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TYPE-5 PHOSPHODIESTERASE INHIBITION IN PREVENTION OF
DOXORUBICIN CARDIOMYOPATHY

A Dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Physiology at Virginia Commonwealth University.

By

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Abstract

TYPE-5 PHOSPHODIESTERASE INHIBITION IN PREVENTION OF DOXORUBICIN
CARDIOMYOPATHY

By Patrick William Fisher, DO, PhD

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
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Virginia Commonwealth University, 2005

Major Director: Rakesh C. Kukreja, PhD
The Eric Lippman Chair of Molecular Cardiology
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Prior studies have demonstrated the effect of diazoxide in protecting against apoptosis via mitochondrial K_{ATP} channel opening *in vitro*. The current investigations are designed to determine if sildenafil, a phosphodiesterase-5 inhibitor and known mitochondrial K_{ATP} channel opener, would protect against chronic doxorubicin cardiomyopathy both *in vivo* and *in vitro*.

Male ICR mice were randomized to 1 of 4 treatments: saline, sildenafil (0.7 mg/kg IP), doxorubicin (5 mg/kg IP), and sildenafil (0.7 mg/kg IP)+doxorubicin. Apoptosis was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and in situ oligo ligation methods. Desmin distribution was determined via immunofluorescence. Bcl-2 was analyzed by Western blot. Left ventricular function was measured in Langendorff mode. Electrocardiographical analysis measured changes indicative of doxorubicin cardiotoxicity (ST-prolongation). *In vitro* studies using adult ventricular cardiomyocytes were exposed to doxorubicin (1 μ M), sildenafil (1 μ M) with or without N^G-nitro-L-arginine methyl ester (L-NAME; 100 μ M), or 5-hydroxydecanoate (5-HD; 100 μ M)) 1 hour before doxorubicin and incubated for 18 hours.

Doxorubicin-treated mice demonstrated increased apoptosis and desmin disruption, which was attenuated in the sildenafil+doxorubicin group. Bcl-2 decreased in the doxorubicin group but was maintained at basal levels in the sildenafil+doxorubicin group. Left ventricular developed pressure and rate pressure product were significantly depressed in the doxorubicin group but attenuated in the sildenafil+doxorubicin group. ST-interval significantly increased in the doxorubicin group over 8 weeks. In the sildenafil+doxorubicin group, ST-interval remained unchanged from baseline. Doxorubicin significantly increased apoptosis, caspase-3 activation, and disruption of mitochondrial membrane potential *in vitro*. In contrast, sildenafil significantly protected against doxorubicin cardiotoxicity; however, protection was abolished by both L-NAME and 5-HD. Cell viability studies using spectrophotometer and flow cytometric techniques

demonstrated that sildenafil did not affect the antitumor efficacy of doxorubicin in PC-3 cells *in vitro*. In fact, flow cytometry data indicate that sildenafil, when combined with doxorubicin, was synergistic in the antineoplastic action of doxorubicin.

Prophylactic treatment with sildenafil prevented apoptosis and left ventricular dysfunction in a chronic model of doxorubicin-induced cardiomyopathy. Moreover, these studies provide relevant clinical data on the safety and efficacy of sildenafil, leading the way for clinical trials in humans receiving doxorubicin chemotherapy.

BACKGROUND

Heart failure remains a leading cause of morbidity and mortality in the United States, affecting approximately 5 million Americans; particularly those age 65 and older. In 1995, Medicare spent an estimated \$3.4 billion dollars for the treatment of heart failure. In 2004, this number increased to \$28 billion dollars. A continually aging population is expected to result in a greater number of people afflicted with heart failure, requiring costly long-term medical management with unpredictable effect on quality of life [1].

Etiologies of heart failure development are numerous and involve complex molecular mechanisms, not entirely understood. However, recent advances have expanded our knowledge and understanding of the cellular and molecular mechanisms involved in the development of heart failure. For example, progress in primary and secondary prevention of coronary artery disease has improved the ability of physicians to target at-risk populations who may benefit from early treatment and lifestyle modifications aimed at reducing myocardial infarction and the development of ischemic cardiomyopathy. In contrast, prevention of nonischemic cardiomyopathy remains a challenge. Moreover, successful treatment is often palliative unless the patient is able to receive an orthotopic heart transplant. Left ventricular assist devices offer promise as a bridge to

transplantation or as destination therapy. However, despite these modern biomedical innovations, a cure is not attainable and both life expectancy and quality of life are unpredictable.

Over thirty years ago, the introduction of the anthracycline antibiotics markedly shifted the momentum in the battle against cancer. Doxorubicin, one of the most widely used anthracyclines, continues to be a powerful weapon in the treatment of many human neoplasms, including Kaposi's Sarcoma, acute leukemias, lymphomas, stomach, breast, and ovarian cancers [2]. Despite its clinical efficacy, doxorubicin is associated with a delayed and progressive cardiomyopathy often presenting more than 20 years after treatment cessation [3,4,11]. The underlying mechanism of chronic doxorubicin-induced cardiomyopathy occurs primarily via the generation of reactive oxygen species (ROS) in the cardiomyocyte mitochondria—a mechanism that is separate from its antineoplastic activity, which occurs primarily through inhibition of topoisomerase II [5]. Additionally, numerous studies involving both *in vitro* and *in vivo* models of heart failure link ROS to cardiomyocyte apoptosis [6-9]. In fact, it is hypothesized that apoptosis plays a role in the development of heart failure via mechanisms that contribute to cardiomyocyte loss, eventually leading to structural changes maladaptive to normal cardiac physiological demands [10]. Over the past three decades, significant research has focused on unraveling this conundrum. More recently, a prospective study evaluating cardiac

abnormalities in childhood survivors of cancer 15 years or more after treatment with doxorubicin, demonstrated an increased incidence of cardiomyopathy at doses much lower than the current acceptable total cumulative dose of approximately 450 mg/m² [11,12]. In fact, the prevalence of severe cardiac dysfunction was found in more than six percent of patients at 15 years post-treatment at doses less than 250 mg/m². Moreover, the mean prevalence of severe cardiac dysfunction in patients receiving more than 250 mg/m² was found to be 19% at 25 years after treatment. Overall, after an average follow-up of 18 years, 39% of childhood cancer survivors treated with doxorubicin exhibited severe cardiac dysfunction. Additionally, severe cardiac dysfunction was identified in 6% of patients who had no prior clinical history of cardiac failure with doses of doxorubicin at or above 150 mg/m² without evidence for threshold. [11]

Despite advances in understanding this disease process at the cellular and molecular level, prophylactic pharmacological agents are lacking. Because of the potency, broad spectrum, and efficacy of doxorubicin in treating many malignancies today, coupled with the difficulty in treating patients with doxorubicin-induced cardiomyopathy, it is imperative that the development of novel approaches aimed at prevention of cardiotoxicity is aggressively pursued. The ideal agent must prevent cardiotoxicity while maintaining effective anti-neoplastic activity

PHARMACOLOGY OF DOXORUBICIN

Mechanism

Doxorubicin is an anthracycline antibiotic, isolated from *Streptomyces peucetius* var *caesius*. There are three major actions that account for the anti-neoplastic activity and toxicity of doxorubicin: (1) high affinity DNA binding via intercalation and interaction with topoisomerase II, ultimately blocking the synthesis of both DNA and RNA; (2) binding to cellular membranes with alteration of fluidity and ion transport; and (3) redox cycling of doxorubicin to its semiquinone free radical with subsequent generation of oxygen radicals. Additionally, several hypotheses have been proposed to explain the acute and chronic cardiotoxicity of doxorubicin; these include formation of free radicals, inhibition of enzymes and proteins, changes in cardiac muscle gene expression, alterations of mitochondrial membrane function, sensitization of Ca^{2+} release from sarcoplasmic reticulum channels, mitochondrial DNA damage, and dysfunction. [12-15,58]

It is thought that doxorubicin alone is responsible for acute cardiotoxicity. In contrast, chronic cardiotoxicity is believed to be a result of chronic, perpetual redox cycling of doxorubicin in the mitochondria—a process that continues long after serum concentrations become undetectable.

Pharmacokinetics

In the clinical setting, doxorubicin is administered by intravenous (IV) injection. The usual dose is 60 mg/m^2 IV every three weeks to a maximum dose of $450\text{-}550 \text{ mg/m}^2$. 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. After IV administration, peak serum concentrations of doxorubicin are attained rapidly. Thirty minutes after infusion, serum concentration drops by approximately 50%. However, significant levels persist for up to 20 hours. The main route for doxorubicin metabolism is via the liver where it undergoes reduction and hydrolysis of ring substituents. The alcohol metabolite of doxorubicin, doxorubicinol, is pharmacologically active. In contrast, the aglycone derivative is an inactive metabolite. The majority of doxorubicin and its metabolites are excreted in the bile with a minute amount excreted in the urine. Enterohepatic recirculation of toxic metabolites occurs during biliary excretion. Therefore, dose reduction needs to be considered in patients with hepatic disease or elevated hepatic transaminases. [16]

CELLULAR AND MOLECULAR MECHANISMS OF DOXORUBICIN CARDIOTOXICITY

Multiple studies substantiate the major role that reactive oxygen species (ROS) play in the development of heart failure both in vivo and in vitro [17,18]. In fact, substantial evidence exists implicating the generation of ROS as the underlying mechanism for the development of dilated cardiomyopathy and heart failure from multiple etiologies including chemotherapy-induced [14,17,18]. Moreover, prior studies have identified the mitochondria as the main target of doxorubicin accumulation in cardiac cells [19]. In fact, mitochondrial concentrations of doxorubicin (5-50 μM) are several-fold greater than simultaneous clinically relevant serum concentrations (0.1-1 μM) [20].

Mitochondrial NADH dehydrogenase is a major contributor to doxorubicin-generated reactive oxygen species (ROS) production via redox cycling of doxorubicin to its semiquinone [21, Figure 1].

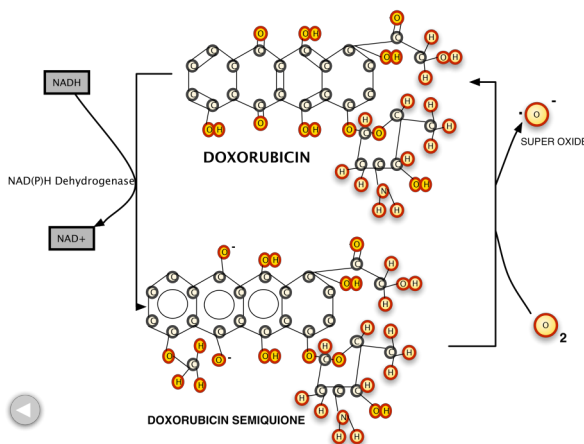


Figure 1A. Redox cycling of doxorubicin & formation of Superoxide.

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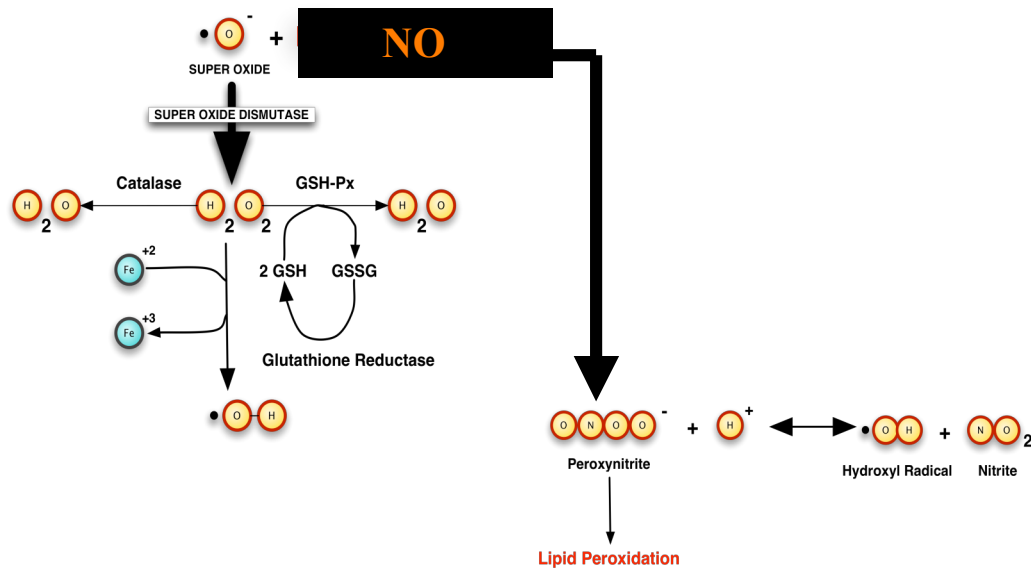


Figure 1B. Redox cycling of doxorubicin and formation of ROS & RNS.

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It is therefore, not surprising that high mitochondrial concentrations of doxorubicin, relative to simultaneous serum concentrations, rapidly perpetuates the formation of free radicals. Compared with other organs such as the liver, the heart possesses a relatively limited supply of catalase and glutathione peroxidase (GSH-Px), key intracellular free radical scavengers. Because of chronic doxorubicin free radical production in the heart, the supply of both GSH-Px and catalase is rapidly expended; thus, creating an environment that promotes hydroxyl radical production [22].

Accordingly, the accumulation of ROS results in dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$), direct activation of the mitochondrial permeability transition pore (MPTP), and release of cytochrome-c with subsequent activation of caspase-9 and caspase-3 followed by DNA fragmentation consistent with apoptosis [34].

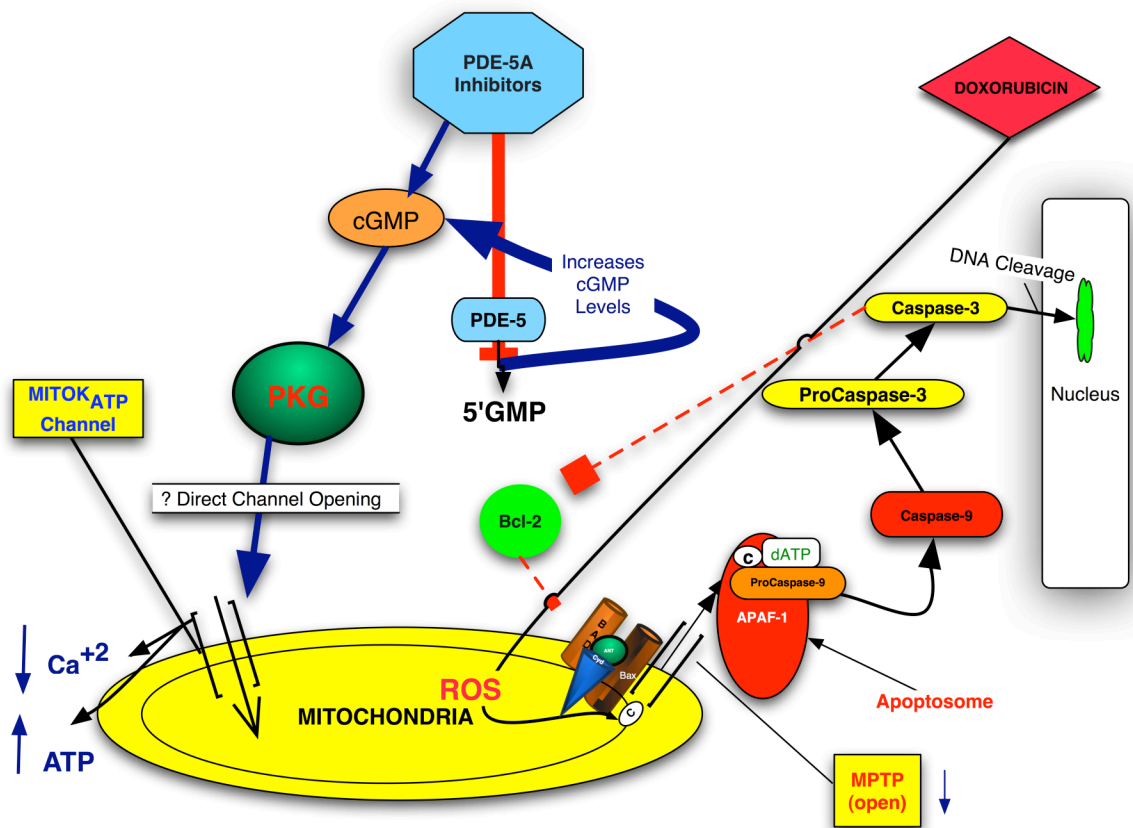


Figure 2. Cell signaling pathways involved in doxorubicin-induced cardiotoxicity.

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APOPTOSIS

It is well known that doxorubicin-induced cardiomyocyte apoptosis occurs via both the extrinsic and intrinsic pathways [23,24]. However, it remains unknown whether one pathway is more important than the other in respect to doxorubicin-induced cardiomyocyte apoptosis. However, recent studies substantiate the significance of the intrinsic pathway of apoptosis in this pathophysiological process [25,26].

Apoptosis, commonly known as programmed cell death, is a regulated cellular process dependent on ATP [27]. It results in cell death for reasons that often are required in maintaining normal physiological function in many species. For example, apoptosis plays an important role in the degeneration of the human thymus gland. Additionally, apoptosis is critical in sloughing of intestinal epithelial tissue, which rapidly turns over multiple times a year in humans [28]. Distinguishing apoptotic cell death from necrotic cell death and DNA repair mechanisms has remained controversial [29]. However, it is now possible to delineate these two very different modes of cell death through the implementation of several sensitive and specific assays. The most well defined method for determining apoptosis from necrosis is by morphological evaluation [30]. Apoptosis is recognized by a series of well-defined morphological changes that differ from necrotic cell death [31]. In apoptosis, common morphological findings include condensed heterochromatin often observed in the perinuclear regions of the cardiomyocyte, cell shrinkage, nuclear pyknosis, and late fragmentation into apoptotic bodies [31]. In contrast, necrotic cell death is

characterized by cell membrane disruption and release of intracellular contents that are toxic to surrounding cells and tissues [31].

Apoptosis contributes to the development of heart failure via mechanisms that contribute to a loss of cardiomyocytes over time, leading to structural changes that often are maladaptive to normal cardiac physiological demands [6,10]. Furthermore, it is also suggested that the major difference between whether cells undergo apoptosis versus necrosis is the cellular availability of ATP [32]. Since apoptosis is an energy dependent process requiring ATP, cells originally destined for programmed cell death may undergo necrotic cell death if depleted of adequate ATP stores [32].

Extrinsic Pathway of Apoptosis [Figure 3]

The extrinsic pathway is initiated via binding of a death ligand to a cell surface receptor. The ligand is usually one of two different types. The Fas ligand (Fas-L), otherwise known as CD95 or Apo-1, is an integral membrane protein found on the surface of another cell (T-Lymphocyte). On the other hand, a ligand may exist as an extracellular protein such as tumor necrosis factor-alpha (TNF- α). Upon ligand-receptor binding, the death-inducing signaling complex (DISC) is formed. For example, the binding of Fas-L to the Fas receptor results in recruitment of the Fas-associated death domain (FADD). Consequently, the recruitment of caspase-8 by FADD results in cleavage of the procaspase to its active form. Activated caspase-8 can directly activate caspase-3 while completely bypassing the mitochondrial death pathway. However, active caspase-8 can also activate the BH3

interacting death domain agonist (Bid), a proapoptotic Bcl-2 (B cell leukemia/lymphoma-2) protein, which links the extrinsic to the intrinsic pathway of apoptosis. Both in vivo and in vitro studies of acute doxorubicin cardiotoxicity have demonstrated involvement of both TNF-alpha and FAS/FAS-L in this process. [33,34, Figure 3]

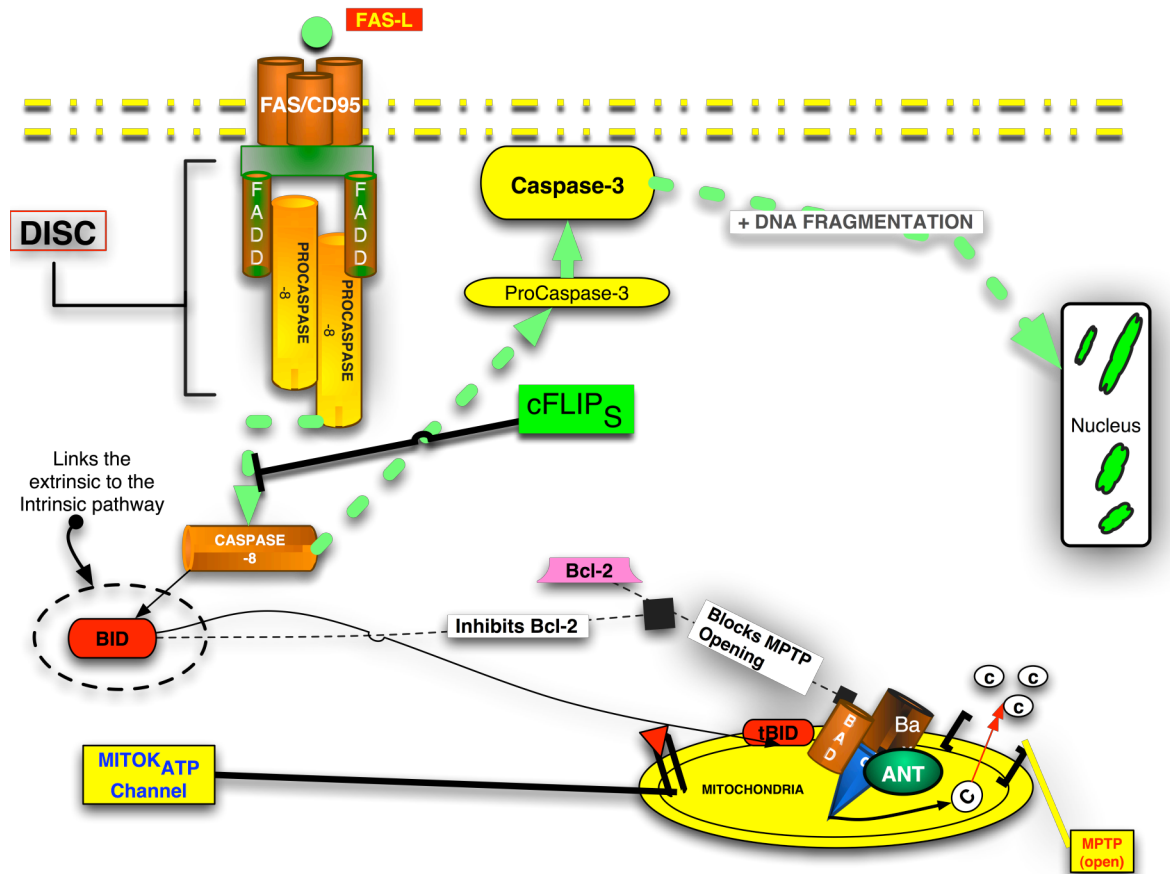


Figure 3. Simplified Version of the Extrinsic Pathway of Apoptosis in Doxorubicin Cardiotoxicity and the Illustration of the Key Link Between Extrinsic and Intrinsic Pathways. © 2005 Patrick W. Fisher, DO, PhD

Intrinsic Pathway of Apoptosis [Figure 4]

The intrinsic pathway of apoptosis is more complex than the extrinsic pathway and is stimulated by a multitude of various extracellular stimuli and intracellular signaling mechanisms [33,36]. Extracellular stimuli include growth-factor withdrawal or complete deficit of trophic factors or nutrients, ionizing radiation, and chemicals such as toxins. Intracellular stimuli include oxidative stress, DNA damage, physical stress on the cytosolic matrix proteins that provide a support or scaffold for cells such as myocytes, or oxidation of fatty acids. The result of activation of the intrinsic pathway of apoptosis is the release of pro-apoptotic proteins, such as cytochrome-c and apoptosis-inducing factor (AIF), into the cytosol with activation of caspases and subsequent DNA fragmentation [36].

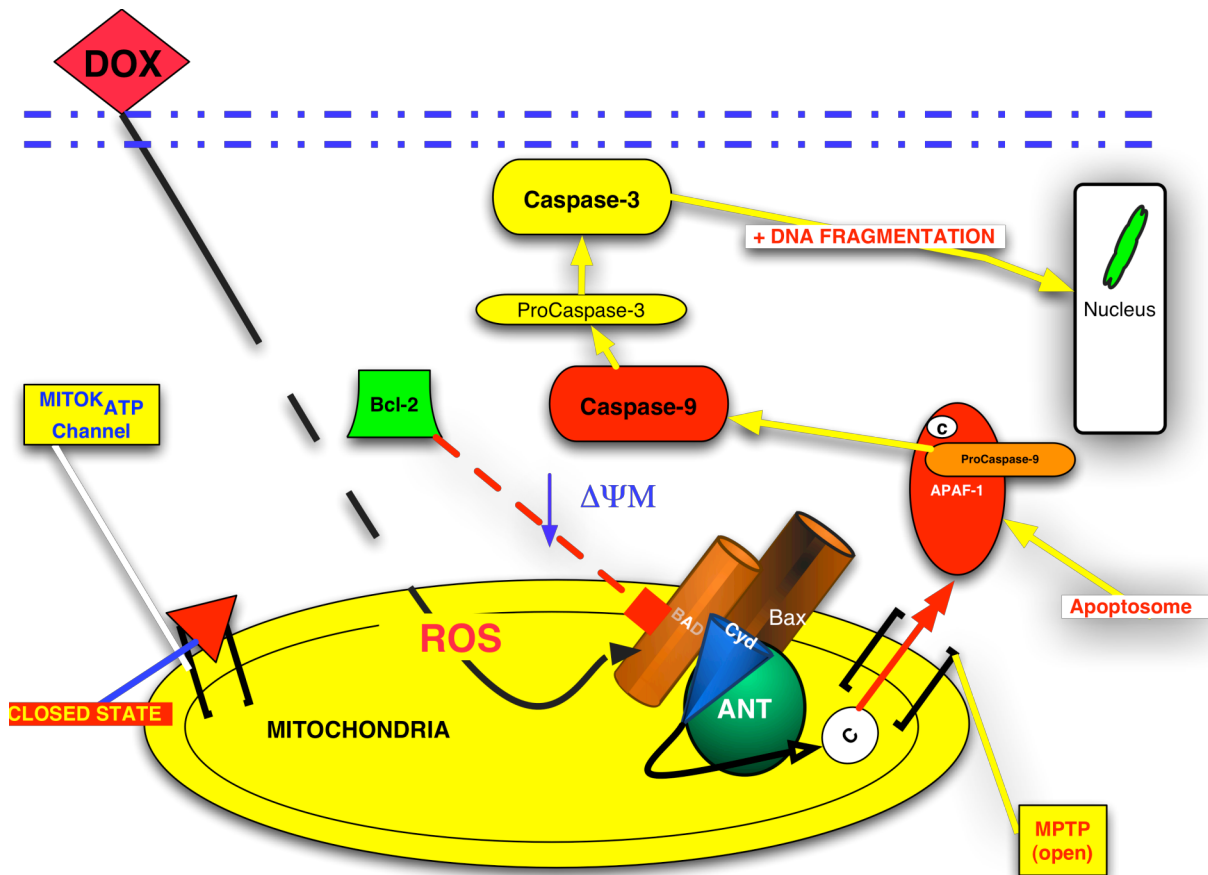


Figure 4. Simplified Illustration of the Intrinsic Pathway of Apoptosis in Doxorubicin Cardiotoxicity. © 2005 Patrick W. Fisher, DO, PhD

KEY MEDIATORS OF APOPTOSIS

Caspases

Caspases belong to the class of cysteine proteases that cleave substrates after aspartic acid residues. They play a critical role in apoptosis. Moreover, caspases are synthesized as inactive zymogens known as procaspases. Procaspases contain an N-terminal prodomain and a C-terminal catalytic domain. The catalytic domain consists of a 20 kDa (p20) and a 10 kDa (p10) subdomain. Caspases can further be divided into upstream versus downstream caspases. Upstream caspases include caspases 2, 8, 9, 10, and 12.

Downstream caspases include caspases 3, 6, and 7. These caspases are then converted to their respective active forms by proteolytic cleavage after aspartic acid residues, located between the prodomain, p20, and p10 subunits. Caspase-3 is a key effector in the apoptotic pathway, amplifying the signal from initiator caspases (such as caspase-8) and signifying full commitment to cellular disassembly. In addition to cleaving other caspases in the enzyme cascade, caspase-3 has been shown to cleave poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C δ , actin, and intermediate filaments such as desmin. Caspase-8 plays a critical role in the early cascade of apoptosis, acting as an initiator of caspase activation; whereas, caspase-9 is an integral component of the intrinsic pathway of apoptosis where it cleaves procaspase-3 to its active form. [35,37]

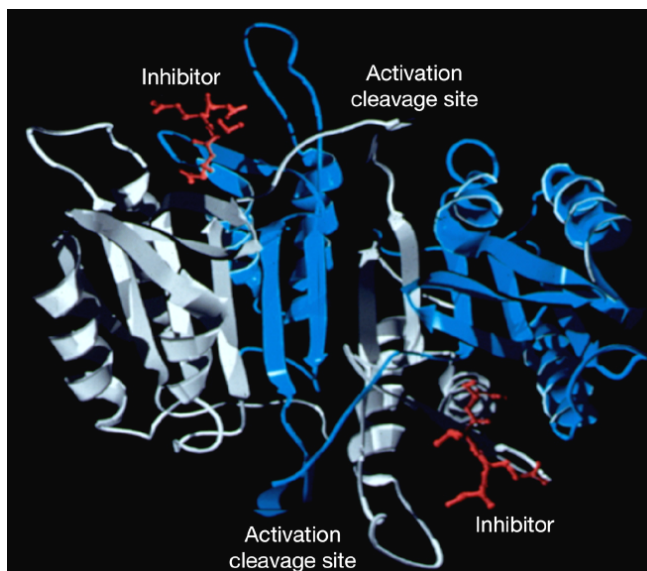


Figure 5. Illustration of caspase-3 and associated subunits.

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In addition, the cleavage of the pro-caspase to its active form has long been regarded as an irreversible marker of commitment to apoptotic cell death. However, more recent studies have demonstrated the ability of NO, at physiological concentrations, to directly, but reversibly, inactivate caspase-3 (activated cleaved form) via *s*-nitrosylation at the cysteine residue on the p17 subunit [68,71].

Bcl-2 Family of Proapoptotic and Antiapoptotic Proteins

Bcl-2 is a key regulator of apoptosis and is essential for proper development, tissue homeostasis, and elimination of exogenous toxic stimuli. Bcl-2 is a 26 kDa, anti-apoptotic protein, which promotes cell survival via interactions with anti-apoptotic Bcl-2 family members. These include Bax, Bak, Bik, Bad, and Bid. One of the major functions of Bcl-2 is prevention of cell death through its ability in blocking the release of cytochrome-*c* from the intramembrane space of the mitochondria. Bax and Bak are multidomain proapoptotic Bcl-2 proteins. Either Bax or Bak is required for all instances of apoptosis mediated via the intrinsic pathway. Their interaction at the mitochondrial membrane contact site contributes to the formation of the voltage-dependent anion channel, or VDAC—facilitating the release of cytochrome-*c* to the cytosol. [38-41]

Cytochrome-*c*

Cytochrome-*c* is an electron transport protein essential in aerobic energy conversion. It is found in the mitochondrial intermembrane space in mammalian species. Cytosolic cytochrome-*c* is a key mediator in the intrinsic pathway of apoptosis. For example, the release of cytochrome-*c* to the cytosol results in the formation of the apoptosome and activation of downstream caspase-3; consequently leading to interaction of activated caspase-3 with nuclear dsDNA resulting in DNA fragmentation characteristic of apoptosis [38,41].

Mitochondrial membrane potential (Ψ_m) & Mitochondrial K_{ATP} Channels

The collapse of $\Delta\Psi_m$ is a prominent feature of apoptosis, representing an irreversible marker of cellular commitment to apoptotic cell death [42]. Moreover, breakdown of the mitochondrial membrane potential ($\Delta\Psi_m$) may precede nuclear signs of apoptosis, and it may be associated with Ca^{2+} homeostasis.

Prior research using the mito K_{ATP} channel opener, diazoxide, demonstrated a link between mito K_{ATP} channel opening and preservation of mitochondrial integrity, maintenance of mitochondrial membrane potential, and inhibition of cytochrome-*c* translocation to the cytosol following in vitro oxidative stress [43]. Moreover, the opening of mito K_{ATP} channels is critical in mediating the cardioprotective effect induced by pathophysiological stressors and pharmacological agents. The activation of these channels is triggered by a drop in tissue ATP levels, which result in preventing the dissipation of the mitochondrial membrane potential and inhibition of apoptosis [43,44].

Furthermore, studies by Marban et al have shown that opening of mitochondrial K_{ATP} channels by diazoxide induced a cardioprotective effect, which was abolished by 5-hydroxydeconate (5HD), an inhibitor of the mito K_{ATP} channel [44]. These findings have been reported in several other studies of ischemia/reperfusion injury in animal models where post-ischemic functional recovery significantly improved and infarct size was reduced [42].

Because of the key role of mitochondrial bioenergetics in cellular respiration and in mitigating apoptosis, it is a promising target for potential development of novel therapeutic applications in cardioprotection.

Intermediate filaments and Mitochondrial Permeability Transition Pore (MPTP)

Formation

Intermediate filaments play an integral role in cellular structure and function. Desmin, an intermediate filament found in cardiomyocytes is localized to the Z-line where it supports cellular integrity and stabilization of actin filaments [Figure 6A]. Moreover, desmin integrates physical contraction of the myofibril via its linkage to adjacent myofibrils. In addition to attachment to the Z-line, desmin also tethers the myofibrils to the sarcolemma, nuclei, and to the mitochondria [Figure 6B]. In fact, desmin attaches to the mitochondrial membrane contact sites, the same location where the VDAC and mitochondrial permeability transition pore (MPTP) are formed [45]. Furthermore, desmin plays a vital role in protecting the structural integrity of the myofibrils during mechanical stress. It is conceivable that disruption of desmin either through repeated strain on the contractile apparatus resulting from impaired contractility or through direct cleavage from activated caspases may contribute to MPTP formation, cytochrome-*c* release, and apoptosis [Figure 6]. [45-51]

Although it is known that cardiomyocyte apoptosis contributes to dilated cardiomyopathy and heart failure, there is increasing evidence that intermediate filaments such as desmin

are involved in this pathological process [45]. Recently, Dinsdale et al [46] demonstrated caspase-cleavage of intermediate filaments during apoptosis, which subsequently formed intracytoplasmic aggregates. Moreover, a study using a transgenic mouse model (desmin -/-) of desmin-related cardiomyopathy (DRM) demonstrated the ability of Bcl-2 overexpression in preventing DRM as evidenced by prevention of cardiomyocyte apoptosis and preservation of cardiac contractility [45]. In addition, Wang et al [47] demonstrated the disruption of desmin and formation of intracytoplasmic aggregates in a mouse model of desmin-related cardiomyopathy. Furthermore, Heling et al [48] illustrated the disorganization and accumulation of desmin in explanted human heart specimens from patients with dilated cardiomyopathy.

Morphological changes including disruption of normal desmin distribution in myocytes as observed in DRM are similar to those seen in other forms of cardiomyopathy and heart failure [49]. Because intermediate filaments participate in the transmission of active force [50], it is plausible that disruption of the filamentous network involving desmin may significantly impair contractile force and result in sarcomere fragility.

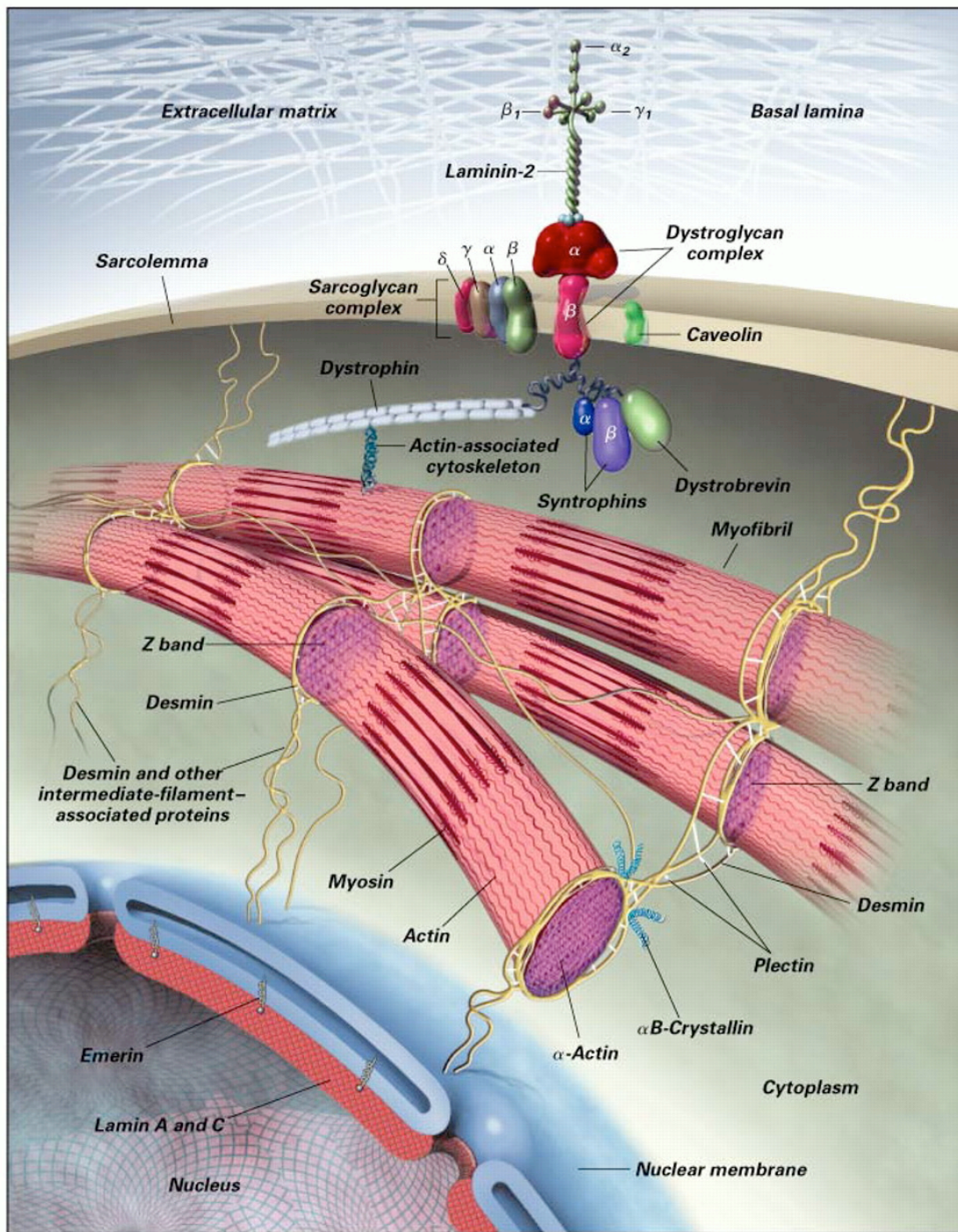


Figure 6A. Main Intermediate Filaments and Cytoskeletal Proteins Linking the Extracellular Matrix with the Structural Muscle Proteins Associated with Mutations Causing Cardiac and Skeletal Myopathy. © 2000 Massachusetts Medical Society, Dalakas, MC et al. *N Engl J Med* 2000; 342: p778.

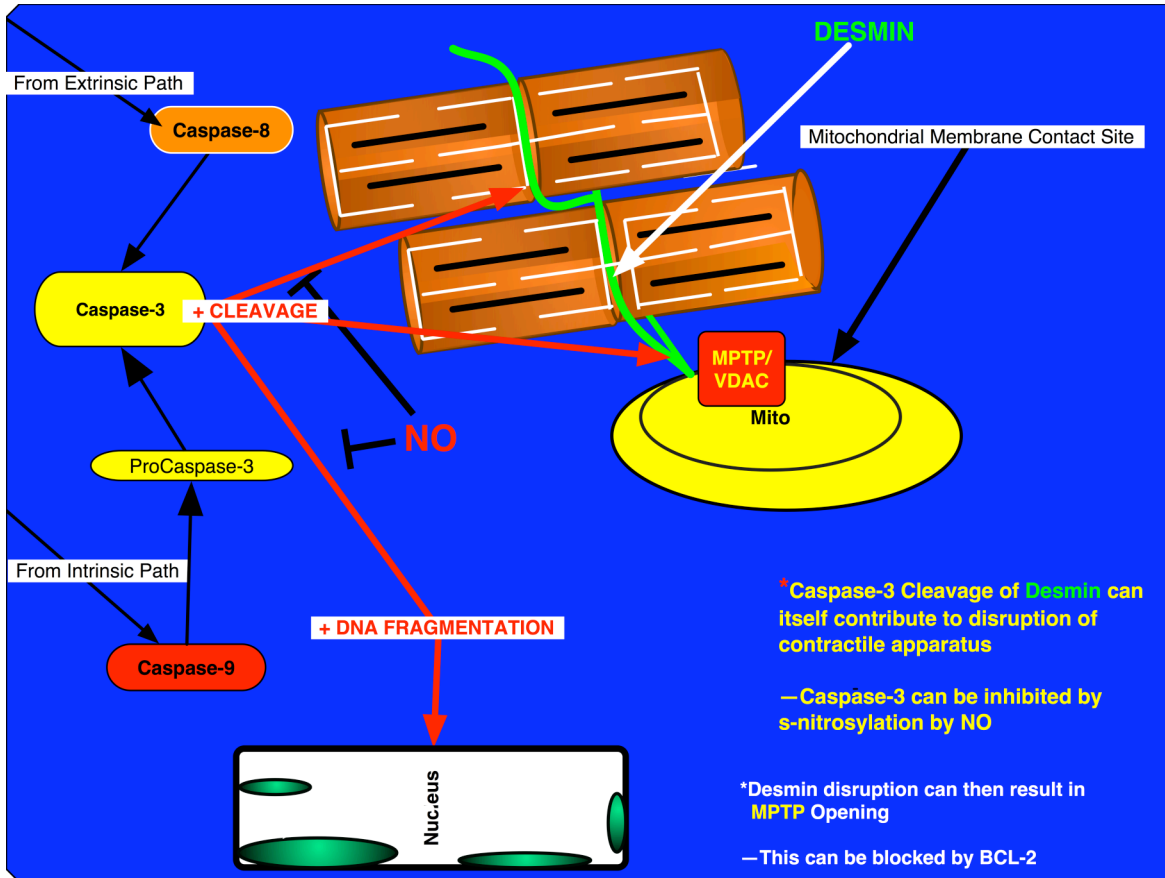


Figure 6B. Role of desmin in myofibrillar integrity. The mechanism of caspase-3 cleavage of desmin is depicted. Caspase-3 cleavage of desmin results in disruption of normal contractile function and propagation of apoptosis via its interaction with the mitochondria at the site of the MPTP (mitochondrial contact sites).

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MECHANISMS FOR PREVENTION OF DOXORUBICIN-INDUCED CARDIOTOXICITY

Free Radical Scavengers & Iron Chelators

Most studies focusing on the development of pharmaceuticals aimed at preventing doxorubicin cardiotoxicity have targeted mechanisms involved in production of free radicals. Despite some success in pre-clinical experiments, many of these agents have significant clinical disadvantages. For example, probucol, a lipid-lowering antioxidant, confers significant protection against doxorubicin-induced cardiotoxicity [56]. However, concerns about its high-density lipoprotein lowering properties and its potential to cause QT-interval prolongation discourage its application in cancer patients [57]. The cytoprotective drug amifostine is less potent than dexrazoxane (Zinecard), an iron chelator, and it does not prevent the mortality and weight loss caused by doxorubicin in spontaneously hypertensive rats [57,58]. In fact, it has also been shown to have some benefit on prevention of acute doxorubicin cardiotoxicity but not on chronic cardiotoxicity [59]. Finally, dexrazoxane, the only cardioprotective drug currently available clinically, only reduces 50% of doxorubicin-related cardiac complications [60]. Moreover, it interferes with the antitumor activity of anthracycline antibiotics and potentiates the hematotoxicity of doxorubicin [60].

Other potential pharmacological agents are free radical scavengers such as melatonin and 5-hydroxymethylrutoside. However, these agents have limitations in respect to dose and frequency and concentration. Furthermore, clinical investigations are necessary to prove their efficacy and safety in humans. [52,58]

Type-5A Phosphodiesterase Inhibition

Type-5 phosphodiesterase enzyme inhibitors are a class of drugs currently used clinically to treat erectile dysfunction and pulmonary hypertension [71]. Because of the mechanism of action, these agents show promise for potential targeting of cellular mechanisms that promote doxorubicin-generated free radicals.

Phosphodiesterase enzymes convert the intracellular second messengers cyclic AMP and cyclic GMP to the corresponding nucleotides AMP and GMP. There are now 11 phosphodiesterase families, many of which exist as splice variants. The cAMP-specific enzymes include phosphodiesterase 4 (PDE4), -7 and -8. The cGMP-specific PDE's are PDE5, -6 and -9, whereas PDE1, -2, -3, -10 and -11 use both cyclic nucleotides [35]. Most known PDE5 inhibitors compete with the substrate cGMP for binding to the protein at the catalytic site. Although cGMP binding to the catalytic site stimulates cyclic-nucleotide binding to the allosteric sites, inhibitors do not elicit the same property, and Ser92 phosphorylation has no effect on inhibitor binding. PDE5 is the primary cGMP-hydrolyzing activity in human corpus cavernosum tissue. [60]

Vasodilatory Effects of cGMP

Intense research in vascular smooth muscle physiology beginning more than two decades ago led to the discovery of sildenafil citrate, the first synthetic PDE-5 inhibitor for the treatment of erectile dysfunction in men. Initial clinical studies using sildenafil focused on its efficacy in treating systemic hypertension and coronary angina. The hypothesis behind these early investigations stemmed from the theory that elevating cGMP levels via PDE-5 inhibition would result in systemic arterial vasodilatation. Although the results from early studies using sildenafil for the treatment of coronary angina were disappointing, the side-effect of penile erection, reported by many patients enrolled in this trial, inspired an exciting new area of research leading to advancement in the treatment of patients with erectile dysfunction [61,62].

Erection or tumescence is a neurovascular reflex mediated by smooth muscle relaxation/contraction in cavernosal tissue [63]. Cavernosal smooth muscle cells are normally in a “contracted state”, mediated via α -adrenergic neural stimuli with subsequent phosphorylation of Ca^{+2} /calmodulin-dependent myosin light chain kinase. In contrast, tumescence is stimulated by parasympathetic CNS output via neural release of acetylcholine. Consequently, NO is released from non-adrenergic, non-cholinergic cavernosal nerves subsequently activating soluble guanylyl cyclase, the enzyme that converts GTP to cGMP. The cyclic nucleotide then stimulates protein kinase G (PKG), which initiates a protein phosphorylation cascade. This results in a decrease in

intracellular levels of calcium ions, leading ultimately to dilation of the arteries that bring blood to the penis and compression of the spongy corpus-cavernosum. This compression contracts veins, reduces outflow of blood, and increases intracavernosal pressure, ultimately resulting in tumescence. A PDE-5 inhibitor will retard enzymatic hydrolysis of cGMP to 5'GMP in the human corpus cavernosum, leading to the same outcome. [63]

Role of Nitric Oxide (NO)

Nitric Oxide is well recognized as a key mediator in cell signaling processes. It is produced from L-arginine through chemical reaction catalyzed by at least four major isoforms of NOS, *i.e.* neuronal (nNOS), inducible (iNOS), endothelial (eNOS), and mitochondrial NOS (mtNOS). In cellular studies, treatment with doxorubicin at 5 μ M for 24 h increased the amount of iNOS protein without affecting either eNOS or nNOS expression in H9C2 rat cardiac cells [105] The reduction in cardiac contractility in animals given doxorubicin at 20 mg/kg i.p., was associated with a 3-4 fold increase in the immunopositivity of myocardial iNOS (33 \pm 18 vs. 9 \pm 2%) and 3-nitrotyrosine formation (56 \pm 24 vs. 0.3 \pm 0.4%) compared with the control group [106]. Pacher *et al* [107] reported a decrease in ejection fraction and cardiac output which coincided with the increase in cardiac nitrotyrosine synthesis in mice 5 days after the administration of doxorubicin at 25 mg/kg (IP). In these studies, iNOS ($^{-/-}$) mice treated with doxorubicin displayed improved cardiac function versus iNOS ($^{+/+}$) litters. These findings suggest that the production of peroxynitrite (ONOO $^{-}$) by anthracyclines via iNOS was the critical mechanism of drug-

induced cell injury in myocytes. In addition, it is suggested that elevated eNOS expression and subsequent NO synthesis in bovine aortic endothelial cells was a result of doxorubicin-induced hydrogen peroxide production.

Pharmacology of Sildenafil

Sildenafil citrate (Viagra®) is an orally administered drug used for the treatment of erectile dysfunction in men. It is a potent type-5 phosphodiesterase inhibitor, which blocks the breakdown of cGMP. In humans, sexual arousal or stimulation results in release of NO from penile vascular tissue increasing cGMP production with a subsequent cascade of events resulting in: (1) vascular smooth muscle relaxation in the corpus cavernosa, (2) engorgement of the lacunae, (3) compression of veins involved in draining blood from cavernosal tissue, and ultimately (4) penile rigidity. [63]

Pharmacokinetics of Sildenafil

After oral administration, sildenafil reaches peak plasma concentration within approximately sixty minutes. Oral administration results in a bioavailability of approximately 40% with 96% protein-bound. The half-life of sildenafil ranges between three to five hours with clinical effects lasting 12 hours on average.

Sildenafil is metabolized by the liver and primarily excreted in the feces with a small amount excreted in urine. The primary hepatic metabolism occurs by microsomal P450 enzymes (isoenzyme 3A4 and to a lesser extent, 2C9). Dose adjustment is warranted in patients with hepatic disease, or who take any potent inhibitors of the P450 3A4 isoenzyme including: (1) ketoconazole, (2) cimetidine, or (3) erythromycin to name a few.

Furthermore, because sildenafil is protein bound, caution should be used in patients taking medicine that is also highly protein bound such as digoxin, amiodarone, or warfarin.

According to Shabsigh [61], the side-effect profile of sildenafil in patients with hepatic impairment or who currently take inhibitors of P450 3A4 isoenzymes did not differ from the general population despite significantly elevated plasma concentrations.

In addition to studies in humans, sildenafil has been shown to enhance nitric oxide (NO)-driven cGMP accumulation in the corpus cavernosum of rabbits without affecting cAMP formation. In the absence of NO drive, sildenafil had no functional effect on rabbit isolated corpus cavernosum but potentiated the relaxant effects of NO on these tissues.

Furthermore, it has been shown that sildenafil causes mild to moderate decreases in systolic and diastolic pressure because of the inhibition of PDE-5 in smooth muscle cells in

the vascular bed. Prior studies in our laboratory confirmed the mild effect of sildenafil on systemic hemodynamics in rabbits. [64]

SPECIFIC AIMS

In the present study, a series of novel investigations designed to examine the effect of PDE-5 inhibitors in preventing doxorubicin-induced cardiotoxicity in mice was proposed. Preliminary studies conducted in our laboratory by Fisher *et al* suggest the viability of our major hypothesis that PDE-5 inhibitors may confer significant protection against doxorubicin-induced cardiomyopathy, when administered in a prophylactic manner. The purpose of this application is to show the effect of sildenafil on doxorubicin-induced cardiomyocyte apoptosis and to attain a better understanding of the potential signaling pathways in the heart that lead to cardioprotection in a chronic model of doxorubicin-induced cardiotoxicity.

Accordingly, the main goals of the present study were:

1. ***To determine whether suppression of PDE-5 with the novel inhibitor, sildenafil, attenuates doxorubicin-induced cardiotoxicity and contractile dysfunction via inhibition of cardiomyocyte apoptosis in the heart.*** The effect of clinically relevant doses of sildenafil on doxorubicin-induced cardiomyocyte apoptosis both *in vivo* and *in vitro*, myocardial contractile dysfunction, and of Bcl-2 protein expression was investigated. It was further that PDE-5 inhibition would inhibit doxorubicin-induced apoptosis via opening of mitochondrial K_{ATP} channels; thereby preventing the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) and preventing opening of the mitochondrial permeability transition pore (MPTP).

2. To investigate if PDE-5 suppression will prevent myofibrillar disarray commonly associated with cardiomyopathies. More specifically, the effect of prophylactic treatment with sildenafil, at clinically relevant doses, on doxorubicin-induced disruption of desmin in cardiomyocytes was examined. Moreover, it was proposed that PDE-5 inhibition would attenuate the disruption of the normal desmin network in the heart, which is vital in maintenance of myofibrillar integrity and myocardial contractility. Sildenafil-induced opening of mitochondrial K_{ATP} channels, preservation of the mitochondrial membrane potential, prevention of the MPTP formation, and subsequent caspase-3 activation followed by DNA damage consistent with apoptosis will demonstrate this. The initial hypothesis suggested that the latter would be prevented by increased Bcl-2 protein expression ultimately inhibiting the translocation of Bax/Bad to the mitochondrial contact site where the MPTP is located.

3. To evaluate the effect of PDE-5 inhibition in preventing chronic doxorubicin-induced cardiotoxicity without affecting doxorubicin's antineoplastic activity. To test this hypothesis, the viability of PC-3 prostate cancer cells *in vitro* after treatment with sildenafil plus doxorubicin, sildenafil alone, and with doxorubicin alone was studied. The initial hypothesis was that the efficacy of doxorubicin in killing PC-3 cells, at a clinically relevant dose (1 μ M), will not adversely affect prophylactic treatment with sildenafil.

The aforementioned studies are the first to demonstrate the protective effect of PDE-5 inhibition in doxorubicin-induced cardiotoxicity in the heart at both the cellular and sub-cellular level. Moreover, this is the first study to demonstrate the prevention of doxorubicin-induced cardiomyopathy coupled with the inability of sildenafil, at clinically relevant doses, in adversely affecting the antineoplastic activity of doxorubicin.

Additionally, these studies provide relevant data setting the foundation for clinical trials in humans receiving doxorubicin chemotherapy for hematological and/or oncological neoplasms.

Furthermore, these studies provide novel insights into expanding the utility of PDE-5 inhibitors their current use in the treatment of erectile dysfunction (ED) in men.

METHODS (*In Vivo* Model)

All animal studies were performed in accordance with the guidelines of the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals”, the American Physiological Society and the Virginia Commonwealth University.

Adult male ICR mice (~33 grams each) were randomized to one of four groups. Group 1 saline only and served as a control. Group 2 received sildenafil (0.7 mg/kg i.p.) one hour prior to the administration of an equivolume of saline in place of doxorubicin. Group 3 received an equivolume of saline one hour prior to doxorubicin (5 mg/kg i.p.). Group 4 received sildenafil (0.7 mg/kg i.p.) one hour prior to administration of doxorubicin (5 mg/kg i.p., Sigma Chemicals, St. Louis, MO). Animals were housed in a 12:12 hour light/dark cycle, temperature-controlled room. Diet consisted of normal mouse chow (Harlan, Indianapolis, IN) and water *ad libitum*.

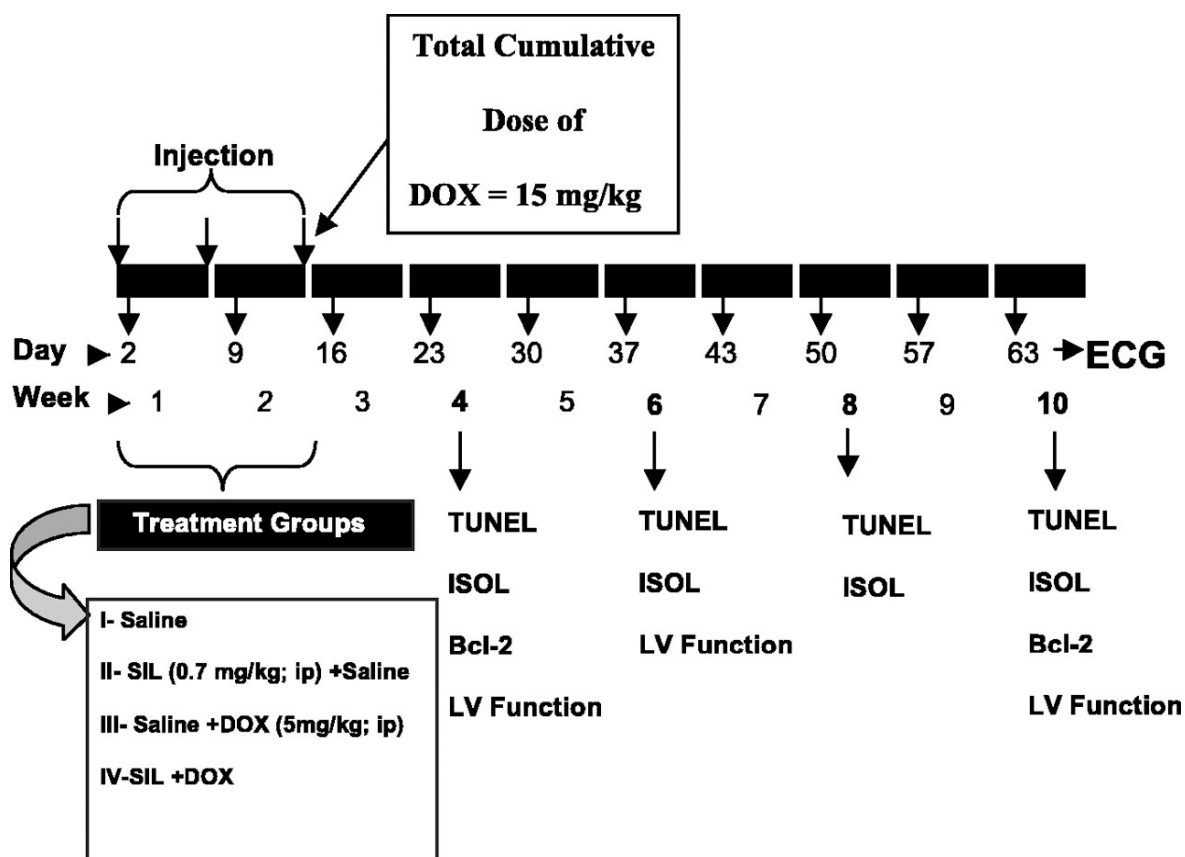


Figure 7. Experimental protocol. Treatment was administered on days 0, 7, and 14. Groups II and IV were administered sildenafil 1 hour before either saline or doxorubicin, respectively. Group III received saline 1 hour before doxorubicin. ECG (lead II) was performed 2 days after each treatment and 1 week thereafter for 8 weeks. LV indicates left ventricular.

Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis was evaluated via the terminal dUTP nick-end labeling method (TUNEL) using the ApopTag® In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The quantification of apoptosis or Apoptotic Index (AI) was determined by counting TUNEL (+) myocyte nuclei from ten random fields per section and expressed as a percentage of total myocyte nuclei. Because the TUNEL assay can detect DNA damage from non-apoptotic stimuli, complementary analysis was conducted using the ApopTag® In Situ Oligo Ligation (ISOL) technique (Chemicon, Temecula, CA). The ISOL method uses T4 DNA ligase to specifically ligate DNAase Type I ends to biotin-labeled hairpin oligonucleotides. The localization of oligonucleotides (labeled) is restricted to regions of chromatin characteristic for apoptosis. The ISOL method does not label nicks, gaps, or ssDNA, 3'recessed ends or 3'overhanging ends longer than one dT base. These techniques have been used together for appropriate labeling of DNA characteristic of apoptosis [56,74-75].

Desmin Immunofluorescence

Distribution of desmin, an intermediate filament important in cellular integrity and myocyte contraction, was analyzed in frozen sections (5 μm) in animals from each of the experimental groups. After appropriate fixation in 4% paraformaldehyde, samples were incubated for one hour with 10% normal goat serum. Next, primary goat anti-desmin antibody (Santa Cruz Biotech, Santa Cruz, CA), diluted 1:50 was applied to each slide and incubated for one hour at room temperature. After several washes, Alexa Fluor 488 donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR), diluted 1:400 was applied to each slide. Samples were incubated for one hour at room temperature. After several washes in 1X PBS, Prolong Gold® Antifade (Molecular Probes, Eugene, OR) was applied followed by mounting with a glass coverslip. Visualization of desmin distribution was accomplished using a Nikon epifluorescent microscope with a 60X oil objective and a FITC filter cube. Image acquisition was obtained using MicroPublisher® 3.3 CCD camera with Q-Capture® Professional image analysis software (QImaging, Burnaby, B.C. Canada).

Analysis of BCL-2 Expression

Mice whole heart proteins were extracted with RIPA buffer (Upstate™, Charlottesville, VA) and proteins were separated on SDS-PAGE and transferred onto 12% nitrocellulose membranes (Bio-Rad, Hercules, CA). Primary antibodies against Bcl-2 (Molecular weight: 28kDa) were followed by secondary rabbit IgG-conjugated horseradish peroxidase antibody according to manufacturer's instructions (Santa Cruz Biotech, Santa Cruz, CA). Antibodies against B-actin (Molecular weight: 39 kDa) were used for determination of protein loading (Santa Cruz Biotech, Santa Cruz, CA). Densitometry was performed using BioQuant® software (BioQuant, Nashville, TN).

Hemodynamics

Animals (n = 6/group) were sacrificed at 2,4 and 8 weeks after the last day of treatment (day 14). After adequate anesthetization using pentobarbital (100 mg/kg i.p), the heart was excised and immediately placed in cold saline (4°C). The heart was then cannulated via the aorta and retrogradely perfused at a constant perfusion pressure equivalent to 100 cmH₂O. All hearts were perfused with modified Krebs-Henseleit (K-H) buffer at 37°C, containing (in mM) 118.5 NaCl, 25.0 NaHCO₃, 3.2 KCl, 1.19 MgSO₄, 1.25 CaCl₂, 1.2 KH₂PO₄, and 11 glucose and was bubbled with 95% O₂-5% CO₂ mixture. The pH was maintained at 7.4. After the heart began spontaneous contraction, a small incision was made in the left atrium. A latex balloon connected to a pressure transducer via polyethylene cannula was inserted through the left atrium and mitral valve into the left

ventricle. The balloon was filled with enough water to increase end-diastolic pressure (EDP) to approximately 10 mmHg. Left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDP), and heart rate (HR) were recorded (Chart 4.0, AD Instruments, Colorado Springs, CO). LVDP was calculated by subtracting EDP from the LVSP. Rate pressure product (RPP), an index of myocardial oxygen demand and workload, was calculated by multiplying LVDP with HR. Coronary flow reserve was measured by timed collection of coronary effluent. Care was taken to maintain temperature of the heart at 37°C.

Electrocardiography

A separate set of four groups (n=6/group) was utilized for the assessment of electrocardiographic (ECG) changes indicative of doxorubicin cardiotoxicity [76,77]. More specifically, this technique will further confirm the presence of doxorubicin-induced cardiotoxicity throughout the course of the investigation (10 weeks) as initially proposed by Fisher. All animals were weighed at baseline and every 7-10 days for 8 weeks prior to the ECG analysis. Animals were anesthetized using pentobarbital (50 mg/kg IP) followed by insertion of electrodes in the left front limb, right front limb, left hind limb, and right hind limb. The electrodes were connected to an electrocardiography module (LDS Life Science, Valley View, CA) and data was recorded for 2-3 minutes per animal. The ST-interval was measured in five consecutive complexes using Ponemah® physiology software (LDS Life Science, Valley View, CA). ST-interval duration was measured at

baseline, 48-72 hours after each dose of doxorubicin (Days 0, 7, and $14 \pm 2-3$ days) and every 7-10 days thereafter until 8-weeks was attained.

METHODS (*In Vitro Model*)

Isolation of Adult Cardiomyocytes

Adult male outbred ICR mice (Harlan, Indianapolis, IN) were used in isolation of ventricular myocytes. Ventricular myocytes were isolated using an enzymatic technique. Briefly, mice (n = 3/experiment) were anesthetized with pentobarbital (100 mg/kg IP) and the heart was quickly removed from the chest. Within 3 minutes, the aortic opening was cannulated and placed onto a Langendorff perfusion system. Next, the heart was retrogradely perfused (37°C) at a constant pressure of 55 mmHg for 5 minutes with a Ca²⁺-free bicarbonate-based buffer containing (in mM): 120 NaCl, 20 NaHCO₃, 5.4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5.6 glucose, 10 2,3-butanedione monoxime, and 5 taurine, which was gassed with 95% O₂-5% CO₂ mixture. The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.5 mg/ml each) and protease type XIV (0.02 mg/ml) to the perfusion buffer and continued for ~15 minutes. Following initial enzymatic digestion, 50 μM Ca²⁺ was added to the enzyme solution and the heart was perfused for an additional 10-15 minutes. Digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating cell dissociation. The cell pellet was resuspended for a 3-step Ca²⁺ restoration procedure (i.e. 125, 250, 500 μM Ca²⁺). Freshly isolated cardiomyocytes were suspended in minimal essential medium (Sigma, Catalogue # M1018, pH 7.35-7.45) containing 1.2 mM Ca²⁺, 12 mM NaHCO₃, 2.5% fetal bovine serum, and 1% penicillin-streptomycin. Cells were plated onto 2-chamber slides,

which were pre-coated with 20 $\mu\text{g/ml}$ mouse laminin in PBS + 1% penicillin-streptomycin for 1 hour. Cardiomyocytes were cultured in the presence of 5% CO₂ for 1 hour in a humidified incubator at 37°C. Myocyte cultures were randomly assigned to one of 12 treatments: (1) control; (2) sildenafil (1 μM); (3) doxorubicin (1 μM); (4) sildenafil (1 μM) one hour prior to doxorubicin (1 μM); (5) 5-Hydroxydecanoate (5-HD), a mitoK_{ATP} channel blocker (100 μM); (6) 5-HD (100 μM) one hour prior to doxorubicin (1 μM); (7) 5-HD (100 μM)+ sildenafil (1 μM); (8) 5-HD (100 μM)+sildenafil (1 μM) one hour prior to doxorubicin (1 μM); (9) NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor (100 μM); (10) L-NAME (100 μM) one hour prior to doxorubicin (1 μM); (11) L-NAME (100 μM)+ sildenafil (1 μM); or (12) L-NAME (100 μM)+ sildenafil (1 μM) one hour prior to doxorubicin (1 μM). Slides were incubated for 18 hours followed by several washes in 1X PBS prior to analysis.

Cardiomyocyte Apoptosis (*In Vitro*)

Cardiomyocyte apoptosis was evaluated via the terminal dUTP nick-end labeling method (TUNEL) using the ApoAlert™ DNA Fragmentation Assay Kit (BD Biosciences, Palo Alto, CA) according to manufacturer's instructions. Equilibration buffer was used in place of working TdT reagent for use as a negative control. DNAase-I was applied and used as a positive control. Analysis was performed using a Nikon epifluorescent microscope with 20x objective. A FITC filter cube was utilized in detection of apoptotic myocyte nuclei. An ultraviolet filter cube was utilized in detection of DAPI-stained myocyte nuclei.

Apoptotic index (AI) was determined from counting TUNEL-positive myocyte nuclei from ten separate fields per treatment and expressed as a percentage.

Active Caspase-3 Detection

Active Caspase-3 staining was determined using the CaspaTag™ In Situ Assay Kit (Chemicon, Temecula, CA) according to manufacturer's instructions. This assay is based on Fluorochrome Inhibitors of Caspases (FLICA). The inhibitor binds covalently to the active caspase. This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspases-3 and -7 (SR-DEVD-FMK), which emits a red fluorescence. The SR-DEVD-FMK probe enters each cell and covalently binds to reactive cysteine residue on the large subunit of the active caspase heterodimer, thereby inhibiting enzymatic activity. The bound labeled reagent is retained within the cell. The red fluorescent signal is a direct measure of active caspase-3 in the cell at the time the reagent was added. After application of CaspaTag™ reagent and Hoechst, cells were immediately examined using a Nikon epifluorescent microscope with rhodamine (Active Caspase-3) and ultraviolet (Hoechst) bandpass filters.

Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Loss of $\Delta\Psi_m$ was assessed using epifluorescent microscopy. Cultured adult mouse ventricular myocytes were stained with 5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1, Biocarta, San Diego, CA) after an 18 hour incubation. Cells were incubated with 2 $\mu\text{g/ml}$ JC-1 for 10 minutes at 37°C. After washing with 1xPBS, cells on chamber slides were scanned with a Nikon epifluorescent microscope using a 20x objective lens. Fluorescence was analyzed using a Texas Red-FITC filter cube. Red emission of the dye represented a potential-dependent aggregation in the mitochondria, reflecting $\Delta\Psi_m$. Green fluorescence represented the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. The ratio of mitochondrial aggregates (red) to the monomeric form of JC-1 (green) was analyzed using Q-Capture® Professional image analysis software (QImaging, Burnaby, B.C. Canada). Myocytes were counted from ten separate fields per group and expressed as a ratio of mitochondrial aggregates to the monomeric form of JC-1.

Cell Viability Assay

In order to determine if sildenafil affects the antitumor efficacy of doxorubicin, a highly effective and accurate technique, using PC-3 prostate cancer cell line was utilized. PC-3 prostate cancer cells (American Cell Culture, Manassas, VA), which are p53-deficient and susceptible to doxorubicin, were cultured in F12-K medium supplemented with 10% fetal bovine serum. Approximately 4,000 PC-3 cells/well were plated into one-half of a 96-well dish while the other half of the 96-well dish was incubated with 8,000 PC-3 cells/well and allowed to attach overnight. Lane 1 consisted of PC-3 cells plus culture media alone. Lane 2 was treated with doxorubicin (80 nM). Lane 3 was treated with doxorubicin (1 μ M). Lane 4 was treated with sildenafil (1 μ M) one-hour before doxorubicin (80 nM). Lane 5 was treated with sildenafil (1 μ M) one-hour before doxorubicin (1 μ M). Lane 6 was treated with sildenafil (1 μ M). Lane 7 was treated with sildenafil (10 μ M). Wells 1-3 & 7-9 of Lane 8 were treated with sildenafil (10 μ M) one-hour before doxorubicin (1 μ M). Wells 4-6 & 10-12 of Lane 8 were treated with PC-3 cells in culture media only. Cell viability or the number of surviving cells was measured 4 days after doxorubicin application with CellTiter-Blue™ Cell Viability Assay according to manufacturer instructions (Promega). CellTiter-Blue™ reagent (20ul/well) was added and incubated for one-hour before recording absorbance at 570nm and 600 nm using a VersaMax microplate reader with SoftMaxPro software (Molecular Devices). The average absorbance (600nm) values of the culture medium background was subtracted from all 570nm values of experimental wells. Next, the 570-600nm absorbance versus concentration of test compound was analyzed. The CellTiter-Blue™ Cell Viability Assay uses the indicator dye,

resazurin, to measure the metabolic activity of cells as an indicator of cell viability. Viable cells, for example, are capable of reducing resazurin to the highly fluorescent resafurin. Because non-viable cells cannot reduce resazurin to resafurin, the fluorescence or absorbance is negligible. The absorbance obtained from this assay is proportional to the number of viable cells.

Flow Cytometry

To confirm our results demonstrating the inability of sildenafil in affecting the antitumor efficacy of doxorubicin *in vitro* using PC-3 cancer cells, we subsequently measured cell viability using flow cytometry (Beckman Coulter Flow Cytometer; 488nm laser). Calibration for doxorubicin autofluorescence and propidium iodide was conducted before analysis. The experimental groups included: control, doxorubicin (1, 2 and 5 μM), sildenafil (1 and 2 μM), doxorubicin (1 μM)+ sildenafil (1 or 10 μM), doxorubicin (2 μM)+sildenafil (1 or 10 μM), and doxorubicin (5 μM)+sildenafil (10 μM). In this approach, instead of using a compound that is actively reduced by viable cells (resazurin), we utilized the impermeable nucleic acid dye, propidium iodide (Sigma-Aldrich), to detect the amount of non-viable cells. Since non-viable cells would have disruption of their nuclear membrane, it is expected that necrotic cells would actively stain with propidium iodide and be detected using flow cytometry. Controls containing doxorubicin only, doxorubicin + propidium iodide, propidium iodide + media, and media only were added as additional controls. Flow cytometric data are depicted in Appendix E.

Statistics

Data are presented as mean \pm SEM. Difference between groups was analyzed with unpaired t test or one-way ANOVA followed by Tukey-Kramer HSD post-hoc test (JMP, Version 5, SAS Institute Inc., Cary, NC). $P < 0.05$ was considered as statistically significant.

RESULTS (*In Vivo*)

Cardiomyocyte apoptosis

Prior studies have implicated cardiomyocyte apoptosis in the development of chronic cardiomyopathy induced by doxorubicin administration [75,76]. The following results indicate the powerful cardioprotection of sildenafil via mitigation of cardiomyocyte apoptosis in the experimental group receiving sildenafil. Data from both TUNEL and ISOL techniques demonstrated significant cardiomyocyte apoptosis in doxorubicin group compared to saline control at 2, 4, 6, and 8-weeks post-treatment ($P < 0.001$). Sildenafil attenuated doxorubicin -induced cardiomyocyte apoptosis when administered one hour before each of three separate treatments with doxorubicin (5 mg/kg IP; 15 mg/kg total cumulative dose). These results were similar to saline control animals [Figure 8 A-D].

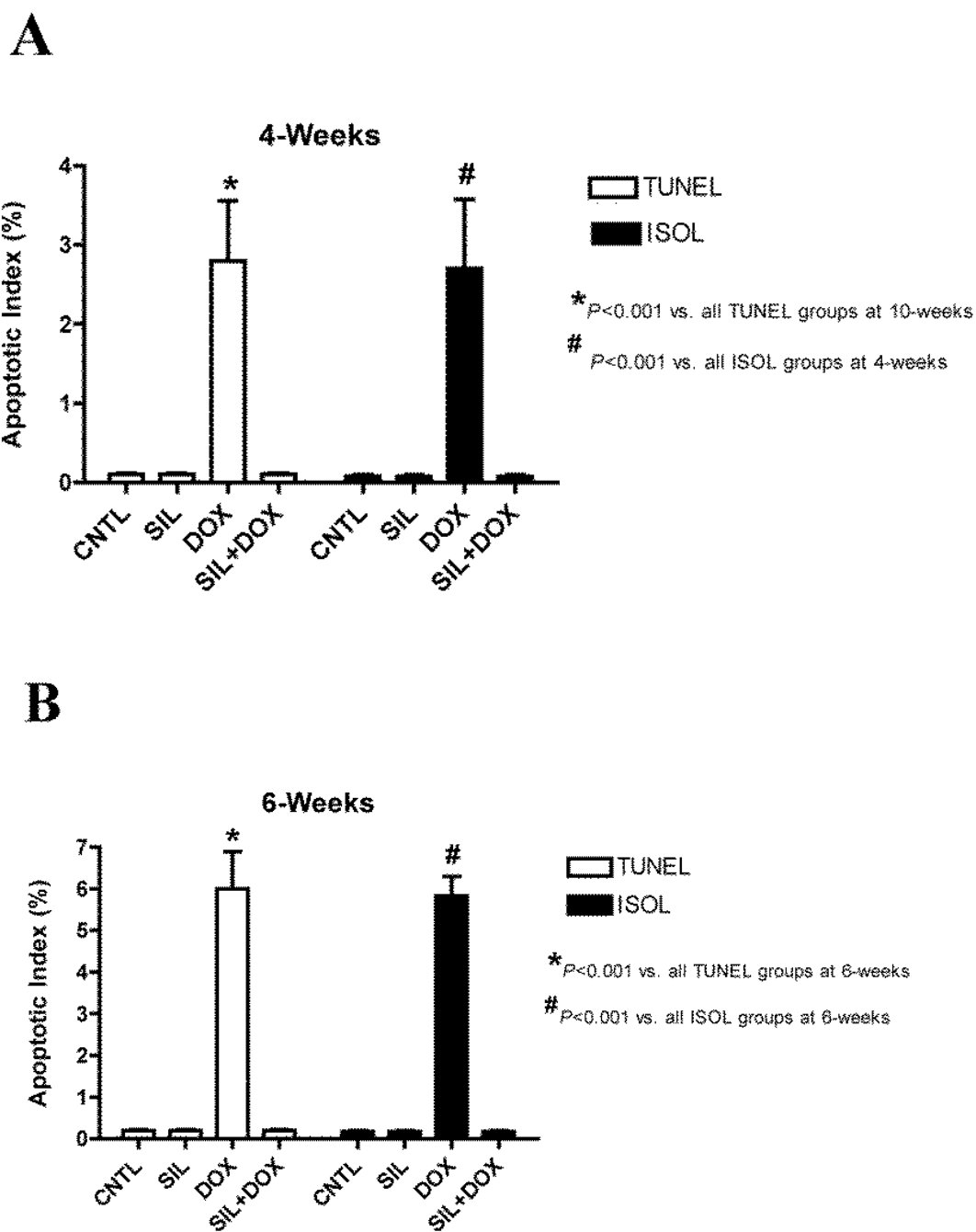


Figure 8A. Apoptotic Index using both TUNEL and ISOL techniques in experimental groups at (A) 4 and (B) 6 weeks post treatment.

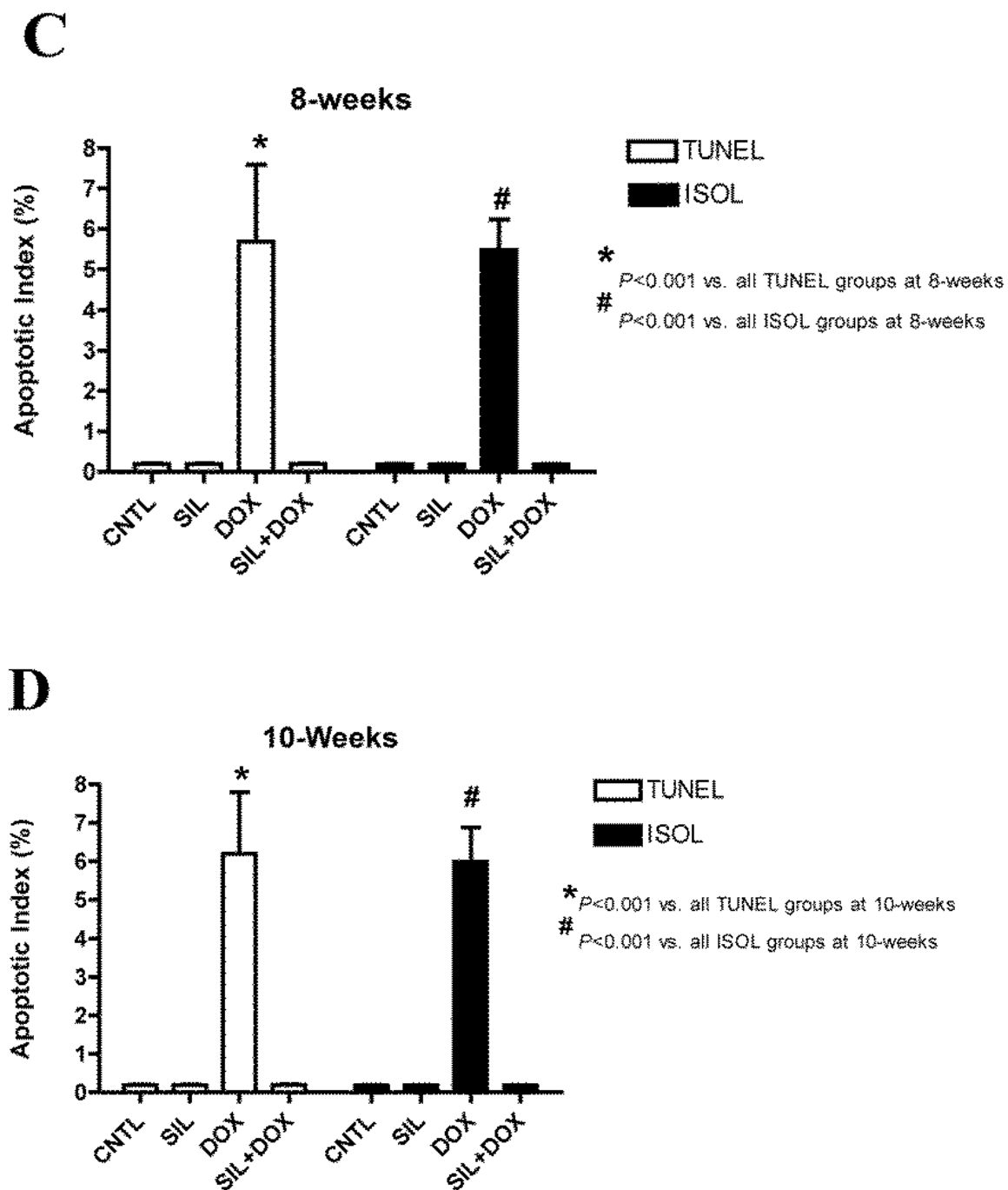


Figure 8B. Apoptotic Index using both TUNEL and ISOL techniques in experimental groups at (C) 8 and (D) 10 weeks.

Bcl-2 expression

The Bcl-2 family of proteins provides maintenance of the integrity of the outer mitochondrial membrane [78]. Pro-apoptotic Bcl-2 family of proteins including Bax, Bak, and t-Bid can integrate into the outer mitochondrial membrane in response to apoptotic stimuli inducing cytochrome-c release via mitochondrial transition permeability pore (MPTP) formation [79, Figure 10]. However, binding of Bcl-2 or Bcl-X_L inhibits membrane integration of pro-apoptotic Bcl-2 family of proteins and subsequent MPTP formation. [80, 81]. In the present study, a significant decrease in Bcl-2 expression was observed at 2-weeks and 8-weeks post treatment in the doxorubicin group compared to both sildenafil + doxorubicin and control group. Moreover, Bcl-2 expression was maintained when sildenafil was given one hour before doxorubicin treatment [Figure 9].

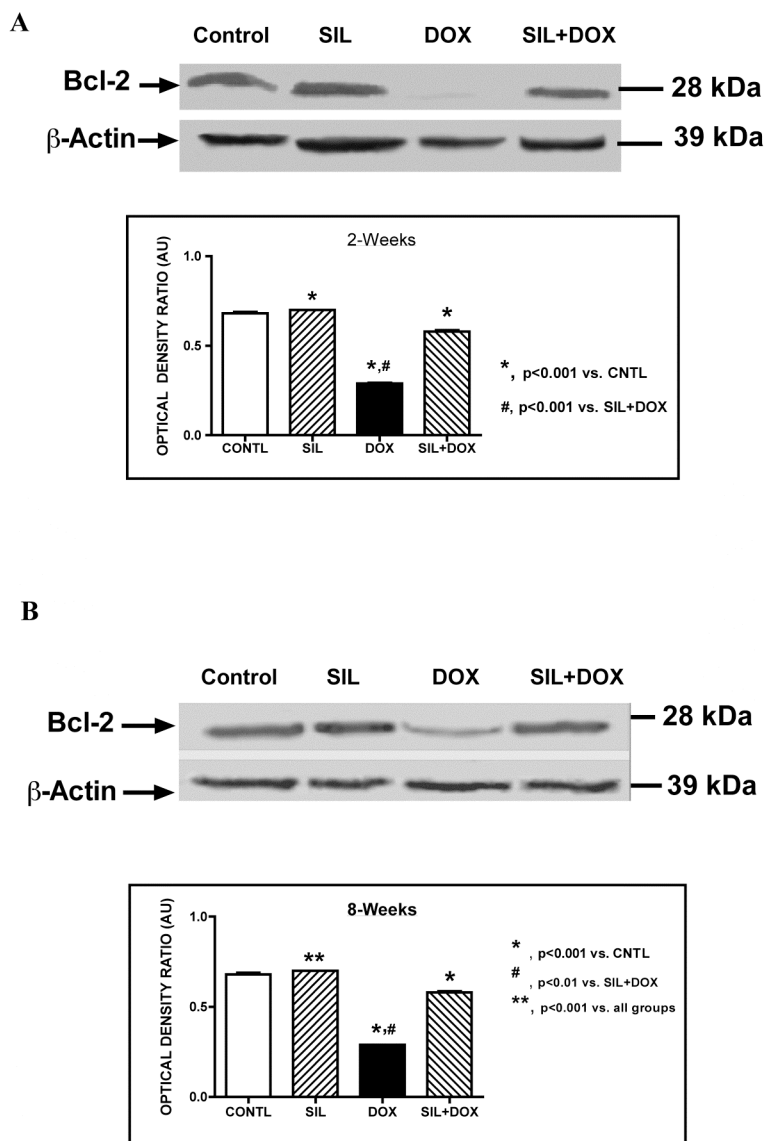


Figure 9. Western Blot of Bcl-2 Protein at 2 Weeks after Treatment (week 4), (A) and 8 weeks after treatment (week 10) (B). Bar Graph represents densitometric quantification from 3 individual hearts per group, which is normalized against the actin level for each sample. Data are Mean \pm SEM. Abbreviations are defined in Figure 7 legend.

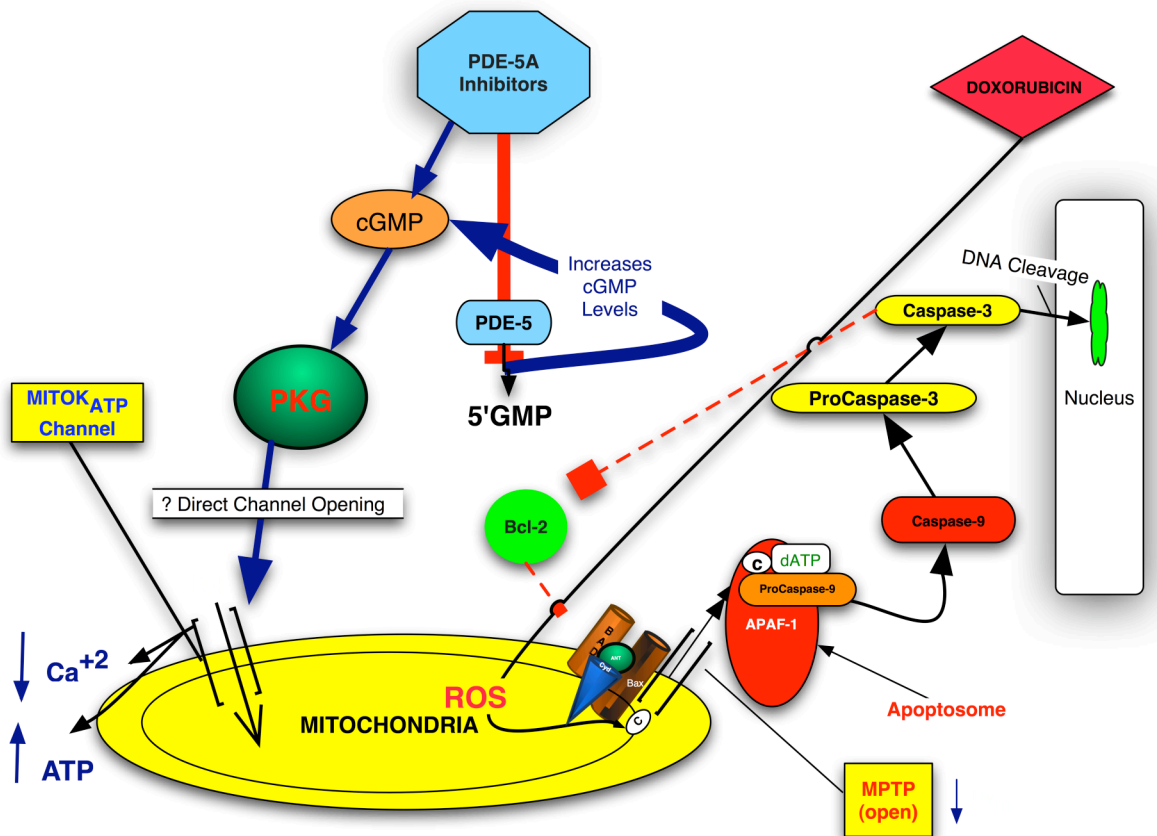


Figure 10. Illustration demonstrating the effect of PDE-5A inhibition on mitochondrial bioenergetics in the presence of doxorubicin. © 2005 Patrick W. Fisher DO, PhD.

Doxorubicin-induced myofibrillar disarray (desmin distribution)

At 8-weeks post treatment, doxorubicin group exhibited myofibrillar disarray as evidenced by abnormal desmin distribution, lack of Z-line integrity, and abnormal cytoplasmic desmin aggregation. In contrast, sildenafil+doxorubicin group displayed normal desmin distribution as evidenced by immunofluorescent staining throughout the entire cytoplasm with clear delineation of Z-lines. This was similar to both control and sildenafil +saline groups. [Figure 11]

Furthermore, PDE-5A expression was significantly reduced in the doxorubicin-only group at 8 weeks post treatment. This reduced expression and lack of localization to the z-line was most marked in the area of the cardiomyocyte where desmin disruption was evident [Appendix B, C].

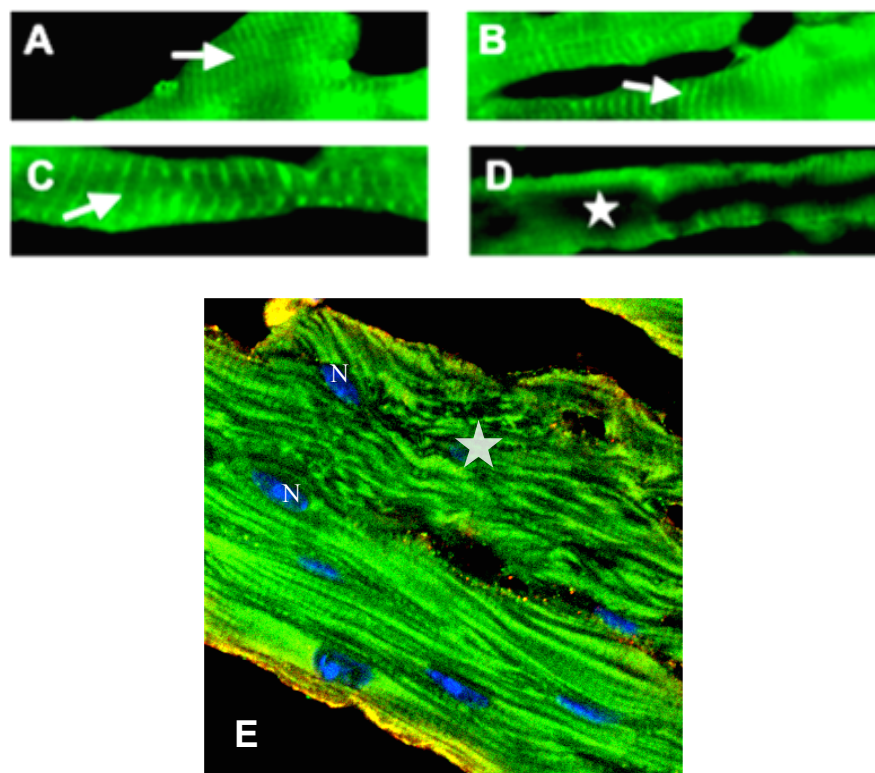


Figure 11. Immunofluorescent staining for desmin (green) in cryo-sections from mice in the saline control (A), sildenafil (B), sildenafil+doxorubicin (C), and doxorubicin (D & E) groups at 8 weeks after treatment. In control, sildenafil, and sildenafil+doxorubicin groups (A, B, C), desmin staining is present throughout the entire cytoplasm and is observed at the Z-lines demonstrated as green striations (arrowheads). In the doxorubicin-treated group (D, E), obvious disruption of the desmin network is present, with loss of Z-line localization. Areas of decreased uptake of anti-desmin antibody are apparent (star). Nucleus (N). Magnification X600; E, image acquired with a Zeiss LSM 510 Confocal Microscope (Figure E: © 2005 Patrick W. Fisher, DO, PhD)

Electrocardiography (ECG)

Prior studies in mice demonstrated a strong correlation between ST-interval duration and doxorubicin-induced cardiotoxicity [76,77]. In contrast to ECG recordings in humans, the ECG (Lead II) in mice does not contain an ST-segment. The T-wave immediately follows the QRS complex [76,77]. Prolongation of the ST-interval in doxorubicin-treated mice is secondary to an increase in action potential duration (APD) [77]. Le Marc et al [82] observed an increase in APD in Purkinje fibers after incubation with doxorubicin. Furthermore, in isolated cardiomyocytes exposed to doxorubicin, Jabr et al [83] observed APD prolongation resulting from doxorubicin-generated ROS. In experimental groups receiving doxorubicin, a significant progressive increase in ST-interval was observed at all time points compared to baseline [Figure 12]. Moreover, the most marked increase in ST-interval occurred between week 4 and week 8. Furthermore, ECG's of the control and sildenafil+ doxorubicin group did not change during the course of the study. Sildenafil significantly protected against ST-interval prolongation throughout the study period.

[Figure 12]

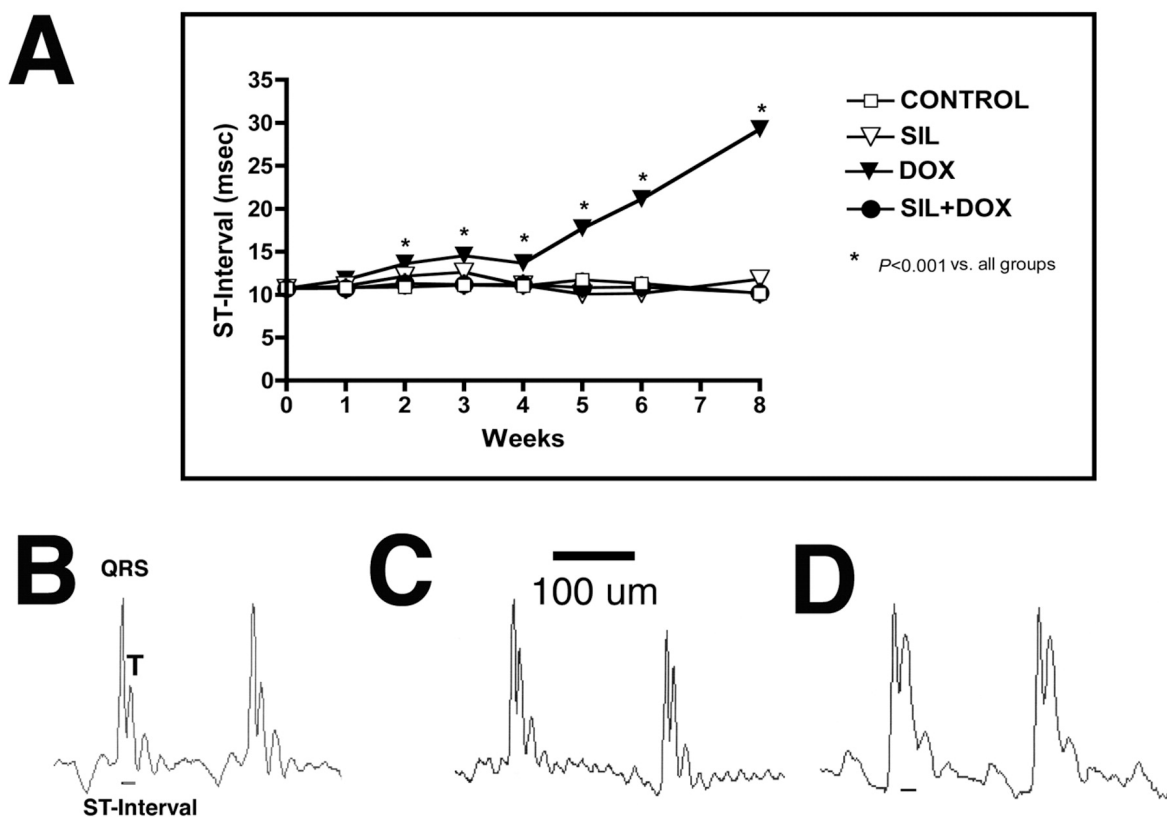


Figure 12. Electrocardiographical Analysis of ST-Prolongation in mice (lead II). Effect of sildenafil on ST-interval prolongation after doxorubicin treatment. ST interval was measured with the use of lead II. A, ST-interval prolongation over time. Representative tracings of control (B), sildenafil+doxorubicin (C), and doxorubicin only (D) are shown. Data are mean \pm SEM (n=6/group). Abbreviations are as defined in Figure 7 legend.

Effect of sildenafil on cardiac function in doxorubicin-treated animals

Our data shows a significant decline in LVDP in the saline+ doxorubicin group compared to control at 2 weeks post treatment (27% vs control; 24% vs sildenafil+ doxorubicin) [Table 1]. Decline in contractility as measured by rate pressure product (RPP) persisted through 8 weeks after treatment cessation in the saline+ doxorubicin group. Animals treated prophylactically with sildenafil before doxorubicin demonstrated RPP that remained unchanged from control over 8-week post-treatment period.

[Figure 13]

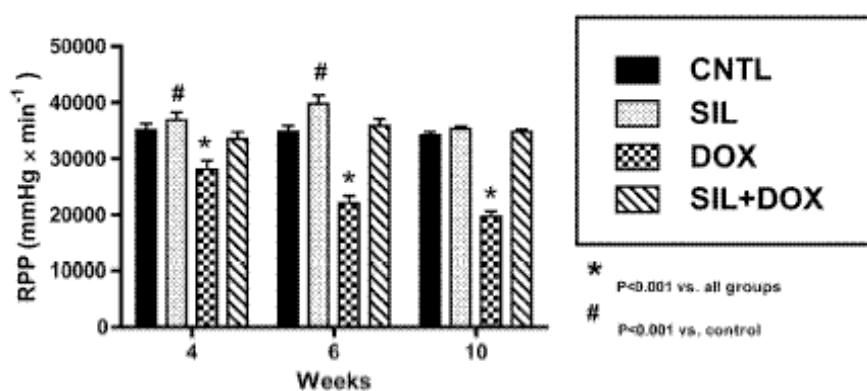


Figure 13. Bar graph representing rate pressure product in mouse hearts via Langendorff mode at 4, 6, and 10-weeks post-treatment. Data are mean \pm SEM ($n = 6$ /group/time point).

Table 1. Hemodynamic Indices

Group	Week 2			Week 4			Week 8		
	HR (bpm)	LVDP (mmHg)	RPP (mmHg•min ⁻¹)	HR (bpm)	LVDP (mmHg)	RPP (mmHg•min ⁻¹)	HR (bpm)	LVDP (mmHg)	RPP (mmHg•min ⁻¹)
Control	384±4	99±3	35116±1121	410±1	92±1	34851±1057	412±8	90±1	34159±651
SIL	393±5	101±3	36905±1342*	399±11	106.6±3	39762±1588*	412±4†	93±1	35296±437*
DOX	439±11*	72±4*	28099±1562*	374±13*	68±4*	22108±1248*†	365±15*†	62±3*†	19716±947*†
SIL+DOX	381±6**	95±3**	33471±1290**	381±11**	102±3	35861±1238**	409±2**	92±1	34827±413**

* $P < 0.05$ vs. control, ** $P < 0.05$ vs. DOX, † $P < 0.001$ vs. Week 2.

Abbreviations: HR= heart rate; LVDP= left ventricular developed pressure; RPP= rate pressure product; SIL= sildenafil+saline; SIL+DOX= sildenafil+doxorubicin; DOX= saline+doxorubicin

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RESULTS (*IN VITRO*)

Cardiomyocyte Apoptosis

Treatment of cardiomyocytes with doxorubicin (1 μ M) for 18 hours resulted in a significant increase in TUNEL (+) nuclei as indicated by Apoptotic Index (AI) of $0.61 \pm 0.09\%$, which was similar to both L-NAME+sildenafil+doxorubicin ($0.62 \pm 0.08\%$) and 5-HD+sildenafil+doxorubicin ($0.60 \pm 0.10\%$) groups. In contrast, a significant inhibition of apoptosis was evident in the sildenafil+doxorubicin ($0.078 \pm 0.031\%$) group, which was similar to control ($0.078 \pm 0.032\%$) [Figure 14]. Additionally, active caspase-3 expression increased in the doxorubicin, sildenafil+L-NAME+ doxorubicin, and 5HD+sildenafil+ doxorubicin groups compared to sildenafil+ doxorubicin and control groups. [Figure 15]

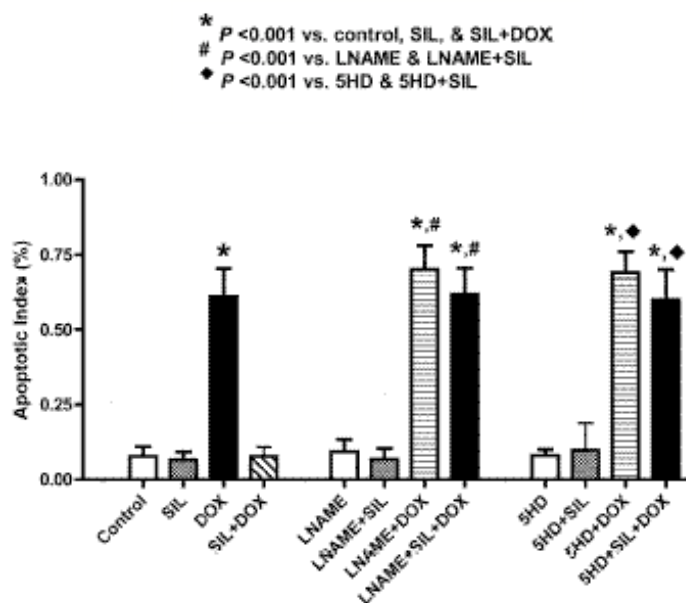


Figure 14. Apoptotic Index of Adult Ventricular Myocytes (TUNEL).

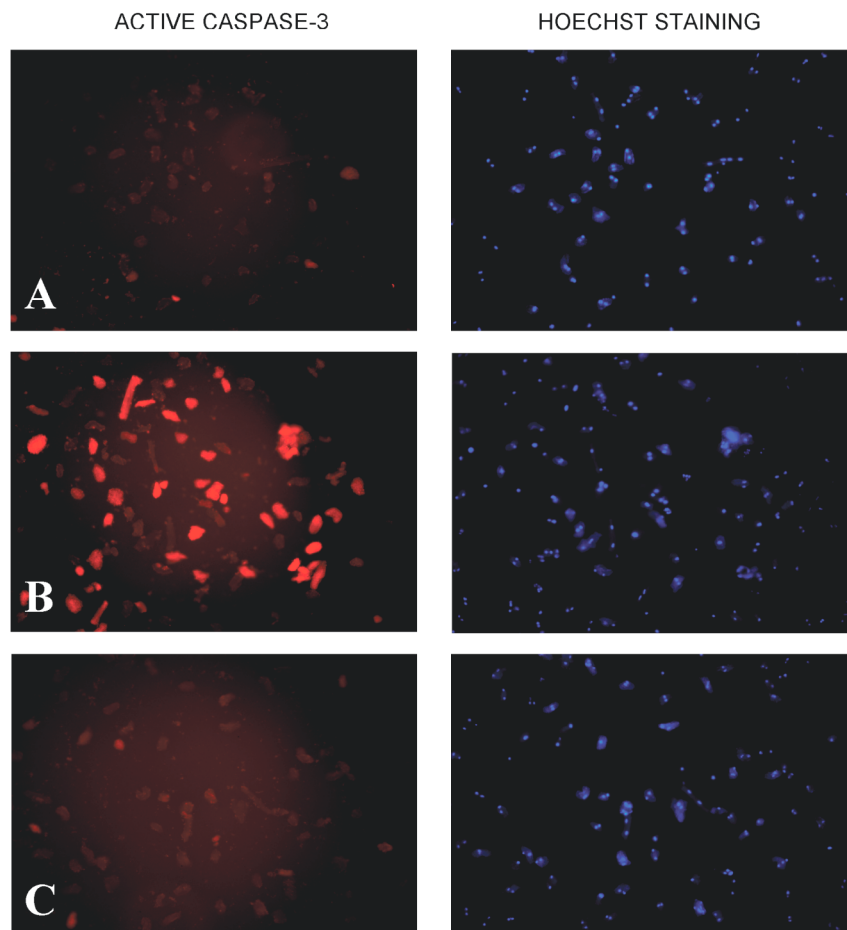


Figure 15A. Activated caspase-3 in adult mouse ventricular myocytes (red; left column) with myocyte nuclei stained with Hoechst (blue; right column). A, Control; B, doxorubicin; C, sildenafil plus doxorubicin; Magnification x200; n=3.

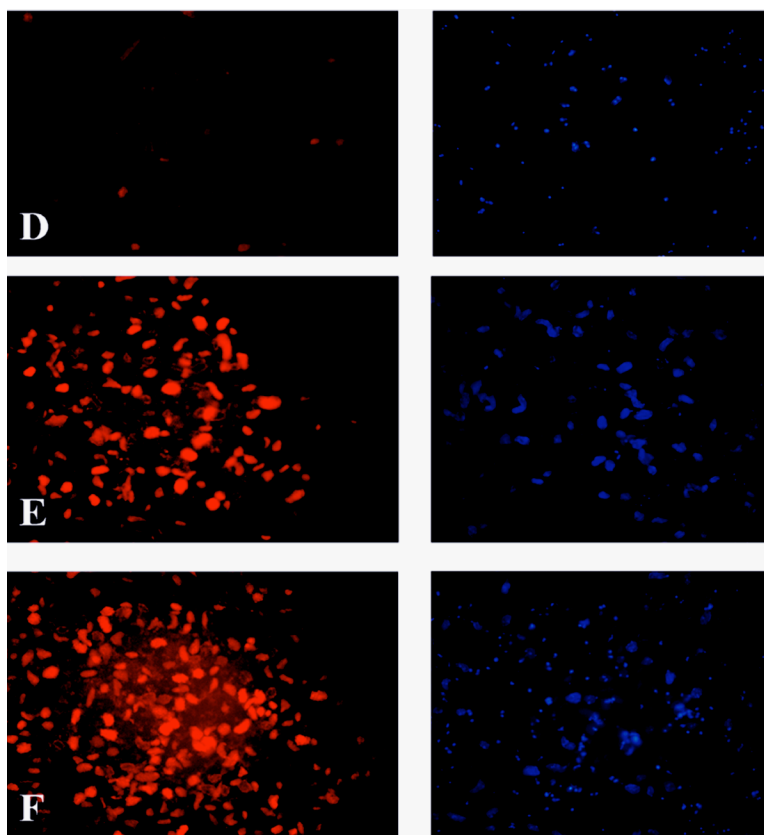


Figure 15B. Activated Caspase-3 in Adult Ventricular Cardiomyocytes

D, sildenafil (1 μM); E, L-NAME (100 μM) plus sildenafil plus doxorubicin; F, 5-HD (100 μM) plus sildenafil (1 μM) plus doxorubicin. Magnification x200; $n=3$.

Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Exposure of adult mouse ventricular myocytes to doxorubicin (1 μM) for 18 hours resulted in dissipation of $\Delta\Psi_m$ as illustrated via JC-1 immunofluorescent staining [Figure 16]. In contrast, myocytes pretreated with sildenafil (1 μM) before treatment with doxorubicin demonstrated preservation of the $\Delta\Psi_m$. The latter result was similar to both control and sildenafil+ doxorubicin groups [Figure 17]. However, dissipation of $\Delta\Psi_m$ occurred in both the L-NAME (100 μM)+sildenafil+ doxorubicin and 5-HD (100 μM)+sildenafil+ doxorubicin groups. [Figure 16]

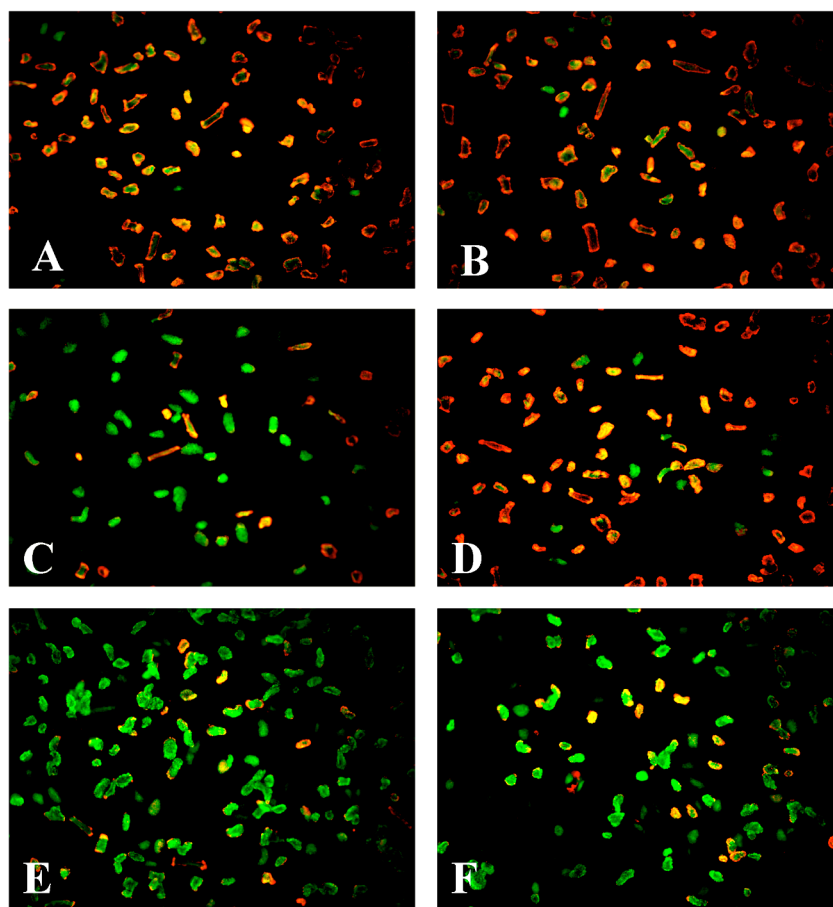


Figure 16. Effect of Sildenafil on $\Delta\Psi_m$ in Adult Ventricular Cardiomyocytes. Red Fluorescence represents the mitochondrial aggregate form of JC-1, indicating intact mitochondrial membrane potential. Green Fluorescence represents the monomeric form of JC-1, indicating dissipation of $\Delta\Psi_m$. A, Control; B, sildenafil (1 μM); C, doxorubicin (1 μM); D, sildenafil (1 μM) plus doxorubicin (1 μM); E, L-NAME (100 μM) plus sildenafil plus doxorubicin; F, 5-HD (100 μM) plus sildenafil plus doxorubicin.

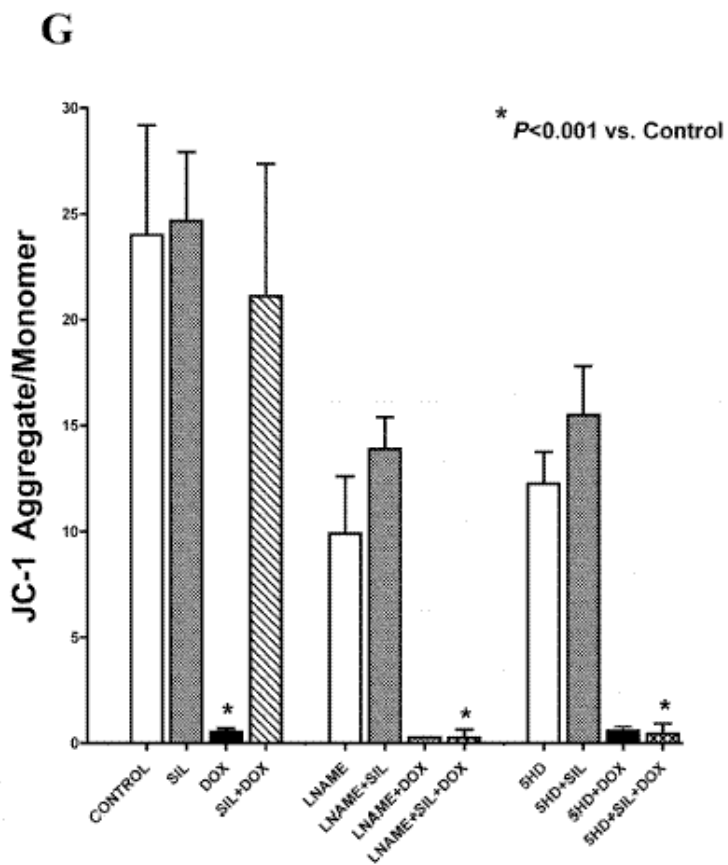


Figure 17. Ratio of Mitochondrial Aggregates to the Monomeric Form of JC-1. Data are expressed as Mean \pm SEM.

Effect of Sildenafil on the Antitumor Efficacy of Doxorubicin

Cell Viability Assay

Using absorbance as a measure of cell viability, the administration of doxorubicin at a concentration of 80 nM did not significantly inhibit the growth of PC-3 cells *in vitro*. In contrast, doxorubicin at a concentration of 1 μ M significantly decreased the ability of PC-3 cells to reduce resaruzin to the highly fluorescent resafurin. In other words, doxorubicin at a concentration of 1 μ M killed approximately 65% of PC-3 cells when administered alone. Additionally, the administration of sildenafil at concentrations of either 1 μ M or 10 μ M one-hour before the administration of doxorubicin, did not effect the antitumor efficacy of doxorubicin in PC-3 cells *in vitro*. Moreover, when sildenafil (1 μ M or 10 μ M) was administered alone, the viability of PC-3 cells was not significantly altered.

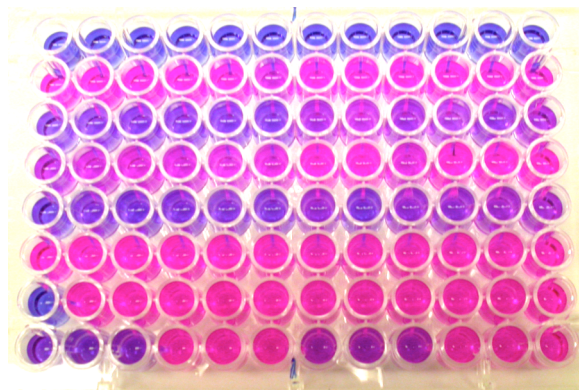


Figure 18. Photograph of 96-well Microplate used in the Cell Viability Assay of sildenafil on the antitumor efficacy of doxorubicin. Lane 1 & Lane 8 (Wells 4-6 & 10-12), Media only; Lane 2, DOX (80 nM); Lane 3, DOX (1 μ M); Lane 4, sildenafil (1 μ M)+DOX (80 nM); Lane 5, sildenafil (1 μ M)+DOX (1 μ M); Lane 6, sildenafil (1 μ M), Lane 7, sildenafil (10 μ M); Lane 8 (Wells 1-3 & 7-9), sildenafil (10 μ M)+DOX 1 μ M).

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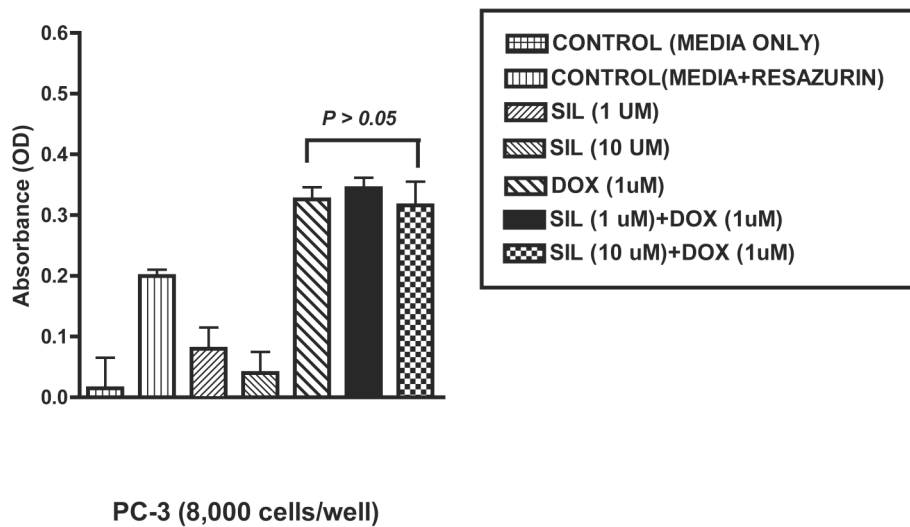
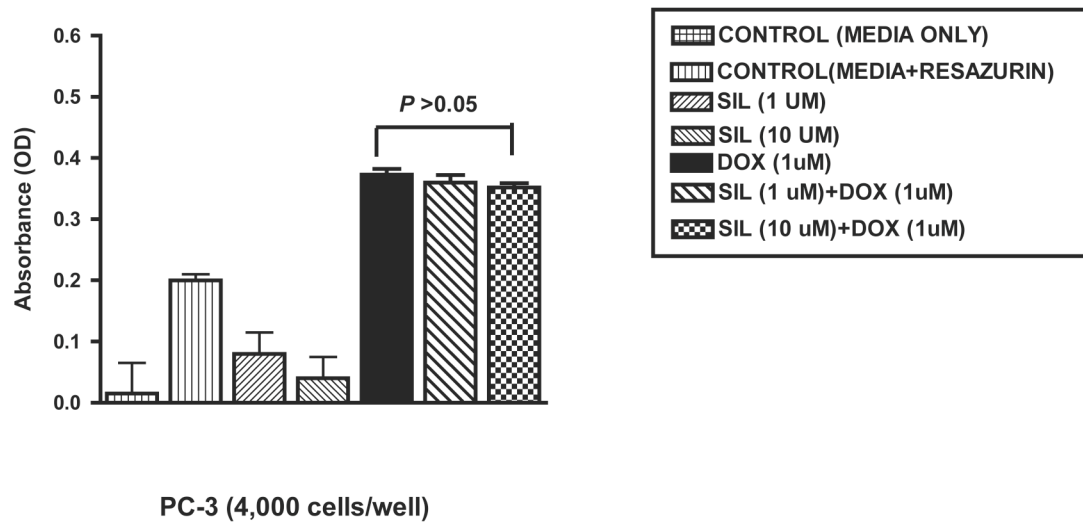


Figure 19. Cell Viability of PC-3 Prostate Cancer Cells (*in vitro*). The absorbance is proportional to the number of viable cells. © 2005 Patrick W. Fisher, DO, PhD.

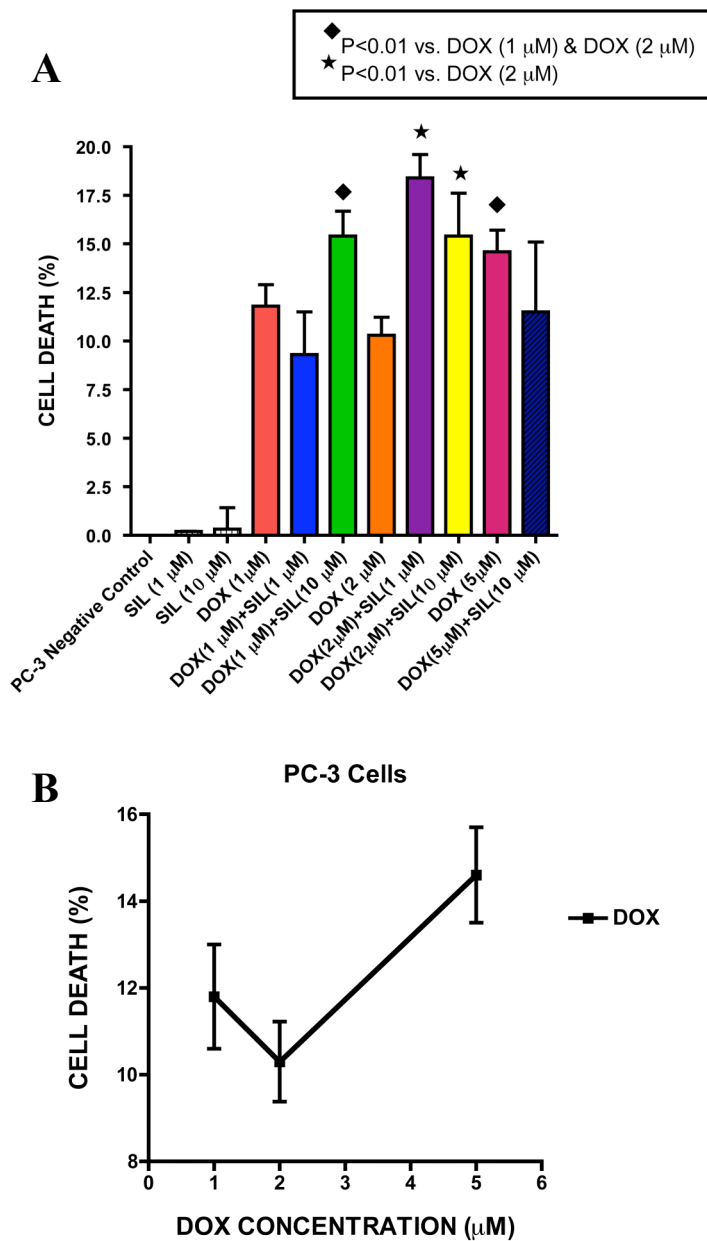


Figure 20. Flow cytometry data assessing the percentage of cell death in PC-3 prostate cancer cells *in vitro* after treatment with varying concentrations of doxorubicin with or without sildenafil, A. Cell death (% of PC-3 prostate cancer cells) versus doxorubicin concentrations (1, 2, and 5 μ M), B. Cell Death was determined using propidium iodide (50 μ g/ml). Subtraction of doxorubicin autofluorescence was performed prior to flow cytometric analysis. Data are Mean \pm SEM. © 2005 Patrick William Fisher, DO, PhD

DISCUSSION

For the first time, it is demonstrated that treatment with clinically relevant doses of sildenafil (0.7 mg/kg IP) one hour prior to doxorubicin resulted in cardioprotection from doxorubicin-induced cardiotoxicity. More specifically, the aforementioned data illustrate the capacity of sildenafil in attenuation of cardiomyocyte apoptosis, maintenance of the $\Delta\Psi_m$, preservation of myofibrillar integrity, prevention of left ventricular dysfunction, and prevention of ST-prolongation consistent with chronic doxorubicin toxicity 8-weeks after the final of three treatments.

The initial hypothesis behind pharmacological preconditioning with sildenafil was that the vasodilatory action of sildenafil could potentially release endogenous mediators of preconditioning such as adenosine or bradykinin from endothelial cells triggering phosphorylation of nitric oxide synthase (NOS) and subsequent release of nitric oxide (NO) [63]. The generation of NO could then serve to activate sGC with increased formation of cyclic guanyl monophosphate (cGMP). Increase in cGMP is believed to be responsible for activation of protein kinase G (PKG) and subsequent opening of mitoK_{ATP} channels in acute and delayed cardioprotection [63]. Previously, it was demonstrated that sildenafil-induced delayed preconditioning was linked to a NOS-dependent mechanism in mice [64]. Moreover, both the acute and delayed cardioprotective effects of sildenafil in an *in vivo* rabbit model were blocked by 5-HD, supporting the significance of mitoK_{ATP} channel opening in sildenafil-induced cardioprotection.

In addition to the present *in vivo* model of sildenafil-induced cardioprotection from doxorubicin as developed by Fisher, an *in vitro* model of adult mouse ventricular myocytes was utilized to further investigate the mechanism of protection by sildenafil. In this study, prophylactic administration of sildenafil inhibited doxorubicin-induced $\Delta\Psi_m$ dissipation, caspase-3 activation, and cardiomyocyte apoptosis. This protection was completely abolished by both L-NAME and 5-HD. These findings infer that sildenafil-mediated protection from doxorubicin-induced cardiomyocyte apoptosis is NOS-dependent and establishes a significant role of mitoK_{ATP} channel opening in sildenafil-induced cardioprotection.

The exact mechanism of NO/cGMP in protection from doxorubicin cardiotoxicity is not fully explicable. It has been shown that doxorubicin-generated H₂O₂ induces a massive increase in eNOS gene transcription followed by generation of extremely high levels of NO favoring potentiation of ROS and reactive nitrogen species [84]. In contrast, exposure to low nonlethal levels of endogenous NO induces adaptive responses by continuous stimulation of sGC with maintenance of basal cGMP levels, rendering cells resistant to lethal concentrations of NO or peroxides [85]. Moreover, it has been reported that physiologically stimulated sGC by NO preserved $\Delta\Psi_m$ and inhibited apoptosis [86,87] and caspase-3 activation [88]. From the current results, it is plausible that pretreatment with sildenafil prior to an onslaught of doxorubicin-generated free radicals augments inherent

cellular adaptive mechanisms mediated by endogenous NO/cGMP, leading to maintenance of mitochondrial bioenergetics and inhibition of apoptosis.

Doxorubicin-induced cardiomyocyte apoptosis occurs via both the extrinsic and intrinsic pathways [89,90]. However, using this model substantiates the significance of the intrinsic pathway of apoptosis in both normal and pathophysiological processes. Prior studies have identified the mitochondria as the main target of doxorubicin accumulation in cardiac cells [91]. Mitochondrial NADH dehydrogenase contributes to doxorubicin-generated ROS production via redox cycling of doxorubicin to its semiquinone [92]. Furthermore, mitochondrial concentrations of doxorubicin (5-50 μ M) are several folds greater than simultaneous clinically relevant serum concentrations (0.1-1 μ M) [93]. Consequently, the relatively limited supply of both catalase and glutathione peroxidase (GSH-Px) is rapidly expended in the heart; thus creating an environment that promotes hydroxyl radical production [94]. Accordingly, the accumulation of ROS results in dissipation of the $\Delta\Psi_m$, direct activation of the MPTP, and cytochrome-c release followed by caspase-3 activation and DNA fragmentation consistent with apoptosis [95].

In the present study, a significant decline in Bcl-2 expression both at 2-weeks and 8-weeks post treatment in the doxorubicin group compared to the sildenafil+doxorubicin group and control was observed, suggesting an important role of Bcl-2 in altering the pathological process leading to end-stage heart failure. Furthermore, significant differences in desmin distribution between the doxorubicin group compared to all other groups was evident

[FIGURE 11, Appendix B]. In the doxorubicin group, desmin distribution was clearly disrupted with areas of decreased staining in the cytoplasm consistent with desmin aggregation. In contrast, sildenafil+doxorubicin group displayed an intact desmin network similar to control. Although it is known that cardiomyocyte apoptosis contributes to dilated cardiomyopathy and heart failure, there is increasing evidence that intermediate filaments such as desmin are involved in this pathological process [96]. Recently, Dinsdale et al [97] demonstrated caspase-cleavage of intermediate filaments during apoptosis. Moreover, a study using a transgenic mouse model (desmin $-/-$) of desmin-related cardiomyopathy (DRM) demonstrated the ability of Bcl-2 overexpression in preventing DRM as evidenced by prevention of cardiomyocyte apoptosis and preservation of cardiac contractility [96]. In addition, Wang et al [98] demonstrated the disruption of desmin and formation of intracytoplasmic aggregates in a mouse model of DRM. Furthermore, Heling et al [99] illustrated the disorganization and accumulation of desmin in explanted human heart specimens from patients with dilated cardiomyopathy. Consistent with findings by Heling [99] and Wang [98], we demonstrated disruption of desmin in the doxorubicin group compared with the sildenafil+doxorubicin and control groups. Moreover, morphological changes including disruption of normal desmin distribution in myocytes as observed in DRM are similar to those seen in other forms of cardiomyopathy and heart failure [100]. Because intermediate filaments participate in transmission of active force [101], it is plausible that disruption of the filamentous network involving desmin may significantly impair contractile force and result in sarcomere fragility. Also, since desmin is known to adhere to the mitochondria in the same location

where the MPTP is formed, it is conceivable that disruption of desmin either through repeated strain on the contractile apparatus resulting from impaired contractility or through direct cleavage from activated caspases may contribute to MPTP formation, cytochrome-c release, and apoptosis.

In the current study, an 8-week post-treatment strategy was utilized, which is adequate in demonstrating many of the pathological findings of chronic doxorubicin-induced cardiotoxicity. Moreover, it provides an animal model that parallels the clinical progression of this disease in humans. In addition, it serves as a unique and powerful model to understand many of the common factors that are shared by most forms of clinical cardiomyopathies.

Results from *in vitro* studies using PC-3 cancer cells testing the effect of sildenafil on the efficacy of the antineoplastic action of doxorubicin provide relevant pre-clinical safety and efficacy data that are required prior to eventual clinical trials in humans.

Because sildenafil has proven to be relatively safe and effective in treating both erectile dysfunction and pulmonary hypertension [102,103], it is conceivable that sildenafil may provide an additional tool to hematologists and oncologists in preventing cardiotoxicity. Moreover, sildenafil prophylaxis during doxorubicin treatment may potentially allow an increase in the dose of doxorubicin beyond the cumulative limitation of 450-600 mg/m² [104], thereby expanding its therapeutic window. Studies using flow cytometry assessing

the cytotoxicity of doxorubicin on PC-3 cancer cells *in vitro*, demonstrate a powerful synergistic effect when sildenafil is combined with doxorubicin. This *in vitro* effect of sildenafil on the antineoplastic action of doxorubicin is presently being evaluated by Fisher et al using an *in vivo* nude mouse model with subcutaneously injected human breast cancer cells known to be susceptible to doxorubicin.

The powerful synergy between doxorubicin and sildenafil in killing PC-3 prostate cancer cells *in vitro* is intriguing. However, it also raises concerns regarding the possibility of potentiating the side effects of doxorubicin in non-cardiac cells where cell turnover is considered a normal physiological process. In the aforementioned chronic mouse model of used in our investigations, no evidence of potentiation of doxorubicin toxicity in non-cardiac cells was observed. Although not investigated in our model, the addition of sildenafil in patients undergoing chemotherapy with doxorubicin may lead to an increase in doxorubicin-induced toxicity in hematopoietic stem cells. This could prove fatal during the induction phase of chemotherapy. Moreover, it may prolong the time that patients remain neutropenic, thereby exposing them to the additional risk of opportunistic infections. Further studies are warranted in examining whether this synergistic effect of sildenafil with doxorubicin is translated to non-neoplastic cells in humans.

The mechanism responsible for the synergy of sildenafil in the presence of doxorubicin in PC-3 prostate cells *in vitro* is not fully explicable. However, numerous studies using PC-3 cell lines in examining mechanisms of chemoresistance have elucidated several intriguing hypotheses—many implicating NO as a key mediator in chemosensitization [114-119]. Moreover, many cancer cells develop chemoresistance by overexpressing Bcl-2 and Bcl-X_L, key mediators of apoptosis under the transcriptional regulation of NF-κB [115]. In fact, Huerta-Yepez et al [115] demonstrated the ability of NO donors in down regulating the expression of Bcl-X_L via inhibition of NF-κB activity resulting in the sensitization of cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This ultimately resulted in activation of the mitochondrial pathway of apoptotic cell death.

Furthermore, there is evidence suggesting that suppression of endogenous nitric oxide may play a key role in hypoxia-induced chemoresistance [119]. Moreover, it has been demonstrated that hypoxia inducible factor (HIF-1) DNA binding is inhibited by NO in the presence of hypoxia [118]. In addition, NO has also been demonstrated to inhibit HIF-1α accumulation in cells under hypoxic conditions [116]. It is therefore not surprising that PC-3 cells contain all three well-known isoforms of nitric oxide synthase (NOS): eNOS, iNOS, and nNOS [115].

In another study by Frederiksen et al [116], exposure of hypoxia to human breast carcinoma cells resulted in decreased endogenous cGMP, further implicating the role of NO in chemoresistance. In fact, in a follow-up study, Frederiksen et al provided further support implicating that administration of low concentrations of NO mimetic agents can prevent acquired drug resistance in PC-3 prostate cancer cells to doxorubicin [114].

In the present studies illustrating the synergistic effect of sildenafil in doxorubicin-induced antineoplastic action in PC-3 cells, it is plausible that the inhibition of PDE-5, maintains or upregulates production of NO to physiological levels thereby “sensitizing” this cancer cell line to doxorubicin-induced cell death. Further studies will help unravel this conundrum and provide a better understanding of the role of PDE-inhibitors in the pathophysiology of cell signaling mechanisms involved in the proliferation of many neoplastic diseases.

Future Investigations

Further studies aimed at attenuating the detrimental effects leading to chronic doxorubicin cardiomyopathy should be investigated. Some of the potential targets include the examination of the transcription factors, heat shock factor-1 and GATA-4, in addition to heat shock proteins and their relationship with cGMP/sGC in cardiomyocytes.

An early event in the cardiotoxic effect of the antitumor drug doxorubicin is GATA-4 depletion, which in turn causes cardiomyocyte apoptosis. Studies by Aries et al [110] indicate that the transcription factor GATA-4 is antiapoptotic and may be vital for the

adaptive stress response of the adult heart. As such, the ability to regulate the genes responsible for apoptosis in the heart via transcription factor modulation offers promise in the area of heart failure [Figure 21]

The transcription factors GATA-4 and GATA-6 regulate cardiomyocyte hypertrophy *in vitro* and *in vivo*. Recent studies have shown that GATA-4 might also play a role in survival of adult cardiac myocytes [110,111]. This transcription factor may potentially regulate pathophysiological conditions such as myocardial ischemia-reperfusion injury, ischemic preconditioning, and environmental and drug-induced cardiomyopathies where apoptosis and survival of cardiac myocytes play an essential role. [112]

Additionally, because of the protective effects of the anti-apoptotic protein, Bcl-2, in preventing doxorubicin-induced cardiomyopathy as demonstrated *in vivo* [113], it would be intriguing to ascertain the effect of PDE-5 inhibition in preserving the DNA-binding activity of GATA-4 and its subsequent ability to preserve basal levels of Bcl-2 following treatment with doxorubicin. Studying the transcription and phosphorylation of GATA-4 *in vivo* can accomplish this.

Moreover, further investigations are warranted that may lead to a better understanding of the role and significance of PDE-5 localization in cardiomyocytes *in vivo* and its relationship to subsequent contractile dysfunction. PDE-5 expression in mouse cardiomyocytes has previously been demonstrated in our laboratory by Das et al.

Moreover, Takimoto et al [112] have also demonstrated PDE-5 expression in frozen sections from adult NOS3 (-/-) mice and their wild-type counterparts. Furthermore, the study by Takimoto et al suggests that the effects of cGMP on PKG-1 activation is dependent on z-band localization of PDE-5A, thus enabling modulation of PKA and subsequent calcium-induced contractility by sildenafil. Moreover, they suggest that acute NOS3 inhibition removes the critical substrate to the PDE-5A complex and thus eliminates the antiadrenergic effect of sildenafil. In fact, they infer that chronic NOS3 inhibition or the use of NOS3 (-/-) mice results in the loss of PDE-5A from the z-band, resulting in the elimination of sildenafil's effectiveness in maintaining contractility even in the presence of exogenous NO. Although intriguing, their rationale for PDE-5A localization to the z-band is not entirely complete. In recent studies by Fisher [Appendix B, C], clear evidence exists demonstrating the loss of PDE-5A expression and lack of localization at the z-band in frozen sections from doxorubicin-only treated mice at 8-weeks post-treatment (total cumulative dose= 15mg/kg IP). It is plausible that decreased contractility from desmin disruption subsequently results in the inability of PDE-5A from localizing to the z-band. Moreover, the ability of caspase-3 in cleavage of desmin at the z-band may be interrelated to the inability of PDE-5A in locating to the z-band.

One hypothesis that requires further investigation is that PDE-5A localization to the z-band is associated with protection of desmin cleavage by activated caspase-3. Because NO is known to inactivate caspase-3 via reversible binding of NO to the active cysteine residue on the large subunit via *s*-nitrosylation, it is plausible to that maintaining myofibrillar

integrity and contractile function at the z-band is coordinated through PDE-5A inhibition with subsequent physiological available “pools” of NO that are available to inactivate the detrimental actions of active caspase-3 on myofibrillar disruption of intermediate filaments.

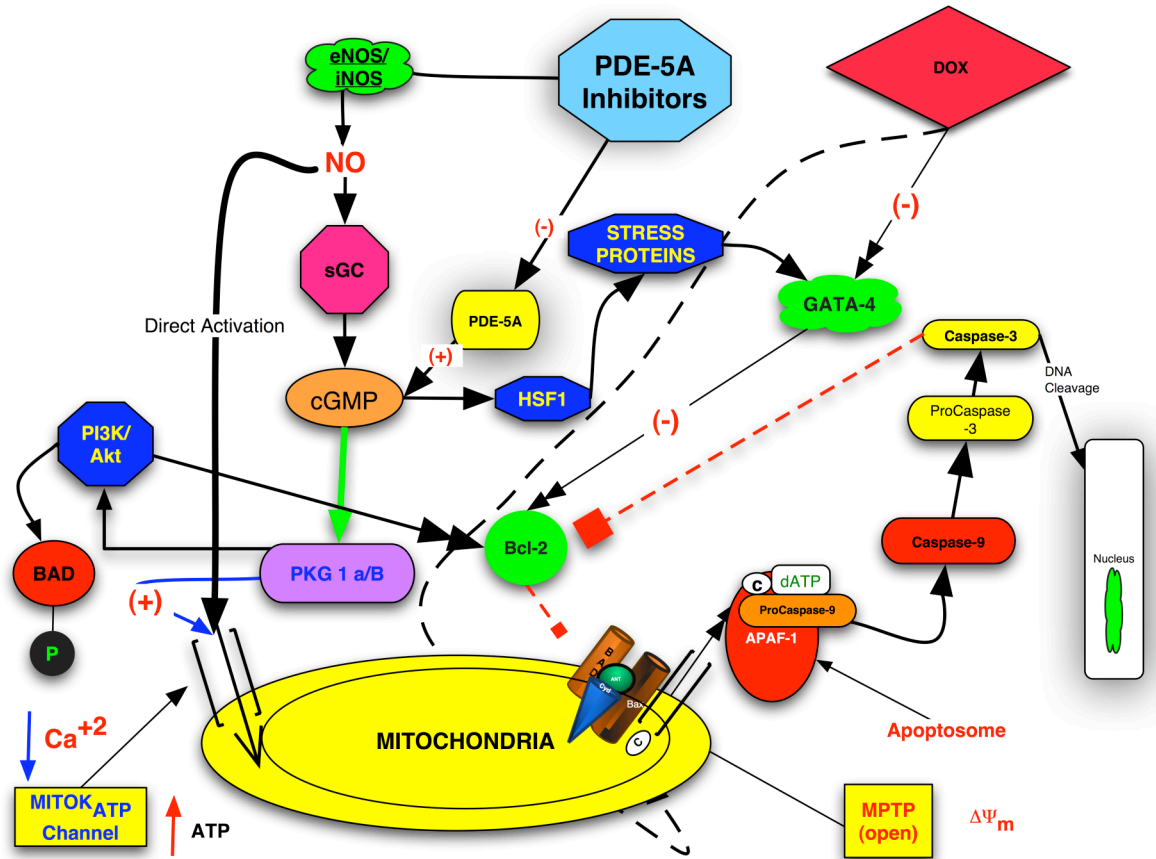


Figure 21. Potential targets for further investigation on the molecular mechanisms of doxorubicin-induced cardiotoxicity.

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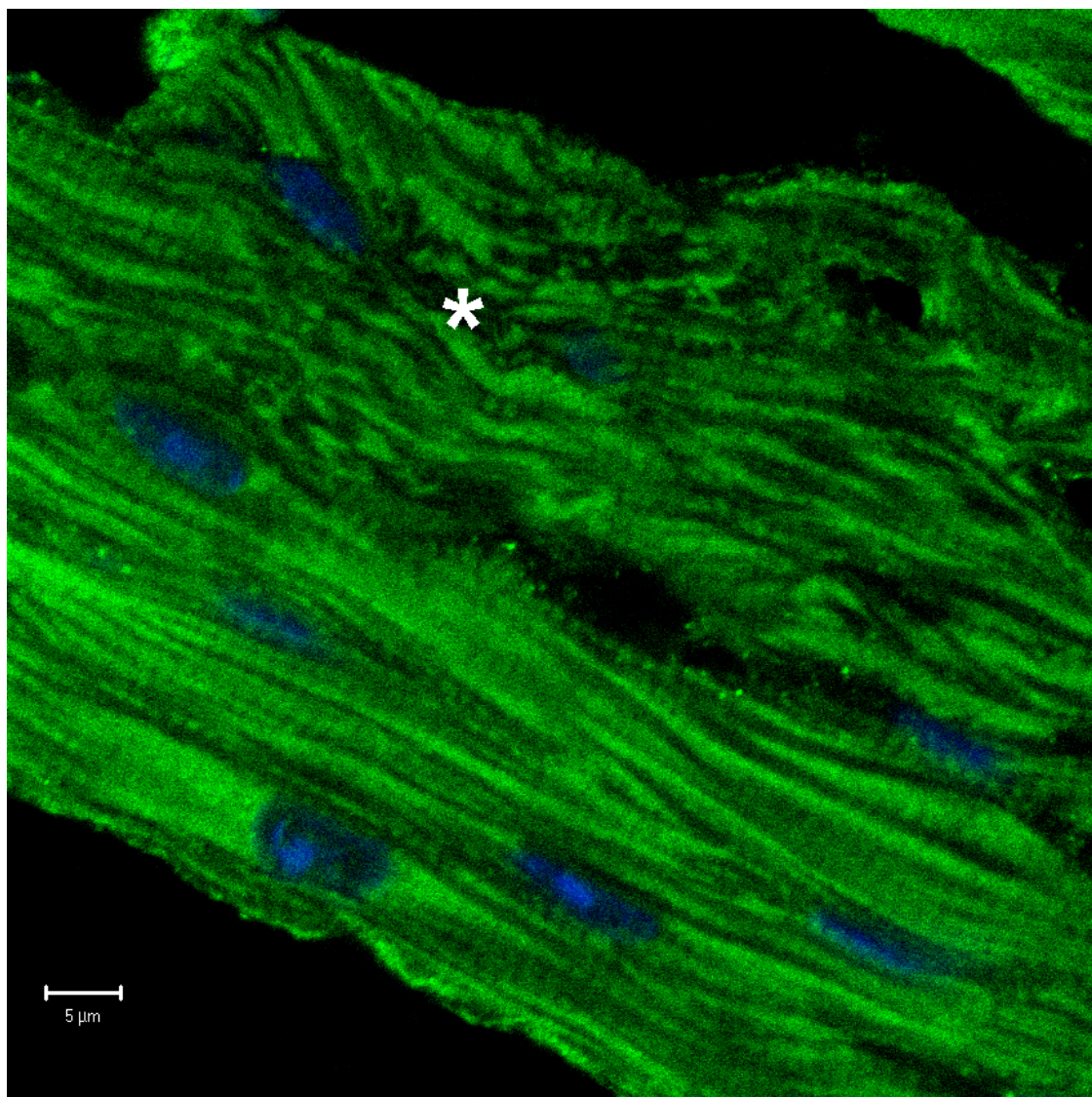
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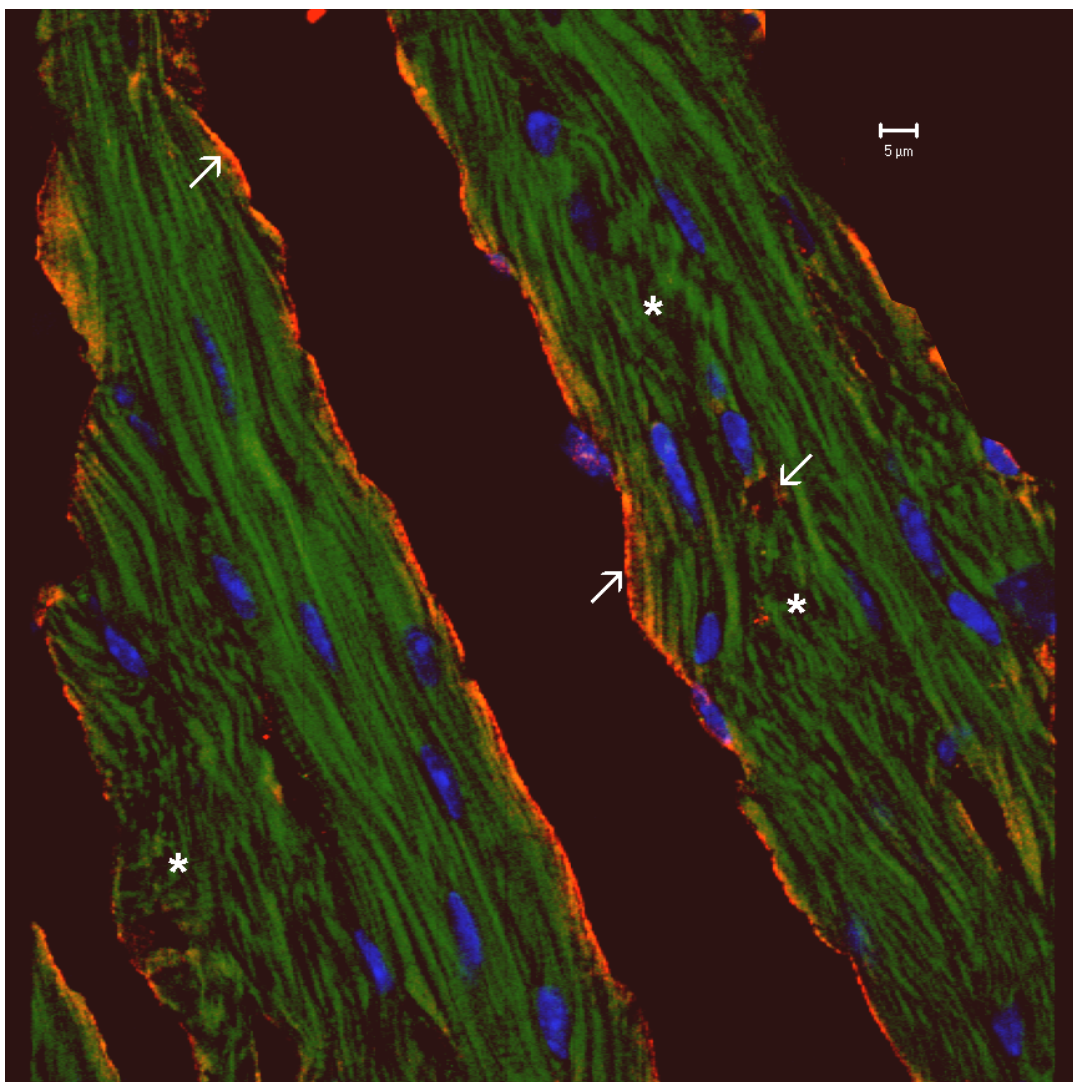
Appendix A



Confocal microscopic image illustrating desmin distribution (green) and DAPI-stained nuclei (blue) in a cryosection from a mouse left ventricle at 8 weeks post treatment with doxorubicin [Total Cumulative dose= 15mg/kg IP].

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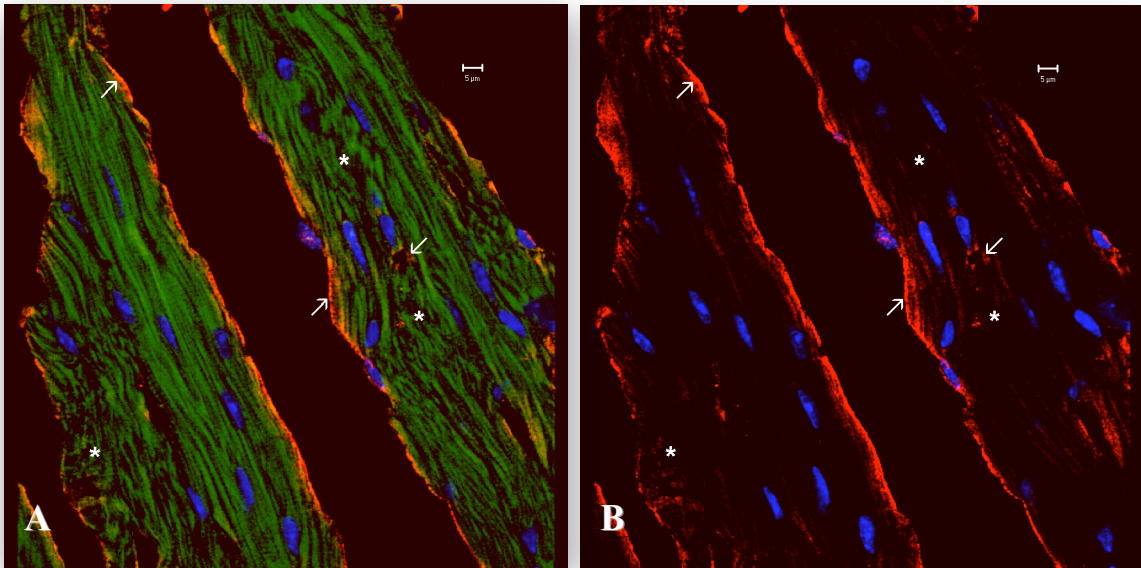
Appendix B



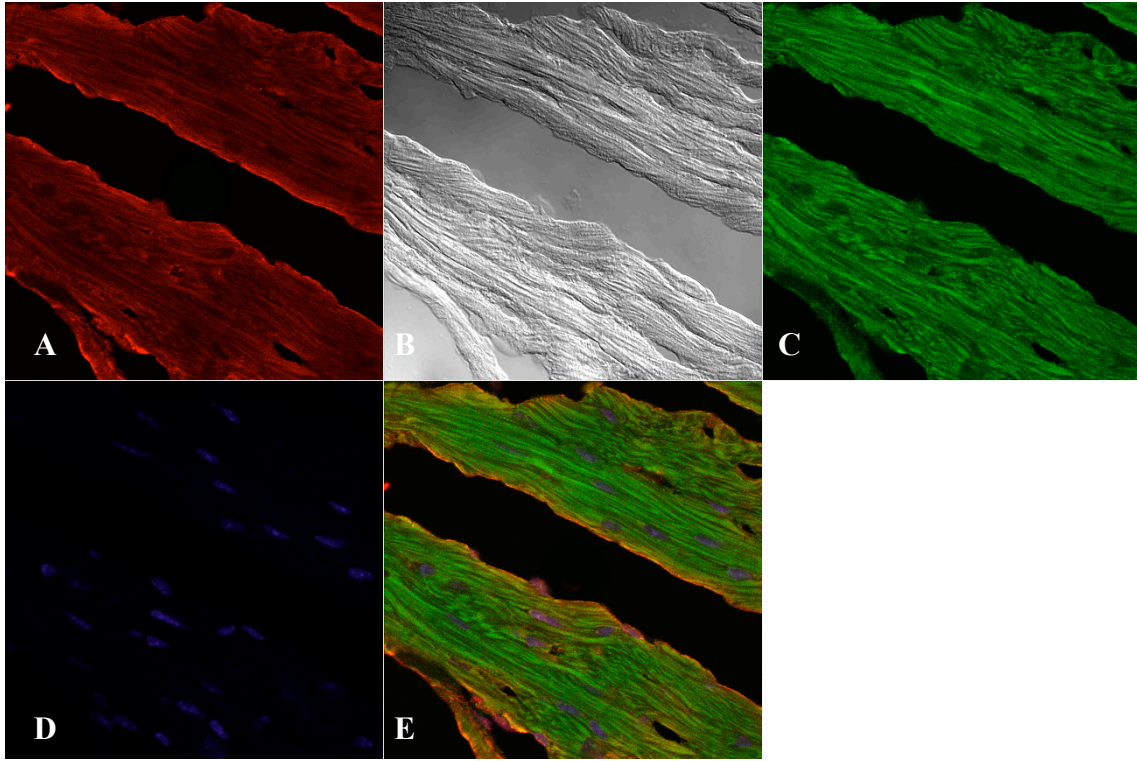
Confocal microscopic image of cryosection from a mouse left ventricle, demonstrating colocalization of desmin (green) with PDE-5A (red)--in a doxorubicin-only treated animal 8 weeks post treatment (Total cumulative dose = 15 mg/kg IP). Nuclei are stained blue with DAPI. * Desmin disruption; PDE-5A localization (arrows).

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Appendix C



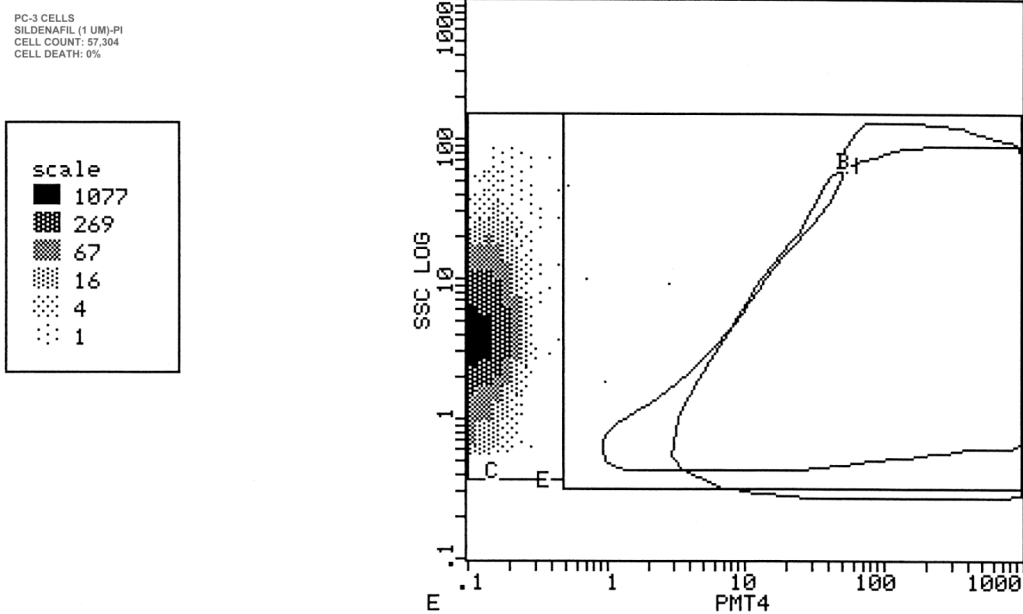
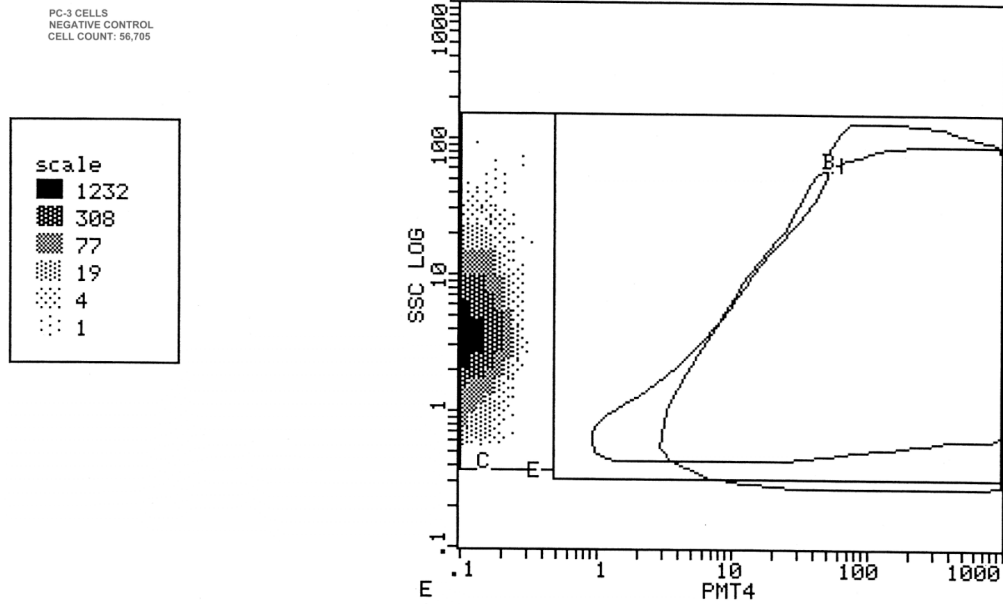
A, Confocal microscopic image of cryosection from a mouse left ventricle, demonstrating colocalization of desmin (green) with PDE-5A (red)--in a doxorubicin-only treated animal 8 weeks post treatment (Total cumulative dose = 15 mg/kg IP). Nuclei are stained blue with DAPI. B, Same image without desmin fluorescence; only PDE-5A and DAPI-stained nuclei are visible. * Desmin disruption; PDE-5A localization (arrows). © 2005 Patrick W. Fisher, DO, PhD.



Confocal microscopic image of cryosection from a mouse left ventricle in a doxorubicin-only treated animal 8 weeks post treatment (Total cumulative dose = 15 mg/kg IP), demonstrating PDE-5A (red), A; Phase image, B; desmin (green), C; Nuclei stained with DAPI (blue), D; and merged image of all three fluorophores, E.

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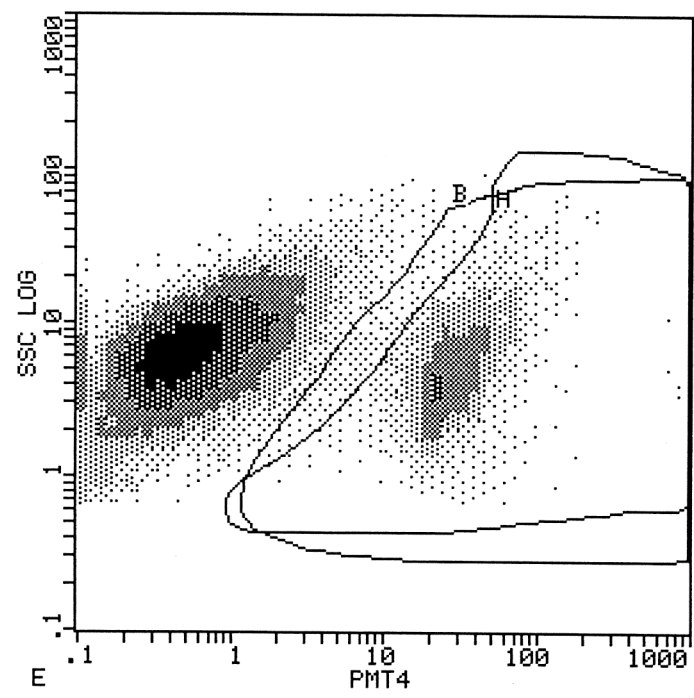
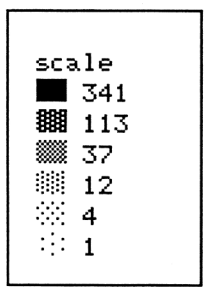
APPENDIX E



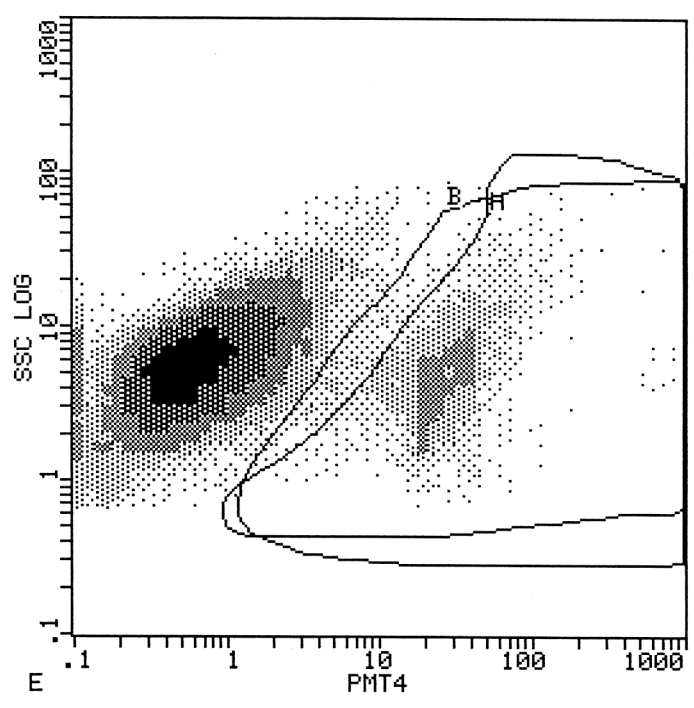
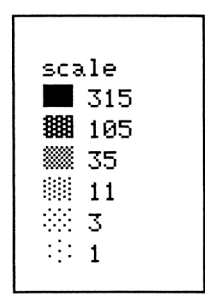
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APPENDIX E (CONTINUED)

PC-3 CELLS
DOX (1 UM)+PROPIDIUM IODIDE
CELL COUNT: 56,672



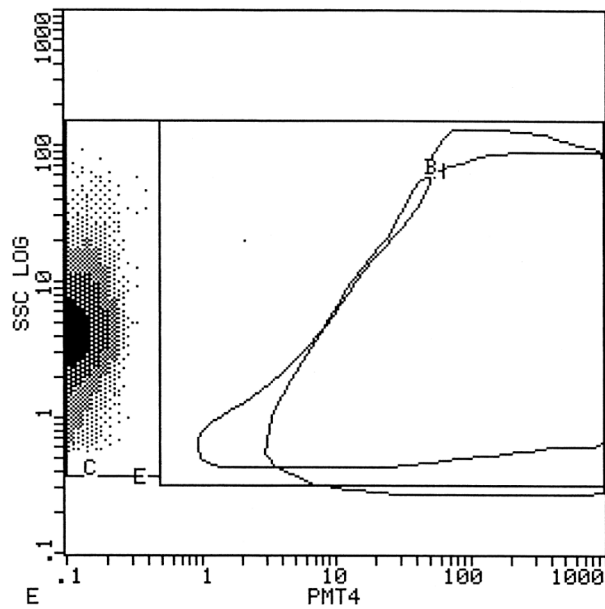
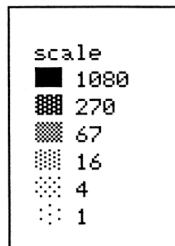
PC-3 CELLS
DOX (1 UM)+SILDENAFIL (1 UM)+PROPIDIUM IODIDE
CELL COUNT: 56,885
CELL DEATH: 9.3%



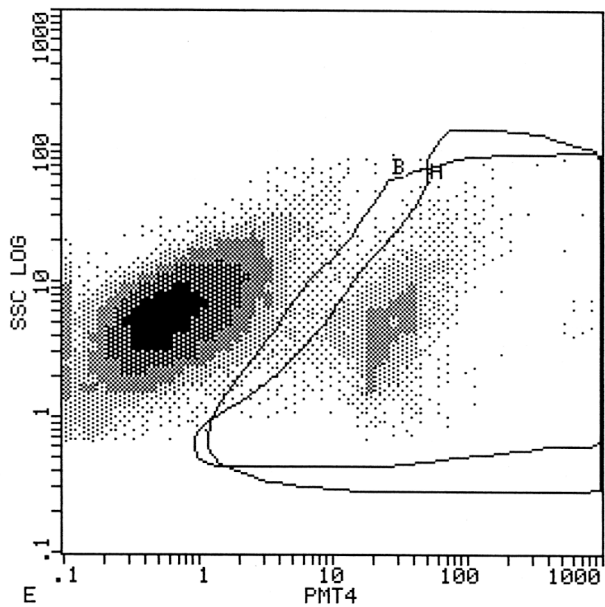
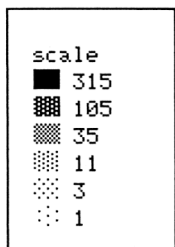
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APPENDIX E (CONTINUED)

PC-3 CELLS
 SILDENAFIL (10 UM)-PI
 CELL COUNT: 57,136
 CELL DEATH: 0%



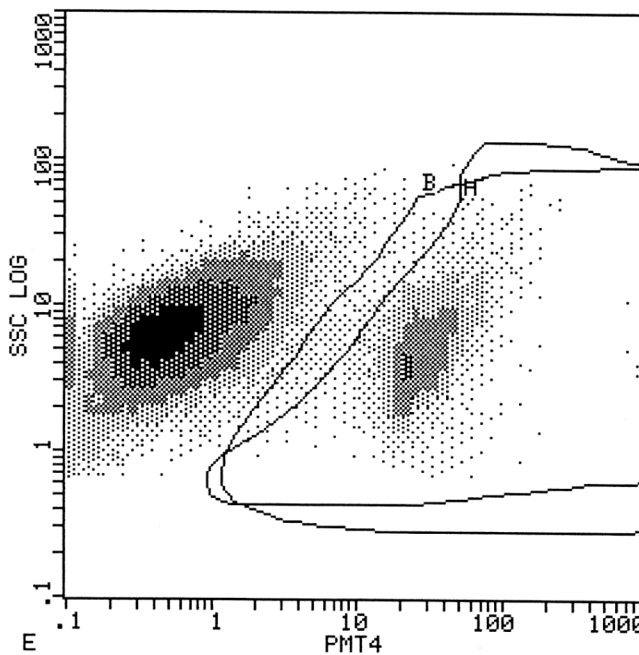
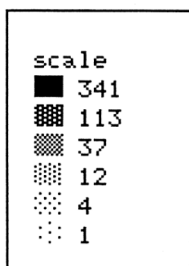
PC-3 CELLS
 DOX (1 UM)+SILDENAFIL (1 UM)+PROPIDIUM IODIDE
 CELL COUNT: 56,885
 CELL DEATH: 9.3%



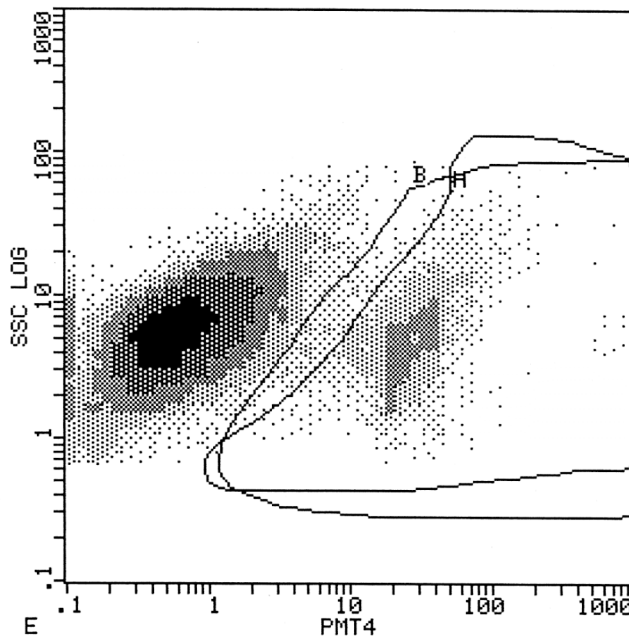
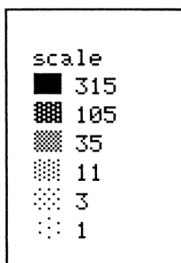
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APPENDIX E (CONTINUED)

PC-3 CELLS
DOX (1 UM)+PROPIDIUM IODIDE
CELL COUNT: 56,672



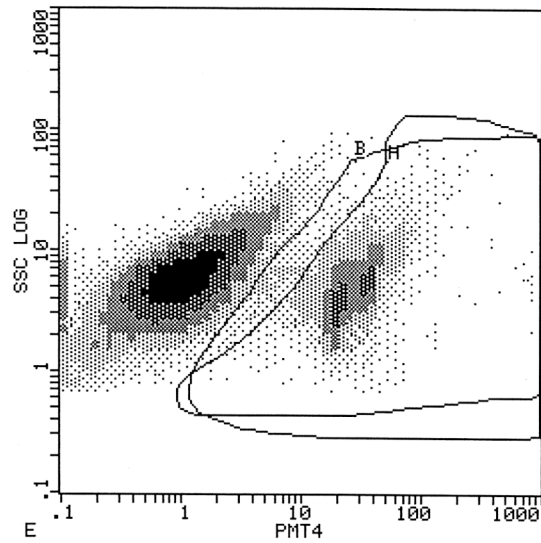
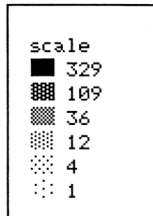
PC-3 CELLS
DOX (1 UM)+SILDENAFIL (1 UM)+PROPIDIUM IODIDE
CELL COUNT: 56,885
CELL DEATH: 9.3%



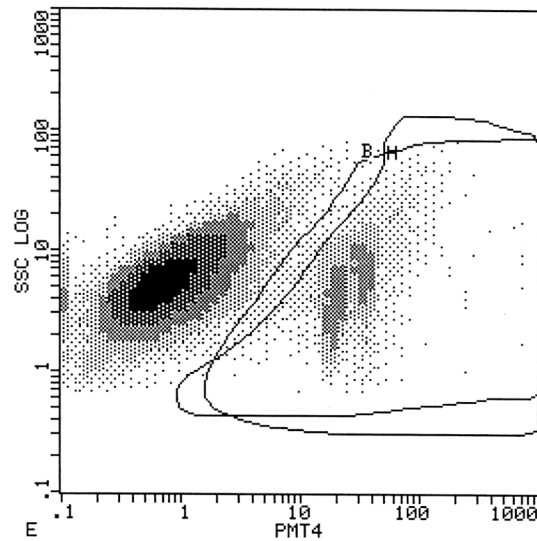
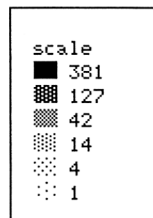
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APPENDIX E (CONTINUED)

PC-3 CELLS
DOX (1 UM)+SILDENAFIL (10 UM)+PI
CELL COUNT: 55,765
CELL DEATH: 15.4%



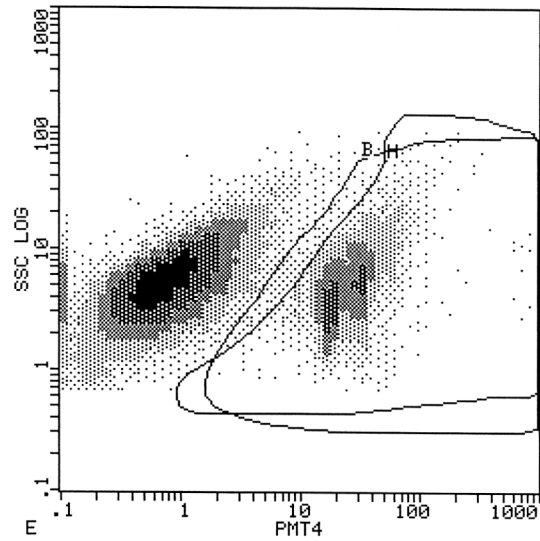
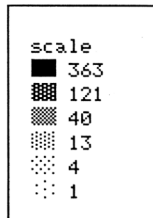
PC-3 CELLS
DOX (2 UM)+PROPIDIUM IODIDE
CELL COUNT: 56,222
CELL DEATH: 10.3%



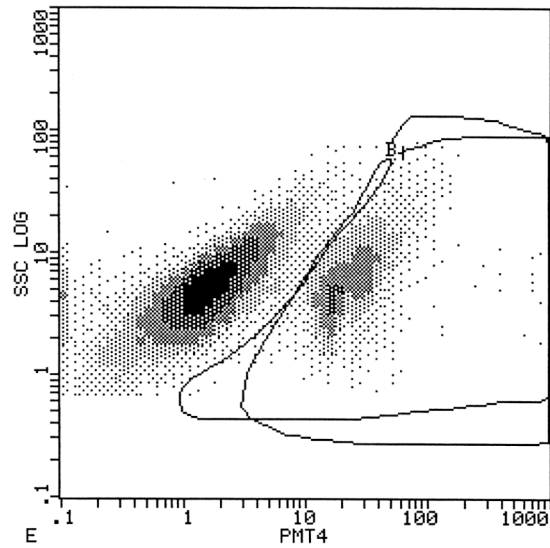
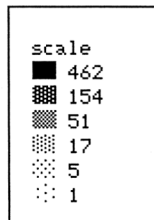
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APPENDIX E (CONTINUED)

PC-3 CELLS
DOX(2 UM)+SILDENAFIL(1 UM)+PI
CELL COUNT: 53,129
CELL DEATH: 18.4%



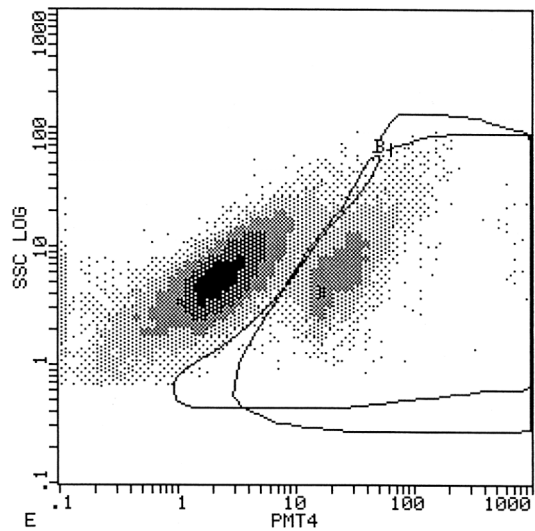
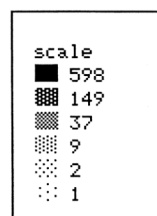
PC-3 CELLS
DOX (5 UM)+PROPIDIUM IODIDE
CELL COUNT: 54,363
CELL DEATH: 14.6%



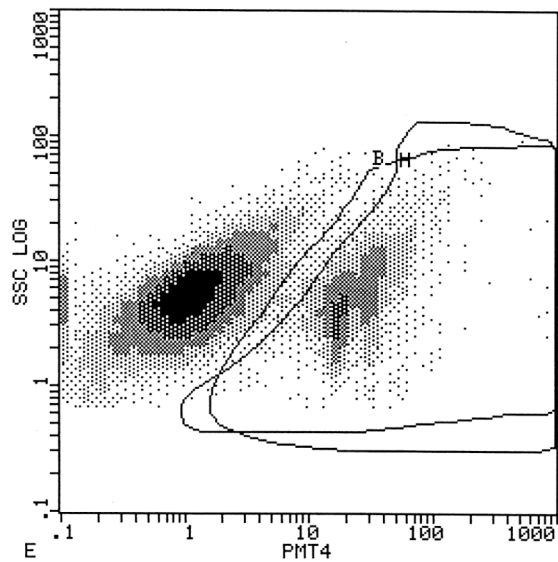
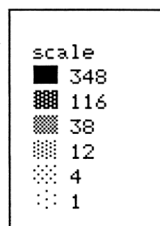
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APPENDIX E (CONTINUED)

PC-3 CELLS
DOX(5 UM)+SILDENAFIL (10 UM)+PI
CELL COUNT: 55,882
CELL DEATH: 11.5%



PC-3 CELLS
DOX(2 UM)+SILDENAFIL (10 UM)+PI
CELL COUNT: 52,587
CELL DEATH: 15.4%



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VITA**Patrick William Fisher, DO, PhD**

E-MAIL: pfisher002@md.northwestern.edu

BORN: January 6, 1972, Evanston Hospital, Evanston, Illinois

CITIZENSHIP: United States of America

PROFESSIONAL LICENSURE (License to Practice Medicine and Surgery)

1. State of California
2. Commonwealth of Virginia

BOARD CERTIFICATION

1. American Board of Internal Medicine (Certification # 211668)
2. National Board of Osteopathic Medical Examiners (Certification #35244)

POST-GRADUATE TRAINING

Stanford University School of Medicine, Stanford, CA 2006-2007

Post-Doctoral Scholar/ Advanced Heart Failure/Heart Transplant Fellow

Virginia Commonwealth University Medical Center, Richmond, VA 2002-2006

Clinical Cardiology Fellow/COCATS Level III Research Track

Northwestern University Feinberg School of Medicine, Evanston, IL 1999-2002

Internal Medicine Residency

GRADUATE EDUCATION

**Virginia Commonwealth University School of Medicine,
Department of Physiology**

Doctor of Philosophy in Physiology, May 21, 2005

Concentration: Molecular and Cellular Cardiology

Dissertation Title: Type-5A Phosphodiesterase Inhibition in the Prevention of Doxorubicin Cardiomyopathy

Kirkville College of Osteopathic Medicine, Kirksville, MO

Doctor of Osteopathic Medicine, June 6, 1999

UNDERGRADUATE EDUCATION

Truman State University, Kirksville, MO

B.S. Biology, Cum Laude, May 7, 1994

HONORS & AWARDS

**National Heart, Lung, and Blood Institute
Post-Doctoral Training Grant (2003-2005)**

Type T32 (HL07537-15); “Cardioprotective Mechanisms of Myocardial Injury”

Principal Investigator: Michael L. Hess, MD

Co-Principal Investigator: Rakesh C. Kukreja, Ph.D.

Phi Kappa Phi National Honorary Society (Spring 2005)-- Nominated by the Department of Physiology at the Virginia Commonwealth University School of Medicine

GlaxoSmithKline Research & Education Foundation for Cardiovascular Disease Young Investigator Award: “New Frontiers in Genomics and Cardiovascular Disease”. (April 8-9, 2005) Philadelphia, Pennsylvania

National Clinical Vignette Award Winner—
American College of Physicians Annual Sessions 2002

Presidential Scholar, Kirksville College of Osteopathic Medicine (1995 & 1996)

Truman State University Dean’s List (6 Consecutive Semesters)

HONOR SOCIETIES:

1. Sigma Sigma Phi National Osteopathic Honor Society
2. National Order of Omega
3. Blue Key Society
4. Alpha Sigma Alpha National Honor Society
5. Gamma Sigma Alpha Honorary Society

REFEREE REVIEWER

1. Circulation
2. Journal of Cardiac Failure
3. American Journal of Kidney Diseases
4. Journal of Thrombosis and Thrombolytics
5. Journal of Clinical Biochemistry
6. Journal of Lasers in Science and Medicine

INVITED LECTURES

- 1. International Society for Heart Research American Section, 27th Annual Meeting (May 12th-15th, 2005)
New Orleans, LA**

Symposium: Role of Phosphodiesterases in Cardiovascular Disease;

**Lecture: Role of PDE-5A Inhibition in Attenuation of Doxorubicin
Cardiomyocyte Apoptosis and Left Ventricular Dysfunction**

- 2. American Heart Association Scientific Sessions 2005, Dallas, TX**

Symposium: Type 5A-Phosphodiesterase Inhibitors in Cardiovascular Disease

**Lecture: Role of PDE-5A Inhibition in Attenuation of Doxorubicin
Cardiomyocyte Apoptosis and Left Ventricular Dysfunction**

- 3. Virginia Commonwealth University School of Medicine, Department of Physiology/Cardiology Annual Lecture Series (MAY 3, 2005)**

**Lecture: Clinical Application of Viagra in Attenuation of Doxorubicin-Induced
Cardiomyocyte Apoptosis and Left Ventricular Dysfunction**

- 4. Virginia Commonwealth University School of Medicine, Department of Physiology Cardiovascular & Exercise Physiology Graduate Course- (Dr. Alexandre Fabiato, Course Director)**

**Lecture: Pathophysiology of Heart Failure: From Bench to Bedside
(March 29-31, 2005)**

RESEARCH EXPERIENCE

**Virginia Commonwealth University School of Medicine, Division of Cardiology,
Richmond, VA (July 2003-July 2006)**

Mentor:

Rakesh C. Kukreja, Ph.D.

The Eric Lippman Chair of Molecular Cardiology
Professor of Medicine, Professor of Biochemistry, Professor of Emergency
Medicine

**Ph.D. Dissertation Title: Type-5A Phosphodiesterase Inhibition on Prevention
of Doxorubicin Cardiomyopathy**

**Feinberg Cardiovascular Research Institute, Northwestern University Feinberg
School of Medicine, Chicago, IL (June 2000-September 2000)**

Mentors:

Francis J. Klocke, MD, MACC Director, Feinberg Cardiovascular Research
Institute, Professor of Medicine, Feinberg School of Medicine, Northwestern
University, Chicago, IL

Robert S. Decker, PhD Professor of Medicine, Professor of Cell and Molecular
Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL

**Investigated the contribution of apoptosis to myocardial contractile
dysfunction in canine models of low-flow ischemia**

**Evanston Northwestern Healthcare, Evanston, IL Division of Cardiology
(9/1999-9/2001)**

1. Congestive Heart Failure Telemanagement Clinical Outcomes Trial

Mentor:

Randall E. Williams, MD Associate Professor of Medicine, Feinberg School of Medicine Northwestern University, Chicago, IL

2. Metoprolol vs. Diltiazem in the Acute Treatment of Ventricular Rate in Atrial Fibrillation or Atrial Flutter Using Combination Intravenous and Oral Therapy: A Double-Blind, Randomized Controlled Clinical Trial Principal Investigator: Patrick W. Fisher, DO

Mentor: David M. Najman, MD Assistant Professor of Medicine, Feinberg School of Medicine Northwestern University, Chicago, IL

The MDAFIB Study Investigator Group: Patrick W. Fisher, DO, Tomasz Kuzniar, MD, PhD, David You, MD, Kyong Oh, MD, Dawn Lombardo, DO, Isabella Rybaltowski, PharmD, Marla N. Fisher, RN, BSN, Rajiv Udani, DO, DJ. Anh, MD, and David M. Najman, MD.

Clinical trial approved December 21, 2000 by the Evanston Northwestern Healthcare Institutional Review Board

Kirksville College of Osteopathic Medicine, Kirksville MO (1999)

Mentor:

Martin Schwarze, DO, FACOI, FACC Cardiology Diagnostics, St. Louis, MO

Developed a two-volume evidence-based manual as an addition to the core cardiology curriculum for students and house-staff at the Kirksville College of Osteopathic Medicine

ABSTRACTS & PRESENTATIONS

1. **Angiotensin II Receptor Blockade with Losartan Reduces Apoptosis and Infarct Size Following Ischemia/Reperfusion in the Rabbit Heart.** Okubo S, **Fisher PW**, Salloum F, Ockaili R, Marwaha VR, Ambrosio G, Hess ML, and Kukreja, RC.

Oral Presentation, March 9th, 2005; ACC 2005 Scientific Sessions, Orlando, FL

2. **Contribution of Apoptosis to the Contractile Dysfunction During Low-Flow Ischemia in the Canine Myocardium.** Robert S. Decker, Marlene L. Decker, **Patrick W. Fisher**, Sakie Nakamura, Kathleen R. Harris, and Francis J. Klocke.

Abstract presented at the International Society for Heart Research XVII World Congress, July 6-11, 2001

3. **Cardiac Conundrum: Complications of a Young Woman Twenty Years After Receiving Doxorubicin and Radiation Treatment for Rhabdomyosarcoma.** **Patrick W. Fisher**, David M. Najman, and Randall E. Williams.

Abstract accepted as a **finalist** for the Heart Failure 2001 Annual Symposium, Loyola University, Stritch School of Medicine, February 9, 2001

Poster presented at “**Challenging Cases in Heart Failure**”, Heart Failure 2001 Symposium, Loyola University, Stritch School of Medicine, February 9, 2001

4. **A Novel Twist To the familial Mediterranean Fever Story: Non-Amyloid Nephrotic Syndrome Due to an Unusual Mutation and Fibrillary Glomerulonephritis.** **Patrick W. Fisher**, Tammy Ho, Robert Goldschmidt, Ronald Semerdjian, and Gregory W. Rutecki.

Selected as a **National Award Winner & Finalist**

American College of Physicians-American Society of Internal Medicine (ACP-ASIM) 2002 Clinical Vignette and Research Competition (April 12-15, 2002) Philadelphia, PA

Poster presented orally before three-judge panel at the ACP-ASIM Annual Session, April 12, 2002, Philadelphia, PA

5. **Why Live on Borrowed Time and ‘Collateral’? Securing Longevity with Preconditioning.** Patrick W. Fisher, David Najman, Timothy A. Sanborn, and Gregory W. Rutecki.

Abstract submitted to the American College of Physicians- American Society of Internal Medicine 2001 Associates Clinical Vignette and Research Competition

6. Fisher PW, Salloum F, Das A, Kukreja RC. **Type-5A Phosphodiesterase Inhibition Using Sildenafil (Viagra) Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin-induced Cardiotoxicity.** J Mol Cell Cardiol 2005 In Press

PUBLICATIONS

1. Decker, Robert S, Decker, Marlene L, **Fisher, Patrick W**, Nakamura, Sakie, Harris, Kathleen R, Klocke, Francis J. **Contribution of Apoptosis to the Contractile Dysfunction During Low-Flow Ischemia in the Canine Myocardium.** *J Mol Cell Cardiol* 2001; 33: A26.
2. **Fisher, PW**, Ho, LT, Goldschmidt, R, Semerdjian, RJ, and Rutecki, GW. **Familial Mediterranean Fever, Inflammation and Nephrotic Syndrome: Fibrillary Glomerulopathy and the M680I Missense Mutation.** *BMC Nephrol*, 2003; (4): p 6.
3. **Fisher PW**, Salloum F, Das A, Hyder H, and Kukreja, RC. **Phosphodiesterase-5 Inhibition with Sildenafil Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin Cardiotoxicity.** *Circulation*, 2005; 111(13): 1601-1610.
4. Kukreja RC, Salloum F, Das A, Ockaili R, Yin C, Bremer Y, **Fisher PW**, Wittkamp M, Hawkins J, Chou E, Kukreja A, Marwaha VR, Xi L. **Pharmacological Preconditioning with Sildenafil: Basic Mechanisms and Clinical Implications.** *Vascular Pharmacol, In Press, Corrected Proof, Available online 30 March 2005*
5. Wexler J, **Fisher PW**, Salloum F, Ockaili R, and Kukreja RC. **Brain-Type Natriuretic Peptide in Pharmacologic Preconditioning: Role of Nitric Oxide and the Mitochondrial K_{ATP} Channel.**

Manuscript pending submission for publication-AJP(Heart-Circ)
6. Okubo S, **Fisher PW**, Salloum F, Ockaili R, Marwaha VR, Guiseppe A, Hess ML, and Kukreja RC. **Angiotensin II Receptor Blockade Reduces Apoptosis and Infarct Size in Ischemia/Reperfusion Injury in Rabbit Hearts.** *JACC* 2005; 45(3) Supplement A: p252A.
7. **Fisher PW**, Salloum F, Das A, Kukreja RC. **Type-5A Phosphodiesterase Inhibition Using Sildenafil (Viagra) Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin-induced Cardiotoxicity.** *J Mol Cell Cardiol*, 2005. In Press.

BOOKS

1. **Patrick W. Fisher** and **Martin Schwarze, DO, FACC**
Clinical Cardiology: Evidence-Based Medicine—A Guide to Advancements in Cardiovascular Medicine and its Application in the Clinical Setting.
© 1999-2000 KCOM Press, Inc.

PROFESSIONAL MEMBERSHIPS

1. American Medical Association
2. American Heart Association-Basic Cardiovascular Science Council
3. International Society for Heart Research
4. American Academy for the Advancement of Science
5. American College of Cardiology
6. Medical Society of Virginia
7. American College of Physicians
8. American Association for Cancer Research (AACR)
9. International Cell Death Society
10. Northwestern University Alumni Association
11. National Postdoctoral Association
12. International Society for Heart and Lung Transplantation
13. Heart Failure Society of America
14. Kirksville College of Osteopathic Medicine Alumni Association

PROFESSIONAL EXPERIENCE

Ad Hoc Committee for the Advancement of Medical Education Chairman (2001-2001)

Evanston Northwestern Healthcare, Evanston, IL

- Developed a proposal to acquire a HARVEY® patient simulator for the Department of Medicine (Spring 2001)
- Purpose: To enhance the physical diagnostic skills of residents, interns, and medical students through the coordination of bedside teaching with the UMEDIC® computer simulator and the HARVEY® patient simulator
- Worked in coordination with the Department of Medicine and the Division of Cardiology in raising \$100,000 for this project
- Developed a core curriculum for the intensive and cardiac care unit rotations (Summer 2001)
- HARVEY® and the UMEDIC® program obtained (December 2001)

Developed a proposal for the establishment of an endowment to provide financial resources to patients in the internal medicine outpatient department who are unable to afford necessary medications (Spring 2002)