The Deoxyfucose-Anthranilate of Esperamicin A1 Confers Intercalative DNA Binding and Causes a Switch in the Chemistry of Bistranded DNA Lesions

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Received July 11, 1994[⊗]

Abstract: We report three as yet undescribed, critical features of the interaction of the enediyne antitumor antibiotic esperamicin A1 (ESP A1) with DNA. First, results from hydrodynamic and spectroscopic studies revealed an intercalative binding mode conferred by the deoxyfucose-anthranilate of ESP A1. An intercalative binding mode is consistent with earlier observations of nucleosome linker-selective DNA damage by ESP A1 [Yu, L. *et al. J. Biol. Chem.* **1994**, *269*, 4144-4151]. While capable of adopting a planar structure, the *N*-(2-methoxyacrylyl)anthranilate group would represent an unusual intercalator, which by itself has no apparent affinity for DNA, yet contributes 1-2 kcal/mol to the binding energetics of ESP A1. Second, bistranded DNA lesions, which consist of a direct strand break opposite an abasic site, represent 20-25% of ESP A1-mediated DNA damage. Finally, sequencing gel analysis and tritium abstraction studies revealed that the deoxyfucose-anthranilate caused a switch in the chemistry of ESP A1-mediated DNA damage from 4′-hydrogen abstraction to 1′. We propose that intercalation of the anthranilate of ESP A1 alters the minor groove position of the diradical form of the drug and causes a switch in the hydrogen abstraction in bistranded lesions; the predominance of single-strand lesions associated with ESP A1 may be the result of the altered positioning or intramolecular quenching of one of the drug radicals.

Introduction

Esperamicin A1 (ESP A1) is a clinically-promising member of the growing enediyne family of antitumor antibiotics, which includes calicheamicin[1] (CAL) and neocarzinostatin[2] (NCS). The cytotoxic effects of these agents are thought to be related to DNA damage produced by a highly-reactive diradical form of the drug that arises by a thiol-mediated electronic rearrangement of the enediyne core (Figure 1). Precise positioning of the diradical in the minor groove leads to abstraction of deoxyribose hydrogen atoms from one or both strands,[3,4] and thus the drugs have the potential to produce double-strand (DS) DNA lesions.[5-8] Indeed, virtually all of the DNA damage produced by CAL consists of DS lesions,[8] thought to arise by strand-specific hydrogen abstraction from the 4′- and 5′-positions of deoxyribose.[3,4] However, previous studies have concluded that ESP A1 causes only single-strand (SS) lesions[6,9] involving 5′-hydrogen abstraction.[10]

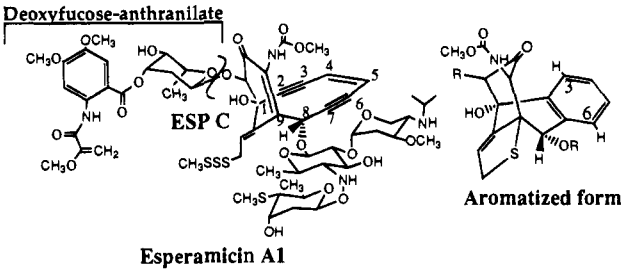

Figure 1. Structures of ESPs A1 and C and their aromatized forms.

Structural differences between CAL and ESP A1 were proposed to account for these latter observations.[10] While these drugs share a common DNA-damaging enediyne core, the terminal carbohydrate-aromatic group of CAL has essentially been apportioned to the opposite side of the core in the form of the deoxyfucose-anthranilate of ESP A1 (Figure 1). Christner *et al.* proposed that the absence of DS lesions and the singular DNA damage chemistry of ESP A1 resulted from anthranilate-mediated quenching one of the drug radicals or malpositioning of the drug in the minor groove.[10] Their conclusions were supported by the occurrence of DS lesions and 4′- and 5′-chemistry with ESP C, an ESP A1 analogue missing the deoxyfucose-anthranilate.

Our recent studies of enediyne-mediated DNA damage in chromatin suggested an additional or alternative role for the anthranilate of ESP A1, that of a novel intercalating agent.[11] While CAL and ESP C were observed to produce DNA damage in both the core and linker DNA of the nucleosome, ESP A1 damage was restricted to the linker region. We proposed that this linker selectivity resulted from intercalative DNA binding

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[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1994.

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by the deoxyfucose-anthranilate group, a hypothesis based on precedent with other intercalating agents.[11]

We now report that ESP A1 binds to DNA by an intercalation-like mechanism mediated by the anthranilate moiety. The presence of the anthranilate causes a switch in the chemistry of DNA damage from 4′-hydrogen abstraction to 1′-chemistry, but it does not abolish the ability of ESP A1 to produce bistranded DNA lesions, which were found to comprise 20−25% of ESP A1-mediated DNA damage. These observations have significant implications for the currently proposed models of ESP A1/DNA interactions.

Materials and Methods

Materials. ESP A1, ESP Z, ESP C, and the methyl glycoside of the deoxyfucose-anthranilate moiety of ESP A1 were prepared as described elsewhere.[9] Aromatized ESP C was prepared by reaction of ESP C with triphenylphosphine (4 molar equiv) in methylene chloride in the presence of 1,3-cyclohexadiene (200 molar excess) as a hydrogen source (4 °C, 12 h). The reaction products were resolved by thin-layer chromatography (TLC) on silica gel plates with a solvent system of 1:9 methanol/chloroform. The product migrating with an R_f of ~0.3 was purified and identified as aromatized ESP C by electrospray-ionization mass spectrometry. In a similar manner, stocks of ESP Z were purified by TLC, and the isolate was characterized by mass spectrometry.

Binding Isotherm. Isotherms for the binding of ESP A1 and ESP C to phage λ DNA were established by separating DNA-bound from unbound drug by ultracentrifugation.[12] Experimental conditions were as follows: 1−20 μM ESP A1 or ESP C; 50−100 μM base pairs (bp) of λ-DNA (type c1857 *Sam7*, Promega); 10 mM HEPES, 1 mM EDTA, 10 mM NaCl, 15% DMF, pH 6.5; a total volume of 80 μL in siliconized 1.5 mL polypropylene MCT tubes (USA/Scientific). The DNA was pelleted by centrifugation at 217 000 × g for 20 min at 22 °C in a Beckman TL100 ultracentrifuge with a TLA.100.3 rotor. The concentration of unbound drug was determined by its absorbance at 320 nm, with the remaining drug assumed to be bound to DNA. The best fit of the data to the neighbor-exclusion model of McGhee and von Hipple[13] was determined by nonlinear regression analysis.

UV Quenching. The effect of DNA binding on the 320 nm absorbance of ESP A1 and ESP C was assessed with sonicated, S1 nuclease-treated calf thymus DNA fragments. Ratios of drug to DNA (bp) ranging from 1:0 to 1:160 were prepared by adding aliquots of DMF stock solutions of the drug (final 5 μM) to a DNA solution (10 mM HEPES, 1 mM EDTA, 10% DMF final, pH 6.5, 0.5−815 μM DNA bp) at room temperature. Extinction coefficients (320 nm) for ESPs A1 and C under these conditions were determined to be ~16 000 and ~5 000 cm^{-1} M^{-1}, respectively.

Plasmid Topoisomer Sedimentation. The effects of ESP A1, ESP C, and NCS on the superhelical state of plasmid pBR322 were examined by sedimentation analysis. A 2 μL volume of a mixture of plasmid pBR322 consisting of 0.47 μg [^{14}C]-labeled supercoiled plasmid (form I DNA) and 0.03 μg [^{3}H]-labeled nicked closed-circular plasmid (form II DNA) was layered on a 170 μL 5−20% sucrose gradient (10 mM HEPES, 1 mM EDTA, 10% DMF, pH 7) containing various concentrations of drug. The sample was subjected to centrifugation at 248 000 × g for 1.5 h at 4 °C in a Beckman TLS-55 rotor. Fractions (8.5 μL) were collected from the top of the tubes, and radioactivity quantitated by scintillation counting. The peak radioactive fraction for each isotope was used to calculate the sedimentation of the form I DNA relative to the form II DNA. As discussed below, ESPs A1 and C were found to be soluble and chemically stable for up to 10 h in the sucrose buffer, and there was no detectable nicking of the plasmid DNA (data not shown).

Viscometry. Viscosity studies were performed with sonicated, S1 nuclease-treated calf thymus DNA (~400 bp average size prepared by Sepharose 6B chromatography) in an Ostwald capillary viscometer mounted in a temperature-controlled water bath (25 °C) in the dark. The flow times (t) of solvent (10 mM HEPES/1 mM EDTA, pH 6.5, 10% DMF) without (t_0) and with DNA (t; 500 μg/ml) were used to calculate the specific viscosity, $\eta = (t - t_0)/t_0$, of the DNA solution without (η_0) or with (η) drug. ESPs A1 and C were added as stock solutions in DMF to give a range of final concentrations up to 77 and 54 μM, respectively; DMF was adjusted to maintain a constant 15% concentration.

Quantitation of ESPA1-Induced DS and SS Lesions in Plasmid DNA. The relative quantities of DS and SS DNA lesions produced by ESP A1 and ESP C were determined by a plasmid topoisomer technique described previously.[8,14] Reaction conditions were as follows: 30 μg/mL pBR322, 10 mM GSH, 50 mM HEPES, 1 mM EDTA, and pH 7, in a volume of 25 or 50 μL. The reaction was initiated by adding 2 μL of drug in methanol and allowed to proceed for 10 min at 37 °C or 1.5 h at 0 °C in the dark; there were no significant differences noted for the two reaction temperatures (data not shown). The reaction mixture was divided into two portions: one kept as control, and one treated with putrescine (100 mM, 1 h, 37 °C) to cleave abasic sites to phosphate-ended fragments.[15,16] Plasmid DNA forms resolved on agarose gels were quantitated by fluorescence photography and densitometry.[8,14]

Sequencing Gel Analysis of ESP-Induced DNA Damage Products. The chemistry of ESP-mediated DNA damage was tentatively identified by sequencing gel analysis.[8,10] The 221 bp *Eco*RI/*Pvu*II fragment of pAP1-3, 5′-[^{32}P] end-labeled at the *Eco*RI site, was treated with either ESP A1 (0.2 μM) or ESP C (1 μM) in a solution containing 50 mM HEPES, 1 mM EDTA, 10 mM GSH, 30 μg/mL calf thymus DNA, pH 7, for 1 h at 0 °C in the dark. Each reaction was divided into three portions, two of which were treated with hydrazine (100 mM, pH 8, room temperature, 1 h) or putrescine as described above. Following ethanol precipitation, equal quantities of radioactivity for each sample were loaded on a 20% sequencing gel, and the fragments visualized by autoradiography.

Tritium Transfer Experiments. Plasmid pAP1-3[14] was labeled with [1′,2′,5-^{3}H]-dCTP or -dTTP and with [5′,5-^{3}H]-dCTP or -dTTP (Amersham and Dupont NEN) by nick translation.[17] Per manufacturers' technical specifications, the radiochemical purity of all labeled nucleotides was >97%, with site specificity of [^{3}H] labels verified by [^{3}H]-NMR. The labeled DNA (18 μM bp) was treated with ESP A1 (0.2 μM) or ESP C (2 μM) under the following conditions: 50 mM HEPES, 10 mM glutathione, 1 mM EDTA, pH 7, 0 °C, 2 h, 50 μL volume. The aromatized drug products present in dried dichloromethane extracts were dissolved in acetonitrile containing 2 μg of aromatized drug standard and resolved by reversed-phase thin-layer chromatography (RP-TLC; C18; J. T. Baker) with acetonitrile or by silica gel TLC (J. T. Baker) with 10% methanol in chloroform. The standard was located under UV light, and radioactivity in scrapings from 5 mm segments along each lane was quantitated by scintillation counting.

Results

Esperamicin-DNA Binding Isotherm. To quantify the interactions of ESP A1 and ESP C with DNA, binding constants with λ DNA (48 502 bp) were estimated. A Scatchard plot of

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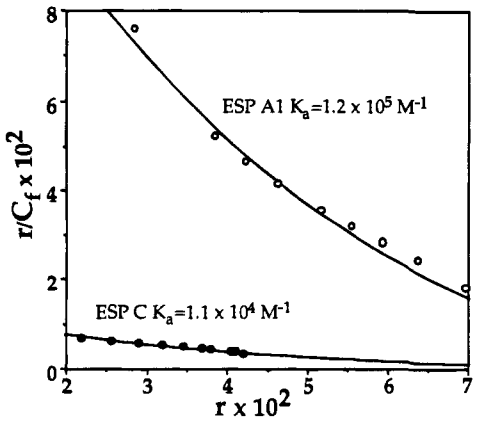

Figure 2. DNA binding isotherms for ESPs A1 (○) and C (●). Concentrations of drug bound per bp of λ DNA (r) and free in solution (C_f) were used to prepare a Scatchard plot. The curves represent the best fit of the data to the site exclusion model of McGhee and von Hippel.[13] The indicated association constants have standard errors of 0.1 M^{-1}, and the calculated values for the size of the binding site (n) and cooperativity parameter (ω) are ~8 and ~0.5, respectively.[13]

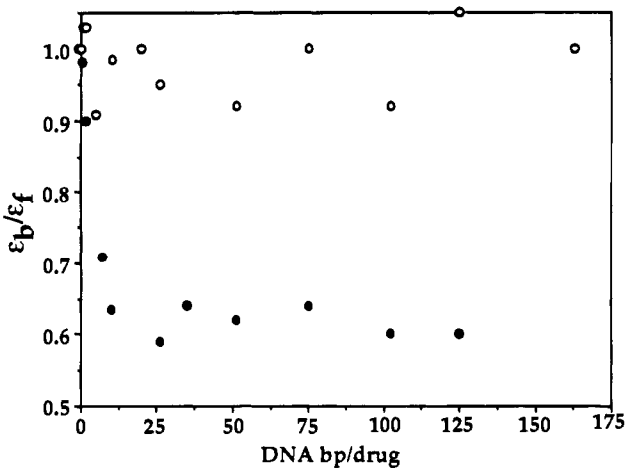

Figure 3. DNA-induced quenching of the 320 nm absorbance of ESP A1. The ratio of the 320 nm absorbance of DNA-bound (ϵ_b) and free (ϵ_f) ESP A1 (●) and ESP C (○) is plotted relative to the ratio of DNA bp to the total concentration of drug. The data are single or duplicate values derived from 3–4 experiments.

the binding data is shown in Figure 2. Binding constants were calculated from the data by applying the excluded site binding model of McGhee and von Hippel.[13] The affinity of ESP A1 for DNA, K_a = 1.2 (±0.1) × 10^5 M^{-1}, was found to be ~10-fold higher than that of ESP C, K_a = 1.1 (±0.1) × 10^4 M^{-1}. This observation is in accord with drug concentrations found to produce similar levels of DNA damage (*vide infra*) and with the binding constants for NCS and CAL in large populations of binding sites.[18,19] The deoxyfucose-anthranilate of ESP A1 thus contributes 1.4 kcal/mol, or about 20%, to the free energy of binding to DNA under the conditions of these experiments (ΔG = 6.3 kcal/mol).

The major impediment to this approach is the limited solubility of the enediynes in aqueous solution. However, the ESPs and CAL at 2–10 μM were found to be soluble in 15% DMF, as judged by the loss of <5% of the drug following a 20 min centrifugation at 217 000 × g; this small loss has been taken into account in the treatment of the binding data. Drug aggregates, if present, should have been significantly smaller than the <0.5 μm particles soluble at 16 000 × g in 5% dimethyl sulfoxide.[20] For our purposes, underestimation of the quantity of DNA-bound drug resulting from an equilibrium between aggregate, free drug, and DNA/drug complex would ensure that the apparent properties of the drug/DNA complex are attributable to bound drug. Given the above, the binding constants must be regarded as estimates for purposes of comparison. Additionally, the drugs were found to be chemically stable under these conditions, as judged by DNA damage assays, TLC, and UV spectroscopy, and there was no detectable degradation of the λ DNA (data not shown).

DNA-Induced Quenching of the UV Absorption of the Anthranilate of ESP A1. In many cases, intercalation alters the spectral properties of a chromophore.[21] With ESP A1, the deoxyfucose-anthranilate contributes ~60–70% to the 320 nm absorbance (ϵ_{320} ~ 16 000 cm^{-1} M^{-1} in 15% DMF), while the core and trisaccharide account for the remaining ~30–40% (ϵ_{320} ~ 5000 cm^{-1} M^{-1} in 15% DMF) (data not shown). As shown in Figure 3, there is a reduction of the 320 nm extinction of ESP A1 as the proportion of DNA-bound drug increases, until a limit is reached at ~10 000 cm^{-1} M^{-1} (ϵ_b/ϵ_f ~ 0.6). This amounts to a 40% reduction in absorbance, which is in accord with other known intercalators.[21] The absorbance of ESP C, however, remains essentially unchanged, suggesting a different electronic environment. These observations are consistent with the known minor groove location of the enediyne core[22] and with intercalation of the anthranilate of ESP A1.

Esperamicin A1 Unwinds Supercoiled DNA. Intercalating agents characteristically unwind the DNA helix upon binding.[23,24] The ability of ESP A1 to alter the helical state of DNA was assessed by the differential sedimentation of negatively-supercoiled plasmid DNA relative to nicked-open circular DNA in the presence of the drugs;[23] the latter DNA form is not significantly affected by intercalation. As shown in Figure 4, increasing concentrations of ESP A1 alter the plasmid sedimentation in a manner consistent with progressive relaxation of negative superhelical tension followed by induction of positive superhelices. However, similar levels of bound ESP C did not alter the sedimentation of the supercoiled plasmid DNA. The behavior of ESP A1 is similar to the unwinding produced by intercalation of the naphthoate of NCS.[19,25]

Esperamicin A1 Increases the Viscosity of DNA Solutions. Another hallmark of intercalation is lengthening of a DNA molecule, which can be detected by an increase in the viscosity of a DNA solution.[24] A plot of the relative viscosity (η/η_0) of a solution of linear DNA fragments as a function of *bound* drug is shown in Figure 5. The results demonstrate that ESP A1, but not ESP C, is capable of increasing the viscosity of the DNA solution. The increase in viscosity produced by ESP A1 was larger than that occurring with ethidium bromide (EtBr), possibly due to the presence of DMF (data not shown). Drugs alone did not alter solvent viscosity (data not shown), and the

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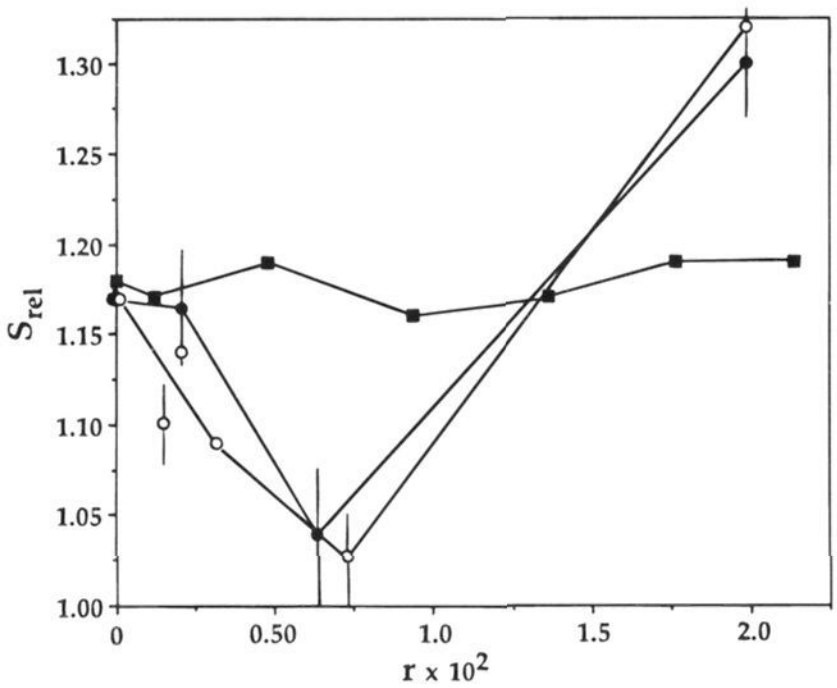

Figure 4. Unwinding of negatively-supercoiled DNA by ESP A1. The sedimentation of negatively-supercoiled plasmid DNA relative to nicked closed-circular DNA (S_{rel}) was determined in the presence of ESP A1 (●; 1−10 μM), ESP C (■; 1−20 μM), and NCS (○; 0.7−10 μM). The ratios of bound drug to DNA bp, r, were calculated from the binding isotherms assuming an initial DNA concentration of 385 μM. The r values are thus a conservative estimate of the quantity of bound drug. Neocarzinostatin serves as a positive control for intercalation.[25] The data points are derived from two experiments; error bars represent variation about the mean for duplicate values.

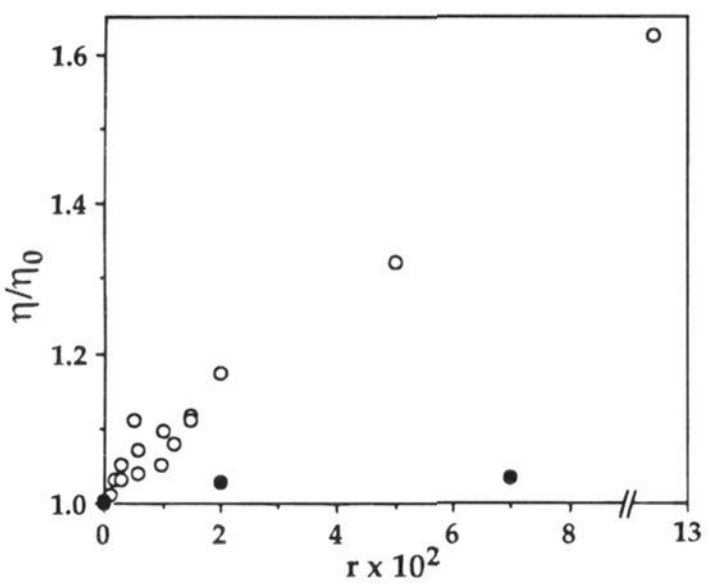

Figure 5. ESP A1 increases the viscosity of a DNA solution. ESP A1 (○) or ESP C (●) was added to solutions of calf thymus DNA (~400 bp average size fragments; 10% DMF), and the relative viscosities (η/η_0) were determined as described in Materials and Methods. The value r represents moles of bound drug per DNA bp; the high concentration of DNA (500 μg/mL) assures that virtually all the drug molecules are bound. Each data point represents an average of 3−5 flow time determinations.

high concentration of DNA (500 μg/mL) ensures that virtually all the ESP A1 and >90% of the ESP C is bound to DNA. It is unlikely that drug-induced changes in viscosity are due to stiffening of the DNA, since the short DNA fragments (~400 bp average size) behave essentially as stiff rods in solution.[26] Neither drug produced significant DNA damage under these conditions (data not shown), thus ruling out DNA fragmentation as a cause of the results obtained with ESP C.

Esperamicin Is Capable of Producing Bistranded DNA Lesions. It was concluded from previous studies that ESP A1 produces few, if any, DS DNA lesions.[6,9,10,27] However, these studies did not account for bistranded lesions consisting of an abasic site opposite a direct stand break. Since these lesions

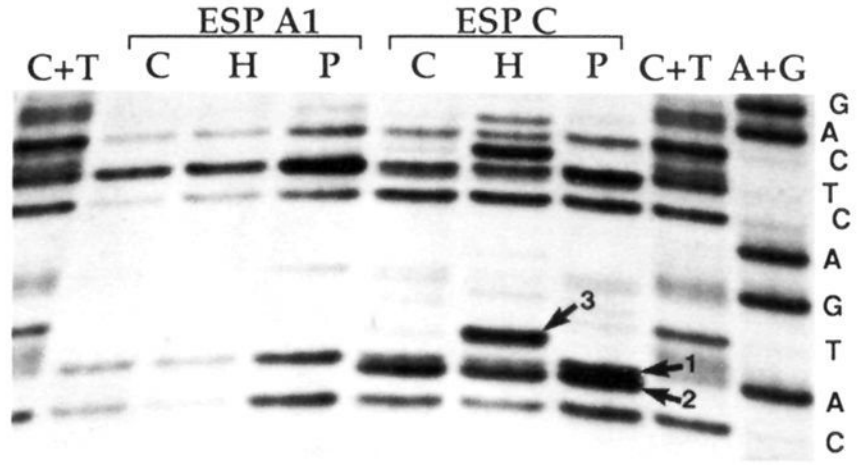

Figure 6. Sequencing gel analysis of DNA damage produced by ESPs A1 and C. The 5′-[^{32}P]-labeled *Hind*III/*Pvu*II fragment of pAP1−3 was treated with ESP A1 (0.2 μM) or ESP C (1 μM) in the presence of 10 mM GSH. One portion of the reaction mixture was kept as control (**C**), while two others were treated with hydrazine (**H**) or putrescine (**P**). The DNA was then resolved on a 20% sequencing gel. The numbered bands denote structures attached to the 3′-ends of the DNA fragments tentatively identified as follows: ***1***, 3′-phosphate; ***2***, 3′-phosphoglycolate; and ***3***, 3′-phosphopyridazine derivative of the 4′-hydroxylated abasic site. G, A + G, and C + T represent Maxam-Gilbert phosphate-ended chemical sequencing fragments;[47] the sequence is noted in the right margin.

represent a large portion of the damage associated with by CAL and NCS,[8,14,28] we undertook plasmid topoisomer studies to define the quantities of DS and SS lesions produced by ESP A1 and ESP C.[8,14] While treatment of plasmid DNA with ESP A1 resulted in a ratio of SS to DS lesions of 24 ± 5 (mean ± SD), exposure of drug-damaged DNA to putrescine increased the proportion of DS lesions from ~4% to ~23% of total damage (SS:DS ratio 3.4 ± 0.3). Since putrescine cleaves abasic sites to phosphate-ended DNA fragments,[14,28] we conclude that the majority (~80%) of ESP A1 DS lesions consist of a putative abasic site opposite a direct strand break. With ESP C, putrescine treatment shifted the ratio of SS to DS lesions from 0.68 ± 0.08 to 0. Thus, like CAL,[8] DNA damage produced by ESP C consists entirely of DS lesions, a majority of which involved abasic sites opposite strand breaks.

Two observations support the conclusion that ESP A1-mediated DS lesions arise by the action of a single drug molecule, rather than closely opposed, independent SS breaks formed by different drug molecules. First, the ratio of DS to SS lesions of ~1:3 persists even at the detection limit of the assay, which is 1 drug molecule for every 10 plasmid molecules (a total of 43 620 bp). More importantly, there is a ~1:1 stoichiometry between ESP A1 molecules present in the reaction and the number of damaged plasmid molecules (data not shown). It is thus unlikely that two drug molecules are attracted to the same or nearby sites in the plasmid DNA.

Sequencing Gel Analysis of Esperamicin-Mediated DNA Damage. The unique shifts in sequencing gel mobility of drug-damaged DNA fragments were employed to characterize the chemistry of ESP-mediated DNA damage.[8,29] Three products were identified for ESP C (Figure 6). One fragment comigrates with the Maxam-Gilbert chemical sequencing products, consistent with the presence of a phosphate on the 3′-end (band no. 1). This product could arise by 5′-chemistry or by adventitious degradation of an abasic site. Band no. 2 in Figure 6 identifies a fragment migrating slightly faster than the 3′-phosphate-ended fragment. This is consistent with the presence of a 3′-phosphoglycolate residue, one of the two products of 4′-hydrogen abstraction observed and characterized previously

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with ESP C, NCS, CAL, and BLM.[10,29-31] Finally, treatment of ESP C-damaged DNA with hydrazine results in a DNA fragment migrating more slowly than the phosphate-ended fragments (band no. 3). Precedent with BLM[32] suggests the presence of a 3′-phosphopyridazine residue, which arises from the reaction of hydrazine with the other product of 4′-chemistry, the 4′-hydroxylated abasic site.[32] Also consistent with the presence of an abasic site, putrescine treatment causes an increase in 3′-phosphate-ended fragments (band no. 1). These three observations confirm the previously noted 4′-chemistry mediated by ESP C.[10]

The spectrum of damage products with ESP A1 is notable for the absence of two products: 3′-phosphoglycolate-ended fragments and the putative phosphopyridazine derivative (Figure 6). These observations rule out significant levels of 4′-hydrogen abstraction by ESP A1 at sites shown in Figure 6 or at *any* damage site on either strand of the ~60 bp *Eco*RI/*Bam*HI region of pAP1-3 (data not shown). Treatment of ESP A1-damaged DNA with putrescine, however, resulted in an increase in the quantity of 3′-phosphate-ended fragments, consistent with the presence of an abasic site. Such a lesion is reminiscent of the 2-deoxyribonolactone-containing abasic site produced by NCS abstraction of the 1′-hydrogen atom of deoxyribose.[33,34]

Our observation of ESP A1-induced abasic sites by three different techniques is inconsistent with the apparent absence of alkali-labile lesions reported by Christner *et al.*[10] Their conclusion was based on sequencing gel analysis of drug-damaged DNA, in which piperidine treatment was not observed to increase the quantity of 3′-phosphate-ended DNA fragments.[10] It is unlikely that this discrepancy is due to sequence context, given the large number of cleavage sites examined in both studies. Furthermore, except for the identity of the activating thiol (glutathione *vs* dithiothreitol), the reaction conditions were similar in both studies. However, two factors support the conclusion that the identity of the thiol will not effect the specificity of hydrogen atom abstraction by ESP A1: (1) precedent with CAL[3,4,8,31,35] and (2) the fact that the chemistry of ESP C-induced DNA damage was identical in the present studies and those of Christner *et al.*[10] One possible explanation for the failure to observe a piperidine-mediated increase in ESP A1-induced 3′-phosphate-ended DNA fragments is lane-to-lane variation in the quantity of radiolabeled DNA loaded on the sequencing gels, as suggested by the reduced intensities of virtually all bands in the piperidine-treated DNA samples compared to control samples in Figure 2 of Christner *et al.*[10] We were very careful to control for the quantity of radioactive material loaded in each gel lane of Figure 6.

Abstraction of 1′-Tritium Labels by ESP A1. The ability of ESP A1 and ESP C to perform chemistry at the 5′- and 1′-positions of deoxyribose was assessed by examining aromatized forms of the drugs for the presence of tritium following reactions of the drugs with tritium-labeled DNA. Given the precedent for 5′-hydrogen abstraction by ESPs A1 and C,[10] it was not surprising that a [5,5′-^{3}H]-cytidine-labeled DNA substrate resulted in comigration of tritiated material with both aromatized drug standards (Figure 7). However, only ESP A1 incorporated [^{3}H] during a reaction with DNA labeled with [5,1′,2′-^{3}H]-

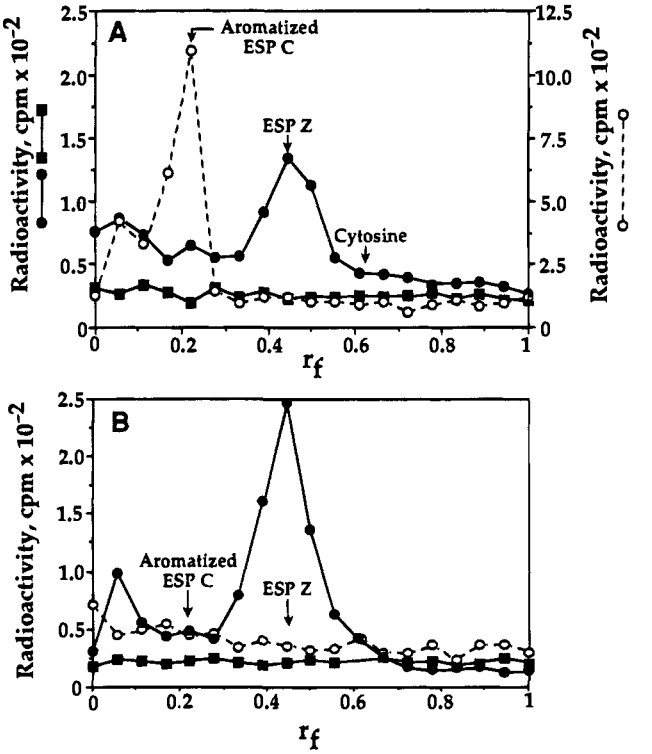

Figure 7. Abstraction of tritium from [^{3}H]-cytidine-labeled DNA by ESPs A1 and C. (*A*) DNA labeled with either [5′,5−^{3}H]-dCTP was treated with ESP A1 (●), ESP C (○), or methanol vehicle (■), and the extracted drug remnants resolved by RP-TLC. Radioactivity in 5 mm segments along the TLC lane was measured by scintillation counting. R_f represents the migration relative to the solvent front. The positions of aromatized ESP A1 (ESP Z), aromatized ESP C, and free cytosine are noted. (*B*) Conditions identical to those in panel A, except the DNA substrate was labeled with [1′,2′,5-^{3}H]-dCTP.

cytidine (Figure 7). ESP A1 was also observed to incorporate tritium in a reaction with [5,1′,2′-^{3}H]-thymidine-labeled DNA, but ESP C was not examined with this substrate (data not shown).

These results suggest that ESP A1, but not ESP C, is capable of performing 1′-chemistry at cytidine. Abstraction of [^{3}H] from either the base or 2′ position of the sugar is highly unlikely, given the minor groove specificity of the ESPs[22] and the enediynes in general. We also observed free [^{3}H]-cytosine base by HPLC analysis of the dichloromethane-extracted reaction mixture (data not shown), which would again be consistent with abasic sites formed by 1′-chemistry. However, 4′-hydrogen abstraction could also contribute to free base in the reaction mixture.

The radioactivity comigrating with the aromatized drug standards could not be attributed to released cytosine base, base propenal, nucleoside 5′-aldehyde degradation products, or other deoxyribose fragments, given their insolubility in dichloromethane (data not shown) and the fact that radioactive products were not observed in the reaction of ESP C with [5,1′,2′-^{3}H]-labeled DNA (Figure 7). Furthermore, the radioactive product(s) comigrated with ESP Z standard in two TLC systems (RP- and silica gel TLC; data not shown).

The presence of intact ESP Z in our experiments (Figure 7) is inconsistent with the observations of Langley *et al.*,[27] who found release of the trisaccharide of ESP A1 following a 3 h reaction with DNA at 37 °C. They proposed that a hydrogen transfer from the C8 position to the C6 radical center resulted in homolytic cleavage C8−O glycosidic bond. However, we have observed progressive loss of ESP Z during such prolonged incubations at 20−37 °C, accompanied by the formation of a species migrating with an R_f of ~0.1 in our TLC system (data

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not shown). This behavior is consistent with an aromatized form of the drug missing part or all of the trisaccharide. It is thus possible that the majority of the trisaccharide cleavage observed by Langley *et al.* resulted from degradation of ESP Z rather than an intramolecular hydrogen transfer.

Discussion

We have described three important features of the ESP A1/DNA interaction: an intercalative binding mode, a significant quantity of bistranded DNA lesions, and an anthranilate-induced switch in the chemistry of DNA damage. These observations suggest that the anthranilate contributes significantly to the biochemistry of ESP A1-induced DNA damage.

Intercalation of the anthranilate is supported by three observations: unwinding of supercoiled DNA, lengthening of the DNA helix, and DNA-induced quenching of the UV absorbance of the anthranilate. Alone, the individual observations are insufficient to establish intercalation, since there are exceptions to each. To our knowledge, however, there is no precedent for all three effects occurring with groove-binding small molecules such as netropsin and irehdiamine[36,37] and cross-linking agents such as cisplatin.[38] Intercalation of the anthranilate is also supported by the observation of nucleosome linker-selective DNA damage produced by ESP A1 in nuclei,[11] presumably due to the constrained dynamics of DNA wrapped around the histone core of the nucleosome.[11]

The structure and physical properties of the anthranilate of ESP A1 make it unusual among intercalating agents. The isolated deoxyfucose-anthranilate group does not interact with DNA (data not shown), yet it contributes 1−2 kcal/mol to the DNA binding energetics of ESP A1, similar to the situation with the naphthoate of NCS.[38] Furthermore, intercalating agents usually consist of polycyclic aromatic systems with either fused or closely tethered ring systems.[39] However, the *N*-(2-methoxyacrylyl)anthranilate group should be capable of adopting a planar structure capable of insertion between base pairs. Indeed, the benzoxazolinate groups present in the enediyne C1027[40−42] and in the chromophore of the antitumor antibiotic, auromomycin,[43] represent planar analogues of the anthranilate of ESP A1, in which the terminal oxygen of the methoxyacrylyl group is bonded to the aromatic ring. Based on our observations with ESP A1, we expect the benzoxazolinate to cause C-1027 and auromomycin to bind to DNA by intercalation.

We have also described two other important features of ESP A1-mediated DNA damage: DS lesions and 1′-chemistry. Previous models for the biochemical role of the anthranilate were based on two conclusions: ESP A1 makes only SS lesions involving 5′-chemistry[9,10] and ESP C produces DS lesions involving both 4′- and 5′-chemistry.[10] Christner *et al.* proposed that the anthranilate inhibited the activity of the C6 radical center, thought to mediate 4′-hydrogen abstraction.[10] However, as we have demonstrated, 20−25% of the damage produced by ESP A1 consists of DS DNA lesions consisting of an abasic site opposite a strand break. Based on this observation, the absence of 4′-chemistry, and the evidence for an abasic site caused by 1′-chemistry, we propose that the anthranilate causes a switch in the deoxyribose hydrogen abstraction from 4′ to 1′ in ESP A1-induced bistranded DNA lesions.

The similarity between ESP A1 and NCS is noteworthy in several respects. Both compounds produce bistranded lesions with 1′-hydrogen abstraction on one strand and 5′-chemistry on the other, with SS lesions outnumbering DS lesions 2−3:1.[15,44,45] While the observation of ESP A1-induced DS lesions rules out complete inhibition of one of the radical centers,[10] partial quenching may explain the observed 3:1 ratio of SS and DS lesions. Again, this is similar to the situation with NCS, in which hydrogen transfer to one of the radical centers from the thiol adduct has been implicated in the predominance of SS over DS lesions.[44−46]

The observed intercalation-like behavior of the anthranilate of ESP A1 confirms our earlier hypothesis based on reactions of ESPs A1 and C with chromatin.[11] Our results also strengthen the validity of one of the three molecular models developed by Langley *et al.* for the ESP A1/DNA complex.[27] The one model favored in these studies, which placed the anthranilate superficially in the major groove, appears incompatible with our observation of a DNA-induced reduction in the 320 nm absorbance attributable to the anthranilate, and the observation of Sugiura *et al.* that a protein lying in the major groove did not interfere with ESP A1-mediated DNA damage in the opposing minor groove.[22] Additionally, these models were based on experimental data available at the time and were thus constructed to account only for SS damage.[27] The observed DS lesions invite further refinement of the intercalative model.

Acknowledgment. The authors are grateful to Koli Taghizadeh (Center for Environmental Health Sciences, MIT) and Pete Wishnok and Sarah Stillwell (Division of Toxicology, MIT) for mass spectrometric analyses. Special thanks are extended to Prof. Leonard Lerman for his advice and encouragement as well as the loan of the capillary viscometer and to Profs. Lerman, John Essigmann, and Joanne Stubbe for critical review of the manuscript. This work was supported in part by NIH Grants CA57633 (P.C.D.), ES02190 (CEHS, MIT), an MIT Sloan Basic Grant (P.C.D.), and the Samuel A. Goldblith Career Development Professorship (P.C.D.). Our thanks to the Research Science Institute for summer internship support (R.H.).

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