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DNA-Reactive Protein Monoepoxides Induce Cell Death and Mutagenesis in Mammalian Cells

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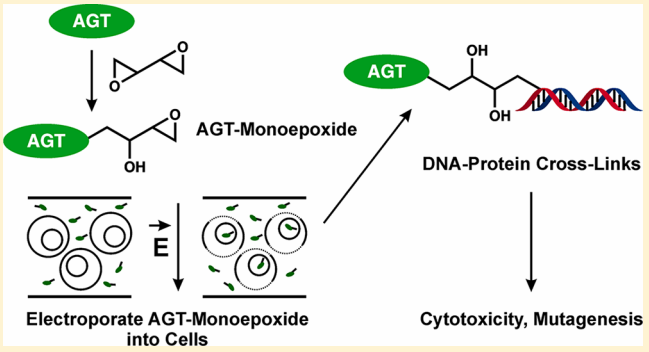
Supporting Information

ABSTRACT: Although cytotoxic alkylating agents possessing two electrophilic reactive groups are thought to act by cross-linking cellular biomolecules, their exact mechanisms of action have not been established. In cells, these compounds form a mixture of DNA lesions, including nucleobase monoadducts, interstrand and intrastrand cross-links, and DNA−protein cross-links (DPCs). Interstrand DNA−DNA cross-links block replication and transcription by preventing DNA strand separation, contributing to toxicity and mutagenesis. In contrast, potential contributions of drug-induced DPCs are poorly understood. To gain insight into the biological consequences of DPC formation, we generated DNA-reactive protein reagents and examined their toxicity and mutagenesis in mammalian cells. Recombinant human O^6-alkylguanine DNA alkyltransferase (AGT) protein or its variants (C145A and K125L) were treated with 1,2,3,4-diepoxybutane to yield proteins containing 2-hydroxy-3,4-epoxybutyl groups on cysteine residues. Gel shift and mass spectrometry experiments confirmed that epoxide-functionalized AGT proteins formed covalent DPC but no other types of nucleobase damage when incubated with duplex DNA. Introduction of purified AGT monoepoxides into mammalian cells via electroporation generated AGT−DNA cross-links and induced cell death and mutations at the hypoxanthine-guanine phosphoribosyltransferase gene. Smaller numbers of DPC lesions and reduced levels of cell death were observed when using protein monoepoxides generated from an AGT variant that fails to accumulate in the cell nucleus (K125L), suggesting that nuclear DNA damage is required for toxicity. Taken together, these results indicate that AGT protein monoepoxides produce cytotoxic and mutagenic DPC lesions within chromosomal DNA. More generally, these data suggest that covalent DPC lesions contribute to the cytotoxic and mutagenic effects of bis-electrophiles.



DNA−protein cross-links (DPCs) are ubiquitous DNA lesions that are formed when proteins become irreversibly trapped on chromosomal DNA. A variety of physical and chemical agents, including formaldehyde,[1] ionizing radiation,[2] UV light, and common anticancer drugs such as nitrogen mustards and platinum compounds, can induce covalent DPCs in cells.[3−5] Because of their unusually bulky size and their ability to disrupt dynamic DNA−protein interactions, DPC lesions are thought to interfere with critical cellular processes of DNA replication, transcription, and repair.

Although DPCs are hypothesized to contribute to the cytotoxicity and mutagenesis of common cross-linking agents,[3] their specific contributions to the cellular effects of DNA-alkylating agents are poorly understood because these compounds also induce other types of DNA damage such as interstrand and intrastrand DNA−DNA cross-links and nucleobase monoadducts. For example, 1,2,3,4-diepoxybutane (DEB), the ultimate carcinogenic metabolite of 1,3-butadiene, forms DPCs[6−8] but is also capable of generating guanine−guanine and adenine−guanine cross-links,[9−11] as well as a number of guanine and adenine monoadducts.[9,12] DPCs are estimated to constitute only 1−3% of total DNA damage following exposure to bis-electrophiles and ionizing radiation,[2,3,13] making it difficult to evaluate their specific role in the cytotoxic and mutagenic effects of these agents.[14,15]

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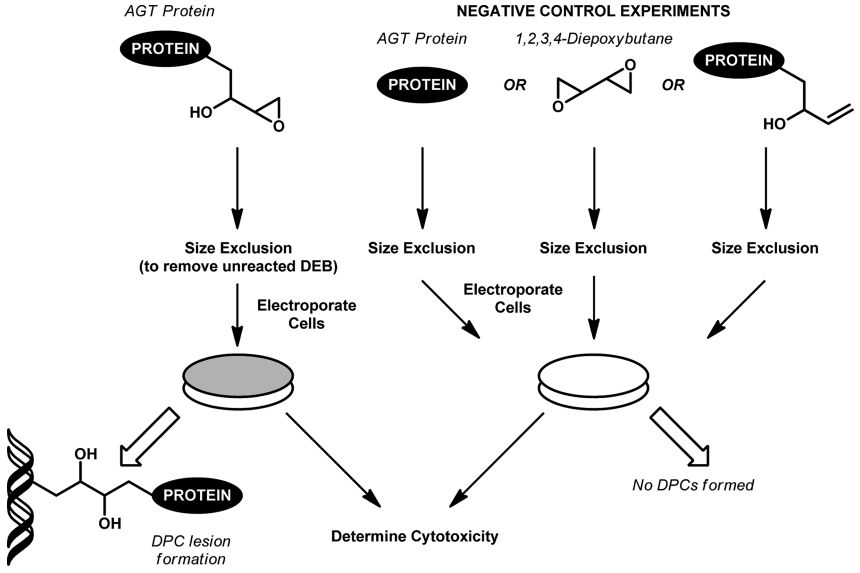
Scheme 1. Experimental Strategy for the Selective Introduction of DNA−Protein Cross-Links (DPCs) into Mammalian Cells



Experimental evidence that supports an important role of DPCs in the biological activity of bis-electrophiles is mounting. For example, it has been reported that the cytotoxicity and mutagenicity of several bifunctional alkylation agents, including 1,2-dibromoethane, dibromomethane, and DEB, are enhanced in bacteria that overexpress human O^6-alkylguanine DNA alkyltransferase (AGT) protein because of the formation of toxic AGT−DNA cross-links.[14−16] AGT is a DNA repair protein that typically protects the human genome from the damaging effects of promutagenic O^6-alkylguanine lesions induced by simple alkylating agents.[17] During the repair reaction, the O^6-alkylguanine nucleotide is flipped out of the base stack into the protein's active site, where it is subject to nucleophilic attack by the activated side chain thiolate anion of Cys^{145}.[18] The irreversible transfer of the O^6-alkyl lesion from the alkylated guanine residue to Cys^{145} of the AGT protein regenerates intact guanine within the DNA duplex. Alkylation at Cys^{145} destabilizes the protein's tertiary structure, thereby targeting AGT for ubiquitination and proteasomal degradation.[19,20]

AGT-mediated enhancement of the genotoxicity of bis-electrophiles in bacteria has been attributed to the formation of toxic AGT−DNA cross-links, which have been characterized by gel electrophoresis and mass spectrometry techniques.[14,21] Liu and colleagues detected the formation of 1,2-dibromoethane-mediated cross-links between guanine bases in DNA and the catalytic cysteine residue (Cys^{145}) of human AGT.[21] Similar results were obtained for 1,2,3,4-diepoxybutane (DEB), which formed butanediol cross-links between the active site cysteine residues of AGT (Cys^{145} or Cys^{150}) and position N-7 of guanine in DNA.[22] The chemical structure of the DEB-induced cross-link has been established as 1-(*S*-cysteinyl)-4-(guan-7-yl)-2,3-butanediol (Cys-N7G-BD).[22]

In theory, AGT−DNA cross-linking can be initiated by DEB reactions with either DNA or the protein to form 2-hydroxy-3,4-epoxybutyl (HEB) intermediates, which can subsequently react with other biomolecules to form covalent DPCs. Kalapila et al. investigated the sequential order of reactivity of the DEB, protein, and DNA to form DPCs using a gel shift assay.[23] Either AGT protein or ^{35}S-labeled DNA duplexes were preincubated with DEB for various lengths of time prior to the addition of the other biomolecule. DPCs were formed regardless of the order of component addition, indicating that cross-linking can originate from DEB reactions at the protein or at the DNA.[23]

The goal of this work was to examine the influence of DPC formation on cell survival and mutagenesis by developing a DNA-damaging agent that was capable of producing DPCs in chromosomal DNA of intact cells but that, through its design, was unable to produce other types of chromosomal DNA lesions (e.g., monoadducts or DNA−DNA cross-links). On the basis of the known ability of AGT to become covalently cross-linked to DNA in the presence of bis-electrophiles,[14,23] it was selected as the basis for the development of a protein reagent capable of selectively inducing DPCs in mammalian cells. Recombinant AGT protein or its variants were treated with DEB to create protein monoepoxides containing DNA-reactive 2-hydroxy-3,4-epoxybutyl (HEB) groups (Scheme 1). AGT treated with 1,2-epoxybut-1-ene (EB) was used as a negative control because the resulting conjugate contains an unreactive double bond instead of the electrophilic epoxide functionality and thus cannot produce DPCs. AGT monoepoxides or the corresponding controls were introduced into mammalian cells via electroporation, and the formation of chromosomal DPCs was demonstrated by mass spectrometry. Furthermore, cell toxicity and mutagenesis were analyzed in cells treated with protein bioconjugates capable of selectively inducing DPCs in the absence of other types of DNA damage.

■ EXPERIMENTAL PROCEDURES

Safety Statement. *DEB is a known human carcinogen and should be handled with caution in a well-ventilated fume hood with appropriate personal protective equipment.*

Chemicals and Reagents. DEB and EB were purchased from Sigma (St. Louis, MO), while mass spectrometry-grade Trypsin Gold was from Promega (Madison, WI). Cys-[^{15}N]-N7G-BD and Cys-N7G-BD were synthesized in our laboratory as reported elsewhere.[22] Adenosine 5′-[γ-^{32}P]triphosphate was obtained from Perkin-Elmer (Boston, MA), and T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Synthetic DNA oligodeoxynucleotides were prepared at the University of Minnesota's Biomedical Genomics Center (Minneapolis, MN). Cell growth media

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were obtained from Invitrogen Life Technologies and supplemented with fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA).

Recombinant Proteins. Recombinant C-terminally histidine-tagged wild-type, variant K125L, and N-terminally histidine-tagged variant C145A AGT proteins were produced and purified as described previously.[24] The hAGT K125L mutation was introduced by polymerase chain reaction (PCR) using a sense primer (5′-GCCCTG*GCCGGC*AACCCCT TAGCCGCGCGAGCAG-3′) for the mutation of Lys[125] to Leu (the mutagenic mismatch is underlined, and the *Ngo*MIV site is shown in italics) and an antisense primer (5′-ACGCCCGGCGGCAACCGAGCG-3′) matching nucleotide 861 to nucleotide 841 in the pQE-hAGT plasmid.[24] The PCR was performed using Accuprime *Pfx* DNA polymerase (Invitrogen) with the pQE-hAGT plasmid as the template under the following conditions: initial denaturation for 2 min at 95 °C followed by 35 cycles of denaturation (15 s at 95 °C), annealing (15 s at 55 °C), and extension (45 s at 68 °C), with a final extension reaction at 72 °C for 5 min. The PCR product (390 bp) was purified using the Qiagen (Chatsworth, CA) gel extraction kit and digested with *Ngo*MIV and *Kpn*I. The 270 bp insert was purified and ligated into the pQE-hAGT plasmid digested with the same enzymes. The plasmid DNA was sequenced to ensure that no additional mutations were introduced during the construction of the pQE-hAGT K125L plasmid.

Cell Culture. HT1080 human fibrosarcoma cells[25] (obtained from the American Type Culture Collection) and Chinese hamster lung (CHL) fibroblast cell line V-79 (obtained from the Coriell Institute for Medical Research, Camden, NJ) were maintained as exponentially growing monolayer cultures in growth medium (Dulbecco's modified Eagle's medium and Ham's F-12 medium, respectively) supplemented with 9% fetal bovine serum. Both cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2.

DEB-Induced Alkylation of AGT Protein for Mass Spectrometric Analysis. Recombinant wild-type, C145A, or K125L AGT protein (2.87 nmol) was incubated with 25 molar equiv of racemic DEB in 10 mM Tris-HCl (pH 7.4) for 2 h at 37 °C (150 μL total volume). Control samples were incubated with buffer or 3,4-epoxy-1-butene (EB) in place of DEB. The reaction mixtures were acidified by the addition of formic acid (1% final concentration) and subjected to size exclusion chromatography to remove unreacted DEB or EB. Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA) were prepared by exchanging the buffer with 0.05% formic acid according to the manufacturer's instructions. Alternatively, DEB-modified AGT proteins were isolated by HPLC using an Agilent 1100 HPLC system. An Agilent Zorbax 300 SB-C3 column (2.1 mm × 150 mm, 5 μm) was eluted with 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B) at a flow rate of 0.2 mL/min. The solvent gradient began at 30% B and was linearly increased to 80% B over 30 min. Under these conditions, both native and modified AGT proteins eluted as a single peak at ∼17 min.

To assess epoxide stability under physiological conditions, DEB-modified proteins were incubated at 37 °C in a water bath. Aliquots containing 0.5 nmol of protein were removed immediately or following incubations for 1, 2, 4, and 24 h and frozen at −20 °C pending HPLC−ESI$^+$-MS analysis as described below. To identify the alkylation sites within the AGT protein and to investigate the fate of the newly introduced epoxide functionalities, aliquots corresponding to 0.5 nmol of protein were diluted to 200 μL by the addition of water and 100 mM ammonium bicarbonate (pH 7.9) (final concentration of 25 mM). Trypsin (2.0 μg) was added to initiate proteolytic digestion, and the samples were incubated overnight at 37 °C. The resulting peptides were dried under vacuum, resuspended in 25 μL of an aqueous 0.5% formic acid/0.01% TFA mixture, and subjected to HPLC−ESI$^+$-MS/MS analysis as described below.

HPLC−ESI$^+$-MS Analysis of AGT Monoepoxides. All mass spectrometric analyses were performed with an Agilent 1100 capillary HPLC ion trap MS system operated in ESI$^+$ mode (m/z 200−2000). For all protein analyses, chromatography was conducted using an Agilent Zorbax SB 300-C18 column (150 mm × 0.5 mm, 5 μm) eluted at a flow rate of 12 μL/min with a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was held at 30% B for the first 5 min, followed by a linear increase to 80% B over 25 min, and further to 95% B over 5 min. Under these conditions, both native and alkylated AGT proteins eluted as a single peak at ∼15.5 min. Deconvolution of the protein charge envelope was performed using commercial software provided with the Agilent ion trap.

For analysis of tryptic peptides, an Agilent Zorbax SB-C18 column (150 mm × 0.5 mm, 5 μm) was eluted with a gradient of 0.1% formic acid and 0.05% TFA in water (A) and 0.1% formic acid and 0.05% TFA in acetonitrile (B) at a flow rate of 15 μL/min. After the solvent composition had been held at 3% B for 3 min, a linear increase from 3 to 5% B over 7 min was employed. The solvent composition was kept at 5% B for 10 min, followed by a linear increase to 35% B over 95 min, and further to 75% B over 10 min. Auto MS^2 was employed to select and fragment the doubly charged ions at m/z 658.4 (unmodified peptide G[136]NPVPILIPCHR[147]), m/z 701.4 [2-hydroxy-3,4-epoxybutyl (HEB) monoadduct on G[136]NPVPILIPCHR[147]], m/z 710.4 [2,3,4-trihydroxybut-1-yl (THB) monoadduct on G[136]NPVPILIPCHR[147]], m/z 834.4 (unmodified peptide V[148]VCSSGAVGNYSGGLAVK[165]), m/z 877.4 (HEB monoadduct on [148]VCSSGAVGNYSGGLAVK[165]), and m/z 886.4 (THB monoadduct on V[148]VCSSGAVGNYSGGLAVK[165]).

Gel Shift Assay. To determine whether AGT monoepoxide can induce covalent DPCs, ^{32}P-end-labeled DNA duplexes (5′-CAGTGACCATCGTTCGTAAC-3′, 40 pmol) were incubated with increasing amounts of C145A AGT monoepoxide (0, 1, 5, 10, 25, and 100 molar equiv) in 10 mM Tris-HCl buffer (pH 7.4) (30 μL final volume) for 1 h at 37 °C. Samples were diluted with water (45 μL final volume), mixed with 4× gel loading buffer (15 μL), and heated at 90 °C for 15 min. The covalent AGT−DNA conjugates were resolved from the unreacted DNA using 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Gel separations were conducted at a constant voltage of 150 V, and the radiolabeled DNA strands were visualized using a Typhoon FLA 7000 phosphorimager (GE Healthcare, Pittsburgh, PA).

To test the effectiveness of the size exclusion method for the removal of unreacted DEB, protein-free solutions of DEB in Tris-HCl buffer (25 mM) were incubated for 15 min at 37 °C, and the samples were split in two. One half of the sample was subjected to size exclusion by standard method, while the other was not. Both samples were then incubated for 2 h at 37 °C in the presence of C145A AGT (4 μg) and duplex ^{32}P-labeled oligodeoxynucleotides (40 pmol), followed by gel electro-

phoresis analysis to detect DPC formation as described above. Additional controls consisted of ^{32}P-labeled oligonucleotide alone and AGT incubated with ^{32}P-labeled oligonucleotide in the absence of DEB.

HPLC−ESI-MS/MS Analysis of Cys-N7G-BD. To confirm that AGT monoepoxides form covalent DPCs, covalent cysteine−guanine cross-links were detected in proteolytic digests. Recombinant C145A or K125L AGT proteins (2.87 nmol) were treated with 25 molar equiv of racemic DEB in 10 mM Tris-HCl (pH 7.4) for 2 h at 37 °C (150 μL total volume). Samples were acidified with formic acid and subjected to size exclusion to remove unreacted DEB using Micro Bio-Spin 6 columns (Bio-Rad) as described above. Protein monoepoxides (final protein concentration of 2.1 μM) were introduced into mammalian cells via electroporation. In brief, HT1080 cells (~10^7 cells per electroporation) were suspended in 500 μL of ice-cold serum-free cell medium (Dulbecco's modified Eagle's medium) and electroporated using a BTX ECM 630 instrument (Harvard Apparatus, Holliston, MA) in 0.4 cm cuvettes using the following conditions: 300 V, 950 μF, 25 Ω (final electroporation volume, i.e., cells plus column eluate, of 550 μL). In control experiments, cells were electroporated in the presence of the eluates obtained from passing DEB solutions through size exclusion columns. Cells were plated onto prewarmed normal growth medium (containing 9% fetal bovine serum) and permitted to recover for 4 h. Chromosomal DNA containing DPCs was isolated by phenol/chloroform extraction, and DNA samples (50 μg) were subjected to neutral thermal hydrolysis (1 h at 37 °C) to release protein−guanine conjugates from the DNA backbone. Proteins were cleaved with trypsin (10 μg of protein in 25 mM ammonium bicarbonate, overnight at 37 °C), and the resulting peptides were dried under vacuum, reconstituted in water, and digested to amino acids using proteinase K (10 μg in 250 μL of H_2O, overnight at 37 °C).

The digests were spiked with a Cys-[^{15}N]-N7G-BD internal standard (500 fmol), followed by off-line HPLC purification to enrich them with Cys-N7G-BD. Chromatographic separation was accomplished with an Agilent Technologies HPLC system (model 1100) incorporating a diode array detector, an autosampler, and a fraction collector fitted with a Supelcosil LC-18-DB (4.6 mm × 250 mm, 5 μm) column (Sigma-Aldrich). The column was eluted at a flow rate of 1 mL/min using a gradient of 15 mM ammonium acetate (pH 4.9) (A) and acetonitrile (B). The solvent composition was changed linearly from 0 to 24% B over 24 min and further to 60% B over 6 min. HPLC fractions containing Cys-N7G-BD and its internal standard (7.5−9 min) were collected, dried under vacuum, and reconstituted in water (25 μL) for HPLC−ESI$^+$-MS/MS analysis.

Quantitative analyses of Cys-N7G-BD were conducted with a Thermo Scientific-Dionex-UltiMate 3000 capillary HPLC (Thermo Scientific Corp., Waltham, MA) system interfaced to a Thermo-Finnigan TSQ Vantage mass spectrometer (Thermo Scientific Corp.). Chromatographic separation was accomplished with a Phenomonex Synergi Hydro-RP C18 column (250 mm × 0.5 mm, 4 μm) eluted with a gradient of 15 mM ammonium acetate (pH 5.0) (A) and an acetonitrile/isopropyl alcohol mixture (1:1) (B) at a flow rate of 10 μL/min. The solvent composition was linearly changed from 4 to 20% B over 8 min, further increased to 30% B over 5 min, and returned to 4% B over 3 min. Under these conditions, Cys-N7G-BD and its internal standard (Cys-[^{15}N]-N7G-BD) eluted at ~7 min. Electrospray ionization was achieved at a spray voltage of 3100 V and a capillary temperature of 270 °C. Collision-induced dissociation was performed with Ar as a collision gas (1.5 mTorr) at a collision energy of 23 V. Instrument parameters were optimized for the maximal response during infusion of a standard solution of Cys-N7G-BD. HPLC−ESI$^+$-MS/MS analysis was performed in the selected reaction monitoring mode by following the neutral loss of guanine from protonated molecules of Cys-N7G-BD (m/z 359.0 $[M + H]^+ \rightarrow m/z$ 208.2 $[M + H - Gua]^+$) and the corresponding mass transition of Cys-[$^{15}N_5$]-N7G-BD (m/z 364.0 $[M + H]^+ \rightarrow m/z$ 208.2 $[M + H - {}^{15}N_5 - Gua]^+$).

Cytotoxicity Experiments. AGT monoepoxides were prepared by treating AGT protein and its variants (4.8 nmol) with 25 molar equiv of racemic DEB (120 nmol) in 10 mM Tris-HCl (pH 7.4) (220 μL total volume) for 2 h at 37 °C EB-modified AGT was prepared analogously (see Experimental Procedures). Samples were acidified with formic acid to prevent protein precipitation and subjected to size exclusion to remove unreacted DEB using Micro Bio-Spin 6 columns (Bio-Rad) as described above. The typical protein recovery from size exclusion was 80% as determined by performing the Bio-Rad Protein Assay.

Protein monoepoxides and EB-modified AGT were introduced into HT1080 cells via electroporation as described above. CHL V-79 cells were treated exactly like HT1080 cells, with an exception that the CHL V-79 cells were electroporated and cultured in Ham's F-12 medium. In control experiments, cells were electroporated in the presence of unmodified AGT protein or column eluates obtained from passing DEB solutions through size exclusion columns.

Immediately following electroporation, cells were plated in prewarmed serum-containing medium and allowed to recover for 24 h under normal growth conditions. Cells were recovered from culture dishes by being exposed to trypsin and counted in a hemocytometer. Cell counts for individual experiments represent the average of two independent counts. The extent of cell death induced by AGT monoepoxides was calculated from the number of viable cells present 24 h postelectroporation in the presence of the protein monoepoxides as compared to the number of viable cells 24 h postelectroporation in the presence of the column eluate from DEB only (no protein) controls. This method was employed to ensure that any cell death observed resulted from exposure to AGT monoepoxides and was not influenced by the presence of residual DEB. Pilot experiments revealed that cell viability was unaffected by the presence of unmodified AGT; therefore, this control was not routinely performed.

Mutation Frequency Assay. The influence of C145A AGT monoepoxides on mutation frequency at the hypoxanthine guanine phosphoribosyltransferase locus was determined via selection for 6-thioguanine-resistant clones.[26] HT1080 cells (~10^7 cells, in duplicate) were electroporated in the presence of Cys^{150} AGT monoepoxide (2 μM) or in the presence of buffer that contained residual DEB remaining following size exclusion (negative control). The cells were then replated and permitted to recover for 8 days at 37 °C. Following trypsinization, 1.5 × 10^6 cells were plated onto 15 cm dishes (three dishes plated per electroporation) in Delbucco's modified Eagle's medium containing 9% FBS and held at 37 °C overnight. The following day, the cell cultures were placed in medium containing 1 mM 6-thioguanine and placed in the 37 °C incubator for 13−15 days to allow for the formation of

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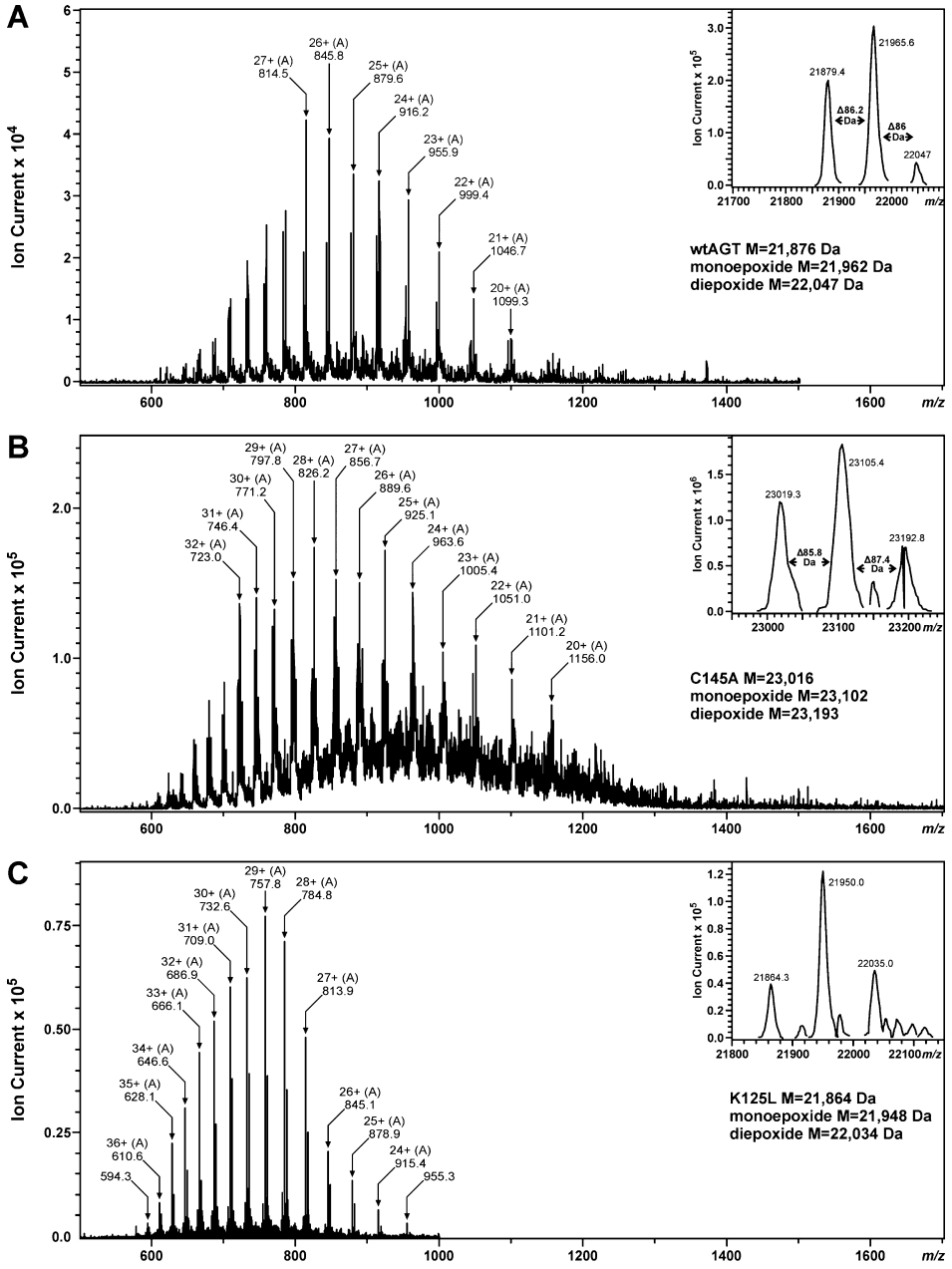

Figure 1. HPLC–ESI$^+$-MS analysis of DEB-induced AGT monoepoxides: wild-type AGT protein (A), C145A AGT (B), and K125L AGT (C). AGT monoepoxides were prepared by incubating recombinant human AGT proteins with 25 molar equiv of DEB for 2 h at 37 °C. Each panel shows the ESI mass spectrum of the protein mixture, while the deconvoluted mass spectrum in shown in each inset.

colonies. Mutation frequency was expressed as the number of 6-thioguanine-resistant colonies per 10^6 cells plated. The results presented are based on pooled data obtained from three independent series of electroporation experiments (i.e., AGT monoepoxide vs eluate control), with each performed in duplicate.

RESULTS AND DISCUSSION

Selective Induction of DPCs in Cell Culture. Previous investigators have proposed that DPC lesions induce cytotoxic and mutagenic outcomes in living cells[7,8,15] However, a serious limitation of these earlier studies is that they employed chemical agents capable of inducing many other types of potentially toxic DNA adducts in addition to DPCs. We now report a novel methodology for introducing DPCs into living cells in the absence of other types of DNA damage (e.g., DNA–DNA cross-links and nucleobase monoadducts). In our approach (Scheme 1), recombinant AGT is incubated with excess DEB to introduce DNA-reactive 2-hydroxy-3,4-epoxybutyl groups (HEB) on the protein. Mass spectrometry analyses have revealed that HEB groups are introduced preferentially at two cysteine thiols (C145 and C150) within the DNA-binding domain of the AGT protein (see below). After any unreacted DEB has been removed by size exclusion, AGT monoepoxides are introduced into cultured mammalian cells via electroporation, inducing chromosomal DPCs. Unlike DPC-inducing agents used previously (e.g., formaldehyde), AGT monoepoxides are unable to produce other types of DNA damage, so any cellular effects observed can be attributed to DPC formation.

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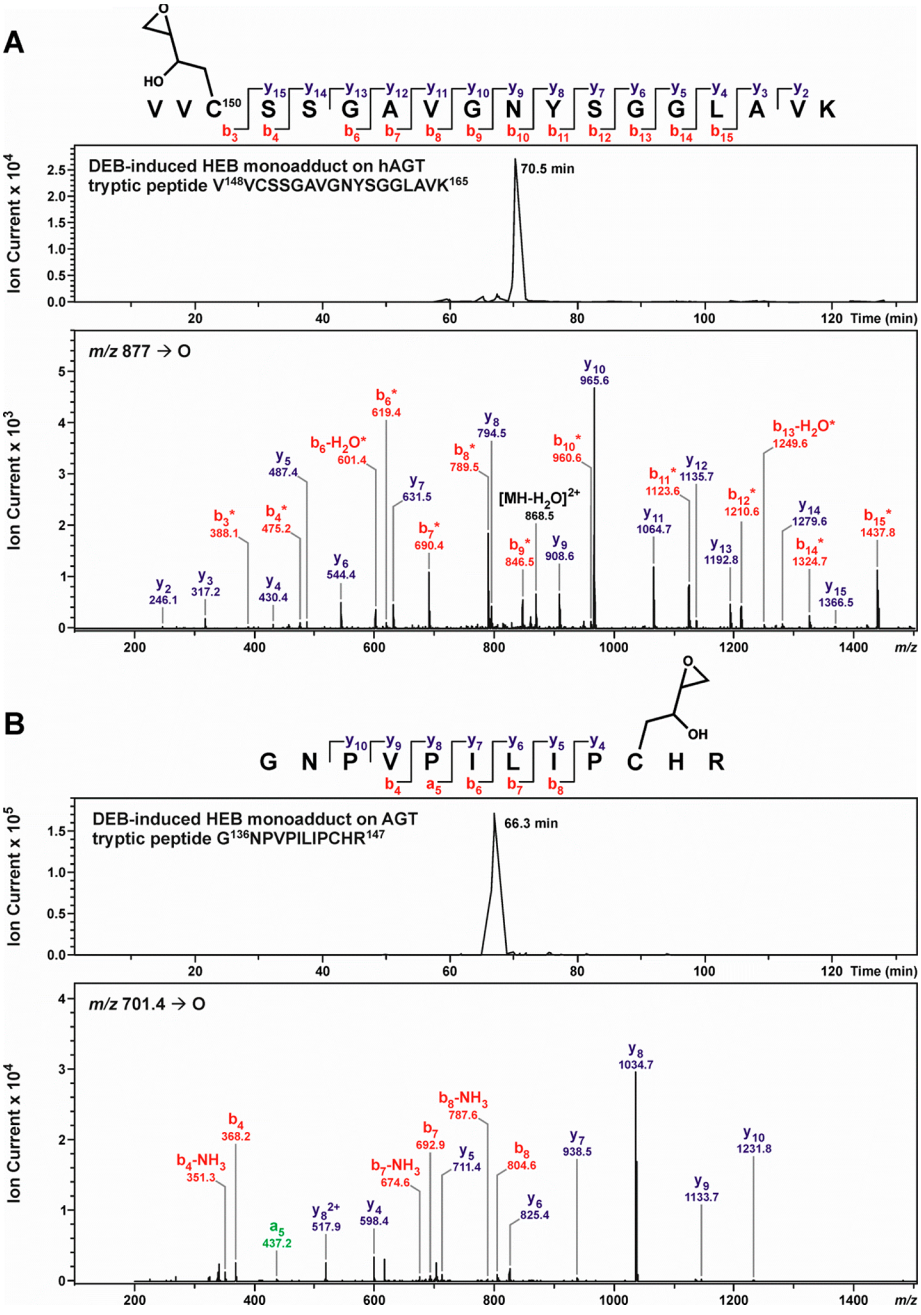

Figure 2. HPLC−ESI$^+$-MS/MS analysis of tryptic peptides containing 2-hydroxyl-3,4-epoxybut-1-yl (HEB) groups. AGT monoepoxides were prepared by incubating recombinant human AGT protein with 25 molar equiv of DEB for 2 h at 37 °C (Figure 1). The resulting proteins were isolated by HPLC and digested to peptides in the presence of trypsin. (A) HPLC−ESI$^+$-MS/MS analysis of AGT tryptic peptide V^{148}VCSSGAVGNYSGGLAVK^{165} containing an HEB monoadduct at Cys^{150}. The top panel shows the extracted ion chromatogram of m/z 877 $[M + 2H]^{2+}$ and the bottom panel the MS/MS spectrum of m/z 877 revealing b and y ion series employed for peptide sequencing. (B) HPLC−ESI$^+$-MS/MS analysis of AGT tryptic peptide GNPVPILIPCHR containing an HEB monoadduct at Cys^{145}. The top panel shows the extracted ion chromatogram of m/z 701.4 $[M + 2H]^{2+}$ and the bottom panel the MS/MS spectrum of m/z 701.4 revealing b and y ion series employed for peptide sequencing. Modified fragment ions are indicated with asterisks. The MS/MS data are consistent with the presence of a 2-hydroxy-3,4-epoxybutyl group on cysteines 145 and 150 of the protein.

Three variants of AGT protein were employed: wild type, C145A active site mutant, and K125L mutant. C145A was selected because whereas alkylation of active site cysteine 145 has been reported to destabilize AGT protein, replacement of this residue with an alanine results in a protein that becomes alkylated solely at position C150 and is relatively more stable.[23] The K125 variant was included because it has been previously reported to be excluded from the nucleus,[27] and therefore, K125L AGT monoepoxide was expected to be nontoxic because it cannot reach DNA.

HPLC−ESI$^+$-MS Characterization of AGT Monoepoxides. AGT monoepoxides were prepared by incubating recombinant human wild-type, C145A, or K125L AGT proteins with excess DEB. Any unreacted DEB was removed by size exclusion or HPLC, and the modified proteins were characterized by mass spectrometry. HPLC−ESI$^+$-MS analysis

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of the wild-type AGT protein following incubation with DEB revealed a single protein peak with a retention time of ~16.6 min, with an ESI^+ spectrum corresponding to the mixture of unreacted wild-type AGT protein (M = 21879 Da) and AGT protein containing a single HEB monoadduct (M = 21966 Da; the observed mass shift of ~86 Da corresponds to the DEB-derived HEB group) (Figure 1A). In addition, a low-abundance species was observed at 22047 Da, corresponding to protein bis-alkylation by two molecules of DEB.

Similar results were obtained for DEB-treated AGT mutants [C145A and K125L (see panels B and C of Figure 1)]. For example, treatment of C145A AGT protein (M = 23019 Da) with DEB produced a bioconjugate containing one HEB monoadduct (M = 23105 Da) or two HEB monoadducts (M = 23193 Da) (Figure 1B). The corresponding masses for DEB-treated K125L AGT protein were found to be 21864 Da (unmodified protein), 21950 Da (K125L protein with one HEB monoadduct), and 22035 Da (two HEB groups) (Figure 1C). The ability of the C145A variant to form two HEB adducts despite the absence of active site cysteine 145 can be explained by reactions of DEB with other amino acid side chains within the protein. AGT proteins that have been treated with 3,4-epoxy-1-butene (EB) were also characterized by ESI^+-MS, revealing the addition of the 2-hydroxy-3,4-buten-1-yl group (Figure S-1 of the Supporting Information).

To identify amino acid residues that are modified by DEB, AGT monoepoxides were digested with trypsin, followed by HPLC–ESI^+-MS/MS analysis of the resulting peptides. Our previous studies have revealed that AGT–DNA cross-linking by DEB takes place mostly at positions Cys^{145} and Cys^{150}.[22] However, DEB-induced AGT monoepoxides have not been previously characterized by mass spectrometry. The mass spectrometer was operated in the auto MS^2 mode to detect and fragment doubly charged ions of G^{136}NPVPILIP<u>C</u>HR^{147} and V^{148}V<u>C</u>SSGAVGNYSGGLAVK^{165} peptides containing 2-hydroxy-3,4-epoxybut-1-yl (HEB, intact monoepoxide) and 2,3,4-trihydroxybut-1-yl (THB, hydrolysis product) groups.

HPLC–ESI^+-MS/MS analysis of tryptic digests of wild-type AGT monoepoxide has revealed the presence of the V^{148}VCSSGAVGNYSGGLAVK^{165} peptide containing a DEB-derived 2-hydroxy-3,4-epoxybut-1-yl (HEB) group (m/z 877.1 $[M + 2H]^{2+}$, calculated M = 1752.2, ΔM = +86) (Figure 2A). The tandem mass spectrum of this peptide exhibited characteristic b- and y-series ions consistent with the presence of the HEB group at Cys^{150}: while the masses of the y_2–y_{15} fragments matched the theoretical values expected for the unmodified peptide, the masses of the b_3–b_{15} ions were shifted by +86 Da (Figure 2A). In addition, we observed the G^{136}NPVPILIP-<u>C</u>HR^{147} peptide containing the HEB group on the active site Cys^{145} (Figure 2B). DEB-modified C145A and K125L variants exhibited similar results (not shown), with the exception that modification of C145 was not observed for the C145A mutant.

Hydrolytic Stability of AGT Monoepoxides. AGT monoepoxides can induce covalent DPCs with chromosomal DNA within the cell nucleus only if the protein epoxide groups are stable enough to persist in cells and enter the nucleus. To evaluate the hydrolytic stability of the HEB group within the DEB-alkylated AGT protein and its variants, isolated protein monoepoxides were incubated at 37 °C. Aliquots were removed following incubation for 0, 1, 2, 3, and 4 h and subjected to mass spectrometric analysis. HPLC–ESI^+-MS analysis of DEB-treated wild-type AGT, C145A AGT, and K125L variants has revealed that samples were essentially unchanged following a 4 h incubation under physiological conditions, suggesting that the epoxide-containing proteins are sufficiently stable to survive in cells long enough to allow for DNA alkylation (see Figure S-2 of the Supporting Information). These results were confirmed by HPLC–ESI^+-MS/MS analysis of tryptic digests, which revealed that the epoxide groups of wild-type, C145A, and K125L proteins were intact following incubation for 4 h under physiological conditions. Small amounts of hydrolyzed peptides were observed by HPLC–ESI-MS/MS analysis (Figure S-3 of the Supporting Information), but they were less abundant than the intact monoepoxides and most likely originated as an artifact during the overnight tryptic digestion.

SDS–PAGE Analysis of AGT Monoepoxide-Induced DPCs. To evaluate the ability of AGT monoepoxides to induce DPCs, ^{32}P-labeled DNA oligodeoxynucleotide duplexes were incubated with an increasing number of equivalents of AGT monoepoxides, and the resulting DPCs were detected as slowly moving bands on a denaturing SDS–PAGE gel (Figure 3). We found that the extent of DPC formation increased as the ratio of molar equivalents of AGT monoepoxide to DNA was increased from 1:1 to 100:1 (Figure 3).

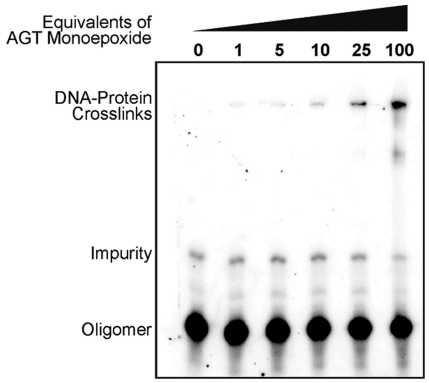

Figure 3. SDS–PAGE analysis of the DPC produced by AGT monoepoxide in vitro. (A) 12% SDS–PAGE analysis of ^{32}P-end-labeled DNA duplexes (5′-GGA GCT GGT GGC GTA GGC-3′ + strand) following incubation with increasing amounts of C145A AGT monoepoxide (0, 1, 5, 10, 25, or 100 molar equiv) for 1 h. The respective mobilities of the unmodified DNA oligomer and then more slowly migrating DNA–protein cross-links are indicated.

The ability of AGT monoepoxide to form covalent DPCs was further confirmed by HPLC–ESI^+-MS/MS detection of cysteine–guanine conjugates (Cys-N7G-BD) in total protein digests. Following incubation with C145A AGT monoepoxide, DNA was subjected to thermal hydrolysis to release protein–nucleobase conjugates, followed by digestion with trypsin and proteinase K to achieve complete proteolytic digestion of proteins to amino acids.[6,22] The resulting amino acid–nucleobase conjugates were enriched by off-line HPLC and analyzed by capillary HPLC–ESI^+-MS/MS analysis using $^{15}N_5$-Cys-N7G-BD as an internal standard. HPLC–ESI^+-MS/MS analysis revealed the formation of Cys-N7G-BD (results not shown).

DNA–Protein Cross-Linking and Cytotoxicity of Protein Monoepoxides in a Mammalian Cell Culture. To assess the ability of AGT–DEB bioconjugates to induce DPCs and produce cytotoxic effects in mammalian cells, human fibrosarcoma (HT1080) cells were electroporated in the presence of C145A-derived AGT monoepoxide. The formation of covalent AGT–DNA conjugates was monitored by HPLC–

ESI$^+$-MS/MS analysis of Cys-N7G-BD conjugates in total proteolytic digests (Figure 4). We found that electroporation in

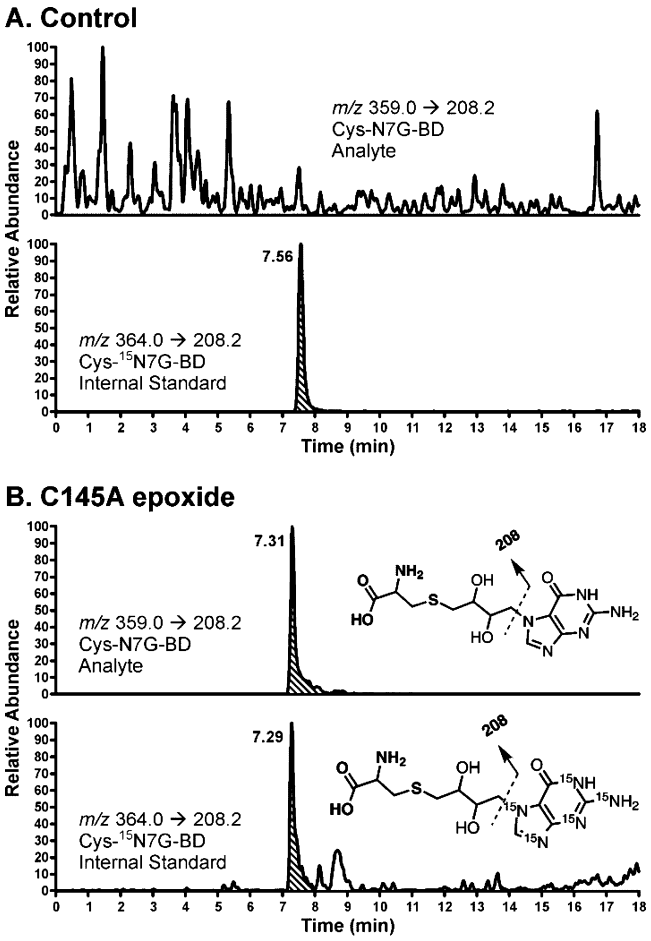

Figure 4. HPLC–ESI$^+$-MS/MS analysis of covalent 1-(*S*-cysteinyl)-4-(guan-7-yl)-2,3-butanediol (Cys-N7G-BD) conjugates in DNA isolated from HT1080 cells electroporated in the presence of C145A AGT monoepoxide. Chromosomal DNA was extracted from treated cells, spiked with the $^{15}N_5$-labeled internal standard, and subjected to thermal hydrolysis and enzymatic digestion of proteins to amino acids to release amino acid–nucleobase conjugates. Samples were subjected to HPLC–ESI$^+$-MS/MS analysis with a triple-quadrupole mass spectrometer. (A) Control cells electroporated with buffer control. (B) Cells electroporated in the presence of C145A AGT monoepoxide.

the presence of C145 AGT monoepoxide induced up to nine AGT–DNA cross-links per 1 million nucleotides in nuclear DNA (Figure 5). In contrast, only background levels of Cys-N7G-BD were observed in cells electroporated in the presence of K125L monoepoxide. As described above, this variant of the AGT protein fails to accumulate in the nucleus of mammalian cells because of its impaired nuclear import and/or lack of retention in the nucleus.[27]

The toxicity of AGT monoepoxides in mammalian cells was quantified by comparing the numbers of cells surviving electroporation in the presence of AGT monoepoxide and in the negative control (cells electroporated in DEB only column eluate). Our pilot experiments revealed that electroporation in the presence of the C145A AGT protein that is lacking an epoxide group did not affect cell viability (data not shown). In contrast, electroporation in the presence of C145A AGT

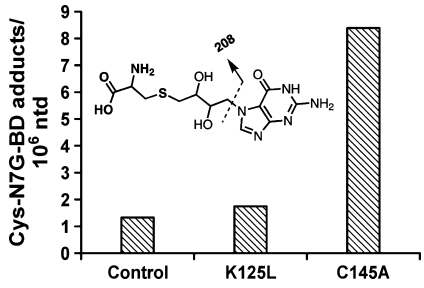

Figure 5. Formation of Cys-N7G-BD conjugates in HT1080 cells electroporated in the presence of AGT monoepoxides. AGT monoepoxides were prepared by treating recombinant C145A and K125L AGT proteins with DEB, and unreacted DEB was removed via size exclusion chromatography as described in Experimental Procedures. Protein monoepoxides were introduced into mammalian cells via electroporation. Following extraction of DPC-containing chromosomal DNA, equal amounts of DNA from each sample were subjected to thermal and enzymatic hydrolysis to release Cys-N7G-BD conjugates. In control experiments, cells were electroporated in the presence of column eluates obtained by passing DEB only solutions through size exclusion columns. The samples were subjected to off-line HPLC prior to HPLC–ESI$^+$-MS/MS analysis. Quantification of Cys-N7G-BD was accomplished using isotope dilution with Cys-$^{15}N_5$-N7G-BD.

monoepoxide (2 μM) reduced HT1080 cell survival by 20 ± 3% (SEM), as compared to controls ($P < 0.01$, *t* test) (Figure 6A). As an additional control, cells were electroporated in the

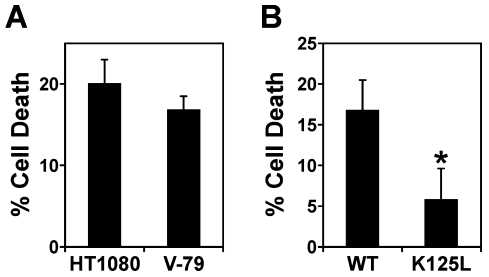

Figure 6. AGT monoepoxide is cytotoxic in mammalian cells. (A) Human HT1080 or hamster V-79 cells (~10^7) were electroporated in the presence of C145A AGT monoepoxides, or protein-free solutions resulting from size exclusion of DEB. The extent of cell death induced by protein monoepoxide was calculated as described in Experimental Procedures. Results depict the average percent killing observed, and the error bar represents the standard error of the mean ($N \geq 3$). (B) V-79 cells (~10^7) were electroporated in the presence of AGT monoepoxide molecules prepared using either wild-type AGT or K125L AGT, and the extent of cell death was determined as described above. Error bars represent the standard error from four independent experiments. Results were statistically significant when $P < 0.02$.

presence of C145A protein that had been reacted with epoxybutene (EB). As discussed above, this generates protein conjugates that contain no DNA-reactive epoxide. As expected, the levels of cell death observed following treatment of EB–AGT conjugates were not significantly greater than those observed in control cells (data not shown).

To confirm the ability of AGT monoepoxides to induce cell death in mammalian cells, analogous series of experiments were performed in Chinese hamster lung (CHL) cell line V-79. As the data in Figure 6A reveal, introduction of C145A AGT monoepoxides into V-79 CHL cells resulted in ~17% cell death relative to control cells electroporated with buffer containing

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any residual DEB that remained after size exclusion. The magnitude of the cytotoxic effect observed in the V-79 cells electroporated with the AGT monoepoxide reagent was statistically significant ($P < 0.05$, t test) and was similar to that observed when HT1080 cells were treated under similar conditions (Figure 6A). These results suggest that both mammalian cell lines are equally sensitive to cell death induced by AGT monoepoxides.

It was previously shown that a modified version of the AGT protein that harbored amino acid substitution K125L was excluded from the nucleus while the wild-type AGT protein was concentrated in the nucleus.[25] We therefore hypothesized that electroporation of cells with DEB-treated K125L AGT (which is excluded from the nucleus) would result in significantly lower levels of toxicity than in cells electroporated with similarly treated wild-type AGT. We chose to use wild-type protein because while there is no reason to anticipate that conversion of cysteine 145 to an alanine would alter the protein's intracellular dynamics, such a change cannot *a priori* be excluded. The results presented in Figure 6B confirm this prediction, showing that while introduction of wild-type AGT monoepoxide into HT1080 cells resulted in approximately 17% cell death, introduction of K125L monoepoxide increased the level of cell death by <6%. The difference in the magnitude of cytotoxicity observed following electroporation with wild-type and K125L AGT monoepoxides was statistically significant ($P < 0.02$, paired t test). In contrast, the levels of cell death observed in cells electroporated with K125L were not significantly greater than those observed in buffer control-treated cells ($P > 0.02$, t test). These results support the interpretation that chromosomal DPCs are responsible for cytotoxicity observed in cells electroporated with protein monoepoxides (Figure 6A).

Mutagenic Effects of AGT Monoepoxides. To determine whether C145A AGT monoepoxides can induce mutations in mammalian cells, we measured the frequency of inactivating mutations within the hypoxanthine guanine phosphoribosyltansferase (HPRT) gene of HT1080 cells electroporated with Cys^{150} AGT monoepoxide (2 μM). Cells electroporated with protein-free buffer containing any residual DEB remaining after size exclusion served as a negative control (see Experimental Procedures for details). As shown in Figure 7, the frequency of inactivating mutations within the HPRT gene in H1080 cells electroporated in the presence of monoepoxide was increased 1.8-fold as compared to that of control-electroporated cells. While the magnitude of this effect was modest, the results were statistically significant ($P < 0.01$, χ^2 test). These results are consistent with the interpretation that AGT protein monoepoxides enter the nucleus producing mutagenic DPC lesions.

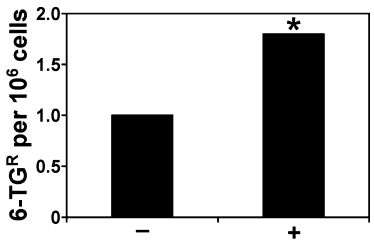

Figure 7. AGT monoepoxide is mutagenic in mammalian cells The frequency of inactivating mutations at the X-linked hypoxanthine guanine phophoribosyltransferase gene was determined using a clonogenic assay that measured 6-thioguanine-resistant colony formation (see Experimental Procedures for details) in HT1080 cells electroporated in the absence (−) or presence (+) of C145A AGT monoepoxide. The observed differences were statistically significant (*$P < 0.01$, χ^2 test).

CONCLUSIONS

While the ability of bulky DPC lesions to induce cell death has been hypothesized in the literature,[3] previous attempts to directly study their biological effects have been hampered by the propensity of cross-linking agents, such as DEB, to form other types of DNA lesions, e.g., DNA−DNA cross-links, in addition to the DPCs. As a result, the extent to which the toxicity observed following treatment of cells with these drugs can be attributed to DPC formation is not clear. In this work, novel protein monoepoxide reagents have been developed that, when introduced in cells, react with chromosomal DNA to form covalent DPC lesions (Scheme 1 and Figure 4). Unlike DEB and other typical cross-linking agents, HEB-functionalized proteins prepared in our studies are not capable of inducing any type of DNA damage other than DPCs. We have shown that protein reagents derived from human AGT protein (Figures 1 and 2) form covalent DPCs *in vitro* and *in vivo* (Figure 4) and are cytotoxic to human fibrosarcoma HT1080 cells and hamster CHL fibroblasts (Figure 6). Furthermore, AGT monoepoxides induced inactivating mutations within the HPRT gene in HT1080 cells (Figure 7). Although the levels of AGT monoepoxide-induced cell death were modest (17−20%), presumably because of the difficulty in delivering protein monoepoxides to the nucleus, the results were reproducible, and the differences between the cytotoxic effects of control and DNA-reactive proteins were statistically significant (Figure 6B). Taken together, these findings support the interpretation that electroporation of cells with epoxide-containing proteins results in the formation of cytotoxic and mutagenic DPC lesions.

Although epoxide-functionalized proteins, when introduced in cells, may also alkylate other biomolecules such as proteins and RNA, several lines of evidence support our hypothesis that the observed cytotoxicity is mediated by alkylation of the chromosomal DNA. We have shown that AGT monoepoxides produce covalent DPCs *in vitro* (Figure 3) and induce mutations in HT1080 cells (Figure 7). Furthermore, our HPLC−ESI-MS/MS results indicate that when electroporated in human cells, protein monoepoxides alkylate chromosomal DNA to form covalent Cys-N7G-BD conjugates (Figure 4). Finally, electroporation results for protein monoepoxides that do not accumulate in the nucleus (K125L AGT variant) indicate that they are substantially less toxic than monoepoxides derived from the wild-type AGT protein (Figure 6B). Taken together, our data indicate that AGT−DEB conjugates produce chromosomal DPCs that are cytotoxic and mutagenic in mammalian cells.

The DNA-reactive protein reagents developed in this work will be useful in exploring the role of specific DNA repair pathways in mediating the cellular response to DPC lesions. We are currently pursuing experiments with cell lines harboring inactivating mutations in known DNA repair genes. Our results indicate that these DNA repair-deficient cell lines exhibit altered sensitivity to the cytotoxic and mutagenic effects of DPC-inducing agents. Currently, there is a lack of agreement in the literature regarding the respective roles of the homologous recombination and nucleotide excision repair pathways in the cellular response to DPCs.[28−30] If homologous recombination

plays a leading role in DPC repair, cells and tissues of patients suffering from Fanconi anemia may be deficient in their ability to repair spontaneous DPCs formed as a consequence of normal cellular metabolism. Consistent with this hypothesis is the recent observation that cells with inactivating mutations in a number of the Fanconi anemia genes are hypersensitive to death induced by formaldehyde, the prototypical DPC-forming agent.[31−33]

In conclusion, our study demonstrates that synthetic DPC-inducing protein reagents are cytotoxic and mutagenic to mammalian cells. Because these protein reagents are incapable of inducing any type of DNA damage other than DPCs, the observed cell death and mutations resulting from electroporation with these agents must be a result of the formation of covalent DPC lesions on the chromosomal DNA. These findings are important because they provide the first direct evidence of DPC-mediated cytotoxicity. We anticipate that these engineered proteins, as well as other monoepoxide molecules prepared from additional protein and peptide substrates, will prove to be useful in the future in efforts to more fully characterize the mechanisms of formation and repair of DPCs in mammalian cells.

■ ASSOCIATED CONTENT

Supporting Information

ESI^+ mass spectra of EB-treated K125L AGT protein (Figure S-1), ESI^+ spectra of DEB-treated K125L AGT before and after a 4 h incubation at 37 °C (Figure S-2), and HPLC−ESI^+-MS/MS analysis of AGT tryptic peptides containing a THB monoadduct at Cys^{145} (Figure S-3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AGT, O^6-alkylguanine DNA alkyltransferase; BSA, bovine serum albumin; CHL, Chinese hamster lung; Cys-N7G-BD, 1-(*S*-cysteinyl)-4-(guan-7-yl)-2,3-butanediol; DEB, 1,2,3,4-diepoxybutane; DPC, DNA−protein cross-link; EB, 3,4-epoxy-1-butene; HEB, 2-hydroxy-3,4-epoxybut-1-yl; HPLC−ESI^+-MS/MS, high-performance liquid chromatography−electrospray ionization tandem mass spectrometry; NER, nucleotide excision repair; THB, trihydroxybutyl; TFA, trifluoroacetic acid.

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