Role of Electrostatics in the Sequence-Selective Reaction of Charged Alkylating Agents with DNA

Gangning Liang,[†,‡] Lance Encell,[†,‡] Marek G. Nelson,[§] Christopher Switzer,*[,§] David E. G. Shuker,[∥] and Barry Gold*[,†,‡]

Eppley Institute for Research in Cancer and Allied Diseases and Department of Pharmaceutical Sciences University of Nebraska Medical Center Omaha, Nebraska 68198-6805 Department of Chemistry University of California—Riverside Riverside, California 92521 MRC Toxicology Unit, University of Leicester Leicester LE1 9HN, U.K.

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We have previously reported that methanediazonium ion ($CH_3N_2^+$), which is the ultimate carcinogen formed from the oxidative metabolism of *N*-nitrosamines, and the hydrolytic decomposition of *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), methylates N7-guanine (N7-G) in duplex DNA with significant sequence selectivity: the difference between a weak and strong N7-G methylation site can differ by 10-fold.[1,2] $CH_3N_2^+$ also shows a sequence-dependent methylation pattern at O^6-G.[3,4] Other carcinogens and antineoplastic drugs, *e.g.*, chloroethylnitrosoureas and nitrogen mustards, which react via positively charged intermediates, show very similar N7-G alkylation patterns.[5] In contrast, neutral alkylating agents, *e.g.*, dimethyl sulfate (DMS), react uniformly at all G's. It has been proposed that fluctuations in the nucleophilicity at N7-G sites are a consequence of sequence-dependent electrostatic potentials in the major groove.[1,6] The observed inhibition of DNA methylation by inorganic and DNA affinity binding cations confirms a role for electrostatics in the alkylation process and is consistent with the association of the cationic alkylating intermediate with DNA prior to covalent modification.[1,2] Ionic strength has no effect on the methylation of DNA by uncharged alkylating agents, *e.g.*, DMS.[1] It is improbable that steric effects are responsible for the N7-methylguanine (N7-mG) pattern produced by $CH_3N_2^+$ because all G's are equally methylated by the much bulkier DMS, and double-stranded DNA is a better substrate for MNU than single-stranded DNA.[7]

To determine if the controlled positioning of cationic groups could be used to modulate the electrostatic potential in the major groove, MNU was reacted with DNA containing 5-(6-amino-hexyl)-2′-deoxycytidine (zwitterionic dC) residues.[8] We report herein that the incorporation of a zwitterionic dC allows regiospecific inhibition of N7-alkylation of G in duplex DNA by MNU (Figure 1). The N7-mG pattern generated by MNU (500 μM) in "normal" oligomer **1**+**2** (100 μM, phosphate) illustrates the sequence selectivity previously described for MNU.[1,2] When a single C is substituted with a zwitterionic dC (Figure 1, oligomer **1**+**3**), there is a specific inhibition (>60%) of methylation at G-8 and G-9, which are 2−3 bp to the 5′-terminus on the complementary strand (Figure 2). Little change in N7-mG formation is seen at G-7 or G-11 which is base-paired to the zwitterionic residue. This is additional evidence that the zwitterionic dC does not disturb Watson−Crick base pairing,[8] since we have previously shown that MNU is less reactive with G's in single-stranded regions and in noncanonical structures.[7] Multiple zwitterionic substitutions (Figure 1, oligomer **1**+**4**) have a more global effect on N7-mG formation, but the relatively small change at G-13 indicates that the impact is still biased toward the 5′-side of the complementary

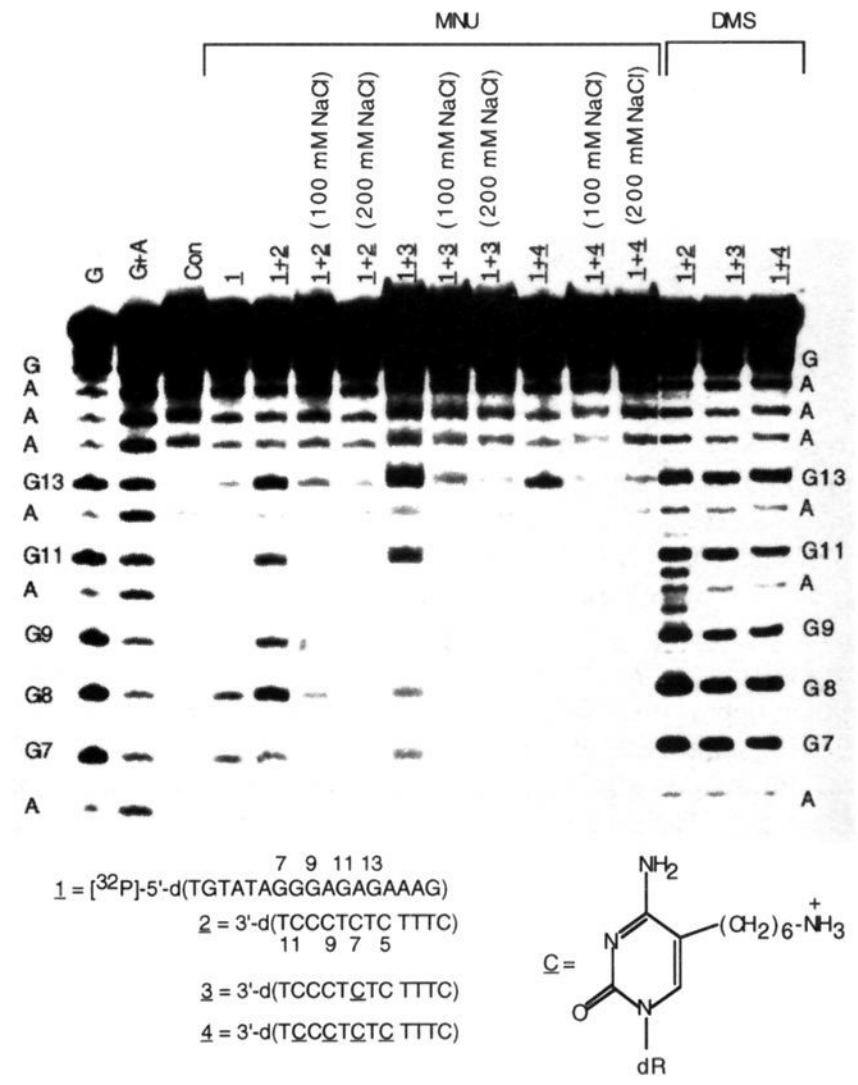

Figure 1. Reaction of MNU and DMS with normal DNA (**1**+**2**) and DNA containing zwitterionic dC residue(s) (**1**+**3** and **1**+**4**). Phosphorimaging results of a denaturing 20% polyacrylamide gel using 5-[^{32}P]-**1** with or without complementary strand **2**, **3**, or **4**. Incubations with MNU (500 μM) were performed at 4 °C in 10 mM sodium cacodylate buffer (pH 7.6) for 16 h. These conditions ensure that the substrate is duplex DNA: the T_M's of **1**+**2**, **1**+**3**, and **1**+**4** are 39.4, 42.8, and 45.4 °C, respectively, in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl.[8] The 5′-overhang on the top strand (oligomer-**1**) was designed to allow detection of strand breaks at G-7, which would be very near the 5′-label and difficult to detect if **1**+**2** was a perfect dodecamer duplex. Incubations with DMS (40 mM) were run in 15 mM sodium cacodylate buffer (pH 8.0), 1 mM EDTA for 1 min at ambient temperature. Upon completion of the reaction, the DNA was precipitated, washed, dried *in vacuo*, and treated with hot piperidine as previously described (Maxam, A.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499−560).

* To whom inquiries should be addressed.

[†] Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center.

[‡] Department of Pharmaceutical Sciences, University of Nebraska Medical Center.

[§] University of California—Riverside.

[∥] University of Leicester.

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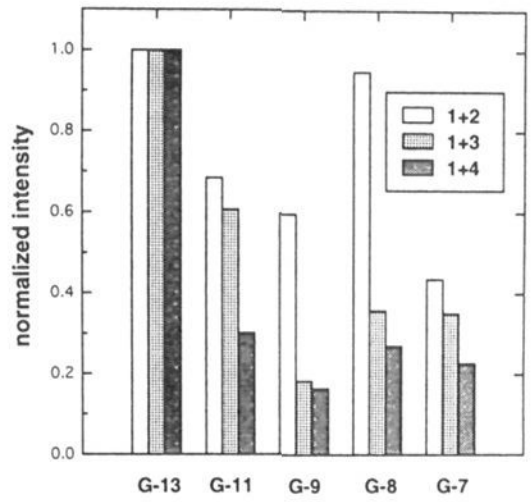

Figure 2. Phosphorimaging-based quantitative analysis of Figure 1. Strand breaks at G-7, G-8, G9, and G-11 from the reaction of MNU with duplexes **1**+**2**, **1**+**3** and **1**+**4** are normalized against G-13, which is the most intense band in each lane.

strand (Figure 2). The fact that there is an additional, albeit small, decrease in N7-mG at G-9 and G-8 in **1**+**4** may suggest that a more general inhibition due to the four zwitterionic residues is possible. This could involve DNA conformational changes.[9] The co-addition of 100 or 200 mM NaCl causes a strong inhibition in the MNU-mediated methylation at all G's in **1**+**2**, **1**+**3**, and **1**+**4**. We have previously noted that ionic strength does not qualitatively alter MNU's methylation pattern.[1] No significant inhibition of G-methylation is seen with the much bulkier methylating agent DMS[10] in **1**+**2** *vs.* **1**+**3** or **1**+**4**. This is clear evidence that the inhibition of DNA methylation by MNU is not due to steric hindrance by the aminohexyl appendage of the zwitterionic residue. Increasing the ionic strength of the incubation medium does not affect DMS-mediated methylation of DNA.[1]

The methylation patterns with MNU are consistent with modeling studies using Arnott B-DNA[11] that show that the extended $(CH_2)_6-NH_3^+$ appendage can reach ~2 bp in the 5′-direction on the complementary strand.[12]

Antibodies were used to quantitate total N3-methyladenine (N3-mA) (minor groove) and N7-mG (major groove) adducts formed from the reaction of **1**+**2** and **1**+**4** with MNU.[13] The data (Table 1) show that multiple zwitterionic residues affect only methylation in the major groove. This contrasts to the inhibitory effect of ionic strength, which causes an equal diminution of products at major and minor groove sites, and on the phosphate backbone.[14]

Table 1. Quantitation of Total N3-Methyladenine (N3-mA) and N7-Methylguanine (N7-mG) Using Monoclonal Antibodies[a]

DNA target	N3-mA[b]	N7-mG[b]
control (**1**+**2**)[c]	nd[d]	0.29 ± 0.11
1+**2**	0.82 ± 0.07[e]	21.76 ± 1.91[f]
1+**4**	0.68 ± 0.04[e]	9.05 ± 2.26[f]

[a] See ref 13 for methods. [b] Millimoles of adduct/mole of DNA. [c] Background signal from unmodified bases in the absence of MNU. [d] Not detected (<0.005). [e] These two values of N3-mA (determined from two independent incubations using four ELISA wells per incubation) are not statistically different from each other using Student's *t* test (*p* value = 0.127). [f] These two values of N7-mG (determined from two independent incubations using four ELISA wells per incubation) are statistically different from each using Student's *t* test (*p* value = 0.026).

In summary, the data represent a unique example of regioselective modulation of DNA damage by zwitterionic substitutions, and they support electrostatic neutralization, and not steric inhibition, being responsible for the inhibition of N7-mG from MNU. The electrostatic potential associated with the grooves is invisible to most physical methods; therefore, probing this property with small charged alkylating molecules provides a unique method to "see" this feature of DNA. Zwitterionic modifications are a valuable tool to understand how molecules recognize this subtle property of DNA.

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(9) Multiple zwitterionic residues alter the helical repeat of DNA as measured by gel mobility studies (J. Maher, personal communication).

(10) The SYBYL (Tripos Associates, St. Louis, MO) calculated van der Waals surface volumes of DMS and $CH_3N_2^+$ are 84.2 and 41.4 Å, respectively.

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(12) In these modeling studies Arnott B-DNA was used (ref 11) and the DNA treated as a rigid aggregate with only the $(CH_2)_6-NH_3^+$ appendage subject to molecular mechanics minimization using the Tripos force field found in SYBYL (Tripos Associates, St. Louis, MO).

(13) Oligomer **1**+**2** or **1**+**4** (70 μM) was reacted with 10 mM MNU in 10 mM sodium cacodylate buffer (pH 8.0) for 48 h at 4 °C. The DNA was then heated at 90 °C for 30 min to release the heat-labile N7-mG and N3-mA adducts. The DNA was precipitated with ice cold 0.1 N HCl, and the supernatant containing the N7-mG and N3-mA lesions was analyzed using antibody-based competitive enzyme-linked immunosorbent analysis (ELISA) as previously described (Prevost, V.; Shuker, D. E. G.; Bartsch, H.; Pastorelli, R.; Stillwell, W. G.; Trudel, L. J.; Tannenbaum, S. R. *Carcinogenesis* **1990**, *11*, 1747−1751. Durand, M.-J.; Shuker, D. E. G. *Carcinogenesis* **1994**, *15*, 957−961).

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