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Relationship of Cross-Linking Potential to Mechanism of Cell Death

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Relationship of Cross-Linking Potential to Mechanism of Cell Death

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Contents

Abstract

Mechlorethamine (HN2), a nitrogen derivative of mustard gas, was the first synthetic anti-tumor chemotherapeutic because it forms covalent cross-links between strands of duplex DNA. HN2 represents a class of bifunctional alkylating agents that are both chemotherapeutic and carcinogenic: diepoxybutane (DEB), the active form of the pro-drug treosulfan, and epichlorohydrin (ECH), a structural hybrid of HN2 and DEB, also form covalent cross-links between DNA. While HN2 and DEB are clinically used as anti-tumor chemotherapeutics, ECH is a structural hybrid of these two compounds not used in a clinical setting. Accordingly, we aimed to understand the relationship between the cross-linking potential of these compounds and their ability to induce cell death (apoptosis). Cytotoxicity rankings were determined by assessing the median lethal dose (LD_{50}) concentrations using MTT Cell Proliferation Assays for 12- and 24-h treatments (HN2 >> DEB > ECH; DEB > HN2 > ECH, respectively). Cross-linking potentials at equimolar concentrations suggest DEB > ECH > HN2 for a 24-h treatment. Finally, these compounds' abilities to induce apoptosis at 12- and 24-h equitoxic concentrations were assayed over 12- and 24-h treatments, suggesting that DEB >> ECH > HN2. Accordingly, we propose a relationship between a compound's ability to form cross-links and the induction of cell death and apoptosis (DEB > ECH > HN2) when considering reactivity and stability of unreacted cross-linkers over time. Future studies will aim to elucidate the cross-linking potential at equitoxic concentrations for each of these three compounds over multiple time frames.

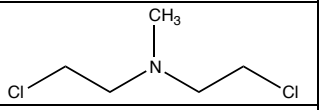
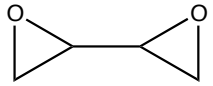
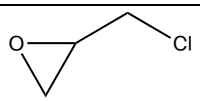
Introduction

DNA Alkylating Agents: Importance, Mechanism, and Cross-Linking

The cytotoxic effects of mustard gas were realized during World War I in its deployment as a chemical weapon against Allied forces. Autopsies of exposed soldiers revealed its ability to severely deplete bone marrow, raising the possibility for its use as an anti-tumor agent to treat Hodgkin's leukemia [1]. The water-soluble nitrogen derivative, mechlorethamine hydrochloride (HN2, Table 1) was later used as a chemotherapeutic in patients with leukemia and shown to decrease tumor size and delay death [2]. HN2 ultimately revolutionized the war on cancer as it became the first synthetic anti-tumor chemotherapeutic used clinically, suggesting the possible use of bifunctional alkylating agents as anti-tumor chemotherapeutics [1,3].

Bifunctional alkylating agents are compounds that use two functional groups (e.g. chloride or epoxide) to alkylate and form covalent, interstrand cross-links in duplex DNA. These cross-links prevent double stranded DNA (dsDNA) from separating, making transcription and replication nearly impossible without specific repair enzymes. Accordingly, the formation of these cross-links is believed to be the source of the cytotoxicity of HN2 and other bifunctional alkylating agents [4].

Table 1. Bifunctional alkylating agents used in this study.

Name	Abbreviation	Structure
Mechlorethamine	HN2	
1,2,3,4-Diepoxybutane	DEB	
(±)-Epichlorohydrin	ECH	

The ability of HN2 to induce DNA damage has been harnessed in the drug Mustargen®, which is currently used topically to treat melanomas. These cross-links are primarily formed between the N7 sites of distal guanosine residues in the sequence 5′-GNC [4]. Unfortunately, in

addition to stopping cell proliferation, HN2 has the potential to cause insertions, rearrangements, and point mutations within the DNA of cancerous and healthy cells alike [5]. As a result, treatment has the potential to lead to an increased risk of developing secondary cancers [6,7].

Another bifunctional alkylating agent used to treat cancer is 1,2,3,4-diepoxybutane (DEB, Table 1). DEB is believed to be the active form of the anti-tumor pro-drug treosulfan (Ovastat), which has been used to treat advanced ovarian cancers [8,9]. Cross-linking DNA similarly to HN2 [10], DEB exerts cytotoxic effects that are also non-specific to cancer cells. DEB's carcinogenicity has been observed in mice and rats [11,12], as well as in humans [13]. A link between industrial workers exposed to 1,3-butadiene (BD), the metabolic precursor to DEB, and a high occurrence of leukemia has been documented [14-18], raising concerns about its dual chemotherapeutic and carcinogenic nature. Its ubiquitous presence in gasoline, automobile exhaust, and cigarette smoke (~20-75 μg/cigarette) are enough to cause cross-linking and induce apoptosis [19], making its exposure to civilian populations especially alarming. A cause for further concern is the approximately four million pounds of BD released from the production of synthetic rubber, polymers, and plastics from US facilities each year [20]. This staggering figure highlights the need to study DEB and its ability to cross-link and induce cell death [21,22] more in depth.

While not currently used as an anti-tumor chemotherapeutic, epichlorohydrin (ECH, Table 1) is a structural hybrid of HN2 and DEB. ECH has also been identified as a suspected mutagen and probable carcinogen [23]. In 2003, approximately 203,900 metric tons of ECH were used in the production of synthetic polymers, epoxy resins, glycerine, and elastomers [24]. ECH forms cross-links similar to HN2 and is also capable of reacting with free bases at the N^6 and N^3 of adenine and O^6 on guanine [25-29]. It has been hypothesized that the 3-atom cross-link

formed by ECH would be too small to cross-link duplex DNA [30]; however, *in vitro* studies performed by our lab [31] and *in vivo* studies on mammalian cell cultures counter this hypothesis [32-35]. Further, in mammalian cell cultures, ECH has been shown to be mutagenic [36], clastogenic [37], and able to induce neoplastic cell transformations [23], papillomas, and carcinomas [38]. Due to cross-link formation, chromosomal aberrations, and cell damage, the risk of developing cancer greatly increases upon exposure to ECH [23,24,39,40]. Each year, an estimated 250,000 industrial workers in the United States will contact ECH, potentially raising their risk of cancer. Therefore, understanding ECH's cross-linking potential and cytotoxicity are important for furthering our understanding of this compound's health risks.

Characterizing the relationship between cross-linking and cytotoxicity for these compounds

The purpose of this study is to understand the abilities of the three compounds (HN2, DEB and ECH) to form interstrand cross-links within double stranded DNA *in vivo* and how the formation of cross-links relates to cytotoxicity and the induction of apoptosis (Figure 1). We determined the median lethal dose (LD_{50}) as a measure of cytotoxicity after 12- and 24-h treatments using the MTT Cell Proliferation assay. The potential for each compound to form interstrand cross-links at equimolar concentrations over 24-h was investigated using an ethidium bromide fluorescence assay. Finally, the apoptotic potential of these compounds was determined by the detection of two hallmark apoptotic proteins, active caspases 3 and 7, using a Caspase-Glo® 3/7 assay. Accordingly, these three tests suggest a relationship between the ability of these compounds to form cross-links and their abilities to induce cell death and trigger an apoptotic response.

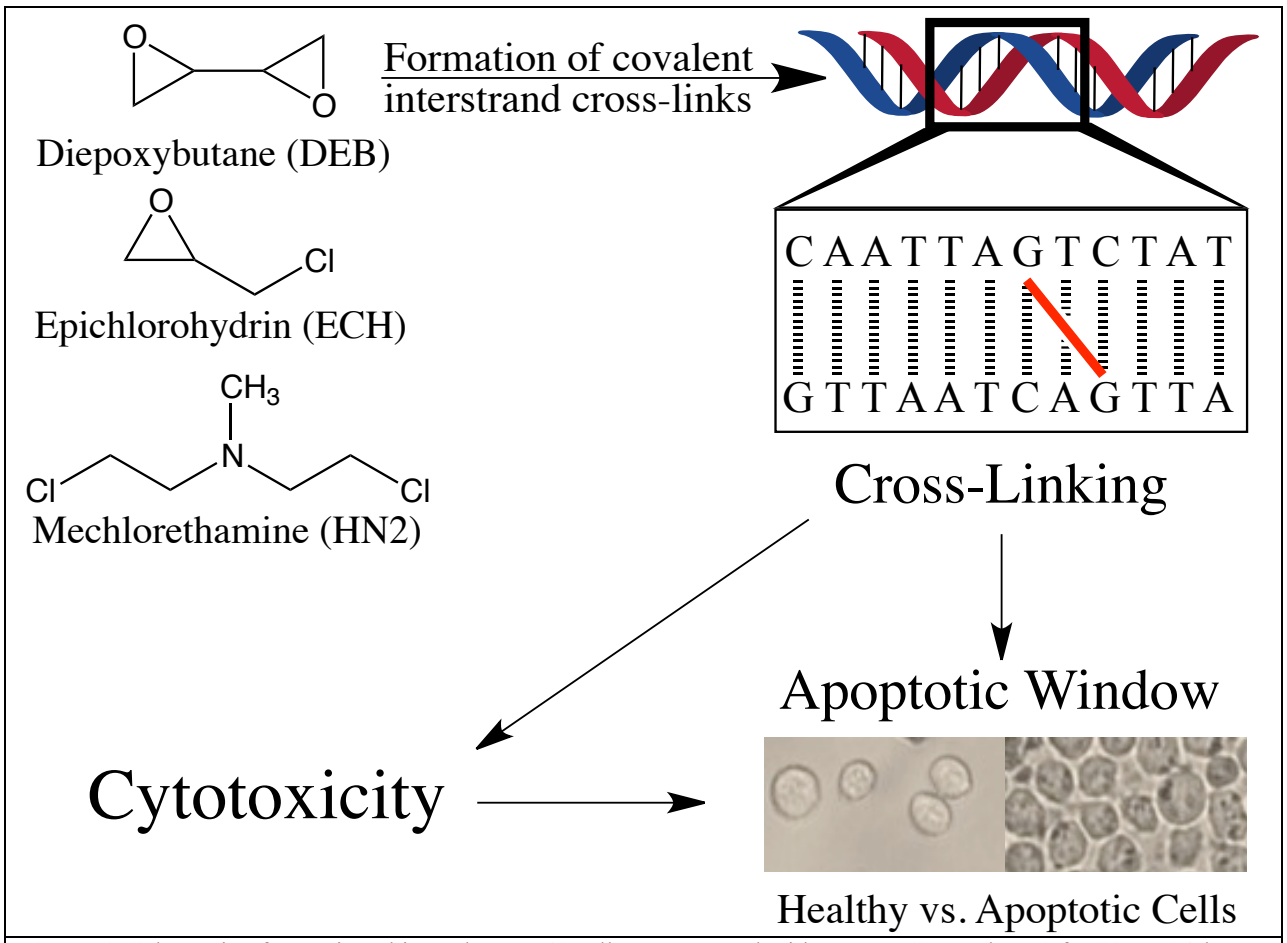


Figure 1. Schematic of overview this study. HL-60 cells were treated with DEB, ECH, and HN2 for 12- or 24-h. Cross-linking potentials were determined using an ethidium bromide fluorescence assay, which was compared to the cytotoxicity as measured through the MTT Cell Proliferation Assay and compounds' ability to trigger an apoptotic response as detected by the Caspase-Glo® 3/7 assay.

Materials And Methods

Cell Lines and Cell Culture

Human acute myeloid leukemia (HL-60) cells (American Type Culture Collection) were cultured in RPMI 1640 (Lonza) containing 10% fetal bovine serum (FBS; Carolina Biologicals) and broad spectrum antibiotics (10,000 I.U./mL Pennicillin and 10,000 μg/mL Streptomycin; Cellgrow) at 37°C, in 5% CO_2. Stocks were maintained at approximately 3.0 - 4.0 x 10^5 cells/mL. Stock viability and density were measured using 2.0 μL Trypan Blue 0.4% stain (Lonza) and 20.0 μL cell suspension. After brief mixing, 20 μL were injected into SD100 Cellometer cell counting chambers and counted using a Cellometer T4 Nexelcom, automated haemocytometer with the accompanying Cellometer Auto T4 Software. The concentration and viability of each stock were determined using the HL-60 cell type setting.

Drug Treatments

Stocks of each bifunctional alkylating agent (1.0 M) were prepared for each trial in either DMSO (DEB and ECH) or 0.1 N HCl (HN2). Treatments were administered such that wells contained 1% v/v alkylating agent in vehicle solution. Cells were aliquoted into 6-well plates to maximize surface area exposure at a concentration of 3.0-4.0 x 10^5 cells/mL. Cells were treated continuously in growth media containing various concentrations of a bifunctional alkylating agent and assayed after 12 or 24 hours. Treated cells were compared relative to a number of controls: negative controls containing cell-free RPMI 1640 media, and a vehicle control with either 1% v/v DMSO or 0.001 N HCl. For the Caspase-Glo 3/7® assay, a positive control of 0.15 μM camptothecin (DNA topoisomerase I inhibitor) in DMSO was prepared and administered 2.5 hours prior to each treatment interval [41-43].

MTT Cell Proliferation Assay

A Cell Quanti-MTT Cell Proliferation Assay (BioAssay Systems) was used for a precise determination of equitoxic (LD_{50}) concentrations over 12- and 24-h treatments. The MTT Cell Proliferation Assay uses the reduction of yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) by succinate dehydrogenase in metabolically active cells to create MTT formazan, a purple precipitate that can be used to spectrophotometrically quantitate metabolically active cell populations [44].

Cells were treated at an initial density of 3.0 – 4.0 x 10^5 cells/mL. After the appropriate incubation interval, triplicates of 18.75 μL reconstituted MTT reagent were added to 100 μL aliquots of treated and untreated cells into a clear bottom, 96-well plate. After a 4 hour incubation at 37^{o}C and 5% CO_2, cells were lysed with 125 μL solubilization buffer and shaken at 120 RPM until the purple precipitate homogenized within the well (3-10 hours). Absorbances of the purple precipitate (570 nm) were measured using a SPECTRAmax M2 plate reader and SoftMax® Pro Software (Molecular Devices).

MTT Cell Proliferation LD_{50} Data Analysis

Absorbance values were electronically transferred to Microsoft Excel 2011. Using a technique described previously by former Millard labmate Christopher Ng, I solved for the viable fraction (VF) of cells by taking the fraction of the blanked (RPMI media) average of an individual treatment divided by the blanked average of the positive control (0.15 μM camptothecin in DMSO), Equation 1. The fit viable fraction [P(c)] was calculated using Equation 2, where "c" is concentration, parameter "a" is the LD_{50} concentration (mM), and parameter "b" is a scaling exponent. The residual sum of squares for a non-linear data set [=SUMXMY2(viable

fraction, fit viable fraction)] was used to determine the quality of fit between the two values in Excel. Using the Solver add-in for Excel, the residual sum of squares was minimized by changing the parameters ($a \geq 0$, $b \geq 0$). Only one parameter could be minimized for each set of 100 iterations through Excel, so each parameter was changed until the "a" parameter stayed constant up to the fourth significant digit. For each parameter and the residual sum of squares, an uncertainty value was calculated using a non-linear least squares macro, SolverAid, from Robert de Levi's MacroBundle12 [45]. This macro "provides uncertainty estimates (standard deviations and the covariance matrix) for Solver-derived parameter values." Accordingly, we were able to calculate the LD_{50} value and its uncertainty for each compound at each time interval.

$$VF = (\text{Sample } A_{570} - \text{Mean Blank } A_{570})/(\text{Mean Control } A_{570} - \text{Mean Blank } A_{570}) \quad (1)$$

$$P(c) = 1/(1+(c/a)^b) \quad (2)$$

Ethidium Bromide Fluorescence Assay

An ethidium bromide fluorescence assay [13] was used to assess the degree of cross-linking for each compound at an equimolar concentration (1.0 μM) over a 24-h treatment (Figure 1). Ethidium bromide is an intercalating agent that binds DNA, fluorescing more when bound to duplex DNA than single stranded DNA (ssDNA). This property can be used to assess the ability of our test compounds to cross-link duplex DNA. By measuring fluorescence before and after a rapid denature-renaturation cycle, we could determine how easily the DNA was able to reanneal. A greater abundance of cross-links will aid in the renaturing of complementary strands of DNA to their initial conformation, thereby increasing the proportion of duplex DNA to ssDNA. In other words, strands that can realign more completely will have more bases that match, permitting greater fluorescence of the ethidium bromide during a second fluorescence reading.

Genomic DNA was extracted from unsynchronized HL-60 cells using a GenCatch Genomic DNA Extraction Kit following the Blood protocol (Qiagen). The concentration of each DNA sample and the ratio of DNA:protein ($A_{260/280}$) was measured using a Nanodrop-1000 Spectrophotometer. Samples were eluted in Tris-EDTA (TE) buffer (pH 9.0) and diluted with TE to 20 ng DNA/μL. 100 μL of extracted genomic DNA and 100 μL ethidium bromide (10 μg/mL) solution containing 20 mM K_2HPO_4 and 0.4 mM EDTA (pH 12.0) were aliquoted into a flat-bottom, black 96-well plate.

Baseline fluorescence readings (RFU) were recorded for each well prior to a rapid denature-renaturation cycle. This cycle consisted of a 5-minute incubation at 100°C, then 3 minutes in a -20°C freezer. A spectrum sweep for emission and excitation wavelength maxima was determined to be 525 nm for excitation and 580 nm for emission, matching literature values.

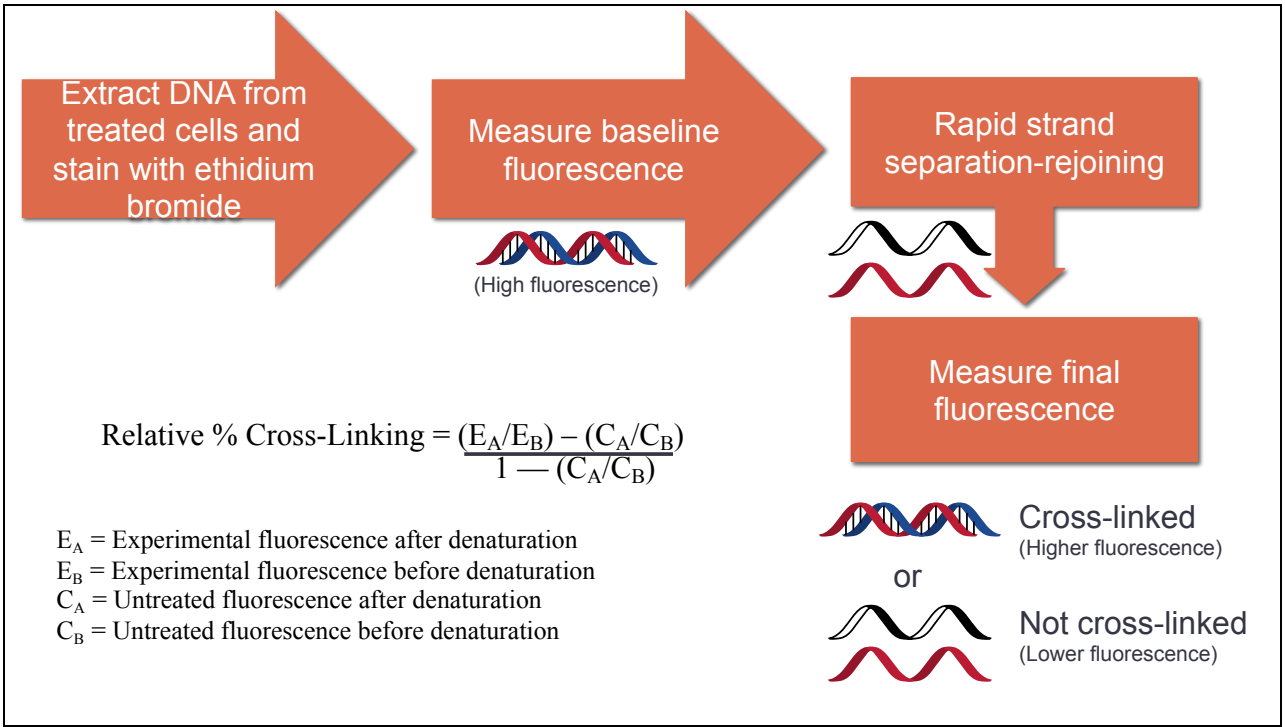


Figure 2. Schematic for ethidium bromide cross-linking assay. Ethidium bromide was excited at 525 nm and fluorescence values were measured at 580 nm.

Fluorescence readings were recorded before and after this heat-chill cycle. The relative percent of cross-linking was calculated using the equation for relative percent cross-linking in Figure 2 [13,44]. By taking the difference between the ratio of experimental to control fluorescences, and dividing that by a theoretically, fully cross-linked sample, we were able to calculate the cross-linking potential of each alkylating agent.

Caspase-Glo® 3/7 Assay

A Caspase-Glo-3/7 assay was used to determine relative levels of apoptosis based on the detection of caspases 3 and 7. This assay uses a proluminescent caspase-3/7 DEVD-aminoluciferin substrate with a proprietary thermostable luciferase to generate a luminescent signal as active caspases 3 and 7 cleave the aminoluciferin from the substrate. Luciferase cleaves the aminoluciferin to create a luminescent signal that is proportional to the abundance of these active caspases, thus quantitating the apoptotic response.

Twelve and 24-h treatments with each cross-linker at both 12- and 24-h LD_{50} concentrations were compared with the positive control for apoptosis (0.15 μM camptothecin in 1% v/v DMSO). Using a 1:1 ratio of caspase reagent to treated cells, treated cells and reconstituted caspase reagent were aliquot onto an opaque white, round-bottom 96-well plate. The plate was mixed at 200 RPM for 30 seconds, then left to incubate for 30 minutes at 37°C and 5% CO_2.

Relative luminescent units (RLU) were measured using a SPECTRAmax M2 plate reader and SoftMax® Pro Software (Molecular Devices). The mean luminescent signal of each condition was blanked with the mean negative control (RPMI 1640-based cell-free media). Relative levels of apoptosis were determined by taking the fraction of the blanked-adjusted

average of an individual treatment divided by the blanked-adjusted average of the positive control. Vehicle controls of 1% v/v DMSO and/or 1% v/v 0.1 N HCl were also assayed.

Standard error mean (S.E.M; bars displayed in Figure 1) were calculated by dividing the standard deviation by the square root of the sample size. Significance between treatment and vehicle controls were determined using a student's t-test for statistical analysis of the variance between two means.

Results

Cytotoxic Potential

Cells react differently to bifunctional alkylating agents based on their position in the cell cycle [41], cell line and type, length of treatment (time), and dosing concentration [46]. Unsynchronized HL-60 cells were treated with varying concentrations of bifunctional alkylating agents. Based on the concentration-dependent response, we were able to establish the median lethal dose (LD_{50}) for each treatment condition.

Table 2. LD_{50} values and ± S.E.M. (mM) for HL-60 cells treated with bifunctional alkylating agents over 12- and 24-h periods. Subscripts indicate uncertainty of the uncertainty value, n = 3.

X-Linker	12 Hour	24 Hour
HN2	$0.000410 \pm 0.000055_2$	$0.0217 \pm 0.0026_3$
DEB	$0.226 \pm 0.011_0$	$0.0174 \pm 0.0008_5$
ECH	$0.492 \pm 0.035_4$	$0.175 \pm 0.016_3$
12-h	HN2 >> DEB > ECH	
24-h	DEB > HN2 > ECH	

The cytotoxicity of each compound is inversely proportional to its LD_{50} value; more potent compounds require a lower concentration to eliminate half the fraction of viable cells. In the case of HN2, the LD_{50} concentration at 12-hours was approximately 550-fold less than DEB and approximately 2,000-fold less than ECH, indicating a potency ranking of HN2 >> DEB > ECH (Table 2). After 24-hours, the LD_{50} of DEB was markedly lower than HN2 and approximately 10-12 fold greater than ECH (DEB > HN2 > ECH; Table 2).

Of note, the LD_{50} values for DEB and ECH decreased (13.0 and 2.8-fold, respectively) between the 12- and 24-h treatments, suggesting that they become slightly more toxic over time. However, the LD_{50} value for HN2 increased 50-fold over the same time interval, suggesting that cytotoxicity, or number of metabolically active cells, decreases over time.

Cross-Linking Potential

It has been proposed previously that a bifunctional alkylating agent's ability to cross-link DNA is the cause of its cytotoxicity [47]. The ethidium bromide fluorescence assay was used to understand how well each compound is able to form cross-links with DNA. Genomic DNA was extracted from unsynchronized treated and untreated cells then stained with ethidium bromide. Because ethidium bromide fluoresces when bound to dsDNA versus ssDNA, comparison of fluorescences before and after a rapid denaturation-renaturation cycle could be used to estimate the relative number of cross-links in a given condition when compared to an untreated control.

Due to time constraints and complications in the protocol, we were only able to obtain data for 24-h treatments with 1.0 μM of each cross-linker. DEB (37.1%) was the most potent cross-linker followed by ECH (14.0%), then HN2 (2.6%), suggesting a cross-linking order of DEB > ECH > HN2. While this does not characterize the compounds at equitoxic (LD_{50})

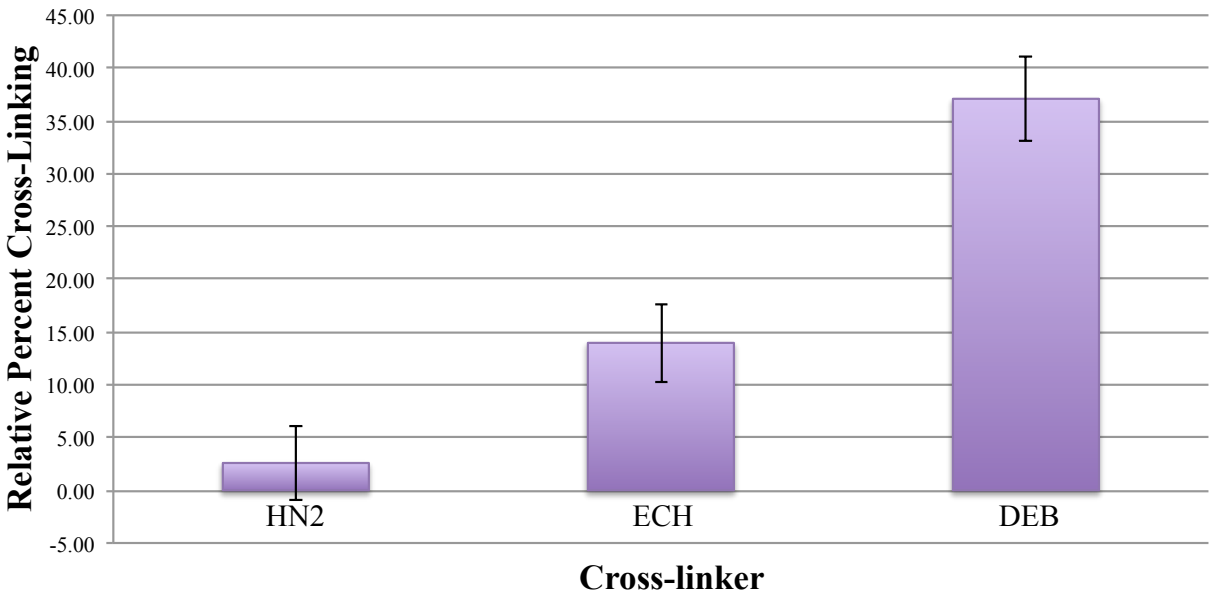


Figure 3. Ethidium bromide fluorescence assay for a 24-h treatment with 1.0 μM of each compound indicates the cross-linking strengths: DEB > ECH > HN2. The formation of covalent, interstrand cross-links prevents DNA from separating, allowing it to reanneal easier and fluoresce more after a rapid denature-renaturation cycle, n = 3.

concentrations, it allows us to speculate these compounds' abilities to enter the cell and react with the DNA over a longer treatment length.

Apoptotic Potential

Apoptotic potentials for each compound were assayed across four conditions where the treatment time (12- and 24-h) and LD_{50} concentration at each time were varied (Figure 4). Compared against vehicle controls, cells treated with DEB showed significant levels of apoptosis induced for three test conditions: both 12- and 24-h LD_{50} concentrations (0.226 mM and 0.0174 mM) during a 24-h treatment ($p < 0.00001$ and $p < 0.00001$) and during a 12-h treatment with the 24-h LD_{50} ($p < 0.00001$). Treatment at these three conditions caused levels of apoptosis that differed significantly from each other: 24-h LD_{50} treated for 24-h > 12-h LD_{50} treated for 12-h > 24-h LD_{50} treated for 12-h ($p < 0.00005$ for each combination).

Treatment with ECH caused significant levels of apoptosis for two test conditions: 24-h LD_{50} for the 12- and 24-h treatments (0.175 mM; $p < 0.05$ and $p < 0.01$), when compared against the vehicle control. Each treatment differed significantly from the other as well, with the 24-h treatment inducing a greater apoptotic response than the 12-h treatment ($p < 0.0005$).

Treatment with HN2, using the 12-h LD_{50} concentration (0.000410 mM) over a 24-h interval, induced significant levels of apoptosis ($p < 0.005$) when compared against its vehicle control. However, the magnitude of this apoptotic response was very low.

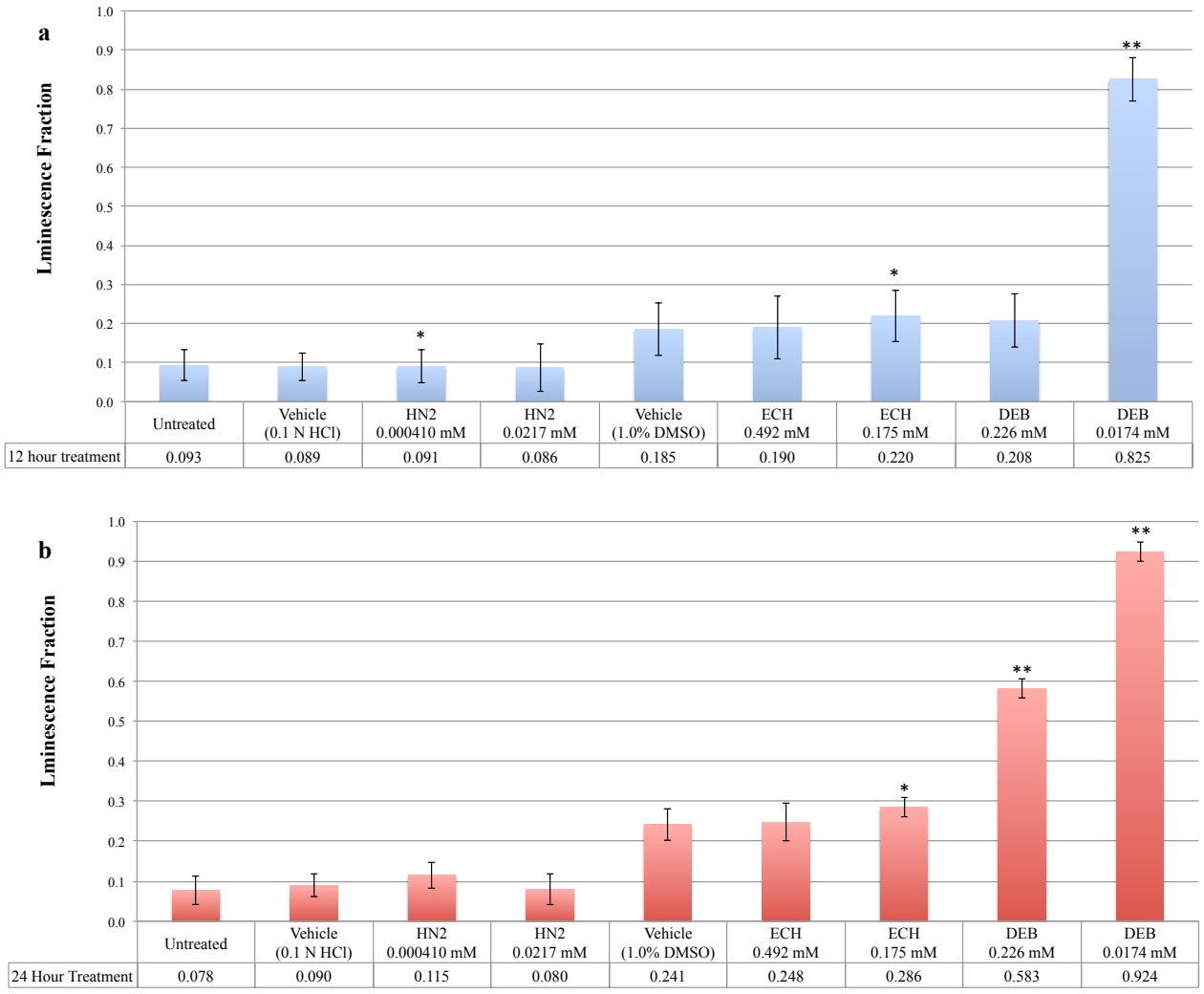


Figure 4. Relative levels of apoptosis induced by LD_{50} concentrations of bifunctional alkylating agents after (a) 12 and (b) and 24-hour treatments. Significant levels of apoptosis were induced for three treatments with DEB, two with ECH, and one with HN2. Asterisks indicate significance (* = $p < 0.005$, ** = $p < 0.00001$) between luminescence fractions of treatment conditions and their respective vehicle controls, n = 3.

Discussion

The ability for our test compounds to enter cells and form interstrand cross-links is believed to be the source of their cytotoxicity. These compounds are highly reactive within the cells, though we focused specifically on their ability to interact with genomic DNA. Accordingly, we sought to characterize their cytotoxicity, specifically their ability to induce apoptosis, at equitoxic (LD_{50}) concentrations with respect to their ability to form these interstrand cross-links.

Cytotoxicity of Bifunctional Alkylating Agents

Equitoxic concentrations of unsynchronized HL-60 cells were determined based on the median lethal dose determined using the MTT assay. During the 12-h treatment, HN2 was markedly more cytotoxic than either DEB (550-fold) or ECH (2,000-fold); however, DEB was more cytotoxic than HN2 during the 24-h treatment and only slightly more so than ECH.

The increase in LD50 concentration for HN2 was surprising. Higher levels of metabolism were detected from cells treated with HN2 over a 24-h treatment than would be theoretically possible considering the 12-h LD_{50} concentration and the 24-36 hour doubling time of HL-60 cells. It is important to recognize that the MTT assay measures the metabolic activity; it does not directly quantitate cell death. Cells that contain cross-linked DNA may be less metabolically active than healthy cells, but they necessarily dead. This discrepancy is noteworthy, since the LD_{50} concentration for HN2 increased 50-fold over the twelve hours between assaying for cytotoxicity, while the LD_{50} for ECH and DEB decreased (~3 and 12-fold, respectively). This trend may be due, in part, to the stability of these compounds once in aqueous solution. HN2 is reported to have a very short half-life in aqueous solution, dependent upon the nature of the reactant [48] and the medium in which it is solvated [49]. A theoretical minimum half-life based on the time it takes HN2 to cyclize into the ethylene imonium ion, the reactive species in HN2's

nucleophilic reactions, is approximately 90 seconds [50]. One source suggests 0.2 mM HN2 has a half-life of approximately 30 minutes [50]. In comparison, DEB and ECH have reported half-lives of 31 ± 4 [51] and 213 [29] hours, respectively. The substantially longer half-life of these compounds may increase their potency over prolonged treatment lengths and slow down the rate of replication. Cells exposed to a compound that breaks down quickly will be exposed to it for less time, allowing them a chance to repair cross-links using DNA excision repair enzymes. This can allow for more potent and targeted treatments while decreasing the chance of collateral damage to neighboring healthy tissues. However, the ability for treated cells to recover quickly after treatment raises the chances of developing secondary or more resilient cancers [52].

Cross-Linking at Equimolar Concentrations Suggests DEB > ECH > HN2

The cross-linking ability of these compounds was measured using the ethidium bromide fluorescence assay using 1.0 μM of each compound over a 24-h treatment. Data from the ethidium bromide fluorescence assay indicate a positive correlation between cross-links formed and trends in cytotoxicity from DEB and ECH in the context of compound half-life. These cross-linking results indicate DEB > ECH > HN2. These results differ from earlier *in vitro* studies conducted within the Millard lab, which suggest HN2 is a more potent cross-linker than DEB [53], and that DEB is a more potent cross-linker than ECH [31].

The potency of these cross-linkers *in vitro* is similar for to their cytotoxicity determined for the 12-h treatment, but not 24-h treatment. This discrepancy is likely due to the compounds' half-lives. Despite ECH's longer half-life, its ability to form cross-links is much less than both DEB and HN2. Therefore, its more concentrated presence in solution over a 24-h is not enough to induce more cross-links than DEB.

While HN2 is the most reactive cross-linker *in vitro*, it is not necessarily the best cross-linker. HN2's ability to form cross-links *in vitro* is less biologically relevant than its half-life when used to treat live cells. Since its half-life is so short, cross-links will only form for a certain period of time before the compound's presence in solution in negligible. The rate of cross-link repair in cells will eventually overcome that of the decreasing rate of cross-link formation. This hypothesis may also help explain why the potency of HN2 decreases over time, while the potency of the epoxides increases. The instability of HN2 over longer periods of time may also help to explain why our *in vivo* cross-linking results (DEB > ECH > HN2) differ from our *in vitro* results (HN2 > DEB > ECH).

Another possible reason for the disparity between HN2's *in vivo* and *vitro* cross-linking potentials are the compounds' ability to permeate the cells and interact with the DNA. Previous studies solvated DEB and ECH in DMSO and HN2 in an acidic aqueous solution. The difference in solvent may ultimately affect the permeability of these compounds into the cell and nucleus, which could affect our results and explain the increased cross-linking observed with DEB and ECH. However, due to time constraints and complications with the ethidium bromide cross-linking protocol, we were unable to test this hypothesis and establish data for equitoxic concentrations over the different time points. These aspects of bifunctional alkylating agent biochemistry may be biologically relevant aspects to consider further.

High Apoptotic Potential of DEB

The induction of apoptosis is important in treating tumors and cancers for two reasons. First, the increased replication rate of a cancer cell means that damage caused that may interrupt cellular replication will slow or stop the rate of proliferation, which provides the immune system

an opportunity to destroy the cancerous cells. Second, triggering apoptosis is a more targeted approach to chemotherapy. Cells with damage induced by chemotherapeutics will be stopped at one of the checkpoints in replication, which can mean it is more likely to trigger an apoptotic response if it is stopped for too long. Therefore, cells that replicate abnormally fast are more likely to be selected against than cells that undergo fewer replications in a given timeframe. In contrast, necrosis is a form of cell death that causes unregulated cell death and can affect both healthy and cancerous cells.

A cell's ability to undergo apoptosis is largely dependent on its type, length and concentration of treatment, and its progression through the cell cycle [46]. Previous studies suggest that DEB is capable of triggering apoptosis [54-58], ECH has an overwhelming ability to induce necrosis [59], and HN2 is able to stimulate both necrosis and apoptosis [48], depending on an apoptotic window, as determined by the treatment concentration (lower concentrations induce apoptosis) and length (shorter treatments of 1-h induce apoptosis) [60]. By assaying for the activity of active caspases 3 and 7, two hallmark proteases of apoptosis, we were able to determine how well these compounds induced apoptosis at specified equitoxic concentrations. Thus, we demonstrated that while there is no direct correlation between cross-linking and compound-induced cytotoxicity (apoptosis), a relationship might exist if the stability of unreacted cross-linkers is taken into account.

DEB showed the most significant levels of apoptosis over both 12- and 24-hour treatments and the greatest number of conditions in which apoptosis was induced (Figure 4). With the exception of the 12-h LD_{50} during a 12-h treatment, DEB showed very significant levels of apoptosis (24-h LD_{50} after 24-h treatment > 24-h LD_{50} after 12-h treatment > 12-h LD_{50} after 24-h treatment > 12-h LD_{50} after 12-h treatment = vehicle control). DEB is largely believed

to be the active form of the pro-drug treosulfan, which is used to treat advanced stages of ovarian cancer. Studies on treosulfan in human acute myeloid leukemia cells, including HL-60, indicate that the physical biological features associated with apoptosis (e.g. membrane blebbing) are clearly present in cells treated with treosulfan after 72 hours, further highlighting the ability of DEB to induce apoptosis [56]. It is likely that we observed apoptosis prior to this time point because DEB is the pro-form of treosulfan. In order for treosulfan to become active and form cross-links as DEB, it must be broken down. On the contrary, since DEB is already the active form and can react immediately it should be able to begin forming cross-links sooner.

Cells treated with the lower, 24-h ECH LD_{50} concentration induced apoptosis over both time intervals. While this challenges the primarily necrotic response reported in previous studies [59], it does support previous work that lower concentrations of bifunctional alkylating agents can induce apoptosis [60]. Finally, cells treated with the 12-h LD_{50} concentration of HN2 for 12-h also induced statistically significant levels of apoptosis when compared to the vehicle control. However, these levels of apoptosis were very small and may have little biological relevance.

DEB Shows Strong Relationship between Cross-Linking and Cytotoxicity/Apoptotic Response

The half-life of a compound must be known to understand how long cross-linking can occur after a cell's initial exposure. Therefore, we propose a relationship between cross-linking and cytotoxicity (induction of apoptosis) for the three test bifunctional alkylating agents over longer treatment lengths.

DEB and ECH each have half-lives that are greater than the length of treatment. Of the initial treatment concentration over 12- and 24-h, DEB (76.5% and 58.5%) and ECH (96.2% and 92.5%) were still largely present in the treatment wells. For these same time intervals, HN2 (6.0

x 10^{-6} % and 3.6 x 10^{-13} %) decayed and had a negligible presence in the treatment wells. Cells exposed to DEB and ECH were able to continue to form cross-links with genomic DNA long after HN2 was broken down and HN2-exposed cells had begun to repair these cross-links. Therefore, while HN2 may be more cytotoxic in shorter doses, DEB and ECH have a prolonged effect that contributes to their ability to form cross-links, ultimately inducing cell death and apoptosis. This property of the epoxides may cause collateral damage in neighboring tissues, representing a clear disadvantage to treatment with such compounds.

Of all three of the test compounds, DEB showed the strongest support for the hypothesis that cross-linking potential is correlated with the induction of apoptosis. Results from ECH also support this hypothesis, since cross-links form over a 24-h period and low, yet significant, levels of apoptosis were induced. Finally, despite HN2's high potency during shorter treatments, it was unable to continually cross-link DNA, likely a result of a theoretically lower presence after a certain period of time. HN2's degradation provides a window of opportunity for the metabolically viable cells to repair cross-linking and continue to divide after treatment. It is possible that HN2's cross-linking and apoptotic potentials may be much greater than DEB's if assays were performed much closer to the time of treatment when a substantial concentration of reactive HN2 was present. However, the short half-life of HN2 limits the window of exposure, which may be an advantage relative to other compounds when it comes to reducing collateral damage in healthy cells nearby but disadvantage because selection favors resiliency to treatment.

Conclusion

In this study, we suggest that a bifunctional alkylating agent's potential to cross-link duplex DNA and to induce cell death by triggering apoptosis are related when considering a compound's stability in solution. The cross-linking potential of these three compounds at equimolar concentrations suggests that DEB was the most effective, followed by ECH, then HN2. The ability of these compounds to cross-link was largely dependent on their ability to permeate the cell and bind DNA, as well as their stability in solution. Therefore, despite HN2's potency during a shorter 12-h treatment, its measured decrease in cytotoxicity after 24-h of treatment and lower levels of apoptotic response may be largely due to its short half-life.

On the contrary, DEB and ECH, each with half-lives longer than the 24-h treatment length, showed greater cross-linking potentials than HN2. Their ability to form cross-links also correlated with their apoptotic potential. DEB was able to induce highly significant levels of apoptosis after 24-h treatment ($p < 0.00001$) and for 12-h of treatment using the higher dose ($p < 0.0005$). Similarly, ECH was able to induce apoptosis at the 24-h LD_{50} concentration during 12- and 24-h treatments (0.175 mM; $p < 0.05$ and $p < 0.01$). While the concentration of ECH at the assaying time, compared to the initial concentration, was greater than that of DEB for both time points, the ability for DEB to induce cell death and apoptosis were greater. This, and the reactivity of DEB over ECH, may explain why the longer half-life of ECH did not correlate with its ability to form more cross-links than DEB.

HN2 had the lowest cross-linking potential at equimolar concentrations, despite inducing nearly as strong a cytotoxic response as DEB after 24-h of exposure. After 24-h, cells treated with HN2 did not contain significant levels of cross-linking. HN2 was still able to elicit a

significant apoptotic signal during the 24-h treatment with the 24-h LD_{50} ($p < 0.005$), though whether this is biologically relevant is unclear.

While preliminary work has been done to understand the cross-linking potential of these compounds at 24-h under equimolar concentrations, future studies should investigate the cross-linking potential at LD_{50} concentrations and use methods to directly quantitate cell death. Additionally, studies should also characterize the half-lives of these compounds in cell media to better understand how well they are able to affect cells during treatment.

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