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

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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 its therapeutic usage. We designed this study to investigate whether 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 three groups ($n = 15$ –24/group) to receive either saline (0.2 ml i.p.), DOX (15 mg/kg, given by a single intraperitoneal injection), or tadalafil (4 mg/kg p.o. daily for 9 days) plus DOX. Left ventricular function was subsequently assessed by transthoracic echocardiography and Millar conductance catheter. Cardiac contractile function was impaired by DOX, and it was significantly improved by cotreatment with tadalafil. Tadalafil attenu-

ated DOX-induced apoptosis and depletion of prosurvival proteins, including Bcl-2 and GATA-4, in myocardium. Cardiac oxidative stress was attenuated and antioxidant capacity was enhanced by tadalafil possibly via up-regulation of mitochondrial superoxide dismutase (MnSOD). Moreover, the 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 human osteosarcoma cells in vitro or its antitumor 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 up-regulation 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 DOX's excellent antitumor 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 doses (Le-

frak et al., 1973; Minotti et al., 2004). Acute DOX cardiotoxicity is clinically manifested as arrhythmia, tachycardia, and arterial hypotension, and chronic symptoms are marked by ventricular dilatation and cardiac dysfunction, eventually leading to heart failure (Lefrak et al., 1973; Fu et al., 1990). 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 the non-specific 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 to DOX-induced cardiomyopathy and heart failure. These include free radical formation (Doroshov and Davies, 1986), lipid peroxidation (Myers et al., 1977), inhibition of protein synthesis (Singal and Iliskovic, 1998), mitochondrial edema and

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ABBREVIATIONS: DOX, doxorubicin; PDE, phosphodiesterase; PKG, protein kinase G; SOD, superoxide dismutase; Cu/ZnSOD, cytosolic SOD1; MnSOD, mitochondrial SOD2; LV, left ventricular; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; HW/TL, heart weight/tibia length ratio; ROS, reactive oxygen species.

vacuolization (Billingham et al., 1978), calcium overloading (Arai et al., 2000), and structural disorganization and death of myocytes (Arola et al., 2000). Several therapeutic strategies such as administration of β -blockers, inhibitors of renin-angiotensin system, free radical scavengers, and antioxidants such as probucol have been used to reduce DOX-induced cardiotoxicity at early stages. The development of anthracycline analogs 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 for treating 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 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 the 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 antitumor effect of DOX. A second goal was to delineate the mechanisms by which tadalafil attenuates DOX-induced cardiotoxicity. Tadalafil is an Food and Drug Administration-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: 1) the pharmacokinetic properties of tadalafil allow for the most sustained PDE-5 inhibition among this class of agents; 2) it is more slowly metabolized than sildenafil and thus probably could be used at lower doses for long-term management of patients receiving DOX for malignant tumors; and 3) 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 min). It is >10,000-fold more potent for PDE-5 than for PDE-1, PDE-2, PDE-3, PDE-4, and PDE-7 enzymes, approximately >9000-fold more potent for PDE-5 than for PDE-8, PDE-9, and 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 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; therefore, it is necessary to follow up with studies using tadalafil, which does not inhibit PDE1C as effectively as sildenafil. Because of these compelling reasons, we chose tadalafil for the current investigation.

Materials and Methods

Animals and Experimental Protocols. Adult male CF-1 mice (~30 g body weight) obtained from Harlan (Indianapolis, IN) were randomized to receive saline (0.2 ml i.p.), DOX (Sigma-Aldrich, St. Louis, MO; 15 mg/kg i.p.), or DOX + tadalafil (4 mg/kg p.o. daily) for 9 days starting 3 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 5 days after DOX treatment. This 5-day post-DOX time point was chosen because it was more than five final half-lives of DOX elimination from both plasma and cardiac tissue in mice (van der Vijgh et al., 1990). The hearts were excised and weighed, and heart weight/tibia length ratio (HW/TL) was calculated after the treatment schedule illustrated in Fig. 1. Some hearts were used 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. This dose was chosen based on the interspecies dose extrapolation scaling to result in plasma concentrations equivalent to a human dose of 20 mg/day. One hour after the last oral dose of tadalafil on the ninth day, the mice were anesthetized with pentobarbital (30 mg/kg i.p.) and blood was collected into vacutainer tubes (BD Diagnostics, Franklin Lakes NJ) containing K_2EDTA . The blood was centrifuged at 600g 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 using fluorescence detection.

Measurement of Left Ventricular Contractile Function and Hemodynamics. Under surgical anesthesia (pentobarbital 50 mg/kg i.p.), a micro-tip pressure-volume catheter transducer (model SPR-1045; Millar Instruments, Inc., Houston, TX) was inserted into the right carotid artery and advanced into left ventricular (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 (ADInstruments, Inc., Colorado Springs, CO), stored, and displayed on a computer. LV systolic and end-diastolic pressures, maximal slope of systolic pressure increment ($+dP/dt_{max}$), diastolic pressure decrement ($-dP/dt_{max}$), 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 by 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, whereas M-mode images were obtained from the parasternal short-axis view at the level of papillary muscles and the apical four-chamber view. LV end-systolic and end-diastolic diameters, fractional shortening, and ejection fraction were calculated by using Vevo Analysis software (version 2.2.3) as described previously (Schiller et al., 1989; Salloum 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 whole heart tissue samples were collected, and the proteins were extracted in a buffer containing 50 mmol/l potassium phosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l phenylmethylsulfonyl fluoride, 5 mmol/l β -glycerophosphate, 2 mmol/l NaF, 2 mmol/l Na_3VO_4 , 10 mmol/l β -mercaptoethanol, 1 μ g/ml pepstatin, and 0.5 μ g/ml leupeptin, (pH 7.0) with a tissue homogenizer. The homogenate was centrifuged at 10,000g for 15 min under 4°C, and the supernatant was recovered. Fifty milligrams of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred onto 12 to 10% nitrocellulose membrane. The membrane was incubated with primary antibodies at a dilution of 1:1000 for each of the

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 fourth day and served as the control group. Group II received an acute single intraperitoneal dose of DOX (15 mg/kg), and group III received tadalafil (4 mg/kg by oral gavage) for 9 days in addition to a single dose of DOX (15 mg/kg *i.p.*) on the fourth day of treatment. Arrows indicate the time points of treatment and measurement of various parameters. **B**, Kaplan-Meier survival curve. **C**, HW/TL. Percentages of surviving mice were plotted and compared by χ^2 test ($n = 15$ – 24 /group, $P < 0.05$). HW/TL is presented as means $\pm$ S.E. ($n = 6$ – 8 /group; *, $P < 0.05$ versus control).

proteins, Bcl-2 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), cytosolic superoxide dismutase (Cu/ZnSOD) (Calbiochem, San Diego, CA), mitochondrial SOD (MnSOD) (Calbiochem), GATA-4 (Sigma-Aldrich), and actin (Santa Cruz Biotechnology, Inc.). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; 1 h at room temperature). Detection of the signals was performed by using LumiPhos reagent (Pierce Chemical, Rockford, IL), and chemical luminescence was detected by using X-OMAT film (Kodak, Rochester, NY). The densitometry quantification was performed with image analysis software from Bioquant (San Diego, CA).

Lipid Peroxidation Assessment. Lipid peroxidation was estimated by measuring malondialdehyde and 4-hydroxyalkenals as described previously (Kang et al., 1996) with a colorimetric assay kit (Bioxytech LPO-586; Oxis International, Foster City, CA). In brief, the heart tissue was homogenized in ice-cold phosphate-buffered saline containing 5 mM butylated hydroxytoluene in acetonitrile. The homogenate was centrifuged at 3000g at 4°C for 10 min. Protein extracts containing clear supernatant (200 μ l) were used for the assay following the manufacturer's instructions.

Cardiomyocyte Apoptosis. Cardiomyocyte apoptosis was evaluated by using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with an ApopTag in situ apoptosis detection kit (Millipore Bioscience Research Reagents, Temecula, CA) according to the manufacturer's instructions. The quantification

of apoptosis was determined by counting the TUNEL-positive myocyte nuclei from five random fields per section and was expressed as percentage of total myocyte nuclei as reported previously (Das et al., 2008).

PKG Activity Assay. Protein kinase G (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. In brief, 10 μ l 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 milligram of protein.

cGMP Measurement. cGMP was quantitatively determined by using a EIA cGMP kit (BIOMOL Research Laboratories, Plymouth Meeting, PA). In brief, the frozen heart tissue was ground to fine powder under liquid nitrogen and then homogenized in ice-cold 5% trichloroacetic acid. Homogenates were centrifuged at 600g for 10 min, and the supernatants were extracted in three volumes of water-saturated ether. After lyophilization 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 CellTiter96Aqueous One So-

lution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol.

In Vivo Antitumor Efficacy Study. Tumors were generated in male nude mice (strain-BALB/cAnNCr-nu/nu from the National Cancer Institute Developmental Therapeutic Program, Bethesda, MD) by subcutaneous injection of OSA-1 sarcoma cells (5×10^6 cells) with 50 μ l of MatriGel matrixes (BD Biosciences Discovery Labware, Bedford, MA). Tumors were permitted to grow to a volume of ~ 200 mm³ over the next 2 weeks, and then the animals were randomly divided into three groups ($n \geq 6$ per group). The control group received phosphate-buffered saline (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, respectively.

Statistical Analysis. Statistical analysis was performed by using Prism software version 4.03 (GraphPad Software Inc., San Diego, CA). Data are presented as mean $\pm$ S.E. The difference between groups was analyzed by analysis of variance followed by Student's Newman-Keuls post hoc test. The χ^2 test was used to compare survival rates between the groups. Statistical differences were considered to be significant at $P < 0.05$.

Results

Administration of tadalafil (4 mg/kg p.o. for 9 days; $n = 6$) resulted in 534 ± 89 ng/ml tadalafil concentration in the plasma of the mice. The group treated with DOX + tadalafil exhibited enhanced survival rates (93.3%) compared with 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 a decrease in the HW/TL ($n = 8$, $p < 0.05$; Fig. 1C).

Left Ventricular Function. LV function was significantly impaired 5 days after DOX treatment. As shown in the representative tracing images (Fig. 2A), echocardiography demonstrated that mice treated with DOX + tadalafil preserved fractional shortening and ejection fraction compared with those treated with DOX ($n = 6$, $p < 0.05$; Fig. 2, B and C). In addition, the LV systolic pressure decreased 36%, $+dp/dt_{\max}$ decreased 63%, $-dp/dt_{\max}$ decreased 57%, and heart rate decreased 30% compared with the controls ($P < 0.05$). In contrast, mice treated with DOX + tadalafil showed improved LV function (i.e., LV systolic pressure, 33%; $+dp/dt_{\max}$, 35%; $-dp/dt_{\max}$, 46%, and heart rate, 27%) 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 the antioxidant enzyme superoxide dismutase (SOD). The cytosolic SOD1 (Cu/ZnSOD) and MnSOD were quantified 5 days after DOX treatment. Tadalafil cotreatment with DOX had no effect on Cu/ZnSOD expression. However, MnSOD expression was increased in mice treated with DOX + tadalafil compared with the control group ($n = 4$ /group, $p < 0.05$; Fig. 4, A and B).

Lipid Peroxidation. Cardiac lipid peroxidation activity in the DOX-treated group was significantly increased by 37.6% compared with the control group ($n = 8$, $p < 0.05$; Fig. 4C). However, the lipid peroxidation in the group treated with DOX + tadalafil was not significantly different from the control group.

Apoptosis and Bcl-2 Expression. Cardiomyocyte apoptosis is implicated as one of the mechanisms underlying

Fig. 2. Transthoracic echocardiography assessment of the effect of tadalafil on ventricular contractile dysfunction caused by DOX. A, representative echocardiographic tracings for each of the three experimental groups. B and C, the averaged data of fractional shortening (B) and ejection fraction (C) in the mice are presented as mean $\pm$ S.E. ($n = 6$ per group; *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX).

DOX-induced cardiomyopathy. Expression of the antiapoptotic protein Bcl-2 was down-regulated in the group treated with DOX compared with the control group (Fig. 5A; $n = 6$, $p < 0.05$). Tadalafil cotreatment significantly preserved the Bcl-2 level. Apoptosis, as assessed by TUNEL-positive nuclei, was increased in the DOX-treated group compared with the control and DOX + tadalafil-treated 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). The tadalafil-treated group showed higher expression of GATA-4 compared with the DOX-treated group ($n = 4$, $p < 0.05$; Fig. 5C).

Cardiac cGMP Level and PKG Activity. Treatment with DOX increased cGMP levels in the heart compared with the saline-treated control ($n = 5$, $p < 0.05$; Fig. 6A). The combined treatment with tadalafil and DOX further augmented cGMP levels compared with DOX alone or control. PKG activity was also increased with DOX compared with control, although this change was insignificant. However, treatment with DOX + tadalafil caused a significant increase

Fig. 3. Effect of tadalafil on hemodynamic function of the heart in DOX-induced cardiomyopathy. Left ventricular hemodynamic parameters: left ventricular systolic pressure (A), left ventricular end-diastolic pressure (B), positive dP/dt (C), negative dP/dt (D), heart rate (E), and mean blood pressure (F) measured with a Millar micro-tip catheter in the aortic artery. Data are expressed as mean $\pm$ S.E. ($n = 6$ per group). *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX.

in PKG activity compared with DOX alone ($p < 0.05$ versus 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 $76.1 \pm 0.7\%$ after 48-h incubation with $1 \mu\text{M}$ DOX. Cotreatment with tadalafil ($10 \mu\text{M}$) and DOX also reduced the cell viability to $76.4 \pm 0.5\%$ 48 h after treatment (Fig. 7A), suggesting that tadalafil did not impede the cell-killing efficacy of DOX in vitro. To further confirm whether tadalafil does not interfere with the in vivo antitumor effect of DOX, we used the OSA-1 tumor xenograft model. As expected, DOX significantly reduced body weight (Fig. 7B), tumor weight (Fig. 7C), and tumor volume (Fig. 7D) compared with the controls after 16 days of DOX treatment ($p < 0.001$, $n = 6$). Tadalafil cotreatment did not change DOX-induced reduction in tumor weight (Fig. 7C), tumor volume (Fig. 7D), and body weight (Fig. 7B). These results suggest that cotreatment with tadalafil does not interfere with the antitumor 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 (Ockaili

Fig. 4. Effect of tadalafil on superoxide dismutases and lipid peroxidation after DOX treatment. A and B, representative Western blots with specific bands of MnSOD (A) and Cu/ZnSOD (B) are shown. Bar graphs show densitometric quantification from four individual hearts per group, which was normalized against actin level for each sample. Data are expressed as mean $\pm$ S.E. *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX.

et al., 2002; Salloum et al., 2006; Sesti et al., 2007; Das et al., 2008). 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 that contributed to improved LV function without interfering with the anticancer efficacy of DOX. These results conceptually support our previous report on 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

Fig. 5. Tadalafil attenuates apoptosis and down-regulation of GATA-4 in DOX-induced cardiomyopathy. A, representative Western blot and densitometric quantification showing Bcl-2 expression ($n = 6/\text{group}$). B, cardiac tissue apoptosis quantified by using TUNEL staining and expressed as apoptotic index for TUNEL-positive cells. Data are expressed as mean $\pm$ S.E. ($n = 6/\text{group}$). *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX group. C, cardiac protein expression of GATA-4 as measured by Western blot analysis. Bar graphs show densitometric quantification from four individual hearts per group, which is normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level for each sample. *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX group.

propose that the class of PDE-5 inhibitors can represent an attractive novel therapeutic approach for managing the clinical concern of 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 up-regulation of MnSOD, a key mitochondrial antioxidant enzyme. Second, we demonstrated that the cGMP/PKG signaling pathway is involved in tadalafil-induced cardioprotection in the setting of DOX-induced cardiomyopathy. Third,

Fig. 6. Tadalafil augments cGMP and protein kinase G after DOX treatment. cGMP level (A) and PKG activity (B) normalized against milligram of protein in the cardiac tissue. Data are expressed as mean $\pm$ S.E. ($n = 5-7/\text{group}$). *, $P < 0.05$ versus control; #, $P < 0.05$ versus DOX group.

tadalafil treatment prevented DOX-induced down-regulation of transcription factor GATA-4. Finally, we provided both in vitro and in vivo evidence for the anticancer efficacy of DOX, which remained unaltered by cotreatment with tadalafil.

We used an oral administration regimen of tadalafil (4 mg/kg for 9 days), which resulted in a plasma concentration of 534 ± 89 ng/ml, which is similar to reported levels in human subjects taking clinically relevant doses of tadalafil (20 mg p.o. daily for 1 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 (Figs. 2 and 3). At the systemic level, DOX administration caused a decrease in HW/TL 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 the 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 anti-

Fig. 7. Effect of tadalafil on the antitumor efficacy of DOX. A, cell viability in human osteosarcoma cancer cell lines (OSA-1) after 48 h of cotreatment with DOX and tadalafil. B–D, effect on body weight (B), tumor weight (C), and tumor volume (D) in sarcoma (OSA-1) tumor xenograft model in BALB/cAnNCr-nu/nu mice after 16 days of tadalafil (4 mg/kg p.o.) administration in DOX-treated mice ($n = 6/\text{group}$). *, $P < 0.001$ versus control.

oxidant properties of PDE-5 inhibitors, particularly in vivo, have not yet been well studied and understood. Fernandes et al. (2008) reported that the physiological concentrations of sildenafil ($<50 \mu\text{M}$) decreased H_2O_2 generation by mitochondria respiring glutamate/malate. Moreover, it was shown that sildenafil decreased superoxide radical generated by a hypoxanthine/xanthine oxidase system without affecting either mitochondrial bioenergetics or Ca^{2+} -induced mitochondrial permeability transition (Fernandes et al., 2008). Most recently, in a rat model of traumatic spinal cord injury, Serarslan et al. (2009) demonstrated that tadalafil reduced the spinal cord injury by increasing tissue/serum levels of nitric oxide and serum activity of SOD.

To further correlate the antioxidant effects of tadalafil with its antiapoptotic protection, we looked at the expression of Bcl-2, which is known to block the 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 compared with the DOX-treated group (Figs. 2 and 3).

GATA-4 is a key transcriptional factor that 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 down-regulation of GATA-4 expression after DOX treatment (Fig. 5C), which confirmed a previous report (Aries et al., 2004). The significant restoration of GATA-4 expression by cotreatment 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 inhibitor-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 mice treated with DOX and those treated with DOX + tadalafil, which is consistent with DOX-induced increases in NO and cGMP levels in vitro (Mykhaylyk et al., 2005). Moreover, we also observed a significant increase in PKG activity and cGMP levels in the mice treated with DOX + tadalafil. Considering the demonstrated role of PKG in protection against ischemia/reperfusion injury (Das et al., 2006, 2008; Salloum et al., 2009), we speculate that this enzyme may have a role in reducing DOX-induced cardiotoxicity through the activation of extracellular signal-regulated kinase and the inhibition of glycogen synthase kinase β .

Finally, we further addressed the possible effect of tadalafil in interfering with the antitumor efficacy of DOX. We used both in vitro OSA-1 cell viability assay and an in vivo xenograft tumor model to rule out such a possibility. Our results suggested that tadalafil did not reduce the cytotoxic efficacy of DOX or interfere with the DOX-induced reduction of tumor volume and weight. Hence, our

results unvaryingly indicated that tadalafil did not impede the antitumor 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 should be performed to demonstrate the protective effect of tadalafil after 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 the attenuation of DOX-induced cardiac dysfunction. Tadalafil activated mitochondrial antioxidative and antiapoptotic mechanisms through up-regulation 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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