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Selective Recognition of the m^{5}CpG Dinucleotide Sequence in DNA by Mitomycin C for Alkylation and Cross-linking

Wendell S. Johnson, Qiao-Yun He and Maria Tomasz*

Department of Chemistry, Hunter College, City University of New York, New York, NY 10021, U.S.A.

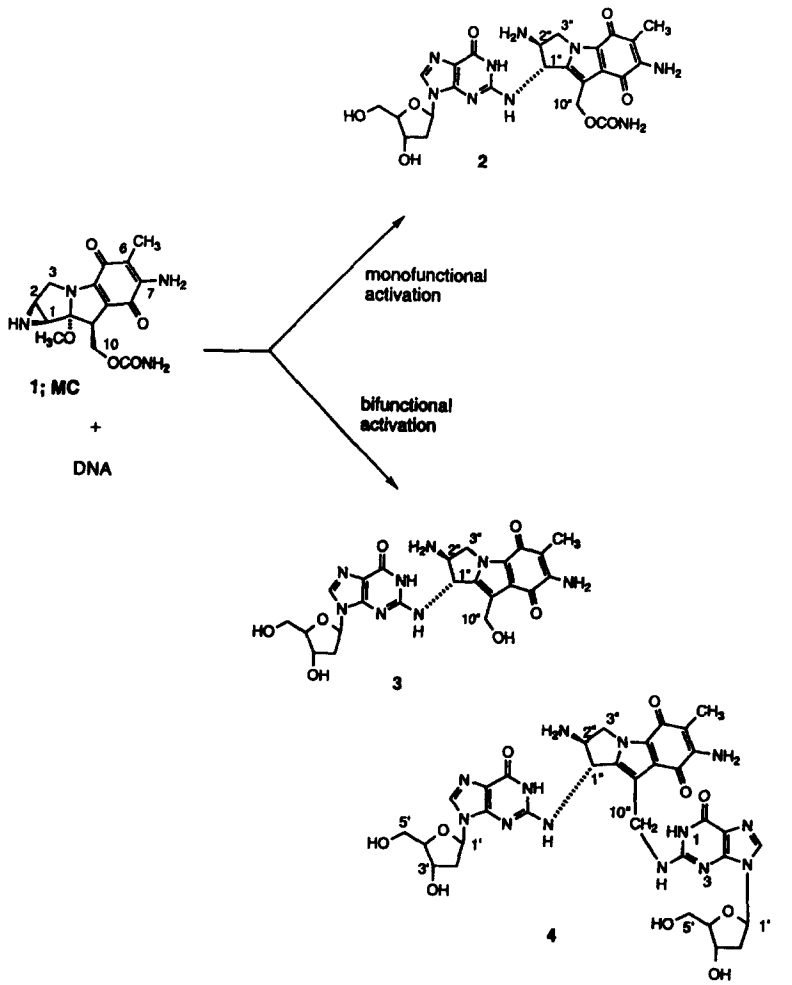
Abstract—The clinically used natural antitumor agent mitomycin C (MC) is known to alkylate DNA monofunctionally and bifunctionally, resulting in the cross-linking of DNA. These reactions occur selectively with guanines at the CpG sequence. We show, confirming a previous report (Millard, J. T.; Beachy, T. M. *Biochemistry* **1993**, *32*, 12850) that cross-linking in oligonucleotides is further enhanced when the cytosines in CpG·CpG are 5-methylated to m^{5}CpG·m^{5}CpG. It is shown, furthermore, that guanines in m^{5}CpG are monoalkylated two- to three-times faster than in CpG indicating that the m^{5}C-induced rate enhancement occurs at the first, monoalkylation step of the two-step cross-linking process. The same MC-DNA adducts are formed in methylated as in non-methylated DNA. The basepaired but not the 5'-flanking, m^{5}C residue is responsible for the enhanced alkylation of guanine. Enzymatically activated or $Na_2S_2O_4$-activated MC shows identical rate-enhancement of alkylation at m^{5}CpG. pBR322 DNA methylated by CpG-methylase was cross-linked two- to three-times more efficiently by MC than non-methylated DNA, indicating that the m^{5}C effect is not an artifact of oligonucleotides. An electronic effect of the 5-methyl group of cytosine transmitted via G·C H-bonding to N^2 of guanine is suggested as responsible for increased reactivity with MC. CpG is severely depleted in mammalian DNA and it is speculated that this factor attenuates MC cytotoxicity in human cells.

Introduction

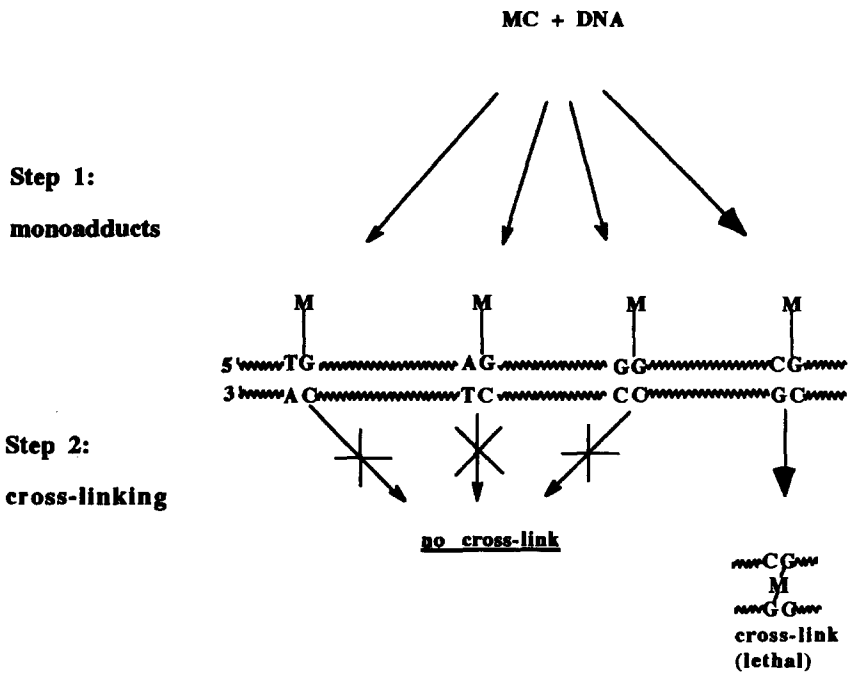
The antitumor antibiotic mitomycin C (**1**; MC[1]) is known to alkylate DNA and cross-link its complementary strands.[2] These related DNA damaging properties are generally considered as the ultimate basis of the antitumor and cytotoxic activity of MC.[3] It has been shown that covalent alkylation of DNA by MC occurs at N^2 of guanines. Monofunctional alkylation proceeds via the C-1 aziridine function, resulting in monoadduct **2**, while bifunctional alkylation involves both the C-1 aziridine and C-10 carbamate, giving rise to a guanine bisadduct, **4** which accounts for the observed cross-linking of DNA by MC (Scheme 1).[4,5] Activation of MC is required via reduction of its quinone to induce its alkylating activity.[2,6] A number of flavoreductases have been identified which activate MC *in vivo*.[3] The adducts **2–4** have been shown to be major products of MC's attack on DNA in cell-free systems as well as in tumor cells[7] and rat liver.[5]

The reactivity of guanines of double-stranded DNA with MC varies with their sequence context. Thus, guanines in the CpG sequence are highly preferred sites of alkylation compared to the other three NpG dinucleotide sequences.[8–10] The 3'-flanking base (N' in the NpGpN' triplet sequence) has only a relatively small modulating effect on the dominant CpG specificity. Remarkably, the DNA interstrand cross-link between two guanines (**4**; product of bifunctional alkylation by MC) is even more specific to CpG: it is formed exclusively between guanines at the CpG·CpG sequence rather than those at GpC·GpC or any other sequence.[11–14] The CpG-specificity of the monoalkylation was explained by formation of a precovalent H-bond at CpG·CpG between the O-10 of the reduced MC and the N^2-H of the guanine in the strand opposite from that of the covalent attack; this facilitates the covalent reaction.[9,10] Recent NMR evidence supports this mechanism.[15] The cross-link (bifunctional alkylation) specificity is reinforced for the same sequence by an independent mechanism, namely the exclusive orientation of the monoadduct in the minor groove which aligns it for CpG·CpG cross-linking only[14,15] (Scheme 2). In view of this one may think[9] that the structure of MC has been shaped by molecular evolution for maximum potency as a DNA cross-linking agent, with minimal reactivity at the non-cross-linkable guanine sites where only the less lethal monoadduct can be formed (Scheme 2).

Recently, it was reported by Millard and Beachy[16] that the efficiency of the cross-linking of synthetic oligonucleotides by $Na_2S_2O_4$-activated MC is increased 1.4- to 2-fold when the cytosine in the requisite CpG sequence is 5-methylated. Since CpG of mammalian DNA is 60–90% methylated[17] m^{5}CpG may be the most frequently cross-linked sequence by MC in the mammalian genome. The mechanism by which the 5-methyl substitution in cytosine accelerates the cross-linking of guanines by MC at the m^{5}CpG·m^{5}CpG sequence was not studied by Millard and Beachy.[16] In order to learn more about this phenomenon we investigated its critical characteristics with respect to the following questions: (i) identity of the covalent MC adducts. (ii) Which of the two consecutive alkylation



Scheme 1. Reaction products of mitomycin C with DNA.



Scheme 2. Preferential recognition of CpG by MC in its first alkylation step (bold headed arrow). A cross-linking event can only occur at this site.

steps (*c.f.* Scheme 2) is affected by the methylation? (iii) Of the two m^5Cs in $m^5CpG{\cdot}m^5CpG$ which one affects the reactivity of a guanine: the 5'-flanking one or the one in the opposite strand? (iv) Is the methylation-induced enhancement observed also under the more natural, enzymatic activation of MC? (v) Is it

operative in native high molecular weight DNA? We show that the reactivity of a guanine towards MC is specifically enhanced by 5-methylation of its basepaired cytosine partner. The enhancement occurs in the first, monoalkylation step which is then reflected in increased DNA cross-linking at the m^5CpG sites. A mechanism of the enhancement, featuring an electronic effect of cytosine methylation is suggested which is experimentally testable. The alkylation by MC presents the first case of increased reactivity of guanine when basepaired with 5-methyl cytosine in DNA.

Results

Monoadduct enhancement at m^5CpG in oligonucleotides

The identity and yield of the MC monoadduct **2** formed in oligonucleotides with reductively activated MC was determined by HPLC of the enzymatic digests of MC-oligonucleotide covalent complexes, according to our developed methodology.[9] Such digests contained the unmodified nucleoside components of DNA and MC-deoxyguanosine adducts; the yields were determined by HPLC peak area measurements. For example, Figure 1a and b depicts the HPLC of the digests of oligonucleotides I and II (Table 1) after their reaction with $Na_2S_2O_4$-activated MC. The same, single adduct **2** was formed with both CpG- and m^5CpG-containing oligonucleotides. Replacement of C with m^5C led to 1.9- to 2.4-fold increases of the yield of **2** (Table 1). The data suggest that replacement of C by m^5C in both strands gives a larger increase than in one strand only (2.4-fold versus 1.9-fold; oligonucleotides III–VI in Table 1).

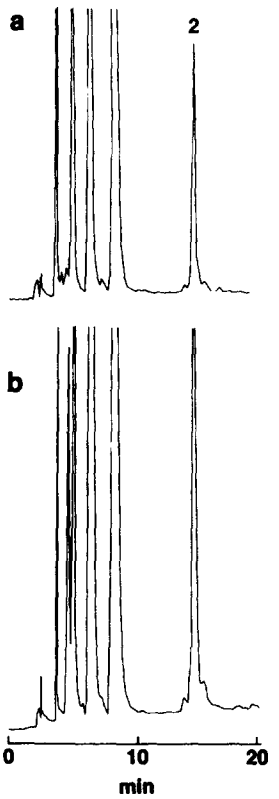


Figure 1. HPLC assay of MC-monoadduct **2** in enzymatic digests of alkylated oligonucleotides. (a) Digest of the alkylation product from oligonucleotide I. (b) Same from oligonucleotide II. Both oligonucleotides were monoalkylated by $Na_2S_2O_4$-activated MC under the standard conditions. HPLC conditions: Beckman ODS C-18 Ultrasphere column, 4.6 x 250 mm; 6–18% acetonitrile/0.03 M sodium phosphate (pH 5.3) gradient in 30 min, at 1 mL min^{-1} flow rate.

Table 1. Mol per cent yields of MC-monoadduct **2** in a series of oligodeoxyribonucleotides having different cytosine methylation status

oligonucleotide duplex		mol % yield of **2** with activation by	
no.	structure	$Na_2S_2O_4$	cytochrome *c* reductase
I	5'-ATATACGTATAT 3'-TATATGCATATA	26	20
II	5'-ATATAm^{5}CGTATAT 3'-TATATGm^{5}CATATA	49 (1.9-fold)[a]	37 (1.85-fold)[a]
III	5'-AATAACGATAAT 3'-TTATTGCTATTA	17, 23 (av[b]:20)	
IV	5'-AATAAm^{5}CGATAAT 3'-TTATT GCTATTA	36, 41 (av:38.5) (1.9-fold)[c]	
V	5'-AATAAC GATAAT 3'-TTATTGm^{5}CTATTA	39 (1.95-fold)[c]	
VI	5'-AATAAm^{5}CGATAAT 3'-TTATTGm^{5}CTATTA	47 (2.4-fold)[c]	
VII	5'-ATTATGGTTATT 3'-TAATACCAATAA	8	
VIII	5'-ATTATG GTTATT 3'-TAATACm^{5}CAATAA	12 (1.5-fold)[d]	
VIII	5'-ATTAT GGTTATT 3'-TAATAm^{5}CCAATAA	6.5 (0.8-fold)[d]	

[a]Increase as compared to no. I.
[b]Average.
[c]Increase as compared to no. III.
[d]Increase as compared to no. VII.

Activation of MC by NADPH:cytochrome *c* reductase resulted in a 1.85-fold increase of yield in the prototype m^5C-containing oligonucleotide II, which was identical to that observed upon MC-activation by $Na_2S_2O_4$ (Table 1).

Monoadduct enhancement at GpG·m⁵CpC and GpG·Cpm⁵C (Table 1)

Monoadduct (**2**) yields are lower at GpG than at CpG.[8,9] Nevertheless, in oligonucleotide VIII a significant yield increase (1.5-fold) was observed when the 5'-C was replaced by m^5C. Replacement of the 3'-C gave no increase; the observed small decrease (1.2-fold) is within experimental error and is, therefore, not significant.

Enhancement of cross-linking of oligonucleotides by MC and m⁵CpG

The yield of cross-linking was determined by an HPLC assay, based on the separation of the cross-linked oligonucleotide from the unmodified parent starting material.[14] This is illustrated in Figure 2a and b, in the case of oligonucleotides X and XI, listed in Table 2. Replacement of C by m^5C in both strands resulted in 1.9-fold enhancement of the yield of the cross-linked oligonucleotide (Table 2). When two tandem CpGs were present the control, C-containing oligonucleotide XII was 72% cross-linked, in accordance with our earlier results.[14] Replacement of the Cs by m^5C (oligonucleotide XIII) did not result in any observable increase over this value, most likely because the rate of the reaction deviates appreciably from pseudo-first order when approaching saturation.[14] The cross-linked oligonucleotides were digested to nucleosides and MC-nucleoside adducts and the digest was analyzed by HPLC.[18] In all cases the identity of the single MC-nucleoside adduct was established as **4** by comparison with authentic standard **4** (data not shown).

Replacement of C by m⁵C at CpG activates specifically the opposite-strand guanine

This was shown by using a special method of digestion of the monoadducted oligonucleotides developed in our laboratory which allows differentiation between monoadducts formed at the two guanines in the same oligonucleotide duplex.[9] The method features the use of nuclease P_1 instead of the usual snake venom diesterase (SVD) for the digestion. Unlike SVD, nuclease P_1 cannot cleave the GpN phosphodiester linkage when G is monoalkylated by MC (adduct **2**), therefore, the endproducts of digestion are the dinucleoside phosphate derivatives of **2**, d-[G(M)pN].[4] The 3'-nucleoside N serves as a marker for the location of the adduct **2** within the oligonucleotide duplex. Oligonucleotides III–VI (Table 3) differing in m^5C

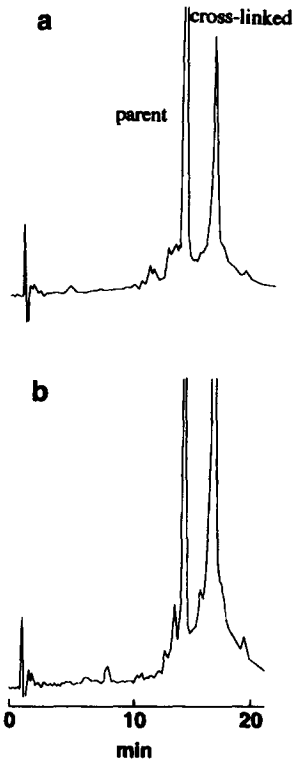


Figure 2. HPLC assay of cross-linked oligonucleotides resulting from bis-alkylation by $Na_2S_2O_4$-activated MC under standard conditions. (a) Reaction mixture of oligonucleotide X. (b) Reaction mixture of oligonucleotide XI. HPLC conditions: Beckman RPSC, C-3 Ultrapore column, 4.6 x 75 mm; 6–18% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) buffer gradient in 24 min at 1 mL min^{-1} flow rate.

Table 2. Cross-linking of oligodeoxyribonucleotides by MC at CpG and m^5CpG sites by MC

oligonucleotide duplex		% yield of
no.	structure	cross-link
X	5'-ATATACGTATAT 3'-TATATGCATATA	30, 31
XI	5'-ATATAm⁵CGTATAT 3'-TATATGm⁵CATATA	65, 56, 53 (Av[a]:58) (1.9-fold)[b]
XII	5'-ATATACGCGTATAT 3'-TATATGCGCATATA	72
XIII	5'-ATATAm⁵CGm⁵CGTATAT 3'-TATATGm⁵CGm⁵CATATA	77 (1.07-fold)[c]

[a]Average.
[b]Increase as compared to X.
[c]Increase as compared to XII.

Table 3. Mol per cent yields of d-[G(M)pN] (dinucleoside phosphate derivatives of monoadduct 2) in oligodeoxyribonucleotides having different cytosine methylation status

oligonucleotide duplex		mol % yield of		yield
no.	structure	d-[G(M)pA][a]	d-[G(M)pT][b]	ratio[c]
III	5'-AATAACGATAAT 3'-TTATTGCTATTA	8	8	1:1
VI	5'-AATAAm^{5}CGATAAT 3'-TTATTGm^{5}CTATTA	21	25	1:1.2
IV	5'-AATAAm^{5}CGATAAT 3'-TTATTG CTATTA	9	19	1:2.1
V	5'-AATAAC GATAAT 3'-TTATTGm^{5}CTATTA	25	12	2.1:1

[a] Alkylation product from upper strand.
[b] Alkylation product from lower strand.
[c] Of d-[G(M)pA] to d-[G(M)pT].

status were submitted to monoalkylation by $Na_2S_2O_4$-activated MC under the standard conditions. Digestion by nuclease P_1/alkaline phosphatase and subsequent HPLC of the digest resulted in the HPLC patterns shown in Figure 3. As expected, two MC-dinucleoside phosphate adducts were formed in all cases, identified as d-[G(M)pT] and d-[G(M)pA] by comparison with authentic standards. Their absolute and relative yields (Fig. 3, Table 3), however, differed dramatically, depending upon the m^{5}C status of the oligonucleotide. Thus, the unmethylated oligonucleotide III produced d-[G(M)pT] and d-[G(M)pA] in equal yields. The oligonucleotide VI, containing m^{5}C in both the upper and lower strands gave three-fold enhanced yields of each, but their yields relative to each other were close to one (1.2). The yields from the hemimethylated oligonucleotides IV and V, however, showed considerable dissymmetry: in IV, the yield of d-[G(M)pT] was 2.1-fold higher than that of d-[G(M)pA] indicating that the guanine in the bottom strand was 2.1-fold more reactive with MC than the guanine in the top strand. In V the opposite was true: the yield of d-[G(M)pA] was 2.1-fold higher than that of d-[G(M)pT], which indicated that in this oligonucleotide the top strand guanine was more reactive. These results show unambiguously that m^{5}C activates its basepaired guanine in the opposite strand while it has no appreciable effect on the 3'-flanking guanine in the same strand.

Enhancement of MC-induced cross-linking in pBR322 DNA upon CpG methylation

This was demonstrated by comparing the yield of cross-linking of the 4362-bp plasmid, pBR322 DNA (linearized) with that of the same DNA after being methylated exhaustively with CpG-methylase (M. SssI methylase). We determined that our methylated DNA had 71.5% of its CpG sequences methylated to m^{5}CpG (see Experimental). The comparison of the yields of cross-linking of the non-methylated and methylated DNA was carried out using a sensitive DNA cross-link assay.[19] In this assay linear, 3'-^{32}P-labeled pBR322 DNA is treated with a cross-linking agent, followed by heat-denaturation and subsequent electrophoresis on non-denaturing 1% agarose gels. Cross-linked DNA migrates as slowly as control native non-denatured DNA on account of its rapid renaturation upon cooling, while the non-cross-linked DNA fraction migrates faster, as two single strands. Both native and methylated labeled DNAs were treated with increasing concentration of MC under anaerobic $Na_2S_2O_4$-activating conditions, resulting in increasing yields of cross-linked DNA (Fig. 4). The per cent yield of cross-linked DNA in the case of the methylated pBR322 was three-fold higher at the lowest MC concentration used (5 μM), two-fold higher at 10 μM MC and less than two-fold higher at higher cross-linking values as cross-link saturation was

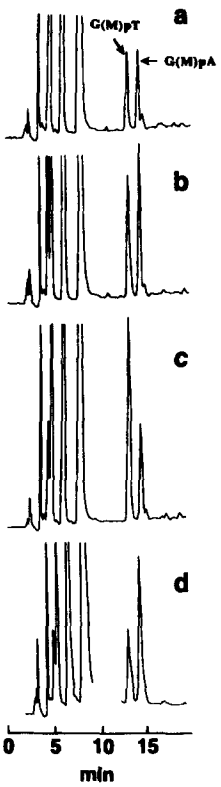


Figure 3. HPLC assay of d-[G(M)pN] (dinucleoside phosphate derivatives of adduct 2) in nuclease P_1-alkaline phosphatase digests of alkylated oligonucleotides. Digest of the alkylation product from (a) oligonucleotide III; (b) oligonucleotide VI; (c) oligonucleotide IV; (d) oligonucleotide V. HPLC conditions: same as in Figure 1.

approached (Fig. 4c). These results demonstrate that 5-methyl cytosine in high molecular weight natural DNA exerts the same activating effect on the alkylation of guanine by MC as in short oligonucleotides.

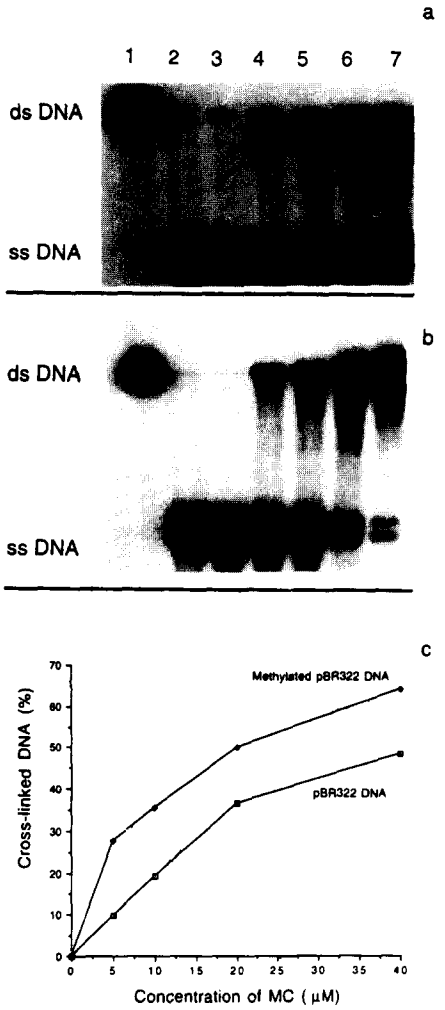


Figure 4. Assay of cross-linking of non-methylated and methylated pBR322 DNA by MC utilizing neutral agarose gel electrophoresis. (a) Autoradiogram of the cross-linking reaction mixture of linearized 3'-[^{32}P]pBR322 DNA (non-methylated). (b) Same, methylated DNA. In both (a) and (b): lane 1, control DNA; lane 2, control DNA, heat-denatured; lanes 3–7, DNA treated with 0, 5, 10, 20 and 40 μM MC and $Na_2S_2O_4$, and subsequently heat-denatured. (c) Plot of % cross-linking as function of MC concentration, calculated from the relative densities of the single-stranded and double-stranded DNA bands, as determined by densitometry.

Discussion

The present work identifies guanine in the m^{5}CpG sequence as the most reactive site in the monoalkylation and cross-linking of duplex DNA by MC. It thus complements a recent report which described this sequence-preference by the cross-linking alone.[16] It is shown that m^{5}CpG surpasses CpG in its affinity to MC, the sequence previously identified as the most preferred target in non-methylated DNA including both types of MC-induced damage.[8–14] A comprehensive summary of the sequence specificities of MC as determined in an oligonucleotide model series is given in Figure 5, assembled from our present and previously obtained data.[9,14] It is seen that the rather impressive CpG specificity of MC monoalkylation is further enhanced (approximately 2-fold) when the cytosine is 5-methylated. Furthermore, for the cross-linking CpG is the exclusive target and methylation of the cytosine further increases its affinity to cross-linking two- to three-fold.

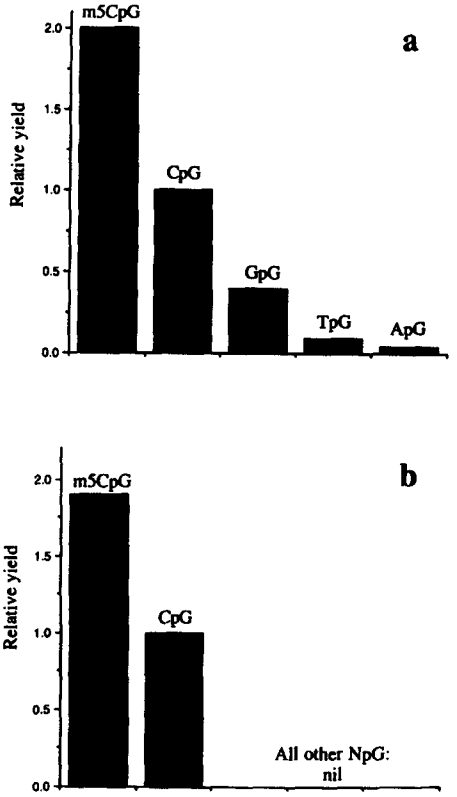


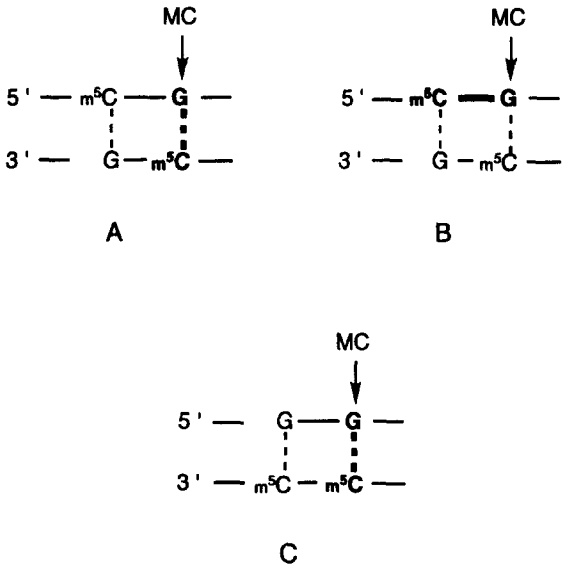
Figure 5. Sequence-specific relative reactivity of MC with guanine in (a) monoalkylation and (b) cross-linking reactions of duplex oligodeoxyribonucleotides. The data in (a) originate partly from Ref. 9 (NpGpT series), and from Table 1. (b) Data from Table 2.

DNA-sequence selectivity, in addition to base selectivity is often observed of DNA-damaging agents.[20] This phenomenon in general is of interest in at least two respects: (i) it is expected to play a role in the biological effects of drugs in various ways (although explicit demonstration of this is lacking, to our knowledge). (ii) It can reveal information about the mechanism of the chemical reaction between the drug and DNA.[20] We shall discuss the case of MC in both of these contexts as follows.

Although the MC targets CpG and m^{5}CpG are very short sequences indeed, they have unique roles and properties in the mammalian genome. CpG occurs at an anomalously low frequency compared to the other 15 dinucleotides; only at one-fifth of the statistically calculated value, i.e. once per every 200 bases on average.[21,22] Many of the CpGs are clustered in G + C-rich regions where they occur at high frequency. Such regions have been identified in repetitive satellite DNA and in non-repetitive DNA of transcription regulatory

sites. Approximately 60–90% of all CpG sites in vertebrate DNA are methylated to m^5CpG.[21] Temporal patterns of methylation–demethylation have been shown to regulate promoter activity and, furthermore, normal development.[23,24] CpG-methylated promoters are transcriptionally silent, by a mechanism in which a repressor protein binds the methylated promoter, thereby blocking its transcription.[24,25] CpG clusters in certain promoters, usually those of constitutively expressed 'housekeeping genes' are never methylated, however ('CpG islands').[21] Tumor tissues may display abnormal CpG methylation patterns.[26] CpG is also a 'hot spot' for spontaneous point mutations.[27] While it is interesting that MC has intrinsic preference for CpG and m^5CpG as targets, no indications exist in the literature that it acts as selective inhibitor of eukaryotic transcription. Increased expression of certain genes by MC, however, has been described[28] which might now be explained by assuming that MC-m^5CpG adducts block the binding sites of m^5CpG-binding proteins, and thereby prevent them from repressing transcription. Primarily, however, MC is regarded as a selective inhibitor of DNA replication[29] and this property is most likely to be critical in the drug's killing of tumor cells. Given this mechanism of action the CpG specificity of MC could very well be a disadvantage in its action as replication inhibitor of human tumor DNA since this target is present only at very low concentration in the total genome. In this respect, it is interesting that Szybalski and Iyer noted 30 years ago[29] that mammalian DNA was surprisingly resistant to cross-linking by MC in comparison to viral and bacterial DNAs under the same conditions, even though some of these non-eukaryotic DNAs had the same G + C content. Since the CpG-frequencies were much higher in those organisms than in mammalian DNA it is likely, in retrospect, that they were responsible for the observed more efficient cross-linking by MC. Designing and testing MC analogs which cross-link human DNA at a more abundant sequence, could probe, in principle, whether altered sequence specificity correlated with increased cytotoxicity in this series of drugs.

What is the chemical mechanism of the enhanced rate of alkylation and cross-linking of guanine by MC upon 5-methylation of cytosine? As we show here, the rate-enhancement is associated with m^5C in the basepair rather than m^5C in the 5'-flanking position (Scheme 3, A). GpG alkylation is also enhanced by m^5C in the partner strand (see Results). The enhancement occurs at the initial, monoalkylation step. The effect is relatively small; the observed two-fold rate enhancement corresponds to a 0.4 kcal mol^{-1} decrease of the activation energy at 25 °C. Millard and Beachy[16] in their report on the m^5C effect on MC cross-linking speculated that its origin lay in either the enhancement of nucleophilicity of the guanine-2-amino group (electronic factor) or a local conformational change, rendering this amino group more accessible (steric factor). Indeed, a local conformational change caused by cytosine methylation in the self-complementary duplex d-(CCAGGCm^5CTGG) in the crystalline state was reported and described[30] as "small and limited to the basepairs directly involved". The m^5C·G basepairs were found pushed slightly toward the minor groove of the helix. In another investigation alteration of the intrinsic DNA bending of three promoters was observed upon methylation of CpG sites.[31] Speculation, however, on any correlation of these observations to the guanine-MC reaction rate would be unwarranted before more specific information becomes available.



Scheme 3. Potential alternative activation of guanine by the basepaired m^5C (A) or by the 5'-flanking m^5C (B). (C) Activation of the 3'-guanine in GpG by its basepaired m^5C. The interactive bases are indicated by bold-face.

Alternatively, electronic factors may be considered as the possible cause of the increased reaction rate by cytosine methylation. Replacement of the H-5 atom of cytosine with the electron-donating methyl substituent increases the pK_a of the base as expected, by a small value: the pK_as of d-m^5C and dC were reported as 4.4 and 4.3,[32] or 4.6 and 4.4,[33] respectively.[34] Molecular polarizability is also increased by the methylation.[35] We hypothesize that the inductive electronic effect of the m^5C methyl group may be transmitted to the 2-amino nitrogen of the partner guanine via the cytosine··O^2/guanine-N^2H H-bond and increases the nucleophilicity of guanine-N^2. This hypothesis could be tested by substituting cytosine with 5-fluorocytosine in oligonucleotides. If it is correct then this should result in the opposite effect from that of 5-methylcytosine: a slower rate of alkylation and cross-linking by MC. Such experiments are in progress in our laboratory. It is consistent with this theory, that alkylations at guanine-O^6 or -N7 are not enhanced by basepairing with m^5C.[36]

Experimental

Materials

Oligonucleotides were synthesized on a DNA synthesizer, Applied Biosystems 380B, using the

cyanoethyl phosphoramidite method. The reagents were purchased from Applied Biosystems Inc., Foster City, CA. The crude products (1-μmol scale, 'trityl-off'), after deprotection by concentrated NH_4OH according to the manufacturer's protocol (Users Bulletin, No. 13, Revised, 1 April 1987) were routinely purified by a passage through a Sephadex G-25 (fine) column (2.5 x 56 cm; 0.02 M NH_4HCO_3 eluant). The homogeneity and nucleoside composition of the oligonucleotides were routinely checked by HPLC, as described previously.[9]

Enzymes used and their sources were as follows: Snake venom diesterase (*Crotalus adamanteus* phosphodiesterase I), *Escherichia coli* alkaline phosphatase (type III-R), Worthington, Freehold, NJ; nuclease P_1, Pharmacia P-L Biochemicals. Purified NADPH: cytochrome *c* reductase from rat liver was a gift of Dr Wayne Backes, Louisiana State Medical School, New Orleans, LA. NADPH was purchased from Boerhinger Mannheim (Indianapolis, IN). Mitomycin C was obtained from Bristol-Myers Squibb Co. (Wallingford, CT).

Reagents for the assay of cross-linking of pBR322 DNA and their sources were the following: pBR322 DNA (supercoiled), Klenow fragment of DNA polymerase I and restriction endonuclease *Eco*RI (GIBCO BRL Life Technologies, Grand Island, NY); [α-^{32}P]dATP (Dupont Technology Division, Wilmington, DE); agarose gel electrophoresis supplies (Sigma, St. Louis, MO); M. SssI methylase, *S*-adenosyl-methionine (New England BioLabs, Beverly, MA).

HPLC was performed using reverse phase columns and a Beckman Model 332 HPLC system, including a Beckman Model 427M integrator and Model 165A absorbance detector, set to 254-nm wavelength. Specific run conditions are described in the appropriate figure legends.

Quantitative analysis of oligonucleotides

This was based on absorbance measurements in 0.1 M Tris, pH 7.0 buffer. The molar extinction coefficients E_{260} of single stranded oligonucleotides were calculated as equal to (number of purines)(14,000)+(number of pyrimidines)(7000).[37] Oligonucleotide concentration can also be expressed in mononucleotide units. For this, E_{260}=10,000 was used as the average molar extinction coefficient of mononucleotide units.

Standard reaction conditions of monoalkylation of oligonucleotides by MC to form monoadduct 2

(i) $Na_2S_2O_4$ as reducing agent: 12-mer duplex oligonucleotides (self-complementary or complementary 1:1 molar mixture) (1 μmol mononucleotide unit/mL) were incubated with MC (4 μmol mL^{-1}) in 0.1 M potassium phosphate buffer, pH 7.5, at 0 °C in an ice bath under stirring, open to the atmosphere. Fresh, anaerobically prepared $Na_2S_2O_4$ solution (40 mM in above buffer) was added at once to 2 μmol mL^{-1} final concentration. After 1 h at 0° the mixture was immediately chromatographed on a 2.5 56 cm Sephadex G-50 column using 0.002 M NH_4HCO_3 as eluant. The first UV-absorbing peak fraction contained the partially alkylated oligonucleotide(s) which was then lyophilized. (ii) NADPH:cytochrome *c* reductase/ NADPH as reducing agent: The same procedure was employed except 8 μmol mL^{-1} NADPH and 6.12 units mL^{-1} NADPH:cytochrome *c* reductase was substituted for $Na_2S_2O_4$ and the reaction was conducted anaerobically by constant purging with argon.

Standard reaction conditions of cross-linking of oligonucleotides by MC

$Na_2S_2O_4$ as reducing agent: the same 12-mer oligonucleotides as above (2 μmol mononucleotide unit/mL) were mixed with 4 μmol mL^{-1} MC in 0.1 M Tris, pH 7.4 buffer and the solution was deaerated and kept anaerobic by constant purging with argon. At 5°C $Na_2S_2O_4$ (0.16 M; fresh anaerobic solution in the same buffer) was added in 5 portions at 10-min intervals, to a final concentration of 6 μmol mL^{-1}. After 1 h the reaction mixture was exposed to air and chromatographed over a 2.5 × 56 cm Sephadex G-25 (fine) column. The void volume fraction, containing cross-linked and unreacted oligonucleotides was lyophilized.

Enzymatic digestion of monoalkylated and cross-linked oligonucleotides to nucleosides and MC-deoxyguanosine adducts 2 or 4

Two A_{260} units of the reaction product were digested with 2 units of SVD and 1 unit of *E. coli* alkaline phosphatase in 0.4 mL of 0.1 M Tris–2 mM $MgCl_2$ pH 8.2 buffer at 45 °C for 4.5 h. The digest was analyzed directly by HPLC (Figs 1 and 2).

Digestion with nuclease P_1/alkaline phosphatase to nucleosides and d-[G(M)pN] adducts

This was accomplished by adding 1.6 units of nuclease P_1 to 2 A_{260} units of oligonucleotide in 0.7 mL dilute aqueous acetic acid, pH 5.5 and incubating the mixture at 37 °C for 2 h. The pH was then adjusted to 8.2 by the addition of 0.5 M Tris, pH 8.2 buffer and 3.7 units of alkaline phosphatase was added. Incubation continued for 2 h at 37 °C.

Calculation of the mol per cent yield of adduct 2 from HPLC analysis of enzymatic digests of alkylated oligonucleotides

The theoretical yield of adduct **2** (mol/mol oligonucleotide duplex) is equal to 0.5 × mol dG/mol oligonucleotide duplex. The 0.5 factor accommodates the fact that only one monoadduct can be formed at a complementary CpG·CpG dinucleotide duplex sequence. From the given molar ratio of dG to dT one

can relate the yield of **2** to dT mol^{-1} oligonucleotide duplex, using the relationship

$$\frac{\text{mol\%}}{\text{yield of } \mathbf{2}} = \frac{\text{area of } \mathbf{2}/24{,}000}{0.5 \times \text{area of dT}/6{,}600} \times \frac{100}{\text{mol dT/mol dG}}$$

where 'area' is the measured area of the corresponding peak in the HPLC; 24,000 and 6,600 are the values of the molar extinction coefficients of **2** and dT, respectively, at 254 nm.[9]

The yields of MC-dinucleoside phosphate adducts d-[G(M)pT] and d-[G(M)pA] were determined analogously to those of **2**, above. The E_{254} values were 30,600 and 37,000, respectively.[9]

Methylation of linearized pBR322 DNA

pBR322 DNA (supercoiled) was linearized by treatment with *Eco*RI restriction endonuclease according to standard protocol.[38] The product was methylated using M. SssI methylase according to a reported procedure.[39]

Determination of the extent of methylation

Methylated pBR322 DNA (25 μg) was digested to nucleosides by DNAse I, SVD and alkaline phosphatase by our standard methodology.[4] The digest was directly analyzed by HPLC using a reversed-phase C18 column (4.6 mm × 25 cm; Beckman Ultrasphere-ODS), eluted with 0–6% CH_3CN in 0.03 M NH_4OAc, pH 7.0 in 25 min, at a flow rate of 1 mL min^{-1}. The molar ratio of m^5dC to dC was determined from the ratio of the areas of the corresponding HPLC peaks, each divided by its molar extinction coefficient E_{254} (6200 and 6300, respectively). The % C methylated in the CpG sequence was calculated from these data using also the known CpG frequency of pBR322 DNA[40] and it was found that 71.5% of CpG was converted to m^5CpG.

Cross-linking of non-methylated and methylated ^{32}P-pBR322 DNA by MC

The linearized DNA (methylated or non-methylated) was 3'-end labeled by [α-^{32}P]dATP and Klenow fragment of DNA polymerase I according to standard protocol.[38] A mixture containing approximately 10 ng [^{32}P]pBR322 DNA, 1000 ng calf thymus DNA and varying amounts of MC (0–1 nmol) in 21 μL 5 mM Tris, pH 7.4–0.8 mM EDTA was deaerated by gentle bubbling with argon for 2 min. A freshly made, deaerated $Na_2S_2O_4$ solution (7.5 mM) (4 μL) in 0.1 M Tris, pH 7.4–2 mM EDTA buffer was added to give final concentrations as 118 μM DNA, 0–40 μM MC and 1.2 mM $Na_2S_2O_4$ in a total volume of 25 μL 20 mM Tris, pH 7.4–1 mM EDTA buffer. The reaction mixture was capped tightly and left at room temperature for 1 h. The reaction was terminated by the addition of an equal volume of 'stop solution' (0.6 M sodium acetate–1 mM EDTA, 300 μg/mL tRNA) and 3 volumes of cold ethanol. The precipitated DNA (–20 °C) was collected, washed and dried by standard procedures.

Assay of cross-linked [^{32}P]pBR322 DNA by neutral agarose gel electrophoresis

This was based on the method developed by Hartley and coworkers[19] and was carried out as we described it previously.[41]

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References and Notes

1. Abbreviations: MC, mitomycin C; SVD, snake venom diesterase; m^5C, 5-methyl cytosine; dG(M), monoadduct **2** (Scheme 1).

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