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The 5'-GNC Site for DNA Interstrand Cross-Linking is Conserved for Diepoxybutane Stereoisomers

Julie T. Millard[*,†], **Trevor C. Hanly**[†], **Kris Murphy**[‡], and **Natalia Tretyakova**[*,‡]
†*Department of Chemistry, Colby College, Waterville ME 04901*

‡*Cancer Center and the Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455*

Abstract

The bifunctional alkylating agent 1,2,3,4-diepoxybutane forms interstrand DNA-DNA cross-links between the N7 positions of deoxyguanosine residues on opposite strands of the duplex. For racemic diepoxybutane, these cross-links predominate within 5'-GNC/3'CNG sequences, where N is any nucleotide. We used denaturing polyacrylamide gel electrophoresis (dPAGE) to examine the role of stereochemistry in the cross-linking reaction, subjecting a restriction fragment to cross-linking with *S,S* DEB, *R,R* DEB, or *meso* DEB. DNA cross-links generated by each isomer were isolated by dPAGE, and the sites of cross-linking were identified by sequencing gel analysis of DNA fragments generated by hot piperidine cleavage. We found that the 5'-GNC consensus sequence of racemic DEB is conserved, but the efficiencies of cross-linking vary, with *S,S* > *R,R* > *meso* DEB. These results help explain the observed differences between the biological activities of DEB stereoisomers.

INTRODUCTION

1,2,3,4-Diepoxybutane (DEB) is a cytotoxic bifunctional alkylating agent that produces mutations and chromosomal aberrations (1). As an active metabolite of 1,3-butadiene, DEB has been implicated in the high incidence of leukemia among workers in the styrene-butadiene industry (2-5). On the other hand, *S,S* DEB is believed to be the active metabolite of the pro-drug treosulfan (L-threitol 1,4 bismethanesulphonate), used in the treatment of advanced ovarian cancer (6).

Because DEB is about two orders of magnitude more mutagenic and cytotoxic than the monoepoxide metabolites of butadiene, its biological activity has been attributed to the formation of interstrand cross-links within DNA (7,8). The isolation of N7-linked guanine-guanine conjugates from DEB-treated DNA (9) originally led to the proposal of interstrand cross-linking at 5'-GC sites, which have the minimal N7-to-N7 distance within canonical B-DNA (10). The structural assignment of the DEB-DNA conjugate as 1,4-*bis*(guan-7-yl)-2,3-butanediol was later confirmed independently (11). However, the preferential site of cross-linking was found to be at 5'-GNC sequences (where N is any base) for racemic DEB within both DNA oligomers and defined sequence nucleosomal core particles (12,13). This core sequence preference is further modulated by the secondary influence of flanking sequences (14).

Most prior studies of DNA cross-linking have been carried out with racemic DEB, although some early reports suggested that the three DEB stereisomers (Chart 1) differ in their biological

[*]Authors to whom correspondence should be addressed. Julie T. Millard Department of Chemistry 5757 Mayflower Hill Drive Colby College Waterville, ME 04901 tel: (207) 859-5757 email: jtmillar@colby.eduNatalia Tretyakova 760E CCRB, Univ. of Minnesota Cancer Center 806 Mayo 420 Delaware Street, SE Minneapolis, MN 55455 tel: (612) 626-3432 email: trety001@umn.edu

activity. *S,S* DEB is the most genotoxic and cytotoxic, followed by *R,R* and then *meso* (15-17). Recently, the different stereoisomers were reported to have distinct reactivities with DNA (18). While *meso* DEB forms comparable amounts of interstrand and intrastrand cross-links, *R,R* DEB induces predominantly interstrand adducts with smaller amounts of instrastrand *bis*-N7-guanine lesions, and *S,S* DEB selectively gives rise to interstrand DNA-DNA lesions (18). These findings are consistent with prior reports that *meso* DEB is a less effective interstrand cross-linker than either *S,S* or *R,R* DEB (15,19).

In this study, we examined the sequence preferences of the three optical isomers of DEB within a 152 base pair (bp) restriction fragment. We found that while the three compounds vary in their efficiencies of forming interstrand cross-links, all stereoisomers share the 5'-GNC consensus site for cross-linking.

EXPERIMENTAL PROCEDURES

Caution: DEB is mutagenic and a suspect human carcinogen and must be handled appropriately. *Cross-Linking Reactions.* Racemic DEB was purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). Optically active (*S,S* and *R,R*) and *meso* DEB were prepared as described previously (18). A 152 bp *Eco*RI-*Rsa*I restriction fragment from the plasmid pXP-11 (20), containing a portion of the *Xenopus borealis* 5S RNA gene and 5'-flanking region, was 3'-end radiolabeled and purified as described previously (21). Cross-linking was initiated by addition of DEB (1 μL of either racemic; *S,S*; *R,R*; or *meso*; final concentration 250 mM) to radiolabeled DNA in 49 μL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.1). Incubation was at 37°C for 30 min, followed by ethanol precipitation (22) and lyophilization.

Analysis of Cross-Linking. DEB-treated samples were dissolved in 10 μL of 5 M aqueous urea/0.1% xylene cyanole and loaded onto a 6% denaturing polyacrylamide gel (19:1 acrylamide/bisacrylamide, 42% urea, 30% formamide, 0.35 mm thick, 41 x 37 cm), run on a Hoefer thermojacketed Poker Face gel stand at circa 60 W and ambient temperature. Gels were dried for analysis via phosphorimagery or left wet for purification of alkylated DNA after autoradiography. Percent cross-linking was determined through volume analysis of the family of low-mobility bands in comparison to the single strands with an Amersham Biosciences STORM 840 phosphorimager.

Piperidine Cleavage of Alkylated DNA. Cross-linked (low mobility bands) and monoalkylated DNA (from the higher mobility, single-stranded region of the gels) were recovered from denaturing polyacrylamide gels by the crush-and-soak procedure (22). These samples were cleaved at sites of guanine N7 alkylation by heating at 90°C in 10% aqueous piperidine for 15 min (23). Control (not alkylated) samples showed virtually no degradation under these conditions.

Sequencing Gel Analysis. Samples were dissolved in 10 μL 5 M urea/0.1% xylene cyanole and loaded onto an 8% denaturing gel (19:1 acrylamide/bisacrylamide, 50% urea) run at circa 60 W and 55°C followed by drying and phosphorimagery.

RESULTS

We used a restriction fragment from the 5S RNA gene of *Xenopus borealis* (Figure 1) to monitor interstrand cross-linking by racemic DEB in comparison to *meso, S,S* and *R,R* DEB. This DNA has fifteen potential cross-linking sites (5'-GNC), and its cross-linking by racemic DEB is well characterized (13). Interstrand lesions were analyzed by 6% denaturing polyacrylamide gel electrophoresis (dPAGE). All DEB stereoisomers and the racemic mixture produced the characteristic low-mobility bands diagnostic of interstrand cross-linking (Figure

2). The pattern of low mobility bands was similar for all agents, although the amount of cross-link varied greatly. Following incubation with 250 mM diepoxide, *S,S* DEB cross-linked about 30% of the total DNA, about twice as much as racemic DEB. *R,R* and *meso* DEB were relatively inefficient at producing interstrand cross-links, with *meso* the least efficient (1.88% cross-linking) of the three isomers.

In order to determine the sites of alkylation for each agent, cross-linked and monoalkylated DNA were extracted from wet denaturing gels and subjected to hot piperidine cleavage. The resulting cleavage products were analyzed via polyacrylamide sequencing gels (Figure 3). For all agents, monoalkylation occurred at every deoxyguanosine residue and cross-linking occurred predominantly at 5'-GNC sites, where N is any base. Traces of cleavage could also be noted at other sites, particularly in the region containing eight consecutive deoxyguanosine residues (G117-G124), suggesting some crosslinking at G-rich sites other than the 5'-GNC consensus sequence. Preferential alkylation at G-rich sites could also lead to monoalkylation of some cross-links, which would contribute to enhanced cleavage in this region. Virtually no high molecular weight material remained, supporting the predominance of cross-linking at piperidine-cleavable sites such as the N7 of guanine.

DISCUSSION

Bifunctional electrophiles include such clinically useful anti-tumor drugs as the nitrogen mustards. Although such compounds have many potential cellular targets, anti-cancer potential has been attributed to interstrand cross-linking (24). Indeed, the efficiency of interstrand cross-linking correlates with cytotoxicity for nitrogen mustards (25) and may be a critical aspect governing the action of other antitumor drugs. On the other hand, intrastrand cross-links can be both cytotoxic and mutagenic (16,26), suggesting that these lesions may contribute to carcinogenic potential.

While *S,S* DEB is the active form of the prodrug treosulfan used to treat ovarian cancer (6), racemic DEB has been linked to ovarian toxicity and carcinogenicity in mice and rats (27). The dissimilar biological activities of DEB stereoisomers suggest that they may possess distinct reactivity towards DNA. Indeed, *S,S* DEB has been recently demonstrated to form more 1,3-interstrand cross-links in 5'-GNC trinucleotides than either *R,R* or *meso* DEB (18). However, sequence preferences for DNA cross-linking by individual DEB stereoisomers have not previously been compared. Bifunctional carcinogens have unique sequence selectivities. For example, nitrogen mustards link distal guanines within a 5'-GNC context (28,29) and mitomycin C targets endogenously methylated ^{Me}CpG sites (30-32), yet interstrand cross-link formation by N,N'-bis(2-chloroethyl)-nitrosourea is independent of local sequence context (33).

Many previous studies of the sequence preferences for interstrand cross-linking by DEB have used a racemic mixture of stereoisomers (12-14,34). 5'-GNC was identified as the principal site of DNA cross-linking by racemic DEB, but 5'-GNNC is a secondary target, reacting at about half the frequency of 5'-GNC. In contrast with early predictions (10), the 5'-GC site is poorly cross-linked despite the relatively short length of the butanediol tether. It is likely that significant DNA distortion is required for the formation of 1,3-interstrand 1,4-*bis*(guan-7-yl)-2,3-butanediol conjugates (34).

In the present study, we explored the role of stereochemistry in the sequence specificity of the interstrand cross-linking reactions of DEB, mapping the sites targeted within a 152 bp restriction fragment previously used for studies with racemic DEB (13). Upon incubation with DEB (*S,S*; *R,R*; *meso*, or racemic), radiolabeled DNA was subjected to dPAGE. Interstrand cross-links appeared as a family of bands with reduced mobility relative to the single strands.

Because denaturing gels resolve interstrand cross-linked isomers (35), the pattern of cross-linking can be diagnostic for the sequence preferences of cross-linking (13).

The observed patterns of interstrand cross-links were similar for all types of DEB but the amount of cross-linking varied widely, with the *S,S* stereoisomer about 15-fold more efficient than either *R,R* or meso DEB. Racemic DEB, as a mixture of all three isomers, had an intermediate efficiency. These trends are consistent with a recent report of the relative efficiencies of cross-linking with the DEB stereoisomers (18) although our absolute numbers differ. These differences could arise from differences in concentration (100 mM versus 250 mM used in these studies), reaction time (3 hours versus 30 min in these studies), and the target DNA (an oligomer containing a single core 5'-GGC site versus the restriction fragment used in these studies). Subtle differences in flanking sequence effects for the stereoisomers, as noted previously for DEB and diepoxyoctane (14), and secondary sequence preferences could also modulate the relative efficiencies of these agents towards a DNA with many different target sites.

Sites of alkylation were mapped through piperidine cleavage of gel-purified monoadducts and cross-links followed by sequencing gel analysis. The intensities of monoalkylation at different deoxyguanosines were comparable for all types of DEB, with slight variations in intensity between different sites. Preferential alkylation was noted with G-rich regions, such as that from G117-124. Preferential alkylation at runs of contiguous guanines has been noted for other N7 alkylators (36-38), including racemic DEB (13). The sequence preferences for cross-linking also appeared to be comparable for the three optical isomers of DEB, with cross-linking predominating at 5'-GNC sites. Although *R,R* DEB was recently reported to form interstrand cross-links at 5'-GC sites, these lesions are formed at only about 20% of the efficiency of 5'-GGC cross-links (18). They would therefore be difficult to detect through gel analysis of a DNA with many different target sites such as used in these experiments.

Fairly uniform monoalkylation by racemic DEB has been noted previously (12,34), indicating that the sequence specificity for cross-linking arises in the second step. That is, after formation of monoadducts, reaction of the second epoxide group occurs preferentially at 5'-GNC sites. We also found similar monoalkylation patterns for each stereoisomer, suggesting that differences in cross-link efficiencies arise from different orientations of reactive groups in stereoisomeric N7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-guanine (N7-HEBG) intermediates (18). In *S,S*- and *R,R*-N7-HEBG, the epoxy oxygen and the 2'-hydroxy group are on the same side of the plane formed by the alkyl chain, while in *meso* N7-HEBG, the epoxy oxygen and the 2'-hydroxy group are on opposite sides of the plane. It has been proposed that hydrogen bonding between the 2'-hydroxy group in *S,S*- and *R,R*-N7-HEBG and the N3 of the 3'-neighboring guanine may position the epoxide ring favorably for the S_N2-type nucleophilic attack by the N7 of the guanine in the opposite strand to form an interstrand cross-link (18). Differences in the efficiencies of this reaction for the two stereoisomers may arise from geometry influencing the strengths of these interactions, with the *S,S* intermediate in better position for the subsequent attack. In contrast, the hydrogen bonding interactions between *R,S*- and *S,R*-N7-HEBG intermediates and the 3'-neighboring guanine may better position the epoxide for an S_N2 attack by the 3'-guanine to form intrastrand lesions with the *meso* isomer. Interstrand cross-links also occur at 5'-GNC sites with the *meso* isomer, although at a reduced efficiency, perhaps due to steric effects. Characterization of the structures of the N7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-guanine intermediates could confirm the molecular basis for the differences in the lesions formed by the optical isomers of DEB. An understanding of the factors governing lesion formation would be helpful to distinguish the therapeutic and mutagenic effects of DEB.

Because the different optical isomers share the same core preference for interstrand cross-linking, observed differences in their biological activities are more likely to arise from cross-linking efficiencies than sequences targeted. The high efficiency of interstrand cross-linking by *S,S* DEB is consistent with its potent cytotoxicity and role in the mechanism of treosulfan anti-tumor potential (6). The relatively poor cross-linkers *R,R* and *meso* DEB would be expected to have reduced cytotoxicity. However, the intrastrand cross-linking capacity of *meso* DEB, and to a lesser degree the *R,R* stereoisomer (18), could contribute to mutagenic effects (39).

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Chart 1.
Structures of DEB stereoisomers: R,R DEB (1a), S,S DEB (1b), and meso DEB (1c).

Figure 1.
Sequence of the 3’-end radiolabeled 152 bp fragment used in these studies (20,21). Potential sites for cross-linking at 5’-GNC sequences are shown in bold. Only one strand is shown for convenience and is numbered from 1 to 152 in the 5’-3’ direction. A single P-32 radiolabel is denoted with an asterisk, although either one or two radiolabels may be incorporated during the fill-in reaction of the *Eco*RI site.

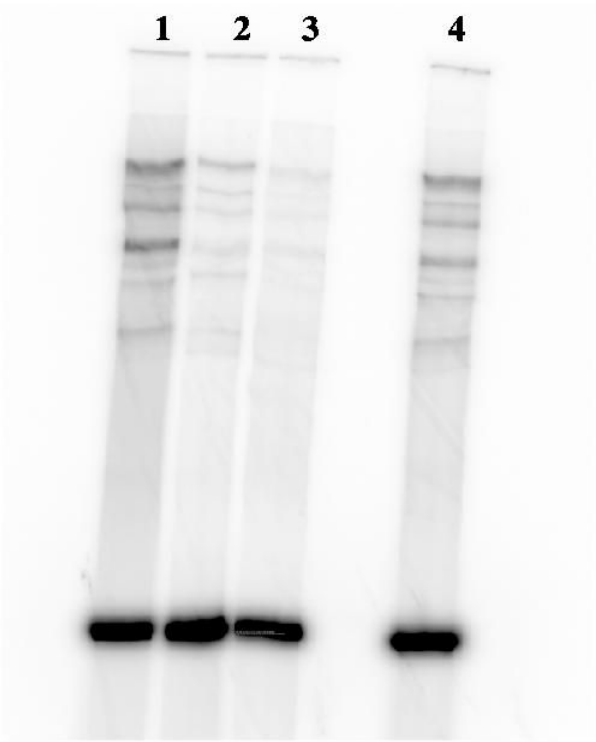

Figure 2.
Interstrand cross-linking patterns of the 152 bp restriction fragment from the 5S RNA gene of *Xenopus borealis* by *S,S* DEB (lane **1)**; *R,R* DEB (lane **2**); *meso* DEB (lane **3**), and racemic DEB (lane **4**). Percent cross-linking is as follows: *S,S* = 28.7%; *R,R*= 2.17%; *meso* = 1.88%; racemic = 15.0%.

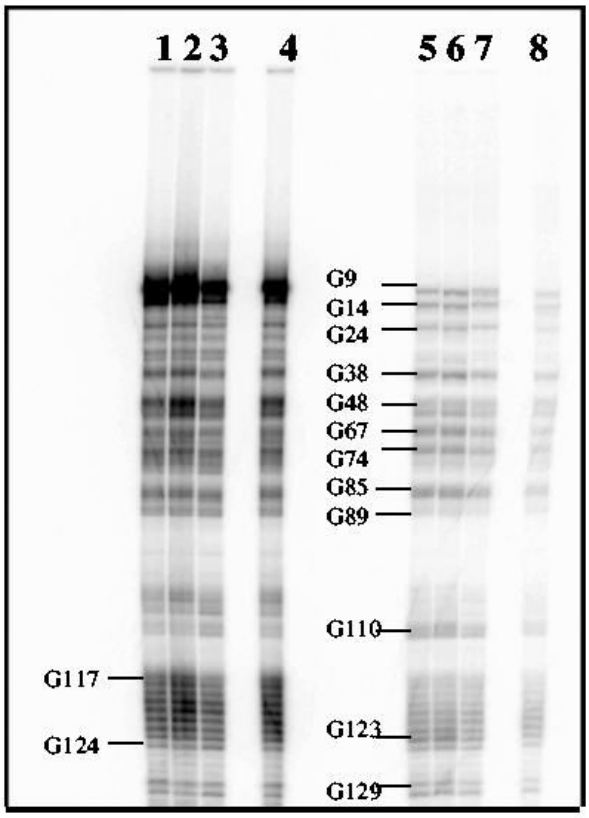

Figure 3.
Piperidine cleavage patterns of monoalkylated (Lanes 1-4) and cross-linked (Lanes 5-8) DNA. Lanes 1 and 6-*S,S* DEB; Lanes 2 and 7-*R,R* DEB; Lanes 3 and 8-*meso* DEB; Lanes 4 and 5-racemic DEB. Landmark deoxyguanosine residues are indicated at the side. Some doubling is visible for low molecular weight fragments because of the possibility of incorporating either one or two deoxyadenosine residues during radiolabeling.