Effects of the Enediyne C-1027 on Intracellular DNA Targets†

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Received June 23, 1994; Revised Manuscript Received October 20, 1994⊗

ABSTRACT: We examined DNA damage induced by the enediyne-containing antitumor antibiotic C-1027 in intracellular nuclear and mitochondrial DNA targets using the episome-containing cell line 935.1. Strand-scission activity of the C-1027 holoantibiotic was measured by the topological forms conversion assay in episomal and mitochondrial DNA, as well as in cell-free plasmid DNA. Genomic DNA damage was quantitated by filter elution analysis. Comparisons were made to the well-characterized enediyne neocarzinostatin. From these studies, mixed single- and double-strand breaks were observed not only in cell-free, plasmid DNA but also in intracellular episomal, mitochondrial, and genomic DNA at low nanomolar concentrations. C-1027 cleaved DNA 285-fold more efficiently in cells than in a cell-free environment, and displayed preference for intracellular DNA species in the following rank order: episome > mitochondrial DNA ≫ genomic. NCS also damaged the non-histone-associated mitochondrial DNA, but not the episome. Cleavage of the 935.1 cell episome by C-1027 occurred at specific sites including the BPV origin of replication and E6/E7 open reading frame regions, as well as the MMTV LTR promoter region.

Antitumor antibiotics containing bioactive enediyne structures have recently received significant attention due to their remarkable DNA-damaging activities. A potent member of this class of compounds is the microbial derivative C-1027. The biological activities of C-1027 include potent cytotoxicity against cultured human cancer cells (Sugimoto et al., 1990; Zhen et al., 1989) and marked growth inhibition of transplantable tumors in mice (Zhen et al., 1989). At the cellular level, C-1027 selectively inhibits nucleic acid synthesis without affecting protein synthesis (Sugimoto et al., 1990; Xu et al., 1990). It also retards cell cycle progression through S-phase and blocks mitosis at the G_2+M boundary (Xu et al., 1990).

The C-1027 holoantibiotic is structurally similar to two other members of the enediyne-containing chromoprotein family, neocarzinostatin (NCS)[1] (Goldberg, 1991) and kedarcidin (Zein et al., 1993), in that it is composed of a single, acidic polypeptide which stabilizes a labile, nonprotein chromophore (Otani et al., 1988; Sugimoto et al., 1990). The chromophore component of C-1027 contains a highly strained nine-membered enediyne core that is similar in structure to that of kedarcidin, whereas it differs from that of NCS both in its structure and in its mechanism of cyclization (Yoshida et al., 1993). Activation of the C-1027 chromophore involves the generation of a biradical species from the enediyne ring via Masamune/Bergman-type cycloaromatization reaction (Sugiura & Matsumoto, 1993). This mechanism is common among the enediyne-containing class of antibiotics (Nicolaou et al., 1993). In contrast to the other enediynes, including NCS, the formation of the bioactive C-1027 species appears to be spontaneous in that it does not require, nor is it affected by, the presence of reducing agents (Sugimoto et al., 1990). The protein moiety of C-1027, in addition to stabilization of the labile chromophore, has been implicated in the targeting to and the internalization at the cell surface of the holocomplex (Sakata et al., 1992; S.-Tsuchiya et al., 1994). Such translocation of the intact holoantibiotic into cells has also been reported for NCS (Oda et al., 1987). Once internalized, the C-1027 apoprotein may be instrumental in escorting the reactive chromophore to its ultimate intracellular destination, DNA (S.-Tsuchiya et al., 1994).

The cytopathological effects ascribed to C-1027 stem from its DNA strand-scission activity (Sugimoto et al., 1990). The C-1027 chromophore binds to A/T-rich sequences in the minor groove of the DNA helix where it produces direct single- (SSBs) and double-strand breaks (DSBs) as well as abasic sites (Sugiura & Matsumoto, 1993; Xu et al., 1994). The resulting SSBs are base-specific lesions that occur at A and T residues (Xu et al., 1994). In contrast, the more lethal DSBs are sequence-specific lesions that occur preferentially in such pentanucleotide sequences as GTTAT/ATAAC (Xu et al., 1994). These breaks occur via C4′ and C5′ hydrogen abstraction from the deoxyribose sugars of the indicated adenylate residues in the complementary strands. The resultant DSB is separated by two bases in a 3′-stagger that is similar to what is produced by NCS, although the latter is specific for select trinucleotide sequences containing GT (Povirk et al., 1988; Dedon & Goldberg, 1990; Dedon et al., 1992). Xu and colleagues (1994) have suggested that, similar to NCS (Hatayama & Goldberg, 1979), double-strand lesions are responsible for the cytotoxic effects exhibited by C-1027. However, the data published thus far on the DNA

† This work was supported by National Cancer Institute Grants CA-52682 and CA-16056.

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1994.

[1] Abbreviations: bp, base pair(s); BPV, bovine papillomavirus; DSB, double-strand break; DTT, dithiothreitol; kbp, kilobase pair(s); MMTV LTR, mouse mammary tumor virus long terminal repeat; NCS, neocarzinostatin; SSB, single-strand break.

0006-2960/95/0434-583$09.00/0

strand-scission mechanism of C-1027 have been garnered only from studies with isolated DNA. Consequently, the purpose of the studies presented herein is to characterize the strand-scission activity of C-1027 holoantibiotic in an intracellular environment.

To examine the selective DNA-damaging effects of C-1027 on defined intracellular targets we employed an episome-containing cell line, 935.1. This model has been used previously in our laboratory to evaluate drug-induced damage both in cultured cells and in tumor-bearing mice (Cullinan & Beerman, 1989; Cullinan et al., 1990, 1991). The high copy number (approximately 200 per cell) and the circular nature of the episome make it a convenient model for the detection of SSBs and DSBs on a histone-associated, nuclear DNA molecule. The 935.1 cell episome was derived from the chimeric plasmid pM19. This construct contains a fragment of pBR322, the 69% transforming fragment of bovine papillomavirus (BPV) type 1, and the *v-Ha-ras* gene, whose expression is driven by the adjacent dexamethasone-inducible mouse mammary tumor virus long terminal repeat (MMTV LTR) promoter (Ostrowski et al., 1983). The 935.1 cell line has also provided a means for comparison of the effects on episomal DNA to those on mitochondrial DNA within the same cell (Vertino et al., 1991). The mitochondrial DNA in this mouse cell line is a small (16.3 kbp), non-histone-associated, double-strand DNA of typically 1000–2000 copies per cell.

In the studies presented here, the 935.1 cell line has provided a facile means for assessing the nature of C-1027-induced DNA damage in cells. The extent of these effects was measured relative to NCS, by changes in DNA topology in episomal and mitochondrial DNA. To determine whether these small, extrachromosomal DNA targets were preferentially affected, damage to genomic DNA was examined via filter elution techniques. From these data, the relative sensitivity of the various DNA targets to C-1027-induced breaks has been determined by comparison of their individual cleavage frequencies. The specificity of C-1027 for the BPV origin and MMTV LTR regions of the episome was assessed as a means of determining the selectivity of the drug for transcriptionally active DNA.

EXPERIMENTAL PROCEDURES

Materials

C-1027, from Taiho Pharmaceutical Co., Ltd. (Tokushima, Japan), was stored as a 1 mg/mL stock solution in water. Neocarzinostatin (NCS) was obtained from Bristol-Myers Co. (Syracuse, NY), as a 2 mg/mL solution in 15 mM sodium acetate (pH 5.0). Stock solutions of both drugs were stored at −20 °C. Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at −20 °C as a 1 mM stock in ethanol, and diluted in medium before use. Dithiothreitol and proteinase K were obtained from Boehringer Mannheim (Indianapolis, IN), and restriction endonucleases and reaction buffers were purchased from either Boehringer Mannheim or GIBCO/BRL (Grand Island, NY). SeaKem GTG agarose was from FMC BioProducts (Rockland, ME). [2-^{14}C]TdR (0.1 mCi/mL, 55 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). [α-^{32}P]-dCTP (10 mCi/mL, 3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). All other chemicals were reagent grade or better, and were purchased from either Sigma Chemical Co. or Fisher Scientific.

The episome-containing 935.1 cell line and the bacterial plasmid pM19, which contains the entire sequence (Ostrowski et al., 1983), were obtained from Dr. Gordon Hager of the National Cancer Institute. The 935.1 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (qualified) supplemented with 10% fetal bovine serum and 1.2 mM L-glutamine. All cell culture reagents were obtained from GIBCO/BRL. The plasmid pAM1 contains the entire mouse mitochondrial genome (Martens & Clayton, 1979), and was provided by Dr. D. A. Clayton of Stanford University.

Methods

Cell-Free DNA Damage. Drug-induced SSBs and DSBs in the superhelical duplex of pM19 plasmid DNA were measured using a topological forms conversion assay. DNA (100 ng at 5 μg/mL) was incubated with the indicated concentration of drug in 10 mM Tris, pH 7.6, for 15 min at 37 °C. For those experiments performed with NCS, 10 mM dithiothreitol (DTT) was included in the reaction buffer as an activating agent. Loading buffer containing 70% sucrose and 0.05% bromophenol blue was added directly at the end of the reaction, and samples were electrophoresed on a 1% agarose gel in a 1 × TAE running buffer (50 mM Tris-HCl, 66 mM acetic acid, and 2 mM EDTA, pH 8.5) at 4.0 V/cm for 75 min at room temperature. Gels were stained with a 0.25 mg/mL ethidium bromide solution and photographed with Polaroid 665 film on a Fotodyne 3−3000 ultraviolet transilluminator. Topological forms conversion was quantitated from film negatives as described below.

Isolation of DNA from Cultured Cells. 935.1 cells were plated at a density of 4 × 10^5 cells per 60-mm dish and allowed to grow for 48 h. Growth medium was replaced with 3 mL of fresh medium, and dexamethasone was added to plates as indicated 60 min prior to the addition of drugs. The drugs were added to the plates and incubated for the indicated amount of time at 37 °C. Following drug treatment, the cell monoloayer was rinsed twice with phosphate-buffered saline and then lysed for 2 h at 37 °C in a lysis buffer containing 1% (w/v) SDS, 10 mM Tris-HCl (pH 7.6), 20 mM EDTA, and 0.2 mg/mL proteinase K (added immediately prior to use). Lysates were triturated from the culture dishes and then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (PCI, 24:24:1; Amresco, Solon, OH) followed by extraction with an equal volume of chloroform. The DNA was precipitated with an equal volume of 2-propanol and 0.2 M sodium chloride, and then resuspended in 120 μL of 10 mM Tris-HCl (pH 7.6)/1 mM EDTA (TE).

Analysis of Topological Forms Conversion. For analysis of episomal DNA damage, purified DNA (1 μg) was separated on a 0.8% agarose gel with a 1 × TAE running buffer at 0.8 V/cm for 18 h at room temperature. Gels were stained with 0.25 mg/mL ethidium bromide and checked for equal loading of DNA per lane using an ultraviolet transilluminator. Using 1 μg of the same purified DNA, mitochondrial DNA was separated on a 0.7% agarose gel with a 1 × TBE running buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.3) containing 5 μg/mL ethidium bromide at 3.5 V/cm for 16 h with constant buffer recircula-

tion at 4 °C. After electrophoresis, equal loading of DNA on the gel was checked by ultraviolet transillumination. For both the episomal and mitochondrial gels, DNA was transferred to Hybond-N nylon membranes (Amersham) by the Southern blot method and then UV-cross-linked. [^{32}P]-pM19 and [^{32}P]pAM1 plasmid DNA probes (specific activities approximately 1×10^9 cpm/μg, each) were prepared by random priming using the DECAprime II DNA labeling kit (Ambion, Austin, TX) and hybridized to episomal and mitochondrial blots, respectively. Blots were exposed to Kodak XAR-5 film at −80 °C using Du Pont Cronex intensifying screens. Relative amounts of topological forms [supercoiled (I), relaxed (II), and linear (III)] were quantitated from negatives (cell-free studies) and X-ray films using a Molecular Dynamics computing laser densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and were expressed as percent of total of the three forms. For calculation of r values in cells, the total amount of DNA was quantitated in samples that had been treated with 40 μg/mL RNase A for 2 h at 37 °C prior to the addition of proteinase K. Nonextracted samples were serially diluted and spotted on plastic wrap stretched over an ultraviolet transilluminator. Ethidium bromide (diluted in TE) was added to a final concentration of 1 μg/mL, and samples were quantitated by comparing their fluorescence to a series of DNA standards. By this method, the total DNA was calculated to be 2.31×10^{-15} mol per 935.1 cell.

Site Specificity of DNA Damage. An adaptation of the indirect end-labeling technique was used to examine the site specificity of DNA damage on the episome (Wu, 1980; Cullinan & Beerman, 1989). After drug treatment, the DNA was purified from 935.1 cells as described above and then digested with *Eco*RI. This enzyme has one recognition site on the episome and generates either full-length linear DNA from the populations of forms I and II or two smaller fragments from the drug-induced form III population. The full-length linear episomal DNA was separated from the smaller drug-damaged fragments by electrophoresis of 10 μg of total DNA on a 1% agarose gel with a 1 × TBE running buffer at 1.2 V/cm for 21 h at room temperature. A series of DNA size markers were generated using pM19 plasmid DNA first digested with *Eco*RI followed by a second restriction endonuclease. As in the 935.1 cell episome experiments, the gels were checked for equal loading of DNA per lane, and the DNA was Southern-blotted to nylon membranes and cross-linked. The blots were probed with the random-primed ^{32}P-labeled *Eco*RI−*Sma*I fragment of the pM19 plasmid (specific activity approximately 1×10^9 cpm/μg). The blots were autoradiographed and scanned as above with a computing densitometer, and the sites of the damage were mapped from the single *Eco*RI site.

Filter Elution. The alkaline and neutral filter elution techniques were performed as previously described (Cullinan & Beerman, 1989; Cullinan et al., 1991), using the methods of Kohn and colleagues (Kohn et al., 1976; Bradley & Kohn, 1979). For neutral elutions, cells were labeled with [^{14}C]-thymidine (55 mCi/mmol; Moravek Biochemicals, Brea, CA), treated with drug, placed on a polycarbonate filter, and lysed with 0.25 mg/mL proteinase K in a 2% SDS/0.25 M Na_4-EDTA solution. Lysis buffer was washed away with 0.02 M EDTA, pH 9.6. DNA was eluted with a neutral buffer (pH 9.6), and fractions were collected at 90 min intervals at a flow rate of 0.033 mL/min. Alkaline elutions differed only in that the 0.02 M EDTA wash buffer was pH 10.3 and the elution buffer was pH 12.1. For both alkaline and neutral elution experiments, the percent DNA retained on the filter was determined from the radioactivity in each fraction. The extent of DNA damage was calculated from the slopes of the first 3 h of the elution curves using previously published X-ray calibration curves from 935.1 cells (Cullinan et al., 1991).

RESULTS

Topological Forms Conversion in Extracellular DNA. Drug-induced cleavage of various DNA targets was quantitated by topological forms conversion assay which measures the conversion of superhelical (form I) DNA to either nicked, circular DNA (form II) or linear (form III) DNA. Because of the probability of more than one hit occurring per molecule at higher levels, decreases in form I DNA of ≤20% were used to determine the frequency of SSBs. Likewise, increases in form III of ≤10% were used to quantitate the frequency of direct DSBs, because at higher levels a DSB may be a composite of proximal SSBs. The conversion of topological forms in Figures 1, 2, and 3 is presented as a function of both drug concentration and relative to the r value (moles of drug per mole of DNA base pairs) for the particular drug. The latter allows for direct comparisons between drug effects in various systems (cell-free, episomal, and mitochondrial DNA), and between multiple drugs within the same system, independent of the amount of DNA. For the topological forms conversion studies described here, the well-characterized enediyne chromoprotein NCS was chosen as the drug to which C-1027 was compared.

In Figure 1, the 12.5 kbp pM19 plasmid was treated with drug to examine cleavage profiles on cell-free DNA. This plasmid is the precursor of the episome contained within the 935.1 cells used in the subsequent experiments. Figure 1A is a representative gel showing the decrease in form I and increases in form II and III topoisomers that occurred with increasing concentration of C-1027 holoantibiotic. Densitometric quantitation of such gels revealed that cleavage of pM19 by C-1027 occurred in a mixed single- and double-strand manner as indicated by the respective decrease in form I and the increase in form III at low drug concentrations (Figure 1B). The earliest detectable damage occurred at 2 nM, and by 250 nM, C-1027 effected complete degradation of the DNA as observed by the absence of all three topological forms (Figure 1A). Assuming a Poisson distribution of lesions, it was determined from the data depicted in Figure 1B that C-1027 produced an average SSB:DSB ratio of 1.1:1. In contrast, NCS holoantibiotic produced a single-strand cleavage profile when DTT was used as the thiol activator (Figure 1C). This was consistent with results previously described for cleavage of PM2 DNA by holo-NCS in the presence of DTT (Beerman et al., 1977).

Single- and Double-Strand Breaks in Episomal DNA. To evaluate the effects of C-1027 on a defined intracellular target, the topological forms conversion assay was used with the episome-containing 935.1 cell line. The 8794 bp episome in these cells differs from the progenitor plasmid (pM19) only by the absence of the pBR322 sequence (Ostrowski et al., 1983). For this series of experiments, forms conversion was detected using the Southern blot method because the episome comprises less than 0.01% of the total DNA

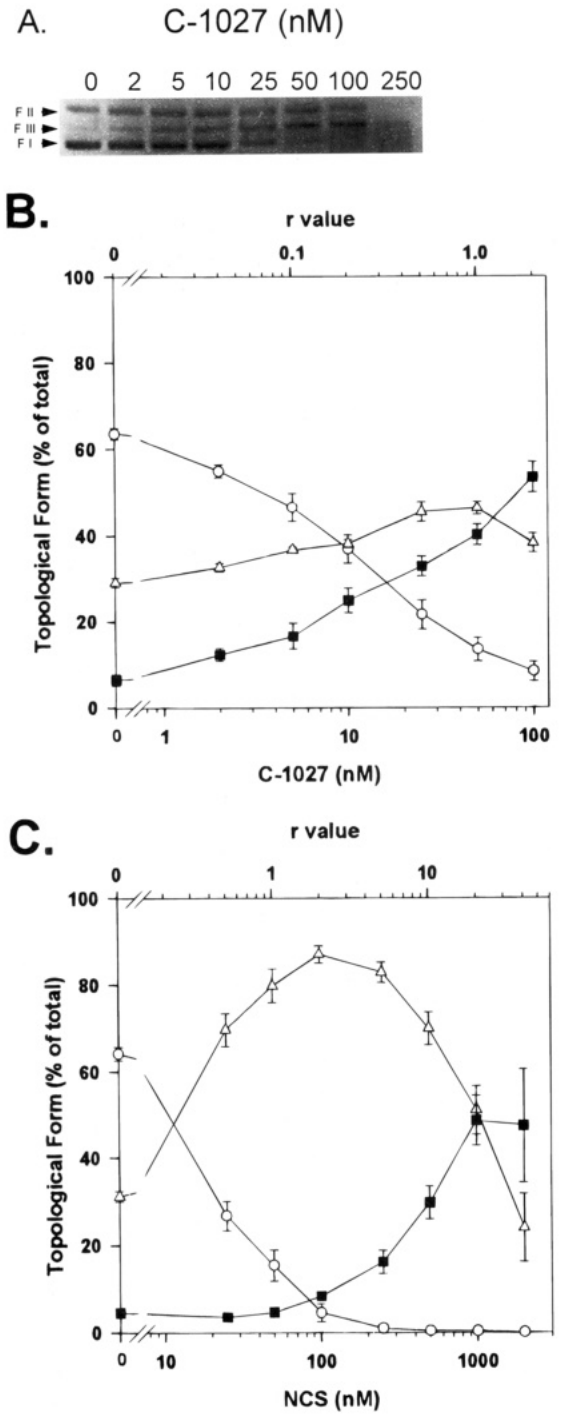

FIGURE 1: Concentration-dependent cleavage of cell-free DNA by C-1027 and NCS. (A) Representative agarose gel pattern of pM19 DNA treated for 15 min with the indicated concentrations of C-1027. DNA stand scission is depicted as a decrease in form I (FI), and an increase in form II (FII) and in form III (FIII). The resultant distributions of topological forms within each lane for treatment with (B) C-1027 and (C) NCS were quantitated using a computing densitometer. The concentrations of supercoiled FI (○), nicked circular FII (△), and linear FIII (■) DNA are expressed as the percent of the total concentration of topoisomers in each sample. Drug to DNA ratios (r values) were calculated using the value 1.54 $\times 10^{-10}$ mol of DNA per 100 ng of pM19. Each data-point is the mean ± SEM ($n = 6-7$).

concentration. Figure 2A shows a representative blot of C-1027-treated, 935.1 cell DNA.[2] As was observed with naked DNA (Figure 1B), exposure of cells to C-1027 produced a mixed pattern of SSBs and DSBs in the episomal DNA (Figure 2B). At concentrations ≤1 nM, simultaneous increases were observed in both forms II and III. From these data, the ratio of single- to double-strand lesions was determined to be 1.4:1. Although this ratio and the overall conversion pattern of the intracellular forms appeared similar to that for the naked DNA (1.1:1), the r values at which form I was decreased by 20% (the level used to determine break frequencies) for cell-free pM19 ($r = 40 \times 10^{-3}$) was over 285-fold greater than that observed in the cell ($r = 0.14 \times 10^{-3}$). In addition, treatment of 935.1 cells with 20 nM C-1027 ($r = 30 \times 10^{-3}$) showed a time-dependent increase in episomal DNA cleavage that continued up through 180 min (Figure 2C). By comparison, NCS-induced intracellular DNA cleavage has been reported to be complete within 30 min (Grimwade & Beerman, 1986). Consequently, the treatment time chosen for the present NCS dose studies was 30 min (Figure 2D). No effect was observed on 935.1 cell episomal DNA integrity at concentrations up to 2 μM ($r = 86 \times 10^{-3}$) (Figure 2D). Increasing the time to 60 min was similarly ineffective (data not shown).

Mitochondrial DNA Cleavage. In addition to the evaluation of episomal DNA damage, the effects of C-1027 on mitochondrial DNA were determined concurrently. As seen in the Southern blot in Figure 3A, C-1027 evoked a dose-dependent conversion of mitochondrial DNA topoisomers in 935.1 cell cultures, with almost total degradation of the DNA occurring at a concentration of 250 nM. Quantitatively, C-1027 produced a profile of mitochondrial DNA cleavage (Figure 3B) that was very similar to that obtained with the episome (Figure 2B): Mitochondrial DNA was cut at low concentrations in a mixed single- and double-strand manner. This was evidenced by an SSB:DSB ratio of 1.6:1, similar to the 1.4:1 ratio obtained with the episome. However, results differed between episomal and mitochondrial DNA from cultured cells in the time-course study (Figure 3C). While 20 nM C-1027 produced a steady increase in the relative proportion of linear episome molecules with time (Figure 2C), mitochondrial form III reached a maximum at 30 min before decreasing through the remaining time (Figure 3C). Concurrently, the amount of form II DNA began to increase after 30 min, and the concentration of form I DNA remained constant after reaching a low at 30 min. Identical results were obtained when the time-course study was conducted using a lower concentration of C-1027 (5 nM) (data not shown). These results indicate that C-1027-induced damage of mitochondrial DNA is complete within 30 min.

A surprising difference in the drug effects on mitochondrial DNA relative to episomal DNA was observed with NCS in 935.1 cells. Whereas NCS produced no changes in episomal DNA topology at concentrations as high as 2 μM (Figure 2D), it did affect measurable cutting of mitochondrial DNA (Figure 3D). At concentrations greater than 100 nM ($r \geq 0.1$), a steady decrease in the proportion of form I was observed concomitant with an increase in linear, form III. This indicated the direct formation of both single- and double-strand lesions in mitochondrial DNA by NCS. From these results, an SSB:DSB ratio of 2.3:1 was determined at a concentration of 250 nM, indicating that NCS efficiently produced direct DSBs as well as SSBs in mitochondrial

[2] The intense bands above form II have been suggested to be replicative intermediates of the episome (Cullinan & Beerman, 1989), or alternatively they may be concatenated episomal DNA.

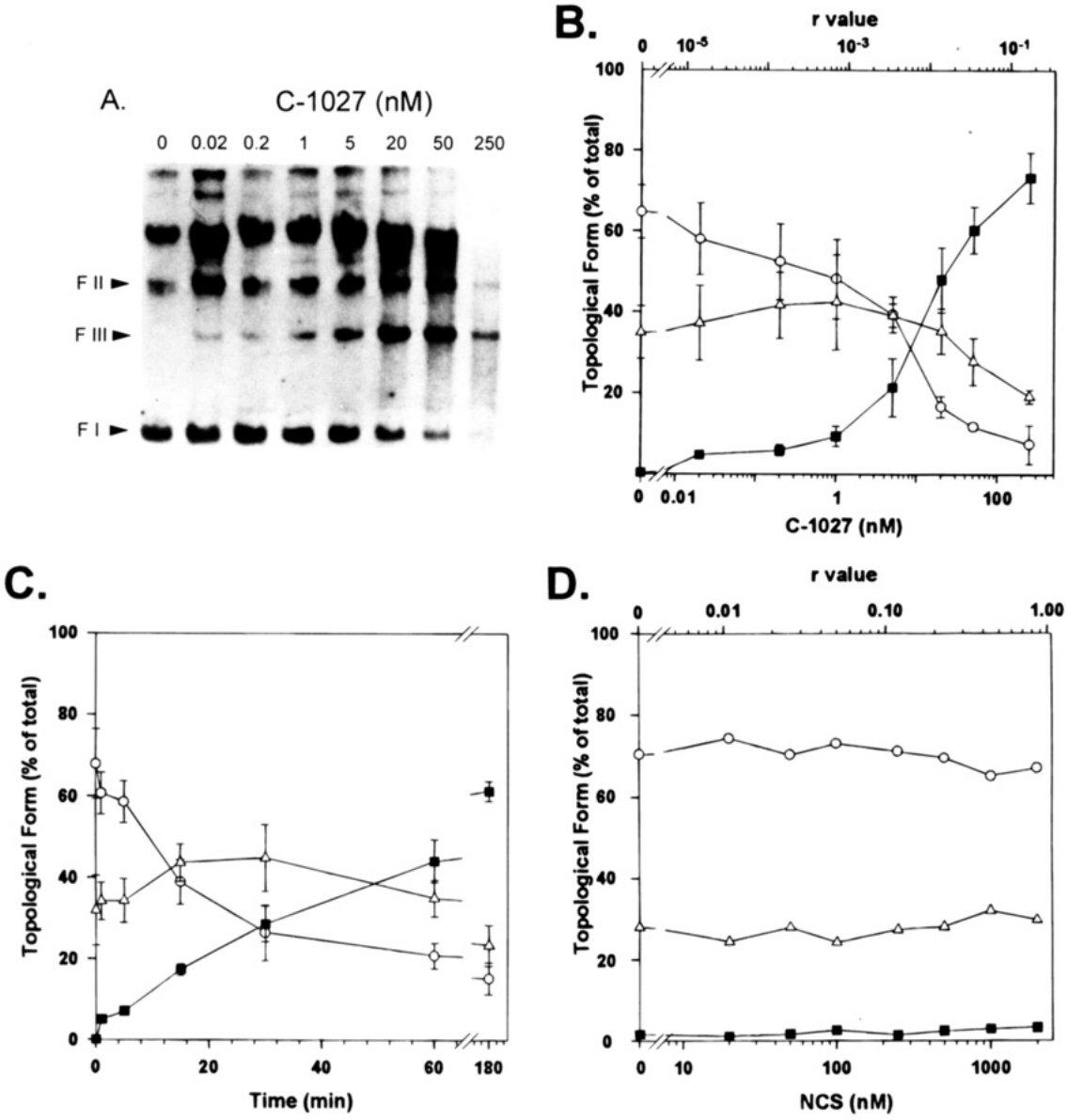

FIGURE 2: Episomal DNA cleavage in cultured 935.1 cells. (A) Southern blot of episomal DNA from 935.1 cells treated for 60 min with the indicated concentrations of C-1027 hybridized to random-primed [^{32}P]pM19. Cleavage of DNA is depicted as a decrease in FI, and an increase in FII and in FIII. (B) Dose effects of 60 min exposure to C-1027. (C) Time effects of exposure to 20 nM C-1027. (D) Dose effects of 30 min exposure to NCS. The concentrations of supercoiled FI (○), nicked circular FII (△), and linear FIII (■) DNA are expressed as the percent of the total concentration of topoisomers in each sample. Drug to DNA ratios (r values) were calculated using the value 2.3 $\times$ 10^{-15} mol of DNA per 935.1 cell. For C-1027, each data point is the mean $\pm$ SEM (n = 3). For NCS, each data point is the mean of duplicate experiments.

DNA. This was particularly significant in light of the fact that NCS had no effect on episomal DNA in the same cells even at a 2 μM concentration.

Comparison of Genomic DNA Damage to That of Defined Targets. Filter elution techniques were employed to quantitate C-1027-induced strand breaks in 935.1 cell genomic DNA, and thereby allow comparison to lesions in the episomal and mitochondrial DNA. The alkaline filter elution profile in Figure 4A indicates that 60 min C-1027 treatment produced SSBs in the bulk DNA of 935.1 cells at concentrations as low as 0.5 nM, and that the number of these breaks increased in a dose-dependent manner. Similarly, DSBs initially were detected by neutral filter elution at 2.5 nM C-1027, and their number continued to increase with concentration (Figure 4B). These latter data were obtained from 15 min drug treatment, and no change in the elution profile was seen at exposure times up to 180 min (data not shown). From these alkaline and neutral elution dose curves, the SSB and DSB frequencies were calculated, and it was determined that 1 nM C-1027 produced 0.17 SSB/10^6 nt and 0.016 DSB/10^6 bp, giving an SSB:DSB ratio of approximately 10:1. In contrast, NCS was markedly less effective than C-1027 in that a comparable SSB frequency (0.19 SSB/10^6 nt) was only obtained at a concentration of 250 nM (data not shown).

An overall comparison of the various C-1027- and NCS-inducd strand-break frequencies is shown in Table 1. C-1027 displayed an approximately 2-fold preference for cleavage of episomal vs mitochondrial DNA, although the relative SSB:DSB ratios were approximately 1:1 in both cases. The SSB frequency was 85-fold greater in episomal than genomic DNA, and more than 640-fold more double-strand lesions were produced in the episome. The 1 nM concentration of C-1027 at which these comparisons were made corresponds

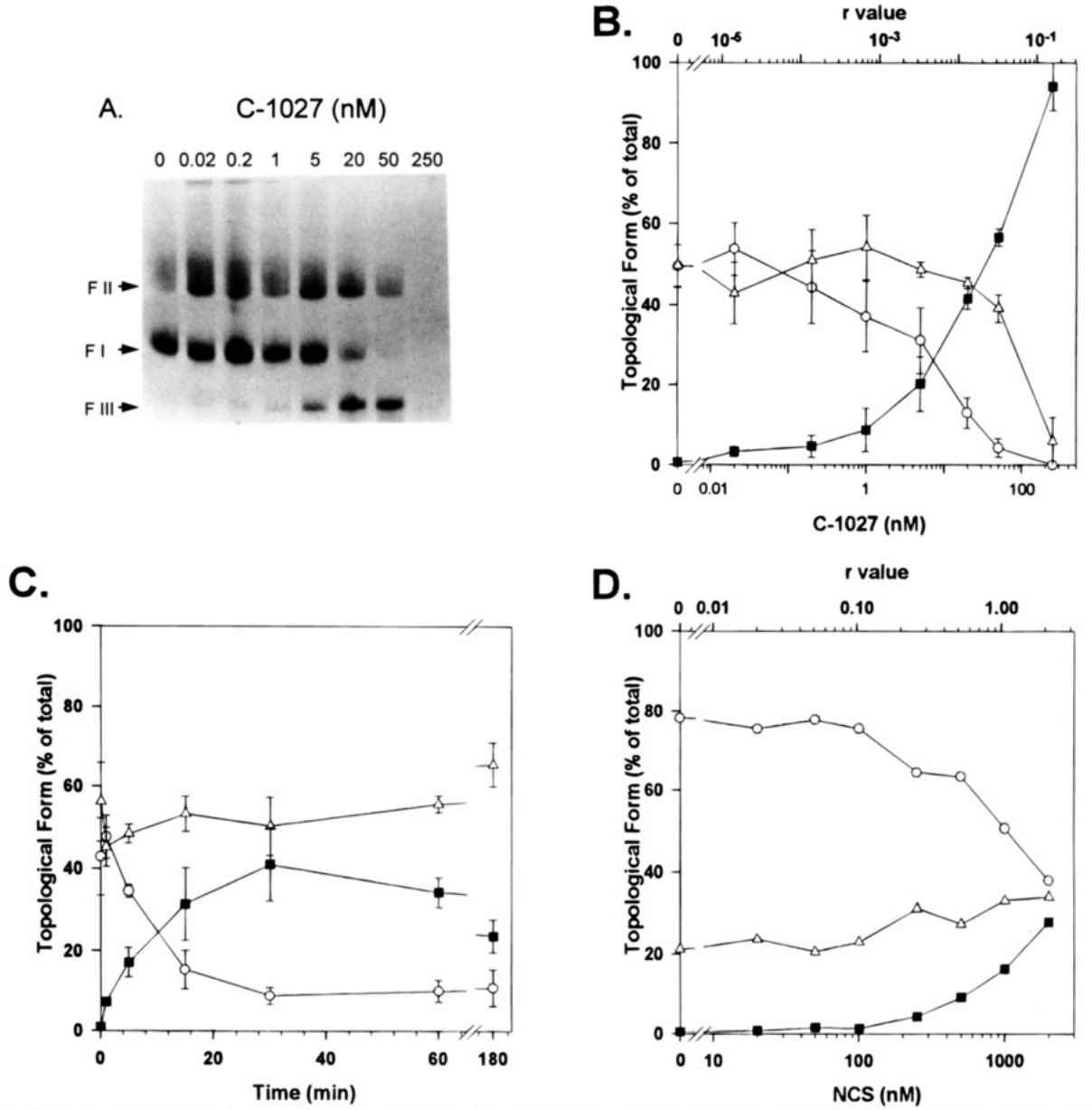

FIGURE 3: Mitochondrial DNA cleavage in cultured 935.1 cells. (A) Southern blot of mitochondrial DNA from 935.1 cells treated for 60 min with the indicated concentrations of C-1027 hybridized to random-primed [^{32}P]pAM1. Cleavage of DNA is depicted as a decrease in FI, and an increase in FII and in FIII. (B) Dose effects of 60 min exposure to C-1027. (C) Time effects of exposure to 20 nM C-1027. (D) Dose effects of 30 min exposure to NCS. The concentrations of supercoiled FI (○), nicked circular FII (△), and linear FIII (■) DNA are expressed as the percent of the total concentration of topoisomers in each sample. For C-1027, each data point is the mean ± SEM (n = 3). For NCS, each data point is the mean of duplicate experiments.

to an r value of 0.67×10^{-3} for episomal and mitochondrial DNA, and 0.83×10^{-3} for genomic DNA. This indicates that the preference for the smaller targets is a real effect and not a function of different drug-to-DNA ratios. The relative r values were likewise held constant for the NCS treatments ($r \cong 0.2$ for all three intracellular targets). As noted previously, NCS produced no detectable damage to episomal DNA yet directly produced both single- and double-strand lesions in mitochondrial DNA. Similar to what was observed with C-1027, NCS cut mitochondrial DNA much more frequently (28-fold) than it did genomic DNA, indicating a preference for the former. However, the NCS concentration at which the mitochondrial and genomic damage occurred was 250-fold greater than that for C-1027.

Specificity of Episomal DNA Cleavage. While it was apparent that C-1027 induced direct DSBs in intracellular DNA, it was not known whether these lesions occurred selectively. Since the conversion of topological forms would only indicate general damage to the episome, the specificity of C-1027 for particular regions of the episome was measured using the indirect end-labeling technique (Wu, 1980; Cullinan & Beerman, 1989). Figure 5A shows a densitometric profile of the subbands effected by C-1027, with the sites of strongest cleavage indicated by arrows in the episome map. These results clearly indicate that the DSBs produced by C-1027 are not random events, but occur in specific regions of the episome. The degree of cleavage at these sites increased with the dose of C-1027, and the most intense bands were measured at the highest concentration tested (20 nM). Of the five prominent cleavage sites, three occurred within functional genetic elements. These included the origin of replication and the E6/E7 coding region of BPV, and the MMTV LTR glucocorticoid-inducible promoter. Activation of the MMTV LTR promoter region by dexamethasone

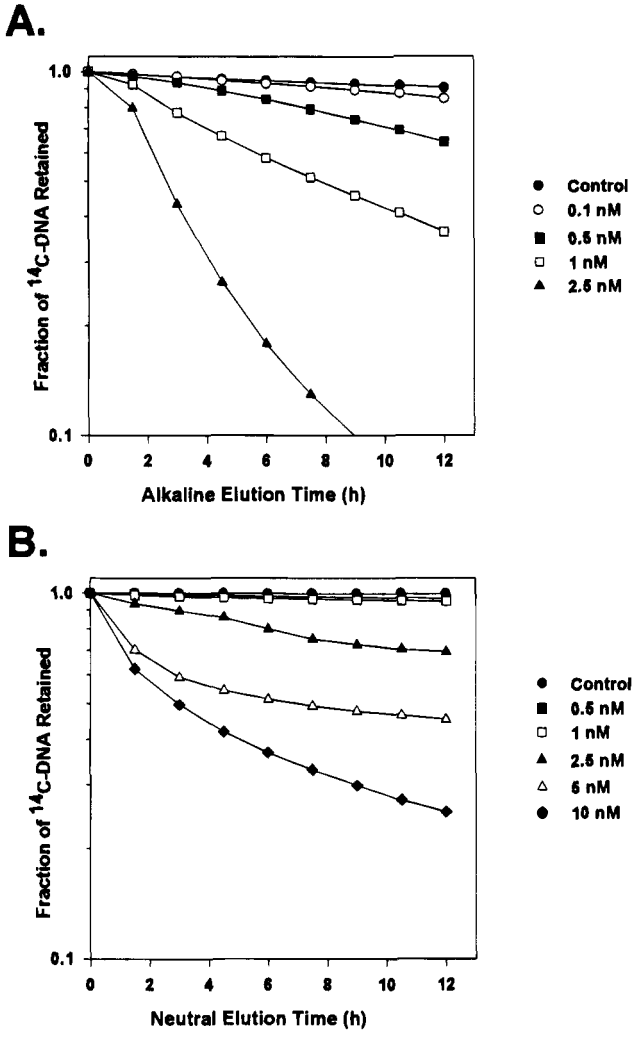

FIGURE 4: C-1027-induced single- and double-strand cleavage of 935.1 cell genomic DNA, as determined by (A) alkaline and (B) neutral filter elution, respectively. [^{14}C]Thymidine-labeled 935.1 cells were drug-treated for 60 and 15 min with the indicated concentrations of C-1027 for alkaline and neutral elution assays, respectively. For both alkaline and neutral elution curves, each point is the mean of 2−5 replicate determinations.

treatment changes its conformation, resulting in the generation of a DNase I-hypersensitive site (Richard-Foy & Hager, 1987), and increases transcription of the downstream *v-Ha-ras* gene (Ostrowski et al., 1983). Consequently, 935.1 cells were pretreated with dexamethasone to determine if transcriptionally activated DNA associated with the MMTV LTR is more susceptible to C-1027-induced damage. This treatment affected neither the specificity nor the intensity of the C-1027-induced cleavage sites (data not shown). Similarly, the contribution of glucocorticoid induction to gross episomal damage was addressed by measuring drug-induced topological forms conversion of episomal DNA (Figure 5B). As with the site preference, no difference was observed in the relative intensities of the three topoisomers whether dexamethasone was present or absent.

DISCUSSION

The present studies were undertaken to examine C-1027 holoantibiotic-induced damage of intracellular DNA targets, using the episome-containing cell line 935.1 as a model. The presence of the episome provided a readily isolated, defined DNA target from which the generation of strand breaks was

Table 1: Comparison of C-1027- and NCS-Induced Intracellular Strand-Break Frequencies[a]

	C-1027		NCS	
DNA	SSB	DSB	SSB	DSB
episomal	14.55	10.30	0.00	0.00
mitochondrial	7.72	4.94	5.42	2.36
genomic	0.17	0.016	0.19	ND

[a] A comparison was made of intracellular single- and double-strand break frequencies induced by 1 nM C-1027 and 250 nM NCS in 935.1 cell episomal, mitochondrial, and genomic DNA. C-1027 treatment times were 60 min, except for the genomic DSB determination which was 15 min. NCS treatment times were 30 min, except for the genomic SSB determination which was 15 min. From the data in Figures 2 and 3, SSB frequencies in episomal and mitochondrial DNA were calculated by determining the fractional decrease in form I divided by the number of nucleotides (17 588 and 32 600 for episomal and mitochondrial DNA, respectively). Similarly, DSB frequencies in episomal and mitochondrial DNA were calculated by determining the fractional increase in form III divided by the number of base pairs (8794 and 16 300 for episomal and mitochondrial DNA, respectively). The genomic break frequencies were determined from the data in Figure 4 (C-1027; NCS alkaline elution profile not shown) by multiplying the rad equivalent damage [185.3 and 213.5 rad for C-1027 and NCS SSBs, respectively; 360.5 rad for C-1027 DSBs (data not shown) (Cullinan et al., 1991)] by 0.9×10^{-9} SSB rad^{-1} nt^{-1} for SSBs, and by 4.5×10^{-11} for DSBs (Kohn et al., 1976; Bradley & Kohn, 1979). For all break frequency determinations, results are expressed as SSB/10^6 nt and DSB/10^6 bp. ND, not determined.

monitored. In addition, the effects of C-1027 on mitochondrial and genomic DNA integrity were quantitated in the same cells. This has allowed for the first time a detailed examination of intracellular DNA damage caused by C-1027, and it is the first study of an enediyne-containing drug's comparative effects on both nuclear and cytoplasmic DNA. Previously, Sugimoto and colleagues (1990) demonstrated that the primary intracellular target for C-1027 is DNA. However, that work was limited to general effects such as cytotoxicity, inhibition of macromolecular synthesis, and general DNA damage in cultured cells. Subsequent studies have shown that C-1027 produces direct SSBs and DSBs in a cell-free environment (Sugiura & Matsumoto, 1993; Xu et al., 1990), thus indicating that the induction of such lesions may be the source of the drug's potent cytotoxic effects. Similar to those cell-free studies, C-1027 was observed in the present studies to cleave intracellular DNA in a mixed single- and double-strand manner. The SSB:DSB ratios of episomal (1.4:1) and mitochondrial (1.6:1) DNA cleavage indicated a nearly equal propensity to generate DSBs as well as SSBs. Similar results were obtained with cell-free pM19 DNA (the plasmid precursor of the 935.1 cell episome) where an SSB:DSB ratio of 1.1:1 was obtained using the C-1027 holoantibiotic. This ratio indicates a higher frequency of DSBs than the 4.2:1 ratio reported by Xu and colleagues (1994), who used isolated C-1027 chromophore to treat pBR322 DNA. This difference may be a consequence of variance in the sequences of the target plasmids utilized.

An important difference between the cell-free and intracellular effects of C-1027 is noted upon comparison of the r values at which degradation of form I takes place in episomal vs cell-free plasmid DNA. A 20% decrease in the relative concentration of intracellular episomal form I DNA is observed at a drug to DNA ratio of 0.14×10^{-3} compared to $r = 40 \times 10^{-3}$ under cell-free conditions. This indicates that C-1027 cleaves DNA 285-fold more efficiently in cells than in a cell-free environment. The reason(s) for this

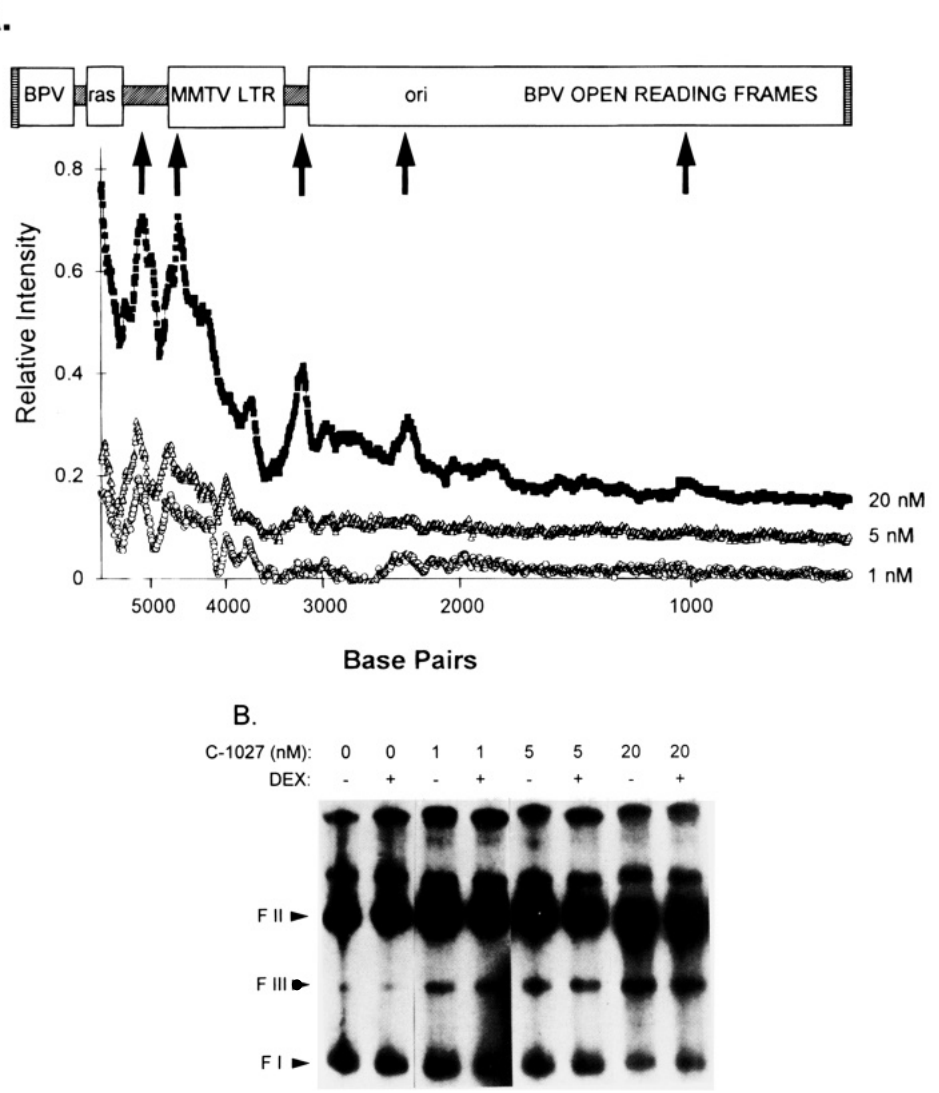

FIGURE 5: Cleavage of episomal DNA in cultured 935.1 cells by C-1027: Site-preference and dexamethasone effects. (A) Densitometric scan of an autoradiogram of drug-induced breaks on the intracellular episome. Cells were treated for 60 min with either 1, 5, or 20 nM C-1027 as indicated in the right margin of the figure. DNA was separated on an agarose gel, and the Southern blot was hybridized to random-primed [^{32}P]*Eco*RI−*Sma*I fragment of the pM19 plasmid. Each graph represents the densitometric pixel density of a lane on the gel minus that of the control lane. The graphs for the 5 and 20 nM C-1027-treated samples were offset from the base line by +0.07 and +0.14 densitometric unit per point, respectively, so as to prevent overlap. Peak positions corresponding to the location of double-strand breaks were determined relative to the migration of size markers prepared by endonuclease restriction of pM19. A genomic map of the episome is illustrated at the top of the figure for orientation of the drug-induced cleavage sites indicated by the arrows. (B) Southern blot of episomal DNA from 935.1 cells treated for 60 min with the indicated concentrations of C-1027 in the presence (+) or absence (−) of dexamethasone. Cleavage of DNA is depicted as a decrease in FI, and an increase in FII and in FIII.

difference is (are) not yet clear. One possibility is that activation of the drug's enediyne core is enhanced within the cell. However, since C-1027 activity does not require activation by thiol (Sugimoto et al., 1990), it is not likely that this effect is due to the presence of cellular thiols such as glutathione. Another possible explanation is that drug−DNA interaction is enhanced in the cell. S.-Tsuchiya et al. (1994) have suggested that the C-1027 apoprotein moiety accompanies the chromophore into the cell and conveys it to the DNA. Therefore, it may be that there are intracellular factors that aid in targeting the protein, and thus the chromophore, to the DNA. Similarly, the chromophore may be dissociated more efficiently from the apoprotein carrier in the cell. Such possibilities await more detailed study.

The preference exhibited by C-1027 for episomal and mitochondrial DNA relative to genomic DNA indicates that cleavage of the DNA duplex by this enediyne chromoprotein is not a random event. A similar preference has been observed in 935.1 cells upon treatment with bleomycin (Cullinan et al., 1991) and the topoisomerase inhibitor *m*-AMSA (Cullinan & Beerman, 1989). Low-dose irradiation of 935.1 cells results in 15-fold greater cleavage of episomal versus genomic DNA, and this effect has been attributed to preferential cleavage of transcribing chromatin (Cullinan et al., 1991). The base and sequence specificity of C-1027-induced damage has previously been demonstrated for the isolated chromophore in studies using cell-free DNA (Xu et al., 1994). It was determined that SSBs occur primarily at A and T residues and that the more lethal DSBs are pentanucleotide sequence-specific lesions occurring at sequences such as GTTAT/ATAAC. Examination of the site specificity of damage determined that there are a number of preferential cleavage sites on the 935.1 cell episome (Figure 5A). These included the BPV origin of replication and the E6/E7 open reading frame regions, as well as the MMTV LTR promoter region. These cleavage sites are significant

in that they are regions of active or potentially active chromatin. NCS (Beckmann et al., 1987) and the protein strand-scission agent bleomycin (Beckmann et al., 1987; Cullinan et al., 1991; Kuo, 1981) have been suggested to selectively cut actively transcribing regions of DNA. Furthermore, treatment of 935.1 cells with bleomycin demonstrated episomal cleavage sites similar to those observed here with C-1027 (Cullinan et al., 1991). Treatment of 935.1 cells with the synthetic glucocorticoid dexamethasone induces a conformational change in this MMTV LTR region resulting in the generation of a DNase I-hypersensitive site (Richard-Foy & Hager, 1987), and increases transcription of the downstream *v-Ha-ras* gene by 20-fold (Ostrowski et al., 1983). In the present studies, dexamethasone treatment had no effect on either the site or the intensity of the episome cleavage by C-1027, suggesting a lack of specificity for the transcriptionally active MMTV LTR region of DNA. However, as noted previously (Cullinan et al., 1991), potential differences in drug damage in the presence and absence of dexamethasone may be masked by effects of the hormone-independent BPV promoter on the MMTV LTR.

It was interesting that NCS induced damage of mitochondrial DNA, but had no effect on episomal DNA even at concentrations as high as 2 μM. Previous studies with an SV40-infected cell model have demonstrated that NCS cleaves intracellular, nuclear DNA. However, the requisite concentration of NCS was 1000-fold greater than that necessary to cleave cell-free DNA (Grimwade & Beerman, 1986). Similar to cell-free DNA which is devoid of nucleosomal proteins, and is cut by NCS, mitochondrial DNA is non-histone-associated. Recent work has indicated that the presence of histone proteins on DNA may hinder, either directly or indirectly, the intercalation of the NCS naphthoate moiety and thereby preclude enediyne-mediated strand scission (Yu et al., 1994). Therefore, the selective cleavage of mitochondrial DNA seen here may have occurred because the non-histone-associated mitochondrial DNA is more susceptible to NCS-mediated damage than in nuclear DNA (*i.e.*, the episome). That NCS cleaves genomic and not episomal DNA may be due to the limited sequence diversity of the episome. The fact that C-1027 is only 2-fold different in its cleavage of nuclear and mitochondrial DNA targets suggests that it associates with DNA by a different mechanism than NCS. This effect may be related to the structural differences in C-1027 compared to either NCS or kedarcidin. The latter two compounds contain naphthoate moieties which are thought to be responsible for their intercalative binding to DNA (Povirk et al., 1981; Zein et al., 1993). In contrast, C-1027 possesses a benzoxazolinate moiety (Otani et al., 1991), which, if it is responsible for intercalative interaction of the drug with the DNA minor groove, may not be affected by the presence of histones.

There appears to be little or no barrier to cellular penetration of the C-1027 chromoprotein complex, as DNA damage occurred in cells at a rapid rate. Initial conversion of episomal (Figure 2C) and mitochondrial (Figure 3C) topological forms was evident within 1 min of addition of C-1027 to cultures of 935.1 cells. However, where damage to the episome was observed to increase continuously with time, mitochondrial DNA cleavage may reverse at later times. The data in Figure 3C indicate that the damage of mitochondrial DNA cleavage may reverse at later times. The data in Figure 3C indicate that the damage of mitochondrial DNA induced by 20 nM C-1027 was complete within 30 min. This was evident from the plateau of form I DNA and the decrease in the percent of form III DNA at times greater than 30 min. That similar data were obtained when this study was repeated using 5 nM C-1027 (data not shown) indicates that these effects were not the result of degradation of forms II and III. The decrease in the relative concentration of form III DNA after 30 min may indicate that C-1027-induced damage of mitochondrial DNA is reversed. There have been reports of the repair of various mitochondrial DNA lesions including alkylation (Myers et al., 1988; Satoh et al., 1988; Pettepher et al., 1991; LeDoux et al., 1993), inter- and intrastrand cross-links (LeDoux et al., 1992; Pasupathy & Pradhan, 1992), and apurinic/apyrimidinic sites (Foury, 1982; Tomkinson et al., 1988). Bleomycin is the only other strand-scission agent whose cleavage effects have been examined on mitochondrial DNA, and unlike the present study where intact cells were treated with C-1027 or NCS, those studies used only isolated mitochondria and cell-free mitochondrial DNA (Lim & Neims, 1987). Since lesion repair was not examined in the present studies, the potential for the repair of mitochondrial DNA strand scission damage awaits future study.

The present studies have indicated that the C-1027 holoantibiotic is a highly potent DNA-damaging agent in cells. It efficiently cleaves nuclear and mitochondrial DNA at low nanomolar concentrations and demonstrates preference for specific regions within the episome. Furthermore, mitochondrial DNA damage by an enediyne-containing strand-scission agent has been examined for the first time. In the future, studies will focus on the relationship between DNA damage and inhibition of replication using defined, intracellular DNA targets.

ACKNOWLEDGMENT

We thank Loretta Gawron for her excellent technical assistance.

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BI941385Z