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Mapping of Altromycin B-DNA Adduct at Nucleotide Resolution in the Human Genomic DNA by Ligation-mediated PCR

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The ligation-mediated PCR was used to map DNA alkylation sites induced by altromycin B at nucleotide resolution in genomic DNA purified from cultured human colon carcinoma. Altromycin B, one of the pluramycin group of antitumor antibiotics, is characterized as intercalator with the added ability to alkylate N7 guanine. DNA adducts formed in genomic DNA were cleaved into DNA strand breaks by hot piperidine treatment, and fragments containing ligatable breaks were then amplified in a single-sided, ligation-mediated PCR. The alkylation sites observed in exon 9 of the p53 gene revealed that the most high reactivity sites for altromycin B were found to be N7 of guanine in a 5′-AG* sequence. Determination of the DNA alkylation sites in naked radiolabeled plasmid DNA also showed that altromycin B preferred N7 of guanine in a 5′-AG* sequence. Thus, it can be concluded that the sequence selective DNA adduct formation induced by the intercalating alkylator, altromycin B, in genomic DNA is similar to that observed in naked plasmid DNA.

Keywords: Altromycin B; Anticancer Agent; DNA Ligation-mediated PCR; DNA-Drug Adduct; Sequence Selectivity.

Introduction

It is widely recognized that a number of anticancer agents elicit anticancer activity by interacting with DNA to form a specific adduct. The investigation of the type and the frequency of DNA adduct is one step towards understanding the action mechanism of anticancer activity. The formation of DNA adducts has been studied previously with naked radiolabeled plasmid DNA or oligonucleotides. However, the distribution of adducts along a human chromosome may be modulated by chromatin structure or nuclear DNA-binding proteins.

Recently, ligation-mediated (LM) PCR has been developed to detect specific DNA-strand breaks in the human genomic DNA at the nucleotide level (Mueller and Wold, 1989; Pfeifer *et al*., 1989). This method significantly increases the sensitivity and specificity of the original genomic sequencing methods. This has made possible its use for *in vivo* footprinting, genomic sequencing, studies on chromatin structure and analysis of DNA damages and repair at single-nucleotide resolution (Denissenko *et al.*, 1996; Mueller and Wold, 1989; Pfeifer *et al*., 1989; 1990; 1993; Tommasi *et al*., 1997).

DNA adducts induced by carcinogen or anticancer agents can be mapped if it is possible to convert them into DNA strand breaks with a 5′-phosphate group. Either chemical or enzymatic cleavage methods can be used. Initially, this technique was used for the detection of UV-induced DNA photoproducts, (6-4) photoproducts and cyclobutane pyrimidine dimer (Pfeifer, 1992; Pfeifer *et al*., 1991). DNA adducts induced by carcinogens such as benzo[a]pyrene, aflatoxin B1, and reactive oxygen species have been mapped in genomic DNA (Denissenko *et al*., 1999; Pfeifer *et al*., 1998; Rodriguez *et al*., 1996; Tang *et al*., 1999). Mapping of DNA adducts induced by anticancer agents, CC-1065 analogs and diaziridinylbenzoquinones, has been performed to explain the action mechanism of anticancer activities *in vivo* (Lee *et al*., 1994a; 1994b).

Altromycin B is a new member of the pluramycin group of antibiotics and is currently being developed at Abbott Laboratories, Chicago, IL (Fig. 1) (Brill *et al*., 1990; Jackson *et al*., 1990). Altromycin B first intercalates between base pairs in duplex DNA to insert the disaccharide in the minor groove and position the alkylating epoxide in the major groove to form altromycin B (N7-guanine)-DNA adduct. (Sun *et al*., 1993). The

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Abbreviations: LMPCR, ligation-mediated PCR.

sequence selective alkylation in the AG* sequence (*denotes the alkylation site) has been observed in oligomer duplex and naked plasmid pCAT DNA (Hansen and Hurley, 1995; Sun *et al*., 1995).

We have used the LMPCR technique in conjunction with chemically induced DNA strand cleavage to determine whether DNA adducts induced by altromycin B in naked plasmid DNA are also observed in genomic DNA. Mapping of DNA alkylation sites induced by altromycin B along the p53 gene of human colon carcinoma DNA demonstrates that DNA adduct formation in genomic DNA is sequence-dependent as observed in naked plasmid DNA.

Materials and Methods

Chemicals and reagents Altromycin B was obtained from Abbott Laboratories, Chicago, IL. T4 DNA ligase, *Taq* DNA polymerase, T7 DNA polymerase (Sequenase), Klenow fragment of DNA polymerase I, pBR322 DNA, and restriction endonucleases were purchased from Promega. [α-^{32}P]dATP was purchased from Amersham.

Cell line HT-29 colon carcinoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum in 25 cm^2 flask until cells reached approximately 80% confluence.

Drug treatment and genomic DNA isolation The drug was dissolved in sterile dimethyl sulfoxide. Cells were exposed to 0.5 ng/μl final concentration of altromycin B and incubated for 4 h at 37°C. Drug treatment was terminated by aspiration of the drug-containing media, and cells were washed twice with phosphate-buffered saline. Cells were lysed, and genomic DNA was isolated as described previously (Pfeifer *et al*., 1993).

DNA strand cleavage at drug alkylation sites Isolated human genomic DNA was resuspended in 50 μl of freshly diluted 1 M piperidine and heated at 92°C for 20 min to quantitatively convert the sites of alkylation into DNA strand cleavage (Je *et al*., 1998; Kohn *et al.*, 1987; Mattes *et al*., 1986). This DNA strand cleavage step produces a 5′-phosphate group at the drug alkylation sites which is required for T4 DNA ligase reaction in a subsequent LMPCR. The DNA was then precipitated in ethanol and lyophilized overnight to completely remove residual piperidine.

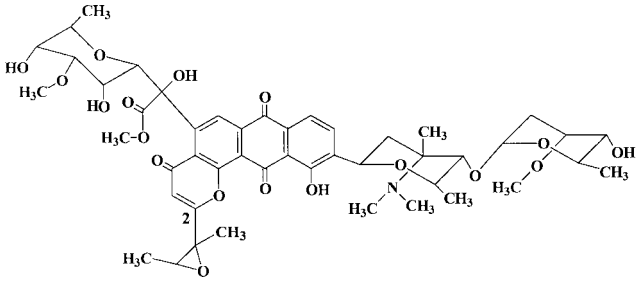

Fig. 1. Chemical structure of altromycin B.

Lyophilized DNA was resuspended in 20 μl of Tris-EDTA (pH 7.5).

Ligation-mediated PCR The LMPCR protocol used for this work can be divided into six steps: 1) DNA strand cleavage at the alkylation site, 2) primer extension of an annealed gene-specific oligonucleotide primer (primer 1), 3) ligation of a universal asymmetric double-stranded linker, 4) PCR amplification using a second gene-specific oligonucleotide (primer 2) and liker primer, 5) primer extension of a radiolabeled third gene-specific oligonucleotide (primer 3), and 6) separation of the DNA fragment on a sequencing gel.

The sequences of the oligonucleotide linker, consisting of a 25-mer annealed to an 11-mer oligonucleotide, and linker primer were as previously indicated (Mueller and Wold, 1989; Pfeifer and Riggs, 1993). LMPCR was performed as described previously (Lee *et al*., 1994a; Mueller and Wold, 1989; Pfeifer and Riggs, 1993).

DNA purified from colon carcinoma cells was treated with base-specific chemical cleaving agents (Maxam and Gilbert, 1980; Pfeifer and Riggs, 1993). Chemically cleaved sequencing samples were run along with the drug-treated sample through LMPCR and were included on the sequencing gels to provide base position markers.

Sequencing analysis The end-labeled PCR product was extracted with phenol/chloroform and precipitated in ethanol. DNA was resuspended in 20 μl of TE, pH 7.5 and 2 μl of DNA was loaded with 8 μl of tracking dye containing 80% formamide, 1 mM EDTA, and xylene cyanol on an 8% polyacrylamide sequencing gel. Gels were transferred to filter paper, dried and then autoradiographed. Drug alkylation sites were determined by using Maxam-Gilbert chemically produced markers as control.

Determination of alkylation sites in end-labeled pBR322 restriction fragment To prepare a 3′ end-labeled DNA fragment of pBR322 *Eco*RI-*Bam*HI (375 bp), plasmid pBR322 (5 mg) was first linearized with *Eco*RI and 3′ end-labeled with Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. After a second digestion with *Bam*HI, the resulting 375 bp fragment of pBR322 was isolated by preparative electrophoresis on an 8% nondenaturing polyacrylamide gel. The drug was incubated with DNA in 20 μl of 10 mM phosphate buffer, pH 7.0, at 37°C for 3 h. Reaction was terminated by ethanol precipitation. To determine alkylation sites, an aliquot of drug-modified DNA was resuspended in freshly diluted 1 M piperidine and heated at 92°C for 20 min to convert quantitatively sites of guanine alkylation into strand breaks (Kohn *et al.*, 1987; Mattes *et al*., 1986).

Results and Discussions

Determination of DNA alkyaltion sites induced by altromycin B in the p53 gene Altromycin B is known to alkylate N7 guanine in a sequence-selective manner in naked plasmid DNA. It was reported that the 5′-AG* sequence was the site with the highest reactivity, although other sequences were also reactive (Sun *et al.*, 1993; 1995). We have investigated whether sequence-selective DNA alkylations induced by altromycin B in naked

plasmid DNA are also observed in genomic DNA. For this purpose, the DNA alkylation sites were mapped within the p53 gene by means of the LMPCR technique.

The drug was treated with human colon carcinoma cell lines and genomic DNA was then purified. The alkylation sites were quantitatively converted into DNA strand breaks by hot piperidine treatment (Kohn *et al.*, 1987; Mattes *et al*., 1986). These DNA strand breaks within the p53 gene were determined by the LMPCR technique in parallel with Maxam-Gilbert genomic sequencing reactions.

Figure 2 is the pattern of alkylation sites induced by altromycin B (lane Alt) in exon 9 of the p53 gene within HT-29 colon carcinoma cells. The bands in drug-treated lane indicate the alkylation sites at specific nucleotide sequences and the bands which show high intensity are considered to reflect high-affinity sites of drug alkylation. Each site was found to correspond to a guanine base with no indication that adenine, cytosine, thymine represent sites of alkylation *in vivo*.

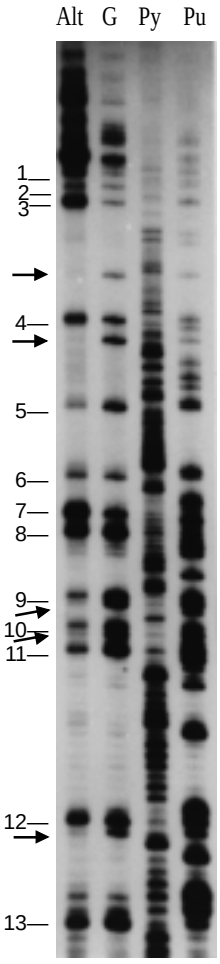

Fig. 2. Autoradiogram of an 8% sequencing gel showing alkylation sites induced by altromycin B in exon 9 of the p53 gene within HT-29 colon carcinoma cells. Alt, Altromycin B; G, guanine-specific reaction; Py, pyrimidine-specific reaction; Pu, purine-specific reaction.

Figure 3 represents the precise sites of alkylation with their neighboring sequences within the p53 gene. The high reactivity sites for altromycin B were observed in the 5′-AG* sequence (double lines, seven out of ten 5′-AG sites). The medium or low reactivity sites were found in the 5′-AG* sequence (single lines, three out of ten 5′-AG sites) and the 5′-TG* sequence (single lines, three out of five 5′-TG sites). It is interesting that alkylation was not observed in three 5′-GG sites and two 5′-TG sites (arrows in the bottom). The absence of alkylation at 5' guanine of the 5′-GG sequence could be explained by the fact that the drug does not prefer to intercalate between guanine and guanine.

Determination of DNA alkylation sites induced by altromycin B in the pBR322 DNA To confirm the sequence selective alkylation of altromycin B in naked plasmid DNA, DNA restriction fragment of pBR322 was prepared and drug alkylation sites were determined by heat-induced DNA strand cleavage assay. Figure 4 shows the strand cleavage patterns in the 3′-end-labeled *Eco*RI/*Bam*HI 375 bp restriction fragments of pBR322. When the drug-treated DNA was heated in the presence of 1 M piperidine (lane Alt), strand cleavage was seen at all guanine residues with different degrees of intensity. Figure 5 summarizes alkylation sites at the nucleotide level in the pBR322 DNA. It is indicated that the 5′-AG* sequence (arrows) was the highly reactive consensus sequence, although other sequences were alkylated. These results are consistent with the previous report that consensus sequence of the most reactive sites were founded in the 5′-AG* sequence (Sun *et al.*, 1993; 1995). The observed 5′-AG* selectivity for guanine alkylation was accounted for by a preferential binding to this

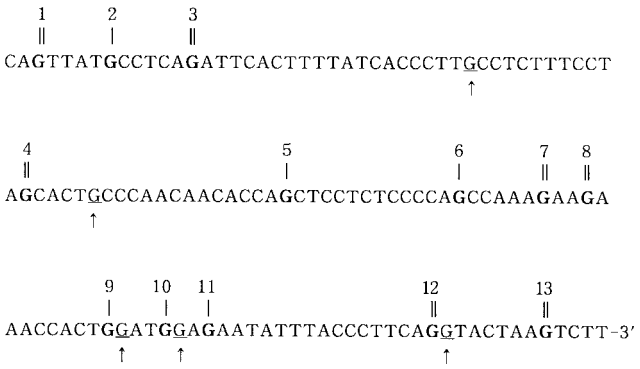

Fig. 3. Alkylation sites induced by altromycin B in exon 9 of the p53 gene within HT-29 human carcinoma cells. Data were obtained from Fig. 2. Double lines represent high-affinity sites of alkylation, and single lines represent medium or low-affinity sites of alkylation. Arrows in the bottom represent nonalkylated guanines. The sequence reported has been assigned the GenBank accession number AF135120.

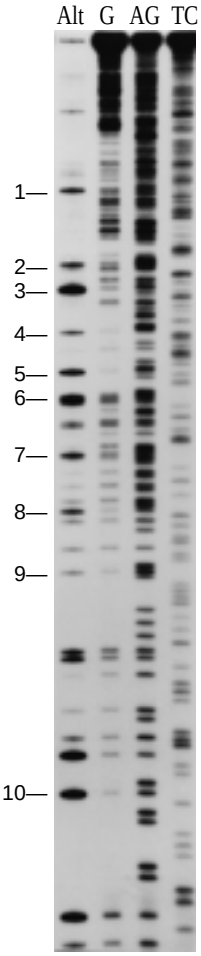

Fig. 4. Autoradiogram of an 8% sequencing gel showing alkylation sites induced by altromycin B in the 3′ end-labeled *Eco*RI/*Bam*HI restriction fragment of pBR322 DNA. Alt, Altromycin B; G, guanine-specific reaction; AG, purine-specific reaction; TC, pyrimidine-specific reaction.

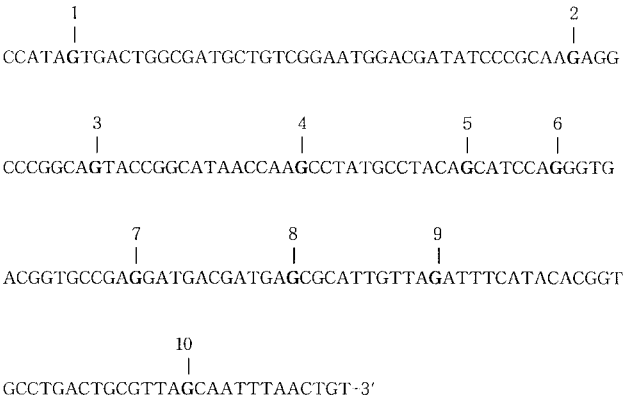

Fig. 5. Alkylation sites induced by altromycin B in the *Eco*RI/*Bam*HI restriction fragment of pBR322 DNA. Data were obtained from Fig. 4. Numbers represent alkylation sites in a 5′-AG sequence.

sequence through a DNA-sugar interaction (Hansen and Hurley, 1995). These results together with LMPCR results clearly demonstrate that sequence selective alkylation of altromycin B in naked plasmid DNA are similar to that observed in genomic DNA.

One of the unique features of altromycin B is the ability to intercalate between base pairs and alkylate N7 of guanine in the major groove of DNA. The mechanism of sequence-selective alkylation of altromycin B in the 5′-AG* sequence was proposed to be as follows: cooperative interaction between sugars and DNA in the minor and major groove dictates the position of the epoxide in the major groove in proximity to N7 of guanine (Sun *et al*., 1995). CC-1065 analogs, the minor groove alkylating agents, also show similar sequence-selective alkylation in naked plasmid DNA and human genomic DNA (Lee *et al*., 1994a; Yoon and Lee, 1998). Thus, it can be speculated that DNA binding mode, whether it is intercalating (altromycin B) or groove binding (CC-1065 analogs), is not an important factor in modulating sequence-selective alkylation in genomic DNA. It is also suggested that histones and other nuclear proteins in cells do not participate in the modulation of the patterns of DNA alkylation.

By contrast, the frequency of UV photoproducts, cyclobutane pyrimidine dimer, and (6-4) photoproducts in genomic DNA has been modulated by nucleosome and other DNA binding proteins (Mitchell *et al*., 1990; Pehrson, 1995; Pfeifer *et al*., 1992; Tornaletti *et al*., 1995). This result infers that the local conformational change in DNA structure in DNA-protein complexes could be a possible dominating factor for the modulation of photoproducts formation (Pfeifer, 1997).

Since LMPCR allows the mapping of DNA adducts at the nucleotide level, its application can be further extended to understand the molecular mechanism of DNA repair and mutagenesis induced by other carcinogens or DNA-binding anticancer agents in the specific human gene.

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