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ORIGINAL ARTICLE

The iron chelator Dp44mT inhibits the proliferation of cancer cells but fails to protect from doxorubicin-induced cardiotoxicity in spontaneously hypertensive rats

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Abstract
Purpose The iron chelator Dp44mT is a potent topoisomerase IIα inhibitor with novel anticancer activity. Doxorubicin (Dox), the current front-line therapy for breast cancer, induces a dose-limiting cardiotoxicity, in part through an iron-mediated pathway. We tested the hypothesis that Dp44mT can improve clinical outcomes of treatment with Dox by alleviating cardiotoxicity.
Methods The general cardiac and renal toxicities induced by Dox were investigated in the presence and absence of Dp44mT. The iron chelating cardioprotectant Dexrazoxane (Drz), which is approved for this indication, was used as a positive control. In vitro studies were carried out with H9c2 rat cardiomyocytes and in vivo studies were performed using spontaneously hypertensive rats.
Results Testing of the GI_{50} profile of Dp44mT in the NCI-60 panel confirmed activity against breast cancer cells. An acute, toxic dose of Dox caused the predicted cellular and cardiac toxicities, such as cell death and DNA damage in vitro and elevated cardiac troponin T levels, tissue damage, and apoptosis in vivo. Dp44mT alone caused insignificant changes in hematological and biochemical indices in rats, indicating that Dp44mT is not significantly cardiotoxic as a single agent. In contrast to Drz, Dp44mT failed to mitigate Dox-induced cardiotoxicity in vivo.
Conclusions We conclude that although Dp44mT is a potent iron chelator, it is unlikely to be an appropriate cardioprotectant against Dox-induced toxicity. However, it should continue to be evaluated as a potential anticancer agent as it has a novel mechanism for inhibiting the growth of a broad range of malignant cell types while exhibiting very low intrinsic toxicity to healthy tissues.

Keywords Anthracyclines · Spontaneously hypertensive rats · Cardiotoxicity · Dexrazoxane · Dp44mT

Introduction

Doxorubicin (Dox) is an anthracycline drug that is commonly used in the treatment of a variety of neoplasms [15]. However, administration of Dox is associated with a dose-limiting cardiac dysfunction in a significant number of patients [1]. Owing to this clinical limitation, considerable effort has been expended in an attempt to attenuate Dox-induced cardiotoxicity without interfering with its antitumor activity.

The mechanism by which Dox induces cardiomyopathy is believed to involve the generation of reactive oxygen species (ROS), in part through an iron-dependent pathway [17]. The heart is especially vulnerable to this ROS generation because cardiac muscle has only low levels of antioxidant enzymes [17]. Thus, while Dox systemically affects the entire body and can increase ROS levels throughout the body [28], the heart is particularly sensitive to these changes. As an iron-dependent mechanism has been implicated in Dox-induced increases in ROS, use of an iron

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chelator may be a logical means to mitigate the detrimental cardiac effects of Dox.

A current clinical strategy for limiting the cardiotoxicity of Dox in women with metastatic breast cancer is combination therapy with the iron chelator dexrazoxane (Drz) [9]. Dezrazoxane is a cell-permeable derivative of EDTA that has no appreciable antitumor activity of its own, but it is able to limit the amount of cardiotoxicity experienced by patients receiving Dox therapy [9]. However, an agent that could function as both an iron chelator to mitigate Dox cardiotoxicity as well as a complementary anticancer therapy would greatly enhance Dox utility and potentially improve patient outcomes.

Di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT) is an iron chelator that has been found to have intrinsic anticancer activity [20, 27]. We have previously reported that, in addition to functioning as an iron chelator, Dp44mT is a potent inhibitor of topoisomerase IIα [20]. Topoisomerase IIα is an essential enzyme in DNA replication that functions to decatenate entwined daughter chromosomes in mitosis. It is highly expressed in tumor tissue [14]. Several of the most commonly used anticancer agents, including Dox and etoposide, function by targeting both topoisomerase IIα and IIβ [14]. Dp44mT would also be advantageous because it selectively targets topoisomerase IIα, which is absent in cardiac tissue. Thus, Dp44mT is a promising candidate for combination therapy with Dox because it could function to enhance the antitumor activity of Dox while at the same time sequestering the iron that is responsible for Dox-induced cardiac dysfunction.

In this study, an extensive characterization of the cardioprotective and cardiotoxic potential of Dp44mT, both alone and in combination with Dox, was evaluated. In addition, results were compared with Drz, the current clinically utilized cardioprotectant.

Methods

Materials

Dp44mT was obtained from Calbiochem (#412520, San Diego, CA). The compounds dexrazoxane and doxorubicin were purchased from Pharmacia Laboratories (Columbus, OH). The γ-H2AX rabbit polyclonal antibody was a gift from Dr. William Bonner [National Cancer Institute (NCI)] [22]. The phospho-S1981 ATM mouse monoclonal antibody was purchased from Cell Signaling (Danvers, MA).

Cell culture

Breast cancer cells (MDA-MB-231 and MCF-7) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM:F12 (1:1) medium containing 5% heat-inactivated fetal calf serum (FCS), 2 mM L-Glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37°C in 5% CO_2. Primary healthy epithelial cells (MCF-12A) were obtained from Biowhittaker Clonetics (Walkersville, MD) and maintained in mammary epithelial cell growth medium (Biowhittaker, Clonetics) [8]. Rat cardiomyocytes (H9c2) were obtained from ATCC and were maintained in DMEM containing 10% FCS, 0.5% glucose (w/v), and 0.2% $NaHCO_3$ (w/v). The doubling time of this cell line under the above culturing conditions is approximately 48 h.

Animals

Male spontaneously hypertensive rats (SHR) (10 weeks of age) were obtained from Harlan Laboratories, Inc. (Indianapolis, IN) and were housed individually in an environmentally controlled room (18–21°C, 40–70% relative humidity, 12-h light/dark cycle). The study commenced after a 7-day acclimation period. Rats were fed Certified Purina Rodent Chow #5002 (Ralston Purina Co., St. Louis, MO) and water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Center for Drug Evaluation and Research, FDA, and conducted in an AAALAC-accredited facility. All procedures for animal care and housing were in compliance with the Guide for the Care and Use of Laboratory Animals, 1996 (Institute of Laboratory Animal Resources).

Animal study design

Nine groups of 5 SHR each were treated with saline, Dp44mT vehicle (saline with 15% polypropylene glycol), Dox (10 mg/kg), Dp44mT (0.1 or 0.3 mg/kg), or Drz (50 mg/kg), alone or in combination. Dp44mT was administered in two doses, one 24 h after the first. When in combination with Dox, Dp44mT was injected 30 min prior to and 24 h after Dox. Dox and Dp44mT were administered intravenously (IV) via the lateral tail vein. Drz was given intraperitoneally (IP) either alone or 30 min prior to Dox. The animals were observed for 14 days following treatment after which they were euthanized by exsanguination under isoflurane anesthesia. At necropsy the heart, a kidney, and samples of liver, lung, spleen, and small intestine were excised and fixed in 10% formalin solution.

Clinical chemistry and hematology analysis

At necropsy, blood was collected from the inferior vena cava into serum collection tubes and centrifuged to obtain serum samples. Clinical chemistry determinations were performed using a VetScan Analyzer (Abaxis, Inc., Union

City, CA). An aliquot of whole blood was also collected in order to monitor various hematological parameters (Abaxis, Inc.).

Cardiac troponin T analysis

An aliquot of 500 µl serum from each animal was maintained at −80°C until analysis could be performed. The serum concentration of Troponin T was determined using the third generation cTnT immunoassay (Elecsys, STAT, Roche Diagnostics, Indianapolis, IN) in the laboratory of Dr. Nader Rifai (Children's Hospital, Harvard Medical School, Boston, MA).

Histopathological studies

Portions of the hearts were embedded in glycol methacrylate plastic resin, sectioned (1 µm) and stained with toluidine blue. Other portions of the heart, the kidney, and other tissues were then embedded in paraffin, cut into 5 µm sections, and stained with hematoxylin and eosin. The grading for cardiac and renal lesions was determined as seen previously [6].

Immunohistochemical studies

The terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay was carried out on sections of formalin-fixed, paraffin-embedded cardiac tissue as described previously. Briefly, the CardioTACS in situ apoptosis detection kit (Trevigen, Gaithersburg, MD) was utilized in concert with the TACS Blue Label detection system (Trevigen).

Assessment of antiproliferative activity

Growth inhibition was assessed by the sulforhodamine B (SRB; Sigma–Aldrich, St. Louis MO) assay as previously described [24]. Resultant IC_{50} values were calculated using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA). Data were obtained from at least three independent experiments.

Immunocytochemical analysis

Cells were grown in chamber slides (Nunc, Naperville, IL) and after treatment were fixed and permeabilized with 4% paraformaldehyde and 70% ethanol as described previously [4, 19]. Slides were stained with the rabbit anti-γ-H2AX (1:500), or mouse antiphosphorylated S1981 ATM primary antibodies (1:500) and Alexa-Fluor 488 anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR). Cells were visualized with green, red, DAPI, or phase contrast filters using a 40× objective on a PCM2000 laser scanning confocal microscope (Nikon, Augusta, GA). Line scans and image analysis was performed using the Image J software package (NIH, Bethesda, MD).

Statistics

Differences in cardiac and vascular lesion scores between groups were compared using the Kruskall-Wallis test (nonparametric analysis of variance). Differences in the growth inhibition, γ-H2AX nuclear foci formation, hematology indices, and serum levels of clinical chemistry analytes were compared using Student's t test. The hematology indices and serum chemistry analytes between groups were compared using Bonferroni's multiple comparison test. A value of $P \leq 0.05$ was considered statistically significant.

Results

Cardioprotective potential and anticancer cytotoxicity profile of Dp44mT

Dp44mT is a potent iron chelator that has been identified as a potential anticancer agent both alone and in combination with other chemotherapeutic compounds [20, 27, 30]. We examined the cardioprotective potential of Dp44mT in the H9c2 rat cardiomyocyte cell line. The proliferative activity of H9c2 cells are decreased in the presence of Dox [23]. Therefore, the influence of Dp44mT on this growth suppressant activity was examined (Fig. 1a). Dp44mT at a concentration of 10 nM was able to significantly attenuate the Dox-induced decrease in H9c2 proliferative activity. After 72 h, the 60% growth inhibition normally seen after 0.1 µM Dox was 40% in the presence of Dox plus Dp44mT. However, at a higher concentration (100 nM for 72 h), Dp44mT itself exerted significant growth inhibition and likewise was not able to alter the growth inhibitory effects of Dox (Fig. 1a, lower graph). The magnitude of growth inhibition caused by exposure of H9c2 cells to 100 nM of Dp44mT alone was 14 and 28% at 48 and 72 h, respectively, compared to 25 and 60% by 0.1 µM Dox alone.

The genotoxicity of Dp44mT and Dox was compared in H9c2 myocytes by evaluating the formation γ-H2AX foci and phosphorylation of ATM at Ser1981 at 1 h after drug exposure. Both of these post-translational modifications are sensitive markers of DNA double-strand break (DSB) formation [3, 19]. Dp44mT was clearly less genotoxic than Dox, as seen by differences in the size and intensity of immunofluorescent foci (Fig. 1b). In addition, the combination of Dox and Dp44mT was less genotoxic than Dox treatment alone.

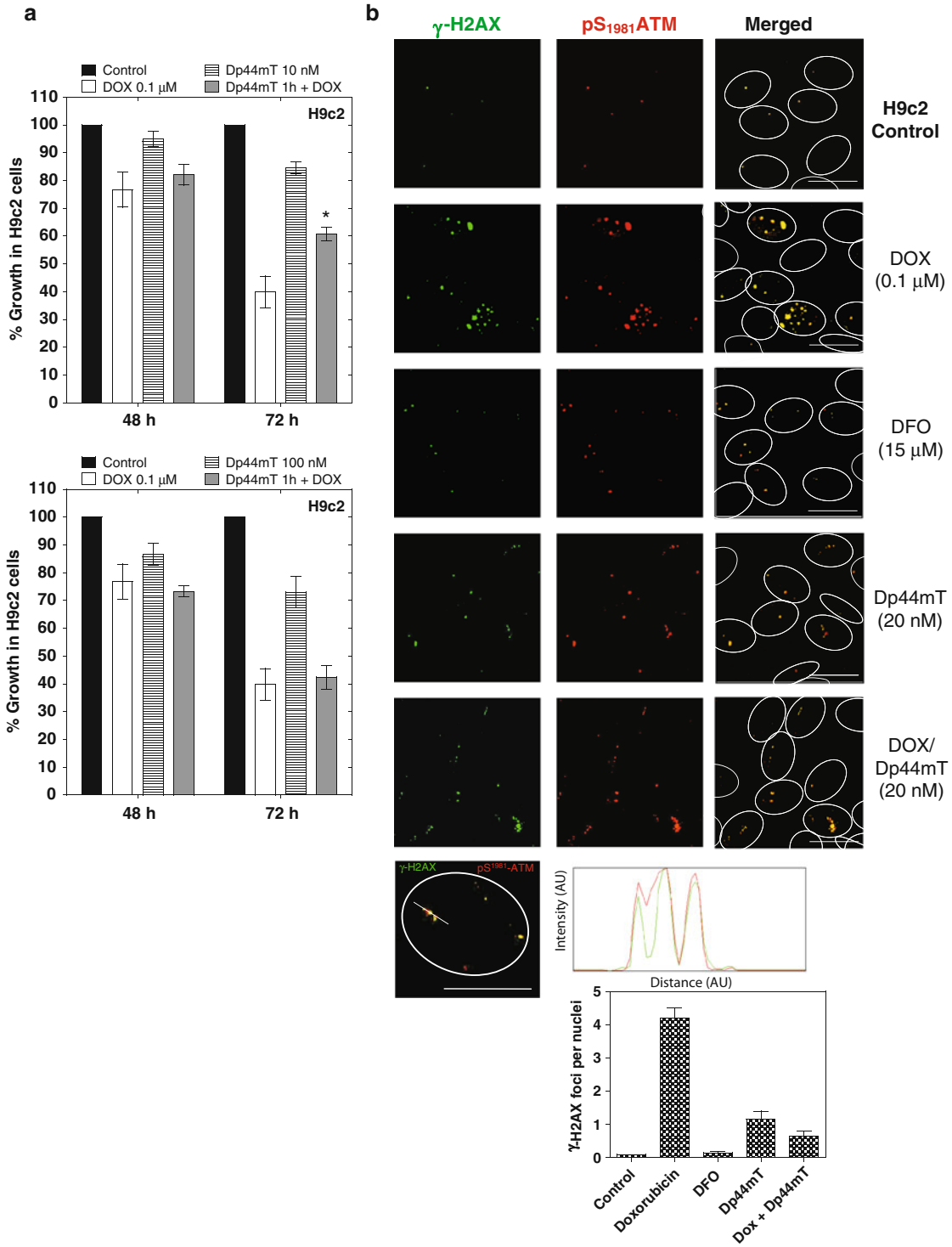


Fig. 1 *In vitro* cardioprotective potential of Dp44mT in combination with Dox. **a** Cell proliferation was measured using an SRB dye-based assay. Exponentially growing H9c2 cells were incubated for 48 or 72 h with either 0.1 μM Dox (*clear bars*), 10 nM Dp44mT (*horizontal bars*, *upper graph*), 100 nM (*horizontal bars*, *lower graph*), or in combination (*gray bars*). When tested in combination with Dox, cells were incubated with Dp44mT 1 h prior to Dox addition. Results are expressed relative to untreated controls (*black bars*). *Error bars* represent the SD of three independent experiments. *Asterisk* denotes statistically significant differences ($P < 0.05$). Significance in **a** is relative to Dox treatment alone. **b** Genotoxicity was determined using confocal imaging to detect γ-H2AX (*green*) and phospho-Serine modified ATM (*red*) in the nucleus. *Yellow spots* in merged images represent individual DNA double-strand break sites. H9c2 cells were either untreated (*top row*), given 0.1 μM Dox (*second row*), 15 μM desferrioxamine (*DFO*, *third row*), 20 nM Dp44mT (*fourth row*), or both Dox and Dp44mT (*fifth row*). The last row shows a single representative enlarged nucleus and line scan analysis indicative of colocalization of red and green signals. On average, 100 cells from at least three separate microscopic fields were examined to determine the number of γ-H2AX foci per nucleus (*Scale bars* = 5 μm) (color figure online)

Table 1 Mean heart, body weights, WBC, RBC, platelet counts, hematocrit values (Hct), and hemoglobin concentration (Hb) obtained in SHR given doxorubicin with or without Drz or Dp44mT for 4 weeks

Treatment	H/FBW%	FBW/IBW	WBC	RBC	Hct	Hb	PLT
Saline	0.404 ± 0.052	1.072 ± 0.019	4.762 ± 1.647	8.440 ± 0.881	45.30 ± 5.510	13.16 ± 1.514	445.8 ± 256.54
Dox	0.404 ± 0.036	0.926 ± 0.028[a]	10.78 ± 0.880[a]	8.078 ± 0.513	42.26 ± 3.560	12.60 ± 0.964	1,813.8 ± 189.84[a]
Drz	0.390 ± 0.047	1.104 ± 0.029	6.936 ± 1.554	8.898 ± 0.312	48.40 ± 1.444	13.84 ± 0.329	408.6 ± 31.72
Drz/Dox	0.414 ± 0.017	1.024 ± 0.029[b]	9.234 ± 1.252	7.390 ± 0.704	41.58 ± 4.328	11.46 ± 0.950[b]	1,501.6 ± 311.7[b]
Dp44mT	0.382 ± 0.008	1.048 ± 0.013	6.196 ± 1.042	9.810 ± 0.313	52.70 ± 2.033	15.14 ± 0.709	533.6 ± 49.58
Dp44mT/Dox	0.330 ± 0.022	0.974 ± 0.073	10.64 ± 0.691[c]	7.296 ± 1.070[c]	37.52 ± 5.441[c]	11.10 ± 1.428[c]	972.0 ± 376.34[c]

[a] Values significantly different from that recorded for saline ($P < 0.01$)

[b] Values significantly different from that recorded for doxorubicin alone ($P < 0.01$)

[c] Values significantly different from that recorded for Dp44mT alone ($P < 0.01$)

Table 2 Mean serum concentrations of albumin, alanine aminotransferase (ALT), urea nitrogen (BUN), creatinine (CRE) measured in SHR given doxorubicin with or without Drz or Dp44mT

Treatment	ALB	ALT	BUN	CRE
Saline	4.32 ± 0.238	48 ± 6.557	22.2 ± 2.387	0.44 ± 0.0894
Dox	1.86 ± 0.270[a]	26 ± 5.523[a]	39.4 ± 8.877[a]	0.78 ± 0.1304[a]
Drz	4.16 ± 0.151	45.2 ± 2.683	22.8 ± 1.304	0.4 ± 0.1000
Drz/Dox	3.32 ± 0.466[b]	43.8 ± 6.140[b]	21.8 ± 3.768[b]	0.34 ± 0.1517[b]
Dp44mT	4.38 ± 0.130	43.2 ± 1.924	21.2 ± 2.168	0.36 ± 0.1140
Dp44mT/Dox	1.54 ± 0.207[c]	24 ± 7.106[c]	49.2 ± 7.396[c]	0.56 ± 0.1673[c]

[a] Values significantly different from those recorded for saline ($P < 0.01$)

[b] Values significantly different from those recorded for doxorubicin alone ($P < 0.01$)

[c] Values significantly different from those recorded for Dp44mT alone ($P < 0.01$)

The tissue-specific anticancer activity of Dp44mT was examined in a number of cancer tissue sub-types using the NCI 60 panel of tumor cell lines. As shown in Supplementary Figure 1, the antiproliferative activity of Dp44mT was most prominent in nonsmall cell lung cancer, melanoma, and breast cancer, with GI_{50} values in the nanomolar range. The growth profile of various other tissue sub-types and cell lines are also depicted. The GI_{50} values for each cell line in the panel are presented in Supplementary Figure 2 and are sorted by tissue type. These results are also in agreement with previous data showing that Dp44mT exerted significant growth inhibition on the breast cancer cell lines MDA-MB-231 and MCF-7 [20].

In vivo general toxicity comparisons

Dp44mT (0.3 mg/kg IV) and Dox (10 mg/kg IV) were given alone and in combination to groups of SHR as described for nude mice with xenografts studied in previous publications [6, 27, 30]. Other groups of rats were given Dox and Drz (50 mg/kg IP) for comparison. All animals were monitored for 2 weeks following treatment.

Tables 1 and 2 summarize the effects of the various treatments on hematological values including white blood cell (WBC), platelet (PLT) and red blood cell (RBC) counts, hematocrit (Hct) and hemoglobin (Hb) levels and clinical chemistry values including creatinine (Cre), blood urea nitrogen (BUN), and alanine transferase (ALT), as well as heart and body weights. Heart weight is shown as a function of final heart weight over final body weight (H/FBW). Overall weight loss is depicted as final body weight divided by initial body weight (FBW/IBW).

SHR treated with either dose of Dp44mT experienced a slight (<5%) decrease in average mean body weight. The 0.3 mg/kg dose of Dp44mT also caused a decrease in the heart weight to final body weight ratio (3%). These changes were smaller than those caused by of the 10 mg/kg dose of Dox (12% decrease in body weight and a 4% decrease in heart to final body weight ratio). Dp44mT treatment caused a significant (84%) decrease in platelets. However, SHR treated with Dox had greater than twofold increases in white blood cell and platelet counts, and a significant decline in red blood cell counts, hematocrit, and hemoglobin concentration.

Dp44mT did not alter Dox-induced decreases in total body and heart weights. Similar results were obtained with 0.1 mg/kg Dp44mT (data not shown). Likewise, Dp44mT also was not able to alter the changes in hematological

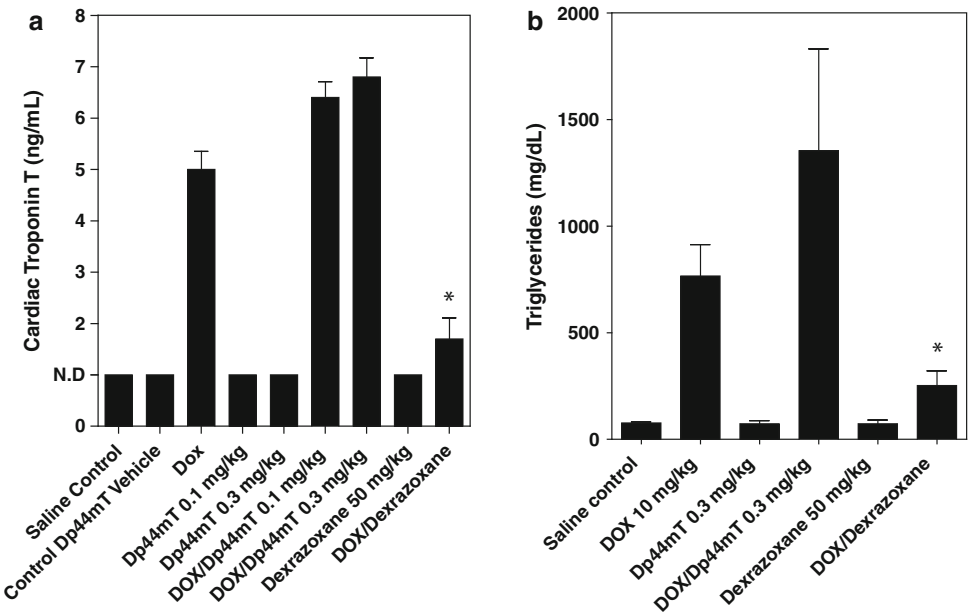


Fig. 2 Serum cardiac Troponin T (cTnT) and triglyceride analysis. Whole blood was centrifuged to obtain serum samples for analysis of triglyceride and cTnT levels. **a** The serum concentration of Troponin T (ng/mL) was determined by immunoassay, and values are expressed as a percentage relative to control. **b** Triglyceride levels (mg/dL) are expressed as the mean value of each treatment group. In each case, *error bars* represent the SD of three independent experiments. *Asterisk* denotes statistically significant difference ($P < 0.05$) compared to doxorubicin-treated animals

parameters resulting from treatment with Dox. In contrast, pretreatment with Drz attenuated all aspects of Dox toxicity. Similar results with Drz have been reported previously [9].

In vivo serum biochemical comparisons

Cardiac Troponin T (cTnT) is a well-characterized marker of cardiac damage [26]. Treatment with Dp44mT alone (at 0.1 or 0.3 mg/kg) had no effect on serum cTnT levels (Fig. 2a). Dox treatment induced a fivefold increase in cTnT serum concentration. The serum level of cTnT was 6.7 times higher in SHR given the combination of Dp44mT and Dox than in control animals with no detectable levels of cTnT (denoted N.D.) (Fig. 2a). The cTnT levels in SHR given Drz alone were similar to those of the control animals. The concentration of cTnT was significantly lower in animals given Drz and Dox than in animals given Dox alone.

The accumulation of cholesterol (especially triglycerides) in the blood of mammals is a well-characterized marker of heart disease [12]. As shown in Fig. 2b, a basal average triglyceride level of <100 mg/dl was found in saline-treated animals. Dox treatment induced a significant elevation of triglyceride levels (~750 mg/dl) [16]. Dp44mT (0.3 mg/kg) treatment alone had no effect on the level of triglycerides. Dp44mT did not alter Dox-induced elevations in serum triglyceride levels. In fact, the triglyceride levels in the SHR that received the combination of Dp44mT and Dox were higher than those observed in SHR given Dox alone (~1,300 mg/dl). Drz had no effect on triglyceride levels alone but was able to significantly attenuate Dox-induced increases in triglycerides.

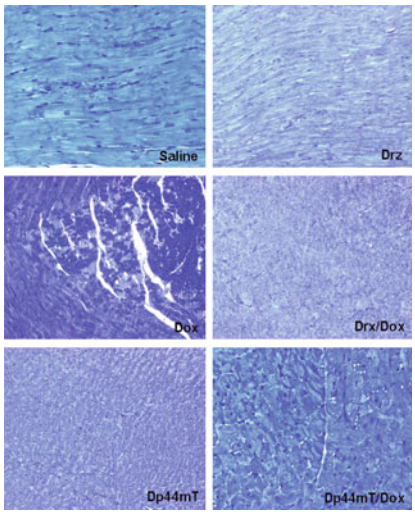
In vivo cardiac and renal tissue histology analysis

In previous studies using SHR, Dox has induced lesions in both cardiac and renal tissue [6]. Representative images from drug-treated tissues (left) and a corresponding table of lesion scores (right) are shown in Fig. 3a and b, respectively. The semiquantitative assessment of lesion severity was based on a scale of 0–3 for the heart and 0–4 for the kidney as described [6]. Cardiac lesions induced by Dox are characterized morphologically by myofibrillar loss and cytoplasmic vacuoles, and Dox-induced renal lesions are characterized by necrosis and degeneration of tubular epithelial cells, tubular dilation, protein casts, and glomerular vacuolization. Treatment of SHR with Dp44mT (either 0.1 or 0.3 mg/kg, data with 0.3 mg/kg shown) and Dox induced the same characteristic lesions as those seen in Dox-cardiorenal toxicity. Dox treatment was associated with a mean cardiac lesion score of 2.2 and a mean renal lesion score of 3.0. Dp44mT induced minor myocardial alterations as a single agent (lesion score of 1.5). In contrast to Dox, Dp44mT had no effect on renal tissue. Drz significantly attenuated the severity of Dox-mediated cardiac and renal lesions. SHR given the combination of Dp44mT and Dox also had less severe lesions (particularly renal) than SHR treated with Dox alone.

Detection of apoptotic bodies in cardiac tissue

Dox has been previously shown to induce apoptosis in cardiac tissue [11]. In this study, the TUNEL assay was used to detect DNA fragmentation in situ on cardiac tissue sections from the SHR animals (Fig. 4). Dox-treated rats exhibited an increase in the number of apoptotic bodies in

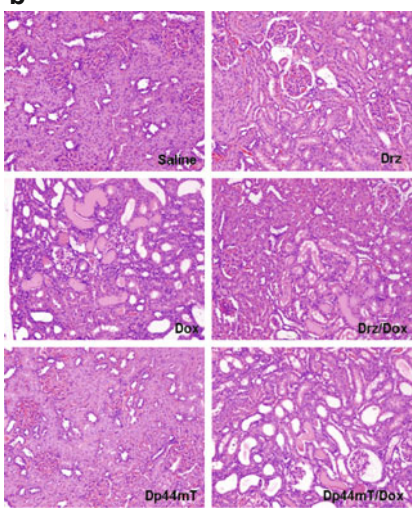
a Cardiac lesions



Treatment *	Total number of animals	Distribution of animals and cardiomyopathy score						
		0	1	1.5	2	2.5	3	Average score
Saline control	5	5	0	0	0	0	0	0
Doxorubicin [a,b]	5	0	1	0	1	2	1	2.2
Dexrazoxane	5	5	0	0	0	0	0	0
Dexrazoxane/doxorubicin [a]	5	1	3	1	0	0	0	0.9
Dp44mT [a]	5	1	2	2	0	1	0	1.5
Dp44mT/doxorubicin [a]	5	0	0	2	3	0	0	1.8

* Animals received dexrazoxane or Dp44mT 30 min prior to dose of doxorubicin
a. Lesion scores significantly more severe than in rats given saline ($p<0.05$)
b. Lesion scores significantly more severe than in rats given combination of dexrazoxane and doxorubicin ($p<0.05$)

b Renal lesions



Treatment *	Total number of animals	Distribution of animals and nephropathy score						
		0	1	1.5	2	2.5	3	Average score
Saline control	5	5	0	0	0	0	0	0
Doxorubicin	5	0	0	0	0	0	5	3
Dexrazoxane	5	5	0	0	0	0	0	0
Dexrazoxane/doxorubicin [a]	5	3	2	0	0	0	0	0.4
Dp44mT	5	5	0	0	0	0	0	0
Dp44mT/doxorubicin [a]	5	0	0	0	3	0	2	2.4

* Animals received dexrazoxane or Dp44mT 30 min prior to dose of doxorubicin
a. Lesion scores significantly more severe than in rats given combination of dexrazoxane and doxorubicin ($p<0.05$)

Fig. 3 Photomicrographs of histological sections. **a** Cardiac plastic sections (1 μm), with toluidine blue stain (200× magnification) and **b** renal tissue paraffin sections (5 μm) with hematoxylin and eosin stain (200× magnification) from SHR treated with saline (*upper left*), Drz alone (*upper right*), Dox alone (*middle left*), Drz+Dox (*middle right*), 0.3 mg/kg Dp44mT alone (*lower left*), and Dp44mT + Dox (*lower right*) are shown. Tables on the right side contain the numerical cardiac (maximum severity score = 3) and renal (maximum severity score = 4) lesion scores. Please refer to the results section for descriptions of the cardiac and renal lesions

cardiac tissue compared to those treated with saline vehicle. Dp44mT as a single agent (0.3 mg/kg) caused a slight increase in apoptotic cells as indicated by the arrows. The combination of Dp44mT and Dox did not attenuate the apoptosis and appeared to produce a synergistic increase in apoptosis detected by TUNEL in cardiac tissue.

Discussion

This study attempted to determine whether the iron chelator Dp44mT was capable of mitigating the well-known iron-mediated cardiotoxicity of Dox. Currently Dox may be administered in combination with the iron chelator Drz in order to reduce its cardiotoxicity [1, 9]. However, in experimental and clinical situations, Drz does not completely suppress the cardiotoxicity of Dox, so there is a need for compounds with superior chemoprotective activity [13, 29]. In response to this unmet medical need, numerous studies have been performed in order to identify an iron chelating agent that is able to both mitigate the cardiotoxicity of Dox while sustaining or enhancing its antitumor effects [5, 6, 28]. Previous research has shown that Dp44mT is a potent iron chelator with greater chelating abilities than other compounds in its class [27]. We have previously reported that Dp44mT is a selective and potent

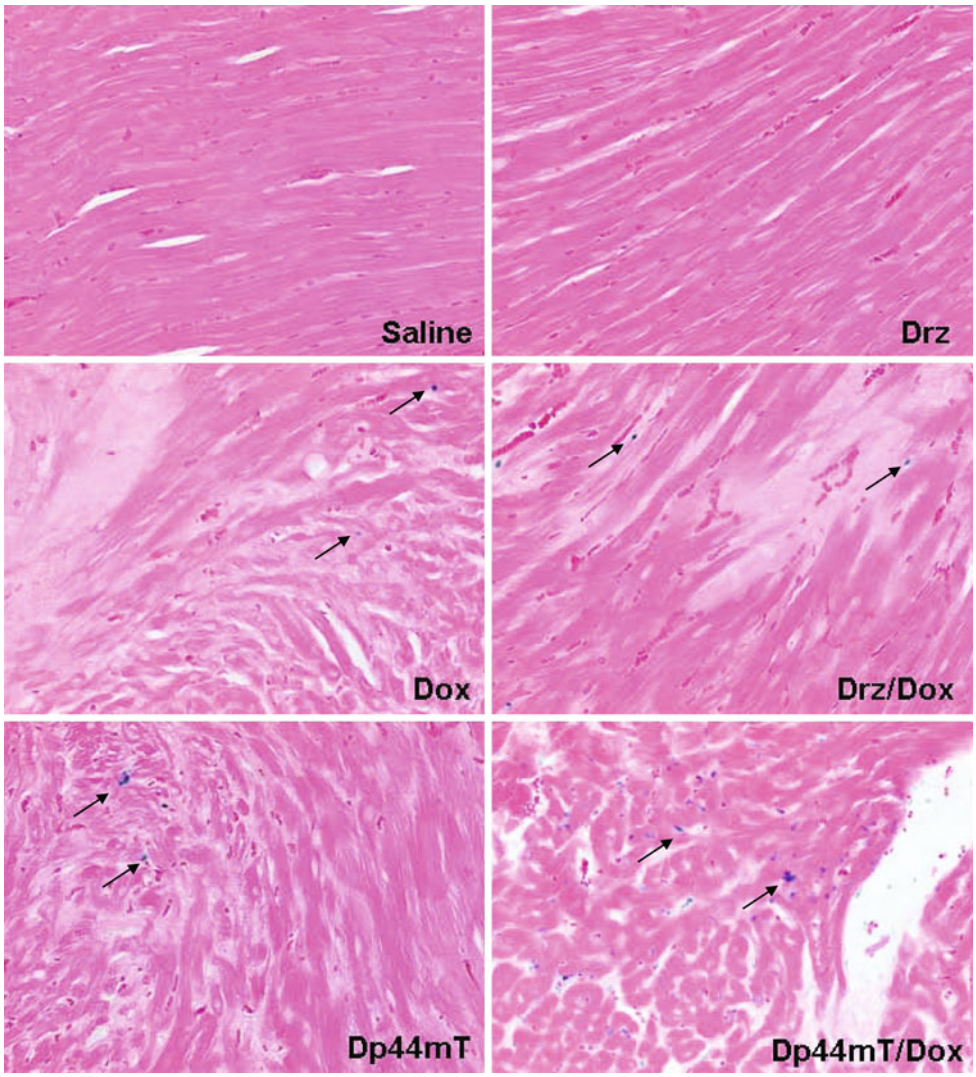


Fig. 4 Immunohistochemical detection of apoptotic cells in the heart (TUNEL staining). Representative images of cardiac tissue treated with Saline, Drz, Dox, Drz + Dox, 0.3 mg/kg Dp44mT, and Dp44mT + Dox are shown (200× magnification). Tissues were embedded in paraffin and sectioned to 5 μm. A positive reaction of DNA fragmentation is indicated by a *blue color* (marked by *arrows*) (color figure online)

inhibitor of topoisomerase IIα, making it a potential anticancer agent [20]. Previous studies in mice showed that Dp44mT can cause low level cardiac lesions [17, 27]. Yet, the lack of topoisomerase IIα in cardiac tissue suggested to us that the level of such damage should be limited, especially when compared to the cardiotoxicity induced by Dox. Thus, we hypothesized that Dp44mT might still provide advantages as a chemoprotectant against Dox-induced cardiotoxicity. Therefore, in order to determine if Dp44mT could be used in combination with Dox to reduce anthracycline-induced cardiotoxicity, both in vitro and in vivo analyses were performed.

Cell-based assays confirmed that Dp44mT had anticancer cytotoxic activity and was relatively nontoxic to healthy rat cardiac cells. In addition, Dp44mT was not, itself, genotoxic and also seemed to inhibit the genotoxicity of Dox in healthy cardiac cells. We note that the use of H9c2 cell line for cellular studies has its limitations compared to freshly isolated cardiomyocytes due to the fact that they are nonprimary cells and are no longer able to beat in culture [7]. Therefore, an in vivo acute Dox treatment animal study was undertaken. The results indicate that while Dp44mT does not exhibit significant toxicity in animals as indicated by cTnT levels, it is not capable of reducing the cardiotoxicity from Dox treatment, and should probably not be pursued in clinical trials as a protective agent. Our results in the SHR and H9c2 cells are in concordance with previous in vitro cellular studies that investigated the iron chelating activity of Dp44mT in the presence of Dox using primary rat cardiomyocytes [5]. Using several measures of cardiac viability, we found that Drz is superior to Dp44mT in mitigating Dox-induced cardiotoxicity.

The fact that Dp44mT is not an effective cardioprotective agent in vivo suggests that the mechanism of Dox-induced toxicity might be more complex than previously thought. While an abundance of evidence has shown that anthracycline-induced cardiotoxicity is mediated through increased reactive oxygen species (ROS), the mechanism

by which these molecules are generated may not be entirely understood [10]. Some evidence has indicates that iron may not be involved in the generation of cytotoxic ROS in response to Dox [16, 28]. In addition, while exercise seems to limit Dox-induced cardiotoxicity, obesity and a high-fat diet exacerbate the effect [25]. These studies indicate that a number of systemic changes in antioxidant and other defense systems may be required to reduce the effects of Dox on the heart. In fact, the potent antioxidant resveratrol was found to reduce some of the cardiotoxicity associated with Dox in an animal model, possibly by increased nitric oxide production [2, 18]. How nitric oxide production prevents cardiac injury remains to be clarified [21]. A recent report suggesting that mitochondrial trafficking is an important component of iron metabolism offers yet another clue to the pathogenesis of Dox-induced toxicity [20, 30]. These findings indicate that research into other mechanisms may prove fruitful in discovering a consistent and more effective way to eliminate the cardiotoxicity of Dox and other anthracycline drugs. Additional studies are needed to investigate intracellular iron uptake and subcellular mechanisms that contribute to Dox's toxicity, and to clarify how iron chelators may or may not mitigate such mechanisms.

While Dp44mT is unlikely to serve as a good combination therapy with Dox, future studies should focus on examining the effectiveness of this compound for its promising anticancer potential. Our current cell-based research and previous animal studies indicate that it may be highly effective against a number of tumor types, and the cardiotoxicity results reported here reveal that Dp44mT is significantly less toxic than Dox. Our studies indicate some tissue cell-selective sensitivity of tumor cells to Dp44mT that could provide a primer for designing further preclinical and clinical studies. Specifically, Dp44mT should be studied as a potential treatment for nonsmall cell lung cancer, melanoma, and breast cancer as these were the tumor cell sub-types that were especially sensitive to this agent.

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Conflict of interest None declared.

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