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Structural Elucidation of DNA-Protein Crosslinks Using Reductive Desulfurization and Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Structural characterization of DNA-protein crosslinks involving cysteine using reductive desulfurization in combination with liquid chromatography-tandem mass spectrometry is highlighted. The novel approach was used to identify hydrolytically stable DNA-protein lesions involving alkylguanine DNA alkyltransferase (AGT).

Keywords

DNA-protein crosslinks; *O*⁶-alkylguanine DNA alkyltransferase; labile and nonlabile DNA adducts; desulfurization; liquid chromatography-tandem mass spectrometry

DNA-protein crosslinks (DPCs) are ubiquitous, highly heterogeneous DNA lesions formed when nuclear proteins become covalently trapped on DNA in the presence of free radicals and *bis*-electrophiles. DPCs can be induced by a wide range of environmental carcinogens, antitumor drugs, lipid peroxidation products, reactive oxygen species, transition metals, UV light, and ionizing radiation.^[1–3] DPC formation disrupts normal DNA-protein interactions and interferes with DNA replication, transcription, and repair, ultimately compromising genetic stability and cell viability.^[4, 5] Accumulation of bulky DPC lesions in cells and tissues can contribute to cardiovascular disease, age-related neurodegeneration, and cancer.^[3, 6–8] Therefore, structural and quantitative analysis of DPC adducts formed endogenously and upon exposure to cross-linking agents is urgently needed to help understand the etiology for these common diseases and to establish the risk factors for their development.

DNA repair protein *O*⁶-alkylguanine DNA alkyltransferase (AGT), also known as *O*⁶-methylguanine DNA methyltransferase (MGMT), has been widely used to investigate DPC formation by common *bis*-electrophiles. AGT is involved in the direct removal of methyl, ethyl, 2-chloroethyl, butyl, benzyl, and 4-(3-pyridyl)-4-oxobutyl groups from the *O*⁶ position of guanine in DNA.^[9, 10] Active site cysteine of the protein (Cys¹⁴⁵) is activated to a thiolate anion *via* a hydrogen bond network around the active site involving His146, Glu172, and a water molecule, greatly increasing its nucleophilicity.^[10] Following binding to the minor groove of DNA, AGT flips out the modified nucleoside out of the base stack and catalyzes *O*⁶-alkyl group transfer from DNA to the active site cysteine residue (Cys¹⁴⁵), restoring normal guanine and producing alkylated protein.

Multiple studies have shown that AGT readily participates in crosslinking reactions with DNA in the presence of *bis*-electrophiles to produce toxic and mutagenic DPC lesions.^[11–14] AGT overexpression in bacteria enhances the toxicity and mutagenicity of dihaloalkanes such as 1,2-dibromoethane (DBE),^[11, 15–18] and the introduction of DNA-reactive AGT monoepoxides into mammalian cells via electroporation leads to mutations and cell death.^[19] It has been proposed that AGT Cys¹⁴⁵ reacts with dihaloalkanes to generate a half mustard, which alkylates DNA via an episulfonium ion intermediate to generate covalent DPCs.^[10, 18, 20, 21] The majority of the resulting lesions (> 80%) are hydrolytically labile adducts involving the N7 position of guanine in DNA, however, hydrolytically stable DPC lesions are also formed. Gel shift studies have revealed that DBE forms covalent cross-links between AGT and all four DNA bases, with the adduct yields in the order G > T > C > A.^[20] However, the structures of hydrolytically stable AGT-DNA adducts have remained elusive.

Structural elucidation of DPC lesions has largely relied on liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MSⁿ) methodologies.^[13, 14, 19, 22] However, ESI MS analysis of DPC conjugates is complicated due to the presence of negatively charged DNA and positively charged proteins within their structure. To facilitate MS detection of DPCs, hydrolytically labile N7 guanine lesions can be released from the DNA backbone as free bases by heating to generate nucleobase-protein adducts.^[13, 14] Subsequent proteolytic digestion of the protein generates nucleobase-peptide

conjugates, which can be readily sequenced by tandem mass spectrometry to identify the cross-linking site.^[13, 14, 18, 20]

This selective hydrolysis approach has been used to examine AGT cross-linking to the *N*7 position of guanine in DNA in the presence of 1,2,3,4-diepoxybutane, epihalohydrins, dihaloalkanes, nitrogen mustards, and platinum compounds.^[13, 14, 21, 23] However, hydrolytically stable DPC adducts may be equally important for the toxicity and mutagenicity of cross-linking agents. For example, the major types of mutations observed in bacterial cells treated with 1,2-dibromoethane (DBE) in the presence of AGT are G→A transitions.^[20] Hydrolytically unstable *N*7 guanine adducts, if converted to the corresponding AP sites, are expected to cause G→T transversions.^[24] This suggests that non-labile DPC adducts at G or C nucleobases may be responsible for the mutagenic properties of DBE-mediated DPCs.

Chowdhury *et al.* have developed a powerful novel approach for structural elucidation of hydrolytically stable AGT-DNA cross-links.^[12] At the heart of their method is reductive desulfurization of the thioether linkage between AGT and DNA to convert cysteine DPCs to the corresponding ethyl-DNA adducts, which can be readily characterized by LC-MSⁿ (Scheme 1). Covalent DNA-ethylene-AGT crosslinks were generated by reacting calf thymus DNA with DBE in the presence of AGT.^[12] Protein component of the resulting DPCs was digested to peptides with trypsin, followed by the release of labile adducts (*N*7G and *N*3A) by thermal depurination and ethanol precipitation of nonlabile DNA-ethylene-peptide crosslinks. The supernatant containing labile nucleobase-ethylene-peptide adducts was subjected to desulfurization using Raney Ni to generate the corresponding nucleobase ethyl adducts (Scheme 1). Similarly, nonlabile DNA adducts were subjected to Raney Ni treatment, followed by enzymatic digestion of DNA to produce 2'-deoxynucleoside ethyl adducts. Both nucleobase ethyl adducts and nucleoside ethyl adducts were characterized by selective reaction monitoring (SRM) by LC-MS/MS and LC-MS/MS/MS, leading to identification of four novel nonlabile covalent DPC adducts to the *N*1, *N*2 and *O*⁶ of guanine and the *N*⁶ of adenine (Scheme 2).^[12]

Although the reductive desulfurization approach^[12] is only applicable to identifying cysteine cross-links and cannot be used to study DPC formation at other nucleophilic side chains of proteins (e.g. lysines, arginines and histidines), it can be used in future studies to evaluate DPC formation to other proteins containing nucleophilic cysteine residues, including histones, HMG box proteins, and proteins involved in DNA repair, replication and transcription. One could envision the application of this methodology to obtain quantitative information regarding total cellular pool of cysteine-containing DPCs in cells and tissues, contributing to our understanding of the biological effects of DPCs. Such *in vivo* studies may benefit from the use of nanoLC-nanospray ionization-tandem mass spectrometry, isotope dilution, and high resolution mass spectrometry (HRMS)/accurate mass detection to improve method accuracy and sensitivity.

The novel hydrolytically stable guanine adducts identified in this study (Scheme 2) may help explain the origins of G → A transitions observed upon DBE treatment of AGT-expressing cells.^[20] As mentioned above, the structural basis of these genetic changes has remained

unknown, despite active research in the area. Future polymerase bypass and mutagenesis studies with site-specifically modified DNA templates are needed to confirm the ability of the newly discovered O^6G , N^1G , and N^2G AGT adducts (Scheme 2) to induce $G \rightarrow A$ transitions. Based on previous studies with other bulky N^2 -G adducts, DPCs at this position are likely to completely block DNA replication.^[25] On the other hand, the ability of O^6 -G to induce $G \rightarrow A$ mutations lesions is well known.^[26] Given the hydrolytic stability of these adducts, it would be important to identify DNA repair mechanisms responsible for their removal in cells.

Finally, although the current study has focused on identifying the cross-linking sites within DNA, it would be interesting to adopt the reductive desulfurization methodology (for example, in combination with iodoacetamide alkylation to label free cysteines) to determine sequence specificity for stable DPC formation within the proteins. This would provide complete information about the cross-linking sites within DNA and protein molecules, allowing for future structural and biological studies of these complex and fascinating lesions.

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Scheme 1.

Structure elucidation of AGT-DNA cross-links induced by DBE using reductive desulfurization in combination with liquid chromatography-tandem mass spectrometry. ^[12]

Scheme 2.

DNA-ethylene-AGT crosslinks of DBE identified by Chowdhury and coworkers^[12]: hydrolytically labile *N*7 guanine adduct (a) and nonlabile adducts at the *O*⁶ guanine (b), *N*1 guanine (c), *N*² guanine (d) and *N*⁶ adenine (e).