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Esperamicins, a class of potent antitumor antibiotics: Mechanism of action*

(DNA breakage/alkaline elution/cytotoxicity)

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ABSTRACT **The esperamicins represent a class of antitumor antibiotics characterized by an unusual chemical core structure and extremely potent cytotoxicity. The mechanism by which these drugs produce cytotoxicity was investigated and found to be related to the formation of single- and double-strand DNA breaks. Using five structurally related analogs, we defined a structure–activity relationship for cytotoxicity in various eukaryotic and DNA-repair-deficient prokaryotic cell lines, for DNA breakage in a human colon carcinoma cell line, and for DNA breakage *in vitro* in pBR322 DNA. Mild reducing agents such as dithiothreitol greatly increased the DNA breakage potency of these analogs *in vitro*. Results suggest that the pendant aromatic chromophore of esperamicin A_1 may contribute to the uptake of the drug into cells but may also hinder double-strand DNA break formation. Little DNA breakage specificity was observed for the drug in a 139-base-pair fragment of pBR322 DNA. Evidence supports a previously proposed mechanism whereby esperamicins may produce the observed DNA breaks through reduction of the methyl trisulfide group to a thiolate anion followed by a Michael addition of the anion across the α,β-unsaturated ketone. This addition may result in the saturation of the bridgehead double bond, thus allowing the two triple bonds to approach each other, causing cyclization of the diyn-ene to form a phenylene diradical. It is likely that this diradical is the active form of the drug responsible for single- and double-strand DNA breakage produced by this class of antitumor agents.**

A family of extremely potent compounds showing broad spectrum antimicrobial and antitumor activity in murine systems has been identified in cultures of *Actinomadura verrucosospora* (1). The elucidation of their complex chemical structures has recently been reported (2, 3). This class of compounds, collectively called the esperamicins, is characterized by the presence of a central core containing a number of unusual features. These include a bicyclo[7.3.1] ring system, an allylic trisulfide attached to the bridging atom, a 1,5-diyn-3-ene as part of the ring system, and an α,β-unsaturated ketone in which the double bond is at the bridgehead of the bicyclic system. Esperamicin A_1 (espA) contains four sugars attached to the bicyclic core and an aromatic chromophore attached to one of the sugars (3), as shown in Fig. 1*A*.

Various analogs of espA have been prepared by chemical hydrolysis, including esperamicin C (espC), which lacks both the 2-deoxy-L-fucose and the aromatic ring moieties; esperamicin D (espD), which is similar to espC but also lacks the thiomethyl hexopyranose moiety; esperamicin E (espE), which consists of the bicyclic core and the hydroxylamino sugar; esperamicin Z (espZ), which is an esperamicin A_1 that has undergone reductive cyclization of the trisulfide and subsequent aromatization of the 1,5-diyn-3-ene structure; and esperamicin X (espX), which is an analog of espZ but lacking the trisaccharide at the C-12 position (see Fig. 1*A*). The structural identifications of all these analogs except espZ have been previously described (2, 3).

It is quite conceivable that espZ could arise from espA by reductive cleavage of the trisulfide, Michael addition to the bridgehead double bond, and aromatization of the diynene (3), as illustrated in Fig. 1*B*. The spontaneous formation of the aromatic ring found in espX and espZ is prevented by the presence of the bridgehead double bond in espA, which does not allow the ends of the diynene to approach one another to permit cyclization unless this double bond is eliminated through Michael addition (3). We have previously proposed that the extreme cytotoxic potency of espA may be due to the generation of a diradical resulting from the ring closure and that this diradical would be capable of H atom extraction from the sugar phosphate backbone of DNA, thus leading to DNA breakage (Fig. 1*B*). This possibility has been explored and a series of studies have been conducted to uncover the molecular basis for the antitumor effects of this class of compounds. Evidence is presented demonstrating that espA interacts with DNA, causing DNA breakage in cells and *in vitro*. Furthermore, partial structure–activity relationships for cytotoxicity and DNA breakage activities in cells and *in vitro* have been generated by using the structural analogs shown in Fig. 1*A*. We have also investigated the effects of espA and its analogs on a series of isogenic strains of *Escherichia coli*, each with specific, genetically engineered DNA repair defects, to confirm DNA breakage as the mechanism by which these drugs produce cytotoxicity.

MATERIALS AND METHODS

Chemicals. espA and its analogs, espC, espD, espE, and espX, prepared as previously described (2, 3), were obtained from the Antitumor Chemistry and Microbiology Department, PRDD, Bristol-Myers Corp., Wallingford, CT. espZ was prepared from 25 mg of espA in 5 ml of methylene chloride and methanol (2:1, vol/vol) by treatment with 0.25 ml of 2-mercaptoethanol for 16 hr at room temperature. Purification was performed by silica gel column and preparative thin-layer chromatography, yielding 10.9 mg of espZ,

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Abbreviations: espA, esperamicin A_1; espC, esperamicin C; espD, esperamicin D; espE, esperamicin E; espX, esperamicin X; espZ, esperamicin Z; sccc, supercoiled covalently closed circular; SSB, single-strand DNA break; DSB, double-strand DNA break.

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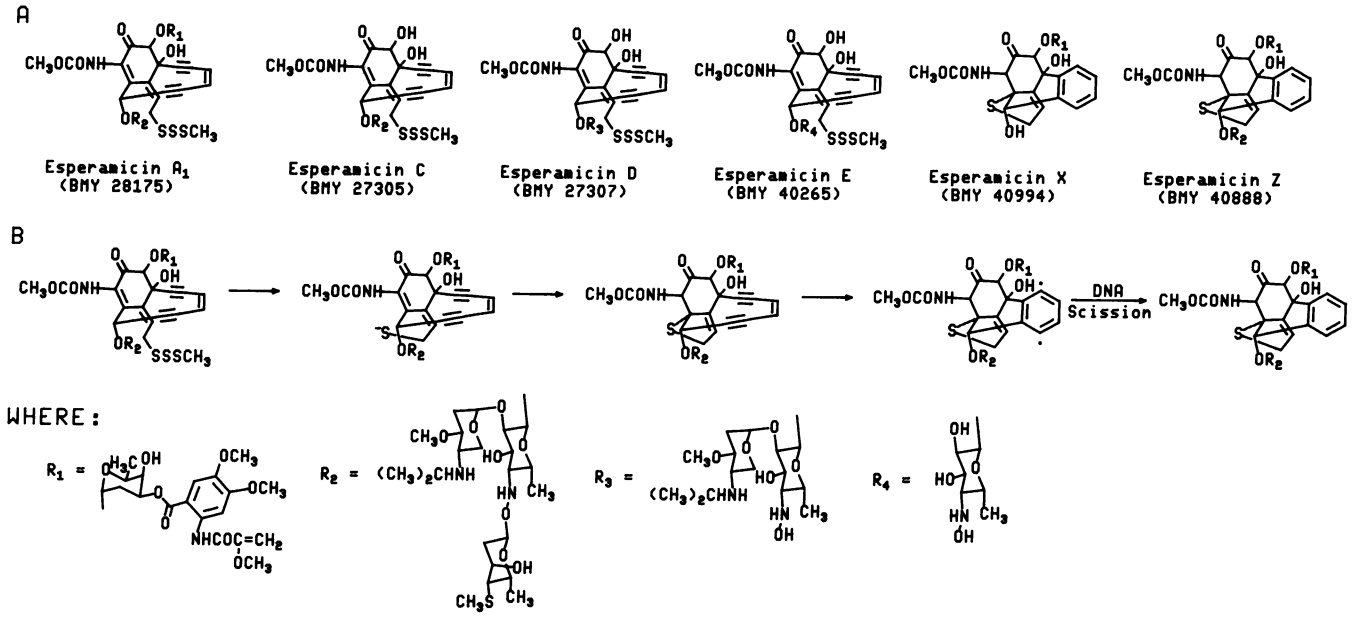

FIG. 1. (*A*) Chemical structures of the esperamicin analogs. (*B*) Model for the mechanism of DNA breakage by esperamicins.

which was identified and characterized by ^{1}H NMR spectroscopy and mass spectrometry. Echinomycin, hedamycin, and mitomycin C were obtained from the Bristol–Myers chemical repository.

Cell Culture. For cytotoxicity testing, anchorage-dependent cell lines (B16-F10, HCT116, H2981, MOSER, and SW900) were grown in Eagle's minimal essential medium (MEM) with Earle's salts (GIBCO) containing 2 mM L-glutamine, penicillin/streptomycin (10 units/ml and 10 μg/ml, respectively), and 10% heat-inactivated fetal bovine serum. For DNA breakage studies the human tumor cell line HCT116 was maintained in logarithmic growth in a cell culture medium consisting of McCoy's 5A medium (modified, GIBCO), supplemented with 2 mM L-glutamine, 0.12 mM L-serine, 0.17 mM asparagine, 1.5 mM sodium pyruvate, 0.625% MEM essential amino acids (GIBCO), 10% fetal calf serum (GIBCO or HyClone), and penicillin/streptomycin (as above). Cells were incubated at 37°C in 5% CO_2 in air at 90% relative humidity.

***In Vitro* Cytotoxicity.** The relative cytotoxic potencies of espA and analogs in eukaryote cell lines were determined by using a microtiter-based dye binding assay. In this procedure, cells were plated in microtiter wells 1 day prior to addition of drug. After a 72-hr incubation in the presence of drug, the cells were fixed with Formalin, air dried, and stained with crystal violet. Dye extracted from the wells was quantified by measuring absorbance of the solubilized dye at 590 nm. A detailed description of this procedure has been published elsewhere (4).

Alkaline Elution Assay for DNA Damage. DNA breaks were quantified by standard alkaline elution techniques (5, 6), using HCT116 cells, prelabeled for 72 hr with [^{14}C]thymidine at 0.01 μCi/ml (1 Ci = 37 GBq) and exposed to various concentrations of drug for 1 hr. Cells were harvested and layered onto polycarbonate filters with reference HCT116 cells prelabeled with [^{3}H]thymidine at 0.1 μCi/ml and exposed to 300 rads (1 rad = 0.01 gray) of γ-radiation as an internal elution standard. Cells were lysed with detergent and the DNA remaining on the filters was slowly eluted by pumping a buffer adjusted to pH 12.1 through the filters. Quantification was achieved by relating slopes of DNA elution curves obtained after drug treatment to those obtained after exposure to different doses of γ-radiation, as previously described (7, 8).

***In Vitro* DNA Assay for DNA Breakage Activity.** Analysis of drug-induced damage to pure supercoiled, covalently closed, circular (sccc) pBR322 DNA was performed in the presence of 1 mM dithiothreitol, for 60 min at 37°C, followed by agarose gel electrophoresis to separate the different DNA products. Incubation of drugs with 0.5 μg of DNA occurred in 30 μl of a buffer consisting of 50 mM Tris·HCl, pH 7.5. The substrate, sccc pBR322 DNA (form I, Boehringer Mannheim) could be converted to nicked, relaxed circular DNA (form II) by the introduction of one single-strand DNA break (SSB) or to linearized DNA (form III) by the introduction of one double-strand DNA break (DSB). The different forms of DNA were separated by electrophoresis for 4 hr at 100 V in 0.9% agarose (Bio-Rad) slab gels buffered with 80 mM Tris base and 40 mM boric acid, pH 8.0. The DNA bands were visualized, by using ethidium bromide binding and the resulting fluorescence produced by UV irradiation.

DNA Cleavage Site Sequence Specificity. The *Hin*dIII/*Nci* I 139-base-pair restriction fragment of pBR322 DNA was isolated and 3′-end labeled at position 33 (adenosine), and in a separate experiment, 5′-end labeled at position 30 (adenosine) with ^{32}P, as previously described (9, 10). The strand scission reactions involving espA and DNA were carried out at 37°C for 10 min in a total volume of 6 μl. The buffered medium (50 mM Tris·HCl, pH 7.5/0.1 mM EDTA) contained approximately 1 μM labeled fragment, in DNA base pairs, 1 mM dithiothreitol, and espA at concentrations of 10^{-4} to 10^{-10} M. The espA–DNA mixture was allowed to equilibrate for 20 min prior to the addition of dithiothreitol. The reactions were terminated by addition of 8 M urea, and the mixture was loaded into a 12% denaturing polyacrylamide gel for electrophoretic analysis. Quantitative autoradiography without an intensifying screen and linear scanning microdensitometry yielded oligonucleotide concentrations as a function of espA concentration and sequence (11). The amount of fragment cleavage was strongly dependent on drug concentration, with about 10^{-9} M drug yielding "single-hit" kinetics on the 139-mer. The establishment of sequence and the numbering system used for the fragment were as earlier described (9, 10).

Microbiological Studies with DNA-Repair-Deficient Bacterial Cells. An isogenic series of *E. coli* strains, each with specific genetically engineered DNA repair defects, was obtained from Michael Volkert (University of Massachusetts Medical School, Worcester), and the cells were maintained in

LBM medium (10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl, 5 g of glucose, and 0.5 mg of thiamine·HCl per liter of 0.1 M potassium phosphate buffer, pH 7.5). When cultures grown at 37°C reached optimal optical density (0.8–0.9 at 600 nm) they were diluted 100-fold with LBM medium, then further diluted 100-fold with LBM medium containing sufficient EDTA to achieve a final concentration of 1 mM EDTA, which was determined to be the optimal concentration for drug uptake without appreciable retardation of cell growth. Each sample was run in duplicate with a standard agent (mitomycin C at concentrations less than 20 μg/ml) included as an internal control on each plate. Plates were shaken using a mini-orbital shaker (Bellco Glass) to avoid settling, and the plates were incubated at 37°C for 18–20 hr. Growth was measured by using a Titertek Multiscan MC microtiter plate reader and filter no. 6 (540 nm).

RESULTS

Cell Proliferation Studies. A survey of the relative sensitivities of various murine and human cell lines to espA confirmed that this drug is an extemely potent antitumor antibiotic, with IC_{50} values in the ng/ml range (Table 1). The espA analogs were considerably less potent, with espE, espX, and espZ showing no cytotoxicity at concentrations up to 12.5 μg/ml. espC was approximately 1/200th as potent and espD was approximately 1/1000th as potent as espA (Table 1). Of the different cell lines tested, HCT116 was clearly the most sensitive cell line and MOSER was the most resistant line, being 25-fold more resistant to this drug class than HCT116 cells were.

DNA Breakage in HCT116 Human Colon Carcinoma Cells. Incubation of HCT116 cells with different concentrations of espA for 1 hr resulted in the dose-dependent formation of DNA breaks that could be easily detected by alkaline elution techniques. Total DNA breaks calculated from the slopes of alkaline elution curves increased from 3 breaks per 10^8 nucleotides upon exposure to espA at 1 pg/ml to 47 breaks upon exposure to 100 pg/ml (Table 2). Above 100 pg/ml, the alkaline elution curve became highly biphasic, with most of the DNA eluting in the first fraction and increased appearance of DNA in the lysis fraction upon exposure to espA at 1 ng/ml. A comparison of the DNA breakage produced by the different esperamicin analogs assessed by alkaline elution is also included in Table 2. In producing DNA breaks in intact cells, potencies relative to espA are approximately 1/200 for espC, 1/3000 for espD, 1/100000 for espE, 1/5000 for espX, and 1/20000 for espZ.

DNA Breakage Produced *in Vitro*. Because of the presence of a trisulfide linkage in the chemical structure of espA, it was conceivable that DNA breakage could be demonstrated *in vitro* in the presence of mild reducing agents. The conversion of sccc DNA (form I) to nicked, relaxed circles (form II), or linearized DNA strands (form III) provides a sensitive and reliable method for not only demonstrating DNA breakage activities *in vitro* but also identifying the types of DNA breakage being produced.

Table 1. Cytotoxicity analysis of esperamicin analogs in eukaryotic cells

Analog	IC_{50}, μg/ml				
	B16-F10	HCT116	H2981	MOSER	SW900
espA	0.0045	0.0003	0.0018	0.0083	0.0022
espC	0.85	0.067	0.71	1.5	1.04
espD	4.6	0.87	2.7	>12	4.9

Relative cytotoxic potencies of the esperamicin analogs in eukaryotic cell lines were determined by using a microtiter-based dye-binding assay. B16-F10 is a murine cell line; the other lines are human. espE, espX, and espZ were not cytotoxic at 12 μg/ml.

Table 2. DNA breakage produced in HCT116 cells by esperamicin analogs at various concentrations

Analog conc., ng/ml	DNA breaks per 10^8 nucleotides					
	espA	espC	espD	espE	espX	espZ
0.001	3.2					
0.010	9.8					
0.030	28.0					
0.10	47					
0.30	—					
3.0		1.1				
10		6.1				
30		27.7				
100		—	1.9			
300			6.8		1.6	
1,000			19.2	3.4	12.3	6.7
3,000			34	6.9	36	
10,000			—	15.7	—	36

HCT116 cells were incubated with analog for 1 hr in cell culture medium prior to alkaline elution. — indicates values were unreliable because of the biphasic nature of the elution curve.

The incubation of espA with pBR322 sccc DNA had no effect on DNA integrity at concentrations below 1.0 μg/ml in the absence of reducing agent (data not shown). However, as shown in Fig. 2, the addition of 1 mM dithiothreitol to the reaction mixture resulted in conversion of the starting material (form I DNA) to DNA containing mostly SSBs (form II DNA) and some noticeable DSBs (form III DNA) at the lowest drug concentration tested (0.01 μg/ml). Almost complete conversion of the substrate to DNA containing either SSBs or DSBs was produced by espA at 0.3 μg/ml, and multiple breakage sites on the DNA fragments were produced by concentrations of drug above 0.3 μg/ml, as evidenced by the presence of smearing (Fig. 2). Other mild reducing agents such as 2-mercaptoethanol and glutathione were also effective towards activating espA to produce DNA breakage (results not shown).

A comparison of the relative DNA breakage produced in pBR322 DNA by the different esperamicin analogs in the presence of 1 mM dithiothreitol is also presented in Fig. 2. espC was clearly more active than espA toward the formation of DSBs, whereas espC was slightly less potent than espA, and espD was about 1/50th as potent as espA in causing the consumption of form I DNA. Concentrations of espE, espX, and espZ as high as 1 μg/ml were inactive toward producing DNA breakage *in vitro* (Fig. 2 and results not shown). These potency differences were not as great as seen in the cytotoxicity and DNA breakage studies with intact eukaryotic cells (Tables 1 and 2), and they likely reflected differences in drug uptake by the various analogs (see *Discussion*). In addition to quantitative differences, differences were also observed between the proportions of SSBs and DSBs produced by the analogs relative to the proportion produced by espA.

DNA Cleavage Site Sequence Specificity. DNA cleavage sites on a pBR322 DNA fragment along with relative breakage frequencies obtained from densitometric scans of selected autoradiographic data are shown in Fig. 3. espA cleaves at all possible nucleotide positions of DNA. However, the number of shorter fragments produced by cleavage when the fragment was 3′-end labeled at position 33 was greater than when it was 5′-end labeled at position 30. Some of the smallest products (12- to 14-mers) produced by espA were found to be electrophoretically identical to the Maxam and Gilbert (12) products. However, other "satellite" products did not have mobilities identical to those of the sequencing-derived products. When the label is on the 5′ end of the molecule, position 30, the cleavage reaction by espA appeared to produce a single product, without satellites, having

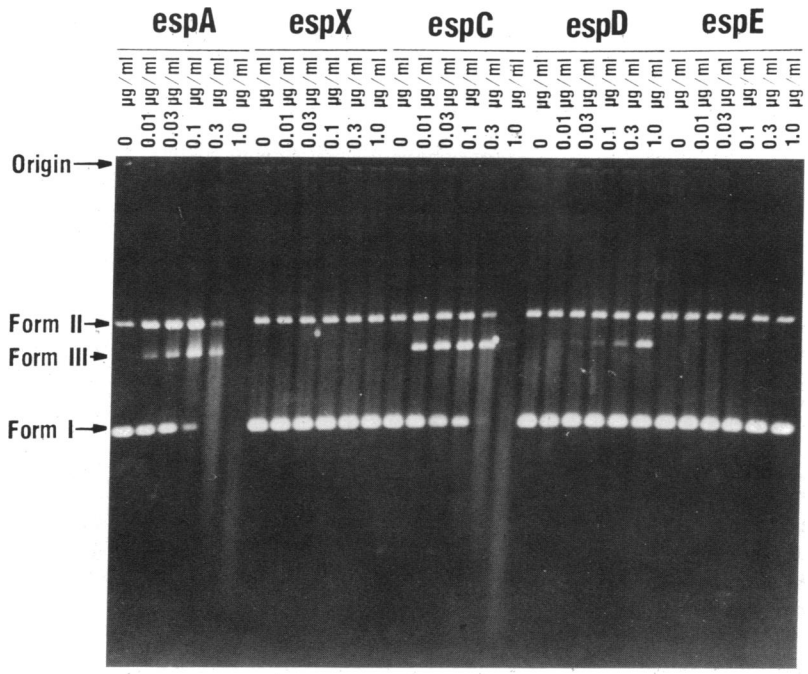

FIG. 2. DNA breakage *in vitro* by esperamicin analogs at various concentrations in the presence of reducing agents. sccc pBR322 DNA (form I) was incubated with espA, espC, espD, espE, or espX at the indicated concentrations in the presence of 1 mM dithiothreitol for 1 hr at 37°C, then subjected to electrophoresis in a 0.9% agarose gel and visualization with ethidium bromide.

a mobility identical to that of the Maxam and Gilbert oligomer.

Cytotoxicity of Esperamicins in Repair-Deficient Bacteria. To gather additional information on the mechanism of action of the esperamicin antitumor antibiotics, we have investigated their effects on a series of isogenic strains of *E. coli*, each with specific, genetically engineered defects in DNA repair. The test used in this study was a microtiter assay based on differential growth inhibition of the parent *E. coli* strain MV1161, with its intact genome, and a set of three isogenic strains (Table 3). The hypersensitivity of a strain deficient in postreplication repair (*recA*$^-$) to the cytotoxic effects of espA would provide an indication of the mechanism by which this agent produces cytotoxicity, if DNA is the primary biological target for this class of drugs.

Activities of different DNA-reactive cytotoxic agents and analogs were compared with the activities of espA and analogs against the parental and mutant strains of *E. coli* (Table 3). The activity of espA paralleled that of mitomycin C and hedamycin in that postreplication-repair-deficient mutants (MV1175 and MV1255) were hypersensitive to these drugs. These results are consistent with results reported by Mamber *et al.* (13) for veractamycins A and B, which appear to be identical to esperamicins A_1 and A_2, respectively (S.F., T.W.D., and J. Veitch, unpublished results). Relative cytotoxicities of the different analogs of esperamicin against the parental and mutant *E. coli* strains revealed that espC showed slightly increased activity against *recA*$^-$ strains (Table 3). The analogs espE, espX, and espZ had no detectable cytotoxicity in this assay.

DISCUSSION

These studies confirm that the esperamicin compounds are extremely potent cytotoxic antitumor agents with a novel mechanism of action (1–3). Similar or identical compounds containing the 1,5-diyn-3-ene structure have been discovered recently in fermentation broths and their structures have been identified: the calichemicins (14, 15), FR-900405 and FR-900406 (16), and the veractamycins PD114,759 and PD115,028 (17).

One possible mechanism by which esperamicins produce cytotoxicity in intact cells is through the formation of DNA breaks (Table 2). Furthermore, we have demonstrated the production of both SSBs and DSBs in sccc pBR322 DNA by espA at concentrations below 10 ng/ml in an *in vitro* system in the presence of 1 mM dithiothreitol (Fig. 2). Other mild reducing agents such as 2-mercaptoethanol, glutathione, ascorbate, and BH_4CN were also capable of activating the compound (data not shown). Similar agents can also activate the chromophore of neocarzinostatin, which has structural and possibly mechanistic similarities to espA (18–20).

A mechanism of action has been proposed involving reduction of the methyl trisulfide group to a thiolate anion followed by a Michael addition of the anion across the α,β-unsaturated ketone, resulting in the elimination of the bridgehead double bond (Fig. 1*B*). The absence of this bridgehead double bond would allow the triple bonds to approach each other, thus favoring cyclization of the 1,5-diyn-3-ene system to create a phenylene diradical. It is proposed that the phenylene diradical is responsible for the DNA breakage (3).

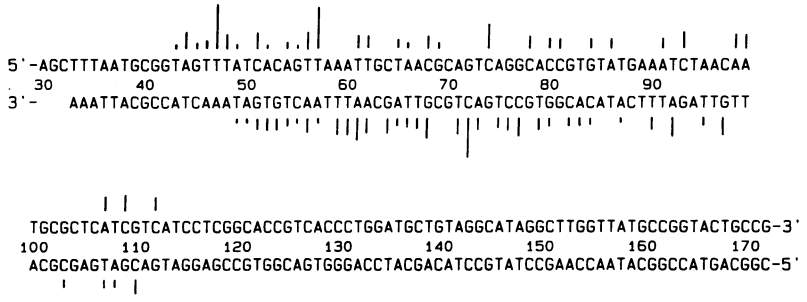

FIG. 3. Sequence specificity of esperamicin in DNA breakage. DNA cleavage frequencies, in histogram format, are presented over the sequence of a 139-base-pair *Hin*dIII/*Nci* I restriction fragment of pBR322 DNA used as the target for the drug. Incubation of the 139-base-pair fragment, labeled on either the 3′ or the 5′ end with [^{32}P]phosphate, with espA in the presence of 1 mM dithiothreitol resulted in DNA fragments, which were separated by gel electrophoresis. Site-specific DNA cleavage relative frequencies were obtained from densitometric scans of autoradiograms of such gels.

Table 3. Antimicrobial activity of standard antitumor agents and esperamicin analogs in DNA-repair-deficit mutants

Drug	IC_{90}, μg/ml			
	MV1161	MV1242	MV1175	MV1255
Echinomycin	5.0	5.0	1.25	1.25
Hedamycin	5.0	1.25	0.63	<0.01
Mitomycin C	1.25	0.16	0.08	<0.01
espA	5.0	5.0	0.63	0.63
espC	1.25	1.25	0.08	<0.01
espD	>20	>20	5.0	2.5

Isogenic strains of *E. coli* lacking specific functional DNA repair genes: MV1161, parental strains; MV1242, *recA6*$^-$; MV1175, *recA56*$^-$; and MV1255, *recA6*$^-$:*recF143*:(*sclR–recA*)*306*$^-$. The IC_{90} values are the averages of the results obtained from duplicate experiments with each condition run in duplicate for each experiment.

The data obtained on the series of analogs analyzed for cytotoxicity of eukaryote cells (Table 1) and repair-deficient bacteria (Table 3) and for DNA breakage in cells (Table 2) and *in vitro* (Fig. 2) allow us to draw some preliminary conclusions about structure–activity relationships in this class of highly potent antitumor antibiotics. The preliminary data suggest that two structural features of the molecule are essential for its biological activity: the diyn-ene group and the intact trisaccharide moiety. For example, espA and espC both displayed equal potencies in the bacterial DNA repair assay (Table 3) and in the *in vitro* DNA breakage assay (Fig. 2) but not in the assays for eukaryote cytotoxicity (Table 1) and DNA breakage assay conducted in intact cells (Table 2). These results suggest that the presence of the deoxyfucose-anthranilate moiety affects drug accumulation in both prokaryotic and eukaryotic cells. This suggestion is supported by the observation of improved antimicrobial activity of espC relative to espA when EDTA is included in the assay buffer, due to increased cell permeability facilitated by the presence of EDTA.

In vitro studies demonstrated that espA produced predominantly SSBs at low drug concentrations, whereas espC and espD, analogs that lack the deoxyfucose-anthranilate moiety, produced predominantly DSBs even at low drug concentrations (Fig. 2). It is possible that the presence of this sugar and attached chromophore on espA may sterically hinder the resulting phenylene diradical from becoming positioned on the DNA in such a way as to allow for the production of a DSB. Thus, it is possible that the presence of the deoxyfucose-anthranilate moiety may favor increased uptake of drug but may also hinder formation of DSBs.

Absence of the methylthio sugar (espD) resulted in a dramatic loss in activity, which was further decreased by the removal of the isopropylamino sugar (espE) (Tables 1–3, Fig. 2). It is quite possible that the sugars serve a role in facilitating the binding of the drug in a proper position relative to the DNA to allow DNA breakage to occur. The diyn-ene group is a key functional group in the esperamicin antitumor antibiotics, as shown by the lack of activities for espX and espZ. These observations fully support the previously proposed mechanism involving reductive cleavage of the trisulfide and cyclization of the diyn-ene (3). Together, these results indicate that espC represents the optimal pharmacophore for this class of agents.

The observation that espA cleaved the 139-base-pair DNA fragment at all nucleotide positions (Fig. 3) strongly indicates that the site of attack is the deoxyribose moiety of the polymer. Although further study will be necessary, the mechanism of cleavage does not appear to involve the release of a diffusable radical species, as is the case for FeEDTA (21, 22). This type of mechanism generally results in cleavage over four to five adjacent sites on the DNA, which does not appear to be the case for these agents (Fig. 3).

A greater number of espA-induced cleavage products were observed when the label was on the 3′ end of the restriction fragment at position 33 than when the label was on the 5′ terminus of the fragment at position 30. This observation is probably due to chemical heterogeneity on the 5′ end of the DNA adjacent to a DNA strand scission site. In this respect, cleavage of DNA by espA in the presence of dithiothreitol appears to be similar to that by the DNA-cleaving chromophore of neocarzinostatin, which is known to leave a 5′ aldehyde, 5′ phosphate, and at least one other product adjacent to its DNA strand scission site (23). When the radiolabel was located on the 5′ end of one strand, position 30, only products that comigrated with Maxam and Gilbert oligomers were observed. This observation strongly suggests that the sugars adjacent to the site of strand scission possess 3′ phosphate groups. Although further work will be necessary, the presence of strong espA cleavage sites at positions 47, 57, and 74 (top strand) suggests that the antibiotic may be specific for pyrimidine sites located two nucleotides in the 3′ direction from a guanine residue.

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