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Insights into Doxorubicin-induced Cardiotoxicity: Molecular Mechanisms, Preventive Strategies, and Early Monitoring

Nadine Wenningmann, Merle Knapp, Anusha Ande, Tanaya R. Vaidya, Sihem Ait-Oudhia

Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida (N.W, M.K, A.A, T.R.V, S.AO).

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Running title: Cardiac Safety of Doxorubicin

Corresponding author: Sihem Ait-Oudhia, MS., PharmD., PhD.

Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida. Address: 6550 Sanger Road. Office #469. Orlando. Florida. 32827. USA. Tel: +1 407-313-7037. Fax: +1 407-313-7030. Email: sihem.bihorel@cop.ufl.edu.

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Non-standard Abbreviations:

AIF: Apoptosis-inducing factor; **Akt:** AKT8 virus oncogene cellular homolog; **ALL**: Acute lymphoblastic leukemia; **ANP**: Atrial natriuretic peptide; **ATP**: Adenosine triphosphate; **BNP**: Brain natriuretic peptide; **CHF**: Chronic heart failure; **cTnT**: cardiac troponin T; **DIC**: Doxorubicin-induced cardiotoxicity; **DOX**: Doxorubicin; **DR**: Death receptors; **eNOS**: Endothelial nitric-oxide synthase; **EPO**: Erythropoietin; **ERK:** Extracellular signal-regulated kinases; **ET-1**: Endothelin 1; **ETC**: Electron transport chain; **FDA**: Food and drug administration; **HER2**: Epidermal growth factor receptor 2; **HPLC**: High performance liquid chromatography; **In-111**: Indium-111; **iPS-CMs**: pluripotent stem cells-derived cardiomyocytes; **LC-MS/MS**: Liquid chromatography with tandem mass spectrometry; **LLOQ**: Lower limit of quantification; **LVEF**: Left ventricular ejection fraction; **MnSOD/SOD2**: manganese superoxide dismutase; **mtDNA**: Mitochondrial DNA; **NADH**:

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Nicotinamide adenine dinucleotide, reduced form; **NADPH**: Nicotinamide adenine dinucleotide phosphate, reduced form; **NFAT**: nuclear factor of activated T-lymphocytes; **NFκB**: Nuclear factor-kappa B; **NOX**: NADPH oxidase; **PARP**: Poly(ADP-ribose) polymerase; **PD**: Pharmacodynamics; **PI3K:** Phosphatidylinositol 3-kinase; **PK**: Pharmacokinetics; **PS**: phosphatidylserine; **PUMA**: p53 upregulated modulator of apoptosis; **RNS**: Reactive nitrogen species; **ROS**: Reactive oxygen species; **SERCA**: sarco/endoplasmic reticulum Ca^{2+} ATPase; **SIRT1**: silent mating type information regulation 2 homolog 1; **TDM**: Therapeutic drug monitoring; **TDM**: Therapeutic drug monitoring; **TNF**: Tumor necrosis factor; **TPO**: Thrombopoietin; **TRAIL**: TNF-related apoptosis inducing ligand; **UHPLC**: Ultra-high performance liquid chromatography; **^{123}I-MIBG**: 123-labeled metaiodobenzylguanidine; **2D-STE**: two-dimensional speckle tracking echocardiography.

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ABSTRACT

Doxorubicin (DOX) is one of the most effective anticancer drugs to treat various forms of cancers, however, its therapeutic utility is severely limited by its associated cardiotoxicity. Despite the enormous amount of research conducted in this area, the exact molecular mechanisms underlying DOX toxic effects on the heart are still an area that warrants further investigations. Here, we reviewed literature to gather the best-known molecular pathways related to DOX-induced cardiotoxicity (DIC). They include mechanisms dependent on mitochondrial dysfunction such as DOX influence on the mitochondrial electron transport chain, redox cycling, oxidative stress, calcium dysregulation, and apoptosis pathways. Further, we discuss the existing strategies to prevent and/or alleviate DIC along with various techniques available for therapeutic drug monitoring (TDM) in cancer patients treated with DOX. Finally, we propose a step-wise flowchart for TDM of DOX, and present our perspective at curtailing this deleterious side effect of DOX.

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I. Introduction

Anthracyclines are widely recognized as a class of effective chemotherapeutic agents to treat different types of cancer since their discovery in the 1960s (Kayser et al., 1999; Matsuoka et al., 2000; Shah, 2009; Octavia et al., 2012; Pendlebury et al., 2017; Qiu et al., 2017; Wei et al., 2017). DOX, a product of Streptomyces peucetius var. caesius, is a prototype agent of anthracycline antibiotics (Blum and Carter, 1974). It is proven efficacious against a wide range of human malignant neoplasms including a variety of solid tumors, breast cancer, Hodgkin's disease, Kaposi's sarcoma, , acute lymphoblastic leukemia, pediatric leukemia, lung cancer, lymphomas and several metastatic cancers (Vejpongsa and Yeh, 2014). Despite its widespread use, DOX therapy demonstrated dose-limiting effects owing to its acute and chronic cardiac toxicity (Gao et al., 2016). Based on small retrospective studies in childhood cancer survivors previously treated with anthracyclines, the cardiotoxicity of anthracyclines can be classified in three categories based on the time of onset. First, acute cardiotoxicity, which occurs after a single course of chemotherapy, and where clinical manifestations appear within two weeks from the end of treatment. Second, the early-onset chronic cardiotoxicity, which develops within one year post-treatment cessation, and usually manifests itself as a dilated and hypokinetic cardiomyopathy leading to heart failure (HF). Third, the late-onset chronic cardiotoxicity, which develops years or even decades after the end of chemotherapy (Cardinale et al., 2015). However, it is worth mentioning that this classification is inappropriate for adult populations since no prospective study has reported regular monitoring of cardiac function in adult patients for more than 3 years. Alternately, based on the stage of disease progression and the clinical manifestations, HF symptoms that are diagnosed several years after anthracycline therapy can be defined as late. The left ventricular ejection fraction (LVEF) reduction that occurs within months post treatment with anthracyclines can be defined as early; while myocardial damage using a biomarker like troponin can be identified during or soon after therapy, and is termed as acute (Cardinale et al., 2015).

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A cumulative treatment dose of > 350 mg/m^2 of free DOX shows a dose-dependent decrease in the LVEF, and at a cumulative dose of 550 mg/m^2, a sharp increase in the prevalence of HF is reported (O'Brien et al., 2004; Volkova and Russell, 2011). It is reported that the percentage of patients with DOX-related congestive heart failure (CHF) increases with an increase in the patients' cumulative dose of DOX therapy (Swain, Whaley and Ewer, 2003). For example, 5% of patients manifest CHF at a cumulative dose of 400 mg/m², which increases to 16% at a dose of 500 mg/m², 26% at a dose of 550 mg/m², and about 48% at doses above 700 mg/m². The toxicity caused by DOX further increases while administered in combination with other targeted agents such as trastuzumab, an anti-epidermal growth factor receptor 2 (HER2) monoclonal antibody, hence remaining a major contributor to chemotherapy-induced heart diseases (Conte et al., 2001; Rochette et al., 2015; Mitry and Edwards, 2016). While DOX-induced acute cardiotoxicity emerges during or immediately after treatment and typically involves reversible hypotension, pericarditis and transient electrocardiographic abnormalities such as non-specific changes in the ST-T waves on an electrocardiogram (ECG), QT prolongation, and vasodilatation (Licata et al., 2000); its chronic cardiotoxicity develops after completion of cumulative dose regimens and results in irreversible cardiomyopathy, steadily progressing towards congestive heart failure (CHF) (Licata et al., 2000; Takemura and Fujiwara, 2007).

The precise molecular mechanisms by which DOX induces cardiac dysfunction are still not completely elucidated. Due to the existence of various and interconnected intracellular signaling pathways triggered by DOX, in this review, we limit our discussion to highlight the essential molecular mechanisms of DIC involving potential pharmacological targets for therapeutic interventions. Our review also focuses on presenting various preventive strategies to lower, and ideally, to overcome cardiotoxicity, and narrate the procedure of therapeutic drug monitoring presently followed in clinical settings.

II. Molecular mechanisms of doxorubicin-induced cardiotoxicity

DOX acts by multiple mechanisms of action. Currently it is unclear, which of these mechanisms are the most responsible for its associated cardiac toxicity. Many articles support the view that the generation of reactive oxygen species (ROS) upon DOX treatment and its consequential lipid peroxidation, calcium dysregulation and intervention in energy transfer could cause heart failure (Tokarska-Schlattner et al., 2006; Takemura and Fujiwara, 2007; Renu et al., 2018). However, the underlying biochemical mechanisms of its toxicity are still not fully elucidated. In the following section, we present the most probable intracellular/signaling mechanisms behind cardiotoxicity, as summarized in **Figure 1**.

A. Generation of reactive oxygen species (ROS)

One of the major mechanisms of DOX-induced cardiotoxicity (DIC) is strongly linked to mitochondrial dysfunction leading to an increased generation of intracellular ROS and oxidative stress. Mitochondria are the most injured intracellular organelles upon cell exposure to DOX. One of the contributing factors for the accumulation of DOX in the inner mitochondrial membrane is its high affinity binding to cardiolipin (Goormaghtigh et al., 1986; Goormaghtigh et al., 1990). Cardiolipin is a phospholipid present in the inner leaflet of the mitochondrial membrane and is known for maintaining mitochondrial structure, function, cardiac energy metabolism, and cell survival (Schlame et al., 2000). Mitochondrial toxicity arising from the cardiolipin bound DOX is majorly mediated through oxidative stress (Minotti et al., 2004). Electrostatic binding between cardiolipin and DOX leads to disruption of the activity of complexes I, III and IV in the electron transport chain (ETC), all of which are known to require cardiolipin in order to maintain maximal activity. In addition, it has been shown that complex I can catalyze reduction of DOX to a semiquinone radical species. This species can be re-oxidized by transfer of an electron to molecular oxygen (O_2), leading to the formation of superoxide anion. This transfer of electrons through DOX can result in the formation of an even stronger bond with cardiolipin leading to further disruption of the ETC (Goormaghtigh et al., 1983; Marcillat et al., 1989). Owing to its chemical structure that consists of a tetracycline moiety

containing a quinone, DOX can also be easily reduced into semiquinone by endothelial nitric-oxide synthase (eNOS) (Delemasure et al., 2006). Different mitochondrial and membrane-bound enzymes can catalyze the conversion of quinone to the semi-quinone state as described earlier. Few flavoprotein oxidoreductases that catalyze the redox quinone cycle of DOX include nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase (complex I of the ETC), NADPH/cytochrome P450, localized at the endoplasmic reticulum, and xanthine oxidase (Delemasure et al., 2006; Takemura and Fujiwara, 2007). The semi-quinone can easily auto-oxidize by transferring an electron to molecular oxygen (O_2), converting back to the parent compound which is available for a new redox cycle, during which superoxide radicals are produced (Delemasure et al., 2006). Out of the superoxide radicals, hydrogen peroxide (H_2O_2) can be formed by manganese superoxide dismutase (MnSOD or SOD2) , which can be further converted into more reactive hydroxyl radicals in the presence of iron or copper (Delemasure et al., 2006).

DOX accumulation in mitochondria leads to enhanced production of ROS and reactive nitrogen species (RNS) as well (Weinstein et al., 2000; Priya et al., 2017). These reactive species in turn cause peroxidation of lipids and oxidative damage to DNA and proteins resulting in mitochondrial DNA (mtDNA) damage, loss of adenosine triphosphate (ATP) levels, peroxidation of cardiolipin, and mitochondrial permeability transition (Mizutani et al., 2005; Tokarska-Schlattner et al., 2006). A close interaction between ROS, mtDNA damage, and the ETC can result in the formation of a vicious loop in two ways: enhanced ROS levels can directly inactivate the ETC and result in further ROS formation (de Oliveira and Niederer, 2016). Alternatively, mtDNA damage caused by increased ROS levels can inhibit ETC proteins aggravating mitochondrial dysfunction and ROS formation (de Oliveira and Niederer, 2016). Altogether, this cycle of events results in the release of cytochrome c as well as the release of additional apoptogenic factors from mitochondria, hence, initiating the apoptotic pathway. Since mitochondria are abundantly present in the energy-demanding cardiac tissue - 20-40% of its cellular volume - the production of free radicals through oxidative metabolism

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in cardiomyocytes upon exposure to DOX is likely high. Hence, making the heart a highly susceptible tissue to DOX-mediated oxidative damage.

B. *Apoptotic pathway*

DOX activates apoptosis by both intrinsic and extrinsic pathways (Nakamura et al., 2000; Priya et al., 2017; Zhao and Zhang, 2017). Mizutani et al showed that human promyelocytic leukemia (HL-60) cells, treated with DOX exhibited an activation of caspase-3 protein resulting in cell death (Mizutani et al., 2005). In this report, they identified a H_2O_2-dependent mechanism at mediating apoptosis through Poly(ADP-ribose) polymerase (PARP), NADPH oxidase (NOX) activation, and increased mitochondrial membrane permeability. Mitochondrial dependent intrinsic pathway plays a key role in DIC. This pathway is activated by up-regulation of pro-apoptotic proteins such as Bax, which promotes the release of cytochrome c from mitochondria, leading to activation of caspase-9 that further causes the activation of effector caspase-3 (Liu et al., 2008b). An elegant study performed in rat cardiomyoblasts (H9c2) demonstrated the sequential events of molecular mechanisms, highlighting the importance of NOX/ROS mediated nuclear factor-kappa B (NFκB) signaling cascade at triggering DOX mediated apoptosis. The extracellular signal-regulated kinases 1 and 2 ($ERK_{1/2}$) and mitogen-activated kinases (MAPK) are also involved in the above signaling cascade modulated by NADPH/ROS system; resulting in an NFκB activation and ultimately cell death. Another finding also reported the involvement of $ERK_{1/2}$/p53 pathway and the activation of the NFκB-dependent p53 upregulated modulator of apoptosis (PUMA) in DOX induced cardiomyocyte apoptosis (Zhang et al., 2016). Similarly, the roles of ERKs and p53 at mediating DOX-induced apoptosis in H9c2 cells and cardiomyocytes are demonstrated in a study by Liu et al (Liu et al., 2008a).

Additionally, DOX is also shown to mediate cardiomyocytes apoptosis through extrinsic pathway mediators such as death receptors (DRs). Zhao et al demonstrated the up-regulation of DRs such as tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), Fas, DR4, and DR5 in DOX treated human induced pluripotent stem cells-derived cardiomyocytes (iPS-CMs) (Zhao and Zhang,

2017). These up-regulated DRs bind to their cognate ligands and trigger the caspase cascade ultimately leading to apoptosis. A TNF-related apoptosis inducing ligand (TRAIL) further augmented DIC, suggesting that serum levels of TRAIL could be used as a predictive biomarker to identify populations at higher risk for DIC (Zhao and Zhang, 2017). However, despite the involvement of caspases activation in mediating apoptosis, caspase inhibitors do not completely prevent cell death. This finding led to the identification of a caspase-independent pathway involving the role of mitochondrial apoptosis-inducing factor (AIF) (Moreira et al., 2014). In this finding, DOX mediated ROS generation increased cathepsin B activity, which mediated AIF release from mitochondria following its interaction with Bax clusters. AIF caused large-scale DNA damage, an enhanced expression of p53, and the activation of PARP1 resulting in caspase independent apoptosis.

In vivo, a study in DOX-treated male Wistar rats indicated that acute DIC involved cardiomyocytes apoptosis as assessed by the TUNEL assay in post-mortem analyses of rat hearts (Arola et al., 2000). However, cumulative doses of DOX did not lead to an additive effect in the percentage of TUNEL-positive cardiomyocytes. Moreover, the percentage of apoptotic cells gradually declined to baseline levels, after 24-48 hours post dosing with a single injection and at follow-up after cumulative dosing, suggesting cardiomyocyte apoptosis to be an acute cardiotoxic effect. In a clinical study, myocardial biopsies of childhood cancer patients demonstrated that DOX treatment may impair myocardial growth as observed by the disproportionately small increase in left ventricular wall thickness in relation to somatic growth in patients. This was attributed to DOX-mediated loss or damage of a critical number of cardiac myocytes resulting in numbers that were far lower than those required for the formation of normal adult myocardial mass (Lipshultz et al., 1991). Similar results were also observed in children with DOX-induced CHF (Goorin et al., 1990). In another clinical study, case reports of two adult patients demonstrated a striking decrease in the number of cardiac myocytes and a degeneration of the remaining myocardial cells in post mortem patho-clinical analyses. Both patients had received a cumulative dose of >700 mg/m^2, which was highly correlated with development of CHF (Lefrak et al., 1973). Taken together, these findings

suggest cardiomyocyte apoptosis to be an important mechanism of cardiac toxicity, especially in children, leading to inadequate ventricular mass development and important cardiac complications in later years. In addition, pre-clinical and clinical evidence are indicative of cardiomyocyte apoptosis as both, an acute and chronic side-effect of DOX-therapy.

C. Calcium dysfunction

Calcium dysregulation is another well-known and established mechanism contributing to DOX cardiotoxicity (Wallace, 2007). Doxorubicinol, the hydroxyl metabolite of DOX is known to affect calcium homeostasis by multiple mechanisms including, the modulation of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) present on sarcoplasmic reticulum (SR), and the sodium/potassium exchanger on sarcolemma (Nicolay et al., 1986; Mitry and Edwards, 2016). Decreased gene expression levels of SR proteins responsible for calcium transport is found to be the underlying cause of altered calcium homeostasis, as observed in a rabbit model of cardiomyopathy following treatment with DOX (Arai et al., 1998). In addition, a comprehensive study examining the role of calcium imbalance at inducing apoptosis identified the role of calcineurin, a calcium dependent phosphatase, at triggering apoptosis mediated through Fas (Kalivendi et al., 2005). The major findings from this study showed that mitochondrial ROS generated from the exposure of rat cardiac cells to DOX led to an increase in cytosolic calcium levels. The latter increase led to a calcineurin-dependent activation of the nuclear factor of activated T-lymphocytes (NFAT), which further enhanced the Fas mediated cardiac cells death. Furthermore, experimental examinations of the calcium/calmodulin dependent protein kinase (CaMKII), revealed its role in disturbing calcium balance through promoting SR calcium leakage (Little et al., 2009; Sag et al., 2011). In mice chronically treated with DOX, heart dysfunction occurred at 15 weeks which may be due to a depressed $[Ca2+]_i$ transient (Llach et al., 2019). In another study, mechanical unloading helped increase functional sarcoplasmic reticulum Ca2+ATPase and improved $[Ca2+]_i$ handling and contractility in rats with DOX-induced cardiomyopathy (Takaseya et al., 2004). These findings implicate that alteration in Ca^{2+} handling in cardiac myocytes precedes clinical signs of heart dysfunction. A comparative short-term vs long term

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exposure to DOX in rats helped delineate the sequence of calcium dysfunction when compared to other mechanisms. Findings from this study concluded that mitochondrial deoxyribonucleic acid (mtDNA) depletion and its associated ETC impairment preceded calcium levels disruption (Lebrecht et al., 2010). Nevertheless, targeting calcium dysfunction still remains a viable approach to treat DOX mediated cardiotoxicity (Agustini et al., 2016; Gao et al., 2016).

D. *Endothelin-1*

Endothelin-1 (ET-1) is potent vasoconstrictor peptide that stimulates a variety of cell types including cardiomyocytes. ET-1 biological effects include vasoconstriction, inflammation, cell division and proliferation, stimulation of free radical formation, and platelet activation (Bohm and Pernow, 2007). ET-1 has been implicated as an important factor in the development of vascular dysfunction, cardiovascular disease, and DIC, specifically at triggering a left ventricle dysfunction (Bien et al., 2007). Its plasma concentrations increase upon DOX treatment in patients and in animal models of cardiomyopathy in both acute and chronic studies (Picard et al., 1998; Sayed-Ahmed et al., 2001; Suzuki and Miyauchi, 2001). A study by Schwebe et al conducted on a murine DOX cardiotoxicity model also showed that the subunits ET_A and ET_B of ET-1 receptors equally contribute to DIC and further presented a signal transduction pathways modulated by ET-1 antagonists (Schwebe et al., 2015). However, in primary neonatal rat cardiomyocytes the ET-1 receptor, specifically through its ET_A subunit, also showed a cytoprotective effect at rescuing DIC at an early phase through the up-regulation of MnSOD (Suzuki and Miyauchi, 2001).

E. *Topoisomerase-II*

Another cellular target of DOX is topoisomerase-II, through which it induces single and double strand breaks in DNA (Tewey et al., 1984). Between the two isoforms of topoisomerases that exist, topoisomerase IIβ is abundantly present in the mitochondria of adult cardiomyocytes. It forms a ternary cleavage complex with DOX and DNA, inducing DNA double strand breaks and cell death (Capranico et al., 1992; Lyu et al., 2007). Further, topoisomerase IIβ specific role at mediating DIC

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has been demonstrated using the cardiomyocyte-specific topoisomerase IIβ knockout mice (Zhang et al., 2012). Topoisomerase IIβ role at mediating DIC is further confirmed by the protective effects of dexrazoxane, the only Food and Drug Administration (FDA) approved drug to prevent DOX induced heart failure (van Dalen et al., 2005; Deng et al., 2014a). Findings from Deng et al. implied that dexrazoxane prevented double strand breaks via topoisomerase IIβ degradation rather than by iron chelation (Deng et al., 2014a). This is the first *in vivo* report that presented transient depletion of topoisomerase IIβ by dexrazoxane confirming previous *in vitro* reports (Lyu et al., 2007; Yan et al., 2009).

With all the above molecular mechanisms leading to doxorubicin-induced cardiotoxicity, its clinical use is limited. Although several mechanisms of DIC were identified, the relative contribution of each of those mechanisms is not yet completely understood. In an attempt to identify the pathways causing acute and chronic DIC, a systems pharmacological approach was utilized by Oliveira et al (de Oliveira and Niederer, 2016). According to the simulations conducted using their systems-based model, ETC inhibition is considered to play a key role at mediating acute cardiotoxicity. This interpretation from a mathematical modeling perspective can also be corroborated by an earlier independent research finding from DOX treated mice through pathway and biochemical analyses (Pointon et al., 2010). While direct mtDNA damage triggered by DOX is responsible for chronic cardiotoxicity at therapeutic doses leading to further irreversible mitochondrial dysfunction.

III. Strategies for prevention of doxorubicin-induced cardiotoxicity

The primary strategies to prevent DIC are: 1) drug administration via continuous infusion or liposome encapsulation, and 2) simultaneous use of a cardio-protective agent such as dexrazoxane along with DOX treatment. Other methods include co-administration of antioxidants or hematopoietic cytokines such as erythropoietin (EPO) and thrombopoietin (TPO). **Table 1** summarizes the various strategies for the prevention and/or alleviation of DIC.

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A. *Altering anthracycline dosing regimen*

Following the evaluation of several dosing schedules in early clinical trials, slow continuous administration rather than large bolus doses of DOX is found to be safer from a cardiotoxicity point of view. Moreover, dose-fractionated weekly schedules of DOX have been found to significantly reduce cardiotoxic events, without compromising efficacy, as compared to the standard three-weekly dosing regimen (Lum et al., 1985). The mechanism is thought to be that, by maintaining lower peak plasma concentrations and hence lower concentrations of DOX in the heart, the intensity of exposure of cardiomyocytes to DOX is attenuated, thus leading to reduction in the occurrence of DIC (Pacciarini et al., 1978; Lum et al., 1985). Prolonged durations of DOX infusions for over 48-96 hours resulted in lower cardiotoxicity, while still demonstrating anti-cancer efficacy (Pacciarini et al., 1978). However, altering the administration time did not yield similar effects in adult patients versus pediatrics. For instance, continuous infusions over 48 hours were found to be a safer alternative in breast cancer women, whereas it did not render any cardio-protection to children with acute lymphoblastic leukemia (ALL) treated with DOX (Legha et al., 1982b; Hortobagyi et al., 1989; Lipshultz et al., 2002).

B. *Liposomal formulation of doxorubicin*

Extensive research has been undertaken to examine modified formulations of DOX like utilizing liposomal encapsulation strategies to overcome its cardio-toxic effects, by several research groups (Tacar et al., 2013; Tahover et al., 2015; van den Hurk et al., 2015). Liposomal formulations allow for direct encapsulation of hydrophilic drug within the aqueous compartment or lipophilic drugs incorporation into the lipid bilayer. Both polyethylene glycol (PEG)ylated and non-PEGylated forms of liposomal DOX formulations are commercially available, among which the PEGylated ones are the most frequently used within the United States (Allen and Cullis, 2013). Doxil is the FDA approved PEGylated DOX liposomal formulation. It is indicated for patients with ovarian cancer, Kaposi's sarcoma, and multiple myeloma following failure of at least one prior chemotherapy (Barenholz,

2012). Liposomal formulations of DOX preferentially accumulate at the tumor site due to the enhanced permeability retention effect and elicit lower peak plasma concentrations of free DOX, thus diminishing cardiotoxic effects (Gabizon et al., 2003). Additionally, PEGylation of liposomes allows for prolonged circulation times in the bloodstream through evasion of uptake by the reticulo-endothelial system, thus allowing retention of efficacy, while maintaining safety by encapsulation of free DOX (Gabizon et al., 2003; Torchilin, 2005). Moreover, a review of phase II and III clinical trials demonstrated that liposomal DOX formulations caused significantly lesser cardiotoxicity while retaining efficacy in breast cancer when used in combination with other chemotherapeutic agents, thus demonstrating potential to substitute liposomal DOX in place of conventional DOX as a safer option to treat cancers (Franco et al., 2018).

C. Antioxidants and iron chelators

Since enhanced oxidative stress is one of the major mechanisms of DIC, concomitant use of antioxidants can help in combating oxidative stress and its associated toxicity. Vitamin C was shown to be one such effective antioxidant in mitigating DOX induced oxidative/nitrosative stress and apoptosis in cardiomyocytes and in rats (Akolkar et al., 2017). Resveratrol, a polyphenolic compound was also found to have both prophylactic and therapeutic benefits in mitigating DOX induced apoptosis and fibrosis in myocardium in DOX treated rats (Shoukry et al., 2017). Bicalein, a bioflavonoid treatment alleviated DIC by inhibition of myocardial oxidative stress and apoptosis in mice (Sahu et al., 2016). Mangiferin, a naturally occurring C-glucosylxanthone is also found to exhibit protective effects in circumventing DIC in rats through its antioxidant capacity (Arozal et al., 2015). It exhibited greater cardio-protection compared to the common anti-oxidants such as Vitamin E and silymarin, which can be attributed to its effect on other mechanisms like calcium regulation (Arozal et al., 2015; Agustini et al., 2016). Amifostine is another cardio-protective agent that is shown to alleviate DIC in perfused isolated rat hearts and in several pre-clinical animal models (Nazeyrollas et al., 1999; Dragojevic-Simic et al., 2004; Potemski et al., 2006). *In vivo*, amifostine gets dephosphorylated into WR-1065, an aminothiol, due to alkaline phosphatases present in the cellular

membrane of small vessels. This aminothiol moiety is then thought to play an antioxidant role in normal tissue, rendering amifostine as a cytoprotectant (Smoluk et al., 1988). Despite its cardio-protective role demonstrated in pre-clinical studies, no conclusive results have been reported with studies in humans (Gallegos-Castorena et al., 2007). Other agents that have shown cardio-protection in response to DOX treatment include α-linolenic acid, melatonin, N-acetylcysteine, and sesame oil majorly through the activation of antioxidant pathways (Arica et al., 2013b; Yu et al., 2013; Govender et al., 2014; Saleem et al., 2014).

In mice studies, probucol, an anti-hyperlipidemic drug, prevented DOX and trastuzumab mediated cardiotoxicity through its antioxidant effects (Walker et al., 2011). Carvedilol, a beta blocker, was shown to prevent DIC when tested in two independent randomized clinical trials conducted in female patients diagnosed with breast cancer, suggesting further investigation for validation of this agent as a suitable prophylactic agent (Tashakori Beheshti et al., 2016; Nabati et al., 2017). However, the exact mechanism of carvedilol cardio-protective effects is not fully known, where its potent antioxidant activity might be the major attribute. Other possible mechanisms include the restoration of SERCA2 promoter activity in myocytes and the blockade of down-regulation of SERCA2 gene expression independent of its beta-blocking activity. In addition, carvedilol anti-apoptotic activity could be another contributing factor to its protection from DIC. Previous studies have shown the positive impact of carvedilol on cardiac mitochondria in vitro, ex-vivo, and in vivo models. Particularly, carvedilol is sugested to act as an inhibitor of mitochondrial complex-I which is known as a cause of DIC (Oliveira et al., 2005), and was demonstrated to be superior than propranolol against DIC, metoprolol for preventing from hydroxyl radical-induced cardiac contractility, and atenolol for preventing from DOX-induced cardiomyocytes apoptosis (Kalay et al., 2006). Considering the above findings, the cardio-protective effects of carvedilol appear to be related to its anti-oxidant and anti-apoptotic properties rather than its beta-blocking activity.

As discussed earlier, dexrazoxane is the only FDA approved cardio-protective agent for prevent DIC. Although it was initially thought to act through chelating to intracellular iron, later it was

discovered to exhibit its cardio-protective effect via its interaction with the topoisomerase II enzyme, thus preventing its binding to DOX (Lyu et al., 2007). A clinical trial conducted in children with ALL revealed the potential use of dexrazoxane at reducing the cardiac injury caused by DOX as evident from its role on reducing the serum concentrations of troponin T (Lipshultz et al., 2004). Despite its proven efficacy in several clinical trials, the American Society of Clinical Oncology still recommends its use to only metastatic breast cancer patients receiving already a cumulative dose of DOX of at least 300 mg/m^2 and who still need further treatment with DOX (Wexler et al., 1996; Swain et al., 1997; Lopez et al., 1998; Hensley et al., 2009; Lipshultz et al., 2010). It is also noteworthy to mention that for any other protective agent such as carvedilol to be approved by the FDA for the indication of cardio-protection from DIC, these agents should exhibit an equal or greater potency than dexrazoxane in a comparative clinical trial (Steiner and Hellmann, 2013).

D. *Hematopoietic Cytokines*

Erythropoietin (EPO) plays a key role in the hematopoiesis and is commonly used for treating anemia (Perreault and Venters, 2018). EPO receptors are found to be expressed in several tissues including the heart, brain, and skeletal muscle (Li et al., 2006b). EPO is proven efficacious *in vitro* as well as *in vivo* in mice by inhibiting apoptosis of cardiomyocytes and heart atrophy along with attenuation of left ventricular dysfunction in mice (Li et al., 2006b). This cardio-protective effect of EPO was investigated in male Wistar rats and was shown to be associated with a decreased oxidative stress and apoptosis of cardiomyocytes (Ammar et al., 2011). A recent communication by Cui et al linked the silent mating type information regulation 2 homolog 1 (SIRT1) to EPO mediated cardio-protection against DIC mediated through mitochondria dysfunction and toxicity (Cui et al., 2017). Similarly, thrombopoietin (TPO) showed cardio-protective effects while examined on H9c2 cells, neonatal rat primary myocytes, and mouse models (Li et al., 2006a). Further investigations in rat models of acute and chronic DOX treatment demonstrated that TPO rescued heart function in both models suggesting its effects to be mediated through modulating protein kinase B (Akt) and ERK pathways (Chan et al., 2011).

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E. Treatment of congestive heart failure

CHF is often irreversible, but also treatable. This can be done in two ways: 1) relieve pressure of the heart, and 2) minimize causing factors of CHF in general. Usually, high blood pressure is reduced by using ACE-inhibitors or angiotensin II receptor agonists. Beta-blockers can also protect the heart from negative effects of the body's own stress hormones. Hence, the heart beats steadier and needs less oxygen. Another option for the treatment of CHF would be using an anti-mineralcorticoid like spironolactone, which reduces fluid retention, and thereby the high blood pressure, thus relieving the heart.

IV. Therapeutic drug monitoring of doxorubicin-induced cardiotoxicity

A potential strategy that can be used for early detection and mitigation of DIC involves the therapeutic drug monitoring (TDM) of patients receiving DOX. Although traditionally, TDM focused on measuring the drug concentrations in plasma to assess the pharmacokinetics (PK) of the drug for designing optimal dosing regimens, in later years it helped to determine the incidence of toxicity and adverse drug reactions (Kang and Lee, 2009). TDM often demands the utility of a combined strategy integrating pharmaceutical, PK and pharmacodynamic (PD) analyses. PD measures such as identifying cardiac biomarkers can aid at monitoring DIC (Pongprot et al., 2012). **Table 2** gives an overview of different techniques that have been studied for the last 20 years and are being used presently. The aim of this review section is to take a closer look at these techniques to summarize the examined methods that are suitable for TDM of DOX with DIC as a dose-limiting toxicity.

As summarized in **Table 2**, various analytical techniques are available for TDM during DOX therapy, with varying degrees of sensitivity. Techniques such as measurements of DOX and doxorubicinol (DOX metabolite), LVEF or endomyocardial biopsy are well established for TDM. Further investigations are warranted for some of the newer methods such as In-111-antimyosin imaging, 123-labeled metaiodobenzylguanidine (^{123}I-MIBG) scintigraphy, or TUNEL assay. Therefore, a combination of guidelines, as outlined in **Figure 2**, and established monitoring-methods

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are required to identify the risk for developing severe cardiac dysfunction in a timely manner, and thus, reduce mortality rates.

Measurement of doxorubicin and doxorubicinol blood concentrations:

Although, DOX is proven cardiotoxic by itself, this serious side effect is amplified by its active metabolite, doxorubicinol (Olson et al., 1988). Despite several analytical high-performance liquid chromatography (HPLC) methods that are presently available for the quantification of both DOX and doxorubicinol in various biological matrices such as human and rat plasma as well as mice tumors, methods to quantify the concentrations of both analytes in mouse tissues are lacking (Arnold et al., 2004; Liu et al., 2008c; Ibsen et al., 2013; Sottani et al., 2013). One of the primary needs for such type of methods is to capture the bio-distribution profile of nano-formulated DOX (e.g. liposomal DOX). DOX delivery via nano-formulations is one of the preventative techniques to successfully reduce this metabolism-dependent cardiotoxicity. Besides diminishing the cardiovascular side effect of DOX through the slow release of free drug from the nanocarrier, nano-formulations are meant to improve free drug availability, delivery, and accumulation at the tumor site (Mazzucchelli et al., 2017). Other metabolites of DOX such as DOX deoxyaglycone, DOX hydroxyaglycone, doxorubicinol hydroxyaglycone are also suspected of increasing cardiac dysfunction by inducing stress or perturbing iron homeostasis (Licata et al., 2000).

HPLC is one example for an established method to analyze the various metabolites mentioned above. Using HPLC, it is possible to tailor plasma concentrations of DOX and its various metabolites in patients undergoing DOX treatment. Furthermore, metabolites of different anthracyclines (daunorubicin, idarubicin, DOX, epirubicin) have been measured by HPLC coupled with fluorescence detection at 233, 254 and 480nm. This technique with a lower limit of quantification (LLOQ) at 0.4 ng/mL and a signal-to noise ratio of 3 is suitable for monitoring chemotherapeutic regimens of cancer patients (Fogli et al., 1999). A far easier, faster, and more cost effective analytical technique is liquid chromatography mass spectroscopy coupled mass spectroscopy (LC-MS/MS),

which allows quantification of DOX and doxorubicinol in different mice bio-matrices such as plasma, liver, kidney or urine with a minimum quantity of samples. The different LLOQ of tested matrices via LC-MS/MS for DOX are summarized in **Table 2**. In addition to HPLC and LC-MS/MS, UHPLC-fluorescence appears to be another simple, fast, and cost effective technique to simultaneously measure large number of samples (about 15 samples per hour) in broad concentration ranges for DOX as well as doxorubicinol. In comparison, LC-MS/MS detection is expensive and requires a long run-time (Perez-Blanco et al., 2014). However, a caveat to this technique is that it only enables quantification of the concentrations of DOX and its metabolites and does not quantify any specific early cardiac markers of DOX-induced heart injury.

Measurement of doxorubicin-induced cardiotoxicity biomarkers:

1. *L eft ventricular ejection fraction (LVEF):*

Ejection fraction (EF) is the percent measurement of blood volume that the left ventricle (LV) pumps during each contraction. In normal conditions, the LV ejects between 50% and 70% of its total blood capacity. A decrease in the LVEF of the heart is an early sign of cardiac dysfunction. It is presumed to be reversible, however, during anthracycline therapy it may progressively evolve into irreversible cardiomyopathy with LVEF < 40%. Thus, LVEF is an important determinant for TDM of DOX-mediated cardiotoxicity (Jain, 2000). It can be measured by various techniques such as radionuclide angiocardiography, echocardiography, and Doppler echocardiography. LVEF analysis is easy to perform and provides reliable and reproducible measures of the LVEF. The latter has been used to detect and diagnose early cardiac alterations due to a variety of chemotherapeutics including DOX (Lu, 2005; Panjrath and Jain, 2006). However, changes in LVEF during DOX therapy may not entirely be due to DOX, but to the presence of other confounding non-cardiac conditions (Jain, 2000). Therefore, measurement of LVEF should not be used as the only method to monitor cardiac function of patients undergoing DOX treatment, but rather be combined with other techniques such as monitoring of plasma concentrations of DOX and its metabolites or measurement of the patient's angiocardiography at rest (**Figure 1**).

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2. *In vivo imaging and scintigraphic techniques:*

Several *in vivo* imaging and scintigraphic techniques have been reported in the literature for the TDM of chemotherapy induced heart failure (de Geus-Oei et al., 2011). These include (i) Indium-111 (In-111) antimyosin imaging, (ii) 123-labeled metaiodobenzylguanidine (^{123}I-MIBG) scintigraphy, and (iii) scintigraphic imaging using Tc-99 annexin (Hiroe et al., 1992; Lekakis et al., 1996; Bennink et al., 2004). All three techniques utilize radioactive labeled substances to identify different cardiac targets. In-111-antimyosin is a radioactive monoclonal antibody targeting intracellular myosin in the cardiac muscle. It was used as an immunoscintigraphic agent for the imaging of myocardial necrosis, hence, evaluating heart conditions related to diffuse myocardial cell injury (Hiroe et al., 1992). Uptake of In-111-antimyosin by the myocardium occurs only when the integrity of the sarcolemma is lost as a result of irreversible cardiac tissue damage (Hiroe et al., 1992). Although, this immunoscintigraphic technique has provided insights into the molecular mechanisms of chemotherapy-induced cardiotoxicity, presently, it is no longer commercially available.

^{123}I-MIBG scintigraphy is reported to be reproducible and sensitive, and is able to detect abnormalities of myocardial adrenergic innervation prior to LVEF decreases. A few minutes after intravenous (I.V.) injection of ^{123}I-MIBG, the LV myocardium can be visualized. Its initial cardiac concentration (15 min after I.V. injection) depends on the cardiac blood flow and reflects both the extra- and intra-vesicular accumulation of ^{123}I-MIBG in cardiac neurons. Generally, 4 hours after I.V. injection, this concentration reaches a constant value equivalent to the adrenergic neuron terminal concentration, used to examine specific cardiac neuron injury and loss of norepinephrine uptake function (Lekakis et al., 1996). In a study conducted on patients with various neoplasms and receiving DOX therapy, ^{123}I-MIBG cardiac uptake was decreased in a DOX dose-dependent manner, which demonstrates the cardiac adrenergic neurotoxic effect of DOX (Ono and Takahashi, 1994). Since the latter occurs much sooner than the decline of the EF, ^{123}I-MIBG scintigraphy remains an attractive option for early detection of drug-related heart failure.

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^{99m}Tc-annexin scintigraphy has long been used to image apoptotic cardiomyocytes. Although, currently, this technique is no longer used as a diagnostic method for chemotherapy-induced cardiac impairment, its application has contributed to a better understanding of myocardial injury at the molecular and cellular levels. Apoptotic cardiomyocytes have been identified in cancer patients during anthracycline therapy (Lefrak et al., 1973; Unverferth et al., 1983; Goorin et al., 1990; Lipshultz et al., 1991). When cardiomyocytes start undergoing apoptosis proteases and sphingomyelinases are activated. This leads to the phosphatidylserine (PS) molecules flipping to th extracellular layer of the cardiomyocyte membrane. At this stage, imaging of apoptotic cardiomyocytes is feasible using ^{99m}Tc-annexin V, which has a high affinity for the exposed PS molecules. These early phases of apoptosis precede the morphological changes of cardiomyocytes' outer membrane layer by forming blebs and vesicles. Additionally, intracellular alterations also occur such as breakdown of the cytoskeleton, cytoskeletal , reduction in the cytoplasm volume, condensation of the nuclear chromatin, and fragmentation of the DNA (Bennink et al., 2004). Several animal models of acute and chronic DOX-induced heart toxicity have been used, and showed an elevated uptake by the myocardium of ^{99m}Tc-annexin V. Animals with longer exposure to DOX showed higher DOX uptake. These results agreed well with findings on DOX cardiotoxicity examined using histopathology and immunohistochemistry techniques (Panjrath and Jain, 2006; Panjrath et al., 2007), and with other toxicity indices. (Bennink et al., 2004). An alternative technique is the TUNEL assay, which allows the characterization of late stages apoptosis by detecting DNA damage (Darzynkiewicz et al., 2008). This technique alone may not be entirely suitable for early TDM of DOX-inducedcardiotoxicity given that only late apoptotic cells can be measured. Therefore, a combination of scintigraphic imaging using ^{99m}Tc-annexin V and TUNEL assay to detect both early and late apoptotic cells may be a useful option for TDM.

3. *Specific clinical soluble cardiac biomarkers:*

Measurement of serum cardiac biomarkers such as cardiac troponins T and I, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) should be considered for TDM of DOX treatment

(Atas et al., 2015). Unlike scintigraphic and imaging techniques that have limited value in early detection of cardiotoxicity driven by chemotherapy, serum concentrations of these cardio-specific proteins released from damaged cardiomyocytes indicates early stages of myocardial injury. Initially, cardiac troponin T (cTnT) was evaluated as a biomarker of DIC in a spontaneously hypertensive rat model (Herman et al., 1998). Results of this study demonstrated the potential for this early cardiac biomarker as a non-invasive evaluation method for clinical use in DOX therapy. Although, cTnT was established as a suitable marker in several *in vivo* models, its application in children receiving DOX therapy is limited due to the lack of acute elevation of cTnT following DOX therapy (Kismet et al., 2004; Koh et al., 2004; Clark et al., 2007).

DOX treatment also results in increase in the brain natriuretic peptide (BNP) levels along with cTnT as demonstrated in a study in breast cancer patients (Advani et al., 2017). Recent evidence has established the superiority of two-dimensional speckle tracking echocardiography (2D-STE) over conventional electrocardiography, coupled with cTnT levels, effectively leading to early detection of cardiotoxicity, thus improving TDM of DOX therapy (Wang et al., 2017). Secretion of natriuretic peptides such as BNP was associated with impaired LV diastolic function during DOX therapy in adult patients with non-Hodgkin's lymphoma (Nousiainen et al., 2002). Natriuretic peptide concentrations were also significantly elevated in the plasma of pediatric cancer patients with LVEF dysfunction, however, they are best correlated with systolic and not diastolic function in contrast to adult patients (Hayakawa et al., 2001). Despite the correlation between natriuretic peptide concentrations and reduced LVEF, monitoring LVEF remains a clinical diagnostic and prognostic gold standard in DOX induced heart failure (Daugaard et al., 2005).

In summary, several techniques have been introduced for TDM of patients treated with DOX and who are at risk of developing DIC. The use of only one of these techniques is not sufficient to reliably detect and/or confirm heart injury as a consequence of DOX therapy. Hence, a combination of these strategies in a step-wise manner as proposed in our flowchart (**Figure 2**) is recommended. This approach will aid in the early diagnosis of cardiotoxicity caused by anthracycline-based therapy and

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will lead to faster clinical intervention and efficient management of this life-threatening adverse event. Finally, pilot studies in long-term survivors of childhood cancer suggest that comprehensive physical activity interventions may also be beneficial in avoiding delayed cardiotoxicity (Scully and Lipshultz, 2007). Further, following these survivors for long-term in a comprehensive clinical program for identifying the late-effects has also been demonstrated to be effective (Scully and Lipshultz, 2007).

V. Discussion and Conclusions

For most cancers, treatment with DOX remains an effective therapeutic option. However, the optimal use of DOX is restricted by its undesired cardiotoxicity and the limited possibilities for its prevention. As DOX is known to cause life-threatening cardiotoxicity in patients receiving cumulative doses of approximately 450-500 mg/m^2, altering the dosing regimens of DOX is perhaps one of the most feasible strategies that can be implemented clinically in order to lessen or even overcome DIC. Some pre-clinical and clinical studies have demonstrated that modification of the dosing schedule of DOX by switching from bolus dosing or short-term infusion to continuous prolonged infusion, or by dose-fractionation, leads to reduction in cardiotoxicity (Pacciarini et al., 1978; Legha et al., 1982b; Greene et al., 1983; Yeung et al., 2002). At present, there are no specific clinical practice guidelines for the management of DIC. Overall, available cardio-protective measures include a combination of natural antioxidants, β-blockers, ACE-inhibitors, angiotensin receptor blockers, diuretics, nitrates, and hydralazine (Gianni et al., 2008), and heart transplantation for end-stage heart failure. Among those, ACE-inhibitors and β-blockers showed best cardio-protective results in patients under DOX treatment (Kalay et al., 2006; Kaya et al., 2013; Cardinale et al., 2015). However, additional clinical studies are necessary to develop alternative pharmacological approaches and clinical practices for the diversified patient population treated with anthracycline based chemotherapy.

Other cardio-protective measures aim at interfering with molecular and cellular mechanisms altered by DOX such as the use of dexrazoxane, an iron-chelating agent. Not only is dexrazoxane the

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only drug with proven cardio-protective effects in cancer patients receiving anthracycline chemotherapy, but it is also the only cardio-protective drug approved by the Food and Drug Administration (FDA) in this population (Cvetkovic and Scott, 2005; Jones, 2008). Although dexrazoxane is a valuable option for prevention of DIC, a higher incidence of severe leukopenia (78% vs 68%; $p < 0.01$) is observed in patients receiving this drug. Hence, further investigations are required to identify agents that can mitigate the debilitating action of DOX on cardiomyocytes, along with an overall improved safety profile.

One of the key challenges that needs to be considered while selecting a cardio-protective agent for overcoming DIC is the parallel evaluation of the anticancer activity of that agent, such that oncological efficacy is not compromised. The key mechanisms of anticancer efficacy of DOX are 1.) DNA-damage caused by intercalation into DNA and disruption of topoisomerase II-mediated DNA repair, and 2.) generation of ROS that cause damage to the cell membrane, proteins, and DNA, resulting ultimately in apoptosis (Thorn et al., 2011). Since these mechanisms largely overlap with those of DIC, there is a risk of loss of oncological efficacy, especially when cardio-protective agents acting through these mechanisms are utilized. For example, in the case of dexrazoxane, a retrospective analysis demonstrated that dosing with dexrazoxane after a cumulative dose of >300 mg/m^2 of DOX in cancer patients, was not only cardio-protective, but also allowed retention of anticancer efficacy. This was in contrast to the treatment group that received dexrazoxane at the initiation of chemotherapy, wherein significant loss of anticancer efficacy was observed as compared to the group that received chemotherapy alone (Swain et al., 1997). Therefore, a potential strategy could include adjusting the timing of administration of cardio-protective agents such that oncological efficacy loss is minimized, while cardio-protective function is retained. Additionally, targeting pathways that are predominant in/specific to DIC, rather than DOX anticancer efficacy, could be another useful strategy to maintain the safety-efficacy balance with DOX-based therapy. For example, DOX is converted to its primary alcohol metabolite, doxorubicinol, via enzymatic conversion by aldo-keto reductase and carbonyl reductases 1 and 3 (Thorn et al., 2011). This metabolite plays a large role in causing

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cardiotoxicity via interference with iron and calcium homeostasis in cardiac tissue, and was demonstrated to be more potent than DOX itself (Olson et al., 1988). On the other hand, in the same study, DOX retained superior potency over doxorubicinol in pancreatic adenocarcinoma cell lines *in vitro*, thus allowing dissociation of the anticancer effects from cardiotoxic effects of DOX. Therefore, inhibition of these metabolizing enzymes may possibly be a useful strategy to provide protection from doxorubicinol-mediated cardiotoxicity (Schaupp et al., 2015). However, further *in vivo* studies and detailed analyses are warranted in order to apply the above discussed strategies.

So far, TDM of DIC is a feasible option to detect the early signs of heart failure in patients treated with DOX, thereby preventing severe cardiac adverse events resulting from continued exposure to DOX. The identification and validation of the earliest detectable, sensitive, and reliable biomarkers for DIC is one of the most active fields of research in translational cancer research and cardiovascular medicine (Jones et al., 2011). With the advent of highly sensitive and automated analytical techniques for the measurement of drugs and metabolites concentrations in blood, as well as the quantification of serum biomarkers discussed in this review, the combination of TDM along with early clinical symptoms of heart failure may be effectively and routinely implemented in the clinic to make DOX a safer chemotherapeutic option.

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Wrote or contributed to the writing of the manuscript: Wenningmann, Knapp, Ande, Vaidya, Ait-Oudhia

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Footnotes:

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Address correspondence to: Sihem Ait-Oudhia, Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida, 6550 Sanger Road. Office #469. Orlando. Florida. 32827. USA. Email: sihem.bihorel@cop.ufl.edu.

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Legend for Figures:

Figure 1: Intracellular signaling pathways altered by doxorubicin action on cardiomyocytes. Figure depicts molecular mechanisms of doxorubicin-mediated cardiomyopathy due to necrosis, apoptosis and autophagy.

Akt: AKT8 virus oncogene cellular homolog. **ATP**: adenosine triphosphate. **Bad**: Bcl-2-associated death promoter. **Bax**: bcl-2-like protein 4. **Bcl-2**: B-cell lymphoma 2. **Casp**: caspase. **CREB**: cAMP response element-binding protein. **EGFR**: epidermal growth factor receptor. **ERK**: extracellular signal–regulated kinases. **ETC**: electron transportation chain. **FasR**: Fas cell surface death receptor. **Hsp 90**: heat shock protein 90. **IRAK**: Interleukin-1 receptor-associated kinase. **JNK**= c. Jun N-terminal kinases. **MEK**: Mitogen-activated protein kinase. **NADH-D**: nicotinamide adenine dinucleotide (NADH) dehydrogenase. **NCX**: sodium-calcium exchanger. **NFκB**: nuclear factor kappa-light-chain-enhancer of activated B cells. **PI3K**: Phosphatidylinositol 3-kinase. **PUMA**: p53 upregulated modulator of apoptosis. **Raf**: Rapidly Accelerated Fibrosarcoma. **Ras**: Rat sarcoma. **RyR**: ryanodine receptor. **SERCA**: sarco/endoplasmic reticulum Ca^{2+}-ATPase. **TLR**: toll-like receptors.

Figure 2: Stepwise flowchart for the therapeutic drug monitoring of patients under doxorubicin treatment for the prevention of doxorubicin-induced cardiotoxicity as a dose limiting toxicity.

ANP: atrial natriuretic peptide. **BNP**: brain natriuretic peptide**. ET-1**: endothelin 1. **HPLC**: high performance liquid chromatography. **LC-MS/MS**: liquid chromatography with tandem mass spectrometry. **LVEF**: left ventricular ejection fraction. **UHPLC**: ultra-high performance liquid chromatography.

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Table 1: Preventive/alleviative strategies for doxorubicin-induced cardiotoxicity

Strategy	Mechanism of action	Benefits	Disadvantages	Model[a]
Altering the dosing regimen of doxorubicin	Lower peak plasma concentrations and hence lower concentrations in cardiac tissue	- Easiest way to intervene - Dose-fractionation and continuous infusion regimens have demonstrated decreased incidence of cardiac events such as CHF, cardiomyopathy and LVEF dysfunction - Anticancer efficacy was demonstrated to be uncompromised	Patient compliance may be an issue with slow continuous infusion regimens and/or increased dosing frequency	Mouse (Pacciarini et al., 1978), Rat (Yeung et al., 2002; Kamendi et al., 2015), Human (Weiss and Manthel, 1977; Chlebowski et al., 1980; Legha et al., 1982a; Torti et al., 1983)
Liposomal formulations of doxorubicin	Reduced circulating concentrations of free doxorubicin in the bloodstream due to liposomal encapsulation	- Lower concentrations of free doxorubicin in cardiac tissue resulting in reduced intensity of cardiac events as compared to conventional doxorubicin treatment	- Skin-related toxicities are commonly observed, such as, hand-foot syndrome (palmar-plantar erythrodysesthesia), rashes, melanotic macules etc. due to the unique PK and tissue distribution properties of PEGylated liposomal DOX - Stomatitis is another major toxic effect observed	Mouse (Forssen and Tökès, 1981; Kanter et al., 1993b), Beagle dog (Herman et al., 1983; Kanter et al., 1993a; Working et al., 1999), Rabbit (Working et al., 1999), Human (Berry et al., 1998; Lotem et al., 2000; Lyass et al., 2000; Safra et al., 2000; O'Brien et al., 2004)
Use of cardio-protective agents[b]				
Dexrazoxane	- Iron chelator that prevents reactive oxygen species (ROS) generation - Topoisomerase II (TOPII) inhibitor that reduces	- Has demonstrated long-term cardio-protective effects - FDA approved cardio-	- Severe myelosuppression due to additive effects with chemotherapeutics - Recommended only after receiving >300	H9c2 rat cardiomyocytes (Lyu et al., 2007; Deng et al., 2014b), Mouse (Deng et al., 2014b), Rat (Herman et al., 2000;

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	doxorubicin-induced DNA damage	protective agent	mg/m^2 of cumulative doxorubicin dose, as interference with anticancer activity has been observed when used with chemotherapy initiation	Lebrecht et al., 2007), Rabbit (Popelová et al., 2009), Human (Bu'Lock et al., 1993; Wexler et al., 1996; Lipshultz et al., 2010)
Erythropoietin	- Anti-atrophic effect on cardiac myocytes and an anti-fibrotic effect on the myocardium - Inhibitory effects on apoptosis of cardiomyocytes - Stimulation of SIRT1 leading to improvement in mitochondrial function and biogenesis	Attenuates left ventricular (LV) dysfunction and enhances LV contractility and cardiac recovery	- Lack of evidence of long-term cardio-protective effects in humans	Perfused isolated rat heart (Ammar et al., 2011), Rat (Ammar et al., 2011), Neonatal mouse ventricular cardiomyocytes (Kim et al., 2008), Mouse (Li et al., 2006b; Kim et al., 2008), AC16 human cardiomyocyte cell line (Cui et al., 2017)
Thrombopoietin	- Inhibitory effects on apoptosis of cardiomyocytes - Promotion of cardiomyocyte survival via Akt and ERK pathway activation			H9c2 rat cardiomyocytes (Li et al., 2006a), Primary neonatal rat cardiomyocytes (Li et al., 2006a), Mouse (Li et al., 2006a), Rat (Chan et al., 2011)
Vitamins A, C and E	Scavengers of free radicals/reactive oxygen and nitrogen species (ROS/RNS) due to antioxidant properties	Reduces short term cardiotoxicity	- Long term cardiotoxicity may not be alleviated due to persistence of mechanisms other than free radical generation in cardiac tissue - Anticancer efficacy may be	Rat (Tesoriere et al., 1994; Puri et al., 2005; Viswanatha Swamy et al., 2011; Akolkar et al., 2017), Rabbit (Milei et al., 1986), Isolated rat cardiomyocytes (Ludke et al.,

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			compromised due to antagonistic effects with doxorubicin - Lack of reports on the effect on anticancer efficacy, especially in human studies	2012; Ludke et al., 2017)
Melatonin				Mouse (Wahab et al., 2000; Liu et al., 2002), Rat (Dziegiel et al., 2002; Oz et al., 2006; Othman et al., 2008), Human (Lissoni et al., 1999)
N-acetylcysteine				Mouse (Doroshow et al., 1981), Rat (Arica et al., 2013a; Farshid et al., 2014), Beagle (Herman et al., 1985), Human (Jo et al., 2013)
Amifostine				Perfused isolated rat heart (Nazeyrollas et al., 1999), Infant rat (Jahnukainen et al., 2001), Rat (Herman et al., 2000; Dragojevic-Simic et al., 2004), Rabbit (Potemski et al., 2006), Human (Gallegos-Castorena et al., 2007)
Mangiferin	- Prevention of $OH^{\cdot}$ radical formation and lipid peroxidation by iron chelation - Regulates intracellular calcium homeostasis			Rat (Arozal et al., 2015; Agustini et al., 2016)
Probucol	Enhancement of endogenous antioxidants such as glutathione peroxidase and			Rat (Siveski-Iliskovic et al., 1994; Siveski-Iliskovic et al., 1995; Kumar et

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	superoxide dismutase			al., 2001), Mouse (Walker et al., 2011)
Carvedilol	- Inhibition of free radical generation due to antioxidant properties - Inhibition of apoptotic pathways in doxorubicin-induced cardiomyocyte death			H9c2 rat heart cell line (Spallarossa et al., 2004), Rat (Santos et al., 2002; Oliveira et al., 2004), Human (Tashakori Beheshti et al., 2016; Nabati et al., 2017)

[a] Refers to experimental models that were utilized to demonstrate benefits or disadvantages of the respective strategies. The list of references in parantheses is not exhaustive, but representative of the studies conducted.

[b] The list of cardio-protective agents is not exhaustive. Some commonly studied/reported agents are listed in this table.

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Table 2: Summary of analytical techniques for measurement of doxorubicin (DOX) and doxorubicinol (DOXL) blood concentrations for therapeutic drug monitoring (TDM)

Methof of measurement	**Measured variable**	**Lower limit of quantification (LLOQ)**
High performance liquid chromatography with fluorescence detection (Fogli et al., 1999)	Daunorubicin, idarubicin, DOX, epirubicin and their 13-dihydro metabolites in human plasma samples	0.4 ng/mL
Liquid chromatography with tandem mass spectrometry (Mazzucchelli et al., 2017)	Concentration of DOX, DOXL in mouse plasma, liver, kidney, tumor, urine	*Tissue* *DOX* *DOXL* Plasma 0.04 0.24 Liver 0.12 0.3 Kidney 0.43 0.32 Tumor 0.52 0.35 Urine 0.025 0.09
Ultra-high performance liquid chromatography - fluorescence (Perez-Blanco et al., 2014)	Concentrations of DOX and DOXL in human plasma	DOX = 8 ng/mL DOXL = 3 ng/mL
Radionuclide angiocardiography (Lu, 2005; Panjrath and Jain, 2006)	Measurement of left ventricular ejection fraction (LVEF)	Not Applicable
Myocardial imaging using 111Inantimyosin antibody (Hiroe et al., 1992)	Uptake of 111In antimyosin antibody in myocardium by immunoscintigraphy in rats to evaluate myocardial damage	Not Applicable
123-labeled metaiodobenzylguanidine (MIBG) scintigraphy (Lekakis et al., 1996)	Cardiac 123I-MIBG uptake to generate adrenergic neuronal imaging in doxorubicin treated patients	Not Applicable
Scintigraphic detection of apoptosis (Bennink et al., 2004)	Assessment of early apoptosis using annexin V scintigraphy in rats	Not Applicable

Figure 1

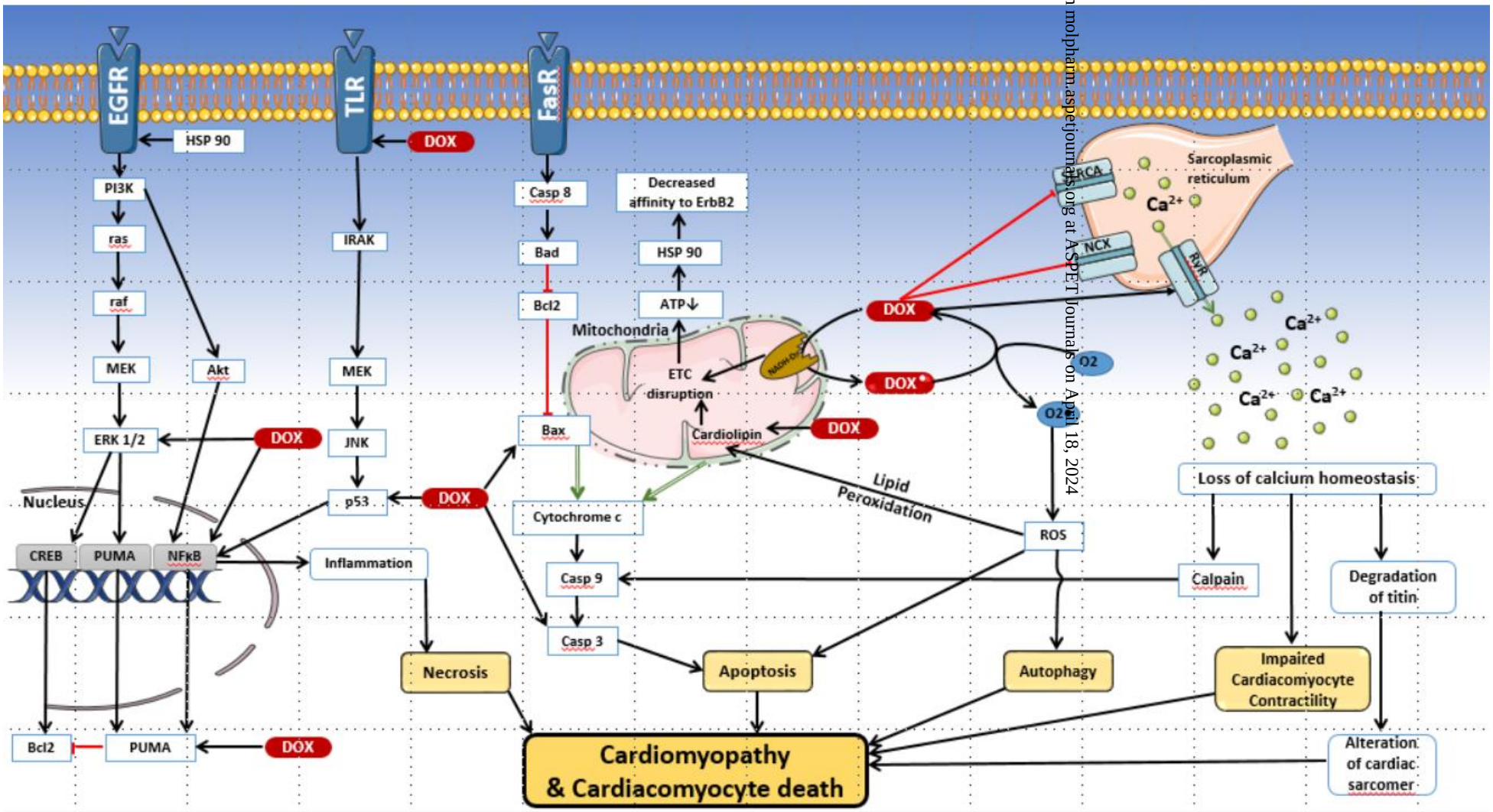

Figure 2