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ORIGINAL ARTICLE

# Early alterations in heart gene expression profiles associated with doxorubicin cardiotoxicity in rats

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**Abstract**
*Purpose* The antineoplastic anthracycline doxorubicin can induce a dose-dependent cardiomyopathy that limits the total cumulative dose prescribed to cancer patients. In both preclinical and clinical studies, pretreatment with dexrazoxane, an intracellular iron chelator, partially protects against anthracycline-induced cardiomyopathy. To identify potential additional cardioprotective treatment strategies, we investigated early doxorubicin-induced changes in cardiac gene expression.
*Methods* Spontaneously hypertensive male rats ($n = 47$) received weekly intravenous injections of doxorubicin (3 mg/kg) or saline 30 min after pretreatment with dexrazoxane (50 mg/kg) or saline by intraperitoneal injection. Cardiac samples were analyzed 24 h after the first ($n = 20$), second ($n = 13$), or third ($n = 14$) intravenous injection on days 1, 8, or 15 of the study, respectively.
*Results* Rats receiving three doses of doxorubicin had minimal myocardial alterations that were attenuated by dexrazoxane. Cardiac expression levels of genes associated with the Nrf2-mediated stress response were increased after a single dose of doxorubicin, but not affected by cardioprotectant pretreatment. In contrast, an early repressive effect of doxorubicin on transcript levels of genes associated with mitochondrial function was attenuated by dexrazoxane pretreatment. Dexrazoxane had little effect on gene expression by itself.
*Conclusions* Genomic analysis provided further evidence that mitochondria are the primary target of doxorubicin-induced oxidative damage that leads to cardiomyopathy and the primary site of cardioprotective action by dexrazoxane. Additional strategies that prevent the formation of oxygen radicals by doxorubicin in mitochondria may provide increased cardioprotection.

**Keywords** Doxorubicin · Anthracycline · Cardiotoxicity · Microarray · Dexrazoxane · Cardioprotection

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## Introduction

The therapeutic use of doxorubicin (DOX), a member of the anthracycline class of chemotherapeutic drugs that inhibit DNA topoisomerases, is associated with a late-onset cardiomyopathy. The cumulative dose and dosing schedule greatly influence the incidence of latent cardiac toxicity induced by DOX [1]. Each dose of DOX is thought to cause subclinical myocardial damage that can cumulatively lead

to clinical manifestations of cardiotoxicity during or after treatment is completed. Children that receive anthracycline therapy may experience reduced cardiac reserve and subsequent congestive heart failure 15–20 years after termination of treatment [1].

Experimental evidence suggests that the pathogenesis of DOX-induced cardiotoxicity involves the production of reactive oxygen species that are promoted by iron [2]. Purine nucleotide flavoproteins catalyze the metabolism of DOX into a quinone intermediate that undergoes one-electron redox cycling, reducing $O_2$ to superoxide anion and $H_2O_2$. Low-molecular-weight iron can decompose $H_2O_2$ into highly toxic hydroxyl radicals. In addition, doxorubicin can form reactive oxygen species non-enzymatically through anthracycline–iron complexes. The earliest ultrastructural damage induced by DOX is seen in mitochondria and the sarcoplasmic reticulum, which contain enzymes (NADH dehydrogenases and P450 (cytochrome) oxidoreductases) that can reduce DOX to the semiquinone [3].

The heart is thought to be more sensitive to doxorubicin toxicity as a result of the large number of mitochondria, an increased amount of NADH dehydrogenase associated with complex I in these mitochondria, the affinity of doxorubicin for the inner mitochondrial membrane phospholipid cardiolipin, and a lower peroxide detoxification capacity than that of the liver [4]. Dexrazoxane (DZR) attenuates DOX-induced injury, both experimentally and clinically, without affecting the chemotherapeutic efficacy of DOX [5]. DZR is hydrolyzed intracellularly to an open ring form (ADR-925) that is a strong chelator of iron and can remove iron from complexes with anthracyclines in vitro [5]. Combination therapy with DZR, at the recommended DZR:DOX ratios of 10:1 or 20:1, allows higher cumulative doses of DOX to be administered to patients. An intraperitoneal injection of DZR 30 min before intravenous injection of DOX reduces DOX cardiotoxicity in rats, mice, and dogs [6]. This treatment regimen attenuates damage but does not completely protect the heart.

The role of iron and reactive oxygen in doxorubicin-induced cardiotoxicity is still under debate [7]. The cardioprotective activity of dexrazoxane supports the involvement of iron but the parent drug is also a catalytic inhibitor of topoisomerase II and is more effective than other iron chelators and cytosolic free radical scavengers such as *N*-acetyl cysteine and vitamin E in attenuating doxorubicin-induced cardiomyopathy in the clinic [reviewed in 1]. Dexrazoxane has been shown to selectively block DNA damage induced by doxorubicin through the beta form of topoisomerase II expressed in differentiated tissues like heart but not through the alpha form of topoisomerase II present in cancer and other proliferating cells [8]. An analog of DZR that chelates iron but lacks topoisomerase II inhibitory activity is not protective against doxorubicin-induced cardiomyopathy in rodents [9]. These and other studies suggest that the mechanism of cardioprotection by dexrazoxane may involve more than iron chelation.

To further investigate the protective effect of DZR, we analyzed early myocardial changes using microarray technology after administering DOX alone or in combination with DZR. The studies were performed in spontaneously hypertensive rats (SHR), which have a greater sensitivity to DOX cardiotoxicity and that display myocardial alterations similar to those that are induced in patients after chronic cancer chemotherapy [10]. In the SHR model, cardiac alterations characterized by myofibrillar loss and cytoplasmic vacuolization are consistently observed after treatment with cumulative doses of 6 mg/kg or more of DOX [10]. In the present study, cardiac gene expression was analyzed 24 h after 1, 2, or 3 injections of DOX given 1 week apart (for a total dose of 3, 6, or 9 mg/kg) and before profound changes in heart histopathology or marked elevations in serum troponins appeared. Ontological analysis of gene expression identified early targets of DOX and their association with cardioprotection by DZR.

## Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Center for Drug Evaluation and Research, US Food and Drug Administration, in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Forty-seven male SHR (Charles River Breeding Laboratories, Wilmington, MA, USA), 10 weeks old, were housed in individual cages and had free access to rodent chow and water. When the rats reached 12 weeks of age, they were divided into four groups of 11–14 animals each (Table 1). Three to five rats from each group were treated once, twice, or three times with DOX and/or DZR at weekly intervals. DOX (3 mg/kg) or saline (0.1 mL/100 g bw) were injected intravenously (iv) into a tail vein 30 min following intraperitoneal (ip) administration of DZR (50 mg/kg) or saline, creating four treatment groups at each time point:

| | |
|---|---|
| Group 1 | Saline (ip) and DOX (iv) |
| Group 2 | DZR (ip) and DOX (iv) |
| Group 3 | DZR (ip) and saline (iv) |
| Group 4 | Saline (ip) and saline (iv) |

The animals were anesthetized with isoflurane and euthanized by exsanguination 24 h after the first, second, or third intravenous injection on days 1, 8, or 15 of the study, respectively. Doxorubicin and dexrazoxane (ICRF-187) were obtained from Pfizer, Inc. (Kalamazoo, MI, USA).

**Table 1** Lesion scores and serum levels of cholesterol, triglycerides and cTnT in spontaneously hypertensive rats given 3 mg/kg doxorubicin with or without 50 mg/kg dexrazoxane weekly for up to 15 days

| Day of study | Group | Rats (*n*) | Initial ip injection | IV injection 30 min later | Cholesterol (mg/dL[a]) | Triglycerides (mg/dL) | cTnT (ng/mL) | Lesion score[a] |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 5 | Saline | DOX | 51 (3) | 20 (8)[b] | ND | 0 (0) |
| | 2 | 5 | DZR | DOX | 51 (4) | 22 (4)[b] | – | 0 (0) |
| | 3 | 5 | DZR | Saline | 57 (6) | 42 (16) | – | 0 (0) |
| | 4 | 5 | Saline | Saline | 55 (4) | 42 (12) | – | 0 (0) |
| 8 | 1 | 3 | Saline | DOX | 60 (1) | 29 (2) | <0.01 | 0 (0) |
| | 2 | 3 | DZR | DOX | 63 (2) | 14 (2) | <0.01 | 0 (0) |
| | 3 | 3 | DZR | Saline | 47 (6) | 34 (5) | <0.01 | 0 (0) |
| | 4 | 4 | Saline | Saline | 54 (4) | 36 (7) | 0.06 (0.06) | 0 (0) |
| 15 | 1 | 3 | Saline | DOX | 233 (56)[b] | 288 (145)[b] | 0.11 (0.04)[b] | 1 (0) |
| | 2 | 3 | DZR | DOX | 60 (7) | 57 (18) | 0.01 (0.02) | 0 (0) |
| | 3 | 3 | DZR | Saline | 47 (5) | 68 (12) | <0.01 | 0 (0) |
| | 4 | 5 | Saline | Saline | 46 (3) | 62 (18) | 0.02 (0.04) | 0 (0) |

*ND* no data

[a] Mean (standard deviation)

[b] Significantly different from Group 4 controls, $P < 0.05$

### Cardiac troponin T assay

Terminal blood samples were collected for biochemical and hematological analysis (Analytics, Inc., Gaithersburg, MD, USA). To measure serum levels of cardiac troponin T (cTnT), whole blood samples were centrifuged, and the serum was frozen at −80°C until assayed. Serum concentrations of cTnT were measured by immunoassay (Elecsys STAT; Roche Diagnostics, Indianapolis, IN, USA) performed at Boston Children's Hospital, Boston, MA, USA.

### Histopathology

At necropsy, the entire heart was removed. A portion of the left ventricle was fixed in phosphate-buffered 10% formalin. The remaining portion (both ventricles and atria) was snap frozen for microarray analysis. For pathologic evaluation, formalin-fixed heart tissue was embedded in glycol methacrylate resin which was sectioned at a thickness of 1 μm and stained with alkaline toluidine blue. The frequency and severity of DOX-induced myocyte alterations visible by light microscopy were assessed semi-quantitatively by a pathologist (J.Z.) blinded to treatment groups. Myocyte damage was scored on a scale of 0–3 indicating the degree of myofibrillar loss and cytoplasmic vacuolization, according to the method of Billingham [10, 11]. Non-cardiac tissues (kidney, spleen, liver, lung, and small intestine) were also excised and fixed in phosphate-buffered 10% formalin. Blocks of tissue from these organs were embedded in paraffin, stained with hematoxylin–eosin, and evaluated by light microscopy.

### Microarray assays and analysis

Frozen heart tissue was thawed in RNALater (Ambion, Austin, TX, USA), randomly diced, and weighed. The tissue was homogenized using a VirTis Tempest rotor–stator homogenizer and total RNA was isolated using the Qiagen Maxi protocol for heart tissue (Valencia, CA, USA). The average yield was about 0.5–1 μg of total RNA per mg heart tissue. RNA was precipitated with $LiCl_2$, washed with 70% ethanol, and rehydrated in DEPC-treated $H_2O$. The RNA was treated with DNase (DNA-Free protocol, Ambion). RNA quality was checked on an Agilent 2100 bioanalyzer.

The cDNA was synthesized from 5 μg of total RNA per sample and used as a template for biotin-labeled cRNA synthesis. cRNA (20 μg) was fragmented and hybridized to Affymetrix RGU34A arrays (Santa Clara, CA, USA). Signal levels were estimated for each time-matched group using the probe logarithmic intensity error (PLIER) algorithm and the default settings in ArrayAssist Lite (Stratagene, La Jolla, CA, USA). All hybridizations had 3′/5′ GAPDH ratios less than 1. One hybridization that was an outlier for noise and percent present call (it was outside the upper and lower adjacent values in a box plot analysis) was removed from further analysis (one Group 2 sample from day 1). The Affymetrix microarray data for this study are available in the EBI ArrayExpress data repository (Accession No. E-MEXP-2071).

An unsupervised method for pattern identification, EPIG (Extracting microarray gene expression Patterns and Identifying co-expressed Genes) [12], was applied using default

criteria ($\log_2$ ratio magnitude > 0.5, SNR > 3, $r$-value > 0.8) to identify groups of genes that are statistically different from controls and whose responses to drug treatments are correlated at each of the three time points. Similar results were obtained using an ANOVA method (the random-variance $F$ test in BRB-ArrayTools version 3.5.0 developed by Dr. Richard Simon and Amy Peng Lam, using an alpha of 0.001). At day 1, 133 significantly changed probe sets were identified using EPIG and 126 by ANOVA, with 66% overlap (of the EPIG gene list with the ANOVA gene list). At day 8, 52 significantly changed probe sets were identified using EPIG and 43 by ANOVA with 91% overlap, and at day 15, 267 significantly changed probe sets were identified using EPIG and 457 by ANOVA with 93% overlap.

The EPIG method identified 6 profiles in the day 1 treatment group, 3 in the day 8 group, and 16 in the day 15 group. Using the $\log_2$-transformed signal ratios relative to the average control (Group 4) value, the similarity between treatment groups within a profile was evaluated with hierarchical clustering distance, using the Euclidian distance of unweighted pair-group method averages or $t$ test comparisons (alpha = 0.05), which produced similar results (data not shown). Profiles were considered DOX-selective if Group 1 (Sal/DOX) samples, but not Group 3 (DZR/Sal) samples, differed significantly from Group 4 (Sal/Sal) samples. Profiles were considered DZR-selective if Group 2 (DZR/DOX) and Group 3 (DZR/Sal) samples differed significantly from Group 1 and Group 4 samples, but not from each other. The effect of DZR treatment on doxorubicin-selective profiles was evaluated based on the hierarchical clustering of Groups 2 with Groups 1 or 3. Profiles with similar DOX or DZR selectivity at the same time point were grouped into the same cluster for functional classification. Pathway analysis on clusters of significantly changed genes was performed using Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com) and DAVID [13, 14]. Enrichment of functional annotations among clusters of genes regulated by DOX or by DZR was evaluated using DAVID, with the RGU34A gene list as background, for annotations with a significance threshold less than 0.05. Functional annotation clustering was restricted to Gene Ontology (GO) category level 4 and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classifiers.

### Reverse transcription quantitative PCR assay

The cDNA was synthesized from total RNA using Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was conducted using 100 ng cDNA and Power SYBR Master Mix (Applied Biosystems) on an ABI Prism 9700HT system (Foster City, CA, USA). ΔCts were normalized to ribosomal protein L7 (RL7) mRNA levels and calibrated to the average ΔCt for the Group 2 controls. The forward (For) and reverse (Rev) primer sequences (5′–3′) for the following gene transcripts were:

Atp2a2 (For) TGCCTTCCAACATCCATCAA, (Rev) TGTTTAGGAAGCGGTTACTCCAGT
Jph2 (For) GTAGAGGAGGTCCCCAACACC, (Rev) GGCCGATGTTCAGCAGGAT
Postn (For) ACAAGCCAACAAAAGGGTTCA, (Rev) ACGGCCTTCTCTTGATCGC
Slc2a4 (For) CCAGTATGTTGCGGATGCTATG, (Rev) ACGGCAAATAGAAGGAAGACGTA
Sqstm1 (For) GTACCCACATCTCCCACCAGAG, (Rev) GAGAGTGACTCAATCAGCCGG

### Statistical methods

Differences in myocardial lesion scores between the treatment groups were assessed with the Mann–Whitney test for nonparametric data. The data met the assumptions of the test. The Tukey–Kramer multiple comparisons test was used to assess differences between the groups in serum chemistry and cTnT values using the InStat (GraphPad Software, San Diego, CA, USA) statistical software package, with alpha set at 0.05. Statistical significance of the log transformed qRT-PCR data was analyzed using a one-way ANOVA with Tukey's post test in GraphPad Prism 3.0.

## Results

### Doxorubicin-induced toxicity

No significant change in body weight was seen in animals 24 h after a single 3 mg/kg dose of DOX. Animals in treatment groups receiving two doses of DOX gained weight. The mean (SD) increase in body weight ranged from 10 (10) g in Group 2 (DZR/DOX) to 26.7 (11.5) g in Group 3 (DZR/Sal). Weight gain for rats receiving three doses was significantly retarded in Groups 1 and 2 at day 15 compared to Groups 3 and 4 ($P < 0.05$). Mean (SD) weight gain at day 15 for Groups 1 through 4 was 7.7 (6.4) g, 9.7 (9.0) g, 38.3 (6.1) g, and 41.8 (10.3) g, respectively. No mortality was observed in any of the treatment groups.

At all 3 time points, white blood cell counts were significantly lower in the DOX-treated groups than in the saline controls (Table 2). Red blood cell counts, hematocrit, and hemoglobin concentrations were significantly lower in Groups 1 and 2 than in Group 4 controls at day 15. In contrast, at day 8, red blood cell counts were significantly higher in Group 1 than in Group 4 (Sal/Sal) controls, and

**Table 2** Changes in selected hematology values in spontaneously hypertensive rats given 3 mg/kg doxorubicin with or without dexrazoxane weekly for up to 15 days

| Day of study | Group | WBC[a] ($\times 10^3$/μL) | RBC ($\times 10^6$/μL) | Hgb (g/100 mL) | HCT (%) |
|---|---|---|---|---|---|
| 1 | 1 | 3.15 (0.72)[b] | 8.18 (0.12) | 13.45 (0.24) | 39.5 (0.6) |
| | 2 | 2.70 (0.43)[b] | 8.10 (0.38) | 13.30 (0.52) | 39.0 (1.9) |
| | 3 | 3.95 (0.75)[b] | 8.31 (0.54) | 13.58 (0.92) | 39.0 (2.7) |
| | 4 | 7.16 (1.21) | 8.51 (0.20) | 13.98 (0.37) | 40.3 (1.1) |
| 8 | 1 | 2.67 (0.38)[b] | 7.01 (0.20)[b] | 13.70 (0.60) | 40.7 (1.4) |
| | 2 | 2.03 (0.42)[b] | 6.31 (0.31) | 12.50 (0.70) | 36.8 (2.1)[b] |
| | 3 | 3.00 (0.56)[b] | 6.41 (0.04) | 13.30 (0.10) | 37.6 (0.3)[b] |
| | 4 | 5.33 (0.24 | 5.5 (0.24) | 14.30 (0.10) | 41.0 (1.1) |
| 15 | 1 | 3.03 (0.49)[b] | 6.32 (0.14)[b] | 12.60 (0.20)[b] | 37.7 (0.7)[b] |
| | 2 | 2.40 (0.23)[b] | 5.88 (0.16)[b] | 12.10 (0.20)[b] | 34.7 (0.9)[b] |
| | 3 | 4.17 (0.68) | 6.48 (0.64) | 13.10 (1.30) | 38.8 (3.7) |
| | 4 | 5.57 (1.56) | 7.4 (0.56) | 15.0 (1.20) | 44.1 (3.5) |

[a] Mean (SD) values. *WBC* white blood cells, *RBC* red blood cells, *Hgb* hemoglobin, and *HCT* hematocrit

[b] Significantly different from Group 4 controls, $P < 0.05$

the hematocrit was significantly lower than controls only in Groups 2 and 3.

Renal lesions were noted exclusively in rats treated with three doses of DOX (Group 1). Lesions included minimal degeneration of the proximal tubule epithelial cells (cytoplasmic vacuoles and hyaline droplet formation) and glomerular vacuolization (overall mean lesion severity score = 1). Serum cholesterol and triglyceride levels were significantly elevated over baseline in Group 1 at day 15, but not at earlier time points or in Group 2 (Table 1). These histopathologic and clinical chemistry results are consistent with those of earlier studies showing that DZR protects against DOX-induced nephrotic syndrome [15].

Previous studies have shown that DOX-induced myocardial lesions are characterized by cytoplasmic vacuolization and myofibrillar loss [11, 15]. In this study, minimal cardiomyocyte damage was observed in groups receiving 3 weekly injections of DOX (Fig. 1c, d). Lesions in all 3 Group 1 animals at 15 days consisted primarily of cytoplasmic vacuolization (lesion score of 1). Similar lesions or other myocardial alterations were not observed in animals pretreated with DZR before DOX administration (Fig. 1e, f) or in animals treated with saline (Fig. 1a, b). At day 15, serum levels of cTnT were slightly elevated in Group 1, but not in Groups 2 or 3 (Table 1).

## Early global changes in gene expression associated with doxorubicin

Microarray technology was used to identify early changes in cardiac gene expression induced by DOX and DZR treatments. Whole-heart samples from individual animals in each treatment group at days 1, 8, and 15 were assayed on Affymetrix RGU34A arrays. The data were analyzed using an unsupervised, profile-based method developed for extracting patterns among co-regulated genes (EPIG) [12]. Genes in profiles that responded similarly to either DOX or DZR treatment were clustered together and analyzed for enrichment in annotations for biological processes or signaling pathways.

Twenty-four hours after a single dose of doxorubicin, 133 probe sets differed significantly between treatment groups and vehicle controls. The 108 probe sets (for 105 genes) that were in profiles of responses specific to DOX treatment were grouped into 3 clusters (Fig. 2a). Cluster I contains a single EPIG profile of 17 genes up-regulated by DOX. Seven genes in this cluster are regulated by nuclear factor erythroid-derived 2-like 2 (NFE2L2, also known as Nrf2) through antioxidant response element (ARE) sites in their promoters or were identified as Nrf2-regulated in studies using knock-out mice [16, 17] (Table 3). This cluster is also enriched for genes involved in xenobiotic metabolism or associated with mitochondria (Table 3). The mitochondrial genes up-regulated by DOX at day 1 are known to be induced in response to stress through Nrf2 (*Hmox1* and *Maoa*) or HIF-1 pathways (*Hmox1* and *Hk2*) [18] and were significantly changed only at the earliest time point.

Clusters II and III contain 88 genes down-regulated by DOX treatment at day 1. About one-third of these gene changes persisted at day 15 with continued weekly dosing (Fig. 2a). Clusters II and III are enriched for genes associated with mitochondria, ion transport (e.g., SERCA2 (*Atp2a2*)), lipid metabolism, muscle contraction, and antigen processing (Table 3).

At day 8, 28 probe sets representing 24 genes were selectively up-regulated by DOX (data not shown). Most of these genes were also changed significantly at earlier or later time points and were also significantly associated with the Nrf2-regulated response to antioxidants and with other injury responses (e.g., cathepsin L, thioredoxin reductase 1). Fewer significantly regulated genes were observed at this time point, as a result of the variability within groups and the low number of animals per group.

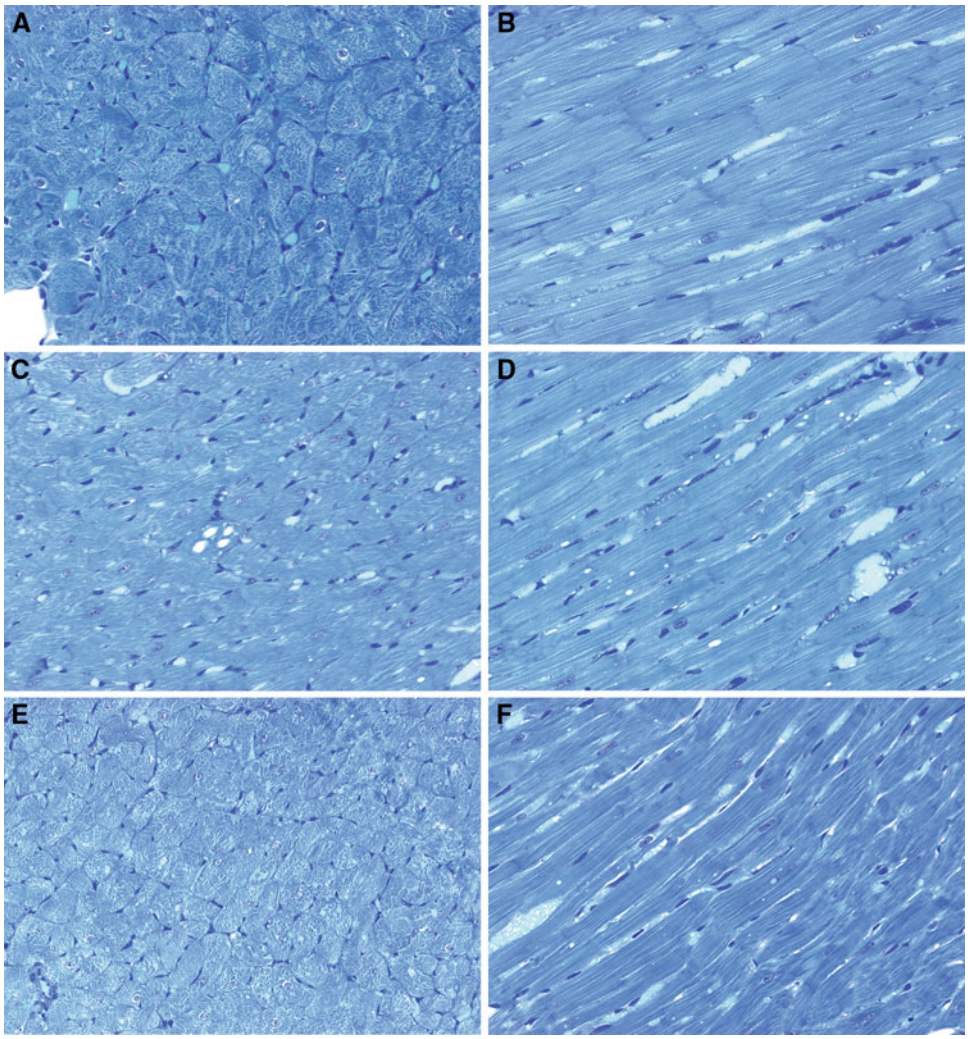


**Fig. 1** Histopathologic changes in the heart after 3 weekly injections. Representative cross-sections (**a**, **c**, **e**) and longitudinal sections (**b**, **d**, **f**) of myocardium from a Group 4 saline-treated control (**a**, **b**), a Group 1 doxorubicin-treated animal pretreated with saline (**c, d**), and a Group 2 doxorubicin-treated animal pretreated with dexrazoxane (**e, f**) (×400). Small vacuoles are observed in the myocardium of Group 1 rats, while no remarkable alterations were seen in Group 2 rats compared with Group 4 saline-treated rats on study day 15

Histopathology indices and the number of significantly changed genes in the heart were both higher at day 15 than at earlier time points. Minimal levels of myocyte vacuolation were detected in Group 1 but not Group 2 at day 15. EPIG profiles of genes were identified in heart samples that were specifically up-regulated by DOX (a total of 42 genes in Clusters I and IV) and specifically down-regulated by DOX (110 genes total in Clusters II, III, V, and VI) (Fig. 2a, b).

Genes in Cluster IV, upregulated after day 15, were enriched for annotations associated with cellular regulation of apoptosis, the inflammatory process, muscle contraction, cellular ion homeostasis, and carbohydrate metabolism (Table 4). Cluster IV includes several genes that are important in myocardial matrix remodeling after injury to the heart, such as matrix metallopeptidase 2 (*MMP2*), its endogenous inhibitor *TIMP1* [19], and the cysteine protease, cathepsin L (*Ctsl*) [20]. Transcript levels of *Ctsl* were significantly up-regulated by DOX at all three time points.

Genes down-regulated by doxorubicin treatment at day 15 were enriched for mitochondrial proteins (28 genes represented by 37 probe sets) (Tables 3, 4), including two key regulators of ATP transfer in mitochondria, adenylate kinase 1 and creatine kinase. Clusters V and VI, limited to genes down-regulated at day 15 of the study, were also enriched in genes in the KEGG pathway for glycolysis–gluconeogenesis and associated with ion homeostasis. The latter group includes the ryanodine receptor (Ryr2), a calcium-release channel in the sarcoplasmic reticulum that is downregulated in response to DOX treatment [21].

One of the patterns extracted at day 15 but not at earlier time points contained genes that were down-regulated in response to DZR treatment but not affected by DOX alone. This cluster of 58 probe sets was enriched in genes for neuroactive receptors (gamma-aminobutyric acid (GABA) receptor subunits (*Gabra3, Gabra4*), leptin receptor (*Lepr*), and thyroid stimulating hormone receptor (*Tshb*)) and includes genes for myosin, ankyrin 3, and several other cytoskeletal proteins (*Msn, Ank3, Myh3, Dlgh2, Mtap1a*).

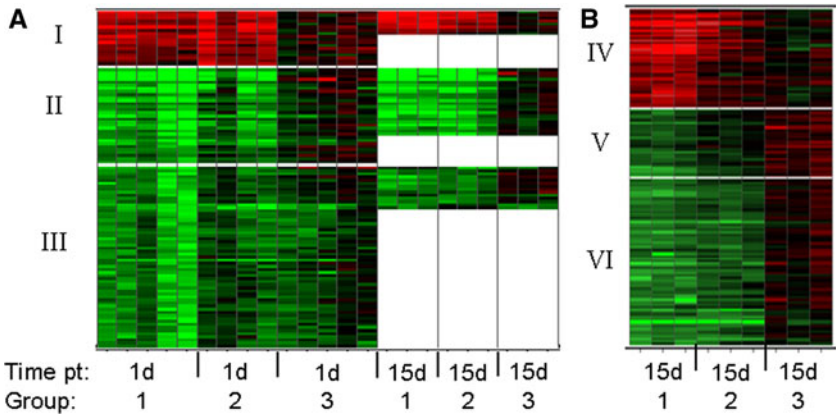


**Fig. 2** Early gene changes in the heart after a single or repeated dose of doxorubicin. EPIG profiles of significantly changed genes were clustered based on direction of change by doxorubicin and effect of dexrazoxane. **a** Clusters I–III of 108 probe sets significantly changed by doxorubicin treatment on study day 1. Cluster I was up-regulated by doxorubicin and not significantly attenuated by dexrazoxane. Clusters II and III were down-regulated by doxorubicin: III was attenuated by DZR and II was not. The 44 probe sets that continued to show regulation by doxorubicin at day 15 are also shown. **b** Clusters IV–VI of 137 genes in 7 EPIG profiles that were significantly changed on study day 15 but not study day 1. Cluster IV was up-regulated by doxorubicin and attenuated by dexrazoxane. Clusters V and VII were down-regulated by doxorubicin; Cluster V was attenuated by dexrazoxane while Cluster VII was not. The heatmap, using a $\log_2$ scale of −1 to 1 represented as *green* to *red*, displays the $\log_2$ ratios for statistically significant probe sets, calculated relative to the Group 4 average, for individual samples within each of the three non-control treatment groups at day 1 and day 15

The mechanism responsible for these observed changes is unclear, but evidence that latent iron deficiency can cause decreases in GABA and other neurotransmitters [22] suggests an indirect effect of iron chelation.

## Effect of dexrazoxane on early gene changes associated with doxorubicin

In this study, DZR reduced the incidence of cardiac damage induced by DOX that presented as myocardial lesions and elevations in serum levels of cTnT at day 15. We examined the effect of DZR on the changes in gene expression induced by DOX before there was a measurable effect on cardiotoxicity (at day 1) and at later time points. For genes in groups based on EPIG profile similarity or functional classification, transcript levels were evaluated for their responsiveness to DZR pretreatment based on hierarchical clustering distance between samples in Groups 1, 2, and 3.

Genes up-regulated by DOX at day 1 (Cluster I), which includes many Nrf2-regulated oxidative stress genes, were not significantly altered by DZR pretreatment (Fig. 2a). Cluster I genes that remained up-regulated by DOX at day 15 also continued to be largely unaffected by cardioprotectant (Fig. 2a). EPIG profiles of genes down-regulated by DOX treatment at day 1 were grouped into two clusters with different responses to DZR. Cluster II genes, which showed only partial or no response to DZR, were enriched in the overlapping ontological categories of ion transport and muscle contraction, and in lipid metabolism. These genes tended to persist in responsiveness to DOX treatment at day 15. DZR pretreatment attenuated the repression by DOX of transcript levels of genes in Cluster III, which was enriched for genes associated with mitochondria and the KEGG pathway of antigen processing and presentation. Most of the gene changes in Cluster III were transient and did not persist at day 15 of the study.

The up-regulation of genes in Cluster IV by DOX at day 15 but not at day 1, which include genes involved in apoptosis, was largely prevented by DZR pretreatment. Genes down-regulated by DOX at day 15 in Clusters V and VI differed in their response to DZR pretreatment. Cluster V, which contains 23 genes that were down-regulated by DOX but are more similar to control levels in groups pretreated with dexrazoxane, is enriched for genes involved in glycolysis-gluconeogenesis. Cluster VI, which contains 60 genes that are generally down-regulated by DOX to a similar extent with or without cardioprotectant pretreatment, is enriched for genes involved in ion homeostasis. Mitochondrial genes in both clusters demonstrated, as a group, less repression by DOX in samples from animals that also received DZR pretreatment.

## Quantitative changes in representative gene markers of doxorubicin-induced cardiac injury

To verify the transcriptional responses to DOX and DZR treatments identified in the heart using microarray technology, qRT-PCR assays were performed for several genes that are representative of different types of cellular responses to DOX-induced cardiac injury. The five selected targets were: (1) sequestosome1 (*Sqstm1*), an Nrf2-regulated

**Table 3** Genes that are statistically significantly changed after a single dose of doxorubicin and in ontological categories enriched in treatment groups used to investigate heart gene expression profiles associated with doxorubicin cardiotoxicity in the rat

| Cluster | Annotation | Gene symbols (Affymetrix Probe Set ID) | Gene names |
|---|---|---|---|
| I (up-regulated) | Nrf2-mediated oxidative stress | Ephx1 (M26125_at), **Gsta3** (K01932_f_at), Gstt2 (rc_AI138143_at), Hmox1 (rc_AI179610_at), Maoa (D00688_s_at, S45812_s_at), **Mgst1** (J03752_at), **Sqstm1** (Y08355cds#2_at) | Epoxide hydrolase, glutathione *S*-transferase alpha 1, glutathione *S*-transferase theta 2, heme oxygenase (decycling) 1, monoamine oxidase A, microsomal glutathione *S*-transferase 1, sequestosome 1 |
| | Mitochondrion | Hk2 (D26393exon_s_at), Hmox1 (rc_AI179610_at), Maoa (D00688_s_at, S45812_s_at) | Hexokinase 2, heme oxygenase (decycling) 1, monoamine oxidase A |
| | Xenobiotic metabolism signaling | Cited2 (rc_AI014091_at), **Gsta3** (K01932_f_at), Gstt2 (rc_AI138143_at), Hmox1 (rc_AI179610_at), **Mgst1** (J03752_at) | Cbp/p300-interacting transactivator, glutathione S-transferase alpha 1, glutathione S-transferase theta 2, heme oxygenase (decycling) 1, microsomal glutathione S-transferase 1 |
| II and III (down-regulated) | Mitochondrion | Acox2 (X95189_at)[a], **Ak1** (D13376_at, rc_AA799299_at), **Alas2** (D86297_at), Amid (rc_AA894233_at), Arg1 (J02720_at)[a], Cox6a1 (rc_AI104520_s_at), Cox8a (L48209_s_at), Cyp11a1 (J05156_s_at), Dci (rc_AA799541_at), Hbb (M94918mRNA_f_at)[a], **Kcnj11** (D86039_at, D86039_g_at), **MGC72973** (M94919mRNA_f_at)[a], **Slc25a20** (rc_AA800120_at), Txn2 (rc_AA799459_at), Ucp2 (AB005143_s_at), Ucp3 (AF030163_s_at) | Acyl-coenzyme A oxidase 2 branched chain, adenylate kinase 1, aminolevulinic acid synthase 2, apoptosis-inducing factor-like mitochondrion-associated inducer of death, arginase 1, cytochrome c oxidase subunit VIa polypeptide 1, cytochrome c oxidase subunit VIIIa, cytochrome P450 family 11 subfamily a polypeptide 1, dodecenoyl-coenzyme A delta isomerase, hemoglobin beta chain complex, potassium inwardly rectifying channel subfamily J member 11, beta-glo, solute carrier family 25 member 20, thioredoxin 2, uncoupling protein 2, uncoupling protein 3 |
| | Ion transport | Atp2a2 (rc_AA957510_s_at), Cngb1 (AJ000515_s_at), Gnai2 (rc_AA875225_at), **Kcnj11** (D86039_at, D86039_g_at), **Pllp** (Z49858_at), **Scn5a** (M27902_at), **Slc11a2** (AF008439_g_at), Slc12a3 (U10097_at), Slc22a1 (rc_AA799679_at), Ucp2 (AB005143_s_at), Ucp3 (AF030163_s_at) | ATPase $Ca^{++}$ transporting (cardiac muscle) slow twitch 2, cyclic nucleotide gated channel beta 1, guanine nucleotide binding protein alpha inhibiting 2, potassium inwardly rectifying channel subfamily J member 11, plasma membrane proteolipid, sodium channel voltage-gated type V alpha polypeptide, solute carrier family 11 member 2, solute carrier family 12 member 3, solute carrier family 22 member 1, uncoupling protein 2, uncoupling protein 3 |
| | Lipid metabolism | **Acox2 (X95189_at),** Dci (rc_AA799541_at), **Dgat1** (rc_AA859529_at), **Pla2g5** (U03763cds_at), Slc27a1 (U89529_at), Ucp3 (AF030163_s_at) | Acyl-coenzyme A oxidase 2 branched chain, dodecenoyl-coenzyme A delta isomerase, diacylglycerol O-acyltransferase 1, phospholipase A2 group 5, solute carrier family 27 (fatty acid transporter) member 1, uncoupling protein 3 |
| | Muscle contraction | Atp2a2 (rc_AA957510_s_at), Myh6 (rc_AI104924_f_at), **Scn5a** (M27902_at) | ATPase $Ca^{++}$ transporting (cardiac muscle) slow twitch 2, myosin heavy polypeptide (cardiac muscle) alpha, sodium channel voltage-gated type V alpha polypeptide |
| | Antigen processing and presentation | Cd74 (X14254cds_at), Hla-dmb (U31599_g_at), Rt1-n2 (L23128_g_at) | CD74 antigen, major histocompatibility complex class II DM beta, RT1 class IB gene H2-TL-like GRC region (N2) |

Gene symbols in bold indicate statistically significant regulation by doxorubicin at days 1 and 15

[a] Annotation based on literature Ref. [37]

**Table 4** Genes that are statistically significantly changed after several weekly doses of doxorubicin and in ontological categories enriched in treatment groups used to investigate heart gene expression profiles associated with doxorubicin cardiotoxicity in the rat

| Cluster | Annotation | Gene symbols (Affymetrix Probe Set ID) | Gene names |
|---|---|---|---|
| IV (up-regulated) | Regulation of apoptosis | Ccl2 (X17053mRNA_s_at), Loc498276 (M32062_at), Mmp2 (U65656_at), Nol3 (U40628_at), Ppp2ca (rc_AI012595_at), Timp1 (rc_AI169327_at, rc_AI169327_g_at) | Chemokine (C–C motif) ligand 2, Fc receptor (IgG) low affinity III, matrix metalloproteinase 2, nucleolar protein 3, protein phosphatase 2 catalytic subunit alpha isoform, tissue inhibitor of metalloproteinase 1 |
| | Inflammatory response | Ccl2 (X17053mRNA_s_at), Ccl20 (AF053312_s_at), Cd14 (AF087943_s_at), Loc498276 (M32062_at) | Chemokine (C–C motif) ligand 2, small inducible cytokine subfamily A20, CD14 antigen, Fc receptor (IgG) low affinity III |
| | Cellular ion homeostasis | Scn1a (M22253_at), Srprb (D38380_g_at) | Sodium channel voltage-gated, type 1 alpha polypeptide, transferrin |
| | Cellular carbohydrate metabolic process | Pfkp (L25387_g_at), Ppp1r2 (S79213_at), Xlkd1 (rc_AI639246_at) | Phosphofructokinase (platelet), protein phosphatase 1 regulatory subunit 2, extracellular link domain-containing 1 |
| | Muscle contraction | Kbtbd10 (rc_AI639444_g_at), Myl1 (L00088expanded_cds#2_at), Ppp1r12a (S74907_at) | Kelch repeat and BTB domain-containing 10, fast myosin alkali light chain, protein phosphatase 1 regulatory subunit 12A |
| V and VI (down-regulated) | Mitochondrion | Acsl1 (D90109_at, rc_AA893242_g_at, rc_AI044900_s_at), Alas1 (J03190_at, J03190_g_at), Atp5s (rc_AA799829_at), Bcat2 (U68417_at), Ckb (M57664_at, M57664_g_at), Cpt2 (J05470_at), Ctsd (X54467_at, rc_AI235585_s_at)[a], Dci (rc_AI170568_s_at), Dlat (rc_AA892485_at), Etfdh (rc_AI176422_at), Gpd2 (U83880UTR#1_g_at), Hmgcl (rc_AI171090_at), Hspe1 (rc_AI170613_at, rc_AI170613_g_at), Isoc2b (rc_AA891524_at), Mfn2 (U41803_at), Mrpl38 (rc_AA891689_at, rc_AA891689_g_at), Mrpl40 (rc_AA799888_at), Mtch2 (rc_AA799279_at), Ndufs8 (rc_AA799479_at), Pdhb (rc_AA892828_at), Supv3l1 (rc_AA799741_at), Tufm (rc_AA866234_at), Vars2 (M98327_at) | Acyl-CoA synthetase long-chain family member 1, aminolevulinic acid synthase 1, mitochondrial ATP synthase regulatory component factor B, branched chain aminotransferase 2 (mitochondrial), creatine kinase (brain), carnitine palmitoyltransferase 2, cathepsin D, dodecenoyl-coenzyme A delta isomerase, dihydrolipoamide S-acetyl transferase, electron-transferring-flavoprotein dehydrogenase, glycerol-3-phosphate dehydrogenase 2, 3-hydroxy-3-methylglutaryl-coenzyme A lyase, heat shock 10 kDa protein 1, isochorismatase domain-containing 2b, mitofusin 2, mitochondrial ribosomal protein L38, mitochondrial ribosomal protein L40, mitochondrial carrier homolog 2, NADH dehydrogenase Fe–S protein 8, pyruvate dehydrogenase beta, suppressor of var1 3-like 1, tu translation elongation factor (mitochondrial), valyl-tRNA synthetase 2 |
| | Glycolysis/ gluconeogenesis | Dlat (rc_AA892485_at), Eno3 (rc_AA851223_at), Pdhb (rc_AA892828_at), Pgam2 (Z17319_at) | Dihydrolipoamide S-acetyl transferase, enolase 3 beta, pyruvate dehydrogenase beta, phosphoglycerate mutase 2 |
| | Cellular ion homeostasis | Fxyd1 (rc_AA799645_g_at), Kcnh2 (Z96106_at), Mt1a (rc_AI102562_at), Ryr2 (U95157_at), Tfrc (M58040_at) | FXYD domain-containing ion transport regulator 1, potassium voltage-gated channel subfamily H member 2, metallothionein 1A, ryanodine receptor 2 (cardiac), transferrin receptor |

[a] Annotation based on literature Refs. [38, 39]

**Table 5** Relative expression levels of selected gene transcripts assayed by qRT-PCR in treatment groups used to investigate heart gene expression profiles associated with doxorubicin cardiotoxicity in the rat

| Day of study | Group | Sqstm1[a] | Slc2a4 | Atp2a2 | Jph2 | Postn |
|---|---|---|---|---|---|---|
| 1 | 1 | 0.77 (0.06)[b] | −1.48 (0.27)[b] | −0.53 (0.07)[b] | −0.69 (0.22)[b] | 0.22 (0.21) |
| | 2 | 0.80 (0.24)[b] | −1.24 (0.50)[b] | −0.26 (0.10) | −0.29 (0.17) | 0.57 (0.25)[b] |
| | 3 | 0.09 (0.23) | −0.01 (0.18) | −0.11 (0.20) | −0.10 (0.15) | −0.06 (0.22) |
| | 4 | −0.003 (0.11) | −0.03 (0.29) | −0.02 (0.25) | −0.03 (0.32) | 0.00 (0.28) |
| 8 | 1 | 1.68 (0.19)[b] | −1.56 (0.18)[b] | ND | ND | ND |
| | 2 | 1.58 (0.13)[b] | −1.22 (0.31)[b] | ND | ND | ND |
| | 3 | 0.47 (0.08) | 0.47 (0.08) | ND | ND | ND |
| | 4 | 0 (0.17) | 0 (0.17) | ND | ND | ND |
| 15 | 1 | 0.98 (0.07)[b] | −1.55 (0.08)[b] | ND | −0.71 (0.09)[b] | 1.01 (0.32)[b] |
| | 2 | 0.85 (0.04)[b] | −1.11 (0.43)[b] | ND | −0.44 (0.07) | 0.33 (0.44) |
| | 3 | 0.14 (0.15) | 0.34 (0.29) | ND | 0.16 (0.11) | −0.11 (0.07) |
| | 4 | −0.01 (0.18) | −0.03 (0.30) | ND | −0.01 (0.14) | −0.004 (0.13) |

*ND* not determined because no statistically significant difference in signal level between Groups 1 and 4 in the microarray data

[a] Mean $\log_2$ ratios (SD) normalized to each sample's RL7 ΔCt and relative to the average test gene control ΔCt for each treatment group

[b] Statistically different from Groups 3 and 4 ($P < 0.01$)

antioxidant response gene that is significantly induced at days 1, 8, and 15 and is in Cluster I; (2) Glut4 (*Slc2a4*), a glucose transporter that is representative of the adult-to-fetal isoform switch in energy use induced by cardiac stress [23] and is included in Cluster II; (3) SERCA2 (*Atp2a2*), the sarcolemmal calcium transporter that is down-regulated in cardiac hypertrophy and heart failure [24] and is also in Cluster II; (4) junctophilin2 (*Jph2*), a sarcolemma–sarcoplasmic reticulum junction protein that is down-regulated under conditions of cardiac hypertrophy [25] and found in Cluster III; and (5) periostin (*Postn*), a myocardial repair protein in Cluster IV.

At days 1, 8, and 15, *Sqstm1* and *Slc2a4* mRNA levels in Groups 1 and 2 differed significantly (up-regulated and down-regulated, respectively) from the levels in Groups 3 and 4, indicating DOX-selectivity and lack of marked attenuation by DZR pretreatment (Table 5). Gene expression levels for both of the sarcolemmal proteins assayed showed reduced response to DOX after DZR pretreatment. *Jph2* mRNA levels were statistically significantly decreased at day 1 and after repeated dosing with doxorubicin. Dexrazoxane pretreatment reduced the effect of DOX on *Jph2* expression at both days 1 and 15. The small but statistically significant decrease in *Atp2a2* mRNA levels only observed after a single DOX injection was also suppressed by DZR pretreatment.

Cardiac *Postn* transcripts levels were increased by doxorubicin and attenuated by DZR pretreatment. Periostin is an adhesion protein that is secreted by myocardial fibroblasts in response to cardiac injury [26]. At day 15, *Postn* mRNA levels increased twofold in Group 1 samples but not in Group 2 samples, in correlation with observations of myocardial damage by histopathology.

## Discussion

Doxorubicin induces a delayed cardiomyopathy that can be reduced in severity by pre-treatment with DZR [1]. In the present study, which was limited to 2 weeks, DOX induced minimal cardiac and renal toxicity that was prevented by contemporaneous administration of DZR. In other studies of SHR where DOX was given at lower doses over a longer time, DZR was also markedly cardioprotective [15]. However, in these studies, the cardiomyopathy was more severe and DZR protection was not complete (a mean lesion score of 2.6 was reduced to 1.0). Analysis of early changes in gene expression in the heart after DOX and DZR co-administration was performed to help investigate cellular targets of DOX that are modulated by DZR and may be involved in the formation of cardiac lesions. We observed immediate and persistent changes in gene expression 24 h after the initial dose of DOX and prior to histopathologic changes in myocardial tissue that consisted primarily of the up-regulation of genes associated with the cellular response to oxidative stress and the down-regulation of genes encoding mitochondrial proteins.

Dexrazoxane treatment attenuated a marked and selective decrease in the levels of many nuclear genes that encode proteins with mitochondrial functions that is caused by doxorubicin treatment. The depressive effect on the expression of genes that comprise the mitochondrial proteome

is persistent and can be observed weeks after prolonged administration of DOX [27]. These results are consistent with evidence that mitochondria are key subcellular targets and the primary sites of free radical formation by doxorubicin [28]. The selective effect of DOX on suppression of mitochondrial gene expression is likely a coordinate and adaptive response to stress mediated by energy-sensing molecules in the heart; for example, by AMP-activated protein kinase, hypoxia-inducible factor 1, Sirt1, or proliferator-activated receptor gamma coactivator 1 (PGC-1) [29]. PGC-1 is a key regulator of energy metabolism and mitochondrial biogenesis in tissues relying on oxidative metabolism for ATP production (reviewed by Hood et al. [30]). The activity of PGC-1, regulated by post-translational modification, modulates adaptive responses, such as the increased expression in skeletal muscle of nuclear genes encoding mitochondrial proteins in response to exercise.

In this study, dexrazoxane had little effect on the induction of the Nrf2 pathway in the heart by doxorubicin and on changes shown in other studies to be part of the reprogramming of cardiac gene expression that occurs in reaction to external stressors (e.g., *Slc2a4*). Increased expression of ARE-regulated genes by doxorubicin has been seen in other rodent studies [31] and quinone-induced oxidative stress has been shown to increase the expression of Nrf2-regulated genes by disrupting the association of Nrf2 with KEAP [32]. The inability of dexrazoxane to block the effect of doxorubicin on ARE-regulated gene induction is surprising but would be consistent with the limited diffusion of highly reactive oxygen free radicals from their site of generation in mitochondria. Stabilization of Nrf2 may be mediated by non-iron catalyzed reactive oxygen species that are generated in mitochondria or the sarcoplasmic reticulum by redox cycling of doxorubicin but are less reactive than hydroxyl radicals. Further studies would be needed to determine the source and type of cellular stress that signals this program of response to oxidative damage in the heart that is a result of doxorubicin treatment.

Cardiomyocyte apoptosis occurs in in vivo models of acute cardiotoxicity induced by short-term administration of DOX [33, 34], but its involvement in the progressive cardiotoxicity induced by long-term DOX treatment is less certain [35]. At day 15 in the present study, DOX produced statistically significant increases in several gene transcripts for proteins that regulate apoptosis, including *Nol3* (Table 4). The *Nol3* gene product (also known as ARC, apoptosis repressor with CARD domain) is expressed predominantly in muscle and brain, and has been shown to be important in promoting cardiomyocyte survival after biomechanical and ischemic stress in studies using ARC knockout mice [36].

Dexrazoxane is hydrolyzed to an open ring form that chelates metal ions and reduces reactive oxygen production by removing iron from complexes with anthracyclines but is also a catalytic inhibitor of topoisomerase II [5]. In this study, dexraxozane treatment alone did not produce a gene signature in the heart that provided strong evidence of its cardioprotective mechanism of action. In the absence of DOX, dexrazoxane treatment was not associated with significant changes in cardiac gene expression until day 15, when repressions in the transcript levels of a set of cardiac genes that may represent an adaptive response to iron chelation were observed.

## Conclusions

Genomic analysis of early, DOX-induced cardiotoxic changes was consistent with a pathogenic mechanism in which DOX generates short-lived radicals in mitochondria that are catalyzed by iron and oxidoreductases. The analysis also identified some additional pathways that might be modified to improve cardioprotection. Dexrazoxane had little effect on DOX's early and robust activation of the Nrf2 pathway, which is mediated through the cytosolic oxidant sensor KEAP, and provided partial protection against changes in genes that are associated with mitochondrial injury. Dexrazoxane, by reducing production of the more damaging, iron-catalyzed types of ROS, blocks the minimal myocardial alterations induced by DOX in the early stages of treatment. Thus, more complete cardioprotection might be achieved with strategies that increase or supplement the action of DZR on reducing the formation of ROS in mitochondria.

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