Cytosine Methylation Enhances DNA Damage Produced by Groove Binding and Intercalating Enediynes: Studies with Esperamicins A1 and C[†]

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Received June 25, 1997; Revised Manuscript Received September 9, 1997[⊗]

ABSTRACT: Methylation of the C5 position of cytosine in CG dinucleotides represents an important element in the control of gene expression in eukaryotic cells. This major groove modification of DNA causes changes in DNA conformation that are recognized by DNA-binding proteins and DNA-damaging chemicals. We have observed that CG methylation affects the DNA damage produced by the enediyne esperamicin A1 and its analog lacking the intercalating anthranilate, esperamicin C. Fragments of the human phosphoglycerate kinase gene (PGK1) and the plasmid pUC19 were methylated with *Sss*I methylase and subjected to damage by esperamicins A1 and C. Damage produced by esperamicin A1 was enhanced 1.5−2-fold near a single CG sequence at position −101 in PGK1 and in a region containing several methylated CG dinucleotides between positions −120 and −131. Esperamicin C-induced damage was enhanced to a similar degree in PGK1 but only at the site that contained multiple CG dinucleotides. There was enhancement of damage for both drugs in the pUC19 fragment at several sites near CG sequences. Analysis of the chemistry of esperamicin-induced DNA damage suggests that cytosine methylation does not affect the identity of drug-abstracted hydrogen atoms. The damage chemistry was also used to identify the DNA binding orientation of the esperamicins. The anthranilate of esperamicin A1 is predicted to intercalate in a CT step four base pairs in a 3′-direction to the CG sequence at −101 in PGK1 and in a CG dinucleotide at the site containing multiple CGs (positions −120 to −131). These observations are consistent with other studies that indicate a long range effect of cytosine methylation on DNA conformation.

Modification of mammalian DNA by methylation at the C5 position of cytosine plays a critical role in both the regulation of gene expression (*1*−*3*) and in cancer (*4*). Given the known effects of cytosine methylation on DNA structure and dynamics, we have examined its effect on DNA damage produced by two structurally related enediyne antitumor antibiotics, esperamicins A1 and C (*5*−*7*).

Cytosine methylation occurs in the underrepresented CG dinucleotides in eukaryotic cells. While 1−2% of the genome consists of clusters of nonmethylated CG sequences, mainly in the 5′-regions of certain genes, approximately 70% of CG dinucleotides are methylated (*2*). The general observation is that transcriptionally active regions are undermethylated in comparison to their inactive counterparts in other cells. There are exceptions to the association of methylation with transcriptional silencing since methylcytosine also occurs in active genes such as p53 (*8*).

Although the mechanism by which cytosine methylation causes changes in gene expression is unclear, it is believed to involve differential recognition of methylated DNA sequences by proteins (*9*) due to changes in DNA conformation or by a direct methyl group−protein interaction. With regard to the former hypothesis, cytosine methylation causes changes in DNA structure and dynamics that include helical unwinding (*10*), increased base stacking and helical stability (*11*), reduction in major groove charge density near the methyl group (*12*), and, in certain sequence contexts, modulation of DNA bending (*13*−*15*). These effects may account for the propensity of methylated CG repeats to induce the formation of Z-DNA (*16*).

The changes in DNA structure and dynamics associated with cytosine methylation also affect the interaction of small molecules with DNA. Both benzo[*a*]pyrenediol epoxide (*17*) and mitomycin C (*18*) show enhanced reactivity with methylated sequences, while damage produced by bleomycin (*19*) and *N*-methyl-*N*-nitrosourea (*20*) is inhibited by cytosine methylation. However, the molecular basis for this altered reactivity remains unclear.

To better understand the role of cytosine methylation in the selection of DNA targets by small molecules, we have undertaken studies with two enediynes, esperamicins A1 and C. Enediynes are extremely potent cytotoxins that produce high levels of double-strand DNA damage by forming a benzenoid diradical intermediate that binds in the minor groove and abstracts hydrogen atoms from the deoxyribose backbone (*21*−*23*). While they share a common mechanism for damaging DNA, enediynes differ in the structure and arrangement of functional groups attached to the enediyne core. For example, esperamicin A1 binds in the minor groove of DNA with intercalation of its anthranilate moiety (Figure 1) (*24*, *25*), while removal of the sugar−anthranilate to produce esperamicin C results in a shift in the chemistry of the DNA damage (*25*, *26*). Despite this structural difference, esperamicins A1 and C have similar sequence selectivities (*5*, *27*). These two analogs thus provide an opportunity to investigate the relationship between cytosine methylation and DNA intercalation.

[†] Supported by NIH/NCI Grants CA64524 and CA72936 (P.C.D.), a Whitaker Fellowship (J.X.), and the Samuel A. Goldblith Professorship (P.C.D.).

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[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1997.

S0006-2960(97)01533-X CCC: $14.00

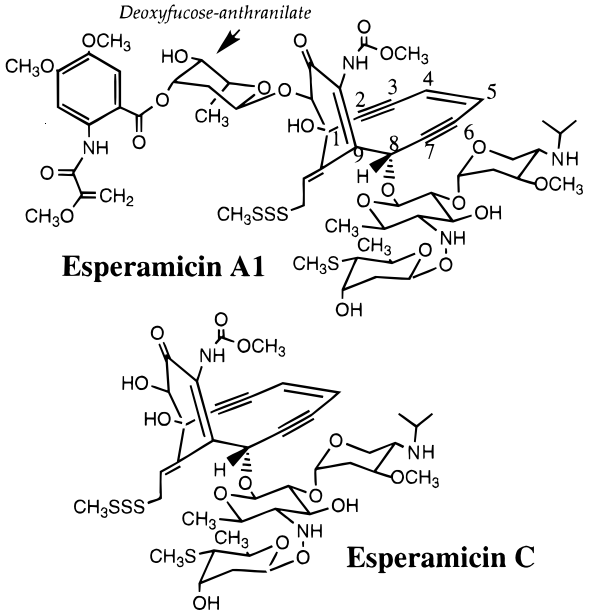

FIGURE 1: Structures of esperamicin A1 and esperamicin C.

We report here that cytosine methylation in CG sequences increases the reactivity of esperamicin A1. However, it was also observed that damage produced by esperamicin C, the nonintercalating analog of esperamicin A1, was enhanced by DNA methylation at some but not all sites noted with esperamicin A1. The results with the esperamicins illustrate the complexity of methylation-induced alterations of DNA conformation and suggest that the altered conformation extends beyond the CG sequence.

EXPERIMENTAL PROCEDURES

Materials. Esperamicin A1 was generously provided by Dr. J. Golik (Bristol-Myers Squibb). Esperamicin C was prepared by acid-catalyzed methanolysis as described elsewhere (*25*). A plasmid (pBSHPGK1) containing a fragment of the upstream region of the human phosphoglycerate kinase gene (PGK1) was provided by Dr. J. Singer-Sam (City of Hope) (*28*). *Sss*I methylase, plasmid pUC19, T4 polynucleotide kinase, and restriction enzymes were obtained from New England Biolabs.

DNA Methylation. Plasmids pBSHPGK1 and pUC19 were digested with *Spe*I and *Sal*I, respectively, and both were 5′-^{32}P end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase or 3′-^{32}P end-labeled by a Klenow fill-in reaction with [α-^{32}P]ddATP (*29*). Following digestion with *Eag*I (pBSHPGK1) or *Pvu*II (pUC19), the 105 or 121 bp labeled fragments, respectively, were purified from a 10% polyacrylamide gel (*29*). A portion of the labeled DNA was subjected to methylation by *Sss*I methylase (6 units) in a 30 μL solution of labeled DNA ($\sim 5 \times 10^5$ cpm), 160 μM *S*-adenosylmethionine, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (pH 7.9). Following a 1 h reaction at 37 °C, the enzyme was inactivated at 65 °C and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. Control reactions were performed with heat-inactivated *Sss*I methylase. The methylation status was confirmed by *Bst*U1 digestion.

Esperamicin Reactions. DNA damage reactions were initiated by adding 2 μL of an esperamicin stock solution in methanol to 98 μL of a solution of ^{32}P-labeled DNA containing 100 μg/mL sonicated calf thymus DNA, 10 mM glutathione, 50 mM HEPES, and 1 mM EDTA (pH 7). The reaction was allowed to proceed for 30 min on ice. DNA fragments containing 5′-end labels were treated with either 100 mM putrescine dihydrochloride for 30 min at 37 °C or 100 mM hydrazine (pH 7) for 30 min at ambient temperature; untreated controls were kept on ice. DNA was finally purified by ethanol precipitation and resolved on 8 or 20% sequencing gels. The dried gels were subjected to phosphorimager analysis (Molecular Dynamics).

RESULTS

Effect of Cytosine Methylation on the Location and Frequency of DNA Damage. The effect of cytosine methylation on esperamicin-induced DNA damage was studied in a cloned segment of the human PGK1 gene (*28*) and in a pUC19 fragment. Cytosines in CG sequences were uniformly methylated using *Sss*I methylase (*30*). As shown in the sequencing gels in Figure 2B and the bar graph in Figure 3A, several esperamicin A1-induced DNA lesions in the PGK1 gene are enhanced in the methylated DNA. There is a reproducible 1.5−2-fold increase in damage produced by esperamicin A1 at several sites that consisted of either CG repeats or, in one case, a single CG sequence. A similar enhancement was also observed in the *Sal*I/*Pvu*II fragment of pUC19, as shown in the bar graph in Figure 3C.

Also apparent in the PGK1 fragment is the expected three-nucleotide 3′-stagger to the damage sites on opposite strands (Figure 3A), which reflects positioning of the diradical intermediate in the minor groove of DNA (*6*, *23*). However, at several locations, including site b in Figure 3A, the damage on one strand is significantly lower in frequency than that on the opposite strand and is not affected by methylation. This suggests that esperamicin A1 produces mainly single-strand lesions on the bottom strand at these sites.

To define the features of esperamicin A1 structure responsible for the increased DNA damage in methylated DNA, we repeated the studies with esperamicin C, an analog of esperamicin A1 missing the deoxyfucose−anthranilate moiety known to be a DNA intercalator (*25*). DNA damage produced by esperamicin C, which has a sequence selectivity similar to that of esperamicin A1 (*25*), was also found to be enhanced by DNA methylation as shown in Figures 2A and 3B,D. While there was no methylation-induced enhancement of esperamicin C-induced DNA damage at the single CG sequence (site d) in Figure 3B, there was a small enhancement near an isolated CG in the pUC19 fragment (Figure 3D). Furthermore, unlike esperamicin A1, esperamicin C did not produce DNA damage in the PGK1 DNA in which one strand sustained significantly more lesions than the other. This is consistent with the exclusive production of bistranded DNA lesions by esperamicin C (*25*).

Orientation of Esperamicins A1 and C at Methylated Damage Sites. The putative binding orientation of esperamicins A1 and C at each damage site can be derived from the chemistry of the damage on each strand. Esperamicin A1 has been shown to abstract a 1′-hydrogen atom from deoxyribose on one strand and a 5′-hydrogen atom from the other strand, while esperamicin C abstracts 4′- and 5′-hydrogen atoms (*25*, *26*).

The damage chemistry can be identified by the unique shifts in the mobility of drug-induced DNA fragments

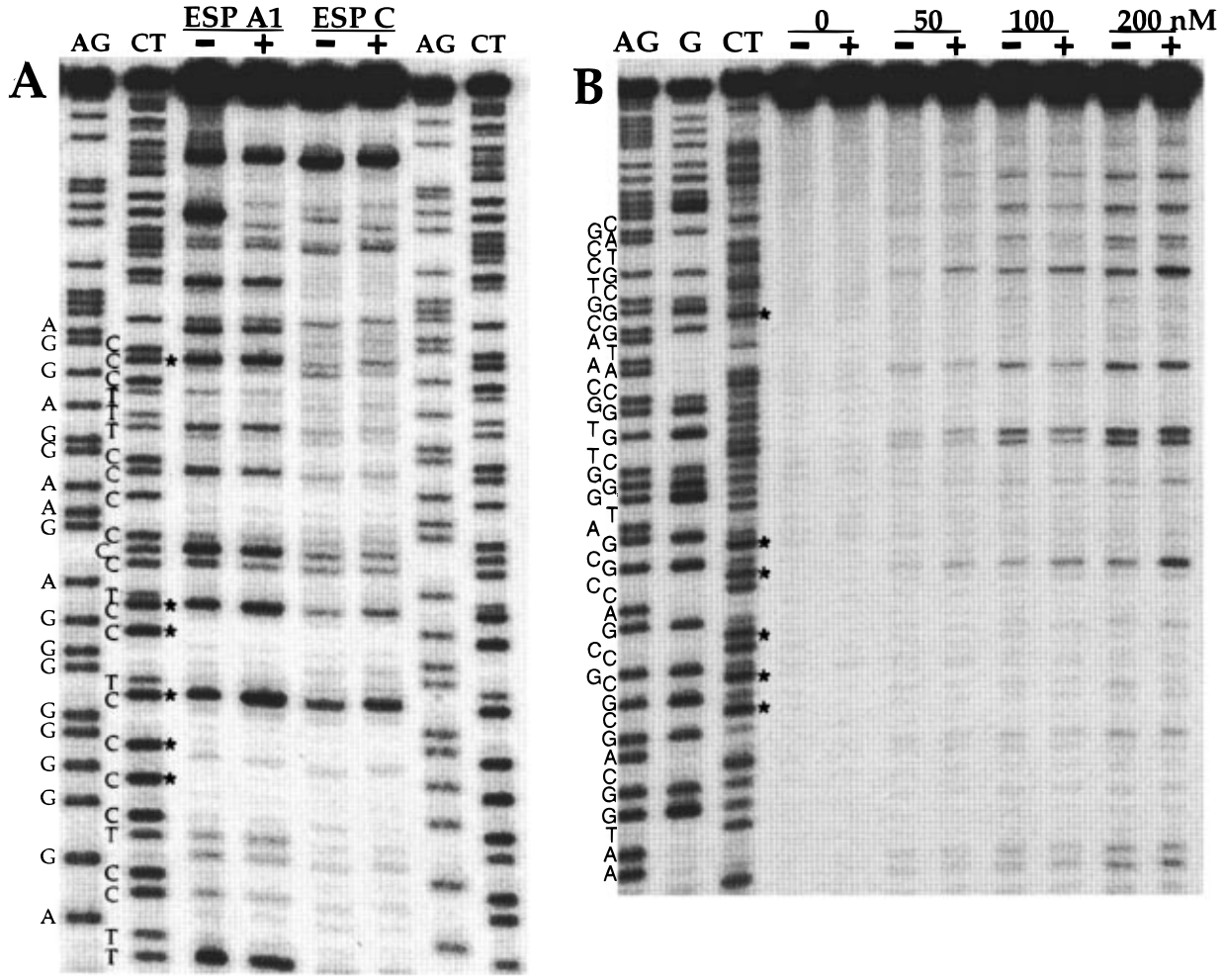

FIGURE 2: Damage produced by esperamicins A1 and C in a cloned fragment of the PGK1 gene containing methylated or unmethylated CG sequences. A (A) 5′- or (B) 3′-^{32}P end-labeled *Spe*I/*Eag*I fragment of the upstream region of the PGK1 gene was methylated with *Sss*I methylase and treated with esperamicin A1 or C as described in Experimental Procedures. Methylated DNA fragments run slightly slower than unmethylated DNA in the sequencing gels. (A) DNA damage produced by esperamicins A1 and C in the 3′-^{32}P end-labeled PGK1 gene fragment. The methylation status is indicated by minus or plus signs, and methylated cytosines are denoted by asterisks; AG and CT denote Maxam−Gilbert sequencing markers (*31*). (B) Esperamicin A1-induced DNA damage in the 5′-^{32}P end-labeled PGK1 fragment. DNA was treated with 0−200 nM esperamicin A1 and resolved on an 8% sequencing gel. The methylation status is indicated by minus or plus signs; methylated cytosines are denoted by asterisks. G, AG, and CT represent lanes containing Maxam−Gilbert sequencing markers. Note that less radioactivity was loaded in the lane containing methylated DNA treated with 100 nM esperamicin A1 than in the other lanes.

containing various sugar residues relative to the phosphate-ended Maxam−Gilbert chemical sequencing standards (*31*). This technique has been used to identify damage chemistry produced by several radical-mediated DNA cleaving agents (*23*, *25*, *26*, *32*−*35*). DNA damage produced by both esperamicins A1 and C in the 3′-^{32}P end-labeled PGK1 fragment was consistent with 5′-hydrogen atom abstraction (Figure 4A). This damage consisted of either a strand break with 5′-nucleoside aldehyde- and 3′-phosphate-ended DNA fragments or a strand break with both 3′- and 5′-phosphate-ended fragments (Figure 4A). The 5′-nucleoside aldehyde, here stabilized by reduction with sodium borohydride (*36*), slows the migration of the DNA fragment by 1−4 nucleotides (Figure 4, asterisks in left panel), while treatment with piperidine cleaves the nucleoside aldehyde to produce a 5′-phosphate-ended fragment (Figure 4A, bands in right panel).

As demonstrated previously, the presence of esperamicin A1-induced 5′-chemistry on one strand is presumed to be accompanied by 1′-hydrogen abstraction, and the resulting 2′-deoxyribonolactone, on the other strand (*25*). This conclusion is supported by the relative resistance of lesions on the strand opposite the 5′-chemistry to cleavage by hydrazine compared to that by putrescine (data not shown) (*25*). Hydrazine converts the keto aldehyde abasic site, arising from 4′-hydrogen atom abstraction, to a pyridazine-ended strand break (*37*), while putrescine cleaves all abasic sites to phosphate-ended fragments (*25*).

Unlike esperamicin A1, analysis of the damage produced by esperamicin C revealed 4′-hydrogen abstraction at sites on the strand opposite that showing 5′-chemistry (Figure 4B), as observed previously (*25*). The resulting damage products consist of either a keto aldehyde abasic site or a strand break with a phosphate on the 5′-end and a phosphoglycolate residue on the 3′-end (*25*). As shown in Figure 4B, the keto aldehyde abasic site reacts with hydrazine to form a strand break with a slow-migrating 3′-phosphopyridazine-ended fragment (asterisks in Figure 4B, lanes 2 and 5) or with putrescine to form phosphate-ended fragments (Figure 4B, lanes 3 and 6).

In all cases, there were no methylation-dependent differences in the relative quantities of deoxyribose degradation products (data not shown). This suggests that cytosine methylation, while it enhances the quantity of DNA damage,

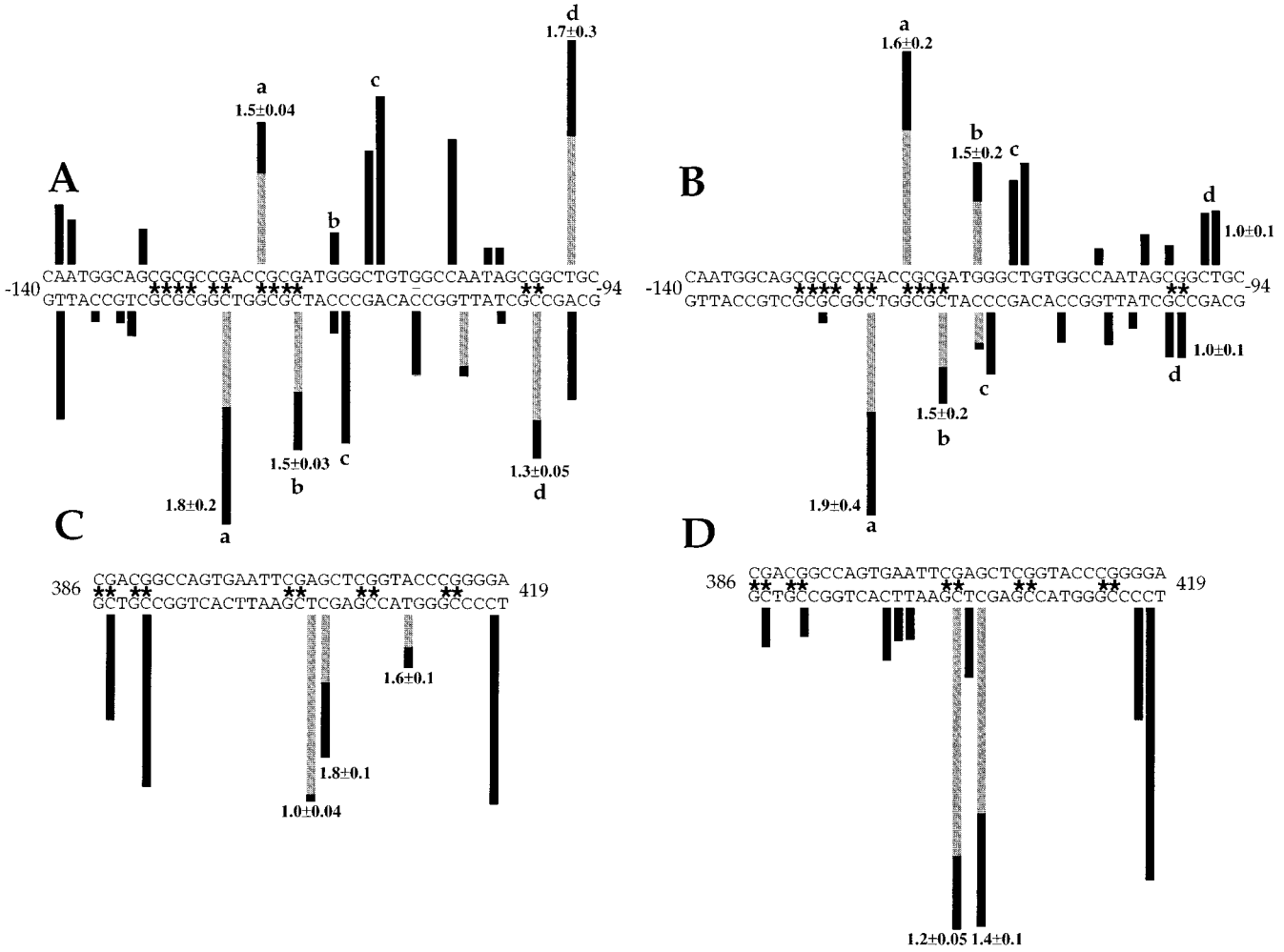

FIGURE 3: Frequency of DNA damage produced by esperamicins A1 (A and C) and C (B and D) in the methylated or unmethylated PGK1 gene (A and B) or the pUC19 fragment (C and D). The height of the bar at each position in the PGK1 sequence is proportional to the damage frequency. Stippled and solid bars represent damage in unmethylated and methylated DNA, respectively; the absence of a stippled pattern indicates that damage frequency was equal in both types of DNA (<10% error as illustrated at sites d in panel B). The numbers above the bars represent average ratios of the damage frequency in methylated DNA to that in unmethylated DNA; in panels A and B, the error is a standard deviation for $n = 3$, and in panels C and D, the error is variation about the mean for $n = 2$. Methylated cytosines are denoted with asterisks. Sites marked with letters a−d are discussed in the text.

does not alter the chemistry of esperamicin-induced DNA lesions.

Having identified the damage chemistry associated with esperamicin C and A1 lesions on each strand, we could define a putative DNA binding orientation of the drugs. The results of these orientation studies are presented in Figure 4C. The orientation of the bound drug is the same for both esperamicins C and A1, with the presumed position of the intercalating anthranilate of esperamicin A1 indicated by the bold line in Figure 4C. The location of the intercalated anthranilate is based on NMR structural studies of the esperamicin A1−DNA complex (*24*).

DISCUSSION

The role of DNA methylation in the selection of DNA targets by small molecules has been the subject of several studies with benzo[*a*]pyrenediol epoxide (*17*), mitomycin C (*18*), bleomycin (*19*), and *N*-methyl-*N*-nitrosourea (*20*). Here, we demonstrate that methylation enhances the damage produced by two equilibrium-binding DNA-cleaving molecules, esperamicins A1 and C. Both enediynes bind in the minor groove and share similar sequence selectivities. However, they differ structurally in that esperamicin C does not possess the intercalating anthranilate of esperamicin A1. The results with these structural analogs illustrate the complexity of methylation-induced alterations of DNA conformation.

There are several effects of cytosine methylation on DNA structure that could influence the interactions of small molecules with DNA. These include helical unwinding (*10*), increased base stacking and helical stability (*11*), reduction in major groove charge density near the methyl group (*12*), and, in certain sequence contexts, modulation of DNA bending (*13*−*15*). Methylation-induced torsional flexibility or helical unwinding could explain the increased binding of esperamicin A1 since intercalators unwind the helix upon binding and prefer to bind to the underwound helix of negatively supercoiled DNA (*38*−*41*). This effect would be consistent with the observation of Denissenko *et al.* (*17*), who found that cytosine methylation increases adduct formation by benzo[*a*]pyrenediol epoxide. However, plasmid unwinding studies with esperamicin C suggest that the drug does not alter DNA twist (*25*). Esperamicin C would thus not be expected to be sensitive to methylation-induced changes in DNA twist unless such changes affected other features of DNA structure, such as minor groove width,

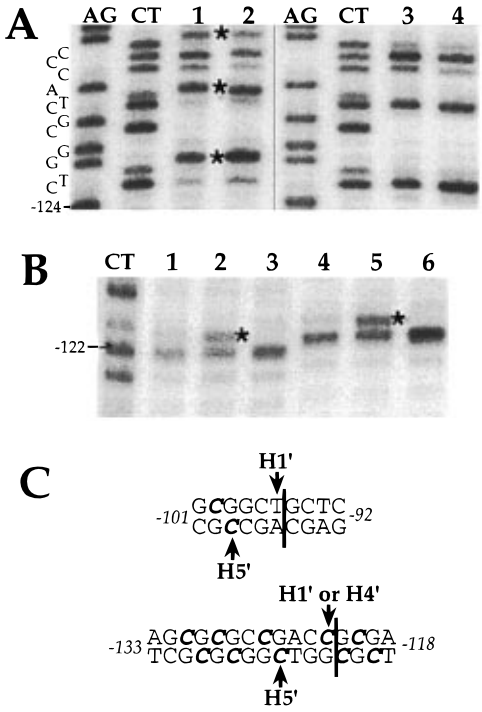

FIGURE 4: Putative orientation of esperamicins A1 and C at sites of methylation-enhanced DNA damage. (A) Demonstration of 5′-hydrogen abstraction by esperamicin A1 in the 3′-^{32}P end-labeled PGK1 fragment on an 8% sequencing gel: lanes 2 and 4, methylated DNA; and lanes 1 and 3, unmethylated DNA. Asterisks in lanes 1 and 2 denote the positions of nucleoside aldehyde-ended DNA fragments (stabilized by reduction with sodium borohydride); the nucleoside aldehyde residues are cleaved by piperidine to yield phosphate-ended DNA fragments in lanes 3 and 4. (B) Demonstration of 4′-hydrogen abstraction by esperamicin C at position −122 in the 5′-^{32}P end-labeled PGK1 fragment on a 20% sequencing gel: lanes 1−3, unmethylated DNA; lanes 4−6, methylated DNA; lanes 1 and 4, drug-treated controls; lanes 2 and 5, hydrazine derivatization (asterisks denote 3′-phosphopyridazine-ended fragments); and lanes 3 and 6, putrescine derivatization (converts abasic sites to phosphate-ended fragments). The methylated DNA fragments migrate more slowly than the unmethylated DNA. (C) Putative orientation of esperamicins A1 and C at methylation sites. The damage chemistry on each strand was used to predict the orientation of bound esperamicins A1 and C at two sites of methylation-enhanced DNA damage. The heavy bars denote the presumed sites of intercalation of the anthranilate of esperamicin A1. Esperamicin C assumes the same orientation as esperamicin A1 in the lower sequence; no methylation enhancement of esperamicin C-induced DNA damage was observed in the upper sequence.

thereby creating a more attractive binding site for the drug. A similar argument could also be made for the methylation-induced increase in cross-linking by the nonintercalating mitomycin C (*18*).

In studies with mitomycin C, Johnson *et al.* (*18*) proposed that the enhancement of mitomycin C cross-linking by cytosine methylation is due to an electronic effect transmitted by hydrogen bonding between the methyl group and the N2 of guanine. It could be argued that such local alterations in minor groove charge density increase the binding of esperamicins A1 and C at position −122 in the PGK1 sequence (Figure 4C, lower sequence). However, this could not be the case at position −96 (Figure 4C, upper sequence), where esperamicin A1-induced damage is enhanced by cytosine methylation while damage produced by esperamicin C is not.

Alternatively, axial flexibility or curvature could be involved in the enhancement of esperamicin C-induced DNA damage by methylation. We have observed that adenine methylation at the 3′-end of a target sequence for calicheamicin γ_1^I, a related enediyne, increases drug-induced DNA damage by 1.5−2-fold (unpublished observations), an effect that may be related to the propensity of calicheamicin γ_1^I to bend its target sequences (*42*). While esperamicin C is a structural analog of calicheamicin γ_1^I missing the terminal sugar and aromatic ring, it does not share calicheamicin's selectivity for the 3′-end of purine tracts (*42*, *43*). Furthermore, calicheamicin damage was not seen to be enhanced by cytosine methylation in PGK1, which is due in part to the fact that damage sites did not occur near CG sequences (data not shown).

Despite the local changes in DNA conformation caused by DNA methylation, the effects appear to be transmitted along the helix over at least several base pairs. Kim *et al.* (*44*) have observed that CG repeats exert long range influences on DNA secondary structure, and it is possible that cytosine methylation affects these long range structural changes. Furthermore, Hertzberg *et al.* (*19*) demonstrated that the effect of methylation on bleomycin cleavage can extend as far as 14 base pairs away from the methylated cytosine, while Hodges-Garcia and Hagerman (*14*) observed that cytosine methylation affects curvature of A tracts up to three base pairs away. As shown in Figure 4C, the anthranilate is predicted to intercalate four base pairs downstream from the methylated CG site in the sequence −99 to −92. This suggests that the methylation-induced changes in DNA conformation extend beyond the CG site to affect drug binding.

The effects of methylation on the selection of DNA targets by esperamicins A1 and C also appear to have a complex dependency on sequence context and drug structure, as has been noted in studies relating DNA curvature to cytosine methylation (*13*, *14*, *45*). The sequence motif XGGGCTGPy at sites c and d in Figure 3A,B provides an interesting example. Damage in this sequence at both sites c and d occurs at C and T, which is consistent with a PuPyPy sequence preference for esperamicins A1 and C (Figure 3; *27*). When X is C, the damage produced by esperamicin A1, but not by esperamicin C, is enhanced by cytosine methylation (site d in Figure 3A,B). This suggests that the anthranilate is sufficient but not necessary for the enhancement of damage at some sites. Another example of the sequence dependence is the observation that several CG sites are not damaged by either drug (positions −131 to −128 in Figure 3A,B) or do not show a methylation-dependent enhancement of damage despite a PuPyPy motif (positions 386−390 in Figure 3C,D). In the DNA sequences studied here, methylation enhanced DNA damage at some sites but it did not create new sites or decrease damage at others. These results suggest that cytosine methylation affects aspects of esperamicin target selection that are yet to be defined, but also that there are other sequence- or structure-dependent factors that determine damage sites for the esperamicins.

In conclusion, we have shown that damage produced by esperamicins A1 and C is enhanced by cytosine methylation, while the chemistry of the damage is not affected. Furthermore, by using intercalating and nonintercalating enediynes that share similar sequence selectivities and a common mechanism of action, we have ruled out intercalation as the primary factor in the methylation-induced increase in DNA

damage. Additional studies are underway to resolve the role of cytosine methylation in enediyne target selection.

ACKNOWLEDGMENT

The authors thank Dr. Judith Singer Sam for generously providing the pBSHPGK1 plasmid, Dr. Jiongru Wu and Aaron Salzberg for their assistance with several of the experiments, Kenneth Moore, Jr., for critical reading of the manuscript, and the other members of the Dedon lab for helpful discussions.

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BI971533W