Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction

Anindita Das, David Durrant, Clint Mitchell, Eric Mayton, Nicholas N. Hoke, Fadi N. Salloum, Margaret A. Park, Ian Qureshi, Ray Lee, Paul Dent, and Rakesh C. Kukreja

We have shown that the potent phosphodiesterase-5 (PDE-5) inhibitor sildenafil (Viagra) induces a powerful effect on reduction of infarct size following ischemia/reperfusion injury and improvement of left ventricular dysfunction in the failing heart after myocardial infarction or doxorubicin (DOX) treatment. In the present study, we further investigated the potential effects of sildenafil on improving antitumor efficacy of DOX in prostate cancer. Cotreatment with sildenafil enhanced DOX-induced apoptosis in PC-3 and DU145 prostate cancer cells, which was mediated by enhanced generation of reactive oxygen species, up-regulation of caspase-3 and caspase-9 activities, reduced expression of Bcl-xL, and phosphorylation of Bad. Overexpression of Bcl-xL or dominant negative caspase-9 attenuated the synergistic effect of sildenafil and DOX on prostate cancer cell killing. Furthermore, treatment with sildenafil and DOX in mice bearing prostate tumor xenografts resulted in significant inhibition of tumor growth. The reduced tumor size was associated with amplified apoptotic cell death and increased expression of activated caspase 3. Doppler echocardiography showed that sildenafil treatment ameliorated DOX-induced left ventricular dysfunction. In conclusion, these results provide provocative evidence that sildenafil is both a powerful sensitizer of DOX-induced killing of prostate cancer while providing concurrent cardioprotective benefit.

Results

Sildenafil Potentiates DOX-Induced Killing of Prostate Cancer Cells. First, we examined the dose-dependent effect of DOX treatment in PC-3 and DU145 cells. Cell growth was reduced in a dose-dependent manner with DOX in both cells (Fig. 1 A and B). Cotreatment with sildenafil resulted in an additive effect on DOX-induced reduction of proliferation (Fig. 1 A and B). Cell killing assessed by trypan blue exclusion assay also confirmed similar additive effect (Fig. 1 C and D). DOX treatment also increased apoptosis as evaluated by TUNEL assays (Fig. 1 E and F). The sildenafil and DOX combination further enhanced apoptosis in PC-3 and DU145 cells, whereas sildenafil alone had no effect. Colony formation assays performed using median dose effect isobologram analysis further corroborated the synergistic effect of sildenafil and DOX in enhancing cell killing (Fig. 2 A). In contrast, DOX treatment induced cell death in the normal prostate epithelial cells (PrEC), which was significantly reduced by sildenafil (Fig. 2 B). Sildenafil treatment alone had no effect on cell death in the PrEC or prostate cancer cells.

Apoptosis was also quantified using Annexin-V-FITC and propidium iodide (PI) staining followed by flow cytometry analysis. The cells in the subpopulations labeled by staining of Annexin-V-FITC (+)/PI (−) were indicative of early apoptotic

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cells, whereas those labeled by Annexin-V-FITC(+)/PI(+) were indicative of late apoptotic/necrotic cells. DOX induced apoptosis after 72 h of treatments in PC-3 (7.52%) and DU145 cells (45.01%) compared with control (3.49% in PC-3 and 5.52% in DU145) (Fig. S1A and B). Cotreatment with sildenafil and DOX increased apoptosis relative to DOX alone in both cell lines (18.71% in PC-3 and 56.82% in DU145 cells) (Fig. S1A and B). Moreover, the combination treatment increased apoptotic cell death in other cancer cell types including sarcoma OSAC-1 (Fig. S1C), ovarian cancers UCI 101 (Fig. S1D), and A2780 (Fig. S1E).

Sildenafil Enhances DOX-Induced Generation of Reactive Oxygen Species. Reactive oxygen species (ROS) generation is the key component of antitumor activity of anthracyclines in a variety of tumor cells (25, 26). We tested whether sildenafil enhances cell killing through increased ROS generation that was measured by exposing DOX- and/or sildenafil-treated cells to the indicator dye dichlorodihydrofluorescein diacetate (H2DCFDA). As expected, DOX increased ROS levels in PC-3 and DU145 cells as indicated by positively stained cells (H2DCFDA green fluorescence) (Fig. 5A and B, and Fig. S2A and B). However, cells exposed to sildenafil and DOX further boosted ROS generation. In contrast, the sildenafil and DOX combination attenuated DOX-induced ROS generation in the PrEC normal cells (Fig. 4A and B). Sildenafil alone had no effect on ROS generation in normal or cancer cells. Furthermore, incubation with a putative antioxidant, mercaptopropionyl glycine (MPG), attenuated the enhanced killing effect of sildenafil and DOX combination in DU145 (Fig. S3A) and PC-3 cells (Fig. S3B).

Sildenafil and DOX Enhance Intrinsic Pathway of Apoptosis. DOX increased caspase-3 activity that was further enhanced by cotreatment with both sildenafil and DOX in PC-3 and DU145 cells (Fig. 5A). Bcl-2 expression was diminished in DU145 cells but remained unaltered in PC-3 cells following treatment with sildenafil and DOX (Fig. 5B and Fig. S4A). Similarly, the expression of the antiapoptotic protein Bcl-xL was reduced with sildenafil and DOX compared with individual treatments or control in both cell lines (Fig. S4B). Bad belongs to the proapoptotic members of the Bcl-2 family and forms a complex with Bcl-xL thereby preventing its antiapoptotic effects. Phosphorylation of Bad impairs its binding to Bcl-xL and therefore abrogates Bad’s proapoptotic effects (27). DOX reduced Bad phosphorylation and sildenafil and DOX further decreased Bad phosphorylation (Fig. 5B and Fig. S4C). DOX induced the proapoptotic protein Bax, which was further enhanced by cotreatment with sildenafil in PC-3 cells (Fig. 5B and Fig. S4D). Overexpression of Bcl-xL inhibited cell death with sildenafil and DOX compared with DOX alone (Fig. 5D). Caspase-9 activity was unchanged with sildenafil although it increased after DOX treatment (Fig. 5C). Caspase-9 activity was further increased with sildenafil and DOX treatment compared with DOX alone. Overexpression of dominant negative caspase 9 (dnCasp9) attenuated the synergistic effect of sildenafil and DOX on cell death compared with cells infected with empty vector (Fig. 5E).

Sildenafil Potentiates DOX-Induced Inhibition of Prostate Tumor Xenograft Growth. Treatment of nude mice carrying PC-3 flank tumors with DOX (1.5 mg/kg, i.p.) reduced tumor volume (Fig. 6A). Sildenafil (5 mg/kg, i.p.) cotreatment potentiated DOX-induced tumor volume reduction (Fig. 6A). The ratio of tumor weight to body weight was also reduced with sildenafil cotreatment (Fig. 6B). Similar results were obtained when sildenafil (10 mg/kg)
The synergy of killing of PC-3 and DU145 by sildenafil and DOX

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Discussion

The high incidence of recurrence and metastasis, as well as the refractory nature of the malignancy to chemotherapy, make hormone refractory prostate cancer one of the most challenging malignancies for therapeutic drug combination studies (28). Surgical resection of the prostate also causes significant risk of erectile dysfunction due to trauma sustained by the cavernosal nerve (29). PDE-5 inhibitors have been shown to improve erectile function in men postradical prostatectomy (30–32). We have established a powerful cardioprotective effect of PDE-5 inhibitors in animal models (33). Moreover, sildenafil improved DOX-induced left ventricular (LV) dysfunction and cardiomyocyte apoptosis (11). In the present study, we provide evidence that sildenafil potentiates DOX-induced killing of androgen-independent human prostate cancer cells in vitro and in vivo. Moreover, sildenafil attenuated DOX-induced cardiac dysfunction in mice bearing prostate tumors. These results suggest that sildenafil may represent a therapeutic approach to improve DOX efficacy in prostate cancer while simultaneously reducing the risk of cardiomyopathy. Our data also show that the sildenafil and DOX combination enhanced the killing of ovarian cancer and sarcoma cells, suggesting a potential utility of sildenafil in chemosensitization in multiple malignancies.

Mitochondrial ROS is the key component of antitumor activity of DOX in tumor cells (25, 26). In the present study, we observed higher levels of intracellular ROS in PC-3 and DU145 cells after treatment with DOX and sildenafil compared with DOX alone. In contrast, however, sildenafil and DOX treatment decreased ROS production in normal cells. Similar to these results, sulindac, a potent anticancer drug, selectively enhanced killing of cancer cells exposed to oxidizing agents via production of ROS (34). It has been suggested that the basic difference in mitochondrial respiration between normal and cancer cells makes cancer cells more sensitive to oxidative stress (35, 36). Exactly how sildenafil sensitizes cancer cells to amplify DOX-mediated ROS generation is not clear but needs to be investigated. Interestingly, low levels of sulindac also induced delayed preconditioning response against...
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we further investigated the mechanisms of cell death induced by dioprotection at the same time.

sulindac in enhancing the antitumor effect while providing cardioprotective effects of cardioprotection including iNOS and HSP27 ischemia/reperfusion injury in the heart through up-regulation of protective effectors of cardioprotection including iNOS and HSP27 (37). In this respect, it appears that sildenafil is very similar to sulindac in enhancing the antitumor effect while providing cardioprotection at the same time.

Because resistance to apoptosis is one of the hallmarks of cancer, we further investigated the mechanisms of cell death induced by sildenafil and DOX. Apoptosis is regulated at points within the intrinsic pathway by pro- and antiapoptotic proteins, which include members of the Bcl-2 family together with mitochondria, cytochrome c, and caspases (38). The regulation occurs by the balance of intrinsic protein levels and/or their localization within intracellular compartments (39). In the present study, the increased apoptosis by sildenafil and DOX was associated with enhanced expression of proapoptotic proteins Bad and Bax and suppression of Bcl-2 and Bcl-xL. Also, this cotreatment regimen dephosphorylated Bad, which may enhance Bad heterodimerization with Bcl-xL, thereby promoting DOX-induced apoptosis. The ectopic overexpression of Bcl-xL in DU145 cells suppressed the lethality of sildenafil with DOX compared with DOX alone, suggesting that down-regulation of Bcl-xL played a significant role in the synergistic interactions between these therapeutic agents. Sildenafil- and DOX-induced cell killing was also associated with increased caspase-3 and caspase-9 activity. Overexpression of dominant negative procaspase 9 in DU145 cells blocked the enhanced cell killing by combined treatment with sildenafil and DOX compared with DOX alone.

Our results show that sildenafil and DOX treatment also caused significant inhibition of tumor growth and enhanced caspase-3 activity as well as apoptosis. The sildenafil and DOX combination also ameliorated DOX-induced cardiac dysfunction, which is consistent with our previous study showing improved LV function with sildenafil in DOX-treated mice (11). In these studies, sildenafil reduced cardiomyocyte apoptosis, maintained mitochondrial membrane potential, preserved myofibrillar integrity, and prevented electrocardiogram ST interval prolongation after DOX treatment.

Similar to other chemotherapeutic agents, the clinical use of DOX is hampered by its cardiotoxic effects (40). It impairs the clinical response and survival of patients (41). Therefore, rendering cancer cells more sensitive to DOX while improving cardiac function would be an efficient approach to enhance its therapeutic

![Fig. 4.](image)

![Fig. 5.](image)
effect. Our results suggest a potential utility of sildenafil in enhancing the antitumor efficacy of DOX while attenuating its cardiotoxic effect in prostate cancer. Clinical studies are warranted to fully define the importance of combined treatment with DOX and sildenafil as therapeutic tool in prostate cancer patients.

Materials and Methods

Cell Growth and Death Assay. Cell proliferation and metabolically active PC-3 and DU145 cells were measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp.) according to manufacturer’s protocol. The percentage of cell death was measured by trypan blue staining.

Apoptosis Assay. Cell apoptosis was analyzed by TUNEL staining using ApopTag Peroxidase In Situ Apoptosis Detection kit (Chemicon International Company). Apoptosis in tumor section was analyzed using In Situ Cell Death Detection kit, TMR red (Roche Diagnostics).

Measurement of ROS. Following 24 and 48 h of treatment with DOX and/or sildenafil, cells were incubated with 50 μM of H2DCFDA (Molecular Probes) in growth medium for 30 min at 37 °C. Cells were rinsed with PBS, and ROS levels were visualized by fluorescence microscope.

Caspase-3 and -9 Activity Assay. Cell viability was measured using CellTiter-Fluor viability assay kit (Promega Corp.) in a 96-well plate. Caspase-3/7 and -9 activities were measured in treated cells using Caspase-Glo 3/7 assay and Caspase-Glo-9 assay kits (Promega Corp.) according to manufacturer’s protocol.

In Vivo Tumor Study. Tumors were generated in athymic male BALB/cAnNCr-nu/nu mice from National Cancer Institute Developmental Therapeutic Program by s.c. injection of PC-3 cells (5 × 106 cells) with 50-μL matrigel matrices (BD Bioscience). Tumors were permitted to grow to a volume of ~200 mm3 over the following 2 wk. The animals received i.p. injections of saline (for control), DOX (1.5 mg/kg) alone, sildenafil (5 mg/kg) alone, or DOX (1.5 mg/kg) and sildenafil (5 mg/kg) everyday, 5 d/wk (Fig. 5/7). Tumor sizes were measured twice weekly. Tumor volume was calculated by ab2/2 where “a” and “b” are the long and short axes of tumor. The animal protocol was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Doppler Echocardiography. Cardiac function in nude mice with tumor xenografts was monitored by Doppler echocardiography using the Vevo770 imaging system (VisualSonics) as previously reported (12).

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**Fig. 6.** Sildenafil (Sild) potentiates DOX-induced inhibition of prostate tumor xenograft growth. Tumor weight and body weight were measured after 18 d of treatment with DOX (1.5 mg/kg, i.p.) and/or Sild (5 mg/kg, i.p.). (A) Tumor growth inhibition (*P < 0.01 vs. control and *P < 0.001 vs. other; n = 8). Sildenafil enhances DOX-induced apoptosis in tumors. (B) Bar diagram showing TUNEL-positive cells (*P < 0.001 vs. control and *P < 0.001 vs. DOX; n = 3). Results are reported as means ± SE.

**Fig. 7.** Sildenafil enhances activity of caspase 3 in tumor. (A) Representative images of the immunohistochemical staining for Alexa 488 labeled cleaved caspase 3 in tumors. (Top) Cleaved caspase 3 (green fluorescence), (Middle) nuclei staining with DAPI, and (Bottom) overlay of both types of staining. Cardiac function was assessed by Doppler echocardiography of mice treated with Sild (10 mg/kg by oral gavage) everyday and DOX (3 mg/kg i.p.) twice per week. (B) LVFS and (C) LVEF (*P < 0.05 vs. control and Sild and **P < 0.01 vs. DOX; n = 8). Results are reported as means ± SE.


