Pharm Res (2014) 31:1644–1655
DOI 10.1007/s11095-013-1267-1

RESEARCH PAPER

Interindividual Variability in the Cardiac Expression of Anthracycline Reductases in Donors With and Without Down Syndrome

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Received: 16 September 2013 / Accepted: 9 December 2013 / Published online: 22 February 2014

ABSTRACT

Purpose The intracardiac synthesis of anthracycline alcohol metabolites (e.g., daunorubicinol) contributes to the pathogenesis of anthracycline-related cardiotoxicity. Cancer patients with Down syndrome (DS) are at increased risk for anthracycline-related cardiotoxicity. We profiled the expression of anthracycline metabolizing enzymes in hearts from donors with- and without- DS.

Methods Cardiac expression of CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 was examined by quantitative real time PCR, quantitative immunoblotting, and enzyme activity assays using daunorubicin. The *CBR1* polymorphism rs9024 was investigated by allelic discrimination with fluorescent probes. The contribution of CBRs/AKRs proteins to daunorubicin reductase activity was examined by multiple linear regression.

Results *CBR1* was the most abundant transcript (average relative expression; DS: 81%, non-DS: 58%), and AKR7A2 was the most abundant protein (average relative expression; DS: 38%, non-DS: 35%). Positive associations between cardiac CBR1 protein levels and daunorubicin reductase activity were found for samples from donors with- and without- DS. Regression analysis suggests that sex, CBR1, AKR1A1, and AKR7A2 protein levels were significant contributors to cardiac daunorubicin reductase activity. *CBR1* rs9024 genotype status impacts on cardiac *CBR1* expression in non-DS hearts.

Conclusions CBR1, AKR1A1, and AKR7A2 protein levels point to be important determinants for predicting the synthesis of cardiotoxic daunorubicinol in heart.

KEY WORDS Aldo-keto reductases · Anthracycline-related cardiotoxicity · Anthracyclines · Carbonyl reductases · Down syndrome

ABBREVIATIONS

aCGH	Array comparative genomic hybridization
ACTB	Actin B
AKR1A1	Aldo-keto reductase family 1, member A1
AKR1C3	Aldo-keto reductase family 1, member C3
AKR7A2	Aldo-keto reductase family 7, member A2
AKRs	Aldo-keto reductases
CBR1	Carbonyl reductase 1
CBR3	Carbonyl reductase 3
CBRs	Carbonyl reductases
DS	Down syndrome
LOD	Limit of detection
LOQ	Limit of quantification

Electronic supplementary material The online version of this article (doi:10.1007/s11095-013-1267-1) contains supplementary material, which is available to authorized users.

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INTRODUCTION

In humans, carbonyl reductases (CBRs) and aldo-keto reductases (AKRs) play predominant roles during the metabolism of more than 30 clinically relevant drugs including the antipsychotic agent haloperidol, the opioid receptor antagonist naltrexone, the antiemetic dolasetron, the anti-inflammatory ketoprofen, and the anticancer anthracyclines doxorubicin and daunorubicin (1–3). The use of anthracyclines for the chemotherapy of a variety of solid and hematological cancers is limited by the development of cardiotoxicity in some patients (4). For example, a study of children ($n=115$) who survived leukemia revealed that 60% of those treated with anthracyclines developed preclinical abnormalities in cardiac structure and function (5). Of note, pediatric cancer patients with Down syndrome (DS, trisomy 21) constitute a population at particularly greater risk for anthracycline-related cardiotoxicity, and a safe dose of anthracyclines for cancer patients with- DS remains to be defined (6,7). For example, children with- DS represent 15% of pediatric acute myeloid leukemia (AML) cases, and a report from the Children's Oncology group has documented clinically symptomatic cardiomyopathy in 17.5% of DS-AML patients treated with an anthracycline-containing regimen (7,8).

The complex pathogenesis of anthracycline-related cardiotoxicity is mediated by a combination of oxidative stress and metabolic perturbations in myocardial tissue that are induced by the C-13 anthracycline alcohol metabolites, whose formation is catalyzed by specific CBRs and AKRs (4,9–12). A growing cumulus of evidence indicates that two monomeric CBRs, namely CBR1 and CBR3, together with the members of the AKRs superfamily AKR1A1, AKR1C3 and AKR7A2 catalyze the synthesis of cardiotoxic C-13 anthracycline alcohol metabolites (e.g., daunorubicinol and doxorubicinol) (13–16). In addition specific *CBRs/AKRs* genetic variants may contribute to the unpredictable pharmacological profile of anthracyclines in cancer patients (17–19). For example, a recent study from the Children's Oncology group described the impact of functional single nucleotide polymorphisms in *CBR1* and *CBR3* on the risk of anthracycline-related cardiomyopathy in childhood cancer survivors (20). Thus, interindividual variability in the expression of CBRs and AKRs would impact the intracardiac formation of cardiotoxic C-13 anthracycline alcohol metabolites, and consequently the pharmacodynamics of anthracycline drugs. Furthermore, the *CBR1* and *CBR3* genes are located in the DS critical region of chromosome 21 (21q21-21q22.3). The altered expression of *CBRs* as a result of the gene dosage effect may contribute to the increased risk of anthracycline-related cardiotoxicity in cancer patients with- DS (21).

In spite of the prominent contributions of CBRs and AKRs towards the pharmacodynamics of anthracycline drugs, reports documenting gene expression levels and protein abundance in cardiac tissue are limited to the analysis of individual samples or pooled tissue samples (13,22,23). Thus, the main goal of this study was to document the extent of interindividual variability in the expression of CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 in a collection of heart samples from donors with- and without- DS. The expression of CBRs and AKRs was examined by quantitative real time PCR (qRT-PCR) with specific primers, quantitative immunoblotting with specific antibodies, and enzyme activity assays using the anthracycline substrate daunorubicin. We also examined the impact of a functional polymorphism in *CBR1* (rs9024), known to impact CBR1 expression and daunorubicinol synthesis in liver, on cardiac *CBR1* gene expression and enzymatic activity for the substrate daunorubicin (21,24,25).

MATERIALS AND METHODS

Human Heart Samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Heart samples from donors with- ($n=9$) and without- DS ($n=30$) were procured from The National Disease Research Interchange (NDRI, funded by the National Center for Research Resources), The Cooperative Human Tissue Network (CHTN, funded by the National Cancer Institute), and the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank. The postmortem to tissue recovery interval was ≤10 h. Samples (2–20 g, myocardium, left ventricle only) were frozen immediately after recovery and stored in liquid nitrogen until further processing. The main demographics from donors with- and without- DS are summarized in Supplementary Material Table I. Down syndrome status (yes/no) and relevant diagnoses (Supplementary Material Table II) were obtained from anonymous medical histories. Heart samples were processed following standardized procedures to isolate DNA and RNA as described (24,26).

Array CGH Analysis

Down syndrome status was confirmed by array comparative genomic hybridization (aCGH). Briefly, genomic DNA (3.0 μg) from test samples and a euploid reference DNA sample were fluorescently labeled and hybridized to high resolution Agilent 244 K aCGH arrays containing +236,000 coding and non-coding human probes. Changes in DNA copy number were determined by evaluating $\log_2$ ratios across whole chromosomes. aCGH assays were performed at the Genomics core facility, Roswell Park Cancer Institute (Buffalo, NY).

Quantitative Real-Time PCR

Cardiac *CBR1*, *CBR3*, *AKR1A1*, *AKR1C3*, and *AKR7A2* mRNA expression was analyzed by qRT-PCR with gene

specific primers (Table I) following the MIQE guidelines (27). Briefly, 5 ng of total RNA was reverse transcribed and amplified using the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Venlo, The Netherlands). Target genes and *ACTB* (reference gene) were amplified in parallel with the following cycling parameters: 50°C for 30 min (reverse transcription), 95°C for 10 min (*Taq* DNA polymerase activation), 40 cycles of 95°C for 15 s (denaturation), 56°C for 30 s (annealing) and 72°C for 30 s (extension). PCR amplification products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide. PCR amplification products were cloned into pCR 2.1- TOPO vectors (Invitrogen, Carlsbad, CA), and their identities were verified by DNA sequencing. Cloned PCR products were diluted to generate standards for calibration curves. In all cases, the regression coefficients of the standard curves were $r^2 \geq 0.95$. The amplification efficiencies of the target genes (e.g., *CBR1*, *AKR1A1*) and the reference gene (*ACTB*) were comparable and ranged between 96 and 110%. For each gene, experimental samples and standards for calibration curves were analyzed in triplicate. For each heart sample, the copy number of the target gene and *ACTB* were calculated using the average Ct values and direct extrapolation from the calibration curves (Fig. 1). In all cases, cardiac mRNA levels were expressed as copy number ratios using the following expression:

$$\text{Copy number ratio} = \frac{\text{Target gene copy number} \times 10^6}{\textit{ACTB}\,\text{copy number}}$$

Quantitative Immunoblotting

Heart tissue cytosols (~50 mg) were obtained using the Minute Cytoplasmic and Nuclear Extraction Kit (Invent Biotechnologies, Eden Prairie, MN) following the manufacturer instructions. Protein concentration was determined using a BCA assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Cytosolic proteins were denatured with NuPAGE LDS sample buffer (Invitrogen) containing NuPAGE sample reducing agent (Invitrogen), and boiled at 70°C for 10 min prior to use. Briefly, cytosolic protein (12.5 μg for CBR1, AKR1A1, AkR1C3 and AKR7A2, and 40 μg for CBR3) and corresponding recombinant protein standards (Abcam, Cambridge, England) were separated by gel electrophoresis using NuPAGE Novex 4 - 12% Bis-Tris precast gels, and transferred onto nitrocellulose membranes using the iBlot Gel Transfer Device (Invitrogen). Membranes were blocked with 5% non-fat milk in 0.2% Tween 20-PBS for 1 h at room temperature and then probed with specific polyclonal anti-human CBR1 (1:3000; Santa Cruz Biotechnology, Dallas, TX), CBR3 (1:500; Santa Cruz Biotechnology), AKR1A1 (1:3000; Abnova, Taipei City, Taiwan), AKR1C3 (1:3000; Abnova), and AKR7A2 (1:3000; Abnova) antibodies overnight at 4°C. Next, membranes were incubated with a secondary goat or rabbit anti-IgG antibodies conjugated with horseradish peroxidase (1:10000; Santa Cruz Biotechnology) for 1.5 h at room temperature. To normalize for protein loading, membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA), and re-probed with anti-GAPDH antibody (1:1000; Santa Cruz Biotechnology). Immunoreactive bands were visualized with the ECL Plus Western blotting substrate (GE Healthcare, Little Chalfont, England) on a ChemiDoc XRS imager (Bio-Rad, Berkeley, CA). Densitometric analysis was performed using Image Lab software (Bio-Rad). Cardiac CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 protein levels were estimated by direct extrapolation from the corresponding recombinant protein standard curves. Detection of recombinant CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 proteins was linear (range: 0.01–0.1 μg; $r^2 > 0.99$). The limits of detection (LOD) and limits of quantification (LOQ) were calculated based on the standard deviation of the band intensity and the slope of the calibration curve (Supplementary Material Fig. 1). Results are as follows: $LOD_{CBR1} = 0.34$ ng, $LOQ_{CBR1} = 0.80$ ng; $LOD_{CBR3} = 0.49$ ng, $LOQ_{CBR3} = 1.49$ ng; $LOD_{AKR1A1} = 0.58$ ng, $LOQ_{AKR1A1} = 1.76$ ng; $LOD_{AKR1C3} = 0.33$ ng, $LOQ_{AKR1C3} = 0.98$ ng; $LOD_{AKR7A2} = 0.44$ ng, $LOQ_{AKR7A2} = 1.34$ ng.

Kinetic Analysis

Anthracycline reductase activity was measured in heart cytosols using the substrate daunorubicin as described previously (21). Validation experiments with pooled heart cytosols

Table I List of PCR Primers for the Amplification of Cardiac *CBRs* and *AKRs* mRNAs

Gene	Forward primer	Reverse primer	Size (bp)
CBR1	5′-TCAAGCTGAAGTGACGATGA-3′	5′-GGTGCACTCCCTTCTTTGTA-3′	239
CBR3	5′-AACCTCATGGGAGAGTGGTG-3′	5′-TCCTCGATAAGACCGTGACC-3′	231
AKR1A1	5′-CCTGGTCAGGTAAAAGCAGC-3′	5′-CCCGCTCAAAGGCATAAGG-3′	286
AKR1C3	5′-CCTCCAGAGGTTCCGAGAAG-3′	5′-GTGGACCAAAGCTTTGAAGTG-3′	173
AKR7A2	5′-GGCCTCTCCAACTATGCTAG-3′	5′-GGCATAGAACCTCAGTCCAAAG-3′	168
ACTB	5′-GGACTTCGAGCAAGAGATGG-3′	5′-AGCACTGTGTTGGCGTACAG-3′	234

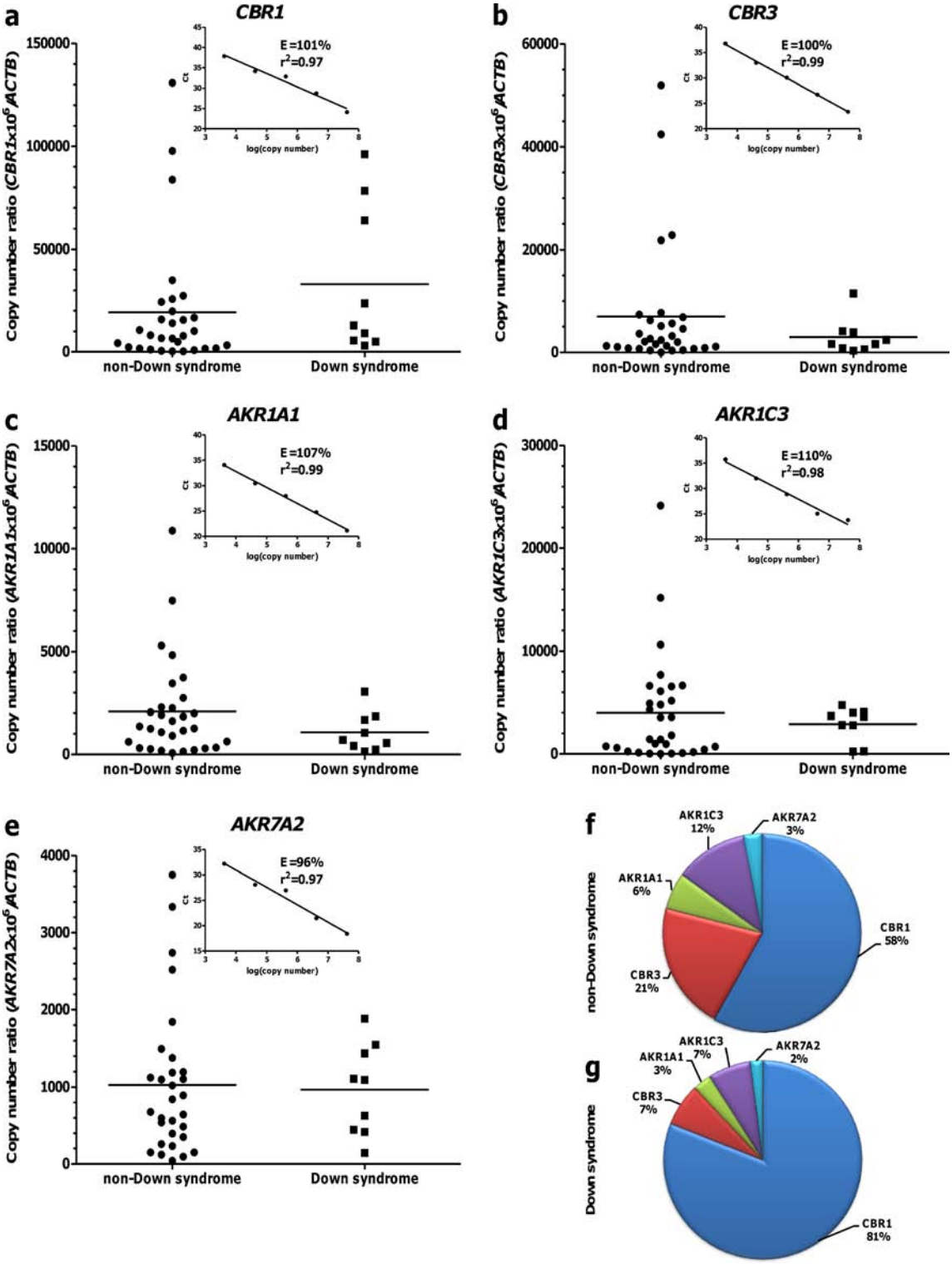

Fig. 1 Cardiac *CBR1*, *CBR3*, *AKR1A1*, *AKR1C3*, and *AKR7A2* mRNA expression in samples from donors with- ($n = 9$) and without- DS ($n = 30$) (**a**-**e**). Each symbol depicts the average of individual samples. *Horizontal lines* indicate group means. Insets show the corresponding calibration curves. Samples and standards for calibration curves were analyzed in triplicates. Relative abundance of cardiac *CBRs* and *AKRs* mRNAs in donors without- (**f**) and with- DS (**g**)

showed that 400 μM daunorubicin [S] ensured conditions of V_{max} (zero-order kinetics). Incubation mixtures (0.5 mL) contained potassium phosphate buffer (0.1 M; pH 7.4), cytosolic protein (400 μg) and daunorubicin (400 μM; Sigma-Aldrich, St. Louis, MO). Mixtures were equilibrated for 5 min at 37°C. Reactions were initiated by the addition of NADPH (200 μM; Sigma). Reaction mixtures were flash frozen with liquid nitrogen after 2 min, and stored at -80°C

until daunorubicinol quantification. Daunorubicinol was quantified using a high performance liquid chromatography (UPLC) fluorescent detection method (28). The metabolite was extracted, and then quantified using a daunorubicinol standard (Toronto Research Chemicals, Toronto, ON) as previously described (29).

CBR1 Genotyping

The *CBR1* polymorphism (rs9024) was investigated by allelic discrimination with fluorescent probes and real-time PCR (Assays-by-Design; Applied Biosystems, Foster City, CA) as described previously (24,30). Rs9024 genotype status in DS samples (i.e., trisomy 21) was determined with a validated allelic discrimination assay as described (21).

Data Analysis

Statistical comparisons were performed using Excel 2007 (Microsoft Office; Microsoft, Redmond, WA) and GraphPad Prism version 4.03 (GraphPad Software Inc., La Jolla, CA). The Kolmogorov–Smirnov test was used to analyze the normality of data sets. The Man-Whitney *U* test or Student's *t* test were used to compare group means. Pearson's coefficient of correlation (r_p) was used to analyze data sets with normal distributions. Comparison tests were considered significant at the $P<0.05$.

Multiple linear regression models were fit using the R programming language (http://www.r-project.org/). Multiple linear regression analysis was restricted to non-DS hearts due to sample size limitations. For the regression modeling, the observations that have a missing value in at least one of the protein expression measurements were eliminated from the data set. CBR3 was not considered for the regression modeling due to the number of missing values. In the union of missing data for the remaining proteins: CBR1, AKR1A1, AKR1C3, and AKR7A2, there were 12 observations with missing values for at least one of the measured proteins. These observations were eliminated for model fitting, and the remaining subset of 18 observations (9 males and 9 females), was used for the regression. The full model was represented as:

$$\text{Daunorubicin Activity} \sim \beta_0 + \beta_1 \times \text{Sex} + \beta_2 \times \text{CBR1} + \beta_3 \times \text{AKR1A1} + \beta_4 \times \text{AKR1C3} + \beta_5 \times \text{AKR7A2} + \varepsilon,$$

where β_j for j = 0, 1, …, 5 represent the regression coefficients for the predictors in the model, β_0 is the intercept, and $\varepsilon \sim N(0,1)$ is an error term. CBR1, AKR1A1, AKR1C3, and AKR7A2 represent the normalized protein concentration (nmol/gram cytosolic protein) in heart. The term Sex was represented as a binary factor.

RESULTS

CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 mRNA Expression in Hearts from Donors With and Without DS

In all cases, DS status from medical records (i.e., trisomy 21) was confirmed by aCGH. Cardiac *CBR1*, *CBR3*, *AKR1A1*, *AKR1C3* and *AKR7A2* mRNA levels in donors with and without DS are shown in Fig. 1a–e. Analysis of the average relative expression levels showed that *CBR1* mRNA is the most abundant transcript in heart samples from donors with (81%) and without DS (58%. Fig. 1f and g). On average, the remaining transcripts showed similar trends in their relative expression levels in samples from donors with (*CBR3* ≈ *AKR1C3* > *AKR1A1* > *AKR7A2*. Fig. 1g) and without DS (*CBR3* > *AKR1C3* > *AKR1A1* > *AKR7A2*. Fig. 1f). The cardiac expression of *CBR1*, *CBR3*, *AKR1A1*, *AKR1C3* and *AKR7A2* mRNA displayed considerable interindividual variability in samples from donors with and without DS (Table II). The cardiac expression of *CBRs* and *AKRs* mRNAs did not significantly differ between samples from donors with and without DS (Table II).

Table II Cardiac *CBR1*, *CBR3*, *AKR1A1*, *AKR1C3*, and *AKR7A2* mRNA Expression in Samples from Donors Without and With DS

Transcript	Non-DS		DS		*P* value
	Copy number ratio		Copy number ratio		
	Mean ± SD	Range	Mean ± SD	Range	
CBR1	19283.8 ±30824.3	245.5-130786.4	33029.3 ± 36187.0	3103.1-96061.0	0.23
CBR3	6975.3 ± 12271.4	0.4-51914.3	2992.8 ± 3465.6	334.5-11487.5	0.46
AKR1A1	2078.4 ± 2396.1	66.0-10865.2	1073.6 ± 955.5	144.2-3048.4	0.42
AKR1C3	3986.8 ± 5264.9	14.3-24131.8	2913.3 ± 1631.6	227.0-4732.8	0.69
AKR7A2	1026.1 ± 955.7	42.3-3747.9	965.8 ± 590.6	143.9-1883.0	0.98

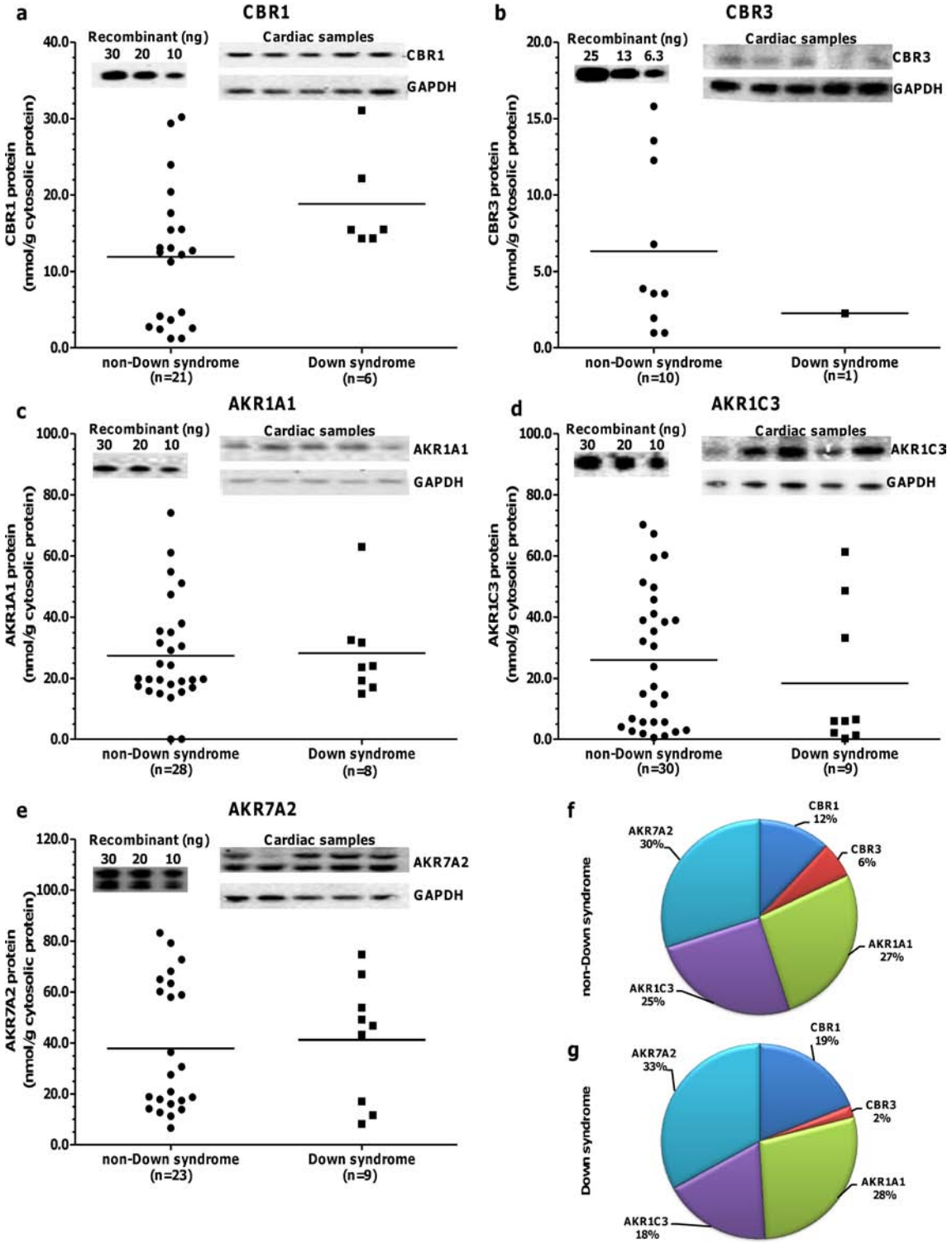

Fig. 2 Cardiac CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 protein expression in samples from donors with and without DS. Each *symbol* depicts the average of individual samples. *Horizontal lines* indicate group means. Samples and standards for calibration curves were analyzed in duplicates for CBR3 and in triplicates for CBR1, AKR1A1, AKR1C3, AKR7A2. Samples exhibiting protein levels below the LOQs were excluded (Supplementary Material Fig. 1). Insets show representative immunoblots for recombinant standards (*left*) and cytosolic CBRs-AKRs plus GADPH (*right*). Relative abundance of cardiac CBRs and AKRs proteins in donors without (**f**) and with DS (**g**)

Table III Cardiac CBR1, CBR3, AKR1A1, AKR1C3, and AKR7A2 Protein Expression in Samples From Donors Without and With DS

Transcript	Non-DS		DS		*P* value
	nmol/g cytosolic protein		nmol/g cytosolic protein		
	Mean ± SD	Range	Mean ± SD	Range	
CBR1	11.9 ± 8.9	1.2–30.2	18.8 ± 6.7	14.3–31.1	0.04
CBR3	6.3 ± 5.5	1.0–15.8	2.3	N/A	N/A
AKR1A1	27.4 ± 17.3	0.0–74.1	28.3 ± 15.4	15.0–63.0	0.86
AKR1C3	26.0 ± 22.4	0.5–70.3	18.4 ± 23.2	0.3–61.4	0.32
AKR7A2	31.0 ± 21.0	5.4–68.1	33.8 ± 19.6	6.8–61.2	0.97

N/A. not applicable ($n = 1$)

CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 Protein Expression in Hearts From Donors With and Without DS

Cardiac CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 protein expression levels in samples from donors with and without DS are shown in Fig. 2a–e. Eight out of 9 heart samples from donors with DS exhibited CBR3 protein levels below the LOQ (Supplementary Material Fig. 2). AKR7A2 showed the highest average expression levels in hearts from both groups, accounting for 33% of total CBRs-AKRs in donors with DS and 30% of total CBRs-AKRs in donors without DS. On average, the pattern of relative cardiac expression was similar in samples from donors with (AKR7A2 > AKR1A1 > AKR1C3 > CBR1 > CBR3. Fig. 2g) and without DS (AKR7A2 > AKR1A1 ≈ AKR1C3 > CBR1 > CBR3. Fig. 2f). Table III shows cardiac CBRs and AKRs protein levels in samples from donors with and without DS. The average cardiac expression of CBR1 was 57% higher in samples from donors with DS than in samples from donors without DS (Fig. 2 and Table III). The expression levels of AKR1A1, AKR1C3 and AKR7A2 did not differ between samples from donors with and without DS (Table III).

Maximal Daunorubicin Reductase Activity in Hearts From Donors With and Without DS

Figure 3a shows maximal daunorubicin reductase activities in hearts from donors with and without DS. Variable levels of daunorubicin reductase activity were observed in cardiac

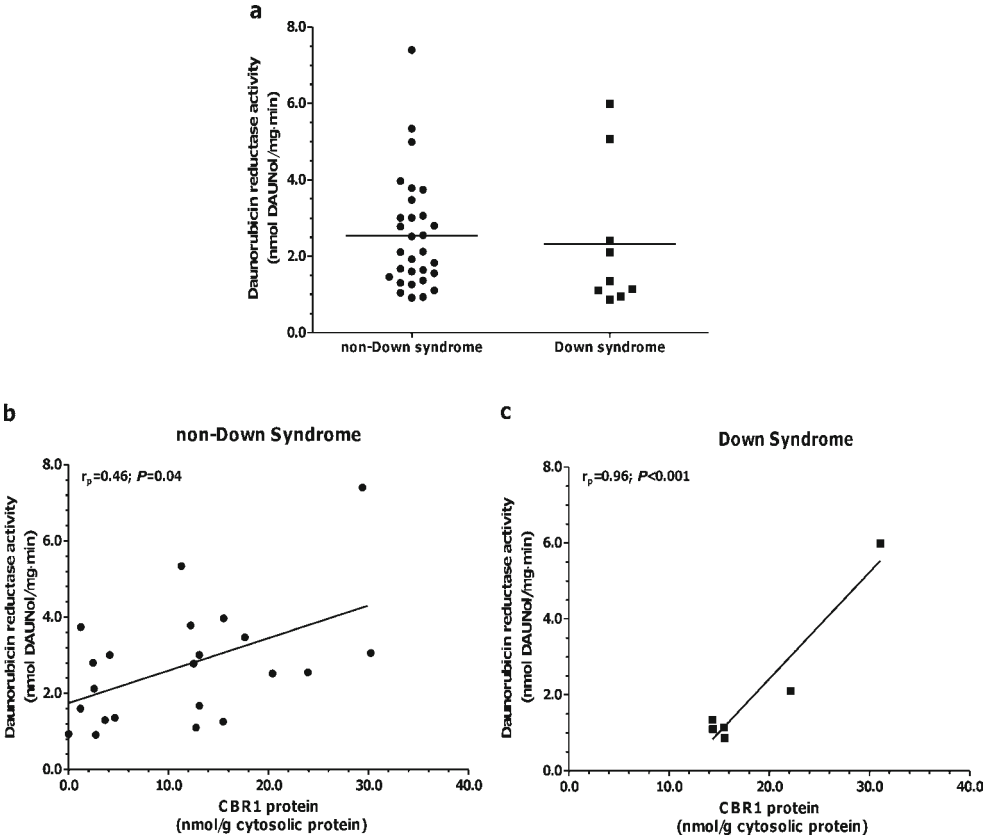

Fig. 3 Daunorubicin reductase activity in donors with ($n = 9$) and without DS ($n = 30$) (**a**). Each *symbol* depicts the average of individual heart samples. Each sample was analyzed in duplicates. Group means are indicated by *horizontal lines*. Linear regression analysis of maximal daunorubicin reductase activity *versus* cardiac CBR1 protein expression in samples from donors without and with DS (**b** and **c**)

samples from donors with DS (range: 0.9–6.0 nmol/mg·min; 7-fold) and without DS (range: 0.9–7.4 nmol/mg·min; 8-fold. Fig. 3a). Statistical comparisons of the means demonstrated that cardiac maximal daunorubicin reductase activities were similar between both groups ($Activity_{non\text{-}DS} = 2.5 \pm 1.5$ nmol/mg·min *vs*. $Activity_{DS} = 2.3 \pm 1.9$ nmol/mg·min, $P = 0.28$. Fig. 3a). Linear regression analysis was used to test for associations between CBRs-AKRs protein levels and cardiac maximal daunorubicin reductase activities (Table IV). There existed a significant positive association between CBR1 protein levels and daunorubicin reductase activity in samples from donors with and without DS (Table IV and Fig. 3b and c).

Our collection included three samples from pediatric donors with DS (Supplementary Material Table III). Age-related changes in the cardiac expression of CBRs/AKRs during the developmental continuum encompassing birth and adolescence may impact the metabolism of anthracycline substrates in pediatric patients. Exploratory analyses revealed no differences in the cardiac expression of *CBRs/AKRs* (mRNA, protein and daunorubicin reductase activity) in samples from pediatric donors in comparison to samples from non-pediatric donors (Supplementary Material Table III).

Multiple linear regression analysis was used to examine the impact of cardiac CBRs/AKRs protein levels on daunorubicin reductase activities in samples from donors without- DS. The results (Supplementary Material Table IV) indicate Sex is the most significant factor ($P < 0.001$), followed by AKR7A2, CBR1, and AKR1A1 ($P < 0.05$). AKR1C3 was not significant and removed from the model in an effort to identify the most parsimonious model of daunorubicin reductase activity (Table V). As expected, the removal of this term did not change the overall model, and the terms that were significant in the full model remained significant in the reduced model (Table V).

CBR1 rs9024 Genotype-Phenotype Associations in Heart Samples From Donors With and Without DS

Tables VI and VII show cardiac *CBR1* expression in samples from donors with and without DS stratified by *CBR1* rs9024 genotype status. No statistical comparisons are shown for donors with DS due to sample size limitations. Visual inspection of the data shown in Fig. 4 suggests the absence of an evident association between rs9024 genotype status and cardiac *CBR1* mRNA and protein expression in samples from donors with DS (Fig. 4a and b). Similarly, visual inspection does not suggest any association between *CBR1* rs9024 genotype status and maximal cardiac daunorubicin reductase activity in samples from donors with DS (Fig. 4c). The analysis of donors without DS showed that, on average, relative *CBR1* mRNA levels tend to be higher in samples with *CBR1* rs9024 homozygous G/G genotype compared to samples with homozygous A/A genotype; the difference between genotype groups did not reach statistical significance (Table VII and Fig. 5a). Further analysis showed a trend towards higher CBR1 protein levels (≈4-fold) in samples from donors with *CBR1* rs9024 homozygous G/G genotype in comparison to samples with homozygous A/A genotype (Table VII and Fig. 5b). In line, heart cytosols from donors with homozygous *CBR1* rs9024 G/G genotypes displayed higher maximal rates of daunorubicinol synthesis (≈1.6-fold) than cytosols from donors with homozygous A/A genotype (Table VII and Fig. 5c).

Table IV Linear Regression Analysis of Cardiac Daunorubicin Reductase Activities *Versus* CBRs/AKRs Protein Levels in Samples From Donors Without and With DS

Protein	Non-DS		DS	
	Pearson correlation coefficient	*P* value	Pearson correlation coefficient	*P* value
CBR1	0.46	0.04	0.96	0.002
CBR3	0.20	0.58	N/A	N/A
AKR1A1	0.00	0.99	−0.03	0.95
AKR1C3	−0.16	0.41	0.32	0.41
AKR7A2	0.28	0.19	−0.22	0.58

N/A. not applicable ($n = 1$)

DISCUSSION

There is a paucity of reports describing the expression of prominent drug metabolizing enzymes in tissues from subjects with DS. Within the context of multi-agent chemotherapy that includes anthracyclines for the treatment of pediatric leukemia, there is still considerable uncertainty about the optimal dose requirements for patients with DS (31). We propose that variability in the cardiac expression of specific CBRs and AKRs may contribute to the unpredictable pharmacodynamics of anthracycline drugs. The goal of this study was to document the expression of CBR1, CBR3, AKR1A1, AKR1C3 and AKR7A2 in a collection of heart samples from donors with and without DS.

Table V Results of the Reduced Multiple Regression Model Fit

Term	β estimate	Std. error	t-stat	*P* value	Sig. codes
Intercept	−2.527	1.023	−2.471	0.028	**
Sex	2.560	0.571	4.481	0.001	****
CBR1	2.380	1.001	2.377	0.033	**
AKR1A1	1.739	0.592	2.939	0.012	**
AKR7A2	1.242	0.357	3.479	0.004	***

The significant codes are as follows: **** for $P < 0.001$, *** for $P < 0.01$, ** $P < 0.05$, * $P < 0.1$. The r^2 is 0.73

Table VI Cardiac *CBR1* mRNA Expression, CBR1 Protein Expression and Daunorubicin Reductase Activity in Hearts from Donors With DS stratified by *CBR1* rs9024 Genotype Status

CBR1 rs9024 genotype (n)	*CBR1* mRNA copy number ratio Mean ± SD	CBR1 protein (nmol/g cytosolic protein) Mean ± SD	Daunorubicin reductase activity (nmol daunol/mg. min) Mean ± SD
AAA (2)	36423.3 ± 38775.3	18.8 ± 4.7	1.5 ± 0.9
AGG (3)	59954.5 ± 9322.3	31.1	4.5 ± 1.9
GGG (4)	11138.5 ± 47942.6	14.7 ± 0.7	1.1 ± 0.2

CBR1, *CBR3*, *AKR1A1*, *AKR1C3* and *AKR7A2* mRNAs are expressed at variable levels in heart samples from donors with and without DS (Fig. 1 and Table II). Of all the transcripts, *CBR3* mRNA exhibited the widest range of expression in samples from donors with (34-fold) and without DS (>1.3×10^5 fold). The cardiac expression of *CBR1* and *CBR3* mRNAs are of particular interest due to the location of the *CBR1* and *CBR3* genes in chromosome 21, and the expected≈1.5-fold increase in gene expression due to "gene dosage effect" in tissue samples with trisomy 21. Studies on transcriptional profiles in the DS setting suggest that the gene-dosage effect for most of the chromosome 21 transcripts (≈70%) is compensated by natural variation in gene expression (32). The compensation effect appears to be dependent upon the cellular context. For example, the expression of *CBR1* and *CBR3* mRNAs is increased in trisomic fibroblasts (≈1.5-fold) but not in trisomic lymphoblastoid cell lines (33). In this study, the expression of *CBR3* mRNA was similar in samples from donors with and without DS. Thus, it appears that natural interindividual variability in *CBR3* mRNA expression compensates the expected gene dosage effect in trisomic heart tissues. In contrast, *CBR1* mRNA expression tended to be higher (1.7-fold) in samples from donors with DS than in samples from donors without DS (Fig. 1 and Table II). Trisomic heart samples also showed a concomitant≈1.6-fold increase in CBR1 protein expression (Fig. 2 and Table III). These findings are in agreement with our previous observation documenting increased cardiac *CBR1* mRNA and CBR1 protein expression in a subset of 4 samples from donors with DS (21). It is assumed that the overexpression of "dosage-sensitive" genes from chromosome 21 is involved in specific DS phenotypes (e.g., cognitive impairments, facial dysmorphisms, and congenital heart disease (34). In this context, our data suggest that *CBR1* overexpression in the cardiac tissue from donors with DS may contribute to phenotypic abnormalities in trisomic hearts.

At the protein level, AKR7A2 was the most abundant anthracycline reductase in hearts from donors with and without DS, representing≈30% of the total reductase content (Fig. 2). In non-DS hearts, linear regression analysis of protein content *versus* maximal enzymatic activity for the substrate daunorubicin suggests that AKR7A2 content accounts for≈ 8% of the total variance in cardiac daunorubicin reductase activity (Table IV). A similar analysis indicates that cardiac CBR1 protein content accounts for≈21% of the variance in daunorubicin reductase activity in samples from donors without DS. The contribution of CBR1 expression to daunorubicin reductase activity in hearts from donors with DS appears to be substantial. In this case, the regression analysis suggests that CBR1 content accounts for≈90% of the variance in cardiac daunorubicin reductase activity in hearts from donors with DS (Fig. 3 and Table IV). These findings suggest that CBR1 protein expression is an important predictor of daunorubicinol synthesis in human heart.

CBR1 rs9024 genotype status has been associated with the hepatic expression of *CBR1* and anthracycline reductase activity for the substrate doxorubicin. In this study, the impact of *CBR1* rs9024 genotype status on cardiac *CBR1* expression was apparent in the group of heart samples from donors without- DS (Fig. 4 and Table VII). Samples with homozygous G/G genotype exhibited higher daunorubicin reductase activity (≈60% increase) than samples with homozygosis for the A allele. At first glance, the impact of *CBR1* rs9024 on cardiac daunorubicin reductase activity may appear relatively modest. However, certain therapeutic regimens such as combination chemotherapies for pediatric acute myeloid leukemia require the administration of several doses of daunorubicin ($n > 5$). In this "chronic" context, the effect size of *CBR1* rs9024 genotype status may become more relevant in terms of favoring the intracardiac build-up of cardiotoxic daunorubicinol. It appears that *CBR1* rs9024 genotype status

Table VII Cardiac *CBR1* mRNA Expression, CBR1 Protein Expression and Daunorubicin Reductase Activity in Hearts from Donors Without- DS Stratified by *CBR1* rs9024 Genotype Status

CBR1 rs9024 genotype (n)	*CBR1* mRNA copy number ratio Mean ± SD	CBR1 protein (nmol/g cytosolic protein) Mean ± SD	Daunorubicin reductase activity (nmol daunol/mg. min) Mean ± SD
AA (2)	2719.1 ± 2206.0	3.1 ± 0.8	1.7 ± 0.6
AG (6)	29148.5 ± 50373.5	11.4 ± 6.4	2.3 ± 0.9
GG (21)	18726.8 ± 25996.4	13.2 ± 9.6	2.7 ± 1.7

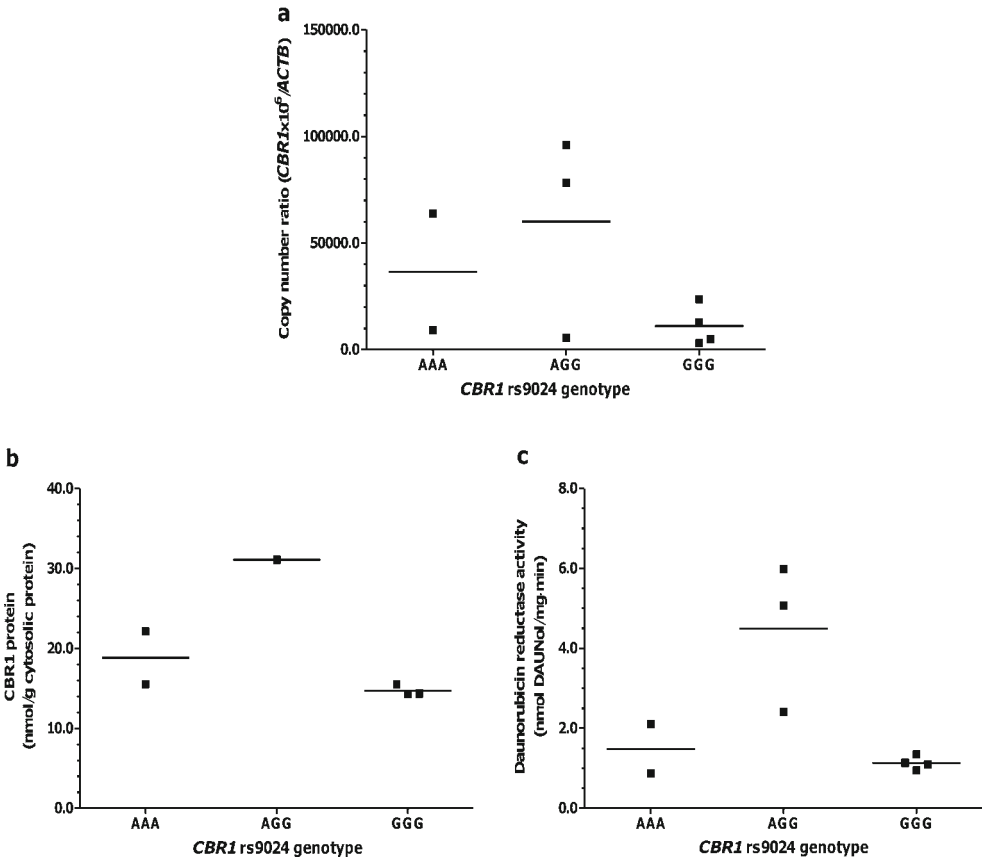

Fig. 4 Impact of *CBR1* rs9024 genotype status on: cardiac *CBR1* expression (**a**), cardiac CBR1 protein expression (**b**), and daunorubicin reductase activity (**c**) in donors with DS. Each *symbol* represents individual heart samples. *Horizontal lines* indicate group means

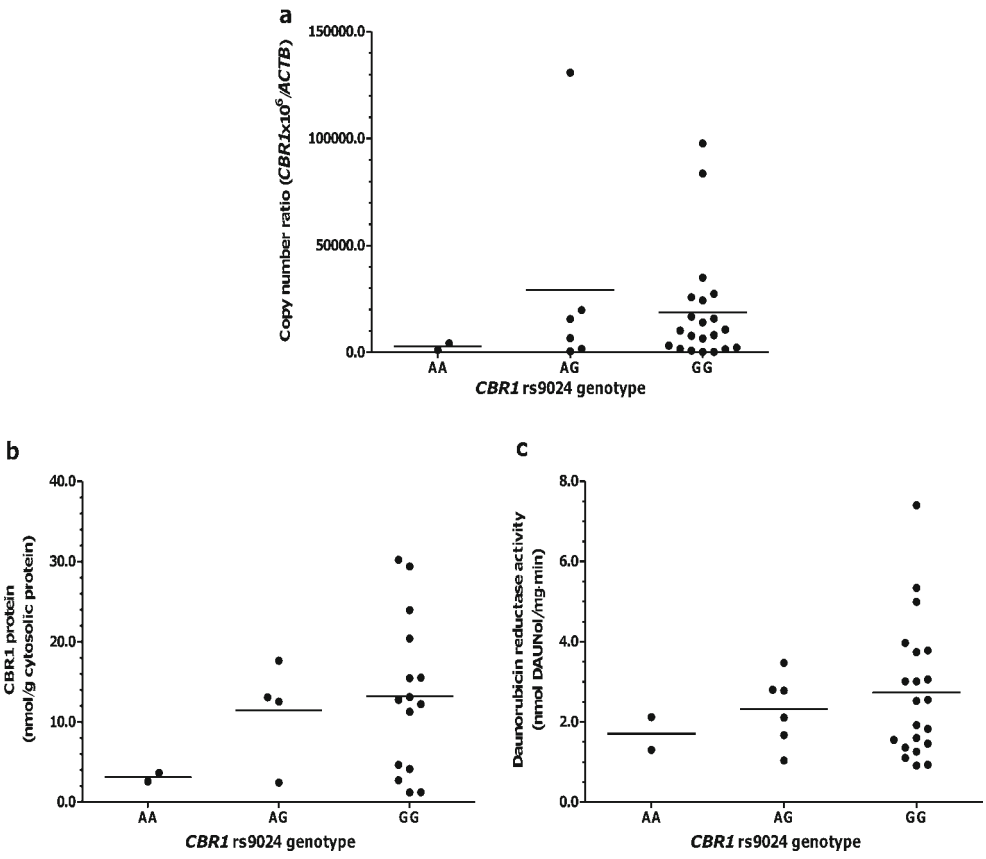

Fig. 5 Impact of *CBR1* rs9024 genotype status on: cardiac *CBR1* expression (**a**), cardiac CBR1 protein expression (**b**), and daunorubicin reductase activity (**c**) in donors without- DS. Each *symbol* represents individual heart samples. *Horizontal lines* indicate group means

does not impact the cardiac expression of *CBR1* in heart samples from donors with DS. A recent study described regulation of *CBR1* expression by microRNAs (hsa-miR-574-5p and hsa-miR-921) dependent on the allele status of the *CBR1* rs9024 (25). New evidence suggests that the expression of specific microRNAs is dysregulated in lymphocytes from subjects with DS (35). Thus, it is plausible that alterations in the cardiac expression of hsa-miR-574-5p and/or hsa-miR-921 in the DS setting would in turn disrupt the regulation of *CBR1* dependent upon rs9024 genotype status. Our laboratory is exploring this intriguing possibility by examining the expression of hsa-miR-574-5p and hsa-miR-921 in hearts and cell lines from donors with and without DS.

There are some limitations in this study, the main one being the relatively small number of cardiac samples from donors with DS. Our procurement rates for samples from donors with DS is low (≈ 1 sample every 9–12 months), even after working with national cooperative resources such as NDRI, CHTN, and the NICHD Brain and Tissue Bank. The scarcity of representative tissue samples from donors with DS continues to impair the execution of large-scale quantitative studies to tackle fundamental topics related to the metabolism and disposition of commonly used drugs in this group of subjects (36,37). Second, studies based on the use of tissue samples from cadaveric donors are prone to many issues including, but not limited to sample degradation, incomplete/null medical histories and/or demographics, use of concomitant medications/drugs/smoking status. Nevertheless, the collection of samples that is the object of this study may still represent an informative window to assess the extent of variability in the cardiac expression of anthracycline reductases in subjects with and without DS.

Weiss highlighted the need for performing comprehensive cardiac drug metabolism studies to characterize the system response at the organ level during pharmacotherapy with common drugs (e.g., anthracyclines) (38). In this study, multiple regression analysis suggests that sex together with CBR1, AKR1A1, and AKR7A2 protein expression account for≈ 70% of the total variance in cardiac daunorubicin reductase activity. Thus, the expression of specific CBRs and AKRs appears to drive the cardiac synthesis of daunorubicinol. Future modeling efforts should be directed towards the identification of distinct cardiac CBR/AKR expression profiles that dictate low and/or high rates of synthesis of cardiotoxic C-13 anthracycline alcohol metabolites. The present study represents a necessary step for the creation of novel quantitative methods to predict the variable pharmacodynamics of anthracyclines in human heart by integrating *genetic* and *phenotypic* data (e.g., CBR/AKR protein abundance, rs9024, miRNA levels, DS status, etc.) for the specific CBRs and AKRs involved in the metabolism of these clinically useful drugs.

ACKNOWLEDGMENTS AND DISCLOSURES

Adolfo Quiñones-Lombraña and Daniel Ferguson contributed equally to this manuscript. This work was supported by the National Institute of General Medical Sciences [GM073646].

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