Reaction of the Antitumor Antibiotic CC-1065 with DNA. Location of the Site of Thermally Induced Strand Breakage and Analysis of DNA Sequence Specificity†

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ABSTRACT: CC-1065 is a unique antitumor antibiotic produced by *Streptomyces zelensis*. The potent cytotoxic effects of this drug are thought to be due to its ability to form a covalent adduct with DNA through N3 of adenine. Thermal treatment of CC-1065–DNA adducts leads to DNA strand breakage. We have shown that the CC-1065 structural modification of DNA that leads to DNA strand breakage is related to the primary alkylation site on DNA. The thermally induced DNA strand breakage occurs between the deoxyribose at the adenine covalent binding site and the phosphate on the 3′ side. No residual modification of DNA is detected on the opposite strand around the CC-1065 lesion. Using the early promoter element of SV40 DNA as a target, we have examined the DNA sequence specificity of CC-1065. A consensus sequence analysis of CC-1065 binding sites on DNA reveals two distinct classes of sequences for which CC-1065 is highly specific, i.e., 5′PuNTTA and 5′AAAAA. The orientation of the DNA sequence specificity relative to the covalent binding site provides a basis for predicting the polarity of drug binding in the minor groove. Stereo drawings of the CC-1065–DNA adduct are proposed that are predictive of features of the CC-1065–DNA adduct elucidated in this investigation.

CC-1065 is an antitumor agent produced by *Streptomyces zelensis* (Hanka et al., 1978; Hurley & Rokem, 1983). The cytotoxicity of CC-1065 is attributed to the ability of this drug to bind covalently to DNA (Swenson et al., 1982; Hurley et al., 1984). Structurally, CC-1065 consists of three subunits: two identical benzodipyrrole units (subunits B and C) and a third subunit containing the DNA-reactive cyclopropane ring (subunit A) which is conjugated with an indolequinone system (Figure 1). X-ray crystallographic studies (Chidester et al., 1981) have shown that CC-1065 is characterized by a pronounced right-handed twist along the long axis of the drug molecule with its hydrophobic groups aligned along the inside edge while the hydrophilic substituents are displayed on the outside edge. Thermal treatment of CC-1065–DNA adducts leads to the release of a base covalently attached to the CC-1065 moiety. This adduct has been purified and identified by ^1H and ^{13}C NMR as the N3 alkyl adenine CC-1065 adduct (Hurley et al., 1984) (Figure 2).

Our initial interest in this antibiotic was stimulated by its significant cytotoxicity in vitro and antitumor activity in vivo (Bhuyan et al., 1982; Martin et al., 1978). CC-1065 is one of the most potent agents tested against L1210 cells in culture. This drug, at 0.05 ng/mL, caused 90% inhibition of growth of L1210 cells. This may be compared to the 90% inhibition of L1210 growth by other cytotoxic antitumor agents in this assay system: actinomycin D at 4 ng/mL and adriamycin at 20 ng/mL (Li et al., 1982). In the tumor cloning assay, CC-1065 caused a ≥50% decrease in the colony-forming units of tumor cells from patients with cancer of lung, breast, pancreas, ovary, etc., at a concentration of 0.1 ng/mL (Bhuyan et al., 1982). It was greater than 100-fold more lethal to B16 and CHO[1] cells in culture than adriamycin, actinomycin D, and *cis*-diamminedichloroplatinum (Bhuyan et al., 1981, 1982). It was also active in vivo against the murine tumors, P388 and L1210 leukemia, B16 melanoma, CD_8F mammary, and colon 26 at doses ranging from 1 to 50 μg/kg (Martin et al., 1978; Neil et al., 1981). CC-1065 caused lethal delayed hepatotoxicity in mice at therapeutic antineoplastic doses (McGovren et al., 1984).

The reaction of CC-1065 with DNA and other macromolecules has been extensively studied. In addition to having an absolute requirement for double-stranded nucleic acid (Li et al., 1982) CC-1065 binds preferentially to AT-containing synthetic polymers (Swenson et al., 1982). Covalently bound CC-1065 does not alter the electrophoretic mobility of supercoiled DNA in agarose gels and thus does not appear to intercalate the DNA helix (Swenson et al., 1982). Alkylation studies and experiments using glucosylated T4 DNA led to the conclusion that CC-1065 binds in the minor groove of DNA (Swenson et al., 1982). This conclusion is also supported by the observation that CC-1065 and netropsin have common binding sites in DNA (Chidester et al., 1981) while CC-1065 and anthramycin have only partial overlap of sites (Swenson et al., 1982). Both netropsin and anthramycin are known to bind in the minor groove, netropsin at AT-rich sites and anthramycin covalently at N2 of guanine (Kolchinskii et al., 1975; Hurley & Petrusek, 1979).

The minor groove covalent binding site (N3 of adenine) for CC-1065 (Hurley et al., 1984) and the right-handed twist of the drug molecule suggested that it might reside in its entirety within the minor groove of DNA. In this situation, CC-1065 might be expected to exhibit DNA sequence specificity, and we have recently demonstrated that CC-1065 forms a defined

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[1] Abbreviations: AP, apurinic; bp, base pair; CHO, Chinese hamster ovary; CPK, Corey, Pauling, Koltum; DMF, dimethylformamide; DSC, 0.015 M NaCl and 0.0015 M sodium citrate, pH 7.4; EDTA, ethylenediaminetetraacetic acid; Pu, purine nucleotide; Py, pyrimidine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

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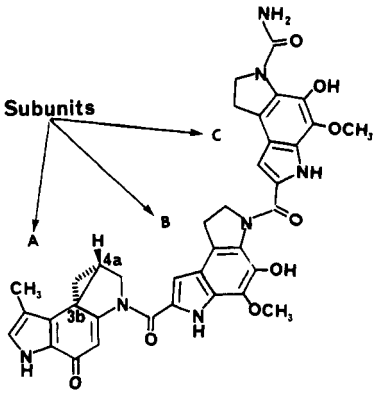


FIGURE 1: Structure of CC-1065 showing the absolute stereochemistry at C3b and C4a deduced during this investigation.

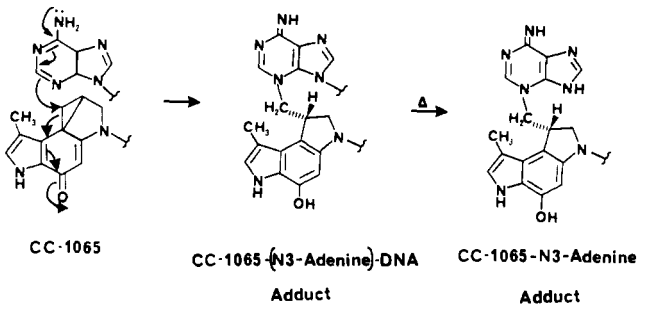


FIGURE 2: Reaction of CC-1065 with DNA to form the CC-1065–(N3-adenine)–DNA adduct, and the structure of the CC-1065–N3-adenine adduct released upon thermal treatment of the CC-1065–DNA adduct (Hurley et al., 1984).

duplex adduct with the 14-mer (5′CGGAGTTAGGGGCG3′) in which the adenine binding sequence is 5′TTA3′ (Needham-VanDevanter et al., 1984). The investigations described in this paper were designed to further examine the DNA-binding specificity of CC-1065 and to provide more direct evidence for the location of the DNA strand breakage that occurs when the adduct is subject to thermal treatment. Since CC-1065 binds preferentially to AT-rich regions, we purposely chose the early promoter element of SV40 DNA for these studies so that we could determine whether CC-1065 might have a preference for a specific regulatory region, such as the TATA box, which might help to explain the remarkable potency of this drug. Not only do the results demonstrate that CC-1065 has a marked DNA sequence selectivity, but they also provide important structural information on the CC-1065–DNA adduct and further insight into possible reasons for the extreme cytotoxic potency of CC-1065. Some of this work has appeared in preliminary form (Swenson et al., 1983).

Materials and Methods

Drugs, Enzymes, and Chemicals. CC-1065 was supplied by The Upjohn Co. (Kalamazoo, MI) and was used without further purification. Polynucleotide kinase from bacteriophage T4 infected *Escherichia coli* was isolated and purified according to published procedures (Richardson, 1971). AP endonuclease (HeLa AP endonuclease II; Kane & Linn, 1981; Mosbaugh & Linn, 1982) was a generous gift of Dale W. Mosbaugh, Department of Chemistry, The University of Texas at Austin. Restriction enzymes were purchased from New England Biolabs. [γ-^{32}P]ATP was synthesized according to published methods (Walseth & Johnson, 1979). Bacteriophage T7, originally from F. W. Studier, was grown and purified according to established procedures (Studier, 1969, 1979). SV40 DNA and ϕX174 RF DNA were purchased from Bethesda Research Laboratories.

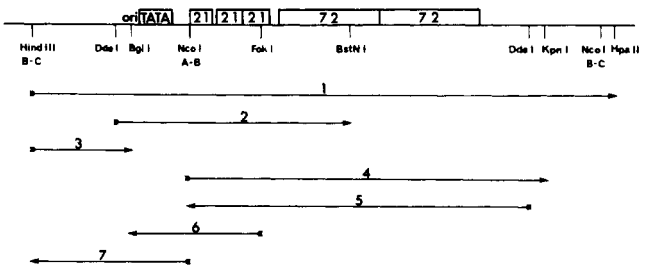


FIGURE 3: Scheme of restriction digestion strategy used to examine the interaction of CC-1065 with the SV40 early promoter. Singly ^{32}P-end-labeled DNA restriction fragments spanning both strands of the SV40 early promoter were obtained by using the restriction enzyme sites depicted. The tail of the arrow represents the 5′ ^{32}P-radiolabeled end. Symbols: ori, origin of replication; TATA, TATA box; 21, 21-base repeats; 72, 72-base repeats.

DNA Sequencing. DNA sequencing was carried out according to the methods of Maxam & Gilbert (1980). SV40 DNA restriction fragments used in this study spanned both strands of the entire SV40 early promoter region and were obtained by using the strategy depicted in Figure 3. The restriction fragment from bacteriophage T7 was obtained by isolating the 401-bp *Taq*I restriction digestion product of the doubly ^{32}P-end-labeled *Mbo*I D fragment. ϕX174 RF DNA was initially cut with *Pst*I, and after it was labeled with [α-^{32}P]dideoxyATP (Amersham) and terminal transferase, it was treated with *Hpa*I and the singly end-labeled 364-base-pair fragment purified. Subsequent analyses demonstrated that the sequence of DNAs used in this study matched exactly the published sequence of SV40 (Reddy et al., 1978; Van Heuverswyn & Fiers, 1979), bacteriophage T7 (Dunn & Studier, 1981), and ϕX174 (Sanger et al., 1977).

Reaction of CC-1065 with DNA and Production of Drug-Related Single-Strand Breaks. A stock solution of CC-1065 was prepared by dissolving a small amount of CC-1065 in 5 drops of DMF. Addition of 1 mL of DSC yielded a viscous suspension that, by using ϵ_{365} = 48 000, was adjusted to 0.14 μmol of CC-1065/mL by addition of DSC. This concentration of CC-1065 is referred to as the stock solution. Serial dilutions (1:10) of the stock were prepared with DSC as a diluent. When stored frozen (−30 °C), these drug solutions were stable for several weeks as judged by ^{1}H NMR. CC-1065 was mixed with radiolabeled DNA in 20 μL of 0.5 × DSC and incubated at 4 °C for the desired length of time (typically 24 h), and unreacted drug was removed by precipitation of the DNA in ethanol, using 20 μL of tRNA (1 mg/mL) as carrier nucleic acid. Ethanol precipitation of DNA removes all noncovalently bound CC-1065. The samples, now containing DNA bound with CC-1065, were resuspended in 100 μL of DSC and heated in sealed vessels at 100 °C (unless otherwise stated) for 30 min. The DNA was then precipitated again in ethanol, washed once with 1 mL of ethanol, dried under vacuum, and electrophoresed as described below. Two additional procedures were carried out in experiments to characterize more fully the CC-1065–DNA adduct.

(i) Piperidine treatment: CC-1065–DNA adducts were prepared as described above. Following precipitation of the DNA in ethanol to remove unreacted drug, the DNA was resuspended in 100 μL of piperidine (freshly diluted 1:10 in deionized water) and heated in a sealed tube at 100 °C for 30 min. The sample was then transferred to a clean tube, lyophilized repeatedly to remove all traces of piperidine, and electrophoresed as described below.

(ii) AP endonuclease treatment: CC-1065–DNA adducts were prepared, heated in DSC, and precipitated as described above. The DNA was then resuspended in 50 μL of 0.05 M

Tris, pH 7.5, and 0.01 M $MgCl_2$ and incubated with 40 units of AP endonuclease II at 37 °C for 1 h. The DNA was then precipitated with ethanol, dried, and electrophoresed as described below.

Electrophoretic Analysis of CC-1065 Binding Sites on DNA. Control lanes of DNA fragments were treated according to the procedures of Maxam & Gilbert (1980). The DNA samples were then resuspended in 80% (v/v) deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue. For samples containing Maxam–Gilbert-treated or CC-1065- plus heat-treated DNA, equal amounts of radioactivity were loaded onto thin denaturing polyacrylamide gels and electrophoresed at 1600 V. Autoradiographs were developed with Kodak XRP-1 film.

Densitometric Analysis of Autoradiograms. A Beckman DU-8 spectrophotometer equipped with a gel scan program was used to quantitate the autoradiographs. Comparative values were determined by integrating the area under the curve.

Results

The observation that thermal treatment of CC-1065–DNA adducts caused conversion of supercoiled DNA to relaxed DNA (Swenson et al., 1982) led us to investigate whether this cleavage reaction could be used as a means to pinpoint the location of CC-1065 binding sites within DNA. A singly ^{32}P-end-labeled restriction enzyme fragment (fragment 1 in Figure 3) from the SV40 DNA genome was used as a template to determine the dependence on dose, temperature, and incubation time of CC-1065-directed DNA breaks.

Dose Dependency of DNA Strand Breakage Produced by CC-1065. Tenfold dilutions of the stock solution of CC-1065 were used to treat aliquots of DNA fragments, which were subsequently heated at 100 °C for 30 min. The results, shown as the complete autoradiogram and also as densitometric tracings at two of the drug concentrations used, are shown in Figure 4A. At the more dilute CC-1065 concentrations (lanes 5 and 6) the majority of the material appears at the high molecular weight end of the gel[2] where band separation is incomplete, but this shifts to lower molecular weight bands that are separable at more concentrated drug solutions (lanes 7–11). The most prominent bands produced by CC-1065-induced strand breaks occur at two sequences, 5′AAAAA and 5′AATTA, and are evident at up to 10^5-fold dilution of the stock solution of CC-1065. These are the most sensitive binding sites in the readable part of the gel (i.e., from the 5′ end to the TATA box). Interestingly, saturation of these two most prominent binding sequences occurs at a 10^3-fold dilution of stock CC-1065, while higher molecular weight material is still evident at higher concentrations of CC-1065. It is likely that at these higher concentrations of CC-1065, efficient covalent drug binding occurs at less reactive sequences and thus precludes any interaction at preferred sites; i.e., the first hit can affect the affinity of subsequent hit sites.

The poly(dA) sequence at the lower portion of the gel shows predominantly one binding site at the 3′ end of the sequence.

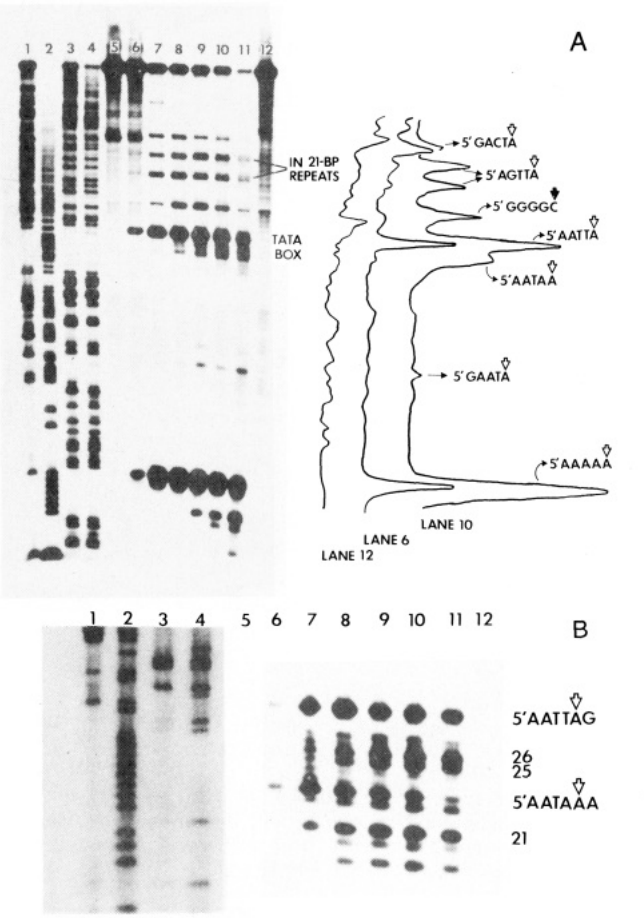


FIGURE 4: Effect of the CC-1065 dose on DNA strand breakage produced by thermal treatment of CC-1065–DNA adducts. (A) The reaction of CC-1065 with SV40 DNA was examined by using fragment 1 depicted in Figure 3. Lanes 1–4, Maxam–Gilbert sequencing reactions: G, A + G, C, and T + C, respectively. Lanes 5–12, CC-1065 treatments. CC-1065 DNA adducts were prepared by incubating the DNA for 24 h at 4 °C with various dilutions of a parent stock solution of CC-1065: lanes 5, 6, 7, 8, 9, 10, and 11 are 1:10^6, 1:10^5, 1:10^4, 1:10^3, 1:10^2, and 1:10 dilutions and undiluted stock solution of CC-1065, respectively. Lane 12 is no drug treatment. The DNA was then precipitated in ethanol, resuspended in DSC, heated (100 °C, 30 min), precipitated again, and electrophoresed as described under Materials and Methods. Densitometric analysis of the autoradiograph was performed as described under Materials and Methods, and representative examples are depicted. The open arrows indicate the adenine that is covalently bound to CC-1065. The solid arrow represents the site of a single-strand break indirectly produced by CC-1065 (see text). (B) The reaction of CC-1065 with the TATA box in the SV40 early promoter was examined by using fragment 2 depicted in Figure 3. Sequencing reactions and drug treatments are as described for panel A.

At 10^3-fold higher concentrations of CC-1065, the 5′ adenine is also receptive to binding by CC-1065, i.e., the fastest migrating bands in lanes 9–11. The 5′AATTA sequence contained within the TATA box is accompanied by a shoulder in the densitometer tracing at 10-fold dilution of the drug, which is due to CC-1065 binding at the sequence 5′AATAA. Figure 4B shows a portion of the autoradiogram from an experiment in which fragment 2 (see Figure 3) has been subjected to various concentrations of CC-1065. The results from this autoradiogram reveal in greater detail the hierarchy of CC-1065 binding sites on one strand around the TATA box. In agreement with the data in Figure 4A, the two most sensitive sites to CC-1065 binding are 5′AATTA and 5′AATAA. Other sites of lower sensitivity occur within the poly(dA) sequence of the TATA box (positions 21, 25, and 26) and on the 5′ side of this sequence (positions 17 and 19). Interestingly,

[2] For this particular fragment (fragment 1 in Figure 3) we consistently found some degradation that was dependent upon heating at 100 °C for 30 min. This results in a smear of bands at the high molecular weight part of this gel (see lanes 5, 6, and 12). While this heat-induced degradation of DNA masks some of the more sensitive CC-1065 binding sites in high molecular weight regions, it did not significantly affect the distribution of binding sites in the lower molecular weight region of this fragment.

not all adenines within the poly(dA) sequence are equally sensitive, and some do not show any CC-1065 binding affinity. A detailed analysis of the pattern of CC-1065 binding on both strands around the TATA box region is given later.

It is clear from the data in Figure 4A that CC-1065 exhibits selectivity in the sequences to which it binds and, following heat treatment, causes strand breakage. It is also clear that different sequences have different affinities for CC-1065. However, as is discussed in more detail later, even the same pentanucleotide binding sequences can respond differently in a quantitative sense. These differences may be due to the proximity of an end of the DNA molecule (there are some quantitative differences between panels A and B of Figure 4 in the region of the TATA box) or to the DNA sequences that flank the binding site of the drug. Nevertheless, quantitative analyses are of significance; the data are highly reproducible, and it should be noted that the sequence 5′AGTTA is equally receptive to CC-1065-induced strand breakage (Figure 4A) when it is part of a longer conserved sequence (two of the three 21-bp repeats of SV40 DNA contain this sequence, and these sites are the most sensitive to CC-1065-induced cleavage so far discovered).

The band between the TATA box and the first of the 21-bp repeats (Figure 4A; see also Figure 8) is interesting from a different perspective. This band is present even in the absence of drug treatment, and strand breakage is located at the cytidine residue within the sequence 5′GGGGCGG. The covalent attachment of CC-1065 is specific for adenine residues (Hurley et al., 1984), and thus the drug should not produce direct strand breakage within this sequence. However, when CC-1065 is bound to the DNA, presumably at distant sites, it dramatically increases the intensity of this band. An analogous effect on other DNA sequences has also been noted with bleomycin-treated DNA (Mirabelli et al., 1983). The basis for this phenomenon is unclear, but it may be related to the changes in overall helix stability, and thus DNA conformation, following drug binding (Swenson et al., 1982). It should be noted that the same sequence (5′GGGCGG), which occurs in the two other 21-bp repeats (see Figure 8), is not a target for either spontaneous or CC-1065-enhanced breakage.

Temperature and Time Dependency of DNA Strand Breakage Produced by CC-1065. We have routinely employed a 24-h incubation of DNA with CC-1065 to ensure that binding was complete; similarly, we have standardized the heating step (to induce strand breakage) for 30 min at 100 °C. In order to show that these conditions are optimal, the temperature and time dependencies of CC-1065-induced breaks were evaluated by using DNA fragment 1 (Figure 3). Following a 24-h incubation at 4 °C of the DNA with CC-1065 and removal of unbound drug, separate aliquots of the modified DNA were heated for 30 min at various temperatures. Integration of the densitometric results demonstrates that, in a 30-min period, significant strand breakage occurs only at temperatures above 70 °C (Figure 5A). The background level of strand breakage observed at lower temperatures is most probably due to the fact that all samples are heated at 90 °C for 1 min in order to denature the DNA prior to loading the sequencing gel. Maximum strand breakage is obtained within 30 min at 100 °C; alternatively, heating at 70 °C for 12 h also leads to complete strand scission (data not shown).

To show that a 24-h incubation of DNA with CC-1065 leads to maximum binding, a 10^3-fold dilution of the stock solution was incubated with DNA (fragment 1, Figure 3) for various

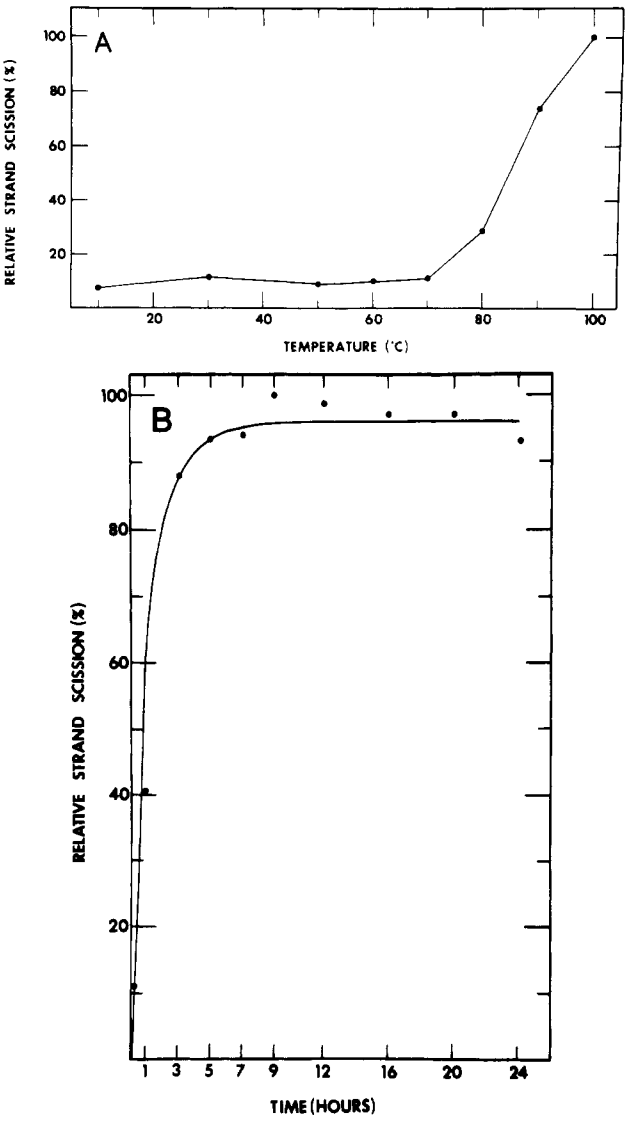


FIGURE 5: Temperature and time dependence of CC-1065-induced breaks. DNA fragment 1 (Figure 3) was treated with a $1:10^3$ dilution of the stock CC-1065 solution and processed as described under Materials and Methods except that in (A) the DNA–CC-1065 adduct was heated for 30 min at various temperatures and in (B) the duration of incubation at 4 °C of DNA and CC-1065 was varied. The data are expressed as the percentage of strand scission at the sequence 5′AAAAA relative to, in (A), 100 °C and, in (B), a 9-h incubation.

times before the drug was removed and strand breakage induced (100 °C for 30 min). The results (Figure 5B) demonstrate that DNA strand breakage at the sequences 5′AAAAA is detectable following the shortest incubation of CC-1065 with DNA examined (1 min) and reaches a maximum between 3 and 5 h. However, other sequences that are less sensitive to CC-1065 binding required longer incubation periods (data not shown).

Relationship of the CC-1065-Directed DNA Strand Break to the Adenine Covalent Binding Site. The isolation of the CC-1065–N3-adenine adduct (see Figure 2) resulting from thermal treatment of CC-1065-modified DNA provided firm evidence for the primary site of covalent modification of DNA by CC-1065 (Hurley et al., 1984). In order to obtain information on the site and a possible mechanism for the thermally induced CC-1065 strand break, the relationship of the position of the strand breakage to the AP site was investigated. Examination of sequencing gels in which CC-1065-induced strand breakage had occurred shows a minor product (Figure 6A, lane 5) that comigrates with the product of an adenine-specific

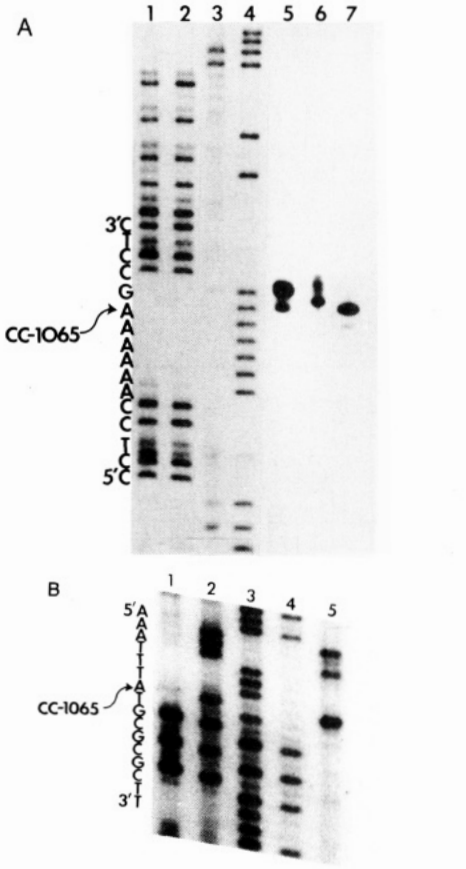


FIGURE 6: (A) Relationship of the adenine to which CC-1065 is covalently bound and the point of CC-1065-induced strand breakage as revealed from a 5′-end-labeled fragment. The relationship between the site of covalent linkage of CC-1065 to DNA and the drug-directed single-strand break was examined by using DNA fragment 3 (Figure 3). Lanes 1–4, Maxam–Gilbert sequencing reactions: C, T + C, G, and A + G, respectively. Lanes 5–7, CC-1065 treatments. CC-1065–DNA adducts were prepared by incubating CC-1065 ($1:10^3$ dilution of stock CC-1065 solution) with DNA for 24 h at 4 °C. After precipitation of the DNA in ethanol to remove unbound drug, samples were treated as follows: In lane 5, thermal treatment was used. The modified DNA was resuspended in 100 μL of DSC and heated at 100 °C for 30 min. In lanes 6 and 7, the sample was resuspended in 100 μL of DSC and heated at 100 °C for 30 min. The DNA was then precipitated in ethanol, dried under vacuum, and treated with either HeLa AP endonuclease II as described under Materials and Methods (lane 6) or piperidine at 100 °C for 30 min (lane 7). Samples were then electrophoresed as described under Materials and Methods. (B) Relationship of the adenine to which CC-1065 is covalently bound and the point of CC-1065 thermally induced strand breakage as revealed by a 3′-end-labeled fragment. A 364-base-pair fragment isolated from φX174 RF DNA 3′ end labeled with [α-^{32}P]dideoxy-ATP was used in this experiment (see Materials and Methods). Lanes 1–4, Maxam–Gilbert sequencing reactions: G, A + G, T + C, and C, respectively. Lane 5, CC-1065 treatment as for lane 5 in panel A.

reaction as defined by the Maxam–Gilbert sequencing protocol. This minor product, which appears in variable amounts, has therefore arisen from cleavage on the 5′ side of the adenine residue that reacted with CC-1065 and is therefore probably a 3′-phosphate-terminated molecule (as are the DNA molecules following chemical cleavage by the methods of Maxam and Gilbert). The major product (Figure 6A, lane 5) is one that migrates more slowly on electrophoresis, suggesting that in this case strand scission occurs to the 3′ side of the adenine that has reacted with CC-1065. This modified adenine is, however, released by heating (Hurley et al., 1984), and thus, the major product of CC-1065-induced strand scission is likely to retain the resulting AP site. If this is indeed the case, then the DNA fragment that produces the major band in lane 5 (Figure 6a) should be susceptible to piperidine degradation. Lane 7 in Figure 6A shows that piperidine treatment of the previously thermally treated CC-1065–DNA adduct quantitatively removes the slower migrating product and produces a single band corresponding to the adenine on the 5′ side of the thermal break. This proves that the covalent binding site (N3 of adenine) for CC-1065 on DNA is on the 5′ side of the thermal cleavage position.

Further evidence that an AP site was responsible for the piperidine-induced break at adenine was obtained by incubating the thermally treated fragment with HeLa AP endonuclease II. The results of this experiment (Figure 6A, lane 6) show that the major point of strand breakage corresponds to a fragment migrating at an intermediate position between the thermal- and piperidine-induced bands. This is to be expected, since it is known that the AP endonuclease produces a strand break on the 5′ side of the AP site and leaves a 3′-hydroxyl (in contrast to the 3′-phosphate resulting from piperidine treatment) on the labeled fragment (Mosbaugh & Linn, 1982).

Evidence for the position of thermal cleavage on the CC-1065–DNA adduct comes from fragments ^{32}P labeled on the 3′ terminus rather than the 5′ end (see Figure 6B). In this case, the chain break nearest the 3′ end of the molecule gave rise to a product that comigrated with the A band in the Maxam–Gilbert sequencing lanes (Figure 6B, lane 5). Thus, the thermal breakage yielded a ^{32}P-3′-end-labeled fragment with a 5′-phosphate terminus, the latter of which is to the 3′ side of the CC-1065-modified deoxyadenosine site. This conclusion is at variance with that of our earlier report (Needham-VanDevanter et al., 1984), which was based on the inability to label, with polynucleotide kinase and [γ-^{32}P]ATP, any of the DNA fragments that result from thermally induced cleavage of a CC-1065–DNA adduct even after phosphatase treatment. The reason(s) for this negative result is (are) unclear, but the data in Figure 6B clearly show electrophoretic comigration of the 3′-end-labeled fragments with a marker DNA that is known to contain a 5′-phosphate (Maxam & Gilbert, 1980).

While the data from the 3′-end-labeling experiment demonstrate that the CC-1065-directed thermal cleavage results in production of a 5′-phosphate, the identity of the 3′-end terminus of the 5′-end-labeled fragment is less clear. Preliminary results suggest that the terminus is a modified deoxyribose resulting from a single β-elimination reaction (Swenson, unpublished results).

Lack of Residual DNA Strand Modification on the Complementary Strand at CC-1065 Covalent Binding Sites. The experiments of Figure 6 conclusively show that the thermally induced DNA strand break occurred on the same strand as the CC-1065–N3-adenine covalent modification site, but they do not reveal whether CC-1065 also modified, or perhaps cleaved, the complementary strand of DNA opposite the CC-1065 lesion. To evaluate this possibility, DNA molecules (fragments 2 and 6 of Figure 3) were prepared where the unique ^{32}P label was on complementary strands. These fragments were modified and heated under standard conditions and examined for DNA strand breaks by gel electrophoresis. As expected from previous experiments, thermal treatment of the CC-1065 adduct produced a single break in fragment 2 on the 3′ side of the adenine in the sequence 5′GGAGTTAGGGG (Figure 7). The complementary sequence showed no detectable residual modification by thermal treatment or thermal plus piperidine treatment. Therefore,

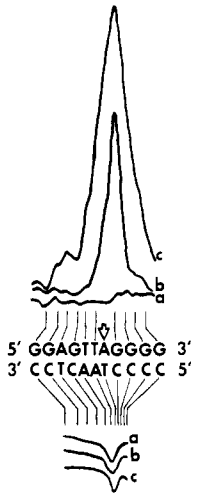


FIGURE 7: Reaction of CC-1065 with overlapping complementary strands of DNA. The reaction of CC-1065 with SV40 DNA was examined in separate experiments using overlapping complementary strands from the 21-bp repeats of the SV40 early promoter region. The DNA restriction fragments used correspond to fragment 2 (for 5'GGAGTTAGGGG) and to fragment 6 (for the complementary 5'CCCCTAACTCC) depicted in Figure 3. CC-1065–DNA adducts were prepared as described in the legend to Figure 4. Densitometric tracings were obtained from autoradiograms and are presented for (a) control sample (DNA heated in the absence of drug treatment) and DNA heated after incubation with (b) $1:10^5$ and (c) $1:10^3$ dilutions of a stock CC-1065 solution. The densitometric tracings for the 5'CCCCTAACTCC sequence are virtually identical and are presented on offset axes for clarity.

Table I: Analysis of the Two Subsets of DNA Sequences Flanking the Covalent Binding Site of CC-1065[a]

		frequencies of occurrence (%)						
	5'	−4	−3	−2	−1	A*	+1	3'
(A) Analysis of 5'PuNTTA3' Sequences								
G		30	33	10	0	0	23	
A		53	33	20	0	100	30	
C		3	17	17	0	0	23	
nT		13	17	53	100	0	23	
Pu		83	67	30	0	100	53	
Py		17	33	70	100	0	47	
GC		33	50	27	0	0	47	
AT		67	50	73	100	100	53	
consensus		Pu	N	T	T	A	N	
(B) Analysis of Poly(A) Sequences								
G		7	13	0	0	0	40	
A		67	67	87	100	100	27	
C		7	7	0	0	0	7	
T		20	7	13	0	0	27	
Pu		73	80	87	100	100	67	
Py		27	20	13	0	0	33	
GC		13	27	0	0	0	47	
AT		87	73	100	100	100	53	
consensus		A	A	A	A	A	N	

[a] A* represents the adenine to which CH-1065 is covalently bound. The frequencies of occurrence of individual bases or base combinations in the regions adjacent to A* are computed from a total of 30 (A) and 15 (B) binding sites on SV40 and bacteriophage T7 DNA which were sensitive to 0.14×10^{-3} to 0.14×10^{-6} μmol of CC-1065/mL. The consensus sequences were determined by χ^2 analysis ($\alpha = 0.005$, part A; $\alpha = 0.05$, part B) using the raw frequency values observed.

CC-1065 does not cleave or leave any detectable residual modification on DNA opposite the primary CC-1065 lesion.

DNA Sequence Specificity of CC-1065. A variety of overlapping restriction enzyme fragments were prepared covering both strands of the control element between the *Hin*dIII B–C junction and the *Hpa*II site on SV40 DNA, as described under Materials and Methods (see Figure 3). In addition to those sequences summarized in Figure 8, other fragments from SV40 and T7 DNA were used in the analysis. In all, over 1000 base pairs were examined for CC-1065 binding sites. Following CC-1065 modification of these fragments and thermal treatment to produce DNA strand breakage, gel electrophoresis was used to determine the DNA sequence specificity. In all cases studied, the adenine covalent binding site is assumed to be on the 5′ side of the position of strand breakage (the minor, variable product has been ignored for this analysis). A catalog of all CC-1065 binding sequences revealed two subsets of sites, in which the sequence specificity always lies to the 5′ of the adenine binding site. The greater number of sequences and those generally more sensitive to CC-1065 belong to the consensus sequence 5′PuNTTA, whereas the subset having fewer examples belongs to the consensus sequence 5′AAAAA. A statistical analysis of the frequency of occurrence of bases at each side of the covalently modified adenine for both subsets is given in parts A and B of Table I.[3] The 5′PuNTTA subset shows an absolute specificity for 5′TA and an almost complete specificity for 5′TTA at 10^4-fold and greater dilutions of stock CC-1065 solutions (0.14 μmol/mL). Interestingly, while there is no specificity for the adjacent base on the 5′ side of the 5′TTA sequence, there is a strong perference for a purine at the next position (see Table IA). With one exception all members of the second subset of binding sites have the sequence 5′AAA at which covalent binding occurs at the 3′ adenine position. There is a less significant preference for adenines at the fourth and fifth base from the adenine covalent binding site, but this should not be overemphasized since the DNA sequences we have studied are overrepresented with poly(dA) tracts relative to other DNAs of the general sequence 5′NNAAA.

The nucleotide sequences of all DNA restriction fragments used in these experiments were examined to locate and identify (1) apparent consensus sequences that *fail* to bind CC-1065 and (2) DNA sequences at which strand breakage occurs due to the presence of CC-1065 but which do *not* fit either of the consensus sequences determined in parts A and B of Table I. Three consensus sequences were located at which CC-1065-induced strand scission was not detected (see Table IIA). These are the adenines at SV40 positions 27, 28, and 3909 and are likely the result of site-exclusion effects produced by nearby CC-1065 binding sites. For example, the CC-1065 adduct at position 3905 may preclude binding and subsequent strand scission at position 3909. In addition to preventing adduct formation, the presence of a nearby CC-1065 adduct may influence the *position* of subsequent CC-1065–DNA adduct formation. For example, it is observed that the 3′-terminal adenine in the 5′AAAAA subset of sequences is the preferred site of covalent binding by CC-1065. However, at the SV40 DNA position 533, CC-1065 selects the middle of a triplet of adenines. This may be due to the presence of a CC-1065 molecule at position 528 which would be expected to occupy the minor groove up to position 532. Further, the complex binding pattern in and around the TATA box (see below) may be due to end-to-end clustering of drug molecules on the DNA. However, with the exception of the examples noted in Table IIA, no other 5′PuNTTA sequences or poly(dA) tracts of three or more adenines were found that failed to bind CC-1065. Furthermore, under the conditions used to generate the data in Table I, there were only three sequences at which drug-related strand scission occurred at *nonconsensus* sequences[4]

[3] A complete list of the actual binding sequences and their location in SV40 DNA and T7 can be obtained from L.H.H.

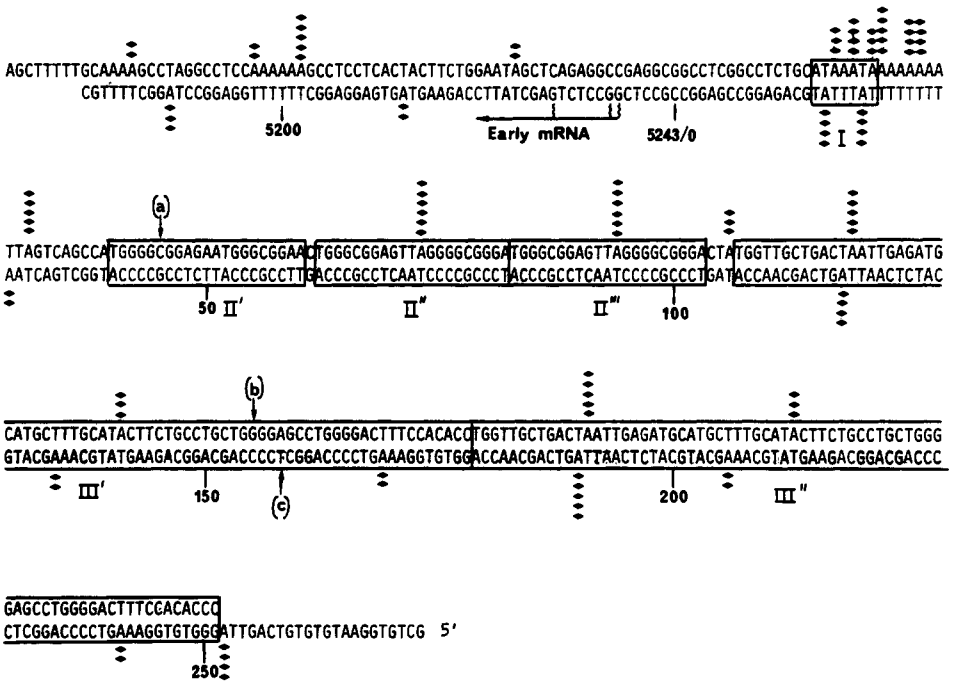


FIGURE 8: DNA sequence specificity of CC-1065 within the early promoter region of SV40. Restriction enzyme fragments were prepared as described under Materials and Methods and were singly end labeled with ^{32}P, incubated with CC-1065 (24 h at 4 °C), and then heated under standard conditions to produce DNA strand breakage. The diamonds (♦) show the CC-1065–adenine covalent binding sites, and the number of diamonds indicates the relative sensitivity to strand breakage by CC-1065; e.g., ♦♦ and ♦♦♦ are $1:10^2$ and $1:10^3$ dilutions, respectively, of a stock solution of CC-1065, which are the maximum dilutions at which strand breakage is still observed. Symbols: I, TATA box; II′, II″, and III‴, 21-base repeat regions; III′ and III″, 72-base repeat regions. (a) Strand breakage site that is enhanced by CC-1065 binding particularly between $1:10^2$ and $1:10^3$ drug dilutions of a CC-1065 stock solution (see also Figure 3). (b) Strand breakage in the poly(G) sequence present in the control. The intensity of this band is relatively weak and is not influenced by CC-1065 binding. A comparable band deriving from the other 72-bp repeat (III″) has not been observed. (c) Strand breakage site at an apparent nondrug binding site. This site for strand breakage is absent in the control and is present only at relatively high concentrations of CC-1065. The equivalent sequence in the other 72-bp repeat (III″) is unaffected by CC-1065.

(Table IIB). It is not clear at this time what factors caused these events.

Sequence Specificity of CC-1065 within the Early Control Element of SV40 DNA. CC-1065 is extremely cytotoxic (Bhuyan et al., 1982; Martin et al., 1978). A partial explanation for the potency of the drug may be its specificity for sequences of DNA that are commonly found in control elements (e.g., the TATA box) in eukaryotic DNA.

Figure 8 summarizes the pattern of CC-1065 binding to the early control element of SV40 DNA. The two perfect 21-base repeat regions contain the most sensitive CC-1065 binding sites (5′AGTTA) so far discovered. This same sequence is found elsewhere in SV40 DNA and at least in the cases examined is a sensitive binding sequence for CC-1065. The 72-base repeat regions contain very sensitive CC-1065 binding sequences on opposite strands that are only one base pair apart (118 and 119). Of the two sequences, only the 5′AATTA (position 119) fits the consensus sequence exactly and may be the preferred site, since although strand breakage can be detected at a 10^5-fold dilution of CC-1065 on both strands, the intensity of the band is markedly greater for the 5′AATTA sequence.[5]

The TATA box region between positions 16 and 31 receives the greatest concentration of hit sites in the early control element of SV40 DNA (10 hits out of 32 positions). The two most sensitive sites to CC-1065 binding within this region are 5′AATTA and 5′AATAA. Furthermore, we can conclude that of these two binding sites, the 5′AATTA sequence is the more sensitive to CC-1065. This conclusion is based upon two observations. First, this binding site will only be evident if the alternative binding site (i.e., 5′AATAA) nearer to the 5′ ^{32}P label is not occupied since cleavage at the latter site would lead to the 5′AATTA being in a nonradioactive and thus undetectable molecule. Second, site exclusion is absolute at the 5′AATTA binding site whereas at the 5′AATAA binding site almost equally sensitive binding sites occur within the distance expected to be occupied by CC-1065. We suggest that after the 5′AATTA site is occupied, other binding sites for CC-1065 are not so strictly enforced. We are, however, unable at this time to offer a firm rationalization as to why the adenines of 18, 23, and 24 are so unreactive toward CC-1065. Conceivably, end-to-end clustering of CC-1065 molecules may be favored. In this way, binding at approximately 5-base-pair intervals may be favored and thereby reduce the likelihood of binding to these particular adenines.

Discussion

The results described in this paper provide important new information on the interaction of CC-1065 with DNA and the selectivity with which CC-1065 is able to discern sequence-dependent features in DNA. The results obtained through this study, taken together with the previously acquired structural information on the CC-1065–DNA adduct, provide an excellent basis to postulate a model for the specific interaction of this drug with DNA.

Structural Modification of DNA by CC-1065 following Chemical and Enzymatic Treatment of the CC-1065–DNA Adduct. Residual modifications of DNA by CC-1065 fol-

[4] In addition to the sequences listed in Table IIB, *enhanced* strand breakage resulting from CC-1065 binding at distant sites has also been noted previously (see discussion of Figure 4A).

[5] We are hesitant to place too much emphasis on this point since it is difficult to relate intensities of bands on opposite strands. Factors such as the location and number of other CC-1065 binding sites can dramatically affect the intensity of a particular strand break.

Table II: Negative Analysis of CC-1065 Binding Sequences in Select Regions of SV40 DNA and T7

(A) Consensus Sequences That Fail To Bind CC-1065

position[a]	DNA sequence flanking the expected (designated ↓)[b] adenine covalent binding site
SV40 27 and 28	5' A A A A A A A A T T A G
SV40 3909	5' T G R G T T T A A A A T

(B) DNA Sequences in Which CC-1065 Induced Strand Breakage That Was Unexpected

position[a]	DNA sequence flanking the unexpected base (designated –) at which strand breakage occurred[c]
SV40 533	5' A A G A C T A A A C C T T A C
	5' G T G T T G G A C C C C A A T
SV40 1020 SV40 158	5' C C C A G G C T C C C C A G C

[a] See footnote to Table I. [b] The diamonds (♦) indicate the position and sensitivity of CC-1065 binding sites (see legend to Figure 8). [c] The diamonds (♦) indicate the position of DNA strand breakage induced by CC-1065.

lowing thermal, piperidine, and AP endonuclease II treatment can only be detected on one strand and include the thermally induced strand break and the AP site. The DNA strand breakage produced as a result of thermal treatment of CC-1065–DNA adducts is located in close proximity to the covalent binding site. Since both events (AP site formation and DNA strand breakage) are induced during thermal treatment of the CC-1065–DNA adduct, it seems likely that they are both related to the same lesion. The data suggest that most likely a single β-elimination at the CC-1065-modified deoxyadenosine produces a backbone strand break yielding a 5′-phosphate and a 3′-modified deoxyribose. Significantly, subsequent treatment with piperidine or AP endonuclease of the previously thermally cleaved CC-1065–DNA adduct does not reveal *new* binding sites for CC-1065 that were not previously revealed by thermal treatment alone. We interpret this to mean that *all* AP sites produced as a result of thermal treatment of the CC-1065–(N3-adenine)–DNA adduct are accompanied by strand breakage at an adjacent or nearby positions.

Effect of the CC-1065–DNA Adduct on the Reactivity of DNA at Sites outside the Immediate Drug Binding Region. Previous studies have shown that CC-1065 produced dramatic stabilization of DNA upon drug binding. For example, CC-1065–DNA adducts have significantly elevated melting temperatures and are far less susceptible to digestion by S1 nuclease and intercalation by ethidium bromide (Swenson et al., 1982). Several observations made during this investigation support the concept that CC-1065 can exert stabilizing and telestability effects (Wells, 1977). As noted in Figure 8 (notes a and c), there are two strand breakage sites in DNA that are influenced *indirectly* by CC-1065 binding at distant positions. In one case (note a in Figure 8), enhancement of background strand breakage occurs at higher drug binding ratios of CC-1065, while in the second case (note b in Figure 8), a DNA strand breakage position occurs, presumably at a non-CC-1065 binding position, that is absent in the non-drug-treated lane. In both cases, destabilization of the helix is occurring at sites remote from the primary drug binding site (telestability effect).

Sequence Specificity of CC-1065. The prime purpose behind selecting the early promoter element of SV40 DNA as the target for determining the DNA sequence specificity of CC-1065 was to investigate the possibility that this region might contain a biologically important sequence that was exquisitely sensitive to CC-1065. Our prior knowledge that CC-1065 showed high specificity for AT polymers (Swenson et al., 1982) suggested that the TATA box might be a prime target for CC-1065. While the TATA box region received multiple hits by CC-1065 (10 possible binding sites in a 16-base-pair region, which is considerably more than the number that any other comparably sized sequence receives that we have examined), other sequences appear to have more sensitive CC-1065 binding sites, e.g., the 5′AGTTA sequence in the 21-bp repeat (see Figure 8). A negative analysis of the DNA sequences screened for CC-1065 binding sites reveals that the lack of CC-1065 binding sites within other SV40 and T7 DNA regions can be explained by the absence of appropriate sequences. Furthermore, there are no consensus sequences that fail to bind drug except where site exclusion by higher affinity binding sites exists at adjacent sequences.

While this study establishes the most sensitive binding sequences in the restriction enzyme fragments examined for SV40 and T7 DNA, it is important to recognize that the sequence specificity may also be dependent upon the topology of the substrate. For example, Mirabelli et al. (1983) have demonstrated the significant effect of changes in topological conformation of DNA on the sequence-specific breakage of DNA by bleomycin and talisomycin. Therefore, some caution must be taken in extrapolating our in vitro linear DNA results with CC-1065 to the intracellular and actual chromatin interactions with this drug.

Development of a Model for the CC-1065–DNA Adduct. The established primary site of covalent binding of CC-1065 through N3 of adenine attaches the drug molecule to the inside of the minor groove of DNA. Furthermore, the right-handed twist of the CC-1065 molecule provides the drug molecule with a suitable pitch to be accommodated comfortably within the minor groove of B-form DNA. Competition experiments with netropsin and anthramycin (see the introduction) support these findings. CPK models of the CC-1065–(N3-adenine)–DNA adduct predict that the drug molecule should cover about five base pairs. However, since only the relative stereochemistry at C3b and C4a is known from X-ray crystallography (Chidester et al., 1981), the polarity of the drug molecule in the minor groove in reference to the covalent binding site cannot be predicted. The data presented in Table I confirm the CPK model prediction that CC-1065 should span about five base pairs, since this is the extent of the DNA sequence specificity (i.e., 5′PuNTTA and 5′AAAAA). Furthermore, the strict orientation of the sequence specificity to the 5′ side of the adenine covalent binding site in both subclasses is predictive of the polarity of drug binding in the minor groove. The CPK model of the CC-1065–(N3-adenine)–DNA adduct can only be accommodated to agree with the polarity of drug binding if the stereochemistry at the C4 position of the CC-1065–DNA adduct molecule is *R*. Therefore, the absolute stereochemistry

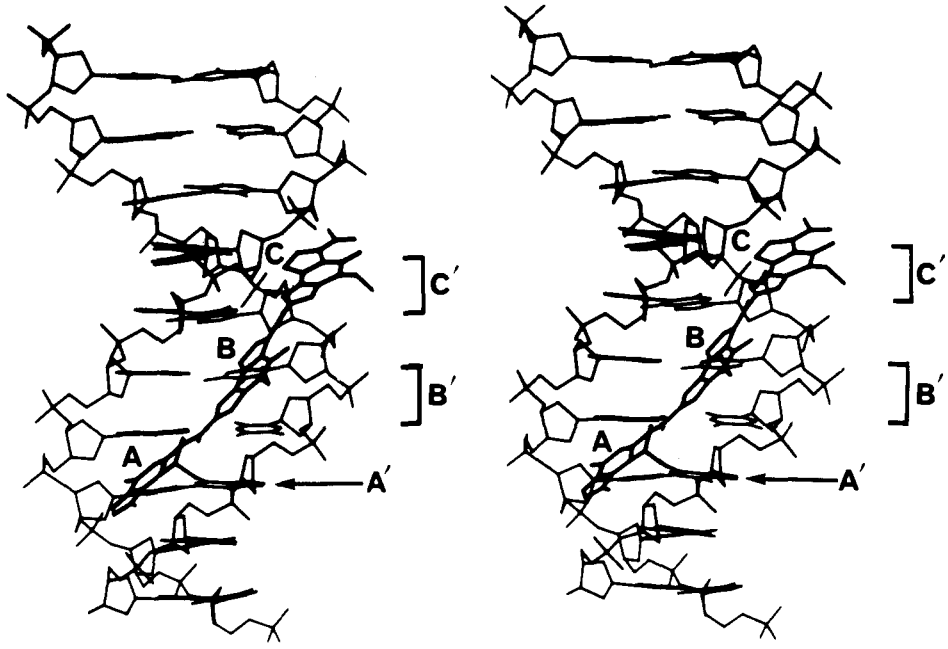


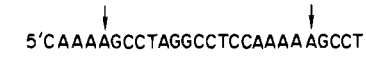
FIGURE 9: Stereo drawings of the CC-1065–DNA adduct. Key: A, B, and C are the subunits of CC-1065; A′ is the adenine covalent binding site for CC-1065; B′ is a pair of highly conserved bases immediately to the 5′ side of the adenine covalent binding site; C′ is a pair of less well conserved bases at the 5′ end of CC-1065 binding site. The base sequence from top to bottom is 5′CGGAGTTAGG3′. For readers having difficulty visualizing this figure in three dimensions, the use of stereo glasses is recommended.

of CC-1065 must be C3b(*R*),C4a(*S*). This is illustrated in Figure 1. To our knowledge, this is the first time that DNA has been used as a chiral reagent to determine the absolute stereochemistry of a second molecule. An interesting consequence of the construction of a CC-1065–(N3-adenine)–DNA adduct in which the unnatural antipode of CC-1065 [C3b(*S*),C4a(*R*)] is used is that the sequence specificity should lie to the 3′ side of the adenine covalent binding site rather than the 5′ side as is the case with the naturally occurring antipode.

Model of the CC-1065–(N3-Adenine)–DNA Adduct and Its Use To Rationalize the Chemical and Biological Consequences of CC-1065 Binding to DNA. Following the construction of the CPK model of the CC-1065–(N3-adenine)–DNA adduct, an Evans and Sutherland computer graphics system was used to prepare a pair of stereo drawings of the same adduct. The docking was carried out between the DNA sequence 5′CGGAGTTAGG and the cyclopropane ring opened CC-1065 molecule depicted in Figure 2. The coordinates of the DNA molecule were taken from a program prepared by Arnott (1972), while the coordinates of the modified CC-1065 were taken predominantly from the X-ray structure prepared by Chidester et al. (1981). After the covalent linkage between C4 of CC-1065 and N3 of adenine was formed, the conformations about the amide linkages between subunits A and B and between subunits B and C were adjusted to reduce the number of close contacts between the DNA and CC-1065 to a minimum. The stereo drawings of this adduct are shown in Figure 9.

Both the CPK model and the stereo drawings of the CC-1065–DNA adduct reveal a remarkably snug fit of the drug molecule within the minor groove of DNA. CC-1065 spans the five-base-pair region specified by the CC-1065 DNA sequence specificity. A careful examination of the CPK model reveals that the floor of the minor groove is in close proximity to the inside edge of the CC-1065 molecule. The width and depth of the minor groove appear just sufficient for CC-1065 to be accommodated without disruption of the helix or protrusion of the drug molecule outside of the minor groove. The closeness of the fit of CC-1065 within the minor groove of DNA is predictive of the considerable DNA sequence specificity exhibited by CC-1065. We speculate that a layer of water molecules could bridge the hydrophilic substituents on the outside edge of CC-1065 with phosphate groups on DNA. This would provide additional stabilizing interactions that would inhibit both DNA melting and intercalation of other agents in the vicinity of the CC-1065 binding site. While hydrogen bonding may facilitate stabilization of the CC-1065–DNA adduct, it is probably not involved in the original binding mechanism (Braithwaite & Baguley, 1980).

The proximity of specific regions of the CC-1065 molecule to groups within the chemically interactive range on the DNA molecule can be used to speculate on the reason for the two subclasses of DNA binding sequences. First, the data in Table I reveal there is an overwhelming preference for AT pairs over GC pairs. This is similar to the data for netropsin and distamycin, which also bind in the minor groove but without a formal covalent linkage. The snug fit of the CC-1065 molecule along the floor of the minor groove is suggestive that the bulky 2-amino group of guanine may discourage binding via steric interference. A striking example of the dramatic effect of substitution of a GC pair for an AT pair on CC-1065 binding to DNA is found in the SV40 DNA sequence between positions 5180 and 5202, i.e.



In this region on SV40 DNA, the binding sequence 5′CAAAA is only receptive to CC-1065 binding at drug concentrations 10^3-fold higher than those that react with the very similar binding sequence 5′AAAAA.

The discovery of the two different consensus sequences for CC-1065 binding sites was unexpected. A comparison of the consensus analysis in parts A and B of Table I reveals that if the base on the 5′ side of the covalently modified adenine is an adenine then the next base will most likely be another adenine and similarly for pairs of thymines. The specificity for the 5′-proximal pair of bases in both consensus sequences is less well-defined. Therefore, it is possible to consider that

the consensus sequence for both subclasses of binding sites consists of three basic units. The first unit (A′) at the 3′ end consists of the alkylation site for CC-1065; the second unit (B′) is a pair of highly conserved bases (AA or TT); the third unit (C′) is a pair of less well conserved bases (AA or PuN). Examination of the stereo drawings of the CC-1065–DNA adduct (Figure 9) reveals that the three units (A′, B′, and C′) making up the consensus sequence overlap with subunits A, B, and C of CC-1065, respectively. Since the subunits of CC-1065 are connected by amide linkages, there is some flexibility in the conformational angles between subunits A and B and between subunits B and C. Moreover, the rigidity within subunit B of CC-1065 which overlaps with the second consensus unit (B′) will specify one of a pair of homoduplexes (AA or TT), depending upon the conformational angle between subunits A and B and between subunits B and C. Presumably, the steric interactions presented by a heteroduplex consisting of 5′AT or 5′TA are less favorable. From the data in Table I, the order of preference for unit B′ for interaction with CC-1065 is 5′TT > 5′AA. The less strict preference for consensus unit C′ makes a similar analysis as carried out for unit B′ less informative.

While these results do not directly provide a single rational basis to explain the extreme cytotoxic potency of CC-1065, at least three possibilities can be suggested. The remarkable DNA sequence specificity of CC-1065 might lead to selective reactions with a critical target within DNA. Although such a target is still unclear, we are screening larger portions of DNA for such binding sites. Alternatively (or additionally), CC-1065 might alter DNA helix or chromatin structure and affect gene expression at critical sites some distance from the actual drug binding position. Finally, the CC-1065–DNA adduct may represent an intractable lesion to DNA repair enzyme processes or one that results in detrimental repair and/or the production of a lethal lesion in DNA. Some of these possibilities are presently being explored in our laboratories.

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