Mapping of DNA Alkylation Sites Induced by Adozelesin and Bizelesin in Human Cells by Ligation-Mediated Polymerase Chain Reaction†

Chong-Soon Lee,*,‡ Gerd P. Pfeifer,§ and Neil W. Gibson‡,∥

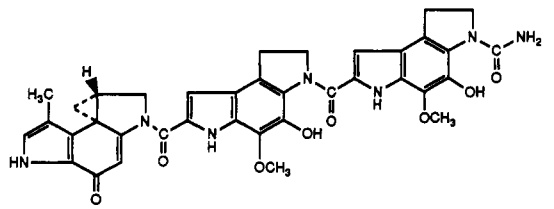
School of Pharmacy and Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033, and Department of Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010

Received December 28, 1993; Revised Manuscript Received March 14, 1994⊛

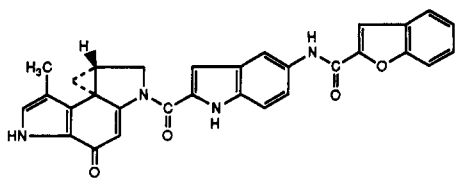
ABSTRACT: In this study, we have mapped the intracellular alkylation sites of adozelesin and bizelesin, two potent analogs of CC-1065, in individual genes at the single-nucleotide level. Human colon carcinoma cells were treated with adozelesin and bizelesin, and the position of adducts were mapped within the PGK-1 and p53 genes by means of ligation-mediated polymerase chain reaction. The monofunctional alkylating agent adozelesin was found to alkylate genomic DNA predominantly within 5′-(A/T)(A/T)A* sequences. Additional sites of alkylation were observed within 5′-(A/T)(G/C)(A/T)A* sequences; however, these were considered to represent sites of medium to low preference. Bizelesin, a bifunctional analog capable of both DNA monofunctional alkylation and DNA interstrand cross-link formation, was also found to alkylate 5′-(A/T)(A/T)A* sequences. Putative bizelesin DNA interstrand cross-link sites indicated that AT-rich sequences are preferred in the intervening sequence between the two cross-linked adenines. Both six- and seven-nucleotide regions were identified as putative sites of DNA interstrand cross-link formation with 5′-TTTTTTA*, 5′-TTTATCA* and 5′-GTACTAA* sequences being preferred. Non-adenine bases are not observed as potential intracellular sites of either DNA interstrand cross-linking formation or monofunctional alkylation. Thus, the patterns of alkylation induced by adozelesin and bizelesin in genomic DNA are similar but not identical to that observed in purified cell-free DNA.

CC-1065 (Figure 1), an extremely potent antitumor antibiotic, alkylates the N3 adenine in a selective manner (Hanka et al., 1978; Martin et al., 1981; Warpehoski, 1992; Hurley et al., 1988; Warpehoski & Hurley, 1988; Boger et al., 1991a–c). Adozelesin and bizelesin, two second-generation analogs of CC-1065, have excellent antitumor activity but are devoid of the delayed hepatotoxicity associated with CC-1065 (McGovren et al., 1984; Li et al., 1991; Lee & Gibson, 1991; Bhuyan et al., 1992a,b). Bizelesin, a bifunctional analog, shows good antitumor efficacy both in vitro and in vivo and is generally 2–30-fold more potent than adozelesin (a monofunctional analog) when tested against human carcinoma cells (Mitchell et al., 1991; Lee & Gibson, 1991). Adozelesin is currently in phase II clinical trials, and bizelesin is currently being developed for phase I clinical trials in humans (Fleming et al., 1992; Burris et al., 1992; McGovren, personal communication).

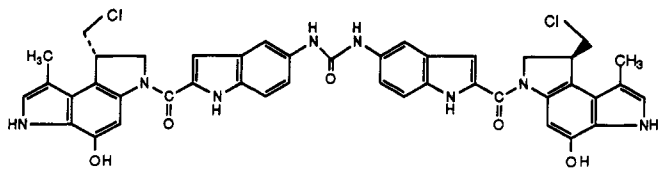
One of the unique features of the cyclopropylpyrroloindole-like compound (CPI)[1] class of molecules is the ability to alkylate the adenine N3 position in the minor groove of DNA in a sequence-specific manner. Indeed, a common consensus sequence of 5′-(T/A)(T/A)A* (where the asterisk indicates the site of alkylation) has been found for the CPI class of molecules. Some minor variations to this sequence preference are associated with variations within the structures of the specific CPI. Adozelesin alkylates DNA selectivity in a manner similar to that observed for CC-1065 (Weiland & Dooley, 1991; Krueger et al., 1991). In contrast, bizelesin (a CPI dimer with two alkylating components) induces either a six- or a seven-nucleotide DNA ISC which is dependent upon the intervening sequences between the two cross-linked



CC-1065



ADOZELESIN



BIZELESIN

FIGURE 1: Structures of CC-1065, adozelesin, and bizelesin.

† This work was supported by Grant CA 59537 from the National Institutes of Health.

* To whom correspondence should be addressed at the Department of Biochemistry, College of Natural Sciences, Yeungnam University, Gyongsan 712-749, Korea.

‡ University of Southern California.

§ Beckman Research Institute of the City of Hope.

∥ Present address: Cancer Research Group, Central Research Division, Pfizer Inc., Eastern Point Rd., Groton, CT 06340.

⊛ Abstract published in *Advance ACS Abstracts*, April 15, 1994.

[1] Abbreviations: CPI, cyclopropylpyrroloindole-like compound(s); LM-PCR, ligation-mediated polymerase chain reaction; PGK1 gene, phosphoglycerate kinase 1 gene; DNA ISC, DNA interstrand cross-links; DNA MA, DNA monofunctional adduct.

0006-2960/94/0433-6024$04.50/0

adenines. The preferred sequence for the formation of DNA ISC was found to be either 5′-TAATTA*-3′ or 5′-TAAA-AA*A*-3′, where the asterisk indicates the location of alkylation on the top strand and the underlined T indicates the location of adenine alkylation on the complementary strand (Lee & Gibson, 1993a; Ding & Hurley, 1991). Surprisingly, bizelesin has also been reported to alkylate non-adenine bases, and this has been explained by its strong precovalent affinity for specific DNA sequences (Sun & Hurley, 1993).

In the present study, we have investigated whether the patterns of alkylation induced by adozelesin or bizelesin in naked DNA are also observed in genomic DNA. For this purpose, the LM-PCR technique has been utilized to map drug-induced DNA alkylation sites in single-copy genes at the nucleotide level in human cells (Mueller & Wold, 1989; Pfeifer et al., 1989, 1993; Garrity & Wold, 1992; Pfeifer & Riggs, 1993). An analysis of alkylation patterns of adozelesin and bizelesin in cellular DNA shows that both compounds retain their ability to alkylate DNA in a sequence-specific manner. The patterns of alkylation induced by adozelesin and bizelesin in genomic DNA are similar but not identical to that observed in purified cell-free DNA.

MATERIALS AND METHODS

Chemicals and Reagents. Adozelesin (U-73 975) and bizelesin (U-77 779) were provided by The Upjohn Co., Kalamazoo, MI. T4 DNA ligase was purchased from Promega. Vent DNA polymerase was from New England Biolabs. T7 DNA polymerase (Sequenase version 2.0), proteinase K, and RNase A were from United States Biochemical.

Cell Lines. HT-29 and BE human colon carcinoma cells were maintained by growing cells at 37 °C as monolayer in Eagle's minimum essential media supplemented with 10% calf bovine serum, gentamycin (0.05 mg/mL), glutamine (0.03 mg/mL), 0.1 mM nonessential amino acids, 0.1 M sodium pyruvate, and 0.02 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

Drug Treatment and DNA Preparation. Adozelesin and bizelesin were dissolved in sterile dimethyl sulfoxide. Cells, at 80% confluency in 25 cm^2 flasks, were exposed to each drug (concentrations are indicated in each figure legend) and incubated for 2 or 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 (Pfeifer et al., 1993; Pfeifer & Riggs, 1993).

DNA Strand Cleavage at Alkylation Sites. Drug-treated genomic DNA was resuspended in 100 μL of 10 mM potassium phosphate buffer (pH 7.0), heated at 92 °C for 30 min, and precipitated in 0.3 M sodium acetate with 2.5 volumes of ethanol. Precipitated DNA was resuspended in 20 μL of TE, pH 7.5. This DNA strand cleavage mechanism produces a 5′-phosphate group at the drug alkylation site. This is required in order that the damaged DNA can serve as a substrate for T4 DNA ligase in a subsequent step.

Sequencing Reaction of Human Genomic DNA. Human genomic DNA was sequenced according to the Maxam–Gilbert protocol (Pfeifer & Riggs, 1993; Maxam & Gilbert, 1980).

Purine-specific reaction: Thirty microliters of formic acid (88%) was added to 30 μL of DNA in TE, pH 8, and the mixture were incubated at 20 °C for 4 min. The reaction was terminated by two consecutive DNA precipitations with sodium acetate and ethanol. The chemically modified genomic DNA was resuspended in 50 μL of 1 M piperidine, heated at 92 °C for 30 min, precipitated in 0.3 M sodium acetate with 2.5 volumes of ethanol, and then lyophilized overnight to remove residual piperidine.

Pyrimidine-specific reaction: Thirty microliters of hydrazine was added to 30 μL of DNA in TE, pH 8, and the mixture was incubated at 20 °C for 8 min. The reaction was terminated by ethanol precipitation and was treated with 1 M piperidine as described above.

Ligation-Mediated PCR. LM-PCR was performed in parallel on drug-treated and chemically sequenced genomic DNA. 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 & Wold, 1989; Pfeifer & Riggs, 1993; Pfeifer et al., 1993). Three primers for the p53 gene, exon 9, upper strand were 5′-AAACG-GCATTTTGAGTGTTAGA (primer 1, T_m = 51 °C), 5′-AAACGGCATTTTGAGTGTTAGACTGGAAAC (primer 2, T_m = 63 °C), and 5′-TGGAAACTTTCCACTTGATAA-GAGGTCCC (primer 3, T_m = 63 °C). The initial extension of primer 1 was carried out at 48 °C for 15 min with Sequenase (version 2.0) as described (Pfeifer & Riggs, 1993; Pfeifer et al., 1993). The DNA polymerase was heat-inactivated at 67 °C for 15 min. The oligonucleotide linker was ligated to the blunt-end, primer-extended molecules at 18 °C for 16 h as described (Pfeifer & Riggs, 1993; Pfeifer et al., 1993). After precipitation of DNA, p53 gene-specific fragments were amplified with Taq DNA polymerase by using linker primer (25-mer) and primer 2 (Pfeifer & Riggs, 1993; Pfeifer et al., 1993). Nineteen cycles of PCR were run as follows: cycle 1, 3.5 min at 95 °C, 2 min at 62 °C, 3 min at 75 °C; cycles 2–18, 1 min at 95 °C, 2 min at 62 °C, 3 min at 75 °C; cycle 19, 1 min at 95 °C, 2 min at 62 °C, 10 min at 75 °C. The different sized amplified DNA products (indicative of the sites of alkylation) were visualized by using 5′-end-labeled primer 3 in a four-cycle extension reaction using Taq DNA polymerase. In brief, 10 pmol of 5′-end-labeled primer 3 was mixed with 2.5 μL of 2.5 mM dNTPs, 0.5 μL of Taq DNA polymerase, 10× buffer, and 0.3 μL (1.5 units) of Taq DNA polymerase in a total volume of 5 μL. This mixture was subsequently added to an ice-cooled 50 μL of the PCR amplification reaction, and four cycles of an extension reaction was performed as follows: cycle 1, 3.5 min at 95 °C, 2 min at 62 °C, 10 min at 75 °C; cycles 2–4, 1 min at 95 °C, 2 min at 62 °C, 10 min at 75 °C. The extension products were separated by electrophoresis on an 8% polyacrylamide gel. The continuity of the purine–pyrimidine sequence ladders shows that the LM-PCR reaction amplifies fragments of disparate size with similar efficiency. Therefore, no apparent sequence bias is introduced by LM-PCR amplification, and the band patterns obtained after exposure of genomic DNA to specific drugs can thus be interpreted with confidence as indicating sites of drug-induced damage.

Alkylation sites induced by each drug within the PGK1 gene were identified using Vent DNA polymerase as described by Garrity and Wold. Three primers for the PGK1 gene were 5′-CGGTGTTCCGCATTCTGC (primer N1, T_m = 54 °C), 5′-TTCTGCAAGCCTCCGGAGCGCAC (primer N2, T_m = 68 °C), and 5′-GCACGTCGGCAGTCGGCTCCC (primer N3, T_m = 71 °C). With this primer set, sequences of the transcribed strand of exon 1 and adjacent untranslated sequences of the PGK1 gene were analyzed. In brief, primer extension was performed by adding 0.3 μL (0.6 unit) of Vent DNA polymerase to DNA samples (3 μg) and 0.6 pmol of primer N1 in 30 μL of reaction buffer containing 40 mM

NaCl, 5 mM $MgSO_4$, 10 mM Tris-HCl (pH 8.9), and 250 μM dNTPs. Primer extension used a thermal cycle of 3 min at 95 °C, 30 min at 45 °C, and 10 min at 76 °C. The samples were immediately iced. After addition of 2 μL of 1 M Tris-HCl, pH 7.5, for pH adjustment, 48 μL of freshly prepared ligation mix (13.33 mM $MgCl_2$, 30 mM DTT, 1.66 mM ATP, and 83.3 μg/mL BSA with 100 pmol of oligonucleotide linker in 250 mM Tris-HCl, pH 7.7) and 1.5 μL (4.5 units) of T4 DNA ligase were added. Ligation reaction was performed at 18 °C for 16 h and stopped by ethanol precipitation of DNA. PCR amplification was performed for 19 cycles in 50 μL of buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$, and 0.1% Triton X-100 (supplied by New England Biolabs) in the presence of 250 μM dNTPs, 10 pmol of linker primer, 10 pmol of primer N2, and 0.5 μL (1 unit) of Vent DNA polymerase. The PCR was run as follows: cycle 1, 3.5 min at 95 °C, 2 min at 66 °C, 3 min at 76 °C; cycles 2–18, 1 min at 95 °C, 2 min at 66 °C, 3 min at 76 °C; cycle 19, 1 min at 95 °C, 2 min at 66 °C, 10 min at 76 °C. Amplified PCR products were detected in a manner similar to that described above for the p53 gene except that 0.3 μL (0.6 unit) of Vent polymerase and Vent polymerase 10× buffer were used with different annealing (69 °C) and extension (76 °C) temperatures. It may be interesting to note that the use of Vent DNA polymerase to obtain LM-PCR product within exon 9 of the p53 gene was not successful.

Sequencing Gel. The end-labeled PCR product was extracted with phenol/chloroform and precipitated in the presence of 1 μL of tRNA (10 mg/mL), 0.3 M sodium acetate, and cold 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 a 8% polyacrylamide sequencing gel [mono:bis(acrylamide) ratio = 29:1, 8 M urea]. The sequence and alkylation sites were visualized by autoradiography.

RESULTS

Determination of Alkylation Sites Induced by Adozelesin and Bizelesin in the p53 Gene. Genomic DNA was purified from drug-treated human colon carcinoma cells, and the sites of alkylation were exposed by heat-induced DNA strand cleavage. Heat treatment at neutral pH produces a fragment with a 5′-phosphate at the nucleotide on the 3′-side of the site of alkylation (Hurley et al., 1984; Reynolds et al., 1985; Warpehoski et al., 1992, 1993b). Such a 5′-phosphate is required in order that the cleaved DNA can serve as a substrate for T4 DNA ligase (see Materials and Methods). An end-labeled gene-specific primer was used to visualize the amplified LM-PCR product (Garrity & Wold, 1992; Pfeifer et al., 1993). The end-labeled LM-PCR product was then electrophoresed on a sequencing gel in parallel with Maxam–Gilbert genomic sequencing reactions. This allowed the mapping of drug-induced alkylation sites. The band continuity and uniformity observed with the Maxam and Gilbert sequencing lanes suggest that there are no inherent artifacts within the method which might lead to problems in analyzing specific drug-induced lesions.

Figure 2 is an autoradiogram of an 8% sequencing gel showing the pattern of alkylation induced by adozelesin (lane 1) and bizelesin (lane 2) in exon 9 of the p53 gene within BE colon carcinoma cells. To pinpoint precisely the sites of alkylation and to identify clearly their neighboring sequences, additional samples identical to that described above were electrophoresed for longer times (panel B). This extends the

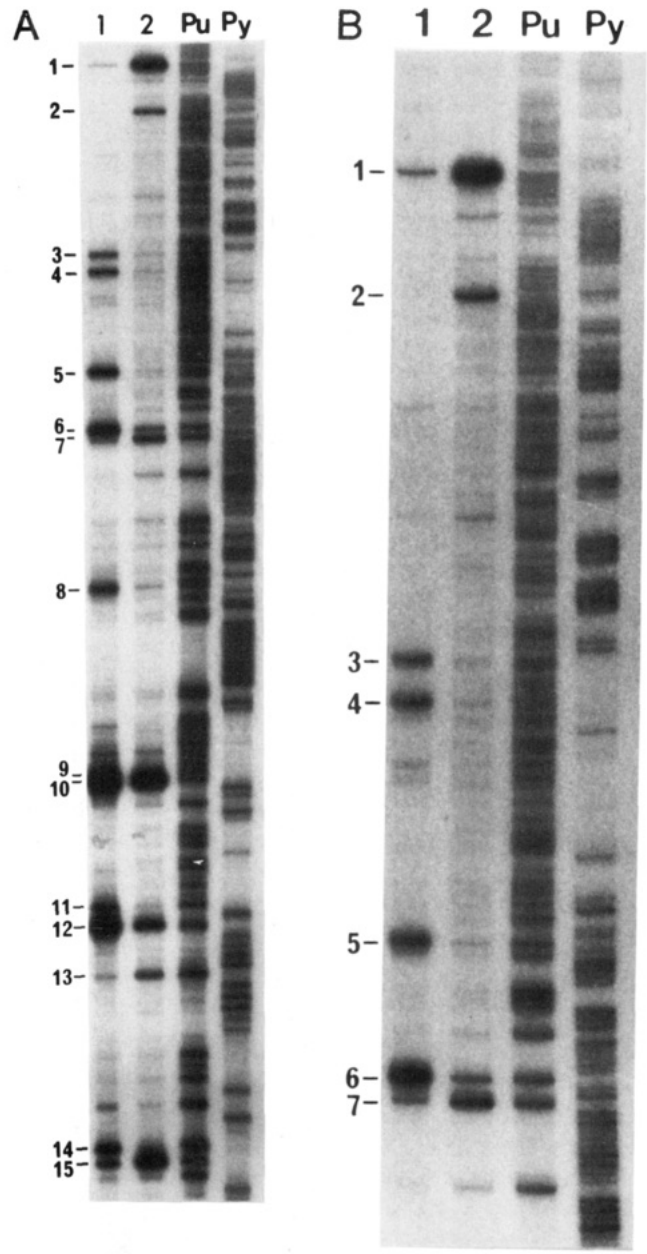


FIGURE 2: (Panel A) Autoradiogram of an 8% denaturing polyacrylamide gel showing alkylation sites induced by adozelesin and bizelesin in exon 9 of the p53 gene within BE colon carcinoma cells. BE cells were treated with 3 μM adozelesin (lane 1) and bizelesin (lane 2) for 4 h, and then genomic DNA was isolated as described under Materials and Methods. Nineteen cycles of PCR were performed, and four cycles of extension reaction were performed using a 5′ end-labeled gene-specific primer to visualize the LM-PCR products. Pu, purine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction. (Panel B) Extended autoradiogram of panel A showing alkylation sites induced by adozelesin (lane 1) and bizelesin (lane 2). Legend as in panel A.

distance between discrete DNA fragments and allows the identification of the drug-induced DNA lesion unequivocally. Bands which show high intensity upon autoradiography are considered to reflect high-affinity sites of drug alkylation. Each site of drug-induced damage was found to correspond to an adenine base with no indication that guanine, cytosine, or thymine represent sites of alkylation in vivo.

Figure 3 summarizes the adozelesin- and bizelesin-induced alkylation sites at the nucleotide level within the p53 gene. Most of the sites of adenine alkylation were found to be common for both adozelesin and bizelesin. However, the affinity of such adenine sites was compound-specific. For

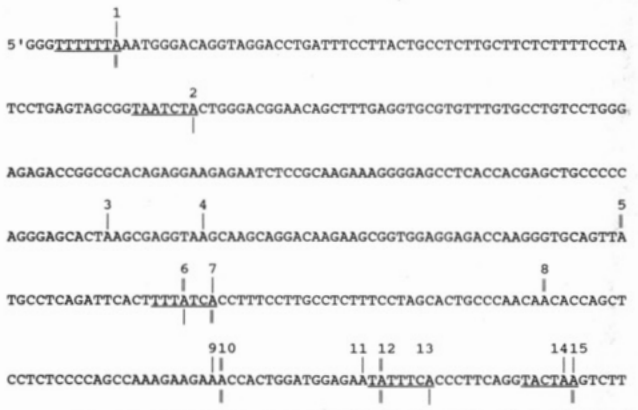


FIGURE 3: Alkylation sites of adozelesin (top) and bizelesin (bottom) in the sequences of the human p53 gene. Data were obtained from Figure 2. Double lines represent high-affinity sites of alkylation, and single lines represent medium- or low-affinity sites of alkylation.

example, a 5′-CTTTTA* sequence (position number 6) was a higher affinity site for adozelesin than a 5′-TTATCA*

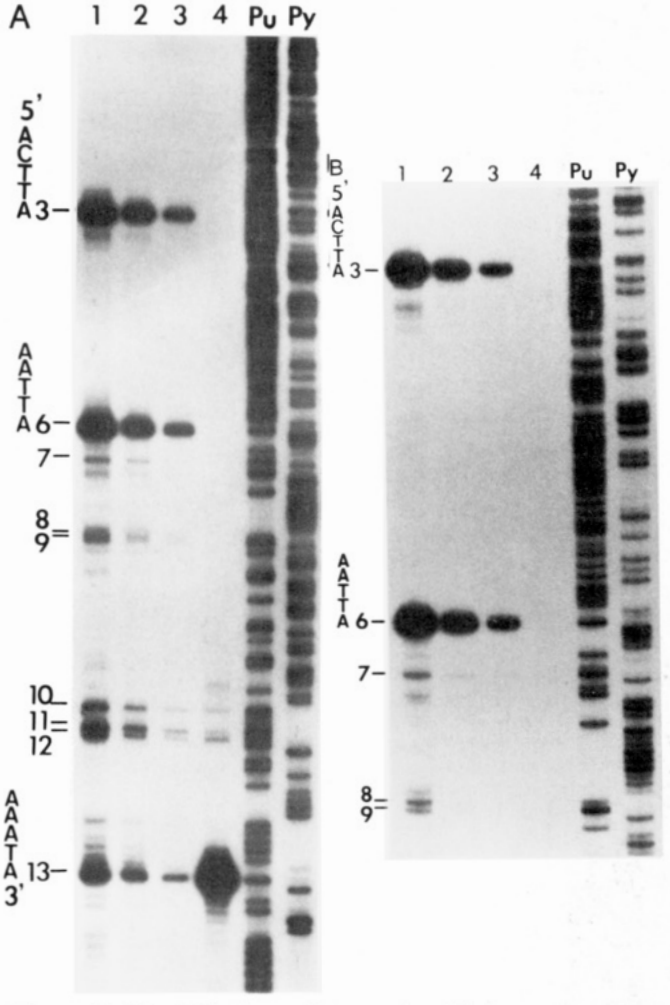


FIGURE 4: (Panel A) Autoradiogram of an 8% denaturing polyacrylamide gel showing alkylation sites induced by adozelesin and bizelesin in the PGK1 gene within BE colon carcinoma cells. BE cells were treated with 3 μM adozelesin (lanes 1–3) and bizelesin (lane 4) for 4 h, and then genomic DNA was isolated as described under Materials and Methods. Nineteen cycles of PCR were performed, and four cycles of extension reaction were performed using a 5′ end-labeled gene-specific primer to visualize the LM-PCR product. Lanes 2 and 3 are half and one-fourth amounts of lane 1, respectively. Pu, purine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction. (Panel B) Extended autoradiogram of panel A showing alkylation sites induced by adozelesin (lanes 1, 2, and 3) and bizelesin (lane 4). Legend as in panel A.

sequence (position number 7), while the reverse was true for bizelesin. The higher affinity of the 5′-TTATCA* sequence rather than the 5′-CTTTTA* sequence for bizelesin-induced alkylation may be explained by the fact that this sequence represents a putative site for DNA ISC formation (Lee & Gibson, 1993a; Sun & Hurley, 1993).

Determination of Alkylation Sites Induced by Adozelesin and Bizelesin in the PGK1 Gene. In order to determine whether the data obtained in the p53 gene are representative of other DNA regions, we have mapped alkylation sites within the PGK1 gene. Figure 4 shows an autoradiogram of the products obtained after 19 cycles of PCR amplification and 4 cycles of subsequent primer extension using a PGK1 gene-specific primer. Panel B represents the extended autoradiogram of the top portion of panel A. In order to locate precisely the sites of adozelesin alkylation, half (lane 2) or one-fourth (lane 3) of the amount of lane 1 was loaded. Adozelesin (lanes 1–3) shows higher affinity sites of alkylation in 5′-ACTTA* (band 3), 5′-AATTA* (band 6), and 5′-AAATA* (band 13) sequences while bizelesin (lane 4) has a high-affinity site only in the 5′-AAATA* sequence (band 13).

Effect of Drug Concentration upon the Analysis of DNA Sequence Selectivity. In an attempt to determine whether the drug concentration in cells could alter the interpretation of sequence-selective alkylation, cells were exposed to a reduced level of drug, and then alkylation sites were determined within the PGK1 gene. The conditions used for this study were 0.3 μM with 2-h incubation, which is a 10-fold lower concentration and a 2-fold lower incubation time than the conditions used in Figure 4. Twenty-four PCR cycles were performed instead of 19 PCR cycles. Figure 5 shows an autoradiogram of the products obtained after four cycles of a subsequent primer extension reaction. Panel B represents the extended autoradiogram of the top portion of the gel shown in panel A. Figure 6, obtained from the results of Figures 4 and 5, represents alkylation sites of adozelesin and bizelesin in the sequences of the PGK1 gene. The alkylation sites of adozelesin (Figure 5, lanes 1 and 2) and bizelesin (Figure 5, lane 3) are identical with those shown in Figure 4 except that some additional bands were observed on the top portion of the gel. This may be due to the fact that the frequency of drug modification is lower and as a result larger DNA fragments are available for amplification after cleavage of damaged DNA. As a consequence of this analysis, additional alkylation sites of bizelesin were observed at 5′-AACTTA* (band 3) and 5′-GAATTA* (band 6). In the case of adozelesin, an additional high-affinity site of alkylation could be seen on the top portion of the gel (Figure 5, panel B). That is, a 5′-TAAAA* sequence (band 2) was found to be a higher affinity site for alkylation than a 5′-ACTTA* sequence (band 3). The overall pattern of alkylation, however, is very similar to that seen with higher drug concentrations.

DISCUSSION

The organization of DNA in cells is quite different from naked DNA with the latter being free of histones and other nuclear proteins. This difference always raises the question as to whether the molecular mechanism of drug–DNA interactions ascertained from studies on naked DNA would also be apparent from studies with human genomic DNA. In an attempt to answer this question, the majority of studies have used human alphoid DNA as a substrate and determined the sites of drug binding at the nucleotide level (Bubbly et al., 1992; Grunberg & Haseltine, 1980; Hartley et al., 1992; Murray & Martin, 1992; Murray et al., 1992). This has

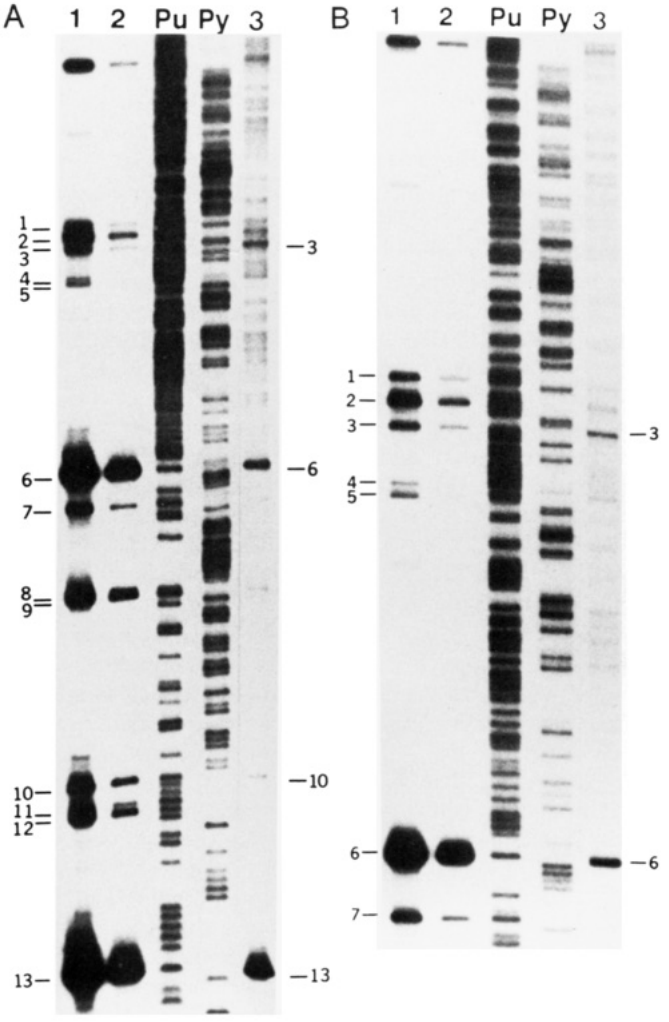


FIGURE 5: (Panel A) Autoradiogram of an 8% denaturing polyacrylamide gel showing alkylation sites induced by adozelesin and bizelesin in the PGK1 gene within BE colon carcinoma cells. BE cells were treated with 0.3 μM adozelesin (lanes 1 and 2) and bizelesin (lane 3) for 2 h, and then genomic DNA was isolated as described under Materials and Methods. Twenty-four cycles of PCR were performed, and four cycles of extension reaction were performed using a 5′ end-labeled gene-specific primer to visualize the LM-PCR product. Lane 2 is half the amount of lane 1. Pu, purine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction. (Panel B) Extended autoradiogram of panel A showing alkylation sites induced by adozelesin (lanes 1 and 2) and bizelesin (lane 3). Legend as in panel A.

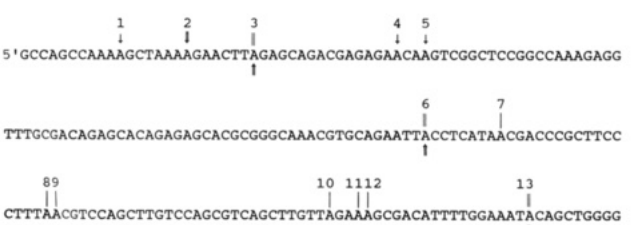


FIGURE 6: Alkylation sites of adozelesin (top) and bizelesin (bottom) in the sequences of the human PGK1 gene. Data were obtained from Figures 4 and 5. Double lines represent high-affinity sites of alkylation, and single lines represent medium- or low-affinity sites of alkylation under the condition of 4-h incubation at 3 μM drug concentration (Figure 4). Double arrows represent high-affinity sites of alkylation, and single arrows represent medium- or low-affinity sites of alkylation sites under the condition of 2-h incubation at 0.3 μM drug concentration (Figure 5).

provided some useful, but limited, data on the nature of drug–DNA interactions in vivo. The introduction of the LM-PCR technique has allowed the detection of UV-induced DNA

Table 1: Summary of DNA Sequence Selectivity of DNA MA Induced by Adozelesin in Human Genomic DNA[a]

sequence	position number
(A) Most Reactive Sequences for Adozelesin	
AGTTA*TG	5 in p53 gene
TTTTA*TC	6 in p53 gene
AACAA*CA	8 in p53 gene
AGAAA*CC	10 in p53 gene
GAATA*TT	12 in p53 gene
TAAAA*GA	2 in PGK gene[b]
ACTTA*GA	3 in PGK gene
AATTA*CC	6 in PGK gene
AAATA*CA	13 in PGK gene
(A/T) (A/T) A*	
(B) Unique Sequences for Adozelesin	
CACTA*AG	3 in p53 gene
AACAA*CA	8 in p53 gene
AAGAA*AC	9 in p53 gene
GAGAA*TA	11 in p53 gene
TACTA*AG	14 in p53 gene
TAGAA*AG	11 in PGK gene
(A/T) (G/C) (A/T) A*	

[a] These data were obtained from Figures 2 and 4. [b] This sequence was obtained from Figure 5. A* represents the adenine to which drug is covalently bound. Sequences were written from 5′ to 3′.

Table 2: Summary of DNA Sequence Selectivity of DNA ISC and MA Induced by Bizelesin in Human Genomic DNA[a]

sequence	position number
(A) Most Reactive Sequences for Formation of DNA MA by Bizelesin	
GAAGAAA*CC	10 in p53 gene
GAGAATA*TT	12 in p53 gene
GAACTTA*GA	3 in PGK gene[b]
AGAATTA*CC	6 in PGK gene[b]
GGAAATA*CA	13 in PGK gene
(A/T) (A/T) A*	
(B) Most Reactive Sequences for Formation of Putative DNA ISC by Bizelesin	
TTTTTTA*AA	1 in p53 gene
TTATCA*CC	7 in p53 gene
GTACTAA*GT	15 in p53 gene
(C) More Reactive Sequences for Bizelesin than Adozelesin	
TTTTTTA*AA	1 in p53 gene
TAATCTA*CT	2 in p53 gene
TTTATCA*CC	7 in p53 gene
TATTTCA*CC	13 in p53 gene
GTACTAA*GT	15 in p53 gene

[a] These data were obtained from Figures 2 and 4. [b] This sequence was obtained from Figure 5. A* represents the adenine to which drug is covalently bound, and the underlined T indicates the possible location of adenine alkylation on the complementary strand. Sequences were written from 5′ to 3′.

damage in single-copy genes at the nucleotide level (Pfeifer et al., 1991, 1992; Tormanen & Pfeifer, 1992; Tornaletti et al., 1993). Thus, a greater understanding of the intracellular mechanisms of DNA reactive molecules can now be achieved.

In this study, we have used LM-PCR to map the alkylation sites induced by adozelesin and bizelesin within DNA in human cells. The pattern of drug alkylation sites mapped within the human PGK1 and p53 genes is summarized in Tables 1 (adozelesin) and 2 (bizelesin). In the case of the monofunctional agent adozelesin, the consensus sequence for most reactive sites is found to be 5′-(A/T)(A/T)A* (Table 1A). This consensus sequence obtained for adozelesin in vivo coincides with that of CC-1065 and its analogs in vitro (Reynolds et al., 1985; Hurley et al., 1988, 1990; Weiland & Dooley, 1991; Boger et al., 1991a–c). In addition, 1 GC bp

has been found to be tolerated at the third or fourth base on the 5′-side of alkylation (Table 1A). Interestingly, a new consensus sequence, 5′-(A/T)(G/C)(A/T)A*, has been found to be an additional alkylation site for adozelesin (Table 1B). This is true for one high-affinity site (position 8 in p53 gene) as well as numerous moderate to low-affinity sites. Such consensus sequences are not preferred alkylation sites for CC-1065. This is in contrast to previous work which catalogued such consensus sequences as *non*alkylating sites for adozelesin (Weiland & Dooley, 1991). The difference between both studies may relate to the fact that Weiland and Dooley's work utilized polymerase stop assays with cell-free plasmid DNA (Weiland & Dooley, 1991). The frequency of adozelesin-induced alkylation within 5′-(A/T)(G/C)(A/T)A* sequences observed here suggests that there are additional mechanisms for DNA sequence recognition by adozelesin in vivo. This finding may lend additional weight to the proposal that the sequence selectivity of CPI is determined by noncovalent interactions between DNA and the middle and right-handed subunits of the drug molecule in addition to the covalent alkylation step mediated by the left-handed subunit (Hurley et al., 1988; Warpehoski & Hurley, 1988; Boger et al., 1991a–c).

Bizelesin, a bifunctional CPI, has been shown to induce either a six- or a seven-nucleotide DNA ISC with the efficiency of formation being dependent upon the intervening sequence between the two cross-linked adenine bases (Lee & Gibson, 1993a; Ding et al., 1991). While the formation of six-nucleotide DNA ISC mimics the sequence selectivity for CC-1065-induced DNA MA, the formation of seven-nucleotide DNA ISC prefers intrinsically bent 5′-TTTTTTA* sequences (Lee & Gibson, 1993a). The pattern of sequence selectivity of bizelesin observed in genomic DNA within cells can be divided into two categories; one relates to sites of DNA MA (Table 2A), and the other relates to putative sites for the formation of DNA ISC (Table 2B). The sequences which are predominantly sites of DNA MA conform to a 5′-(A/T)(A/T)A* consensus sequence (Table 2A). These data support the notion that the alkylation pattern of bizelesin in human cellular DNA is based upon the inherent sequence selectivity associated with monofunctional alkylation, such as that observed with adozelesin in vitro and in vivo (Lee & Gibson, 1993a; this study). Our analysis indicated that 5′-TTTTTTA*, 5′-TTTATCA*, and 5-GTACTAA* (Table 2B) sequences conform to putative sites of DNA ISC formation, in which the possible cross-linking adenine site on the complementary strand was underlined at T. Although the formation of DNA ISC is determined by the sequence of both DNA strands in vitro (Lee & Gibson, 1993a; Sun & Hurley, 1993), definitive proof for the formation of DNA ISC in genomic DNA is difficult to obtain. However, it is clear that bizelesin prefers AT-rich regions for DNA ISC and 1 GC bp may be tolerated between the two adenines involved in the putative DNA ISC. When the higher affinity sites of bizelesin than adozelesin in the p53 gene are analyzed (Table 2C), all such sites would allow the formation of a putative six- or seven-nucleotide DNA ISC (Lee & Gibson, 1993a; Sun & Hurley, 1993).

The alkylation of guanine, cytosine, and thymine by bizelesin has recently been reported to occur within sequences of cell-free plasmid DNA (Sun & Hurley, 1993). Alkylation, however, of bases other than adenine has not been observed in this study. Therefore, the level of alkylation at non-adenine bases in human cells may be extremely low in comparison with the level of adenine alkylation and thus prevent detection by LM-PCR. It is also possible that the organization of DNA into chromatin in cells might modulate sequence selectivity or affect drug alkylation at certain sites. The data suggest that information obtained in a cell-free system provides a solid but not complete understanding of the events which occur within cells. In conclusion, drug alkylation sites in human cellular DNA have been mapped in single-copy genes at the nucleotide level. The mechanism by which such sequence-specific lesions elicit cell death remains to be clarified.

REFERENCES

Bhuyan, B. K., Smith, K. S., Adams, E. G., Petzold, G. L., & McGovren, J P. (1992a) *Cancer Res. 42*, 5687–5692.

Bhuyan, B. K., Smith, K. S., Adams, E. G., Wallace, T. L., Von Hoff, D. D., & Li, L. H. (1992b) *Cancer Chemother. Pharmacol. 30*, 348–354.

Boger, D. L., Munk, S. A., & Zarrinmayeh, H. (1991a) *J. Am. Chem. Soc. 113*, 3980–3983.

Boger, D. L., Munk, S. A., Zarrinmayeh, H., Ishizaki, T., Haught, J, & Bina, M. (1991b) *Tetrahedron 47*, 2661–2682.

Boger, D. L., Zarrinmayeh, H. J., Munk, S. A., Kitos, P. A., & Suntornwat, O. (1991c) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1431–1435.

Bubley, G. J., Ashburner, B. P., Balk, S. P., Ara, G., Varshaney, A., & Teicher, B. A. (1992) *BioTechniques 12*, 694–698.

Burris, H., Earhart, R., Kuhn, J., Shaffer, D., Smith, L., Weiss, G., Kasunic, D., Radbury, G., Campbell, L., & Von Hoff, D. (1992) *Proc. Am. Assoc. Cancer. Res. 33*, 520.

Ding, Z.-M., & Hurley, L. H. (1991) *Anti-Cancer Drug Design 6*, 427–452.

Flemimg, G. F., Ratain, M. J., O'Brien, S. M., Vogelzang, N. J., & Earhart, R. H. (1992) *Proc. Am. Assoc. Cancer. Res. 33*, 265.

Garrity, P. A., & Wold, B. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1021–1025.

Grunberg, S. M., & Haseltine, W. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6546–6550.

Hanka, L. J., Dietz, A., Gerpheide, S. A., Kuentzel, S. L., & Martin, D. G. (1978) *J. Antibiot. 31*, 1211–1217.

Hartly, J. A., Bingham, J. P., & Souhami, R. L. (1992) *Nucleic Acids Res. 20*, 3175–3178.

Hurley, L. H., Reynolds, V. L., Swenson, D. H., Petzold, G. L., & Scahill, T. A. (1984) *Science 226*, 843–844.

Hurley, L. H., Lee, C.-S., McGovren, J. P., Mitchell, M. A., Warpehoski, M. A., Kelly, R. C., & Aristoff, P. A. (1988) *Biochemistry 27*, 3886–3892.

Hurley, L. H., Warpehoski, M. A., Lee, C.-S., McGovren, J. P., Scahill, T. A., Kelly, K. C., Wicnienski, N. A., Gebhard, I., & Bradford, V. S. (1990) *J. Am. Chem. Soc. 112*, 4633–4649.

Krueger, W. C., Li, L. H., Moscowitz, A., Prairie, M. D., Petzold, G. L., & Swenson, D. H. (1985) *Biopolymer 24*, 1549–1572.

Lee, C.-S., & Gibson, N. W. (1991) *Cancer Res. 51*, 6586–6591.

Lee, C.-S., & Gibson, N. W. (1993a) *Biochemistry 32*, 2592–2600.

Lee, C.-S., & Gibson, N. W. (1993b) *Biochemistry 32*, 9108–9114.

Li, L. H., Swenson, D. H., Schpok, S., Kuentzel, S. L., Dayton, B. D., & Krueger, W. C. (1982) *Cancer Res. 42*, 999.

Li, L. H., Kelly, R. C., Warpehoski, M. A., McGovren, J. P., Gebhard, I., & DeKoning, T. F. (1991) *Invest. New Drugs 9*, 137–148.

Li, L. H., DeKoning, T. F., Kelly, R. C., Krueger, W. C., McGovren, J. P., Padbury, G. E., Petzold, G. L., Wallace, T. L., Ouding, R. J., Prairie, M. D., & Gebhard, I. (1992) *Cancer Res. 52*, 4904–4913.

Martin, D. G., Biles, C., Gerpheide, S. A., Hanka, L. J., Krueger, W. C., McGovren, J. P., Mizsak, S. A., Neil, G. L., Stewart, J. C., & Visser, J. (1981) *J. Antibiot. 34*, 1119–1125.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499.

McGovren, J. P., Clarke, G. L., Pratt, E. A., & DeKoning, T. F. (1984) *J. Antibiot. 37*, 63–70.
Mitchell, M. A., Kelly, R. C., Winnienski, N. A., Hatzenbuhler, N. T., & Williams, M. G. (1991) *J. Am. Chem. Soc. 113*, 8994–8995.
Mueller, P. R., & Wold, B. (1989) *Science 246*, 780–786.
Murray, V., & Martin, R. F. (1985) *J. Biol. Chem. 260*, 10389–10391.
Murray, V., Motyka, H., England, P. R., Wickham, G., Lee, H. H., Denny, W. A., & McFadyen, W. D. (1992) *Biochemistry 31*, 11812–11817.
Pfeifer, G. P., & Riggs, A. D. (1993) *Methods in Mol. Biol. 15*, 153–168.
Pfeifer, G. P., Stegerwald, S. D., Mueller, P. R., Wold, B., & Riggs, A. D. (1989) *Science 246*, 810–813.
Pfeifer, G. P., Drouin, R., Riggs, A. D., & Holmquist, G. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1374–1378.
Pfeifer, G. P., Drouin, R., Riggs, A. D., & Holmquist, G. P. (1992) *Mol. Cell. Biol. 12*, 1798–1804.
Pfeifer, G. P., Singer-Sam, J., & Riggs, A. D. (1993) *Methods Enzymol. 225*, 567–583.
Reynolds, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6228–6237.
Sun, D., & Hurley, L. H. (1993) *J. Am. Chem. Soc. 115*, 5925–5933.
Tormanen, V. T., & Pfeifer, G. P. (1992) *Oncogene 7*, 1729–1736.
Tornaletti, S., Rozek, D., & Pfeifer, G. P. (1993) *Oncogene 8*, 2051–2057.
Warpehoski, M. A. (1992) in *Advances in DNA Sequence Specific Agents* (Hurley, L. H., Ed.) Vol. 1, pp 217–246, JAI Press Inc., Greenwich, CT.
Warpehoski, M. A., & Hurley, L. H. (1988) *Chem. Res. Toxicol. 1*, 315–333.
Warpehoski, M. A., Harper, D. E., Mitchell, M. A., & Monroe, T. J. (1992) *Biochemistry 31*, 2502–2508.
Weiland, K. L., & Dooley, T. P. (1991) *Biochemistry 30*, 7559–7565.