Evidence in *Escherichia coli* that N3-Methyladenine Lesions Induced by a Minor Groove Binding Methyl Sulfonate Ester Can Be Processed by both Base and Nucleotide Excision Repair†

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ABSTRACT: It has been previously reported that a neutral DNA equilibrium binding agent based on an *N*-methylpyrrolecarboxamide dipeptide (lex) and modified with an *O*-methyl sulfonate ester functionality ($MeOSO_2$-lex) selectively affords N3-methyladenine lesions. To study the interaction of the neutral lex dipeptide with calf thymus DNA, we have prepared stable, nonmethylating sulfone analogues of $MeOSO_2$-lex that are neutral and cationic. Thermodynamic studies show that both the neutral and monocationic sulfone compounds bind to DNA with K_b's of 10^5 in primarily entropy-driven reactions. To determine how the cytotoxic N3-methyladenine adduct generated from $MeOSO_2$-lex is repaired in *E. coli*, $MeOSO_2$-lex was tested for toxicity in wild-type *E. coli* and in mutant strains defective in base excision repair (*tag* and/or *alkA* glycosylases or *apn* endonuclease), nucleotide excision repair (*uvrA*), and both base and nucleotide excision repair (*tag*/*alkA*/*uvrA*). The results clearly demonstrate the cellular toxicity of the N3-methyladenine lesion, and the protective role of base excision glycosylase proteins. A novel finding is that in the absence of functional base excision glycosylases, nucleotide excision repair can also protect cells from this cytotoxic minor groove lesion. Interaction between base and nucleotide excision repair systems is also seen in the protection of cells treated with *cis*-diamminedichloroplatinum(II) but not with anti-(±)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

Most carcinogenic and antineoplastic DNA alkylating agents afford a diverse array of DNA modifications. This heterogeneity in products includes reactions at the four different bases, at multiple sites on the same base, and reactions that can be moderately sequence-dependent (*1*−*9*). The resulting "mixture" of damaged DNA makes it difficult to dissect out the impact of specific types of DNA lesions on mutagenesis and/or toxicity, which are two distinct biological endpoints. Elucidation of the mutational and cytotoxic contribution of different DNA lesions is of paramount importance for both mechanistic and pragmatic reasons because effective treatment of cancers with combination radiotherapy/chemotherapy (including alkylating agents) can cause secondary leukemias in a significant number of patients (*10, 11*). Therefore, the design of effective cytotoxic antineoplastic agents that do not generate mutations would be an important improvement in cancer chemotherapy. In addition, an understanding of how specific lesions are repaired is necessary to understand tumor cell resistance to DNA-damaging antineoplastic drugs.

Because it is generally thought that 3-alkyladenine is a cytotoxic but not promutagenic lesion (*12*−*14*), the design of reagents to chemically introduce 3-alkyladenine modifications into DNA was undertaken. In our work, we have employed the neutral equilibrium binding lex[1] dipeptide (see Figure 1 for structures) solely as a vehicle to deliver alkyl groups to DNA: once the alkylation reaction has taken place, the peptide is designed to dissociate from the DNA. This dissociation occurs because the alkyl sulfonate ester func-

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[1] Abbreviations: Aag, mammalian alkyladenine−DNA glycosylase; AlkA, *E. coli* N3-methyladenine−DNA glycosylase II; BER, base excision repair; BPDE, anti-(±)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene; cis-Pt, *cis*-diamminedichloroplatinum(II); lex, lexitropsin (*N*-methylpyrrolecarboxamide-based information reading peptide); $MeOSO_2$-lex, [1-methyl-4-[1-methyl-4-(3-(methoxysulfonyl)-propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane; 3-MeA, N3-methyladenine; 7-MeG, N7-methylguanine; $MeSO_2$-lex, [1-methyl-4-[1-methyl-4-(3-(methanesulfonyl)propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane; $MeSO_2$-lex+, 3-[1-methyl-4-[1-methyl-4-(3-(methanesulfonyl)propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]-1-(*N*,*N*-dimethylamino)propane; MMS, methyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; NER, nucleotide excision repair; Tag, *E. coli* 3-methyladenine−DNA glycosylase I.

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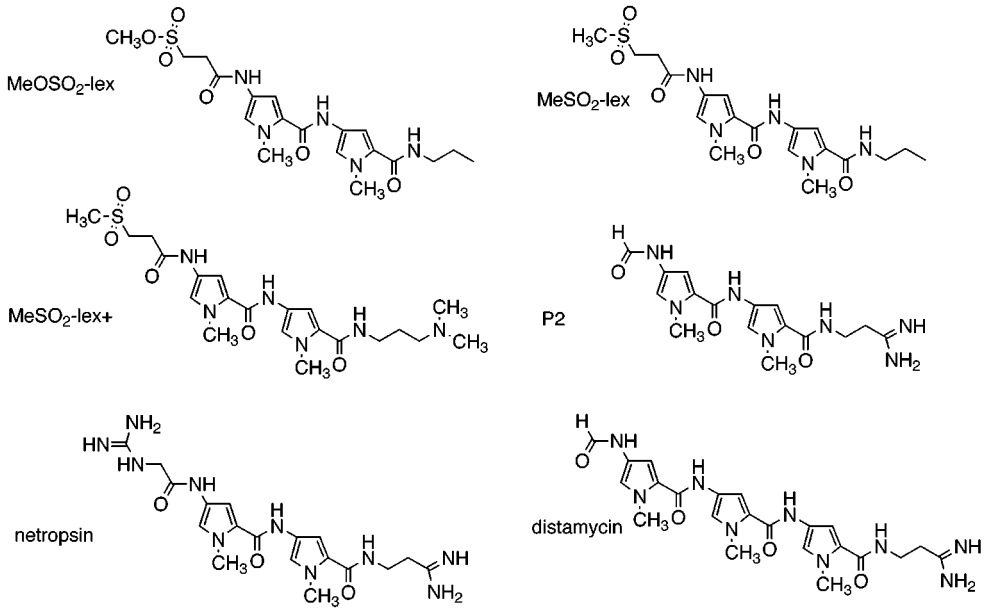

FIGURE 1: Structures of compounds.

tionality is converted into a sulfonate anion upon reaction with nucleophiles. In fact, the neutral lex peptide is a relatively weak equilibrium binder that cannot be chemically footprinted on DNA (*15*).

This delivery approach using the neutral lex dipeptide has been successful for the methylating agent $MeOSO_2$-lex that sequence selectively methylates double-stranded DNA in vitro based on its equilibrium binding properties (*16*): $MeOSO_2$-lex affords approximately 99% of the minor groove adduct 3-MeA (*17*), with little of the major groove lesions, 7-MeG (*17*) and O^6-methylG (*18*). Finally, $MeOSO_2$-lex shows enhanced cytotoxicity over MMS in wild-type and Aag null mouse ES cells (*19*, *20*). Cells in which poly(ADP-ribose) polymerase is inhibited are also highly responsive to $MeOSO_2$-lex (*21*).

In the present report, we characterize the affinity binding properties of the neutral lex peptide versus a cationic analogue, and detail the cytotoxicity of $MeOSO_2$-lex in wild-type *E. coli* and in base excision and/or nucleotide excision repair mutants.

EXPERIMENTAL PROCEDURES

Hazardous Procedures. $MeOSO_2$-lex and MMS should be considered toxic and/or carcinogenic, and handled accordingly.

All reagents and solvents were of the highest grade commercially available and used without further purification. $MeOSO_2$-lex was prepared as previously described (*16*). *E. coli* strains were generously provided by T. O'Connor (City of Hope National Medical Center, Duarte, CA), L. Samson (Harvard School of Public Health, Boston, MA), B. Sedgwick (ICRF, Herts, U.K.), and the *E. coli* Genetic Stock Center (Yale University, New Haven, CT).

Synthesis of [1-Methyl-4-[1-methyl-4-(3-(methanesulfonyl)-propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane ($MeSO_2$-lex). 3-(Methanesulfonyl)propanoic acid (152 mg, 1 mmol) and 1,1-carbonyldiimidazole (162 mg, 1 mmol) were dissolved in 10 mL of DMF, and the resulting solution was stirred at room temperature for 2−3 h at which time [1-methyl-(1-methyl-4-amino-pyrrole-2-carboxamido)-pyrrole-2-carboxamido]propane (200 mg, 180 μmol) (*15*) in 10 mL of DMF was added. The reaction solution was stirred at room temperature for 24 h and the solvent removed under reduced pressure. The crude product was purified by flash silica chromatography (EtOAc−MeOH, 9:1) to give 80 mg (28% yield): TLC (silica, EtOAc−MeOH, 9:1) r.f., 0.6; ^{1}H NMR (DMSO-d_6) δ 10.03 (s, 1 H, CONH), 9.87 (s, 1 H, CONH), 7.97 (t, 1 H, CONH), 7.17 (s, 2 H, pyrrole CH's), 6.85 (s, 1 H, pyrrole CH), 6.82 (s, 1 H, pyrrole CH), 3.82 (s, 3 H, NCH_3), 3.77 (s, 3 H, NCH_3), 3.39 (t, 2 H, SCH_2), 3.12 (m, 2 H, $SCH_2C\underline{H}_2$), 3.00 (s, 3 H, S-CH_3), 2.73 (m, 2 H, $C\underline{H}_2CH_2CH_3$), 1.47 (m, 2 H, $C\underline{H}_2CH_3$), 0.85 (t, 3 H, $CH_2C\underline{H}_3$); FAB/MS (Xe, 9 keV) *m*/*z* (intensity) 438.2 (55, M+1), 379.1 (30, $-NHCH_2CH_2CH_3$).

Synthesis of 3-[1-Methyl-4-[1-methyl-4-(3-(methanesulfonyl)propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]-1-(N,N-dimethylamino)propane ($MeSO_2$-lex+). 3-(Methanesulfonyl)propanoic acid (152 mg, 1 mmol) and 1,1-carbonyldiimidazole (162 mg, 1 mmol) were dissolved in 10 mL of DMF, and the resulting solution was stirred at room temperature for 2−3 h. To this was added 3-[1-methyl-(1-methyl-4-amino-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-1-(*N*,*N*,-dimethylamino)propane (300 mg, 1 mmol) (*15*) in 10 mL of DMF and the reaction solution stirred at room temperature for 24 h. The reaction solution was concentrated under reduced pressure and the crude product purified by flash silica chromatography (EtOAc−concentrated NH_4OH, 99:1) to afford 218 mg (52% yield): TLC (silica, EtOAc−concentrated NH_4OH, 99:1) r.f., 0.16; ^{1}H NMR (DMSO-d_6) δ 10.05 (s, 1 H, CONH), 9.87 (s, 1 H, CONH), 8.08 (t, 1 H, CONH), 7.17 (s, 2 H, pyrrole CH's), 6.85 (s, 1 H, pyrrole CH), 6.80 (s, 1 H, pyrrole CH), 3.83 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.40 (t, 2 H, SCH_2), 3.17 (m, 2 H, $SCH_2C\underline{H}_2$), 3.01 (s, 3 H, S-CH_3), 2.71 (m, 2 H, $C\underline{H}_2CH_2CH_2N(CH_3)_2$), 2.23 (m, 2 H, $C\underline{H}_2N(CH_3)_2$), 2.12 (s, 6 H, $(NCH_3)_2$), 1.60 (m, 2 H, $CH_2C\underline{H}_2CH_2$); FAB/MS (Xe, 9 keV) *m*/*z* (intensity) 481.2 (100, M+1), 379.1 (15, $-NHCH_2CH_2CH_3$).

Thermodynamic Binding Studies. Poly d(AT)·poly d(AT) was purchased from Pharmacia (Piscataway, NJ) and calf thymus DNA from Sigma (St. Louis, MO). Each polymer sample was dissolved in 10 mM phosphate buffer (pH 7.0) containing 1 mM Na_2EDTA and dialyzed exhaustively against the same buffer. The concentration of each compound in solution was determined spectrophotometrically using the following molar extinction coefficients: $MeSO_2$-lex, $MeSO_2$-lex+, and netropsin, $\epsilon_{296} = 21\,500\ M^{-1}\ cm^{-1}$; distamycin, $\epsilon_{303} = 34\,000\ M^{-1}\ cm^{-1}$; poly d(AT)·poly d(AT), $\epsilon_{260} = 6500\ M^{-1}\ cm^{-1}$ in phosphate; and calf thymus DNA, $\epsilon_{260} = 6550\ M^{-1}\ cm^{-1}$ in phosphate. All measurements were performed in buffer solutions consisting of 10 mM sodium phosphate, 1 mM Na_2EDTA adjusted to pH 7.

Absorbance versus temperature profiles (melting curves) for the duplexes and the ligand-saturated polymers (1 lex molecule per 10 phosphates) were measured at 260 nm with a thermoelectrically controlled Perkin-Elmer 552 spectrophotometer. The temperature was scanned at a heating rate of 0.5 °C/min. These melting curves provide transition temperatures (T_M) which are the midpoint of the helix−coil transition of the free and ligand-bound duplexes (*22, 23*). Ligand association constants (K_b) are calculated from the observed increase in the thermal stability of the bound duplex relative to the free duplex (ΔT_M) by the following relation (*24*):

$$\Delta T_M = (RT^\circ_M T_M/n\Delta H_{hc}) \ln(1 + K_b a_L)$$

where R is the universal gas constant, T°_M and T_M are the transition temperatures of the free and bound duplexes, respectively, n is the neighbor exclusion parameter equal to 5 base pairs, ΔH_{hc} is the helix−coil transition enthalpy of the free duplex (measured by differential scanning calorimetry), and a_L is the activity of the free ligand (assumed equal to half of the total concentration of ligand in the solution at the T_M). The resulting ligand binding affinities are extrapolated to the temperature of interest by the van't Hoff equation:

$$\partial \ln K_b/\partial(1/T) = -\Delta H_b/R$$

where ΔH_b is the binding enthalpy measured by titration calorimetry and assumed independent of temperature, i.e., $\Delta C_p = 0$.

Binding heats were measured with an Omega Titration Calorimeter (Microcal Inc., Northampton, MA) (*25*). Ligand solutions were used to titrate each polymer sample with a 100 μL syringe with mixing done by rotating the syringe at 400 rpm. The concentration of $MeSO_2$-lex, $MeSO_2$-lex+, distamycin, or netropsin in the syringe was generally 10−25 times higher than the DNA solution in the reaction cell. Typically 10−14 injections of 5 μL each were made in a single titration analysis. The reference cell, acting as a thermal reference, was filled with water. The instrument was calibrated by means of a known standard electrical pulse. The binding heats of the different compounds were measured from the heat evolved by the initial injections, corrected for the dilution heat and normalized by the concentration of bound ligand.

Stability of 3-MeA in Duplex DNA. The HPLC-purified oligomer 5′-d(TTCTCCTTTACGTCT)-3′ was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolab, Beverly, MA) using standard methods (*26*). The end-labeled oligomer (3 nmol) was annealed to an excess (15 nmol) of 5′-d(GGAGACGTAAAGGAG)-3′. The resulting duplex was incubated with 0 (control) or 250 μM $MeOSO_2$-lex for 4 h at room temperature in 50 mM sodium cacodylate buffer (pH 7.0) containing 5 mM EDTA, precipitated with 3 M NaOAc (pH 5.3) and EtOH, and then resuspended in 10 mM sodium cacodylate buffer (pH 7.0). The control and treated duplex solutions were each separated into two aliquots. One aliquot was incubated at room temperature, and two 10 μL samples were removed at 12, 24, 48, and 72 h. The other aliquot was incubated at 37 °C, and two 10 μL samples were removed for analysis at 6, 12, 24, and 48 h.

For the room temperature and 37 °C incubations, a sample for each time point was subjected to neutral thermal hydrolysis (90 °C for 15 min to depurinate and/or depyrimidinate any thermally labile adducts), precipitated with NaOAc and EtOH, washed with cold 75% ethanol, and then treated with hot piperidine to convert abasic sites into single-strand breaks. The other sample was precipitated, washed, and directly treated with hot piperidine to generate strand breaks preferentially at 7-MeG and at abasic sites generated from the hydrolysis of the 3-MeA adduct. In all cases, the piperidine was removed in vacuo. Each sample was resuspended in formamide, heat-denatured at 90 °C for 1 min, and then cooled in ice/water prior to electrophoresis at 50 W on a 20% polyacrylamide gel containing 7.8 M urea and Tris−EDTA borate buffer (pH 8.3) for 2 h. Maxam−Gilbert G and G+A lanes were included as reference markers. The location and quantitation of the bands on the gel was performed using a Molecular Dynamics Phosphor Imager (Sunnyvale, CA).

Table 1: *E. coli* Strains

strain	genotype	antibiotic resistance	reference
AB1157	wild type	none	*57*
BW-535	*ap* endonuclease	kanamycin	*58*
MV1902	*alkA*	chloramphenicol	*59*
MV1932	*alkA/tag*	none	*59*
AB2500	*uvrA*	streptomycin	*60*
CO10	*alkA/tag/uvrA*	none	*61*

Toxicity Studies. E. coli cultures (Table 1) were started from the frozen stocks and grown overnight in the LB media with (when indicated) the appropriate antibiotic. An aliquot of the overnight culture 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 tubes were centrifuged to pellet the bacteria; the pellets were washed free of LB media with phosphate buffer (pH 7.0) and then resuspended in citrate buffer (pH 5.5). The $MeOSO_2$-lex, MMS, or cis-Pt in 100 μL of 95% EtOH solvent, BPDE in 10 μL of DMSO or solvent alone (control), was added to the tubes and incubated in a shaking incubator (200 rpm) at 37 °C for 1 h. 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 plated on LB agar with/or without 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.

Table 2: Thermodynamics of Neutral and Cationic Lex Binding to DNA

compound	DNA substrate	ΔT_M (°C)	K_b (M^{-1})	ΔG_b (kcal/mol)	ΔH_b (kcal/mol)	$T\Delta S$ (kcal/mol)
CH_3SO_2-lex	calf thymus DNA	0.5	3.0×10^5	−7.3	0.7	8.0
	poly d(A·T)	0.2	8.0×10^5	−7.9	−1.4	6.5
CH_3SO_2-lex+	calf thymus DNA	0.8	5.3×10^5	−7.7	−2.4	5.3
	poly d(A·T)	13.0	5.0×10^7	−10.3	−11.2	−0.9
distamycin	calf thymus DNA	10.8	7.3×10^7	−10.5	−14.0	−3.5
	poly d(A·T)[a]	29.3	3.5×10^9	−12.8	−19.1	−6.3
	poly d(C·G)[a]	−	3.3×10^5	−7.4	−5.1	2.3
netropsin	calf thymus DNA	15.7	3.7×10^7	−10.1	−8.1	2.0
	poly d(A·T)[b]	45.0	2.3×10^9	−13.1	−12.2	0.9
P2	poly d(A·T)[c]	−	−	−9.1	−11.2	−2.1

[a] Ref *62*. [b] Ref *24*. [c] Ref *32*.

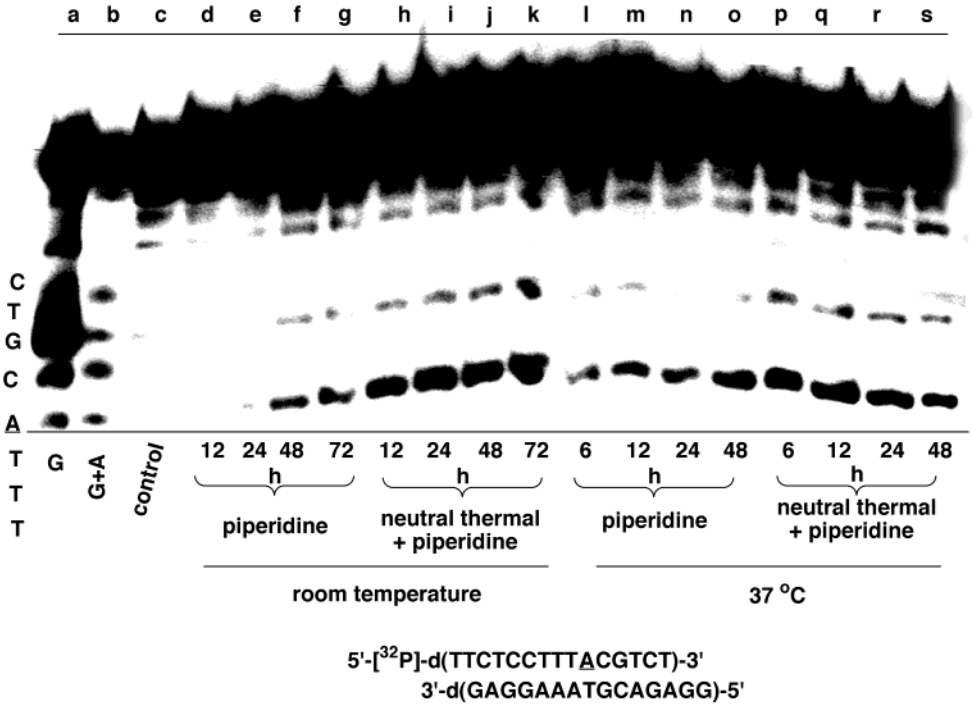

FIGURE 2: Stability of 3-MeA in double-stranded DNA at room temperature and 37 °C: lanes a−c, G, G+A and control lanes, respectively; lanes d−g, 250 μM $MeOSO_2$-lex-treated DNA incubated at room temperature for 12, 24, 48, and 72 h, respectively, and then treated with hot piperidine; lanes h−k, 250 μM $MeOSO_2$-lex-treated DNA incubated at room temperature for 12, 24, 48, and 72 h, respectively, and then heated at 90 °C at pH 7.0 and followed by treatment with hot piperidine; lanes l−o, same as lanes d−g except incubation carried out at 37 °C; lanes p−s, same as lanes h−k except incubation carried out at 37 °C.

RESULTS

To study the thermodynamics of the binding of the lex dipeptide to DNA, two sulfone derivatives were prepared (Figure 1) to serve as stable surrogates for the reactive $MeOSO_2$-lex. The charged analogue with a cationic carboxyl terminus ($MeSO_2$-lex+) was synthesized in addition to the neutral compound to determine the impact of cationic charge on the binding using titration microcalorimetry and T_M measurements. To directly compare our thermodynamic results, the independent and observed variables, K_b and ΔH_b, along with the dependent and calculated variables, $\Delta G°_b$ and $T\Delta S_b$, are shown in Table 2. The free energy values are calculated by the standard thermodynamic relation: $\Delta G°_b = -RT \ln K_b$. The entropies are from the equation: $\Delta G°_b = \Delta H_b - T\Delta S_b$; all values refer to a common temperature of 20 °C. Similar binding affinities of $\sim 10^5$ M^{-1} are obtained for the interaction of the neutral $MeSO_2$-lex with both polymers and for the monocationic $MeSO_2$-lex+ with calf thymus DNA; however, a 100-fold increase in K_b is observed for the interaction of the monocationic lex with poly d(AT)· poly d(AT). These binding data parallel the results on the heats of binding: lower heats are obtained with the neutral lex while a higher exothermic heat is obtained with the charged compound.

The stability of 3-MeA in duplex DNA was determined at room temperature and 37 °C by reacting $MeOSO_2$-lex with a duplex oligomer having a unique lex binding site containing a single A residue in the labeled (top) strand (Figure 2). This generates DNA that is selectively modified with 3-MeA at a single site, although some methylation at the G in the top strand is also observed (Figure 2). The rate of hydrolytic depurination of the adduct is followed by incubating the methylated DNA in pH 7.0 buffer at room temperature or 37 °C. At different time points, the DNA was directly treated with piperidine to convert abasic sites (and 7-MeG), but not 3-MeA lesions, into strand breaks. Similarly, methylated

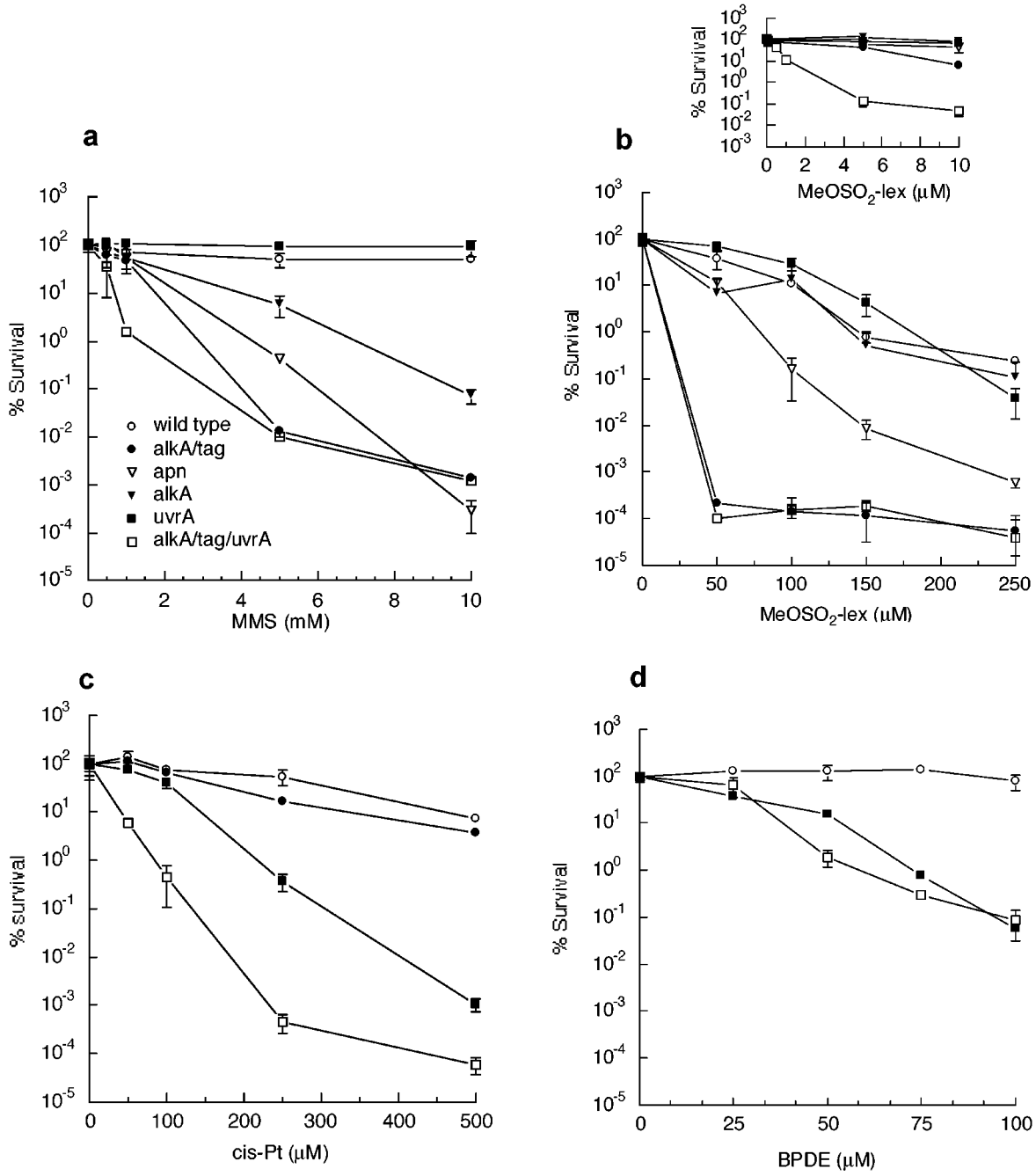

FIGURE 3: Toxicity (relative to control) of MMS (a), $MeOSO_2$-lex (b), cis-Pt (c), and BPDE (d) in wild-type *E. coli* and repair mutants: ○, wild type; ●, *alkA/tag* mutant; ▽, *apn* mutant; ▼, *alkA* mutant; □, *alkA/tag/uvrA* mutant; ■, *uvrA* mutant.

DNA at the same time points was subjected to neutral thermal hydrolysis followed by hot piperidine to convert 3-MeA, 7-MeG, and abasic sites in strand breaks. The direct piperidine treatment gives the extent of abasic sites from the loss of 3-MeA due to "spontaneous" hydrolysis, while the neutral thermal + piperidine treatment provides the total level of 3-MeA and abasic sites. The results indicate that at room temperature no detectable depurination of 3-MeA occurs until 24 h, but even at 72 h there is still a significant amount of adduct left on the DNA (Figure 2, lanes d−g vs h−k). At 37 °C, depurination of 3-MeA is detectable at 6 h but not complete even at 48 h (Figure 2, lanes l−o vs p−s).

The effect of $MeOSO_2$-lex and the corresponding non-peptide analogue MMS on the growth of wild-type *E. coli* and a series of DNA repair mutants was determined. Because of limited solubility in water, $MeOSO_2$-lex was added to the bacteria as a 10% solution in 95% EtOH with no observable toxicity due to the solvent. Other studies to determine the contribution, if any, of the dipeptide moiety to the toxicity of $MeOSO_2$-lex showed that after hydrolysis of the alkyl sulfonate functionality it is not toxic in the *alkA/tag* mutant at doses as high as 100 μM (data not shown).

MMS has little effect in WT or *uvrA* mutant bacteria even at concentrations as high as 10 mM; however, there is a very reproducible dose-dependent increase in toxicity in the *alkA* mutant (Figure 3a). Sensitivity to MMS is further enhanced in the *apn* mutant and even greater in the *alkA/tag* double mutant. In *E. coli* that are defective in both BER and NER

(*alkA*/*tag*/*uvrA*), there is a significant increase in MMS toxicity relative to the *alkA*/*tag* mutant that is most clearly seen at ≤1 mM MMS.

In contrast to MMS, the toxicity of $MeOSO_2$-lex in WT *E. coli* and the *alkA* mutant is equivalent: 250 μM $MeOSO_2$-lex causes a 2 log decrease in survival in both strains (Figure 3b). The *apn* mutant shows a 2 log increase in sensitivity to $MeOSO_2$-lex relative to WT and *alkA* mutant cells. The *alkA*/*tag* double mutant is an additional 4−5 logs more sensitive to $MeOSO_2$-lex than the *apn* mutant (at the 50 μM dose). The difference between *alkA* and *alkA*/*tag* mutants indicates the protective role of the constitutively expressed Tag glycosylase. The *uvrA* mutant, which is defective in NER but proficient in BER, shows no sensitivity to $MeOSO_2$-lex. However, the *alkA*/*tag*/*uvrA* triple mutant is >3 logs more sensitive to $MeOSO_2$-lex than the *alkA*/*tag* mutant at the 5 μM dose with toxicity observable at 1 μM concentration (Figure 3b, inset).

The toxicities of cis-Pt (Figure 3c) and BPDE (Figure 3d) were determined in order to characterize agents that yield DNA damage repaired by NER (*27*, *28*). The *uvrA* mutant was sensitive to cis-Pt, while no toxicity is seen in WT or the *alkA*/*tag* mutant. However, there is an enhancement in cytotoxicity in the *alkA*/*tag*/*uvrA* triple mutant by 250 μM cis-Pt, indicating that there is some protection via BER from the effects of platination due to the presence of the glycosylases (Figure 3c). The toxicity of BPDE is the same in *uvrA* and the *alkA*/*tag*/*uvrA* mutants, consistent with the bulky DNA lesions produced by BPDE being predominantly repaired by NER, even at high levels of DNA modification (Figure 3d).

DISCUSSION

The lex delivery dipeptide is by design uncharged which differs from naturally occurring analogues that are monocationic (e.g., distamycin) or dicationic (e.g., netropsin) (Figure 1). The neutral $MeOSO_2$-lex molecule was conceived to have sufficient affinity for DNA to allow efficient bimolecular transfer of the methyl group from the sulfonate ester to DNA, but not so high as to have any biological activity related to the affinity binding properties of the dipeptide. Complexation of *N*-methylpyrrolecarboxamide-based peptides with DNA is stabilized by a combination of interactions including hydrogen bonding and electrostatic and van der Waals interactions (*29*−*31*). Comparison of the thermodynamic data in Table 2 for $MeSO_2$-lex, $MeSO_2$-lex+, P2, distamycin, and netropsin shows that deep penetration of a ligand in the minor groove of B-DNA or poly d(AT)·poly d(AT) is accompanied by both high binding affinities and high exothermic enthalpies (*22*, *23*). This contrasts with the binding of the ligands to poly d(GC)·poly d(GC). The reason for the strong preference of the different peptides for A·T-rich regions is the presence of the amino group on the N^2-position of guanine that sterically blocks the stable association of the pyrrole ligands with the atoms that line the floor of the minor groove (*30*, *31*).

Biologically, $MeOSO_2$-lex represents a successful compromise: it sequence selectively methylates DNA (*16*), but the lex dipeptide shows no toxicity in *E. coli* (data not shown). Moreover, the neutral compound has increased lipophilicity and should have improved cell penetration over structurally related charged molecules. The thermodynamic studies reported herein indicate that the charged $MeSO_2$-lex+ and uncharged $MeSO_2$-lex have similar overall binding affinities for calf thymus DNA; however, a difference is observed at alternating A·T runs in a synthetic heteropolymer. Despite the similar binding affinities to calf thymus DNA and ΔG's, a fundamental difference is seen in the nature of the forces that contribute to the overall observed free energy change. The driving force is primarily entropic for the binding of the neutral lex and monocationic molecules to calf thymus DNA. The same is true for the binding of $MeSO_2$-lex to poly d(AT)·poly d(AT). In contrast, the binding is enthalpic in the case of the monocationic lex to poly d(AT)·poly d(AT), which is similar to that reported for the binding of P2, a two-ring analogue of distamycin (Table 2) (*32*).

The extent of binding to poly d(AT)·poly d(AT) for ligands with two methylpyrrole groups depends on the overall cationic charge of the ligand while their heats of interaction are independent of charge. The heat of distamycin binding, which contains an extra methylpyrrole group, is much larger than that seen for any of the dipeptides. In essence, the presence or absence of cationic charge does not influence the enthalpic driving force. However, the exothermicity of the heat indicates the extent of penetration of the ligand into the minor groove (*22*, *23*). The lower binding affinities and heats measured in the interaction of the neutral lex with DNA and poly d(AT)·poly d(AT), and of the monocationic lex with calf thymus DNA, indicate that the ligands do not penetrate deeply into the minor groove at most affinity binding sites; i.e., their K_b's with DNA are in the same range as those for distamycin and netropsin with poly d(GC)·poly d(GC). In contrast, the monocationic $MeSO_2$-lex+ binds deep in the minor groove of poly d(AT)·poly d(AT) where only highly preferred affinity binding sites are present. The binding reactions of $MeSO_2$-lex to calf thymus DNA and the heteropolymer, and $MeSO_2$-lex+ to calf thymus DNA, are accompanied by favorable entropy terms, which are due mainly to a release of water molecules. The thermodynamic studies confirm that the lex dipeptide is a weaker binder than analogues with a cationic terminus. Despite the lower K_b, $MeOSO_2$-lex sequence selectively methylates DNA (*16*) while having no biological activity of its own. Moreover, the neutral design eliminates any head-to-tail lex complexes of lex that can bind to DNA in a 2:1 stoichiometry (*33*).

In *E. coli* there are two glycosylases (i.e., Tag and AlkA) that excise 3-alkyladenine lesions as part of the BER system. The Tag protein is highly selective for 3-MeA and is constitutively expressed, while AlkA, which has a broader substrate specificity, is constitutively expressed at low levels but can be induced approximately 20-fold by specific DNA lesions as part of the adaptive response pathway (*34*−*37*). In mammalian systems, only one glycosylase (Aag) for the removal of 3-MeA has been identified, and it is homologous in its glycosylase activity to AlkA (*38*−*40*): both proteins excise a wide variety of lesions, e.g., 3-MeA, N3-methylG, $1,N^6$-ethenoA, etc. (*41*, *42*). To complete the repair process, the abasic site created by the glycosylase must be sequentially and coordinately processed by AP endonuclease, DNA polymerase, and DNA ligase (*43*). Accordingly, mutant bacteria that cannot perform one or more of these steps in

the BER pathway are sensitive to the toxic and/or mutagenic activity of many DNA alkylating agents.

In a previous study comparing the survival of bacteria exposed to $MeOSO_2$-lex and MMS, it was demonstrated that an *alkA*/*tag* mutant is considerably more sensitive to both drugs relative to wild-type bacteria (*44*). However, it required 100-fold more MMS than $MeOSO_2$-lex to obtain the same decrease in survival in the *alkA*/*tag* mutant. These results in BER-deficient bacteria are consistent with the enhanced toxicity of $MeOSO_2$-lex in null *Aag* mouse ES cells (*19*). The difference in toxicity of $MeOSO_2$-lex relative to MMS roughly parallels the in vitro abilities of the two compounds to generate 3-MeA lesions (*17*). In addition to the quantitative difference, there is one other variation between the two methylating agents. The *alkA* mutant is reproducibly more sensitive to MMS than wild-type bacteria (*44* and this study); however, this is not observed with $MeOSO_2$-lex. High concentrations of MMS can induce the transcription of alkA (*45*, *46*), possibly via direct methylation of the Ada protein (*46*). This activation of the adaptive response pathway (*34*−*37*) could account for difference in the sensitivity of the *alkA* mutant to MMS relative to $MeOSO_2$-lex. This implies that MMS generates a quantitatively minor or weakly toxic lesion other than 3-MeA (e.g., 7-MeG) that is not a substrate for the Tag protein, but is for AlkA, which is constitutitively expressed at low levels in uninduced cells (*47*).

It is generally thought that 3-alkyladenine lesions are exclusively removed from DNA due to the fast kinetics of BER, and in wild-type cells this is probably the case. However, in the current study we demonstrate that the combined BER/NER mutant shows extraordinary sensitivity to $MeOSO_2$-lex with cell survival down 6 logs at 50 μM and toxicity observed even at doses as low as 1 μM. A similar scenario is seen in the MMS-treated *E. coli*: there is a 2 log increase in toxicity in the *alkA*/*tag*/*uvrA* relative to the *alkA*/*tag* mutant. This effect is seen at 1 mM MMS, whereas the same level of toxicity in the *alkA*/*tag*/*uvrA* mutant requires only 3 μM $MeOSO_2$-lex. Again, these results are consistent with the relative levels of 3-MeA generated by $MeOSO_2$-lex vs MMS (*17*). It is known that proteins associated with bacterial (*48*), mammalian (*49*, *50*), and yeast (*51*) NER can repair abasic sites. This raises the question about the increase in toxicity in the NER/BER mutant being due to a failure to repair abasic sites rather than a role for the direct repair of 3-MeA adducts. In the sensitive *alkA*/*tag* double mutant, the 3-MeA lesion is definitely responsible for toxicity because the required glycosylases are not present to process it. Also, stability studies at room temperature and 37 °C show that depurination of 3-MeA from double-stranded DNA is not very rapid. The time it takes to perform the toxicity assay is approximately 24 h. In this period, formation of abasic sites due to nonenzymatic hydrolysis of 3-MeA will be limited. Therefore, the toxicity observed in the *alkA*/*tag* mutant is primarily due to the 3-MeA adduct. Moreover, the *apn* mutant is not as sensitive to $MeOSO_2$-lex as the *alkA*/*tag* mutant. In the case of the *alkA*/*tag*/*uvrA* triple mutant, the same explanation is valid as stated for the *alkA*/*tag* mutant: there is accumulation of 3-MeA because glycosylases are not present to process it and because it only slowly depurinates to generate abasic sites. Thus, the sensitivity of the triple *alkA*/*tag*/*uvrA* mutant to $MeOSO_2$-lex and MMS (at much higher concentrations) is a consequence of the persistence of 3-MeA rather than the inability to repair abasic sites; i.e., the role of uvrA protein in protecting BER-defective cells is related to its effect on the repair of 3-MeA.

Good substrates for NER are proposed to be covalently modified nucleobases that distort DNA structure (*52*). Quantitatively it is not yet clear whether 3-MeA would be characterized as a good substrate for NER, and little is known about how 3-alkyladenine lesions affect local DNA structure. The introduction of a positive charge on A by methylation at the N3-position may not have a significant impact on structure if this minor groove adduct behaves analogous to 7-methylguanine (*53*). This subject in presently under investigation.

In the course of our investigations, we observed that cis-Pt, which was used as a "positive" control for an NER substrate, also demonstrates overlap or synergy between the BER and NER pathways that is only observable in the BER/NER mutant. In this case, NER is probably the predominant repair pathway, but cells defective in both NER and BER are significantly more sensitive to the cytotoxicity of cis-Pt lesions. Recently, it has been reported that cis-Pt adducts inhibit the repair of 1,N^6-ethenoA because the adducts bind to the human Aag, although the cis-Pt lesions are not removed by the human glycosylase (*54*). 1,N^6-EthenoA is an excellent substrate for the glycosylases encoded by the Aag and AlkA genes (*41*, *42*). Based on our results, we propose that the binding of AlkA or Tag glycosylases to cis-Pt lesions enhances adduct repair by NER or some other pathway. This contrasts with the glycosylases directly excising adducts from DNA. Alternatively, the glycosylases may physically disguise lesions and diminish their toxicity. The former explanation is consistent with the inability of BER to directly repair cis-Pt adducts (*54*) and our results that show that there is no difference in cis-Pt toxicity between the *alkA*/*tag* and wild-type cells. The suggestion that the binding of alkA to cis-Pt lesions can "hide" their toxicity is not consistent with the known effect of HMG box proteins that also bind to cis-Pt intrastrand cross-links. HMG box proteins significantly increase (not decrease) the toxicity of cis-Pt presumably because complexation prevents repair of cytotoxic lesions (*55*, *56*). If, and how, binding of glycosylases to a particular cis-Pt lesion helps the efficiency of repair is unclear at this time.

In contrast to the results with cis-Pt, the toxicity of BPDE, which generates bulky adducts that are considered classical substrates for NER, is only enhanced in the *uvrA* mutant, indicating that there are limits to the lesions recognized and/or repaired by the AlkA glycosylase.

REFERENCES

1. *Chemical Carcinogens* (1984) 2nd ed., Vols. I and II, ACS Monograph *182* (Searle, C. E., Ed.) American Chemical Society, Washington, D.C.
2. Beranek, D. T., Weis, C. C., and Swenson, D. H. (1980) *Carcinogenesis 1*, 595−605.
3. Den Engelse, L., Menkveld, G. J., De Brij, R.-J., and Tates, A. D. (1986) *Carcinogenesis 7*, 393−403.
4. Ludlum, D. B. (1990) *Mutat. Res. 233*, 117−126.
5. Bodell, W. J. (1999) *Chem. Res. Toxicol. 12*, 965−970.
6. Kohn, K. W., Hartley, J. A., and Mattes, W. B. (1987) *Nucleic Acids Res. 15*, 10531−10549.
7. Hartley, J. A., Gibson, N. W., Kohn, K. W., and Mattes, W. B. (1986) *Cancer Res. 46*, 1943−1947.

8. Wurdeman, R. L., and Gold, B. (1988) *Chem. Res. Toxicol.* 1, 146−147.
9. Wurdeman, R. L., Church, K. M., and Gold, B. (1989) *J. Am. Chem. Soc. 111*, 6408−6412.
10. Henry-Amar, M., and Dietrich, P. Y. (1993) *Hematol. Oncol. Clin. North Am. 7*, 369−387.
11. Povirk, L. F., and Shuker, D. E. (1994) *Mutat. Res. 318*, 205−226.
12. Karran, P., Lindahl, T., Ofsteng, I., Evensen, G. B., and Seeberg, E. (1980) *J. Mol. Biol. 140*, 101−127.
13. Evensen, G., and Seeberg, E. (1982) *Nature 296*, 773−775.
14. Boiteaux, S., Huisman, O., and Laval, J. (1984) *EMBO J. 3*, 2569−2573.
15. Church, K. M., Wurdeman, R. L., Zhang, Y., Chen, F.-X., and Gold, B. (1990) *Biochemistry 29*, 6827−6838.
16. Zhang, Y., Chen, F.-X., Mehta, P., and Gold, B. (1993) *Biochemistry 32*, 7954−7965.
17. Kelly, J., Inga, A., Chen, F.-X., Dande, P., Shah, D., Monti, P., Aprile, A., Burns, P. A., Scott, G., Abbondandolo, A., Gold, B., and Fronza, G. (1999) *J. Biol. Chem. 274*, 18327−18334.
18. Encell, L., Shuker, D. E. G., Foiles, P. G., and Gold, B. (1996) *Chem. Res. Toxicol. 9*, 563−567.
19. Engelward, B., Dreslin, A., Christensen, J., Huszar, D., Kurahara, C., and Samson, L. (1996) *EMBO J. 15*, 945−952.
20. Engelward, B. P., Allan, J. M., Dreslin, A. J., Kelly, J. D., Gold, B., and Samson, L. D. (1998) *J. Biol. Chem. 273*, 5412−5418.
21. Tentori, L., Vernole, P., Madaio, R., Portarena, I., Levati, L., Lacal, P. M., Turriziani, M., Dande, P., Gold, B., Bonmassar, E., and Graziani, G. (2000) *Leukemia 14*, 1451−1459.
22. Marky, L. A., and Breslauer, K. J. (1987) *Biopolymers 26*, 1601−1620.
23. Marky, L. A., and Breslauer, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4359−4363.
24. Marky, L. A., and Kupke, D. W. (1989) *Biochemistry 28*, 9982−9998.
25. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L.-N. (1989) *Anal. Biochem. 179*, 131−137.
26. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol. 65*, 499−560.
27. Sancar, A. (1994) *Science 266*, 1954−1956.
28. Batty, D. P., and Wood, R. D. (2000) *Gene 241*, 193−204.
29. Patel, D. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6424−6428.
30. Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., and Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376−1380.
31. Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A., and Wang, A. H.-J. (1989) *Biochemistry 28*, 310−320.
32. Breslauer, K. J., Ferrante, R., Marky, L. A., Dervan, P. B., and Younquist, R. S. (1988) in *Structure and Expression*: *DNA and its Drug Complexes* (Sarma, R. H., and Sarma, M. H., Eds.) Vol. 2, pp 273−290, Academic Press, New York.
33. Pelton, J. G., and Wemmer, D. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723−5727.
34. Lindahl, T. (1976) *Nature 259*, 64−66.
35. Laval, J. (1977) *Nature 269*, 829−832.
36. Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeppu, Y. (1988) *Annu. Rev. Biochem. 57*, 133−157.
37. Samson, L., and Cairns, J. (1977) *Nature 267*, 281−283.
38. O'Connor, T. R., and Laval, J. (1991) *Biochem. Biophys. Res. Commun. 176*, 1170−1177.
39. Engelward, B. P., Boosalis, M. S., Chen, B. J, Siciliano, M. J., and Samson, L. D. (1993) *Carcinogenesis 14*, 175−181.
40. Chakravarti, D., Ibeanu, G. C., Tano, K., and Mitra, S. (1993) *J. Biol. Chem. 266*, 15710−15715.
41. Dosanjh, M. K., Roy, R., Mitra, S., and Singer, B. (1994) *Biochemistry 33*, 1624−1628.
42. Singer, B., and Hang, B. (1997) *Chem. Res. Toxicol. 10*, 713−732.
43. Wilson, S. H., and Kunkel, T. A. (2000) *Nat. Struct. Biol. 7*, 176−178.
44. Dinglay, S., Gold, B., and Sedgwick, B. (1988) *Mutat. Res. 407*, 109−116.
45. Takahashi, K., and Kawazoe, Y. (1987) *Biochem. Biophys. Res. Commun. 144*, 447−453.
46. Takahashi, K., Kawazoe, Y., Sakumi, K., Nakabeppu, Y., and Sekiguchi, M. (1988) *J. Biol. Chem. 263*, 13490−13492.
47. Karran, P., Hjelmgren, T., and Lindahl, T. (1982) *Nature 296*, 770−773.
48. Snowden, A. Y., Kow, Y. W., and Houten, B. V. (1990) *Biochemistry 29*, 7254−7259.
49. Reardon, J. T., Bessho, T., Kung, H. C., Bolton, P. H., and Sancar, A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9463−9468.
50. Huang, J.-C., Hsu, D. S., Kazantsev, A., and Sancar, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12213−123217.
51. Swanson, R. L., Morey, N. J., Doetsch, P. W., and Jinks-Robertson, S. (1999) *Mol. Cell. Biol. 19*, 2929−2935.
52. Hess, M. T., Schwitter, U., Petretta, M., Giese, B., and Naegeli, H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6664−6669.
53. Azaz-Nikpay, K., and Verdine, G. L. (1994) *Chem. Biol. 1*, 235−240.
54. Kartalou, M., Samson, L. D., and Essigmann, J. M. (2000) *Biochemistry 39*, 8032−8038.
55. McA'Nulty, M. N., Whitehead, J. P., and Lippard, S. J. (1996) *Biochemistry 35*, 6089−6099.
56. McA'Nulty, M. N., and Lippard, S. J. (1996) *Mutat. Res. 362*, 75−86.
57. Rebeck, G. W., and Samson, L. (1991) *J. Bacteriol. 173*, 2068−2076.
58. Cunningham, R., Saporito, S., Spitzer, S., and Weiss, B. (1986) *J. Bacteriol. 168*, 1120−1127.
59. Volkert, M., Nguyen, D., and Beard, C. (1986) *Genetics 112*, 11−26.
60. Howard-Flanders, P., Boyce, R. P., and Theriot, L. (1966) *Genetics 53*, 1119−1136.
61. Mattes, W. B., Lee, C.-S., Laval, J., and O'Connor, T. R. (1996) *Carcinogenesis 17*, 643−648.
62. Rentzeperis, D., Marky, L. A., and Kupke, D. W. (1992) *Biopolymers 32*, 1065−1075.

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