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PRODUCT ANALYSES IN DNA STRAND SCISSION BY ANTITUMOR ANTIBIOTIC ELSAMICIN A

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Summary: Elsamicin A is an antitumor antibiotic with fascinating chemical structure and a good candidate for pharmaceutical development. Molecular mechanism of DNA backbone cleavage mediated by Fe(II)-elsamicin A has been examined. Product analysis using DNA sequencing gels and HPLC reveals the production of damaged DNA fragments bearing 3'-/5'-phosphate and 3'-phosphoglycolate termini associated with formation of free base. In addition, hydrazine-trapping experiments indicate that C-4' hydroxylated abasic sites are formed concomitant with DNA degradation by Fe(II)-elsamicin A. The results lead to the conclusion that the hydroxyl radical formed in Fe(II)-elsamicin A plus dithiothreitol system oxidizes the deoxyribose moiety via hydrogen abstraction predominantly at the C-4' carbon of the deoxyribose backbone and ultimately produces strand breakage of DNA.

Antitumor antibiotics with novel structures as DNA targeting molecules could be better templates for new chemotherapeutic agents and biotechnology tools. Elsamicin A consisting of chartarin core and aminosugar residue falls within such antibiotics (Figure 1) (1, 2). The water soluble property of the drug makes it a good candidate for pharmaceutical development (3). Indeed, the antibiotic currently undergoes phase II clinical trials. Some efforts to understand the key features responsible for antitumor activity of this family of antibiotics indicate that DNA molecule is one important target in the action mode (4-8). Their fascinating structures as DNA targeting molecules and their importance for chemotherapy stimulated us to study the detailed mechanism of its action to DNA at the molecular level. We previously reported single-strand cleavage in DNA by elsamicin A in the presence of ferrous ion and dithiothreitol. The preferential cutting sites of the DNA strand scission are on the bases adjacent to the 3'-side of guanine residues, particularly 5'-GG site. Several features of the DNA-elsamicin A complex have been proposed: the aglycon portion binds preferentially to 3'-side of guanine by intercalation and is the direct participant in recognizing G-C base pair, the aminosugar residue is engulfed by the minor groove of host DNA and facilitates the stabilization of the DNA-elsamicin A complex, and the

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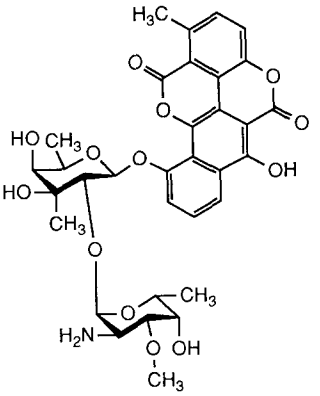

Figure 1. Chemical structure of elsamicin A.

2-amino group of guanine residue of host DNA plays an important role in DNA-elsamicin A interaction (9).

However, the chemistry of DNA degradation by elsamicin A remains to be understood. An understanding of DNA-cleaving process is surely necessary to achieve its full potential as a template for second-generation drugs. The experiments reported here were designed to elucidate the molecular mechanism for the DNA strand scission by Fe(II)-elsamicin A.

Materials and Methods

Materials: Elsamicin A was a kind gift from Bristol-Meyers Squibb Research Institute (Tokyo, Japan). Plasmid pBR322 DNA was isolated from *Escherichia coli* C600. Poly(dG-C), bacterial alkaline phosphatase, polynucleotide kinase, DNase I, and restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). Distilled water was purified through a Sybron Nanopure II System. All other chemicals used were of commercial reagent grade.

^{32}P-End-Labeling of DNA Restriction Fragments: To achieve generalization of the experimental results, various pBR322 DNA restriction fragments were used: *Sal* I-*Hha* I, *Bam* HI-*Sph* I, and *Hin* dIII-*Eco* RI fragments. The fragments were 5'- or 3'-labeled at the *Sal* I, *Bam* HI, and *Hin* dIII cuts, respectively. The 5'-end was labeled with polynucleotide kinase and [γ-^{32}P]ATP, after treatment with bacterial alkaline phosphatase (10). The 3'-end was labeled with [α-^{32}P]dTTP or [α-^{32}P]dGTP by using the filling-in function of Escherichia coli DNA polymerase I large fragment. After digestion with the second enzyme, the singly labeled fragment was purified on a nondenaturing 5 % polyacrylamide gel.

Conditions of DNA Cleavage Reactions: A standard reaction sample contained elsamicin A (10 μM) or bleomycin (1 μM) with a trace of ^{32}P-end-labeled pBR322 DNA fragment and sonicated calf thymus carrier DNA (40 μg/mL) in a total volume of 20 μL of 20 mM Tris-HCl buffer (pH 7.5), ferrous sulfate (equivalent concentration to each drug), and 1 mM dithiothreitol. The cleavage reactions were allowed to proceed at 37 °C for 5 min. To stop the reactions, Na_2EDTA (20 mM) and ice-cold ethanol were added to the samples.

Analysis on a High Resolution DNA Sequencing Gel: Each lyophilized sample was dissolved in 5 μL of formamide containing 0.01 % bromophenol blue, and then loaded into 15 % polyacrylamide gel containing 7 M urea. Electrophoresis was performed at 2000 V and 25 °C for 2.5 hr.

Analyses of DNA Termini on DNA Sequencing Gels: The presence of phosphoryl groups on the 5'-termini of the breaks was tested with T4 polynucleotide kinase to remove 3'-phosphoryl groups (11). DNA product from the standard cleavage reactions was precipitated with ethanol to remove the drugs, dissolved in 20 μL of H_2O, denatured at 90 °C for 5 min, and chilled on ice. Twenty μL of 20 mM Tris-HCl buffer (pH 6.6) containing 20 mM magnesium chloride and 10 mM β-mercaptoethanol were added and followed by 6 units of T4 polynucleotide kinase. The reaction sample was incubated at 37 °C for 1 hr. The presence of phosphoryl groups on the 3'-termini was examined by using bacterial alkaline phosphatase. The drug-treated DNA was dissolved in 50 μL of 100 mM Tris-HCl (pH 8.0). Two units of phosphatase were added and the sample was incubated at 65 °C for 30 min.

Hydrazine Treatment: Subsequently to the standard drug reaction, each lyophilized sample was dissolved in 0.1 M hydrazine hydrochloride (pH 7.0) and incubated at 90 °C for 5 min.

HPLC Analysis of Released Cytosine: The reaction mixture (total volume, 50 μL) contained 3 nmol(bp) of poly(dG-C), various amount of elsamicin A, 20 mM Tris-HCl buffer (pH 7.5), 1 mM dithiothreitol, and 1 mM ferrous sulfate. After incubation at 37 °C for 30 min, each sample was directly subjected to HPLC analysis for determination of spontaneously released cytosine. The analysis was carried out on a μBondapack C18 column (Waters Associates), and the elution was performed with 5 mM ammonium acetate (pH 5.5) at a flow rate of 1 mL/min.

Results and Discussion

Analyses of DNA Termini on High Resolution DNA Sequencing Gels: The termini of the DNA fragments induced by Fe(II)-elsamicin A was tentatively determined by comparing the electrophoretic mobility of the fragments with the known DNA cleavage products on a sequencing gel. For characterization of 3'-ends of the breaks, the 5'-end-labeled pBR322 restriction fragment was treated with Fe(II)-elsamicin A and analyzed on a 15 % sequencing gel (Figure 2). The reaction yielded the two distinguishable kinds of the products (lane 3 of Figure 2). The slower migrating band of each doublet exhibited the electrophoretic mobility identical to the chemically produced Maxam-Gilbert markers, thus suggesting the presence of 3'-phosphate termini. The faster moving band of each doublet ran identically with the fragment bearing 3'-phosphoglycolate termini produced at 5'-GN sites by Fe(II)-bleomycin (see lanes 3 and 4) (12-15). The observations strongly suggest that the fragments bearing 3'-phosphoglycolate and 3'-phosphate termini are produced in the DNA cleavage reaction with Fe(II)-elsamicin A. The estimated amount of the 3'-phosphoglycolate termini was less than that of the phosphate termini in 5'-GN sites. The chemistry at 5'-end of the same fragment on the opposite strand was similarly assessed by analyzing the 3'-end-labeled fragment treated with Fe(II)-elsamicin A on a 15 % sequencing gel. The species comigrated with the Maxam-Gilbert marker, namely 5'-phosphate product.

The presence of phosphoryl group on the 3'-termini was confirmed by using T4 polynucleotide kinase which effectively acts as 3'-phosphatase at a low pH (11). Comparison of lanes 4 and 5 in Figure 3 reveals that one of the bands of each doublet disappears on kinase treatment and a new band appears. The disappeared band comigrated with the Maxam-Gilbert marker (compare lanes 4 and 3), while the newly appeared band comigrated with the fragment

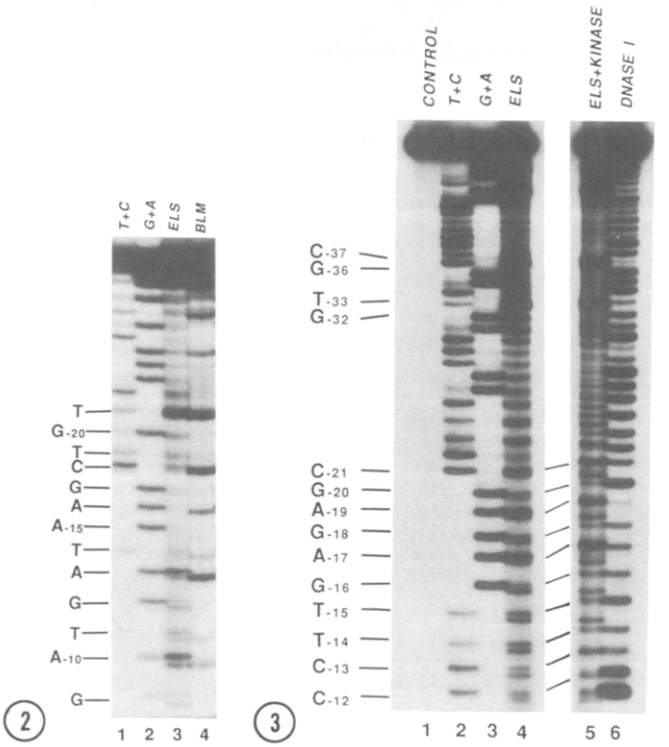

Figure 2. Analysis of 3'-termini on a 15 % DNA sequencing gel. The pBR322 DNA restriction fragments labeled at the 5'-terminus were incubated with 15 μM Fe(II)-elsamicin A (lane 3), or 1 μM Fe(II)-bleomycin (lane 4). Lanes 1 and 2 show the Maxam-Gilbert C+T and G+A ladders, respectively.

Figure 3. Analysis of 3'-termini on a 15% DNA sequencing gel. The pBR322 DNA restriction fragments labeled at 5'-end were incubated with 15 μM Fe(II)-elasmicin A (lane 4), 15 μM Fe(II)-elsamicin A followed by T4 polynucleotide kinase (lane 5), or DNase I (lane 6). Lanes 1-3 show intact DNA alone, the Maxam-Gilbert C+T, and G+A reactions, respectively.

produced in the DNase I reaction (see lanes 5 and 6). The faster moving band of each doublet, presumed as 3'-phosphoglycolate product, was resistant to the kinase treatment. Indeed, the results support the assignment that the slower moving band of each doublet is a DNA fragment bearing 3'-phosphoryl group. The presence of phosphoryl group on the 5'-termini was similarly confirmed by using bacterial alkaline phosphatase. In summary, Fe(II)-elsamicin A produces 3'-phosphoglycolate and 3'-/ 5'-phosphate termini in its DNA cleavage reaction.

Detection of C-4' Hydroxylated Abasic Sites: In the DNA damage induced by Fe(II)-bleomycin and neocarzinostatin, the C-4' hydroxylated abasic product has been identified from the experimental evidence involving its conversion to pyridazine derivative (16, 17). We examined whether such abasic product is formed or not in the DNA strand scission by Fe(II)-elsamicin A reaction (Figure 4). When the DNA fragments treated with Fe(II)-elsamicin A were incubated with hydrazine, the new band appeared with slower mobility than the

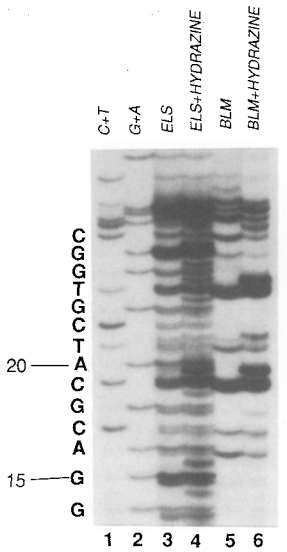

Figure 4. Detection of C-4' hydroxylated abasic sites. A portion of the standard reaction mixture was treated with hydrazine prior to gel electrophoresis (lanes 4 and 6). Lanes 3 and 4 show the reaction sample treated by Fe(II)-elsamicin A. Lanes 5 and 6 indicate the sample treated by Fe(II)-bleomycin. Lanes 1 and 2 show C+T and G+A ladders. The pyridazine derivatives of C-4' hydroxylated products migrate slower than the corresponding phosphate products.

corresponding 3'-phosphate band (see lanes 3 and 4). The band is reasonably considered to bethe pyridazine derivative, because the hydrazine-treatment of the products derived from the bleomycin reaction gave the identically migrating band (lane 6).

Formation of Free Base: The productions of 3'-phosphate termini and C-4' hydroxylated abasic sites could predict the formation of free base. To clarify this point, poly(dG-C) was used as a substrate for the cleavage reaction by elsamicin A. The reaction mixtures were directly analyzed by using HPLC. The HPLC profile evidently showed the formation of product eluted at 3.0 min coincident with authentic cytosine. Figure 5 reveals an apparent correlation between the formation of released cytosine and the concentration of elsamicin A.

Probable Mode of DNA Damage Mediated by Elsamicin A: On the basis of ESR spin trapping and selective DNA cutting experiments, it was previously demonstrated that in the presence of ferrous ion and dithiothreitol chartarin core generates hydroxyl radical near deoxyribose residues (9). We now identified several products including the DNA fragments bearing 5'-/3'-phosphate and 3'-phosphoglycolate termini, C-4' hydroxylated abasic sites, and free base. The formations of these products indicate to be integrated into a reaction scheme as summarized in Figure 6. Initial abstraction of hydrogen atom probably occurs at the C-4' position of the deoxyribose ring in analogy with DNA damage of bleomycin. The hydrogen atom abstraction from this position generates a carbon radical on C-4' position of the deoxyribose.

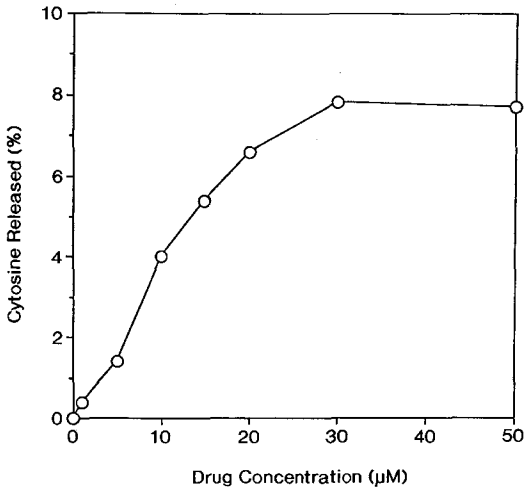

Figure 5. Production of free cytosine. Three nmol(bp) of poly(dG-C) were treated with various concentrations of Fe(II)-elsamicin A at 37 ℃ for 30 min. Each reaction mixture was analyzed by HPLC equipped with μBondapack C18 column, and the amount of cytosine was determined by UV-absorption.

Addition of O_2 to the 4'-carbon-centered radical gives rise to a peroxyl radical which degrades DNA strand to produce 5'-phosphate-ended and 3'-phosphoglycolate-ended fragments. In the absence of O_2, the carbon radical on the deoxyribose is hydroxylated to produce free base and

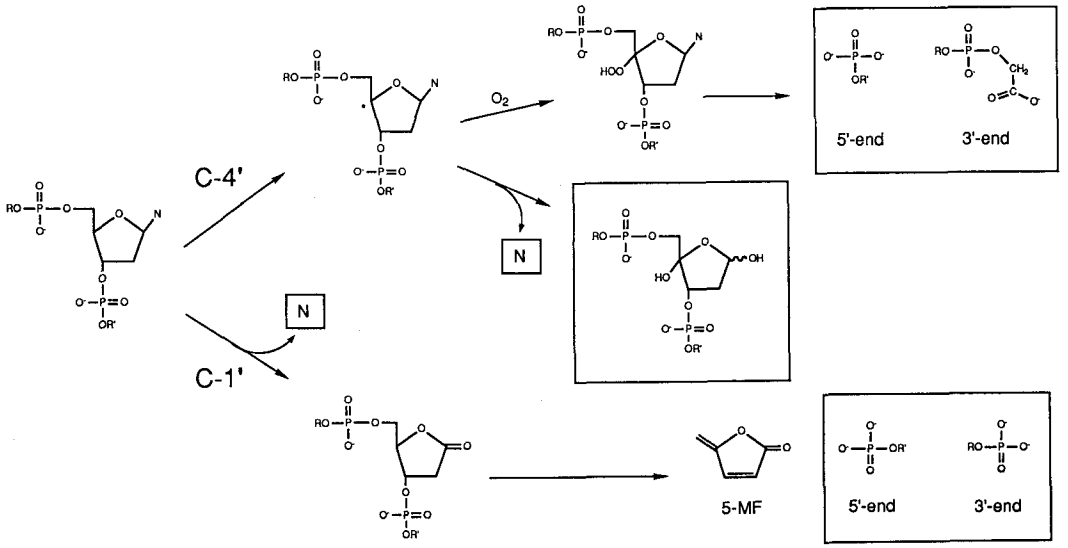

Figure 6. Proposed DNA cleavage mode of Fe(II)-elsamicin A. The products identified are shown by the squares.

C-4' hydroxylated abasic sites in host DNA. Indeed, the product distribution was influenced by O_2 condition. The ratio between 3'-phosphoglycolate and C-4' hydroxylated abasic product changed from 40.5 : 59.5 in usual condition to 47.9 : 52.1 in O_2 bubbling condition (data from densitometric tracing of phosphoglycolate- and pyridazine derivative-bands in 5'-GG site).

The above-mentioned C-4' hydrogen abstraction can reasonably account for the formation of all products presented here except of 3'-phosphate product. Possible reaction pathway involving 3'-phosphate product appears to be due to C-1' abstraction chemistry. In nuclease activity of phenanthroline-copper ion, the elegant studies of Sigman and coworkers showed that hydrogen atom abstraction from the C-1' position, could result in 5'- and 3'-phosphate termini in parallel with release of free base (18, 19). An oxidized 1'-carbon radical produces the hemiaminal which 2'-hydrogen is considered to be acidic. Under physiological condition, therefore, 3'-α,β-unsaturated deoxyribonolactone-ended and 5'-phosphate-ended fragments are produced. Under basic condition, the 3'-α,β-unsaturated deoxyribonolactone-ended fragment undergoes a second elimination to yield 3'-phosphate termini and 5-methylenefranone. Although the DNA cleavage reaction with Fe(II)-elsamicin A gave free base and 5'/3'-phosphate products, it is unclear whether 5-methylenefranone and metastable 3'-α,β-unsaturated deoxyribonolactone are produced or not.

We previously reported the alteration of elsamicin A-induced DNA cleavage by the pretreatments of host DNA with minor groove binders and the similarity of its DNA binding affinity to T4 phage and (T4dC) DNAs (9). The results suggest the interaction of elsamicin A in minor groove of Watson-Crick double helical DNA. This proposal is strongly supported by our finding described here, because hydrogen atom at C-4' carbon is situated in the minor groove of DNA helix. The present information is valuable to an understanding the DNA binding mode of elsamicin A, and also to a design of more effective anticancer drugs.

Acknowledgments

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