Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6706–6710, August 1992
Biochemistry

Neocarzinostatin acts as a sensitive probe of DNA microheterogeneity: Switching of chemistry from C-1′ to C-4′ by a G·T mismatch 5′ to the site of DNA damage

(sequence specificity/bistranded oxidative damage/G·A mismatch/misonidazole/hydrazine reaction)

LIZZY S. KAPPEN AND IRVING H. GOLDBERG*

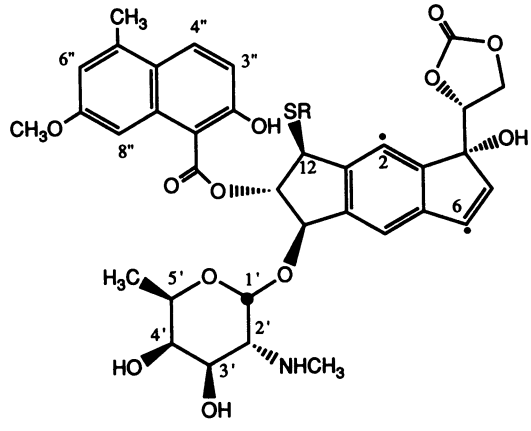
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Communicated by Daniel Nathans, April 23, 1992 (received for review March 5, 1992)

ABSTRACT **The diradical form of thiol-activated neocarzinostatin chromophore resides in the minor groove of DNA, where it has access to hydrogen atoms at the C-5′, C-1′, and C-4′ positions of deoxyribose on each strand. In a dodecamer oligodeoxyribonucleotide containing the sequence AGC·GCT, a bistranded lesion staggered two nucleotides in the 3′ direction, is generated that consists primarily of an abasic site (2′-deoxyribonolactone) at the C due to 1′ chemistry and a direct strand break at the T due to 5′ chemistry. Sequencing-gel analysis reveals that 72% of the damage at the C results from 1′ chemistry with minor lesions consisting of a strand break due to 5′ chemistry (15%) and 4′ chemistry (<2%) and an abasic site (4′-hydroxylation product) (12%) due to 4′ chemistry. Replacement of the G·C base pair 5′ to the C by a G·T wobble mismatch results in a remarkable switching of the chemistry of damage at the C from C-1′ to C-4′. The 1′ chemistry is almost eliminated and replaced by 4′ chemistry, so that the latter accounts for 64% of the damage, mainly in the form of the 4′-hydroxylation product (abasic site) and a smaller amount of the DNA fragment with a phosphoglycolate at the 3′ end (strand break). Substitution of the radiation sensitizer misonidazole for dioxygen markedly enhances partitioning of the 4′ chemistry in favor of the glycolate-containing product. On the complementary strand the G·T mismatch results in an increase in 4′ chemistry at the T residue, but 5′ chemistry remains the main mechanism. When a G·A mismatch is inserted 5′ to the C, there is a marked decrease in all damage at this site without detectable switching of chemistry. These results show that the diradical form of thiol-activated neocarzinostatin chromophore acts as sensitive probe of DNA microheterogeneity.**

The enediyne-containing chromophore of the antitumor antibiotic neocarzinostatin (NCS-Chrom) undergoes activation by thiol adduction at C-12 to generate a diradical species with radical centers at C-2 and C-6 (structure 1; for review, see ref. 1). The indacene diradical resides in the minor groove of duplex DNA, positioned there by intercalative binding of the naphthoate moiety and electrostatic and other interactions of the amino sugar. Sequence-dependent bistranded lesions consisting of strand breaks and abasic sites are formed predominantly at AGC·GCT and AGT·ACT sequences, due to the abstraction of hydrogen atoms by presumably a single drug molecule from the deoxyribose of each of the DNA strands. This involves mainly 1′ chemistry at the C of AGC, 4′ chemistry at the T of AGT, and 5′ chemistry at the other T residues on the complementary strands (Scheme I).

In addition to sequence-dependent DNA microstructure, other factors also influence the composition of the DNA damage products generated. The structure and reducing potential of the activating thiol have been found to affect the ratio of single-stranded to double-stranded lesions (2, 3) and to alter the partitioning of the products of 4′ chemistry between breaks with 3′-phosphoglycolate ends and 4′-hydroxylated abasic sites (4–6). Further, the partitioning of the chemistry between the 4′ and 5′ carbons also varies with thiol structure in single-stranded (not double-stranded) lesions at AGT·ACT sequences (5, 6). Finally, it is possible to alter the kinetics and extent of 4′ or 5′ chemistry at the T of AGT·ACT by placing deuterium at either the 4′ or 5′ positions (5). Shuttling of the hydrogen atom abstraction reaction between the two positions can be explained by isotope selection effects.



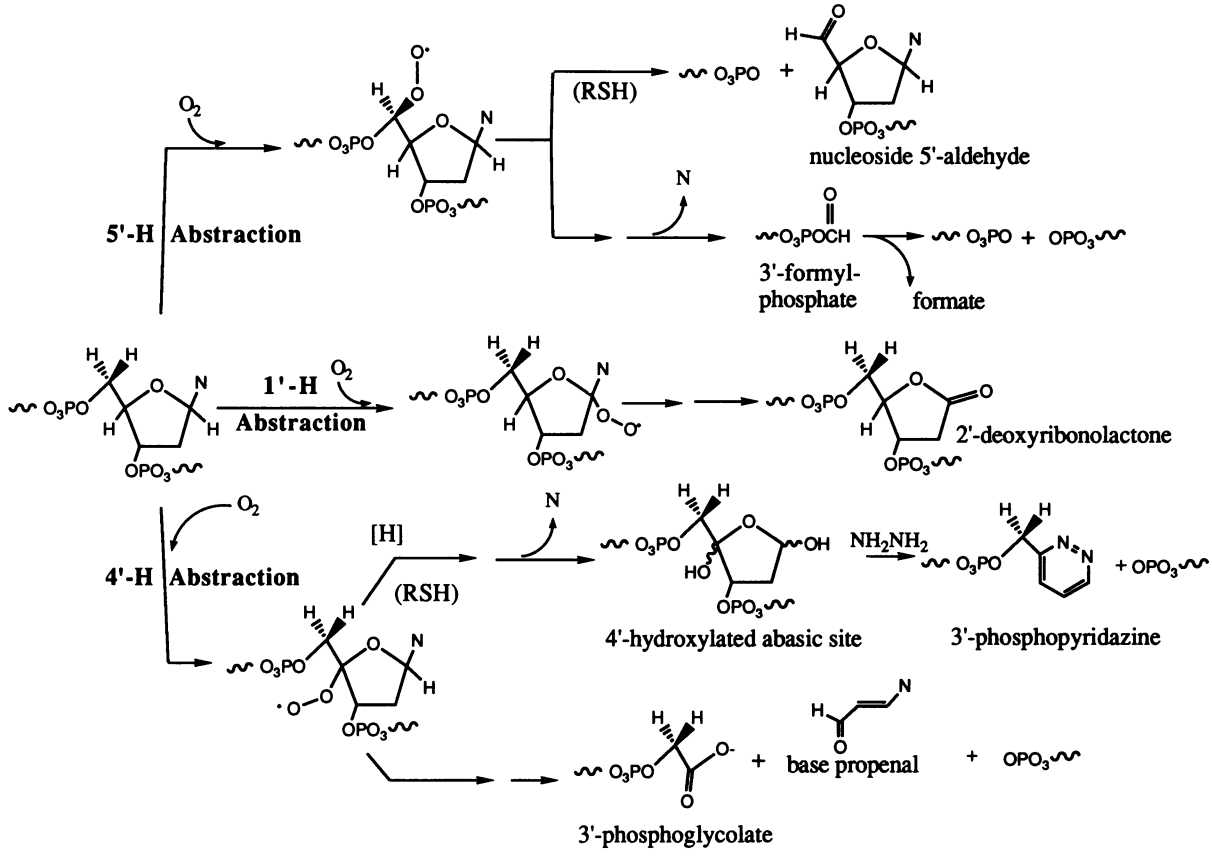
Structure 1

Even minor DNA base changes, such as elimination of the 2-amino group of the guanine residue, result in significant quantitative alterations in the extent of the lesions at the expected damage sites (7). Further, global effects have also been observed, since sequence differences some distance from the attack site influence the extent of the DNA damage reaction at a particular site and the size of isotope selection effect associated with the hydrogen abstraction reaction (4). These results have led to a model of active drug–DNA interaction in which the precise chemistry of DNA damage generated by NCS-Chrom is determined to a considerable degree by the geometry of the DNA at the site of interaction (1). We now report a striking example of this phenomenon wherein a G·T mismatch immediately 5′ to the C of AGC induces switching of the chemical mechanism from mainly 1′ chemistry to predominantly 4′ chemistry at the C residue. In addition, at the T on the complementary strand, 4′ chemistry increases at the expense of 5′ chemistry. These findings

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: NCS-Chrom, neocarzinostatin chromophore.

*To whom reprint requests should be addressed.



Scheme I

indicate that NCS-Chrom acts as a sensitive probe of DNA microheterogeneity.

MATERIALS AND METHODS

Neocarzinostatin and misonidazole were obtained from Kayaku Antibiotics (Tokyo) and Hoffman–La Roche, respectively. Oligodeoxyribonucleotides were purchased from Oligos Etc. (Guilford, CT) or Chem Genes (Waltham, MA). Oligomers were 5'-end-labeled with ^{32}P and purified by electrophoresis on a 20% polyacrylamide gel containing 8.3 M urea (5).

Drug Reaction. NCS-Chrom was extracted from the holoantibiotic by methanol in the presence of sodium citrate (8). ^{32}P-labeled positive strands were first annealed to the negative strands (1:2 ratio) by heating in the 2× reaction buffer (see below) at 90°C for 2 min and slow cooling. After addition of glutathione and H_2O required to make up the final reaction volume, the mixture was chilled in ice before adding the drug. A control containing no drug received an equal volume of methanol (maximum final concentration, 10%). A standard reaction mixture (30–90 μl) contained 22 mM Tris·HCl (pH 8.0), 1 mM EDTA, 248 μM (phosphate) ^{32}P-labeled positive strand annealed with twice as much negative strand, 3 mM glutathione, and 31 μM NCS-Chrom. The reaction was allowed to proceed in the dark in ice for 30 min. Reactions under anaerobic conditions were performed in a vessel containing a side arm, as described (8). All components except thiol were placed in the main reaction chamber. After removal of O_2, the reaction was started by addition of thiol (final concentration, 2 mM) from the side arm. When misonidazole (20 mM) was present, it was placed in the main chamber from the start.

Alkali or Hydrazine Treatment. Duplicate aliquots of the drug reaction mixture (8–20 μl) were dried in a Speed-Vac concentrator. One of the resulting pellets was heated in 100 μl of 1 M piperidine (90°C, 30 min). After drying, the sample was dissolved in 15 μl of H_2O and dried. H_2O addition and drying were repeated three times. A third aliquot of the reaction mixture was directly treated with hydrazine hydrochloride at pH 8.0 (50 mM) for 2 hr at room temperature and dried.

Product Analysis. The dried sample pellets were redissolved in 80% (vol/vol) formamide containing 1 mM EDTA and marker dyes and electrophoresed on a 20% urea-containing polyacrylamide gel with the gel maintained at room temperature. The gel band intensities were quantitated by scanning the autoradiograms (preflashed and lightly exposed) using an LKB Ultroscan Laser densitometer.

RESULTS

The first bistranded lesion generated by NCS-Chrom to be described consists of an abasic site at the C of AGC·GCT and a strand break at the T on the complementary strand (7, 9, 10). This bistranded lesion, staggered by 2 nucleotides in the 3' direction, is produced mainly by abstraction of a hydrogen atom from C-5' of the T residue by the carbon-centered radical at C-6 of activated NCS-Chrom (11) with the formation of a strand break having a 3'-phosphate and a 5'-thymidine 5'-aldehyde and by abstraction of a hydrogen atom from C-1' of the C residue by the radical at C-2 (refs. 7 and 12; S. M. Meschwitz, R. G. Schultz, G. W. Ashley, and I.H.G., unpublished results) to generate an abasic site consisting of a 2'-deoxyribonolactone moiety (13). This reaction is oxygen-dependent and involves peroxyl radical intermediates of DNA deoxyribose (Scheme I). Virtually all of the abasic lesions at the C residue (10), but only a portion of the breaks at the T residue (7, 11), are involved in the bistranded lesions. The abasic lesion at the C with its complementary

break at the T in AGC·GCT has been found to be the predominant premutagenic lesion causing GC → AT transitions in λ phage DNA treated with NCS-Chrom (14).

To determine the influence of local structural changes on the chemistry of DNA damage at the C of AGC, duplex oligodeoxynucleotides containing the stable mismatches G·T and G·A 5′ to the attack site were tested as substrates for glutathione-activated NCS-Chrom (Fig. 1). Treatment of the dodecamer d(CCAGC[5]GAGCG[10]CG·CGC[15]GCTCG[20]-CTGG), 5′-end-labeled with ^{32}P on the positive strand, with NCS-Chrom results in the formation of piperidine-sensitive breaks at C^5 and C^9 (compare Fig. 1, lanes 3 and 4). A much slower moving band (asterisk, lane 3) disappears upon piperidine treatment, presumably having been converted to material containing a 3′-phosphate end with a mobility identical to that of the chemically produced marker. The slow-moving band represents the portion of the abasic 2′-deoxyribonolactone-containing product that underwent a single β-elimination on its 3′ side to form a DNA fragment with a 3′ sugar moiety (15). That this product results from 1′ chemistry was shown by the earlier finding of a substantial isotope selection effect on its formation when deuterium was substituted at the 1′ position (L.S.K., I.H.G., J. W. Kozarich, and J. Stubbe, unpublished data). Most of the abasic sites, however, are in full-length molecules with intact phosphodiester linkages (7). In both lanes 3 and 4, very faint bands (arrow) can be seen that move slightly faster than the bands containing a 3′-phosphate end. In earlier experiments, this mobility has been found to be characteristic of a DNA fragment with a 3′-phosphoglycolate end ("glycolate") resulting from 4′ chemistry (5, 16, 17). To determine the extent of the putative 4′ chemistry, the drug reaction products were treated with hydrazine, which is known to react with the 4′-hydroxylated abasic site generated by bleomycin (18, 19) and NCS-Chrom (5, 6, 20) to form a 3′-pyridazine derivative ("pyridazine") with elimination of the DNA fragment with a 3′-phosphate end (Scheme I). The pyridazine derivative (dashed arrow) migrates more slowly than the fragment with the 3′-phosphate end (Fig. 1, lane 5). The presence of glycolate bands and pyridazine bands (after hydrazine treatment) indicates that the lesions at C^5 and C^9 include products

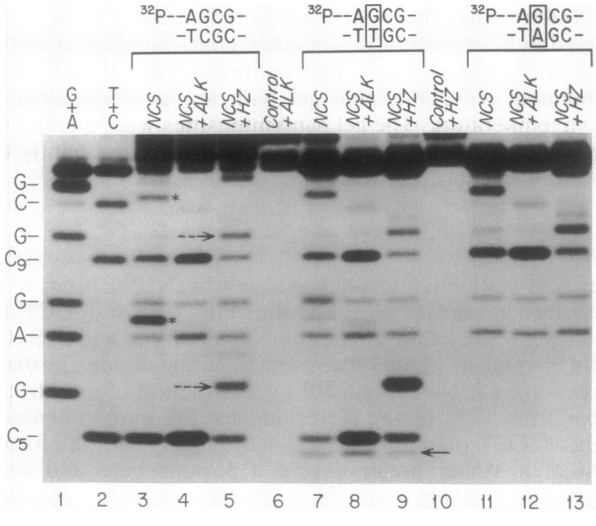


FIG. 1. Effect of G·T or G·A mismatches on NCS-Chrom-induced lesions at the C of AGC. 5′-^{32}P-labeled positive strand (CCAGC[5]GAGC[9]GCG), either as a perfectly matched duplex or as one having a mispaired base on the negative strand 5′ to the C^5, was treated with NCS-Chrom (NCS) and then with alkali (ALK) or hydrazine (HZ). G+A and T+C represent Maxam and Gilbert markers. Lanes 6 and 10 are no-drug controls. Arrow, glycolate derivative; dashed arrow, pyridazine derivative; *, partial elimination product of C-1′ attack.

Table 1. Distribution of NCS-Chrom-induced chemistry at C in AGC[5] with and without a G·T mismatch

Substrate	% of total damage 5′	4′	1′
[^{32}P]AGC- ::: -TCG-	15	13	72
[^{32}P]AGC- ::: -TTG-	28	64	8

Data are derived from gel analysis of NCS-Chrom-treated oligomer duplexes as in Fig. 1. 5′ chemistry is measured as the intensity of the PO_4 band in the absence of alkali. The intensity of PO_4 after alkali treatment includes breaks due to 5′ chemistry and those generated from abasic lesions resulting from 4′ and 1′ attack. This, combined with any glycolate (the second 4′ attack product), accounts for the total damage at C^5. Pyridazine plus glycolate represent total 4′ chemistry. The difference between the total and the sum of the 4′ and 5′ chemistry is taken as the 1′ damage. Values are the mean from three experiments.

due to 4′ chemistry. Scanning of lighter exposures of the gel in Fig. 1 shows that ≈13% of the chemistry at C^5 and C^9 involve 4′ chemistry, mainly as the 4′-hydroxylated abasic site; the main damage (72%) is the 2′-deoxyribonolactone-containing abasic site resulting from 1′ chemistry (Table 1). The remainder (15%) of the damage resulting in direct strand breaks is presumably due to 5′ chemistry; this analysis does not distinguish the 5′-nucleoside 5′-aldehyde from the 3′-formylphosphate pathway of 5′ chemistry (see Scheme I and ref. 1). These results are consistent with an earlier effort to relate cytosine base release at an AGC with 2′-deoxyribonolactone formation; in this analysis the latter moiety accounted for at least 60% of the base release (13).

When a G·T mismatch was present 5′ to the C of AGC at C^5, there was some decrease in the total damage at the C residue (≈33%) and this was accompanied by a marked increase in glycolate and 4′-hydroxylation abasic site formation (pyridazine) (Fig. 1, lanes 7–9, and Table 1). All but 11% (8% of the total lesion) of the piperidine-sensitive abasic site can be accounted for as 4′ chemistry (Table 1). Sixty-four percent of the total lesion is due to 4′ chemistry; the remainder is almost entirely due to 5′ chemistry, which increases to 28%. Consistent with this finding is the substantial decrease in the asterisk-marked band representing the partial elimination product of the 2′-deoxyribonolactone-containing lesion (compare Fig. 1, lanes 3 and 7). The lesion at C^9, which lacks the G·T mismatch, is unchanged (Fig. 1); the lesion with the G·T mismatch (data not shown) is similar to that at C^5, when it possesses the mismatch. The asterisk-marked bands at C^9 also behave in the expected manner. The decrease in the glycolate band upon hydrazine treatment (5, 6) has been attributed to the reduction of a glycolate precursor and its conversion to the 4′-hydroxylation product (6).

The placement of a G·A mismatch at the same position 5′ to C^5 results in almost total elimination of all reaction at the C^5 site but not at the other site C^9 (lanes 11–13). In fact, the total reaction, without switching of chemistry, appears to be increased at C^9, probably because more NCS-Chrom is available for binding at this site.

In studies of the 4′ chemistry occurring at the T of AGT, it had been found that substitution of the radiation sensitizer misonidazole for dioxygen alters the partitioning of the 4′ chemistry so that the glycolate product increases at the expense of the pyridazine product (5, 6). By using this observation as a diagnostic test for the involvement of 4′ chemistry, the appropriate products were characterized after a drug reaction conducted under anaerobic conditions in the presence of misonidazole. As shown in Fig. 2, the glycolate band is increased in all reactions involving the C residue of

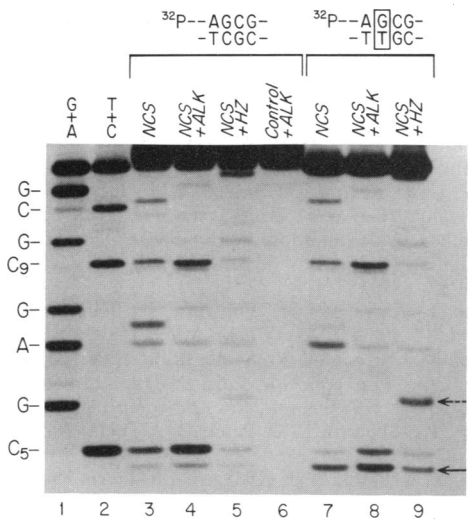


FIG. 2. Effect of misonidazole on the distribution of NCS-Chrom-induced lesions at the C of AGC in the presence and absence of a G·T mismatch. Reactions similar to those in Fig. 1 were performed under anaerobic conditions in the presence of misonidazole (20 mM). In reactions with NCS-Chrom in the absence of misonidazole, there was virtually no damage at any site (data not shown). Arrow, glycolate derivative; dashed arrow, pyridazine derivative.

AGC, especially with the substrate containing the G·T mismatch, where it becomes the major product (Fig. 2, lane 8).

In view of the almost complete switching of DNA damage chemistry from C-1′ to C-4′ in association with the G·T mismatch 5′ to the lesion, it was of interest to study the staggered lesion on the complementary strand. In the absence of the G·T mismatch, direct strand breakage at T^{22}, represented by a DNA fragment with a 3′-phosphate end, is almost entirely due to 5′ chemistry; a trace of 4′ chemistry, as revealed by a very faint glycolate band and a weak pyridazine band, can be detected (Fig. 3, lanes 3–5). Damage due to 4′ chemistry at T^{22} increases significantly upon substitution of the G·T mismatch but remains much less than that generated by 5′ chemistry (lanes 7–9). It is interesting that T^{21} of the G·T mismatch is a barely detectable damage site. With the G·A mismatch, there was essentially no damage at T^{22}, similar to the finding on the complementary strand (data not shown).

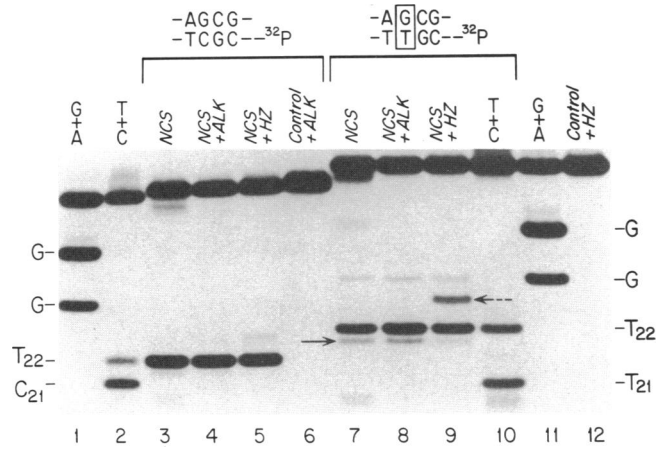


FIG. 3. NCS-Chrom-induced lesion on the complementary strands of the normal duplex and the G·T mispaired duplex. In experiments similar to those in Fig. 1, the negative strands of the duplexes have a 5′ ^{32}P label. Arrow, glycolate derivative; dashed arrow, pyridazine derivative.

DISCUSSION

A G·C → G·T or G·A pair substitution destabilizes the DNA helix much less than other base-pair mismatches (21). Whereas G·T mismatches are good substrates for repair, G·A mismatches are relatively poorly recognized by the repair systems (22). The structures of the G·T and G·A mismatches have been extensively studied using NMR (21, 23, 24) and single-crystal x-ray analysis (25–27). These mismatches result in little alteration in the conformation of the DNA backbone or in the global conformation of the double helix. Displacement of the guanine of the G·T and G·A base pairs into the minor groove results in a loss of the pseudosymmetry about the glycosyl bond that Watson–Crick base pairs have relative to a vector joining the C-1′ carbons of the two sugar residues. Of the two mismatches the G·T wobble pair is much more asymmetric, resulting in substantial changes in the DNA minor groove so that the quasiequivalence of certain functional elements, such as the hydrogen bond acceptors present on the purine N-3 and pyrimidine O^2 of the Watson–Crick base pair, is altered (25, 27). The largest perturbation in the rise per base pair also occurs at steps involving G·T mismatches (27).

How these changes in DNA microheterogeneity relate to the switching of chemistry (G·T mismatch) or lack of reactivity (G·A mismatch) by activated NCS-Chrom remains to be determined. The marked decrease in all damage 3′ to the G·A mismatch suggests that the drug fails to bind at this site, possibly due to alteration at the intercalation site. The projection of the guanine of G·T into the minor groove of DNA (24, 27), where the indacene diradical form of the drug abstracts hydrogen atoms from nearby C-1′, C-4′, and C-5′ deoxyribose sites, might be expected to modify the orientation of the radical centers vis-à-vis these sites. Since C-1′ lies deeper in the minor groove than C-4′, it is possible that protrusion of the guanine keeps the drug in a more superficial position in the minor groove so that it attacks C-1′ poorly but is better situated for reacting with C-4′. This may also account for the relative increase in chemistry at C-5′, whose prochiral H_S resides at the outer edge of the minor groove. In earlier computer-based modeling studies of the complex formed between the post-activated (glutathione) form of NCS-Chrom and AGC·GCT, in which the naphthoate intercalates between the A·T and G·C base pairs, it was found that the distances between the radical at C-2 of NCS-Chrom and the C-4′ and C-1′ of the C residue are 4.00 Å and 4.10 Å, respectively (28). Clearly factors other than merely the distance between the drug radical and the attack site are responsible for the predominantly C-1′ attack ordinarily found at this sequence. Upon substitution of a G·T or G·A mismatch 5′ to the lesioned nucleoside, new structural and possibly functional determinants have been introduced that must significantly affect the damage chemistry. As a result of the G·T mismatch attack at C-1′ almost disappears and that at C-4′ goes from being a minor lesion to becoming the major lesion. Preliminary experiments similar to those reported here but involving the bistranded lesions at the T residue of AGT·ACT show that G·T mismatches 5′ to the lesions cause shifts in damage chemistry (from C-5′ to C-4′), but not to the extent noted in this report.

These studies clearly demonstrate that NCS-Chrom is a sensitive probe of DNA microstructure. Several options exist for the abstraction of hydrogen atoms from minor groove accessible sites on the deoxyribose by the radical centers of activated drug. Relatively minor changes in DNA local geometry result in substantial changes in the pattern of hydrogen atom abstraction. The relation, if any, between the recognition elements for DNA repair and NCS-Chrom interaction merits further exploration.

This work was supported by U.S. Public Health Service Research Grant CA 44257 from the National Institutes of Health.

1. Goldberg, I. H. (1991) *Acc. Chem. Res.* **24,** 191–198.
2. Dedon, P. C. & Goldberg, I. H. (1990) *J. Biol. Chem.* **265,** 14713–14716.
3. Dedon, P. C. & Goldberg, I. H. (1992) *Biochemistry* **31,** 1909–1917.
4. Frank, B. L., Worth, L., Jr., Christner, D. F., Kozarich, J. W., Stubbe, J., Kappen, L. S. & Goldberg, I. H. (1991) *J. Am. Chem. Soc.* **113,** 2271–2275.
5. Kappen, L. S., Goldberg, I. H., Frank, B. L., Worth, L., Jr., Christner, D. F., Kozarich, J. W. & Stubbe, J. (1991) *Biochemistry* **30,** 2034–2042.
6. Dedon, P. C., Jiang, Z.-W. & Goldberg, I. H. (1992) *Biochemistry* **31,** 1917–1927.
7. Kappen, L. S., Chen, C.-Q. & Goldberg, I. H. (1988) *Biochemistry* **27,** 4331–4340.
8. Kappen, L. S. & Goldberg, I. H. (1985) *Nucleic Acids Res.* **13,** 1637–1648.
9. Povirk, L. F. & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3182–3186.
10. Povirk, L. F., Houlgrave, C. W. & Han, Y. (1988) *J. Biol. Chem.* **263,** 19263–19266.
11. Meschwitz, S. M. & Goldberg, I. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3047–3051.
12. Kappen, L. S., Goldberg, I. H., Wu, S. H., Stubbe, J., Worth, L. & Kozarich, J. W. (1990) *J. Am. Chem. Soc.* **112,** 2797–2798.
13. Kappen, L. S. & Goldberg, I. H. (1989) *Biochemistry* **28,** 1027–1032.
14. Povirk, L. F. & Goldberg, I. H. (1986) *Nucleic Acids Res.* **14,** 1417–1426.
15. Kuwabara, M., Yoon, C., Goyne, T., Thederahn, T. & Sigman, D. S. (1986) *Biochemistry* **25,** 7401–7408.
16. Takeshita, M., Grollman, A. P., Ohtsubo, E. & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5983–5987.
17. Giloni, L., Takeshita, M., Johnson, F., Iden, C. & Grollman, A. P. (1981) *J. Biol. Chem.* **256,** 8608–8615.
18. Sugiyama, H., Xu, C., Murugesan, N., Hecht, S. M., van der Marel, G. A. & van Boom, J. H. (1988) *Biochemistry* **27,** 58–67.
19. Steighner, R. J. & Povirk, L. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8350–8354.
20. Saito, I., Kawabata, H., Fujiwara, T., Sugiyama, H. & Matsuura, T. (1989) *J. Am. Chem. Soc.* **111,** 8302–8303.
21. Patel, D. J., Kozglowski, S. A., Ikuta, S. & Itakura, K. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 2663–2670.
22. Modrich, P. (1987) *Annu. Rev. Biochem.* **56,** 435–466.
23. Hare, D., Shapiro, L. & Patel, D. J. (1986) *Biochemistry* **25,** 7445–7456.
24. Gao, X. & Patel, D. J. (1988) *J. Am. Chem. Soc.* **110,** 5178–5182.
25. Kneale, G., Brown, T., Kennard, O. & Rabinovich, D. (1985) *J. Mol. Biol.* **186,** 805–814.
26. Brown, T., Hunter, W. N., Kneale, G. & Kennard, O. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2402–2406.
27. Hunter, W. N., Brown, T., Kneale, G., Anand, N. N., Rabinovich, D. & Kennard, O. (1987) *J. Biol. Chem.* **262,** 9962–9970.
28. Galat, A. & Goldberg, I. H. (1990) *Nucleic Acids Res.* **18,** 2093–2099.