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Effect of Ionic Strength and Cationic DNA Affinity Binders on the DNA Sequence Selective Alkylation of Guanine N7-Positions by Nitrogen Mustards

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ABSTRACT: Large variations in alkylation intensities exist among guanines in a DNA sequence following treatment with chemotherapeutic alkylating agents such as nitrogen mustards, and the substituent attached to the reactive group can impose a distinct sequence preference for reaction. In order to understand further the structural and electrostatic factors which determine the sequence selectivity of alkylation reactions, the effect of increased ionic strength, the intercalator ethidium bromide, AT-specific minor groove binders distamycin A and netropsin, and the polyamine spermine on guanine N7-alkylation by L-phenylalanine mustard (L-Pam), uracil mustard (UM), and quinacrine mustard (QM) was investigated with a modification of the guanine-specific chemical cleavage technique for DNA sequencing. For L-Pam and UM, increased ionic strength and the cationic DNA affinity binders dose dependently inhibited the alkylation. QM alkylation was less inhibited by salt (100 mM NaCl), ethidium (10 μM), and spermine (10 μM). Distamycin A and netropsin (100 μM) gave an enhancement of overall QM alkylation. More interestingly, the pattern of guanine N7-alkylation was qualitatively altered by ethidium bromide, distamycin A, and netropsin. The result differed with both the nitrogen mustard (L-Pam < UM < QM) and the cationic agent used. The effect, which resulted in both enhancement and suppression of alkylation sites, was most striking in the case of netropsin and distamycin A, which differed from each other. DNA footprinting indicated that selective binding to AT sequences in the minor groove of DNA can have long-range effects on the alkylation pattern of DNA in the major groove.

Bis(2-chloroethyl)methylamine (mechlorethamine, nitrogen mustard) was the first clinically effective anticancer agent (Gilman & Philips, 1946), and derivatives such as L-phenylalanine mustard (melphalan, L-Pam),[1] cyclophosphamide, and chlorambucil are still among the most useful clinical agents (Haskel, 1985). Covalent binding may occur at many nucleophilic sites within nucleic acids and proteins, but DNA is probably the most important target with reaction predominantly at the N7-position of guanine (Lawley, 1966; Singer, 1975). The requirement for antitumor activity of two alkylating groups within the mustard molecule suggests that the activity arises from the formation of cross-links between macromolecular sites (Lawley, 1966; Kohn et al., 1966; Kohn, 1980). DNA interstrand cross-links and DNA–protein cross-links have been observed in intact cells (Ewig & Kohn, 1977), and evidence for intrastrand cross-links has been obtained indirectly (Chun et al., 1969).

A modification of the Maxam and Gilbert (1980) guanine-specific chemical cleavage technique for DNA sequencing has allowed a direct examination of the guanine N7 reaction of alkylating agents at the individual base level in purified DNA (Mattes et al., 1986a). Large variations in alkylation intensities existed among guanines in a DNA sequence following treatment with nitrogen mustards (Mattes et al., 1986b; Kohn et al., 1987), *N*-alkyl-*N*-nitrosoureas (Hartley et al., 1986; Wardeman & Gold, 1988), and triazenes (Hartley et al., 1988b). The most striking finding was that most agents reacted preferentially in runs of guanines, the degree of preference being much greater than would be expected from the number of guanines alone. This correlated well with the molecular electrostatic potential at the guanine N7-position imposed by the nearest-neighbor base pairs (Pullman & Pullman, 1981) and suggests that the specific biological effects of such compounds may depend on preferential reaction at GC-rich genomic locations (Mattes et al., 1988). In addition, some nitrogen mustards (in particular uracil and quinacrine mustards, Figure 1) showed distinctly different reaction patterns from other mustards, indicating that the substituent attached to the reactive group could impose a distinct sequence preference for reaction (Mattes et al., 1986), and models to explain this have been proposed (Kohn et al., 1987).

In order to understand further the structural and electrostatic factors which determine the sequence selectivity of alkylation reactions, the present study was undertaken to investigate the effect of cationic DNA binders on guanine N7-

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[1] Abbreviations: L-Pam, L-phenylalanine mustard (melphalan); UM, uracil mustard; QM, quinacrine mustard; MPE, methidiumpropyl-EDTA; MNU, *N*-methyl-*N*-nitrosourea; EDTA, ethylenediaminetetraacetic acid.

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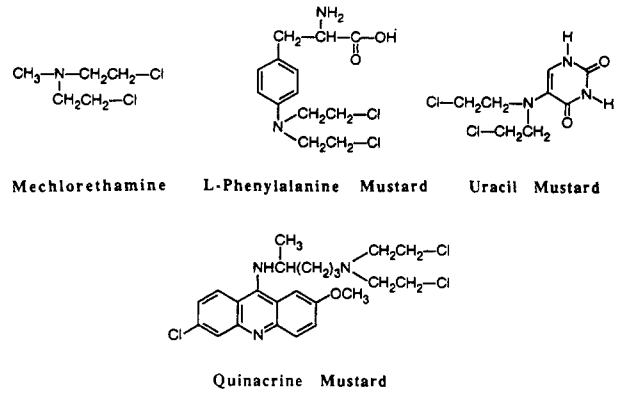

FIGURE 1: Structures of the nitrogen mustards used in this study.

alkylation in the major groove of DNA. Increased ionic strength has been shown to produce the expected general reduction in reaction rates but does not alter the rank order of reactivity of guanines to nitrogen mustards (Kohn et al., 1987; Hartley et al., 1988a) although the degree of selectivity was altered in some cases. Recently, methylation of DNA by *N*-methyl-*N*-nitrosourea (MNU) was shown to be dose dependently inhibited by NaCl, ethidium bromide, distamycin A, or spermine, but the pattern of guanine N7-alkylation was not altered by salt or the cationic DNA affinity binders (Wurdeman & Gold, 1988). In contrast, however, the present study clearly indicates that cationic DNA binders can quantitatively and qualitatively alter the pattern of alkylation of DNA by nitrogen mustards.

Materials and Methods

Chemicals. Mechlorethamine (HN2) was obtained from Sigma Chemical Co., L-phenylalanine mustard (Melphalan, L-Pam) from the Wellcome Foundation, uracil mustard (UM) from The Upjohn Co., and quinacrine mustard (QM) from Fluka Chemical Co. Netropsin was obtained from Serva Biochemicals, distamycin A and spermine were from Sigma, and ethidium bromide and piperidine were from BDH. Electrophoresis-grade acrylamide and bis(acrylamide) were purchased from Sigma; ultrapure urea and agarose were from BRL. [γ-^{32}P]ATP (5000 Ci/mmol) was from New England Nuclear and pBR322 plasmid DNA from Pharmacia P-L Biochemicals. MPE was obtained from Professor P. Dervan, California Institute of Technology.

Enzymes. Restriction enzymes *Hin*dIII and *Sal*I, T4 polynucleotide kinase (PNK), and bacterial alkaline phosphatase (BAP) were obtained from BRL.

Buffers. TEA is 25 mM triethanolamine and 1 mM EDTA, pH 7.2. TBE electrophoresis buffer is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.3. Alkylation stop solution is 0.6 M sodium acetate, 20 mM EDTA, and 100 μg/mL tRNA. BAP buffer is 10 mM Tris-HCl and 120 mM NaCl, pH 8. PNK buffer is 60 mM Tris-HCl 15 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 35 μM ATP, pH 7.8. *Hin*dIII buffer is 50 mM Tris-HCl, 10 mM $MgCl_2$, and 50 mM NaCl, pH 8, and *Sal*I buffer is 100 mM Tris-HCl, 10 mM $MgCl_2$, and 150 mM NaCl, pH 7.6.

Preparation of End-Labeled DNA. pBR322 DNA was linearized by reaction with *Hin*dIII (2 units/μg, 37 °C, 1 h), dephosphorylated with BAP (3 units/μg, 65 °C, 1 h), and purified by standard phenol/chloroform extraction and ethanol precipitation reactions (Maniatis et al., 1982). The DNA was labeled at 5′ ends with T4 PNK as described by Maxam and Gilbert (1980). The DNA was further cut with *Sal*I (2 units/μg, 37 °C, 1 h) and the 675 base pair fragment isolated by preparative electrophoresis on 0.8% agarose gels.

Alkylation Reactions. Labeled DNA (~50000 cpm/sample) was incubated with the nitrogen mustard in TEA buffer in a total volume of 50 μL for 60 min at 20 °C. Where appropriate, 100 mM NaCl was included in the reaction mixture. In experiments involving the cationic DNA affinity binders, these were added to the DNA in TEA buffer and incubated for 30 min at 20 °C prior to the addition of the alkylating agent. The reaction was terminated by the addition of 50 μL of cold alkylation stop solution and DNA recovered by precipitation with three volumes of 95% ethanol. The DNA was resuspended in 0.3 M sodium acetate–1 mM EDTA and ethanol precipitated again and the pellet was washed with cold ethanol prior to vacuum drying.

The salt-free DNA pellet was resuspended in freshly diluted 1 M piperidine and incubated at 90 °C for 15 min to convert quantitatively sites of guanine N7-alkylation into strand breaks (Mattes et al., 1986a). Samples were lyophilized, resuspended in formamide loading buffer, heated at 90 °C for 1 min, and chilled in an ice bath prior to loading onto the gel.

Polyacrylamide Gel Electrophoresis. Electrophoresis was achieved on 0.4 mm × 80 cm × 20 cm 6% polyacrylamide gels containing 8 M urea. Running time was approximately 3 h at 3000 V, 55 °C. Gels were transferred to filter paper and dried. Following autoradiography, relative band intensities were determined by microdensitometry with an LKB Ultrascan-XL laser densitometer. The extent of alkylation for a given dose of drug was determined by comparison of the integrated area of the band corresponding to the full-length fragment for the treated sample with that for untreated sample (after adjustment for total counts loaded) and by use of the absolute value of the natural logarithm of that ratio to give the average number of breaks per molecule (Boles & Hogan, 1984).

DNA Footprinting. DNA was reacted with netropsin or distamycin A in 30 μL of 10 mM Tris-HCl–50 mM NaCl, pH 7.4, for 30 min at 20 °C. MPE and $Fe(NH_4)_2(SO_4)_2$ (freshly prepared) were added to a final concentration of 10 mM and DTT was added to 2 mM, and the mixture was incubated for a further 30 min. The reaction mixture was frozen and lyophilized prior to polyacrylamide gel electrophoresis.

Results

Guanine N7-Alkylation by Nitrogen Mustards. The three nitrogen mustards L-Pam, UM, and QM (Figure 1) were chosen in the present study since their patterns of guanine N7-alkylation have been studied in detail and differ from each other in a consistent and predictable way (Kohn et al., 1987). This can be seen by comparing the pattern of alkylation of singly end-labeled pBR322 DNA on denaturing polyacrylamide gels (following quantitative conversion of sites of guanine N7-alkylations to strand breaks by treatment with hot piperidine) for L-Pam (Figure 2, lane b), UM (Figure 2, lane f; Figure 3, lane a), and QM (Figure 3, lane g). A detailed examination of the specificities confirms the pattern observed previously: (a) Alkylation by L-Pam at low ionic strength correlated with the molecular electrostatic potential at the guanine N7 imposed by the nearest-neighbor base pairs resulting in a preferential alkylation in runs of guanines, e.g., Figure 2, lane b, pairs of guanines at bases 329, 330 and 355, 356 [in this L-Pam is representative of most nitrogen mustards (including mechlorethamine, Figure 1)]. (b) UM showed a pattern similar to that of L-Pam except for a preferential reaction in the sequence 5′-Py*G*C-3′ (e.g., Figure 2, lane f, bases 300 and 303, both 5′-C*G*C-3′, and Figure 3, lane a, bases 160 and 169, both 5′-T*G*C-3′). (c) QM alkylates primarily

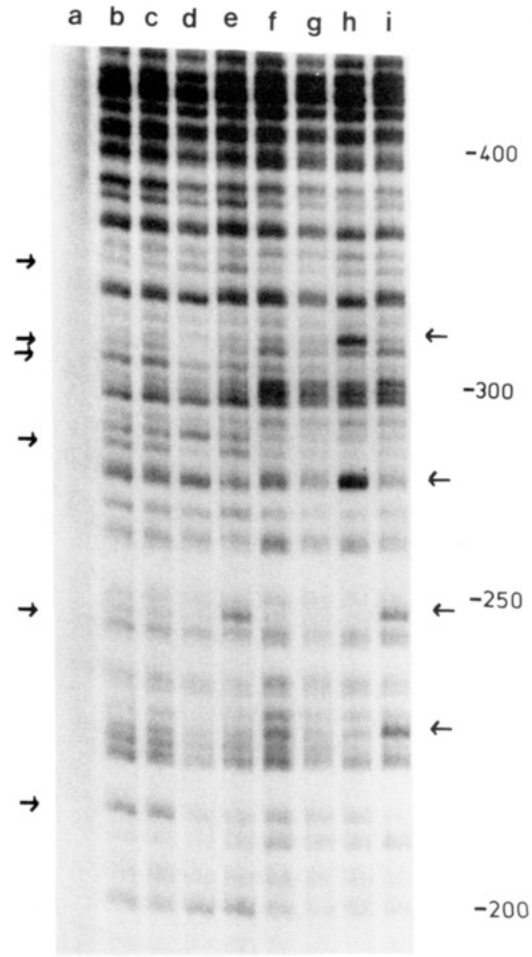

FIGURE 2: Autoradiogram of 6% denaturing polyacrylamide gel showing piperidine-induced single-strand breaks in a 675 base pair fragment of pBR322 DNA caused by guanine N7-alkylation by the nitrogen mustards L-Pam and UM in the presence or absence of cationic DNA affinity binder ethidium bromide, distamycin A, or netropsin. 5′-^{32}P end-labeled DNA was incubated with the nitrogen mustard for 1 h at 20 °C in TEA buffer. Where appropriate, cationic DNA affinity binders were incubated with the DNA for 30 min prior to addition of the nitrogen mustard. Lane a, control, unalkylated DNA; lanes b–e, 100 μM L-Pam; lanes f–i, 25 μM UM. Lanes c and g, 10 μM ethidium bromide; lanes d and h, 100 μM distamycin A; lanes e and i, 100 μM netropsin. Numbers indicate the base number (see Figure 7) and arrows indicate major differences for L-Pam (on left) and UM (on right).

Table I: Effect of Ionic Strength and Cationic DNA Affinity Binders on Total Guanine N7-Alkylation by Nitrogen Mustards

	% inhibition of guanine N7-alkylation[a]		
	L-PAM	UM	QM
sodium chloride (100 mM)	91	93	8
ethidium bromide (10 μM)	63	77	53
distamycin A (100 μM)	72	66	0[b]
netropsin (100 μM)	72	59	0[b]
spermine (10 μM)	82	90	33

[a] Calculated by comparing the integrated areas of bands from polyacrylamide gels corresponding to the full-length fragment for treated versus untreated samples, adjustment being made for total counts loaded per lane (see Materials and Methods). [b] Enhancement of overall alkylation was obtained in several experiments.

at 5′-*G*GPu-3′ and 5′-*G*TPu-3′ sequences (e.g., Figure 3, lane g).

Quantitative Effect of Ionic Strength and Cationic DNA Affinity Binders. The alkylation of guanine N7 by the nitrogen mustards was examined quantitatively in the presence

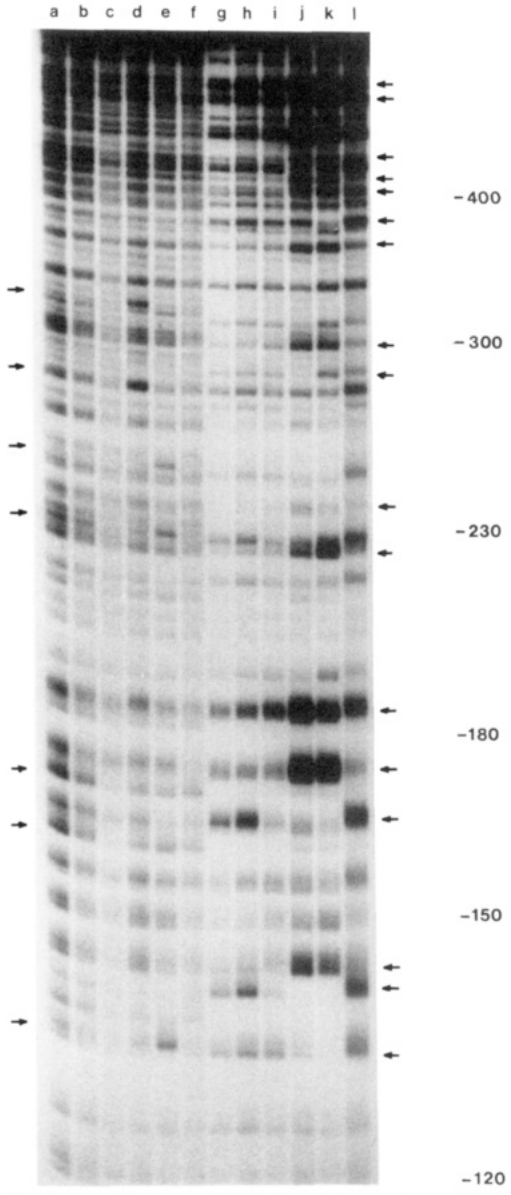

FIGURE 3: Autoradiogram of 6% denaturing polyacrylamide gel showing piperidine-induced single-strand breaks in a 675 base pair fragment of pBR322 DNA caused by guanine N7-alkylation by the nitrogen mustards UM (lanes a–f) and QM (lanes g–l) in the presence or absence of high salt or the cationic DNA affinity binder ethidium bromide, distamycin A, netropsin, or spermine. 5′-^{32}P end labeled DNA was incubated with the nitrogen mustard for 1 h at 20 °C in TEA buffer. Where appropriate, cationic DNA affinity binders were incubated with the DNA for 30 min prior to addition of the nitrogen mustard. Lanes b and h, 100 mM NaCl; lanes c and i, 10 μM ethidium bromide; lanes d and j, 100 μM distamycin A; lanes e and k, 100 μM netropsin; lanes f and l, 10 μM spermine. Doses of UM were 25 μM except for lane b, 125 μM. QM dose was 125 nM. Numbers indicate the base number (see Figure 7), and arrows indicate major differences for UM (on left) and QM (right).

of salt (100 mM NaCl), the intercalator ethidium bromide (10 μM), the minor groove AT-specific binders distamycin A and netropsin (100 μM), and the biological polyamine spermine (10 μM). This was calculated by comparing the integrated areas of bands from polyacrylamide gels corresponding to the full-length fragment for the treated sample with that for untreated sample as described under Materials and Methods. Alkylation by L-Pam and UM was quantitatively inhibited by salt and the cationic DNA affinity binders (Table I). In this respect, 10 μM ethidium bromide and spermine were as affective as 100 μM minor groove binder. In contrast, QM was inhibited less by ethidium bromide and spermine, very

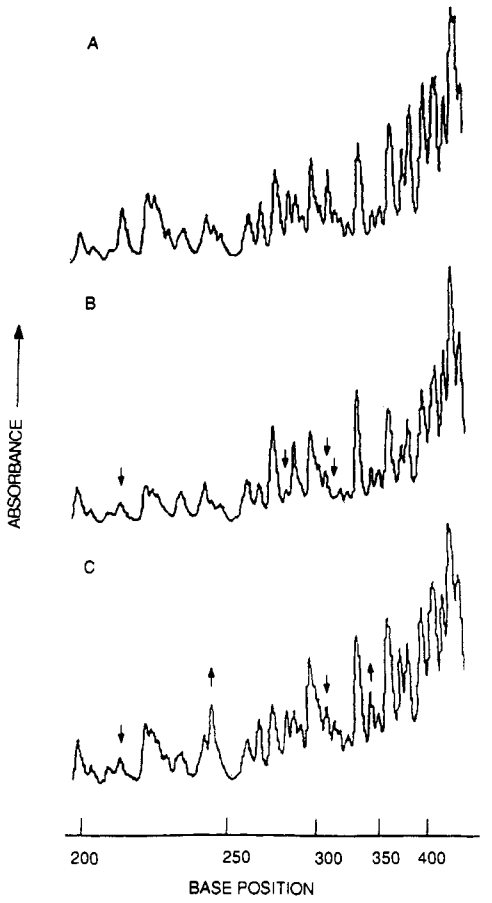

FIGURE 4: Densitometric traces of autoradiogram (Figure 2) showing the effect of distamycin A and netropsin on the pattern of guanine N7-alkylation by L-Pam. Trace A corresponds to Figure 2, lane b (100 μM L-Pam alone), trace B to Figure 2, lane d (L-Pam + 100 μM distamycin A), and trace C to Figure 2, lane e (L-Pam + 100 μM netropsin). Arrows correspond to major differences in the alkylation pattern, down arrows show suppression of alkylation, and up arrows show enhancement. The base sequence is seen in Figure 7.

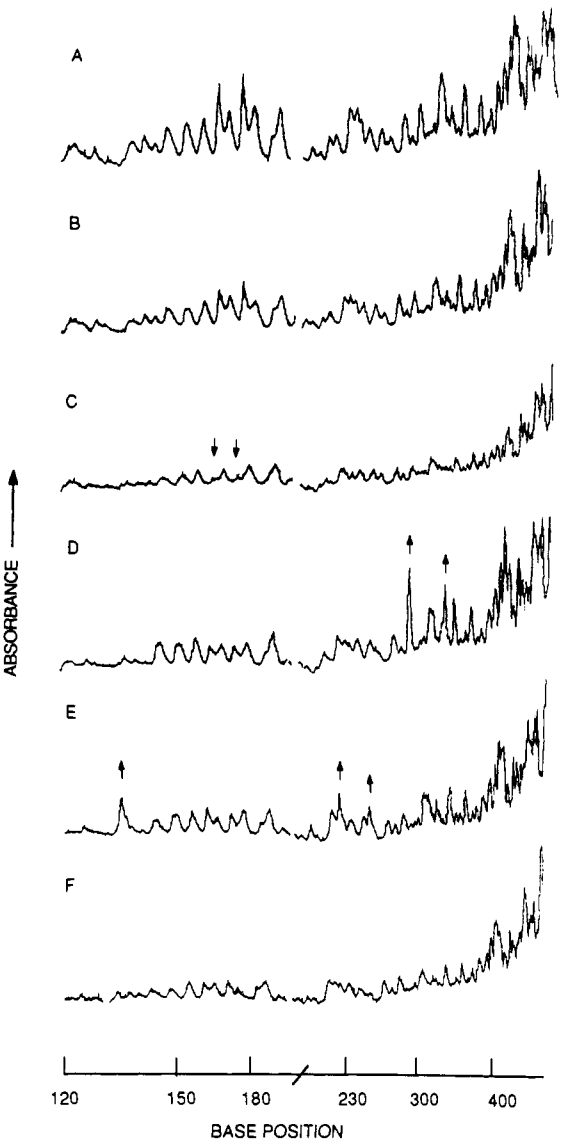

FIGURE 5: Densitometric traces of autoradiogram (Figure 3) showing the effect of ionic strength, ethidium bromide, distamycin A, netropsin, and spermine on the pattern of guanine N7-alkylation by UM. Trace A correspond to Figure 3, lane a (25 μM UM alone), trace B to Figure 3, lane b (125 μM UM + 100 mM NaCl), trace C to Figure 3, lane c (25 μM UM + 10 μM ethidium bromide), trace D to Figure 3, lane d (25μM UM + 100 μM distamycin A), trace E to Figure 3, lane e (25 μM UM + 100 μM netropsin), and trace F to Figure 3, lane f (25 μM UM + 10 μM spermine). Arrows correspond to major differences in the alkylation pattern, down arrows show suppression of alkylation, and up arrow show enhancement. Base sequence is seen in Figure 7.

little by salt (<10%), and not at all by distamycin A and netropsin. In fact, enhancement of the overall extent of alkylation by QM was observed in the presence of distamycin A and netropsin in several experiments.

Qualitative Effect of Ionic Strength and Cationic DNA Affinity Binders. The pattern of guanine N7-alkylation by nitrogen mustards was examined qualitatively in the presence of salt and the cationic affinity binders [polyacrylamide gels (Figures 2 and 3) and corresponding densitometric traces (Figure 4, L-Pam; Figure 5, UM; Figure 6, QM)]. The base number corresponds to the pBR322 sequence, the relevant portion of which is shown in Figure 7. In the gels and densitometric traces, arrows corresponds to the major differences observed in the presence of cationic affinity binders compared to the corresponding low ionic strength lane. Salt did not alter the rank order of reactivities for any of the mustards tested. The pattern of alkylation by L-Pam was least affected by the affinity binders. Ethidium bromide (Figure 2, lane c) and spermine (data not shown) had no effect. Distamycin A and netropsin (Figure 2, lanes d and e), however, gave a different pattern of bands compared to that in the low ionic strength lane, which was different for each of the minor groove binders. The differences were small, however, and restricted to a few sites [see arrows on gel (Figure 2) and densitometry (Figure 4)] but were reproducible in several experiments. Distamycin A (Figure 4, trace B) suppressed alkylation at a number of sites (down arrows) whereas netropsin (trace C) produced suppression at two of these sites but more striking enhancement of alkylation (up arrows) at two sites (in particular base 247).

UM was affected by all the affinity binders (gel, Figures 2 and 3; densitometry, Figure 5). In the case of ethidium and spermine this was restricted to a suppression of the UM-preferred sites (e.g., bases 160 and 169). This is most striking for ethidium (Figure 5, trace C). It is also evident that distamycin A and netropsin altered the pattern of alkylation by UM, which again differed for each of the minor groove binders (Figure 2, lanes h and i; Figure 3, lanes d and e). The major effect was strong enhancement at two sites for distamycin A (Figure 5, trace D) and enhancement at three different sites for netropsin (Figure 5, trace E).

The effect on QM was most striking (gel, Figure 3; densitometry, Figure 6). With ethidium bromide this was restricted to three major sites (Figure 6, trace C). For dista-

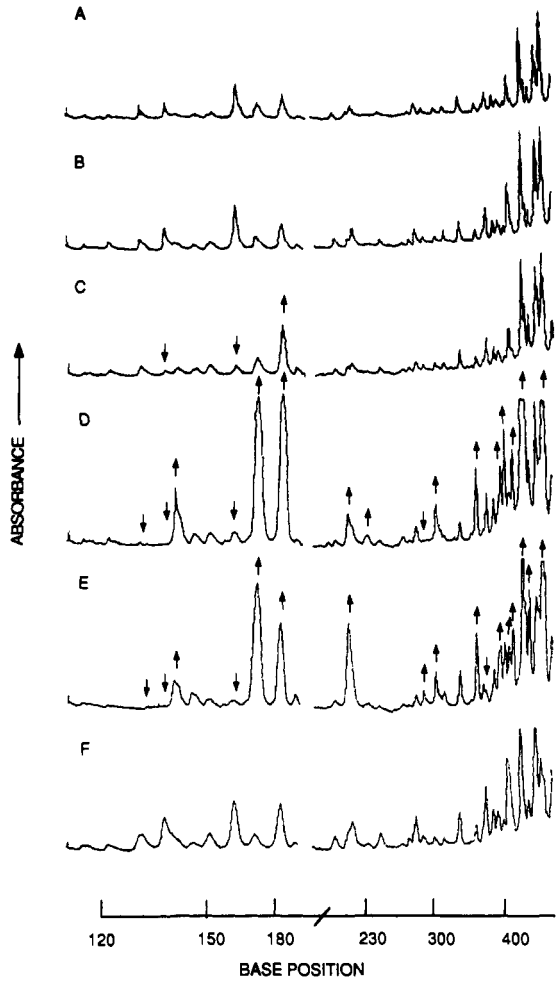

FIGURE 6: Densitometric traces of autoradiogram (Figure 3) showing the effect of ionic strength, ethidium bromide, distamycin A, netropsin, and spermine on the pattern of guanine N7-alkylation by QM. Trace A correspond to Figure 3, lane g (125 nM QM alone), trace B to Figure 3, lane h (QM + 100 mM NaCl), trace C to Figure 3, lane i (QM + 10 μM ethidium bromide, trace D to Figure 3, lane j (QM + 100 μM distamycin A, trace E to Figure 3, lane k (QM + 100 μM netropsin), and trace F to Figure 3, lane l (QM + 10 μM spermine). Arrows correspond to major differences in the alkylation pattern, down arrows show suppression of alkylation, and up arrows show enhancement. Base sequence is seen in Figure 7.

110	120	130	140	150
GCGCTCATCG	TCATCCTCGG	CACCGTCACC	CTGGATGCTG	TAGGCATAGG
160	170	180	190	200
CTTGGTTATG	CCGGTACTGC	CGGGCCTCTT	GCGGGATATC	GTCCATTCCG
210	220	230	240	250
ACAGCATCGC	CAGTCACTAT	GGCGTGCTGC	TAGCGCTATA	TGCGTTGATG
260	270	280	290	300
CAATTTCTAT	GCGCACCCGT	TCTCGGAGCA	CTGTCCGACC	GCTTTGGCCG
310	320	330	340	350
CCGCCCAGTC	CTGCTCGCTT	CGCTACTTGG	AGCCACTATC	GACTACGCGA
360	370	380	390	400
TCATGGCGAC	CACACCCGTC	CTGTGGATCC	TCTACGCCGG	ACGCATCGTG

FIGURE 7: DNA sequence used in the present study.

mycin A (trace D) and netropsin (trace E) the alkylation pattern was completely changed. Some sites are strongly (or completely) suppressed (e.g., base 140); others are strongly enhanced (e.g., bases 183 and 184).

Comparison of QM Alkylation Sites to Sites of Distamycin A and Netropsin Binding. Since distamycin A and netropsin binding to AT-rich sequences in the minor groove of DNA was seen to markedly effect QM alkylation, the exact binding sites

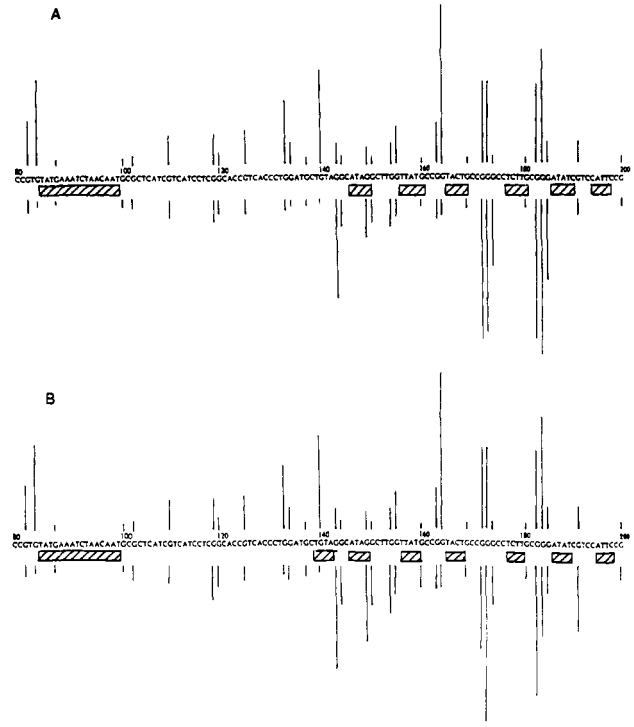

FIGURE 8: Schematic diagram of the pattern of guanine N7-alkylation by QM either alone or in the presence of distamycin A and netropsin. Bars indicate the relative intensity of guanine alkylations scaled to the highest alkylation in each case. QM alone (from Figure 3, lane g) is above the sequence in both panels A and B. QM + distamycin A (from Figure 3, lane j) is below the sequence in panel A, and QM + netropsin (from Figure 3, lane k) is below the sequence in panel B. Hatched boxes correspond to the sites of binding of the minor groove binders as determined by DNA footprinting (see Materials and Methods).

of the minor groove binders were examined on the same DNA by MPE footprinting. The major binding sites are seen as hatched boxes in Figure 8 for distamycin A (panel A) and netropsin (panel B). The alkylation patterns for QM (scaled to the strongest sites in each case) are seen both in the absence (above sequence) and in the presence (below) of the affinity binders. It is clear that distamycin A and netropsin recognize essentially the same sites on the DNA (with only one extra recognition site for netropsin at bases 139–142). It is also evident that the guanine N7-position in the major groove is affected many bases away from the minor groove binders; e.g., suppression at guanines 140 and 132 is 6 and 13 bases away from the nearest distamycin binding site.

Discussion

DNA is probably the most important cellular target for alkylation by nitrogen mustards with reaction predominantly at the N7-position of guanine. The present study clearly demonstrates that the pattern of guanine N7-alkylation produced by nitrogen mustards can be quantitatively inhibited by the presence of salt and various cationic DNA affinity binders. This has been shown previously for methylation of DNA by MNU (Rajalakshmi et al., 1978; Wurdeman & Gold, 1988) and is consistent with the screening of the DNA electrostatic potential by salt and the affinity binders. The reactive group in the activated form of nitrogen mustards is a positively charged aziridinium ion which would be drawn toward more electronegative regions in the DNA (Levins & Papanastassiou, 1965; Wiliamson & Witten, 1967). The strong electrostatic attraction at low salt between such an intermediate and the polyanionic DNA backbone (Pullman & Pullman, 1981) would be inhibited by the presence of salt

and the cationic DNA binders. Theoretical evaluations of the effect of netropsin, distamycin A, and spermine suggest that binding induces long-range modification of the electrostatic properties of the DNA which would result in an altered reactivity of DNA toward alkylating agents (Zakrzewska & Pullman, 1983, 1985; Pullman & Zakrzewska, 1985).

In addition to quantitative alterations in the alkylation pattern produced by nitrogen mustards, distinct qualitative alterations were observed in the present study. This was most striking in the case of distamycin A and netropsin and suggests that, in addition to affecting the electrostatic properties of the DNA, binding of these agents can produce structural changes which produce an altered accessibility at certain sites. Netropsin and distamycin A are structurally similar antiviral antibiotics, and DNA footprinting with MPE reveals similar specificities for AT-rich regions recognizing site sizes of four and five base pairs, respectively (Van Dyke et al., 1982; Harshman & Dervan, 1985; Figure 8). Several studies indicate alterations in DNA structure resulting from binding of these agents. X-ray analysis revealed binding of the drugs in the minor groove of B-DNA through hydrogen bonding and hydrophobic interactions and that binding of netropsin widens the minor groove by several angstroms and the helical axis of the DNA is bent by 8° per molecule bound (Kopka et al., 1985). Distamycin A apparently introduces similar alterations in the DNA structure as determined by solution NMR studies (Klevit et al., 1986). It has also been reported from solution studies that bound netropsin winds the DNA helix by about 10° (Snounou & Malcolm, 1983). The binding of these sequence-specific drugs can locally alter the DNA structure of a promotor bearing a substitution of nonalternating A-T base pairs such as to lead to the specific activation of transcription (Bruzik et al., 1987).

Although all of these studies indicate alterations in DNA structure that could presumably lead to an altered reactivity of sites in the major groove, none show significant differences between netropsin and distamycin A to sufficiently explain the quite distinct effects of the two agents on the guanine N7-position in the major groove as demonstrated in the present study. Differences were observed in the magnitude of the specific activation of transcription in which the effect observed with distamycin A was significantly more pronounced than that observed for netropsin (Bruzik et al., 1987). Significant differences were observed however between the two compounds with DNase I and micrococcal nuclease footprinting (Portugal & Waring, 1987). Although a similar binding of netropsin and distamycin A to AT-rich sequences was revealed, distinctly different patterns of enhanced cleavage by DNase I were evident in the presence of the ligands. Such enhanced rates of cleavage are attributed to DNA structural variations induced in the vicinity of the ligand binding site, most probably involving changes in the width of the minor groove (Fox & Waring, 1984).

The alkylation pattern for nitrogen mustards observed in the present study differed depending on the group attached to the alkylating moiety and did not appear to be predictable at the primary sequence level. The alkylation by simple nitrogen mustards such as L-Pam (Figure 2) and mechlorethamine (data not shown) is similarly affected by the affinity binders, and these agents are representative of most agents in this class of chemotherapeutic drugs. The unique preferences of UM, analyzed in detail and modeled previously (Kohn et al., 1987), are lost in the presence of the cationic DNA binders, most strikingly in the case of ethidium bromide. The resulting patterns differ from that of L-Pam suggesting that the uracil group can influence the ultimate alkylation preference. QM is capable of intercalating into DNA prior to covalently reacting with the guanine N7-position. A rapid initial noncovalent binding is indicated by the unusually low concentrations required for reaction. Of all the mustards studied, QM shows the largest discrimination among different guanines and is qualitatively affected most by the cationic DNA affinity binders. In contrast, however, the overall extent of alkylation by QM is inhibited much less by salt and the affinity binders (even stimulated in the case of distamycin A and netropsin), suggesting that the unique sequence specificity of this compound is not due to long-range electrostatic interactions. The stimulation of QM alkylation by distamycin A and netropsin suggests that minor groove binder-induced DNA structural changes result in increased accessibility for quinacrine intercalation. No sequence dependence was observed previously for the inhibition of MNU methylation by distamycin A (Wurdeman & Gold, 1988), but this may be due to the small size and easy accessibility of the methyldiazonium ion.

Several nitrogen mustards, such as L-Pam, are used clinically in the treatment of cancer. It is therefore important to understand the factors that can effect the alkylation of DNA by such agents. Clearly the results presented here with ethidium bromide suggest that the alkylation of DNA by nitrogen mustards in drug combinations containing intercalating agents should not be assumed to be the same as in a single-agent case. Intercalation of ethidium into DNA extends and unwinds the DNA helix with an unwinding angle of 26° (Gale et al., 1981). Such simple intercalators have little sequence specificity although NMR and conformational energy minimization evidence suggests some preference for 3′–5′ pyrimidine–purine sequences (Kastrup et al., 1978; Lybrand & Kollman, 1985). In general, the simple intercalators have not been successfully footprinted, but DNase I footprinting of ethidium at low temperatures has been achieved and demonstrated a preference for alternating purine and pyrimidine sequences (Fox & Waring, 1987). Although DNA structural changes could not be analyzed in that study, DNase I footprinting of the more complex intercalating drug actinomycin D revealed conformational changes in the DNA (Fox & Waring, 1984). Spermine is present in relatively high concentrations in cells, and at concentrations at which it is stimulatory to several enzymatic processes (Janne et al., 1975; Tabor & Tabor, 1976), it is inhibitory to DNA alkylation. Distamycin A has been shown recently to antagonize the inhibition of topoisomerase II by anticancer drugs (Fessen & Pommier, 1988) and indicates that distamycin A induced DNA structural alterations affect topoisomerase II DNA interactions. Binding of distamycin A can also selectively inhibit the binding of regulatory proteins to DNA (Broggini et al., 1989).

In conclusion, increased ionic strength and the presence of cationic DNA affinity binders can quantitatively, and in some cases qualitatively, alter the pattern of guanine N7-alkylation by nitrogen mustards. The result differed with both the nitrogen mustard and the cationic DNA affinity binder. Most strikingly, specific binding to AT sequences in the minor groove of DNA can have long-range effects on the alkylation pattern of DNA in the major groove.

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