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Excision Repair of 2,5-Diaziridinyl-1,4-Benzoquinone (DZQ)-DNA Adduct by Bacterial and Mammalian 3-Methyladenine-DNA Glycosylases

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The mechanisms of anticancer activity of 2,5-diaziridinyl-1,4-benzoquinone (DZQ) are believed to involve the alkylation of guanine and adenine bases. In this study, it has been investigated whether bacterial and mammalian 3-methyladenine–DNA glycosylases are able to excise DZQ-DNA adduct with a differential substrate specificity. DZQ-induced DNA adduct was first formed in the radiolabeled restriction enzyme DNA fragment, and excision of the DNA adduct was analyzed following treatment with homogeneous 3-methyladenine–DNA glycosylase from *E. coli*, rat, and human, respectively. Abasic sites generated by DNA glycosylases were cleaved by the associated lyase activity of the *E. coli* formamidopyrimidine–DNA glycosylase. Resolution of cleaved DNA on a sequencing gel with Maxam-Gilbert sequencing reactions showed that DZQ-induced adenine and guanine adducts were very good substrates for bacterial and mammalian enzymes. The *E. coli* enzyme excises DZQ-induced adenine and guanine adducts with similar efficiency. The rat and human enzymes, however, excise the adenine adduct more efficiently than the guanine adduct. These results suggest that the 3-methyladenine–DNA glycosylases from different origins have differential substrate specificity to release DZQ-DNA lesions. The use of 3-methyladenine–DNA glycosylase incision analysis could possibly be applied to quantify a variety of DNA adducts at the nucleotide level.

Keywords: Anticancer Agent; Aziridinylbenzoquinone; Base Excision Repair; Drug-DNA Adduct; 3-Methyladenine–DNA Glycosylase.

Introduction

The anticancer agent 2,5-diaziridinyl-1,4-benzoquinone (DZQ) is a diaziridinyl quinone compound (Fig. 1). The aziridinyl groups are responsible for the bifunctional alkylation of DNA, while the quinone moeity places DZQ in the class of bioreductive alkylators by virtue of its ability to undergo bioreductive activation. The alkylating activity of DZQ is enhanced by a reducing environment since the aziridine ring in the resulting hydroquinone is readily protonated, thereby facilitating ring opening (Berardini *et al*., 1993; Gibson *et al*., 1992; Haworth *et al*., 1993). Opening of the aziridinyl ring permits N7 guanine alkylation with sequence selectivity similar to nitrogen mustard (Hartley *et al*., 1991). Reduced DZQ by either DT-diaphorase or ascorbic acid showed new sites of adenine alkylation in 5′-(A/T)AA sequences and enhanced guanine alkylation in 5′-GC sequences (Lee *et al*., 1992). Identical sequence-selective alkylation was observed in the p53 and PGK1 genes of the HT-29 colon carcinoma cell line, as measured by ligation-mediated PCR (Lee *et al*., 1994). The preferred nucleotide sequences for the formation of DNA interstrand cross-links via two N7 guanine alkylation were 5′-GC and 5′-GNC sequences (Berardini *et al*., 1993; Haworth *et al*., 1993).

3-Methyladenine (3-meAde)–DNA glycosylase is one of the base excision repair enzymes which remove a wide range of DNA lesions by hydrolysis of the glycosylic bond (Seeberg *et al*., 1995; Wyatt *et al*., 1999). The resulting abasic sugar is incised by apurinic/apyrimidinic (AP) endonucleases and then restored to the original undamaged DNA. There are two 3-meAde–

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Abbreviations: AP site, apurinic/apyrimidinic site; DZQ, 2,5-diaziridinyl-1,4-benzoquinone; Fpg protein, formamidopyrimidine-DNA glycosylase; 3-meA, 3-methyladenine; 3-meG, 3-methylguanine; 7-meG, 7-methylguanine.

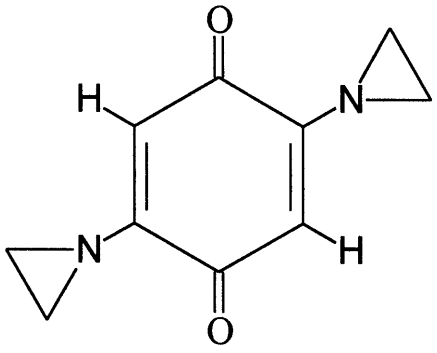

Fig. 1. The structure of DZQ.

DNA glycosylases in *Escherichia coli*. The constitutively expressed Tag specifically removes 3-meA, whereas the alkylation-inducible AlkA removes 3-meA, 7-meG, and a variety of other adducts (Singer and Hang, 1997; Wyatt *et al*., 1999). The coding sequences for 3-meAde–DNA glycosylase proteins from different eucaryotic organisms, including human cells, have been cloned, and the corresponding proteins were purified to homogeneity (Berdal *et al*., 1990; Chakravarti *et al*., 1991; Chen *et al*., 1989; Engelward *et al*., 1993; Memisoglu and Samson, 1996; O'Connor, 1993; O'Connor and Laval, 1990; 1991; Roy *et al*., 1994; Samson *et al*., 1991; Santerre and Britt, 1994). The human gene product was homologous to the amino acid sequence of a murine 3-meAde–DNA glycosylase (O'Connor and Laval, 1990). The mammalian enzymes have a very broad spectrum of substrate range including 3-meA, 7-meG, 3-meG, 3-ethyladenine, 7-ethylguanine, 8-hydroxyguanine, cyclic etheno adducts, 7-hydro-8-oxoguanine, and hypoxanthine (Bessho *et al*., 1993; Dosanjh *et al*., 1994; Singer and Hang, 1997; Wyatt *et al*., 1999). Eucaryotic 3-meAde–DNA glycosylases have also been shown to protect against the cell-killing effects of chemotherapeutic nitrosourea, nitrogen mustard, and mitomycin C (Allan *et al*., 1998; Engelward *et al*., 1996; Matijasivec *et al*., 1993; McHugh *et al*., 1999).

It has been previously found that purified rat and human 3-meAde–DNA glycosylases excise bulky adenine and guanine adducts damaged by nitrogen mustards less efficiently than the *E. coli* AlkA protein *in vitro* (Matijasivec *et al*., 1996; Mattes *et al*., 1996); however, purified rat and human 3-meAde–DNA glycosylases were not able to excise guanine adducts damaged by hepsulfam, while the *E. coli* AlkA protein completely excises them (Je *et al*., 1998). In this study, the substrate specificity of 3-meAde–DNA glycosylases from *E. coli*, rat, and human has been studied to excise DNA lesions induced by DZQ. Excision of drug-modified bases was monitored by DNA sequencing gel analysis following treatment of the DNA adduct with 3-meAde–DNA glycosylase and the subsequent formamidopyrimidine–DNA glycosylase (Fpg protein). The result showed that bacterial and mammalian 3-meAde–DNA glycosylases exhibited a difference in substrate recognition.

Materials and Methods

Chemicals and reagents A Klenow fragment of DNA polymerase I, pBR322 DNA, and restriction endonucleases were purchased from Promega. [α-^{32}P]dATP was purchased from Amersham.

Purification of 3-meAde–DNA glycosylases of *E. coli*, rat, and human The AlkA protein was purified to apparent homogeneity from *E. coli* JM105 cells harboring pALK10 expressing the *alkA* gene (O'Connor and Laval, 1990). Recombinant full-length human and rat 3-meAde–DNA glycosylase proteins were purified to apparent homogeneity (O'Connor, 1993; O'Connor and Laval, 1991). One unit of 3-meAde–DNA glycosylase activity is defined as 1 pmol of methylated base released per minute at 37°C from [^{3}H]dimethylsulfate-DNA. The Fpg protein was purified to apparent homogeneity and used as AP endonuclease to create strand breaks at AP sites generated by the 3-meAde–DNA glycosylase action (O'Connor and Laval, 1989). One unit of the Fpg protein is defined as 1 pmol of formamidopyridine in 5 min at 37°C. These enzymes were kindly provided by T. R. O'Connor.

Preparation of a restriction fragment of pBR322 DNA First pBR322 plasmid DNA was linearized with *Eco*RI and then it was radiolabeled at the 3′ end using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. DNA was digested with the second *Bam*HI to produce a singly end-labeled 375-bp fragment which was then isolated on an 8% nondenaturing polyacrylamide gel.

Drug modification of DNA Radiolabeled DNA was resuspended in 50 µl of 10 mM phosphate buffer, pH 6.0, and treated with the final concentration of 100 µM of DZQ at 37°C for 1 h. The reaction was terminated by ethanol precipitation of DNA.

Chemical-induced DNA strand cleavage at alkylation sites To determine the sites of N7-guanine alkylation, an aliquot of drug-modified DNA was heated in 44 µl of freshly diluted 1 M piperidine at 92°C for 20 min to convert quantitatively the N7 guanine adduct into DNA strand breaks (Mattes *et al*., 1986). To determine the sites of both N7 guanine and N3 adenine alkylations, an aliquot of drug-modified DNA was heated in 40 µl of 10 mM potassium phosphate buffer, pH 7.0 at 92°C for 20 min and then reheated in freshly prepared 44 µl of 1 M piperidine at 92°C for 20 min (Reynolds *et al*., 1985).

Excision assay of the drug-modified bases by 3-meAde–DNA glycosylases of *E. coli*, rat, and human To determine the base excision of the adduct by the AlkA protein, 5 µl (5 units) of freshly diluted enzyme was added to 45 µl of reaction buffer containing 70 mM Hepes, pH 7.6, 0.5 mM EDTA, 10 mM β-mercaptoethanol, and 5% glycerol. It was then incubated at 37°C for 15 min to release the DNA adduct. To induce DNA

strand cleavage at apurinic sites generated by the AlkA protein, 5 μl (0.5 units) of the Fpg protein and 5.5 μl of 1 M KCl were added and the reaction buffer was incubated at 37°C for an additional 15 min. For the base excision of adduct by rat and human 3-meAde–DNA glycosylases, 5 μl (5 units) of each enzyme and 5 μl (0.5 units) of the Fpg protein were added to 50 μl of reaction buffer containing 100 mM KCl, 70 mM Hepes, pH 7.6, 0.5 mM EDTA, 10 mM β-mercaptoethanol, and 5% glycerol. This was then incubated at 37°C for 15 min. All reactions were terminated by phenol/chloroform extraction and ethanol precipitation of DNA.

Sequencing gel analysis Precipitated DNA samples were resuspended in a tracking dye containing 80% formamide, 1 mM EDTA, and xylene cyanol, heated at 90°C for 2 min, chilled in an ice bath, and then subjected to sequencing electrophoresis in parallel with Maxam and Gilbert base-specific reactions (Maxam and Gilbert, 1980). Electrophoresis was achieved on 8% polyacrylamide gels containing 8 M urea at 30 W. The gels were transferred to filter paper, dried, and then autoradiographed.

Results and Discussion

In order to determine the ability of the purified *E. coli*, rat, and human 3-meAde–DNA glycosylases to release the DZQ-DNA adduct, a drug-modified DNA fragment was reacted with the 3-meAde–DNA glycosylase. The abasic sites generated by the DNA glycosylase were cleaved by the lyase activity of the Fpg protein and the resulting cleaved DNA was resolved on an 8% sequencing gel.

Figure 2 shows excision patterns of DZQ-DNA adduct by *E. coli,* rat, and human 3-meAde–DNA glycosylases with the additional Fpg protein. The site of N7 guanine alkylation was determined by hot 1 M piperidine treatment (lane P). The bands observed in this lane correspond to the guanine-specific Maxam–Gilbert sequencing reaction (G). Some additional faint bands corresponding to adenine were also detected, since apurinic sites are sensitive to strand cleavage by piperidine treatment (lane P) or the Fpg proteins (see lane 7). Both the N3 adenine and N7 guanine alkylation sites were determined by heat treatment at neutral pH and subsequent hot 1 M piperidine treatment (lane Δ + P). The enhanced additional bands observed in this lane when compared to those in lane P correspond to the alkylation sites of the N3 adenine (arrows) within the consensus sequence 5′-(A/T)AA.

The reactions of DNA samples with *E. coli*, rat, and human 3-meAde–DNA glycosylase proteins and the subsequent Fpg protein treatment (lanes 1, 3, and 5, respectively) show that all the adenine and guanine adducts were released by DNA glycosylase when compared to each 3-meAde–DNA glycosylase protein alone (lanes 2, 4, and 6, respectively). This result shows all the enzymes originating from *E. coli*, rat, and human are able to release adenine and guanine adducts.

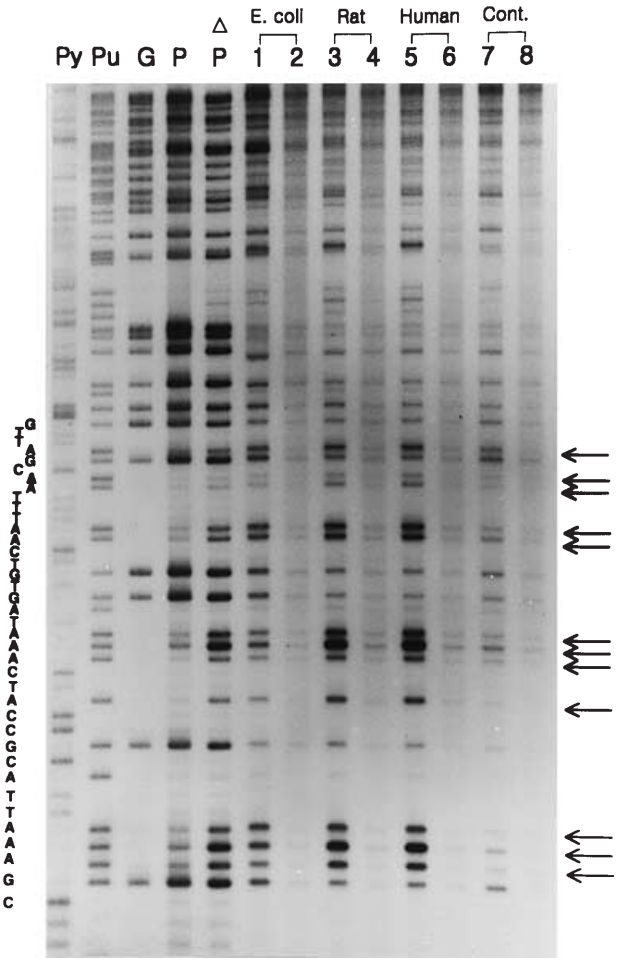

Fig. 2. Autoradiogram of an 8% sequencing gel showing that the N3 adenine and guanine adducts formed by DZQ are substrates for 3-meAde–DNA glycosylase originating from *E. coli* (lanes 1 and 2), rat (lanes 3 and 4), and human (lanes 5 and 6). A 3′-end-labeled 375-bp DNA fragment was modified with DZQ and then reacted with 3-meAde–DNA glycosylase and Fpg protein (lanes 1, 3, and 5), 3-meAde–DNA glycosylase alone (lanes 2, 4, and 6), or Fpg alone (lane 7). Lane P, hot 1 M piperidine treatment; lane Δ + P, heat treatment at neutral pH followed by hot 1 M piperidine treatment: lane 1, *E. coli* 3-meAde–DNA glycosylase and Fpg; lane 2, *E. coli* 3-meAde–DNA glycosylase; lane 3, rat 3-meAde–DNA glycosylase and Fpg; lane 4, rat 3-meAde–DNA glycosylase; lane 5, human 3-meAde–DNA glycosylase and Fpg; lane 6, human 3-meAde–DNA glycosylase; lane 7, Fpg; lane 8, no enzyme. Py, pyrimidine-specific reaction; Pu, purine-specific reaction; G, guanine-specific reaction. The arrows indicate the DZQ-modified adenine adduct.

The comparison of the excision efficiency of the mammalian enzymes with the *E. coli* enzyme is interesting in terms of the difference in substrate recognition. Mammalian enzymes (lanes 3 and 5) excise the adenine adduct (see arrows) with intensity equivalent to or slightly stronger than chemically induced DNA strand cleavage (lane Δ + P). However, the intensity of the bacterial enzyme excision (lane 1)

at the adenine adducts is much lower than chemically induced DNA strand cleavage. This difference was only observed at the adenine adduct, not at the guanine adduct. These results suggest that the mammalian enzymes are more efficient than the bacterial enzyme in excising DZQ-induced adenine adduct. This differential substrate specificity may be consistent with the fact that the human and rat sequences of cDNA are highly homologous but display only limited homology to the bacterial sequence (Chakravarti *et al*., 1991; O'Connor and Laval, 1991; Samson *et al*., 1991). Thus, it can be expected that mammalian and bacterial enzymes have different mechanisms to recognize the DNA adduct (Lau *et al*., 1998).

Another interesting observation is that the repair enzyme-mediated DNA cleavage assay of the DZQ-DNA adducts is as sensitive as the heat-induced DNA strand cleavage assay. This observation may imply a possibility that a 3-meAde–DNA glycosylase incision method can be applied to identify the formation and repair of a variety of DNA adducts at both nucleotide and gene levels. In fact, the method using *E. coli* UvrABC nuclease incision analysis has been successfully applied to quantify a variety of DNA adducts at the nucleotide level (Kohn *et al*., 1992; Li *et al*., 1995; Tang *et al*., 1992; 1994).

In summary, base excision repair enzyme 3-meAde–DNA glycosylases from different sources release the DZQ-induced adenine adduct with a differential substrate specificity. This may result from differences in the sequence homology of enzymes from different sources (Fridberg *et al*., 1995). Whether such a repair system could modulate the potency of cytotoxic drugs and thus play a role in cancer chemotherapy is an intriguing speculation.

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