DNA Damage and Cytotoxicity Induced by Minor Groove Binding Methyl Sulfonate Esters†

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ABSTRACT: Minor groove specific DNA equilibrium binding peptides (lex) based on *N*-methylpyrrolecarboxamide and/or *N*-methylimidazolecarboxamide subunits have been modified with an O-methyl sulfonate ester functionality to target DNA methylation in the minor groove at Ade/Thy- and/or Gua/Cyt-rich sequences. HPLC and sequencing gel analyses show that the Me-lex compounds all selectively react with DNA to afford N3-alkyladenine as a major adduct. The formation of the N3-alkyladenine lesions is sequence-dependent based on the equilibrium binding preferences of the different lex peptides. In addition to the reaction at adenine, the molecules designed to target Gua/Cyt sequences also generate lesions at guanine; however, the methylation is not sequence dependent and takes places in the major groove at the N7-position. To determine if and how the level of the different DNA adducts and the sequence selectivity for their formation affects cytotoxicity, the Me-lex analogues were tested in wild type *Escherichia coli* and in mutant strains defective in base excision repair (*tag* and/or *alkA* or *apn*). The results demonstrate the importance of 3-methyladenine, and in some cases 3-methylguanine, lesions in cellular toxicity, and the dominant protective role of the DNA glycosylases. There is no evidence that the sequence specificity is related to toxicity.

DNA damage plays a critical role in the generation of cytotoxicity and mutagenicity elicited by many carcinogens, as well as by clinically used antineoplastic agents (*1*, *2*). While mutagenesis and cell death do not necessarily overlap mechanistically, many DNA lesions can induce both biological endpoints. This point is dramatically exposed in the clinical setting where a significant incidence of secondary cancers is attributed to the treatment of patients with antineoplastic agents for their primary cancer (*3*, *4*). Regardless, DNA remains an extremely attractive target for anticancer agents. Therefore, it is imperative to identify and eliminate the formation of promutagenic lesions while maintaining those that selectively induce cytotoxicity since virtually all anticancer drugs find their way to "normal" tissues prone for transformation, e.g., hematopoietic cells in the bone marrow.

On the basis of previous studies, there is strong evidence that minor groove 3-alkylpurine adducts are highly cytotoxic polymerase blocking lesions (*5*−*7*). Accordingly, we designed reagents to selectively introduce 3-MeAde[1] modifications into DNA by attaching sulfonate esters to affinity binding di- and tripeptides (referred to as lex) composed of *N*-methylpyrrolecarboxamide subunits (*8*) (see Figure 1 for structures). These lex peptides are structurally based on minor groove binding natural products, i.e., netropsin and distamycin, but they have been modified so that their affinity binding to DNA is significantly reduced. This was done by eliminating the cationic group(s) on one or both termini of the natural compounds. The result is a lex dipeptide that recognizes the same affinity binding sites as distamycin or netropsin, but with K_b's that are 100-fold lower (*9*).

Therefore, the lex dipeptide transiently binds to DNA with selectivity for A/T-rich sequences and in so doing places the reactive alkylating agent near the floor of the minor groove. For example, the methylating agent Me-lex$^{Py/Py}$ (Figure 1) affords > 90% of the minor groove adduct

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[1] Abbreviations: Aag, mammalian alkylpurine-DNA glycosylase; AlkA, *E. coli* N3-methyladenine-DNA glycosylase II; Apn, AP endonuclease; BER, base excision repair; DMS, dimethyl sulfate; lex, lexitropsin (information reading peptide); Me-lex$^{Im/Im}$, [1-methyl-4-[1-methyl-4-(3-(methoxysulfonyl)propanamido)imidazole-2-carboxamido]-imidazole-2-carboxamido]-ethane; Me-lex$^{Im/Py}$, [1-methyl-4-[1-methyl-4-(3-(methoxysulfonyl)propanamido)imidazole-2-carboxamido]-pyrrole-2-carboxamido]propane; Me-lex$^{Py/Py}$, [1-methyl-4-[1-methyl-4-(3-(methoxysulfonyl)propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane; 3-MeAde, N3-methyladenine; 3-MeGua, N3-methylguanine; 6-MeGua, O^6-methylguanine; 7-MeGua, N7-methylguanine; MMS, methyl methanesulfonate; Tag, *E. coli* 3-methyladenine-DNA glycosylase I.

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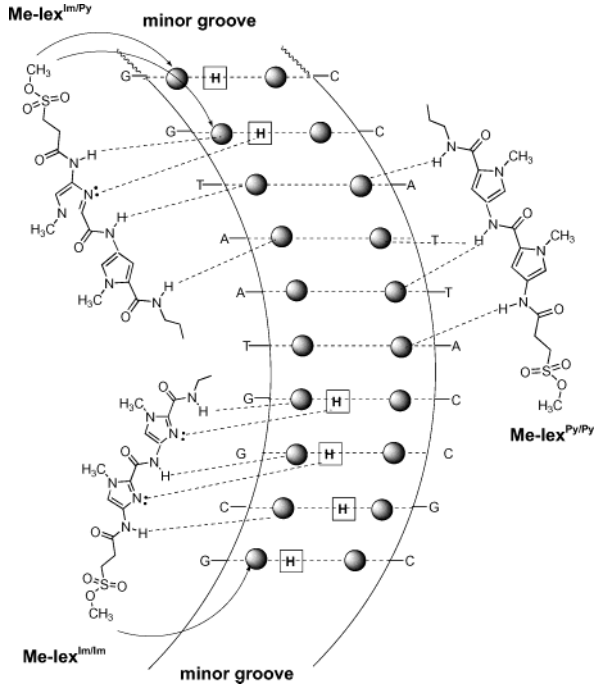

FIGURE 1: Structures of Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and Me-lex$^{Im/Im}$, and their potential interaction with DNA in the minor groove: solid spheres (○) represent H-bond acceptor atoms on Ade (N-3) and Thy (O-2); [H] represents the N^2-amino H-bond donor group on Gua; dashed line (- - -) indicates H-bonds and arrow (→) indicates transfer of methyl group from alkylating agent to N-3 of Ade or N3 of Gua.

3-MeAde with low concomitant formation of the major groove lesions, 7-MeGua and 6-MeGua (*10*−*12*). Due to the increase in 3-MeAde formation, Me-lex$^{Py/Py}$ is significantly more cytotoxic than MMS in *E. coli* (*9*, *13*) and mouse ES cells (*14*). Consistent with the high levels of 3-MeAde, the toxicity of Me-lex$^{Py/Py}$ is dramatically increased in Tag/AlkA glycosylase deficient *Escherichia coli* mutants (*9*, *13*) and in Aag$^{-/-}$ ES cells (*14*). There is also an enhancement of Me-lex$^{Py/Py}$-induced toxicity in leukemic cells made deficient in poly(ADP-ribose) polymerase with aminobenzamide (*15*, *16*). Remarkably, in the same cells there is also a pronounced switch in the mechanism of cell death from necrosis to apoptosis accompanied by poly(ADP-ribose) polymerase inhibition (*16*).

In the present report, we have extended our work to analogues of Me-lex designed to affinity bind to regions containing Gua/Cyt base pairs. Their synthesis is described, along with a quantitative and qualitative characterization of their in vitro reactions with DNA. The toxicity of the compounds in wild type *E. coli* and BER mutants are also presented. The results show that the compounds designed to target the N3-position of Gua methylate DNA at Gua, but the position of adduction is in the major groove. The minor groove 3-MeAde lesion remains a major product in all cases. In addition, overall toxicity of the Me-lex compounds is related to the level of 3-MeAde (and 3-MeGua) formation, but not to any differences in sequence specificity.

EXPERIMENTAL PROCEDURES

Hazardous Procedure. The methyl sulfonate esters and 1-methyl-3-*p*-tolyltriazene should be considered toxic and potential human carcinogens, and handled accordingly.

All chemicals were of the highest purity available and used without further purification except where noted. ^{1}H NMR spectra were recorded on a Varian Unity-500 spectrometer. Mass spectra were determined on a Kratos MS-50 instrument at the Midwest Center for Mass Spectrometry in the Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE. Me-lex$^{Py/Py}$ was prepared as previously described (*8*). *E. coli* strains were generous gifts from T. O'Connor (City of Hope National Medical Center, Duarte, CA), L. Samson (Massachusetts Institute of Technology, Cambridge, MA), and B. Sedgwick (ICRF, Herts, UK).

*Synthesis of Ethyl 1-methylimidazole-2-carboxylate (**2**).* Compound **2** was synthesized following the general procedure described by Krowicki and Lown (*17*).

*Synthesis of Ethyl 1-methyl-4-nitroimidazole-2-carboxylate (**3**).* **3** was synthesized by nitrating **2** (*18*).

*Synthesis of 1-Methyl-4-nitroimidazole-2-carboxylic acid (**4**).* Compound **4** was synthesized as described by Krowicki and Lown (*17*).

*Synthesis of (1-Methyl-4-nitroimidazole-2-carboxamido)-ethane (**5**).* To **4** (5.13 g, 30 mmol) in a dry flask flushed with Ar was added THF (100 mL) followed by diisopropylethylamine (12 mL). The mixture was stirred at room temperature for 10 min at which time pivaloyl chloride (4 mL, 32 mmol) was added and stirring continued for an additional 30 min. Ethylamine (25 mL of 2 M solution in THF) was added and the reaction mixture was stirred for 3−4 h under Ar. The solvent was removed in vacuo and ice−water was added to precipitate the product, which was filtered and dried to give pure **5** (5.5 g, 93% yield): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 3.25 (q, 2H), 4.05 (s, 3H), 8.59 (s, 1H), 8.8 (br, t, 1H); MS (FAB) *m*/*z* 199.083, $C_7H_{11}N_4O_3$ $(M+H)^+$.

*Synthesis of [1-Methyl-4-(1-methyl-4-nitroimidazole-2-carboxamido)imidazole-2-carboxamido]ethane (**6**).* **5** (5.5 g, 27.8 mmol), 10% Pd/C (2.75 g), cyclohexene (55 mL), and 95% EtOH (280 mL) were refluxed overnight. The catalyst was removed by filtration and washed with 100 mL of EtOH. The filtrate was dried in vacuo to afford the amine, which was taken up in anhydrous THF (48 mL) under Ar. In a separate reaction, **4** (4.7 g, 27.8 mmol) was placed in a dry flask, flushed with Ar, and sealed with a rubber septum. THF (87 mL) and diisopropylethylamine (11.2 mL) were injected through the septum. The mixture was stirred at room temperature for 10 min at which time pivaloyl chloride (3.66 mL) was added and stirring was continued for an additional 30 min. After the amine solution was slowly added, the reaction was stirred at room temperature for an additional 12 h under Ar. The solvent was removed in vacuo and ice water added to precipitate product. The product was filtered and dried to give pure **6** (6.456 g, 73% yield): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 3.2 (q,2H), 3.94 (s, 3H), 4.05 (s, 3H), 7.5 (s, 1H), 8.23 (br, t, 1H), 8.6 (s, 1H), 10.2 (s, 1H); MS (FAB) *m*/*z* 322.12, $C_{12}H_{16}N_7O_4$ $(M+H)^+$.

*Synthesis of [1-Methyl-4-[1-methyl-4-(2-propenamido)-imidazole-2-carboxamido]-imidazole-2-carboxamido]-ethane (**7**).* **6** (1 g, 3.12 mmol), 10% Pd/C (500 mg), cyclohexene (10 mL), and 95% EtOH (88 mL) were refluxed overnight. The catalyst was removed by filtration and washed with 100 mL of EtOH. The filtrate was concentrated to give the free amine, which was dissolved in anhydrous THF (75 mL) containing diisopropylethylamine (2 mL). The solution

was flushed with Ar, cooled to −40 °C and acryloyl chloride (0.312 mL, 3.12 mmol) added dropwise and stirring continued for 4 h at −20 °C. The solvent was removed on a rotary evaporator and ice water was added to precipitate the product. The product was filtered and dried to give pure **7** (730 mg, 78% yield): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 3.2 (q, 2H), 3.94 (s, 3H), 4.05 (s, 3H), 5.7 (d, 1H), 6.22 (d, 1H), 6.5 (m, 1H), 7.5 (s, 1H), 7.62 (s, 1H), 8.23 (br, t, 1H), 9.55 (s, 1H), 10.6 (s, 1H); MS (FAB) *m*/*z* 346.163, $C_{15}H_{20}N_7O_3$ $(M+H)^+$.

*Synthesis of [1-Methyl-4-[1-methyl-4-(3-sulfopropanamido)imidazole-2-carboxamido]-imidazole-2-carboxamido]-ethane (**8**).* **7** (344 mg, 1 mmol) was suspended in water (30 mL) and a 45% aqueous solution of ammonium bisulfite (2 mL) and 30% hydrogen peroxide (0.6 mL) were added. The solution was refluxed vigorously until the starting material had disappeared (12−20 h) as measured by TLC (silica gel, EtOAc). The solvent was removed in vacuo and the residue was dissolved in a minimal amount of water. The solution was acidified using concentrated HCl to precipitate the sulfonic acid. The mixture was kept in the cold for at least 5 h to ensure complete precipitation, and the acid filtered, washed with a small amount of ice water, and dried to give pure **8** (345 mg, 80% yield): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 2.62 (t, 2H), 2.7 (t, 2H), 3.2 (q, 2H), 3.94 (s, 3H), 3.96 (s, 3H), 7.5 (s, 1H), 7.62 (s, 1H), 8.23 (br, t, 1H), 9.55 (s, 1H), 10.6 (s, 1H); MS (FAB) *m*/*z* 450, $C_{15}H_{21}N_7O_6S_1Na$ $(M+Na)^+$.

*Synthesis of [1-Methyl-4-[1-methyl-4-(3-(methoxysulfonyl)-propanamido)-imidazole-2-carboxamido]imidazole-2-carboxamido]ethane (**9**, Me-lex$^{Im/Im}$).* **8** (133 mg, 0.31 mmol) was suspended in anhydrous dioxane (20 mL) and stirred for 1 h under Ar. In a separate flask, 3-methyl-*p*-tolyltriazene (100 mg) was dissolved in 2 mL of anhydrous dioxane under Ar and then added to a cold suspension of **8**. The reaction mixture was stirred at 55 °C for 2 h with protection from light. The solvent was removed in vacuo and the residue purified by flash chromatography (silica gel, EtOAc), to give pure **9** (70 mg, 51% yield): ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 2.8 (t, 2H), 3.2 (q, 2H), 3.6 (t, 2H), 3.94 (s, 3H), 3.96 (s, 3H), 7.5 (s, 1H), 7.62 (s, 1H), 8.23 (br, t, 1H), 9.55 (s, 1H), 10.6 (s, 1H); MS (FAB) *m*/*z* 464, $C_{16}H_{23}N_7O_6SNa$ $(M+Na)^+$.

*Synthesis of (1-methyl-4-nitropyrrole-2-carboxamido)propane (**10**).* Compound **10** was synthesized as described earlier (*7*).

*Synthesis of [1-Methyl-4-(1-methyl-4-nitroimidazole-2-carboxamido)pyrrole-2-carbox-amido]propane (**11**).* A suspension of **10** (0.26 g, 1.23 mmol) and 10% Pd/C (0.5 g) in 20 mL of 95% EtOH containing 5 mL of freshly distilled cyclohexene was refluxed until complete disappearance of **10** (6 h) as indicated by TLC (EtOAc). The catalyst was removed by filtration and washed with 50 mL of EtOH. The filtrate and washings were concentrated by rotary evaporation to afford the amine which was dried under vacuum overnight and then taken up (without further purification) in anhydrous THF (10 mL) under Ar. In a separate reaction, **4** (0.207 g, 1.21 mmol) was placed in a dry flask under Ar and THF (10 mL), diisopropylethylamine (0.5 mL) and pivaloyl chloride (0.154 mL, 1.25 mmol) were added and the mixture stirred for 30 min. The amine solution was then added dropwise (using a cannula) and the reaction was stirred at room temperature overnight when a yellow precipitate fell out of solution. The solution was cooled in ice and the precipitate was filtered out and washed with cold ethanol to give pure **12** (0.288 g, 70% yield): ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H), 1.47 (m, 2H), 3.11 (m, 2H), 3.79 (s, 3H), 4.03 (s, 3H), 7.00 (d, 1H), 7.23 (d, 1H), 8.02 (t, 1H), 8.59 (s, 1H), 10.78 (s, 1H).

*Synthesis of [1-Methyl-4-[1-methyl-4-(2-propenamido)-imidazole-2-carboxamido]pyrrole-2-carboxamido]propane (**12**).* A suspension of **11** (0.22 g, 0.66 mmol) and 10% Pd/C (0.5 g) in 20 mL of 95% EtOH containing 5 mL of freshly distilled cyclohexene was refluxed for 6 h (until complete disappearance of **11** by TLC). The catalyst was removed by filtration and washed with 50 mL of EtOH. The filtrate and washings were concentrated by rotary evaporation to afford the amine which was dried under vacuum overnight and then taken up (without further purification) in anhydrous THF (10 mL) under Ar. Diisopropylethylamine (0.46 mL) was added, the mixture stirred at room temperature for 30 min and then cooled to −40 °C. Acryloyl chloride (0.08 mL, 0.99 mmol) was added dropwise with stirring and the mixture allowed to warm to −20 °C. Stirring was continued for 4 h at −20 °C and then the mixture was concentrated by rotary evaporation and the desired product purified by silica gel (column equilibrated with 0.1% triethylamine in ethyl acetate using ethyl acetate as the mobile phase) to afford **12** (0.163 g, 69% yield): ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H), 1.47 (m, 2H), 3.11 (m, 2H), 3.78 (s, 3H), 3.94 (s, 3H), 5.69 (dd, 1H), 6.22 (dd, 1H), 6.49 (dd, 10 Hz, 1H), 6.92 (d, 1H), 7.19 (d, 1H), 7.52 (s, 1H), 7.99 (t, 1H), 9.88 (s, 1H), 10.54 (s, 1H), 10.78 (s, 1H).

*Synthesis of [1-Methyl-4-[1-methyl-4-(3-sulfopropanamido)imidazole-2-carboxamido]-pyrrole-2-carboxamido]propane (**13**).* **12** (0.2 g, 0.56 mmol) was dissolved in 1.5 mL of DMF and 8.5 mL of water was added to form a white suspension. To the suspension was added 45% ammonium bisulfite (1.2 mL) and 30% H_2O_2 (0.1 mL) and the mixture was refluxed until the starting material had disappeared (3−4 h) as indicated by TLC (EtOAc). The solvent was removed by rotary evaporation and the white solid left behind was extracted several times with methanol. The methanol extracts were combined, the solvent removed by rotary evaporation, and the remaining solid dissolved in a minimal amount of water. The solution was cooled to 4 °C, acidified (to pH 2) using concentrated HCl, and allowed to sit for 2 h at 4 °C. The solid that fell out of the solution was filtered out, washed with a small amount of cold water, and dried to give pure **13** (0.197 g, 80% yield): ^{1}H NMR (DMSO-d_6) δ 0.84 (t, 3H), 1.47 (m, 2H), 2.81 (t, 2H), 3.10 (m, 2H), 3.42 (t, 2H), 3.78 (s, 3H), 3.92 (s, 3H), 6.92 (s, 1H), 7.19 (s, 1H), 7.41 (s, 1H), 7.99 (s, 1H), 9.91 (s, 1H), 10.46 (s, 1H).

*Synthesis of [1-Methyl-4-[1-methyl-4-(3-(methoxysulfonyl)-propanamido) imidazole-2-carboxamido]pyrrole-2-carboxamido]propane (**14**, Me-lex$^{Im/Py}$).* **13** (0.136 g, 0.31 mmol) was suspended in anhydrous dioxane (20 mL) and stirred for 1 h under Ar. In a separate flask, 3-methyl-p-tolyltriazine (0.1 g) was dissolved in 2 mL of anhydrous dioxane under Ar and then added to the suspension of **13**. The reaction mixture was stirred at 55 °C for 2 h with protection from light. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (silica gel, EtOAc) to give pure **15** (0.087 g, 62% yield): ^{1}H NMR (CD_3-OD) δ 0.96 (t, 3H), 1.60 (m, 2H), 2.89 (t, 2H), 3.24 (m,

Table 1: List of *E. coli* Strains

strain	genotype	antibiotic resistance	ref
AB1157	wild type	none	*42*
BW535	*apn*	kanamycin	*43*
MV1902	*alkA*	chloramphenicol	*44*
GC4803	*alkA/tag*	kanamycin	*45*

2H), 3.58 (t, 2H), 3.86 (s, 3H), 3.90 (s, 3H), 4.02 (s, 3H), 6.78 (d, 1H), 7.22 (d, 1H), 7.38 (s, 1H), 7.99 (s, 1H); MS (FAB) *m*/*z* 455.1697, $C_{18}H_{27}N_6O_6S$ $(M+H)^+$, 454.1631, $C_{18}H_{26}N_6O_6S$ $(M)^+$.

Adduct Analysis. The three different Me-lex compounds and MMS were reacted with 2 mM calf thymus DNA in 10 mM cacodylate buffer (pH 7.0) containing 10% DMSO for 24 h in the absence or presence of 100 μM netropsin. The DNA was directly subjected to neutral thermal hydrolysis (90 °C for 15 min) to liberate 7-MeGua, 3-MeGua and 3-MeAde and then the apurinic DNA was removed by precipitation after cooling, with the addition of cold 0.1 N HCl (*19*). The supernatant containing the methylated purines was collected and analyzed on reverse phase HPLC: column, 4.6 × 250 mm YMC C18; flow rate, 1 mL/min; mobile phase, solvent 4% MeOH in 0.1 M NaOAc, pH 5.0. UV (270 nm) was used to quantitate 3-MeAde and 3-MeGua. 7-MeGua was quantified using either an ESA electrochemical (EC) detector (guard cell at 850 mV, and analytical cell E_1 at 400 mV and E_2 at 800 mV) or by UV (270 nm).

Sequencing DNA Lesions. The isolation and end-labeling of the Parvovirus restriction fragment (*20*) used in these studies, and the procedures to sequence N-alkylpurine lesions using neutral thermal hydrolysis followed by treatment with alkali have been previously described (*8*). In some experiments the DNA was directly treated with piperidine to selectively remove the 7-MeGua lesion from DNA (*21*).

DNA Degradation. pBR322 plasmid was incubated with the Me-lex compounds for 1 h at room temperature. The DNA was either not treated, or heated at 90 °C for 20 min and then treated with 1 M piperidine. The fragmentation of the plasmid DNA Forms-I and -II was determined using agarose gel electrophoresis. The disappearance of the full-length plasmid DNA (Forms-I and -II) as a function of the concentration of alkylating agent was plotted and the initial slope calculated.

Toxicity Studies. E. coli cultures (Table 1) were started from the frozen stocks and the experiments performed as previously described in detail (*9*). In brief, the *E. coli* cultures were grown overnight in the LB media with (when indicated) the appropriate antibiotic. An aliquot of the overnight culture

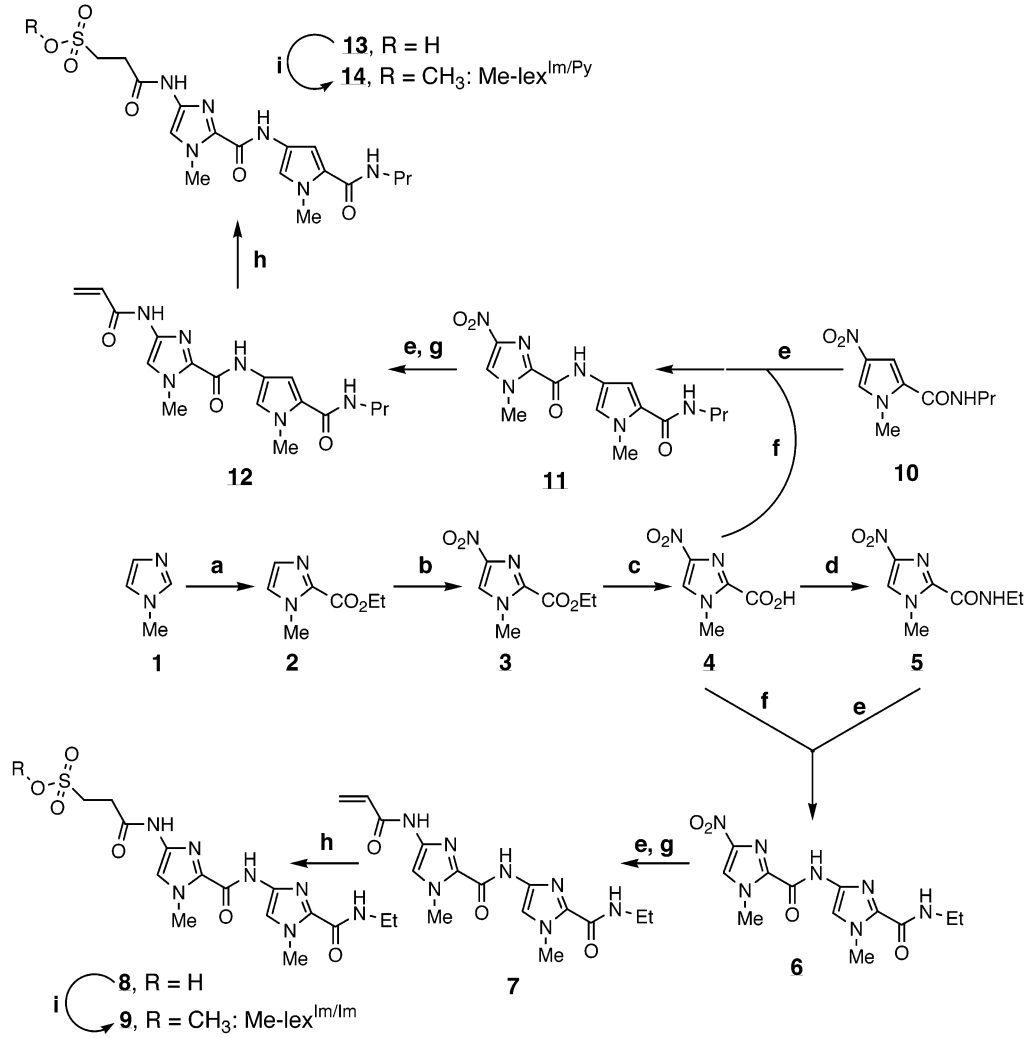

FIGURE 2: Synthesis of Me-lex$^{Im/Im}$ (*9*) and Me-lex$^{Im/Py}$(*14*): a, ClCOOEt, Et_3N; b, HNO_3; c, aqueous NaOH; d, pivaloyl chloride; ethylamine; e, Pd/C, cyclohexene; f, pivaloyl chloride; g, acryloyl chloride; h, ammonium bisulfite/H_2O_2; i, 1-methyl-3-*p*-tolyltriazene.

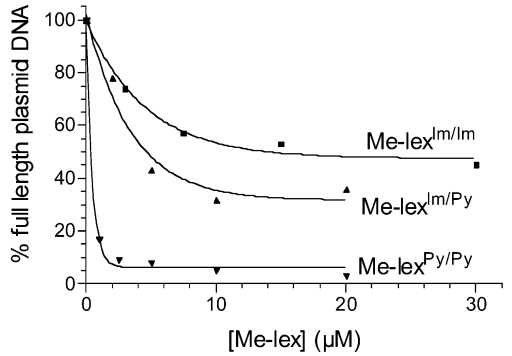

FIGURE 3: Plot of full-length plasmid DNA (total of forms-I and -II) remaining after exposure to Me-lex$^{Py/Py}$ (▼), Me-lex$^{Im/Py}$ (▲), and Me-lex$^{Im/Im}$ (■) as a function of concentration. The data were fitted using a nonlinear one-exponential decay equation.

was transferred to fresh LB media and grown until it reached log phase. A 1-mL aliquot of log phase culture was dispensed into 2.0 mL-screw-cap tubes. The log phase cultures were washed free of LB media with phosphate buffered saline and pelleted by centrifugation. The pellets were then resuspended in Tris-EDTA buffer (pH 7.5) incubated with Me-lex or MMS in 1.0 mL of 95% EtOH for 1 h. The controls were incubations without alkylating agent. After the incubation period, the bacteria were pelleted by centrifugation, washed free of agent using phosphate buffer, and resuspended in 1 mL of saline. Serial dilutions were made and bacteria were plated on LB agar with the appropriate antibiotic. The plates were incubated overnight at 37 °C for colony development and then counted manually to determine the percent survival. All experiments were performed in, at least, triplicates.

RESULTS

Synthesis. The preparation of Me-lex$^{Py/Py}$ has been reported (*8*). The synthesis of Me-lex$^{Im/Im}$ and Me-lex$^{Im/Py}$ precursors generally followed synthetic procedures (Figure 2) previously outlined (*8*, *17*, *18*). Not previously reported is the conversion in 50-60% yield of the sulfonic acids into the methyl sulfonate esters by treatment with methyl-*p*-tolyltriazene. The Me-lex compounds are stored as solids at −20 °C under an inert atmosphere, and solutions are made up just prior to use.

Fragmentation of DNA. The ability of the different Me-lex compounds to generate alkali labile sites in pBR322 plasmid DNA was measured by the combined loss of full-length Form-I and Form-II DNA as a function of Me-lex concentration (Figure 3). The disappearance of full-length plasmid measures the formation of single strand breaks that is related to the formation of heat labile N-alkylpurine lesions. The results show the following order for the induction of DNA damage: Me-lex$^{Py/Py}$ > Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$.

Methyl Adduct Quantitation. The yields of 7-MeGua, 3-MeGua, and 3-MeAde from the reaction of the three Me-lex compounds and MMS with calf thymus DNA were determined using HPLC with UV and EC detection (Table 2). EC detection was used for 7-MeGua because this peak elutes in the shoulder of unmodified Ade, which makes quantitation by UV problematic, especially at low levels of adduct. Methylation at the O^6-position of Gua was not measured in these experiments since it has been previously demonstrated that neither Me-lex (*10*) nor MMS (*19*) yields a significant amount of this adduct. The formation of the adducts follows a dose−response for all three Me-lex compounds and MMS.

Table 2: Adduct Levels from Reaction of Me-lex Compounds with Calf Thymus DNA[a]

compound	conc (μM)	netropsin (μM)	adduct level (μmol/mol DNA) 3-MeAde	3-MeGua	7-MeGua
Me-lex$^{Py/Py}$	250		8827 ± 489	105 ± 20	1094 ± 63[b]
	125		5365 ± 103	46 ± 4	521 ± 45[b]
	100		4913 ± 151	66 ± 4	385 ± 9[c]
	50		2771 ± 86	35 ± 10	170 ± 15[c]
	250	100	641 ± 18	31 ± 3	983 ± 37[b]
	100	100	108 ± 4	18	349 ± 12[c]
Me-lex$^{Im/Py}$	500		2994 ± 50	124 ± 26[d]	3143 ± 10[c]
	250		1362	65	1931[c]
	125		714	37	965[c]
	500	100	1944 ± 64	195 ± 22[d]	2656 ± 97[c]
Me-lex$^{Im/Im}$	500		997 ± 128	36 ± 1[e]	1986 ± 51[c]
	250		461 ± 8	19 ± 3	1067 ± 69[c]
	125		239 ± 5	9	630[c]
	500	100	1132 ± 18	71 ± 5[e]	1625 ± 51[c]
MMS	5000		716 ± 21	66 ± 5	4370 ± 233[c]
	500		91 ± 8	not detected[f]	592 ± 143[c]
	5000	100	473 ± 18	38 ± 7	4134 ± 220[c]
	500	100	71 ± 1	not detected	757 ± 15[c]

[a] DNA (2 mM) incubated with Me-lex or MMS for 24 h at room temperature in 10 mM sodium cacodylate buffer (pH 7.0) containing 10% DMSO. [b] UV detection. [c] Electrochemical detection. [d] Values are significantly different from each other ($p = 0.053$). [e] Values are significantly different from each other ($p = 0.008$). [f] Limit of detection is ≥7 μmol/mol DNA.

On the basis of an equimolar dose (500 μM), the total of all three lesions (3-MeAde, 3-MeGua, and 7-MeGua) normalized against MMS is 39:9:4:1 for Me-lex$^{Py/Py}$ > Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$ > MMS, respectively (Table 2). These results are consistent with the trend seen in the DNA degradation assay (Figure 3). The yield of the minor groove adducts (3-MeAde + 3-MeGua) follows a similar normalized order of 274:34:11:1 for Me-lex$^{Py/Py}$ > Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$ > MMS, respectively. Analysis for the formation of the major groove adduct gives a normalized ratio of 5:3:3:1, respectively, for Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$ ≅ Me-lex$^{Py/Py}$ > MMS.

Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$ were designed to equilibrium bind in the minor groove at sequences containing Gua-Cyt base pairs and to methylate the N3-position of Gua. The latter does not occur to any significant extent as all three Me-lex compounds and MMS afford similar low levels of the 3-MeGua lesion (Table 2). In addition, both Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$ yield significant amounts of 7-MeGua, and if the adduct levels are corrected for concentration, both are actually more efficient than MMS in methylating the major groove site. In fact, even Me-lex$^{Py/Py}$, which so effectively methylates Ade in the minor groove, generates more 7-MeGua than MMS after correction for concentration.

Netropsin and distamycin are competitive inhibitors of Me-lex$^{Py/Py}$ equilibrium binding (*8*, *11*), and the effect of the former well-characterized minor groove ligand on the methylation of DNA by the Me-lex compounds and MMS is reported in Table 2. Netropsin inhibits the formation of 3-MeAde and 3-MeGua from Me-lex$^{Py/Py}$. The level of 3-MeAde and 3-MeGua dropped to an average of 96 and

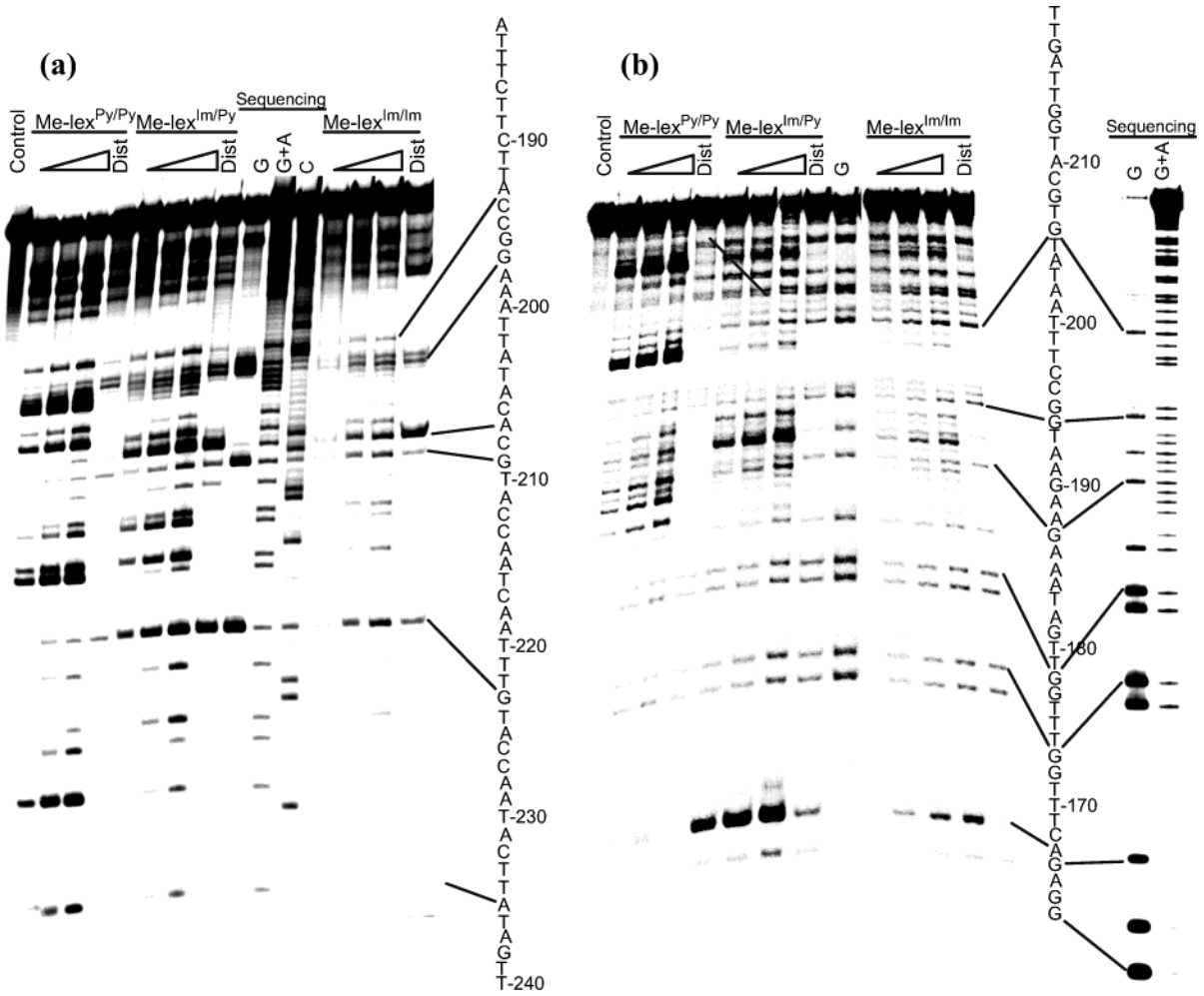

FIGURE 4: Dose response for the alkylation of DNA by Me-lex$^{Py/Py}$ (75, 150, 300 μM), Me-lex$^{Im/Py}$ (250, 500, and 1000 μM) and Me-lex$^{Im/Im}$ (250, 500, and 1000 μM) in Watson (a) and Crick (b) strands of 5′-end-labeled 85-bp restriction fragment. Concentration of distamycin, when indicated, is 100 μM in the presence of the highest concentration of each alkylating agent.

72%, respectively, of the levels measured in the absence of netropsin. However, netropsin did not change the yield of 7-MeGua. Netropsin caused a smaller reduction in 3-MeAde and 7-MeGua produced from Me-lex$^{Im/Py}$and actually increased the level of 3-MeGua by almost 60%. An increase (2-fold) in 3-MeGua was also observed upon incubating netropsin with Me-lex$^{Im/Im}$. Netropsin in combination with MMS causes a modest reduction in the minor groove lesions 3-MeAde (28%) and 3-MeGua (42%), with no change in 7-MeGua.

Sequence Specificity. To understand if the different Me-lex compounds alkylated DNA at sequences predicted by their structures, they were reacted with a 5′-[^{32}P]-labeled 85-bp (Figure 4) restriction fragment using the concentrations and incubation times shown in the legend. Sites of N-alkylpurine lesions were characterized by neutral thermal hydrolysis (pH 7.0 at 90 °C) to release the adducted purine base followed by treatment of the resulting apurinic sites with hot piperidine to generate strand breaks (*21*). The position of strand breaks were determined using denaturing polyacrylamide gel electrophoresis and Maxam−Gilbert G and G+A marker lanes (*21*). To distinguish between 3- and 7-alkylpurines, the neutral thermal hydrolysis step was omitted and the DNA was directly treated with piperidine (*21*). This treatment selectively converts 7-MeGua, but not 3-MeAde or 3-MeGua, into strand breaks. The gels were analyzed by phosphorimaging.

The intensities of the cleavage bands induced by all three Me-lex compounds are concentration (Figure 4) and time dependent (data not shown). Moreover, the bands represent the sequence selectivity of the agents since there is abundant uncleaved (i.e., unmodified) DNA at the top of each lane. The sequence specificity of Me-lex$^{Py/Py}$, which has been previously studied (*8*, *11*), shows a strong preference for Ade that are in Ade/Thy-rich sequences. Due to competitive inhibition for equilibrium binding sites, the co-addition of distamycin (Figure 4) or netropsin (data not shown) results in a strong inhibition of cleavage at Ade (*8*, *11*).

Me-lex$^{Im/Im}$ was designed to preferentially equilibrium bind to Gua-rich sequences due to the ability of the N-3 position of the imidazole to H-bond to the exocyclic N^2-amino of Gua (*17*, *18*, *22*, *23*). However, Me-lex$^{Im/Im}$ shows no sequence-specific alkylation at Gua: all Gua sites are methylated with only minor differences in intensities. On the basis of the HPLC analysis (Table 2), the formation of strand breaks at Gua is attributed to methylation in the major groove at the 7-position. Consistent with this interpretation is the qualitatively and quantitatively similar cleavage patterns at Gua in the sequencing gels using either direct piperidine treatment of the methylated DNA that generates

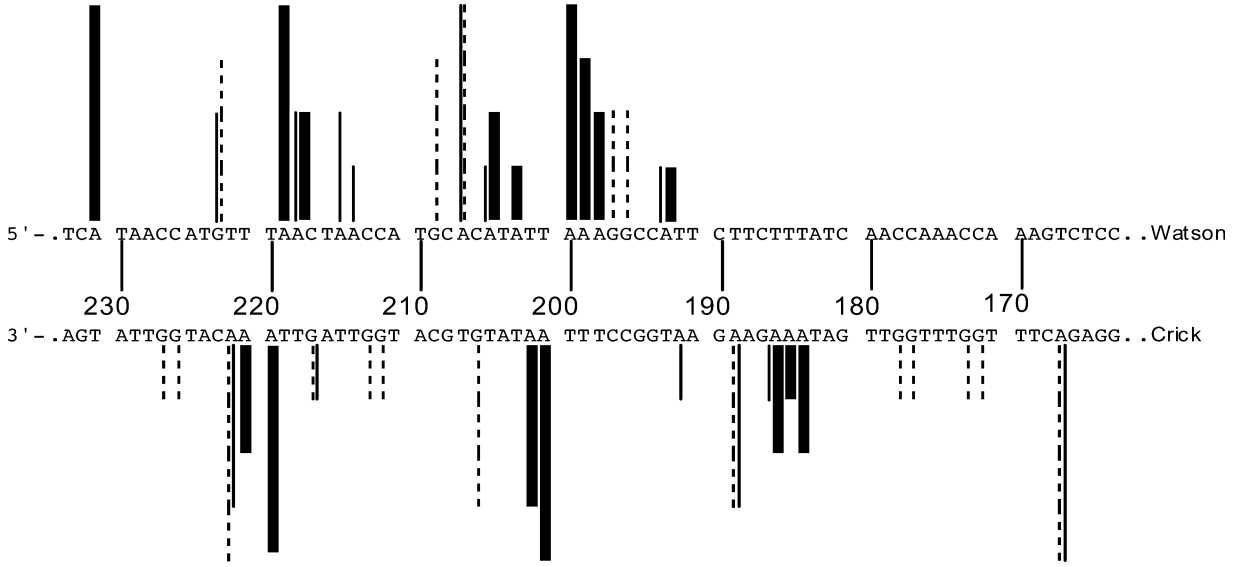

FIGURE 5: Location and intensity of methylation sites induced by Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and Me-lex$^{Im/Im}$ in Watson and Crick strands of 85-bp fragment: thick solid bars, 75 μM Me-lex$^{Py/Py}$; solid lines, 250 μM Me-lex$^{Im/Py}$; and dashed lines, 500 μM Me-lex$^{Im/Im}$. Line length indicates the relative strength of the cleavage bands for each individual compound. Because sites below Cyt^{190} on the Watson strand were not resolved on the gels, no information on alkylation is provided.

strand breaks at 7-MeGua (data not shown) or neutral thermal hydrolysis followed by piperidine, which cleaves all three adducts. Thus, all of the data are consistent with the bands at Gua being predominantly derived from nonspecific formation of 7-MeGua lesions. The same result for Gua cleavage sites is seen in lanes in which the DNA was reacted with Me-lex$^{Im/Py}$ (Figure 4). The only deviation is at Gua^{223} (Figure 5, Watson strand) which is hyperreactive with Me-lex$^{Im/Py}$ relative to the other Gua residues. Note that the intensities of the bands in Figure 5 are normalized for each compound and for each strand; therefore, direct quantutative comparisons between compounds cannot be made.

In addition to the sequence independent formation of 7-MeGua, Me-lex$^{Im/Im}$ shows a sequence selective methylation pattern at Ade with 5′-ACAT (Figure 5, Ade^{207} Watson strand) or 5′-AGAA (Figure 5, Ade^{189} Crick strand) being preferred cleavage sites. However, no bands are formed at Gua in 5′-GCAT or 5′-GGAA sequences, where the Ade that is a good target for methylation is replaced by a Gua. It appears that despite the equilibrium binding properties of the lex$^{Im/Im}$ peptide, Gua is particularly refractory to minor groove methylation. The preference for methylation of Ade adjacent to Gua/Cyt base pairs is consistent with the predicted equilibrium binding of the lex$^{Im/Im}$ dipeptide (*22*, *23*). Moreover, the Ade cleavage sites formed from Me-lex$^{Im/Im}$ are distinct from Me-lex$^{Py/Py}$ but overlap with Me-lex$^{Im/Py}$ (Figure 5). The co-addition of distamycin weakens the intensities of some Ade and Gua bands suggesting that only a subset of lex$^{Im/Im}$ binding sites coincide with the Ade/Thy sequences preferred by distamycin. These qualitative results are consistent with the quantitative HPLC analysis.

The 3-MeAde pattern generated by Me-lex$^{Im/Py}$ (Figure 5), which is distinct from Me-lex$^{Py/Py}$ frequently overlaps with Me-lex$^{Im/Im}$, and many, but not all, Ade that are methylated are adjacent to Gua/Cyt base pairs. In addition, the co-addition of distamycin does not universally inhibit methylation at Ade sites: the Ade bands that remain intense are flanked by Gua/Cyt base pairs and would not be predicted to be preferred distamycin binding sites. For this hybrid compound, affinity binding places the methyl group at mixed sequences near N3-Ade and N3-Gua positions, but only the former are methylated.

Toxicity. The effects of Me-lex$^{Py/Py}$, Me-lex$^{Im/Im}$, Me-lex$^{Im/Py}$, and MMS on the growth of wild type *E. coli* and base excision repair mutants were determined to relate in vitro adduct formation with in vivo toxicity (Figure 6). The *alkA/tag* mutant cannot remove 3-MeAde from the genome, while the *alkA* mutant retains the Tag protein that efficiently and selectively removes 3-MeAde from DNA (*2*, *25*). The *apn* mutant cannot process the abasic sites that arise by the initial glycosylase removal of the *N*-methylpurine lesions.

The Me-lex compounds were incubated with the bacteria as a 10% solution in 95% EtOH, with no observable toxicity due to the solvent alone. In previous studies, we demonstrated that the dipeptide moiety is not responsible for the toxicity of Me-lex$^{Py/Py}$ (*9*). In fact, concentrations as high as 1 mM of an ethyl analogue of Me-lex$^{Py/Py}$ (i.e., ethyl-lex$^{Py/Py}$), which is a very poor alkylating agent, is not toxic in *E. coli* (data not shown). This implicates DNA methylation as the critical factor in toxicity of the Me-lex compounds.

The strain most sensitive to the methylating agents is the *alkA/tag* mutant, and the relative toxicity of the methylating agents follows the order: Me-lex$^{Py/Py}$ > Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$ > MMS (Figure 6). The same qualitative pattern of toxicity with the different methylating agents is seen in less sensitive bacteria strains with the sensitivity of the different repair backgrounds being: *alkA/tag* $\gg$ *apn* $\gg$ *alkA* $\cong$ (or >) wt. One significant difference between the strains is observed in the sensitivity of the *alkA* mutant to the higher concentrations of Me-lex$^{Im/Py}$ and MMS. For Me-lex$^{Py/Py}$, there is no difference between wild type and the *alkA* strains at the concentrations studied. The solubility of Me-lex$^{Im/Im}$ limited the concentrations that could be tested.

DISCUSSION

Among the numerous adducts formed from the reaction of alkylating agents with DNA, 3-MeGua remains one of

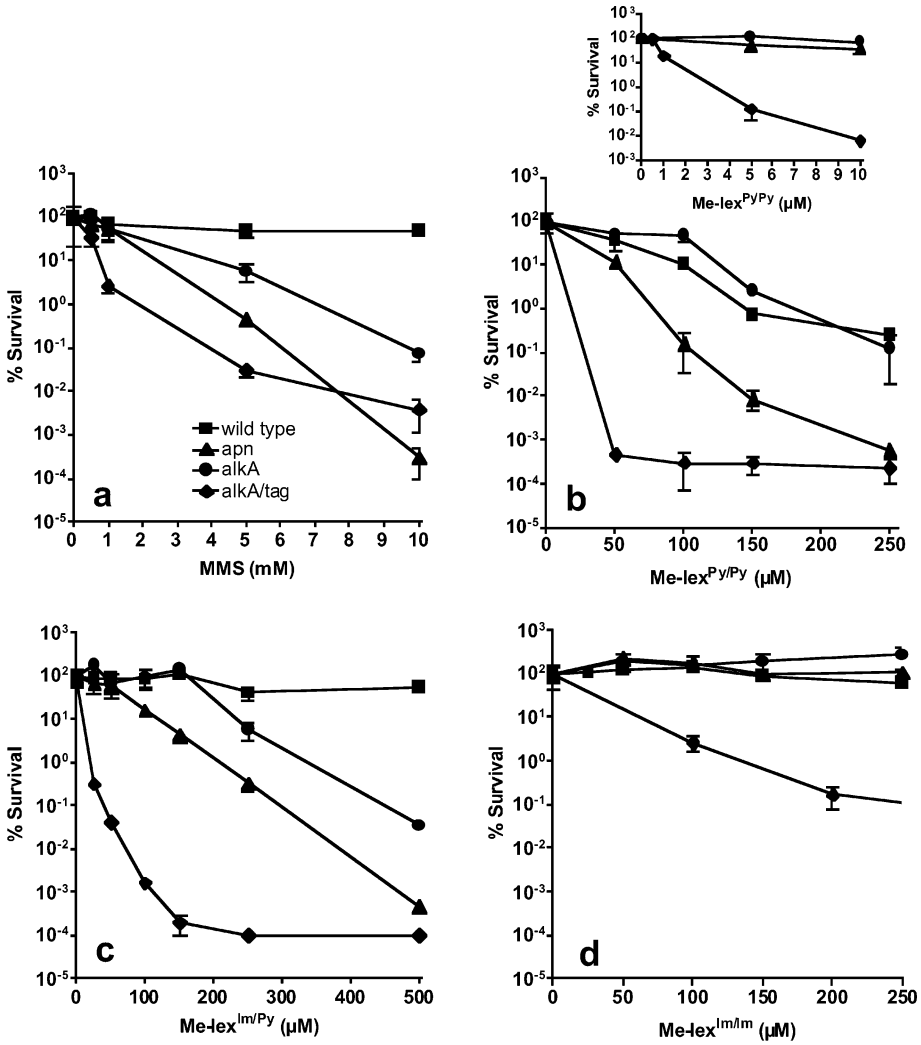

FIGURE 6: Toxicity of (a) MMS, (b) Me-lex$^{Py/Py}$ with insert for toxicity at low concentrations, (c) Me-lex$^{Im/Py}$, and (d) Me-lex$^{Im/Im}$ in wild type *E. coli* and repair mutants: ■, wild type; ◆, *alkA/tag* mutant; ▲, *apn* mutant; ●, *alkA* mutant.

the lesions whose biological properties are poorly understood. The in vivo $t_{1/2}$ of 3-MeGua in DNA is 3.6 ± 0.3 h in *E. coli*, as compared to an in vitro $t_{1/2}$ of 105 h in double-strand DNA (*26*). In contrast to 3-MeGua, the in vitro $t_{1/2}$ of 3-MeAde in double-strand DNA is 24 h (*27*). 3-MeGua is formed in low abundance in DNA treated with alkylating agents: the yield of 3-MeGua is normally < 0.01% of 7-MeGua from diffusible methylating agents (*19*). The calculated charge densities on N3-Gua and N3-Ade are equivalent, while the 3-Gua site is the least accessible site in B-DNA (*28*). Therefore, steric hindrance is probably the main contributor to the low yields of 3-MeGua from most methylating agents.

On the basis of previous studies, Me-lex$^{Py/Py}$ almost exclusively affords thermally labile lesions at Ade that are in, or immediately adjacent to, the dipeptide's minor groove equilibrium binding sites (*8*, *10*, *11*). Because the alkylation by Me-lex$^{Py/Py}$ is significantly reduced by the co-addition of the minor groove binders, distamycin and netropsin, the alkylation process involves an initial association of the lex$^{Py/Py}$ moiety with its minor groove binding site followed by transfer of the alkyl group from the sulfonate ester to accessible nucleophilic atoms in the minor groove. The reason that lex$^{Py/Py}$ does not bind to G:C sequences is a consequence of a steric interaction between the exocyclic N^2-amino group of Gua that protrudes into the minor groove and sterically blocks binding of the pyrrole subunit (*29*, *30*). Me-lex$^{Im/Im}$ and Me-lex$^{Im/Py}$, because of the incorporation of imidazole subunit(s), were designed to position the methylating functionality at G/C rich sequences (*17*, *18*) and, thereby enhance the production of 3-MeGua. However, a similar absolute yield of 3-MeGua was observed for all four methylating agents. The low yield of 3-MeGua, even when a Gua is immediately adjacent to a binding site of lex$^{Im/Py}$ and lex$^{Im/Im}$ indicates that the steric problem cannot be readily overcome by an equilibrium binding delivery strategy. The yield of 3-MeGua produced from Me-lex$^{Im/Py}$and Me-lex$^{Im/Im}$ increased in the presence of netropsin, which significantly decreases the level of this same lesion with Me-lex$^{Py/Py}$ and MMS. We suggest that the occupation of the minor groove at Ade:Thy-rich regions causes an increased concentration of Me-lex$^{Im/Py}$and Me-lex$^{Im/Im}$ at less favored sites that contain Gua, and that this increase results in a modest ≤2-fold increase in 3-MeGua.

The results show that Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$ afford 3-MeAde in a sequence-selective manner that is distinct from Me-lex$^{Py/Py}$. The main difference is that Ade residues adjacent to Gua or Cyt are more reactive targets for methylation by Me-lex$^{Im/Im}$ and Me-lex$^{Im/Py}$. For example, Ade^{218} in the 85-bp fragment Watson strand (Figure 5) is a preferred site for

Me-lex$^{Im/Py}$, while Ade^{219} is targeted by Me-lex$^{Py/Py}$. Ade^{218} is in an Ade:Thy-rich region that is adjacent to a 3′-C:G base pair. This is consistent with the lex$^{Im/Py}$ peptide binding toward the 3′-side of the Ade/Thy-rich run near Ade^{219}. Similarly, Ade^{207} which is flanked on both sides with a C:G base pair is a strong site for methylation by Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$ but is barely methylated by Me-lex$^{Py/Py}$. In general, the sequence selectivity at Ade is consistent with the lex$^{Im/Py}$ and lex $^{Im/Im}$ peptides recognizing their targeted G:C/A:T sequences, but as discussed above, the inaccessibility of the 3-Gua position remains a barrier to the transfer of the alkyl group.

In contrast to the potent inhibition of Me-lex$^{Py/Py}$ methylation of DNA by distamycin, which is a competitive inhibitor for the lex$^{Py/Py}$ affinity binding sites, the alkylation by Me-lex$^{Im/Py}$ is only partially blocked. This is reasonable since the distamycin and lex$^{Im/Py}$ peptides have different binding preferences. A clear example of this is shown in Figure 4a where Ade^{207} that is flanked by Cyt's in the sequence 5′-ATGC(A^{207})CATAT is a strong methylation site by Me-lex$^{Im/Py}$(but not for Me-lex$^{Py/Py}$) and methylation is not inhibited by distamycin.

The formation of 7-MeGua (Table 2) in a nonsequence selective pattern (Figure 4) from the different Me-lex compounds is of interest. For Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$, the yields are quite high compared to equimolar concentrations of MMS. This is attributed to the Me-lex molecules acting as nonspecific methylating agents, similar to MMS and DMS that predominantly react at 7-Gua (*19*). The increased potency of the Me-lex$^{Im/Py}$ and Me-lex$^{Im/Im}$ compounds may be due to nonspecific hydrophobic interactions of the less polar peptides and the groove of DNA. The origin of the overall alkylating activities of the three Me-lex compounds (Me-lex$^{Py/Py}$ > Me-lex$^{Im/Py}$ > Me-lex$^{Im/Im}$) can be attributed to a combination of the sequence preferences (A/T vs G/C) and binding affinities of the lex peptides since the introduction of imidazole for pyrrole markedly reduces ligand binding constants as well as sequence specificity (*22*, *31*, *32*).

Toxicity. In *E. coli* there are two glycosylases (Tag and AlkA) that excise 3-MeAde lesions as part of the BER system. The Tag protein is highly selective for 3-MeAde and is constitutively expressed (*24*, *25*), while AlkA, which has a broader substrate specificity (*33*, *34*), is constitutively expressed at low levels, but can be induced approximately 20-fold (*35*, *36*) as part of the adaptive response pathway. To complete the repair process, the abasic site created by the glycosylase must be sequentially processed by AP endonuclease, dRP lyase, DNA polymerase, and DNA ligase (*37*, *38*). Accordingly, mutant bacteria that cannot perform one or more of these BER steps are sensitive to the toxicity caused by methylation of DNA. In previous studies comparing the survival of bacteria exposed to Me-lex$^{Py/Py}$, it was demonstrated that an *alkA*/*tag* mutant is considerably more sensitive relative to wild-type bacteria (*9*, *13*). The results in BER deficient bacteria are consistent with the enhanced toxicity of Me-lex$^{Py/Py}$ in null *Aag*$^{-/-}$ mouse ES cells that cannot hydrolyze 3-MeAde from DNA (*14*).

The toxicities of Me-lex$^{Im/Py}$and Me-lex$^{Im/Im}$ in *E. coli* are significantly lower than Me-lex$^{Py/Py}$. The relationship between 7-MeGua, 3-MeAde, and 3-MeGua levels formed in vitro, and the concentration of methylating agent required to reduce survival in the BER deficient cells (*alkA/tag*) to 1% (LD$_{99}$) is plotted in Figure 7. We have chosen 1% survival because this value is within the dose−response curve for all the compounds. Using another level of toxicity would not change the conclusions. The results indicate that there is a strong correlation between 3-MeAde levels and toxicity for the Me-lex analogues and MMS. As expected, there is no correlation between 7-MeGua and toxicity since this major groove lesion is considered benign, persists in cells over several generations, and is not a good substrate for BER glycosylases (*5*, *26*, *34*, *39*).

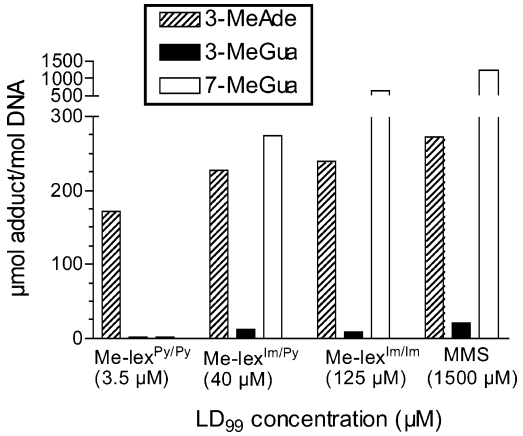

FIGURE 7: Relationship between 3-MeAde, 3-MeGua, and 7-MeGua adduct levels formed at concentrations of Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, Me-lex$^{Im/Im}$, and MMS required for 1% cell survival in *alkA/tag* mutant *E. coli*.

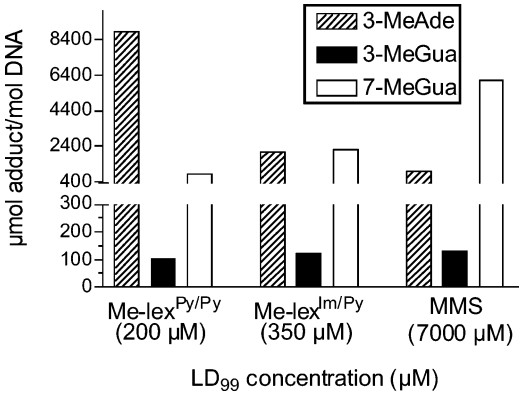

FIGURE 8: Relationship between 3-MeAde, 3-MeGua, and 7-MeGua adduct levels at concentrations of Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and MMS required for 1% cell survival in *alkA* mutant *E. coli*.

Toxicity of 3-MeGua. The only suggestion that a lesion other than 3-MeAde may contribute to the toxicity of the methylating agents is the enhanced sensitivity of the *alkA* mutant to higher doses of MMS (>5 mM) and Me-lex$^{Im/Py}$ (>250 μM). This effect has been previously noted (*9*) and suggests that an adduct not efficiently repaired by the Tag protein, but which is a substrate for AlkA, is produced by these two compounds. The observation that the toxicity is only seen at high doses is also consistent with the need to form sufficient amounts of a normally minor lesion. 3-MeGua meets these criteria. The relationship between the concentration of Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and MMS required to reduce *alkA*mutant survival to 1% (LD$_{99}$) was extrapolated from the toxicity data (Figure 6) and is shown in Figure 8. Me-lex$^{Im/Im}$ was not included in the analysis since its solubility limited using the high concentrations required to see toxicity in the

alkA mutant. The LD_{99} in the *alkA* mutant were estimated to be 200, 350, and 7000 μM, respectively, for Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and MMS. The relative concentrations are very different from those calculated in the *alkA/tag* mutant (Figure 7), a result consistent with different adducts being responsible for toxicity in the two mutants. The relative amounts of 3-MeAde, 3-MeGua, and 7-MeGua generated at these concentrations (Figure 8) were then estimated based on the in vitro data in Table 2. A previous comparison between in vitro and in vivo methylation patterns in DNA by DMS and *N*-methyl-*N*-nitrosourea showed a strong correspondence (*19*). There is clearly no relationship between the amount of 3-MeAde and toxicity, which is understandable since 3-MeAde is rapidly and efficiently repaired by the constitutive Tag protein (*24*, *25*). There is also no relationship between 7-MeGua lesions and toxicity, which was anticipated for the reasons mentioned above. However, there is a strong correlation between 3-MeGua and toxicity in the *alkA* mutant which cannot efficiently remove this lesion since Tag releases 3-MeGua approximately 70-fold slower than AlkA (*40*). The average in vitro level of 3-MeGua from the three compounds at concentrations that reduce survival to 1% in the *alkA/tag* cells is 80 $\pm$ 9 μmol/mol DNA. If 3-MeGua is the unidentified lesion responsible for the toxicity of high concentrations of MMS and the Me-lex compounds in the Tag proficient background, then it would be expected that the levels of 3-MeGua in the *alkA* mutant would be in the same range as the levels of 3-MeAde in the *tag/alkA* double mutant. This is based on the assumption that both lesions are toxic due to their interference with DNA polymerases (*7*). From the in vitro data, the calculated average amount of 3-MeAde to induce the same level of toxicity from Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$ and MMS in the *alkA/tag* double mutant is 209 $\pm$ 43 μmol/mol DNA. Considering the estimations made, the correspondence of 3-MeGua to toxicity is reasonable and supports the proposal that 3-MeGua is the unidentified lesion that is a substrate for the AlkA protein (*7*, *9*, *41*).

The sensitivities of the different repair defective bacteria to Me-lex$^{Py/Py}$, Me-lex$^{Im/Py}$, and Me-lex$^{Im/Im}$ are qualitatively similar: cells that cannot remove the 3-MeAde lesion show the most toxicity when exposed to the compounds. Since the different Me-lex compounds and MMS all have different sequence selectivities, the site of methylation, at least in *E. coli*, has no impact on the mechanism of toxicity.

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