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DNA Interstrand Cross-Linking Activity of (1-Chloroethenyl) oxirane, a Metabolite of β-chloroprene

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Abstract

With the goal of elucidating the molecular and cellular mechanisms of chloroprene toxicity, we examined the potential DNA cross-linking of the bifunctional chloroprene metabolite, (1-chloroethenyl)oxirane (CEO). We used denaturing polyacrylamide gel electrophoresis to monitor possible formation of interstrand cross-links by CEO within synthetic DNA duplexes. Our data suggest interstrand cross-linking at deoxyguanosine residues within 5′-GC and 5′-GGC sites, with the rate of cross-linking depending on pH (pH 5.0 > pH 6.0 > pH 7.0). A comparison of the cross-linking efficiencies of CEO and the structurally similar cross-linkers diepoxybutane (DEB) and epichlorohydrin (ECH) revealed that DEB > CEO ≥ ECH. Furthermore, we found that cytotoxicity correlates with cross-linking efficiency, supporting a role for interstrand cross-links in the genotoxicology of chloroprene.

Introduction

Chloroprene is a petrochemical produced in large scale worldwide, at about 4.0×10^6 kg/year (1). Its principal use is for the production of polychloroprene (Neoprene), a solvent-resistant elastomer used to make adhesives, automotive parts, wetsuits, and other consumer goods (2). The main source of exposure to chloroprene is occupational, with exposure of the general public thought to be very low (2,3). Although there is only inconsistent evidence of chloroprene-induced carcinogenesis in humans, correlation between occupational exposure and elevated liver and lung cancer risk has been reported (4,5). However, chloroprene is carcinogenic to rats and mice when given by inhalation, with comparable carcinogenic potency to butadiene in mice (6). Based on sufficient evidence for carcinogenicity in animals but inadequate evidence in humans, the International Agency for Research on Cancer has classified chloroprene as a possible human (Group 2B) carcinogen (7).

Chloroprene is metabolized by microsomes from rats, mice, and humans primarily to the monoepoxide (1-chloroethenyl)oxirane (CEO; **1**, Chart 1) (1,5). CEO is mutagenic in the Ames test, implicating this metabolite in the genotoxicity of chloroprene (8). Furthermore, CEO reacts with DNA, with major adducts derived from reaction with N7 of deoxyguanosine and N3 of deoxycytidine (5,9). The structurally similar compound epichlorohydrin (ECH; **2**,Chart 1) likewise alkylates the N7 position of deoxyguanosine to form interstrand cross-links (10), suggesting the likelihood of DNA cross-linking by CEO.

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Our objective was to investigate possible DNA cross-linking activity of CEO, which could contribute to the genotoxicity of chloroprene. We also compared the cross-linking efficiency, DNA sequence preferences, and cytotoxicity of CEO to two established cross-linkers, ECH and diepoxybutane (DEB; **3**, Chart 1). Our data support interstrand cross-linking at deoxyguanosine residues by CEO at 5′-GC and 5′-GGC sites, a similar sequence preference to ECH (10). We also found a correlation between cross-linking efficiency and cytoxicity, with DEB > CEO > ECH. These findings suggest a role for CEO interstrand cross-links in the genotoxicity of chloroprene.

Experimental Procedures

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

Preparation of Racemic (1-Chloroethenyl)oxirane

We modified a simple two-step literature procedure for synthesis of CEO as follows. The intermediate product 2-(1,2-dichloroethyl)oxirane was prepared as described previously (1). In the second step (11), 5.27 g of KOH (93.6 mmol) was added to a two-necked 100-mL round-bottom flask equipped with an addition funnel and a short-path distillation head. KOH was melted at 120°C using an oil bath, and then 7.5 g of 2-(1,2-dichloroethyl)oxirane (53.2 mmol) was added dropwise with rapid stirring. After complete addition of oxirane intermediate, the bath temperature was raised to 200°C. The desired product **1** distilled into the receiving flask. The distillate was dried using Na_2SO_4 and then filtered, affording 3.20 g (58% yield; >90% pure[1]) of CEO as a colorless liquid. ^{1}HNMR for major isomer (500 MHz, $CDCl_3$): δ 2.90-2.95 (m, 2 H), 3.5-3.52 (m, 1 H), 5.49 (d, 1 H, J=1.8 Hz), 5.29 d, 1 H, J=1.8 Hz). ^{13}CNMR (500 MHz, $CDCl_3$): δ 47.5 (C-3), 52.5 (C-2), 115.0 (C-2′), 138 (C-1′).

Preparation of Cross-Linked DNA Duplexes

Oligonucleotides (Integrated DNA Technologies, Inc.) were purified via 20% denaturing polyacrylamide gel electrophoresis (19:1 acrylamide: bisacrylamide; 40% urea) followed by UV shadowing and the crush-and-soak procedure (12). Duplexes radiolabeled on the 3′- or 5′-terminus were prepared as described previously (10). Racemic DEB and ECH were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI); CEO was prepared as described above. To 50 μg of radiolabeled duplex DNA in buffer, 250 mM (2 μL DEB, 2 μL ECH, or 2.3 μL CEO) of cross-linker was added to achieve a total volume of 100 μL. All reactions were run at 37°C, and TE buffer (10 mM Tris-Cl, 1 mM EDTA), 0.1 M MES buffer, and 0.1 M sodium acetate buffer were used for cross-linking reactions at pH 7.0, pH 6.0, and pH 5.0, respectively. Previously determined optimal reaction times were used for ECH (6 h) (10) and DEB (45 min) (13). CEO reaction times (typically 4 - 6 h) were determined for each duplex via time trials, in which aliquots were removed and ethanol precipitated during the course of the reaction.

Separation of Cross-Links

Cross-links were separated from single strands via 20% denaturing polyacrylamide gels (19:1 acrylamide/bisacrylamide, 50% urea) run at 60 W and ambient temperature. Gels were dried for analysis via phosphorimagery (Amersham Biosciences STORM 840) or left wet for purification of cross-links, which have approximately half the mobility of single strands, via crush-and soak (12) after autoradiography. Percent cross-linking was determined through volume analysis of the low-mobility bands in comparison to total DNA (ImageQuant software).

[1]As determined via GC-MS, impurities included 3-4-dichloro-1-butene (<3%), 2-chlorobenzoic acid (<5%), and 2-(1,2-dichloroethyl) oxirane (~2%).

Piperidine Cleavage of Alkylated DNA

Gel-purified cross-linked DNA was cleaved at sites of guanine N7 alkylation by heating at 90° C in 10% aqueous piperidine for 30 min, followed by lyophilization and water washing (14). Piperidine-cleavage products were analyzed via 25% sequencing gels (19:1 acrylamide/bisacrylamide, 50% urea) run at 60 W and 55°C. After drying and phosphorimaging, the relative intensities of bands corresponding to cleavage at guanine residues were determined through volume analysis.

Cytotoxicity Assays

We used Trypan blue exclusion (15) to determine the cytotoxicities of DEB, ECH, and CEO in 6C2 chicken erythro-progenitor cells. Briefly, confluent cells were grown (37°C, 5% CO_2) at a 1:10 dilution with MEM Richter's modification with L-glutamine until the cell count reached between 1.00×10^5 and 1.00×10^6 cells per 10 mL of culture. 1.0-mL aliquots were removed into a 6-well plate with 4 mL fresh media and incubated overnight (37°C, 5% CO_2). CEO, DEB, or ECH at concentrations ranging from 0 - 1.00 mM was added to different wells, and the cells were incubated for 24 h (37°C, 5% CO_2). Twenty-microliter aliquots were taken from each well and mixed with 2 μL Trypan blue (0.2% stock), dyed samples were monitored for viable cells with an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience), and the viable fraction was plotted versus concentration. Determinations were made in duplicate, with the Solver tool in Excel used to optimize the values of a and b in the equation $y=1/[1+[x/a]^b]$ to achieve the best-fit dose-response curve, with a being the LD_{50} (median lethal dose). Standard deviations were determined with SolverAid (16).

Results

Determination of Optimum pH and Time for CEO Cross-linking

To optimize reaction conditions for CEO cross-linking we used **Duplex 1** (Table 1), containing a central 5′-GGC site previously shown to be cross-linked efficiently by ECH (10). Radiolabeled **Duplex 1** was incubated with CEO at pH 5.0, 6.0, or 7.0 and aliquots removed hourly for 8 hours. Reaction products were resolved using denaturing polyacrylamide gel electrophoresis (dPAGE), with low mobility bands presumed to be interstrand cross-links. Cross-link production increased with time at pH 5.0 and 6.0 (Figure 1), with significant amounts of low-mobility product forming only after several hours. No cross-links were detected at pH 7.0 (Figure 2). This combination of optimal pH (5.0) and reaction time (~ 6 h) is similar to the conditions previously established for ECH (10).

Corroboration of Interstrand Cross-Linking

In order to confirm that the low-mobility bands seen in Figure 1 are interstrand cross-links, we prepared **Duplex 2** (Table 1), which is similar to **Duplex 1** but has strands that differ in length. Two sets of cross-linking reactions were run at pH 5.0, one with the short strand radiolabeled and the other with the longer strand radiolabeled. The gel mobility of the presumed cross-link was the same regardless of which strand was radiolabeled, supporting our assignment of the low-mobility band as a cross-link rather than a single-stranded product (Figure 3). Furthermore, these low-mobility bands co-migrated with the low-mobility bands of samples treated with the established cross-linker DEB (17). The diffuse nature of the low mobility bands produced by CEO suggests heterogeneity in the location of these cross-links (18).

Relative Cross-linking Efficiencies of CEO, DEB, and ECH

We compared the cross-linking efficiency of CEO to that of ECH and DEB using **Duplex 3** (Table 1), which has a central 5′-GGC site, the DEB consensus sequence for cross-linking (13). Radiolabeled duplexes were independently incubated with CEO, DEB, or ECH under

optimal reaction conditions (pH 5.0, 37°C) and analyzed via dPAGE. The phosphorimagery volume analysis results (Table 2) revealed that DEB had a significantly higher efficiency than either CEO or ECH. Additionally, CEO was more efficient than ECH, although this difference was not significant at the Bonferronin-corrected level of 0.05. Therefore, the trend for cross-linking follows the order DEB > CEO > ECH, although the latter difference is not statistically significant.

Sequence Specificity of CEO

In order to establish the sequence specificity of CEO, we did two separate experiments. In the first, we compared cross-linking of **Duplex 3**, with its central 5′-GGC site, to **Duplex 4** (Table 1), which has a central 5′-GC site. The 5′-GC sequence, which has the minimal N7-to-N7 interstrand distance, was originally proposed to be the sole target of cross-linkers with chains of five or fewer atoms (19). The formation of CEO cross-links was comparable for **Duplex 3** and **Duplex 4** (Table 3), suggesting that there is no significant difference in CEO cross-linking at 5′-GC and 5′-GGC sites. Similar findings were observed for ECH, consistent with literature reports that it cross-links these sequences about equally (10). In contrast, DEB, which has a strong preference for 5′-GGC sites in comparison to 5′-GC sites (13,17), had an almost three-fold preference for **Duplex 3** relative to **Duplex 4**.

For the second experiment, we designed **Duplex 5** (Table 1), which contains both a 5′-GC and a 5′-GGC site. Presenting both sites within the same duplex ensured identical reaction conditions for the two sequences. **Duplex 5** was radiolabeled on either the 5′- or 3′-end of the top strand and then treated with DEB, CEO, or ECH under optimum reaction conditions. Cross-links were purified from wet denaturing polyacrylamide gels for piperidine cleavage at sites of deoxyguanosine alkylation at N7 positions. Cleavage products were resolved on sequencing gels and analyzed via phosphorimagery to determine cross-linking efficiencies at each site. We averaged data for 5′- and 3′- radiolabeling experiments to minimize the effects of over-alkylation, which inflates the abundance of fragments closest to the radiolabeled end.

Our data confirmed that CEO, like ECH (10), displays a reduced sequence preference relative to DEB (Table 4). As expected from its established consensus sequence for cross-linking at 5′-GGC sequences (13,17), DEB showed an 18-fold preference for the first residue within the 5′-GGC site. In contrast, CEO and ECH both partitioned about equally between the 5′-GGC and 5′-GC sites. Although the two deoxyguanosine residues on the bottom strand were cleaved approximately equally after piperidine treatment of purified cross-link radiolabeled on that strand (data not shown), some cross-link remained intact. This observation is suggestive of some cross-linking at sites other than deoxyguanosine residues, such as terminal residues, which are often hyperreactive (18).

Cytotoxicity Studies

In order to determine a possible relationship between interstrand cross-linking and cytotoxicity for CEO, we used the Trypan blue exclusion assay to obtain cell survival curves for CEO, DEB, and ECH in 6C2 chicken erythro-progenitor cells (Figure 4). With a 24-h treatment, cytotoxicity followed the order DEB (LD_{50} = 0.025 mM ± 0.064) > CEO (LD_{50} = 0.14 mM ± 0.03) > ECH (LD_{50} = 0.59 mM ± 0.14), supporting a relationship between interstrand cross-linking and cytotoxicity. This finding is consistent with previous reports that the interstrand cross-linking ability of bifunctional alkylating agents often, although not always, correlates with cytotoxicity (20).

Discussion

The goal of this study was to investigate the DNA cross-linking potential of (1-chloroethenyl) oxirane, CEO, a metabolite of the possible human carcinogen chloroprene. Structurally similar bifunctional alkylating agents such as DEB, ECH, and the nitrogen mustards have been demonstrated to form interstrand cross-links between deoxyguanosine residues on opposite strands (17,10,21,22). Initially, we screened for CEO cross-linking with a duplex containing a central 5′-GGC site previously established to be efficiently cross-linked by ECH (10). Low-mobility bands suggestive of interstrand cross-links were resolved via dPAGE, with the intensity of these bands increasing with decreasing pH. Although we did not detect cross-links at pH 7.0, their formation after longer incubation times *in vivo* is likely. Even a small number of interstrand cross-links can be lethal because of blockage of DNA replication and transcription. Indeed, it has been proposed that as few as 20 interstrand cross-links in the mammalian genome can result in cell death (23).

In general, cross-linking is a two-step process with formation of monoadducts followed by closure to cross-links (24). The acid catalysis of CEO cross-linking, similar to that observed for ECH (10), is consistent with a similar mechanism in which the initial reaction is at the epoxide. Our data suggest that CEO monoalkylation proceeds by nucleophilic attack on the protonated epoxide by N7 of a deoxyguanosine residue, leading to *N7*-(3-chloro-2-hydroxy-3-buten-1-yl)-guanine. Indeed, this proposed monoadduct is the major product formed in the reaction of calf thymus DNA with CEO (5). Formation of a cross-link could then proceed via nucleophilic attack by a second deoxyguanosine on the opposite strand after protonation of the terminus of the butenyl group (Scheme 1). While the presence of a chlorine substituent might be expected to impede this step, recent experimental evidence supports the feasibility of this reaction (25).

For some of the duplexes in these experiments, we observed multiple low-mobility bands after CEO treatment, suggesting heterogeneous products (e.g., Figure 3). Terminal residues of synthetic DNA duplexes are often hyperreactive, leading to diffuse or multiple cross-linking bands for agents with flexible sequence specificity (26). In general, low-mobility bands with the greatest retardation correspond to centrally cross-linked products (24), a fact that we confirmed for our duplexes through piperidine cleavage and subsequent analysis of the fragments. We observed a decrease in low-mobility bands and the appearance of products corresponding to cleavage at deoxyguanosine residues following treatment with piperidine, which supports alkylation at N7, consistent with previous reports for calf thymus DNA treated with CEO (5,9). However, some low-mobility products were not cleaved by piperidine, suggesting linkage at other sites as well.

Comparison of the cross-linking efficiencies of CEO, ECH, and DEB revealed that DEB was several times more efficient at cross-linking the duplex containing its consensus sequence, 5′-GGC. CEO was modestly more efficient than ECH. DEB also required much shorter incubation times (45 min) than CEO (4-6 h) and ECH (6 h). We therefore rank the cross-linking efficiencies of these compounds as DEB > CEO ≥ ECH.

Reminiscent of ECH, CEO appears to be somewhat flexible in its sequence requirements for cross-linking, as confirmed by our findings for **Duplexes 3**, **4**, and **5**. Whereas DEB has a strong preference for the 5′-GNC sequence relative to 5′-GC (17), CEO and ECH cross-linked distal deoxyguanosine residues at these sites about equally, despite the relatively short alkyl chain lengths of these compounds. Interestingly, early reports proposed that cross-linking between N7 of guanine would be precluded for agents of less than seven carbon atoms in length (27).

In conclusion, we have demonstrated that CEO is a DNA cross-linker, targeting 5′-GC and 5′-GGC sites comparably. Furthermore, this compound is significantly cytotoxic to 6C2 cells.

The correlation between cross-linking activity and cytotoxicity suggests that cross-linking is a contributor to the genotoxic effects of chloroprene.

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Abbreviations

CEO	[1](1-chloroethenyl)oxirane
ECH	epichlorohydrin
DEB	diepoxybutane
TE	10 mM Tris buffer, 1 mM EDTA [pH 7.0]
MES	2-(N-morpholino)ethanesulfonic acid
dPAGE	denaturing polyacrylamide gel electrophoresis
LD_{50}	median lethal dose

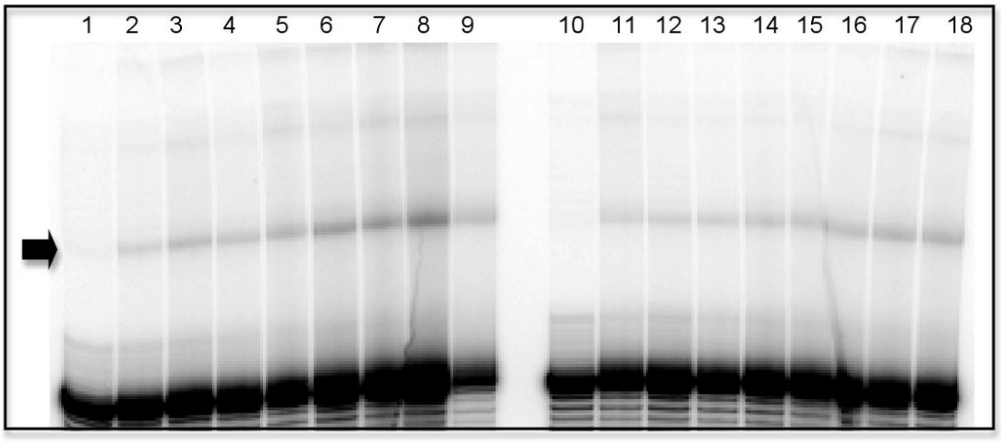


Figure 1.
Reaction of CEO and **Duplex 1** over time at pH 5.0 (Lanes 1-9) and pH 6.0 (Lanes 10-18). Reaction aliquots were taken hourly from time = 0 h (Lanes 1 and 10) to 8 h (Lanes 9 and 18). Presumed interstrand cross-links appear as low-mobility bands (arrow).

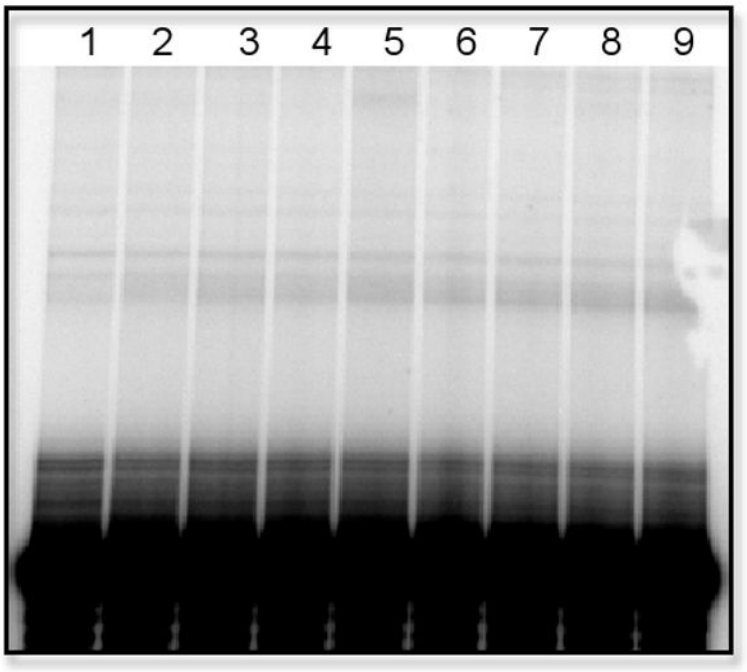


Figure 2.
Reaction of CEO and **Duplex 1** over time at pH 7.0. Reaction aliquots were taken hourly from time = 0 h (Lane 1) to 8 h (Lane 9).

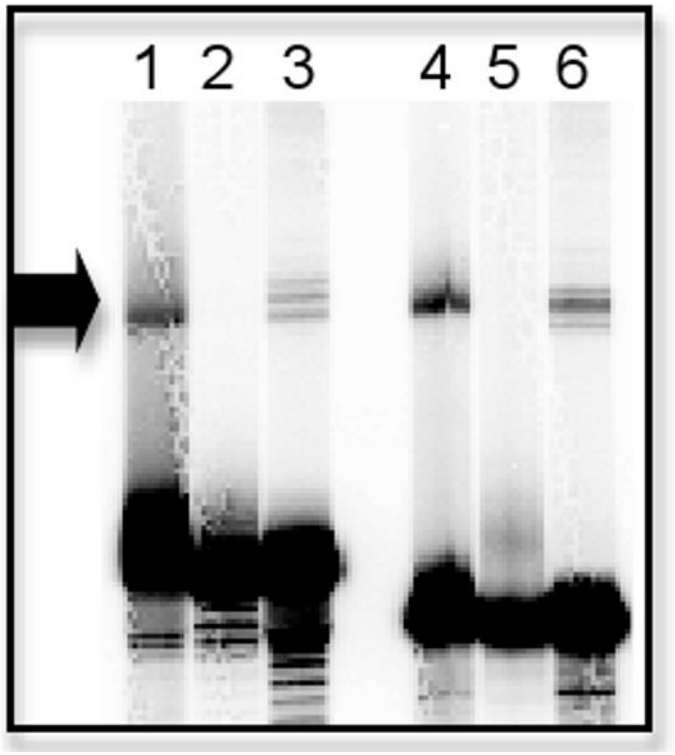


Figure 3.
Cross-linked **Duplex 2**, with strands of differing lengths. Lanes 1-3 have the long strand radiolabeled; lanes 4-6 have the short strand radiolabeled. Lanes 1 and 4 are DEB products; Lanes 2 and 5 are controls (no cross-linker); Lanes 3 and 6 are CEO products. Presumed cross-links (arrow) have the same mobility independent of which strand is labeled.

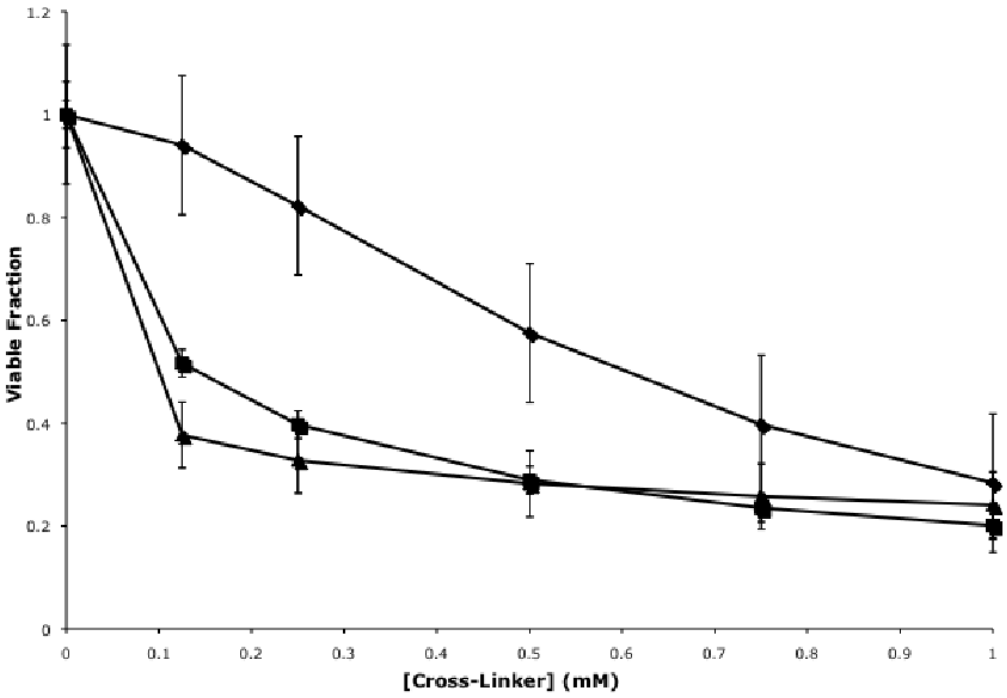


Figure 4.

Survival curves for 6C2 cells treated with CEO (-■-), DEB (-▲-), or ECH (-◆-). Viable cells were determined by the Trypan blue exclusion assay as described in Experimental Procedures. Data are the averages from two trials per compound with standard deviations reported as error bars. The following LD_{50} values were calculated from the best-fit lines: DEB, 0.025mM ± 0.064; CEO, 0.14mM ± 0.03; ECH, 0.59mM ± 0.14. Despite the relatively high standard deviation, the LD_{50} value for DEB was reproducible to within ~12% in a separate set of experiments conducted in triplicate.

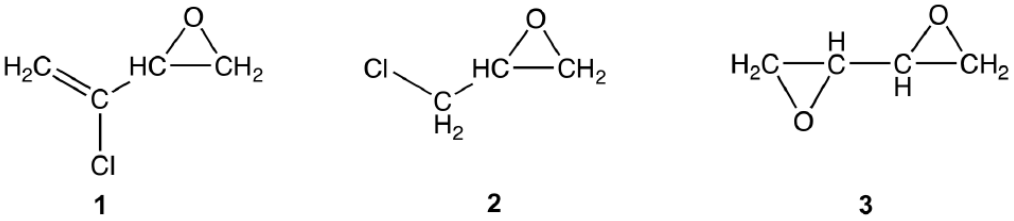
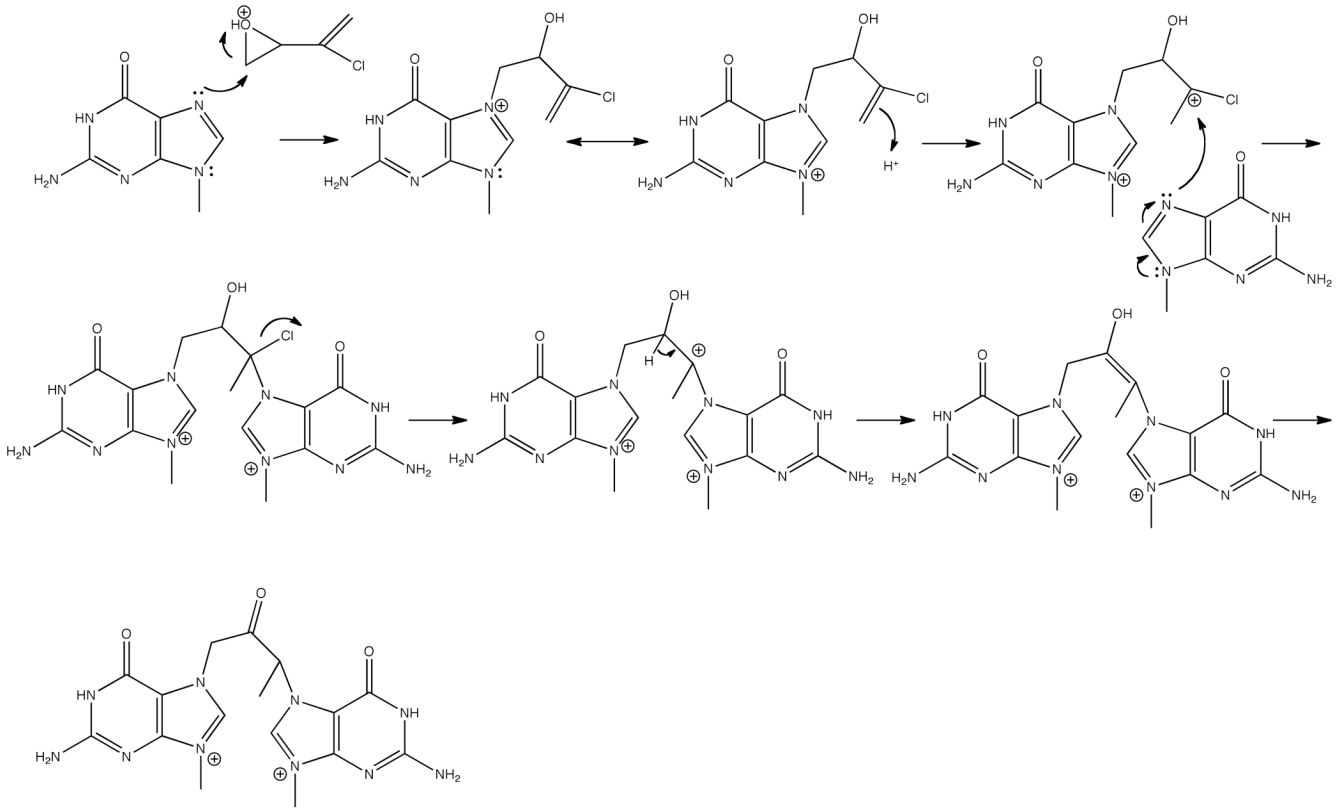


Chart 1.
Cross-linkers used in this study.



Scheme 1.
Possible mechanism for CEO cross-linking at deoxyguanosine residues.

Table 1

DNA oligomers used in these studies.

Duplex 1	5′CAGTATATATTTATAGGCTATATTTATATT 3′ 3′ATATATAAATATCCGATATAAATATAAGTC 5′
Duplex 2	5′CAGAACATATATTTATAGGCTATATTTATATTCCC3′ 3′GTATATAAATATCCGATATAAATATAAGGG 5′
Duplex 3	5′TATATATTTATAGGCTATATTTATATT3′ 3′ATATATAAATATCCGATATAAATATAA5′
Duplex 4	5′TATATATTTATAAGCTATATTTATATT3′ 3′ATATATAAATATTCGATATAAATATAA5′
Duplex 5	5′CAGTATATATTTGGCTATTAGCATTTATAAT3′ 3′ATATATAAACCGATAATCGTAAATATTATAA5′

Table 2

Mean percentages of cross-linking (% XL) for **Duplex 3**, which contains a central 5′-GGC site, with CEO, DEB, and ECH. Data for three replicate trials were averaged.

	CEO	DEB	ECH
Mean % XL	7.4	21.2	5.3
SD	1.5	4.8	1.4

Table 3

Mean percentages of cross-linking (% XL) for **Duplex 4**, which contains a central 5′-GC site, with CEO, DEB, and ECH. Data for three replicate trials were averaged.

	CEO	DEB	ECH
Mean % XL	5.6	7.7	4.1
SD	0.9	2.3	0.7

Table 4

Mean relative percentages of cross-linking as a function of nucleotide position within **Duplex 5** (containing both a GC and a GGC site). Relative intensities of piperidine cleavage at bolded deoxyguanosines from separate experiments in which the top strand was either 5′- or 3′-end-radiolabeled were averaged (three replicate trials of each).

	CEO cross-linking		DEB cross-linking		ECH cross-linking	
nucleotide	mean%	SD	mean%	SD	mean%	SD
GC	34.0	6.9	5.5	5.2	25.6	13.7
G**G**C	29.3	3.8	5.7	2.9	39.4	4.6
GGC	36.7	4.0	88.8	8.0	35.1	10.6