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Mechanisms of Cytotoxicity of Diepoxybutane, Epichlorohydrin and 1-(Chloroethenyl)oxirane

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Mechanisms of Cytotoxicity of Diepoxybutane, Epichlorohydrin and 1-(Chloroethenyl)oxirane

By Rebecca D. Kamins

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

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Mechanisms of Cytotoxicity of Diepoxybutane, Epichlorohydrin and 1-(Chloroethenyl)oxirane

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_____ Date

VITAE

Rebecca Dora Kamins was raised in Westport, Connecticut. She is the daughter of Harold and Margaret Kamins and has one sibling, Michael Andrew Kamins, who is studying mechanical engineering at Bucknell University. She graduated from Staples High School in the spring of 2005. It was there that she developed an interest in scientific research under the tutelage of Dr. Alfred J. Scheetz.

In the fall of 2005, Rebecca matriculated at Colby College as a member of the class of 2009. She has participated in many clubs, including being the president of the student EMTs for three years. Rebecca spent time working in a molecular biology lab at Columbia University before entering the Millard Research Group in the spring of 2008. Rebecca has pursued majors in Mathematical Sciences and Chemistry with a concentration in Cell and Molecular Biology/Biochemistry with the hopes of attending medical school in the future.

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ABSTRACT

Diepoxybutane (DEB), epichlorohydrin (ECH), and 1-(chloroethenyl)oxirane (COX) are small molecules that can form DNA interstrand cross-links. All three of these compounds are, or are metabolites of, industrial chemicals linked to cancer. Additionally, DEB is a metabolite of treosulfan, an anti-cancer drug. We are trying to determine why some of these compounds cause cancer and some can also cure cancer. To do so, we are attempting to link cytotoxicity, cross-linking efficiency, and apoptotic potential of the three compounds to see if any correlations exist. The purpose of this project was to quantify apoptotic potential of DEB, ECH and COX. This was done first by determining caspase activity. These studies showed that in terms of apoptotic potential, DEB>ECH>COX. Later studies quantified apoptosis using flow cytometry with Annexin V/propidium iodide staining. It was determined from these studies that DEB has the strongest apoptotic potential, and while it seems COX>ECH, more work must be done to be sure. If it is determined that DEB>COX>ECH, this would correlate directly to cross-linking efficiency as determined in other studies. Future work may use this correlation to mechanistically explain how some compounds cure cancer while others cause cancer.

INTRODUCTION

Annually, the National Cancer Institute alone spends nearly 6 billion dollars on elucidating the mechanisms of and finding treatments for cancer.¹ In 2008, it was estimated that over 1.4 million people nationwide were newly diagnosed, and nearly half a million people died from various forms of the disease, with lung cancer as the biggest culprit.^{2,3} In 2005, cancer accounted for 22.8% of all deaths nationwide, a figure only surpassed by heart disease. Unlike with heart disease, cerebrovascular diseases, influenza and pneumonia, cancer is still responsible for nearly as many deaths per 100,000 people as it was in 1950. The other diseases mentioned claim less than half as many victims as they did over 50 years ago, and the only decreases in cancer rates have occurred in the past ten years.³ We are finally at the brink of solving the puzzle of cancer, and continued research is essential to a long-term decrease in the rates of people who suffer from the dreadful disease.

Cancer can be caused by a plethora of factors, both internal and external. People are exposed to risk factors, which when experienced alone or in combination, may lead to cancer. Some of the most common risk factors are age, tobacco use, unprotected exposure to UV radiation, exposure to toxic chemicals, poor diet, inactivity, and genetics.⁴ Cancer can occur anywhere in the body, and the specific name of the disease is based on its origin. The main characteristic of the disease is uncontrolled cell division. This can be caused either be uncontrolled mitosis, or by the inability of cells to die.⁵

Normally, cells are meant to recycle regularly, with between 50 and 70 billion cells committing daily suicide in a normal adult.⁶ These cells may be damaged, abnormal,

or merely in excess.⁵ This form of cellular death is called apoptosis, and is tightly regulated

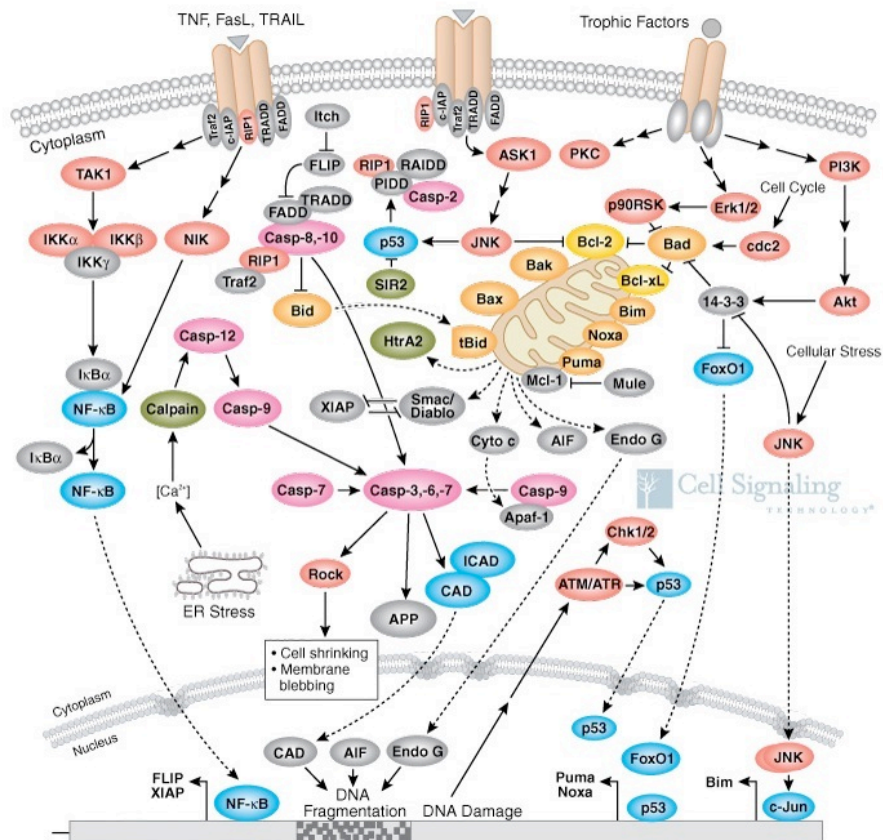


Figure 1: Apoptosis cascade.⁴² Central to this cascade are the caspase family of proteins (pictured in pink). The cascade can be initiated by stress to the endoplasmic reticulum, external triggers, and cellular stress. Ultimately, the process causes DNA fragmentation, membrane blebbing, and cell shrinkage.

so that cells do not either die too rapidly or too slowly, and a happy homeostasis is maintained.⁵⁻⁹ When a cell is signaled to die, either externally via a receptor that activates a “death domain”, or internally due to damage, a cascade that activates otherwise inactive zymogens occurs (Figure 1).⁵⁻⁹ The caspase family of proteins, with 13 involved members, is at the center of this cascade. Specifically, caspase-8 is directly activated by cleavage after an aspartate residue by the death domain and goes on to stimulate

mitochondrial release of cytochrome c from the intermembrane space, which in turn stimulates caspase-3 and -7, a feature that we will exploit in our studies.⁶⁻⁹ Ultimately, caspases are responsible for the cell shrinkage, membrane blebbing, and nuclear degradation that are physical characteristics of this amazing form of cellular control (Figure 2).^{6, 8, 9}

Cells may not always undergo apoptosis. Often, when direct damage occurs, cells will go through necrosis, which is considered the “messy” form of cell death, as the result is the bursting of cells (Figure 2).¹⁰ It has been found that a p53 peptide is able to stimulate necrosis in human breast cancer cells, while leaving healthy cells alone.¹¹ Determining whether or not compounds stimulate apoptosis or necrosis is essential in fully understanding their effects and potential uses for treatment.

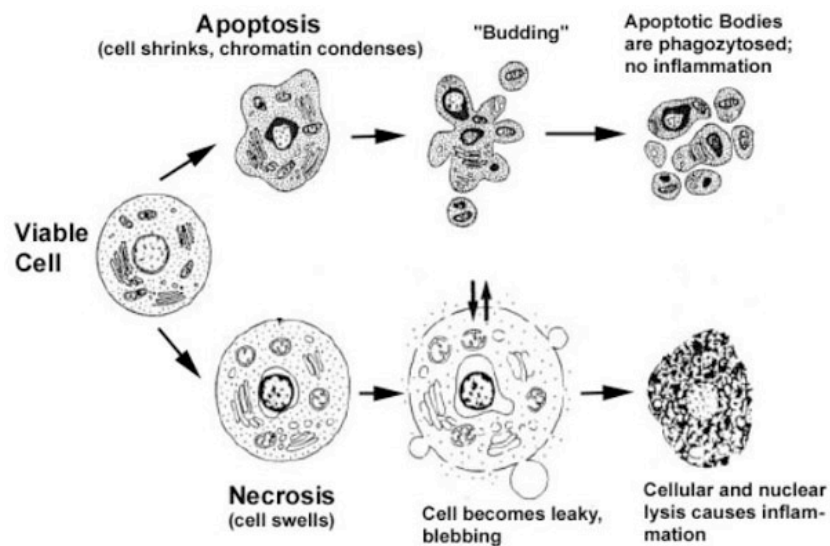


Figure 2: Morphological changes that a cell goes through once death is initiated.⁴³ Top shows apoptosis, which is associated with a large number of cellular changes. Necrosis on the other hand is known as the “messy” form of death, characterized by the lysis of the cell membrane.

When cancer occurs, there is often something wrong with the cell’s ability to undergo apoptosis.^{5-7, 9} Most cancers arise from a series of mutations, which are either

spontaneous or caused by environmental factors. DNA and specific nucleotides may undergo deamination, hydrolysis, formation of dimers, or develop nicks in their backbone, for example. If external chemicals are involved, bases may be deaminated or alkylated, the latter being the focus of the Millard Lab. Alkylation of DNA by a bifunctional agent first involves reaction of one side of the compound with a base. Following this, the other functional group may react with water to form a monoadduct, react with a protein to form a DNA-protein cross-link, bind to the same strand of DNA, making an intrastrand cross-link, or bind to the opposite strand, creating an interstrand cross-link (Figure 3).¹²

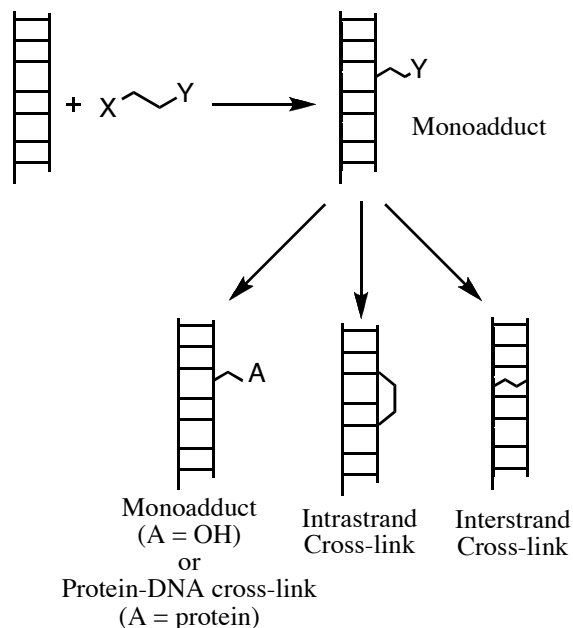


Figure 3: Mechanisms through which bifunctional alkylating agents can alkylate DNA.

Normally, human cells can undergo mismatch repair, base-excision repair, nucleotide-excision repair or direct repair to remedy any defect. However, if these

aberrations are not resolved, or if the repair mechanism fails at replacing a damaged base with the correct one, the mutation may become permanent and cancer may result.⁷ This happens more often when two lesions occur close to each other and on opposite strands, so that neither template is perfect.¹² In addition, repair, replication and gene expression may fail completely if alkylation results in covalently linked strands of DNA.^{12, 13}

The link between mutagenesis and carcinogenesis is significantly greater when a mutation occurs either in a proto-oncogene or a tumor suppressor gene. Oncogenes form as a result of chromosomal rearrangements, chemicals, and radiation that mutate the original sequences of genes, called proto-oncogenes, that are involved in either division or signal transduction. These mutations are dominant, and any mutation will result in tumor development. Mutations to tumor suppressor genes, which are responsible for restraining cell division, may also result in tumor development. However, unlike with oncogenes, these mutations are recessive, and both chromosomes must have a mutation in order to develop cancer. Being born with one defective gene puts you at risk for certain cancers, as you only need one additional mutation, but is not a death sentence. Often, many mutations are necessary before a cell completely loses control and creates a tumor.⁷

Examples of two proto-oncogenes and one tumor suppressor gene that often give rise to cancers after mutations are *bcl-2*, *ras* and *p53*, respectively. It was found in 1988 that the *bcl-2* gene, which normally halts apoptosis by acting before cytochrome c is released from the mitochondria, is overexpressed in many cancers due to mutations and posttranscriptional regulatory networks.^{5, 6} *Ras*, which is mutated in over 20% of cancers, normally helps to transduce external signals involved in growth and differentiation.^{14, 15} Mutations can affect many aspects of the gene and resulting protein,

but often alter one of three critical amino acids involved in its GTPase activity. This results in the accumulation of a large number of active ras proteins, and growth and differentiation is stimulated autonomously.^{14, 15} *p53* is known as the “guardian of the human genome.”⁵ It is responsible for blocking cell division or inducing apoptosis by releasing cytochrome c upon DNA damage. 55-70% of all cancers exhibit a damaged *p53* gene.^{5, 7, 11}

Many chemotherapy drugs that are on the market today have been found to work by stimulating apoptosis in tumors.^{5, 9} The hope is that once cells are induced to undergo apoptosis, the excess or damaged cells will proceed to die. Therapies that target apoptosis have a wide range of mechanisms of action and include DNA cross-linking agents, DNA fragmenting agents, antimetabolites, intercalating agents, kinase inhibitors, and hormones. Unfortunately, it has been found that in cancers characterized by oncogene activation and tumor suppressor gene mutation such as those mentioned above, these drugs are unable to reverse the effects of their downstream mutations.⁵ Thus, it is important to know where in the cascade the drug is stimulating apoptosis to maximize its effectiveness.

Many chemicals that are often found to be carcinogenic also have been shown to have anti-cancer properties, both *in vivo* and *in vitro*. The first chemical shown to exhibit this behavior was nitrogen mustard after autopsies of soldiers exposed to the gas in World War I showed potential therapeutic effects from the chemical.^{16, 17} Later, nitrogen mustard was proved to be a bifunctional agent that exhibits its effects by cross-linking strands of DNA, something that is particularly cytotoxic because it can physically inhibit transcription and DNA replication.^{9, 12, 13, 16, 17} Many other drugs have been discovered to

have similar properties in that they work beautifully at targeting tumor cells, but then increase the patient's chance of developing additional tumors later in life.^{18, 19} Thus, these compounds are of particular interest to many researchers.

Since the discovery of nitrogen mustard, many additional chemicals have been discovered to work by cross-linking DNA. This includes, but is by no means limited to, diepoxybutane, epichlorohydrin and 1-(chloroethenyl)oxirane, which are all classified as probable carcinogens (Figure 4). These three chemicals are the current focus of the Millard lab, and we are trying to find out as much as we can about how they exhibit their effects with the hope of finding a link between cross-linking efficiency, cytotoxicity and apoptotic potential.

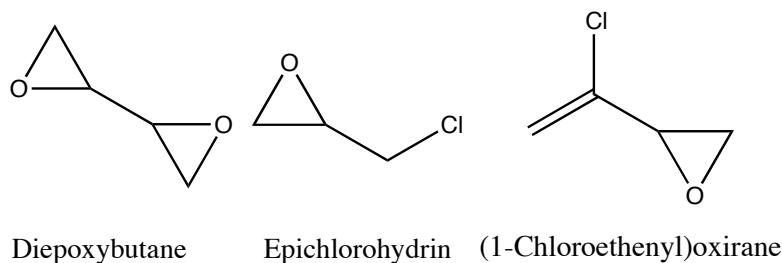


Figure 4: Diepoxybutane, epichlorohydrin and (1-chloroethenyl)oxirane, the three bifunctional alkylating agents currently at the center of research in the Millard Group.

Diepoxybutane (DEB) is a potent metabolite of 1,3-butadiene, a colorless gas that is a byproduct of ethylene production, is used in the production of plastics, rubber and latex, and can be found in cigarette smoke and automotive emissions.^{20, 21} DEB is also a metabolite of treosulfan, an anti-cancer drug.²² The Millard lab previously determined that DEB preferentially forms interstrand cross-links at distal deoxyguanosines at 5'-GNC sites in duplex DNA.²³ Total damage in cultured cells was

shown to be comparable in both nuclear and mitochondrial DNA.²⁴ Exposure to diepoxybutane has been linked to the increase in the incidence of hematopoietic and lymphatic cancers, and inhalation exposure to DEB is highly carcinogenic, even for weeks after exposure stops.^{25, 26}

Epichlorohydrin (ECH) is an epoxide commonly used in the production of epoxy resins, glycerin, elastomers, insecticides, agricultural chemicals, and other specialty chemicals, and is classified as a probable carcinogen.^{21, 27} Epichlorohydrin exposure has been found to increase the risk of lung cancer, in addition to causing chromosomal aberrations and breaks.^{28, 29} Previous work in the Millard lab has shown that ECH cross-links deoxyguanosines at both 5'-GNC and 5'-GC sites.²¹ Total damage in cultured cells was found to be greater in nuclear DNA than in mitochondrial DNA.²⁴

(1-Chloroethenyl)oxirane (COX) is a primary metabolite of β -chloroprene, a monomer used in the production of polychloroprene (neoprene) and other synthetic rubbers.^{25, 30} Chloroprene has been found to be carcinogenic via inhalation, with a major source of the toxicity originating from its epoxide metabolites.²⁵ COX has recently been shown to have DNA cross-linking activity, although its sequence preference has not yet been elucidated.³¹

Currently, we are trying to determine the effects of DNA packaging on cross-linking by these agents. Mitochondrial DNA (mtDNA) is protected by a membrane, but not by associated proteins. Nuclear DNA (nDNA) on the other hand, has a very intricate system of packaging that allows nearly one meter of DNA, the approximate length of the chicken genome, to fit within a nucleus that is less than 15 micrometers wide.^{7, 13, 32, 33} In eukaryotic cells, most nDNA is not free, but is instead associated with histones. There are

five types of histone proteins, H1, H2A, H2B, H3, and H4. Additionally, avian erythrocytes have been found to have an additional histone, H5, that functions like H1 in humans.³⁴ All histones are positively charged to ease binding to negatively charged DNA. The latter four proteins are found in duplicate and form a core octamer of one (H3,H4)₂ tetramer and two (H2A, H2B) dimers. Each histone has a N-terminal tail that helps guide nDNA around the proteins. DNA is wound left-handedly around this core approximately 1.65 times, with 147 base pairs involved in the loops (Figure 5a). The phosphate backbone is connected to the histone proteins through multiple hydrogen bonds. These bonds often form in the minor groove, and while they are not sequence specific, tend to be rich in A=T pairs, due to their relative flexibility. This complex of histones and DNA is called a nucleosome and exhibits symmetry across a dyad axis. Portions of linker DNA, often between 20 and 70 bp, separate individual nucleosomes and make this level of packing resemble “beads on a string” (Figure 5a). H1 binds the end of this linker DNA to the middle of the core DNA and stimulates the next level of packing by bringing adjacent nucleosomes closer together (Figure 5b). This initiates packing of nucleosomes into a solenoid, which is also called the 30 nm fiber (Figure 5c). The 30 nm fiber is then looped around a chromosomal scaffold that helps pack the nDNA into its final form.^{7, 13,32}

nDNA that is packed into histones has an altered structure from free DNA. Not only do the histone proteins physically block replication, recombination, repair and transcription, but they also cause a 45° curvature in the DNA per turn around the octamer.^{13, 32} Additionally, the three turns of DNA in the center are underwound, with 10.7 bp/turn, but the rest is overwound, with 10.0 bp/turn. These differences may ultimately affect the reactivity of nDNA bound to histones as compared to free DNA.^{13, 35}

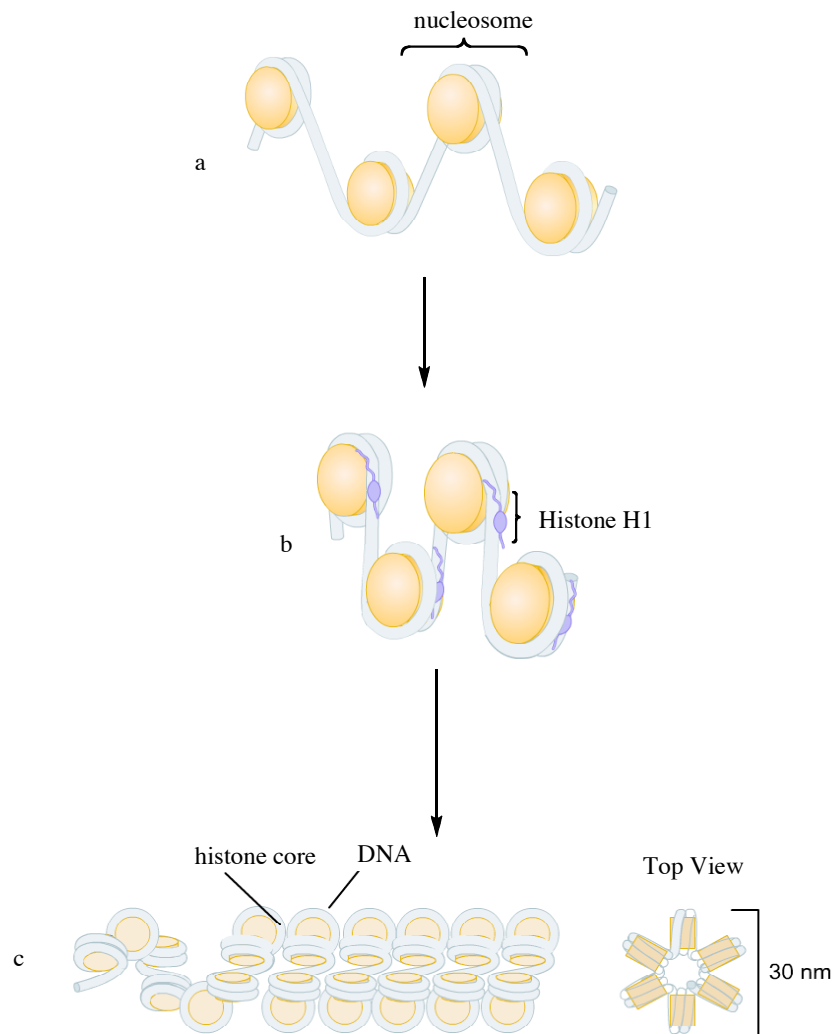


Figure 5: DNA condensation in humans. (a) First, DNA loops around a histone core to form a structure resembling “beads on a string.” (b) Histone H1 then condenses this string further and initiates formation of (c) a solenoid.³²

Previous studies done in the Millard lab showed that at concentrations of 250 mM, DEB and ECH cause equivalent damage to condensed and uncondensed DNA. Additionally, DEB damages mtDNA much more rapidly than does ECH. One of the goals of this research is to determine whether or not these patterns are found at concentrations closer to 10 μ M, which is amount of treosulfan in patients treated with the

drug.³⁶ Additionally, we are particularly interested in COX and how its reactivity *in vivo* compares to the related compounds DEB and ECH.

Before we can focus on the targets of each cross-linker, we needed to conduct apoptosis studies. As part of the apoptosis cascade, endonucleases are produced to digest the cell's DNA.^{6, 8, 9} We needed to ensure that these endonucleases were not interfering with our DNA, so that any damage observed was due to the direct effects of the treatment. In order to measure apoptosis, an assay testing for caspase -3 and -7 was used. In the presence of these caspases, a substrate is cleaved and light is produced by luciferase (Figure 6).³⁷ Luminescence is thus directly proportional to caspase activity. These assays were used to ensure that further experiments would not be confounded by apoptotic endonucleases. We tested for caspase activity in cells treated with DEB, ECH and COX, concentrations ranging from 10 μ M to 1 mM and time points ranging from 2 to 24 h.

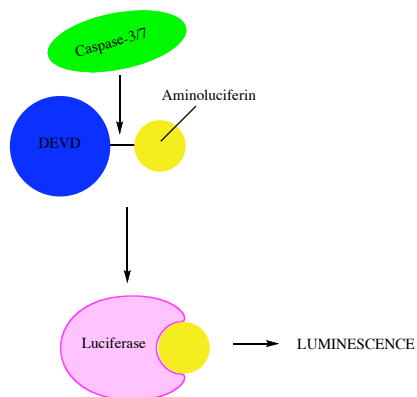


Figure 6: Caspase-3/7 Assay. Caspase cleaves a substrate to release aminoluciferin. The aminoluciferin is then consumed by luciferase, causing luminescence.³⁴

After determining the minimum treatment length and concentration in which caspase is expressed, we could study the DNA targets of each chemical at lower doses and treatment times. We used real time PCR, which fluorescently tags PCR products, allowing quantification of PCR products as they are amplified.³⁸ This technique allows for easy detection of minor changes in the amount of undamaged starting DNA so that even a small amount of damage can be detected. Our studies focused on an unexpressed, condensed portion of the β -globin gene, an expressed portion of the folate receptor gene, and a portion of mtDNA.

Final studies used flow cytometry to quantify cell death. Although it is not known whether ECH and COX induce cell death via necrosis or apoptosis, recent studies in human lymphoblasts demonstrated that DEB induces apoptosis via a mitochondrial pathway.³⁹ In order to measure the proportion of cells undergoing apoptosis and necrosis in cells exposed to DEB, ECH and COX, flow cytometry was used with propidium iodide and Annexin V staining. Propidium iodide (PI) intercalates into DNA, causing red fluorescence. However, PI cannot enter apoptotic cells, as membrane integrity is maintained. On the other hand, necrotic cells have lost membrane integrity, and fluoresce red in the presence of PI (Figure 7).¹⁰ Annexin V binds to phosphatidylserines, which are normally distributed solely on the inner cell leaflet of the plasma membrane. However, an important part of the apoptosis cascade involves translocase activity to flip these molecules out, so that they are exposed. Once exposed on the outer membrane, Annexin V conjugated with FITC causes cells in apoptosis, and necrotic cells with compromised membrane integrity, to fluoresce green (Figure 7).^{10, 40} When cells are stained with PI and Annexin V-FITC, necrotic cells show high fluorescence for both, apoptotic cells only

show high green fluorescence corresponding to Annexin V, and healthy cells show no fluorescence (Figure 7). A chart of red versus green fluorescence for each cell is then created. Regions can be drawn to mark levels of death, with the lower left quadrant corresponding to healthy cells, lower right to apoptotic cells, and upper right to necrotic cells (Figure 8). Proportions of cells in necrosis and apoptosis can then be determined by counting cells in each region.^{10, 41}

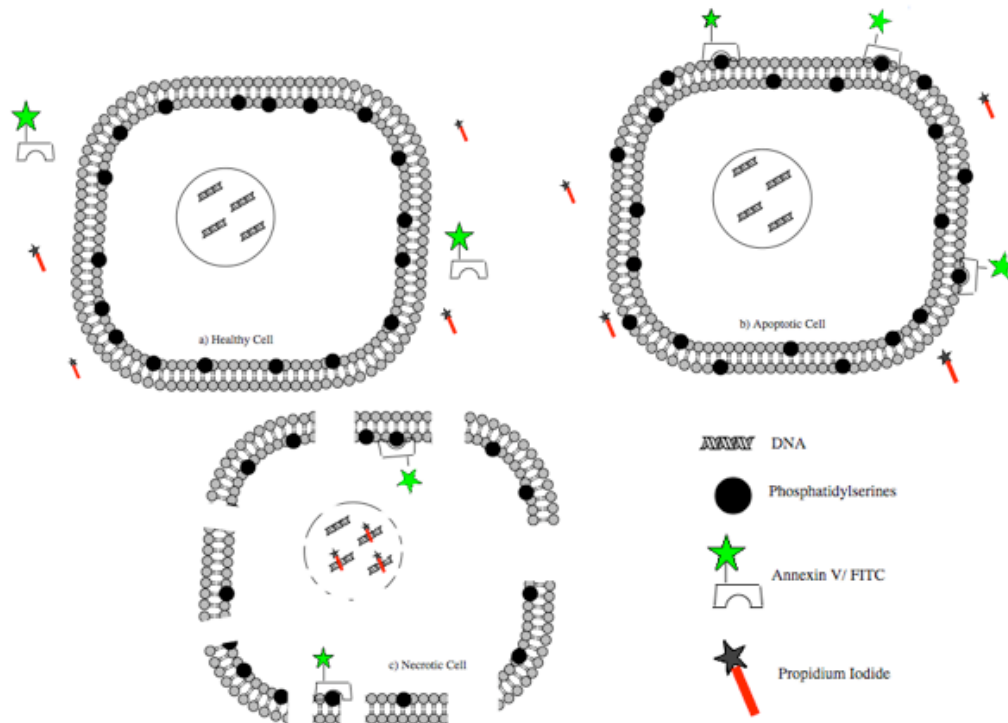


Figure 7: Annexin V and propidium iodide binding in varying types of death. (a) Healthy cells exclude both Annexin V or PI, and neither binds. (b) During apoptosis, phosphatidylserines are exposed on the outer membrane, allowing Annexin V binding and green fluorescence. However, membrane integrity is maintained and PI cannot enter the cell. (c) In necrosis, membrane integrity is lost and both Annexin V and PI can bind to their respective targets, and green and red fluorescence is detected.

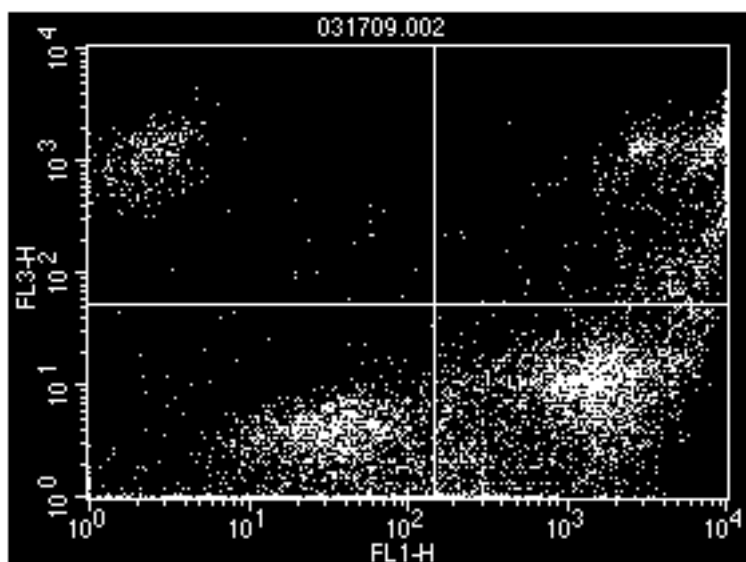


Figure 8: Flow Cytometry scattergram, with red versus green fluorescence. Cells treated with 10 μ M camptothecin for 4 h. Healthy cells do not show any fluorescence and remain in the lower left quadrant. Apoptotic cells fluoresce green only, and are found in the lower right quadrant. Necrotic cells show both red and green fluorescence and are found in the upper right quadrant.

The results of this study will complement other work in the lab on the relationship between cross-linking efficiency and cytotoxicity of DEB, ECH and COX. Previous work has shown a correlation between cross-linking, cytotoxicity and apoptotic potential for a family of platinum-containing compounds.⁴¹ Understanding these relationships for our family of epoxides may provide insight into carcinogenicity versus anti-tumor potential.

MATERIALS AND METHODS

Caution: DEB, ECH and COX are suspect carcinogens and must be handled appropriately.

Cell Culture

Chicken 6C2 (erythro-progenitor) cells were cultured in Richter's modified Eagle's medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, 1 M Hepes buffer, 100 units/L penicillin, 100 µg/mL streptomycin, and 50 µM β-mercaptoethanol, all of which were run through a sterile filter. Cells were then incubated at 37 °C with 5% CO₂ and passaged to fresh media in a 1:5 (1mL cells: 4 mL fresh media), 1:10 (1 mL cells: 9 mL fresh media) or 1:20 (0.5 mL cells: 9.5 mL fresh media) dilution every few days, or when they achieved between 80 and 90% confluence.

Treatment

Cells at 75-85% confluence were passaged into 6-well plates with a total volume of 5mL fresh media prior to treatment with the cross-linkers. These cells were then incubated with 1 µM to 1 mM DEB, ECH and COX for a maximum of 24 h (37°C, 5% CO₂). In addition, 5 mL control cells were left untreated. When positive controls for apoptosis were needed, 5mL cells were treated with camptothecin (1 to 50 µM, maximum 24 h, positive control for apoptosis).⁴⁴

Assessment of Apoptosis via Caspase Activity Assays

Caspase-3/7 activity was monitored in cells treated as outlined above. At various time intervals between 2 and 24 h, 100 μ L cells (less than 20,000 cells) were collected in microcentrifuge tubes. Samples were centrifuged at 250 x g for 5 min, the pellets were washed with 1x phosphate buffered saline solution (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl [pH 7.4]), centrifuged again, and suspended in 100 μ L PBS. Samples were then transferred to a 96-well plate, and 100 μ L of caspase-3/7 reagent (Promega) was added for a total volume of 200 μ L. Cell blanks were made up of either 100 μ L cells or PBS with either 100 μ L reagent or 100 μ L additional PBS (total of four blanks). Caspase activity was measured by luminescence every 15 to 30 minutes for circa 90 min (Molecular Devices Spectra Max M2).

Isolation of DNA

After cell treatment with DEB, ECH, and COX as outlined above, between 500 μ L and 1.5 mL cells (less than 5×10^6 cells) were transferred to microcentrifuge tubes and spun at 500 x g for 5 min. The supernatant was removed and cells were resuspended in 500 μ L PBS. Isolation was carried out according to the procedures included in the Qiagen DNeasy Blood and Tissue Kit. This kit has a special spin column that is designed to bind DNA from lysed cells. Several buffers are then used to wash the column. Pure DNA is extracted by a final centrifugation with 200 μ L elution buffer. DNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer.

Assessment of DNA Damage Using Real-Time PCR

Primers were previously designed by the Millard Lab to amplify mitochondrial DNA, a portion of the folate receptor gene (expressed, FR), and a portion of highly condensed chromatin at the 3' terminus of the β -globin gene (unexpressed).²⁴ Primer sequences were synthesized by Integrated DNA Technologies and are as follows.

Mitochondrial : 5'- CTCCCAGCCCCATCCAACATCTCTGCTTGATGAAA and 5'-
TAACGGTGGCCCCTCAGAATGATATTTGGCCCCA.

FR: 5'- AAAGTACTACGCCTGGAAGAAGAGA and 5'-
ATTCAGAAATGGATCATGAACAAAC.

Unexpressed: 5'- AGTACTGCCGTGTGTTTGCTC and 5'-
TACAGCCCTCTCAGCAAGTAA.

Full Sequences of amplified regions are seen in Figure 9. PCR reactions (25 μ L) contained 12.5 μ L (1x) Qiagen 2x QuantiFast SYBR Green PCR Master Mix (containing HotStarTaq *Plus* DNA Polymerase, QuantiFast SYBR Green PCR Buffer, dNTP mix, SYBR Green I, and ROX), 1 μ M each primer, and 15 ng template DNA. RNAase free water was added to bring the total volume up to 25 μ L. The thermal cycling was completed in a real-time PCR cycler (Applied Biosciences StepOne Real-Time PCR System) with the following sequence: 5 min at 95 °C, 40 cycles at 10 s 95 °C and 30 s at 60 °C. Included at the end was a melt curve built into the software. Fluorescent data were collected during the 60 °C phase, and the amount was plotted against cycle number. Cycle thresholds (C_T) were assessed by determining the cycle at which fluorescence exceeded 3.5 units.

Prior to experimentation, untreated cells were cultured and PCR was performed at each locus, as outlined above, using 50, 25, 12.5, and 6.25 ng DNA. Amount of DNA was then plotted against average C_T and it was determined that 15 ng appeared in the linear portion of the data for each locus, so this was an appropriate amount of DNA to use in the experiments.

Assessment of Apoptosis via Flow Cytometry

Flow cytometry was performed on cells treated as outlined above. Additionally, 500 μL cells ($\sim 1 \times 10^6$ cells/mL) were incubated at 56 °C for 45 min to induce necrosis. After each treatment, 5 mL cells were spun at 250 x g for 5 min and then resuspended in 2 mL cold PBS. Cell suspensions were then counted to ensure presence of sufficient cells to make a final concentration of 1×10^6 cells/mL in 100 μL . Cells were then prepared for flow cytometry using an Invitrogen Vybrant Apoptosis Assay Kit #3. The 5X annexin-binding buffer included in the kit was diluted to 1X, and a 1 mg/mL stock of propidium iodide (also included) was diluted in the 1X buffer to a final concentration of 100 $\mu\text{g/mL}$ (referred to as the working solution). Cells were then spun at 250 x g for 5 min and resuspended in the 1X buffer to a final concentration of $\sim 1 \times 10^6$ cells/mL (at least 100 μL needed for each assay). A 100 μL cell suspension was transferred to a flow cytometry tube and 5 μL Annexin V and 1 μL working solution were added. Cells were then incubated at room temperature for 15 min. Four hundred microliters 1X buffer was added, and the cells were kept on ice until analysis. Cell viability was determined via flow cytometry (BD Biosciences FACScalibur with CellQuest software). FL-3 (>575 nm, Texas red fluorescence) versus FL-1 (530 nm, green fluorescence) plots were then

created. The percentage of cells experiencing apoptosis was then calculated from these scattergrams.

MITOCHONDRIAL LOCUS (376 bp)

CTCCCAGCCCCATCCAACATCTCTGCTTGATGAAATTTTCGGCTCCCTATTAGC
AGTCTGCCTCATGACCCAAATCCTCACCGGCTACTACTAGCCATGCACTACA
CAGCAGACACATCCCTAGCCTTCTCCTCCGTAGCCACACTTGCCGGAACGTA
CAATACGGCTGACTCATCCGGAATCTCCACGCAAACGGCGCCTCATTCTTCTT
CATCTGTATCTTCCTTCACATCGGACGAGGCCTATACTACGGCTCCTACCTCT
ACAAGGAAACCTGAAACACAGGAGTAATCCTCCTCCTCACACTCATAGCCAC
CGCCTTTGTGGGCTATGTTCTCCCATGGGGCCAAATATCATTCTGAGGGGCCA
CCGTTA

UNEXPRESSED LOCUS, 3' to β -globin gene (398 bp)

AGTACTGCCGTGTGTTTGCTCCGTAATAGTCTTGCTGGTTTACCGCACAGAAG
GTTTCATCCATTCCAGATGATTTTGTAAAGGGGAGCGACTTGAAAATGGGGA
GCTAAATGCTCTCAGCCCTCTCAATTTTCCAGCTTATTCTGCTCATGAAATCCT
TAACTTGCAATATATCCTCCAGAGAATAGGAAAACCCACATCTAACTGTTC
AACTGCAGATAGATAAATATCAGTGCTGAATTTGTGTGCTGATTCAAGTGAA
TGCTTATATCAATTCCCAGGAATGAGCCACACGTAATCCCTATGAAAGAACC
AGTGAAGCAGTCATTTAATGGCTGTGGGTCAGCGACTTGAATGAGCTCATTTC
TTGAGCCTTTTACTTGCTGAGAGGGCTGTA

OPEN LOCUS, within FR gene (451 bp)

AAAGTACTACGCCTGGAAGAAGAGATCCTCTCCTGCCAGGATGGAGGACGTG
ACTCCTGAGGCAGGCAGAGCTGCGTGTGCTGTGCCACGGCTCACCTGCTGC
TGGCCCTCGTGCTGCTCACTGCAGGCTGGGGGTCCTATGGGTGGGGGTCCCTG
TGACAGCTCCCTGAAGGGATGGGGCTTCTCTTTCTGCAAAGGAGGAGGCTGC
TCCCGTGGCATGAGTGGCACTTGGTCTGTTCAATTTGCTTGTGTGCCCCAGTG
GATGCTCAGCATGGACTGTGGTGTGGACTTTGATGCCCCTGCTGCTTCCAG
CTGGTGGAGGCAGTGATTTCCCATCCTTTGTGTCTGAGCTCCTTCTGTCTTCCC
ACCCTGTGTTTCTGCCCATAGGACAGGTGCTGATGGCTGTGCTCTGGGTGGG
TAGTGTTTGTTCATGATCCATTTCTGAAT

Figure 9: Sequences of PCR products for each locus probed.²⁴

RESULTS

Caspase Assay

Levels at which DEB, ECH and COX exhibit their cytotoxic effects and induce apoptosis in 6C2 erythro-progenitor cells were determined by monitoring caspase-3/7 activity over a range of concentrations and time points. This was done using a proluminescent caspase-3/7 substrate that generates a luminescent signal when in the presence of caspase -3 or -7. Concentrations ranged from 1 μ M to 1 mM of each toxin. Caspase activity was assessed at time points between 2 and 24 h. Using camptothecin at various concentrations as a positive control, it was determined that luminescence between 950 and 9000 relative light units (RLU) is a positive indicator of apoptosis. Each trial also included several negative controls with cells and caspase reagent, just cells, buffer (phosphate-buffered saline solution) and caspase reagent, or just buffer. If the control cells with caspase were found to be within the ranges mentioned above, it was determined that the cells had overgrown in their wells and the assay was deemed unreliable. It was determined using the negative controls that values below 400 RLU could be attributed to noise inherent in the luminometer.

In addition to looking at raw luminescence values, data were analyzed by dividing the luminescence of the sample by the luminescence of the control (Table 1). This allowed complete sets of data to be analyzed by observing whether or not this value was found to be greater than 1. Values greater than 2, along with raw luminescence of 950, indicated apoptosis.

Cells that were treated with 250 μ M and those treated with 500 μ M DEB showed apoptosis after 24 h, cells treated with 1 mM ECH showed apoptosis after 4 hours, and

cells treated with 1 mM COX showed apoptosis at 22 h (Table 2 and Figure 10). However, the luminescence signals of the 500 μ M DEB were slightly lower than 950 RLU, and time points between 12 and 24 h must be collected in order to adequately determine apoptosis. There was no significant apoptosis observed in treatments less than 250 μ M (Table 2 and Figure 11). This assay proved to be quite sensitive to time, as the caspase signal peaks at the times given, but was not present at intervals several hours afterwards. For the samples treated with 1 mM COX, the luminescence signal appears to be especially sensitive to time. Qualitatively, after the 22 h time interval, the cells appeared smaller, and membrane blebbing was seen, even though the cells no longer showed caspase activity at 24 h. These cells resembled those of the camptothecin control several hours after peak luminescence.

Table 1: Luminescence (RLU) of the treated cells divided by the untreated control cells. Values shown are of dosages of DEB, ECH and COX shown to induce apoptosis over the control. Numbers in italics are greater than 2, and with the exception of 500 μ M DEB at 24 h, which does not correspond to luminescence above 950 RLU, denote apoptosis. An asterisk (*) denotes no data for that particular concentration or time.

Dosage
250 μ M

500 μ M

1 mM

		Lumir S ₀ Lumir C
Time (h)	DEB	1
4	1.14	
12	0.90	
24	3.52	
4	0.88	
12	0.90	
24	2.48	
4	4.22	
6	1.31	
12	*	
18	*	
22	*	
24	0.83	

Table 2: Luminescence (RLU) of the treated cells divided by the untreated control cells. Values shown are of dosages insufficient to induce apoptosis for DEB, ECH and COX. The one value that is above 2, which corresponds to 100 μ M ECH at 6 h, is disregarded because the control cells showed high luminescence. An asterisk (*) denotes no data for that particular concentration or time.

Dosage	Time (h)	Luminescence of Sample/ Luminescence of Control		
		DEB	ECH	COX
25 μ M	24	1.18	1.32	1.18
50 μ M	3	0.76	0.87	0.66
	6	1.08	1.04	1.13
	9	1.02	0.77	0.95
	12	0.81	1.86	1.03
	24	1.34	0.70	0.51
100 μ M	4	0.77	0.72	*
	6	1.44	2.23	*
	12	*	1.23	*
	24	1.42	0.85	*

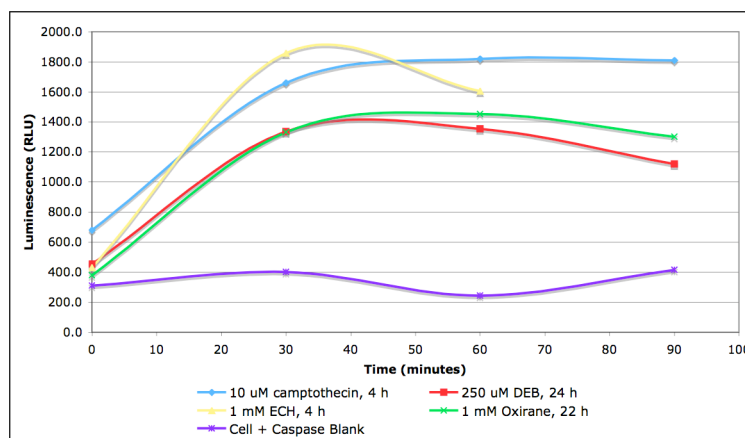


Figure 10: Luminescence (RLU) vs. time in luminometer (min) for samples that yielded positive indications of apoptosis. Also included is a camptothecin at 10 μ M for 4 h as a positive control, and untreated cells with the caspase reagent as a negative control. Clearly, apoptosis can be considered with luminescence above 950 RLU, and any values around 400 RLU resemble normal, healthy cells.

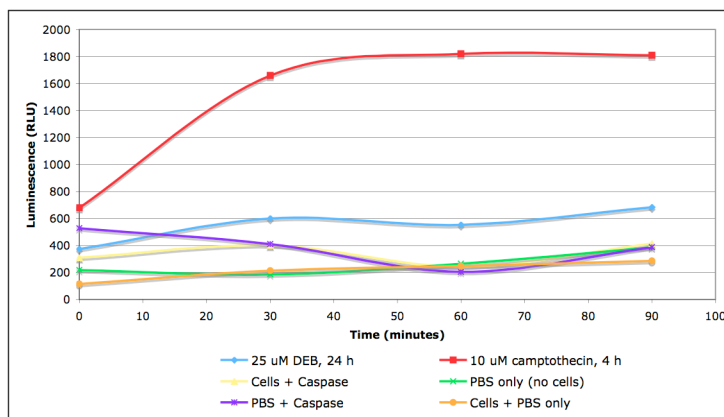


Figure 11: Luminescence (RLU) vs. time in luminometer (min) for samples that yielded negative indications of apoptosis. These include DEB at 25 μ M and 24 h, and all of the negative controls described, untreated cells with caspase reagent, untreated cells with just PBS, PBS with caspase reagent and PBS alone. Also included is a camptothecin at 10 μ M for 4 h as a positive control. This shows that luminescence below 400 RLU can be attributed to noise inherent in the machine, and that samples with apoptosis induction luminesce in the range of 1000 RLU.

It was also established that when using longer trials, it is necessary to begin treatment soon after transferring cells to the 6-well plates, instead of waiting 24 hours until cells reached between 80 and 90% confluence, as was done previously. This is because the cells are easily overcrowded and continue to multiply after treatment. Negative controls have been shown to exhibit apoptosis after 48 hours of growth (24 hours of “preparation” and 24 hours of “treatment”). Using a 1:10 dilution of 80% confluent cells and then treating between 4 and 6 hours later qualitatively yielded the healthiest control cells after 24 hours.

Real Time PCR

Real time PCR was performed on chicken erythro-progenitor cells exposed to various concentrations of DEB, ECH and COX for several time points. Before assessing for

damage due to our cross-linking agents, standard curves were generated using varying amounts of starting DNA template from healthy, untreated cells. All three loci, unexpressed nDNA, expressed nDNA and mtDNA showed a region where the cycle threshold is proportional to amount of template (Figure 12). These linear regions were curve fitted to linear trendlines using Microsoft Excel. The unexpressed and mtDNA loci are linear with lower amounts of template, while the expressed loci requires greater than 12.5 ng to have a linear relationship. Thus, our studies were performed with 15 ng starting template DNA, which fell within the linear region of all three loci.

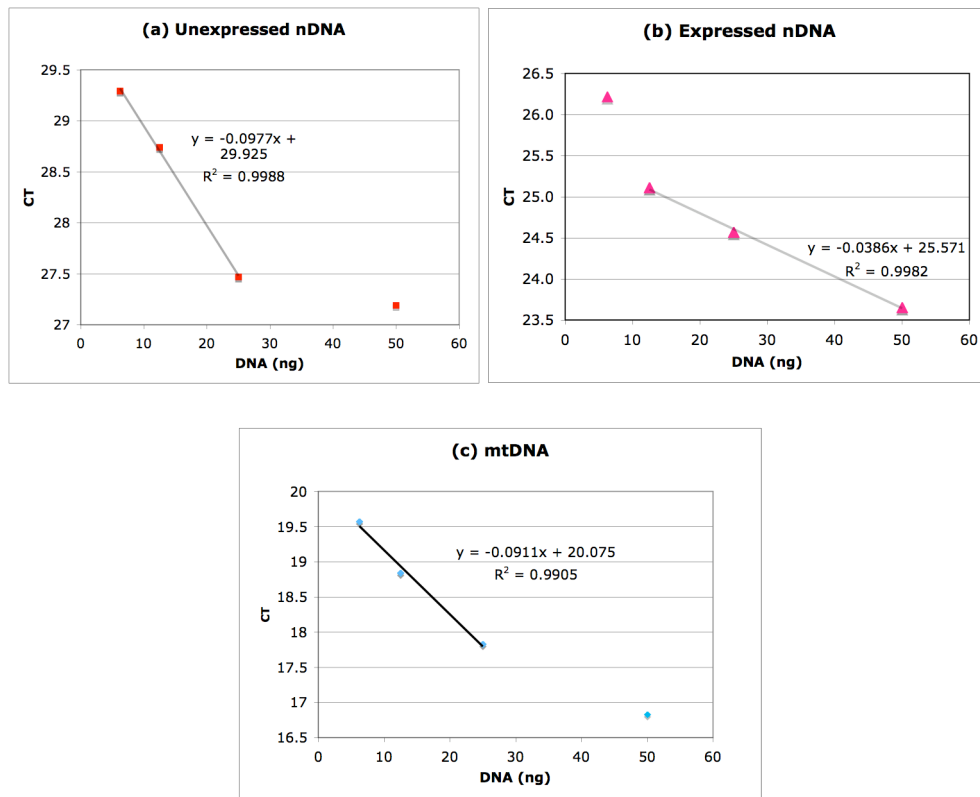


Figure 12: Standard curves generated using variable amounts of starting template DNA. Samples were amplified using real time PCR, and the cycle at which there is an appreciable amount of fluorescence was determined (cycle threshold, C_T). Curves were generated at three loci: (a) unexpressed nDNA, (b) expressed nDNA, (c) mtDNA. All three curves show linear regions that have been approximated using linear regression in Microsoft Excel.

We tested treatments of 10 μM for 24 h, 100 μM for 12, 18 and 24 h, and 1000 μM for 2 h, and looked at regions of expressed nDNA, unexpressed nDNA and mtDNA. Damage was assessed by subtracting the cycle threshold of the untreated control from the threshold of the sample. Samples with a difference greater than 0.5 were considered significant (Table 3). It was determined that there was some evidence of damage in the unexpressed region from 1 mM DEB and ECH after 2 h. DEB also damaged mtDNA in the same conditions. Additionally, damage at the expressed locus by 1 mM ECH and COX was seen after 2 h. However, these results had high standard deviations and were not always reproducible. Thus, we concluded that the regions that we were amplifying were too short, especially at such small concentrations of our cross-linkers. Further studies are needed with new amplicons in order to determine whether or not these results are accurate.

Table 3: Real Time PCR summary. Dosages that indicated significant damage are highlighted in blue. Otherwise, no concentrations were significant to induce damage at the specified locus.

	Unexpressed	Expressed	mtDNA
DEB	1 mM, 2 h	1 mM, 2h	1 mM, 2 h
ECH	1 mM, 2 h	none	none
COX	none	1 mM, 2 h	none

Flow Cytometry

In order to quantify the proportion of cells undergoing apoptosis after treatment with DEB, ECH and COX, flow cytometry with propidium iodide/Annexin V staining was performed. Percentage of cells undergoing apoptosis or necrosis due to treatment was

determined by subtracting the percentage of cells in the right upper and lower quadrants from the percentage in the corresponding untreated population's quadrants. Cells treated with camptothecin or subjected to heat shock were assessed to serve as a benchmark for treatment with our cross-linkers (Figure 13). It was determined that after treatment with 1 μ M camptothecin for 24 h, 16% of cells were induced to be apoptotic, while 47% were necrotic. After 10 μ M camptothecin for four hours, apoptosis was induced in 40% of cells, with no additional necrotic cells as compared to untreated cells. After 100 μ M camptothecin treatment for two hours, 17% of cells showed apoptosis induction and 6% were necrotic.

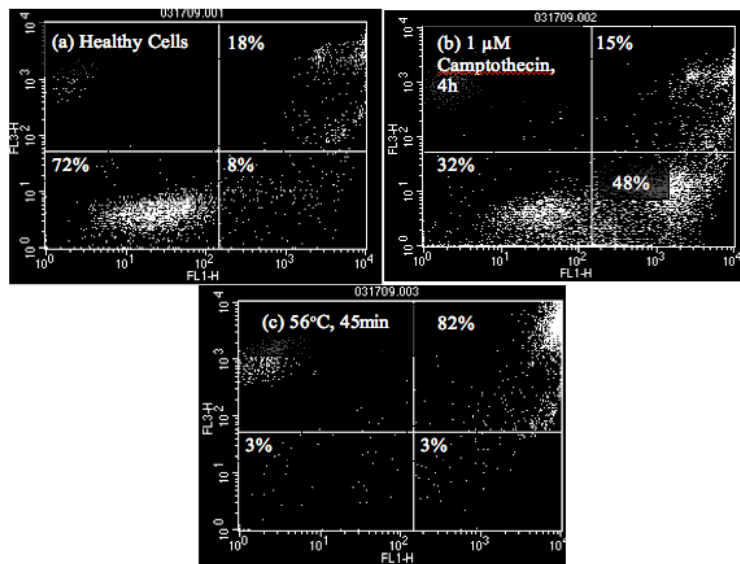


Figure 13: Types of cell death as observed using flow cytometry with Annexin V (FL1)/propidium iodide (FL3) staining in 6C2 cells. (a) Healthy, untreated cells. (b) Cells induced to undergo apoptosis by camptothecin. (c) Cells induced to become necrotic after heat shock. Percentage of cells in each quadrant is indicated.

Cells were treated with 1, 10, 100 or 1000 μ M of each cross-linker for 4, 6, 12, or 24 h before assessment via flow cytometry. Preliminary results indicated that a 24 h treatment induced apoptosis in was induced in 6.02% of cells treated with 1 mM COX

and 10.31% of cells treated with 1 mM DEB after 24 h (Figure 15d). The sample treated with DEB showed that an additional 5% of cells were necrotic over the untreated (data not shown). Additionally, after 12 h, 1 μ M ECH showed 6% of cells to be apoptotic and 14% to be necrotic, 10 μ M DEB showed 6% of cells to be apoptotic and 5% to be necrotic, and 10 μ M COX induced apoptosis in 6% of cells and necrosis in 5% (Figure 15c).

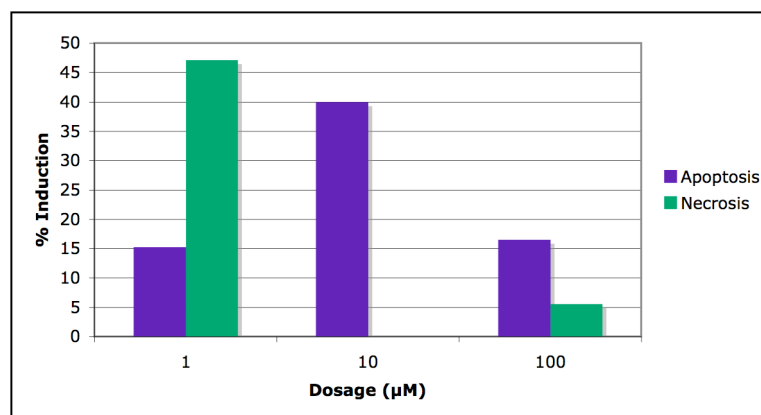


Figure 14: Percent of apoptotic (purple) or necrotic (green) induction after treatment with 1 μ M camptothecin for 24 h, 10 μ M for 4 h, or 100 μ M for 2 h.

However, another set of data collected after treatment with each compound at each dose for 24 h showed different results, with the exception of the 1000 μ M DEB sample, which still induced apoptosis in \sim 10% of cells (Figure 15e-f). These experiments must be repeated in order to determine the accurate pattern of apoptosis induction.

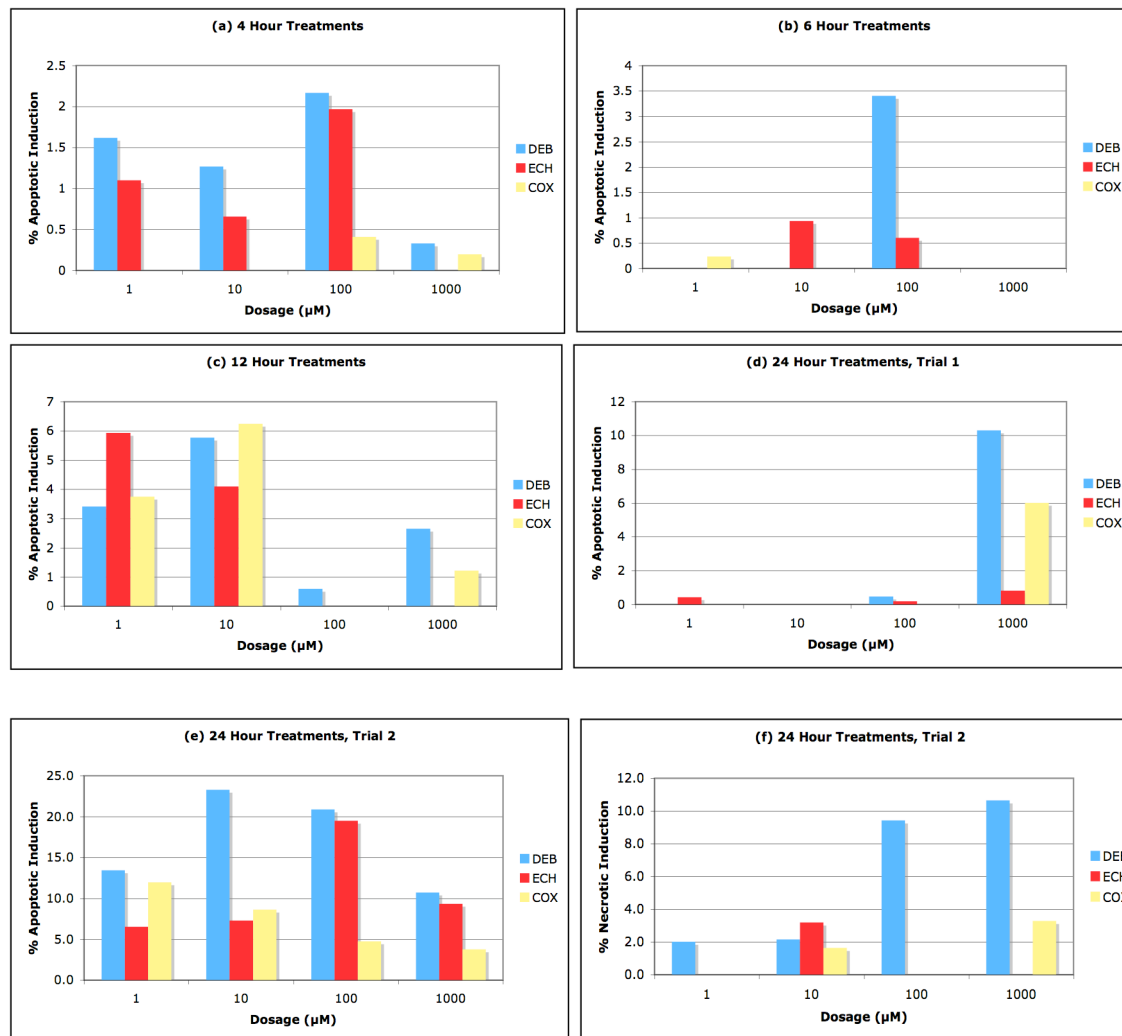


Figure 15: Percentage of cells with apoptosis or necrosis induced above untreated cells after treatment with DEB, ECH or COX. Duration of treatment is indicated.

DISCUSSION

Previous and current work in the Millard research group has focused on determining cytotoxicity, cross-linking efficiency and apoptotic potential at high concentrations of DNA alkylating agents for short incubation times. This research has been focused on more physiological concentrations of DEB, ECH, and COX. The studies used chicken

erythro-progenitor cells because many alkylating agents are linked to development of leukemia and other hematopathologies.^{18, 19, 25, 26}

It was first determined that DEB induces apoptosis, as determined by caspase activity, at 250 and 500 μ M, concentrations much lower than those observed in ECH and COX. Previous studies in the Millard lab indicate apoptosis at concentrations as low as 50 μ M.²⁴ However, at 1mM, ECH shows caspase activity after four hours, while COX requires 22. The conditions required to induce apoptosis in COX are nearly four times those required in DEB. Thus, initial studies established that in terms of apoptotic potential, DEB>>ECH>COX.

Many chemotherapeutic agents exert their effects by inducing apoptosis in cells.^{25,26} This guides cells that are not dying normally, such as tumor cells, to perish. Thus, it may be that the increased apoptotic potential in DEB explains why its parent compound, treosulfan, is used as an anti-cancer drug, while ECH and COX are not.

Our results are also consistent with the idea of an apoptotic window within which caspase activity is present. It is known that the apoptosis cascade can take as little as 60 min, and if the assay is not performed within this window, the caspase has been degraded and cells will not luminesce, even if they have died from such a cascade.⁸ This result was especially evident in the 1 mM COX treated cells. COX was assayed at 4 h, 12 h and 24 h. At 24 h, the cells appeared to be blebbing, and it looked like there was an unusual amount of cellular debris around any remaining cells. However, these cells did not luminesce in their assay. When repeating the trial at 18 h and 22 h, the cells showed evidence of apoptosis at the latter. Thus, the cells must peak at 22 h, and the caspase has been degraded by 24 h.

Determining when the apoptotic caspase cascade is induced is particularly important for the real time PCR studies. It is known that following caspase production endonucleases degrade DNA.^{6, 8, 9} Thus, we wanted to ensure we used concentrations in PCR well below those significant enough to induce apoptosis. Additionally, previous studies at 250 mM doses of DEB and ECH show that ECH prefers mitochondrial DNA to nuclear DNA, while DEB does not have a preference.²⁴

At concentrations well below both of these criteria, it was determined that no appreciable damage could be observed in our three amplified regions, expressed nDNA, unexpressed nDNA and mtDNA. When moving to larger doses, it was observed that at 1 mM for two hours, DEB caused damage at all three loci, while ECH and COX only damaged forms of nDNA. However, 1 mM was enough to induce caspase activity at longer incubation times, so it is worrisome that these results could be produced by endonucleases present in the apoptosis cascade. Additionally, it is possible that damage was not observed because the amplified regions were too short to gain evidence of significant damage at such small concentrations of cross-linking agents. Future work should be done to design primers for a longer region that is more susceptible to damage.

Further studies must be done on DNA targets of these agents because learning whether or not bifunctional alkylating agents have a preference for mitochondrial over nuclear DNA will help to elucidate their mechanisms and may result in a link between cytotoxicity, cross-linking efficiency and apoptotic potential. There are many differences between nuclear DNA, which is often wrapped around histones, and mitochondrial DNA, which is double stranded and circular. In addition, during cell division, the nuclear membrane disappears, while the mitochondria DNA is always surrounded. Furthermore,

damage to mtDNA has been implicated in apoptosis.⁴⁵ All of these factors may contribute to a molecule's target preference, and it is necessary to know this preference before possible mechanisms can be determined.

To conclude, studies were done to quantify apoptosis induction in cells treated with DEB, ECH and COX. This was done using flow cytometry to test for the appearance of phosphatidylserines using Annexin V staining, and membrane compromise by propidium iodide. It was determined in preliminary experiments that at concentrations of 1, 10, and 100 μM , less than 6% of cells were apoptotic at any given time point. When concentrations were raised to 1000 μM , apoptosis was seen only in cells treated with DEB and COX. DEB induced apoptosis in almost two times as many cells as COX, leading to the conclusion that in terms of apoptotic potential, $\text{DEB} > \text{COX} > \text{ECH}$. However, this does not match with all of the flow cytometry results, and further studies must be done to determine whether or not ECH or COX has greater apoptotic potential.

Additional experiments were done on cells treated with the full range of each compound for 24 h. These results vary from those described above in that apoptosis is seen after each treatment. The results still indicate that DEB is the strongest apoptosis inducer, with the ability to induce apoptosis in nearly 25% of cells treated with 10 μM of the compound. Additionally, like in the preliminary results, ~10% of cells are apoptotic after treatment with 1000 μM DEB. However, it is difficult to discern whether COX or ECH should be considered a stronger apoptotic inducer. Cells treated with COX underwent more apoptosis at lower concentrations, but ECH was able to induce a higher percentage of cells to die this way at higher doses. Thus, at low concentrations,

COX>ECH, but at high concentrations, ECH>COX. In all cases, camptothecin is still seen as having a stronger apoptotic potential.

Our results were consistent with the idea proposed by members of the Millard group that there exists an apoptotic “window” after which cells can no longer be detected as being apoptotic. These cells have entered into a later stage of apoptosis, characteristic of nuclear condensation and membrane blebbing.¹⁰ We saw this in both the caspase assay and in flow cytometry. In the caspase assay, activity was seen at 22 h after a 1 mM COX treatment, but was not seen at 24 h. When looking at the camptothecin controls of flow cytometry, cells treated with 1 μ M for 24 h showed more cells in the necrotic region than in the apoptotic. Additionally, in the 24 h DEB samples (Figure 15 e,f) the proportion of cells undergoing apoptosis and necrosis had an inverse relationship. Thus, it seems that after reaching maximum apoptosis, cells begin to die, caspase is degraded, and the membrane becomes compromised, allowing for PI fluorescence.

Overall, it has been determined that in terms of apoptotic potential, DEB>COX>ECH. While these results are not fully consistent with those from the caspase assay, flow cytometry is a much more precise procedure, as it is able to count each cell individually. These results do correlate with cross-linking efficiency of our compounds, meaning there may be a mechanistic origin to how the compounds function *in vivo*.³¹ Furthermore, DEB has been found to be the least cytotoxic of our three compounds. Since it has the highest apoptotic potential, it can be seen why it is the only one of the three currently being used as an anti-cancer drug. The amount of apoptosis induced after treatment with 10 μ M DEB during the second trial exceeds the 15% induction found after treatment with 58 μ M cisplatin for 24 h.⁴¹ It is possible that

treosulfan, the parent compound of DEB, may have a higher anti-cancer potential as it seems to be a stronger apoptotic inducer. The goal of any chemotherapeutic agent is to kill cancerous cells, often by inducing apoptosis, without affecting healthy cells too much. Both of these goals are achieved with DEB, but not with ECH or COX.

CONCLUSIONS

The purpose of this study was to determine the relative apoptotic potentials of DEB, ECH and COX. While results are not fully conclusive at this time, it appears that DEB is able to induce apoptosis at both lower concentrations and in higher proportions than ECH and COX. This was determined using an assay for caspase activity in addition flow cytometry. The relationship between ECH and COX is unclear at this point, but flow cytometry results imply that COX is able to induce apoptosis at lower concentrations than ECH. Overall, we see that DEB, the only compound of ours that is a metabolite of an anti-cancer drug, is the only one able to induce significant amounts of apoptosis in chicken erythroprogenitor cells. Further work must be done to repeat all of the flow cytometry work so that results can be confirmed. Additionally, apoptotic potential should be determined at the LD₅₀ values once those have been obtained. Overall, these results are promising, and it is hoped that when coupled with cytotoxicity and cross-linking results, a pattern will emerge that can help us predict future compounds to study.

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