Alteration in DNA Cross-Linking and Sequence Selectivity of a Series of Aziridinylbenzoquinones after Enzymatic Reduction by DT-Diaphorase†

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ABSTRACT: DT-diaphorase (DTD) mediated reduction of a series of 2,5-bis-substituted-3,6-diaziridinyl-1,4-benzoquinones was found to increase the level of DNA interstrand cross-linking (ISC) formed at neutral pH with an enhancement observed as the pH was decreased to 5.8. The analogues used were symmetrically alkyl-substituted carbamoyl ester analogues of AZQ (D1–D7), 3,6-diaziridinyl-1,4-benzoquinone (DZQ), the 2,5-dimethyl derivative (MeDZQ), and a 2,5-bis[(2-hydroxyethyl)amino] analogue (BZQ). At pH 5.8, the level of DNA ISC induced by enzymatic reduction was as follows: DZQ > MeDZQ ≫ D1 (methyl) > D3 (*n*-propyl) > D2 (AZQ; ethyl) > D5 (*n*-butyl) > D7 (*sec*-butyl) > D4 (isopropyl) D6 > (isobutyl). A similar trend was observed at pH 7.2. The level of DNA ISC induced by BZQ, which is not a substrate for DTD, was not increased by enzymatic reduction. Dicumarol, a known inhibitor of DTD, was capable of inhibiting the DNA ISC induced by these quinones upon enzymatic reduction. MeDZQ and DZQ reacted with guanines, as measured by Maxam and Gilbert sequencing, with a sequence selectivity similar to that of the nitrogen mustard class of antitumor agents. Enzymatic reduction of DZQ and MeDZQ by DTD was found to alter their sequence-selective alkylation. Reduced DZQ showed enhanced guanine alkylation in 5′-GC-3′ sequences and new sites of adenine alkylation in 5′-(A/T)AA-3′ sequences. Reduced MeDZQ only showed new sites of adenine alkylation at 5′-(A/T)AA-3′ sequences but no enhancement of guanine alkylation. The new sites of adenine alkylation were found to be inhibited in the presence of magnesium and rapidly converted into apurinic sites. Chemical reduction of DZQ by ascorbic acid altered the sequence-selective pattern of alkylation in a manner similar to that observed for enzymatic reduction. Ascorbic acid was unable to reduce MeDZQ. These data show that DTD mediated reduction can effect the sequence selectivity of alkylation and DNA interstrand cross-link induction by aziridinylbenzoquinones but that the alteration in sequence selectivity was compound specific.

Enzymatic reduction of antitumor quinones increases their cytotoxicity in vitro and is thought to be responsible for their antitumor activity in vivo (Butler & Hoey, 1987; Powis, 1987). In particular, the aziridinylbenzoquinones, such as AZQ[1] and BZQ (Figure 1), have been utilized clinically even though their mechanisms of reduction and action have not been clearly elucidated (Khan & Driscoll, 1979; Bender et al., 1983; Hard et al., 1985). Quinones such as AZQ and BZQ can undergo both one- and two-electron reduction (Gutierrez, 1989; Dzielendziak et al., 1990). One-electron reduction of quinones generates semiquinone radicals, which, depending upon their redox potential, may interact with oxygen to produce superoxide and other reactive oxygen species (Gutierrez, 1989). Two-electron reduction converts the parent quinone to its hydroquinone derivative and in the case of AZQ and mitomycin C generates a reactive intermediate which is capable of alkylating and cross-linking DNA (Siegel et al., 1990a,b).

We have previously suggested that the two-electron reductase NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase; DTD) may be an important contributor to the cytotoxicity and DNA damage induced by a series of aziridinylbenzoquinones in the HT-29 human colon carcinoma cell line (Pacheco et al., 1991). This cell line is rich in DTD, and we found that a general trend existed between the ease of reduction of the quinone and the cytotoxicity observed (Pacheco et al., 1991). A more detailed study found that purified DTD from HT-29 cells metabolized AZQ and that this metabolism was NADH dependent and could be inhibited by dicumarol (Siegel et al., 1990a). On the basis of these results, we hypothesized that two-electron reduction of aziridinylbenzoquinones may be responsible for the observed cytotoxicity and DNA damage (Siegel et al., 1990a).

In the present study, we have utilized purified rat hepatic DTD and investigated the ability of this enzyme to activate

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[1] Abbreviations: DZQ, 3,6-diaziridinyl-1,4-benzoquinone; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone; BZQ, 2,5-bis[(2-hydroxyethyl)amino]-3,6-diaziridinyl-1,4-benzoquinone; DTD, DT-diaphorase; DNA ISC, DNA interstrand cross-linking; EDTA, ethylenediaminetetraacetic acid; bp, base pairs; NADH, nicotinamide adenine dinucleotide (reduced form); TEA, triethanolamine. Other abbreviations are 2,5-bis-substituted-3,6-diaziridinyl-1,4-benzoquinone analogues where the varying substituents are as follows: D1, methyl; D2, ethyl (AZQ); D3, *n*-propyl; D4, isopropyl; D5, *n*-butyl; D6, isobutyl; D7, *sec*-butyl.

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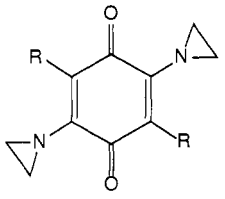

B		COMPOUND
$NHCH_2CH_2OH$		BZQ
H		DZQ
CH_3		MeDZQ
$NHCOOCH_3$		D1
$NHCOOC_2H_5$		D2 (AZQ)
$NHCOOC_3H_7$	(*n*-propyl)	D3
$NHCOOC_3H_7$	(*i*-propyl)	D4
$NHCOOC_4H_9$	(*n*-butyl)	D5
$NHCOOC_4H_9$	(*i*-butyl)	D6
$NHCOOC_4H_9$	(*sec*-butyl)	D7

FIGURE 1: Structures of the aziridinylbenzoquinones investigated.

selected quinones to a species capable of alkylating and cross-linking DNA. We have also investigated the pH dependency of such reactions and found that a greater degree of enzymatically induced cross-linking is observed at acidic pH. DNA ISC can be detected at neutral pH but requires the presence of DTD. In general, the extent of DNA ISC was reduced as the size of the alkyl substituent at the C2 and C5 positions of the quinone ring was increased. The most intriguing result observed was that both enzymatic and chemical reduction enhances DZQ induced alkylation at 5′-GC-3′ sequences with a preference for thymine 5′ of the guanine and creates new sites of alkylation at 5′-(A/T)AA-3′ sequences. Enzymatic reduction of MeDZQ, but not chemical reduction, was found to generate new sites of alkylation at 5′-(A/T)AA-3′ sequences with no alteration in the pattern of guanine alkylation being observed. Our results suggest that DTD reduces the parent quinone to the hydroquinone and it is this species which is responsible for the induction of DNA ISC and observed alteration in DNA sequence selectivity.

Materials and Methods

Chemicals and Reagents. AZQ was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. All other quinones were synthesized according to published methods (Dzielendziak & Butler, 1989; Dzielendziak et al., 1990). NADH (grade IV) and dicumarol were obtained from Sigma. pBR322 DNA, Klenow fragment of DNA polymerase I, and restriction endonucleases *Eco*RI, *Taq*I, and *Bam*HI were purchased from Bethesda Research Laboratories. [α-^{32}P]dATP was purchased from NEN. All other reagents were at least of analytical grade.

Purification of Rat Hepatic DT-Diaphorase. Rat hepatic DTD was purified according to a previously published method (Hojeberg et al., 1981) from uninduced rats, yielding material with a specific activity of 660 nmol/min (μg of protein)$^{-1}$. This material was supplied to us by Dr. P. Preusch.

In Vitro DNA Interstrand Cross-Linking Assay. DNA ISC was measured by the method of Hartley et al. (1991b). Briefly, pBR322 plasmid DNA was linearized by digestion with *Eco*RI and 3′-end-labeled with [α-^{32}P]dATP (3000 Ci/mmol) and Klenow fragment of DNA polymerase I. Unincorporated ATP was removed by precipitation of the DNA which was then resuspended in sterile distilled water. Approximately 10 ng of end-labeled DNA was incubated with each quinone in either 10 mM potassium phosphate buffer (pH 5.8, 6.6, and 7.8) or 25 mM triethanolamine, pH 7.2. Each buffer contained 100 μM NADH, 0.173 μg of DTD, and 1 mM EDTA in a final volume of 50 μL. Dicumarol (20 μM) was used to study the effect of inhibiting DTD activity. Incubations were for 1 h at 25 °C. Reactions were terminated by ethanol precipitation of DNA. Prior to electrophoresis, the samples were dissolved in 20 μL of strand-separation buffer (30% dimethyl sulfoxide, 50% glycerol, 1 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol), heated at 90 °C for 2 min, and chilled immediately in an ice water bath (Hartley et al., 1991b). Control nondenatured samples were not subjected to heating. Samples were then loaded into a 1.0% agarose gel (20 cm long) and electrophoresed for 14–16 h at 40 V. The gel and running buffer contained 40 mM Tris, 20 mM acetic acid, and 2 mM EDTA (pH 8.1). Gels were dried at 80 °C onto filter paper and autoradiographed. The percentage of cross-linked (double-stranded) DNA relative to non-cross-linked (single-stranded) DNA was determined by densitometry of the autoradiograph using a LKB ultrascan XL laser densitometer.

DNA Alkylation and Sequencing Analysis. pBR322 plasmid DNA was digested with *Eco*RI and 3′-end-labeled with [α-^{32}P]dATP. This DNA was then digested with *Bam*HI and *Taq*I separately, and resulting single end-labeled 375- and 344-bp fragments were isolated on a 5% acrylamide gel. DNA was drug treated as described above for the DNA interstrand cross-link assays and incubated for 1 h at 25 °C (pH 5.8) or 37 °C (pH 7.2). In certain experiments, 10 mM Mg^{2+} was added to study the inhibition of alkylation in the major groove (Kohn et al., 1987; Prakash et al., 1990). After termination of the drug reaction by ethanol precipitation, the DNA pellet was resuspended in freshly diluted 1 M piperidine and heated at 90 °C for 20 min to convert quantitatively sites of guanine N7 alkylation into strand breaks (Mattes et al., 1986a). Heat treatment at 90 °C for 20 min, in the presence of 10 mM sodium hydroxide, was utilized in order to detect the presence of apurinic sites in the alkylated DNA. Samples were precipitated, resuspended in alkaline tracking dye containing 80% formamide and 10 mM sodium hydroxide and 1 mM EDTA, heated at 90 °C for 2 min, chilled on an ice bath, and then subjected to sequencing electrophoresis in parallel with Maxam and Gilbert base-specific reactions (Maxam & Gilbert, 1980). Electrophoresis was achieved on 0.4 mm × 31 cm × 38.5 cm 6% polyacrylamide gels containing 8 M urea. Running time was approximately 3 h at 1500 V, 50 °C. Gels were transferred to filter paper, dried, and then autoradiographed.

Results

Figure 2 shows the ability of a series of aziridinylbenzoquinones (50 μM) to induce DNA ISC in a linearized pBR322 DNA fragment as measured by an agarose gel assay. The presence of DNA ISC is indicated by the appearance of double-stranded DNA. The two bands observed in the denatured samples in these agarose gels represent each individual single strand (both end-labeled) of the DNA which have different mobilities. Both BZQ and DZQ were capable of inducing DNA ISC without enzymatic reduction when incubated with DNA in 25 mM triethanolamine at pH 7.2 (panel A). Under these buffer conditions, however, all other quinones required reduction by DTD prior to DNA ISC being detected (panel B). The level of DNA ISC induced by BZQ, as measured by densitometry, in either the presence or absence of DTD was 58% and 65%, respectively, suggesting that BZQ is not a substrate for DTD (Figure 2).

MeDZQ and DZQ were previously found to be the most cytotoxic of these quinones against the HT-29 cell line (Pacheco et al., 1991), and a more detailed study of these compounds was warranted. Figure 3 shows that as the concentration of DZQ and MeDZQ was increased the level of DTD mediated DNA ISC was also found to be increased at pH 7.2. The level of DZQ induced DNA ISC was consistently greater than that observed with MeDZQ at all concentrations. A significant level of DNA ISC could still be detected when 2.5 μM DZQ or 5–10 μM MeDZQ was incubated in 25 mM TEA, pH 7.2, with DTD. Dicumarol (20 μM), a known inhibitor of DTD, was found to inhibit the ability of the en-

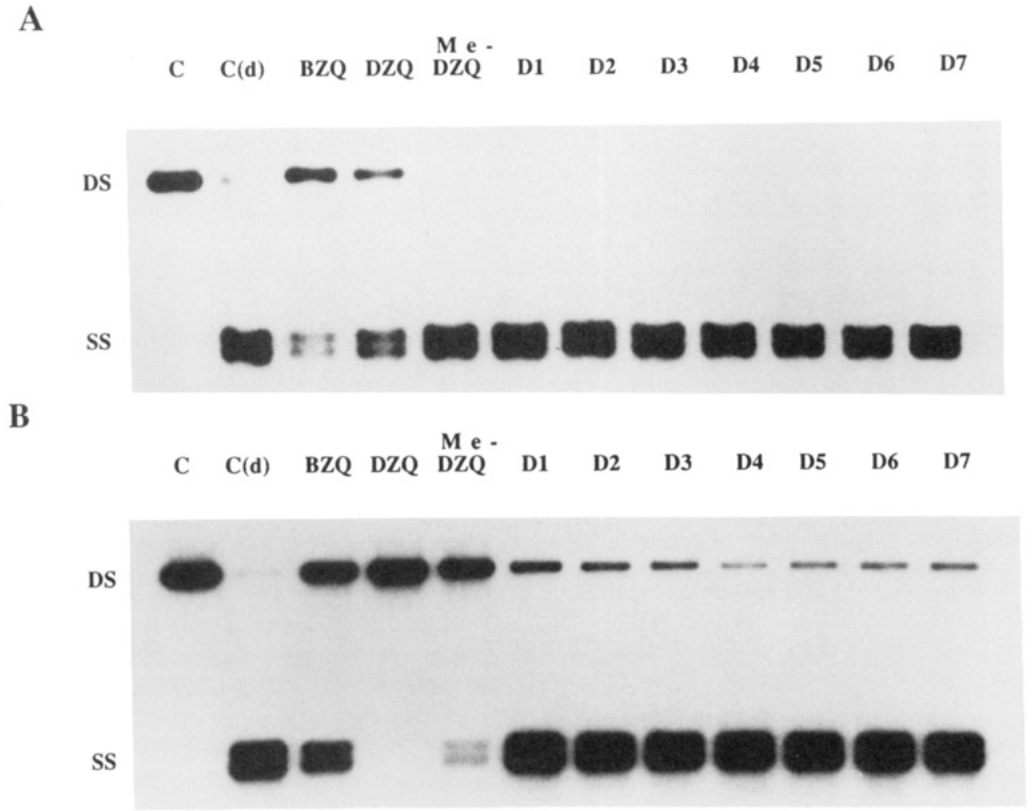

FIGURE 2: Autoradiogram of a 0.8% neutral agarose gel showing DNA ISC formation in linear pBR322 DNA by a series of aziridinylbenzoquinones. DNA was incubated for 2 h in 25 mM TEA, pH 7.2, with 50 μM drug without (panel A) or with (panel B) 0.173 μg of DTD. Lane C is the control, nondenatured DNA, and lane C(d) is denatured DNA in the absence of drug. DS and SS are double- and single-stranded DNA, respectively.

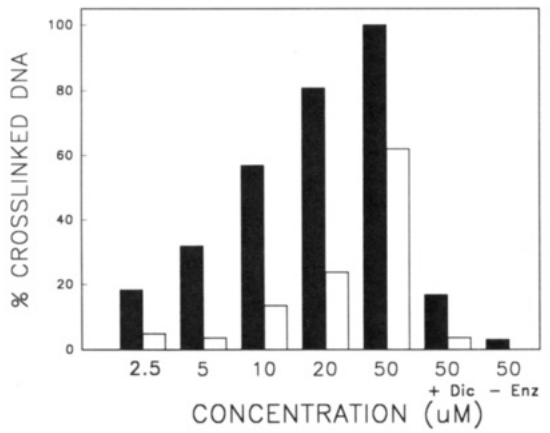

FIGURE 3: Concentration dependence of DTD mediated DNA ISC by DZQ (solid bars) and MeDZQ (open bars) in pBR322 DNA. DNA was incubated with the indicated concentration of drug for 1 h in 25 mM TEA, pH 7.2, at 25 °C. Apart from the panel indicated with (50 – Enz), all incubations contained 0.173 μg of DTD. The percentage of cross-linked DNA was determined by densitometric analysis.

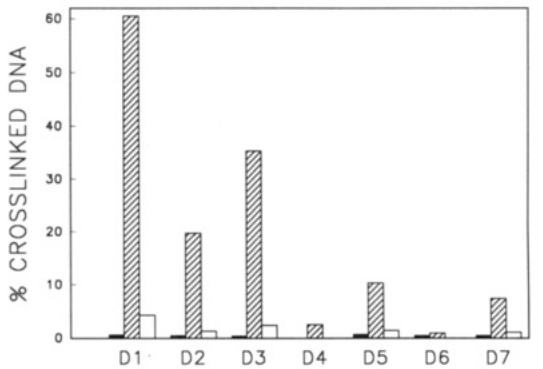

FIGURE 4: pH and enzymatic dependence of DNA ISC induced by a series of aziridinylbenzoquinones in pBR322 DNA. DNA was incubated in 10 mM potassium phosphate, pH 5.8 (hatched bars) or pH 6.6 (open bars), for 1 h in the presence of 20 μM drug and 0.173 μg of DTD. In the pH 5.8 incubations, 20 μM dicumarol, a known DTD inhibitor, was added (solid bars). The percentage of cross-linked DNA was determined by densitometric analysis.

zyme to reduce either DZQ or MeDZQ to a species capable of cross-linking DNA. Figure 3 also shows that in the absence of enzyme DZQ was capable of inducing DNA ISC whereas MeDZQ was not.

Figure 4 shows the level of DNA ISC induced, upon enzymatic reduction at different pH's, by the alkyl-substituted carbamoyl ester aziridinylbenzoquinone compounds D1–D7 (20 μM). As the pH was decreased from 7.8 to 5.8, the level of DNA ISC was found to be increased significantly. Indeed, no DNA ISC was detected in 10 mM potassium phosphate at pH 7.8 (data not shown), with a low level detected at pH 6.6 and higher levels observed at pH 5.8. Dicumarol completely inhibited the ability of DTD to reduce all compounds to a species capable of cross-linking DNA at pH 5.8 (Figure 4).

Figure 5 shows that a similar pH dependency was observed for the induction of DNA ISC by DZQ and MeDZQ upon enzymatic reduction; that is, a significantly greater quantity of DNA ISC was observed at pH 5.8 than was observed at either pH 6.6 or 7.8. In addition, the formation of DNA ISC was enhanced in the presence of DTD (lane 2) although DNA ISC could be observed in the absence of DTD (lane 1), particularly in the case of DZQ. Dicumarol was able to inhibit the formation of MeDZQ induced DNA ISC at all pH's tested (lane 3). When taken together with Figure 4, the ability of each quinone to induce DNA ISC upon enzymatic reduction

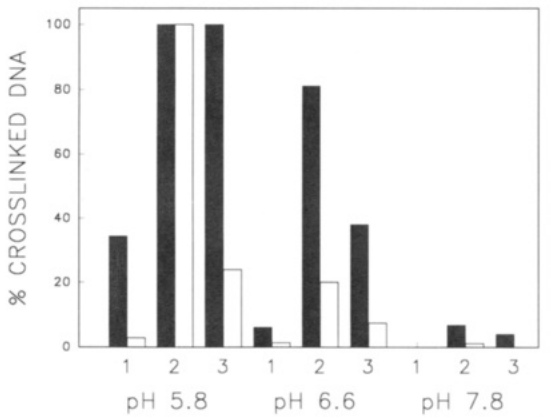

FIGURE 5: pH and enzymatic dependence of DNA ISC induced by DZQ (2.5 μM, solid bars) and MeDZQ (10 μM, open bars) in pBR322 DNA. DNA was incubated in 10 mM potassium phosphate (pH 5.8, 6.6, or 7.8) for 1 h in the absence (lane 1) or presence (lane 2) of 0.173 μg of DTD. The ability of 20 μM dicumarol to inhibit DTD mediated DNA ISC was also determined (lane 3). The percentage of cross-linked DNA was determined by densitometric analysis.

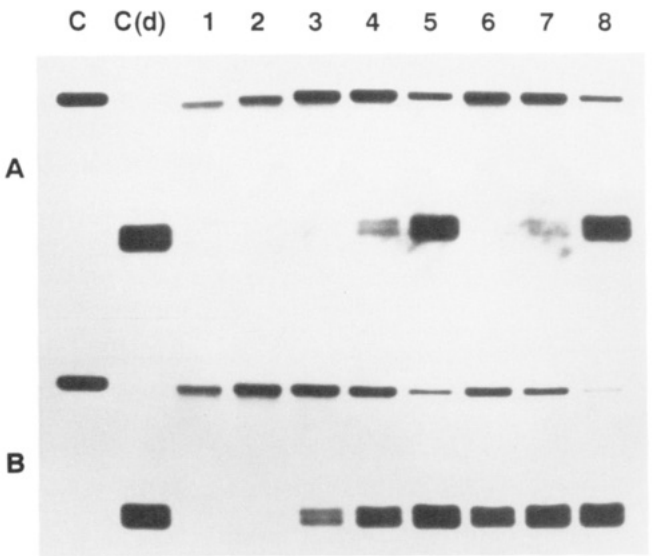

FIGURE 6: Autoradiogram of a 1% agarose gel showing the concentration dependence of DNA ISC induced by DZQ (panel A) and MeDZQ (panel B) after enzymatic reduction (0.173 μg of DTD) in 10 mM potassium phosphate, pH 5.8, in either the absence (lanes 1–5) or presence (lanes 6–8) of 20 μM dicumarol. Concentrations: lanes 1 and 6, 2.5 μM; lane 2, 1.25 μM; lanes 3 and 7, 0.25 μM; lane 4, 0.125 μM; lanes 5 and 8, 0.025 μM. Lane C was nondenatured DNA; lane C(d) was denatured DNA.

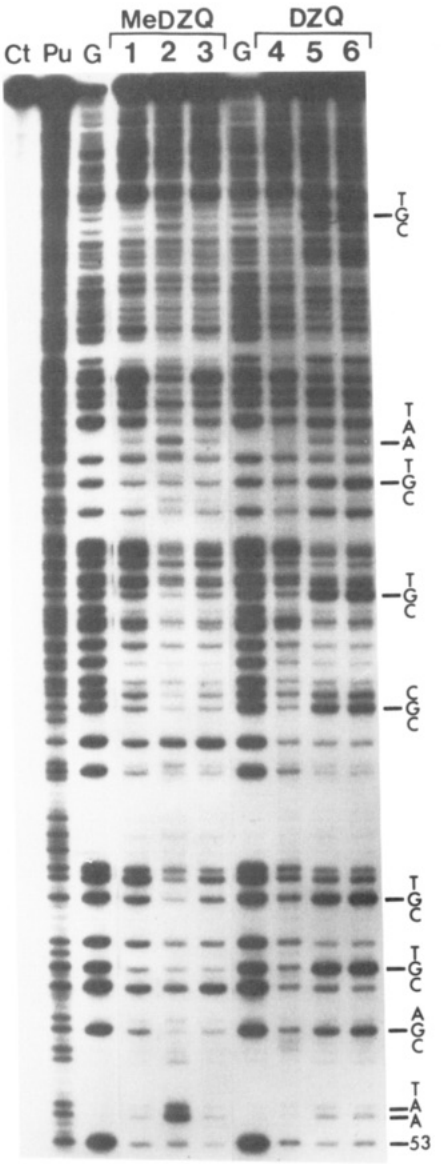

FIGURE 7: Autoradiogram of a 6% denaturing polyacrylamide gel showing piperidine-induced DNA strand cleavage in a 375-bp fragment of pBR322 DNA caused by purine alkylation by either MeDZQ (lanes 1–3) or DZQ (lanes 4–6). The reactions shown in lanes 1–3 were carried out in 10 mM potassium phosphate (pH 5.8, 25 °C), and in lanes 4–6 they were carried out in 25 mM TEA (pH 7.2, 37 °C) for 1 h: lane 1, 200 μM; lane 2, 100 μM with 0.173 μg of DTD; lane 3, 100 μM with 2 mM ascorbic acid; lane 4, 100 μM; lane 5, 10 μM with 0.173 μg of DTD; lane 6, 10 μM with 2 mM ascorbic acid. Lane Ct was the control; lane Pu was the purine-specific reaction; lane G was the guanine-specific reaction.

at pH 5.8 could be ranked in the following order: DZQ > MeDZQ $\gg$ D1 > D3 > D2 > D5 > D7 > D4 > D6. A similar trend was observed at pH 6.6 and 7.2 although the level of DNA ISC was markedly reduced (Figures 2 and 4).

Figure 6 shows the concentration dependence of DZQ and MeDZQ induced DNA ISC at pH 5.8. At this acidic pH, a significant level of DNA ISC could still be detected with 25 nM DZQ and MeDZQ. The ability of dicumarol to inhibit the formation of DNA ISC could be observed at these lower concentrations. As was observed in Figure 5, the level of DNA ISC formed after enzymatic reduction of 2.5 μM DZQ was apparently not inhibited by dicumarol (Figure 6). This may be due to the fact that DZQ upon reduction induces more than 1 cross-link per fragment and that inhibition by dicumarol does occur but does not reduce the number of cross-links per fragment to less than 1. The increased electrophoretic mobility and decreased intensity of cross-linked DNA observed in DZQ (lanes 1 and 2) and MeDZQ (lane 1) may be due to DNA fragmentation resulting from the generation of reactive oxygen species (Gutierrez, 1989) and/or heat-labile adenine alkylation (see Figures 8 and 9).

The pattern of sequence-selective alkylation of DZQ and MeDZQ in a 375-bp *Eco*RI–*Bam*HI fragment of pBR322 DNA is shown in Figure 7. Piperidine-induced strand cleavage suggests that DZQ and MeDZQ preferentially alkylate guanines within DNA. Reduction of DZQ, by either DTD (lane 5) or 2 mM ascorbic acid (lane 6), results in an enhanced sequence selectivity of alkylation at 5′-GC-3′ sequences, with a preference for a thymine 5′ of the guanine. Although the intensity of alkylation is increased, a similar pattern of sequence selectivity is observed upon enzymatic reduction when the pH is lowered from pH 7.2 to 5.8 (data not shown). A similar enhancement in the intensity of alkylation of MeDZQ at 5′-GC-3′ sequences upon reduction by

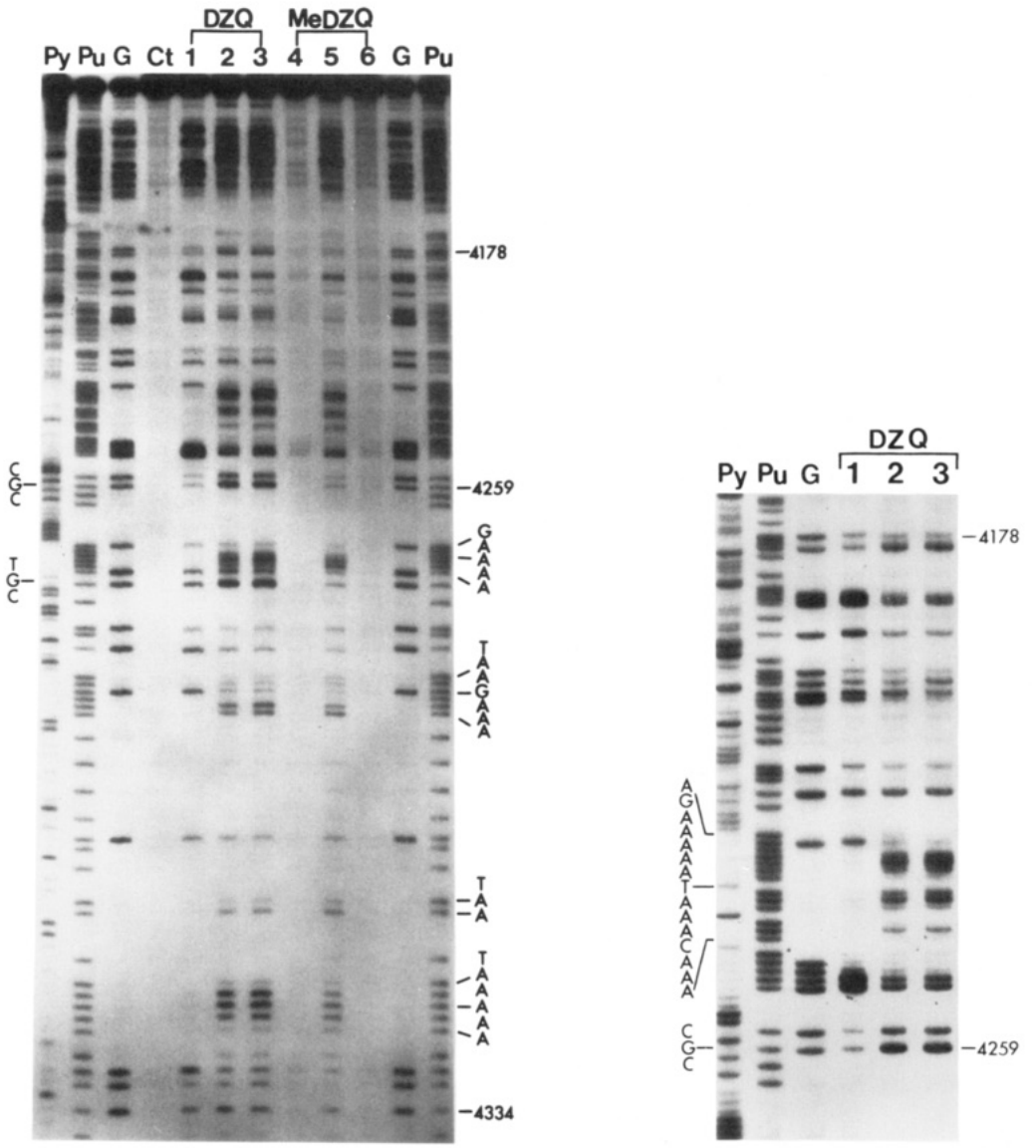

FIGURE 8: (Left) Autoradiogram of a 6% denaturing polyacrylamide gel showing piperidine-induced DNA strand cleavage in a 344-bp fragment of pBR322 DNA caused by purine alkylation by either DZQ (lanes 1–3) or MeDZQ (lanes 4–6). The reactions were carried out in 25 mM TEA (pH 7.2, 37 °C) for 1 h: lane 1, 100 μM; lane 2, 10 μM with 0.173 μg of DTD; lane 3, 10 μM with 2 mM ascorbic acid; lane 4, 200 μM; lane 5, 100 μM with 0.173 μg of DTD; lane 6, 100 μM with 2 mM ascorbic acid. Ct was the control lane; Py was the pyrimidine-specific reaction; Pu was the purine-specific reaction; lane G was the guanine-specific reaction. (Right) Expanded autoradiogram of the left panel (4178–4259) showing piperidine-induced DNA strand cleavage in a 344-bp fragment of pBR322 DNA caused by purine alkylation by DZQ: lane 1, 100 μM; lane 2, 10 μM with 0.173 μg of DTD; lane 3, 10 μM with 2 mM ascorbic acid.

DTD (lane 2) and ascorbic acid (lane 3) was not observed. It is important to note that ascorbic acid, as determined by spectrophotometric analysis, was unable to reduce MeDZQ, nor was it able to convert MeDZQ to a species which can cross-link DNA under the conditions employed in this study (data not shown). One interesting observation was that both DZQ and MeDZQ upon enzymatic reduction appeared to alkylate adenines in 5′-TAA-3′ sequences in this fragment (Figure 7).

To study the sequence dependence and the frequency of adenine alkylation in more detail, the pattern of alkylation in a 344-bp *Eco*RI–*Taq*I fragment of pBR322 DNA was also studied. This fragment was chosen because it had numerous runs of adenines and also contained one 5′-TGC-3′ and two 5′-CGC-3′ sequences. Alkylation of both of these sequences by DZQ, but not MeDZQ, was enhanced upon reduction (Figure 8, left panel), and this is consistent with the observations shown in Figure 7. New sites of alkylation, when DZQ and MeDZQ are reduced by DTD (lane 2 and 5) and when DZQ is reduced by ascorbic acid (lane 3), at 5′-(A/T)AA-3′ sequences were confirmed. The inability of ascorbic acid to reduce MeDZQ is evident from lane 6 (Figure 8, left panel), where no alkylation is observed. The following preferences at 5′-(A/T)AA-3′ sequences were observed. If a guanine or cytosine preceded the 5′ adenine, no alkylation at this site was then observed. Alkylation at the centrally located adenines in runs of adenines was more enhanced in comparison to the 5′ or 3′ flanking adenines (Figure 8). In addition to new alkylation sites at 5′-(A/T)AA-3′ sequences, another alkylation site at 5′-TA-3′ sequences was observed, but this constitutes a minor alkylation event. An expanded view of the upper part (4178–4259) of the left panel of Figure 8 is shown in the right panel. One interesting observation of the patterns of guanine alkylation observed within this fragment (Figure 8, right panel) was that the nonreduced quinone preferred the two guanines located at the 3′ end of 5′-$(G)_n$ sequences, where $n \geq 3$. A summary of the alterations in sequence selectivity of DZQ and MeDZQ after enzymatic reduction by DTD is shown in Table I. The degree of alkylation induced by DZQ at 5′-TGC-3′

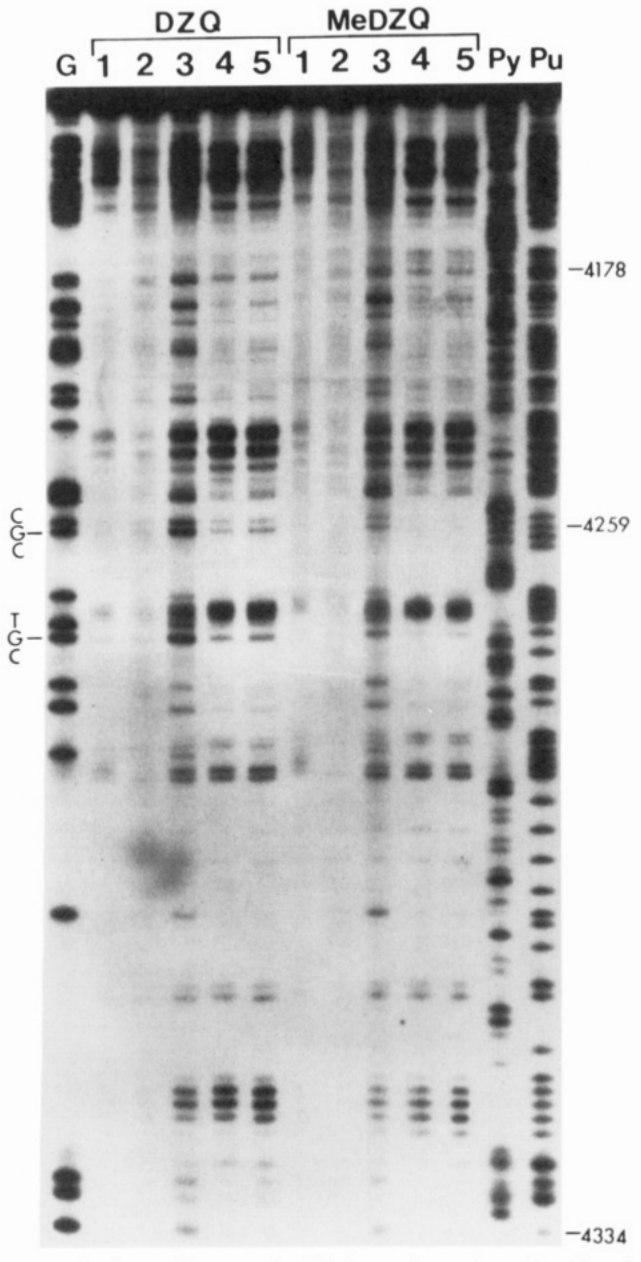

FIGURE 9: Autoradiogram of a 6% denaturing polyacrylamide gel showing the major groove alkylation and rapid depurination in 5′-(A/T)AA-3′ sequences in a 344-bp fragment of pBR322 DNA. DNA was treated with 10 μM DZQ or 100 μM MeDZQ with 0.173 μg of DTD in 25 mM TEA (pH 7.2, 37 °C) for 1 h in the absence (lanes 1, 3, 4, and 5) or presence (lane 2) or 10 mM Mg^{2+}. Drug-treated samples were heated at 90 °C for 20 min in 1 M piperidine (lanes 2 and 3) or 10 mM sodium hydroxide (lane 4) or 10 mM potassium phosphate, pH 7.2 (lane 5). Precipitated DNA was resuspended in tracking dye containing 80% formamide and 1 mM EDTA.

sequences was greater than 5′-CGC-3′ > 5′-AGC-3′ = 5′-GGC-3′ sequences.

To determine whether these new sites of adenine alkylation occurred in the major or minor groove of DNA, we investigated the pattern of alkylation in the presence of 10 mM Mg^{2+}. Mg^{2+} has been shown to decrease the level of alkylation within the major groove but not the minor groove of DNA (Kohn et al., 1987; Prakash et al., 1990). Figure 9 shows that the pattern of alkylation obtained with either MeDZQ or DZQ could be inhibited by 10 mM Mg^{2+} (lane 2). We also found that the presence of 10 mM Mg^{2+} inhibited the level of alkylation observed with the major groove alkylating agent nitrogen mustard but not the minor groove alkylating agent CC-1065 (data not shown). Piperidine treatment (lane 3), which can produce DNA strand cleavage at apurinic sites in addition to sites of guanine N7 alkylation (Mattes et al., 1986a), allowed the visualization of both guanine and adenine alkylation. Breaks at sites of adenines were detected, and to a lesser extent at guanines, when alkylated DNA was heated at 90 °C in 10 mM sodium hydroxide (pH 12.0) (lane 4). A similar pattern of cleavage was observed when alkylated DNA was heated at 90 °C at pH 7.2 (lane 5). In order to avoid the cleavage of apurinic sites by sodium hydroxide which is present in our alkaline loading dye, a neutral loading dye was utilized in this set of experiments.

DISCUSSION

The results presented within this paper show that purified rat hepatic DTD is capable of reducing a series of aziridinylbenzoquinones to species capable of cross-linking DNA. The induction of cross-links by these quinones is predominantly an enzymatic event with the level of cross-linking increasing as the pH of the incubation buffer is decreased from 7.8 to 5.8. Even under the most acidic conditions used here (pH 5.8), quinone-induced DNA ISC required the presence of DTD. Two compounds, DZQ and BZQ, were found to cross-link DNA without enzymatic reduction. The level of DNA ISC induced by DZQ, however, but not that by BZQ, was increased upon enzymatic reduction. This is consistent with the fact that DZQ is a substrate for DTD, whereas BZQ is not.

Enzymatic reduction and acid-catalyzed activation are thought to result in the formation of the hydroquinone species. The change in electron distribution observed when the nonaromatic quinone is reduced to the aromatic hydroquinone facilitates aziridine ring opening (Butler et al., 1988; Lusthof et al., 1989). Acid-assisted aziridine ring opening of the hydroquinone species would then be expected to enhance the levels of alkylation and DNA ISC (Lusthof et al., 1989). A general feature observed within this study was that the extent of DNA ISC was reduced as the sizes of the alkyl substituents at the C2 and C5 positions of the quinone ring were increased. Indeed, the ease of reduction of these quinones closely followed both their in vitro cytotoxicity and their ability to cross-link DNA (Pacheco et al., 1991).

The guanine N7 position is the most electronegative position within DNA (Pullman & Pullman, 1981), and the majority of alkylating agents which act via aziridine ring formation (i.e., the nitrogen mustards) react with runs of guanines with a preference for those centrally located within such sequences (Mattes et al., 1986b). Nitrogen mustards may form either DNA intra- or interstrand cross-links, and a requirement for 5′-GNC-3′ sequences in the formation of DNA ISC has recently been determined (Ojwang et al., 1989; Millard et al., 1990). Nonreduced DZQ and MeDZQ were found to react with DNA preferentially at runs of guanines, but their sequence selectivity was found to be altered upon enzymatic reduction. In the case of DZQ the parent quinone is capable of alkylating guanines within DNA, whereas the reaction of the reduced hydroquinone species with 5′-GC-3′ sequences is enhanced. Molecular modeling analysis has suggested that upon intercalation the two hydroxyls of the hydroquinone are in an excellent position to hydrogen bond with the cytosine O2 and C4-NH_2 groups (Hartley et al., 1991a). This hydrogen bonding, by fixing the hydroquinone in position, allows the reactive carbon of the aziridine ring to alkylate the nearby guanine N7 position. Substitution of the hydrogens at the C2 and C5 positions of the quinone ring with methyl groups abolishes this phenomena (i.e., compare the DZQ results with MeDZQ).

Table I: Analysis of the Altered Sequence Selectivity of DZQ and MeDZQ after Enzymatic Reduction[a]

	frequencies of alkylation	
	DZQ	MeDZQ
(A) Figure 7[b]		
highly enhanced sequences	4 5′-TGC	NO[c]
moderately enhanced sequences	2 5′-CGC	NO
	3 5′-AGC	NO
	2 5′-GGC	NO
alkylated new sequences	2 5′-TAA	2 5′-TAA
(B) Figure 8		
highly enhanced sequences	1 5′-TGC	NO
moderately enhanced sequences	2 5′-CGC	NO
alkylated new sequences	4 5′-TAA	4 5′-TAA
	11 5′-AAA	11 5′-AAA

[a] These data were obtained from the results shown in Figures 7 and 8. All the 5′-NGC sequences examined were enhanced by DZQ, not by MeDZQ, after enzymatic reduction. Underlined bases represent the enhanced or new alkylation site of DZQ and MeDZQ. Among the moderately enhanced sequences, the 5′-CGC sequence was the higher affinity site for alkylation than 5′-(A/G)GC sequences. [b] For accuracy, only the lower two-thirds of the gel shown in Figure 7 was analyzed. [c] NO, not observed.

Another interesting feature of the sequence-selective alkylation patterns was the observation that DTD reduction of both DZQ and MeDZQ resulted in a species which was capable of alkylating adenines. This result is consistent with the fact that when DZQ was reduced electrochemically the greatest degree of binding was observed with poly(dA)·poly(dT) (Lusthof et al., 1990). In contrast, BZQ, which is not a substrate for DTD, shows the greatest degree of binding with poly(dG)·poly(dC) (Butler et al., 1989). This new site of adenine alkylation occurred at runs of adenine although alkylation at the 5′ adenine was not observed when it was preceded by either a guanine or a cytosine. In order to gain additional mechanistic information regarding the site of adenine alkylation, DNA was incubated with either DZQ or MeDZQ in the presence of 10 mM Mg^{2+}. Previous work has suggested that magnesium can diminish the level of alkylation within the major groove but not within the minor groove of DNA (Kohn et al., 1987; Prakash et al., 1990). We found that the level of alkylation induced by DZQ and MeDZQ was inhibited by magnesium. Thus, it is assumed that the site of adenine alkylation is the adenine N7 position in the major groove of DNA. We cannot completely rule out the possibility that the adenine N3 site is being alkylated. We do observe that cleavage at sites of adenine alkylation continue to be detected when alkylated DNA was exposed to heat either at neutral or alkaline pH. This suggests that alkylation of adenine, irrespective of the site of alkylation, results in an adduct which rapidly depurinates. Piperidine can then break DNA at such apurinic sites (Mattes et al., 1986a). The mechanism responsible for this depurination event, however, is not clear at this time.

Several antitumor quinones are thought to require bioreductive activation in order to elicit their antitumor activity in vivo. We have previously implicated the two-electron reductase DTD as being an extremely important cellular enzyme involved in the activation of both AZQ and mitomycin C (Siegel et al., 1990a,b). In this study, we have clearly shown that purified rat hepatic DTD is capable of reducing a series of aziridinylbenzoquinones to species that are capable of alkylating and cross-linking DNA. Enzymatic reduction increases the level of DNA ISC and alters the pattern of sequence-selective alkylation, but these alterations are compound specific. In conclusion, our results strongly support the hypothesis that DTD is involved in the intracellular activation of antitumor quinones to DNA reactive and cytotoxic species.

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