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## Metabolism of Doxorubicin to the Cardiotoxic Metabolite Doxorubicinol Is Increased in a Mouse Model of Chronic Glutathione Deficiency: A Potential Role for Carbonyl Reductase 3

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### Abstract

Doxorubicin is highly effective at inducing DNA double-strand breaks in rapidly dividing cells, which has led to it being a widely used cancer chemotherapeutic. However, clinical administration of doxorubicin is limited by off-target cardiotoxicity, which is thought to be mediated by doxorubicinol, the primary alcohol metabolite of doxorubicin. Carbonyl reductase 1 (CBR1), a well-characterized monomeric enzyme present at high basal levels in the liver, is known to exhibit activity toward doxorubicin. Little is known about a closely related enzyme, carbonyl reductase 3 (CBR3), which is present in the liver at low basal levels but is highly inducible by the transcription factor Nrf2. Genetic polymorphisms in *CBR3*, but not *CBR1*, are associated with differential cardiac outcomes in doxorubicin treated pediatric patients. *Cbr3* mRNA and CBR3 protein are highly expressed in the livers of *Gclm*<sup>-/-</sup> mice (a mouse model of glutathione deficiency) relative to wild type mice. In the present study, we first investigated the ability of CBR3 to metabolize doxorubicin. Incubations of doxorubicin and purified recombinant murine CBR3 (mCBR3) were analyzed for doxorubicinol formation using HPLC, revealing for the first time that doxorubicin is a substrate of mCBR3. Moreover, hepatocytes from *Gclm*<sup>-/-</sup> mice produced more doxorubicinol than *Gclm*<sup>+/+</sup> hepatocytes. In addition, differentiated rat myoblasts (C2C12 cells) co-cultured with primary *Gclm*<sup>-/-</sup> murine hepatocytes were more sensitive to doxorubicin-induced cytostasis/cytotoxicity than incubations with *Gclm*<sup>+/+</sup> hepatocytes. Our results indicate a potentially important role for CBR3 in doxorubicin-induced cardiotoxicity. Because there is likely to be variability in hepatic CBR3 activity in humans (due to either genetic or epigenetic influences on its expression), these data also suggest that inhibition of CBR3 may provide protection from doxorubicinol cardiotoxicity.

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## Keywords

Doxorubicin; glutathione deficiency; pharmacogenetics; cardiotoxicity; carbonyl reductase

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## Introduction

1.1 The anthracycline doxorubicin (also known as Adriamycin) is indicated for a broad range of malignant neoplasms, including blood cancers, carcinomas, and sarcomas. Its central role in hematological cancer regimens also makes it one of the most widely prescribed chemotherapeutics in children [1,2]. Despite the general effectiveness of doxorubicin in destroying cancer cells, its use is dose-limited by off-target complications, namely cardiomyopathies [1,3]. A dose-dependent increase in cardiomyopathy risk is observed in patients; approximately 50–60% of patients given high doses of doxorubicin develop cardiomyopathies [1,4]. The risk of developing cardiomyopathy resulting from administration represents a major impediment to the continued use of doxorubicin in chemotherapy regimens. Elimination of doxorubicin-associated cardiotoxicity would thus represent a major advance in the clinical setting, especially in the context of chronic doxorubicin administration.

Doxorubicinol, an alcohol metabolite of doxorubicin, has been implicated in the cardiotoxicity observed in doxorubicin-treated patients, largely due to doxorubicinol targeting  $\text{Na}^+/\text{K}^+$  channels present in cardiac sarcolemma [5,6]. The NADPH-dependent two-electron reduction of doxorubicin to doxorubicinol is shown in Figure 1. Carbonyl reductase 1 (CBR1) has been shown to catalyze the reduction of doxorubicin to doxorubicinol [7]. However, another carbonyl reductase, CBR3, has been less well characterized [8]. Interestingly, in childhood cancer patients treated with doxorubicin, a relatively common polymorphism in the *CBR3* gene (present in ~30% of Caucasians) encodes for a non-synonymous amino acid change (V244M), which is associated with decreased risk of developing cardiomyopathy, while a polymorphism in the gene encoding *CBR1* (1096 G>A) is not associated with differential cardiomyopathy risk [3].

Furthermore, another *CBR3* variant (11 G>A) has been shown to influence the relative expression of CBR3—and subsequent doxorubicinol formation—in a cohort of Southeast Asian breast cancer patients [9]. Though the importance of specific CBR3 variants remains controversial, currently available data taken as a whole suggest an important role for this protein in doxorubicin-induced cardiotoxicity [3,9,10,11]; factors such as tissue-specific expression, polymorphisms present in other genes, patient age, duration of treatment, dosage, and co-therapies, among others, likely influence the relative role of CBR3.

While CBR1 and CBR3 share high amino acid identity (~78%) and are both NADPH-dependent, the endogenous substrate(s) and function(s) of these enzyme appear distinct, and the endogenous role of CBR3 remains unknown. We were initially drawn to CBR3 due to our lab's interest in the tripeptide glutathione (GSH), an abundant low-molecular weight antioxidant thiol within cells. GSH synthesis is rate-limited by the conjugation of glutamate to cysteine by glutamate cysteine ligase (GCL), which is composed of catalytic (GCLC) and modifier (GLCM) subunits. The level of GSH synthesized in the livers of mice lacking two

copies of *Gclm* (*Gclm*  $-/-$ ) is only ~10% relative to that of wild-type mice [12]. To compensate for low GSH levels, *Gclm*  $-/-$  mice have up-regulated a number of genes, especially those involved in antioxidant defense. *Cbr3* mRNA is the most highly up-regulated gene in the livers of *Gclm*  $-/-$  mice. On average, primary transcripts of *Cbr3* are increased approximately 10-fold relative to *Gclm*  $+/+$  mice, a trend mirrored in another model of thiol insufficiency—conditional hepatic knockout of *Txnrd1* [13,14]. This is especially relevant in the context of doxorubicin metabolism, given the liver's critical role in xenobiotic biotransformation and detoxification.

While we are currently working to identify the endogenous substrate(s) of CBR3, which currently remain unknown, we present here evidence that doxorubicin is an exogenous substrate of mouse CBR3, a previously undocumented finding. We demonstrate a significantly higher rate of doxorubicinol formation in doxorubicin-treated *Gclm*  $-/-$  mouse hepatocytes relative to *Gclm*  $+/+$  mouse hepatocytes, and also show that differentiated rat myoblasts (C2C12 cells) co-cultured with primary *Gclm*  $-/-$  mouse hepatocytes are more sensitive to doxorubicin-induced changes in cell growth and/or viability relative to those co-cultured with *Gclm*  $+/+$  mouse hepatocytes. Our findings are important for the ongoing elucidation of mechanisms of doxorubicin-induced cardiotoxicity, and, given the central role of doxorubicin use in chemotherapy regimens, our results may prove clinically relevant upon further investigation.

## Materials and Methods

### 2.1 Chemicals

All reagents for cell culture and biochemical analyses were purchased from Life Technologies (Carlsbad, CA) and/or Sigma-Aldrich (Saint Louis, MO), unless otherwise noted.

### 2.2 Purification of recombinant murine CBR3

The *Cbr3* expression plasmid was made in two steps. First, the complete open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using a RIKEN cDNA clone as template and primers that placed a Nde1 site overlapping the AUG start codon. The PCR product was blunt end-ligated into Sma1-cut pBluescript II KS (Stratagene California, La Jolla, CA). The Nde1 site and a downstream *Hind3* site in the polylinker were then used to move the ORF into a similarly cleaved pET28a expression vector (EMD Millipore, Billerica, MA). The plasmid was shuttled to BL21, and a 500 ml culture was induced by addition of 1 mM isopropyl thiogalactopyranoside when it reached an A600 of 0.6. Six hours later, cells were collected by centrifugation, and disrupted by sonication (five one-minute bursts, on ice) in 20 ml of extraction buffer (300 mM NaCl, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7), containing 20 mM imidazole and protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Lysate was clarified by centrifugation (15 min at 15,000 rpm, SS-34 rotor) and mixed on a rotating wheel with a 1 ml slurry of Talon resin (Clontech Laboratories, Inc., Mountain View, CA). After collecting the resin by centrifugation and washing twice with 50 ml extraction buffer containing 20 mM imidazole, the resin was transferred in small volume of washing buffer to a nickel mini-column, and protein was

eluted in extraction buffer containing 150 mM imidazole. The first milliliter of eluate contained the highest concentration of recombinant protein and was used for gel and enzymatic analyses.

### 2.3 Animals

All procedures for animal use were in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals and were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). Female *Gclm*  $-/-$  mice were derived by homologous recombination techniques in mouse embryonic stem (ES) cells, as previously documented [12]. Mice were anesthetized with 0.02 ml/g of a mixture of ketamine and xylazine prior to liver perfusion and/or liver excision.

### 2.4 Liver excision and homogenization

Livers were excised from anesthetized mice and placed in TES/SB buffer (20 mM Tris, 1 mM ethylenediaminetetraacetic acid, 250 mM sucrose, 20 mM sodium borate, 2 mM serine) with protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) on ice, and homogenized. Homogenates were then centrifuged at 9000  $\times$  g for 20 minutes at 4°C; the resulting supernatant was extracted and prepared for dialysis.

### 2.5 Primary murine hepatocyte isolation

Mouse livers were perfused and hepatocytes isolated by cannulating the hepatic portal vein and perfusing with Hanks' Balanced Salt Solution (HBSS) containing 0.5 mM ethylene glycol tetraacetic acid (EGTA) for 4 minutes, after which livers were perfused with 15 mg TH Research Grade Liberase (Roche, Madison, WI) per 100 ml perfusion medium (William's E medium supplemented with 2 mM L-glutamine, 10 mM HEPES (pH 7.55), and 5 ug/ml ITS+ Premix (BD Biosciences, Bedford, MA)) for 6 minutes. Digested liver lobes were immediately placed in William's E Medium (supplemented with 2 mM L-glutamine, 5 ug/ml ITS+ Premix, 100 U penicillin and 100 ug/ml streptomycin, and 25 mM dexamethasone; WECM), on ice and then centrifuged at 130  $\times$  g for 5 minutes at 4 °C. Following centrifugation, enriched cell pellets were resuspended in Hepatozyme medium (Life Technologies) supplemented with 2 mM L-glutamine, 100 U penicillin and 100 ug/ml streptomycin. Hepatocyte yields were assessed by trypan blue exclusion using a hemacytometer. A typical perfusion yielded approximately 20–30 million hepatocytes per liver.

### 2.6 Cell culture

All cells were cultured in 5% CO<sub>2</sub>/95% air in a humidified incubator at 37 °C .

**2.6.1 Primary hepatocytes**—Murine hepatocytes were seeded on collagen (25 ug/well)-coated six well plates (Corning Inc., Corning, NY) at a density of ~500,000 cells/well. After 4 hours incubation at 37 °C to allow for attachment, fresh WECM was added to each well

**2.6.2 C2C12 culture and differentiation**—Undifferentiated mouse C2C12 myoblasts were cultured in 75 cm<sup>2</sup> flasks at 37 °C for 24 hours using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 IU penicillin plus

100 µg/ml streptomycin . C2C12 cells were then trypsinized and reseeded onto 6-well Transwell Permeable Supports (0.4 µm polyester membrane; Corning Inc., Corning, NY), at a density of 75,000 cells/well, at which time medium was switched to DMEM supplemented with 2% horse serum and 100 U penicillin plus 100 µg/ml streptomycin (differentiation medium) to allow for differentiation. Differentiation medium was replaced every 24 hours for 3 days.

## 2.7 Treatment and co-culture of primary mouse hepatocytes with C2C12 cells

After four hours incubation at 37 °C in Hepatozyme to allow for attachment, primary mouse hepatocytes were given 2 ml of fresh WECM, and treated with 0 (vehicle control), 2, 5, or 10 µM doxorubicin hydrochloride (dissolved in dimethyl sulfoxide (DMSO) vehicle; volume of DMSO did not exceed 0.2% for any treatment). Concomitantly, transwell inserts containing differentiated C2C12 cells were placed in wells containing hepatocytes cultured on the bottom of the wells. Medium within each insert was replaced with fresh differentiation medium (1.5 ml/insert). Underlying doxorubicin-treated hepatocytes and overlying C2C12 cells were co-cultured for 18 hours following treatment, after which time metabolic and toxicological endpoints were assessed.

## 2.8 High pressure liquid chromatography

HPLC analyses for hepatocyte lysates, culture media, and liver homogenate incubations were performed using a Shimadzu LC6A reverse-phase HPLC, with an RFIOAxI fluorescence detector and a C-R5A integrator (Kyoto, Japan). Samples were loaded in 50 mM monobasic potassium phosphate buffer (pH 2.5) and eluted under isocratic conditions, with the mobile phase containing 27% acetonitrile, 73% 50 mM monobasic potassium phosphate (pH 2.5) and a flow rate of 1 ml/minute through a 150-mm Synergi 5u Hydro – RP 80A column (Phenomenex, Torrance, CA). HPLC analysis of purified mCBR3 incubations was performed on a Waters 2690 Separations Module, with a Waters 474 Scanning Fluorescence Detector (Waters Corporation, Milford, MA). A slightly modified gradient to more effectively separate doxorubicin and doxorubicinol peaks was employed—22% Acetonitrile, 78% in 50 mM monobasic potassium phosphate (pH 2.5) and a flow rate of 1 ml/minute through a 150-mm Synergi 5u Hydro – RP 80A column (Phenomenex, Torrance, CA). Excitation and emission wavelengths were 470 nm and 590 nm, respectively, and Waters Millenium Software V2.0 was used for data processing (Waters Corporation, Milford, MA).

### 2.8.1 Detection of doxorubicinol in media and primary mouse hepatocytes—

Following 18 hours doxorubicin treatment, 5% 5-sulfosalicylic acid (SSA) was added to culture media in microfuge tubes, on ice, for 10 min and then centrifuged at 14,000 x g for 2 minutes at room temperature. Resulting supernatants were analyzed by HPLC. For hepatocytes, cells were washed with phosphate-buffered saline and trypsinized for 15 min at 37 °C using 0.25% trypsin/2.21 mM EDTA in HBSS (Cellgro, Corning Inc., Corning, NY). They were then removed from the dishes by scraping, triturated 3–5 times and transferred to microfuge tubes. Next, hepatocytes were centrifuged for five minutes at 130 x g, 4°C, resuspended in TES/SB and sonicated on ice. Cell lysates were centrifuged for 2 minutes at 14,000 x g (4°C) and the resulting supernatant was added to 5% SSA, incubated on ice for

15 minutes, and centrifuged again at 14,000 x g (room temperature) for 2 minutes. Resulting supernatants (pH=2.5) were used for HPLC analyses.

**2.8.2 Detection of doxorubicinol formation in liver homogenates**—Aliquots of liver cytosolic fractions prepared by centrifugation of liver homogenates at 9000 x g (S9 fraction) from female *Gclm* +/+ and *Gclm* -/- mice were placed in 3.0 mL dialysis cassettes (Thermo Scientific, Waltham, MA) overnight in a solution of 100 mM monobasic potassium phosphate, pH 7.4. The following day, 20 µl of the dialyzed liver S9 fraction (approximately 50–70 mg/ml concentration) were transferred to microfuge tubes containing 15 µl of 10 mM nicotinamide adenine dinucleotide phosphate (NADPH) in 100 mM monobasic potassium phosphate (pH 7.4), 1.5 µl of 1 mg/ml doxorubicin hydrochloride (in DMSO), and 113.5 µl of 100 mM monobasic potassium phosphate buffer (pH 7.4). Samples were briefly vortexed, then placed on a rocker in the dark at 37 °C, and allowed to incubate for one hour. Next, 50 µl of 5% SSA was added to each sample, on ice, for 15 minutes, and samples were centrifuged at 14,000 x g for 2 minutes. Resulting supernatants were used for HPLC analysis.

**2.8.3 Detection of doxorubicinol formation using purified recombinant murine CBR3**—Incubations with purified recombinant mouse CBR3 (mCBR3), followed the same protocol as incubations with liver homogenates. Briefly, 1 µl of 55 mM mCbr3, 1 µl of 1 mg/ml doxorubicin, 15 µl of 10 mM NADPH, and 133 µl 100 mM monobasic potassium phosphate buffer (pH 7.4) were briefly vortexed, then placed on a rocker in the dark at 37 °C, and allowed to incubate for one hour. Next, 50 µl of 5% SSA was added to each sample, on ice, for 15 min, and samples were centrifuged at 14,000 X g for 2 minutes. Resulting supernatants were used for HPLC analysis. For control experiments, the same protocol was followed, with the additional step of boiling mCBR3 for ten minutes prior to incubation with doxorubicin and NADPH.

## 2.9 Liquid Chromatography/Mass Spectrometry (LC/MS)

*Gclm* WT and KO murine liver homogenates were incubated with doxorubicin (1 µg/150 µl total incubation volume) for 60 minutes at 37 °C in the dark, as described in section 2.8.2 of Methods. Following incubation and derivitization, samples were analyzed using LC/MS to confirm the presence of the doxorubicinol metabolite. Samples were run on an Agilent Technologies (Santa Clara, CA) MS QQQ Mass Spectrometer using a Poroshell SB-C18 (3.0mm x 50mm x 2.7 µ) column at 0.4 ml/minute flow rate (0.1% formic acid buffer). An internal standard of epirubicin (4'-epimer of doxorubicin) was used to differentiate between doxorubicinol and doxorubicin. The mass to charge (*m/z*) ratio for the precursor ions of doxorubicin and epirubicin were 544.2, and 546.1 for doxorubicinol (determined using standards of each compound). The *m/z* of the product ions were 361.1 and 397.1 for epirubicin and doxorubicin, respectively, and 363.1 and 399.2 for doxorubicinol. Analyses were performed in triplicate for each *Gclm* genotype.

## 2.10 Assessment of C2C12 cell viability

Cellular cytostasis and/or cytotoxicity of C2C12 cells following 18 hour co-culture with doxorubicin-treated primary mouse hepatocytes was determined spectrophotometrically by



measuring the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as previously described [15].

### 2.11 Protein isolation

Total protein concentration in cell lysates from primary mouse hepatocytes and S9 liver homogenate fractions was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc., Hercules, CA), in 96-well microtiter plates, following the manufacturer's protocol.

### 2.12 Western Blotting

Western blotting was performed using standard techniques. Briefly, ~15 ug protein per sample were separated by electrophoresis using 12% Tris-Glycine mini-gels in a Novex XCell *SureLock* Mini-Cell (Life Technologies, Carlsbad, CA). Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA). Membranes were blocked with 5% milk in tris-buffered saline with Tween 20 (TBS-T) and incubated with primary antibodies raised against mouse Cbr3 (1:1000 dilution; rabbit polyclonal antisera; GFM, unpublished),  $\beta$ -actin (1:1000 dilution; Cell Signaling, Beverly, MA), and Gclm (1:50000; rabbit polyclonal antisera, as described previously [12]), followed by incubation with horseradish peroxidase-conjugated secondary rat anti-rabbit antibody (Millipore, Billerica, MA), diluted 1:10,000 in 5% milk TBS-T.

### 2.13 Statistical Analyses

Data are expressed as the mean and SEM, unless otherwise noted. Data were analyzed by one- or two-way ANOVA, followed by a Dunnett's post-hoc test (for cytotoxicity experiments), or a Student's *t*-test (for pair-wise comparisons) using a statistical software package (Prism, GraphPad, Inc., San Diego, CA). Differences yielding a *p* value of less than 0.05 were considered statistically significant.

## Results

### 3.1 Carbonyl reductase 3 levels in the livers of Gclm $-/-$ and Gclm $+/+$ mice

Western blotting of primary hepatocyte lysates and dialyzed liver homogenates (S9 fraction) confirmed that CBR3 protein is highly up-regulated in the livers of *Gclm*  $-/-$  mice relative to *Gclm*  $+/+$  mice (Figure 2).

### 3.2 Assessment of the ability of mCBR3 to metabolize doxorubicin to doxorubicinol

We report here that doxorubicin is a substrate of murine CBR3. Incubation of purified mCBR3 with doxorubicin and NADPH, revealed the formation of doxorubicinol with HPLC analysis, at the expected elution time of approximately 10 minutes (Figure 3).

### 3.3 Doxorubicinol formation in primary mouse hepatocytes and liver homogenates

Primary *Gclm*  $-/-$  mouse hepatocytes treated for 18 hours with 2, 5, or 10  $\mu$ M doxorubicin had significantly higher levels (~2.5- to 3.5-fold) of both cellular and extracellular doxorubicinol than *Gclm*  $+/+$  mouse hepatocytes, as measured by HPLC (Figure 4;  $n=12$ , *p*

< 0.001 for all doses). A graded dose-response of doxorubicinol formation was observed with increasing doxorubicin treatment (Figure 4). Additionally, dialyzed liver S9 homogenates (cytosolic + microsomal fraction) from *Gclm*<sup>-/-</sup> mice produced approximately 2.5-fold more doxorubicinol than S9 fractions from *Gclm*<sup>+/+</sup> homogenates (Figure 5; n=4, *p* < 0.05).

### 3.4 LC/MS Analysis of doxorubicinol formation in liver homogenates

Analysis of doxorubicinol formation using mass spectrometry confirmed the identity of the putative doxorubicinol peak observed in HPLC analyses. Additionally, following the trend observed in HPLC analyses, 2- to 3-fold more doxorubicinol was formed in incubations containing *Gclm*<sup>-/-</sup> liver homogenate relative to *Gclm*<sup>+/+</sup> liver homogenate (Figure 6).

### 3.5 Co-culture of differentiated mouse myoblasts with doxorubicin-treated primary murine hepatocytes

C2C12 cells differentiated on transwell inserts were placed on top of primary *Gclm*<sup>-/-</sup> or *Gclm*<sup>+/+</sup> mouse hepatocytes and then treated with doxorubicin to assess the effect of hepatocyte genotype on the production of compound(s) affecting myocyte viability, an *in vitro* proxy of liver-mediated metabolism resulting in cardiotoxicity. Significantly decreased C2C12 cell viability and/or cell growth (as measured by the MTT assay) was observed in C2C12 cells co-cultured with primary *Gclm*<sup>-/-</sup> hepatocytes relative to those co-cultured with primary *Gclm*<sup>+/+</sup> hepatocytes (Figure 7; n=6 per dose and genotype, *p* < 0.001 by two-way ANOVA and Dunnet's post-hoc analysis).

## Discussion

4. 1 In this study, we investigated the potential role of a poorly characterized enzyme, mouse CBR3, in doxorubicin metabolism. Preliminary epidemiological data implicate human CBR3 polymorphisms as being important in predicting doxorubicinol formation [3,9,16,17]. Because doxorubicinol is believed to contribute significantly to cardiotoxicity in cancer patients given doxorubicin as part of chemotherapy regimens, further investigation into the role of CBR3 in doxorubicin metabolism was warranted [6].

We first tested the ability of purified recombinant mCBR3 to metabolize doxorubicin. Our results indicate that murine CBR3 is indeed able to convert doxorubicin to doxorubicinol, a previously undocumented finding. The Nrf2-regulated CBR3 enzyme [18] is highly up-regulated in *Gclm*<sup>-/-</sup> mice (a model of glutathione deficiency) relative to *Gclm*<sup>+/+</sup> mice. Accordingly, we evaluated the ability of primary mouse hepatocytes and liver homogenates from *Gclm*<sup>-/-</sup> and *Gclm*<sup>+/+</sup> mice to produce doxorubicinol. *Gclm*<sup>-/-</sup> hepatocytes produced significantly higher levels of doxorubicinol following 18 hours of doxorubicin treatment in a dose-dependent fashion, relative to *Gclm*<sup>+/+</sup> hepatocytes. Additionally, dialyzed liver homogenates from *Gclm*<sup>-/-</sup> mice produced significantly higher amounts of doxorubicinol following incubation with doxorubicin.

Following these observations, we expanded our focus to a physiologically-relevant *in vitro* system to model cardiotoxicity induced by doxorubicin treatment. We used differentiated C2C12 cells (a murine myoblast cell line) as a surrogate for cardiomyocytes, and co-



cultured them with primary murine hepatocytes from *Gclm*  $-/-$  and *Gclm*  $+/+$  mice. As anticipated, a greater impact on C2C12 cell viability/numbers was observed in co-cultures with doxorubicin-treated *Gclm*  $-/-$  mouse hepatocytes relative to cultures with *Gclm*  $+/+$  mouse hepatocytes. Moreover, there was an inverse relationship between doxorubicin concentration and cell numbers and/or viability in these *in vitro* cell culture studies.

Because of their enhanced CBR3 expression, *Gclm*  $-/-$  mice are likely to exhibit increased sensitivity to doxorubicin-induced cardiomyopathy, relative to *Gclm*  $+/+$  mice, a hypothesis that we are currently in the process of investigating with *in vivo* studies..

Ultimately, the overarching goal of this research is to reduce doxorubicin-induced cardiomyopathy to a level at which doxorubicin administration is not dose-limited by off-target cardiotoxicity. Doxorubicin continues to be and will likely remain a widely used chemotherapeutic central to numerous chemotherapy regimens [1]. Despite the discovery and use of targeted, less toxic therapies for specific cancer types (e.g., Trastuzumab), little progress has been made in reducing off-target cardiotoxicity induced by anthracyclines since their clinical introduction nearly 40 years ago [1,19]. Our findings not only implicate CBR3 in doxorubicin metabolism, but underscore the potential importance of CBR3 inhibition in the context of doxorubicin-containing therapies. Finally, if validated in appropriate pre-clinical models, such inhibition would represent a novel advancement in the treatment of a wide variety of cancers.

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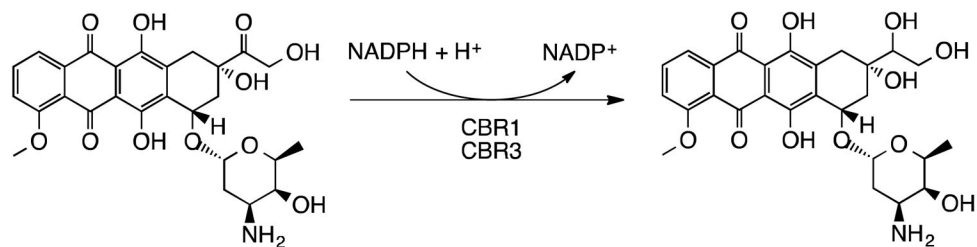
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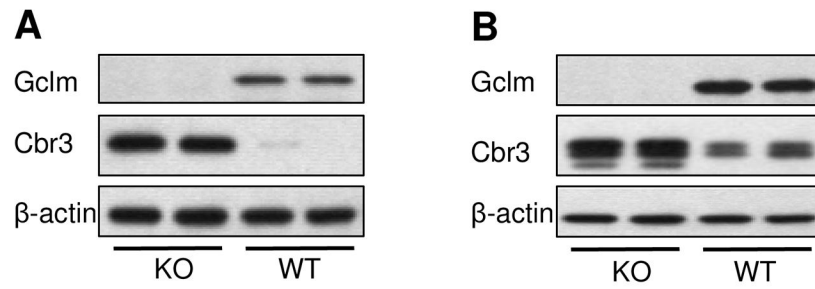
**Highlights**

- Doxorubicin is a substrate of murine Cbr3
- *Gclm*  $-/-$  hepatocytes express higher levels of Cbr3 than *Gclm*  $+/+$  hepatocytes
- More doxorubicinol is made by *Gclm*  $-/-$  hepatocytes than *Gclm*  $+/+$  hepatocytes
- *Gclm*  $-/-$  hepatocytes sensitize co-cultured C2C12 cells to doxorubicin toxicity

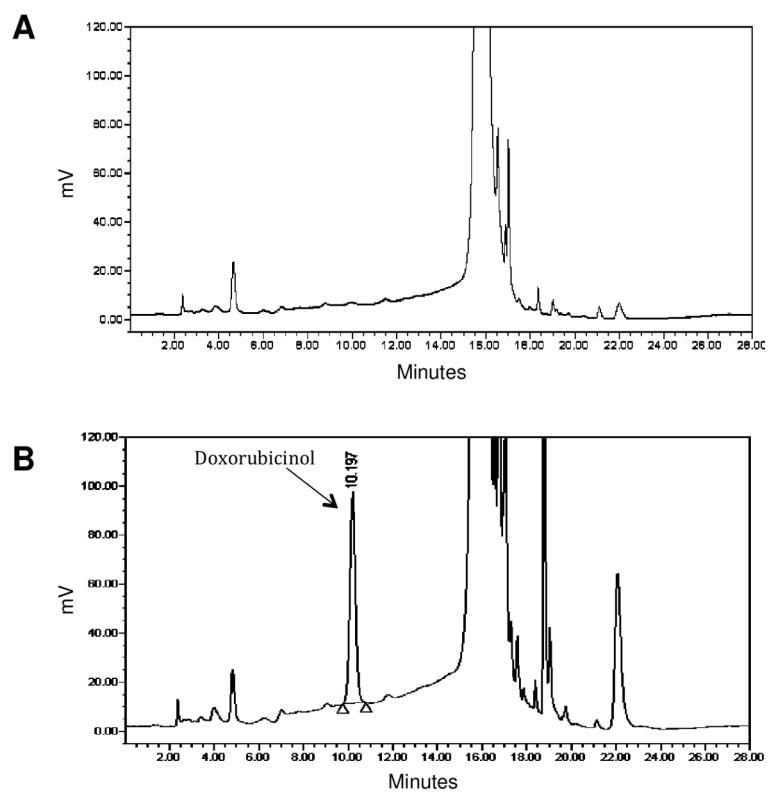


**Figure 1.**

Two-electron reduction of doxorubicin to the putative cardiotoxic alcohol metabolite, doxorubicinol, at the 13<sup>th</sup> carbon. NADPH-dependent monomeric carbonyl reductase CBR1 is known to mediate this reaction. Here, we demonstrate that this reaction is also carried out by a protein that shares high sequence identity with Cbr1, carbonyl reductase 3.

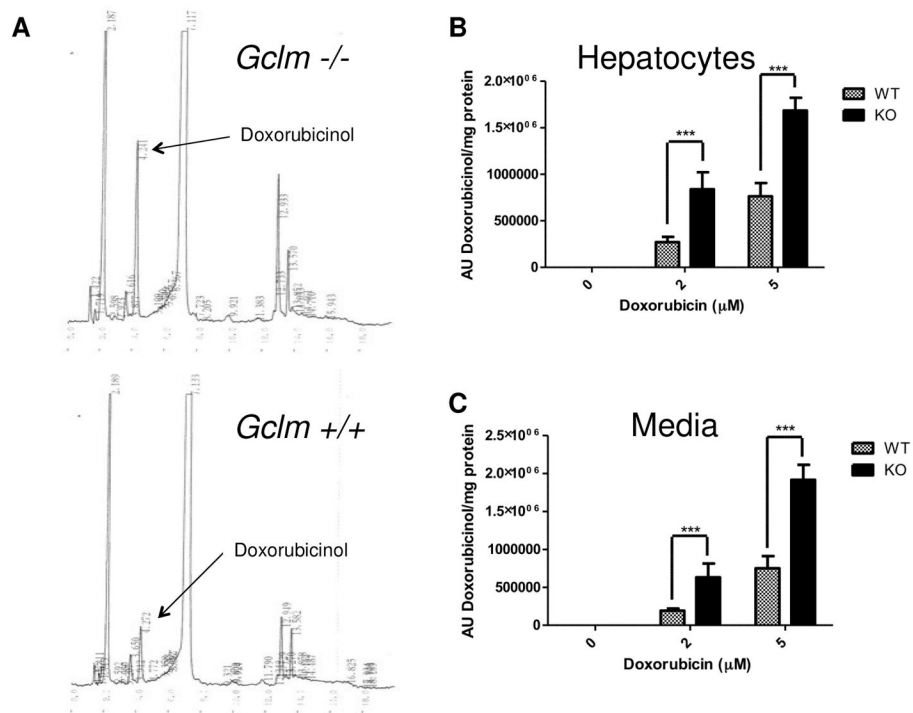


**Figure 2.** Representative Western blot analyses of GCLM, CBR3, and  $\beta$ -actin (loading control) proteins in (A) primary murine hepatocytes and (B) dialyzed liver homogenates from *Gclm*<sup>-/-</sup> and *Gclm*<sup>+/+</sup> mice.

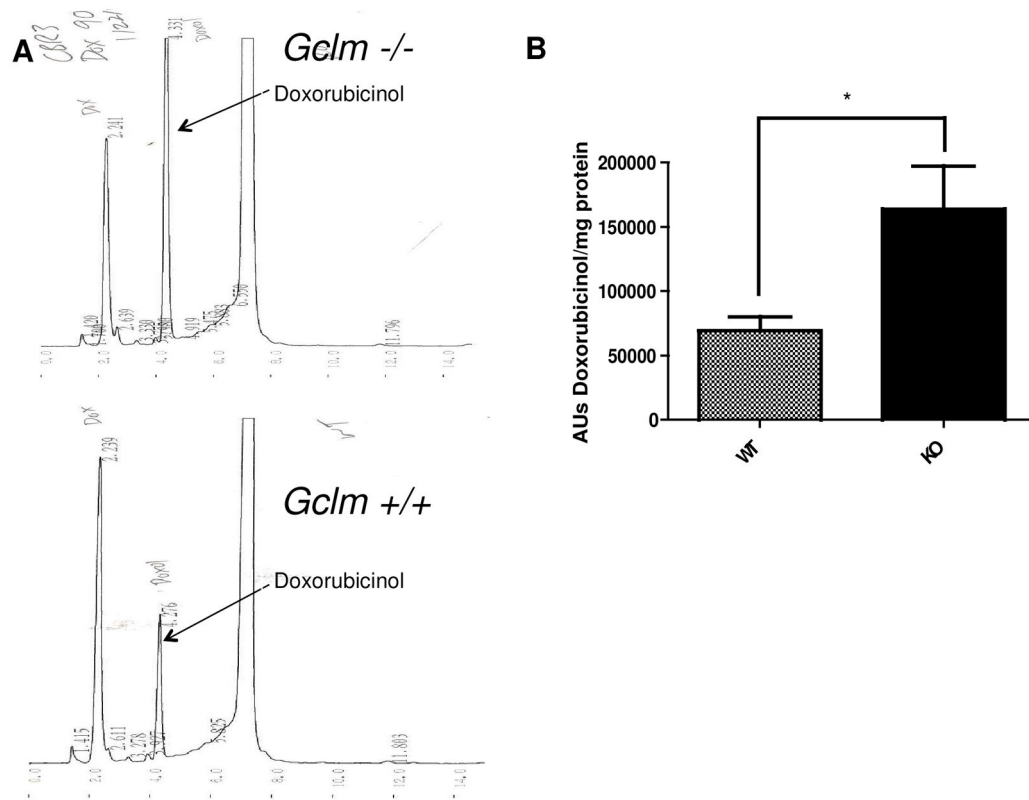


**Figure 3.** HPLC chromatograms showing doxorubicinol formation with (A) catalytically inactive and (B) catalytically active purified mCBR3. Doxorubicinol eluted at ~10 minutes, while doxorubicin eluted at ~15 minutes.

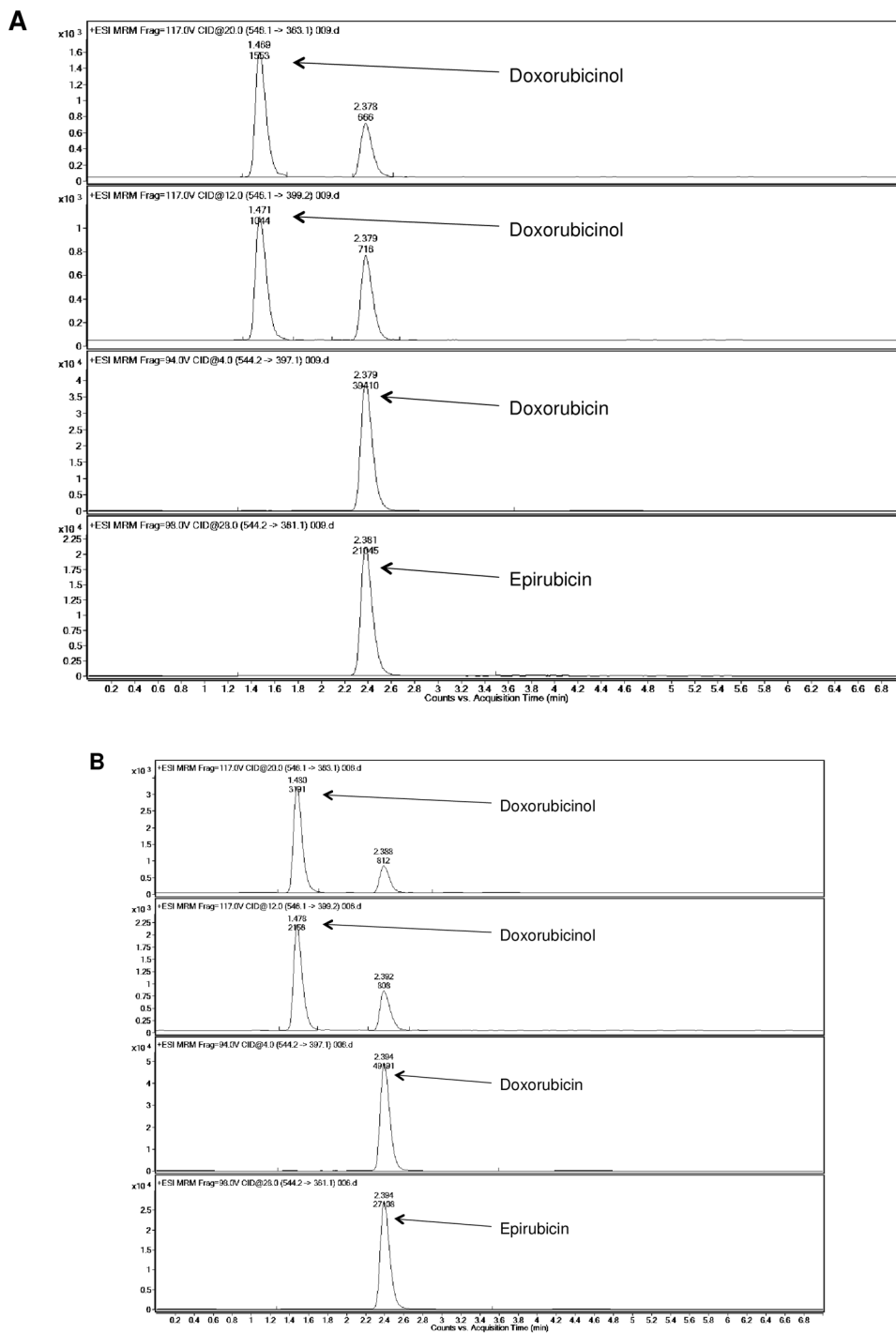




**Figure 4.** Doxorubicinol formation in *Gclm* <sup>-/-</sup> and *Gclm* <sup>+/+</sup> hepatocytes. (A) Representative HPLC chromatograms of doxorubicinol formation in *Gclm* <sup>-/-</sup> (top panel) and *Gclm* <sup>+/+</sup> (bottom panel) hepatocytes. (B) Quantification by HPLC of doxorubicinol formation (area units, AU) in hepatocytes and (C) media taken from doxorubicin treatments; n=12 for each treatment. \*\*\* indicates  $p < 0.001$  for Student's *t*-test relative to wild-type for each dose.



**Figure 5.** Doxorubicinol formation by *Gclm*  $-/-$  and *Gclm*  $+/+$  liver homogenates. (A) Representative HPLC chromatograms of doxorubicinol formation in *Gclm*  $-/-$  (top panel) and *Gclm*  $+/+$  (bottom panel) dialyzed liver homogenates. (B) Quantification by HPLC of doxorubicinol formation (area units, AU) in dialyzed liver homogenates following incubation with 50 mM doxorubicin;  $n=4$  per genotype. \*indicates  $p < 0.05$  for Student's  $t$ -test relative to wild-type.



**Figure 6.** LC/MS analysis of liver homogenate-doxorubicin incubations. Confirmation of doxorubicinol formation in (A) *Gclm* +/+ and (B) *Gclm* -/- liver homogenates. Precursor ions for doxorubicin and epirubicin had a m/z of 544.2, while the precursor ion for doxorubicinol had a m/z of 546.1. The mass-to-charge ratio of the product ions for

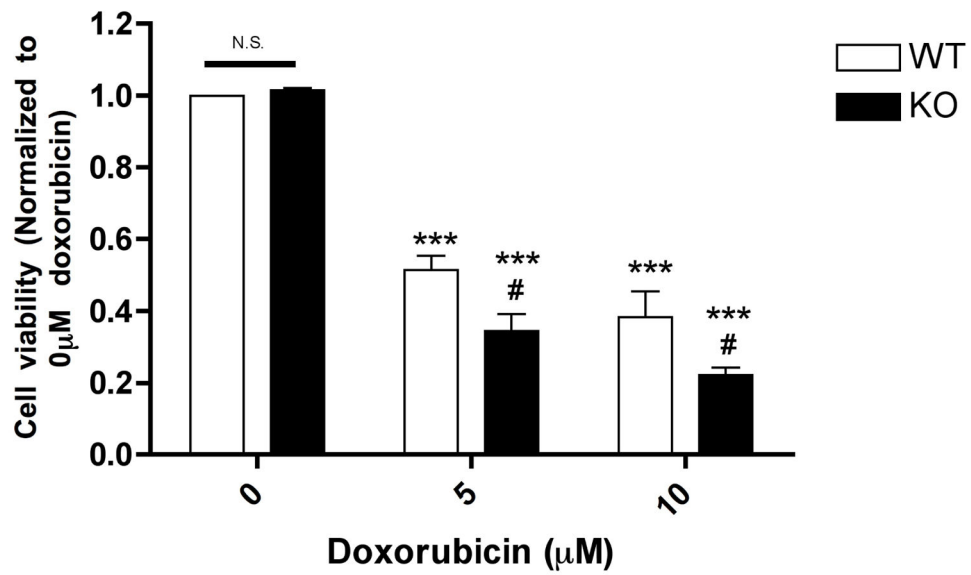
doxorubicin and epirubicin were 397.1 and 361.1, respectively, while doxorubicinol had two product ions with m/z ratios of 399.2 and 363.1.

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**Figure 7.** Cytotoxicity in C2C12 cells following co-culturing with either *Gclm*  $-/-$  or *Gclm*  $+/+$  primary hepatocytes. Two-way ANOVA analysis, \*\*\* indicates  $p < 0.001$  relative to controls, # indicates  $p < 0.001$  relative to wild-type at each; representative of two independent experiments,  $n=3$  per dose and genotype per experiment; n.s. - not significant.