Long-acting Phosphodiesterase-5 Inhibitor Tadalafil Attenuates Doxorubicin-Induced Cardiomyopathy without Interfering with Chemotherapeutic Effect

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List of nonstandard abbreviations: DOX, Doxorubicin; PDE, phosphodiesterase; PKG, protein kinase G; SOD, super oxide dismutase

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ABSTRACT

Doxorubicin (DOX) is one of the most effective anticancer drugs. However, its cardiotoxicity remains a clinical concern that severely restricts the therapeutic usage of DOX. We designed this study to investigate if tadalafil - a long-acting phosphodiesterase-5 (PDE-5) inhibitor protects against DOX-induced cardiotoxicity. We also sought to delineate the cellular and molecular mechanisms underlying tadalafil-induced cardioprotection. Male CF-1 outbred mice were randomized into 3 groups (n=15-24/group) to receive either Saline (0.2 ml, i.p.), or DOX (15 mg/kg, given by a single i.p. injection) or tadalafil (4 mg/kg, p.o. daily for 9 days) plus DOX. Left ventricular function was subsequently assessed by trans-thoracic echocardiography and Millar conductance catheter. Cardiac contractile function was impaired by DOX, which was significantly improved by co-treatment with tadalafil. Tadalafil attenuated DOX-induced apoptosis and depletion of pro-survival proteins including Bcl-2 and GATA-4 in myocardium. Cardiac oxidative stress was attenuated and antioxidant capacity was enhanced by tadalafil possibly via upregulation of MnSOD. Moreover, tadalafil treated group demonstrated increased cardiac cGMP level and protein kinase G (PKG) activity. Tadalafil did not interfere with the efficacy of DOX in killing of human osteosarcoma cells in vitro or its anti-tumor effect in vivo in tumor xenograft model. We conclude that tadalafil improved left ventricular function and prevented cardiomyocyte apoptosis in DOX-induced cardiomyopathy through mechanisms involving upregulation of cGMP, PKG activity and MnSOD level, without interfering with the chemotherapeutic benefits of DOX.
INTRODUCTION

Doxorubicin (DOX) is an antineoplastic anthracycline widely used in the therapy of various malignant tumors including leukemias, lymphomas, and solid tumors such as ovarian, breast, lung, cervical and uterine cancers (Hortobagyi, 1997). However, despite its excellent anti-tumor efficacy, dose-dependent cardiotoxic side effects of DOX have been a major clinical concern limiting its therapeutic usage (Singal et al., 2000). It has been shown that DOX induces irreversible cardiomyopathy and heart failure in >30% patients receiving 500 mg/m² or higher cumulative dose (Lefrak et al., 1973; Minotti et al., 2004). Acute DOX cardiotoxicity is clinically manifested as arrhythmia, tachycardia and arterial hypotension while, chronic symptoms are marked by ventricular dilatation and cardiac dysfunction eventually leading to heart failure. (Fu et al., 1990; Lefrak et al., 1973) The heart failure caused by DOX is characterized by damage resulting from the disintegration of the myofibrillar array, mitochondrial injury, and cardiomyocyte apoptosis leading to the loss of the myofibrils (Billingham et al., 1978). Reduction in fractional shortening and abnormalities in nonspecific T wave and ST-T segment of EKG are typically observed in DOX-induced ventricular dysfunction (Friess et al., 1985).

A variety of mechanisms have been suggested to contribute in DOX induced cardiomyopathy and heart failure. These include free radical formation (Doroshow and Davies, 1986), lipid peroxidation (Myers et al., 1977), inhibition of protein synthesis (Singal and Iliskovic, 1998), mitochondrial edema and vacuolization (Billingham et al., 1978), calcium overloading (Arai et al., 2000), structural disorganization and death of myocytes (Arola et al., 2000). Several therapeutic strategies like administration of β-blockers, inhibitors of renin-angiotensin-system, free radical scavengers and
antioxidants such as probucol have been employed to reduce DOX-induced cardiotoxicity at early stages. The development of anthracycline analogues and alternative methods of drug delivery such as liposomal and nanosomal encapsulated DOX are some of the promising approaches aimed at improving the antitumor efficacy and attenuating the toxic effects of DOX. However, despite various therapeutic interventions adapted to protect the heart against DOX-induced cardiotoxicity, all of these approaches have been limited by their pronounced side effects and demerits (Granger, 2006). At present, cardiac transplantation remains as the only definitive option to treat DOX-induced heart failure in later stages (Thomas et al., 2002). Hence, there is an ongoing need to further investigate and develop efficient therapeutic agents to combat DOX-induced cardiac damage.

Tadalafil is a potent long acting selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase-5 (PDE-5), which hydrolyzes and eliminates cGMP in cells. cGMP causes smooth muscle relaxation and increases blood flow (Rotella, 2002). Several studies from our laboratory have shown that PDE-5 inhibitors induce powerful cardioprotective effect during ischemia/reperfusion injury (Ockaili et al., 2002; Kukreja et al., 2003; Salloum et al; 2003). We also demonstrated that short acting PDE-5 inhibitor, sildenafil (Viagra®) attenuates cardiac dysfunction in DOX-induced cardiomyopathy (Fisher et al., 2005). In the present study, we hypothesized that tadalafil (Cialis®) may also provide protection against DOX induced cardiotoxicity. Our first goal was to demonstrate that tadalafil induces cardioprotective effect without interfering with the anti-tumor effect of DOX. A second goal was to delineate the mechanisms by which tadalafil attenuates DOX induced cardiotoxicity. Tadalafil is an FDA-approved drug that targets the same enzyme as sildenafil i.e., PDE-5 and has a number of
properties that could make it the preferred drug for treatment. These include: (a) the pharmacokinetic properties of tadalafil allow for the most sustained PDE-5 inhibition among this class of agents; (b) it is more slowly metabolized than sildenafil and thus likely could be used at lower doses for long-term management of patients receiving DOX for malignant tumors; (c) Tadalafil is the only PDE-5 inhibitor whose activity is unaffected by food and it has a relatively short time to onset of action (16–17 minutes). It is >10,000 fold more potent for PDE-5 than for PDE-1, PDE-2, PDE-3, PDE-4 and PDE-7 enzymes., ~ >9,000 fold more potent for PDE-5 than for PDE-8, PDE-9, PDE-10 and ~700 fold more potent for PDE-5 than for PDE-6 (Kuan and Brock, 2002). At present it seems that the doses of sildenafil currently being used in the cardiac hypertrophy and heart failure studies are high enough to also inhibit PDE1C in the heart (Takimoto et al., 2005; Vandeput et al., 2009). Therefore, it is not entirely clear which molecular target characterizes the beneficial effects of Viagra® on cardiac dysfunction and therefore it is necessary to follow up with studies using tadalafil, which does not inhibit PDE1C as effectively as sildenafil. Due to these compelling reasons, we chose tadalafil for the current investigation.
METHODS

Animals and experimental protocols
Adult male CF-1 mice (~30 g body weight) obtained from Harlan Sprague Dawley (Indianapolis, IN) were randomized to receive: Saline (0.2 ml, i.p.), DOX (Sigma, 15 mg/kg; i.p.) or DOX+tadalafil (4 mg/kg, p.o. daily) for 9 days starting three days before DOX treatment (n=15-24/group). In this study we used a single dose of DOX at 15 mg/kg i.p. which has been reported to be cardiotoxic (Abd-Allah et al., 2002). The mice were hemodynamically characterized five days after DOX treatment. This 5-day post-DOX time point was chosen as it was > 5 final half-lives of DOX elimination from both plasma and cardiac tissue in mice (van der Vijgh et al., 1990). The hearts were excised, weighed and heart weight/tibia length ratio (HW/TL) was calculated after the treatment schedule as illustrated in Figure 1. Some hearts were utilized for molecular, biological analysis as described below. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Measurement of tadalafil in plasma
Mice were treated with tadalafil (4 mg/kg, p.o.) for 9 days. The 4 mg/kg p.o. was chosen based on the interspecies dose extrapolation scaling to result in plasma concentrations equivalent to human dose 20 mg/day. One hour after the last oral dose of tadalafil on the 9th day, the mice were anesthetized with pentobarbital (30 mg/kg, i.p.) and blood was collected into BD Diagnostics vacutainer® tubes containing K2EDTA (Franklin Lakes NJ, USA). The blood was centrifuged at 600 g for 15 min at 4°C and the plasma was collected and stored at -80°C. The concentration of
tadalafil in the plasma was measured by high-performance liquid chromatography (HPLC) method utilizing fluorescence detection.

**Measurement of LV contractile function and hemodynamics**

Under surgical anesthesia (pentobarbital 50 mg/kg, i.p.), a micro-tip pressure-volume catheter transducer (Millar instruments Inc., Houston, TX; Model SPR-1045) was inserted into the right carotid artery and advanced into LV cavity. After stabilization for 15 to 20 min, the signals were continuously recorded with a MPVS-300 system (Millar Instruments) coupled with a Powerlab 8/30 converter (AD Instruments, Colorado Springs, CO), stored and displayed on a computer. LV systolic and end-diastolic pressures, maximal slope of systolic pressure increment (+dP/dt\text{max}) and diastolic pressure decrement (-dP/dt\text{max}), heart rate, and aortic blood pressure were recorded on a beat-by-beat basis.

**Echocardiography**

Under light anesthesia (pentobarbital 30 mg/kg, i.p.), Doppler echocardiography was performed using the Vevo 770™ imaging system (VisualSonics, Inc., Toronto, Canada) which is equipped with a 30-MHz mechanical scan probe to obtain high resolution two-dimensional images. B-mode images were obtained in the plane containing aortic and mitral valves, while M-mode images were obtained from parasternal short-axis view at the level of papillary muscles and the apical four-chamber view. Left ventricular (LV) end-systolic and end-diastolic diameters (LVESD and LVEDD), fractional shortening, ejection fraction were calculated using Vevo Analysis software (version 2.2.3) as described previously (Schiller et al., 1989; Salloumn et al., 2008). M-mode
measurements of LV dimensions were averaged from three cycles. The investigators performing echocardiography were blinded to the treatment status.

**Western blot analysis**

Five days after DOX treatment the whole heart tissue samples were collected and the proteins were extracted in a buffer containing (in mmol/l): 50 potassium phosphate, 1 EDTA, 1 EGTA, 0.2 PMSF, 5 beta-glycerophosphate, 2 NaF, 2 Na₃VO₄, 10 β-mercaptoethanol, 1µg/ml peptatin, and 0.5 µg/ml leupeptin, (pH 7.0) with a tissue homogenizer. The homogenate was centrifuged at 10,000 g for 15 min under 4°C and the supernatant was recovered. 50 mg of protein from each sample was separated by SDS-PAGE and transferred onto 12-10% nitrocellulose membrane. The membrane was incubated with primary antibodies at a dilution of 1:1000 for each of the respective proteins, i.e. Bcl-2 (Santa Cruz Biotechnology), Cu/ZnSOD (Calbiochem), MnSOD (Calbiochem), GATA-4 (Sigma) and actin (Santa Cruz Biotechnology). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, 1h at room temperature). Detection of the signals was performed using LumiPhos™ reagent (Pierce) and chemical luminescence was detected using X-omat film. The densitometry quantification was performed with Bioquant image analysis software.

**Lipid peroxidation Assessment**

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) and 4-hydroxyalkenals as described previously (Kang et al., 1996) using a colorimetric assay kit (Bioxytech LPO-586, Oxis International, Foster City, CA). Briefly, the heart tissue was homogenized in ice cold PBS containing 5mmol/l butylated hydroxytoluene in acetonitrile. The homogenate was centrifuged at
3000 g at 4°C for 10 min. 200 µl of clear supernatant containing protein extracts were used for the assay following the manufacturer’s instruction.

**Cardiomyocyte apoptosis**

Cardiomyocyte apoptosis was evaluated using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method with an ApopTag *in situ* apoptosis detection kit (Chemicon) according to the manufacturer’s instruction. The quantification of apoptosis was determined by counting the TUNEL-positive myocyte nuclei from 5 random fields per section and was expressed as percentage of total myocyte nuclei as previously reported (Das et al., 2008).

**PKG activity assay**

PKG activity was assayed by colorimetric analysis with a CycLex cGMP-dependent protein kinase assay kit (MBL International, Woburn, MA) in the whole heart lysate. Briefly, 10 µl of clear supernatant collected after cardiac tissue homogenization was used to measure the PKG activity as described in the manufacturer’s protocol (Das et al., 2008). Spectrophotometric absorbance was measured at 450 nm and the results were normalized per mg of protein.

**cGMP measurement**

cGMP was quantitatively determined using a BIOMOL EIA cGMP kit. Briefly, the frozen heart tissue was ground to fine powder under liquid nitrogen and then homogenized in cold 5% trichloroacetic acid. Homogenates were centrifuged at 600 g for 10 min and the supernatants were extracted three volumes of water-saturated ether. After lypholization of the aqueous
extracts, the dry extracts were dissolved in assay buffer and cGMP was measured according to the manufacturer’s protocol.

**In vitro cancer cell viability assay**

The inhibitory effects of DOX and tadalafil on proliferation and viability of OSA-1 human osteosarcoma cells were measured by CellTiter96®AQueous One Solution Cell Proliferation Assay (Promega Co.) according to manufacturer’s protocol.

**In vivo anti-tumor efficacy study**

Tumors were generated in male nude mice (strain-BALB/cAnNCr-nu/nu from the NCI Developmental Therapeutic Program) by subcutaneous injection of OSA-1 sarcoma cells (5×10^6 cells) with 50 µl MatriGel matrixes (BD Bioscience, Bedford, MA). Tumors were permitted to grow to a volume of ~200 mm^3 over the following 2 weeks, and then animals were randomly divided into three groups (n ≥ 6 per group). The control group received PBS (0.2 ml) daily by oral gavage. Other groups received DOX (3 mg/kg, i.p. twice a week for 16 days) or tadalafil (4 mg/kg, p.o. daily for 16 days) plus DOX. Tumor size was measured twice a week and tumor volume was calculated by ab^2/2 where “a” and “b” are the long and short axes of tumor.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (version 4.03). Data are presented as mean ± SE. The difference between groups was analyzed using ANOVA followed by Student Newman-Keuls post-hoc test. The Chi-square test was used to compare survival rates between the groups. Statistical differences were considered to be significant at P value of <0.05.
RESULTS

Administration of tadalafil (4 mg/kg, p.o. for 9 days, n=6) resulted in 534 ± 89 ng/ml of tadalafil concentration in the plasma of the mice. The DOX+ tadalafil treated group exhibited enhanced survival rates (93.3%) as compared to the DOX group (79.2%, p<0.05, n=15-24/group, Fig.1B) during the 9 day experimental protocol. The decreased survival rate in the DOX group was also associated with decrease in the ratio of heart weight and tibial length (HW/TL) (n=8, p<0.05, Fig. 1C).

Left ventricular function

LV function was significantly impaired five days after DOX treatment. As shown in the representative tracing images (Fig. 2A) echocardiography demonstrated that DOX+tadalafil treated mice preserved fractional shortening (FS) and ejection fraction (EF) as compared to DOX-treated group (n=6, p<0.05, Fig. 2B and C). In addition, the LV systolic pressure decreased 36%, +dp/dt_{max}, 63%; -dp/dt_{max}, 57%; heart rate, 30% as compared with the controls (P<0.05). In contrast, DOX+tadalafil treated mice showed improved LV function (i.e. LV systolic pressure, 33%; +dp/dt_{max}, 35%; -dp/dt_{max}, 46%; heart rate, 27%;) when compared with the group treated with DOX alone (n=6, p<0.05, Fig. 3).

Cu/ZnSOD and MnSOD expression

We investigated whether tadalafil plays a role in the regulation of antioxidant enzyme, superoxide dismutase (SOD). The cytosolic SOD1 (Cu/ZnSOD) and mitochondrial SOD2 (MnSOD) were quantified 5 days after DOX treatment. Tadalafil co-treatment with DOX had no
effect on Cu/ZnSOD expression. However, MnSOD expression was increased in DOX+tadalafil treated as compared to the control group (n=4/group, p<0.05, Fig. 4A and 4B).

**Lipid peroxidation**

Cardiac lipid peroxidation activity in the DOX group was significantly increased by 37.6% as compared to the control group (n=8, p<0.05, Fig. 4C). However, the lipid peroxidation in the DOX+tadalafil group was not significantly different from control group.

**Apoptosis and Bcl-2 expression**

Cardiomyocyte apoptosis is implicated as one of the mechanisms underlying DOX-induced cardiomyopathy. Expression of the antiapoptotic protein, Bcl-2 was downregulated in DOX group as compared with the control (Fig. 5A; n=6, p<0.05). Tadalafil co-treatment significantly preserved Bcl-2 level. Apoptosis, as assessed by TUNEL positive nuclei was increased in the DOX group compared with control and DOX+ tadalafil groups (n=6, p<0.05, Fig. 5B).

**GATA-4 expression**

GATA-4 is a member of the GATA family of zinc finger transcription factors, which plays important roles in transducing nuclear events that modulate cell lineage differentiation during development in the heart. GATA-4 was reduced in DOX-treated mice as reported previously (Li et al., 2007). Tadalafil treated group showed higher expression of GATA-4 when compared to DOX group (n=4, p<0.05, Fig. 5C).
Cardiac cGMP level and PKG activity

Treatment with DOX increased cGMP level as compared to the saline treated control in the heart (n=5, p<0.05, Fig. 6A). The combined treatment with tadalafil and DOX further augmented cGMP levels as compared with DOX alone or control. PKG activity was also increased with DOX as compared with control although this change was insignificant. However, DOX+tadalafil treatment caused significant increase in PKG activity as compared with DOX alone (p< 0.05 vs DOX, n=7, Fig. 6B).

Effect of tadalafil on DOX-induced cancer cell killing and xenograft growth

In human osteosarcoma (OSA-1) cancer cell lines, the percentage of cell viability was reduced to 76.1 ± 0.7% following 48 hrs incubation with 1 µmol/l DOX. Co-treatment with tadalafil (10 µmol/l) and DOX also reduced the cell viability to 76.4 ± 0.5% 48 hrs after the treatment (Fig. 7A), suggesting that tadalafil did not impede the cell-killing efficacy of DOX in vitro. To further confirm if tadalafil does not interfere with the in vivo anti-tumor effect of DOX, we used the OSA-1-tumor xenograft model. As expected, DOX significantly reduced the body weight (Fig. 7B), tumor weight (Fig. 7C) and tumor volume (Fig. 7D) as compared to the controls after 16 days of DOX treatment. (p<0.001, n=6). Tadalafil co-treatment did not change DOX-induced reduction in tumor weight, volume (Fig. 7C and 7D) and body weight (Fig. 7B). These results suggest that co-treatment with tadalafil does not interfere with the anti-tumor efficacy of DOX in vivo.
DISCUSSION

We and others have demonstrated that PDE-5 inhibitors including sildenafil, vardenafil and tadalafil induce anti-ischemic effect in the heart in various animal species (Das et al., 2008; Ockaili et al., 2002; Salloum et al., 2006; Sesti et al., 2007). In the present study, for the first time we show that the long-acting PDE-5 inhibitor tadalafil protects against DOX-induced cardiotoxicity in mice. Tadalafil activated mitochondrial antioxidative and antiapoptotic mechanisms which contributed to improved LV function without interfering with the anti-cancer efficacy of DOX. These results conceptually support our previous report on the sildenafil-induced cardioprotection in a chronic model of DOX-induced cardiomyopathy (Fisher et al., 2005). Furthermore, considering the specificity of this drug, these studies suggest that PDE-5 is the molecular target for attenuating DOX cardiotoxicity. We therefore propose that the class of PDE-5 inhibitors can represent attractive novel therapeutic approach for managing the clinical concern of the DOX-induced cardiotoxicity in patients.

In the present study, we have made several significant advances in understanding the mechanisms of protection against LV dysfunction caused by DOX. First, we have shown that tadalafil reduced myocardial oxidative stress via upregulation of MnSOD - a key mitochondrial antioxidant enzyme. Second, we demonstrated that cGMP/PKG signalling pathway is involved in tadalafil-induced cardioprotection in the setting of DOX-induced cardiomyopathy. Third, tadalafil treatment prevented DOX-induced downregulation of transcription factor GATA-4. Finally, we provided both in vitro and in vivo evidence for the anti-cancer efficacy of DOX, which remained unaltered by co-treatment with tadalafil.
We employed an oral administration regimen of tadalafil (4 mg/kg for 9 days), which resulted in plasma concentration of 534±89 ng/ml similar to the reported levels in the human subjects taking clinically relevant doses of tadalafil (20 mg p.o. daily for a week) (Forgue et al., 2006). The mouse model of cardiotoxicity induced by a single dose of DOX (15 mg/kg i.p.) has also been used by other investigators (Abd-Allah et al., 2002). We observed severe LV systolic and diastolic dysfunction after DOX administration which is significantly improved by tadalafil (Fig. 2 and 3). At the systemic level, DOX administration caused decrease in HW/TL ratio, and survival rate in the mice (Fig. 1). These detrimental effects of DOX were partially attenuated by tadalafil.

More importantly, our results show that treatment with tadalafil inhibited DOX-induced increase in lipid peroxidation - a marker of oxidative stress (Fig. 4C). The increased generation of reactive oxygen species (ROS) with subsequent lipid peroxidation has been considered as a major pathogenic factor in DOX-induced cardiomyopathy. Antioxidant enzymes including Cu/ZnSOD and MnSOD play a critical role in detoxification of ROS. Tadalafil did not affect the regulation of cytoplasmic Cu/ZnSOD but MnSOD was significantly increased. These data imply that mitochondrial elimination of ROS (by virtue of increased MnSOD) contribute to the cardioprotective effects of tadalafil during DOX-toxicity. Previous studies have also shown that MnSOD overexpression can exert cardioprotection against DOX-induced injury and ischemia-reperfusion injury (Yen et al., 1996). The antioxidant properties of PDE-5 inhibitors, particularly in vivo have not yet been well studied and understood. Fernandes et al reported that the physiological concentrations of sildenafil (<50 µmol/L) decreased both H2O2 generation by mitochondria respiring glutamate/malate. Moreover, it was shown that sildenafil decreased superoxide radical generated by hypoxanthine/xanthine oxidase system, without affecting either
mitochondrial bioenergetics or Ca$^{2+}$-induced mitochondrial permeability transition (Ferandes et al., 2008). Most recently, in a rat model of traumatic spinal cord injury Serarslan et al demonstrated that tadalafil reduced the spinal cord injury via increasing tissue/serum levels of nitric oxide and serum activity of SOD (Serarslan et al., 2009).

To further correlate the antioxidant effects of tadalafil with its anti-apoptotic protection, we looked at the expression Bcl-2, which is known to block mitochondrial pathway of apoptosis. DOX caused a significant decrease in cardiac Bcl-2 expression and tadalafil treatment completely preserved the level of Bcl-2 (Fig. 5A), thereby suggesting mitochondrial protection against apoptosis. The depletion of Bcl-2 in the DOX group was associated with a significant increase in TUNEL-positive apoptotic cells which was also decreased by tadalafil (Fig. 5B). The decrease in cardiomyocyte apoptosis may at least partially explain the improvement of LV contractile function in the tadalafil-treated mice as compared with the DOX alone group (Fig. 2 and 3).

GATA-4 is a key transcriptional factor which plays a pivotal role in the regulation of cardiac protein expression and in turn controls embryonic development, cardiomyocyte differentiation, and stress responsiveness of the heart. It was recently shown that cardiac-specific deletion of GATA-4 resulted in a progressive and dose-dependent deterioration in cardiac function and dilation in adulthood (Oka et al., 2006). In response to pressure overload, the GATA-4 deficient mice developed rapid decompensation and heart failure. These detrimental phenotypes were associated with increased cardiomyocyte apoptosis (Oka et al., 2006). Our results also showed significant downregulation of GATA-4 expression after DOX treatment (Fig. 5C), which confirmed the previous reports (Aries et al., 2004). The significant restoration of
GATA-4 expression by co-treatment with tadalafil suggests that this transcription factor may have effectively contributed to the cardioprotective effect in the setting of DOX-induced toxicity.

PDE-5 inhibitors are well known to increase cGMP levels and activate the cGMP/PKG dependent signaling pathway in the heart, which in turn plays a critical role in PDE-5 inhibitors-induced cardioprotection against ischemia-reperfusion injury (Das et al., 2008). However, the role of cGMP/PKG signaling in protection against DOX-induced cardiotoxicity is not clear. In the present study, we observed an increase in cGMP levels in the DOX as well as DOX+tadalafil treated mice which are consistent with DOX-induced increase in NO and cGMP levels in vitro (Mykhaylyk et al., 2005). Moreover, we also observed a significant increase in the PKG activity and cGMP levels in the DOX+tadalafil treated mice. Considering the demonstrated role of PKG in protection against ischemia/reperfusion injury (Das et al., 2008; Salloum et al., 2009; Das et al., 2006), we speculate that this enzyme may have a role in reducing DOX induced cardiotoxicity through activation of ERK and inhibition of glycogen synthase kinase 3β.

Finally, we further addressed the possible effect of tadalafil in interfering with the anti-tumor efficacy of DOX. We employed both in vitro OSA-1 cell viability assay and in vivo xenograft tumor model to study to rule out such a possibility. Our results suggested that Tadalafil neither reduced the cytotoxic efficacy of DOX nor interfered with the DOX-induced reduction of tumor volume and weight. Hence, our results unvaryingly indicated that tadalafil did not impede the anti-tumor efficacy of DOX (Fig. 7).

Nevertheless, the current study has several limitations. First, the potential mediators that we identified were based on the association between the molecular changes (such as cGMP, PKG, GATA-4, and MnSOD) and the cardioprotective effects induced by tadalafil. Further studies are warranted to confirm their cause-and-effect relationship between these molecules and
tadalafil-induced cardioprotection. Second, this study focused on acute cardiomyopathy caused by a single high dose of DOX to provide the proof of concept for the protective effect of tadalafil. Future studies would be performed to demonstrate the protective effect of tadalafil following chronic treatment with low doses of DOX, a drug regimen used for treating cancer patients.

In conclusion, our studies provide valuable new information about the efficacy of tadalafil in attenuation of DOX-induced cardiac dysfunction. Tadalafil activated mitochondrial antioxidative and antiapoptotic mechanisms through upregulation of cGMP, PKG activity and MnSOD level, without interfering with the chemotherapeutic benefits of DOX. Thus, prophylactic treatment with tadalafil might become a promising therapeutic intervention, if substantiated by further clinical studies in patients.
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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Experimental protocol and effect of tadalafil on survival and body weight. (A) Mice were randomized into three groups. Group I received saline (0.2 ml, i.p.) on the 4th day and served as control group. Group II received an acute single intraperitoneal dose of DOX (15 mg/kg) while group III received tadalafil 4 mg/kg by oral gavages for 9 days in addition to a single dose of DOX (15 mg/kg i.p.) on the 4th day of treatment. Arrows indicate the time points of treatment and measurement of various parameters. (B) Kaplan-Meier survival curve, (C) ratio of heart weight to tibia length (HW/TL). Percentages of surviving mice were plotted and compared by chi squares test (n=15-24/group, P<0.05). HW/TL were presented as means ± S.E. (n=6-8/group, *P<0.05 vs Control).

Fig. 2. Trans-thoracic echocardiography assessment of the effect of tadalafil on ventricular contractile dysfunction caused by DOX. The top graph shows the representative echocardiographic tracings for each of the 3 experimental groups (A). The averaged data of fractional shortening (B) and ejection fraction (C) in the mice are presented as mean ± SE (n=6 per group; *P<0.05 vs Control, #P <0.05 vs DOX).

Fig. 3. Effect of tadalafil on hemodynamic function of the heart in DOX-induced cardiomyopathy. Left ventricular hemodynamic parameters, (A) Left ventricular systolic pressure, (B) left ventricular end-diastolic pressure, (C) positive and (D) negative dP/dt, (E) heart rate and (F) mean blood pressure measured with Millar micro-tip catheter in the aortic artery. Data are expressed as mean ± SE (n=6 per group), *P<0.05 vs Control, #P <0.05 vs DOX.
Fig. 4. Effect of tadalafil on superoxide dismutases and lipid peroxidation after DOX treatment. Representative western blots with specific bands of (A) MnSOD and (B) Cu/ZnSOD are shown. Bar graphs show densiometric quantification from 4 individual hearts per group, which was normalized against actin level for each sample. Data were expressed as mean ±SE. *P<0.05 vs Control. (C) Lipid peroxidation activity was quantified using a commercial kit. Data are represented as mean ± S.E. (n=8/group) *P<0.05 vs Control, #P <0.05 vs DOX.

Fig. 5. Tadalafil attenuates apoptosis and downregulation of GATA-4 in DOX-induced cardiomyopathy. (A) Representative western blot and densitometric quantification showing Bcl-2 expression (n=6/group). (B) Cardiac tissue apoptosis quantified using TUNEL staining and expressed as apoptotic index for TUNEL-positive cells Data are expressed as mean ± SE; (n=6/group). *P<0.05 vs Control; #P <0.05 vs DOX group. (C) Cardiac protein expression of GATA-4 as measured by western blot analysis. Bar graphs show densiometric quantification from 4 individual hearts per group, which is normalized against GAPDH level for each sample. *P<0.05 vs Control; #P <0.05 vs DOX group.

Fig. 6. Tadalafil augments cGMP and protein kinase G following DOX treatment. (A) cGMP level and (B) PKG activity normalized against mg protein in the cardiac tissue. Data are expressed as mean ± SE; (n=5-7/group).*P<0.05 vs Control; #P <0.05 vs DOX group, n=5-7/group.
Fig. 7. Effect of tadalafil on the anti-tumor efficacy of DOX. (A) Cell viability in human osteosarcoma cancer cell lines (OSA-1) following 48 hours of co-treatment with DOX and Tadalafil. (B) Effect on body weight, (C) tumor weight and (D) tumor volume in sarcoma (OSA-1) tumor xenograft model in BALB/cAnNCr-nu/nu mice following 16 days of tadalafil (4 mg/kg, p.o.) administration in DOX treated mice (n=6/group). *P<0.001 vs Control.
Fig. 1

A

LV Function
Tad
DOX or Saline

Days
1 2 3 4 5 6 7 8 9

Treatment Groups

I. Control (Saline, 0.2 ml; i.p.)
II. Doxorubicin (DOX, 15 mg/kg; i.p.)
III. Tadalafil (Tad, 4 mg/kg; p.o. for 9 days) + DOX (15 mg/kg; i.p.)

B

Survival rate (%)

Control
DOX
DOX + Tad

No. of days of treatment

C

Heart weight / Tibial length (mg/mm)

Control * DOX DOX + Tad

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**Fig. 2**

A) Control, DOX, and DOX + Tad

B) Bar graph showing Fractional Shortening (%)

C) Bar graph showing Ejection Fraction (%)

* indicates statistically significant difference from control
# indicates statistically significant difference from DOX
Fig. 3

A. LVSP (mmHg)

B. LVEDP (mmHg)

C. +dp/dt (mmHg/s)

D. -dp/dt (mmHg/s)

E. Heart rate (beats/min)

F. Mean Blood Pressure (mmHg)
Fig. 4

A

Control DOX DOX + Tad

MnSOD (25 kDa)

Actin (43 kDa)

Ratio of MnSOD / Actin

Control DOX DOX + Tad

B

Control DOX DOX + Tad

Cu/ZnSOD (16 kDa)

Actin (43 kDa)

Ratio of Cu/ZnSOD / Actin

Control DOX DOX + Tad

C

Control DOX DOX + Tad

Lipid Peroxidase Activity

* #
Fig. 5

A

Control DOX DOX + Tad

Bcl-2 (26 kDa)

Actin (43 kDa)

Ratio of Bcl-2 / Actin

Control DOX DOX + Tad

* #

B

Apoptotic Index (%)

Control DOX DOX + Tad

* #

C

Control DOX DOX + Tad

GATA-4 (44 kDa)

GAPDH (37 kDa)

Ratio of GATA-4 / GAPDH

Control DOX DOX + Tad

* #