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Naturally Occurring Variants of Human CBR3 Alter Anthracycline In Vitro Metabolism[S]

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ABSTRACT

Doxorubicin (DOX) and daunorubicin (DAUN) are anthracycline anticancer agents; however, considerable interpatient variability exists in their pharmacokinetics. This interpatient variability is attributed in part to altered metabolism by nonsynonymous single-nucleotide polymorphisms (ns-SNPs) in genes encoding the carbonyl reductases. This study examines the effect of seven naturally occurring ns-SNPs in the *CBR3* gene on in vitro metabolism of anthracyclines to doxorubicinol and daunorubicinol. Kinetic assays measure metabolite levels by high-performance liquid chromatography separation with fluorescence detection by use of purified, histidine-tagged, human CBR3 wild type and variant enzymes. The V224M, C4Y, and V93I variants resulted in significantly reduced maximal reaction velocity (V_{max}) for both anthracyclines compared with the wild-type enzyme, whereas the M235L variant had significantly reduced V_{max} for DOX only. Significant increases in substrate affinity were found for the V244M variant with DAUN, as well as the C4Y and V93I variants with DOX. The catalytic efficiency values for the V244M, C4Y, and V93I variants were significantly lower than the wild type for DAUN and DOX. Furthermore, DOX was observed to be a better substrate than DAUN for the wild-type enzyme and its variants. HapMap analysis indicated that a haplotype carrying the C4Y and V244M mutations may occur in some individuals in the 11 ethnic populations studied in the HapMap project. Our preparation of the double mutant indicated a significant reduction in activity compared with the wild-type enzyme and single-mutant preparations. These findings suggest that commonly occurring ns-SNPs in human CBR3 significantly alter the in vitro metabolism of DOX and DAUN.

Carbonyl reductases (CBRs) are members of a highly divergent superfamily of NADPH-dependent short-chain dehydrogenases reported to metabolize a range of endogenous and exogenous carbonyl-containing compounds, including steroid hormones, aflatoxins, antipsychotics, anti-inflammatories, and prostaglandins (Matsunaga et al., 2006; Hoffmann and Maser, 2007). CBRs are classified as phase I biotransformation enzymes, which play a major role in converting aldehyde- and ketone-containing compounds to their corresponding hydroxy metabolites. This conversion facilitates elimination from the body either directly or via phase II conjugation reactions.

CBR1, CBR3, and CBR4 are the three isoforms of CBRs currently found in humans (Matsunaga et al., 2006; Hoffmann and Maser, 2007; Oppermann, 2007; Endo et al., 2008). This study focuses on CBR3, which is widely distributed in human tissues (Wirth and Wermuth, 1992; Forrest and Gonzalez, 2000), and is of particular interest for its role in the metabolism of the anthracycline drugs, daunorubicin (DAUN) and doxorubicin (DOX), two of the most effective antineoplastic agents ever developed (Licata et al., 2000; Lakhman et al., 2005; Blanco et al., 2008). DAUN plays an essential role in the treatment of patients with acute myeloid and lymphoblastic leukemias, whereas DOX is used in treating a broad range of cancers, such as non-Hodgkin's lymphoma, breast,

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ABBREVIATIONS: CBR, carbonyl reductase; DOX, doxorubicin; DAUN, daunorubicin; DOXol, doxorubicinol; DAUNol, daunorubicinol; SNP, single-nucleotide polymorphism; ns-SNP, nonsynonymous single-nucleotide polymorphism; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; FXa, Factor Xa; PCR, polymerase chain reaction; IPTG, β-D-1-thiogalactopyranoside; LOD, logarithm of odds ratio; Ni-NTA, nickel-nitrilotriacetic acid affinity.

lung, and ovarian cancers, and soft tissue carcinomas (Hunault-Berger et al., 2001; Fassas and Anagnostopoulos, 2005; Cortés-Funes and Coronado, 2007).

Therapies involving these anthracyclines have resulted in increased life expectancy, but both have been linked to the onset of life-threatening cardiotoxicity that eventually leads to irreversible complications such as reduced left ventricular ejection fraction or congestive heart failure (Barry et al., 2007; Deng and Wojnowski, 2007; Menna et al., 2007). The frequency of chronic cardiotoxicity and mortality rate are correlated with anthracycline dose and show interpatient variability (Deng and Wojnowski, 2007). Moreover, the risk of chronic cardiotoxicity increases when DOX and DAUN are used in combination with other anticancer drugs, such as herceptin, paclitaxel, vincristine, and cyclophosphamide (Wojtacki et al., 2000; Mordente et al., 2001; Danesi et al., 2002; Floyd et al., 2005; Gianni et al., 2007). Although the cause of the interpatient variation in DAUN- or DOX-induced adverse effects is unknown, allelic variation in the form of nonsynonymous single-nucleotide polymorphisms (ns-SNPs) in the genes expressing enzymes that metabolize these anthracyclines may be one contributing factor.

The purpose of this study is to improve our understanding of the effect of genetic variation in the human *CBR3* gene on the in vitro metabolism of DOX and DAUN and the standard test substrate menadione. There are seven documented ns-SNPs in the human *CBR3* gene (National Center for Biotechnology Information Database), with allele frequencies ranging from 1.7 to 53.3% in specific ethnic populations (Table 1). Although the catalytic properties are documented for the V244M mutant enzyme with DOX as a substrate (Lakhman et al., 2005; Blanco et al., 2008), none is reported for DAUN. There is no information on the catalytic properties of the six remaining enzyme variants in the presence of either anthracycline. This study compares the wild type and the seven known variants of human CBR3 for the in vitro metabolism of DAUN and DOX with the corresponding carbon-13 alcohol metabolites, daunorubicinol (DAUNol) and doxorubicinol (DOXol). With use of purified, bacterially expressed, human histidine-tagged enzymes, we demonstrate that the C4Y, V244M, and V93I variants result in significantly reduced maximal reaction velocity (V_{max}) for both DOX and DAUN, whereas the M235L variant has significantly reduced V_{max} for DOX only compared with the wild-type enzyme. Furthermore, the V244M variant exhibits a significant increase in affinity (K_m) for DAUN, whereas the C4Y and V93I variants exhibit significant increases in K_m for DOX compared with the wild-type enzyme. In addition, we observed that DOX is a better substrate than DAUN for the wild-type and variant isoforms of CBR3, as shown by increases in catalytic efficiency (k_{cat}/K_m). Although reports in the literature are conflicting on the ability of CBR3 to metabolize menadione, we found it to be a poor substrate for the enzyme.

Analysis using the HapMap database indicated the possibility of four haplotypes, each containing two ns-SNPs. Although no frequency data were available for any of the combinations, the C4Y+V244M haplotype had the highest linkage disequilibrium parameter scores. Kinetic analysis of DOX and DAUN metabolism by this double mutant demonstrated significant reduction in activity compared with the wild-type enzyme and single-mutant preparations.

TABLE 1

Allele frequencies of the nonsynonymous single-nucleotide polymorphic variant enzymes of human CBR3

Variants and allele frequencies were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). In addition, the chromosome sample count (*n*) is given for each allele frequency.

Variant	NCBI db-SNP rs-Number	Allele Frequencies
		% (n)
P131S	rs16993929	European = 30.0 (120)
V244M	rs1056892	European = 30.0 (120)
		Chinese = 33.3 (90)
		Japanese = 30.0 (90)
		African = 52.5 (120)
C4Y	rs8133052	European = 48.3 (120)
		Chinese = 53.3 (90)
		Japanese = 47.7 (88)
		African = 23.7 (118)
M235L	rs4987121	African = 2.5 (120)
L84V	rs9282628	European = 2.2 (46)
		African = 11.8 (34)
V93I	rs2835285	European = 1.7 (120)
D239V	rs11701643	Not available

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). High-performance liquid chromatography (HPLC)-grade acetonitrile, agar, ammonium persulfate, formic acid, ethanol, glycine, glycerol, glacial acetic acid, imidazole, and Tris were purchased from Thermo Fisher Scientific (Waltham, MA). 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 MBI Fermentas (Hanover, MD), respectively. Tween 20 was purchased from EMD Biosciences (San Diego, CA), and DNaseI was provided by Roche Diagnostics (Manheim, Germany). Klenow fragment, T4 ligase, Factor Xa, and restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Doxorubicinol was obtained from Qventas Inc. (Branford, CT). Pooled human liver cytosol was purchased from BD Biosciences.

Molecular Cloning of Human CBR3 Gene and Creation of the Genetic Variants. The human CBR3 wild-type coding region was excised from a pOTB7 recombinant plasmid (MGC-3489; American Type Culture Collection, Manassas, VA) by use of BlpI (blunt end with Klenow fragment) and XhoI, and subcloned into NheI (blunt end)-XhoI sites of the pET28a prokaryotic expression vector (Novagen, EMD Biosciences) with T4 ligase. This construct gave rise to a human CBR3 enzyme with an amino-terminal His_6 tag separated from the enzyme by a 15-amino acid residue linker. A Factor Xa (FXa) cleavage site was inserted at the amino terminus between the linker and *CBR3* gene by use of the QuikChange Site-Directed Mutaganesis Kit (Stratagene, La Jolla, CA) with the 5′-GCTAGCTCAGGCATAGAAGGAAGAATGTCGTCCTGC-3′ (forward) and 5′-CGAGGACGACATTCTTCCTTCTATGGCTGAGCTAGC-3′ (reverse) primers. The pET28a-variant constructs were created by site-directed mutagenesis using the primers listed in Table 2.

The QuikChange polymerase chain reaction (PCR) amplification protocol for site-directed mutagenesis was modified as follows: two separate 50-μl reactions (one for each primer) were subjected to 10 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1.5 min, and elongation at 68°C for 6.5 min. A 25-μl aliquot of each PCR amplification reaction was combined with 0.75 μl of PfuTurbo DNA polymerase (Stratagene). This reaction was subjected to 18 cycles of the same PCR protocol as above. All constructs were verified by

TABLE 2
Primers for creating nonsynonymous single-nucleotide polymorphic variants of human CBR3 by use of site-directed mutagenesis
The base pair mutation is given beneath each variant in square brackets. In addition, the mutated base pair is underlined in the forward and reverse primer sequences.

Variant	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
P131S [C→T]	CTGCCGATAATGAAAT<u>C</u>TCATGGGAGAGTGG	CCACTCTCCCATGAG<u>A</u>TTTCATTATCGGCAG
V244M [G→A]	GAAAGACAGCATCAGGACT<u>A</u>TGGAGGAGGGGGCTGAGAC	GTCTCAGCCCCCTCCTCCAT<u>A</u>GTCCTGATGCTGTCTTTC
C4Y [G→A]	GAATGTCGTCCT<u>A</u>CAGCCGCGTGGCG	CGCCACGCGGCTGT<u>A</u>GGACGACATTC
M235L [A→T]	GACCAGTGAAGACAGACT<u>T</u>GGATGGGAAAGACAG	CTGTCTTTCCCATCCA<u>A</u>GTCTGTCTTCACTGGTC
L84V [C→G]	CAAGGAGTACGGGGGGG<u>T</u>CAATGTACTGGTCAAC	GTTGACCAGTACATTGA<u>C</u>CCCCCCCGTACTCCTTG
V93I [G→A]	CAACAACGCGGCC<u>A</u>TCGCCTTCAAGAG	CTCTTGAAGGCGAT<u>G</u>GGCCGCGTTGTTG
D239V [A→T]	GACATGGATGGGAAAG<u>T</u>CAGCATCAGGACTGTG	CACAGTCCTGATGCTG<u>A</u>CTTTCCCATCCATGTC

dideoxy sequencing at the University of British Columbia Nucleic Acid Protein Service unit.

Expression and Purification of Recombinant Human CBR3 Wild-Type and Variant Enzymes. The pET28a constructs of the CBR3 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 broth agar (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl) 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 Luria-Bertani broth 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 5 h, after which the cultures were harvested by centrifugation (4000*g* for 20 min at 4°C). The cells were lysed with lysozyme, and the recombinant His_6-tagged proteins were extracted and purified by use of nickel-nitrilotriacetic acid affinity (Ni-NTA) chromatography (Bains et al., 2008).

Protein purity was assessed by running elution fractions on 18% SDS-polyacrylamide gels that were stained with SYPRO Ruby (Invitrogen Canada, Inc., Burlington, ON) overnight (16 h). After staining, the protein was detected by use of a Storm 840 Molecular Dynamics Imager (GE Healthcare, Little Chalfont, Buckinghamshire, UK) 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). After 18% SDS-PAGE, 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). The membranes were blocked in Odyssey blocking buffer, and the enzyme was detected with use of a MaxPab polyclonal mouse anti-human CBR3 antibody (Abnova Corporation, Taipei City, Taiwan) (diluted 1:2500) as the primary antibody and IRDye 800CW goat anti-mouse IgG as the secondary antibody (diluted 1:5000) (LI-COR Biosciences). Both primary and secondary antibodies were in blocking buffer containing 0.1% Tween 20. The bound secondary antibody was detected using the Odyssey Infrared Imaging system (LI-COR Biosciences).

Kinetic Analysis of CBR3 Wild-Type and Variant Enzymes. The enzyme activities of the purified His_6-tagged CBR3 wild-type and variant enzymes were measured at 37°C with use of a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific) 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 with use of menadione as the test substrate (Lakhman et al., 2005). In brief, purified protein (3 μg) was incubated with 1 mM NADPH and menadione (20–100 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 by use of 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 (10–700 μM) with purified enzyme (3 μg) for 30 min in a total volume of 150 μl of buffer containing 100 mM potassium phosphate, pH 7.4, and 1 mM NADPH at 37°C. The samples were mixed at 250 rpm. Protein concentrations were based on the Bradford protein assay with 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 centrifugation at 10,000*g* for 10 min at 4°C to remove protein. The supernatant was removed for HPLC analysis. The procedures for HPLC separation and fluorescence detection of DOX and DAUN and their carbon-13 hydroxy metabolites, DOXol and DAUNol were carried out as described previously (Bains et al., 2008).

The kinetic constants of V_{max} and K_m were determined by fitting rate measurement data by use of nonlinear least-squares fitting to 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 mass for the His_6-tagged CBR3 and variant proteins of 34 kDa. The values for k_{cat}/K_m were also calculated. After 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 a Tukey-Kramer multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

Expression and Purification. The expression of the His_6-tagged recombinant human CBR3 wild-type enzyme was confirmed by Western blot analysis, which showed a band with mobility corresponding to the calculated molecular mass of the tagged CBR (~34 kDa) (Fig. 1). Total protein staining of the SDS-PAGE gel demonstrated that the wild-type fraction was purified from its transformed bacterial lysate 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. Expression and purification of the seven variants also produced similar results as the wild-type enzyme (Supplemental Fig. 1).

Kinetic Characterization of Wild-Type and Variant Enzymatic Activities with Menadione. We conducted

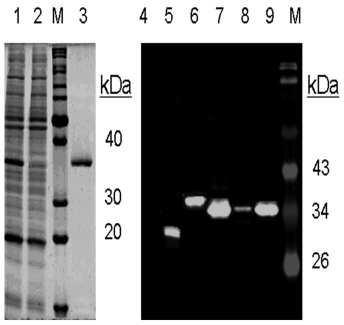

Fig. 1. Purification of human recombinant His_6-tagged CBR3 wild-type enzyme. Left, gel stained with SYPRO Ruby after SDS-PAGE showing purified protein fraction (lane 3; 2 μg), free of contaminating proteins from the bacterial lysate (lane 1; 10 μg of total protein). Removal of contaminating proteins is observed in the flow-through fraction from QIAGEN purification procedures [Ni-NTA column flow-through (lane 2; 10 μg of total protein)]. Right, Western blot detection of transformed lysate (lane 7) and purified protein fractions (lane 9), confirms expression of the desired CBR protein with mobility at the expected molecular mass (~34 kDa). Little immunoreactivity was detected in the flow-through fraction (lane 8), suggesting that the majority of the enzyme was bound to the Ni-NTA resin before their elution. Human liver cytosol (lane 5; 10 μg of total protein) and GST-tagged CBR3 partial recombinant protein (Abnova Corporation; lane 6; 2 μg of total protein) were used as positive controls for antibody immunoreactivity. No antibody immunoreactivity is observed for untransformed bacterial lysate (lane 4; 10 μg of total protein). M refers to the molecular mass markers.

Michaelis-Menten kinetic studies with menadione, a reported CBR3-specific substrate (Lakhman et al., 2005), in the presence of the CBR3 wild type enzyme (Fig. 2). The kinetic parameters of the wild-type enzyme are reported in Table 3. These values differ substantially from those reported in the Lakhman study, which also used a His_6-tagged CBR3 enzyme (Table 3), indicating that our CBR3 enzyme preparation was comparatively less efficient at metabolizing menadione. In addition, we performed these assays with higher concentrations of cofactor (1.5 and 2 mM); however, the kinetic parameter values did not differ from those reported in this study with 1 mM NADPH.

The His_6 tag and amino acid linker were removed with FXa, as described previously (Bains et al., 2009), to determine whether this modification on the amino terminus of the enzyme disrupted activity in our preparation. This was not the case, because the kinetic parameters did not differ significantly from the tagged enzyme (Table 3). It should be noted here that human CBR1 was used as a positive control for enzyme activity with menadione. The CBR1 enzyme was His_6-tagged as described in the study by Bains et al. (2009). CBR1 expression, purification, and activity assays with menadione were performed by use of the same protocols as described in the current study. The values obtained for the kinetic parameters of human CBR1 are shown in Table 3, which were similar to the values reported previously in Bains et al. (2009).

Miura et al. (2008) recently tried to reproduce the results of the Lakhman menadione study with use of the same assay conditions and obtained kinetic parameters that are consistent with our results (Table 3). In addition, a study by El-Hawari et al. (2009) reported that the wild-type human CBR3 was inactive toward menadione as a substrate. Taken together, these data indicate that menadione is a poor substrate for CBR3. Nonetheless, the metabolism of menadione demonstrated that our purified human CBR3 wild-type enzyme preparations were functional.

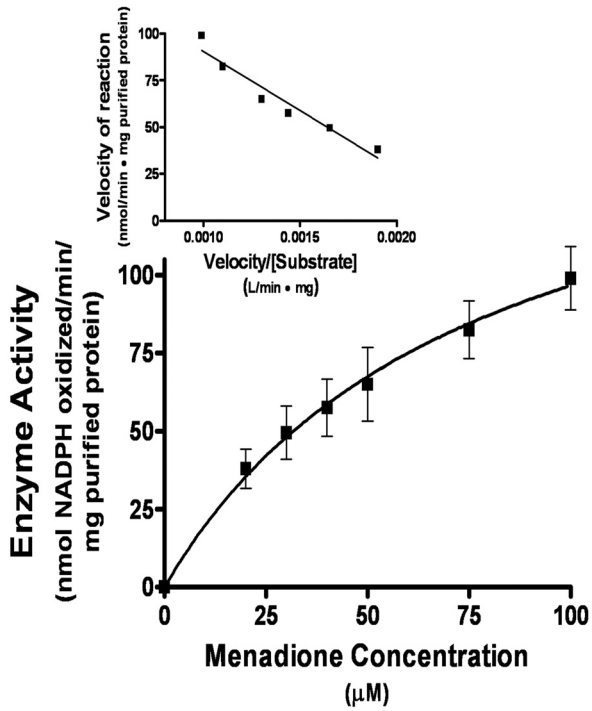

Fig. 2. In vitro enzymatic activities for the purified His_6-tagged CBR3 wild type with 20 to 100 μM test substrate menadione. Three independent batches of the enzyme were purified, and assays were performed in triplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n = 9) with the background levels subtracted. The background levels represented the reaction buffer, enzyme, and NADPH cofactor without the addition of the test substrate. The Eadie-Hofstee plot for the wild type is shown at the top of the activity plot ($r^2 > 0.94$).

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 CBR3 enzyme on the reduction of anthracycline drugs, we measured the in vitro formation of the major alcohol metabolites. A 30-min incubation period was found to be in the linear range of enzymatic activity for each of the concentrations of DOX and DAUN used to conduct the enzymatic assays (CBR3 wild-type enzyme; 3 μg of purified protein). In addition, the concentration of cofactor (1 mM NADPH) was sufficient for maximal enzymatic activity during this 30-min incubation period. Higher concentrations of cofactor were also examined (1.5 and 2 mM), but the enzymatic rates associated with these concentrations were not significantly different from that of 1 mM (data not shown).

Full chromatographic resolution of DAUNol, DOXol, DAUN, DOX, and idarubicin (internal standard) was achieved for all chemical standards and in vitro samples (Bains et al., 2008). 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 His_6-tagged CBR3 wild-type and variant enzymes with DOX generated a single new chromatographic peak that was identified as DOXol. Likewise, 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. There were no detectable peaks at the DAUNol or DOXol retention time in the absence of the CBR3 protein. In addition to the enzymatic

TABLE 3

Kinetic constants for menadione metabolism by His_6-tagged and untagged recombinant human CBR3 wild-type enzyme

Values correspond to the mean ± S.D. obtained from three experiments performed with three independent enzyme preparations (n = 9). Also presented are the kinetic constants for the His_6-tagged CBR1 wild-type human enzyme, which serves as a positive control for enzyme activity with menadione. In addition, the kinetic constants are shown from the studies by Lakhman et al. (2005) and Miura et al. (2008).

Kinetic Parameters	CBR3 Wild Type (Tagged)	CBR3 Wild Type (Untagged)	CBR1 Wild Type (Tagged)	Lakhman et al.	Miura et al.
V_{max}, nmol/min·mg protein	170 ± 29	149 ± 31	582 ± 43	19,600 ± 2000	126[b]
K_m, μM	76 ± 23	60 ± 15	20 ± 6	25 ± 3	54 ± 10
k_{cat}, s^{-1} [a]	0.096 ± 0.014	0.084 ± 0.016	0.33 ± 0.02	9.9	0.071
k_{cat}/K_m, s^{-1} M^{-1}	1320 ± 290	1410 ± 400	17,400 ± 2800	402,000	1300

[a] k_{cat} calculated for His_6-tagged CBRs and untagged CBR3 from molecular mass of 34 and 30.9 kDa, respectively.
[b] Calculated V_{max}.

assays performed in this study, we incubated enzyme only as well as enzyme and substrate without the addition of cofactor, as controls. The production of DOXol and DAUNol from these controls were below the limit of detection (25 nM) using HPLC fluorescence. Therefore, we conclude the production of the metabolites is the result of enzymatic processes.

Michaelis-Menten kinetic properties were determined for the wild type and each of the variant forms of human CBR3, by use of DAUN (Fig. 3; Table 4) and DOX (Fig. 4; Table 5) as substrates. To determine whether the tag and linker influenced the activity of the purified CBR3 enzyme, we removed both with FXa. The V_{max}, K_m, k_{cat}, and k_{cat}/K_m values of the wild-type His_6 tagged CBR3 enzyme using DAUN as substrate were not significantly different from those of the untagged wild-type enzyme, demonstrating that the amino acid linker and His_6 tag had no effect on activity. Therefore, the tag and linker were not removed with FXa before conducting the assays with the variants. The C4Y, V244M, and V93I variants reduced V_{max} for DAUN metabolism significantly but did not alter the K_m (Table 4). The k_{cat}/K_m values obtained from DAUN metabolism with these three variant enzymes were significantly lower than the wild type (29–43% decrease).

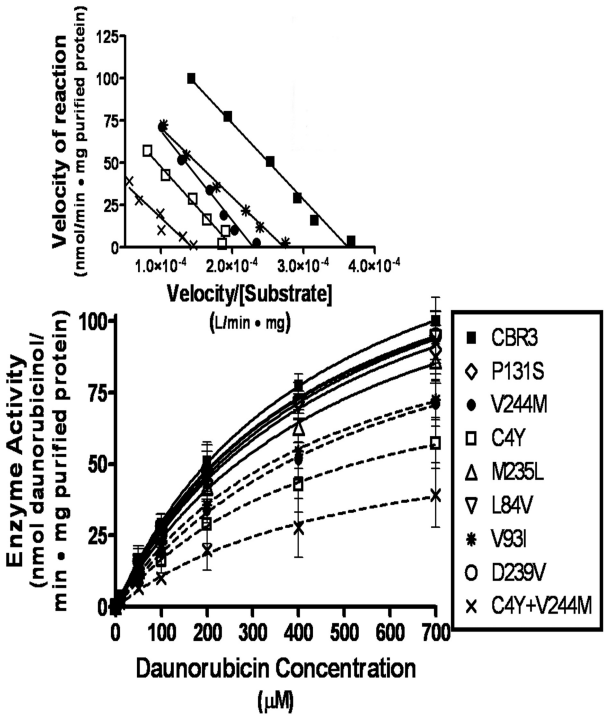

Fig. 3. In vitro enzymatic activities for the purified His_6-tagged CBR3 wild type and variants with 10 to 700 μM daunorubicin. Activities were measured by following the rate of daunorubicinol production. Three independent batches of each enzyme were purified, and assays were performed in triplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n = 9). Dotted lines refer to variants that have significant differences in kinetic parameter values compared with the wild type. Eadie-Hofstee plots for the wild type, C4Y, V93I, and V244M variant enzymes, along with the C4Y+V244M double variant, are shown at the top of the activity plot. For clarity, plots of the remaining variant enzymes, although similar, are not shown. ($r^2 > 0.91$ for all plots).

With DOX as a substrate, the kinetic parameter values of the His_6-tagged and untagged CBR3 wild-type enzymes were not significantly different from each other (Table 5). As a result, the tag and linker were not cleaved from the amino terminus of the variants for subsequent assays. The C4Y, V244M, and M235L variants reduced V_{max} significantly without affecting the K_m. In addition, the V93I variant significantly affected both V_{max} and K_m. The k_{cat}/K_m values for the V244M, C4Y, and V93I variants were significantly lower than the wild type, ranging from 41 to 66%. Eadie-Hofstee plots for the CBR3 wild-type and variant enzymes verified linearity at the same concentrations of DAUN and DOX used to conduct the assays ($r^2 > 0.91$ for all plots). Furthermore, we observed that DOX is a better substrate for the CBR3 wild-type and variant enzymes compared with DAUN, as shown by the significant increases in V_{max}, k_{cat}, and k_{cat}/K_m, and a significant decrease in K_m. However, the V93I variant demonstrated a significant increase in K_m for DOX compared with DAUN.

In addition, we examined the effect of the combined C4Y+V244M double variant because these two ns-SNPs are in linkage disequilibrium according to the International HapMap Project, which uses three parameters of linkage analysis in human populations: Lewontin's disequilibrium coefficient (D′), the correlation coefficient (r^2), and the logarithm of odds ratio (LOD) score. The D′, r^2, and LOD score values range from 0.921 to 1, 0.226 to 0.616, and 5.01 to 19.22, respectively, for the 11 ethnic groups studied in the HapMap project. By use of the tools on Haploview (version 4.2; http://www.broadinstitute.org/haploview/haploview), we found that the double variant occurs at estimated frequencies ranging from 0 to 0.9% in the ethnic populations studied in HapMap. The His_6-tagged C4Y+V244M enzyme was expressed and purified similarly to the wild-type and variant enzymes (Supplemental Fig. 1) and examined for its ability to metabolize DAUN and DOX (Figs. 3 and 4). This double variant resulted in a significant decrease in V_{max} compared with the CBR3 wild-type enzyme (59 and 55% decrease for DAUN and DOX, respectively), whereas the K_m values were unchanged for both substrates. As a result, the k_{cat}/K_m values for DAUN (64% lower than the wild type) and DOX (58% lower) were also significantly reduced (Tables 4 and 5). In addition, three other haplotypes were found in the HapMap

TABLE 4

Kinetic constants for daunorubicin metabolism by recombinant CBR3 wild-type and variant allele enzymes

Values for the His_6-tagged CBR enzymes, and the untagged CBR3 wild-type enzyme, correspond to mean ± S.D. obtained from three experiments performed with three independent enzyme preparations ($n = 9$) for each isoform.

Enzyme	V_{max}	K_m	k_{cat} [a]	k_{cat}/K_m
	nmol/min·mg protein	*μM*	s^{-1}	$s^{-1}\,M^{-1}$
CBR3	166 ± 5	461 ± 25	0.094 ± 0.003	204 ± 13
P131S	157 ± 15	503 ± 88	0.088 ± 0.009	180 ± 24
V244M	130 ± 9*	587 ± 75	0.074 ± 0.005*	128 ± 22*
C4Y	95 ± 7*	471 ± 63	0.054 ± 0.004*	116 ± 19*
M235L	149 ± 14	524 ± 91	0.084 ± 0.008	165 ± 33
L84V	161 ± 8	499 ± 50	0.091 ± 0.005	185 ± 28
V93I	120 ± 7*	472 ± 47	0.068 ± 0.004*	145 ± 16*
D239V	156 ± 7	454 ± 38	0.088 ± 0.004	197 ± 19
C4Y + V244M	68 ± 15*	527 ± 36	0.038 ± 0.009*	73 ± 16*
CBR3 untagged	159 ± 16	440 ± 29	0.080 ± 0.008	182 ± 21
CBR1	2990 ± 245*	57 ± 15*	1.69 ± 0.14*	31,500 ± 7150*

* significantly different from His_6-tagged wild-type CBR3 ($P < 0.05$).
[a] k_{cat} calculated for His_6-tagged CBRs and untagged CBR3 from molecular mass of 34 and 30.9 kDa, respectively.

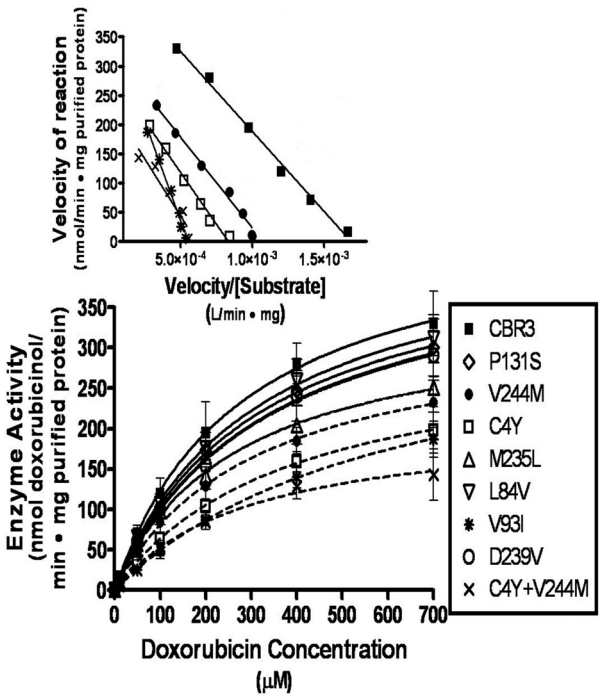

Fig. 4. In vitro enzymatic activities for the purified His_6-tagged CBR3 wild type and variants with 10 to 700 μM doxorubicin. Activities were measured by following the rate of doxorubicinol production. Three independent batches of each enzyme were purified and assays were performed in triplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n = 9). Dotted lines refer to variants that have significant differences in kinetic parameter values compared with the wild type. Eadie-Hofstee plots for the wild-type, C4Y, V93I, V244M, and M235L variant enzymes, along with the C4Y+V244M double variant, are shown at the top of the activity plot. For clarity, plots of the remaining variant enzymes, while similar, are not shown ($r^2 > 0.91$ for all plots).

project; however, their linkage disequilibrium scores were comparatively lower than the C4Y+V244M double variant, and as a result were not studied (C4Y+P131S: $D' = 1$, $r^2 = 0.01$–0.06, LOD score = 0.06–2.06; P131S+V244M: $D' = 1$, $r^2 = 0.006$–0.179, LOD score = 0.17–5.41; and V93I+V244M: $D' = 0.271$, $r^2 = 0.003$, LOD score = 0.07).

Furthermore, we compared the kinetic parameter values between the wild-type human CBR3 and CBR1 isoforms in the presence of DAUN and DOX (Tables 4 and 5). DAUN was shown to be a significantly better substrate for CBR1 than CBR3, as seen by a 18-fold increase in both V_{max} and k_{cat}, a 8-fold decrease in K_m, and a 154-fold increase in k_{cat}/K_m. However, DOX is a better substrate for CBR3 than CBR1, as demonstrated by significant increases (1.5-fold) in V_{max} and k_{cat} values. Western blots were used to examine the relative abundance of both CBRs in pooled human liver cytosol (Fig. 5). The blots were performed as described previously, with the addition of a MaxPab polyclonal mouse anti-human CBR1 primary antibody (Abnova Corporation) (diluted 1:2500). Immunoreactive CBR bands were quantified by densitometry with use of the Odyssey Infrared Imaging System. The level of CBR1 is approximately 10-fold higher than CBR3 in human liver cytosol (CBR1: 6.10 ± 1.40% of total protein; CBR3: 0.59 ± 0.09%).

Discussion

The focus of this in vitro study was to examine the effect of naturally occurring allelic variations on the metabolic activity of human CBR3 by use of the anthracyclines DOX and DAUN as substrates. The metabolism of the standard test substrate menadione with the wild-type enzyme was also assessed. Enzymatic assays were conducted by use of purified His_6-tagged CBR3 wild-type and mutant human enzymes expressed in *E. coli*. Removal of the His_6 tag had no significant effect on enzyme activity. The water-soluble metabolites DOXol and DAUNol were quantified because previous studies have acknowledged them to be the major metabolites in cancer patients who are receiving treatment with either DOX or DAUN, respectively (Lipp and Bokemeyer, 1999; Plebuch et al., 2007). We demonstrated that DOX and DAUN were both readily converted to their respective alcohol metabolites by the CBR3 wild-type enzyme, but DOX was a better substrate for human CBR3 than DAUN. To our knowledge, this is the first demonstration that human CBR3 metabolizes DAUN in vitro. In addition, we confirmed a report by Blanco et al. (2008) that DOX was metabolized by CBR3, but the metabolic activity of our human CBR3 was considerably higher. Both our study and that of Blanco et al. (2008) were in disagreement with an in vitro study by Kassner et al. (2008), who found that a histidine-tagged human CBR3 does not metabolize DOX. The reason for the discrepancy between these results is not known.

Several of the ns-SNP mutations altered the activity of CBR3. With DAUN as a substrate, the V224M, C4Y, and V93I variants, along with the C4Y+V244M double variant,

TABLE 5

Kinetic constants for doxorubicin metabolism by recombinant CBR3 wild-type and variant allele enzymes

Values for the His_6-tagged CBR enzymes, and the untagged CBR3 wild-type enzyme, correspond to mean ± S.D. obtained from three experiments performed with three independent enzyme preparations (n = 9) for each isoform.

Enzyme	V_{max}	K_m	k_{cat} [a]	k_{cat}/K_m
	nmol/min·mg protein	μM	s^{-1}	$s^{-1}\,M^{-1}$
CBR3	466 ± 22	278 ± 30	0.264 ± 0.013	965 ± 151
P131S	439 ± 24	317 ± 38	0.249 ± 0.013	797 ± 121
V244M	334 ± 16*	311 ± 33	0.189 ± 0.009*	567 ± 47*
C4Y	308 ± 10*	382 ± 25	0.175 ± 0.006*	458 ± 27*
M235L	345 ± 23*	270 ± 42	0.196 ± 0.014*	745 ± 151
L84V	452 ± 24	310 ± 36	0.256 ± 0.013	842 ± 130
V93I	352 ± 16*	613 ± 48*	0.199 ± 0.009*	327 ± 24*
D239V	432 ± 35	334 ± 46	0.245 ± 0.020	743 ± 116
C4Y + V244M	211 ± 19*	299 ± 23	0.120 ± 0.011*	402 ± 47*
CBR3 untagged	430 ± 32	261 ± 41	0.215 ± 0.016	839 ± 123
CBR1	319 ± 29*	273 ± 47	0.180 ± 0.016*	682 ± 153

* significantly different from His_6-tagged wild-type CBR3 ($P < 0.05$).

[a] k_{cat} calculated for His_6-tagged CBRs and untagged CBR3 from molecular mass of 34 and 30.9 kDa, respectively.

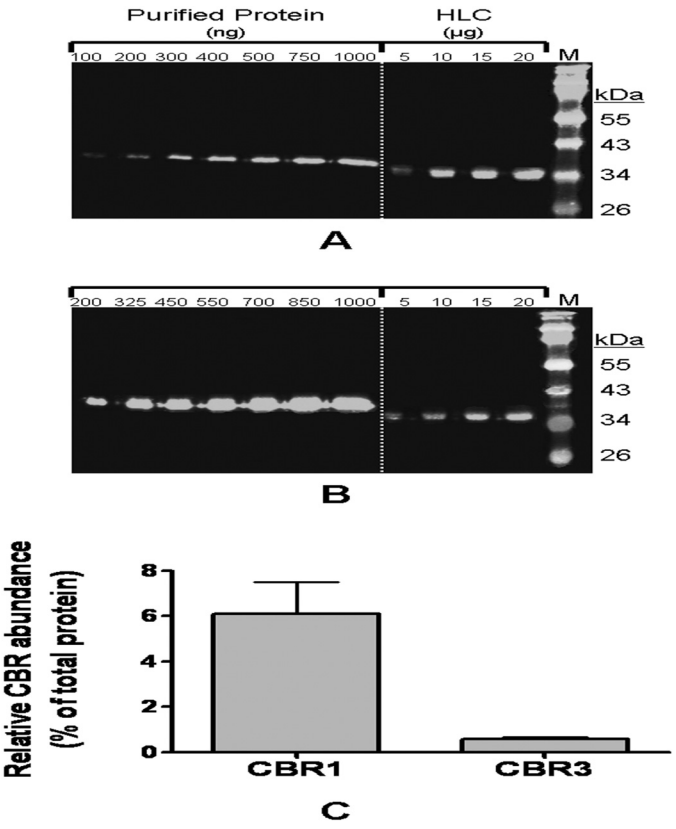

Fig. 5. Relative abundance of CBR1 and CBR3 isoforms in pooled human liver lysate (HLC). Cytosols (range, 5–20 μg of total protein) were run on 18% SDS-PAGE gels and subjected to Western blotting for detection of the CBR1 (A) and CBR3 (B) proteins. Densitometry of CBR immunoreactive bands was carried out on the purified proteins (range, 100–1000 ng) to establish a standard curve to determine the amount of CBR in the HLC. Relative abundance is reported as a percentage of total protein in HLC. All experiments were repeated three times, and the relative abundance for CBR1 and CBR3 is shown in the bar graph (C) as mean ± S.D. (n = 3).

resulted in significantly reduced V_{max} (22–59% decrease), k_{cat} (21–60% decrease), and k_{cat}/K_m (29–64% decrease). By use of DOX as a substrate, these variants had significantly reduced V_{max} (24–55% decrease), k_{cat} (25–55% decrease), and k_{cat}/K_m (41–66% decrease). In addition, the M235L variant had a significant decrease for V_{max} and k_{cat} (26% for both). Furthermore, the V93I variant exhibited a significant increase in K_m (2.2-fold).

The study by Blanco et al. (2008) also demonstrated that the bacterially expressed and His_6-tagged V244M human variant had reduced in vitro CBR3 activity with use of DOX as substrate. The reduction in activity was 60% (0.05 versus 0.14 nmol of DOXol produced per min·mg protein at 500 μM DOX, variant versus wild type). We repeated the enzyme assay using their conditions (5 h; 500 μM DOX, 200 μM NADPH) with our purified CBR3 wild-type and V244M variant; the rates we obtained were 83- and 156-fold higher, respectively (11.4 ± 0.5 and 8.3 ± 0.8 nmol DOXol produced per min·mg protein), indicating a greater activity for both our wild-type and V244M variant preparations. We repeated these assays by using our concentration of NADPH (1000 μM) and found that the V_{max} increased to 26.8 ± 1.6 and 21.8 ± 0.6 nmol of DOXol produced per min·mg protein for the CBR3 wild-type and V244M variant enzymes, respectively. These higher rates of DOXol production suggest that the cofactor was rate-limiting at the NADPH concentrations used by Blanco and colleagues. Therefore, we believe that the rates in our in vitro studies used to generate the Michaelis-Menten curves are a better representation of CBR3 metabolism of these anthracyclines.

Four of the seven human CBR3 alleles (C4Y, V93I, and V244M, and the C4Y+V244M double variant) have significantly altered DOX and DAUN metabolism (referring to the k_{cat}/K_m values), whereas the effects of the four alleles (L84V, P131S, M235L, and D239V) were not statistically significant in our in vitro studies. The location of these alleles are shown in a three-dimensional structure of the human CBR3 enzyme (Fig. 6), along with the residues involved in catalysis; Tyr194 acts as catalytic acid/base, Ser140 stabilizes the substrate, Lys198 forms hydrogen bonds with the nicotinamide ribose moiety and lowers the pK_a of the Tyr194 hydroxyl group to promote proton transfer to the carbonyl group of the substrate, and Asn114 stabilizes the position of Lys198 (Jörnvall et al., 1995; Filling et al., 2002). Of the mutations that significantly affect the metabolic activity of CBR3 when DOX or DAUN is the substrate, only the V93I mutation is physically located in close proximity to the catalytic and cofactor binding sites (Fig. 6). The change from valine to isoleucine at this position has been proposed to disrupt side chain interaction with Asn114 [The Structural Genomics Consortium, crystal structure of human carbonyl reductase 3 complexed with $NADP^+$, RSCB Protein Data Bank ID 2HRB (http://www.

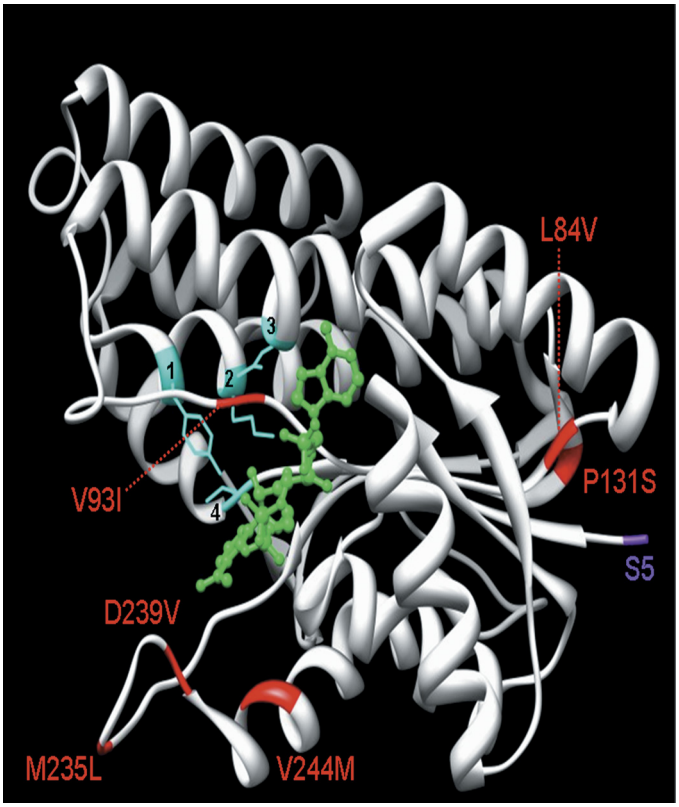

Fig. 6. A three-dimensional molecular structure of human carbonyl reductase 3 wild-type enzyme complexed with the cofactor, $NADP^+$ (green) (Protein Data Bank ID 2HRB). The ns-SNPs are shown (red) along with the four catalytic residues [(1) Tyr194, (2) Lys 198, (3) Asn114, and (4) Ser140] (blue). Because the amino acid sequence begins at position 5 with serine (purple), there is no C4Y ns-SNP illustrated. This molecular graphic image was produced by use of the UCSF Chimera program (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, CA).

sgc.ox.ac.uk/)]. This disruption to the active site may lead to decreased metabolism of DOX and DAUN. Further studies are needed to provide a more definitive explanation with respect to how the other variants alter CBR3 activity. At present, two studies have investigated the impact of the V244M variant on the catalytic site of human CBR3 (Lakhman et al., 2005; Blanco et al., 2008); however, the results are conflicting. The study by Lakhman et al. (2005) indicated that the V244M mutant resulted in increased activity by use of menadione as a substrate, whereas the study by Blanco et al. (2008) demonstrated significantly reduced activity with DOX as a substrate in agreement with our studies.

Fan et al. (2008) investigated the effect of the C4Y and V244M alleles on DOX metabolism among patients with breast cancer in a Southeast Asian population. Individuals with the C4Y allele were found to have significantly lower DOXol production compared with the wild-type population, a result consistent with our in vitro studies using the C4Y mutant enzyme. However, DOXol production by the V244M allele was significantly higher in patients with breast cancer compared with the wild-type population, a result inconsistent with our and Blanco's in vitro studies using the V244M mutant. It would seem that further investigations are required to clarify these discrepancies.

Besides looking at the differences in enzyme activity between the wild-type and variant enzymes with either DAUN or DOX, our studies demonstrated that DOX is a better substrate than DAUN for both the wild-type and variant forms of human CBR3, as shown by a 2.3- to 3.2-fold increase in V_{max} and k_{cat}, 1.2- to 1.9-fold decrease in K_m (except for the V93I variant, which has a 1.3-fold increase in K_m), and a 2.3- to 4.7-fold increase in k_{cat}/K_m. Previous studies have shown that DAUN is a better human CBR1 substrate than DOX (Oppermann 2007; Bains et al., 2009). This is interesting because both human CBR1 and CBR3 enzymes are identical in length (277 amino acids) and share 72% amino acid sequence identity and 85% similarity (El-Hawari et al., 2009). Despite the high amino acid sequence identity, the human CBR1 and CBR3 enzymes distinguish between two substrates that differ only at the carbon-14 position (DOX is hydroxylated, but DAUN is not).

Significant reductions in enzyme activity by the C4Y, V93I, M235L, and V244M variants may contribute to the interpatient variability seen with the development of serious cardiac side effects in patients treated with either DOX or DAUN. If reductases such as CBR3 play a major role in anthracycline-induced cardiotoxicity, we propose that this condition is largely the result of accumulation of the parent drug.

In conclusion, this study demonstrates that a His_6-tagged CBR3 enzyme, the activity of which does not differ from that of the untagged enzyme, is efficient at metabolizing DAUN and DOX, with higher specificity for the latter. In addition, we observed that menadione was a poor substrate for CBR3, contrary to the study by Lakhman et al. (2005). We have also shown that the V244M, C4Y, and V93I variants along with the C4Y+V244M haplotype significantly reduce reductase activity toward both anthracyclines, whereas the M235L variant significantly reduces reductase activity toward DAUN. The data collected beg the question of whether individuals bearing one or more of these mutations in human CBR3 are at higher risk for developing cardiac side effects after treatment with either of these anthracyclines. This issue can be addressed with clinical association studies, which are currently underway in our laboratory. We realize that there are single-nucleotide polymorphisms (SNPs) in the noncoding regions of the human CBR3 gene, including untranslated regions, introns, and promoter regions, which may influence the amount of protein produced. Even though a study by Zhang and Blanco (2009) found that two common SNPs in the CBR3 promoter [−725 T>C (rs2239566, q = 12.7–22.5%) and −326 T>A (rs8132243, q = 2.4–17.5%)] did not modify gene promoter activity or significantly alter hepatic CBR3 mRNA levels compared with the wild type, there are a number of common SNPs in the noncoding regions that remain to be investigated. The clinical studies that we have initiated will examine these SNPs and the ns-SNPs in the human CBR enzymes to determine whether there is a correlation with cardiotoxicity.

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References

Bains OS, Karkling MJ, Grigliatti TA, Reid RE, and Riggs KW (2009) Two non-synonymous single nucleotide polymorphisms of human carbonyl reductase 1 demonstrate reduced *in vitro* metabolism of daunorubicin and doxorubicin. *Drug Metab Dispos* **37:**1107–1114.

Bains OS, Takahashi RH, Pfeifer TA, Grigliatti TA, Reid RE, and Riggs KW (2008)

Two allelic variants of aldo-ketoreductase 1A1 exhibit reduced *in vitro* metabolism of daunorubicin. *Drug Metab Dispos* **36:**904–910.
Barry E, Alvarez JA, Scully RE, Miller TL, and Lipshultz SE (2007) Anthracycline-induced cardiotoxicity: course, pathophysiology, prevention and management. *Expert Opin Pharmacother* **8:**1039–1058.
Blanco JG, Leisenring WM, Gonzalez-Covarrubias VM, Kawashima TI, Davies SM, Relling MV, Robison LL, Sklar CA, Stovall M, and Bhatia S (2008) Genetic polymorphisms in carbonyl reductase 3 gene CBR3 and the NAD(P)H:quinone oxidoreductase 1 gene NQO1 in patients who developed anthracycline-related congestive heart failure after childhood cancer. *Cancer* **112:**2789–2795.
Cortés-Funes H and Coronado C (2007) Role of anthracyclines in the era of targeted therapy. *Cardiovasc Toxicol* **7:**56–60.
Danesi R, Fogli S, Gennari A, Conte P, and Del Tacca M (2002) Pharmacokinetic pharmacodynamic relationships of the anthracycline anticancer drugs. *Clin Pharmacokinet* **41:**431–444.
Deng S and Wojnowski L (2007) Genotyping the risk of anthracycline-induced cardiotoxicity. *Cardiovasc Toxicol* **7:**129–134.
El-Hawari Y, Favia AD, Pilka ES, Kisiela M, Oppermann U, Martin HJ, and Maser E (2009) Analysis of the substrate-binding site of human carbonyl reductases CBR1 and CBR3 by site-directed mutagenesis. *Chem Biol Interact* **178:**234–241.
Endo S, Matsunaga T, Kitade Y, Ohno S, Tajima K, El-Kabbani O, and Hara A (2008) Human carbonyl reductase 4 is a mitochondrial NADPH-dependent quinone reductase. *Biochem Biophys Res Commun* **377:**1326–1330.
Fan L, Goh BC, Wong CI, Sukri N, Lim SE, Tan SH, Guo JY, Lim R, Yap HL, Khoo YM, et al. (2008) Genotype of human carbonyl reductase CBR3 correlates with doxorubicin disposition and toxicity. *Pharmacogenet Genomics* **18:**621–631.
Fassas A and Anagnostopoulos A (2005) The use of liposomal daunorubicin (DaunoXome) in acute myeloid leukemia. *Leuk Lymphoma* **46:**795–802.
Filling C, Berndt KD, Benach J, Knapp S, Prozorovski T, Nordling E, Ladenstein R, Jörnvall H, and Oppermann U (2002) Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J Biol Chem* **277:**25677–25684.
Floyd JD, Nguyen DT, Lobins RL, Bashir Q, Doll DC, and Perry MC (2005) Cardiotoxicity of cancer therapy. *J Clin Oncol* **23:**7685–7696.
Forrest GL and Gonzalez B (2000) Carbonyl reductase. *Chem Biol Interact* **129:**21–40.
Gianni L, Salvatorelli E, and Minotti G (2007) Anthracycline cardiotoxicity in breast cancer patients: synergism with trastuzumab and taxanes. *Cardiovasc Toxicol* **7:**67–71.
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.
Hunault-Berger M, Milpied N, Bernard M, Jouet JP, Delain M, Desablens B, Sadoun A, Guilhot F, Casassus P, and Ifrah N (2001) Daunorubicin continuous infusion induces more toxicity than bolus infusion in acute lymphoblastic leukemia induction regimen: a randomized study. *Leukemia* **15:**898–902.
Jörnvall H, Persson B, Krook M, Atrian S, Gonzàlez-Duarte R, Jeffery J, and Ghosh D (1995) Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34:**6003–6013.
Kassner N, Huse K, Martin HJ, Gödtel-Armbrust U, Metzger A, Meineke I, Brockmöller J, Klein K, Zanger UM, Maser E, et al. (2008) Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metab Dispos* **36:**2113–2120.
Lakhman SS, Ghosh D, and Blanco JG (2005) Functional significance of natural allelic variant of human carbonyl reductase 3 (CBR3). *Drug Metab Dispos* **33:**254–257.
Licata S, Saponiero A, Mordente A, and Minotti G (2000) Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and carbonyl reduction. *Chem Res Toxicol* **13:**414–420.
Lipp HP and Bokemeyer C (1999) Anthracyclines and other intercalating agents, in *Anticancer Drug Toxicity: Prevention, Management and Clinical Pharmacokinetics* (Lipp HP ed) pp 81–113, Marcel Dekker, New York.
Matsunaga T, Shintani S, and Hara A (2006) Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug Metab Pharmacokinet* **21:**1–18.
Menna P, Recalcati S, Cairo G, and Minotti G (2007) An introduction to the metabolic determinants of anthracycline cardiotoxicity. *Cardiovasc Toxicol* **7:**80–85.
Miura T, Nishinaka T, and Terada T (2008) Different functions between human monomeric carbonyl reductase 3 and carbonyl reductase 1. *Mol Cell Biochem* **315:**113–121.
Mordente A, Meucci E, Martorana GE, Giardina B, and Minotti G (2001) Human heart cytosolic reductases and anthracycline cardiotoxicity. *IUBMB Life* **52:**83–88.
Oppermann U (2007) Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu Rev Pharmacol Toxicol* **47:**293–322.
Plebuch M, Soldan M, Hungerer C, Koch L, and Maser E (2007) Increased resistance of tumor cells to daunorubicin after transfection of cDNAs coding for anthracycline inactivating enzymes. *Cancer Lett* **255:**49–56.
Wirth H and Wermuth B (1992) Immunohistochemical localization of carbonyl reductase in human tissues. *J Histochem Cytochem* **40:**1857–1863.
Wojtacki J, Lewicka-Nowak E, and Leśniewski-Kmak K (2000) Anthracycline-induced cardiotoxicity: clinical course, risk factors, pathogenesis, detection and prevention—review of the literature. *Med Sci Monit* **6:**411–420.
Zhang J and Blanco JG (2009) Identification of the promoter of human carbonyl reductase 3 (CBR3) and impact of common promoter polymorphisms on hepatic CBR3 mRNA expression. *Pharmacol Res* **26:**2209–2215.

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