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DNA Sequence Specificity of Mitomycin Cross-Linking†

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ABSTRACT: Using a gel electrophoresis assay, we show that the target DNA sequence cross-linked by *N*-methylmitomycin A, its aziridinomitosene, and mitomycin C is CpG, in strong preference over GpC. The yield per CpG site increases as the number of successive CpG sequences increases. Molecular modeling reveals no systematic difference between the energies of mitomycin cross-links at CpG in comparison with GpC. However, the distance between guanine amino groups in CpG sequences is nearly the same as the distance in the cross-linked adduct, whereas the amino group separation at GpC sites is substantially larger in the starting DNA than in the adduct. We suggest that the favorable placement of the second reaction center in CpG greatly accelerates the second step in the cross-linking reaction. As shown by a competition assay, mitomycins bind A·T and G·C sequences noncovalently equally well, even though the only sequence that yields appreciable cross-linking is CpG. *N*-Methylmitomycin A and its aziridinomitosene are found to be better cross-linking agents than is mitomycin C.

Since their discovery in 1956 (Hata et al., 1956), mitomycins have received much attention due to their potency as chemotherapeutic agents (Remer, 1956) although most of the work has been focused on one member of the group, mitomycin C (MC).[1] MC has three major functional groups: quinone, carbamate, and aziridine, but the drug is not active in the quinone form (Figure 1). Activation requires either enzymatic or chemical reduction (Iyer & Szybalski, 1964a), or mild acidic treatment (Tomasz & Lipman, 1979), converting MC to alkylating agents capable of monofunctional and/or bifunctional covalent interaction with DNA (Iyer & Szybalski, 1964; Mercado & Tomasz, 1972). MC acts directly on DNA to inhibit DNA synthesis (Goldberg & Friedman, 1971), thus preventing cell division and diminishing cell viability. Although monofunctional alkylation occurs 10–20 times more frequently than bifunctional alkylation (Iyer & Szybalski, 1964; Weissbach & Lisio, 1965), cross-linking has been considered to be the source of the lethal effect on cancer cells (Iyer & Szybalski, 1967). More recently, monofunctional alkylation has been reported to cause significant DNA damage (Mercado & Tomasz, 1972; Small et al., 1976; Kinoshita et al., 1971). Besides damage caused by alkylation, superoxide radicals formed upon reoxidation of the mitomycin hydroquinone induce single-strand breakages (Lown & Beigleiter, 1976; Tomasz, 1976).

Characterization of the DNA–mitomycin complex has been difficult, since early studies showed that only a small fraction of added mitomycin incorporates into DNA, about one per several hundred nucleotides (Weissbach & Lisio, 1965). In addition, the activated intermediate is short-lived and difficult to isolate (Iyer & Szybalski, 1964; Patrick et al., 1964). In spite of these problems, investigators have been able to determine the functional groups involved in mitomycin–DNA binding. Iyer and Szybalski hypothesized that the cross-linking of DNA by MC involved C_1, after loss of the methoxy group and subsequent opening of the aziridine ring, and C_{10}, after displacement of the carbamoyl group (Iyer & Szybalski, 1963, 1964). Support for Iyer and Szybalski's proposal has been provided by many investigators. Both Tomasz and Mercado

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[1] Abbreviations: MC, mitomycin C; NMA, *N*-methylmitomycin A; MS, the aziridinomitosene of NMA (Danishefsky & Egbertson, 1986).

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Table I: Oligomers Used[a]

sequence (5′ to 3′)	abbreviation[b]	no. of CpG	no. of GpC	curve[c]
GCGCGCGCGCGC	GC	(5)[d]	6	G
ATATATATATATAT	AT			
ATATATGTATATA	13GT			
TATATAGATATAT	13GA			
TATATATGGGTATATAT	17G			
TATATATAAATATATAT	17A			
ATATATGCTATATA	14G		1	G
ATATATCGTATATA	14C	1		C
ATATATGCATATAT	14TG		1	
ATATATCGATATAT	14TC	1		
TATATAGCTATATA	14AG		1	
TATATACGTATATA	14AC	1		
ATATATGCGCTATATA	16G	(1)	2	G
ATATATGCGCGCTATATA	18G	(2)	3	G
ATAAATCGCGCGTAAATA	18C	3	(2)	C
ATATCGTTCGTTCGTATA	18CG	3		
ATAAATCGCGCGCGCGTAAATA	22C	5	(4)	C

[a] Complementary strands are omitted. [b] Abbreviated names are used throughout the paper. [c] G or C indicates the corresponding curve in Figure 3. [d] Values in parentheses give the smaller of the number of GpC or CpG in alternating sequences. The convention used in Figure 3 is to plot the data against the larger of these two numbers, curves C and G, respectively, depending on whether the number of CpG or GpC is larger.

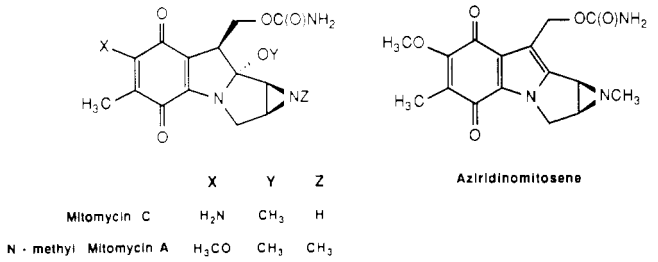

FIGURE 1: Mitomycin C (MC), *N*-methylmitomycin A (NMA), and the aziridinomitosene (MS) derived from NMA by loss of the methoxy substituent.

(1972) and Otsuji and Murayama (1972) found that derivatives without the aziridine ring or carbamoyl group were less active than MC in cross-linking DNA. Hashimoto et al. (1983) showed that C_1 is one of the binding sites when they isolated mononucleotides covalently bound to the 1-position of a mitosene. Most recently, isolation of deoxyguanosine–MC adducts and a deoxyguanosine–MC cross-linked adduct by Tomasz et al. (1986, 1987) confirmed that the active sites are C_1 and C_{10}.

Although there has been some uncertainty concerning the functional group on guanine that reacts with MC, covalent binding of activated MC to DNA at guanine residues is well-known; depurinating conditions removed bound MC from DNA and apurinic acid did not bind MC (Tomasz et al., 1974). Direct proof that guanine is the essential base for MC–DNA interaction was not obtained until recently when Tomasz et al. (1986, 1987) reported the isolation and structure of deoxyguanosine–MC mono and cross-linked adducts. In this work, MC was shown to be attached to the 2-amino group of guanine.

These findings leave unresolved the possible base sequence specificity of mitomycin reaction, the issue to which our experiments are directed. In particular, we ask whether sequences containing guanine residues differ in their reactivities for cross-linking, and whether adjacent guanosines are essential for cross-linking.

In the search for more effective drugs, many analogues and derivatives have been synthesized (Remer, 1976) to further our understanding of the mode of action and structure–activity relationship of MC. *N*-Methylmitomycin A (NMA) and its aziridinomitosene (MS) containing more active functional groups (Figure 1) are thought to be more active than MC. Using electrophoresis to separate mitomycin cross-linked DNA from un-cross-linked DNA, we were able to compare the cross-linking ability of the three drugs and verify the expected relationship.

Materials and Methods

Materials

Stock Solutions. Sodium dithionite (Fisher Scientific Co.) (60 mM) was dissolved in deaerated water and used within 30 min after preparation. Mitomycin C (Bristol Pharmaceutical Co.), *N*-methylmitomycin A, and the aziridinomitosene of NMA (kindly supplied by Samuel Danishefsky and Melissa Egbertson) were dissolved in DMSO (0.015 M) and stored at 4 °C. Calf thymus DNA (Sigma Chemical Co.) was dissolved in water (2 μg/μL). Gel loading buffer contained 0.1% Bromophenol Blue, 0.1% xylene cyanol, 25% glycerol, 0.001 M EDTA, and 0.02 M sodium hydroxide.

Oligomers were synthesized on an Applied Biosystems DNA synthesizer and purified by Waters HPLC. Table I lists the oligomers used in our studies. They were labeled with [γ-^{32}P]ATP and hybridized with the complementary strand after labeling.

Methods

Preparation of Mitomycin–DNA Complex. Samples of approximately 10 μL containing 10 mM sodium phosphate (pH 4.5 or 7.5), [γ-^{32}P]ATP-labeled double-helical oligomer (≈10000 cpm, 20 ng), 1.5 mM (in bp) calf thymus DNA, and 1.5 mM mitomycin were deaerated by flushing with nitrogen gas for 1 min. The reducing agent sodium dithionite was then added, to a final concentration of 6.0 mM. Bubbling of nitrogen gas was stopped, but the sample was capped tightly and left for 1 h. The reaction was performed in an ice bath. We routinely used carrier calf thymus DNA in these experiments since it is a potential competitor for the drug, and its presence helps to ensure a more reproducible concentration of the reactive species. At relative drug excess (1.5 mM drug, 1.5 mM bp) the competition effects are modest, and similar results were obtained when calf thymus DNA was excluded.

Gel Electrophoresis. Ten microliters of gel loading buffer was added to samples. The samples were denatured by heating at 90 °C for 3 min. About 5–10 μL of sample was removed and loaded on a 20% polyacrylamide gel (90 mM TBE, 7 M urea) and electrophoresed at 30 W for 1 h. The gel was autoradiographed overnight at −70 °C. Oligomers cross-linked by mitomycin run more slowly than the parent compound, with

Table II: Percentage of Cross-Linking: CpG vs GpC

	% cross-linking[a]					
	NMA		MS		MC	
	pH 4.5	pH 7.5	pH 4.5	pH 7.5	pH 4.5	pH 7.5
XCpGY						
TCGT (14C)[b]	5.3	1.8	5.1	1.7	2.9	0.9
TCGA (14TC)	5.7	1.8	5.7	1.9	3.1	1.5
ACGT (14AC)	2.6	1.9	2.5	1.7	1.1	0.8
XGpCY						
TGCT (14G)	0.4	0.2	0.2	0.1	0.2	0.1
TGCA (14TG)	0.4	0.2	0.4	0.3	0.3	0.2
AGCT (14AG)	0.2	0.2	0.2	0.2	0.2	0.1

[a] From Cerenkov counting of region of the gel where cross-linked material is expected. Cross-linking percentages of less than 0.5% (GpC sequences) are probably not significant, since no corresponding band could be seen in overexposed autoradiograms (Figure 2). [b] Abbreviation from Table I.

an electrophoretic mobility similar to that of analogous oligomers cross-linked by psoralen (results not shown).

Quantitation of Results. Cross-linked DNA and non-cross-linked DNA bands were cut out and counted by Cerenkov counting with a scintillation counting spectrometer from Packard. The percentage of cross-linked DNA was determined from the counts in the two bands. Repetition of experiments indicated that the numbers obtained were reproducible within about 10% of the measured value.

Structural Analysis. The molecular mechanics program AMBER (Assisted Model Building with Energy Refinement) was employed for structural analysis of NMA-cross-linked oligomers. The parameters are obtained from Rao et al. (1986) and Remers et al. (1986).

Binding Competition. The purpose of these experiments was to assess the strength of noncovalent binding of the drug to both reactive and nonreactive DNA sequences. Experiments were done under two conditions: (a) For experiments in the presence of an inhibitor, reactions were carried out as described above with the following changes. Ten-microliter reaction solutions containing 10 mM sodium phosphate (pH 4.5), [γ-^{32}P]ATP-labeled oligomer 22C, 0.15 mM unlabeled oligomer 22C, 0.15 mM mitomycin, and 0, 0.0375, 0.075, 0.15, 0.30, and 0.60 mM oligomer 17A (which contains no guanosine) were deaerated under nitrogen gas for 1 min. The reducing agent sodium dithionite was added to 0.6 mM final concentration. Procedures for electrophoresis and quantitation of results were as described above. (b) For experiments in the absence of an inhibitor, the procedure was similar but oligomer 17A was omitted. Reactions were performed with 0, 0.0375, 0.075, 0.15, 0.30, and 0.60 mM unlabeled oligomer 22C and a small amount of labeled oligomer 22C. In both cases a and b, the use of low concentrations of drug required low amounts of dithionite in order to keep constant the ratio of drug to reductant. Because of the instability of solutions of this reagent, the total cross-linking yield could differ by as much as a factor of 2 from one series of experiments to another. However, it was found that comparative values within a series were reproducible to within about 10%.

Results

Optimum Reaction Conditions. By varying the relative concentrations, we found that the millimolar ratio of sodium dithionite to total base pairs of DNA to drug which gives optimal cross-linking is approximately 6.0:1.5:1.5; the concentration of labeled oligomer is only in the micromolar range. As described in detail in a forthcoming paper (Cera et al., 1989), a 2–3-fold molar excess of dithionite to drug is required for optimal cross-linking. Drug concentrations beyond a few millimolar are difficult to use reliably, because the large amount of dithionite needed tends to produce acidic pH drift in the solution. Cross-linking is maximized with freshly prepared sodium dithionite. Reduction of mitomycins with sodium dithionite prepared an hour earlier decreased cross-linking significantly.

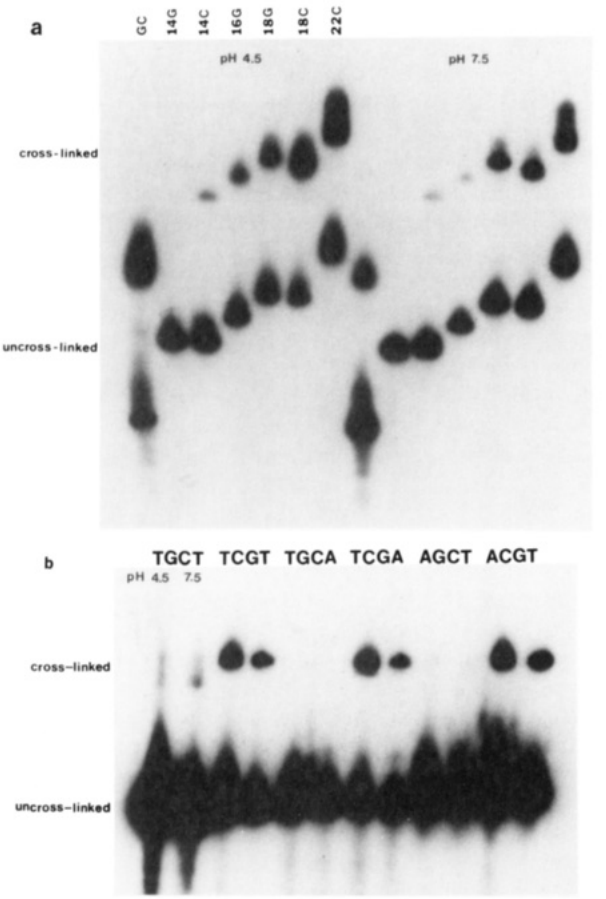

FIGURE 2: Separation of cross-linked and un-cross-linked DNA by electrophoresis. Cross-linked DNA has a reduced mobility. (a) Autoradiogram showing MS-cross-linked oligomers with increasing number of CpG sequences. Refer to Table I for identification of the sequences. (b) Overexposed autoradiogram of oligomers 14G (TGCT), 14C (TCGT), 14TG (TGCA), 14TC (TCGA), 14AG (AGCT), and 14AC (ACGT), containing either 1 GpC or 1 CpG sequence. Reaction at two pH values, 4.5 and 7.5, is shown for each oligomer.

pH Dependence. The amount of cross-linking increases with decreasing pH for all three drugs. A comparison of the results shows that the amount of cross-linking at pH 4.5 is greater than at pH 7.5 for all three drugs, although the quantitative effect of pH varies with drug and DNA sequence, for unknown reasons (Figure 2, Table II). Intermediate pH values gave intermediate results.

Table III: Percentage of Cross-Linking: Oligomers 18

	NMA		MS		MC	
	pH 4.5	pH 7.5	pH 4.5	pH 7.5	pH 4.5	pH 7.5
18G	42.1	10.5	39.3	8.3	13.7	7.6
18C	60.5	22.5	55.3	16.6	19.7	14.0
18CG	50.5	15.5	48.4	13.7	16.2	8.9

Dinucleotide Sequence Responsible for Cross-Linking. Since MC is thought to alkylate and cross-link guanine, we attempted to cross-link oligomers containing a variety of sequences. The cross-linking results with these oligomers (Figure 2 and Table II) confirm that A·T paris are not responsible for DNA–mitomycin cross-linking. The inability of mitomycins to cross-link oligomers 13GT, 13GA, 17G, and 17A is in agreement with the finding (Tomasz et al., 1987) that guanines in complementary strands act as cross-linking sites. Furthermore, comparison of the 14-mers containing CpG and GpC between flanking A·T sequences clearly implicates CpG as the dinucleotide responsible for cross-linking. The overexposed autoradiograms in Figure 2 indicate that cross-linking cannot be detected in sequences that contain GpC without CpG. This result is not dependent on whether the flanking bases are A or T; however, variation of the flanking bases affects measured values by up to a factor of 2 (Table II).

CpG Pairs in Sequence. Increasing the number of CpG sites in an alternating sequence enhances cross-linking of oligomers, as shown in Figure 3; for visual clarity the data are plotted against whichever is the larger of the number of CpG (curve C) or GpC (curve G) sites in the oligomer. The amount of cross-linking increases more than linearly with the number of CpG sites in sequence when the number is small: the curves (curve C) for oligomers 14C, 18C, and 22C, containing 1, 3, and 5 CpG sites, respectively, rise steeply between 1 and 3 sites. These results clearly show that an extended alternating G-C sequence has greater reactivity per CpG site than is found for an isolated CpG sequence. The percentage of cross-linked oligomers 14G and 18G containing 1 and 3 GpC sites, respectively, is lower than the percentage of cross-linked oligomers 14C and 18C, as expected if the reactive site is CpG but not GpC. (14G and 18G contain 0 and 2 CpG sites, respectively, 1 less than in 14C and 18C.) The shape of the curves for oligomers 14G, 16G, and 18G (curves G) is similar to that of curves C. Oligomer GC, which lacks flanking bases for the alternating G-C tract and contains 5 CpG sites, produces 15% less cross-linking than oligomer 22C, which also contains 5 CpG sites, but is flanked by A·T sequences.

Interrupting and Flanking Sequences. The cross-linking of oligomer 18CG containing 3 CpG sites interrupted by pTpTp is less than found for oligomer 18C possessing 3 CpG sites, but more than oligomer 18G containing 3 GpC or 2 CpG sites in sequence (Table III). Thus, cross-linking is affected not only by the total number of CpG pairs but also by their arrangement in an oligomer.

Comparison of Activities of Mitomycins. The maximum amount of cross-linking by each drug is found with oligomer 22C at pH 4.5 (Figure 3). The percentage of NMA- and MS-cross-linked oligomer 22C is similar and is twice as much as obtained with MC. Analogous comparative reactivities are seen with shorter oligomers. MS, though as active as NMA, is more difficult to reduce; the standard reduction potential of NMA is ≈0.2 volts compared to −0.4 V for MS and MC (Remers et al., 1979). In comparing the ability of mitomycins to cross-link DNA, it should be recognized that reactivity is dependent on activation conditions—pH, ratio of drug to DNA to reducing agent used, reduction potentials of the drugs, and sequence composition of the DNA.

Table IV: Sequences Whose Energy Was Minimized by Using the AMBER Program

sequence[a]	abbreviated name	sequence[a]	abbreviated name
$(TA_2GC(TA)_2$	AGCT	$(AT)_2GC(TA)_2$	TGCT
$(TA)_2CG(TA)_2$	ACGT	$(AT)_2CG(TA)_2$	TCGT
$(AT)_2GC(AT)_2$	TGCA	$(CG)_5$	CG
$(AT)_2CG(AT)_2$	TCGA		

[a] A cross-link is introduced at the CpG or GpC sequence in the middle of the fragment.

Table V: Energies of Minimized DNA Structures and Average Base Pairs per Helical Turn

sequence[a]	E_{tot}	ΔE_{tot}	bp/turn
BTCGT	13.12		10.42
TCGT	−5.99	−19.11	10.35
BTGCT	11.79		10.42
TGCT	−3.07	−14.89	10.28
BTCGA	7.48		10.46
TCGA	−7.59	−15.07	10.38
BTGCA	6.38		10.46
TGCA	−9.81	−16.19	10.32
BACGT	9.12		10.40
ACGT	−7.61	−16.73	10.32
BAGCT	10.36		10.39
AGCT	−6.60	−16.96	10.26
BGC	−17.54		10.39
CG	−37.25	−19.71	10.33
GC	−33.62	−16.08	10.26

[a] Un-cross-linked strucures are indicated by the letter "B" before the sequence (BXGCY), and sequences cross-linked by NMA use their abbreviated names (Table IV). E_{tot} is the calculated energy (kcal/mol), and ΔE_{tot} is the calculated energy difference between the final adduct and the starting DNA helix.

Table VI: Helical Repeat Angles[a]

BACGT		ACGT	
A-C	35.72	A-C	48.95
C-G	33.32	C-G	5.30
G-T	35.46	G*-T	50.28
BAGCT		**AGCT**	
A-G	35.41	A-G	20.94
G-C	34.0	G*-C	66.41
C-T	35.49	C-T	20.12

[a] Helical screw advance in degrees at each dinucleotide calculated for the energy-minimized structures without (left) and with (right) cross-linking.

AMBER *Calculations.* Mono and di adducts of mitomycin with DNA have previously been modeled by using the AMBER program developed by Kollman and co-workers (Rao et al., 1986; Remers et al., 1986). We used their parameters and a similar computational approach to compare the energies of structures in which mitomycin is cross-linked at the N2 position of guanosine in a number of sequences. We sought to determine whether the strongly preferred cross-linking reactivity of CpG over GpC is a reflection of differences in the energy changes, ΔE_{tot}, upon reaction with the two sequences. The results of our molecular modeling and energy refinements studies are summarized in Figure 4 and Tables IV–VI. The calculations show no systematic difference of ΔE_{tot} between CpG and GpC sequences; the values were similar (±5 kcal) for the two and dependent on the nature of the flanking bases. Since entropy terms are not included in these calculations, it remains possible that sequence preference is related to overall reaction free energy. However, it is more probable that sequence discrimination, which must, for an irreversible binding

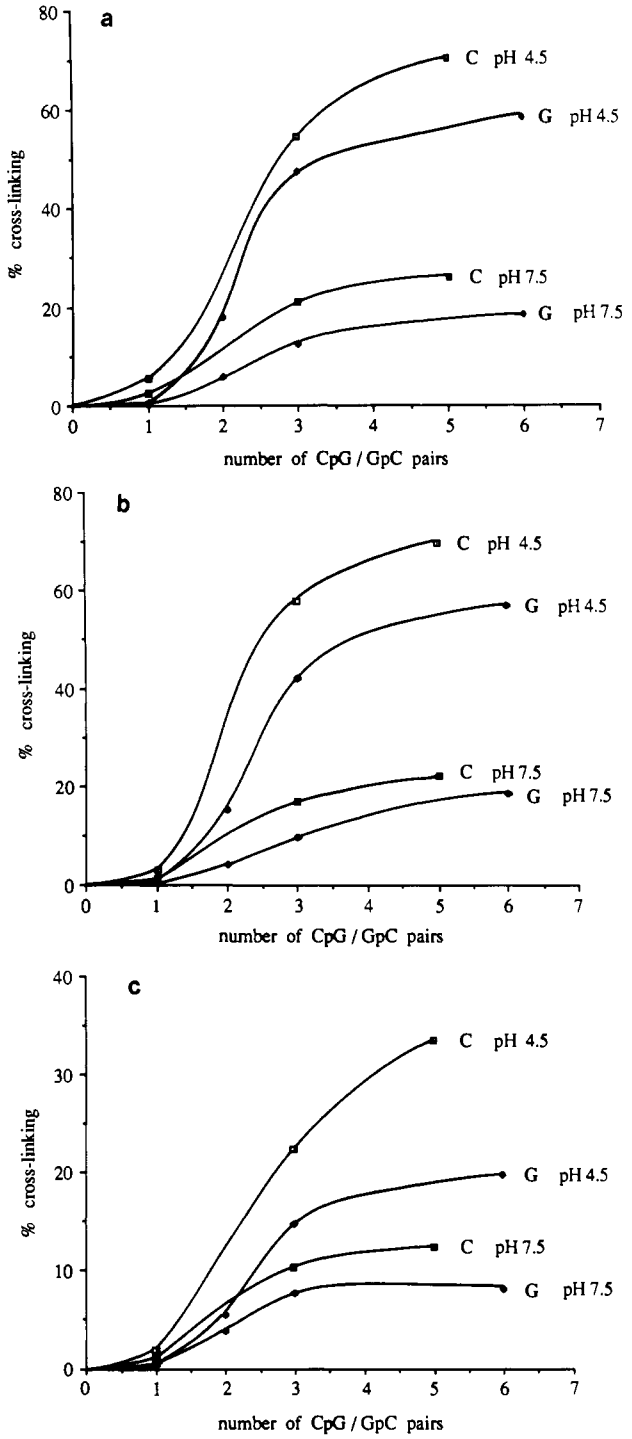

FIGURE 3: Cross-linking vs number of CpG or GpC pairs in sequence. (a) Cross-linking with NMA; (b) cross-linking with MS; (c) cross-linking with MC. Members of curve C: Oligomers 14C, 18C, and 22C. Members of curve G: Oligomers 14G, 16G, 18G, and GC.

reaction, reflect relative free energies in a critical reaction transition state, is not correlated to the overall thermodynamic parameters.

We compared the energy-minimized structures of the oligomers cross-linked at CpG and GpC sites with that of non-cross-linked oligomer (Figure 4, Table V). Comparison shows the helical twist angles increasing, decreasing, and increasing around the cross-linked CpG site in contrast to decreasing, increasing, and decreasing about the cross-linked GpC site. Although the helical repeat angles (Table VI) are different for free and cross-linked DNA around the cross-linking site, the average helical repeats for cross-linked and non-cross-linked

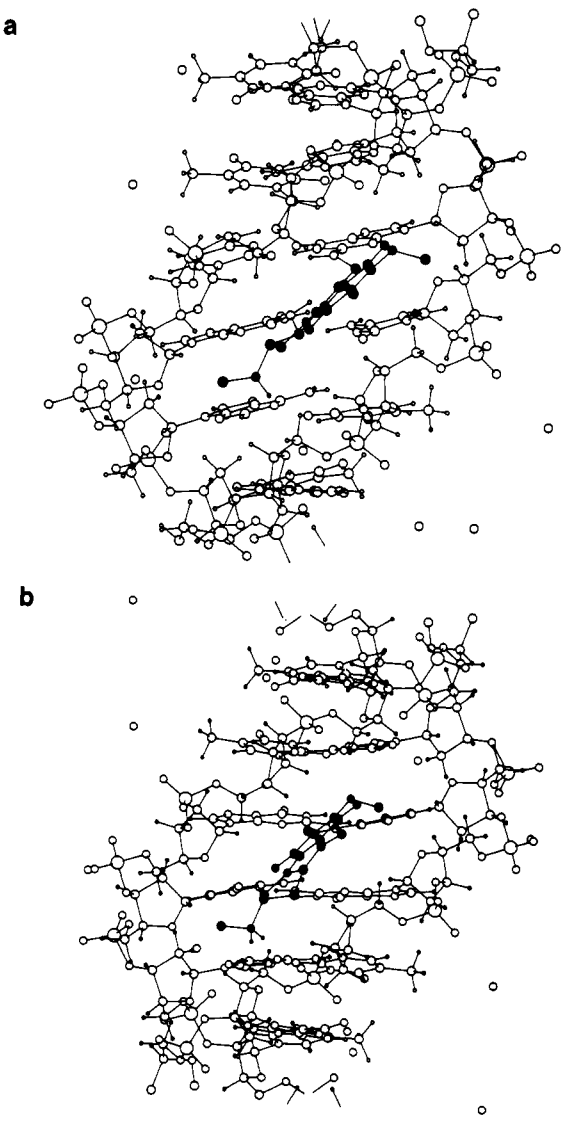

FIGURE 4: Energy-minimized DNA structures: (a) sequence AGCT; (b) sequence ACGT. Cross-linking is imposed at either the GpC (a) or CpG (b) site.

oligomers are similar—10.35 ± 0.1 bp. In both sequences, the modified G (G*) bases are found to be less tilted than G, and C-G* or G*-C base pairs are twisted slightly more than free DNA. Cross-linking distorts primarily the local structure at the site of adduct formation.

Binding Competition. The objective of these experiments was to investigate the possibility that A·T sequences might bind noncovalently with the drug, even though they are unable to cross-link. This was done by determining the extent of inhibition of cross-linking that results from addition of a competing but non-cross-linking oligonucleotide, in this case in the absence of carrier calf thymus DNA. The analysis is of necessity indirect, since we assess formation of a noncovalent complex by modulation of the reaction yield of the cross-linked species. However, there is no simple way to measure directly the equilibrium dissociation constant for DNA binding by the transient reduced drug which is responsible for cross-linking. In parallel experiments we studied the reaction yield as a function of the concentration of reactive sites, which allows determination of an apparent dissociation constant analogous to the Michaelis constant in enzyme kinetics.

The model used is simple and approximate. The reaction is assumed to proceed by formation of an initial noncovalent complex D*G, where D* is the reduced drug and G is a CpG

site (in oligomer 22C) reactive for cross-linking. In addition, allowance is made for drug sequestration by nonreactive nucleotide sites A (in oligomer 17A and potentially the flanking nucleotides in oligomer 22C):

$$\begin{aligned} D^* + G &\underset{}{\overset{K_G}{\rightleftharpoons}} D^*G \rightarrow \text{cross-linked product} \\ D^* + A &\overset{K_A}{\rightleftharpoons} D^*A \end{aligned} \quad (1)$$

The dissociation constants K_A and K_G refer to noncovalent binding of the reduced drug to nonreactive and reactive sites, respectively. We take the reaction rate for formation of cross-linked product to be proportional to the concentration of the species D*G. All forms of the drug are subject to the competing degradation reactions which produce non-cross-linked products; free drug and the species D*A lead only to these products. In addition, we use the fact that the yield of cross-linked product is much smaller than the starting amount of nucleotides present, so that the reaction may be considered to be pseudo first order. Under these conditions, the overall reaction yield is proportional to the rate under the starting concentration conditions at time $t = 0$. The coupled equilibrium scheme above was solved numerically by an iterative approach, and the parameters K_A and K_G were adjusted for best fit (using a minimum least-squares criterion) to the relative reaction yields within a given experimental series which compares reactivity as a function of concentration.

The concentrations of reactive and nonreactive sites G and A are expressed in terms of dinucleotides. For simplicity, and because of the low levels of binding, possible neighbor exclusion effects between bound drugs are neglected. One unknown feature in the model is the extent to which A·T nucleotides flanking the reactive CpG sites should be considered to be competitors: they may also serve to help concentrate the reduced drug in the neighborhood of the reactive sites. We left the fraction of the flanking sites that are competitive as an unknown in the analysis; the fit to experimental data was not highly sensitive to that parameter. The results shown assume that about half of the flanking dinucleotide sites are competitive and half are not. The parameters that emerge from this analysis should be regarded as order-of-magnitude estimates only. However, the ability of nonreactive sites to bind in a similar manner to the reactive sites is a definite conclusion of our analysis.

Figure 5a shows the influence of the millimolar concentration of competitor oligonucleotide 17A on the millimolar yield of cross-linked oligonucleotide 22C at a fixed concentration of drug (mitomycin C). The results show clearly the inhibitory effect of an oligonucleotide containing only unreactive A·T pairs. Figure 5b shows a similar plot giving the dependence of reaction yield on reactive oligonucleotide 22C concentration, in the absence of competitor 17A and at fixed drug concentrations. We take the observed approach to saturation of the reaction yield as evidence for saturation of the reactive complex D*G.

The noncovalent dissociation constants derived from fitting the theoretical model (solid curves in Figure 5) to the experimental data are approximately 0.6 mM^{-1}, with no significant difference for K_G and K_A. (Note that the reciprocal dissociation constants are measured in dinucleotide concentrations, which are substantially higher than oligonucleotide concentrations; for example, our simple model assumes that oligomer 17A contains 16 dinucleotide binding sites and oligomer 22C contains 5 reactive CpG sites.) We conclude that the preferential reaction of mitomycin with guanosine cannot be explained on the basis of selectivity at the noncovalent binding step. In addition, we found no significant variation of the noncovalent binding constants for the other drugs studied (NMA and MS).

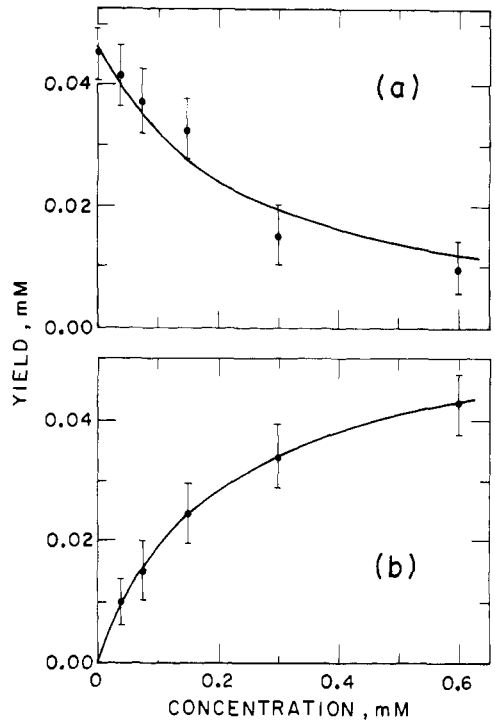

FIGURE 5: Final concentration of cross-linked oligonucleotide 22C as a function of the initial concentration of (a) inhibitor oligonucleotide 17A or (b) starting concentration of oligomer 22C. In both cases the starting concentration of drug was 0.15 mM, and the dithionite concentration was 0.6 mM. In (a) the starting concentration of oligomer 22C was 0.15 mM. The solid line is a theoretical calculation based on reaction scheme 1, with $K_G = 0.57$ mM^{-1} and $K_A = 0.57$ mM^{-1}, and assuming that oligomer 22C contains 5 competitive, non-cross-linking binding sites. The estimated error in these interrelated parameters is roughly a factor of 2.

Discussion

It has long been known that mitomycin cross-linking of DNA requires G·C base pairs (Iyer & Szybalski, 1964). Tomasz et al. (1987) showed that adjacent guanines on opposite strands provide a cross-linking site. We have confirmed their result and demonstrated in addition that CpG is the dominant cross-linking site, in strong preference over GpC. No significant cross-linking is found when G is preceded by any nucleotide other than C. Furthermore, cross-linking per CpG site rises when there are several such sites in series, possibly implying a modified helical structure in alternating G-C tracts, and indicating special reactivity of the drug with such tracts.

Using the AMBER program, we compared the energy of oligomers cross-linked at CpG and GpC sites in a B-DNA geometry. In some cases (TCGT and TCGA) the calculated energy difference between drug adduct and starting DNA (ΔE_{tot}) is larger (more negative) for CpG sequences than for the corresponding GpC sequences. The same is not true for ACGT, however, which our experimental results showed to be preferred over AGCT. We conclude that, according to the calculation, CpG and GpC are energetically similar sites, and preferential cross-linking cannot be rationalized by energy minimization of the structure of the final product.

One view of DNA reactivity emphasizes the relative electrostatic potential of various sites on DNA in determining reactivity with electrophillic agents (Pullman et al., 1982). We used the results of Pullman et al. (1979) to estimate differences

in the electrostatic potential at the reactive guanine amino groups in GpC and CpG sequences and found that these calculations did not yield a consistent correlation between cross-linking reactivity and estimated negative potential.

It seems likely that kinetic factors are dominant in distinguishing the reactivities of CpG and GpC sites for mitomycin. Creation of a cross-link requires that a monofunctionally attached mitomycin react with a neighboring guanine amino group more rapidly than with solvent. The rate of this process is likely to depend on the proximity of the second amino group to a monofunctionally attached mitomycin. We found in examining the structures refined by AMBER that the distance between amino groups in the adduct is substantially closer to the value in the free DNA helix for CpG sites than for GpC. For example, the N to N distance is 3.49 Å in the adduct with ACGT and 3.62 Å in the starting B-DNA helix. In contrast, the adduct distance is 3.06 Å in AGCT and 4.36 Å in the starting DNA. The shortened distance in the adduct is not associated with a large change in energy of the final minimized structure (in this force field), but it is plausible that the rate of conformational change is insufficient to produce reaction within the short lifetime of the activated intermediate. According to this hypothesis, all sites should be reactive in monoadduct formation, but CpG excels as the cross-linking site because of ideal placement of the second reactive group.

It has been shown that MC reacts with the DNA minor groove (Tomasz et al., 1987), although computer modeling experiments have been able to fit MC into both the major and the minor grooves (Remers et al., 1986; Rao et al., 1986). The dissociation constants K_A and K_G that we determined from the binding competition experiments are similar for each of the three drugs, suggesting that the reactive reduced forms of the mitomycins differ little in their noncovalent binding. In addition, the similarity of K_A and K_G values indicates that the reactive CpG site has no special advantage over A·T sequences in noncovalent interaction. With an apparent binding affinity of roughly 1 mM^{-1}, the transient reactive species has only a modest DNA binding affinity.

Though mitomycins bind noncovalently to A·T as well as G·C sites, cross-linking occurs only at CpG sequences. Cross-linking which involves noncovalent binding or recognition of specific sequences in DNA followed by alkylation requires the presence of active functional groups on both mitomycins and bases of DNA. At A·T sequences, reactive sites for alkylation are unavailable, so mitomycins will only bind and not cross-link DNA. We found NMA and MS to be more active than MC even though their noncovalent dissociation constants are similar; it is probable that MS and NMA are better cross-linking agents because they possess more active functional groups.

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Registry No. NMA, 18209-14-8; MS, 15973-07-6; MC, 50-07-7; mitomycin, 1404-00-8.

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