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Sequence-Specific, Strand-Selective, and Directional Binding of Neocarzinostatin Chromophore to Oligodeoxyribonucleotides†

Seok Ho Lee and Irving H. Goldberg*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
Received July 26, 1988; Revised Manuscript Received October 21, 1988

ABSTRACT: The sequence-specific interaction of neocarzinostatin chromophore (NCS-C) has been evaluated with a series of synthetic oligodeoxyribonucleotides of defined sequences containing the most preferred nucleotide cleavage site, T or A, or both. NCS-C preferentially cleaves T or A residues in the sequence GN_1N_2, where N_2 is T or A. Greater cleavage occurs on the strand enriched with G residues, provided that they are adjacent to other G residues, but not at N_1. These results are compatible with a model for drug binding in which the naphthoate moiety of NCS-C preferentially intercalates at GN_1. This is accompanied by electrostatic binding interaction provided by the positively charged amino sugar moiety so as to place the reactive bicyclo[7.3.0]dodecadienediyne epoxide moiety in an appropriate orientation in the minor groove enabling, upon thiol activation, attack at C-5′ of T or A. At certain sequences, such as GCT·AGC, a similar binding mode is also able to generate abasic lesions at the C residue on the opposite strand, forming a bistranded lesion. Although the reactions with glutathione generally show the same strand selectivity and sequence specificity as those with dithiothreitol, the former is usually more efficient than the latter.

The labile nonprotein chromophore of neocarzinostatin (NCS-C)[1] (Napier et al., 1979, 1981; Albers-Schönberg et al., 1980; Hensens et al., 1983; Edo et al., 1985a,b, 1986; Myers et al., 1988), an antitumor antibiotic produced by *Streptomyces carzinostaticus*, is solely responsible for its DNA-damaging activity. NCS-C binds to DNA by an intercalative mechanism and has a relatively strong affinity for DNA ($K_d = 10^{-6}$ M) (Povirk & Goldberg, 1980; Povirk et al., 1981; Dasgupta et al., 1985) with two types of binding sites (Povirk et al., 1981; Dasgupta & Goldberg, 1985). Its binding stoichiometry (r_b) for the high-affinity binding sites is 0.12 (approximately one NCS-C per four base pairs), and that for the low-affinity binding sites is 0.23 (approximately one NCS-C per two base pairs). Thiol-activated NCS-C abstracts a hydrogen atom from the C-5′ of deoxyribose at T and A residues (Kappen & Goldberg, 1985), forming either O_2-dependent single-stranded nicks with nucleoside 5′-aldehyde at the 5′-end of the break (Kappen et al., 1982; Kappen & Goldberg, 1983) or O_2-independent stable adducts on the deoxyribose of these same residues (Povirk & Goldberg, 1982a,b, 1984, 1985a).

NCS-C consists of three main structural subunits: a substituted naphthoic acid, an amino sugar (*N*-methyl-α-D-fucosamine), and a bicyclo[7.3.0]dodecadienediyne epoxide (Figure 1). We previously proposed that the naphthoic acid moiety intercalates DNA in the minor groove and that the positively charged amino sugar moiety interacts with the negatively charged sugar phosphate backbone of DNA (Napier & Goldberg, 1983). These two anchors assist in the juxtaposition of the bicyclo[7.3.0]dodecadienediyne epoxide moiety with the deoxyribose of chiefly T residues in the minor groove of DNA (Goldberg, 1986). The epoxide, or its equivalent, has been found to be relatively important in its biological activity, probably in a cooperative manner with the highly strained unsaturated bicyclo[7.3.0]dodecadienediyne moiety (Lee & Goldberg, 1988), whereas the cyclic carbonate (1,3-dioxolan-2-one) moiety appears to play a role in the passage of NCS-C through cellular and nuclear membranes (Napier et al., 1981; Lee & Goldberg, 1988). However, neither the nucleotide sequences for the preferential intercalative binding by NCS-C nor its relationship with major cleavage sites, which mainly occur at T residues, has been identified so far. In the presence of β-mercaptoethanol, the preferred dinucleotides for the NCS-C-induced cleavage of DNA restriction fragments occur in the relative probability order of AT > CT > TT > GT, where T residues are the target sites (Takeshita et al., 1981).

A recent hypothetical model, based on theoretical computations on the binding of NCS-C to a limited number of self-complementary tetramers (CGCG, GCGC, TATA, and ATAT), proposes that purine-(3′,5′)-pyrimidine sites are more favorable for intercalation than pyrimidine-(3′,5′)-purine in the minor groove (Chen et al., 1987). Unfortunately, however, it fails to provide a clear picture of how the binding mode

† This work was supported by U.S. Public Health Service Research Grants CA 44257 and GM 12573 from the National Institutes of Health.

[1] Abbreviations: NCS, neocarzinostatin (holoprotein); NCS-C, neocarzinostatin chromophore; DTT, dithiothreitol; GSH, glutathione; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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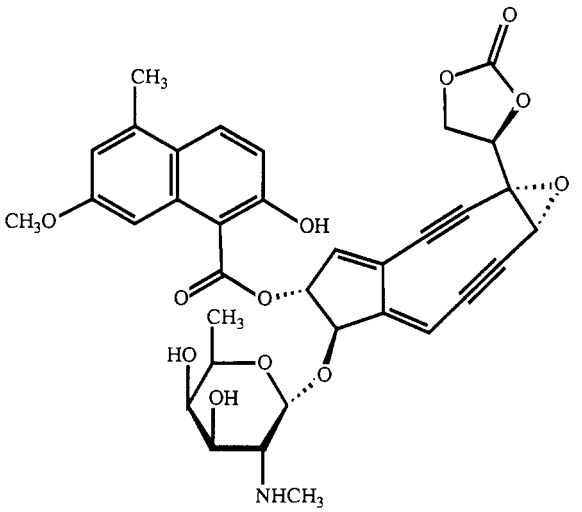

FIGURE 1: Neocarzinostatin chromophore (NCS-C). The absolute stereochemistry at C-10 and C-11 is depicted as *R,R* in accord with a model proposed by Schreiber and Myers (Schreiber & Kiessling, 1988; Myers et al., 1988).

relates to the attack site specificity.

We have investigated a series of synthetic oligodeoxyribonucleotides, mostly hexamers of self-complementary and non-self-complementary duplexes of designed sequence, to determine the nature of specificity and selectivity of NCS-C in its binding and cleavage of DNA, as part of an effort to obtain insight into the mechanism of action of this agent. It is well established that the relative stability of a DNA duplex depends primarily on its base sequence, that is, the identity of the nearest-neighbor bases. Therefore, thermodynamically, the relative stability of a DNA duplex can be considered to be the sum of its nearest-neighbor base-pair interactions (Breslauer et al., 1986). As a result, the stabilities of the duplex structures in the oligodeoxyribonucleotides under study, which are dictated by the overall relative stability of nearest-neighbor bases, are expected to be reasonably comparable despite variations in their base sequences within the series. Also, these oligodeoxyribonucleotides are believed to be of B-DNA structure under the experimental conditions employed (Saenger et al., 1986; Kubasek et al., 1986; Nilges et al., 1987a,b; Aboul-ela et al., 1988). Since there is an overwhelming specificity of NCS-C for the target site (T > A ≫ C > G) and also a minimum length of oligodeoxyribonucleotides (hexamers) needed to provide stable duplexes with few options for high-affinity binding to a site of about four base pairs, the binding of NCS-C was anticipated to occur at or around its normal target site. On the basis of these considerations, it should be possible to correlate directly the degree of binding specificity with that of cleavage.

Materials and Methods

All procedures involving NCS-C were carried out in the dark at 0 °C. NCS-C was stored in methanol buffered with 20 mM sodium citrate–citric acid, pH 4.5, at −70 °C in brown vials until use.

Neocarzinostatin Chromophore. NCS-C (ϵ_{341} 8800 M^{-1} cm^{-1}; S. H. Lee and I. H. Goldberg, unpublished data) was extracted from NCS in clinical ampules (Kayaku, Tokyo, Japan) for several hours in methanol after dialysis against distilled water and subsequent lyophilization in the presence of 20 mM sodium citrate–citric acid, pH 4.5, as described previously (Povirk et al., 1981). Its concentration was determined by UV spectrophotometry (ϵ_{341} 10 800 M^{-1} cm^{-1}; Povirk et al., 1981), after it was reconstituted with an excess of NCS apoprotein (ϵ_{278} 14 400 M^{-1} cm^{-1}; Povirk et al., 1981).

Oligodeoxyribonucleotides. A series of synthetic oligodeoxyribonucleotides was prepared manually with 1*H*-tetrazole-activated N-protected 5′-*O*-(4,4′-dimethoxytrityl)deoxyribonucleoside 3′-(2-cyanoethyl *N,N*-diisopropyl phosphoramidites) and long-chain alkylamine-controlled pore glass–deoxyribonucleoside columns (Matteucci & Caruthers, 1981; Sinha et al., 1984). The bound protected oligonucleotide was treated with concentrated NH_4OH at 50 °C for 16 h. The free deprotected oligonucleotide was dried, dissolved in 2 mL of H_2O, and then extracted three times with diethyl ether. The lyophilized crude oligonucleotide was purified by high-performance reversed-phase thin-layer chromatography on HETLC-RPSF (Analtech) using 0.1 M triethylamine–H_2CO_3, pH 7.0, in 10–30% methanol by stepwise developments. The gel band containing the oligonucleotide was extracted three times with 50% aqueous methanol, and the combined extract was lyophilized. An aliquot (0.5 OD; A_{260}) of oligonucleotide was then labeled at the 5′-end with [γ-^{32}P]ATP (1000–3000 Ci/mM; New England Nuclear) by use of T_4 polynucleotide kinase (New England Biolabs). The reaction mixture was passed through Nensorb 20 nucleic acid purification cartridges (Du Pont), and the labeled oligonucleotide was recovered with 20% aqueous ethanol. It was further purified by electrophoresis using a 0.8-mm 20% polyacrylamide sequencing gel containing 7 M urea. The radioactive band, identified by autoradiography, was extracted into 0.2 M triethylamine–H_2CO_3, pH 7.5. The supernatant was collected by centrifugation at 6000 rpm and passed through Sep-Pak C_{18} cartridges (Waters Associates). The oligonucleotide product was eluted with 3 mL of 30% CH_3CN in 0.1 M triethylamine–H_2CO_3 (Lo et al., 1984). The sequence was verified by the Maxam and Gilbert sequencing technique (Maxam & Gilbert, 1980; Maniatis et al., 1982; Banaszuk et al., 1983). These markers were also used to confirm the cleavage reaction products of NCS-C.

Aerobic, Single-Strand Cleavage by NCS-C. A typical reaction mixture contained the following in 50 μL: 15 μM duplex oligonucleotide (1000 Bq), 45 μM NCS-C in 10 μL of methanol, thiol (either 10 mM DTT or 20 mM GSH), 5 mM sodium citrate–citric acid, pH 4.5, and 50 mM Tris-HCl, pH 8.0. Non-self-complementary oligonucleotides were mixed with the corresponding complementary strands to give duplexes prior to use (Smith, 1983), but self-complementary oligonucleotides were used directly. After an oligonucleotide in 2 mM sodium citrate–citric acid, pH 4.5, was cooled to 0 °C for 1 h, Tris-HCl and a thiol (either DTT or GSH) were added. The mixture was incubated for 10 min before NCS-C was added to initiate the reaction at 0 °C in the dark. The reaction mixture was subsequently lyophilized. Reactions were normally carried out twice in duplicates for each thiol.

Abasic, Alkali-Labile Lesions by NCS-C. A total of 5 μL of 1.1 M NaOH was added to the above reaction mixture in a total volume of 50 μL to give a final concentration of 0.1 M. The mixture was heated at 90 °C for 30 min.

Electrophoresis. Sequencing gel electrophoresis using an 0.8-mm 20% polyacrylamide gel containing 7 M urea was performed in a buffer containing 100 mM Tris, 100 mM H_3BO_3, and 5 mM Na_2EDTA at 400 V overnight after a prerun for 30 min at 1000 V.

Autoradiography. Autoradiography of the gel was done on XAR-5 films (35 × 43 cm), and the films were subjected to densitometry (UltroScan XL Laser Densitometer, LKB).

Statistical Analysis. Mean values of the data obtained from densitograms were used in the tables. Standard deviations

Table I: Strand Selectivity in Non-Self-Complementary Oligonucleotides[a]

strand		% cut					
		A		B		A/B	
A	B	DTT	GSH	DTT	GSH	DTT	GSH
GGATGG	CCATCC	28.8	49.5	6.1	13.1	4.7	3.8
GGATGC	GCATCC	29.8	45.2	12.9	20.1	2.3	2.3
GGATCG	CGATCC	30.6	22.4	16.8	14.3	1.8	1.6
GCATGG	CCATGC	22.6	33.7	12.3	17.1	1.8	2.0
CGATGG	CCATCG	19.6	43.1	7.5	21.5	2.6	2.0
CGATGC	GCATCG	10.7	18.5	9.4	13.3	1.1	1.4
GGTAGG	CCTACC	13.6	36.8	7.2	19.7	1.9	1.9
GGTAGC	GCTACC	12.5	21.1	12.6	23.2	1.0	0.9
GGTACG	CGTACC	20.5	28.0	11.4	14.8	1.8	1.9
GCTAGG	CCTAGC	18.3	32.6	9.0	13.5	2.0	2.4
CGTAGG	CCTACG	8.0	27.1	9.3	23.6	0.9	1.1
CGTAGC	GCTACG	7.1	9.0	19.8	25.9	0.4	0.3

[a] Percent cut indicates the combined cleavage at N_3 and N_4 for each strand; N_4 and N_3 represent the fourth and the third nucleotides, respectively, from the 5′-end; A/B indicates the ratio of combined cleavages at strand A over strand B.

Table II: Target Base Specificity in Non-Self-Complementary Oligonucleotides[a]

strand		N_4/N_3				A/B			
		A		B		N_3		N_4	
A	B	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH
GGATGG	CCATCC	19.4	17.1	2.4	2.3	0.8	0.7	6.3	5.1
GGATGC	GCATCC	20.4	22.1	2.1	2.1	0.3	0.3	3.3	3.2
GGATCG	CGATCC	14.8	7.8	7.6	5.6	1.0	1.2	1.9	1.6
GCATGG	CCATGC	2.2	2.3	3.4	3.3	2.5	2.5	1.6	1.8
CGATGG	CCATCG	20.8	17.3	5.2	3.0	0.7	0.4	3.0	2.5
CGATGC	GCATCG	18.0	18.9	3.4	3.2	0.3	0.3	1.4	1.7
GGTAGG	CCTACC	2.6	2.8	0.7	1.0	0.9	1.0	3.3	2.7
GGTAGC	GCTACC	2.6	4.2	0.7	0.9	0.5	0.3	1.8	1.6
GGTACG	CGTACC	3.6	4.5	2.3	2.7	1.3	1.3	2.0	2.1
GCTAGG	CCTAGC	0.4	0.6	0.6	0.9	2.4	3.0	1.5	1.8
CGTAGG	CCTACG	2.3	2.4	1.1	1.1	0.5	0.7	1.2	1.6
CGTAGC	GCTACG	6.0	8.5	1.0	1.5	0.1	0.1	0.6	0.5

[a] N_4/N_3 indicates the ratio of cleavage at N_4 and N_3 for each strand; A/B indicates the ratio of cleavage for strand A and strand B at each nucleotide indicated.

were usually within 10% for cleavage of more than 5%. However, standard deviations for cleavage of less than 2% were somewhat variable in a range of 5–40%. Also, the *t* test showed that 95% confidence limits for the N_4/N_3 ratio normally fell within 5–20%.

Results

Strand Selectivity in Non-Self-Complementary Oligonucleotides. Cleavage patterns of both strands of non-self-complementary hexamers by NCS-C were analyzed, after each strand was separately labeled with ^{32}P at the 5′-end and then annealed to its respective cold complementary strand, to determine if there was any degree of preference for either strand within the same nucleotide duplex. Table I shows variable degrees of preferential cleavage at one strand over the other in these duplexes.

The order of strand selectivity, represented by the ratio of total cleavage at both A and T between complementary strands, A/B, is GGATGG > GGATGC = CGATGG > GGATCG = GCATGG > CGATGC when N_3N_4 was AT. However, the order of strand selectivity when N_3N_4 is TA is somewhat different, although it followed a similar trend: GGTAGG = GGTACG = GCTAGG > GGTAGC = CGTAGG > CGTAGC. The following two factors seem to play a crucial role in determining strand selectivity: (1) the difference in the number of G residues between complementary strands and (2) the unique environment at the binding site for NCS-C relative to its target site, which can be interpreted in terms of the importance of the trinucleotides with the sequence GNT, where NCS-C intercalates in between GN and cleaves at T.

Correlation between Strand Selectivity and Sequence Specificity in Non-Self-Complementary Oligonucleotides. Sequence specificity (base specificity), represented by the ratio N_4/N_3, in non-self-complementary hexamers was also studied (Table II). Generally higher N_4/N_3 is observed within the strands of higher strand selectivity, regardless of the exact nature of N_3N_4 (AT or TA), especially when the ratio represents T/A rather than A/T. This is particularly true within the $N_1N_2ATN_5N_6$ (AT) series compared with the $N_1N_2TAN_5N_6$ (TA) series. This result suggests that the trinucleotide GN_1N_2 (N_2 as the target nucleotide) plays a decisive role in determining whether there is either enhanced or depressed cleavage at A or T, depending on where they are located (either N_1 or N_2 in GN_1N_2). The preferred targets are GAT and GCA in the AT series and GCT and GTA in the TA series where the third nucleotide at the 3′-end is attacked by NCS-C. Also, within GN_1N_2, T holds a clear edge over A at N_2.

Effect of Preferred Trinucleotide GN_1N_2 on the Se-

quence-Dependent Cleavage at T and A. The cleavage patterns of hexamers produced by NCS-C with either AT or TA at N_3N_4 are illustrated in Table III. For convenience, Table III is shown in two different ways: In Table III-1, the hexamers are arranged in such a way that each pair represents positional isomers of either G or C at a single nucleotide. In Table III-2, they are gathered in groups of common quartets at $N_2N_3N_4N_5$, for example, GATG, GATC, CATG, and CATC.

Higher base specificity in terms of the N_4/N_3 ratio is noticeable in hexamers with quartet sequences ($N_2N_3N_4N_5$) of GN_3N_4G and GN_3N_4C compared with those of CN_3N_4C and CN_3N_4G. The order of base specificity observed is GATG $\geq$ GATC > CATG = CATC = GTAG $\geq$ GTAC > CTAG = CTAC. In the TA series, N_4/N_3 is generally smaller than that in the AT series due to the inherent preference for T over A by NCS-C as the target nucleotide.

At least two parameters should be considered with respect to the base specificity of cleavage: (1) the base specificity of the cleavage by NCS-C is very closely associated with the sequence specificity of the binding of NCS-C to oligonucleotides, and (2) the order of preferential cleavage at the target base is T > A $\gg$ C > G. Nevertheless, base specificity has to be redefined as site specificity when the preferred trinucleotides GN_1N_2 are under consideration, due to the fact that N_2 is always the target nucleotide within GN_1N_2. However, it seems that good intercalative binding of NCS-C in between GN_1 does not necessarily lead to a good cleavage at N_2, unless N_2 is either T or A.

In general, hexamers with sequences of $N_1GATN_5N_6$ give rise to greater overall cutting relative to their counterparts with $N_1CATN_5N_6$, although clustering of G residues at the 5′-end, in particular, has a tendency to improve its status as a substrate for NCS-C (Table III-1). $N_1GCTN_5N_6$, where A has been replaced by C, was the best substrate when DTT was used, although less so with GSH (Table IV). Hexamers of the TA series are generally poorer substrates for NCS-C than those of the AT series (Table III-1).

Long-range effects of sequence also appear to be important. Thus, the influence of the bases at N_1 and N_6 was found in the hexameric series with oligonucleotides having common sequences at the four consecutive nucleotides $N_2N_3N_4N_5$, such as GATG, GATC, CATG, CATC, etc. Again, clustering of G residues on one strand (or clustering of C residues on the complementary strand) gave dramatically enhanced cleavage (Table III-2).

However, it is the trinucleotide GN_1N_2, where N_2 is the target site for the cleavage, that is the preferred sequence for the binding and action of NCS-C, with GN_1T always better than GN_1A. Among the three possible nucleotides, A, C, or G, for N_1 in GN_1N_2, G at N_1 is the least preferred (Table IV). Although the degree of cleavage varies somewhat depending on the exact location of GN_1N_2 within the same hexamer, the results clearly indicate that it is N_2 of GN_1N_2, whether it is T or A (N_3 or N_4 of hexamers in this series), which is the more favorable target of NCS-C cleavage. Nevertheless, considerably higher cleavage was observed with N_2 of GN_1N_2 as T, instead of A. The order of preference observed is GCT $\geq$ GAT > ACT = GGT = AGT.

Effect of Oligodeoxyribonucleotide Length on the Interaction with NCS-C. To determine whether the increased length of oligonucleotides improves their status as substrates for NCS-C, a single nucleotide was added at each end of the hexamer CGATCG to give octamers. However, the result did not provide a clear difference (data not shown).

Effect of Target Site Location on Cleavage. The results also showed a clear positional effect for the target site of NCS-C (either T or A), so that N_3 was cleaved approximately two-thirds as much as N_4 in hexamers: 10.0% and 14.6%, respectively, with DTT and 26.9% and 37.0%, respectively, with GSH, when GGTGGG and GGGTGG were employed; 1.7% and 1.8%, respectively, with DTT and 4.0% and 6.3%, respectively, with GSH, when CCACCC and CCCACC were employed.

Thiols. Both DTT and GSH were employed to compare the effects of the two thiols as essential cofactors in the DNA cleavage reaction by NCS-C. GSH was chosen as the naturally abundant thiol present in living cells to mimic in vivo conditions. The results show that GSH is superior to DTT in most cases, except for GGATCG (Table I). Although the two thiols were not qualitatively different in most cases, as determined by the ratio N_4/N_3 (Tables III and IV), the results on some hexamers such as GGATCG (Table III), GGCTGG, GAGTGG, GACTGG, GGGTGG, and CCACTC (Table IV) imply that the mode of interaction of these thiols may not be identical at least in some sequences.

Abasic, Alkali-Labile Lesion. The duplex $^{5'}$GGCTGC$^{3'}$·$^{5'}$GCAGCC$^{3'}$, labeled at the 5′-end of each strand separately and then annealed to its respective cold complementary strand, was studied to correlate direct single-strand breaks with abasic alkali-labile site formation. Results show that direct breaks at T in strand GGCTGC occurred twice or more often than abasic site formation at C* in strand GCAGC*C (55.8% versus 26.9% when GSH was used and 24.9% versus 3.9% when DTT was used). A far greater difference in efficiency between GSH and DTT was observed in forming abasic sites (26.9% versus 3.9%) than in generating direct breaks (55.8% versus 24.9%).

Discussion

Sequence-specific interaction of DNA intercalating agents such as actinomycin D (Phillips & Crothers, 1986; Wilson et al., 1986), daunomycin (Wang et al., 1987; Chaires et al., 1987), echinomycin (Ughetto et al., 1985), or triostin A (Wang et al., 1984) has been well documented. Even simple intercalators such as ethidium are known to be able to discriminate between different nucleotide sequences (Fox & Waring, 1987). Intercalation generally occurs between pyrimidine-(3′,5′)-purine: daunomycin (daunorubicin), echinomycin, and triostin A intercalate betwen CG, whereas actinomycin D intercalates between GC. NCS-C binding to DNA also involves an intercalative mechanism (Povirk et al., 1981; Dasgupta et al., 1985), but an obvious sequence requirement had not been observed. Further, at certain abnormal DNA structures, such as single-base bulges, enhanced binding of NCS-C (as well as certain other intercalating agents) at the bulge was found to be sequence independent (Williams & Goldberg, 1988).

A theoretical model of the binding of NCS-C, based on the finding that A was found most often on the 5′-side of target T in DNA restriction fragments (Takeshita et al., 1981) and on computations of various energy terms for tetramers of alternating sequences, CGCG, GCGC, TATA, and ATAT (Chen et al., 1987), proposed that the drug preferentially intercalates purine-(3′,5′)-pyrimidine, especially at AT, rather than pyrimidine-(3′,5′)-purine in the minor groove. This model involves cleavage at the site of intercalation. On the other hand, their calculations show that the interaction energy between the bases and the individual moieties of NCS-C, such as the aromatic substituted naphthoic acid, the amino sugar, and the bicyclo[7.3.0]dodecadienediyne epoxide, around the intercalation site favors GC over AT. The preference for

Table III[a]

	% cut							
	N_3		N_4		$N_3 + N_4$		N_4/N_3	
strand	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH
(Part 1) Relationship of Sequence to Target Site Specificity								
GGATGG	1.4	2.7	27.4	46.8	28.8	49.5	19.4	17.1
GGATGC	1.4	2.0	28.4	43.3	29.8	45.2	20.4	22.1
GGATCG	1.9	2.5	28.6	19.9	30.6	22.4	14.8	7.8
GGATCC	1.3	3.3	21.2	46.0	22.5	49.2	16.2	14.0
GCATCC	4.2	6.5	8.7	13.6	12.9	20.1	2.1	2.1
CGATCC	2.0	2.1	14.8	12.1	16.8	14.3	7.6	5.6
CCATCC	1.8	3.9	4.3	9.1	6.1	13.1	2.4	2.3
CCATGC	2.8	4.0	9.5	13.1	12.3	17.1	3.4	3.3
CCATCG	1.2	5.4	6.3	16.1	7.5	21.5	5.2	3.0
CCATGG	2.8	9.4	9.0	24.7	11.9	34.2	3.2	2.6
GCATGG	7.0	10.2	15.6	23.5	22.6	33.7	2.2	2.3
CGATGG	0.9	2.4	18.7	40.8	19.6	43.1	20.8	17.3
GCATGC	5.2	5.4	9.4	13.9	14.6	19.3	1.8	2.6
GCATCG	2.1	3.1	7.3	10.2	9.4	13.3	3.4	3.2
CGATGC	0.6	0.9	10.2	17.6	10.7	18.5	18.0	18.9
CGATCG	2.6	1.0	12.5	14.9	15.1	15.9	4.8	15.1
GGTAGG	3.8	9.8	9.8	27.0	13.6	36.8	2.6	2.8
GGTAGC	3.5	4.0	9.0	17.1	12.5	21.1	2.6	4.2
GGTACG	4.5	5.1	16.0	22.9	20.5	28.0	3.6	4.5
GGTACC	4.0	7.3	10.3	31.6	14.3	39.0	2.6	4.3
GCTACC	7.6	12.2	5.0	11.0	12.6	23.2	0.7	0.9
CGTACC	3.5	4.0	7.9	10.8	11.4	14.8	2.3	2.7
CCTACC	4.3	9.8	3.0	9.9	7.2	19.7	0.7	1.0
CCTAGC	5.5	7.0	3.5	6.5	9.0	13.5	0.6	0.9
CCTACG	4.5	11.3	4.8	12.3	9.3	23.6	1.1	1.1
CCTAGG	5.4	9.3	3.1	7.3	8.6	16.6	0.6	0.8
GCTAGG	13.0	20.7	5.2	11.9	18.3	32.6	0.4	0.6
CGTAGG	2.4	8.1	5.6	19.1	8.0	27.1	2.3	2.4
GCTAGC	10.3	8.1	6.2	7.2	16.5	15.3	0.6	0.9
GCTACG	9.7	10.4	10.0	15.5	19.8	25.9	1.0	1.5
CGTAGC	1.0	0.9	6.1	8.0	7.1	9.0	6.0	8.5
CGTACG	2.4	3.3	5.9	9.6	8.3	12.9	2.5	2.9
(Part 2) Effect of Remote Base Substitution at N_1 and N_6								
GGATGG	1.4	2.7	27.4	46.8	28.8	49.5	19.4	17.1
GGATGC	1.4	2.0	28.4	43.3	29.8	45.2	20.4	22.1
CGATGG	0.9	2.4	18.7	40.8	19.6	43.1	20.8	17.3
CGATGC	0.6	0.9	10.2	17.6	10.7	18.5	18.0	18.9
GGATCG	1.9	2.5	28.6	19.9	30.6	22.4	14.8	7.8
GGATCC	1.3	3.3	21.2	46.0	22.5	49.2	16.2	14.0
CGATCG	2.6	1.0	12.5	14.9	15.1	15.9	4.8	15.1
CGATCC	2.0	2.1	14.8	12.1	16.8	14.3	7.6	5.6
GCATGG	7.0	10.2	15.6	23.5	22.6	33.7	2.2	2.3
GCATGC	5.2	5.4	9.4	13.9	14.6	19.3	1.8	2.6
CCATGG	2.8	9.4	9.0	24.7	11.9	34.2	3.2	2.6
CCATGC	2.8	4.0	9.5	13.1	12.3	17.1	3.4	3.3
GCATCG	2.1	3.1	7.3	10.2	9.4	13.3	3.4	3.2
GCATCC	4.2	6.5	8.7	13.6	12.9	20.1	2.1	2.1
CCATCG	1.2	5.4	6.3	16.1	7.5	21.5	5.2	3.0
CCATCC	1.8	3.9	4.3	9.1	6.1	13.1	2.4	2.3
GGTAGG	3.8	9.8	9.8	27.0	13.6	36.8	2.6	2.8
GGTAGC	3.5	4.0	9.0	17.1	12.5	21.1	2.6	4.2
CGTAGG	2.4	8.1	5.6	19.1	8.0	27.1	2.3	2.4
CGTAGC	1.0	0.9	6.1	8.0	7.1	9.0	6.0	8.5
GGTACG	4.5	5.1	16.0	22.9	20.5	28.0	3.6	4.5
GGTACC	4.0	7.3	10.3	31.6	14.3	39.0	2.6	4.3
CGTACG	2.4	3.3	5.9	9.6	8.3	12.9	2.5	2.9
CGTACC	3.5	4.0	7.9	10.8	11.4	14.8	2.3	2.7
GCTAGG	13.0	20.7	5.2	11.9	18.3	32.6	0.4	0.6
GCTAGC	10.3	8.1	6.2	7.2	16.5	15.3	0.6	0.9
CCTAGG	5.4	9.3	3.1	7.3	8.6	16.6	0.6	0.8
CCTAGC	5.5	7.0	3.5	6.5	9.0	13.5	0.6	0.9
GCTACG	9.7	10.4	10.0	15.5	19.8	25.9	1.0	1.5
GCTACC	7.6	12.2	5.0	11.0	12.6	23.2	0.7	0.9
CCTACG	4.5	11.3	4.8	12.3	9.3	23.6	1.1	1.1
CCTACC	4.3	9.8	3.0	9.9	7.2	19.7	0.7	1.0

[a] Percent cut indicates the fractional cleavage at N_3, N_4, or N_3 plus N_4; N_4/N_3 indicates the ratio of cleavage at N_4 and N_3. The oligonucleotides are paired with each other with single positional isomers in part 1 and are grouped according to the quartets at $N_2N_3N_4N_5$ in part 2.

Table IV: Effect of Various Base Substitutions on Cleavage by NCS-C[a]

	% cut								ratio					
	N_2		N_3		N_4		N_5		N_4/N_3		N_4/N_2		N_5/N_3	
strand	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH	DTT	GSH
GGCTGG			1.9	1.3	36.8	43.3			19.7	34.5				
GGATGG			1.4	2.7	27.4	46.8			19.4	17.1				
GGGTGG			0.7	0.8	14.6	37.0			21.9	47.1				
GACTGG	1.2	0.5	1.6	1.8	26.0	32.2			16.0	18.0	21.3	60.2		
GGCTGG			1.9	1.3	36.8	43.3			19.7	34.5				
GCGTGG			1.0	1.9	19.6	27.5			19.3	14.3				
GAGTGG	0.8	1.2	1.4	2.4	14.3	41.7			10.5	17.3	16.8	36.3		
GGGTGG			0.7	0.8	14.6	37.0			21.9	47.1				
CCACCC			1.7	4.0	0.5	0.4			0.3	0.1				
CCATCC			1.8	3.9	4.3	9.1			2.4	2.3				
CCAGCC			2.8	2.9	0.8	0.9			0.3	0.3				
CCAGCC			2.8	2.9	0.8	0.9			0.3	0.3				
CCAGTC			1.2	1.4	0.7	0.9	16.3	23.9	0.5	0.6			13.5	17.1
CCACCC			1.7	4.0	0.5	0.4			0.3	0.1				
CCACTC			1.0	1.2	0.6	1.3	14.7	25.5	0.6	1.0			14.1	21.1
CCACGC			2.8	3.5	1.5	2.3	1.1	1.2	0.5	0.7				

[a] Percent cut indicates the fraction of cleavage at the particular nucleotide; The various ratios indicate the fractional cleavage between the two nucleotides.

purine-(3′,5′)-pyrimidine at the intercalation site, GC in particular, is in agreement with our results showing GN_1 in GN_1N_2 as the preferred intercalation site of NCS-C, but unlike the Pullman model, our proposal involves cleavage at N_2 not N_1.

Results obtained from non-self-complementary oligonucleotides clearly indicate that NCS-C can preferentially cleave one strand over its complementary strand. This selectivity can be seen from the ratio A/B which represents the ratio of the combined cleavage at A and T in each of the complementary strands. The magnitude of A/B reflects the difference in the number of G residues between the two complementary strands. It is also dependent on how the site of sequence-specific binding and the site of cleavage are located relative to each other, as in GN_1N_2 where NCS-C presumably intercalates between GN_1 and cuts at N_2. In addition, the ratio is by and large higher in oligonucleotides with AT than in those with TA at N_3N_4.

In the case of non-self-complementary hexamers, the sequence specificity is also greater in the preferred strand A compared with strand B (ratio N_4/N_3 in Table II). This generally results from enhanced cutting at nucleotide N_4 and slightly depressed cutting at nucleotide N_3 in strand A compared with that in strand B. The hexamers, such as GCATGG and GCTAGG (ratio N_4/N_3 of strand A in Table II), indicate the importance of G in GN_1N_2 (GCA and GCT), compared with CGATGG and CGTAGG, respectively, in determining the preferred target site. However, GGGTGG was not as good a substrate as hexamers with A or C at N_3 instead of G, as in GGATGG or GGCTGG (Table IV). The presence of a higher number of G residues within one strand and at the 5′-end tends to make an oligonucleotide a better substrate for NCS-C.

The important role of G as the second next nucleotide to the 5′-end from the target nucleotide (that is, GN_1N_2 where N_2 is the target nucleotide) (Figure 2) in terms of both sequence specificity and extent of cleavage in hexamers is quite evident (Tables III and IV). The variations in the ratio N_4/N_3 clearly indicate the effect of base substitution from C to G at N_2. In the $N_1N_2ATN_5N_6$(AT) series, this substitution enhances the absolute cleavage at T and suppresses that at A. In the $N_1N_2TAN_5N_6$ (TA) series, it enhances the absolute cleavage at A and suppresses that at T. Therefore, it is evident

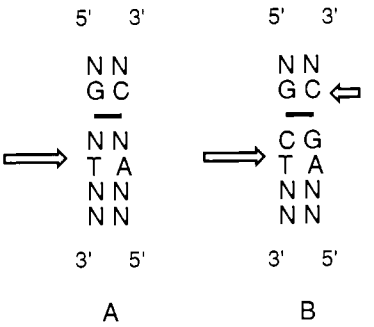

FIGURE 2: Interaction of NCS-C with oligonucleotides. The duplex A contains the preferred trinucleotide GNT, where NCS-C intercalates GN and cleaves at T. The duplex B contains preferred sites for both single-strand nicks (^{5}GCT^3) and abasic, alkali-labile lesions (^{5}AGC^3). The solid bars represent the proposed intercalation sites for NCS-C. The long arrows represent major single-strand nicks at the 5′-side of the T residues indicated. The short arrow represents an abasic, alkali-labile lesion at the C residue indicated. On the basis of the data presented in the paper, it is reasonable to speculate that the intercalation site accounts for the cleavage at T, but whether the same binding complex should account for both lesions remains to be determined.

that regardless of the exact nature of N_3 and N_4, whether A or T, N_4 is cleaved more when N_2 is changed from C to G, and an exactly opposite effect is seen at N_3. This explains why GCATGG and GCTAGG show distinctive results as to low ratio N_4/N_3 (Table II). In the AT series, substitution of N_2 from C to G improves the overall absolute cleavage due to the fact that the hexamers now have the best target nucleotide T in position as GAT.

The question arises as to what feature of the preferred trinucleotide, GN_1N_2, such as the GN_1 intercalation site or the N_2 cleavage site (or both) is more important in the initial recognition by NCS-C of its DNA binding site. Possibilities include the following: (1) The proper intercalative binding of the substituted naphthoic acid moiety in between GN_1 initiates the process. NCS-C may not, however, be able to produce single-strand nicks at N_2 unless it is a suitable nucleotide such as T or A, although the binding of NCS-C at GN_1 might be adequate. (2) The target nucleotide N_2 is recognized first by the bicyclo[7.3.0]dodecadienediyne epoxide moiety, followed by the intercalative interaction of the substituted naphthoic acid moiety, presumably assisted by hy-

drogen bonding and van der Waals interaction of the amino sugar moiety. The intercalative binding at GN_1 may not take place properly if the substituted naphthoic acid moiety cannot be positioned well. (3) The entire trinucleotide GN_1N_2 is recognized. The respective entities of GN_1 and N_2 are equally important in the initial recognition process.

The ability of poly(dA-dT), but not poly(dG-dC), to protect against the cleavage of [^{3}H]DNA by NCS-C (Poon et al., 1977) implies that the nature of target site N_2 is important in NCS-C binding. At the same time, the unequal degree of cleavage at various T residues in DNA restriction fragments [reviewed in Goldberg (1986)] and in the hexamers studied here indicates that the sequence of the surrounding nucleotides is important in the drug–DNA interaction. There exists preference for a purine base over C to give GN_1N_2 or AN_1N_2 or purine-(3′,5′)-pyrimidine such as GC, GT, AC, or AT to give GCN_2, GTN_2, ACN_2, or ATN_2 for the hot-spot trinucleotides (N_2 as the cleaved nucleotide) in providing the most preferred intercalative and hydrogen-bonding interaction site for NCS-C. This is also exemplified by the finding that the major adduct of NCS-C to poly(dA-dT) found after enzymatic digestion is on the A in poly(dA-dT) instead of on the T in a ratio of approximately 3 to 1 (Povirk & Goldberg, 1982a, 1984; S. H. Lee and I. H. Goldberg, unpublished data), although T is usually the best target site for cleavage. This result suggests that NCS-C intercalates in between AT of poly(dA-dT), but its target nucleotide is not the same T but the next A to the 3′-end from the intercalation site. Also, with a DNA restriction fragment, NCS-C was found to be adducted to the T in GCT (Povirk & Goldberg, 1985). Evidently, in the case of poly(dA-dT), the attack preference for T is overridden by its prebinding at the better intercalation site. This is another demonstration that prebinding of NCS-C is required for the DNA damage reaction to take place. In summary, it appears that the overall structure of GN_1N_2 is important in determining optimal interaction with NCS-C.

NCS-C-induced abasic lesions, which are alkali-labile cleavage sites, occur frequently at the C in trinucleotide AGC (Povirk & Goldberg, 1985; Kappen et al., 1988). The complementary strand, GCT, corresponds to the NCS-C hot spot for DNA cleavage in our study. Evidence has been presented to show that each abasic site at the C in AGC is accompanied by a direct break at the T, two nucleotides to the 5′-side, on the complementary strand of the same duplex, forming closely opposed bistranded lesions (Povirk & Houlgrave, 1988; Povirk et al., 1988). On the other hand, not all breaks at the same T are accompanied by abasic site formation on the opposite strand (Kappen et al., 1988). Similarly, we have found more direct single-strand nicking (55.8% with GSH and 24.9% with DTT) at T of GGCTGC than abasic alkali-labile site formation (26.9% with GSH and 3.9% with DTT) at C* on its complementary strand CCAGC*C. It seems likely that both lesions result from the concerted interaction of a single molecule of activated NCS-C, which has been proposed to be a bifunctional diradical species (Kappen & Goldberg, 1985; Myers, 1987). The diradical species of NCS-C presumably abstracts one hydrogen atom from C-5′ of T at the site of direct strand breakage and the other from C-1′ (Kappen et al., 1988; Kappen & Goldberg, 1989) of C at the site of formation of the abasic lesion. This mechanism is supported by the finding that two hydrogens from DNA are incorporated into the activated drug during the DNA damage reaction (Chin et al., 1988). A diradical mechanism has also been invoked to explain the bistrand lesions produced by the related diynene antibiotic calicheamicin (Zein et al., 1988). However, the finding that NCS-C generates abasic sites less frequently than single-strand breaks, even within $^{5'}GCT^{3'}\cdot^{5'}AGC^{3'}$, remains to be explained with regard to the above mechanism.

Even though GSH is generally more efficient than DTT as a cofactor in the NCS-C-induced cleavage of hexamers, as shown in Table III-1, it exerts the same strand selectivity and base specificity as does DTT in most cases. This result suggests that NCS-C first binds DNA specifically and then NCS-C is activated by thiol resulting usually in the same strand selectivity and base specificity for DTT and GSH. Since GSH, a tripeptide, is bulkier, more flexible, and possesses more functionalities capable of providing specific interaction with DNA, it may provide a unique sequence-dependent interaction with oligonucleotides, in particular since the thiol becomes part of the active drug species by adduction. The superiority of GSH over DTT in generating abasic lesions at the C of trinucleotide AGC (Povirk & Goldberg, 1985; Kappen et al., 1988; duplex $^{5'}GGCTGC^{3'}\cdot^{5'}GCAGCC^{3'}$ in this study) is another example of the unique quality displayed by the natural cellular thiol.

Acknowledgments

We are grateful to Dr. Andrew H.-J. Wang at the Department of Biology, Massachusetts Institute of Technology, for helpful discussions. The technical expertise of Jeanne O. Thivierge throughout this investigation is greatly appreciated. We thank Dr. Loren D. Williams for assistance with the statistical analysis.

Registry No. NCS-C, 79633-18-4; GGATGG·CCATCC, 118043-11-1; GGATGC·GCATCC, 118043-14-4; GGATCG·CGATCC, 118043-16-6; GCATGG·CCATGC, 118043-19-9; CGATGG·CCATCG, 118043-22-4; CGATGC·GCATCG, 118043-24-6; GGTAGG·CCTACC, 118043-27-9; GGTAGC·GCTACC, 118043-30-4; GGTACG·CGTACC, 118043-33-7; GCTAGG·CCTAGC, 118102-13-9; CGTAGG·CCTACG, 118043-37-1; CGTAGC·GCTACG, 118070-61-4.

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