Dantrolene Attenuates Cardiotoxicity of Doxorubicin Without Reducing its Antitumor Efficacy in a Breast Cancer Model

Valentina K. Todorova[*], Eric R. Siegel[†], Yihong Kaufmann[*], Asangi Kumarapeli[‡], Aaron Owen[*], Jeanne Y. Wei[§], Issam Makhoul[¶] and V. Suzanne Klimberg[*,1]

[*]Department of Surgery, University of Arkansas for Medical Sciences, Little Rock, USA; [†]Department of Biostatistics, University of Arkansas for Medical Sciences, Little Rock, USA; [‡]Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, AR, USA; [§]Department of Geriatrics, University of Arkansas for Medical Sciences, Little Rock, USA; [¶]Division of Medical Oncology, University of Arkansas for Medical Sciences, Little Rock, USA

Abstract

Dysregulation of calcium homeostasis is a major mechanism of doxorubicin (DOX)-induced cardiotoxicity. Treatment with DOX causes activation of sarcoplasmic reticulum (SR) ryanodine receptor (RYR) and rapid release of Ca^{2+} in the cytoplasm resulting in depression of myocardial function. The aim of this study was to examine the effect of dantrolene (DNT) a RYR blocker on both the cardiotoxicity and antitumor activity of DOX in a rat model of breast cancer. Female F344 rats with implanted MAT B III breast cancer cells were randomized to receive intraperitoneal DOX twice per week (12 mg/kg total dose), 5 mg/kg/day oral DNT or a combination of DOX + DNT for 3 weeks. Echocardiography and blood troponin I levels were used to measure myocardial injury. Hearts and tumors were evaluated for histopathological alterations. Blood glutathione was assessed as a measure of oxidative stress. The results showed that DNT improved DOX-induced alterations in the echocardiographic parameters by 50%. Histopathologic analysis of hearts showed reduced DOX induced cardiotoxicity in the group treated with DOX + DNT as shown by reduced interstitial edema, cytoplasmic vacuolization, and myofibrillar disruption, compared with DOX-only—treated hearts. Rats treated with DNT lost less body weight, had higher blood GSH levels and lower troponin I levels than DOX-treated rats. These data indicate that DNT is able to provide protection against DOX cardiotoxicity without reducing its antitumor activity. Further studies are needed to determine the optimal dosing of DNT and DOX in a tumor-bearing host.

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Introduction

Doxorubicin (DOX) is an anthracycline antibiotic commonly used for treatment of various malignancies. DOX can cause unpredictable and sometimes irreversible cardiac toxicity, which remains a major limitation in cancer chemotherapy [1,2]. DOX-induced cardiotoxicity is cumulative-dose—dependent and begins with the first dose of chemotherapy [3,4]. The acute/subacute cardiovascular complications of DOX are characterized by various atrial and ventricular arrhythmia [5,6], whereas the cumulative chronic cardiotoxicity is typically manifested as irreversible cardiomyopathy, which, in turn, leads to congestive heart failure [3].

The mechanism of DOX-induced cardiotoxicity is not well understood, making it difficult to predict or prevent cardiotoxicity in the individual patients [7]. It is believed that DOX-induced cardiotoxicity is a result of the summation and mutual feedback of diverse processes [8,9], with the major mechanism considered to be the oxidative stress [10,11]. Redox-related metabolic transformation of DOX results in overproduction of reactive oxygen species (ROS) and nitrogen species [12]. ROS alter calcium homeostasis in various muscle cell types via disruption of normal sarcoplasmic reticulum (SR) function, including activation of Ca^{2+}ryanodine receptors/ Ca^{2+-}releasing channels (RYRs) to induce Ca^{2+} release from SR and inhibition of the Ca^{2+} ATPase pump (SERCA) [13,14]. Treatment with high concentrations of ryanodine [15], or Ca^{2+} chelators [16] inhibits DOX-mediated ROS production. These reports indicate that increased DOX/ROS-mediated abnormal Ca^{2+} release may play an important role in DOX-induced cardiotoxicity. It has also been found that RYRs have several sites for binding DOX and the binding occurs regardless of whether the channel is open or closed [17]. DOX can bind and open RYR2 which contributes to the release of Ca^{2+} from SR and increases the levels of Ca^{2+} in the cytoplasm, resulting in activation of contractile proteins and initiation of muscle contraction [18]. It has been suggested that Ca^{2+} uptake into SR and restoration of SR Ca^{2+} levels is inhibited by redox modification of SERCA2A by the DOX metabolite doxorubicinol, leading to calcium overload [19].

Dantrolene (DNT) is a postsynaptic muscle relaxant that lessens excitation-contraction coupling in muscle cells. It is commonly used to treat malignant hyperthermia, a genetic predisposition to excessive intracellular Ca^{2+} release on exposure to volatile anesthetics. DNT was able to block both RYRs [20] and to correct the abnormal Ca^{2+} in experimentally induced heart failure [21]. Rat experimental studies have tested and compared single intraperitoneal (i.p.) doses of DNT at 5 mg/kg and 10 mg/kg [22,23] and intravenous (i.v.) doses of DNT at 3 mg/kg, 10 mg/kg, and 20 mg/kg [24]. DNT at a dose of 5 mg/kg was able to protect against DOX-induced cardiotoxicity [25], isoproterenol cardiotoxicity [22], and whole body irradiation [26].

DNT and its analog azumolene were suggested as potent inducers of cell death in tumor cell culture [27] because of antioxidant properties [28]. However, there are no published studies on the effect of DNT in a tumor-bearing host. Therefore, the present study aimed to obtain preliminary data on the efficacy of DNT as an adjuvant to chronic DOX chemotherapy in a tumor-bearing host using a rat breast cancer model [29,30].

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the Central Arkansas Veterans Healthcare System (CAVHS), where the animals were housed, treated, and sacrificed. All procedures and experiments complied with the guidelines to minimize animal suffering.

Animal Model

Female Fisher344 rats (NCI, Frederick, MD) weighing 140–150 g were used. The rats were maintained two/cage in standard cages in the animal care facility and housed under controlled conditions of a 12-hours light/dark cycle at 21 °C. Food and water were provided ad libitum. Orthotopic breast cancer was created by injecting $1 \times 10\hat{\ }6$ 13762 MAT B III rat mammary adenocarcinoma cells (ATCC, Manassas, VA) in 0.2 ml saline into the mammary fat pad of rats anesthetized with 2% isoflurane/oxygen, as described in our previous publications [30,31]. After tumors reached 5–6 mm in diameter, the rats were randomly assigned into the following treatment groups: (1) DOX-treated ($n = 14$) group received intraperitoneally (i.p.) 2 mg/kg/injection of doxorubicin hydrochloride (Sigma Chemical Co., St. Louis, MO) diluted in saline twice a week for 3 weeks (6 doses for a total dose of 12 mg/kg); (2) DNT-treated group ($n = 14$) received by oral gavage 5 mg/kg/day dantrolene sodium (Revonto, Novartis, Greenville, NC) diluted in water for 3 weeks; (3) DOX + DNT ($n = 14$) group received DOX and DNT as described for DOX and DNT groups; and (4) tumor growth control group injected with saline and gavaged with water ($n = 10$); and (5) naïve tumorfree controls for body weight change comparison ($n = 5$). Rat body weights were recorded daily and tumor size was measured using calipers. Animals were sacrificed after the completion of the treatment. Blood was withdrawn via heart puncture with a heparin-containing syringe. Samples from tumors and the left ventricle (LV) were fixed in neutral buffered formaldehyde (4% wt/ vol). Tumor volumes were calculated according to the formula: $V = 0.5 \times (ab\hat{\ }2)$, where "a" is the longer diameter and "b" is the shorter diameter of the tumor [31].

Echocardiography

Cardiac pathophysiological alterations were examined by echocardiography before tumor implantation and after completion of treatment using ultrasound imaging system Vevo 770 High-Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada) with an RMV 707B Scanhead, as described previously [30].

Histopathological Assessment of Cardiac and Tumor Tissue

Tissue samples from the left ventricle were fixed in 10% neutral buffered formalin and processed for paraffin-embedded sections of 4 μm thickness. Sections were stained with hematoxylin and eosin (H&E) for routine light microscopic examination. The myocardium was scored using a semiquantitative method as described [32,33] for the severity and extent of cardiomyopathy, specifically for cytoplasmic vacuolization, edema, vascular congestion and myofibrillar degeneration. The latter parameter encompassed assessing for hypereosinophilic cytoplasm, loss of striations and pyknosis or karryorhexis of cardiomyocytes. Briefly, the above changes were graded as no change (0), mild (1), moderate (2) and marked (3) to facilitate recognition of trends in lesional severity. Formalin-fixed and paraffin-embedded sections were also prepared also from representative breast tumors from the study groups.

GSH Determination

GSH concentrations of whole blood were determined by a standard enzymatic recycling procedure, as previously described [34]. Briefly, 500 μl of heparinized blood was mixed with equal volumes of 10% 5-sulfosalicylic acid and centrifuged at 5 °C at $3000 \times g$ for 15 min. Ten μL of the supernatant was added to 1 ml of reaction mix (0.2 mmol/L NADP, 0.6 mmol/L 5,5-dithio-bis-2-nitrobenzoic acid and 1.33 units GSH reductase) and the absorbance was measured at 412 nm. The data were expressed as nmol/g tissue and μmol/L.

Plasma Troponin Measurement

Plasma concentrations of cardiac troponin I (cTnI) were determined using Rat Cardiac Troponin-I ELISA kit (Life Diagnostics, West Chester, Pa). The results were expressed as ng/ml.

Statistical Analysis

Data were analyzed statistically using the MIXED Procedure from SAS v9.2 (The SAS Institute, Cary, NC). Briefly, each rat had its body-weight change, its tumor-volume change, and all of its cardiac-function responses calculated as the measured value at sacrifice minus the measured value at baseline. The resulting within-subject changes and responses were analyzed via one-way ANOVAs with post hoc pairwise comparisons for whether the amount of change or response differed significantly between treatment groups. Before the one-way ANOVAs, the changes in body weight and tumor volume were examined via likelihood-ratio test for variance heterogeneity between treatment groups (no significant variance heterogeneity was detected). All statistical hypothesis tests were two-sided and used a $P < 0.05$ significance level despite the multiple comparisons, in order not to inflate Type II (false negative) error in this modestly powered pilot study.

Results

Effect of DNT on DOX-Induced Body Weight Loss and Survival

The body weight change, which is a good indicator of the general health status of laboratory animals, is presented in Figure 1. The means of body weight for the baseline between the groups were not significantly different. Tumor-control rats and rats in the DNT group lost less body weight in comparison with the groups treated with DOX alone or DOX + DNT (group means ± SDs of body-weight loss were 14 ± 4 g for the tumor controls, 25 ± 5 g for the DOX group, 18 ± 6 g for the DNT group, and 23 ± 8 g for the DNT + DOX group). This finding was associated with a 40% mortality in the group of rats treated with DOX alone, compared with only 16% mortality in the DNT + DOX group and 0% mortality in the group treated with DNT alone during the 3-week study period (not shown).

Echocardiographic Assessment of Cardiac Physiological Alterations

To determine whether DNT can provide cardioprotection during chronic DOX treatment of tumor-bearing rats, we used echocardiography to measure changes in cardiac output (CO) and stroke volume (SV) before and after tumor implantation. Heart rate was similar between the groups and was not significantly affected by tumor presence or treatments (260−325 beats/min). Once the tumor implants had engrafted, we measured CO and SV in the rats before and after their assigned treatment. Changes in CO and SV after treatment (denoted ΔCO and ΔSV, respectively) are presented in Figure 2. The results showed that DOX treatment significantly impaired cardiac function (means ± SDs of −15.1 ± 14.3 mil/min for ΔCO and −16.3 ± 39.0 μl for ΔSV). In contrast, DNT preserved both CO and SV in DNT treatment alone (2.8 ± 17.6 ml/min for ΔCO and 11.3 ± 38.4 μl for ΔSV) and in DOX + DNT treatment (−1.1 ± 10.8 ml/min for ΔCO and 5.5 ± 40.1 μl for ΔSV). ANOVA post hoc analysis of the treatment-group differences in ΔCO showed that the 17.9-ml/min difference in DNT versus DOX was statistically significant ($P = 0.0041$), and that the 14.0-ml/min difference in DNT + DOX versus single-agent DOX was also statistically significant ($P = 0.022$), but that the 3.9-ml/min difference between DNT + DOX versus DNT alone was not ($P = 0.52$). However, the same analysis of treatment group difference in ΔSV showed that none of the groups differed significantly from each other in the amount of stroke-volume degradation (P-values of 0.088, 0.17, and 0.72, respectively, for DNT versus DOX, DOX + DNT versus DOX alone, and DOX + DNT versus DNT alone).

Histopathological Examination

DOX-treated cardiac tissue is characterized by excessive myofibrillar degeneration, swelling of sarcoplasmic reticulum (SR) as well as
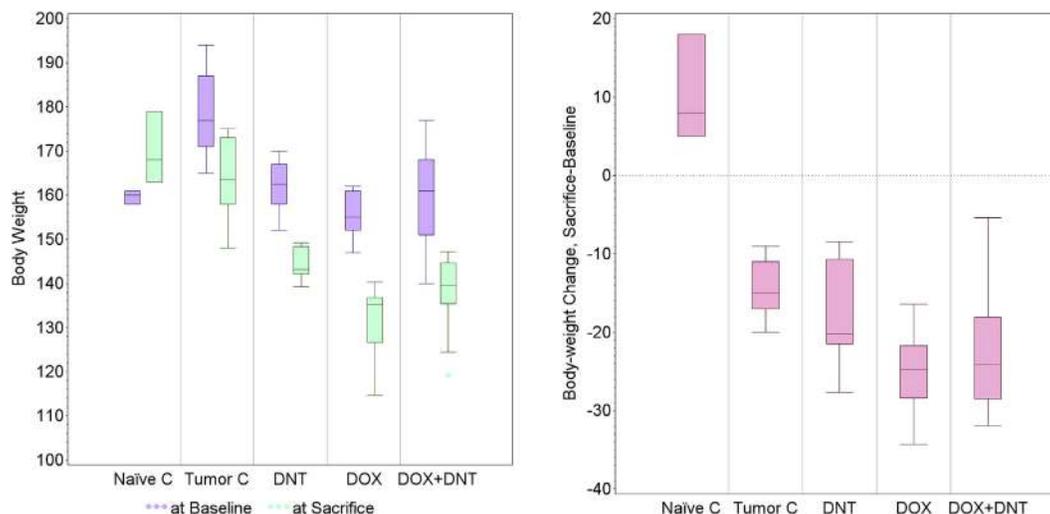

Figure 1. Effects of chronic DOX, DNT, and DOX + DNT on body weight loss from baseline of surviving rats with implanted breast tumors. Bottoms and tops of boxes respectively show first and third quartiles; horizontal lines dividing boxes show medians, and symbols inside boxes show means. $n = 5$ Naive controls (both time points), $n = 10$ Tumor controls (both time points), $n = 14$ in the DOX group at baseline ($n = 11$ at sacrifice), $n = 14$ in the DNT group (both time points), and $n = 14$ in the DOX + DNT group at baseline ($n = 12$ at sacrifice).
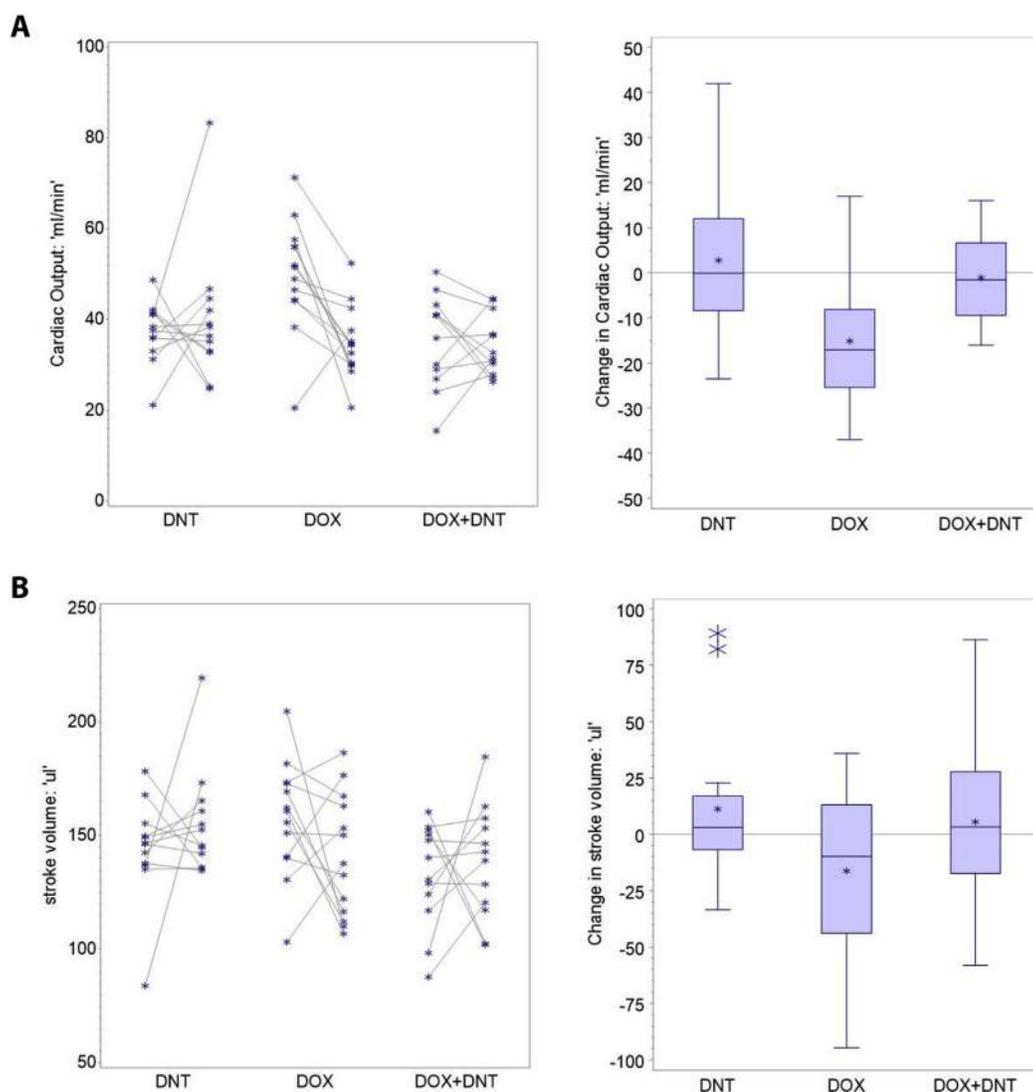
Figure 2. Changes in cardiac output (A) and stroke volume (B) before the treatment ($n = 14$/group) and after the treatment of the surviving rats with breast tumors ($n = 13$/DOX group; $n = 12$/DNT group; $n = 12$/DOX + DNT group) treated with chronic DOX, DNT and DOX + DNT.

mitochondria, myocyte vacuolization, granulation of cytoplasm, and hypereosinophilia [17]. We have applied a semiquantitative assessment of the histopathological alterations of the hearts of the surviving rats treated with chronic DOX, DNT and DOX + DNT. The results indicate that DNT reduced DOX-induced cardiotoxicity in the group treated with DOX + DNT as shown by reduced interstitial edema, cytoplasmic vacuolization and myofibrillar disruption, compared with DOX only treated hearts (Figure 3). However, when compared with drug-free tumor controls, single-agent DNT leads to increases in histopathology scores for all of the examined markers.

cTnI Plasma Concentration

Previously we have found that DOX administration caused increase in the circulating level of cTnI in this rat model of breast cancer [30]. The results from this study showed that, at the end of the experiment, when compared with that in the DOX group, the cTnI concentration in the plasma of rats treated with DNT was 30% lower ($P = 0.028$), whereas the cTnI concentration in the plasma of rats

treated with DNT + DOX was 27% lower ($P = 0.11$) (Figure 4). However, when the groups' cTnI plasma concentrations were compared with that in the untreated tumor-control group, they were 68% higher ($P = 0.0057$) in the DNT group, and 77% higher ($P = 0.030$) in the DNT + DOX group (Figure 4). These data suggest that the amount of tumor burden and weight loss can induce cardiotoxicity. These data also correlate with the results from the echocardiography showing that DNT can decrease DOX-induced severe cardiotoxicity, even in the presence of tumor.

GSH Concentration

GSH (glutamyl-cystein-glycine) is a sulfhydryl (-SH) antioxidant, ubiquitous in living organisms, that has multiple functions, including scavenging oxidants and detoxifying toxic substances [35,36]. Reduced glutathione (GSH) can be converted to oxidized glutathione (GSSG) during oxidative stress and the ratio GS/GSSG is considered a marker for oxidative stress [37]. The results from this study (Figure 5) showed that rats treated with DNT had strongly elevated
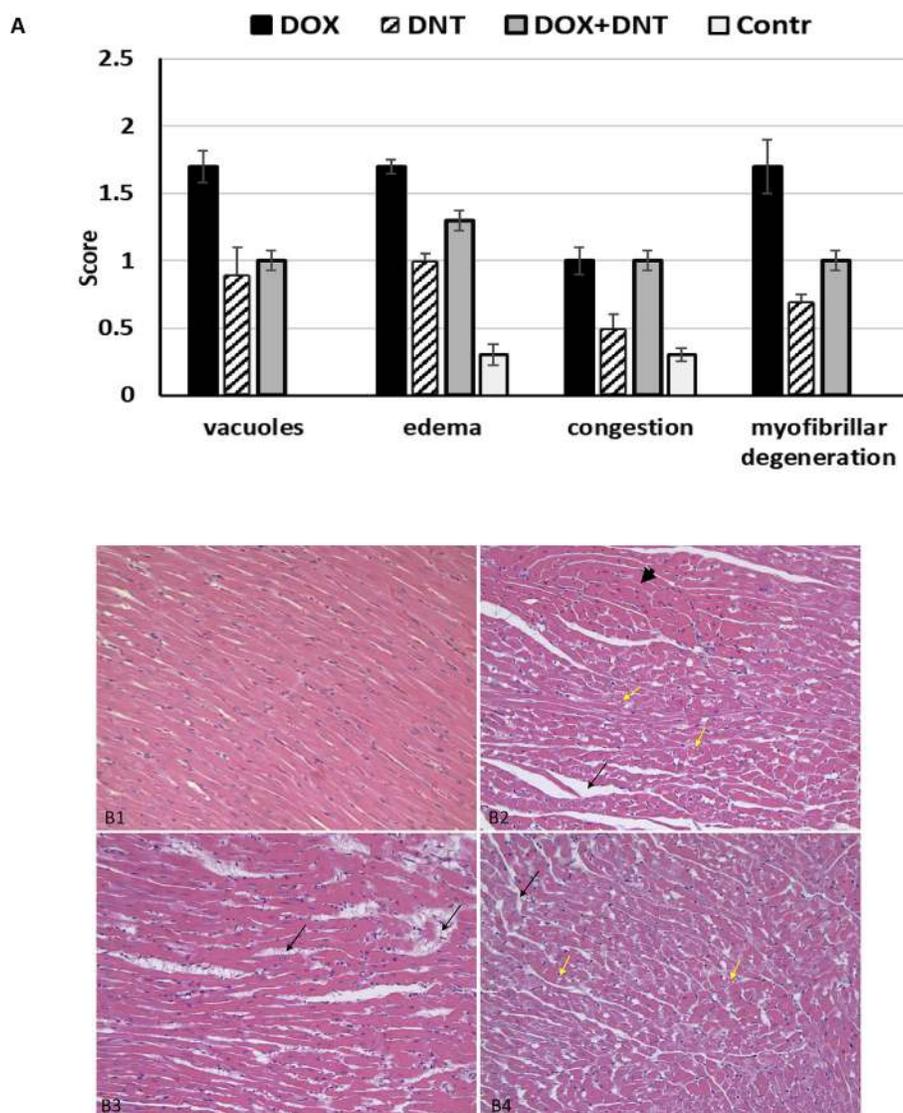
Figure 3. Histopathological alterations in the hearts of the surviving rats with breast tumors treated with chronic DOX ($n = 9$), DNT ($n = 14$) or DOX + DNT ($n = 12$) versus no tumor-controls. A. Results from a semiquatitative assessment of histopathological alterations (mean ± SE, $n = 9$/DOX group; $n = 10$/DNT group; $n = 10$/DOX + DNT group; $n = 5$/control group. *$P < 0.05$ DOX vs all. B. Composite image of representative histopathologic changes in myocardium of the study groups: B1: normal myocardial histology in naïve controls; B2 cytoplasmic vacuoles (yellow arrows), edema (black arrow) and eosinophilic myocytes with pyknotic nuclei (thick black arrow) in rats treated with DOX; B3: edema (black arrows) in rats treated with DNT + DOX; B4: cytoplasmic vacuoles (yellow arrows) and mild edema (black arrow) in rats treated with DNT. Hematoxylin—eosin stain, magnification 200×.

levels of GSH and GSH/GSSG in comparison with rats treated with DOX, whereas rats treated with a combination of DOX + DNT had only mildly elevated levels. Compared with DOX rats, DNT rats has 95% higher mean GSH levels ($P < 0.0001$) and 63% higher mean GSH/GSSG ratios ($P = 0.0014$), whereas DNT + DOX rats had 31% higher mean GSH levels ($P = 0.019$) and 24% higher mean GSH/GSSG ratios ($P = 0.13$).

Effect of DNT on Antitumor Efficacy of DOX

Figure 6 shows the means for tumor volumes of the rats treated with DOX alone, DNT alone, DOX + DNT and control tumors. At this dose, when compared with DNT alone, DOX alone reduced tumor growth by 62% ($P = 0.0003$) whereas DOX + DNT reduced it by 48% ($P = 0.0033$), but the 14% difference in tumor-growth reduction by DOX alone versus DOX + DNT was not statistically

significant ($P = 0.39$). The histopathological examination of the tumors showed increased presence of necrosis in the tumors of rats treated with DOX + DNT in comparison with DNT group, which was comparable with the necrosis detected in tumors of rats treated with DOX alone.

Discussion

DOX-induced cardiotoxicity may not be detected for many years and remains a life-long threat. Approximately 10% of patients treated with DOX or its derivatives develop cardiac complications up to 10 years after the cessation of chemotherapy [12]. As many as 65% of patients with a childhood malignancy treated with DOX have echocardiographic evidence of left ventricular contractile abnormalities as adults [7,38]. Cardiovascular disease is the major cause of competing mortality in women with early breast cancer, and women
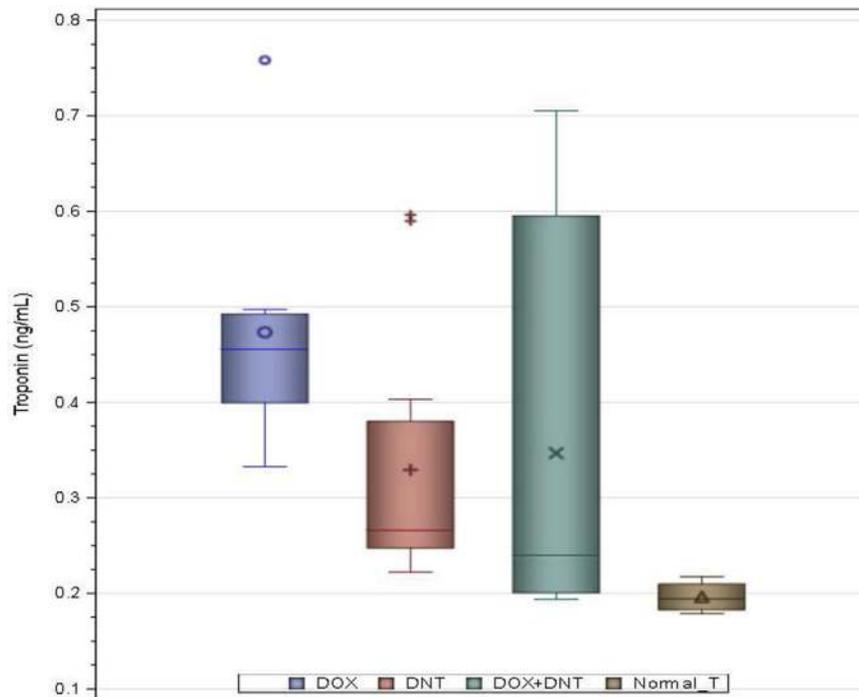
Figure 4. Average plasma cTnI concentration in the surviving rats treated with chronic DOX ($n = 8$), DNT ($n = 12$), DOX + DNT ($n = 11$) and control group ($n = 4$). Bottoms and tops of boxes, respectively, show first and third quartiles; horizontal lines dividing boxes show medians, and symbols inside boxes show means.

Figure 5. Blood GSH levels (A), GSSG (B) and ratio GSH/GSSG (C) in the surviving rats treated with chronic DOX ($n = 8$), DNT ($n = 13$), DOX + DNT ($n = 11$) and control group ($n = 3$). Bottoms and tops of boxes respectively show first and third quartiles; horizontal lines dividing boxes show medians, and symbols inside boxes show means.
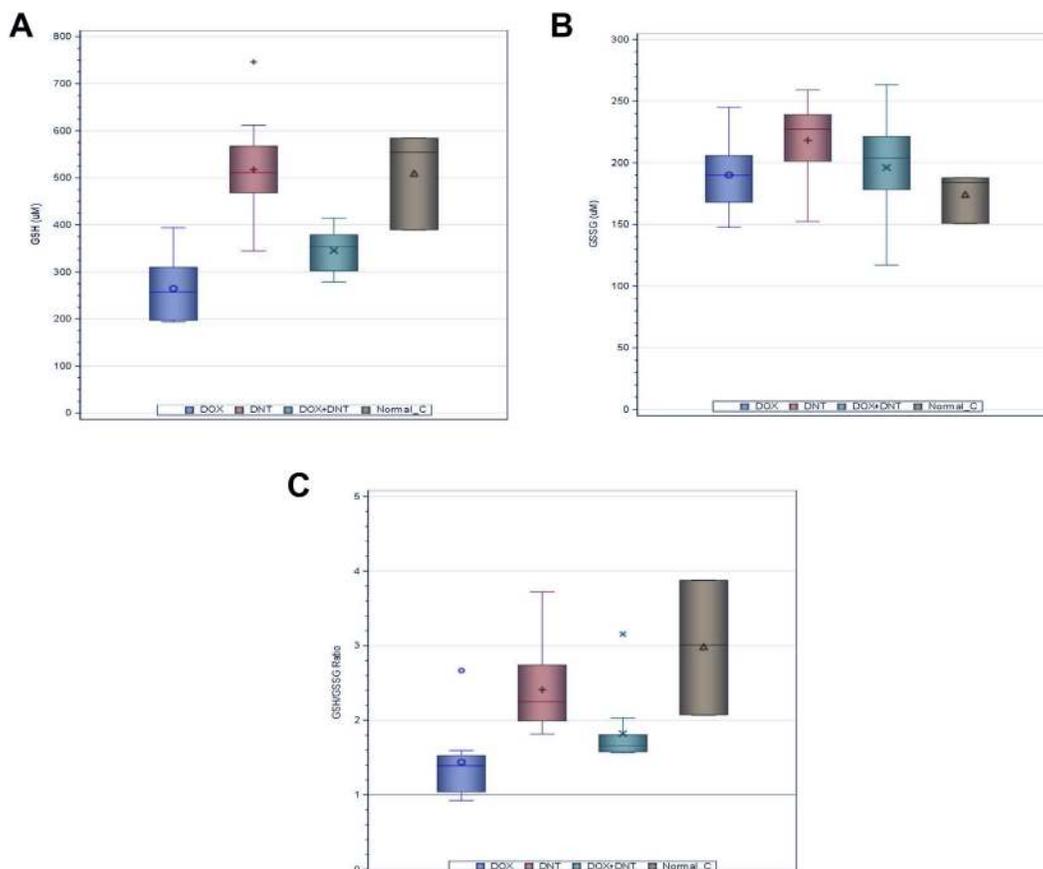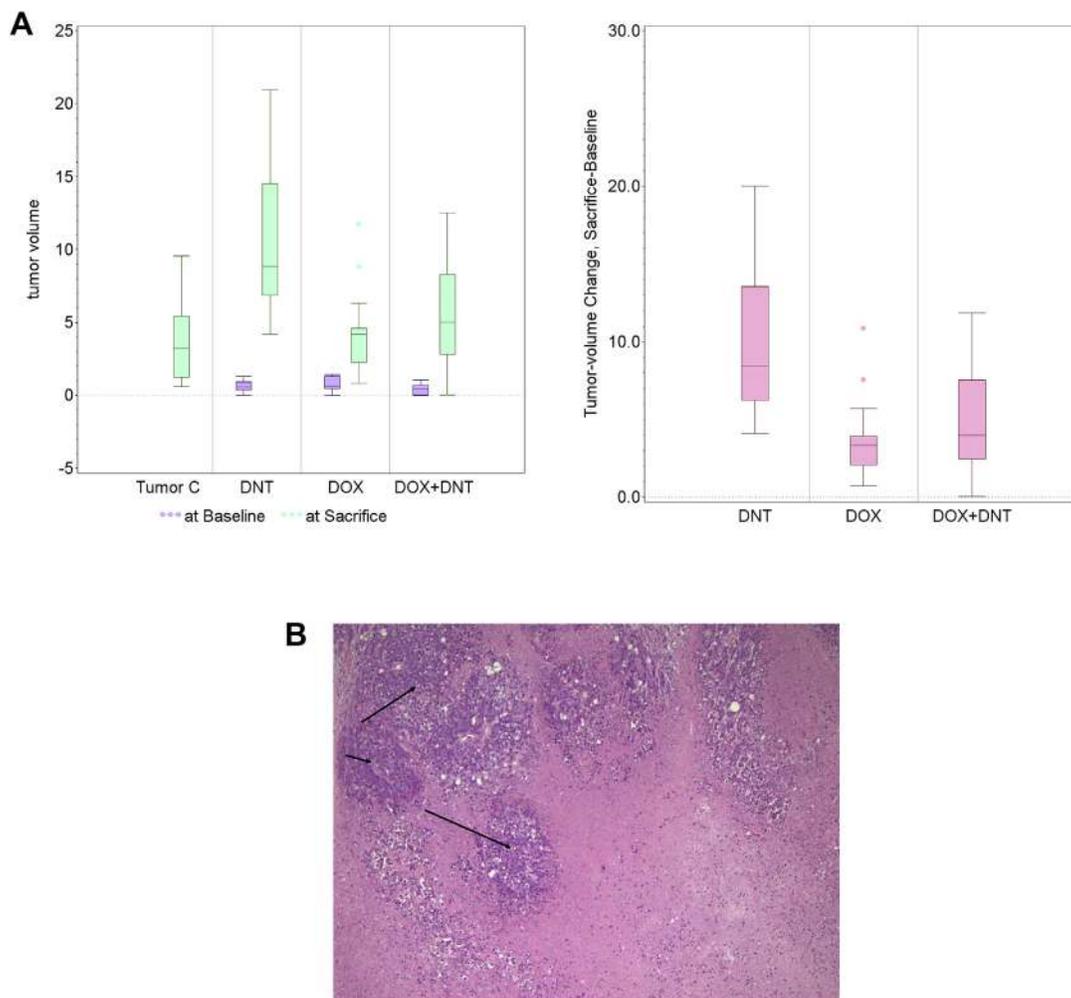
Figure 6. Effect of chronic DOX, DNT and DOX + DNT on breast tumor growth in surviving rats. Tumor volumes of rats with treated with chronic DOX ($n = 9$), DNT ($n = 14$) and DOX + DNT ($n = 12$-versus controls with untreated tumor ($n = 10$) (A). Representative image of tumor necrosis in a rat treated with DOX (B). Arrows show viable tumor nests in a background of prominent tumor necrosis. Hematoxylin and eosin stain, magnification 200×.

with breast cancer have an excess risk of cardiovascular diseases relative to age-matched women without a history of breast cancer [39,40]. The role of ROS in the pathogenesis of DOX-induced cardiotoxicity and heart failure has provided a basis for coadministration of antioxidants to counteract cardiotoxicity, but have not been successful. Dexrazoxane is the only approved drug used in clinical settings as cardioprotective agent, but its clinical use has been limited because of reports showing a possible interference with antitumor activity of DOX and the potential risk of a second malignancy [41–43]. The search for new approaches to prevent DOX-induced cardiotoxicity remains a critical issue in both oncology and cardiology.

DNT, an antagonist of RYRs is an FDA-approved drug for clinical treatment of malignant hyperthermia and several other disorders associated with dysregulation of Ca^{2+} homeostasis, such as neuroleptic malignant syndrome, muscle spasticity, ecstasy intoxication, and Alzheimers [44–47]. Because DOX is a widely used chemotherapy agent, efforts are directed towards reducing its off-target toxicity without alteration of anticancer efficacy.

In this study we have used our rat breast cancer model [30,48,31] which closely resembles human breast cancer to examine the

cardioprotective actions of DNT against chronic DOX, a scenario similar to the clinical situation. An important finding of this study was that DNT was able to protect the heart from DOX toxicity in the presence of tumors, as evident by preservation of CO and SV, without affecting DOX antitumor efficacy. However, single treatment with DNT at this dose led to the increase in histopathological scores of heart vacuolization, edema, and myofibrillar degeneration in comparison with the untreated tumor controls. This observation indicates that the dose used in this study may be too high and the dose–response needs to be determined in the future studies.

It has been found that malignant disease negatively affects cardiac function in patients and animal models independently of cardiotoxicity of anticancer treatment, because of the oxidative and inflammatory responses elicited by the cancer [49–51]. Our previous studies using the same rat model of breast cancer showed the damaging effects of the tumor presence on the heart function [31,29].

Malignancy depletes host GSH levels resulting in increased treatment-related toxicity [36,52]. The levels of GSH and GSH/GSSG were significantly decreased in the blood of the patients with breast cancer compared with those of the control subjects [53]. DOX and other chemotherapeutic agents generate high levels of ROS in

cancer patients resulting in increased degree of oxidative stress [54]. A single dose of 5 mg/kg DNT was able to protect rat heart against an acute DOX (20 mg/kg) dose [25]. Similarly, a single dose of 5 mg/kg DNT was reported to protect rat heart against injury induced by isoproterenol [22] and whole body irradiation. DNT inhibited DOX-mediated ROS production in vitro in rat cardiomyocytes [15]. In the present study, DNT-treated control rats had higher levels of blood GSH and GSH/GSSG in comparison with rats treated with DOX.

Decrease in food intake and weight loss are the most common and serious health problems in patients with cancer undergoing chemotherapy [55]. A number of studies, including ours [29] have shown that DOX administration induces significant body weight loss and suppressed food intake and water consumption [56,57]. This study showed that DOX treatment was associated with a significant body weight loss from the baseline because of the suppressed food intake and water consumption, and a 40% of the animals in this group did not survive until the end of the experiment. In contrast, DNT-treated groups increased food and water consumption, and lost less body weight. None of the rats treated with DNT alone died during the experiment. This finding is translatable to humans because up to 80% of cancer patients develop cachexia characterized by reduced food intake, involuntary weight loss and wasting muscle mass [58,59] and this is associated with more toxicity from chemotherapy [60,61]. DNT supplementation in the present study was able to decrease cardiac toxicity even as it improved the catastrophic loss of body weight in the group of rats treated with DOX alone. Moreover, none of the rats in the DNT group died as a result of the treatment, in comparison with 40% mortality in DOX group and 16% in the DNT + DOX group. One limitation of this study was that we have determined the cardioprotective effects of a single dose of DNT that was based on data reported in the literature. This single dose exposure limits the interpretation of the benefits as well as the side effects of DNT. Future studies will benefit from a dose response assessment of DNT. Furthermore, we did not measure and correlate the effects of DNT with calcium levels in the treated rats. Despite its limitations, this study showed for the first-time that DNT has the potential as an adjuvant in cancer therapy.

Conclusions

The combination of chemotherapy has the ability to minimize the toxicity limitations of some chemotherapy agents and to increase clinical efficacy [62]. Recent reports suggest that currently available anticancer drugs are more effective when combined with Ca^{2+} channel blockers [63] which can also block multidrug resistance of cancer cells [64] and therefore can increase the sensitivity of cancer cells to treatment [65,66]. DOX is a powerful chemotherapy agent used for treatment of various malignancies; however, cardiac toxicity continues to compromise its clinical application [67]. The search for new approaches to prevent DOX-induced cardiotoxicity remains a critical issue in both oncology and cardiology [68]. The collective results from this study suggest that DNT may be a valuable drug for protection against DOX-induced cardiotoxicity in cancer patients while maintaining DOX antitumor efficacy. These results lay the foundation for further studies to determine the optimal DNT parameters (e.g., frequency, dosages), delivery mechanism (e.g., oral versus intraperitoneal or intravenous) and its effect on tumor response/resistance.

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