DMD Fast Forward. Published on February 9, 2009 as doi:10.1124/dmd.108.024711
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DMD #24711

# Two non-synonymous single nucleotide polymorphisms of human carbonyl reductase 1 demonstrate reduced *in vitro* metabolism of daunorubicin and doxorubicin

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**RUNNING TITLE:** Genetic variants of *CBR1* reduce anthracycline metabolism

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Number of Text Pages: 27

Number of Tables: 2

Number of Figures: 4

Number of References: 34

Number of Words for Abstract: 246 (max 250)

Introduction: 652 (max 750)

Discussion: 1044 (max 1500)

**ABBREVIATIONS:** CBR, carbonyl reductase ; DAUN, daunorubicin; DAUNol, daunorubicinol; DOX, doxorubicin; DOXol, doxorubicinol; FXa, Factor Xa; HPLC, high performance liquid chromatography; Ni-NTA, nickel-nitrilotriacetic acid; ns, non-synonymous; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 6x-His, six histidine

## ABSTRACT

Carbonyl reductases (CBRs) are a group of metabolic enzymes belonging to the short chain dehydrogenase family with NADPH-dependent oxidoreductase activity. These enzymes are known to metabolize the anthracyclines doxorubicin (DOX) and daunorubicin (DAUN). Both DOX and DAUN are highly effective in cancer therapy; however, there is considerable interpatient variability in adverse effects seen in patients undergoing treatment with these drugs. This may be attributed to altered metabolism associated with non-synonymous single nucleotide polymorphisms (ns-SNPs) in the genes encoding for CBRs. In this study, we examine the effect of the V88I and P131S mutations in the human *CBR1* gene on the metabolism of anthracyclines to their respective major metabolites, doxorubicinol (DOXol) and daunorubicinol (DAUNol). Kinetic studies using purified, histidine-tagged, recombinant enzymes in a high-performance liquid chromatography-fluorescence assay demonstrated that the V88I mutation leads to a significantly reduced maximal rate of activity ($V_{max}$), 2090±112 nmol/min•mg purified protein for DAUN and 257±11 nmol/min•mg for DOX, compared to the wild-type, 3430±241 and 364±37 nmol/min•mg for DAUN and DOX, respectively. In the case of the P131S mutation, a significant increase in substrate affinity ($K_m$) was observed for DAUN only, 89±13 μM, compared to the wild-type, 51±13 μM. In the presence of either anthracycline, both variants exhibited a 20-40% decrease in catalytic efficiency ($k_{cat}/K_m$) compared to the wild-type enzyme. Therefore, the ns-SNPs generating both these mutations may alter bioavailability of these anthracyclines in cancer patients and should be examined in clinical studies as potential biomarkers for DAUN and DOX induced adverse effects.

## INTRODUCTION

Short chain dehydrogenases encompass a highly divergent superfamily of enzymes with the carbonyl reductases (CBRs) being prominent members of this family. CBRs are cytosolic, NADPH-dependent oxidoreductases that metabolize a broad spectrum of endogenous and exogenous carbonyl-containing compounds, including aflatoxins, prostaglandins and steroids (Hoffmann and Maser, 2007). They are classified as Phase I biotransformation enzymes that are involved in the conversion of carbonyl groups to alcohols for the purposes of increasing their water solubility which facilitates their elimination from the body directly or *via* conjugation reactions.

Although carbonyl reductases are found in nearly all phyla (i.e., bacteria, yeast, plants and insects), the most studied is human carbonyl reductase 1 (CBR1) (Oppermann, 2007; Hoffmann and Maser, 2007). CBR1 is widely distributed in human tissues such as the liver, heart, stomach, kidney, small intestine, cerebellum, and smooth muscle fibers (Wirth and Wermuth, 1992; Forrest and Gonzalez, 2000). This enzyme is of particular interest clinically as it plays a major role in metabolism of the anthracyclines doxorubicin (DOX) and daunorubicin (DAUN) (Licata et al., 2000; Gonzalez-Covarrubias et al., 2007). These two drugs are the most widely used anthracyclines in oncological practice. DOX is a vital component in the treatment of non-Hodgkin's lymphoma, childhood solid tumors, soft tissue carcinomas, and breast cancer, while DAUN occupies a central position in treating acute leukemias (Hunault-Berger et al., 2001; Fassas and Anagnostopoulos, 2005; Cortes-Funes and Coronado, 2007; Deng and Wojnowski, 2007). While DAUN and DOX-based chemotherapies have contributed to improved life expectancy, both have been linked to the development of serious life-threatening adverse events with a high patient variability (Minotti et al., 1999; Mordente et al., 2001; Danesi

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et al., 2002; Barry et al., 2007; Menna et al., 2007; Scully and Lipshultz, 2007). A prominent example is chronic cardiotoxicity, which can ultimately lead to irreversible complications such as congestive heart failure, a life long threat with a mortality rate that can exceed 10% in children treated with ≥300 mg/m$^2$ of any anthracycline and 26% in adults treated with ≥550 mg/m$^2$ DOX (Deng and Wojnowski, 2007). Both the frequency of chronic cardiotoxicity and mortality rate are associated with increased dosage of the anthracyclines. In addition, the risk of chronic cardiotoxicity increases with the concurrent use of other anticancer drugs, such as herceptin, paclitaxel and cyclophosphamide (Minotti et al., 1999; Wojtacki et al., 2000; Danesi et al., 2002; Gianni et al., 2007).

The underlying cause(s) of interpatient variation in DAUN or DOX induced adverse drug events may be due to altered enzyme activity associated with non-synonymous nucleotide polymorphisms (ns-SNPs) of the *CBR* genes, particularly human *CBR1*. There are two documented ns-SNPs, one producing a change from a valine to isoleucine at amino acid position 88 (V88I; SNP ID: rs1143663 from National Centre for Biotechnology Information Database; *q* = 2.1%), and the other from a proline to serine at amino acid position 131 (P131S; SNP ID: rs41557318; *q* = 2.2%). Although there is evidence on the catalytic properties of the V88I enzyme with DAUN (Gonzalez-Covarrubias et al., 2007), none have been reported for DOX. Also, there is no information on the catalytic properties of the P131S variant in the presence of either anthracycline. Therefore, our goal for this study is to compare the wild-type and variant enzymes for their ability to metabolize DAUN and DOX to their corresponding carbon-13 alcohol metabolites, daunorubicinol (DAUNol) and doxorubicinol (DOXol). Using purified, bacterially-expressed, recombinant, human histidine-tagged enzymes, we demonstrated that the V88I variant resulted in significantly reduced metabolic activity for both DOX and DAUN,

while the P131S gene product exhibited a significant increase in affinity with DAUN compared to the wild-type enzyme. We also found that DAUN is a better substrate than DOX for both the wild-type and variant enzymes. The metabolic/affinity differences seen with the mutant V88I and P131S enzymes compared with that of the wild-type could result in increased concentrations of DOX or DAUN in cancer patients and the increased occurrence of adverse side effects.

## MATERIALS AND METHODS

### Chemicals and enzymes

Agarose, chloramphenicol, daunorubicin, doxorubicin, idarubicin, kanamycin sulfate, lysozyme, menadione, methanol, potassium phosphate ($KH_2PO_4$), RNaseI, sodium phosphate ($NaH_2PO_4$), N,N,N',N' tetramethylethylenediamine and NADPH were supplied by Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile, agar, ammonium persulfate, formic acid, ethanol, glycine, glycerol, glacial acetic acid, imidazole, and Tris were purchased from Fisher Scientific Co. (Fair Lawn, NJ). NaCl and yeast extract were ordered from EMD Chemicals Inc. (Darmstadt, Germany). Bacto tryptone and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from BD Biosciences (Franklin Lakes, NJ) and Fermentas Inc. (Hanover, MD), respectively. Tween 20 was purchased from EMD Biosciences (La Jolla, CA), and DNaseI was provided by Boehringer Manheim GmbH (Manheim, Germany). Klenow fragment and T4 ligase were obtained from Fermentas (Burlington, ON). Factor Xa and restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Doxorubicinol was obtained from Qventas Inc. (Branford, CT).

### Molecular cloning of human *CBR1* gene and creation of the genetic variants

The *CBR1* coding region, which was excised from a pOTB7 recombinant plasmid (MGC-1920, American Type Culture Collection (ATCC), Manassas, VA) using BssHII (blunt end with

Klenow fragment) and XhoI, was subcloned into NheI (blunt end)-XhoI sites of the pET28a prokaryotic expression vector (EMD, Novagen, San Diego, CA) with T4 ligase. These constructs gave rise to a human CBR1 enzyme with a 6x-His tag separated by an 18-amino acid residue linker on the amino terminus. A Factor Xa (FXa) site was inserted at the amino terminus between the linker and *CBR1* gene using the QuikChange® Site-Directed Mutaganesis Kit (Stratagene, La Jolla, CA) with the 5' - CCCCGTTCAGCCATAGAAGGAAGAATGTCGTCCGGC - 3' (forward) and 5' - GCCGGACGACATTCTTCCTTCTATGGCTGAACGGGG - 3' (reverse) primers. Afterwards, the pET28a-V88I and pET28a-P131S constructs were created by site directed mutagenesis with the following primers: (a) V88I: 5' – CTGGACGTGCTGATCAACAACGCGG – 3' (forward) and 5' – CCGCGTTGTTGATCAGCACGTCCAG – 3' (reverse); (b) P131S: 5' – CTCCCTCTAATAAAATCCCAAGGGAGAGTGG – 3' (forward) and 5' – CCACTCTCCCTTGGGATTTTATTAGAGGGAG – 3' (reverse). A modified site directed mutagenesis procedure was used in which two separate 50 µl reactions (one for each primer) were subjected to 10 cycles of denaturation at 95$^{o}$C for 1 min, annealing at 60$^{o}$C for 1.5 min, and elongation at 68$^{o}$C for 6.5 min. Afterwards, 25 µl of each reaction were combined and 0.75 µl PfuTurbo® DNA polymerase (Stratagene) was added. This reaction was subjected to 18 cycles using the same PCR protocol. All constructs were verified by dideoxy sequencing at the University of British Columbia Nucleic Acid Protein Service unit.

**Expression and purification of recombinant human CBR1 wild-type and variant enzymes**

The pET28a constructs of the CBR1 wild-type and variants were heat-shock transformed into *Escherichia coli* BL21 (DE3) pLysS competent cells and expressed under the control of an IPTG-inducible T7 polymerase. Cells were plated on Luria-Bertani (1% bacto-tryptone, 0.5%

yeast extract, 0.5% NaCl) broth agar supplemented with 50 µg/ml kanamycin sulfate and 25 µg/ml chloramphenicol for selection. Colonies were randomly picked and cultured overnight at 37°C in 3 ml of LB with kanamycin and chloramphenicol at the same concentrations stated previously. Cultures were expanded to 800 ml and grown at 37°C until an $OD_{600}$ of 0.5 was reached. IPTG was added to a final concentration of 1 mM and cells were allowed to grow for an additional 3 hrs. The cultures remaining after the induction were harvested by centrifugation (4000*g* for 20 min at 4°C), and the recombinant 6x-His tagged proteins were purified using nickel-nitriloacetic acid affinity (Ni-NTA) chromatography and elution with imidazole according to the procedure described in Bains et al. (2008).

Protein purity was assessed by running elution fractions on 18% SDS-polyacrylamide gels, which were stained with SYPRO® Ruby (Invitrogen Canada, Inc., Burlington, Ontario) overnight (16 hours). After staining, the protein was detected using a Storm 840 Molecular Dynamics Imager (GE Healthcare, Piscataway, NJ) at excitation and emission wavelengths of 450 and 520 nm, respectively.

Western blot analyses of the purified fractions were conducted according to the procedure described by Odyssey™(LI-COR Biosciences, Lincoln, NE). Following 18% SDS-polyacrylamide gel electrophoresis, proteins were transferred at 20 V in Towbin's buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol) overnight (at 4°C) to a Hybond-C Extra nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membranes were blocked in Odyssey blocking buffer, and the enzyme was detected using a monoclonal mouse anti-human CBR1 (Abnova® Corporation, Taipei City, Taiwan) antibody (diluted 1:3000) as the primary antibody and IRDye 800CW goat anti-mouse IgG as the secondary antibody (diluted 1:5000) (LI-COR). Both primary and secondary antibodies were in blocking buffer containing 0.1%

Tween 20. The bound secondary antibodies were detected using the Odyssey™ Infrared Imaging system (LI-COR).

**Kinetic analysis of CBR1 and variant enzymes**

The enzyme activities of the purified 6x-His-tagged CBR1 wild-type and variant enzymes were measured at 37°C using a Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA) by following the initial rate of NADPH oxidation at excitation and emission wavelengths of 355 and 460 nm, respectively. The assays were conducted as described previously using menadione as the test substrate (Gonzalez-Covarrubias et al., 2007). In brief, purified protein was incubated with 1 mM NADPH and menadione (20-150 µM) in a reaction mixture of 150 µl of 100 mM potassium phosphate, pH 7.4. Protein amount and incubation times were selected for each enzyme and substrate concentration to ensure that measured rates were in the linear range of the enzyme kinetic curve. In these assays, the concentration of 95% ethanol, which was required to dissolve the substrate, was kept below 4% (v/v) in the final reaction mixture. Readings were collected at 20-s intervals for 1.5 h with shaking between each reading. Maximal rates were calculated from the Ascent program (version 2.6) using a 5-min interval (15 total readings) with the steepest slope. Enzymatic activity (nanomoles of NADPH consumed per minute per milligram of purified protein) was calculated from these rates using a standard curve constructed from the fluorescence measurements of solutions of known NADPH concentrations.

Activity measurements for the reduction of the anthracyclines were performed by incubating either DOX or DAUN (1-400 µM) with purified enzyme in a total volume of 150 µl containing 100 mM potassium phosphate, pH 7.4, and 1 mM NADPH at 37°C. Protein concentrations were based on the Bradford protein assay using bovine serum albumin as a

standard. The reaction was stopped by adding 300 µl of ice-cold acetonitrile, which contained idarubicin as an internal standard, followed by vortex mixing and centrifuging at 10,000*g* for 10 min at 4°C to remove protein. The supernatant was removed for high performance liquid chromatography (HPLC) analysis. The procedures for HPLC separation and fluorescence detection of DOX and DAUN and their carbon-13 hydroxy metabolites, DOXol and DAUNol, were consistent with the protocol in Bains et al. (2008).

The kinetic constants of maximal rate of reaction ($V_{max}$) and substrate affinity ($K_m$) were determined by fitting rate measurement data using nonlinear least-squares fitting of a Michaelis-Menten hyperbola (GraphPad Prism version 4.0; GraphPad Software Inc., San Diego, CA). The turnover values ($k_{cat}$) were calculated from $V_{max}$ values using the apparent molecular weight for the 6x-His-tagged CBRs and variant proteins of 34 kDa. Catalytic efficiency ($k_{cat}/K_m$) was also calculated. Following Michaelis-Menten data analysis, Eadie-Hofstee plots were created to check for deviation from linearity with changing substrate concentrations.

### Statistical analysis

Statistical analyses were performed using GraphPad Instat® (version 3.6; GraphPad Software Inc., San Diego, CA). Results were expressed as means ± S.D. Enzyme activities were compared using a one-way analysis of variance followed by Tukey-Kramer multiple comparisons tests. Differences were considered significant at $p<0.05$.

## RESULTS

### Expression and purification

The expression of the recombinant 6x-His-tagged recombinant human CBR1 wild-type along with the V88I and P131S enzymes was confirmed by Western blot analysis showing bands

DMD Fast Forward. Published on February 9, 2009 as DOI: 10.1124/dmd.108.024711
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with mobility corresponding to the calculated molecular mass of the tagged CBRs (~34 kDa) (Fig. 1). Total protein staining of the SDS-PAGE gels demonstrated that these fractions were successfully purified from their respective transformed bacterial lysates as no other proteins were detected (Fig. 1). A majority of the pure enzyme was recovered in the 50 mM imidazole elution fractions; no further protein was eluted with 100 or 250 mM imidazole (data not shown).

**Kinetic characterization of wild-type and variant enzymatic activities with menadione**

Our initial goal was to determine if the purified human CBR1 protein was active and, if so, to determine the kinetic properties of the protein and the effect of the ns-SNP mutations on these properties. To accomplish this Michaelis-Menten kinetic studies were conducted using menadione as a standard test substrate since it is specific for the CBR1 enzyme (Gonzalez-Covarrubias et al., 2007). In the presence of menadione, the 6x-His-tagged CBR1 wild-type enzyme was found to have a maximal rate of reaction ($V_{max}$) of 537±61 nmol NADPH consumed/min•mg purified protein (Fig. 2; Table 1). In comparision the V88I variant with the same substrate had a $V_{max}$ that was significantly lower, 334±9 nmol/min•mg protein; however, no significant difference in $V_{max}$ was seen with the P131S variant, 581±68 nmol/min•mg protein . These values for the CBR1 wild-type and V88I variant were approximately two-fold higher than the $V_{max}$ values previously reported in the literature for bacterially expressed recombinant human 6x-His-tagged CBR1 and V88I variant form, 220±27 and 168±16 nmol/min•mg, respectively (Gonzalez-Covarrubias et al., 2007). Greater protein stability following purification as well as our use of fluorescence detection, a more sensitive method of measuring the rate of NADPH oxidation, over UV-visible spectrophotometry may account for the differences in value of the kinetic parameters between these studies. Both studies illustrate that the V88I variant enzyme has significantly decreased metabolic activity using menadione as substrate ($p < 0.05$).

No significant difference was detected with the $K_m$ values between the wild-type and both variant isoforms. $k_{cat}/K_m$ values for the V88I and P131S variant were found to be 20% and 40% lower than the wild-type, respectively. Eadie-Hofstee plots for the CBR1 wild-type, V88I and P131S enzymes verified linearity at the same concentrations of menadione used to conduct the assays ($r^2$ = 0.91 for CBR1 wild-type; $r^2$ = 0.99 for V88I; $r^2$ = 0.97 for P131S).

Following the kinetic studies with menadione, the CBR1 wild-type and variant enzymes were exposed to Factor Xa (FXa) for 6 h at 23$^o$C to remove the tag and linker. The native enzyme was separated from the tag and linker by mixing the cleaved preparation with Ni-NTA resin and filtering the mixture. The tag and linker were retained by the resin and the purified enzyme was collected in the filtrate. Western blot analysis demonstrated this treatment completely removed the tag and linker from the wild-type and variant enzymes (data not shown). The metabolic activities of tagged and native (untagged) enzymes were compared. Using 150 µM menadione, there were no differences in enzymatic activity between the tagged and native enzyme (CBR1: 504±48 versus 479±36 nmol/min•mg protein for tagged and un-tagged, respectively; V88I: 365±24 versus 343±30 nmol/min•mg protein; P131S: 484±51 versus 463±42 nmol/min•mg protein), suggesting that the amino acid linker and 6x-His tag engineered on the amino terminus of the wild-type and mutant gene products has no effect on enzyme activity. Based on these findings, the uncleaved wild-type and variant enzymes were used for subsequent activity assays involving the anthracyclines.

**Kinetic characterization of wild-type and variant enzymatic activities with DOX and DAUN**

To evaluate the impact of the single amino acid substitutions in the human CBR1 enzyme on the reduction of the anthracycline drugs, we measured the formation of the major alcohol

metabolites *in vitro*. Full chromatographic resolution of DAUNol and DOXol from DAUN and DOX, respectively, and idarubicin (internal standard) was achieved for all chemical standards and *in vitro* samples. DOXol, DOX, DAUNol, DAUN, and idarubicin were observed to elute at 4.5, 5.5, 6.0, 6.8, and 7.3 min, respectively. Incubation of the 6x-His-tagged CBR1 wild-type and V88I variant enzyme with DOX generated a single new chromatographic peak that was identified as DOXol. Similarly, incubation with DAUN generated a single new chromatographic peak that was identified as DAUNol. The identification of the metabolite peaks was confirmed by incubation of DOX and DAUN with human liver cytosol and the generation of compounds that had identical chromatographic behaviors, as well as correspondence in retention time of the metabolite peak from DOX incubations with that for the chemical standard of DOXol. There were no detectable peaks at the DAUNol or DOXol retention time in the absence of protein.

Michaelis-Menten kinetic studies properties were determined for the wild-type and variant forms of the human CBR1, using both DAUN and DOX, separately, as substrates. With DAUN (Fig. 3A), the $V_{max}$ and $K_m$ of the wild-type CBR1 enzyme were 3430±241 nmol/min•mg and 51±13 μM, respectively (Table 2). The V88I variant reduced the $V_{max}$ significantly (2090±112 nmol/min•mg) but did not alter the $K_m$ (Table 2). In contrast, the P131S variant exhibited a significant increase in $K_m$, 89±13 μM, but did not alter the $V_{max}$ (Table 2). With DOX as a substrate (Fig. 3B), the $V_{max}$ and $K_m$ for the CBR1 wild-type enzyme were 364±37 nmol/min•mg and 287±57 μM, respectively (Table 2). The V88I variant resulted in a 30% reduction in the $V_{max}$ for DOX metabolism, 257±11 nmol/min•mg, which is a significant difference from the wild-type. The $k_{cat}/K_m$ values obtained from DAUN metabolism with both variant enzymes were 40% lower than the wild-type. With DOX, the $k_{cat}/K_m$ values for the V88I and P131S variants were 20% and 40% lower than the wild-type, respectively. Eadie-Hofstee

plots for the CBR1 wild-type and variant enzymes verified linearity at the same concentrations of DAUN and DOX used to conduct the assays ($r^2 > 0.94$ for all plots). In addition, we observed that DAUN is a better substrate than DOX for the wild-type and the variant enzymes as shown by the significant decrease in $V_{max}$ (V88I) as well a significant increase in $K_m$ (P131S).

**DISCUSSION**

Living organisms are regularly exposed to a broad spectrum of compounds containing carbonyl functional groups such as aldehydes, ketones, carboxylic acids and quinones, which are endogenous or exogenous in nature. This functional group is of particular concern to human health since it is highly reactive and can induce oxidative stress or interact with nucleophilic centers of proteins and nucleic acids (Matsunaga et al., 2006). In order to combat the negative effects brought about by carbonyl compounds, organisms have evolved several biotransformation enzyme systems, one of them being the CBRs (Hoffmann and Maser, 2007; Oppermann, 2007).

In humans, CBR1 is one of the most extensively studied carbonyl reductases and has been shown to metabolize drugs containing carbonyl groups, including DOX and DAUN. Treatment with these anthracyclines has played a significant role in improving life expectancy in cancer patients by halting the division of cancer cells. Unfortunately, the mechanism by which they function remains puzzling; a variety of hypotheses have been proposed including: induction of apoptosis, RNA synthesis inhibition, DNA intercalation, and interference with DNA unwinding or DNA strand separation (Minotti et al., 2004; Takemura and Fujiwara 2007).

We conducted *in vitro* metabolic assays on DOX and DAUN using purified CBR1 wild-type and V88I and P131S mutant enzymes expressed in *E. coli*. The water-soluble metabolites, DOXol and DAUNol, were quantified because previous studies have acknowledged them to be

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the major metabolites in cancer patients (Lipp and Bokemeyer, 1999; Plebuch et al., 2007). We demonstrated that DOX and DAUN are both readily metabolized to their respective alcohol metabolites with the CBR1 wild-type having a significantly higher $V_{max}$ for both anti-cancer drugs compared to the V88I variant and a significantly lower $K_m$ for DAUN compared to the P131S variant. Also, the CBR1 wild-type enzyme and both variants metabolize DAUN far more efficiently than DOX; there is an 8 to 11-fold increase in $V_{max}$, 4 to 7-fold decrease in $K_m$ and a 40 to 50-fold increase in $k_{cat}/K_m$. The metabolic preference of the CBR1 wild-type enzyme and the variant forms for DAUN over DOX is interesting and is consistent with what we observed for DAUN metabolism with another metabolic reductase, aldo-keto reductase 1A1 (Bains et al., 2008). Clearly the metabolic activity of these two reductases can differ substantially in spite of the minor difference in chemical structure between these two drugs.

Our results with the V88I variant in the presence of DAUN are consistent with those reported by Gonzalez-Covarrubias and colleagues (2007), which used cofactor (NADPH) consumption to demonstrate that the $V_{max}$ was significantly decreased in the V88I variant compared to the wild-type enzyme. While co-factor usage is an indirect test of substrate conversion, these authors confirmed this kinetic difference by directly measuring DAUNol levels using HPLC-fluorescence at a single DAUN concentration (500 μM); which demonstrated that the variant enzyme produced 47% less metabolite than the wild-type. These authors suggested that the V88I mutation has an impact on NADPH binding affinity due to its close proximity to the cofactor site (Fig. 4). Their finding demonstrates that the valine to isoleucine substitution leads to a lower affinity for the cofactor. Since NADPH is a vital cofactor in the metabolic conversion of DOX and DAUN to their major alcohol metabolites, this might be the basis for the significant decrease in the levels of DOXol and DAUNol produced with the V88I variant

enzyme. The P131S mutation, albeit located further from the active and cofactor binding sites compared to the V88I mutation (Fig. 4), may alter the conformation of one or both sites which could explain why there was a significant increase in $K_m$ for DAUN compared to the wild-type enzyme while no change was observed for DOX. However, further studies are needed to provide a more definitive explanation.

Reductions in (i) metabolic activity for DOX and DAUN by the V88I variant and (ii) substrate affinity for DAUN by the P131S variant, may explain the interpatient variability in the serious side effects seen in cancer patients treated with either of these anthracyclines. We propose that these adverse events are the result of excessive build up of parent drug. An increasing amount of data has shown that these drugs accumulate in mitochondria of cardiomyocytes and interfere with enzyme function of the electron transport chain (Hasinoff et al., 2003; Wallace 2003; Ohara et al., 2007). For example, complex I of the electron transport chain acts on DOX and DAUN such that the quinone moiety within the tetracycline ring undergoes redox cycling between the quinone and semiquinone states. The electrons generated during this cycling are captured by oxidizing agents, including oxygen, leading to the formation of reactive oxygen species (ROS) (Singal et al., 2000; Kim et al., 2005; Wold et al., 2005; Takemura and Fujiwara 2007). ROS are a major concern since they are capable of mitochondrial respiratory chain disruption, lipid peroxidation, and DNA disintegration, all of which may be the underlying causes of the life threatening side effects seen in some patients undergoing DOX and DAUN treatment (Wojtacki et al., 2000). The heart appears to be the most susceptible to the harmful effects associated with ROS due to myocardial tissue having low levels of endogenous antioxidant enzymes, such as superoxide dismutases and glutathione peroxidases, to neutralize these free radicals (Takemura and Fujiwara 2007). ROS can also be generated during Fenten

reactions following the formation of complexes involving anthracyclines and intracellular free iron. This has been demonstrated in studies with the free radical scavenger, dexrazoxane, an intracellular iron chelator that binds free iron ($Fe^{2+}$ and $Fe^{3+}$), thus preventing site specific iron-based oxygen radical damage (Wu and Hasinoff, 2005). Studies have revealed that co-administration of dexrazoxane with DOX has resulted in cardioprotection (Wexler et al., 1996; Swain et al., 1997; Wu and Hasinoff, 2005).

In conclusion, this study demonstrates that a 6x-His tagged CBR1 enzyme, whose activity does not differ from that of the untagged enzyme, is efficient at metabolizing DOX and DAUN, with higher specificity for the latter. We have also shown that the V88I variant significantly reduces reductase activity toward both anthracyclines and the P131S variant significantly reduces substrate affinity of DAUN. Therefore, the ns-SNPs in the human *CBR1* gene generating the V88I and P131S mutations may prove to be clinically useful genetic biomarkers for guiding anthracycline therapy in cancer patients to minimize adverse effects.

**ACKNOWLEDGMENTS**

The authors extend their gratitude to Mr. R.H. Takahashi, Mr. A. Szeitz, Dr. R.C. Mottus, Dr. J.M. Lubieniecka, Dr. T.A. Pfeifer and Dr. J.W. Hodgson (University of British Columbia) for their advice and technical expertise.

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**FOOTNOTES**

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR).

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## FIGURE LEGENDS

**Figure 1**—Purification of human recombinant (A) 6x-His-tagged CBR1 wild-type, (B) 6x-His-tagged V88I variant, and (C) 6x-His-tagged P131S enzymes. (***Left***) Gels stained with SYPRO® Ruby following SDS-PAGE showing purified protein samples, CBR1 wild-type and V88I variant (lane 3; 700 ng), free of contaminating proteins from bacterial lysates (lane1; 1 µg total protein). Removal of contaminating proteins is observed in fractions from Qiagen purification procedures [Ni-NTA column flow through (lane 2; 1 µg total protein)]. (***Right***) Western blot detection of transformed lysates (lane 6) and purified protein samples (lane 8; 700 ng), confirms expression of the desired CBR protein with mobility at the expected molecular weight (~34 kDa). No immunoreactivity was detected in the flow through samples (lane 7) suggesting that the enzymes were bound to the Ni-NTA resin prior to their elution. Human liver cytosol was used as a positive control for antibody immunoreactivity (lane 5; 10 µg total protein). No antibody immunoreactivity is observed for untransformed bacterial lysate (lane 4; 10 µg total protein). M refers to the molecular weight ladder.

**Figure 2**—*In vitro* enzymatic activities for the purified 6x-His-tagged CBR1 wild-type and V88I variant with 20 to 150 µM of the test substrate menadione as measured by following the initial rate of NADPH oxidation. Two independent batches of each enzyme were purified and assays were performed in triplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n=6) with the background levels subtracted. The background levels represented the reaction buffer, enzyme and NADPH cofactor only, without the addition of the test substrate. Eadie-

Hofstee plots for the wild-type and variant enzyme are shown to the right of the activity plot ($r^2$ > 0.91).

**Figure 3—***In vitro* enzymatic activities for the purified 6x-His tagged CBR1 wild-type and variants with (A) 0 to 400 μM of daunorubicin and (B) 0 to 400 μM of doxorubicin. Activities are measured by following the rate of daunorubicinol and doxorubicinol production. Two independent batches of each enzyme were purified and assays were performed in triplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n=6). Eadie-Hofstee plots for the wild-type and variant enzyme are shown to the right of the activity plot for each anthracycline ($r^2$ > 0.94 for each plot).

**Figure 4—**3-D molecular structure of human carbonyl reductase 1 wild-type enzyme complexed with the cofactor, NADP, and substrate (5R,10S)-5-{[(carboxymethyl)amino]carbonyl}-7-oxo-3-thia-1,6-diazabicyclo[4.4.1]undecane- 10-carboxylic acid (DDD) (both in green). The structure was provided by Bateman and colleagues (2007). Both ns-SNPs are also illustrated with the V88I mutation (orange) most proximal to the co-factor binding compared to the P131S mutation (yellow). The valine and proline are at positions 87 and 130 in the model, respectively, since amino acid numbering does not include initiator methionine. This molecular graphic image was produced using the UCSF Chimera program (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, CA).

DMD Fast Forward. Published on February 9, 2009 as DOI: 10.1124/dmd.108.024711
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## TABLES

**Table 1—**Kinetic constants for menadione metabolism by recombinant CBR1 wild-type and variant allele enzymes. Values correspond to mean ± S.D. obtained from three experiments performed with two independent enzyme preparations (n=6) for each isoform.

| KINETIC PARAMETER | CBR1 wild-type | V88I variant | P131S variant |
|---|---|---|---|
| $V_{max}$ (nmol/min ● mg protein) | 537±61 | 334±9* | 581±68 |
| $K_m$ (μM) | 24±10 | 18±2 | 45±15 |
| $k_{cat}$ ($s^{-1}$)[a] | 0.30 | 0.19 | 0.33 |
| $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) | $12.8 \times 10^3$ | $10.5 \times 10^3$ | $7.3 \times 10^3$ |

* Significantly different from wild-type ($p<0.05$)

[a] $k_{cat}$ calculated from $M_r$ 34000

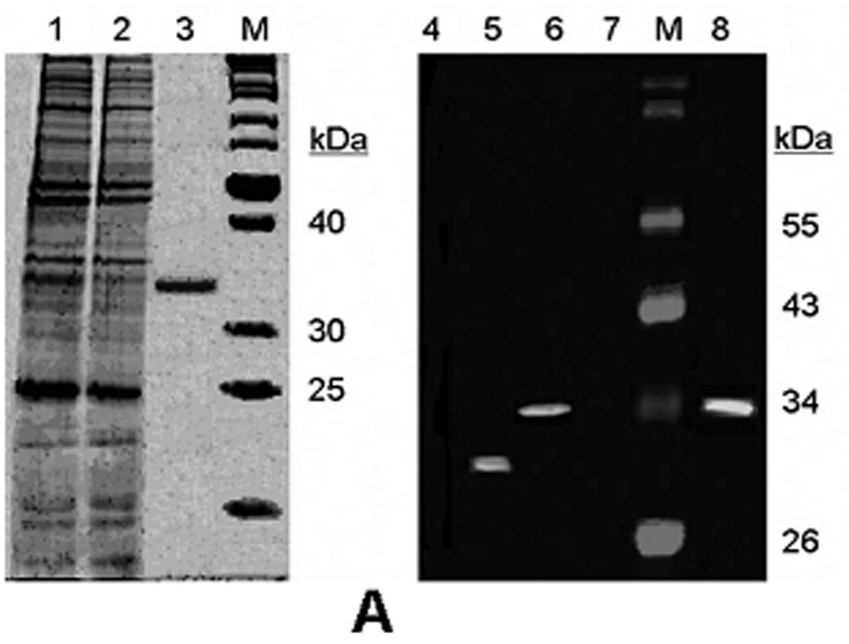
**Table 2—**Kinetic constants for DAUN and DOX reduction by recombinant CBR1 wild-type and variant allele enzymes. Values correspond to mean ± S.D. obtained from three experiments performed with two independent enzyme preparations (n=6) for each isoform.

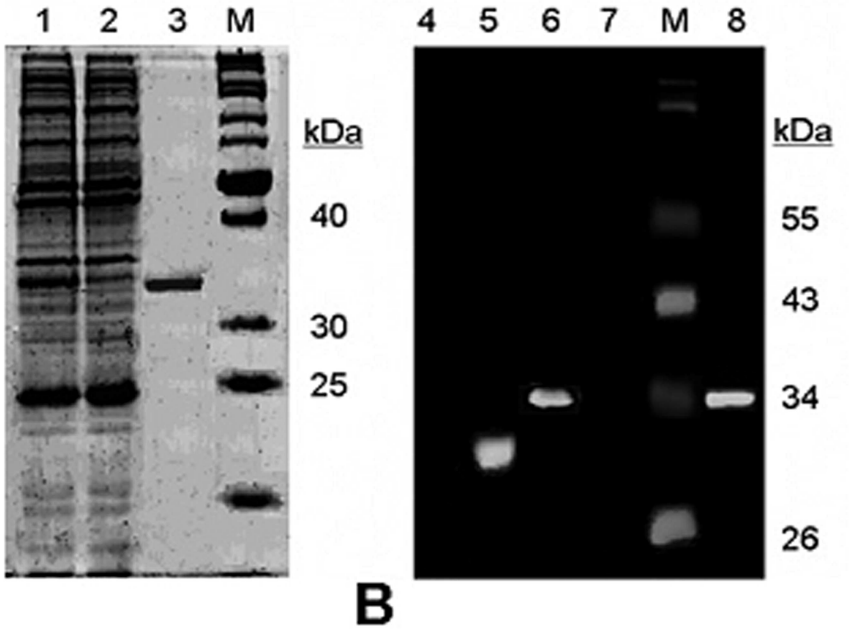
| SUBSTRATE | KINETIC PARAMETER | CBR1 wild-type | V88I variant | P131S variant |
|---|---|---|---|---|
| DAUN | $V_{max}$ (nmol/min • mg protein) | 3430±241 | 2090±112* | 3570±176 |
| | $K_m$ (μM) | 51±13 | 51±10 | 89±5* |
| | $k_{cat}$ ($s^{-1}$)[a] | 1.94 | 1.19 | 2.02 |
| | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) | $37.8 \times 10^3$ | $23.1 \times 10^3$ | $22.6 \times 10^3$ |
| DOX | $V_{max}$ (nmol/min • mg protein) | 364±37 | 257±11* | 325±40 |
| | $K_m$ (μM) | 287±57 | 335±26 | 325±72 |
| | $k_{cat}$ ($s^{-1}$)[a] | 0.21 | 0.15 | 0.18 |
| | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) | $7.2 \times 10^2$ | $4.4 \times 10^2$ | $5.7 \times 10^2$ |

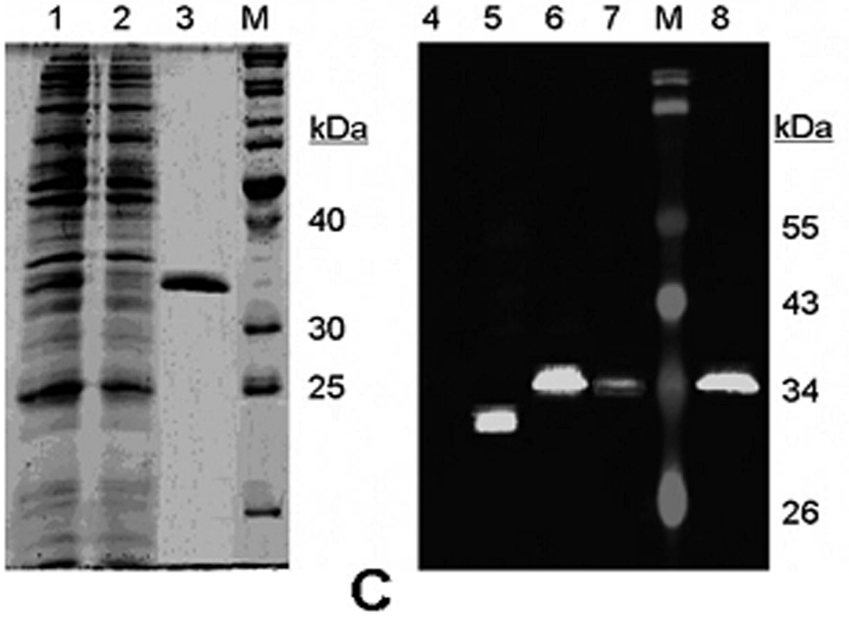
* Significantly different from wild-type ($p<0.05$)

[a] $k_{cat}$ calculated from $M_r$ 34000

**Figure 1**



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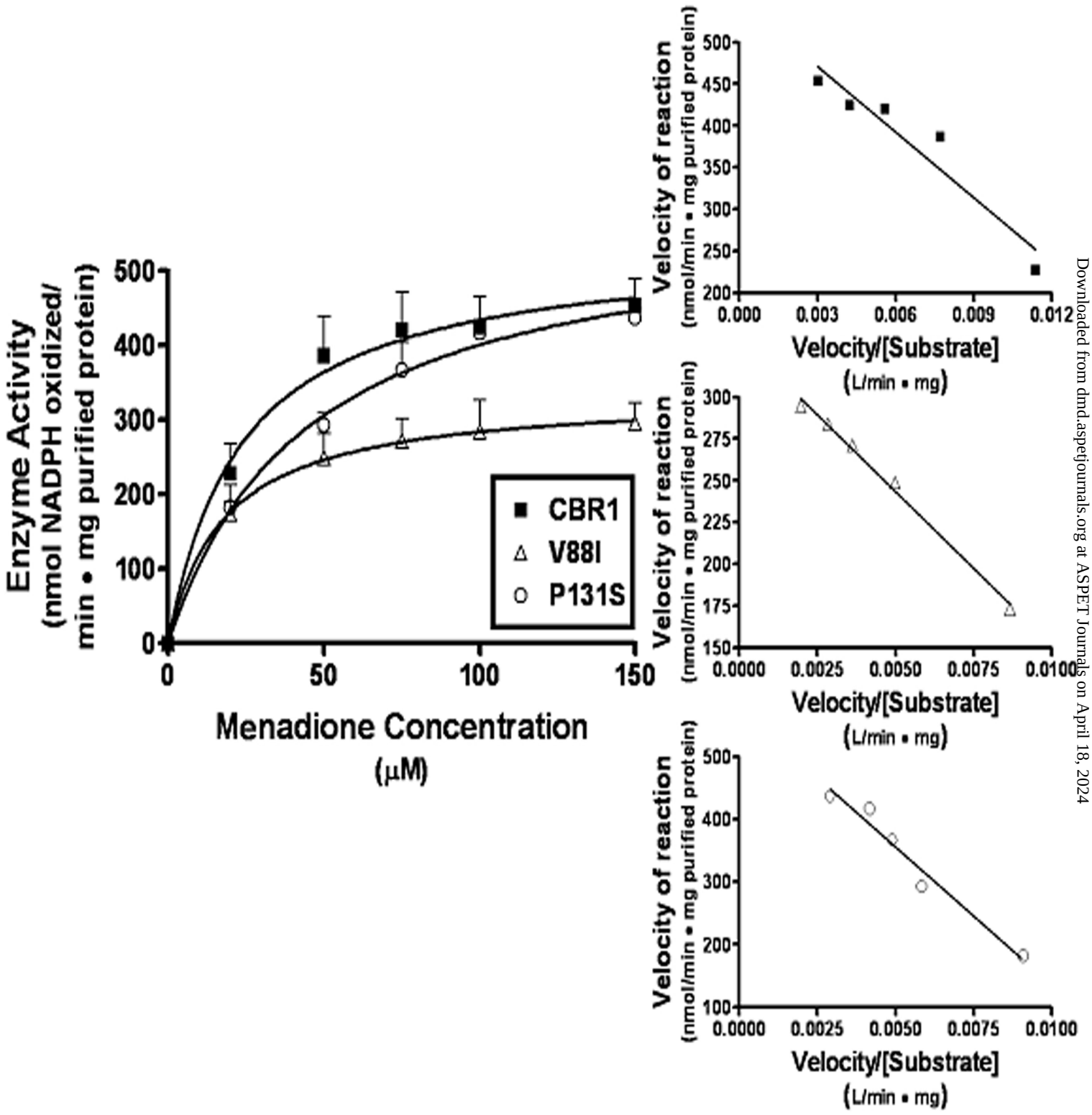

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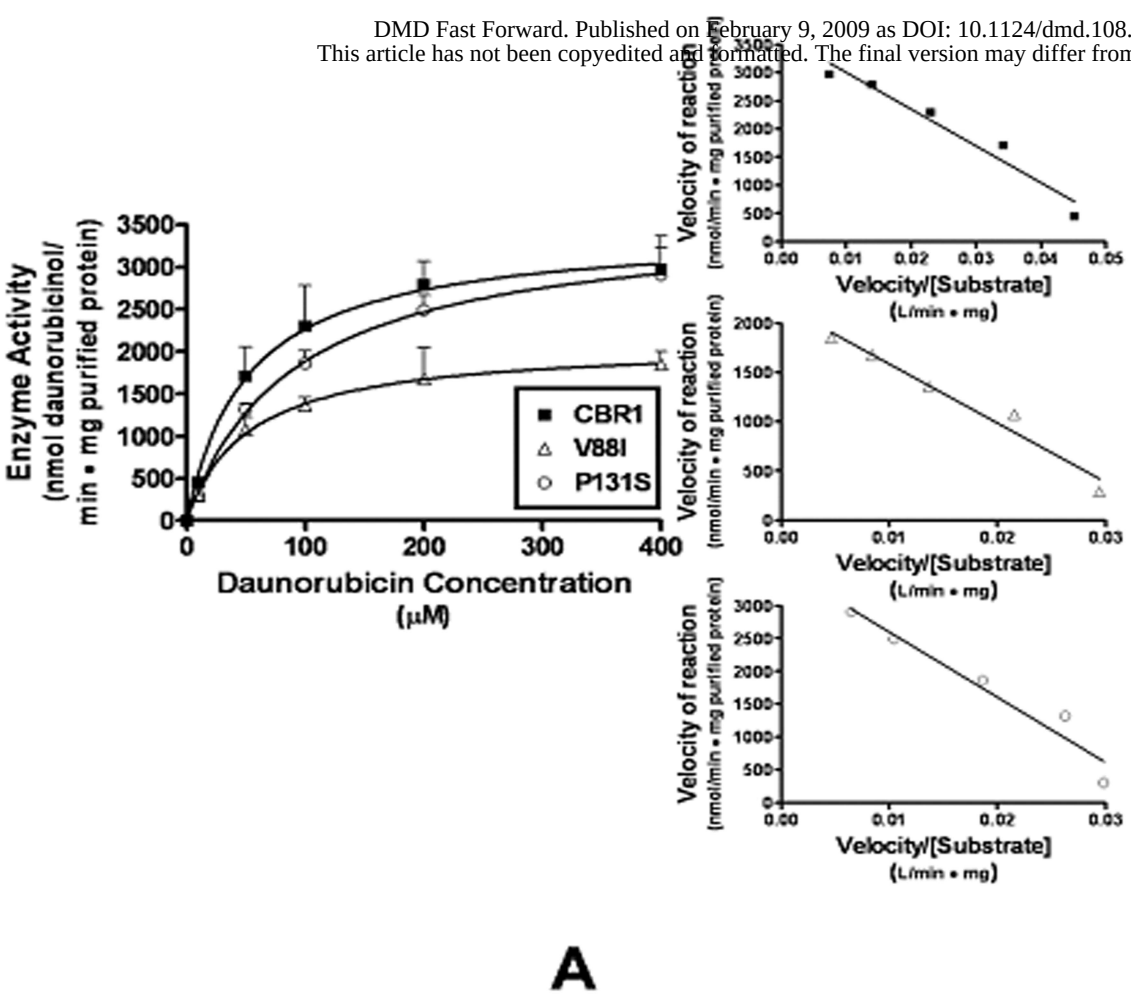
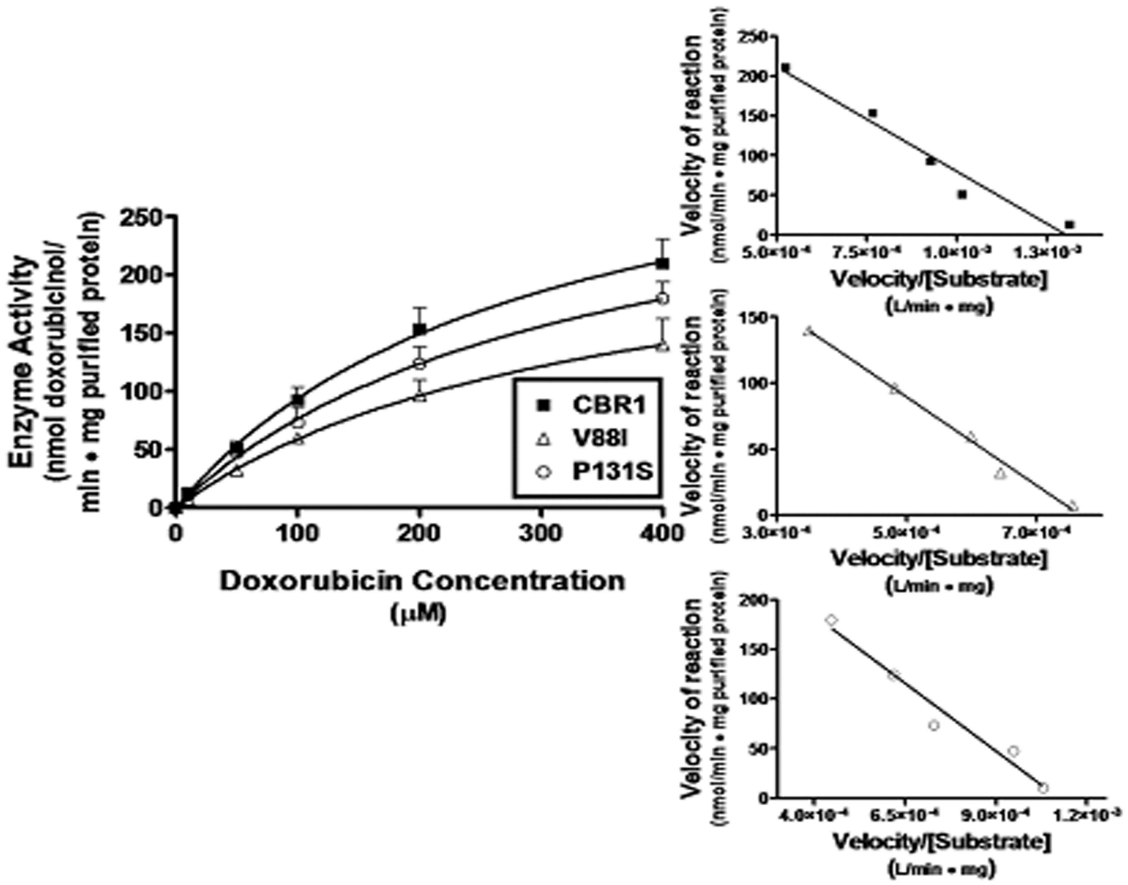



A




B

**Figure 3**

Figure 4

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