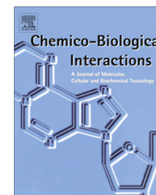
Chemico-Biological Interactions 234 (2015) 154–161

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



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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A R T I C L E I N F O

Article history:
Available online 21 November 2014

Keywords:
Doxorubicin
Glutathione deficiency
Pharmacogenetics
Cardiotoxicity
Carbonyl reductase

A B S T R A C T

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*−/− 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*−/− mice produced more doxorubicinol than *Gclm*+/+ hepatocytes. In addition, differentiated rat myoblasts (C2C12 cells) co-cultured with primary *Gclm*−/− murine hepatocytes were more sensitive to doxorubicin-induced cytostasis/cytotoxicity than incubations with *Gclm*+/+ 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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1. Introduction

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 Na^{+}/K^{+} channels present in cardiac sarcolemma [5,6]. The NADPH-dependent two-electron reduction of doxorubicin to doxorubicinol is shown in Fig. 1. Carbonyl reductase 1 (CBR1) has been shown to catalyze the reduction of doxorubicin to doxorubicinol [7]. However,

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http://dx.doi.org/10.1016/j.cbi.2014.11.010
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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 [3,9–11]; 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.

2. 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 pBluescipt 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 β-D-1-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_2HPO_4, 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,

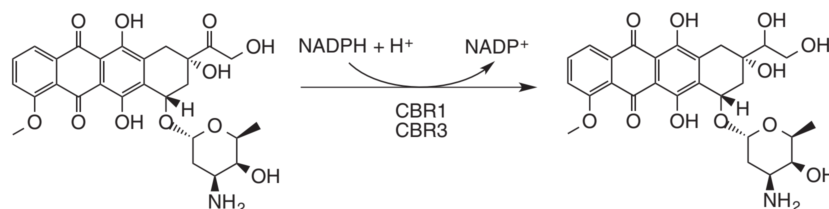

Fig. 1. Two-electron reduction of doxorubicin to the putative cardiotoxic alcohol metabolite, doxorubicinol, at the 13th carbon. NADPH-dependent monomeric carbonyl reductase CBR1 is known to mediate this reaction. Here, we demonstrate that this reaction is also carried out by a protein that shares high sequence identity with Cbr1, carbonyl reductase 3.

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