Influence of Thiol Structure on Neocarzinostatin Activation and Expression of DNA Damage†

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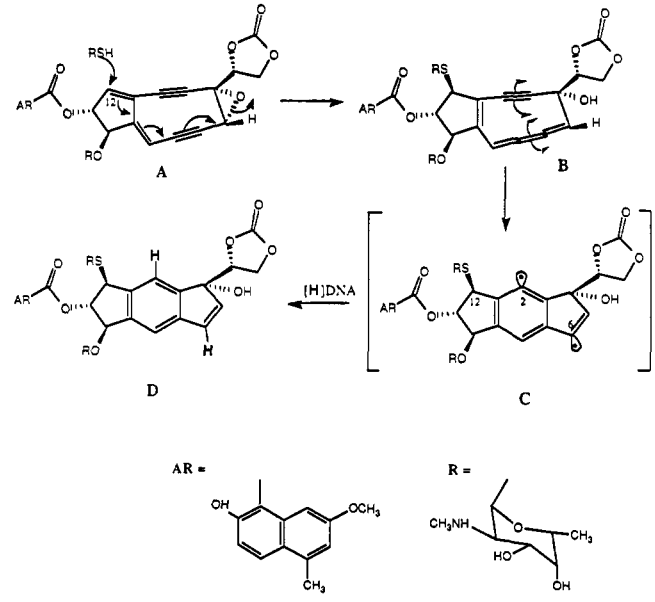
ABSTRACT: Neocarzinostatin (NCS) is an enediyne antitumor antibiotic that cleaves DNA following a thiol-induced electronic rearrangement to a diradical form. Structure–function studies with 11 thiol-containing compounds were undertaken to clarify the role of the thiol in NCS-mediated DNA damage. The rates of activation of NCS in the presence of DNA with the various thiols approximated a Brønsted relation ($\beta = 0.43$, $r^2 = 0.86$), which suggests that the basicity/nucleophilicity of the thiol is important to NCS activation. However, an additional contribution to NCS activation may arise from the affinity of the thiol for DNA, since there is a correlation between the concentration of thiol producing maximal DNA damage, assessed by quantitating the topologic forms of plasmid pBR322 following treatment with NCS, and the apparent ability of the thiol to bind to DNA by hydrophobic or electrostatic interactions. The overall second-order rate constants for the activation of NCS were found to be inversely correlated with the thiol optima; a plot of the former versus the reciprocal of the optimal thiol concentration revealed a first-order rate constant of activation of 0.013 s^{-1} in the presence of DNA. This indicates that maximal DNA damage occurs when NCS is activated with a half-life of 52 s, a relatively slow rate of activation that suggests that NCS binds to DNA before undergoing activation by thiol. Finally, an analysis of strand breaks in pBR322 shows that thiols possessing a carboxylate moiety produce larger quantities of bistranded DNA lesions than their esterified or non-carboxylate-containing counterparts.

The nonprotein chromophore of neocarzinostatin (NCS),[1] the first described member of the enediyne family of antitumor antibiotics (Goldberg, 1991) that includes calicheamicin (Lee et al., 1991), esperamicin (Long et al., 1989), and dynemicin (Sugiura et al., 1990), is the DNA-cleaving portion of the holoantibiotic produced by *Streptococcus neocarzinostaticus*. These agents share a common mechanism of action in spite of significant structural diversity: nucleophilic attack by a thiol results in a Bergman-type electronic rearrangement (Bergman, 1973) to form a diradical species that, positioned in the minor groove, cleaves DNA by abstracting hydrogen atoms from the deoxyribose sugar [for a review of enediyne activity, see Dedon and Goldberg (1992)]. The pathway for the activation of NCS is illustrated in Scheme I, which highlights one of the crucial roles of the thiol in the mechanism of action of NCS.

The bulk of DNA damage produced by NCS arises from the addition of molecular oxygen to the NCS-induced carbon-centered radicals of the deoxyribose sugar to form peroxyradicals. The breakdown of a particular peroxyradical results in the formation of unique products as shown in Scheme I of the accompanying paper (Dedon et al., 1992). C1′-hydrogen atom abstraction ultimately yields a putrescine-cleavable 2′-deoxyribonolactone abasic site (Kappen & Goldberg, 1989), while C4′-chemistry partitions to form either a strand break with 3′-phosphoglycolate- and 5′-phosphate-ended fragments or a 4′-hydroxylated abasic site (Scheme I of Dedon et al., 1992; Saito et al., 1989; Frank et al., 1991; Kappen et al., 1991). The latter lesion is cleaved by putrescine to form phosphate-ended fragments (Lindahl & Andersson, 1972; Povirk & Houlgrave, 1988) or by hydrazine to form a 3′-phosphopyridizine derivative (Sugiyama et al., 1988; Kappen et al., 1991). Finally, removal of the C5′-hydrogen results in a strand break with either a 5′-nucleoside aldehyde and 3′-phosphate (Kappen et al., 1982; Kappen & Goldberg, 1983) or a 5′-phosphate and a labile 3′-formylphosphate-ended DNA fragment (Kappen & Goldberg, 1984; Chin et al., 1987).

Scheme I: Mechanistic Proposal for the Cycloaromatization of NCS to the Diradical State[a]



[a] Based on the proposal of Myers (1987).

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[1] Abbreviations: NCS, neocarzinostatin chromophore; GSH, glutathione; MPA, 3-mercaptopropionate; TG, thioglycolate; CYS, cysteine; MMPA, methyl 3-mercaptopropionate; CSM, cysteamine; E-CYS, ethyl cysteine; MTG, methyl thioglycolate; BME, 2-mercaptoethanol; DTT, dithiothreitol; HTP, 4-hydroxythiophenol; SS, single strand; DS, double strand; AP, apurinic/apyrimidinic; SD, standard deviation.

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The role of the thiol in DNA damage produced by NCS appears to be complicated but is currently thought to involve three major functions: drug activation, positioning of the diradical intermediate in the minor groove of DNA, and reduction of radicals generated in the DNA sugars. The proposed mechanism of activation of NCS involves nucleophilic attack by a single thiol at the C12 position of the native chromophore (Scheme I) (Hensens et al., 1983; Povirk & Goldberg, 1983; Myers & Proteau, 1989). The concentration and physical characteristics of the thiol should thus affect the rate of bimolecular activation of the drug. One of the unresolved questions about the mechanism of DNA damage produced by NCS is whether the drug undergoes activation by the thiol when bound to the DNA or when free in solution.

Following the activation step, the thiol is a covalent adduct of the DNA-cleaving, diradical form of the drug. It is conceivable that the steric bulk, charge, and polarity of the thiol could influence the positioning of the radicals relative to the abstractable deoxyribose hydrogen atoms in the minor groove. In a presumably drug-independent manner, the thiol is then thought to play a role in the reduction of the peroxyradicals and subsequent hydroperoxides that form by addition of molecular oxygen to the NCS-induced carbon-centered radicals of the damaged deoxyribose (Povirk & Goldberg, 1983; Chin et al., 1984; Kappen et al., 1991). The ability of the thiol to reduce these intermediates, determined by the thiol's redox potential, charge and bulk, may influence the ultimate distribution of sugar breakdown products. Additionally, the thiol may also serve to quench the radicals formed on both NCS and the deoxyribose sugar, thus reducing the quantity of DNA damage under conditions of excess thiol (Kappen & Goldberg, 1978).

In vitro studies indicate that any of a number of different thiol-containing compounds (see Figure 1 for structures) can serve as activator, including 2-mercaptoethanol (BME) (Poon et al., 1977; Kappen & Goldberg, 1978; Boye et al., 1984), 4-hydroxythiophenol (HTP) (Saito et al., 1989), dithiothreitol (DTT) (Kappen & Goldberg, 1978), and glutathione (GSH) (Kappen et al., 1980). GSH is thought to serve as the activating thiol in vivo, since reduction of GSH levels results in a decrease in NCS-mediated cytotoxicity and DNA damage (DeGraff & Mitchell, 1985; DeGraff et al., 1985; Kappen et al., 1987). The different thiol activators appear to have unique effects on both the quantity and quality of NCS-mediated DNA damage. Studies of DNA damage in vitro demonstrate that, compared to BME, GSH produces a larger quantity of direct double-strand breaks (DS) (Poon et al., 1977; Dedon & Goldberg, 1990). This relationship holds for the two most frequent sites of bistranded DNA damage produced by NCS: AGT·ACT and AGC·GCT, in which BME not only produces fewer bistranded lesions (Povirk & Goldberg, 1985; Povirk & Houlgrave, 1988; Dedon & Goldberg, 1990) but also produces a different distribution of sugar breakdown products (Dedon et al., 1992; Kappen et al., 1991).

In this and the accompanying paper, we have undertaken a series of structure–function studies with a variety of different thiols (Figure 1) to begin to dissect the many roles of the thiol in NCS-mediated DNA damage. The present work quantitates the NCS/thiol-mediated SS and DS breaks in plasmid pBR322 and examines the kinetics of activation of NCS by the thiols.

Experimental Procedures

Materials. Neocarzinostatin chromophore was isolated from the holoantibiotic (Kayaku Antibiotics Research Co., Ltd.) as described (Kappen et al., 1980) and stored at −70 °C in

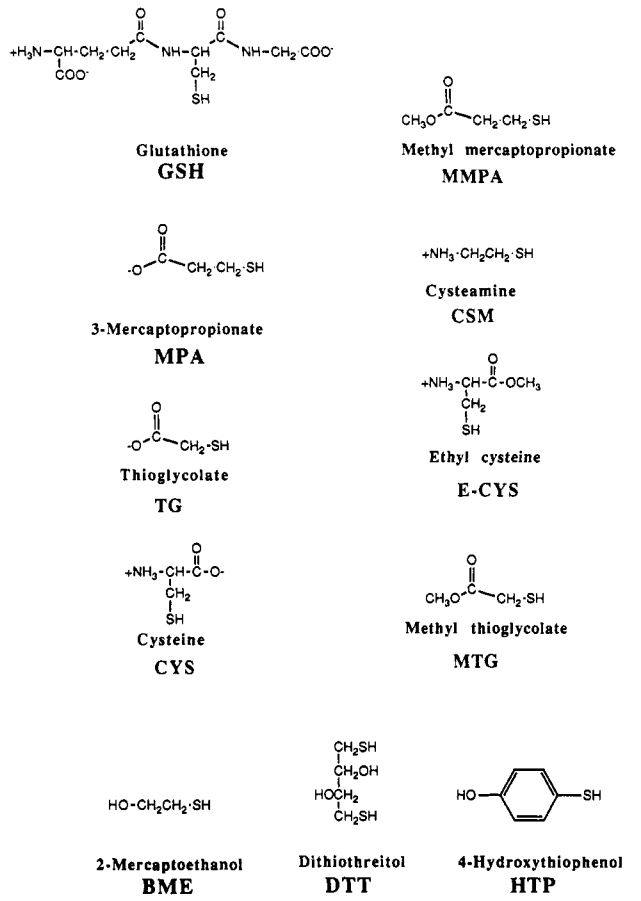

FIGURE 1: Thiol structures and abbreviations.

methanol at a stock concentration of 456 μM. Plasmid pBR322 was obtained from Boehringer Mannheim and BRL and consisted of 85–90% supercoiled plasmid and 10–15% form II DNA.

Determination of the Thiol Concentration Causing Maximal NCS-Mediated Damage in pBR322. Drug/DNA reactions consisted of 30 μg/mL pBR322, 6 mM EDTA, 50 mM HEPES, pH 7.4, and thiol, added as either aqueous (GSH, TG, MPA, CYS, CSM, E-CYS, BME, and DTT) or methanolic (MMPA, MTG, and HTP) solutions. NCS was added to a final concentration of 10 nM to start the reaction, which was allowed to proceed for 1.5 h at 0 °C. This concentration of NCS is well below that which results in "one-hit" kinetics. To cleave abasic sites, and thus to express them as strand breaks, either hydrazine (1 M, pH 8), for 4′-hydroxylated abasic sites (Sugiyama et al., 1988; Kappen et al., 1991), or putrescine (1 M, pH 8), for all abasic sites (Lindahl & Andersson, 1972; Povirk & Houlgrave, 1988), was added to a final concentration of 100 mM and the solution was incubated at room temperature or 37 °C, respectively, for 1 h. The topologic forms in 0.2 μg of drug-treated plasmid DNA were resolved on a 1% agarose gel. Densitometry of the negative images of the ethidium bromide-stained gel (LKB Ultrascan XL) showed linear variation of signal with DNA concentration over the DNA range studied (Dedon & Goldberg, 1990). An adjustment was made to the form I signal given its 70% fluorescence intensity compared with forms II and III (Lloyd et al., 1978). The final concentration of methanol from the thiol and NCS was never more than 4% of the reaction solution.

Quantitation of SS and DS Breaks Produced by Thiols and NCS in pBR322. The fluorescence photographic technique was again employed to determine the quantities of SS and DS

breaks produced by NCS in the presence of various thiols. Reactions were exactly as described for the thiol optimization studies, except that the thiol was present at its optimal concentration and the NCS concentration was varied between 2.5 and 20 nM. Following the 1.5-h treatment with NCS at 0 °C, hydrazine or putrescine was added to 100 mM and cleavage of abasic sites was allowed to proceed for 1 h at room temperature or 37 °C, respectively, as described above.

The number of SS and DS breaks per molecule of plasmid were determined by assuming a Poisson distribution for the purposes of comparison (Povirk et al., 1977). The quantity of total strand breaks, the sum of the number of SS (n_{ss}) and DS (n_{ds}) breaks, was calculated from the fraction of form I DNA remaining (f_I) as $f_I = e^{-(n_{ss}+n_{ds})}$, while DS breaks were calculated from the fraction of form III DNA (f_{III}) resulting from drug treatment: $f_{III} = n_{ds}e^{-n_{ds}}$. Again, the fluorescence of form I DNA was corrected for reduction in ethidium staining compared to forms II and III DNA. Plots of the SS versus the DS breaks per plasmid molecule were linear for all the thiols.

Kinetics of Activation of NCS in the Presence of DNA. The rate of loss of NCS from an aqueous solution containing thiols can be written as a two-termed rate law:

$$-d[NCS]/dt = k_{obsd}[NCS][RSH/RS^-] + k_2[NCS] = k_3[NCS]$$

where k_{obsd} is the second-order rate constant for the activation of NCS by thiol, k_2 is the pseudo-first-order rate constant for the degradation of NCS in aqueous solution (Povirk & Goldberg, 1980), and k_3 is the overall first-order rate constant when $[RSH/RS^-] \gg [NCS]$. The rate constants for the activation of NCS by the various thiols in the presence of DNA were determined by monitoring the production of the thiol–NCS adduct that fluoresces at 430 nm upon excitation at 340 nm; native NCS also fluoresces under these conditions but with 7-fold lower intensity than the thiol-inactivated form (Povirk & Goldberg, 1980). Degradation of NCS in aqueous solution produces a species with excitation and emission maxima of 380 and 490 nm, respectively (Povirk & Goldberg, 1980). By monitoring thiol–NCS adduct formation only, k_3 becomes the pseudo-first-order rate constant for NCS activation, since degradation of NCS (k_2) is not observed.

The pseudo-first-order rate constants, k_3, were determined for several thiol concentrations from the integrated rate equation

$$\ln (F_\infty - F_t) = \ln F_\infty - k_3 t$$

where F_∞ is the maximum 430-nm fluorescence produced during the reaction, which is proportional to the starting concentration of NCS, and F_t is the fluorescence at time t; $(F_\infty - F_t)$ is proportional to the concentration of NCS at time t. The rate constant was determined for each thiol in a reaction consisting of 2 mL of 30 μg/mL sheared calf thymus DNA and 75 mM HEPES, pH 7.4, to which thiol was added as described above. A 5-μL aliquot of NCS (45.6 μM) was then added to start the reaction (final [NCS] = 110 nM), which was monitored at room temperature with a spectrofluorometer (Perkin-Elmer 512). The $t = \infty$ fluorescence value was taken as that resulting from at least a 5-min reaction for each of the thiols at the following concentrations: GSH, 5 mM; MPA, 10 mM; CYS, 5 mM; TG, 30 mM; MMPA, 10 mM; CSM, 0.5 mM; E-CYS, 5 mM; MTG, 1 mM; BME, 10 mM; DTT, 3 mM. Plots of $\ln (F_\infty - F_t)$ versus time were linear for all the thiols at all concentrations studied, and examples of such plots are shown in Figure 4A. Thiol was always at least 3 orders of magnitude more concentrated than NCS, and dilutional effects caused by addition of the thiol were never more than 3%. There was no detectable change in fluorescence over 5 min of reaction in the absence of thiol.

The pseudo-first-order rate constant for the degradation of NCS, k_2, was determined in a manner similar to that described above for thiol activation, except that fluorescence was monitored at 490 nm with excitation at 380 nm, and the reaction occurred without added thiol.

Results

Characteristics of NCS-Mediated DNA Damage in Plasmids. The lesions produced by NCS in DNA consist of either direct strand breaks or alkali-labile abasic sites. NCS-mediated damage in plasmid pBR322 was characterized by quantitation of the agarose-resolved forms I, II, and III DNA (supercoiled, nicked circular, and linear forms, respectively) produced by a range of NCS concentrations (5–20 nM). The numbers of SS and DS breaks per plasmid molecule were determined for each NCS concentration by assuming a Poisson distribution for NCS-induced lesions, and the number of breaks per plasmid molecule per micromolar NCS was derived from the linear plots of strand breaks per plasmid versus NCS concentration (data not shown). Figure 2 shows the quantities of total, SS, and DS breaks for eleven different thiols at their optimal concentrations (vide infra), with strand breaks expressed under three different conditions: direct breaks alone and in combination with abasic sites expressed as strand breaks by hydrazine or putrescine. The results with direct SS and DS breaks ignores the presence of abasic sites produced by NCS, of which two types are known to form, as discussed earlier. The 2′-deoxyribonolactone abasic site produced by C1′-chemistry has been shown to occur at the C of AGC as part of a bistranded lesion (Kappen et al., 1988; Povirk & Houlgrave, 1988; Kappen & Goldberg, 1989). The other type of abasic site, the 4′-hydroxylated abasic site generated by C4′-chemistry (Saito et al., 1989; Frank et al., 1991; Kappen et al., 1991), occurs in both SS and bistranded lesions involving the T of both AGT and TGT (see Dedon et al., 1992), the predominant sites for direct DS breaks with NCS (Dedon & Goldberg, 1990). To reveal the presence of abasic sites as strand breaks, NCS-treated pBR322 DNA was exposed to either hydrazine or putrescine. Hydrazine reacts with the C4′-hydroxylation product to form a pyridazine derivative at the 5′-end of the cleavage site (Sugiyama et al., 1988), while putrescine reacts with virtually all abasic sites to produce strand breaks (Lindahl & Andersson, 1972; Povirk & Houlgrave, 1988). In control experiments with AGT- and AGC-containing restriction fragments treated with NCS, it was determined that hydrazine cleaved predominantly the 4′-hydroxylated abasic sites, while putrescine cleaved these sites as well as the 2′-deoxyribonolactone abasic sites (data not shown). Both agents were similar in the efficiency of their reaction with the 4′-hydroxylated abasic site (data not shown). The two abasic sites are formed in roughly equal proportions for the 11 thiols.[2]

As expected, the majority of NCS-mediated damage in plasmid DNA was SS in nature under all three conditions of abasic site expression (Figure 2). The SS breaks fell within a 1.7-fold range for the different thiols, while DS breaks varied over a 25-fold range, which suggests that the nature of the thiol affects the production of DS breaks more than SS breaks.

[2] Hydrazine treatment produced an average increase in total strand breaks of 18% ± 7% for the 11 thiols, while putrescine caused an increase of 35% ± 13% (errors are SD). The two values are significantly different with $p < 0.01$ by t test.

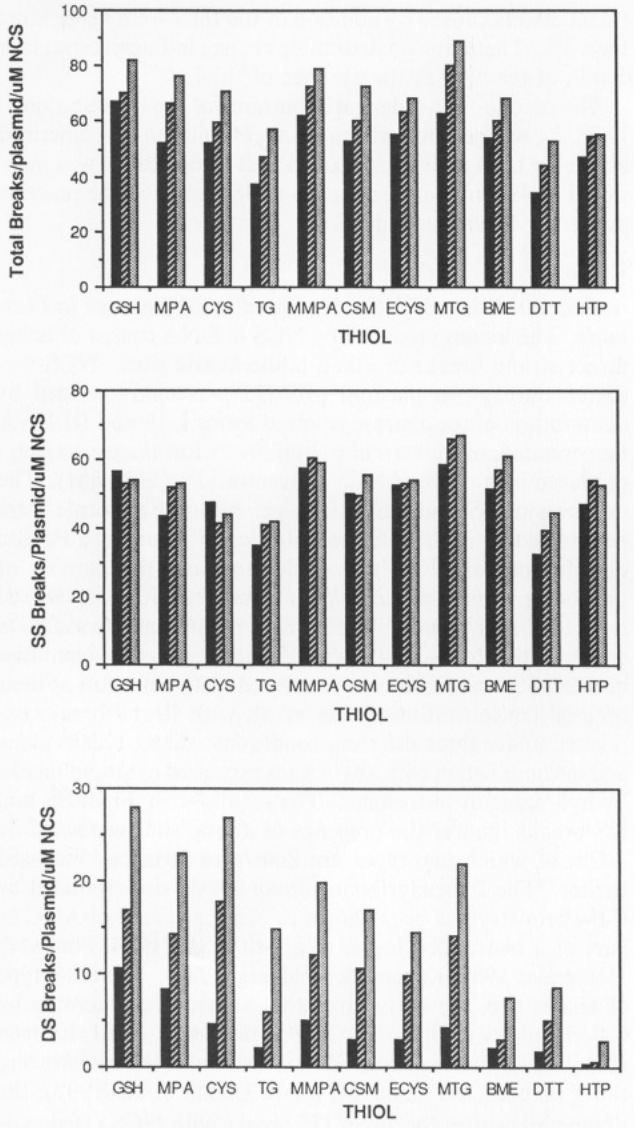

FIGURE 2: Effect of the thiol activator on NCS-mediated DNA damage in plasmid pBR322. NCS-mediated strand breaks were determined in plasmid DNA under three different conditions: direct strand breaks, solid bars; total breaks after hydrazine treatment, hatched bars; and total breaks after putrescine treatment, stippled bars. Total damage is the sum of SS and DS breaks. The values presented are averages from 2–4 experiments, with the thiols at their optimal DNA-damaging concentrations. See text for details.

While the quantity of SS breaks produced by the various thiols changed little under the three conditions of abasic site expression, there was a large increase in the quantity of DS breaks: 2.6-fold (±0.9 SD) and 4.8-fold (±1.4 SD) following treatment with hydrazine and putrescine, respectively. The increases in DS breaks largely account for the consistent increases in total breaks caused by both hydrazine (increases ranging from 5 to 28% for the 11 thiols)[2] and putrescine (22–55%)[2] treatment of DNA samples treated with NCS; DNA samples not treated with NCS show no changes upon exposure to hydrazine or putrescine under the conditions employed here (data not shown).

Ratios of SS to DS breaks for the different thiols at their optimal concentrations (vide infra) are listed in Table I and examples of the plots, all of which are linear, are shown in Figure 3B. Previously, the SS:DS ratios for *direct* breaks with BME and GSH were determined to be 41 and 6, respectively, following a 5-min cleavage reaction (Dedon & Goldberg, 1990). As will be discussed in detail in the accompanying paper, this short reaction time prevented the full expression

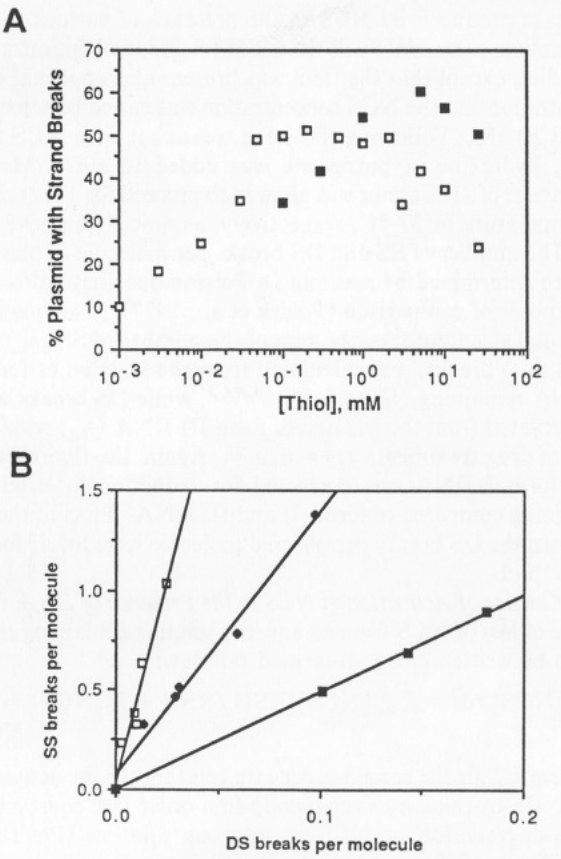

FIGURE 3: Determination of optimal thiol activator concentrations and SS:DS ratios for NCS-mediated damage in pBR322. (A) The quantity of NCS-induced strand breaks (SS + DS) in plasmid DNA was determined for different concentrations of the thiol activator following hydrazine treatment of the damaged DNA as described in Materials and Methods. Results are shown for GSH (■) and CSM (□). (B) The number of NCS-induced SS and DS breaks per plasmid molecule was determined by the application of a Poisson distribution to the quantity of forms I, II, and III DNA determined by fluorescence photography, as described in Materials and Methods. Results are shown for NCS/DTT-induced damage revealed under the following conditions: direct strand breaks (control) (□), strand breaks after hydrazine treatment (◆), and strand breaks after putrescine treatment (■). The DTT concentration was 5 mM.

of direct DS breaks due to the slow formation of 3′-phosphoglycolate-ended fragments ($t_{1/2} \sim 12$ min). The reaction time was extended to 1.5 h in the present work, which is enough time for complete expression of such direct strand breaks. This resulted in an increase in the quantity of DS breaks produced by GSH and BME, lowering the ratios to 5.3 and 22, respectively (Table I).

Determination of the Concentration of Thiol Producing Optimal DNA Damage. Since previous studies have demonstrated that maximal NCS-mediated DNA damage occurs at an optimal thiol concentration (Kappen & Goldberg, 1978), the concentration of each thiol that produced maximal direct and hydrazine-sensitive DNA damage was determined. Plasmid pBR322 was treated with 10 nM NCS and thiol at several different concentrations, the 4′-hydroxylated abasic sites were cleaved with hydrazine (vide supra), and the agarose-resolved plasmid forms were quantitated by fluorescence photography. Examples of the plots of the quantity of damaged plasmid (forms II + III) versus thiol concentration are shown in Figure 3A for glutathione (GSH) and cysteamine (CSM), for which maximal DNA damage was produced by concentrations of 5 and 0.2 mM, respectively. The optimal concentration, listed for each thiol in Table I, was determined to be the concentration of thiol giving maximal DNA damage

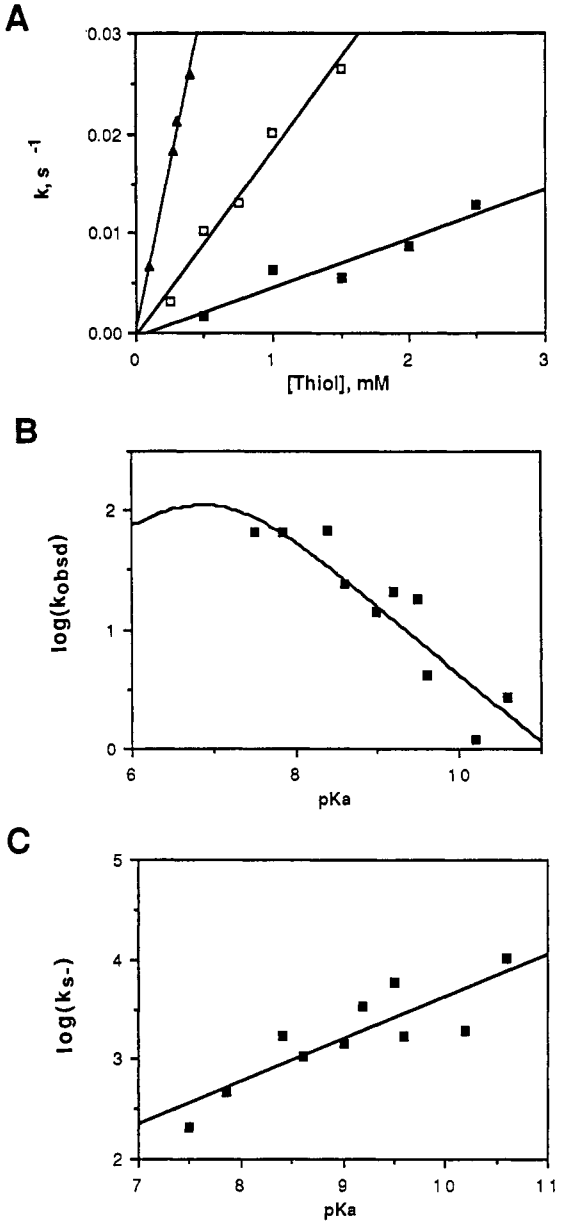

FIGURE 4: Second-order rate constants for activation of NCS by thiol in the presence of DNA: A Brønsted relation between pK_a and k_{s-}. (A) The psuedo-first-order rate constants for the activation of NCS at different thiol concentrations are plotted against the thiol concentration to derive the overall second-order activation rate constants (see Materials and Methods). The results are shown for MMPA (□), MPA (■), and E-CYS (▲). (B) Plot of log k_{obsd} vs thiol pK_a for the activation of NCS. The line was generated from log $k_{obsd} = -0.65 + 0.43pK_a - \log 10^{pK_a-7} + 1)$. (C) Plot of log k_{s-} vs thiol pK_a for NCS activation. Least-squares fitting of the data generated a line with the following equation: log $k_{s-} = -0.65 + 0.43pK_a$. See text for details.

or the first concentration occurring past the shoulder of a curve that reaches a plateau. Cleavage of additional abasic sites (C1′-mediated) with putrescine resulted in a 2–20% increase in DNA damage over hydrazine treatment without affecting the optimal thiol concentration (data not shown).

Values for the optimal thiol concentrations range over 2 orders of magnitude, which indicates significant differences between the individual thiols with respect to NCS-mediated DNA damage. The values determined here are in agreement with optimal thiol concentrations for BME (10 mM) and DTT (5 mM) determined by Kappen and Goldberg (1978) for the acid solubilization of linear DNA, but their value for CYS differs significantly from the optimum of 0.5 mM determined here. In the earlier studies, Kappen and Goldberg used the

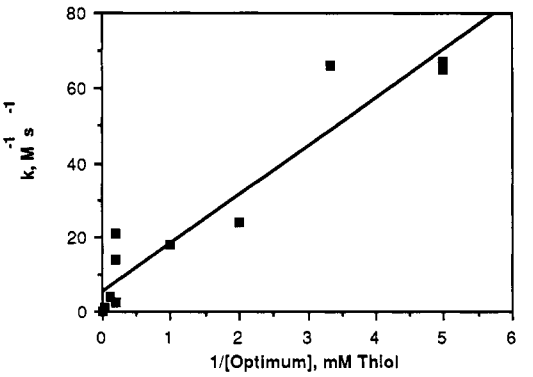

FIGURE 5: Relationship between the concentration of thiol producing optimal NCS-mediated DNA damage and the overall second-order rate constant for the activation of NCS in the presence of DNA.

holoantibiotic at pH 8, the latter condition probably resulting in a significant negative charge for CYS ($pK = 8.6$ for the sulfhydryl of CYS).

The Nature of the Thiol Affects the Rate of Activation of NCS. The second-order rate constants for activation of NCS by the different thiols, k_{obsd}, are shown in Table I. The constants, determined at 23 °C and pH 7 in the presence of DNA, were calculated from the slopes of plots of the pseudo-first-order rate constants, k_3, versus their determining thiol concentrations, as demonstrated in Figure 4A for MMPA, MPA, and E-CYS. The values in Table I for GSH and BME are in reasonable agreement with the rate constants of 28.2 M^{-1} s^{-1} and 1.9 M^{-1} s^{-1}, respectively, determined at pH 8 with 32 μg/mL calf thymus DNA (V. Favaudon and I. H. Goldberg, unpublished observations).

Of particular interest here is the relationship between the basicity of the thiol and its rate of NCS activation. The rate constant for NCS activation by thiolate anion, k_{s-}, can be calculated from plots of k_3 versus the concentration of thiolate determined from the thiol's pK_a (shown in Table I); the relationship $k_{s-} = k_{obsd}(10^{pK_a-7} + 1)$ yields similar values (Whitesides et al., 1977; Szajewski & Whitesides, 1980). A plot of log k_{s-} for each thiol versus the thiol pK_a results in straight line, which reflects a Brønsted relationship with $\beta_{nuc} = 0.43$ ($r^2 = 0.86$), as shown in Figure 4C.

A plot of the rate of activation for each thiol, except HTP, versus the reciprocal of the concentration of the thiol that produces optimal DNA damage is shown in Figure 5. A strong correlation ($r^2 = 0.96$) is apparent between these two parameters, and the slope represents an optimal first-order rate constant for activation of 0.013 s^{-1}, which gives a half-life for the reaction of 52 s. Degradation of NCS, on the other hand, occurs with a rate constant of 0.0049 s^{-1}, which gives a half-life of 141 s. This value is in reasonable agreement with the degradation rate constant of 0.0064 s^{-1} determined with 47 mg/mL calf thymus DNA at pH 7 by Povirk et al. (1981).

Discussion

Thiol Nucleophilicity and Apparent DNA Affinity Affect the Rate of NCS Activation. The members of the enediyne family all possess a common mechanistic feature: a Bergman-type cyclization of the enediyne structure to produce a diradical intermediate (Bergman, 1973; Zein et al., 1988; Long et al., 1989; Myers & Proteau, 1989; Sugiura et al., 1990). The cyclization reaction is held in check by a high activation energy barrier imposed by a carbon–carbon double bond at the bridgehead of calicheamicin, esperamicin, and dynemicin and by the C12–C1 unsaturation in NCS [see Scheme I; for review see Dedon and Goldberg (1992)]. Attack by the thiol

Table I: Thiol Concentration Optima, Rates of NCS Activation, and Strand Break Ratios[a]

thiol	optimum concn[b] (mM)	thiol pK_a[c]	k_{obsd}[d] (M^{-1} s^{-1})	k_s-[e] (M^{-1} s^{-1})	SS:DS[f] direct	hydrazine	putrescine
GSH	5	9.0	14	1.4×10^3	5.3	3.7	2.2
MPA	5	10.6	2.7	1.0×10^4	5.3	4.7	2.7
CYS	0.5	8.6	24	1.0×10^3	8.8	3.1	2.3
TG	25	10.2	1.2	1.9×10^3	11	5.7	2.3
MMPA	1	9.5	18	5.9×10^3	11	6.2	3.4
CSM	0.2	8.4	67	1.7×10^3	17	5.7	3.8
E-CYS	0.3	7.5	66	2.1×10^2	18	6.8	4.1
MTG	0.2	7.8	65	4.6×10^2	16	7.3	3.3
BME	10	9.6	4.2	1.7×10^3	22	17	7.7
DTT	5	9.2	21	3.4×10^3	38	14	5.2
HTP	0.2	6.5	nd[g]	nd[g]	104	100	30

[a] Structures of the different thiols are shown in Figure 1. The groupings of the thiols are described in the text. [b] Concentration of thiol producing maximal NCS-mediated damage in plasmid DNA after hydrazine treatment; see text for details. [c] The pK_a values are averages of literature values from the following sources: GSH, references in Szajewski and Whitesides (1980) and Crampton (1974); MPA, Irving et al. (1964); CYS, references in Crampton (1974) and Danehy and Noel (1960); TG, Sekine et al. (1974) and Danehy and Noel (1960); MMPA, Hupe and Jencks (1977); CSM, Danehy and Noel (1960) and references in Crampton (1974); E-CYS, references in Crampton (1974); MTG, Hupe and Jencks (1977) and references in Crampton (1974); BME, Hupe and Jencks (1977); DTT, Whitesides et al. (1977); HTP, estimated from Hupe and Jencks (1977) and Whitesides et al. (1977). [d] Observed second-order rate constant for activation of NCS in the presence of DNA, pH 7. [e] Second-order rate constants for thiolate anion activation of NCS in the presence of DNA, pH 7. [f] Ratio of SS to DS breaks produced by the thiol and NCS in pBR322 DNA; plasmid DNA was subsequently treated with the indicated agent to reveal abasic sites as strand breaks. [g] Not determined; spectral properties of HTP prevented spectroscopic determination of rate constants.

at the C12 position of NCS in a Michael-type addition lowers the energy barrier and allows spontaneous aromatization to the diradical form (Myers & Proteau, 1989; Snyder, 1989; Magnus et al., 1990). The nucleophilicity/basicity of the thiol should thus have a significant influence on the rate of activation of NCS, a hypothesis supported by the finding of a Brønsted relation with $\beta = 0.43$ (Figure 4C). This value for β is similar to that found for other thiol reactions, including thiol–disulfide interchange, $\beta \approx 0.4$–0.5 (Whitesides et al., 1977; Wilson et al., 1977; Szajewski & Whitesides, 1980); *p*-nitrophenyl acetate, $\beta = 0.38$ (Ogilvie et al., 1964); and *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide, $\beta = 0.42$ (Sekine et al., 1974). The curve in Figure 4B suggests that thiols with pK_a's lower than ~7 will probably not be more efficient activators of NCS under physiologic conditions, except possibly HTP and other aromatic thiols, for which the reactivity in thiol–disulfide interchange reactions is higher than for alkyl thiols of equal basicity (Wilson et al., 1977). Despite the well-characterized free-radical addition of thiols to unsaturated bonds (Griesbaum, 1970) and the fact that NCS can be activated in a radical-mediated process by carboxyl radical (Favaudon et al., 1985), it seems unlikely that a radical-mediated process is responsible for the attack by thiols at the C12 of NCS given the absence of a radical initiation mechanism.

While the nucleophilicity/basicity of the thiol probably contributes significantly to the activation of NCS, an examination of the thiol concentrations producing maximum DNA damage suggests that other factors may also be involved. The thiol optima are inversely correlated with the k_{obsd} for NCS activation ($r^2 = 0.96$), as shown in Figure 5, but they do not correlate well with the thiol pK_a's: CYS, CSM, ECYS, MTG, and HTP have similar optimal concentrations of 0.2–0.5 mM, while their pK_a values range over 2.5 units (Table I). One possible factor that may be influencing NCS activation in the presence of DNA is the nature of the physical interaction of the thiol with DNA. There appears to be a correlation between the concentration optima and an apparent affinity of the thiols for DNA: the positively charged (E-CYS and CSM) and hydrophobic thiols (MTG, MMPA, and HTP) have significantly lower concentration optima than their negatively-charged and water-soluble counterparts (compare to BME, DTT, TG, MPA, and GSH in Table I). One complicating factor in this relationship is that structural features that enhance the basicity of the thiols (the presence of an amino group in the alkyl thiols and the esterification of carboxylate moieties) also impart positive charge or hydrophobicity.

In the case of the water-insoluble thiols, a hydrophobic interaction may be causing high concentrations of the thiols to accumulate on or near the DNA. Such hydrophobic effects appear to be an important element in model ligand/receptor interactions in aqueous media, with strong correlations between the water insolubility of the ligand and the association constant for binding to the hydrophobic cavity of the host (Shepodd et al., 1988). The relatively low dielectric environment of the minor groove compared to the bulk solvent in aqueous solution (Marky & Breslauer, 1987) has been implicated as a driving force for the interaction of a number of small nonpolar molecules with DNA, such as acetone (Rokita et al., 1990), netropsin (Markey & Breslauer, 1987), and the water-insoluble enediynes such as calicheamicin and neocarzinostatin (Zein et al., 1990; Ding & Ellestad, 1991).

A more definitive DNA-binding affinity is apparent with the positively charged thiols (CSM and E-CYS). Like the DNA-binding polyamines, the aminothiol CSM has been shown to bind strongly to DNA and, at high concentration, to stabilize the B geometry, possibly by virtue of an electrostatic interaction between the amine and the phosphate groups (Liquier et al., 1983). This ability of CSM, for example, to concentrate on DNA and its proposed localization along the edges of the grooves may explain why the basicity of a particular thiol does not entirely correlate with its optimal DNA-damaging concentration. In support of the hypothesized correlation between positive charge and low optimum concentration, Kappen and Goldberg (1978) found that the radiation protector *S*-(2-aminoethyl)isothiuronium bromide, which rearranges spontaneously to form the positively-charged 2-mercaptoethylguanidine in neutral aqueous solution, displayed a significantly lower optimum concentration (0.5–1 mM) than BME or DTT (10 and 5 mM, respectively).

Activation of NCS Appears To Occur after the Drug Binds to DNA. Whatever the physical basis, whether it is basicity or DNA affinity, a relationship exists between the thiol optima and the NCS activation rate constants. This is probably an important feature of NCS activity, since a plot of these parameters is linear ($r^2 = 0.96$) with a slope that represents a first-order rate constant of 0.013 s^{-1} (Figure 5). Maximal

DNA damage is thus produced when NCS is activated at an optimal rate, that is, when it is activated with a half-life of 52 s under the conditions employed here. This rate of activation stands in sharp contrast to the rate of binding of NCS to DNA determined by Dasgupta et al. (1985): 288 s^{-1}, or $t_{1/2}$ = 0.002 s (adjusted for DNA concentration and temperature). NCS has a strong affinity for DNA, with an equilibrium binding constant of 5×10^6 M^{-1} (Povirk et al., 1981), and binds in what appears to be a two-step process, with either external and intercalative binding modes or two types of intercalative binding (Dasgupta et al., 1985). Since the drug binds to DNA much more rapidly than it is activated by thiol, it seems reasonable to conclude that NCS is activated after it has bound to DNA rather than when it is free in solution; the addition of NCS *after* the thiol in the kinetics experiments gives the thiol an opportunity to activate NCS before it binds to DNA. At which stage of drug binding the activation occurs, before or after it has assumed its most stable orientation, cannot be determined from the data. DNA appears to act as a sort of catalyst for its own destruction by providing an environment that brings together both the thiol activator and the NCS. Nature has compensated for the reduced DNA binding affinity of GSH, the activator of NCS in vivo, by providing a relatively high concentration of the thiol of 1–5 mM in most eukaryotic cells (Metzler, 1977).

The requirement for an optimal rate of activation can be rationalized by considering that, since the rate of degradation of NCS to a nondamaging species ($t_{1/2}$ = 141 s under the conditions of the experiments here) would compete successfully with the activation step, a thiol concentration lower than the optimal level would reduce the total quantity of DNA damage.

Thiol Structure as It Affects NCS Activity. A second important set of relationships can be found by comparing the SS:DS ratios to the structures of the different thiols (see structures in Figure 1). The thiols can be divided into three broad groups on the basis of the magnitude of the SS:DS ratios as shown in Table I: group 1, MPA, GSH, CYS, and TG; group 2, MMPA, CSM, E-CYS, and MTG; and group 3, BME, DTT, and HTP. In order of increasing SS:DS ratios (decreasing quantities of DS breaks), the groups can be arranged as follows: group 1 > group 2 > group 3. These groupings and their relative order holds for all three types of bistranded lesions, that is, for direct DS breaks, hydrazine-sensitive DS breaks (4′-hydroxylated abasic sites), and putrescine-sensitive DS breaks (both 4′-hydroxylated and 2′-deoxyribonolactone abasic sites).

These three groups differ from each other structurally by at least one significant feature: the presence or absence of a carboxylate group. The thiols producing the greatest quantity of bistranded lesions, group 1, all possess a carboxylate moiety. Removing or esterifying the carboxyl group results in the thiol structures comprising group 2, with a concomitant reduction in the quantity of bistranded lesions produced when these thiols activate NCS (compare MPA with MMPA, CYS and CSM and E-CYS, and TG with MTG). Group 3 thiols, those producing the fewest bistranded lesions, possess no charged moieties and are structurally different not only from each other but also from the thiols of groups 1 and 2. It has been hypothesized that a negative charge on the thiol contributes to the formation of glycolate (Frank et al., 1991; Kappen et al., 1991). However, it appears that a simple net negative charge is not solely responsible for the small SS:DS ratios of the group 1 thiols, since the amino acid CYS has a net charge of nearly zero at physiologic pH and produces more DS breaks than its positively charged ethyl ester.

While it is impossible to determine from the present data what role the thiol carboxyl group plays in the DNA damage produced by NCS, it is possible that there is an ionic interaction between the amino sugar of NCS and the negatively-charged carboxyl group of the covalently-bound thiol that influences the orientation of the drug in the minor groove. Alternatively, repulsive interactions between the carboxylate and the phosphate of the DNA backbone could provide optimum orientation of the activated drug to produce bistranded lesions. Molecular modeling and dynamics studies with NCS binding at AGC·GCT, one of the trinucleotide sequences involved in bistranded lesions with NCS (Kappen et al., 1988; Povirk & Houlgrave, 1988), suggest that the presence of GSH as an NCS adduct forces the drug deeper into the minor groove than the structure without the thiol present (Galat & Goldberg, 1990). This orientation places the C2 radical of the drug very close to the C1′-hydrogen atom of the C of AGC and creates an ideal situation for the generation of a bistranded lesion. It is also possible that variability in the relative quantities of SS and DS lesions may reflect internal quenching of the C2 radical of the drug by hydrogen abstraction from the adducted thiol, as discussed in the accompanying paper (Dedon et al., 1992).

Abasic Sites as SS and DS Lesions. Treatment of plasmid DNA with NCS results in the formation of two types of abasic site: the 4′-hydroxylated abasic site and the 2′-deoxyribonolactone. When these sites are converted to strand breaks with hydrazine and putrescine, there is an increase in the total number of strand breaks in the plasmid DNA (Figure 2). The increase in total strand breaks reflects not only expression of abasic sites present in bistranded lesions (now apparent as DS breaks) but also expression of abasic sites that occur as SS lesions as well. If all abasic sites occurred only in bistranded lesions, hydrazine and putrescine treatment would not cause an increase in total breaks, which is the sum of SS and DS breaks, but only a conversion of a SS break into a DS break. These findings suggest that abasic sites can occur as SS lesions as well as in bistranded DNA damage. This is in agreement with the finding of abasic sites at the T of AGT in restriction fragments [see Dedon et al. (1992)], as well as with the observations of Bose et al. (1980) in NCS-treated ColE1 DNA, that an apurinic/apyrimidinic endonuclease from human lymphocytes increased total strand breaks by a factor of 1.6 compared to direct strand breaks. Povirk et al. (1988) found smaller increases (5–10%) in total breaks when NCS-treated ColE1 DNA was treated with either putrescine or AP endonucleases, and they concluded that abasic sites occur virtually only as bistranded lesions.

Abasic sites appear to play an important role in the bistranded lesions produced by the different thiols. The SS:DS ratios reflect changes in the quantities of both SS and DS breaks under the various conditions of abasic site expression, but it has been demonstrated that changes in the quantities of DS breaks contribute most to the decrease in the ratios with both hydrazine and putrescine treatment (Figure 2). The nature of the thiol appears to affect both the SS:DS ratios and the quantity of abasic sites produced by NCS. HTP, for example, produces amounts of SS breaks similar to the other thiols (Figure 2) but produces far fewer bistranded lesions, the majority of which are 2′-deoxyribonolactone-containing sites. The latter conclusion is based on the very small change in DS breaks produced by hydrazine treatment, which cleaves mainly 4′-hydroxylated abasic sites, compared to the larger change produced by putrescine, which cleaves all abasic sites (Figure 2). This is in agreement with analysis of NCS-me-

diated damage at the AGT·ACT site, where HTP produces few if any bistranded lesions (Dedon et al., 1992). DTT, on the other hand, produces few direct bistranded breaks, consistent with the reduced quantity of 3′-phosphoglycolate residues it produces in damage at the T of AGT (Dedon et al., 1992), but it does cause bistranded lesions with both types of abasic site (Table I). As will be demonstrated in the accompanying paper, the thiol can influence the partitioning of breakdown products resulting from C4′-hydrogen abstraction, thus influencing the relative quantities of strand breaks and abasic sites formed by C4′-chemistry (Dedon et al., 1992) and the ratio of SS:DS breaks in plasmid DNA.

In summary, structure–function studies with various thiol activators of NCS suggest that thiol nucleophilicity and what appears to be an affinity of the thiol for DNA are both significant features of NCS activation. Furthermore, it appears that NCS is activated after it has bound to DNA and that the rate of activation determines the extent of DNA damage produced by NCS. Abasic sites appear to play a major role in NCS-mediated bistranded DNA damage but are also present as isolated SS lesions. Finally, the carboxylate moiety of the thiol activator may play a significant role in the production of bistranded lesions by NCS.

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Neocarzinostatin-Mediated DNA Damage in a Model AGT·ACT Site: Mechanistic Studies of Thiol-Sensitive Partitioning of C4′ DNA Damage Products†

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ABSTRACT: Double-strand (DS) DNA damage caused by neocarzinostatin (NCS) has been studied in the trinucleotide AGT·ACT sequence in an AP-1 transcription factor binding site. There are strong similarities between bistranded lesions produced at AGT·ACT and AGC·GCT, including the fact that DS lesions outnumber SS lesions on the AGT and AGC strands, while SS exceed DS on the ACT and GCT strands. Structure–function studies revealed that a variety of different thiols produced bistranded lesions in this model by predominantly C4′-hydrogen atom abstraction (84–93%) at the T of AGT and C5′-hydrogen atom abstraction (87–91%) at the T of ACT. Single-strand (SS) lesions were found to represent a variable mixture of C4′ and C5′ chemistry. The C4′-hydroxylated abasic site occurred in both SS and DS lesions at both sites and accounted for most of the DS damage at AGT (60–83%); the remaining damage consisted of 3′-phosphoglycolate- and 3′-phosphate-ended fragments. The nature of the thiol was found to affect the partitioning of the breakdown products arising from C4′ and, to a lesser extent, C5′ hydrogen atom abstraction. Production of 3′-phosphoglycolate residues, restricted mainly to the T of AGT in bistranded lesions, correlated with the incidence of direct DS breaks in the AGT·ACT model and in plasmid DNA and appeared to be influenced by the reducing power of the thiol activator. Furthermore, hydrazine and sodium borohydride both inhibited the formation of glycolate, an effect that was exploited to determine the rate constant for 3′-phosphoglycolate formation: 0.06 min^{-1} at 0 °C, pH 7.4. Under anaerobic conditions, the nitroaromatic radiation sensitizer misonidazole caused a large increase in glycolate production in both SS and DS lesions formed by NCS, which suggests that the formation of 3′-phosphoglycolate, like 3′-formylphosphate generated by C5′ chemistry, involves an oxyradical intermediate. The pathways for DNA damage involving C4′ and C5′ hydrogen atom abstraction thus share many common features, several of which are consistent with a mechanism for the production of NCS-mediated bistranded lesions at AGT·ACT that involves a tetraoxide bridge joining the lesions on opposite strands of DNA.

The enediyne core of the antibiotic antitumor agent neocarzinostatin (NCS)[1] binds to DNA via the minor groove and, following nucleophilic attack by a thiol, rearranges to form a bicyclic diradical species that abstracts hydrogen atoms from the deoxyribose sugar of DNA [see Scheme I in the accompanying paper (Dedon & Goldberg, 1992); for review, see Goldberg (1991)]. The carbon-centered radicals add molecular oxygen to form peroxyradicals, which undergo degradation to unique sugar fragments depending on the location of the abstracted hydrogen (Scheme I of this paper). At the C4′-position, breakdown of the peroxyradical partitions to form a 4′-hydroxylated abasic site (Saito et al., 1989; Frank et al., 1991; Kappen et al., 1991) or to produce a strand break with a 3′-phosphoglycolate residue and a 5′-phosphate (Frank et al., 1991; Kappen et al., 1991). Similarly, the strand break resulting from the degradation of the C5′-peroxyradical involves either a nucleoside 5′-aldehyde at the 5′-end and a 3′-phosphate (Kappen et al., 1982; Kappen & Goldberg, 1983) or a labile 3′-formylphosphate-ended DNA fragment and a 5′-phosphate (Kappen & Goldberg, 1984; Chin et al., 1987) (Scheme I). Abstraction of the C1′-hydrogen atom, the third

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[1] Abbreviations: NCS, neocarzinostatin chromophore; GSH, glutathione; MPA, 3-mercaptopropionate; TG, thioglycolate; CYS, cysteine; MMPA, methyl 3-mercaptopropionate; CSM, cysteamine; E-CYS, ethyl cysteine; MTG, methyl thioglycolate; BME, 2-mercaptoethanol; DTT, dithiothreitol; HTP, 4-hydroxythiophenol; SS, single strand; DS, double strand; AP, apurinic/apyrimidinic; bp, base pair.

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