DNA에 결합하는 항암제의 작용기전

이 종 순(영 남 대)

Mechanism of Action of Anticancer Drug Aziridinylbenzoquinones: Involvement of DT-diaphorase

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The abbreviations used are: DTD, DT-diaphorase or NAD(P)H:quinone oxidoreductase; DZQ, 3,6-diaziridinyl-1,4-benzoquinone; MeDZQ, 2,5-dimethyl 3,6-diaziridinyl-1,4-benzoquinone; LM-PCR, Ligation-mediated polymerase chain reaction; PGK1 gene, phosphoglycerate kinase-1 gene;

ABSTRACT

Aziridinylbenzoquinones such as 3,6-diaziridinyl-1,4-benzoquinone (DZQ) and its 2,5-methyl analog (MeDZQ) require bioreductive activation in order to elicit their anticancer activities. To determine the involvement of DTD in the activation of these drugs, we have used a ligation-mediated polymerase chain reaction to map the intracellular alkylation sites in a single copy gene at the nucleotide level. We have performed this analysis in two human colon carcinoma cells, one proficient (HT-29) and one deficient (BE) in DT-diaphorase (DTD) activity. In the DTD proficient HT-29 cell line, DZQ and MeDZQ were found to alkylate both 5'-(A/T)G(C)-3' and 5'-(A/T)A-3' sequences. This is consistent with the nucleotide preferences observed when DZQ and MeDZQ are activated by purified DTD to reactive metabolites capable of alkylating DNA *in vitro* [Lee, C.-S., Hartley, J. A., Berardini, M. D., Butler, J., Siegel., D., Ross, D., & Gibson, N. W. (1992) Biochemistry, 31: 3019-3025]. Surprisingly in the DTD-deficient BE cell line a pattern of alkylation induced by DZQ and MeDZQ similar to that observed in the DTD-proficient HT-29 cells was observed. This suggests that reductive enzymes other than DTD can be involved in activating DZQ and MeDZQ to DNA reactive species in vivo.

INTRODUCTION

A major two-electron reductase in biological system is DTD (1). This cytosolic flavoenzyme has been shown to play an important role in bioreductive activation of aziridinylbenzoquinones such as diaziquone and in particular DZQ and MeDZQ (Fig. 1) (2-6). In particular, DZQ and MeDZQ have been found to be more cytotoxic to the DTD proficient HT-29 human colon carcinoma cell line than to the DTD deficient BE human colon carcinoma cell line (7). This increased cytotoxicity to the HT-29 cell line has been hypothesized to be due to the ability of DTD to activate DZQ and MeDZQ to DNA reactive species intracellularly (7).

In vitro DZQ and MeDZQ reacted with guanines with a sequence selectivity similar to that of the nitrogen mustard class of antitumor agents (8). DTD-mediated reduction of DZQ and MeDZQ was capable of altering their sequence-selective alkylation *in vitro* (9). DZQ and MeDZQ reduced by purified rat hepatic DTD showed new sites of adenine alkylation in 5'-(A/T)<u>AA</u>-3' sequences, while only reduced DZQ showed enhanced guanine alkylation in 5'-<u>G</u>C-3' sequences (9). The nucleotide preferences for the formation of DNA interstrand cross-links by reduced DZQ and MeDZQ were found to be at 5'-<u>G</u>C-3' and 5'-<u>G</u>NC-3' sequences, respectively (10, 11).

In the present study, we have used a ligation mediated-polymerase chain reaction (LM-PCR) to map the genomic alkylation sites induced by DZQ and MeDZQ intracellularly in single copy

genes at the single nucleotide level (12-17). To dissect out the involvement of DTD in the intracellular activation of DZQ and MeDZQ, we have used a DTD proficient (HT-29) and a DTD deficient (BE) human colon carcinoma cell line (3). The results show that the pattern of alkylation sites observed in the DTD-proficient HT-29 cell line is predicted from *in vitro* studies (9). Surprisingly the pattern of alkylation sites observed in the DTD-deficient BE cell line was not consistent with that expected from the nonreduced quinone molecule. These results suggest that other reducing enzymes in addition to DTD may be involved in the intracellular activation of the aziridinylbenzoquinones, DZQ and MeDZQ, to DNA-reactive metabolites.

MATERIALS AND METHODS

Cell Culture.

HT-29 and BE human colon carcinoma cells were maintained by growing cells at 37 °C as monolayer as described previously (18).

Drug Treatment and Genomic DNA Preparation.

All compounds were dissolved in sterile dimethyl sulfoxide. Cells, at 80% of confluence, were exposed to 25 μM concentration of each drug and incubated for 4 h at 37 °C. Drug treatments were terminated by aspiration of the drug-containing media and cells were washed twice with phosphate buffered saline (PBS). Cells were lysed and genomic DNA was isolated as described previously (15, 16).

DNA Strand Cleavage at Alkylation Sites.

DNA was redissolved in 100 μL of freshly diluted 1 M piperidine solution and heated at 90 °C for 30 min to quantitatively convert the sites of alkylation into DNA strand breaks (9).

Sequencing Reaction of Human Genomic DNA.

Human genomic DNA was sequenced according to the Maxam-Gilbert protocol (15, 19, 20).

Ligation Mediated-PCR.

LM-PCR was performed in parallel on drug treated and chemically sequenced genomic DNA as decribed (19).

RESULTS

In order to determine whether DTD was involved in the intracellular activation of DZQ and MeDZQ, their alkylation sites were investigated at single nucleotide level in human colon carcinoma cells which differ markedly in DTD activity. After cells were treated with drug, genomic DNA was purified and alkylation sites were cleaved by hot 1 M piperidine treatment. Since piperidine treatment produces a 5' phosphate at the drug alkylation sites, the strand cleaved DNA can serve as a substrate for a subsequent ligation reaction with the oligonucleotide linker. Ligated genomic DNA was then amplified by PCR and an end-labeled gene-specific third primer was used to visualize the LM-PCR product. The end-labeled LM-PCR product was electrophoresed on a sequencing gel in parallel with the end-labeled LM-PCR product of Maxam-Gilbert genomic sequencing reactions.

Fig. 2 shows an autoradiogram of a sequencing gel that shows the alkylation sites of DZQ and MeDZQ in exon 9 of the p53 gene within HT-29 (HT) and BE (BE) cells. The continuity and uniformity of the purine-pyrimidine sequence ladders suggest that sequence bias was not introduced by the LM-PCR technique. Thus, the bands observed in drug treated lanes indicate the drug alkylation sites at specific nucleotide sequences and increased band intensity reflects an increased frequency of alkylation. Surprisingly, a similar pattern was observed whether the alkylation sites were mapped in the DTD-proficient HT-29 or the

DTD-deficient BE cell line (compare lanes DZQ and MeDZQ). The only difference we observed between two cell lines was a slightly reduced reactivity of DZQ at sites 16 and 17 in BE cells (Fig. 2). DZQ and MeDZQ were found to preferentially alkylate the guanine in 5'-(A/T)G(C)-3' and the adenine in 5'-(A/T)A-3' sequences. When experiment was performed with cells incubated with only Dimethyl sulfoxide, no DNA damage was detected (data not shown). Presentation of the data shown in Fig. 2 as either high or low affinity sites of alkylation is shown in Fig. 3. In this manner the nucleotide sequence surrounding the sites of alkylation can be clearly delineated and are as described above.

To determine whether this pattern of alkylation was unique to exon 9 of the p53 gene or whether this represented a general trend, we have mapped the alkylation sites induced by DZQ and MeDZQ in the PGK1 gene. Fig. 4 shows an autoradiogram of a sequencing gel that shows the alkylation sites of DZQ (D) and MeDZQ (M) in the PGK1 gene in the BE cell. DZQ and MeDZQ were also found to preferentially alkylate guanine and adenine at specific sequences. This preference for DZQ and MeDZQ alkylation of specific nucleotide sequences in the BE cells was identical to that observed in HT-29 cells (data not shown). The data observed within the PGK1 gene is also expressed as either high or low affinity sites of alkylation (Fig. 5).

Table 1 listed all the alkylation sites observed in exon 9 of the p53 gene and also in the PKG1 gene of both HT-29 and BE cells. This form of analysis suggests that a consensus sequence

for DZQ and MeDZQ alkylation occurs either in 5'-(A/T)G(C)-3' or in 5'-(A/T)A-3' sequences. This was true whether their alkylation sites were mapped in DTD-proficient HT-29 or DTD-deficient BE cell lines.

DISCUSSION

Aziridinylbenzoquinones have been shown to be substrates for metabolism by DTD, and an increase in drug-induced DNA interstrand cross-linking and cytotoxicity has been observed in DTD-proficient cell lines when compared to DTD-deficient cell lines (2-7, 9-11). A general trend exists between the ease of reduction of the quinone and the cytotoxicity observed. DZQ and MeDZQ are the most potent aziridinylbenzoquinones toward the DTD-proficient HT-29 cell line. The initial rate of reduction of DZQ is greater than that for MeDZQ yet MeDZQ is more cytotoxic to HT-29 cells (7).

A major difference in the fate of the reduced metabolites that may help explain the observed differences in cytotoxicity between DZQ and MeDZQ is the manner in which they alkylate DNA. DZQ and MeDZQ react with guanines, as measured by Maxam and Gilbert sequencing (20), with a sequence selectivity similar to the nitrogen mustard class of antitumor agents (8). Enzymatic reduction of DZQ and MeDZQ by DTD, however, is found to alter their sequence selective alkylation (9). Reduced DZQ shows enhanced guanine alkylation in 5'-<u>G</u>C-3' sequences and new sites of adenine alkylation in 5'-(A/T)<u>A</u>A-3' sequences. Reduced MeDZQ shows only new sites of adenine alkylation at 5'-(A/T)<u>A</u>A-3' sequences but no enhancement of guanine alkylation (9). This work has been performed *in vitro* and the relevance of such studies to the cellular scenario is not clear.

In this study, we have investigated the nucleotide

preferences for alkylation in genomic DNA of two specific aziridinylbenzoquinones, DZQ and MeDZQ. Thus, we have been able to directly compare the ability of each drug to alkylate DNA both *in vitro* and *in vivo* and as a result we have been able to probe the role that DTD may play in the bioreductive activation of such quinones to DNA reactive and cytotoxic species intracellularly.

Our results show that in the DTD-proficient HT-29 cells DZQ and MeDZQ induced alkylation sites are as predicted from *in vitro* studies. In contrast, the data obtained in the DTD-deficient BE cells

Recent attempts to determine the sequence selectivity of DNA alkylating agents in human cells have been successful but have been limited to the study of alphoid DNA (21-25). Recently, the introduction of a LM-PCR method has allowed the amplification and detection of UV-induced DNA damage in a single copy gene at the single-nucleotide level (26-29). are not as expected based on our *in vitro* analysis. Both DZQ and MeDZQ alkylate DNA within these DTD-deficient cells in a manner which resembles bioreductive activation by DTD and not that expected from the parent quinone. Given that the BE cells contain a mutation in the *DTD* gene (30) and that there is no DTD enzymatic activity (3), another mechanism must be responsible for the data obtained. Although cytochrome P-450 reductase and xanthine dehydrogenase are known to participate in the reduction of numerous quinones; such as mitomycin C, indoloquinone EO9, and dinitrophenylaziridine CB 1954 (31-37), it is not known whether

those enzymes can activate DZQ and MeDZQ. This is considered likely, however, becuse DZQ is shown to be metabolized by xanthine oxidase (38); thus the data obtained in the BE cell line may reflect the pattern of alkylation of either a hydroquinone or semiquinone species. At this time it is not known whether semiquinones are capable of preferentially alkylating DNA at specific nucleotide sequences.

In conclusion, it is apparent from this work that great care must be taken when extrapolating data obtained *in vitro* to the intracellular scenario. One cannot assume that because a particular compound is a substrate for a particular enzyme that this is the mechanism by which this agent is activated to DNA reactive and cytotoxic species within cells. Mapping of drug-induced alkylation sites by LM-PCR provides a method to directly determine the DNA binding potential and sequence selectivity of antitumor drug within target cells.

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Table 1. *Summary of the DNA sequence selectivity of DZQ and MeDZQ observed within the human p53 (exon 9) and PGK1 genes*[a]

Sequence	Site in Region[b] Analyzed	Sequence	Site in Region Analyzed
DZQ: Guanine Alkylation		**MeDZQ: Guanine Alkylation**	
GTGCA	site 2 in PGK gene	TTGTC	site 7 in PGK gene
GGGTG	site 1 in p53 gene	GGGTG	site 1 in p53 gene
GTGCA	site 2 in p53 gene	GTGCA	site 2 in p53 gene
ATGCC	site 4 in p53 gene	ATGCC	site 4 in p53 gene
TTGCC	site 9 in p53 gene	TTGCC	site 9 in p53 gene
TAGCA	site 11 in p53 gene	TAGCA	site 11 in P53 gene
CTGCC	site 12 in p53 gene	CTGCC	site 12 in p53 gene
CAGCC	site 15 in p53 gene	CAGCC	site 15 in p53 gene
(A/T)G(C)		**(A/T)G(C)**	
DZQ: Adenine Alkylation		**MeDZQ: Adenine Alkylation**	
TAACG	site 4 in PGK gene	AAACG	site 1 in PGK gene
TAACG	site 6 in PGK gene	TTACC	site 3 in PGK gene
AAAGC	site 9 in PGK gene	TAACG	site 4 in PGK gene
ATACA	site 10 in PGK gene	TTAAC	site 5 in PGK gene
TTATC	site 7 in p53 gene	TAACG	site 6 in PGK gene
CAACA	site 13 in p53 gene	TTATA	site 8 in PGK gene
CAACA	site 14 in p53 gene	TTATG	site 3 in p53 gene
CAAAG	site 16 in p53 gene	AGATT	site 5 in p53 gene
AAAGA	site 17 in p53 gene	TCACT	site 6 in p53 gene
GAAAC	site 18 in p53 gene	TTATC	site 7 in p53 gene
AAACC	site 19 in p53 gene	TCACC	site 8 in p53 gene
(A/T)A		CTAGC	site 10 in p53 gene
		CAACA	site 13 in p53 gene
		CAACA	site 14 in p53 gene
		CAAAG	site 16 in P53 gene
		AAAGA	site 17 in p53 gene
		AAACC	site 19 in p53 gene
		(A/T)A	

[a]The sequences are written 5' to 3'. [b]Sites in region analyzed are from Figs. 2 and 3 (*p53* gene), and Figs. 4 and 5 (*PGK1* gene). The underlined base indicates the site of alkylation.

FIGURE LEGENDS

Fig. 1. Structures of DZQ and MeDZQ.

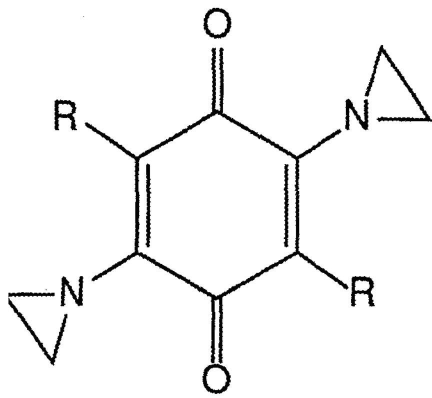
Fig. 2. Autoradiogram of an 8% denaturing polyacrylamide gel showing alkylation sites of DZQ and MeDZQ in exon 9 of the *p53* gene within HT-29 and BE colon carcinoma cell lines. HT-29 (HT) and BE cells (BE) were treated with 25 μM DZQ and MeDZQ and then genomic DNA was isolated. LM-PCR was performed and the products, obtained upon a subsequent primer extension reaction using an end-labeled gene specific primer to exon 9 of the *p53* gene, were electrophoresed to map the drug-induced alkylation sites. Pu, purine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction.

Fig. 3. Analysis of alkylation sites of DZQ (top) and MeDZQ (bottom) in the human *p53* gene. Double lines, high affinity sites of alkylation; single lines, low affinity sites of alkylation.

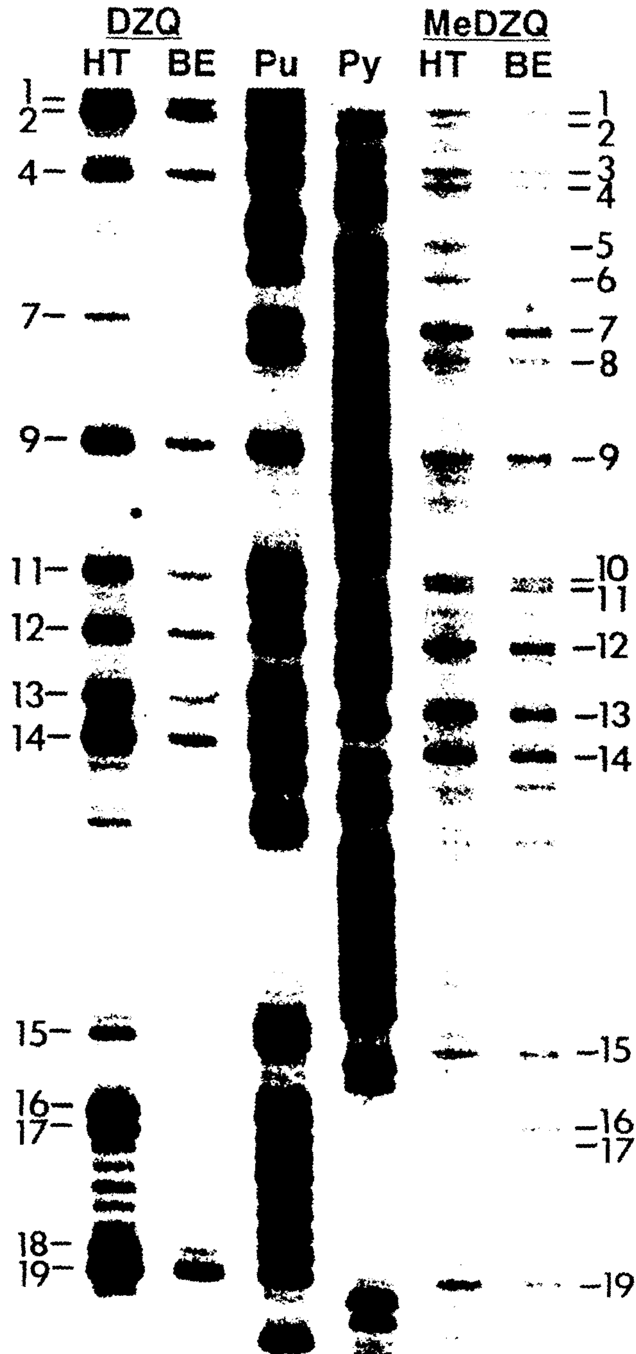
Fig. 4. Autoradiogram of an 8% denaturing polyacrylamide gel showing alkylation sites of DZQ and MeDZQ in the *PGK1* gene of BE cells. BE cells were treated with 25 μM DZQ (D) and MeDZQ (M) and then genomic DNA was isolated. LM-PCR was performed and the products, obtained upon a subsequent primer extension reaction using a *PGK1*

specific end-labeled primer, were electrophoresed to map the drug-induced alkylation sites. Pu, purine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction.

Fig. 5. Alkylation sites of DZQ (top) and MeDZQ (bottom) within the human *PGK1* gene. Double lines, high affinity sites of alkylation; single lines, low affinity sites of alkylation.



R	COMPOUND
H	DZQ
CH_3	MeDZQ



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     1  2        3  4        5     6      7   8           9            1011   12
CCAAGGGTGCAGTTATGCCTCAGATTCACTTTTATCACCTTTCCTTGCCTCTTTCCTAGCACTGC

 13  14                      15   1617     1819
CCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACT-3'
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    1  2         3         4                     56           7
       ‖                   |                      |
CAAACGTGCAGAATTACCTCATAACGACCCGCTTCCCTTTAACGTCCAGCTTGTCCAGCGTCAGC
    |            ‖         |                     ‖ ‖          |

   8      9                 10
          |                 |
TTGTTATAGAAAGCGACATTTTGGAAATACAGCT-3'
     |
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