C1027 Chromophore, a Potent New Enediyne Antitumor Antibiotic, Induces Sequence-Specific Double-Strand DNA Cleavage†

Yong-jie Xu,‡,§ Yong-su Zhen,§ and Irving H. Goldberg*,‡

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Tiantan, Beijing 100050, China

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ABSTRACT: C1027, a new macromolecular antitumor antibiotic produced by a *Streptomyces* strain, shows highly potent cytotoxicity to cultured cancer cells and marked DNA cleaving ability. The structure of its chromophore, responsible for most of the biological activities of the antibiotic, was recently determined and found to contain a nine-membered enediyne. In contrast to other enediyne antibiotics, such as neocarzinostatin, calicheamicin, esperamicin, and recently found kedarcidin, C1027 damages duplex DNA even in the absence of thiols. The DNA damage caused by C1027 includes double-strand breaks, single-strand breaks, and abasic sites. Experiments with plasmid DNA and ^{32}P-end-labeled restriction fragments demonstrated that the chromophore, extracted from the protein-containing holoantibiotic, interacts in the DNA minor groove and cleaves double-helical DNA with a remarkable sequence-selectivity causing direct double-strand breaks. The double-strand cleavage sites, occurring predominantly at CTTTT/AAAAG, ATAAT/ATTAT, CTTTA/TAAAG, CTCTT/AAGAG, and especially GTTAT/ATAAC, consist of five nucleotide sequences with a two-nucleotide 3′-stagger of the cleaved residues (cutting sites are underlined). The chemical structures of the damaged residues at the GTTAT/ATAAC cleavage site suggest a model in which a C1027-induced double-strand break results from abstraction, by a single molecule of the diradical form of the chromophore, of a C4′ hydrogen atom from the A residue of GTTAT and a C5′ hydrogen atom from the A of ATAAC on the opposite strand. Single-strand breaks, which are mainly produced at adenylate and thymidylate residues, appear to be separate events presumably resulting from different binding modes of the drug to DNA. The highly sequence-selective double-strand breaks induced by C1027 chromophore and their resistance to repair may account for the potent cytotoxicity of the antibiotic.

C1027, a new macromolecular antitumor antibiotic produced by *Streptomyces globisporus* C1027 (Hu et al., 1988), is extremely cytotoxic to cultured cancer cells (IC_{50} = 1.5 × 10^{-17}–3.2 × 10^{-16} M) and highly active against a panel of transplantable tumors in mice (Zhen et al., 1989). It consists of a labile chromophore, which is responsible for most of the biological activities, and a noncovalently bound apoprotein (Otani et al., 1988). This constitution is similar to that of other macromolecular antitumor antibiotics such as neocarzinostatin, macromomycin, actinoxanthin, and the recently found kedarcidin (Xu et al., 1991; Otani 1993). The protein moiety of C1027 has a single polypeptide chain cross-linked by two disulfide bonds with a molecular mass of 10 500 Da (Otani et al., 1991a,b). The chromophore (**1**, Figure 1) has been recently identified as a member of the potent enediyne family of antitumor antibiotics (Minami et al., 1993; Iida et al., 1993), which at present includes neocarzinostatin chromophore (Goldberg, 1991), the calicheamicins (Lee et al., 1991), the esperamicins (Long et al., 1989), the dynemicins (Sugiura et al., 1990), and kedarcidin chromophore (Leet et al., 1993). Like other enediyne agents, antibiotic C1027 is believed to exert its biological actions through the induction of cellular DNA damage (Xu et al., 1990).

Of the types of DNA damage induced by neocarzinostatin and the free radicals generated by other means, such as ionizing radiation, double-strand (DS) cleavage appears to be the most lethal (Hatayama & Goldberg, 1979; von Sonntag, 1987). Enediyne antitumor antibiotics are unique for their potential to produce bistranded DNA damage. In spite of significant structural diversity, these compounds share a common mechanism of action (Figure 1): they undergo an electronic rearrangement to form a benzenoid diradical upon activation, which is positioned in the minor groove of DNA so as to damage DNA by abstracting hydrogen atoms from the deoxyribose sugar on both strands (Dedon & Goldberg, 1992). Reaction of the resulting deoxyribose carbon-centered radicals with molecular oxygen initiates a process that results in a variety of chemically modified abasic sites and strand breaks consisting of DNA fragments with sugar residues attached to the 3′- or 5′-ends. This novel mechanism of DNA damage has important implications for their application as potent cancer chemotherapeutic agents. Neocarzinostatin chromophore cleaves double-stranded DNA predominantly at GT steps, especially the AGT/ACT trinucleotide sequences (Dedon & Goldberg, 1990). On the other hand, DS breaks produced by calicheamicin occur with a tetranucleotide sequence specificity mainly at the TCCT/AGGA site (Zein et al., 1988).

Unlike other enediyne compounds, antibiotic C1027 shows prominent damage of duplex DNA even in the absence of thiols or reducing agents. The DNA damage induced by C1027 includes single-strand (SS) and double-strand (DS) breaks and abasic sites (Xu et al., 1992). Recently, Sugiura and Matsumoto 1993) reported that the preferential cutting sites of holoantibiotic C1027 are at adenylate and thymidylate residues with the frequency A≥T≫C>G and that the major damage appears to be strand scission caused by hydrogen abstraction from the C4′ position of the T and A residues.

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* To whom correspondence should be addressed.

‡ Harvard Medical School.

§ Chinese Academy of Medical Sciences.

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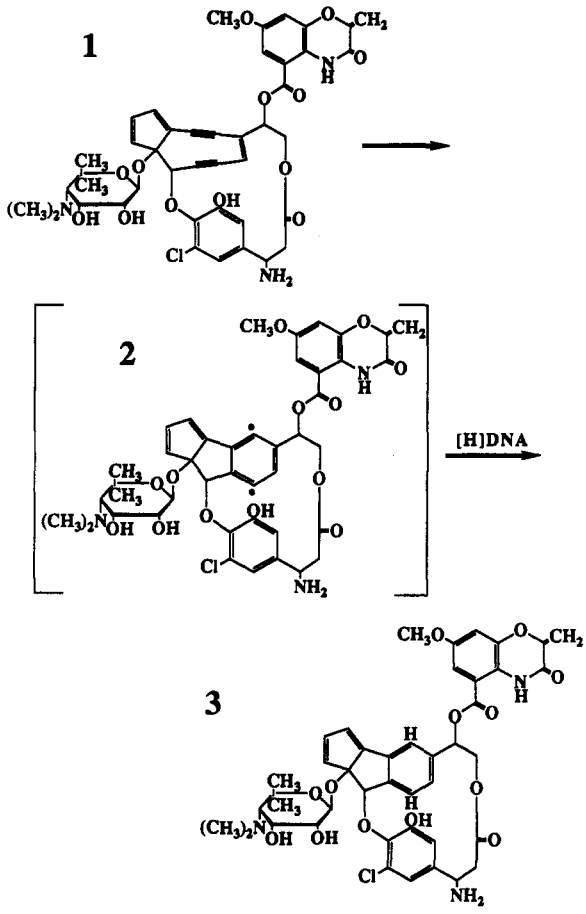

FIGURE 1: Chemical structure of C1027 chromophore (**1**) and proposed mechanism for its cycloaromatization to the diradical state (**2**).

Although DS breakage was also found, these lesions were, with only one possible exception, not specifically analyzed for their sequence-specificity. Rather, the sequence-specificity of the DS lesions was mainly inferred from the sequencing of the separate complementary DNA strands. We have carried out a study on the DS breaks produced by C1027 chromophore in plasmids and DNA restriction fragments and find sequence-specificities that are different from those reported by Sugiura and Matsumoto (1993). Further, the DS breakage involves C5′ chemistry at one site of the two-nucleotide staggered lesion, in addition to C4′ chemistry at the lesion on the complementary strand.

MATERIALS AND METHODS

Materials. Lyophilized antibiotic C1027 was purified from the fermentation broth of the producing *Streptomyces* strain as previously described (Otani et al., 1988). C1027 chromophore was extracted from the holoantibiotic with cold methanol in the presence of sodium citrate and stored in the dark at −70 °C (Shao & Zhen, 1992; Otani et al., 1991a,b). Plasmids (>90% in supercoiled form) pAP 1–3 (Dedon et al., 1992), generously provided by Dr. Peter C. Dedon, and pBR322 were purified by standard procedures (Ausubel et al., 1989). Calf thymus DNA was purchased from Sigma, sonicated, extensively dialyzed against Tris buffer, and ethanol-precipitated prior to use. Other reagents were obtained as follows: pBR322 DNA–*Msp*I digest, restriction enzymes, and T4 polynucleotide kinase, New England Biolabs; Klenow fragment, Boehringer Mannheim; radiochemicals, New England Nuclear DuPont; and reagent-grade chemicals, Sigma and Aldrich.

Quantitation of Strand Breaks Produced by C1027 Chromophore in pBR322. A standard reaction mixture containing 25 μg/mL pBR322 DNA and 20 mM Tris·HCl, pH 7.6, was chilled to 0 °C in ice before the drug reaction was started by the addition of C1027 chromophore in methanol (maximum final concentration was 10%). After 30 min in ice, 0.5 μg of pBR322 was electrophoresed on a slab of 1.0% agarose gel containing TBE (Tris–borate–EDTA) buffer, pH 8.0. The negative image of the ethdium bromide-stained gel was scanned by an LKB Ultrascan XL laser densitometer. 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 number of DS and SS breads in pBR322 was calculated from the relative quantities of the three plasmid forms produced by C1027 chromophore by assuming a Poisson distribution of DNA cutting sites (Povirk et al., 1977).

Preparation of Labeled Restriction Fragments. The 247 bp *Hin*dIII/*Bsi*HKAI fragment of pBR322 and the 307 bp *Ava*II/*Apa*LI and the 270 bp *Eco*RI/*Pvu*II fragments from pAP 1–3 plasmid were separately 3′-^{32}P and 5′-^{32}P end-labeled at one restriction site (underlined) (Ausubel et al., 1989). The 5′-end was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase after treatment with bacterial alkaline phosphatase, and the 3′-end was labeled with *E. coli* DNA polymerase I large fragment. After digestion with the second restriction enzyme, the singly labeled fragment was purified by polyacrylamide gel electrophoresis.

Drug/DNA Reaction. The DNA cleaving reaction was initiated by adding C1027 chromophore (1 μM) to a prechilled mixture of 20 μg/mL calf thymus DNA and end-labeled DNA fragments (~6 × 10^5 cpm) in 20 mM Tris·HCl buffer (pH 7.6). The reaction was allowed to proceed in the dark at 0 °C for 10 min and then stopped by adding ice-cold ethanol to precipitate the DNA. Similarly, DNA was treated with bleomycin A_2 in the presence of equimolar $Fe(NH_4)_2(SO_4)_2$ and 10 mM 2-mercaptoethanol. When studying the chemistry, a portion of the DNA/C1027 reaction solution with 3′-end-labeled fragments was treated with sodium borohydride to reduce the 5′-nucleoside aldehyde as previously reported (Kappen & Goldberg, 1983).

Analysis of DS Cleavage Sites. When the DNA/drug reaction was stopped, the precipitated DNA was rinsed twice with 70% ethanol and dried in a SpeedVac concentrator. DS cleavage fragments produced by C1027 chromophore were resolved on nondenaturing 12% polyacrylamide gels (230 V, room temperature). 5′-End-labeled pBR322 DNA–*Msp*I digest was used as a molecular size marker. The resolved bands were excised from the gel by alignment of an autoradiogram. The cleavage fragments in each band were eluted by the diffusion method (Ausubel et al., 1989) and then analyzed on 8 or 15% polyacrylamide sequencing gels (Maxam & Gilbert, 1980). After electrophoresis, the gel was dried onto filter paper and exposed to X-ray films. The band location was determined by comparison with Maxam–Gilbert sequencing standards. DS breakage products were quantified by scanning the autoradiograms with the densitometer. To characterize the chemistry, alkali treatment was carried out by heating the dried DNA pellets in 50 μL of 1 M piperidine (90 °C, 30 min), as described previously (Kappen & Goldberg, 1992).

RESULTS

SS and DS Cleavage in pBR322. Incubation of form I (supercoiled form) pBR322 DNA with C1027 chromophore at 0 °C for 30 min resulted in the conversion of form I to form

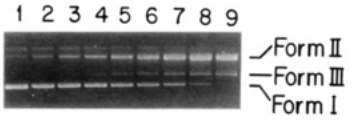

FIGURE 2: Agarose gel electrophoretic patterns of DNA strand scission by C1027 chromophore. pBR322 plasmid DNA (0.5 μg) was incubated with C1027 chromophore at the following drug concentrations: 0.0125, 0.025, 0.05, 0.1, 0.125, 0.25, 0.5, and 1 μM in lanes 2–9, respectively. The reaction conditions were as described under Materials and Methods. Lane 1 shows intact DNA alone.

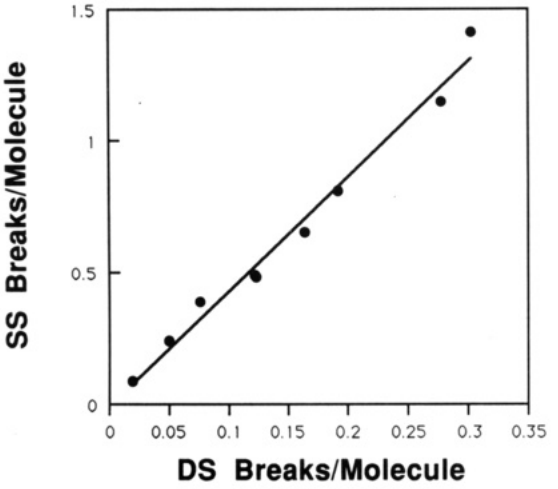

FIGURE 3: Quantitation of DS and SS breaks produced by C1027 chromophore in pBR322 DNA. The number of SS and DS breaks was calculated from the relative quantities of three topological forms (Figure 2) by assuming a Poisson distribution of DNA breaks. Results are expressed as breaks per pBR322 plasmid molecule.

II (nicked circular form resulting from one SS break in form I) and form III (linear duplex resulting from a DS break in form I or from a SS break in form II in the complementary strand opposite a previous nick). Higher concentrations of the drug exerted more marked cleaving effects (Figure 2). In contrast to other enediyne compounds, C1027 chromophore did not require thiols or reducing agents for efficient DNA cleavage (data not shown). A time course study at one concentration of the chromophore showed that DNA cleavage was essentially complete within 5 min of incubation at 0 °C (data not shown). This was different from holo-C1027, which showed essentially no activity at 0 °C but potent DNA breakage at 37 °C, as also found by Sugiura and Matsumoto (1993); further, incubation with the isolated chromophore at 37 °C only slightly increased the accumulation of forms II and III DNA. When pBR322 DNA was incubated with 0.25 μM C1027 chromophore, the changes of forms were as follows: at 0 °C, form I, 35.9 ± 5.5%; form II, 49.9 ± 2.4%; and form III, 14.1 ± 3.3%; at 37 °C, form I, 29.7 ± 6.0%; form II, 53.9 ± 4.1%; and form III, 16.4 ± 2.2% (data are based on three different experiments). The number of DS and SS breaks produced by C1027 chromophore in pBR322 was determined by assuming that the cutting followed a Poisson distribution. Figure 3 shows that the average ratio of DS to SS breaks in the absence of thiols is 1:4.2, which is slightly higher than that (1:5.3) of neocarzinostatin with glutathione as an activator (Dedon & Goldberg, 1992). To reveal the presence of abasic sites as strand breaks, C1027-treated pBR322 DNA was exposed to putrescine which reacts with virtually all abasic sites to produce strand breaks (Povirk & Houlgrave, 1988). After treatment with putrescine, the ratio was increased to about 1:1. This result indicates that about half of the DNA damage induced by C1027 are DS lesions.

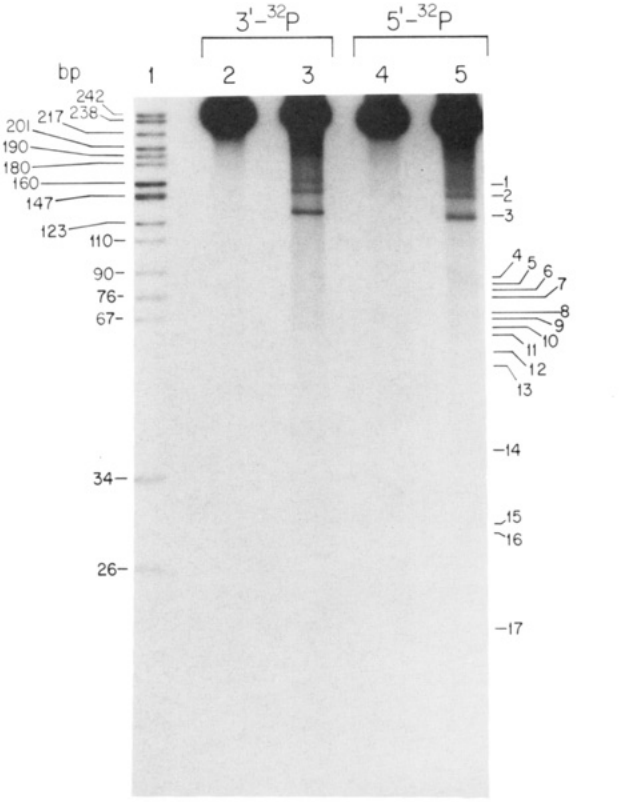

FIGURE 4: DS breaks produced by C1027 chromophore. The 247-bp *Hind*III/*Bsi*HKAI fragment of pBR322 was separately 3′- and 5′-^{32}P end-labeled at the *Hind*III site and treated with C1027 chromophore (1 μM) or drug vehicle (10% methanol; lanes 2 and 4) in 20 mM Tris buffer, pH 7.6. The drug-treated DNA was resolved on a 12% nondenaturing polyacrylamide gel. The 3′-^{32}P fragments are two nucleotides longer, so run lightly slower than the 5′-end-labeled counterparts. Molecular size markers are 5′-^{32}P-labeled *Msp*I-digested pBR322 (lane 1). Lanes 2 and 4, 3′-^{32}P- and 5′-^{32}P-labeled DNA control; lanes 3 and 5, drug-treated 3′-^{32}P- and 5′-^{32}P-labeled DNA. Bands representing sites of DS cleavage are numbered (1–17). The faint bands (4–17) on the gel are more readily detected by gel scanning.

Identification of DS Cleavage Sites. To identify the DS cutting sites, we examined the reaction of C1027 chromophore with several 3′-^{32}P and 5′-^{32}P end-labeled restriction fragments in the presence of sonicated calf thymus DNA. An example of the electrophoretic analysis of DS cleavage sites is shown in Figures 4 and 5 for the 247 bp *Hind*III/*Bsi*HKAI fragment from pBR322. C1027 chromophore-treated DNA was first subjected to electrophoresis on a nondenaturing polyacrylamide gel (Figure 4). The bands containing the products of DS cleavage, marked in Figure 4, were excised and eluted, and the identity of the nucleotides at isolated DS cleavage sites was determined by sequencing gel analysis (Figure 5A,B). The cleavage bands migrating slightly ahead of the Maxam–Gilbert sequencing standard markers of 5′-end-labeled fragment and some extra bands on the 3′-end-labeled fragment sequencing gel were due to different 3′- and 5′-terminal chemical structures (vide infra). The bands migrating ahead of the major bands with extremely low intensity represent SS breaks. Figure 6 shows a summary of the results of the DS cleavage analyses on three DNA restriction fragments. The cleavage sites are indicated by arrows, and the size of the arrows represents the intensity of the autoradiographic signal from bands in the nondenaturing gels. The absolute intensity of the band was normalized to the band with the largest intensity for each restriction fragment examined. In spite of separate analysis of each strand, the intensity of cleavage at each site was consistent between strands. Our results showed

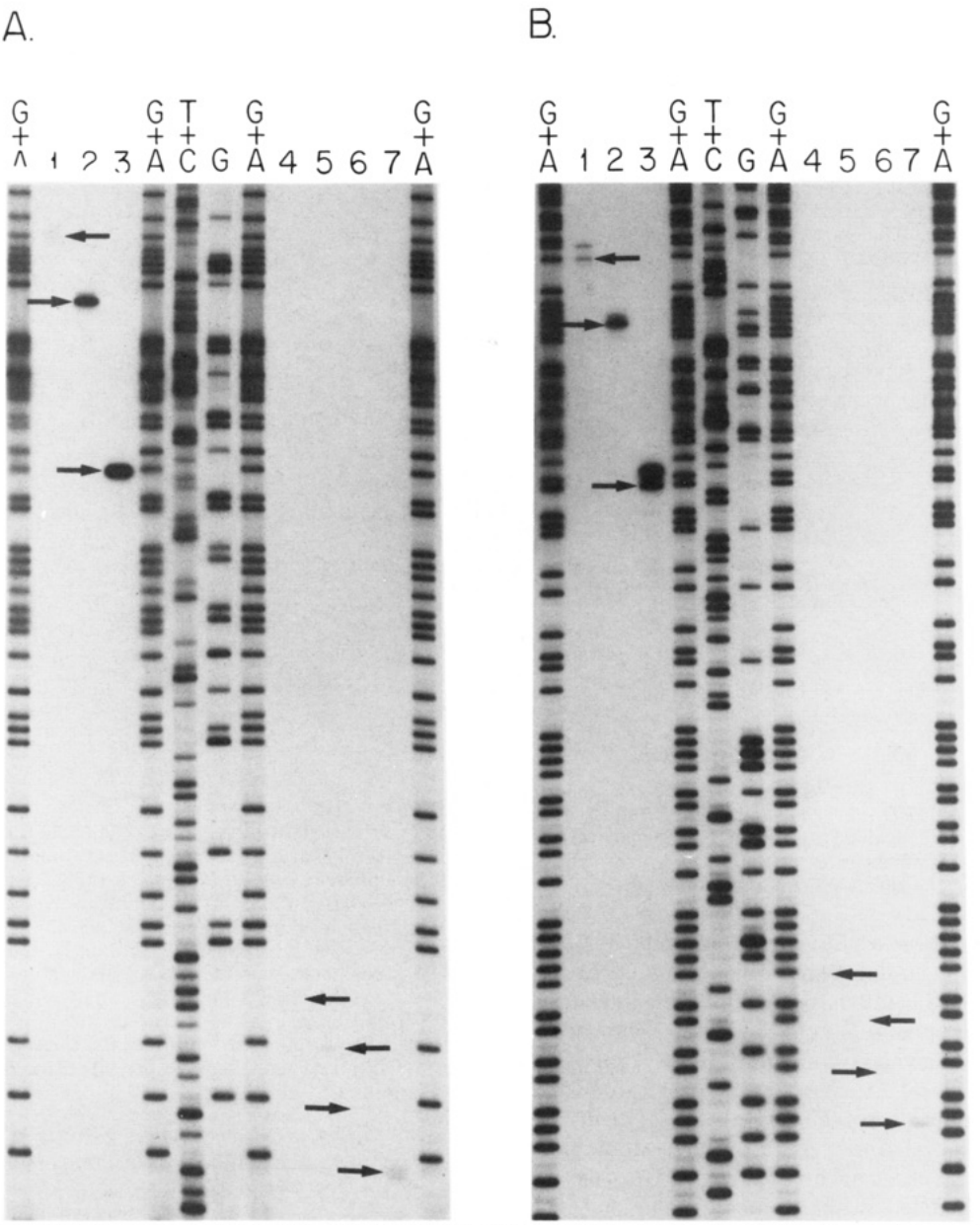

FIGURE 5: Sequence determination of C1027 chromophore-induced DS break sites. The 5′- and 3′-^{32}P-labeled *Hind*III/*Bsi*HKAI DNAs, (A) and (B), respectively, were treated with C1027 chromophore and then resolved on a 12% nondenaturing gel. DNA fragments from the bands (numbered in Figure 4) representing DS breaks were excised and resolved on an 8% sequencing gel. The A+G, C+T, and G lanes are Maxam–Gilbert markers. Lanes 1–7 correspond to the indicated bands in the nondenaturing gel (Figure 4). The cutting sites are indicated by arrows. The bands slightly ahead of Maxam–Gilbert markers on the sequencing gel of the 5′-^{32}P-labeled fragment (A) and some slower bands on that of 3′-^{32}P-labeled fragments (B) are probably caused by the different 5′- and 3′-terminal structures (see text).

that the most preferred DS cleavage sequence induced by C1027 chromophore was GTTAT/ATAAC. Other preferred cutting sites were found at CTTTT/AAAAG and to a lesser degree at ATTAT/ATAAT, CTTTA/TAAAG, and CTCTT/AAGAG sequences (cleaved residues are underlined). It is apparent that the attack site consists of three base pairs, with the 3′-nucleotides as cleavage sites. In addition, the base pair immediately 3′ to each of the cleavage sites is critical in determining the extent of cleavage. By contrast, TAT/ATA and some AGA/TCT-containing sites, which were identified by Sugiura and Mutsumoto (1993) as "preferred attacking sites", either were not cleaved or were very weakly cleaved in the restriction fragments we used (Figure 6). DNA isolated from bands corresponding to parent-length fragments containing SS breaks was also examined on sequencing gels. In general, SS breaks were formed mainly at A and T residues (data not shown) as reported (Sugiura & Matsumoto, 1993). Both DS cleavage and SS cleavage have the same base attack site specificity. At the most intensely DS cutting site, SS break sites were found at the A of GTTAT and at the A and T of the ATAAC sequence on the opposite strand. The former break could also be due to the breakdown of an abasic site. The other prominent feature of this DS cleavage study was that the two components of the DS break were staggered in the 3′-direction by two nucleotides on each strand.

Characterization of the Chemistry of Cleavage. To characterize the chemistry at the 5′-ends of the DS breaks produced by C1027 chromophore, 3′-end-labeled *Ava*II/*Apa*LI fragment from plasmid pAP 1–3 was treated with the drug and resolved on a nondenaturing gel, and the DS cleavage fragment from the GTTAT/ATAAC site (band 11, Figure 6) was analyzed on an 8% sequencing gel. Figure 7A (lane 2) shows that there was a band relative to the A residue of the ATAAC site that migrated about 2.5 nucleotides more slowly than the 5′-phosphate-ended DNA Maxam–Gilbert

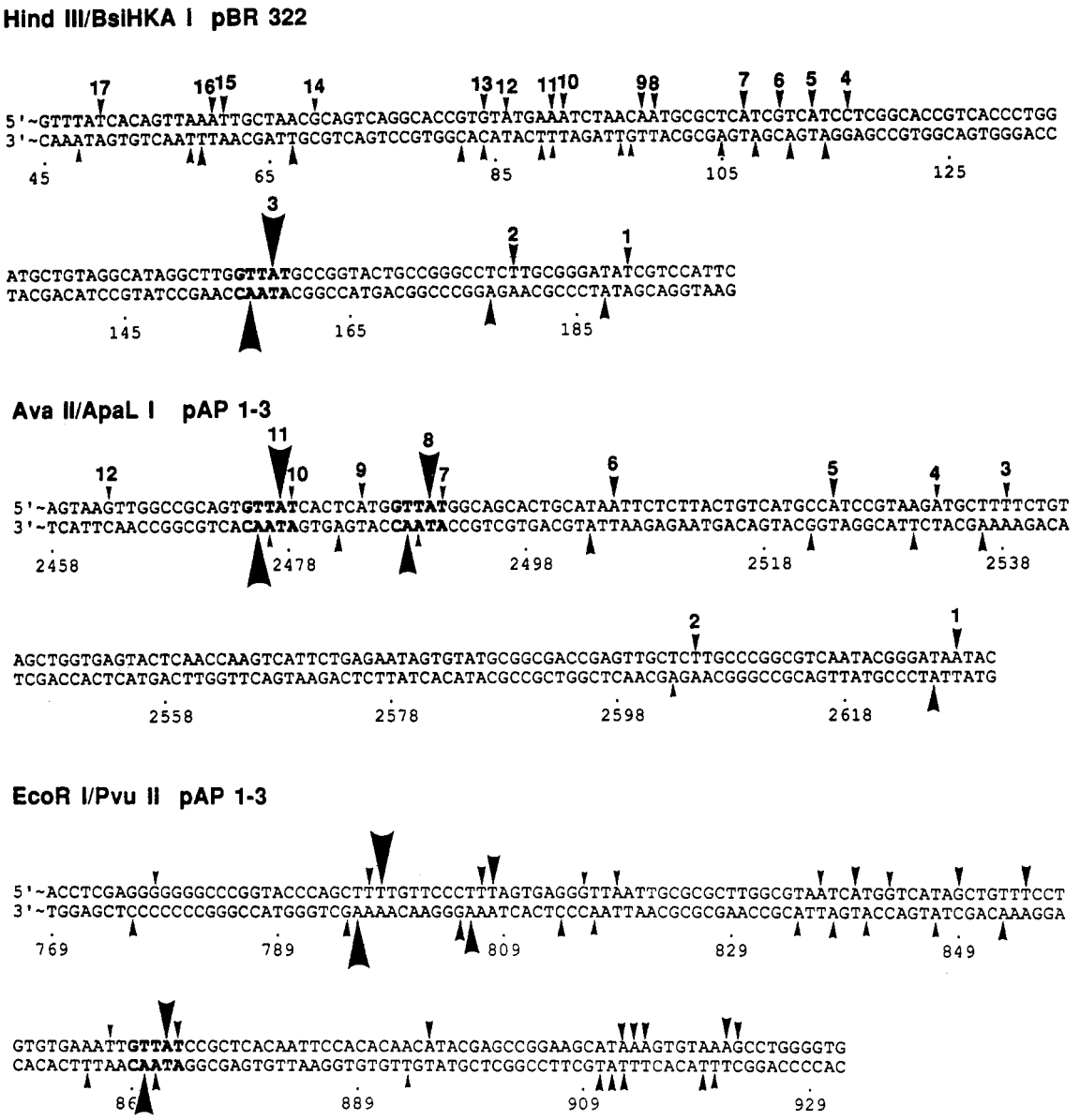

FIGURE 6: Histograms of DS cleavages sites on portions of DNA restriction fragments. The arrows indicate the preferred sites of attack by C1027 chromophore on each strand. Their sizes are proportional to the intensity of cleavage at each site as shown in Figure 4. The sequences printed in boldface type indicate the most preferred GTTAT/ATAAC sites. Only the region of each restriction fragment analyzed for DS breaks is shown.

marker. The electrophoretic mobility of this band is consistent with that of a DNA fragment that contained a nucleotide 5′-aldehyde at its 5′-end (Kappen & Goldberg, 1983, 1993a). The structure of this band was confirmed by piperidine treatment, which converted the fragment to a 5′-phosphate end (lane 3). Reduction with $NaBH_4$ made the fragment migrate slightly ahead of the aldehyde position, and presumably the resulting alcohol protected it from piperidine-induced degradation (lanes 4 and 5). The reduced amount of aldehyde relative to phosphate in lane 2 of Figure 7A is due to its decomposition during the processes of workup. Scanning data from lane 4, in which the cleavage products were stabilized immediately by $NaBH_4$ reduction after drug reaction, showed that there was predominance of the aldehyde residue relative to the phosphate species (83.5% versus 16.5%). Similarly, nucleoside 5′-aldehyde was observed at all ATAAC sites studied and at other cutting sites such as AAAAG, ATAAT, and CATAA (data not shown). This indicates that DNA sequence-specificity, at least in part, determines the specific chemical mechanism of deoxyribose damage occurring at the cutting sites. At the most preferred ATAAC cutting sites, two SS breaks were found at A and T of ATAAC (lane 1). The lack of effect of $NaBH_4$ reduction and piperidine treatment (data not shown) on their mobilities and the coincidence with standard markers suggest that the fragments have a 5′-phosphate end.

The bleomycin–iron complex is well-known to cleave DNA by C4′ hydrogen abstraction and to form 3′-phosphoglycolate termini and 4′-hydroxylated abasic sites (Giloni et al., 1981; Stubbe & Kozarich, 1987). Treatment of 5′-^{32}P-labeled *Ava*II/*Apa*LI pAP 1–3 restriction fragment with bleomycin A2 produced bands migrating slightly ahead of the phosphate-ended fragment at the T of the GT and AT steps (lane 3, Figure 7B). These bands are diagnostic of a 3′-phosphoglycolate terminus. The band between T and A standard markers (lane 3, Figure 7B) is likely a slow band generated by β-elimination of the 3′-phosphate from a ring-opened 4′-keto abasic site (Stubbe & Kozarich, 1987; Kozarich et al., 1989). To determine the chemistry of the cutting site by C1027 chromophore on the opposite strand (GTTAT), the DS

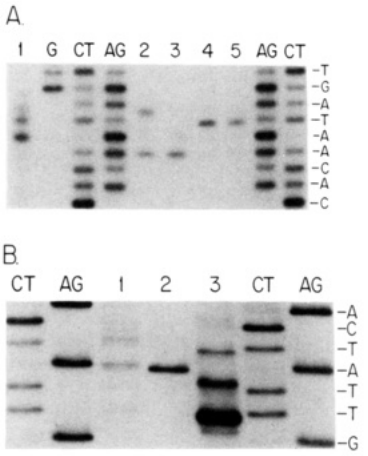

FIGURE 7: Chemical structure analysis of C1027 chromophore-induced DS and SS cleavage sites. (A) 3′-^{32}P-Labeled *Ava*II/*Apa*LI restriction fragment from pAP1-3 was incubated with C1027 chromophore, and the DNA cleavage products were resolved on a 12% nondenaturing gel. The isolated DS cleavage fragment at the ATAAC site, as shown in Figure 6, band 11, and fragments with SS breaks (DNA at the origin of the nondenaturing gel) were analyzed on an 8% sequencing gel. The G, CT, and AG lanes are Maxam–Gilbert markers. Lane 1, C1027 chromophore-treated SS-nicked DNA; lane 2, ATAAC DS cleavage fragment; lane 3, same as lane 2 except treated with 1 M piperidine at 90 °C for 30 min; lane 4, same as lane 2 except treated with $NaBH_4$ immediately after the drug reaction; lane 5, same as lane 4 except treated with piperidine as in lane 3. (B) C1027 chromophore-treated 5′-^{32}P-labeled *Ava*II/*Apa*LI pAP1-3 was resolved on a 12% nondenaturing gel, and the isolated DS cleavage and SS-nicked fragments were resolved on a 15% sequencing gel. The outer CT and AG lanes are Maxam–Gilbert standards. Lane 1, C1027 chromophore-treated SS-nicked DNA; lane 2, C1027 chromophore-treated DS fragment at the GTTAT site (Figure 6, band 11 isolated from *Ava*II/*Apa*LI pAP1-3); lane 3, bleomycin-treated DNA (no reisolation of bands).

cleavage fragment from 5′-end-labeled drug-treated DNA (band 11, Figure 6) was analyzed on a 15% sequencing gel. A band corresponding to A of the GTTAT migrated slightly faster than the phosphate-ended fragment (lane 2, Figure 7B); its electrophoretic mobility is consistent with the formation of a fragment with a 3′-phosphoglycolate end such as is produced by abstraction of the C4′ hydrogen. In order to compare the differences in the cleavage mechanism between SS and DS cleavage, DNA containing SS breaks and remaining at the origin of the nondenaturing gel was analyzed on the same sequencing gel. Similar to neocarzinostatin (Dedon & Goldberg, 1990), C1027 chromophore-induced SS breaks at the GTTAT site consisted mainly of 3′-phosphate, not 3′-phosphoglycolate, ends, whereas DS cleavage products had 3′-phosphoglycolate ends on one strand.

DISCUSSION

The enediyne antitumor antibiotics are currently the focus of intense research activity in the fields of chemistry, biology, and medical sciences because of their unusual molecular structure and potent biological activities. Neocarzinostatin (Goldberg, 1991) and calicheamicin (Lee et al., 1991) are typical examples of such compounds which bind in the DNA minor groove and induce DS damage in a thiol-dependent reaction. Like other enediyne compounds, antibiotic C1027 probably exerts its extremely potent cytotoxicity on cancer cells by inducing cellular DNA damage (Xu et al., 1990). Previous work showed that this antibiotic induced DNA damage, including DS and SS breakage and abasic site formation, even in the absence of thiols or other reducing agents (Xu et al., 1992). Recently, Sugiura and Matsumoto (1993) reported that C1027 preferentially cuts DNA at adenylate and thymidylate residues and that the frequency of bases attacked by antibiotic C1027 (A≥T≫C>G) differs from that of neocarzinostatin (T>A≫C>G), calicheamicin γ1 (C≫T>A≅G), esperamicin A1 (T>C>A>G), and bleomycin (C>T>A>G). The major damage appears to be strand scission caused by hydrogen abstraction from the C4′ position of the deoxyribose of T and A (Sugiura & Matsumoto, 1993). Our results, as shown in Figure 2, indicate that the chromophore extracted from the holoantibiotic maintains its full DNA cleaving ability in the absence of thiols. Calculation based on the optical density data derived from agarose gel experiments with pBR322 DNA indicates that, at lower drug concentrations, the ratio of DS to SS breaks induced by C1027 chromophore was about 1:4, which is slightly higher than for neocarzinostatin with glutathione as a thiol activator (1:5). Expression of abasic site damage as breaks by putrescine treatment further increases the ratio so that total DS damage (breaks plus abasic sites) is equivalent to SS lesions. These data suggest that the direct DS breaks, as well as total DS damage, can be induced by a single C1027 chromophore. Direct evidence supporting this view comes from cleavage studies with 5′- and 3′-end-labeled restriction fragments in which DNA fragments with DS breaks were isolated on nondenaturing gels. Moreover, there is a distinct sequence requirement for DS breakage. The most preferred DS cleavage site of C1027 was found at the GTTAT/ATAAC sequence that is distinct from the DS cleavage sites caused by other enediyne antibiotics such as neocarzinostatin (AGT/ACT) and calicheamicin γ1 (AGGA/TCCT). Other sequences such as CTTTT/AAAAG, ATAAT/ATTAT, CTTTA/TAAAG, and CTCTT/AAGAG are also preferred by C1027 chromophore for DS cleavage. Further, the base pair just 3′ to each of the cleavage sites was found to have a marked influence on the extent of cleavage.

The other striking result from this study is that the chemistry at a DS break consists of both C4′ and C5′ hydrogen abstraction reactions. At the A residue of the GTTAT sequence, C4′ hydrogen abstraction was supported by the predominance of a 3′-phosphoglycolate-ended fragment, while at the A of ATAAC on the complementary strand the presence of a 5′-aldehyde-ended residue indicated cleavage by the mechanism of C5′ hydrogen abstraction. This mixture of chemistry is also found at the DS cutting sites induced by neocarzinostatin (Dedon & Goldberg, 1990) and calicheamicin γ1 (Hageland et al., 1992; Zein et al., 1988). Neocarzinostatin chromophore cleaves double-stranded DNA predominantly at GT steps, especially at AGT/ACT trinucleotide sequences. At this cleavage site, a C5′ hydrogen atom from the T of ACT and the C4′ hydrogen atom of the T of AGT are abstracted by a single molecule of the diradical form of the NCS chromophore (Dedon & Goldberg, 1990; Meschwitz & Goldberg, 1991), leaving a two-nucleotide 3′-stagger of the cleaved residues. On the other hand, DS breaks produced by calicheamicin occur with a tetranucleotide sequence-specificity mainly at a TCCT/AGGA site. The chemistry of calicheamicin-induced DNA cleavage involves removal of the C5′ hydrogen from the C of TCCT and the C4′ hydrogen from the nucleotide of the 3′-NNAGGA (Zein et al., 1988; De Voss et al., 1990), which results in a three-nucleotide 3′-staggered lesion. As also noticed by Sugiura and Matsumoto (1993), the specific DS cleavages by C1027 were staggered by two nucleotides between the two strands, which is different from that by calicheamicin γ1 but similar to the DS lesions induced by neocarzinostatin. Calicheamicin γ1 lacks an

intercalating moiety and hence causes DS breaks three base pairs apart on the two DNA strands. The 3′-asymmetry of the DS cleavage sites produced by C1027 and the competition with netropsin (data not shown), a known minor groove binder, strongly suggest the association of the chromophore molecule in the minor groove of DNA.

In antibiotics containing bicyclic enediyne structures, the diradical species derived from rearrangement of the enediyne moiety is believed to be responsible for the DNA damaging properties (Goldberg, 1991; Lee et al., 1991). As for C1027, it seems likely that when the chromophore binds to the minor groove of DNA at the most preferred sequence, GTTAT/ATAAC, one of the benzenoid diradicals (**2**, Figure 1) attacks C4′ of the A residue of GTTAT to form a 3′-phosphoglycolate, and the other radical center of the chromophore attacks C5′ of the A residue of ATAAC on the opposite strand to form a nucleoside 5′-aldehyde, generating a DS break. Since hydrazine and putrescine treatments increase DS breakage at this site, presumably the 4′ chemistry also produced significant amounts of 4′-hydroxylated abasic sites, as occurs with bleomycin and neocarzinostatin (Giloni et al., 1981; Dedon et al., 1992). Further, the sequence-specificity of these lesions was unaffected by these treatments (unpublished data). The base-specific SS breaks induced by C1027 are predominantly at the A and T residues. The differences between the mechanisms of DS and SS cleavage are presumably due to distinct binding modes of the chromophore in DNA or to selective quenching of one of the carbon-centered radicals. DS cleavage is of more significance than SS cutting in terms of mutagenicity and cytotoxicity. SS breaks are rapidly repaired, whereas persistent DS breaks can be related to cell-killing (Hatayama & Goldberg, 1979; von Sonntag, 1987). The sequence-specific DS damages caused by C1027 may account for its potent antitumor activities both in vitro and in vivo.

It is noteworthy that in the DNA cutting reaction, C1027 chromophore does not require thiols or other nucleophiles for its activation. Nucleophilic activation is known to play an important role in drug activation of other enediyne antibiotics for the cleavage of duplex DNA. Sugiura and Matsumoto (1993) showed that DNA cutting by holo-C1027 was negligible at 0 °C and markedly enhanced by heating at 37 °C and proposed a mechanism for its activation involving heating (Sugiura & Matsumoto, 1993). However, as shown here, isolated C1027 chromophore is fully active at 0 °C, implying that it is the release of chromophore from the holoantibiotic that requires the higher temperature. Recently, it has been found that neocarzinostatin chromophore cleaves a DNA bulge at a specific site in a thiol-independent reaction (Kappen & Goldberg, 1993a,b). It has been shown that neocarzinostatin undergoes base-catalyzed spontaneous conversion by an intramolecular reaction to a diradical species (Hensens et al., 1994) and that one of the drug radical centers (at C2) is quenched intramolecularly under the influence of the bulged DNA structure (Hensens et al., 1993). The other radical center (at C6) selectively abstracts the hydrogen atom from C5′ of deoxyribose, resulting in a SS break at the target site. Whether or not DNA structure plays a role in C1027 activation which leads to the destruction of DNA itself needs to be further studied. The preferred DS cutting sites, such as GTTAT/ATAAC, CTTTT/AAAG, and ATTAT/ATAAT, clearly indicate that C1027 tends to bind at A/T-rich sequences. Since the A/T-rich regions have very different microstructures from GC-containing sequences (Trifonov & Ulanovski, 1988; Price & Tullius, 1993), the nature of the specific interaction of C1027 chromophore with DNA should be of interest for future research.

In conclusion, the unusual DNA sequence-specificity and the staggered nature of the cleavage sites on the complementary strands suggest a unique interaction of this new enediyne compound with DNA. The experimental data indicate that the diradical of the enediyne moiety, when positioned in the minor groove at specific sequences, abstracts C4′ and C5′ hydrogens of deoxyriboses on both strands, generating DS breaks. It appears that SS breaks which are mainly produced at adenylate and thymidylate residues are base-specific, while DS cleavage shows five-nucleotide sequence-specificity. The extremely potent cytotoxicity of the antibiotic seems to be caused by its ability to introduce sequence-specific DS cleavage on DNA.

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