Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3047–3051, April 1991
Biochemistry

Selective abstraction of ^2H from C-5′ of thymidylate in an oligodeoxynucleotide by the radical center at C-6 of the diradical species of neocarzinostatin: Chemical evidence for the structure of the activated drug–DNA complex

(^1H NMR/^2H isotope effect/bistranded oxidative DNA damage/sequence specificity)

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Communicated by Charles C. Richardson, January 4, 1991 (received for review November 21, 1990)

ABSTRACT **Use has been made of the mechanism of DNA deoxyribose damage by the ene-diyne-containing chromophore of the antitumor antibiotic neocarzinostatin to provide chemical evidence for the structure of the activated drug–DNA complex. Radical centers at C-2 and C-6 of the diradical form of the glutathione-activated chromophore abstract hydrogen atoms from C-1′ of the C residue and C-5′ of the T residue in AGC·GCT to generate a bistranded lesion consisting of an abasic site at C and a strand break at T. This laboratory has proposed a molecular model for the drug–DNA interaction in which the naphthoate moiety of the chromophore intercalates between A·T and G·C, placing the diradical core in the minor groove, so that the radical centers at C-6 and C-2 are close to C-5′ of T and C-1′ of C, respectively. To determine which radical center abstracts one of the hydrogen atoms from C-5′, the self-complementary oligodeoxynucleotide GCAGCGCTGC was synthesized with ^2H at both 5′ positions of the T residue and treated with glutathione-activated chromophore. Sequencing-gel electrophoresis showed that drug attack was limited to the T and C residues and that abstraction of ^2H from C-5′ exhibited a small isotope selection effect of 1.25. ^1H NMR spectroscopic examination of the reacted chromophore, isolated by HPLC, indicated that ^2H was selectively abstracted by C-6, providing experimental corroboration of the model and further elucidating the chemical mechanism. Since direct strand breakage at the T residue exceeds (44% more) abasic site formation at the C residue, other models of drug–DNA interaction leading to only single-strand breaks are also considered.**

The DNA-damaging antitumor antibiotic neocarzinostatin (NCS) uniquely consists of a biologically active highly strained diacetylene-containing chromophore noncovalently complexed with its carrier apoprotein (for review, see ref. 1). The chromophore (2) structure is comprised of three subunits (3–5): a 5-methyl-7-methoxynaphthoate and a 2,6-dideoxy-2-(methylamino)galactose interlinked by a C_{12} subunit containing a bicyclo[7.3.0]dodecadiyne system bearing an epoxide and a cyclic carbonate moiety (Fig. 1, structure **1**). Evidence has been presented that the ene-diyne bicyclic core of the chromophore binds in the minor groove of DNA with the naphthoate moiety intercalated between the DNA base pairs and the amino sugar moiety interacting with the DNA backbone (11–13). Damage to the DNA occurs when activation of the chromophore by thiol (or sodium borohydride) occurs by nucleophilic addition at C-12 and epoxide opening, followed by rearrangement of the bicyclic ene-diyne core into a diradical species (structure **2**) (NCS*) with radical centers at C-2 and C-6 (3, 6–9) that, depending on DNA microstructure, attack minor-groove-accessible carbons at C-5′ (10, 14), C-1′ (15–17), and C-4′ (18–21) of deoxyribose (Fig. 1). The carbon-centered radical generated on the deoxyribose by abstraction of a hydrogen atom by NCS* adds dioxygen to form a peroxyl radical species that eventuates in either a strand break or an abasic site.

Although single-strand breaks are base specific (thymine > adenine >> cytosine > guanine), there appears to be no clear-cut sequence specificity (22); double-stranded lesions, however, are sequence-specific and appear to result from the bistranded interaction of a single NCS molecule. The double-stranded lesion responsible for most GC to AT transitions in the *cI* gene of λ phage (23) consists of an abasic site at the C residue and a strand break at the T residue in the sequence AGC·GCT (15–17, 24, 25). These lesions result from NCS* attack at C-1′ of the C residue with the formation of 2-deoxyribonolactone and at C-5′ of the T residue with the formation of thymidine 5′-aldehyde. The other described bistranded lesion consists of either a strand break or an abasic lesion due to 4′ chemistry at the T residue of the GT step in the sequence AGT·ACT and a strand break with thymidine 5′-aldehyde formation on the complementary strand due to 5′ chemistry (19). In both types of double-stranded damage the lesions on the DNA strands are separated by two nucleotides in the 3′ direction, diagnostic of minor-groove interactions.

Molecular model building based on energy minimization and molecular dynamics simulations has led to a proposal for the NCS*–DNA complex responsible for the bistranded lesions at AGC·GCT in which the naphthoate moiety intercalates between the A·T and G·C base pairs with its diradical core oriented toward the 3′ end of the plus strand (26) (Fig. 2). This model predicts that C-6 of NCS* abstracts one of the two hydrogen atoms at C-5′ of the T residue (distance of 3.3 Å) and C-2 abstracts the hydrogen atom at C-1′ of the C residue (distance of 4.1 Å). To test this proposal and to further elucidate the mechanism of cleavage of DNA by NCS*, we have synthesized the self-complementary oligodeoxynucleotide GCAGCGCTGC with deuterium at both 5′ positions of the T residue and followed its abstraction into NCS* by ^1H NMR.

MATERIALS AND METHODS

NCS chromophore was extracted from the holoantibiotic and stored in methanol (324–555 μM) at −70°C as described (10). Oligodeoxynucleotides were synthesized using phosphoramidite chemistry. All synthetic intermediates were purified by silica gel chromatography.

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Abbreviations: NCS, neocarzinostatin; NCS*, diradical species of neocarzinostatin; GSH, glutathione.

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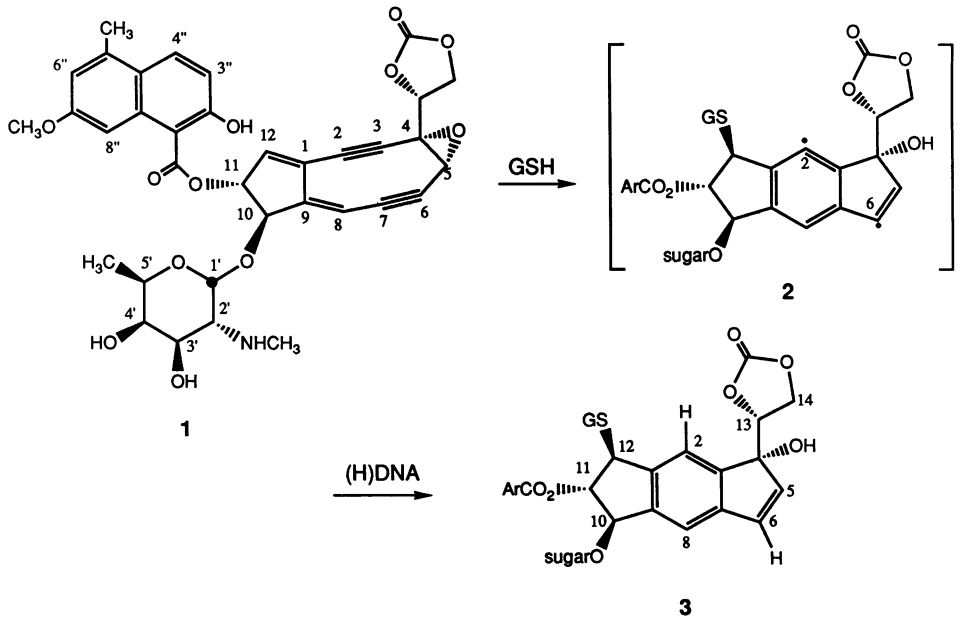

FIG. 1. Structure of NCS and its proposed mechanism of activation and action (3–10). Ar, aromatic.

Synthesis of [5′-$^2H_2$]Thymidine 3′-Phosphoramidite. 3′-*O*-Acetylthymidine was oxidized to the corresponding 5′-*tert*-butyl carboxylic ester according to the method of Corey and Samuelsson (27) in 65% yield. Reductive deuteriation of the 5′ ester group and concomitant removal of the 3′ acetate with $LiEt_3B^2H$ were performed by a modification of the procedure of Brown *et al.* (28). [5′-$^2H_2$]Thymidine was obtained in 60% yield with >98% incorporation of deuterium at both 5′ positions (H_r and H_s, the hydrogen atoms projecting away from and toward minor groove, respectively), as indicated by ^1H NMR. Preparation of [5′-$^2H_2$]thymidine for incorporation into the oligodeoxynucleotide involved protection of the 5′-hydroxyl with dimethoxytritylchloride (29) (78% yield) followed by phosphitylation of the 3′-hydroxyl with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (30) (79% yield).

Reaction of 5′-^{32}P-End-Labeled Oligodeoxynucleotides with NCS Chromophore. Chemically synthesized oligodeoxynucleotides GCAGCGCT^{8}GC containing either 5′ protium or deuterium at T^8 were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (31). The labeled oligodeoxynucleotides were purified on a 20% denaturing polyacrylamide gel.

The self-complementary radiolabeled oligomers (3×10^3 cpm) were first annealed to an unlabeled oligomer (2.0 A_{260} units) in a 2× reaction buffer (see below) by heating at 80°C for 2 min and subsequent slow cooling to room temperature. The annealed oligomer was then distributed for drug reactions, diluted with the amount of water required to make up the final volume, and chilled for 20 min on ice. Glutathione (GSH) was then added followed by NCS chromophore. The reaction was allowed to proceed at 0°C in the dark for 1 hr. A standard reaction mixture (50 μl) contained 25 mM Tris·HCl (pH 8.0), 1 mM EDTA, 150 μM DNA phosphate, 5 mM GSH, and 8.1 μM NCS chromophore. Control reaction mixtures contained no drug but received an equal volume of methanol (final maximum, 15%).

Analysis of Cleavage by Gel Electrophoresis and Determination of Isotope Effect. Analysis of strand breakage required lyophilization of two samples of each reaction mixture, one of which was treated with 0.1 M NaOH at 90°C for 30 min, neutralized with 0.1 M HCl, and relyophilized. The residue from each sample was dissolved in 80% (vol/vol) formamide containing 1 mM EDTA and marker dyes and electrophoresed on a 20% polyacrylamide sequencing gel (32). Gel-band intensities were quantitated by scanning an autoradiogram with an LKB Ultroscan laser densitometer. The kinetic isotope effect at T^8 was determined from the ratio of the integrated peak areas at that position (33). C^5 was used as an internal control.

NCS Chromophore Oligodeoxynucleotide Reactions. A standard reaction mixture (2.8–5.6 ml) contained 25 mM Tris·HCl (pH 8.0), 1 mM EDTA, [5′-^{1}H]- or [5′-^{2}H]T^8 in the oligomer

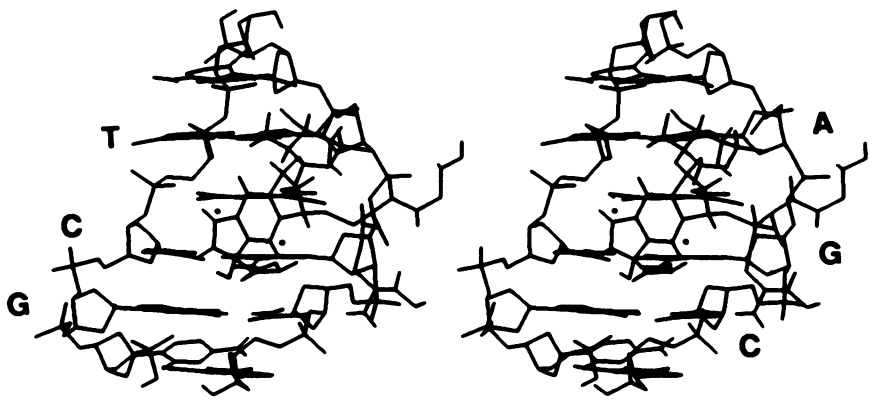

FIG. 2. Stereodrawings of optimized structure of reacted form of NCS complexed with AGC·GCT containing oligodeoxynucleotide. C-2 and C-6 of NCS are labeled with a dot. GSH is attached to C-12 of NCS. Modified from ref. 26.

(1.0 mM DNA phosphate; 221 μg/ml), 5 mM GSH, and 50 μM NCS chromophore. To ensure the presence of duplex DNA, the oligomer was kept at 80°C for 2 min and allowed to cool slowly to room temperature in 2× reaction buffer (50% total reaction volume). The amount of water required to make up the final volume was added and the mixture was chilled to 0°C before addition of GSH and chromophore. After incubation at 0°C for 90 min, methanol was removed *in vacuo*. The resulting mixture was frozen and lyophilized to dryness overnight.

Isolation and Characterization of the NCS Chromophore Product. The residue from the drug reaction was redissolved in a small amount of water (100–300 μl) before analysis by reverse-phase HPLC on a Rainin Microsorb C_{18} (5 μm, 1.0 × 25 cm) column. The column was eluted first for 10 min with aqueous 5 mM ammonium acetate (pH 5.0), followed by an 80-min convex gradient of 0–80% methanolic 5 mM ammonium acetate (pH 5.0) at a flow rate of 3 ml/min. Fractions (3 ml) were collected. The major fluorescent (excitation at 340 nm/emission at ≥418 nm) peak, which eluted at 55 min, was collected and lyophilized after removal of methanol *in vacuo* at 0°C. Characterization of the compound included ^1H NMR and mass spectroscopy. ^1H NMR spectra were recorded on a Varian VXR 500S spectrometer in $C^2H_3O^2H/C^2H_3CO_2{}^2H$, 10:1 (vol/vol), at 25°C (sample concentration, ≈100 μM).

RESULTS

Due to the lability of the native NCS chromophore, even in the presence of DNA and even, more importantly, due to the fact that the structure of the species of the drug actually responsible for DNA damage (NCS*, structure **2**) differs substantially from the preactivated form (Fig. 1, structure **1**), it is not possible to obtain direct structural evidence for the nature of the drug–DNA complex by traditional NMR or crystallographic studies. Accordingly, we have resorted to the use of chemical means to obtain such information and to elucidate the mechanism of hydrogen atom abstraction. In earlier experiments using ^1H NMR and fast atom bombardment mass spectroscopy, DNA was shown to be the source of the hydrogen atoms that are incorporated into the C-6 and C-2 positions of NCS (9). Since selectively deuteriated DNA was not available at the time, a reverse isotope labeling experiment was carried out in deuteriated solvent and activating agent (sodium borodeuteride). It was found that, whereas both C-2 and C-6 were fully deuteriated in the absence of DNA, in its presence both positions abstracted nonexchangeable protium atoms from the DNA. These experiments, however, could not distinguish from where in the heterogeneous DNA the protium originated or which radical center interacted with which particular attack site (C-5′, C-1′, or C-4′). This type of information is essential to clarify the underlying mechanism and to verify the proposed molecular model for the drug–DNA interaction.

To this end we prepared an oligodeoxynucleotide containing a single bistranded attack site that was labeled with ^2H at both C-5′ positions of its T residue. To optimize incorporation into the drug from this site, it was important that the oligonucleotide not have other significant sites of attack. Information on this point was obtained by studying the ability of the self-complementary oligodeoxynucleotide GCAGCGCTGC, containing either ^1H or ^2H at C-5′ of the T residue and 5′-end-labeled with ^{32}P, to act as a damage substrate for NCS by DNA sequencing gel electrophoresis. Under the conditions of the reaction (0°C, 25 mM Tris·HCl), the 10-mer is expected to exist almost entirely as a duplex rather than as a hairpin structure (34). As shown in Fig. 3, with GSH (5 mM) as the activating thiol, there is direct strand breakage at the T residue and alkali-dependent cleavage at the C residue of AGC. Little, if any, breakage occurs at the other residues. The direct breakage at the T residue is about 1.8-fold greater than the alkali-dependent cleavage at the C residue. Since the drug reaction was carried out under one-hit kinetic conditions, by quantitating the breakage at the T residue of the ^1H and ^2H substrate by densitometry, it is possible to calculate a small isotope selection effect (k_{1H}/k_{2H} = 1.25). This value is in agreement with similar calculations at T residues at which 5′ abstraction chemistry occurs (20, 21) and is considerably smaller than that at C-1′ of C (k_{1H}/k_{2H} = 4.0) (17).

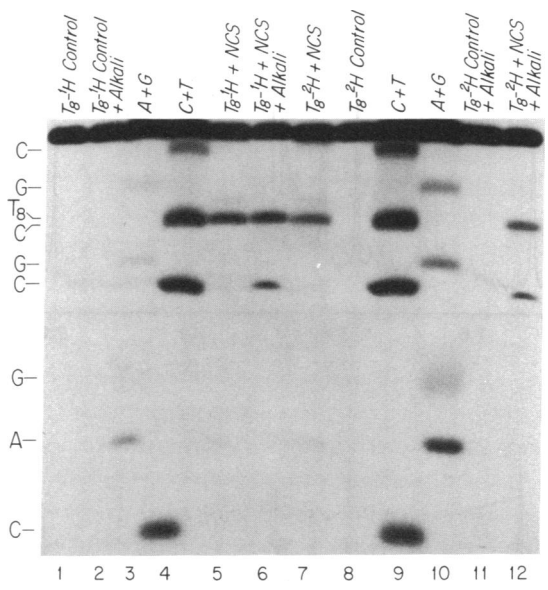

FIG. 3. Direct strand breakage at T^8 and alkali-dependent cleavage at C^5 in $GCAGC^5GCT^8$*GC by NCS; isotope effect on cleavage at T^8. The 5′-^{32}P-end-labeled $GCAGC^5GCT^8$*GC in duplex form was incubated with 8.1 μM NCS and 5 mM GSH in a standard reaction mixture. Lanes: 1, 2, 5, and 6, [5′-$^1H_2$]T^8; 7, 8, 11, and 12, [5′-$^2H_2$]T^8; 6 and 12, alkali-treated drug reactions for [5′-$^1H_2$]T^8 and [5′-$^2H_2$]T^8, respectively; 1 and 2, controls (−drug) with and without alkali, respectively, for the unlabeled oligomer; 8 and 11, similar controls were performed for the labeled oligomer; 3, 4, 9, and 10, Maxam–Gilbert markers.

To determine the site in NCS* responsible for hydrogen atom abstraction from C-5′ of the T residue by ^1H NMR, the drug-damage reaction with the ^1H- or ^2H-labeled 10-mer was scaled-up and the reacted NCS chromophore was isolated by HPLC. Fig. 4 and Table 1 show partial ^1H NMR spectra and assignments, respectively, of the reacted chromophore (structure **3**). The ^1H NMR spectrum (Fig. 4*A*) of the chromophore from the reaction with the protium-containing 10-mer shows that both H-6 and H-5 resonances appear as doublets centered around 6.95 ppm and 6.33 ppm, respectively, with a coupling constant of 5.9 Hz. The H-2 resonance appears as a sharp singlet at 7.68 ppm. The chromophore product isolated from the reaction mixture containing the ^2H-labeled 10-mer displayed an ^1H NMR pattern (Fig. 4*B*) that was different in only one respect. All resonances were the same except for H-5, which now appeared as a mixture of a doublet centered around 6.33 ppm (J = 5.9 Hz) and a singlet at 6.33 ppm. The latter accounted for ≈30% (error in measurement of <5%) of the total resonances at H-5, as determined by integration data. This result indicates partial incorporation of deuterium into the C-6 position of the drug. Consistent with this interpretation is the finding that integration of the H-6 resonance was 30% (error in measurement of <5%) less in the reaction with the ^2H-labeled substrate. The H-6 resonance appeared as a doublet, but at a decreased intensity. On the other hand, integration of the H-2 resonance showed no detectable difference in the two reactions.

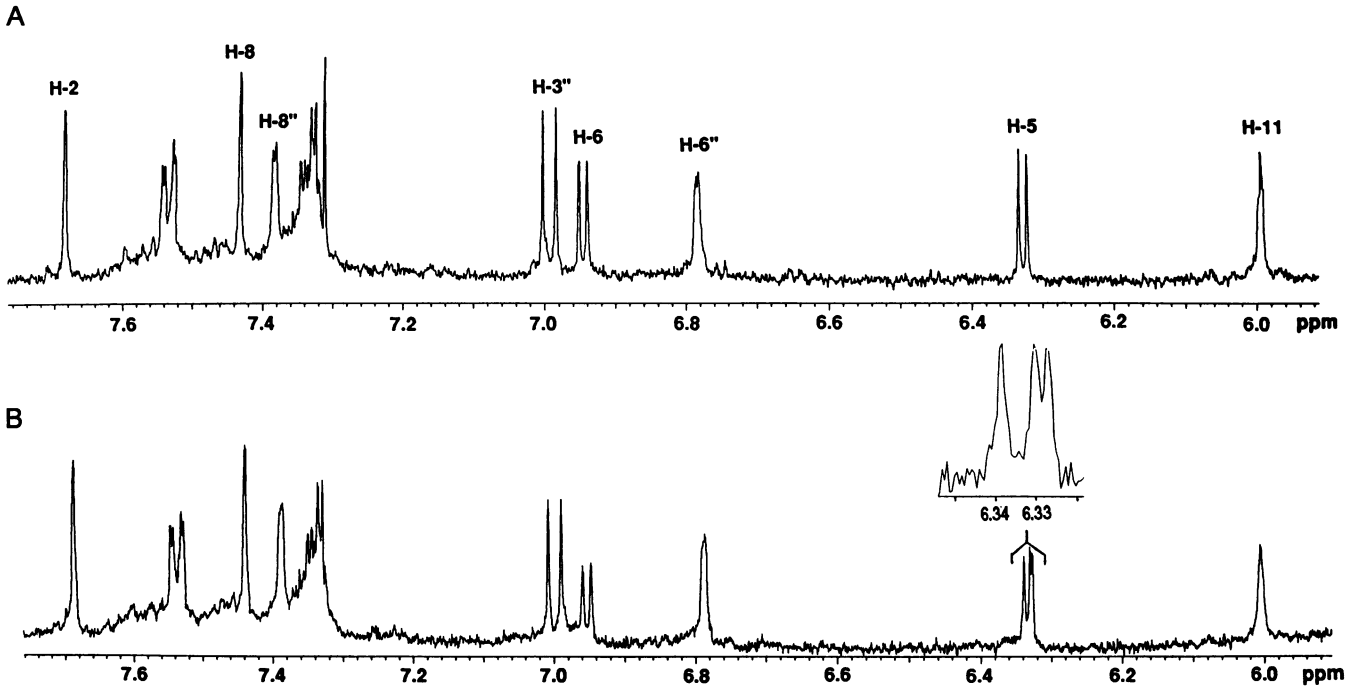

FIG. 4. Partial 500-MHz ^1H NMR spectra for NCS chromophore product (structure 3) isolated from a reaction mixture containing 5 mM GSH with unlabeled (*A*) and ^2H-labeled oligomer GCAGCGCT^8*GC (*B*). The unidentified peaks in the region of 7.3–7.55 ppm could be due to either the amide protons of GSH or to oligomer degradation products.

These results indicate that ^2H from C-5′ of the T residue was selectively abstracted by the radical center at C-6 of NCS*. The incomplete incorporation of ^2H at C-6 could be due to the competitive quenching of this radical center by the GSH used as the drug-activating agent. This action might be enhanced somewhat by the ^2H isotope selection effect. Given the relatively high molar ratio of thiol to drug (100:1) in the damage reaction, it is possible that some of the hydrogen atom incorporation at C-6 comes from the exchangeable hydrogen of the unlabeled GSH (5, 8). However, a 5 times decrease in the GSH concentration (1 mM) did not significantly alter the extent (33%) of ^2H incorporation into C-6 as determined by the measurements cited above. The possibility that carbon-bound hydrogen in GSH is responsible for the chromophore-incorporated ^1H (9) has not been eliminated. The absence of a cleavage band representing a 3′-phosphoglycolate-ended fragment [and thus 4′ chemistry (19–21)] (Fig. 3) suggests that C-4′ is not a significant source of hydrogen atom incorporation at C-6.

Table 1. Partial ^1H NMR assignments of NCS chromophore product (3) isolated from reaction with GSH and oligomer

Assignment	Chemical shift, ppm	Multiplicity
H-4″	8.02	d, J = 9.2
H-2	7.68	s
H-8	7.43	s
H-8″	7.38	d, J = 2.2
H-3″	6.99	d, J = 9.2
H-6	6.95	d, J = 5.9
H-6″	6.78	d, J = 2.2
H-5	6.33	d, J = 5.9; or s*
H-11	5.99	br s
H-1′	5.77	d, J = 3.6
H-10	5.69	br s
H-13	4.70	dd, J = 6.0, 8.5
H-12	4.61	br s
H-14a	4.52	t, J = 8.5
H-14b	4.42	dd, J = 6.0, 8.5
H-3′	4.03	dd, J = 3.0, 10.9
Ar-OCH_3	3.43	s
N-CH_3	2.95	s
Ar-CH_3	2.56	s
5′-CH_3	1.19	d, J = 6.6

Spectra were recorded in $C^2H_3O^2H/C^2H_3CO_2{}^2H$, 10:1 (vol/vol), and assignments were made by comparison of data with previously reported data for the methylthioglycolate compound (3, 5, 7). Chemical shifts are given in ppm with CH_3OH at 3.30 ppm as reference. s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; J, coupling constant in Hz; Ar, aromatic.

*H-5 appears as a mixture of a singlet and a doublet in the compound isolated from the reaction with GSH and ^2H-labeled oligomer.

DISCUSSION

The ^1H NMR results provide chemical evidence that the C-6 radical center of NCS* is solely responsible for abstraction of a hydrogen atom from C-5′ of the thymidine in the AGC·GCT sequence. Presumably the radical center at C-2 is responsible for abstraction of the C-1′ hydrogen atom of the C residue on the complementary strand. These conclusions corroborate the prediction made by the molecular modeling studies and eliminate other energetically less favorable, but still possible, models involving sites of drug intercalation permitting C-2 of NCS* to attack at C-5′ of the T residue (26). The experiments reported here do not, of course, distinguish which of the two prochiral hydrogen atoms at C-5′ is abstracted by NCS*. To obtain this information chirally labeled substrate will have to be prepared. Based on the molecular model of DNA, it appears likely that H_s, the hydrogen atom projecting toward the minor groove, is the one abstracted.

The approach taken in this work provides direct chemical evidence, based on mechanism, for the nature of the activated drug–DNA complex. Not only are these results important for their mechanistic implications in terms of the DNA damage reaction but also, because of the structural analysis limitations stated above, these results may be the only way to clarify the sequence-dependent binding interaction between drug and DNA. Although the thiol-reacted chro-

mophore (structure 3), which most closely resembles NCS* (structure 2) in structure, binds to DNA as determined by UV and fluorescence measurements (35), efforts to demonstrate stable complex formation with short oligodeoxynucleotides of defined structure have not so far been successful (unpublished data). The weaker interaction of NCS chromophore with oligodeoxynucleotides than with DNA restriction fragments of greater length may explain why higher concentrations of drug are required to produce comparable strand breakage (15) and may be responsible for the incomplete labeling of C-6 of the chromophore with ^2H from C-5′ of the substrate.

The role of thiol in the drug action is complicated by the fact that it serves both as activator and as a reductant in the expression of the DNA damage (1, 10). Further, by becoming a covalent part of the active species of the drug, it may play a critical role in the DNA sequence selection process and in determining whether 5′, 1′, or 4′ chemistry ensues. This formulation is consistent with the observation that GSH is far superior to several other thiols in forming the bistranded lesion at AGC·GCT (15, 24) and in generating double-strand breaks at AGT·ACT (19). The model depicted in Fig. 2 for the former lesion suggests that the GSH adduct at C-12 of the chromophore forces the diradical core deeper into the minor groove, where C-1′ resides, to enable formation of the abasic lesion at the C residue (26). These results imply that it may be NCS* [or its proposed cumulene precursor (6, 8)] that is the actual DNA-sequence-seeking species.

The finding that direct cleavage at the T residue exceeds indirect cleavage (alkali labile) at the C residue (Fig. 3 and ref. 15) raises the possibility that ^2H abstraction from C-5′ by the radical center at C-6 is in part due to a different mode of drug–DNA binding that leads only to direct single-strand breaks. Since every abasic site at the C residue is accompanied by a break at the T residue on the complementary strand (25), ≈44% of the latter could be due either to a different binding mechanism or to the same mechanism that leads to the bistranded lesion but, as discussed earlier (26), with abasic site formation being less efficient for either chemical or geometric reasons. On the other hand, it is also conceivable that relatively less damage is expressed at the C residue because it is repaired more readily by scavenging thiol. If a different energetically allowable binding mode is involved, such as intercalation at the G·C and C·G base pairs with the diradical core extending toward the 5′ end of the plus strand (26), it is still necessary that C-6 of NCS* attack C-5′ of the T residue, since there is no detectable ^2H at C-2. In this case the diradical core would have to be rotated so that it is almost perpendicular to the helix axis, resulting in a distance of 3.47 Å between C-6 of NCS* and C-5′ of T (26).

We are grateful to Dr. Jonathan P. Lee for his assistance in obtaining the ^1H NMR spectra and for helpful discussions. The 500-MHz NMR spectrometer used in this study was funded through a National Institutes of Health shared instrumentation grant (1 S10 RR04862) and an award from the National Health Resources Foundation. This work was supported by U.S. Public Health Service Research Grants CA 44257 and GM 12573 from the National Institutes of Health.

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