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The Effect of C(5) Cytosine Methylation at CpG Sequences on Mitomycin–DNA Bonding Profiles

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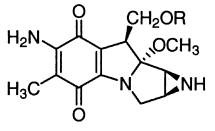
Abstract—Recent studies have documented that cytosine C(5) methylation of CpG sequences enhances mitomycin C (**1**) adduction. The reports differ on the extent and uniformity of **1** modification at the nucleotide level. We have determined the bonding profiles for mitomycin monoalkylation in two DNA restriction fragments where the CpG sequences were methylated. Three mitomycin substrates were used and two different enzymatic assays employed to monitor the extent of drug modification at the individual base sites. Drug–DNA modification was accomplished with **1** and 10-decarbamoylmitomycin C (**2**) under reductive ($Na_2S_2O_4$) conditions and with *N*-methyl-7-methoxyaziridinomitosene (**3**) under nonreductive conditions. The UvrABC incision assay permitted us to quantitate the sites of drug adduction, and the λ-exonuclease stop assay provided a qualitative estimation of drug–DNA modification consistent with the UvrABC data. We learned that C(5) cytosine methylation (m^5C) enhanced the extent of overall DNA modification. Using the UvrABC endonuclease assay, we found that modification by **1** increased 2.0 and 7.4 times for the two DNA restriction fragments. Analysis of the modification sites at the nucleotide sequence level revealed that guanine (G) was the only base modified and that the overall increased level of DNA adduction was due to enhanced modification of select m^5CpG* (G* = mitomycin (mitosene) adduction sites) loci compared with CpG* sites: the largest differences reached two orders of magnitude. Significantly, not all CpG* sites underwent increased drug adduction upon C(5) cytosine methylation. The effect of C(5) cytosine methylation on the drug adduction profiles was less pronounced for G* sites located within dinucleotide sequences other than CpG*. We observed that DNA methylation often led to slightly diminished adduction levels at these sites. The different m^5CpG* adduction patterns provided distinctive sequence-selective bonding profiles for **1**–**3**. We have attributed the large differences in guanine reactivity to DNA structural factors created, in part, by C(5) cytosine methylation. The significance of these findings in cancer chemotherapy is briefly discussed. © 2001 Published by Elsevier Science Ltd.

An important paradigm in cancer chemotherapy is the selective inactivation of DNA in tumorigenic cells.[1] Among the DNA-targeting reagents, mitomycin C (**1**) is unique.[2] Enzymatic reduction of **1**[3] provides both mono- and bis(cross-linked)-alkylated DNA adducts. Both DNA modifications are likely responsible for the antitumor activity of **1**.[3a,f–h] Of these two, it is the cross-linked adduct that has aroused the most interest.[3] Interstrand cross-linking of complementary DNA strands is expected to inhibit DNA replication and subsequent cell proliferation.

DNA minor-groove mitomycin C mono- and bis-alkylation transformations occur only at the 2-amino group of guanine[4,5] and proceed with sequence selectivity. Interstrand DNA cross-linking takes place exclusively at 5′CpG*•5′CpG* sequences, where G* is the site of mitomycin C adduction.[5–7] This sequence is also the preferred site for monoalkylation events with other dinucleotide guanine-containing sequences (5′NpG*) modified to a lesser extent.[8–10] Recently, we demonstrated that the extent of DNA modification and the sequence selectivity for CpG* sites were dramatically enhanced for mitomycin C monoalkylation processes upon C(5) methylation of cytosine units (m^5C) within CpG steps.[11] Using



1 R = C(O)NH$_2$
2 R = H

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the 188-bp *Stu*I-*Mse*I fragment of the APRT exon III gene from CHO cells, we found that two of the three m^5CpG* sites were modified upon treatment with **1** and $Na_2S_2O_4$ and that adduction at these two sites exceeded all other NpG* sequences by ≥19:1. Interestingly, the remaining m^5CpG* site was not appreciably modified (<2%). We further showed that C(5) cytosine methylation led to a 12-fold increase in the extent of mitomycin C modification at one of the three CpG* sites over the corresponding site in unmethylated DNA.

The effect of C(5) cytosine methylation on mitomycin C alkylation processes has been previously investigated.[11–15] Millard and Beachy first showed, using synthetic oligonucleotides, that **1** cross-linking transformations were enhanced 1.4–2.0-fold upon C(5) cytosine methylation.[12] Similarly, Tomasz and co-workers found a 1.9-fold enhancement in cross-linking for oligonucleotide duplexes containing m^5CpG sequences on complementary strands.[13] These investigators also demonstrated that replacement of C with m^5C within CpG sequences increased the yield of mitomycin C monoalkylation adducts 1.9- to 2.4-fold. Both Millard[12] and Tomasz[13,15] have discussed the potential importance of m^5CpG sites in genomic DNA and the effect of drug modification at these sites. The CpG sequence occurs only one-fifth of the statistical value in the mammalian genome.[16,17] Nonetheless, sequences containing 5′CpG are present in unusually high amounts in DNA promoter regions, housekeeping genes, and proto-oncogenes. In these instances, the cytosine residues are largely unmethylated. This pattern contrasts to the situation in bulk vertebrate DNA and tissue-specific genes, where most (60–90%) of the cytosines are methylated at the C(5) position.[16] These contrasting methylation patterns have been proposed to function as control elements for gene expression[18] since cytosine methylation alters DNA structure and affects protein–DNA binding.[19] Tumor cells exhibit different DNA methylation patterns compared with normal cells.[20] It is known that some tumor cells display unusually high levels of DNA methylase,[21] and that transformed cells have regions of DNA hypomethylation and hypermethylation.[22,23] These patterns of methylated and unmethylated DNA provide a unique DNA mosaic that may contain structural and functional information necessary for cell maintenance.[20]

Little is known about whether cytosine methylation of CpG steps lead to a *uniform* increase in the levels of mitomycin C modification at these sites. Tomasz and co-workers suggested that the enhanced mitomycin C adduction levels at m^5CpG* compared with CpG* sites are promoted by the electron-releasing C(5) methyl substituent present on the *complementary* cytosine base.[15] Their studies indicated that this electronic effect would lead to the 2- to 3-fold increase in **1** modification at observed m^5CpG* sites.[13,15] Recently, Dannenberg and Tomasz reported calculations using density functional theory at the B3LYP/D95** level that support this hypothesis.[24] Our initial study on the mitomycin C adduction patterns using the 188-bp *Stu*I-*Mse*I fragment from the CHO APRT exon III gene indicated that C(5) cytosine methylation did not lead to a uniform increase in **1** adduction at the *three* m^5CpG* sites.[11] The significance of C(5) cytosine methylation in chemical biology led us to determine the effect of C(5) cytosine methylation on the mitomycin alkylation patterns at guanine sites embedded in different DNA structural contexts. Two different DNA restriction fragments were used and two different methods were employed to detect the mitomycin-DNA lesions at the nucleotide level. The results of these two assays were compared, and we report that C(5) cytosine methylation within CpG steps led to large increases in drug modification in select cases at these sites, but the extent of modification varied markedly with overall DNA sequence. We found further that C(5) cytosine methylation was sufficient to convert mitomycin C modification sites from minor to major alkylation sites. Our findings suggested that multiple factors contribute to the enhanced levels of mitomycin C adduction at m^5CpG* sites and that cytosine methylation may alter local DNA structure affecting drug adduction.

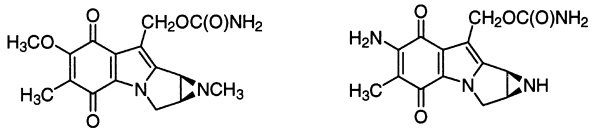
Results

Design and scope of studies

Our approach was to compare the mitomycin monoalkylation patterns for corresponding pairs of methylated and unmethylated DNA restriction fragments containing multiple CpG sites. Selective C(5) cytosine methylation at 5′ CpG sites was accomplished with S-adenosyl methionine (SAM) and bacterial *Sss*I methylase.[25] The Maxam-Gilbert chemical sequencing reactions provide a measure of the extent of cytosine methylation at CpG sites.[26] It has been shown that under those conditions C(5) cytosine methylation blocks hydrazine-mediated cleavage of the phosphodiester bond 3′ to cytosine sites.[27] We did not detect any radiolabeled fragments corresponding to 5′ m^5C fragments in the methylated DNA samples and estimate that C(5) methylation at these sites was nearly complete.

We chose to use the *Bst*NI-*Eco*RI 129-bp and the *Bst*NI-*Nar*I 146-bp restriction fragments from a pBR322 plasmid.[8,9] Both fragments have been used to determine the sequence specificity of mitomycin C monoalkylation processes.[8,9] The 3′ end-labeled (upper strand) 129- and (lower strand) 146-bp DNA restriction fragments were modified with mitomycin C (**1**) and 10-decarbamoylmitomycin C[28] (**2**) under chemical reductive conditions ($Na_2S_2O_4$) and with *N*-methyl-7-methoxyaziridinomitosene[29] (**3**) under nonreductive conditions. The mitomycin (mitosene)–DNA adduction experiments were carried out under conditions that render, on average, fewer than one adduct per DNA fragment.[8,30] Previously, we demonstrated that upon sequential addition of limiting amounts of $Na_2S_2O_4$ to mitomycin C the principal DNA alkylating agent is 7-aminoaziridinomitosene[31] (**4**) and that the DNA-bonding profiles of **3** alone and reductively activated **1** were comparable.[30] We also showed that with limiting amounts of $Na_2S_2O_4$ only the C(1) position in mitomycin C is activated.[32] This finding was confirmed by comparing the bonding profiles for

$Na_2S_2O_4$-activated **1** with those of $Na_2S_2O_4$-activated **2** transformations. Compound **2** can only bond to DNA at the C(1) site in the drug because the carbamate group is absent at the C(10) position.[8–10,33]



3 **4**

Our primary method to monitor mitomycin bonding profiles was the UvrABC endonuclease assay.[34] UvrABC is an important repair enzyme found in *Escherichia coli* and is capable of detecting, incising, and then repairing DNA lesions.[34–36] We have shown that this multi-subunit endonuclease typically hydrolyzes the seventh 5′ phosphodiester bond and the fourth 3′ phosphodiester bond to the mitomycin-modified guanine providing a distinctive incision pattern.[9,30,33] Moreover, this enzyme incises mitomycin monoalkylation lesions, independent of sequence, with equal efficiency in both methylated and unmethylated DNA samples, allowing the quantification of mitomycin bonding profiles.[11,33] The UvrABC/DNA molar ratio was $\geqslant 6$.

The potential significance of C(5) cytosine methylation on mitomycin C bonding profiles warranted our use of a second method to monitor drug–DNA monoalkylation. We asked whether the λ-exonuclease stop assay[36,37] was a suitable method. Earlier, we used this enzyme assay to provide information concerning the preferred sites of anthramycin[36] and mitomycin C[8] transformations in unmethylated DNA restriction fragments. λ-Exonuclease digests DNA from the 5′ end,[37] and stop sites are typically observed two-to-three bases 5′ to the presumptive drug–guanine (G*) adduct.[8,36] In this assay, quantitative determination of the relative bonding affinities for the various sites *within* the DNA restriction fragment is less straightforward since λ-exonuclease often encounters multiple stop sites as it progresses toward a drug–DNA adduct.[8,37]

Mitomycin (mitosene)-DNA modification profiles for the BstNI-EcoRI 129-bp restriction fragment

UvrABC assay. Figure 1(a) shows the autoradiogram of the high-resolution denaturing polyacrylamide gel after UvrABC digestion of the unmethylated and methylated 3′ end-^{32}P-labeled 129-bp DNA fragments modified with mitomycin C (0.2 mM) treated with $Na_2S_2O_4$, 10-decarbamoylmitomycin C (0.9 mM) treated with $Na_2S_2O_4$, or with *N*-methyl-7-methoxyaziridinomitosene (1.5 mM). The Maxam–Gilbert chemical sequencing reactions are given in lanes 1–4. Lane 2 shows the radiolabeled fragments generated upon hydrazine treatment of the unmethylated DNA to give selective cleavage at thymidine and cytosine sites. Lane 3 is the corresponding reaction for the m^{5}CpG DNA. Comparison of lanes 2 and 3 shows four distinct T/C sequencing bands in lane 2 that are absent in lane 3. These bands correspond to the CpG sites (*C) in the central DNA reading region (upper strand) of the 129-bp fragment. Cleavage patterns upon **3**, **1** and **2** modifications are given in lanes 7 and 8, 9 and 10, and 11 and 12, respectively. In each pair of mitomycin-modified lanes, the first corresponds

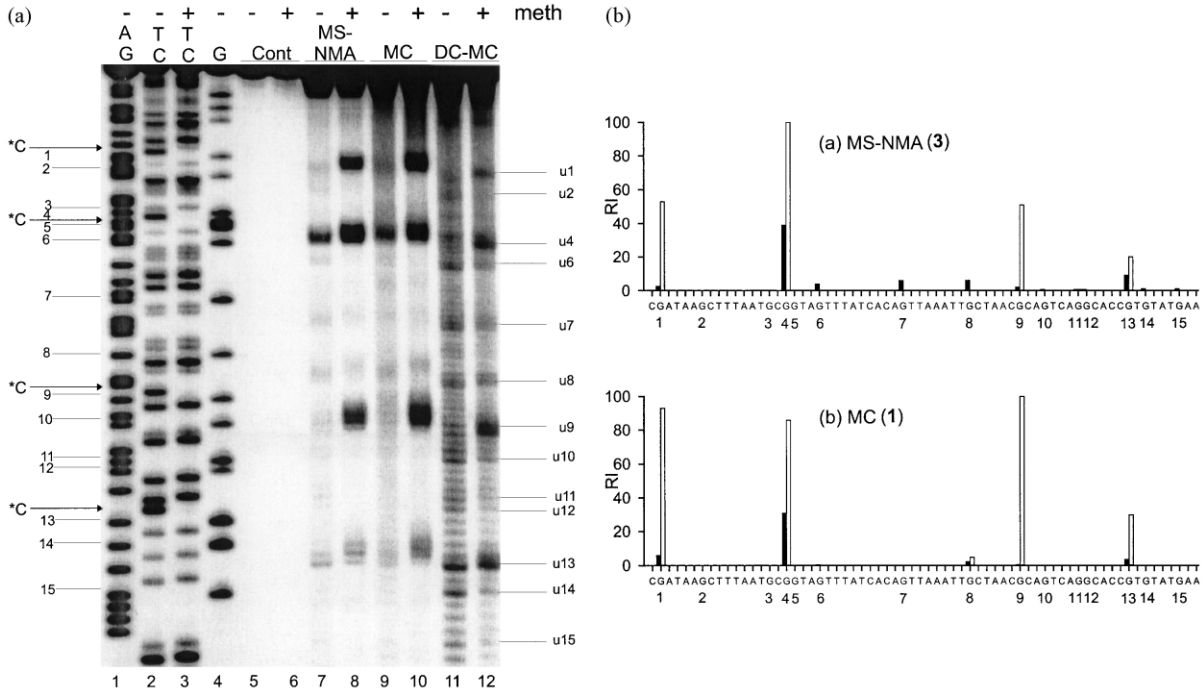


Figure 1. (a) Autoradiogram of the effect of m^{5}CpG (C(5) cytosine methylation at CpG sites) on UvrABC cutting of **3** (1.5 mM), **1** (0.2 mM), and **2** (0.9 mM) modified 3′-labeled *Bst*NI-*Eco*RI 129-bp fragment from pBR322 (upper strand). *C represents methylated cytosines. Guanine residues are labeled 1–15 and the corresponding UvrABC incision bands are labeled u1–u15. Meth: methylation; symbol +/−: with and without C(5) cytosine methylation; (b) Quantification of the effect of m^{5}CpG on UvrABC cutting of (a) **3** and (b) **1** modified *Bst*NI-*Eco*RI 129-bp fragment from pBR322 (upper strand). Shaded bar: methylated DNA; solid bar: unmethylated DNA. The guanines are numbered the same as in (a). RI: relative intensity.

with the unmethylated DNA and the second with the C(5) cytosine methylated DNA. Approximately equal amounts (±10%) of radioactivity per sample were loaded onto the gel. Inspection of the autoradiogram revealed several prominent bands within each lane. These bands have been assigned to the UvrABC incision sites that were induced by the mitomycin (mitosene)–DNA lesions according to the guanine number on the left side of the gel. The incision bands have been labeled u1–u15. Two possible incisions sites (u3 and u5) were not detected. The bonding sites were less distinct (Fig. 1(a), lanes 11 and 12) in the 10-decarbamoylmitomycin C (**2**) reaction, and a ladder-like set of bands appeared that corresponded to random DNA cleavage. Comparison of the unmethylated and the methylated DNAs for each mitomycin substrate (lane 7 versus 8, 9 versus 10, and 11 versus 12) showed that DNA methylation led to enhanced drug adduction at select sites. In both the mitomycin C (**1**) and *N*-methyl-7-methoxyaziridinomitosene (**3**) modifications we observed two incision sites (u1 and u9) in the methylated DNA that were largely undetected in the unmethylated DNA.

Densitometric scanning of Figure 1(a) permitted us to determine the bonding profiles for **1** and **3** adduction and to quantitate the effect of m^5CpG methylation on mitomycin modification (Fig. 1(b)). The two histograms in Figure 1(b) have been normalized to the most intense band in each *pair* of reactions (unmethylated and methylated DNAs) for the two mitomycin substrates. The corresponding histogram for **2** adduction is not provided because of the error introduced by background, nonspecific DNA cleavage bands. Inspection of the histograms in Figure 1(b) documented the increased level of drug–DNA modification that accompanied DNA methylation. Comparison of the individual cleavage profiles for the mitomycin (mitosene)-modified unmethylated and methylated DNAs (Fig. 1(b), lane 7 versus 8; lane 9 versus 10) show that the selectivity for specific CpG* sites for **3** and **1** modification in unmethylated DNA (lanes 7 and 9) was markedly enhanced upon DNA C(5) cytosine methylation (lanes 8 and 10).

λ-Exonuclease stop assay

The autoradiogram for the 129-bp DNA fragment after drug modification and λ-exonuclease treatment is provided in Figure 2(a) and the corresponding histograms in Figure 2(b). We expected several differences between the drug-DNA adduction patterns observed using this assay and the UvrABC nuclease method. First, the mitomycin modification sites accessible in the λ-exonuclease stop assay do not exactly correspond to those observed in the UvrABC nuclease assay because of the different DNA cleavage pathways for the two enzyme systems.[8,9] Second, we have shown that while UvrABC nuclease incises drug-DNA adducts formed at methylated and unmethylated CpG sites with equal efficiency and does not incise unmodified DNA, λ-exonuclease DNA digestion is influenced by the drug-DNA adduct and the DNA local structure.[36] Third, the processive nature of λ-exonuclease DNA digestion[8,36] does not permit the determination of the relative bonding distribution *within* the DNA restriction fragment using this assay. As a result, the **1** and **3** bonding intensities observed using the λ-exonuclease assay do not always correspond to those detected by the UvrABC method for guanine sites common to both assays. Nonetheless, we

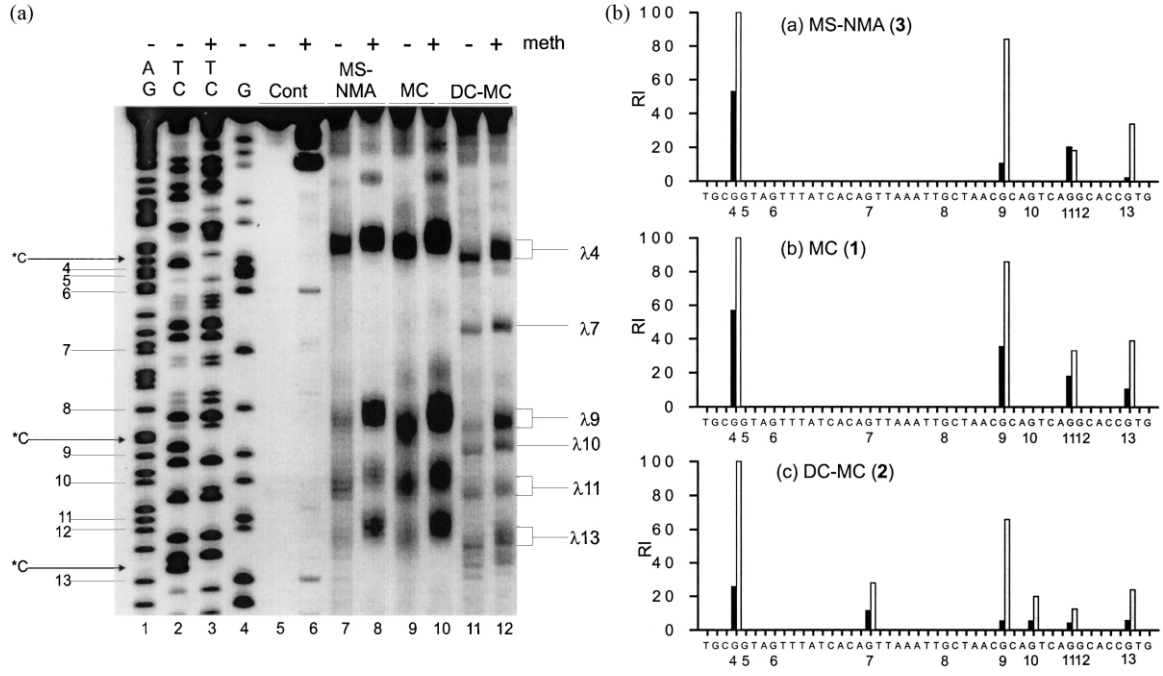


Figure 2. (a) Autoradiogram of the effect of m^5CpG on λ-exonuclease digestion of **3** (1.5 mM), **1** (0.45 mM) and **2** (0.9 mM) modified 3′-labeled *Bst*NI-*Eco*RI 129-bp fragment from pBR322 (upper strand). Guanine residues are labeled 1–13 and the corresponding λ-exonuclease stop sites are labeled λ1–λ13. All other symbols are the same as in Figure 1(a). (b) Quantification of the effect of m^5CpG on λ-exonuclease digestion of (a) **3**, (b) **1**, and (c) **2** modified *Bst*NI-*Eco*RI 129-bp fragment from pBR322 (upper strand). Shaded bar: methylated DNA; solid bar: unmethylated DNA. The guanines are numbered the same as in (a). RI: relative intensity.

have utilized the results from the λ-exonuclease assay to provide a *qualitative* comparison of the extent of drug adduction at identical guanine sites in unmethylated and methylated DNA samples. Side-by-side comparison of the **1** and **3** bonding profiles for the unmethylated and the methylated DNA restriction fragments reinforced two results found in the UvrABC experiments. First, C(5) cytosine DNA methylation led to enhanced levels of DNA modification. Second, specific CpG* sites (λ9, λ13) that were only weakly and moderately modified in the unmethylated DNA underwent extensive adduction when converted to m^5CpG sites.

Mitomycin (mitosene)-DNA modification profiles for the BstNI-NarI 146-bp restriction

Fragment using the UvrABC and λ-exonuclease assays. We conducted a comparable study with the *Bst*NI-*Nar*I 146-bp DNA fragment. Only the results in the well-separated region of this fragment are shown. Figure 3(a) shows the UvrABC cutting results of methlylated and unmethylated DNA fragment modified with reductively activated **1** and **3**. Figure 4(a) contains the corresponding autoradiogram showing the λ-exonuclease stop sites. Studies using two different concentrations of **1** (0.1 and 0.15 mM) documented that the bonding intensities for the stop sites in the unmethylated and methylated DNA increased with drug concentration (data not shown). Figure 4(a) also contains the bonding profile for 10-decarbamoylmitomycin C (**2**) after treatment with $Na_2S_2O_4$. Comparison of the **2** and **1** bonding profiles documented that the patterns were similar and that C(5) cytosine methylation led to enhanced DNA modification for both **1** and **2**.

The histograms for the 146-bp fragment after UvrABC and λ-exonuclease treatment are provided in Figures 3(b) and 4(b), respectively. The results confirmed many of the findings obtained with the 129-bp fragment. First, we observed that DNA C(5) cytosine methylation led to a substantial increase in the extent of mitomycin (mitosene) DNA modification. Second, the preference for mitomycin (mitosene)-adduction at CpG* sites increased upon conversion of DNA CpG steps to m^5CpG units. Third, C(5) cytosine methylation led to significant increases in the extent of adduction at most, but not all, CpG sequences. Fourth, comparison of the λ-exonuclease drug bonding patterns for reductively activated **2** in the unmethylated and the methylated DNA samples with those for reductively activated **1** (Figs 2(b) and 4(b)) indicated that the deletion of the C(10) carboxamide group in **1** did not alter the strong preference for CpG* modification.

Comparison of the UvrABC and λ-exonuclease stop assays

Examination of the UvrABC and λ-exonuclease histograms (Fig. 1(b) versus 2(b), Fig. 3(b) versus 4(b)) for common G* modification sites showed that while both assays provided similar information concerning the effect of DNA methylation on mitomycin (mitosene) adduction some differences did exist. In particular, a few CpG* drug modification sites that displayed large increases upon C(5) cytosine methylation in the UvrABC assay did not show comparable increases in the λ-exonuclease assay (e.g., Figs 3(b) and 4(b): G6). Similarly, the large increases detected in the λ-exonuclease assay for C(5) cytosine methylation were not always observed in the UvrABC assay (e.g., Figs 3(b) and 4(b): G1).

Review of the autoradiograms from the λ-exonuclease experiments revealed an interesting phenomenon (Figs 2(a) and 4(a)). The intensities for the radiolabeled DNA fragments corresponding to the central mitomycin (mitosene)–CpG* sites were noticeably lower than the corresponding terminal drug–CpG* adducts. This phenomenon was not observed in our earlier study using λ-exonuclease.[8] We suspect that it is a function of the enzymatic activity of λ-exonuclease. It is known that

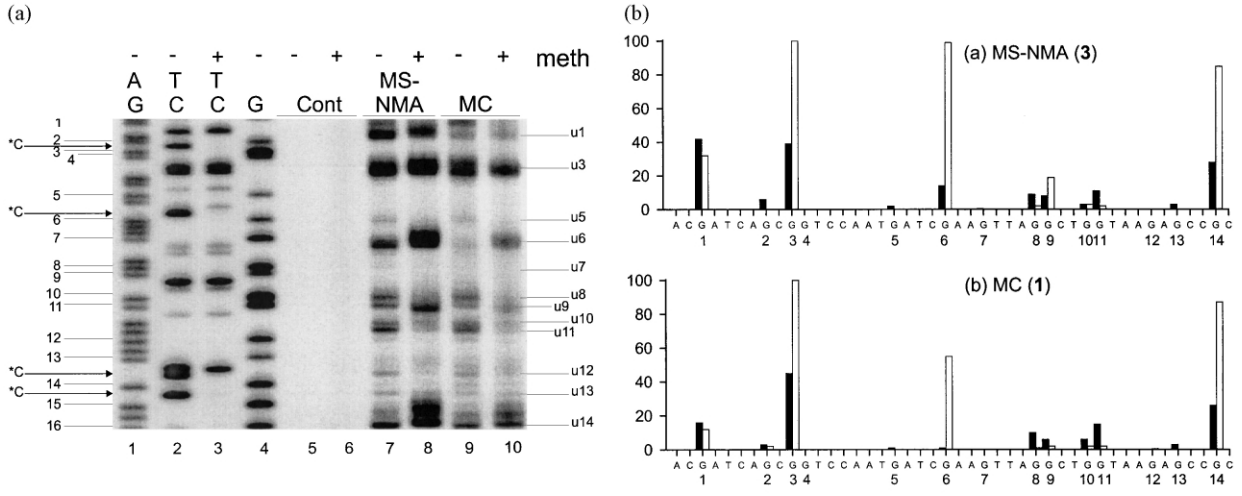


Figure 3. (a) Autoradiogram of the effect of m^5CpG on UvrABC cutting of **3** (1.5 mM) and **1** (0.15 mM) modified 3′-labeled *Bst*NI-*Nar*I 146-bp fragment from pBR322 (lower strand). Guanine residues are labeled 1–16 and the UvrABC incision bands are labeled u1–u14. All other symbols are the same as in Figure 1(a); (b) Quantification of the effect of m^5CpG on UvrABC cutting of (a) **3** and (b) **1** modified *Bst*NI-*Nar*I 146-bp fragment from pBR322 (lower strand). Shaded bar: methylated DNA; solid bar: unmethylated DNA. The guanines are numbered the same as in (a). RI: relative intensity.

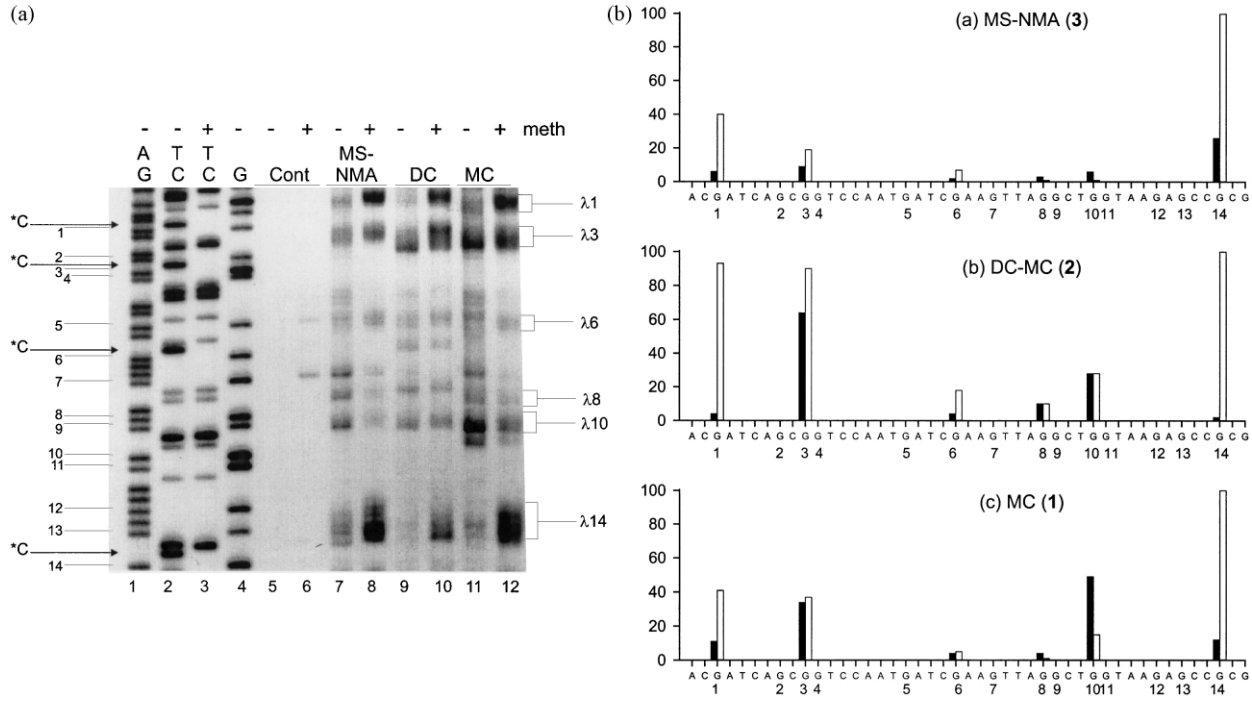


Figure 4. (a) Autoradiogram of the effect of m^5CpG on λ-exonuclease digestion of **3** (1.5 mM), **2** (0.45 mM) and **1** (0.1 mM) modified 3′-labeled *Bst*NI-*Nar*I 146-bp fragment from pBR322 (lower strand). Guanine residues are labeled 1–14 and the corresponding λ-exonuclease stop sites are labeled λ1–λ14. All other symbols are the same as in Figure 1(a); (b) Quantification of the effect of m^5CpG on λ-exonuclease digestion of (a) **3**, (b) **2** and (c) **1** modified 3′-labeled *Bst*NI-*Nar*I 146-bp fragment from pBR322 (lower strand). Shaded bar: methylated DNA; solid bar: unmethylated DNA. The guanines are numbered the same as in (a). RI: relative intensity.

λ-exonuclease always digests DNA from the 5′ end and that digestion occurs more efficiently with double-stranded than with single-stranded DNA.[36] It is likely that the mitomycin lesions located in the central region may be under-recognized if DNA strand denaturation occurred before the processive enzyme reached the drug adduct.

Discussion

Analysis of the bonding profiles for 1-, 2-, and 3-DNA adduction

We employed the UvrABC incision and λ-exonuclease stop assays to demonstrate the effect of C(5) cytosine methylation on mitomycin–DNA modification. We have used these methods previously to show that mitomycin-DNA processes occur selectively at CpG* sites.[8,11,30,33] The autoradiograms showed that after modification with **1**, **2**, or **3** the UvrABC nuclease cutting and the λ-exonuclease stop sites for the *un*methylated 129- and 146-bp DNA fragments were in excellent agreement with those previously reported.[8,9,30,33]

Many of the mitomycin (mitosene)–DNA adduction patterns previously noted for unmethylated DNA[8,9,30,33] were observed with the methylated DNA samples. In particular, we found that monoadduction primarily occurred at m^5CpG* sites. Furthermore, the preference for these sites was reinforced in the methylated DNA compared to the unmethylated samples. The enhancement was large and led to an increase in drug–DNA adduction efficiency and in mitomycin (mitosene) selectivity for m^5CpG* sites.

Table 1 summarizes the impact of DNA methylation at CpG* sites on mitomycin monoalkylation transformations. Listed are the observed increases in DNA modification by **1**–**3** at *all* the guanine (G*) sites in the methylated DNA samples ('Effect of DNA methylation on G* modification'); they are compared with the corresponding unmethylated DNA fragments. The data for each assay are given separately. The increases provide a measure of the effect of DNA methylation on the efficiency of mitomycin (mitosene) adduction. Also listed in Table 1 are the average increases and the range of increases observed for mitomycin modification at *only* CpG* sites upon C(5) cytosine methylation ('Effect of DNA methylation on CpG* modification'). Finally, we report the effect of DNA methylation at guanine (G*) sites, other than CpG* ('Effect of DNA methylation on NpG* modification (N≠C)').

The values listed in Table 1 showed that while the two assays provided qualitatively similar results there were differences. Several factors may have contributed to the variations. First, the bands generated after UvrABC cutting were more distinct than those generated by λ-exonuclease permitting us to more accurately quantify the UvrABC cleavage sites. Second, it appears that the UvrABC assay was more sensitive than the λ-exonuclease assay and thereby allowed detection of minor drug lesions. Similar observations have been reported when these two assays were used to detect anthramycin–DNA adducts.[36] Finally, we determined that, under the conditions

Table 1. The effect of C(5) cytosine methylation of CpG sequences on mitomycin adduction

Mitomycin substrate	Effect of DNA methylation on G* modification[a]		Effect of DNA methylation on CpG* modification[b]		Effect of DNA methylation on NpG* modification (N≠C)[c]	
	UvrABC assay	λ-Exo assay	UvrABC assay	λ-Exo assay	UvrABC assay	λ-Exo assay
*Bst*NI-*Eco*RI 129 base pair						
MC (**1**)	7.4	2.1	> 32 (2.8– > 100)	2.6 (1.8–3.7)	0.4[d]	1.8
10-DCMC (**2**)	—[e]	4.3	—[e]	6.8 (3.9–12)	—[e]	2.9
MsNMA (**3**)	3.1	2.7	13 (2.2–26)	8.5 (1.9–16)	—[f]	0.9
*Bst*NI-*Nar*I 146 base pair						
MC (**1**)	2.0	1.9	15.3 (0.8–55)	3.6 (1.1–8.3)	0.2	0.3
10-DCMC (**2**)	—[e]	3.0	—[e]	19.9 (1.4–50)	—[e]	1.0
MsNMA (**3**)	2.1	3.2	3.4 (0.8–7.1)	4.0 (2.1–6.7)	0.6	0.2

[a]Total normalized intensities for G* modification at all sites in methylated DNA/total normalized intensities for G* modification at all sites in unmethylated DNA.
[b]The average per site increase in the normalized intensity for CG* modification in methylated DNA versus the same site in the unmethylated DNA:

$$\frac{\Sigma\left(\frac{mCG_1}{CG_1}+\frac{mCG_2}{CG_2}+\cdots\right)}{nCG_n}.$$

The numbers in parentheses show the range in observed increases.
[c]Total normalized intensities for G* modification at all sites other than CpG* sites in methylated DNA/Total normalized intensities for G* modification at all sites other than CpG* sites in unmethylated DNA.
[d]Value corresponds to a single modification site.
[e]Not determined.
[f]No detectable intensities were observed for G* modification sites other than CpG* loci in the methylated DNA.

employed in the UvrABC assay, DNA incision was independent of the sequence context in both unmethylated[33] and methylated[11] DNA samples. No comparable information concerning the corresponding λ-exonuclease process is available. These concerns led us to rely on the UvrABC assay for quantitative information concerning the effect of C(5) cytosine methylation on mitomycin (mitosene) monoalkylation processes. Nonetheless, the λ-exonuclease assay provided a qualitative assessment of the effect of DNA methylation on drug bonding processes that was consistent with the UvrABC data.

Effect of C(5) cytosine methylation on the extent of overall DNA modification

We find that C(5) cytosine methylation of CpG sites in the 129- and 146-bp fragments was virtually complete and led to increases in the overall extent of mitomycin (mitosene) adduction at guanine sites. The calculated increase for reductively activated **1** for the two DNA fragments using the UvrABC assay was 7.4- and 2.0-fold, and for the λ-exonuclease stop assay it was 2.1- and 1.9-fold. Similarly, we observed for **3**-DNA experiments a 2.1- to 3.2-fold increase in DNA modification using the UvrABC and λ-exonuclease stop assays. We determined the corresponding values for reductively activated **2** using only the λ-exonuclease assay. For the 129- and 146-bp fragments, we found 4.3- and 3.0-fold increases in DNA modification, respectively, upon DNA methylation. These values fall within the ranges observed for **1** and **3**, indicating that the relative increases in the extent of DNA adduction upon C(5) cytosine methylation was not affected by removing the C(10) carboxamide moiety.

The findings in Table 1 tells why C(5) cytosine methylation led to significant increases in *overall* mitomycin (mitosene)-DNA adduction. Listed in Table 1 is the observed average increase in drug modification at only CpG* sites upon conversion to m^5CpG* sequences. The numbers varied with the mitomycin (mitosene), the DNA, and the assay. The large variation was expected given the limited number of CpG* sites monitored and the differences in the two assays. Nonetheless, we found significant increases in the extent of mitomycin (mitosene) adduction at many CpG* sites using both assays. This enhanced reactivity of specific m^5CpG* sites toward drug modification is responsible for the observed overall increased efficiency of mitomycin (mitosene) adduction for methylated DNA samples compared with unmethylated samples.

Table 1 data provided support for the findings of Millard,[12] Tomasz,[13,15] and our recent report.[11] Both Millard[12] and Tomasz[13] showed that mitomycin C oligonucleotide cross-linking transformations were enhanced by 1.8- to 2.0-fold upon C(5) cytosine methylation. Furthermore, Tomasz reported that mitomycin C-DNA monoalkylation transformations increased by a comparable margin.[13] Similarly, we observed an average 4.7-fold increase for **1** and 2.6-fold increase for **3** using the UvrABC assay data. The current study also showed that while C(5) cytosine methylation led to an *average* increase in mitomycin C–DNA adduction, the increase at the *individual* m^5CpG* sites was not uniform. We observed different levels of increased adduction that spanned two orders of magnitude.

Our studies indicated that an additional factor may have contributed to the finding that C(5) cytosine methylation led to only an overall 2.6- to 4.7-fold increase in mitomycin (mitosene) DNA adduction compared with unmethylated DNA. For $Na_2S_2O_4$-mediated mitomycin

C-DNA and *N*-methyl-7-methoxyaziridinomitosene-DNA transformations we found that adduction at most non-CpG sites in methylated DNA samples was reduced compared with the corresponding sites in the unmethylated DNA (Table 1: UvrABC analysis; Figs 1(B) and 3(B)). It is likely this effect may reduce the extent of the *total* amount of drug modification in the methylated DNA samples.

Effect of C(5) cytosine methylation on the sequence selectivity of DNA modification

An important finding in our analyses of the UvrABC histograms for the 129- and 146-bp fragments (Figs 1(B) and 3(B)) was that C(5) cytosine methylation led to significant enhancements in the DNA sequence selectivity for drug adduction to provide a distinctive drug bonding profile. In our earlier study, we showed that mitomycin C preferentially modified CpG* sites compared with TpG*, GpG*, and ApG* sites by factors of 2.9, 4.5, and 5.6:1, respectively.[9] Analysis of the histograms (Figs 1(B), 2(B), 3(B), and 4(B)) showed that upon C(5) cytosine methylation this sequence selectivity for CpG* sites in reductively activated mitomycin C transformations increased > 10-fold at select sites. Similar enhancements were observed in the reductively activated 10-decarbamoylmitomycin C (**2**) reactions and the *N*-methyl-7-methoxyaziridinomitosene (**3**) adduction experiments.

What are the contributing factors for this enhanced selectivity and why does mitomycin adduction for m^5CpG* sites vary within any given DNA fragment? Clearly, most of the increased sequence selectivity stems from the enhanced levels of CpG* site adduction (Table 1). Several hypotheses have been presented to account for the effect of C(5) cytosine methylation on drug modification. Millard and Beachy proposed that the 1.4- to 2.0-fold preference for DNA cross-linking in methylated DNA compared with unmethylated DNA can be accounted for by local charge effects that enhance the nucleophilicity of the guanine 2-amino residue, by local DNA conformational changes that make guanine sites more accessible toward mitomycin modification, or by both.[12] Indeed, C(5) cytosine methylation induces DNA structural changes. These include a cruciform extrusion from a double helix structure, increases in DNA helical pitch, and conversion of DNA from the B to the Z form.[38,39] Correspondingly, Tomasz and co-workers suggested that the C(5) methyl group exerts an electronic effect that is transmitted to the complementary guanine residue leading to an increase in guanine reactivity toward mitomycin C adduction.[13,15,24] We suspect that charge and electronic effects coupled with hydrophobic factors[40] do contribute to the enhancements in mitomycin adduction at m^5CpG* sites compared with CpG* sequences, but they can explain neither the large increases observed nor the transformation of 'silent' or unreactive CpG* sites to 'hot' sites for mitomycin modification.

In Table 2, we list the 8 CpG* sites in the 129- and 146-bp DNA fragments accessible to UvrABC endonuclease analysis and the observed increase in drug adduction upon C(5) cytosine methylation when the intensities of the methylated sample are compared with the unmethylated DNA sample ('m^5CpG Increase'). We also report the five flanking upstream and downstream nucleotides surrounding the CpG* sites. We found that (a) three CpG* sites (sequences 1, 3, and 7) showed large enhancements (m^5CpG Increase: ⩾10-fold average for the combined **1** and **3** experiments) in guanine modification upon DNA methylation; (b) four CpG* sequences (sequences 2, 4, 6, and 8) underwent moderate enhancements (2.4- to 6.1-fold) upon DNA methylation; and (c) one sequence (sequence 5) showed lower levels of adduction upon C(5) cytosine methylation (0.8-fold). Analysis of the upstream and downstream mono- and di- nucleotide DNA residues flanking the CpG* modification site for the eight sequences showed that other than for sequence 5, a higher percentage of A/T residues surrounded the most extensively modified sites. These empirical observations suggest that the surrounding DNA bases influence the effect that C(5) cytosine methylation has on the local DNA structure and drug adduction and that this effect exceeds previous predictions.[12]

Table 2. The relative bonding affinities of **1** and **3** at CpG* sites

Sequence no.	Sequence	**1**-$Na_2S_2O_4$ m^5CpG Increase[a]	**3** m^5CpG Increase[a]
*Bst*NI-*Eco*RI 129-*bp*			
1	5′ A T C A T **C G** A T A A G 3′	16	21
2	5′ T A A T G **C G** G T A G T 3′	2.8	2.6
3	5′ G C T A A **C G** C A G T C 3′	> 100	26
4	5′ G G C A C **C G** T G T A T 3′	10	2.2
*Bst*NI-*Nar*I 146-*bp*			
5	5′ C G T G A **C G** A T C A G 3′	0.8	0.8
6	5′ A T C A G **C G** G T C C A 3′	2.2	2.6
7	5′ A T G A T **C G** A A G T T 3′	55	7.1
8	5′ A G A G C **C G** C G A G C 3′	3.3	3.0

[a]The increase (decrease) in the normalized intensity for mitomycin (mitosene)–CpG* modification in the methylated DNA versus the corresponding site in the unmethylated DNA. The values used have been normalized to the most intense band for each *pair* of methylated and unmethylated DNA experiments (UvrABC endonuclease analysis).

Hurley and co-workers have reported the effect of neighboring DNA sequences on drug adduction. These researchers showed that the efficiency of pluramycin DNA alkylation depended upon the length of the 5′ A tract preceding the N(7) guanine alkylation site.[41] For altromycin B, maximal adduction was observed for three adenines demonstrating that minor groove geometry[42] can markedly affect the reactivity of the pluramycin with the guanine alkyation site. We note, however, that both Millard[12] and Tomasz[13] reported only modest increases in adduction upon DNA methylation for mitomycin C-DNA cross-linking reactions at CpG* sites within oligonucleotides preceded by an A/T-rich sequence.

A second factor contributed to the striking sequence selectively for **1**–**3** modification of specific m^5CpG sites in the methylated DNA samples. Analysis of the extent of NpG* bonding, where N = A, G, or T, showed that DNA methylation in most instances led to slightly *lower* levels of drug modification (Table 1). This was always the case when the sites of mitomycin (mitosene)

adduction were determined using the UvrABC endonuclease assay and in half the cases when the λ-exonuclease assay was used. A similar observation was noted in our earlier study using the 188-bp *Stu*I-*Mse*I DNA fragment and the UvrABC endonuclease assay, and we have suggested that DNA structural changes induced by C(5) cytosine methylation can hinder the interaction of **1** and **3** with surrounding non-CpG sites.[11]

Conclusions

Use of two different enzymatic assays has demonstrated that C(5) cytosine methylation within select CpG* sequences led to enhanced levels of mitomycin modification at these sites. We observed that DNA methylation led to increased levels of DNA adduction, increased selectivity for drug modification at m^5CpG* sites versus other NpG* residues, and the activation of normally unreactive CpG* sites toward mitomycin modification processes. Significantly, C(5) cytosine methylation did not always lead to enhanced mitomycin (mitosene) adduction at m^5CpG* loci. We observed that while C(5) cytosine methylation produced significant increases in mitomycin adduction at most CpG* sites, others were not affected. Relative increases in drug modification at CpG* sites upon DNA methylation varied by two orders of magnitude and appeared to be influenced, in part, by the surrounding DNA sequence. The enhanced reactivity of select m^5CpG* sites to mitomycin C adduction led to a distinctive drug bonding pattern.

Since it has been found that the methylation pattern of tumor cells is different from normal cells we predict that mitomycin C adduction patterns will be distinctive and unique between these two types of cells. The significance of these bonding profiles in cancer chemotherapy is undetermined and is an important issue that requires resolution if we are to understand how mitomycin C expresses its activity at the molecular level.

Experimental

Materials

Mitomycin C was supplied by Bristol-Myers Squibb Co. (Wallingford, CT), and mitomycin A was obtained from Kyowa Hakko Kogyo Co. Ltd. (Osaka, Japan). 10-Decarbamoylmitomycin C was prepared using the procedure of Kinoshita and co-workers with modification.[28] *N*-Methyl-7-methoxyaziridinomitosene was prepared according to the report by Danishefsky and Egbertson.[29] $Na_2S_2O_4$ was purchased from Fisher Scientific Co. Restriction enzymes, DNA polymerase (Klenow fragment), *Sss*I methylase and pBR322 plasmid were obtained from New England BioLabs. NACS Prepacs Cartridge (syringe format) and λ-exonuclease were purchased from BRL. All other chemicals and electrophoretic materials were obtained from either Sigma Chemical Co. or Bio-Rad Laboratories. The [α^{32}P]dTTP (specific activity approximately 3000 Ci/mmol) was purchased from DuPont-New England Nuclear.

DNA Fragments isolation and ^{32}P end labeling

The pBR322 plasmid was first digested with *Bst*NI, and the bands corresponding to the 1857- and 383-bp fragments were isolated from 1.4% agarose gel and cleaned by passing through a NACS Pac followed by ethanol precipitation. The 1857-bp fragment was labeled at the 3′ termini in the presence of [α ^{32}P]dTTP and Klenow fragment (5 units) in 10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, and 7.5 mM DTT at 22 °C (30 min). The labeled DNA fragment was precipitated with ethanol and digested with *Eco*RI. The 129-bp fragment was purified by electrophoresis on a 5% polyacrylamide gel. The 383-bp fragment was labeled at both the upper and the lower 3′ ends with [α ^{32}P]dTTP, precipitated, and then digested with *Nar*I. The resulting 146-bp fragment (labeled at the lower strand 3′ end) was purified as above.

DNA C(5) cytosine methylation

C(5) Cytosine methylation of the DNA restriction fragments was carried out in the same manner as previously described.[11]

Mitomycin C (1)- and 10-decarbamoylmitomycin C (2)-DNA bonding with sodium dithionite

Specified amounts of drugs were added to the radiolabeled DNA in 25 mM Tris·HCl buffer (pH 7.4) to give the desired final concentration. The solutions were deaerated with argon (15 min), and then freshly prepared, deaerated, aqueous $Na_2S_2O_4$ solutions (1 equiv for 0.1, 0.15 and 0.2 mM **1**, 0.6 equiv for 0.45 mM **1**, and 1 equiv for 0.45 and 0.9 mM **2**) were added in three incremental portions (20 min) to give a total volume of 100 μL. The reactions were maintained under argon (1 h) at 0 °C for **1** and 22 °C for **2**.

N-Methyl-7-methoxyaziridinomitosene (3)-DNA bonding

The mitosene was added to the radiolabeled DNA in 25 mM Tris·HCl buffer, pH 7.4, to a final concentration of 1.5 mM. The solution (100 μL) was incubated at 37 °C for 30–60 min. The unreacted drug was removed by phenol-chloroform extraction.

Purification of UvrA, UvrB, and UvrC proteins

UvrA, UvrB, and UvrC proteins were isolated from *E.coli* K12 strain CH292 (recA, end A/FlacI^q) carrying plasmids pUNC45 (*uvr*A), pUNC211 (*uvr*B), and pDR3274 (*uvr*C).[43] The methods of purification were the same as described previously.[35h]

UvrABC nuclease reactions

The UvrABC nuclease reactions were carried out in a reaction mixture as previously described.[11]

λ-Exonuclease digestion of mitomycin (mitosene)-modified DNA

Modified DNAs were resuspended in the λ-exonuclease buffer (67 mM glycine/KOH, pH 9.4, 2.5 mM $MgCl_2$).

*Eco*R1 linearized pBR322 plasmid (2 μg) was added to each sample, followed by λ-exonuclease (8.7 units), and the solutions incubated at 37 °C (15 min). The reaction was stopped by the addition of 2 vol ethanol and the DNA precipitated.

DNA sequencing, DNA sequencing gel electrophoresis, and autoradiography

The 3′ end-^{32}P-labeled DNA fragment was sequenced by the method of Maxam and Gilbert.[26] The ^{32}P-labeled fragments with or without various enzyme treatments were suspended in sequencing tracking dye (80% v/v deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue), heated at 90 °C (3 min), and quenched in an ice bath. The samples were applied to an 8% denaturing sequencing gel in parallel with Maxam–Gilbert sequencing reactions. After electrophoresis, the gels were exposed to Kodak X-Omat AR film or Fuji RX film with an intensifying screen at −70 °C.

Densitometric scanning

The intensities of UvrABC nuclease and λ-exonuclease incision bands were determined with Bio-image Open Windows Version 3 System consisting of a Howteck Scanmaster 3+ and whole band analysis software.

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