Sequence-specific Double-strand Breakage of DNA by Neocarzinostatin Involves Different Chemical Mechanisms within a Staggered Cleavage Site\*  
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Direct double-strand breaks in DNA have been implicated in cellular lethality of the antitumor antibiotic neocarzinostatin, but the mechanism of their formation has not been elucidated. Evidence is presented that neocarzinostatin causes sequence-specific direct double-strand breaks whose formation is strongly influenced by the activating thiol. Seven-fold more double-strand breaks result when glutathione rather than 2-mercaptoethanol is used to activate the drug to its putative diradical form, while the sequence specificity of cleavage remains the same. These data explain ear-lier inconsistencies in the ratios of double-strand to single-strand breaks obtained from in vitro and in vivo studies. Double-strand cleavage sites, occurring predominantly at GT steps, especially AGT-AGT, consist of trinucleotide sequences with a two-nucleotide 3'-stagger of the cleaved residues. The chemical structures of the cleavage sites suggest a model in which a neocarzinostatin-induced double-strand break results from abstraction of a C5' hydrogen atom from the T of ACT and the C4' hydrogen atom of the T of AGT by a single molecule of the diradical form of the drug. Single-strand breaks at these sites occur as separate events with attack at the C5' hydrogens. These findings permit the generalization that single-strand breaks produced by neocarzinostatin show a base preference but no clear sequence specificity, while bi-stranded lesions are sequence-specific in nature.

Although double-strand (ds)\(^1\) breaks produced in cellular DNA by the antitumor antibiotic neocarzinostatin appear to be responsible for its lethality for cells (1), the mechanism of their formation has remained unclear, and no evidence has been offered that ds breaks are sequence dependent (2). Single-strand (ss) breaks, the predominant lesions produced by the neocarzinostatin chromophore (NCS), arise by the abstraction of the C5' hydrogen atom from the deoxyribose  

sugar of mainly T and A residues by a putative thiol-activated diradical form of the drug bound in the minor groove of DNA by intercalation of the attached naphthoate moiety (see Refs. 3 and 4 for review and structures (5)). The damage at ss breaks consists of a phosphate at the 3'-end and a nucleoside 5'-aldehyde at the 5'-end of the cleaved fragments (6). There appears to be a base preference but no clear-cut sequence specificity for the ss breaks (7, 8). In the absence of evidence of sequence specificity, NCS-induced ds breaks have been attributed to the random coincidence of nonspecific ss breaks at closely opposed T and A residues (1, 9, 10). Bistranded lesions consisting of a direct break on one strand and an abasic lesion on the other, however, are sequence-specific (predominantly at AGC-GCT) and appear to result from the action of a single molecule of NCS (11-13). It seems reasonable, therefore, to expect that the diradical form of NCS might also produce direct ds breaks dependent on particular sequences. Based on this expectation we have undertaken a study of the ds breaks produced by NCS in the plasmids pUC13 and pBR322.

EXPERIMENTAL PROCEDURES

Materials—NCS chromophore was isolated from the bolaobiotic (Kayaku Antibiotics) as described (14) and stored at -70 °C in methanol at a stock concentration of 412 μM. Plasmid pUC13 (Boeh-inger Mannheim) was purified from Escherichia coli (strain HB101) by standard procedures (15). Plasmid pBR322 was >90% supercoiled. Quantitation of NCS-induced Strand Breaks in pBR322—A solution of pBR322 (80 μg/ml) containing 50 μM HEPES, 2 mM EDTA, pH 7.5, and either 1 or 5 μM glutathione (GSH) or 10 μM 2-mercaptoethanol (2-ME) was chilled to 0 °C and NCS added to start the reaction. After 5 min, DNA (0.2 μg) was resolved on a 1% agarose gel containing either TBE (Tris borate-EDTA) (15) or HAE (40 mM sodium acetate, 2 mM EDTA, pH 7.5) buffer. Den-sitometry of the negative images of the etched bromide-stained gel (LKB Ultrascan XL laser densitometer) showed a linear variation of signal with DNA concentration over the DNA range studied. An adjustment was made to the form I signal given its 70% fluorescence intensity compared with forms II and III (16).

Preparation and Labeling of Restriction Fragments—Restriction fragments were isolated from pUC13 and pBR322. Two fragments were used without purification: the 316-bp HindIII/TaqI fragment of pBR322 and the 459-bp HindIII/TaqI fragment of pUC13; the other end-labeled sites produced by digestion with the second enzyme were 10 bp or less in length and thus did not affect the results.

Drug Treatment of Restriction Fragments—DNA cleavage was initiated by adding NCS to 1 μM to a solution of 50 μM HEPES, 2 mM EDTA, 30 μg/ml calf thymus DNA, 1 mM GSH, pH 7.5, and end-labeled DNA (5-10 × 10⁶ cpm) at 0 °C. After 5 min, the reaction was terminated. Similarly, DNA was treated with 1 μM bleomycin A₅, 3 mM Fe(NH₄)₂(SO₄)₉, and 10 μM 2-ME in the absence of EDTA. NaBH₄ was employed to reduce the 5'-nucleoside aldehyde residues as described previously (6).

Purification and Analysis of ds Break Sites—Drug-treated DNA was resolved on nondenaturing 8% polyacrylamide gels (200 V, 23 °C). The resolved bands were cut from the gel by alignment of an autoradiogram. The DNA in each band was eluted by the crude and soak method (15) and resolved on 8 or 20% polyacrylamide sequencing gels (17). The band location was determined by comparison with Maxam-Gilbert sequencing standards (17).
Neocarzinostatin-induced Double-strand Breaks

RESULTS

Quantitation of ds and ss Breaks in NCS-treated pBR322—
The ratio of ds to ss breaks caused by NCS chromophore in 
pBR322 was determined by assuming that the cutting fol-
lowed a Poisson distribution, as employed for strand cleavage 
caused by bleomycin (18). Fig. 1 shows that the ratio of ds to 
ss cuts with GSH as the activator is 1.6 for both 1 and 5 mM 
GSH, while with 2-ME the ratio drops to 1:41.

Identification of ds Break Sites—An example of the electro-
phoretic analysis of ds cleavage sites is presented in Fig. 2 for 
HindIII/TaqI pBR322. NCS-mediated ds cleavage fragments 
were resolved on nondenaturing polyacrylamide gels (Fig. 2A). 
The bands, numbered in Fig. 2A, were excised and the identity 
of nucleotides at isolated break sites determined by sequenc-
ing gel analysis (Fig. 2B). Occasionally, extremely low inten-
sity bands representing ss cuts were apparent migrating ahead 
of the major DNA band. Fig. 3 shows the summary of the ds 
cleavage analysis; the cleavage site is indicated by an arrow. 
One of the most prominent features of this cleavage survey is 
the 3' stagger of 2-3 bp in the cleavage sites on each strand. 
The following list contains all sequences encountered at ds 
breaks along with the ratio of sequence copies cut to the total 
number of sequence copies present: AGT ACT, 18/18; ACA 
TGT, 10/19; AAA TAT, 3/17; AGC CTG, 2/3; AGA TA 
TCT, 3/6; ACC GGT, 3/12; TAT ATA, 1/11. The cleaved 
residues are underlined. The size of the arrows in Fig. 3 
represents the intensity of the autoradiographic signal from 
bands in the nondenaturing gels normalized to the largest 
signal for each strand. Thus, each arrow is proportional to 
the intensity of cutting at each site. The average (±S.D.) 
normalized cutting intensity at each of the above sites for all 
restriction fragments is as follows: 0.75 ± 0.25 (n = 36); 0.34 
± 0.23 (n = 20); 0.36 ± 0.26 (n = 6); 0.22 ± 0.14 (n = 4); 0.14 
± 0.06 (n = 6); 0.09 ± 0.04 (n = 6); 0.1 (n = 1), respectively. 
Sequences containing GT steps are especially good ds cleavage 
sites. The most frequent and most intensely cut site is AGT. 
ACT, for which a single direct ds break was noted by Povirk 
et al. (11) in a study of bistranded lesions involving abasic 
sites at C residues of AGC sequences. In spite of separate 
analyses for each strand, the intensity of cutting at each site 
is consistent between strands.

The sequence specificity and cutting intensity were com-
pared for GSH and 2-ME by treating 3'-32P-labeled HindIII/ 
TaqI pBR322 treated with NCS or methanol was resolved on a nondenaturing gel (see “Experimental 
Procedures”). The 3'-32P fragments are two nucleotides longer 
so run slightly slower than the 5'-32P counterparts. Lane 1 5'-32P-
labeled aX174 digested with HaeIII (size markers); lane 2, control 3'- 
32P-labeled DNA; lane 3, NCS-treated 3'-32P-labeled DNA; lane 4, 
control 5'-32P-labeled DNA; lane 5, NCS-treated 5'-32P-labeled DNA. 
A, DNA from bands excised from the 5'-32P-labeled DNA nondena-
turing gel were resolved on an 8% sequencing gel. Lanes numbered 
5-9 correspond to the indicated bands in the gel shown in A. The 
central four lanes are Maxam-Gilbert standards.

Characterization of the Chemistry of Cutting—To better 
characterize the chemistry at the 5'-ends of the ds breaks 
produced by NCS in the presence of GSH, 3'-end-labeled 
HindIII/TaqI pBR322 was treated with NCS, resolved on a 
nondenaturing gel, and the ds break fragment from an ACT 
site (band 9 from Fig. 2A) analyzed on an 8% sequencing gel. 
It is apparent from Fig. 4A that there is a band (lane 1) at 
the T residue of the ACT site that migrates 3-4 bp slower 
than the 5'-phosphate-ended DNA (band migrating with 
Maxam-Gilbert CT standard). This band was degraded to 6'- 
phosphate-ended material upon treatment with alkali (Fig. 
4A, lane 2), which is consistent with its identity as a nucleo-
site 5'-aldehyde (6). To prevent possible elimination of the

Fig. 2. Sequence determination at NCS-induced ds break 
sites. A, 3'- and 5'-32P-labeled HindIII/TaqI pBR322 treated with 
NCS or methanol was resolved on a nondenaturing gel (see “Experi-
mental Procedures”). The 3'-32P fragments are two nucleotides longer 
so run slightly slower than the 5'-32P counterparts. Lane 1 5'-32P-
labeled aX174 digested with HaeIII (size markers); lane 2, control 3'- 
32P-labeled DNA; lane 3, NCS-treated 3'-32P-labeled DNA; lane 4, 
control 5'-32P-labeled DNA; lane 5, NCS-treated 5'-32P-labeled DNA. 
B, DNA from bands excised from the 5'-32P-labeled DNA nondena-
turing gel were resolved on an 8% sequencing gel. Lanes numbered 
5-9 correspond to the indicated bands in the gel shown in A. The 
central four lanes are Maxam-Gilbert standards.

analyses for each strand, the intensity of cutting at each site 
is consistent between strands.

The sequence specificity and cutting intensity were com-
pared for GSH and 2-ME by treating 3'-32P-labeled HindIII/ 
BglII pUC13 with NCS in the presence of either thiol and 
resolving the DNA on nondenaturing (ds breaks) and se-
quencing gels (ss and ds breaks) (data not shown). The band 
patterns obtained from either type of gel analysis are the same 
for both thiols. Under conditions where ss breakage for the 
two thiols is equivalent, the intensity of ds breaks produced 
with GSH is 5-fold stronger on average than with 2-ME. The 
relative intensities of ss and ds cleavages from these gels, 
along with the strand break ratio of 41:1 determined for 2-
ME from Fig. 1, can be used to calculate a strand break ratio 
of 8:1 for GSH, which is in agreement with and provides 
independent verification of the ratio of 6:1 for GSH derived 
from Fig. 1.

Characterization of the Chemistry of Cutting—To better 
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HindIII/TaqI pBR322 was treated with NCS, resolved on a 
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site (band 9 from Fig. 2A) analyzed on an 8% sequencing gel. 
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site 5'-aldehyde (6). To prevent possible elimination of the

DS Breaks/Molecule

Fig. 1. The effect of thiol activator on NCS-induced ds and 
ss breaks. Supercooled pBR322 was treated with NCS activated by 
10 mM 2-ME (A), 1 mM GSH (B), or 5 mM GSH (C). The agarose 
gel-resolved topologic forms were quantitated (see "Experimental 
Procedures") and strand breakage assumed to follow a Poisson dis-
tribution for comparative purposes. Results are expressed as cleavage 
events per molecule of plasmid DNA.
Neocarzinostatin-induced Double-strand Breaks

FIG. 3. Summary of the ds cleavages produced by NCS. Consensus sequences for ds break sites were determined from gels as shown in Fig. 1. Cleavage sites in each strand are indicated by arrows, which are proportional in size to the intensity of cleavage at each site (see "Experimental Procedures"). Asterisks indicate AGT, ACT sites. Only the region of each restriction fragment analyzed for ds breaks is shown.

nucleoside aldehyde during work-up, which explains the reduced amount of aldehyde relative to phosphate in lane 1 of Fig. 4A, and thus to rigorously establish the ratio of aldehyde to phosphate-ended material, NCS cleavage products were reduced immediately with NaBH₄ after the drug reaction (Fig. 4A, lanes 3 and 4). Upon reduction, the fragment migrates slightly ahead of the aldehyde position, and its resistance to alkali further establishes the cleavage product as a nucleoside 5'-aldehyde (6). There is a predominance of the aldehyde residue relative to the phosphate species (80% versus 20% by densitometry of a lighter exposure). Similarly, nucleoside 5'-aldehyde was observed at all ACT sites studied. Only phosphate-ended material was observed at AGT sites in 3'-end-labeled ds break fragments (data not shown).

The chemistry at the 3'-end of the same cleavage site on the opposite strand (the AGT site) was assessed by analyzing the ds break fragment produced from 5'-end-labeled NCS-treated DNA on an 8% nondenaturing gel, and the isolated ds break fragment at the ACT site shown in Fig. 2A, lane 3 (band 9) resolved on an 8% sequencing gel. The outer CT and AG lanes are Maxam-Gilbert sequencing standards. Lane 1, ACT ds break fragment; lane 2, same as lane 1 except treated with 0.5 M piperidine at 90 °C for 20 min; lane 3, same as lane 1 except reduced with NaBH₄ immediately after the drug reaction; lane 4, same as lane 3 except treated with piperidine as in lane 2. Variation in the band intensities in the different lanes is due to variability of DNA recovery during work-up. B, NCS-treated 5'-32P-labeled HindIII/TaqI pBR322 was resolved on an 8% nondenaturing polyacrylamide gel and the isolated ds break fragment resolved on a 20% sequencing gel. The first three lanes are Maxam-Gilbert standards. Lane 1, bleomycin-treated DNA (no reisolation of bands); lane 2, NCS-treated ss-nicked DNA (DNA at the origin of a nondenaturing gel); lane 3, ds break fragment (band 9 from lane 5 of Fig. 2A).

Whether there might be differences in the cleavage mechanism between ss and ds cutting sites, a 20% sequencing gel was used to compare the 5',32P-labeled HindIII/TaqI pBR322 ds break fragments with the purely ss breaks present in the band migrating at the origin of the nondenaturing gel, which consists of uncut and ss-nicked DNA. The glycolate-ended fragment represents only 10% of the material from the DNA with only ss cuts, while the ds cut DNA consists of 25% phosphate-ended material and 75% glycolate-ended species (Fig. 4B, lanes 2 and 3). The percentages of glycolate- and phosphate-ended material are 43 and 57%, respectively, in the unfraccionated reaction products (data not shown).

DISCUSSION

NCS-induced ds breaks can result from random independent cleavage at T and A residues at closely opposed sites, by...
the preferred cleavage by a second drug molecule opposite an existing ss break site, or by a concerted reaction involving simultaneous bistranded cleavage at a single site by a single drug molecule. The finding of high ratios (30 to 50:1) of ds to ss breaks under *in vitro* conditions with NCS using 2-ME as the thiol activator (9, 20) has led to the conjecture that ds breaks result from the random placement of ss breaks at closely opposed sites and that a specific concerted mechanism for ds breakage may not exist. However, *in vivo* experiments in mammalian cells where GSH is the thiol have given a ratio of 5:1 (1, 21) in excellent agreement with the results shown in Fig. 1. This finding along with the observed linearity of the variation of the ds:ss break ratios with drug concentration suggests that ds cleavages with GSH as activator are caused by a single event or interdependent events and not by the coincidence of random ss breaks on opposite strands. The structure of the lesion produced at a ds break suggests a concerted bistranded mechanism. Not only is there a distinct sequence requirement for ds breakage, usually containing a GT step, but six of seven sites are trinucleotide sequences with cleavage at T or A residues on opposite strands staggered 2 bp in the 3' direction from each other. This geometry (plus the equivalent of 1 base pair occupied by the intercalated drug (23)) places the attacked sugar residues directly across the minor groove from each other and, according to molecular modeling studies, provides optimum access for the two radical centers of a single molecule of NCS to the C4' and C5' of the cleaved residues of both strands (23). Support for this model also comes from the particular mix of chemical structures, involving minor groove attack sites (C4' and C5'), noted at the cleavage sites. The preponderance of 5'-aldehyde (80%) formed at the T of ACT suggests that C5' attack is the major mechanism of cleavage at this site (6). At the GT step of AGT and TGT, however, C4' hydrogen atom abstraction is supported by the predominance of a putative glycolate-ended fragment in ds cleavage at this site, consistent with the recently demonstrated C4' deuterium isotope selection effect in the formation of this product at GT steps and the effect of various thios on its formation. With regard to the effect of thios on the C4' chemistry of NCS, the reduction in ds breakage with 2-ME is consistent with the findings that the use of neutral thiol, such as 4-hydroxythiophenol (24) and 2-ME, is associated with C4' attack involving almost exclusive abasic site formation with little direct strand breakage at a GT step. It is also of relevance that the other described lesion generated by NCS, consisting of an abasic site at the C residue (underlined) of ACC-GCT and a strand break at the T residue, is also staggered two nucleotides to the 3' side and appears to result from hydrogen atom abstraction from the C1' of the C residue (12, 13, 25) and from C5' of the T residue (11, 12) by a single diradical form of the drug, in accord with molecular modeling studies (23). The formation of this lesion is also greater when GSH, rather than 2-ME, is the activating thiol (12, 26).

Of considerable interest is the near absence of glycolate from AGT ss cleavage sites and the presence of 3'-phosphate as the major species at these sites. This indicates that ss and ds breakage involve different mechanisms at the T residue AGT. These results are consistent with a model in which NCS in one orientation may be positioned for concerted attack at C5' of ACT and at C4' of ACT, resulting in a direct ds break, while a slightly different orientation of the diradical would result in only C5' hydrogen abstraction and a ss break at the AGT or ACT. It is also possible that ss and ds breaks are due to two different binding modes, perhaps associated with different intercalation sites, such as has been proposed to account for the excess formation of direct breaks at the T residue in AGC-GCT compared with abasic lesions (23).

The lone tetranucleotide ds break site that appeared in these studies, AGCT-AGCT, may be an exception to the single event ds lesion being proposed. The sequence is self-complementary, raising the possibility of the cooperative binding of two drug molecules with ss cleavages of each strand.

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REFERENCES


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