Probing DNA Single Strands for Single-Base Bulges with Neocarzinostatin Chromophore†

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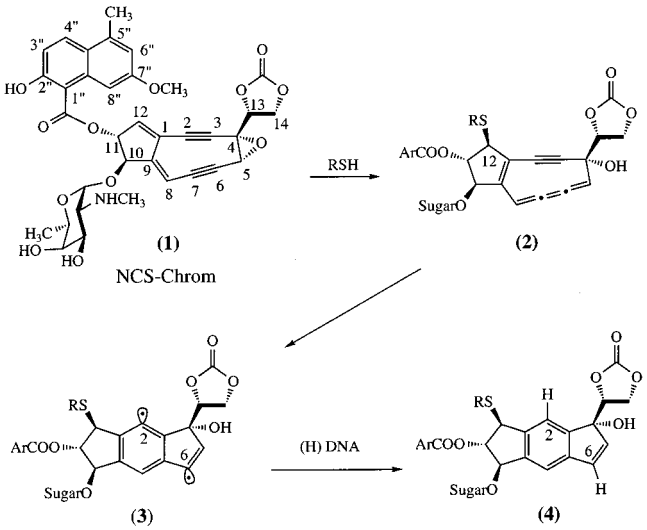
ABSTRACT: Neocarzinostatin chromophore (NCS-Chrom) induces strong cleavage at a single site (C3) in the single-stranded and 5′ ^{32}P-end-labeled 13-mer GCCAGATTTGAGC in a reaction dependent on a thiol. By contrast, in the duplex form of the same 13-mer, strand cleavage occurs only at the T and A residues, and C3 is not cleaved. To determine the minimal structural requirement(s) for C3 cleavage in the single-stranded oligomer, several deletions and mutations were made in the 13-mer. A 10-mer (GCCAGAGAGC) derived from the 13-mer by deletion of the three T residues was also cleaved exclusively at C3 by NCS-Chrom, generating fragments having 5′ phosphate ends. That the cleavage at C3 is initiated by abstraction of its 5′ hydrogen is confirmed in experiments using 3′ ^{32}P-end-labeled 10-mer. The competent 13-mer and 10-mer were assigned hairpin structures with a stem loop and a single bulged out A base, placing C3 across from and 3′ to the bulge. Removal of the bulged A base from the 13-mer and the 10-mer resulted in complete loss of cutting activity, proving that it is the essential determinant in competent substrates. Studies of thiol post-activated NCS-Chrom binding to the DNA oligomers show that the drug binds to the bulge-containing 13-mer (K_d = 0.78 μM) and the 10-mer (K_d = 1.11 μM), much more strongly than to the 12-mer (K_d = 20 μM) and the 9-mer (K_d = 41 μM), lacking the single-base bulge. A mutually induced-fit between NCS-Chrom and the oligomer resulting in optimal stabilization of the drug−DNA complex is proposed to account for the site-specific cleavage at C3. These studies establish the usefulness of NCS-Chrom as a probe for single-base bulges in DNA.

The antitumor drug neocarzinostatin chromophore (NCS-Chrom; Scheme 1, **1**) is unique among the family of enediyne drugs in its versatility to induce a variety of lesions in DNA in the presence or absence of a thiol activator (*1*−*3*). The naphthoate moiety of NCS-Chrom intercalates into duplex DNA via the minor groove, while its epoxybicyclo-[7,3,0]-dodecadiendiyne part binds to the minor groove through hydrophobic and electrostatic interactions. Thiol activation of the drug involves nucleophilic attack at C-12 to form a labile cumulene intermediate which undergoes a spontaneous Bergman cyclization to the biradical (Scheme 1) (*4*). The biradical abstracts hydrogen atoms from the C-5′, C-4′, and C-1′ positions of the DNA sugar to generate carbon-centered radicals, which in the presence of oxygen lead to strand breaks and/or abasic sites, and in the absence of oxygen form drug−DNA adducts (*1*). Single-strand breaks, which occur predominantly at T and A residues, are initiated by C-5′ hydrogen abstraction and generate fragments having phosphate at the 3′ end and a nucleoside aldehyde at the 5′ end (*5, 6*). Attack at the C-4′ position, which occurs mainly at the T residues of GT steps, results in 4′-hydroxylated abasic products (*7*) and/or 3′-phosphoglycolate-ended fragments (*8, 9*). C-1′ hydrogen abstraction at the C residues of AGC sequences produces a deoxyribonolactone-containing abasic site (*10*), which is the source of alkali-sensitive breaks (*11*). In the bistranded lesions involving C of AGC (or T of AGT) sequences, the radical centers at C-2 and C-6 of NCS-Chrom abstract hydrogen from the two complementary strands (*12*). Support for this model comes from the NMR structure of a complex formed between thiol post-activated drug and an oligomer duplex containing such potential cleavage sites (*13*). Another interesting property of this drug is its ability to alter the chemistry of attack at a particular base when a mismatched base pair is placed in its vicinity, as has been

Scheme 1: Acitivation Mechanism of NCS-Chrom by Thiol



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demonstrated in the switching of chemistry at the C of AGC from 1′ to 4′, when a G·C base pair to its 5′ was substituted with a wobble G·T mismatch (*14*). In addition, NCS-Chrom induces strong site-specific cleavage in DNA across from (*15*, *16*) or at a bulge itself (*17*−*19*) depending upon the drug activation mechanism. Thus, by virtue of its ability to induce a variety of lesions in DNA, including base-specific single-strand breaks and sequence-specific double-strand lesions, each involving different attack sites on the DNA sugar depending upon the microstructure and the local geometry of the drug−DNA complex, NCS has proven to be a remarkably sensitive molecular probe of nucleic acid structures.

Single-stranded DNA is not generally a substrate for the drug in the thiol-dependent reaction except in cases where there are duplex regions generated by folding of the same strand or by partial complementarity between two strands of the same oligomer. In the course of our investigations with several DNA substrates, it was found that NCS-Chrom, in a thiol-dependent reaction, induced strong cleavage in certain single-stranded oligomers at a single site, which is not a cleavage site in its duplex form. This appeared to be due to the drug's ability to recognize and/or induce some specific structure(s) in the single-stranded oligomer. The present study was undertaken to identify the structural determinants for such cleavage hot spots.

MATERIALS AND METHODS

Nucleic Acid Substrates. Oligodeoxyribonucleotides were purchased from commercial sources and purified on a 15% sequencing gel. Radioactive materials and enzymes were from New England Nuclear and New England Biolabs, respectively. Oligomers were 5′ or 3′ end-labeled with ^{32}P using [γ-^{32}P]ATP and cordycepin 5′-triphosphate, respectively, by standard procedures (*20*) and purified by electrophoresis on a 15% sequencing gel. The gel slices were soaked in 1 M triethylammonium bicarbonate, pH 7.5, and the eluted DNA was recovered using a Sep-Pak cartridge (Waters).

Drug Reaction. Neocarzinostatin powder (holo-NCS) was obtained from Kayaku Antibiotics (Tokyo). NCS-Chrom was extracted from the holoantibiotic by cold methanol containing 0.5 M acetic acid by a procedure similar to that described by Myers (*21*). The chromophore was stored at −70 °C and protected against light. Single strands or two complementary strands of the oligomers (unlabeled strand in 2−3-fold excess) were annealed by heating in the reaction buffer at 90 °C for 2 min and slow-cooling to room temperature. After the addition of glutathione, the mixture was cooled on ice for 15 min prior to the addition of NCS-Chrom. The final reaction contained 25−30 mM Tris-HCl, pH 8−8.50, 1.0 mM EDTA, ^{32}P-labeled oligomer, 2.5−3 mM glutathione, DNA, and NCS-Chrom at concentrations given in the figure legends. The reaction was allowed to proceed in the dark for 1 h on ice. Maximal final methanol concentration was 10%.

Treatment of Drug-Treated DNA and Product Analysis. To reveal any alkali-labile lesions in the drug-treated DNA, portions of the reaction mixture were dried, and the pellet was redissolved in 1 M piperidine and heated at 90 °C for 30 min. The sample was dried in a Speed Vac concentrator followed by H_2O addition to the pellet and drying 3 times.

Table 1: Oligonucleotides Used as Sustrates for NCS-Chrom[a]

1.	5′-GCCAGATTTGAGC	(GCC13)
2.	5′-GCTCAAATCTGGC	(GCT13)
3.	5′-GCCAGAGAGC	(GCC10)
4.	5′-GCTCTCTGGC	(GCT10)
5.	5′-GCCAGATTTGGC	(GCC12)
6.	5′-GCCAGAGGC	(GCC9)
7.	5′-GCCTGGGAGC	(GCC10A)
8.	5′-GCCAGAGTGC	(GCC10B)
9.	5′-GACAGAGATC	(GCC10C)
10.	5′-GGCAGAGACC	(GCC10D)
11.	5′-GCTAGAGAGC	(GCC10E)

[a] In parentheses is shown the abbreviated representation.

Reduction of the nucleoside aldehyde ends on the DNA was carried out with sodium borohydride and oxidation by treatment with sodium hypoiodite as previously described (*6*). To determine strand cleavage, portions of the reaction mixture were dried, and the sample pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes and analyzed on a 15% sequencing gel. The gel band intensities were quantitated on a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA). Chemical cleavage reactions specific for T+C and G+A were done using hydrazine and formic acid, respectively, as described by Maxam and Gilbert (*22*).

Drug Binding to DNA. This assay is based on the distinct fluorescent properties of the glutathione post-activated NCS-Chrom (NCSi-glu) and its fluorescence quenching by DNA (*1*). NCSi-glu was prepared by treating the drug with glutathione in Tris-HCl buffer, pH 8.3, on ice, and the product was purified by HPLC using procedures similar to those previously reported (*13*). Fluorescence measurements were done using a SPEX Fluoro Max-2 at 3 °C. To the cuvette containing a solution of NCSi-glu (5 nM) in 10 mM phosphate buffer, pH 7.5, were added increasing amounts of DNA solutions, and the fluorescence reading (excitation, 360 nm; emission, 440 nm) was recorded after an equilibrium had been reached. The dissociation constants were calculated from curve-fitting with Kaleidagraph using the equation:

$$i/i_0 = 1 + (\Delta i/2i_0)\{[T_0] + [DNA] + K_d - [([T_0] + [DNA] + K_d)^2 - 4[T_0][DNA]]^{1/2}\}$$

where $[T_0]$ is the initial concentration of the fluorescent probe, i is its fluorescence intensity at equilibration, i_0 is the initial intensity, [DNA] is the concentration of the DNA, and Δi is the total change in intensity per drug unit from the free state to the total bound state.

RESULTS

Strand Cleavage in a Single-Stranded Oligomer. When a 5′ ^{32}P-end-labeled 13-mer, GCCAGATTTGAGC (Table 1, GCC13), which had been annealed with its complementary 13-mer (GCT13) to form a duplex, was treated with NCS-Chrom in the presence of glutathione, strand cleavage occurs mainly at T and A residues (Figure 1, lane 8), a result consistent with previous studies using duplex DNA (*1*). By contrast, when the same 13-mer, as a single strand, was the substrate, the drug induces strong cleavage at a single site, C3 (lane 5), generating a fragment with a phosphate at its 3′ end, as indicated by its mobility coincident with that of the

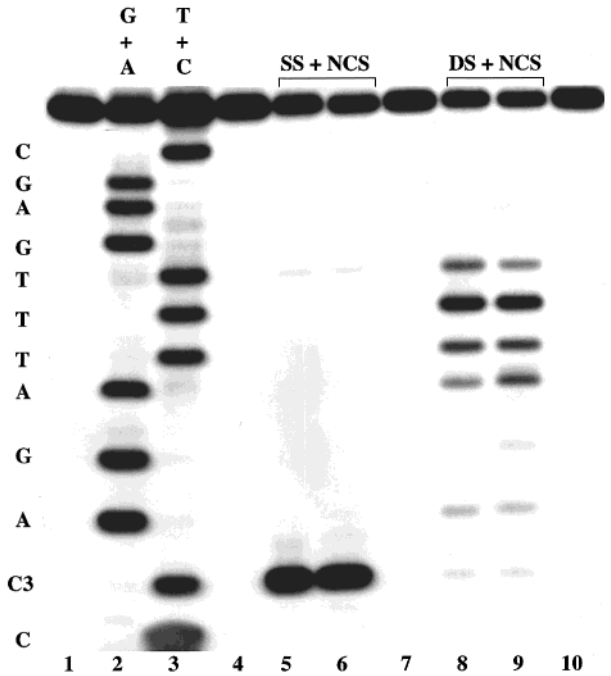


FIGURE 1: Strand cleavage by NCS-Chrom in the single-stranded (SS) and double-stranded (DS) 13-mer (GCC13). Annealing of 5′ ^{32}P-end-labeled 13-mer GCCAGATTTGAGC alone or a mixture of it with its complementary 13-mer, drug treatment of the DNA in the presence of thiol, subsequent alkaline treatment, and gel analysis were as described under Materials and Methods. ^{32}P-labeled 13-mer (12 μM) in SS or DS form was treated with 58.5 μM NCS-Chrom. Lanes 1 and 10 are controls of SS and DS forms, respectively, without the drug but treated with piperidine; lanes 2 and 3, Maxam−Gilbert markers; lanes 4 and 7, controls of SS and DS forms, respectively; lane 5, SS 13-mer + NCS; lane 6, SS 13-mer + NCS + piperidine; lane 8, DS 13-mer + NCS-Chrom; lane 9, DS 13-mer + NCS + piperidine. NCS refers to NCS-Chrom in this and subsequent figures.

Maxam−Gilbert marker for chemical cleavage at C3. To reveal any alkali-labile lesions as strand breaks, the drug-treated DNA was subjected to hot alkali treatment. The lack of any increase in the intensity of the C3 band (lane 6) precludes C-1′ or C-4′ attack and suggests that the damage at C3 is initiated by abstraction of its 5′ hydrogen by the drug. In the absence of a thiol, there was no strand cleavage at any residue (data not shown). Further, in the duplex form of the 13-mer, there is only a very faint band at the C3 position (lane 8). These results suggest that the selective targeting of C3 in the single 13-mer strand is due to some unique structural feature(s) in the DNA not found in the duplex and that the drug stabilizes or promotes formation of the involved structure.

Cleavage in a Single-Stranded 10-mer and Chemistry of Damage. To determine the minimal structure required in single-stranded oligomers to be cleavage substrates for NCS-Chrom, several oligomers of varying sequence and length were tested in the standard cleavage reaction. A 10-mer, GCCAGAGAGC, derived from the 13-mer of Figure 1 by deletion of three T residues (Table 1, GCC10), was labeled with ^{32}P at its 5′ end and treated with the drug in its single-stranded and duplex form in the presence of glutathione. As shown in Figure 2 (lane 4), the drug induces efficient cleavage exclusively at C3, giving rise to fragments with 3′ phosphate ends, suggesting that the damage at C3 results from 5′ hydrogen abstraction. On the other hand, in the

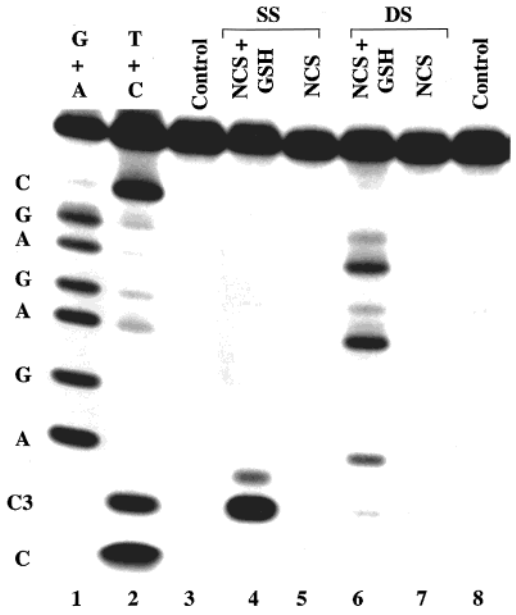
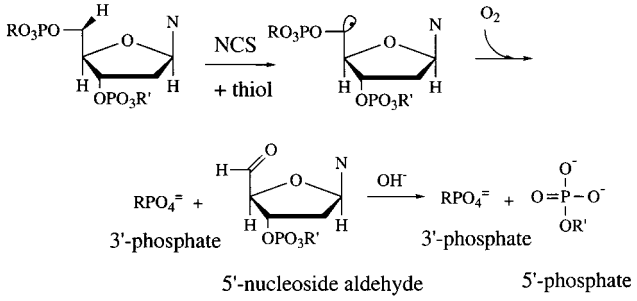


FIGURE 2: Strand cleavage by NCS-Chrom in the 10-mer (GCC10). 5′ ^{32}P-end-labeled GCCAGAGAGC in (SS) or (DS) form (28.5 μM) was treated with NCS-Chrom (48.7 μM) as in Figure 1. Lanes 1 and 2, Maxam−Gilbert markers; lane 3, SS 10-mer + thiol; lane 4, SS 10-mer + NCS + thiol; lane 5, SS 10-mer + NCS without thiol; lane 6, DS 10-mer + NCS + thiol; lane 7, DS 10-mer + NCS without thiol; lane 8, DS control + thiol.

Scheme 2: Chemistry of DNA Cleavage by Thiol-Activated NCS-Chrom



duplex form of the 10-mer, moderate cleavage occurs only at the A residues, and C3 is not a target (lane 6). In the absence of thiol, there is no damage (lanes 5 and 7). Alkaline treatment of the drug-treated DNA does not enhance the intensity of the C3 band (data not shown), a result indicative of the absence of C-1′ and C-4′ attack.

The proposed chemistry of strand cleavage at C3 (Scheme 2) is further confirmed in experiments where 3′ ^{32}P-end-labeled single-stranded 10-mer (GCC10) was treated with NCS-Chrom in the standard cleavage reaction (Figure 3). Previous work (*6*) has shown that DNA fragments having nucleoside aldehyde at their 5′ ends migrate in gel two bases slower than their 5′ phosphate-ended counterparts. This is also the case with small oligomers, except that the relative distance in gel mobility between the two fragments varies with their length and sequence. The fragment cleaved off the 10-mer has a mobility coincident with that of the parent oligomer (lane 5). As expected, hot alkaline treatment breaks down the nucleoside aldehyde to 5′ phosphate to generate a fragment which migrates at the same position as the marker for C3 cleavage (lane 6). Conversion of nucleoside aldehyde to 5′ hydroxyl by reduction (lane 7, arrow) and to 5′ carboxyl

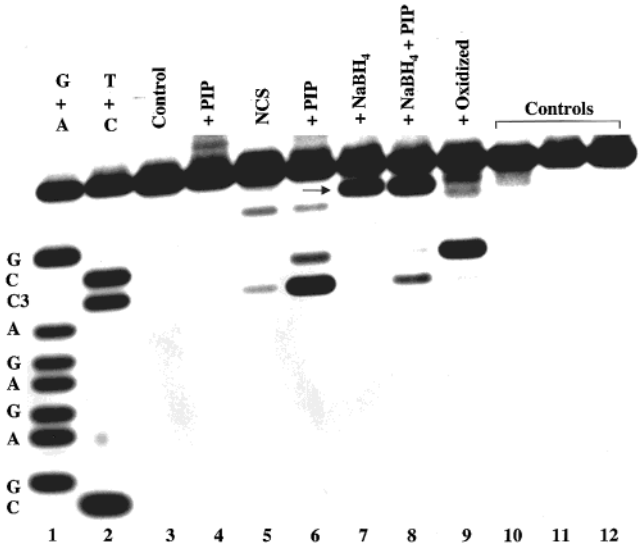
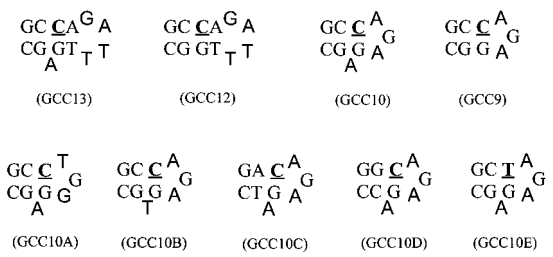


FIGURE 3: Chemistry of strand cleavage by NCS-Chrom in SS 10-mer (GCC10). 3′ ^{32}P-end-labeled GCCAGAGAGC was treated with NCS-Chrom under the same reaction conditions as in Figure 2. Aliquots of the reaction mixture were subjected to reduction, oxidation, and alkali treatment as described under Materials and Methods. Controls refer to the 10-mer that was not treated with the drug, but it had gone through other postdrug treatments, and PIP refers to piperidine. Lanes 1 and 2, Maxam−Gilbert markers; lane 10, oxidized; lane 11, reduced; lane 12, reduced followed by piperidine-treated samples of control.

Scheme 3: Proposed Folding Pattern of Single-Stranded Oligonucleotides[a]



[a]Underlining indicates the base at which cleavage occurs.

by oxidation (lane 9) gives rise to fragments with distinctly different mobilities. The reduced product, having a 5′ hydroxyl group, is unchanged upon alkali treatment (lane 8). These results establish that the C3 band is exclusively the product of 5′ hydrogen abstraction.

Proposed Structure and Supporting Evidence. The unexpected finding of a single strong cleavage site in a single strand of DNA (Figures 1−3) immediately suggested the possibility that cleavage was occurring at a site opposite a single-base bulge (*15*). Accordingly, we tentatively assigned the 13-mer (GCC13) and the 10-mer (GCC10) a folded structure (Scheme 3) which has a base paired stem region, a loop comprised of three or more bases, and a bulged out A residue which is across from the target C3. To test the validity of the proposed structures, the bulge A base was removed from the competent 13-mer and 10-mer, and the resulting 12-mer (GCC12) and 9-mer (GCC9) were compared with the parent oligomers in strand cleavage. In experiments shown in Figure 4, using 5′ ^{32}P-end-labeled oligomers, NCS-Chrom induces strong cleavage at C3 in both the 13-mer and the 10-mer (lanes 2 and 6), but there is no damage in the 12-mer and the 9-mer (lanes 4 and 8). Since the incompetent 12-mer and 9-mer differ from the competent

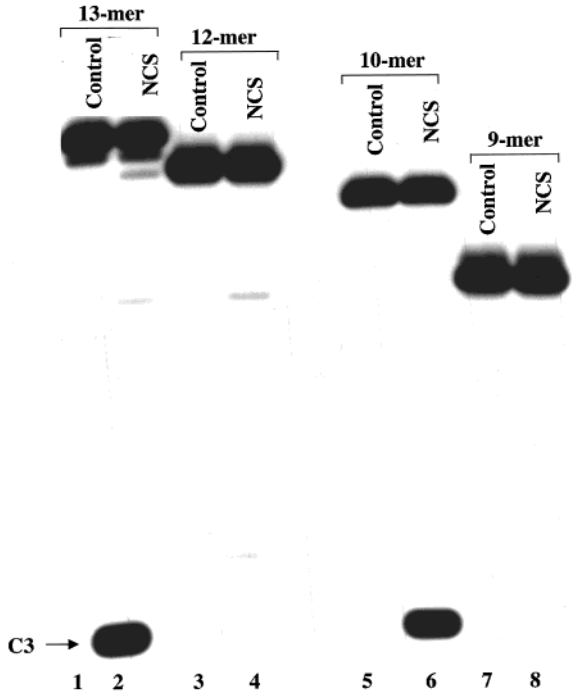


FIGURE 4: Comparison of strand cleavage by NCS-Chrom in single-stranded oligomers with and without the extra base. 5′ ^{32}P-end-labeled oligomers GCCAGATTTGAGC (GCC13, lanes 1 and 2) and GCCAGATTTGGC (GCC12, lanes 3 and 4) (each 13 μM) were treated with NCS-Chrom (58.5 μM) in the presence of thiol. Similarly, 5′ ^{32}P-end-labeled GCCAGAGAGC (GCC10, lanes 5 and 6) and GCCAGAGGC (GCC9, lanes 7 and 8) (each 28.5 μM) were treated with NCS-Chrom (49.4 μM) in the presence of thiol. Lanes 1, 3, 5, and 7 are controls without the drug.

13-mer and 10-mer, respectively, only in the absence of the bulge residue, we conclude that the determinant for C3 cleavage in the latter is the presence of the extra unpaired base. Several sequence changes were made in the competent 10-mer (GCC10), and the resulting oligomers, in their single-stranded form, were tested for cleavage by NCS-Chrom. The results (data not shown) showed that 10-mers having substitution of the AGA loop by TGG, bulge base A by a T, and C2·G9 by A2·T9 or G2·C9 (Table 1 and Scheme 3) were all efficiently cleaved exclusively at C3. The 10-mer with a T3·G7 mismatch (Table 1, no. 11) at the target site was cleaved at T3. This suggests that the cleavage specificity is bulge-dependent and independent of sequence.

Binding of NCS-Chrom to DNA Oligomers. The selective cleavage of bulged DNA (Figures 1−4) at the site 3′ opposite the bulge may be due to kinetic cleavage efficiency and/or binding. The binding constants for the drug were determined in an assay based on the fluorescence quenching of the glutathione post-activated drug upon DNA binding. The data show that the single-stranded oligomers that are capable of folding into single-base bulge-containing hairpin structures bind the drug much more strongly (GCC13, $K_d = 0.78$ μM; GCC10, $K_d = 1.11$ μM) than their counterparts lacking the bulge (GCC12, $K_d = 20$ μM; GCC9, $K_d = 41$ μM) (Figure 5). These results suggest that the presence of the bulge is essential for effective drug binding and support the cleavage data. The structure of GCC10 with only a single C3·G7 base pair proximal to the AGA hairpin and the A bulge is expected to be structurally metastable and to be in a dynamic equilibrium with a folded form where C3·G7 is open. The strong binding of the drug to the competent 10-mer is likely

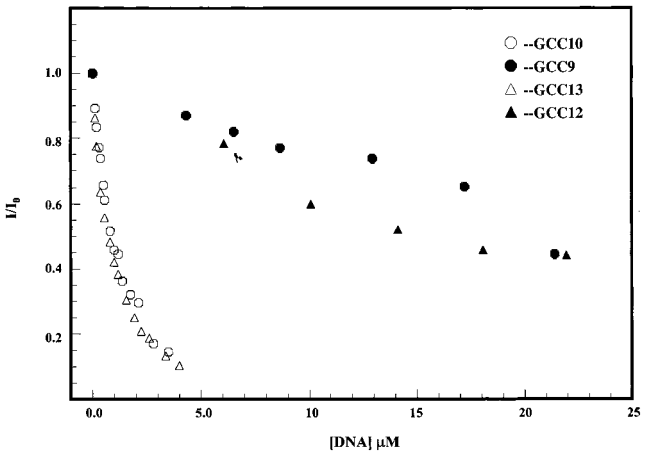
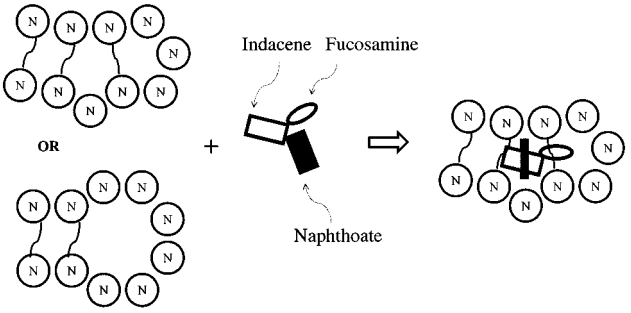


FIGURE 5: Isothermal binding curves of NCSi-glu to oligonucleotides. The procedure has been described under Materials and Methods.

Scheme 4: Schematic Model of Mutual Induced-Fit Binding of NCSi-glu to Single-Stranded DNA[a]



[a]The three structural components of NCS-Chrom are indicated by arrows.

attributed to a mutually induced-fit resulting in the stabilization of the drug−DNA complex (Scheme 4). In this model, the intercalation of the naphthoate moiety of the drug into the pocket bounded by the bulge base and its flanking base pairs induces a conformational change in the drug and DNA so as to accommodate each other and confer stability to the complex.

DISCUSSION

Bulges in DNA have been implicated as intermediates in frame shift mutagenesis (*23*−*25*), as targets for DNA repair enzymes (*26*−*28*), and as products of slipped mispairing during DNA replication (*29*). Triplet repeats which can form multibase bulges are believed to be involved in neurodegenerative diseases such as Huntington's disease (*30*). Because of their significance in many biological processes, structures of bulge-containing DNAs and their local geometry have been the subject of much interest (*31*−*33*). NMR spectroscopic studies of DNA oligomer duplexes containing single-base bulges revealed that the equilibrium between a nucleotide bulge intercalating in or looping out of the helix depends on the temperature, the identity of the base, and the sequence of the base pairs flanking the bulge (*31*, *33*). Bulges can also cause kinks (*34*) and bending in the DNA helix (*35*). The structural diversities and variations in the local geometry caused by the presence of bulges affect the binding of ligands to DNA. It has been shown that DNA intercalators such as ethidium preferentially bind to the bulged regions (*36*−*38*). Recently a novel octahedral Co^{II} complex has been developed as a probe for DNA bulges (*39*). There is also a report on the selective binding of 2-acylamino-1,8-naphthyridine to a single G bulge in duplex DNA through base pairing (*40*).

We have previously shown that NCS-Chrom induces site-specific cleavage in a folded single-stranded DNA containing a two-base bulge (*17*−*19*). This reaction is substantially different from that reported in the present study in several respects. In the two-base bulge-specific reaction, cleavage is at the bulge itself, and the drug activation does not require a thiol. The drug activation in this case involves a base-catalyzed intramolecular nucleophilic attack of C-1″ at C-12 to generate, via a spirolactone cumulene intermediate, a 2,6-didehydroindacene biradical which is responsible for abstraction of the 5′ hydrogen of the target residue (*41*, *42*). Further, a novel DNA bulge-specific drug end product is generated in the reaction (*17*, *41*, *42*). By contrast, in the thiol-promoted reaction, the final drug product is the same in the presence or absence of DNA. The solution structure by NMR spectroscopy of a complex formed between the wedge-shaped isostructural form of the active spirolactone diradical species in the base-catalyzed reaction and a DNA oligonucleotide containing a two-base bulge reveals that the binding of the reactive drug species occurs via the major groove to the triangular prism pocket formed by the two looped-out bases and the neighboring base pairs with which the two rings of the wedge-shaped drug molecule stack (*43*). We have also shown that synthetic analogues of the spirolactone diradical bind specifically to DNA containing a two-base bulge at submicromolar concentrations (*44*).

The results obtained in this study are in general agreement with those reported with model duplex DNA constructs having a single-base bulge where NCS-Chrom in a thiol-dependent reaction induced specific cleavage on the strand opposite the bulge at a position 3′ to the bulge (*15*). The cleavage, which involved 5′ chemistry, was more efficient with a single purine bulge than with one consisting of a pyrimidine or more than one purine (*16*). Taken together, the results show that NCS-Chrom is a uniquely versatile drug that can recognize diverse bulge structures (single-base or two−three-base) in folded single-stranded DNAs and cause site-specific damage by way of different radical species generated by different modes of drug activation. The very high level of selectivity for a single-base bulge target site described here has not been found in earlier studies (*15*, *16*). This probably reflects the ability of the activated drug to induce an optimal binding structure from the otherwise disorganized, un-base-paired region of the single-stranded DNA (see Scheme 4). The induced-fit nature of the interaction between drug and DNA to form bulged structures is a general phenomenon and has also been observed with the two-base bulge binding form of NCS-Chrom (X. Gao, A. Stassinopoulos, J. Jie, S. Bare, Y. Kwon, G.-S. Hwang, and I. H. Goldberg, unpublished data).

The tight binding data combined with the high cleavage efficiency (Figures 1−5) indicate that the 10-mer (GCC10) is an excellent substrate to study the intercalation-based interaction between NCS-Chrom and a single-base bulge-containing oligomer and to probe the molecular basis for bulge recognition. In fact, recent NMR spectroscopic studies (X. Gao, Z. Xi, L. S. Kappen, and I. H. Goldberg,

unpublished data) confirm that the thiol-activated drug binding is at the bulge of the oligomer.

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