Characterization of the Canine Anthracycline-Metabolizing Enzyme Carbonyl Reductase 1 (cbr1) and the Functional Isoform cbr1 V218

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Received March 13, 2015; accepted April 27, 2015

ABSTRACT

The anthracyclines doxorubicin and daunorubicin are used in the treatment of various human and canine cancers, but anthracycline-related cardiotoxicity limits their clinical utility. The formation of anthracycline C-13 alcohol metabolites (e.g., doxorubicinol and daunorubicinol) contributes to 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 Escherichia 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 $K_{\text{m}}$: 188 ± 144 μM versus cbr1 V218 $K_{\text{m}}$: 527 ± 136 μM, $P < 0.05$, and cbr1 D218 $V_{\text{max}}$: 646 ± 3615 nmol/min per milligram versus cbr1 V218 $V_{\text{max}}$: 1559 ± 2623 nmol/min per milligram, $P < 0.01$). Canine cbr1 also metabolized menadione (cbr1 D218 $K_{\text{m}}$: 104 ± 50 μM, $V_{\text{max}}$: 2034 ± 307 nmol/min per milligram). 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 in 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 hematologic 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 metabolite products from their parent anthracyclines, with formation catalyzed by short-chain dehydrogenases reductases (SDR), namely carbonyl reductases (CBRs), and members of the aldo-keto reductase (AKR) family (Mordente et al., 2001). Most prominent for the metabolism of anthracyclines appear to be CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 (Quiñones-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). As in 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; Oostierbaan 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, in 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 (Fig. 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 bisulfite, NADPH, dimethyl sulfoxide, monobasic potassium phosphate, dibasic potassium phosphate, phosphate-buffered saline (PBS), rutin hydrate, daunorubicin hydrochloride, and acetonitrile were purchased from Sigma-Aldrich. 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'-AGCAAACACGCCCTTGCA-3', (reverse) 5'-CTCTAAGGGTTCTGTTG-3'. The polymerase chain reaction (PCR) product was cloned into a pcR2.1 vector with TOPO TA (Invitrogen/Life Technologies, Grand Island, NY), and transformed into chemically competent TOP10 Escherichia coli cells following the manufacturer’s instructions. Constructs were then isolated using the Geneaid High-Speed Plasmid Mini Kit (New Taipei City, Taiwan) and sequenced with an ABI PRISM 3130 XL Genetic Analyzer (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 below).

Cloning and Expression of Canine cbr1. 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'-AGGAAATTCTCATATGCTGCAGCCTCCTCGGTGG-3', reverse-Xho I: 5'-ATCCGGCTCGAAGTTATCCTAAAGGGTTCTGTTG-3'). The resulting PCR products were then purified and double-digested with NheI and XhoI restriction enzymes. The digested fragments were then inserted into a NheI/XhoI predigested pET2a 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, and were then processed separately.

Daunorubicinol Quantification. The enzymatic activity of recombinant canine cbr D218 and cbr 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), with 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 –80°C until processing. Daunorubicin and daunorubicinol in reaction mixtures was quantified using an ultra-performance liquid chromatography fluorescence detection method as previously described (Quiñones-Lombrana et al., 2014).

Kinetic Analysis. The enzymatic activities of recombinant canine cbr D218 and cbr 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-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 (Bio-Tek Instruments, Winooski, VT). Initial enzymatic velocity (V0) was determined by linear regression (ΔAbs/min), followed by conversion to nanomoles NADPH/min using the molar absorption coefficient for NADPH (6202 M−1cm−1), and automatic path-length determination by Gen5 software (Bio-Tek).
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 (Km and Vmax) 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., La Jolla, 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 (Ki) 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 (R2) with alternative models (noncompetitive, uncompetitive). Enzymatic turnover (kcat) was determined using the calculated Vmax and the known enzyme concentrations within the reaction mixtures, assuming a canine cbr1 molecular weight of 32.8 kDa (kcat = Vmax/[E]). Enzymatic activity data were expressed as the mean ± S.D. (n = 6) of two separate protein preparations with reactions performed in triplicate.

Results

Identification of a Nonsynonymous 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 nonsynonymous single nucleotide polymorphism (SNP), occurring at chromosome 31 (NC_006613.3) nucleotide position 31,320,945 (canine cbr1, exon 3, T>A; Fig. 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 6xHis-tag (Fig. 3A). The recombinant cbr1 proteins were also detected as single bands with an anti-human CBR1 antibody that exhibits cross reactivity with canine cbr1 (Fig. 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 (Fig. 4A). The kinetics of canine cbr1 were described well with the one-site Michaelis-Menten equation (cbr1 D218, R2 = 0.98, and cbr1 V218, R2 = 0.99). The variant isoform cbr1 V218 displayed a significantly lower Km for daunorubicin than wild-type cbr1 D218 (P < 0.05. Table 1). The variant cbr1 V218 also displayed a significantly higher Vmax for daunorubicin than cbr1 D218 (P < 0.01; Table 1).

Menadione enzyme kinetics were assessed by monitoring the consumption of NADPH cofactor and could be described well by a simple one-site Michaelis-Menten equation (R2 = 0.98; Fig. 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 (Km, P = 0.6967, and Vmax, P = 0.9792; Table 1).
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 (Fig. 4, C and 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 $1.84 \pm 1.02$ μM and $1.38 \pm 0.47$ μM for cbr1 D218 and cbr1 V218, respectively.

**Discussion**

In regard to the use of anthracyclines for cancer chemotherapy in both humans and canines, similar life-threatening, treatment-limiting cardiotoxic side effects are 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/m² (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/m² 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 toward anthracycline-related cardiotoxicity, and 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: 1) the mechanisms of C-13 alcohol metabolite formation in relevant tissues in canine patients treated with anthracyclines, 2) sources of genetic or interbreed variability affecting anthracycline metabolism, and 3) potential facilitators of anthracycline-related cardiotoxicity. Notably, C-13 alcohol metabolites, the major anthracycline metabolites, have been shown to have significantly less antitumor activity than parent anthracyclines (Mordente 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 and was found to be the primary doxorubicin reductase in human liver and the most prominent CBR expressed in heart tissue (Kassner et al., 2008; Quinones-Lombrana et al., 2014).

A previous sequencing endeavor conducted by our laboratory sought to identify functional variants within the canine cbr1 gene (proximal 5’ untranslated region, exons 1–3 and flanking intronic sequences) from samples spanning seven of the most popular canine breeds in the United States (n = 97) (Cheng et al., 2012). This previous study sequenced 13 samples from Beagle dogs. The nonsynonymous variant identified in the single Beagle donor commercial cDNA sample procured for this 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 (Km) was found to be 2.8-fold larger in the variant recombinant enzyme (V218) compared with the wild-type (D218). Additionally, the maximal enzyme velocity (Vmax) was found to be 2.41-fold larger in the variant recombinant enzyme compared with 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 be explained in part by the common amino acid sequence features of canine cbr1 with human CBR1. Sequence alignments reveal that canine cbr1 displays the NADPH-sensing glycine residues (glycine12-16-18) typical of an SDR-type enzyme (Hoffmann and Maser, 2007) (Fig. 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) (Fig. 5). A cysteine residue in human CBR1 observed at position 227 previously identified to be important for catalysis of some nonglutathione-bound substrate binding is also observed in the canine protein sequence (Tinguey 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

### Table 1: Canine cbr1 D218 and cbr1 V218 enzyme kinetics constants

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (μM)</th>
<th>Vmax (nmol/min per milligram)</th>
<th>kcat (min⁻¹)</th>
<th>Vmax/Km (mg/min)</th>
<th>kcat/Km (mg/min × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunorubicin</td>
<td>188 ± 144</td>
<td>6446 ± 3615</td>
<td>211</td>
<td>34.2</td>
<td>19.6</td>
</tr>
<tr>
<td>Menadione</td>
<td>104 ± 50</td>
<td>2034 ± 507</td>
<td>67</td>
<td>34.2</td>
<td>19.6</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>527 ± 136</td>
<td>15539 ± 2623</td>
<td>509</td>
<td>29.4</td>
<td>17.3</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Amino acid sequence alignment of human CBR1 and canine cbr1. Cofactor sensing residues are highlighted in gray 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).
Canine Carbonyl Reductase 1, an Anthracycline Reductase

several pathophysiological pathways, including inhibition of CBR1 (Kaiserova et al., 2007; Carquist 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 with 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 with 9% for animals 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 pharmacogenomic 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 explored further in canine DNA panels to determine the frequency of the variant allele in different breeds. Studies are warranted to examine whether other relevant pharmacological substrates of human CBR1 used in veterinary medicine, such as haloperidol and dolasetron, are also metabolized by canine cbr1.

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.

References

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