Modulation of neocarzinostatin-mediated DNA double strand damage by activating thiol: deuterium isotope effects

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Received November 5, 1991; Revised and Accepted January 16, 1992

ABSTRACT
The neocarzinostatin chromophore causes double-strand damage at AGC sequences on DNA by concomitant 1'-oxidation at C and 5'-oxidation at the T on the complementary strand. The extent of this damage is dependent upon the structure of the thiol used for activation. Deuterium isotope effects suggest that this dependence on thiol structure may be due to internal quenching of one radical site of the activated chromophore by the hydrogen atoms of the thiol sidechain. The 12-mer d[GCAAGCGCTTGC] is treated with the neocarzinostatin chromophore and either sodium thioglycolate or [2-2H2]-thioglycolate, and the distribution of strand breaks is determined by gel electrophoresis. Two isotope effects are noted: an overall sequence-independent effect in which deuterated thioglycolate increases total strand damage by a factor of 2, and a sequence-specific effect by which deuteration increases the proportion of alkali-sensitive strand damage at C6 by an additional factor of 1.5. Methyl thioglycolate shows essentially identical behavior to that of thioglycolate anion, ruling out electrostatic effects as major contributors to the effect of thiol structure on the mode of DNA damage observed. A model for NCSC action consistent with these results is discussed.

INTRODUCTION
The neocarzinostatin chromophore (NCSC) has been shown to cause a variety of types of damage on DNA *in vitro*, including single-strand breaks, double-strand breaks, and covalent adducts.1-5 The chemistry of NCSC is extremely complex, yet some generalizations may be made. The primary lesion formed by NCSC is a single-strand break resulting from 5'-oxidation. This occurs with high specificity at thymidine residues, although a preferred sequence context has not been observed.6 Direct double-strand breaks are rare, although sequences such as AGC show moderate levels of concomitant 1'-oxidation of the C and 5'-oxidation of the T on the complement strand.5,7-9 The 1'-oxidation chemistry leads to a alkali-sensitive apyrimidinic site, and a double-strand break is observed after treatment at high pH. The lesion resulting from 1'-oxidation has been demonstrated to be a ribonolactone residue.8 Other minor types of DNA damage have been noted, such as 4'-oxidation.10-12 All types of damage require activation of the NCSC, typically by addition of a thiol.

The partitioning between damage types shown by NCSC is dependent upon the thiol used for activation.12,13 The peptide thiol glutathione is quite good at inducing alkali-sensitive double strand damage at AGC sequences, while dithiothreitol (DTT) supports this mode of damage only poorly. There are at least three possible explanations for this observation: (1) the different NCSC-thiol adducts have different binding properties to the target DNA, such that the NCSC-DTT adduct is not properly positioned for double-strand damage; (2) DTT may be able to repair one of the two DNA radicals more efficiently than glutathione due to the presence of a second, nearby thiol; or (3) double-strand damage requires chemistry by both radical sites on the activated drug, and formation of the NCSC-DTT adduct may lead to quenching of one drug radical site faster than double-strand damage can be initiated.

We report here results of experiments designed to investigate the last hypothesis. Substitution of the methylene hydrogen atoms of thioglycolate with deuterium enhances alkali-dependent damage at the C of AGC, indicating a role for the thiol sidechain in enhancing or suppressing double-strand cleavage by NCSC. Deuteration also results in a large increase in total DNA damage, consistent with thiol quenching of radicals leading to strand damage being a significant component of the overall efficiency of NCSC.

MATERIALS & METHODS
Materials
Neocarzinostatin was purified from cultures of *Streptomyces carzinostaticus* var. neocarzinostaticus F-51 (ATCC 15944) according to the procedure of Samy et al.14 The isolated neocarzinostatin showed identical spectroscopic and DNA damaging properties to those of an authentic sample generously provided by Kayaku Co., Ltd. (Tokyo). NCSC was extracted from the antibiotic using 0.1 M acetic acid in methanol.15
DNA oligomers were synthesized by automated cyanoethyl phosphoramidite synthesis on an Applied Biosystems 380B synthesizer and purified by anion-exchange chromatography at pH 12 on Sepharose QFF and desalted by reversed-phase HPLC (C18, water-methanol). The oligomer was 5'-end labeled with $^{32}$P-ATP and T4 polynucleotide kinase using standard procedures.\textsuperscript{16} After electrophoretic purification, the labeled oligomer was eluted from the crushed gel slice and precipitated with ethanol.

Sodium [2-$^2$H$_2$]-thioglycolate was prepared from sodium thioglycolate (Sigma Chemical Co.) by exchange in 98 atom%-$^2$H$_2$O.\textsuperscript{17} NMR and 70-eV electron impact mass spectrometric analysis indicated that >90% of the protons had been exchanged for deuterons after 4 days at 160°C. Due to the rapid oxidation of sodium thioglycolate, stock solutions were purged with N$_2$ and stored at 4°C under inert atmosphere. A thiol concentration determination was performed immediately prior to each NCSC cleavage reaction.\textsuperscript{18} Methyl [2-$^2$H$_2$]-thioglycolate was prepared by esterification of sodium [2-$^2$H$_2$]-thioglycolate in CDCl$_3$/CH$_3$OD and D$_2$SO$_4$, and was purified by vacuum distillation. This material was analyzed by $^1$H-NMR and 70eV EI-MS.

**Electrophoresis**

Electrophoresis was performed at 50°C on 20% polyacrylamide sequencing gels (1:20 crosslinking) containing 8.3 M urea. Sequencing lanes (G+A and T) were used as markers.\textsuperscript{19} Quantitation of radioactive bands was achieved using a Molecular Dynamics phosphorimager and ImageQuant software.

**Cleavage reactions**

A 1-µL aliquot of 0.18 mM NCSC in 0.1 M acetic acid/methanol was added in the dark to an ice-cold mixture of 90 pmol DNA strands and 9 nmol of thiol dissolved in 9 µL of 0.2 M triethylammonium bicarbonate, pH 8. After 15 min on ice, the reaction mixture was taken to dryness in a Speed-Vac centrifugal concentrator. Reactions being analyzed for alkali-sensitive cleavage were treated with 10 µL of 4 M aqueous NH$_3$ in a sealed tube at 90°C for 30 min prior to evaporation. The dried mixture was taken up in water and redried in order to remove traces of buffer. The dried sample was then dissolved in 5 µL of formamide loading buffer and analyzed by gel electrophoresis. Gels were washed briefly with dilute acetic acid and air dried between sheets of cellophane prior to imaging.

**RESULTS**

Treatment of the self-complementary DNA dodecamer d(GCAAGCGCTTGC)$_2$ (4.5 µM in duplex) with 18 µM NCSC and 900 µM thiol at pH 8.0 gives rise to 14–38% total strand damage, depending on the thiol used. Under these conditions, approximately 60% of the duplex is cleaved with NCSC based on a dissociation constant of 10 µM (S.E. McAfee, unpublished data). This gives a maximal efficiency of damage from complexed NCSC as approximately 60%, in agreement with previous observations.\textsuperscript{20} The major strand breaks (Fig. 1) occur at the two thymidine residues, T9 and T10, in agreement with previous reports on the base specificity of NCSC.\textsuperscript{6} The ratio of damage at T9/T10 is approximately 2.5:1 regardless of whether glutathione, DTT, or thioglycolate is used for activation. Cleavage at C6 is minor in comparison, although the intensity of this band varies with the nature of the activating thiol (Table 1). Glutathione consistently gives the highest amount of damage at C6, while DTT gives the lowest amount. Sodium thioglycolate is intermediate in its ability to support damage at C6. Methyl thioglycolate supports damage at C6 comparably to sodium thioglycolate.

Careful electrophoresis of the reaction mixtures at low temperature shows the presence of a band migrating slightly faster than full-length DNA. This band shows the characteristics of abasic-site formation.\textsuperscript{5} Treatment of the samples with aqueous ammonia prior to electrophoresis results in loss of this band and a concomitant increase in the intensity of the C6 band (Table 1).

**Table 1. Site selectivity of total strand breaks on d(GCAAGCGCTTGC) by NCSC activated by different thiols.**

<table>
<thead>
<tr>
<th>thiol</th>
<th>%C6</th>
<th>%T9</th>
<th>%T10</th>
<th>%DNA cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutathione</td>
<td>4.8 (0.1)</td>
<td>44.4 (2.4)</td>
<td>29.8 (1.0)</td>
<td>31.4 (1.2)</td>
</tr>
<tr>
<td>+alkali</td>
<td>9.2 (0.5)</td>
<td>56.9 (2.8)</td>
<td>23.2 (1.2)</td>
<td>38.0 (3.4)</td>
</tr>
<tr>
<td>DTT</td>
<td>10.0 (0.1)</td>
<td>44.1 (1.8)</td>
<td>18.6 (0.5)</td>
<td>24.8 (2.9)</td>
</tr>
<tr>
<td>+alkali</td>
<td>2.5 (0.2)</td>
<td>55.7 (3.2)</td>
<td>18.1 (0.8)</td>
<td>31.0 (2.7)</td>
</tr>
<tr>
<td>TG</td>
<td>5.0 (1.0)</td>
<td>52.9 (2.1)</td>
<td>27.1 (2.8)</td>
<td>12.1 (3.4)</td>
</tr>
<tr>
<td>+alkali</td>
<td>7.3 (0.4)</td>
<td>52.5 (6.9)</td>
<td>21.6 (1.0)</td>
<td>14.8 (3.6)</td>
</tr>
<tr>
<td>d$_2$-TG</td>
<td>5.3 (0.8)</td>
<td>54.6 (0.8)</td>
<td>23.8 (1.3)</td>
<td>26.8 (2.9)</td>
</tr>
<tr>
<td>+alkali</td>
<td>10.8 (0.7)</td>
<td>48.5 (7.2)</td>
<td>19.2 (1.9)</td>
<td>35.1 (4.0)</td>
</tr>
<tr>
<td>Me-TG</td>
<td>2.9 (0.2)</td>
<td>58.4 (1.3)</td>
<td>24.2 (1.3)</td>
<td>49.1 (2.2)</td>
</tr>
<tr>
<td>+alkali</td>
<td>5.4 (0.4)</td>
<td>56.7 (1.9)</td>
<td>24.8 (1.1)</td>
<td>52.8 (4.2)</td>
</tr>
</tbody>
</table>

*TG = sodium thioglycolate, d$_2$-TG = [2-$^2$H$_2$]-thioglycolate, and Me-TG = methyl thioglycolate. The results are the average of 3 (glutathione, DTT) or 5 (TG, d$_2$-TG, and Me-TG) experiments, with standard deviations given in parentheses. Percentages given for individual sites are proportions of the total damage occurring at that site.
Table 2. Absolute percentages of total DNA cleavage on d[GCAAGCGCTTGC] by NCSC activated by thioglycolate or [2-H2]-thioglycolate.

<table>
<thead>
<tr>
<th>site</th>
<th>[3H]-TG % cleavage</th>
<th>[2H]-TG % cleavage</th>
<th>H/H/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6</td>
<td>1.3 (0.4)</td>
<td>4.4 (1.0)</td>
<td>0.29</td>
</tr>
<tr>
<td>T9</td>
<td>9.1 (2.5)</td>
<td>19.4 (2.7)</td>
<td>0.47</td>
</tr>
<tr>
<td>T10</td>
<td>3.8 (1.4)</td>
<td>7.2 (0.9)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Standard deviations are given in parentheses. The results are the average of five separate experiments, with two electrophoretic quantitations for each experiment. Reactions were treated with aqueous NH3 at 90°C for 30 min prior to electrophoresis to insure complete cleavage of abasic sites. Percentages given are the ratio of radioactivity in the indicated band relative to the total radioactivity in the lane.

1). It is not certain at the present time if the cleavage observed at C6 prior to base treatment is due to uncontrolled breakdown of an abasic site during the cleavage reaction or electrophoresis or to competing 5'-oxidation chemistry at this site, as the gels are run at high temperature and in the presence of Tris buffer, conditions under which the abasic sites are known to be somewhat unstable.5 When the gels are run at 10°C, the results are essentially unchanged: damage at C6 makes up 3.2% (s.d. 0.7) of the total damage prior to and 7.5% (s.d. 0.3) after alkali treatment when thioglycolate is used. With [2-2H2]-thioglycolate as activator, C6 damage is 5.5% (s.d. 1.3) of the total damage prior to and 11.1% (s.d. 0.7) after alkali treatment. It is therefore likely that the background damage at C6 observed prior to alkali treatment occurs via direct strand cleavage rather than abasic site formation. While this is a larger amount of such damage at C than typically observed, we have noted that thioglycolate appears to promote much less selective strand damage than glutathione or DTT (R.G. Schultz, unpublished data).

A comparison of NCSC damage to d[GCAAGCGCTTGC]2 in the presence of thioglycolate and [2-2H2]-thioglycolate was made. Electrophoresis of the reaction mixtures prior to and after treatment with aqueous ammonia revealed the same pattern of damage discussed above. However, C6 damage made up a greater proportion of the total NCSC-mediated strand damage with deuterated thioglycolate than with nondeuterated thioglycolate. Comparison of five sets of experiments gives an isotope effect of 0.68 on damage at C6 upon deuteration of the thiol, based on the proportion of total damage. Calculation of isotope effects at T9 and T10 revealed effects of at most 1.1 at each site.

 Unexpectedly, the total amount of DNA damage observed increased 2-fold with deuterated thioglycolate. This is made clear in comparison of the absolute band intensities at each site (Table 2). The standard deviation of these numbers is substantially higher than that of the percentage of total strand damage, due primarily to variations in sample loading between gel lanes. It is clear, however, that there is a marked increase in efficiency with deuterated thioglycolate.

**DISCUSSION**

NCSC (I, Scheme 1) is most efficient at causing single-strand breaks on DNA by oxidation of the 5'-positions of the deoxyriboseyl groups, yet double-strand damage can be a significant fraction of the total damage at special sequences and may constitute a significant part of the biological effects of this antitumor drug. Povirk and Goldberg have suggested that DNA double-strand damage caused by NCSC at the susceptible site AGC results from simultaneous damage on opposing strands by the activated NCSC biradical.7,13 DNA labeling experiments have indicated that the double-strand damage observed in the sequence AGC occurs via 1'-oxidation at the C residue and 5'-oxidation at the T on the complementary strand.9 Computer modeling studies have indicated that such a damage pattern may result from a binding mode of NCSC in which the naphthoate group is intercalated and the diyne-epoxide group lies in the minor groove.21 For a given intercalation site, this model predicts that the radical at the 2-position of activated NCSC (2, Scheme I) is involved in 1'-oxidation while the 6-position radical is involved in 5'-oxidation. It may be argued on chemical grounds that the 6-position radical is more likely to be responsible for the more prevalent 5'-oxidation, as it is sterically less hindered and thus kinetically more reactive than the 2-position radical. Recent results of Meschowitz and Goldberg demonstrate specific transfer of deuterium from the 5'-position of DNA to the 6-position of the activated drug.22 No deuterium transfer was noted to the 2-position, in agreement with this model. In these experiments, only 30% deuterium was transferred to the 6-position, however, and quantitation of the drug recovered was not reported. The source of the 70% protium at the 6-position has not been determined. As there is always more single-strand damage than double-strand damage in these experiments, it is difficult to correlate this degree of label incorporation specifically with double-strand damage.

This structural model provides an intriguing hypothesis for the infrequency of double-strand cleavage seen with NCSC relative to related drugs like calicheamicin.23 Activation of NCSC appears to occur by nucleophilic addition of the thiol to the alkene group adjacent to what will become the 2-position radical (Scheme 1).24,25 Double-strand damage would be effectively prevented by hydrogen atom transfer from the thiol side chain to the 2-position radical (Scheme 2). (Since preparation of this manuscript, this hypothesis has also been put forth by I.H. Goldberg [1991] Accs. Chem. Res. 24, 191–198.) For drugs such as calicheamicin and esperamicin, the activating thiol does not become covalently attached to the activated drug, and such internal quenching would not occur.26–29

This hypothesis predicts that an isotope effect should be observed on double-strand cleavage if the thiol side chain were
deuterated, such that the proportion of double-strand damage would increase upon thiol deuteration. Specifically, if the 2-position radical is involved with 1'-oxidation as predicted, a selective increase should be observed on damage at C6 of d[GCA-AAGCGCTTGC].

Glutathione is best able to support double-strand damage by NCSC, but synthesis of the required deuterated material is not trivial. We have therefore investigated the simple thiol sodium thioglycolate, which can be readily deuterated on the methylene carbon by exchange with D₂O. This thiol supports double-strand damage on d[GCA-AAGCGCTTGC], with 7% of the total strand damage occurring as alkali-sensitive breaks at C6. Rapid air oxidation of thioglycolate makes it imperative that the thiol concentration of working stocks be determined immediately prior to use in cleavage reactions, especially as we have noted that aqueous solutions of sodium thioglycolate and of sodium [2-2H₂]-thioglycolate appear to oxidize at different rates. Titration using 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, is a convenient procedure for this quantitation.

Use of methylene-deuterated thioglycolate increases the proportion that damage at C6 makes of the total strand damage by a factor of 1.5. This is in agreement with the predictions of our hypothesis, and provides support for the idea that internal radical quenching may be a factor controlling partitioning between single- and double-strand damage by NCSC at susceptible sequences. A sequence-independent increase in DNA damage of 2-fold is also noted with methylene-deuterated thioglycolate. It is not possible to cleanly separate the two isotope effects at C6, where the absolute amount of damage increases by a factor of 3.4 with [2-2H₂]-thioglycolate (Table 2). The change in distribution of damage sites (Table 1) is strongly suggestive of two separate increases of 1.5-fold and 2-fold at this position, however.

It should be noted that these results are at variance with the results of model studies using NCSC and methyl thioglycolate in organic media in the absence of DNA. Under these conditions, the putative diradical intermediate 2 abstracts deuterium from the solvent (9:1 tetrahydrofuran-D₈; CD₂COOH) to equivalent extents at the 2- and 6-positions, as determined by the NMR spectra of the purified aromatized thiol adduct. There may be several reasons for this discrepancy, foremost being that the model reaction was run in the presence of a very high concentration of radical quencher (organic solvent). This would suppress internal quenching of the type proposed here. Secondly, the thiol sidechain may well adopt a different conformation relative to the drug radical when constrained in the minor groove of DNA. Work is currently underway to examine deuterium transfer from thiol to the 2-position radical when NCSC is bound to DNA.

The selectivity of the sequence-dependent isotope effect for increasing alkali-sensitive damage at C6 supports a model in which the 2-position radical of activated NCSC is responsible for 1'-oxidation, while the 6-position radical is involved with 5'-oxidation. This is in agreement with the model of Galat and Goldberg and the experimental results of Meschwitz and Goldberg. In contrast, the model proposed by Hawley et al. predicts the opposite result, and is inconsistent with these experimental results.

The sequence-independent increase in total DNA damage observed upon thiol sidechain deuteration supports a mechanism in which the degree of DNA damage depends upon the lifetime of the NCSC diradical species in the presence of excess activating thiol. Slowing the rate of intermolecular quenching by thiol should increase the amount of hydrogen atom abstraction from DNA. It is also possible that there is an isotope effect on repair of NCSC generated DNA radicals, such that the deuterated thiol is less efficient at repairing such radicals and overall chain damage increases. Regardless of the exact mechanism, our results support a pathway in which this radical quenching arises via abstraction of a methylene hydrogen of the thioglycolate rather than the sulfhydryl hydrogen as might be anticipated. This possibility has been suggested by Chin et al. based on the degree of deuteration incorporation into NCSC when the drug was activated by methyl thioglycolate in D₂O as well as earlier model work.

These isotope effects demonstrate the potential for modulation of double-strand damage by the activating thiol, although by no means settle the question of thiol structure and its relationship to NCSC chemistry. There clearly may be other factors involved in this competition. It has been noted that AGT sequences are also strong sites of double strand damage, and that the partitioning of damage chemistry at GT sequences is strongly dependent upon the nature of the thiol: acidic thiols such as glutathione and mercaptopropionate appear to support damage at GT sites to a greater extent than neutral thiols such as mercaptoethanol and 4-hydroxythiophenol. It is thus possible that electrostatic effects may be important in determining the mode of NCSC binding to DNA, and thereby the type of damage observed. We have found that the neutral ester methyl thioglycolate is only slightly less effective than the anionic thioglycolate at supporting damage at C6. The charge of the activating thiol does not appear to be as critical in the double strand damaging complex at AGC sequences. Although these results are for a single thiol concentration, we have found that the percentage of 1'-damage at C is essentially independent of thioglycolate concentration over a wide range (R.G. Schultz, unpublished data). It thus appears unlikely that the observed independence of the efficiency of 1'-damage at this AGC site on thiol charge will vary significantly with thiol concentration. Steric interactions might also result in an orientation of the thiol sidechain in the bound complex which is unfavorable for internal radical quenching. On the other hand, it is also possible that thiol structure may play a role in the repair of NCSC generated DNA radicals, and thus may control the observed strand cleavage chemistry in an indirect manner. Elucidation of factors which govern the type of damage caused by NCSC will greatly aid our ability to design new, specific antitumor agents.

**ACKNOWLEDGEMENTS**

This work was supported in part by grant # 88-48 from the Illinois Division of the American Cancer Society.
REFERENCES