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Selective Abstraction of ^2H from C-1′ of the *C* Residue in AGC·ICT by the Radical Center at C-2 of Activated Neocarzinostatin Chromophore: Structure of the Drug/DNA Complex Responsible for Bistranded Lesion Formation†

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ABSTRACT: Glutathione-activated neocarzinostatin chromophore (NCS-Chrom) generates bistranded lesions at AGC·GCT sequences in DNA, consisting of an abasic site at the *C* residue and a strand break at the *T* residue on the complementary strand, due to hydrogen atom abstraction from C-1′ and C-5′, respectively. Earlier work showed that ^2H from C-5′ of *T* was selectively abstracted by the radical center at C-6 of activated NCS-Chrom, supporting a proposed model of the active-drug/DNA complex. However, since under the conditions used breaks at the *T* exceeded their inclusion in bistranded lesions, it was not clear what fraction of the hydrogen transfer represented bistranded lesions. Since virtually all abasic sites at the *C* are part of a bistranded lesions, hydrogen transfer from C-1′ of *C* into the drug should reflect only the bistranded reaction. Accordingly, a self-complementary oligodeoxynucleotide 5′-GCAGCICTGC-3′ was synthesized in which the *C* contained ^2H at the C-1′ position. In order to eliminate an ^2H isotope effect on the transfer and to increase the extent of the bistranded reaction, an I residue was substituted for the G opposite the *C* residue. Sequencing gel electrophoretic analysis revealed that under one-hit kinetics, 37% of the damage reaction was associated with abasic site (alkali-labile break) formation at the *C* residue and 48% with direct strand breaks at the *T* residue. Thus, 74% of the damage involved a bistranded lesion. ^1H NMR spectroscopic analysis of the reacted chromophore showed that ^2H had been selectively transferred into the C-2 position to the extent of ~22%. Among other possible sources of hydrogen transfer, accounting for the low ^2H incorporation from DNA, are the hydrogen of the solvent (methanol/water), and possibly other components, present in the reaction mixture (intermolecular) and the carbon α to the sulfur of the adducted glutathione (intramolecular). The finding that solvent deuterium selectively quenches the radical at C-2 of NCS-Chrom in the presence of DNA helps to explain the excess of single-stranded over double-stranded lesions. The results in this report provide experimental support for an earlier proposed model [Galat, A., & Goldberg, I. H. (1990) *Nucleic Acids Res. 18*, 2093–2099] for the activated NCS-Chrom/DNA complex that generates bistranded lesions at AGC·GCT.

The cytotoxic and mutagenic properties of the enediyne-containing chromophore of the antitumor antibiotic neocarzinostatin (**1**, Scheme I) arise from its ability to produce double-stranded lesions in DNA (Goldberg, 1991). These lesions are believed to result from simultaneous abstraction of minor groove accessible hydrogen atoms from the deoxyribose backbone of opposing strands of the DNA by the thiol-activated diradical form of the drug (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985; Hensens et al., 1983; Myers, 1987; Hensens & Goldberg, 1989; Chin et al., 1988). The first double-stranded lesion to be identified (Povirk & Goldberg, 1985; Povirk et al., 1988; Kappen et al., 1988) occurs at the

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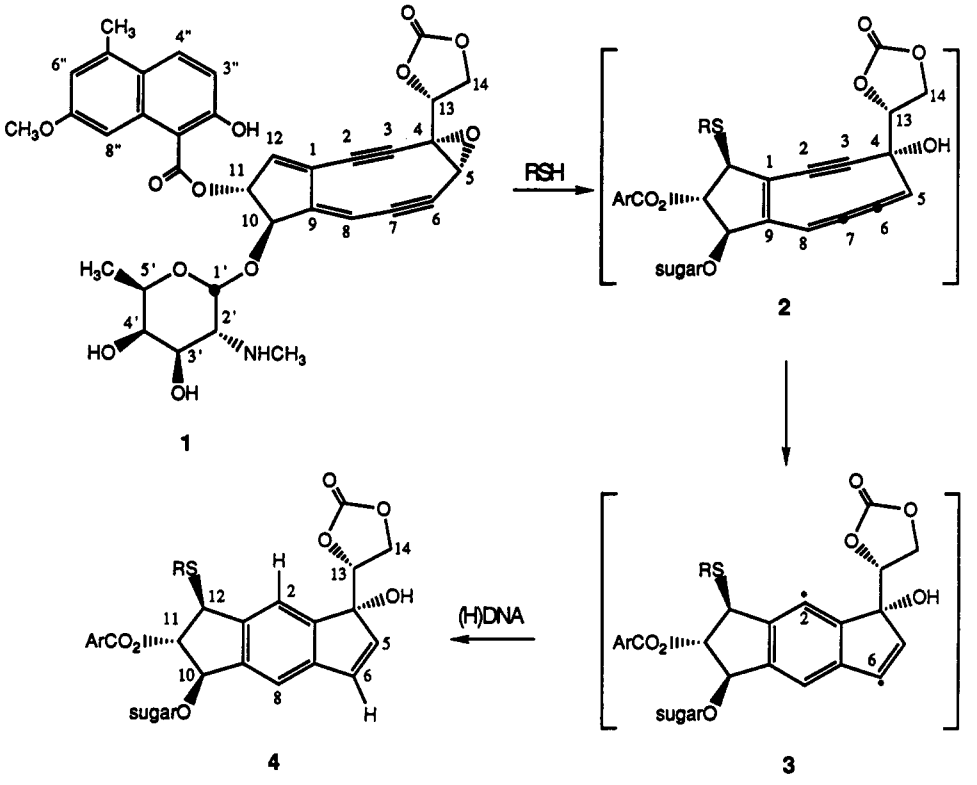
Scheme I: Proposed Mechanism of Activation and Action of NCS-Chrom (Myers, 1987)



sequence AG*C*·GC*T* and has been shown to be responsible for GC to AT transitions in λ phage (Povirk & Goldberg, 1986). This lesion involves 5′-hydrogen atom abstraction from the *T* residue to produce a strand break and, on the opposite strand, 1′-hydrogen atom abstraction from the *C* residue, resulting in an abasic site consisting of a 2-deoxyribonolactone moiety (Kappen et al., 1988; 1990; Kappen & Goldberg, 1989).

Recently, we have shown (Meschwitz & Goldberg, 1991), using deuterium transfer studies and ^{1}H NMR, that the C-6 radical center of the glutathione-activated drug is solely responsible for the abstraction of the hydrogen atom from the 5′-position of the *T* residue in the self-complementary oligodeoxynucleotide GCAG*C*GC*T*GC. These results, along with molecular modeling studies (Galat & Goldberg, 1990), led us to propose a structure of the activated-drug/DNA complex at the AG*C*·GC*T* bistranded attack site in which the naphthoate group is intercalated between the A:T and G:C base pairs with the diradical core oriented toward the 3′ end of the (+) strand. This orientation places the C-6 radical center in close proximity to the C-5′ position of the *T* residue and the C-2 radical center close to the C-1′ position of the *C* residue. However, other energetically allowable binding modes are possible which still allow for abstraction of the 5′-hydrogen atom from the *T* residue by the C-6 radical center (Galat & Goldberg, 1990). Further, since a substantial fraction of the direct strand breaks at the *T* residue are not ordinarily part of double-stranded lesions (Kappen et al., 1988; Meschwitz & Goldberg, 1991) and since the transfer of deuterium from C-5′ into C-6 of NCS-Chrom is incomplete (~33%) (Meschwitz & Goldberg, 1991), we cannot be certain what fraction of the transfer represents double-stranded lesions.

In order to unequivocally establish the structure of the activated-drug/DNA complex involved in generating double-stranded lesions at AG*C*·GC*T*, we have taken advantage of the finding that abasic sites at the *C* residue are virtually always part of a double-stranded lesion (Povirk et al., 1988) and have examined the transfer of deuterium from the C-1′ position of the *C* residue into the glutathione-activated drug. Presented herein are results which provide further evidence for the orientation of the drug in the minor groove at the AG*C*·GC*T* bistranded attack site.

MATERIALS AND METHODS

Materials. NCS-Chrom was extracted from the holoantibiotic (Kayaku Antibiotics Research Co., Ltd.), as described (Kappen & Goldberg, 1985), and stored at −70 °C in methanol (555–930 μM). Oligodeoxyribonucleotide GCAG*C*IC*T*GC, containing protium at the C-1′ position of the *C* residue, was purchased from Oligos Etc. Oligomer GCAG*C*IC*T*GC (*C* = [1′-^{2}H]2′-deoxycytidine) was chemically synthesized using standard cyanoethylphosphoramidite methodology (see below).

Synthesis of [1′-^{2}H]2′-Deoxycytidine. [1′-^{2}H]2′-Deoxycytidine was synthesized from [1′-^{2}H]arabinose, which was labeled using classical techniques (96% incorporation of deuterium selectively at C-1′ as determined by ^{1}H and ^{13}C NMR; details are available in Supplementary Material). [1′-^{2}H]Arabinose was converted first into 3′,5′-dibenzoyl-[1′-^{2}H]2′-deoxyuridine in 28% overall yield (Sanchez & Orgel, 1970; Holy, 1972) and then into 3′,5′-dibenzoyl-2′-deoxycytidine in high yield by displacement of the 4-*O*-sulfonate by NH_3. Subsequent benzoylation on N^4 and saponification of the benzoate esters yielded N^4-benzoyl-[1′-^{2}H]2′-deoxycytidine, which was converted into the protected phosphoramidite for oligo synthesis using standard protocols (Jones, 1985; Sinha et al., 1984) (complete experimental details are provided in Supplementary Material).

Oligomer Synthesis. The decamer GCAG*C*IC*T*GC (*C* = [1′-^{2}H]2′-deoxycytidine) was prepared on a 10-μmol scale by the syringe technique (Tanaka & Letsinger, 1982) using commercial phosphoramidite reagents (OmniChem, Wheaton, IL). After deprotection, the crude oligomer was purified by anion exchange chromatography on Sepharose QFF using a

linear gradient from 0.1 to 1.0 M NaCl in 10 mM NaOH. Fractions containing product were neutralized with KH_2PO_4 and then desalted by reversed-phase HPLC (H_2O/CH_3OH) to yield approximately 3 μmol of purified oligomer. The final material was analyzed by reversed-phase HPLC (0.1 M triethylammonium acetate, pH 6.4/CH_3CN).

Reaction of 5′-^{32}P-End-Labeled Oligomers with NCS-Chrom. Chemically synthesized oligomer GCAG*C*IC*T*GC, containing either 1′-protium or deuterium at *C*, was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase using standard procedures (Sambrook et al., 1989). The labeled oligonucleotides were purified on a 20% denaturing polyacrylamide gel.

The self-complementary radiolabeled oligomers (6×10^4 cpm) were first annealed to the corresponding unlabeled oligomer (2.0 A_{260} units) in a 2× reaction buffer (see below). To ensure duplex DNA, the mixture was kept at 80 °C for 2 min and then cooled slowly 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 (40 μL), and chilled for 20 min on ice. Glutathione (GSH) was then added, followed by NCS-Chrom. The reaction was allowed to proceed at 0 °C in the dark for 1 h. A standard reaction mixture contained 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 μM DNA phosphate, 1 mM GSH, and 7.5 μM NCS-Chrom. Control reaction mixtures contained no drug but received an equal volume of methanol (final maximum, 10%).

Analysis of Cleavage by Sequencing Gel Electrophoresis. Analysis of total strand breakage required treatment with alkali. Aliquots of each reaction mixture were taken in duplicate and lyophilized to dryness. One sample was then treated with 1 M piperdine at 90 °C for 30 min, lyophilized, and then washed with 10 μL of water and relyophilized three times. The residue from each sample was resuspended in 80% formamide loading buffer containing marker dyes and electrophoresed overnight on a 20% polyacrylamide gel with Maxam–Gilbert sequencing markers. Gel band intensities were quantitated by scanning an autoradiogram with an LKB Ultroscan laser densitometer.

Reaction of ^{2}H-Labeled Oligodeoxynucleotides with NCS-Chrom. A standard reaction mixture (1.6–3.0 mL) contained 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM GSH, 50 (or 93) μM NCS-Chrom, and 1.0 (or 1.86) mM DNA phosphate (thiol to drug ratio was either 20 or 12). To ensure the presence of duplex DNA, the oligomer, in a 2× reaction buffer (50% total reaction volume), was kept at 80 °C for 2 min and allowed to cool slowly to room temperature. The amount of water required to make up the final volume was added, and the mixture was chilled to 0 °C on ice before the addition of GSH and the chromophore. After incubation at 0 °C for 90 min in the dark, the mixture was frozen and lyophilized to dryness overnight.

Isolation and Characterization of the Reacted Drug Product. The residue from the drug reaction was redissolved in a small amount of water and purified by reverse-phase HPLC on a Rainin Microsorb C_{18} (5 μm, 1.0 × 25 cm) column. The column was first eluted 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 at a flow rate of 3 mL/min. Fractions (3 mL) were collected and followed by fluorescence (excitation at 340 nm/emission at >418 nm). The fractions corresponding to the major fluorescent peak (55–56 min) were pooled, frozen, and lyophilized overnight after removal of methanol in vacuo. ^{1}H

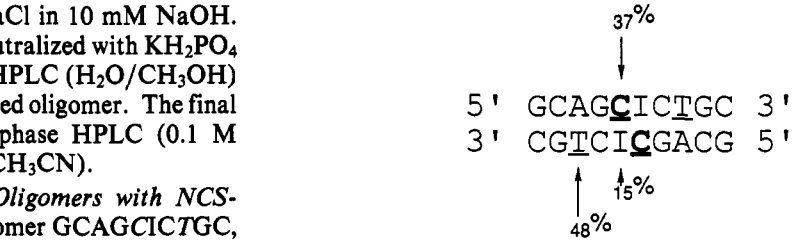


FIGURE 1: Positions and relative proportions of cleavage of self-complementary deuterium-labeled decamer **5** (150 μM) by NCS-Chrom (**1**, 7.5 μM) in the presence of 1 mM GSH at 0 °C in 25 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Percentages were obtained by scanning densitometry of sequencing gels.

NMR spectra were recorded on a Varian VXR 500S spectrometer in CD_3OD/CD_3CO_2D, 10:1 (vol/vol), at 25 °C. ^{1}H NMR data were acquired using 40–45° pulses with a repetition time of 3.277 s. Using these parameters, it was estimated that the steady-state magnetization of the system was nearly completely relaxed at the beginning of each transient. The extent of deuterium transfer into C-2 was determined by integrating the signal for H-2 against a value representative of one proton derived from the mean of the remaining signals in Figure 2 (excluding H-6). All other protons, including H-6, showed the expected independence from DNA deuteriation. Relative intensity comparisons were also made between the deuterium-labeled and unlabeled (control) NCS-Chrom products.

RESULTS AND DISCUSSION

The oligonucleotide chosen for this study was the self-complementary decamer GCAG*C*IC*T*GC (**5**), which was specifically labeled with deuterium at the C-1′ position of the *C* residue of AG*C*. The deuterium isotope effect (k_H/k_D; Kozarich et al., 1989) associated with NCS-Chrom-induced abasic site formation at the *C* residue of C-1′ deuterium-labeled AGC·GCT-containing oligonucleotides has been shown to range from 3.5 to 4.0, depending on the sequence of oligonucleotide (Kappen et al., 1990). However, substitution of the G residue opposite the *C* residue with an I residue virtually eliminates the deuterium isotope effect on abasic site formation (k_H/k_D = 1.24) and enhances attack (4-fold) at the *C* residue of AG*C* in decamer **5** [as found earlier for a different AGC-containing oligonucleotide (Kappen et al., 1988, 1990)], as determined from alkali-induced cleavage patterns on DNA sequencing gels (data not shown). Therefore, we were assured that labeling of the C-1′ position of the *C* residue with deuterium in decamer **5** would not significantly interfere with the transfer of deuterium from the *C* residue into the drug. The loss of a deuterium isotope effect upon substitution of an I residue for a G residue is explainable if enhanced abasic site formation is caused by an increase in the relative rate of hydrogen atom abstraction by the activated drug versus its dissociation from the DNA (Kappen et al., 1990).

In order to assess the ability of the deuterium-labeled AG*C*IC*T*-containing decamer **5** to act as a damage substrate in the reaction with NCS-Chrom, the oligomer was 5′-end-labeled with ^{32}P and incubated with NCS-Chrom in the presence of glutathione. Scanning densitometry of sequencing gels provided the relative proportions of the cleavage products after piperidine treatment, as shown in Figure 1. Cleavage at the deuterium-labeled *C* residue amounted to 37% of the observed products, while cleavage at the *T* residue amounted to 48% of the observed cleavage products. Since every abasic

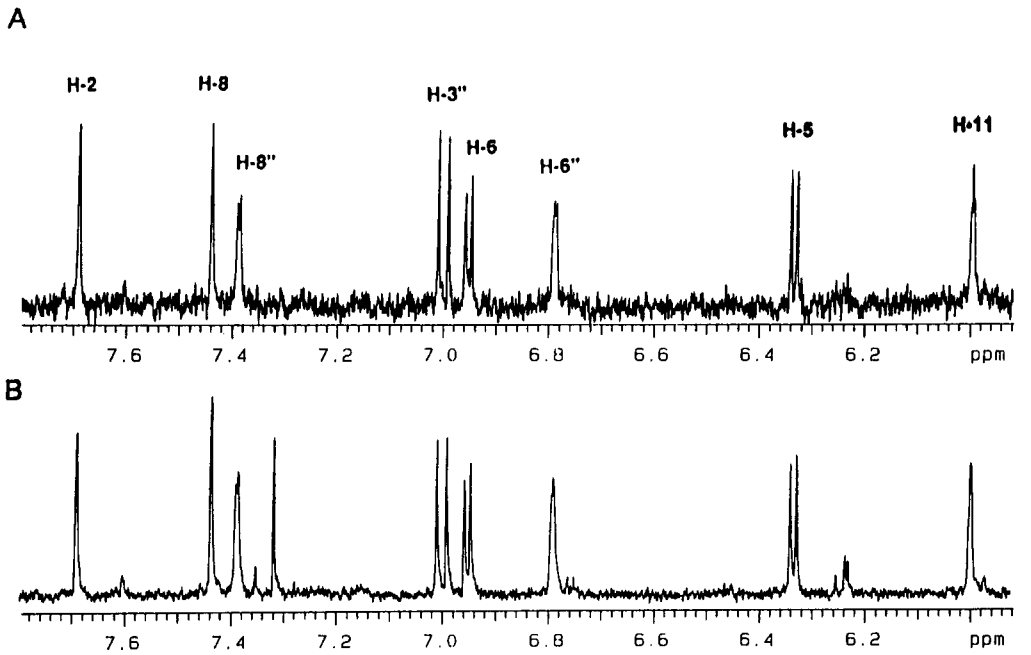


FIGURE 2: Partial 500-MHz ^{1}H NMR spectra of NCS-Chrom product (**4**) isolated from a reaction mixture containing 1 mM GSH (thiol/drug = 12) with unlabeled (A) and deuterium-labeled (B) decamer **5**. The unidentified peak at 7.32 in spectrum B is a contaminant resulting from HPLC purification, seen only occasionally with both deuterium-labeled and unlabeled samples.

site at the *C* residue is accompanied by a direct strand break at the *T* residue on the opposite strand (Povirk et al., 1988) (or in this case, the same strand due to the self-complementary nature of the decamer), we can assume that, of the 48% that represents cleavage at the *T* residue, 37% is part of the AG*C*ICT bistranded lesion. Therefore, 74% of the cleavage of decamer **5** by the activated drug occurs at the AG*C*ICT bistranded attack site, presumably by a single molecule of NCS-Chrom. In addition to the 11% which represents single-stranded cleavage at the *T* residue, there is also a small amount of cleavage (15%) at the I residue.

Once it had been established that the AG*C*ICT trinucleotide sequence in decamer **5** was a favorable bistranded attack site, the transfer of deuterium from the C-1′ position of the *C* residue into the putative diradical form of the drug (**3**) was followed by ^{1}H NMR. Both deuterium-labeled and unlabeled decamer **5** (1 mM in DNA phosphate) were treated with NCS-Chrom (50 μM) and GSH (1 mM) in parallel incubations, and the reacted drug products were isolated by HPLC. The ^{1}H NMR spectra of both products (**4**) are shown in Figure 2.

That deuterium was *selectively abstracted* from the C-1′ position of the *C* residue by the C-2 radical center of the drug is evident for two reasons. First, there is a decrease in the intensity of the H-2 resonance in the spectrum of the drug product isolated from the reaction with the labeled decamer **5** (spectrum B, Figure 2) relative to the H-2 resonance in the spectrum of the drug product isolated from the reaction with unlabeled decamer **5** (spectrum A, Figure 2), indicating the presence of deuterium at the C-2 position. Second, the fact that the H-5 resonance in spectrum B remains as a sharp doublet indicates the absence of deuterium at the C-6 position of the drug.

Integration of the relative peak intensities revealed the extent of transfer of deuterium into the C-2 radical center to be ~22% (average of six separate experiments, error in measurement of <5%). Several explanations exist for the incomplete transfer. It was found that when unlabeled decamer **5** is treated as above, but in fully deuteriated medium (10% CD_3OD in D_2O; thiol to drug ratio of 20), ~35% deuterium is incorporated into the C-2 position, but none into C-6, of the drug (average of four separate experiments, error in measurement <5%). Interestingly, it appears that the C-6 position is selectively shielded from the solvent by DNA, since in its absence (in 100% CD_3OD) deuterium incorporation into the drug from solvent virtually obliterates *both* C-6 and C-2 peaks. The selective quenching of the radical at C-2 by solvent may be quantitatively important in determining the excess of single-stranded over double-stranded lesions generated by NCS-Chron (Dedon & Goldberg, 1992). The sulfhydryl hydrogen has been shown to quench the radical centers of the thiol-activated chromophore at high ratios of thiol to drug in the absence of DNA in organic solvent (Myers et al., 1988; Myers & Proteau, 1989). Lowering of the thiol to drug ratio to as low as 6, however, had little, if any, effect on the incorporation of deuterium from C-1′ of DNA into C-2. Further, since earlier studies using similar conditions showed that little, if any, deuterium was incorporated into either position from exchangeable hydrogen of the solvent (Hensens et al., 1983; Chin et al., 1988; Hensens & Goldberg, 1989), it seems likely that the ~35% incorporation into C-2 noted above results mainly from abstraction of the carbon-bound deuterium of CD_3OD.

In addition to solvent as a source of the hydrogen incorporated in the C-2 position of the drug, a recent finding has shown that at relatively low thiol to drug ratios, hydrogen atom is abstracted from the carbon α to the sulfur of a drug bound thiol into the C-2 position of the drug, both in the presence and in the absence of DNA (Chin & Goldberg, 1992). A similar internal transfer has been found for a monocyclic NCS analogue (Wender & Tebbe, 1991). Further, a deuterium-isotope effect on the formation of the abasic lesion at the C of AGC occurs with sodium [2-$^2H_2$]thioglycolate, also indicative of internal quenching of the C-2 radical center of the activated chromophore by the hydrogen atoms of the thiol side chain (McAfee & Ashley, 1992). The extent of deuterium transfer into the C-2 radical center of NCS-Chrom from the carbon α to the adducted thiol was shown to range from 20 to 30% (Chin & Goldberg, 1992). Therefore, it is likely that, under the conditions used for the deuterium transfer

studies described here, some of the hydrogen incorporated into the C-2 position of the drug is a result of intramolecular quenching of the radical by the thiol at C-12. Thus, concerning the question of incomplete transfer of deuterium from decamer **5** into NCS-Chrom, solvent hydrogen (probably mainly carbon-bound) and the hydrogen on the carbon α to the C-12 bound thiol could account for as much as 65% of the hydrogen abstracted by the C-2 radical of the activated drug. The remaining hydrogen probably originates from some other, as of yet unidentified, component in the reaction mixture.

In conclusion, we have demonstrated that the C-1′ hydrogen atom of the *C* residue in the AG*C*·IC*T* bistranded attack site is selectively abstracted by the C-2 radical center of the glutathione-activated NCS-Chrom. These results provide additional support for the proposed structure of the activated-drug/DNA complex at the AG*C*·GC*T* bistranded attack site and argue against other possible energetically allowable binding modes (Meschwitz & Goldberg, 1991; Galat & Goldberg, 1990). In addition, this study confirms the hypothesis that the two radical centers at C-2 and C-6 of a single NSC-Chrom molecule act, at least macroscopically, in a concerted fashion to produce double-stranded lesions.

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SUPPLEMENTARY MATERIAL AVAILABLE

Experimental details of the synthesis and ^{1}H and ^{13}C NMR spectra of deuterated arabinose and N^4-benzoyl-2′-deoxy-[1′-^{2}H]cytidine (10 pages). Ordering information is given on any current masthead page. A copy of this material will be provided by G.W.A. upon request.

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