Hydroxyl Radical Footprinting of Calicheamicin. Relationship of DNA Binding to Cleavage[†]

Stanley C. Mah, Craig A. Townsend,* and Thomas D. Tullius*

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

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ABSTRACT: The binding to DNA by calicheamicin ϵ (CLMϵ), the rearranged and reduced product of the diynene antitumor antibiotic calicheamicin $\gamma_1{}^I$ (CLM$\gamma_1{}^I$), was studied using the method of hydroxyl radical footprinting. The drug binding sites determined in this way were compared to locations of double-stranded DNA cleavage by thiol-activated CLM$\gamma_1{}^I$. The results of these experiments show that CLMϵ lies in the minor groove in an extended conformation protecting approximately four nucleotides on each strand of DNA. Sites of CLMϵ binding correlate to sites of CLM$\gamma_1{}^I$ cleavage with protection by CLMϵ occurring mainly to the 3′ side of the site of C5′ hydrogen abstraction. From these results, it is possible to propose global structures of the drug/DNA complexes such that the oligosaccharide side chain is arrayed to the 3′ side of the site of C5′ hydrogen abstraction. This conclusion is entirely consistent with the results of recent atom-transfer experiments [Hangeland, J. J., De Voss, J. J., Heath, J. A., Townsend, C. A., Ding, W.-D., Ashcroft, J., & Ellestad, G. A. (1992) *J. Am. Chem. Soc. 114,* 9200–9202]. Somewhat greater protection on the strand undergoing C5′ hydrogen abstraction was observed to the 5′ side of the site of attack owing presumably to proximity of the methyl carbamate portion of the drug with DNA. Overall, binding is seen where cleavage is seen in accord with thermodynamics of drug association to DNA being important in determining the sites of cleavage.

The calicheamicins (CLM)[1] (Lee et al., 1987a,b) are antitumor antibiotics belonging to the emerging class of diynene-containing natural products that includes neocarzinostatin (Edo et al., 1985, and references therein), the esperamicins (Golik et al., 1987a,b), dynemicin (Konishi et al., 1990), and kedarcidin (Leet et al., 1992). The $\gamma_1{}^I$ form of CLM is notable for its ability to cause predominantly double-stranded cleavage of DNA in vitro at nanomolar concentrations, with a strong preference reported for TCCT and related sequences (Zein et al., 1988). Among the members of this family of mechanistically related cleaving agents, CLM and kedarcidin show the greatest sequence selectivity in their reactions with DNA. Interestingly, despite their distinct structures, while kedarcidin is chiefly a single-strand cutter, both recognize similar sequences in DNA (Zein et al., 1993). The origins of this selectivity and the nature of the noncovalent interactions between CLM and DNA are questions of fundamental importance. In this paper we compare sites of calicheamicin binding, determined by the hydroxyl radical footprinting method (Tullius & Dombroski, 1986; Dixon et al., 1991), with sites of cleavage to yield both structural and comparative reactivity information for several CLM/DNA complexes. We present direct experimental evidence for the location and overall conformation of the oligosaccharide side chain relative to the DNA helix.

The proposed mechanism of DNA scission by CLM involves bimolecular reaction of the allylic methyltrisulfide of **1** with a reductant (which is presumably the thiolate anion of glutathione in vivo) to form the reactive dihydrothiophene **2** (De Voss et al., 1990a) (Scheme 1). Variable-temperature NMR studies indicate that the sulfur chemistry associated with the formation of **2** is rapid and complex and that the lifetime of **2** at 37 °C is on the order of 4–5 s (De Voss et al., 1990a). The dihydrothiophene undergoes an electrocyclization reaction, first characterized by Bergman in a simpler system (Bergman, 1973), to give the 1,4-didehydrobenzene **3**. This diradical, when positioned within and spanning the minor groove, has been proposed as the reactive entity responsible for hydrogen abstraction from the two strands of the DNA. The spent form of calicheamicin, CLMϵ (**4**), is the product of hydrogen atom transfer from DNA to **3**. The resulting DNA radicals are believed to react with dissolved oxygen to lead on to strand scission.

Support for this complex mechanism of activation and DNA strand scission has been gathered from a variety of experimental observations. On the basis of extensive studies by Goldberg and co-workers (Kappen & Goldberg, 1983), who examined the high-resolution gel electrophoretic behavior of DNA cleavage products produced by neocarzinostatin, the Lederle group proposed that calicheamicin abstracts a C5′ hydrogen atom from one strand of the duplex to yield 3′ phosphate and 5′ aldehyde nucleoside ends (Zein et al., 1988). Cleavage on the opposite strand was seen to take place three bases in the 3′ direction. The 3-bp offset of cleavage sites, as well as the ability of netropsin to protect against cutting by CLM, was strongly suggestive of minor groove binding (Zein et al., 1988).

Atom-transfer experiments have unambiguously demonstrated the direct removal of nonexchangeable DNA hydrogens by the putative diradical intermediate **3**. In the first of these experiments (Zein et al., 1989b) CLM$\gamma_1{}^I$ (**1**) was reacted in

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* To whom correspondence should be addressed.

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[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; CLM, calicheamicin; HPLC, high-pressure liquid chromatography.

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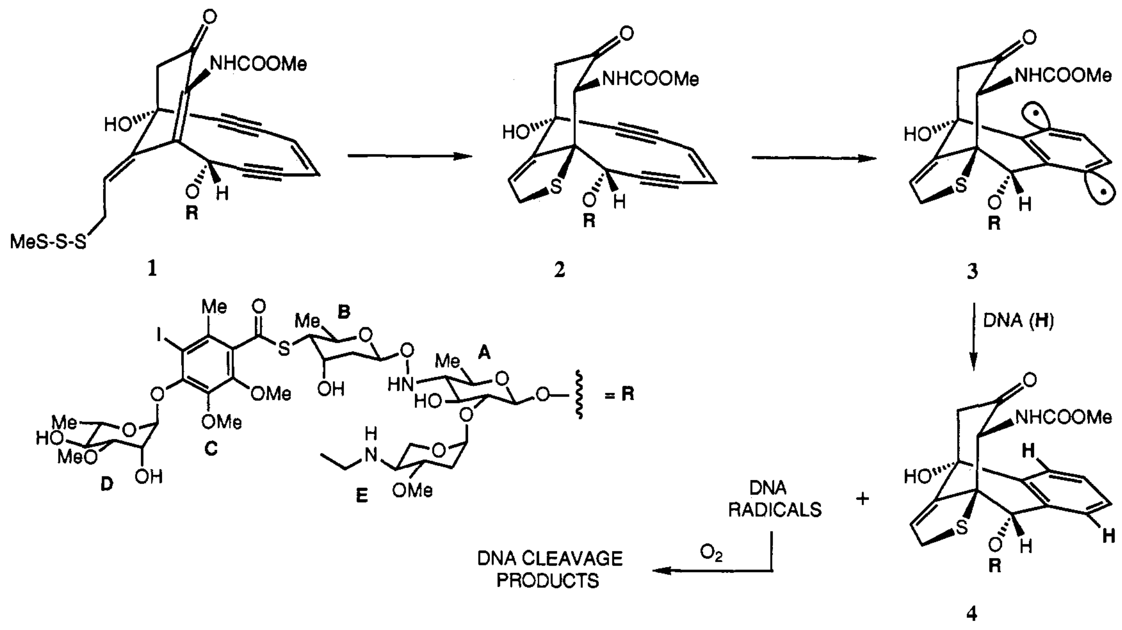
Scheme 1



a deuteriated medium in the presence or absence of calf thymus DNA. Under the former conditions solvent-derived deuterium was observed in the recovered CLMε at sites corresponding to the proposed diradical 3, whereas in the presence of DNA only hydrogen was detected in the reduced molecule. For a subsequent series of experiments, short oligonucleotides were prepared, each containing the sequence TCCT, a particularly favorable site for cleavage (De Voss et al., 1990b). Specific deuterium labeling of the 5′ cytidine (C) led to efficient transfer of isotope to only one of the two radical sites in the bound dehydrobenzene 3, that nearer to the attached oligosaccharide side chain. This important result confirmed the proposed direct C5′ hydrogen abstraction suspected on the basis of electrophoretic mobilities of the resulting DNA fragments, and it revealed a single orientation of CLM in the minor groove with the oligosaccharide extending toward the 3′ side of the TCCT cleavage site in the dodecamers tested. Additional atom-transfer experiments (Hangeland et al., 1992) were carried out by labeling the complementary strand with deuterium and showed abstraction of the C4′ hydrogen from an appropriately placed dC. Transfer of label occurred exclusively to the opposite diradical site seen above, completely in keeping with a single orientation of the drug bound to DNA. These experiments, however, while they indicate direction, do not otherwise define the location of the side chain with respect to the DNA helix.

In the present study we have used hydroxyl radical footprinting of CLMε (4) to define the structure of the calicheamicin/DNA complex at several different DNA sequences. The structure of CLMε, which itself is incapable of cleaving DNA, closely resembles the reactive 1,4-diyl 3 thought to be responsible for the initiation of strand cleavage. Hydroxyl radical footprinting was first developed to investigate protein/DNA complexes (Tullius & Dombroski, 1986) and DNA structure (Tullius & Dombroski, 1985) with subsequent application to the distamycin and actinomycin drug/DNA complexes (Churchill et al., 1990). Previously, hydroxyl radical footprints of the DNA-binding drugs distamycin and netropsin (Portugal & Waring, 1987), mithramycin (Cons & Fox, 1989a,b), and actinomycin and nogalamycin (Fox, 1988) have been obtained. Relative to traditional footprinting reagents such as DNase I, the hydroxyl radical has the virtues of being small in size and cleaving with little sequence selectivity. These advantages lead to high-resolution footprints of proteins and drugs and yield data at single-nucleotide resolution. Of particular importance for small molecule/DNA interactions where binding may be relatively weak, the EDTA·Fe(II) protocol (Dixon et al., 1991) causes minimal, if any, structural perturbations to DNA or the DNA/ligand complexes. We have exploited the power of this method to afford the first experimental evidence for the global structure(s) of CLM/DNA complexes with respect to their corresponding sites of cleavage.

EXPERIMENTAL PROCEDURES

Materials. CLMγ_1^{I} was kindly provided by Dr. G. A. Ellestad (Lederle Laboratories). CLMγ_1^{I} was stored as a solid at −20 °C or in solution at a concentration of 10 μM in 100% ethanol at −20 °C.

Preparation and Purification of CLMε. CLMγ_1^{I} (10 μL of an 18 mM solution) was added to a solution of tributylphosphine (90 μL, 100-fold molar excess diluted in ethanol at −78 °C) to form the dihydrothiophene intermediate (De Voss et al., 1990a). The reaction was allowed to proceed for 30 min. Subsequently, 1–2 μg of calf thymus DNA was added at room temperature to provide a source of hydrogen for the reactive diradical intermediate to form CLMε. CLMε was purified by HPLC (Hitachi L-6200 Intelligent Pump interfaced with an L-6000 diode array detector) using a reverse-phase column (250 × 4.6 mm Spherex C-18; Phenomenex). A linear gradient of 42.5 mM triethylamine at pH 6 in 15% acetonitrile to 20 mM triethylamine in 60% acetonitrile, run over 25 min, was used to separate CLMε (retention time, 25 min) from residual CLMγ_1^{I} (retention time, 35 min). CLMε was dried in a Speed-Vac concentrator (Savant) and stored as a solid at −20 °C. CLMε is relatively unstable and was dissolved in acetonitrile shortly before use.

Radioactive Labeling of DNA. The 215-bp *Eco*RI–*Nar*I restriction fragment from plasmid pUC18 was singly end-labeled with radioactive phosphorous at the 3′ end of either restriction site using standard procedures and purified by nondenaturing polyacrylamide gel electrophoresis (Maniatis et al., 1989).

Cleavage of DNA by Calicheamicin $\gamma_1{}^I$. The reaction conditions for cleavage of DNA by CLM$\gamma_1{}^I$ were modified slightly from those previously published (Zein et al., 1989). CLM$\gamma_1{}^I$, at a final concentration of 100 nM, was allowed to react with 6–10 fmol of radiolabeled duplex DNA molecules (5000 cpm, determined using a Geiger counter) in a final volume of 50 μL for 30 min at room temperature. The DNA was precipitated in ethanol, rinsed with 70% ethanol, and lyophilized.

Hydroxyl Radical Footprinting. For footprinting, 2 μL of a ~1 mM solution of CLMϵ in acetonitrile was added to 5 μL of water (purified using a Milli-Q system) containing 5000 cpm of 3′-radiolabeled DNA (~10 fmol of duplex DNA molecules). Binding was allowed to proceed for 10–15 min at room temperature before initiation of hydroxyl radical cleavage. One microliter each of the three reagents used to generate the hydroxyl radical was added separately to the side of the tube containing the sample. The hydroxyl radical cleavage reaction was initiated by briefly spinning the reagents into the reaction mixture in a microcentrifuge. The final concentrations of the reagents in the reaction mixture were 2 mM Fe(II), 4 mM EDTA, 0.3% H_2O_2, and 20 mM L-ascorbate (Tullius et al., 1987). These concentrations are somewhat higher than the standard conditions for hydroxyl radical cleavage of DNA (Dixon et al., 1991) because of the presence of acetonitrile, which acts to scavenge the hydroxyl radical. For cleavage of DNA by the hydroxyl radical in the absence of CLMϵ, 2 μL of 100% acetonitrile was added. Reactions were quenched with thiourea, and the DNA was precipitated, rinsed, and lyophilized as above.

Gel Electrophoresis. Samples were resuspended in a formamide–dye mixture (Maniatis et al., 1989), heated to 90 °C, placed on ice, and loaded onto an 8% denaturing polyacrylamide gel. Products of the Maxam–Gilbert guanine-specific DNA sequencing reaction (Maxam & Gilbert, 1980) were run in parallel on the gel as size markers. The autoradiographs shown in Figures 1 and 2 were obtained by exposure of X-ray film (Kodak XAR-5) to dried gels at room temperature. For scans of gel lanes (Figures 3 and 4), the dried gels were exposed to a phosphor-imaging plate (Molecular Dynamics) at room temperature. The plate was scanned by a Model 400E PhosphorImager (Molecular Dynamics). Plots of scans were made using Microsoft Excel.

RESULTS

Hydroxyl Radical Footprinting. We have used the hydroxyl radical as a chemical footprinting reagent to define the binding sites of CLMϵ (the reductive rearrangement product of CLM$\gamma_1{}^I$) within the *Eco*RI–*Nar*I restriction fragment from pUC18, which contains several sites at which CLM$\gamma_1{}^I$ cleaves. We also attempted to footprint CLM$\gamma_1{}^I$ itself, but the footprinting reagents activated CLM$\gamma_1{}^I$ and led to DNA cleavage. We then compared the binding sites of CLMϵ defined by footprinting with sites of DNA cleavage by CLM$\gamma_1{}^I$. CLMϵ is a propitious choice for this comparison since it is very close in structure to the reactive diradical intermediate of CLM$\gamma_1{}^I$ (**3**) believed to cleave DNA.

Figures 1 and 2 show the results of two hydroxyl radical footprinting experiments on CLMϵ, in which either the top DNA strand was 3′-radiolabeled at the *Eco*RI site (Figure 1) or the bottom strand was 3′-radiolabeled at the *Nar*I site (Figure 2). The products of CLM$\gamma_1{}^I$-induced cleavage of the same radiolabeled DNA molecules were also separated by electrophoresis in parallel for direct comparison with the footprints. Four regions of DNA that are protected by CLMϵ from radical attack are visible in Figures 1 and 2. These footprints all occur at sites that are cleaved by the active form of calicheamicin. The four footprints are labeled A–D on the top strand and correspondingly A′–D′ on the complementary strand.

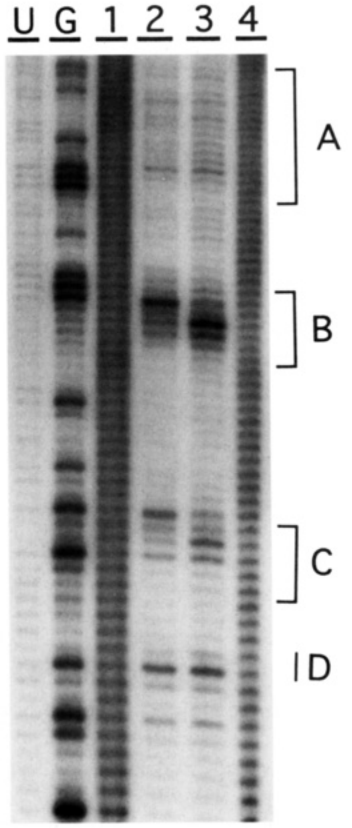


FIGURE 1: Autoradiograph of a DNA sequencing gel with (U) uncut DNA and the products of (G) Maxam–Gilbert G-specific reactions, (1) hydroxyl radical cleavage of DNA, (2) CLM$\gamma_1{}^I$ cleavage of DNA, (3) CLM$\gamma_1{}^I$ cleavage of DNA with subsequent alkali treatment, and (4) hydroxyl radical cleavage of DNA bound by CLMϵ. The *Eco*RI–*Nar*I fragment of pUC18 was 3′-end-labeled at the *Eco*RI site. Four sites of protection of DNA by CLMϵ are designated A, B, C, and D.

The intensities of the bands in the gel lanes were quantitated by PhosphorImager analysis. One-dimensional scans of the lanes are shown in Figures 3 and 4. The cutting pattern of the DNA free in solution is substantially even, with a few small variations in cleavage intensity which are likely due to structural heterogeneity inherent in the DNA. These variations might include changes in the dimensions of the minor groove in short runs of adenines, which have been shown to affect hydroxyl radical cleavage intensities (Burkhoff & Tullius, 1987). The cutting pattern of the complex shows regions that are protected from hydroxyl radical attack by bound CLMϵ.

Of the four sites of protection seen in Figure 3, site A is the largest footprint, approximately 15 or 16 nucleotides long. The sequence TAA in the center of this footprint and the region to the 3′ end of site A are less protected from hydroxyl radical attack than the other nucleotides in this site. On the opposing strand, protection by CLMϵ is seen at two regions within A′. Five nucleotides at the 5′ end of A′, AACTT, are protected by CLMϵ. The sequence GCC at the 3′ end of site A′ also is protected, though not apparently as strongly as the 5′ region of site A′. The sequence AATC at the center of site A′ exhibits no detectable protection by CLMϵ from hydroxyl radical attack on this strand.

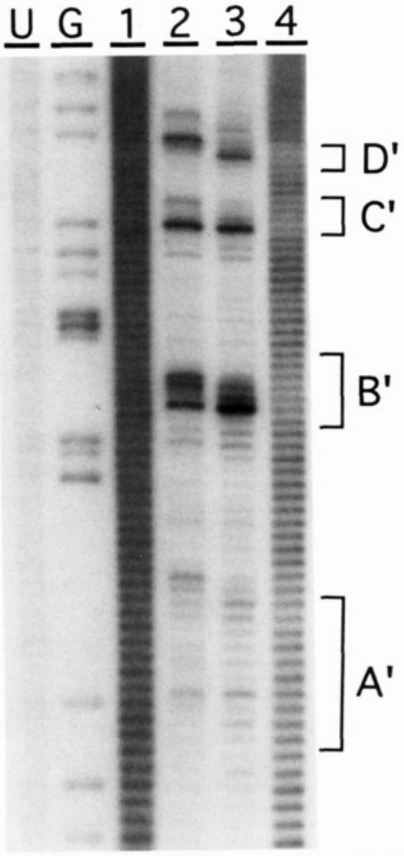


FIGURE 2: Autoradiograph of a DNA sequencing gel with (U) uncut DNA and the products of (G) Maxam–Gilbert G-specific reactions, (1) hydroxyl radical cleavage of DNA, (2) CLM$\gamma_1{}^{I}$ cleavage of DNA, (3) CLM$\gamma_1{}^{I}$ cleavage of DNA with subsequent alkali treatment, (4) hydroxyl radical cleavage of DNA bound by CLMϵ. The *Eco*RI–*Nar*I fragment of pUC18 was 3′-end-labeled at the *Nar*I site. Four sites of protection of DNA by CLMϵ complementary to the sites in Figure 1 are designated A′, B′, C′, and D′.

In the segment of the restriction fragment that we examined, sites B and B′ are cleaved by CLM$\gamma_1{}^{I}$ to the greatest extent (Figures 3 and 4). We also observe strong hydroxyl radical footprints of CLMϵ at B and B′. These footprints are equal in intensity, and each spans seven nucleotides. The footprints are offset by one nucleotide in the 3′ direction from one strand to the other, indicative of protection across the minor groove of DNA (Drew & Travers, 1984). One difference between the footprints on the two strands is a subtle decrease in the level of protection by CLMϵ at the 5′ end of site B′.

Hydroxyl radical footprints of CLMϵ also are observed at cleavage sites C and D and on the complementary strand at C′ and D′. Five nucleotides are protected at C and five or six nucleotides at C′. The degree of protection at C′ appears to be slightly greater than at C. The footprint at D is weaker than the corresponding protection at D′ and covers at most 2 or 3 nucleotides. This footprint is also weak compared to the footprint at site C, even though the level of cutting by CLM$\gamma_1{}^{I}$ is comparable at sites C and D. The footprint at D′ is strong, similar to the level of protection seen at C′.

Cleavage of DNA by CLM$\gamma_1{}^{I}$. Calicheamicin abstracts a different deoxyribose hydrogen atom from each DNA strand at a double-stranded cleavage site (De Voss et al., 1990; Hangeland et al., 1992). On one strand a C5′ hydrogen is removed, while on the other the hydrogen attached to C4′ is abstracted. Previous work has exploited the observation of single origins and termini of atom transfer to define the orientation of the CLM$\gamma_1{}^{I}$ molecule relative to its DNA cleavage site (De Voss et al., 1990; Hangeland et al., 1992). These experiments establish that the calicheamicin side chain is directed to the 3′ side of the nucleotide from which a C5′ hydrogen is abstracted. A diagram of this orientation is shown in Figure 5. This determination was made for the case of CLM$\gamma_1{}^{I}$-induced cleavage of a particular oligonucleotide containing the sequence TCCT at its center. If CLM adopts the same orientation at other sites, simply determining at which nucleotide abstraction of a C5′ hydrogen occurs would permit alignment of the molecule in its complexes with DNA at those sites.

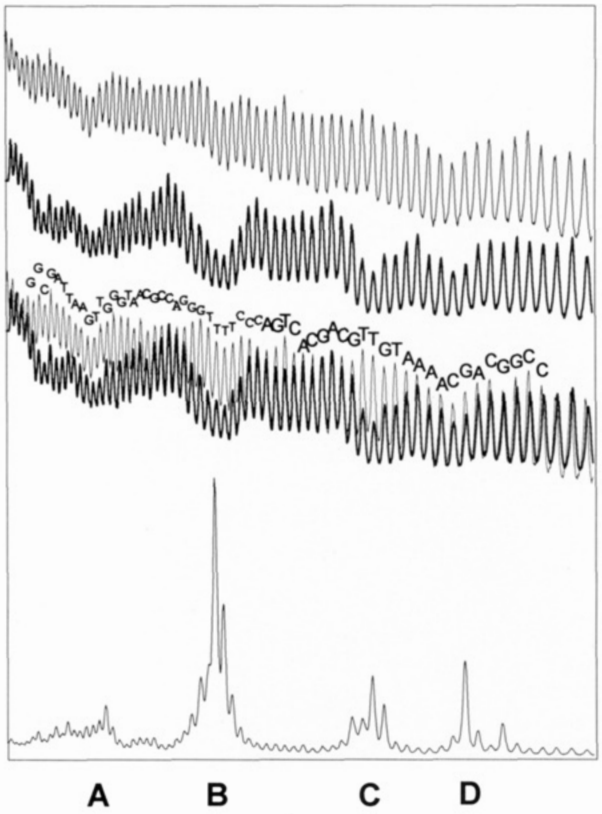


FIGURE 3: One-dimensional scans of the lanes from the gel shown in Figure 1. The top scan is from hydroxyl radical cleavage of free DNA (lane 1). The second scan is from hydroxyl radical cleavage of DNA bound by CLMϵ (lane 4). An overlay of the first two scans is shown to visualize the regions of protection by CLMϵ. The bottom scan is from CLM$\gamma_1{}^{I}$ cleavage of DNA with subsequent alkali treatment (lane 3).

Sites of C5′ hydrogen abstraction may be conveniently identified by analysis of the end remaining on the DNA strand after calicheamicin cleavage by chemical treatment and subsequent denaturing gel electrophoresis (Zein et al., 1988). Abstraction of a C5′ hydrogen leaves an aldehyde nucleoside at the 5′ end of the DNA at the site of cleavage. The aldehyde nucleoside can be eliminated by treatment with base, resulting in a 5′ phosphate end (Kappen & Goldberg, 1983). DNA fragments containing 5′ aldehyde nucleoside ends migrate on DNA sequencing gels as if they were two nucleotides longer than the base-treated fragments, which can be directly compared to Maxam-Gilbert DNA sequencing markers (Kappen & Goldberg, 1983; Zein et al., 1988). An increase in the mobility of a band after treatment of the DNA sample with base thus identifies a nucleotide that was attacked by CLM$\gamma_1{}^{I}$ through C5′ hydrogen abstraction.

In the restriction fragment we studied, CLM$\gamma_1{}^{I}$ cleaves most at site B (Figures 3 and 4). PhosphorImager analysis shows that the extent of cutting at C and D is approximately 3-fold lower than at site B, and cleavage at A is approximately 1 order of magnitude lower than at site B. One major and

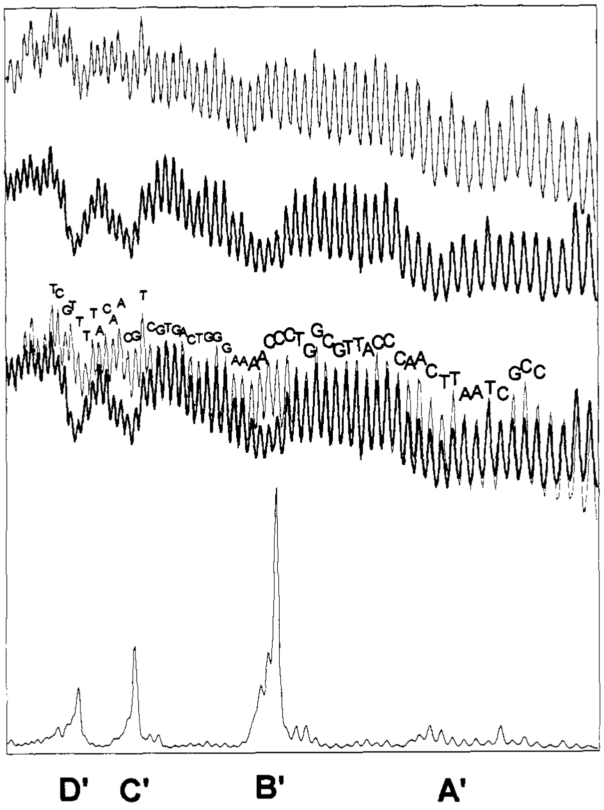


FIGURE 4: One-dimensional scans of the lanes from the gel shown in Figure 2. The top scan is from hydroxyl radical cleavage of free DNA (lane 1). The second scan is from hydroxyl radical cleavage of DNA bound by CLMε (lane 4). An overlay of the first two scans is shown to visualize the regions of protection by CLMε. The bottom scan is from CLM$\gamma_1{}^I$ cleavage of DNA with subsequent alkali treatment (lane 3).

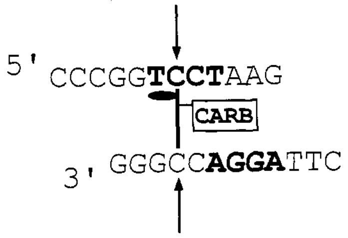


FIGURE 5: Diagram illustrating the orientation of CLM relative to its cleavage site. The bottom strand is shifted by three nucleotides in the 5′ direction to align nucleotides that are directly across the minor groove from one another. Cleavage would occur at the nucleotides marked by arrows. The carbohydrate tail and methyl carbamate (filled oval) are indicated. The TCCT and base-paired AGGA sequences are in bold for reference.

several minor cleavages occur at sites B and B′. At site B, the most intense cleavage occurs within a T_4 tract embedded in a 7-bp-long homopurine•homopyrimidine stretch. This cleavage is the result of abstraction of a C5′ hydrogen, because the band on the sequencing gel increases in mobility after base treatment. The corresponding cut on the other strand occurs at the cytidine three nucleotides in the 3′ direction, consistent with cleavage across the minor groove of DNA. We also observe a minor cleavage site which overlaps the major site at B and B′. In the minor site, CLM$\gamma_1{}^I$ removes the C5′ hydrogen from the purine-containing strand in a homopurine•homopyrimidine stretch, and not from the pyrimidine strand as was seen for the major site. We conclude from this analysis that at B/B′ two cleavage sites occur, overlapping by one nucleotide and oriented in a head-to-head manner.

The cleavages induced by CLM$\gamma_1{}^I$ at sites C and D (and at the corresponding positions C′ and D′ on the complementary strand) are similar in intensity. At site C, a C5′ hydrogen is abstracted from a thymine in the sequence TTGT. The molecule, therefore, extends to the 3′ side of the cleavage at site C. In the nearby cleavage at D/D′, C5′ hydrogen abstraction occurs on the complementary strand at site D′, within a pyrimidine-rich stretch (TTTT). Thus at site D/D′ CLM extends to the 5′ side of the cleavage at D and is directed toward site C.

Several weak cleavages occur at sites A and A′, spanning 14 or 15 bp. This cluster of cleavage sites corresponds to the broad hydroxyl radical footprints observed at A and A′. Two of the cleavages appear to be more intense than the others. The two regions within A/A′ which are most protected by CLMε from hydroxyl radical attack correspond to the two stronger cleavage sites. The DNA sequence at site A/A′ consists of an 8-bp A/T-rich region flanked by three or four GC base pairs on either side.

Comparison of Cleavage Sites with Footprints. A summary of the cleavage and footprinting data is presented in Figure 6. At sites C and D, it is easy to see that C5′ hydrogen abstraction occurs near the 5′ end of the footprint on the pyrimidine-rich strand. The position of cleavage on the opposite strand occurs at the 3′ end of the footprint. We infer that in each instance the aryl-linked carbohydrate side chain of CLM is positioned to the 3′ side of the site of C5′ hydrogen abstraction and to the 5′ side of the cut on the opposing strand.

A similar comparison of cleavage sites and footprints at B/B′ is complicated by the presence of two overlapping cleavage sites with different orientations. We conclude that the length of the footprints at B/B′ is a consequence of the head-to-head orientation of two binding sites having a 1-bp overlap.

When all of the data are compared, the degree of protection of DNA by CLMε from hydroxyl radical attack does not correlate fully with the degree of cleavage by CLM$\gamma_1{}^I$. The footprints at sites B and B′ are comparable in their intensities to the footprints observed at other sites examined, with the exception of site D. However, the level of cleavage by CLM$\gamma_1{}^I$ at sites B and B′ is about 3 times greater than at sites C and D. Similarly, the degree of cutting at A and A′ is lower in relation to the degree of protection.

DISCUSSION

Orientation of CLM Relative to DNA. Hydroxyl radical footprinting of the complex of CLMε with DNA, combined with analysis of CLM$\gamma_1{}^I$-induced DNA cleavage, provides physical evidence that the aryl tetrasaccharide of CLM lies in the minor groove of DNA. Since CLMε protects four or five nucleotides on each strand of DNA from hydroxyl radical attack, it likely adopts an extended conformation. In support of this conclusion, space-filling CPK models and computer modeling of the calicheamicin/DNA complex show that CLM, when extended, is of sufficient length to interact with four or five nucleotides on each strand of duplex DNA. NMR studies of CLMε in solution have shown that the molecule is predominantly in an extended conformation that has been suggested to be preordered for DNA binding (Walker et al., 1990).

The hydroxyl radical footprinting data for the CLMε/DNA complex supports the conclusions from atom-transfer studies concerning the orientation of CLM$\gamma_1{}^I$ at its cleavage site (De Voss et al., 1990; Hangeland et al., 1992). Those experiments demonstrated that deuterium labels incorporated at the C5′

FIGURE 6: Summary of hydroxyl radical footprinting and CLM$\gamma_1{}^I$ cleavage data. Arrows indicate positions of CLM$\gamma_1{}^I$ cleavage. The size of the arrows approximates the relative extent of cleavage and is consistent within each strand. Bars indicate regions of protection by CLMϵ, and the thickness of the bars reflects the relative degrees of protection within each strand. The nucleotides suffering C5′ hydrogen abstraction by CLM$\gamma_1{}^I$ are marked 5′.

and C4′ positions of the deoxyribose ring of DNA are each transferred to single carbon atoms of the CLM diradical **3**. This complementary deuterium transfer can occur only if the aryl tetrasaccharide of CLM is oriented to the 3′ side of the nucleotide that is attacked at its C5′ hydrogen. Our footprinting experiments show that protection by CLMϵ from hydroxyl radical attack consistently occurs to the 3′ side of the site of C5′ hydrogen abstraction and to the 5′ side of the cut on the opposing strand in a variety of sequences (Figure 6). Interestingly, work by Christner et al. (1992) on a related enediyne, esperamicin C, suggests that this DNA cleaver is oriented in a bidirectional manner relative to its double-stranded DNA cleavage sites. This difference in orientation between the two enediynes most likely stems from the greater sequence specificity of CLM$\gamma_1{}^I$ relative to that of the esperamicin family of enediynes.

Relationship of Binding to Cleavage. Previous work from several laboratories has shown that isolated segments of CLM cannot fully define the binding and cleavage sites of the drug itself. Drak et al. (1992) found that the racemic aglycon alone makes nonspecific cuts along DNA in a single-stranded fashion. CLM-T, which consists of the aglycon and sugars A and E, cleaves the same sequence of DNA from pUC18 that we study here in a double-stranded manner, though again with no sequence specificity (Walker et al., 1992). However, other truncated versions of CLM$\gamma_1{}^I$ lacking either the 4-ethylamino sugar or the terminal rhamnose maintain the sequence specificity of CLM$\gamma_1{}^I$, though with decreased levels of cleavage (Zein et al., 1989a). Work by Aiyar et al. (1992) and Nicolaou et al. (1992) has demonstrated that the oligosaccharide alone can bind to DNA sequence-specifically. However, the binding site defined by the carbohydrate moiety in the absence of the aglycon is shifted in relation to the binding site defined by CLM$\gamma_1{}^I$ cleavage. It therefore appears that the conformational rigidity of CLM that is imparted by the combination of the aglycon and the carbohydrate moieties and the interactions with DNA made by the whole molecule are necessary for the observed sequence-specific double-stranded cleavage of duplex DNA by the intact drug.

Our experiments demonstrate that CLMϵ binds only to sites which are cleaved by CLM$\gamma_1{}^I$. However, there is not a strict correlation between the extent of binding of CLMϵ as determined by hydroxyl radical footprinting and the amount of cleavage of DNA by CLM$\gamma_1{}^I$. Site A/A′ (Figures 3 and 4), which is cleaved relatively lightly by CLM$\gamma_1{}^I$, is protected from hydroxyl radical to an extent comparable to regions which are cleaved much more strongly by CLM$\gamma_1{}^I$. Similarly, site B/B′ is cleaved quite strongly but shows protection comparable in extent to C/C′ and D/D′. These differences could owe to small kinetic factors that favor or disfavor reaction by a factor of 3–10. Structural conditions (e.g., local alignment of the diynene in the minor groove, groove width, orientation of the deoxyribose C–H bonds undergoing reaction, ease of helix deformation, and so forth) may enhance or inhibit the rate of hydrogen abstraction from the DNA backbone at some binding sites.

Alternatively, the small differences observed between extents of protection in footprinting studies and strength of cleavage could owe to the structural variations between CLMϵ and the form of the drug activated for DNA cleavage, **2**. However, the most likely origin of these differences is that the concentration of CLM$\gamma_1{}^I$ used for cleavage experiments is chosen to ensure single-hit kinetics and is therefore quite low, whereas footprinting studies are carried out at much higher concentrations of CLMϵ. Because the binding sites are nearly saturated at the high drug concentrations used for footprinting, small differences in affinity for the various sites will be difficult to discern in these footprints. Nonetheless, cleavage is seen only where binding is seen, but not in its absence. Therefore, an important determinant of cleavage selectivity is likely the thermodynamics of the prior association of CLM$\gamma_1{}^I$ and/or its activated form **2** with certain DNA sequences (De Voss et al., 1990a). A similar conclusion has been reached by Kahne on other grounds (Walker et al., 1992). These equilibria are presumably established rapidly relative to the rearrangement of the dihydrothiophene **2** and lead on to observed oxidative damage at these positions.

The Methyl Carbamate Group of Calicheamicin. In footprints C and D′, protection from damage by the hydroxyl radical extends one nucleotide to the 5′ side of C5′ hydrogen abstraction (Figure 6), while the sites of C4′ abstraction on the opposite strands occur right at the edges of the footprints. Examination of a three-dimensional model of the CLM/DNA complex suggests an explanation for this observation. If the aglycon of CLM is positioned within the minor groove of DNA so that it is poised to abstract a C5′ hydrogen from one strand and a C4′ hydrogen from the other strand, the methyl carbamate moiety of the aglycon covers the nucleotide just to the 5′ side of C5′ hydrogen abstraction. This interaction does not occur on the complementary strand, accounting for the slight asymmetry of the footprint.

Sequence Selectivity of CLM$\gamma_1{}^I$. Previous reports have emphasized the preference of CLM$\gamma_1{}^I$ for cleavage of TCCT and related sequences (Zein et al., 1988), or more generally, cleavage within homopurine·homopyrimidine tracts, as has been observed in this laboratory and elsewhere (Walker et al., 1992). Some are stretches of oligo(dA)·oligo(dT), or oligo(dG)·oligo(dC) abutting oligo(dA)·oligo(dT) which maintain a homopurine·homopyrimidine sequence. Sequences consisting of a mixture of purines on one strand and pyrimidines on the other strand also are sites of binding and cleavage. The sequence TTGT, which occurs in site C/C′, is an exception to this generalization. However, isolated stretches of oligo(dG)·oligo(dC) are not cleaved by CLM$\gamma_1{}^I$ (unpublished results).

Schreiber and co-workers proposed, on the basis of computer modeling of a CLM/DNA complex, that interactions between the iodine atom of CLM$\gamma_1{}^I$ and the 2-amino groups of guanines in the minor groove are critical for sequence-specific cleavage of the TCCT sequence (Hawley et al., 1989). However, too many guanines in a sequence clearly is unfavorable. The lack of cleavage within oligo(dG)·oligo(dC) sequences suggests that the presence of at least four consecutive guanines disrupts the interactions between CLM and DNA necessary for DNA cleavage. A sequence with a mixture of purines on one strand and pyrimidines on the other, in which the 2-amino groups of guanines are presumably favorably situated, can allow binding and cleavage of DNA by CLM$\gamma_1{}^I$. An important experimental observation that differs with this model is that some sequences which do not contain GC base pairs (for example, TTTT in site B) are cleaved quite efficiently by CLM$\gamma_1{}^I$ (Figures 3 and 4; Walker et al., 1992). In such cases our footprinting experiments have shown that CLM is directed along the T-tract, so no interaction with a guanine 2-amino group is possible. Thus, the significance of the proposed interaction between the iodine atom of CLM$\gamma_1{}^I$ and a 2-amino group of guanine (Hawley et al., 1989) is likely a minor factor in determining the sites of cleavage.

CONCLUSIONS

Calicheamicin $\gamma_1{}^I$ is notably selective in its cleavage of DNA. Among observed sites of cleavage, runs of four or more pyrimidines have been found to be particularly favorable where the chief site of reaction is typically the penultimate 5′ pyrimidine. By monitoring the electrophoretic behavior of 5′- and 3′-radiolabeled fragments resulting from these cleavages, it is possible to assign those bearing a 5′ aldehyde terminus and, therefore, determine loci of C5′ hydrogen abstraction by the activated form of CLM. We have demonstrated by comparisons of hydroxyl radical footprints afforded by CLMϵ with fragment identifications from CLM$\gamma_1{}^I$-induced cleavage that the molecule is oriented with respect to DNA in a nonrandom manner. The aryl-linked oligosaccharide side chain of calicheamicin is directed to the 3′ side of the site of C5′ hydrogen abstraction. The size of these footprints and the comparable degree of protection on each strand strongly suggest that the oligosaccharide side chain exists in an extended conformation within the minor groove. This picture of calicheamicin binding in the minor groove is striking in its uniformity and shows only small differences between the relative extents of cleavage by CLM$\gamma_1{}^I$ and the degrees of protection by CLMϵ. These variations may owe to slight structural differences between CLMϵ (**4**) and the activated form of the drug (**2**) responsible for binding, rearrangement to the reactive 1-4 diyl **3**, and subsequent rapid abstraction of DNA hydrogens to initiate strand scission. Moreover, the significantly greater concentrations of CLMϵ used to footprint the drug will more nearly saturate binding sites, tending to equalize their apparent intensities whereas cleavage experiments are done at 4 or 5 orders of magnitude lower concentration. Nevertheless, DNA cleavage occurs only where binding is seen, indicating that thermodynamic effects of drug interactions with DNA are important in the cleavage selectivity of calicheamicin.

ADDED IN PROOF

Walker and Kahne have reported NMR spectroscopic results recently (Walker et al., 1993), demonstrating that calicheamicin $\gamma_1{}^I$ binds to the octamer d(GTGACCTG) in an extended conformation in the minor groove. These observations are fully in keeping with our conclusions from hydroxyl radical footprinting experiments with calicheamicin ϵ.

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