Exclusive Production of Bistranded DNA Damage by Calicheamicin†

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ABSTRACT: Bistranded DNA damage produced by the enediyne antitumor antibiotic calicheamicin (CAL) was examined in plasmid DNA and in a model site for CAL-mediated DNA damage containing the sequence AGGA·TCCT. CAL was observed to produce bistranded DNA damage to the virtual exclusion of single-strand (SS) lesions. Without expression of drug-induced abasic sites as strand breaks, CAL activated by 10 mM glutathione produced equal numbers of DS and SS breaks in plasmid DNA. However, cleavage of drug-induced abasic sites with hydrazine and putrescine resulted in a dramatic increase in the appearance of bistranded damage, with ratios of DS to SS breaks of ~6:1 and ~24:1, respectively. A similar bias for DS lesions was noted in CAL-mediated damage in the AGGA·TCCT model sequence, in which evidence for a 4′-hydroxylated abasic site was observed on the AGGA strand. These model DNA systems revealed three important features of the bistranded damage produced by CAL. First, the major lesion produced by CAL is a bistranded lesion consisting of an abasic site on one strand and a direct strand break on the other. Second, 3′-phosphoglycolate residues resulting from CAL-induced C4′-hydrogen abstraction at the C of AGGATC were restricted to bistranded lesions. This unusual behavior of the C4′-chemistry of both CAL and the related neocarzinostatin (NCS) is consistent with an intermediate in the partitioning that is dependent on bistranded lesions. Finally, 4-hydroxythiophenol activated CAL to produce a ratio of DS to SS lesions of ~10:1, as opposed to the ~1:30 ratio noted previously for NCS. This is consistent with the absence of a thiol adduct in the activated form of CAL, compared to NCS. The observed predominance of bistranded lesions (>95%) may explain the potent cytotoxic and clastogenic activity of CAL.

Of the DNA lesions produced by free radicals, those involving damage to both strands appear to be the most lethal [reviewed in von Sonntag (1987) and Frankenberg-Schwager (1990)]. Members of the growing family of enediyne antitumor antibiotics are unique for their potential to produce bistranded DNA damage [reviewed in Dedon and Goldberg (1992b,c)]. These agents undergo an electronic rearrangement to form a putative diradical intermediate (Figure 1), positioned in the minor groove, which abstracts hydrogen atoms from the deoxyribose moieties on both strands of DNA. Reaction of the resulting carbon radicals with molecular oxygen starts a degradation process that yields a variety of chemically-modified abasic sites and strand breaks consisting of DNA fragments with sugar residues attached to the 3′- and 5′-ends.

In spite of this potential for a single drug molecule to produce damage on both strands at a single site, bistranded lesions have been found to account for only one-third of the DNA damage produced by neocarzinostatin (NCS)[1] and calicheamicin (CAL) (Drak et al., 1991; Walker et al., 1992; Dedon & Goldberg, 1992a). The findings with CAL, however, were made without consideration of the presence of drug-induced abasic sites. Such lesions account for a significant fraction of NCS-mediated DNA damage (Povirk & Goldberg, 1985; Kappen et al., 1988, 1990; Kappen & Goldberg, 1989; Frank et al., 1991; Dedon et al., 1992; Dedon & Goldberg, 1992a).

In this report, evidence is presented that CAL produces bistranded DNA damage to the virtual exclusion of SS lesions (>95%) both in a large population of cleavage sites in plasmid DNA and in a single model cleavage site for CAL-mediated DNA damage. The majority of bistranded lesions in both systems consisted of abasic sites opposite direct strand breaks. CAL-mediated DS lesions appear to be less affected by the nature of the activating thiol than those produced by NCS, while 3′-phosphoglycolate residues produced by both drugs are limited to DS breaks. These observations are discussed in relation to the potent cytotoxic activity of CAL.

MATERIALS AND METHODS

Chemicals. Calicheamicin γ_1^I was generously provided by Dr. George Ellestad of Lederle Laboratories, and bleomycin (BLM), by Dr. Terrance Doyle of Bristol-Myers Squibb. Reagents were obtained as follows: pBluescript (pBSCT), from Stratgene; pUC19, from New England Biolabs; reagent grade chemicals, from Sigma and Aldrich; restriction enzymes, T4 polynucleotide kinase, and Klenow fragment, from Promega and New England Biolabs; and radiochemicals, from Amersham and New England Nuclear.

Optimization of Glutathione Concentration. The concentrations of glutathione (GSH) and 4-hydroxythiophenol (HTP) that produced maximal CAL-mediated DNA damage were determined by densitometric quantitation of drug-induced plasmid topologic forms as described elsewhere (Dedon & Goldberg, 1992a). CAL (final concentration of 3 nM) was added to a solution containing a plasmid DNA and a range of thiol concentrations, and the reaction was allowed to proceed for 2 h at 0 °C. Abasic sites were cleaved by exposure to 100 mM putrescine for 1 h at 37 °C (Lindahl & Andersson, 1972; Povirk & Goldberg, 1985; Dedon & Goldberg, 1992a).

Quantitation of SS and DS Breaks Produced by CAL/GSH in pBSCT. Quantitation of CAL-mediated DNA damage in the presence of 10 mM GSH and under various conditions of abasic site expression was accomplished as described previously (Dedon & Goldberg, 1992a). Following

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[1] Abbreviations: AP, apurinic/apyrimidinic; BLM, bleomycin; bp, base pair; CAL, calicheamicin γ_1^I; DS, double strand; ESP, esperamicin; GSH, glutathione; HTP, 4-hydroxythiophenol; NCS, neocarzinostatin; SS, single strand.

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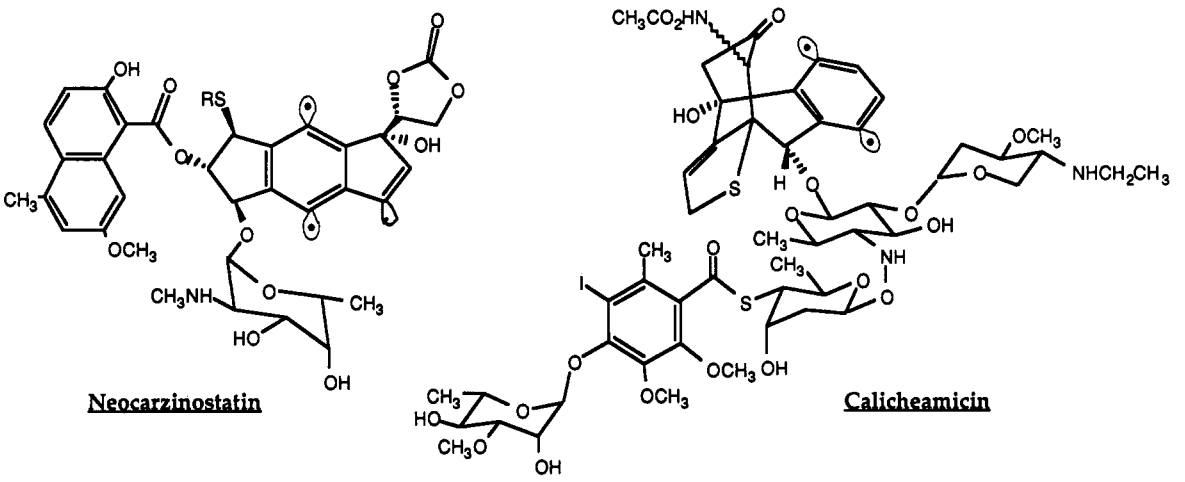

FIGURE 1: Structures of the putative diradical forms of neocarzinostatin and calicheamicin.

FIGURE 2: Model site for calicheamicin-mediated bistranded lesions in pUC19. The heavy bar marks the AGGA·TCCT site, and arrows denote damage sites.

the drug reaction, the mixture was split into three aliquots, one of which was kept as a control. One of the two remaining aliquots was treated with hydrazine, which cleaves mainly 4′-hydroxylated abasic sites (Sugiyama et al., 1988; Frank et al., 1991; Kappen et al., 1991; Dedon & Goldberg, 1992a; Dedon et al., 1992); the other, with putrescine, to cleave all abasic sites (Lindahl & Andersson, 1972; Povirk & Goldberg, 1985). Each reagent was added to a final concentration of 100 mM, and the mixtures were incubated at room temperature or 37 °C, respectively, for 1 h.

Preparation of Labeled pUC19 DNA. 5′- and 3′-[^{32}P] end-labeling of the *Hin*dIII site of pUC19 (Figure 2) was performed by standard methods (Ausubel et al., 1989) and was followed by cleavage with *Pvu*II. The 141-bp *Hin*dIII/*Pvu*II fragment was isolated from a polyacrylamide gel as described elsewhere (Dedon et al., 1992).

Drug/DNA Reactions. DNA cleavage was initiated by adding CAL to a final concentration of 0.2 μM to a solution of end-labeled DNA (5 × 10^4–1 × 10^5 cpm), 30 μg/mL calf thymus DNA, 10 mM GSH, 5 mM EDTA, and 50 mM HEPES, pH 7.0. The reaction was allowed to proceed for 2 h at 0 °C. In experiments with 5′-[^{32}P]-labeled DNA, either hydrazine or putrescine was then added to cleave abasic sites as described above, followed by ethanol precipitation. With 3′-[^{32}P]-labeled DNA, a portion of the reaction mixture was treated with sodium borohydride to reduce nucleoside 5′-aldehydes, as described previously (Dedon et al., 1992a).

Gel Analysis of Cleavage Products. Resolution of drug-treated DNA on nondenaturing polyacrylamide gels, isolation of DNA in bands corresponding to SS and DS breaks, and examination of the DNA on sequencing gels were accomplished as described elsewhere (Dedon et al., 1992). The quantities of DNA damage products were determined by PhosphorImager analysis (Molecular Dynamics).

RESULTS

CAL-Mediated Damage in Plasmid DNA. The dual role of the thiol in drug activation and reduction of damaged deoxyribose necessitated the determination of the optimal thiol concentrations for CAL-mediated DNA damage. The optimal concentrations for GSH and HTP were found to be 10 and 1 mM, respectively (Figure 3). These values for CAL compare favorably with the 5 mM GSH and 0.2 mM HTP optima

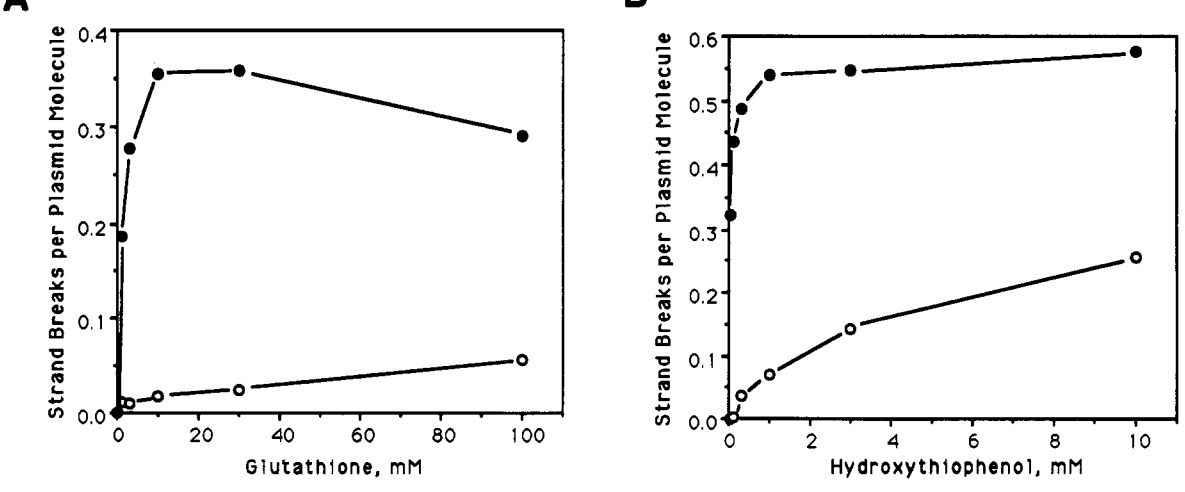

FIGURE 3: Determination of thiol concentrations that produce maximal calicheamicin-mediated DNA damage in pBluescript. Following treatment of plasmid DNA with 3 nM calicheamicin and varying concentrations of thiol, abasic sites were expressed as strand breaks by cleavage with putrescine. Plasmid forms (I–III; see Figure 4) were quantitated and strand breaks per plasmid molecule determined as described in Materials and Methods. Single-strand breaks, ○; double-strand breaks, ●. (A) Glutathione; (B) 4-hydroxythiophenol. The figure depicts results from one experiment.

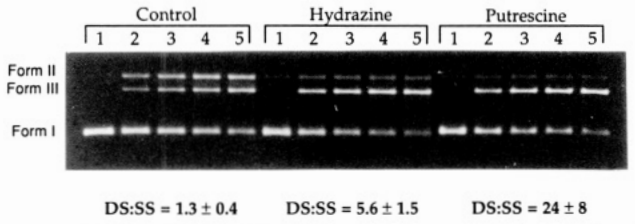

FIGURE 4: Quantitation of double- and single-strand lesions produced by calicheamicin and glutathione in pBluescript under various conditions of abasic site expression. Supercoiled plasmid DNA (form I) was treated with calicheamicin in the presence of 10 mM glutathione at the following drug concentrations: 0, 1, 2, 3, and 5 nM in lanes 1–5, respectively. Abasic sites were left intact (control) or cleaved with either hydrazine or putrescine, as described in the text. Values represent mean ± SD for three to five experiments.

noted previously for NCS (Dedon & Goldberg, 1992a).

The numbers of SS and DS breaks produced by CAL and GSH were calculated from the relative quantities of the three plasmid DNA forms produced by drug treatment (Figure 4) by assuming a Poisson distribution of DNA lesions (Povirk et al., 1977; Povirk & Houlgrave, 1988; Dedon & Goldberg, 1992a). In these experiments, the molar ratio of drug to plasmid ranges from 1:16 to 1:3, and the DNA damage produced by CAL is nearly quantitative (data not shown). These "single-hit" conditions are consistent with the generation of DS lesions by a single molecule of CAL, rather than random coincidence of nonspecific SS lesions.

In control reactions, CAL produced a DS:SS ratio of 1.3:1, which is in reasonable agreement with the values of ~1:2 determined by Drak et al. (1991) and Walker et al. (1992). However, when the CAL-treated DNA is exposed to hydrazine and putrescine, the DS:SS ratio rises to 5.6:1 and 24:1, respectively (Figure 4). Putrescine has been demonstrated to cleave all known abasic sites (Lindahl & Andersson, 1972; Povirk & Houlgrave, 1988), while hydrazine reacts with 4′-hydroxylated abasic sites produced by bleomycin (BLM) and NCS to form a strand break with a 3′-phosphopyridazine derivative on one end (Sugiyama et al., 1988; Kappen et al., 1991; Frank et al., 1991; Dedon et al., 1992).

CAL-Mediated DNA Damage in a Model Site for Bistranded Lesions. The polylinker region of plasmid pUC19 contains an AGGA·TCCT site (Figure 2), one of the sequences noted by Zein et al. (1988) as a frequent site for CAL-mediated DNA damage. The production of bistranded lesions in this model is demonstrated in Figure 5, in which CAL-treated DNA with no damage or with only SS breaks is resolved from DS-break fragments on a nondenaturing polyacrylamide gel. It is apparent that the AGGA·TCCT sequence is the major site for bistranded lesions in this DNA fragment. This observation is consistent with that of Hangeland et al. (1992), in which CAL produced damage on both strands in an oligonucleotide AGGA·TCCT site; however, no attempt was made in these studies to differentiate SS from DS breaks.

The utility of this model stems from the ability to examine the chemistry of the damage on both sides of the cleavage sites on both strands by placement of a ^{32}P label at either the *Hin*dIII or the *Eco*RI site. The nature of the DNA damage produced by CAL at this site is shown in a sequencing gel in Figure 6A for the 5′-[^{32}P]-labeled *Hin*dIII/*Pvu*II fragment. The mobility of the band migrating slightly faster than the phosphate-ended fragment corresponding to damage at the C of AGGATC (band II in Figure 6A) is similar to the 3′-phosphoglycolate-ended fragment generated by BLM at this site (data not shown) [BLM chemistry is reviewed in Stubbe and Kozarich (1987)] and by NCS at GT steps (Dedon & Goldberg, 1990a; Frank et al., 1991; Kappen et al., 1991; Dedon et al., 1992). This band has been observed by other groups in CAL-mediated DNA damage (Zein et al., 1989; Walker et al., 1992; Hangeland et al., 1992) and is consistent with the predominant CAL-mediated C4′-hydrogen abstraction noted at this site in the deuterium-labeling studies of

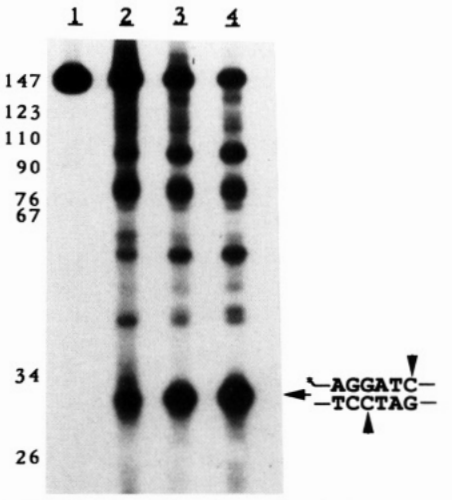

FIGURE 5: Double-strand breaks produced by calicheamicin and glutathione in the model AGGA·TCCT site in pUC19. The 141-bp *Hin*dIII/*Pvu*II fragment of pUC19 was 5′-[^{32}P]-labeled at the *Hin*dIII site and treated with calicheamicin (0.2 μM for lanes 2–4) or drug vehicle (methanol; lane 1) in the presence of 10 mM glutathione. The DNA was then treated with hydrazine (lane 3) or putrescine (lane 4) or left untreated (lane 2) and was resolved on a 12% nondenaturing polyacrylamide gel. The numbers in the left margin represent the sizes (bp) of 5′-[^{32}P]-labeled *Msp*I-digested pBR322 molecular weight standards. The position of the double-strand break in the AGGA·TCCT sequence is shown in the right margin.

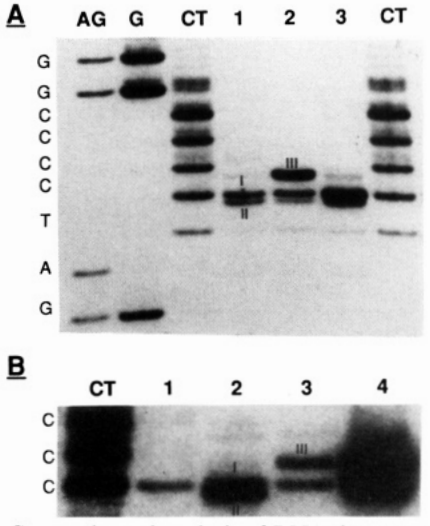

FIGURE 6: Sequencing gel analysis of DNA damage produced by calicheamicin at the C of AGGATC in the model site for bistranded lesions in pUC19. (A) The 5′-[^{32}P]-labeled *Hin*dIII/*Pvu*II fragment was treated with 0.2 μM calicheamicin and 10 mM glutathione. The DNA was subsequently treated with either hydrazine (lane 2) or putrescine (lane 3) or left untreated (lane 1). Lanes marked AG, G, and CT represent Maxam–Gilbert sequencing standards; the sequence is noted in the left margin. (B) 5′-End-labeled DNA was treated as described in panel A and either treated with hydrazine (lanes 3 and 4) or left untreated (lanes 1 and 2). The DNA was resolved on a 12% nondenaturing polyacrylamide gel (see Figure 5), and the DNA fragments representing double-strand breaks (lanes 2 and 4) and single-strand breaks (lanes 1 and 3) were excised and resolved on a 20% sequencing gel. Damaged deoxyribose residues in panels A and B are tentatively identified as follows: (I) 3′-phosphate, (II) 3′-phosphoglycolate, and (III) 3′-phosphopyridazine.

Hangeland et al. (1992). The accompanying phosphate-ended fragment probably represents a small quantity of C5′-chemistry, also observed by Hangeland et al. (1992).

Following treatment of the drug-damaged DNA with hydrazine, a band appears that migrates ~1 nucleotide more slowly than the corresponding phosphate-ended fragment (band III in Figure 6A). This behavior is consistent with the 3′-phosphopyridazine-ended fragment formed by reaction of a 4′-hydroxylated abasic site with hydrazine, as observed for BLM and NCS (Sugiyama et al., 1988; Kappen et al., 1991; Dedon et al., 1992). The abasic site was anticipated, given the precedent for partitioning of BLM- and NCS-mediated C4′-chemistry to form either the abasic site or a strand break with 3′-phosphoglycolate- and 5′-phosphate-ended fragments (Stubbe & Kozarich, 1987; Saito et al., 1989; Frank et al., 1991; Kappen et al., 1991). As expected, treatment of the CAL-damaged DNA with putrescine results in the conversion of abasic sites to phosphate-ended DNA strand breaks entirely (lane 3, Figure 6A) (Lindahl & Andersson, 1972; Povirk & Houlgrave, 1988).

3′-[^{32}P] labeling of the *Hin*dIII/*Pvu*II fragment of pUC19 revealed the presence of damage at the 5′-C of TCCT consistent with C5′-hydrogen abstraction: nucleoside 5′-aldehyde residues and 5′-phosphate-ended fragments (data not shown). This has also been observed previously by Zein et al. (1988) and Walker et al. (1992).

Comparison of SS and DS Breaks Produced by CAL at AGGA·TCCT. To identify chemical differences between SS and DS damage products, drug-treated DNA was resolved on a nondenaturing polyacrylamide gel (Figure 5). DNA isolated from bands corresponding to DS breaks and parent-length DNA (SS lesions, direct breaks opposite abasic sites, and uncut material) was examined on a sequencing gel (Figure 6B; overexposed to show SS breaks). Two features of the damage at the AGGA·TCCT site are apparent in these studies. First, the relative quantities of SS and DS lesions changed as a function of the expression of abasic sites by hydrazine and putrescine, as in the plasmid model. On the AGGA strand, the ratio of DS:SS breaks in control samples (abasic sites still intact) was ~10:1 and rose to ~40:1 with hydrazine treatment. Similarly large differences were also noted on the TCCT strand (data not shown). Recovery of both full-length DNA and DS DNA fragments from polyacrylamide gel slices was nearly quantitative (data not shown).

The chemistry also differed for the two types of DNA damage. Both SS and DS lesions involved C4′-hydrogen abstraction, as suggested by the putative phosphopyridazine residue present in both lesions (band III, lanes 3 and 4, Figure 6B). However, only DS lesions possessed the other product of the partitioning of C4′-chemistry: 3′-phosphoglycolate residues (band II, lane 2, Figure 6B).

DISCUSSION

The bifunctional nature of the enediyne diradical forms suggests the facile production of bistranded DNA lesions. Consistent with this hypothesis are the observations of DS-break fragments by electrophoretic analysis (Zein et al., 1988), of CAL-mediated abstraction of site-specific deuterium labels from either strand of a model AGGA·TCCT cleavage site (Hangeland et al., 1992), and of CAL-mediated DNA cleavage kinetics that fit a DS-break model (Kishikawa et al., 1991). However, the quantitation of CAL-induced SS and DS breaks in plasmid DNA by Drak et al. (1991) and Walker et al. (1992) suggested that DS lesions represented at most one-third of the DNA damage. The reason for this apparently low yield of bistranded lesions stems from the presence of drug-induced abasic sites, which were not considered in the two studies. We have observed drug-induced abasic sites to be a major component of CAL-mediated bistranded DNA damage: hydrazine- and putrescine-sensitive lesions opposite direct strand breaks represent ~95% of the damage in plasmid DNA. There are two likely candidates for these abasic sites: the 2-deoxyribonolactone-containing site and the 4′-hydroxylated abasic site. The former is produced by C1′-hydrogen abstraction by NCS (Kappen & Goldberg, 1989), and while CAL does not appear to produce such a lesion in the AGGA·TCCT sequence (Hangeland et al., 1992), it may be part of CAL-mediated damage in other DNA sequences. When bistranded lesions with abasic sites opposite strand breaks are taken into account along with direct DS breaks, it is clear that DNA damage produced by CAL in a large population of cleavage sites consists almost exclusively (>95%) of bistranded lesions.

This bias toward DS lesions was also noted in the model AGGA·TCCT sequence, where approximately two-thirds of the bistranded lesions appeared to consist of a 4′-hydroxylated abasic site at the C of AGGATC accompanied by a strand break produced by C5′-chemistry at the 5′-C of TCCT. The presence of the C4′-derived abasic site was expected, given the precedent for such a lesion in the C4′-chemistry of NCS and BLM (Sugiyama et al., 1988; Saito et al., 1989; Kappen et al., 1991; Dedon et al., 1992) and the evidence for C4′-hydrogen abstraction by CAL (Hangeland et al., 1992). Evidence for an alkaline-labile species generated by C4′-hydrogen abstraction has also recently been obtained with the esperamicins (ESP) (Christner et al., 1992).

The kinetic analysis of CAL-mediated SS and DS break formation performed by Kishikawa et al. (1991) needs to be considered in light of the present observation of abasic sites as a major component of CAL-mediated bistranded DNA damage. In their studies, Kishikawa et al. examined the quantities of the three topologic forms of PM2 DNA generated by CAL treatment, a technique similar to the plasmid studies performed in the present work. They conclude that the kinetic data for CAL-mediated DNA strand break formation fits a DS-break model, with which the present studies are in general agreement. However, CAL-induced abasic sites were not considered in their analysis, and thus the derived rate constants apply only to the formation of *direct* DS breaks, the minor bistranded lesion noted for CAL in the present study and noted previously for NCS (Dedon et al., 1992). The results of the present study suggest that direct "cleavage" of DNA (i.e., immediate formation of strand breaks) does not necessarily reflect the true extent of DNA damage produced by agents such as CAL. Kinetic analyses of enediyne-mediated DNA damage must therefore account for the formation of abasic sites.

The observed partitioning of CAL-mediated C4′-chemistry at the AGGA site displayed the unusual behavior noted previously with NCS: 3′-phosphoglycolate residues were restricted to DS lesions (Figure 6B) (Dedon & Goldberg, 1990; Dedon et al., 1992). This differential partitioning is consistent with the proposed involvement of a DS lesion-dependent intermediate, such as the tetroxide bridge proposed for NCS-mediated bistranded lesions (Dedon et al., 1992).

The predominance of DS DNA lesions in CAL-mediated DNA damage may explain the potent cytotoxicity and clastogenicity associated with this agent at picomolar concentrations (Zein et al., 1988; Zhao et al., 1990; Lee et al., 1991), since DS lesions appear to correlate best with the

lethality of ionizing radiation and NCS (Bonura & Smith, 1976; Hatayama & Goldberg, 1979). Of the two types of DS lesion produced by CAL, direct DS breaks and abasic sites opposite closely-opposed strand breaks, the latter may be more toxic. Such lesions produced by NCS appear to be resistant to cleavage by repair-related AP endonucleases (Povirk & Goldberg, 1985; Povirk & Houlgrave, 1988; Povirk et al., 1988). The lethality associated with the production of a large quantity of DS lesions by CAL may thus be compounded by the persistence of a majority of them in vivo.

Why do other enediynes not produce larger relative quantities of bistranded lesions? The quantities of SS and DS lesions produced by dynemicin (Sugiura et al., 1990) have not been extensively characterized. However, recent studies with the esperamicin (ESP) compounds suggest a role for the carbohydrate side chains of CAL/ESP-type enediynes in the formation of bistranded lesions (Christner et al., 1992). The native A_1 form of ESP, which appears to produce mainly C5′-hydrogen abstraction (Christner et al., 1992), has been shown to cause more SS than DS lesions (Long et al., 1989). Removal of the deoxyfucose–anthranilate moiety of ESP A_1 generates a species (ESP C) that produces a greater proportion of DS breaks (Long et al., 1989; Christner et al., 1992), now involving both C5′- and C4′-chemistry (Christner et al., 1992). Two mechanisms have been proposed for this effect of the deoxyfucose–anthranilate group: it may cause altered positioning of the diradical in the minor groove, or it may specifically quench one of the radical centers of the drug, specifically, the radical mediating C4′-hydrogen abstraction. In either case, the presence of this group appears to prevent abstraction of a hydrogen atom from one strand of DNA, resulting in the formation of mainly SS lesions.

In the case of NCS, the predominance of SS lesions appears to be related in part to the thiol as a covalent adduct of the activated drug. Such an adduct may influence the positioning of the drug in the minor groove of DNA and the subsequent abstraction of hydrogen atoms from one strand (Dedon & Goldberg, 1992a). Furthermore, it has been demonstrated that one of the NCS radicals is quenched by intramolecular transfer of hydrogen from the thiol adduct (Wender & Tebbe, 1991; McAfee & Ashley, 1992; Chin & Goldberg, 1992).

The thiol appears to play a different role in the activity of CAL, which may account for the higher level of CAL-mediated DS lesions. The thiol does not appear to form a covalent adduct with CAL during activation and, thus, should not influence the positioning of the CAL diradical on DNA. Additionally, the absence of a thiol adduct also suggests that CAL may be less susceptible to thiol-mediated quenching of drug radicals. The experiments with HTP are consistent with these hypotheses. HTP as an activator of CAL was associated with a DS:SS ratio of ~10:1 (Figure 3B), while activation of NCS by HTP resulted in a DS:SS ratio of ~1:30 (Dedon & Goldberg, 1992). The similar chemistries of deoxyribose degradation associated with NCS and CAL suggest that the difference in the relative quantities of SS and DS lesions results from the presence or absence of a thiol adduct. Interestingly, the production of DS lesions by CAL appeared to be more sensitive to thiol concentration than production of SS lesions (Figure 3). It is possible that quenching of one of the CAL radicals by thiols accounts for this phenomenon, but the thiol sensitivity may also represent alterations in the expression of deoxyribose damage.

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