Molecular Pharmacology Fast Forward. Published on December 18, 2013 as DOI: 10.1124/mol.113.090043

MOLPHARM#2013/090043

PDE5 inhibitors enhance chemotherapy killing in gastrointestinal/genitourinary cancer cells.

Laurence Booth, Jane L. Roberts, Nichola Cruickshanks, Adam Conley, David E. Durrant, Anindita Das, Paul B. Fisher, Rakesh C. Kukreja, Steven Grant, Andrew Poklepovic and Paul Dent

Departments of Biochemistry and Molecular Biology (NC, LB, JR, AC, PD), Cardiology (AD, DD, RCK), Medicine (SG, AP), Human and Molecular Genetics (PBF), Virginia Commonwealth University, 401 College St., Richmond, VA 23298.
Abstract

The present studies determined whether clinically relevant phosphodiesterase 5 (PDE5) inhibitors interacted with clinically relevant chemotherapies to kill gastrointestinal / genitourinary (GI/GU) cancer cells. In bladder cancer cells, regardless of H-RAS mutational status, at clinically achievable doses, PDE5 inhibitors interacted in a greater than additive fashion with doxorubicin / mitomycin C / gemcitabine / cisplatin / paclitaxel to cause cell death. In pancreatic tumor cells expressing mutant active K-RAS PDE5 inhibitors interacted in a greater than additive fashion with doxorubicin / gemcitabine / paclitaxel to cause cell death. The most potent PDE5 inhibitor was sildenafil. Knock down of PDE5 expression recapitulated the combination effects of PDE5 inhibitor drugs with chemotherapy drugs. Expression of c-FLIP-s did not significantly inhibit chemotherapy lethality but did significantly reduce enhanced killing in combination with sildenafil. Over-expression of BCL-XL suppressed individual and combination drug toxicities. Knock down of CD95 or FADD suppressed drug combination toxicity. Combination toxicity was also abolished by necrostatin or RIP1 knock down. Treatment with PDE5 inhibitors and chemotherapy drugs promoted autophagy which was maximal at ~24 h –post treatment, and 3-methyl adenine or knock down of Beclin1 suppressed drug combination lethality by ~50%. PDE5 inhibitors enhanced and prolonged the induction of DNA damage as judged by Comet assays and γH2AX and CHK2 phosphorylation. Knock down of ATM suppressed γH2AX and CHK2 phosphorylation and enhanced drug combination lethality. Collectively our data demonstrate that the combination of PDE5 inhibitors with standard of care chemotherapy agents for GI/GU cancers represents a novel modality.
Introduction

The majority of bladder cancers are defined as transitional cell carcinomas; carcinomas of the epithelial cells that are the inner lining of the bladder, including squamous cell and adenocarcinoma. In the United States there are approximately ~73,000 cases of bladder cancer diagnosed every year with ~15,000 deaths, with the majority of patients presenting with superficial bladder tumors (Stage 0 or Stage 1) (ACS: Cancer Facts and Figures, 2013; Hudson and Herr, 1995). Surprisingly, even in patients with disseminated disease > T1, there is the possibility of long-term complete response with multi-modal therapies (Torti and Lum, 1984; Catalona et al, 1987; Thrasher and Crawford, 1993).

In patients with superficial disease, the morphological differentiation status of the tumor cells also determines their response to the most widely used therapy against bladder cancer, the immuno-therapy Bacillus Calmette-Guerin (BCG). Of patients who present with poorly differentiated superficial carcinoma cells and who have a complete initial response to BCG, ~20% show disease progression at 5 years. However in individuals who do not initially fully respond to BCG there is a > 90% likelihood of disease progression and metastasis (Lacombe et al, 1996; Stein et al, 1998; Lamm et al, 1991; Quek et al, 2005). Several modalities are routinely used to treat bladder cancer patients including BCG, urethral surgery and cystectomy, intra-vesicle chemotherapy with mitomycin C (MITO), gemcitabine (Gemzar) and doxorubicin (DOX), and systemic chemo-/radio-therapy with gemcitabine (Gemzar) (Witjes et al, 1998; Malmstrom et al, 1999; Singal et al, 2000; Rivera et al, 2008; Shen et al, 2008; Grivas et al, 2013). DOX usefulness has been limited due to myelo-suppression and cardio-toxicity. Clearly novel approaches to use DOX, which will reduce normal tissue toxicity, would be helpful. Collectively, these chemotherapeutic approaches have been shown to provide at least 80-90% of patients with an enhanced 5 year disease free survival.
The erection dysfunction drugs sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) inhibit phosphodiesterase 5 (PDE5), the predominant phosphodiesterase enzyme in the corpus cavernosum which is essential for the regulation of vascular smooth muscle contraction through elevation of cGMP levels (Bender and Beavo, 2006). PDE5 inhibitors also protect the heart against ischemia/reperfusion injury and DOX-induced cardiomyopathy. The cardioprotective effect of PDE5 inhibitors is attributed to suppressing apoptosis and necrosis. Downstream of reduced PDE5 function, the effects of elevated cGMP levels include enhanced expression of nitric oxide synthase (NOS) enzymes, particularly endothelial NOS (eNOS) and inducible NOS (iNOS) and activation of protein kinase C isoforms and protein kinase G (Das et al, 2004; Das et al, 2005; Ockaili et al, 2002; Salloum et al, 2003; Salloum et al, 2006; Fisher et al, 2005; Salloum et al, 2008; Das et al, 2008; Das et al, 2009). Thus increased NOS levels lead to increased nitric oxide (NO) production.

PDE5 expression is also increased in multiple human carcinoma cell types including breast, colon, bladder and lung cancers (Bender and Beavo, 2006; Karami-Tehrani et al, 2012; Eggen et al, 2012; Zhang et al, 2012). At high non-physiologic levels sildenafil and vardenafil suppress tumor cell growth and induce caspase-dependent apoptosis in B-CLL cells (Sarfati et al, 2003). The PDE5 inhibitors sildenafil and vardenafil are also multi-drug resistance transporter inhibitors suggesting they may be useful in the treatment of CNS localized diseases where drug penetration across the blood brain barrier is an issue (Chen et al, 2012). More recently we have shown in prostate cancer cells and flank tumors that high concentrations of PDE5 inhibitors (~10 μM) enhance DOX lethality, and protect the heart from DOX toxicity (Das et al, 2010). Mitochondrial ROS is one key component of anti-tumor activity of DOX in tumor cells. In prostate cancer cells reactive oxygen species / reactive nitrogen species (ROS/RNS) levels in DOX and sildenafil treated cells were greater than those in either PDE5 inhibitor alone or DOX alone.
The present studies were designed to determine whether PDE5 inhibitors interacted with standard of care cancer chemotherapeutic agents to kill gastrointestinal / genitourinary (GI/GU) tumor cells in vitro. It is known that nitric oxide (NO) donors such as exisulind inhibit bladder carcinogenesis and that PDE5 inhibitors increase NO levels (Piazza et al, 2001). Our data show that PDE5 inhibitors interact in an on-target fashion with DOX, MITO and Gemzar to kill bladder cancer cells and do so through increased death receptor signaling mediated by caspase 8 and increased autophagy mediated by RIP-1.
**Materials and Methods.**

**Materials.**

Phospho-/total- antibodies were purchased from Cell Signaling Technologies (Danvers, MA) and Santa Cruz Biotech. (Santa Cruz, CA). All drugs were purchased from Selleckchem (Houston, TX). Commercially available validated short hairpin RNA molecules to knock down RNA / protein levels were from Qiagen (Valencia, CA). Antibody reagents, other kinase inhibitors, caspase inhibitors cell culture reagents, and non-commercial recombinant adenoviruses have been previously described (Cruickshanks et al, 2012; Park et al, 2008; Zhang et al, 2008; Bareford et al, 2011). Cell death assays were performed using both trypan blue exclusion and the Millipore Scepter system with 60 μm tips (Billerica, MA).

**Methods.**

*Cell culture and in vitro exposure of cells to drugs.* All established cancer lines were cultured at 37 °C (5% (v/v CO₂) *in vitro* using RPMI supplemented with 5% (v/v) fetal calf serum and 10% (v/v) Non-essential amino acids. For short-term cell killing assays and immunoblotting, cells were plated at a density of 3 x 10³ per cm² and 24 h after plating were treated with various drugs, as indicated. *In vitro* small molecule inhibitor treatments were from a 100 mM stock solution of each drug and the maximal concentration of Vehicle (DMSO) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study.

*Cell treatments, SDS-PAGE and Western blot analysis.* Cells were treated with various drug concentrations, as indicated in the Figure legends. SDS PAGE and immunoblotting was performed as described in refs. (Cruickshanks et al, 2012; Park et al, 2008; Zhang et al, 2008; Bareford et al, 2011).
Recombinant adenoviral vectors; infection in vitro. We generated and purchased previously noted recombinant adenoviruses as per refs. (Cruickshanks et al, 2012; Park et al, 2008; Zhang et al, 2008; Bareford et al, 2011). Cells were infected with these adenoviruses at an approximate m.o.i. as indicated in the Figure / Legend (usually 50 m.o.i.). Cells were incubated for 24 h to ensure adequate expression of transduced gene products prior to drug exposures.

Assessment of ROS Generation. Cancer cells were plated in 96 well plates. Cells were pre-incubated with dihydro-DCF (5 mM for 30 minutes). Fluorescence measurements were obtained 0–30 minutes after drug addition with a Vector 3 plate reader. Data are presented corrected for basal fluorescence of vehicle-treated cells at each time point and expressed as a –Fold increase in ROS levels.

Detection of cell death by Trypan Blue, Hoechst, TUNEL and flow cytometric assays. Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37 °C. Cell death assays were performed as described in refs. (Cruickshanks et al, 2012; Park et al, 2008; Bareford et al, 2011).

Assessment of autophagy. Cells were transfected with a plasmid to express a green fluorescent protein (GFP) tagged form of LC3 (ATG8). For analysis of cells transfected with the GFP-LC3 construct, the GFP-LC3 - positive vesicularized cells were examined under the X40 objective of a Zeiss Axiovert fluorescent microscope (Cruickshanks et al, 2012; Park et al, 2008; Bareford et al, 2011).
**Plasmid transfection.**

**Plasmids:** Cells were plated as described above and 24 h after plating, transfected. Plasmids (0.5 μg) expressing a specific mRNA or appropriate vector control plasmid DNA was diluted in 50 μl serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 μl Lipofectamine 2000 (Invitrogen), was diluted into 50 μl of serum-free and antibiotic-free medium. Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 min. This mixture was added to each well/dish of cells containing 200 μl serum-free and antibiotic-free medium for a total volume of 300 μl and the cells were incubated for 4h at 37°C. An equal volume of 2X medium was then added to each well. Cells were incubated for 48h, then treated with drugs. To assess transfection efficiency of plasmids we used a plasmid to express GFP and defined the percentage of cells being infected as the percentage of GFP+ cells. For all cell lines the infection efficiency was > 70%.

**siRNA:** Cells were plated in 60 mm dishes from a fresh culture growing in log phase as described above, and 24 h after plating transfected. Prior to transfection, the medium was aspirated and 1 ml serum-free medium was added to each plate. For transfection, 10 nM of the annealed siRNA, the positive sense control doubled stranded siRNA targeting GAPDH or the negative control (a “scrambled” sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used (predominantly Qiagen, Valencia, CA; occasional alternate siRNA molecules were purchased from Ambion, Inc., Austin, Texas). Ten nM siRNA (scrambled or experimental) was diluted in serum-free media. Four μl Hiperfect (Qiagen) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temp for 10 min, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37 °C for 2 h. One ml of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37 °C for 24-48 h before re-plating (50 x 10^3 cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0-48 h). Trypan blue exclusion assays and SDS PAGE / immunoblotting analyses were then performed at the indicated time points.
Data analysis. Comparison of the effects of various treatments was performed using one way analysis of variance and a two tailed Student’s t-test. Synergy was measured by the Method of Chou and Talalay; Combination Index (CI) values of less than 1.00 were considered synergistic. Differences with a p-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points from multiple experiments (± SEM).
Results

Initial studies examined whether there was a lethal interaction between FDA approved PDE5 inhibitors such as sildenafil and standard of care chemotherapeutic agents for bladder cancer including mitomycin C, doxorubicin, cisplatin and gemcitabine. Sildenafil enhanced the lethality of mitomycin C, doxorubicin, cisplatin and gemcitabine in bladder cancer cell lines in short-term survival assays (Figures 1A-1D (p < 0.05)). The toxic interaction of PDE5 inhibitors with chemotherapeutic agents was not just restricted to bladder cancer cells, as in pancreatic cancer cells sildenafil also enhanced the lethality of doxorubicin, paclitaxel and gemcitabine (Figure 1E (p < 0.05)).

Sildenafil is not the only FDA approved PDE5 inhibitor, with the chemically related vardenafil and chemically dissimilar tadalafil also being approved for use. Parallel combinatorial killing data to that using sildenafil were obtained using the PDE5 inhibitors vardenafil and tadalafil (Figures 2A and 2B (p < 0.05)). In long-term colony formation assays sildenafil enhanced the lethality of doxorubicin, mitomycin C and gemcitabine in an apparently greater than additive fashion (Figures 2C-2E (p < 0.05)). As measured by the Method of Chou and Talalay (Chou and Talalay, 1984), the range of Combination Index values for each of these panels were: Figure 2C, 0.36-0.19; Figure 2D, 0.58-0.43; Figure 2E, 0.65-0.55. As the measured Combination Indexes were less than 1.00, our data tend to argue that we were observing a synergy of drug interaction in terms of cell killing.

The PDE5 inhibitors we have used can also have inhibitory effects on other PDE enzymes. Thus we wished to determine whether inhibition of PDE5 itself was playing any role in the observed combinatorial killing effect. Knock down of PDE5 using three different siRNA molecules enhanced chemotherapy toxicity in vitro (Figures 3A-3C (p < 0.05)). Thus regardless of H-/K-RAS mutational status, or side-effects on other
PDE isoforms, the drug combination of PDE5 inhibitor plus chemotherapy agent resulted in greater than additive killing effects in tumor cells.

We next attempted to determine the mechanisms by which the drug combination killed cells; i.e. the mitochondrial / caspase 9 intrinsic pathway; the caspase 8 extrinsic pathway; autophagy; or necrosis. Expression of dominant negative caspase 9 reduced overall killing but did not prevent sildenafil enhancing chemotherapy toxicity (Figures 4A-4C). However, over-expression of either the mitochondrial protective protein BCL-XL or the caspase 8 inhibitor c-FLIP-s prevented sildenafil enhancing chemotherapy toxicity. Similar data were obtained in another GU cell type; prostate cancer cells (Figure 4D). Comparable data to that in bladder and prostate tumor cells were obtained when sildenafil was combined with paclitaxel and gemcitabine in GI pancreatic cancer cells (Figure 4E and 4F). The data obtained expressing the caspase 8 inhibitor c-FLIP-s or the mitochondrial protective protein BCL-XL suggests that both death receptor and aberrant mitochondrial signaling are part of the killing process. In agreement with the protective effect of the caspase 8 inhibitor c-FLIP-s, knock down of death receptor expression (CD95) or the linker protein Fas-associated death domain protein (FADD) expression suppressed the toxicity enhancing activity of sildenafil (Figures 4G-4I). The combination of sildenafil with chemotherapies increased plasma membrane surface levels of the death receptor CD95, demonstrating that the sildenafil and chemotherapy drug combinations induced receptor activation (Figure 5).

Based on the known actions of the individual drugs, treatment of cells with chemotherapy and/or sildenafil is expected to increase the levels of ROS and RNS. Incubation of cells with the nitric oxide synthase inhibitor N(G)-nitro-L-arginine (L-NAME) or the ROS quenching agent N-acetyl cysteine (NAC) significantly reduced the increase in CD95 plasma membrane localization by 67% +/- 8 and 79% +/- 9, respectively (p < 0.05).
Prior studies by our laboratory have shown that activation of the death receptor CD95 can increase the vesicularization of an expressed LC3-GFP fusion protein and elevate LC3II processing, both suggestive of autophagosome formation (autophagy). In the present studies combined exposure of cells to sildenafil and chemotherapy resulted in enhanced LC3-GFP vesicularization that correlated with enhanced processed LC3II levels and reduced levels of p62 and LAMP2, all indicative of autophagy flux i.e. formation of vesicles followed by their fusion with acidic endosomes and finally protein digestion (Figure 6A; 24 h time point, observe lane below arrow). Treatment of cells with the small molecule inhibitor of the class III PI3K vps34 (3-methyl adenine), an enzyme essential for vesicle formation, suppressed the lethality of sildenafil and chemotherapy at the 6 h time point, whereas the small molecule inhibitor of receptor interacting protein 1 (RIP-1) (necrostatin; inhibition of necrotic cell killing) abolished killing by all agents at this time (Figure 6B). Knock down of the autophagy regulatory protein Beclin1 suppressed the induction of LC3-GFP vesicles and reduced killing by the drug combinations (Figures 6C-6E, data not shown). Knock down of RIP-1, in a manner similar to necrostatin, abolished killing by all agents at this time (Figure 6C-6E). In general agreement with these findings, knock down of RIP-1 partially, though significantly, reduced the levels of autophagy caused by the drug combination treatments (Figure 6F). This suggests that downstream of CD95, RIP-1 may stimulate cell death through autophagy dependent and independent pathways.

Standard of care chemotherapy agents such as doxorubicin are known to cause DNA damage as part of their killing mechanism. Sildenafil rapidly and significantly enhanced the amount of DNA damage caused by doxorubicin or mitomycin C as judged in Comet assays (Figures 7A – images and 7B – quantified tail moments). The drug combination significantly increased the phosphorylation of γH2AX 6 h after exposure (Figure 7B, upper blots). Of greater interest was that at the 12 h time point where doxorubicin –induced γH2AX phosphorylation was declining, that of the drug combination was increasing. The phosphorylation of γH2AX is thought to be regulated via the ataxia telangiectasia mutated (ATM) protein. As previously
published by ourselves and others, knock down of ATM reduced drug-induced $\gamma$H2AX phosphorylation (data not shown; Golding et al, 2007; Booth et al, 2013). Knock down of ATM increased the toxicity of doxorubicin and of mitomycin C (Figure 7C). Knock down of ATM further enhanced the toxicity of sildenafil combined with doxorubicin or mitomycin C. Knock down of ATM suppressed doxorubicin-induced phosphorylation of CHK2 within 6 h (Figure 7D). The drug combination modestly activated the JNK1/2, ERK1/2 and AKT signal transduction pathways within 6 h; inhibition of JNK1/2 signaling suppressed killing and inhibition of MEK1 and AKT enhanced killing (Figure 7E).

PDE5 inhibitors increase the levels of cGMP and of nitric oxide (NO) in cells. NO regulates many cellular processes including the protection of normal tissues from noxious stimuli; however high levels of NO combined with high levels of ROS in tumor cells can result in the generation of the toxic molecule peroxynitrate (ONOO-). Thus we determined whether the generation of ROS / NO was involved in the toxic interaction between doxorubicin and sildenafil. Doxorubicin / mitomycin C and sildenafil interacted to increase the levels of ROS, as judged using DCFH (Figure 7F). Knock down of CD95 suppressed the production of ROS and knock down of ATM increased ROS production. Cells were incubated in the presence of the nitric oxide synthase inhibitor L-NAME. L-NAME or the ROS quenching agent NAC abolished the toxic interaction between doxorubicin and sildenafil (Figure 7G).
Discussion.

The present studies were performed to determine whether clinically relevant PDE5 inhibitors interacted with standard of care chemotherapy agents to kill bladder and pancreatic cancer cells. PDE5 inhibitors, regardless of whether the bladder cancer cell expressed a mutant active H-RAS protein, interacted with standard of care chemotherapy agents in a greater than additive fashion to kill bladder tumor cells. Similar findings were made in pancreatic cancer cells that express a mutant active K-RAS protein. The interaction between PDE5 inhibitors and standard of care chemotherapy agents was dependent on activation of the extrinsic pathway / death receptors. However, although inhibition of caspase 8, through c-FLIP-s, or mitochondrial protection using BCL-XL abolished the drug interaction, inhibition of caspase 9 only partially reduced overall killing. This implied another signal was emanating from the death receptor to cause killing and to enhance mitochondrial dysfunction. Thus downstream of CD95, we discovered that knock down of RIP-1 also was found to profoundly suppress the induction of both autophagy and tumor cell killing by the drug combination. Molecular inhibition of autophagy was partially protective. Hence the ability of PDE5 inhibitors to facilitate cytotoxic chemotherapy killing; killing that acts through autophagy and mitochondrial dysfunction, utilizes both the caspase 8 and RIP-1 pathways downstream of death receptors in bladder cancer (T24) cells (Figure 8).

Prior studies have shown that activation of CD95 can stimulate the induction of LC3-GFP vesicle formation and processing to LC3II (Zhang et al, 2008). Treatment of cells with sildenafil and doxorubicin increased LC3-GFP vesicle numbers and LC3II processing as well as lowering the levels of p62 / LAMP2, all indicative of autophagic flux. Knock down of Beclin1 reduced cell killing to a lesser extent than knock down of RIP-1 strong suppressed death. The small molecule inhibitor of RIP-1, necrostatin, blocks the death process known as “necroptosis,” a non-apoptotic cell death pathway. Inhibition of RIP-1 / necroptosis has been shown by others to not inhibit FAS-L -induced apoptosis nor does it alter the classical appearance of
apoptotic morphology (Liedtke et al, 2011). In our hands we observed necrotic nuclei and nuclei that had a mixed morphology of necrosis and apoptosis. As both caspase 8, RIP-1 and elevated autophagy are downstream of death receptors our data tends to suggest that RIP-1 / autophagy signaling complements caspase 8 signaling in our specific drug combination in bladder cancer cells (Figure 8). These findings are in contrast to those which argue autophagy and apoptosis can be antagonistic e.g. Amir et al, 2013. Clearly, our data in the present manuscript using agents which cause a DNA damage response are different from those using other non-DNA damaging drugs. Thus the mechanisms by which sildenafil can stimulate this form of killing will need further investigation.

One initial concern over our studies was that PDE5 inhibitors were enhancing the toxicity of multiple chemotherapies, implying that we may be observing an off-target phenomenon with respect to PDE5 inhibitor action. However, knock down of PDE5 using three different siRNAs argued that loss of PDE5 function did enhance chemotherapy toxicity. In addition, use of L-NAME and NAC argued that drug combination killing required the generation of reactive nitrogen / oxygen species, most probably downstream of elevated cGMP concentrations. Loss of ATM function is known to increase basal levels of ROS (Rosato et al, 2010). In our system knock down of ATM did not appreciably increase basal levels of ROS production but did facilitate the induction of ROS following exposure to chemotherapy or chemotherapy plus sildenafil. To our surprise was that knock down of CD95 suppressed the overall induction of ROS by all agents, except sildenafil; and that L-NAME and NAC both suppressed CD95 activation as judged by plasma membrane localization. These findings argue that CD95 by mechanisms unknown regulates ROS production shortly following drug combination exposure. Additional studies outside the scope of the present manuscript will be required to further understand the biology by which nitric oxide / ROS / cGMP signaling and the CD95 pathway interact.
A frequent mode of cell killing by “traditional” cytotoxic chemotherapies involves the damaging of DNA, and the subsequent inability of cells to fully repair this DNA damage. With doxorubicin, an agent that causes single and double stranded DNA breaks, we used two read-outs for DNA damage, the Comet assay and \( \gamma H2AX \) phosphorylation. By both measures, drug treatment caused DNA damage. The amount of damage caused by the chemotherapeutic drug was enhanced and prolonged by sildenafil. Sildenafil itself did not cause any DNA damage, and chemically this drug would not be expected to damage DNA. It is known that protein nitration can alter the formation of DNA repair complexes as well as regulate the phosphatases which regulate protein complex formation and our data argues that sildenafil treatment results in greater levels of DNA damage that are not repaired over time (Jones et al, 2009; Sturla et al, 2005). In general agreement with this hypothesis we note that the amount of cell killing observed in colony formation assays is much greater than that observed in short-term death assays at 24 h, indicating that prolonged DNA damage is resulting in an inability of cells to form colonies. Further studies beyond the scope of the present manuscript will be required to define how changes in protein repair complex formation are altered by sildenafil co-exposure.

The ATM protein controls multiple aspects of cell biology after DNA damage including cell cycle arrest, activation of signaling pathways, activation of apoptosis pathways, and DNA repair protein complex formation (and as previously described; ROS production) (Golding et al, 2007; Booth et al, 2013). We noted that not only was \( \gamma H2AX \) phosphorylation increased by the drug combination of sildenafil and doxorubicin compared to DNA damaging agent alone, but so too was phosphorylation of the ATM downstream target, CHK2 (Duong et al, 2013). In general it is believed that beyond a certain threshold, DNA damage –induced activation of ATM/CHK2 in a cell switches from an arrest/survival outcome to a programmed cell death outcome (Scafoglio et al, 2013). CHK2 can signal cell cycle arrest through CDC25C/CDC2, suppress CDC25A expression which leads to elevated ERK1/2 activity and cyto-protection, and shift the apoptotic
rheostat towards cell survival through modulation of reactive oxygen species levels and the transcriptional
regulation of pro- and anti-apoptotic genes (Roos and Kaina, 2006; Antoni et al, 2007; Stolz et al, 2011;
Lavin and Kozlov, 2007; Ward et al, 2001). Of note is that the PP2C family protein phosphatase Wip-1 is
induced by CHK2, which then acts to dephosphorylate and inactivate ATM and CHK2 i.e. a feedback loop
(Lu et al, 2005). In the presence of elevated ROS and RNS levels, however, any elevation in Wip-1 activity
will be somewhat negated due to covalent modification of the phosphatase by elevated ROS/RNS levels
(Sturla et al, 2005; Fang et al, 2003). This will permit CHK2 activity to be prolonged and pro-apoptotic, as
we observed in our time course. Further studies into the biology of Wip-1 in our system will require
additional investigation beyond our present studies.

Our prior studies in prostate cancer cells demonstrated, regardless of PTEN expression, that sildenafil and
doxorubicin interacted to suppress tumor growth in vivo. The present studies have focused in greater detail
on in vitro mechanism, and animal studies in bladder and pancreatic tumor cells are a planned future
direction. It is intriguing to wonder, however, whether, based on the known vascular response to sildenafil
exposure, i.e. vessel dilation, we also may also cause an increased penetration of our chemotherapy agents
into a tumor itself, which will also result in a greater level of tumor cell killing. Additional in vivo studies
measuring both antitumor effects and chemotherapy agent concentrations in the tumor using GC/MS will be
required to prove or refute this hypothesis.

In conclusion, our findings strongly argue that at physiologically achievable concentrations in the 1-3 μM
range, obtainable after 50-200 mg doses of sildenafil, clinically relevant PDE5 inhibitors enhance the
lethality of multiple well established chemotherapy agents (Milligan et al, 2002; Kanjanawart et al, 2011).
The drug interaction was dependent in part on expression of death receptors i.e. CD95, and was observed in
multiple tumor cell types. At present, based on our prior studies in prostate cancer cells, a Phase I trial is
open at VCU Massey Cancer Center combining doxorubicin with sildenafil with an assessment end-point of measuring cardio-toxicity (Das et al, 2010; NCT01375699). In this study sildenafil is given only proximal to the period of doxorubicin infusion (DOX being infused every 21 days). This transient level of dosing would reduce the likelihood of negative sequelae caused by prolonged exposure to PDE5 inhibitors. Hence, the present studies further argue for the addition of PDE5 inhibitors to multiple existing treatment regimens in bladder and pancreatic cancer patients. Further laboratory based and clinical studies will be required to understand more fully the mechanisms of drug interaction and the clinical utility of this therapeutic approach.
Authorship Contribution

Participated in research design: Dent, Kukreja, Grant, Poklepovic, Das, Durrant

Conducted experiments: Booth, Roberts, Cruickshanks, Conley.

Contributed new reagents: Fisher

Performed data analysis: Dent

Wrote or contributed writing of the manuscript: Dent.


MOLPHARM#2013/090043


Footnotes
Support for the present study was funded from PHS grants from the National Institutes of Health [R01-CA141704, R01-CA150214, R01-DK52825]; the Department of Defense [W81XWH-10-1-0009]. PD is the holder of the Universal Inc. Professorship in Signal Transduction Research. The authors have no conflicts of interest to report.
Figure Legends

Figure 1. The PDE5 inhibitor sildenafil interact with established cytotoxic chemotherapy agents to kill multiple bladder cancer cell lines. (A) Bladder cancer cells (HT-1376; J82; T24) were treated with mitomycin C (MITO 100-200 nM) and/or sildenafil (SIL, 2.0 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control. (B) Bladder cancer cells (HT-1376; J82; T24) were treated with doxorubicin (DOX 200-400 nM) and/or sildenafil (SIL, 2.0 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control. (C) Bladder cancer cells (HT-1376; J82; T24) were treated with cisplatin (CDDP 1000-2000 nM) and/or sildenafil (SIL, 2.0 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control. (D) Bladder cancer cells (HT-1376; J82; T24) were treated with gemcitabine (Gemzar 25-50 nM) and/or sildenafil (SIL, 2.0 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control. (E) Bladder and Pancreatic cancer cells (T24, PANC-1, Mia Paca2, AsPC-1) were treated with gemcitabine (Gemzar 25 nM) and/or paclitaxel (TAX, 10 nM) and/or sildenafil (SIL, 2.0 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control.

Figure 2. PDE5 inhibitors enhance doxorubicin or mitomycin C toxicity. (A) Bladder cancer cells (HT-1376; J82; T24) were treated with doxorubicin (DOX 400 nM) and/or vardenafil (VAR, 0.5 μM) and/or tadalafil (TAD, 2 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control. (B) Bladder cancer cells (HT-1376; J82; T24) were treated with mitomycin C (MITO 200 nM) and/or vardenafil (VAR, 0.5 μM) and/or tadalafil (TAD, 2 μM). Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in vehicle control.
greater than corresponding value in vehicle control. (C) J82 cells were plated as single cells in sextuplicate (250-500 cells per well). Twelve hours after plating cells were treated with vehicle, sildenafil (SIL, 1-4 μM), doxorubicin (DOX, 100-400 nM) or the combination in a fixed dose ratio. Cells were treated with drugs for 24 h followed by culture in drug free media for ~10 days. Colonies were fixed, stained and counted (n = 3, +/- SEM) * p < 0.05 less than DOX alone value. (D) J82 cells were plated as single cells in sextuplicate (250-500 cells per well). Twelve hours after plating cells were treated with vehicle, sildenafil (SIL, 1-3 μM), mitomycin C (MITO, 50-150 nM) or the combination in a fixed dose ratio. Cells were treated with drugs for 24 h followed by culture in drug free media for ~10 days. Colonies were fixed, stained and counted (n = 3, +/- SEM) * p < 0.05 less than MITO alone value. (E) Mia Paca 2 cells were plated as single cells in sextuplicate (250-500 cells per well). Twelve hours after plating cells were treated with vehicle, sildenafil (SIL, 1-3 μM), Gemcitabine C (Gemzar, 50-150 nM) or the combination in a fixed dose ratio. Cells were treated with drugs for 24 h followed by culture in drug free media for ~10 days. Colonies were fixed, stained and counted (n = 3, +/- SEM) * p < 0.05 less than Gemzar alone value.

**Figure 3. Knock down of PDE5 expression enhances doxorubicin or mitomycin C lethality.** Bladder cancer cells (HT-1376; J82; T24) were transfected with scrambled siRNA (siSCR) or one of three different siRNA molecules to knock down expression of PDE5 (si#1, si#2, si#3: Panels A-C). Thirty six h after transfection cells were treated with vehicle, mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than corresponding value in siSCR control.

**Figure 4. The toxic interaction between PDE5 inhibitors and chemotherapy is blocked by over-expression of BCL-XL or c-FLIP-s.** Bladder cancer cells (HT-1376; J82; T24) were infected with empty vector
adenovirus (CMV) or three other viruses to express dominant negative caspase 9 (dn casp 9); BCL-XL; c-FLIP-s: Panels A-C. Thirty six h after infection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), gemcitabine (Gemzar, 50 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 less than corresponding value in CMV control. (D) Prostate cancer cells (DU145) were infected with empty vector adenovirus (CMV) or three other viruses to express dominant negative caspase 9 (dn casp 9); BCL-XL; c-FLIP-s): Thirty six h after infection cells were treated with vehicle, sildenafil (SIL, 2 μM), doxorubicin (DOX, 200 nM), as indicated. PC-3 prostate cancer cells that lack PTEN and have constitutive AKT activity were used as an internal control. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 less than corresponding value in CMV control. (E and F) Pancreatic cancer cells (PANC-1, Mia Paca2) were infected with empty vector adenovirus (CMV) or three other viruses to express dominant negative caspase 9 (dn casp 9); BCL-XL; c-FLIP-s treated with vehicle, sildenafil (SIL, 2 μM), and/or gemcitabine (Gemzar, 50 nM) and/or paclitaxel (TAX, 10 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM). (G, H, I) Bladder cancer cells (HT-1376; J82; T24) were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of CD95 or FADD (siCD95, siFADD). Thirty six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), gemcitabine (Gemzar, 50 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 less than corresponding value in siSCR control.

Figure 5. The toxic interaction between PDE5 inhibitors and chemotherapy is associated with increased plasma membrane levels. Bladder T24 cells were grown in chamber slides. Twenty four h after plating cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM),
gemcitabine (Gemzar, 50 nM), as indicated. Cells were fixed 6 h after exposure and the cell surface levels of CD95 under each condition determined by immunohistochemistry.

**Figure 6. Sildenafil and chemotherapy–induced lethality is mediated through RIP-1 and increased autophagy.** (A) **Lower:** T24 cells were transfected with a plasmid to express LC3-GFP. Twenty-four h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), gemcitabine (Gemzar, 50 nM), as indicated. Cells were examined 6 h and 24 h after treatment using a fluorescent microscope and the mean number of LC3-GFP+ vesicles determined in > 40 cells (n = 3, +/- SEM). **Upper:** T24 cells were treated with vehicle, sildenafil (SIL, 2 μM), doxorubicin (DOX, 200 nM), as indicated. Cells were isolated 6 h and 24 h after treatment and immunoblotting performed to examine the phosphorylation / expression of the indicated proteins (n = 3). (B) T24 cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), as indicated. In parallel cells were treated with vehicle, necrostatin (1.0 μM) or 3-methyl adenine (3MA, 10 mM). Cells were isolated after 6 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 less than corresponding value in VEH control. (C, D, E) Bladder cancer cells (HT-1376; J82; T24) were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of RIP-1 or Beclin1 (siRIP-1, siBeclin1). Thirty-six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), or doxorubicin (DOX, 200 nM), as indicated. Cells were isolated after 12 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 less than corresponding value in siSCR control. (F) T24 cells were transfected to express LC3-GFP and with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of RIP-1 (siRIP-1). Thirty-six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), or doxorubicin (DOX, 200 nM), as indicated. Cells were examined 24 h after treatment using a fluorescent microscope and the mean number of LC3-GFP+ vesicles determined in > 40 cells (n = 3, +/- SEM).
Figure 7. Sildenafil increases and prolongs chemotherapy–induced DNA damage; knock down of ATM enhances drug combination toxicity. (A). T24 cells were grown in soft agar, and treated as indicated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), as indicated, for 6 h. Cells were subjected to electrophoresis and stained. Images are a representative (n = 4). (B) Graph: The tail moments of cells imaged in panel (A) were measured and plotted (n = 4, +/- SEM) # p < 0.05 greater than corresponding value in vehicle treated cells. Blot: T24 cells were treated with vehicle, sildenafil (SIL, 2 μM), doxorubicin (DOX, 200 nM), as indicated. Cells were isolated 6 h and 12 h after exposure and the phosphorylation of γH2AX determined, with the –Fold increase in phosphorylation shown (n = 3, +/- SEM). (C) T24 cells were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of ATM (siATM). Thirty six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), or doxorubicin (DOX, 200 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than DOX/MITO only treated cells; ## p < 0.05 greater than corresponding value in siSCR control. (D) and (E) BLOT: T24 cells were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of ATM (siATM). Thirty six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM) and/or doxorubicin (DOX, 200 nM), as indicated. Cells were isolated after 6 h and the expression and the phosphorylation of the indicated proteins determined (n = 3). GRAPH: T24 cells were transfected with an empty vector plasmid (CMV) or plasmids to express dominant negative MEK1 and dominant negative AKT. Twenty four h after transfection cells were as indicated treated with the JNK inhibitory peptide (JNK-IP, 10 μM) and then treated with vehicle or sildenafil (SIL, 2 μM) and doxorubicin (DOX, 200 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 greater than SIL+DOX in CMV cells; * p < 0.05 less than corresponding value in CMV. (F) T24 cells were plated in 96 well plates in phenol red free media. T24 cells were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down expression of ATM (siATM) or CD95 (siCD95). Thirty six h after transfection cells were treated with vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), or doxorubicin (DOX, 200 nM), as indicated. Cells were
incubated with dihydro-DCF (5 mM for 10 minutes). Fluorescence measurements were obtained 6 h and 12 h after drug addition with a Vector 3 plate reader. Data are presented including basal fluorescence of vehicle-treated cells at each time point (n = 3 +/- SEM). # p < 0.05 greater than DOX/MITO only treated cells; ¶ p < 0.05 greater than corresponding value in siSCR control; * p < 0.05 less than sildenafil / doxorubicin alone. (G) Bladder cancer cells (HT-1376; J82; T24) were treated with vehicle or L-NAME (10 mM) or NAC (10 mM) followed 30 min later by vehicle, sildenafil (SIL, 2 μM), mitomycin C (MITO, 100 nM), doxorubicin (DOX, 200 nM), gemcitabine (Gemzar, 50 nM), as indicated. Cells were isolated after 24 h and viability determined by trypan blue exclusion (n = 3, +/- SEM).

Figure 8. A schematic of how PDE5 inhibitors and DNA damaging drugs interact to kill T24 cells. PDE5 inhibitors such as sildenafil inhibit PDE5 that leads to increased cGMP levels, increased iNOS expression and elevated levels of NO. DNA damaging drugs stimulate the production of ROS, that is counteracted by ATM. ROS and NO generate peroxynitrite which inhibits the protein tyrosine phosphatase that inhibits CD95. CD95 becomes activated, stimulating: (a) the extrinsic apoptosis pathway; (b) through RIP-1 an autophagy pathway that also leads to cell killing.
Figure 6

A. Mean number of LC3 vesicles per cell

B. Percentage cell death

C. Percentage cell death

D. Percentage cell death

E. Percentage cell death

F. Mean number of LC3-GFP vesicles per cell
Figure 8