Evidence in *Escherichia coli* that N3-Methyladenine Lesions and Cytotoxicity Induced by a Minor Groove Binding Methyl Sulfonate Ester Can Be Modulated *in Vivo* by Netropsin†

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ABSTRACT: The use of DNA equilibrium binding molecules to transfer alkyl groups to specific positions on DNA is an approach to generating cytotoxic DNA damage while avoiding the formation of promutagenic lesions that increase the risk for the development of secondary cancer. We have previously reported that *in vitro* a neutral DNA equilibrium binding agent based on an *N*-methylpyrrolecarboxamide dipeptide (lex) and modified with an *O*-methyl sulfonate ester functionality (Me-lex) selectively affords N3-methyladenine lesions in >90% yield relative to the formation of other adducts. While *in vitro* interactions between the lex dipeptide and DNA have been thoroughly studied, *in vivo* interactions are more difficult to elucidate. We report herein the relationship between the *in vivo* formation of N3-methyladenine and toxicity in wild-type and base excision repair defective mutant *Escherichia coli*. In addition, it is demonstrated that both N3-methyladenine adduction and cytotoxicity can be inhibited *in vivo* with netropsin, a potent competitive inhibitor of binding of lex to DNA. The results show a clear relationship between the levels of N3-methyladenine and toxicity in an *alkA/tag* glycosylase mutant that cannot remove the adduct from its genome. For methyl methanesulfonate, which does not sequence selectively methylate DNA, a relationship between the formation of N3-methyladenine and toxicity is also observed. However, netropsin affects neither the level of N3-methyladenine nor the toxicity of methyl methanesulfonate in *E. coli*.

Damaging DNA is a critical event in both the carcinogenicity and cytotoxicity induced by carcinogens and many clinically used antineoplastic agents (*1*, *2*). In some cases, the two biological effects are coupled because specific types of DNA damage are both mutagenic and cytotoxic. An example of this is O^6-alkylguanine, which can miscode for thymine when present in the template strand during DNA replication (*3*−*6*) and induce apoptosis through a process that requires mismatch repair (*7*−*10*). Drugs that yield O^6-alkylguanine DNA lesions, e.g., streptozotocin, temozolamide, cyclophosphamide, *N*,*N*-bis(2-chloroethyl)nitrosourea, and *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea, are used in the treatment of cancer (*2*, *11*). A serious side effect of such treatment is the future risk of developing therapy-related cancers (*12*, *13*). Since DNA damage-induced cell death remains an attractive strategy against rapidly growing tumor cells, we have embarked on an effort to design molecules that form DNA adducts that are cytotoxic but not mutagenic. The lesion on which we have focused is N3-methyladenine (3-MeA),[1] which seems to fit these criteria (*14*−*20*). To generate this lesion, a minor groove DNA equilibrium binding *N*-methylpyrrolecarboxamide-based dipeptide (lex) has been modified with a traditional alkylating agent, i.e., methyl sulfonate ester (*21*). The compound, (1-methyl-4-{1-methyl-4-[3-(methoxysulfonyl)propanamido]pyrrole-2-carboxamido}pyrrole-2-carboxamido)propane (Me-lex, Figure 1), yields 3-MeA in >90% yield based on the total amount of adducts that is formed (*18*, *21*, *22*). Because of the delivery of the alkylating agent by the dipeptide, the covalent modification of DNA is highly sequence dependent and can be inhibited *in vitro* by distamycin and netropsin due to competition with Me-lex for the same minor groove

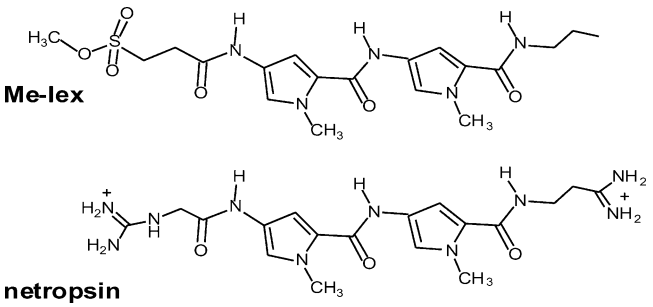

FIGURE 1: Structures of Me-lex and netropsin.

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[1] Abbreviations: AlkA, *E. coli* N3-methyladenine DNA glycosylase II; lex, lexitropsin (*N*-methylpyrrolecarboxamide-based information reading peptide); Me-lex, (1-methyl-4-{1-methyl-4-[3-(methoxysulfonyl)propanamido]pyrrole-2-carboxamido}pyrrole-2-carboxamido)propane; 3-MeA, N3-methyladenine; 3-MeG, N3-methylguanine; 7-MeG, N7-methylguanine; MMS, methyl methanesulfonate; Tag, *E. coli* 3-methyladenine DNA glycosylase I; WT, wild-type.

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equilibrium binding sites (*18*, *21*). In the work reported herein, we demonstrate that Me-lex-induced formation of 3-MeA in wild-type and base excision repair (*alkA/tag*) mutant *Escherichia coli* can be efficiently inhibited by the preincubation of the cells with netropsin. Concomitant with the reduction in the number of adducts is a marked reduction in the toxicity of Me-lex. A similar effect of netropsin on adduct level and toxicity is not seen with methyl methanesulfonate (MMS).

EXPERIMENTAL PROCEDURES

Hazardous Procedures. Me-lex and MMS should be considered toxic and/or carcinogenic, and should be handled accordingly.

Materials. All chemicals were purchased from Aldrich Chemicals (Milwaukee, WI) or Sigma Chemicals (St. Louis, MO), unless stated otherwise. Me-lex was prepared as previously described (*21*) and stored as a dry solid under an N_2 atmosphere at −80 °C until it was used. Solutions of Me-lex, MMS, and netropsin were made immediately before use, and any unused material was discarded. The 3-MeA-OV albumin coating antigen was prepared as previously described (*24*−26). The antibody against 3-MeA was a gift from D. Shuker (The Open University, London, U.K.). *E. coli* strains were provided by T. O'Connor (City of Hope National Medical Center, Duarte, CA) and L. Samson (Massachusetts Institute of Technology, Cambridge, MA).

Toxicity Studies. E. coli cultures of the wild type (AB1175) and *alkA/tag* mutant (GC4803) were started from the frozen stocks and grown overnight in LB medium with (when indicated) the appropriate antibiotic. An aliquot of the overnight culture was transferred to fresh LB medium 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 medium with phosphate-buffered saline and pelleted by centrifugation. The pellets were then resuspended in Tris-EDTA buffer (pH 7.5) and preincubated with netropsin (25 μM) in 10 μL of DMSO (or just DMSO solvent) in a shaking incubator (200 rpm) at 37 °C for 0.5 h followed by incubation with Me-lex or MMS in 1.0 mL of 95% EtOH for 1 h. The controls that were included were incubations with (i) solvent(s) containing neither the alkylating agent nor netropsin and (ii) solvent containing netropsin but no alkylating agent. After the incubation period, the bacteria were pelleted by centrifugation, washed free of the agent using phosphate buffer, and resuspended in 1 mL of saline. Serial dilutions were made and bacteria 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, triplicate.

In Vivo Adduct Studies. Aliquots of the two *E. coli* strains containing $\sim 3 \times 10^8$ cells of log phase cultures were washed free of LB medium with phosphate buffer and pelleted by centrifugation. The pellet was suspended in 44.5 mL of 10 mM Tris-EDTA buffer (pH 7.5) and preincubated with netropsin (25 μM) dissolved in 500 μL of DMSO or just 500 μL of DMSO solvent (control) at 37 °C for 0.5 h followed by incubation with Me-lex or MMS in 5.0 mL of 95% EtOH for 1 h. The controls that were included were similar to those described for the toxicity studies. After the incubation period, the bacteria were pelleted by centrifugation, washed free of compounds using phosphate buffer, and resuspended in 350 μL of water. To the bacterial pellet suspended in water was added 320 μL of 10% sucrose in 50 mM Tris-HCl (pH 8) followed by addition of 80 μL of freshly prepared lysozyme (10 mg/mL) in 10 mM Tris-HCl (pH 8) and 100 μL of EDTA (10 mM, pH 8). The contents of the tube were mixed and placed on ice for 30 min. Then 10 μL of 10% SDS was added followed by 100 μL of 1 M NaCl. The contents of the tube were shaken and incubated at room temperature for 30 min, and then 10 μL of RNase (10 mg/mL) was added and the mixture incubated for 3 h at room temperature with shaking. After the RNase treatment, 10 μL of proteinase K (20 mg/mL) was added and incubated overnight with shaking. The DNA was purified using phenol/chloroform extraction, and the isolated DNA was dissolved in 1 mL of 10 mM sodium cacodylate buffer (pH 7.0) containing 1 mM EDTA by shaking for 72 h at 4 °C.

A 100 μL aliquot of the DNA solution was used to quantitate the DNA (in duplicate) by the Burton assay (*27*). *N*-Methylpurine adducts were released from DNA by heating at 70 °C for 8 h at pH 7 (*28*). The polymeric DNA was precipitated using 90 μL of cold 1 N HCl, and the supernatant containing the *N*-methylpurines was removed and concentrated *in vacuo*. The *N*-methylpurines in the supernatant were separated on an HPLC system equipped with a UV detector [YMC ODS-18 AQ column (4.6 mm × 250 mm), mobile phase of 82% water, 8% MeOH, and 10% NH_4OAc (0.1 M, pH 4)]. The fractions containing the DNA adducts (3-MeA and 7-MeG) were collected, dried in a vacuum centrifuge, and dissolved in water.

The ELISA quantification of 3-MeA was performed at room temperature using a 96-well plate (Nunc-Immunoplate Maxisorp F96, Fischer) format so that each sample or standard was distributed in three wells. Wells on the outer edge of the plate were not used. The plates were coated at room temperature overnight with 750 ng of 3-MeA-OV coating antigen in 100 μL of coupling buffer [15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.0)]. The plates were washed twice (200 μL each) with ELISA buffer [0.9% saline, 10 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, and 0.1% dried milk (w/v)] and blocked for 1 h with an additional 200 μL of ELISA buffer to saturate unbound sites in the wells. The plates were washed with ELISA buffer, and 100 μL of primary antibody (anti-3-MeA antibody, final dilution of 1:120000 in ELISA buffer) was added to each well and the mixture incubated for 1 h. In the competitive ELISA used to quantitate 3-MeA, samples and authentic standards were preincubated with the primary antibody for 30 min prior to addition of the mixture of the antibody and inhibitors to the wells. The plates were washed (5 × 150 μL) with TBS-Tween [10 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, and 0.9% saline/0.05% Tween 20 (v/v)], and 100 μL of secondary antibody (horseradish peroxidase-conjugated goat anti-mouse antibody, diluted 1:500 in ELISA buffer) was added to each well and the mixture incubated for 1 h. The plates were again washed with TBS-Tween (5 × 150 μL), and 100 μL of peroxidase substrate [100 μL of 15 mg/mL ABTS and 3.5 μL of 30% hydrogen peroxide in 10 mL of citric acid buffer (pH 4.0)] was added to each well. The color that developed was spectrophotometrically quantitated at 405 nm using an

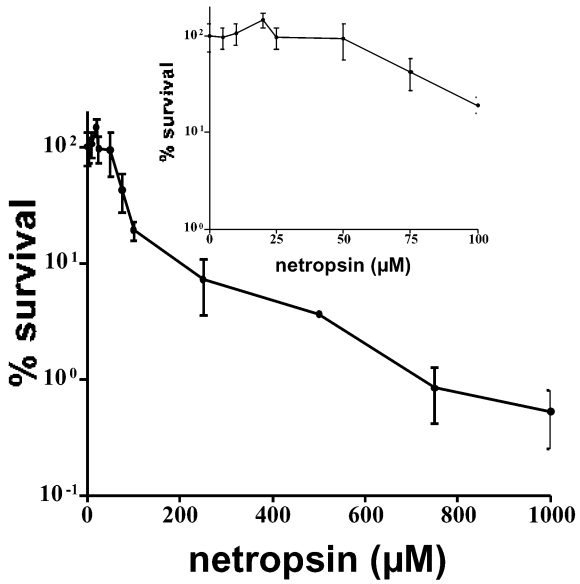

FIGURE 2: Toxicity of netropsin in *alkA/tag E. coli*. The inset shows the response at low netropsin concentrations.

$E_L X \times 808$ Ultra microplate reader (Bio-Tek Instruments, Inc.). For each plate, a standard inhibition curve was generated. Inhibition values obtained from the samples containing unknown amounts of 3-MeA were fitted to the standard curve for adduct quantitation using Prism (GraphPad Software, San Diego, CA).

The 7-MeG was analyzed with a reverse phase HPLC system (4.6 mm × 250 mm YMC C18 column, flow rate of 1 mL/min, mobile phase of 0.1 M NaOAc and 4% MeOH at pH 5.0 and 40 °C) coupled with an ESA electrochemical detector (guard cell at 850 mV and analytical cell E_1 at 500 mV and E_2 at 800 mV).

In Vitro Adduct Studies. Calf thymus DNA (200 μM) in Tris-EDTA buffer (pH 7.5) was preincubated with 150 μM netropsin dissolved in 500 μL of DMSO or just 500 μL of DMSO solvent in a shaking incubator (200 rpm) at 37 °C for 0.5 h followed by incubation with Me-lex or MMS in 95% EtOH for 1 h. After the treatment, the DNA was precipitated, washed, and dissolved in 1 mL of 10 mM sodium cacodylate buffer (pH 7.0) containing 1 mM EDTA. An aliquot was used to quantitate DNA by UV, and the remaining solution was heated at 90 °C for 15 min to selectively release *N*-alkylpurines from DNA. The solution was then treated with ice-cold 0.1 N HCl to precipitate the DNA and the supernatant collected and analyzed for 3-MeA, 3-MeG, and 7-MeG using reverse phase HPLC with detection at 270 nm.

RESULTS

Toxicity of Netropsin. To determine if the minor groove ligand netropsin, which effectively blocks methylation of DNA by Me-lex *in vitro*, could be used to selectively inhibit both the *in vivo* toxicity and DNA damage induced by Me-lex, a dose−response experiment was performed for a 1.5 h incubation with netropsin in the absence of any alkylating agent (Figure 2). Netropsin, which is a dipeptide with two cationic termini, shows a dose response in *alkA/tag* cells. On the basis of the dose−response curve, survival was not affected at concentrations of ≤75 μM. Since 25 μM netropsin is well below the toxic threshold in both WT and *alkA/tag* mutant cells, this dose was used in the studies involving Me-lex and MMS.

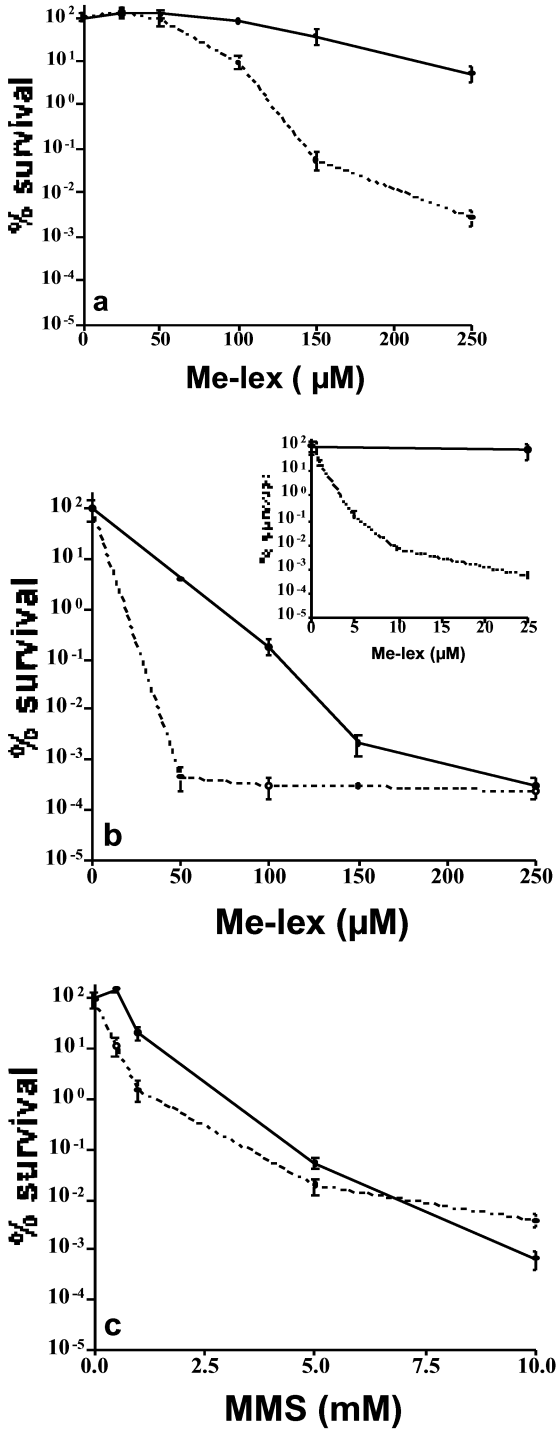

FIGURE 3: Toxicity of Me-lex and MMS in *E. coli* in the absence (○, dashed line) or presence of 25 μM netropsin (●, solid line): (a) Me-lex in WT *E. coli*, (b) Me-lex in the *alkA/tag* mutant (the inset shows the response at low Me-lex concentrations), and (c) MMS in the *alkA/tag* mutant.

Effect of Netropsin on the Toxicity of Me-lex. As previously reported, the toxicity of Me-lex in wild-type (Figure 3a) and *alkA/tag* mutant (Figure 3b) *E. coli* is dose-dependent and plateaus ($\leq 10^{-3}$% survival) near 25 μM in the double mutant, and 250 μM in wild-type cells (*23*, *29*). A similar qualitative pattern is observed with MMS in the *alkA/tag* cells, except

Table 1: *In Vivo* Adduct Levels (micromoles per mole of DNA) Produced by Me-lex and MMS in Wild-Type and *alkA/tag* Mutant *E. coli*[a]

compound	dose (μM)	netropsin[b] (25 μM)	3-MeA	
			wild type	*alkA/tag*
Me-lex	5	−	nd[c]	4.88 ± 1.36
	5	+	nd[c]	nd[c]
	10	−	nd[c]	11.30 ± 0.76
	10	+	nd[c]	nd[c]
	25	−	nd[c]	29.36 ± 10.87
	25	+	nd[c]	nd[c]
	50	−	nd[c]	54.90 ± 16.50
	50	+	nd[c]	4.05 ± 1.44
	100	−	51.37 ± 8.22	136.77 ± 15.01
	100	+	nd[c]	1.69 ± 0.31
	150	−	81.08 ± 8.41	171.14 ± 17.56
	150	+	nd[c]	2.43 ± 0.13
	250	−	122.10 ± 9.68	nd[c]
	250	+	nd[c]	nd[c]
MMS	5000	−	nd[c]	1.28 ± 0.18
	5000	+	nd[c]	3.48 ± 2.09
	10000	−	−	3.30 ± 0.42
	10000	+	−	5.35 ± 1.88

[a] The bacteria (3×10^8) were treated with Me-lex for 1 h. [b] Netropsin or solvent (control) was added to the bacteria for 30 min prior to the addition of Me-lex. [c] Not determined.

much higher doses are required to achieve the same level of toxicity (Figure 3c). Because MMS causes no toxicity at concentrations as high as 10 mM in wild-type *E. coli*, no studies were done with MMS in this strain (*23*, *29*).

The effect of 25 μM netropsin on the toxicity of Me-lex is shown in Figure 3. In wild-type bacteria, netropsin can quantitatively inhibit the toxicity induced by concentrations as high as 250 μM Me-lex (Figure 3a). This corresponds to almost 4-log protection. A similar effect is seen in the *alkA/tag* double mutant where netropsin affords more than 5-log protection at 25 μM Me-lex and 4-log protection at 50 μM Me-lex (Figure 3b). At the higher doses of Me-lex, the protection conferred by netropsin decreases to where survival is down to $<10^{-3}$% at 250 μM Me-lex in the presence (or absence) of netropsin. In contrast, no inhibition of toxicity by netropsin is seen with MMS in the *alkA/tag* cells (Figure 3c). The dose of MMS needed to reduce cell survival to 0.1% is at least 600-fold higher than for Me-lex in the *alkA/tag* mutant.

Effect of Netropsin on in Vivo DNA Methylation by Me-lex. To determine if there is a relationship between the toxicity of Me-lex in the two strains and the levels of DNA damage, the bacteria (3×10^8 log phase cells) were treated under the same conditions used in the toxicity studies. Similar studies were also carried out in the presence of netropsin. The genomic DNA was isolated after a 1 h exposure of the cells to the methylating agent, and levels of 3-MeA and N7-methylguanine (7-MeG) were determined by HPLC separation using ELISA (3-MeA) and electrochemical (7-MeG) quantification. There is a dose response for the formation of 3-MeA from Me-lex in both WT and *alkA/tag* strains in the absence of netropsin (Table 1). The average decrease in adduct levels observed in repair competent WT relative to *alkA/tag* cells is 2.4-fold at the 1 h time point with the different concentrations of Me-lex (Table 1). However, a comparison of cell survival against Me-lex concentration indicates that it requires approximately 30-fold more Me-lex to obtain the same 0.1% survival in WT versus glycosylase mutant cells. This produces by calculation an initial requirement of 35-fold more 3-MeA based on the adduct levels formed in the *alkA/tag* cells in the absence of any repair. No effort was made to quantify the levels of 7-MeG produced by Me-lex in the bacteria since previous studies indicated that it would be well below our level of detection (10 μmol of 7-MeG/mol of DNA) at the concentrations of Me-lex that were used (*18*, *22*).

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

[Me-lex] (μM)	netropsin[b] (150 μM)	adduct level (μmol/mol of DNA)		
		3-MeA	3-MeG	7-MeG
300	−	5657 ± 1521	100 ± 33	437 ± 84
300	+	60 ± 14	14 ± 4.4	483 ± 272
900	−	7232 ± 1356	179 ± 77[c]	769 ± 183
900	+	1554 ± 346	145 ± 38[c]	3721 ± 651

[a] DNA (200 μM) incubated with Me-lex for 1 h at 37 °C. [b] Netropsin or solvent (control) was added to the DNA for 30 min prior to the addition of Me-lex. [c] Not statistically different from each other.

Adduct levels were determined in *alkA/tag* mutant cells at 5 and 10 mM MMS. The yield of 3-MeA in the *alkA/tag* mutant is low, but appears to be dose-related as is the cytotoxicity (Table 1). The level of 7-MeG could be quantified at the highest concentration, but the adduct was below detection ($\leq$10 μmol/mol of DNA) at a concentration of 5 mM. Adduct levels were not determined for MMS in the WT cells since the compound causes no toxicity at doses below 10 mM in these cells (*23*, *29*). The ratio of 7-MeG to 3-MeA is 13 at 10 mM MMS in *alkA/tag* cells, which is similar to previous determinations (*30*, *31*).

Adduct levels were also determined in experiments where netropsin was co-incubated with the *alkA/tag* bacteria for 30 min prior to the addition of Me-lex or MMS. The effect of netropsin is very different for the two methylating agents (Table 1). Netropsin causes an increase, which is not statistically different, in the amount of 3-MeA formed from MMS in the *alkA/tag* mutant cells. In contrast, the 3-MeA levels generated by Me-lex in the presence of netropsin are significantly reduced by 92% at 50 μM Me-lex and 99% at both 100 and 150 μM in mutant *E. coli.* At all concentrations of Me-lex, the level of 3-MeA in the presence of netropsin falls to an average of 2.7 ± 1.2 μmol/mol of DNA. Similar to the results for 3-MeA, similar levels of 7-MeG are formed from MMS in the absence and presence of netropsin: 43.10 ± 39.30 and 40.95 ± 15.29 μmol of 7-MeG/mol of DNA, respectively.

Effect of Netropsin on in Vitro DNA Methylation by Me-lex. Because some of the lesions, such as 3-MeG and 7-MeG, form at such low levels at the concentrations of Me-lex and/or MMS used in the toxicity studies, it was not possible to measure them *in vivo*. Therefore, a limited *in vitro* study was conducted using conditions designed as much as possible to mimic the *in vivo* experiments. Me-lex (300 and 900 μM) was reacted with 200 μM DNA for 1 h in the absence or presence of 150 μM netropsin. The concentrations were selected on the basis of the estimated DNA concentration in the cell culture flask (33 μM), the relative amounts of netropsin and alkylating agent, and the need to scale up (~6-fold) the reaction to obtain sufficient adducts to measure. The results of these studies are shown in Table 2. At the low dose of Me-lex, the co-addition of netropsin inhibits

formation of 3-MeA by 99% and 3-MeG by 86%. The yield of 7-MeG is not affected by the presence of netropsin. The extent of 3-MeA inhibition is similar to that observed *in vivo* (Table 1). At 900 μM Me-lex, the extent of inhibition of 3-MeA is 79%. The level of 3-MeG is lowered (19%) which is not statistically different ($p > 0.4$) from the level without netropsin. At this high concentration of Me-lex, the amount of 7-MeG actually increases almost 5-fold with the addition of netropsin.

DISCUSSION

We have previously reported on the *in vitro* methylation of DNA by Me-lex (*18*, *21*, *22*) and its cytotoxicity in *E. coli* (*23*, *29*) and mammalian cells (*17*, *20*). The predominant product formed both *in vivo* and *in vitro* is 3-MeA (>90%); however, low levels of N3-methylguanine (3-MeG) and 7-MeG are also detected *in vitro* (*18*), and we assume that these minor lesions will also be present *in vivo*. The cytotoxicity associated with 3-MeA is attributed to its ability to stall DNA polymerases (*16*) because the methyl group blocks the required contact between the electronegative N3-purine position and highly conserved arginine residues found to date in all DNA polymerases (*32*−35). For the same reason, 3-MeG is probably as cytotoxic as 3-MeA, although it is repaired less efficiently than 3-MeA (*36*, *37*). In mammalian cells, the stalling of DNA polymerase by 3-MeA initiates subsequent biological effects, including cell cycle arrest, apoptosis, and sister chromatid exchange (*38*). The AlkA protein, which repairs 3-MeG, can remove 3-MeA from single-stranded DNA, which may be the relevant activity at a blocked replication fork (*39*). The Tag protein does not have this single-stranded activity, and it is not known whether the mammalian homologue of AlkA, i.e., AAG/MPG/APG, can repair 3-MeA in single-stranded DNA.

The data presented here show that Me-lex efficiently generates 3-MeA in bacterial cells, which is consistent with its potent toxicity in *E. coli* as compared to other nonequilibrium binding methylating agents such as MMS (*23*, *29*). A direct comparison of 3-MeA levels in cells that cannot repair this lesion shows that for an equimolar amount of Me-lex and MMS there is more than 3 orders of magnitude more 3-MeA formed from Me-lex. For example, 50 μM Me-lex yields 55 μmol of 3-MeA/mol of DNA, while a 5 mM dose of MMS yields approximately 1 μmol of 3-MeA/mol of DNA, a difference of 5500-fold. Similar ratios are seen at higher Me-lex doses. This difference in adduct levels between the two methylating agents roughly correlates to the toxicity of the two compounds, whereby 5 mM MMS generates 1.3 μmol of 3-MeA/mol of DNA, causing a 3−4 log decrease in the level of survival, while 5 μM Me-lex yields 4.9 μmol of adduct/mol of DNA and a similar decrease in the level of survival. Despite the very different array of adducts produced by MMS (*30*, *31*) and Me-lex (*18*, *21*, *22*), the results show that at approximately the same level of 3-MeA both methylating agents induce a level of toxicity that is close to the same in the *alkA/tag* mutant cells. In wild-type cells, MMS is not cytotoxic at concentrations as high as 10 mM because these cells can rapidly repair the low levels of 3-MeA (and possibly 3-MeG) that are formed from MMS. It should be noted that at these high, but noncytotoxic, concentrations of MMS, significant amounts of 7-MeG are formed. This lesion, which is not removed by the Tag protein and is inefficiently removed by AlkA, can persist in the genome after cell division and is not considered cytotoxic (*14*, *36*, *39*, *40*). Our results, in combination with previous studies (*14*−*19*, *23*, *29*), are consistent with the toxicity of MMS and Me-lex being mainly due to the formation of 3-MeA.

The difference in 3-MeA levels in wild-type and *alkA/tag* mutant cells is on average only 2.4-fold at the 1 h time point for the different concentrations of Me-lex. A previous comparison between the wild type and a *tag* mutant using dimethyl sulfate reported a similar ratio (3-fold) at a 30 min time point after removal of the methylating agent (*14*). However, it requires 30-fold more Me-lex in WT cells than in *alkA/tag* cells to induce similar levels of toxicity. Therefore, the protection afforded by base excision repair is not apparent in the adduct levels measured within 1 h of treatment with the methylating agent.

The effect of minor groove binders on *in vitro* DNA methylation by Me-lex has been previously reported (*18*, *21*, *23*), but no study has been performed *in vivo*. The binding constant (K_b) of netropsin is approximately 100-fold higher (10^7 vs 10^5) than of the neutral lex dipeptide (*23*). Therefore, it is reasonable that 25 μM netropsin can quantitatively inhibit *in vivo* DNA methylation by Me-lex at doses as high as 150 μM. In fact, the *in vivo* (Table 1) and *in vitro* (Table 2) effects of netropsin appear to be nearly identical with potent inhibition of 3-MeA and no decrease in the amount of 7-MeG.

We note that in the *alkA/tag* cells the toxicity of Me-lex in the presence of netropsin shows a dose response (Figure 3b), but the levels of 3-MeA are not statistically different at the different concentrations (Table 1). It is possible that at these high concentrations of Me-lex, 3-MeG may contribute to the toxicity. The *in vitro* studies demonstrate that the inhibitory effect of netropsin on 3-MeG is dose-dependent, and there is no inhibition at 900 μM (Table 2). As discussed above, the *in vitro* Me-lex concentration of 900 μM mimics the concentration of 150 μM used *in vivo*. If the *in vivo* ratio of 3-MeA to 3-MeG is similar to the ratio of 40:1 observed *in vitro* (Table 2), then at 150 μM Me-lex >4 μmol of 3-MeG/mol of DNA will be produced (based on the *in vitro* results) in the presence of netropsin. Therefore, it is possible that the combination of 3-MeG (dose-dependent) and the residual 3-MeA (dose-independent) can account for the observed toxicity of Me-lex in the presence of netropsin.

The observation that the level of 3-MeA and the cytotoxicity in MMS-treated bacteria are not sensitive to the presence of netropsin is consistent with the dominant biological role of 3-MeA in MMS toxicity. The reason that 3-MeA is not affected by netropsin is because MMS randomly methylates the DNA in both the major (7-MeG) and minor (3-MeA and 3-MeG) grooves (*41*). Since minor groove methylation by MMS is not restricted to A/T rich sequences, as is the case for Me-lex, MMS is not sensitive to the occupation of minor groove A/T sites by netropsin. It was recently suggested that the toxicity of MMS in mammalian cells was not due to 3-MeA, but rather to the formation of 7-MeG, because the authors did not observe a decrease in toxicity upon the co-incubation of netropsin with MMS (*42*). This interpretation assumed that netropsin, which binds to DNA in a sequence selective manner, universally blocks minor groove methylation by the non-sequence specific methylating agent. This

is clearly not the case as shown in previous *in vitro* studies (*43*) and in the *in vivo* data reported here. The results presented in our study do not rule out the involvement of other lesions (e.g., 3-MeG) in the toxicity of MMS, but they do indicate that 3-MeA plays a dominant role in *E. coli*. However, there is ample evidence in mammalian systems that toxic BER intermediates other than 3-MeA may accumulate depending on the level of DNA damage and the capacity of the cell to perform the different steps in BER (*44*). Therefore, the role of individual lesions can vary, and in some cases, initiation of repair can actually sensitize cells to toxicity (*45*).

The adduct level at 3.5 μM Me-lex, which is the concentration that reduces the level of survival to 1%, is ca. 3.4 μmol of 3-MeA adduct/mol of DNA in a cell that cannot repair 3-MeA. If there is 0.017 pg (5×10^{-17} mol of phosphate) of DNA in rapidly dividing *E. coli* (*46*), there will be approximately 100 3-MeA lesions/cell at 3.5 μM. If a Guassian distribution of adducts among the treated bacteria and some spontaneous depurination of 3-MeA to repairable abasic sites are assumed, it will actually require <100 3-MeA adducts/cell to kill 99% of the cells. While <100 lesions afford almost quantitative killing in the glycosylase mutant cells, it requires approximately 35-fold more Me-lex to produce the same effect in WT cells, which calculates to almost 2000 3-MeA adducts/cell based on the wild-type concentration response (Table 1). Therefore, the glycosylases in wild-type cells can remove approximately 2000 3-MeA lesions per cell genome. This repair capacity is consistent with the resistance of wild-type cells to concentrations of $\leq$100 μM Me-lex, and previous reports that 3-MeA could not be detected in WT cells treated with DMS (*36*, *47*). A Me-lex concentration of 100 μM will produce ~1500 3-MeA lesions, and the WT cells can remove them via the AlkA and Tag glycosylases to prevent toxicity. It has been estimated that there are 200 Tag molecules per wild-type *E. coli* cell (*37*), so each protein molecule must turn over an average of <10 adducts.

In summary, we have shown that the level of DNA damage and the resulting toxicity by a DNA methylating agent can be selectively modulated *in vivo* by a competitive inhibition process. We believe that this constitutes the first clear example of *in vivo* control of a specific type of DNA damage. We hope to further exploit this approach, in combination with other sequence, groove, and site specific DNA alkylation agents, to more precisely control *in vivo* DNA damage. The data also support the central role of 3-alkylpurines in the cellular toxicity induced by DNA methylating agents.

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