Demonstration of the asymmetric effect of CC-1065 on local DNA structure using a site-directed adduct in a 117-base-pair fragment from M13mp1

(DNase I "footprinting"/*Alu* I digestion)

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Communicated by Peter B. Dervan, June 11, 1987 (received for review January 7, 1987)

ABSTRACT Using DNase I and *Alu* I endonuclease analysis of a site-directed CC-1065-[*N*³-adenine]DNA adduct in a 117-base-pair fragment from M13mp1 DNA, we have demonstrated that CC-1065 produces an asymmetric effect on DNA conformation that extends more than one helix turn to the 5' side of the covalently modified adenine. CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*, which is believed to mediate its cytotoxic effects through covalent binding to DNA. Previous studies have demonstrated that CC-1065 binds covalently to *N*³ of adenine and lies within the minor groove of DNA spanning a 4-base-pair sequence to the 5' side of the modified adenine. DNase I footprinting of this site-directed CC-1065-DNA adduct on the noncovalently modified strand shows that inhibition of cleavage occurs over a 12-base region, which is bordered on the 3' side by a site of 2-fold enhancement of cleavage. On the covalently modified strand a much less pronounced inhibition/enhancement pattern of cleavage occurs as far as 11 bases to the 5' side of the covalently modified adenine. While *Hae* III is able to cleave the DNA on both strands on the 3' side of the covalently modified adenine, *Alu* I is only able to cleave the covalently modified strand on the 5' side of the adduct. By taking into account the recently published structure of DNase I, we are able to interpret these results and develop a model for the effect of CC-1065 on local DNA structure. In this model, we propose selective drug-induced distortion of the covalently modified strand as a consequence of the alkylation of adenine by CC-1065.

CC-1065 is an extremely potent antitumor agent produced by *Streptomyces zelensis* (1). The structure was solved by x-ray crystallography (2), and a variety of synthetic routes to the subunits of CC-1065 have been reported. Recent reviews of the synthesis, pharmacological activity and toxicity (3), and the mechanism of action of CC-1065 (4) have appeared. Previous studies have shown that CC-1065 alkylates duplex DNA to form a *N*³-adenine adduct (5) (Fig. 1). The drug molecule lies within the minor groove of DNA spanning a 4-base-pair (bp) region to the 5' side of the covalently modified adenine for which a predominantly A+T sequence selectivity exists (6). Thermal treatment of CC-1065-DNA adducts leads to loss of the CC-1065-*N*³-adenine adduct and DNA backbone strand breakage directly to the 3' side of the covalently modified adenine (5, 6) (see Fig. 1). CC-1065 has been shown to produce depletion of NAD levels in repair proficient and deficient (xeroderma pigmentosum) human cells, which appears to be related to poly(ADP) ribosylation and persistent DNA strand breakage (7).

We have recently developed a method to construct a CC-1065-[*N*³-adenine]DNA adduct in a 117-bp restriction

enzyme fragment from M13mp1 DNA at A-6229 (8). This method is based on a knowledge of the sequence specificity of CC-1065 and its selectivity to bind to duplex DNA in a relatively nondistortive manner. We now wish to report the unexpected and unusual effect that covalent binding of CC-1065 to DNA has on DNA local structure as revealed by both DNase I "footprinting" and a unidirectional strand-selective inhibition of restriction enzyme cleavage.

MATERIALS AND METHODS

Materials

CC-1065 was obtained from Upjohn. Electrophoretic reagents (acrylamide, bisacrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine) were from Bio-Rad. DNase I and bacterial alkaline phosphatase were from Sigma. T4 polynucleotide kinase, DNA polymerase I (large fragment), and restriction endonucleases *Bst*NI, *Msp* I, *Alu* I, and *Hae* III were from New England Biolabs. Methidiumpropyl-EDTA (MPE) was a generous gift of P. Dervan (California Institute of Technology). Materials used for construction of the site-directed CC-1065-DNA adduct have been reported previously (8). X-ray film, intensifying screen, and developing chemicals were from Kodak.

Methods

Construction of the Site-Directed CC-1065-DNA Adduct. The construction of a site-directed CC-1065 adduct into a 117-bp DNA restriction fragment isolated from the *lac* insert of M13mp1 was performed exactly as described (8).

Characterization of CC-1065-Modified DNA Fragments. *MPE footprinting.* The footprinting of the site-directed CC-1065-DNA adduct was performed as described (8).

DNase I footprinting. The site-directed CC-1065-DNA adduct was footprinted with DNase I by a modification of the method of Low *et al.* (9), in which DNA was suspended in 10 μ l of 10 mM Tris-HCl (pH 7.5). A freshly diluted DNase I solution (2 μ l of 0.35 unit/ml in 20 mM NaCl/5 mM MgCl₂) was added. After 30 min, 10 μ l of tracking dye containing 80% formamide, 10 mM EDTA, and 0.1% xylene cyanol was added to stop the reaction.

3'-End-Labeling of (-)-Strand of 117-bp Fragment. The 250-bp fragment obtained from an *Msp* I digestion of M13mp1 replicative form DNA was dissolved in 30 μ l of 10 mM Tris-HCl, pH 8.0/5 mM MgCl₂/7.5 mM dithiothreitol/0.1 mCi of [α -³²P]dCTP (1 Ci = 37 GBq). One unit of the DNA pol I (large fragment) was added and incubated for 20 min at

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Abbreviation: MPE, methidiumpropyl-EDTA.

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FIG. 1. Reaction of CC-1065 with N^3 of adenine in DNA and products from thermal cleavage reaction (5, 6). The exact nature of the species generated on the 5' side of the strand break is unknown.

room temperature. One microliter of a 10 mM dGTP chase was added and incubated another 10 min. After ethanol precipitation, secondary digestion by *Bst*NI was carried out to obtain the 117-bp fragment.

DNase I Footprinting of the Non-Site-Directed Adduct. A CC-1065 solution (0.5 μ l of 2.8 μ M stock) was added to 5 μ l of the 3'-end-labeled 117-bp DNA fragment and incubated for 2 hr at 37°C. DNase I digestion was carried out at room temperature for 30 min with 1 μ l of a freshly prepared solution (0.35 unit/ml in 20 mM NaCl/5 mM MgCl₂). Reactions were stopped with 5 μ l of tracking dye as described.

Restriction Endonuclease Digestion. The site-directed CC-1065-DNA adduct was 10-fold overdigested with either *Alu* I or *Hae* III restriction endonucleases by incubation of 4000 dpm of unmodified or CC-1065-modified DNA in 1 μ g of unlabeled M13mp1 replicative form DNA with 5 units of restriction endonuclease for 2 hr at 37°C in 10 μ l of reaction buffer. Reactions were terminated by addition of 10 μ l of tracking dye as for DNase I treatments described above.

DNA Sequencing Reactions. Singly 5' or 3' ³²P-labeled DNA fragments were sequenced using the Maxam and Gilbert base-specific cleavage method (10) and suspended in alkaline tracking dye for subsequent polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Analyses of MPE, DNase I, and restriction endonuclease digestions were performed by electrophoresis of products on 8% denaturing polyacrylamide gels, as described (8), adjacent to Maxam-Gilbert DNA sequencing reactions.

RESULTS

The ability to construct a site-directed CC-1065-DNA adduct in a 117-bp restriction enzyme fragment from M13mp1 DNA provided us with the opportunity to probe the effect of CC-1065 on local DNA structure without the complications of multiple adducts or ambiguity in assigning the site of covalent modification. A combination of MPE-Fe(II) footprinting (11) and thermal strand break analysis allows both the position of covalent binding and the overlap of the drug molecule on DNA to be determined (8).

DNase I Footprinting of the Site-Directed CC-1065-DNA Adduct in the 117-bp Restriction Enzyme Fragment. DNase I has been previously used to locate drug-binding sites on DNA and to monitor the influence of drugs on local DNA structure (12-14). In the latter respect, DNase I is a more sensitive probe than MPE-Fe(II) (15). Fig. 2 shows the results of DNase I footprinting of the site-directed CC-1065-DNA adduct on the 117-bp fragment. Lanes 1-4 are derived from a single 5' ³²P label on the covalently modified (+)-strand (8).

The site of covalent attachment (A-6229) is revealed by the thermally induced strand break (8) (lane 1). Lanes 5-9 are from a single 5' ³²P label on the noncovalently modified (-)-strand. The location of the CC-1065 molecule on this strand is revealed by MPE-Fe(II) footprinting (11) (compare lanes 5 and 6). DNase I footprinting of each of the two CC-1065-modified 5' ³²P-labeled duplex molecules shows a markedly different inhibition pattern between the two strands (compare lane 2 with 3 and lane 8 with 9). The (-)-strand shows a clear inhibition of DNase I cleavage extending well beyond the CC-1065 MPE-Fe(II) footprint (bracket Y in Fig. 2). A large enhanced DNase I cleavage site at G-6221 with smaller enhancements at 1 and 2 bases to the 3' side (T-6220 and A-6218) are also visible at the 3' end of the inhibition zone. Analysis of the (+)-strand to the 3' side of the covalent binding site (A-6229) is complicated by the attachment of the CC-1065 molecules to fragments on this side of the CC-1065 adduct, which results in inhibition of their migration relative to non-drug-modified fragments. Consequently, only the portion of the gel to the 5' side of A-6229 can be analyzed for comparative DNase I cutting. Some significant changes in DNase I cutting occurs in this region including an enhancement at T-6218 and a new band at C-6222.

DNase I Footprinting of Non-Site-Directed CC-1065 DNA Adducts on the (-)-Strand of the 117-bp Restriction Enzyme Fragment. To ensure that the striking inhibition/enhancement pattern observed on the (-)-strand was both reproducible and not a peculiarity of the binding sequence at A-6229, a multiple CC-1065-modified fragment was prepared. Fortunately, the M13mp1 fragment we had used for the construction of the site-directed adduct has all of the high-affinity CC-1065 binding sites on the (+)-strand (8). Consequently, although the analysis of the (+)-strand is blurred by the presence of CC-1065 molecules at multiple sites (results not shown), the (-)-strand is amenable to analysis. Fig. 3 shows the results of DNase I footprinting of the 3' ³²P-labeled non-site-directed CC-1065-DNA adduct on the (-)-strand of the 117-bp fragment. The use of a 3' ³²P label produces a more readily interpreted result than the 5' label, since the DNase I cleavage site with a 3' label corresponds to the product of a Maxam and Gilbert reaction. Four CC-1065-binding sites are found in the readable portion of the gel and these are bracketed A-D. Alongside the brackets are the actual CC-1065-binding sequences, which correspond to the (+)-strand with the covalently modified adenine denoted with an asterisk. For each CC-1065 binding site, a zone of DNase I cutting inhibition 13-15 bases long (A', B', C', D' bars in Fig. 3) straddles the drug-binding site with a site of DNase I enhancement (arrows in Fig. 3) located at the 3' terminus of the inhibition zone. A comparison of the inhibition/

FIG. 2. DNase I footprinting of 5' ^{32}P -labeled (+)- and (-)-strands of a site-directed CC-1065-DNA adduct on a 117-bp fragment from M13mp1 DNA. Lanes: 1-4, 5'-(+)-labeled DNA; 5-9, 5'-(-)-labeled DNA. Lanes 1, 3, 6, and 8, CC-1065-DNA adduct present at A-6229 of (+) drug strand. Lane 1, thermal treatment (90°C for 30 min) to induce strand breakage at drug-binding site. Lanes 2, 3, 8, and 9, DNase I digestion. Lanes 5 and 6, MPE·Fe(II) digestion (11). Lanes 4 and 7, Maxam-Gilbert purine- and pyrimidine-specific cleavage. The bracket Y identifies the region across the groove corresponding to inhibition of DNase I on the (-)-strand. T-6218, C-6222, and G-6221 are positions of enhanced DNase I cleavage.

enhancement pattern for the CC-1065-binding site B in Fig. 3, which corresponds to the site-directed CC-1065 adduct in Fig. 2, shows a reasonably close match. The inhibition zone appears to be somewhat larger for non-site-directed adduct (13-15 vs. 12 bases), but the main DNase I enhancement site is at the same base location. Significantly, the general pattern of inhibition/enhancement holds for all four CC-1065 binding sites on the (-)-strand and, consequently, we conclude that the results of the site-directed CC-1065-DNA adduct are representative of CC-1065 lesions on DNA.

Alu I and Hae III Restriction Enzyme Digestion of the 117-bp Fragment Containing the Site-Directed CC-1065-DNA Adduct. The 117-bp fragment containing the site-directed CC-1065-DNA adduct has single restriction enzyme cleavage sites for *Alu I* and *Hae III*, 14 and 12 bp to the 5' and 3' side of the covalently modified adenine, respectively (Fig. 2). This permitted an analysis of the effect of CC-1065 on restriction enzyme recognition and catalytic activity. The results in Fig. 4 show that while *Hae III* is able to cut both the (-)- and (+)-strands in the presence of CC-1065 (lanes 6 and 12), *Alu I* is only able to cut the (+)-strand in the presence of CC-1065 (lanes 4 and 10). The slower migration of the fragments in lanes 2 and 6 relative to lanes 1 and 5 is due to the presence of CC-1065 at A-6229.

DISCUSSION

CC-1065, like other covalent minor groove-binding agents such as the pyrrolo[1,4]benzodiazepines (16), is considerably more potent as a cytotoxic agent than the noncovalent minor groove binders distamycin and netropsin (4). Consequently, we can conclude that simple occupation of the minor groove

is not sufficient for high potency, but some inherent property of the covalent modification of DNA is also required. Furthermore, since CC-1065 and the distamycin group have similar sequence specificities (9), cytotoxic potency is not apparently related to A+T versus G+C specificity, a fact further substantiated by the G+C specificity of the pyrrolo[1,4]benzodiazepines (17). Consequently, it is of considerable importance to elucidate the structural (steric and electronic) features of the CC-1065-[*N*³-adenine] DNA adduct that might mediate the potent cytotoxic effects of CC-1065 and possibly other minor groove covalent binders. Several properties of these DNA adducts might be important in this regard. For example, the stabilizing but relatively nondistortive nature of the CC-1065-DNA adduct might be a potent cytotoxic combination of properties of a drug-DNA adduct. This type of DNA lesion might be difficult to detect and/or repair, or, alternatively, covalent binding might predispose the DNA in the near vicinity of the adduct to assume an unusual or anomalous form that has detrimental biological consequences. The availability of a site-directed CC-1065-DNA adduct in a 117-bp restriction enzyme fragment from M13mp1 has allowed us to probe the effect of drug binding on the local DNA structure.

The results presented in Figs. 2-4 demonstrate that covalent binding of CC-1065 to DNA induces an asymmetric effect on DNA structure as revealed by the strand-selective inhibition of DNase I and *Alu I* cleavage. Several features of the drug-induced changes in the DNase I cleavage pattern are significant. This includes the strand-selective inhibition of cleavage spanning a 12-bp region on the (-)-strand and the presence of enhanced sites of cleavage on both (+)- and (-)-strands in the direction of drug overlap from the covalent

FIG. 3. DNase I footprinting of 3' ^{32}P -labeled (–)-strand of a non-site-directed CC-1065–DNA adduct on a 117-bp fragment from M13mp1 DNA. Lanes: 1 and 2, Maxam and Gilbert purine- and pyrimidine-specific cleavage; 3 and 4, subjected to DNase I digestion. Lane 3, control (no drug modification); lane 4, CC-1065 modification at sites indicated by brackets A, B, C, and D. These brackets correspond to the CC-1065-binding sequences (8), which are identified as they correspond to the covalently modified (+)-strand, with the covalent modified adenine denoted by an asterisk. DNase I-enhanced cleavage sites are indicated by arrows; A8, etc., corresponding to the CC-1065-binding site and distance between the covalent binding site and the enhancement site. Vertical bars, zones of DNase I inhibition corresponding to each drug-binding site—i.e., A' corresponding to CC-1065 binding site A.

binding site (A-6229) (Fig. 5). Superficially, the selective inhibition of *Alu* I cleavage on the same strand as DNase I inhibition suggests that drug modification may cause a selective conformational change in the structure of the (–)-strand. However, as we discuss below, a consideration of the structures and mechanisms of cleavage of DNA by DNase I leads to a quite different interpretation of these data.

The structure of DNase I at 2.0 Å resolution (18) permitted Suck and Oefner to propose a mechanism for binding to and cutting of DNA. One feature of this model, which has

FIG. 4. Denaturing electrophoretic analysis of *Hae* III and *Alu* I digestion of the site-directed CC-1065–DNA adduct in the 117-bp fragment from M13mp1 DNA. Lanes: 1–6, 5' ^{32}P -(+)-labeled DNA; 7–12, 5' ^{32}P -(–)-labeled DNA. Odd-numbered lanes, no drug modification; even-numbered lanes, CC-1065 modification of adenine 6229 of the (+)-strand. Lanes 3, 4, 9, and 10, 10-fold overdigestion with *Alu* I; lanes 5, 6, 11, and 12, 10-fold overdigestion with *Hae* III. For an explanation of slower migration of bands in lanes 2 and 6, see text.

recently been confirmed (18) by x-ray structure analysis of a DNase I–DNA complex, is penetration of an exposed loop of the protein into the minor groove, 2–3 bp to the 5' side of the DNase I cutting site. Consequently, steric interactions in the minor groove between the covalently bound CC-1065 and the exposed loop of the protein predict inhibition of DNase I cleavage between G6222 and T6226 on the (–)-strand and between T6228 and G6232 on the (+)-strand [A⁽⁺⁾ and A^(–) in Fig. 5]. This accounts partially for the inhibition zone on the (–)-strand and for the visible part (T-6228) of the inhibition zone on the (+)-strand. A second important feature of the mechanism of DNase I binding to DNA is van der Waals interactions, which occur between the protein and two phosphates on the opposite strand to the cutting site, 5–6 bp on the 5' side. Consequently, the observed inhibition of DNase I cutting between A6227 and T6233 on the (–)-strand could result from a drug-induced change in the conformation of the (+)-strand between C6222 and T6228, which could prevent the correct alignment of DNase I required for cleavage in this region (Fig. 5, brackets B' and B"). In contrast, the normal or sometimes enhanced cleavage by DNase I on the (+)-strand to the 5' side of the covalent binding site implies that the backbone conformation of the (–)-strand in the vicinity of the drug-binding site is not adversely affected. Unlike the results of previous studies with noncovalently binding drugs (9, 14), the CC-1065-induced changes in DNase I cleavage pattern observed here are generally not staggered 2–3 bases in the 3' direction across the strands as would be expected if these represent changes purely in groove width.

Based on the interpretation of the DNase I footprinting experiments and the selective inhibition of *Alu* I cleavage on the (–)-strand, we tentatively conclude that CC-1065 produces a selective conformation change in the structure of the (+)-strand, which extends perhaps as far as 11 bases to the 5' side of the covalent modification site. Since the drug-induced changes on the (–)-strand seem far less significant, we suggest that the duplex DNA in the vicinity of CC-1065 lacks the identical antiparallel strand commonly associated with A-, B-, and Z-DNA. Since covalent binding of CC-1065 to DNA appears to require a deformation of DNA structure in the vicinity of the adduct, it is then possible that the relative propensity of different sequences to undergo this conformational change may play a role in sequence selectivity for covalent binding. Such an argument has been put forward based on theoretical modeling to account for the experimentally determined sequence specificity of the pyrrolo[1,4]benzodiazepines (21). The manner in which these drug-induced conformational changes in DNA structure relate to the biological potency of CC-1065 remains to be determined.

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