Doxorubicin-induced cardiomyopathy: an update beyond oxidative stress and myocardial cell death

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The clinical use of doxorubicin is limited by the total cumulative dose due to its dose-related cardiac toxicities. The incidence of doxorubicin-induced cardiotoxicity ranges from more than 4% at a cumulative dose of 500–550 mg/m² to greater than 36% in patients receiving a cumulative dose ≥ 601 mg/m² [1]. To minimize the risk, the recommended dose in patients 18 years of age or older should be limited to 550 mg/m², and for those who are younger than 18 years old the maximum cumulative dose is capped at 300 mg/m² [2]. It is noteworthy that despite receiving the recommended doses of doxorubicin, the cardiotoxicity continues to be observed in some individuals, suggesting involvement of genetic components.

Mechanisms of doxorubicin-induced cardiotoxicity have been a matter of investigations. Cumulative findings point to the direction that cardiomyopathy induced by doxorubicin is an on-going, complex, multifactorial process. For instance, doxorubicin is metabolized by aldoke to reductase to yield a...
metabolite, doxorubicinol. This doxorubicinol concentrates in cardiomyocytes and inhibits Ca\(^{2+}\) uptake into sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2A) causing a considerable delay in the activation and inactivation of cardiomyocyte contractility [3]. The shift of cellular calcium distribution into the cytoplasm may also induce apoptosis of the cardiomyocytes. Additionally, doxorubicinol interferes with iron homeostasis via an inhibition of iron regulatory proteins (IRPs) that modulate the expression of transferrin receptor and ferritin [4]. Several lines of evidence also indicate that doxorubicin-induced cardiotoxicity involves the formation of highly reactive radicals e.g., superoxide radical anion (\(\cdot \text{O}_2\)) and hydroxyl radical (\(\cdot \text{OH}\)). In regard to their detoxifying capacity, cardiomyocytes possess limited levels of catalase. Exposure of cardiomyocytes to doxorubicin causes an inhibition of both catalase and glutathione peroxidase (GPx) activities [5], leaving the cardiomyocytes with inadequate means of detoxifying hydrogen peroxide (H\(_2\)O\(_2\)). In the presence of endogenous ferrous ions (Fe\(^{2+}\)), H\(_2\)O\(_2\) is being reduced to \(\cdot \text{OH}\) by means of the Fenton reaction, creating oxidative stress condition. In this article, the readers will find a brief review and updates on the effects of doxorubicin on cardiomyocytes and endothelial cells, and a possible interplay between these two cell types that contributes to the developing process of cardiomyopathy.

**Effects of doxorubicin on cardiomyocytes**

Cardiomyopathy is a condition in which the cardiac muscles are damaged, leading to cardiac dysfunctions. Histological studies have revealed that doxorubicin causes myofibril disruption, cytoplasmic vacuolization, and mitochondrial membrane disruption [6]. Mechanistically, several mechanisms that cause damages to cardiomyocytes have been proposed, and most of which appear to stem from oxidative stress initiated by one-electron redox cycling of the quinone moiety within the chemical structure of doxorubicin (Figure 1). This oxidative stress-mediated cardiotoxicity theory has been supported by an attenuation of cardiac tissue damage in transgenic mice overexpressing the antioxidant enzyme superoxide dismutase 2 (SOD2) compared with their wild-type littermates following doxorubicin [7].

**Oxidative stress induced by doxorubicin redox cycling**

Doxorubicin is effectively accumulated in the nuclei and mitochondria of cardiomyocytes [8]. The doxorubicin concentration in mitochondria was reported to be a hundred times higher than that in the plasma [9]. The accumulation of doxorubicin in mitochondria is most likely due to its ability to form complex with cardioliopin [10]. Additionally, cardiac mitochondria have been estimated to occupy approximately 40% of the total intracellular volume of cardiomyocytes [11], positioning the mitochondria as major organelles responsible for a generation of highly reactive oxygen species (ROS) via redox cycling of the doxorubicin (Figure 1).

Due to the redox cycling between quinone and semiquinone form of doxorubicin, substantial amounts of ROS, especially \(\cdot \text{O}_2\), can be generated. This redox cycling process is mediated by reduced nicotinamide adenine dinucleotide (NADH)- or NAD(P)H-dependent enzymes e.g., NADH dehydrogenase and NADPH-dependent glutathione reductase NAD(P)H. Large amount of \(\cdot \text{O}_2\) generated from this futile redox cycling of doxorubicin then get converted to H\(_2\)O\(_2\); this reaction requires protons and is catalyzed by SOD2. Subsequently, the H\(_2\)O\(_2\) is rapidly converted to water and oxygen. However, in the presence of ferrous ion (Fe\(^{2+}\)), H\(_2\)O\(_2\) can undergo the Fenton reaction, yielding the highly reactive \(\cdot \text{OH}\). This scenario, in fact, has been observed in doxorubicin-treated patients where cardiac mitochondrial iron level from patients with doxorubicin-induced cardiomyopathy are higher than that in patients with other types of cardiomyopathies or normal cardiac function [12]. Additionally, it has been well recognized that doxorubicin and doxorubicinol can interfere with iron homeostasis by inactivating IRPs [4], resulting in a release of iron molecules from the [4Fe–4S] cluster of cytoplasmic aconitase. These unbound ion molecules therefore are available to participate...
in the Fenton reaction, and a generation of ·OH guarantee. Co-administration of dexrazoxane, an iron chelating agent, has been shown to significantly reduce the incidence of adverse cardiac events in cancer patients treated with doxorubicin-containing regimens \[13\]. This cardioprotective effect of dexrazoxane reassures the involvement of Fe\(^{2+}\) in mediating oxidative stress during the course of doxorubicin therapy.

In addition to ROS, reactive nitrogen species (RNS) e.g., peroxynitrite (ONOO\(^{-}\)) also being generated in cardiac tissue following a doxorubicin treatment in mice \[14\]. The formation of ONOO\(^{-}\) in cardiomyocytes is most likely through the chemical reactions between \(\text{O}_2\) generated from mitochondria and nitric oxide (NO) (Figure 2). NO may diffuse from nearby endothelial cells. It is also possible that NO is being produced locally by cardiomyocytes as evidenced by increased expression of inducible nitric oxide synthase (iNOS) following an exposure to doxorubicin \[5\]. Both ONOO\(^{-}\) and ·OH interact with mitochondrial proteins and membrane lipids. These protein and lipid modifications usually lead to mitochondrial dysfunction and significant damages to the cardiac tissue \[15\]. The ·OH also reacts to membrane lipids and causes lipid peroxidation and a production of lipid aldehydes, including a highly reactive 4-hydroxy-2-nonenal (HNE). This HNE formation in heart tissues can be detected as early as in 3 hours following doxorubicin administration \[16\]. This is in-line with the levels of proteins adducted by HNE in cardiac mitochondria of mice treated with doxorubicin \[17\].

HNE is a potent electrophile and is one of the most toxic aldehydes generated during lipid peroxidation. HNE interferes with protein structures and functions by forming Michael adducts with histidine, cysteine, and lysine residues. Also, HNE is able to form Schiff bases with lysine residues \[6, 18\]. As a result, accumulation of HNE can be toxic to cells. HNE is partially detoxified by conjugation with GSH to form a glutathione-conjugated HNE (GS-HNE). This reaction is catalyzed by the enzyme glutathione-S-transferases (GST). The formation of GS-HNE was first described by Ishikawa et al \[19\] in which he demonstrated the formation and saturable efflux of GS-HNE in the isolated HNE-perfused rat heart. This saturable efflux of GS-HNE is believed to be mediated by ATP-binding cassette transport protein, subfamily C member 1 (ABCC1), which is also known as multidrug-resistance associated protein 1 (MRP1 for human; Mrp1 for rodent) (Figure 3).

**A potential cardioprotective role of multidrug-resistance associated protein 1**

MRP1, originally discovered by Cole et al \[20\], transports both conjugated, e.g., estradiol 17-(β-D-glucuronide)
(E217G) and cysteinyl leukotriene C4 (LTC4), and non-conjugated substrates, e.g., vincristine, in the presence of GSH [21]. MRP1 is expressed in several organs including the heart, and is believed to be involved in tissue defense. We have demonstrated that MRP1 expression levels in cardiac tissue increase following doxorubicin treatment [6]. Because MRP1 effluxes both doxorubicin and GS-HNE, we have postulated that a loss of function mutation of MRP1 influences efflux of these cytotoxic compounds, and thereby could play a pivotal role in promoting functional impairment of the myocardium following treatment with doxorubicin. This hypothesis have led us to study the effects of MRP1 in context of doxorubicin-induced toxicity. Our first set of studies focused on the MRP1 variant that has been associated with the doxorubicin-induced cardiotoxicity. The second set of studies focus on the effects doxorubicin in Mrp1 deficient mice.

The contribution of a genetic component in doxorubicin-induced cardiotoxicity has been suggested by the great variation in individual sensitivity to doxorubicin. Some patients are highly tolerant to doxorubicin at doses higher than 1000 mg/m2 while others develop cardiac toxicity at doses less than 400 mg/m2 [22]. Our first set of studies was influenced by the published reports in which Conrad et al [23] identified several MRP1 variants from Caucasian volunteers. Among those several variants, they found two non-synonymous MRP1 single nucleotide polymorphisms (SNPs) i.e. R433S and G671V, which may affect MRP1 transport activities. This finding is in-line with the prediction made by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), a web tool that predicts a possible impact of amino acid substitutions on structure and function of human proteins. Based on a single amino acid substitution, both R433S and G671V variants are predicted to be ‘probably damaging’ variants. In the R433S-MRP1, the amino acid 433 is located in the second transmembrane spanning domain. The functional study of R433S-MRP1 variant revealed a two-fold decrease in the transport of LTC4. However, cells expressing the R433S-MRP1 variant were two-fold more resistant to doxorubicin than those expressing the wild-type MRP1 [24]. As for the G671V, the amino acid 671 is only six positions away from the conserved Walker A motif in the nucleotide binding domain. The G671V variant showed no difference in the in vitro transport activities for LTC4 and estradiol E217G. The sensitivity of cells expressing G671V variant to doxorubicin was not documented in their reports [23, 24]. However, in one clinical study, patients with the G671V variant showed increased doxorubicin-induced acute cardiac toxicity [25]. Based on this report, a single amino acid changes from glycine to valine at the position 671 of MRP1 transporter protein appears to affect its transport function for certain substrates, and that could be doxorubicin and GS-HNE.

Despite more water soluble and less reactive than HNE, intracellular accumulation of GS-HNE remains toxic by mean of its ability to inhibit GST and subsequently delay the detoxification process of HNE, ensuring toxicity. Therefore, it is possible that patients who carry G761V-MRP1 variant may have a higher risk of doxorubicin-induced cardiotoxicity due to the decrease in MRP1 ability to excrete GS-HNE from the cardiac tissue. Our studies provided evidence that (1) cells overexpressing G671V variant were more sensitive to doxorubicin than cells that were overexpressing wild-type MRP1, and (2) the G671V variant’s ability to transport GS-HNE was markedly decreased by 85% in the G671V-MRP1 variant relative to the wild-type MRP1 [26]. Together with the clinical study conducted by Wojnowski et
al [25], we believe that MRP1 plays a significant role in protecting cardiac tissue via elimination of GSH-conjugated electrophilic compounds such as HNE. In other words, lacking MRP1 may potentiate doxorubicin-induced cardiotoxicity.

In our second set of studies, we used the Mrp1-deficient mice to directly address the role of Mrp1 in protecting the heart against doxorubicin-induced cardiotoxicity. We have postulated that deficiency of Mrp1 enhances doxorubicin-induced cardiotoxicity due to intracellular accumulation of GS-HNE. To prove our hypothesis, we injected the Mrp1-deficient or wild-type mice with a single dose of doxorubicin (15 mg/kg), and quantified GS-HNE retention in the whole heart homogenates. We also analyzed the expression of relevant antioxidant enzymes, GSH, and glutathione disulfide. To our surprise, deficiency in Mrp1 only modestly increased GS-HNE retention in the hearts of Mrp1-deficient mice vs. wild-type mice. This could be due to alternative detoxification pathways of HNE. Besides GSH conjugation, HNE can also be oxidized to 4-hydroxy-2-nonenoi acid or reduced to 1,4-dihydroxy-2-nonenone. GS-HNE can also be metabolized to mercapturic acid conjugates [27, 28]. In addition, there was no significant difference between Mrp1-deficient vs. wild-type mice in the expression levels of antioxidant enzymes (catalase, SOD1, SOD2, SOD3, and glutathione reductase). However, the amount of GSH in the hearts of Mrp1-deficient mice was higher than that of wild-type littermates. Despite increased GSH levels in the heart, doxorubicin induced significantly more nuclear injury in Mrp1-deficient vs. wild-type mice [29]. Thus, we concluded that elevated GSH was not sufficient to protect the cardiac injury induced by doxorubicin. Our studies also suggest that Mrp1 may play a pivotal role in guarding nuclei of the cardiomyocytes, and perhaps it may have additional functions other than efflux of its known substrates.

Finally, Zhang et al [30] recently published the chronic model of doxorubicin-induced cardiotoxicity in Mrp1-deficient mice. In this model, the mice were injected with 2 mg/kg of doxorubicin, twice a week for 5 weeks. The key finding in their study was that the deficiency in Mrp1 potentiated doxorubicin cardiotoxicity as measured by echocardiography, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) positive of myocardial tissues, and elevated ventricular dysfunction biomarker, BNP (brain natriuretic peptide). In comparison to wild-type mice, chronic doxorubicin treatment causes more severe left
ventricular dysfunction in Mrp1-deficient mice. Higher BNP expressions and more apoptotic bodies also were observed in the Mrp1-deficient vs. wild-type mice, once again, supporting the role of Mrp1 in protecting cardiotoxicity mediated by oxidative stress.

**Effects of doxorubicin on endothelial cells**

Effects of doxorubicin on endothelial cells are less documented compared to its effects on cardiomyocytes. Nonetheless, there are both preclinical and clinical data to support that doxorubicin does cause adverse effects on coronary endothelium [31, 32]. Similar to cardiomyocytes, the endothelial dysfunction appears to be mediated by oxidative stress [33]. Utilizing human endothelial cells (EA.hy926), Wojcik et al [34] have demonstrated that doxorubicin accumulated in nuclei of the cells at the concentration as low as 50 nM following 24 hours incubation. Increased generation of ROS, decreased level of GSH, and alteration in endothelial elasticity were also detected at concentrations higher than 50 nM. Studies also demonstrated that doxorubicin increased endothelial cell apoptosis indicated by an activation of caspase-3 [35]. Besides ROS production, the derangements in nitric oxide synthesis (NOS) is considered as a potential mechanism of endothelial dysfunction caused by doxorubicin [36]. Finally, it has been demonstrated that doxorubicin induced activation of NFκB in both cardiomyocytes and endothelial cells [37]. This doxorubicin-mediated activation of NFκB in endothelial cells subsequently induces NFκB-dependent production of endothelial cytokines such as interleukin (IL)-1α, IL-2, and IL-6, and is depending on the focal adhesion kinase (FAK) in endothelial [38]. These inflammatory mediators may play an important role in myocardial dysfunction.

**Interplay between endothelial cells and cardiomyocytes**

Myocardial work is tightly controlled by the coronary blood flow and heart rate. In the situation where doxorubicin causes cardiomyocytes death, the cumulative loss of these cardiomyocytes compromises myocardial contractility and subsequently, reduces cardiac output (CO). To sustain CO and adequately perfuse vital organs, the sympathetic nervous system and the renin–angiotensin–aldosterone system are activated. These compensatory mechanisms lead to increased sympathetic tone, release of angiotensin-II (AngII), endotelinls, vasopressin, aldosterone, and an impairment of NO release. These biochemical changes result in an increase in preload and afterload, and thereby a sustained CO. However, if the compensatory mechanisms continue, increased ventricular wall stress will eventually lead to a left ventricular hypertrophy and cardiomyopathy (Figure 4).

In molecular levels, doxorubicin is known to activate NFκB-dependent cytokine productions in endothelial cells and cardiomyocytes [37, 38]. It has been demonstrated that an activation of NFκB is necessary for adaptive cardiac hypertrophy [39]. Activation of NFκB, however, requires bone marrow kinase in chromosome X (Bmx) [40]. This relationship is of considerable interest. It implies that there is an interaction between Bmx and NFκB signaling molecules. Recently published data indicate that Bmx is essential for myocardial hypertrophy and remodeling [41], an underlying pathogenesis of cardiomyopathy. Bmx is expressed specifically in endothelial cells and not in cardiomyocytes, yet, Bmx deficiency prevents pressure overload–induced cardiac hypertrophy [42], further indicating a cross-communication between endothelial cells and cardiomyocytes.

Activation of Bmx leads to subsequent activation of signal transducer and activator of transcription 3 (STAT3) and an upregulation of IL-1, IL-6, IL-8, tumor necrosis factor receptor-2, vascular endothelial growth factor, and matrix metalloproteinase 2 [41, 43, 44]. Reciprocally, binding of AngII to angiotensin 1 receptor (AT), leads to a phosphorylation of Bmx. The phosphorylated Bmx in turn phosphorylates STAT3 [41]. These interactions provide evidence for an existing and complex cross-talk between NFκB and Bmx–STAT3 signaling pathways. STAT3 has been shown to be important for stress-induced cardiac remodeling [45] via mammalian target of rapamycin (mTOR). Activation of mTOR stimulates protein translation through phosphorylation of eIF4E-binding protein and S6 kinase, leading to cell growth.

Lastly, the therapeutic effects of angiotensin-converting enzyme inhibitors (ACEIs) [46] and angiotensin receptor blockers (ARBs) [47] in doxorubicin-induced cardiotoxicity have been reported. The protective effects of ACEIs and ARBs in doxorubicin models further highlight and support the interplay between endothelial cells and cardiomyocytes presumably via Bmx–STAT3 signaling pathway [41]. In summary, the activation of NFκB-Bmx–STAT3 axis could be an underlying mechanism for doxorubicin-induced cardiomyopathy, and future study is warranted.

**Future direction**

Mechanism of doxorubicin-induced cardiomyopathy is complex and multifactorial. Nonetheless, it is well accepted that the pathophysiology and pathogenesis of the cardiomyopathy is driven by oxidative stress initiated by doxorubicin and its metabolite, doxorubicinol. Oxidative stress is the product of an imbalance between oxidants and antioxidants. Therefore, rebalancing the cellular oxidative
status by reducing the generation of oxidants while increasing the production of antioxidant enzymes and proteins is essential. Due to a cross-talk between endothelium and cardiomyocyte in response to oxidative stress, it may be necessary to prevent oxidative stress in both cellular compartments. Hence, in developing prophylactic strategies for doxorubicin-induced cardiomyopathy, we may have to consider a combination therapy approach that acts on both endocardium and myocardium without compromising the therapeutic efficacy of cancer chemotherapeutic agents. The specific targets may include mitochondria, nuclei, and membrane receptors involved in redox regulation.

**Conflicting interests**

The authors have declared that no competing interests exist.

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**Abbreviations**

HNE: 4-hydroxy-2-nonenal; ABCC1: ATP-binding cassette transport protein subfamily C member 1 (ABCC1); ALA: alpha-lipoic acid; AngII: angiotensin-II; Asc⁺: ascorbyl radical; Asch²⁺: ascorbate anion; Bmx: endothelial bone marrow kinase in chromosome X; CO: cardiac output; DHLA: dihydrolipoic acid; Fe²⁺: Ferrous iron; GSH: glutathione; GPx: glutathione peroxidase; LVH: left ventricular hypertrophy; MRP1: Multidrug-resistance associated protein 1; NFκB: nuclear factor κ-B; NO: nitric oxide; ·O₂⁻: superoxide radical anion; ·OH: hydroxyl radical; ONOO⁻: peroxynitrate; RAAS: renin–angiotensin–aldosterone system; ROS: reactive oxygen species; RNS: reactive nitrogen species; SOD2: superoxide dismutase 2; SNS: sympathetic nervous system; STAT3: signal transducer and activator of transcription 3; T-OH: tocopherol.

**References**

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