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Recognition of Bulged DNA by a Neocarzinostatin Product via an Induced Fit Mechanism

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Abstract—The binding of the wedge-shaped isostructural analogue of the biradical species of the chromphore of antitumor antibiotic neocarzinostatin to sequence-specific bulged DNAs results in alterations in ellipticity of the DNAs. Circular dichroism (CD) spectroscopic results suggest that the drug specifically recognizes bulges of DNA via a combination of conformational selection and induced fit, not by binding to a preorganized site. Analysis of circular dichroism spectra indicates that the degree of induced fit observed is primarily a consequence of optimising van der Waals contacts with the walls of the bulge cavity. The effective recognition of the bulge site on duplex DNA appears to depend to a significant extent on the bent groove space being flexible enough to be able to adopt the geometrically optimal conformation compatible with the wedge-shaped drug molecule, rather than involving 'lock and key' recognition. The spectroscopic results indicate a change of DNA conformation, consistent with an allosteric binding model. Spectroscopic studies with various bulged DNAs also reveal that the binding strength directly correlates with the stability of the bulge structures.

Introduction

Many ligands have been discovered to possess a composite specificity involving recognition of both primary and secondary structural features of nucleic acids.[1] Few ligands, however, exhibit tertiary structure specificity.[2,3] Conformational recognition has been implicated in many biological processes which regulate some important transcription and translation steps.[4] Despite many studies probing the interactions of ligands with nucleic acid duplexes, relatively little is known about ligand binding to bulge-containing forms of nucleic acids.[5,6] Neocarzinostatin chromophore (NCS-chrom), a potent antitumor antibiotic, has long been studied as a model of sequence-specific intercalative DNA (cleaving) binding ligand.[7] It has been well established that NCS-Chrom binds in the minor groove of duplex DNA by intercalation of its naphthoate moiety and specific base pair recognition by its 2′-*N*-methyl sugar moiety.[8,9]

NCS-chrom, however, differs from other members of the enediyne family[10] in its ability to attack specifically and exclusively a single residue at a two-base bulge of certain DNA sequences under the influence of general base catalysis.[11,12] The reaction with DNA bulges is initiated by an intramolecular nucleophilic attack of C1″ at C12 of NCS chrom **1**, resulting in the sequential formation of the labile spirolactone-containing intermediates cumulene **1c** and biradical **1d** (Scheme 1). The biradical **1d** or rather its derivative **1e**[12] is ultimately responsible for DNA damage by hydrogen atom abstraction at the bulged site and production of **3**. Compound **2**, produced by the general-base-catalyzed activation of NCS chrom in the absence of DNA, is a stable structural analogue of the labile biradical species and binds specifically at the bulge site.[13] Further, this specific recognition was shown by NMR analysis to result from an unusual binding mode by **2**[14] via the major groove to a two-base bulged oligodeoxynucleotide. The wedge-shaped **2**, with its planar naphthoate and indacene ring systems, held rigidly at a 60° angle to each other by the connecting spirolactone, fits snugly in the triangular prism bulge binding pocket formed by the two looped-out bulge bases and the neighboring base pairs.[14]

A recent study[12] elucidating the detailed mechanism of the base-catalyzed transformation of NCS-chrom clearly shows the possible roles of bulged DNA in promoting the activation reaction. Evidence was provided

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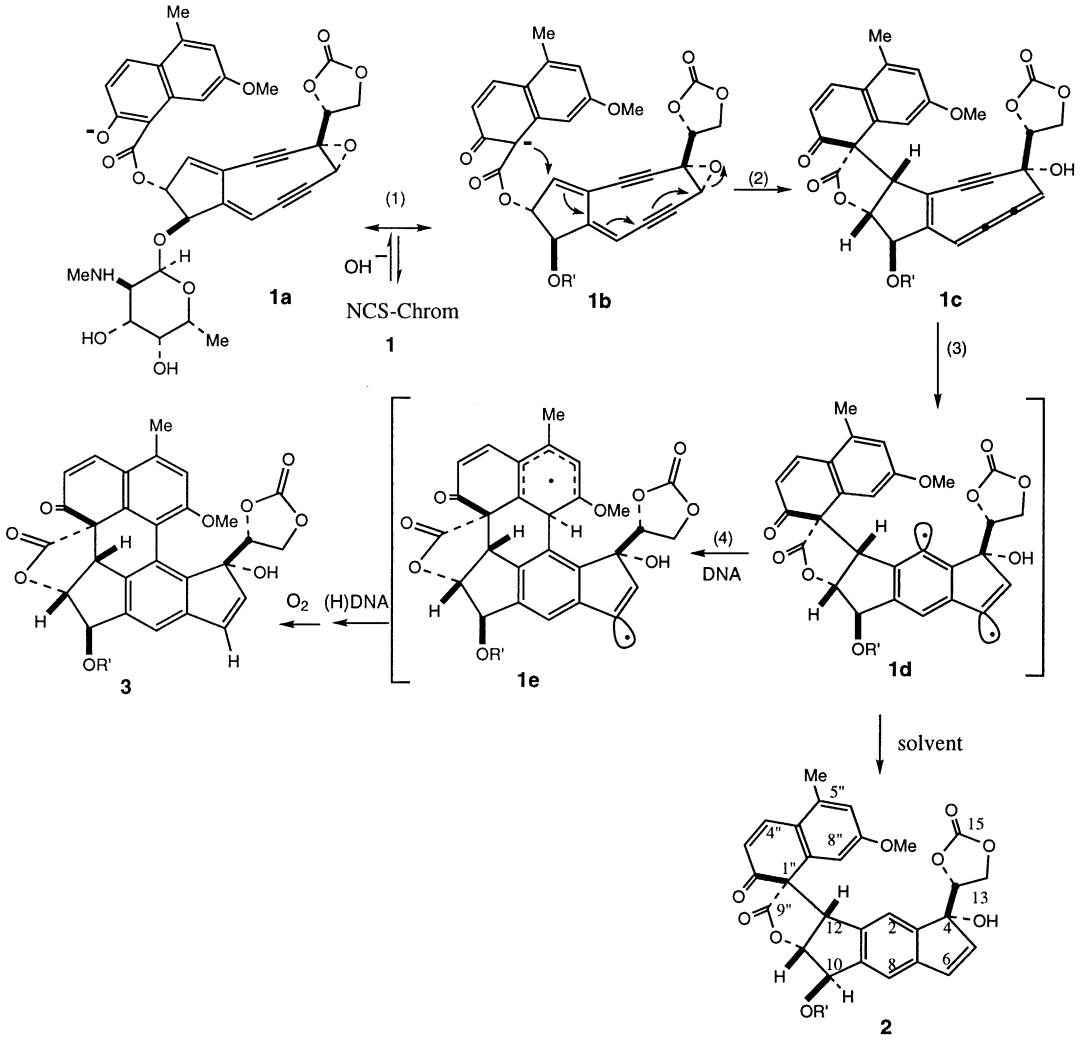

Scheme 1. Proposed thiol-independent mechanism of NCS-chrom-induced cleavage of bulged DNA.

that nucleobases in the DNA bulge are not required to form an effective bulge pocket but enhance the binding of the wedge-shaped activated drug molecule. Study of the effect of solvent (methanol) concentration on NCS-chrom degradation indicates that bulged DNA acts to assist the intramolecular quenching of the radical at C2 by C8 of the naphthoate moiety by excluding solvent from the binding pocket, thus preventing the formation of spirolactones **2** and by blocking radical polymerization. Subsequent prediction was made that the DNA bulge may also induce a conformational change in the drug to promote the intramolecular reactions.

The present study was undertaken to fully understand the dynamics of the unusual recognition at the tertiary structure level and to determine the exact roles the bulged DNA and the drug play in the binding process in terms of conformation. What is the consequence in terms of the conformation of bulged DNA upon specific binding of **2**? This paper presents evidence that the drug analogue induces significant conformational change in bulged DNA so as to enable it to serve as a binding host for the drug. The circular dichroism spectral profiles reflect the binding of the drug to bulged DNA. Further, the difference CD spectra generated from drug–DNA complex show that the conformations of bulged DNA are significantly perturbed, but to a different degree depending on the different sequences of the bulge strands. This reflects the relative orientation of the drug derivative with respect to DNA. The conformational flexibility of bulged DNA facilitates incorporation of the induced-bent drug molecule at the bulge, optimizing the binding. A dynamic and cooperative induce-fit mode is suggested for the bulged DNA recognition. These results indicate that structural alterations in the DNA can be recognized by a specific ligand via conformational alterations in the drug.

Results and Discussion

Fluorescence analysis of drug–DNA complex formation

We have earlier reported[13] that the fluorescence of drug **2** is strongly quenched upon binding to DNA BG22. In this study, we extend our fluorescence binding titrations with other bulge-containing DNAs (Fig. 1) used in the CD experiments. Figure 2 shows the effect of these DNAs on the emission spectrum of compound **2**. As seen in Figure 2A, the non-bulge hairpin oligonucleotide DS8-

L-8 containing a chemical linker to form the hairpin fails to quench the drug fluorescence, even at a ratio of 6:1, ruling out the possibility of drug binding at the hairpin loop site. On the other hand, increasing concentrations of its bulge-containing counterpart, BG8-L-10, results in a significant fluorescence quenching (Fig. 2B) up to a maximum of ca. 60%, without any perceptible shift in fluorescence maximum (500 nm). A similar fluorescence quenching pattern was found upon addition of the more stable 26 mer bulged DNA BG26, which possesses a four nucleobase hairpin. This DNA structure, containing longer duplex regions than BG8-L-10, is, as expected, a more effective quencher (Fig. 2C). BG28, which extends the stem with an alternating base pair, results in even stronger binding: $K_d = 1.74$ μM. The dissociation constants of the complexes formed between **2** and the DNAs in Figure 1 are shown in Table 1. Figure 2D shows the fluorescence ratio F_0/F for the drug as a function of the DNA concentration, manifesting the binding (quenching) efficiency with respect to bulge formation.

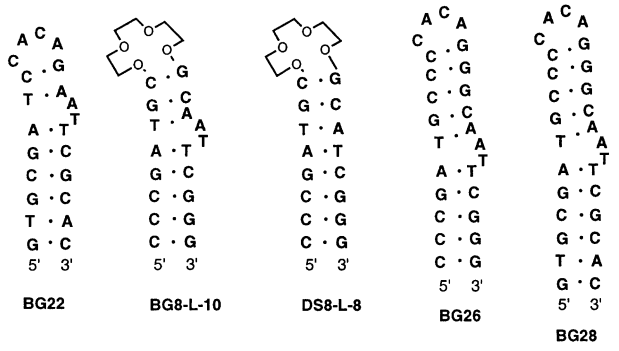

Figure 1. Structures of bulge-containing DNA oligomer binding hosts.

CD studies of conformations of drug–DNA complex

CD spectropolarimetry was used to monitor conformational transitions as the ligand–polynucleotide complex formed. Ligands such as drug molecules acquire an induced CD signal when they form complexes with a polynucleotide. To gain insight into the chiral nature and the dynamics of bulged DNA binding to a drug, the bulge containing oligonucleotides shown in Figure 1 were selected as binding hosts for **2**. Figure 3 shows the circular dichroism titration spectra of bulged DNA. Figure 3A gives the titration spectra of DNA BG8-L-10 and **2** from 200 to 320 nm. The changes in the CD spectra of DNA and the drug upon addition of increasing amounts of DNA are shown in Figure 3A. The CD spectra of drug with different concentrations of DNA reveal an isoelliptic point at 255 nm. Figure 3B gives the titration spectra of DNA BG26 and **2** from 200 to 320 nm. The

Table 1. Dissociation constants (K_d) of bulged DNA with drug

DNA bulges	K_d (μM)
DS 8-L-8	∞
BG 8-L-10	6.13
BG22	2.90
BG 26	1.82
BG 28	1.74

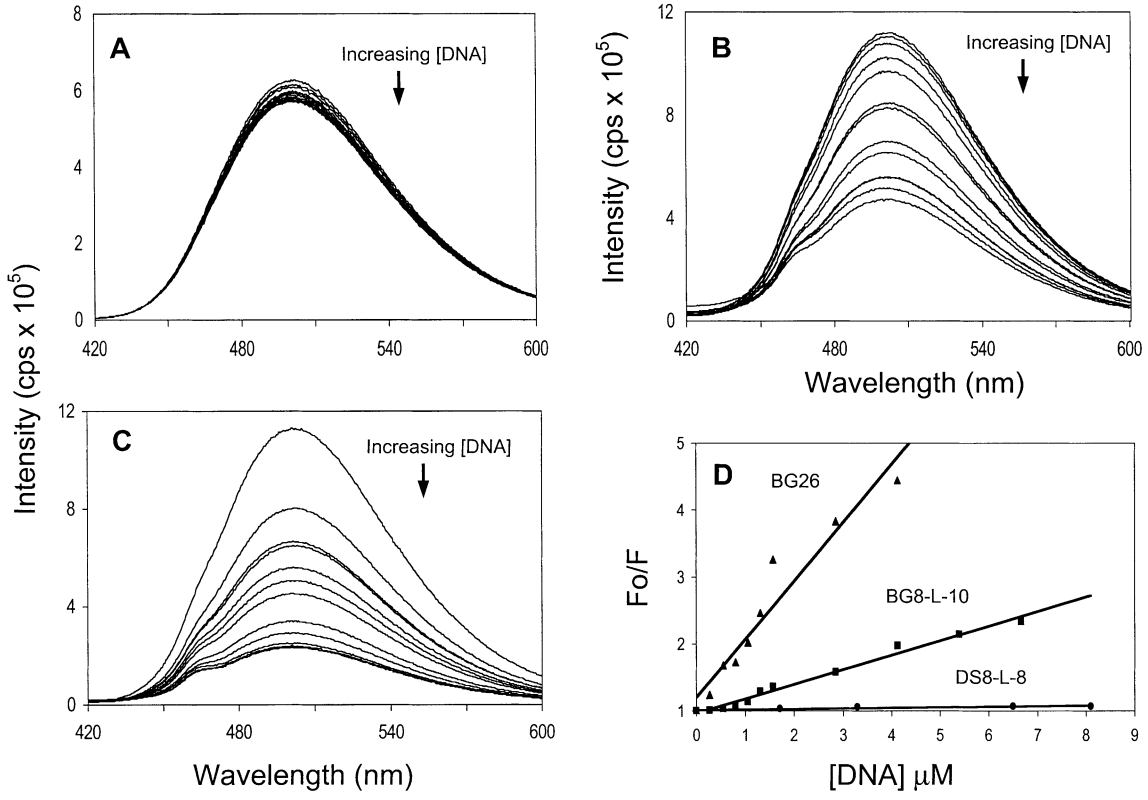

Figure 2. Fluorescence quenching spectra of drug NCS-**2** by DNA. A 0.57 μM drug is mixed with the DNAs: (A) DS8-L-8 from 0, 1.7, 3.3, 6.5, 8.1, 9.7, 11.3, 12.9, 14.5, 16.0, 17.6 to 9.25 μM; (B) BG8-L-10 from 0, 0.27, 0.54, 0.80, 1.05, 1.30, 1.56, 2.85, 4.13, 5.39, 6.67, 7.86 to 9.25 μM; and (C) BG26 from 0, 0.27, 0.54, 0.80, 1.56, 2.85, 4.13, 5.39, 6.67 to 7.89 μM; containing pH 5.2, 5 mM sodium acetate buffer. Emission fluorescence intensity was measured at 5 °C with the excitation at 400 nm. (D) is the fluorescence ratio F_0/F for NCS-**2** as a function of the DNA concentration.

changes in the CD spectra of DNA and the drug upon addition of increasing amounts of DNA are also shown in Figure 3B. The CD spectra of drug with different concentrations of DNA consistently reveal an isoelliptic point at 255 nm, indicating the formation of a drug–DNA complex. Figure 3B also shows a gradual red shift of the peak of the negative band from 229 to 233 nm. The insert in Figure 3B shows the single-wavelength titration curves obtained from the CD amplitudes at 230 nm as a function of molar concentration of added DNA to the drug. As can be seen from the insert, the negative CD amplitude at 230 nm approaches saturation. The interaction of the drug with bulged DNA elicited considerable changes in its CD spectra.

Figure 4A shows the CD spectra of the native DNA (curve a) and its altered DNA (curve b) after subtracting the drug-alone spectrum from the complex, assuming that the conformation of the drug is not significantly altered since the molecular model of **2** is fairly rigid. The non-bulged hairpin DS8-L-8 was also examined with drug. There was no overall ellipticity change detected from the difference spectra of DNA in Figure 4A, ruling out the possibility of conformational change.

Figure 4B shows the CD spectra of BG8-L-10 (curve a) and its altered DNA (curve b) complex with the drug. The spectrum of drug-subtracted DNA compared to that of the native DNA has been significantly changed, giving distinct negative peaks at 220 and 238 and a positive peak at 287 nm, indicating an alteration in the DNA conformation. The altered DNA spectrum (curve b) in Figure 4B shows a large decrease in the positive band at 268 nm, and the positive peak is red-shifted by 19 nm. These changes above 260 nm may imply a change in the winding angle for the DNA complex by the drug.[15] The decreased long wavelength band is reminiscent of spectra of dehydrated DNAs in a non-standard B conformation, suggesting a more dehydrated form.[15]

With BG26, greater negative ellipticity changes are generated in the altered DNA spectrum (curve b) from the drug–DNA complex in Figure 4C, which indicates that the longer stem secured the bulge formation and makes for a better complex, inducing larger conformational changes. The increased band intensities of the BG26-altered DNA spectrum throughout the spectral region from 210 to 260 nm suggest an overall bending of the DNA backbone.[15] A red shift is observed indicating that a tightly binding complex was formed. Figure 4D shows the CD spectra of DNA with a hairpin loop composed of four nucleobases BG22 and its altered DNA (curve b) from the complex. The curve b of altered BG22 exhibits a red shift (from 275 to 282 nm) of the positive band and negative ellipticity changes on the DNA difference spectrum.

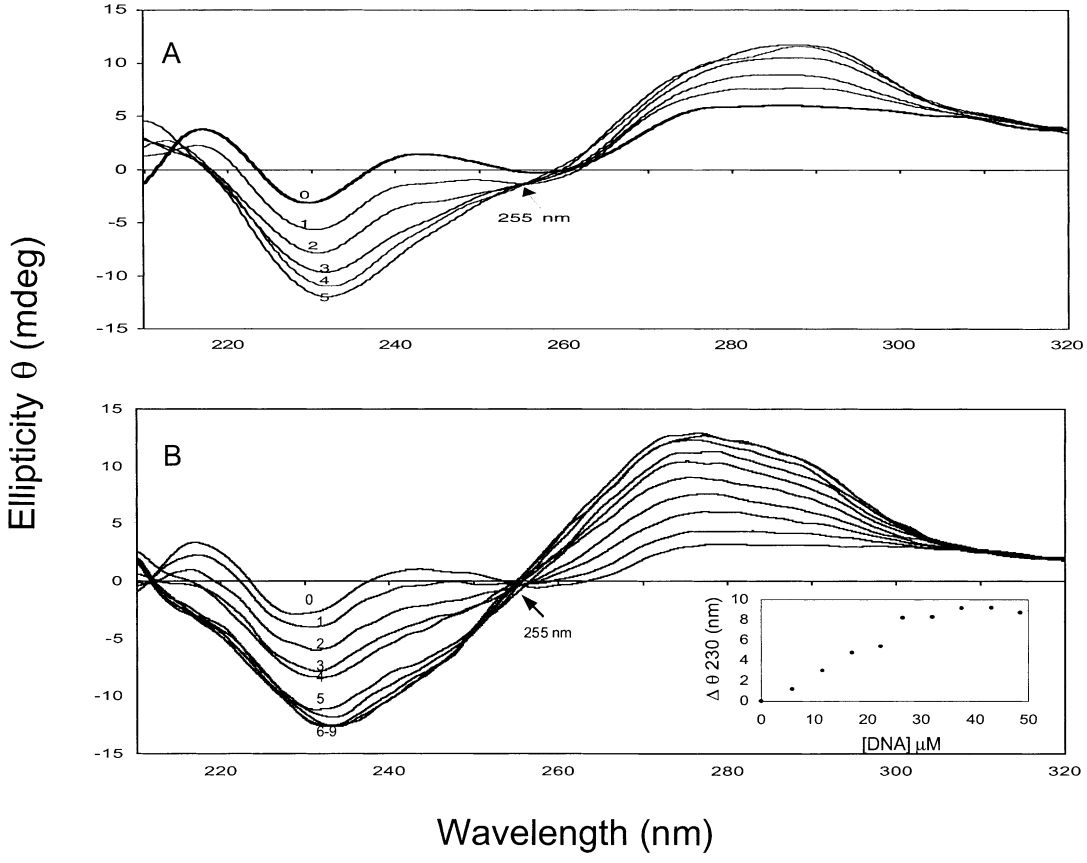

Figure 3. Circular dichroism titration spectra of complex of bulged DNA with drug **2**. (A) Drug **2** and its complexes with bulged DNA BG8-L-10 at various concentrations: 0 (0), 11 (1), 22 (2), 32 (3), 42 (4), and 47 μM (5). (B) Drug **2** and its complexes with bulged DNA BG26 at various concentrations: 0 (0), b (1), 12 (2), 17 (3), 22 (4), 27 (5), and 32 μM (6). (B) All CD measurements were recorded from 210 to 320 nm at 5 °C on a Jasco-810 spectropolarimeter with a 19.4 μM drug–DNA complex containing pH 5.20, 5 mM sodium acetate buffer with 1 mM NaCl. The insert shows the single-wavelength titration curves obtained from the CD amplitudes at 230 nm as a function of molar concentration of added DNA to the drug.

Conclusion

Structural interpretation of the enhanced binding of NCS drug product 2 to the bulged DNA

Nucleic acids are capable of forming complex three-dimensional structures (e.g., bulges and hairpins) that are ubiquitous components of the complex folded assembly. These higher-ordered nucleic acid structures are often associated with important biological functions.[16] The behavior of bulges in DNA duplexes is not completely understood although it depends on many factors, including the sequence context, temperature, the length of the bulge region, and so on.[16] Binding of ligands may influence the property of bulged DNA structures. Our experiments demonstrate an 'induced fit' mechanism in the recognition event of the drug altering the conformation of bulged DNA. The binding of hydrophobic drug **2** reduces the degree of hydration in the major groove and then induces a conformational change in the host-bulged DNA; shifting the bulged distortion away from the original location. The reorganization of the host DNA provides a major-groove surface that is complementary to the reoriented drug molecule. These features reflect the ability of the NCS-drug intermediate to alter both the conformations of the DNA and its own inherent twist to achieve the proper phasing of its aromatic sub-units with the edges of the base pairs. This highly cooperative 'induced fit' binding behavior appears to enhance both hydrogen bonding interactions and van der Waals contacts between the ligand and atoms lining the walls of the major groove. Such hydrogen bonds and van der Waals contacts are generally energetically favorable and maximized through the structural accommodations providing the drug–DNA complex with a source of enhanced stability.

This study also indicates that **2** is a model compound that possesses the geometry for mimicking natural helical bases. The DNA bulge may also induce a slight conformational change in the drug (data not shown) to promote the intra-molecular reaction. Ellipticity alterations strongly suggest the stacking interactions caused by the two rigidly held ring systems of drug **2** form a molecular wedge that penetrates the binding pocket and immobilizes the flexible bulge.[14] A considerable degree of induced fit is involved in drug recognition; the drug appears to stabilize a flexible major groove rather than fitting into a preorganized binding site. The reorganization of the host DNA provides a major-groove surface that is complementary to the bending drug molecule (U-shape) (Fig. 5). NMR measurements also suggest that the binding of **2** promotes the formation of the bulge binding pocket since the unbound DNA strand does not possess structure at the potential bulge site (X. L. Gao, A. Stassinopoulos, J. Ji, S. Bare, Y. Kwon and I. H. Goldberg, unpublished data). A 'closing jar' process of binding is suggested in Figure 5.

There are three possible simplistic models for this cooperative binding since CD only provides the global conformational state of the complex. Figure 6 schematically shows the possible models. Model A assumes that

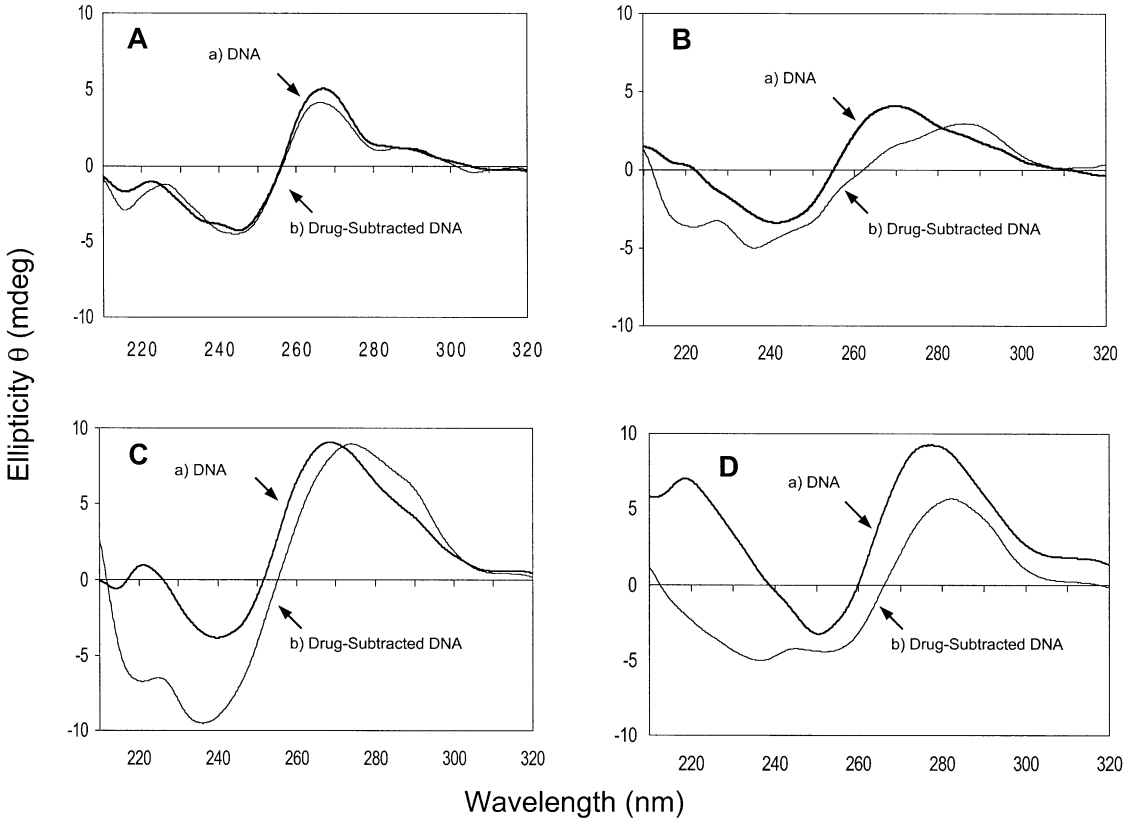

Figure 4. Circular dichroism spectra of DNAs alone (a) and DNAs plus drug after subtraction of drug alone (b). All CD measurements were recorded with a 24.5 μM drug–DNA (1:1) complex containing pH 5.20, 5 mM sodium acetate buffer with 1 mM NaCl. DNA used are (A) DS8-L-8, (B) BG8-L-10, (C) BG26, and (D) BG22.

the binding of the drug to DNA induces some conformational change in the drug lattice providing enhanced binding affinity. In model B, the binding of the drug to DNA is assumed to induce some conformational change in the DNA lattice, with the result that the drug will bind with somewhat greater affinity. In model C the drug binds to an unperturbed lattice, but when bound contiguously they induce some conformational change in the lattice which is favorable for binding and gives a greater affinity. The specificity of only oligodeoxynucleotides[11] capable of forming certain bulged structures as substrates for this base-catalyzed reaction can be attributed to the more effective binding. The optimal bulge structures[17–20] are necessary to warrant the cooperative interactions.

Finally, this study provides evidence that reveals the tertiary structure (conformation) recognition process of **2**. A long-range effect of conformational change is propagated away from the initial bulged site due to the binding of the drug. This finding may be helpful in understanding the possible effect of the drug on a bulged DNA. In addition, NCS-chrom has been demonstrated as a unique ligand capable of forming different complexes at minor and major grooves with different DNA targets. Such extremely dynamic major groove binding could provide insight into designing more specific agents to recognize altered DNA. Several research groups have been inspired by this fascinating mode of recognition specificity to synthesize **2** analogues,[21] which may be used as ligands for selective control of gene function. A thorough understanding of the tertiary DNA recognition process of the natural enediyne compounds will allow further developments in the area of drug design of the synthetic enediynes. The second-generation enediynes may find future application not only in cancer chemotherapy but also as antiviral agents.

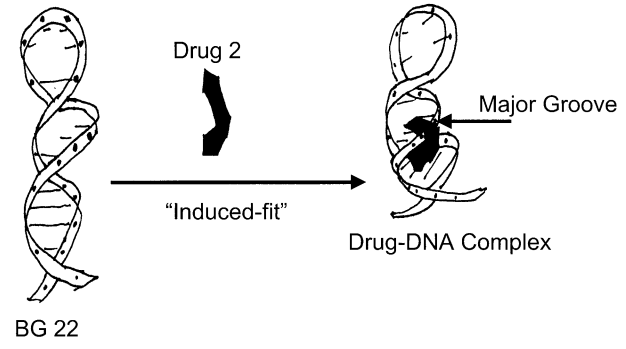

Figure 5. Schematic representation for the process of binding of **2** to DNA.

Experimental

Materials

Oligonucleotides. Oligonucleotides were synthesized at the Nucleic Acid Facility of University of Pennsylvania using standard cyanoethyl phosphoramidite chemistry, followed by purification with reverse-phase HPLC. A 9-*O*-dimethoxy-trityltriethyleneglycol 2-cyanoethyl *N*,*N*-diisopropylchloro-phosphoramidite (X) was used for the linker incorporation. DNA concentrations (all expressed per mole of oligonucleotide) were determined by UV absorbance using the following extinction coefficients (mM^{-1} cm^{-1}) $\varepsilon_{260} = [(nA \times 15.4) + (nC \times 7.3) + (nG \times 11.7) + (nT \times 8.8) + (nX \times 10.8)] \times 0.9$. Annealing of strands to form duplexes containing bulges was accomplished by heating the solution to 95 °C and then cooling overnight to room temperature followed by refrigeration for 30 min to ensure the formation of bulges. Figure 1 shows the putative structures of the oligonucleotides used in this study.

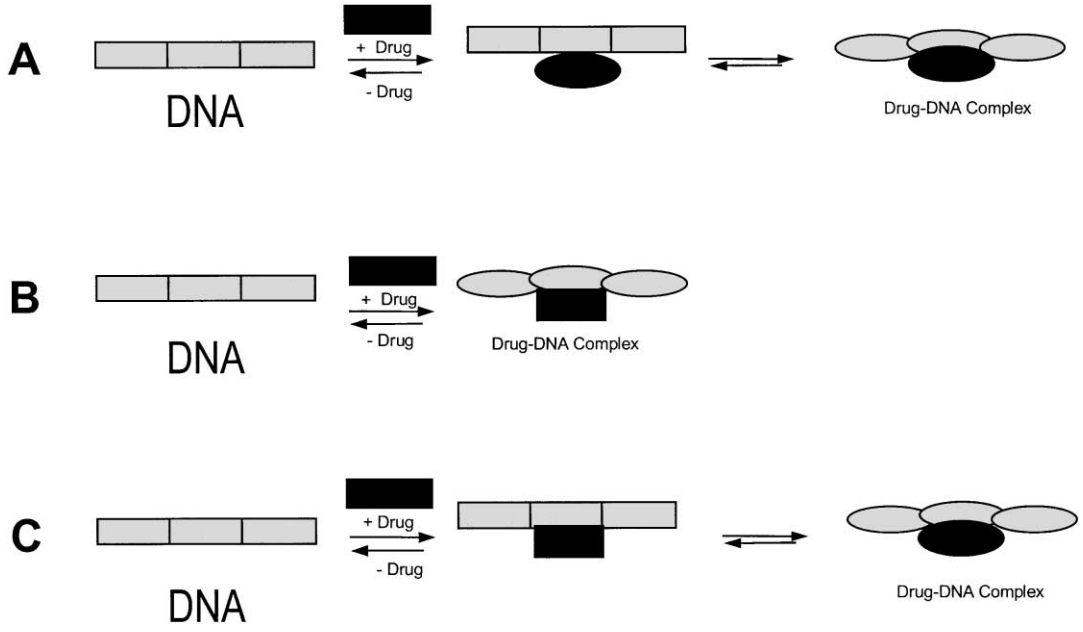

Figure 6. Three models for the cooperative binding of **2** to DNA (see text for explanation).

Drug preparation. NCS-chrom was isolated from the holoantibiotic (gift from Dr. Sugiyama) with 20 mM sodium citrate in methanol and stored at −70 °C as described.[12] Drug product **2** was generated by incubation of NCS-Chrom (80–100 μM) in 50 mM Tris–HCl, pH 8.5, 50% ethanol in the absence of DNA at 0 °C for 1 h. Following lyophilization of the reaction mixture, the dried sample was redissolved in 100 mM ammonium acetate at pH 4 and subjected to HPLC on a reverse-phase C-18 column. Isolation of **2** was accomplished by using a linear gradient of 40–65% solvent B/solvent A over a 40-min period [solvent A, aqueous 5 mM ammonium acetate (pH 4); solvent B, methanolic 5 mM ammonium acetate (pH 4); flow rate, 1 mL/min].

Fluorescence spectroscopy. Fluorescence emission spectra were measured on a Jobin Yvon-Spex FluoroMax-2 luminescence spectrophotometer. The emission spectra of the drug were obtained in the range 420–600 nm upon excitation at 400 nm, using a slit width of 0.5 and 2.0 mm each, and a scan speed of 100 nm/min at 5 °C. Fluorescence titration experiments were performed by keeping the drug concentration (3.1 μM) constant and stoichiometrically varying the DNA concentration from 2.95 M to 25 μM containing pH 5.2, 5 mM sodium acetate. The dissociation constants (K_d) of drug binding to bulged DNA were calculated from the fluorescence titration curves. Scatchard analysis was done according to the equation:

$$\frac{\nu}{[\mathrm{DNA}]} = \frac{1}{K_d}(N - \nu) \quad (1)$$

where K_d is the dissociation constant of DNA–drug complex, ν is the fraction of the total DNA bound to drug, and N is the number of binding sites. Assuming there is one binding site, $N = 1$. The K_d values can be obtained from the linear regression best fit to the data.

Circular dichroism (CD) spectropolarimetry. All CD measurements were performed at 5 °C on a Jasco-810 Spectropolarimeter, using a water-jacketed quartz cylindrical cell of 1 mm pathlength whose temperature was adjusted with a circulating bath. The cell compartment was continuously purged with dry N_2. Individual 150 μL samples were prepared containing 19.4 μM drug and DNA at concentrations ranging from 11 to 47 μM with pH 5.2, 5 mM sodium acetate and 1 mM NaCl. The spectra were recorded at a bandwidth of 1.0 nm and measured at every 0.2 nm over a range of 210–320 nm. Conformations of bulged DNA bound drug were obtained by subtracting the drug-only CD signal from that of the complex made by 24.5 μM of DNA mixed with 24.5 μM drug.

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