Replacement of the Bizelesin Ureadiyl Linkage by a Guanidinium Moiety Retards Translocation from Monoalkylation to Cross-Linking Sites on DNA

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Abstract: In this contribution we demonstrate that Bizelesin can translocate along the minor groove of DNA from a kinetically favored monoalkylation site to a thermodynamically favored cross-linking site. This translocation is prevented in compounds that have a charged guanidino linkage substituting for the ureadiyl linkage. Furthermore, the manipulative interplay of Bizelesin and the target sequence 5′-TAATTA (Seaman, F. C.; Chu, J.; Hurley, L. H. *J. Am. Chem. Soc.* **1996**, *118*, 5383−5395) that is required to produce a suitably rearranged product for cross-linking is prevented by the substitution of a guanidino for the ureadiyl linkage. A structural basis involving hydrogen bonding of the guanidino linkage with phosphates on the backbone of DNA is proposed to account for the absence of translocation, the slow conversion of monoalkylated to cross-linked species, and the non-rearranged cross-linked product.

Introduction

Bizelesin (Figure 1), a synthetic derivative developed from the potent antitumor antibiotic (+)-CC-1065,[1] was designed as a DNA−DNA interstrand-cross-linking agent to increase the DNA sequence selectivity and efficacy of (+)-CC-1065.[2] Because Bizelesin is mechanistically unique and has improved antitumor efficiency in animal models, it is being evaluated in phase I clinical trials as a possible chemotherapeutic candidate.[3]

Bizelesin, like most cyclopropa[*c*]pyrrolo[3,2-*e*]indol-4(5*H*)-ones (CPIs), covalently modifies N3 of adenine by electrophilic addition.[4] The Bizelesin monoalkylation reaction increases DNA bending in a manner similar to that of (+)-CC-1065.[5] In contrast, the cross-linking reaction (Figure 1) with the sequences 5′-TAATTA* and 5′-TAAAAA* (underlined T indicates that the adenine on the opposite strand is cross-linked by Bizelesin, and asterisked A represents the alkylated adenine) reduces the intrinsic bending of DNA, straightening the DNA.[6]

In our initial sequence selectivity study on Bizelesin, an analysis of the monoalkylating vs cross-linking sequence specificity was made.[7] An unexpected finding was the over-representation of cross-linked adducts in the mixture of cross-linked and monoalkylated adducts. This could be rationalized on the basis of the relative thermodynamic stability of cross-linked vs monoalkylated species and the ability of the monoalkylated reaction to reverse.[8] Although the sequence specificity of cross-linking was more promiscuous than that of monoalkylation, the highly reactive cross-linking sites were largely AT specific. NMR-derived models and structures for two of the most favored Bizelesin cross-linking sites, 5′-TAATTA*[9] and 5′-T$(A)_4$A*,[6d] have been determined, and in both cases, unlike the monoadduct, the cross-linked adducts are straight DNA molecules. For 5′-T$(A)_4$A* the straight form of the A-tract is trapped out by the cross-linking reaction,[6b] and for 5′-TAATTA* the sequence undergoes a proposed drug-manipulated rearrangement of the central two AT base pairs to achieve a straight DNA structure suitable for the cross-linking reaction.[9b]

In the present study we have addressed two important problems related to proposed mechanisms[7] for achieving the higher than expected proportion of cross-linked species. First, we have designed experiments to determine directly whether the monoalkylation reaction can reverse so that Bizelesin can accumulate as cross-linking species. Second, we have examined the structurally related guanidino (Gu) and cyanoguanidino (CyGu) compounds (Figure 1) to determine the importance of the ureadiyl linkage in the cross-linking reaction.

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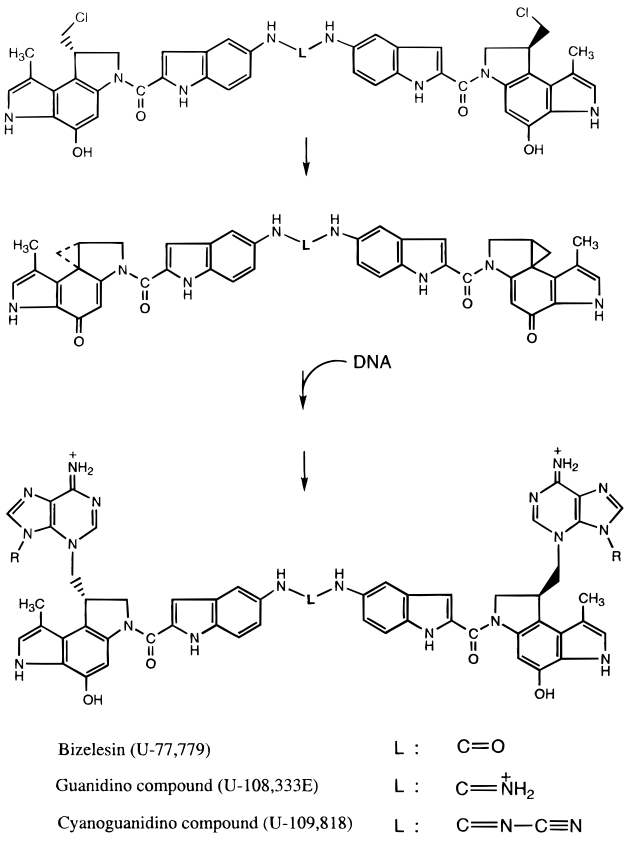

Figure 1. Structures of Bizelesin and its analogues, the guanidino (Gu) and cyanoguanidino (CyGu) compounds, and the reaction of Bizelesin and its analogues to form the DNA-reactive cyclopropyl derivative and their subsequent reaction with adenines of opposite DNA strands to give the cross-linked adducts.

Chart 1. Oligomer DNA Sequences Used in This Study

Overlapped sequence

5′-TGGCCGAAATTAATTACGGGT-3′
3′-GGCTTTAATTAATGCCCAACC-5′

TAATTA Sequence

5′-TGGCCGGCGTAATTAGGGCCC-3′
3′-GGCCGCATTAATCCCGGGACC-5′

Results

Bizelesin Translocates from the Kinetically Favored Monoalkylation Site (5′-AAATTA*) to the Thermodynamically Favored Cross-Linking Site (5′-TAATTA*) in the Overlapped Sequence 5′-AAATTA*ATTA*-3′. A. Bizelesin Forms Either Monoalkylated or Cross-Linked DNA Adducts in the Duplex Sequence 5′-AAATTA*ATTA*-3′. A highly favored sequence for cross-linking by Bizelesin is 5′-TAATTA*-3′.[2,6a−c] Replacement of the 5′-T with an A allows for monoalkylation only. Consequently, combining the exclusive monoalkylation site AAATTA* and the preferred cross-linking site TAATTA* on the same sequence produces 5′-AAA TTA*ATTA*-3′ (overlapped sequence in Chart 1). Since Bizelesin spans six or seven base pairs and interacts directly with the minor groove of the DNA duplex, only one Bizelesin molecule can occupy either the monoalkylation or cross-linking site. By incubating the overlapped sequence with Bizelesin and measuring the rate of formation of the two alkylation products, the kinetically and thermodynamically favored sites can be determined.

In order to first isolate and then identify the two alkylated species of DNA, the overlapped sequence was labeled on both strands in separate experiments and modified with Bizelesin. The drug-modified species were then separated on a denaturing gel based on the differing mobilities of the monoalkylated and cross-linked adducts. As shown in the autoradiogram in Figure 2A, drug modification of the duplex DNA labeled at the top strand produced three species (1−3) and modification of duplex DNA labeled at the bottom strand generated two species (4 and 5). To identify the various drug-modified species, the radiolabeled DNA that generated the bands in the autoradiogram was eluted from the gel and subjected to the thermally induced strand breakage assay.[10] It should be noted that exposure of drug-modified DNA to alkaline conditions such as in piperidine causes a reversal of DNA alkylation, resulting in an increased amount of unmodified DNA.[8b] For this reason, the strand breakage assays were performed without piperidine. However, when piperidine is omitted from the assay, incomplete degradation occurs, which results in strand scission products that have slower mobilities than the corresponding Maxam−Gilbert adenine sequencing reaction (see Figure 2B).[11] The results of the strand breakage assays shown in Figure 2B indicate that bands 1 and 4 are unmodified DNA species, while the DNA from bands 3 and 5, which showed identical mobilities in Figure 2A, was modified at the cross-linking sites. Two alkylation sites on the bottom strand were modified (5′-TAATTA*A*TTT-3′), but the two sites could not be clearly discerned in the autoradiogram due to the overlapping of the incomplete degradation of the product from the 6-base-pair-cross-linked adduct with the completely degraded product from the 7-base-pair adduct. However, when the strand scission products were subjected to high-resolution electrophoresis, the major product was the 6- rather than the 7-base-pair-cross-linked species (data not shown). Minor portions of the DNA obtained from bands 3 and 5 did not appear to be drug modified (top band in Figure 2B). This unmodified DNA is probably due to reversible dealkylation of the drug−DNA adduct during the elution step.

Although the strand breakage assay indicated that band 2 resulted in a mixture of unmodified, cross-linked, and monoalkylated DNA, the major product is the monoalkylated species. The different products shown in the strand breakage assay of the DNA that was produced from band 2 must be the result of the redistribution of Bizelesin during the elution step of the monoalkylated adduct. When the elution time of the monoalkylated adduct was increased, the levels of cross-linked and unmodified DNA also increased (data not shown), which supports our contention that drug redistribution occurs during the elution step. These data suggested to us that the monoalkylated product is unstable and may be a transition product from unmodified to cross-linked DNA. The data also indicate that the eluted cross-linked adduct, which did not convert to monoalkylated adduct, is more thermodynamically stable than the monoalkylated adduct.

B. Monoalkylation Is Kinetically Favored whereas Cross-Linking Is Thermodynamically Favored in the Overlapped Sequence. To confirm our assumption concerning the instability of the monoalkylated product and the stability of the cross-linked product, we carried out a time-course experiment designed to analyze the relative kinetics of the alkylation reactions. In this experiment the overlapped sequence radiolabeled on the top strand was incubated with Bizelesin, and then

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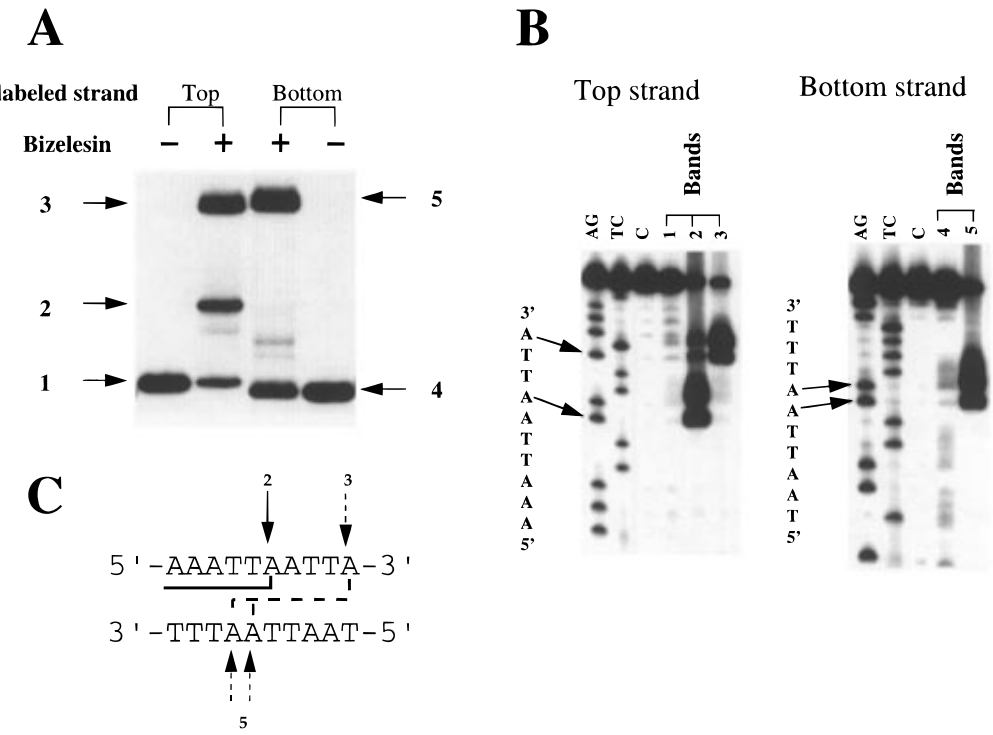

Figure 2. Isolation of two Bizelesin-alkylated species and verification of their alkylation sites. (A) Samples labeled at the top (+) and bottom (−) strands of the overlapped sequence were modified with Bizelesin and separated on a 20% denaturing polyacrylamide gel. (B) DNA from the bands was eluted, and a strand breakage assay[10] was performed to determine the alkylation sites. The numbers at the top of each lane correspond to those of bands from Bizelesin-modified lanes in panel A. Arrows and sequence to the left of the gel indicate alkylation sites. AG and TC, sequencing lanes; C, control without Bizelesin modification. (C) Alkylation sites of the overlapped sequence. Solid and dotted arrows indicate monoalkylation and cross-linking sites, respectively.

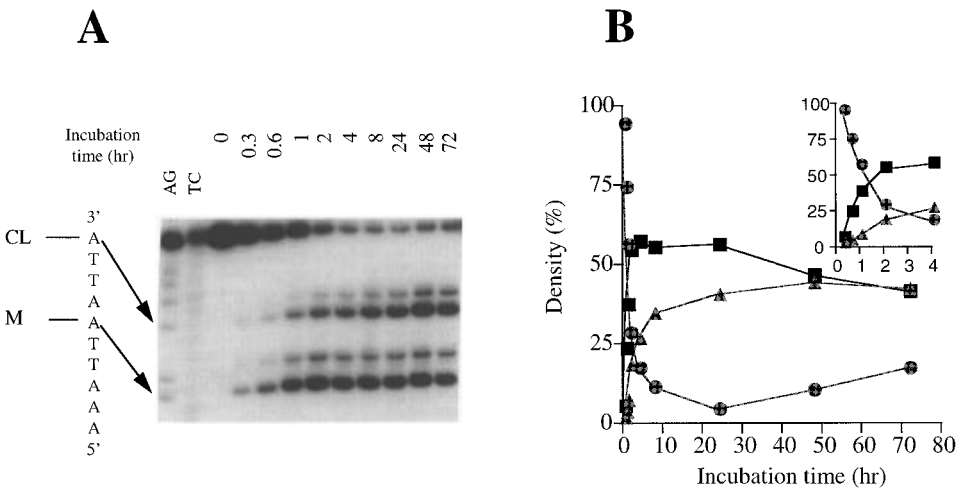

Figure 3. Kinetics of Bizelesin modification on the overlapped sequence. (A) Strand breakage assays of the time-course Bizelesin modification experiment of the overlapped sequence. The numbers at the top of each lane indicate incubation time in hours. CL and M indicate cross-linked and monoalkylated adenines, respectively. (B) Densitometric analysis of alkylation sites. Percentile densities of unmodified DNA (●), monoalkylation site adduct (■), and cross-linking site adduct (▲) were plotted over time. Inset: Detail plot for the short time range.

strand breakage assays were performed at various time points. As indicated in the previous section, the unstable character of the monoalkylated adduct hindered the isolation of a single pure species from a gel. Therefore, the alkylation profiles for adducts in the reaction mixture were determined rather than for the isolated species. The gel analysis of the time-course experiment and the densitometric analysis of alkylation sites are shown in Figure 3, panels A and B, respectively. As shown, the DNA was rapidly monoalkylated, but the percentage of monoalkylated product (squares in Figure 3B) decreased slightly over the 72-h period. In contrast, the cross-linking site was more slowly alkylated, with the percentage of cross-linked product (triangles in Figure 3B) increasing over the 72-h period. These results confirm that monoalkylation occurs more rapidly than cross-linking and that the monoalkylated adduct is less stable than the cross-linked adduct over time. The data from Figure 3B also indicate that the percentage of unmodified DNA (circles) decreased dramatically during the first 24 h of incubation but then gradually increased during the remainder of the incubation period, suggesting that reversible alkylation had occurred and that some of the product was unable to react with DNA again. The decrease in monoalkylated DNA together with the increase in both cross-linked and unmodified DNA over the 24-h period suggests that monoalkylated DNA can convert to either cross-linked or unmodified DNA. These data support our contention that in the overlapped sequence the monoalkylation of DNA

by Bizelesin may be an intermediate step from unmodified to cross-linked DNA.

C. Pathway of Drug from the Monoalkylation to the Cross-Linking Site in the Overlapped Sequence Is Primarily through Translocation. When the overlapped sequence is modified with Bizelesin, the drug preferentially alkylates the monoalkylation site during the early reaction period, but then the unstable monoalkylated adduct slowly reverses and converts to the stable cross-linked adduct. Two possible transfer mechanisms were considered for how the drug molecule moves from the monoalkylation site to the cross-linking site. In the diffusion mechanism, free drug is released from the minor groove of the DNA duplex and diffuses to the cross-linking site or back to the monoalkylation site. In the second mechanism, called translocation, the drug molecule is released from the monoalkylation site but remains localized on DNA and moves along the minor groove before alkylating the DNA at the cross-linking site. To differentiate between these two possibilities, unlabeled competitor DNA was added to the incubation mixture. If diffusion is responsible for the conversion, the addition of competitor DNA would significantly reduce the accumulation of the cross-linked DNA adduct by diluting out the drug released from the minor groove. If translocation is the predominant pathway, competitor DNA would not affect the accumulation of the cross-linked DNA adduct.

Radiolabeled overlapped DNA that had previously been modified with Bizelesin and then treated to remove the free drug was incubated both with and without the unlabeled overlapped sequence competitor DNA. Figure 4 shows the gel analysis of the experiment and densitometric profiles of each species in the absence and presence of competitor. The data in Figure 4B, which shows each species as a percentage of the total DNA in the reaction, indicate that at time zero 22% of the DNA was unmodified, 43% was monoalkylated, and 35% was cross-linked. In the absence of competitor DNA (open symbols), as the incubation time increased, the amount of monoalkylated DNA decreased dramatically (from 43% to 22% after 6 days) and the amount of cross-linked DNA increased significantly (from 35% to 53% after 6 days). The amount of unmodified DNA was also slightly increased (from 22% to 25% after 6 days). These data suggest that the increase in the amount of cross-linked DNA was principally caused by the released drug generated from the reversible dealkylation of monoalkylated DNA. When competitor DNA was added to the reaction (closed symbols), the formation of cross-linking site adduct was not appreciably affected, indicating that the transfer to the cross-linking site adduct is independent of competitor DNA and suggesting that cross-linking proceeds by a mechanism that does not involve diffusion outside the minor groove, i.e., by translocation. The small decrease in the amount of cross-linking site adduct observed in the presence of competitor DNA may have resulted from a minor loss of the cross-linking adduct due to reversible dealkylation at the cross-linking site. Meanwhile, the addition of competitor DNA drastically affected the amount of unmodified DNA at the end of the incubation period. Presumably, any Bizelesin resulting from the reversal of monoalkylation that diffuses from the DNA is trapped by the competitor DNA. In summary, these data suggest that the transition of Bizelesin−DNA adduct formation from the monoalkylation site to the cross-linking site was accomplished principally by the translocation mechanism.

D. Substitution of the Ureadiyl Linkage of Bizelesin with a Gu Rather than a CyGu Linker Prevents Translocation to the Cross-Linking Site. In order to determine the importance of the ureadiyl linkage in mediating the translocation reaction from the kinetically favored monoalkylating site to the thermodynamically favored stable cross-linking site, the Gu and CyGu compounds were synthesized.[12] The time-course monoalkylation and cross-linkage of all three compounds are shown in Figure 5. While Bizelesin and the CyGu compound monoalkylate and cross-link the two available sites with about the same efficiency, the Gu compound alkylates the monoalkylation site with even greater efficiency but only alkylates at the cross-linking site at a very low efficiency. In subsequent experiments, translocation from the monoalkylation to the cross-linking site was demonstrated for both the Bizelesin and CyGu compound, but the Gu compound failed to undergo the translocation reaction (unpublished results).

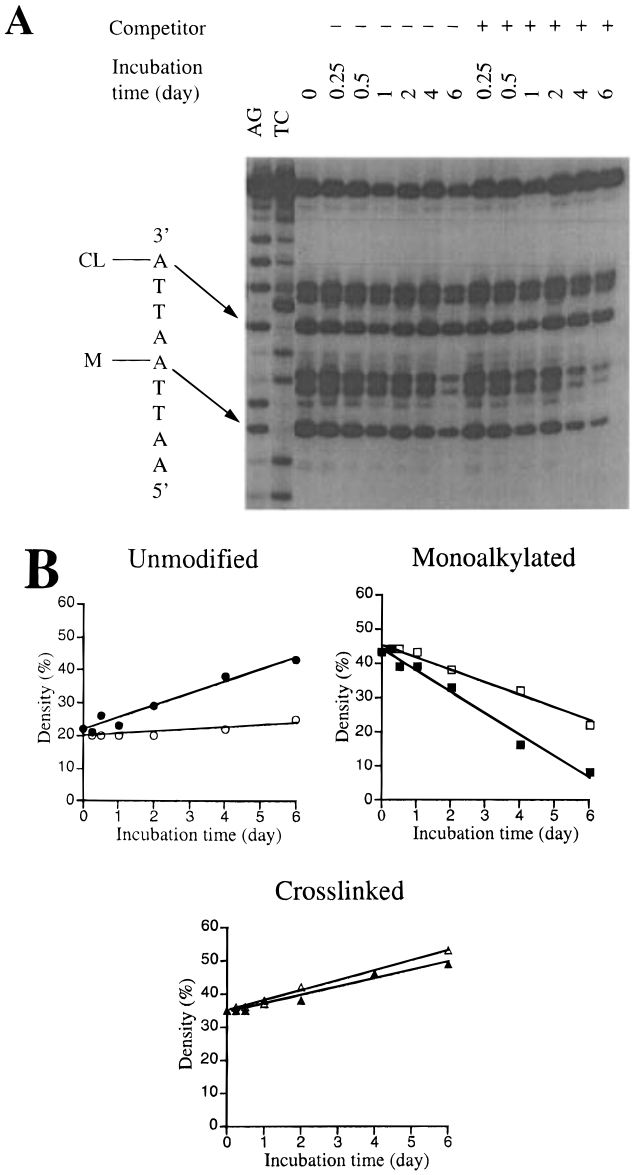

Figure 4. Effect of competitor DNA on translocation of alkylated adducts. (A) Strand breakage assays of Bizelesin-modified overlapped sequence in the absence and presence of overlapped sequence competitor DNA. The initial reaction mixtures (at time 0) were prepared by Bizelesin modification of the overlapped sequence DNA (see Experimental Procedures) and incubated without (−) or with (+) cold competitor DNA at 37 °C. The numbers at the top of each lane indicate incubation time in days. (B) Densitometric analyses of transition of alkylation adducts in the absence (open symbol) and presence (closed symbol) of competitor DNA. Percentile densities of unmodified DNA (circle), monoalkylated adduct (square), and cross-linked adduct (triangle) were plotted over time.

(12) Xiong, H.; Kelly, R. The Pharmacia Upjohn Co., unpublished results.

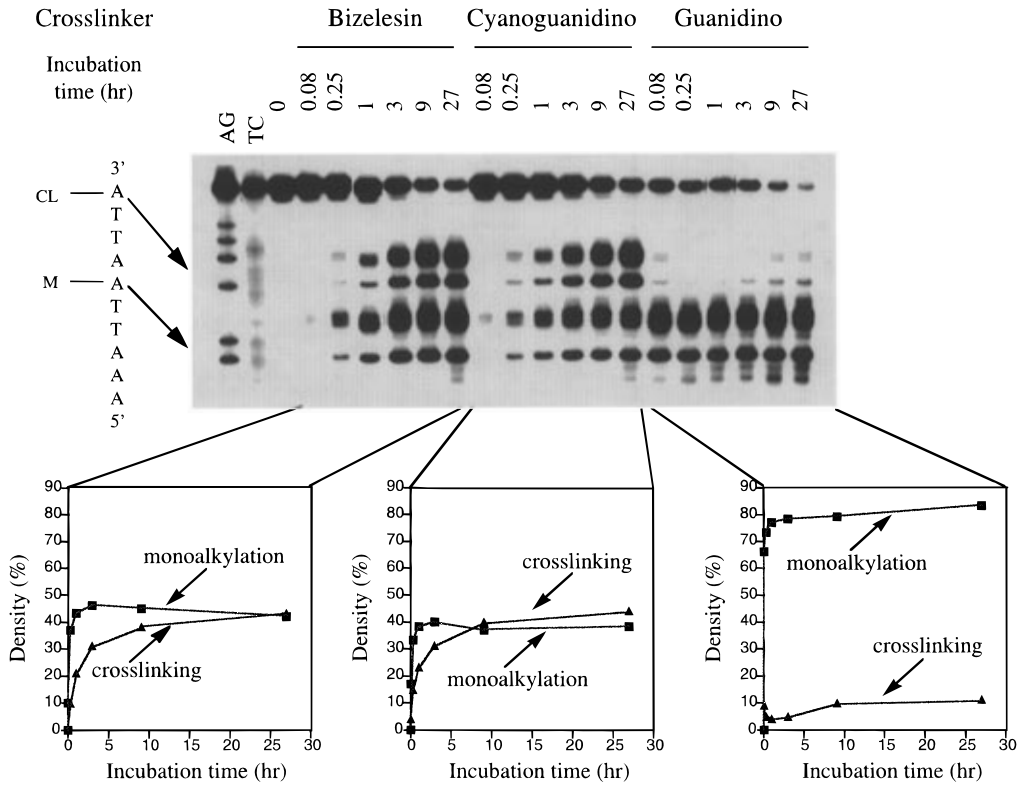

Figure 5. Comparison of alkylation of three CPI cross-linkers on the overlapped sequence. Reaction mixtures at the different times were subjected to a strand breakage assay and analyzed on a 16% polyacrylamide denaturing gel. Percentile densities of monoalkylation site (■) and cross-linking site (▲) adducts were plotted over time.

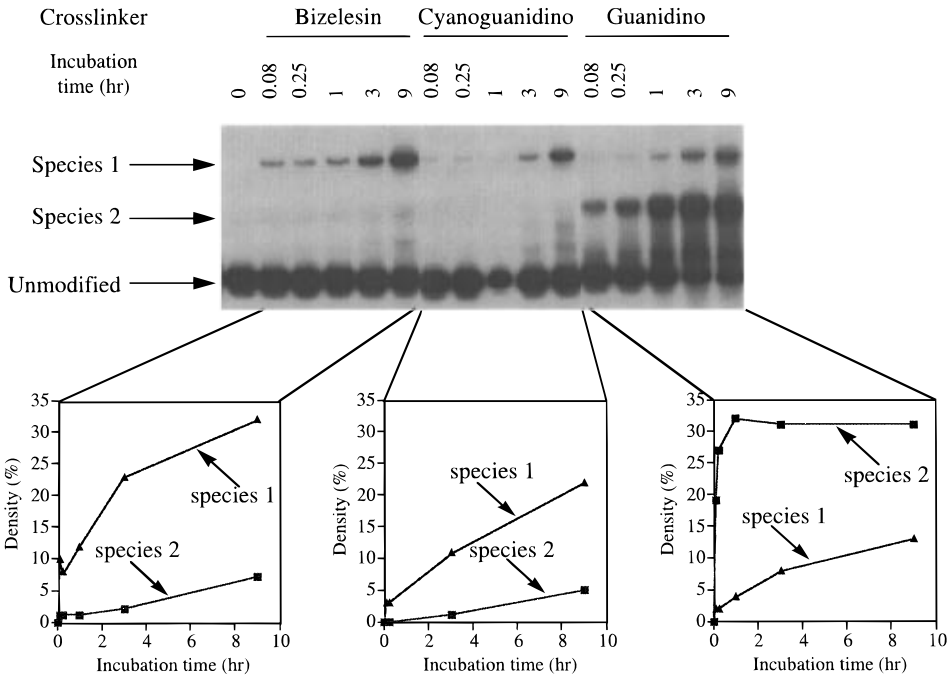

Figure 6. Comparison of alkylation of three CPI cross-linkers on the TAATTA sequence. After alkylation at different time periods, reaction mixtures were separated on a 16% polyacrylamide denaturing gel. Percentile densities of species 1 (cross-linking; ▲) and species 2 (monoalkylating; ■) were plotted over time.

While the Kinetics of Cross-Linking and Products of the Reaction of Bizelesin and the CyGu Compound with 5′-TAATTA* Appear Similar, They Are Dramatically Different from Those of the Gu Compound. A. While All Three Compounds Cross-Link the Sequence 5′-TAATTA*, Only in the Case of the Gu Compound Is the Conversion of Monoalkylating to Cross-Linking Species Rate Limiting. To address directly why the sequence 5′-TAATTA* is only very poorly cross-linked by the Gu compound, the kinetics of the reaction of each of the three compounds with this isolated sequence (TAATTA sequence in Chart 1) was determined. In comparison to Bizelesin and the CyGu compound, the extent of cross-linking after 9 h by the Gu compound is much less (13% vs 22% and 32%) (Figure 6). In contrast, the overall kinetics of alkylation, predominantly in the form of monoalkylation, is much greater for the Gu compound; i.e., after 15 min

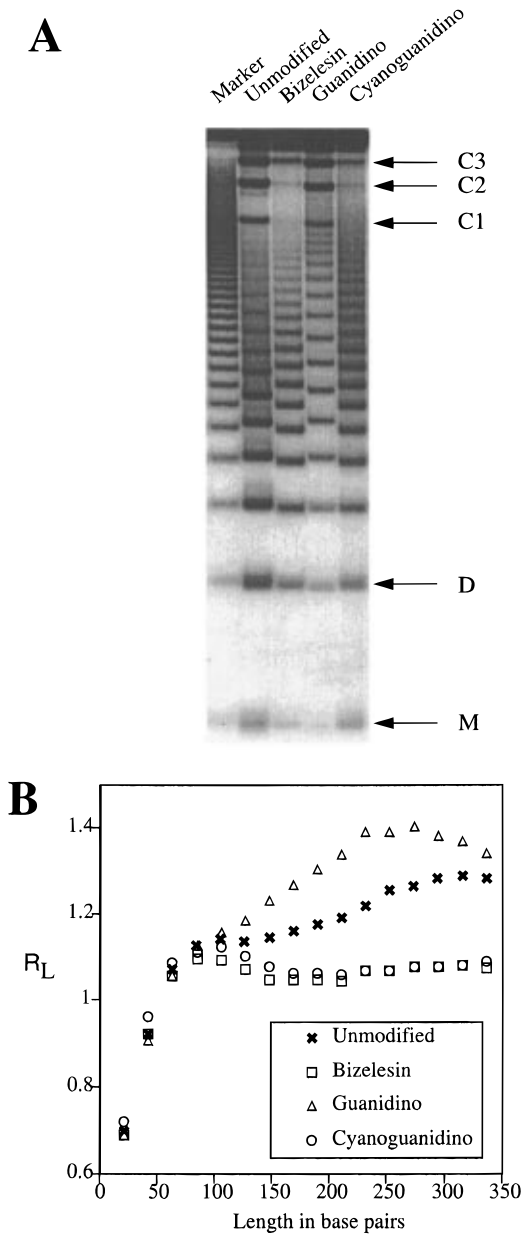

Figure 7. Comparison of relative mobilities of cross-linked adducts. (A) Autoradiogram of ligation products of the TAATTA sequence-cross-linked DNA adducts modified with three CPI cross-linkers. M, D, and C1−C3 indicate monomer, dimer, and circular forms of ligation products, respectively. (B) Plot of relative value (R_L) versus total length of oligomers in base pairs.

the Gu total alkylation is >25%, while for Bizelesin and the CyGu compound there is about 9% and 3% total alkylation, respectively. Clearly, while Bizelesin and the CyGu compound appear to have similar monoalkylation and cross-linking reactivities in the sequence 5′-TAATTA*, the Gu compound has quite distinct reactivities.

B. While Bizelesin and the CyGu Compounds Straighten the 5′-TAATTA* Sequence after Cross-Linking, the Gu Compound Increases the Bending of This Sequence. In order to examine the overall structural effects of cross-linking 5′-TAATTA* by Bizelesin and the CyGu and Gu compounds, 21-base-pair oligomers containing the cross-linking sequence were modified in parallel experiments with each of these three compounds. The cross-linked oligomers were purified and then incubated with T4 DNA ligase prior to examination of their gel mobility on a nondenaturing gel (see Figure 7A). In comparison to the unmodified ligated 21-mer, which shows gel mobility characteristics of a moderately bent DNA structure (expressed as an R_L value[13] of about 1.2), the Bizelesin- and CyGu-modified oligomers show reduced R_L values (1.0), while the Gu-modified 21-mer oligomer shows an enhanced R_L value (1.4). The absence of low molecular weight circular DNA (C1 and C2 at the top of the gel, indicated by arrows) in the Bizelesin and CyGu lanes also supports the straightening of the intrinsic bending associated with the unmodified DNA, while the Gu compound retains the circularized DNA species.

Two-Dimensional ^{1}H-NMR and Molecular Modeling Reveal the Structural Origin of the Differences between the Gu- and Bizelesin-Cross-Linked Species. In order to determine the structural differences between the Gu-cross-linked species and the apparently similar Bizelesin- and CyGu-cross-linked species, a combined two-dimensional ^{1}H- and ^{31}P-NMR and molecular dynamics study on the Gu-cross-linked duplex d(CGTAATTA*CG)$_2$ was carried out, and a comparison to the previously reported Bizelesin and unmodified duplex was made.[9] On the basis of this comparison, we hoped to gain some insight into those structural features of the Gu-cross-linked species that give rise to the slower kinetics of conversion of monoalkylation to cross-linking species and the inability of the Gu species to translocate from the monoalkylation site to the cross-linking site.

A. Gu-Cross-Linked Species Maintains Normal Base Pairing within the Duplex in Contrast to the Bizelesin-Cross-Linked Species, Which Has a Partially Rearranged or Base-Paired Opened Form. A comparison of the aromatic base H8/H2 to H1′ and H3′ NOESY cross-peak spectral regions for the unmodified duplex and Bizelesin- and Gu-cross-linked species is shown in Figure 8. It is clear that while the Bizelesin species shows, as previously demonstrated, connectivities indicative of a Hoogsteen base-paired AT-step region, the Gu-cross-linked species resembles the uniformly Watson−Crick base-paired B-form DNA of the unmodified duplex. In both the symmetrical Gu adduct (Figure 8A) and the unmodified duplex spectra (Figure 8B), the AT-step adenine, 5AH8, displays the standard cross-connectivity to its attached sugar's H1′ (intraresidue) and the 5′-side 4AH1′ (interresidue). The 5AH2 signal shows the expected interstrand (e.g., 7TH1′) and intrastrand (e.g., 5AH1′) H1′ connectivities. In contrast, the symmetrical Bizelesin-cross-linked structure yields cross-peaks typical of *syn*-oriented AT-step adenines (Figure 8C). An intense cross-peak links 5AH8 to 5AH1′, consistent with base rotation from *anti*- to *syn*-orientation. Equally consistent are the 5AH2 major groove contacts expressed as weak cross-peaks with the 5′-side 4A residue.

B. Modeling of the Gu-Cross-Linked Species Reveals Strong Hydrogen Bonding of the Gu Protons to Phosphates on Both Strands of DNA. The ^{31}P-NMR chemical shifts (based on ^{31}P−^{1}H correlation data) for the unmodified 10-mer and the two adducts (Figure 9) also indicate a retention of a 2-fold axis of symmetry in the adducts. The Bizelesin- and guanidino analogue-cross-linked 10-mers differ in the patterns of chemical shift divergence from the corresponding signals of the unmodified duplex. Whereas the most upfield-shifted ^{31}P-NMR signals of the Bizelesin adduct occur in the 5′-AATT region, this region is considerably less affected in the guanidino analogue adduct, wherein the major chemical shift divergence occurs for the phosphate group between 7T and 8A (most upfield-shifted ^{31}P-NMR signal). The proximity of this phosphate moiety (of strands 1 and 2) to the guanidino linker suggests that the unusual 7T−8A ^{31}P-NMR chemical shift results from the torsional and/or electrostatic effects of the hydrogen-bonding interaction of

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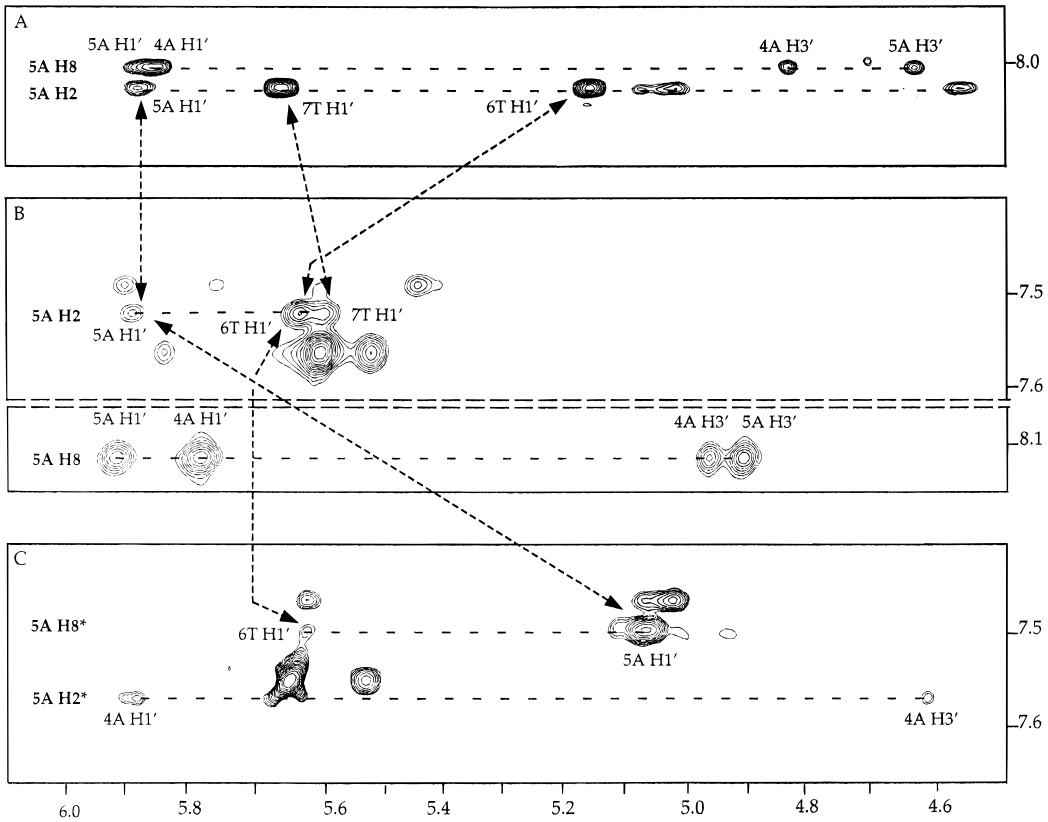

Figure 8. Comparison of the AT-step adenine's H2/H8 to deoxyribose H1′ and H3′ NOESY cross-peaks for the Gu analogue adduct (A), the unmodified 10-mer duplex (B), and the Bizelesin adduct (C). AT-step adenine minor groove base proton (either H8 or H2) cross-peaks to equivalent DNA sugar protons are connected by dotted lines for the three spectral expansions. Asterisks indicate *syn*-oriented 5A bases typical of Hoogsteen base pairing.

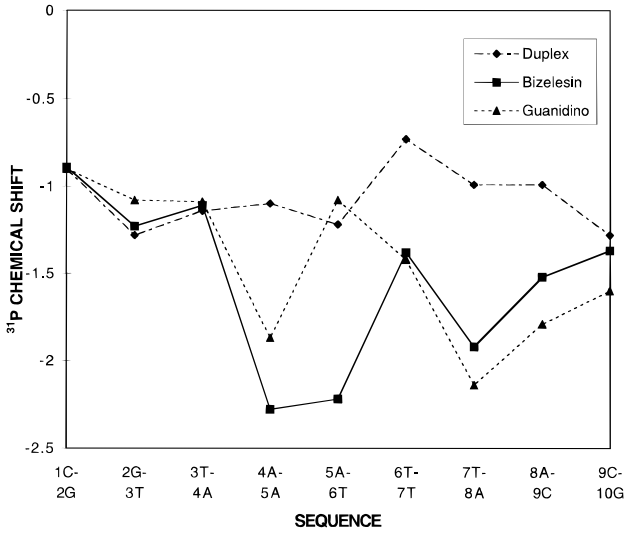

Figure 9. Comparison of the ^{31}P-NMR chemical shifts for the unmodified 10-mer, Bizelesin 10-mer adduct, and guanidino analogue 10-mer adduct. Chemical shifts (vertical axis) are plotted against the 10-mer base sequence (horizontal axis).

the negatively charged phosphate group and the positively charged guanidinium moiety. To examine this, solvated molecular dynamics was conducted to observe the interactions of potential hydrogen bond drug donors and DNA acceptors: the Bizelesin ureadiyl linker and the Gu analogue's positively charged Gu linker differ in that the former contains a pair of hydrogen bond donors directed into the minor groove of the 5′-AATT region while the latter contains, in addition to the same pair of internally directed donor atoms, a pair of externally directed donors. Whereas the ureadiyl linker is limited to minor groove hydrogen-bonding targets (e.g., thymine O2 or adenine N3), the Gu linker can target these same acceptors together with acceptors accessible to the external donors. Preliminary restrained molecular dynamics (solvated, AMBER 4.1) of the Gu analogue−10-mer-cross-linked adduct results in a stable structure (Figure 10, left) with a symmetrical array of bridging water molecules between the Gu external donors and the 7T−8A phosphate oxygen acceptors of the two strands (Figure 10, right).

Discussion

The inspiration for this investigation was our earlier observation that Bizelesin-cross-linked species were overrepresented in comparison to monoalkylation species in experiments in which restriction enzyme fragments were incubated with Bizelesin.[7] We had postulated that one source of this overabundance was the greater thermodynamic stability of cross-linked species than the monoalkylated species coupled with the inherent reversibility of the monoalkylated product.[8b] In this contribution we have directly tested the postulate that the monoalkylated species at a non-cross-linked site can reverse and move to a cross-linked site. We also examine whether or not the regenerated Bizelesin can translocate along the DNA helix from the monoalkylation to the cross-linking site. Last, we evaluate the importance of the ureadiyl linker region of Bizelesin in monoalkylation reversal, translocation, and cross-linking reaction.

In order to test the reversibility of monoalkylation and the transfer to a cross-linking site, the overlapped sequence shown in Chart 1 was designed. Alkylation at the monoalkylation site was faster than at the cross-linked site (7.4×10^{-3} s^{-1} vs 2.6×10^{-3} s^{-1}, see Figure 4B) at the initial stage, but over an extended period of time, the amount of monoalkylation species

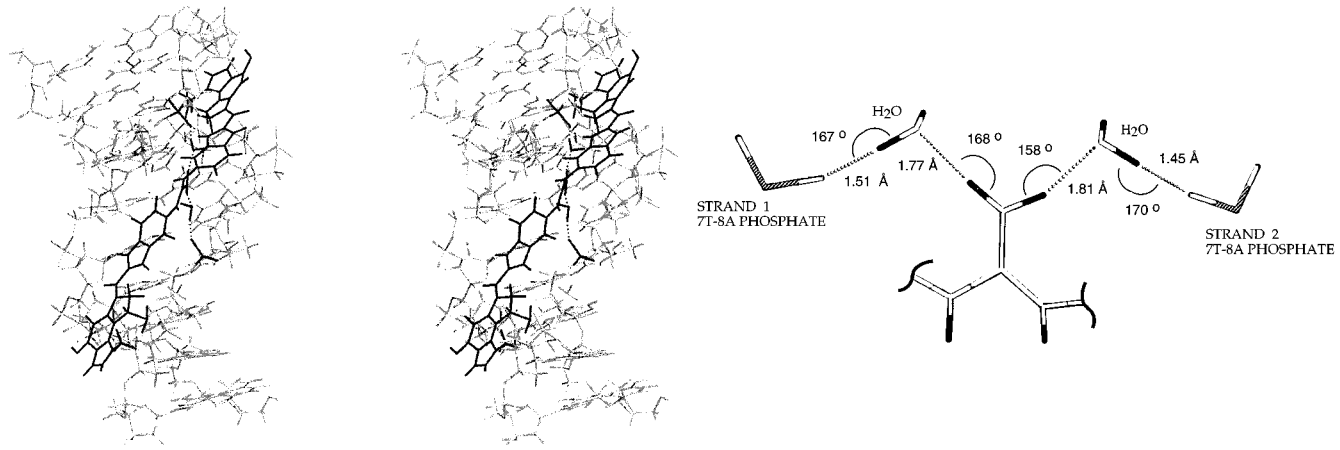

Figure 10. (Left) Stereodiagram derived from the final coordinates produced by the rMD (solvated) analysis (Amber 4.1: Pearlman, D. A.; Case, D. A.; Caldwell, J. W.; Ross, W. S.; Cheatham, T. E.; Ferguson, D. M.; Seibel, G. L.; Singh, C.; Weiner, P. K.; Kollman, P. A. AMBER 4.1, University of California, San Francisco, 1995) of the Gu analogue 10-mer adduct. The two hydrogen bond donors of the Gu linker portion of the drug (bold) are each bridged by a water molecule (bold) to the phosphate oxygen acceptor moiety (bold) of opposite DNA strands (hydrogen-bonding bridge indicated by dotted lines). (Right) Portion of rMD (solvated) structure depicting the two water molecules bridging the guanidinium linker unit to the 7T−8A phosphate oxygens (O1P) of strands 1 and 2. Angles (in degrees) indicate the angle formed by donor−H−acceptor and distances (Å) refer to hydrogen−acceptor distance.

decreased and the cross-linked species increased. Direct transfer was demonstrated by starting with a set amount of monoalkylated species in the absence of free drug and following the time-course loss from the monoalkylation site and accumulation of the cross-linked species. Since the presence of the same-sequence unlabeled DNA had little effect on the accumulation of cross-linked species, it appears likely that Bizelesin translocates along the minor groove from the monoalkylation site to the adjacent cross-linked site rather than diffusing away from the DNA before rebinding in the minor groove. This mechanism for transfer from one site to another may not be universal, since in non-overlapped sites separated by two GC base pairs, translocation seemed to be a minor pathway and the accumulation of cross-linked species was reduced (S.-J. Lee and L. H. Hurley, unpublished results).

The importance of the ureadiyl linkage in the translocation from monoalkylated to cross-linked site was examined by substituting either a CyGu or the positively charged Gu linkage for the ureadiyl linkage (see Figure 1). While the neutral CyGu linkage substitution did not drastically affect the translocation from monoalkylation to cross-linked sites or, apparently, the structure of the cross-linked product, the charged Gu species did not translocate and had an altered cross-linked structure. Examination of the isolated Gu-cross-linked 5′-TAATTA* sequence showed a bent DNA without the typically central Hoogsteen base-paired adduct found in Bizelesin-cross-linked 5′-TAATTA*. Moreover, the kinetics of the monoalkylating and cross-linking reactions of Bizelesin and the Gu compound were quite different. For Bizelesin the slow reaction is the monoalkylation step, while for the Gu compound the cross-linking reaction is the slow step.

A structural analysis revealed that the positively charged Gu group is probably hydrogen bonded via two water molecules to phosphates on opposite strands of DNA (Figure 9), and at the monoalkylation step this likely restricts the internal motion of the central base pairs, precluding rearrangement to the open or Hoogsteen conformations, a process that permits the rapid cross-linking by Bizelesin of a straight DNA. This then accounts for the slow cross-linking reaction, because the Gu-substituted linker has to cross-link an energetically unfavorable bent DNA structure.

Implications for DNA Sequence Recognition by DNA−DNA Interstrand Cross-Linkers. In a previous contribution[7] we speculated that the thermodynamic stability of Bizelesin-cross-linked species coupled with the facile reversibility[8b] of the monoalkylated species could give rise to the unexpected accumulation of cross-linked species. Direct evidence for this process has been achieved in this contribution, although the example is limited to a purely AT region and the monoalkylation and cross-linking sites are overlapped. Presumably, the design of even more facile monoalkylation reversal agents would further exaggerate this phenomenon. However, it is likely that the propensity to reverse is also sequence dependent, as is the forward reaction.

In a recent publication[9b] we have suggested that Bizelesin achieves cross-linking of the 5′-TAATTA* sequence by the manipulative interplay of the drug and target sequence. Specifically, the ureadiyl linkage assists in the rearrangement of the central base pairs of the 5′-TAATTA*-cross-linked sequence to achieve a rapid second alkylation reaction. This rearrangement is necessary to restore a normal B-form DNA at the cross-linking site, which is made unfavorable for reaction by propagation of the bent DNA properties to this site.[14] Further evidence for this proposal is provided by the contrasting cross-linking kinetics and products found with the Gu-substituted compound and Bizelesin. First, the slow step in the overall formation of Gu-cross-linked species is the cross-linking reaction, in contrast to Bizelesin, where monoalkylation is the slow step. Second, the cross-linked Gu-substituted product does not contain the rearranged DNA, suggesting the direct involvement of the ureadiyl linkage in facilitating this process. Presumably, the hydrogen bonding of the charged Gu linkage to the phosphate backbone of the duplex restricts the ability of the duplex to undergo this rearrangement.

Finally, in another recent publication we have demonstrated that more complex linkers, such as a diamide pyrrole linker (Figure 11), can specify recognition of mixed AT−GC sequences.[15] Presumably, now armed with the insight provided by the effect of charged Gu linkages in preventing the manipulative interplay of drug and DNA, we can design even more complex linkers that prevent rearrangement of DNA in

(14) Seaman, F. C.; Chu, J.; Hurley, L. H. *J. Am. Chem. Soc.* **1996**, *118*, 5383−5395.

(15) Park, H.-J.; Kelly, R. C.; Hurley, L. H. *J. Am. Chem. Soc.* **1996**, *118*, 10041−10051.

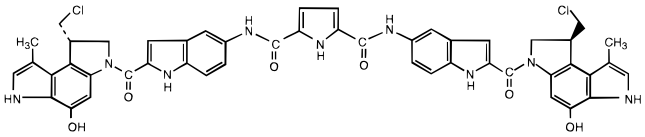

Figure 11. Structure of the DNA−DNA cross-linker containing the diamide pyrrole linker from ref 15.

one region while facilitating rearrangement in a second region prior to cross-linking.

Experimental Procedures

Chemicals. Bizelesin was supplied by The Pharmacia Upjohn Co., Kalamazoo, MI, and the Gu and CyGu compounds were synthesized at Pharmacia Upjohn.[12] Electrophoretic reagents (acrylamide, bisacrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine, Tris, and boric acid) were purchased from J. T. Baker, TN. T_4 polynucleotide kinase, T_4 DNA ligase, [γ-^{32}P]ATP, and X-ray film were purchased from Amersham. Reagents used to prepare the NMR buffer, sodium phosphate (99.99%) and sodium chloride (99.99%), were purchased from Aldrich. HPLC water and methanol were purchased from Baxter Scientific and Fisher, respectively.

Preparation of the 5′ ^{32}P-End-Labeled Oligomer Duplex. Oligonucleotides (Chart 1) were synthesized on an automated DNA synthesizer (Applied Biosystems 381A) using the phosphoramidite method[16] and purified on a 20% denaturing polyacrylamide gel after deprotection with ammonium hydroxide at 55 °C overnight. Individual strands of oligomers were labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase, annealed with unlabeled complementary strand in annealing buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.5), and purified on an 8% nondenaturing polyacrylamide gel. After detection on an autoradiogram, DNA duplex bands were excised from the gel, crushed with a blade, and extracted by shaking overnight with annealing buffer.

Isolation of Alkylated DNA Adducts. A 50−100-ng portion of the same radioactive count of samples labeled at the top or the bottom strand of the overlapped sequence (Chart 1) was modified with 10 μM Bizelesin at room temperature (pH 9.0) for 24 h and recovered by ethanol precipitation. Modified DNA was dissolved in alkaline dye (80% formamide, 10 mM NaOH, 0.025% xylene cyanol, 0.025% bromophenol blue) and isolated on a 20% denaturing polyacrylamide gel, keeping the gel temperature between 40 and 50 °C. DNA from the bands was eluted by shaking for 3 h at room temperature with distilled water and precipitated by ethanol.

Determination of Alkylation Sites and Level of Modification on DNA. Drug modification sites were determined by the thermally induced strand breakage assay,[10] performed at 95 °C for 30 min without piperidine to minimize reversible dealkylation. Strand breakage products were dried, dissolved in alkaline dye, and separated on a 16% or 20% sequencing gel parallel to Maxam−Gilbert sequencing lanes. To obtain quantitative information, the autoradiograms were scanned with a laser densitometer (LKB 2202) coupled to a recording integrator (LKB 2220) or a PhosphorImager (Molecular Dynamics 445 SI).

(16) Gait, M. J., Ed. *Oligonucleotide Synthesis−A Practical Approach;* IRL Press: Oxford, England, 1984.

Kinetics of Drug Modification Reaction. DNA (10 ng) labeled at the top strand was incubated with 10 μM drug in 10 mM Tris-HCl, 0.1 M NaCl at room temperature at pH 7.5 or 9.0 for the indicated period. Drug modification was terminated by adding the excess amount of calf thymus DNA and performing a strand breakage assay.

Incubation of Alkylated DNA Mixture in the Presence and Absence of Competitor DNA. DNA (10 ng) labeled at the top strand of the overlapped sequence was modified with 10 μM Bizelesin at pH 7.5 and room temperature for 24 h. The reaction was stopped, and unbound drug molecules were removed by extensive organic solvent extractions with phenol/chloroform, butanol, and chloroform/isoamyl alcohol followed by ethanol precipitation. The initial reaction mixture of drug-modified DNA adducts and unmodified DNA was incubated with and without the same amount of unlabeled competitor DNA at 37 °C in annealing buffer for up to 6 days. The aliquot of the sample was taken at the indicated time period and stored at −70 °C until the final sampling.

Ligation of the Cross-Linked DNA Adduct. DNA (10 ng) labeled at both top and bottom strands of the TAATTA sequence (Chart 1) was modified with 10 μM drug at 37 °C for 24 h. After isolation on a 16% polyacrylamide denaturing gel, the cross-linked DNA adducts were ligated at 4 °C for 24 h using T_4 DNA ligase. Ligation products were separated on an 8% native gel at room temperature.

Adduct Preparation and Purification. Synthesis and purification of the self-complementary 10-mer [d(CGTAATTACG)$_2$] was previously described.[9a] Preparation of the Gu analogue 10-mer adduct was as previously described for the corresponding Bizelesin adduct.[9a] Additional purification was achieved by reverse-phase HPLC using Rainin C18 and C8 Dynamax-300A preparative columns (21.4 mm × 250 mm). The solvent gradient progressed from a buffer solution of 15 mM sodium phosphate (pH 6.95) toward a solvent of CH_3CN (100%) with a flow rate of 5 mL/min. The percentage of the latter solvent increased according to a regimen of 0.0% (0 min), 45% (5 min), 65% (50 min), and 100% (60 min).

Proton NMR Experiments. One- and two-dimensional 500 MHz ^{1}H-NMR data sets in D_2O-buffered solution (pH 6.8−7.0) were recorded on a Bruker AMX 500 FT NMR spectrometer. Proton chemical shifts of the ca. 6 mM-buffered solution were recorded in parts per million (ppm) and referenced relative to external TSP (1 mg/mL) in D_2O (HOD signal was set to 4.751 ppm). Phase-sensitive two-dimensional NOESY spectra were obtained at 27 °C (TPPI) for two mixing times, 100 and 200 ms, using a presaturation pulse to suppress the HOD signal. All spectra were acquired with 16 scans at each of 1024 t_1 values, spectral width of 10.002 ppm, and repetition time of 10 s between scans. During data processing, a shifted squared sine bell function (shift = 90°) was used in both ω_1 and ω_2 dimensions. The fid in ω_1 was zero-filled to 2K prior to Fourier transformation to give a 2K × 2K spectrum.

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