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

Daniel C. Ferguson, Qiuying Cheng, and Javier G. Blanco

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, The State University of New York at Buffalo, Buffalo, New York

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_m : $188 \pm 144 \mu\text{M}$ versus cbr1 V218 K_m : $527 \pm 136 \mu\text{M}$, $P < 0.05$, and cbr1 D218 V_{max} : $6446 \pm 3615 \text{ nmol/min per milligram}$ versus cbr1 V218 V_{max} : $15539 \pm 2623 \text{ nmol/min per milligram}$, $P < 0.01$). Canine cbr1 also metabolized menadione (cbr1 D218 K_m : $104 \pm 50 \mu\text{M}$, V_{max} : $2034 \pm 307 \text{ nmol/min per milligram}$). Rutin acted as a competitive inhibitor for the reduction of daunorubicin (cbr1 D218 K_i : $1.84 \pm 1.02 \mu\text{M}$, cbr1 V218 K_i : $1.38 \pm 0.47 \mu\text{M}$). 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 metabolic 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-Lombraña et al., 2014).

Research in this report was supported by awards from the National Institutes of Health National Institute of General Medical Sciences [R01GM073646] and the AKC Canine Health Foundation [Grant 1972]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the founding sources.

dx.doi.org/10.1124/dmd.115.064295.

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; 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, 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

ABBREVIATIONS: AKR, aldo-keto reductase; CBR, carbonyl reductase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDR, short chain dehydrogenase reductase; SNP, single nucleotide polymorphism.

Fig. 1. 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 dog.

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 (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'-AGCAAACACGCCCTTGACAG-3', (reverse) 5'-TCCTAAAGGGTTCCTGG-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 details in Results). 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*. 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'-GAGGAATTCCATATGTCTGCAGCTCCCGCTGG-3', and reverse-*Xho* I: 5'-ATCCGCTCGAGTTATCCTAAAGGGTTCCTGG-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 6xHis-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 (DE3) pLysS-competent *E. coli* (Invitrogen/Life Technologies) by heat shock, plated onto LB broth agar supplemented with kanamycin (50 μ g/ml), and grown overnight at 37°C. Randomly selected colonies were then cultured in 3 ml of LB broth supplemented with kanamycin (50 μ g/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 μ M) 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 Cobalt 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). The purity of concentrated proteins was assessed by SDS-PAGE on a NuPAGE Novex 4–12% Bis-Tris precast gel (Life Technologies) with Coomassie 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 Biotechnology, Dallas, TX). Briefly, 5 μ g of recombinant canine *cbr1* (*cbr1* D218 and *cbr1* V218), and 1 μ g of recombinant human CBR1 (Abcam, Cambridge, MA) were separated on a NuPAGE Novex 4–12% Bis-Tris precast gel (Life Technologies). Proteins were transferred to a nitrocellulose membrane using the iBlot transfer device (Life Technologies). Membranes were blocked with 5% nonfat 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 4°C, followed by goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:10000, Santa Cruz Biotechnology) 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 μ l) were assembled in 2 ml cryogenic vials (Sigma-Aldrich) in 0.1 M potassium phosphate buffer (pH 7.4), with recombinant protein (6 μ g) and daunorubicin (5–325 μ M). The mixtures were equilibrated to 37°C for 3 minutes, followed by the addition of NADPH to a final concentration of 300 μ M. 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 *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-Covarubias et al., 2007). Briefly, reaction mixtures (200 μ l) 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 μ g), and menadione (5–500 μ M). The mixtures were then equilibrated to 37°C for 3 minutes, followed by the addition of NADPH to a final concentration of 250 μ M. 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 (V_0) was determined by linear regression ($\Delta Abs_{340}/\text{minute}$), followed by conversion to nanomoles NADPH/min using the molar absorption coefficient for NADPH (6220 $M^{-1}cm^{-1}$), 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 μ l) contained 0.1 M potassium phosphate buffer (pH 7.4), recombinant protein (6 μ g), rutin (2–25 μ M), daunorubicin (5–300 μ M), and NADPH (250 μ M). 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., 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 (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) with alternative models (noncompetitive, 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 $\pm$ 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, $R^2 = 0.98$, 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 by monitoring the consumption of NADPH cofactor and could be described well by a simple one-site Michaelis-Menten equation ($R^2 = 0.98$; 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 (K_m , $P = 0.6967$, and V_{max} , $P = 0.9792$; Table 1).

Fig. 2. DNA sequence alignment of wild-type (top) and variant (bottom) canine *cbr1*. A single nucleotide polymorphism occurring at nucleotide position 31,320,945 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).

Fig. 3. (A) Coomassie blue-stained SDS-PAGE of recombinant canine cbr1 D218 (5 µg) and cbr1 V218 (5 µg). (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 with a molecular weight marker (MWM).

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 (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 ± 1.02 µM and 1.38 ± 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.

Fig. 4. (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 $\pm$ S.D. of two separate protein preparations analyzed in triplicate ($n = 6$).

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 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 pharmaceutical 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

- Astra LI, Hammond R, Tarakji K, and Stephenson LW (2003) Doxorubicin-induced canine CHF: advantages and disadvantages. *J Card Surg* **18**:301–306.
- Buranakarl C, Kijtaowornrat A, Chansaisakorn W, Trisirirotj M, Ngamdarnongkiet C, Taecholarn T, Sonpee J, and Chanwathik W (2014) Changes in electrophysiology, heart rate variability and proteinuria in clinical dogs treated with doxorubicin chemotherapy. *Thai J Vet Med* **44**:307–316.
- Carlquist M, Frejd T, and Gorwa-Grauslund MF (2008) Flavonoids as inhibitors of human carbonyl reductase 1. *Chem Biol Interact* **174**:98–108.
- Carr IM, Camm N, Taylor GR, Charlton R, Ellard S, Sheridan EG, Markham AF, and Bonthron DT (2011) GeneScreen: a program for high-throughput mutation detection in DNA sequence electropherograms. *J Med Genet* **48**:123–130.
- Cheng Q, Sanborn C, Ferguson D, and Blanco JG (2012) DNA sequence variants in the carbonyl reductase 1 (*cbr1*) gene in seven breeds of *Canis lupus familiaris*. *Genet Mol Res* **11**:1109–1116.
- Endo S, Matsunaga T, Nagano M, Abe H, Ishikura S, Imamura Y, and Hara A (2007) Characterization of an oligomeric carbonyl reductase of dog liver: its identity with peroxisomal tetrameric carbonyl reductase. *Biol Pharm Bull* **30**:1787–1791.
- FitzPatrick WM, Dervisis NG, and Kitchell BE (2010) Safety of concurrent administration of dexrazoxane and doxorubicin in the canine cancer patient. *Vet Comp Oncol* **8**:273–282.
- Forrest GL, Gonzalez B, Tseng W, Li X, and Mann J (2000) Human carbonyl reductase overexpression in the heart advances the development of doxorubicin-induced cardiotoxicity in transgenic mice. *Cancer Res* **60**:5158–5164.
- Gillings S, Johnson J, Fulmer A, and Hauck M (2009) Effect of a 1-hour IV infusion of doxorubicin on the development of cardiotoxicity in dogs as evaluated by electrocardiography and echocardiography. *Vet Ther* **10**:46–58.
- Gonzalez-Covarrubias V, Ghosh D, Lakhman SS, Pendyala L, and Blanco JG (2007) A functional genetic polymorphism on human carbonyl reductase 1 (CBR1 V88I) impacts on catalytic activity and NADPH binding affinity. *Drug Metab Dispos* **35**:973–980.
- Gonzalez-Covarrubias V, Kalabus JL, and Blanco JG (2008) Inhibition of polymorphic human carbonyl reductase 1 (CBR1) by the cardioprotectant flavonoid 7-mono-hydroxyethyl rutoside (monohER). *Pharm Res* **25**:1730–1734.
- Hara A, Nakayama T, Deyashiki Y, Kariya K, and Sawada H (1986) Carbonyl reductase of dog liver: purification, properties, and kinetic mechanism. *Arch Biochem Biophys* **244**:238–247.
- Hoffmann F and Maser E (2007) Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Metab Rev* **39**:87–144.
- Iwata N, Inazu N, and Satoh T (1990) Immunological and enzymological localization of carbonyl reductase in ovary and liver of various species. *J Biochem* **107**:209–212.
- Jörnvall H, Persson B, Krook M, Atrian S, González-Duarte R, Jeffery J, and Ghosh D (1995) Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34**:6003–6013.
- Kaiserová H, Simunek T, van der Vijgh WJ, Bast A, and Kvasnicková E (2007) Flavonoids as protectors against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochim Biophys Acta* **1772**:1065–1074.
- Kassner N, Huse K, Martin HJ, Gödel-Ambrust U, Metzger A, Meineke I, Brockmöller J, Klein K, Zanger UM, and Maser E, et al. (2008) Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metab Dispos* **36**:2113–2120.
- Loveless H, Arena E, Felsted RL, and Bachur NR (1978) Comparative mammalian metabolism of adriamycin and daunorubicin. *Cancer Res* **38**:593–598.
- Mauldin GE, Fox PR, Patnaik AK, Bond BR, Mooney SC, and Matus RE (1992) Doxorubicin-induced cardiotoxicosis. Clinical features in 32 dogs. *J Vet Internal Med* **6**:82–88.
- Minotti G, Menna P, Salvatorelli E, Cairo G, and Gianni L (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* **56**:185–229.
- Mordente A, Meucci E, Martorana GE, Giardina B, and Minotti G (2001) Human heart cytosolic reductases and anthracycline cardiotoxicity. *IUBMB Life* **52**:83–88.
- Notredame C, Higgins DG, and Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**:205–217.
- Olson LE, Bedja D, Alvey SJ, Cardounel AJ, Gabrielson KL, and Reeves RH (2003) Protection from doxorubicin-induced cardiac toxicity in mice with a null allele of carbonyl reductase 1. *Cancer Res* **63**:6602–6606.
- Oosterbaan MJ, Dirks RJ, Vree TB, and van der Kleijn E (1984) Pharmacokinetics of anthracyclines in dogs: evidence for structure-related body distribution and reduction to their hydroxy metabolites. *Pharm Res* **1**:33–38.
- Quiñones-Lombraña A, Ferguson D, Hageman Blair R, Kalabus JL, Redzematovic A, and Blanco JG (2014) Interindividual variability in the cardiac expression of anthracycline reductases in donors with and without Down syndrome. *Pharm Res* **31**:1644–1655.
- Ratterree W, Gieger T, Pariaut R, Saelinger C, and Strickland K (2012) Value of echocardiography and electrocardiography as screening tools prior to Doxorubicin administration. *J Am Anim Hosp Assoc* **48**:89–96.
- Selting KA, Lana SE, Ogilvie GK, Olmstead A, Mykles DL, Bright J, Richardson KL, Walton JA, Monnet E, and Fettman MJ (2004) Cardiac troponin I in canine patients with lymphoma and osteosarcoma receiving doxorubicin: comparison with clinical heart disease in a retrospective analysis. *Vet Comp Oncol* **2**:142–156.
- Simon D, Nolte I, Eberle N, Abbrederis N, Killich M, and Hirschberger J (2006) Treatment of dogs with lymphoma using a 12-week, maintenance-free combination chemotherapy protocol. *J Vet Intern Med* **20**:948–954.
- Stothard P (2000) The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* **28**:1102–1104.
- Tinguely JN and Wermuth B (1999) Identification of the reactive cysteine residue (Cys227) in human carbonyl reductase. *Eur J Biochem* **260**:9–14.
- Von Hoff DD, Layard MW, Basa P, Davis HL, Jr, Von Hoff AL, Rozenzweig M, and Muggia FM (1979) Risk factors for doxorubicin-induced congestive heart failure. *Ann Intern Med* **91**:710–717.

Address correspondence to: Dr. Javier G. Blanco, Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, 470 Kapoor Hall, Buffalo New York, 14214-8033. E-mail: jgblanco@buffalo.edu