Analysis of the Monoalkylation and Cross-Linking Sequence Specificity of Bizelesin, a Bifunctional Alkylation Agent Related to (+)-CC-1065

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Abstract: The sequence specificity of bizelesin, an interstrand DNA–DNA cross-linker related to the monoalkylating compound (+)-CC-1065, was studied using restriction enzyme fragments. Bizelesin, like (+)-CC-1065, forms monoalkylation adducts through N3 of adenine but can also form DNA–DNA cross-links six base pairs apart on opposite strands. Compared to many other minor groove cross-linking compounds, bizelesin is very efficient at cross-linking DNA. There is a higher than expected proportion of cross-linked adducts based upon the relative number of cross-linked vs monoalkylated adducts. This is rationalized based upon the relative thermodynamic stability of the cross-linked vs monoalkylated species. Where bizelesin monoalkylation occurs, the sequence specificity is significantly higher than those of (+)-CC-1065 and other monoalkylating (+)-CPI analogs. The bizelesin GC tolerance at cross-linking sites is twice as high as for the monoalkylation sites. This increased GC tolerance can be largely explained by the covalent immobilization of the second alkylation arm at sequences that are not normally reactive toward CPI monoalkylation compounds but are made reactive due to a proximity effect. This same rationale can be used to explain the reactivity of the second alkylation arm of bizelesin with guanine, cytosine, and thymine on some sequence. There are some sequences that appear to be unusual in their reactivity with bizelesin in that bizelesin formed cross-linking spanning seven base pairs, and bizelesin forms monoakylation adducts on guanine. In these cases, it is proposed that bizelesin may trap out rare conformational forms during the second alkylation step, or bizelesin may alkylate unusual sites due to the strong precovalent affinity of bizelesin for those sites.

Introduction

The underlying mechanisms for sequence recognition of DNA by small molecules are of considerable interest in molecular recognition and antitumor drug development.[1] Drugs that covalently modify DNA are consistently at or near the top of the list of the more potent cytotoxic compounds.[2] These compounds often show considerable sequence selectivity beyond what might be intuitively obvious from examination of their structures and molecular weights.[2] Among the mechanisms mediating this sequence selectivity are sequence dependent molecular electrostatic potential,[3] conformational flexibility,[4] catalytic functional group juxtapositions,[5] and precovalent binding affinities.[6] Of particular interest to us is how the sequence selectivity of monoalkylation of DNA by a compound such as (+)-CC-1065 relates to the sequence selectivity of cross-linking compounds that are derived from the appropriate joining of two suitably positioned monoalkylation moieties of the same type. The compound used in this study, bizelesin, is such a synthetically derived compound designed and synthesized by Upjohn scientists.[7] The two main questions we have addressed in this study are whether the sequence selectivity of bizelesin can be predicted from a knowledge of the sequence selectivity of monoalkylation compounds such as (+)-CC-1065, and what unique properties the cross-linking compounds may possess relative to the monoalkylation compounds.

Bizelesin (Figure 1) is an antitumor agent that is a synthetically derived dimeric analog of (+)-CC-1065, a potent antitumor antibiotic produced by *Streptomyces zelensis*.[8] Like (+)-CC-1065 and other cyclopropylpyrrolindoles (CPIs), bizelesin alkylates N3 of adenine with concomitant opening of cyclopropane ring of the CPI moiety[9] and is able to cross-link at 5′-TAATTA sequences[7] (Figure 2). Therapeutically, bizelesin shows high antitumor efficacy both *in vitro* and *in vivo* to various kinds of tumor cells,[10] and it does not show the delayed toxicity associated with (+)-CC-1065.[11] Its pattern of inhibition of macromolecular synthesis is different from any of the monoalkylation analogs, and bizelesin has been accepted into the NCI Decision Network (McGovren, J. P., private communication). As a first step toward understanding the molecular basis for these unique biochemical and biological characteristics of bizelesin, we have compared the molecular interactions of bizelesin and CPI monoalkylation analogs with DNA, the presumed principal target of both groups of compounds. In a previous study, employing a very limited number of defined oligomers, one of which contained a highly reactive cross-linking sequence for bizelesin, it has been shown that bizelesin produces a stable interstrand cross-linked species and appeared to show an increased sequence selectivity relative to its monoalkylating analogs.[9]

In the present study we have investigated in more detail the sequence selectivity of bizelesin by screening about 4.0 Kb of

(1) (a) Dickerson, R. E.; Kopka, M. L.; Pjura, P. E. In *DNA Ligand Interactions*; Guschlbauer, W., Saenger, W., Eds.; Plenum Press: 1987; pp 45–62. (b) *Advances in DNA Sequence Specific Agents*; Hurley, L. H., Ed.; JAI Press Inc.: Greenwich, 1992; Vol. 1.
(2) Warpehoski, M. A.; Hurley, L. H. *Chem. Res. Toxicol.* **1988**, *1*, 315.
(3) Kohn, K. W.; Hartley, J. A.; Mattes, W. B. *Nucleic Acids Res.* **1987**, *15*, 10531.
(4) Zakrzewska, K.; Pullman, B. *J. Biomol. Struct. Dyn.* **1986**, *4*, 127.
(5) Buckley, N. *J. Am. Chem. Soc.* **1987**, *109*, 7918.
(6) Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2700.
(7) Mitchell, M. A.; Kelly, R. C.; Wicnienski, N. A.; Hatzenbuhler, N. T.; Williams, M. G.; Petzold, G. C.; Slighton, J. L.; Siemieniak, P. R. *J. Am. Chem. Soc.* **1991**, *113*, 8994.
(8) (a) Warpehoski, M. A. In *Advances in DNA Sequence Specific Agents*; Hurley, L. H., Ed.; JAI Press Inc.: Greenwich, 1992; Vol. 1 pp 217–246. (b) Reynolds, V. L.; McGovren, J. P.; Hurley, L. H. *J. Antibiotics* **1986**, *39*, 319. (c) Hurley, L. H.; Needham, VanDevanter, D. K. *Acc. Chem. Res.* **1986**, *19*, 230. (d) Warpehoski, M. A. *Drugs of the Future* **1991**, *16*, 131.
(9) Ding, Z.-M.; Hurley, L. H. *Anti-Cancer Drug Design* **1991**, *6*, 427.
(10) For example, a single iv injection of bizelesin at 10 μg/Kg cured (30-day survivors) 50% of the mice. For comparison, adozelezin [(+)-ABC″ in Figure 1] increased the life span of such mice by 94% at an optimum dose of 100 μg but with no cures.
(11) (a) Reynolds, V. L.; McGovren, J. P.; Hurley, L. H. *J. Antibiotics* **1986**, *39*, 319. (b) Warpehoski, M. A.; Bradford, V. S. *Tetrahedron Lett.* **1988**, *29*, 131.

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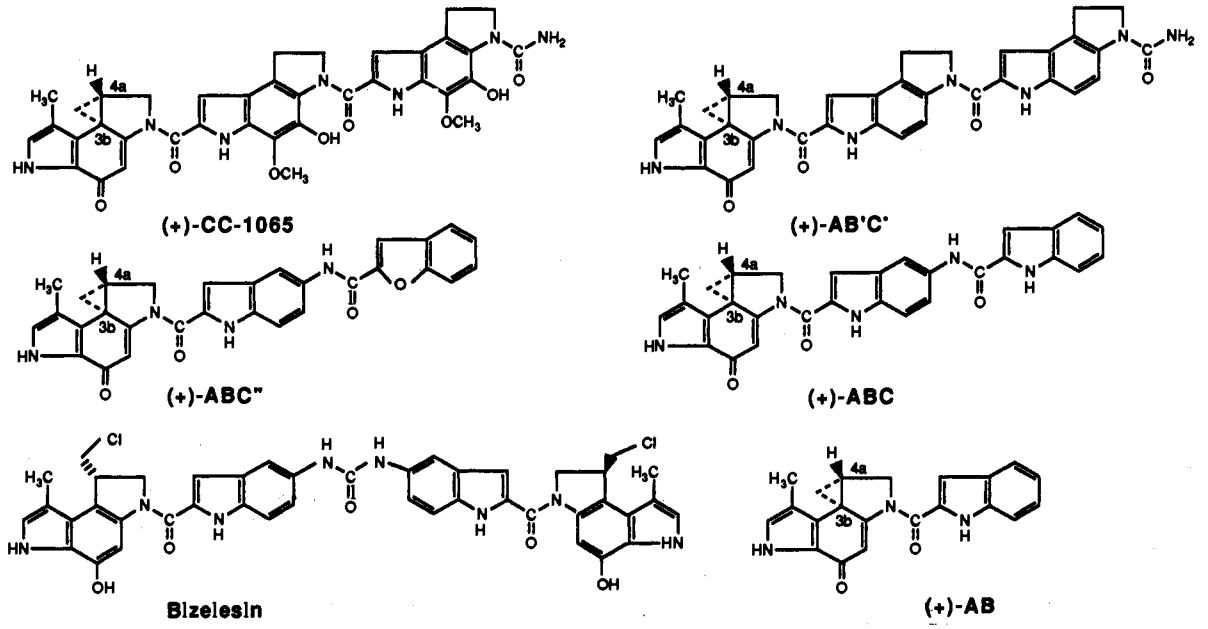

Figure 1. Structures of bizelesin and its monoalkylating analogs (+)-CC-1065, (+)-AB'C', (+)-ABC", (+)-ABC, and (+)-AB.

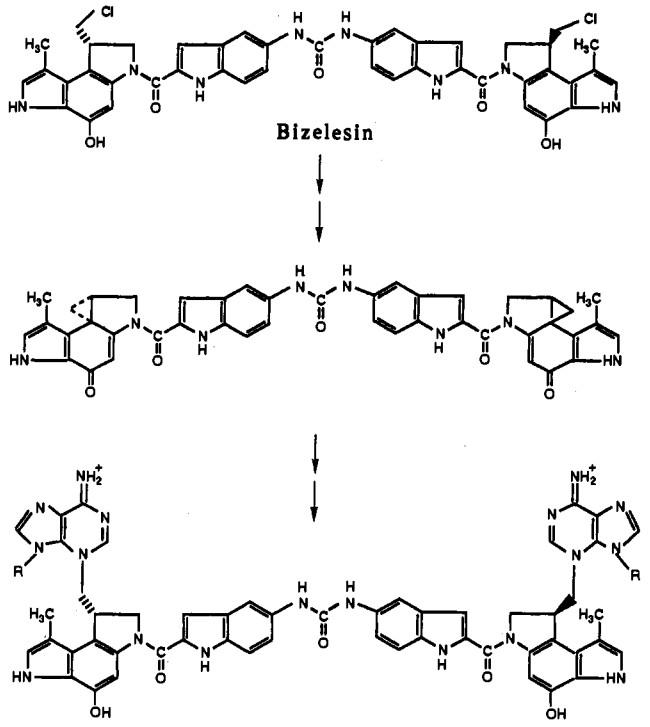

Figure 2. Proposed reaction of bizelesin to form the DNA-reactive CPI and its subsequent reaction with DNA.

DNA from restriction fragments generated from plasmids or viral genomic DNA for monoalkylation and cross-linking sites. Some of these fragments were chosen purposely to address specific questions. As a consequence of this investigation, highly reactive cross-linking or monoalkylating sequences have been identified on the restriction fragments. An analysis of the potential cross-linking sites and monoalkylation sites on these fragments shows that the cross-linking sites have a higher tolerance for GC base pairs (bps) than the monoalkylation sites and that alkylation appears to be favored for certain cross-linking sites over monoalkylation sites. There is also evidence for an effect of drug concentration on specific alkylation sites where these are overlapping. Alkylation at nonadenine bases is also evident within certain specific sequence contexts.

Results

Comparison of the DNA Sequence Specificity of Bizelesin with Monoalkylating Cyclopropylpyrroloindoles. The gel electrophoretic results of a thermal strand breakage assay[12] following drug treatment of the (+)- and (−)-strands of a 134-bp fragment (bp of duplex region) from plasmid pSP6/T3 (Figure 3) with (+)-CC-1065, (+)-AB'C', (+)-ABC", (+)-ABC, (+)-AB, and bizelesin (for structures, see Figure 1) reveal an initial comparison of the sequence selectivity of these various alkylating molecules (lanes 1–6, respectively, in Figure 3). While there are some sites on both (+)- and (−)-strands that are alkylated by all of the drugs to about the same extent (i.e., 5'-CCAATA* and 5'-ACTAAA* on the (−)-strand),[13] other sites (e.g., 5'-AATTCA* on the (+) strand) show considerable difference in reactivity both between individual monoalkylation compounds (e.g., (+)-CC-1065 vs (+)-ABC) and in comparison to the cross-linking compound bizelesin. As we have noted previously,[14] while (+)-CC-1065 and (+)-AB'C' have quite similar sequence selectivities, these drugs also alkylate some sites that are either more or less reactive to (+)-AB and (+)-ABC; meanwhile, (+)-ABC" appears to be related to both subtypes of molecules because it shares most of the bonding sites of both groups of drugs. Unexpectedly, it was found that (+)-CC-1065, (+)-AB'C', and (+)-ABC" could alkylate the guanine on the sequence 5'-GCGTTG*-3' [(−)-strand], while (+)-ABC, (+)-AB, and bizelesin lack the ability to alkylate that site.[15] However, most important from the perspective of this study are the differences in sequence specific alkylation that exist between bizelesin and the monoalkylating CPIs. While bizelesin is most like (+)-ABC and (+)-AB in sequence selectivity, in certain instances it differs from these compounds quite dramatically. For example, bizelesin appears unique in bonding at 5'-TTCAGA* and 5'-TAGTGA* [(+)-strand] and 5'-AATTAA* [(−)-strand] sequences. Some sites are notably much more reactive to bizelesin than the monoalkylating CPIs [e.g., 5'-TAATTA* and 5'-AATTCA* on the (+)-strand].

A quantitative comparison of the sequence selectivity of bizelesin and (+)-AB derived from the results shown in Figure

(12) Reynolds, V. L.; Molineux, I. J.; Kaplan, D. J.; Swenson, D. H.; Hurley, L. H. *Biochemistry* **1985**, *24*, 6228.

(13) The asterisk here and elsewhere indicates the covalently modified base.

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

(15) This is the first report of (+)-CC-1065 modification of guanine, and a more complete description of these results has been submitted for publication (H. J. Park, D. Sun, and L. H. Hurley).

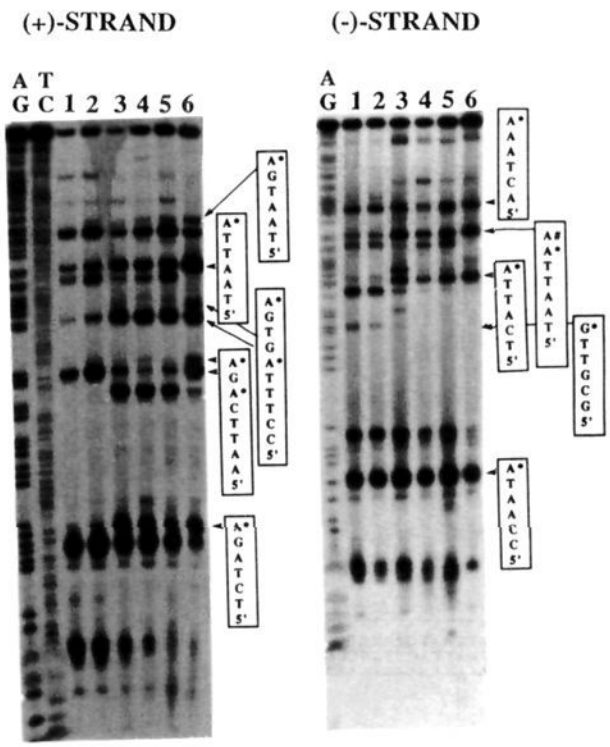

Figure 3. Comparison of sequence selectivity of bizelesin and its monoalkylating analogs on the (+)- and (−)-strands of a 134-bp restriction fragment. 5′ ^{32}P-labeled restriction fragment (134 bps) was modified with 1.4 μM of (+)-CC-1065, (+)-AB′C′, (+)-ABC″, and (+)-ABC, and 14 μM of (+)-AB and bizelesin for 2 days at room temperature. Asterisks indicate the covalently modified adenine or guanine. AG and TC represent the purine- and pyrimidine-specific chemical cleavage reaction. Lanes 1–6 reveal the strand breakage products[12] of the 134-bp restriction fragment previously modified with (+)-CC-1065, (+)-AB′C′, (+)-ABC″, (+)-ABC, (+)-AB, and bizelesin, respectively.

3 is presented in Figure 4, parts A and B, respectively. An examination of this comparative data shows that the most reactive alkylation sites of (+)-AB (Figure 4B) do not correspond to the most reactive alkylation sites of bizelesin (Figure 4A), although many of the sites are common. Significantly, bizelesin bonding sites with high reactivity are often found *six bps* to the 3′ side of thymine and are therefore potential interstrand A–A cross-linking sites (e.g., 5′-TAATTA*), and where unique bizelesin alkyklation sites are found (e.g., 5′-TAGTGA* and 5′-TTCA-GA*), these are also interstrand cross-linking sites. In one case, a small amount of 7-bp potential interstrand cross-linking sequence [5′-TAATTAA* in the (−)-strand] was identified, which was confirmed by nondenaturing gel analysis employing oligomeric DNA.[16] (+)-AB reacts uniquely at some sites (5′-GGGTTA* and 5′-GCTCGAA*) where monoalkylation is precluded for bizelesin. This suggests that the monoalkylation sequence selectivity of bizelesin is higher than for monoalkylation (+)-CPIs (see later).

Concentration Dependency of Sequence Alkylation by Bizelesin. To determine in a quantitative manner the hierarchy of bizelesin alkylation sites on the same fragments as used in the previous experiment, a series of five bizelesin solutions spanning a 10^4-fold dilution were reacted individually with this 134-bp fragment. The bizelesin alkylation sites at each of the concentrations were scored, and the results are diagrammatically portrayed in Figure 5. The most sensitive sites (>0.014 μM) for alkylation by bizelesin are those shown with five circles (5′-TAATTA*) and those least sensitive (>140 μM) with one circle (e.g., 5′-CTCGAA*). Based upon this analysis, the most sensitive alkylation sites are those associated with the potential cross-linked sequence (5′-TAAT-TA*). In previous experiments using nondenaturing gel analysis with a 21-bp sequence containing this sequence, this site was confirmed to be an interstrand cross-linked sequence.[9] Of the monoalkylation sites shown on this fragment, four out of a total of eleven alkylated adenines on the upper (+)-strand are six bps from an adenine on the opposite strand and are thus potential interstrand cross-linked sites. The second most reactive site for alkylation after 5′-TAATTA* is 5′-CCTTTA*, which is a monoalkylation site that is reactive at a 10-fold lower drug concentration. There is a potential cross-linking site (* in Figure 5) that was observed to be unreactive toward bizelesin in this experiment.[18] Cross-linking at guanine is not an automatic consequence of covalent immobilization at adenine six bp removed on the opposite strand. The ability to cross-link at guanine is a function of the intervening sequence.

Bizelesin Alkylation at Non-Adenine Bases and the Effect of Drug Concentration and Alkylation in AT Sequences. Based upon the analysis of the results of the previous experiments using the 134-bp fragment (see before), a second restriction fragment was selected in which longer runs of AT bps containing *overlapping mono and cross-linking sites* were selected for study. Using this restriction fragment, the question of priority for bizelesin between these sites was examined (see Figure 6). In this sequence at the higher molecular weight end of the gel, the sequence 5′-TAAAAA*TA* has both high reactivity monoalkylation (5′-AAAATA*) and cross-linking sites (5′-TAAAAA*). The results shown in the expansion of this region (shown to the right of the main gel in Figure 6) clearly reveal that at low drug concentrations both sites are alkylated approximately equally (lanes 2 and 3 in Figure 6); whereas, at higher concentrations, only the monoalky-lation site at the 3′ end of the AT tract is alkylated. A possible explanation for this concentration-dependent shift in alkylation sites is that the competition for precovalent binding sites at the higher drug concentration results in end-to-end drug binding in this AT-rich region and preclusion of bonding at the TAAAAA* sequence.

In this same fragment at an intermediate molecular weight in the gel (Figure 6), two non-adenine alkylation sites (5′-TCCGCG* and 5′-TTCCGC*) appear to be reactive to bizelesin at a 10-fold higher concentration than the most reactive adenine alkylation sites. At lower molecular weight in the gel, a series of four mono- and/or cross-link alkylation sites is present, in which the potential cross-linkage site, 5′-TATTTA*, is the most reactive.

Comparison of the Alkylation Reactivity of A_5 and A_7 Tracts and Cross-Linking Sequences in the TATA Box to Bizelesin and Monoalkylating CPIs. A further restriction fragment was chosen for examination because it contained both isolated A_5 and A_7 tracts without the potential for competition with interstrand cross-linking and a TATA box sequence (5′-TTTTTATTTA), which has the potential for monoalkylation and interstrand cross-linking. The first question addressed was how bizelesin compares to the monoalkylating compounds in sequence selectivity and reactivity when *only* monoalkylation is possible. The results (Figure 7) show that bizelesin and (+)-ABC″ both show selectivity for the 3′-terminal adenine and its 5′-neighbor in the A_7 tract (sites A and B), but bizelesin has an overall lower reactivity. However, bizelesin predominantly alkylates the 3′-terminal adenine (site A), whereas (+)-ABC″ is equally reactive to both the 3′-terminal and its 5′-neighbor adenine, and (+)-AB is most reactive to the 5′-neighbor adenine of the 3′-terminal adenine (site B). Overall, this result indicates that bizelesin is similar to (+)-CPI compounds in preferentially recognizing the 3′-terminal adenine in A-tracts, presumably due to their intrinsic bent DNA structure and

(16) However, a comparison of the reactivity of the six- and seven-bp sequence in a 21-bp oligomer containing this same sequence confirmed the lower reactivity of the seven-bp site in comparison to the six-bp sequence 5′-TAATTA* (Sun, D.; Hurley, L. H. Unpublished results).
(17) Park, H. J.; Sun, D.; Hurley, L. H. Unpublished results.
(18) However, in a subsequent experiment using a longer incubation time, this sequence (5′-TTTAGTGA*) was shown to be reactive.

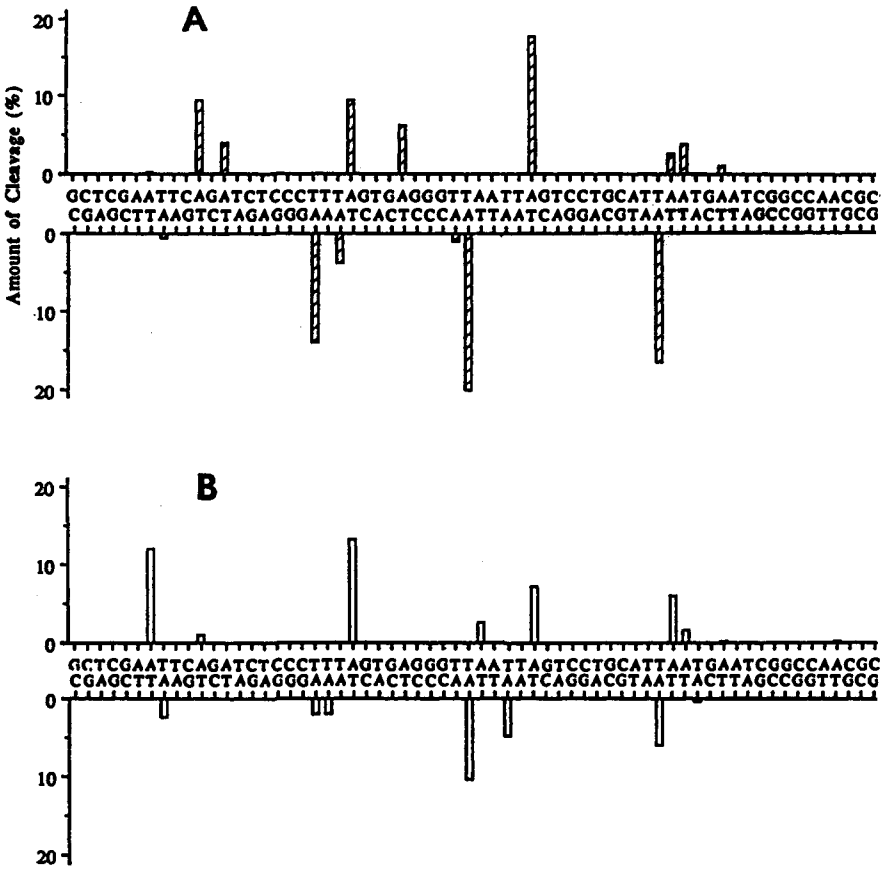

Figure 4. Diagrammatic representation of a comparison of the sequence selectivity of bizelesin (A) and (+)-AB (B) on the 134-bp restriction fragment. The data are from Figure 3 and densitometric measurements were made using an LKB 2202 Ultrascan XL laser densitometer to compute the percentage of the strand breakage at a given site relative to the total strand breakage. Only that portion of the 134-bp fragment between the 34th and the 98th base is shown, although the analysis is based upon alkylation sites over the entire fragment.

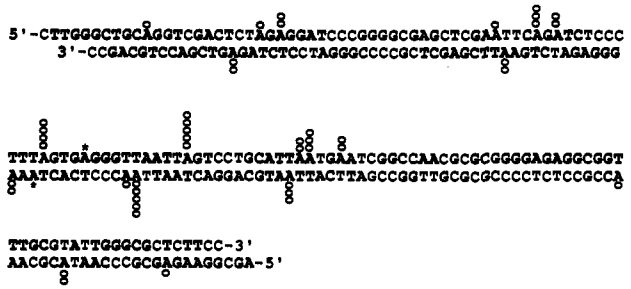

Figure 5. Summary of observed bizelesin strand breakage (alkylation) sites on a 134-bp restriction fragment from plasmid pSP6/T3. Restriction enzyme fragments were prepared as described under Materials and Methods and were singly 5′ end labeled with ^{32}P, incubated with bizelesin (24 h at 20 °C), and heated for 30 min at 95 °C to produce DNA strand breakage. Minimum concentrations at which the bizelesin alkylation sites were observed in this experiment were 0.014 μM (five circles), 0.14 μM (four circles), 1.4 μM (three circles), 14 μM (two circles), and 140 μM (one circle).

associated junction sites.[19] Although all the compounds tested reacted with 5′-TAAAA*, this sequence was less reactive than the sequence 5′-AAAAA*. We suspect this is due to the higher intrinsic bending of A_5 over the TA_4 tract. Both monoalkylating CPIs show the expected low reactivity with 5′-CAGAA* and 5′-AAGTA* sequences, and bizelesin was unreactive to both sequences. A comparison of the results of the alkylation of the TATA box region by bizelesin and the monoalkylating CPIs reveals that bizelesin shows both more reactivity and selectivity for this region, both in terms of adenine alkylated on the A-rich strand (only those adenines 6 bp away from the available adenines of the T-rich strand) and the ability to form cross-linking of DNA. The lower reactivity of the monoalkylatng CPIs toward this region is presumably due to the reactivity of these drug molecules within the A-tract on the opposite strand, which then competes for the alkylation sites on this strand. In the case of bizelesin, the alkylation of the opposite strand to that shown in Figure 7 promotes the alkylation of the other strand to form interstrand cross-linking. In conclusion, the apparent high reactivity of both of these sites in the TATA box region can be explained by examining the alkylation pattern of the opposite strand, which shows high reactivity adenines six bps away from both bizelesin cross-linkage sites.

Relative Cross-Linking vs Monoalkylation Efficiency of Bizelesin and the Consensus Sequence Analysis of Bizelesin Monoalkylation and Cross-Linking Sites. A previous analysis of a catalog of (+)-CC-1065 alkylation sites on restriction fragments from SV40 and T7 DNA had revealed that the sequences 5′-PuNTTA* and 5′-AAAAA* were most reactive at the lowest drug concentration.[12] A more recent study under slightly different experimental conditions has identified the sequence 5′-(T/A)-(T/A)-A* as the consensus.[20] While this is the first study in which the hierarchy of sequence bonding sites for bizelesin have been determined using restriction fragments from plasmid DNA, a previous study using a 21-bp oligomer[9] showed a preference for the cross-linking sequence 5′-TAATTA*. In the present investigation, a total of about 4.0 Kb of DNA from restriction fragments obtained from pBR322, SV40, pCAT, and pSP6/T3 have been screened for bizelesin covalent bonding sites using four different concentrations of drug molecules (0.014, 0.14, 1.4, and 14 μM). Typical autoradiograms showing the results of the thermal strand

(19) Sun, D.; Lin, C. H.; Hurley, L. H. *Biochemistry*, in press.

(20) Hurley, L. H.; Warpehoski, M. A.; Lee, C.-S.; McGovren, J. P.; Scahill, T. A.; Kelly, R. C.; Mitchell, M. A.; Wicnienski, N. A.; Gebhard, I.; Johnson, P. D.; Bradford, V. S. *J. Am. Chem. Soc.* **1990**, *112*, 4633.

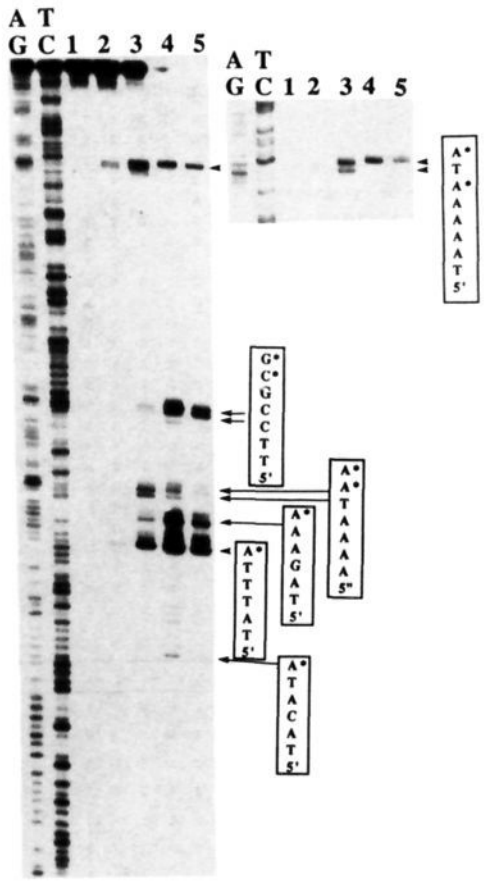

Figure 6. Concentration dependency of bizelesin (alkylation) sites on DNA following thermal treatment of bizelesin–DNA adduct. The DNA in panel A is an Ear I-Hind III fragment (235 bps) from plasmid pBR322 that was 5′ end-labeled on the top strand at the Ear I site. The reaction mixtures (10 μL) consisted of 10 mM NaCl, 10 mM Tris-HCl (pH 7.6), about 20 ng of DNA, and the indicated amount (see below) of drug molecules and were incubated at room temperature for 2 days. AG and TC represent the purine and pyrimidine specific chemical cleavage. Lanes 1–5 contain 0, 0.014, 0.14, 1.4, and 14 μM of bizelesin, respectively. The inset to the right of the main gel is the same experiment, in which the gel electrophoresis was run for a longer period of time to resolve this region.

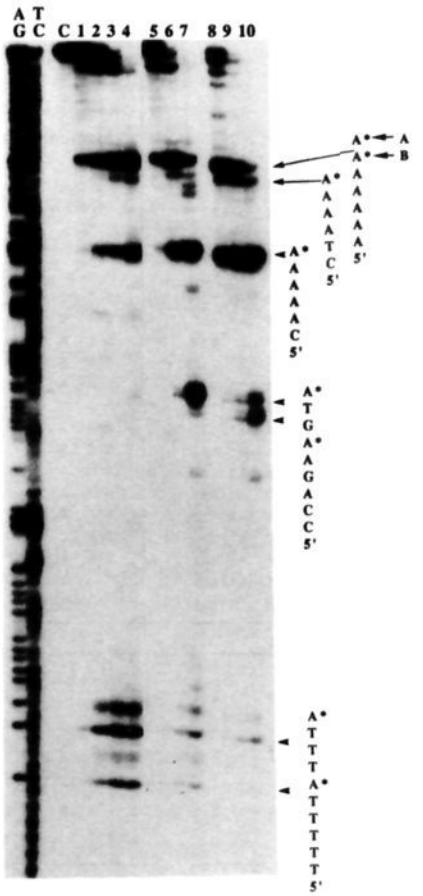

Figure 7. Comparison of the reactivity of bizelesin, (+)-ABC″, and (+)-AB. The DNA in this experiment is an Nco I-Hpa II fragment (304 bps) from plasmid pCAT that was 5′ end-labeled on the top strand at the Nco I site. Lanes 1–4 contain 0.014, 0.14, 1.4, and 14 μM of bizelesin, respectively; lanes 5–7 contain 0.0014, 0.014, and 0.14 μM of (+)-ABC″, respectively; and lanes 8–10 contain 0.014, 0.14, and 1.4 μM of (+)-AB, respectively. The clustered bonding sites at the top region of the autoradiogram were resolved by running the same sample on electrophoresis for a longer period of time.

breakage assay following bizelesin modification of restriction fragments are shown in Figures 6 and 7. In most cases, only one of the two strands was examined for bizelesin alkylation sites.

Overall, a total of 77 bizelesin adenine alkylation sites were found on the 4.0 Kb of DNA at drug concentrations evaluated between 0.014 and 14 μM (Table I, parts A and B). Of these sites, half were potential cross-linkage sites (i.e., had a thymine six bps to the 5′-side of the alkylated base), while the remaining 39 sites were most probably monoalkylation sites. However, the relative frequency of monoalkylation vs cross-linking sites on duplex DNA are underestimated by about 50%, since only one strand of DNA is monitored, and while this reveals *all* the potential cross-linking sites, only the monoalkylation sites on one strand are detected. Therefore, we *estimate* a total of 78 monoalkylation sites (67%) in a combined total of 116 cross-linking and monoalkylation sites on duplex DNA. Based upon the overall GC/AT ratio in these sequences (50/50), we would have only expected a 25% alkylation of potential cross-linking vs monoalkylation sites instead of the 33% found. These results imply that there are factors that predispose potential cross-linking over monoalkylation sites to alkylation. Based upon the observation that the percentage of A vs G vs C at a position six bps to the 5′-side of the alkylated base is 21% vs 14% vs 14%, respectively, this excess of potential cross-linking sites cannot be explained by a precovalent binding preference for AT bps at this position that predisposes bizelesin for cross-linking. Other factors possibly responsible for this enrichment of cross-linking sites relative to monoalkylation sites are discussed later (see Discussion).

The monoalkylating and potential cross-linking (i.e., having a thymine six nucleotides to the 5′-side of the alkylated adenine) sites of bizelesin were divided into three groups based either upon their comparative alkylation reactivity or the nature of the base alkylated: i.e., Tables I, parts A, B, and C represent high reactivity (0.014–0.14 μM), medium reactivity (>0.14 μM but <14 μM), and alkylation of non-adenine bases, respectively. In each table individual nucleotide sequences are listed that include the covalently modified base plus bases five and one nucleotides to the 5′- and 3′-sides of this adenine base, respectively. In Tables I, parts A and B, a sequence analysis for the cross-linking and monoalkylation sites in each group is also provided. For potential cross-linking sequences in Table I, parts A and B, the overall GC content between the two covalently bound adenines (positions +4, +3, +2, and +1) is 13.8% and 40%, respectively. For the equivalent positions the GC content for the monoalkylation sequences (i.e., those sequences lacking a suitable adenine six bps away to cross-link) is 7.1% and 19.1% for high and medium reactivity sites, respectively, which, within experimental error, is in both cases about 50% of that of the cross-linking sites. The simplest explanation for this is that alkylation at one adenine site in a highly reactive monoalkylation sequence (e.g., 5′-TTA*,

Table I

A. High Reactivity Sites[a]

Cross-linking Sites

+5'T	A	A	T	T	A*	A3'
+5'T	A	A	T	T	A*	G3'
+5'T	A	T	T	T	A*	G3'
5'T	A	T	T	T	A*	T3'
+5'T	T	T	T	T	A*	T3'
+5'T	A	G	T	T	A*	A3'
+5'T	A	A	A	A	A*	T3'
5'T	T	T	A	A	A*	G3'
+5'T	T	T	A	A	A*	T3'
5'T	C	A	A	A	A*	T3'
+5'T	A	G	A	A	A*	A3'
5'T	T	T	T	A	A*	A3'
5'T	A	T	T	A	A*	T3'
5'T	C	A	A	T	A*	A3'
5'T	A	C	A	T	A*	G3'
5'T	T	T	G	C	A*	T3'
5'T	T	T	C	T	A*	C3'
5'T	C	A	C	A	A*	A3'

	+5	+4	+3	+2	+1	0	-1
A	0	9	6	7	8	18	7
G	0	0	2	1	0	0	4
T	18	6	9	8	9	0	6
C	0	3	1	2	1	0	1
AT	18	15	15	15	17	18	13
GC	0	3	3	3	1	0	5
GC/N	[13.8%]						

Monoalkylating Sites

5'C	C	T	T	T	A*	G3'
5'G	T	A	T	T	A*	A3'
5'C	A	A	T	T	A*	G3'
5'A	A	A	A	A	A*	T3'
5'C	A	A	A	A	A*	G3'
5'A	A	A	A	T	A*	G3'
5'A	A	A	A	T	A*	A3'
5'G	A	A	A	T	A*	A3'
5'G	A	A	A	T	A*	G3'
5'A	A	A	T	A	A*	A3'
5'A	A	A	T	A	A*	C3'
5'A	C	A	T	A	A*	G3'
5'A	A	G	T	A	A*	A3'
5'A	T	T	G	T	A*	G3'

	+5	+4	+3	+2	+1	0	-1
A	8	10	11	6	6	14	5
G	3	0	1	1	0	0	7
T	0	2	2	7	8	0	1
C	3	2	0	0	0	0	1
AT	8	12	13	13	14	14	6
GC	6	2	1	1	0	0	8
GC/N	[7.1%]						

B. Medium Reactivity Sites[b]

Cross-linking Sites

5'T	C	A	T	T	A*	A3'
5'T	C	C	T	T	A*	C3'
+5'T	G	A	T	T	A*	C3'
5'T	G	T	A	A	A*	C3'
5'T	A	C	A	T	A*	T3'
5'T	C	T	A	T	A*	G3'
5'T	G	G	T	A	A*	C3'
5'T	A	A	T	G	A*	A3'
5'T	A	A	C	A	A*	C3'
5'T	T	T	G	G	A*	G3'
5'T	T	T	G	G	A*	C3'
5'T	A	T	G	T	A*	T3'
5'T	A	T	T	G	A*	A3'
5'T	T	A	T	C	A*	G3'
5'T	T	A	T	C	A*	T3'
5'T	G	A	C	T	A*	A3'
5'T	G	T	G	T	A*	T3'
5'T	G	G	T	C	A*	G3'
5'T	G	C	T	G	A*	C3'
5'T	C	C	A	G	A*	A3'

	+5	+4	+3	+2	+1	0	-1
A	0	5	7	4	3	20	5
G	0	7	2	4	6	0	4
T	20	4	7	10	8	0	4
C	0	4	4	2	3	0	7
AT	20	9	14	14	11	20	9
GC	0	11	6	6	9	0	11
GC/N	[40.0%]						

Monoalkylating Sites

5'G	C	A	T	T	A*	A3'
5'A	T	A	T	T	A*	C3'
5'A	T	A	T	T	A*	T3'
5'C	A	T	T	T	A*	T3'
5'A	C	A	T	T	A*	A3'
5'G	A	G	T	T	A*	G3'
5'G	C	T	T	T	A*	A3'
5'A	G	G	T	T	A*	A3'
5'G	C	A	A	A	A*	A3'
5'G	C	A	A	A	A*	G3'
5'C	G	A	A	A	A*	G3'
5'C	T	A	A	A	A*	T3'
5'C	C	A	A	T	A*	C3'
5'C	A	A	A	T	A*	G3'
5'G	T	A	A	T	A*	C3'
5'C	G	T	A	A	A*	C3'
5'G	G	A	T	A	A*	C3'
5'A	G	T	T	A	A*	G3'
5'C	T	T	T	A	A*	C3'
5'C	T	C	G	A	A*	T3'
5'G	A	A	G	T	A*	G3'
5'A	A	A	G	T	A*	A3'
5'A	A	A	T	C	A*	C3'
5'A	A	T	T	C	A*	G3'
5'A	T	T	T	C	A*	C3'

	+5	+4	+3	+2	+1	0	-1
A	9	8	14	8	9	25	6
G	8	5	2	3	0	0	7
T	0	7	8	14	13	0	4
C	8	5	1	0	3	0	8
AT	9	15	22	22	22	25	10
GC	16	10	3	3	3	0	15
GC/N	[19.1%]						

C. Unusual Adduct Sites[c]

Cross-linking Sites

5'T	A	T	A	T	G*	T3'
+5'T	T	T	T	T	G*	G3'
5'T	T	T	T	T	G*	C3'
+5'T	A	A	T	T	G*	T3'
5'T	C	C	G	C	G*	C3'
5'T	G	T	T	G	G*	G3'
5'T	A	T	A	G	G*	C3'
5'T	A	T	C	T	G*	G3'
5'T	C	T	C	C	T*	T3'
5'T	C	T	G	A	G*	G3'
5'T	T	C	C	G	C*	G3'

Monoalkylating Sites

5'A	A	A	T	T	G*	C3'
5'A	T	A	T	T	G*	G3'
5'A	A	G	T	T	G*	T3'
5'C	T	G	T	T	G*	G3'
5'A	A	C	T	T	G*	C3'

[a] These sequences can be modified with bizelesin at the lower concentration than 0.14 μM bizelesin. [b] These sequences can be modified with bizelesin at the range from 0.14 to 14 μM bizelesin. [c] These sequences can be modified with bizelesin at the lower concentration than 14 μM bizelesin. + These sequences have been confirmed to be cross-linked sites.

TAA*, ATA*, or TAA*) directs the alkylation at a second adenine six bps away, even in normally less desirable alkylation sequences (e.g., 5′-CTA*, 5′-TGA*, 5′-GTA*, and 5′-GCA*), which consequently dictates a higher GC tolerance at cross-linking sites. This explanation may account for the apparent high reactivity of 17 of the 18 sequences listed in Table IA under cross-linking. Of these 17 sites, 15 conform to the 5′-(A/T)(A/T)A* consensus sequence analysis for monoalkylation at the covalently modified adenine. The remaining sequence, 5′-TCACAA*, may have some special structural or dynamic features that confer its intrinsic reactivity. In Table IA, under monoalkylation sites, all of these sequences except two, 5′-ATTGTA* and 5′-AAGTAA*, conform to the consensus sequence 5′-(A/T)(A/T)(A/T)A*, which requires one more (A/T) base pair compared to the consensus sequence 5′-(A/T)(A/T)A* found previously[20] to be reactive to monoalkylation CPIs, indicating that one more (A/T) bp is required to accommodate the A and B subunits of the nonalkylating leg of bizelesin inside the minor groove without steric hindrance.

Analysis of the medium reactivity sites for potential cross-linking (Table IB) shows that these sites have a greater than a 2-fold tolerance for GC pairs than the high reactivity cross-linking sites. Seven of the 20 sites conform to the 5′-(A/T)(A/T)A* consensus sequence for monoalkylation of the reactive adenines, and a further eight have a consensus monoalkylation site on the opposite strand. These numbers are in contrast to the 14 and two in 19 similar cross-linking sites listed in Table IA. The remaining five medium reactivity sites lack consensus sequences at either end of the cross-linking sequence, and three of these sites contain three GC bps.

The monoalkylation sites of medium reactivity (Table IB), in contrast to the equivalent reactivity cross-linking sites, conform mostly (19 of 25 sites) to the 5′-(A/T)(A/T)A* consensus

sequence analysis for monoalkylation. In comparison to the high reactivity monoalkylation sites, a much greater proportion (six of 25 vs one of 14) contains one GC pair in the +1 or +2 positions.

All of the sequences in Table IC are alkylated at *non-adenine* bases: predominantly G in potential cross-linking sequences and exclusively G in the monoalkylation sequences. Of the sequences in the cross-linked category, six of the 11 sequences have a consensus monoalkylation sequence 5′-(A/T)(A/T)A* at the adenine alkylation site. Not surprisingly, four of these sequences are entirely AT, except for the alkylated base. However, what is quite unexpected is that one of the alkylated sequences is entirely made up of GC bps, except for one of the alkylated bases, which is adenine (5′-TCCGCG*).

The non-adenine monoalkylation group consists of five sequences in which the consensus is 5′-A(A/T)PuTTG*. The special reactivity of guanine in this group is of course not dependent upon a suitable positioned adenine on the opposite strand six bps distant from the guanine but must be related to a special characteristic of this sequence.

Discussion

Antitumor agents such as the nitrogen mustards, mitomycin C, *cis*-Pt, nitrosoureas, and carzinophilin have the capability to form intra- or interstrand cross-links on DNA.[22] The interstrand cross-linking of mitomycin C and the nitrogen mustards is proposed to be responsible for the high potency of these compounds.[23] Not unexpectedly, bizelesin is considerably more potent than monoalkylating CPIs and shows different biological properties to the clinical candidate adozelesin. It has been proposed by Hopkins[24] that DNA–DNA interstrand cross-linking occurs preferentially at locations that will result in minimal distortion of DNA. The implication being that cross-links will form most readily at sites where the minimal energy is required to convert a monoadduct to a cross-linked site. With the agents so far investigated, the cross-links are most often formed at sites directly adjacent to each other or separated by one bp. However, with the recent design and synthesis of cross-linking agents based upon monoalkylation compounds such as the CPIs,[7] pyrrolo(1,4)-benzodiazepines,[25] and *cis*-Pt[26] a series of agents forming interstrand cross-links at greater interhelical distances (four to six bps) has emerged. With this larger span of cross-linked sequences comes the possibility of expressing the minimum energy cross-linked structure in more conformationally varied ways and perhaps finding exceptions to the general idea that preferred cross-linking sequences are those that are minimally distorted. For example, the energy of the transition state for formation of a cross-linked structure involving a particular sequence that can exist in a rare and unusual conformation may be much less than for a second sequence of quite similar sequence that cannot assume this conformation and thereby provide a kinetically favored reaction at the first site. Therefore, in addition to quantitation of the proportion of mono vs cross-link species formed on DNA with bizelesin, and determining the consensus sequence selectivity for both types of adducts, we were also interested in identifying especially reactive cross-linking sequences, or perhaps less reactive sequences, that had unexpected cross-linked sequence lengths or contexts, since these might well represent unusual DNA conformations kinetically entrapped by bizelesin, which are not normally observed in significant amounts.

In previous studies at Upjohn[7] and Texas,[9] it has been shown that interstrand cross-linking occurs with high efficiency in the sequence 5′-TAATTA* on adenines six bps apart, which is in accord with expectations based upon a previous knowledge of CPI monoalkylation specificity and molecular modeling. This present, more expansive study using restriction fragments not only provides results that are in accord with this observation but also extends this finding to encompass some less expected results. For example, as expected, interstrand cross-linked by bizelesin occurs preferentially at adenines that are positioned six bps away from a CPI alkylation site on the opposite strand having the consensus sequence 5′-(A/T)(A/T)A*. However, in isolated cases, a potental seven-bp cross-linkage site for bizelesin was identified. Subsequent unpublished oligomer studies have confirmed this 7-bp cross-linkage site.[16,17] In addition, we find that alkylation also occurs at non-adenine bases, most frequently guanine and most often six bp away from an adenine in the CPI consensus sequence. This non-adenine alkylation is presumbly mediated by the covalently immobilized second alkylation arm of bizelesin placed in proximity to a normally unreactive base. In subsequent studies, using oligomers of defined sequence, cross-linking at these sites has been confirmed, and the alkylation at the non-adenine base has been shown to be the second step and occurs much slower than the monoalkylation reaction.[15] Based upon the pattern of strand breakage for 3′- and 5′-labeled fragments that are similar to N3-adenine, alkylation is strongly suspected to occur at N3 of guanine and O2 of cytosine.

A quantitation of the mono vs cross-linking sites shows that there is a somewhat higher proportion of potental cross-linkage sites than expected, based upon a strict statistical analysis; i.e., the percentage of potential cross-linkage to monoalkylation sites was found to be 33% to 67% vs the expected 25% to 75%. A full explanation for this excess of cross-linking sites will have to await a more complete analysis; however, there are at least three possible reasons. First, many of the "cross-linkage sites" are identified only as *possible* cross-linkage sites, because they have an adenine six bps to the 5′-side on the opposite strand. Not all cross-linkage sites have been confirmed by nondenaturing gel analysis (see Table I, parts A and B, and ref 27). Second, the analysis may be biased because some restriction fragments were chosen that had AT-rich regions amenable to cross-linking, and third, the inherently greater instability of monoalkylation vs cross-linking over long periods of time may produce a bias toward the thermodynamically stable cross-links rather than the kinetically favored monoalkylation sites and thus an apparent preference for cross-linkage sites.[28,29] As an extreme case, the *unique* reactivity of some sequences (e.g., 5′-TAGTGA* in Figure 4) to cross-linking by bizelesin in the *absence* of monoalkylation at either of the sites may be rationalized if we assume that the covalent product of monoalkylation is thermodynamically unstable. In this case, it wil not accumulate at the monoalkylation site and will not be visible in the strand breakage assay. However, at potential cross-linking sites, if the monoalkylation product is trapped by the cross-linking reaction, which leads to a thermodynamically more stable product, this will accumulate at monoalkylation sites, which are individually unfavorable.[29] While the futile reversibility of monoalkylation by CPIs has been noted previously, its significance in providing unique cross-linking sites for bizelesin has not been predicted. Thus, it is possible to envisage a strategy of designing cross-linkers that will uniquely form cross-links in the absence

(21) Lin, C. H.; Hill, G. C.; Hurley, L. H. *Chem. Res. Toxicol.* **1992**, *5*, 167.

(22) (a) Pratt, W. B.; Ruddon, R. W. *The Anticancer Drugs*; Oxford University Press: New York, 1979. (b) Gale, E. F.; Cundliffe, E.; Reynolds P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Mechanism of Antibiotic Action*, 3rd ed.; Wiley: New York, 1981. (c) Armstrong, R. W.; Salvati, M. E.; Nguyen, M. *J. Am. Chem. Soc.* **1992**, *114*, 3144.

(23) (a) Kohn, K. W. *Bioscience* **1981**, *31*, 593. (b) Kohn, K. W. In *Development of Target-Oriented Anticancer Drugs*; Cheng, Y.-C., Goz, B., Minkoff, M., Eds.; Raven Press: New York, 1983; pp 181–188.

(24) Hopkins, P. B.; Millard, J. T.; Woo, J.; Weidner, M. F.; Kirchner, J. J.; Sigurdsson, S. T.; Raucher, S. *Tetrahedron* **1991**, *47*, 2475.

(25) Bose, D. S.; Thompson, A. S.; Ching, J.; Hartley, J. A.; Berardini, M. D.; Jenkins, T. C.; Neidle, S.; Hurley, L. H.; Thurston, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 4939.

(26) Qu, Y.; Farrell, N. *J. Am. Chem. Soc.* **1991**, *113*, 4851.

(27) The sequences marked with the symbol (+) were confirmed as cross-linkage sites by nondenaturing gel analysis.[25]

(28) Ding, Z.-M.; Sun, D.; Fan, J.-Y.; Hurley, L. H. Manuscript in preparation.

(29) We thank an insightful referree for pointing out this special case.

of monoalkylation adducts where the thermodynamic stability of the mono and cross-linked adducts differ in the manner described here. While we cannot discount the possibility that the analysis is biased by the first two reasons cited above, we suspect that the thermodynamic factos may play the more important role. Experiments using specifically designed oligomers have been carried out to test this important principle.

An analysis of sequence selectivity of monoalkylation and cross-linking sites by bizelesin leads to some important conclusions. First, bizelesin is more sequence selective than its monoalkylating CPI analogs in the monoalkylation reaction. The consensus sequence analysis obtained from Table I, parts A and B, has revealed that bizelesin is much more reactive toward the sequence 5′-(A/T)(A/T)(A/T)A* than the consensus sequence of (+)-CC-1065 (e.g., PuNTTA* and (A/T)(A/T)A*). This implies that one more (A/T) bp is required for the accommodation of the AB subunit, which is not involved in the alkylation reaction. The preference for an (A/T) bp pair over a (GC) bp pair at the fourth base from the 5′-side of modified adenine also implies that the precovalent binding interaction through a minor groove involving van der Waals contacts of the other AB subunit is important in fine-tuning the sequence selectivity of monoalkylation with bizelesin. In comparison with monoalkylation CPIs, bizelesin alkylates a number of *unique sites*, which generally occur six bps to the 3′-side of a thymine. These are presumably driven by covalent immobilization of the second alkylation arm in proximity to a normally unreactive adenine sequence. In addition, there are a number of sites that are *much more* reactive to bizelesin than monoalkylation by CPIs. Many of these sites are more reactive by virture of having two suitably spaced adenines (six bps apart) both contained in highly reactive alkylation sequences. Thus, although these sites appear to be selective for bizelesin, their special reactivity can usually be explained by a highly reactive monoalkylation site at one end of the bizelesin followed by an extremely rapid second alkylation at the second site increasing the apparent extent of reaction at each site by 2-fold. This observation may in part account for the apparent higher selectivity of bizelesin in comparison to CPIs observed in a previous report.[9]

An analysis of the GC tolerance of cross-linking vs monoalkylation sites of bizelesin reveals that in both the high and medium reactivity sites there is a 2-fold higher tolerance for GC pairs in potential cross-link sites. However, once it is recognized that if alkylation has occurred at one favored monoalkylation site, the second alkylation is driven by a covalently immobilized arm at the second site, which may be normally unfavorable, the GC tolerance for at least most of the cross-linking sites can be explained. Likewise, the previously discussed alkylation at unfavorable bases such as guanine, cytosine, and thymine can also be rationalized. In these situations, cross-linking sequence specificity is lower than monoalkylation.

(+)-CC-1065 was the initial lead compound that provided the inspiration for the development of other monoalkylation CPIs belonging to both the (+)-series (natural configuration of the cyclopropyl group) and the (−)-series (unnatural enantiomer), and now for the interstrand cross-linking compounds represented by bizelesin. (+)-CC-1065 has been characterized as minor groove alkylating compound whose sequence selectivity is primarily determined not by the sequence recognizing noncovalent binding but by the sequence requirements inherent in the covalent bonding step itself.[14] The molecular processes involved in this recognition have been described previously[2,8a] and have been compared from a structural perspective to a mechanism-based or suicide-enzyme inactivation.[8a] Subsequent studies[20] involving the unnatural (−)-series compounds have revealed the importance of minor groove binding interactions unique to CC-1065 and AB′C′ in optimizing the critical hydrophobic interactions that are required to stabilize the unnatural (−)-CPI, which has less favorable precovalent binding interactions with DNA than (+)-series compounds. Bizelesin is similar to (+)-ABC in its sequence selective monoalkylation potential, having the naturally evolved (+)-CPI unit linked to a nonoptimized noncovalent binding moiety. However, the cross-linking reaction is much more promiscuous both in terms of base specificity and sequence context, presumably because of the covalent immobilization of the second alkylating arm in proximity to normally unreactive bases and sequences and also because of the thermodynamic principles described before. In the optimum situation for cross-linking, an adenine in a consensus sequence 5′-(A/T)(A/T)A* provides approximately the same critical DNA interactions found in a high reactivity monoalkylation site, and, consequently, this second step is very fast in comparison to the first step, e.g., 5′-TAATTA*. In less favorable binding sequences (e.g., 5′-TGA*), most probably due to less inherent conformation flexibility, the cross-linking step is slow in comparison to the monoalkylation. Last, where the second base alkylated is not adenine, presumably the less electronically or sterically favored site on guanine, cytosine, or thymine contributes significantly to the slower rate of alkylation.

While most of the putative cross-linked sequences can be rationalized based upon the established reactivity of monoalkylating CPIs and through covalent immobilization of a second alkylating arm, there are a number of unexpected findings. First, some of the unusual cross-link sites, such as 5′-TCCGCG* and 5′-TTCCGC*, even though they have medium or low reactivity, are unexpected sites for potential cross-linking, especially in the absence of monoalkylation products at either end of the six-bp sequence. Second, the high cross-linking reactivity of sequences such as 5′-TTAGTTA, which is a confirmed seven-bp cross-linked sequence,[17] suggests that these sequences may adopt special conformers to allow bizelesin to span these distances. Some of these unusual cross-linked sequences are being explored in more detail using structural and kinetic tools, and at least in one case (5′-TAATTA*) high field NMR studies have shown that the cross-linked sequence contains Hoogsteen base pairs or is base pair opened in the central AT step.[31] Last, the impact of the reversibility of bizelesin-monoalkylated and cross-linked DNA is a consideration in determining the relative proportion of different adducts and their sequence specificity at different times after initial exposure to drug. In unpublished work, we have demonstrated that bizelesin cross-linking is reversible, and the kinetics are both sequence and base dependent.[28]

Materials and Methods

Bizelesin and (+)-CC-1065 and its monoalkylation analogs were obtained from The Upjohn Company. Electrophoretic reagents (acrylamide, bisacrylamide, ammonium persulfate, and *N,N,N′,N′*-tetramethylethylenediamine) were from BioRad. Bacterial alkaline phosphatase was from Sigma. T4 polynucleotide kinase and restriction endonucleases were from New England Biolabs. Plasmid DNA, pBR322, was isolated by the alkaline lysis method[32] and further purified in cesium chloride gradients. SV40 viral DNA was from New England Biolabs, Vector plasmid pSP6/T3 was from BRL, and plasmid pCAT was from Promega.

End-Labeling of Restriction Fragments of Plasmid DNA. Plasmid DNA was routinely digested with the first restriction endonuclease followed by dephosphorylation with bacterial alkaline phosphatase treatment.[12] Restriction fragments (for origin, see figure legends and supplementary material) were purified by phenol/$CHCl_3$ extraction and ethanol precipitation and kinated with [γ-^{32}P]ATP and polynucleotide kinase. 5′-End-labeled restriction fragments were digested with a second restriction endonuclease to generate a unique end-labeled restriction fragment, and the desired fragments were separated on an 8% nondenaturing polyacrylamide gel electrophoresis. The wet gel was exposed onto autoradiographic film, and the desired DNA fragment was located

(30) Warpehoski, M. A.; Harper, D. E.; Mitchell, M. A.; Monroe, T. J. *Biochemistry* **1992**, *31*, 2502.
(31) Seaman, F.; Hurley, L. H. Submitted for publication.
(32) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning. A Laboratory Manual*; Cold Springs Harbor Laboratory: Cold Springs Harbor, NY, 1982; p 122.

and excised. The DNA was removed from the gel by crushing and vortexing at room temperature overnight in 10 mM Tris-HCl (pH 7.6) and 10 mM NaCl.

Drug Treatments of Isolated DNA and Determination of Drug-Bonding Sites. Single 5′-end-labeled restriction fragments were incubated with drugs in 10 mM Tris-HCl, 10 mM NaCl, pH 7.6, for 2 days, unless otherwise indicated, and unreacted drug molecules were removed by phenol/$CHCl_3$ extraction followed by ethanol precipitation. DNA pellets were dissolved in double-distilled water and subjected to thermal treatment at 95 °C for 15 min to induce DNA strand breakage at the drug modification site.[9] After thermal treatment, samples were lyophilized, and the DNA pellets were redissolved in alkaline sequencing dye solution (80% foramide, 10 mM NaOH) and applied to 8% denaturing sequencing gel electrophoresis. After electrophoresis, gels were dried over DE81 paper and exposed X-ray film.

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Supplementary Material Available: SC1 and SC2: Summary of cleavage sites for bizelesin on the sequence of restriction fragment used in the experiment described in Figures 6 and 7, respectively. The numbering system used was as described in the manufacturer's manual (Promega). The relative intensity of cleavage is represented by the number of symbols above a given site. One symbol indicates the medium reactive sites, two symbols indicate highly reactive sites. SC3: Diagrammatic representation of restriction fragments used in the study of sequence selectivity of bizelesin. The arrow head indicates the 3′-side and the end of the arrow indicates the 5′-side of restriction fragments. The 5′-end of restriction fragments were labeled with [γ-^{32}P] using polynucleotide kinase and [γ-^{32}P] ATP (4 pages). Ordering information is given on any current masthead page.