Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

HHS Public Access

Author manuscript

DNA Repair (Amst). Author manuscript; available in PMC 2021 June 04.

Published in final edited form as:

DNA Repair (Amst). 2020 May ; 89: 102840. doi:10.1016/j.dnarep.2020.102840.

Cross-Linking of the DNA Repair Protein O^6-Alkylguanine DNA Alkyltransferase to DNA in the Presence of Cisplatin

Xun Ming[a], **Erin D. Michaelson-Richie**[a], **Arnold S. Groehler IV**[d], **Peter W. Villalta**[b], **Colin Campbell**[c], **Natalia Y. Tretyakova**[a,*]

[a]Department of Medicinal Chemistry and the Masonic Cancer Center, University of Minnesota, Minneapolis, MN, 55455, USA

[b]Mass Spectrometry Core at the Masonic Cancer Center, University of Minnesota, Minneapolis, MN, 55455, USA

[c]Department of Pharmacology, University of Minnesota, Minneapolis, MN, 55455, USA

[d]Center for Genomic Integrity, Institute for Basic Science, Ulsan, Republic of Korea

Abstract

1,1,2,2- *Cis*-diamminedichloroplatinum (II) (cisplatin) is a chemotherapeutic agent widely used in the clinic to treat various cancers. The antitumor activity of cisplatin is generally attributed to its ability to form intrastrand and interstrand DNA-DNA cross-links via sequential platination of two nucleophilic sites within the DNA duplex. However, cisplatin also induces DNA- protein lesions (DPCs) that may contribute to its biological effects due to their ability to block DNA replication and transcription. We previously reported that over 250 nuclear proteins including high mobility group proteins, histone proteins, and elongation factors formed DPCs in human HT1080 cells treated with cisplatin (*Ming et al. Chem. Res. Toxicol.* 2017, 30, 980–995). Interestingly, cisplatin induced DNA-protein conjugates were reversed upon heating, by an unknown mechanism. In the present work, DNA repair protein O^6-alkylguanine DNA alkyltransferase (AGT) was used as a model to investigate the molecular details of cisplatin-mediated DNA-protein cross-linking and to establish the mechanism of their reversal. We found that AGT is readily cross-linked to DNA in the presence of cisplatin. HPLC-ESI$^+$-MS/MS sequencing of tryptic peptides originating from dG-Pt-AGT complexes revealed that the cross-linking occurred at six sites within this protein including Glu[110], Lys[125], Cys[145], His[146], Arg[147], and Cys[150]. Cisplatin-induced Lys-Gua cross-links (1,1-*cis*-diammine-2-(5-amino-5-carboxypentyl)amino-2-(2'-deoxyguanosine-7-yl)-platinum(II) (dG-Pt-Lys) were detected by HPLC-ESI$^+$-MS/MS of total digests of modified protein in comparison with the corresponding authentic standard. Upon heating, dG-Pt-AGT complexes were subject to platination migration from protein to DNA, forming *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ cross-links which were detected by HPLC-ESI$^+$-MS/MS. Our results provide a new insight into the mechanism of cisplatin-mediated DNA-protein cross-linking and their dynamic equilibrium with the corresponding DNA-DNA lesions.

*To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, 2231 6th Street SE, 2-147 CCRB, Minneapolis, MN 55455, USA; Tel: 612-626-3432; Fax: 612-624-3869; trety001@umn.edu.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Keywords

mass spectrometry; DNA-protein cross-links; Cisplatin; AGT

Introduction

Reversible DNA-protein interactions play an important role in normal cell function. Nuclear protein binding to regulatory sequences within DNA controls DNA replication, gene expression, and mediates responses to DNA damage [1–3]. Any interruption of these dynamic interactions can have serious consequences for cell viability and genetic stability [4]. For example, proteins can become covalently trapped on chromosomal DNA as a result of exposure to physical and chemical agents such as formaldehyde [5, 6], ionizing radiation [7], and anticancer drugs [8–14]. The resulting irreversible DNA-protein cross-links (DPCs) are expected to block normal DNA-protein interactions, potentially contributing to toxicity, cancer, and neurodegenerative diseases [4, 15, 16].

The role of DPC lesions in biological effects of *bis*-alkylating drugs is not well understood due to their inherent complexity and the propensity of these agents to induce other types of DNA damage. For example, the anticancer drug 1,1,2,2-*cis*-diamminedichloroplatinum (II) (cisplatin) forms interstrand and intrastrand DNA-DNA cross-links [17], monoadducts [18], and DPCs [19] (Scheme 1). While DNA-DNA cross-linking by cisplatin is well characterized and is thought to be responsible for its anticancer mechanism [20], only limited information is available about the corresponding DNA-protein cross-links. DPCs are estimated to constitute $< 1\%$ of total DNA damage following exposure to cisplatin [21] and constitute a highly heterogeneous and complex type of DNA damage, making it difficult to evaluate their role in the cytotoxic and mutagenic effects of platinum drugs [22, 23].

We recently conducted a mass spectrometry based proteomics study of cisplatin induced DNA–protein cross-links in human fibrosarcoma (HT1080) cells [24]. Over 250 nuclear proteins cross-linked to chromosomal DNA following treatment with cisplatin were identified, including high mobility group (HMG) proteins, histone proteins, and elongation factors [24]. Interestingly, cisplatin induced DPCs were reversible, with proteins quantitatively released from DNA upon heating. In the present study, we employed O^6-Alkylguanine DNA alkyltransferase (AGT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as model proteins to investigate the structural basis for cisplatin-mediated DPC formation and the mechanism of their reversal upon heating. AGT and GAPDH were previously identified as proteins that form DPC in cisplatin treated cells [24].

Materials and Methods

Chemicals and Reagents.

Cisplatin, dG, and human recombinant glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Sigma-Aldrich (St. Louis, MO). Boc-L-Lys-OH was obtained from Fluka (Buchs, Switzerland), and mass spectrometry-grade Trypsin Gold was purchased from Promega (Madison, WI). Adenosine 5'-[γ–^{32}P]-triphosphate was obtained

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

from Perkin-Elmer (Boston, MA). T4 polynucleotide kinase and proteinase K were purchased from New England Biolabs (Beverly, MA). Synthetic DNA oligodeoxynucleotides were prepared at the University of Minnesota Microchemical Facility (Minneapolis, MN). Recombinant *C*-terminal histidine-tagged hAGT was produced as described previously [25]. *Cis*-1,1-diammine-2-chloro-2-(2'-deoxyguanosine-7-yl)-platinum (II) (dG-Pt-Cl) monoadducts and *cis*-1,1-diammine-2,2-*bis*-(2'-deoxyguanosine-7-yl)platinum (II) (dG-Pt-dG) conjugates were produced according to previous reports [26].

1,1-diammine-2-(5-amino-5-carboxypentyl)amino-2-(2'-deoxyguanosine-7-yl)-platinum(II) (dG-Pt-Lys).—Cisplatin (10 mg, 33.33 μmol) was dissolved in 1 mL of 10 mM Tris-HCl buffer (pH 7.2). $AgNO_3$ (11.3 mg, 66.7 μmol) was added, and the solution was kept in the dark with stirring for 4 h at room temperature. Following centrifugation, to the filtrate was added 2'-deoxyguanosine (dG) (9 mg, 33.3 μmol) and Boc-L-Lysine (8.2 mg, 33.3 μmol), and the resulting mixture was incubated for 48 h at 37 °C. The precipitate was isolated by filtration, and the supernatant was separated by semi-preparative HPLC on a Supelcosil LC-18-DB column (25 cm × 10 mm, 5 μm) eluted with a linear gradient of acetonitrile (B) in 15 mM ammonium acetate, pH 4.9 (A). The solvent composition was changed from 0 to 24% B in 24 min and further to 60% in 6 min. Under these conditions, Boc-protected dG-Pt-Lys eluted at 20.4 min. ESI^+-MS/MS (dG-Pt-Lys-Boc): *m/z* 741.3 $[M]^+ \rightarrow$ *m/z* 724.2 $[M - NH_3]^+$, 624.5 $[M - NH_3 - Boc]^+$. Following HPLC purification, the Boc protective group was removed by incubation in 10% TFA (0.5 mL) at room temperature for 30 min. The deprotected dG-Pt-Lys was purified using the same HPLC method. Under these conditions, dG-Pt-Lys eluted at 8.6 min. UV: λ_{max} 260nm, λ_{min} 280 nm (pH 4.9); ESI^+-MS/MS (dG-Pt-Lys): *m/z* 641.2 $[M]^+ \rightarrow$ *m/z* 624.2 $[M - NH_3]^+$, *m/z* 508.1 $[M - NH_3 -$ deoxyribose $+ H]^+$, and *m/z* 357.1 $[M - NH_3 -$ deoxyguanosine$]^+$.

Denaturing PAGE of Cisplatin-Induced DNA-Protein Crosslinks.

DNA 18-mer, 5′-GGA GCT GGT GGC GTA GGC-3′ (200 pmol), was 5′-end-labeled with ^{32}P in the presence of $[\gamma-^{32}P]$ATP and T4 polynucleotide kinase by standard methods [27], purified by 12% denaturing PAGE, and desalted by size exclusion chromatography. The radiolabeled DNA was mixed with equimolar amounts of the complementary strand (5'-GCCTACGCCACC AGCTCC-3’) in the annealing buffer (10 mM Tris pH=8, 50mM NaCl, 50 μl), heated in a heating block at 95° C for 10 min, and slowly cooled down overnight. The ^{32}P-labeled duplex (0.93 nmol) was incubated with O^6-alkylguanine DNA alkyltransferase (AGT) (2.0 μg) or human recombinant glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (2.0 μg) in the presence of 1–100 mol equiv of cisplatin (1, 5, 10, and 20 nmol, respectively) for 3 h at 37 °C. The reaction mixtures were separated by 12% SDS-PAGE, and the radiolabeled products were visualized using a Packard Cyclone Phosphoimager (Packard BioScience, Meridan, CT).

Reaction of Synthetic dG-Pt-Cl Monoadduct with Recombinant AGT Protein.

Human recombinant wild-type AGT protein or its variant (C145A/C150S_AGT) (2.3 nmol) was incubated with 10 equiv of synthetic dG-Pt-Cl (23 nmol) in 10 mM Tris-HCl buffer (pH 7.4) for 4 h at 37 °C. Any unreacted dG-Pt-Cl was removed by size exclusion chromatography using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA), in which the

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

buffer was exchanged to 0.1% TFA in H_2O following manufacturer's instructions. The cross-linked protein was isolated by HPLC using an Agilent 1100 HPLC system equipped with a DAD UV detector. Agilent Zorbax 300 SB-C3 column (2.1 × 150 mm, 5 μm) was eluted with 0.1% TFA in water (A) and 0.1% TFA in 4:1 acetonitrile/water (B) at a flow rate of 0.4 mL/min at 10 °C. The solvent composition began at 0% B and was linearly changed to 15% B over 15 min and further to 50% from 15 to 25 min. The column was washed at 50% B for 15 min and re-equilibrated to 0% B for 2 min. Under these conditions, the modified AGT proteins eluted as a single peak at ~ 30 min, and the unreacted AGT protein eluted as a single peak at ~ 32 min. HPLC peaks containing modified AGT protein were collected and dried under vacuum, followed by HPLC-ESI$^+$-MS analysis as described below.

Trans-Platination Reactions of dG-Pt-AGT in the Presence with dG.

HPLC-purified AGT-Pt-dG cross-links (~25 μg) were incubated with 10 molar equivalent of dG (~1.1 nmol) in SDS buffer (1% SDS, 10 mM Tris-HCl, 1% glycerol, 50 μM EDTA, pH 8.5) at 70 °C for 10 min or 60 min. Control experiments were conducted in the presence of dG at 37 °C for overnight or in the absence of dG at 70 °C for 10 min. Proteins were subjected to size exclusion chromatography as described above, followed by HPLC-ESI$^+$-MS analysis. To detect *cis*-1,1-diammine-2,2-*bis*-(2'-deoxyguanosine-7-yl)-platinum (II) (dG-Pt-dG) cross-links, the solution was passed through Amicon Ultra-0.5 mL Centrifugal Filters (10K MWCO, Millipore, Temecula, CA) to remove proteins before HPLC-ESI$^+$-MS/MS analysis as described below.

Tryptic Digestion of Platinated AGT.

Control or platinated AGT protein (~ 50 μg) was digested with trypsin (2 μg) in 25 mM ammonium bicarbonate buffer (pH 7.9) for 8 h at 37 °C. Samples were dried, desalted by ZipTip C18 purification (ZipTip C18 Pipette Tips, Millipore, Temecula, CA), and finally reconstituted in 0.1% formic acid (25 μL) prior to MS analysis as described below.

Total Digestion of Platinated AGT to Amino Acids.

Tryptic peptides (from ~50 μg of protein) were filtered through Microcon YM-10 membrane filters to remove trypsin. Proteinase K (20 μg) was added to the filtrate, and proteolysis proceeded at 37 °C for 24 h. Samples were dried and subjected to off-line HPLC separation using an Agilent Technologies HPLC system (1100 model) incorporating a diode array detector and a Supelcosil LC-18-DB (4.6 × 250 mm, 5 μm) column (Sigma-Aldrich, St. Louis, MO). The column was eluted at a flow rate of 1 mL/min using 15 mM ammonium acetate, pH 4.9 (A) and acetonitrile (B). The solvent composition was changed linearly from 0 to 24% B over 24 min and further to 60% B in 6 min. HPLC fractions containing dG-Pt-Lys (5–7 min) were collected, dried under vacuum, and reconstituted in 25 μL of 15 mM ammonium acetate buffer for HPLC-ESI$^+$-MS/MS analysis.

Mass Spectrometry.

HPLC-ESI$^+$-MS analysis of modified AGT proteins was performed with an Agilent 1100 capillary HPLC-ion-trap MS system operated in ESI$^+$ mode (*m/z* 200–2000). For whole protein mass spectrometry, chromatography was achieved using an Agilent Zorbax Extend

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

SB 300-C8 column (150 × 0.3 mm, 3.5 μm) eluted at a flow rate of 12 μL/min with a mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The solvent composition was held at 30% B for the first 5 min, followed by a linear increase to 80% B over 25 min, and further to 95% B in 5 min. Using these conditions, dG-Pt-Cl-modified AGT proteins (dG-Pt-AGT) eluted ~14.5 min. Deconvolution of the protein charge envelope was performed using the commercial deconvolution software.

AGT tryptic peptides were analyzed by HPLC-ESI^+-MS/MS with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer in line with an Eksigent NanoLC-Ultra 2D HPLC system, a nanospray source, and Xcalibur 2.1.0 software for instrument control. Peptide mixtures (8 μL) were loaded on a Symmetry C18 trapping column (180 μm × 20 mm, Waters, Milford, MA) using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow composition of 95% A and 5% B at 5 μL/min for 3 minutes. Following trapping, the flow was reversed and decreased to 0.3 μL/min. The peptides were eluted off the trap column and onto a capillary column (75 μm ID, 10 cm packed bed, 15 μm orifice) created by hand packing a commercially purchased fused-silica emitter (New Objective, Woburn MA) with Zorbax SB-C18 5 μm separation media (Agilent, Santa Clara, CA). The gradient program started at 5% B, followed by a linear increase to 60% B over 60 min, and further to 95% B in 5 min. Liquid chromatography was carried out at an ambient temperature. Centroided MS-MS scans were acquired using an isolation width of 2.5 *m/z*, an activation time of 30 ms, an activation Q of 0.25, 35% normalized CID collision energy, and 1 microscan with a max ion time of 100 ms for each MS/MS scan. The mass spectrometer was calibrated prior to each analysis, and the spray voltage was adjusted to endure a stable spray. Typically, the tune parameters were as follows: spray voltage of 1.6 kV, a capillary temperature of 275 °C, and an S-lens RF Level of 50%. Peptide MS/MS spectra were collected using data dependent scanning in which one full scan mass spectrum was followed by eight MS/MS spectra. Dynamic exclusion was enabled for 60 s and singly charged species were excluded.

Spectral data were analyzed using Thermo Proteome Discoverer 1.2 (ThermoScientific, San Jose, CA) that linked raw data extraction, database searching, and probability scoring. The raw data were directly uploaded, without any format conversion, to search against human AGT protein FASTA database (http://www.uniprot.org/uniprot/P16455) combined with its reversed counterpart by using the SEQUEST algorithm [28, 29]. Search parameters included trypsin specificity and up to 2 missed cleavage sites. The dG-Pt-AGT conjugates were expected to experience fragmentation at the ESI^+ source by a loss of one or two amino groups (due to the fragile nature of coordination between platinum and $-NH_3$ ligands) or a loss of 2-deoxyribose, as well as the possible breakage of the glycosodic bond of dG. Furthermore, dG-Pt-Cl -induced platination at the *N*-donor residues (*N*-terminus, histidine, lysine, or arginine), *S*-donor residues (cysteine or methionine), and *O*-donor residues (threonine, tyrosine, aspartic acid, or glutamic acid) were specified as the following dynamic modifications to identify spectra of modified peptides: (A) cross-link to dG: +496.115 Da (*dG + Pt + 2NH*$_3$), +479.087 Da (*dG + Pt + NH*$_3$*, a loss of –NH*$_3$), or +462.061 Da (*dG + Pt, a loss of 2-NH*$_3$); (B) cross-link to guanine: +379.059 Da (*Gua + Pt + 2NH*$_3$), +361.030 Da (*Gua + Pt + NH*$_3$*, a loss of –NH*$_3$), or +345.005 Da (*Gua + Pt, a loss of 2-NH*$_3$). The following criteria were implemented for all of the identified peptides: mass tolerance 300 ppm, Xcorr 2.5, Cn 0.5, and RSp (preliminary score rank) 3. Peptide sequences with

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

MS/MS spectra not meeting these criteria were removed from the final target list. Platinated peptides were included in the final lists only if they exhibited good quality MS/MS spectra (at least 40% of the observed MS/MS ions should match the theoretical b^+ or y^+-type peptide fragment ions) and with both platinated and non-platinated fragment ions whose relative abundances were unambiguously higher than the baseline.

HPLC-ESI$^+$-MS/MS of dG-Pt-Lys conjugates was conducted with a Thermo-Finnigan TSQ Vantage mass spectrometer in line with an Eksigent MicroAS autosampler and nanoLC 2D HPLC pump, a heated ESI source, and a Xcalibur 1.4 software for instrument control. Chromatographic separation was accomplished using a Hypercarb column (100 mm × 0.5 mm, 3 μm, ThermoScientific, Waltharm, MA) eluted with a gradient of 15 mM ammonium acetate (A) and 1:1 acetonitrile:water with 1% formic acid (B) at a flow rate of 13 μL/min. The gradient program began at 2% B, followed by a linear increase to 8% B in 10 min, further to 80% B in 18 min, and finally back to 2% B in 2 min. Using this gradient, dG-Pt-Lys eluted at ~14.5 min. ESI was achieved at a spray voltage of 3.2 kV and a capillary temperature of 200°C. CID was performed with Ar as a collision gas (1.0 mTorr) at a collision energy of 25V. The MS parameters were optimized for maximum response during infusion of a standard solution of dG-Pt-Lys and may vary slightly between different experiments. HPLC-ESI$^+$-MS/MS analyses were performed in the selected reaction monitoring (SRM) mode using the transitions corresponding to major fragment ions observed upon CID fragmentation of dG-Pt-Lys using a triple quadrupole mass spectrometer (*m/z* 641.3 $[M]^+ \rightarrow$ 508.2 $[M - NH_3$ – deoxyribose $+ H]^+$, and 340.1 $[M - 2NH_3$ – deoxyguanosine$]^+$).

HPLC-ESI$^+$-MS/MS analysis of dG-Pt-dG cross-links was performed using an Agilent 1100 series capillary LC Ion Trap MS system operated in the ESI$^+$ mode. Auto MS^2 was used to isolate and fragment the $[M]^+$ ion of dG-Pt-dG (*m/z* 762.3). Chromatographic separation was achieved using a Zorbax SB-C18 column (150 mm × 0.5 mm, 5 μm) eluted at a flow rate of 15 μL/min. The mobile phase consisted of 15 mM ammonium acetate, pH 4.9 (A) and acetonitrile (B). The solvent composition was held with a linear gradient of 0 – 15% B over the course of 30 min.

Results

SDS-PAGE Analysis of Cisplatin-Induced DNA-Protein Cross-Links.

To examine the ability of cisplatin to cross-link proteins to DNA, recombinant O^6-alkylguanine DNA alkyltransferase (AGT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins were incubated with 5'–^{32}P-end labeled DNA duplexes in the presence of increasing amounts of cisplatin, followed by SDS-PAGE analysis. The results presented in Figure 1 reveal a slow migrating species corresponding to protein-DNA conjugates when duplex DNA was exposed to cisplatin in the presence of AGT or GAPDH. No such product was formed in control experiments in which either protein or oligonucleotide was omitted (Lanes 1–3, Figures 1A and 1B). Cross-linking of AGT to DNA by cisplatin displayed a pronounced concentration dependence, with 2–5% of recombinant protein being cross-linked to DNA depending on treatment levels (Lanes 4–8, Figure 1A).

Author Manuscript

HPLC-ESI$^+$-MS Analysis of dG-Pt-Cl Monoadduct-Induced AGT-dG Cross-Links: Whole Protein Results.

Initial platination of DNA by cisplatin leads to the formation of DNA monoadducts, which retain one reactive Pt functionality (Scheme 1). These monoadducts can subsequently react with nucleophilic sites within proteins, giving rise to DNA-protein cross-links. To gain insight into the nature of cisplatin-induced AGT-DNA linkages, recombinant AGT protein was incubated with synthetic *cis*-1,1-diammine-2-chloro-2-(2'-deoxyguanosine-7-yl)-platinum (II) (dG-Pt-Cl) as a model of monoplatinated DNA (4 h at 37 °C). Following size exclusion chromatography to remove the bulk of unreacted dG-Pt-Cl, AGT-dG cross-links were separated from unmodified AGT by reverse phase HPLC (Scheme 2 and Figure 2). Three HPLC peaks were observed (Figure 2). HPLC-ESI$^+$-MS/MS analyses revealed that the first peak eluting at 4.5 min corresponded to unreacted dG-Pt-Cl, the second peak (30.1 min) contained AGT-dG cross-links, and the third peak (32.1 min) was due to unreacted AGT protein (Figures 2 and 3). ESI$^+$ spectrum of unreacted AGT contains multiple *m/z* signals corresponding to the various charge states of the protein (+17 – +37) (Figure 3A). Deconvolution of the mass spectrum results in a molecular weight of 21,878 Da, which matches the theoretical value of 21,877 Da (Figure 3A). A similar spectrum was observed for the peak eluting at 32.1 min (Figure 2), with the exception of a small additional signal at 22,355 Da corresponding to AGT protein containing a single cisplatin cross-link to dG ($AGT + Pt + dG + NH_3$, $M = 22355$ Da) (Figure 3B). ESI$^+$ analysis of the material eluting at 30.1 min (Figure 2) reveals the presence of AGT containing a single platinum cross-link to dG ($AGT + Pt + dG + 2NH_3$, M = 22372 Da), two platinum cross-links to dG ($AGT + 2Pt + 2dG + 2NH_3$, $M = 22832$ Da), and a triple platinum cross-link to dG ($AGT + 3Pt + 3dG + 4NH_3$, $M = 23328$ Da) (Figure 3C). Taken together, those results indicate that AGT protein can be covalently modified by dG-Pt-Cl to form cross-links at a minimum of three different sites of the protein.

To determine whether the cross-linking reaction involved active site cysteine residues of AGT, cross-linking experiments were repeated using C145A/C150S AGT active site mutant. The calculated molecular weight of the C145A/C150S_AGT variant is 22996 Da because of the addition of the N-terminal histidine tag (MRGSHHHHHHGS) (See Supporting Information, S-1). A similar HPLC trace (Figure 2B) was obtained for C145A/C150S_AGT variant that had been treated with synthetic dG-Pt-Cl monoadduct under the same condition as described above. HPLC-ESI$^+$-MS analysis of the HPLC fractions revealed that the peak at 31.6 min contained C145A/C150S_AGT-dG cross-links, including C145A/C150S_AGT containing a single platinum cross-link to dG ($AGT + Pt + dG + 2NH_3$, M = 23493 Da), two platinum cross-links to dG ($AGT + 2Pt + 2dG + 3NH_3$, $M = 23968$ Da). ESI$^+$ analysis of the material eluting at 34.5 min indicated a single protein species with a deconvoluted mass of 22,996.2 Da, which is consistent with the theoretical value (data not shown). Comparison of Figure 2A with Figure 2B in terms of the relative peak areas of modified proteins against unmodified proteins showed that wild-type AGT produced more adducts than the C145A/C150S_AGT variant, suggesting that Cys^{145} and Cys^{150} are involved in cross-linking.

Author Manuscript

Peptide Mapping by HPLC-ESI$^+$-MS/MS.

Our ESI$^+$-MS results for dG-Pt-Cl-treated AGT protein detected AGT species containing one-, two-, or three- platinum cross-linked to dG (Figure 3C), suggesting the presence of at least three distinct cross-linking sites within the protein. Further insight into the identities of AGT amino acid residues responsible for reaction with dG-Pt-Cl was provided by HPLC-ESI$^+$-MS/MS analysis of tryptic digests of the platinated protein as described below.

Proteolytic digestion of native AGT protein with trypsin provided good protein sequence coverage (82%, Table 1). Only one of the predicted tryptic peptide, representing amino acids 37–96, was not detected due to its large size. In order to locate platinated sites within the protein, dG-Pt-Cl modified AGT (from HPLC peak at 30.1 min at Figure 2, see ESI$^+$-MS spectrum in Figure 3C) was subjected to tryptic digestion, followed by nanoHPLC-nanospray ESI$^+$-MS/MS on an Orbitrap Velos mass spectrometer. Spectral data were analyzed using Thermo Proteome Discoverer 1.2 software to search against human AGT protein FASTA database. Because the cross-linked moiety (*dG-Pt-2NH$_3$*) is likely to experience fragmentation in the ESI$^+$ source by a loss of one or two amino groups or a loss of 2-deoxyribose, multiple *m/z* values were specified for each target peptide: (A) cross-link to dG: +496.115 Da (*dG + Pt + 2NH$_3$*), +479.087 Da (*dG + Pt + NH$_3$, a loss of –NH$_3$*), or +462.061 Da (*dG + Pt, a loss of 2 –NH$_3$*); (B) cross-link to guanine: +379.059 Da (*Gua + Pt + 2NH$_3$*), +361.030 Da (*Gua + Pt + NH$_3$, a loss of –NH$_3$*), or +345.005 Da (*Gua + Pt, a loss of 2 – NH$_3$*). Furthermore, dG-Pt-Cl monoadduct-induced platination at the *N*-donor residues (*N*-terminus, histidine, lysine, or arginine), *S*-donor residues (cysteine or methionine), and *O*-donor residues (threonine, tyrosine, aspartic acid, or glutamic acid) were specified as potential dynamic modifications. The following stringent criteria were further implemented for all of the identified peptides: mass tolerance   300 ppm, Xcorr   2.5, Cn   0.5, and RSp (preliminary score rank)   3. Peptides sequences with MS/MS spectra not obeying with these criteria were removed from the final target list.

HPLC-ESI$^+$-MS/MS analyses detected a prominent, triply charged peptide at *m/z* 801.12 ([M+3H]$^{3+}$) corresponding to AGT residues F^{108}GEVISYQQLAALAGNPK^{125} containing a cisplatin-dG cross-link (calculated M = 2401.28, M = 496.11 Da (*dG + Pt + 2NH$_3$*), Table 2 and Figure 4A). When this peptide was subjected to collision-induced dissociation (CID), the resulting MS/MS spectrum was consistent with the presence of *dG-Pt-2NH$_3$* adduct at Glu^{110} (Table 2 and Figure 4A). While the m/z values of y_2, y_3, y_5-y_{10} and y_{12} fragments were in agreement with theoretical values for unmodified peptide, the mass of the y_{17} fragment was increased by 495.11 Da (observed M = 2255.05 Da, calculated M = 1759.93 Da for the unmodified y_{17} peptide fragment). The b_3^{2+}-b_7^{2+} and b_9^{2+}-b_{15}^{2+} fragments also contained the *dG-Pt-2NH$_3$* moiety as indicated by the 496.11 Da mass increase, suggesting the adduct resides at either the 2nd or the 3rd residue of the peptide (G^{109} or E^{110}). Since Gly is a non-nucleophilic residue, these results are suggestive of Glu^{110} participation in AGT-dG cross-linking by cisplatin.

The second cross-linking site was located upon detection of the peptide F^{108}GEVISYQQ LAALAGNPKAAR^{128} containing a single platinum-dG adduct (*m/z* 850.12 [M+3H]$^{3+}$; calculated M = 2547.35 Da, M = 344.00 Da (*Gua + Pt*)) (Table 2 and Figure 4B). The cross-linking site was mapped to Lys^{125} based on the MS/MS fragmentation patterns,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

especially the diagnostic y_3, y_4 and b_{18} ions at *m/z* 317.18, 395.15 and 1116.51, respectively (Figure 4B). The y_3 ion mass matched the theoretical value for unmodified peptide, while the y_4 and b_{18} both experienced a mass shift corresponding to guanine-platinum adduct, which suggested that the cross-linking took place at Lys^{125}.

Significant cross-linking was observed at three active site residues directly involved in AGT catalysis: Cys^{145} (Figure 4C), Arg^{147} (Figure 4D), His^{146} (Figure 4E), and Cys^{150} (Figure 4F). While Cys^{145} serves as alkyl acceptor during AGT repair reaction, His^{146} acts as a base to deprotonate Cys^{145}. Arg^{147}, together with Pro^{144} and Leu168, provides a hydrophobic slot for His^{146}. In this hydrophobic environment, His^{146} accepts a hydrogen bond from water and donates one to the negatively charged Glu^{172} carboxylate, which pairs with Arg^{146} within a salt bridge [30]. HPLC-ESI$^+$-MS/MS analyses revealed doubly charged ions corresponding to a guanine-cisplatin cross-link-containing the $G^{136}NPVPILIPCHR^{147}$ peptide (*m/z* 838.88 $[M+2H]^{2+}$, calculated $M = 1675.74$ Da, $M = 361.03$ Da ($Gua + Pt + NH_3$), Table 2). CID of the doubly charged ion of the peptide (*m/z* 838.88 $[M+2H]^{2+}$, calculated $M = 1675.74$ Da, $M = 361.03$ Da ($Gua + Pt + NH_3$)) produced an MS/MS spectrum containing *b*- (b_3, b_4, and b_6–b_{10}) and *y*-series ions (y_1, y_2, y_5, y_6, y_8, and y_{10}), consistent with the presence of a guanine-platinum adduct at Arg^{147} (Figure 4D).

Figure 4F depicts the CID-MS^2 spectrum of the ions at *m/z* 682.65 ($[M+3H]^{3+}$), corresponding to the platinated peptide $V^{148}VCSSGAVGNYSGGLAVK^{165}$ containing a $Pt(NH_3)_2$-guanine adduct. The observed *b*- and *y*-series ions y_2–y_3, y_5–y_{11}, and y_{13} ions are in agreement with the fragmentation of unmodified peptide, whereas *m/z* 340.62 (b_3^{2+}) and 626.73 (b_{10}^{+}) both experience +379.10 Da ($Gua + Pt + 2NH_3$) mass increase (Figure 4F), making it possible to assign the modification site to Cys^{150} of the AGT protein.

Taken together, our nanoHPLC-nanospray MS/MS results for tryptic digests of platinated AGT provide evidence for six dG-Pt-Cl binding sites: Glu^{110}, Lys^{125}, Cys^{145}, His^{146}, Arg^{147}, and Cys^{150} (Table 2 and Figure 4). A crystal structure of human AGT protein bound to DNA is shown in Figure 5 (PDB 1T39). As is apparent from the crystal structures, Cys^{145}, His^{146}, and Arg^{147} are located directly in the AGT active site pocket ($I^{143}PCHRV^{148}$). The side chain of Glu110 is in a close proximity to the protein active site. Lys^{125} is located at the DNA-binding domain of the protein, which interacts with double stranded DNA *via* helix-turn-helix (HTH) motif. Mutations at Lys^{125} (i.e. Lys125Ala) have been previously shown to significantly disrupt the interaction of wild type AGT with DNA, indicating Lys^{125} is essential for DNA binding [30, 31].

Platination Migration from DNA-Pt-Protein to DNA-Pt-DNA.

In our earlier global proteomics study, we observed that unlike other types of DPC adducts, cisplatin-induced DPCs could be reversed by heating [24]. One interesting property of platinum-induced adducts is that the Pt-S and Pt-N coordination bonds are potentially reversible, making it possible to observe “platination migration” from one nucleophilic site within biomolecules to another [32–35]. For example, Reediji and colleagues employed synthetic *S*-guanosyl-L-homocystine (sgh) as a model compound to examine intramolecular migration of platinum from cysteine thiol to the N7 of guanosine [32]. Similarly, Sadler’s group investigated the intermolecular displacement of Pt-S bound in $Pt(dien)^{2+}$ model

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

compound by N7-guanosine 5'-monophosphate [33]. In 2000, Reediji *et al* further demonstrated that the sulfur atom of the platinum-thioether adduct can be substituted by guanine nucleobase within oligonucleotides [34].

To investigate the possibility that AGT-DNA cross-links may undergo platination migration to form guanine-guanine cross-links to release the intact AGT protein, HPLC-purified dG-AGT conjugates (dG-Pt-AGT) were incubated with excess dG at 70 °C for varying times, followed by capillary HPLC-ESI$^+$-MS analysis of the products (Scheme 2). HPLC-ESI$^+$-MS analysis of the HPLC-purified dG-Pt-AGT confirmed that AGT was completely modified by dG-Pt-Cl, with no unreacted AGT protein present (Figure 3C). Deconvoluted ESI$^+$ MS spectra revealed AGT protein bearing one ($AGT + Pt + dG + NH_3$, M = 22355 Da), two ($AGT + 2Pt + 2dG + 2NH_3$, M = 22832 Da) or three-platinum adducts cross-linked to dG ($AGT + 3Pt + 3dG + 4NH_3$, M = 23328 Da) (Figure 3C). In contrast, HPLC-ESI$^+$-MS analysis of dG-Pt-AGT cross-links incubated in the presence of free dG revealed the presence of free AGT protein (M = 21879 Da) (Figure 6A). When the incubation time was extended to 60 min, the unreacted AGT species (M = 21877 Da) became the dominant species (Figure 6B). In contrast, no unmodified AGT species was observed when dG-Pt-AGT complexes were heated in the absence of dG (Figure 6C).

Further experimental evidence for platination migration from protein to DNA was obtained by direct detection of dG-Pt-dG conjugates. HPLC-ESI$^+$-MS/MS of AGT-DNA cross-links after heating detected a prominent peak co-eluting with the authentic standard of *cis*-1,1-diammine-*bis*-(2'-deoxyguanosine-7-yl)-platinum (II) (dG-Pt-dG, *m/z* 762.3) in samples treated with dG for 60 min at 70 °C (Figure 7) but not in control samples incubated either in the presence of dG at 37 °C overnight or in the absence of dG (results not shown). MS/MS fragmentation of *m/z* 762.3 yielded the product ions corresponding to the loss of ammonia (*m/z* 745.2 $[M\text{-}NH_3]^+$), the removal of 2'-deoxyribose and ammonia (*m/z* 629.1 $[M\text{-}NH_3\text{-}dR+H]^+$), and the loss of one dG (*m/z* 495.1 $[M\text{-}dG]^+$). This compound had the same MS/MS fragmentation pattern and HPLC retention time as a synthetically prepared standard of dG-Pt-dG (Figure 7). In contrast, no dG-Pt-dG cross-link peak was detected from the control sample in which no dG was added (results not shown). Taken together, our results indicate that cisplatin-induced DNA-protein cross-links can be transformed into DNA-DNA cross-links upon heating, releasing intact proteins.

A similar platination migration experiments were conducted with HPLC-purified C145A/C150S AGT-dG conjugates. However, HPLC-ESI$^+$-MS analysis of dG-Pt-C145A/C150S_AGT cross-links incubated in the presence of free dG revealed that only a small amount of free C145A/C150S_AGT protein (M = 22996 Da) was released from the cross-links, even after heating at 70 °C for 60 min (Figure S-2). This is consistent with platination migration from sulfhydryl chains of Cys^{145} and Cys^{150} to the N7 position of guanine.

Capillary HPLC-ESI$^+$-MS/MS Analysis of dG-Pt-Lys Conjugates in Total Protein Digests.

To confirm that cisplatin-induced AGT-DNA cross-linking involves covalent modification of proteins and DNA, HPLC-ESI$^+$-MS/MS analysis of the total enzymatic digests of platinated protein was performed. Synthetic 1,1-*cis*-diammine-2-(5-amino-5-carboxypentyl)amino-2-(2'-deoxyguanosine-7-yl)-platinum (II) (dG-Pt-Lys) was used as an

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

authentic standard. Following cross-linking reaction, AGT protein was subjected to complete digestion in the presence of trypsin and proteinease K, followed by HPLC-ESI$^+$-MS/MS analysis of the resulting amino acid mixtures in parallel with authentic dG-Pt-Lys.

Representative extracted ion chromatograms for capillary HPLC-ESI$^+$-MS/MS analysis of dG-Pt-Lys conjugates in digests of cisplatin AGT-dG cross-links are shown in Figure 8. HPLC-ESI$^+$-MS/MS of the digest mixtures detected a prominent peak (Figure 8B) co-eluting with the authentic standard of dG-Pt-Lys (Figure 8A). These data confirm that cisplatin-induced DNA-protein cross-linking can take place between the N7 position of guanine in DNA and the ε-amino group of lysines in AGT protein.

Discussion

AGT is an important DNA repair protein that removes promutagenic DNA *O*^{6}-alkylguanine lesions formed as a result of exposure to chemotherapeutic drugs and environmental toxins [36]. AGT transfers the *O*^{6}-alkyl group from *O*^{6}-alkylguanines in DNA to a cysteine residue within the protein active site (Cys^{145}), thus restoring normal guanine [31]. Crystal structures of AGT-DNA complexes reveal that during the alkyl transfer reaction, the alkylated nucleotide is flipped out of the DNA base stack to enter the AGT active site [31].

AGT is readily cross-linked to DNA in the presence of various *bis*-electrophiles, probably due to its high affinity for DNA and the remarkable nucleophilicity of AGT Cys^{145}, which is activated to a thiolate anion *via* a hydrogen bonding network in the protein active site [37]. As a result, the cytotoxicity and mutagenicity of *bis*-electrophiles such as 1,2-dibromoethane, dibromomethane, and 1,2,3,4-diepoxybutane (DEB), are enhanced in bacteria over-expressing human AGT protein due to the formation of toxic AGT-DNA cross-links [38, 39].

Based on the known ability of AGT to form toxic DPC lesions [39, 40], this protein was selected for our *in vitro* studies of DNA-protein cross-linking by cisplatin. Gel shift experiments with wild type and mutant AGT proteins revealed multiple sites of AGT can participate in cross-linking (Scheme 2, Figures 1–3). Subsequent HPLC-ESI$^+$-MS/MS sequencing of tryptic peptides originating from AGT treated with dG-Pt-Cl monoadduct demonstrated that the cross-linking can occur at three sites within this protein, including Glu^{110}, Lys^{125}, and Arg^{147} (Figure 4A – C). Finally, the exact chemical structure of the amino acid-nucleoside conjugates was established as 1,1-*cis*-diammine-2-(5-amino-5-carboxypentyl) amino- 2-(2'-deoxyguanosine-7-yl)- platinum (II) (dG-Pt-Lys) based on the HPLC-ESI$^+$-MS/MS analysis of amino acid-nucleoside conjugates in total protein digests in comparison with the corresponding authentic standard (Figure 8).

Our experiments were conducted with dG-Pt-Cl as a model of monoalkylated DNA because platination of N7 position of guanine accounts for over 98% among all the DNA-damage induced by cisplatin [18]. However, similar specificity is expected for AGT-DNA cross-linking in cells, resulting in N7-guanine-AGT cross-links. Our laboratory previously employed mass spectrometry-based methods to characterize AGT-DNA cross-linking by antitumor nitrogen mustards [11] and diepoxybutane (DEB) [41]. For both *bis*-electrophiles,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

cross-linking took place specifically at the two active site cysteine residues within the protein: Cys^{145} and Cys^{150} [11, 41]. Additional sites within AGT were identified to be involved in cisplatin-induced cross-linking, including the non-thiol side chains of Glu^{110}, Lys^{125}, and Arg^{147}. This is indicative of a higher reactivity of cisplatin towards proteins and a different cross-linking chemistry characteristic for platinum compounds. Indeed, our recent mass spectrometry-based proteomics experiments of cisplatin-induced DPCs identified over 250 proteins covalently trapped to chromosomal DNA [24].

Previous studies investigated the reactions of model proteins such as cytochrome C [42], myoglobin [43], insulin [44, 45], ubiquitin [46], and human serum proteins [47] with platinum-based drugs. These researchers identified multiple Pt binding sites at *N*-donor residues (His and Lys), *S*-donor residues (Cys and Met), and *O*-donor residues (Thr, Tyr, Asp, and Glu). This is consistent with our results for AGT-dG cross-linking (Table 2 and Figure 4).

Crystal structures of human AGT protein reveal two distinct domains: the *N*-terminal domain (residues 1–85) and the *C*-terminal domain (residues 86–207) [31]. The *N*-terminal domain is composed of three β-sheets separated by two α-helixes, whereas the *C*-terminal domain contains two β-sheets and five α-helixes [31] (Figure 5). The *C*-terminal domain is involved in DNA-binding *via* the helix-turn-helix (HTH) motif and is responsible for DNA repair function. AGT active site contains the conserved active site cysteine motif ($I^{143}PCHRV^{148}$), and the O^6-alkylguanine-binding channel, that are critical for nucleotide flipping and alkyl transfer reaction [30, 48].

The observed specificity of cisplatin-mediated cross-linking at Glu^{110}, Lys^{125}, Arg^{147}, Cys^{145}, and Cys^{150} of the AGT protein may be a result of their proximity to the AGT active site. These residues are found in the *C*-terminal domain of AGT (Figure 5) [30]. Arg^{147} is a nucleophilic active site residue that is located in the immediate proximity to DNA in the AGT-DNA complex (Figure 5) [30]. While the side chain of Glu^{110} is close to the reactive site pocket ($I^{143}PCHRV^{148}$) in space, Lys^{125} is directly located in the DNA-binding domain (Figure 5). In contrast, the other three cysteines within AGT, Cys^5, Cys^{24} and Cys^{62} are not identified as the primary cross-linking sites, confirming our hypothesis that cross-linking could potentially take place at residues in close proximity DNA or directly involved in DNA-binding.

Our results reveal a potentially transient nature of dG-AGT cross-links, which can be converted to dG-dG cross-links upon heating (Figures 6 and S-1). Historically, there has been a long-term debate over the competition of purine bases, protein side chains, and glutathione for coordination sites of platinum [49]. The Hard Soft Acid Base (HSAB) theory predicts that the preferential binding ligands for platinum are *S*-containing biomolecules such as methionine and cysteine residues of proteins or glutathione [50]. This should leave little opportunity for platinum to coordinate with *N*-donor ligands in DNA, since numerous *S*-donor biomolecules present in cytosol (*e.g.* glutathione) should capture the drug before it enters the nucleus. Indeed, *in vitro* studies have demonstrated kinetically favored affinity of cisplatin toward S over N [33]. However, it is generally accepted that the ultimate biological

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

target of cisplatin is DNA due to the ability of this drug to form DNA intra- and interstrand cross-links between N7 of the purine bases [20].

To determine how cisplatin can induce DNA-DNA cross-links despite its potential reactions with glutathione, peptides, and proteins in the cytosol before reaching the nucleus, Reedijk [32] and Sadler [33] conducted intra- and intermolecular competition experiments with *S*-ligands and nucleobases. These studies detected platination migration from *S*-ligands to the N7 of guanine. Deubel *et al* [49] employed density functional theory to calculate Pt-L bond energy in $[Pt(NH_3)_3L]^{2+}$ complexes, where L represents peptide side chains, sulfur-containing protein agents, and guanine bases of DNA. These results revealed that platinum prefers *N*-ligands over *S*-ligands in terms of orbital interactions, electrostatics, and intramolecular hydrogen binding [49].

In summary, our results are consistent with previous studies indicating that there is a balance between Pt-S and Pt-N binding, where Pt-S binding is kinetically favored but Pt-N binding is more thermodynamically stable [35]. As a result, platinum initially bound to proteins may further react with DNA to yield Pt-DNA complexes and to release intact proteins. Indeed, our results presented above (Figures 6 and 7) provide evidence that AGT-DNA cross-links can rearrange to DNA-DNA cross-links, with N7-G winning the competition for the coordination sites of cisplatin upon heating of dG-AGT complexes. Although platination migration was not observed upon incubation at physiological conditions for 1 h, further studies are needed to establish whether platination migration from AGT to DNA takes place in cells [34].

In conclusion, our study demonstrates that cisplatin is capable of sequentially platinating nucleophilic sites within dG and AGT protein to form covalent cross-links between the N7 position of guanine and several residues within AGT including Glu^{110}, Lys^{125}, Cys^{145}, His^{146}, Arg^{147}, and Cys^{150}. To our knowledge, this is the first report of specific, structurally defined DNA-protein cross-linking involving cisplatin and a first observation of platination migration from DPC to DNA. These results are important because cisplatin-mediated DPC formation is likely to contribute to both on-target and off-target toxicity of this drug.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Professor Anthony E. Pegg (Penn State University) for generously providing AGT protein, Brock Matter (Masonic Cancer Center, University of Minnesota) for assistance with MS experiments, Jason A. Kuchar (ThermoScientific) and Pratik Jagtap (Minnesota Supercomputing Insitute, University of Minnesota) for help with proteomic data analyses, Prof. Christine Chow and Bett Kimutai (Wayne State Univ.) for help with MS/MS data interpretation, and Robert Carlson for preparing figures for this manuscript. Funding for this research was provided by NIH grants R01-ES023350 and R01-CA-1006700 and a faculty development grant from the University of Minnesota Academic Health Center. X.M. and E.M.R. and were partially supported by the NIH Chemistry-Biology Interface Training Grant (T32-GM08700), University of Minnesota Masonic Cancer Center, and University of Minnesota Graduate School.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Abbreviations

Cisplatin	1,1,2,2-*cis*-diamminedichloroplatinum (II)
AGT	*O*[6]-alkylguanine DNA alkyltransferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
dG-Pt-Lys	1,1-*cis*-diammine-2-(5-amino-5-carboxypentyl)amino-2-(2'-deoxyguanosine-7-yl)-platinum(II)
DEB	1,2,3,4-diepoxybutane
DPC	DNA-protein cross-link
d(GpG)	*cis*-[Pt(NH_3)$_2${d(GpG)}] adducts
HPLC-ESI$^+$-MS/MS	high performance liquid chromatography electrospray ionization tandem mass spectrometry

Bibliography

[1]. Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, Krop I, Schlissel MS, Feeney AJ, van Roon M, et al., E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements, Cell, 79 (1994) 885–892. [PubMed: 8001125]

[2]. Dynlacht BD, Regulation of transcription by proteins that control the cell cycle, Nature, 389 (1997) 149–152. [PubMed: 9296491]

[3]. Accili D, Arden KC, FoxOs at the crossroads of cellular metabolism, differentiation, and transformation, Cell, 117 (2004) 421–426. [PubMed: 15137936]

[4]. Barker S, Weinfeld M, Murray D, DNA-protein crosslinks: their induction, repair, and biological consequences, Mutation Research, 589 (2005) 111–135. [PubMed: 15795165]

[5]. Shaham J, Bomstein Y, Meltzer A, Kaufman Z, Palma E, Ribak J, DNA-protein crosslinks, a biomarker of exposure to formaldehyde—*in vitro* and *in vivo* studies, Carcinogenesis, 17 (1996) 121–126. [PubMed: 8565120]

[6]. Qiu H, Wang Y, Exploring DNA-binding proteins with *in vivo* chemical cross-linking and mass spectrometry, Journal of Proteome Research, 8 (2009) 1983–1991. [PubMed: 19714816]

[7]. Barker S, Weinfeld M, Zheng J, Li L, Murray D, Identification of mammalian proteins cross-linked to DNA by ionizing radiation, Journal of Biological Chemistry, 280 (2005) 33826–33838.

[8]. Ewig RA, Kohn KW, DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells, Cancer Research, 38 (1978) 3197–3203. [PubMed: 150940]

[9]. Kloster M, Kostrhunova H, Zaludova R, Malina J, Kasparkova J, Brabec V, Farrell N, Trifunctional dinuclear platinum complexes as DNA–protein cross-linking agents, Biochemistry, 43 (2004) 7776–7786. [PubMed: 15196020]

[10]. Baker JM, Parish JH, Curtis JPE, DNA-DNA and DNA-protein crosslinking and repair in *Neurospora crassa* following exposure to nitrogen mustard, Mutation Research, 132 (1984) 171–179. [PubMed: 6239978]

[11]. Loeber R, Michaelson E, Fang Q, Campbell C, Pegg AE, Tretyakova N, Cross-linking of the DNA repair protein *O*[6]-alkylguanine DNA alkyltransferase to DNA in the presence of antitumor nitrogen mustards, Chemical Research in Toxicology, 21 (2008) 787–795. [PubMed: 18324787]

[12]. Loeber RL, Michaelson-Richie ED, Codreanu SG, Liebler DC, Campbell CR, Tretyakova NY, Proteomic analysis of DNA–protein cross-linking by antitumor nitrogen mustards, Chemical Research in Toxicology, 22 (2009) 1151–1162. [PubMed: 19480393]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[13]. Michaelson-Richie ED, Ming X, Codreanu SG, Loeber RL, Liebler DC, Campbell C, Tretyakova NY, Mechlorethamine-induced DNA–protein cross-linking in human fibrosarcoma (HT1080) cells, Journal of Proteome Research, 10 (2011) 2785–2796. [PubMed: 21486066]

[14]. Groehler A, Villalta PW, Campbell C, Tretyakova N, Covalent DNA–protein cross-linking by phosphoramide mustard and nornitrogen mustard in human cells, Chemical Research in Toxicology, 29 (2016) 190–202. [PubMed: 26692166]

[15]. 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, 8 (1987) 135–140. [PubMed: 3477283]

[16]. Tretyakova NY, Groehler A, Ji S, DNA–protein cross-links: Formation, structural identities, and biological outcomes, Accounts of Chemical Research, 48 (2015) 1631–1644. [PubMed: 26032357]

[17]. Goggin M, Loeber R, Park S, Walker V, Wickliffe J, Tretyakova N, HPLC–ESI$^+$-MS/MS analysis of N7-guanine–N7-guanine DNA cross-links in tissues of mice exposed to 1,3-butadiene, Chemical Research in Toxicology, 20 (2007) 839–847. [PubMed: 17455958]

[18]. Jamieson ER, Lippard SJ, Structure, recognition, and processing of cisplatin–DNA adducts, Chemical Reviews, 99 (1999) 2467–2498. [PubMed: 11749487]

[19]. Chválová K, Brabec V, Kašpárková J, Mechanism of the formation of DNA–protein cross-links by antitumor cisplatin, Nucleic Acids Research, 35 (2007) 1812–1821. [PubMed: 17329374]

[20]. Jung Y, Lippard SJ, Direct cellular responses to platinum-induced DNA damage, Chemical Reviews, 107 (2007) 1387–1407. [PubMed: 17455916]

[21]. Sherman SE, Lippard SJ, Structural aspects of platinum anticancer drug interactions with DNA, Chemical Reviews, 87 (1987) 1153–1181.

[22]. Zwelling LA, Anderson T, Kohn KW, DNA-protein and DNA interstrand cross-linking by cis- and trans-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity, Cancer Research, 39 (1979) 365–369. [PubMed: 570092]

[23]. Zwelling LA, Bradley MO, Sharkey NA, Anderson T Kurt W. Kohn, Mutagenicity, cytotoxicity and DNA crosslinking in V79 Chinese hamster cells treated with cis- and trans-Pt(II) diamminedichloride, Mutation Research, 67 (1979) 271–280. [PubMed: 481452]

[24]. Ming X, Groehler A, Michaelson-Richie ED, Villalta PW, Campbell C, Tretyakova NY, Mass spectrometry based proteomics study of cisplatin-induced DNA–protein cross-linking in human fibrosarcoma (HT1080) cells, Chemical Research in Toxicology, 30 (2017) 980–995. [PubMed: 28282121]

[25]. Bender K, Federwisch M, Loggen U, Nehls P, Rajewsky MF, Binding and repair of O^6-ethylguanine in double-stranded oligodeoxynucleotides by recombinant human O^6-alkylguanine-DNA alkyltransferase do not exhibit significant dependence on sequence context, Nucleic Acids Res, 24 (1996) 2087–2094. [PubMed: 8668540]

[26]. Eastman A, Separation and characterization of products resulting from the reaction of cis-diamminedichloroplatinum(II) with deoxyribonucleosides, Biochemistry, 21 (1982) 6732–6736. [PubMed: 6891601]

[27]. Sambrook J, Fritsch EF, Maniatis T, Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[28]. Yates JR, Eng JK, McCormack AL, Schieltz D, Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database, Analytical Chemistry, 67 (1995) 1426–1436. [PubMed: 7741214]

[29]. Yates JR, Eng JK, McCormack AL, Mining genomes: Correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases, Analytical Chemistry, 67 (1995) 3202–3210. [PubMed: 8686885]

[30]. Daniels DS, Mol CD, Arvai AS, Kanugula S, Pegg AE, Tainer JA, Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical base binding, EMBO J, 19 (2000) 1719–1730. [PubMed: 10747039]

[31]. Daniels DS, Woo TT, Luu KX, Noll DM, Clarke ND, Pegg AE, Tainer JA, DNA binding and nucleotide flipping by the human DNA repair protein AGT, Nature Structural & Molecular Biology, 11 (2004) 714–720.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[32]. van Boom SSGE, Reedijk J, Unprecedented migration of [Pt(dien)]2+(dien = 1,5-diamino-3-azapentane) from sulfur to guanosine-N7 in S-guanosyl-L-homocysteine (sgh), Journal of the Chemical Society, Chemical Communications, (1993) 1397–1398.

[33]. Barnham KJ, Djuran MI, del Socorro Murdoch P, Sadler PJ, Intermolecular displacement of S-bound L-methionine on platinum(II) by guanosine 5′-monophosphate: implications for the mechanism of action of anticancer drugs, Journal of the Chemical Society, Chemical Communications, (1994) 721–722.

[34]. Teuben J-M, Reedijk J, Reaction of DNA oligonucleotides with [Pt(dien)GSMe]2+ (GSMe =S-methylated glutathione) and cis-[Pt(NH3)2(GSMe)2]2+: evidence of oligonucleotide platination via sulfur-coordinated platinum intermediates, Journal of Biological Inorganic Chemistry, 5 (2000) 463–468. [PubMed: 10968617]

[35]. Reedijk J, Why does cisplatin reach guanine-N7 with competing S-donor ligands available in the cell?, Chemical Reviews, 99 (1999) 2499–2510. [PubMed: 11749488]

[36]. Pegg AE, Repair of *O*[6]-alkylguanine by alkyltransferases, Mutation Research, 462 (2000) 83–100. [PubMed: 10767620]

[37]. Guengerich FP, Principles of covalent binding of reactive metabolites and examples of activation of bis-electrophiles by conjugation, Archives of Biochemistry and Biophysics, 433 (2005) 369–378. [PubMed: 15581593]

[38]. Liu L, Hachey DL, Valadez G, Williams KM, Guengerich FP, Loktionova NA, Kanugula S, Pegg AE, Characterization of a mutagenic DNA adduct formed from 1,2-dibromoethane by *O*[6]-alkylguanine-DNA alkyltransferase, Journal of Biological Chemistry, 279 (2003) 4250–4259.

[39]. Valadez JG, Liu L, Loktionova NA, Pegg AE, Guengerich FP, Activation of *bis*-electrophiles to mutagenic conjugates by human *O*[6]-alkylguanine-DNA alkyltransferase, Chemical Research in Toxicology, 17 (2004) 972–982. [PubMed: 15257623]

[40]. Kalapila AG, Loktionova NA, Pegg AE, Alkyltransferase-mediated toxicity of 1,3-butadiene diepoxide, Chemical Research in Toxicology, 21 (2008) 1851–1861. [PubMed: 18712882]

[41]. Loeber R, Rajesh M, Fang Q, Pegg AE, Tretyakova N, Cross-linking of the human DNA repair protein *O*[6]-alkylguanine DNA alkyltransferase to DNA in the presence of 1,2,3,4-diepoxybutane, Chemical Research in Toxicology, 19 (2006) 645–654. [PubMed: 16696566]

[42]. Zhao T, King FL, Direct determination of the primary binding site of cisplatin on cytochrome c by mass spectrometry, Journal of the American Society for Mass Spectrometry, 20 (2009) 1141–1147. [PubMed: 19286393]

[43]. Zhao T, King FL, A mass spectrometric comparison of the interactions of cisplatin and transplatin with myoglobin, Journal of Inorganic Biochemistry, 104 (2010) 186–192. [PubMed: 19945168]

[44]. Moreno-Gordaliza E.a., Cañas B, Palacios M.a.A., Gómez-Gómez MM, Top-down mass spectrometric approach for the full characterization of insulin–cisplatin adducts, Analytical Chemistry, 81 (2009) 3507–3516. [PubMed: 19323565]

[45]. Moreno-Gordaliza E, Canas B, Palacios MA, Gomez-Gomez MM, Novel insights into the bottom-up mass spectrometry proteomics approach for the characterization of Pt-binding proteins: The insulin-cisplatin case study, Analyst, 135 (2010) 1288–1298. [PubMed: 20411195]

[46]. Zhao T, King FL, Mass-spectrometric characterization of cisplatin binding sites on native and denatured ubiquitin, Journal of Biological Inorganic Chemistry, 16 (2011) 633–639. [PubMed: 21365334]

[47]. Will J, Wolters DA, Sheldrick WS, Characterisation of cisplatin binding sites in human serum proteins using hyphenated multidimensional liquid chromatography and ESI tandem mass spectrometry, ChemMedChem, 3 (2008) 1696–1707. [PubMed: 18855968]

[48]. Tubbs JL, Pegg AE, Tainer JA, DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by *O*[6]-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy, DNA Repair, 6 (2007) 1100–1115. [PubMed: 17485252]

[49]. Deubel DV, On the competition of the purine bases, functionalities of peptide side chains, and protecting agents for the coordination sites of dicationic cisplatin derivatives, Journal of the American Chemical Society, 124 (2002) 5834–5842. [PubMed: 12010058]

[50]. Pearson RG, Recent advances in the concept of hard and soft acids and bases, J Chem Educ, 64 (1987) 561–567.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

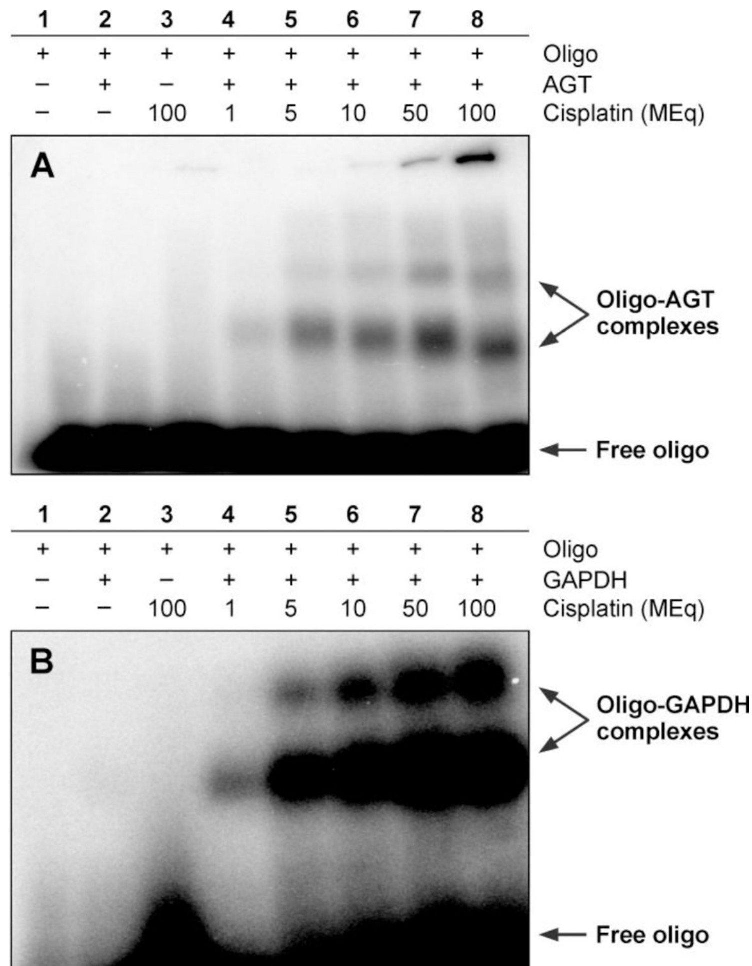


Figure 1.

Detection of cisplatin-induced DNA-protein cross-links by gel electrophoresis. 12% SDS-PAGE analysis of ^{32}P-endlabeled DNA duplexes (5'-GGA GCT GGT GGC GTA GGC-3' +strand) following incubation with (**A**) recombinant human AGT or (**B**) recombinant GAPDH protein in the presence of 1 (lane 4), 5 (lane 5), 10 (lane 6), 50 (lane 7) or 100 (lane 8) molar equivalents of cisplatin. Free duplex DNA (labeled “Free oligo”) migrates to the bottom of the gel, whereas DNA-protein cross-links display substantially reduced mobility.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

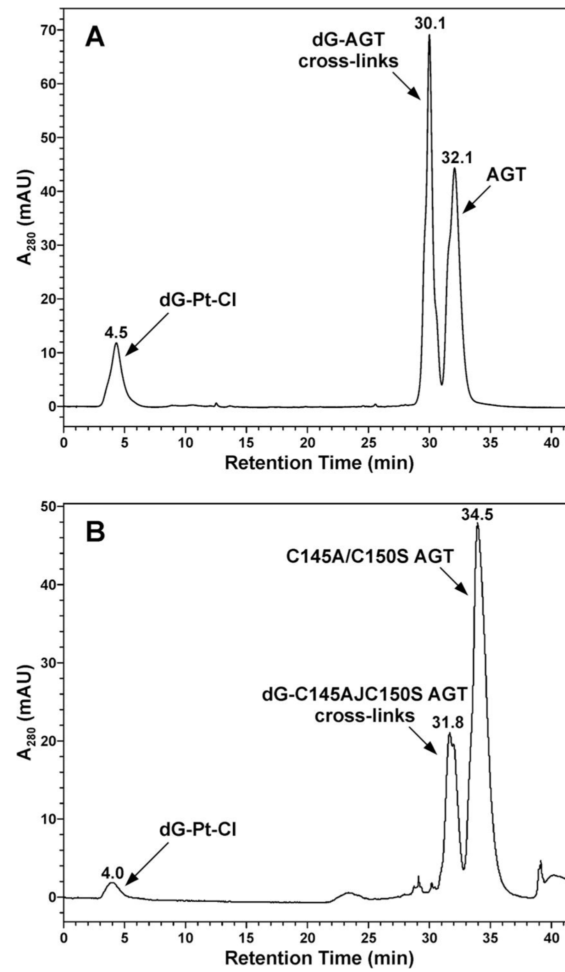


Figure 2.

HPLC separation of reaction mixtures, following incubation of recombinant AGT protein with dG-Pt-Cl, as models for monoalkylated DNA to induce cross-linking. AGT-dG conjugates were identified by HPLC-ESI^{+}-MS as shown in Figure 3.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

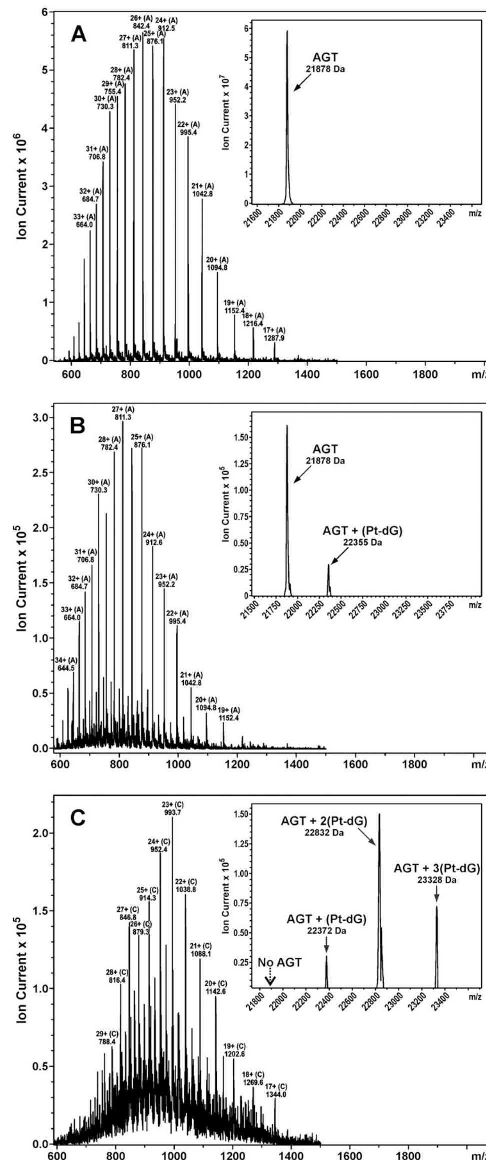


Figure 3.

HPLC-ESI$^+$-MS and deconvoluted spectra (inset) of (**A**) unreacted AGT protein, (**B**) dG-Pt-Cl monoadduct-treated AGT protein that had been separated by HPLC with retention time 32.1 min in Figure 2, and (**C**) dG-Pt-Cl monoadduct-treated AGT protein that had been separated by HPLC with retention time 30.1 min in Figure 2. Unmodified AGT (calculated, $M = 21876$ Da; observed, $M = 21878$ Da), AGT containing a single platinum cross-link to dG (calculated, $M = 22354$ Da; observed, $M = 22355$ Da or calculated, $M = 22371$ Da; observed, $M = 22372$ Da), AGT containing two platinum cross-links to dG (calculated, $M = 22832$ Da; observed, $M = 22832$ Da), and AGT containing three platinum cross-links to dG (calculated, $M = 23327$ Da; observed, $M = 23328$ Da).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

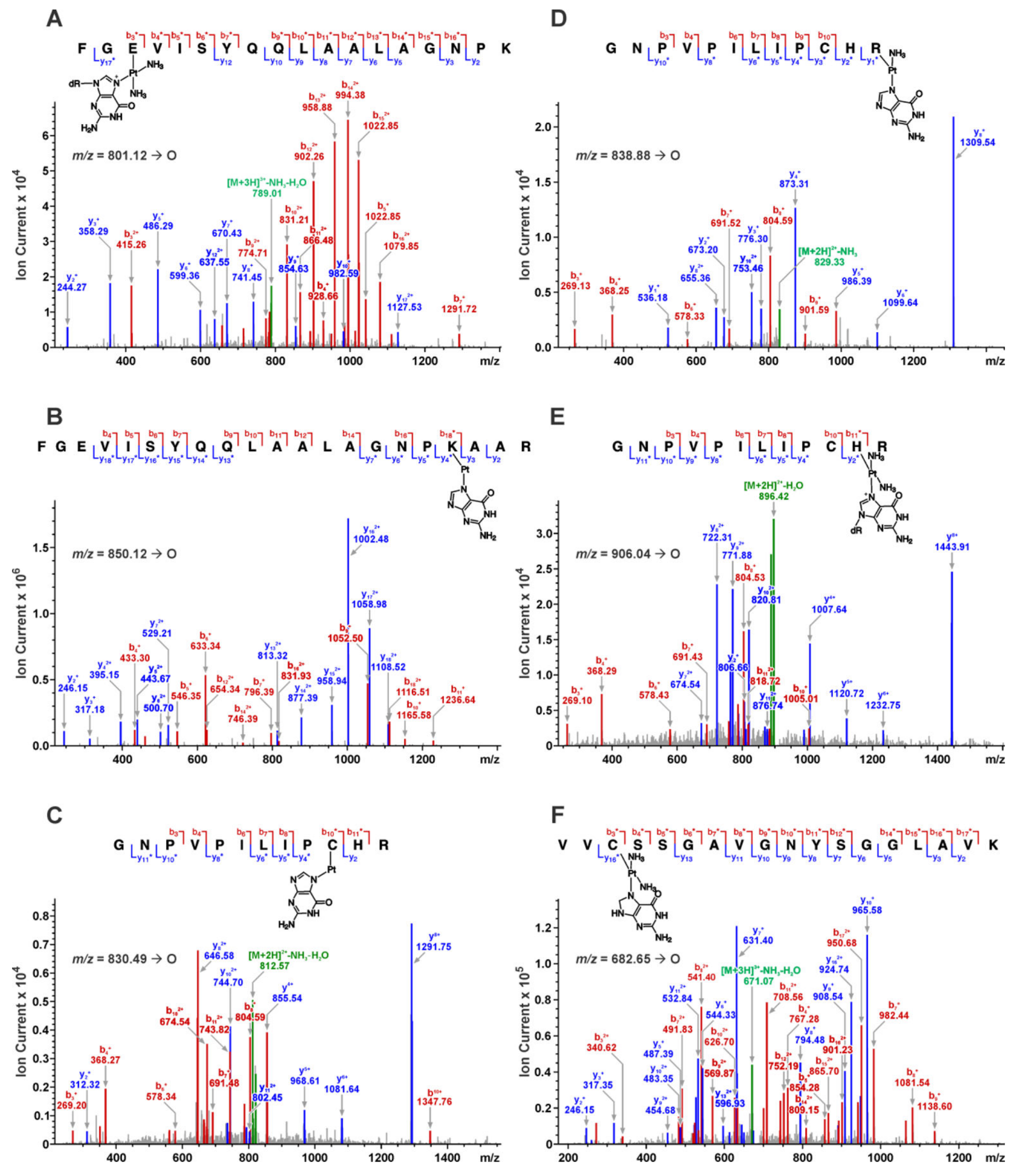


Figure 4.
HPLC-ESI$^+$-MS/MS analysis of tryptic peptides derived from cisplatin-induced AGT-dG conjugates at Glu^{110}, Lys^{125}, Cys^{145}, His^{146}, Arg^{147}, and Cys^{150}. Fragment ions containing a cross-linked platinum adduct are indicated by "*".

Author Manuscript

Figure 5.

Crystal structure of human AGT protein bound to DNA (PDB 1T39) showing the sites of cisplatin-mediated DNA-protein cross-linking.

Author Manuscript

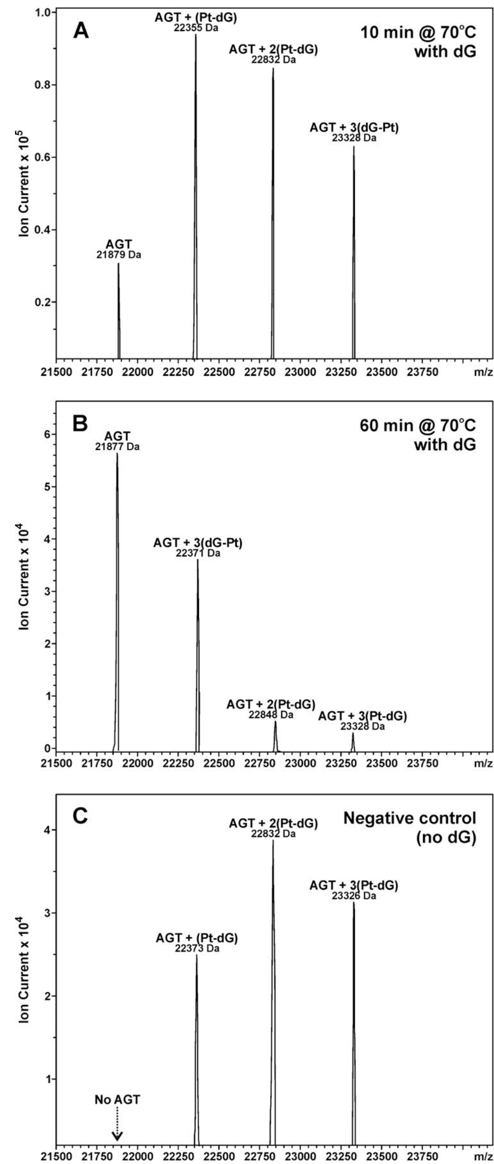


Figure 6.

Platination migration from AGT to dG. (**A**) Deconvoluted ESI$^+$-MS spectra of dG-Pt-AGT that was incubated with dG for 10 min at 70 °C. (**B**) Spectra of dG-Pt-AGT that was incubated with dG for 60 min at 70 °C. (**C**) Spectra of dG-Pt-AGT complexes was incubated in the absence of dG for 10 min at 70 °C.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

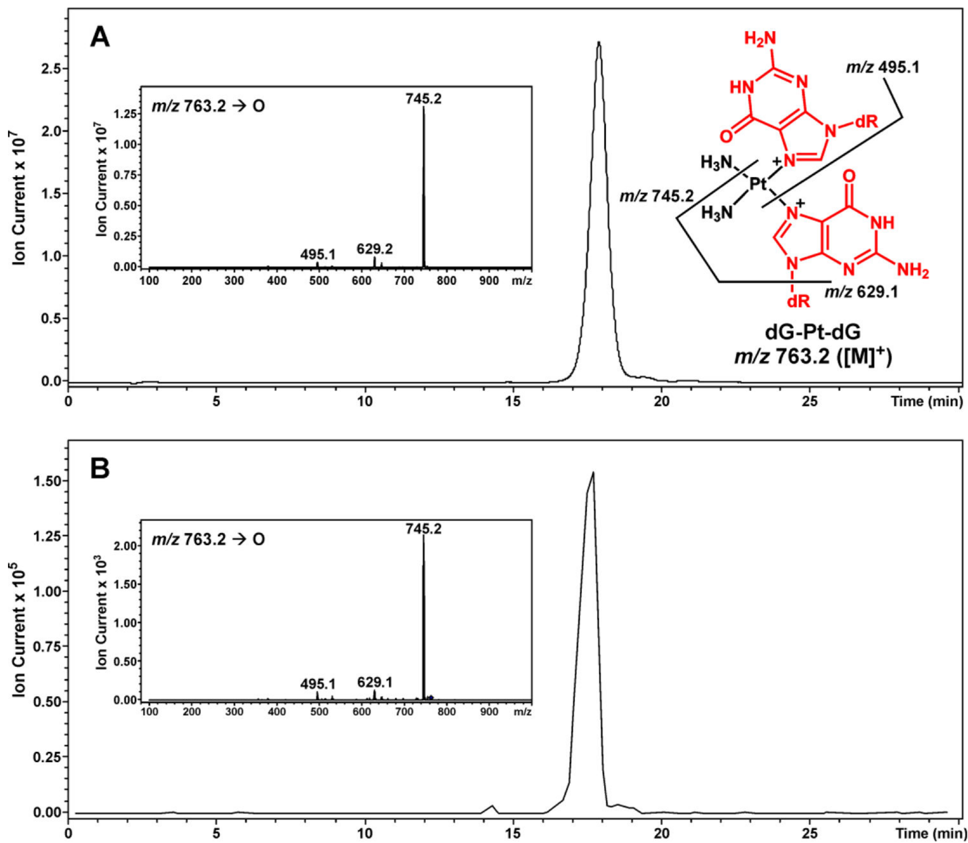


Figure 7.

HPLC-ESI$^+$-MS/MS analysis of dG-Pt-dG conjugates produced as a result of platination migration from dG-Pt-AGT cross-links to dG. Extracted ion chromatogram of dG-Pt-dG (*m/z* 763.2 $[M]^+$). Inset: MS/MS fragmentation. (**A**) Synthetic dG-Pt-dG; (**B**) Sample treated with dG for 60 min at 70 °C

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

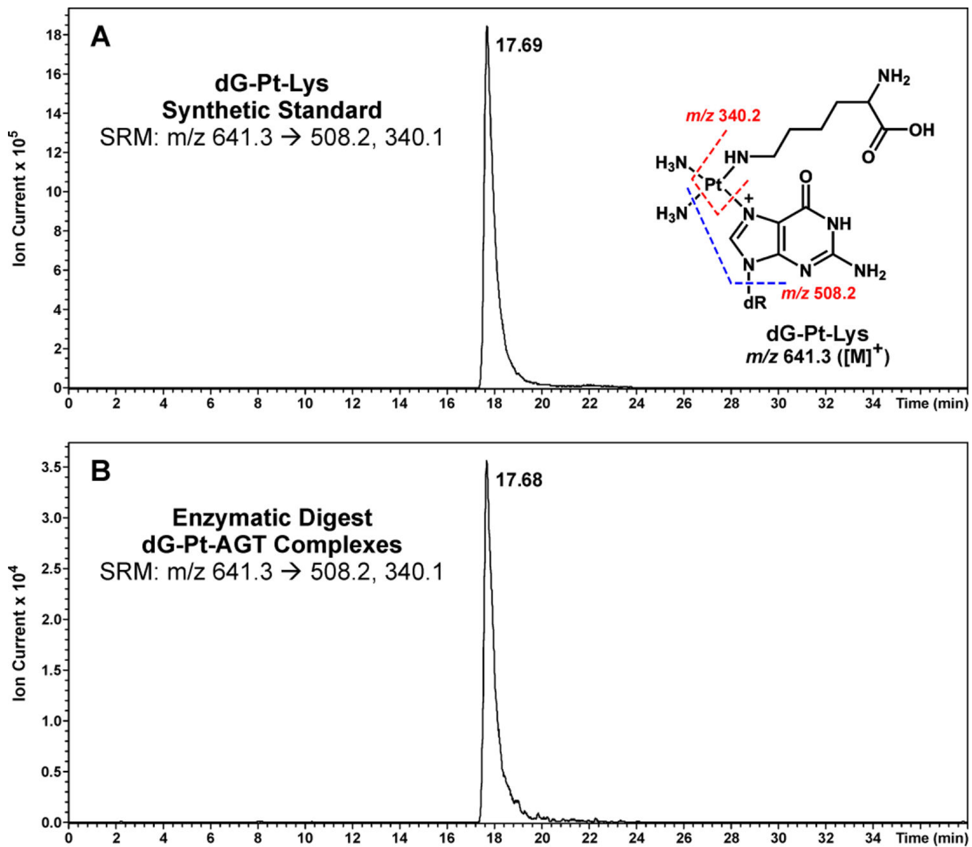


Figure 8.

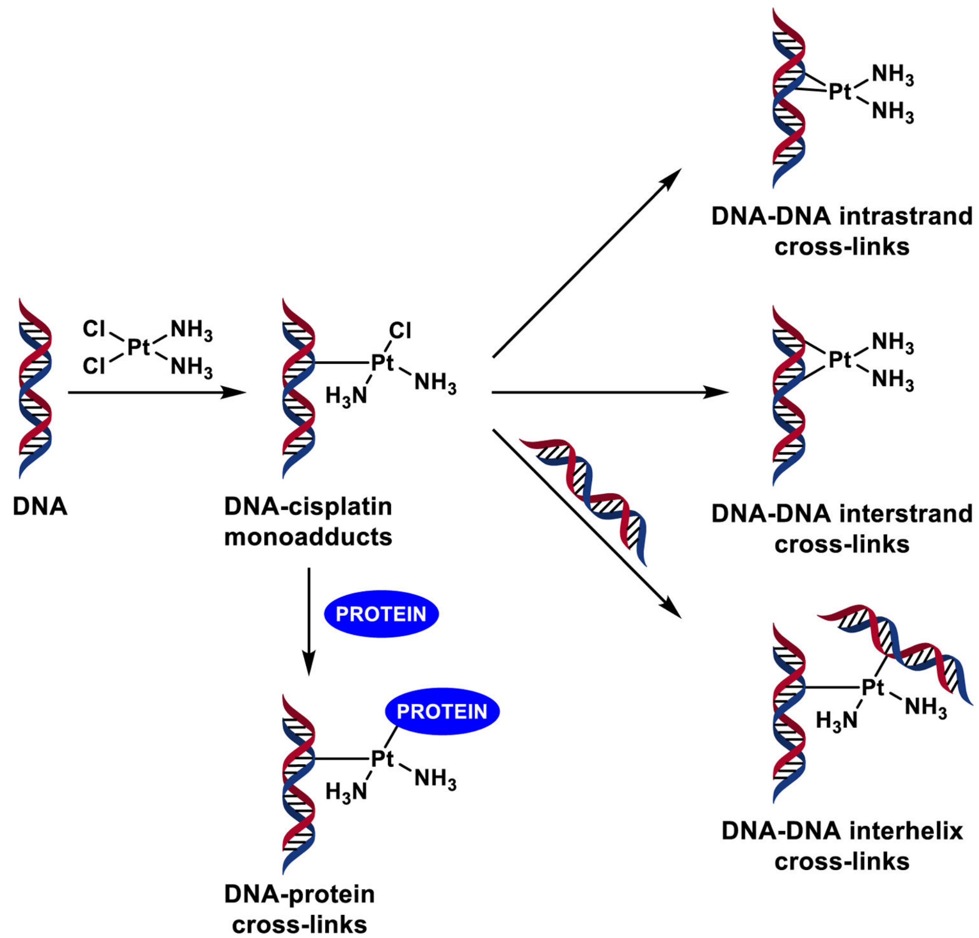
HPLC-ESI^{+}-MS/MS analysis of dG-Pt-Lys conjugates in total proteolytic digests of AGT treated with dG-Pt-Cl to generate cross-links. Following HPLC purification of dG-Pt-AGT complexes, they were subjected to enzymatic digestion to release amino acid-nucleobase conjugates. (**A**) Synthetic dG-Pt-Lys; (**B**) Enzymatic digests of dG-Pt-AGT complexes.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



Scheme 1.

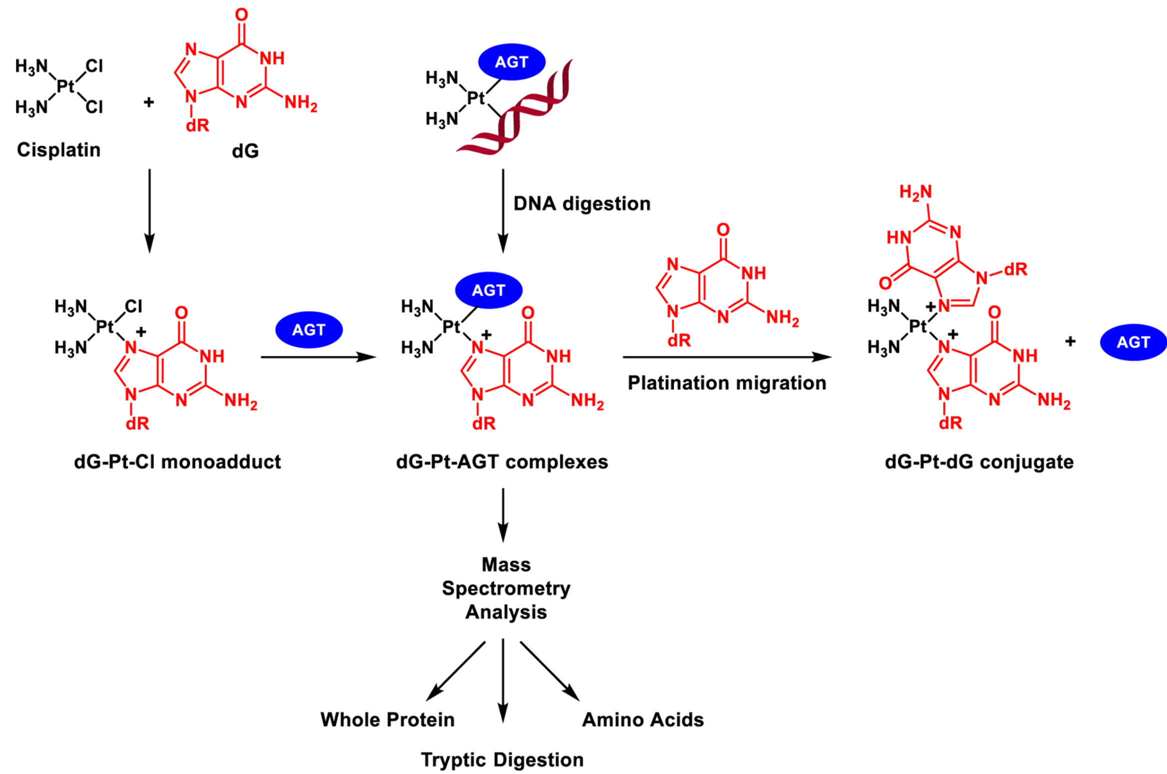
Formation of bifunctional DNA adducts by cisplatin.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



Scheme 2.

Mass spectrometry-based approach employed to characterize AGT-DNA cross-links of cisplatin and platination migration from AGT protein to dG to release intact AGT and form G-G cisplatin cross-links.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1.

HPLC-ESI$^+$-MS/MS analysis of AGT tryptic peptides (unmodified protein).

Position	Peptide sequence	$[M + H]^+_{calculated}$	$[M + 2H]^{2+}_{calculated}$	$[M + 3H]^{3+}_{calculated}$	Observed ions
1–8	MDKDCEMK	998.38	499.6939	333.4652	500.2
9–18	RTTLDSPLGK	1,086.60	543.8039	362.8718667	363.21, 544.31
10–18	TTLDSPLGK	930.5	465.7539	310.8385333	466.26
19–32	LELSGCEQGLHEIK	1,554.77	777.8889	518.9285333	519.27, 778.39
33–36	LLGK	429.28	215.1439	143.7652	215.43
37–96	GTSAADAVEVPAPAAVLGGPEPLMQCTAW LNAYFHQPEAIEEFPVPALHHPVFQQESFTR	6,468.13	3234.5689	2156.7152	ND *
97–101	QVLWK	673.40	337.2039	225.1385333	337.21
102–107	LLKVVK	699.51	350.2589	233.8418667	350.26
108–125	FGEVISYQQLAALAGNPK	1,905.00	953.0039	635.6718667	636.01, 935.51
126–135	AARAVGGAMR	959.52	480.2639	320.5118667	320.51, 480.26
129–135	AVGGAMR	661.35	331.1789	221.1218667	331.18
136–147	GNPVPILIPCHR	1,314.72	657.8639	438.9118667	658.37
148–165	VVCSSGAVGNYSGGLAVK	1,666.84	833.9239	556.2852	834.43
166–175	EWLLAHEGHR	1,246.61	623.8089	416.2085333	416.55, 624.32
176–193	LGKPGLGGSSGLAGAWLK	1,667.93	834.4689	556.6485333	566.99
194–207	GAGATSGSHHHHHH	1,428.60	714.8039	476.8718667	715.34

* Not detected.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2.

Platinated peptide sequences of AGT after treatment with dG-Pt-Cl followed by HPLC-ESI$^+$-MS/MS analysis of the resulting tryptic digests. The assigned platinum binding site is given as bold italic type within peptide sequence.

Peptide sequence	Pt adduct	Pt adduct mass (Da)	Charge	SEQUEST parameters		Ions*	MS/MS spectrum
				Xcorr	Cn		
F^{108}G***E***VISYQQLAALAGNPK^{125}	+(Pt-dG-2NH_3)	495.11	+3	4.53	1.0	34/68	Figure 2.4A
F^{108}GEVISYQQLAALAGNP***K***AAR^{128}	+(Pt-Gua)	344.00	+3	3.39	1.0	28/60	Figure 2.4B
G^{136}NPVPILIP***C***HR^{147}	+(Pt-Gua)	344.00	+2	2.54	1.0	22/44	Figure 2.4C
G^{136}NPVPILIPC***H***R^{147}	+(Pt-Gua-NH_3)	361.03	+2	3.07	1.0	20/50	Figure 2.4D
G^{136}NPVPILIPCH***R***^{147}	+(Pt-dG-2NH_3)	495.11	+2	3.05	1.0	16/35	Figure 2.4E
V^{148}V***C***SSGAVGNYSGGLAVK^{165}	+(Pt-Gua-2NH_3)	378.05	+3	4.95	1.0	30/68	Figure 2.4F

*Ratio of the assigned *b* and *y* ions to the total number of all possible fragment ions.