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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · APRIL 2009

Impact Factor: 3.53 · DOI: 10.1021/tx800481j · Source: PubMed

Development of an Ultraperformance Liquid Chromatography/Mass Spectrometry Method To Quantify Cisplatin 1,2 Intrastrand Guanine−Guanine Adducts

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Received December 19, 2008

Platinum chemotherapeutic agents have been widely used in the treatment of cancer. Cisplatin was the first of the platinum-based chemotherapeutic agents and therefore has been extensively studied as an antitumor agent since the late 1960s. Because this agent forms several DNA adducts, a highly sensitive and specific quantitative assay is needed to correlate the molecular dose of individual adducts with the effects of treatment. An ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay for quantification of 1,2 guanine−guanine intrastrand cisplatin adducts [CP-d(GpG)], using $^{15}N_{10}$ CP-d(GpG) as an internal standard, was developed. The internal standard was characterized by MS/MS, and its concentration was validated by inductively coupled plasma mass spectrometry. Samples containing CP-d(GpG) in DNA were purified by enzyme hydrolysis, centrifugal filtration, and HPLC with fraction collection prior to quantification by UPLC-MS/MS in the selective reaction monitoring mode [*m*/*z* 412.5→248.1 for CP-d(GpG); *m*/*z* 417.5→253.1 for [$^{15}N_{10}$] CP-d(GpG)]. The recovery of standards was >90%, and quantification was unaffected by increasing concentrations of calf thymus DNA. This method utilizes 25 μg of DNA per injection. The limit of quantification was 3 fmol or 3.7 adducts per 10^8 nucleotides, which approaches the sensitivity of the ^{32}P postlabeling method for this adduct. These data suggested that this method is suitable for in vitro and in vivo assessment of CP-d(GpG) adducts formed by cisplatin and carboplatin. Subsequently, the method was applied to studies using ovarian carcinoma cell lines and C57/BL6 mice to illustrate that this method is capable of quantifying CP-d(GpG) adducts using biologically relevant systems and doses. The development of biomarkers to determine tissue-specific molecular dosimetry during treatment will lead to a more complete understanding of both therapeutic and adverse effects of cisplatin and carboplatin. This will support the refinement of therapeutic regimes and appropriate individualized treatment protocols.

Introduction

Platinum chemotherapeutics are used in the treatment of many types of cancer including breast, ovarian carcinoma, colorectal, and metastatic cancers. In the first generation of platinum chemotherapeutics is *cis*-diamminedichloroplatinum II (cisplatin), which is an effective treatment for several cancers. The anticancer activity of cisplatin is attributed in part to the formation of inter- and intrastrand cross-links in DNA, which inhibit gene transcription and DNA replication, thereby blocking protein synthesis and cell proliferation (*1*−*3*). Unfortunately, cisplatin treatments have been accompanied by several side effects, such as neuropathy and gastrointestinal and renal toxicity. Recently, capacity for repair of cisplatin adducts has been linked to the severity of peripheral neuropathy in patients (*4*). Additionally, the development of cisplatin resistance prevents its use in some cancer patients.

Cisplatin is an inorganic compound in which the platinum atom is bound to two amine groups and has two chloride leaving groups. Cisplatin undergoes a nonenzymatic conversion in physiologic solutions to active derivatives via displacement of chloride leaving groups (*5*). The activated cisplatin binds to DNA forming mono adducts, which ultimately form inter- and intrastrand cross-linking DNA adducts. The cisplatin-derived 1,2 guanine−guanine intrastrand [CP-d(GpG)][1] cross-links are the most prevalent, compromising ∼65% of the adducts formed in vivo (*5*−*8*). Because DNA adducts are excellent biomarkers for internal dose, many researchers have attempted to evaluate the role of Pt−DNA adducts in vivo. Common methods used to measure Pt−DNA adducts include antibody probes, ^{32}P postlabeling, atomic absorption, and mass spectrometry. Unfortunately, antibody-based assays are prone to false positives due to cross-reactivity and have nonlinear responses (*9*−*11*), and ^{32}P postlabeling methods, while able to detect as little as

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[1] Abbreviations: CP-d(GpG), cisplatin 1,2 guanine−guanine intrastrand cross-link; ICP-MS, inductively coupled mass spectrometry; LOQ, limit of quantification; ctDNA, calf thymus DNA; UPLC, ultraperformance liquid chromatography; SPE, solid-phase extraction; SCX, strong cation exchange; HESI, heat-assisted electrospray ionization; RSD, relative standard deviation; LOD, limit of detection; dRibpdRib, deoxyribose-phosphate-deoxyribose.

10.1021/tx800481j CCC: $40.75
Published on Web 03/27/2009

0.087 fmol adduct per μg DNA, are labor intensive and utilize radioactivity; both methods do not provide structural confirmation of adducts (*12, 13*). Atomic absorption spectroscopy measures total platinum but lacks sufficient sensitivity for routine clinical application (*14*−*18*). Inductively coupled plasma mass spectrometry (ICP-MS) also measures total platinum with higher sensitivity, allowing application to clinical samples. One group has reported an ability to approach the sensitivity of the ^{32}P postlabeling method using ICP-MS. However, they further reported that this level of sensitivity cannot be obtained with the addition of HPLC speciation, which is necessary for quantification of the individual adducts (*19*). Previously, a capillary HPLC-ICP-MS method was reported to measure CP-d(GpG) adducts specifically; this method reported a LOQ of ~1 adduct per 10^6 nucleotides, which is insufficient to measure samples treated with <500 μM cisplatin (*20*). Reported in the literature are mutagenesis assays with <10 μM cisplatin; therefore, greater sensitivity is needed to ensure direct comparisons of the formation and/or persistence of individual cisplatin adducts and the induction of mutations. LC-MS provides structural confirmation of the DNA adducts, without extensive labor or the use of radioactivity. There are some reports of LC-MS methods for platinum−DNA intrastrand adducts; however, each published report lacks an internal standard, which is essential for accurate and reproducible quantification (*21, 22*).

Our method utilizes an internal standard and approaches the sensitivity of the ^{32}P postlabeling method with a limit of quantification (LOQ) of 0.12 fmol CP-d(GpG) per μg DNA or 3.7 adducts per 10^8 nucleotides, requiring only 25 μg of DNA on column. To advance the understanding of the formation and distribution of cisplatin DNA lesions in vitro and in vivo, we report the development of a highly sensitive stable isotope dilution mass spectrometry method for the accurate quantification of CP-d(GpG) cross-links.

Experimental Procedures

Caution: *Cisplatin is carcinogenic and should thus be handled in an approved laboratory fume hood, and personal protective equipment (i.e., gloves and laboratory coat) should be worn.*

Chemicals. Unless otherwise stated, all chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA), along with Gentra PureGene components for DNA isolation. The unlabeled oligonucleotide was prepared by Operon (Huntsville, Alabama). The heavy labeled oligonucleotide used in the synthesis of the internal standard was made by Silantes GmbH (Munich, Germany). Reagents for cell culture studies were purchased from GIBCO Invitrogen (Carlsbad, California), with the exception of the fetal bovine serum, which was purchased from Sigma-Aldrich.

Preparation of CP-d(GpG) Analyte Standard. An analyte standard was synthesized for optimization and standardization during method development. All platination reactions were carried out with 40 mM aquated derivatives of the platinum complex obtained by overnight stirring in the dark at room temperature of a solution containing cisplatin and 1.98 equiv of silver nitrate. The solution was then filtered through a 0.2 μm Gelman Acrodisk CR PTFE syringe filter (Fisher), and 10 μL aliquots were stored at −80 °C. Immediately before each experiment, an aliquot of cisplatin solution was thawed at 50 °C for 10 min and diluted (1:40 volume ratio) with HPLC water, taking care to avoid light exposure. Diluted aquated cisplatin and 2′-deoxyguanylyl (3′,5′)-2′-deoxyguanosine (4:1 molar ratio) were incubated at 37 °C for 24 h.

HPLC cleanup was performed using an Agilent 1100 HPLC with a Phenomenex Clarity 3 μm Oligo-RP (100 mm × 4.6 mm) column and a Hewlett-Packard 1040A photodiode array detector. A gradient of 100 mM triethylamine acetate (TEAA) (buffer A1) and 100% methanol (buffer B) was operated at 1 mL/min, starting at 0% B with linear increases to 30% B by 15 min and 70% B by 16 min, followed by a decrease to 0% B by 17 min and a 3 min column re-equilibration. All peaks thought to contain cisplatin cross-links were collected, and aliquots were characterized by UV on a Thermo BioMate 5. Standards were quantified using the extinction coefficient for d(GpG) (ε_{260} = 21600) (*23, 24*). The stock solutions were stored in 10 mM ammonium acetate, pH 4, at −20 °C. Dilutions of the stock were made in 10 mM ammonium acetate for MS analyte standard curves.

ICP-MS was utilized to confirm the concentration of standards that had been estimated from the UV absorbance and extinction coefficient. Samples of 10−96 pmol, as measured by UV, were diluted to 4 mL in 2% nitric acid in HPLC water for measurement by ICP-MS.

The analytical standards were characterized by LC-MS as follows: a Phenomenex Luna 3 μm C18(2) column (2.0 mm × 150 mm) was operated using a linear gradient of 10 mM ammonium acetate plus 0.1% acetic acid (pH 4.5) (buffer A2) to 50% methanol (buffer B) over 28 min and returning to 0% methanol at 30 min. The positive and negative full scan electrospray mass spectra (*m*/*z* 100−1000) were acquired on a Finnigan TSQ^{Deca} ion trap mass spectrometer.

Preparation of $^{15}N_{10}$ CP-d(GpG) Internal Standard. An internal standard was synthesized to ensure accurate and precise quantification of cisplatin adducts. To determine appropriate ratios of incubation times for platination, an unlabeled oligonucleotide was purchased from Operon with the sequence ACTGGTCATGGTACTGGT. Once optimal conditions were established, Silantes was contracted to synthesize a ^{15}N fully labeled oligonucleotide of the same sequence. ^{15}N labeling was confirmed by mass spectrometry after enzymatic hydrolysis. This ^{15}N-labeled oligo was rehydrated in HPLC water for a final concentration of 0.55 nmol/μL. To make the internal standard, a 60 nM concentration of the oligo was reacted with aquated cisplatin in HPLC water utilizing a drug to oligo molar ratio of 24:1 at 37 °C for 72 h in the dark.

To separate the adducted nucleotide from the surrounding unmodified nucleosides, the samples next underwent an enzymatic hydrolysis based upon the method previously reported by Eastman (*25*). Briefly, 50 μg aliquots of platinated oligo, 320 μL of 50 mM sodium acetate/10 mM magnesium chloride, and 0.02 kunitz unit of DNase I were incubated for 4 h at 37 °C; next, 2 units of nuclease P1 was added, and incubation continued for 16−20 h. Finally, 41 μL of 1 M Tris-HCl, pH 9, and 5 units of alkaline phosphatase were added, and incubation continued for 4 h, followed by Microcon-3 spin columns (Millipore) to remove enzymes. All incubations were performed in the dark. Sample enrichment and characterization were performed as described above for the analyte CP-d(GpG). After the concentration was determined by UV and confirmed by ICP-MS, 500 μL aliquots containing 100 fmol per μL in buffer A2 were stored at −80 °C for use as an internal standard (Table 1).

Platination and Preparation of Calf Thymus DNA (ctDNA). ctDNA was used to validate the ultraperformance liquid chromatography (UPLC)-MS/MS method. Sigma ctDNA

Table 1. Validation of Standard Concentrations by UV and ICP-MS[a]

sample type	pmol CP-d(GpG) (UV)	ppb Pt (ICP-MS)	pmol CP-d(GpG) (ICP-MS)	ICP-MS (% RSD)
IS	13	0.7	13.2	5.5
AS 1	9.6	0.6	11.3	5.6
AS 2	57.6	2.1	42.2	1.9
AS 3	96	3.5	72.6	1.5

[a] Samples were prepared in triplicate for ICP-MS in 2% nitric acid based upon initial UV concentrations; $*n = 3$.

was rehydrated to 1 mg/mL aliquots and stored at −20 °C. ctDNA (150 μg) was reacted with 100 nM aquated cisplatin and brought to a total volume of 1 mL in deionized H_2O at 37 °C for 24 h. DNA was enzymatically digested in the same manner as the internal standard. Because of the increased number of samples, solid-phase extraction (SPE) columns were initially used for sample enrichment instead of HPLC.

SPE cleanup was performed using both strong cation exchange (SCX) and C18 columns. After enzymatic hydrolysis, samples were adjusted to pH 3 by the addition of 0.8 vol of 50 mM HCl. Next, LiChrolut SCX columns (VWR) were placed on a vacuum manifold, conditioned with two additions each of 1 mL of water, 1 mL of methanol, and 1 mL of water, and then equilibrated with 1 mL of 50 mM Tris HCl, pH 3. The samples were applied to the columns, which were then washed four times with 2 mL of 5 mM sodium formate, pH 6. Platinated adducts were then eluted with two additions of 0.5 mL of 250 mM ammonium hydroxide. To remove salt prior to MS analysis, Maxi-Clean 300 mg C18 cartridges (Alltech) were utilized. First, the eluent was adjusted to pH 7 by addition of 130 μL of 50 mM HCl. Next, the C18 cartridges were conditioned and equilibrated using two additions of 2 mL of water, 2 mL of methanol, and 2 mL of 10 mM ammonium acetate. The pH-adjusted sample was then applied to the column, which was washed with three additions of 2 mL of 10 mM ammonium acetate. Platinum adducts were eluted with two additions of 0.5 mL of 10 mM ammonium acetate in 50% methanol. After SPE, enriched samples were dried via centrifugal lyophilization. Samples were rehydrated using 10 mM ammonium acetate, pH 4, in 10% methanol, placed in vials for analysis, dried once again by centrifugal lyophilization, and finally brought to identical running volumes through the addition of 40 μL of 10 mM ammonium acetate, pH 4. Samples were then either stored at −80 °C or immediately placed in the MS injector tray to begin adduct quantification. During method development, we found that the CP-d(GpG) adduct is most stable under specific conditions. Unsuccessful attempts were made to perform loop injections using purified CP-d(GpG) with water and methanol as buffers. It was ultimately determined that CP-d(GpG) was the most stable with a salt-containing buffer between pH 4 and 4.5. We achieved this using 10 mM ammonium acetate with 0.1% glacial acetic acid in place of water as a storage and running buffer. Because of limited recovery during SPE (∼20%), it was determined that HPLC with fraction collection was more suitable for sample purification after enzyme hydrolysis. The changes in pH and multiple buffers required the use of SCX SPE followed by a C18 cartridge to remove sodium salt prior to MS quantification. This may have affected the stability of the adduct, thereby reducing the overall recovery by SPE.

CP-d(GpG) was purified from DNA hydrolysate by HPLC fractionation utilizing an Agilent 1200 system consisting of a G1312B binary pump SL, a G1379B degasser, a G1316B thermostatted column compartment SL, a G1215C diode array SL, a G1367C/G1330B thermostatted high-performance autosampler SL, and a G1364C/G1220B analytical scale fraction collector to separate adducts from unmodified nucleosides. Therefore, a Phenomenex Clarity 3 μm Oligo-RP (100 mm × 4.6 mm) column, 10 mM ammonium acetate in 0.1% glacial acetic acid (buffer A2), and methanol (buffer B) were operated with a linear gradient for 30% B over 20 min, then to 70% B in 2 min, and finally decreasing 0% B in 1 min and re-equilibration for 2 min prior to the next injection. Fractions containing platinum adducts were collected from 6.85 to 8.85 min. Fractions were dried via centrifugal lyophilization and rehydrated using a mix of 50/50 buffers A2 and B, placed in MS vials, and once again dried by centrifugal lyophilzation. Samples were either stored at −20 °C or immediately rehydrated in 40 μL of buffer A2 for UPLC tandem mass spectrometric analysis.

UPLC-MS/MS Method. Quantitative LC-MS/MS data were obtained using a Waters Acquity UPLC coupled to a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer. A heated electrospray ionization (HESI) interface was operated in positive ionization mode. The analyzer was operated in selective reaction monitoring (SRM) mode, monitoring the loss of phosphate, two deoxyriboses, and two amines from CP-d(GpG) (m/z 412.5 to 248.1, $z = 2$) and [$^{15}N_5$]-CP-d(GpG) IS (m/z 417.5 to 253.1, $z = 2$). Samples were kept at 4 °C during analysis, and the sample injection volume was 20 μL. An HSS T3 (2.1 mm × 100 mm; 1.8 μm) UPLC column (Waters) was conditioned with aqueous 0.1% glacial acetic acid (A3) and methanol (B) at a flow rate of 200 μL/min. Cisplatin adducts were eluted with a linear gradient of 1−10% B over 10 min, then to 50% B in 1 min, followed by a 3 min re-equilibration at 1% B. The LC flow was diverted to waste for the first 4 min of the gradient. Instrument conditions were optimized for maximum signal of CP-d(GpG) by direct infusion and on column injections of analyte standard. MS settings were as follows: electrospray voltage (3000 V), capillary temperature (285 °C), HESI temperature (250 °C), sheath and auxiliary gas pressures (35 and 30 arbitrary units), collision energy (25 V), and Q2 collision gas pressure (1.5 mTorr).

Quantification. Analyte standard or 50 μg of platinated DNA was spiked with 500 fmol of internal standard and enzymatically hydrolyzed as described in the analyte standard section. Samples were then processed by SPE columns or HPLC and prepared for MS quantification as described in the ctDNA section.

ICP-MS. Aliquots of analyte and/or internal standard using 10−96 pmol CP-d(GpG) were brought to a final volume of 4 mL in 2% nitric acid. A Varian 820-MS Inductively Coupled Plasma-Mass Spectrometer was used to determine Pt concentration. Samples were introduced via a concentric glass nebulizer with a free aspiration rate of 0.4 mL/min, a Peltier-cooled double pass glass spray chamber, and a quartz torch. A peristaltic pump carried samples from a SPS3 autosampler (Varian) to the nebulizer. All standards and samples were in 2% nitric acid, prepared with milliQ water. Prior to each experiment, optimization of the instrument's operating parameters was performed using a tuning solution (Spectropure, Arlington, TX) diluted to 5 ppb each of Ba, Be, Ce, Co, In, Pb, Mg, Tl, and Th. Ion optics and plasma parameters were optimized to maximize sensitivity while minimizing interferences. Pt standards were prepared by serial dilution of a solution containing 10 ppm Pt (Inorganic Ventures Inc., Lakewood, NJ). A four-point calibration curve was made over a concentration range of 0.5−8 ppb Pt. The two most abundant isotopes of Pt were monitored, ^{194}Pt

(33.0% abundance) and ^{195}Pt (33.8% abundance). A 200 ppb dilution of the tuning solution was used for monitoring ^{115}In as the internal standard. Data acquisition was done using peak hopping with a dwell time of 50 ms, one point per peak, 20 scans/replicate, and five replicates per sample.

Treatment of Ovarian Carcinoma Cells. Human ovarian carcinoma cell lines A2780 and A2780/CP70 were graciously provided by Dr. Thomas C. Hamilton (Fox Chase Cancer Center). The parent cell line A2780 is sensitive to cisplatin, while the A2780/CP70 line exhibits stable cisplatin resistance developed from chronic exposure to increasing concentrations of cisplatin as described previously (*26*). Cells were maintained as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum (heat inactivated), 2 μM L-glutamine, insulin (0.25 units/mL), penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) at 37 °C and 5% CO_2.

In both the dose−response and the time−course studies, 2 × 10^6 cells were plated in T175 flasks and allowed to grow for 3 days. The growth medium was removed, and cells were rinsed twice with phosphate-buffered saline (PBS) before serum-free medium plus 12.5−250 μM cisplatin or PBS was applied. After 1 h of incubation, the cisplatin-containing medium was removed, and the cells for the dose response were trypsinized and collected. Cells for the time−course assay were rinsed with PBS, and complete medium was reapplied for an additional 3 or 7 h before trypsinization and collection. DNA was isolated from cells with the Gentra PureGene cell kit, as recommended by the manufacturer.

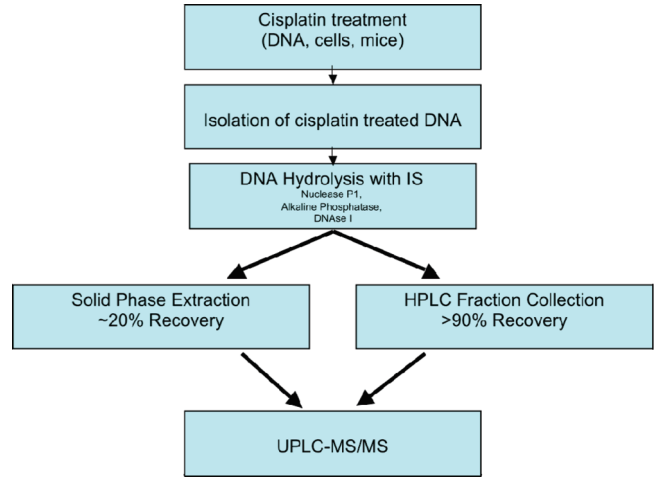
Study Conditions for C57/BL6 Mice. Male C57/BL6 mice (n = 3) were injected i.p. with 7 mg/kg cisplatin. On days 1 and 3 after injection, the mice were euthanized by carbon dioxide anoxia; portions of the kidney, liver, and colon were snap-frozen and stored in a −80 °C freezer, after removing sections for histopathology. The numerical scale of histology scoring and description is as follows: 0 = absent or no significant lesions (NSL), 1 = minimal lesions (<5%), 2 = mild lesions (5−25%), 3 = moderate lesions (25−50%), and 4 = marked/severe lesions (>50%).

We used the FDA's dose calculator[2] to better understand how the cisplatin dose in our mouse study (7 mg/kg) relates to a low human dose (50 mg/m^2) received in the clinic. Using an adult mouse weighing 20 g, the total dose at 7 mg/kg cisplatin received would be 0.14 mg, which is equal to 21.11 mg/m^2. Conversely, a dose of 50 mg/m^2 would require 0.33 mg or 16.58 mg/kg.

DNA Isolation from Tissues. DNA was extracted using Gentra PureGene kit reagents with a significantly modified protocol. Because of the reduced amount of colon tissue available for processing, all volumes in the procedure below were reduced by half when isolating DNA from this tissue. Briefly, frozen kidney (400 mg), liver tissue (400 mg), or colon tissue (150 mg) was thawed in 6 mL of ice-cold PBS (pH 7.4). The tissue samples were homogenized with a Tehran homogenizer (Wheaton Instruments, Millville, NJ). After centrifugation at 1000*g* for 15 min, the pellet was washed with 6 mL of homogenization buffer. The nuclear fraction was collected by centrifugation and was reconstituted in 6 mL of cell lysis buffer. Proteinase K (400 U/mL, 150 μL) was added to the sample and incubated overnight at 4 °C. The following morning, samples were placed on ice. Proteins were then extracted by the addition of 2 mL of protein precipitation solution followed by centrifugation at 2000*g* for 10 min and the collection of the supernatant. Nucleic acids were precipitated from the supernatant using 6 mL of isopropanol. The nucleic acids were collected by centrifugation, rinsed with 6 mL of 70% ethanol, and allowed to air-dry. The nucleic acid pellet was reconstituted in 6 mL of cell lysis solution supplemented with RNase A (0.8 KeU/mL, 27 μL) to digest RNA. After 30 min of incubation at 37 °C, another protein precipitation was performed, and the supernatant was collected. DNA was precipitated using 6 mL of isopropanol, collected by centrifugation, and rinsed with 70% ethanol. The DNA was resuspended in 400 μL of HPLC grade water, and its concentration and purity were estimated by UV spectrometry. The DNA solution was stored at −80 °C until CP-d(GpG) adduct analysis.

[2] http://www.fda.gov/cder/cancer/animalframe.htm.

Scheme 1. Preparation and Quantification of CP-d(GpG) Adducts by UPLC-MS/MS[a]



[a] Cisplatin-treated DNA is isolated. Next, internal standard is added, and enzyme hydrolysis is performed. Following hydrolysis, SPE or HPLC enrichment separates the platinated adduct from nucleosides, enzymes, and sodium salts. Finally, the CP-d(GpG) adduct is quantified by UPLC-MS/MS.

Results

Characterization of the CP-d(GpG) Analyte Standard. The CP-dGpG was characterized by UV and MS. Analyte standards were examined for purity after synthesis using full scan and SRM MS to ensure correct derivatization of our compound. Quantification of adducts was performed using a Waters Acquity UPLC coupled to a Thermo Finnigan TSQ Quantum Ultra MS. Standard curves using the synthesized analyte and internal standards were run using the UPLC-MS/MS method shown in Scheme 1.

Negative and positive ionization efficiencies were evaluated because CP-d(GpG) adducts exist in solution as zwitterions caused by the negative phosphate and positive amine groups. The examination showed better ionization in the positive ion mode. Also, a platinum-specific isotopic cluster consisting of 5 (two major and three minor) isotopes aided product identification (Figure 1). The MS/MS scans were originally performed in the positive ionization mode using several transitions to account for the loss of the deoxyribose-phosphate-deoxyribose (dRibpdRib) moiety as well as one or two amines ([M-dRibpdRib-NH_2]$^+$ or [M-dRibpdRib-2NH_2]$^+$) in both its singlet (m/1 824.0 to 513.1 or 497.2) and doublet (m/2 412.5 to 256.5 or 248.1) states. Figure 2 shows a representative chromatogram in which we measured both the singlet and the doublet charge states. Through the comparison of peak areas, it was determined that MS/MS quantification of the CP-d(GpG) adducts was most

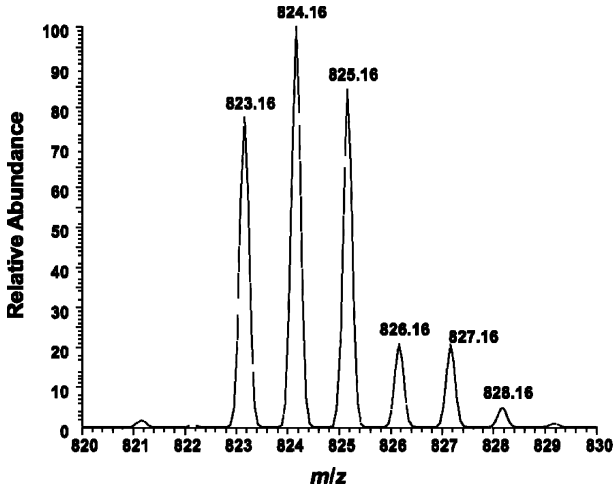


Figure 1. MS isotope simulation of CP-d(GpG). There are three major masses for this adduct due to isotopes.

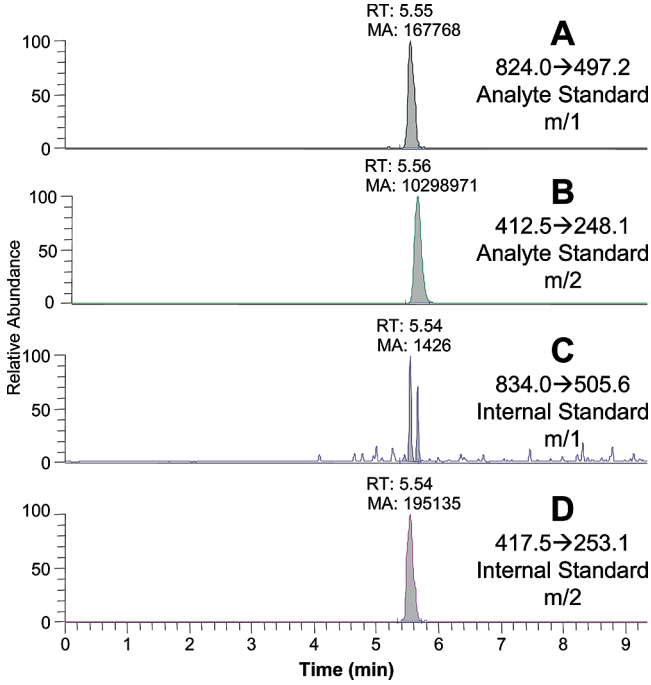


Figure 2. Representative SRM ion chromatograms comparing the use of singly (m/1) vs doubly (m/2) charged state during MS/MS analysis of CP-d(GpG) analyte (A and B) and internal standards (C and D). Parts A and C show MS/MS fragmentation in the singly charged state, while parts B and D show MS/MS fragmentation in the doubly charged state. The chromatogram has been cropped, as no other quantifiable peaks are observed.

sensitive using the doublet charge state. Additionally, the optimal fragmentation involved the loss dRibpdRib and two amines ([M-dRibpdRib-2NH_2]$^+$; analyte *m*/*z* 412.5 to 248.1).

Synthesis and Characterization of ^{15}N CP-d(GpG) Internal Standard. An unlabeled oligonucleotide with identical sequence to the ^{15}N-labeled oligonucleotide was used in the determination of platination efficiency (as confirmed by ^{32}P gels, data not shown) to ensure optimal synthesis of final ^{15}N CP-dGpG adducts. Next, the internal standard was characterized in the same manner as the analyte. Figure 3 shows a positive MS full scan of the unlabeled internal standard after digestion and SPE cleanup. After conditions of adduct synthesis had been optimized, the ^{15}N CP-d(GpG) internal standard was prepared accordingly. As expected, the internal standard was most sensitive in the doubly charged state (internal standard *m*/*z* = 417.5) and shared the same optimal fragmentation as the analyte ([M-dRibpdRib-2NH_2]$^+$; internal standard *m*/*z* = 417.5 to 253.1).

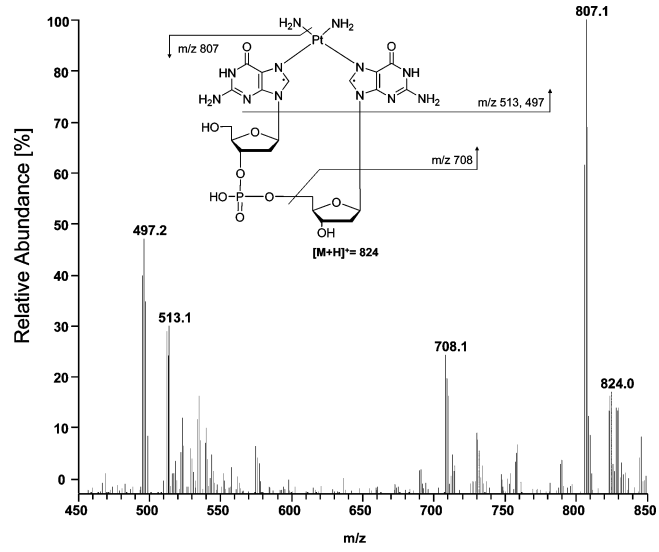


Figure 3. Full-scan positive ion MS spectrum *m*/*z* 450−850 showing the in source fragmentation of CP-d(GpG). The main ions observed were [M-NH_2]$^+$ *m*/*z* 807.1, [M-dRibpdRib-NH_2]$^+$. *m*/*z* 497.2, [M-dRibpdRib]$^+$ *m*/*z* 513.1, and [M-deoxyribose]$^+$ *m*/*z* 708.1. All fragment ions contained the Pt characteristic isotopic profile.

Method Accuracy and Precision. A calibration curve was run using various concentrations of analyte standards and a constant amount of 500 fmol of internal standard. Method precision was first assessed by processing four replicates of known concentrations of analyte standard by SPE. Intraday precision (r^2 = 0.99) was determined using 0, 5, 10, 50, and 100 fmol of CP-d(GpG). Interpreparation precision (r^2 = 0.99) was determined using replicates processed on separate days and run by MS on different days. Interday precision (r^2 = 0.99) was determined using replicates processed on the same day and run by MS on different days. The limit of detection (LOD) was determined using a solution of authentic standard CP-d(GpG). The LOD with a signal-to-noise of >2 was 1 fmol CP-d(GpG) per injection; therefore, the LOQ was set to be three times the LOD [3 fmol of CP-d(GpG) per injection] with a signal-to-noise of >6. Initially, UV measurements were used to determine the concentration of the analyte and internal standard. ICP-MS was used to validate the platinum adduct concentration obtained by UV. Final standard concentrations were adjusted by a factor of 1.3 based upon ICP-MS data. Table 1 shows a comparison of the data obtained by each method. To determine the effect of DNA concentration on the quantification of CP-d(GpG), 0−200 μg of ctDNA was added to samples containing 100 fmol of analyte and 500 fmol of internal standard and processed through the method. No effect of DNA concentration on the quantification of CP-d(GpG) was observed (data not shown). Recovery experiments were also performed using 35, 140, and 700 fmol of analyte standard when using the SPE sample enrichment process vs no SPE enrichment. Sample recovery with SPE enrichment was ∼20% (data not shown). Later additional recovery experiments were performed using analyte standard prepared using HPLC with fraction collection vs no HPLC enrichment. Sample recovery increased to >90% (data not shown).

Quantification of CP-d(GpG) in ctDNA. This method was validated using platinated ctDNA that was diluted with blank ctDNA. Aliquots of 0, 25, 50, and 100 μg of platinated ctDNA were placed in eppendorf tubes with 500 fmol of internal

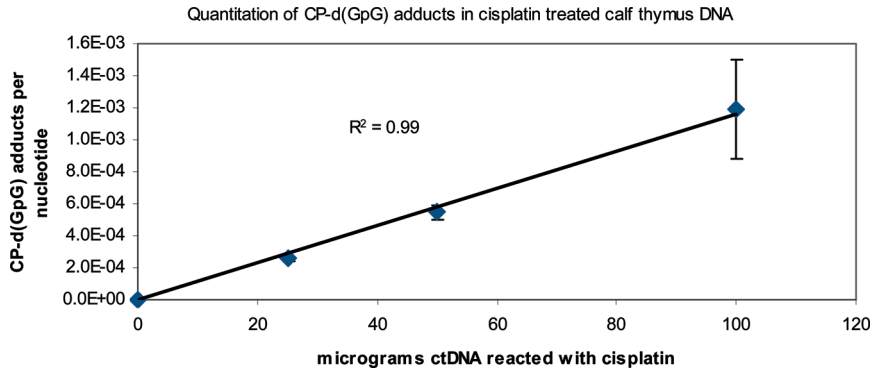


Figure 4. Quantification of CP-d(GpG) in platinated ctDNA. Platinated ctDNA was diluted with blank ctDNA. Aliquots of 0, 25, 50, and 100 μg of platinated ctDNA were placed in eppendorf tubes with 500 fmol of internal standard, and the corresponding amount of blank ctDNA was added to bring the total amount per tube to 100 μg. Samples were processed through the hydrolysis method using HPLC cleanup, and total adducts were quantified by UPLC-MS/MS.

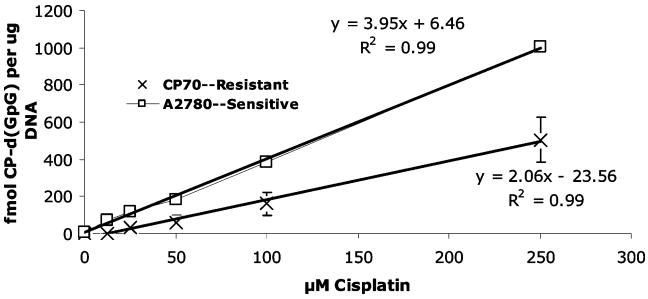


Figure 5. Quantification of CP-d(GpG) in human ovarian carcinoma cells. Two isogenic cell lines, one sensitive (A2780) and one resistant (CP70) to cisplatin, were treated with increasing (12.5−250 μM) doses of cisplatin. Where no error bars are visible, they are smaller than the symbol.

Table 2. Quantification of CP-d(GpG) in C57/BL6 Mice 3 Days Post i.p. Injection of 7 mg/kg Cisplatin

	fmol/μg DNA			
	BL6 1	BL6 2	BL6 3	average
kidney	53	57	67	59 ± 7
liver	21	22	16	20 ± 3
colon	3	6	5	5 ± 2

standard, and the corresponding amounts of blank ctDNA were added to bring the total amount per tube to 100 μg. These samples were processed through the hydrolysis method using HPLC cleanup, and total adducts were quantified by UPLC-MS/MS. As expected, a linear response was observed (Figure 4).

Dose Response of Cisplatin Adducts in Ovarian Carcinoma Cells. An isogenic pair of ovarian carcinoma cell lines (A2780 and CP70), which were originally created to mimic the process of acquired cisplatin resistance, was used in our validated assay. We observed a 2-fold difference between the sensitive A2780 and the resistant CP70 cell lines, which was in line with previous estimates of relative adduct amounts in those two cell lines (Figure 5) (*27*).

Determination of CP-d(GpG) Adducts in Mouse Tissues. The C57/BL6 mouse strain was used to demonstrate CP-d(GpG) accumulation in vivo after an i.p. injection with 7 mg/kg cisplatin. Three days postinjection, the greatest amount of adducts was observed in the kidney, followed by the liver and colon (Table 2). In the same mice, histopathology slides of the kidney showed marked tubular nephrosis/apoptosis with moderate tubular vacuolation/degeneration, while the liver showed moderate centrilobular fatty change and the colon showed minimal crypt cell necrosis/apoptosis (Table 3).

Table 3. Histopathology of C57/BL6 Mice 3 Days Post i.p. Injection of 7 mg/kg Cisplatin[a]

	BL6 1	BL6 2	BL6 3
liver			
hepatic necrosis	0	0	0
centrilobular fatty change	3	3	2
inflammation	0	0	0
colon			
crypt necrosis/apoptosis	1	1	1
kidney			
tubular nephrosis/apoptosis	4	4	3
tubular vacuolation/degeneration	3	3	2
tubular mineralization	2	0	2
inflammation	0	0	0

[a] Key: 0 = absent or no significant lesions, 1 = minimal lesions, 2 = mild lesions, 3 = moderate lesions, and 4 = marked lesions.

Discussion

Method Development and Validation. During the development of this method, it was necessary to modify the sample cleanup procedure after enzymatic hydrolysis from SPE to HPLC. SPE was preferred because it allowed for a higher throughput processing of samples and allowed several types of platinated adducts to be collected simultaneously. However, increased recovery was important when considering our LOD as well as the reduced amount of adduct that may be formed when studying lower and biologically relevant doses of cisplatin. Changing the method to utilize an HPLC cleanup with an autosampler and fraction collector increased recovery from 20 to >90%.

Atomic absorption has often been used to determine total platinum adduct levels in both in vitro and in vivo studies. ICP-MS, which like atomic absorption measures total platinum, has been reported to be more sensitive than atomic absorption (*19*). The oligonucleotide used for the synthesis of the internal standard was designed to form only the CP-d(GpG) adduct, unlike in vivo where other adducts such as CP-d(ApG) may also be formed during DNA platination. To validate the concentration of analyte and internal standard used in our assay, ICP-MS was employed. Using normal conditions, the lowest amount of CP-d(GpG) that could be measured was 11.3 pmol or 0.6 ppb Pt, resulting in a relative standard deviation (RSD) of 5.6%. To decrease error, 42.2 pmol or 2.1 ppb Pt was necessary for the RSD to decrease to 1.9%. Thus, we feel that 2.1 ppb represents the lower level for precise determination of CP-d(GpG) by ICP-MS.

A capillary LC-MS/MS method for CP-d(GpG) adduct quantification was previously developed in our laboratory, which

utilized an internal standard with a chemical structure close to, but not identical to, that of the CP-d(GpG) adduct. While it allowed the use of 10-fold less DNA than atomic absorption, the LOQ for this method was 3 pmol.[3] Therefore, sensitivity needed to be improved 20−25-fold to determine the levels of platinum adducts in cell culture at physiologically relevant doses of cisplatin. Our current method has increased sensitivity by 1000-fold with a LOQ of 3 fmol. The use of UPLC-MS/MS in the SRM mode, coupled with the utilization of a chemically identical stable isotope internal standard, provides a higher level of specificity and accuracy of quantification than previous methods.

Quantification of CP-d(GpG) in Vitro and in Vivo. The method was applied to measure the formation of CP-d(GpG) in treated ctDNA, as well as the accumulation of CP-d(GpG) adducts in ovarian carcinoma cell lines (A2780, CP70) and in mice. In treated calf thymus samples diluted with blank ctDNA, a linear response was observed as expected. Consequently, a study using an in vitro cell culture model allowed further insight as to whether this method could be used to determine CP-d(GpG) adducts at biologically relevant concentrations of cisplatin.

The cisplatin sensitive A2780 and resistant CP70 cell lines had been shown to have a 2-fold difference in adduct formation, but this determination was obtained when treated with >250 μM cisplatin (*27*), because previous methods were unable to measure specific platinum adducts at more biologically relevant levels of cisplatin and/or doses that allowed cells to continue to proliferate. Investigators have commonly used adduct data obtained using toxic, but not necessarily pharmacologically relevant, doses of cisplatin and have assumed a linear relationship when making their conclusions. The present UPLC-MS/MS method allowed analysis of CP-d(GpG) at more pharmacologically relevant doses and has shown that the dose−response relationship remains linear at these lower doses of cisplatin.

The studies were then extended to the analysis of CP-d(GpG) adduct levels in C57/BL6 mice that had received i.p. injections of 7 mg/kg, which is ~21 mg/m^2 cisplatin. Histopathology of the kidney, liver, and colon showed treatment-related effects, and the method was able to quantify CP-d(GpG) adducts formed in those tissues on days 1 and 3. Reed et al. reported up to 0.248 fmol per μg cisplatin intrastrand adducts [CP-d(GpG) and CP-d(ApG)] in blood samples obtained from testicular cancer patients that had been treated with 40 mg/M^2 cisplatin for 5 days (*28*). If we assume that 75% of these adducts are CP-d(GpG) based upon the ratios of total cisplatin DNA adducts as reported by Fichtinger-Schepman, we would be able to quantify most of these adducts using our method (*8*). More recently, Brouwers et al. used ICP-MS to determine platinum levels in DNA extracts of peripheral blood cells from gastric cancer patients treated with 60 mg/m^2 cisplatin during a 4 h infusion. They were able to detect 0.182−16.6 fmol platinum per μg DNA (*19, 29*). When measuring these same samples using ^{32}P-postlabeling, they found 0.161−14.1 fmol CP-d(GpG) and 0−1.78 fmol CP-d(ApG) adducts per μg DNA. Together, these data would suggest that our method would be able to quantify adducts in tissues obtained from patients undergoing cisplatin-based chemotherapy. Recently, several groups have been using systems biology and functional genomics to gain information about interindividual differences in drug toxicity (*30*−*32*). Studies are currently underway to link our DNA adduct results in mice with phenotypic anchoring of genomic data across multiple strains of mice.

[3] Unpublished data.

UPLC-MS/MS Method. Cisplatin is an effective treatment for cancer; however, because of neuropathy and gastrointestinal and renal toxicity, research has been performed to make less toxic analogues. One analogue, carboplatin, has more tolerable toxicity and has been shown to be comparable therapeutically in some cancers, while cisplatin was therapeutically superior in others (*33*). Because of the decreased toxicities, carboplatin is currently more commonly used in chemotherapeutic regimes. Both cisplatin and carboplatin bind to DNA and ultimately form chemically identical DNA cross-links. Therefore, this assay can be used to detect the 1,2 guanine−guanine adducts formed by either compound.

The 1,2 intrastrand guanine−guanine and adenine−guanine adducts comprise over 90% of the total adducts formed by cisplatin exposure (*8, 34*). This method can also be expanded to include quantification of the 1,2 adenine−guanine adduct. However, this method is not suitable for the measurement of all cisplatin DNA adducts. For instance, the 1,3 intrastrand guanine−guanine adduct and the interstrand guanine−guanine adduct once separated from the DNA backbone cannot be differentiated by mass spectrometry; therefore, a different methodology would be needed for those adducts.

In summary, the presented UPLC-MS/MS method has a LOQ of 0.12 fmol CP-d(GpG) per μg DNA or 3.7 adducts per 10^8 nucleotides. This level of sensitivity approaches or equals that of ^{32}P-postlabeling methods (*13, 35*). We have shown that we are able to measure adducts formed in vitro and in vivo at doses of cisplatin that are more relevant for use during biological studies of cisplatin instead of high dose toxicity studies previously required for adduct quantification. As mentioned in the introduction, there have been several other methods developed to measure platinum−DNA adducts. None of the previously published methods included an internal standard. During our studies, we utilized 500 fmol of IS based on our initial method development and cell culture studies, at which time in vivo adduct values were not certain. In future clinical studies, lower IS amounts could be used. Our internal standard has an identical chemical structure to our analyte of interest and differs by 10 mass units because of the stable isotope labeling of the guanines. The use of an internal standard is necessary to correct for error that can be caused during sample preparation or due to differences in equipment sensitivity between runs. Our method is the first to use a stable isotope-labeled internal standard for mass spectrometric quantification of cisplatin−DNA adducts.

Acknowledgment. We acknowledge financial support from NIH Grants R42-ES011746, T32-ES07126, P30-ES10126, R01-CA84480, and U54-CA119343. We thank the Roswell Park Pharmacokinetics/Pharmacodynamics Core Facility for platinum measurements with Atomic Absorption Spectrophotometry. We also thank Patricia Upton for her editorial assistance. We are also thankful to Silantes for their rapid custom synthesis of the ^{15}N-labeled oligonucleotide.

References

(1) Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J., and Sancar, A. (1994) HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc. Natl. Acad. Sci. U.S.A. 91*, 10394–10398.

(2) Reardon, J. T., Vaisman, A., Chaney, S. G., and Sancar, A. (1999) Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. *Cancer Res. 59*, 3968–3971.

(3) Zamble, D. B., Mu, D., Reardon, J. T., Sancar, A., and Lippard, S. J. (1996) Repair of cisplatin−DNA adducts by the mammalian excision nuclease. *Biochemistry 35*, 10004–10013.

(4) Dzagnidze, A., Katsarava, Z., Makhalova, J., Liedert, B., Yoon, M. S., Kaube, H., Limmroth, V., and Thomale, J. (2007) Repair capacity for platinum-DNA adducts determines the severity of cisplatin-induced peripheral neuropathy. *J. Neurosci. 27*, 9451–9457.
(5) Eastman, A. (1987) The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol. Ther. 34*, 155–166.
(6) Eastman, A. (1982) Separation and characterization of products resulting from the reaction of *cis*-diamminedichloroplatinum(II) with deoxyribonucleosides. *Biochemistry 21*, 6732–6736.
(7) Eastman, A. (1983) Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)platinum(II). *Biochemistry 22*, 3927–3933.
(8) Fichtinger-Schepman, A. M., Vendrik, C. P., Dijk-Knijnenburg, W. C., de Jong, W. H., van der Minnen, A. C., Claessen, A. M., van der Velde-Visser, S. D., de Groot, G., Wubs, K. L., and Steerenberg, P. A. (1989) Platinum concentrations and DNA adduct levels in tumors and organs of cisplatin-treated LOU/M rats inoculated with cisplatin-sensitive or -resistant immunoglobulin M immunocytoma. *Cancer Res. 49*, 2862–2867.
(9) Terheggen, P. M., Floot, B. G., Lempers, E. L., van Tellingen, O., Begg, A. C., and den Engelse, L. (1991) Antibodies against cisplatin-modified DNA and cisplatin-modified (di)nucleotides. *Cancer Chemother. Pharmacol. 28*, 185–191.
(10) Tilby, M. J., Johnson, C., Knox, R. J., Cordell, J., Roberts, J. J., and Dean, C. J. (1991) Sensitive detection of DNA modifications induced by cisplatin and carboplatin in vitro and in vivo using a monoclonal antibody. *Cancer Res. 51*, 123–129.
(11) Liedert, B., Pluim, D., Schellens, J., and Thomale, J. (2006) Adduct-specific monoclonal antibodies for the measurement of cisplatin-induced DNA lesions in individual cell nuclei. *Nucleic Acids Res. 34*, e47.
(12) Blommaert, F. A., and Saris, C. P. (1995) Detection of platinum-DNA adducts by ^{32}P-postlabelling. *Nucleic Acids Res. 23*, 1300–1306.
(13) Pluim, D., Maliepaard, M., van Waardenburg, R. C., Beijnen, J. H., and Schellens, J. H. (1999) ^{32}P-postlabeling assay for the quantification of the major platinum-DNA adducts. *Anal. Biochem. 275*, 30–38.
(14) Bassett, E., King, N. M., Bryant, M. F., Hector, S., Pendyala, L., Chaney, S. G., and Cordeiro-Stone, M. (2004) The role of DNA polymerase eta in translesion synthesis past platinum-DNA adducts in human fibroblasts. *Cancer Res. 64*, 6469–6475.
(15) Gupta-Burt, S., Shamkhani, H., Reed, E., Tarone, R. E., Allegra, C. J., Pai, L. H., and Poirier, M. C. (1993) Relationship between patient response in ovarian and breast cancer and platinum drug-DNA adduct formation. *Cancer Epidemiol. Biomarkers Prev. 2*, 229–234.
(16) Poirier, M. C., Reed, E., Shamkhani, H., Tarone, R. E., and Gupta-Burt, S. (1993) Platinum drug-DNA interactions in human tissues measured by cisplatin-DNA enzyme-linked immunosorbent assay and atomic absorbance spectroscopy. *Environ. Health Perspect. 99*, 149–154.
(17) Reed, E., Ozols, R. F., Tarone, R., Yuspa, S. H., and Poirier, M. C. (1987) Platinum-DNA adducts in leukocyte DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. *Proc. Natl. Acad. Sci. U.S.A. 84*, 5024–5028.
(18) Shamkhani, H., Anderson, L. M., Henderson, C. E., Moskal, T. J., Runowicz, C. D., Dove, L. F., Jones, A. B., Chaney, S. G., Rice, J. M., and Poirier, M. C. (1994) DNA adducts in human and patas monkey maternal and fetal tissues induced by platinum drug chemotherapy. *Reprod. Toxicol. 8*, 207–216.
(19) Brouwers, E. E., Tibben, M. M., Pluim, D., Rosing, H., Boot, H., Cats, A., Schellens, J. H., and Beijnen, J. H. (2008) Inductively coupled plasma mass spectrometric analysis of the total amount of platinum in DNA extracts from peripheral blood mononuclear cells and tissue from patients treated with cisplatin. *Anal. Bioanal. Chem. 391*, 577–585.
(20) Garcia, S. D., Montes-Bayon, M., Aguado, O. L., Blanco-Gonzalez, E., Sierra, L. M., and Sanz-Medel, A. (2008) In vivo detection of DNA adducts induced by cisplatin using capillary HPLC-ICP-MS and their correlation with genotoxic damage in *Drosophila melanogaster*. *Anal. Bioanal. Chem. 390*, 37–44.
(21) Iijima, H., Patrzyc, H. B., Dawidzik, J. B., Budzinski, E. E., Cheng, H. C., Freund, H. G., and Box, H. C. (2004) Measurement of DNA adducts in cells exposed to cisplatin. *Anal. Biochem. 333*, 65–71.
(22) Le Pla, R. C., Ritchie, K. J., Henderson, C. J., Wolf, C. R., Harrington, C. F., and Farmer, P. B. (2007) Development of a liquid chromatography-electrospray ionization tandem mass spectrometry method for detecting oxaliplatin-DNA intrastrand cross-links in biological samples. *Chem. Res. Toxicol. 20*, 1177–1182.
(23) Fasman, G. D. (1975) *Handbook of Biochemistry and Molecular Biology*, CRC Press, Cleveland, OH.
(24) Cantor, C. R., Warshaw, M. M., and Shapiro, H. (1970) Oligonucleotide interactions. III. Circular dichroism studies of the conformation of deoxyoligonucleotides. *Biopolymers* 1059–1077.
(25) Eastman, A. (1986) Reevaluation of interaction of *cis*-dichloro(ethylenediamine)platinum(II) with DNA. *Biochemistry 25*, 3912–3915.
(26) Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., and Ozols, R. F. (1987) Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res. 47*, 414–418.
(27) Parker, R. J., Eastman, A., Bostick-Bruton, F., and Reed, E. (1991) Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J. Clin. Invest. 87*, 772–777.
(28) Reed, E., Ozols, R. F., Tarone, R., Yuspa, S. H., and Poirier, M. C. (1988) The measurement of cisplatin-DNA adduct levels in testicular cancer patients. *Carcinogenesis 9*, 1909–1911.
(29) Banger, K. K., Lock, E. A., and Reed, C. J. (1996) Regulation of rat olfactory glutathione *S*-transferase expression. Investigation of sex differences, induction, and ontogenesis. *Biochem. Pharmacol. 52*, 801–808.
(30) Roberts, R. B., Arteaga, C. L., and Threadgill, D. W. (2004) Modeling the cancer patient with genetically engineered mice: Prediction of toxicity from molecule-targeted therapies. *Cancer Cell 5*, 115–120.
(31) Chesler, E. J., Miller, D. R., Branstetter, L. R., Galloway, L. D., Jackson, B. L., Philip, V. M., Voy, B. H., Culiat, C. T., Threadgill, D. W., Williams, R. W., Churchill, G. A., Johnson, D. K., and Manly, K. F. (2008) The Collaborative Cross at Oak Ridge National Laboratory: Developing a powerful resource for systems genetics. *Mamm. Genome 19*, 382–389.
(32) Harrill, A. H., and Rusyn, I. (2008) Systems biology and functional genomics approaches for the identification of cellular responses to drug toxicity. *Expert Opin. Drug Metab. Toxicol. 4*, 1379–1389.
(33) Lokich, J., and Anderson, N. (1998) Carboplatin versus cisplatin in solid tumors: An analysis of the literature. *Ann. Oncol. 9*, 13–21.
(34) Eastman, A. (1987) The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol. Ther. 34*, 155–166.
(35) Blommaert, F. A., Dijk-Knijnenburg, H. C., Dijt, F. J., den Engelse, L., Baan, R. A., Berends, F., and Fichtinger-Schepman, A. M. (1995) Formation of DNA adducts by the anticancer drug carboplatin: Different nucleotide sequence preferences in vitro and in cells. *Biochemistry 34*, 8474–8480.

TX800481J