Changes in Apoptotic Gene Expression Induced by DNA Cross-Linkers

Jordanne B. Dunn
Colby College

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Changes in Apoptotic Gene Expression Induced by DNA Cross-Linkers

Jordanne B. Dunn

A Thesis Presented to the Department of Chemistry, Colby College, Waterville, ME
In Partial Fulfillment of the Requirements for Graduation With Honors in Chemistry

Submitted May, 2009
Changes in Apoptotic Gene Expression Induced by DNA Cross-Linkers

Jordanne B. Dunn

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Date
Vitae

Jordanne Brooke Caitlin Dunn was raised in Manchester, Maine. She is the youngest of two children of Juanita V. Dunn, a retired elementary schoolteacher, and James P. Dunn, a dentist, and a sister to Jared I.I. Dunn, owner of Jared I. Dunn, L.L.C. She attended Manchester Elementary School, Maranacook Community High School, and entered the Colby College class of 2009 in the fall of 2005.

Jordanne pursued a major in chemistry during her Colby career. She conducted biochemistry research with Dr. Julie T. Millard during her junior and senior years. She enjoyed participating in Colby Cares about Kids and the Colby Outing Club. Highlights of her time at Colby include participating in varsity level cross-country running and Nordic skiing, ski patrolling at Sugarloaf/USA, and shadowing local radiologist and Colby alumna, Dr. Eric Stram (’89). She plans to pursue a career in dentistry after graduating from Colby. Jordanne graduated with a Bachelor of Arts with honors in Chemistry: Biochemistry and a minor in Mathematics.
Acknowledgments

I would like to first and foremost thank Dr. Julie T. Millard for her help, guidance, and patience in the completion of this honors project. Working in her laboratory was one of the most rewarding experiences I have had at Colby. Her guidance has taught me the rewards of hard work and it has helped me to enhance my critical thinking. This project would not be possible without her guidance and enthusiasm for biochemical research.

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Thank you to my friends and loved ones for your continuous support and encouragement. Thank you for understanding why I was never left the science buildings and always being there when I needed someone.

A special thank you is owed to my parents, Juanita and James Dunn, for their love, support, and constant encouragement. You have always encouraged me to take on any challenge and made everything that I do possible. Thank you to my brother, Jared Dunn, for always making me laugh and being willing to go on any adventure.
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Abstract

The Millard Research Laboratory is interested in the cytotoxic mechanisms of the bifunctional alkylators diepoxybutane (DEB), epichlorohydrin (ECH), and (1-chloroethenyl) oxirane (COX). Studies performed in the laboratory examine the dual nature of these DNA cross-linking compounds that can act as carcinogens or anti-cancer agents. The mechanisms through which these compounds induce cell death are explored in this study. Cells either undergo cell death due to necrosis or apoptosis. HL-60 cells were treated with varying concentrations of DEB, ECH, or COX. A caspase 3/7 assay was used to test for induction of apoptosis in the treated cells at varying incubation times. It was concluded that DEB induces apoptosis in HL-60 cells treated with 100 µM for 24 hours. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was then used to explore the changes in gene expression of various genes involved in apoptosis signaling. The results were inconclusive as to specific genes involved in DEB induced apoptosis, but the data does suggest that apoptosis is induced by a mitochondrial-mediated apoptosis signaling pathway.
Introduction

In the United States men have a slightly less than a 1 in 2 risk of developing cancer and women have a little more than 1 in 3 risk as of 2008. Cancer is the general term used to define a group of diseases characterized by unrestrained growth and spread of abnormal cells. Diagnostics have been improving in order to identify cancer cells earlier than in the past, and treatments have been improving as well. Cancer is treated with chemotherapy, radiation, hormone therapy, biological therapy, targeted therapy, and surgery. Chemotherapeutic research is a highly active field in order to develop targeted drugs for cancer.¹

Some of the first chemotherapeutic drugs were from the mustard gas family, based upon the discovery of the lymphotoxic action of nitrogen mustards during World War I. It was discovered that nitrogen mustards produce various types of DNA cross-links.² DNA cross-linking compounds can act as carcinogens or an anti-cancer agents, and thus, the molecular mechanisms through which these compounds induce damage to cells is of great interest. The cytotoxic mechanisms of the bifunctional alkylators diepoxybutane (DEB), epichlorohydrin (ECH), and (1-chloroethenyl)oxirane (COX) are of particular interest in the Millard Research Laboratory (Figure 1).

![Chemical structures](https://via.placeholder.com/150)

*Figure 1:* Chemical structures of diepoxybutane, epichlorohydrin, and (1-chloroethenyl)oxirane.
Exposure to 1,3 butadiene, a gaseous hydrocarbon, has been shown to have carcinogenic effects, although it is an important industrial chemical used in production of polymers and synthetic rubber.\textsuperscript{3} The metabolites of 1,3 butadiene include 1,2-epoxybutene, produced via cytochrome P450-mediated oxidation. Studies have shown that inhalation of 1,3 butadiene induces point mutations at both A:T and G:C base pairs.\textsuperscript{4-6} The 1,2-epoxybutene intermediate can feed into many different metabolic pathways, one of which is oxidation to diepoxybutane. Diepoxybutane is 100-fold more mutagenic than 1,2-epoxybutene, an observation attributed to its formation of DNA interstrand cross-links. In general, the cross-linking compounds of interest are known to alkylate the N7 guanine position on opposite strands of DNA and form an interstrand cross-link. DEB reacts with synthetic DNA oligomers to form interstrand cross-links preferentially at 5’GNC sites, where N is either a guanine or cytosine residue.\textsuperscript{7, 8} DEB performs a double epoxide ring-opening reaction at the N7 position of guanine residues (Figure 2) to cross-link DNA.\textsuperscript{9}

Epichlorohydrin is commonly used in the manufacturing of epoxy resins, glycerine, and elastomers.\textsuperscript{10} ECH has been classified as a potential human carcinogen and it has been shown that exposure to ECH appears to increase the risk of lung cancer.\textsuperscript{10, 11} ECH cross-links 5’GNC and 5’GC sequences approximately equally.\textsuperscript{12} ECH is believed to cross-link with one epoxide ring opening, where the now negatively charged oxygen performs an $S_N2$ reaction, releasing chloride and forming a new epoxide ring. The N7 of a guanine residue nucleophilically attacks the ECH epoxide ring, creating a covalent interstrand cross-link (Figure 3).\textsuperscript{12}
Figure 2. The structure of a cross-link formed by diepoxybutane between two guanine residues on opposing strands of DNA.

Figure 3. The presumed structure of a cross-link formed by epichlorohydrin between two guanine residues on opposing strands of DNA.

Chloroprene is commonly used in the manufacturing of polychloroprene, a solvent-resistant elastomer. The primary route of exposure to chloroprene is inhalation. The main metabolite of chloroprene is (1-chloroethenyl)oxirane (COX) and there is evidence that this product forms DNA adducts. Preliminary results indicate that COX targets both 5’-GGC and 5’-GC sites approximately equally. The rate of COX cross-linking increases as pH decreases, suggesting acid catalysis. The mechanism for DNA cross-linking is believed to be similar to that of ECH.
DEB, ECH, and COX are high-volume industrial chemicals that are potential carcinogens. It is important to study the molecular mechanisms through which these compounds exert their effects due to common exposure to these compounds. These compounds have all been shown to form interstrand DNA cross-links. However, the mechanisms through which these compounds induce cell death is unknown.

Cells undergo cell death either due to necrosis, which is induced by physical or chemical injury, or apoptosis, which is programmed cell death. Necrosis is the “messy” form of cell death since it affects groups of cells and there is no regulation. Necrosis occurs when cells are exposed to extreme variance from physiological conditions. Phenomena leading to necrosis include ischemia, heat, toxins, and trauma. These affect more than one cell, so groups of cells die, causing local inflammation at the point of injury. Necrosis is characterized by the loss of cell membrane integrity and eventual lysis of the cell and its organelles. The cell remnants from necrosis are phagocytosed by macrophages.

Apoptosis occurs when the cell is programmed to die via complicated signaling pathways (Figure 4). Apoptosis is induced via activation of cell surface receptors or events such as DNA damage. Caspases are part of a highly conserved protein family that is central to the apoptotic pathway. Caspases are proteases activated after a cell has received a signal instructing it to undergo apoptosis. The key components that the caspases break down include DNA repair enzymes and structural proteins in the cytoskeleton. Caspases can also activate other enzymes that degrade other parts of the cellular machinery by cleaving an inhibitory sequence on these enzymes. There is a loss of nuclear membrane integrity after disruption. Signals are sent to the plasma...
membrane of the cell during the apoptotic process, where phagocytes recognize these signals. Phagocytic cells clear the apoptotic cells from the surrounding tissues, preventing the local inflammation seen in necrotic tissue.

Figure 4. Apoptosis signaling pathways.\textsuperscript{21}

Cell stress and DNA damage can induce apoptosis; however, in other cases apoptosis can be signaled via cell surface receptors called death receptors. Death receptor ligands include Fas ligand, tumor necrosis factors (TNF), and TNF-related apoptosis inducing ligand (TRAIL). TNF is one of the most well studied physiological inducers of
apoptosis. TNF is produced by T-cells and activated macrophages in response to infection. TNF binds to a TNF receptor on the plasma membrane, allowing the binding of tumor necrosis factor receptor associated death domain (TRADD) to a death domain on the receptor. TRADD can either recruit TNF-associated factor 2 (TRAF2) to activate the Jun N-terminal kinase (JNK) pathway or associate with Fas associated protein with death domain (FADD), leading to apoptosis. FADD recruits and cleaves pro-caspase 8. Fas induces apoptosis with a similar mechanism to the TNF receptor as they both signal through a Fas receptor. FADD can be recruited directly to the death domain on a Fas receptor, initiating apoptosis. TRAIL also signals apoptosis in a similar way when it binds to its receptors. Apoptosis induced by signaling via death receptors often results in caspase 8 or 10 activation. Caspase 8 or 10 then cleaves other pro-caspases, activating effector caspases, such as caspase 3, caspase 7, and caspase 6. Caspases 3, 7, and 6 are then responsible for cleavage of the key cellular proteins.

Apoptosis is an important part of normal development and is often disrupted by oncogenic mutations. Cellular proliferation is unchecked in cells where apoptosis is disrupted, leading to the formation of tumor cells. Damage in the apoptosis signaling pathways can make the tumor cells resistant to some cancer treatments, and thus, understanding apoptosis is of great interest for developing future treatments. Many cytotoxic anti-cancer agents induce apoptosis, which is important to note since the defects in apoptosis in tumor cells may contribute to some treatment failure. Information about the molecular mechanisms of either anti-cancer drugs or carcinogens can therefore be exploited to develop new forms of cancer treatments. The molecular mechanisms of DNA
cross-linkers are of particular interest to the Millard Research Laboratory since DEB is the active form of the anti-cancer drug treosulfan.32

A study performed by Brockman et al. investigated the apoptotic potential of seven simple oxiranes of varying structures by utilizing a caspase 3 fluorescence assay. The apoptosis testing was carried out during exponential growth for all assays in L929 fibroblasts. The order of relative apoptotic potential was determined to be: (highest to lowest) diepoxybutane > styrene oxide > phenyl glycidyl ether > epichlorohydrin > glycidol > epoxybutane > epoxycyclohexane. The relative cytotoxicity was determined to be: diepoxybutane > epichlorohydrin > phenyl glycidyl ether > styrene oxide > glycidol > epoxybutane > epoxycyclohexane.33 Therefore, based on these findings, we expected DEB to have a greater apoptotic potential than ECH in HL-60 cells. A similar caspase assay was used in this project to determine if apoptosis is induced by DEB, ECH, or COX.

The goal of this project was to determine the molecular mechanism through which the cross-linking compounds of interest cause cell death. Caspase activation is an irreversible commitment towards cell death, and thus, testing for caspase activity can help determine if a compound induces apoptosis. Caspase activity was monitored in cells treated with varying concentrations of DEB, ECH, and COX for varying incubation periods using a Caspase-Glo® assay (Figure 5).17
Figure 5. Schematic of Caspase-Glo® 3/7 Assay.\textsuperscript{17}

Following the determination of conditions that induce apoptosis, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to study the changes in apoptotic gene expression in a real-time experiment. Real time PCR is used to quantify the accumulation of product after each cycle using an intercalating fluorescent dye. When coupled with a reverse transcription step, it can be used to look at the changes in gene expression. Upregulation of a gene results in increased expression of one or more genes, and thus, the proteins coded for by these genes. Upregulation of a gene can be characterized by an increase in transcription, and as a result, an increase in messenger RNA (mRNA). mRNA is translated by ribosomes into proteins within the cell, so by studying the amount of mRNA in treated and untreated cells via qRT-PCR, it can be determined which apoptotic genes are upregulated (Figure 6). This knowledge will help to elucidate the molecular mechanism through which DNA cross-linkers induce cell death.
Figure 6. Schematic of protein synthesis.
Experimental Procedures

Cell Culture: HL-60 (Human promyelocytic leukemia) cells were grown in HyQ® RPMI-1640 medium with 2.05 mM L-glutamine (Fisher Scientific). The medium was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown in T25 culture flasks maintained at 37°C in 5% CO₂. Cells at a concentration of 4 x 10⁵ cells/mL in HL-60 cell suspension were passaged by 1:10 (1 mL cell suspension and 9 mL media) dilutions to maintain the cell line.

The genetic composition of the cells mutates after a number of passages, and for HL-60 cells the cell line loses its integrity after 20 passages. New cell lines were started from stocks in liquid nitrogen storage. A vial was removed from the liquid nitrogen cooler and transported in dry ice. The vial was thawed in a 37°C water bath and the vial was wiped with 70% ethanol before opening. The contents of the vial were transferred to a 15 mL conical Falcon tube and 9 mL of HL-60 media was added drop-wise with occasional mixing of the suspension. The cells were centrifuged at 250 x g for 5 min and the supernatant was removed and discarded. The pellet was re-suspended in 10 mL of HL-60 media and transferred to a T-25 flask at 37°C and 5% CO₂.

Caspase Assay: The presence of caspase enzymes was determined using a Caspase-Glo® 3/7 Assay from Promega. Cells at 85-95% confluence were passaged at 1:10 (1 mL cell suspension and 9 mL media). The following day cells were plated into six-well plates by adding 1 mL of cell suspension, varying amounts of the cross-linking agent, and cell culture media to achieve a total volume of 5mL. The vehicle for the cross-linking agent was HL-60 cell culture media. The amount of media varied based upon on the
concentration of cross-linking reagents with which the cells were treated. A cell control was also plated by adding 1 mL of cell suspension and 4 mL of cell culture media to one of the wells. No cross-linking agent was added to the cell control. The day after plating the HL-60 cells were treated with 1 µM, 10 µM, 50 µM, 100 µM, and 1 mM DEB (Aldrich), ECH (Aldrich), camptothecin (Matt Stein), or COX (Brian Wadugu). Initially, caspase activity was measured 1h, 4 h, and 24 h after addition of agent. If caspase activity was detected during this initial screen, the experiment was repeated and caspase activity measured at 1 h, 4 h, 8 h, 12 h, and 24 h. At each time interval, 100 µL of the treated cells were collected in a microcentrifuge tube and centrifuged at room temperature (25°C) for 5 min at 1500 rpm. The supernatant was discarded and the pellet was resuspended in 100 µL of 1x phosphate buffered saline (PBS). The cells were centrifuged again at room temperature for 5 min at 1500 rpm. The supernatant was removed and the pellet was resuspended in 100 µL of 1x PBS. The sample was transferred to a 96 standard opaque well plate and 100 µL of Caspase-Glo® 3/7 reagent was added to each well that contained samples. A Molecular Devices Spectra Max M2 luminometer was used to measure the luminescence.

**RNA Purification:** Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen); regents specified below were part of this kit. The concentration of cells in culture was determined using a Nexcelom Biosciences Cellometer Auto T4, and 2 mL of cell suspension was transferred to a 15 mL conical Falcon tube. The cell suspension was centrifuged for 5 min at 1500 rpm, and the supernatant was discarded. The cell pellet was resuspended in 800 µL of RNAprotect Cell Reagent. The suspension was transferred to two microcentrifuge tubes and centrifuged at 5000 x g for 5 min. The supernatant was
discarded and 350 µL of buffer RLT plus was added if the number of cells was less than 5 x 10⁶ and 600 µL of buffer RLT plus was added if the number of cells was between 5 x 10⁶ and 1 x 10⁷. The solution was vortexed and the lysate was pipetted directly onto a QIAshredder spin column. The column was centrifuged for 2 min at 20,000 x g to homogenize the cell lysate. The flow-through was transferred to a gDNA Eliminator spin column, which was centrifuged at 10,000 x g for 30 s to remove genomic DNA. The column was discarded and 1 volume of 70% ethanol was added to the flow-through to provide appropriate binding conditions for RNA. The sample was transferred to an RNeasy spin column and centrifuged for 15 sec at 10,000 x g, binding the total RNA to the column membrane. If the sample volume was greater than 700 µL, then successive aliquots were centrifuged in the same column. The column was washed by adding 700 µL of Buffer RW1, centrifuging for 15 s at 10,000 x g, and discarding the flow-through. The column was washed again with the addition of 500 µL of Buffer RPE, centrifuging for 15 sec at 10,000 x g, and discarding the flow-through. The column was washed with another addition of 500 µL of Buffer RPE and centrifuged for 2 min at 10,000 x g. The RNeasy spin column was transferred to a new 2 mL collection tube and centrifuged at 20,000 x g for 1 min to eliminate any possible carryover of Buffer RPE or if any residual flow-through remained on the outside of the spin column. The RNeasy spin column was transferred to a new 1.5 mL collection tube and 30-50 µL of RNase-free water was added. The column was centrifuged for 1 min at 10,000 x g to elute the RNA. The NanoDrop was used to measure the concentration of RNA, and the sample was stored at -20 °C for later use.
Table 1. Names and abbreviations of primers used from Realtimeprimer.com

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Real time Quantitative Reverse Transcriptase PCR (qRT-PCR), Comparative C<sub>T</sub>

Experiments: PCR reactions (25 µL) contained 12.5 µL of 2x Quantifast SYBR Green RT-PCR Master Mix, 0.1 µM primer, 0.25 µL of Quantifast RT Mix, and 2.5 µL total of forward and reverse primers. The primers used for this study were from the Human
Apoptosis Gene PCR Array by Realtimeprimer.com (Table 1). The PCR was run using an Applied Biosystems Step One Real Time PCR system. The amplicon, a small piece of DNA selected for by a primer, was amplified from cDNA via a protocol that consisted of the following thermal cycling sequence: 50°C for 10 min, 95°C for 5 min, 50 cycles of 95°C for 10 s and 58°C for 45 s, 95°C for 15 s, and 60°C for 1 min. Following thermal cycling the threshold was set to 0.783351 using the StepOne software and C_T values were calculated automatically. The threshold is the point at which a reaction reaches a fluorescent intensity above the background noise of the instrument. C_T stands for cycle threshold, which is the cycle number at which the amplified product reaches the threshold level.

**Data Analysis:** The data from qRT-PCR consist of C_T values for untreated and treated cells for both an endogenous control and each gene of interest. The endogenous control used in this study was ß-actin. ß-actin is a housekeeping gene, which means that it is highly conserved and the expression of ß-actin should not change between un-treated and treated cells. An endogenous control helps correct for any error induce by the instrument. The ∆C_T value was calculated by subtracting the C_T value for untreated cells from the C_T value for treated cells for the endogenous control and each gene of interest. The ∆∆C_T value was calculated by subtracting the ∆C_T value for the endogenous control from the ∆C_T value for a gene of interest. The ∆∆C_T value was calculated for each gene of interest, where a positive ∆∆C_T indicates downregulation and a negative ∆∆C_T indicates upregulation.
Results

Caspase Assay:

Testing for caspase activity in cells can help determine if a compound induces apoptosis since caspase activation is an irreversible commitment towards cell death. Previous studies have shown that DEB induces caspase 3/7 mediated-apoptosis in TK6 lymphoblasts, indicating that a caspase 3/7 assay may be useful to determine if similar DNA cross-linkers induce apoptosis. A Caspase-Glo® 3/7 Assay was used to monitor caspase activity for cells treated with 1 µM, 10 µM, 50 µM, 100 µM, and 1mM DEB, ECH, and COX for varying lengths of time (Figures 7-12). Camptothecin (campto) is known to induce apoptosis in treated cells, and therefore it was used as a positive control for the caspase 3/7 assay.

Figure 7: The caspase assay for a 24 hr treatment with 10 µM and 100 µM camptothecin, DEB, and ECH. (D9 = cell control, D10 = 10 µM campto, D11 = 10 µM DEB, D12 = 10 µM ECH, E9 = blank control, E10 = 100 µM campto, E11 = 100 µM DEB, E12= 100 µM ECH). Only 10 µM camptothecin, 100 µM DEB, and 100 µM camptothecin displayed significant fluorescence RLU (greater than 4000).
Figure 8: The caspase assay for a 24 hr treatment with 50 µM and 1 mM camptothecin, DEB, and ECH. (A9 = cell control, A10 = 50 µM campto, A11 = 50 µM DEB, A12 = 50 µM ECH, B9 = blank control, B10 = 1 mM campto, B11 = 1mM DEB, B12 = 1mM ECH). Only 1 mM ECH had significant fluorescence.

Figure 9: The caspase assay for a 24 hr treatment with 100 µM DEB and 1 mM ECH. (C1 = cell control, C2 = 100 µM campto, C3 = 100 µM DEB, D1 = blank control, D2 = 1 mM campto, D3 = 1 mM ECH). Samples with significant fluorescence were 1 mM camptothecin, 100 µM camptothecin, and 100 µM DEB.
Figure 10: The caspase assay for a 24 hr treatment with 1 mM ECH. (A9 = blank control, A10 = cell control, A11 = 1 mM campto, A12 = 1 mM ECH). None of the samples showed significant fluorescence.

Figure 11: The caspase assay for a 12 hr treatment with 100 µM and 1 mM camptothecin, DEB, ECH, and oxirane. (D1 = cell control, D2 = 100 µM campto, D3 = 100 µM DEB, D4 = 100 µM ECH, D5 = 100 µM oxirane, E1 = blank control, E2 = 1 mM campto, E3 = 1 mM DEB, E4 = 1 mM ECH, E5 = 1 mM oxirane). Samples with significant fluorescence were 1 mM DEB and 100 µM camptothecin.
Apoptosis was induced in HL-60 cells treated with 100 µM DEB for 24 hours and 1 mM DEB for 12 hours (Figure 7, E11; Figure 9, C3, Figure 12, D9; Figure 11, E3). Caspase activity was seen in cells treated with 1mM ECH for 24 hours (Figure 8, B12). However, in subsequent trials no caspase activity was seen, and therefore, ECH questionably induces apoptosis at a concentration of 1mM for 24 hours.

*Real time qRT-PCR Comparative C<sub>t</sub> experiments:*

Real time qRT-PCR was used to study the changes in apoptotic gene expression in order to elucidate the genetic mechanism through which DEB, the most apoptotic of our cross-linkers, induces cell death. Thirty-five different genes involved in apoptosis were studied using a different primer set for each gene of interest and comparing each to an
endogenous control, β-actin. Initially, trials to determine the ideal concentration of RNA in a reaction mixture for each gene of interest were performed. The trials were conducted by testing varying RNA concentrations, using RNA purified from HL-60 cells treated with 100 µM DEB for 24 h. RNA concentrations of 0.28, 2.8, and 28 ng/µL per reaction mixture were analyzed using qRT-PCR to determine the ideal RNA concentration for the amplification of a gene of interest. The concentration trials also helped to determine which genes of interest were amplified in HL-60 cells (Table 2).

**Table 2.** Results from concentration trials for each gene of interest.

<table>
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<tr>
<th>Target Name</th>
<th>Amplified in HL-60 cells</th>
<th>Target Name</th>
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The ideal concentration of RNA in a reaction mixture was determined to be 28 ng/µL, since higher concentrations of RNA could be obtained during RNA purification. The genes of interest that are highlighted in Table 1 were amplified in treated HL-60 cells, and thus, it was these genes that were studied further.
Comparative $C_T$ qRT-PCR experiments were used to explore the changes in gene expression induced by DEB under conditions found to cause apoptosis. Each experiment involved un-treated and treated cells (100 µM DEB for 24 h) and were assayed for an endogenous control and for various genes of interest. The threshold value was set after the experiment had been completed. The $C_T$ values were then obtained for each gene of interest and compared to the endogenous control. The $\Delta C_T$ and $\Delta \Delta C_T$ values were calculated for each gene of interest. A positive $\Delta \Delta C_T$ indicates downregulation of a gene and negative $\Delta \Delta C_T$ indicates upregulation of a gene (Figure 13).

Figure 13. Results from comparative $C_T$ qRT-PCR experiments.
The comparative $C_T$ experiments revealed that APAF, BAG1, BAG4, BAK1, BAX, BCL2A1, BCL2L11, BCL2L2, BIRC4, CASP1, and CASP5 were upregulated. ATM and CASP7 were run in duplicate, however, the results from the two trials conflict. BCL10 was downregulated. It could not be determined if BOK is upregulated or downregulated since the error for BOK was so high that the $\Delta\Delta C_T$ spans both positive and negative values.
Discussion

The goal of this project was to determine the molecular mechanism through which the cross-linking compounds of interest cause cell death. A caspase assay was used to determine the conditions under which apoptosis was induced by the different cross-linking compounds. HL-60 cells treated with 100 µM DEB for 24 hours were shown to be in apoptosis, and thus, the focus of this project was the molecular mechanism through which DEB induces apoptosis. Quantitative RT-PCR was used to explore the changes in gene expression of genes involved in apoptosis in order to determine the molecular mechanism.

The caspase assay tests for the presence of caspase enzymes, and their presence indicates that apoptosis was induced by the cells. The luminescence values for cells treated with varying concentrations and time of incubation were compared to a cell control and camptothecin, which was previously shown to induce apoptosis. Cells were treated with camptothecin, DEB, ECH, or COX at concentrations ranging from 1 µM to 1mM for varying incubation time periods.

The initial screen of DEB at each of the varying concentrations showed caspase activity in cells treated with 100 µM DEB (Figure 7, E11) for 24 hours. The luminescence values for DEB were compared to those for the camptothecin and cell controls to determine if apoptosis was induced. The values for 100 µM DEB at 24 hours were similar to the values for camptothecin and well above those for the cell control, indicating that caspase activity was present in these cells. Subsequent trials with 100 µM DEB duplicated the previous results (Figure 9, C3; Figure 12, D9). In the third trial the luminescence values for cells treated with 100 µM DEB for 24 hours were noticeably lower than previous trials. However, they were still comparable to the values for cells
treated with 100 µM camptothecin (Figure 12, D8). The lower values may be attributed to cells that were almost a month old and had undergone almost twenty passages. After twenty passages the cells start to mutate and grow faster, and thus, there were more cells at the 24-hour incubation than usual. Caspase activity was also seen in cells treated with 1 mM DEB for 12 hours (Figure 11, E3).

The initial screen of ECH at each of the varying concentrations showed caspase activity only in cells treated with 1 mM ECH (Figure 8, B12) for 24 hours. Generally, cells treated with 1 mM camptothecin for 24 hours show caspase activity, but no caspase activity was seen for 1 mM camptothecin in the initial screen. This anomaly may be attributed to older cells that had undergone many passages. Another trial with 1 mM ECH attempted to duplicate this result. However, caspase activity was not seen in a second trial (Figure 9, D3). A third trial with 1 mM ECH was conducted, taking out aliquots of the treated cells at 1 hr, 4 hrs, and 24 hrs. Caspase activity was not seen in this trial (Figure 10, A12). However, the camptothecin control did not show caspase activity for any of the incubation time periods, suggesting a flaw in the experiment. Further investigation revealed that none of the cells in the six-well plates were growing, including the cell control. The HL-60 cells were not proliferating as fast as they normally did so this trial was not used to determine if caspase activity is present in cells treated with 1mM ECH for 24 hours. Caspase activity was not seen in another trial with 1 mM ECH at 24 hours (Figure 12, E10).

The caspase activity for COX was tested at concentrations that were previously shown to induce apoptosis in cells by DEB and then working up to the concentrations at which ECH had questionably induced apoptosis. Cells were treated with 100 µM and 1
mM COX and were assayed at incubation times of 1 hr, 4 hrs, 12 hrs, and 24 hrs. Caspase activity was not seen for cells treated with COX at these concentrations and no other trials with COX have been performed thus far. Further experimentation is necessary to determine if ECH and COX induce apoptosis in HL-60 cells. Also, flow cytometry may be used to quantify the number of apoptotic cells in a population.

The next step in this work was to establish which apoptotic genes are upregulated in cells treated with DEB versus untreated cells via real time PCR. The upregulated genes may reveal the molecular mechanism through which DEB causes cell death. Initially, trials to determine the ideal concentration of RNA in a reaction mixture for each gene of interest were performed. Comparative C<sub>T</sub> qRT-PCR experiments were then used to explore the changes in gene expression.

The ideal concentration of RNA in a reaction mixture was determined to be 28 ng/µL. It is possible that a higher concentration of RNA would work better. However, RNA purification was not able to yield concentrations high enough to test over 28 ng/µL in the reaction mixture. Thirty-five different genes of interest were analyzed using comparative C<sub>T</sub> qRT-PCR experiments. APAF was shown to be upregulated in treated HL-60 cells. This gene codes for a protein that initiates apoptosis via a mitochondrial-regulated apoptosis signaling pathway. Activation of APAF causes pro-caspase 9 to be cleaved, activating caspase 9, which starts a caspase cascade. The caspase cascade commits the cell to apoptosis. Upregulation of APAF indicates that DEB induces apoptosis through a mitochondrial-mediated signaling pathway.

ATM was upregulated in treated HL-60 cells. ATM is an important cell cycle checkpoint kinase that regulates downstream proteins including p53, CHK2, and many
others. ATM is part of a pathway that signals for apoptosis following DNA damage or genotoxic stress.\textsuperscript{39, 40} However, a duplicate trial for ATM showed that ATM was downregulated in treated cells. No conclusions can be made about ATM due to the discrepancies between duplicate trials.

BAK1, upregulated in treated cells, belongs to the BCL2 protein family, and thus, it moves to the mitochondria where it induces apoptosis. BAK1 accelerates the opening of the mitochondrial voltage-dependent anion channel, leading to a release of cytochrome c.\textsuperscript{41} BAX also showed upregulation in treated cells. BAX interacts with the mitochondrial voltage-dependent anion channel, leading to the release of cytochrome c.\textsuperscript{41} This is further support for a mitochondrial pathway.

Several other members of the BCL-2 protein family were explored next. The proteins from this family can either act as anti- or pro-apoptotic regulators and are involved in mitochondrial-regulated apoptosis. The members of this family explored in this study were BCL10, BCL2A1, BCL2L11, BCL2L2, and BOK. BCL10, downregulated in treated cells, contains a caspase recruitment domain and its deregulation contributes to tumor formation.\textsuperscript{42} BCL2A1, upregulated in treated cells, reduces the amount of cytochrome c released from the mitochondria and therefore inhibits apoptosis.\textsuperscript{43} BCL2L11 was upregulated in treated cells and this gene activates apoptosis by interacting with other members of the BCL2-protein family.\textsuperscript{44, 45} BCL2L2 was upregulated in treated cells and it has been shown to inhibit apoptosis under cytotoxic conditions.\textsuperscript{44, 45} BOK is also known to be a pro-apoptotic factor.\textsuperscript{44, 45} The error for BOK is large, and thus, nothing can be concluded about the change in expression in HL-60 cells. There were changes in the gene expression for the other members of the BCL-2 protein family, further supporting
mitochondrial-regulated apoptosis; however, the upregulation and deregulation of these
genes is not what would be expected. The upregulation of BCL2L11 and the
downregulation of BCL10 was expected, but the upregulation of BCL2A1 and BCL2L2
was not. It is surprising that BCL2A1 and BCL2L2 are upregulated in apoptotic cells
since they inhibit apoptosis.

BAG1 showed upregulation in treated cells. BAG1 is a membrane protein that
inhibits a step in a signaling pathway leading to apoptosis. BAG1 binds to BCL2 and
enhances the anti-apoptotic effects of BCL2.46 BAG4 also showed upregulation in treated
cells. BAG4 is a member of the BAG1-related protein family and also functions as anti-
apoptosis protein. BAG4 regulates downstream signaling by associating with the death
domain of a tumor necrosis factor receptor.47 Therefore, it is somewhat odd that these
proteins would be upregulated in apoptotic cells.

BIRC4 belongs to a family of proteins that inhibits apoptosis. BIRC4 binds to
tumor necrosis factor receptor associated factors TRAF1 and TRAF2 to inhibit apoptosis,
and it was upregulated in treated cells.48 It is surprising that BIRC4 is upregulated in
apoptotic cells.

CASP1, CASP5, and CASP7 are members of the caspase family and they were
analyzed next. CASP1 induces apoptosis and activates the precursor of interleukin-1.49
CASP5, upregulated in treated cells, is an inflammatory caspase and it was upregulated
with a ΔΔCT value of -1.755 ± 0.15.50 Pro-Caspase 7 is cleaved by caspase 3 and 10 and it
is activated when the cell receives signals to induce apoptosis.49 Initially CASP7 was
shown to be upregulated; however, a duplicate trial showed downregulation. Conclusions
cannot be drawn about the change in gene expression of CASP7 due to conflicting results.
Upregulation of members of the caspase family indicate that apoptosis is induced in HL-60 cells by DEB.

BCL2, BIRC3, and BIRC5 were analyzed by comparative $C_T$ qRT-PCR, however, the amplified products were below the detection limit of the qRT-PCR instrument. Nothing can be drawn from the $\Delta\Delta C_T$ values because the amplified products cannot be distinguished from the instrument noise. Previous trials had shown detectable amplification for these genes of interest, but the $C_T$ values were past forty cycles. The reagents in the reaction mixtures often do not last beyond forty amplification cycles.

The results from the comparative $C_T$ qRT-PCR experiments were inconclusive due to discrepancies among data sets. However, the data does indicate that DEB induces apoptosis by a mitochondrial-mediated apoptosis signaling pathway consistent with previous reports. Further work needs to be done to duplicate these data sets in order to make any conclusions about the upregulation of any specific genes of interest. The next step would be analyze the genes coding for tumor necrosis factors and p53 in order to help piece together the signaling pathway involved in DEB-induced apoptosis.
Conclusions

HL-60 cells treated with DEB at 100 µM for 24 hours and 1 mM for 12 hours undergo apoptosis. The data suggest that DEB has a higher apoptotic potential than COX and ECH in HL-60 cells. Real time qRT-PCR results indicate that DEB induces apoptosis via a mitochondrial-mediated apoptosis signaling pathway. Future work includes analyzing other genes of interest and duplicating the comparative C_T experiments to obtain consistent data sets in order to verify which genes of interest have been upregulated.
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