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ORIGINAL ARTICLE

Dexrazoxane protects against doxorubicin-induced cardiomyopathy: upregulation of Akt and Erk phosphorylation in a rat model

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Abstract
Purpose Dexrazoxane (DZR), a clinically approved cation chelator, is effective in reducing doxorubicin (DOX)-induced heart damage, yet its cardioprotective mechanism is not fully understood. We aimed to investigate the effects of DZR on the activation of Akt and Erk 1/2 signals in a rat model of DOX-induced cardiomyopathy.
Methods Male Sprague–Dawley rats received weekly DOX injection (2.5 mg/kg) for 6 weeks, with or without DZR pretreatment at a dose ratio of 20:1. The ventricular functions of these animals were monitored at week 6, 9 and 11 by echocardiography. At week 11, their heart morphology was studied by light and electron microscopy. Phosphorylation of Akt and Erk in heart tissues was measured by Western blot analysis.
Results DOX caused myocardial damage with compromised left ventricular function, increased myocardium injury and reduced phosphorylation of Akt and Erk. DZR exerted a significant cardioprotective effect in terms of improved fractional shortening, cardiac output and cardiomyopathy score at one or more time points. We also provided the first evidence that dexarazoxane-treated animals had increased levels of Akt and Erk activation, whilst total Akt and Erk remained unchanged.
Conclusions Our results showed that the cardioprotective effect of dexarazoxane has been sustained beyond the treatment period. The data also suggested that activation of the Akt and Erk signaling pathways was regulated in the course of DOX-induced cardiomyopathy and protection by DZR.

Keywords Dexrazoxane · Doxorubicin · Cardioprotection · Akt · Erk1/2

Ping Xiang and Hai Yan Deng have equal contribution to this study.

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Introduction

Doxorubicin (DOX), an anthracycline drug, is one of the most effective antineoplastic agents developed for the treatment of solid tumors and hematologic malignancies. However, its clinical use is limited by cardiac damage which can occur at any stage during or following treatment. DOX-induced cardiotoxicity is dose-related and essentially irreversible. Patients who receive a cumulative dose of more than 500 mg/m^2 of DOX have increased risk of developing

cardiac toxicity, including cardiomyopathy and congestive heart failure [24]. It has been suggested that the anticancer effects and cardiotoxicity of DOX do not follow identical pathways [1, 14, 27]. The mechanism of DOX-induced cardiotoxicity is multifactorial and involves the induction of lipid peroxidation and generation of free radicals. The increase in oxidative stress and depletion of endogenous antioxidants trigger the intrinsic mitochondria-dependent apoptotic pathways. The heart is particularly vulnerable to free radical injury, likely related to low activities of protective enzymes, such as superoxide dismutase, relative to other tissues [4, 13, 21].

Dexrazoxane (DZR), belonging to a class of bis(2,6-dioxopiperazines), has been proven clinically effective in reducing DOX-induced cardiotoxicity and recently approved as an antidote for alleviating tissue damage due to accidental anthracycline extravasation [6]. In a multicenter randomized phase III trial, DZR significantly reduced the occurrence and severity of anthracycline-induced cardiotoxicity in breast cancer patients who were at increased risk of cardiac dysfunction, without compromising the antitumor efficacy of the chemotherapeutic regimen [20]. The molecular mechanism underlying DZR-associated cardioprotection remains unclear. Recent studies have shown that DZR specifically abolished DNA and mitochondrial damage signals induced by DOX [15, 19]. In vivo, DZR permeates the cell membrane and is rapidly hydrolyzed to its metal ion-binding metabolite, ADR-925, thus decreasing anthracycline-iron binding and formation of reactive oxygen species [7]. However, many other iron chelators or free radical scavengers failed to rescue DOX-induced cardiotoxicity [19].

Signaling through phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and the p42/p44 extracellular signal-regulated kinases (Erk 1/2) pathways have been shown to play important roles in heart development and diseases [26, 28, 29]. The phosphorylation cascades of Akt and Erk 1/2 are referred to as the Reperfusion Injury Salvage Kinase (RISK) pathways which mediate cell survival during ischemia heart reperfusion injury [9]. Activation of RISK pathways result in down-regulation of proapoptotic proteins and protection of mitochondrial integrity. DOX-induced damage and apoptosis of cardiomyocytes have been associated with down-regulation of Akt and Erk 1/2 activation in in vitro and in vivo animal models [3, 17, 18, 22, 25]. In a previous study, we demonstrated that DZR has protective activity against DOX-induced toxicity in rat H9C2 myoblastic cell line and primary neonatal rat cardiomyocytes in culture [16]. In this study, we established an in vivo rat model of DOX-induced cardiomyopathy and further investigated the protective effect of DZR on cardiac damage and the activation of the Akt and Erk phosphorylation signals.

Materials and methods

Male Sprague–Dawley rats (Laboratory Animal Services Centre, The Chinese University of Hong Kong) were fed a regular rat chow and housed in a normal night–day rhythm under standard conditions of temperature and humidity. At 170–180 g of body weight, the rats were divided into three experimental groups: Control (saline), DOX (Ebewe Pharma Ges, Austria; 2.5 mg/kg body weight), and DOX + DZR (2.5 mg/kg DOX + 50 mg/kg DZR). DOX was given by intravenous injection into the tail vein once weekly for 6 consecutive weeks. DZR (Chiron, Amsterdam, The Netherlands) was administered by intraperitoneal injection 30 min prior to each dose of DOX. The body weight of the animals was monitored weekly. Eleven weeks after the first injection, the animals were sacrificed by cervical dislocation. The study was approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong, Hong Kong.

Two-dimensional echocardiography

Transthoracic echocardiography was performed to evaluate left ventricle (LV) function as described previously [16]. At 2 days before DOX injection (baseline), week 6, 9 and 11, the rat was anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg). The chest was shaved, and two-dimensional echocardiography was performed using the echocardiographic system (Sonos 7500, Philips Ultrasound, Bothell, Wash) with a 7.5–12 MHz probe. M-mode echocardiography of the LV at the papillary muscle level were preformed, guided by two-dimensional short-axis images. Left ventricular end-diastolic dimensions (LVEDD) and left ventricular end-systolic dimensions (LVESD) were measured on the M-mode tracings and were averaged from three cardiac cycles. The LV fractional shortening (% FS) was automatically calculated as $[(\mathrm{LVEDD} - \mathrm{LVESD})/\mathrm{LVEDD}] \times 100$.

Cardiomyopathy score and mitochondrial ultrastructure

At week 11 after echocardiography, all rats were killed and their hearts immediately excised. The heart tissue was fixed in 4% formaldehyde, and 4 μm-thick paraffin sections were stained with hematoxylin-eosin for histological examination. The severity of DOX-induced myocardial damage was evaluated by an investigator in a blinded manner using light microscopy. The degree of damage was scored from 0 to 3 according to the percentage of vacuolization and myofibrillar loss in eight randomly assigned areas of each section and two sections per heart as described [16]. Some cardiac specimens were immersion-fixed overnight in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), postfixed for 1 h with 1% osmium tetroxide, dehydrated through a graded ethanol series, and

embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in an electron microscope (CM120, Philips, The Netherlands).

Western blot analysis

For protein isolation, frozen heart tissues were powdered in liquid nitrogen and then suspended in radioimmunoprecipitation assay lysis buffer (Sigma Chemical Co., St Louis, MO, USA). The samples were homogenized and the lysates were centrifuged at 14,000 rpm for 15 min, and the supernatant was stored at −80°C. The protein concentration was measured by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as a standard. The isolated protein was subjected to SDS–PAGE and transferred to polyvinylidene difluoride membrane (Amersham International, Buckinghamshire, England). The activation of Akt and Erk was assessed by specific antibodies against phospho-Akt, total Akt, phospho-Erk and total Erk (all from Cell Signaling Technology, Inc., Boston, MA, USA). The blots were visualized by means of chemiluminescence (ECL, Amersham), and the signals quantified by densitometry. The membranes were reprobed with α-Tubulin which served as the loading control. Protein levels were normalized to total Akt or Erk and α-Tubulin.

Statistical analysis

The survival rates of animals were compared by the log rank test. Effects of DOX and DOX + DZR on body weight were analyzed using multilevel modeling to compare the longitudinal rate of change between the three groups. The models were fitted by the method of restricted iterative generalized least-squares algorithm of MLn for Windows software package, Version 2.0 (Institute of Education, University of London, London, UK). The likelihood ratio test was used to assess the statistical significance of the estimates at 5% level. Heart function parameters at baseline and various time points, heart weight and signal expression at week 11 were analyzed by the Mann–Whitney test. Cardiomyopathy scores were analyzed by the Kruskal–Wallis ranking test and Mann–Whitney test. The levels of significance were adjusted by Bonferroni adjustment of multiple comparisons. All data are presented as mean ± standard deviation (SD).

Result

Survival, body weight and heart/body weight

Two rats that received DOX and one rat in the DOX + DZR group died at week 10, probably due to severe cardiotoxicity because they had plenty of ascites. There were no statistical significances in the mortality rates of the three groups of animals.

There were significant differences in the trend of body weights of animals in the DOX-treated group compared with those in the Control groups ($P < 0.001$) or the DOX + DZR group ($P < 0.001$) (Fig. 1). The differences were more pronounced from 6 to 9 weeks after the initial treatment. The heart-to-body ratios at week 11, however, were similar among the three groups (Control group 2.66 ± 0.16 mg/g; DOX 2.8 ± 0.45 mg/g; DOX + DZR 2.77 ± 0.52 mg/g).

Echocardiographic assessment of cardiac function

All echocardiographic parameters were similar among the three groups of animals at baseline (Table 1, Fig. 2). At week 6 after DOX treatment, there were significant differences in LVEDD ($P < 0.001$), cardiac output (CO; $P < 0.001$) and LVEDD/BW ($P < 0.001$) in DOX-treated animals compared to Control animals. Pretreatment with DZR saw increased CO ($P = 0.004$) compared with the DOX group. The cardiac output index per unit body weight appeared to be decreased in the DOX group (Table 1; $P = 0.022$), compared with that of the Control group. The DOX + DZR group had slightly higher cardiac output index ($P = 0.041$) than the DOX group. At week 9 (i.e., 3 weeks after DOX and DZR treatment were stopped), one batch of animals (DOX, $n = 6$; DOX + DZR, $n = 7$, Control, $n = 5$) were also scanned for cardiac function. Results showed significant improvement in fractional shortening (FS; DOX 34.2 ± 5.7% vs. DOX + DZR 43.3 ± 3.1%, $P = 0.008$)

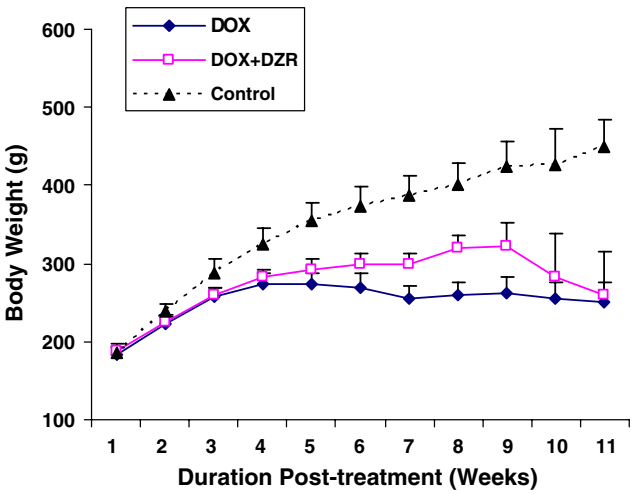

Fig. 1 Effect of DOX and DZR on body weight of animals. Male Sprague–Dawley rats were injected with DOX once weekly for 6 weeks with or without DZR pretreatment. Control animals ($n = 10$) received saline only. DOX-treated animals ($n = 14$) had compromised growth rate in terms of body weight ($P < 0.001$), whereas DOX + DZR-treated animals ($n = 13$) had improved body weight, particularly at week 6–9 ($P = 0.002$)

Table 1 Cardiac function by serial echocardiography

Time point	Group	LVEDD (mm)	LVESD (mm)	HR (beats/min)	FS (%)	CO (mL/min)	CO/BW (mL/min/g)	LVEDD/BW (cm/kg)
Baseline	DOX	5.2 ± 0.4	2.7 ± 0.2	302 ± 16	48.9 ± 2.0	87.4 ± 20	0.50 ± 0.1	3.0 ± 0.3
	DOX + DZR	5.3 ± 0.4	2.7 ± 0.2	307 ± 24	49.9 ± 1.8	97.8 ± 23	0.56 ± 0.1	3.0 ± 0.2
	Control	5.2 ± 0.3	2.6 ± 0.2	310 ± 26	49.5 ± 2.8	90.1 ± 16	0.52 ± 0.1	3.0 ± 0.3
6 weeks	DOX	5.5 ± 0.4*	3.1 ± 0.4	255 ± 31*	44.6 ± 4.2	83.6 ± 17*	0.32 ± 0.1	2.1 ± 0.1*
	DOX + DZR	6.0 ± 0.5$^{\#}$	3.3 ± 0.4	265 ± 23	45.4 ± 4.2	113 ± 28$^{\#}$	0.38 ± 0.1	2.0 ± 0.2
	Control	6.4 ± 0.4	3.5 ± 0.4	283 ± 25	46.4 ± 3.8	143 ± 17	0.37 ± 0	1.7 ± 0.1
11 weeks	DOX	6.7 ± 0.5*	4.6 ± 0.5*	216 ± 32*	31.8 ± 3.6*	98.1 ± 21*	0.41 ± 0.1	2.8 ± 0.4*
	DOX + DZR	6.0 ± 0.5$^{\#}$	3.4 ± 0.5$^{\#}$	214 ± 52	43.4 ± 5.7$^{\#}$	90.9 ± 33	0.36 ± 0.1	2.5 ± 0.4$^{\#}$
	Control	7.3 ± 0.4	4.1 ± 0.4	268 ± 21	44.1 ± 3.5	186 ± 28	0.41 ± 0.1	1.6 ± 0.1

Values are mean ± SD in the DOX (n = 15), DOX + DZR (n = 13) and Control (n = 10) animal groups

LVEDD left ventricular internal dimensions at end-diastole; *LVESD* left ventricular internal dimensions at end-systole; *HR* heart rate; *FS* fractional shortening; *CO* cardiac output

* DOX vs. Control, $P < 0.025$; $^{\#}$ DOX vs. DOX + DZR, $P < 0.025$ at same time points

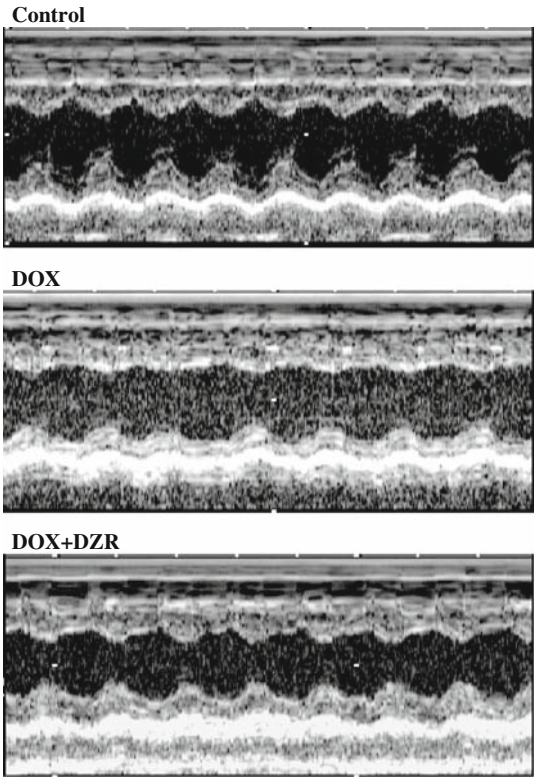

Fig. 2 Representative M-mode echocardiograms of experimental animals at week 11. DOX treatment resulted in significant left ventricular cardiac impairment as indicated by two-dimensional M-mode tracing of wall motion, compared to Control animals. The DOX + DZR animals had significantly improved cardiac functions

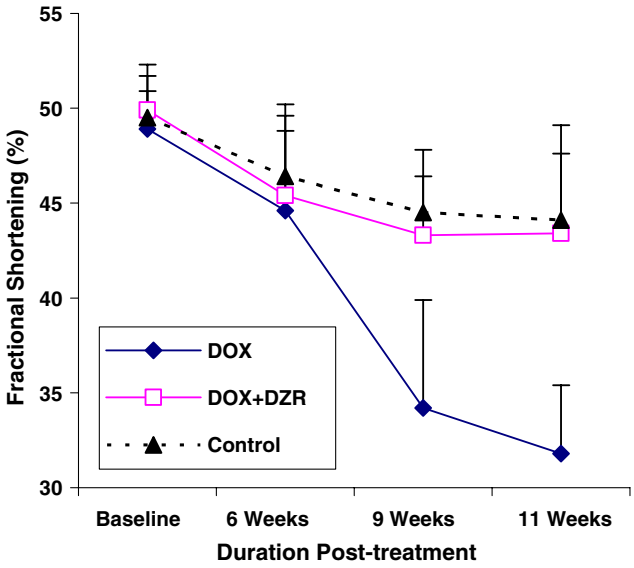

Fig. 3 Fractional shortening levels of experimental animals. Echocardiographic analysis of animals at baseline, 6, 9 and 11 weeks post-treatment with DOX showed significantly impaired FS in DOX-treated animals compared with the Control group at week 9 and 11 ($P < 0.01$). Increased FS was observed in the DOX + DZR group compared with the DOX group ($P < 0.01$)

(Fig. 3) and CO (DOX 97.8 ± 18.8 mL/min vs. DOX + DZR 126 ± 18.5 mL/min, $P = 0.02$) with DZR pre-treatment. The FS and CO of DOX + DZR animals were not significantly different from those of Control animals (FS = 44.5 ± 3.3%; CO = 151 ± 21.6 mL/min) at this stage. At week 11, FS ($P < 0.001$) of the DOX + DZR group remained to be higher than the DOX group, whereas there was no longer significant difference of CO between the two groups. FS of DOX + DZR animals were similar to those of Control animals but CO levels were lower than the Controls ($P < 0.001$). The cardiac output index was similar among the three groups of animals at week 9 or 11.

Light and electron microscopy of myocardium

There were significant differences in the cardiomyopathy score among the three groups of animals at week 11

(Table 2; $P < 0.001$). The myocardial pathology associated with DOX treatment included myofibrillar loss and cytoplasmic vacuolization (Fig. 4a–c). The DOX-treated group had more severe lesions compared with the control group ($P = 0.001$), and the DOX + DZR-treated group had reduced scores of cardiomyopathy ($P = 0.004$) compared with those in the DOX-treated animals. Ultrastructural changes associated with DOX-induced cardiomyopathy included swelling and vacuolization of mitochondria, and damage of the sarcotubular system (Fig. 4e). The DOX + DZR group showed less damage in myofibril arrangement, sarcotubular system and the mitochondria (Fig. 4f). There was no significant difference between the DOX + DZR group and the Control group ($P = 0.058$).

Activated Akt and Erk phosphorylation in DZR-treated myocardium

At week 11, protein expressions of total Akt and Erk 1/2 in the myocardium of Control animals were relatively high (Fig. 5). The respective phosphorylation kinases were also fairly active as observed in the p-Akt and p-Erk levels. In the DOX group, phosphorylation of Akt was reduced to 19% of those in Control animals, whereas a much milder reduction (91% of Control) was observed in the DOX + DZR group (DOX vs. DOX + DZR, $P < 0.001$). The phosphorylation of Erk 1/2 in the DOX-treated myocardium decreased to 28% of the Control, but was increased to 50% in the DOX + DZR group ($P = 0.032$).

Discussion

We established a rat model of chronic cardiomyopathy induced by repeated administration of DOX for 6 weeks. We also investigated the effects of pretreatment with DZR on cardioprotection by assessing outcomes at medium to long term (6–11 weeks). Our data showed that DOX-treated animals had reduced body weight and compromised cardiac functions in terms of heart rate (HR), FS, CO and LVEDD/BW at one or more time points. The damage severity appeared to progress with time, even after DOX and DZR treatment were stopped at week 6. Cardiomyopathy injuries were again observed in the heart morphology in terms of myofibrillar and mitochondrial structures scored under light and electron microscopy. Our results are in line with those reported by us [16] and others [3, 11, 15, 23] on acute and chronic animal models of DOX-induced cardiomyopathy. At a dose ratio of 20:1 of DZR:DOX [12], our

Table 2 Cardiomyopathy scores of experimental animals

Group	No. of animals	Cardiomyopathy score					
		0	1	1.5	2	2.5	3
DOX*	9	0	0	3	3	1	2
DOX + DZR§	6	1	3	2	0	0	0
Control	5	4	1	0	0	0	0

The Kruskal–Wallis test showed that there were differences among the three groups ($P < 0.001$). There was a significant increase of cardiomyopathy score in DOX-treated animals compared with control animals (Mann–Whitney test, $^{*}P = 0.001$) and reduction of score in DOX + DZR-treated animals ($^{\S}P = 0.004$) compared with the DOX-treated animals

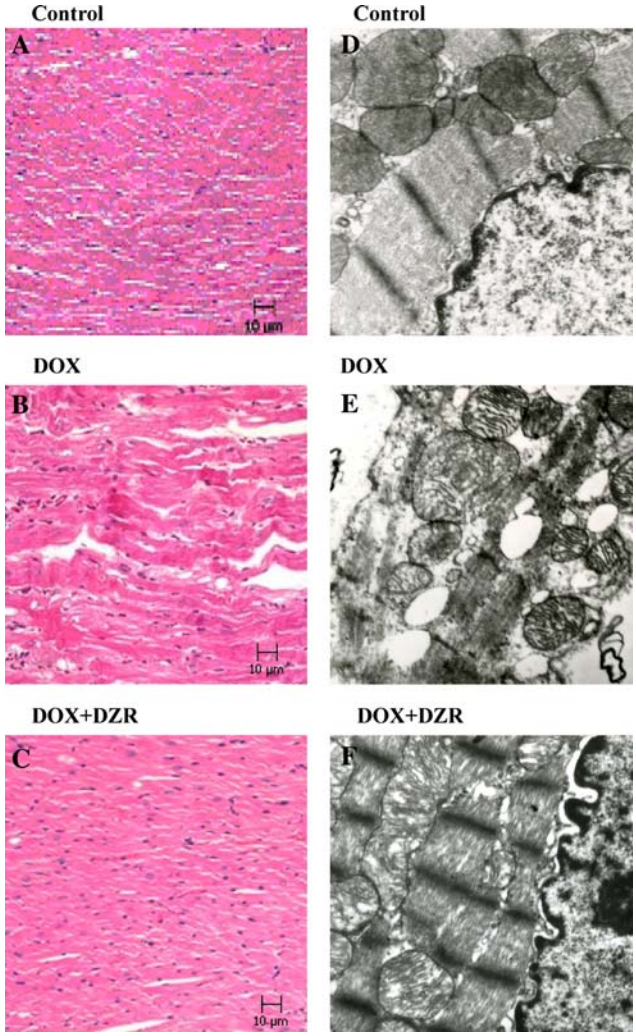

Fig. 4 Representative images on histopathology of rat myocardium. Left ventricular heart sections were stained with hematoxylin–eosin (**a**–**c** magnification ×200): **a** Control rat had normal myocardium morphology; **b** heart tissue damage in DOX-treated animals as indicated by increased cytoplasmic vacuolization and myofibrillar loss; **c** heart myocardial lesions were significantly reduced in animals that received DZR treatments. The electron-microscopic ultrastructure showed significant differences among the three groups (**d**–**f** magnification ×12,000): **d** Control rat had normal ultrastucture of the myocardium; **e** DOX-induced morphological damages, including swelling and vacuolization of mitochondria, dilatation of sarcotubular system, were observed; **f** DOX + DZR animals had regular myofibril arrangement, sarcotubular system and mitochondria

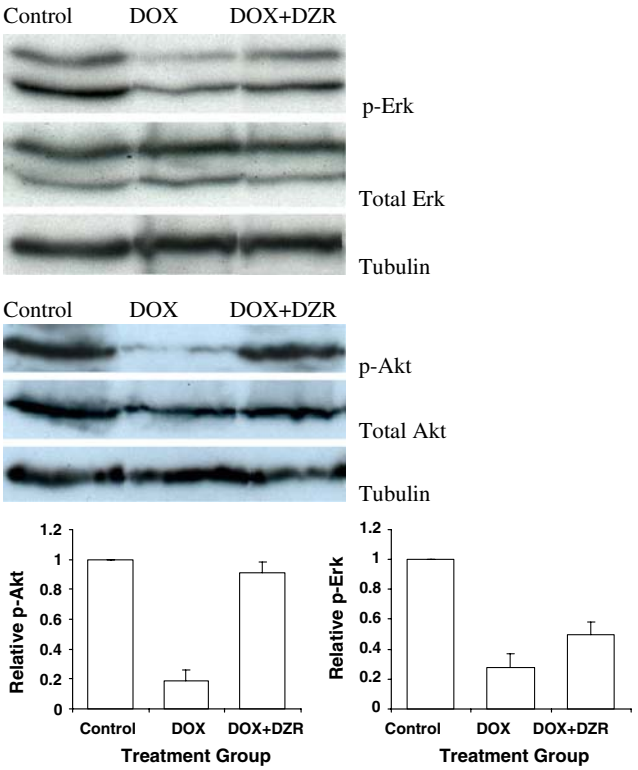

Fig. 5 Western blot analysis of heart tissues for Akt and Erk phosphorylation. Protein expressions of total Akt, p-Akt, total ERK1/2, p-Erk and α-Tubulin from heart tissues were examined at week 11. Values are presented as the ratio of phospho-specific kinases to total kinases, relative to proteins in the Control animals. Akt and Erk phosphorylation were compromised in DOX-treated myocardium and were significantly recovered in the DOX + DZR-treated animals ($P < 0.05$). Data are mean ± SD from three hearts

data showed that pretreatment with DZR had a cardioprotective effect against DOX-induced cardiac damage. The effects were particularly apparent on the FS data (Fig. 3) which are significant at 9 and 11 weeks when damages in the DOX-treated groups were most noticeable (Table 2). The cardioprotective activity of DZR was also demonstrated in the cardiomyopathy scores (Table 2), morphology and ultrastructure of heart tissues (Fig. 4). The preservation of mitochondrial integrity in the DZR-treated animals (Fig. 4d–f) was in line with reported DOX-induced mitochondrial toxicity and protective mechanism of DZR [1, 15]. Published data have shown that DZR-only treatment on Control animals without subjection to DOX-induced damage had normal cardiomyopathy score and histological features [12, 30]. Héon et al. reported that DZR-only treatment did not alter the protein expression of apoptotic and oxidative markers in heart tissues of young male rats [10].

Mitochondrial membrane integrity has been shown to be regulated by the interaction of a panel of pro- and antiapoptotic protein signals through heterodimerization and kinase phosphorylation activities. Akt and Erk activation pathways are known to be protective against the progression of cardiomyocyte apoptosis. Activation of the PI3 K/Akt signals could phosphorylate the proapoptotic proteins, such as Bad, Bim and Bax, resulting in their inactivation. Another action of Akt is the phosphorylation and inhibition of caspase 9, an initiator caspase of the intrinsic pathway [2, 8]. On the other hand, phosphorylation of Ras/Mek1/Erk1/2 could activate protein kinase p90RSK, which phosphorylates Bad and other proapoptotic proteins, resulting in cell survival [26, 28]. In our study, p-Akt and p-Erk levels were reduced in DOX-treated heart tissues whereas total Akt and Erk levels were similar when compared to Control tissues. The in vivo effects of DOX on the Akt and Erk signal pathways remained not fully understood. The mechanism is possibly related to the severity of heart damage and timing of post-DOX insult. Our results are in line with those of Lou et al. [18], who demonstrated that Erk 1/2 was upregulated at early time points (peaked at 4 h) of DOX administration, followed by a decline in the heart failure stage (3 weeks). Li et al. [17] reported in a mouse model after a single treatment with DOX that Erk phosphorylation was markedly inhibited, whereas Akt activation remained unchanged. However, using a female rat model, Gabrielson et al. [3] observed increased p-Akt in DOX-treated animals and suggested that injury above a certain threshold level cannot be rescued by ErbB2 or Akt activation. In human hypertensive patients with heart failure, González et al. [5] reported p42/p44 MAPK and P13 K/Akt activation were decreased when compared with nonheart-failure hypertensive individuals. To our knowledge, this is the first report showing DZR-regulated phosphorylation of Akt and Erk in DOX-induced cardiomyopathy. We suggest that these survival/antiapoptotic pathways are related to the cardioprotective effect of DZR against DOX damage.

In the acute phase of DOX insults (4 days), Child et al. [1] reported mitochondrial apoptosis occurring as a result of oxidative stress. However, adaptive responses such as increases of respiratory P/O ratio and Bcl-2:Bax ratio also took place in parallel. Together with the observed upregulation of Erk 1/2 at early time points of DOX administration [18], but compromised Erk and Ark phosphoryation at later stages of heart damage, the Erk and Akt pathways appear to play important roles in the pathophysiology of DOX-induced cardiomyopathy. In our model of chronic DOX-injury, it is apparent that extensive or even permanent damage has occurred to the mitochondria, leading to inferior cardiac functions. Since the heart tissues were analyzed 5 weeks after termination of both DOX and DZR treatments, it appeared that the mitochondrial protection activities of DZR have remained beyond the treatment period. Considering that the CO and output index were relatively higher at week 6 compared with those at week 11, it would be rewarding in future studies to investigate time-related

kinetics of Akt and Erk activation and whether the extension of DZR treatment after termination of DOX would have additional effects on prevention of further deterioration of the myocardium. In summary, the present study presented the first evidence that the Akt and Erk signaling pathways were regulated in the course of DOX-induced cardiomyopathy and protection by DZR. Better understanding of the mechanism of DZR on cardioprotection would enhance the rationale of its clinical use and benefit the development of other cardioprotective agents.

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