Mechanism and Base Specificity of DNA Breakage in Intact Cells by Neocarzinostatin†

Lizzy S. Kappen, Thomas E. Ellenberger, and Irving H. Goldberg*
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: When electrophoresed on an agarose gel, the DNA isolated from neocarzinostatin- (NCS-) treated HeLa cells migrates in a ladder of discrete bands indicative of preferential breakage in the linker region of the nucleosomes. The 5′-termini of the drug-induced DNA strand breaks were characterized by (1) reduction of the nucleoside 5′-aldehyde ends to 5′-hydroxyls followed by incorporation of ^{32}P from [γ-^{32}P]ATP by polynucleotide kinase and (2) treatment of the DNA with hot alkali and alkaline phosphatase prior to the kinase assay to give the total 5′-termini. In DNA isolated from NCS-treated cells, nucleoside aldehyde accounts for 30–45% of the drug-generated 5′ ends; the remainder have PO_4 termini. By contrast, 5′-terminal nucleoside aldehyde in DNA cut with the drug in vitro exceeds 80% of the total 5′ ends. Of the ^{32}P representing nucleoside aldehyde in DNA from NCS-exposed cells, 77% is in TMP; the rest is in AMP $\gg$ CMP > GMP, a distribution in excellent agreement with that obtained for in vitro drug-treated DNA. DNA sequencing experiments, using the 340 base pair alphoid DNA fragment isolated from drug-treated cells, show that the pattern of breakage produced by NCS within a defined sequence of DNA in intact cells is similar to that in the in vitro reaction, with a preferential attack at thymidylate residues, but a much higher concentration of the drug was required to cause comparable breakage in intact cells. Furthermore, in cells depleted of glutathione, DNA strand breakage was greatly reduced, confirming thiol involvement in the activation of the drug in cells, as in the in vitro reaction.

The antitumor antibiotic neocarzinostatin (NCS)[1] consists of a highly labile nonprotein chromophore noncovalently complexed with an apoprotein (Napier et al., 1979). The full biological activity of the drug resides in the nonprotein chromophore; the apoprotein protects it from degradation and serves as its carrier (Kappen et al., 1980; Kappen & Goldberg, 1980; Koide et al., 1982). The chromophore consists of four subunits: 2-hydroxy-7-methoxy-5-methylnaphthoate and 2,6-dideoxy-2-(methylamino)galactose linked to a $C_{15}H_8O_4$ substituent consisting of an ethylene cyclic carbonate group and a highly strained ether epoxide attached to a novel bicyclo[7.3.0]dodecadyine system (Hensens et al., 1983; Edo et al., 1985). The involvement of DNA as the primary target in the action of NCS is manifested by its selective inhibition of DNA synthesis in sensitive cells and its induction of single-strand breaks in the DNA of mammalian cells, unscheduled DNA synthesis in lymphocytes, DNA repair synthesis in HeLa cells and in isolated nuclei, chromosome aberrations in mammalian cells, and mutagenesis in *Escherichia coli* [reviewed in Goldberg et al. (1981) and Goldberg (1986)]. NCS chromophore complexes with isolated DNA by an intercalative mechanism (Dasgupta & Goldberg, 1985; Goldberg, 1986) and damages DNA in a thiol- and oxygen-dependent reaction (Goldberg et al., 1981; Goldberg, 1986). Using this in vitro system, it has been shown that DNA damage results mainly in single-strand breaks, almost exclusively at thymidylic and deoxyadenylic acid residues (Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978; Takeshita et al., 1981), accompanied by the generation of a phosphate at all the 3′ ends of the breaks and a nucleoside 5′-aldehyde (>80%) or a phosphate at the 5′-termini (Kappen & Goldberg, 1978, 1983; Kappen et al., 1982). In addition, NCS releases bases (T > A $\gg$ C > G) (Goldberg et al., 1981) and forms novel covalent DNA adducts (Povirk & Goldberg, 1982, 1985).

While much data have accumulated on the in vitro model reaction, little is known of the mechanisms by which NCS damages DNA in intact cells. In the present study, we have examined the DNA damage products, the specificity of DNA strand breakage, and the involvement of thiol in the action of NCS in intact HeLa cells. Furthermore, using the highly reiterated alphoid sequence (340 bp), which comprises 1% of the total human genome (Wu & Manuelidis, 1980), we made a direct comparison of the sites of DNA damage at the level of individual nucleotides either with purified DNA or with the same DNA in the living cell (Lippke et al., 1981) exposed to the drug. The results show that the biochemical features of the DNA damage by NCS in cells closely resemble those of the cell-free system.

Materials and Methods

Materials and their suppliers are as listed: bacterial alkaline phosphatase, Bethesda Research Laboratories; all other enzymes, New England Biolabs; radioactive materials and NENSorb, New England Nuclear; BSO, Chemical Dynamics Corp.; cell culture media and serum, Grand Island Biological Co.; Sephacryl S-1000, Sigma. NCS was a gift from Dr. W. T. Bradner (Bristol Laboratories).

HeLa cells were grown in spinner cultures in minimum essential medium supplemented with 7% fetal bovine serum.

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[1] Abbreviations: NCS, neocarzinostatin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); BSO, buthionine sulfoximine; PBS, 0.15 M NaCl/0.01 M sodium phosphate, pH 7.2; SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.4; SDS, sodium dodecyl sulfate; holo-NCS, native NCS containing apoprotein and chromophore; PEI, poly(ethylenimine).

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NCS chromophore was extracted from the native drug as previously described (Kappen & Goldberg, 1985).

In Vitro DNA Cutting. In vitro reaction of DNA with NCS chromophore was as previously described (Kappen & Goldberg, 1985), except that the steps to achieve anaerobiosis were omitted and the reaction was performed in regular vessels. In small reaction volumes (<50 μL), chromophore was added to DNA in 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA followed by the addition of thiol. Both procedures gave identical results in all subsequent product analyses, although prebinding of the drug to DNA at acidic pH offers more stability to the drug.

Depletion of Glutathione in Cells. HeLa cells were incubated with 1 mM BSO for 21 h at 37 °C. The cells (1.5×10^6) were washed with phosphate-buffered saline (PBS), and the cell pellet obtained by centrifugation at 10000*g* for 10 min was vigorously vortexed with 0.5 mL of 0.6% sulfosalicylic acid. After 20 min in ice, the samples were centrifuged (10000*g*, 10 min), and portions of the supernatant were assayed for glutathione by the glutathione reductase procedure of Tietze (1969).

Drug Treatment of Cells and DNA Isolation. Cells were washed with PBS and suspended in the same buffer [$(2–3) \times 10^6$ cells/mL] for drug treatment. The cells were chilled in ice prior to the addition of NCS chromophore. The control cells, not treated with the drug, received an equal volume of methanol containing an equivalent amount of citrate buffer (4 mM) present in the drug. After 5 min in ice, the cells were stirred at 37 °C for 30–40 min. In experiments where the DNA was used in the phosphatase/kinase assay, the cells had been labeled with low levels of [*methyl*-^{3}H]thymidine (0.3 μCi/mL, 77.1 Ci/mmol) in order to determine the recovery of DNA.

The procedure used for the isolation of DNA following drug treatment differed slightly depending upon the subsequent analysis to which the DNA was subjected. In experiments where the 5′-termini at the breaks were determined by the phosphatase/kinase assay, the cells, after two washes with SSC, were taken up in 50 mM Tris-HCl, pH 8, containing 100 mM NaCl and 50 mM EDTA and were lysed by the addition of SDS to 0.5%. After 15 min of stirring at room temperature, proteinase K (final concentration 50 μg/mL) was added, and the lysate was incubated at 37 °C for 2 h. The samples were then extracted twice with SSC-saturated phenol followed by $CHCl_3$/isoamyl alcohol (19:1 v/v). DNA was collected by ethanol precipitation (Kappen & Goldberg, 1983) and was dissolved in 10 mM NaCl, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0, for a 16-h dialysis against the same buffer. The dialysates were then digested at 37 °C successively with 100 μg/mL RNase for 1 h and proteinase K for 2 h. The samples were extracted with SSC-saturated phenol and $CHCl_3$/isoamyl alcohol as described above. DNA was recovered by ethanol precipitation.

In experiments where the 340 bp alphoid DNA (α-DNA) fragment was isolated for end labeling, the drug-treated cells, after three washes with PBS, were suspended in 50 mM Tris-HCl, pH 7.5, and 20 mM EDTA and were lysed with SDS (final concentration 0.3%). After the addition of proteinase K to 100 μg/mL, the lysate was passed through a 23-gauge needle 3 times and incubated at 37 °C for 2 with a second addition of proteinase K at half-time. The digest was then extracted twice with phenol/$CHCl_3$/isoamyl alcohol (50:50:1) and twice with ether. DNA recovered by two ethanol precipitations was digested with RNase (100 μg/mL) in 10 mM Tris-HCl/1 mM EDTA, pH 7.9 at 37 °C, for 45 min. This was followed by *Eco*RI digestion (14 h, 37 °C) and separation of the 340 bp α-DNA by electrophoresis on a 1.5% low-melting agarose gel. The DNA was recovered from the melted gel by phenol extraction and ethanol precipitation.

Determination of Nucleoside 5′-Aldehyde in DNA. The 5′-terminal nucleoside aldehyde at the breaks in DNA were first reduced with $NaBH_4$ to 5′-CH_2OH groups essentially as previously described (Kappen & Goldberg, 1983). The DNA was freed of salts either by ethanol precipitation or by passing through NENSorb columns. When NENSorb columns (upper size limit ~50000 bp) were used, DNA from control cells was always ethanol-precipitated.

5′-Terminal hydroxyl groups were quantitated by phosphorylation using [γ-^{32}P]ATP (2–4 Ci/mmol) and polynucleotide kinase under conditions similar to those described (Kappen & Goldberg, 1983). 5′-PO_4-ended termini were first dephosphorylated by treatment of DNA with bacterial alkaline phosphatase (at 65 °C, 40 min) in Tris-HCl, pH 8.0, prior to the kinase assay. When the assay involved a pretreatment of the DNA with NaOH, the samples were neutralized with HCl before the phosphatase and kinase reactions.

To determine the distribution among the four nucleotides of the 5′-terminal ^{32}P incorporated into the reduced and the unreduced DNA (labeled at a specific activity of [γ-^{32}P]ATP 40 times higher than that in the standard assay), the DNA was denatured by heating in 0.1 M NaOH (90 °C, 10 min), neutralized, and digested with nuclease S_1 in 25 mM sodium acetate, pH 4.5, and 0.2 mM $ZnCl_2$ to the level of mononucleotides which were separated by thin-layer chromatography on PEI–cellulose as described (Poon et al., 1977). After autoradiography, the spots on the chromatograms corresponding to each of the mononucleotides were cut out for determination of radioactivity.

Determination of the Specificity of Strand Breakage by DNA Sequencing. The 340 bp α-DNA was labeled with ^{32}P at the 3′ end by using [α-^{32}P]dATP and the Klenow fragment of *E. coli* DNA polymerase I. To introduce ^{32}P at the 5′ end, the DNA was first treated (37 °C, 30 min) with bacterial alkaline phosphatase in 50 mM Tris-HCl, pH 8.0, phenol-extracted, and ethanol-precipitated prior to the reaction involving [γ-^{32}P]ATP and polynucleotide kinase. The labeled DNA fragment was freed of unreacted nucleotides with NENSorb columns.

The 340 bp α-DNA from drug-treated cells, unlike that from control cells, is contaminated with nucleosomes (see Results). To obtain singly end-labeled fragments of each strand free of non-α sequences, ^{32}P-labeled 340 bp α-DNA was hybridized to a single-stranded M13 DNA recombinant containing either the (+) or the (−) strand of the α fragment. A similar approach has been used by Murray and Martin (1985) to determine sequence specificity of bleomycin-induced DNA damage in cells. For this purpose, the 340 bp α-DNA from HeLa cells was inserted into a M13 vector by an established procedure (Messing, 1983). The recombinants containing the (+) or the (−) strand of the insert were selected, and their single-stranded DNA was prepared. The presence of the insert and its orientation were confirmed by DNA sequencing using the dideoxynucleotide method with ^{35}S-labeled deoxynucleotide triphosphates (Sanger et al., 1977). The hybridization reaction involved heating the double-stranded ^{32}P-end-labeled 340 bp α-DNA in 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA at 100 °C for 2 min before mixing with M13 DNA in a buffer that gives a final concentration of 16 mM Tris-HCl, pH 8.3, and 4 mM $MgCl_2$. The samples were then left at 50 °C for 30 min, followed by 30 min at room

Table I: ^{32}P Incorporation into 5′-Termini of Breaks in DNA Isolated from NCS-Treated Cells[a]

	^{32}P incorporated (cpm)			
	$-NaBH_4$		$+NaBH_4$	
	−phosphatase	+phosphatase	−phosphatase	+phosphatase
control cells	337	1565	0	1522
NCS-treated cells	45	17962	5320	17613

[a] The cells were exposed to 31 μM NCS chromophore before isolation of DNA as described under Materials and Methods. Both reduced and unreduced DNA had been heated in 0.1 M NaOH (30 min, 90 °C) before treatment with alkaline phosphatase. Polynucleotide kinase dependent ^{32}P incorporation from [γ-^{32}P]ATP into 1 μg of DNA was determined. A non-enzyme-dependent average background of 998 cpm has been subtracted from all values.

temperature. ^{32}P-Labeled α-DNA strands hybridized to M13 DNA were separated from the unhybridized fragments by chromatography on a column (48 × 1 cm) of Sephacryl S-1000 in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 50 mM NaCl. The fractions were concentrated by lyophilization, and the DNA was ethanol-precipitated. The DNA pellet was then dissolved in distilled H_2O. The amount required for DNA sequencing was lyophilized, and the pellet was taken up in the gel loading solution. In experiments where the DNA had to be heated in alkali prior to analysis on a sequencing gel, the lyophilized DNA was redissolved in 15 μL of 0.1 M NaOH/0.5 mM EDTA and was heated at 90 °C for 20 min. After neutralization with HCl, the samples were again lyophilized before being taken up in the loading solution. Preparation of the standard markers for base-specific cleavage and the analysis of DNA on sequencing gels were as described (Maxam & Gilbert, 1977).

Results

Previous work has shown that the breaks in DNA produced by NCS in an in vitro reaction dependent on O_2 and a thiol have at their 5′-termini a nucleoside 5′-aldehyde (>80%) or a phosphate (<20%) (Kappen et al., 1982; Kappen & Goldberg, 1983). Analysis involved a direct measurement of the nucleoside aldehyde by high-pressure liquid chromatography after either (1) its release from the DNA by enzyme digestions or (2) its reduction on the DNA by $NaBH_4$ to a 5′-hydroxyl followed by introduction of ^{32}P into the 5′-hydroxyl groups from [γ-^{32}P]ATP by polynucleotide kinase. In the present study, we chose the latter method to measure the nucleoside aldehyde on the DNA isolated from cells exposed to NCS. To quantitate the 5′-PO_4 end groups, DNA was also treated with alkaline phosphatase to dephosphorylate the 5′-phosphoryl ends; the 5′-hydroxyl groups so generated were reesterfied with ^{32}P in the kinase reaction. If the DNA had been heated in alkali, a treatment that degrades the nucleoside aldehyde by β-elimination reactions to release the base and a sugar fragment with the generation of a 5′-phosphoryl terminus, the nucleoside aldehyde present in the unreduced DNA or in the reduced DNA from incomplete reduction will also be accounted for in the phosphatase/kinase assay. Thus, ^{32}P incorporation dependent on (a) only kinase accounts for 5′-hydroxyl ends, (b) reduction and kinase is due to the nucleoside aldehyde, (c) both phosphatase and kinase measures 5′-PO_4 termini.

Table I summarizes the data obtained with DNA isolated from NCS-treated cells. There is no significant ^{32}P incorporation into the unreduced DNA in the absence of phosphatase treatment. This shows there are few free 5′-hydroxyl groups at the breaks. Reduction of the same DNA caused a substantial increase in ^{32}P incorporation in the absence of phosphatase, confirming the generation of 5′-hydroxyl termini by reduction of the putative nucleoside aldehyde that was present at the 5′-termini. This constitutes ~33% of the ^{32}P measured after the DNA was subjected to treatments that should offer full expression of all the 5′-termini. The amount of nucleoside aldehyde determined by this procedure varied from 30% to 45% in several assays ($n = 6$) using different batches of DNA. The variation appears to result from different degrees of reduction and enzyme reaction (see Discussion). As expected, in both reduced and unreduced DNA, the total 5′-termini determined after hot alkali and phosphatase treatment are the same. In DNA isolated from cells not treated with the drug, ^{32}P incorporation under all conditions is very low.

Table II: Distribution among Bases of the 5′-Terminal ^{32}P Incorporated into DNA Isolated from Control and NCS-Treated HeLa Cells[a]

	% of total cpm (^{32}P)			
	T	A	C	G
(A) Reduction Dependent				
control	22.7	17.8	23.7	35.6
NCS	77.5	19.3	2.2	1
(B) Total 5′-Termini after Alkali Treatment				
control	31	25.8	9.6	33.6
NCS	28	18.4	13.8	39.7

[a] DNA isolated from control and NCS-exposed cells as in Table I was used. Prior to 5′ end labeling in the polynucleotide kinase dependent reaction, DNA in (A) was reduced with $NaBH_4$ and then denatured in 0.1 M NaOH (10 min, 90 °C); in (B), unreduced DNA was heated in 0.1 M NaOH (30 min, 90 °C) before alkaline phosphatase treatment at 65 °C. Digestion of the ^{32}P-labeled DNA to mononucleotides and their separation were carried out as described under Materials and Methods. One hundred percent radioactivity on the chromatogram represents the values (cpm) given in parentheses: (A) control (261), NCS (3198); (B) control (1773), NCS (9332). Radioactivity present in each of the mononucleotides from an equivalent amount of unreduced DNA [control (160), NCS (504)] and nonphosphatase-treated DNA [control (654), NCS (657)] has been subtracted from (A) and (B), respectively, before calculating the percentages.

The distribution of the incorporated ^{32}P among the four nucleotides in experiments similar to those in Table I is shown in Table II. In DNA isolated from NCS-exposed cells, 77.5% of the reduction- and kinase-dependent ^{32}P is in TMP. The rest is distributed in AMP ≫ CMP > GMP. Since the phosphorylated 5′-hydroxyls in this case arise from the 5′-terminal nucleoside aldehyde, this distribution reflects that of nucleoside aldehyde in the DNA. In the same DNA, under conditions (without reduction, but with hot alkali and phosphatase) where the incorporated ^{32}P is associated with the nearest 3′ neighbor of the nucleoside aldehyde and the 5′-terminal nucleotide at the spontaneously produced PO_4 ends (thus reflecting the total nucleotide 3′ to the one attacked by NCS), there is only a slight preference for G and T over A and C. In the control DNA where the small amount of breakage is mainly due to shearing in the isolation process, there appears to be no significant preference for any base. Taken together, these results show that the preferential attack by NCS at the T residues in DNA in intact cells results in the production of nucleoside aldehyde.

The base specificity of DNA breakage in cells was further confirmed in DNA sequencing experiments using the 340 bp DNA fragment of the α sequence isolated from NCS-exposed cells. When electrophoresed on an agarose gel, the DNA from drug-treated cells, unlike that from control cells, migrates in a ladder of discrete bands (each a multiple of a monomer unit of ~175 bp) (Figure 1). Such a nucleosome ladder results from the preferential breakage in the linker region of the

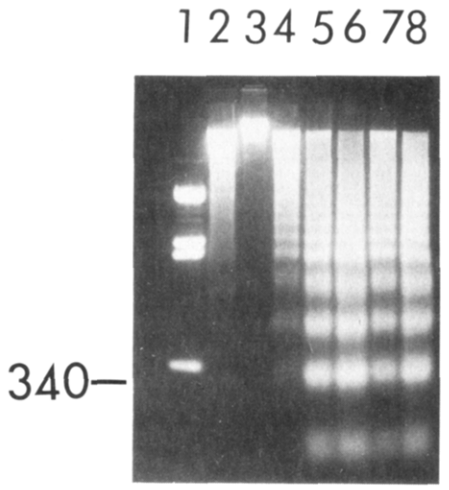

FIGURE 1: Agarose gel electrophoresis of DNA isolated from HeLa cells exposed to NCS chromophore. Drug treatment of cells and processing of DNA were as described under Materials and methods. Five micrograms of DNA was analyzed on a 1.5% agarose gel. (Lane 1) marker (*Bst*NI digest of pBR322 DNA); (lanes 2 and 3) DNA from control cells digested (2) with and (3) without *Eco*RI; (lanes 4, 5, and 7) cells treated with the drug at 9.5, 28.4, and 21.3 μM, respectively; (lane 6) as in lane 5 plus *Eco*RI; (lane 8) as in lane 7 plus *Eco*RI.

nucleosomes in chromatin. No discrete bands, only a smear, were seen when isolated HeLa DNA damaged by NCS in vitro was similarly analyzed (not shown). *Eco*RI digestion of the DNA generates a 340 bp fragment of the α sequence which in the case of the drug-treated DNA, unlike that from the control (lane 2), is contaminated with the nucleosomal background.

To locate the sites of single-strand breaks induced by the drug within the α sequence, the 340 bp α-DNA was end labeled with ^{32}P (3′ or 5′) and further purified by hybridization with an M13 recombinant DNA containing the (+) or the (−) strand of the 340 bp α fragment. The purified α-DNA strand, (−) or (+), was then analyzed on a sequencing gel. To compare with the drug-induced breakage in the same DNA in vitro, the purified α-DNA strand from the control 340 bp (in the hybridized form) was treated with the drug and run parallel with standard markers for base-specific cleavage, also prepared from the same control. Figure 2 shows an analysis with 3′ end-labeled DNA (+ strand) where the samples had also been heated in alkali to convert all nucleoside aldehyde ends to 5′-phosphoryl ends. It can be seen that in intact cells NCS caused breaks (lane 2) preferentially at T residues (e.g., T_{43}, T_{44}, T_{47}, T_{48}, T_{55}, T_{56}, and T_{57}) and to a lesser extent at A's (e.g., A_{38}) and still less at C's. G's are not attacked. A comparison with the in vitro cutting (lane 3) reveals that the pattern of DNA breakage obtained in intact cells and in vitro is quite similar with respect to base specificity and relative attack intensities ($T_{43} > T_{44}$, $T_{47} = T_{48}$, $T_{55} < T_{56}$, T_{57}). Similar correspondence in base attack specificity was also obtained when the (−) strand was analyzed on sequencing gels.

In vitro reactions (not shown) were also set up in which (1) purified HeLa DNA was cut with NCS chromophore in vitro before isolation of the 340 bp fragment and then processed as in the case of the 340 bp fragment from drug-treated cells to locate the breaks, and (2) a singly end-labeled 248 bp DNA fragment derived from the control 340 bp α-DNA by *Eco*RI* digestion (^{32}P-labeled strand of which corresponds to the ^{32}P-strand that hybridizes with the M13 DNA in Figure 2) was cut with NCS and directly analyzed. The breakage patterns obtained in these two cases were quite similar to that obtained when the α-DNA from untreated cells was hybridized

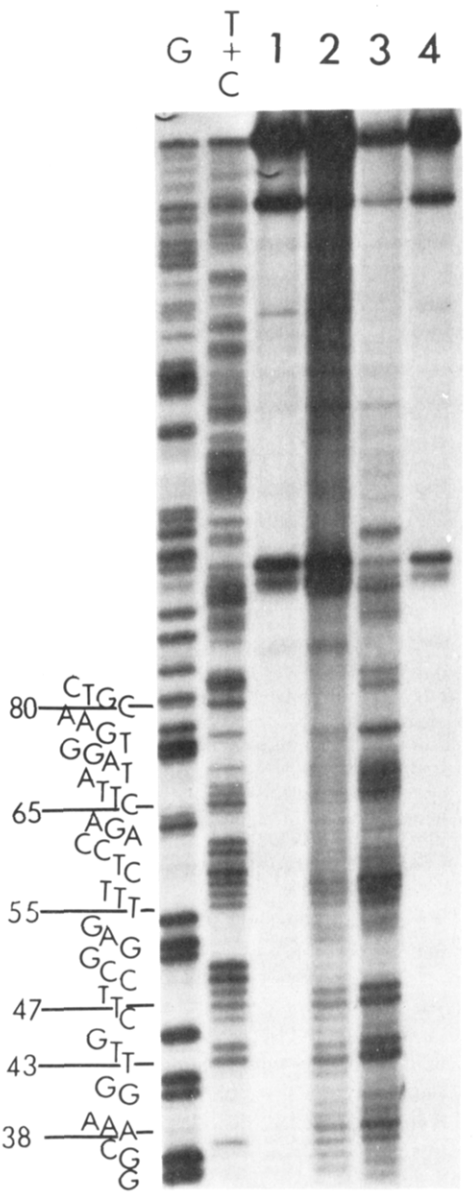

FIGURE 2: Comparison of DNA breakage by NCS chromophore in intact cells and in purified DNA. Cells were treated with 66 μM drug before isolation of the 340 bp α-DNA. The 3′ end ^{32}P-labeled α-DNA fragments from control and drug-treated cells were hybridized with M13 recombinant DNA containing the (−) strand of the α-DNA insert and were analyzed on an 8% sequencing gel. Lanes 1 and 2 represent DNA from control and drug-treated cells, respectively; (lane 3) control DNA as in lane 1 was treated with NCS chromophore (3 μM) at a DNA level of 4.7 μg/mL; (lane 4) minus drug control for the in vitro reaction. Samples (1–4) have been heated in NaOH prior to gel analysis. Lanes G and T + C show the pattern of cleavage in control DNA as in lane 1 by the chemical reactions (Maxam & Gilbert, 1977).

with M13 DNA and then damaged by NCS in vitro. Although an accurate determination of the drug:DNA ratio required to produce a particular degree of DNA breakage in intact cells and in the in vitro reaction cannot be determined from Figure 2, in separate experiments, where total HeLa DNA was treated with NCS chromophore prior to the isolation of the 340 bp α-DNA, we found that about 25–50-fold as much drug was required in intact cells to obtain a comparable signal on the sequencing gel.

In sequencing gels, DNA strands with 5′-terminal nucleoside aldehyde run slower than their counterparts with 5′-PO_4 (Kappen & Goldberg, 1983). With a 3′ end-labeled DNA, therefore, one would expect to see in the absence of alkali treatment two bands for attack at any particular base: one

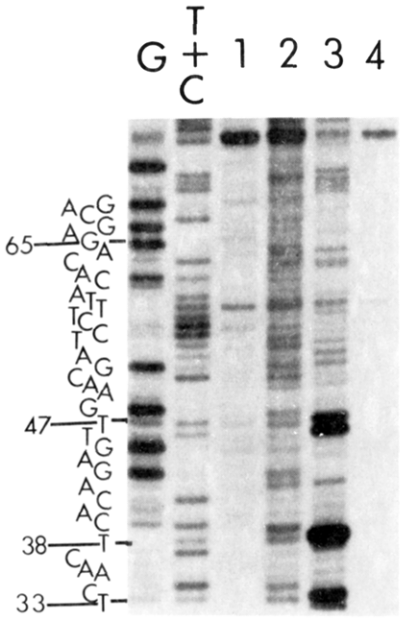

FIGURE 3: Comparison of DNA breakage by NCS chromophore in intact cells and in purified DNA. 340 bp α-DNA, isolated from control and drug (69.6 μM)-treated cells, was ^{32}P labeled at the 5′ end. The (+) strand, purified by hybridization with the M13 DNA as in Figure 2, was analyzed on an 8% sequencing gel. Lanes 1 and 2 show the pattern obtained with DNA from the control and the drug-treated cells, respectively. (3) Control as in lane 1 treated in vitro with NCS chromophore as described in Figure 2; (4) minus drug control for the in vitro reaction. Lanes G and T + C show the standard chemical cleavage pattern obtained for the purified control (+) strand.

each for PO_4 and nucleoside aldehyde ended termini. In experiments similar to those in Figure 2, but without alkali treatment of the DNA prior to gel analysis (not shown), DNA from drug-treated cells gave a pattern of breakage identical with the one obtained in Figure 2, indicating that all fragments produced had 5′-PO_4 termini. The in vitro drug reaction, on the other hand, produced two bands for breakage at each site: the major band of 5′-nucleoside aldehyde ended oligonucleotide and a minor one of 5′-PO_4 termini consistent with earlier findings (Kappen & Goldberg, 1983). This suggests the possibility that the labile nucleoside aldehyde had broken down (to generate 5′-PO_4 termini) in the processing of the DNA before gel analysis. This possibility was confirmed in experiments where a 3′ end-labeled restriction fragment (275 bp), cut with NCS chromophore in vitro, was subjected to the same treatments that the 340 bp α-DNA from drug-treated cells had gone through from its isolation to gel analysis. We found that the intensity of the "nucleoside aldehyde bands" steadily decreased (10–20%) with each of the eight steps.

When the DNA breakage in the α sequence was examined with a ^{32}P label at the 5′ end of the strand (Figure 3), the base specificity of attack in intact cells (lane 2) again closely matched that obtained in the in vitro scission (lane 3) with a preference for T residues over A $\gg$ C > G. A difference between the two reactions, however, is the appearance of two bands (e.g., positions of T_{33}, T_{38}, T_{46}, and T_{47}) at each attack site in the in vivo reaction: one coincident with and the other slower than that obtained for attack at the same site in the in vitro drug reaction and in the standard chemical cleavage, both of which are known to produce 3′-phosphoryl termini (Kappen & Goldberg, 1978; Hatayama et al., 1978; Maxam & Gilbert, 1977). The slower band in this case most likely represents an oligonucleotide with a 3′-hydroxyl.

The possibility of a thiol requirement for DNA damage by NCS in intact cells as in the in vitro reaction was examined

Table III: Effect of Glutathione Depletion on DNA Breakage in HeLa Cells by NCS[a]

	^{32}P incorporated (cpm)			
	control cells		glutathione-depleted cells	
	−phosphatase	+phosphatase	−phosphatase	+phosphatase
−NCS	136	599	142	357
+NCS	821	22298	1360	7492

[a] DNA was isolated after treatment of cells with and without NCS chromophore (34.8 μM) for 30 min at 37 °C; it was heated in 0.1 M NaOH (30 min, 90 °C) prior to the treatment with alkaline phosphatase. Polynucleotide kinase dependent ^{32}P incorporation into 1.2 μg of DNA was measured. A non-enzyme-dependent average background of 1429 cpm has been subtracted from all values.

by depleting the cells of glutathione, the major intracellular nonprotein thiol, and by comparing the DNA damage in normal and thiol-depleted cells. Treatment of cells with BSO, an inhibitor of γ-glutamylcysteine synthetase, one of the enzymes in the biosynthesis of glutathione (Meister & Anderson, 1983), reduced their glutathione content from a normal level of 2–4 μg (per 1 × 10^6 cells) to less than 5 ng, the limit of detection by the assay method used. Concurrent with the thiol depletion, there is a marked decrease (67%) in DNA breakage (Table III). A similar reduction in DNA breakage was also seen when the DNA breakage at low levels of the drug was measured by alkaline sucrose gradient analysis (not shown). These results thus show a dependence on glutathione in DNA breakage by NCS in cells.

Discussion

In intact cells, NCS, like bleomycin (Murray & Martin, 1985), breaks DNA preferentially at the nucleosomal linker regions. Similar findings have been reported for NCS in isolated nuclei (Kuo & Samy, 1978) and in chromatin (Beerman et al., 1983; Hatayama & Yukioka, 1982), but these systems, unlike intact cells, required the addition of a thiol. One main difference between the effect of NCS chromophore in vitro and in intact cells, as has also been reported for other DNA-damaging agents such as ionizing radiation (Lippke et al., 1981), nitrogen mustard (Grunberg & Haseltine, 1980), and bleomycin (Murray & Martin, 1985), is the need in the latter for a much higher level of the agent to inflict the same degree of DNA damage. In the case of NCS, this may be largely due to the inactivation of the highly labile chromophore before it reaches the target DNA in the nucleus, although other causes such as the shielding effect of the nuclear proteins on the DNA may also play a role. Although chromophore is stabilized by binding to HeLa cells (Kappen & Goldberg, 1980), protection by its apoprotein is much more efficient than that by other proteins (Kappen & Goldberg, 1980).

In intact cells, holo-NCS, at the levels used in these studies, is far less effective than the chromophore [holo-NCS at 100 μM did not produce a nucleosomal ladder (not shown)] in producing DNA damage, although in previous work both were found to be equally efficient at low levels in inhibiting DNA synthesis (Kappen et al., 1980). Thus, there appears to be a leveling off of DNA breakage in cells by holo-NCS; this effect, most pronounced at high drug levels, has also been observed previously in experiments where DNA strand breakage was measured by alkaline sucrose gradient analysis (Goldberg et al., 1981). This difference between chromophore and holo-NCS in vivo, but not in the in vitro reaction, may reflect a difference in the availability of the drug to the cell, which in the case of holo-NCS is determined by the rate of release of

the chromophore from its apoprotein. Although it is not yet known with certainty whether holo-NCS is transported across the membrane before it dissociates to release the chromophore or whether the dissociation of holo-NCS occurs at the cell surface and chromophore is then taken in, one can predict that the apoprotein with its strong affinity for the chromophore (dissociation constant, $K_d = 10^{-10}$; Goldberg, 1986) would compete very effectively with cellular uptake by rebinding the chromophore, especially at high levels of drug. In support of an uptake process involving dissociation of the chromophore from the apoprotein at the cell surface is our finding that cell lines simultaneously resistant to several small-molecule anticancer agents due to defective accumulation mechanisms are also resistant to the action of NCS (and its chromophore) (unpublished data).

The chemistry of DNA damage by NCS in intact cells is qualitatively similar in several respects to that in the simple in vitro system using purified DNA. As in the in vitro reaction (Kappen & Goldberg, 1983), the breaks produced by NCS in cells also have nucleoside aldehyde at their 5′-termini (Table I), but unlike the in vitro damage where over 80% of the breaks have a 5′-terminal nucleoside aldehyde only 30–45% of the breaks in cells are found to have 5′-nucleoside aldehyde. This difference is most likely attributable to the limitations of the method used to quantitate the nucleoside aldehyde rather than the true situation. The assay involves three separate reactions (i.e., chemical reduction and enzymatic dephosphorylation and rephosphorylation) all of which require for maximal efficiency single strandedness in the DNA. DNA damage in the in vitro reaction is so extensive (Kappen & Goldberg, 1983) as to result in much smaller fragments (15–25% acid solubilized), arising mostly from double-strand breaks as a result of several single-strand breaks placed within a few base pairs. In small DNA fragments, the 5′-terminal aldehyde should be more accessible to reduction than that at a nick in high molecular weight DNA. Since heat or alkali treatment, two commonly used procedures to denature the DNA prior to the enzymatic assays, would also decompose the nucleoside aldehyde to generate a 5′-PO_4, it is not possible to distinguish between the 5′-PO_4 spontaneously produced by the drug and that generated from the degradation of the nucleoside aldehyde that escaped reduction. On the basis of these limitations, it is reasonable to conclude that the actual amount of 5′-terminal nucleoside aldehyde in DNA damaged in vivo by NCS is significantly greater than that accounted for by the assays in Table I. Despite these quantitative differences, it should be noted that the distribution pattern among the nucleotides of the ^{32}P (reduction dependent) incorporated into the DNA damaged in vivo (Table II) is in excellent agreement with that obtained for in vitro damage where the nucleoside aldehyde distribution was T (73%), A (19%), C (4.7%), and G (3.3%) (Kappen & Goldberg, 1983).

DNA sequencing experiments also show that the base specificity of DNA breakage in the intact cell is the same as in the in vitro reaction, the preferred site of attack being T > A ≫ C > G. In contrast to the in vitro reaction where a 3′ end-labeled DNA gives mainly fragments with 5′-nucleoside aldehyde termini (the mobility of which increases on decomposition, e.g., by alkali-induced β-elimination reactions), the in vivo drug treatment produced fragments all with the faster mobility and presumably with 5′-PO_4 termini. While the absence of nucleoside aldehyde may be attributable to its decomposition in the processing of the DNA for DNA sequencing, the question still remains as to whether the remainder (~70%, Table I) of the 5′-termini had PO_4 spontaneously produced or were products of nucleoside aldehyde removal inside the cell, possibly by an enzymatic repair reaction. It seems likely that the slower of the two bands produced when DNA from NCS-treated cells is labeled with ^{32}P at the 5′ end is due to the removal of the phosphate at the 3′ end of the break by the alkaline phosphatase treatment prior to labeling.

The formation of nucleoside 5′-aldehyde by NCS both in intact cells and in vitro suggests that the molecular mechanism of DNA damage is the same. We have shown that in the in vitro reaction thiol-activated NCS abstracts a hydrogen from C-5′ in DNA to form a presumptive carbon-centered radical intermediate in DNA damage (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985). Dioxygen adds to this lesion, forming a peroxyl intermediate that degrades to generate a strand break and a nucleoside 5′-aldehyde at the 5′ end; in the absence of oxygen, the 5′-carbon-centered radical reacts with the same DNA-bound drug to form a stable covalent adduct between drug and DNA deoxyribose. Spontaneous production of 5′-phosphoryl ends, on the other hand, is reminiscent of the generation of gaps with 5′-PO_4 end groups by NCS in an anaerobic reaction in vitro in which nitroaromatic compounds, such as misonidazole, substitute for O_2 (Kappen & Goldberg, 1984).

Although very high levels of NCS nick DNA in the absence of a thiol in the cell-free system (Goldberg et al., 1981; Chin & Goldberg, 1986), addition of thiol stimulates the reaction at least a thousandfold. In cells, one would expect glutathione, present at millimolar levels (Meister & Anderson, 1983), to be the main activator for NCS. Recent work of DeGraff et al. (1985) has shown that the cytotoxicity of NCS was markedly reduced in glutathione-depleted cells. In the present study, we find that DNA breakage in cells is also glutathione dependent. The residual DNA breakage (33%, Table III) obtained under conditions where cellular glutathione was reduced by more than 99.9% might be due to traces of (undetectable levels) (1) glutathione and/or other cellular thiols activating the drug and/or (2) a non-thiol-dependent DNA breakage by NCS. The amount of breakage, however, appears to be too high to be accounted for by the latter without the involvement of some other cellular factor(s), such as an endonucleolytic activity. Since NCS can undergo autoxidation to generate reduced forms of oxygen (Povirk & Goldberg, 1983; Chin & Goldberg, 1986), it is possible that they can produce DNA damage in intact cells, especially at low cellular glutathione levels, although they appear not to be involved in the in vitro DNA damage reaction (Chin & Goldberg, 1986). Since NCS binds to DNA by an intercalative mechanism, it is conceivable that some of the breaks are protein-associated breaks similar to those reported for other intercalators where topoisomerase II appears to mediate the production of intercalator-induced breaks (Zwelling et al., 1981; Pommier et al., 1984; Tewey et al., 1984; Minford et al., 1986). This notion is compatible with a recent report (Samy et al., 1986), using the alkaline elution method for measuring DNA strand breakage, in which protein-associated breaks were detected in DNA cut in vivo with NCS or auromomycin, the latter an antibiotic that resembles NCS in certain respects (Kappen et al., 1980). It is also possible that the covalent linkage of protein at the strand breaks produced by NCS results from chemical interaction with the DNA aldehyde moieties and/or with DNA sugar-based radical intermediates.

In conclusion, the action of NCS in intact cells resembles that in the in vitro system in its activation by a thiol, in its base specificity of DNA breakage, and, to a large extent, in

the nature of the products of the reaction. This feature should make NCS a useful DNA cleaving agent for studying DNA structures in intact cells.

Registry No. NCS, 9014-02-2; glutathione, 70-18-8.

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