Published in final edited form as:

Environ Mol Mutagen. 2009 July ; 50(6): 502–514. doi:10.1002/em.20491.

Effect of *O*⁶-Alkylguanine-DNA Alkyltransferase on Genotoxicity of Epihalohydrins

Aley G. Kalapila, Natalia A. Loktionova, and Anthony E. Pegg*

Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Abstract

The effect of *O*⁶-alkylguanine-DNA alkyltransferase (AGT) on the toxicity and mutagenicity of epihalohydrins was studied. AGT is a DNA repair protein that protects cells from agents that produce genotoxic *O*⁶-alkylguanine lesions by transferring the alkyl group to an internal cysteine residue (Cys¹⁴⁵ in human AGT) in a single-step. This cysteine acceptor site is highly reactive and epihalohydrins reacted readily with AGT at this site with a halide order of reactivity of Br > Cl > F. AGT expression in bacterial cells caused a very large increase in the mutagenicity and cytotoxicity of epibromohydrin. The mutations were almost all G:C to A:T transitions. Epichlorohydrin also augmented AGT-mediated mutagenesis but to a lesser extent than epibromohydrin. *In vitro* experiments showed that AGT was covalently cross-linked to DNA in the presence of epibromohydrin and that this conjugation occurred predominantly at Cys¹⁴⁵, and to a smaller extent at Cys¹⁵⁰, a less reactive residue also located within the active site pocket. Two pathways yielding the AGT-DNA adduct were found to occur. The predominant mechanism results in an AGT-epihalohydrin intermediate, which, facilitated by the DNA binding properties of AGT, then reacts covalently with DNA. The second pathway involves an initial reactive DNA-epihalohydrin intermediate that subsequently reacts with AGT. Our results show that the paradoxical AGT-mediated increase in genotoxicity which has previously been shown to occur with dihaloalkanes, butadiene diepoxide and nitrogen mustards, also occurs with epihalohydrins and is likely to contribute to their toxicity and mutagenicity.

Keywords

alkyltransferase; epihalohydrins; epibromohydrin; epichlorohydrin; DNA-protein cross-links

INTRODUCTION

Exposure to endogenous and exogenous alkylating agents can trigger substantial cytotoxicity, mutagenicity and carcinogenicity via alkyl adducts at the guanine-*O*⁶ position. These adducts are repaired by the action of the unique DNA repair protein, *O*⁶-alkylguanine-DNA alkyltransferase (AGT)

*Correspondence to: Anthony E. Pegg, Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033. aep1@psu.edu.

(Pegg, 2000; Margison and Santibáñez-Koref, 2002; Margison et al., 2003; Tubbs et al., 2007). AGT binds to DNA and brings about the transfer of the alkyl group from O^6 -alkylguanine to a cysteine residue [Cys¹⁴⁵ in human AGT (hAGT)] in a single-step, stoichiometric, irreversible reaction. Biochemical and structural studies of the hAGT reaction have provided significant insight into this mechanism of DNA repair (Daniels et al., 2000; Daniels et al., 2004; Duguid et al., 2005; Zang et al., 2005; Tubbs et al., 2007; Hu et al., 2008). AGT uses a helix-turn-helix motif to bind substrate DNA through the minor groove. The target alkylated guanine deoxyribonucleoside is flipped out from the base stack into the hAGT active site pocket via a 3' phosphate rotation, which is promoted by Tyr¹¹⁴ and stabilized by an Arg finger (Arg¹²⁸). The displaced alkylated guanine deoxyribonucleoside is positioned within the active site such that the alkyl group is in close proximity to the side-chain of Cys¹⁴⁵, which has a very high reactivity (Guengerich et al., 2003) due to its activation to a thiolate anion by a Glu¹⁷²-His¹⁴⁶-water-Cys¹⁴⁵ hydrogen bond network (Daniels et al., 2000; Tubbs et al., 2007). Attack by this Cys¹⁴⁵ then leads to the alkyl transfer reaction, which is facilitated by reduction of the negative charge on the repaired guanine via the donation of a hydrogen bond from the -OH of Tyr¹¹⁴ to N3 of the O^6 -methylguanine substrate (Daniels et al., 2000; Daniels et al., 2004; Tubbs et al., 2007). Another Cys residue (Cys¹⁵⁰) located on the outer perimeter of the active site pocket of hAGT, does not participate in the repair mechanism and can be mutated to Ser without affecting the DNA binding or subsequent alkyl transfer reaction (Rasimas et al., 2003).

Since AGT is highly protective against the genotoxic effects of many alkylating agents (Pegg, 2000; Margison and Santibáñez-Koref, 2002; Margison et al., 2003; Tubbs et al., 2007), the observation by Abril and colleagues (Abril et al., 1995; Abril et al., 1997) that *Escherichia coli* cells overexpressing hAGT or Ogt, an *E. coli* homolog of AGT, exhibited enhanced cell-killing and mutations when exposed to the bis-electrophiles, 1,2-dibromoethane or dibromomethane, was unexpected. Studies by Liu et al. validated these findings while also providing a possible mechanism of action (Liu et al., 2000; Liu et al., 2002a). 1,2-Dibromoethane reacts with AGT at the Cys¹⁴⁵ alkyl acceptor site to generate a S-(2-bromoethyl) intermediate, which rearranges into a highly reactive half-mustard. Due to its DNA-binding ability, AGT brings this intermediate into close contact with DNA where it reacts and forms a covalent adduct, thus cross-linking AGT to DNA. Depurination of the adduct or further processing to reduce the size of the protein-DNA conjugate could facilitate mutagenesis during subsequent rounds of replication. A similar mechanism of protein-DNA cross-linking was observed for other dihaloalkanes containing bromine or iodine as well as BrCH₂Cl and Br(CH₂)₂Cl. Dichloroalkanes, however, were ineffective at generating such AGT-DNA adducts (Liu et al., 2004a; Valadez et al., 2004).

Based on these data, Guengerich suggested that other *bis*-electrophiles might also interact with AGT to cause DNA damage in a similar way (Valadez et al., 2004; Guengerich, 2005). This suggestion was supported by experiments showing that AGT expression in both *E. coli* and *Salmonella typhimurium* cells augmented cytotoxicity and mutagenesis on exposure to 1,3-butadiene diepoxide (Valadez et al., 2004). Further studies of the interaction of 1,3-butadiene diepoxide with hAGT and DNA demonstrated

some differences compared to the mechanism for AGT-mediated dihaloalkane toxicity described above (Loeber et al., 2006; Kalapila et al., 2008). The diepoxide was shown to interact not only with Cys¹⁴⁵ but also with the Cys¹⁵⁰ residue on hAGT; adducts were generated either by an initial reaction between AGT and the compound followed by attack on DNA or via formation of a diepoxide-DNA adduct which then reacted with AGT. Recently, it has been shown that AGT can also be linked to DNA in these ways via a reaction with nitrogen mustards (Loeber et al., 2008).

The epihalohydrins are another class of bifunctional electrophiles. These epoxides (Fig. 1) have a halogen substituent on the carbon atom adjacent to the oxirane ring. The presence of the heterocyclic ring confers significant ring strain, and hence they are highly reactive compounds. For this reason, they are widely used in chemical industries as intermediates for various synthetic products such as resins, gums, paints, varnishes, manufacture of glycerol and curing propylene-based rubbers (Kolman et al., 2002). Consequently, there is a possibility of occupational exposure during the production of these compounds and during the synthesis of their end products.

Both epichlorohydrin (1,2-epoxy-3-chloropropane, ECH) and epibromohydrin (1,2-epoxy-3-bromopropane, EBH) have been shown to be direct-acting alkylating agents reacting with nucleophilic sites in cellular macromolecules (Ehrenberg and Hussain, 1981) and inducing DNA damage (Kolman et al., 1997; Mlejnek and Kolman, 1999). In *S. typhimurium* tester strains specific for base pair substitutions, ECH and EBH have demonstrated enhanced mutagenesis (Stolzenberg and Hine, 1979; Thier et al., 1995). Furthermore, *Drosophila melanogaster* exposed to EBH became more prone to somatic mutations and recombination (Chroust et al., 2007). *In vitro* studies with ECH have indicated that it reacts with DNA primarily at ring nitrogen atoms. The most prevalent adduct is at the *N7*-position of guanine because of the nucleophilicity and steric availability of that position (Kolman et al., 2002). However, evidence does exist for alkylation of DNA at alternative sites, mainly the *N1* and *N3*-positions of adenine and the *N3*-position of cytosine (Sund and Kronberg, 2006). Due to their instability, several of these adducts undergo chemical transformation yielding secondary DNA damage, which could likely be responsible for some of the genotoxicity seen with these compounds in cell culture (Qian and Dipple, 1995; Koskinen and Plna, 2000; Plna et al., 2000).

Because of their bifunctional nature it appeared possible that AGT-mediated DNA damage might also contribute to the cytotoxicity and mutagenicity of these epihalohydrins. In this paper, we describe experiments to test this hypothesis. The incidence of mutations after treatment with EBH was greatly increased by the presence of wild type hAGT (wt-hAGT) and there was a significant, albeit lower, increase when ECH was used. EBH and, to a lesser extent, ECH inactivated hAGT *in vitro*. Furthermore, the data demonstrated that AGT-DNA cross-links were generated by EBH either by the initial reaction of EBH with DNA or via the formation of a reactive derivative of wt-hAGT that subsequently reacted with DNA. Studies with hAGT mutants showed that residue Arg¹²⁸ (whose side chain is critical for DNA binding) was essential for the increase in mutations and that the

majority of mutations were due to hAGT-DNA adducts at the Cys¹⁴⁵ active site residue although Cys¹⁵⁰ could also participate.

MATERIALS AND METHODS

Materials

EBH (98% purity), ECH (98% purity), epifluorohydrin (EFH) (98% purity) and rifampicin (97% purity) were from Sigma Aldrich Chemical Co. (St. Louis, MO). Isopropyl β -D-thiogalactopyranoside (IPTG), ampicillin, and kanamycin were purchased from Fisher Scientific (Pittsburgh, PA). *Dra* III was from New England Biolabs (Ipswich, MA). T4 polynucleotide kinase (PNK) was obtained from Promega (Madison, WI). Adenosine 5'-[γ -³⁵S]thiotriphosphate triethyl ammonium salt ([γ -³⁵S]ATP γ S) was from GE Healthcare Life Sciences (Piscataway, NJ). All the oligodeoxyribonucleotides used for these studies were synthesized by Integrated DNA Technologies (Coralville, IA). The DNA substrate containing *O*⁶-methylguanine was prepared from calf thymus DNA which had been methylated by reaction with N[³H]methyl-N-nitrosourea as described (Kanugula et al., 1995).

Bacterial strains and plasmids

Table 1 shows the bacterial strains used in this study. The *E. coli* TRG8 strain has enhanced cell membrane permeability to exogenous chemicals (Xu-Welliver et al., 1998). The bacterial cells were derived from the strain GWR109, which was the generous gift of Dr. Leona Samson (Department of Molecular & Cellular Toxicology, Harvard School of Public Health, Boston, MA). GRW109 and TRG8 cells have the *ogt* and *ada* genes replaced by a kanamycin resistance cassette (Rebeck and Samson, 1991). The proteins used in the study include wt-hAGT (Crone et al., 1996), and its mutants R128A (Kanugula et al., 1995), C145A (Crone et al., 1996), C150S (see below), R128A/C145A (Kalapila et al., 2008) and C145A/C150S (Kalapila et al., 2008). Due to the altered active site, the C145A mutants are unable to perform the normal repair function of AGT. Mutating the Arg¹²⁸ residue interferes with the DNA binding properties of the repair protein. The Cys¹⁵⁰ residue, although located on the boundaries of the active site of AGT, does not participate in the repair function of AGT. The wt-hAGT and its mutants were expressed in *E. coli* TRG8 cells using pINIII-A3(*lpp*^{P-5}) vector which is resistant to ampicillin (Duffaud et al., 1987). All the cells were maintained under constant kanamycin (50 μ g/ml) and ampicillin (100 μ g/ml) selection. Western blot analysis was used to ensure equal expression levels of all hAGT proteins.

In order to construct pIN-C150S, pIN-C145A/C150S plasmid DNA was digested with *Dra* III, which cuts immediately after the Cys¹⁴⁵ codon in the hAGT sequence and 150 nucleotides after the stop codon in the pIN vector. The 333 bp fragment containing the C150S mutation was isolated, ligated with pIN-hAGT plasmid digested with *Dra* III, and transformed into *E. coli* TRG8 cells. The presence of a single C150S mutation was verified by sequencing.

The *S. typhimurium* strain YG7108 (Table I) is a derivative of TA1535 that lacks both *ada* and *ogt* genes, which are substituted by kanamycin and chloramphenicol resistance cassettes (Yamada et al., 1995; Yamada et al.,

1997). YG7108 cells were transformed either with empty pIN vector or pIN-hAGT as described (Valadez et al., 2004) and maintained under kanamycin and ampicillin selection (50 µg/ml each).

Measurement of cell survival and mutations in *E. coli* and *S. typhimurium*

The *E. coli* TRG8 cells transformed with either empty pIN vector or the pIN-hAGT, pIN-R128A, pIN-C145A, pIN-R128A/C145A, pIN-C150S, pIN-C145A/C150S plasmids were used to measure toxicity and mutagenicity of epihalohydrins as described previously (Kalapila et al., 2008). Briefly, exponentially growing cells in LB broth ($OD_{600nm} = 0.5-0.6$, 37°C) were exposed to 150 µM IPTG for 30 min to induce expression of wt-hAGT or its mutants. Cells were pelleted, resuspended in M9 medium and treated with epihalohydrins (0.5 M stock solutions in DMSO) at 37°C for 30 min with shaking. Cells were washed with M9 medium and resuspended in LB broth. Aliquots of diluted cultures were plated on ampicillin/kanamycin supplemented LB plates in order to measure cell survival. To determine mutation rates, the forward mutation assay which tests for acquired resistance to the antibiotic rifampicin (Rif^{res}) was used. Rif^{res} occurs as a result of mutations in the *rpoB* gene of *E. coli*, which alters the β subunit of RNA polymerase (Garibyan et al., 2003). The cells were left growing overnight and plated on LB plates supplemented with rifampicin (100 µg/ml). To calculate mutation frequency, the number of colonies on the rifampicin-supplemented plates was expressed per 10⁸ surviving cells that grew on the LB plates without rifampicin.

Cell survival and mutation rates of *S. typhimurium* YG7108 cells transformed with either empty pIN vector or pIN-hAGT were determined as previously described (Valadez et al., 2004). The protein expression was induced by 200 µM IPTG at 37°C for 30 min prior to exposure to epihalohydrins for 30 min (stock solutions in DMSO). Cell survival was estimated on LB plates supplemented with ampicillin and kanamycin (50 µg/ml each). Mutants were measured by the reversion from dependence on histidine (hisG⁺). Aliquots of 100 µl of undiluted cultures were plated on Vogel-Bonner minimal plates with excess biotin and trace amount of L-histidine. The mutation frequency of hisG⁺ revertants was calculated as the number of colonies grown on the selective medium over 10⁸ survivors grown on the nonrestrictive plates.

Rif^{res} mutation analysis

Rif^{res} TRG8 colonies, from the mutagenesis experiments described above, were isolated and used as DNA template for PCR amplification using primers specific for a region of the *rpoB* gene, as described previously (Liu et al., 2004a; Kalapila et al., 2008). The size of the DNA fragment (~700 base pairs) was verified by electrophoresis in a 0.8% agarose gel in Tris-acetate-EDTA buffer. The PCR products were purified using the QiaQuick PCR purification kit from Qiagen and sequenced to determine the mutation site.

Protein Purification

The recombinant hAGT-C145A and hAGT-C145A/C150S mutant proteins have an N-terminal (His)₆-tag that replaces the terminal M- with the sequence MRGS(H)₆GS- (Edara et al., 1996). The wt-hAGT and hAGT-C145S recombinant proteins used for the AGT inactivation studies and the gel

electrophoresis studies have a C-terminal (His)₆-tag replacing residues 202–207 (–PPAGRN) with –HHHHHH (Liu et al., 2002b). All these recombinant proteins were cloned into a pQE expression plasmid (Edara et al., 1996; Liu et al., 2002b) and purified as described previously (Fang et al., 2005). Recombinant N-terminal (His)₆-tagged antizyme protein was purified as described previously (Mackintosh et al., 2000).

AGT inactivation studies

The inactivation of purified wt-hAGT by epihalohydrins was measured as previously described (Nelson et al., 2004). The purified hAGT protein (20 ng) was incubated with the epihalohydrins in 0.5 ml of 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM dithiothreitol and 50 µg hemocyanin for 30 min. The residual AGT activity was determined after incubation with [³H]methylated calf thymus DNA substrate for 30 min at 37°C by measuring the [³H]methylated protein formed and collected on nitrocellulose filters.

Gel Electrophoresis analysis of EBH-mediated hAGT-DNA cross-linking *in vitro*

The 16-mer oligonucleotide used for these studies was 5′-d[(GGA)₅G]-3′ (Liu et al., 2002a). Analysis of hAGT-DNA cross-linking was done as described previously (Kalapila et al., 2008). Briefly, the oligo was radiolabeled at its 5′-end with [γ-³⁵S]ATPγS using PNK (Promega Corporation, Madison, WI). The C-terminal tagged wt-hAGT and C145S mutant, the N-terminal tagged C145A mutant and C145A/C150S double mutant, and antizyme protein (2 µg) were incubated in separate 10 µl reaction mixtures along with 25 mM EBH, 2 pmol [³⁵S]-labeled oligo, and 20 pmol unlabeled oligo in 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 5 mM dithiothreitol at 37 °C for 3 h. Following this incubation, 1 µl of 10% SDS solution and 2 µl of 5x SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 10% SDS, 0.5% bromophenol blue, and 50% glycerol) were added to the reaction mixture. The samples were incubated at room temperature for 10–15 min prior to separation by 12% SDS-PAGE. The gels were stained with Coomassie blue and dried (results not shown). Intensities of oligonucleotide bands were examined and analyzed using both autoradiography as well as a Molecular Dynamics PhosphorImager SI. The amount of oligo in the lower-mobility hAGT complex forms was quantified using the ImageQuant application software (ImageQuant v5.2, GE Healthcare Life Sciences, Piscataway, NJ).

Study of the order of reactivity hAGT with EBH and DNA leading to the formation of hAGT-DNA adducts

A time-course gel electrophoresis analysis was conducted, as described previously (Kalapila et al., 2008), to determine whether EBH reacted (a) with wt-hAGT first to form a compound-protein intermediate that subsequently reacts with DNA, or (b) whether the preliminary reaction entailed a DNA-EBH intermediate that ultimately yields hAGT-DNA cross-links. Similar experiments were also conducted using the hAGT-C145S mutant. For (a), 2 µg aliquots of either C-terminal tagged wt-hAGT or C145S protein were incubated for various lengths of time (0, 10, 30, or 60 min) at 37 °C with 25 mM EBH. Following this preincubation, 2 pmol of [³⁵S]-labeled oligo and 20 pmol unlabeled oligo were added to the reaction mixture, incubated at 37 °C for an additional 3 h, and run on 12% SDS-PAGE. For (b), [³⁵S]-labeled oligo and unlabeled oligo were preincubated at 37 °C for different lengths of time (0, 10, 30, or 60 min) with 25 mM EBH. Each sample was passed through the

MicroSpin G-25 columns (GE Healthcare Life Sciences, Piscataway, NJ) to remove any remaining unreacted free compound prior to the addition of the hAGT protein. Following this, C-terminal tagged wt-hAGT or C145S protein was added and the reaction was allowed to proceed for another 3 h before separation by SDS-PAGE. The gels were dried and analyzed as described above.

RESULTS

AGT-mediated toxicity and mutagenicity of EBH and ECH

The effect of AGT on the cytotoxicity and mutagenicity of EBH was examined by expressing hAGT in either *E. coli* TRG8 cells (Fig. 2) or *S. typhimurium* YG7108 cells (Fig. 3). These strains have no endogenous AGT since both the *ada* and *ogt* genes are inactivated (Rebeck and Samson, 1991; Yamada et al., 1995; Yamada et al., 1997). EBH produced minimal cytotoxicity (>90% survival) at up to 5 mM in *E. coli* TRG8 cells containing an empty vector or expressing inactive hAGT mutants (C145A and C145A/C150S). There was a significant loss of survival when a plasmid encoding wt-hAGT was used (Fig. 2A).

Mutations caused by EBH were determined using the forward assay to rifampicin resistance (Garibyan et al., 2003). An enormous increase in mutation frequency (c. 1000-fold) was observed with wt-hAGT expression when compared to empty vector (Fig. 2B). The C145A mutant was much less efficient at inducing mutations than wt-hAGT but was still able to increase mutations by c. 100-fold. The C145A/C150S double mutant was much less effective with only a slight increase (c. 3 – 4.5 fold) over the empty vector control. In separate experiments, TRG8 cells transformed with the C150S mutant exhibited similar number of mutants to the cells expressing wt-hAGT (Fig. 4A). All these increases in mutation rates were statistically significant compared to the pIN control strain when analyzed by two-way ANOVA (Fig. 2B) and unpaired t-test (Fig. 4A). These results are broadly similar to those found in the same *E. coli* strains treated with 1,3-butadiene diepoxide except that EBH demonstrated even greater mutagenicity in TRG8 cells expressing hAGT (Kalapila et al., 2008).

The DNA-binding properties of hAGT are clearly needed for the augmented mutagenic potential of EBH. Strains expressing the R128A or R128A/C145A mutants showed little or no increase in mutations above those seen with the pIN vector (Fig. 4A). The side chain of Arg¹²⁸ is essential to stabilize the hAGT-DNA complex and the small side chain of the Ala¹²⁸ replacement in this mutant is unable to do this (Kanugula et al., 1995; Daniels et al., 2000; Daniels et al., 2004).

Expression of wt-hAGT or the C150S mutant also increased the number of mutations caused by ECH in *E. coli* TRG8 cells (Fig. 4B). This increase, however, was much less than seen following EBH exposure (Fig. 2B and 4A). As with EBH, loss of DNA-binding ability due to the R128A mutation prevented the increase in mutations with ECH exposure (Fig. 4B). ECH produced minimal cytotoxicity at up to 5 mM in *E. coli* TRG8 cells expressing wt-hAGT and all the mutants tested including C150S (results not shown).

Results obtained from similar experiments using *S. typhimurium* YG7108 cells also showed that wt-hAGT significantly increased the cytotoxicity (Fig. 3A) and mutations (Fig. 3B) caused by EBH. In these cells the enhanced hAGT-mediated mutation rate in YG7108 was only c. 3 to 10-fold higher when compared to the pIN strain (Fig. 3B). There are many possible reasons for this difference. For examples: the reporter mutation used in *S. typhimurium* was a His⁺ reversion that requires a change at a specific G:C pair as compared to the more general *rpoB* forward mutation assay used in *E. coli*; and the hAGT expression level is likely to be higher in the *E. coli* strain used.

Expression of wt-hAGT in YG7108 cells did not enhance the cytotoxicity of ECH, but led to a moderate increase in mutations compare to cells with empty pIN vector (results not shown). Exposure to EFH affected neither cytotoxicity, nor mutations in those cells (results not shown).

***RpoB* mutation spectra induced by EBH in *E. coli* in the presence and absence of hAGT**

Tables II and III show an analysis of the mutation spectra at the *rpoB* locus in the *E. coli* TRG8 cells with and without hAGT following exposure to 2 mM EBH. Mutations were observed at 16 sites (Table II). At the 2 mM dose of EBH used, the frequency of mutations was increased more than 1400-fold by the presence of hAGT (Table III). The presence of the C145A mutant increased mutations by 17-fold and the C145A/C150S double mutant increased mutations by 4.6 fold (Table III).

In cells lacking hAGT expression, the mutations seen were 56% transitions and 44% transversions and 67% were at G:C sites. When wt-hAGT or the C145A mutant was expressed, the vast majority (89–90%) of mutations were transitions at G:C sites (Table II). There was some difference, however, in the sites of these base changes, since with wt-hAGT the mutations were mostly located at the specific codons of the *rpoB* gene encoding Arg⁵²⁹ (5'-CGT-3') and Ser⁵³¹ (5'-TCC-3') whereas with C145A expression the mutations were predominantly at the Ser⁵³¹ (5'-TCC-3') and Asp⁵¹⁶ (5'-GAC-3') codons (Table II). Mutations in the *E. coli* TRG8 cells transformed with the C145A/C150S double mutant also showed enhanced reactivity at G:C base-pairs but with a greater proportion of G:C to T:A transversions and a possible hotspot at the Asp⁵¹⁶ codon.

Inactivation of AGT by epihalohydrins

Incubation of purified hAGT with EBH *in vitro* led to a rapid loss of activity with 50% decrease within 30 min of exposure to 0.1 mM (Fig. 5). ECH, while also capable of inactivating AGT, was much less potent requiring about 0.4 mM for 50 % reduction. EFH, requiring 1.5 mM, demonstrated considerably lower efficiency at inactivating hAGT. These results suggest that these compounds react with the highly reactive cysteine acceptor site located at position Cys¹⁴⁵ in hAGT (Guengerich et al., 2003).

Formation of hAGT-DNA adducts by EBH

Since EBH is a bifunctional compound, the reaction with AGT may generate a reactive species capable of covalently cross-linking with DNA as previously demonstrated for dihaloalkanes and butadiene diepoxide (Liu et al., 2002a; Liu et al., 2004b; Valadez et al., 2004; Loeber et al., 2006; Kalapila et al.,

2008). This possibility was examined by the incubation of EBH, hAGT and a 16-mer oligonucleotide, 5'-d[(GGA)₅G]-3', labeled with [³⁵S] at its 5'-end. The products of this reaction were then analyzed by 12% SDS-PAGE, conditions which disrupt the non-covalent interactions between AGT and DNA. A band corresponding to a covalent AGT-DNA adduct was observed. The position of this band was consistent with a 1:1 complex (Fig. 6). When the gels were exposed for a longer time there was also a faint band at higher MW corresponding to two AGT molecules conjugated with the 16-mer oligo (results not shown). The AGT-oligo was not seen in the absence of EBH or when an irrelevant control protein (antizyme) of similar size was used instead of AGT (Fig. 6).

In order to test whether the AGT-DNA cross-link was formed at Cys¹⁴⁵, mutant proteins in which this residue was changed to a Ser or an Ala were used. These mutant proteins also produced a shift in [³⁵S]-oligonucleotide mobility, comparable to results observed with wt-hAGT protein. Since this indicated that another site could also be used for cross-linking, we tested the double mutant with altered Cys¹⁴⁵ and Cys¹⁵⁰ residues (C145A/C150S), the latter being the only other Cys in the active site pocket of AGT. The covalent AGT-DNA adduct formation was greatly reduced by this mutant indicating the Cys¹⁵⁰ is the most important second site for EBH-mediated conjugation.

These results are broadly similar to those found for butadiene diepoxide (Loeber et al., 2006; Kalapila et al., 2008) but differ from 1,2-dibromoethane and dibromomethane, which form AGT-DNA adducts only at Cys¹⁴⁵ (Liu et al., 2002a; Liu et al., 2004b). The very weak formation of adducts with the C145A/C150S protein and EBH suggests that complex formation may also occur at another cysteine or possibly lysine residue on AGT in these *in vitro* experiments. However, such studies, which utilize high concentrations of AGT, suggest that binding to sites aside from Cys¹⁴⁵ and Cys¹⁵⁰ are unlikely to occur significantly under intracellular conditions with lower AGT levels and many competing nucleophiles.

It was established that 1,2-dibromoethane-induced hAGT-DNA adduct formation occurred via a reactive intermediate generated from an initial reaction between DBE and hAGT (Liu et al., 2002a; Liu et al., 2004b). Later studies have shown that 1,3-butadiene diepoxide and nitrogen mustards can cross-link AGT to DNA in a manner similar to that seen with DBE, but also via an initial reaction with DNA (Loeber et al., 2006; Kalapila et al., 2008; Loeber et al., 2008). The results shown in Fig. 6 indicate that EBH does react rapidly with AGT, which would allow the former pathway. However, since EBH is known to form DNA adducts directly (Koskinen and Plna, 2000; Romano et al., 2007), the second pathway could also contribute to the EBH-mediated AGT-DNA cross-linking. Experiments to investigate this possibility and to establish the stability of the reactive intermediates involved were carried out by initially incubating EBH with either hAGT or with the oligo for up to 60 min and then adding the third component to allow the reactive intermediate to form the cross-link in a further 3 h period before separation by SDS-PAGE. For the experiments involving pre-incubation with oligo, the samples were run through spin columns in order to remove any excess of unreacted EBH. Consequently, by the time the

protein was added, the only species left in the reaction mix should have been the [³⁵S]-oligo and any [³⁵S]-oligo-EBH intermediate.

Fig. 7 shows results obtained when wt-hAGT or the C145S mutant protein was pre-incubated with EBH for differing lengths of time after which the [³⁵S]-labeled 5'-d[(GGA)₅G]-3' oligo was added to the sample and reacted for another 3 h. When wt-hAGT was used, the reactive complex formed was very unstable resulting in low yields of hAGT-DNA adducts after 30–60 min of preincubation. This indicates the instability of the reactive intermediate, which can react with water in the absence of DNA, as well as the rapidity of the reaction between hAGT and EBH at Cys¹⁴⁵, as expected from our data demonstrating the *in vitro* inactivation of hAGT by EBH (Fig. 5). In contrast, when the C145S mutant protein was used, the amount of hAGT-DNA conjugation increased over the entire 60 min preincubation period indicating that reaction with the Cys¹⁵⁰ site occurs at a slower rate and generates a more stable complex.

When EBH was preincubated with the oligonucleotide prior to protein addition, the amount of hAGT-DNA conjugation increased up to 30 min of preincubation and was then constant over the next 30 min (Fig. 8) suggesting that the formation of the EBH-DNA intermediate is relatively slow and that the complex is fairly stable in aqueous solution. Although there was no difference between wt-hAGT and C145S in the rate of cross-link formation, the greater band intensity observed with the wt-hAGT suggested that the Cys¹⁴⁵ site reacts far more readily with the EBH-DNA adduct than the Cys¹⁵⁰ residue.

DISCUSSION

Our studies extend the range of bifunctional agents whose toxicity and mutagenicity are increased in the presence of AGT to include EBH and ECH. The gel electrophoresis analyses demonstrated that exposure to EBH can lead to AGT-DNA cross-links via either the initial reaction of the epihalohydrin with the AGT protein (Fig. 9, Pathway 1) or with DNA (Fig. 9, Pathway 2). Once formed, the reactive EBH-AGT intermediate can either react with DNA (Pathways 1A1 and 1B1) or water or other cellular nucleophiles (Pathways 1A2 and 1B2). The adduct formed between EBH and Cys¹⁵⁰ was much more stable in an aqueous environment (Pathway 1B2) than the adduct formed at Cys¹⁴⁵ (Pathway 1A2). The altered environment of Cys¹⁵⁰ in the active site pocket may contribute to the increased intermediate stability at this residue. The Cys¹⁵⁰ site is situated next to residues (Tyr¹¹⁴ and Ser¹⁵¹) necessary for DNA binding of the repair protein that are on the outer boundary of the active site pocket (Loeber et al., 2006), whereas Cys¹⁴⁵ exists within an extensive hydrogen bond network, along with Tyr¹⁵⁸, His¹⁴⁶ and Glu¹⁷², at the bottom of a solvent accessible groove (Tubbs et al., 2007).

These covalent AGT-DNA adducts seen *in vitro* are likely primarily responsible for the genotoxicity observed in our bacterial model systems. Such covalent conjugation could be demonstrated *in vitro* with both wt-hAGT and the C145A mutant, suggesting that reaction can occur at either Cys¹⁴⁵ or Cys¹⁵⁰. The experiments conducted with *E. coli* TRG8 cells indicated that the AGT-mediated mutagenicity was not reduced by the use of the C150S mutant, but C145A protein produced significantly less

mutations (Fig. 2A). This implies that the reaction with Cys¹⁴⁵ to form the hAGT-DNA cross-link is the critical reaction intracellularly. The low pK_a of the Cys¹⁴⁵ residue, due to its activation by the linked Glu¹⁷²-His¹⁴⁶-water-Cys¹⁴⁵ hydrogen bond network, probably facilitates the reaction via pathway 1A (Guengerich et al., 2003; Daniels et al., 2004; Tubbs et al., 2007). It is possible that with an available Cys¹⁴⁵, cross-linking at the much less reactive Cys¹⁵⁰ site does not occur since there was no significant difference in mutations between cells expressing wt-hAGT and those expressing the C150S mutant.

The increase in cytotoxicity of epihalohydrins mediated via AGT is a less sensitive assay than the increase in mutations but the results of the studies on cytotoxicity (Fig. 2A) with wt-hAGT and the active site mutants are also in agreement with the above hypothesis. Cell survival decreased with wt-hAGT following EBH exposure but not with the C145A mutant. Preliminary experiments also indicated greater cell-killing with the C150S mutant strain (results not shown) suggesting that the Cys¹⁴⁵ residue as a cross-link site has greater impact on cell survival than Cys¹⁵⁰. This enhanced cytotoxicity is most probably due to the blocking of replicative polymerases by covalent hAGT-DNA crosslinks whereas the amplified mutations are likely a result of (mis)repair and error-prone bypass of these bulky lesions. The cytotoxicity combined with the mutagenesis data indicates that conjugation at the more reactive Cys¹⁴⁵ is the critical interaction in the cell with regard to genotoxic outcomes.

Additionally, it is noteworthy that another outcome of the facile reaction between EBH and the Cys¹⁴⁵ acceptor site of hAGT is that the repair protein is inactivated and can no longer take part in the removal of alkylation damage. Thus, by inactivating AGT, exposure to EBH would render cells more susceptible to genotoxic alkylation damage at the guanine-*O*⁶ site by environmental mutagens.

The epihalohydrin-mediated formation of AGT-DNA cross-links and its subsequent intracellular implications on cytotoxicity and mutagenesis differ, in two ways, from those following dihaloalkane exposure. The reaction with EBH can occur with the C145A mutant at the Cys¹⁵⁰ site and these *in vitro* findings correlate well with the data in *E. coli* TRG8 cells showing a significant increase in mutations with the C145A expressing strain (Fig. 2). Experiments with dihaloalkanes demonstrated the complete inability of the C145A mutant protein to cross-link with DNA and subsequently potentiate mutagenesis (Liu et al., 2002a; Liu et al., 2004a; Liu et al., 2004b). Secondly, AGT-DNA conjugation can occur via an initial EBH-DNA intermediate followed by reaction with AGT. Dihalalkane-mediated cross-linking, however, occurs solely via a preliminary reaction with AGT. In these respects, the epihalohydrins exhibit similar characteristics to 1,3-butadiene diepoxide (Loeber et al., 2006; Kalapila et al., 2008) and nitrogen mustards (Loeber et al., 2008), which can also react with C145A and can also form AGT-DNA complexes via both initial reaction with AGT and initial reaction with DNA.

When *E. coli* TRG8 cells expressing wt-hAGT were exposed to similar doses of EBH or 1,3 butadiene diepoxide (Kalapila et al., 2008), exposure to EBH gave rise to a much larger number of mutations (by c. 25-fold). Our *in vitro*

results suggest a possible explanation for this. In the absence of DNA, the wt-hAGT-EBH intermediates were much more stable than those generated with butadiene diepoxide (Kalapila et al., 2008). The stability of both the protein-compound and compound-DNA intermediates detected by gel electrophoresis may translate to their persistence intracellularly, thus permitting greater chance for reaction to yield AGT-DNA adducts and consequently, enhanced genotoxicity.

A previous publication using *S. typhimurium* strain TA-100 reported that EBH induced mutations (Stolzenberg and Hine, 1979). This strain contains low levels of endogenous AGT, which may have contributed to the mutant formation. We observed a significant mutation rate increase with concomitant decrease in cell survival in the hAGT-expressing *S. typhimurium* strain YG7108 (Fig. 3) compared to the empty vector strain. It remains unclear, why even in the absence of hAGT expression, EBH produced mutations in *S. typhimurium* YG7108 cells but not in *E. coli* TRG8 cells in our experiments.

The *in vitro* inactivation of purified hAGT by epihalohydrins clearly showed that their reactivity follows the halide order Br > Cl > F (Fig. 5). Our results with *E. coli* and *S. typhimurium* cells confirmed the same order of reactivity and are in agreement with data obtained for dihaloalkanes (Valadez et al., 2004). Experiments with *S. typhimurium* YG7108 cells demonstrated that expression of wt-hAGT did not significantly increase the cytotoxicity of ECH or the cytotoxicity and the mutagenicity of EFH (results not shown). ECH did exhibit an AGT-mediated mutagenic effect in *E. coli* TRG8 cells (Fig. 4B) but this was not as great as that seen with EBH (Fig. 2B and Fig 4A), supporting the *in vitro* inactivation data (Fig. 5).

Prior work with ECH has indicated that the epihalohydrins are capable of inducing a wide variety of DNA damage via direct reaction (Kolman et al., 2002; Romano et al., 2007) but there is limited information on the mutation profile of either EBH or ECH. *In vitro* analyses have demonstrated the formation of epihalohydrin induced DNA adducts at nitrogen atoms of guanine and adenine bases (Singh et al., 1996; Sund and Kronberg, 2006; Romano et al., 2007). In the absence of any significant number of mutations in EBH-treated cells without AGT we cannot definitively assign the mutations seen in the empty vector expressing TRG8 cells to EBH. However, AGT expression in these cells facilitated a massive increase in mutations at G:C base-pairs. With wt-hAGT expression, virtually all of these mutations were G:C to A:T transitions, which is consistent with the predominant site of attack on DNA being at guanines. Depurination of AGT-DNA-N7-guanine adducts similar to those characterized from dihaloalkane-treated cells expressing wt-hAGT (Liu et al., 2002a; Liu et al., 2004a; Liu et al., 2004b) is unlikely to be the primary pre-mutagenic event with EBH-induced AGT mediated toxicity since such depurination would be expected to generate G:C to T:A transversions rather than the transitions which are observed. Even with dihaloalkanes, a substantial fraction of the mutations were G:C to A:T transitions (Liu et al., 2002a; Liu et al., 2004a; Liu et al., 2004b). Currently, we have not elucidated the adduct that leads to such mutations. An adduct at the N²-position of guanine would be a possibility but it seems likely that some proteolytic cleavage of the AGT protein from the DNA cross-link

would be required to allow bypass polymerases to copy past the lesion in an error-prone manner.

The mutation spectrum obtained for the TRG8 cells expressing C145A also showed an increased bias toward G:C to A:T transitions implying similarities between the structural and chemical nature of the cross-link and parallel mechanisms of mutagenesis at both the Cys¹⁴⁵ and Cys¹⁵⁰ sites in the presence of EBH. However, the mutation frequency of G:C to A:T transitions were almost 100-fold decreased than seen with wt-hAGT-TRG8 cells (Table III), supporting the hypothesis that reactivity and subsequent cross-linking at the Cys¹⁵⁰ residue is much less compared to the Cys¹⁴⁵ site. We have not investigated the possibility of sequence specificity in the *in vitro* formation of AGT-DNA cross-links mediated via EBH but the results of the mutagenesis studies suggest that there may be such specificity leading to two mutagenic hotspots at the codons corresponding to Arg⁵²⁹ and Ser⁵³¹ for wt-hAGT and Asp⁵¹⁶ as well as Ser⁵³¹ for the C145A mutant. Two of these hotspot mutations (Asp⁵¹⁶ and Ser⁵³¹) are within a 5'-GGA-3' context suggesting a possible sequence bias for AGT-DNA adduct formation following EBH exposure. Given these results, it is possible that if cross-linking and subsequent processing of adducts occurs at Cys¹⁵⁰, it may preferentially occur at 5'-GGA-3' sites, whereas AGT conjugation at Cys¹⁴⁵ does not necessarily have a sequence bias.

An increased incidence of mutations at G:C base-pairs was also seen with expression of the C145A/C150S double mutant (Table III). This small but statistically significant increase may be due to cross-linking at a third site but could also be due to the hAGT mutant, with its functional DNA binding properties, binding to EBH-induced DNA alkylation damage and shielding it from other repair pathways.

Overall, we have shown that epihalohydrins can induce alkyltransferase-mediated genotoxicity with a halide order of reactivity Br > Cl > F. Unlike the dibromoalkanes that have been extensively studied in our laboratory, these epoxide compounds exert their hAGT-mediated toxicity by generating protein-DNA cross-links at both the Cys¹⁴⁵ active site and neighboring Cys¹⁵⁰ residue in a manner broadly similar to butadiene diepoxide and nitrogen mustards. AGT-DNA adduct formation specific for the Cys¹⁴⁵ active site and Cys¹⁵⁰ residue can originate from two different predecessors: an AGT-compound intermediate or a compound-DNA intermediate. These two distinct mechanisms are likely responsible for the heightened lethality and mutagenic potential of these adducts intracellularly. Additionally, it is important to emphasize that another outcome of this reaction is that the Cys¹⁴⁵ active site of hAGT is no longer accessible for removal of alkylation damage. Thus, the repair protein can no longer fulfil its normal cellular function, which can contribute to enhanced genotoxicity of these environmental mutagens *in vivo*.

Acknowledgments

This work was supported in part by United States Public Health Service Grants RO1 CA-018137 and RO1 CA-071976. We thank Chungun Du for technical assistance.

References

- Abril N, Luque-Romero FL, Prieto-Alamo MJ, Margison GP, Pueyo C. Ogt alkyltransferase enhances dibromoalkane mutagenicity in excision repair-deficient *Escherichia coli* K-12. *Mol Carcinogenesis* 1995;12:110–117.
- Abril N, Luque-Romero FL, Prieto-Alamo MJ, Rafferty JA, Margison GP, Pueyo C. Bacterial and mammalian DNA alkyltransferases sensitize *Escherichia coli* to the lethal and mutagenic effects of dibromoalkanes. *Carcinogenesis* 1997;18:1883–1888. [PubMed: 9363995]
- Chroust K, Pavlova M, Prokop Z, Mendel J, Bozkova K, Kubat Z, Zajickova V, Damborsky J. Quantitative structure-activity relationships for toxicity and genotoxicity of halogenated aliphatic compounds: wing spot test of *Drosophila melanogaster*. *Chemosphere* 2007;67:152–159. [PubMed: 17113125]
- Crone TM, Goodtzova K, Pegg AE. Amino acid residues affecting the activity and stability of human *O*⁶-alkylguanine-DNA alkyltransferase. *Mutat Res* 1996;363:15–25. [PubMed: 8632775]
- Daniels DS, Mol CD, Arvai AS, Kanugula S, Pegg AE, Tainer JA. Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical binding. DNA damage reversal revealed by mutants and structures of active and alkylated human AGT. *Embo J* 2000;19:1719–1730. [PubMed: 10747039]
- Daniels DS, Woo TT, Luu KX, Noll DM, Clarke ND, Pegg AE, Tainer JA. Novel modes of DNA binding and nucleotide flipping by the human DNA repair protein AGT. *Nat Struct Mol Biol* 2004;11:714–720. [PubMed: 15221026]
- Duffaud GD, March PE, Inouye M. Expression and secretion of foreign proteins in *Escherichia coli*. *Methods Enzymol* 1987;153:492–507. [PubMed: 3323807]
- Duguid EM, Rice PA, He C. The structure of the human AGT protein bound to DNA and its implications for damage detection. *J Mol Biol* 2005;350:657–666. [PubMed: 15964013]
- Edara S, Kanugula S, Goodtzova K, Pegg AE. Resistance of the human *O*⁶-alkylguanine-DNA alkyltransferase containing arginine at codon 160 to inactivation by *O*⁶-benzylguanine. *Cancer Res* 1996;56:5571–5575. [PubMed: 8971155]
- Ehrenberg L, Hussain S. Genetic toxicity of some important epoxides. *Mutat Res* 1981;86:1–113. [PubMed: 7015118]
- Fang Q, Kanugula S, Pegg AE. Function of domains of human *O*⁶-alkylguanine-DNA alkyltransferase. *Biochemistry* 2005;44:15396–15405. [PubMed: 16285744]
- Garibyan L, Huang T, Kim M, Wolff E, Nguyen A, Nguyen T, Diep A, Hu K, Iverson A, Yang H, et al. Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. *DNA Repair (Amst)* 2003;2:593–608. [PubMed: 12713816]
- Guengerich FP, Fang Q, Liu L, Hachey DL, Pegg AE. *O*⁶-alkylguanine-DNA alkyltransferase: Low pK_a and reactivity of cysteine 145. *Biochemistry* 2003;42:10965–10970. [PubMed: 12974631]
- Guengerich FP. Principles of covalent binding of reactive metabolites and examples of activation of bis-electrophiles by conjugation. *Arch Biochem Biophys* 2005;433:369–378. [PubMed: 15581593]
- Hu J, Ma A, Dinner AR. A two-step nucleotide-flipping mechanism enables kinetic discrimination of DNA lesions by AGT. *Proc Natl Acad Sci USA* 2008;105:4615–4620. [PubMed: 18353991]
- Kalapila AG, Loktionova NA, Pegg AE. Alkyltransferase-mediated toxicity of 1,3-butadiene diepoxide. *Chem Res Toxicol* 2008;21:1851–1861. [PubMed: 18712882]
- Kanugula S, Goodtzova K, Edara S, Pegg AE. Alteration of arginine-128 to alanine abolishes the ability of human *O*⁶-alkylguanine-DNA alkyltransferase to repair methylated DNA but has no effect on its reaction with *O*⁶-benzylguanine. *Biochemistry* 1995;34:7113–7119. [PubMed: 7766621]

- Kolman A, Spivak I, Naslund M, Dusinska M, Cedervall B. Propylene oxide and epichlorohydrin induce DNA strand breaks in human diploid fibroblasts. *Environ Mol Mutagen* 1997;30:40–46. [PubMed: 9258328]
- Kolman A, Chovanec M, Osterman-Golkar S. Genotoxic effects of ethylene oxide, propylene oxide and epichlorohydrin in humans: update review (1990–2001). *Mutat Res* 2002;512:173–194. [PubMed: 12464351]
- Koskinen M, Plna K. Specific DNA adducts induced by some mono-substituted epoxides *in vitro* and *in vivo*. *Chem Biol Interact* 2000;129:209–229. [PubMed: 11137062]
- Liu L, Xu-Welliver M, Pegg AE. The role of human *O*⁶-alkylguanine-DNA alkyltransferase in promoting 1,2-dibromoethane-induced genotoxicity in *Escherichia coli*. *Mutat Res* 2000;452:1–10. [PubMed: 10894884]
- Liu L, Pegg AE, Williams KM, Guengerich FP. Paradoxical enhancement of the toxicity of 1,2-dibromoethane by *O*⁶-alkylguanine-DNA alkyltransferase. *J Biol Chem* 2002a;277:37920–37928. [PubMed: 12151404]
- Liu L, Xu-Welliver M, Kanugula S, Pegg AE. Inactivation and degradation of *O*⁶-alkylguanine-DNA alkyltransferase after reaction with nitric oxide. *Cancer Res* 2002b;62:3037–3043. [PubMed: 12036910]
- Liu L, Hachey DL, Valadez G, Williams KM, Guengerich FP, Loktionova NA, Kanugula S, Pegg AE. Characterization of a mutagenic DNA adduct formed from 1,2-dibromoethane by *O*⁶-alkylguanine-DNA alkyltransferase. *J Biol Chem* 2004a;279:4250–4259. [PubMed: 14645247]
- Liu L, Williams KM, Guengerich FP, Pegg AE. *O*⁶-alkylguanine-DNA alkyltransferase has opposing effects in modulating the genotoxicity of dibromomethane and bromomethyl acetate. *Chem Res Toxicol* 2004b;17:742–752. [PubMed: 15206895]
- Loeber R, Rajesh M, Fang Q, Pegg AE, Tretyakova N. Cross-linking of the human DNA repair protein *O*⁶-alkylguanine DNA alkyltransferase to DNA in the presence of 1,2,3,4-diepoxybutane. *Chem Res Toxicol* 2006;19:645–654. [PubMed: 16696566]
- Loeber R, Michaelson E, Fang Q, Campbell C, Pegg AE, Tretyakova N. Cross-linking of the DNA repair protein *O*⁶-alkylguanine DNA alkyltransferase to DNA in the presence of antitumor nitrogen mustards. *Chem Res Toxicol* 2008;21:787–795. [PubMed: 18324787]
- Mackintosh CA, Feith DJ, Shantz LM, Pegg AE. Overexpression of antizyme in the hearts of transgenic mice prevents the isoprenaline-induced increase in cardiac ornithine decarboxylase activity and polyamines, but does not prevent cardiac hypertrophy. *Biochem J* 2000;350:645–653. [PubMed: 10970775]
- Margison GP, Santibáñez-Koref MF. *O*⁶-Alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. *BioEssays* 2002;24:255–266. [PubMed: 11891762]
- Margison G, Povey AC, Kaina B, Santibáñez-Koref MF. Variability and regulation of *O*⁶-alkylguanine-DNA alkyltransferase. *Carcinogenesis* 2003;24:625–635. [PubMed: 12727789]
- Mlejnek P, Kolman A. Effects of three epoxides-ethylene oxide, propylene oxide and epichlorohydrin-on cell cycle progression and cell death in human diploid fibroblasts. *Chem Biol Interact* 1999;117:219–239. [PubMed: 10190577]
- Nelson ME, Loktionova NA, Pegg AE, Moschel RC. 2-Amino-*O*⁴-benzylpteridine derivatives: potent inactivators of *O*⁶-alkylguanine-DNA alkyltransferase. *J Med Chem* 2004;47:3887–3891. [PubMed: 15239666]
- Pegg AE. Repair of *O*⁶-alkylguanine by alkyltransferases. *Mutat Res* 2000;462:83–100. [PubMed: 10767620]
- Plna K, Osterman-Golkar S, Nogradi E, Segerback D. ³²P-post-labelling of 7-(3-chloro-2-hydroxypropyl)guanine in white blood cells of workers occupationally exposed to epichlorohydrin. *Carcinogenesis* 2000;21:275–280. [PubMed: 10657968]
- Qian C, Dipple A. Different mechanisms of aralkylation of adenosine at the 1- and N6-positions. *Chem Res Toxicol* 1995;8:389–395. [PubMed: 7578925]

- Rasimas JJ, Kanugula S, Dalessio PM, Ropson IJ, Pegg AE. Effects of zinc occupancy on human *O*⁶-alkylguanine-DNA alkyltransferase. *Biochemistry* 2003;42:980–990. [PubMed: 12549918]
- Rebeck GW, Samson L. Increased spontaneous mutation and alkylation sensitivity of *Escherichia coli* strains lacking the *ogt* *O*⁶-methylguanine-DNA repair methyltransferase. *J Bacteriol* 1991;173:2068–2076. [PubMed: 2002008]
- Romano KP, Newman AG, Zahran RW, Millard JT. DNA interstrand cross-linking by epichlorohydrin. *Chem Res Toxicol* 2007;20:832–838. [PubMed: 17441735]
- Singh US, Decker-Samuelian K, Solomon JJ. Reaction of epichlorohydrin with 2'-deoxynucleosides: characterization of adducts. *Chem Biol Interact* 1996;99:109–128. [PubMed: 8620562]
- Stolzenberg SJ, Hine CH. Mutagenicity of halogenated and oxygenated three-carbon compounds. *J Toxicol Environ Health* 1979;5:1149–1158. [PubMed: 393836]
- Sund P, Kronberg L. Reaction of epichlorohydrin with adenosine, 2'-deoxyadenosine and calf thymus DNA: identification of adducts. *Bioorg Chem* 2006;34:115–130. [PubMed: 16549090]
- Thier R, Muller M, Taylor JB, Pemble SE, Ketterer B, Guengerich FP. Enhancement of bacterial mutagenicity of bifunctional alkylating agents by expression of mammalian glutathione S-transferase. *Chem Res Toxicol* 1995;8:465–472. [PubMed: 7578934]
- Tubbs JL, Pegg AE, Tainer JA. DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by *O*⁶-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. *DNA Repair (Amst)* 2007;6:1100–1115. [PubMed: 17485252]
- Valadez JG, Liu L, Loktionova NA, Pegg AE, Guengerich FP. Activation of *bis*-electrophiles to mutagenic conjugates by human *O*⁶-alkylguanine-DNA alkyltransferase. *Chem Res Toxicol* 2004;17:972–982. [PubMed: 15257623]
- Xu-Welliver M, Kanugula S, Pegg AE. Isolation of human *O*⁶-alkylguanine-DNA alkyltransferase mutants highly resistant to inactivation by *O*⁶-benzylguanine. *Cancer Res* 1998;58:1936–1945. [PubMed: 9581836]
- Yamada M, Sedgwick B, Sofuni T, Nohmi T. Construction and characterization of mutants of *Salmonella typhimurium* deficient in DNA repair of *O*⁶-methylguanine. *J Bacteriol* 1995;177:1511–1519. [PubMed: 7883708]
- Yamada M, Matsui K, Sofuni T, Nohmi T. New tester strains of *Salmonella typhimurium* lacking in *O*⁶-methylguanine-DNA methyltransferases and highly sensitive to mutagenic alkylating agents. *Mutat Res* 1997;381:15–24. [PubMed: 9403026]
- Zang H, Fang Q, Pegg AE, Guengerich FP. Kinetic analysis of steps in the repair of damaged DNA by human *O*⁶-alkylguanine-DNA alkyltransferase. *J Biol Chem* 2005;280:30873–30881. [PubMed: 16000301]

X = F, EFH

X = Cl, ECH

X = Br, EBH

Fig. 1. Structures of epihalohydrins: epibromohydrin(1, 2-epoxy-3-bromopropane, EBH), epichlorohydrin (1, 2-epoxy-3-chloropropane, ECH), epifluorohydrin (1, 2-epoxy-3-fluoropropane, EFH)

Fig. 2. Effect of AGT on survival and mutations in *E. Coli* TRG8 cells treated with EBH

TRG8 cells transformed with empty pIN vector (pIN, circles, solid black line), wt-hAGT (hAGT, squares, solid black line), mutant C145A (C145A, triangles, dotted line), or double mutant C145A/C150S (C145A/C150S, diamonds, dotted line) were treated for 30 min with 0–5 mM EBH. Cytotoxicity (panel A) and mutation frequency (panel B) were determined as described in Materials and Methods. The results represent the average of 5–7 measurements $\pm$ S. D. Note that the scale is broken between 60 and 1000 mutations/ 10^8 survivors. Two-way ANOVA was used to analyze the effect of wt-hAGT or mutant hAGT expression on response to EBH exposure: Results for survival (panel A) pIN vs hAGT: $P < 0.0001$; pIN vs C145A: $P = 0.05$; pIN vs C145A/C150S: $P = 0.6$; hAGT vs C145A: $P < 0.0001$; C145A vs C145A/C150S: $P = 0.37$. Results for mutation (Panel B): pIN vs hAGT: $P < 0.0001$; pIN vs C145A: $P < 0.0001$; pIN vs C145A/C150S: $P < 0.0001$; hAGT vs C145A: $P < 0.0001$; C145A vs C145A/C150S: $P = 0.002$.

Fig. 3. Comparison of survival and mutations in *S. typhimurium* YG7108 cells treated with EBH

YG7108 cells were transformed with empty pIN vector (circles) or wt-hAGT (squares), and treated for 30 min with 0–10 mM EBH prior to determination of cytotoxicity (panel A) and mutation frequency of hisG⁺ revertants (panel B) as described in Materials and Methods.

Fig. 4. Comparison of the mutations in *E. coli* TRG8 cells treated with EBH or ECH TRG8 cells transformed with wt-hAGT, mutant C150S, empty pIN vector, mutant R128A and double mutant R128A/C145A were treated for 30 min with 2 mM or 5 mM of the epihalohydrin as indicated. The mutation frequency was determined as described in Materials and Methods. Panel A shows results with EBH. The bars represent the average of 3–6 experiments $\pm$ S. D. An unpaired t-test was used to analyze the effect of each dose of EBH: pIN vs wt-hAGT at 2 mM EBH ($P = 0.0005$); pIN vs wt-hAGT at 5 mM EBH ($P < 0.0001$); pIN vs C150S at 2 mM EBH ($P = 0.003$); pIN vs C150S at 5 mM EBH ($P < 0.0001$); pIN vs R128 at 2 mM EBH ($P = 0.45$); pIN vs R128 at 5 mM EBH

($P = 0.93$); pIN vs R128/C145A at 2mM EBH ($P = 0.1$); pIN vs R128/C145A at 5 mM EBH ($P = 0.35$); wt-hAGT vs C150S at 2 mM EBH ($P = 0.19$); wt-hAGT vs C150S at 5 mM EBH ($P = 0.56$). Panel B shows results with ECH. The results for ECH are the average 2–3 experiments.

Fig. 5. Inactivation of AGT by epihalohydrins *in vitro*

Purified recombinant hAGT protein (20 ng) was incubated with the epihalohydrins at the concentrations shown in 0.5 ml of 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM dithiothreitol and 50 μ g hemocyanin for 30 min. The residual AGT activity was determined and plotted as described in Materials and Methods as a percentage of the remaining AGT activity against inhibitor concentration.

Fig. 6. Covalent binding of AGT to DNA in the presence of EBH

Aliquots of 2 μ g of wt-hAGT (wt), the C-terminal tagged C145S mutant (C145S), the N-terminal tagged C145A mutant (C145A), the N-terminal tagged C145A/C150S mutant (C145A/C150S), or N-terminal tagged antizyme (AZ) was incubated with 5'-[35 S]-d[(GGA) $_5$ G]-3' with or without 25 mM EBH at 37°C for 3 h. The mixtures were separated by 12% SDS-PAGE. Coomassie blue staining (results not shown) was used to ensure equal amounts of protein loading in each lane.

Fig. 7. Effect of pre-incubation of EBH with wt-hAGT or C145S on formation of covalent DNA adducts

Aliquots of 2 μ g of wt-hAGT (hAGT) or mutant C145S protein (C145S) were pre-incubated with 25 mM EBH for 0, 10, 30 and 60 min at 37°C. The 5'-[³⁵S]-d[(GGA)₅G]-3' oligo was then added to the sample and reacted for an additional 3h at 37°C. The mixtures were examined by 12% SDS-PAGE. Panels A and B show autoradiograph results obtained with hAGT and C145S proteins respectively. The densities of the band corresponding to the AGT-oligo complex were assessed using ImageQuant and were calculated as a percentage of the band-density at the 0-time point. Panel C shows the change in adduct formation by plotting the percentages of complex remaining against the lengths of pre-incubation. This experiment was repeated 4 times. The data shown are from one representative experiment.

Fig. 8. Effect of pre-incubation of EBH with DNA on formation of covalent DNA adducts with wt-hAGT or C145S

The 5'-[³⁵S]-d[(GGA)₅G]-3' oligo was pre-incubated with 25 mM EBH for 0, 10, 30 or 60 min at 37°C. The samples were then passed through a MicroSpin™ G-25 columns to get rid of any excess unreacted compound. 2 µg of wt-hAGT (hAGT) or mutant C145S protein (C145S) was then added to the sample and reacted for an additional 3h at 37°C. The mixtures were examined by 12% SDS-PAGE. Panels A and B show autoradiograph results obtained with hAGT and C145S protein respectively. The densities of the band corresponding to the AGT-oligo complex were assessed using ImageQuant and were calculated as a percentage of the band-density at the 0-time point. Panel C shows the change in adduct formation by plotting the percentages of complex remaining against the lengths of preincubation. This experiment was repeated 3 times. The data shown are from one representative experiment.

Fig. 9. Schemes for the mechanism of AGT-mediated toxicity of epihalohydrins AGT-DNA cross-links at the Cys¹⁴⁵ and Cys¹⁵⁰ residues induced by epihalohydrins, EBH and ECH, can occur via 2 pathways. Pathway 1: hAGT reacts with the epihalohydrin to form a reactive AGT- epihalohydrin intermediate at either Cys¹⁴⁵ (1A) or to a lesser extent at Cys¹⁵⁰ (1B). The reactive intermediate can react with water (1A2 or 1B2) but if placed in proximity to DNA via the AGT binding DNA affinity, it can form a covalent AGT-DNA cross-link at Cys¹⁴⁵ (1A1) or at Cys¹⁵⁰ (1B1). Pathway 2: The epihalohydrin can react with DNA to generate a reactive epihalohydrin-DNA adduct. This adduct can react with water as shown (2C) or possibly other cellular nucleophiles within its proximity to form a stable DNA adduct, or it can react with AGT to form an adduct at either Cys¹⁴⁵ as shown (2A) or Cys¹⁵⁰ as shown (2B).

Bacterial Strains

TABLE I

Strain	Chromosomal Genotype	Reference
<i>E. coli</i> TRG8	As GWR109 (B/r, <i>his thy Δada-alkB ogt-1::Kan^r</i>)	(Rebeck and Samson 1991; Xu-Welliver et al., 1998)
<i>S. typhimurium</i> YG107	As TA1535 (<i>hisG46 rfa ΔuvrB</i>) but <i>Δada_{ST}::Kan^r</i> and <i>Δogt_{ST}::Cam^r</i>	(Maron and Ames 1983; Yamada et al., 1995)

Table II
Frequency and site of mutations in the *rpoB* gene produced by 2 mM EBH in *E. coli* TRG8 cells containing plasmids expressing no hAGT, wt-hAGT, C145A mutant hAGT, or C145A/C150S mutant hAGT

Base Change	Amino Acid Change	5'-Sequence-3'	Number of mutations with hAGT expression indicated			
			No hAGT	Wt-hAGT	C145A	C145A/C150S
G:C to A:T	D516N	TTT ATG <u>G</u> AC CAG AAC	1	5	14	27
G:C to A:T	H526Y	ATT ACG <u>C</u> AC AAA CGT	7	3	2	1
G:C to A:T	R529C	AAA CGT <u>C</u> GT ATC TCC	4	0	0	0
G:C to A:T	R529H	AAA CGT <u>C</u> G <u>T</u> ATC TCC	2	24	7	1
G:C to A:T	S531F	CGT ATC T <u>C</u> C GCA CTC	10	25	25	6
G:C to A:T	P564L	GAA ACC C <u>C</u> T GAA GGT	4 (28/52)	0 (57/64)	2 (50/55)	0 (35/53)
G:C to T:A	Q513K	CTG TCT <u>C</u> AG TTT ATG	0	1	1	1
G:C to T:A	D516Y	TTT ATG <u>G</u> AC CAG AAC	6	0	2	12
G:C to T:A	R529S	AAA CGT <u>C</u> GT ATC TCC	1	0	0	4
G:C to T:A	S531Y	CGT ATC T <u>C</u> C GCA CTC	0 (7/52)	1 (2/64)	1 (4/55)	0 (17/53)
A:T to T:A	Q513L	CTG TCT <u>C</u> AG TTT ATG	6	1	1	0
A:T to T:A	D516V	TTT ATG <u>G</u> AC CAG AAC	0	1	0	0
A:T to T:A	H526L	ATT ACG <u>C</u> AC AAA CGT	8	0	0	0
A:T to T:A	I572F	GGT CTG <u>A</u> TC AAC TCT	2 (16/52)	0 (2/64)	0 (1/55)	0 (0/53)
A:T to G:C	Q513R	CTG TCT <u>C</u> AG TTT ATG	1 (1/52)	3 (3/64)	0 (0/55)	0 (0/53)
A:T to C:G	Q513P	CTG TCT <u>C</u> AG TTT ATG	0 (0/52)	0 (0/64)	0 (0/55)	1 (1/53)

Table III

Summary of mutations in the *rpoB* gene produced by 2 mM EBH in *E. coli* TRG8 cells containing plasmids expressing no hAGT, wt-hAGT, C145A mutant hAGT, or C145A/C150S mutant hAGT

AGT expressed Mutation	None (pIN vector)			Wt-hAGT			C145A			C145A/C150S		
	Total	%	Frequency*	Total	%	Frequency*	Total	%	Frequency*	Total	%	Frequency*
<i>Transitions</i>												
G:C to A:T	29	55.8	1	60	93.8	2265	50	90.9	26.5	35	66	5.2
A:T to G:C	28	53.8	1	57	89.1	2152	50	90.9	26.5	35	66	5.2
	1	2	0.03	3	4.7	113	-	-	-	-	-	-
<i>Transversions</i>												
G:C to T:A	23	44.2	0.7	4	6.2	151	5	9.1	2.7	18	34	2.6
G:C to C:G	7	13.4	0.2	2	3.1	75.5	4	7.3	2.2	17	32.1	2.5
A:T to T:A	-	-	-	-	-	-	-	-	-	-	-	-
A:T to C:G	16	30.8	0.5	2	3.1	75.5	1	1.8	0.5	-	-	-
	-	-	-	-	-	-	-	-	-	1	1.9	0.1
Mutants Collected	52			64			55			53		
EBH-induced mutation frequency*			1.7			2416			29.2			7.8

* Mutations/10⁸ survivors