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Disulfiram and Thimerosal

Synergistically Induce Apoptosis in Prostate Cancer Cells

A thesis submitted in partial satisfaction

of the requirements for the degree

Master of Science in Physiological Science

by

Elaine Lee Hsia

2013

ABSTRACT OF THE THESIS

Disulfiram and Thimerosal
Synergistically Induce Apoptosis in Prostate Cancer Cells

By

Elaine Lee Hsia

Master of Science in Physiological Science

University of California, Los Angeles, 2013

Professor R. James Barnard, Co-chair

Professor Jiaoti Huang, Co-chair

Prostate cancer is the second leading cause of cancer death in western men. With limited treatment options for patients with castration-resistant prostate cancer, novel approaches are needed. We previously found a novel combination of two compounds that synergistically decreased prostate cancer cell viability at low concentrations. The goal of this project was to explore the mechanism by which the combination treatment leads to synergistic cell death. Molecular experiments were performed through Western blots, cell viability assays, and pull-down assays to examine cell proliferation, apoptosis, cellular redox status, JNK protein levels, and sumoylation. We found that the drug combination synergistically induces apoptosis mediated by increasing levels of reactive oxygen species. The combination treatment leads to enhanced

JNK activity, which likely derives from increased sumoylation of JNK. We found a potential sumoylation site whose mutation results in drastic reduction of sumoylation of JNK.

The thesis of Elaine Lee Hsia is approved.

Dwayne De Angelo Simmons

R. James Barnard, Co-Chair

Jiaoti Huang, Co-chair

University of California, Los Angeles

2013

TABLE OF CONTENTS

LIST OF FIGURES	vi
ACKNOWLEDGEMENTS	vii
INTRODUCTION	1
METHODS	4
RESULTS	7
Disulfiram and thimerosal synergistically inhibit cell proliferation in prostate cancer cell lines	7
Disulfiram and thimerosal synergistically induce apoptotic cell death mediated by redox Regulation in prostate cancer cells	9
Jnk plays a role in disulfiram and thimerosal mediated cell death	10
Disulfiram can regulated sumoylation in vivo and in vitro	13
Disulfiram and thimerosal can synergistically induce jnk level and activity through sumoylation.....	15
DISCUSSION	17
REFERENCES.....	22

LIST OF FIGURES

Figure 1A. Disulfiram and thimerosal dose-response curve.....	8
Figure 1B. Disulfiram and thimerosal synergize.....	8
Figure 2A. Disulfiram and thimerosal co-treatment leads to apoptosis.....	9
Figure 2B. Disulfiram and thimerosal increase oxidative stress.....	10
Figure 3A. Disulfiram and thimerosal lead to increased levels of phosphorylated JNK.....	11
Figure 3B. Dosage curve of JNK inhibitor on PC3 cell viability.....	12
Figure 3C. Inhibition of JNK in disulfiram and thimerosal treated cells prevents cell death.....	12
Figure 4. Disulfiram and thimerosal increases sumoylation.....	13
4A. Disulfiram and thimerosal increases sumo-1 conjugation.....	13
4B. Disulfiram and thimerosal increases sumo-2/3 conjugation.....	13
Figure 4C. Effect of disulfiram and thimerosal on pro-sumo-1 and pro-sumo-2 cleavage by SENP1.....	14
Figure 5A. Disulfiram and thimerosal treatment leads to stabilization of JNK.....	16
Figure 5B. JNK can be sumoylated at amino residue 160.....	17
Figure 6. Mechanistic model of disulfiram and thimerosal-mediated apoptosis.....	21

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INTRODUCTION

Prostate cancer is the second leading cause of cancer related death in men in the United States (Jemal et al. 2011). With current drug therapy options for advanced prostate cancers that can at most only extend life up through a few years due to eventual resistance development to the drugs used, novel approaches are needed to expand treatment options. Recently, we performed a high-throughput screen of 5,000 small molecule compounds from a library containing more than 1,000 U.S. Food and Drug Administration (FDA)-approved drugs as well as purified natural products and other compounds with known safety profiles that led to the identification of disulfiram and thimerosal as a novel synergistic combination that induces cell death in prostate cancer cells. The goal of this project was to explore the mechanism by which this combination treatment leads to synergistic cell death.

Disulfiram is prescribed as treatment for alcohol abuse and is known for its anti-alcoholism effects through its inhibition of aldehyde dehydrogenase (Vallari and Pietruszko 1982). Disulfiram has been reported to have anti-cancer effects in a variety of cancer cell lines including breast cancer, melanoma, leukemia, myeloma, lung cancer, cervical adenocarcinoma, colorectal cancer, glioblastoma, and prostate cancer (Chen et al. 2006, Brar et al. 2004, Wickstrom et al. 2007, Wang et al 2003, Triscott et al. 2012, Iljin et al. 2009). While several Phase I clinical trials have recently been completed in using disulfiram to treat liver cancer metastases, melanoma metastases, lung cancer metastases, and non-metastatic recurrent prostate cancer, it still remains to be seen whether the potent effects *in vitro* can be replicated in patients (www.clinicaltrials.gov, identifiers: NCT00742911, NCT00256230, NCT00312819, NCT01118741). It has been proposed that disulfiram's anti-cancer activity may arise from its ability to inhibit DNA methyltransferases, proteasomes, P-glycoproteins, NFκB, and

angiogenesis (Lin et al. 2011, Chen et al. 2006, Loo and Clarke 2000, Wang et al. 2003, Brar et al. 2004). Additionally, disulfiram has been reported to alter cellular redox status to elicit apoptosis (Morrison et al 2010, Yip et al. 2011, Cen et al. 2002).

Thimerosal is an ethylmercury-containing compound with antibacterial and antifungicidal properties that has been used as a preservative in various antiseptic products and in childhood vaccines (Geier et al. 2007). In the laboratory setting, thimerosal is used as a sulfhydryl reagent and can cause release of calcium from the intracellular stores in many cell types (Thorn et al. 1992, Hecker et al. 1989, Martin et al. 1991, Hatzelmann et al. 1990). Thimerosal has been shown to induce oxidative stress and cause apoptosis as a result of mitochondrial related mechanisms, activation of p38 MAP kinase and caspase-3 pathways, and suppression of the PI3K/Akt/Survivin pathway (Lee et al. 2006, Triscott et al. 2006, Makani et al. 2002, Humphrey et al. 2005, Yel et al 2005, Liu et al. 2007, Baskin et al. 2003, Li et al. 2012).

Reactive oxygen species (ROS) are generated in the cell through various processes such as mitochondrial respiration and function as a normal part of cellular signaling. While ROS has the potential to damage DNA, proteins, and lipids, ROS levels are normally kept in check by intracellular antioxidant systems consisting of non-enzymatic antioxidants and enzymatic antioxidants such as glutathione, superoxide dismutases, and catalases (Martindale and Holbrook 2002). Increased ROS generation has been associated with the malignant transformation of cells and tumor development through DNA damage affecting genomic stability and gene expression (Fruehauf and Meyskens 2007). In contrast to the tumorigenicity of elevated ROS, recent studies have shown that pushing the ROS levels even higher, exceeding the antioxidant capacity of the cells, leads to apoptosis (Huang et al. 2000, Pelicano et al. 2003). Thus, agents that increase oxidative stress may hold promise as anticancer therapies (Wondrak 2009).

Mitogen-activated protein kinase (MAPKs) pathways are involved in the regulation of a wide variety of cellular processes including proliferation, differentiation, stress adaptation, and apoptosis. MAPKs are divided into three major groups: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and the p38 kinases. The p38 and JNK pathways, also called the stress-activated protein kinase (SAPK) pathways, are known to be activated by stimuli such as cytokines, radiation, osmotic shock, mechanical injury, heat stress, and oxidative damage (Martindale and Holbrook 2002). The activation of the SAPKs under stress appears to be important in promoting apoptosis in several cell types. Of the SAPKs, c-jun kinase (JNK) is thought to play a key role in activating the mitochondria-dependent apoptotic pathway (intrinsic pathway), but dispensable for apoptosis induced by the activation of death receptors (extrinsic pathway) (Benhar and Engelberg 2002).

SUMO (small ubiquitin-related modifier) proteins are a family of reversible post-translation modification proteins similar in structure to ubiquitin. In humans, the family consists of SUMO-1, -2, and -3. SUMO proteins are expressed in an immature pro-form and must be cleaved after an invariant Gly-Gly motif at the C-terminal end by sentrin specific protease (SENPs) before they can be conjugated to substrates. Conjugation to substrates in sumoylation occurs through a series of enzymes involving an E1 activating enzyme AOS1-UBA2, an E2 conjugating enzyme UBC9, and an E3 ligase, in a process similar to ubiquitination. In addition to processing immature SUMO proteins, SENPs also deconjugate SUMO proteins (Geiss-Friedlander and Melchior 2007). Sumoylation is regulated by stresses including heat shock, osmotic and high oxidative stress (Bossis and Melchior 2006). The functional consequences of sumoylation are varied and can affect properties such as the stability, localization, or activity of the target protein (Geiss-Friedlander and Melchior 2007).

In this study we examined the molecular mechanism behind the synergistic effects of disulfiram and thimerosal on cell death in prostate cancer cell lines. These studies demonstrate that disulfiram and thimerosal increase oxidative stress, which causes apoptosis involving caspase-8 and leads to a global increase of sumoylation. We also show that JNK is activated and provide evidence for sumoylation of JNK at a specific amino acid residue that can help stabilize the JNK protein.

METHODS

Cell lines and reagents

Cells were maintained at 37°C in 5% CO₂. PC3 and 293T cells were cultured in Dulbecco's modified Eagle's medium (ATCC). C4-2 cells and Du145 were cultured in RPMI. Both types of medium were supplemented with 10% fetal bovine serum, streptomycin, and penicillin. The cells were seeded at a density of 1.2 x10⁵ cells/well in a 6-well dish 1 day prior to treatment. Thimerosal was purchased from (Sigma) and was dissolved into ddH₂O. Disulfiram was purchased from (Aldrich) and dissolved in DMSO. InSolution JNK inhibitor II was purchased from Calbiochem as well as Cycloheximide.

Transfection

293T cells were transfected in BioT transfection reagent (Bioland Inc.) with 0.5µg of DNA (for a 24-well plate) according to the manufacturer's instructions. Plasmids used were either his-tag JNK (pcDNA3-6xHis JNK1a2), mJNK (pcDNA3-6xHis-JNK1a2-KR), or Flag-sumo-1 (Xinhua Feng, MD Anderson, UT) or control vector (PBSK). Media was changed to complete medium 24 hours post-transfection. Cells were collected in PBS using a micropipette

48 hours post-transfection, kept on ice, pelleted and lysed in 1% NP40 lysis buffer (50mM Tris-HCl pH7.5, 200mM NaCl, 20mM KCl, 50mM NaF, 1mM EGTA, 1mM DTT, glycerol, ddH₂O, and 1x protease inhibitors), spun down again at 4°C.

Cell Viability Assay

Cell proliferation was measured by either the CellTiter-Glo Cell Viability Assay (Promega) or the WST-1 Assay (Cayman chemical).

CellTiter-Glo Cell Viability Assay – Cells were seeded into 96 well plates, treated with disulfiram and thimerosal, the assay reagents added, and then the plate read for luminescence according to the manufacturer's instructions.

WST1 assay – final concentration of 0.1mM WST1 with 2µM 1-methoxy PMS was used. Cells were left in these compounds for 3-4 hours before 100uL from the wells were transferred into a separate plate for the reading at OD 420nm.

SDS-PAGE and Western Blot

Cells were collected in PBS using a scraper and then stored at -80°C until use. Cells were lysed in 1% NP40 lysis buffer (50mM Tris-HCl pH7.5, 200mM NaCl, 20mM KCl, 50mM NaF, 1mM EGTA, 1mM DTT, glycerol, ddH₂O, and 1x protease inhibitors). For examination of overall sumoylation of sumo-1, sumo-2/3, and p-JNK, sodium orthovanadate (10mM) and beta-glycerophosphate (100mM) was added to the lysis buffer. Lysate was spun down at 12,000g for 10 minutes. Supernatant was removed and 2x sample buffer with DTT was added.

The mixture was boiled for 8 minutes at 95°C before separation by SDS-PAGE. Proteins were transferred to a PVDF membrane. The PVDF was blocked with 5% nonfat dry milk (nfdm) in

phosphate-buffered saline for 30 minutes prior to incubation with primary antibody overnight at 4°C in either 5% nonfat dry milk or 5% BSA. Membranes were washed in TBST (Tris buffered saline 0.1% Tween-20) for 30 minutes and then incubated with the appropriate secondary antibody (goat, anti-rabbit 1:8000 or goat, anti-mouse 1:8000) in nfdm for 30 minutes. After the final wash (1 hour), bands were detected with enhanced chemiluminescence either Femto or Dura (Pierce). Anti-flag-HRP 1:2000 (Sigma) was used for the detection of SUMO-1 in the JNK sumoylation assay. Rabbit anti-SUMO-1 antibody 1:1000 (Cell Signaling) was used to detect SUMO-1 conjugation in the PC3 cell line. The other primary antibodies used and their dilutions are as follows: Rabbit anti-SUMO-2/3 antibody 1:1000 (Cell Signaling), Mouse anti-Caspase-8 antibody 1:1000 (Cell Signaling). Rabbit anti-p-JNK antibody 1:500 (GenScript), and Rabbit anti-SAPK/JNK antibody 1:500 (GenScript).

***In vitro* SENP activity assay**

A 2x reaction buffer was created using 100mM Tris pH8.0, 40mM NaCl, 2mM DTT, and ddH₂O. SENP1 catalytic domain (Boston Biochem) was diluted 100x in 10% glycerol and 1x reaction buffer. The diluted SENP1 catalytic domain was mixed with drug compounds in 1x reaction buffer at 37°C for 10 minutes and then either His₆-pro-sumo-1 substrate (Boston Biochem) or His₆-pro-sumo-2 substrate (Boston Biochem) in 1x reaction buffer was added for 20 minutes at 37°C, boiled, and then loaded into 20% gel.

JNK sumoylation assay

Cell pellets were lysed in 115 µL of RIPA buffer (20mM KCl, 200mM NaCl, 1% IGEPAL, 0.1%SDS, 0.5% sodium deoxycholate (in water), 50mM Tris pH 7.5, 50mM NaF,

50mM NEM, 1x protease inhibitors, 1mM DTT, ddH₂O) and centrifuged at 4°C for 10 minutes. 100 µL of the supernatant was removed and added to 400 µL of guanidine buffer (7M Guanidine hydrochloride, 25mM NaPO₃ buffer at pH 7.5, 12.8 mM imidazole, 0.5mM DTT, 1µg/ml PMSF) at room temperature. Samples were incubated with 10µL Ni-NTA agarose beads slurry for 2 hours at room temperature. Beads were spun down and supernatant was removed. Beads were washed with 20000µL of urea wash buffer (6M) three times before addition of sample buffer and boiling at 95°C for 10 minutes. Then supernatant was loaded into an 8% SDS PAGE gel followed by an immunoblot analysis with anti-flag-HRP antibody.

RESULTS

Disulfiram and thimerosal synergistically inhibit cell proliferation in prostate cancer cell lines

To validate the synergistic effects of disulfiram and thimerosal on prostate cancer cell viability, we studied the effect of disulfiram and thimerosal in PC3 and C4-2 prostate cancer cells (Data for C4-2 not shown). Cells were treated singly with disulfiram or thimerosal or their combination at varying concentrations. The results show that the combination's effect in PC3 cells reduced viability more than either compound used alone at low concentrations (Fig.1A, B).

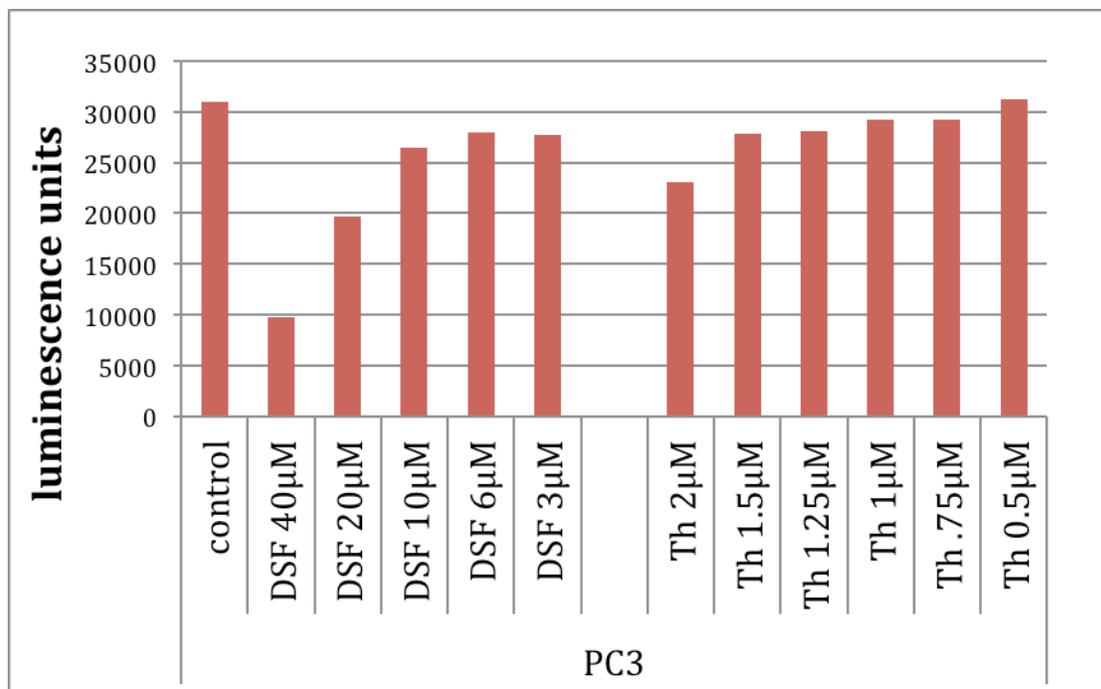


Figure 1A. Disulfiram and thimerosal dose-response curve. Disulfiram (DSF) and thimerosal (Th) shows synergy in PC3 cells. PC3 cells were seeded at 2000/well in a 96 well plate in singlets. DMSO was added to one well as a control. Cells were treated for 24 hours before reading with the plate reader.

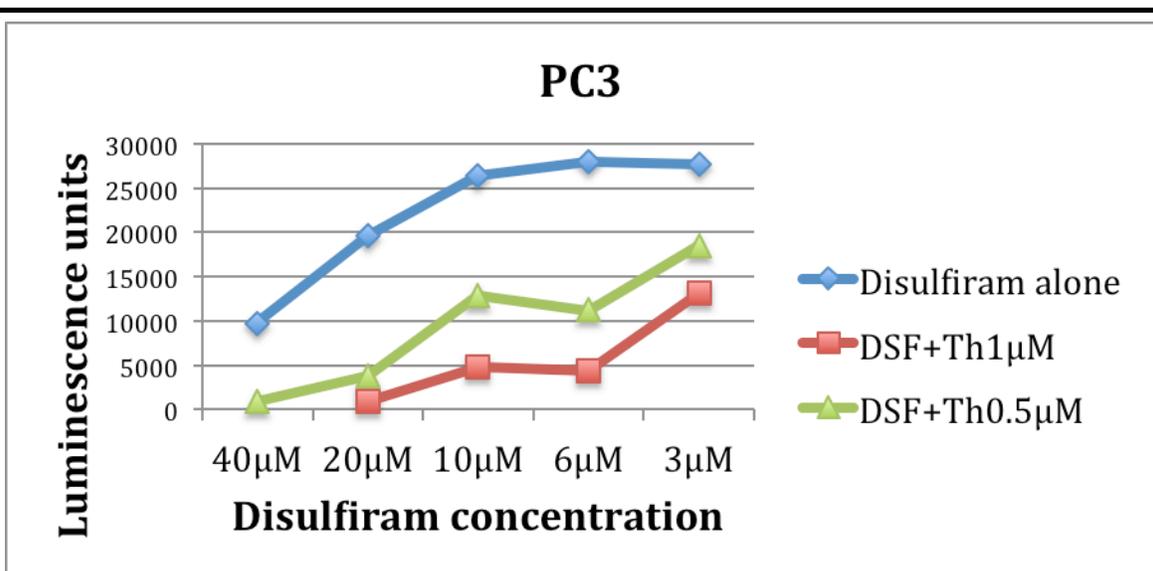
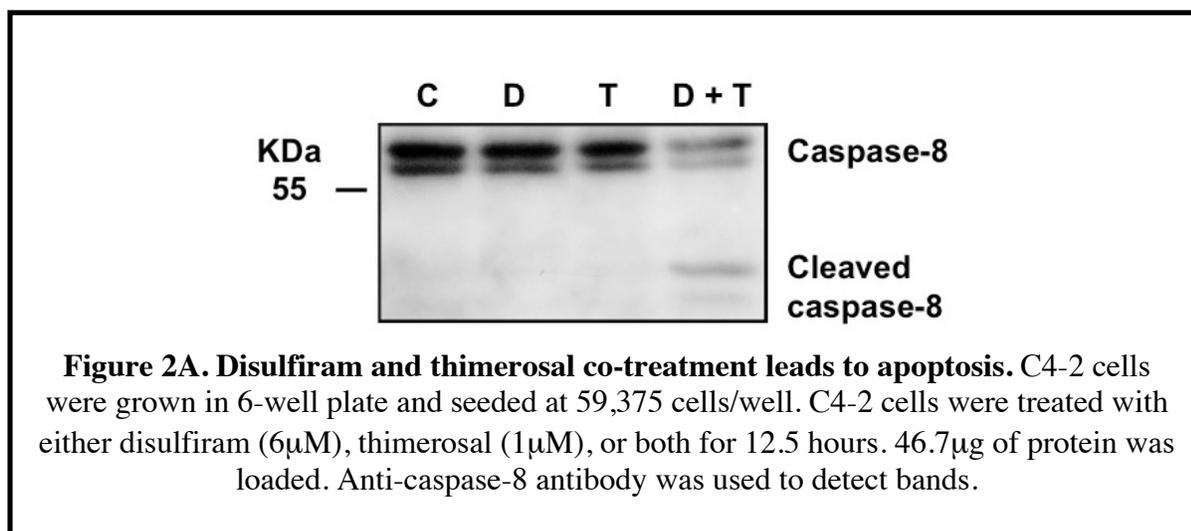


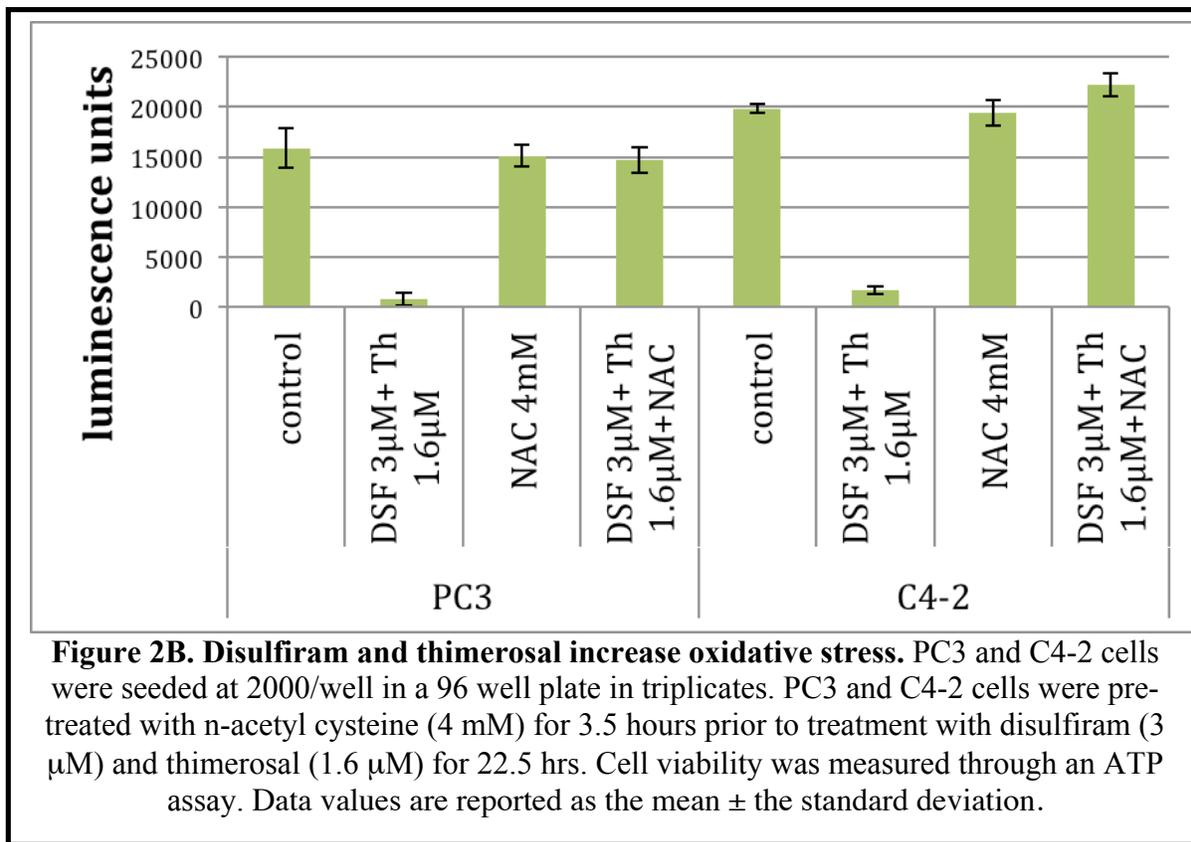
Figure 1B. Disulfiram and thimerosal synergize. Cell viability assay was performed through an ATP measurement method. PC3 cells were seeded at 2000/well in a 96 well plate. Cells were treated for 24 hours before reading with the plate reader. *Thimerosal alone values excluded from graph for 1mM and 0.5mM because both of these values lie above the data points shown on the graph for disulfiram alone.

Disulfiram and thimerosal synergistically induce apoptotic cell death mediated by redox regulation in prostate cancer cells

To identify whether disulfiram and thimerosal co-treatment induces apoptosis, caspase-8 cleavage was examined through western blot analysis. Cells were treated for 12.5 hours with disulfiram (6 μ M) and thimerosal (1 μ M) alone and in combination in C4-2 cells. Neither disulfiram nor thimerosal alone was able to induce caspase-8 cleavage. However, cleavage is clearly seen with the disulfiram and thimerosal combination. This data suggests that disulfiram and thimerosal co-treatment induces apoptosis (Fig. 2A).



We examined the role of oxidative stress in thimerosal and disulfiram-mediated apoptosis. PC3 and C4-2 cells were pre-treated with N-acetyl Cysteine (NAC 4 mM) for 3.5 hours and then incubated with disulfiram (3 μ M) and thimerosal (1.6 μ M) for 22.5 hours (Fig. 2B). In both PC3 and C4-2 cells, pre-treatment with NAC prevented the effects of the disulfiram and thimerosal combination treatment. This suggests that the combination treatment promotes apoptosis by increasing ROS levels.



JNK plays a role in disulfiram and thimerosal mediated cell death

The effect of disulfiram and thimerosal on JNK activity was examined. Both single treatments of disulfiram (6µM) and thimerosal (1µM) show an increase in phosphorylated JNK relative to the de-phosphorylated JNK. With the disulfiram (6µM) and thimerosal (1µM) combination treatment, a marked increase in phosphorylated JNK relative to de-phosphorylated JNK was seen. Total JNK is seen to decrease with single and combination treatments (Fig. 3A).

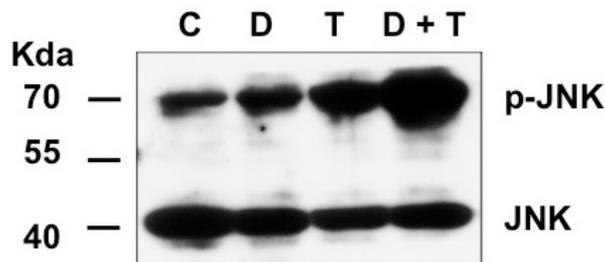
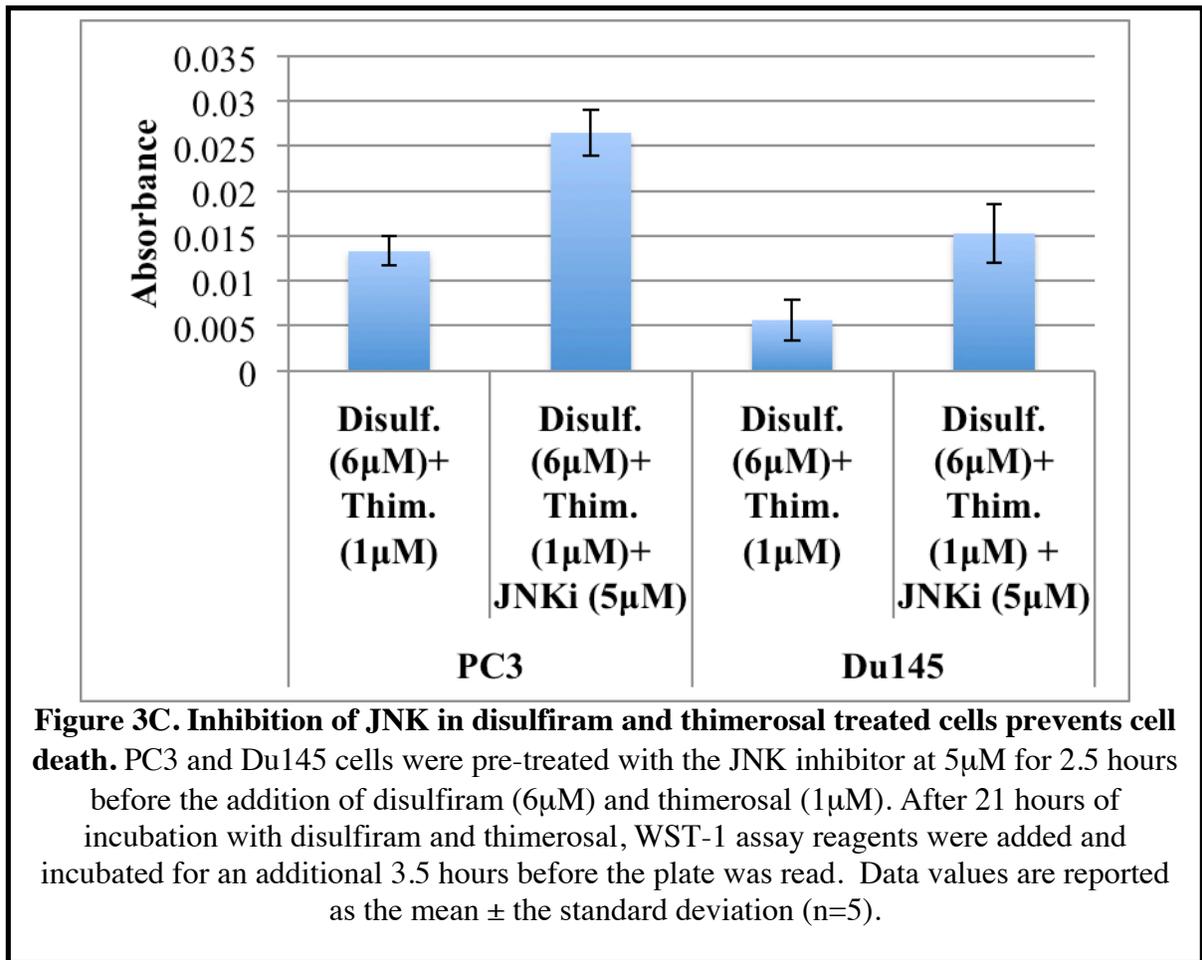
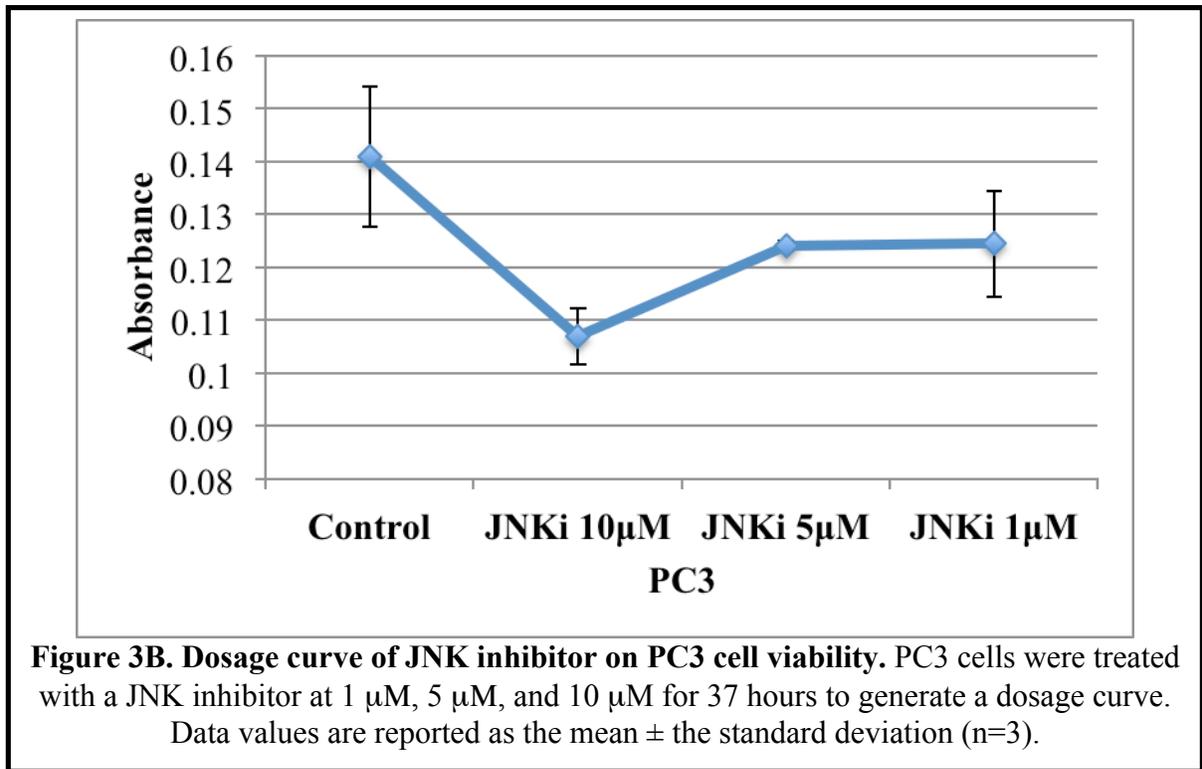


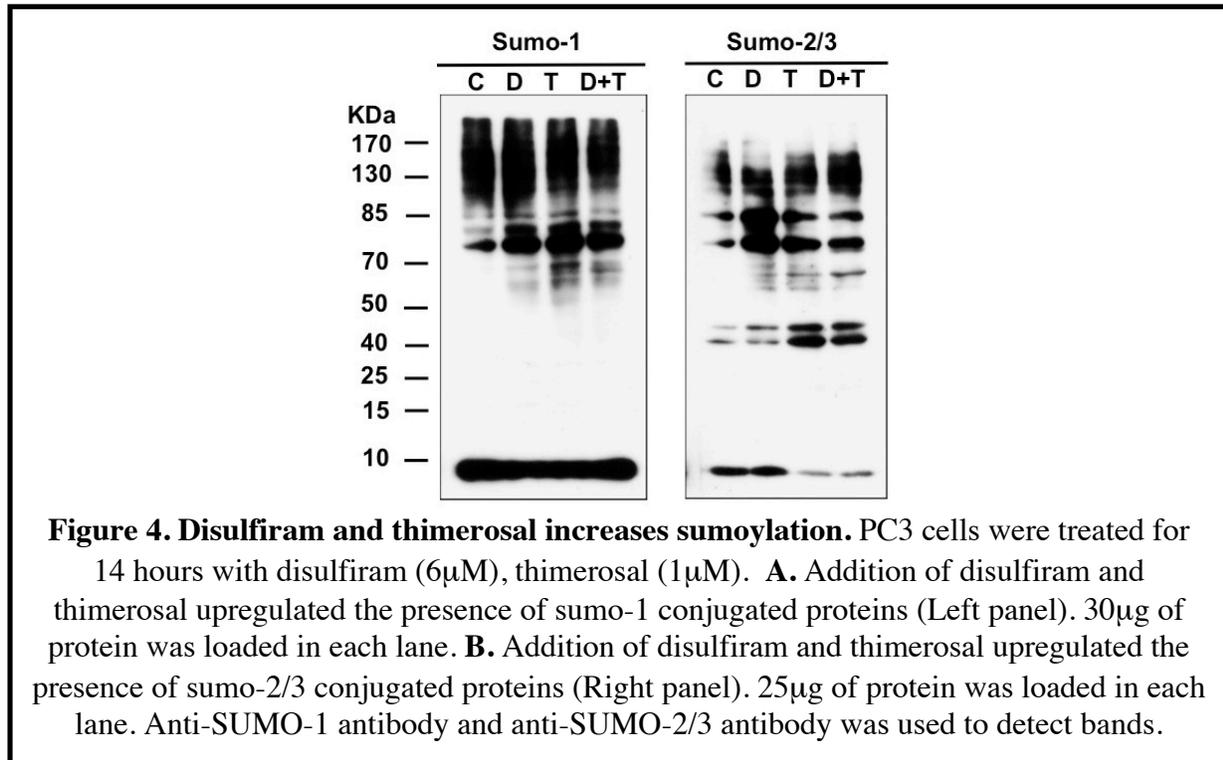
Figure 3A. Disulfiram and thimerosal leads to increased levels of phosphorylated JNK. PC3 cells treated with disulfiram (6 μ M), thimerosal (1 μ M) for 14 hours. 30 μ g protein loaded. 2.48×10^5 cells were seeded per well into a 6-well plate. Anti-p-JNK antibody was used to detect bands.

To examine the role of JNK in cell death mediated by disulfiram and thimerosal, we tested the effect of a chemical JNK inhibitor. PC3 and Du145 cells were seeded at 2000/well and pre-treated with the JNK inhibitor at 5 μ M for 2.5 hours before the addition of disulfiram (6 μ M) and thimerosal (1 μ M) (Fig. 3C). The concentration of the inhibitor was chosen based on a dosage curve we generated (Fig. 3B). Inhibition of JNK was seen to antagonize the effects of disulfiram and thimerosal on cell viability in both cell lines (Fig. 3C).

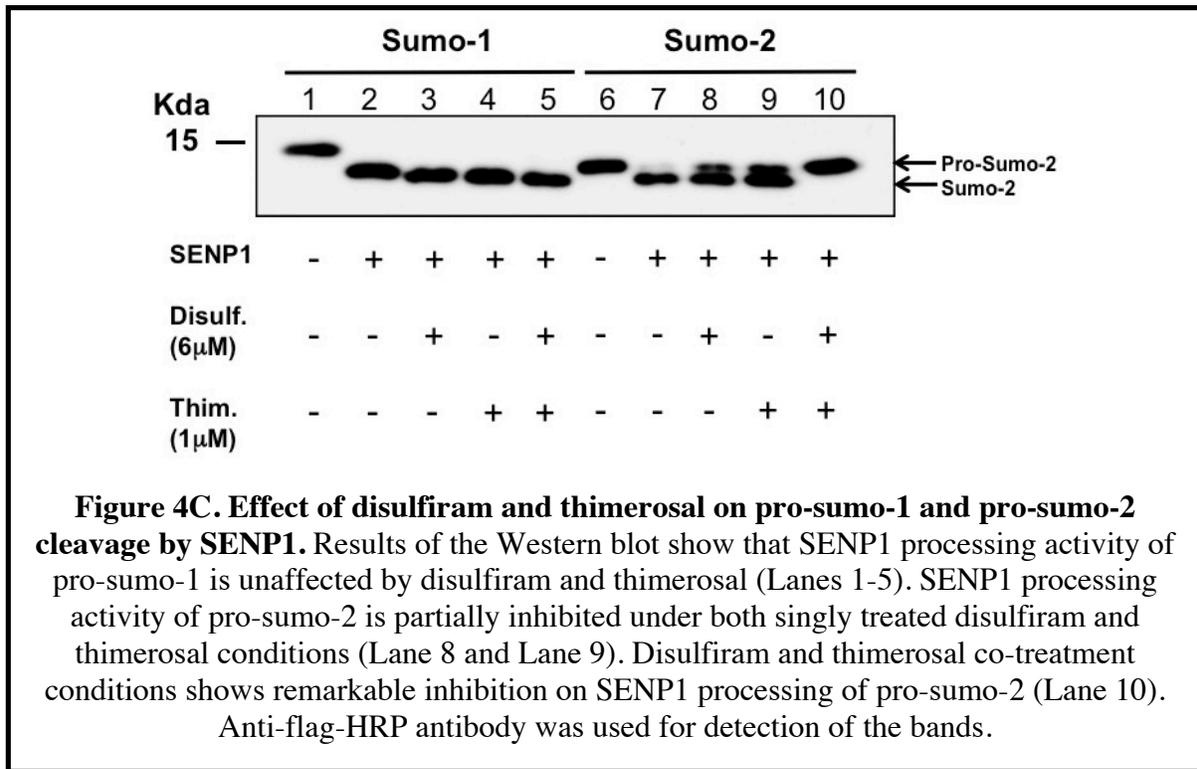


Disulfiram and thimerosal can regulate sumoylation in vivo and in vitro

It has been reported that oxidative stress can induce sumoylation (Manza et al. 2004, Saitoh and Hinchey 2000, Zhou et al. 2004). We examined SUMO-1 and SUMO-2/3 levels in the treated cells. PC3 cells were seeded at 2.48×10^5 cells per well into 6-well plate and treated for 14 hours with disulfiram ($6 \mu\text{M}$) and , thimerosal ($1 \mu\text{M}$). SUMO-1 conjugation increased in all treatment groups compared to the untreated control group. The thimerosal and combination treatment groups had slightly higher levels of conjugation than the disulfiram only group (Fig. 4A). A similar pattern was seen with SUMO-2/3 conjugation (Fig. 4B).



Sentrin-specific proteases (SENPs) are involved in processing the precursor SUMO proteins into their mature form and in deconjugating sumoylated proteins involving SUMO-1, SUMO-2, and SUMO-3 (Geiss-Friedlander and Melchior 2007). Since disulfiram and thimerosal affect SUMO-1 and SUMO-2/3 conjugation, we next wanted to know if the combination might do so by affecting SENPs. Thus, we examined the effects of disulfiram and thimerosal on SENP1 protease activity. Using an *in vitro* assay where SENP1CD was mixed with or without the drug treatment followed by addition of the substrate, either His₆-pro-SUMO-1 or His₆-pro-SUMO-2, we determined that the addition of disulfiram and thimerosal had no effect on SENP1's ability to cleave pro-SUMO-1 (Fig. 4C). However, with the addition of disulfiram and thimerosal singly, pro-SUMO-2 cleavage was partially inhibited and with combination of the two treatments, pro-SUMO-2 cleavage was fully inhibited.



Disulfiram and Thimerosal can synergistically induce JNK level and activity through sumoylation

SUMO modifications have been reported to increase the sumoylated protein's stability in some cases (Geiss-Friedlander and Melchior 2007). We hypothesized that an increase in the level of p-JNK could be the result of increased stability provided by sumoylation. To evaluate whether JNK stability was increased under disulfiram and thimerosal combination treatment conditions, we examined the protein half-life of JNK using cycloheximide (CHX) treatment. Cells were treated with either CHX (20 μ g/ml) alone or disulfiram (6 μ M) and thimerosal (1 μ M) for 22 hours before addition of CHX. JNK in disulfiram and thimerosal treated cells shows enhanced stability (Fig. 5A).

To confirm sumoylation of JNK we performed a pull-down assay. To test whether JNK is sumoylated at the predicted lysine 160, a mutant with lysine to arginine mutation was used. 293T cells were transfected with His₆-JNK+SUMO-1, His₆-mJNK+SUMO-1, SUMO-1, or His₆-JNK. JNK was pulled down using Ni-NTA agarose beads. An immunoblot was performed using an anti-Flag antibody (Fig. 5B). Whole lysate was loaded into an SDS-PAGE gel and immunoblotted with an anti-SAPK/JNK antibody as a loading control (Fig. 5B). Absence of sumoylation was seen with cells transfected with His₆-JNK indicating that sumoylation levels of His₆-JNK in the cells were too low to be detected by the anti-SUMO-1 antibody. Bands were seen with cells transfected with SUMO-1, but because His₆-JNK was not transfected, we considered those to be background. His₆-mJNK+SUMO-1 showed similar bands, which we also considered as background. In His₆-JNK+SUMO-1, presence of an extra band around 130KDa was seen. This data suggests that sumoylated JNK has a size of about 130KDa and that the lysine 160 is a likely sumoylation site of JNK.

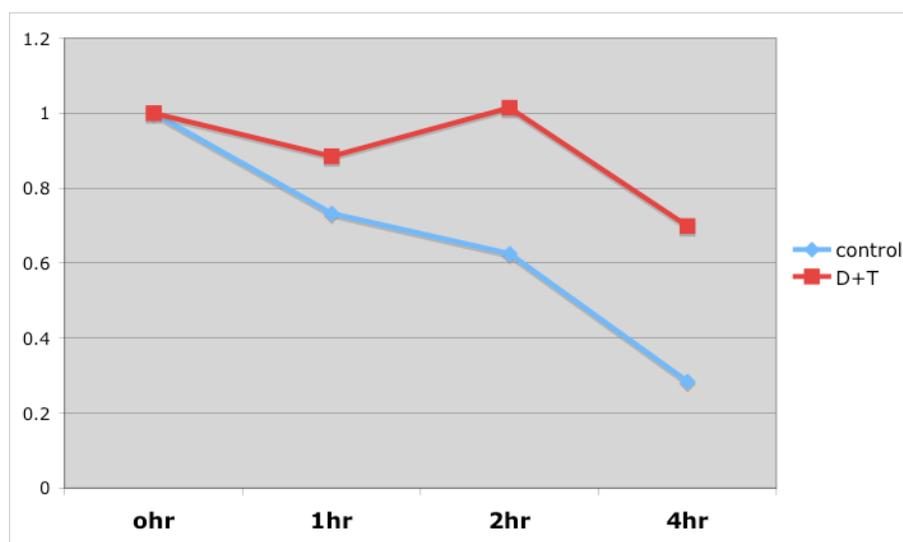
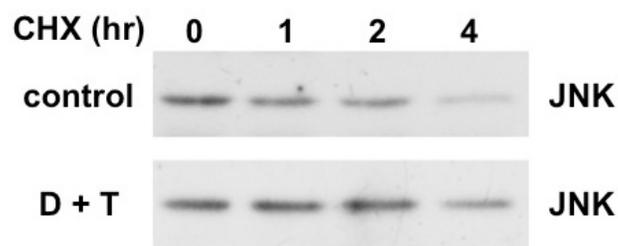
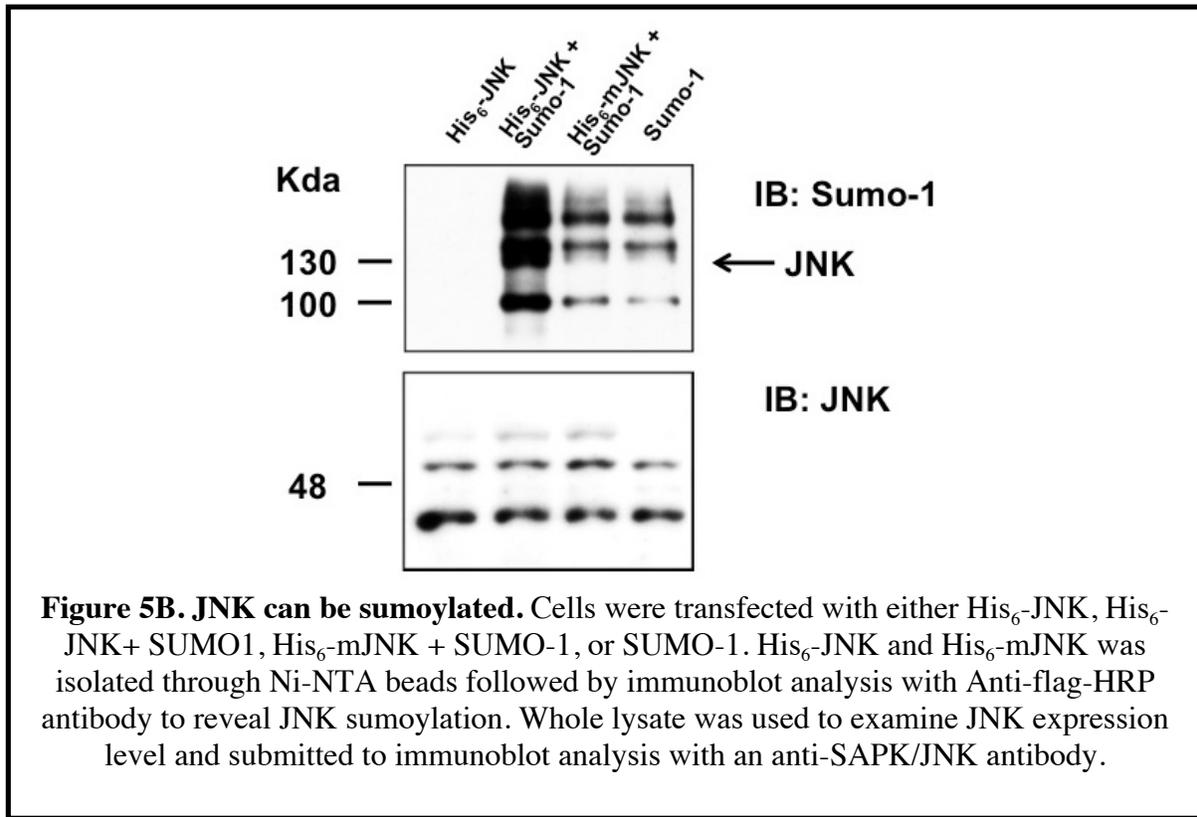


Figure 5A. Disulfiram and thimerosal treatment leads to stabilization of JNK. C4-2 cells grown in a 12-well plate at 8×10^4 cells/well. Cells were treated with either CHX ($20 \mu\text{g}/\text{mL}$) alone or disulfiram ($6 \mu\text{M}$) and thimerosal ($1 \mu\text{M}$) for 22 hours before addition of CHX to examine the effects of the combination treatment on JNK stability. Cells were incubated for the indicated time periods before an Immunoblot analysis of JNK. Anti-SAPK/JNK antibody was used for the detection of JNK. JNK in disulfiram and thimerosal treated cells show enhanced stability. Intensity was calculated using the Photoshop method (Densitometry) and then graphed.



DISCUSSION

We found disulfiram and thimerosal to be a novel synergistic combination that works well at low concentrations. We examined the molecular mechanism behind the synergistic cell death caused by the combination in prostate cancer cells. Toxicity from chemotherapy causes various side effects in patients. While lowering drug doses lessens the side effects of the treatment, lower doses are less effective. However, when drugs are used in the right combinations even at low doses, combinatorial effects can be achieved allowing for both inhibition of tumor growth and reduced side effects. A recent clinical trial showing promise in pairing bevacizumab, an anti-angiogenesis agent, and cyclophosphamide, a cytotoxic agent, for patients with recurrent ovarian cancer demonstrates the validity of this idea (Garcia et al. 2008). Our study demonstrates that disulfiram can be successfully paired with thimerosal to induce

prostate cancer cell death. The potential use of disulfiram as a chemotherapy agent is especially appealing because of its status as an already FDA-approved drug and its affordability compared to most chemotherapy drugs currently on the market (Cvek 2012). Other possible agents that synergize with disulfiram are still being studied. Recently, one group found that sunitinib, a drug used clinically to treat patients with metastatic renal carcinoma and gastrointestinal cancer, synergizes with disulfiram in prostate cancer (Ketola et al. 2012).

We show that disulfiram and thimerosal cause prostate cancer cells to undergo apoptosis involving cleavage of caspase-8 (Fig. 2A). Procaspase-8 activation generally occurs through the mitochondria-mediated pathway or a death receptor-mediated pathway.⁷ In the death receptor-mediated pathway, signals such as Fas ligand (FasL) or tumor necrosis factor (TNF) bind to the Fas or TNF receptor in the plasma membrane to cause Fas to bind with Fas-associated death domain (FADD) or the TNF receptor (TNFR) to bind to TRADD. This leads to FADD aggregation and emergence of the death effector domain (DED). The DED recruits procaspase-8, which is then proteolytically processed into its active form consisting of a small and large unit. In the mitochondria-mediated pathway, after cytochrome c is released from the mitochondria into the cytosol, caspase-6 activates procaspase-8 (Fan et al. 2005). In disulfiram and thimerosal induced apoptosis, whether caspase-8 activation is triggered by the mitochondria-mediated pathway or the cell death receptor-mediated pathway remains to be explored as there is evidence for the role of either pathway in other cases of drug-induced apoptosis (Fulda and Debatin 2000).

Adding NAC blocks the cytotoxic effects of the disulfiram and thimerosal combination treatment thus showing the combination's ability to bring about cell death through alteration of redox status (Fig. 2B). It is therefore not surprising that disulfiram and thimerosal when used together has such a significant effect on ROS levels. Disulfiram has been found to induce the

level of mature miR-17* which suppresses critical primary mitochondrial antioxidant enzymes such as manganese superoxide dismutase, glutathione peroxidase-2, and thioredoxin reductase-2. (Xu et al. 2010). Moreover, thimerosal can act as a sulfhydryl reagent and an oxidizing agent due to the presence of Hg^{2+} in its chemical structure (Elferink 1999). These properties would allow thimerosal to deplete the pool of cellular glutathione through oxidation of glutathione (GSH) into glutathione disulfide (GSSG) thus affecting the cell's capacity to deal with ROS (Lushchak 2012).

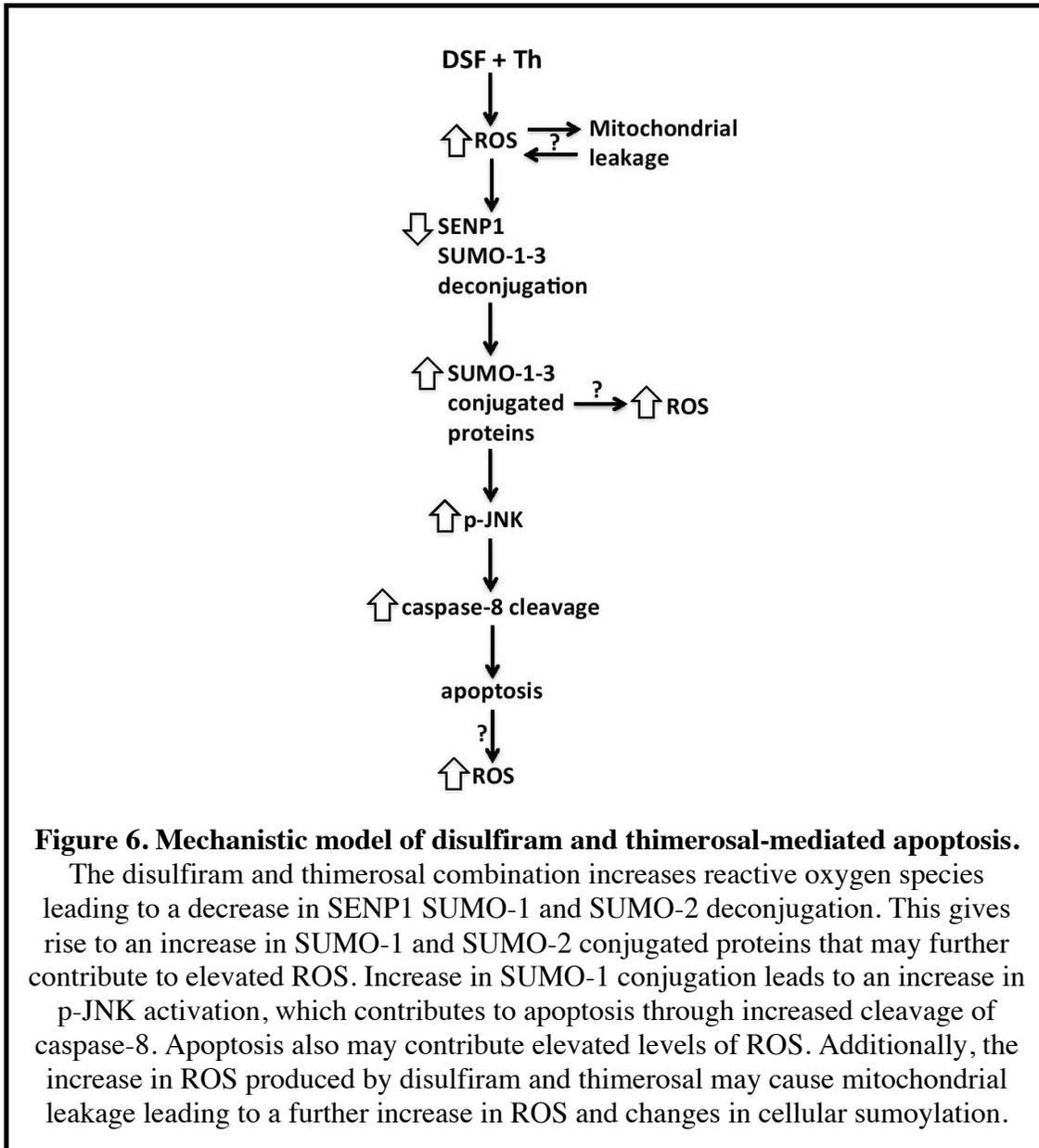
The SAPK pathways are major transducers that signal cell death or survival in response to oxidative stress. Activation of JNK has been reported to be involved in either promoting growth and proliferation or signaling for apoptosis (Maroni et al. 2004). In examining the role of JNK in response to disulfiram and thimerosal-mediated stress, we found that the levels of phosphorylated JNK increase with the combination treatment of disulfiram and thimerosal. We also found a decrease in total JNK. The apparent decrease in total JNK can be explained by modification of JNK that the antibody was unable to detect. Chemical inhibition of JNK activity prior to addition of the combination treatment reduced cell death. These findings support the role of JNK activation in cell death.

It has been reported that a high concentration of H_2O_2 (100mM) increases SUMO-1 and SUMO-2 conjugation while H_2O_2 at 1 mM has opposite effects on SUMO conjugation (Bossis and Melchior 2006). In our study we found an increase in conjugation of SUMO-1 and SUMO-2/3 when cells were treated with disulfiram and thimerosal at 6 μM and 1 μM respectively, suggesting that the combination of these two compounds might result in a higher level of ROS as in the case of 100mM H_2O_2 .

We show that SENP1 is able to cleave pro-SUMO-1 into its mature form under single and combination treatment conditions while SENP1 is inhibited from cleaving pro-SUMO-2 when either drug is present and most inhibited when the combination is present. SENP1 has been reported to process pro-SUMO-1 more efficiently than pro-SUMO-2 (Xu and Au 2005). It has been suggested that the lower affinity of SENP1 for pro-SUMO-2 is due to poor complementarity between the protein-protein interface of SUMO-2 and SENP1 (Shen et al. 2006). Coincidentally, SENP1 contains a catalytic triad that includes a cysteine group that disulfiram and thimerosal may affect. It is conceivable that the lower affinity SENP1 has for pro-SUMO-2 in addition to the interference by disulfiram and thimerosal at the Cys⁶⁰² of the catalytic triad give rise to the difference in effects disulfiram and thimerosal exert on SENP1's processing ability.

We found that JNK can be conjugated to SUMO-1 under conditions of oxidative stress (Fig. 5B). Feligioni et al. 2011 has also reported oxidative stress inducing sumoylation of JNK via SUMO-1. Additionally, we have found evidence that JNK is stabilized by sumoylation. We also found through mutation of a predicted sumoylation site on JNK that lysine 160 is likely to be the SUMO-1 attachment site. To our knowledge, this is the first report showing that the predicted sumoylation site on JNK is indeed a sumoylation site. Taken together, these data suggest that the combination through an increase of ROS production might regulate cellular sumoylation through a decrease of deconjugation of SUMO moiety on the target proteins, resulting in an at least an increase of JNK level and its activity. In turn, increased JNK activity contributes to apoptosis through increased production of cleaved caspase-8. At the moment, it is not clear whether increased ROS production is a result of increased cellular sumoylation/apoptosis or a cause of cell apoptosis through regulation of mitochondria leakage of

free radicals that initiate the cascade leading to changes of cellular sumoylation or both. Perhaps this dual activity can explain the clear synergistic potency of the combination of these two compounds on induction of cell death (Fig. 6).



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