Studies on the Bonding Specificity for Mitomycin C–DNA Monoalkylation Processes

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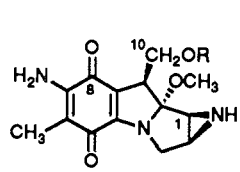
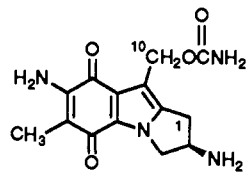
Abstract: Information on the DNA-bonding and sequence specificity for mitomycin C (**1a**) and select derivatives under limiting reducing conditions has been gleaned by using the λ exonuclease stop assay and DNA restriction fragments. This procedure demonstrated that covalent modification of DNA by mitomycin C occurred preferentially at guanine residues within 5′CG and 5′GG sequences. The observed selectivity was shown to proceed at the monoalkylation stage and was independent of the second (cross-linking) drug-bonding event. The bonding properties of mitomycin C have been compared with anthramycin, an antineoplastic agent of comparable size. The results of this investigation are discussed in light of findings previously reported for the bonding specificity of mitomycin C to DNA. Potential explanations are offered for the observed guanine specificity as well as the preference for 5′CG and 5′GG sequences within the duplex DNA.

Mitomycin C (**1a**) is an antineoplastic agent of major clinical significance.[1] Mechanisms have been advanced which suggest that drug function proceeds by initial reductive activation of **1a**



1a, R = C(O)NH$_2$
b, R = H



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followed by covalent bonding[2] of the activated mitomycin species to DNA.[3] Drug attachment is believed to proceed sequentially at carbons-1 and -10 in **1a** leading to the production of DNA cross-linked products. Cytotoxicity and antitumor activity have been associated with the formation of the interstrand linked adducts.[3a,f] Extensive studies have provided information concerning key aspects of the events that precede each of the DNA-bonding steps.[4-12] These investigations lend general support for the prescient hypothesis of Iyer and Szybalski.[3a] Studies have also been directed toward elucidating the mechanism of the DNA–drug interaction process.[3a,4a,13-25] Recently, a series of carefully constructed and insightful investigations have been reported that provide specific details concerning these transformations.[26-32] In

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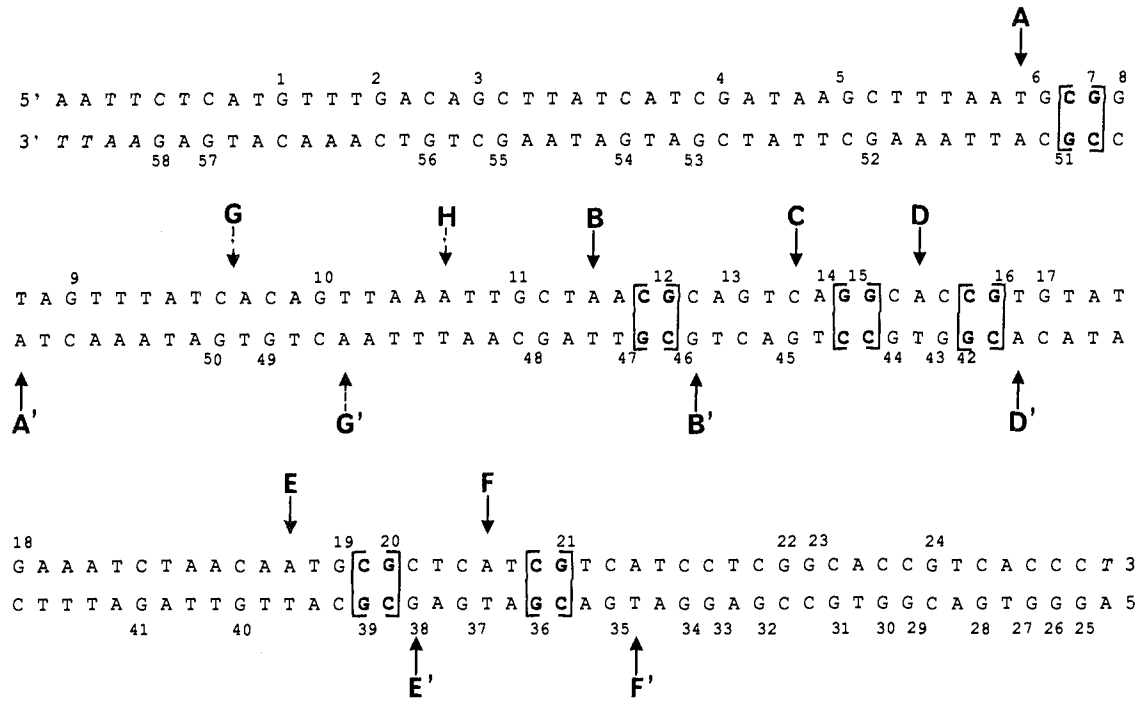

Figure 1. DNA sequence of both strands of the 129-bp fragment of interest from pBR322 plasmid showing the λ-exo stop sites (arrows) after treatment with mitomycin C (**1a**) under reductive conditions. Data are from Figures 2 (upper strand) and 4 (lower strand). The putative mitomycin-bonding sites are placed in brackets. Bases placed in italics are those filled in during 3'-end-labeling procedure. The radiolabel was introduced with [α-^{32}P]dTTP.

1987, Tomasz, Nakanishi, and co-workers reported that the major products isolated upon exhaustive enzymatic hydrolysis of mitomycin C modified DNA were mono- and bis- (cross-linked) deoxyguanosine–mitomycin C adducts.[26a] Structural analysis indicated that drug attachment occurred at the 2-amino group of the purine residue. Confirmation of the proposed site of drug attachment within the cross-linked adduct has been provided by Patel and his research associates using a synthetic oligomer and ^{1}H NMR spectroscopy.[27] Information concerning the DNA base sequence specificity for drug bonding has been secured by several groups.[22,28-32] Crothers and co-investigators using a gel electrophoresis assay and synthetic double-stranded DNA oligomers have shown that reductively activated **1a** preferentially *cross-linked* 5'CG sequences rather than 5'GC sequences.[29] A similar conclusion was arrived at by Hopkins and co-workers employing a single-hit DNA random cleavage method with select synthetic DNA duplexes[30] and by Tomasz and co-investigators using synthetic oligomers and a HPLC assay.[26d,e] Finally, two research teams have confirmed that drug attachment preferentially occurred at guanine in mitomycin-modified DNA restriction fragments.[31,32] Phillips and co-workers employed an in vitro transcription method,[31] whereas Rupp and co-investigators utilized the *Escherichia coli* UvrABC endonuclease incision assay[32] to monitor the site of mitomycin bonding.

In this paper, we demonstrate that employment of the λ exonuclease (λ-exo) stop assay[33,34] with mitomycin C (**1a**) and decarbamoylmitomycin C[35] (**1b**) modified DNA restriction fragments revealed that the *initial* covalent drug-bonding event at carbon-1 in **1a** proceeded at preferred base sequences and was independent of the second drug-bonding step. This observation was surprising in light of recent projections that have suggested that the base sequence selectivity displayed by the antineoplastic agent was primarily controlled by either the cross-linking event[29] or by steric interactions that occur between the carbon-10 carbamate group of the activated drug and bases proximal to the alkylation site.[26e]

Results

The site of mitomycin bonding was determined by locating the stop sites induced by the processive enzyme, λ-exo.[34] The first DNA restriction fragment employed was a 129 base pair sequence (Figure 1) isolated from pBR322 plasmid. In the initial reactions the upper strand 3'-end-labeled duplex DNA was treated in separate experiments with mitomycin C (**1a**), decarbamoylmitomycin C[35] (**1b**), and 2,7-diaminomitosene[4b] (**2**). The autoradiograms for the sequencing gels produced for **1a** and **1b** are presented in Figures 2 and 3, respectively. Compounds **1b** and **2**, unlike **1a**, are DNA *mono*alkylating agents. The deletion of the carbon-10 carbamoyl group in **1b** prevented reaction with the genetic material at this site. Correspondingly, the aziridine ring-cleaved compound **2** can react with DNA only at carbon-10.[24] Several different sodium dithionite mediated procedures[24,26,29,30] were employed to activate the drug. In most instances, a *single* stoichiometric equivalent of a freshly prepared aqueous solution of sodium dithionite was incrementally added (three installments) over a 60-min time period to a deaerated solution containing the mitomycin derivative (i.e., **1a**, **1b**, and **2**) and DNA. These conditions proved sufficient to activate the carbon-1 site in **1a** (Figure 2) and **1b** (Figure 3), but not the carbon-10 position in **2** (data not shown). In the case of mitomycin C, this protocol led to insignificant amounts of interstrand cross-linked adducts as evidenced by the absence of noticeable amounts of radioactive material in the sequencing gel with electrophoretic mobilities slower than the starting denatured 129 base pair fragment (portion of gel for Figure 2 not shown).

The inability to detect drug–DNA bonding for 2,7-diaminomitosene (**2**) with 1 equiv of sodium dithionite led us to use higher stoichiometric amounts (4 equiv) of the reductant for both **1a** and **2**. Previous studies have demonstrated that drug modification in **1a** proceeded initially at carbon-1 followed by carbon-10, and that substitution processes at the former site are considerably more efficient than those at the latter position.[3a,5b,c,12d,e,13] Two protocols were adopted.[24,29] They differed principally in the length of time (30 versus 60 min) and the temperature (20 versus 0 °C) utilized for the drug-bonding process. Both procedures gave comparable results. Use of 4 equiv of

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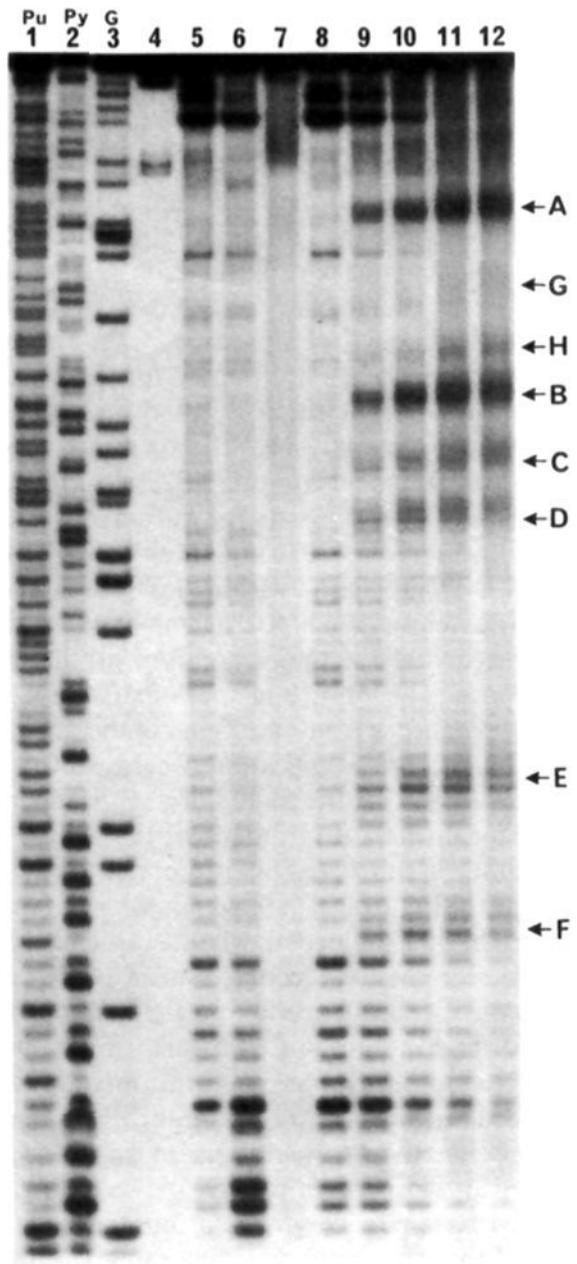


Figure 2. Autoradiogram of λ-exo stop sites of mitomycin C (**1a**) modified 3′-labeled 129-bp fragment from pBR322 plasmid (upper strand). Lanes 1–3, Maxam–Gilbert chemical sequencing reactions of AG(Pu), TC(Py), and G, respectively; lane 4, DNA without drug treatment; lane 5, λ-exo digestion of DNA without prior drug modification; lane 6, λ-exo digestion of DNA in the presence of **1a** (1.5 mM) but in the absence of sodium dithionite; lane 7, DNA treated with mitomycin C (1.5 mM) and sodium dithionite (1.5 mM); lanes 8–12, λ-exo digestion of **1a**-bound DNA at 0.015, 0.15, 0.45, 0.90, and 1.50 mM final concentration of **1a**, respectively, after reductive activation with 1 equiv of sodium dithionite.

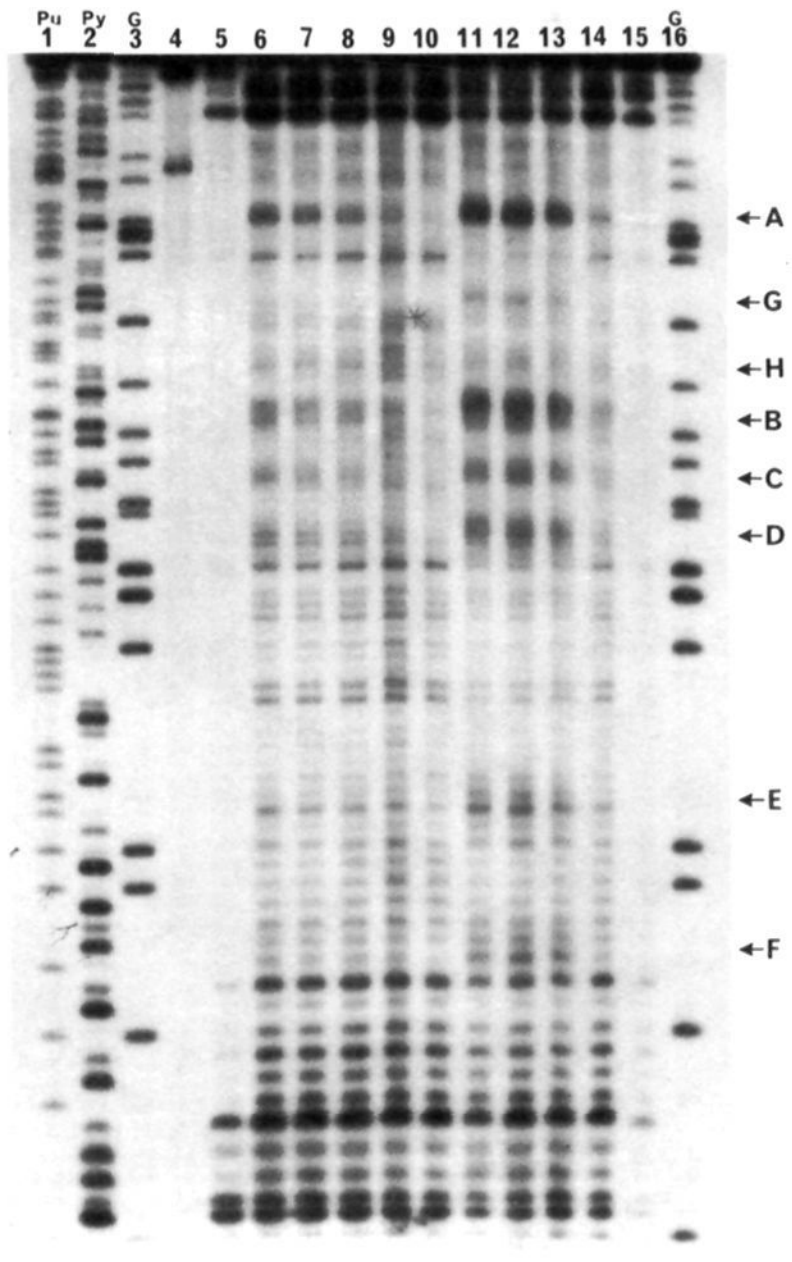


Figure 3. Autoradiogram of λ-exo stop sites of decarbamoylmitomycin C (**1b**) modified 3′-labeled 129-bp fragment from pBR322 plasmid (upper strand). Lanes 1–3 and 16, Maxam–Gilbert chemical sequencing reactions of AG(Pu), TC(Py), G, and G, respectively; lane 4, DNA without drug treatment; lane 5, λ-exo digestion of DNA without prior drug modification; lanes 6–10, λ-exo digestion of **1b**-bound DNA at 1.50, 0.90, 0.45, 0.15, and 0.015 mM final concentration of **1b**, respectively, after reductive activation with 1 equiv of sodium dithionite in the presence of tRNA as carrier; lanes 11–15, λ-exo digestion of **1b**-bound DNA at 1.50, 0.90, 0.45, 0.15, and 0.015 mM final concentration of **1b**, respectively, after reductive activation with 1 equiv of sodium dithionite in the absence of tRNA as carrier.

sodium dithionite for the activation of mitomycin C (**1a**) led to the appearance of extensive amounts of radioactive material in the sequencing gel corresponding to DNA adducts having higher molecular weight than the initial denatured 129 base pair (data not shown). The observed diffuse bands have been tentatively attributed to the formation of DNA–mitomycin C interstrand cross-linked adducts.[29] Surprisingly, use of a comparable procedure (4 equiv of sodium dithionite) to activate **2** did not lead to the appearance of any new pronounced stop sites after λ-exo digestion, suggesting that no significant drug–DNA modification had occurred in these experiments.[24]

The autoradiogram for the denatured sequencing gel obtained in the mitomycin C mediated reactions with the pBR322 restriction fragment under limiting reducing conditions (1 equiv) is shown in Figure 2. Few natural stop sites were observed with unmodified DNA after λ-exo digestion (Figure 2, lane 5). Administration of **1a** led to the appearance of six prominent new stop sites (Figure 2, lanes 8–12). These sites are labeled A–F, respectively, in Figure 2. The intensity of bands A–C as visualized in the autoradiograms increased with increasing drug concentration throughout the dose range of **1a** employed. A similar trend was observed for bands D–F except that the intensity of the band corresponding to the highest dose of mitomycin C utilized appeared to be somewhat diminished. In addition to bands A–F, two faint new stop sites were detected in the autoradiogram, which increased with increasing mitomycin C concentration. These are labeled G and H, respectively, in Figure 2. In no cases did we note a distinctive loss of radioactive material in the lower molecular weight region of the gel as the dose of **1a** was increased. This pattern was observed for anthramycin and is expected in light of the processive nature of the λ-exo assay.[34] We have attributed the absence of this feature in the present study to the inefficiency of the mitomycin C–DNA bonding process, the narrow span of drug doses employed, and the apparent high base sequence selectivity exhibited by **1a**. These factors should act in concert to limit the number of mitomycins bound to any given DNA restriction fragment.

A comparable pattern was observed in the autoradiogram obtained from the decarbamoylmitomycin C (**1b**) modified DNA experiment (Figure 3). Two different protocols were employed for drug bonding. In one procedure (Figure 3, lanes 6–10) tRNA was used as a carrier to ensure that the effective concentration of DNA remained constant within this series of experiments and to aid in the precipitation of the modified DNA, while in the second method (Figure 3, lanes 11–15) no tRNA was utilized. Both procedures gave comparable results, although the bands observed for the stop sites with the latter procedure were more intense. The autoradiogram indicated the appearance of six new, distinct stop sites (labeled A–F) as well as two faint stop sites (labeled G and H) after λ-exo digestion, which increased with increasing drug dose. Significantly, the electrophoretic mobilities

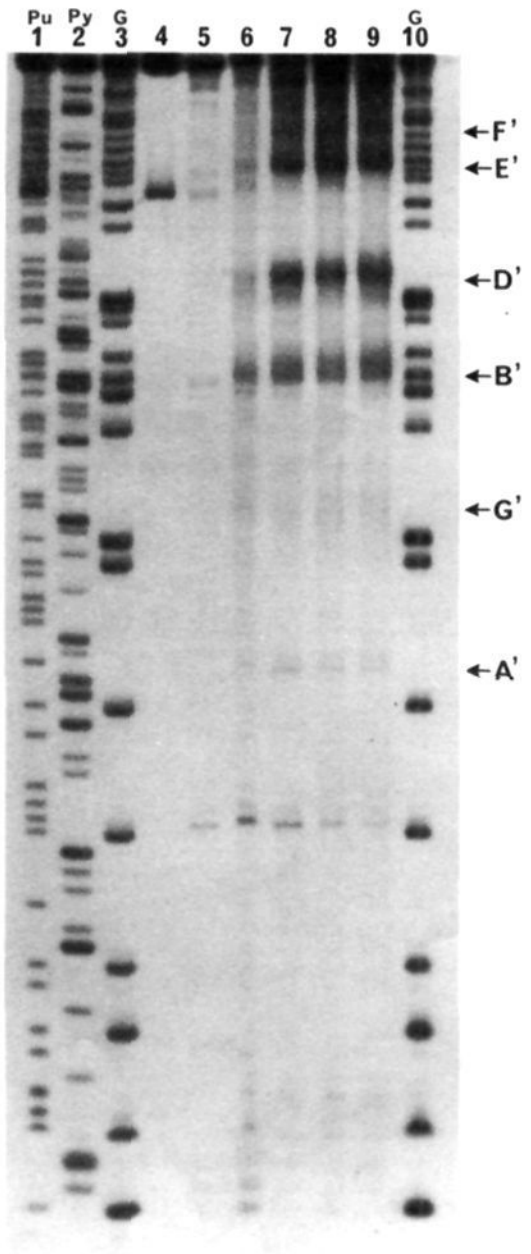


Figure 4. Autoradiogram of λ-exo stop sites of mitomycin C (**1a**) modified 3′-labeled 129-bp fragment from pBR322 plasmid (lower strand). Lanes 1–3, 10 Maxam–Gilbert chemical sequencing reactions of AG(Pu), TC(Py), G, and G, respectively; lane 4, DNA without drug treatment; lane 5, λ-exo digestion of DNA without prior drug modification; lanes 6–9, λ-exo digestion of **1a**-bound DNA at 0.15, 0.45, 0.90, and 1.50 mM final concentation of **1a**, respectively, after reductive activation with 1 equiv of sodium dithionite.

of bands A–H in Figure 3 corresponded to the stop sites A–H induced by mitomycin C (Figure 2).

Identification of the drug-bonding sites A–H in the pBR322 restriction fragment was aided by the initial preparation of a double-stranded DNA fragment, which spanned the 129 base pairs of interest and which contained a 3′-^{32}P label on the complementary strand. The autoradiogram of the sequencing gel obtained after treatment of this modified DNA with reductively activated mitomycin C (**1a**) is displayed in Figure 4. Six new, pronounced λ-exo stop sites (labeled A′, B′, and D′–G′) were observed in the autoradiogram for the **1a**-treated DNA, which increased with increasing mitomycin C concentration (Figure 4, lanes 6–9).[36] Correspondingly, no dose-dependent bands were detected after λ-exo digestion of the 2,7-diaminomitosene-(**2**) treated DNA (data not shown).

The results obtained from the mitomycin C (**1a**)–DNA modified experiments using the complementary 3′-labeled 129 base pair fragments from pBR322 plasmid are summarized in Figure 1. The distinct stop sites A–F and A′, B′, and D′–F′ observed in each of these studies are indicated by the solid arrows. The less intense stop sites G, H, and G′ are presented by broken arrows. Identification of the individual λ-exo stop sites was accomplished upon inspection of the bands produced in the autoradiograms after treatment of the labeled DNA base pair fragments with relatively low amounts of either **1a** or **1b** (i.e., Figure 2, lane 9; Figure 3, lanes 13 and 14; Figure 4, lane 6; Figure 6, lanes 9 and 10). Significantly, each of the pronounced stop sites appeared two to three bases 5′ to a presumptive mitomycin C–guanine adduct located on one of the two strands. This offset in the stop site from the mitomycin-bonding locus may emanate from a drug-induced conformational change in the DNA, an interaction of λ-exo with the modified mitomycin at the drug-bonding site, or an alteration of the electrophoretic mobility of the DNA-cleaved product after mitomycin modification. A comparable although smaller dislocation (one to two bases) has been noted for the λ-exo stop sites induced in anthramycin-modified DNA.[34]

A consensus sequence analysis of the six major stop sites A–F in the 129 base pair fragment from pBR322 plasmid suggests that a preferential base sequence exists for mitomycin C (**1a**) as well as decarbamoylmitomycin C (**1b**) bonding. Five of the six stop sites (A, B, and D–F) appeared within close proximity of a 5′CG sequence. No attempt was made to determine the relative affinities of **1a** and **1b** for each of the major bonding sites A, B, and D–F in light of the processive nature of the λ-exo assay. Enzymatic digestion is known to occur from the 5′-end of the duplex DNA.[33,34] Under conditions of limited digestion, we suspect that mitomycin-bonding sites located at the 5′-end of any given strand should be more apparent in the autoradiograms than those situated at the corresponding 3′-end.[37] This projection accounts for the patterns observed in Figures 2–4, in which the most intense bands corresponded to higher molecular weight radioactive materials. The remaining stop site C occurred near a 5′GG sequence. Significantly, no 5′CG sequence within the reading frame of the gel appeared to remain unmodified as determined by the λ-exo assay. The proposed 5′CG and 5′GG mitomycin-bonding sites have been placed within brackets in Figure 1.

The sequence selectivity detected for mitomycin C (**1a**)– and decarbamoylmitomycin C (**1b**)–DNA bonding with the pBR322-derived base pair fragment prompted our inquiry into the generality of this phenomenon. Accordingly, a 142 base pair sequence from bacteriophage φX174 RF 1 DNA was selected for study (Figure 5). This fragment was utilized by Komano and co-workers to determine the DNA sequence specificity for mitomycin C bonding under conditions where drug activation was accomplished by an *aerobic* $NaBH_4$ reductive procedure.[22] In this study, the location of **1a** bonding was ascertained by the induction of heat-labile sites in DNA fragments.

The autoradiogram obtained after λ-exo digestion of the mitomycin C modified φX174 RF 1 3′-end-labeled 142 base pair fragment is presented in Figure 6 along with a comparable study using anthramycin in place of **1a**. In the case of mitomycin C, drug activation was accomplished with 1 equiv of sodium dithionite. Few new stop sites were revealed in the mitomycin C experiments (Figure 6, lanes 9–12). Three of these were dose dependent and are labeled A–C. The remaining sites D–F exhibited an apparent decrease in radioactive material at the highest dose of **1a** employed. The location of the six stop sites in the starting duplex DNA are given by the solid arrows in Figure 5. All six of the mitomycin C induced stop sites A–F are in close proximity to either 5′CG or 5′GG sequences. These bases have been placed within brackets in Figure 5. This apparent bonding selectivity pattern reinforces the observations ascertained with the 129 base pair fragment from pBR322 plasmid. Significantly, several 5′CG and 5′GG sequences remained unaffected by mitomycin C treatment. This observation suggests that a preference in bonding may exist at each of these two different recognition sites due to differences in the local environment (i.e., DNA base composition and DNA structure). Information concerning the mitomycin C bonding processes was also provided upon analysis of the λ-exo stop sites induced after DNA–anthramycin modification (Figure 6, lanes 13–16). Several new drug dose dependent

(36) In this set of experiments, no stop site was detected corresponding to site C using the upper strand 3′-^{32}P-labeled DNA (Figure 2). It is not known whether this phenomenon is associated with the proposed 5′GG-bonding locus.

(37) For a comparable observation with exonuclease III, see: Hurley, L. H.; Reck, T.; Thurston, D. E.; Langley, D. R.; Holden, K. G.; Hertzberg, R. P.; Hoover, J. R. E.; Gallagher, G.; Faucette, L. F.; Mong, S.-M.; Johnson, R. K. *Chem. Res. Toxicol.* **1988**, *1*, 258.

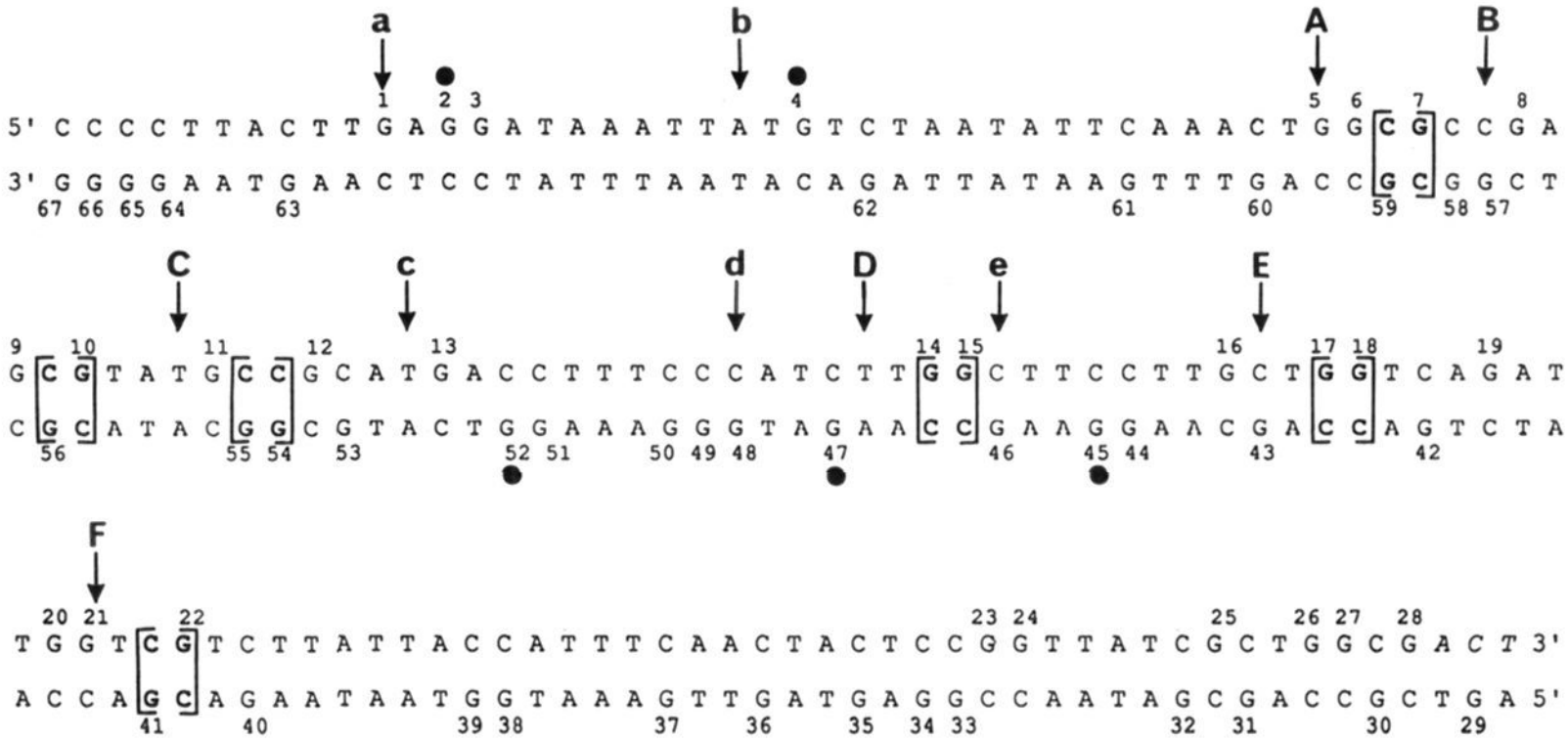


Figure 5. DNA sequence of both strands of 142-bp fragment of interest from ϕX174 RF 1 DNA showing the λ-exo stop sites after treatment with mitomycin C (**1a**) under reductive conditions (capital letters), and anthramycin (lower case letters). Data are from Figure 6. The putative mitomycin C bonding sites are placed in brackets and the putative anthramycin-bonding sites are indicated by solid circles. Bases placed in italics are those filled in during the 3′-end-labeling procedure. The radiolabel was introduced with [α-^{32}P]dTTP.

sites were observed. These stop sites are indicated by the letters a–e in Figure 6, and their potential bonding sites in the duplex DNA are denoted by solid circles in Figure 5. Anthramycin is known to bond specifically at the exocyclic 2-amino group of guanine residues within the minor groove of DNA with drug bonding proceeding preferentially at 5′PuGPu sequences.[2,37] Comparison of the mitomycin C (Figure 6, lanes 9–12) with the anthramycin (Figure 6, lanes 13–16) experiments indicated that drug bonding proceeded more efficiently with anthramycin than with mitomycin C. For example, the λ-exo stop assay detected the anthramycin-bonding sites after treatment of the DNA with a 0.2 μM solution of anthramycin, while the mitomycin C bonding sites were revealed only after treatment of the DNA with a 0.45 mM solution of mitomycin C. This difference in reactivity has been attributed (in part) to the need of an external reductant for mitomycin C activation.

The 142 base pair fragment from ϕX174 RF 1 DNA was used to directly compare the mitomycin C–DNA sequence information obtained from the induction of heat-labile sites in drug-modified DNA (activation of **1a** with $NaBH_4$ in air)[22] with the corresponding data using the λ-exo stop assay (activation of **1a** with sodium dithionite under anaerobic conditions) (Figure 7). In the former method, the concentration of mitomycin C employed varied from 0.025 to 0.20 mM (Figure 7, lanes 5–8); while in the latter procedure, the concentration of mitomycin C ranged from 0.15 to 1.5 mM (Figure 6, lanes 9–12; Figure 7, lanes 10 and 11). The low levels of DNA modification observed with the $NaBH_4$ reductive procedure required us to apply higher amounts of radioactive material in the sequencing gel for these experiments versus those reactions in which DNA bonding was monitored by using the λ-exo stop assay. The results obtained in the $NaBH_4$-mediated experiments (Figure 7, lanes 5–8) mirrored the observations reported by Komano and co-workers.[22] Numerous heat-labile sites were revealed after treatment with mitomycin C. This result differed from the observation secured with the λ-exo assay (Figure 7, lanes 10 and 11) in which few drug-dependent stop sites were detected. Significantly, the heat-induced labile sites found with the $NaBH_4$ protocol closely matched those observed from the Maxam–Gilbert guanine-specific chemical cleavage procedure[38] (Figure 7, lanes 3 and 9). The intensities of the radioactive bands in lanes 5–8 (Figure 7) for the $NaBH_4$ experiments varied, as did the intensities for the radioactive

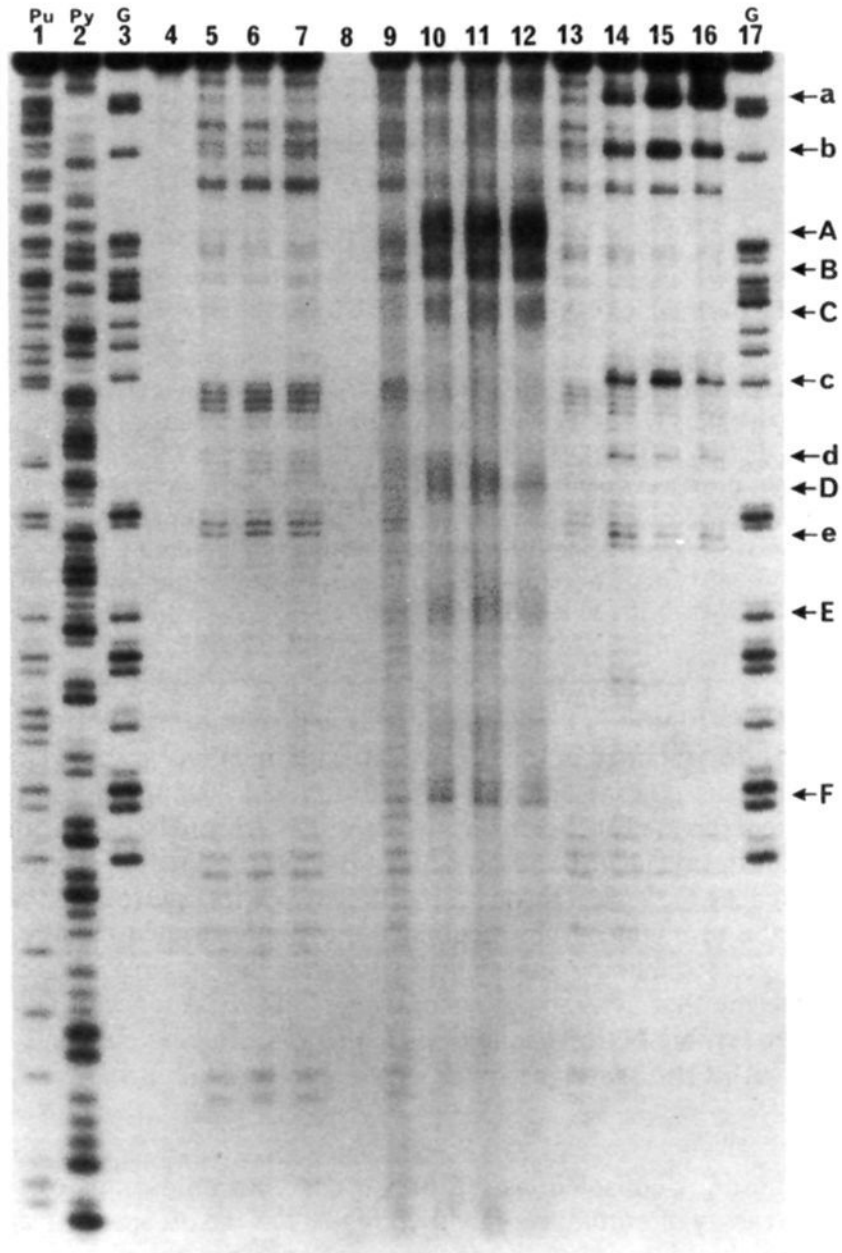


Figure 6. Autoradiogram of λ-exo stop sites of mitomycin C (**1a**) and anthramycin-modified 3′-labeled 142-bp fragment from ϕX174 RF 1 DNA. Lanes 1–3 and 17, Maxam–Gilbert chemical sequencing reactions of AG(Pu), TC(Py), G, and G, respectively; lane 4, DNA without drug treatment; lane 5, λ-exo digestion of DNA without prior drug modification; lane 6, DNA treated with **1a** (1.5 mM); lane 7, DNA treated with sodium dithionite (1.5 mM); lane 8, blank; lanes 9–12, λ-exo digestion of **1a**-bound DNA at 0.15, 0.45, 0.9, and 1.5 mM final concentration of **1a**, respectively, after reductive activation with 1 equiv of sodium dithionite; lanes 13–16, λ-exo digestion of anthramycin-modified DNA at 0.02, 0.2, 2, and 10 μM final concentration of anthramycin, respectively.

(38) (a) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499. (b) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.

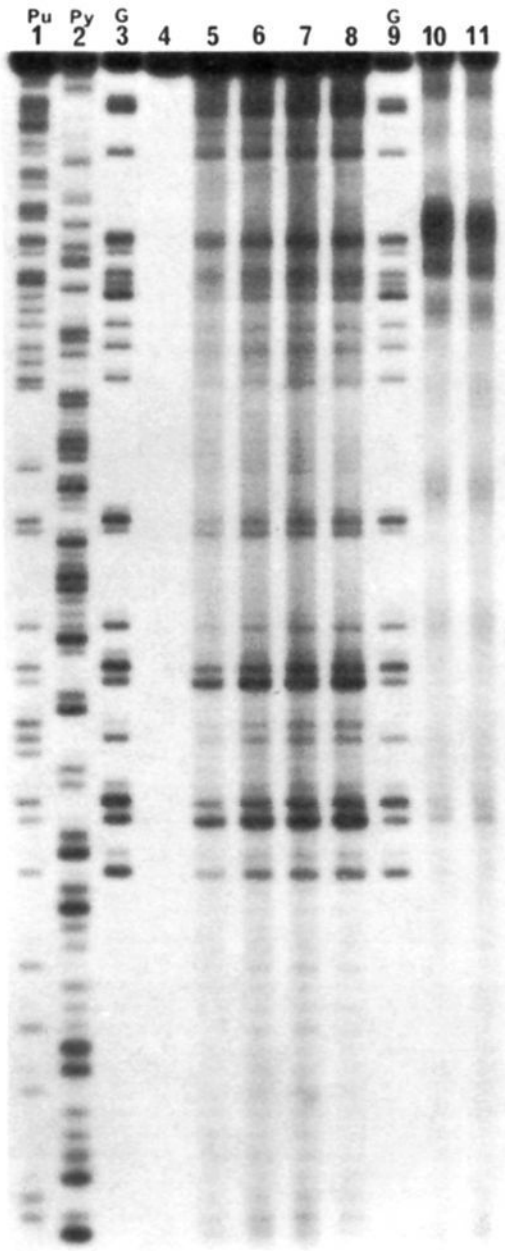


Figure 7. Autoradiogram of 3′-labeled 142-bp fragment from ϕX174 RF 1 DNA after treatment with mitomycin C (**1a**) in the presence of sodium borohydride. Lanes 1–3 and 9, Maxam–Gilbert chemical sequencing reactions of AG(Pu), TC(Py), G, and G, respectively; lane 4, DNA without drug treatment; lanes 5–8, DNA treated with **1a** in the presence of sodium borohydride (5 equiv) at 0.025, 0.05, 0.1, and 0.2 mM of **1a**, respectively; lanes 10 and 11, λ-exo digestion of **1a**-bound DNA at 1.5 and 0.9 mM final concentration of **1a**, respectively, after reductive activation with 1 equiv of sodium dithionite.

products generated from the Maxam–Gilbert guanine-specific reaction (Figure 7, lanes 3 and 9). Furthermore, the pattern of intensities obtained in the two sets of experiments (Figure 7, lanes 5–8 versus lanes 3 and 9) was not identical. Komano and co-workers reported that comparative densitometric analyses indicated that mitomycin C bonding occurred preferentially at 5′PuGT sequences.[22] Visual inspection of lanes 5–8 in Figure 7 did not reveal a pronounced base sequence specificity for drug bonding. The gel sequencing information secured in the present study suggested that activtion of mitomycin C in the presence of DNA under aerobic $NaBH_4$ conditions led to specific guanine bonding but with little sequence specificity.

Discussion

The most conspicuous feature in the autoradiograms of the λ-exo assay of mitomycin-modified DNA was the *absence* of many drug-induced stop sites. The majority of guanine sites in the DNA were unaffected after mitomycin C (**1a**) or decarbamoylmitomycin C (**1b**) treatment. All 12 pronounced mitomycin-induced stop sites (Figures 1 and 5) occurred within close proximity of either a 5′CG or 5′GG sequence. Guanine residues within 5′TG and 5′AG sequences were largely unaffected. Further analysis of the putative 5′CG stop sites revealed that in most instances a pyrimidine base was present on the 3′-side of the putative 5′CG-bonding locus. Unfortunately, the present study does not provide definitive information of whether a preferred trinucleotide (i.e., 5′CGPyr) sequence exists for mitomycin bonding.[39] Several factors contribute to this uncertainty. First, the dislocation of the λ-exo stop sites from the presumptive guanine-bonding loci, the inherent ambiguity in assigning which strand mitomycin modification has taken place, and the diffuse nature of select radioactive bands[40] all contribute to our inability to unequivocally assign the bonding site in several cases. Second, analysis of the nucleotide that flanked the 3′-side of all 5′CG sites within the reading frame of the sequencing gels for both DNA fragments indicated that a higher percentage of pyrimidine versus purine bases existed at this site. Third, no data are available on whether specific mitomycin–base adducts were unable to stall λ-exo digestion. Accordingly, we believe that information on whether drug-bonding specificity extends beyond the observed 5′CG sequence must await the implementation of more sensitive procedures to detect mitomycin–DNA modified adducts, as well as the use of additional DNA fragments containing different sequences. A comparable analysis of the bases that surround the putative 5′GG-bonding locus indicated that in most cases pyrimidine bases flanked the 5′GG sequence. The generality of this finding requires further verification.

The observed preference for 5′CG sequences in DNA for mitomycin C monoalkylation transformations compared favorably with the results obtained by the Crothers,[29] Hopkins,[30] and Tomasz[26c–e] research teams for the DNA sequence specificity displayed by the mitomycins in cross-linking synthetic oligomers. Our findings, however, differ in the underlying causes responsible for this phenomenon. Crothers has suggested that base selectivity stems from the *cross-linking* event. Molecular modeling experiments revealed that while little differences existed in the total energies for the cross-linked adduct occurring at 5′GC versus 5′CG sequences, a substantial reduction in the distance between the guanine amino groups occurred upon formation of the cross-linked adduct in the former sequence when compared to either the starting DNA or the corresponding mitomycin cross-link at the latter sequence.[29] This analysis suggests that the *monoalkylation* of guanine residues within the duplex DNA by the mitomycins should be independent of the neighboring base sequences that flank the guanine site.[41]

Our studies, however, strongly imply that a pronounced base sequence preference exists for the mitomycin C *monoalkylation* step under limiting reducing conditions. In behalf of this contention we note the distinctive pattern of λ-exo stop sites observed for mitomycin C with the two different DNA fragments, the correlation of these sites with 5′CG and 5′GG sequences, and the identity of the mitomycin C and decarbamoylmitomycin C autoradiograms with the 129 base pair DNA from pBR322 plasmid. A previous study provides qualitative support for this hypothesis. Phillips and co-workers have shown through the use of a transcription assay that reductive activation (xanthine oxidase/NADH) of mitomycin C leads to the formation of covalent adducts solely at guanine sites on the DNA.[31] No pronounced base sequence specificity for mitomycin C bonding was noted, however. Our inspection of the relative occupancy of the **1a** transcriptional blockage sites indicates that their data are in agreement with the information secured by use of the λ-exo stop site assay. Of the 12 highest occupancy sites detected in the transcription assay, 11 of these corresponded to transcripts that were terminated one nucleotide prior to a guanine residue present on the coding strand.

(39) Several previous investigations have attempted to address this key issue.[17b,26e,29] Recently, it has been suggested that covalent bonding proceeds preferentially at PuCGPyr·PuCGPyr sequences on the basis of the high yields obtained for the formation of the cross-linked adduct in synthetic oligonucleotides containing this sequence.[26e] Likewise, extensive cross-linked adducts were observed in synthetic oligomers containing tandem CG·CG sequences.[29] Alternatively, an earlier study that focused on the DNA monoalkylation event noted no base specificity for the base residing on the 3′-side of the guanine-bonding locus.[17b]

(40) The apparent 5′CG base sequence preference for mitomycin C bonding may contribute to the diffuseness of the bands observed in several cases. Drug–DNA modification may have randomly taken place at both guanine sites that occur on the complementary DNA strands within this base sequence.

(41) Crothers and co-workers have also reported that mitomycins noncovalently bind A·T and G·C sequences with comparable efficiencies.[29]

In 8 of these 11 transcripts the base 5′ to the presumptive guanine-bonding site was a cytosine, while in the remaining 3 it was a guanine. Prominent but less populated bonding sites were detected for the other two possible 5′NG dinucleotide sequences. Finally, there was only one 5′CG sequence within the reading frame of the coding strand that was not extensively effected by mitomycin C treatment.[42]

The results of the λ-exo study were not in agreement with two previous investigations, however. In a report that appeared in 1989, the 187 base pair *Eco*RI/*Eco*RV fragment of pBR322 plasmid was treated with high concentrations of *N*-methylmitomycin A and 4 equiv of sodium dithionite.[32] Information concerning the position of drug bonding was determined by observing the sites of DNA incision induced by *E. coli* UvrABC endonuclease. Analysis of the autoradiograms obtained in this study indicated that DNA modification had proceeded at 30 different guanine sites within the DNA duplex. Most of the bonding sites were assigned to *N*-methylmitomycin A–DNA monoadducts, and no distinct sequence selectivity was noted for the formation of these lesions. The DNA fragment utilized in the UvrABC endonuclease study was nearly identical with that employed by us in the λ-exo assay (Figure 1). Interestingly, one of the projected recognition sites (Figure 1, guanines 21 and 36) detected in our investigation remained unaffected by *N*-methylmitomycin A treatment. The current data do not permit us to provide an explanation for the observed differences in these two studies. We do note that the conditions employed in the mitomycin-bonding step varied. In the UvrABC endonuclease investigation, higher drug concentrations and extensive reducing conditions were utilized, while in the λ-exo protocol, lower amounts of drug and limiting reducing conditions were employed. Moreover, we suspect that inherent differences exist in the reactivity of *N*-methylmitomycin A and mitomycin C, as well as the sensitivity of the UvrABC and λ-exo enzyme systems to recognize drug–DNA lesions. The high sequence selectivity observed with the λ-exo assay was also not consistent with the information derived from the aerobic $NaBH_4$-mediated procedure for the induction of heat-labile sites in mitomycin C modified DNA. Komano and co-workers have claimed that the latter technique provides a sensitive method to determine the sequence specificity for mitomycin C–DNA bonding.[22] Using this protocol, we observed that **1a** bonding proceeded specifically at guanine sites (Figure 7, lanes 5–8). However, no pronounced patterns for the guanine modification sites were noted within the DNA, and no appreciable evidence was found for the suggestion that **1a** modification preferentially occurred at 5′PuGT sequences. Reduction of the molarity of **1a** did not lead to an increase in mitomycin C sequence selectivity. The similarity of the patterns observed in the $NaBH_4$-mediated experiments (Figure 7, lanes 5–8) versus that obtained with the Maxam–Gilbert guanine-specific chemical reactions (Figure 7, lanes 3 and 9) suggested that mitomycin C under the employed reductive conditions (aerobic $NaBH_4$) functioned as a relatively nondiscriminating guanine alkylating agent, and that subsequent reexposure of the mitomycin-modified DNA to $NaBH_4$ in air led to heat-induced oxidative (i.e., hydroxyl radicals) strand cleavage providing the electrophoretic pattern of radioactive materials observed in the sequencing gel.[43]

(42) An important difference in the λ-exo and in vitro transcription assays was the enhanced number of mitomycin C–DNA bonding sites detected with the latter method. Several factors may have contributed to this finding. Among these are the following: (1) mitomycin C–DNA modification may have proceeded to a greater extent with the xanthine oxidase/NADH (transcription assay) reductive procedure than with the sodium dithionite (λ-exo assay) activation method (1 equiv); (2) the reduction (ionization) state and reactivity of the activated mitomycin C complex may differ in the two procedures; and (3) the sensitivity of the transcription technique to detect mitomycin–DNA modification sites may be greater than that of the λ-exo stop assay.

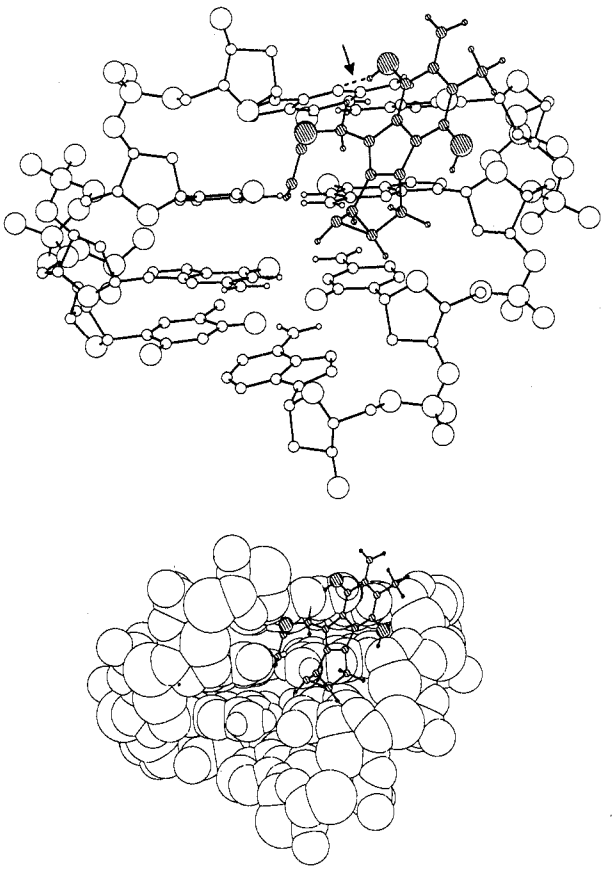
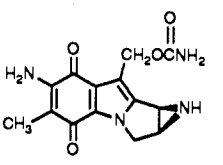


Figure 8. Idealized representation of reductively activated mitomycin C species in the presence of the tetranucleotide duplex CGCA·TGCG *prior* to covalent bonding at carbon-1 in mitomycin C (shaded figure), showing the formation of the key hydrogen bond (arrow) between the carbon-8 hydroxyl group in the activated mitomycin hydroquinone species and the N(3) pyrimidine nitrogen atom of the guanine residue on the complementary strand: (a, top) ball and stick model; (b, bottom) space-filling model.

The results derived from the λ-exo stop site assay provide evidence that the initial covalent mitomycin C–DNA bonding transformation proceeded preferentially at 5′CG and 5′GG sequences within the duplex DNA. We suspect that the affinity of the drug for this dinucleotide sequence is further reinforced by the second, cross-linking event. The factors responsible for the monoalkylation base sequence specificity have not been discerned. Recently, it has been suggested that although the second step (cross-linking event) is primarily responsible for the stringent 5′CG sequence requirement for mitomycin C processes, a bonding preference does exist at the monoalkylation level as well.[26e] The presumptive factor for the latter selectivity has been attributed to the steric interactions encountered by the carbon-10 carbamate group of **1a** with the bases appearing opposite to the bases that flank the putative guanine-bonding site. In addition, the preferred

(43) Little information is known about the mechanism of $NaBH_4$-mediated reductions of **1a**. This permits us only to speculate on the likely source for the observed difference in guanine selectivity between the two reductive methods (i.e., anaerobic, sodium dithionite versus aerobic, $NaBH_4$). In one scenario, treatment of **1a** with $NaBH_4$ in air leads to the formation of *reduced* aziridinomitosene, which then is oxidized to give aziridinomitosene (i).[6] Compound i is expected to rapidly react with nucleophilic species (i.e., guanine residues) to give the corresponding carbon-1 alkylated product.



i

bonding orientation within the minor groove is proposed to benefit from the formation of two hydrogen bonds between the carbon-10 carbamate group in **1a** and the DNA.[44] Our finding that mitomycin C and decarbamoylmitomycin C displayed comparable DNA-bonding properties eliminated the notion that the sequence selectivity stems from secondary interactions of the carbon-10 carbamate group in **1a** with the duplex DNA. Alternative interactions are conceivable. One scenario is depicted in Figure 8 for the bonding process that proceeds at 5'CG sequences. In this hypothesis, DNA modification is promoted by the formation of a hydrogen bond between the carbon-8 hydroxyl group in the activated mitomycin hydroquinone (semiquinone) species and the N(3) pyrimidine nitrogen atom of the guanine residue on the *complementary* 5'CG strand *prior* to the carbon-1 alkylation event.[45] We suspect that drug complexation may be accompanied by an induced local DNA conformational distortion. Formation of this hydrogen bond should promote the monoalkylation of DNA by **1** by inhibiting alternative, competitive drug processes (i.e., mitomycin electrophilic transformations).[46] Moreover, alignment of the antineoplastic agent in this fashion should also facilitate the subsequent, selective cross-linking reaction[26e,29,30] by positioning the carbon-10 group in **1a** close to the N(2) amino group of the second guanine unit and by inhibiting electrophilic processes proceeding at this site as well.[46] A similar phenomenon can be suggested for the λ-exo stop sites that were observed in close proximity to 5'GG base sequences. In this scenario, a hydrogen bond is envisioned to occur between the carbon-8 hydroxyl group in the activated mitomycin hydroquinone (semiquinone) and the N(3) pyrimidine nitrogen atom of an adjacent guanine residue present on the *same* strand in which the drug–DNA monoalkylation process takes place. In support of this notion, Tomasz and co-workers have demonstrated that mitomycin C *intra*strand cross-links are generated within poly(dG)·poly(dC).[26a]

Conclusions

Use of the λ-exo assay and DNA restriction fragments has provided new information concerning the critical carbon-1 mitomycin C–DNA bonding event. Analyses of the autoradiograms revealed that modification of the genetic material proceeded at select sequences. This result suggests that drug–DNA interactions exist at an early stage of the DNA modification process that are sequence specific. The identification of these interactions constitutes an important objective in future studies focusing on the mode of action of this clinically useful antineoplastic agent.

Experimental Section

Materials. Mitomycin C (**1a**) was supplied by Bristol-Myers Squibb Co. (Wallingford, CT) and was used without further purification. 10-Decarbamoylmitomycin C (**1b**) was prepared by a modified procedure of Kinoshita and co-workers,[35] while 2,7-diaminomitosene[4b] (**2**) was obtained by catalytic reduction (PtO_2, H_2) of **1** at pH 5.5. Stock solutions of mitomycin C (**1a**), decarbamoylmitomycin C (**1b**), and 2,7-diaminomitosene (**2**) in DMSO were prepared and stored frozen (−20 °C) until use. HPLC analyses of the stock solutions prior to the drug–DNA bonding studies indicated that no decomposition of the drug occurred under these conditions. Anthramycin was a generous gift of Dr. Laurence Hurley, College of Pharmacy, University of Texas at Austin. Sodium dithionite was obtained from Fisher Chemical Co. pBR322 plasmid was prepared and purified by established procedures with slight modification. The double-stranded φX174 RF I DNA was obtained from New England Biolabs. Restriction enzymes and the λ exonuclease (λ-exo) were purchased from BRL and DNA polymerase I (Klenow) was obtained from New England Biolabs. The [α-^{32}P]dTTP was secured from Du Pont NEN, and all electrophoretic materials (acrylamide, bisacrylamide, TEMED, urea, and ammonium persulfate) were purchased from Bio-Rad.

Isolation, and 3'-^{32}P End Labeling of DNA Restriction Fragments. DNA from pBR322 Plasmid. Primary restriction digest of pBR322 plasmid was performed with *Bst*NI and the band corresponding to the 1857 base pair fragment was isolated from a 1.4% agarose gel. The isolated 1857 base pair fragment was then cleaned by passing through a NACS pack and precipitated with ethanol. The fragment was labeled on the upper strand 3'-termini in the presence of [α-^{32}P]dTTP (50 μCi) and DNA polymerase I (1 unit). The labeled DNA was precipitated with ethanol and then digested with *Eco*RI. The desired 129 base pair fragment was purified by polyacrylamide gel electrophoresis using a 5% preparative gel and TBE electrophoresis buffer.

The 3' lower strand ^{32}P-labeled 129 base pair fragment from pBR322 plasmid was prepared by primary digestion of the pBR322 plasmid with *Eco*RI. The linearized DNA was incubated at room temperature (30 min) with dATP, [α-^{32}P]dTTP, and DNA polymerase I (1 unit), and then dTTP was added and the incubation period continued for an additional 10 min.

DNA from Bacteriophage φX174 RF 1. Double-stranded φX174 RF I DNA was initially digested with *Hae*III to provide a 194 base pair fragment, which after isolation and subsequent digestion with *Hin*fI furnished a 140 base pair fragment. This fragment was labeled at the 3'-termini in the presence of dATP, dCTP, [α-^{32}P]dTTP, and DNA polymerase I (1 unit). The resulting 3'-end-labeled 142 base pair fragment was purified by electrophoresis on a preparative 5% polyacrylamide gel.

Drug Bonding with Sodium Dithionite. DNA–Mitomycin Monolinking Reactions. Specified amounts of either **1a** or **1b** were added to the radiolabeled DNA in 25 mM Tris-HCl buffer, pH 7.4, to give the desired final drug concentration. The solutions were deaerated with argon (15 min), and then freshly prepared, deaerated, aqueous sodium dithionite solutions (total 1 equiv) were added in three incremental portions (20 min). The reactions were maintained at 20 °C (1 h) under argon and then exposed to air.

DNA–Mitomycin C (1a) Cross-Linking Reaction. A solution of **1a** (1.5 mM) and DNA in 25 mM Tris-HCl, pH 7.4, was deaerated with argon (1 min), and then freshly prepared sodium dithionite in deaerated water was added in one portion to a final concentration of 6 mM (4 equiv). The bubbling of argon gas was stopped, and the mixture was incubated on ice (1 h) in a tightly sealed tube. Unreacted **1a** was removed by precipitating the DNA with ethanol and then the pellet was washed with 80% ethanol.

Attempted DNA–2,7-Diaminomitosene (2) Bonding in the Presence of Sodium Dithionite. Method A: same as DNA–mitomycin monolinking reactions. Method B: same as DNA–mitomycin cross-linking reaction. Method C: A mixture of **2** (0.15, 0.45, 0.9, and 1.5 mM final concentration) and DNA in water was deaerated with argon (15 min), and then freshly prepared sodium dithionite (4 equiv) in deaerated water was added in one portion. The purple color disappeared and the reaction mixture was incubated at room temperature under argon (30 min). Oxygen was then bubbled through the solution (2 min), the purple color reappeared, and the DNA was precipitated with ethanol.

DNA–Anthramycin Bonding.[34] Anthramycin was dissolved in ethanol and the drug concentration was determined by the observed absorbance at 333 nm with a UV–vis spectrophotometer. The drug was diluted appropriately in ethanol and then dried in a vacuum centrifuge in separate aliquots to give a final concentration of 0.02, 0.2, 2, and 10 μM. DNA in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) was added to the tubes containing dried drug, then incubated at room temperature (3 h), and then precipitated with ethanol.

λ-Exo Digestion of Mitomycin-Modified DNA. By use of the procedure described by Tang and co-workers,[34] modified DNAs were resuspended in the λ-exo buffer (67 mM glycine/KOH, pH 9.4, 2.5 mM $MgCl_2$). *Eco*RI linearized pBR322 plasmid (2 μg) was added to each sample, followed by λ-exo (15–50 units). The solutions were then incubated at 37 °C (30 min). The reactions were terminated by the addition of ethanol and the DNA precipitated.

Treatment of DNA with Mitomycin C (1a) in the Presence of Sodium Borohydride. The protocol specified by Komuno and co-workers[22] was employed in these experiments. The standard reaction mixture (100 μL) contained DNA, **1a** (0.025, 0.05, 0.1, and 0.2 mM final concentration), Tris-HCl, pH 7.1 (25 mM), and a freshly prepared solution of sodium borohydride (5 equiv). The solution was then incubated at 37 °C (15 min). The reaction was stopped by the addition of 4 μL of 0.5 M EDTA, 2 μL of 1 mg/mL tRNA, 10 μL of φX174 M sodium acetate (pH 5.5), and ethanol. The resulting pellet was washed with 70% ethanol, dried, and resuspended in 40 μL of 10 mM Tris-HCl, pH 8.1, and the suspension was heated at 90 °C for 5 min. The heat-treated DNA was reprecipitated with ethanol.

DNA Sequencing, DNA Sequencing Gel Electrophoresis, and Autoradiography. 3'-End-labeled DNA fragments were sequenced by the chemical methods of Maxam and Gilbert.[38] Samples of the radiolabeled DNA fragments with or without λ-exo treatment were suspended in sequencing tracking dye (80% v/v deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), heated to 90 °C (3 min), and then quenched in an ice bath. Equal amounts of

(44) A similar hydrogen-bonding picture has been predicted by Kollmann, Remers, and co-workers based on their extensive computational studies.[25]

(45) This orientation permits several additional hydrogen bond interactions to take place between the activated mitomycin species and the DNA backbone.

(46) Kohn, H.; Hong, Y. P. *J. Am. Chem. Soc.* **1990**, *112*, 4596.

radioactivity per sample were then loaded onto a 0.4-mm denaturing polyacrylamide gel (8% 19:1 acrylamide/bisacrylamide, 50% urea), and electrophoresed with TBE buffer (100 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 1400 V for 2-3 h. After electrophoresis the gels were exposed to Kodak X-Omat RP film with intensifying screen at -70 °C. Analyses of the autoradiograms demonstrated that the sequences of the DNAs used in this study matched the published sequences[47] of pBR322 plasmid and ϕX174 RF 1 DNA.

(47) pBR322 plasmid: (a) Sutcliffe, J. G. *Nucleic Acids Res.* **1978**, *5*, 2721. (b) Sutcliffe, J. G. *Cold Spring Harbor Symp. Quant. Biol.* **1979**, *43*, 77. ϕX174 RF 1 DNA: Sanger, F.; Coulson, A. R.; Friedmann, T.; Air, G. M.; Barrell, B. G.; Brown, N. L.; Fiddes, J. C.; Hutchison, C. A.; Slocombe, P. M.; Smith, M. *J. Mol. Biol.* **1978**, *125*, 225.

Acknowledgment. We express our deep appreciation to both Dr. Laurence Hurley (University of Texas—Austin) and Dr. Moon-shong Tang (University of Texas M. D. Anderson Cancer Center-Science Park) for the extensive help and valued thoughts that they have provided throughout this investigation. We thank Dr. B. M. Pettitt and Mr. P. Schiltz for their help in generating the idealized representation for the process involved in mitomycin-DNA monoadduct formation (Figure 8). Special thanks are given to Dr. A. M. Casazza and the Bristol-Myers Squibb Co. for the generous gift of mitomycin C. We gratefully acknowledge the National Institute of Health (Grant R01CA29756) and the Robert A. Welch Foundation (Grant E-607) for their support of our research programs.

Toward Chemical Ribonucleases. 2. Synthesis and Characterization of Nucleoside-Bipyridine Conjugates. Hydrolytic Cleavage of RNA by Their Copper(II) Complexes

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Abstract: As part of our program to develop chemical ribonucleases that cleave RNA by phosphodiester hydrolysis, a systematic study of covalently linked nucleoside-2,2'-bipyridine (bpy) conjugates is described. 2'-Deoxythymidine was attached at both its 3'- and 5'-positions to bpy derivatives by using phosphoramidite chemistry, yielding after deprotection ammonium 2'-deoxythymidine 3'-[4-(4'-methyl-2,2'-bipyridin-4-yl)butyl phosphate] (**8**) and triethylammonium 2'-deoxythymidine 5'-[4-[4'-methyl-2,2'-bipyridin-4-yl]butyl phosphate] (**11**). 2'-Deoxyuridine was attached to a modified bpy via derivatization of the uracil ring at C-5, giving 5-[3-[[2-[[4-(4'-methyl-2,2'-bipyridin-4-yl)-1-oxobutyl]amino]ethyl]amino]-3-oxopropyl]-2'-deoxyuridine (**16**). These conjugates and the intermediate bpy derivatives were fully characterized by mass spectrometry and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. The ability of the bpy moieties to bind Cu(II) was demonstrated spectroscopically. The copper(II) complexes of **8**, **11**, and **16** were shown to hydrolyze RNA at 37 °C and neutral pH. The difference in reactivity of **8**, **11**, and **16** provides the basis for optimizing the activity of hydrolytic chemical nucleases.

Introduction

Oligonucleotides covalently linked to metal complexes have been employed in a variety of studies that capitalize on the selective binding ability of DNA and the properties of metal complexes. Thus, oligonucleotides can be directed in a Watson-Crick fashion toward complementary, single-stranded nucleic acids, or via triple-helix formation toward double-stranded DNA targets.[1] The properties that metal complexes can provide include reactivity, i.e., oxidative cleavage behavior,[2] and fluorescence, for labeling purposes.[3] Among the most elegant examples in this area are the "chemical nucleases", which cleave nucleic acids in a sequence-directed manner, and which are composed of a single-stranded oligonucleotide linked to a redox-active metal complex such as Cu(II)(*o*-phenanthroline)$_2$,[4] Fe^{II}EDTA,[5] or iron porphyrins.[6] Cleavage is thought to be effected by metal-bound or free hydroxyl radicals.

Cleavage of DNA or RNA via hydrolysis of the phosphodiester backbone would have distinct advantages over its oxidative counterpart. Hydrolysis would not require redox cofactors to mediate the chemistry nor would highly reactive oxene or oxy radical species be generated. In addition, hydrolytic manipulation of nucleic acid polymers would generate fragments that are chemically competent for ligation to other oligonucleotides by routine enzymatic reactions. Accordingly, there has been considerable interest in developing DNA and RNA hydrolysis catalysts and in probing the mechanism of metal-catalyzed hydrolysis of phosphate esters. Most of these studies have used activated *p*-nitrophenyl phosphate esters or phosphate anhydrides as substrates.[7] However, there are examples of metal-promoted hy-

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0002-7863/91/1513-283$02.50/0