Title Page:

Characterization of the canine anthracycline metabolizing enzyme carbonyl reductase 1 (cbr1) and the functional isoform cbr1 V218

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1

Running Title Page:

Canine carbonyl reductase 1, an anthracycline reductase

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Text Pages: 17

Tables: 1

Figures: 5

References: 31

Abstract: 250

Introduction: 736

Discussion: 1137

Abbreviations: SDR, short chain dehydrogenase reductase, CBR, carbonyl reductase, AKR, aldo-keto reductase, CBR1, carbonyl reductase 1

Abstract

The anthracyclines doxorubicin and daunorubicin are utilized in the treatment of various canine cancers. Anthracycline-related cardiotoxicity limits their clinical use in humans and dogs. The formation of anthracycline C-13 alcohol metabolites (e.g. doxorubicinol and daunorubicinol) contributes towards the development of anthracycline-related cardiotoxicity. The enzymes responsible for the synthesis of anthracycline C-13 alcohol metabolites in canines remain to be elucidated. We hypothesized that canine carbonyl reductase 1 (cbr1), the homolog of the prominent anthracycline reductase human CBR1, would have anthracycline reductase activity. Recombinant canine cbr1 (molecular weight: 32.8 kDa) was purified from *E. coli*. The enzyme kinetics of "wild-type" canine cbr1 (cbr1 D218) and a variant isoform (cbr1 V218) were characterized with the substrates daunorubicin and menadione, as well as the flavonoid inhibitor rutin. Canine cbr1 catalyzes the reduction of daunorubicin to daunorubicinol, with cbr1 D218 and cbr1 V218 displaying different kinetic parameters (cbr1 D218 K_m: 188 ± 144 µM vs cbr1 V218 K_m: 527 ± 136 µM, $P < 0.05$, and cbr1 D218 V_{max}: 6446 ± 3615 nmol/min*mg vs cbr1 V218 V_{max}: 15539 ± 2623 nmol/min*mg, $P < 0.01$). Canine cbr1 also metabolized menadione (cbr1 D218, K_m: 104 ± 50 µM, V_{max}: 2034 ± 307 nmol/min*mg). Rutin acted as a competitive inhibitor for the reduction of daunorubicin (cbr1 D218 K_i: 1.84 ± 1.02 µM, cbr1 V218 K_i: 1.38 ± 0.47 µM). These studies show that canine cbr1 metabolizes daunorubicin and provide the necessary foundation to characterize the role of cbr1 towards the variable pharmacodynamics of anthracyclines in canine cancer patients.

Introduction

The anthracyclines doxorubicin and daunorubicin are chemotherapeutics relied on for the treatment of a variety of cancers in humans, most notably hematological malignancies in children. Unfortunately, the utility of anthracyclines has long been hampered by the development of irreversible cardiotoxicity in some patients (Von Hoff et al., 1979). A suspected mechanism for the development of anthracycline-related cardiotoxicity in humans, termed the "unifying hypothesis", states that the combination of semiquinone iron-catalyzed redox cycling, and the two-electron reduction of parent anthracyclines to C-13 alcohol metabolites synergistically aid in the development of the toxicity (Mordente et al., 2001).

In humans, the C-13 alcohol metabolites doxorubicinol and daunorubicinol are the major metabolic products from their parent anthracyclines, with formation catalyzed by short-chain dehydrogenases reductases (SDR), namely carbonyl reductases (CBRs), and family members of the aldo-keto reductases (AKRs)(Mordente et al., 2001). Most prominent towards the metabolism of anthracyclines appear to be CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 (Quinones-Lombrana et al., 2014).

Anthracyclines are also used in the treatment of many canine cancers, including lymphomas, lymphosarcoma, hemangiosarcoma, osteosarcoma, mammary tumors and squamous cell carcinomas (Simon et al., 2006). Similarly to humans, the phenomenon of anthracycline-related cardiotoxicity is observed in canine patients. The average incidence of anthracycline-related cardiotoxicity in canine patients has been reported at 17.7% (Gillings et al., 2009). More specifically, congestive heart failure in canines receiving anthracyclines has been observed clinically in 2 - 9% of patients (FitzPatrick et al., 2010; Ratterree et al., 2012). The mortality rate of canine patients experiencing congestive heart failure is 100% (Mauldin et al., 1992). The C-13 alcohol metabolite doxorubicinol has been quantified in canine plasma following doxorubicin treatment, and anthracycline reductase activity has been previously

observed in subcellular fractions of canine heart tissue (Loveless et al., 1978; Oosterbaan et al., 1984).

The exact enzymes responsible for the synthesis of anthracycline C-13 alcohol metabolites in canines treated with anthracyclines remain to be identified. The role of individual CBRs, specifically cbr1, towards the metabolism of anthracyclines in dogs remains unexplored. We hypothesized that the canine homolog of human CBR1 (i.e., canine cbr1) results in a protein responsible for the synthesis of anthracycline C-13 alcohol metabolites in dogs (Figure 1). The aim of this study was to investigate the enzyme kinetics of canine cbr1. Thus, we characterized the enzyme kinetics of recombinant "wild type" canine cbr1 (cbr1 D218) with the prototypical CBR substrate menadione and anthracycline daunorubicin. In addition, the enzyme kinetics of a novel functional variant isoform (cbr1 V218) were characterized, with the intent of further comparing potentially critical residues between the protein homologs. Lastly, the inhibitory effect of the common dietary flavonoid rutin on canine cbr1 activity was documented.

Materials and Methods

Chemicals and Enzymes

All primers were custom designed and supplied by Sigma Aldrich (St. Louis, MO). Restriction enzymes NheI and XhoI, as well as T4 DNA Ligase were supplied by New England Biolabs (Ipswich, MA). Kanamycin sulfate, LB agar, LB broth, isopropyl-β-D-thiogalactoside, lysozyme, DNase I, menadione sodium bisulfate, NADPH, dimethyl sulfoxide, monobasic potassium phosphate, dibasic potassium phosphate, phosphate buffered saline, rutin hydrate, daunorubicin hydrochloride, and acetonitrile were purchased from Sigma Aldrich (St. Louis, MO). Daunorubicin and menadione stock solutions were prepared in 0.1 M potassium phosphate buffer (pH 7.4). Rutin stock solutions were prepared in dimethyl sulfoxide.

Canine cDNA Sample and Amplification of cbr1

Canine liver cDNA from a single adult male Beagle (3 years old) was purchased from Zyagen (San Diego, CA). A 909 base pair fragment containing the canine *cbr1* open reading frame was amplified using the GC-RICH PCR System (Roche, Indianapolis, IN) with custom primers: (forward) 5'-AGCAAACACGCCCTTGCAG-3' (reverse) 5'-TCCTAAAGGGTTCCGTGG-3'. The PCR product was cloned into a pcR2.1 vector with TOPO TA (Invitrogen, Carlsbad, CA), and transformed into chemically competent TOP10 *E. coli* cells following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Constructs were then isolated using the Geneaid High-Speed Plasmid Mini Kit (New Taipei City, Taiwan) and sequenced with an ABI PRISM 3130 XL GeneticAnalyzer (Applied Biosystems, Foster City, CA) using combinations of product and vector specific primers. DNA sequencing revealed a single nucleotide polymorphism which results in an amino-acid change of aspartic acid to valine at position 218 of canine cbr1 (see further details in the Results section). pcR2.1 constructs containing each allele, either coding for canine cbr1 isoform D218 or V218, were then processed separately.

Cloning and Expression of Canine cbr1

6

An 897 base pair fragment containing the canine *cbr1* open reading frame was amplified from the pcR2.1 construct templates with custom primers containing restriction enzyme sites (Forward-Nde I: 5'-GAGGAATTCCATATGTCTGCAGCCTCCCGCGTGG-3', and reverse-Xho I: 5'-ATCCGCTCGAGTTATCCTAAAGGGTTCCGTGG-3'). The resulting PCR products were then purified and double digested with Nhe I and Xho I restriction enzymes. The digested fragments were then inserted into a Nhe I/Xho I predigested pET28a expression plasmid (EMD Millipore, Darmstadt, Germany) using T4 ligase. The resulting expression plasmids contained the 883 base pair insert of canine *cbr1* D218 or *cbr1* V218, positioned to include an N-terminal 6x His-tag. The identities of the inserts were confirmed by DNA sequencing using combinations of vector and canine *cbr1* specific primers.

Purified pET28a-cbr1 D218 and pET28a-cbr1 V218 constructs were transformed into BL21 (DES) pLysS competent E. *coli* (Invitrogen, Carlsbad CA) by heat shock, plated onto LB broth agar supplemented with kanamycin (50 µg/ml), and grown overnight at 37°C. Randomly selected colonies were then cultured in 3 ml of LB broth supplemented with kanamycin (50 µg/ml) for 16 hours at 37°C with shaking (250 rpm). Cultures were then expanded to 1 liter and grown until an optical density of 0.3 was reached (OD_{600}=0.3). Protein synthesis was then induced with isopropyl-β-D-thiogalactoside (500 µM) for 2 hours at 37°C with shaking. Bacterial cell pellets were harvested by centrifugation and stored at -80°C until further processing.

Bacterial pellets were reconstituted and lysed in 3 ml of B-PER reagent (Thermo Fisher Scientific, Rockford, IL) supplemented with lysozyme, DNase I, and Halt Protease Inhibitor Cocktail, EDTA Free (Thermo Fisher Scientific) for 45 minutes at 4°C with slight agitation. His-tagged recombinant canine cbr1 proteins were isolated and purified using HisPur Colbalt Spin Columns (Thermo Fisher Scientific) following the manufacturer's instructions. The eluted proteins were further concentrated, and the elution buffer was simultaneously replaced with PBS, using Pierce Concentrator PES 10K MWCO Spin Columns (Thermo Fisher Scientific,).

7

The purity of concentrated proteins was assessed by SDS-PAGE on a NuPAGE Novex 4 - 12% Bis-Tris precast gel (Invitrogen, Carlsbad, CA) with Coomasie staining (Bio Rad, Hercules, CA). The concentration of eluted proteins was determined using the Pierce BCA Protein Assay (Thermo Fisher Scientific), with bovine serum albumin as a standard.

The identity and molecular weight of the recombinant proteins were confirmed by Western blotting using an anti-human CBR1 IgG antibody with cross reactivity to canine cbr1 (Santa Cruz Biotech., Dallas, TX). Briefly, 5 µg of recombinant canine cbr1 (cbr1 D218 and cbr1 V218), and 1 µg of recombinant human CBR1 (Abcam, Cambridge, MA) were separated on a NuPAGE Novex 4-12% Bis-Tris precast gel (Invitrogen). Proteins were transferred to a nitrocellulose membrane using the iBlot transfer device (Invitrogen). Membranes were blocked with 5% non-fat dry-milk (Bio Rad) in PBS for 1 hour at room temperature. Membranes were then incubated with rabbit anti-human CBR1 primary antibody (1:2000) overnight at 4ºC, followed by goat anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody (1:10000, Santa Cruz Biotech) for 1 hour at room temperature. Immunoreactive bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) on a ChemiDoc XRS gel documentation system with Image Lab software (Bio Rad).

Daunorubicinol Quantification

The enzymatic activity of recombinant canine cbr1 D218 and cbr1 V218 for the anthracycline substrate daunorubicin was determined by direct quantification of the C-13 alcohol metabolite daunorubicinol from reaction mixtures. Reactions (300 µl) were assembled in 2 ml cryogenic vials (Sigma Aldrich) in 0.1 M potassium phosphate buffer (pH 7.4), recombinant protein (6 µg), and daunorubicin (5 - 325 µM). The mixtures were equilibrated to 37°C for 3 minutes, followed by the addition of NADPH to a final concentration of 300 µM. Reaction mixtures were maintained at 37°C for 1 minute. Reaction mixtures were quenched by the addition of ice cold acetonitrile, frozen in liquid nitrogen, and stored at -80ºC until processing.

8

Daunorubicin and daunorubicinol in reaction mixtures was quantified using an ultra-performance liquid chromatography (UPLC) fluorescent detection method as previously described (Quinones-Lombrana et al., 2014).

Kinetic Analysis

The enzymatic activities of recombinant canine cbr1 D218 and cbr1 V218 for the substrate menadione were determined by monitoring the rate of oxidation of NADPH using a method similar to that reported previously (Gonzalez-Covarrubias et al., 2007). Briefly, reaction mixtures (200 µl) were assembled in a clear flat-bottom 96 multi-well Costar plate (Sigma Aldrich) with 0.1 M potassium phosphate buffer (pH 7.4), recombinant protein (6 µg), and menadione (5 - 500 µM). The mixtures were then equilibrated to 37°C for 3 minutes, followed by the addition of NADPH to a final concentration of 250 µM. Immediately following the addition of NADPH, the absorbance at 340 nm was monitored every 3 seconds for 2 minutes using a Synergy HT plate reader (BioTek, Winooski, VT). Initial enzymatic velocity (V_0) was determined by linear regression (ΔAbs_{340}/minute), followed by conversion to nanomoles NADPH/minute using the molar absorption coefficient for NADPH (6220 $M^{-1}cm^{-1}$), and automatic path length determination by Gen5 software (BioTek).

Canine cbr1 Inhibition

Kinetic analyses were performed in an identical manner as described for the substrate menadione. The inhibitory effects of the naturally occurring flavonoid rutin on canine cbr1 activity was assessed essentially as previously described (Gonzalez-Covarrubias et al., 2008). Reaction mixtures (200 µl) contained 0.1 M potassium phosphate buffer (pH 7.4), recombinant protein (6 µg), rutin (2-25 µM), daunorubicin (5 - 300 µM), and NADPH (250 µM). The final dimethyl sulfoxide concentration was adjusted to 0.1% (v/v) in all reaction mixtures.

Data Analysis

Enzyme kinetic parameters (K_m and V_{max}) for daunorubicin and menadione were calculated by nonlinear regression using a one-site Michaelis-Menten binding model in GraphPad Prism (version 4.03; GraphPad Software Inc., San Diego, CA). Kinetic parameters were compared using GraphPad's built-in algorithm utilizing the F-test with a cut-off of $p < 0.05$ for statistical significance. The enzyme inhibition constant (K_i) for rutin was calculated by nonlinear regression using simultaneous fitting of a competitive inhibition Michaelis-Menten model to data points corresponding to activity at specified rutin concentrations in GraphPad Prism. Model fit was assessed by visual predictive checks and comparison of best fit values (R^2) compared to alternative models (non-competitive, uncompetitive). Enzymatic turnover (k_{cat}) was determined using the calculated V_{max} and the known enzyme concentrations within the reaction mixtures, assuming a canine cbr1 molecular weight of 32.8 kDa ($k_{cat}=V_{max}/[Et]$). Enzymatic activity data were expressed as the mean $\pm$ SD (n = 6) of two separate protein preparations with reactions performed in triplicate.

Results

Identification of a non-synonymous SNP in Canine cbr1

A liver cDNA sample from a 3 year old male Beagle dog was used to amplify the open

reading frame of *cbr1* (NCBI RefSeq: XM_847582.3). Sequence analysis revealed a non-

synonymous single nucleotide polymorphism (SNP), occurring at chromosome 31

(NC_006613.3) nucleotide position 31,320,945 (canine *cbr1,* exon 3, T>A. Figure 2). This SNP

results in an amino acid change of aspartic acid (D) to valine (V) at position 218 of the amino-

acid chain (NCBI RefSeq: XP_852675.1). Full DNA sequence analysis confirmed that this SNP

was the only alteration occurring within the amplified *cbr1* open reading frame. Colonies

containing the "wild-type" open reading frame (cbr1 D218), and variant open reading frame

(cbr1 V218) were selected and processed separately for subsequent studies.

Recombinant Canine cbr1 *Production*

The purity and identity of the recombinant cbr1 D218 and cbr1 V218 proteins were

confirmed by SDS-PAGE and Western blotting, with molecular weight analysis. Eluted and

concentrated recombinant proteins appeared as distinct single bands with a molecular weight of

approximately 32.8 kDa. The observed molecular weight was consistent with the predicted

molecular weight of canine cbr1 with a 6x His-tag (Figure 3a). The recombinant cbr1 proteins

were also detected as single bands with an anti-human CBR1 antibody that exhibit cross

reactivity with canine cbr1 (Figure 3b).

Kinetic Characterization of Canine cbr1

The enzyme kinetics of canine cbr1 V218 and cbr1 D218 for the anthracycline substrate

daunorubicin were determined by direct quantification of the product daunorubicinol (Figure 4a).

The kinetics of canine cbr1 were described well with the one-site Michaelis-Menten equation

(cbr1 D218, R^2 = 0.98, and cbr1 V218, R^2 = 0.99). The variant isoform cbr1 V218 displayed a

significantly lower K_m for daunorubicin than "wild-type" cbr1 D218 (P < 0.05. Table 1). The

variant cbr1 V218 also displayed a significantly higher V_{max} for daunorubicin than cbr1 D218 (P < 0.01. Table 1).

Menadione enzyme kinetics were assessed monitoring the consumption of NADPH cofactor, and could be described well by a simple one-site Michaelis-Menten equation (R^2 = 0.98, Figure 4b). Unlike daunorubicin, the enzyme kinetics parameters for the prototypical carbonyl reductase substrate menadione did not differ significantly between "wild-type" cbr1 D218 and the variant cbr1 V218 (K_m, P = 0.6967, and V_{max}, P = 0.9792, Table 1).

Canine cbr1 *Inhibition by Rutin*

The inhibitory effect of the common dietary flavonoid rutin (range: 0 - 25 µM) on daunorubicin reductase activity was assessed by monitoring the consumption of the NADPH cofactor in reactions with substrate concentrations ranging from 5 to 300 µM (Figure 4c & d). The inhibition of cbr1 D218 and cbr1 V218 by rutin was best described with a single site competitive inhibition equation (cbr1 D218, R^2 = 0.77, and cbr1 V218, R^2 = 0.87). Alternative inhibition models (noncompetitive and mixed-type) did not improve model fits. The enzyme inhibitory constants of rutin were determined to be 1.84 ± 1.02 µM and 1.38 ± 0.47 µM for cbr1 D218 and cbr1 V218 respectively.

Discussion

In regards to the use of anthracyclines for cancer chemotherapy in both humans and canines, a similar life-threatening, and treatment limiting cardiotoxic side effect is observed. Studies that monitored heart function following weekly intracoronary doses of doxorubicin (5 - 15 mg) showed that the development of anthracycline-related cardiotoxicity in dogs is dose-dependent (Astra et al., 2003). Cardiotoxicity has been observed clinically in 55% of canine lymphoma (n = 13) and osteosarcoma (n = 31) patients receiving cumulative doses of doxorubicin of 150 mg/m^2 (Selting et al., 2004). However, cardiac abnormalities (i.e., QTc prolongation) have been observed in canine cancer patients treated weekly with substantially lower doses of doxorubicin (12 - 25 mg/m^2 i.v.) by as early as the second dose (Buranakarl et al., 2014). Thus, there does not appear to be an apparent safe dose of anthracyclines to recommend in the treatment of canine cancers.

The formation of C-13 alcohol metabolites has long been assumed to be a contributor towards anthracycline-related cardiotoxicity, of which many relevant humans enzymes have been identified and characterized, notably CBR1 (Gonzalez-Covarrubias et al., 2007). To date only a handful of SDR enzymes have been identified or characterized in canines (Loveless et al., 1978; Hara et al., 1986; Iwata et al., 1990; Endo et al., 2007).

Our work here characterizes the first true CBR enzyme in canines, cbr1. Enzyme kinetic analyses reveal that canine cbr1 does in fact catalyze the reduction of daunorubicin to the C-13 alcohol metabolite daunorubicinol. This finding may provide a target enzyme for better understanding: a) the mechanisms of C-13 alcohol metabolite formation in relevant tissues in canine patients treated with anthracyclines, b) sources of genetic or interbreed variability effecting anthracycline metabolism, and c) potential facilitators of anthracycline related cardiotoxicity. Notably, C-13 alcohol metabolites, the major anthracycline metabolite in humans (~70%), have been shown to have significantly less antitumor activity than parent anthracyclines

(Mordente et al., 2001; Minotti et al., 2004). Therefore, changes in metabolism as a result of genetic polymorphisms could not only affect the observed toxicity, but also the pharmacodynamics of the parent drugs. In humans the CBR1 enzyme is ubiquitously expressed in all tissues, was found to be the primary doxorubicin reductase in human liver, and most prominent CBR expressed in heart tissue (Kassner et al., 2008; Quinones-Lombrana et al., 2014).

A previous sequencing endeavor conducted by our lab sought to identify functional variants within the canine *cbr1* gene (proximal 5' untranslated region, exons 1 - 3 and flanking intronic sequences) from samples spanning 7 of the most popular canine breeds in the United States of America (n = 97) (Cheng et al., 2012). This previous study sequenced 13 samples from Beagle dogs. The non-synonymous variant identified in the single Beagle donor commercial cDNA sample procured forthis work, resulted in an amino acid substitution of aspartic acid to valine at position 218 that has not been observed in any of the samples from our previous study. While the frequency of this nucleotide variant is unknown, the functional impact may reveal several higher order protein structural similarities between canine and human CBR1, which may in turn prove useful for future studies of the protein homologs. The canine *cbr1* gene (XM_847582.3), located on chromosome 31, encodes for a predicted enzyme of 277 amino acids, displaying 88.45% identity and 92.42% similarity with human CBR1 (Stothard, 2000). The daunorubicin Michaelis-Menten constant (K_m) was found to be 2.8-fold larger in the variant recombinant enzyme (V218) compared to the "wild-type" (D218). Additionally, the maximal enzyme velocity (V_{max}) was found to be 2.41-fold larger in the variant recombinant enzyme compared to the wild-type. These changes in affinity and capacity were not observed for the prototypical CBR1 substrate menadione, suggesting the effect of the variant on enzyme kinetics is substrate specific. This may in part be explained by the common amino acid sequence features of canine cbr1 with human CBR1. Sequence alignments reveal that canine cbr1

14

displays the NADPH sensing glycine residues (Glycine12-16-18), typical of an SDR-type enzyme (Hoffmann and Maser, 2007) (Figure 5). Additionally, the common SDR catalytic triad (Serine-Tyrosine-Lysine) is present in the canine amino acid sequence in identical arrangement to that of human CBR1 enzyme (Jornvall et al., 1995) (Figure 5). A cysteine residue in human CBR1 observed at position 227 previously identified to be important for catalysis of some non-glutathione bound substrate binding is also observed in the canine protein sequence (Tinguely and Wermuth, 1999). The close proximity of the V218 variant to critical active site amino acid residues, and change from an acidic (aspartic acid) to nonpolar (valine) residue could then be hypothesized to influence the binding interaction between daunorubicin and catalytic residues within the binding pocket of canine cbr1. This finding is encouraging, and may help to further guide the development or selection of new candidate inhibitors of carbonyl reductase 1 in humans and canines alike. Consistent with SDR type enzymes, and human CBR1, both wild-type and variant canine cbr1 displayed inhibition of daunorubicin activity by the flavonoid rutin. Previously, flavonoids were explored as candidate cardioprotectants against anthracycline related cardiotoxicity by targeting several pathophysiological pathways, including inhibition of CBR1 (Kaiserova et al., 2007; Carlquist et al., 2008). The finding that canine cbr1 is also inhibited by the common flavonoid rutin justifies the continued exploration of natural and synthetic flavonoids as potential alleviators of anthracycline related cardiotoxicity in canines.

CBR1 expression and activity has been correlated with the development of cardiotoxicity through the synthesis of C-13 alcohol metabolites in genetically engineered mouse models. For example, the mean survival time in mice overexpressing human *CBR1* in heart tissue was 5 weeks after weekly doses of doxorubicin (5 mg/kg i.p.), compared to a mean survival of 12 weeks for control animals identically treated (Forrest et al., 2000). Similarly, a murine *Cbr1* knock-out model demonstrated that mice with only one functional *Cbr1* allele displayed 82% survival 18 weeks after a single 20 mg/kg i.p. dose of doxorubicin, compared to 9% for animals

15

with two functional *Cbr1* alleles (Olson et al., 2003). The canine cbr1 V218 variant characterized here could be hypothesized to result in increased formation rate of C-13 alcohol metabolites in heart tissue potentially increasing the risk of toxicity. We propose that the investigation of pharmacogenomics determinants for canine cbr1 may contribute to the development of strategies to identify canine patients or breeds "at risk" prior to anthracycline treatment.

Future studies must detail the expression and subcellular location of canine cbr1 and other CBRs and AKRs in tissues relevant to the pharmacodynamics of anthracyclines (e.g., heart and liver). In addition, the *cbr1* D218V polymorphism should be further explored in canine DNA panels to determine the frequency of the variant allele in different breeds. Studies are warranted to examine whether other relevant pharmaceutical substrates of human CBR1, used in veterinary medicine, such as haloperidol and dolasetron are also metabolized by canine cbr1.

16

Acknowledgments:

Authorship Contributions:

Participated in research design: Ferguson, Cheng, Blanco

Conducted experiments: Ferguson, Cheng

Performed data analysis: Ferguson, Cheng

Wrote or contributed to the writing of the manuscript: Ferguson, Cheng, Blanco

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Footnotes:

Figure Legends:

*Figure 1***:** DNA sequence alignment of "wild-type" (top) and variant (bottom) canine *cbr1*. A single nucleotide polymorphism occurring at nucleotide position 31,32,945 of canine chromosome 31 (NC_006613.3), indicated by arrows, results in an amino acid change of aspartic acid to valine at position 218. Sequence alignments were constructed in GeneScreen (Carr et al., 2011).

Figure 2: A) Coomasie blue stained SDS-PAGE of recombinant canine cbr1 D218 (5 µg) and cbr1 V218 (5µg). B) Western blot of recombinant canine cbr1 (5 µg each) and human CBR1 (1 µg) probed with anti-CBR1 primary antibody. In both situations molecular weights of observed bands were compared to a molecular weight marker (MWM).

*Figure 3***:** A) Enzyme kinetics of daunorubicinol formation for canine cbr1 D218 and cbr1 V216 with the substrate daunorubicin. Experiments were performed by measuring the formation of daunorubicinol in reaction mixtures. B) Enzyme kinetics of canine cbr1 D218 and cbr1 V216 with the substrate menadione. Experiments were performed by measuring the consumption of cofactor NADPH. C) Enzyme inhibition of canine cbr1 D218 daunorubicin activity with the flavonoid rutin. Experiments were performed by measuring the consumption of cofactor NADPH. D) Enzyme inhibition of canine cbr1 V218 daunorubicin activity with the flavonoid rutin. Experiments were performed by measuring the consumption of NADPH cofactor. In all kinetic experiments each point represents the mean ± SD of two separate protein preparations analyzed in triplicate (n = 6).

*Figure 4***:** Amino acid sequence alignment of human CBR1 and canine cbr1. Cofactor sensing residues are highlighted in grey and catalytic residues in black. Position of the amino acid substitution detected in canine cbr1 V218 is bolded. Sequence alignments were constructed in T-Coffee (Notredame et al., 2000).

Tables:

TABLE 1

Canine cbr1 D218 and cbr1 V218 enzyme kinetics constants

Substrate	D218				V218			
	K_m	V_{max}	k_{cat}[a]	V_{max}/K_m	K_m	V_{max}	k_{cat}[a]	V_{max}/K_m
	µM	nmol/min*mg	min^{-1}	mg/min x 10^{-3}	µM	nmol/min*mg	min^{-1}	mg/min x 10^{-3}
Daunorubicin[b]	188 ± 144	6446 ± 3615	211	34.2	527 ± 136	15539 ± 2623	509	29.4
Menadione[b]	104 ± 50	2034 ± 307	67	19.6	118 ± 51	2041 ± 334	67	17.3

[a]Calculated assuming a MW of 32800 g/mol

[b]Values represent the mean ± SD from two experiments performed in triplicate with independent protein preparations
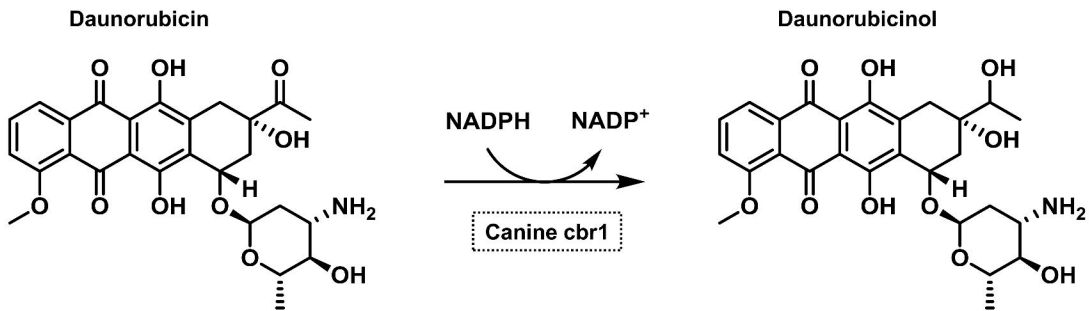
Figure 1

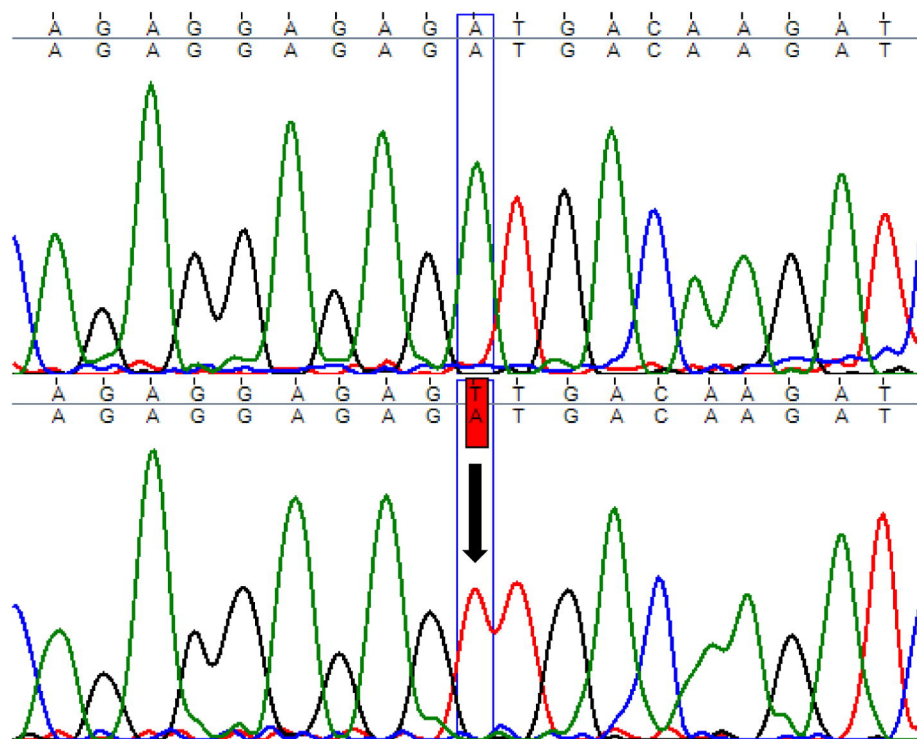**Daunorubicin** → **Daunorubicinol**

NADPH   NADP⁺

Canine cbr1

Figure 2

Figure 3

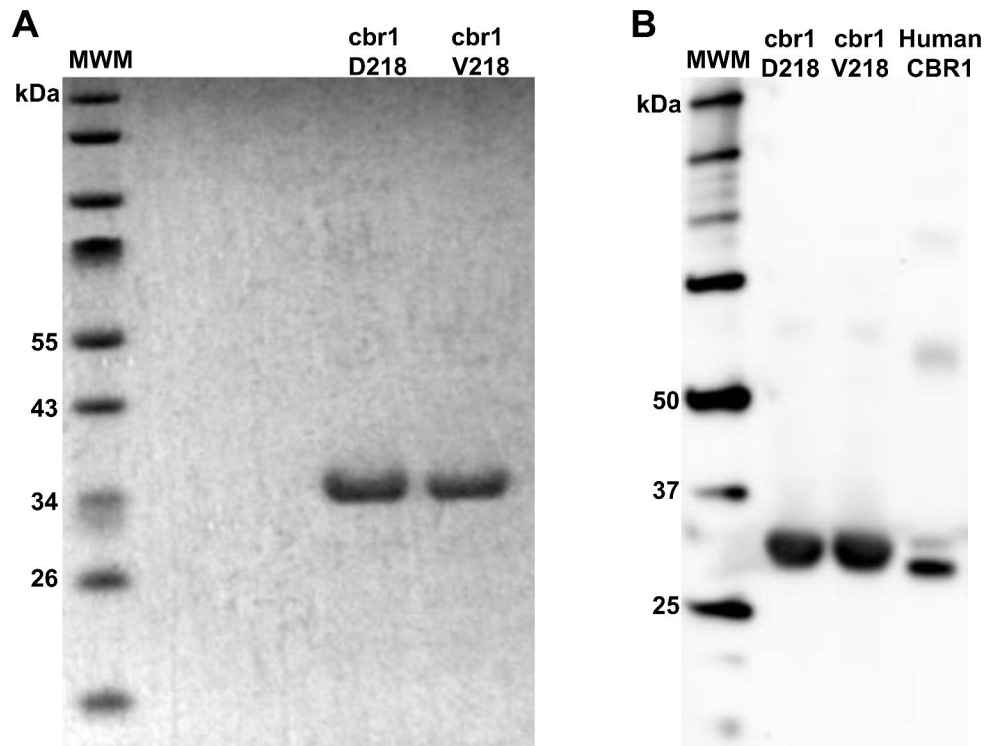# Figure 4
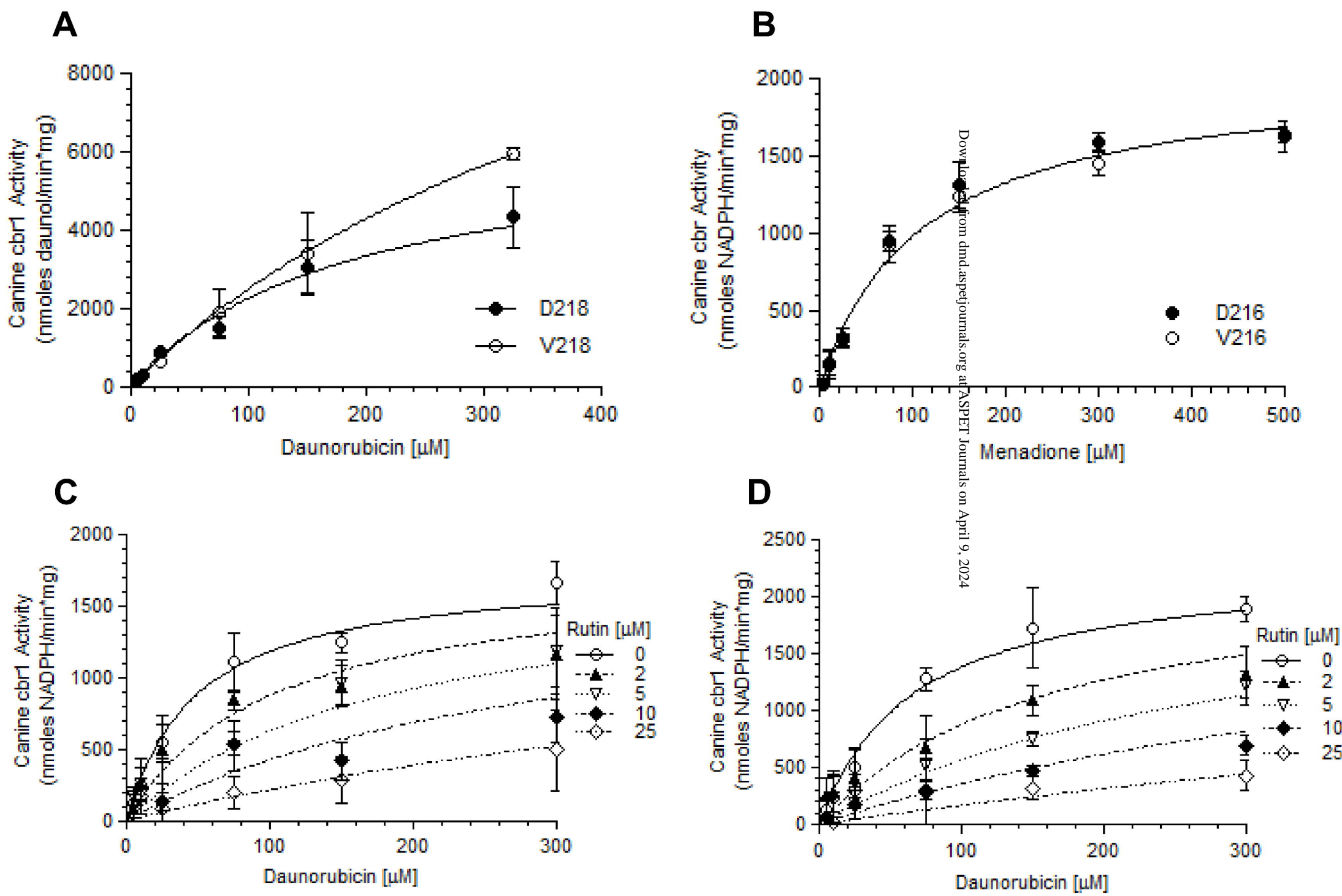
Figure 5

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Human_CBR1   MSSGIHVALVTGGNKGIGLAIVRDLCRLFSGDVVLTARDVTRGQAAVQQLQAEGLSPRFHQLDIDDLQSIRALRDFLRKE 80
Canine_CBR1  MSAASRVALVTGANKGIGFAIARELCRQFSGDVVLTARDEARGRAAVQQLQAEGLSPRFHLLDIDDLQSIRALRDFLRKE 80
             **:. :*****.*****:**.*:*** ********** *:**:***************** *****************

Human_CBR1   YGGLDVLVNNAGIAFKVADPTPFHIQAEVTMKTNFFGTRDVCTELLPLIKPQGRVVNVSSIMSVRALKSCSPELQQKFRS 160
Canine_CBR1  YGGLDVLVNNAGIAFKTNDPTPFHIQAEVTMKTNFFGTRDVCTELLPLMKPQGRVVNVSSVVSVRALKSCSPELQQKFRS 160
             ****************. .******************************:*********::*******************

Human_CBR1   ETITEEELVGLMNKFVEDTKKGVHQKEGWPSSAYGVTKIGVTVLSRIHARKLSEQRKGDKILLNACCPGWVRTDMAGPKA 240
Canine_CBR1  EAITEEELVGLMNKFVEDTKKGVHRNEGWPDNAYGVTKIGVTVLSRIHARKLSEQRRDKILLNACCPGWVRTDMAGPRA 240
             *:*********************:.****. .*************************:.*******************:*

Human_CBR1   TKSPEEGAETPVYLALLPPDAEGPHGQFVSEKRVEQW 277
Canine_CBR1  PKSPEEGAETPVYLALLPSDAEGPHGEFLMEKKVEQW 277
             .*************** .*******:*:: **:****
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