Sequence Preference for Strand Cleavage of Gapped Duplexes by Dynemicin A: Possible Mechanism of Sequence-Dependent Double-Stranded Breaks†

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ABSTRACT: A double-stranded DNA cleavage mechanism by a novel enediyne type antitumor antibiotic, dynemicin A, has been investigated through sequence-dependent strand breakage of a series of duplexes containing a single nucleotide gap. We found that (1) dynemicin A breaks specifically at the 3′-shifted position by one base opposite the gap, (2) the strong cleavage is detected at 5′-Pu_Pu/3′-PyPuPy sequences, and (3) dynemicin H (aromatized form of dynemicin A) gives only a small inhibition effect (20%) on the cleavage of gapped duplex by dynemicin A. The long half-life of aromatization of dynemicin A (118 min, in the presence of DNA) obtained from HPLC analysis provides enough time for the second cleavage. The present results strongly indicate a two-step mechanism for the double-stranded DNA scission of dynemicin A. Namely, this double-stranded break is caused by two drug molecules, each of which cuts one DNA strand.

Dynemicin A, isolated from the fermentation broth of *Micromonospora chersina*, is characterized as a unique hybrid antitumor antibiotic containing typical chemotypes, an enediyne unit and an anthraquinone ring. The antibiotic shows a potent antitumor activity *in vitro* and *in vivo* with low toxicity (Konishi et al., 1989, 1990). Like other enediyne-containing antibiotics, it is believed that the bioactivity is due to its ability to cause DNA strand breaks. The DNA-damaging activity is significantly enhanced by the presence of reducing agents such as NADPH and thiols (Sugiura et al., 1990) or by visible light irradiation (Shiraki & Sugiura, 1990). Under these conditions, the anthraquinone moiety is reduced to undergo opening of the epoxide, causing a significant conformational rearrangement in the drug molecule. This conformational change brings the two acetylene bonds closer together and leads to the Bergman cyclization of the enediyne unit to form a benzenoid diradical. The reactive radical species, when positioned in the minor groove of DNA, can abstract hydrogen atoms from the deoxyribose backbone of DNA and yield the inactivated dynemicin H. The elegance of the action mechanism of dynemicin A can be seen in Figure 1A, which features a cascade of reactions. On the basis of the molecular mechanism of dynemicin A, a series of enediyne model compounds of dynemicin A type equipped with triggering and modulating devices have also been designed and synthesized (Nicolaou et al., 1993; Wender et al., 1993; Elbaum et al., 1995).

It has been shown that other enediyne type antitumor antibiotics, such as neocarzinostatin chromophore (Dedon & Goldberg, 1992), calicheamicin (Dedon et al., 1993), and C-1027 chromophore (Sugiura & Matsumoto, 1993; Xu et al., 1994), induce double-stranded cleavage. However, the previous computer modeling study predicted that dynemicin A does not cause the DNA double-stranded breaks concomitantly but instead produces single-stranded breaks (Langley et al., 1991). On the other hand, our previous study using nicked DNA substrate proposed that dynemicin A can cause double-stranded breaks cooperatively (Kusakabe et al., 1993). Therefore, it is of interest whether the double-stranded cleavage pattern elicited by the drug is a two-step or a one-step process (Figure 1B). Since double-stranded breaks are more resistant to repair than single-stranded breaks (Saito & Andoh, 1973; Hatayama & Goldberg, 1979), such double-stranded cleaving behavior is important for an understanding of its potent cytotoxicity.

Here, we clarify this cooperative double-stranded cleavage mechanism by using synthetic oligonucleotides designed to contain a single nucleotide gap. The study evidently reveals that the double-stranded break is caused by two dynemicin A molecules and that the second step includes sequence-selective cleavage. Indeed, the present work with the gapped duplexes has a bright prospect of some applications to future work.

MATERIALS AND METHODS

Drugs and Chemicals. Dynemicins were kind gifts from Bristol-Myers Squibb Research Institute (Tokyo, Japan). Adriamycin was offered by F. Arcamone (Farmitalia). Plasmid pBR322 DNA was isolated from *Escherichia coli* C600, and T4 polynucleotide kinase was purchased from Takara (Kyoto, Japan). Distilled water was purified through a Sybron Nanopure II system. All other chemicals used were of commercial reagent grade.

Preparation and Labeling of Oligonucleotides. Oligonucleotides used in this study were synthesized on an Applied Biosystems 391 synthesizer. The oligonucleotides were then deprotected with ammonium hydroxide at 55 °C for 10 h and purified by HPLC on a reverse-phase column. Some oligonucleotides were labeled at the 5′-terminus with [γ-^{32}P]-ATP by using T4 polynucleotide kinase. After purification on a denaturing 15% polyacrylamide gel, each oligonucle-

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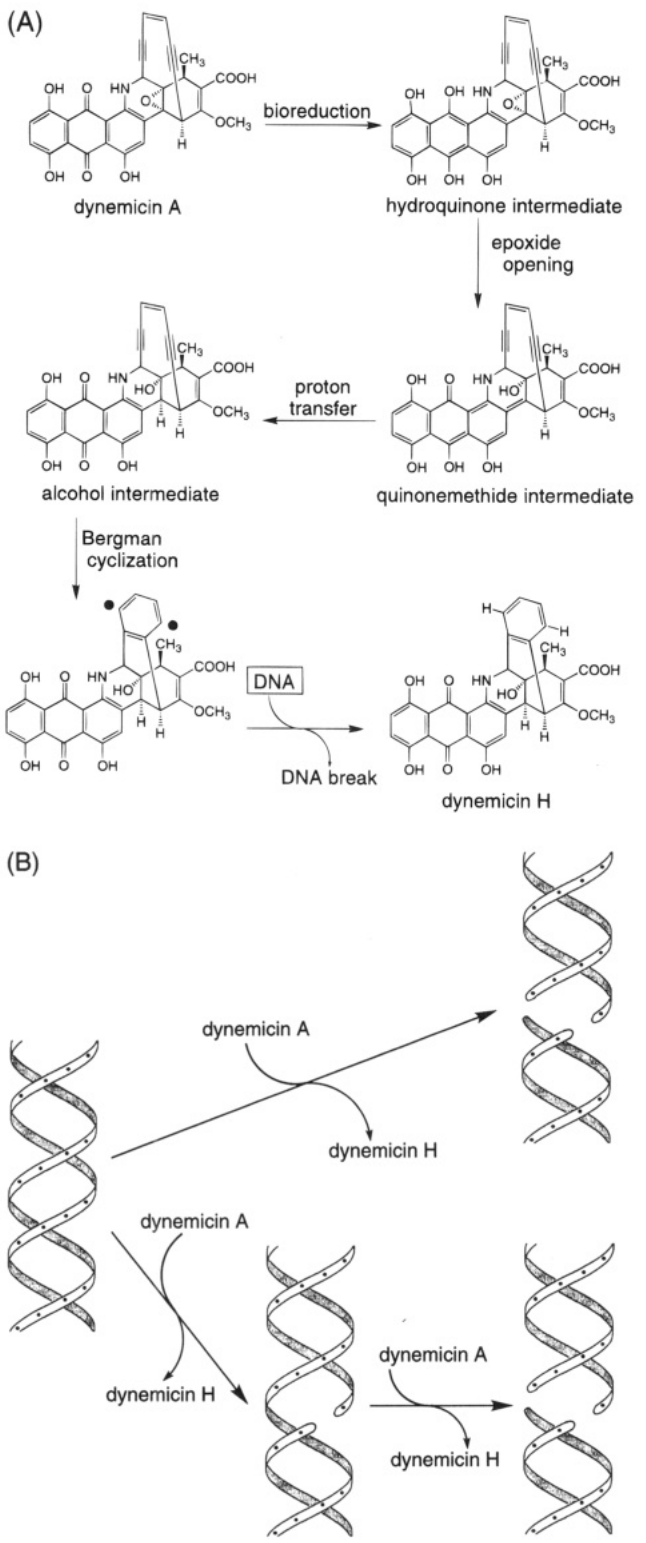


FIGURE 1: (A) Chemical structures of dynemicins and proposed mechanism for its cycloaromatization through the diradical state. (B) Possible fashions of double-stranded cleavage by dynemicin A.

otide was heated at 90 °C for 5 min, slowly cooled to room temperature, and then allowed to reanneal at 4 °C for at least 30 min before the drug reaction.

Cleavage of Oligonucleotides by Dynemicin A. A standard sample solution (20 μL) contained 20 mM Tris-HCl buffer (pH 7.5), 2.0 mM EDTA, 200 mM NaCl, 10% (v/v) dimethyl formamide, a trace of the 5′-end-labeled oligonucleotide (<3 pmol), and cold oligonucleotides (ca. 100 pmol) for annealing. Dynemicin A (50 μM) and methyl thioglycolate (20 mM) were added to the reaction sample, and then the reaction was carried out at 4 °C for 15 h. The reaction was stopped by addition of ice-cold ethanol and 0.3 M sodium acetate, and the DNAs were recovered by ethanol precipitation. After preincubation of the DNA with some intercalators (50 μM) such as 1,4-dihydroxyanthraquinone, 1-aminoanthraquinone, adriamycin, proflavine, actinomycin D, dynemicin H, or dynemicin L at 4 °C for 30 min, the dynemicin A-induced DNA cleavage was investigated and compared with the standard cleavage of gap-containing DNA by dynemicin A. The samples were dissolved in 3 μL of formamide containing 0.01% bromophenol blue and loaded onto a high-resolution denaturing 15% polyacrylamide gel containing 7 M urea in TBE buffer (89 mM Tris−borate and 2 mM Na_2EDTA, pH 8.0). Electrophoresis was performed at 2000 V for 90 min. DNA sequencing was performed by the Maxam−Gilbert method (Maxam & Gilbert, 1980). Autoradiography of the gels was carried out at −80 °C overnight on Fuji medical X-ray film, and the autoradiograms were scanned with a laser densitometer (LKB Model 2222 Ultro-Scan XL).

Identification of Single- and Double-Stranded Damage Sites. A standard sample was incubated at 4 °C for 15 h and directly taken up for electrophoresis on a native 5% polyacrylamide gel. Electrophoresis was performed at 50 V and 4 °C for 20 h. Each band was excised from the gel by alignment with an autoradiogram. The DNA in a band was eluted by diffusion and resolved on a high-resolution DNA sequencing gel.

HPLC Analysis of Reaction between Dynemicin and Methyl Thioglycolate. Aromatization of dynemicin A (50 μM) with methyl thioglycolate (20 mM) was performed in the presence or absence of calf thymus DNA (150 μM base pairs) as described above. After the reaction sample was incubated at 37 °C for 0, 15, 30, 60, or 120 min, the solution was poured into water (30 μL) and extracted twice with ethyl acetate (50 μL). The organic layers were combined, washed twice with water (100 μL), and then evaporated in vacuo. These samples were separated by HPLC on an A301-3S-3-120A ODS column (Yamamura Kagaku, Kyoto; 4.6 × 100 mm) with methanol/0.15% KH_2PO_4 (pH 3.5) (75:25, v/v) as the solvent. The eluate was monitored at 569 nm.

Assay for Damage of Supercoiled DNA. The cleavage of plasmid pBR322 DNA was carried out in a total volume of 20 μL containing 20 mM Tris-HCl buffer (pH 7.5) and 10% (v/v) dimethyl formamide. The final concentration of dynemicin A was 10 μM in each sample. Inactivation of the drug was monitored after the preincubation of dynemicin A with methyl thioglycolate (10 mM) at 37 °C for 0, 15, 30, 60, 120, or 240 min. Then plasmid pBR322 DNA (0.4 μg) was added to each sample, and the samples were incubated at 37 °C for 30 min. The reactions were stopped by addition of 0.3 M sodium acetate and cold ethanol, and DNAs were recovered by ethanol precipitation. Each sample was resuspended in 10 μL of loading buffer containing 0.05% bromophenol blue and 10% glycerol, and then heated at 65 °C for 1 min. The samples were loaded onto a 1% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL) in TBE buffer, and then electrophoresis was performed at 100 V for 90 min. The gel was photographed with Polaroid 665 film. The resulting forms of the plasmid were quantitated by measuring the intensities of the DNA bands on the negative films.

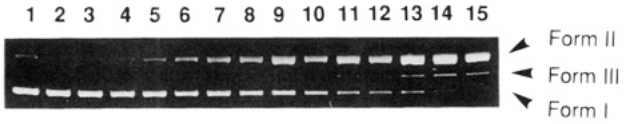


FIGURE 2: Agarose gel electrophoretic patterns of pBR322 DNA (31.4 μM base pairs) nicking by dynemicin A. The concentration of dynemicin A was 0, 0.01, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0, 10.0, 15.0, and 20.0 μM for lanes 1−15, respectively. The cleavage reaction was initiated by addition of 10 mM methyl thioglycolate, and then the samples were incubated at 37 °C for 15 min.

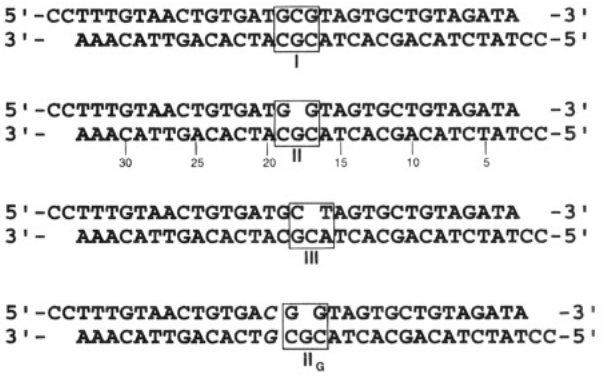
RESULTS AND DISCUSSION

Cooperative Double-Stranded Breaks by Dynemicin A. Double-stranded cleavage of DNA by dynemicin A can be explained by the following two models: (i) the diradical species of one activated dynemicin molecule simultaneously abstracts two hydrogens from duplex DNA, and (ii) the double-stranded break is caused by two drug molecules, each of which cuts one DNA strand; that is, only one of the two radicals abstracts hydrogen from deoxyribose of DNA (Figure 1B).

Figure 2 shows typical gel electrophoretic patterns for strand scission of supercoiled pBR322 DNA by dynemicin A. Seemingly, the drug induces a stepwise conversion of covalently closed circular (form I) → nicked circular (form II) → linear duplex (form III) DNAs rather than a direct change of form I → form III DNAs. Although kinetic analysis of the data appears to indicate that single-stranded breaks dominate rather than simultaneous double-stranded breaks, we could not obtain an exact model concerning the stepwise formation of double-stranded breaks produced by dynemicin A from the kinetic data.

We previously showed that the drug appears to cause typical double-stranded cuts concomitantly at the sequences, 5′-ATAT/3′-TATA, 5′-ACAT/3′-TGTA, and 5′-ACGT/3′-TGCA (Sugiura et al., 1990; Shiraki & Sugiura, 1990). These staggered cutting sites can also be explained by considering the symmetry of the 5′-PuPyPuPy/3′-PyPuPyPu double-stranded dinucleotide (Langley et al., 1991). In order to solve this problem, the 5′- or 3′-end-labeled 100-base-pair (bp) (*Eco*RI−*Hae*III) G4 gene F/G space fragment treated with dynemicin A was electrophoresed on a native gel. To determine the precise site of single- and double-stranded cutting, the original and the double-stranded cleavage bands were eluted and run on a sequencing gel (data not shown). The origin contained a single-stranded cleavage site, which is consistent with the selectivity of previous work (Shiraki & Sugiura, 1990). The double-stranded cleavage sites were remarkably weak, and hence we could hardly identify these sites. This fact indicates that single-stranded breaks are preferred over simultaneous double-stranded breaks and govern the sequence-selective cleavage. The previous computer modeling study of the dynemicin−DNA intercalation complex also predicted that dynemicin A does not cause the double-stranded DNA breaks concomitantly but instead produces a single-stranded break (Langley et al., 1991). On the other hand, our study using nicked DNA substrate has thrown out a hint that dynemicin A can cause the cooperative double-stranded breakage of DNA (Kusakabe et al., 1993). However, this nicked substrate does not reflect the dynemicin-induced damaging site. The previous product analyses of DNA fragments cleaved by dynemicin A revealed

Chart 1



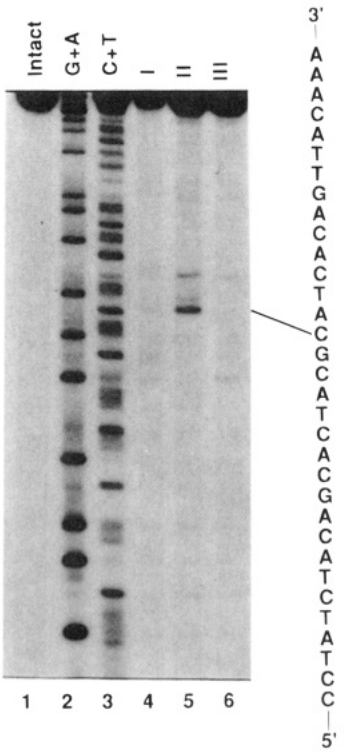


FIGURE 3: Cleavage of duplexes I−III by dynemicin A. Lanes 4, 5, and 6 present the cleavage of oligonucleotides I, II, and III, respectively. Lanes 1, 2, and 3 show intact DNA, the Maxam−Gilbert sequencing ladder for G+A, and that for C+T, respectively.

that the cleavage reaction leads to generation of a single nucleotide "gap" in DNA (Shiraki et al., 1992). Here, the term gap is used to denote the site of a single-stranded cleavage in a DNA duplex.

In order to investigate the exact cooperative double-stranded manner, the following systematic DNA preparations were utilized. We designed duplexes II and III as typical models of a single nucleotide gap which would be produced by dynemicin A (Chart 1). The duplexes II and III contain a gap in the middle of these oligonucleotides, and in the duplex III the gap of II is shifted to the 3′-side by one base. To examine cleavage sites opposite the gap, the opposite strand was labeled at the 5′-terminus and analyzed on a sequencing gel. As shown in lane 5 of Figure 3, dynemicin A cleaved the duplex II specifically at the 3′-shifted position by one base opposite the gap (C-19 position) as compared to the control (lane 4) of duplex I. This result suggests that the dynemicin-induced double-stranded breaks occur as a cooperative event. In addition, removal of the gap in the two oligonucleotides resulted in the loss of the specific

Table 1: Dynemicin A-Induced Cleavage Activities for Various Gap-Containing DNAs

entry	N N[a] NNN	relative intensities (%)[b]
II	G G CGC	**100**
IV	G G CAC	15
V	G G CCC	11
VI	G G CTC	14
VII	A G TGC	**62**
VIII	A A TGT	32
IX	G A CGT	22
X	G C CGG	34
XI	C G GGC	25
XII	C C GGG	9
XIII	A A TAT	**80**
XIV	G A CAT	25
XV	A G TAC	**68**

[a] The gap-containing DNA duplexes used in this study are as follows:

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5'-CCTTTGTAACTGTGATN NTAGTGCTGTAGATA  -3'
3'-  AAACATTGACACTANNNATCACGACATCTATCC-5'
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where **N** is the site of cleavage. [b] Relative DNA cleavage intensities were obtained from densitometric estimation of gel autoradiogram, and the errors were ±3%.

cleavage (data not shown). The fact is indicative of the importance of the gapped structure.

Sequence Preference for Strand Cleavage of Gapped Duplexes by Dynemicin A. Duplex III, which similarly contains a gap but differs from duplex II in the gap position, was also examined in the expectation of a shift of the cleavage site. However, no specific cleavages were detected in duplex III (Figure 3, lane 6). This result suggests that sequences around a gap affect the rate of cleavage in the opposite strand. To explore the effect of sequences around a gap and to get a general rule of sequence selectivity, we constructed duplexes IV−XV in a systematic fashion as shown in Table 1. The cleavage patterns of duplexes II and IV−XV are given in Figure 4, and Table 1 collects their relative cleavage intensities at the nucleotides opposite the gaps. The ratio (1:3) of dynemicin A to the gapped duplexes (base pairs) was the same for all of the cutting experiments, and also no interaction was observed between the drug and the single-stranded oligonucleotides. Table 1 clearly reveals that dynemicin A selects certain gapped sequences in its cleavage reaction. Strong cutting was observed in duplexes II, VII, XIII, and XV. Duplex II exhibited the greatest cleavage intensity. Weak cutting was also detected in duplexes VIII, IX, X, XI, and XIV. The above-mentioned duplexes have in common a 5′-Pu_Pu/3′-PyPuPy sequence with two exceptions, duplexes X and XI. In particular, 5′-G_G/3′-CGC and 5′-A_A/3′-TAT sequences gave potent cleavage. Although duplex IV also shares such a sequence, it is of interest that the cutting opposite the gap was significantly weak.

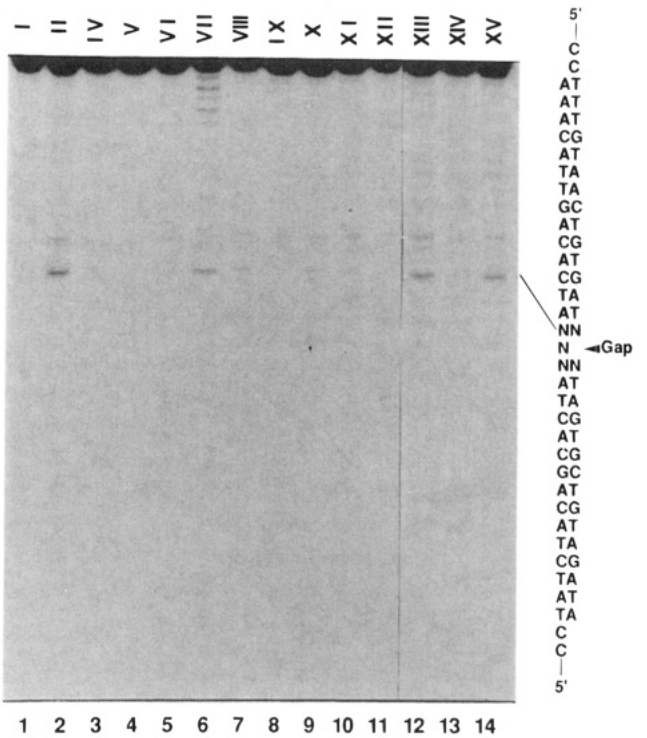

FIGURE 4: Cleavage of gap-containing duplexes. Sequence-dependent cleavages were seen at a position shifted to the 3′-side by one base opposite the gap.

Influence of the sequence outside the gapped site may not be ignored. So, we constructed duplex II_G, which contains a sequence similar to that of duplex II, but the A_{20}-T pair of duplex II is changed into a G_{20}-C pair (Chart 1). Neither strong cutting nor cleavage position in duplex II was altered in the case of duplex II_G. This result also suggests that both cleaving rate and site are significantly dependent on the sequence of the gapped site, which consists of two base pairs and an unpaired base.

The above-mentioned synthesized duplexes contain gaps with 3′- and 5′-hydroxyl termini. On the other hand, our previous analyses of the terminal structures of dynemicin A-cleaved DNA fragments showed the presence of negatively charged phosphates at both the 3′- and 5′-termini (Shiraki et al., 1992). Therefore, the effect of charged phosphates on a gap structure was investigated. The introduction of a phosphate into the 5′-end facing the gap of duplex XIII was accomplished by using T4 polynucleotide kinase. The 3′-terminal phosphate was introduced by periodate/alkali cleavage of the ribouracil attached to the 3′-end (Fraenkel-Conrat & Steinschneider, 1967). Comparison of cutting intensity between duplex XIII and its phosphorylated derivative evidently indicated that the introduction of phosphates did not affect the cleaving rates (data not shown). Accordingly, structural changes upon phosphorylation at the gapped site are not essential to the present reaction, and also the charge of phosphates may be neutralized by NaCl contained in the reaction cocktail.

The sequence preference for strand breaks of the gapped site can be confirmed in our previous work, in which the strong cutting sites are in accordance with the cleavage information of the present gapped model (Figure 5; Shiraki & Sugiura, 1990). The results suggest that, in spite of the presence or absence of gaps, binding modes are unchanged and the same sequences are recognized. However, the gap

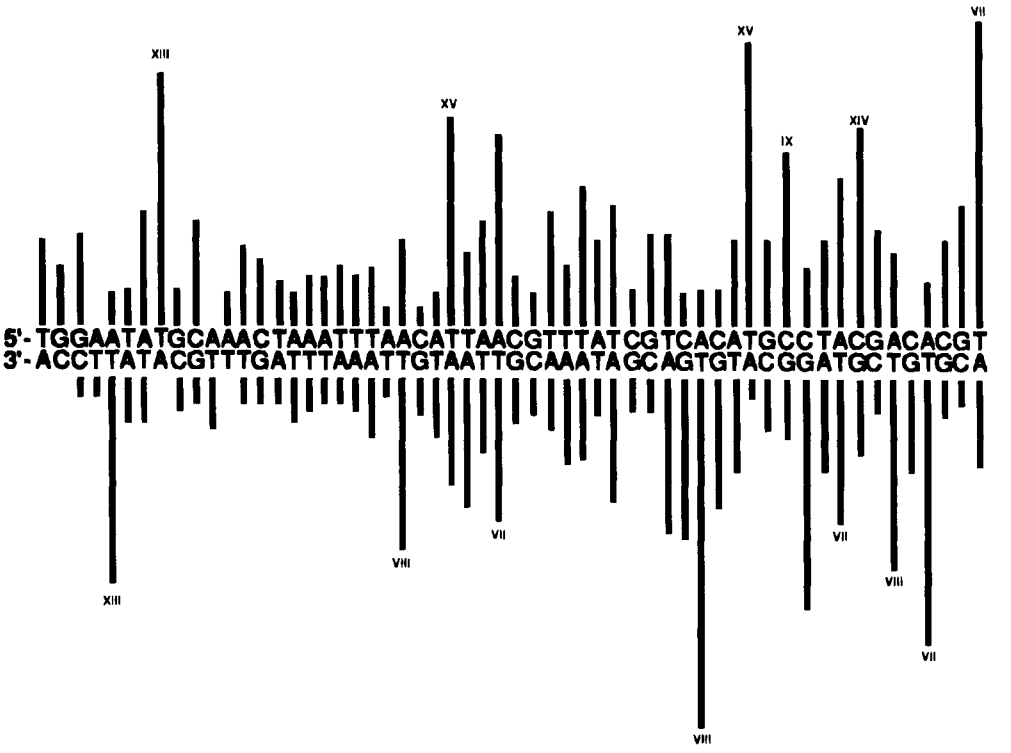

FIGURE 5: Histogram of G4 gene F/G space fragment cutting sites by dynemicin A in the presence of NADPH (Shiraki & Sugiura, 1990). Numbers indicate the second cleavage sites identified in Table 1.

increases the flexibility at this site, e.g., fraying of the ends, and enhances binding at this site through intercalation.

The dynemicin A molecule probably first causes strand scission in an intact DNA duplex by a selective process, producing single-nucleotide gaps (first breaks; this step determines the sequence selectivity). In the following cleavage by a second dynemicin A molecule, however, specific cutting occurs opposite certain gaps, leading to the sequence-dependent double-stranded cleavage (second breaks). Namely, two drug molecules rather than one produce cooperatively double-stranded breaks. Apparent double-stranded scission induced by the drug is important in explaining its potent biological activity, because double-stranded DNA breaks seem to have a lethal effect (Saito & Andoh, 1973; Hatayama & Goldberg, 1979). The efficiency of double-stranded DNA cleavage depends upon the rate of formation of gaps and the affinity of dynemicin A for the preferential gaps. The gaps produced by other drugs such as bleomycin (Keller et al., 1987) could provide extra binding sites for dynemicin A, leading to enhanced double-stranded scission. This cooperative mode is of great promise in the combination of therapy and design of new drugs targeted at DNA.

Aromatization Rate of Dynemicin A and DNA Binding Affinity of Dynemicin H. Cooperative double-stranded breaks require at least two dynemicin A molecules. If the drug is converted quickly into aromatized dynemicins, cooperative double-stranded cleavage proceeds with difficulty. In order to examine this point, the stability of dynemicin A in the reaction buffer was checked with two strategies. First, the rate of aromatization in the presence or absence of calf thymus DNA was monitored by HPLC. The remaining quantity of dynemicin A was estimated in this experiment, and indeed the quantity of dynemicin A slowly decreased (Figure 6). We obtained the rate constant of this aromatization (KaleidaGraph). Although the reaction process is actually second-order, we treated it as pseudo-first-order because of the approximately constant concentration of methyl thioglycolate during this reaction. Aromatization of dynemicin A in the presence of DNA occurs with a rate

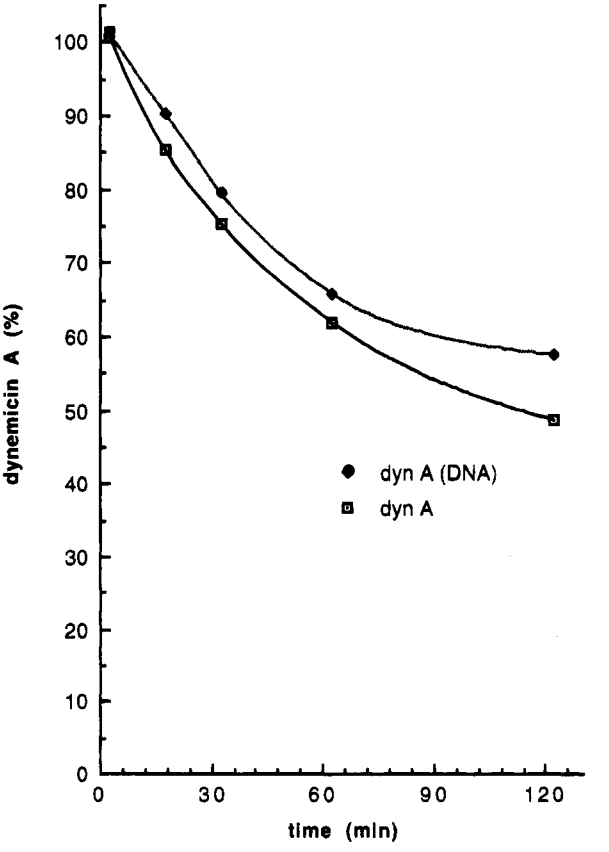

FIGURE 6: Rate of aromatization of dynemicin A with methyl thioglycolate in the presence or absence of calf thymus DNA.

Table 2: Rate Constants and Half-Lives for the Aromatization of DynemicinA in the Presence (+) or Absence (−) of DNA

DNA	k (s^{-1})[a]	$t_{1/2}$ (min)
+	9.83×10^{-5}	118
−	1.25×10^{-4}	92.5

[a] Lines were calculated according to the formula $[A] = [A]_0 e^{-kt}$, where [A] is the quantity of dynemicin A (%). This formula was obtained from the reaction dynemicin A $\xrightarrow{k}$ dynemicin H.

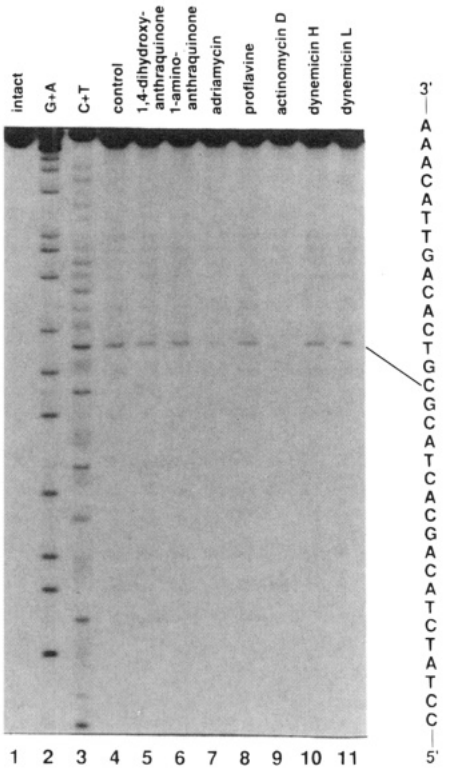

FIGURE 7: Cleavage of duplex II_G by dynemicin A after pretreatment with 1,4-dihydroxyanthraquinone (lane 5), 1-aminoanthraquinone (lane 6), adriamycin (lane 7), proflavine (lane 8), actinomycin D (lane 9), dynemicin H (lane 10), and dynemicin L (lane 11). Lane 4 shows dynemicin A-induced oligonucleotide cleavage for control. Lanes 1, 2, and 3 indicate intact DNA, C+T, and G+A of the Maxam−Gilbert sequencing reactions, respectively.

constant of 9.83×10^{-5} s^{-1} and also has a half-life of 118 min. The long half-life span provides enough time for cooperative second cleavage. In the absence of DNA, on the other hand, the rate constant and half-life are 1.25×10^{-4} s^{-1}, and 92.5 min, respectively (Table 2). It is of interest that the DNAs slow down the aromatization of the drug, while in the case of calicheamicin DNAs accelerate the rate of aromatization (Zein et al., 1989; De Voss et al., 1990). Second, we investigated the time-dependent inactivation of dynemicin A. The experiment showed that DNA cleavage activity still remains after 60 min of preincubation.

After a first DNA cleavage event, one can imagine that the aromatized dynemicin may significantly influence strand scission opposite the gap by a second dynemicin A. Therefore, we investigated the effect of dynemicin H on the dynemicin A-induced cleavage for gap-containing duplex II_G. Figure 7 shows the cleavage of duplex II_G after pretreatment with some intercalators. Actinomycin D (100%) and adriamycin (90%) strongly inhibited the cutting opposite the gap by dynemicin A. In contrast, pretreatment with aromatized dynemicins H and L gave only small effects (20 and 10%, respectively) on the cleavage of this substrate by dynemicin A (Table 3), suggesting that dynemicin H has a lower affinity for the single nucleotide gapped DNA produced by the cleavage reaction of the first dynemicin A molecule.

Table 3: Relative Inhibition of Some Intercalators for Gap-Containing DNA Cleavage by Dynemicin A

intercalator	inhibition (%)[a]
1,4-dihydroxyanthraquinone	30
1-aminoanthraquinone	40
adriamycin	90
proflavine	50
actinomycin D	100
dynemicin H	20
dynemicin L	10

[a] Relative DNA cleavage frequencies were obtained from densitometric estimation of gel autoradiograms, with an error of ±5%.

Although it has not been established whether dynemicin A intercalates before or after its activation, the intermediates of dynemicin A metabolism, such as hydroquinone, quinonemethide, or alcohol, appear to intercalate prior to aromatization, because the anthraquinone moiety of the drug is not easily reduced after intercalation, and the aromatized form also has lower affinity for DNA. Previous work on the reduction of anthracyclines showed that the quinonemethide has a half-life of 53 s, 63 s, and longer for daunomycin (Kleyer & Koch, 1984), adriamycin (Boldt et al., 1987), and menogaril (Boldt et al., 1988), respectively. Therefore, the long half-life of dynemicin A and the low DNA binding affinity of dynemicin H also support the proposed cooperative double-stranded cleavage fashion of dynemicin antibiotic.

In conclusion, the present gap-containing DNA cleavage by dynemicin A clearly shows a two-step mechanism for characteristic double-stranded DNA scission by dynemicin A. This double-stranded break is caused by two drug molecules, each of which cuts one DNA strand. Of special interest is the fact that 5′-Pu_Pu/3′-PyPuPy sequences such as 5′-G_G/3′-CGC and 5′-A_A/3′-TAT are preferentially cut by dynemicin A.

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