VIP DNA Damage Very Important Paper

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Detection and Characterization of 1,2-Dibromoethane-Derived DNA Crosslinks Formed with O^6-Alkylguanine-DNA Alkyltransferase**

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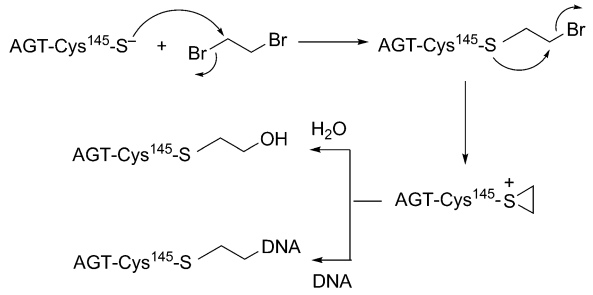
O^6-Alkylguanine-DNA alkyltransferase (AGT, MGMT) is involved in the repair of the promutagenic lesion O^6-methyl (Me) guanine and, to a lesser extent, O^4-Me thymine. AGT constitutes an important repair system against the mutagenic, carcinogenic, and toxic effects of simple alkylating agents. The repair mechanism involves a direct transfer of the alkyl group from the O6 atom of guanine to the active-site cysteine (Cys145) of the AGT protein in a stoichiometric, direct-damage-reversal pathway.[1,2] Apart from methyl groups, AGT can also repair larger alkyl groups at the O6 atom of guanine, including ethyl (Et), 2-chloroethyl, butyl, benzyl, and pyridyloxobutyl.[1,2]

Although AGT is a repair protein, it has been shown to paradoxically augment the toxicity of 1,2-dibromoethane (DBE, also called ethylene dibromide; Scheme 1).[3] Over-



Scheme 1. Mechanism of the formation of DBE-derived crosslinks by AGT.

expression of human AGT (hAGT) or AGTs from other species enhances the mutagenicity and lethality of DBE in *Escherichia coli* and mammalian cells.[3] This unusual enhancement of DBE toxicity stems from the increased reactivity of the active-site Cys145 residue that readily reacts with the bis(electrophile) DBE to form a half-mustard. The half-mustard intermediate subsequently cyclizes to an episulfonium ion that can react to form a covalent DNA-ethylene-AGT crosslink (based on the demonstrated chemistry with glutathione (GSH); Scheme 1).[4] Formation of a DNA–protein crosslink (DPC) is facilitated by the DNA-binding properties of AGT.[1] In prokaryotes, DPCs are repaired by both nucleotide excision repair and homologous recombination (HR), while in eukaryotes HR appears to be the major pathway.[5]

DBE has been widely used as a gasoline additive, as well as in pesticides and soil fumigants. DBE is carcinogenic in rats and toxic and mutagenic in microorganisms, plants, insects, and humans. Following reports of its toxicity, use of DBE has been drastically reduced. Earlier studies with DBE indicated that products formed through microsomal oxidation and by the action of GSH transferase (GST) may be responsible for its toxicity, apart from the AGT pathway. Similar to AGT, the detoxication pathway involves GST catalysis of the reaction of GSH with DBE to form a GSH-half-mustard (*S*-(2-bromoethyl)GSH) and finally the toxic episulfonium ion intermediate. The episulfonium ion reacts rapidly with DNA to form various guanine and adenine adducts.[6] Of these, a heat-labile guanyl-*N*7 adduct, *S*-[2-(*N*7-guanyl)ethyl]GSH, accounts for approximately 95% of total DNA adducts and is associated with G:C to A:T transition mutations.[6] hGST was also shown to enhance the mutagenicity of DBE in *Salmonella typhimurium* tester strains TA100 and TA1535.[7] Although the various bioactivation pathways of DBE are well characterized, the extent to which each pathway (activation by microsomal oxygenases, GST, or AGT) contributes to DBE-induced genotoxicity is still not clear.

The DNA adducts and the spectrum of mutations resulting from GSH-DBE episulfonium ion have been extensively studied.[6,8] In contrast, detection and structural characterization of crosslinks resulting from the AGT-DBE episulfonium ion remain mostly unexplored. To the best of our knowledge, the *N*7-guanyl adduct is the only DNA-ethylene-AGT crosslink that has been detected to date.[9] Using gel-shift assays with synthetic poly A, T, C, or GC oligonucleotides, Liu et al.[9] showed that the AGT-DBE episulfonium ion is capable of crosslinking to all four bases, with the apparent preference (based on gel-shift assays) being G > T > C > A. In addition, the major type of mutation observed in *S. typhimurium* (YG7108) and *E. coli* TRG8 cells was a G:C to A:T transition mutation.[9] Typically, labile adducts (e.g., guanyl-*N*7) will produce G:C to T:A transversions as a result of depurination and the A-coding rule.[10] The fact that DBE (in the presence of AGT) produces G:C to A:T transition mutations[9] also suggests the presence of other adducts.

The structural characterization of DPCs has been largely limited to labile adducts owing to the ease with which the

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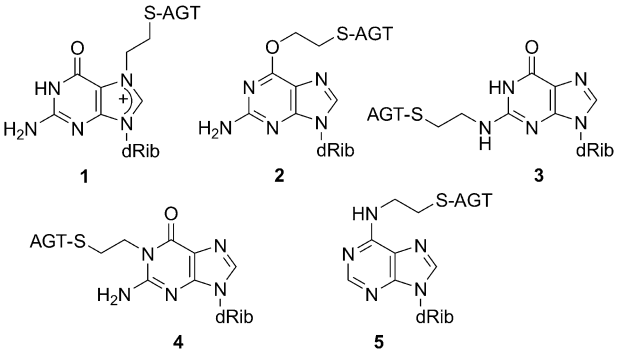
alkylated protein can be separated from DNA. In contrast, the structural characterization of nonlabile covalent DPCs, for example, with AGT, is challenging. The available methods for the characterization of DNA modifications involve digestion of the DNA to nucleosides, followed by LC-MS^n analysis and comparison with authentic standards. In the case of DNA-ethylene-AGT crosslinks, digestion of the DNA and protein to nucleosides and amino acids is inhibited because of steric hindrance for the hydrolases. Chemical methods of digestion of complexes are generally too harsh to preserve the linkages for structural characterization. MS methods of detection are optimized either for nucleic acids or protein/peptides (negative vs. positive electrospray ionization (ESI)) and, therefore, in the case of DPCs the presence of one negates the other.

Herein we report the detection and structural characterization of various DNA-ethylene-AGT crosslinks using LC-tandem mass spectrometric and chemical approaches. With these approaches, one labile and four nonlabile covalent DPCs at guanine and adenine (the N2, O6, and N1 atoms of guanine and N6 of adenine, structures **2**–**5**, Scheme 2) have been systematically identified.



Scheme 2. Structures of DNA-ethylene-AGT crosslinks. dRib = 2'-deoxyribose

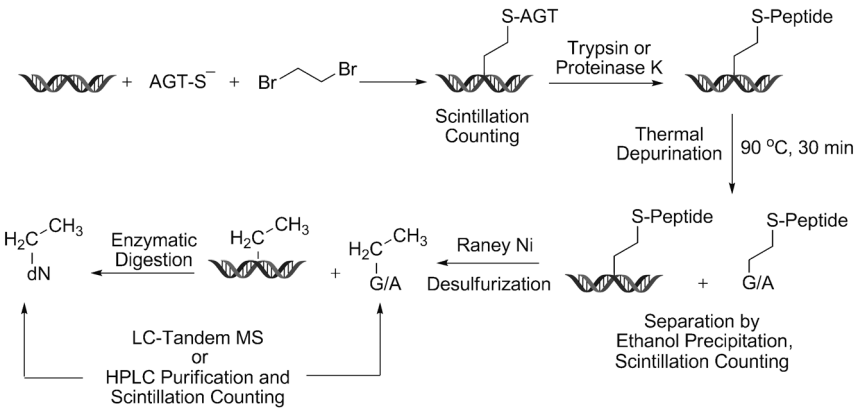
Our initial goal was to determine the formation of any nonlabile covalent DPCs formed in the presence of AGT and DBE. We previously reported an LC-tandem MS (LC-MS/MS or LC-MS^n)-based approach for the detection of oligonucleotide-peptide crosslinks resulting from trypsin digestion of an oligonucleotide-alkyl-AGT complex.[11] Although DPCs are resistant to nuclease and protease digestions, trypsin can efficiently digest the oligonucleotide-alkyl-AGT complex to a 12-mer peptide.[9] Accordingly, we reacted a 15-base-pair GC-rich double-stranded synthetic oligonucleotide with AGT in the presence of DBE. The results clearly indicated that the AGT-DBE episulfonium ion forms nonlabile crosslinks in DNA (Figures S1 and S2, Supporting Information),.

The above results confirmed the presence of nonlabile crosslinks but did not provide any information regarding the base specificity of the DPCs. In collision-induced dissociation (CID), the DNA bases mostly dissociate as neutral species. However, the possibility that the presence of the attached peptide might allow the base to dissociate as a charged species led us to search for peptide-ethylene-base species in the CID spectra. Contrary to our expectation, we did not find any fragment signal corresponding to peptide-ethylene-G or peptide-ethylene-C (m/z 1450 or 1490 for C or G adducts, respectively) in the case of the GC-rich oligonucleotide (Figure S1 e, Supporting Information). However, when the experiment was repeated with a double-stranded $T_5G_2T_4$ oligonucleotide (designed for preferential fragmentation at G compared to T, thereby increasing the yields of the various fragments resulting from G), a signal with m/z 1450 and corresponding to the fragment signal of peptide-ethylene-G was detected (Figure S2, Supporting Information).

We also performed a similar experiment with a double-stranded (12-base pair) $(AT)_6$ oligonucleotide. Unlike for the GC-rich oligonucleotide, in the CID spectrum we found a signal at m/z 1474.6 that corresponds to the adenine connected to the 12-mer peptide through the ethylene linkage (Figure S4, Supporting Information). Together, these results indicate that the AGT-DBE episulfonium ion can form nonlabile crosslinks at G and A in DNA.

The LC-tandem MS approach with oligonucleotides established the presence of nonlabile DNA-ethylene-AGT crosslinks on G and A residues, but it did not provide any information regarding the structures of the DPCs. Moreover, the use of small oligonucleotides has limited capability in dealing with the effects of sequence variation. In an attempt to elucidate the structure of the DPC, we realized that the complex would need to be degraded to the single-nucleoside level. Accordingly, we utilized a reductive desulfuration approach to convert DNA-ethylene-peptide crosslinks into DNA-Et adducts.[12] Reductive desulfurization is a well-established chemical procedure for breaking molecules by attack on a thioether or other sulfur atom. Raney nickel was first employed by Bougault et al.,[12a,13a] and nickel boride ($NaBH_4/NiCl_2$) is also effective.[12b,13b,c] We previously utilized nickel boride in the initial characterization of the major DBE-GSH DNA adduct by converting it to *N*7-EtG by treatment with nickel boride.[12b] In this case, we used a Raney nickel procedure that had been applied by Hecht et al.[12a] to tRNA, because of the issues of solubility (in aqueous buffer) and the adsorption of DNA and nucleosides on the nickel. The complex was first digested with either trypsin or proteinase K to a small peptide, followed by removal of the peptide by reductive desulfuration with Raney nickel. This process leaves only an Et group (derived from the DBE part of the DPC) on the DNA, which can then be readily digested to the nucleoside level with nucleases and phosphatases for characterization by LC-MS and comparison with authentic standards. To include all possible sequence variations, sheared calf thymus DNA was used (Scheme 3). A study with a set of three nucleosides containing an ethylene-GSH modification indicated yields of 89–91 % for the reduction with Raney nickel (Table S3, Supporting Information).

DNA was incubated with DBE and AGT to generate DPCs and then subjected to various procedures, described in the experimental section (Supporting Information). LC-MS/MS analysis of the final reaction product using the m/z 280→164 transition (characteristic neutral loss of −116 for deoxyribonucleosides) for EtdA adducts gave a peak at t_R 3.6 min. CID of the m/z 164 signal gave a fragment ion at m/z 136,



Scheme 3. Flow chart for the detection and quantitation of various DPC and DNA adducts.

consistent with an authentic sample of N^6-EtdA (**5**; Figure S5, Supporting Information). Together, these results clearly indicate the formation of the DNA-ethylene-AGT crosslink at the N6 atom of dA in the DNA (although the presence of an initial crosslink at the N1 atom of dA followed by Dimroth rearrangement cannot be excluded).

In principle, there are three potential sites in dG (N^2, $N1$, O^6) that could form nonlabile DPCs with the AGT-DBE episulfonium ion. Authentic standards of these three ethyl-dG adducts were used in the LC method: t_R $N1$-EtdG (**4**) 3.2 min, N^2-EtdG (**3**) 3.6 min, and O^6-EtdG (**2**) 4.0 min. LC-MS^3 of the deglycosylated MH^+-116 fragments showed very distinct fragmentation patterns: major ion m/z 163 for N^2-EtdG and m/z 152 for $N1$-EtdG and O^6-EtdG. LC-MS/MS analysis using the m/z 296→180 transitions gave a peak at t_R 3.6 and a very small peak at t_R 4.1 min. CID of the m/z 180 fragments gave a peak at t_R 3.6 min with a major fragment ion at m/z 163 and another peak at t_R 4.1 min, with a major fragment ion at m/z 152. Based on the t_R ($\approx$3.2, 3.5, and 4.0 min for $N1$-, N^2-, and O^6-EtdG, respectively) and fragmentation of the authentic standard (Figures S6 and S7, Supporting Information), the peaks at t_R 3.6 and 4.1 min are N^2-EtdG and O^6-EtdG, respectively. These peaks were not seen in control reactions where either AGT or AGT and DBE are omitted (Figure S9, Supporting Information). From the LC-tandem MS results we conclude that DPCs form at the N2 and O6 positions of dG in the DNA. The finding that the O^6-guanyl-AGT is not cleaved by AGT itself is consistent with the lack of AGT activity on an O^6-dG butyl crosslink we reported earlier.[11b]

Calf thymus DNA was treated with ^{14}C-DBE and AGT as described previously (Scheme 3). An aliquot of the precipitated DNA-$(^{14}CH_2)_2$-AGT complex was analyzed by scintillation counting and showed that the reaction with AGT gave significantly higher ($\approx$6 nmol) levels of DPCs (both labile and nonlabile) compared to the control reactions (Figure 1 a). The reaction mixtures were subsequently digested with proteinase K and precipitated with ethanol to remove the digested peptides, and heated at 90 °C for 30 min to hydrolyze the labile DNA-ethylene-peptide complexes. Of the total DNA-ethylene-AGT complexes formed, around 80 % were found to be labile and 20 % nonlabile (Figure 1 a,c). To determine if DPCs are also formed at $N3$-dA, the precipitate was subjected to reductive desulfuration with Raney nickel to generate $N7$-EtdG and/or $N3$-EtdA adducts (Scheme 3). The only labile adduct detected was the $N7$-dG one, and the $N3$-dA adduct was not present. Further, no evidence for an $N3$-adenyl-linked tryptic peptide was seen with LC-MS^n of the soluble fraction.

The precipitate, containing the nonlabile adducts, was subjected to desulfurization with Raney nickel and DNA digestion to convert DNA-$(^{14}CH_2)_2$-peptide complexes into Et adducts (see above). The various Et adducts were separated by HPLC after spiking with carrier $N1$-, N^2-, and O^6-EtdG and N^6-EtdA adducts (monitoring A_{260}; Figure S10, Supporting Information). The radioactivity associated with the various adducts in the DNA + AGT + DBE samples was significantly higher than in the control samples of DNA + DBE. The yields of the various adducts were in the order N^6-EtdA $\approx$ N^2-EtdG > O^6-EtdG $\approx$ $N1$-EtdG. These results are consistent with the LC-MS data; in the LC-MS^3 analysis the yield of the O^6-EtdG adduct was just above the detection limit and the $N1$-EtdG could not be detected, but with ^{14}C-DBE both adducts were detected.

Mutational studies involving overexpression of the repair protein AGT in the presence of DBE in cell culture systems show G:C to A:T transition mutations.[9,14] The observation that only about 20 % of the mutations in bacteria can be attributed to depurination,[9] although around 80 % of the adducts are labile (Figure 1), suggests that the nonlabile

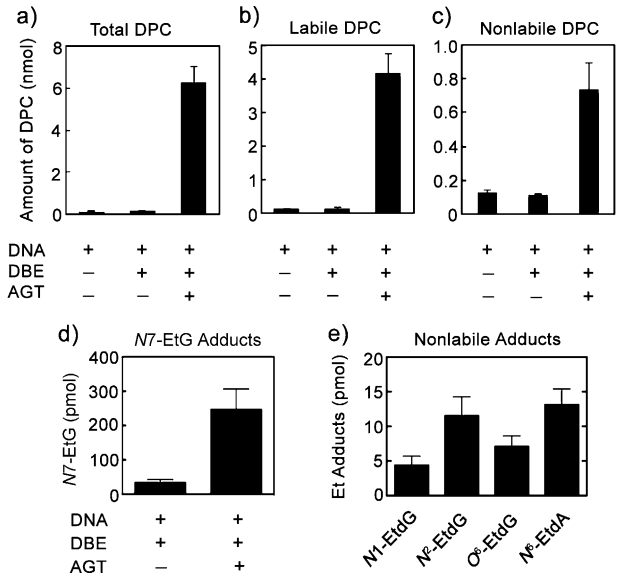


Figure 1. Yields of the various types of DPCs formed in the presence of AGT and ^{14}C-DBE. a) Total DPCs (labile and nonlabile). b) Labile DPCs. c) Nonlabile DPCs. d) Relative yields of labile DPCs ($N7$-G adducts measured as $N7$-EtG). e) Yields of various nonlabile DPCs ($N1$-dG, N^2-dG, and O^6-dG and N^6-dA) measured as their corresponding Et adducts (n = 3, ± SD).

GPCs are much more mutagenic. In vitro primer extension assays with intact proteins have not been reported, to our knowledge. However, primer extension assays with DNA–peptide crosslinks have revealed that translesion polymerases can bypass four to ten amino acid/peptide adducts.[15] Recent work from our group with DNA-butudiene diepoxide-GSH crosslinks at the N6 position of dA (the major adduct detected in vivo) has shown that a number of polymerases can also bypass it with high fidelity.[16] Electroporation of an hAGT-butadiene diepoxide conjugate into mammalian cells caused both cytotoxicity and mutations,[17] further establishing the relevance of the AGT pathway in bioactivation. The mutations resulting from DPCs may be due to misincorporation by translesion polymerases or HR.[5,18]

Knowing the chemical structures of the DPCs is extremely important to understand the mechanisms of mutagenesis. By using a combination of LC-tandem MS, chemical modification, and radioisotope approaches, we have detected and characterized the structures of various DNA-ethylene-AGT crosslinks. Three nonlabile modifications were found at the N1, N2, and O6 atoms of guanine and one at the N6 atom of adenine (structures **2**–**5**), consistent with our previous reports with the GSH-DBE episulfonium ions.[6]

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