Short Communication

Expression of the Anthracycline-Metabolizing Enzyme Carbonyl Reductase 1 in Hearts from Donors with Down Syndrome

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ABSTRACT:
Cancer patients with Down syndrome (DS) are susceptible to developing anthracycline-related cardiotoxicity. The pathogenesis of anthracycline-related cardiotoxicity has been linked to the intracellular synthesis of alcohol metabolites by carbonyl reductase 1 (CBR1). CBR1 is located in the DS critical region (21q22.12). The expression of CBR1 in hearts from individuals with DS has not been characterized. This study documented CBR1 expression in hearts from donors with DS (n = 4) and donors without DS (n = 15). The DS samples showed 1.8-fold higher CBR1 mRNA levels compared to the non-DS samples (levels in DS samples were 3.3-relative fold, and those in non-DS were 1.8-relative fold; p = 0.012). CBR1 protein levels were 1.9-fold higher in DS samples than in non-DS samples (13.5 ± 7.7 versus 7.2 ± 3.9 nmol/g cytosolic protein, respectively; p = 0.029). CBR1 activity for daunorubicin was 1.7-fold higher in DS samples than in non-DS samples (3.8 ± 0.1 versus 2.3 ± 0.2 nmol daunol/min · mg, respectively; p = 0.050). CBR1 1096G>A (rs9024) affects CBR1 activity, and one heart trisomic for the variant A allele (A/A/A) exhibited low enzymatic activity. These findings suggest that increased CBR1 expression in the hearts of individuals with DS may contribute to the risk of anthracycline-related cardiotoxicity.

Introduction
Down syndrome (DS) is the most common genetic cause of mental retardation and originates from the presence of an extra copy of chromosome 21, although in roughly 5% of documented DS cases, a partial trisomy leads to the condition (Amano et al., 2004; Patterson, 2009). Children with DS have a 10- to 20-fold increased risk of developing acute myeloid leukemia (AML) compared to sex- and age-matched children without DS. Treatment regimens for pediatric AML rely heavily on the use of the anthracycline daunorubicin. However, the therapeutic benefit of chemotherapy with daunorubicin is mitigated by the development of cardiotoxicity in some patients. Risk factors for anthracycline-related cardiotoxicity include total cumulative exposure, younger age at cancer diagnosis, radiation therapy to the heart region, and female sex. Epidemiological studies have identified pediatric cancer patients with DS as a population at particularly greater risk for both acute and chronic anthracycline-related cardiotoxicity (Krischer et al., 1997; Grenier and Lipshultz, 1998; Ravindranath et al., 2005). For example, Krischer et al. (1997) found that Trisomy 21 was associated with an increase in the relative risk for anthracycline-related cardiomyopathy of 3.4-fold. A report from the Children’s Oncology Group documented clinically symptomatic cardiomyopathy in 17.5% of patients with DS-AML who were treated with anthracyclines (O’Brien et al., 2008).

The pathogenesis of anthracycline-related cardiotoxicity has been linked to the synthesis of anthracycline C-13 alcohol metabolites by cardiac carbonyl reductase 1 (CBR1) activity (Minotti et al., 2004). Transgenic mice overexpressing human CBR1 in heart exhibited high levels of intracardiac doxorubicinol and increased signs of myocardial damage after doxorubicin administration (Forrest et al., 2000). In contrast, mice with a null Cbr1 allele (Cbr1−/−) had decreased doxorubicinol plasma levels and significantly decreased incidence of anthracycline-related cardiotoxicity compared to mice with two active Cbr1 alleles (Cbr1+/+) (Olson et al., 2003). The CBR1 gene is located in the DS critical region (21q22.12), and an early report noted that CBR1 expression is subjected to gene-dosage effects in lymphoid cells with Trisomy 21 (Lemieux et al., 1993). Elevated CBR1 protein levels have been noted in several regions of the brain in individuals with DS (Balcz et al., 2001). There is a paucity of reports documenting cardiac CBR1 expression in donors with DS. Thus, the goal of the present study was to examine cardiac CBR1 expression in DS samples and non-DS samples. CBR1 mRNA and CBR1 protein levels were investigated by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) and quantitative immunoblotting, respectively. Cardiac CBR1 activity was measured for the substrate daunorubicin.

We recently identified a relatively common single-nucleotide polymorphism in the 3′- untranslated region (UTR) of CBR1 (minor allele, q = 12.5% in whites; CBR1 1096G>A, rs9024) that affects the synthesis of doxorubicinol in liver. Liver samples with the CBR1 1096G>A homozygous G/G genotype exhibited 1.5-fold higher doxorubicinol synthesis rates than samples with the heterozygous G/A genotype (Gonzalez-Covarrubias et al., 2009). Thus, we explored whether CBR1 1096G>A genotype status affects CBR1 expression in hearts from individuals with DS.

Materials and Methods
Heart Samples. The Institutional Review Board of the State University of New York at Buffalo approved this research. Heart samples (20–100 g, left

ABBREVIATIONS: DS, Down syndrome; CBR1, carbonyl reductase 1; AML, acute myeloid leukemia; UTR, untranslated region; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; aCGH, comparative genomic hybridization arrays.
ventricle) from donors with DS (n = 4) and donors without DS (n = 15) were procured from the National Disease Research Interchange (Philadelphia, PA). DS status (yes/no) was obtained from medical records. Tissue procurement protocols have been open since December 2007 and February 2008 for non-DS samples and DS samples, respectively, and included the following criteria: no current diagnosis of cancer, myocardial infarction, and/or congestive heart failure, no evidence of sepsis, and no history of chemotherapy and/or radiation within the last year. Postmortem to tissue recovery interval was ≤10 h. Heart samples were frozen immediately after recovery and stored in liquid nitrogen until further processing. To process heart samples, we followed standardized procedures to isolate RNA and DNA as described previously (Gonzalez-Covarrubias et al., 2009). Cytosols were obtained by differential ultracentrifugation as described previously (Mordente et al., 2003).

**Comparative Genomic Hybridization Arrays.** Genomic DNA was used to probe high-resolution comparative genomic hybridization arrays (aCGH) as described previously (Snijders et al., 2001; Cowell et al., 2004). Arrays contained ~6000 sequenced bacterial artificial chromosome clones, which provided a resolution of 700 kilobases across the genome. Changes in DNA copy number were determined by evaluating log2 ratios across whole chromosomes.

**QRT-PCR.** Total RNA from liver (200 ng) was reverse transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (QIAGEN, Valencia, CA). CBR1 mRNA levels were determined by the comparative quantitation method as described previously (Gonzalez-Covarrubias et al., 2009). Individual β-actin mRNA levels were used for normalization. Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of CBR1 mRNA in each sample was automatically calculated with a comparative quantitation algorithm (iQ5 Optical System Software, version 2.0; Bio-Rad Laboratories, Hercules, CA).

**Quantitative Immunoblotting.** Cytosolic CBR1 protein levels were quantitated as described previously (Gonzalez-Covarrubias et al., 2009). In brief, cytosols (150 µg) and recombinant CBR1 standards (0.03, 0.05, 0.10, 0.15, and 0.30 µg; Abcam Inc., Cambridge, MA) were loaded into 12% protein gels and separated by electrophoresis. Proteins were probed with a specific polyclonal anti-human CBR1 antibody (1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a secondary rabbit anti-IgG antibody conjugated with horseradish peroxidase (1:10000; Sigma-Aldrich, St. Louis, MO). Immunoreactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). CBR1 band intensity values (pixels/mm2) were quantitated with a ChemiDoc XR gel documentation system (Bio-Rad Laboratories). Cardiac CBR1 levels were estimated by extrapolation from the calibration curves of recombinant CBR1. Detection of CBR1 was linear (range 0.03–0.30 µg; r2 > 0.93).

**Kinetic Analysis.** Validation experiments with pooled heart cytosols showed that 400 µM daunorubicin [S] ensured conditions of Vmax and/or maximal CBR1 activity (zero-order kinetics). Thus, maximal CBR1 activities (reaction rates) were directly proportional to the amount of cytosolic CBR1 enzyme. Cytosolic and recombinant CBR1 standards (0.03, 0.05, 0.10, 0.15, and 0.30 µg; Abcam Inc., Cambridge, MA) were loaded into 12% protein gels and separated by electrophoresis. Proteins were probed with a specific polyclonal anti-human CBR1 antibody (1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a secondary rabbit anti-IgG antibody conjugated with horseradish peroxidase (1:10000; Sigma-Aldrich, St. Louis, MO). Immunoreactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). CBR1 band intensity values (pixels/mm2) were quantitated with a ChemiDoc XR gel documentation system (Bio-Rad Laboratories). Cardiac CBR1 levels were estimated by extrapolation from the calibration curves of recombinant CBR1. Detection of CBR1 was linear (range 0.03–0.30 µg; r2 > 0.93).

**CBR1 Genotyping.** The CBR1 1096G>A (rs9024) polymorphism was investigated by allelic discrimination with fluorescence probes and real-time PCR (Assays-by-Design; Applied Biosystems, Foster City, CA). The two possible heterozygous conditions (G/A/G and G/G/A) in DS samples were detected by allele quantification. DNA samples with known CBR1 1096G>A genotypes were used to generate standards for calibration curves. The theoretical ratios of the variant allele were 0, 12.5, 20.0, 33.3, 50.0, 66.7, 80.0, 87.5, and 100% (Cheng et al., 2005). Genotyping reactions were run as described previously (Gonzalez-Covarrubias et al., 2009). CBR1 1096G>A genotypes were extrapolated from the calibration curves depicting normalized fluorescent signal versus percentage of allele A.

Data Analysis. Statistics were computed with Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism, version 4.03 (GraphPad Software Inc., San Diego, CA). The Mann-Whitney test was used to compare group means. Differences were considered to be statistically significant at p < 0.05.

**Results**

First, aCGH analysis was performed on DNA isolated from DS samples to confirm Trisomy 21. An analysis of log2 ratios demonstrated that all DNA samples from donors with DS exhibited a third copy of chromosome 21 (Fig. 1A). Further examination revealed that all DS DNA samples carried three copies of CBR1 (Fig. 1B). Next, cardiac CBR1 mRNA expression in DS and non-DS samples was examined by QRT-PCR. On average, cardiac CBR1 mRNA levels were 1.8-fold higher in DS samples than in non-DS samples (CBR1 mRNADS 3.3 ± 0.5 relative fold, versus CBR1 mRNA nond-DS 1.8 ± 0.2 relative fold; p = 0.012) (Fig. 1C). Increased cardiac CBR1 mRNA expression dictated higher CBR1 protein levels in cytosols from DS samples compared to non-DS samples (CBR1 proteinDS 13.5 ± 7.7 nmol/g cytosolic protein, versus CBR1 protein nond-DS 7.2 ± 3.9 nmol/g cytosolic protein; p = 0.029) (Fig. 1D).

To further examine the impact of Trisomy 21 on cardiac CBR1 expression, we measured CBR1 activity for the anthracycline substrate daunorubicin. Cardiac CBR1 activity demonstrated typical one-site Michaelis-Menten kinetics for daunorubicin (Km = 0.2 mmol/min · mg; Vmax, 202.0 ± 50.6 µmol; Fig. 1G). In line with the CBR1 mRNA and CBR1 protein expression data, maximal CBR1 activity, expressed as the rate of synthesis of daunorubicinol, was 1.7-fold higher in DS samples than in non-DS samples (CBR1 activityDS 3.8 ± 0.1 nmol daunol/min · mg, versus CBR1 activity nond-DS 2.3 ± 0.2 nmol daunol/min · mg; p = 0.050) (Fig. 1H).

Expression of CBR1 in hearts from donors with DS varied between individuals at the mRNA (13.3-fold, range 0.3–4.0 relative fold), protein (5.5-fold, range 3.0–16.4 nmol/g cytosolic protein), and activity levels (3.5-fold, range 1.1–3.9 nmol daunol/min · mg). Likewise, non-DS samples showed variable CBR1 expression at the mRNA (1.9-fold, range 2.2–4.1 relative fold), protein (3.9-fold, range 6.0–23.6 nmol/g cytosolic protein), and activity levels (3.1-fold, range 1.6–4.9 nmol daunol/min · mg).

Next, we examined CBR1 1096G>A genotype status in the DS samples. The calibration curve of normalized fluorescent signal versus ratio of the variant allele A showed a linear relationship (r2 = 0.83). Table 1 shows cardiac CBR1 expression stratified by CBR1 1096G>A genotype status.

**Discussion**

This study documents increased CBR1 expression in DS samples. On average, DS samples showed increased CBR1 mRNA (1.8-fold), CBR1 protein (1.9-fold), and CBR1 activity (1.7-fold) compared to non-DS samples. These observations are in agreement with the expected 1.5-fold increase in CBR1 expression dictated by the gene-dosage effect (Lemieux et al., 1993; Amano et al., 2004). Our findings may provide insights concerning the higher incidence of anthracycline-related cardiotoxicity in cancer patients with DS compared to those in patients without DS (Krischer et al., 1997; Ravindranath et al., 2005; O’Brien et al., 2008). Studies have demonstrated that the development of cardiomyopathy correlates with myocardial accumulation of alcohol metabolites (Forrest et al., 2002; Olson et al., 2003; Minotti et al., 2004). Thus, increased CBR1 expression in hearts from donors with DS would affect the pathogenesis of cardiotoxicity due to the synthesis of relatively high levels of cardiotoxic daunorubicinol.

The relatively small number of DS samples is the main limitation of this study. Heart tissue from donors with DS is scarce, and procurement rates are low (~1 sample/7.3 months), even for cooperative resources.

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such as the National Disease Research Interchange. Stringent criteria were used for tissue procurement and analysis to minimize experimental variability. Thus, our findings may provide a representative picture of cardiac CBR1 expression in the hearts of individuals with DS.

We recently identified a relatively common polymorphism in the 3′-UTR of CBR1 (CBR1 1096G>A, rs9024) that affects hepatic CBR1 expression at the protein and activity levels. CBR1 protein expression levels were 1.7-fold higher in liver samples with the...
suggest that increased CBR1 expression in samples from individuals with DS. Our findings indicate that the polymorphic site at the 3′/H11032 of CBR1 affects the binding of specific microRNA to the polymorphic site at the 3′-UTR of CBR1. Functional studies are ongoing to evaluate whether candidate microRNAs regulate CBR1 expression in various cell types. Additional genotype-phenotype studies with larger sample sizes are needed to define the impact of CBR1 1096G>A on cardiac CBR1 expression.

This pilot study represents the first characterization of cardiac CBR1 expression in samples from individuals with DS. Our findings suggest that increased CBR1 expression and activity dictated by the gene-dosage effect would affect the risk of anthracycline-related cardiotoxicity in cancer patients with DS.

### Table 1

<table>
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<th>Status</th>
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<th>G/G/G</th>
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N/A, not applicable.

### References


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