0026-895X/92/030531-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:531-536

Relationship between DT-Diaphorase-Mediated Metabolism of a Series of Aziridinylbenzoquinones and DNA Damage and Cytotoxicity

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Received February 4, 1992; Accepted June 9, 1992

SUMMARY

A series of 2,5-bis-substituted 3,6-diaziridinyl-1,4-benzoquinones have been tested for their ability to be reduced by the two-electron NAD(P)H:(quinone acceptor) oxidoreductase [DT-diaphorase (DTD); EC 1.6.99.2]. Symmetrically alkyl-substituted carbamoyl ester analogs of 2,5-ethyl(carboethoxyamino)3,6-diaziridinyl-1,4-benzoquinone [AZQ], 3,6-diaziridinyl-1,4-benzoquinone (DZQ), and its 2,5-dimethyl derivative (MeDZQ) were tested. The rate of reduction by DTD was DZQ > MeDZQ > *n*-butyl- (D5) > *sec*-butyl- (D7) > *n*-propyl- (D3) > methyl- (D1) > ethyl- (AZQ) > *i*-butyl- (D6) > *i*-propyl- (D4) substituted derivatives. The hydroxyethylamino analog (BZQ) was not a substrate for DTD. The order of toxicity to HT-29 human colon carcinoma cells (at 1-log cell kill) was MeDZQ > DZQ > BZQ > D1 > D5 > AZQ > D7 > D3 > D6 > D4. Dicumarol, a known inhibitor of DTD, was capable of inhibiting the cytotoxicity of DZQ, MeDZQ, AZQ, D3, D4, D5, D6, and D7, with little inhibition of D1 cytotoxicity. Alkaline elution assays suggested that DZQ induced DNA strand breaks, whereas MeDZQ induced DNA interstrand cross-links in HT-29 cells. The formation of both classes of lesions was inhibited by dicumarol. DZQ and MeDZQ were 5–6-fold less cytotoxic to the DTD-deficient BE cell line, whereas BZQ was more cytotoxic to this cell line than the HT-29 cell line. BZQ was capable of inducing dicumarol-insensitive DNA interstrand cross-links in both cell lines. In summary, these data show a trend between the rate of reduction by DTD of an analog and its ability to induce cytotoxicity in HT-29 cells, and they support a role for DTD in the bioreductive activation of AZQ and its analogs.

Aziridinylbenzoquinones, such as diaziquone (AZQ), are thought to require enzymatic reduction in order to display antitumor activity (1). Enzymatic reduction of such quinones can take place via both one- and two-electron mechanisms (1, 2). 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 (1–4). These reactive oxygen species are then capable of fragmenting DNA and may be responsible, in part, for the cytotoxicity observed with quinones (5, 6). Two-electron reduction converts the parent quinone to its hydroquinone derivative and, in the case of antitumor quinones, generates a reactive intermediate that is capable of alkylating and cross-linking DNA (1–7). Previous work with AZQ has shown that it is the formation of DNA interstrand cross-links and not DNA strand breaks that is the most toxic lesion to cells (4, 5). Thus, the toxicity of antitumor quinones may be more dependant upon the levels of two-electron reductases than one-electron reductases.

We have previously suggested that the two-electron reductase NAD(P)H:(quinone acceptor) oxidoreductase (DTD) may be an important contributor to the cytotoxicity and DNA damage induced by AZQ in two human colon carcinoma cell lines (4). AZQ was found to induce DNA interstrand cross-links in the DTD-rich HT-29 cell line (4). This cell line was more sensitive to AZQ than was the DTD-deficient BE cell line. Extensive metabolism of AZQ was observed in HT-29 cytosol (4). This metabolism was NADH dependent and, in the same manner as observed for DNA interstrand cross-linking, could be inhibited by dicumarol. Purified DTD from HT-29 cells was also found to metabolize AZQ (4). The levels of DTD in tumor cells may,

This work was supported by National Institutes of Health Grant CA-51210 to D.R. and N.W.G., by NATO Grant 0143/89 to N.W.G. and J.A.H., and by a Cancer Research Campaign (Great Britain) grant to J.B.

ABBREVIATIONS: AZQ, 2,5-ethyl(carboethoxyamino)3,6-diaziridinyl-1,4-benzoquinone; DZQ, 3,6-diaziridinyl-1,4-benzoquinone; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone; BZQ, 2,5-dihydroxyethylamino-3,6-diaziridinyl-1,4-benzoquinone; D1–D7, substituted 2,5bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone analogs, where D1 = methyl, D2 = ethyl (AZQ), D3 = *n*-propyl, D4 = *i*-propyl, D5 = *n*-butyl, D6 = *i*-butyl, and D7 = *sec*-butyl; DTD, DT-diaphorase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

thus, determine the clinical outcome in patients treated with AZQ. Unfortunately, AZQ is a relatively poor substrate for DTD, and the impetus for the present work was to identify compounds that may be better substrates and, thus, have a greater potential for antitumor activity in tumors with DTD activity.

In the present study, we have utilized purified rat hepatic DTD to determine the substrate activity of a series of substituted aziridinylbenzoquinones. We have studied the cytotoxicity and DNA damage induced by these quinones in two human colon carcinoma cell lines, which differ markedly in DTD activity. Our results suggest that DTD is an important enzyme in terms of activating aziridinylbenzoquinones to DNA-reactive and cytotoxic species.

Materials and Methods

Chemicals and reagents. AZQ was supplied from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. All other quinones were synthesized according to published methods (8–10). NADH (grade IV) and dicumarol were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of at least analytical grade.

Cell lines. HT-29 and BE human colon carcinoma cells were maintained by growth at 37°, in monolayers, in Eagle's minimum essential medium supplemented with 10% calf serum, 0.05 mg/ml gentamicin, 0.03 mg/ml glutamine, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and 0.02 M HEPES.

Drug treatment. All quinones and dicumarol were dissolved in sterile dimethylsulfoxide immediately before treatment with cell cultures. Cells were pretreated with dicumarol (20 μM), 30 min before the addition of quinone, and dicumarol was present during the quinone treatment. Control cultures were treated with dimethylsulfoxide alone, with the concentration never exceeding 2% (v/v). Drug treatment of cell cultures was performed by removing medium, washing the cells with Hanks' balanced salt solution, and replacing the medium with 0.02 M HEPES-buffered Hanks' balanced salt solution (pH 7.2). In all assays, cells were exposed to the quinone for 2 hr at 37°, and the treatments were terminated by aspiration of the drug-containing buffer and replacement with fresh medium.

In vitro cytotoxicity assays. Inhibition of the colony-forming ability of BE and HT-29 human colon carcinoma cells was assessed as described previously (4). Cells were exposed to each drug, either in the presence or in the absence of 20 μM dicumarol, for 2 hr at 37°, as described above.

Alkaline elution assays. Analysis of the DNA damage induced in human tumor cells by each quinone tested was performed by the technique of alkaline elution. Details of the experimental conditions used for these assays are identical to those previously described and will not be elaborated further (4, 11). In all experiments, internal standards were [^{14}C]thymidine-labeled L1210 cells irradiated with 6 Gy of ^{137}Cs γ-rays, in the cold. For all cross-linking assays, control and drug cells were also irradiated with 6 Gy. Drug treatments were identical to those described above.

Purification of rat hepatic DTD. Rat hepatic DTD was purified from uninduced rats according to the method of Hojeberg *et al.* (12), yielding material with a specific activity of 660 nmol/min/μg of protein. This material was supplied to us by Dr. P. Preusch (National Institutes of Health, Bethesda, MD).

Spectrophotometric analysis of quinone reduction. Spectral analysis of the aerobic and anaerobic reduction of each quinone by DTD was carried out using a Hewlett Packard HP8452 diode array spectrophotometer. Anaerobic reductions were performed using nitrogen-saturated buffers in sealed cuvets. Reactions were initiated by addition of quinone, using a Hamilton syringe, through a rubber septum. All reactions were carried out in a final volume of 1 or 2 ml of 25 mM Tris·HCl (pH 7.4) containing 0.2 mM NADH, with or without 1.73 μg of purified DTD, at 25°. The initial rate of reduction of each quinone by DTD was assessed by measuring the decrease in the absorption of NADH at the isobestic point of the quinone, as determined by the reaction of the quinone with sodium borohydride. Each quinone was added in either 10 or 20 μl of dimethylsulfoxide, and dimethylsulfoxide was present in all blanks. The nonenzymatic rate of NADH reduction of each quinone was negligible. Concentrations of quinone and NADH were determined based upon literature extinction coefficients, when available (1, 4, 13). For comparative purposes, the initial rates of reduction of all quinones are expressed relative to that of the D5 analog (19.1 nmol of NADH removed/min/μg of protein).

Results

Purified rat hepatic enzyme was utilized to examine the substrate activity of a series of substituted aziridinylbenzoquinones. Enzymatic reduction of each quinone, as measured by UV/visible spectrophotometry under aerobic conditions, required either NADH or NADPH and could be inhibited by dicumarol (a known inhibitor of DTD) and by prior boiling of the enzyme. The efficiency of the initial rates of reduction varied quite markedly, and for clarity the values expressed in Table 1 have been normalized to that for the D5 analog. In terms of the initial rate of reduction, the order was DZQ > MeDZQ > D5 > D7 > D3 > D1 > D2 (AZQ) > D6 > D4. BZQ was not a substrate for rat DTD. The nonenzymatic reduction of each quinone by NADH was negligible under the assay conditions used. The initial rates of reduction determined under anaerobic conditions were found to parallel closely the data obtained under aerobic conditions (Table 1).

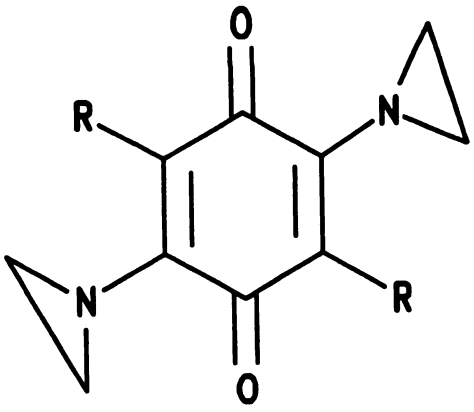
The importance of DTD to the cytotoxicity of a series of AZQ analogs was determined by using the DTD-rich human colon carcinoma HT-29 cell line. When ranked in terms of potency for 1-log cell kill, the order was MeDZQ > DZQ > BZQ > D1 > D5 > D2 > D7 > D3 > D6 > D4. When HT-29 cells were pretreated with 20 μM dicumarol, a greater concentration of each quinone was required to produce a 1-log cell kill (see Table 1). One exception was noted. The cytotoxicity of BZQ, which is not a substrate for DTD, to the HT-29 cell line was increased by dicumarol pretreatment.

Due to the high degree of cytotoxicity to the HT-29 cell line associated with DZQ, MeDZQ, and BZQ, we have studied these compounds in more detail, in both our cytotoxicity assays and our alkaline elution assays. Fig. 1 shows the ability of DZQ, MeDZQ, and BZQ to inhibit the colony-forming ability of the DTD-rich HT-29 and the DTD-deficient BE cell lines. DZQ and MeDZQ were 5–6-fold more cytotoxic to the HT-29 cell line than the BE cell line, a result consistent with the fact that they require activation by DTD. In addition, dicumarol protected HT-29 cells from the cytotoxicity induced by either DZQ (2.5-fold) or MeDZQ (3.26-fold). BZQ, which is not a substrate for DTD, was found to be 3–4-fold more cytotoxic to the DTD-deficient BE cell line than the HT-29 cell line. Dicumarol had no effect upon BZQ cytotoxicity to BE cells but actually potentiated the cytotoxicity observed against the HT-29 cell line. This is in direct contrast to the ability of dicumarol to protect HT-29 cells from the cytotoxicity of azridinylbenzoquinone analogs that are substrates for DTD.

In an attempt to understand the cytotoxicity observed, we have examined the ability of DZQ, MeDZQ, and BZQ to induce DNA damage in HT-29 and BE cells, by the technique of

TABLE 1

Relative rates of anaerobic and aerobic reduction by DTD of a series of quinone substrates and 90% inhibitory concentrations



R group	Abbreviation	Relative rate of reduction by DTD (normalized to D5)[a]		HT-29 IC_{90}[b]	Ratio of IC_{90} with 20 μM dicumarol to IC_{90}[c]
		Anaerobic	Aerobic		
				μM	
H	DZQ	Not done	10.73	0.1	2.50
CH_3	MeDZQ	Not done	1.82	0.075	3.26
$NHCH_2CH_2OH$	BZQ	Not a substrate		3.0	0.62
$NHCOOCH_3$	D1	0.30	0.32	4.6	1.13
$NHCOOC_2H_5$	D2 (AZQ)	0.14	0.25	7.6	1.33
$NHCOOC_3H_7$ (*n*-propyl)	D3	0.49	0.52	9.0	1.32
$NHCOOC_3H_7$ (*i*-propyl)	D4	0.04	0.02	22.0	1.32
$NHCOOC_4H_9$ (*n*-butyl)	D5	1.00	1.00	5.5	6.25
$NHCOOC_4H_9$ (*s*-butyl)	D6	0.12	0.07	19.5	1.35
$NHCOOC_4H_9$ (*i*-butyl)	D7	0.66	0.56	8.4	1.61

[a] Initial rates of reduction, as determined by NADH oxidation, normalized to the value for D5 (19.1 and 17.8 nmol of NADH removed/min/μg of protein for aerobic and anaerobic, respectively). For experimental details, see Materials and Methods.
[b] Concentration of each quinone required to produce a 1-log cell kill in HT-29 cells.
[c] Increase in concentration of each quinone that is required to produce a 1-log cell kill of HT-29 colon carcinoma cells in the presence of 20 μM dicumarol.

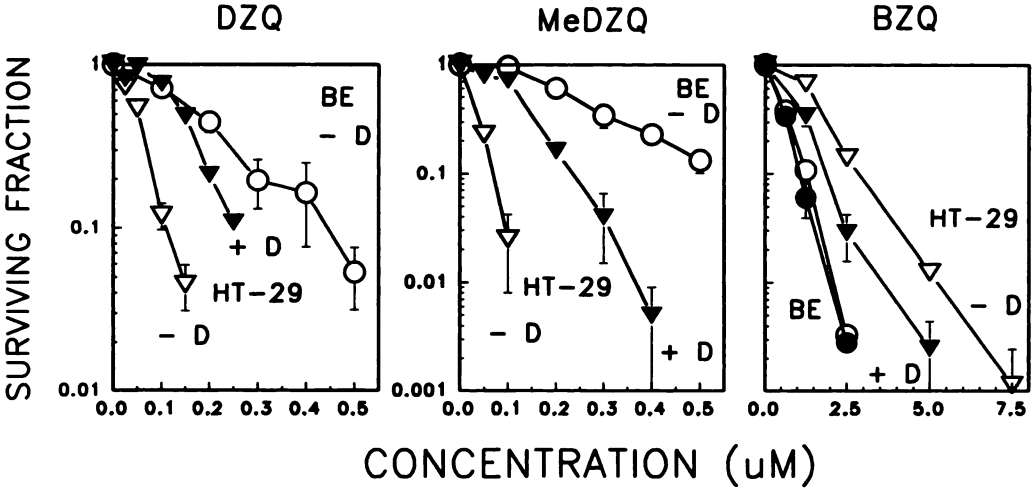


Fig. 1. Inhibition of colony-forming ability of HT-29 and BE human colon carcinoma cells exposed to DZQ (*left*), MeDZQ (*center*), and BZQ (*right*). Colony-forming ability was measured, as described in Materials and Methods, in HT-29 (*inverted triangles*) or BE (*circles*) cells exposed to quinone, either in the absence (*open symbols*) or in the presence (*closed symbols*) of 20 μM dicumarol (*D*). Results are mean ± standard deviation of triplicate determinations.

alkaline elution. Fig. 2 shows that DZQ was capable of inducing DNA strand breaks in HT-29 cells, in a concentration-dependent manner, and that MeDZQ was incapable of inducing DNA strand breaks. These assays were performed 4 hr after a 2-hr drug treatment, conditions previously used for the analysis of AZQ-induced DNA damage in HT-29 and BE cells. The formation of DZQ-induced strand breaks was inhibited by dicumarol pretreatment. No alteration in the single-strand break alkaline elution profiles was observed with MeDZQ after dicumarol pretreatment.

Fig. 3 shows alkaline elution assays designed to detect the presence of DNA interstrand cross-links, also at 4 hr after a 2-hr drug treatment. No evidence of DZQ-induced DNA interstrand cross-linking was detected. In fact, the increased rate of elution of the drug-treated cells, relative to the irradiated control cells, is probably due to the DNA strand breaks produced at the same time after drug treatment. The alkaline elution profiles obtained after dicumarol pretreatment, however, suggested that DZQ is capable of inducing DNA interstrand cross-links but that the ability to detect such lesions in these cells is masked by the presence of DNA strand breaks. In contrast to these results, MeDZQ was capable of inducing DNA

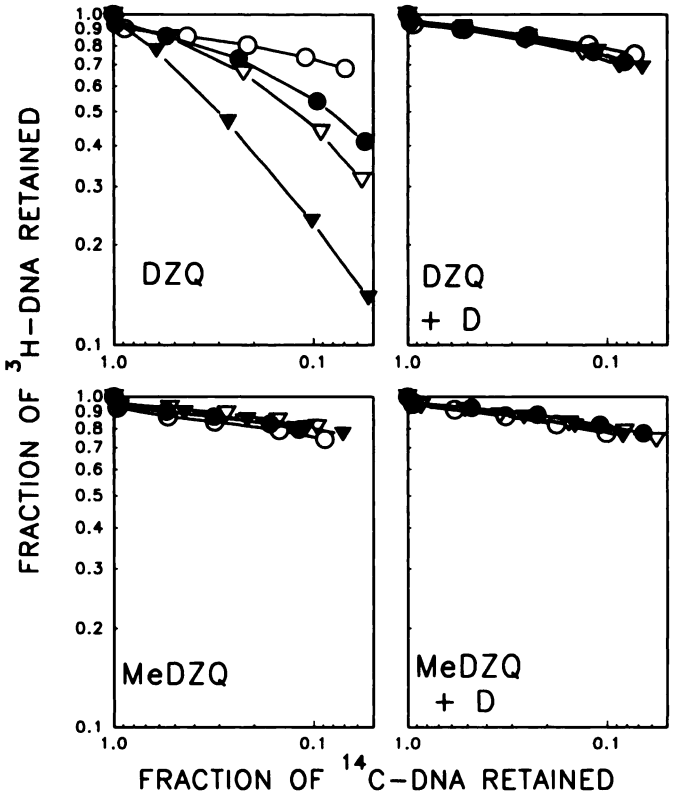


Fig. 2. Alkaline elution assays for DNA single-strand breaks in HT-29 human colon carcinoma cells exposed to either DZQ (*upper*) or MeDZQ (*lower*). DNA strand breaks were measured as described in Materials and Methods, in the presence (*right*) or absence (*left*) of 20 μM dicumarol (*D*). Drug treatments were as follows: dimethylsulfoxide controls (○) and 0.1 μM (●), 0.2 μM (▽), and 0.4 μM (▼) quinone. These profiles are representative of at least two independent experiments performed in duplicate.

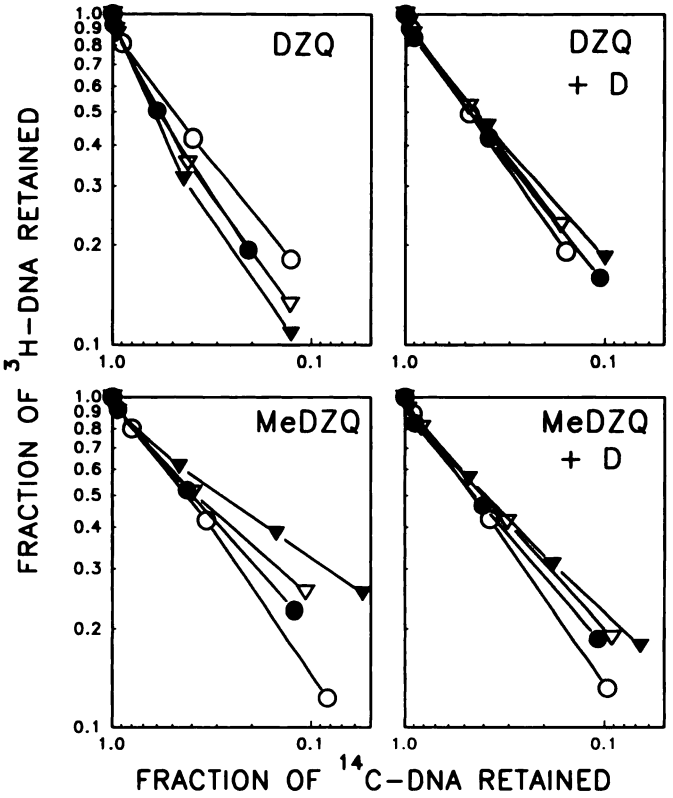


Fig. 3. Alkaline elution assays for DNA interstrand cross-linking in HT-29 human colon carcinoma cells exposed to either DZQ (*upper*) or MeDZQ (*lower*). DNA interstrand cross-links were measured as described in Materials and Methods, in the presence (*right*) or absence (*left*) of 20 μM dicumarol (*D*). Drug treatments were as follows: dimethylsulfoxide controls (○) and 0.1 μM (●), 0.2 μM (▽), and 0.4 μM (▼) quinone. These profiles are representative of at least two independent experiments performed in duplicate.

interstrand cross-links in HT-29 cells, and the formation of these lesions could be inhibited by pretreatment with dicumarol (Fig. 3).

Fig. 4 shows the ability of BZQ to induce DNA interstrand cross-links in a concentration-dependent manner. These assays were also performed 4 hr after a 2-hr drug treatment. Consistent with the cytotoxicity data, the level of DNA interstrand cross-links was found to be higher in the BE cells than the HT-29 cells.

Discussion

In this study, the rate of reduction, by purified rat hepatic DTD, of a structural series of AZQ analogs and their ability to induce cytotoxicity in HT-29 human colon carcinoma cells were examined. The HT-29 cell line contains high levels of DTD and is, therefore, a good model system to use for such studies. We have also used the BE human colon carcinoma cell line, which contains negligible DTD activity, for comparative purposes.

Our data clearly demonstrate that aziridinylbenzoquinones that are efficiently reduced by DTD, such as DZQ and MeDZQ, are potent at inducing cytotoxicity in HT-29 cells, whereas very poor substrates for DTD, such as the *i*-propyl (D4) and the *i*-butyl (D6) derivatives, are relatively ineffective at inducing toxicity in HT-29 cells. The remaining five substituted carbamoyl ester derivatives of AZQ examined (D1, D2, D3, D5, and D7) exhibited intermediate toxicity to HT-29 cells and, correspondingly, demonstrated intermediate rates of reduction by DTD. The ability of dicumarol to inhibit aziridinylbenzoquinone-induced toxicity in HT-29 cells and the greater cytotoxicity exhibited by these compounds for the DTD-rich HT-29 cell line, compared with the DTD-deficient BE cell line (4) (see above), support a role for DTD in bioreductive activation.

BZQ is not a substrate for DTD and can readily undergo acid-assisted aziridine ring opening without reduction (7). This compound, therefore, serves as an excellent control to examine events unrelated to DTD-mediated activation. BZQ was more toxic to the DTD-deficient BE cell line than to HT-29 cells and, interestingly, was more toxic to HT-29 cells than were any of the substituted carbamoyl ester derivatives of AZQ (D1-D7). In contrast to the results obtained with quinones that are substrates for DTD (see Table 1), dicumarol was found to potentiate the cytotoxicity of BZQ to HT-29 cells.

A closer examination of the relationship between rate of reduction of an analog by DTD and cytotoxicity to HT-29 cells demonstrates the lack of any quantitative correlation. This was particularly evident in the consideration of the group of five analogs that demonstrated intermediate toxicity to HT-29 cells. Structure-activity studies utilizing D1–D7 have been performed previously by using four cell lines with undefined and low reductive capacity, relative to the HT-29 cell line (9). These data showed a relationship in two cell lines, but not in two others, between the ease of reduction of the analogs (as defined

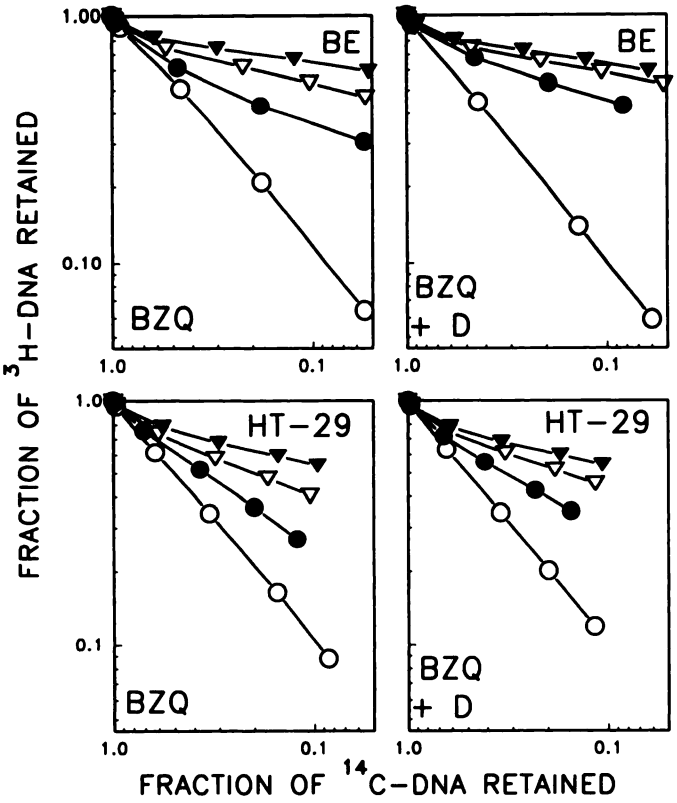


Fig. 4. Alkaline elution assays for DNA interstrand cross-linking in BE (*upper*) and HT-29 (*lower*) human colon carcinoma cells exposed to BZQ, either in the absence (*left*) or in the presence (*right*) of 20 μM dicumarol (*D*). Drug treatments were as follows: dimethylsulfoxide controls (○) and 2.5 μM (●), 5 μM (▽), and 7.5 μM (▼) BZQ. These profiles are representative of at least two independent experiments performed in duplicate.

by one-electron reduction potentials), and the resultant ability of semiquinone radicals to be formed in cells, and cytotoxicity. In HT-29 cells, however, our previous data have suggested a role in the toxic process for two-electron reduction of AZQ to its hydroquinone derivative (4). Because the hydroquinone form of AZQ is in equilibrium with the semiquinone (1), these data are not necessarily contradictory. Considering that the ability of an aziridinylbenzoquinone, once reduced, to exert a cytotoxic effect would be expected to depend on many factors, the lack of a facile relationship between the ease of reduction of an analog by DTD and cytotoxicity is, perhaps, not surprising. Variables such as the rate of aziridine ring opening, rate of reoxidation, diffusion to the site of damage, the type, site, and repair of DNA damage, and competing reactions with other nucleophiles could all modulate the cytotoxic process. It is also a possibility that different results would be obtained using purified HT-29 DTD, rather than rat hepatic DTD. Rat and human DTD differ in their ability to reduce the DNA cross-linking agent CB 1954 (14), so a difference in the ease of reduction of a particular analog that is dependent on the source of enzyme cannot be discounted. Despite these potential problems, our data show a clear trend between the ability to be reduced by purified rat hepatic DTD and cytotoxicity to the DTD-rich human HT-29 cells. These data suggest that examination of the ease of reduction by DTD is a viable approach in the design of aziridinylbenzoquinone analogs for use against tumors with high DTD activity.

The ability of AZQ analogs to be reduced by DTD could not be predicted on the basis of physical constants, such as one-electron reduction potential. One-electron reduction potentials are in the range of −57 mV to −260 mV and proceed in the order D1 > DZQ > D2 > D5 > D3 > D4 > D7 > D6 (9, 15). The one-electron reduction potential of BZQ is −376 mV (15), and a value for MeDZQ has not been reported. Two-electron potentials should follow the same trend as one-electron potentials (16) and, clearly, a simple relationship does not exist between reduction potential and rate of reduction by DTD. This is in agreement with earlier work, where it was reported that there was no simple correlation between the half-wave reduction potentials of quinone epoxides (17) and hydroxy-, methyl-, methoxy-, and glutathionyl-substituted naphthoquinone derivatives (18) and their rates of reduction by DTD. In addition, no correlation was found, in the latter study, between partition coefficient and rate of reduction by DTD. The reasons for this are unclear, and predictive determinants of the ability of compounds to serve as substrates for DTD remain to be established.

Using compounds that either were very good substrates for DTD (DZQ and MeDZQ) or were not substrates (BZQ), we have probed the role of DTD in induction of DNA damage and investigated the type of damage produced. A surprising finding was that DZQ induced DNA strand breaks in HT-29 cells. Previous work with AZQ (4) and MeDZQ (Fig. 2) showed that neither of these compounds could induce DNA strand breaks, when tested under identical conditions. It is important to emphasize that DZQ does form DNA interstrand cross-links but, because of methodological aspects of the alkaline elution assay, the quantity of DNA strand breaks formed masks their detection. For instance, pretreatment with dicumarol, which inhibits the formation of DNA strand breaks, is not sufficient to prevent the formation of DNA interstrand cross-links. This is consistent with the MeDZQ data, where dicumarol pretreatment inhibits but does not abolish the induction of DNA interstrand cross-links.

The mechanism of induction of DNA strand breaks by DZQ is presumably due to the reoxidation of the hydroquinone and the subsequent generation of reactive oxygen species (19). Interestingly, this process would be expected to be pH dependent, and we have shown previously with AZQ that reoxidation of the hydroquinone is more rapid at pH 7.8 than at pH 7.4 (4). Clearly, alkylation of DNA via the hydroquinone and aziridine ring opening is a competing reaction, with respect to autoxidation of the hydroquinone and induction of strand breaks. Consistent with this is the fact that AZQ is more toxic to cells under hypoxic conditions, which limit reoxidation, than under oxic conditions (19). With DZQ, but not with AZQ or MeDZQ, the rate of reoxidation is sufficiently rapid to overwhelm cellular antioxidant defenses in HT-29 cells and to result in DNA fragmentation. Cytotoxicity associated with reoxidation and DNA strand break induction does not appear to be as pronounced as that observed after the induction of DNA cross-links. MeDZQ, which cross-links but does break DNA, is more cytotoxic than DZQ, even though DZQ is a better substrate for DTD. This is in agreement with previous work by Szmigierio *et al.* (5), who showed that the formation of DNA interstrand cross-links was the major lesion in cells, with respect to AZQ-induced cytotoxicity, in four cell lines.

The implications of these data for drug design may well be significant. Clearly, in the case of bioreductive alkylating

agents, the nature and amount of enzymatic reducing systems in cells will have an influence on the cytotoxic effect. If one considers only DTD activity, our data show that rate of reduction of a series of aziridinylbenzoquinones by DTD is an important determinant of cytotoxicity but is by no means the sole determinant. The type of DNA damage induced is also critical. Consequently, the relationship between reoxidation of the hydroquinone generated by DTD, with resultant oxygen radical generation and DNA fragmentation, and rate of aziridine ring opening and subsequent alkylation and DNA cross-linking would be expected to be an important parameter. Because of the above, BZQ would be predicted to be a good candidate for tumors with low or no detectable reductase activity. It has been reported that certain colon tumors and non-small cell lung tumors have high DTD activity, relative to surrounding normal tissue (20, 21). Based upon our data, MeDZQ would be an excellent lead candidate for the design of new agents to be tested against such tumors.

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