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Alkyltransferase-mediated Toxicity of 1,3-Butadiene Diepoxide

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Abstract

Human *O*⁶-alkylguanine-DNA alkyltransferase (hAGT) expression increases mutations and cytotoxicity following exposure to 1,3-butadiene diepoxide (BDO) and hAGT-DNA cross-links are formed in the presence of BDO. We have used hAGT mutants to investigate the mechanism of cross-link formation and genotoxicity. Formation of a hAGT-DNA conjugate in vitro was observed with C145S and C145A mutant proteins but was considerably diminished with the C145A/C150S double mutant confirming that cross-linking primarily involves either of these two cysteine residues, which are located in the active site pocket of the protein. Cross-link formation by BDO occurred both via (a) an initial reaction of BDO with hAGT followed by attack of the reactive hAGT complex on DNA, and (b) the initial reaction of BDO with DNA followed by a reaction between hAGT and the DNA adduct. These results differ from those with 1,2-dibromoethane (DBE) where Cys¹⁴⁵ is the only site of attachment and pathway (b) does not occur. The complex formed between hAGT at Cys¹⁴⁵ and BDO was very unstable in aqueous solution. However, the BDO-hAGT complex at Cys¹⁵⁰ exhibited stability for more than 1 h. The effect of hAGT and mutants on BDO-induced genotoxicity was studied in *E. coli* using the forward assay to rifampicin resistance. Both mutations and cell killing were greatly increased by wild type hAGT and there was a smaller but significant effect with the C145A mutant. The R128A mutant and R128A/C145A and C145A/C150S double mutants were ineffective supporting the hypothesis that the formation of hAGT-DNA cross-links is responsible for the enhanced genotoxicity detected in this biological system. In the absence of hAGT, there were equal proportions of G:C to A:T transitions, G:C to T:A transversions and A:T to T:A transversions. Wild type hAGT expression yielded significantly greater G:C to A:T and A:T to G:C transitions, whereas C145A mutant expression resulted in more transitions and transversions at A:T base-pairs.

Introduction

*O*⁶-alkylguanine-DNA alkyltransferases (AGT¹) are a widely distributed family of proteins that provide protection against the cytotoxic, mutagenic and carcinogenic outcomes induced by alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-methyl-*N*-nitrosourea that form DNA adducts at the guanine-*O*⁶ position (1–4). However, the presence of either bacterial or mammalian AGTs have been shown to paradoxically increase the genotoxic effects of α,ω -dihaloalkanes such as 1,2-dibromoethane (DBE) (5–10). A plausible explanation for this increase was proposed by Liu *et al.* (8) who suggested that the highly reactive cysteine acceptor site of AGT (Cys¹⁴⁵ in hAGT) reacts with the dihaloalkane to generate a half-mustard intermediate. The reactivity of this intermediate facilitates its reaction with DNA yielding covalent hAGT-DNA cross-links. This mechanism was supported by a

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¹List of Abbreviations: AGT, *O*⁶-alkylguanine-DNA alkyltransferase; hAGT, human AGT; wt-hAGT, wild type hAGT; DBE, 1,2-dibromoethane; DBM, dibromomethane; BDO, 1,3-butadiene diepoxide; MS, mass spectroscopy; PNK, T4 polynucleotide kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IPTG, isopropyl β -D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction.

variety of studies including the demonstration that the C145A mutant of hAGT was ineffective at generating this reactive intermediate, the labelling of hAGT at Cys¹⁴⁵ by reaction with [¹⁴C]DBE, the formation of a covalent adduct between hAGT and DNA after incubation with DBE and the subsequent identification by mass spectroscopy (MS) of an N7-guanine linked peptide from hAGT (8,10,11).

Although the studies elucidating the mechanism of AGT-mediated genotoxicity were conducted with DBE and dibromomethane (DBM), it was found that hAGT expression enhanced the production of mutations and cell killing by a variety of other bifunctional compounds. These included α,ω -disubstituted dihaloalkanes with Br or I and methylene chain length at least up to 5 as well as BrCH₂Cl, Br(CH₂)₂Cl and 1,3-butadiene diepoxide (BDO) (12). Given these findings as well as results by Liu *et al.* (8,10–12), it was suggested that these bifunctional agents might induce genotoxicity by forming protein-DNA cross-links in a similar manner (13).

BDO, a known carcinogen, is a bifunctional alkylating agent that has been shown to induce numerous genotoxic effects in a variety of *in vitro* and *in vivo* biological test systems. It is the metabolite of 1,3-butadiene, an important industrial compound and environmental pollutant (14,15). BDO was found to cause direct inactivation of AGT suggesting that it can react with the active site of the protein (12) and Loeber *et al.* demonstrated *in vitro* covalent complex formation between wild-type hAGT protein (wt-hAGT) and DNA in the presence of BDO (16). However, their studies also revealed that cross-link formation could be detected when incubating BDO and DNA with the C145A mutant. This is in contrast to similar experiments with DBE that indicated an absolute requirement for the Cys¹⁴⁵ residue to observe covalent linkage of hAGT to DNA (8). Consequently, they proposed that the mechanism of hAGT-DNA conjugation following exposure to BDO occurred via an alternate sequence of events than seen with DBE wherein a reactive intermediate is generated from an initial reaction between BDO and DNA followed by nucleophilic attack by the Cys¹⁴⁵ or Cys¹⁵⁰ residues in hAGT (16). This mechanism was supported by results of capillary HPLC-electrospray ionization MS analysis of tryptic peptides from the complex which revealed that BDO was capable of cross-linking at Cys¹⁴⁵ and Cys¹⁵⁰ residues of the protein (16). Recently, similar adduct formation with hAGT via either Cys¹⁴⁵ or Cys¹⁵⁰ was detected after hAGT proteins *in vitro* or cultured cells expressing hAGT were treated with anti-tumor nitrogen mustards that are known to react with DNA (17).

Our experiments were designed to study, in greater detail, the effect of hAGT on augmenting the genotoxicity of BDO and to determine the sequential order of reactivity of the three components (BDO, hAGT and DNA) leading to covalent hAGT-DNA cross-link formation. We also assessed the relative importance of reaction with different Cys residues on the enhanced mutations and cell-killing following exposure to BDO and used mutants of hAGT at Arg¹²⁸, which interfere with DNA binding (4,18,19), to examine the importance of the hAGT affinity for DNA.

Materials and Methods

Chemicals and Reagents

BDO (97% purity), rifampicin (97% purity) and DBE (99% purity) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Isopropyl β -D-thiogalactopyranoside (IPTG), ampicillin and kanamycin were obtained from Fisher Scientific (Pittsburgh, PA). Adenosine 5'-[γ -³⁵S]thiotriphosphate, triethyl ammonium salt ([γ -³⁵S]ATP γ S) was obtained from GE Healthcare Life Sciences (Piscataway, NJ). T4 polynucleotide kinase (PNK) was from Promega (Madison, WI). All oligodeoxyribonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Construction of plasmids for expression of C145A/C150S and R128A/C145A Mutant hAGT Proteins

Mutant hAGT proteins were made using the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) according to manufacturer instructions. The template for construction of N-terminal (His)₆-tagged C145A/C150S-hAGT double mutant protein was a pQE-30 vector encoding the N-tagged C145A-hAGT mutant (20). The pIN-C145A/C150S plasmid used for studies of the effect of hAGT expression in *E. coli* was constructed using a pINIII-A3(*lpp*^P-5) expression vector (21) containing the C145A-hAGT coding sequence. The primers were as follows: 5'-CCCACAGAGTGGTC**AG**CAGCAGCGGAGC-3' (sense) and 5'-GCTCCGCTGCT**GCT**GACCACTCTGTGGG-3' (anti-sense, mutated codon in bold). Similarly, the pIN-R128A/C145A plasmid was constructed using pIN-R128A-hAGT as template (18) and primers 5'-CCCCATCCTCATCCCG**G**CCCACAGAGTGGTCTGC-3' (sense) and 5'-GCAGACCACTCTGTGG**G**CCGGGATGAGGATGGGG-3' (anti-sense, mutated codon in bold). The entire coding sequence was checked to ensure that only the desired mutations were present.

Protein Purification

All the recombinant proteins used for these studies were cloned into a pQE30 expression plasmid (20,22) and expressed in XL-1 Blue cells (Stratagene, La Jolla, CA) prior to purification. Recombinant N-terminal tagged hAGT-C145A mutant and hAGT-C145A/C150S double mutant have an N-terminal (His)₆-tag in a sequence replacing the terminal M- with the sequence MRGS(H)₆GS- (20). Recombinant C-terminal tagged wild type hAGT (wt-hAGT) and the hAGT-C145S mutant have a C-terminal (His)₆-tag replacing residues 202-207 (-PPAGRN) with-HHHHHH (22). All proteins were purified as described previously (23). Recombinant N-terminal (His)₆ tagged antizyme was purified as described previously (24).

Gel Electrophoresis Analysis of hAGT reaction with Oligonucleotides and BDO

The 16-mer oligonucleotide used for this was 5'-d(GGAGGAGGAGGAGGAG)-3' (8). This oligo was 5'-end labelled with [γ -³⁵S]ATP γ S using PNK (Promega Corporation, Madison, WI). Ten μ g of oligo, 20 units of PNK and 10 μ L of [γ -³⁵S]ATP γ S (250 μ Ci) were incubated in 50 μ L of 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol at 37°C for 4 h. The kinase was then inactivated by heating at 68°C for 20 min. The labelled 16-mer was separated from unincorporated nucleotides and [γ -³⁵S]ATP γ S by centrifugation using MicroSpin™ G-25 columns (GE Healthcare Life Sciences, Piscataway, NJ). Radioactivity of the 16-mer was confirmed using both autoradiography and scintillation counting. The C-terminal tagged wt-hAGT and C145S mutant, the N-terminal tagged C145A mutant and C145A/C150S double mutant, and antizyme protein (2 μ g) were incubated in separate 10 μ L assay volumes along with 25 mM BDO, 2 pmol [³⁵S]-labelled oligo and 20 pmol unlabelled oligo in AGT Buffer (50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 5 mM dithiothreitol) at 37°C for 3 h. Following this incubation, 1 μ L of 10% SDS solution and 2 μ L of 5 $\times$ 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 briefly centrifuged and 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 were quantified using the ImageQuant application software (ImageQuant v5.2, GE Healthcare Life Sciences, Piscataway, NJ).

Study of the Order of Reactivity of hAGT with BDO or DBE and DNA Leading to Formation of hAGT-DNA cross-links

A time-course gel electrophoresis analysis was done to determine whether BDO and DBE reacted primarily with wt-hAGT protein first to form an intermediate that subsequently reacts with DNA, or whether the initial reaction occurred between DNA and mutagen followed by reaction with the hAGT protein. A similar experiment was also performed to investigate whether the reaction between the hAGT-C145S mutant and BDO occurred in a similar or different manner. Aliquots of 2 µg of 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 BDO or DBE in AGT Buffer. After this pre-incubation, 2 pmol of [³⁵S]-labelled oligo and 20 pmol unlabelled oligo were added to the reaction mixture to a final volume of 10 µL. Each sample was then incubated at 37°C for an additional 3 h prior to separation by 12% SDS-PAGE. The gels were then dried and intensities of oligonucleotide bands were analyzed using both autoradiography as well as a Molecular Dynamics PhosphorImager SI. The density of the major lower mobility form of [³⁵S]-oligo at the zero time point was assessed using ImageQuant and was assumed to be 100%. The density of this band for each additional time-point measured was also quantified using ImageQuant and calculated as a percentage of that at the zero time point. This percentage of remaining complex was plotted against the preincubation time.

The second part of this study involved an initial preincubation of the [³⁵S]-oligo with the compound followed by addition of protein. The assay volumes were proportionally increased from 10 µL to 30 µL in order to ensure that an appropriate volume of sample was loaded onto the spin columns. Six pmol of [³⁵S]-labelled oligo and 60 pmol unlabelled oligo were pre-incubated at 37°C for different lengths of time (0, 10, 30 or 60 min) with 25 mM BDO or DBE. 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 addition of protein. Following this, 6 µg of C-terminal tagged wt-hAGT or C145S protein was added, to a final reaction volume of 30 µL, and the reaction was allowed to proceed for another 3 h before separation by 12% SDS-PAGE. The gels were dried and analyzed as described above.

Bacterial Strains and Plasmids

The TRG8 bacterial strain used in these experiments was derived from the GWR109 cells, which were the generous gift of Dr. Leona Samson (Department of Molecular & Cellular Toxicology, Harvard School of Public Health, Boston, MA). TRG8 cells were selected based on altered cell membrane permeability so as to allow for greater permeability to exogenous chemicals (25). The TRG8 strain has its *ogt* and *ada* genes replaced with a kanamycin resistance cassette and the cells were constantly maintained under kanamycin selection. The wt-hAGT protein, R128A and C145A mutant and R128A/C145A and C145A/C150S double mutant protein coding regions were expressed in *E. coli* using the pINIII-A3(*lpp*^{P-5}) expression vector (21). Studies in which large amounts of the purified recombinant protein were used have shown that mutation of Cys¹⁴⁵ to Ala or Ser totally abolishes the ability of AGT to repair *O*⁶-methylguanine in DNA (19,26) and neither the R128A/C145A or C145A/C150S double mutant have any AGT activity. These plasmids containing sequences encoding hAGT or mutant as well as the empty pIN vector were transformed into TRG8 cells by standard electroporation procedures as described previously (9). The transformed strains were selected for using ampicillin resistance. Western blot analysis was used to ensure equivalent protein expression levels of all hAGT proteins in the TRG8 cells.

Measurement of Cell Survival and Mutations in *E. coli*

The TRG8 cells, transformed with either empty pIN vector or the pIN-hAGT, pIN-C145A, pIN-R128A, pIN- R128A/C145A, pIN-C145A/C150S plasmids, were grown in 5 mL of LB medium supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin at 37°C with

constant shaking until the cultures reached an $OD_{600nm} = 0.5-0.6$. At this point, 150 μ M IPTG was added to each culture to induce hAGT expression and they were again incubated at 37°C with gentle agitation for an additional 30 min. The cells were pelleted by centrifugation for 15 min at room temperature at 5000 rpm, and resuspended in 5 mL of M9 media (27). Aliquots (1 mL) of each culture were treated with 0–5 mM BDO or up to 2% dimethyl sulfoxide (DMSO), as a vehicle control, for 30 min at 37°C with shaking. The cells were pelleted, washed with 2 mL of M9 salts and finally resuspended in 1 mL aliquots of LB media. Aliquots (25 μ L) of the cultures (in 1:250,000 to 1:2500 dilution) were plated onto LB plates supplemented with 100 μ g/mL ampicillin and 50 μ g/mL kanamycin to measure cell survival (10). The plates were incubated at 37°C for 24 h. The survival rates of the mutagen-treated cells were expressed as a percentage of the DMSO-treated cells that survived. The forward mutation marker used for these experiments was resistance to the antibiotic, rifampicin (Rif^{res}). Rif^{res} occurs as a result of mutations in the *rpoB* gene of *E. coli*, which alters the β subunit of RNA polymerase (28). Estimation of Rif^{res} mutation frequency was made by first growing the mutagen treated cultures overnight in 1 mL of LB supplemented with 100 μ g/mL ampicillin and 50 μ g/mL kanamycin. *RpoB* gene mutants (Rif^{res} mutation frequency) were derived by plating undiluted cell suspensions (50 μ L–1 mL) of these overnight cultures onto LB plates supplemented with 100 μ g/mL rifampicin (10). The plates were incubated at 37°C for 36–48 h. Simultaneously, 25 μ L aliquots of these overnight cultures (in 1:250,000–1:2500 dilution) were plated onto LB plates (with ampicillin and kanamycin) lacking rifampicin. For mutation frequency, the number of colonies on the rifampicin-supplemented plates was expressed per 10^8 surviving cells that grew on the LB plates containing only ampicillin and kanamycin.

Analysis of Rifampicin-resistant Mutants

In order to analyze the types of mutations induced at the *rpoB* gene locus by BDO in the TRG8 cells, individual Rif^{res} mutant clones were picked, suspended in 50 μ L of deionized water and vortexed vigorously. 2 μ L aliquots of these suspensions were used as DNA template in a polymerase chain reaction (PCR) to amplify a region of the *rpoB* gene. The primers used for this PCR reaction were 5'-TGGCCTGGTACGTGTAGA-3' (forward primer) and 5'-AACCAGCGGCTTATCAGC-3' (reverse primer). The PCR cycling conditions were as follows: initial melting (92 °C, 4 min) 30 cycles of denaturation (92°C, 30 s), annealing (52° C, 30 s) and extension (72°C, 1 min) followed by a last extension step at 72°C for 5 min (10). 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.

Results

Initial experiments using DBE led to the hypothesis that hAGT-mediated toxicity of this bifunctional agent was due to the interaction of DBE with the highly reactive Cys¹⁴⁵ that serves as the alkyl acceptor residue when AGT repairs methylated DNA (8,10,12). Genotoxicity of BDO is also enhanced by hAGT (10). However, using *in vitro* analysis, Loeber *et al.* (16) showed that BDO was capable of covalent hAGT-DNA adduct formation at both the Cys¹⁴⁵ and Cys¹⁵⁰ residues. In order to determine the relative importance of these sites, we have used wt-hAGT and mutants at Cys¹⁴⁵ and Cys¹⁵⁰ to study BDO-induced hAGT-DNA adduct formation *in vitro* and the effect of hAGT on killing and mutagenesis in cells.

Formation of hAGT-DNA adducts by butadiene diepoxide

We analyzed the ability of wt- and mutant hAGT proteins to induce covalent linkage with DNA in the presence of DBE and BDO using an oligodeoxyribonucleotide 5'-d[(GGA)₅G]-3' that was labelled at its 5'-end with [³⁵S]. Altered mobility of the [³⁵S]-oligo and subsequent appearance of a new radiolabeled-DNA band on 12% SDS-PAGE was used to identify the

conjugated form of hAGT. Non-covalent interactions of hAGT with DNA are disrupted by SDS making this type of study suitable for detection of covalent hAGT-DNA adducts. A band corresponding to a hAGT-oligo cross-link was formed when BDO was present but was not seen in the absence of BDO (Figure 1). The position of the major DNA band is consistent with one hAGT protein being adducted to one oligonucleotide. Our data also demonstrate bands corresponding to larger complexes of weaker intensity, which have also been detected with similar studies from our laboratory in which DBE was used (8). These are likely to be due to the attachment of two or more molecules of hAGT to the oligo (8). No covalent protein-DNA adduct was formed when antizyme, an irrelevant protein involved in polyamine metabolism (24), which contains six cysteines and is of similar molecular weight as hAGT, was substituted for hAGT. When the mutants C145S or C145A were used instead of wt-hAGT the hAGT-oligo cross-link was still detected (Figure 1) indicating that the adduct can be formed at a site other than Cys¹⁴⁵. This site is likely to be Cys¹⁵⁰ since the band was greatly reduced when the C145A/C150S double mutant was used. [The slightly lower mobility of the C145A- and the C145A/C150S- adducts compared to the wt-hAGT- and C145S-adducts is due to the fact that the former have a 12 amino acid extension containing the (His)₆-tag at the N-terminus whereas the latter have a C-terminal (His)₆-tag that replaces the last 6 amino acids.] These results contrast with previous studies with DBE when no significant hAGT-DNA conjugation occurs with the C145A mutant demonstrating that virtually all of the reaction appears to be at this site (8,10). They do however support findings by Loeber *et al.* indicating hAGT-DNA cross-link formation at both the Cys¹⁴⁵ and Cys¹⁵⁰ sites (16). The conjugate formation with the double mutant *in vitro*, which occurs at a much lower level than with wild type or the single mutants, may be due to the reactivity of BDO with other surface residues on hAGT because the double mutant protein still has DNA binding properties. However, MS analysis and peptide mapping studies have shown that hAGT-DNA cross-links, in the presence of BDO, were only detected at Cys¹⁴⁵ and Cys¹⁵⁰ residues (16).

Sequential order of reactivity of hAGT with BDO or BDE and DNA

The scheme suggested for the mechanism of hAGT-mediated toxicity of DBE involved the initial reaction of DBE with hAGT at Cys¹⁴⁵ and the subsequent reaction of the half mustard AGT-Cys¹⁴⁵-S⁺-(CH₂)₂ formed in this reaction with DNA (8). In the absence of DNA, the highly reactive intermediate can react with water to form AGT-Cys¹⁴⁵-S-(CH₂)₂OH which is no longer reactive. BDO is a much more reactive compound overall than DBE and is capable of directly forming a broad spectrum of DNA adducts (29–34). Therefore, it was suggested that initially a reactive BDO-DNA adduct is formed which then reacts with hAGT to form the hAGT-oligo cross-link (16). This was investigated by preincubating two out of the three components (either compound with hAGT or compound with oligo), needed for AGT-oligo complex formation, for 0–60 min. Following this pre-incubation time, the missing component (DNA or protein) was added and the reaction was allowed to proceed prior to running the samples on SDS-PAGE. For the experiments involving pre-incubation with compound and oligo, the samples were run through spin columns, in order to remove any residual unreacted compound. 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-adduct intermediate.

Figure 2 shows results obtained when hAGT or the C145S mutant protein was first incubated with BDO for differing lengths of time, after which the [³⁵S]-labelled 5'-d[(GGA)₅G]-3' oligo was added to the sample and the cross-linking reaction allowed to take place. With hAGT and BDO (Figure 2A), there was substantial hAGT-oligo cross-link formation when the oligo was added immediately after the mixing of the BDO and hAGT protein. There was much less cross-link formation after a 10 min preincubation and a progressive decline with time such that only ~7% of the zero-time-point complex was produced after 1 h of pre-incubation (Figure 2C). This result is very similar to that seen when DBE was used in place of BDO when, as previously

reported (8), the ability of the intermediate of reaction between DBE and hAGT to react with DNA rapidly declined with time (Figure 2D). The findings with the C145S mutant reacted with BDO were quite different. In this case, the formation of the C145S-oligo cross-link was only slightly reduced after a 1 h incubation (Figure 2B and 2C). These results suggest that the BDO-hAGT intermediate formed at Cys¹⁵⁰ is quite stable and resistant to reaction with water whereas that formed at the Cys¹⁴⁵ site is rapidly decomposed in aqueous solution.

Figure 3 shows results obtained when the oligo was incubated with BDO or DBE prior to protein addition. There was no formation of a hAGT-oligo cross-link when DBE was used supporting the original hypothesis that the conjugate formation requires an initial reaction between DBE and hAGT (Figure 3D). With both wt-hAGT and the C145S mutant, the formation of a protein-oligo cross-link actually increased with time of preincubation with oligo and BDO. This supports the hypothesis that both of these proteins can react with a oligo-BDO adduct and that this intermediate accumulates during the preincubation period (Figure 3A and B).

Effect of hAGT and Mutants on Genotoxicity and Mutations Induced by BDO in *E. coli*

In order to evaluate the significance of the cross-linking via the Cys¹⁵⁰ site, hAGT-mediated genotoxicity of BDO was studied in the *E. coli* strain TRG8 lacking endogenous AGT activity. TRG8 cells that express wild-type hAGT, the C145A mutant or the C145A/C150S double mutant from the pIN vector were used (Figure 4). The expression of wild-type hAGT significantly enhanced cell killing by BDO in a dose-dependent manner (Figure 4A) in agreement with previous results (12). The presence of the C145A mutant also increased cell killing by BDO although to a lesser extent than wt-hAGT. C145A/C150S double mutant expression, however, did not yield significantly different results compared to cells with an empty pIN vector. The effects of these hAGT proteins on the incidence of mutations in response to BDO was measured using the forward assay to rifampicin resistance (28). The background mutation spectrum of the TRG8 *E. coli* did not reveal induction toward any specific class of base pair substitutions and were comparable to those published previously (28) (results not shown). BDO alone produced only a small increase in mutations at the doses tested but the incidence of *rpoB* Rif^{res} mutations was greatly increased by the presence of wt-hAGT (Figure 4B). There was a substantial but much lower increase in mutations in cells expressing the C145A mutant. C145A/C150S double mutant expression, however, did not yield an enhanced mutation rate (Figure 4B).

Importance of DNA Binding properties of hAGT in Formation of BDO-mediated hAGT-crosslinks

The Arg¹²⁸ residue in hAGT plays an important role in DNA binding by hAGT (18,19,35). The basic side chain of this residue is inserted into the DNA helix to replace the flipped out nucleoside substrate. As shown in Figure 5, mutation of this residue to Ala totally abolished the increase in mutations and cell killing caused by wt-hAGT or the C145A mutant hAGT in BDO-treated cells.

Molecular Analysis of Rifampicin Resistant Mutants in *E. coli* treated with BDO

The Rif^{res} mutation spectra at the *rpoB* locus are shown in Table 1 and Table 2. Table 1 shows the mutations at sites within this locus and Table 2 shows a summary comparing relative proportions of each class of base-pair substitutions observed with each type of hAGT-expressing strain. In the absence of hAGT, most mutations caused by BDO occurred at G:C base pairs (~70% of total mutants) with equal proportions of G:C to A:T transitions and G:C to T:A transversions. The remainder of the Rif^{res} mutants were A:T to T:A transversions. This pattern was comparable to the cells expressing the C145A/C150S mutant (Table 2) and is consistent with published analyses for mutants initiated in a variety of cell culture models,

which have demonstrated comparable reactivity for both G:C and A:T base-pairs with noted enhancement of G:C to A:T transitions and A:T to T:A transversions. Minor increases in G:C to T:A transversions have also been detected (36–41).

Expression of wt-hAGT led to a significant increase in G:C to A:T transitions ($p=0.02$ versus pIN; Fisher's exact test) and A:T to G:C transition mutations caused by BDO. The latter were not seen in control cells without hAGT expression or with cells expressing the C145A/C150S double mutant. Overall, wt-hAGT expression clearly increased transition mutations ($p=0.007$ for hAGT versus pIN; Fisher's exact test). This was not the case with the C145A mutant where the ratio of transition to transversion mutations was not different from that of cells without hAGT ($p=0.44$ for C145A versus pIN; Fisher's exact test). However, the presence of the C145A protein did cause an increased preference for both transitions and transversion mutations at A:T base pairs ($p=0.032$ for C145A versus pIN; Fisher's exact test) (Table 2). After BDO treatment, the spectrum of *rhoB* mutations obtained with TRG8 cells expressing the C145A/C150S mutant were very similar to the pIN-TRG8 strain. There were no statistically significant differences between the C145A/C150S double mutant and pIN-TRG8 profiles as analyzed using the Fisher's exact test.

Discussion

Our results confirm and extend previous studies demonstrating that BDO exposure can yield covalent cross-links between hAGT and DNA which, in turn can increase both the cytotoxicity and the incidence of mutations caused by this compound (12,16). The incubation of hAGT with *N*7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-deoxyguanosine led to the formation of a butanediol cross-links at both Cys¹⁴⁵ and Cys¹⁵⁰ (16). Loeber *et al.* (16) therefore proposed that an hAGT-DNA cross link was generated via an initial reaction of BDO with DNA followed by subsequent reaction of the BDO-DNA intermediate with hAGT at either cysteine. Our results not only confirm that cross-linking can occur in this manner but also indicate that an initial reaction of BDO with hAGT followed by covalent addition of DNA can yield hAGT-DNA adducts, as previously demonstrated for DBE and DBM (8,10,11).

Our SDS-PAGE analyses with BDO, [³⁵S]-labelled oligo and the C145A or C145S mutants and C145A/C150S double mutant forms of hAGT (Figure 1) validate the finding that hAGT-DNA cross-linking can occur at both the Cys¹⁴⁵ and Cys¹⁵⁰ residues of the repair protein (16). This is in contrast to DBE- or DBM-mediated AGT-DNA adducts, which occur solely at the Cys¹⁴⁵ active site of the repair protein (8,10). *Bis*-electrophile induced adduct formation at Cys¹⁴⁵ of hAGT is not surprising given the extremely low pK_a of the residue and its significant reactivity toward electrophiles (42). This reactivity is due to the presence of a hydrogen bond network involving Glu¹⁷²:His¹⁴⁶:water:Cys¹⁴⁵ that effectively generates a thiolate anion at Cys¹⁴⁵ (4,35). The Cys¹⁵⁰ site on hAGT is located in the active site pocket but does not show significant reactivity with model electrophiles (42) and does not react with DBE or DBM. However, adducts from BDO such as the *N*7-(2'-hydroxy-3',4'-epoxybut-1'-yl)-deoxyguanosine are sufficiently reactive to form a covalent linkage if they come near to hAGT. The DNA binding capacity of hAGT is likely to bring about this proximity to DNA adducts and Cys¹⁵⁰ is located at the edge of the substrate-binding pocket close to the DNA-binding surface (4,35). A variety of adducts of BDO with DNA have been reported in addition to the *N*7-guanine adduct (32,33) including reactions at other *N*-positions of guanine and the *N*3, *N*⁶ and *N*7 of adenine (30,33,36). hAGT has little sequence specificity and although only *O*⁶-alkylguanine adducts are maintained in the active site pocket for repair, unmodified bases and other DNA adducts at guanine and other bases are likely to be located near to Cys¹⁵⁰.

There are three other cysteine residues in hAGT (Cys⁵, Cys²⁴, Cys⁶²) and other potential nucleophilic sites that could be linked to DNA in the presence of BDO. However, as shown in

Figure 1, the reaction with the C145A/C150S double mutant hAGT protein is limited even under *in vitro* incubation conditions where the concentration of hAGT and DNA is very high. Additionally, our studies show that expression of the C145A/C150S mutant hAGT in bacterial cells does not increase cell killing and mutations after treatment with BDO (Figure 4 and Figure 5). Since the DNA binding capacity of the C145A/C150S mutant hAGT is not impaired (43), our results indicate that the increases in mutations and killing seen with wt-hAGT and C145A mutant are due to DNA-protein cross-link formation rather than non-covalent hAGT-DNA interactions which can shield adducts from repair. [There is a precedent for this type of shielding since the C145A mutant prevents NER-mediated repair of *O*⁶-methylguanine (44) and wt-hAGT reduces repair of *O*⁴-methylthymine (45).]

The intermediate formed by BDO in reaction with wt-hAGT in the absence of DNA was extremely unstable and there was minimal DNA-hAGT cross-link formation following addition of DNA one hour after the reaction (Figure 2). This intermediate could occur at either Cys¹⁴⁵ or Cys¹⁵⁰ but the adduct formed at Cys¹⁵⁰ when the C145S mutant was used to prevent reaction at Cys¹⁴⁵ was quite stable over a one hour incubation. These findings suggest that only minimal hAGT-DNA cross-links actually occur at Cys¹⁵⁰ when Cys¹⁴⁵ is available. The most likely explanation for this is that the rapidity of adduct formation at the highly activated Cys¹⁴⁵ as well as steric considerations prevent concurrent cross-linking at the Cys¹⁵⁰ site *in vitro*. Furthermore, this implies that DNA-AGT cross-links formed at the Cys¹⁵⁰ site occur predominantly via the pathway involving an initial BDO-DNA intermediate. It is clear that the reactive BDO-DNA adduct is relatively stable and can react to form a covalent complex with hAGT at Cys¹⁴⁵ or to a lesser extent with Cys¹⁵⁰ (Figure 3). The stability of this BDO-DNA adduct would allow adequate time for reaction with hAGT within the cell where levels of the protein may be low. The lesser reaction with Cys¹⁵⁰ would be consistent with its diminished reactivity as compared to Cys¹⁴⁵ described above.

Valadez *et al.* (12) found that the presence of hAGT in either *E. coli* TRG8 or *S. typhimurium* YG7108 increased cell-killing by BDO and significantly increased mutations in an assay measuring production of his⁺ revertants (which requires a transition mutation in the HisG46 gene at a G:C pair at the second position of a CCC codon encoding Pro to CTC encoding Leu). Our results using the *rpoB* forward mutation assays with wt-hAGT expression in *E. coli* TRG8 cells agree with these results. There was a large increase in G:C to A:T transition mutations (Table 1 and Table 2). We also found that the C145A mutant was able to enhance mutations and cell killing by BDO (Figure 4) which is in contrast to similar assays that demonstrated complete absence of cell-killing and mutations in TRG8 cells expressing C145A-hAGT upon exposure to either DBE or DBM (8,11). However, our results are consistent with the finding that BDO treatment can lead to a covalent hAGT-DNA adduct at Cys¹⁵⁰. Although the increase in mutations brought about by C145A-hAGT was less than with wt-hAGT, there was still at least a 10-fold increase compared to no hAGT expression or the C145S/C150S double mutant (Figure 4 and Figure 5).

The reactivity of BDO toward DNA has been studied extensively (16,40). The compound is known to primarily alkylate *N*7-guanine (32,34) but adduct formation has also been detected on other sites in guanine and on adenine at *N*3, *N*⁶, and *N*7 (30,33,36). Furthermore, novel BDO-induced bifunctional adducts involving the cross-linking of neighboring DNA-bases have also been reported recently (29,46). The *N*7-guanine adduct is known to be unstable and susceptible to spontaneous depurination. Error-prone bypass and subsequent preferential misincorporation of an adenine opposing the abasic site is likely responsible for the G:C to T:A transversion mutations (47,48) seen with our pIN-TRG8 cells. Similarly, the highly labile *N*3-adenine and *N*7-adenine adducts may give rise to abasic sites and adenine incorporation would generate A:T to T:A transversions. Thus far, no definitive mechanism has been provided for the large proportion of G:C to A:T transitions detected following BDO exposure in a number

of biological systems (39,40,49) including our TRG8 bacterial model but given the plethora of established BDO mono- and bifunctional guanine adducts (29,30,34), it is feasible that one of these lesions may be prone to thymine mis-incorporation via by-pass polymerases yielding G:C to A:T transitions.

Expression of wt-hAGT greatly increased the production of G:C to A:T transition mutations by BDO. This mutagenic pattern has previously been observed in both DBE and DBM treated TRG8 cells expressing hAGT (8,11). The large increase in such mutations caused by BDO when hAGT is present is therefore likely to be due to the reaction of the reactive-hAGT complex with DNA. The adduct causing this base pair substitution has not been definitively identified but one possibility would be an AGT-DNA adduct on the *N*²-position of guanine. Binding of the hAGT protein to the minor groove of duplex DNA (19) may facilitate reaction with the exocyclic amino group on the guanine base, which is also situated within the minor groove. Furthermore, it is known that protein-DNA cross-links are degraded by protease activity prior to repair or copy by by-pass polymerases (50,51). Such protein degradation of the adduct at the *N*²-position may be necessary to allow DNA replication.

Remarkably, A:T to G:C transition mutations, which were not seen in the absence of hAGT, were induced by BDO when either wt-hAGT or C145A-hAGT was present. The origin of these mutations is not clear but hAGT does interact at thymine residues even though repair of *O*⁴-methylthymine is very slow (45,52,53). Furthermore, on examination of BDO-mediated AGT-DNA complex formation *in vitro* with guanine and thymine-rich oligonucleotides, we observed comparable reactivity at both bases (results not shown). It is therefore possible that a significant number of cross-links are formed via a linkage to thymine. We also cannot exclude the possibility that these A:T to G:C mutations are due to adducts at adenine bases. Since BDO can alkylate *N*3 and *N*7 positions on adenine (30,33), these adducts may be susceptible to reaction with Cys¹⁴⁵ or Cys¹⁵⁰. Alternatively, BDO may react directly with wt-hAGT at either site to generate an activated intermediate capable of reacting at adenine bases.

Although the percentage of BDO-induced G:C to T:A transversion mutations was decreased by the presence of hAGT the absolute number of such mutations was greatly increased by c. 20-fold (Table 2). These mutations can readily be explained by previous MS studies, which revealed an *N*7-guanine adduct upon covalent linkage of hAGT to DNA in the presence of two different *bis*-electrophiles, DBE (10) and BDO (16). As mentioned above, *N*7-guanine adducts are prone to depurination yielding abasic sites, the predecessor to G:C to T:A transversions (10,16,47). The increase in mutation frequency could be due to either greater lability of the large protein-*N*7-adduct or to an increased production of such adducts via the reaction of the unstable-hAGT-DNA complex. A similar mechanism would account for the c. 40-fold increase in A:T to T:A transversions since BDO is known to alkylate *N*3 and *N*7 of adenine forming labile adducts. Depurination appears to be more likely as a source of the A:T to T:A transversions than direct miscoding across from a damaged adenine base, which would require mispairing of adenine with another adenine (54).

There was an increase in mutations at G:C sites in the BDO-treated cells expressing the C145A-hAGT mutant when compared to the empty vector or C145A/C150S-hAGT double mutant but the increase was much less than with the wt-hAGT, which is consistent with the lower reactivity of the Cys¹⁵⁰ residue for the formation of AGT-DNA cross-links. There was also clearly a rise in mutations at A:T base pairs when the C145A-hAGT mutant was expressed. Due to the location of the Cys¹⁵⁰ residue on the outer edge of the active site pocket it is possible that bases do not need to be flipped out of the DNA helix in order for a reaction and subsequent cross-linking to occur at Cys¹⁵⁰. The results with the R128A-hAGT mutant and R128A/C145A-hAGT double mutant shown in Figure 5 indicate clearly that for mutations to be formed as a result of crosslinking at either site hAGT:DNA binding is essential.

In summary, our studies confirm that the formation of protein-DNA crosslinks involving hAGT may contribute significantly to the toxicity and mutagenicity of BDO. Although our studies were carried out in bacterial cells expressing relatively high levels of AGT, there was a c. 50–100 fold increase in Rif^{res} mutation frequency following 1–2 mM BDO exposure when hAGT was present. Even if the effect in mammalian cells is reduced due to a lower level of AGT and to the presence of alternate pathways to deal with DNA-protein crosslinks it is quite plausible that AGT status would affect the response to BDO and related agents. Recently, Loeber et al. (17) have identified mechlorethamine-induced AGT-DNA conjugates from AGT-expressing CHO cells but not control cells, demonstrating that nitrogen mustards can cross-link the AGT protein to DNA in the presence of other nuclear proteins.

Our results also demonstrate that there are some notable differences in the mechanism of cross-link formation with BDO as compared to dihaloalkanes. The induction of DNA-protein crosslinks with BDO can occur at the Cys¹⁴⁵ active site and at the Cys¹⁵⁰ residue, which is located on the outer perimeter of the active site pocket, adjacent to the DNA binding region of hAGT. The position of this residue suggests that unlike conjugation at the active site, the base may not need to be flipped out of the duplex structure in order for hAGT-DNA adduction to occur at Cys¹⁵⁰. Secondly, a significant part of cross-linking by BDO is likely to occur via reaction of hAGT with adducts already present in DNA. Addition of a protein to such adducts may render them more resistant to repair and more likely to cause mutation or cell death. The fact that the C145A hAGT mutant, which is totally inactive as a repair protein (44,52), is able to take part in these reactions is in agreement with the suggestion made by Guengerich (13,55) that other proteins with sites susceptible to electrophilic attack and DNA binding properties might also enhance genotoxicity of bis-electrophiles.

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Figure 1.

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

Figure 2.

Effect of pre-incubation of BDO or DBE with wt-hAGT or C145S mutant on formation of cross-links between hAGT and DNA. Aliquots of 2 μ g of wt hAGT or mutant C145S protein were pre-incubated with 25 mM BDO or DBE for 0, 10, 30 and 60 min at 37°C. The 5'-[35 S]-d-[(GGA)₅G]-3' oligo was then added and the sample was reacted for an additional 3 h at 37°C. The formation of cross-links was then analyzed as in Figure 1. Panels A and B show autoradiograph results obtained with hAGT and C145S protein respectively. The densities of the major lower mobility form of [35 S]-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 rate of reduction 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. Panel D shows the results of pre-incubation of wt-hAGT with 25 mM DBE.

Figure 3.

Effect of preincubation of BDO or DBE with oligo on formation of cross-links between wt-hAGT or C145S mutant hAGT and DNA. The 5'-[³⁵S]-d[(GGA)₅G]-3' oligo was preincubated with 25 mM BDO for 0, 10, 30 and 60 min at 37°C. The samples were then passed through a MicroSpin™ G-25 columns to remove any unreacted compound. Aliquots of 2 μg of wild-type hAGT (hAGT) or mutant C145S protein (C145S) was then added to the sample and reacted for an additional 3 h at 37°C. The formation of cross-links was then analyzed and quantified as in Figure 2. Panels A and B show autoradiograph results obtained with hAGT and C145S protein respectively. 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 3 times. The data shown are from one representative experiment. Panel D shows the results of pre-incubation of the oligo with 25 mM DBE.

Figure 4.

Effects of BDO and hAGT expression on survival and mutation of *E. coli* TRG8 cells. Cells transformed with either empty pIN vector (pIN, white square, solid black line), wt-hAGT (hAGT, filled square), mutant C145A (C145A, filled triangle,) or double mutant C145A/C150S (C145A/C150S, white triangle) were treated with 0–5 mM of BDO for 30 min. The cytotoxicity (Panel A) and mutation frequency (Panel B) was determined as described under Methods. The results represent the average of 5–7 measurements. Two-way ANOVA was used to analyze the effect of wt-hAGT or mutant-hAGT expression on response to *bis*-electrophile exposure. Results for survival (Panel A): pIN versus hAGT, $P < 0.0001$; pIN versus C145A, $P = 0.004$; hAGT versus C145A, $P = 0.005$; pIN versus C145A/C150S: $P = 0.17$. Results for mutation

(Panel B): pIN versus hAGT, $P < 0.0001$; pIN versus C145A: $P < 0.0001$; hAGT versus C145A, $P < 0.0001$; pIN versus C145A/C150S, $P = 0.16$.

Figure 5.

Effect of R128A mutation on wt-hAGT and C145A mutant hAGT-mediated survival and mutation of *E. coli* TRG8 cells treated with BDO. Cells transformed with either empty pIN vector (pIN, white square, solid black line), wt-hAGT (hAGT, filled square), mutant C145A (C145A, filled triangle), mutant R128A (black circle, solid black line) or double mutant R128A/C145A (white circle) were treated with 0–5 mM of BDO for 30 min. The cytotoxicity (Panel A) and mutation frequency (Panel B) was determined as described under Methods. The scale on panel B is truncated to show the results with the C145A, R128A and R128A/C145A values more clearly but data for wt-hAGT is plotted fully in Figure 4.

Table 1

Frequency and site of Rif^{res} mutations in the *rpoB* gene produced by 1 mM BDO in pIN⁻, hAGT⁻, C145A- and C145A/C150S-expressing *E. coli* TRG8.

Base change	Amino acid change	Sequence	No hAGT	Number of mutations with hAGT expression indicated Wt-hAGT C145A C145A/C150S
G:C to A:T	D516N	5'-TTTATGGACGAGAAC-3'	0	4
G:C to A:T	H526Y	5'-ATTACGCACAAACGT-3'	3	23
G:C to A:T	R529C	5'-AAACGTCTGATCTCC-3'	2	0
G:C to A:T	R529H	5'-AAACGTCTGATCTCC-3'	0	3
G:C to A:T	S531F	5'-CGTATCTCCGCACTC-3'	11	5
G:C to A:T	P564L	5'-GAAACCCCTGAAGGT-3'	1	0
			(17/47)	(35/57)
				(26/64)
				(17/56)
G:C to T:A	Q513K	5'-CTGTCTCAGTTTATG-3'	4	3
G:C to T:A	D516Y	5'-TTTATGGACGAGAAC-3'	8	4
G:C to T:A	R529S	5'-AAACGTCTGATCTCC-3'	4	1
G:C to T:A	R529L	5'-AAACGTCTGATCTCC-3'	0	0
G:C to T:A	S531Y	5'-CGTATCTCCGCACTC-3'	1	2
			(17/47)	(10/57)
				(7/64)
				(16/56)
A:T to T:A	L511Q	5'-AGCCAGCTGTCTCAG-3'	0	1
A:T to T:A	Q513L	5'-CTGTCTCAGTTTATG-3'	8	0
A:T to T:A	D516V	5'-TTTATGGACGAGAAC-3'	5	5
A:T to T:A	H526L	5'-ATTACGCACAAACGT-3'	0	1
A:T to T:A	L572F	5'-GGTCTGATCAACTCT-3'	0	0
			(13/47)	(7/57)
				(20/64)
				(20/56)
A:T to G:C	Q513R	5'-CTGTCTCAGTTTATG-3'	0	1
A:T to G:C	H526R	5'-ATTACGCACAAACGT-3'	0	4
			(0/47)	(5/57)
				(2/64)
				(0/56)
A:T to C:G	Q513P	5'-CTGTCTCAGTTTATG-3'	0	0
			(0/47)	(0/57)
				(9/64)
				(3/56)

Table 2
Summary of mutations produced by 1 mM BDO in *E. coli* expressing hAGT, C145A or C145A/C150S.

AGT expressed Mutation	None (pIN vector)			hAGT			C145A			C145A/C150S		
	Total	%	Frequency*	Total	%	Frequency*	Total	%	Frequency*	Total	%	Frequency*
Transitions	17	36	0.5	40	70	47	28	44	3.2	17	30	0.4
G:C to A:T	17	36	0.5	35	61	41	26	41	3	17	30	0.4
A:T to G:C	-	-	-	5	9	6	2	3	0.2	-	-	-
Transversions	30	64	0.8	17	30	20	36	56	4	39	70	0.9
G:C to T:A	17	36	0.5	10	18	12	7	11	0.8	16	29	0.3
G:C to C:G	-	-	-	-	-	-	-	-	-	-	-	-
A:T to T:A	13	28	0.3	7	12	8	20	31	2.2	20	36	0.6
A:T to C:G	-	-	-	-	-	-	9	14	1	3	5	0.1
Mutants Collected	47			57			64			56		
BDO-induced mutation frequency*			1.3			67			7.2			1.3

* Mutations/10⁸ survivors (see Figure 4).