Two Structurally Related Diaziridinylbenzoquinones Preferentially Cross-Link DNA at Different Sites upon Reduction with DT-Diaphorase†

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ABSTRACT: The nucleotide sequence preferences for the formation of interstrand cross-links induced in DNA by 2,5-diaziridinyl-1,4-benzoquinone (DZQ) and 3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone (MeDZQ) were studied using synthetic duplex oligonucleotides and denaturing polyacrylamide gel electrophoresis (PAGE). Reaction of these bifunctional alkylating agents with a DNA duplex containing several potential cross-linking sites resulted in the formation of cross-linked DNAs with different electrophoretic mobilities. Analysis of the principal cross-linked products by piperidine fragmentation revealed that the preferential site of cross-linking was altered from a 5′-GNC to a 5′-GC sequence upon reduction of DZQ to the hydroquinone form by the enzyme DT-diaphorase. In contrast, the reduced form of MeDZQ was found to preferentially cross-link at 5′-GNC sites within the same sequence. These preferences were confirmed in duplex oligonucleotides containing single potential cross-linking sites. Additional minor cross-linked products were characterized and revealed that DZQ and MeDZQ are both capable of cross-linking across four base pairs in a 5′-GNNC sequence.

The cellular target for many bioreductive antitumor alkylating agents such as mitomycin C and AZQ[1] is believed to be DNA. Enzymatic reduction of the quinone group by cellular enzymes leads to the formation of reactive metabolites capable of producing a variety of cytotoxic DNA lesions (Gutierrez, 1989; Powis, 1989). One-electron reduction of the quinone group can lead to the formation of semiquinone radicals and the production of DNA single strand breaks (Begleiter & Leith, 1990). Antitumor quinones also have the potential to undergo reduction to generate a species capable of alkylating and cross-linking DNA (Dzielendziak et al., 1990). DNA cross-linking is thought to be an important mechanism by which diaziridinylbenzoquinones exert their cytotoxic effect (Siegel et al., 1990; Gibson et al., 1992). Enzymatic or chemical reduction of the nonaromatic quinones to the aromatic hydroquinone alters the electron distribution and facilitates aziridine ring opening. Protonation of the aziridine ring also catalyzes ring opening, and thus an increase in DNA cross-linking has been observed with both the quinone and hydroquinone at acidic pH (Hartley et al., 1991; Lee et al., 1992).

Previous studies have shown that the level of DNA interstrand cross-links (ISC) induced in isolated DNA by a series of AZQ analogues was pH dependent and was increased upon reduction by ascorbic acid (Hartley et al., 1991) or by DT-diaphorase (Lee et al., 1992). In contrast to AZQ, the level of DNA ISC induced by DZQ and MeDZQ was increased to a much greater extent upon reduction by DT-diaphorase. In addition, while the quinone and hydroquinone forms of several structurally similar aziridines exhibited relatively nonspecific alkylation of guanine N7 positions, the reduced form of DZQ reacted preferentially at guanines in 5′-GC sequences and more specifically at 5′-TGC sequences (Hartley et al., 1991; Lee et al., 1992). A model was proposed to account for the unique reaction of the hydroquinone form of DZQ at 5′-GC sites (Hartley et al., 1991) which suggested that the unique selectivity may result in an altered sequence preference for DNA interstrand cross-linking.

Although a single mechanism cannot account for all of the observed cytotoxic effects of these compounds, it is clear that DNA interstrand cross-linking is important in this respect and that DT-diaphorase-mediated two-electron reduction of the quinone group influences the sequence-specific alkylation and cross-linking activity of these agents (Siegel et al., 1990; Lee et al., 1992). In the present study, the nucleotide sequence preferences for the formation of DNA interstrand cross-links induced by DZQ and MeDZQ in synthetic oligonucleotides were investigated by isolating a number of electrophoretically distinct cross-linked products and examining the pattern of guanine N7 alkylation upon piperidine fragmentation.

MATERIALS AND METHODS

Chemicals. DZQ and MeDZQ were synthesised by the reported general methods (Petersen et al., 1955) and stock solutions were 20 mM in DMSO. Electrophoresis grade acrylamide, bis-acrylamide, piperidine, and NADH (grade III) were obtained from Sigma. Ultrapure urea was purchased from Bethesda Research Laboratories (BRL). T4 polynucleotide kinase (PNK) was purchased from Northumbria Biologicals, and [γ-^{32}P]ATP (5000 Ci/mmol, 10 mCi/mL) was from Amersham International. Oligonucleotides were synthesized on a 1.0-mmol scale using cyanoethyl chemistry and fully deprotected with hydroxyl groups on both the 5′- and 3′-ends. Purified rat hepatic DT-diaphorase, with a specific acty of 660 nmol/min/μg of protein, was kindly

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[1] Abbreviations: AZQ, 2,5-diaziridinyl-3,6-bis(carbethoxyamino)-1,4-benzoquinone (diaziquone); DZQ, 2,5-diaziridinyl-1,4-benzoquinone; MeDZQ, 3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone; NADH, nicotinamide adenine dinucleotide (reduced form).

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provided by Dr. D. Ross of the University of Colorado. All other reagents were of analytical grade.

Buffers. T4 kinase buffer is 40 mM Tris (pH 7.6), 50 mM $MgCl_2$, 25 mM dithiothreitol, and 5 μM dATP. TEA is 25 mM triethanolamine and 1 mM EDTA, pH 5.8. TBE is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.3. Alkylation stop solution is 0.6 M sodium acetate, 20 mM EDTA, and 0.1 mg/mL yeast tRNA. Loading buffer is 98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol, and 0.025% bromophenol blue.

Preparation of 5'-End-Labeled Oligonucleotides. Approximately 150 μg of each synthetic oligonucleotide was purified by denaturing PAGE (20% polyacrylamide, 19:1 acrylamide/bis-acrylamide, 7 M urea, 0.4 mm × 50 cm × 20 cm), using an 8-well comb. The gel was run at 3000 V at 55 °C until the xylene cyanol had migrated approximately 8 cm. The gel was transferred to Saran Wrap and placed onto a fluorescent TLC plate (Merck HPTLC silica gel, 60 F254 precoated, 10 × 20 cm). Bands were visualized using short-wave UV light and excised from the gel. The gel slices were crushed and soaked in 1 mL of 150 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA overnight at 37 °C. The eluent was loaded onto a prewet Sep-Pak C_{18} cartridge (Waters), and the DNA on the column was washed with 10 mL of ultrapure water. The desalted sample was eluted with 1.5 mL of 60% aqueous methanol, and the oligonucleotide concentration was estimated by measuring the absorbance at 260 nm. The methanol was evaporated and the remaining sample was lyophilized and resuspended at 1 μg/μL in ultrapure water. Purified single-stranded DNA (5 μg) was 5'-end-labeled with [γ-^{32}P]ATP (5000 Ci/mmol, 10 mCi/mL) and T4 polynucleotide kinase. Unincorporated ATP was removed by passing the DNA through a Sephadex P6 spin column (Bio-Rad). Labeled DNA was then lyophilized and resuspended in 20 μL of ultrapure water. Unlabeled complementary DNA (5 μg) was combined with the labeled strand and annealed by heating the mixture to 90 °C and slowly cooling to room temperature.

For the single-site oligonucleotides A–C, approximately 2.5 μg of the self-complementary duplexes A and B and 1.25 μg of each strand of noncomplementary duplex C were 5'-end-labeled as described above. After removal of unincorporated ATP, the noncomplementary strands of duplex C were combined, and each duplex was annealed by heating to 65 °C and then cooling slowly to 4 °C.

Preparation of Cross-Linked DNA Duplexes. Singly end labeled DNA duplex I (2.5 μg) was reacted with either 100 μM DZQ or 100 μM MeDZQ in TEA buffer containing 100 μM NADH and 0.173 μg of DT-diaphorase in a final volume of 50 μL. DZQ (500 μM) was also reacted with duplex I under the same conditions in the absence of NADH and enzyme. Reactions identical to those described above were carried out on duplex II. After 2 h at 37 °C, 2 μL of each reaction mixture was removed and the volume adjusted to 10 μL with water. The DNA was precipitated upon addition of 10 μL of alkylation stop solution and 3 vol of 95% ethanol. The DNA pellet was dried and stored at −20 °C for later analysis. The remaining reaction mixtures were stopped by addition of an equal volume of alkylation stop solution and ethanol-precipitated. Pellets were dried under vacuum, resuspended in 10 μL of loading dye, heated to 90 °C for 2 min, and chilled in an ice–water bath. Samples were subjected to denaturing PAGE (20% polyacrylamide, 19:1 acrylamide/bis-acrylamide, 7 M urea) at 55 °C and 3000 V until the xylene cyanol had migrated 15 cm. The DNA was visualized by autoradiography, and cross-linked material in addition to untreated single-stranded DNA was excised from the gel. DNA was eluted from the gel slices (ca. 0.5 × 1.0 cm) using the "crush and soak" procedure described above.

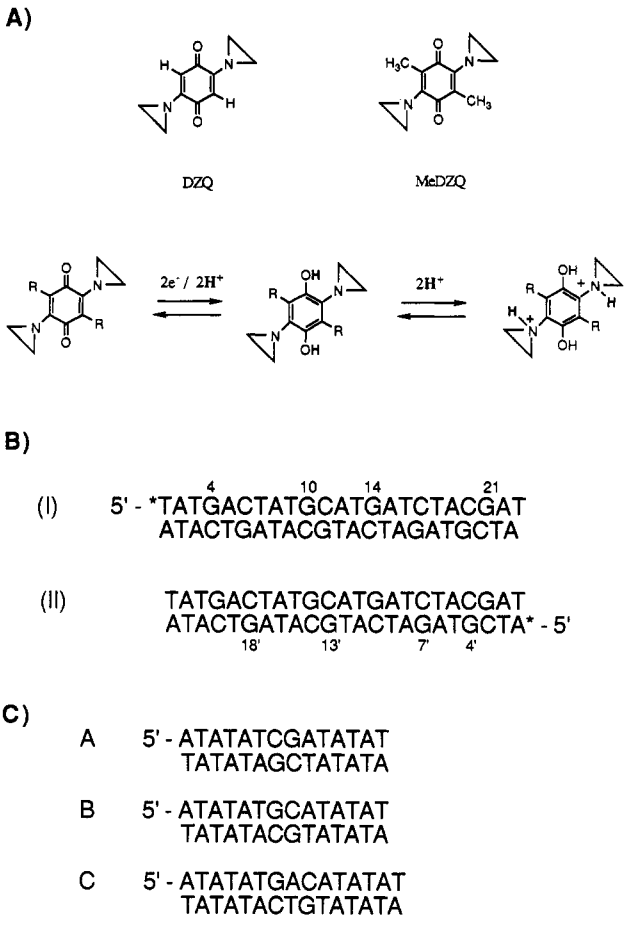

FIGURE 1: (A) Schematic diagram showing the reduction of DZQ and MeDZQ. (B) Duplex oligonucleotides used in this study labeled either on the top strand (I) or the bottom strand (II). (C) Single cross-linking site oligonucleotides.

Table I: Percent Contribution of Each Band to Total Cross-Linked DNA for Each Drug Treatment

treatment	band	% contribution[a]
DZQ−	1	13.3 (3.6)
	2	22.5 (7.0)
	3	28.5 (4.7)
	4	17.1 (3.6)
	5	18.6 (6.4)
DZQ+	1	16.3 (1.6)
	2	20.8 (4.6)
	3	30.5 (3.7)
	4	31.7 (3.8)
MeDZQ+	1	13.9 (2.9)
	2	52.5 (3.1)
	3	21.9 (2.1)
	4	11.7 (3.1)

[a] Results are the average value obtained from duplexes I and II and are the means and standard deviations (brackets) from three independent experiments.

Oligomers A–C were treated with either DZQ or MeDZQ in TEA buffer for 2 h at 8 °C in a final volume of 50 μL. Reactions were terminated and DNA precipitated as described above. Samples were resuspended in loading buffer and heated to 65 °C prior to immersion in an ice–water bath. Electrophoresis was as described and was allowed to run until the dye front had migrated approximately 10 cm.

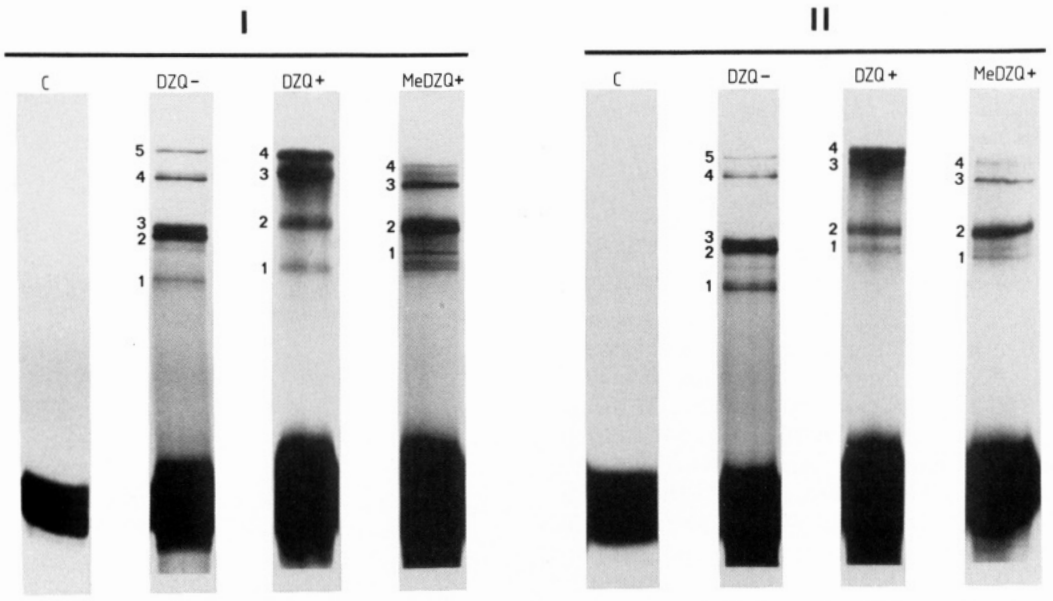

FIGURE 2: Autoradiogram of a 20% denaturing PAGE gel showing cross-links formed in duplexes I and II by DZQ and MeDZQ. C lanes are control untreated DNA. DZQ− lanes are 500 μM DZQ, pH 5.8, unreduced. DZQ+ lanes are 100 μM DZQ, pH 5.8, plus DT-diaphorase. MeDZQ+ lanes are 100 μM MeDZQ, pH 5.8, plus DT-diaphorase. Numbers to the left of the lanes identify the position of the band excised from the gel.

Determination of Sites of Guanine N7 Alkylation. Cross-linked, alkylated, and unmodified DNA were treated with freshly diluted 10% aqueous piperidine for 15 min at 90 °C to convert quantitatively sites of guanine N7 alkylation to strand breaks (Mattes et al., 1986). Samples were lyophilized, and the pellets were resuspended in 15 μL of ultrapure water and relyophilized. Samples were dissolved in loading buffer, heated to 90 °C for 2 min, and immersed in an ice–water bath before they were loaded onto a denaturing polyacrylamide gel (20% polyacrylamide, 19:1 acrylamide/bis-acrylamide, 7 M urea). Approximately 10 000 cpm of cross-linked material was loaded in each lane. Electrophoresis was at 55 °C and 3000 V until the xylene cyanol had migrated approximately 8 cm. Bands were identified by reference to Maxam–Gilbert guanine-specific and Fe/EDTA hydroxyl radical chemical cleavage reactions on unmodified singly end labeled duplex DNA (Maxam & Gilbert, 1980; Weidner et al., 1989, 1990).

Densitometry. Autoradiographs were scanned using a Pharmacia LKB Ultroscan-XL enhanced laser densitometer, and plots were normalized to the highest absorbance value in each lane.

RESULTS

A 23 base pair duplex oligonucleotide (Figure 1) labeled either on the top strand (duplex I) or the bottom strand (duplex II) was treated with DZQ and MeDZQ for 2 h at 37 °C under reducing or nonreducing conditions as described in Materials and Methods. Five-fold lower drug conditions were used under reducing conditions because of the greater drug reactivity. Alteration of the drug concentration did not effect the product distribution (data not shown). Product mixtures were ethanol–precipitated and analyzed using 20% denaturing PAGE. Under these conditions, DNA strands that are covalently cross-linked by the drug are unable to denature completely and migrate with reduced mobility through the gel compared to unmodified DNA and DNA containing monoadducts. Figure 2 shows several cross-linked products of different electrophoretic mobilities produced upon treatment of I and II with DZQ and MeDZQ. No cross-linked products could be detected for unreduced MeDZQ under these conditions (data not shown). Cross-linked products, numbered in order of migration, were excised from the gel, and the relative amount of DNA in each band was determined by microdensitometry of the autoradiogram (Table I). Cross-linked DNA constituted less than 1% of total DNA in each reaction mixture.

Cross-linked DNAs were treated with hot piperidine to cleave quantitatively at sites of guanine N7 alkylation, and the resulting fragments were analyzed by 20% denaturing PAGE (Figure 3) and the autoradiograms analyzed by densitometry (Figure 4). No trace of the initial cross-linked product could be detected after aqueous piperidine treatment, suggesting that the drug was covalently linked between two guanine N7 positions on opposite strands of the DNA duplex. The presence of a trailing band migrating above fragments cleaved at guanine residues was sometimes observed, which was found to be due to incomplete piperidine cleavage and which disappeared upon extended treatment (data not shown). The position of guanine N7 alkylation on cross-linked DNA was determined by comparison with guanine-specific chemical cleavage of the corresponding singly end labeled, unmodified DNA (Figures 3 and 4, G lanes). To determine the site of cross-linking for each drug treatment, fragmentation patterns generated from duplex I cross-linked DNAs were compared to duplex II cross-linked DNAs of corresponding electrophoretic mobility. For example, bands 2 and 3 were the major cross-linked products (>50%) resulting from the reaction of unreduced DZQ with I and II (Figure 2, Table I).

Fragmentation of the DNA in bands 2 and 3 revealed extensive (and in the case of band 2 almost exclusive) alkylation of G4 on duplex I and G18′ on duplex II, suggesting a dG to dG DNA interstrand cross-link at the 5′-GNC sequence (Figures 3A and 4A). However, upon reduction of DZQ to the hydroquinone, a decrease in the electrophoretic mobility of the major cross-linked products (band 4, 32%, and band 3, 30%) was observed. Piperidine cleavage of the major product band 4 showed exclusive alkylation on G10 and G13′, indicating preferential cross-linking at the 5′-GC sequence (Figures 3B and 4B). Band 3 gave a more complex cleavage pattern and, upon extended electrophoretic separation, was found to consist of more than one band (data not shown). This

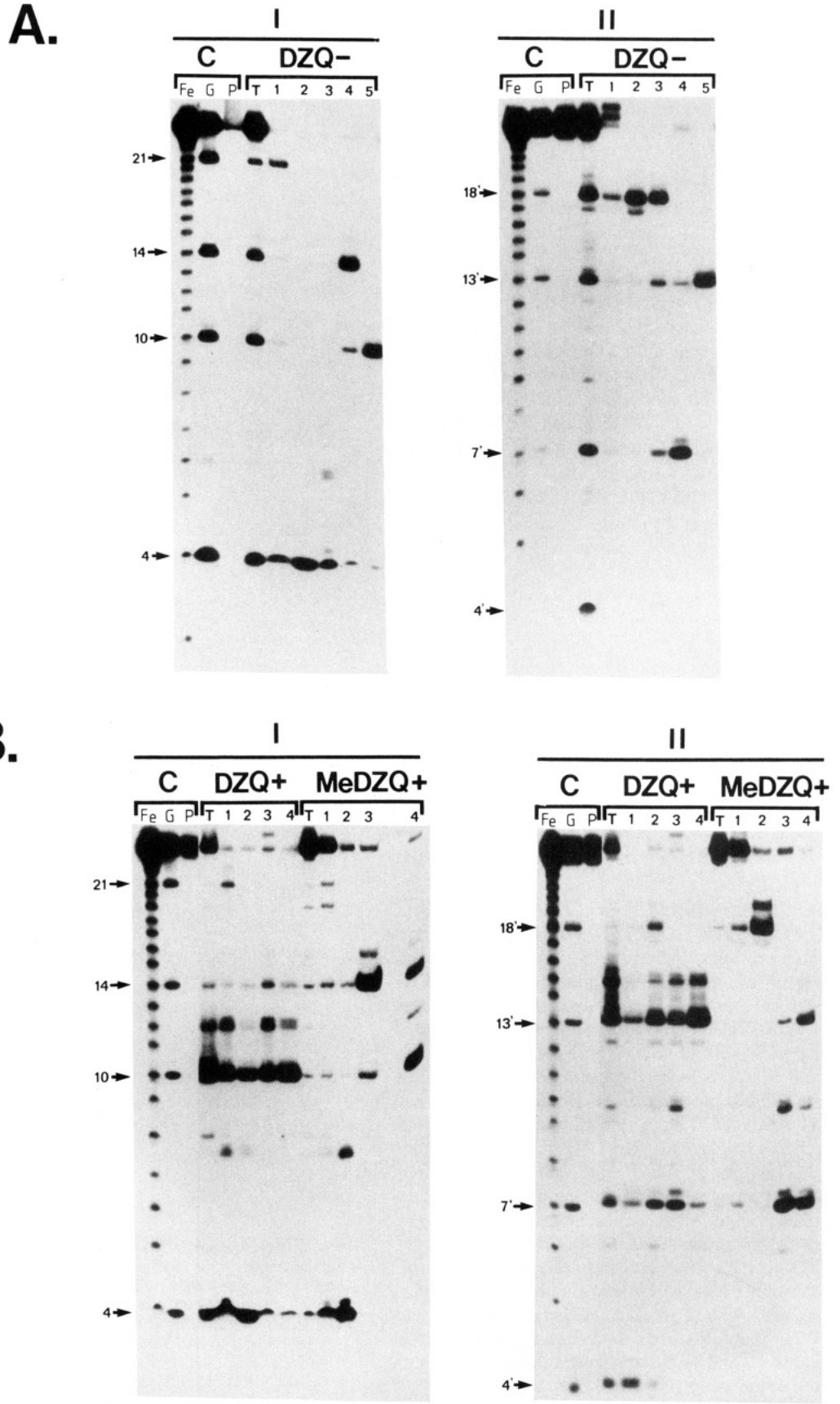

FIGURE 3: (A) Portion of a 20% denaturing polyacrylamide gel showing piperidine-induced fragmentation of cross-linked DNAs I and II isolated from the gel shown in Figure 2. C indicates control lanes, where Fe is control DNA treated with Fe/EDTA/H_2O_2/ascorbic acid showing the position of each base. G is guanine-specific cleavage of control DNA, and P is piperidine treatment of unalkylated DNA. T shows fragments produced upon piperidine treatment of total reaction products prior to separation by denaturing PAGE. Numbered lanes are cross-linked DNAs with different mobilities and correspond to the numbered bands in Figure 2. Panel A shows patterns produced from I and II DNAs cross-linked by 500 μM DZQ, pH 5.8, under nonreducing conditions (DZQ–). Panel B shows patterns produced from I and II DNAs cross-linked with 100 μM DZQ (DZQ+) and 100 μM MeDZQ (MeDZQ+) at pH 5.8 in the presence of DT-diaphorase. Numbers to the left of the gel indicate the position of the guanine from the 5′-end label. Guanines in strand II are distinguished by a prime after the number.

was not the case for band 4, which was clearly the single major cross-linked product. In contrast to reduced DZQ, the major cross-linked product formed upon reaction of reduced MeDZQ with I and II (band 2, 53%) showed alkylations at G4 and G18′, indicating cross-link formation at the 5′-GNC site within the sequence (Figures 3B and 4C).

In order to assess the sequence specificity of total guanine N7 alkylations, DNA containing cross-links and monoadducts was isolated prior to the separation of reaction products and treated with piperidine. The resulting fragments were then separated by denaturing PAGE (Figures 3 and 4, T lanes). Total alkylation products formed upon reaction of unreduced DZQ with I and II showed little preferential alkylation of guanines. However, upon reduction of DZQ to the hydroquinone, a strong preferential alkylation of G10 and G13′ was observed. Reduced MeDZQ was found to have less guanine N7 alkylation sequence specificity than reduced DZQ. Analysis of total alkylation fragments in Figure 3 shows substantial amounts of full-length single-stranded DNA, inidicating that many duplexes are not alkylated and that most DNA duplexes contain only one alkylation.

Fragmentation patterns produced from all cross-linked DNAs were analyzed by linear densitometry and compared in Figure 4. In general, cross-linked DNAs of different electrophoretic mobilities displayed unique fragmentation patterns upon treatment with hot piperidine. Those cross-linked DNAs that have increased mobility through a denaturing gel showed prominent alkylations at guanines near the ends of the DNA duplex. Band 1 DNAs (Figure 4) show alkylation of guanines near the ends of both duplexes I and II in each drug treatment. Similar findings by others have shown that the mobility of mitomycin C cross-linked DNAs through a denaturing gel was a function of the position of the cross-link from the 5′-radiolabel (Weidner, 1990; Millard, 1991). Those cross-linked products with the greatest electrophoretic mobility were shown to be terminally cross-linked (Millard et al., 1991).

A summary of cross-linked sites induced by DZQ and MeDZQ is listed in Table II in the order of preference. Band 1 DNAs which are minor products in each case and which may be the result of terminal cross-links are excluded. The occurrence of more than one fragment upon treatment of some fractions of cross-linked DNAs with piperidine indicates heterogeneity with respect to the position of the guanine alkylation and thus precludes the exact assignment of the cross-link site. This is particularly evident in DNA duplexes cross-linked by reduced DZQ where alkylations at G10 and G13′ are present in all cross-linked DNAs due to the strong preferential alkylation at the 5′-TGC sites. In addition, complex fragmentation patterns could result from more than one cross-linked product with a similar migration through the denaturing polyacrylamide gel as is the case for band 3 from reduced DZQ (see above). Nevertheless, analysis of the alkylation patterns produced by less abundant structurally homogeneous cross-linked products clearly indicated that unreduced DZQ could produce a cross-link at the 5′-GC site (band 5, Figures 3A and 4A), but only in relatively low yield (18%). More interesting, however, was the finding that unreduced DZQ (band 4, Figures 3A and 4A) and reduced MeDZQ (band 3, Figures 3B and 4C) exhibited significant alkylation of G14 and G7′, suggesting the formation of a cross-link across four base pairs at the 5′-GNNC sequence.

The differing sequence preferences for cross-linking by DZQ and MeDZQ were confirmed in oligonucleotides containing only a single potential cross-link site (A–C, Figure 1C). Figure

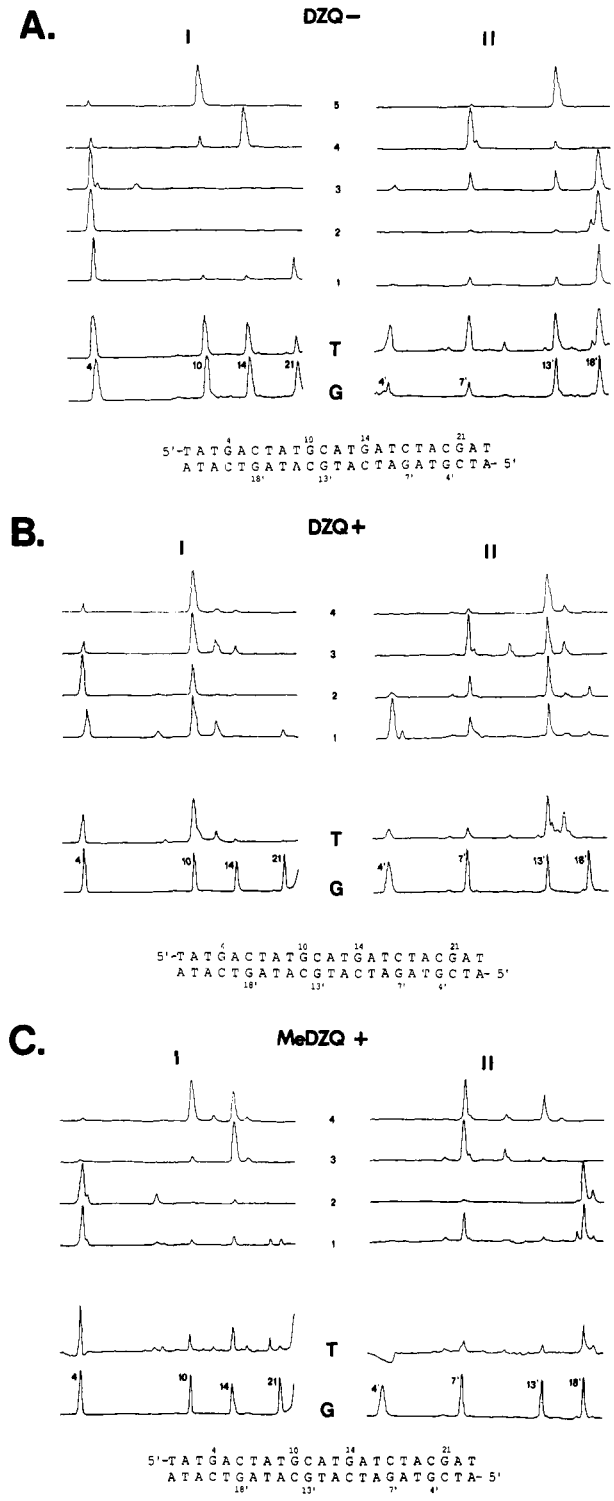

FIGURE 4: Representative densitometry of piperidine-induced fragmentation patterns comparing I and II cross-linked DNAs of corresponding electrophoretic mobilities. Numbers between the traces indicate the mobility of the cross-linked product as shown in Figure 2. T indicates total alkylations. G shows guanine-specific sequencing lanes of control DNA. Numbered peaks show the position of guanine from the 5′-end label. Panel A compares I and II DNAs cross-linked with 500 μM DZQ under nonreducing conditions. Panels B and C show I and II DNAs cross-linked with 100 μM DZQ and 100 μM MeDZQ, respectively, under reducing conditions.

5 shows the separation of products generated from the reaction of the compounds with oligomers A–C under reducing and nonreducing conditions. In the case of nonreduced DZQ and reduced MeDZQ, cross-linking was only observed in duplex C containing the 5′-GNC sequence. In contrast, the reduced

Table II: Summary of the Cross-Linking Sites of DZQ and MeDZQ Listed in Order of Preference

treatment	band[a]	cross-link site
DZQ–	3 (28.5)	5′-GNC
	2 (22.5)	5′-GNC
	5 (18.6)	5′-GC
	4 (17.1)	5′-GNNC
DZQ+	4 (31.7)	5′-GC
	3 (30.5)	?[b]
	2 (20.8)	?
MeDZQ+	2 (52.5)	5′-GNC
	3 (21.9)	5′-GNNC
	4 (11.7)	?

[a] Band number from Figure 2 with percent contribution to total cross-linked products in parentheses. [b] ? denotes that exact assignment of the cross-link site was not possible.

form of DZQ produced cross-links in duplex B containing the 5′-GC sequence, and to a lesser extent in duplex C (5′-GNC). No cross-linking was observed for either compound in duplex A (5′-CG) nor for nonreduced MeDZQ in any of the oligonucleotides. These data confirm the sequence preferences observed for these compounds in the 23 base pair duplex containing several potential cross-linking sites described above.

DISCUSSION

The reaction of DZQ and MeDZQ with a duplex oligonucleotide containing a number of potential cross-linking sites results in the formation of several cross-linked products with different electrophoretic mobilities. Analysis of the principal cross-linked products by piperidine fragmentation showed that reduction of DZQ from the quinone to the hydroquinone by DT-diaphorase alters the sequence preference for DNA interstrand cross-link formation between guanine N7 positions from a 5′-GNC to a 5′-GC site. In contrast, the reduced form of MeDZQ was shown to preferentially cross-link DNA at 5′-GNC sites. These results, which were confirmed in single cross-linking site oligonucleotides, lend support to molecular modeling studies that have suggested that intercalation of the hydroquinone form of DZQ at 5′-GC sites is facilitated by hydrogen bonding of the hydroxyl groups with the O2 and C4-NH_2 groups of cytosine. This allows the reactive aziridine carbon atoms to be in a favorable position above the plane of the aromatic ring for covalent bond formation to the guanine N7 position (Hartley et al., 1991). Stabilization of the intercalated hydroquinone may account for the preferential monoalkylation of guanines in 5′-GC sequences and subsequent cross-link formation at these sites. This idea is supported by the findings of others who report that preferential cross-link formation takes place at locations that will result in the minimum distortion of B-DNA (Hopkins et al., 1991).

A general feature of this study was the observation that, for a given drug treatment, cross-linked DNAs of different electrophoretic mobilities gave rise to unique fragmentation patterns upon treatment with hot aqueous piperidine. With respect to unreduced DZQ, the order for preferential cross-linking was 5′-GNC > 5′-GC, 5′-GNNC, as shown in Table II. Distribution of total guanine N7 fragments (cross-linked and monoadducted DNA) showed no preferential alkylation of guanines (T lanes, Figure 4A). However, reduction of DZQ to the hydroquinone significantly enhanced the total alkylation of guanines at 5′-TGC sequences, as observed previously (Hartley et al., 1991). It is interesting to note that G10 and G13′, both at 5′-TGC sites, are alkylated in all DNAs cross-linked by reduced DZQ (Figure 4B, bands 1–4). Analysis of the major cross-linked product (band 4, Figure 4B) shows that this fraction contained a homogeneous population of DNAs cross-linked by reduced DZQ at G10 and G13′, indicating a dG to dG cross-link at the 5′-GC site.

Cross-linked DNAs differing in the position of the cross-link but having similar electrophoretic mobilities may account for the occurrence of more than one fragment upon treatment of certain fractions of cross-linked DNAs with piperidine. Such heterogeneous mixtures may also arise from cross-linked DNAs that to not differ in the position of the cross-link, but in which a certain fraction of these cross-linked DNAs contain accompanying monoalkylations. Preferential alkylation by reduced DZQ may account for the appearance of more than

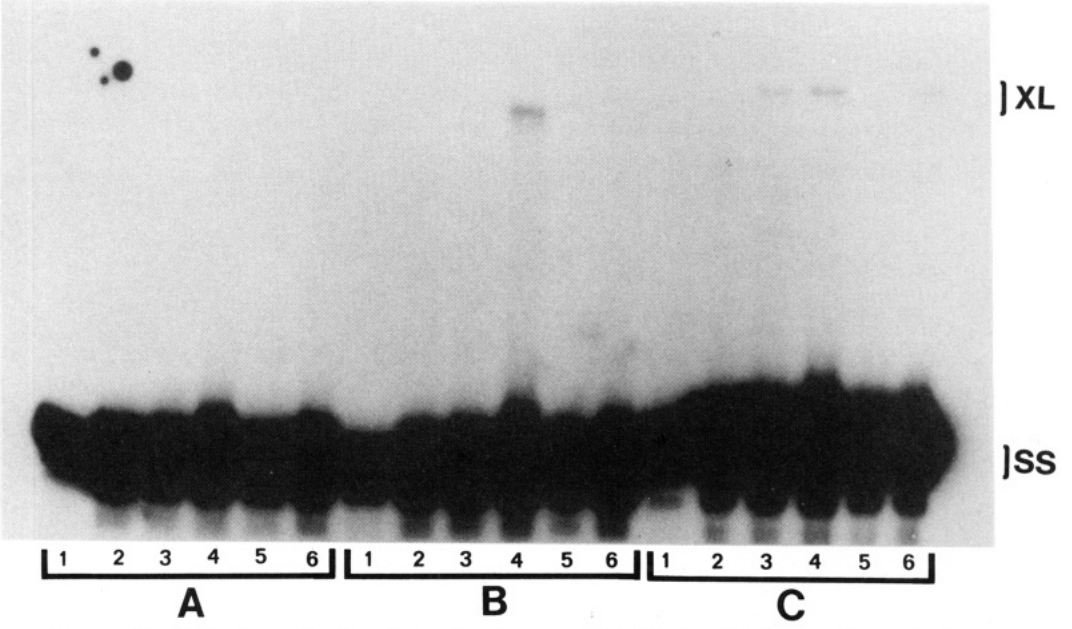

FIGURE 5: Autoradiogram of a 20% polyacrylamide gel showing the cross-linking induced in duplex oligonucleotides A, B, and C by DZQ and MeDZQ under reducing and nonreducing conditions. Duplexes were treated for 2 h at 8 °C. In each case, lane 3 is 500 μM unreduced DZQ, lane 4 is 100 μM reduced DZQ, lane 5 is 500 μM unreduced MeDZQ, and lane 6 is 100 μM reduced MeDZQ. Lanes 1 and 2 are untreated DNA.

one fragment in bands 1–3 in Figure 4B. For example, for band 2 DNA in Figure 4B, fragments resulting from cleavage at G4, G10, G7′, and G13′ may represent a cross-link through G10 and G13′, but a fraction of these cross-linked duplexes contain monoalkylations at G4 and G7′. However, preferential alkylation of G13′ may actually mask a cross-link between G4 and G18′ since only alkylations closest to the 5′-radiolabel are detected. In the case of band 3 in Figure 4B, extended electrophoresis revealed more than one product. The cleavage pattern may thus imply a mixture of duplexes cross-linked at 5′-GC and 5′-GNNC sites. Clearly, the unequivocal assignment of a single cross-link site is precluded for such heterogeneous fractions of cross-linked DNAs.

The DNA interstrand cross-linking sequence specificity of several bifunctional alkylating agents has been investigated by others. Although it has been proposed that the distances of guanine N7 positions in 5′-GC sequences of duplex DNA (7.5 Å) were in favorable proximity for cross-linking by the reactive groups of mechlorethanmine (five atoms apart), it was found that cross-linking between two guanine N7 positions of duplex DNA at 5′-GNC sites was preferred (Millard et al., 1990; Hopkins et al., 1991). It has also been shown that reductively activated mitomycin C cross-links were formed predominantly at 5′-CG sites, while cisplatin was found to preferentially cross-link guanines at 5′-GC sequences using an Fe(II)/EDTA/hydrogen peroxide/ascorbic acid analysis (Hopkins et al., 1991). In the present study, the unreduced form of DZQ and the reduced form of MeDZQ were found to preferentially cross-link at the 5′-GNC site of a duplex oligonucleotide in a manner similar to that of mechlorethamine. However, the cross-link distance in DZQ and MeDZQ is much greater (ten atoms) than in mechlorethamine, which allows the formation of cross-links spanning four base pairs in a 5′-GNNC sequence which is not observed with cisplatin or mitomycin C. A low level of cross-linking at a 5′-GNNC sequence has been suggested for mechlorethamine (Hopkins et al., 1991).

Clearly, reduction of DZQ to the hydroquinone by DT-diaphorase results not only in an altered sequence preference for guanine N7 monoalkylations but also influences the ultimate formation of DNA interstrand cross-links between guanine N7 sites. This is not the case with other 2,5-diaziridinyl-1,4-benzoquinones which contain more bulky site groups in the 3 and 6 positions. It is not clear whether the properties of DZQ would be maintained if one side group remains as hydrogen while the other is modified. Compounds of this type would have the advantage that, depending on the structure of the side groups, the redox capability of the quinone, and hence the ease of formation of the hydroquinone, could be modified. Alternatively, the DZQ molecule could be attached to sequence-specific ligands to produce alkylating agents with enhanced or altered sequence selectivities. Nonsymmetrical compounds of both types are being synthesized.

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