The Design of Agents To Control DNA Methylation Adducts. Enhanced Major Groove Methylation of DNA by an *N*-Methyl-*N*-nitrosourea Functionalized Phenyl Neutral Red Intercalator

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An *N*-methyl-*N*-nitrosourea (MNU) moiety [$CH_3N(N{=}O)C({=}O)NH-$] linked to the C4′-position of the 5-substituted phenyl ring of phenyl neutral red (PNR), 2-methyl-3-amino-5-[*p*-[[2-[(*N*-nitroso-*N*-methylcarbamoyl)amino]ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium chloride (MNU-PNR), has been synthesized as an approach to design a molecule that will deliver alkylating agents with some preference to guanine (Gua) in the major groove of DNA. The PNR nucleus was chosen because previous studies suggested the following: (1) PNR binds with a slight preference for G/C rich sequences; and (2) PNR intercalates into DNA from the major groove with the 5-phenyl ring pointing out into the major groove (Müller, W., Bünemann, H., and Dattagupta, N. (1975) *Eur. J. Biochem.* **54**, 279−291). It is demonstrated that MNU-PNR yields 2.6 and 6.0 times more *N*7-methylguanine (7-MeGua) than MNU at low salt (10 mM Tris buffer) and high salt (10 mM Tris buffer + 200 mM NaCl), respectively. It is also shown that the ratio of 7-MeGua (a major groove adduct) to *N*3-methyladenine (a minor groove adduct) is approximately 5 times higher for MNU-PNR than for MNU. The yield of the 7-MeGua adduct is decreased by the coaddition of a nonmethylating analogue of MNU-PNR or NaCl, but *increased* in the presence of the minor groove intercalator, ethidium bromide. Using a ^{32}P-end-labeled restriction fragment, the enhanced methylation by MNU-PNR at 7-Gua is confirmed, and it is demonstrated that the sequence-dependent formation of 7-MeGua from MNU-PNR is the same as that seen with MNU. UV, circular dichroism, and viscosity studies are consistent with MNU-PNR binding to DNA via an intercalation-based process.

Introduction

Research directed toward understanding the role(s) of different types of DNA modifications in cytotoxicity and mutagenicity is of ongoing interest (*1*, *2*). Because most alkylating agents, including carcinogens and antineoplastic agents, modify DNA at multiple sites, it is not easy to dissect the contribution of individual DNA lesions toward the two biological end points. The issue of adduct complexity has generally been addressed in two ways. The preparation of site-specifically alkylated DNA targets, which can be transfected into cells, provides information on their miscoding (promutagenic) properties upon *in vivo* replication (*3*). This approach requires that the adduct be relatively stable so that it is compatible with the chemical or biological procedures used to introduce lesions into DNA, and the subsequent transfection into cells. The other method is to determine how cells defective in a particular type of repair pathway respond, in terms of cytotoxicity and/or mutagenicity, to an alkylating agent (*4*−*8*). Both strategies have provided remarkable insights into the consequences of DNA alkylation. The knowledge gained from such studies can be useful in the design of antineoplastic drugs that are cytotoxic but not mutagenic (*2*).

We have taken a somewhat different approach to elucidate the biological roles of different adducts in cytotoxicity and mutagenicity, by designing compounds that will sequence and/or groove selectively modify DNA *in vitro* and *in vivo*. Previously, minor and major groove equilibrium binding compounds appended with alkylating agents have been prepared that significantly alter the agent's "normal" alkylation pattern and groove specificity (*9*−*19*). As a consequence of their unusual alkylating properties, some of these compounds have shown interesting biological activities (*8*). In the current work, the goal was to design a methylating agent that would preferentially increase the yield of the N7-methylguanine (7-MeGua),[1] the chemically unstable major groove lesion.

Intercalation is one of the basic mechanisms by which small molecules equilibrium bind to DNA (*20*). For reasons not clear, the predominant route of entry of intercalators into the DNA base pair stack is via the minor groove. One of the proposed exceptions to this

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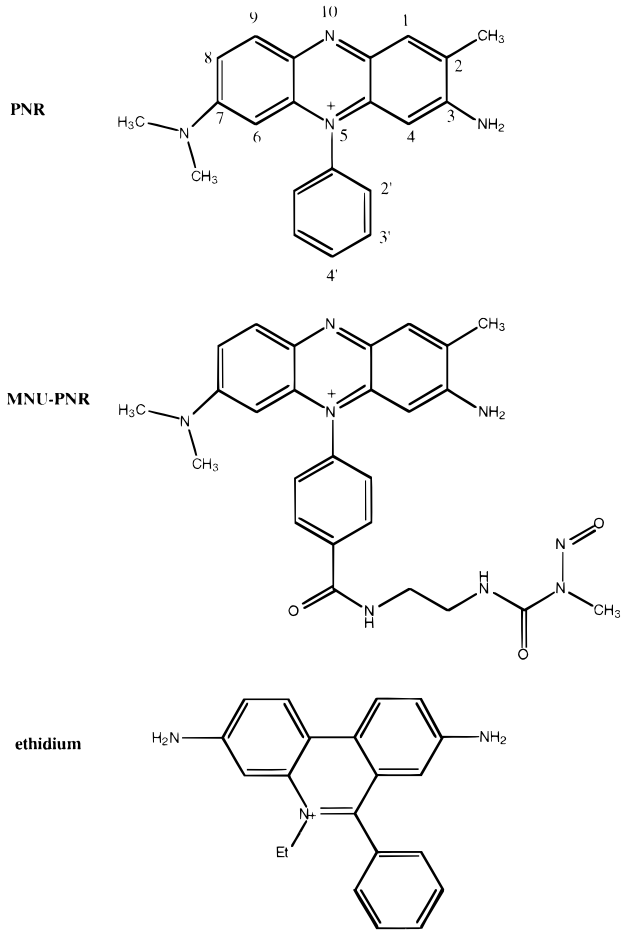

Figure 1. Structure of PNR, MNU-PNR, and ethidium.

generalization is the intercalating dye 2-methyl-3-amino-5-phenyl-7-(dimethylamino)phenazenium chloride (phenyl neutral red, PNR) (see Figure 1 for structures) that has been reported to associate with the major groove of DNA (*21*−*23*). The width of PNR (∼9.5 Å from the carbon of the exocyclic 2-methyl to the ring C-8) physically prevents minor groove access. In addition to its proposed intercalation from the major groove, PNR binds with some preference to G:C rich regions (*21*). Using this knowledge on groove orientation and binding preference, an *N*-methyl-*N*-nitrosourea [$CH_3N(N{=}O)NH-$] functionalized derivative of PNR was synthesized with the goal of preferentially delivering a methylating agent to the major groove of DNA. Since *N*-methylnitrosoureas (MNU) hydrolyze at physiological pH to the highly reactive alkylating agent methanediazonium ion (*24*−*26*), it was anticipated that the regiospecific generation of methanediazonium in the major groove would lead to an increase in the yields of major groove adducts, *e.g.*, 7-MeGua, relative to minor groove adducts, *e.g.*, *N*3-methyladenine (3-MeAde). The association of this dye with DNA is described along with its DNA methylating characteristics.

[1] Abbreviations: CD, circular dichroism; EB, ethidium bromide; EI-MS, electron impact mass spectrometry; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast-atom-bombardment mass spectrometry; 3-MeAde, *N*3-methyladenine; 7-MeGua, *N*7-methylguanine; MNU, *N*-methylnitrosourea; MNU-PNR, 2-methyl-3-amino-5-[*p*-[[2-[(*N*-nitroso-*N*-methylcarbamoyl)amino]ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium chloride; MU-PNR, 2-methyl-3-amino-5-[*p*-[[2-[(*N*-methylcarbamoyl)amino]ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium chloride; PNR, 2-methyl-3-amino-5-phenyl-7-(dimethylamino)phenazenium chloride (phenyl neutral red).

Experimental Section

Caution. *MNU-PNR and MNU should be considered toxic and carcinogenic, and treated accordingly.*

General Procedures. ^{1}H NMR were recorded on a Varian XL-300 spectrometer and 2D-NMR techniques (COSY and phase sensitive DQCOSY) were used in structural assignments. Mass spectral data were performed on an AEI MS-9 or Kratos MS-50 spectrometer utilizing electron impact (EI-MS) or fast-atom-bombardment (FAB-MS) ionization techniques. IR spectra were obtained on an Mattson Alfa Centauri FT-IR, CD spectra on a Jasco J710 spectropolarimeter, and UV spectra on a Varian DMS 300 spectrophotometer. Analytical and preparative silica TLC employed 0.25 and 2.0 mm Merck Kieselgel glass plates. All flash column chromatography was performed with 40 μm silica gel.

Materials. All chemical and biochemical reagents were purchased from Aldrich Chemicals (Milwaukee, WI) and Sigma Chemicals (St. Louis, MO) and used without further purification unless stated otherwise. DMF was passed over a column of 4 Å molecular sieves and dried by sequential azeotropic distillation with benzene and reduced pressure distillation from BaO. DMSO was freshly distilled from CaH_2 after initial drying over 4 Å molecular sieves. Ethylenediamine was dried over KOH and distilled. Salmon sperm DNA (Sigma) used in the DNA methylation studies was dialyzed against 100 mM Tris-HCl (pH 8.0) buffer overnight.

Molecular Modeling. MNU-PNR was constructed and minimized using the Dreiding force field (*27*). The coordinates for the bis-intercalator ditercalinium complexed with 5′-d(CGCG)$_2$ were imported from the Brookhaven Data Base (*28*) into Sybyl (Tripos Associates, St. Louis, MO). Ditercalinium was chosen because it enters the DNA base pair stack from the major groove (*28*, *29*). The ditercalinium molecule was deleted as was one of the two intercalation sites. The remaining d(CG)$_2$ duplex and MNU-PNR were treated as separate aggregates in all the calculations. The MNU-PNR was aligned with the intercalation site and slowly moved into the DNA stack using a distance constraint while monitoring van der Waals energies. When the van der Waals energy of the system started to rise, the analysis was halted. The resulting structure was then minimized using the Kollman Unified-Atom force field (*30*) with no restraints. This process was repeated to obtain the lowest energy structure shown in Figure 2. A similar analysis was done with MNU-PNR approaching the same intercalation site from the minor groove (data not shown).

Synthesis (see Figure 3). (A) *N*-(2-Aminoethyl)-4-aminobenzamide (3). Cyclohexene (10 mL) and Pd/C 10% (1.0 g) were added to a stirred solution of nitro derivative **2** (2.09 g, 10 mmol) in absolute EtOH (50 mL), and the mixture was heated to reflux for 72 h. The solvent was concentrated *in vacuo* to afford compound **3** as a yellow oil (62%): TLC (MeOH/CH_2Cl_2/AcOH, 1:4:trace) R_f 0.20; NMR (DMSO-d_6) δ 2.62 (t, 2 H, CH_2-NH_2), 3.20 (d-t, 2 H, CH_2NHCO), 6.54 (d, 2 H, aryl CH), 7.60 (d, 2 H, aryl CH), 8.00 (t, 1 H, CON*H*CH$_2$); UV (H_2O) λ_{max} (log ϵ) 276 nm (3.78); EI-MS *m*/*z* 180 (M + 1), 179 (M), 164 (M − NH_2), 121 (M − $NHCH_3$), 93.

(B) *N*-(*N*-Carbobenzoxy-2-aminoethyl)-4-aminobenzamide (4). A solution of **3** (1.6 g, 8.9 mmol) and Et_3N (5 mL) in MeOH (15 mL) was cooled to 0 °C, and benzyl chloroformate (1.6 g, 9 mmol) was slowly added. After the addition at 0 °C, the reaction was maintained at room temperature for 16 h. The solvent was evaporated and the residue dissolved in CH_2Cl_2 (25 mL), washed with water (3 × 15 mL), dried (Na_2SO_4), and concentrated to give crude product **4**. The pure compound was obtained after flash silica column chromatography (benzene/CH_2Cl_2, 3:7) in 72% yield: mp 128−130 °C; TLC (MeOH/CH_2Cl_2, 1:19) R_f 0.6; NMR (DMSO-d_6) δ 3.17 (d-t, 2 H, CH_2NHCO), 3.29 (d-t, 2 H, CH_2NHCO$_2$), 5.05 (s, 2 H, OCH_2Ar), 6.55 (d, 2 H, aryl CH), 7.37 (m, 5 H, aryl CH), 7.59 (d, 2 H, aryl CH), 8.05 (t, 1 H, CON*H*); UV (MeOH) λ_{max} (log ϵ) 276 nm (4.05); FAB-MS *m*/*z* 314 (M + 1), 313 (M^+), 277, 241, 210, 185.

(C) *N*,*N*-Dimethyl-4,4′-diaminodiphenylamine Chloride (7). *N*,*N*-Dimethyl-*p*-phenylenediamine (**5**, 210 mg, 1 mmol)

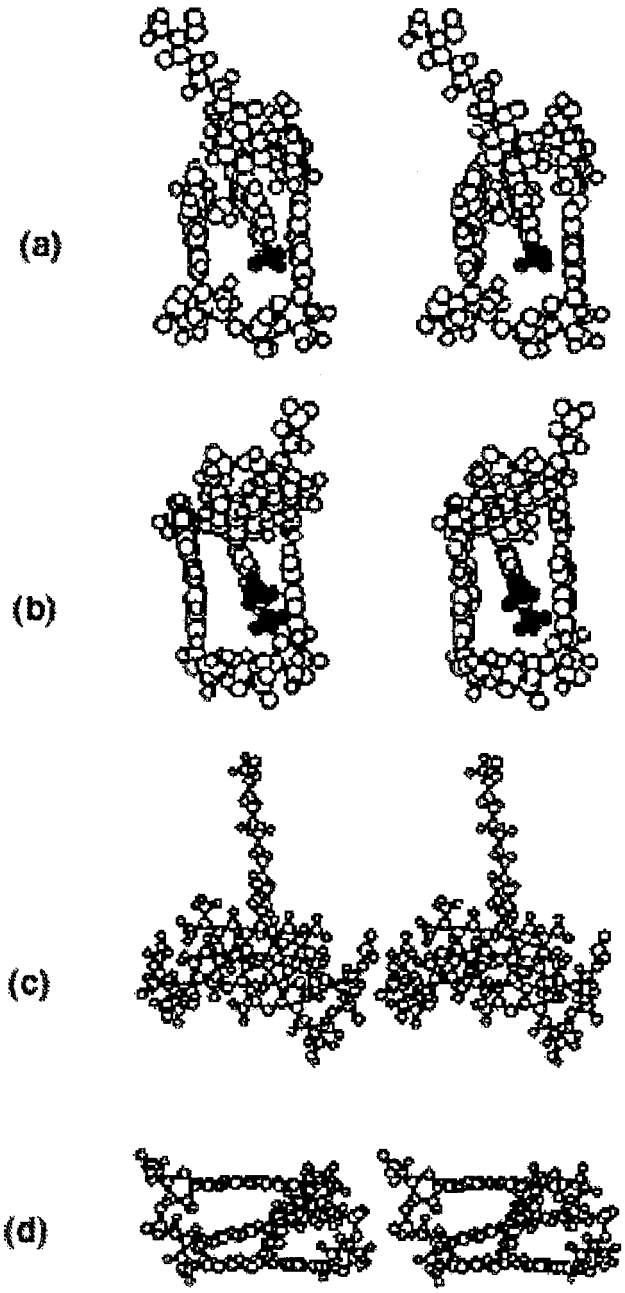

Figure 2. Relaxed stereoscopic views of MNU-PNR docked into 5′-d(CG)$_2$ intercalation site from ditercalinium−DNA complex (*28*): (a) from the major groove highlighting the position of the C-2 methyl (filled-in atoms) of PNR and the backbone; (b) from the major groove highlighting the position of the *N*7-(dimethylamino) group (filled-in atoms) of PNR and the backbone; (c) looking down the helical axis at major groove complex; and (d) major groove MNU-PNR intercalated complex viewed from the major groove.

and *o*-toluidine hydrochloride (**6**, 144 mg, 1 mmol) were dissolved in H_2O (20 mL), and then $Na_2Cr_2O_7$ (600 mg) in 5 mL of H_2O was added slowly with stirring. The green precipitate that formed was collected and thoroughly washed with H_2O and then dried to afford **7** in 67% yield: mp >300 °C; TLC (MeOH/CH_2Cl_2, 1:4), R_f 0.5; NMR (DMSO-d_6) δ 1.95 (s, 3 H, CH_3Ar), 2.92 (s, 6 H, (CH_3)$_2$N), 6.80 (m, 7 H, aryl CH); FAB-MS *m*/*z* 241 (M + 1), 240 (M).

(D) 2-Methyl-3-amino-5-[*p*-[[2-(*N*-carbobenzoxyamino]-ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium Chloride (8). To a stirred suspension of **7** (241 mg, 1 mmol) in water (25 mL) adjusted to pH 5.0 was added **4** (362 mg, 11.6 mmol) in MeOH (70 mL). A pH of 5.0 was maintained with HCl and the mixture heated to boiling. After 10 min the color had changed from green to blue and then to violet-purple. The reaction mixture was cooled and concentrated and the crude product purified by silica column chromatography (MeOH/CH_2Cl_2, 3:47) to give phenyl neutral red derivative **8** in 5% yield: mp >300 °C; TLC (MeOH/CH_2Cl_2, 1:9) R_f 0.45; IR (KBr disc) 3833, 3771, 2983, 1700, 741 cm^{-1}; NMR (DMSO-d_6) δ 2.30 (s, 3 H, CH_3Ar), 3.04 (s, 6 H, (CH_3)$_2$N), 3.25 (q, 2 H, CH_2NHCO), 3.40 (q, 2 H, CH_2NHCO$_2$), 5.03 (s, 2 H, OCH_2Ar), 5.62 (s, 1 H, aryl CH), 5.92 (s, 1 H, aryl CH), 7.35 (m, 5 H, aryl CH), 7.40 (t, 1 H, CONH), 7.52 (d, 1 H, aryl CH), 7.74 (d, 2 H, aryl CH), 7.80 (s, 1 H, aryl CH), 8.03 (d, 1 H, aryl CH), 8.32 (d, 2 H, aryl CH), 8.90 (t, 1 H, CONH); UV (MeOH) λ_{max} 551 nm; FAB-MS *m*/*z* 550 (M + 1), 416, 369, 135.

(E) 2-Methyl-3-amino-5-[*p*-[(2-aminoethyl)carbamoyl]-phenyl]-7-(dimethylamino)phenazenium Chloride (9). The protected phenyl neutral red derivative **8** (40 mg, 72.4 μmol) was dissolved in absolute EtOH (5 mL) containing anhydrous HOAc (2 mL), Pd/C (10%, 20 mg), and cyclohexene (5 mL). The reaction mixture was stirred for 48 h under Ar, the catalyst removed by filtration, and the residue washed with EtOH (3 × 5 mL). The combined filtrate and washings were concentrated to give crude product which was purified by silica column chromatography using MeOH with HOAc (0.1%) as eluant. Amine **9** was obtained in 70% yield: mp >300 °C; TLC (MeOH/CH_2Cl_2, 1:4), R_f 0.2; IR (KBr disc) 3524, 3431, 3137, 3076, 2952, 1607, 1514, 1344, 1190, 1020, 865, 757 cm^{-1}; NMR (DMSO-d_6) δ 2.32 (s, 3 H, CH_3Ar), 2.80 (t, 2H, CH_2NH$_2$), 3.07 (s, 6 H, (CH_3)$_2$N), 3.80 (m, 2 H, CH_2NHCO), 5.66 (s, 1 H, aryl CH), 5.95 (s, 1 H, aryl CH), 7.54 (s, 1 H, aryl CH), 7.76 (d, 2 H, aryl CH), 7.91 (s, 1 H, aryl CH), 8.07 (d, 1 H, aryl CH), 8.37 (d, 2 H, aryl CH), 8.92 (t, 1H, CONH); UV (MeOH) λ_{max} (log ϵ) 549.9 nm (4.21); FAB-MS *m*/*z* 416 (M + 1), 415 (M$^+$), 353.

(F) 2-Methyl-3-amino-5-[*p*-[[2-[(*N*-methylcarbamoyl)-amino]ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium Chloride (MU-PNR). A solution of **9** (50 mg, 120 mmol) in 50% aqueous dioxane (4 mL) and Et$_3$N (12 mg, 120 μmol) was added to a solution of 1,2,2,2-tetrachloroethyl *N*-methylcarbamate (31) (25 mg, 120 μmol) in dioxane (2 mL) at 0 °C. The reaction mixture was stirred for 8 h and concentrated *in vacuo*, and product MU-PNR, after purification by silica column chromatography (MeOH/CH_2Cl_2, 1:19), was obtained in 38% yield: TLC (MeOH/CH_2Cl_2, 2:23) R_f 0.40; IR (KBr disc) 3462, 3354, 2937, 1623, 1530, 1190, 587 cm^{-1}; NMR (DMSO-d_6) δ 2.31 (s, 3 H, CH_3Ar), 2.56 (d, 3 H, CH_3NH), 3.06 (s, 6 H, (CH_3)$_2$N), 3.24 (m, 4 H, CH_2's), 5.63 (s, 1 H, aryl CH), 5.95 (s, 1 H, aryl CH), 6.07 (q, 1 H, CON*H*CH$_3$), 6.40 (t, 1 H, CH$_2$N*H*), 7.53 (d, 1 H, aryl CH), 7.74 (d, 2 H, aryl CH), 7.87 (s, 1 H, aryl CH), 8.02 (d, 1 H, aryl CH), 8.38 (d, 2 H, aryl CH), 9.08 (t, 1 H, CON*H*CH$_2$); UV (MeCN) λ_{max} (log ϵ) 548 nm (4.33), 214 (4.31); FAB-MS *m*/*z* 473 (M + 1), 472 (M$^+$), 458 (−CH$_3$), 443 (−NHCH$_3$).

(G) 2-Methyl-3-amino-5-[*p*-[[2-[(*N*-nitroso-*N*-methylcarbamoyl)amino]ethyl]carbamoyl]phenyl]-7-(dimethylamino)phenazenium Chloride (MNU-PNR). A solution of **9** (25 mg, 60 mmol) in dry DMF (2 mL) containing diisopropylamine (742 mg, 26 mmol, 50 μL) was added to a solution of 1,2,2,2-tetrachloroethyl *N*-methyl-*N*-nitrosocarbamate (*31*) at −5 °C under Ar and the solution stirred for 7 h at this temperature. The solvent and volatile reaction byproducts were then evaporated *in vacuo* at 35 °C, and the resulting solid material was triturated with MeOH (0.5 mL) and product MNU-PNR isolated by precipitation from MeOH with EtOAc and washing with the same solvent. Yield, 41%: TLC (MeOH/CH_2Cl_2/HOAc, 3:47: trace) R_f 0.30; IR (KBr disc) 3431, 2983, 2353, 1623, 1530, 1344, 1090, 1020 cm^{-1}; NMR (DMSO-d_6) δ 2.35 (s, 3 H, CH_3Ar), 3.08 (s, 6 H, (CH_3)$_2$N), 3.12 (s, 3 H, CH_3N), 3.60 (m, 4 H, CH_2's), 5.64 (s, 1 H, aryl CH), 5.98 (s, 1 H, aryl CH), 7.55 (d, 1 H, aryl CH), 7.78 (d, 2 H, aryl CH), 7.95 (s, 1 H, aryl CH), 8.08 (d, 1 H, aryl CH), 8.35 (d, 2 H, aryl CH), 8.90 (b-s, 1 H, CON*H*), 9.08 (b-s, 1 H, CON*H*); UV (MeOH) λ_{max} (log ϵ) 549 nm (4.34); FAB-MS *m*/*z* 502 (M + 1), 472 (−NO), 443 (M − NNOCH$_3$), 415 (M − CONNOCH$_3$); high resolution FAB-MS found 501.23650 (+0.4 ppm from value calculated for $C_{26}H_{29}O_3N_8$).

Adduct Analysis by HPLC/UV. Salmon sperm DNA (1 mM phosphate) was reacted with MNU (1 mM) or MNU-PNR (1 mM)

at room temperature for 24 h in Tris-HCl buffer (10 mM, pH 8.0), with or without 200 mM NaCl. The DNA was precipitated from the reaction mixture with 3 M NaOAc and EtOH, and cooling in ice. The precipitated DNA was washed with EtOH, redissolved in 500 μL of 10 mM Tris buffer (pH 7.0), and then heated at 90 °C for 30 min to release 3- and 7-alkylpurines (*32*). The partially apurinic DNA was precipitated at 0 °C by adding 0.1 volume of 0.1 N HCl and the supernatant analyzed on HPLC using a Whatman Partisil-10 SCX strong cation exchange column with 175 mM ammonium formate buffer (pH 3.0) as the eluant at a flow rate of 1 mL/min. The 7-MeGua and 3-MeAde compounds were monitored by UV detection at 270 nm. Authentic standards were used to confirm the retention time of the two adducts and to calculate response factors. To correct for DNA recovery, the yields of the two adducts are presented as a ratio of adduct to unmodified Ade. Ade was measured by removing an aliquot of the DNA immediately before the neutral thermal hydrolysis (90 °C for 30 min at pH 7.0) and treating it with 0.1 N HCl at 70 °C for 30 min to depurinate the DNA. After precipitation of the apurinic DNA, the supernatant was filtered and analyzed by HPLC to quantitate Ade using the same conditions described above for the adducts.

3-MeAde Analysis by Competitive ELISA. The MNU or MNU-PNR (1 mM) was incubated with salmon sperm DNA (100 μM phosphate) in 10 mM Tris-HCl buffer (pH 8.0) for 24 h at room temperature. At the end of the incubation, the reactions were heated at 90 °C for 30 min to release 3-MeAde (and other heat labile adducts) from the DNA. After cooling in an ice bath, the partially apurinic DNA was precipitated with ice cold 0.1 N HCl (*32*). The supernatant was concentrated *in vacuo* and the residue dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 2.5 mM $MgCl_2$, and 0.1% bovine serum albumin. Samples were assayed for 3-MeAde using a specific antibody in a competitive ELISA (*33*). The *N*3-methyl-N^6-[5-(carboxypentyl)ovalbumin]adenine used as the coating antigen was prepared as previously described (*33*). Standard ELISA inhibition curves using authentic 3-MeAde adduct were generated simultaneously with the samples for each 96 well plate. The standard curve was calculated using SigmaPlot 4.01 (Jandel Scientific, San Rafael, CA) by least squares fitting of the data to the equation:

$$P = Y_{MIN} + (Y_{MIN} - Y_{MAX})(I^n/K^n)/(1 + I^n/K^n)$$

where P is the % inhibition, I is the amount of inhibitor (3-MeAde), Y_{MIN} and Y_{MAX} are the y-axis values where the curve plateaus, n is the Hill coefficient, and K is the dissociation constant (*34*). The adduct levels are expressed as 3-MeAde/Ade where Ade is based on the 100 μM phosphate DNA concentration used in the incubation.

Methylation of Restriction Fragment. A 5′-end-labeled 576 bp fragment was prepared from pBSCPV A1 plasmid containing the promotor region for the coat protein of the canine parvovirus (*35*) by sequential *Nco*I endonuclease digestion, treatment of the linearized DNA with alkaline phosphatase, phosphorylation with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase, and restriction with *Hin*dIII endonuclease (*36*). The labeled fragment was purified on 5% polyacrylamide gel prior to use. The restriction fragment (80 000 cpm) and sonicated calf thymus DNA (final concentration, 100 μM phosphate) were dissolved in 10 mM sodium cacodylate buffer (pH 7.8) containing the desired concentration of NaCl or DNA affinity binder. This DNA solution was incubated at 37 °C for a specified time with freshly prepared solutions of MNU or MNU-PNR. The reactions were terminated by cooling in ice water and precipitation of the DNA with NaOAc and EtOH. The DNA was washed with cold 70% EtOH and dried *in vacuo*. Strand breaks in the modified DNA were generated by neutral thermal hydrolysis (*37*) as previously described (*14*). The DNA was suspended in loading buffer (80% deionized formamide, 50 mM Tris−borate (pH 8.3), 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) and denatured by heating at 90 °C for 1 min and cooling in ice. The DNA was placed into wells on top of a 12% polyacrylamide (7.8 M urea) denaturing gel and the gel run at 75 W (∼55 °C). The standard Maxam−Gilbert Gua and Gua+Ade reaction lanes were included as sequence markers (*36*). The gels were either: (a) exposed to Kodak X-OMAT AR film at −70 °C and the resulting autoradiogram analyzed using a Shimadzu CS-9000 scanning densitometer; or (b) analyzed using a Molecular Dynamics PhosphorImager.

UV−Vis Studies. The UV−vis spectra of MU-PNR in the presence and absence of salmon sperm DNA were obtained in 10 mM Tris-HCl buffer (pH 8.0) with or without 200 mM NaCl at room temperature.

CD Studies. The CD spectra (220−330 nm) of salmon sperm DNA (200 μM nucleotide) in the absence and presence of 76 μM MU-PNR in 13 mM Tris-HCl buffer (pH 8.0), with and without 200 mM NaCl, were obtained (32 scans/run) at 20 °C.

DNA Viscosity Studies. Viscometry experiments were performed using a 10 mL Cannon-Ostwald No. 80-100 viscometer. Calf thymus DNA (15 mg) (Sigma Type 1, highly polymerized) was suspended in 100 mL of 5 mM Tris-HCl buffer (pH 7.5) containing 1 mM NaCl and sonicated for 4 h. Final DNA concentrations were determined by UV at 260 nm (ϵ, 6600). The melting temperature of this DNA was 55 °C, which agrees with literature references (*38*). DNA solutions (350 μM phosphate) were filtered into the viscometer, and the viscometer was maintained at 27 °C by immersion in a thermostated water bath. Flow times were measured with a stop watch and experiments run in triplicate to give standard deviations of <0.1 s. The flow times of the buffer and buffered DNA were 90.6 and 101.1 s, respectively. The compounds (MU-PNR, ethidium bromide) were dissolved in MeOH (5% final concentration by volume) and added to the viscometer with a Hamilton syringe. In the concentration range of compound studied, the MeOH had no effect on DNA viscosity. The compound−DNA solutions were mixed by gentle bubbling with air. The solutions reached equilibrium within 3−4 min after addition of compound. Flow times for buffer, DNA, and compound−DNA complex were fitted to the equation for relative helix extension of Cohen and Eisenberg (*39*, *40*):

$$L/L_0 = \{[t_{compd} - t_{buffer}(V)]/[t_{DNA} - t_{buffer}(V)]\}^{1/3}$$

where L_0 and L are the apparent length of DNA in the absence and presence of compound, respectively, t_{compd} is the flow time for the compound−DNA complex, t_{DNA} is the flow time for pure DNA, and t_{buffer} is the flow time for buffer with a given volume V. All plots were forced through the origin intersection corresponding to L/L_0 in the absence of compound.

Results

Modeling. The intercalation complex of MNU-PNR with DNA was modeled using the atomic coordinates from the Brookhaven Protein Data Bank of an intercalation site derived from the crystal structure of ditercalinium−d(CGCG)$_2$ (*28*, *29*). The ditercalinium was deleted and remaining DNA used as a host for MNU-PNR. A final minimum energy structure (the 5′-CG-3′:3′-GC-5′ region of the DNA that is not involved in the intercalation complex has been removed for viewing) is shown in Figure 2, and it is clear that the 2-methyl (Figure 2a) and *N*7-(dimethylamino) (Figure 2b) groups prevent the dye from penetrating any further into the base pair stack. In the perspective looking down the helix axis (Figure 2c), it is apparent that the dye can interact with the π-systems of the Cyt that are on opposing strands in the CpG intercalation site. It should be noted that stabilization of the dye with DNA via $\pi-\pi$ stacking interactions is not well modeled in Sybyl. The same interstrand base pair overlap is seen in at a modeled (dG)$_2$:(dC)$_2$ dimer: the 5′-base from the two complementary strands is stacked with the PNR nucleus

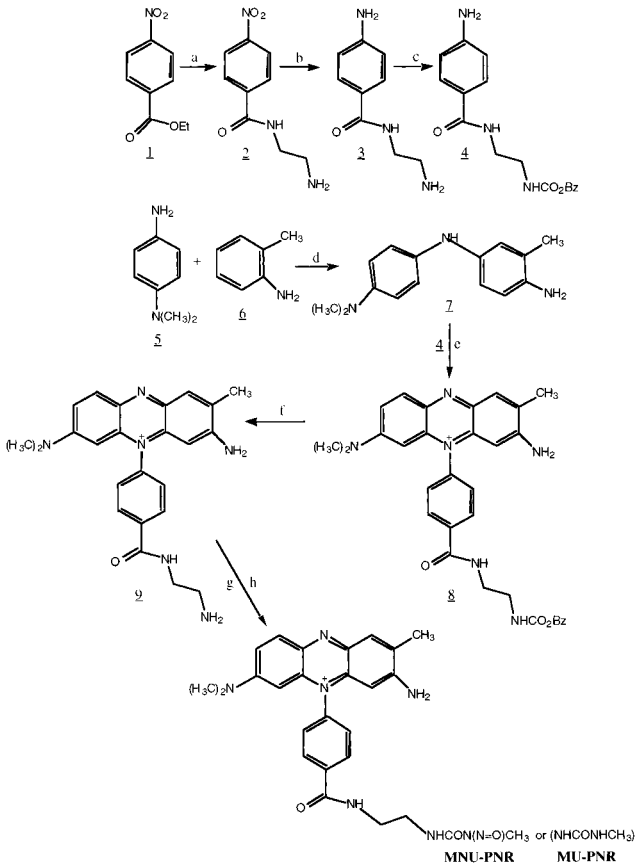

Figure 3. Synthesis of MNU-PNR: a, $NH_2CH_2CH_2NH_2$, Δ; b, Pd/C; c, $PhCH_2OCOCl$; d, $Na_2Cr_2O_7$; e, pH 5.0, Δ; f, Pd/C; g, $Cl_3CCH(Cl)OC(=O)NHCH_3$ for **10**; h, $Cl_3CCH(Cl)OC(=O)N(N=O)CH_3$ for **11**.

(data not shown). This is also true if the MNU-PNR is flipped around so the C2-methyl of the compound is closer to the Cyt rather than the Gua (the latter being the arrangement shown in Figure 2). During the energy minimization a slight twisting of the PNR nucleus with respect to the duplex base pairs occurred because of the C2-methyl group. The opposite stacking scenario would occur if the dye entered the helix via the minor groove; however, due to the steric effect of the C2-methyl and 7-(*N*,*N*-dimethylamino) groups, it is not possible to dock the MNU-PNR molecule into the DNA stack from the minor groove side. As shown in the different perspectives in Figure 2, the *N*-alkyl-*N*-nitrosourea moiety is in an extended conformation and somewhat outside the cylinder defined by the long axis of the helix. Obviously, the aliphatic linkage between the nitrosourea and the aromatic system is somewhat flexible and can move into the major groove.

Synthesis. Compounds MU-PNR and MNU-PNR were prepared as outlined in Figure 3 using an approach based, in part, on a previously described method for the synthesis of PNR analogues (*23*). As anticipated, the formation of the phenazenium ring system (**8**) by the nonregiospecific condensation of **4** with **7** was a particularly low yield step, and purification of the final product from side products was difficult. Numerous efforts to improve this step in the synthesis were unsuccessful. The derivatization of **9** with 1,2,2,2-tetrachloroethyl *N*-methylcarbamate, obtained by the reaction of $CH_3NH_3^+Cl^-$ with 1,2,2,2-tetrachloroethyl chloroformate, gave the urea derivative MU-PNR in ~40% yield. This compound was used as a stable surrogate for MNU-PNR in the DNA equilibrium binding studies. Alternatively, the condensation of **9** with 1,2,2,2-tetrachloroethyl *N*-methyl-*N*-nitrosocarbamate, prepared by the nitrosation of 1,2,2,2-tetrachloroethyl *N*-methylcarbamate, gave the desired nitrosourea product MNU-PNR in a similar 40% yield. The purity of the compound is estimated to be greater than 95% based on ^1H NMR analysis (data not shown). This route allows for the addition of the unstable nitrosourea functionality in the final step of the synthesis and is amenable to the synthesis of [^3H or ^{14}C]methyl-labeled material (*41*).

Sequence Selective Methylation of DNA. The autoradiogram of the 576 bp fragment sequencing gel shows that there is a dose response for the 50 and 100 μM concentrations of MNU-PNR (Figure 4A, lanes e and f). The time course for the reaction of MNU-PNR with DNA is shown in Figure 4A (lanes f−i) along with the uncorrected densitometry values for the 24 h time points (Figure 4B). The band corresponding to G_{271} was used in the time course analysis because it is well resolved and isolated from other G's. A similar analysis using the band for G_{277} affords equivalent results.

The cleavage of DNA by 100 μM MNU-PNR is diminished (15% at the 24 h time point, Figure 4B) by the coaddition of 500 μM MU-PNR, the nonmethylating analogue (Figure 4A, lane j). In contrast, 500 μM ethidium bromide enhances the intensity of the bands at the 24 h time point by 30% (Figure 4A, lane n; Figure 4B). The effect of 200 mM NaCl is to decrease DNA methylation by 50% at 24 h (Figure 4A, lanes r *vs* f; Figure 4B).

The 7-MeGua pattern for 750 μM MNU after a 24 h incubation is also shown (Figure 4A, lanes v−y). If the intensity of the G_{271} band for MNU, determined by densitometry, is divided by 7.5 to correct for the difference in doses between MNU (750 μM) and MNU-PNR (100 μM), the results indicate that on an equimolar basis MNU-PNR yields ~2.6 times more 7-MeGua than MNU. The dose response for the formation of 7-MeGua by MNU is linear under the experimental conditions used from 50 μM through 2 mM (*26*, *42*) Direct comparison of the same concentration of MNU-PNR and MNU in the restriction fragment confirms that DNA methylation at 7-Gua by MNU-PNR is 2−7 times that seen with MNU at the 500 and 1000 μM concentrations (Figure 5). This analysis is based on data using G_{271} and G_{277}. The enhanced methylation for MNU-PNR is higher at the lower dose, suggesting that PNR equilibrium binding sites are saturated at the 1 mM concentration. This determination of the relative yield of 7-MeGua from MNU-PNR and MNU compares favorably to that calculated directly by HPLC analysis of the adducts after hydrolysis of DNA treated with the two agents (see below). MU-PNR (500 μM) inhibits MNU-mediated strand breaks by ~30%, while 500 μM ethidium bromide and 200 mM NaCl inhibit by ~50% and 70%, respectively (Figures 4A, lanes w−z, and 4B).

The sequence selectivity for the formation of 7-MeGua is basically the same for MNU-PNR and MNU. This is most obvious at the $G_{281-283}$ run (Figure 4A). Coincubation of MU-PNR or NaCl with MNU-PNR does not alter the cleavage pattern.

Methyl Adduct Analysis. The yields of 7-MeGua and 3-MeAde, based on HPLC/UV analysis, from the reaction of MNU and MNU-PNR with salmon sperm DNA, in the absence and presence of 200 mM NaCl, are shown in Table 1. The adducts were released from the DNA by

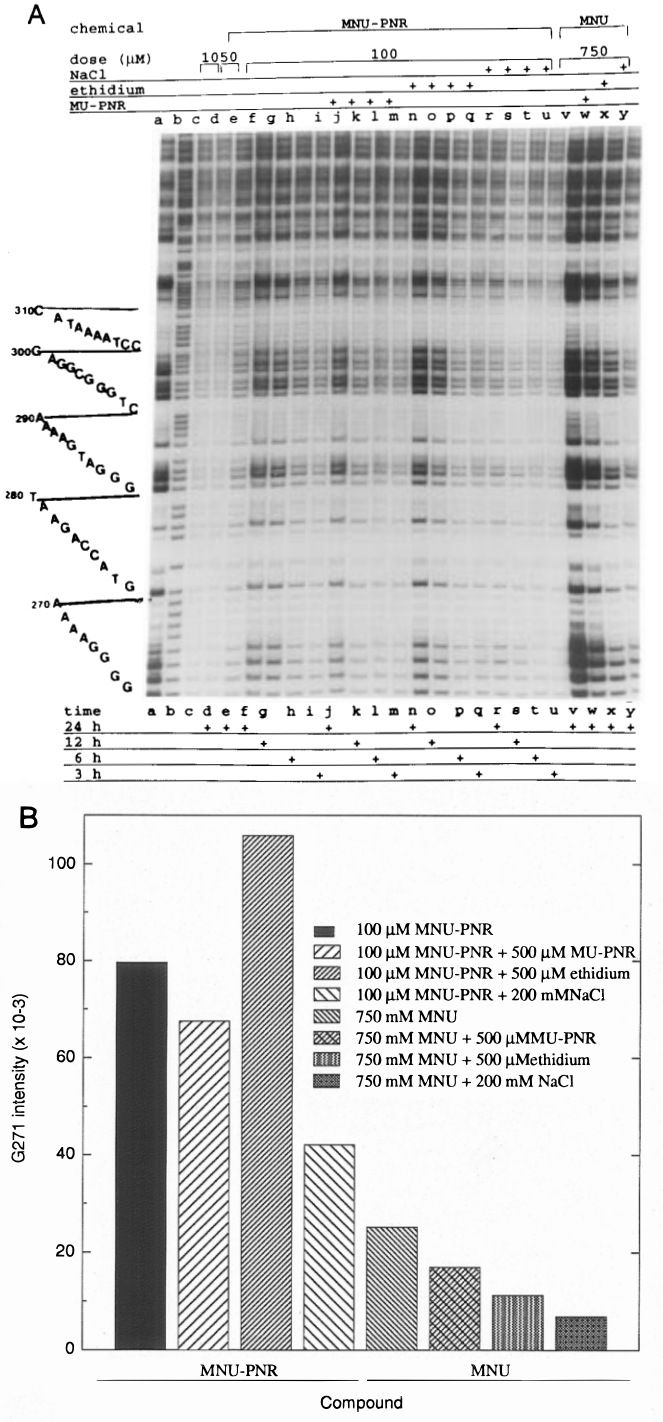

Figure 4. (A) Reactions of MNU-PNR with 576 bp restriction fragment. Autoradiogram of polyacrylamide sequencing gel: lane a, Gua; lane b, Gua+Ade; lane c, control; lane d, 10 μM MNU-PNR; lane e, 50 μM MNU-PNR; lanes f−i, 100 μM MNU-PNR with 24, 12, 6, and 3 h incubation times, respectively; lanes j−m, 100 μM MNU-PNR + 500 μM MU-PNR with 24, 12, 6, and 3 h incubation times, respectively; lanes n−q, 100 μM MNU-PNR + 500 μM ethidium bromide with 24, 12, 6, and 3 h incubation times, respectively; lanes r−u, 100 μM MNU-PNR + 200 mM NaCl with 24, 12, 6, and 3 h incubation times, respectively; lanes v−y, 750 μM MNU alone or with 500 μM MU-PNR, 500 μM ethidium bromide, or 200 mM NaCl, respectively, with 24 h incubation. (B) Quantitation of 7-Gua methylation of DNA at 24 h time point by 100 μM MNU-PNR in the absence or presence of 500 μM MU-PNR, 500 μM ethidium bromide, or 200 mM NaCl: uncorrected densitometric analysis of bands corresponding to Gua_{271} from panel A (lanes f, j, n, and r). Also shown are the concentration corrected (see text) intensities of the bands for 7-MeGua formation from MNU in the absence or presence of 500 μM MU-PNR, 500 μM ethidium bromide, or 200 mM NaCl from panel A (lanes v−y).

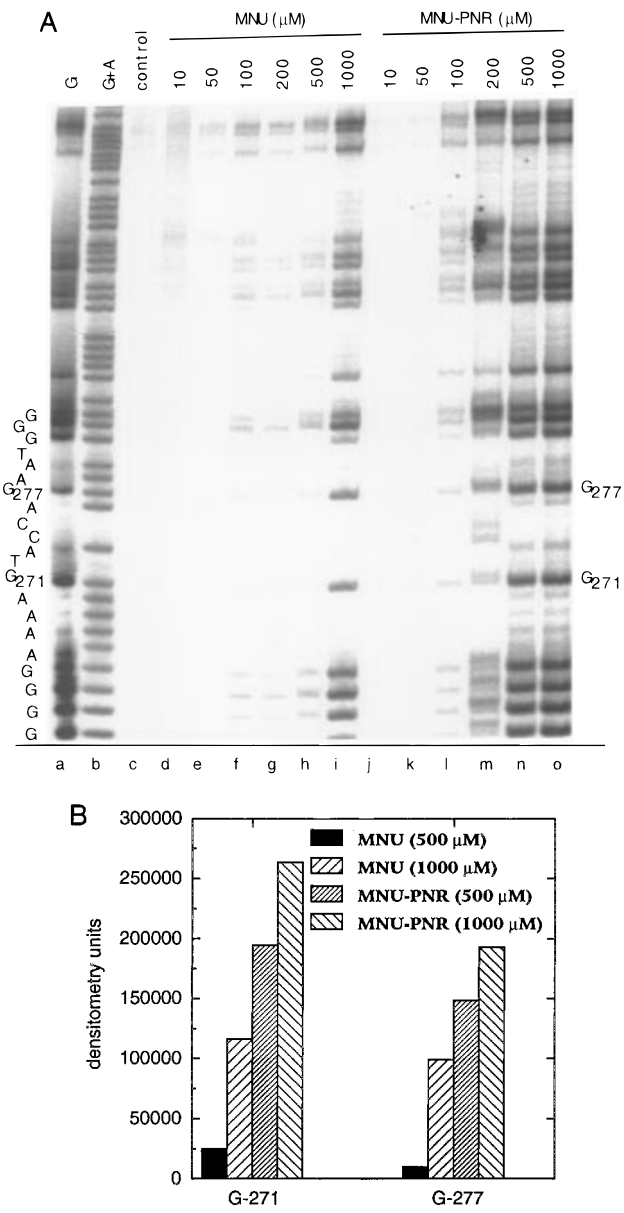

Figure 5. (A) Reactions of MNU-PNR with 576 bp restriction fragment. Autoradiogram of polyacrylamide sequencing gel: lane a, Gua; lane b, Gua+Ade; lane c, control; lanes d−i, 10, 50, 100, 200, 500, and 1000 μM MNU; lanes j−o, 10, 50, 100, 200, 500, and 1000 μM MNU-PNR. (B) Uncorrected densitometry data for 7-MeGua formation from MNU (lanes h and i) and MNU-PNR (lanes n and o) at Gua_{271} and Gua_{277} from panel A.

neutral thermal hydrolysis, and separated and identified by HPLC analysis using UV detection (270 nm) that was optimized to analyze for the minor 3-MeAde adduct (*43*). The results are expressed as a ratio of adduct to unmodified Ade to correct for any difference in DNA recovery between experiments. The highest level of DNA modification is <1 adduct/20 Ade, so there is no saturation of potential alkylation sites.

The ratios of the yields of 7-MeGua to 3-MeAde from MNU are 7.8 and 6.7 at low and high salt, respectively (Table 1). These values compare well with previous studies that report 7-MeGua/3-MeAde ratios averaging ~7.5 (*32*). The ratio of 7-MeGua/3-MeAde from MNU-PNR increases ~5-fold to 37.2 at low salt. The increase at high salt is ~2-fold. The results also show that MNU-PNR generates 2.8 times more 7-MeGua than MNU based on an equimolar dose. This outcome is very close to the difference calculated from the sequencing gel data

Table 1. Yields of 7-MeGua and 3-MeAde Adducts from the Reaction of Salmon Sperm DNA with MNU and MNU-PNR

compd	method	NaCl (mM)	7-MeGua[a]	3-MeAde[a]	7-MeGua/ 3-MeAde
MNU[b]	HPLC[c]	0	1.63	0.21	8
		200	0.20	0.03	7
MNU-PNR[b]	HPLC	0	4.51	0.12	38
		0	4.01	0.11	37
		200	1.20	0.08	15
		200	1.19	0.08	15
MNU[d]	ELISA[e]	0	nd[f]	2.28	
		200	nd	0.31	
MNU-PNR[d]	ELISA	0	nd	0.86	
		200	nd	0.63	

[a] Yields are expressed as mmol of adduct/mol of Ade. [b] [DNA] = 1 mM; [methylating agent] = 1 mM. [c] HPLC analysis with UV detection. [d] [DNA] = 0.1 mM; [methylating agent] = 1 mM. [e] Antibody/ELISA analysis. [f] Not determined.

(see above). In the presence of 200 mM NaCl, the effectiveness of MNU-PNR over MNU to methylate 7-Gua increases to 6-fold using the HPLC/UV data from the DNA digestion or the sequencing gels.

Because the levels of 3-MeAde were at the limit of UV detection, studies were done using a more sensitive monoclonal antibody to 3-MeAde in a competitive ELISA determination. These studies were done using 1 mM MNU or MNU-PNR and 0.1 mM DNA (1/10 the concentration of DNA used in the UV analysis) in order to obtain a clear signal with the ELISA. The results (Table 1) verify that MNU generates approximately 2.5 times more 3-MeAde than MNU-PNR. The data also show that DNA methylation at 3-Ade by MNU is much more sensitive to the inhibitory effect of salt. There is a 70% decrease in yield of the 7-MeGua adduct from MNU-PNR in the presence of salt, which is much larger decrease than that observed for 3-MeAde (25% decrease). In the ELISA studies the ratio of methylating agent to DNA was 10:1 *vs* 1:1 for the HPLC/UV analysis. Also, in the HPLC/UV analysis 3-MeAde levels are expressed as adduct/unmodified Ade, where Ade was experimentally verified by HPLC analysis. In the ELISA work, 3-MeAde is expressed as adduct/Ade, where Ade levels were calculated from the initial DNA concentration used in the incubation. It is for this reason that the apparent levels of adduct are 10-fold higher in the antibody data (Table 1).

UV−Vis and CD Spectra. In order to obtain physical evidence for the association of MNU-PNR with DNA, the stable urea derivative MU-PNR was incubated with DNA and the visible spectrum followed as a function of increasing the [DNA]/[compound] (data not shown). The effect of the addition of DNA to MU-PNR, with or without 200 mM NaCl, is a red shift of 5 nm from 555 to 560 nm with a concomitant 22% decrease in the absorbance at the highest compound to DNA ratios. The addition of 200 mM NaCl to MU-PNR did not alter the UV−vis spectrum.

The CD spectrum of 200 μM salmon sperm DNA in 13 mM Tris buffer shows a clear B-form conformation, and the coaddition of 7.6 μM MU-PNR increases the intensities of the positive (~40%) and negative (~25%) Cotton bands (Figure 6). When the spectrum was run in the presence of 200 mM NaCl, the intensity of the long wavelength band increased by 20% in the presence of MU-PNR, but there was virtually no change in the 247 nm band. The CD spectrum of the DNA in the absence

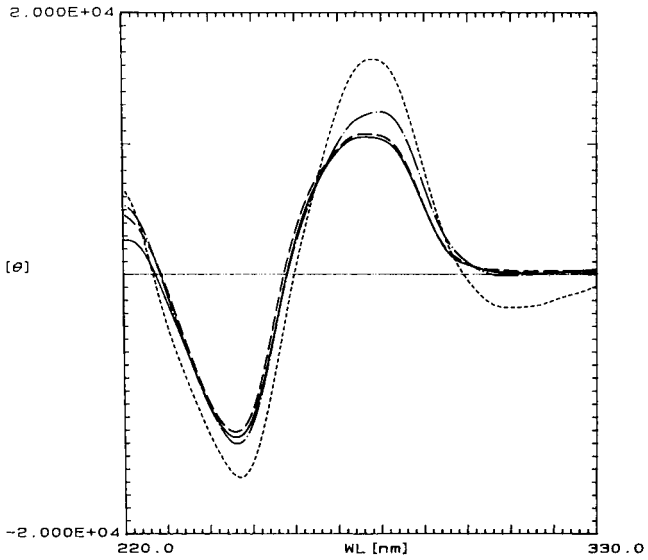

Figure 6. CD spectra of 7.6 μM MU-PNR with 200 μM (phosphate) salmon sperm DNA in 13 mM pH 8.0 Tris-HCl buffer: (—) DNA; (− −) DNA + 200 mM NaCl; (- - -) DNA + MU-PNR; (- · -) DNA + MU-PNR + 200 mM NaCl.

Table 2. Effect of Ethidium Bromide (EB) and MU-PNR on the Viscosity of Calf Thymus DNA

compd	NaCl (mM)	slope[a]	r[c]
EB	0	0.1814	0.9970
	200	0.1704	0.9959
MU-PNR	0	0.1207	0.9969
	200	0.1295	0.9975
EB + MU-PNR	0	0.1066	0.9856

[a] Calculated from formula shown in Experimental Section. [b] Correlation coefficient. [c] Mixture of equimolar amounts of EB and MU-PNR added to DNA.

of compound is not changed by the addition of salt. MU-PNR also induces a small red shift of approximately 3 nm in the positive and negative bands at both low and high ionic strength.

DNA Viscosity Studies. The results for the viscometry studies using calf thymus DNA are shown in Table 2. The slopes of the lines (L/L_0 *vs* [compound]/[DNA-phosphate]) indicate that ethidium bromide (slope = 0.1814, r = 0.9970) (r, correlation coefficient) is more effective at lengthening the DNA than MU-PNR (slope = 0.1207, r = 0.9969). The coaddition of 200 mM NaCl with either compound has no significant effect on the slopes: ethidium bromide (0.1704, r = 0.9959) and MU-PNR (0.1295, r = 0.9975). The addition of the same concentrations of a 1:1 mixture of MU-PNR and ethidium bromide to the DNA results in a decrease in the slope (0.1066, r = 0.9856) as compared to either compound alone.

Discussion

PNR/DNA Complex. The proposed orientation of intercalated PNR (Figure 2) is rare; most small intercalating molecules enter the DNA stack from the minor groove. This placement of PNR was originally inferred from DNA binding studies (in phosphate buffer containing 180 mM NaCl) using a number of PNR analogues and is based on both steric and electronic arguments (*21*, *23*). It was also shown, using DNA's with different G:C/A:T content, that PNR has a modest preference for G:C rich DNA (*21*). The modeling of PNR with a diter-

calinium−DNA derived intercalation site (*28*) confirms that the PNR nucleus is too wide, because of the 2-methyl and *N*7-(dimethylamino) groups, to penetrate into duplex DNA from the minor groove and attain any stacking with the flanking bases (Figure 2).

The viscosity, CD, and UV data are consistent with the intercalation of MNU-PNR into DNA, although the exact structure of the MU-PNR/DNA complex can only be inferred from the molecular modeling studies and other modes of groove binding and cannot be ruled out. The viscosity and spectroscopic studies show that the effects of MU-PNR are independent of ionic strength, which is also congruous with a hydrophobic interaction of the compound with DNA. Previously, an acrylate linker has been substituted onto the C4′-position of PNR in order to exploit the preference of PNR for G:C sequences in an affinity column strategy to selectively bind G:C rich DNA's (*23*). The acrylamide analogue of PNR has a similar binding affinity and G:C base pair preference as PNR.

DNA Methylation. The hydrolytic decomposition of MNU-PNR and MNU yields the same methanediazonium ion intermediate, and this is consistent with the similarity between the 7-Gua methylation pattern from MNU-PNR and MNU. As mentioned above, the absolute yield of 7-MeGua from MNU-PNR is approximately 2.5- and 6-fold higher than for MNU, at low and high salt, respectively (Table 1). Moreover, the 7-MeGua to 3-MeAde ratio, which reflects adduction in the major *vs* minor groove, is approximately 5- and 2-fold higher at low and high salt, respectively. We propose that the primary difference between MNU-PNR and MNU, in terms of DNA methylation, is the regioselective generation of methanediazonium ion in, or near, the major groove, because of the formation of an intercalation complex. It is also possible that a nonspecific electrostatic association, and the ∼2-fold preference of PNR for G:C over A:T regions, can enhance the formation of Gua adducts (*21*, *23*, *45*).

The effect of ionic strength provides an important insight into the route of delivery of reactive intermediate to DNA. The methylation of DNA at 7-Gua by both MNU and MNU-PNR is decreased by the addition of salt even though salt does not affect PNR intercalation as measured by viscosity, UV, or CD. This result can be explained by two nonexclusive mechanisms. First, a decrease in methylation by methanediazonium ion is expected at high salt based on previous studies with MNU and other charged alkylating agents (*14*, *26*, *42*, *46*−*53*). This phenomenon has been attributed to the competitive inhibition of the electrostatic attraction between DNA and positively charged alkylating agents by inorganic and equilibrium binding cations. However, neither the nature of the cation nor the ionic strength qualitatively affects the DNA alkylation pattern of MNU (*26*, *42*), and the same result is seen with MNU-PNR. A second possibility is that at low ionic strength there is a nonspecific electrostatic association between charged PNR dye and DNA, and this association can increase the concentration of methanediazonium ion generated in the vicinity of DNA (*44*). Along this line, a chlorambucil nitrogen mustard has been conjugated to a polyamine (*54*). This polycationic conjugate cross-links DNA through major groove sites 10^4 times more efficiently than chlorambucil, and it is assumed that the electrostatic attraction between the polyamine and the DNA contributes to this increased reactivity. Therefore, the inhibitory salt effect seen with MNU-PNR can also be explained by inhibition of nonspecific electrostatic binding of the cationic methanediazonium ion and/or the cationic PNR dye to DNA (*44*).

At the present time, we cannot differentiate between the two mechanisms for salt inhibition. However, the observation that DNA methylation at 7-Gua and 3-Ade by MNU-PNR is less sensitive to changes in ionic strength than MNU is consistent with the delivery of methanediazonium ion to DNA via an intercalated precursor. The bias in the methylation pattern to major groove adduction, *i.e.*, the 7-MeGua/3-MeAde ratio of >30:1 *vs* to 8:1 for MNU, is also fitting with the MNU-PNR/DNA complex shown in Figure 2 since *a priori* there is no reason to assume that electrostatic interactions of PNR with DNA would favor major groove complexation and/or adduction.

The increase in the MNU-PNR-mediated formation of 7-MeGua upon coaddition of ethidium bromide and the decrease with MU-PNR are additional evidence that the MNU-PNR complex with DNA is not simply electrostatic since, if that were the case, both cationic intercalators would have the same effect on DNA methylation. It should be noted that ethidium bromide inhibits the yield of 7-MeGua with MNU by ∼50% (Figure 4A, lanes r and s). We suggest that MU-PNR, because of its structural similarity to MNU-PNR, is a true competitive inhibitor of MNU-PNR intercalation, while ethidium, because it enters the helix from the minor groove (*55*, *56*), is not. In fact, it is possible, albeit speculative, that the enhanced methylation with MNU-PNR in the presence of ethidium is because ethidium, by entering via the minor groove, can provide a major groove intercalation site for the former without any additional significant conformational change in the DNA. The observation that an equimolar combination of MU-PNR and ethidium bromide (while maintaining the same [total compound]/[DNA] ratios) affords less DNA lengthening than the individual molecules (Table 2) could be explained by the suggested synergistic mode of binding with the ethidium association dominating the viscosity results. In the co-occupation model, it is anticipated that the slope would be close to a value of 0.1 or approximately 50% of that measured when ethidium bromide is incubated with DNA alone at the same compound/DNA ratio (slope ≈ 0.18). The initial formation of the intercalation site by the ethidium from the minor groove followed by entrance of the PNR from the major groove is reasonable since ethidium has a log K_D of −5.7 *vs* −3.6 for PNR and the kinetics of ethidium binding, as measured by NMR, is slow relative to PNR (*45*). Other possible explanations of the viscosity data that require a decrease in available compound through some interaction between the ethidium and PNR molecules are not consistent with the observed increase in DNA methylation when the two compounds are added together. We are not aware of any evidence for the cooperativity model for major and minor groove intercalators; however, there is an analogy for this process if one assumes that a stacked bulged base crudely mimics an intercalator. The preferential intercalation of methidiumpropyl-EDTA-Fe(II) and 9-aminoacridine at bulge sites has been reported (*57*, *58*).

A well-known example of an intercalator that is used to deliver a DNA damaging agent to DNA is the methidium nucleus appended with an iron chelating EDTA (*59*). This reagent, which is thought to bind in the minor groove, generates oxygen-mediated DNA cleavage with

little sequence specificity and is widely used as a footprinting agent (*60*). In other elegant studies, aniline mustards have been linked to the 9-position of the intercalator 9-aminoacridine so that the mustard and acridine become covalently linked to DNA (*13*, *61*, *62*). The results of this work show that the DNA−acridine interactions, presumably through a minor groove interaction, affect the sequence selectivity of DNA alkylation by the nondiffusible mustard group. Unlike the typical nitrogen mustards that alkylate Gua, these intercalator-linked mustards react significantly with Ade sites, although complete characterization of the Ade adducts has not been reported.

Selective methylation of DNA at 7-Gua has also been described. Iversen and Dervan prepared deoxyuracil triphosphate appended with a methylthioether group and introduced the substituted base into ss-DNA using Klenow (*16*). The ss-DNA was hybridized to a complementary strand, and the methylthioether functionality activated by the addition of cyanogen bromide. The resulting cyanomethanesulfonium ion methylated the complementary strand selectively at 7-Gua. In a more straightforward approach, synthetic oligomers have been site-specifically modified with 7-MeGua by the incorporation of 7-Me-dGTP into a gap using DNA polymerase (*63*). While these methods sequence selectively afford 7-MeGua lesions, they obviously cannot be used to generate this lesion *in vivo*.

In summary, the data presented show that the use of PNR to deliver a small diffusible alkylating agent to DNA can cause a significant change in the methylation pattern with a 5-fold increase in the ratio of major to minor groove lesions. The data also demonstrate that an intercalation complex between MNU-PNR and DNA is most likely responsible for the change in the methylation pattern due to an increase in the amount of methanediazonium ion formed near the major groove of DNA. One of the reasons that the groove specificity for DNA methylation is not higher from MNU-PNR is due to the involvement of the "diffusible" methanediazonium ion. The employment of bimolecular methylating agents, *e.g.*, methyl sulfonate esters (*15*), should increase the major to minor groove methylation ratio. Other future work will involve the analysis of how the nature and length of the tether between the alkylating agent and intercalator affect equilibrium binding and methylation, and the extension of the molecular design to include potential antineoplastic molecules derivatized with cross-linking agents.

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