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Mechlorethamine-Induced DNA-Protein Cross-Linking in Human Fibrosarcoma (HT1080) Cells

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Abstract

Antitumor nitrogen mustards, such as *bis*(2-chloroethyl)methylamine (mechlorethamine), are useful chemotherapeutic agents with a long history of clinical application. The antitumor effects of nitrogen mustards are attributed to their ability to induce DNA-DNA and DNA-protein cross-links (DPCs) that block DNA replication. In the present work, a mass spectrometry based methodology was employed to characterize *in vivo* DNA-protein cross-linking following treatment of human fibrosarcoma (HT1080) cells with cytotoxic concentrations of mechlorethamine. A combination of mass spectrometry-based proteomics and immunological detection was used to identify 38 nuclear proteins which were covalently cross-linked to chromosomal DNA following treatment with mechlorethamine. Isotope dilution HPLC-ESI⁺-MS/MS analysis of total proteolytic digests revealed a concentration-dependent formation of *N*-[2-(*S*-cysteinyl)ethyl]-*N*-[2-(guan-7-yl)ethyl]methylamine (Cys-N7G-EMA) conjugates, indicating that mechlorethamine cross-links cysteine thiols within proteins to N-7 positions of guanine in DNA.

Keywords

mass spectrometry; DNA-protein cross-links; nitrogen mustards; western blot; DNA repair

Introduction

DNA-protein cross-links (DPCs) can be caused by exposure to a broad range of chemical and physical agents, including transition metals,¹ ionizing radiation,² and endogenous aldehydes.³ DPCs can also be induced by chemotherapeutic agents, such as platinum drugs,⁴ alkylnitrosoureas,⁵ and nitrogen mustards.⁶ As compared to other types of DNA adducts, DPCs are unusually bulky and complex lesions whose structures and composition are not well characterized, and their biological consequences are not well understood. Because of their considerable size and their ability to block normal DNA-protein interactions, DPCs interfere with critical cellular processes, including DNA replication, transcription, chromatin

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Supplementary Information: Experimental details for mass spectrometry analysis of tryptic peptides and Cys-N7G-EMA; cytotoxicity results for mechlorethamine treated HT1080 cells, representative HPLC-UV trace for enzymatic digests of DNA isolated from mechlorethamine-treated HT1080 cells. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

remodeling, and DNA repair.⁷ If left unrepaired, DPCs may persist through several cycles of replication, resulting in permanent DNA alterations and damaging cytotoxic and mutagenic effects.^{7,8}

Antitumor nitrogen mustards, e.g. *bis*(2-chloroethyl)methylamine (mechlorethamine), chlorambucil, melphalan, and cyclophosphamide, have a long history of clinical use in treatment of cancer.⁹ Nitrogen mustards are bifunctional alkylating agents containing two *N*-(2-chloroethyl) groups that can spontaneously cyclize under physiological conditions. The resulting aziridinium ions are highly reactive and are capable of alkylating nucleophilic sites within DNA and proteins to form DPCs and DNA-DNA cross-links (Scheme 1).¹⁰ Nitrogen mustard-induced DNA-DNA cross-links are well characterized and are thought to play a prominent role in their antitumor effects.^{11–14} In contrast, little is known about the composition and cellular abundance of the corresponding DNA-protein lesions.^{15–17}

The ability of nitrogen mustards to cross-link proteins to DNA was first reported in the 1970s by Ewig and Kohn, who employed alkaline elution methodology to investigate DNA damage in mouse leukemia L1210 cells exposed to mechlorethamine.¹⁶ These studies detected the formation of bulky, proteinase-sensitive DNA lesions hypothesized to be DPCs.¹⁶ Thomas *et al.* subsequently confirmed that mechlorethamine treatment resulted in covalent binding of nuclear proteins to chromosomal DNA through alkylated purine residues.¹⁵ Further studies revealed that while these DNA-protein complexes were resistant to separation by detergents and high salt buffers, the proteins were released from the DNA under acidic conditions that induce depurination of modified nucleobases.¹⁵ However, these primarily biophysical methods could not identify the proteins participating in DPC formation or quantify the extent of cross-linking.

More recently, our laboratory used a mass spectrometry-based approach to demonstrate that the recombinant human DNA repair protein *O*⁶-alkylguanine DNA-alkyltransferase (AGT) becomes cross-linked to DNA *in vitro* in the presence of two representative nitrogen mustards, mechlorethamine and chlorambucil. Peptide sequencing revealed that the cross-linking involved two active site residues of the AGT protein, Cys¹⁴⁵ and Cys¹⁵⁰.¹⁸ We then employed an affinity capture methodology coupled with mass spectrometry and immunological detection to identify 53 human proteins that became cross-linked to biotinylated DNA duplexes *in vitro* in the presence of mechlorethamine.¹⁹

In the present work, we sought to characterize mechlorethamine-mediated DPC formation *in vivo* (cultured human cells). Unlike our previous *in vitro* experiments with synthetic DNA duplexes, studies in cells involve intact chromatin and normal DNA-protein interactions, potentially increasing the efficiency of DPC formation and capturing physiologically relevant interactions. A simple isolation strategy consisting of a modified phenol/chloroform extraction in the presence of proteasome inhibitors was developed to enable selective purification of proteins covalently trapped on DNA and the removal of the bulk of non-covalently attached proteins (Scheme 2). This approach was coupled with thermal hydrolysis of DNA to release protein-guanine conjugates, electrophoretic separation of the proteins, mass spectrometry-based proteomics, and western blotting. A total of 38 cross-linked proteins were identified, including a number of nuclear proteins that play important roles in DNA repair, transcriptional regulation, and chromatin remodeling, e.g. poly(ADP-ribose) polymerase 1, DNA-(apurinic- or apyrimidinic-site) lyase, x-ray cross-complementing protein 1, tumor suppressor p53-binding protein 1, high mobility group protein 1, and nucleophosmin. Finally, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI⁺-MS/MS) analyses of total proteolytic digests revealed a concentration-dependent formation of *N*-[2-(*S*-cysteinyl)ethyl]-*N*-[2-(guan-7-yl)ethyl]methylamine (Cys-N7G-EMA) conjugates in

HT1080 cells treated with cytotoxic concentrations of the drug. Taken together, these results indicate that antitumor nitrogen mustards such as mechlorethamine are capable of forming covalent DPCs *in vivo* involving a range of nuclear proteins via sequential alkylation of nucleophilic amino acid side chains within proteins and guanine nucleobases in DNA. These findings are significant because the super-bulky DPC lesions are likely to contribute to the cytotoxic and mutagenic effects of nitrogen mustards drugs.

Experimental section

Safety statement

Phenol and chloroform are toxic chemicals that should be handled with caution in a well-ventilated fume hood with appropriate personal protective equipment.

Chemicals and reagents—Mechlorethamine hydrochloride, phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, dithiothreitol (DTT), iodoacetamide, chloroform, ribonuclease A, nuclease P1, and alkaline phosphatase were purchased from Sigma (St. Louis, MO). Mass spectrometry-grade Trypsin Gold was purchased from Promega (Madison, WI). Proteinase K was obtained from New England Biolabs (Beverly, MA). Primary polyclonal antibodies specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), matrin-3, poly(ADP-ribose) polymerase 1 (PARP), DNA-(apurinic- or apyrimidinic-site) lyase (Ref-1), nucleophosmin (B-23), and tumor suppressor p53 binding protein 1 (53BP1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary polyclonal antibody to ATP-dependent DNA helicase 2 (Ku) and the monoclonal antibody specific for x-ray cross-complementing protein 1 (XRCC-1) were purchased from Lab Vision/NeoMarkers (Fremont, CA). The primary monoclonal antibody against AGT was purchased from Millipore (Temecula, CA). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma (St. Louis, MO). Cys-N7G-EMA and Cys-[¹⁵N]-N7G-EMA were prepared as described previously.¹⁸

Cell culture—Human fibrosarcoma (HT1080) cells²⁰ were obtained from the American Type Culture Collection. The cells were maintained as exponentially growing monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 9% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells expressing recombinant human AGT (CHO-AGT) were generously provided by Professor Anthony E. Pegg (Pennsylvania State University), and were maintained as exponentially growing monolayer cultures in α -minimal essential medium supplemented with 9% FBS and 1 mg/mL G-418. Both cell lines were maintained in a humidified incubator at 37°C with 5% CO₂.

Assay for cytotoxicity as a result of mechlorethamine exposure—The effect of mechlorethamine on cell survival was determined *via* a direct cell count assay. HT1080 cells were plated in Dulbecco's modified Eagle's medium containing 9% FBS at a density of 5×10^5 cells/dish and permitted to adhere overnight. On the following morning, cells (in triplicate) were treated with mechlorethamine (0, 10, 25, 50, or 100 μ M) for 3 h at 37°C. Following treatment, drug-containing media was replaced with drug-free media, and the cells were allowed to recover for 18 h, followed by cell counting in a haemocytometer. Cytotoxicity was expressed as the number of cells surviving mechlorethamine treatment relative to buffer-treated controls (Supplementary Figure S-1).

Isolation of proteins cross-linked to chromosomal DNA by mechlorethamine—To analyze DPC formation in mammalian cells exposed to mechlorethamine, HT1080 or CHO-AGT cells were treated with mechlorethamine (0, 10, 25, 50, or 100 μ M) for 3 h at 37°C. Following exposure, the cells were washed with ice cold phosphate-buffered saline

(PBS) and re-suspended in PBS to a final density of $\sim 2 \times 10^6$ cells/mL. To isolate nuclei, cells were lysed by adding an equal volume of 2X cell lysis buffer (20 mM Tris-HCl/10 mM MgCl_2 /2% v/v Triton-X100/0.65M sucrose), incubated on ice for 5 min, and centrifuged at 2,000g for 10 min at 4°C. The nuclear pellet was re-suspended in a saline-EDTA solution (75 mM NaCl/24 mM EDTA/1% (w/v) SDS, pH 8.0) containing RNase A (10 $\mu\text{g/mL}$) and a protease inhibitor cocktail (1 mM PMSF; 1 $\mu\text{g/mL}$ pepstatin; 0.5 $\mu\text{g/mL}$ leupeptin; 1.5 $\mu\text{g/mL}$ aprotinin) to a concentration of $\sim 5 \times 10^6$ nuclei/mL and incubated for 2 h at 37°C with gentle shaking. To isolate chromosomal DNA containing covalent DPCs, nuclear lysates were extracted by the addition of two volumes of Tris-buffer saturated phenol. The resulting white emulsion was centrifuged at 1,000g for 15 min at room temperature. The aqueous layer and the interface material were subjected to a second extraction with two volumes of Tris-buffer saturated phenol:chloroform (1:1). DNA was precipitated with cold ethanol. Samples were centrifuged at 4,000 g for 20 min at 4°C, and the resulting DNA pellet was washed with ice cold 70% ethanol, air dried, and reconstituted in 1 mL MilliPore H_2O . DNA concentrations were estimated by UV spectrophotometry. DNA amounts and its purity were determined by quantitation of dG in enzymatic hydrolysates as described below.

Enzymatic digestion of DNA and dG quantitation by HPLC—In order to quantify the DNA isolated from HT1080 cells and to detect any RNA contamination, approximately 5 μg aliquot of DNA from each sample was taken and subjected to neutral thermal hydrolysis (1 h at 70°C) to release protein-guanine conjugates from the DNA backbone. Partially depurinated DNA was digested to 2'-deoxynucleosides in the presence of nuclease P1 (1 U), alkaline phosphatase (10 U), and 45 ng coformycin (to prevent deamination of dA) in 5 mM ZnCl_2 /50 mM ammonium acetate (pH 5.3) buffer for 20 h at 37°C. Enzymatic digests were passed through Amicon Ultra-0.5 mL Centrifugal Filters (10K MWCO, Millipore, Temecula, CA) to remove proteins prior to HPLC-UV analysis.

An Agilent Technologies HPLC System (1100 model) was equipped with a diode array UV detector and an autosampler. Chromatographic separation of nucleosides was achieved using a Zorbax SB-C8 column (4.6 $\times$ 150 mm, 5 μm , from Agilent Technologies, Palo Alto, CA) eluted with a gradient of 150 mM ammonium acetate (A) and acetonitrile (B). Solvent composition was initially held at 0% B for 2 min, followed by a linear increase to 3% B over 13 min, and further to 30% B over 3 min, where it was held for the final 7 min. UV absorbance was monitored at 260 nm. Using this method, dG eluted as a sharp peak at ~ 13.5 min. The amounts of dG present in each sample were determined by comparing the its HPLC peak area to a calibration curve constructed by injecting of known dG amounts.

Mass spectrometric identification of cross-linked proteins—To identify cellular proteins that become covalently attached to chromosomal DNA in mechlorethamine-treated cells, HT1080 cells ($\sim 10^7$ cells, in triplicate) were treated with 25 μM mechlorethamine or solvent control for 3 h at 37°C, and chromosomal DNA containing covalently cross-linked proteins was isolated by phenol/chloroform extraction and quantified as described above. DNA (30 μg) was subjected to neutral thermal hydrolysis to release protein-guanine conjugates, dried under vacuum, and reconstituted in 50 μL of 1X NuPAGE Sample Buffer (Invitrogen, Carlsbad, CA). Proteins were separated using 12% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA) and stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA). The gel lanes were divided into five sections encompassing the entire molecular weight range, and the gel sections were further diced into ~ 1 mm pieces. The proteins contained within the gel pieces were subjected to in-gel tryptic digestion as described previously.²¹ In brief, gel pieces were rinsed with 25 mM ammonium bicarbonate, and the protein thiols were subjected to reduction with DTT (300 mM) and alkylation with iodoacetamide. The gel pieces were then dehydrated by incubation with acetonitrile, dried under vacuum, and reconstituted in 25 mM ammonium bicarbonate buffer. Mass spectrometry-grade trypsin (2–

3 µg) was added, and the samples were digested overnight at 37°C. The resulting tryptic peptides were extracted with 60% acetonitrile containing 0.1% aqueous formic acid, evaporated to dryness, and reconstituted in 0.1% formic acid. HPLC-ESI⁺-MS/MS analysis of tryptic peptides was conducted on a Thermo LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA) as described previously^{19,22} and detailed in Supplement S-1.

Western blot analysis of identified proteins—HT1080 cells (~10⁷) were treated with mechlorethamine (0, 10, 25, 50, or 100 µM) for 3 h at 37°C. Chromosomal DNA, along with any covalently bound proteins, was extracted and quantified as described above.

Approximately 15 µg of DNA from each sample was subjected to neutral thermal hydrolysis (1 h at 70°C) to release protein-guanine conjugates from the DNA backbone. Proteins were separated by 12% SDS-PAGE and transferred to Trans-blot nitrocellulose membranes (Bio-Rad, Hercules, CA). Following blocking in Tris-buffered saline (TBS) containing 5% (w/v) bovine serum albumin, the membranes were incubated with the primary antibody against target protein for 3 h at room temperature, rinsed with TBS buffer, and incubated overnight at 4°C with the corresponding alkaline phosphatase-conjugated secondary antibody. The blots were washed and developed with SIGMA Fast BCIP/NBT (Sigma, St. Louis, MO) according to manufacturer's instructions. The developed blots were scanned as image files. ImageJ software (available free of charge from the NIH website, www.ncbi.nlm.nih.gov) was used to quantify the optical densities of the protein bands. The efficiency of DNA-protein cross-linking *in vivo* was approximated by comparing signal intensities of the protein which was co-purified with chromosomal DNA (corresponding to cross-linked protein) and the intensity of the corresponding protein band present in the whole cell protein lysate (representing total cellular protein). Experiments with Chinese hamster ovary cells expressing human AGT protein (CHO-AGT) were conducted analogously following treatment with 0, 1, 5, 10, 25, or 50 µM mechlorethamine.

Preparation of whole cell protein extracts—HT1080 cells (~10⁷) were harvested, washed three times with ice cold PBS, and centrifuged at 1,000g for 5 min at room temperature. The cell pellet was re-suspended in 2 mL lysis buffer (50 mM NaH₂PO₄/300 mM NaCl/10 mM imidazole, pH 8.0) containing a protease inhibitor cocktail (1 mM PMSF; 1 µg/mL pepstatin; 0.5 µg/mL leupeptin; 0.75 µg/mL aprotinin) and incubated on ice for 5 min prior to sonication (3 × 15 s, 1 min on ice in between). The resulting cellular lysate was centrifuged at 80,000g for 20 min at 4°C, and cellular proteins were isolated in the clear supernatant.

Isotope dilution HPLC-ESI⁺-MS/MS analysis of Cys-N7G-EMA in cells exposed to mechlorethamine—HT1080 cells (~10⁶) were treated with mechlorethamine (0, 10, 25, 50, or 100 µM for 3 h at 37°C, in triplicate). Chromosomal DNA containing DPCs was isolated by the phenol/chloroform extraction procedure described above, and DNA samples (5 µg) were subjected to neutral thermal hydrolysis (1 h at 37°C) to release protein-guanine conjugates of mechlorethamine from the DNA backbone. Proteins were subjected to tryptic digestion (1 µg trypsin in 25 mM ammonium bicarbonate, overnight at 37°C), and the resulting peptides were further digested to amino acids in the presence of proteinase K (10 µg in 250 µL H₂O, overnight at 37°C). Cys-[¹⁵N]-N7G-EMA conjugated were quantified by isotope dilution HPLC-ESI-MS/MS as described in Supplement S-1.

Results

Cytotoxicity Experiments

To establish the effects of mechlorethamine treatment on cell viability, HT1080 cells were treated with 0, 10, 25, 50, or 100 µM concentrations of the drug for 3 h. Following overnight

incubation in a drug free media, cells were counted, and the cytotoxicity was measured as the percentage of cells surviving mechlorethamine treatment as compared to untreated controls. Treatment with mechlorethamine resulted in significant decrease in cell numbers, with approximately 50% cell death observed following treatment with 50 μ M mechlorethamine (Supplementary Figure S-1). These values are consistent with previous findings of Hardej and Billack,²⁴ who studied mechlorethamine cytotoxicity in various human cell types and found that LC50 values ranged between 6 and 1,000 μ M following 24 h exposure, depending on the cell type.

Strategy for the Isolation of DPCs from Mammalian Cells Treated with Mechlorethamine

To enable mass spectrometry-based identification of DNA-protein cross-links from mechlorethamine-treated HT1080 cells, a method was required for isolating covalent DPC lesions while removing any non-covalently bound proteins. Our initial attempts employed commercial DNAzol® reagent (Invitrogen), a proprietary chaotrope detergent intended for the isolation of genomic DNA from a variety of sources.²⁵ DNAzol has been previously employed by Barker et al. to isolate DPCs induced by ionizing radiation.² However, our attempts to use DNAzol to isolate mechlorethamine-induced DPCs yielded poor results due to the limited solubility of DPC-containing DNA (results not shown). Therefore, an alternative isolation method was sought.

In our optimized strategy (Scheme 2), cell nuclei are lysed in the presence of proteasome inhibitors. Chromosomal DNA containing covalently cross-linked proteins is isolated using a modified phenol/chloroform extraction method which incorporates repeated extraction steps in the presence of SDS to remove any non-covalently bound proteins. We found that this method consistently provides good DNA yields while minimizing RNA contamination. To ensure DNA purity and to quantify its amounts, DNA aliquots were taken and digested in the presence of nuclease P1 and alkaline phosphatase. The resulting 2'-deoxynucleosides were analyzed by HPLC with UV detection. As shown in a representative chromatogram shown in Supplementary Figure S-2, no RNA ribonucleosides were detected in enzymatic digests, indicating that our protocol is effective at removing RNA. DNA concentrations were determined by HPLC-UV analysis of dG using a calibration curve obtained by injecting known amounts of standard.²⁶

Concentration-Dependent Formation of DPCs in Human Cell Cultures Following Mechlorethamine Treatment

To determine whether mechlorethamine treatment results in DPC formation, human fibrosarcoma (HT1080) cells ($\sim 10^7$) were treated with increasing concentrations of the drug (0, 10, 50, or 100 μ M) for 3 hours. Chromosomal DNA containing any covalent DPCs was isolated by the modified phenol/chloroform extraction methodology described above. Equal amounts of DNA from each sample (15 μ g) were heated with SDS-containing gel loading buffer to release protein-guanine conjugates, which were resolved by 12% SDS-PAGE (Scheme 2, Figure 1). Total nuclear extract proteins extracted from the same number of cells were analyzed in parallel to estimate cross-linking efficiency (not shown). The proteins were visualized using SimplyBlue SafeStain (Figure 1A). We found that the intensities of protein bands increased with increasing drug concentrations (lanes 4–6 in Figure 1A), reaching ~ 1.6 % cross-linking following treatment with 100 μ M mechlorethamine (Figure 1B). Small amounts of protein were also present in gel lane containing control DNA from untreated cells, probably a result of endogenous DPC formation (lane 3 in Figure 1A). Based on these results, 25 μ M mechlorethamine was selected for proteomics experiments.

Identification of Cross-linked Proteins by Mass Spectrometry-Based Proteomics

To determine the identities of the proteins participating in mechlorethamine-mediated DPC formation, HT1080 cells ($\sim 10^7$ cells, in triplicate) were treated with 25 μ M mechlorethamine, while control cells were incubated in standard media. Following DNA extraction by the modified phenol/chloroform extraction method described above (Scheme 2), ~ 30 μ g of DNA from each sample was taken and subjected to thermal hydrolysis to induce depurination of N7-guanine adducts. As mentioned above, this treatment releases DPCs from the DNA backbone in the form of protein-guanine conjugates (Scheme 2), simplifying protein identification by mass spectrometry.¹⁹

The resulting proteins were separated by SDS-PAGE (Figure 2). Distinct protein bands were observed for mechlorethamine-treated samples (Figure 2B), while the untreated samples exhibited only weak protein signals (Figure 2A). Protein bands in the molecular weight range of 20–250 kDa were excised from the gel and subjected to in-gel tryptic digestion.²¹ The resulting peptides were extracted from the gel and subjected to HPLC-ESI⁺-MS/MS analysis for protein identification. As shown for two representative peptides in Figure 3, MS/MS analysis of tryptic peptides yielded characteristic b- and y-series fragment ions that were used to determine amino acid sequence and to identify the corresponding proteins. Database searching and parsimony analysis of the MS/MS spectral data resulted in identification of 34 proteins that co-purified with chromosomal DNA from mechlorethamine-treated cells (Table 1). All protein identifications are supported by at least two unique peptides. Furthermore, only proteins which exhibited significantly increased ion counts in treated samples (2 $\times$ or greater than in controls) were included in the list.

As shown in Table 1, molecular weights of the identified proteins are generally consistent with their positions on the gel, although a few proteins were also present in higher molecular weight fractions than expected, probably a result of ternary DNA-protein-protein conjugate formation in the presence of mechlorethamine. Of the identified proteins listed in Table 1, 23 (67.6%) are classified as nuclear proteins by the GO database available *via* the European Bioinformatics Institute (<http://www.ebi.ac.uk/QuickGO>) (Figure 4A). These include nucleophosmin, matrin-3, and DEK. This is not surprising, considering that nuclear proteins are localized in the vicinity of DNA and are available for mechlorethamine induced cross-linking. Furthermore, many nuclear proteins have a high affinity for DNA (see below). An additional 11 proteins (32.4%) are classified as cytoplasmic, and the remaining 5 (14.7%) are membrane-bound proteins. Cross-linking of these proteins to DNA is not unexpected, as many of these proteins are known to have additional cellular roles, potentially explaining their presence in the nucleus.

DPC-forming proteins were further classified according to their GO annotations relating to their molecular functions and biological processes (Figures 4B and 4C). We found that a large portion of the identified proteins (23 proteins, or 67.6%) are classified as nucleic acid binding proteins (Table 1 and Figure 4B). By comparison, DNA and RNA-binding proteins comprise only 10.3% of total nuclear proteome of human fibroblasts.²⁷ Many of the identified proteins (14, or 41.2%) play a role in transcriptional regulation, e.g. transcriptional factors, activators, and repressors (Figure 4C). This group includes high mobility group protein HMG-I,²⁸ Bcl-2 associated transcription factor 1,²⁹ and SON.³⁰ An additional 29.4% of proteins are involved in RNA processing, including zinc finger Ran-binding domain-containing protein-2³¹ and transformer-2 protein homolog β .³² Since our samples had minimal, if any, RNA contamination (Supplementary Figure S-2), it is likely that these proteins are also capable of binding to DNA.

It is important to note that many of the identified proteins function in more than one cellular compartment due to their varied roles in multiple biological processes. This observation is

reflected in their GO annotations, where the majority of proteins are counted in multiple GO categories. For example, Bcl-2 associated transcription factor 1 is a DNA-binding transcriptional repressor that is believed to play a role in tumor suppression *via* the induction of apoptosis.²⁹ As a result, this protein is listed under two GO annotation categories, e.g. transcriptional regulation and apoptosis (Figure 4C). Conversely, the available GO annotations may not always take into account protein's secondary cellular localizations, biological processes, and molecular functions.

Western blot analysis of cross-linked proteins

To confirm the results of mass spectrometry analyses and to discover additional proteins participating in DPC formation, proteins co-purified with chromosomal DNA from mechlorethamine-treated cells were subjected to western blot analysis. Commercial antibodies against B23, 53BP1, GAPDH, PARP, Ref-1, Ku, AGT, and XRCC-1 were employed. These proteins were selected because they were either among the gene products identified by mass spectrometry analyses (B23 and 53BP1, Table 1) or have been previously found to form mechlorethamine-induced DPC in our earlier *in vitro* studies employing cell free protein extracts (GAPDH, PARP, Ref-1, Ku, AGT, and XRCC-1).¹⁹ Equal amounts of DNA (15 μ g) isolated from HT1080 cells treated with increasing concentrations of mechlorethamine (10, 25, 50, or 100 μ M) were taken and heated with SDS-containing gel loading buffer (15 min at 90°C) to release protein-guanine conjugates from the DNA backbone (Scheme 2). The resulting proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for western blot analysis using commercial antibodies against specific target proteins.

Western blotting experiments confirmed the identities of two gene products identified from mass spectrometry based proteomics: protein B23 (nuclophosmin) and 53BP1 (Figure 5A). In addition, a concentration-dependent DPC formation involving four additional proteins, GAPDH, PARP, Ref-1, and XRCC-1, was observed. Among these, PARP displayed the greatest cross-linking efficiency, with approximately 0.8 % of total protein becoming cross-linked to DNA following treatment with 10 μ M mechlorethamine (Figure 5B). In contrast, no DPC formation involving AGT and Ku was detected (results not shown). We hypothesize that our inability to detect DPCs involving AGT protein in human cells was due to its low cellular abundance. Indeed, significant AGT-DNA cross-linking was detected when the experiment was repeated using Chinese hamster ovary (CHO) cells over-expressing human AGT protein (Figure 6).

HPLC-ESI⁺-MS/MS Analysis of Cys-N7G-EMA Conjugates as Evidence for DPC Formation

To enable absolute quantification of mechlorethamine-induced DPCs, HT1080 cells ($\sim 10^6$) were treated with 0, 10, 25, 50, or 100 μ M mechlorethamine, and the chromosomal DNA was extracted as described above (Scheme 2). Equal DNA amounts (5 μ g) were taken from each sample and subjected to neutral thermal hydrolysis to release protein-guanine conjugates from the DNA backbone. Proteins were enzymatically digested to amino acids, and the resulting digests were subjected to off-line HPLC purification and HPLC-ESI⁺-MS/MS analysis of Cys-N7G-EMA by isotope dilution with Cys-[¹⁵N]-N7G-EMA as described previously.¹⁸

Representative extracted ion chromatograms for HPLC-ESI⁺-MS/MS analysis of Cys-N7G-EMA in samples from mechlorethamine-treated and control HT1080 cells are shown in Figure 7. Cys-N7G-EMA was detected DNA samples from mechlorethamine-treated cells (Figure 7B), but not from untreated cells (Figure 7A), consistent with conjugate formation as a result of mechlorethamine treatment. Furthermore, adduct amounts increased linearly with increasing concentrations of mechlorethamine, with approximately 2.1 ± 0.7 per 10^6 dG

adducts formed following treatment with 50 μ M mechlorethamine (Figure 8). These data indicate that mechlorethamine-induced DNA-protein cross-linking can take place between the side chain sulfhydryls of cysteine residues in proteins and the N7-position of guanine bases in chromosomal DNA. While we cannot exclude the possibility that other nucleophilic amino acids, such as those of lysine and arginine, also participate in mechlorethamine-induced DNA-protein cross-linking, these findings are consistent with our *in vitro* studies which revealed the formation of cysteine-guanine conjugates in the presence of mechlorethamine.¹⁹

Discussion

Many clinically important antitumor drugs and genotoxic carcinogens are *bis*-electrophiles capable of sequentially alkylating nucleophilic sites on DNA and proteins to form macromolecular DNA-protein cross-links (e.g. mechlorethamine in Scheme 1). We have developed a simple and effective protocol for the isolation and characterization of covalent DPC lesions from cultured cells exposed to cross-linking agents (Scheme 2). By combining this methodology with mass spectrometry-based proteomics and immunoblotting, we examined DNA-protein cross-linking in human fibrosarcoma HT1080 cells exposed to mechlorethamine. A total of 38 proteins were found to form cross-links to chromosomal DNA in the presence of mechlorethamine (Table 1 and Figure 5). These proteins encompass a variety of cellular functions, including transcriptional regulation, RNA processing, cell signaling, apoptosis, and DNA damage response. The majority of the identified proteins are known nucleic acid binding proteins which are present in the nucleus (Figure 4). We also identified several targets which are classified as RNA-binding proteins (Figure 4C). However, since these samples did not contain RNA as confirmed by HPLC-UV analysis (Supplementary Figure S-2), these proteins are hypothesized to have additional DNA-binding capabilities.

In an earlier study, Barker *et al.* employed DNAzol extraction of DPCs with mass spectrometry-based proteomics to identify 29 proteins which became cross-linked to chromosomal DNA as a result of exposure to ionizing radiation.² Among these were proteins involved in cell structure and architecture, cell cycle, chromatin regulation, transcriptional regulation, and RNA splicing. Of these proteins, vimentin was the only protein that was also detected among mechlorethamine-induced DPCs (Table 1). Vimentin is an intermediate filament protein which, along with microtubules and actin, contributes to the cytoskeleton of eukaryotic cells.³² High cellular abundance of this protein likely facilitated its identification in both proteomics screens. The remaining proteins were distinct for IR and nitrogen mustard-induced DPC formation. A likely explanation for the observed differences between the proteins targeted for cross-linking by ionizing radiation and mechlorethamine is the different mechanisms of cross-linking involved. While radiation-induced DPC formation is relatively non-specific and often involves tyrosine residues,² mechlorethamine-mediated DPCs predominantly involve nucleophilic cysteine residues.^{18,19}

Twelve of the proteins identified in the present work, including 53BP1, matrin-3, high mobility group protein HMG-I, and nucleophosmin (B23), were previously detected among DNA-protein cross-links induced by formaldehyde in human acute promyelocytotic leukemia cells.³³ Qiu and Wang employed formaldehyde-induced cross-linking to study DNA-protein interactions by reversibly trapping covalent DNA-protein complexes, enabling the identification of 780 DNA-binding proteins.³³ Of these, 39.1% were classified as nuclear proteins, and 40.8% were annotated as cytoplasmic proteins. Many proteins were classified as both nuclear and cytoplasmic. DNA-binding proteins comprised 14.2% of the identified proteins, while RNA-binding proteins made up 20.0% of total proteins. This is consistent

with our study which also observed a number of RNA-binding proteins among mechlorethamine-induced DPCs (Table 1).

Six of the proteins identified in the present *in vivo* study (matrin-3, nucleophosmin (B23), GAPDH, Ref-1, PARP, and XRCC-1) were also among the proteins that formed DPCs when protein extracts from human cervical carcinoma (HeLa) cells were incubated with biotinylated DNA duplexes in the presence of mechlorethamine.¹⁹ All six are known to be DNA-binding proteins involved in chromatin remodeling, translation, DNA replication, DNA repair, RNA metabolism, transcriptional regulation, and apoptosis. The remaining proteins were not observed in both experiments. This may be explained by differences in protein expression profiles in HeLa and HT1080 cells²⁷ and by the nature of DNA employed in these two experiments. Our earlier *in vitro* studies involved short synthetic DNA 18-mers derived for a region of the *K-ras* gene. These duplexes lacked nucleosomal structure and were unlikely to participate in sequence-specific interactions with DNA-binding proteins.¹⁹ In contrast, experiments described in the present report employed intact human cells, where DNA is organized in chromatin and involves specific interactions between DNA and proteins, potentially facilitating DPC formation. Indeed, ~ 10-fold lower concentrations of mechlorethamine were required to achieve the same extent of cross-linking in intact cells as compared to *in vitro* experiments.¹⁹

In conclusion, this study demonstrates that the treatment of human fibrosarcoma cells with clinically relevant concentrations of mechlorethamine induces DNA-protein cross-links to a variety of cellular proteins, including those participating in chromatin remodeling, translation, DNA replication, DNA repair, RNA metabolism, transcriptional regulation, and apoptosis. The numbers of DPC lesions in treated cells varied between 1 and 5 cross-links per 10⁶ nucleotides, depending on drug concentration (Figure 8). Many of the proteins participating in DPC formation can be characterized as low abundance nuclear proteins involved in cellular processes that place them in close proximity to chromosomal DNA.

If not repaired, bulky DPC lesions are expected to have serious consequences for the cell, blocking the progression of protein complexes involved in critical cellular processes, such as DNA replication, chromatin remodeling, transcription, and DNA repair, and ultimately resulting in cytotoxic and mutagenic effects.⁷ Several possible mechanisms for DPC repair have been proposed, including proteolytic degradation, nucleotide excision repair, and homologous recombination repair.⁷ Quievryn and Zhitkovich have shown that FA-induced DPCs may involve an initial proteolytic step, followed by nucleotide excision repair of the resulting DNA-peptide cross-links.³⁴ Nakano *et al.* studied DPC repair in bacteria and mammalian cells and found that nucleotide excision repair is involved in the removal of DPCs involving small proteins and peptides, whereas homologous recombination is responsible for the repair of cross-links involving larger proteins.^{35,36} Further studies are currently underway in our laboratory to establish the biological consequences of DNA-protein cross-links induced by antitumor nitrogen mustards in human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AGT	O ⁶ -alkylguanine DNA alkyltransferase
B23	protein B23 (nucleophosmin)
CHO	Chinese hamster ovary cell line

Cys-N7G-EMA	<i>N</i> -[2-(<i>S</i> -cysteinyl)ethyl]- <i>N</i> -[2-(guan-7-yl)ethyl]methylamine
Cys-[¹⁵N] N7G-EMA	<i>N</i> -[2-(<i>S</i> -cysteinyl)ethyl]- <i>N</i> -[2-([¹⁵ N]-guan-7-yl)ethyl]methylamine
DPC	DNA-protein cross-link
DTT	dithiothreitol
FBS	fetal bovine serum
FDR	false discovery rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	glutathione
HPLC-ESI⁺-MS/MS	high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry
Ku	ATP-dependent DNA helicase subunit 2
bis(2-chloroethyl)methylamine (mechlorethamine)PARP	poly(ADP-ribose) polymerase I
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonyl fluoride
Ref-1	DNA-(apurinic- or apyrimidinic-site) lyase
TBS	Tris-buffered saline
XRCC-1	x-ray cross-complementing protein I
53BP1	tumor suppressor p53-binding protein

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Reference List

1. Zhitkovich A, Voitkun V, Kluz T, Costa M. Utilization of DNA-protein cross-links as a biomarker of chromium exposure. *Environ. Health Perspect.* 1998; 106 Suppl 4:969–974. [PubMed: 9703480]
2. Barker S, Weinfeld M, Zheng J, Li L, Murray D. Identification of mammalian proteins cross-linked to DNA by ionizing radiation. *J. Biol. Chem.* 2005; 280:33826–33838. [PubMed: 16093242]
3. Murata-Kamiya N, Kamiya H. Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA. *Nucleic Acids Res.* 2001; 29:3433–3438. [PubMed: 11504881]
4. Kloster M, Kostrehunova H, Zaludova R, Malina J, Kasparkova J, Brabec V, Farrell N. Trifunctional dinuclear platinum complexes as DNA-protein cross-linking agent. *Biochemistry.* 2004; 43:7776–7786. [PubMed: 15196020]
5. Ewig RA, Kohn KW. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. *Cancer Res.* 1978; 38:3197–3203. [PubMed: 150940]

6. Baker JM, Parish JH, Curtis JP. DNA-DNA and DNA-protein crosslinking and repair in *Neurospora crassa* following exposure to nitrogen mustard. *Mutat. Res.* 1984; 132:171–179. [PubMed: 6239978]
7. Barker S, Weinfeld M, Murray D. DNA-protein crosslinks: their induction, repair, and biological consequences. *Mutat. Res.* 2005; 589:111–135. [PubMed: 15795165]
8. Oleinick NL, Chiu SM, Ramakrishnan N, Xue LY. The formation, identification, and significance of DNA-protein cross-links in mammalian cells. *Br. J. Cancer Suppl.* 1987; 8:135–140. [PubMed: 3477283]
9. Gilman A, Philips FS. The Biological Actions and Therapeutic Applications of the B-Chloroethyl Amines and Sulfides. *Science.* 1946; 103:409–436.
10. Rajski SR, Williams RM. DNA Cross-Linking Agents as Antitumor Drugs. *Chem Rev.* 1998; 98:2723–2796. [PubMed: 11848977]
11. Kohn KW, Hartley JA, Mattes WB. Mechanisms of DNA sequence selective alkylation of guanine-N7 positions by nitrogen mustards. *Nucleic Acids Res.* 1987; 15:10531–10549. [PubMed: 3697095]
12. Rink SM, Solomon MS, Taylor MJ, Rajur SB, McLaughlin LW, Hopkins PB. Covalent structure of a nitrogen mustard-induced DNA interstrand cross-link: An N⁷-to-N⁷ linkage of deoxyguanosine residues at the duplex sequence 5'-d(GNC). *J. Am. Chem. Soc.* 1993; 115:2551–2557.
13. Osborne MR, Wilman DE, Lawley PD. Alkylation of DNA by the nitrogen mustard bis(2-chloroethyl)methylamine. *Chem. Res. Toxicol.* 1995; 8:316–320. [PubMed: 7766817]
14. Povirk LF, Shuker DE. DNA damage and mutagenesis induced by nitrogen mustards. *Mutat. Res.* 1994; 318:205–226. [PubMed: 7527485]
15. Thomas CB, Kohn KW, Bonner WM. Characterization of DNA-protein cross-links formed by treatment of L1210 cells and nuclei with bis(2-chloroethyl)methylamine (nitrogen mustard). *Biochemistry.* 1978; 17:3954–3958. [PubMed: 568484]
16. Ewig RA, Kohn KW. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res.* 1977; 37:2114–2122. [PubMed: 558823]
17. Ross WE, Ewig RA, Kohn KW. Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. *Cancer Res.* 1978; 38:1502–1506. [PubMed: 565677]
18. Loeber R, Michaelson E, Fang Q, Campbell C, Pegg AE, Tretyakova N. Cross-linking of the DNA repair protein O⁶-alkylguanine DNA alkyltransferase to DNA in the presence of antitumor nitrogen mustards. *Chem. Res. Toxicol.* 2008; 21:787–795. [PubMed: 18324787]
19. Loeber RL, Michaelson-Richie ED, Codreanu SG, Liebler DC, Campbell CR, Tretyakova NY. Proteomic analysis of DNA-protein cross-linking by antitumor nitrogen mustards. *Chem. Res. Toxicol.* 2009; 22:1151–1162. [PubMed: 19480393]
20. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer.* 1974; 33:1027–1033. [PubMed: 4132053]
21. Shin NY, Liu Q, Stamer SL, Liebler DC. Protein targets of reactive electrophiles in human liver microsomes. *Chem. Res. Toxicol.* 2007; 20:859–867. [PubMed: 17480101]
22. Michaelson-Richie ED, Loeber RL, Codreanu SG, Ming X, Liebler DC, Campbell C, Tretyakova NY. DNA-protein cross-linking by 1,2,3,4-diepoxybutane. *J. Proteome Res.* 2010; 9:4356–4367. [PubMed: 20666492]
23. Zhang B, Chambers MC, Tabb DL. Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. *J. Proteome. Res.* 2007; 6:3549–3557. [PubMed: 17676885]
24. Hardej D, Billack B. Ebselen protects brain, skin, lung and blood cells from mechlorethamine toxicity. *Toxicol. Ind. Health.* 2007; 23:209–221. [PubMed: 18429381]
25. Barker S, Murray D, Zheng J, Li L, Weinfeld M. A method for the isolation of covalent DNA-protein crosslinks suitable for proteomics analysis. *Anal. Biochem.* 2005; 344:204–215. [PubMed: 16091282]
26. Tretyakova N, Matter B, Jones R, Shallop A. Formation of benzo[a]pyrene diol epoxide-DNA adducts at specific guanines within *K-ras* and *p53* gene sequences: stable isotope-labeling mass spectrometry approach. *Biochemistry.* 2002; 41:9535–9544. [PubMed: 12135376]

27. Fountoulakis M, Tsangaris G, Oh J, Maris A, Lubec G. Protein profile of the HeLa cell line. *J Chromatogr. A*. 2004; 1038:247–265. [PubMed: 15233540]
28. Abba S, Krishna S, Thanos D, Maniatis T, Strominger JL, Ono SJ. Functional roles of the transcription factor Oct-2A and the high mobility group protein I/Y in HLA-DRA gene expression. *Journal of Experimental Medicine*. 1995; 182:487–500. [PubMed: 7629508]
29. Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. *Mol. Cell Biol*. 1999; 19:4390–4404. [PubMed: 10330179]
30. Sun CT, Lo WY, Wang IH, Lo YH, Shiou SR, Lai CK, Ting LP. Transcription repression of human hepatitis B virus genes by negative regulatory element-binding protein/SON. *J. Biol. Chem*. 2001; 276:24059–24067. [PubMed: 11306577]
31. Adams DJ, van der WL, Mayeda A, Stamm S, Morris BJ, Rasko JE. ZNF265-a novel spliceosomal protein able to induce alternative splicing. *J. Cell Biol*. 2001; 154:25–32. [PubMed: 11448987]
32. Tacke R, Tohyama M, Ogawa S, Manley JL. Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing. *Cell*. 1998; 93:139–148. [PubMed: 9546399] Fuchs E, Weber K. Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem*. 1994; 63:345–382. [PubMed: 7979242]
33. Qiu H, Wang Y. Exploring DNA-binding proteins with *in vivo* chemical cross-linking and mass spectrometry. *J. Proteome. Res*. 2009; 8:1983–1991. [PubMed: 19714816]
34. Quievryn G, Zhitkovich A. Loss of DNA-protein crosslinks from formaldehyde-exposed cells occurs through spontaneous hydrolysis and an active repair process linked to proteasome function. *Carcinogenesis*. 2000; 21:1573–1580. [PubMed: 10910961]
35. Nakano T, Morishita S, Katafuchi A, Matsubara M, Horikawa Y, Terato H, Salem AM, Izumi S, Pack SP, Makino K, Ide H. Nucleotide excision repair and homologous recombination systems commit differentially to the repair of DNA-protein crosslinks. *Mol. Cell*. 2007; 28:147–158. [PubMed: 17936711]
36. Nakano T, Katafuchi A, Matsubara M, Terato H, Tsuboi T, Masuda T, Tatsumoto T, Pack SP, Makino K, Croteau DL, Van Houten B, Iijima K, Tauchi H, Ide H. Homologous recombination but not nucleotide excision repair plays a pivotal role in tolerance of DNA-protein cross-links in mammalian cells. *J. Biol. Chem*. 2009; 284:27065–27076. [PubMed: 19674975]

Figure 1.

Concentration-dependent formation of DNA-protein cross-links in HT1080 cells treated with mechlorethamine. Cells were treated with 0–100 μ M mechlorethamine for 3 h, and chromosomal DNA containing cross-linked proteins was isolated by modified phenol/chloroform extraction in the presence of proteasome inhibitors. Proteins (from 15 μ g DNA) were released from DNA by thermal hydrolysis, separated by SDS-PAGE, and visualized by staining with SimplyBlue SafeStain (A). Densitometric analysis of protein bands in the 25–250 kDa molecular weight region in comparison with total protein extracts was used to approximate the extent of DNA-protein cross-linking (B).

Figure 2.

SDS-PAGE analysis of samples employed in the proteomics studies of mechlorethamine-induced DPCs. HT1080 cells ($\sim 10^7$) were treated with 0 (A) or 25 μM mechlorethamine for 3 h (B). Following modified phenol/chloroform extraction of DNA in the presence of proteasome inhibitors and thermal hydrolysis to release proteins, the cross-linked proteins were separated by 12% SDS-PAGE and visualized by staining with SimplyBlue SafeStain. Proteins present in the 20 – 250 kDa molecular weight range were excised from the gel, subjected to in-gel tryptic digestion, and analyzed by HPLC-ESI⁺-MS/MS.

Figure 3.
Representative HPLC-ESI⁺-MS/MS spectra of tryptic peptides used in the identification of DPCs involving matrin-3 (A), and zinc finger Ran-binding domain-containing protein 2 (B).

Figure 4. GO annotations for proteins involved in mechlorethamine-induced DPC formation in human HT1080 cells: cellular distributions (A), molecular functions (B), and biological processes (C). The number of proteins in each category is labeled on the charts.

Figure 5.

Western blot analysis of mechlorethamine-induced DPCs in HT1080 cells. Following treatment with 0 (lane 1), 10 (lane 2), 25 (lane 3), or 50 μ M mechlorethamine (lane 4), DNA and covalently cross-linked proteins were isolated by phenol/chloroform extraction. Samples were normalized for DNA content, and proteins from 15 μ g DNA were released by thermal hydrolysis, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Western blotting was performed using primary antibodies specific for 53BP1, B23, GAPDH, PARP, Ref-1, and XRCC-1 (A). The efficiency of DPC formation in the presence of mechlorethamine was estimated by densitometric analysis of protein bands in DPC samples and a whole cell protein lysate control (B).

Figure 6.

Western blot analysis of mechlorethamine-induced AGT-DNA cross-links in Chinese hamster ovary (CHO) cells expressing human AGT protein. Following treatment with 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 25 (lane 5) or 50 μ M mechlorethamine (lane 6) for 3 hours, DNA was isolated by modified phenol/chloroform extraction in the presence of proteasome inhibitors. DNA aliquots (15 μ g) were subjected to thermal hydrolysis to release proteins, which were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using a primary antibody against AGT.

Figure 7. HPLC-ESI⁺-MS/MS analysis of Cys-N7G-EMA conjugates in total proteolytic digests of mechlorethamine-induced DPCs. HT1080 cells were treated with mechlorethamine to induce DNA-protein cross-linking. Following extraction of DPC-containing chromosomal DNA, the cross-linked proteins were subjected to thermal and enzymatic hydrolysis to release amino acid-nucleobase conjugates. Digest mixtures were spiked with isotopically labeled internal standard (Cys-¹⁵N₅-N7G-EMA) to enable the direct quantitation of Cys-N7G-EMA. Shown in the figure are extracted ion chromatograms corresponding to HT1080 cells incubated in the absence of mechlorethamine (negative control) (A); and samples treated with 10 μM mechlorethamine (B).

Figure 8.

Concentration dependent formation of Cys-N7G-EMA in mechlorethamine-treated HT1080 cells. HT1080 cells were exposed to 0, 10, 25, 50, or 100 μ M mechlorethamine for 3 h. Following extraction of DPC-containing chromosomal DNA, equal DNA amounts from each sample were subjected to thermal and enzymatic hydrolysis to release amino acid-nucleobase conjugates. The samples were subjected to offline HPLC to enrich for Cys-N7G-EMA prior to HPLC-ESI⁺-MS/MS analysis. Quantification of Cys-N7G-EMA was accomplished using isotope dilution with Cys-¹⁵N₅-N7G-EMA. Error bars represent the standard error of three independent experiments.

Scheme 1.
Formation of DPCs by antitumor nitrogen mustards

Scheme 2.
Strategy for the isolation and analysis of DPCs from mechlorethamine-treated mammalian cell cultures.

Table 1
Proteins that become cross-linked to DNA in the presence of mechlorethamine in human fibrosarcoma HT1080 cells.*

Swiss-Prot ID	Protein	% Coverage	Peptide Sequences	Total Spectra	Primary Cellular Function	No. of Cysteines	Protein MW
Q7Z4V5	Hepatoma-derived growth factor-related protein 2, Isoform 1	3	2	11	DNA Damage Response/DNA Repair	3	74137
Q12888	Tumor suppressor p53-binding protein 1, Isoform 1	1	2	4		36	213574
P15144	Aminopeptidase N	4	3	3	Cellular Homeostasis/Cell Cycle	7	109408
P12270	Nucleoprotein TPR	2	3	5		7	267161
Q14568	Putative heat shock protein HSP 90-alpha A2	10	3	4		2	39365
P78316	Nucleolar protein 14, Isoform 1	2	2	2	RNA Processing/mRNA Splicing	12	97668
Q9Y3B9	Ribosomal RNA-processing protein 15	8	2	2		3	31353
Q07955	Splicing factor, arginine/serine-rich 1, Isoform ASF-1	7	2	3		2	27613
Q8TF01	Splicing factor, arginine/serine-rich 18, Isoform 1	3	2	2		0	92577
Q16629	Splicing factor, arginine/serine-rich 7, Isoform 1	21	5	18		4	27367
P62995	Transformer-2 protein homolog β	17	6	13		2	33666
Q9Y2W2	WW domain-binding protein 11	6	3	7		9	13446
Q95218	Zinc finger Ran-binding domain-containing protein 2, Isoform 1	7	2	4		8	37404
P05556	Integrin β -1, Isoform β -1A	11	8	15	Cell Signalling/Motility/Architecture	56	86191
P46821	Microtubule-associated protein 1B	5	11	18		21	270489
P07196	Neurofilament light polypeptide	10	6	15		1	61385
P08670	Vimentin	73	55	308		1	53520
Q9N9F8	Bcl-2-associated transcription factor 1, Isoform 1	13	13	51	Transcriptional Regulation/Translation	2	106122
P17096	High mobility group protein HMG-I/HMG-Y, Isoform HMG-I	38	4	10		0	11545
P52926	High mobility group protein HMGI-C	33	3	5		0	11832
P43243	Matrin-3	10	7	24		9	94492
Q9NWH9	Modulator of estrogen induced transcription, Isoform b	3	3	6		5	117017
P17480	Nucleolar transcription factor 1, Isoform UBF1	5	3	5		6	89406
P06748	Nucleophosmin, Isoform 2	17	6	29		3	32575
Q9UQ88	PTSLRE serine/threonine-protein kinase CDC2L2, Isoform SV1	3	2	2		4	90974
P35659	Protein DEK	10	5	25		5	42543

Swiss-Prot ID	Protein	% Coverage	Peptide Sequences	Total Spectra	Primary Cellular Function	No. of Cysteines	Protein MW
Q96T23	Remodeling and spacing factor 1	2	2	2		27	163821
P18583	SON protein, Isoform F	2	4	5		7	263715
Q969G3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1, Isoform 1	6	2	4		1	46649
Q5BKZ1	Zinc finger protein 326, Isoform 1	7	4	8		6	65654
P62979	40S ribosomal protein S27a	16	3	10		6	9418
B4DIQ5	cDNA FLJ59211, highly similar to Glucosidase 2, Subunit β	6	3	9	Unknown	16	60134
A8MQ38	Putative uncharacterized protein NOP2	4	4	5		12	93983
Q86VM9	Zinc finger CCCH domain-containing protein 18, Isoform 1	7	6	13		4	106378

*. 25 μ M mechlorethamine for 3 h