LC/MS/MS. The chromatography system used was a Waters Acquity System (UPLC) with a BEH C18 (1.7 μ , 50 X 2.1 mm) column (Waters, Milford, MA) maintained at 60°C. Mobile phase A was 100% water with 0.1% formic acid. Mobile phase B was 100% acetonitrile with 0.1% formic acid. Initial mobile phase composition was 95% A at a flow rate of 0.6 ml/min with a linear gradient operated to 5% A. The UPLC was interfaced to an API 4000 Qtrap (Applied Biosystems, Carlsbad, CA) mass spectrometer operated in the ESI positive ionization mode using selective reaction monitoring.

Statistical Analysis: One-way analysis of variance (ANOVA) was performed on all calculated values using SAS[®] PROC GLM (version 9.1.2). T-tests were constructed in the context of the ANOVA model to compare the (log) means for the populations. Significance was assessed at the 5% level.

Results and Discussion

Following an IV dose to CC, CT or TT dogs, there was a trend towards increased exposure (AUC) of phenacetin in the TT dogs, albeit not statistically significant. Acetaminophen exposure (AUC) was variable, but did show a decrease in the TT animals, which again was not statistically significant and was not reflected in the CT dogs. The metabolite:parent ratio was about 2-fold lower in the TT dogs, but the trend towards a lower M/P ratio was not reflected in the heterozygous CT dogs either [Table 1]. The mean total body clearance of phenacetin was extremely high and not statistically different between the groups. These clearance values are 8-times the hepatic blood flow of the beagle dog, suggesting the potential for multiple elimination pathways other than hepatic. The extremely high clearance achieved after the 15 mg/kg IV dose, is consistent with those found in the literature (Souhay et al., 1975), where

the clearance of phenacetin in beagles was reported to be 10 times greater than hepatic blood flow. Renal elimination of phenacetin may explain, in part, the rapid clearance of phenacetin. (Duggin et al., 1976).

The pharmacokinetic parameters of phenacetin following a 15 mg/kg oral dose to CC, CT or TT dogs were calculated by noncompartmental analysis [Table 2]. In CYP1A2 deficient (TT) dogs, phenacetin showed less than a 2-fold increase in mean oral exposure of phenacetin (AUC_{0-8h}), as compared to wild type (CC) or heterozygous (CT) dogs. The C_{max} and AUC of acetaminophen were similar in all dogs regardless of their genotype [Fig. 2; Table 2]. When comparing metabolite to parent ratios, the mean acetaminophen: phenacetin ratio was 1.7-fold less in TT vs. CC dogs.

A second oral study at a lower dose of phenacetin (5 mg/kg) was conducted to avoid the potential for saturating metabolic or transporter-mediated clearance that may have occurred in the 15 mg/kg study. Due to similarities in phenacetin and acetaminophen exposure obtained in CT and CC dogs in the 15 mg/kg study, the 5 mg/kg study did not include the heterozygous group of dogs. Phenacetin exposures following a 5 mg/kg dose yielded similar trends to those observed after the 15 mg/kg dose. Exposure of phenacetin in the TT dogs was again only 2-fold greater as compared to the CC dogs [Fig. 2]. There was however, a greater difference in the amount of acetaminophen generated between the CC and TT groups of dogs. The mean acetaminophen: phenacetin ratio in CC dogs was 2.34, as compared to 0.54 in TT dogs. This 4.7-fold reduction in the metabolite to parent ratio in TT dogs, as compared to CC dogs, was considered statistically significant ($p < 0.05$).

Although phenacetin and acetaminophen pharmacokinetics in both studies demonstrated modest differences in oral exposure between CC and TT genotyped dogs, this difference was not considered dramatic and suggests that the metabolism of phenacetin to acetaminophen is not a robust pathway for measuring CYP1A2-mediated activity in the dog. Phenacetin O-deethylation to acetaminophen is a common reaction to measure the activity of CYP1A2 *in vitro*, but it is known to display kinetics indicative of multiple metabolic pathways, such as CYP1A1 (Venkatakrishnan et al., 1998). In addition to CYP1A metabolic pathways, phenacetin has been shown to be metabolized by other CYP isoforms. Recent studies suggest that dog CYP2A13, which is predominantly present in the liver,

catalyzes phenacetin O-deethylation to acetaminophen. The phenacetin to acetaminophen intrinsic clearance was found to be similar for dog CYP2A13 (0.92 $\mu\text{L}/\text{min}/\text{pmol}$) and CYP1A2 (1.1 $\mu\text{L}/\text{min}/\text{pmol}$) (Zhou et al., 2010). Although the intrinsic clearance of phenacetin through the CYP1A2 and CYP2A13 pathways are similar, the overall *in vivo* metabolic disposition of phenacetin to acetaminophen will also be governed by the relative amounts of each enzyme present in the liver which is unknown at this time.

Despite the fact that a greater difference in the acetaminophen:phenacetin ratio was observed after a 5 mg/kg dose of phenacetin, thus supporting the saturation theory, the lower phenacetin dose still did not produce a robust difference in overall acetaminophen produced compared to the CYP1A2 substrates studied by Mise and Tenmizu. While our study demonstrates a trend towards a decline in acetaminophen production, a less than 2 fold difference was observed between the CYP1A2 deficient and wild type groups; whereas Mise and Tenmizu observed 5.2 and 24.7-fold differences in oral exposure of unchanged drug between TT and CC dogs. In summary, *in vivo* studies were conducted to assess the metabolic disposition of phenacetin and formation of acetaminophen in dogs having normal CYP1A2 activity vs. those deficient in CYP1A2 activity. Data obtained in CYP1A2 deficient dogs supports recent *in vitro* data by Zhou et al. and strengthens the conclusion that the phenacetin to acetaminophen pathway is not selective for CYP1A2 activity in the dog. These results, particularly at the 5 mg/kg phenacetin dose, show that CYP1A2 is a significant enzyme responsible for the formation of acetaminophen from phenacetin; however, it is not likely to be the only enzyme or pathway responsible for the elimination of phenacetin or generation of acetaminophen. Combined, the previously reported and current results indicate that phenacetin O-deethylation to acetaminophen is not a robust or selective reaction to measure CYP1A2 activity in dogs.

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Authorship Contributions

Participated in research design: Whiterock, Sinz

Conducted experiments: Whiterock, Orcutt

Performed data analysis: Morgan, Whiterock

Wrote or contributed to writing the manuscript: Whiterock, Sinz, Lentz, Morgan

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Legends for Figures

Figure 1. Phenacetin O-deethylation to form acetaminophen

Figure 2. Mean plasma concentration-time profiles of phenacetin in CYP1A2 genotyped dogs

(A) concentrations of phenacetin after 15 mg/kg PO dose and (B) generation of acetaminophen after a 15 mg/kg phenacetin dose. (C) concentrations of phenacetin after 5 mg/kg PO dose and (D) generation of acetaminophen after a 5 mg/kg phenacetin dose. (●) CC (wild type), (■) CT (heterozygous) and (▲) TT (CYP1A2 deficient). *Note: CT group was not dosed at 5 mg/kg.*

Pharmacokinetic parameters of phenacetin and acetaminophen after oral dosing of phenacetin at 5 and 15 mg/kg.

Table 1 Pharmacokinetic parameters of phenacetin and acetaminophen after IV dosing of phenacetin at 15 mg/kg.

		<u>Phenacetin</u>					<u>Acetaminophen</u>		AUC metabolite:parent
		AUC _{last} (nM*h)	T _{1/2} (h)	MRT (h)	V _{ss} (L)	CL (mL/min/kg)	C _{max} (nM)	AUC _{last} (nM*h)	
CC (wild type)	Mean	3290	0.8	0.7	11	264	621	1162	0.34
	SD	1462	0.3	0.2	3	110	386	692	0.07
CT (heterozygous)	Mean	4235	0.4	0.7	15	344	1231	2797	0.59
	SD	1083	0.003	0.03	4	91	778	2235	0.35
TT (CYP1A2 deficient)	Mean	4823	0.5	0.8	15	292	246	601	0.13
	SD	662	0.09	0.03	2	42	171	435	0.17

No significant difference found between groups.

Table 2 Pharmacokinetic parameters of phenacetin and acetaminophen after oral dosing of phenacetin at 5 and 15 mg/kg.

	Dose (mg/kg)		<u>Phenacetin</u>		<u>Acetaminophen</u>		AUC metabolite:parent
			C _{max} (nM)	AUC _{last} (nM*h)	C _{max} (nM)	AUC _{0-8h} (nM*h)	
CC (wild type)	5	Mean	533	345*	480**	792	2.34*
		SD	166	113	187	321	0.66
	15	Mean	1543**	2084*	1056	1349	0.73
		SD	168	559	533	637	0.47
TT (CYP1A2 deficient)	5	Mean	843	887	218	441	0.54
		SD	309	231	95	158	0.27
	15	Mean	2301	4037	795	1749	0.43
		SD	309	237	164	293	0.09
CT (heterozygous)	15	Mean	1780	2336*	838	1549	0.75
		SD	574	780	377	706	0.46

*Significantly different from TT (CYP1A2 deficient) population, $p \leq 0.05$

**Marginal significance in difference from TT (CYP1A2 deficient) population, $p=0.08$

Figure 1

Figure 2