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CO-ADMINISTRATION OF SILDENAFIL POTENTIATES DOXORUBICIN-INDUCED
APOPTOSIS IN PROSTATE CANCER: THE ROLE OF NF- κ B

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

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Richmond, Virginia
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List of Abbreviations

AIPC	Androgen-Independent Prostate Cancer
ANOVA	Analysis of Variance
Apaf-1	Apoptosis activating factor
AR	Ankyrin repeats
BH	BCL-2 homology domain
CHF	Congestive Heart Failure
DNR	Daunorubicin
DOX	Doxorubicin
ED	Erectile dysfunction
EPI	Epirubicin
Fadd	Fas-receptor associated death domain
GnRH	Gonadotropin-releasing hormone
cGMP	Cyclic guanosine monophosphate
sGC	soluble guanylate cyclase
GTP	Guanosine triphosphate
·OH	Hydrogen Peroxide
H ₂ O ₂	Hydroxyl Radical
IDA	Idarubicin
MPTP	Mitochondrial permeability transition pore
NEMO	NF-κB essential modifier
NIK	NF-κB inducing kinase
NF-κB	Nuclear Factor-kappa B
NO	Nitric Oxide
eNOS	Endothelial Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
NLS	Nuclear localization signal
O ₂ ⁻	Superoxide anion
PDE5	Phosphodiesterase-5
PKC	Protein Kinase C
PKG	cGMP-dependent protein kinase
PSA	Prostate Specific Antigen
RHD	c-Rel homology domain
ROS	Reactive Oxygen Species
TNFα	Tumor necrosis factor α
ΔΨ _m	Membrane potential

Abstract

CO-ADMINISTRATION OF SILDENAFIL POTENTIATES DOXORUBICIN-INDUCED APOPTOSIS IN PROSTATE CANCER: THE ROLE OF NF- κ B

By Sarah H. Hassanieh, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Rakesh C. Kukreja
Professor, Department of Internal Medicine, Division Cardiology

Our recent studies have shown that that erectile dysfunction (ED) drugs including Sildenafil (Viagra), Vardenafil (Levitra) and Tadalafil (Cialis) enhance killing of several types of cancer cells by anticancer drug, Doxorubicin (DOX). We observed increased cell death by apoptosis in response to the combined treatment with ED drugs and DOX. However, the mechanism of such enhancement of cell death by combined treatment of ED drugs and DOX is not fully understood. Nuclear factor- κ B (NF- κ B) is an oxidant-sensitive transcription factor that plays a critical role in the immediate-early activation of a multitude of genes that have been documented to play critical role in programmed cell death (apoptosis). NF- κ B activation has been shown to block apoptosis and its inhibition

improves existing anti-oncogenic therapy such as chemotherapy. In the present study, we tested the hypothesis whether combined treatment of prostate cancer cells, PC3, with Sildenafil plus DOX would attenuate the activation of NFκB by inhibiting translocation of the p65 and p50 subunits to the nucleus and by phosphorylation of cytosolic IκB. In addition, we investigated the effect of DOX and DOX plus Sildenafil on the expression of BCL family of proteins which play critical role in apoptosis. We treated PC3 cells with 1.5 μM DOX with or without 10 μM Sildenafil for 6 hours and 72 hours. The nuclear translocation of p65 and p50 and expression of BCL family of proteins was determined by western blot analysis. Our results show that combined treatment of DOX and Sildenafil significantly reduced the nuclear translocation of p65 and p50 as compared with DOX alone ($P < 0.05$). This correlated with the significant reduction in the expression of Bcl-2, Bcl_{xL} and phosphorylation of BAD. These data provide an important mechanism by which Sildenafil treatment augments the apoptotic potential of DOX in PC3 cancer cells.

Introduction

Prostate cancer

Prostate cancer is the most common solid tumor malignancy in men and the second most deadly cancer in men of all ages in the United States (De Marzo et al. 2007). One out of five men is at risk of developing prostate cancer during a lifetime course, and 1 in 33 will die (Jemal et al. 2007). Localized prostate cancer is initially treated with curative intent by radiation therapy or surgery. If these treatment options fail or the cancer metastasizes, the next step is androgen deprivation treatment by bilateral orchiectomy, downregulating gonadotropin-releasing hormone (GnRH) receptors with agonist and antagonist, or reducing the hypothalamic GnRH secretion by estrogen administration (Damber and Aus. 2008). Androgen ablation reduces symptoms in about 70–80% of patients with advanced prostate cancer, but most tumors relapse within 2 years and progress to an androgen-independent phase. Patients with androgen-independent prostate cancer (AIPC) experience fatigue, increased bone pain, and a rise in serum prostate specific antigen (PSA) (Fossa et al. 2002). No cure exists for AIPC, and it is in dire need of new treatment strategies.

Doxorubicin:

The anthracycline antibiotics continue to be the most effective cancer chemotherapeutic agents ever since their discovery in 1960s. Doxorubicin (DOX) and Daunorubicin (DNR) were the first two anthracyclines isolated from the actinobacteria *Streptomyces peucetius* ATCC 29050. As shown in figure 1, DNR and DOX are derived from 1-propionyl-CoA and 9-malonyl-CoA to produce a decaketide that is converted to aklanonic acid (Jiang and Hutchinson. 2006). Next, aklanonic acid is converted to DNR proceeding through the biosynthetic intermediates ϵ - rhodomycinone and rhodomycin D. DOX is finally achieved by DNR hydroxylation at C-14 (Tang et al. 1996). Although the structures DOX and DNR share a close homology to one another, they are used to treat different cancers (Minotti et al. 2004). DNR is indicated in the treatment of acute lymphoblastic or myeloblastic leukemias, while DOX has a broader spectrum activity- used for the treatment of a variety solid tumors such as those of the breast, bile ducts, endometrial tissue, the esophagus and liver, osteosarcomas, soft-tissue sarcomas and non-Hodgkin's lymphoma (Wiernik and Dutcher. 1992; Murphy GP. 1995). Despite their efficiency as chemotherapeutic agents, they pose a threat to normal health tissues in the form of congestive heart failure (CHF) and chronic cardiomyopathy. This sparked the search for a better DOX resulting in about 2000 anthracycline analogues with only a few earning clinical approval.

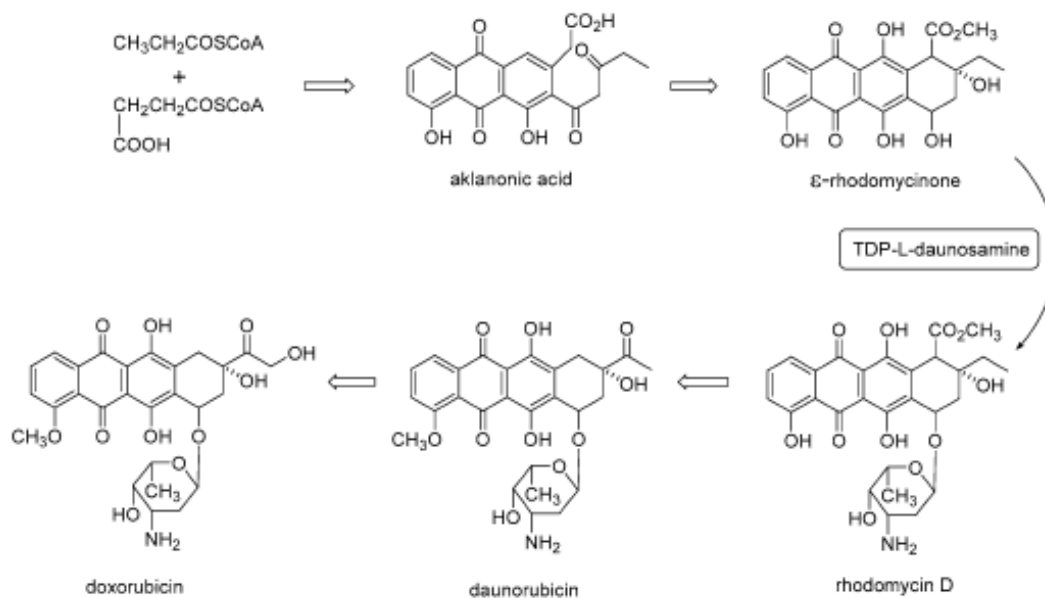


Figure 1: Daunorubicin and Doxorubicin Biosynthesis in *S. peuceletii* (Tang et al. 1996).

The most notorious analogues are Idarubicin (IDA) and Epirubicin (EPI) and can be obtained from DNR and DOX, respectively by removal of the 4-methoxy group in ring D from DNR and by an axial-to-equatorial epimerization of the hydroxyl group at C-4' in daunosamine (sugar moiety displayed in the chair configuration in figure 2) of DOX. Whether IDA and EPI are successful in alleviating the cardiomyopathies is unclear and awaits further assessment; therefore, DOX and DNR remain to be the best chemotherapeutic drugs ever developed.

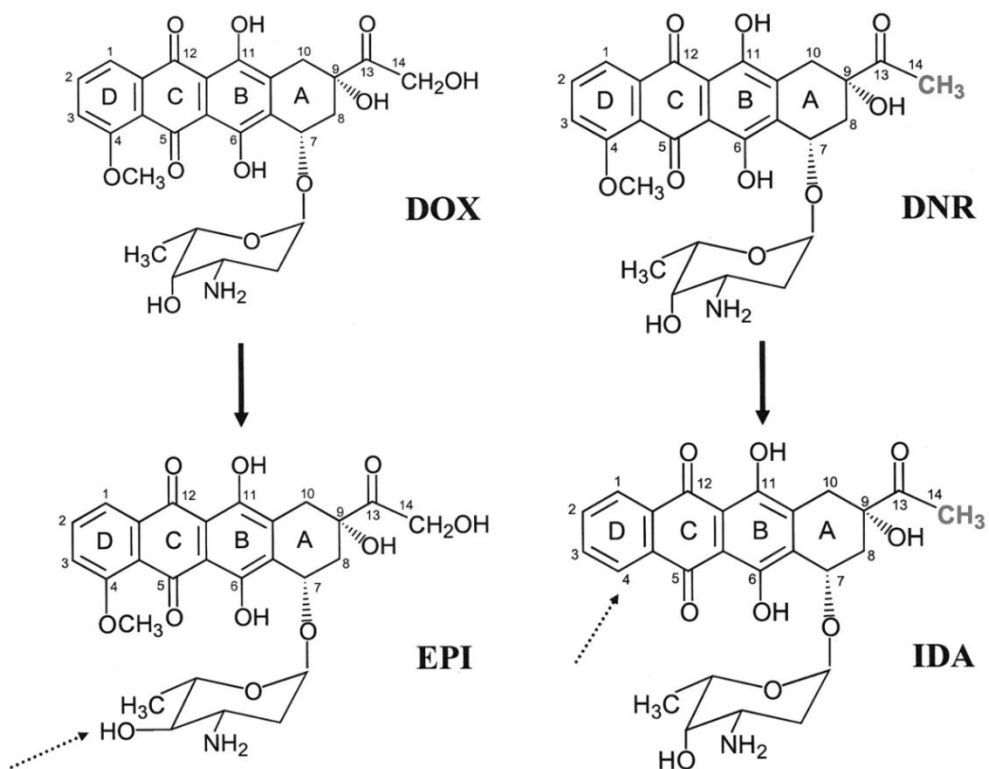


Figure 2: Chemical structures of DOX DNR EPI AND IDA anthracyclines. The side chains (marked in grey) of DNR or IDA terminate with a methyl in place of a primary alcohol compared with DOX or EPI. The dotted arrows point to the structural modifications in EPI compared with DOX (axial-to-equatorial epimerization of the hydroxyl group at C-4' in daunosamine), or in IDA compared with DNR (lack of the methoxy group at C-4 in ring D) (Minotti et al. 2004).

Mechanisms of anthracycline cytotoxicity remain controversial. However, the possible mechanisms include: 1) interference of macromolecular biosynthesis by DNA intercalation; 2) generation of free radicals and lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA strand separation and DNA helicase; 6) direct membrane effects; 7) induction of DNA damage through inhibition of topoisomerase II; 8) apoptosis induction (Gewirtz. 1999). Of the most accepted

mechanisms that have been proven in a variety of cancer cell lines are the inhibition of topoisomerase II and the free radical generation (Minotti et al. 1999).

Topoisomerases are enzymes that modify the structure of DNA, causing transient single or double strand breaks that are subsequently repaired after changing the twisting structure of the molecule. These enzymes are very important in cell division and reproduction, as are the transcriptional activity that occurs during many cellular processes. The anthracyclines act as topoisomerase II poisons by forming an irreversible ternary complex (enzyme-DNA-anthracycline) instead of the physiological reversible binary complex (enzyme-DNA) (Zucchi and Danesi. 2003). This ternary complex causes DNA damage specifically, protein associated persistent (rather than transient) strand breaks in DNA leading to apoptosis (Gewirtz. 1999; Zucchi and Danesi. 2003; Sander and Hsieh. 1983).

DOX and other anthracyclines can also cause DNA damage generating reactive oxygen species (ROS) and hydroxyl radicals ($\cdot\text{OH}$) in a mechanism that involves one electron redox cycling (figure 3) along with the release of low molecular weight iron (Fe(II)) from intracellular stores (Minotti et al. 2004). Electrons are supplied from NAD(P)H-oxidoreductases such as cytochrome P450 of the endoplasmic reticulum and nuclear envelope and endothelial nitric oxide synthase to the quinone moiety in ring C in DOX to form a semiquinone, which will regenerate into its parent quinone by reducing molecular oxygen to ROS superoxide anion ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2) (Minotti et al. 1999; Vasquez-Vivar et al. 1997). In addition, the semiquinone can

oxidize the bond between ring A and daunosamine to form a 7-deoxyaglycone, which has the ability to permeate the cell membrane and exert damaging effects by introducing ROS.

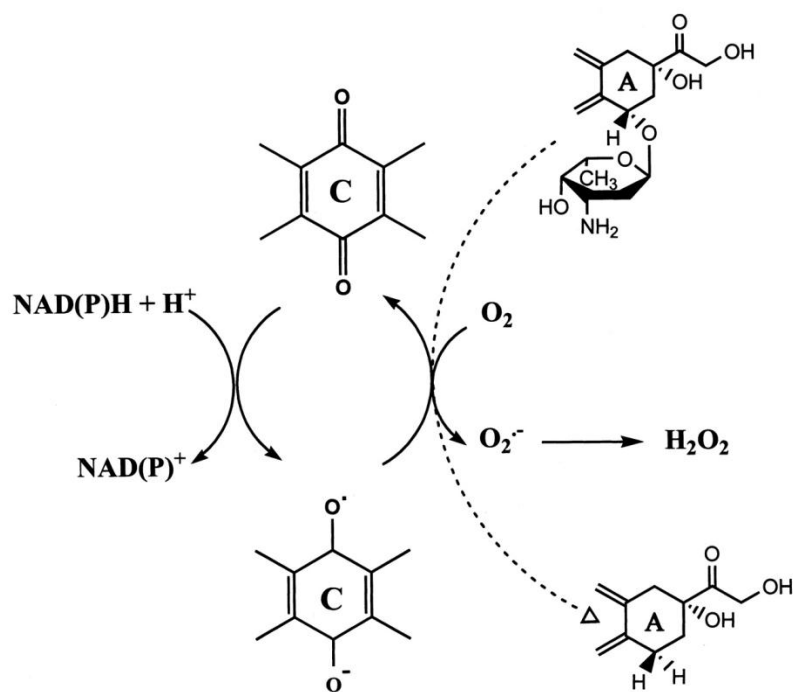


Figure 3: One-electron redox cycling of anthracyclines (Minotti et al. 2004).

Moreover, the accompanied Fe(II) release from one electron cycling augments the damaging effects, with the formation of 3:1 drug (DOX)-Fe(II) complexes that decompose H₂O₂ using the available O₂^{•-} into highly reactive OHs (Minotti et al. 1999). It has been suggested that the oxidative damage in tumor cells is attributed to anthracyclines at supraclinical doses, while topoisomerase II inhibition prevails at doses < 5 μM (Gewirtz. 1999). It is conceivable; however, that cumulative anthracycline dosage overtime will introduce cardiac risk due to the formation of ROS in the

cardiomyocyte mitochondria (Myers. 1998). The build-up of ROS in cardiomyocytes results in dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome *c* release eventually leading to apoptosis (Childs et al. 2002).

In the clinical setting, DOX is administered intravenously with a usual dose of 60 mg/m² every three weeks, with a dose limit of 450-550 mg/m² (Minotti et al. 2004). However, smaller, more frequent doses (once per week) are commonly used depending on type of malignancy, overall chemotherapeutic regimen, prior history of anthracycline exposure, and cardiac risk factors. Peak serum concentrations of DOX are reached quickly following intravenous injection and fall by approximately 50% after thirty minutes. However, significant levels persist for up to 20 hours. DOX is mainly metabolized hepatically by reduction and hydrolysis of its ring substituents. The alcohol metabolite of DOX, Doxorubicinol, is pharmacologically active, whereas the aglycone derivative is inactive. Anthracyclines are mainly excreted in the bile with a small amount excreted in the urine. Because, toxic metabolites are re-circulated in the process of biliary excretion, dose reduction needs to be considered in patients with hepatic disease or elevated hepatic transaminases (Minotti et al. 2004).

Doxorubicin-induced apoptosis in cardiac cells:

It is now recognized that apoptosis is the mechanism underlying Doxorubicin-induced dilative cardiomyopathy and CHF. DOX has been shown to induce apoptosis in a variety of cardiomyocyte cell lines through numerous receptor-mediated (extrinsic) and mitochondrial (intrinsic) apoptotic pathways (Minotti et al. 2004). With respect to the

extrinsic apoptotic pathway, DOX strongly increased recombinant Fas ligand (rFasL) apoptosis in neonatal rat cardiomyocytes, supported by an increase in caspase-8 activity (Yamaoka et al. 2000). Fas ligand is a member of the TNF family of cytokines that when binds to its receptor recruits Fadd (Fas receptor-associated death domain) protein leading to activation of initiator caspases (eg, caspase-8) as shown in figure 4 (Kalyanaraman et al. 2002). Caspase-8 will then cleave and activate effector caspases (eg, caspase-3), which will degrade or activate cellular protein to eventually lead to cell death.

With respect to the intrinsic apoptotic pathway, it has been well-documented that DOX upregulates Bax (pro-apoptotic protein), which triggers cytochrome *c* release by facilitating mitochondrial channel opening (Wang et al. 1998b). This leads to the formation of the apoptosome, which is a complex of apoptosis activating factor (Apaf-1), cytochrome *c*, and pro-caspase-9 resulting in the cleavage and activation of caspase-9. Furthermore, Bax homodimers can be associated with the mitochondria, and they have the ability to directly activate caspase-3 and other potential apoptotic pathways (Reed. 1995). Interestingly, Bax can be directly activated by p53, because it contains a p53 binding site. It has been demonstrated that H₂O₂ and 'O₂⁻ generated from redox cycling of DOX can activate p53 (Miyashita and Reed. 1995)

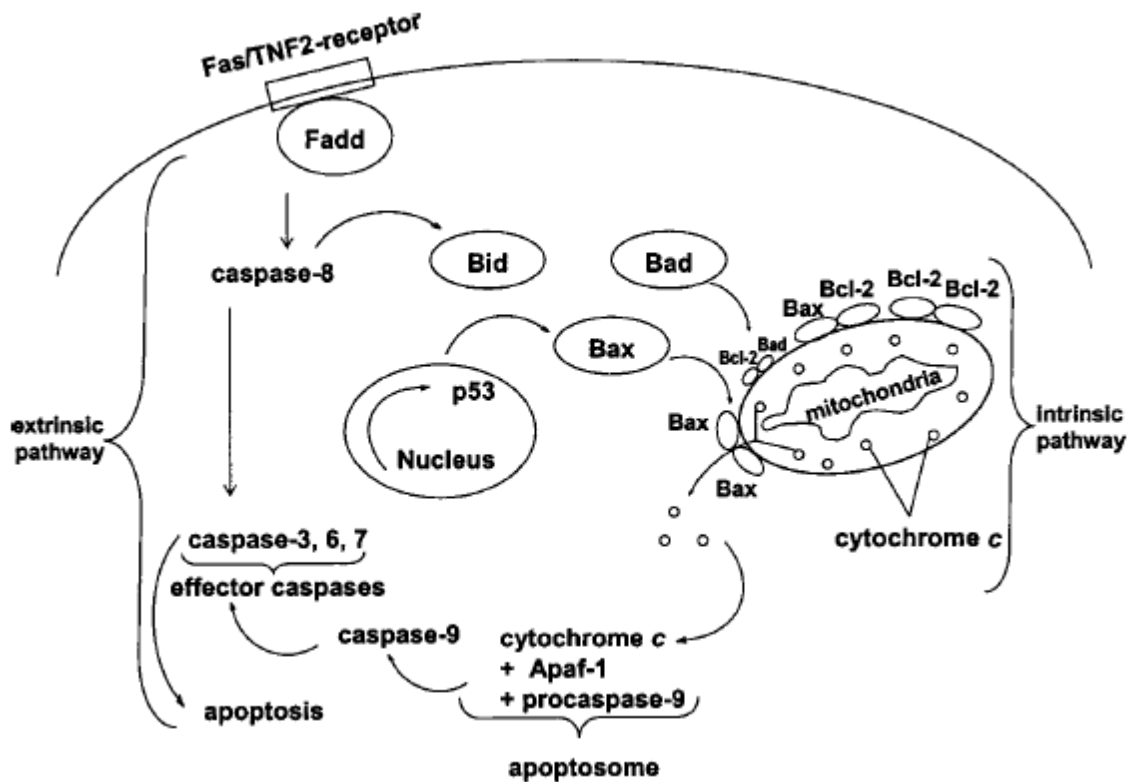


Figure 4: Extrinsic and intrinsic Doxorubicin-induced apoptotic pathways in cardiac cells (Kalyanaraman et al. 2002).

BCL-2 family proteins are pivotal players in the regulation of apoptosis that function to either induced programmed cell death (pro-apoptotic) or promote cell survival (anti-apoptotic). These proteins contain at least one of four conserved α -helical BCL-2 homology domains (BH1, BH2, BH3, and BH4) (Gross et al. 1999). BCL_{xL} and BCL-2 are survival proteins that contain all four domains. BAD, a BH3-only pro-apoptotic protein is susceptible to phosphorylation two serine sites (Ser-112 and Ser-136), which results in its inactivation (Zha et al. 1996). In its phosphorylated state, BAD is sequestered by in the cytosol by the phosphoserine docking protein, 14-3-3 (Downward.

1999). When it is dephosphorylated, it translocates to the mitochondria, where it can form heterodimers with survival proteins BCL-2 or BCL_{xL}, thereby inhibiting them (Gross et al. 1999; Zha et al. 1996; Zhou et al. 2000). This promotes cell death by leading to opening of mitochondrial permeability transition pore (MPTP) and release of cytochrome *c* (Halestrap et al. 2000).

On the other end of the spectrum, survival proteins (eg. BCL_{xL} and BCL-2), which repress apoptosis, become down-regulated (Kalyanaraman et al. 2002). In a normal cell, the mitochondrial membrane permeability is stabilized by the association of BCL-2 homodimers (Reed. 1995). There is evidence that the protection of mitochondrial permeability is lost due to the sequestration of BCL-2 homodimers by the formation of BCL-2/Bax heterodimer (Herrmann et al. 1996). This action is attributed to H₂O₂ generated from redox cycling of DOX, again with evidence linking p53 to Bax activation (Huang et al. 2000).

Phosphodiesterase-5 Inhibitors:

Cyclic guanosine monophosphate (cGMP) is an important regulator of wide variety of physiological processes in many different tissues of the body (Rybalkin et al. 2003). Smooth muscle relaxation is achieved when nitric oxide (NO) activates soluble guanylate cyclase (sGC), an enzyme that converts guanosine triphosphate (GTP) to cyclic cGMP. This results in the activation of cGMP-dependent protein kinase (PKG), which goes on to phosphorylate proteins leading to a decrease in calcium influx (Das et al. 2008). This

process is brought to an end with the breakdown cGMP to GMP by phosphodiesterase-5 (PDE5). Inhibition of PDE5 activity allows the relaxation cycle to be prolonged and enhanced (Rybalkin et al. 2003). Sildenafil citrate (Viagra) is a vasoactive drug that has been used in the treatment of erectile dysfunction (ED) (Boolell et al. 1996). Sildenafil works by inhibiting PDE5, leading to the accumulation of cGMP allowing vasodilatation in the *corpus cavernosum* (Das et al. 2005).

Sildenafil can elicit its actions any place where there is PDE-5; therefore, it can be used to treat problems other than ED. Our laboratory has demonstrated that Sildenafil can induce cardioprotection through opening of mitochondrial K_{ATP} channels in rabbits, a phenomenon known as preconditioning (Ockaili et al. 2002). Activation of PKG in the cardiomyocytes leads to the opening of mitochondrial K_{ATP} channels, which partially compensates for the loss in membrane potential (Kukreja et al. 2005). This allows protons to be extruded, which forms an H^+ electrochemical gradient for ATP synthesis and Ca^{2+} transport. We have also shown that Sildenafil-induced cardioprotection occurs by activation of Protein Kinase C (PKC) (Das et al. 2004).

NO has been recently shown to play a significant role in Sildenafil induced cardiac preconditioning against apoptosis and necrosis in mice cardiomyocytes (Das et al. 2005). NO is synthesized from L-arginine by one of three isoforms of NO synthase (NOS): endothelial (e), neuronal (n), and inducible (i) NOS (Moncada and Higgs. 1993; Moncada et al. 1991). NO derived from eNOS constitutively expressed, releasing NO in the nM range in response to intracellular Ca^{2+} transients (Stefano et al. 2000). iNOS functions in the cardiovascular and immune system and is highly regulated by cytokines

(Moncada and Higgs. 1993). NO serves as a heme ligand and is capable of binding the heme iron of sGC leading to enhanced formation of cGMP (Denninger and Marletta. 1999). With regards to preconditioning with Sildenafil, an upregulation of both iNOS and eNOS mRNA and protein expression promoting NO release offers a potent cardioprotective effect against ischemia/reperfusion (I/R) injury (Das et al. 2005; Salloum et al. 2003)

Prevention of Doxorubicin-Induced Cardiomyopathy with Sildenafil:

As discussed above, anti-cancer drugs (such as DOX) may cause severe cardiotoxicity that ultimately lead to cardiomyopathy. The cardiotoxic effects of DOX continue to be the major restraint in the current cancer chemotherapy. Our laboratory recently demonstrated that *in vivo* treatment of mice with Sildenafil before administration of DOX conferred protective effects against Doxorubicin-induced cardiotoxicity (Fisher et al. 2005). Sildenafil pretreatment attenuated myocyte apoptosis, maintained mitochondrial membrane potential, preserved myofibrillar integrity, alleviated left ventricular dysfunction and ST-segment prolongation. Similarly, in the isolated cardiomyocytes, DOX treatment caused significant increase in apoptosis, caspase-3 activation, and disruption of mitochondrial membrane potential, all of which were attenuated by Sildenafil. In addition, the protective effects were abolished by either L-NAME (an inhibitor of NOS) or 5-HD (a blocker of mitoK_{ATP}), indicating the participation of NO and mitoK_{ATP} in mediating the protective effects of Sildenafil against Doxorubicin-induced cardiomyopathy (Fisher et al. 2005). Interestingly, in a rat model of hypertensive

cardiomyopathy induced by chronic NOS inhibition, oral treatment of Sildenafil reduced both the arterial blood pressure and peripheral vascular resistance (Ferreira-Melo et al. 2006). In addition, a smaller total area of myocardial lesions and decreased accumulation of necrotic and fibrotic tissue was observed. cGMP was proposed to be the central mediator for the anti-cardiomyopathic effects of Sildenafil in these studies.

NF- κ B: structure and activation

The NF- κ B (nuclear factor-kappa B) family of transcription factors is a critical regulator of various target genes involved in numerous cellular functions, such as apoptosis, cell proliferation, inflammatory response, nervous system functioning (Malek et al. 2007). There are five members of the NF- κ B family: p50/p105 (NF- κ B1), p52/p100 (NF- κ B2), p65 (RelA), RelB, and c-Rel that arrange to form homo- or heterodimers that may be transcriptionally active or repressive (Ghosh et al. 1998). Transcriptionally inactive structures are the p50 homodimer and the p52 homodimer, whereas those combinations that are activators of transcription consist of the p50/p65, p50/c-rel, and p65/c-rel heterodimers as well as the p65 homodimer (Ghosh et al. 1998). Of the most prominent and the most relevant to this present study is the p50/p65 dimer.

NF- κ B/Rel proteins contain a conserved 300 amino acid rel homology domain (RHD), shown in figure 5, which happens to be the site of dimerization, DNA binding, interaction with inhibitory proteins, the I κ B family (Ghosh et al. 1998). The Rel proteins (Rel A RelB, and c-Rel) contain a variable C-terminal transactivation domain. P105 and p100, respectively the precursors of p50 and p52, contain this region but it is absent in

p50 and p52 after post-translation or co-translational processing through mechanisms that are not fully understood (Ghosh et al. 1998; Ghosh and Karin. 2002). It is known that processing involves the proteolysis of the ankyrin repeats (AR) on p105 and p100, which occurs constitutively in p105 and in a regulated manner in p100 (Ghosh and Karin. 2002). It should be noted that p50 and p52 repress due to their lack of this transactivation domain (Ghosh et al. 1998).

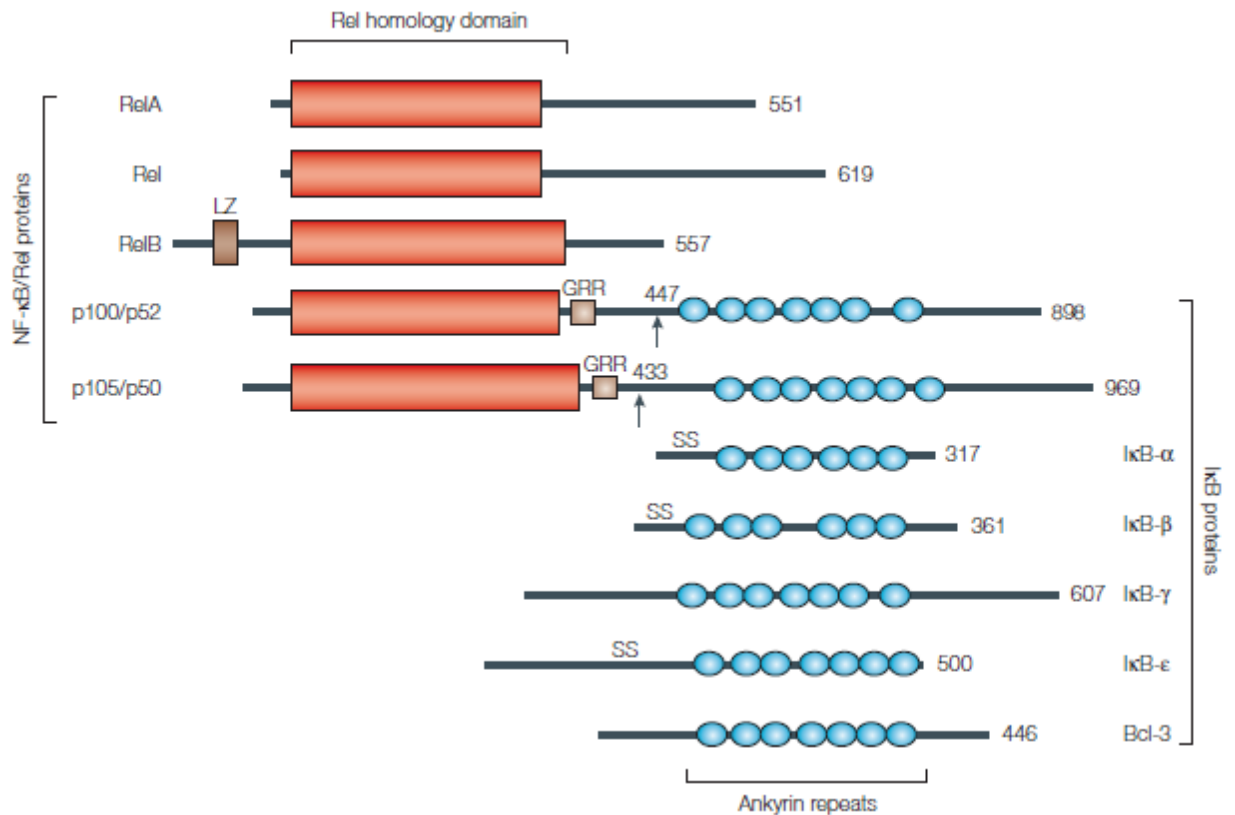


Figure 5: Rel/NF-κB and IκB family of proteins. The number of amino acids in each protein is shown on the right. The arrows point to the C-terminal residues of p50 and p52 (following processing of p105 and p100, respectively). members of the NF-κB family share the c-Rel homology domain (RHD, rectangle), whereas share six to seven ankyrin repeats (AR, beads) GRR, glycine-rich repeat; IκB, inhibitor of NF-κB; LZ, leucine zipper; NF-κB, nuclear factor-κB; SS, two conserved serines in IκB (Karin et al. 2004).

Proteins of the Rel-family bind specific areas called κ B sites on DNA via their RHD (Ghosh et al. 1998); of interest is the binding of p50/p65 heterodimer.

Dimerization occurs at the C-terminal domains where two identical β -sheets interdigitate (Huang et al. 1997). The dimer contacts the major groove of DNA via the N-terminal region of the RHD (Toledano et al. 1993). Taking a closer look, arginines and lysines on p50 and p65 interact with the guanines of the κ B sites. There are a variety of κ B sites specific for the multitude of homo- or heterodimer combinations among the Rel proteins. These are pentameric sites with a consensus sequence of 5'-GGGRNYYYCC-3' (R is an unspecified purine; Y is an unspecified pyrimidine; and N is any nucleotide) (Chen et al. 1998). In particular, the Ig- κ B site 5'-GGGACTTCC-3', of which the 5-base-pair subsite GGGAC subsite is bound by p50 and a 4-base-pair subsite TTCC is bound by p65.

NF- κ B is an inducible transcription factor. On receiving an appropriate signal, it dissociates from I κ B and is able to act rapidly without having to synthesize new proteins. This is the hallmark of NF- κ B action which makes it ideal for situations, such as stress and inflammation, in which the cell has to respond quickly.

In most vertebrate cells, NF- κ B is bound to an inhibitor, I κ B and held in the cytoplasm (Ghosh et al. 1998). I κ B is a family of inhibitory molecules that includes 7 known mammalian members: I κ B α , I κ B β , I κ B ϵ , I κ B γ , BCL-3 and the pre-cursor Rel proteins p100 and p105 (Ghosh and Karin. 2002). Docking of the ARs on I κ B proteins to the RHDs of NF- κ B proteins achieves the cytoplasmic retention that renders NF- κ B inactive (Karin et al. 2004). In normal quiescent cells, virtually no dissociation of the

complex is seen in the absence of stimulation, rendering the I κ B kinase, IKK, complex inactive (Karin and Ben-Neriah. 2000). The IKK complex contains two kinase subunits, IKK α and IKK β , and a regulatory subunit IKK γ or NF- κ B essential modifier (NEMO).

There are a number ways to activate liberate NF- κ B from its inhibitor. The I κ B members interact at their ankyrin repeats with the RHD of NF- κ B, such that the nuclear localization signal (NLS) in the RHD of NF- κ B is masked. The most comprehensively studied pathway for activation is called the classical or canonical pathway for NF- κ B activation. It involves the phosphorylation of I κ B α on two serine (32 and 36) residues by IKK due to cell stimulation resulting in the ubiquitination and subsequent proteolysis of I κ B α through the E3 ligase β -transducing repeat containing protein (β -TrCP). Once NF- κ B is free from I κ B α , it translocates to the nucleus where it is able to regulate the transcription of responsive NF- κ B genes (Zandi et al. 1997; Yates and Gorecki. 2006). Some of these target genes are involved in immune response (TNF α , IL-1, MCP-1, and COX2), cell proliferation (cyclin D1, cyclin D2, c-MYC, and JUNB), cell survival (Bcl-X_L, Bcl-2, and angiogenesis (VGEF, IL-6 and IL-8).

Recently a non-canonical pathway, involved in B-cell mediated responses and adaptive humoral immunity has emerged (Karin et al. 2004; Pomerantz and Baltimore. 2002). In this pathway, NF- κ B activation is independent of IKK complex but requires IKK α . The scheme of the non-canonical pathway involves the phosphorylation and subsequent processing of p100/Rel B to p52/Rel B. In this mechanism IKK α is activated by NF- κ B inducers such as lymphotoxin- β (LT β), B-cell activating factor (BAFF),

receptor activator of NF- κ B (RANK), or by the interaction of IKK α with NF- κ B inducing kinases (NIK) (Yates and Gorecki. 2006).

NF- κ B and Apoptosis:

Depending on the stimulus and cell type, NF- κ B regulates gene expression of target genes that can either promote or block apoptosis. It has been shown that the activation of NF- κ B is necessary for p53 mediated apoptotic cell death (Ryan et al. 2000). It has also been shown that NF- κ B is essential in order for Fas (CD95), a cell surface protein, to induce apoptosis in activated lymphocyte (Chan et al. 1999). Not only does NF- κ B have a crucial role in mediating apoptosis, but it can also promote cell survival. It has been reported that in tumors, such as breast, melanoma, colon, pancreas, thyroid, and prostate, NF- κ B is essential for tumor development (Nunez et al. 2008). NF- κ B can promote tumor cell survival and growth by inducing genes that encode cyclinD1, which will phosphorylate Rb leading the cells into S phase of the cell cycle and by upregulating c-myc (Hinz et al. 1999; Karin et al. 2002). Furthermore, NF- κ B can also facilitate cancer progression by activation genes that stimulate angiogenesis and metastases such as MMP9, uPa, IL-8 and VEGF (Karin et al. 2002).

In many circumstances, NF- κ B activation mediates chemoresistance by offering protection from apoptosis (Huang et al. 2000; Wang et al. 1999a; Yamamoto and Gaynor. 2001; Wang et al. 1999b). Therefore, efforts are underway to developing methods to impinge on this resistance by blocking this aberrant activity. Chemotherapy, tumor necrosis factor α (TNF α), and other anticancer stimuli may further activate NF- κ B, and

this induction has been reported to play a pivotal role in resistance in vitro by suppressing the apoptotic potential of these stimuli (Wang et al. 1996; Beg and Baltimore. 1996).

NF-κB and Prostate Cancer:

NF-κB modulates the expression of genes for many normal and necessary cell functions; however in a number of human cancers-especially in prostate cancer, it is aberrantly activated (Paule et al. 2007). Interestingly, the degree of its activity varies with cancer cell type with a strong correlation to androgen dependency. For instance, NF-κB activity is high in androgen-independent cell lines such as PC3 and DU-145 cells, but it is low in the androgen sensitive cell line LNCaP. With respect to prostate cancer, it appears that persistent expression of NF-κB is associated with constitutive activation of IKK, which contributes to increased phosphorylation of IκBα. Not much is known about the signaling pathways that result in IKK activation; however, it appears that activation of upstream kinases, such as NIK may play a pivotal role (Paule et al. 2007). Nevertheless, this aberrant activity appears to be problematic in cancer treatment.

One of the most frequent causes of DOX chemotherapy failure is resistance. It has been demonstrated that the over-expression of anti-apoptotic genes are due to NF-κB activation (Patel et al. 2000). In some cancer cell lines, desensitization to chemotherapy occurs through the induction in NF-κB (Lee et al. 2003). In cardiomyocytes, it appears that the role of NF-κB activation with regard to apoptosis is reversed. It has been shown that DOX-generated H₂O₂ induces apoptotic cell death as a result of NF-κB activation in endothelial cells and cardiomyocytes (Wang et al. 2002). It has been reported that H₂O₂

modulates the phosphorylation of IKK α leading to further degradation of I κ B α (Li et al. 2001). Therefore, inhibition of NF- κ B activity would be advantageous not only in sensitizing cells to DOX-induced apoptosis, but also promoting cell survival in cardiomyocytes.

While effective treatments exist for prostate cancers that are hormone sensitive, hormone-independent tumors have none. The NO/soluble guanylate cyclase (sGC) pathway has been reported to be impaired in the androgen-independent cell lines PC3 and DU145 (Chen et al. 1998), and PDE5 has been shown to be upregulated in human carcinomas, including prostate cancers (PC3 and LCAP cell lines) (Zhu and Strada. 2007). Moreover, involvement of this PDE5 has been proposed to influence antiproliferation and caspase-dependent proapoptotic mechanisms in multiple carcinomas (Zhu et al. 2005; Sarfati et al. 2003).

Goals of the Present Study:

Previous studies from our laboratory assessed the cell viability after treatment with DOX and Sildenafil in three different cancer cell lines OSA-1, a sarcoma cell line, UCI-101 and A2780, ovarian cancer cell lines. We observed that Sildenafil enhanced DOX-induced antiproliferation and apoptosis with an increase in BAX/BCL-2 ratio and caspase-3. Since DOX is known to activate NF- κ B in various cancer cells and blocking NF- κ B sensitizes cells to DOX-induced apoptosis, we postulated that Sildenafil when used in combination with DOX would reduce NF- κ B activation thereby strongly potentiating apoptosis in PC3 cells. Specifically, we investigated the combined effect of

DOX and Sildenafil in attenuating the activation of NF- κ B. We studied the effect of Sildenafil plus DOX on inhibition of translocation of p65 and p50 subunits to the nucleus, phosphorylation of cytosolic I κ B α , and its resultant influence on apoptosis. In addition, we investigated the effect of DOX and DOX plus Sildenafil on the expression of BCL family of proteins which play critical role in apoptosis. The studies were carried out *in vitro* in PC-3 cell line.

Material and Methods

Materials:

DOX was obtained from Sigma Chemical Co. PS (Pen-Strep) was obtained from Invitrogen. F-12K Medium was obtained from ATCC (American Type Cell Collection). Cell titer96@AQueous one solution cell proliferation assay kit and 5X Reporter Lysis Buffer were obtained from Promega Co. FBS (Fetal Bovine Serum) was purchased from Gemini Bioproduct. Bad, pBad (Ser112), BCL_{xL}, IκBα, pIκBα (Ser32), p65, p50, α-tubulin, and actin antibodies were purchased from SantaCruz Biotechnology. NF-κB Transfactor extraction kit was obtained from Clontech (631930 and 631921). Sildenafil was kindly provided by Pfizer Inc. Secondary antibodies (anti-mouse, anti-rabbit, and anti-goat) were purchased from Amersham Biosciences. ECL western blotting detecting reagents were purchased from GE Healthcare. SuperSignal West Femto Maximum Sensitivity Substrate was obtained from Pierce.

Cell Culture:

PC-3 cells were obtained from the American Type Cell Collection. Cells were grown in F-12K medium containing 10% FBS and 1% PS at 37°C in a humidified incubator with 5% CO₂ and 95% O₂.

Cell Viability Assay:

Cells (Approximately 15,000 cells/well) were plated on 96 wells plate 24 hours before the treatments. Cells were treated with 1.5 μ M of DOX and /or 10 μ M of Sildenafil for 72 hours. Cell viability was assessed using Cell titer96@AQ_{ueous} one solution cell proliferation assay kit. Plates were read at the absorbance of 590 nM.

Western Blot:

For NF- κ B and I κ B α , nuclear extractions were prepared using Clontech Extraction Kit isolated from cells after 6 hours of treatment. Protein was run on a 10% gel for NF- κ B and 12.5% gel for I κ B α . To measure pBad, Bad, BCL_{xL}, total soluble protein was extracted from the cells using Reporter Lysis Buffer (Promega) by a repeated freeze and thaw method. Protein (total soluble, cytoplasmic, or nuclear) was estimated by Bradford assay using Bio-Rad protein assay kit. For NF- κ B, 30 μ g of protein from each sample was separated by 10% SDS-PAGE Criterion Precast Gel and 150 μ g of protein was run in a 12.5% gel for pI κ B α and I κ B α . For pBad, Bad, BCL_{xL}, 150 μ g of protein was separated on a 15% gel. Gels were run at 165V for 1.5 hours with TGS buffer Tris-Glycine-SDS). The proteins were transferred onto nitrocellulose membrane for 1.5 hours at 400 AMPS at 4°C with TG buffer (Tris-Glycine). The blot was incubated with 5%

nonfat dry milk in TBST (10mM Tris-HCL, pH 7.4, 10mM NaCl, and 0.1% Tween 20) for 1 hour. Thereafter, the blot was incubated with rabbit polyclonal primary antibodies for BCL_{xL}, pBad, pIκBα, p65, p50; mouse monoclonal antibodies for IκBα and Bad; and goat polyclonal primary antibody of α-tubulin and actin at a dilution of 1:1000 for α-tubulin and actin, 1:250 for BCL_{xL}, pBad and pIκBα, and 1:500 for p65, p50 in 5% nonfat dry milk in TBST for overnight at 4°C. The membranes were washed three times with TBST at RT. The membranes were then incubated with anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000 (1:1000 for BCL_{xL}, pBAD, BAD, pIκBα, and IκBα) for 1 hour. The membranes were washed again and then developed using an ECLTM Western blot detection reagents and exposed to X-ray film. SuperSignal West Femto Maximum Sensitivity Substrate was used to develop BCL_{xL}, pBAD, BAD, pIκBα, and IκBα. The bands were scanned and quantified by densitometric analysis (Image J).

Statistical analysis and densitometry. To quantitate differences in images of protein expression, films were scanned and densitometry performed using NIH Image 1.73V software. For all statistical comparisons, GraphPad Prism software (Version 4.03) was used. For multiple comparisons, analysis of variance using *Newman-Keuls post test*. $p < 0.05$ was considered to be statistically significant.

Results

Perspective

Previously, we have shown by a Cell Viability Assay that Sildenafil enhances DOX-induced killing of PC3 cells. Figure 6 shows a 43% decrease in cell viability following 72 treatment of 1.5 μ M DOX and 10 μ M Sildenafil compared with DOX alone.

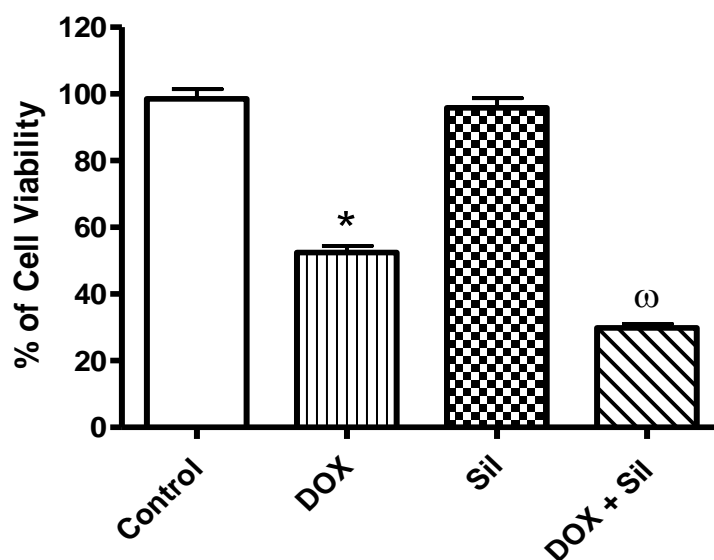


Figure 6: Cell Viability Assay after 72 hrs of treatment of PC3 cells with 1.5 μ M DOX and 10 μ M Sildenafil (Sil); n = 6; means \pm S.E., one-way ANOVA, Newman-Keuls post test. * $P < 0.001$ vs. Control $\omega P < 0.001$ vs. DOX

This encouraged us to investigate the rationale behind Sildenafil action on Doxorubicin-induced apoptosis. Since DOX is known to activate NF- κ B in diverse cancer cells, it was of interest to determine whether Sildenafil co-administration along with DOX treatment is augmenting cell death through the inhibition of NF- κ B.

DOX showed a significant induction NF- κ B in the nuclear translocation of both the p65 and p50 subunits of PC3 cells treated for 6 hours with 1.5 μ M DOX and 10 μ M Sildenafil compared to the control (figures 7 and 8). It was found that combination treatment of DOX and Sildenafil significantly reduced the nuclear translocation of p65 as compared with DOX alone by 45% ($P < 0.05$) and p50 by 37% ($P < 0.05$). The decline of NF- κ B was supported by the western blot for p-I κ B α , which showed a trend in the increase of the ratio of p-I κ B α / I κ B α compared to the control. The phosphorylation of I κ B α was reduced even further with DOX/ Sildenafil combination treatment as compared to DOX administration alone (figure 9).

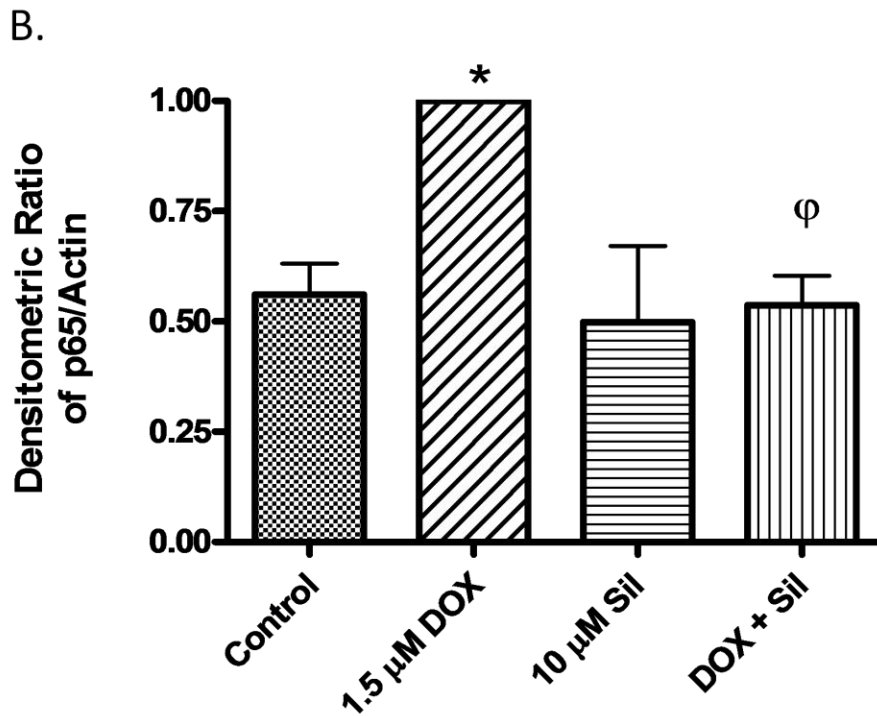
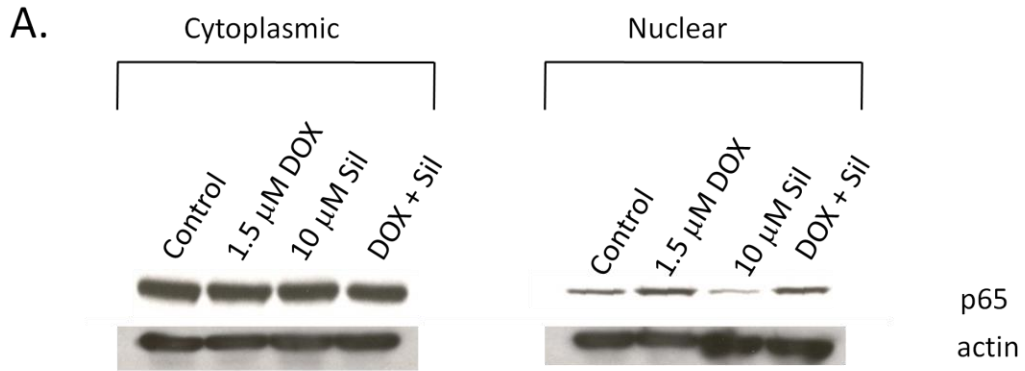


Figure 7: Nuclear translocation of the p65 subunit of NF- κ B in PC3 cells after 6 hrs treatment with 1.5 μ M DOX and 10 μ M Sildenafil (Sil). A: Western blot. B: Densitometric depiction. $\phi P < 0.05$ vs. DOX and $*P < 0.01$ vs. Control. $n = 4$; means \pm S.E., one-way ANOVA, Newman-Keuls post test.

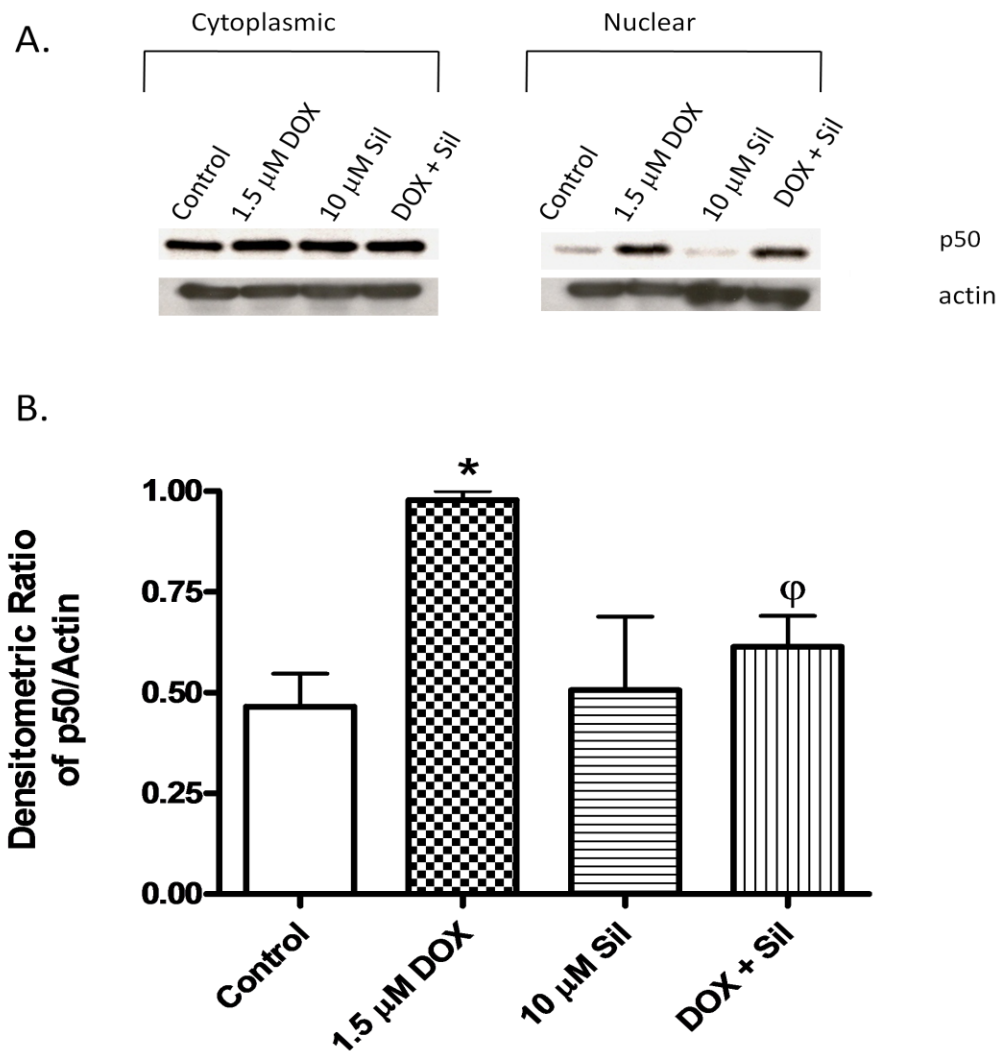


Figure 8: Nuclear translocation of the p50 subunit of NF- κ B in PC3 cells after 6 hrs treatment with 1.5 μ M DOX and 10 μ M Sildenafil (Sil) A: Western blot. B: Densitometric depiction. n = 4; means \pm S.E., one-way ANOVA, Newman-Keuls post test. $\phi P < 0.05$ vs. DOX and * $P < 0.05$ vs. Control.

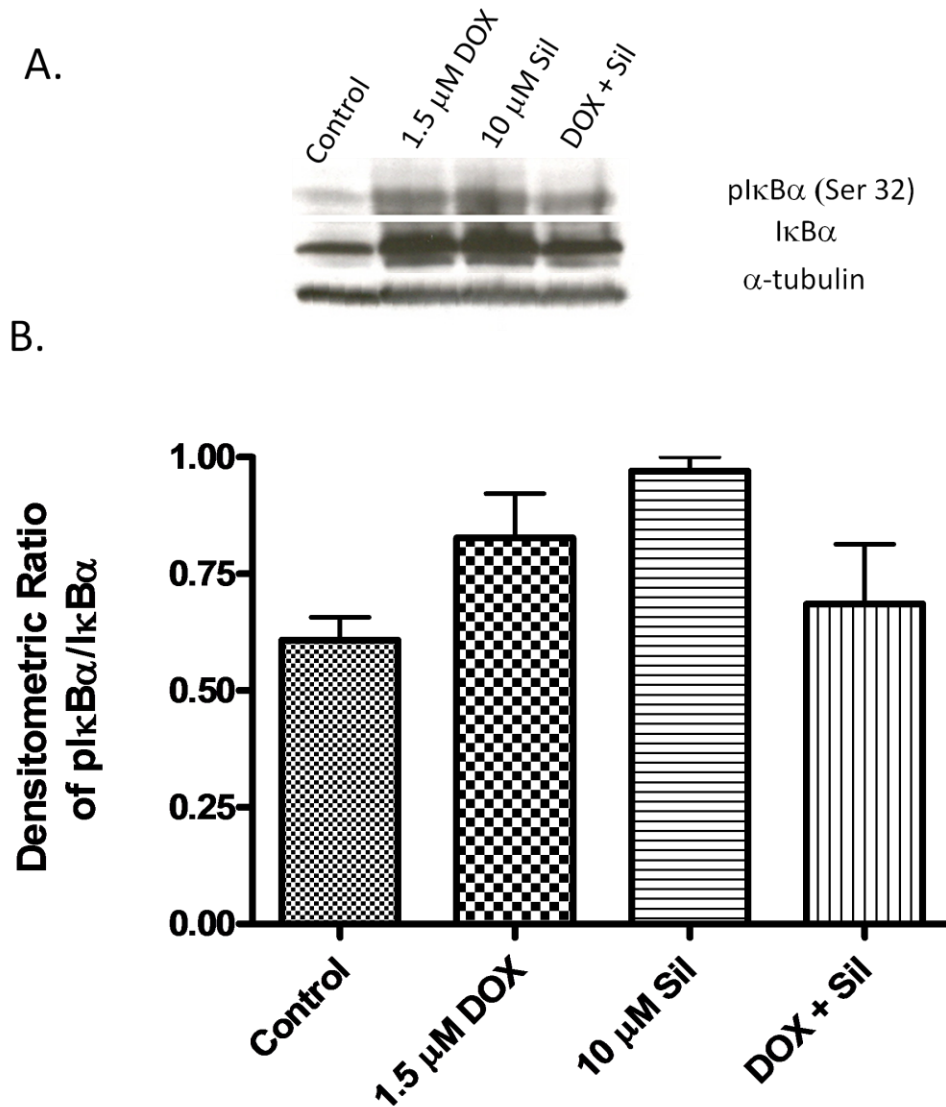


Figure 9: p-I κ B α protein levels in PC3 cells after 6 hrs treatment with 1.5 μ M DOX and 10 μ M Sildenafil (Sil). A: Western blot. B: Densitometric depiction. n = 4; means \pm S.E., one-way ANOVA, Newman-Keuls post test.

Apoptotic Indexes:

Since NF- κ B is has been shown to play a pivotal role in the regulation of cellular growth and apoptosis, we looked at the expression of proteins that are suppressors of apoptosis and those that inducers of apoptosis (Beg and Baltimore. 1996; Wang et al. 1998a). For these studies, PC3 cells were harvested 72 hours after drug treatments. We observed a 52% ($P < 0.001$) reduction in BCL-2 protein levels was observed with DOX alone. Treatment of Sildenafil in combination with DOX reduced the densitometric ratio of BCL-2 / α -tubulin further from 0.449 ± 0.013 (DOX alone) to 0.329 ± 0.035 ($P < 0.05$) (figure 10). DOX treatment alone had an overall trend of higher levels of BCL_{xL} as compared with control (figure 11). Sildenafil co-treatment with DOX significantly reduced BCL_{xL} protein levels by 33% ($P < 0.05$). Phosphorylation of BAD (pBAD) was significantly reduced by 26% ($P < 0.05$) in DOX treated PC3 cells as compared to the control (figure 12). Treatment of Sildenafil in combination with DOX reduced the densitometric ratio of pBAD /BAD further augmented the reduction in the BAD phosphorylation from 0.609 ± 0.089 to 0.341 ± 0.043 ($P < 0.05$).

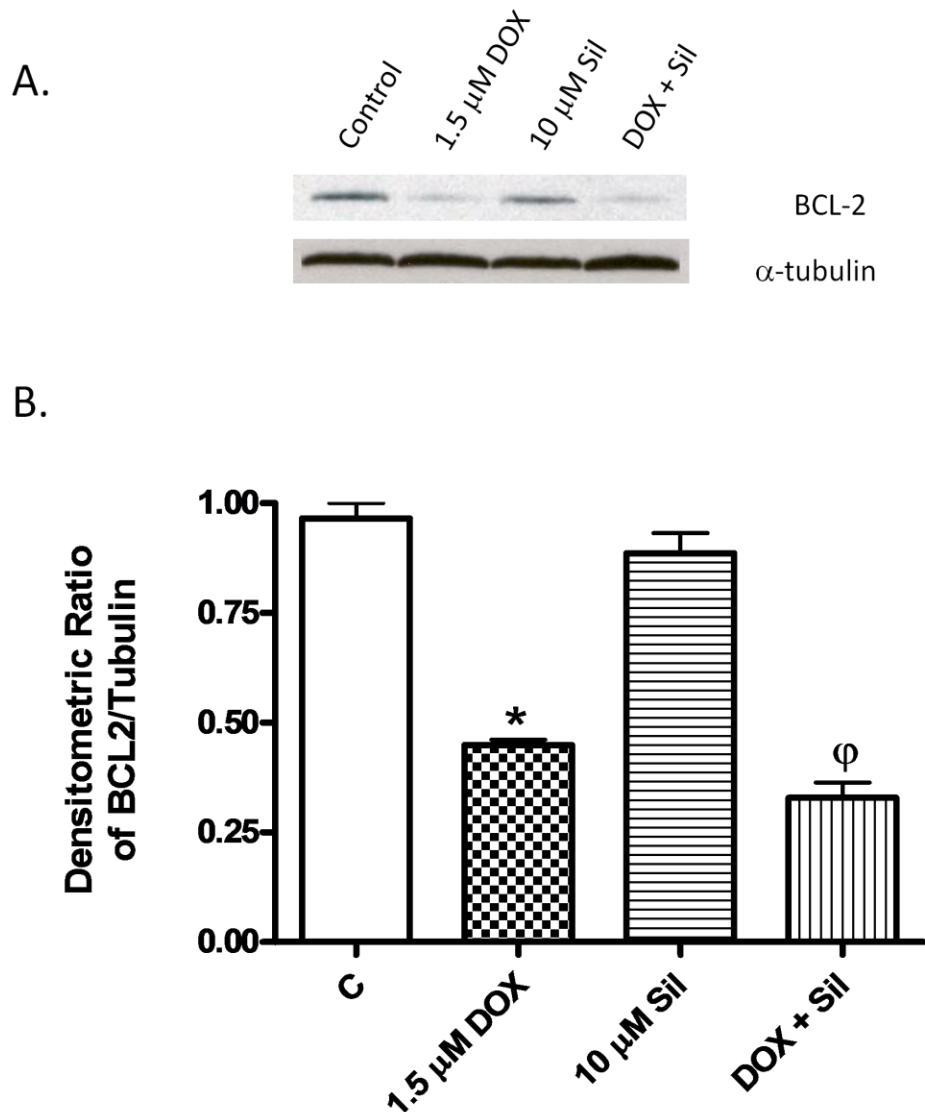


Figure 10: BCL-2 levels in PC3 cells after 6 hrs treatment with 1.5 μ M DOX and 10 μ M Sildenafil (Sil). A: Western blot. B: Densitometric depiction. n = 4; means \pm S.E., one-way ANOVA, Newman-Keuls post test. $\phi P < 0.05$ vs. DOX and $*P < 0.001$ vs. Control

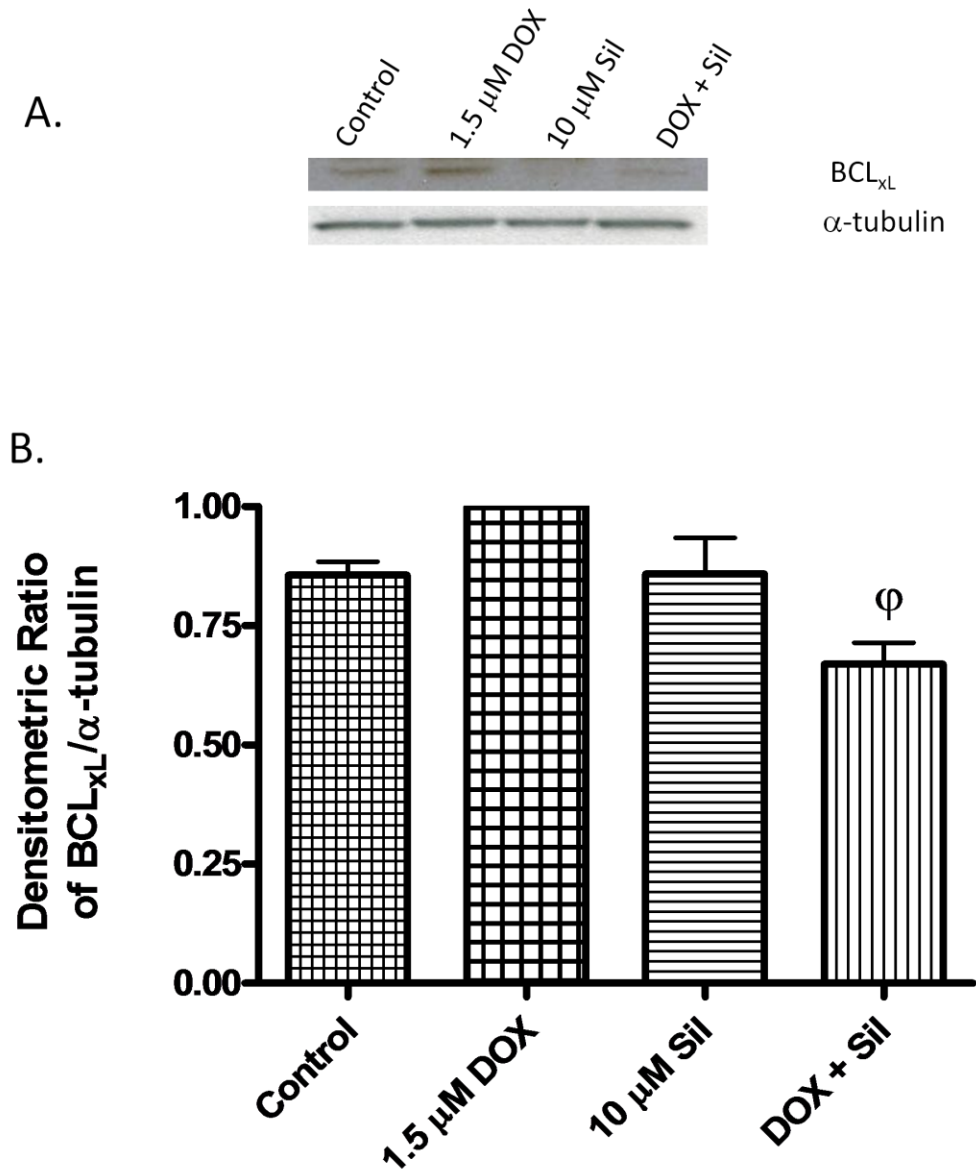


Figure 11: BCL_{xL} protein levels in PC3 cells after 6 hrs treatment with 1.5μM DOX and 10μM Sildenafil (Sil). A: Western blot. B: Densitometric depiction n = 4; means ± S.E., one-way ANOVA, Newman-Keuls post test. ^φP < 0.05 vs. DOX.

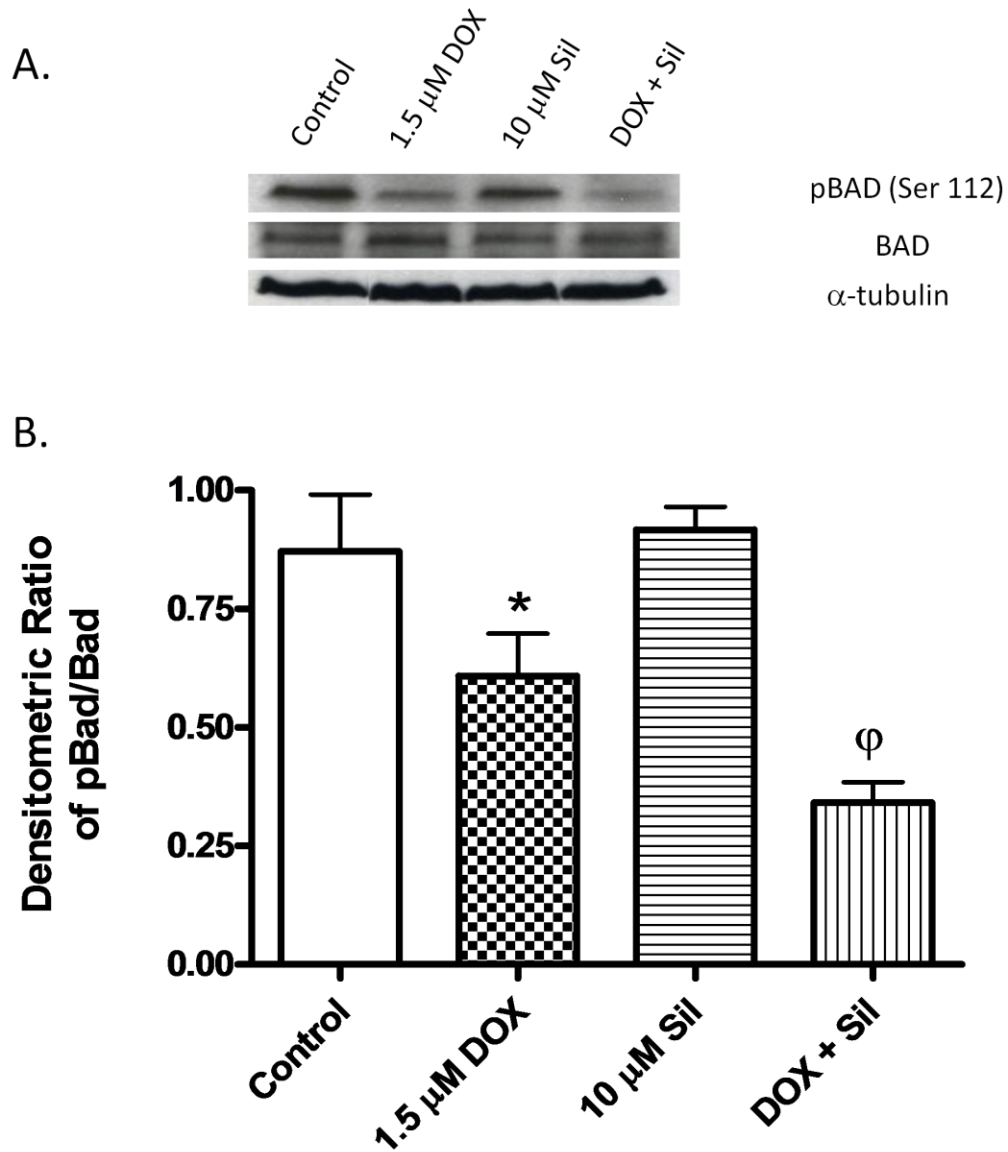


Figure 12: pBad levels in PC3 cells after 6 hrs treatment with 1.5 μ M DOX and 10 μ M Sildenafil (Sil). A: Western blot. B: Densitometric depiction. $n = 4$; means \pm S.E., one-way ANOVA, Newman-Keuls post test. $\phi P < 0.05$ vs. DOX and $*P < 0.05$ vs. Control.

Discussion

Perspective

Prostate cancer can be treated by androgen ablation, because the solid tumors are androgen-dependent. However, PC frequently progresses to a lethal, androgen-independent form, AIPC, which has limited treatment options.

Several studies have shown that PDE-5 inhibitors precondition the heart against I/R in vivo and in vitro (Das et al. 2005; Ockaili et al. 2002; Das et al. 2004; Salloum et al. 2003; Bremer et al. 2005; Salloum et al. 2007; Salloum et al. 2008). The PDE-5 inhibitor, Sildenafil preconditions the heart against I/R through the generation of NO by activation of eNOS and iNOS, activation of PKC, and opening of the mitochondrial K_{ATP} channels (Das et al. 2005; Ockaili et al. 2002; Das et al. 2004; Salloum et al. 2003). Sildenafil can also offer protection against apoptosis and necrosis through a NOS-dependent upregulation of BCL-2/Bax in cardiomyocytes (Das et al. 2005). Moreover, recent studies from our lab have shown that Sildenafil protects the heart and cardiomyocytes against I/R injury through a novel signaling cascade involving protein kinase G (PKG)-dependent activation of ERK and inhibition of glycogen synthase kinase 3 β (Das et al. 2008). Sildenafil also inhibits DOX-induced cardiomyocyte apoptosis by preventing the loss of $\Delta\Psi_m$, caspase-3 activation, and increasing the levels of BCL-2 (Fisher et al. 2005).

In addition to the above cited studies on the cardioprotective effect of PDE-5 inhibitors, unpublished studies from our laboratory have provided a powerful evidence that Sildenafil in combination with DOX significantly decreased cell viability in several cancer cell lines including sarcoma (OSA-1), ovarian (UCI-101 and A2780) and prostate (PC-3) (see figure 6). Similar combination of DOX plus Sildenafil decreased the size of tumor in ovarian cancer xenograft model (Kukreja RC et al. 2008). Moreover, Sildenafil treatment caused higher distribution of DOX in the tumors which is a favorable pharmacokinetic feature for improving the efficacy of DOX.

Since DOX is known to activate NF- κ B in various cancer cells and blocking NF- κ B sensitizes cells to DOX-induced apoptosis, we hypothesized that Sildenafil when used in combination with DOX would attenuate NF- κ B activation that may lead to potentiation of apoptosis in PC3 cells. Accordingly we carried out experiments to study the combined effect of DOX and Sildenafil in attenuating the activation of NF- κ B by measuring the translocation of p65 and p50 subunits to the nucleus, phosphorylation of cytosolic I κ B α , and its resultant influence on apoptosis. In addition, we investigated the effect of DOX and DOX plus Sildenafil on the expression of BCL family of proteins which play critical role in apoptosis. Our results show that the combined treatment of DOX and Sildenafil significantly reduced the nuclear translocation of p65 and p50 as compared with DOX. This correlated with a reduction in the phosphorylation of the expression of BCL2, BCL $_{xL}$ and pBAD. Our results suggest that Sildenafil treatment

augments the apoptotic potential of DOX by decreasing NF- κ B activation in PC3 cancer cells.

NF- κ B is a pleiotropic nuclear transcription factor that controls the expression of a myriad of genes involved in immunity, inflammation, and carcinogenesis (Wulczyn et al. 1996; Barnes and Karin. 1997). NF- κ B is inhibited by I κ B family of proteins which are susceptible to phosphorylation by IKKs following the appropriate stimulus.

However, in some cancer cells, NF- κ B is known to be constitutively activated which has been associated with low I κ B- α expression and high I κ B- α degradation (Bargou et al. 1996). Depending on the circumstance, is capable of promoting apoptosis or promoting cell survival. In cancer cells, it generally appears that constitutive NF- κ B activation is in favor of cell survival.

Indeed, it is well established that DOX and other anthracyclines induce NF- κ B activation (Bian et al. 2001). Since this induction of NF- κ B may confer resistance in cancer, many studies have taken effort to inhibit NF- κ B activation. For example, it was demonstrated that soy isoflavone pre-treatment inactivates NF- κ B and may contribute to increased growth inhibition and apoptosis by Cisplatin, Docetaxel, and Doxorubicin in prostate, breast, lung, and pancreatic cancer cells (Li and Wogan. 2005). Therefore it was of interest to determine whether the PDE5 inhibitor, Sildenafil will block the induction of NF- κ B and whether this inhibition will potentiate apoptosis. Our results demonstrate that Sildenafil in combination with DOX does significantly reduce the nuclear translocation of the p65 and p50 of NF- κ B subunits as compared to cells that are

treated with DOX alone (figures 7 and 8). I κ B α sequesters NF- κ B in the cytosol by masking its NLS. This inhibition is removed by phosphorylation and subsequent degradation of I κ B α by IKK. We observed an overall increase in the reduction in p-I κ B α following DOX treatment and a reduction in the levels of p-I κ B α following combination treatment of DOX plus Sildenafil. The results for p-I κ B α ; however, were not statistically significant.

While it is known that NF- κ B is constitutively expressed in androgen-independent PC3 cells; here we show that DOX serves to further augment NF- κ B levels as demonstrated with the upregulation in p50, 65, as well as p-I κ B α following DOX treatment. Sildenafil appears might be chemosensitizing PC3 cells to DOX chemotherapy by modulating the activity of NF- κ B.

NF- κ B - Apoptosis Connection:

Since NF- κ B is known to promote anti-apoptotic proteins in cancer cells by upregulating an array of survival genes, we therefore assessed the expression of BCL family of proteins, particularly the BCL-2 and BCL_{xL} and phosphorylated BAD. Our goal was to show whether this induction of NF- κ B in PC3 cells as a result of DOX treatment would enhance survival mechanism and whether we can diminish these levels using combination treatment with Sildenafil plus DOX. We found that DOX treatment alone had the higher levels of BCL_{xL} as compared with control in PC3 cells (figure 11). These data suggest a possible connection of NF- κ B with the regulation of apoptosis in

PC3 cells. In this respect, it appears that the induction of NF- κ B in PC3 cells is favoring cell survival evidenced by the increase in BCL_{xL} protein levels. However, co-treatment of Sildenafil with DOX significantly reduced BCL_{xL} as compared with DOX alone. This implies that Sildenafil is undermining PC3 cell survival and sensitizing them to DOX chemotherapy.

It is of interest to examine the phosphorylation of BAD and its relation to BCL-2 and BCL_{xL}. BAD is a pro-apoptotic protein that interacts with cell survival proteins, such as BCL-2 and BCL_{xL} to inhibit them. This ultimately leads to cell death by opening mitochondrial permeability transition pore, encouraging the release of cytochrome *c* and activation of the apoptosome. This death agonist is regulated by kinases that phosphorylate BAD on at least one of two serine residues, Ser¹¹² or Ser¹³⁶ by cAMP-dependent protein kinase (PKA) or protein kinase B (PKB; also known as Akt) respectively (Bonni et al. 1999; Datta et al. 1997) in response to survival-promoting factors, such as nerve growth factor, insulin-like growth factor-1, and interleukin-3 (IL-3) (Zha et al. 1996; Datta et al. 1997; Yang et al. 1995). In the present study, we observed a significant decrease in the ratio of pBAD/BAD with DOX, and a further decline in these levels by co-treatment with Sildenafil plus DOX (figure 12). This decrease in pBAD/BAD ratio indicates DOX is activating BAD facilitating the removal of the inhibition so that it may function as a death agonist and potentially inhibit the cell promoting BCL-2 family proteins. According to our results, this is in accordance with BCL-2 but not for BCL_{xL}. Co-administration of Sildenafil along with DOX significantly reduced both of the survival proteins, BCL-2 and BCL_{xL}, which is in agreement with

reduced BAD phosphorylation favoring apoptosis. These findings demonstrate that combined treatment with Sildenafil along with DOX is further potentiating the apoptotic effect of DOX.

Apoptosis has been shown to be induced by NO in a concentration-dependent manner by activating caspase-3 (Lin et al. 1998). NO has the ability to potentiate apoptosis in various cell types through mechanisms that involve p53 accumulation, poly (ADP ribose) polymerase (PARP) induction, and BCL-2 down-regulation (Li and Wogan. 2005; Umansky et al. 2001; Li et al. 2006). Interestingly, NO has been shown to restrain the DNA binding of activity of NF- κ B (Matthews et al. 1996). It is conceivable that NO may potentiate apoptosis by modulating NF- κ B. Thus, Sildenafil activation of nitric oxide synthase may be responsible for the reduction in the nuclear translocation of the p65/50 dimer. Thus, Sildenafil co-administration appears to be potentiating the therapeutic efficacy of DOX by inhibiting NF- κ B.

Future Directions

PDE-5 is found to be upregulated in prostate cancer (LNCAP and PC3), and ED is encountered following all forms prostate cancer therapy (Rambhatla et al. 2008). Sildenafil not only serves to treat ED but also may increase the efficacy of DOX as a chemotherapeutic agent as suggested by the studies from our laboratory. Therefore, it would be more advantageous to use Sildenafil during anti-oncogenic therapies to potentiate cancer cell death and protect against cardiotoxicity rather than utilize it for the sole purpose to treating ED post cancer therapy.

Clinicians generally employ multiple chemotherapeutic agents in order to gain better treatment outcome; however, this leads to systemic cytotoxicity which often results in detrimental side effects (Daniels et al. 2006). Therefore, utilizing a non-chemotherapeutic drug along with a chemotherapeutic agent would achieve targeted inactivation of NF- κ B, which may lead to more DOX-induced apoptosis of cancer cells without the harmful side-effects. Since Sildenafil has been in clinical use for over ten years, its co-treatment with a chemotherapeutic agent, such as DOX may potentiate the destruction of malignant cells and tissues without further contribution of toxicity of non-cancerous tissues.

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VITA

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