

MOLECULAR DOSIMETRY OF 1,2 GUANINE-GUANINE INTRASTRAND CROSS
LINKS OF CISPLATIN BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY
TANDEM MASS SPECTROMETRY

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ABSTRACT

IRENE M. BASKERVILLE-ABRAHAM: Molecular Dosimetry of 1,2 Guanine-Guanine Intrastrand Cross Links of Cisplatin by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry
(Under the direction of James A. Swenberg and Stephen G. Chaney)

Cisplatin has been extensively studied as an antitumor agent since the late 1960s. However the mode of action for the efficacy and adverse effects of cisplatin are poorly understood. It was previously believed that the cisplatin1,2 intrastrand guanine-guanine [CP-d(GpG)] cross link was likely responsible for much of the cytotoxic actions of the compound. But current techniques prevented accurate and specific adduct quantification using pharmacologically relevant concentrations of cisplatin. Therefore, the development of a highly sensitive and specific method to measure the CP-d(GpG) cross link was begun. Using this technique, this dissertation aimed to study the role of CP-d(GpG) in acquired resistance and different genetic profiles.

The developed mass spectrometry method is able to measure 3.7 adducts per 10^8 nucleotides using 25 μg of DNA per injection. Preliminary results indicated that the method was sensitive enough to quantify adducts in ovarian carcinoma cells using as little as 12.5 μM cisplatin. It was also able to quantify adducts the kidney, liver and colon tissues of mice that had been

given 7 mg/kg cisplatin by i.p. injection. Our hypothesis was that the density of CP-d(GpG) cross links would serve as a useful biomarker for efficacy and/or toxicity of cisplatin. Research was conducted to understand CP-d(GpG) formation in ovarian carcinoma cell lines as well as in 8 inbred strains of mice.

Results indicate that the dose response relationship for adduct formation in our isogenic cisplatin sensitive and resistant cell lines remains linear, when using lower more pharmacologically relevant doses of cisplatin. In mice, adducts were most concentrated in the kidney. Of the 8 inbred strains tested the C57BL/6J mice were the most sensitive and FVB/NJ least sensitive to cisplatin treatment. Toxicity, as determined by histopathology, did not correlate with CP-d(GpG) molecular dosimetry. However, this lack of correlation may be due to the design of the mouse study, as such many suggestions for future animal studies are given. Based on reported concentrations of platinum DNA adducts clinical samples, the sensitivity and specificity of our method could provide additional insight as to the role of CP-d(GpG) adduct formation in cancer patients being treated with cisplatin.

To God and my family

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Shortly after graduation from Virginia State University, a fellow alumna and I were interviewed for a publication. We were one of 15 people in the US accepted into the Interdisciplinary Biomedical Sciences Program at the University of North Carolina at Chapel Hill. A feat that was very unlikely for one let alone two people from our small Historically Black University. When asked how I excelled at VSU and my plan for UNC-CH, I replied, "Persistence is key." Now that we are both alumnae of Carolina, I look back at the statement and realize that while persistence was key, I could not have stayed persistent without a strong support system. My heart felt thanks goes to my family, especially my parents, brother, husband and cousin Renae for keeping me sane and supporting me through the ups and downs of graduate school. Also, I offer gratitude to friends, past and present members of the Swenberg Lab, the UNC-AGEP program and church family at World Overcomers' for loving support, extracurricular fun and many mentoring opportunities. To my committee, thank you for guidance through this process. I also recognize the opportunities given to me by Dr. Sharon Milgram as director of the IBMS program and appreciate her continued confidence in me. Dr. Stephen Chaney, thank you for agreeing to mentor and advise me when I changed my research focus to work with platinum chemotherapeutics. Finally, I thank Dr. James Swenberg for agreeing to serve as my advisor and allowing me the flexibility to pursue a research project that matched changed interests.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAS atomic absorption spectroscopy

AG adenine guanine

ALD1 aldehyde dehydrogenase 1

amol attomole

ANOVA analysis of variance

ApG adenine-phosphate-guanine

BRAC1 breast cancer 1

cis-DDP cisplatin

CP cisplatin

CP-d(GpG) cisplatin 1,2 guanine-guanine intrastrand cross link

ctDNA calf thymus DNA

d(ApG) deoxyadenine-phosphate-deoxyguanine

DFS disease free survival

d(GpG) deoxyguanine-phosphate-deoxyguanine

DNA deoxyribonucleic acid

dRibpdRib deoxyribose-phosphate-deoxyribose

ELISA Enzyme-Linked Immunosorbent Assay

ERCC1 excision repair cross-complementing factor 1

FDA Federal Drug Administration

fmol femtomole

GG guanine guanine

GpG guanine-phosphate-guanine

GST-P1 glutathione S-transferase pi

h hour

HESI heated assisted electrospray ionization

HMG high mobility group

HNSCC head and neck squamous cell carcinoma

HPLC high performance liquid chromatography

ICP-MS inductively coupled mass spectrometry

IgM immunoglobulin M

IS internal standard

i.v. intravenous

LC liquid chromatography

LC-ESI-MS liquid chromatography electrospray ionization mass spectrometry

LOD limit of detection

LOQ limit of quantitation

mg/kg milligram per kilogram

mg/m² milligram per meter squared

mg/mL milligram per milliliter

min minute

mL/min milliliter per minute

mM millimolar

mRNA messenger ribonucleic acid

MS mass spectrometry

m/z mass-to-charge ratio

N7 Nitrogen 7

NER nucleotide excision repair

nM nanomolar

nmol nanomole

NMR nuclear magnetic resonance

NSL no significant lesions

oligo oligonucleotide

OS overall survival

PBS phosphate buffered saline

PCNA Proliferating Cell Nuclear Antigen

ppb parts per billion

Pt platinum

Pt-DNA platinum-DNA adduct

PTFE polytetrafluoroethylene

Q2 quadrupole 2

QTLs quantitative trait loci

RNA ribonucleic acid

RSD relative standard deviation

SCX strong cation exchange

SNPs single nucleotide polymorphisms

SPE solid phase extraction

SRM selective reaction monitoring

$t_{1/2}$ half life

TP transplatin

TpT thymine-phosphate-thymine

TSQ triple stage quadrupole

trans-DDP transplatin

µg microgram

µL microliter

µM micromolar

UPLC ultra performance liquid chromatography

UPLC-HESI-MS/MS ultra performance liquid chromatography heat assisted electrospray ionization tandem mass spectrometry

UV ultraviolet

WBC white blood cell

CHAPTER 1

INTRODUCTION

1.1 Significance

Cis-diamminedichloroplatinum(II) (cisplatin) is a widely used chemotherapeutic agent (Figure 1.1). Cisplatin (CP) is an inorganic compound in which the platinum atom is bound to two amine groups and has two chloride leaving groups. This compound undergoes a non-enzymatic conversion in physiologic solutions to active derivatives via displacement of leaving groups. This leads to the formation of mono, inter- and intra-strand DNA adducts. The formation of these CP-DNA adducts is believed to be necessary for the anticancer activity of platinum drugs. Of DNA adducts formed, the intrastrand cross links are the most prevalent, at over 95% (1,2). These intrastrand cross links are formed at the N7 position of adjacent AGs or GGs (Figure 1.2). The repair of these DNA adducts occurs primarily by the nucleotide excision repair (NER) pathway (3). Replication and transcription are inhibited upon the binding of these agents to DNA. Each compound also forms adducts with glutathione, proteins and other nucleophiles (4-6). The DNA adducts are recognized by both high mobility group (HMG)-domain and damage recognition proteins. The HMG-domain proteins specifically recognize 1, 2 intrastrand DNA cross links of cisplatin adducts (7,8) and inhibit their repair, leading to increased drug efficacy (9).

The mode of action for the efficacy and adverse effects are poorly understood. Current research postulates that the target of these compounds is DNA and the resulting cytotoxicity of these agents causes cell death, via apoptosis or necrosis. If cell death does not occur, unrepaired adducts may lead to mutations, which can initiate secondary cancers. Many studies have been performed regarding platinum resistance and its relationship with genetic polymorphisms, alterations in gene expression and protein levels (10-13). Another aspect that needs to be considered is the idea of repair of adducts versus the tolerance of adducts. Most studies of resistance mechanisms do not make comparisons between cells lines on a per-adduct basis. Therefore, one cannot discriminate between differences due to decreased uptake/increase repair and a real difference in gene expression in response to an equal number of adducts. In the case of tolerance, a reduction in cytotoxicity or increased dose at which a cytotoxic endpoint becomes apparent is observed in comparison to equivalent measurements in the absence of the tolerance mechanism of interest. However, there is not a sensitive and specific method allowing investigators to discriminate between alterations caused by inter-individual differences in gene/protein expression and adduct burden. The development of biomarkers to determine tissue-specific molecular dosimetry during various chemotherapeutic treatments will lead to a more complete understanding of both therapeutic and adverse effects. This will support the refinement of therapeutic regimens and appropriate individualized treatment protocols. Our research provides a sensitive and specific way to quantify these adducts that will enhance pharmacogenomic data that are indexed to the adduct burden achieved in either

tumor tissue or surrogate markers such as circulating lymphocytes. Ultimately this will lead to more efficacious, less toxic individualized targeted therapies, and provide improved indicators of prognosis.

1.2 DNA, the Primary Cellular Target

When entering the body by *i.v.*, the high concentration of chloride ions (100 mM) suppresses hydrolysis and maintains cisplatin in a nonreactive state. The limiting factor for accumulating platinum in cells is its concentration. It was once believed that the uptake of cisplatin was not saturable and was only by passive diffusion. While passive diffusion still plays a role in cisplatin uptake, recent research suggest that carrier mediated transport may occur in some cell types. Once inside the cell, the diminished chloride ion concentration (~20 mM) allows hydrolysis to occur, resulting in an active aquated form, which reacts readily with cellular targets.

Proteins, RNA, and DNA are among the cellular components that react with cisplatin. Studies of the effects of platinum compounds upon these components were performed to gain more insight as to the primary target of cisplatin. One study used HeLa cells in conjunction with a colony forming assay to examine the amount of platinum bound to macromolecules (14). A calculation was performed utilizing a set amount of platinum bound for DNA, RNA and proteins versus the number of platinum atoms bound in the surviving cells. The study showed that 22 platinum atoms were bound per DNA molecule compared to one Pt per 8 mRNA, one per 30 rRNA, one per 1500 tRNA, and one per 1500 protein molecules. Another study

using HeLa cells was performed with ^{195}Pt -radiolabeled cisplatin showing that as little as 1 out of 3×10^5 protein molecules and 1 out of 1000 RNA molecules contained a platinum atom (15). However, in DNA they observed that nine platinum atoms were bound per molecule. This experimental evidence supports the generally accepted belief that DNA is the primary target of cisplatin in cells.

1.3 DNA Adduct Formation

With DNA established as the primary target of cisplatin in cells, investigators next investigated the binding interaction to characterize the adducts formed. Because platinum compounds that are ineffective as therapeutic agents only bind monofunctionally, protein-DNA, intra-, and inter-strand cross links were thought to be important for biological activity because of their bifunctional conformation. To gain more insight, Fitchinger-Schepman performed an enzymatic digestion of salmon sperm DNA followed by chromatographic separation of the products and NMR analysis to identify the major DNA adducts of cisplatin (16). The major products were 1,2-intrastrand cross links involving adjacent bases, with cis- $[\text{Pt}(\text{NH}_3)_2\{\text{d}(\text{GpG})\}]$ or CP-d(GpG) comprising 47-50% of the adducts formed and cis- $[\text{Pt}(\text{NH}_3)_2\{\text{d}(\text{ApG})\}]$ or CP-d(ApG) comprising another 23-28%. Additionally, 8-10% of the digested products contained 1,3-intrastrand cross links involving non-adjacent guanines (CP-GNG) and interstrand adducts, and another 2-3% of the products were due to the monofunctional binding to guanine. In each case, platinum was bound to the N7 atom of purine bases. Eastman performed a similar study using DNA modified by $[\text{}^3\text{H}]\text{-}[\text{Pt}(\text{en})\text{Cl}_2]$, which was believed to have a similar adduct profile

to cisplatin and found 65% CP-d(GpG), 25% CP-d(ApG), and 6% CP-GNG adducts (17).

These *in vitro* experiments provided evidence that the 1,2-intrastrand adducts were the major adducts formed by cisplatin. The DNA adduct formation was then examined in cells using immunochemical assays. Some studies have attempted to correlate the level of intrastrand adducts to treatment response. In one such study, lymphocytes taken from cancer patients treated with cisplatin displayed a similar adduct profile to the *in vitro* studies (18).

To aid in the determination of clinical relevance of cisplatin DNA adducts, one should also consider its clinically ineffective isomer, transplatin (TP), which forms different types of DNA cross links. Each compound forms bifunctional DNA adducts that bind to the N7 positions of guanine and adenine. However, due to its stereochemistry, transplatin is unable to form 1,2-intrastrand d(GpG) or d(ApG) adducts. Enzymatic digestion studies of DNA treated with transplatin show the formation of 50% dG-TP-dC, 40% dG-TP-dG, and 10% dG-TP-dA (19). Thus, treatment with transplatin leads to the formation of 1,3-intrastrand and interstrand cross links. Cisplatin binding to DNA is kinetically controlled. As mentioned previously, a chloride ligand hydrolyzes when cisplatin enters cells, forming cis- $[\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{H}_2\text{O})]^+$ or monoaquated cisplatin. This hydrolysis reaction is the rate limiting step for DNA binding, with a half life of ~2h. Monoaquated cisplatin then binds to an N7 atom of a guanine base, which displaces the water molecule ($t_{1/2} \sim 0.1$ h), forming a monofunctional adduct. Closure of the monofunctional adduct to form a bifunctional adduct involves hydrolysis of the second chloride ligand, with a half life

of ~2h. If we once again compare cisplatin to transplatin, we find that the first hydrolysis step and binding to DNA to form monofunctional adducts occurs at a similar rate. Therefore, a different rate of closure to form the bifunctional adduct by hydrolysis of the 2nd chloride ligand taken together with its inability to form 1,2 intrastrand cross links, may explain the difference in adduct formation and therefore biological activity between these isomers. Studies on this subject are at odds, some suggest rates to be similar ($t_{1/2} \sim 3$ h) (20), while others argue that transplatin forms bifunctional adduct much more slowly ($t_{1/2} > 24$ h) (21). Differences in study design, such as the length of DNA duplex and the concentration of platinum adducts, can affect the rate of closure. Overall, this implies that the slow formation of interstrand and 1,3 intrastrand cross links combined with the inability of transplatin to form 1,2-intrastrand cross links, play a large role in its difference in clinical efficacy.

The processes just described suggest that the 1,2 intrastrand adducts of cisplatin may be important to its anticancer activity. These major adducts are formed both *in vitro* and *in vivo*. Furthermore, the clinically ineffective transplatin cannot form these cross links. Also previously mentioned was the positive correlation of the level of these cisplatin adducts to treatment response. Numerous studies have shown CP-d(GpG) as the most prevalent cisplatin DNA adduct. Therefore, our studies have focused on developing a method to quantify the 1,2 guanine-guanine DNA adduct of cisplatin to aid in the determination of the role it may play in the cisplatin anticancer mechanism.

1.4 Rationale for Development of New Mass Spectrometric Method

Our research is based upon the premise that DNA is the intracellular target of cisplatin chemotherapy. Therefore the formation of 1,2- intrastrand DNA adducts is an important component of the mode of action. The development of a specific and sensitive mass spectrometric method will provide crucial molecular dosimetry data and allow better understanding of the mode of action for these compounds. Studies have attempted to evaluate the role of Pt-DNA adducts *in vivo*; however, the sensitivity and specificity of the methods limited the quality of data obtained. Common methods used to measure Pt-DNA adducts include antibody probes, ³²P post labeling, atomic absorption spectroscopy, inductively coupled plasma mass spectrometry, and electrospray ionization mass spectrometry.

1.4.1 Antibody Probe Based Methods

There are several assays utilizing antibody probes to determine the level of platinum adducts. However these assays are often cross reactive and have a non-linear dose response, making accurate quantitation of specific adducts difficult. The first immunoassay for the detection of cisplatin adducts was a radioimmunoassay (22). However experiments with carcinogen-DNA antisera exhibited a 10- to 100-fold increase in sensitivity when utilized in ELISA, therefore to gain sensitivity, a new study was performed using an antibody elicited against cisplatin modified DNA in an ELISA inhibition assay (23-25). In this study, a polyclonal antibody was used to detect total cisplatin adducts formed *in vivo* and cited a lower limit of detection (LLOD) of 0.2-0.3 fmol total platinum adducts/μg DNA. However, once the authors

compared their data to similar studies performed with lower concentrations of cisplatin and atomic absorption spectroscopy (AAS) to measure total Pt, they postulated that their antibody may not detect all of the platinum associated with DNA *in vivo*. This was due to the ELISA assay showing lower adduct levels (0.27 and 10 fmol/ μ g DNA) as compared to AAS (10-45 fmol of Pt per μ g DNA). Later, a comparison by Fichtinger-Schepman of two different ELISA assays to determine cisplatin-DNA adducts in the blood cells of cisplatin-treated cancer patients would show more shortcomings of the Poirier designed antibody (26). While Poirier et al had determined the adducts in native DNA with an antiserum raised against highly modified cisplatin DNA, Fichtinger-Schepman et al. assayed the various adducts after chromatography of enzymatically digested DNA samples using antibodies raised against synthetic haptens mimicking the Pt-containing digestion products (25,26). When identical human samples were analyzed by both methods, 14-300-fold higher adduct levels were found with the Fichtinger-Schepman method and AAS further confirmed the results of the Fichtinger-Schepman assay (26). As mentioned previously, AAS showed a difference of up to a factor of 1000 in the original Poirier study, further emphasizing the importance of carefully selecting an appropriate antibody. Furthermore, when Fichtinger-Schepman used the Poirier method to analyze samples modified to a level similar to that of cisplatin-treated patients, no adducts could be detected. Next, a monoclonal antibody to detect DNA modification by cisplatin and carboplatin *in vivo* and *in vitro* was published in 1991 (27). This assay cited quantitation of cisplatin-induced DNA adducts at 3 nmol Pt/g DNA or 1 pt adduct per 10^6 bases when using ICR4 (the monoclonal antibody) in a competitive

ELISA assay. This method could detect below this level, but cross-reactivity of unmodified DNA sequences complicated quantitation. Terheggen et al attempted to design antibodies (NKI-A68, A10, A39) which detect specific cis-DDP DNA adducts (CP-d(ApG), CP-d(GpG) and CP-dGMP), as well as one against total cisplatin modified DNA (NKI-A59) to aid the studies of binding of cisplatin to cellular DNA (28). The antibodies for individual adducts were deemed suitable for platinum-DNA adduct analysis of digested DNA in ELISA assays. However, the cross reactivity of these antibodies prevent specific quantitation of any one of these adducts. Several other investigator developed monoclonal and polyclonal antibodies in an attempt to gain more sensitivity and specificity (29-31). As technology changed, investigators moved from using their antibodies in comparative ELISA to techniques involving microscopy (32). One group was able to design an antibody with high sensitivity to cisplatin-DNA adduct involving adjacent guanine residues, however immuno-recognition was influenced by the surrounding DNA sequence (33). Most recently, Liedert et al. used monoclonal antibodies (Mab R-C18 and R-B3) to recognize Pt-d(GpG) and Pt-d(ApG) using an immuno-cytological assay. Their assay quantifies drug induced lesion in individual cell nuclei (34). While their method may be useful for histological studies, cross reactivity of this antibody still prevents specific quantification of individual cisplatin DNA adducts.

1.4.2 ³²P Postlabeling Based Methods

In 1990 Mustonen and Hemmiki published their application of a ³²P-postlabeling technique to detect DNA adducts formed by cisplatin and other methylating agents

(35). This method did not differentiate between the individual cisplatin adducts and was used *in vitro*. Four years later, Hemmiki and Forsti modified this method to enable the detection of cisplatin adducts in platinated calf thymus DNA (36). Next in 1995, Blommaert and Saris developed a sensitive version of ^{32}P -postlabeling that allowed the detection of the *in vitro* and *in vivo* bifunctional intrastrand crosslinks, Pt-d(ApG) and Pt-d(GpG) of cisplatin and carboplatin (37). This method added strong cation exchange chromatography after the enzymatic digestion of platinated DNA to separate the DNA adducts from unplatinated products. Because the platinated dinucleotides were poor substrates for polynucleotide kinase, the samples were deplatinated with cyanide. After excess cyanide was removed using Sep-pak C18 cartridges, the resulting dinucleotide monophosphates, d(GpG) and d(ApG), were postlabeled. The detection limit of this assay was 1 adduct per 10^7 nucleotides using 10 μg of DNA. This procedure was found to have good correlation with other methods, such as AAS and ELISA, for platinum DNA adduct detection *in vitro* and *in vivo* with both cis- or carboplatin (37). This group then studied the formation of platinum DNA adduct *in vitro* in calf thymus DNA and in cell culture using cisplatin, lobaplatin, and oxaliplatin through comparison of AAS and their new sensitive ^{32}P -postlabeling method (38). They found that cisplatin formed a substantially higher rate of adducts in comparison to lobaplatin and oxaliplatin. However, no conclusions could be reached as to the cytotoxicity of the Pt-d(GpG) and Pt-d(ApG) adducts because their ratios in ovarian carcinoma (A2780) cells were not significantly different for any of the platinum compounds. Welters et al. published an improvement to the Blommaert and Saris method in 1997 (39). Their improvements

included the addition of TpT as an internal standard, which had equally efficient ^{32}P -labeling as GpG and ApG. This internal standard was added immediately after isolation of the Pt-adducts from digested DNA samples. Another improvement was to adjust the pH of the DNA digests to ~ 3 prior to strong cation exchange chromatography to assist in the isolation of the Pt-adduct on the basis of a positive charge. They also decreased the amount of cyanide used for deplatination therefore removing the interference with the labeling step. This method was used to determine adduct levels in cisplatin-treated DNA, DNA from cisplatin-treated cultured cells, tumor xenografts from cisplatin-treated mice, and from white blood cells and tumor tissues from cisplatin-treated patients. Samples with high levels of adducts showed significant correlation with atomic absorption spectroscopy while those with low levels showed correlation with specific antibodies used in an ELISA assay (39). Further improvements by this group increased the sensitivity to 87 and 53 amol per μg DNA (40).

In the late 1990s, several groups started to use this method to determine platinum DNA adduct formation in clinical studies. Because the response to cisplatin therapy was assumed to be related to the formation of platinum DNA adducts, Welters et al. decided to measure platinum adducts prior to therapy using ^{32}P -postlabeling after *ex vivo* cisplatin treatment of head and neck squamous cell carcinoma (HNSCC) xenografts and of tumor biopsies from patients with HNSCC and testicular cancer (41). They observed that higher adduct levels during the one hour exposure to 10 to 80 μM cisplatin were associated with better responses. During the following five hour drug free incubation only adducts in the testicular

cancer samples persisted, which is of interest since platinum therapy is curative for testicular cancer. These results show analysis of DNA adducts following *ex vivo* drug treatment to be one possibility for a predictive assay for patients who may undergo platinum based therapy. Another group used ^{32}P -postlabeling to test the predictive value of cisplatin-DNA adduct levels in HNSCC patients treated with cisplatin and concurrent radiation (42). Adducts were quantified in normal and tumor tissues. Adduct levels were correlated with treatment outcome. Patients with higher GG adduct levels (>median) in primary tumor had significantly better disease free survival (DFS) than patients with lower (< or = median) adduct levels ($p = 0.02$). For overall survival (OS), a non-significant trend was observed; again in favor of patients with higher adduct levels ($p = 0.06$). Therefore in this study, cisplatin-DNA adduct formation in primary tumor appears to be predictive for DFS in HNSCC. Recently, this group used ^{32}P -postlabeling to show the lack of a correlation of formation of cisplatin-DNA adducts between normal (WBC and buccal cells) and tumor (biopsy) tissue (43). This suggests that cisplatin-DNA adducts can be used as a predictive test in anticancer platinum therapy, if the correct tissue is used. It would be of interest to see if normal tissue (other than WBC or buccal cells) could be used effectively for a predictive test. For instance, animal studies could be performed to learn whether tumor and normal tissue from the same organ type form and retain cisplatin-DNA adducts to the same extent or not.

In summary, the ^{32}P -postlabeling shows a high level of sensitivity for CP-d(ApG) and CP-d(GpG) adducts, which has made it useful for determining platinum adduct levels in clinical studies. However, it is a time consuming method and

requires the use of radioactivity. Additionally, there are several possible sources of error in this method. First, the internal standard used in this method is added late in sample preparation and is not platinated or structurally identical to measured adduct. Furthermore, adducts must be deplatinated before labeling, causing possible loss of adducts (37,40,44). Finally, there is no structural confirmation of the platinated adduct, which may lead to over or under estimation of adducts. This method has shown good correlation to atomic absorption spectroscopy and antibody probe based assays, which are both less sensitive than this method.

1.4.3 Methods Measuring Total Platinum

1.4.3.a Atomic Absorption Spectroscopy

Atomic absorption spectroscopy (AAS) has long been used to determine the amount of total platinum in a given sample. A recent PubMed search of cisplatin and atomic absorption spectroscopy resulted in over 300 papers. In 1976, Litterst et al. used AAS to study distribution of a single *i.v.* dose of cisplatin in female beagle dogs (45). Platinum concentration was measured in the plasma, bile, urine, as well as sixteen tissues. This was one of the first *in vivo* studies of pharmacokinetics of cisplatin. The data from this study suggested that cisplatin bound tightly to plasma albumin, was rapidly excreted through the urine, and stayed bound longer in the kidney, liver, ovary, and uterus.

AAS requires a large amount of DNA and/or high concentration of platinum for determination of total platinum and therefore is not sensitive enough for use with most clinical samples (46-50). As mentioned previously, Fitchtinger-Schepman et al.

found the following distribution of cisplatin adducts: CP-d(GpG) 47-50% , CP-d(ApG) 23-28%, CP-GNG 8-10%, and 2-3% were due to monofunctional binding to guanine (16). Calculations using these ratios are used with AAS to obtain estimates of individual adduct formation. Furthermore, the high concentration of platinum sometimes required researchers to assume a linear extrapolation in order to correlate molecular dose and effect in experiments using more biologically relevant concentrations of cisplatin.

Another PubMed search of cisplatin, atomic absorption spectroscopy and adducts led to papers showing the AAS method in comparison to new methods, such as ELISA, ³²P-postlabeling, HPLC-ICP-MS, in which each can measure individual platinum adducts. One example is a paper by Welters et al, in which they were studying the pharmacodynamics of cisplatin in head and neck cancer using AAS and ³²P-postlabeling (51). Sensitivity to cisplatin was correlated with total platinum and CP-DNA adduct levels were determined *in vivo* in xenografted tumor tissues in mice and *in vitro* in cultured tumor cells of HNSCC. They found significant correlations between total platinum levels, measured by AAS, and tumor response to cisplatin therapy *in vivo* and *in vitro*. However, sensitivity of the *in vivo* tumors did not coincide with the corresponding cell lines. Interestingly, a significant correlation was found between the CP-d(ApG) levels and sensitivity to cisplatin both *in vitro* and *in vivo*, using ³²P-postlabeling. These correlations suggest that the CP-d(ApG) adduct is responsible for cytotoxicity in this model system. It would be interesting to revisit this study with our method to see if the observed correlation remains or if it was possibly due to an overestimation of CP-d(ApG) adducts by ³²P-postlabeling.

1.4.3.b Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) measures total platinum with higher sensitivity than AAS, allowing its application to clinical samples. One group has reported an ability to approach the sensitivity of the ^{32}P -postlabeling method using ICP-MS (52). However, they further reported that this level of sensitivity could not be obtained with the addition of HPLC speciation, which is necessary for quantification of the individual adducts. Previously, a capillary HPLC-ICP-MS method was reported to measure CP-d(GpG) adducts specifically; this method reported a limit of quantitation (LOQ) of ~ 1 adduct per 10^6 nucleotides, however it lacked adequate sensitivity to measure samples treated with $<500 \mu\text{M}$ cisplatin (53). Mutagenesis assays using $<10 \mu\text{M}$ cisplatin have been reported in the literature (54). Therefore, greater sensitivity is needed to ensure direct comparisons of the formation and/or persistence of individual cisplatin adducts and the induction of mutations. During the development of the UPLC-HESI-MS-MS method we found that for ICP-MS to be effectively used for trace analysis (< 1 ppb) or measurement of cisplatin adducts (<20 picomoles or <6 per 10^6 nucleosides) a clean room and ultra sensitive mode must be utilized. While both AAS and ICP-MS technologies are useful in screening for DNA adducts, measurements of the distribution of mono CP adducts, inter- or intra- strand CP-cross links would allow the correlation of specific CP adducts with biological effects.

1.4.4 Liquid Chromatography Electrospray Ionization Mass Spectrometry

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) measurement of DNA adducts provides structural confirmation of the analyte during analysis, without extensive labor or use of radioactivity. There are several 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 (55, 56). An internal standard is an important tool for mass spectrometry. Our laboratory uses stable isotope labeled compounds for internal standards. Using stable isotopes allows the use of a standard that has an identical chemical structure to the analyte of interest, the only difference being an increased final mass. We then use our internal standard to add a known amount of adduct prior to each sample work-up and therefore have a control for error or loss in processing. During mass spectrometric quantitation, the internal standard has an identical fragmentation pattern and retention time as the analyte.

In summary, the most recent antibody assay is able to detect CP-d(GpG) or CP-d(ApG) in individual cells, however cross reactivity prevents this from being a specific method for adduct quantification. ^{32}P -postlabeling is very sensitive and can quantify specific platinum DNA adducts with a limit of detection of 1 adduct per 10^8 nucleotides. However its use of radioactivity, lack of internal standard and inability to provide structural conformation limit its usefulness. Atomic absorption spectroscopy measures only total platinum and requires large amounts of DNA and/or a high concentration of platinum for consistent quantification. ICP-MS also measures total platinum and is more sensitive than AAS with a limit of detection of 1

adduct per 10^6 nucleotides. However optimal sensitivity requires the use of a clean room. Finally, previously published LC-MS methods for platinum DNA adducts have not utilized an internal standard. Due to the technical limitations listed for each method, previously obtained data on cisplatin distribution and formation of intrastrand CP cross links *in vivo* may be insufficient to draw valid conclusions. To advance our understanding of the formation and distribution of cisplatin cross links in different organs and within tissues (tumor vs. non-tumor) a stable isotope dilution mass spectrometry method was established for the accurate quantification of cisplatin derived 1,2 intrastrand cross links.

1.5 Potential Role of Platinum-DNA Cross Links upon Acquired Resistance

Currently, the complete mechanism leading to acquired resistance of cisplatin is not known. However, the generally accepted intracellular mechanisms leading to cisplatin acquired resistance include: increased detoxification by thiols (e.g. glutathione, metallothionein), improved repair of and tolerance to nuclear lesions, and increased uptake/decreased efflux (diminished accumulation) of cisplatin. Using our UPLC-MS/MS method to quantitate CP-d(GpG) adducts, we can further study the mechanism related to the improved repair and tolerance to these nuclear lesions.

The ability to measure the density of specific adducts, using doses of cisplatin that are closer to those being used in the clinic will provide stronger rationale during the elucidation of the mode of action for efficacy and resistance. We chose to focus our *in vitro* studies upon ovarian carcinoma resistance. A review by Helm and

States tells us that most women are not diagnosed until the disease has already metastasized from the ovaries and ovarian cancer is associated with an overall 5 year survival of little more than 50% (57). Cisplatin has been the most effective therapy of ovarian cancer for the last 4 decades. Women with intrinsically resistant tumors have a very poor prognosis. Even though the majority of patients initially respond to platinum chemotherapy, many will ultimately develop disease that acquires resistance to cisplatin.

Several models, other than ovarian carcinoma, exist for the study of cisplatin resistance. Therefore in this section, we present selected studies, which use platinum DNA adduct formation as one endpoint. One group treated sensitive and *in vitro* acquired resistance human small-cell lung carcinoma cells lines for 4 hours with 166-500 μM cisplatin (58). They found a correlation between resistance factor and level of glutathione. Also, a dose-related trend was observed for the level of Pt-DNA binding, Pt-GG adduct content and amount of interstrand cross links. Atomic Absorption Spectroscopy was utilized to determine the amount of total platinum, as well as the amount of CP-GG adduct after digestion and separation by Mono Q column, that required 1×10^7 and 5×10^7 cells, respectively, for quantitation. Their overall conclusion was that a glutathione-induced decrease of reactive platinum in resistant cells was responsible for the lower net platination and therefore reduced toxic Pt-DNA adduct formation. In 1996, Johnson et al treated sensitive and resistant human hepatoma cell lines for 4 hours using 0-200 micromolar cisplatin (59). When comparing cisplatin efflux, total Pt-DNA adduct and interstrand cross link formation they found decreased intracellular accumulation to be the major

cisplatin resistance mechanism for hepatoma cells. Vendrik et al studied cisplatin sensitivity and resistance in tumor and kidney tissues from LOU/M rats, in which an IgM immunocytoma cell line was grown (60). These rats were treated intravenously with 1mg/kg cisplatin. After 24 hours they noted a significant decrease in platinum content and in adducts of tumors, but not in the kidney. However, once the results were corrected for dilution due to continued tumor growth after the initial dosing, it was determined that the mechanism of resistance was not likely due to differential CP uptake or efficiency of adduct formation/repair. While the authors, during their investigation excluded several possible mechanisms, they did not suggest a specific mechanism for resistance in their model system. Most recently, Fokkema et al. studied this phenomena using human germ cell and small-cell lung cancer cell lines, which were either sensitive, intrinsically resistant or had acquired resistance (61). In this study, platinum-DNA adducts were measured immunohistochemically using an antibody that recognized CP-d(GpG) and the interstrand cross links. Analysis of the data showed no differences in initial Pt-DNA adduct levels between any of the cell lines. This suggests that the platinum resistance in these cell lines is based on adduct tolerance or increased repair. Looking at all of these studies, one can see why there has been no agreement as to the cause of cisplatin resistance. It may be that the mechanism of resistance is different based on tissue and/or tumor type.

Several studies have shown that expression of genes, such as ERCC1 and BRCA1, which affect the repair of Pt-DNA adducts, is altered in cells with differing resistance to platinum chemotherapeutics (62-66). One such study measured Pt-DNA adducts as well as ERCC1 expression in resistant human carcinoma cell lines

after treating with lactacystin, a selective inhibitor of the ubiquitin pathway and degradation of proteins by the proteasome (67). Treatment with lactacystin increased cisplatin toxicity, enhanced DNA platination and decreased repair of cisplatin-DNA adducts. Also, lactacystin dramatically reduced the steady-state mRNA expression and the rate of transcription of the DNA repair gene ERCC1. This response to lactacystin ultimately shows that ERCC1 and BRCA1 expression is likely to be higher with increased cisplatin resistance. However in each of these studies, atomic absorption was used to measure repair/removal of Pt-DNA adducts. As previously discussed this technique measures total platinum and is not sensitive enough for use at clinically relevant levels of treatment. Therefore, newer techniques would be required to quantify platinum DNA-adduct formation in order to test this hypothesis through clinical studies. Many proteomic studies of platinum resistance in gynecologic cancer have also been performed (68-70). In these cases, correlations of proteins such as ALD1 and PCNA (using mRNA expression) and resistance have been made, but without taking into account Pt-DNA adduct formation or repair. To elucidate the role of Pt-DNA adduct formation in acquired resistance to CP, we have measured Pt-DNA adducts formed at various doses and time points in ovarian carcinoma cell lines selected for their sensitivity (A2780) or cell culture acquired resistance (A2780/CP70) to platinum therapy.

1.6 Potential Role of Platinum-DNA Cross Links upon Genetic Response to Cisplatin

1.6.1 Genetics and Cisplatin

Because the human population is genetically diverse, it is possible for individuals to have different responses to pharmaceutical agents. One such genetic difference is a polymorphism, or multiple alleles of a gene within a population, which can express different phenotypes. In our case, examples of phenotypes could be sensitivity or resistance to cisplatin treatment. Patterns of single nucleotide polymorphisms (SNPs) can be used to identify haplotypes or sets of closely linked genetic markers present on one chromosome, which tend to be inherited together. Haplotype mapping can then be used to find quantitative trait loci (QTLs) or regions of DNA that are associated with a particular phenotypic trait. These QTLs can be used to identify candidate genes, which may be responsible for a phenotype. Through techniques such as QTL mapping and gene expression profiling, pharmacogenomics researchers are studying drug related phenotypes, so that personalized medicine becomes a reality.

SNPs of several genes, such as ERCC1, BRCA and GST-P1, have been suggested to have an effect on the efficacy of cisplatin therapy. In one study, cell lines from people of European or African descent were used to identify genetic variants and gene expression contributing to cisplatin-induced cytotoxicity (71). Using their whole genome approach, they found 17 representative SNPs that contributed to cisplatin-induced cytotoxicity by affecting expression of 26 gene in both populations. Even though SNPs are being identified, QTL mapping has not

been widely done for cisplatin. A literature search yielded only two papers in this area. One used yeast as a model system to study the genetic variation in the cysteine biosynthesis pathway (72). The other paper used lymphoblastoid cells, which they ultimately found to be unsuitable for use in QTL mapping (73). In the latter study, their determination that lymphoblastoid cells are not suitable is of concern because the HapMap project is comprised mostly of this cell type.

1.6.2 Total Body Distribution Animal Studies

Only two total body distribution studies have been identified which use cisplatin. These studies performed with mice or dogs use radiolabeled cisplatin or measured total platinum by atomic absorption spectroscopy and have shown the highest concentration of cisplatin in the kidney (74, 75). This is not surprising because of the nephrotoxicities that have been observed during treatment, as well as the kidney being the major excretory organ for this compound. However, in these studies high amounts of cisplatin were also observed in the liver, even though no significant liver toxicity was reported (74, 75). This may be due to the high concentration of glutathione and other thiols in the liver, which may bind cisplatin as a mechanism of detoxification. There were also measureable levels of platinum found in other tissues, including the gastrointestinal tract.

1.6.3 Clinical Studies---Pharmacogenetics

Variability of individuals in the toxicity and efficacy of chemotherapeutic agents has been observed in the clinic (76-78). Dr. Haider Ali, under the direction of

Dr. David Threadgill, has developed a model to study these phenomena using a panel of 8 strains of inbred mice and 7 chemotherapeutic agents, one of which is cisplatin. Accurate quantification of Pt-DNA adducts is needed to understand the relationship between adduct accumulation and gene expression. As mentioned in the previous section, animal distribution studies show the highest concentration of cisplatin in the kidney, show high levels in the liver and were able to detect some platinum in the gastrointestinal tract. Therefore in our study, we have used liver, colon and kidney of the same murine strains to determine the molecular dosimetry of intrastrand CP-d(GpG) cross links. The ultimate goal being to directly correlate the burden of Pt-d(GpG) adducts in tissues using our assay to the gene expression data previously obtained using the same study design. This comparison will allow the discrimination between species-to-species differences in DNA damage response and in biodistribution of cisplatin.

1.7 OBJECTIVE OF DISSERTATION RESEARCH

Platinum chemotherapeutics are used in the treatment of lymphoma, ovarian carcinoma, breast and colorectal cancer. Cisplatin is an effective anti-cancer agent. However, its use is hindered by adverse effects and the development of resistance. The mode of action for the efficacy and adverse effects are poorly understood. Cisplatin is known to bind to cellular nucleophiles, such as DNA and proteins. Binding to DNA results in the formation of intra- and interstrand cross links, which are repaired to some extent by nucleotide excision repair. The goal of this work is to

study the role of cisplatin-DNA intrastrand cross links in acquired resistance and different genetic profiles.

The 1,2 guanine-guanine intrastrand adduct is the most prevalent adduct formed when DNA is reacted with cisplatin. Many studies have examined the role of cisplatin adducts upon toxicity, however the methods available often measured total platinum instead of directly quantifying individual adducts. Furthermore, these methods required more sensitivity to enable data collection during experiments that utilize moderate to low doses of cisplatin. *Our hypothesis is that the density of intrastrand CP-d(GpG) cross links will serve as a useful biomarker for efficacy and/or toxicity of cisplatin.*

1.7.1 Specific Aims

1. To develop an ultra sensitive and specific mass spectrometry method for characterization and quantification of the cisplatin 1,2 d(GpG) intrastrand adduct.
2. To determine the relationship between acquired resistance and formation and persistence of the CP-d(GpG) adducts using sensitive (A2780) and resistant (CP70) ovarian carcinoma cell lines.
3. To determine the density of CP-d(GpG) adducts in kidneys, livers, and colons obtained from eight strains of inbred mice.

FIGURES

Figure 1.1

Chemical Structure of Cisplatin

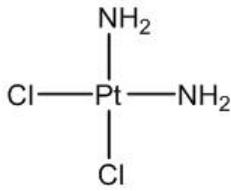


Figure 1.2

Platinum-DNA Cross Links of Cisplatin.

Underlined bases indicate bound cisplatin.

1,2 Intrastrand GG [CP-d(GpG)]

TTAGGTCTCT
AATCCAGAGT

1,2 Intrastrand AG [CP-d(ApG)]

TCTAGTTCTA
AGATCAAGAT

1,3 Intrastrand GG (CP-GNG)

TCTGGCAAC
AGACACGTTG

Interstrand GG (dG-CP-dG)

TTGATCATAT
AACTTGTATA

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CHAPTER 2

DEVELOPMENT OF AN ULTRA PERFORMANCE LC/MS METHOD TO QUANTIFY CISPLATIN 1,2 INTRAstrand GUANINE-GUANINE ADDUCTS

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2.1 Abstract

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 anti-tumor 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 ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay for quantification of 1,2 guanine-guanine

intrastrand cisplatin adducts [CP-d(GpG)], using $^{15}\text{N}_{10}$ CP-d(GpG) as an internal standard, was developed. The internal standard was characterized by MS/MS and its concentration was validated by ICP-MS. 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 (SRM) mode (m/z 412.5 \rightarrow 248.1 for CP-d(GpG); m/z 417.5 \rightarrow 253.1 for [$^{15}\text{N}_{10}$] CP-d(GpG)). 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 C57BL/6J 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.

2.2 Introduction

Platinum chemotherapeutics are used in the treatment of many types of cancer including breast, ovarian carcinoma, colorectal and metastatic cancers. The

first generation of platinum chemotherapeutics, cis-diamminedichloroplatinum II (cisplatin), 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, 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 non-enzymatic 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)] cross links are the most prevalent, comprising ~65% of the adducts formed *in vivo* (5-8). Since 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, ³²P postlabeling, atomic absorption and mass spectrometry. Unfortunately, antibody based assays are prone to false positives due to cross reactivity and have a non-linear responses (9-11) and ³²P postlabeling methods, while able to detect as little as 0.087 fmol adduct per μg DNA, are labor intensive, utilize radioactivity and 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 postlabelling 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 $<500 \mu\text{M}$ cisplatin. (20). Reported in the literature are mutagenesis assays with $<10 \mu\text{M}$ 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.

2.3 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 lab coat) should be worn.*

2.3.1 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 Genra 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 (St. Louis, MO).

2.3.2 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 equivalents of silver nitrate. Solution was then filtered through a 0.2 μm Gelman Acrodisk CR PTFE syringe filter (Fisher), and 10 μL aliquots 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 clean-up was performed using an Agilent 1100 HPLC with a Phenomenex Clarity 3 μ Oligo-RP (100x4.6mm) 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) ($\epsilon_{260}=21,600$) (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.

Inductively coupled plasma mass spectrometry (ICP-MS) was utilized to confirm the concentration of standards that had been estimated from the UV absorbance and extinction coefficient. Samples of 10 to 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 μ C18(2) column (2.0x150 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.

2.3.3 Preparation of ¹⁵N₁₀ 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 ¹⁵N fully labeled oligonucleotide of the same sequence. ¹⁵N labeling was confirmed by mass spectrometry after enzymatic hydrolysis. This ¹⁵N labeled oligo was rehydrated in HPLC water for a final concentration of 0.55 nmol / μ L. To make the internal standard, 60 nM 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 μ g aliquots of platinated oligo, 320 μ L of 50mM sodium acetate/10mM magnesium chloride and 0.02 kunitz unit of DNase I were incubated for 4h at 37 °C, next 2 units of nuclease P1 were

added and incubation continued for 16-20h. Finally 41 μ L 1M Tris-HCl pH 9 and 5 units alkaline phosphatase were added and incubation continued for 4h, 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 concentration was determined by UV and confirmed by ICP-MS, 500 μ L aliquots containing 100 fmol per μ L in buffer A2 were stored at -80° C for use as internal standard (Table 1).

2.3.4 Platination and Preparation of Calf Thymus DNA

Calf thymus DNA (ctDNA) was used to validate the UPLC-MS/MS method. Sigma ctDNA was rehydrated to 1 mg/mL aliquots and stored at -20° C. ctDNA (150 μ g) was reacted with 100 nM aquated cisplatin and brought to a total volume of 1mL in deionized H₂O 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 columns were initially used for sample enrichment instead of HPLC.

Solid phase extraction (SPE) clean-up 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 50 mM HCl. Next, LiChrolut SCX columns (VWR) were placed on a vacuum manifold, conditioned with two additions each of 1 mL water, 1 mL methanol, and 1 mL water, and then equilibrated with 1 mL 50 mM Tris HCl pH 3. The samples were applied to the columns, which were then washed four times with 2 mL 5 mM sodium formate pH 6. Platinated adducts were then

eluted with two additions of 0.5 mL 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 μ L 50 mM HCl. Next, the C18 cartridges were conditioned and equilibrated using two additions of 2 mL water, 2 mL methanol, 2 mL 10 mM ammonium acetate. The pH adjusted sample was then applied to the column, which was washed with three additions of 2 mL 10 mM ammonium acetate. Platinum adducts were eluted with two additions of 0.5 mL 10 mM ammonium acetate in 50% methanol. After solid phase extraction, 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 μ L 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 at a between pH 4-4.5. We achieved this using 10mM 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 strong cation exchange 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 μ Oligo-RP (100x4.6 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 min to 8.85 min. Fractions were dried via centrifugal lyophilization and rehydrated using a mix of 50/50 Buffer A2 and B, placed in MS vials, and once again dried by centrifugal lyophilization. Samples were either stored at -20° C or immediately rehydrated in 40 μ L Buffer A2 for UPLC tandem mass spectrometric analysis.

2.3.5 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 2 amines from CP-d(GpG) (m/z 412.5 to 248.1, z=2) and [¹⁵N₅]-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 x 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).

2.3.6 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 solid phase extraction columns or HPLC and prepared for MS quantification as described in the calf thymus DNA section.

2.3.7 Inductively Coupled Plasma Mass Spectrometry

Aliquots of analyte and/or internal standard using 10 to 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.

2.3.8 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 μ M L-glutamine, insulin (0.25 units/mL), penicillin (100 units/mL), streptomycin (100 μ g/mL), and amphotericin B (0.25 μ g/mL) at 37° C and 5% CO₂.

In both the dose-response and time-course studies, 2 x 10⁶ cells were plated in T175 flasks and allowed to grow for three days. The growth medium was removed and cells were rinsed twice with PBS before serum-free medium plus 12.5-250 μ M cisplatin or PBS was applied. After 1 h 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 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.

2.3.9 Study Conditions for C57BL/6J Mice

Male C57BL/6J 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. 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%), 4= Marked/severe lesions (>50%).

We used the FDA's dose calculator (<http://www.fda.gov/cder/cancer/animalframe.htm>) to better understand how the cisplatin dose in our mouse study (7 mg/kg) relates to a low human dose (50 mg/m²) 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/m². Conversely, a dose of 50 mg/m² would require 0.33 mg or 16.58 mg/kg.

2.3.10 DNA Isolation from Tissues

DNA was extracted using Genra PureGene kit reagents with a significantly modified protocol. Because of 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 (400 mg) or colon tissue (150 mg) were thawed in 6 mL ice-cold phosphate-buffered saline (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 homogenization buffer. The nuclear fraction was collected by centrifugation and was reconstituted in 6 mL 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 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 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 cell lysis solution supplemented with RNase A (0.8 KeU/mL, 27 μ L) to digest RNA. After 30 min incubation at 37° C, another protein precipitation was performed and supernatant collected. DNA was precipitated using 6 mL isopropanol, collected by centrifugation and rinsed with 70% ethanol. The DNA was resuspended in 400 μ L HPLC grade water and its concentration and purity estimated by UV spectrometry. The DNA solution was stored at –80° C until CP-d(GpG) adduct analysis.

2.4 Results

2.4.1 Characterization of the CP-d(GpG) Analyte Standard

The cisplatin 1,2 intrastrand guanine-guanine adduct (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 2.1.

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 (2 major and 3 minor) isotopes aided product identification (Figure 2.1). The MS/MS scans were originally performed in the positive ionization mode using several transitions to

account for the loss of the 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 2.2 shows a representative chromatogram in which we measured both the singlet and doublet charge states. Through the comparison of peak areas, it was determined that MS/MS quantification of the CP-d(GpG) adducts was most 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).

2.4.2 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 2.3 shows a positive MS full scan of the unlabeled internal standard after digestion and SPE clean-up. 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 shares the same optimal fragmentation as the analyte ($[M-dRibpdRib-2NH_2]^+$; internal standard $m/z=417.5$ to 253.1).

2.4.3 Method Accuracy and Precision

A calibration curve was run using various concentrations of analyte standards and constant amount of 500 fmol internal standard. Method precision was first assessed by processing four replicates of known concentrations of analyte standard by solid phase extraction. Intraday precision ($r^2=0.99$) was determined using 0, 5, 10, 50, 100 fmol 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 solution of authentic standard CP-d(GpG). The limit of detection with a signal to noise of >2 was 1 fmol CP-d(GpG) per injection, therefore the limit of quantification (LOQ) was set to be 3 times the limit of detection (3 fmol 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 2.1 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 calf thymus DNA was added to samples containing 100 fmol analyte and 500 fmol 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 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.

2.4.4 Quantification of CP-d(GpG) in Calf Thymus DNA

This method was validated using platinated calf thymus DNA that was diluted with blank calf thymus DNA. Aliquots of 0, 25, 50, and 100 µg platinated calf thymus DNA were placed in eppendorf tubes with 500 fmol internal standard and the corresponding amounts of blank calf thymus DNA was added to bring the total amount per tube to 100 µg. These samples were processed through the hydrolysis method using HPLC clean-up and total adducts were quantified by UPLC-MS/MS. As expected, a linear response was observed (Figure 2.4).

2.4.5 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 two fold difference between the sensitive A2780 and resistant CP70 cell lines, which was in line with previous estimates of relative adduct amounts in those two cell lines (Figure 2.5)(27).

2.4.6 Determination of CP-d(GpG) Adducts in Mouse Tissues

The C57BL/6J mouse strain was used to demonstrate CP-d(GpG) accumulation *in vivo* after an *i.p.* injection with 7 mg/kg cisplatin. Three days post

injection, the greatest amount of adducts were observed in the kidney, followed by the liver and colon (Table 2.2). 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 2.3).

2.5 Discussion

2.5.1 Method Development and Validation

During the development of this method it was necessary to modify the sample clean-up procedure after enzymatic hydrolysis from solid phase extraction to HPLC. SPE was preferred because it allowed for a higher throughput processing of samples, and allowed several types of platinum adducts to be collected simultaneously. However, increased recovery was important when considering our limit of detection 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 clean-up 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 limit of quantification for this method was 3 pmol. Therefore, sensitivity needed to be improved 20- to 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 limit of quantification 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.

2.5.2 Quantification of CP-d(GpG) *in vitro* and *in vivo*

The method was applied to measure the formation of CP-d(GpG) in treated calf thymus DNA, 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 calf thymus DNA, 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 two-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 C57BL/6J mice that had received *i.p.* injections of 7 mg/kg, which is $\sim 21 \text{ mg/m}^2$ 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 microgram cisplatin intrastrand adducts [CP-d(GpG) and CP-d(ApG)] in blood samples obtained from testicular cancer patients which 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/m² cisplatin during a 4 h infusion. They were able to detect 0.182-16.6 fmol platinum per microgram DNA (19,29). When measuring these same samples using ³²P-postlabelling they found 0.161-14.1 fmol CP-d(GpG) and 0-1.78 fmol CP-d(ApG) adducts per microgram 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 inter-individual 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.

2.5.3 UPLC-MS/MS Method

Cisplatin is an effective treatment for cancer, however due to neuropathy, 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 and therefore a different methodology would be needed for those adducts.

In summary, the presented UPLC-MS/MS method has a limit of quantification 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 quantitation. 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 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.

FIGURES

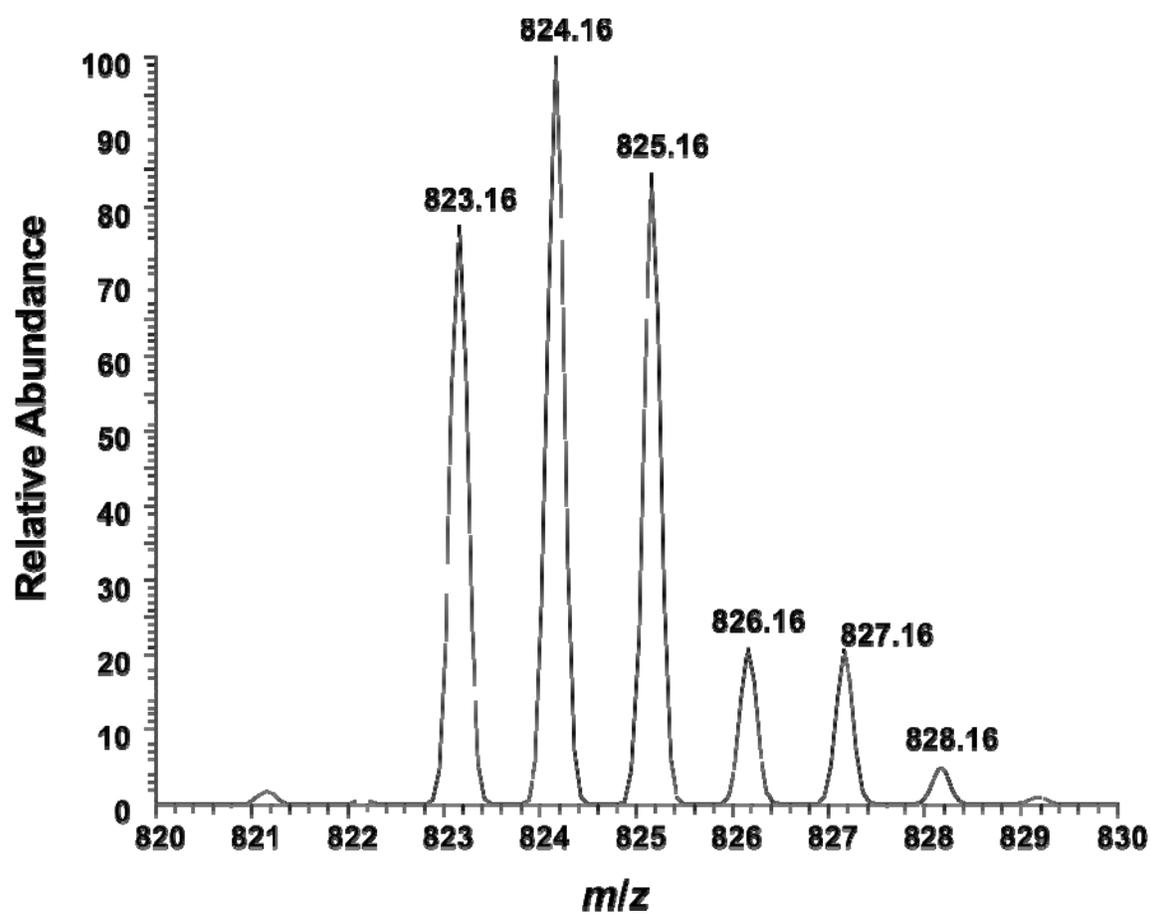


Figure 2.1 MS Isotope Simulation of CP-d(GpG)

There are three major masses for this adduct due to isotopes.

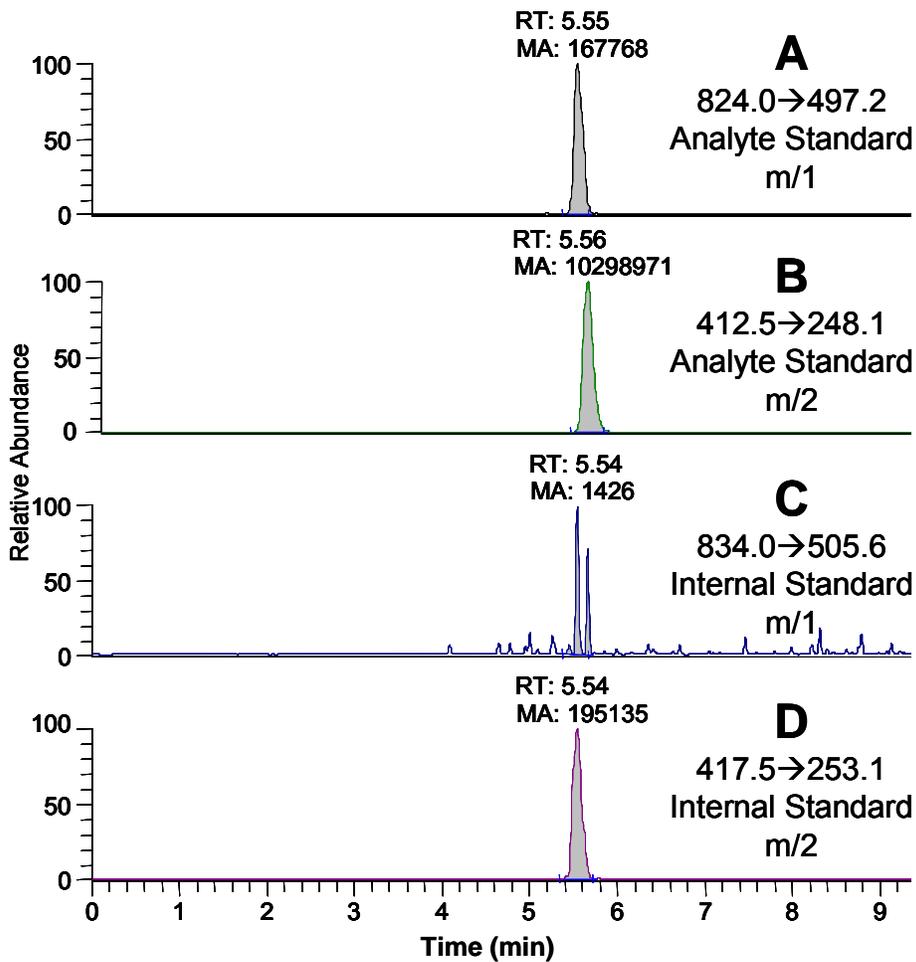


Figure 2.2 Representative SRM Ion Chromatograms comparing the Use of Singly (m/1) vs. Doubly (m/2) Charged State

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). A and C show MS/MS fragmentation in the singly charged state, while B and D show MS/MS fragmentation in the doubly charged state. The chromatogram has been cropped as no other quantifiable peaks are observed.

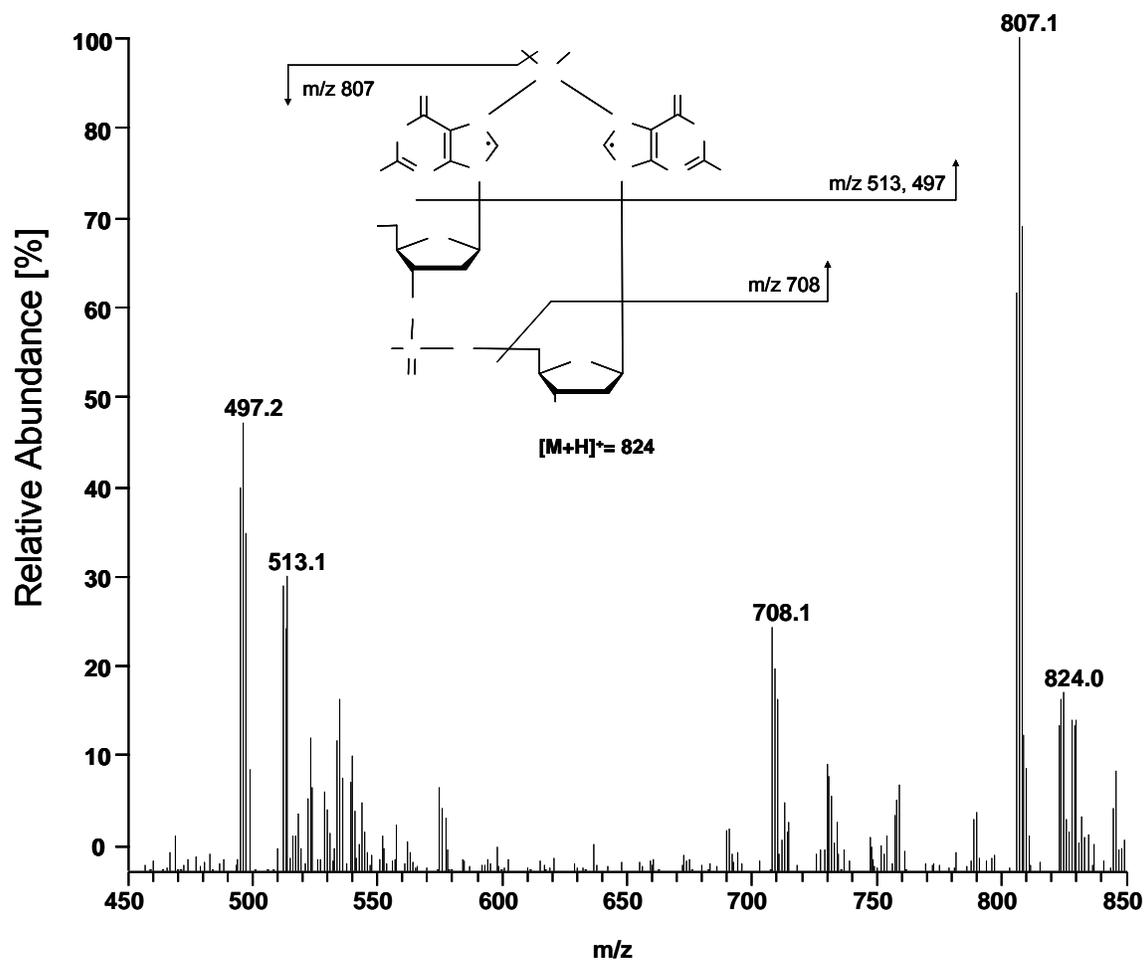


Figure 2.3 Full scan positive ion MS spectrum m/z 450 – 850

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.

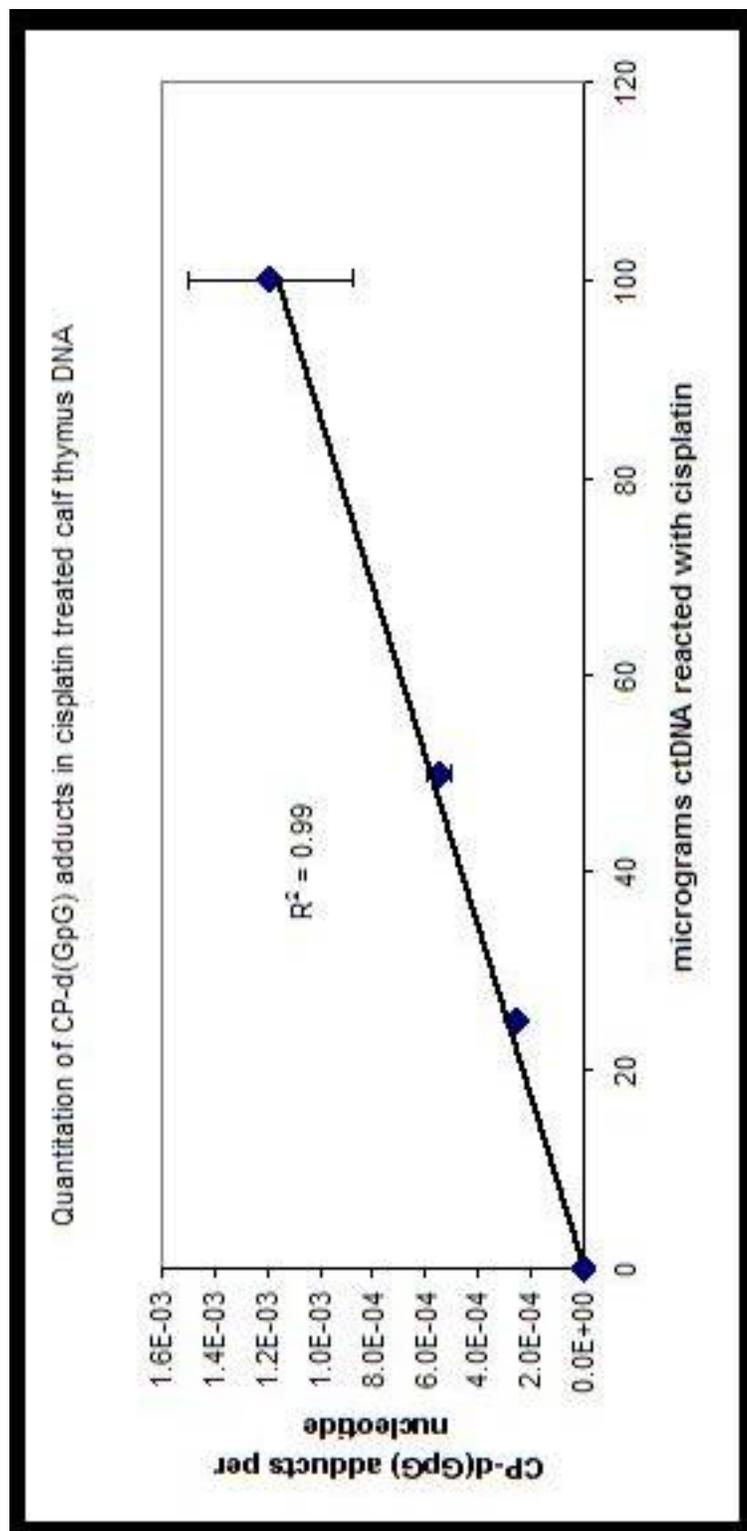


Figure 2.4 Quantification of CP-d(GpG) in platinated ctDNA

DNA was diluted with blank calf thymus DNA. Aliquots of 0, 25, 50, and 100 µg platinated calf thymus DNA were placed in eppendorf tubes with 500 fmol internal standard and the corresponding amount of blank calf thymus DNA was added to bring the total amount per tube to 100 µg. Samples were processed through the hydrolysis method using HPLC clean-up and total adducts were quantified by UPLC-MS/MS.

1,2 Guanine-Guanine Intrastrand Adducts of Ovarian Carcinoma Cells by UPLC-MS/MS

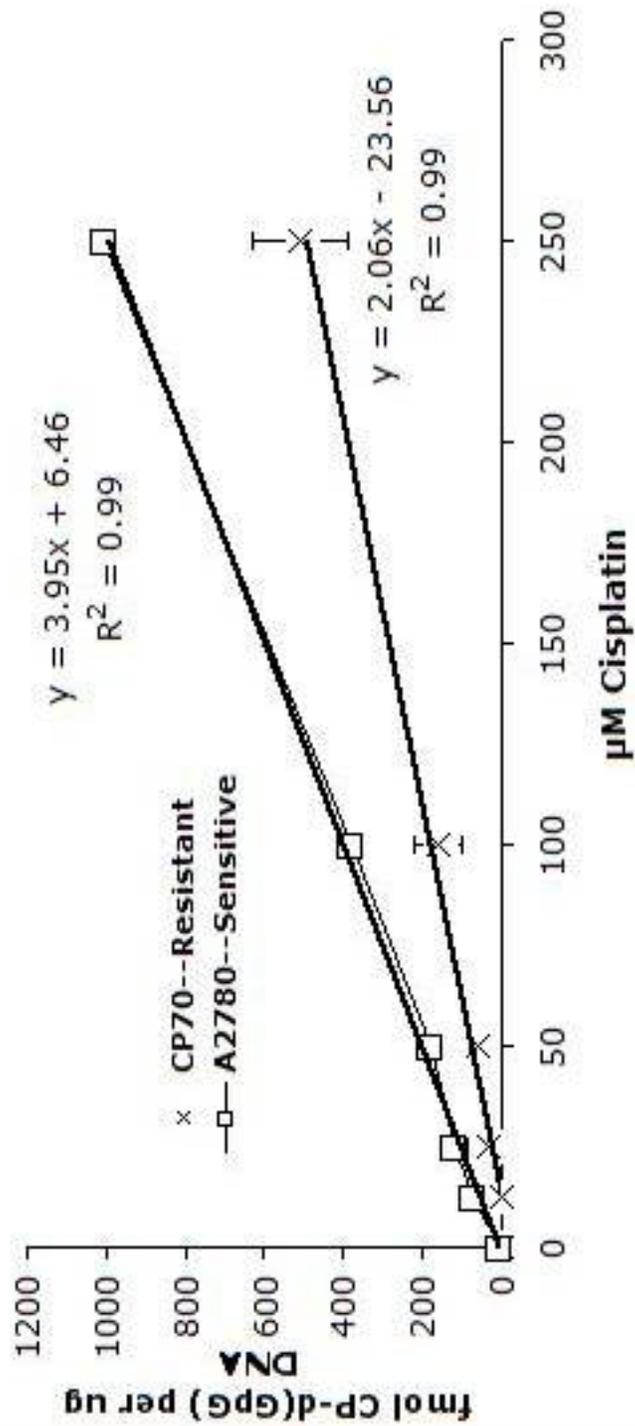
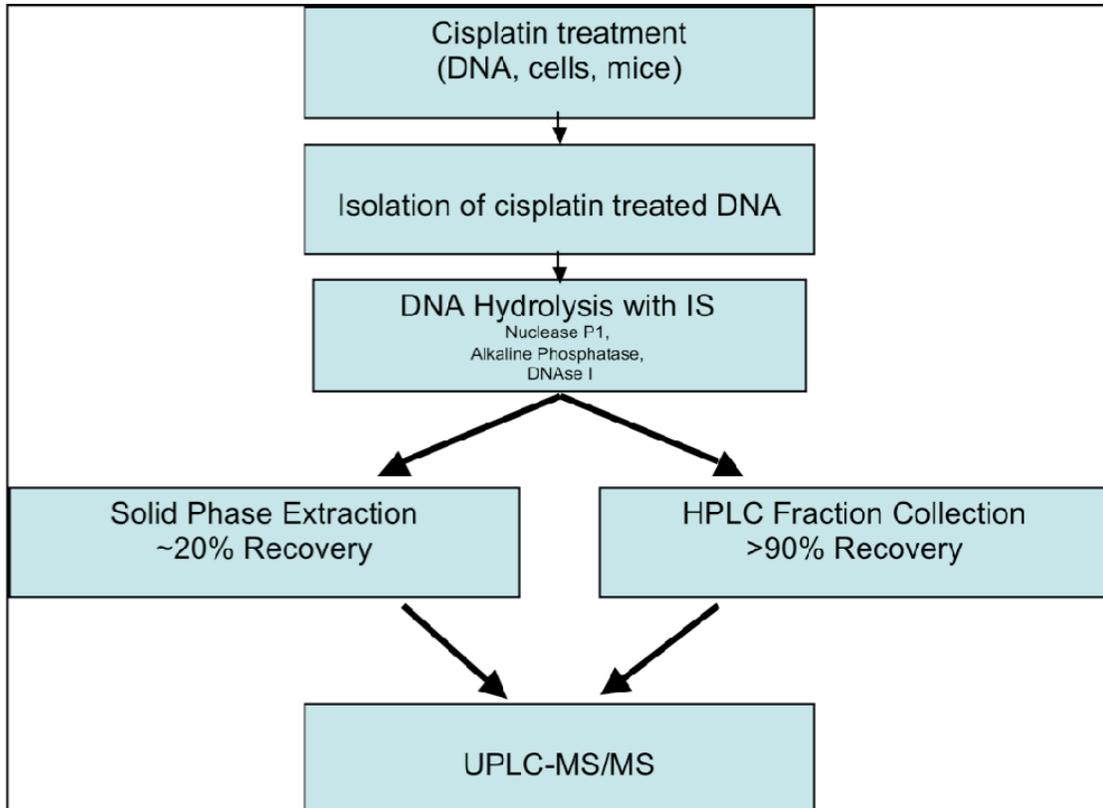


Figure 2.5 Quantification of CP-d(GpG) in human ovarian carcinoma cells.

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.

SCHEMES



Scheme 2.1 Preparation and quantification of CP-d(GpG) adducts by UPLC-MS/MS.

Cisplatin treated DNA is isolated. Next, internal standard is added and enzyme hydrolysis is performed. Following hydrolysis, solid phase extraction 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.

TABLES

Sample type	pmol CP-d(GpG)	ppb Pt	pmol CP-d(GpG)	ICP-MS
	[UV]	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

*n=3

Table 2.1 Validation of Standard Concentrations by UV and ICP-MS.

Samples were prepared in triplicate for ICP-MS in 2% nitric acid based upon initial UV concentrations.

	BL6 1 (fmol/ μ g DNA)	BL6 2 (fmol/ μ g DNA)	BL6 3 (fmol/ μ g DNA)	Average (fmol/ μ g DNA)
Kidney	53	57	67	59 \pm 7
Liver	21	22	16	20 \pm 3
Colon	3	6	5	5 \pm 2

Table 2.2 Quantification of CP-d(GpG) in C57BL/6J mice Three Days post *i.p.* injection of 7mg/kg Cisplatin

	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

0 = absent or no significant lesions

1= minimal lesions

2= mild lesions

3= moderate lesions

4= marked lesions

Table 2.3 Histopathology of C57BL/6J Mice Three Days post *i.p.* injection of 7mg/kg Cisplatin

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CHAPTER 3

STRAIN DIFFERENCES IN TOXICITY AND MOLECULAR DOSIMETRY FOLLOWING CISPLATIN ADMINISTRATION TO MICE

3.1 Introduction

Cisplatin was approved for clinical use in the late 1970s and remains an efficacious chemotherapeutic agent. However, it does not work for every person or for every cancer. Some patients who undergo platinum-based chemotherapy have intrinsic or acquired resistance to treatment. Several of the hypotheses regarding the development of resistance to cisplatin chemotherapy have been discussed in the introduction of this thesis. Our goal for this study was to evaluate strain differences in cisplatin toxicity and molecular dose of cisplatin adducts using the UPLC-MS/MS biomarker developed in Chapter 2. In this way we can use CP-d(GpG) as a biomarker to determine the molecular dose of cisplatin adducts, allowing us to distinguish between strain differences in which genetics affects sensitivity to Pt-DNA adducts from strain-related differences in the pharmacokinetics and pharmacodynamics of cisplatin. Information about CP-d(GpG) adduct burden in specific tissues and strains may be essential for interpretation of gene expression studies aimed at ultimately identifying a phenotypic marker for cisplatin sensitivity. This information coupled with quantitative trait loci mapping and the sequencing of the human genome, could provide valuable information about why some individuals

are more responsive to platinum based chemotherapy and ultimately help researchers to design more individualized therapies for patients.

Because there has been variability in observed toxicity and efficacy of chemotherapeutic agents when used in the clinic, the major goal of pharmacogenomics is to develop individualized therapies for patients. Dr. Haider Sayed Ali, under the direction of Dr. David Threadgill, developed a model to study these phenomena using a panel of 8 strains of inbred mice and 7 chemotherapeutic agents, one of which was cisplatin. Evaluations of gene expression profiling using RNA from the liver and colon of each strain have been completed. The gene expression study did not include an evaluation of the kidney, histopathology or molecular dosimetry endpoints. The UPLC-HESI-MS/MS method to quantitate CP-d(GpG) adducts was applied to DNA from tissues of these eight inbred strains of mice to gain further understanding of the relationships between adduct accumulation and toxicity.

Previous tissue distribution studies used radiolabeled cisplatin or measured total platinum by atomic absorption spectroscopy and have shown the highest concentration of cisplatin in the kidney (1,2). This is not surprising because of the nephrotoxicities that have been observed during treatment in the clinic, as well as the kidney being the major excretory organ for this compound. High amounts of cisplatin were also observed in the liver, even though no significant liver toxicity was reported (1,2). This may be due to the high concentration of glutathione and other thiols in the liver, which may bind cisplatin as a mechanism of detoxification. Anti-neoplastic agents such as cisplatin can also cause toxicity in the gastrointestinal

tract due to the rapid turnover of cells in this region. A few studies have reported toxicity and/or the formation of cisplatin adducts in the intestine after cisplatin treatment (2-4). Intestinal damage can be tracked through the observation of reduction in crypt cell survival as shown in Rebillard et al (4). Measureable concentrations of platinum have also been found in the gastrointestinal tract during body distribution studies in rodents and dogs after cisplatin treatment (1, 2). Due to the presence of platinum or adducts, coupled with the nephro and gastrointestinal related toxicities, we chose to use liver, colon and kidney of the same murine strains to determine the molecular dosimetry of intrastrand CP-d(GpG) cross links. Here we evaluate whether the CP-d(GpG) adducts were a suitable biomarker for toxicity, as determined by histopathology.

3.2 Materials and Methods

3.2.1 Study Conditions

Male inbred mice of eight different strains (FVB/NJ, C3H/HEJ, 129S1/SvImJ, A/J, BALB/CJ, C57BL/6J, BTBR T+ TF/J and DBA/2J ; n=3) were injected *i.p.* with 7 mg/kg cisplatin by Dr. Haider Ali. The use of inbred mice in this study allows each strain to genetically represent one individual. 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. Numerical scale of histology scoring and description was as follows:

0= absent or no significant lesions (NSL), 1= minimal lesions (<5%), 2= mild lesions (5-25%), 3= moderate lesions (25-50%), 4= marked/severe lesions (>50%).

We used the FDA's dose calculator¹ to compare the cisplatin dose in our mouse study (7 mg/kg) to a low human dose (50 mg/m²) 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/m² in humans. Conversely, a dose of 50 mg/m² would require 0.33 mg or 16.58 mg/kg in mice.

3.2.2 DNA Isolation from Tissues

DNA was extracted from frozen kidney (400mg), liver (400mg) or colon tissue (150mg) using Gentra PureGene reagents with a modified protocol, as described in Chapter 2. Because of reduced amount of colon tissue available for processing, all volumes in the procedure were reduced by half when isolating DNA from this tissue. The DNA solution was stored at -80° C until CP-d(GpG) adduct analysis.

3.2.3 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 single reaction monitoring

¹<http://www.fda.gov/cder/cancer/animalframe.htm>

(SRM) mode, monitoring the loss of phosphate, two deoxyriboses and 2 amines from CP-d(GpG) (m/z 412.5 to 248, $z=2$) and [$^{15}\text{N}_5$]-CP-d(GpG) IS (m/z 417.5 to 253, $z=2$). Samples were kept at 4° C during analysis, and the sample injection volume was 20 μL . An HSS T3 (2.1 mm x 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 $\mu\text{L}/\text{min}$. 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).

3.2.4 Quantification

50 μg of platinated murine DNA from each mouse tissue was spiked with 500 fmol of Internal Standard. Next, to separate the adducted nucleotide from the surrounding unmodified nucleosides, the samples underwent an enzymatic hydrolysis based upon the method previously reported by Eastman (5) as described in Chapter 2. The samples were transferred to MS vials, dried by centrifugal lyophilization and rehydrated in 100 μl 10 mM ammonium acetate in 0.1% glacial acetic acid (Buffer A2). CP-d(GpG) was purified from DNA hydrolysate by HPLC

fractionation as described in Chapter 2. Samples were either stored at -20° C or immediately rehydrated in 40 µL Buffer A2 for UPLC tandem mass spectrometric analysis.

3.3 Results

3.3.1 Distribution of CP-d(GpG) Adducts in Eight Inbred Mouse Strains

In two experiments, the trend for distribution of CP-d(GpG) was kidney >liver>colon across all strains (Figures 3.1-3.3). Because the range of overall adduct levels in the kidney of the different strains ranged from 5-60 fmol CP-d(GpG) per ug DNA in the 1st experiment and 8-27 fmol CP-d(GpG) per ug DNA in the 2nd experiment we analyzed the data further to gain a better understanding of the apparent variability. Statistics was performed to identify any significance of CP-d(GpG) molecular dosimetry by strain, tissue or days after dosing. An ANOVA for each experiment showed a strain and tissue related significance for the first experiment (C57BL/6J p=0.0001, DBA/2J p=0.002, kidney p=0.0001, liver p=0.003) and a tissue but no strain related significance for the second experiment (kidney p=0.0001 and liver p=0.03).

Due to small numbers of animals and differences between experiments 1 and 2, limited conclusions can be drawn. In terms of increased or decreased total CP-d(GpG) adducts by tissue and strain for experiment 2 as compared to experiment 1, we see the following trends in Figure 3.3. For C57BL/6J and DBA/2J, the adduct levels were lower in the second experiment for both day 1 and day 3 and for every

organ studied. For FVB/NJ and 129S1/SvImJ, the adduct levels were higher in the colon in the second experiment for both day 1 and 3, while other tissues had no change. While for C3H/HEJ, A/J and BALB/CJ mice the results were mixed.

Next examining the data to determine within an experiment the increase or decrease in CP-d(GpG) from day 1 to 3, we observe the following trends. For FVB/NJ mice, the kidney was found to increase in both experiments. C3H/HEJ mice, had a decrease in liver adducts in the first experiment. A/J mice, had an increase in colon adducts in the second experiment. BALB/CJ mice, experienced a decrease in kidney and liver adducts in the first experiment, while the colon adducts increased in the second experiment. C57BL/6J mice had an increase in kidney adducts in the second experiment. Finally, DBA/2J mice had an increase in colon adducts in the first experiment. Otherwise, there were no observed changes in CP-d(GpG) adducts from day 1 to 3, in either experiment.

It is important to note that the colon specimens from FVB/NJ, C3H/HEJ, 129S1/SvImJ, A/J, and BALB/CJ that were collected with experiment 1 were processed together with the tissues from experiment 2. This knowledge, added to the data summarized above as well as the overall colon tissue concentrations of CP-d(GpG) as shown in Figure 3.3.3 suggests that the variability observed between experiments is likely due to either a change in the dosing solution or mouse colony's treatment of a pinworm infestation during the 1st experiment, not in our methodology for adduct determination as described in Chapter 2.

3.3.2 Histological Observations

Tables 3.1 and 3.2 contain the cumulative histology scores for all strains in each experiment. Figure 3.4 compares histology lesions to concentration of CP-d(GpG) adducts on day three of both experiments, while Figure 3.5 shows histology examples of controls and observed kidney and colon damage caused by cisplatin treatment. The terms and total scores per group used to describe the severity of histological lesions in this section are: 0=no significant lesions (NSL), 1-3=minimal lesions (<5%), 4-6=mild lesions (5-25%), 7-9=moderate lesions (25-50%), 10-12=marked lesions (>50%).

Control animal tissues were only collected for histology during the second experiment. Overall scoring of slides was similar in both experiments, but with stronger kidney effects observed in the 2nd experiment. One exception to this was DBA/2J mice which had less overall damage in the 2nd experiment. However, all strains in both experiments showed evidence of nephrotoxicity on day 3. Another difference in histology between the two experiments was the presence of minimal liver lesions in the C3H/HEJ mice on day 1 and moderate liver lesions due to centrilobular fatty change in the C57BL/6J mice on day three of the first experiment. Otherwise the liver had no lesions associated with treatment in either experiment on either day. Because there was minimal toxicity observed in the liver due to treatment the following paragraphs will be limited to descriptions of the kidney and colon histology.

In the first experiment (Table 3.1), only three strains showed any significant kidney lesions related to treatment 24 hours after dosing. On day one, the

129S1/SvImJ, C57BL/6J had minimal lesions due to tubular vacuolation/degeneration, BTBR T+ TF/J mice had minimal lesions due to apoptosis/necrosis, while DBA/2J mice developed mild kidney lesions due to tubular vacuolation/degeneration. By day three, all strains had developed a minimal to moderate level of kidney lesions due to apoptosis/necrosis with some instances of tubular vacuolation/degeneration and in each case the severity of lesions had increased from the level observed on day 1. Three strains (A/J, C3H/HEJ, FVB/NJ) had minimal apoptotic/necrotic lesions, two (129S1/SvImJ, BALB/CJ) mild apoptotic/necrotic lesions and 3 (C57BL/6J, BTBR T+ TF/J, DBA/2J) moderate apoptotic/necrotic lesions with some tubular vacuolation/degeneration. In experiment two (Table 3.2) histologic changes due to treatment were observed for the DBA/2J mice at a minimal level on day 1 for the kidney due to tubular vacuolation/degeneration, none of the other strains showed any significant kidney lesions on day one. By day three all strains had mild to moderate severity of kidney apoptotic/necrotic lesions, with three strains (129S1/SvImJ, A/J, BALB/CJ) showing minimal tubular mineralization. One strain (129S1/SvImJ) had moderate apoptotic/necrotic lesions with mild tubular vacuolation/degeneration and minimal tubular mineralization, one strain (C57BL/6J) had marked apoptotic/necrotic lesions while all other strains had mild apoptotic/necrotic kidney lesions. Comparing the two experiments to one another in terms of kidney lesions, a lower severity was observed in experiment two on day 1, however on this day in both experiments DBA/2J mice had the greatest damage. If we then compare the two experiments on day three, we see that in experiment one there was less severe kidney damage

overall and that only the strain with the greatest severity of kidney lesions (C57BL/6J) also has the most severe lesions in experiment two. Control kidneys for experiment two showed no significant lesions.

In the colon on day one of the first experiment, four strains (129S1/SvImJ, BALB/CJ, BTBR T+ TF/J, DBA/2J) had minimal apoptotic/necrotic lesions and four strains (A/J, C57BL/6J, C3H/HEJ, FVB/NJ) had mild apoptotic/necrotic lesions. By day three, two strains (A/J, FVB/NJ) remained mild; all others had minimal colon apoptotic/necrotic lesions. On day one of the second experiment, one strain (C57BL/6J) was mild while all others had minimal apoptotic/necrotic lesions. By day three, two strains (A/J, FVB/NJ) were mild and all others had minimal apoptotic/necrotic lesions. Comparing the severity of these lesions across experiments, we see that on day three the A/J and FVB/NJ mice had mild apoptotic/necrotic colon lesions in both experiments while all other strains were minimally affected. In experiment one we see two strains (C57BL/6J, C3H/HEJ) decrease in severity from day one to three while all others maintain their level of severity. In experiment two we see one strain (C57BL/6J) decrease and two strains (A/J, FVB/NJ) increase in severity from day one to three while all others maintain their level of severity. There was an observable increase in lesions on day 1 compared to controls in the colon of all strains, except FVB/NJ. A/J, C57BL/6J and FVB/NJ appeared to be the most sensitive to colon lesions in both experiments. All observed colon lesions were diagnosed as necrosis or apoptosis in the crypt. No lesions were apparent in the villi. In control colons, one strain (FVB/NJ) showed minimal lesions; all others had no significant lesions.

3.4 Discussion

Histology endpoints of apoptosis/necrosis (all tissues) and tubular vacuolation/degeneration (kidney) were important aspects of our study because they allowed observation of differences in the toxicity and concentration of platinum adducts. While adducts were observed to be highest in the most damaged tissue, no overall correlation could be found between increased/decreased histological lesions and changes in adduct distribution (Figure 3.4). Furthermore, there was considerable variability between experiments. Therefore, the data from the two experiments could not be combined to increase the sample size. Because colon DNA of most strains (excluding C57BL/6J and DBA/2J) from the first experiment were processed and quantified with the tissues obtained for the second experiment, the observed variability does not appear to be with the UPLC-MS/MS assay. It is possible that the variability comes from some factor associated with the dosing of the animals. Several important issues were identified that need to be considered in future studies.

This study design contained several confounders that affect the analysis of the results. First, because the dose was given by *i.p.* injection, we cannot be sure of the actual dose received by the mice. One study shows error rates of 11-12% when a trained professional performs a one man *i.p.* injection, due to misplaced injections that were most often partly injected into the lumen of the intestine (6). Second, during necropsy we noticed that the mice in the second experiment appeared much sicker than those from the first experiment. Also, one mouse died and several were close to death by day three in the 2nd experiment. The histology slides also show

more apoptosis/necrosis in the 2nd experiment than the first. One variable that changed from the first experiment to the second was the stock solution of cisplatin used for dosing. The first experiment used cisplatin that had been used previously for the dosing of the mice for the gene expression study, while in the second experiment a new solution of cisplatin was prepared. Therefore, there may have been differences in the preparation of the stock solution for each mouse experiment, which could have affected the potency of the cisplatin solutions. Based upon our histology data, the newer cisplatin may have been more potent than the cisplatin used in dosing the 1st experiment, leading to the observed increased toxicity in the 2nd experiment which manifested as more apoptosis and necrosis.

What is poorly understood is the lower molecular dose of the DNA cross links in the second experiment. One possible explanation could be the increased cell loss due to necrosis, resulting in less adduct being available for quantification in the tissues. This explanation would not, however, be the case for liver, as necrosis was minor in that tissue. Dosing solution preparation and animal dosing was not performed in or by our laboratory. Therefore, another explanation could be that changes in stock solution preparation led to differences in relative amounts of intact and aquated cisplatin in the first and second stock solutions, which would affect the percent platinum incorporation into cellular DNA and protein. Jones et al has shown aquated cisplatin to be 3 times as nephrotoxic as cisplatin itself in rats (7). In his experiments, the aquated form of cisplatin was prepared by placing cisplatin in water, which was then allowed to stand at room temperature for two weeks. Since the dosing solution used for the first experiment was what remained from previous

dosing of animals for the gene expression study, these conditions could have been unknowingly reproduced. For the second experiment, the previous stock dosing solution was gone. Therefore, new cisplatin was ordered and a new solution made immediately before dosing. This by itself could have caused a greater formation of aquated cisplatin, leading to the greater amount of DNA adducts in the first experiment. Alternatively, preparation of the cisplatin in a saline and then non-saline solution could cause similar differences.

One factor unrelated to dosing that may have affected the study was the infestation of pinworms in the mouse facility during the first experiment. It is possible that the medication given to eradicate the outbreak affected the interaction of cisplatin in those mice. In this case, the pinworm treatment may have protected against kidney toxicity, while increasing overall adduct formation. When examining this possibility, it is also possible that the pinworm treatment caused the liver toxicity observed in the first experiment, as no liver toxicity occurred in the second experiment.

With all of this taken into account, based upon both the histology and CP-d(GpG) data obtained in these experiments, the C57BL/6J mice appear to be the most sensitive and FVB/NJ least sensitive with respect to the adduct burden and toxicity in the kidney. It is possible that formation and/or repair of CP-d(GpG) adducts could be strain specific. But due to the lack of correlation of adduct formation between our two experiments, we are unable to conclusively make such a determination. Furthermore, because of the lack of correlation between histopathology and adduct burden, it is possible that the CP-d(GpG) adduct may not

be the adduct driving toxicity. Since the highly toxic interstrand cross links have been primarily linked to cytotoxicity in dividing cells, it is more likely that the primary adducts resulting in kidney toxicity are Pt-protein or Pt-membrane lesions (8). However, it is also possible with a more carefully designed study that the CP-d(GpG) adduct burden and histology would correlate.

We could find no consistent difference in adduct concentration for day 1 versus day 3. In some strains adducts increased, while in others adduct levels decreased with time. This is of concern because one would expect a decrease in platinum DNA adducts between days one and three due to repair and cell death. This study does offer the first reported measurement of CP-d(GpG) adducts in the kidney, liver and colon of mice. While our method was able to detect adducts in all tissues of all strains, with an n=3 statistical outliers could not be eliminated, therefore a larger sample size will be necessary to confirm if observed trends are significant in either experiment. For greater statistical power the sample size for a study of this type should be at least 6 or optimally 9 mice per group. Additionally because of the confounders noted in this report, dosimetry, histology and gene expression studies should all be done in a single experiment.

Our newly developed method for the quantification of CP-d(GpG) adducts was sensitive enough to measure adducts formed using biologically relevant doses of cisplatin. In this study, the dose of cisplatin given is equivalent to ~21 mg/m², which is less than half of a typical clinical low dose of cisplatin. Using the molecular dosimetry of these adducts in the kidney we have found the C57BL/6J and 129S1/SvImJ strains to be most sensitive and the FVB/NJ strain to be the least

sensitive. However, these designations did not match histopathology scoring. If we had been able to use our biomarker to identify sensitive and resistant strains of mice, we could have begun looking at the previously obtained gene expression data for genes that match the trends observed with the adduct data. Such a correlation between adduct burden and gene expression, might have allowed researchers to discriminate between species-species differences in DNA damage response and in the biodistribution of cisplatin. One confounder to using the previously obtained gene expression data is that the kidney was not one of the organs collected for gene expression analysis. Because of the nephrotoxic actions of cisplatin, this tissue should be included in future combined molecular dosimetry/gene array studies of cisplatin. Future genomic studies can be performed to search for haplotypes or gene expression patterns that may be associated with cisplatin-sensitive or resistant phenotypes.

A study of this nature has been shown to be of use in understanding acetaminophen toxicity. Heinloth et al. showed gene expression perturbations at subtoxic doses of acetaminophen that may have indicated low level cellular injury in the liver that was not detected by histopathology or clinical chemistry (9). When they increased the dose of acetaminophen to a toxic level the same genes showed a more exaggerated response, leading to the belief that gene expression profiling can be used to identify markers of low level cellular injury. Another study used oxidative stress biomarkers to provide phenotypic anchors for gene expression profiling of acetaminophen-induced oxidative stress (10).

In our case, the profiling could be used to detect markers that precede the development of resistance to cisplatin treatment. For the best ultimate translation of this and future mouse studies to the clinic, a larger sample size is required to ensure proper identification of alleles and a definite phenotypic classification. Additionally, because of the inconsistencies between our experiments we conclude that phenotypic markers must be determined using the same animals for both biomarkers and gene expression and using larger numbers of animals per group. To reduce the size of a study that would follow our suggested model, one could focus upon the strains found to be most sensitive (C57BL/6J) and resistant (FVB/NJ) to cisplatin kidney toxicity in our experiments. Because the mode of action for cisplatin is not well understood, a complex system of cellular molecular pathways is likely to be playing a role in an individual's response to cisplatin-based chemotherapy. With properly designed pharmacogenomic studies combined with systems biology, individualized medicine is on the horizon. However, it is also clear that a high degree of consistency must be required in the dosing and evaluation of endpoints.

FIGURES

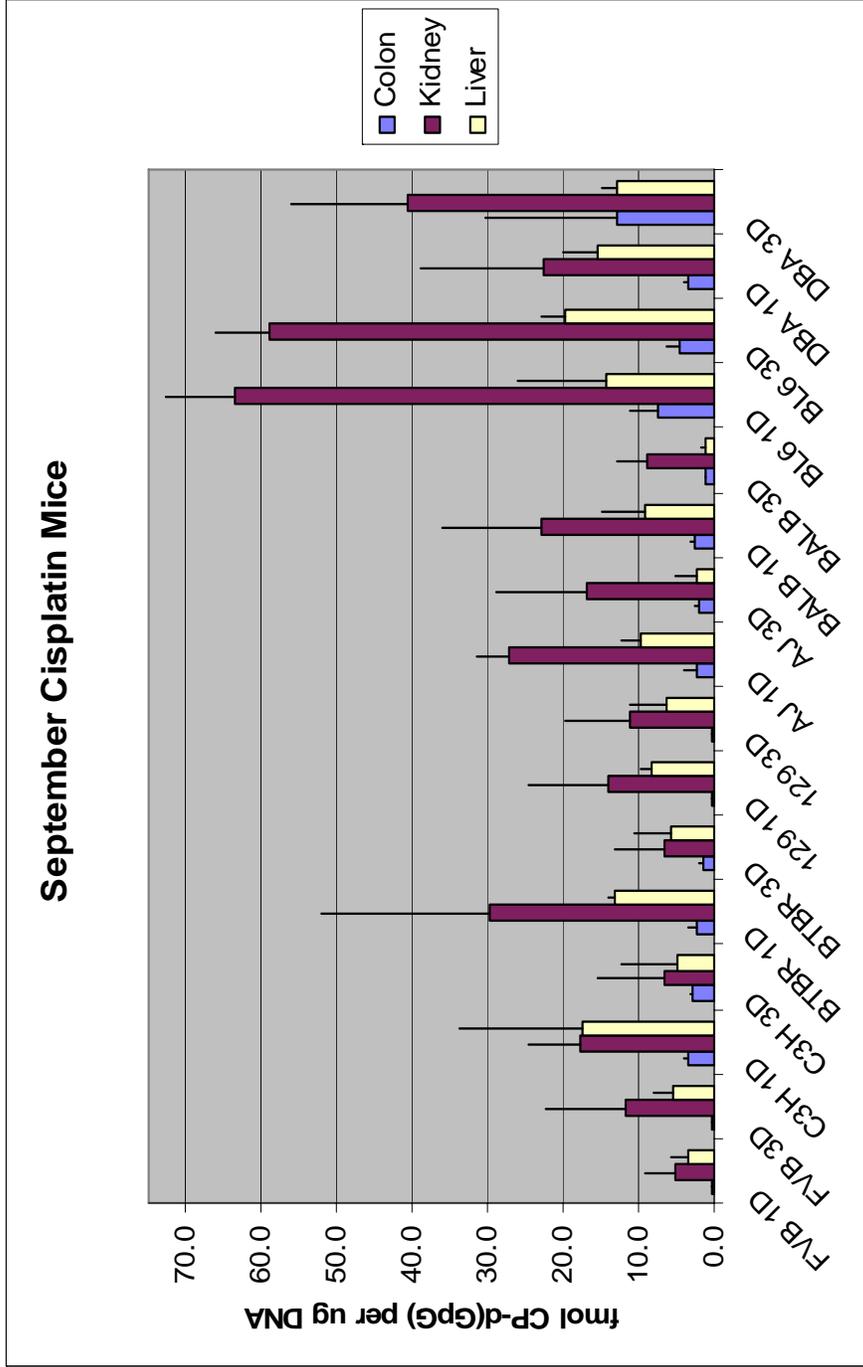


Figure 3.1.1 First experiment of Molecular Dosimetry of CP-d(GpG) with one and three day trends

Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of eight inbred mouse strains after an *i.p.* injection of 7mg/kg cisplatin.

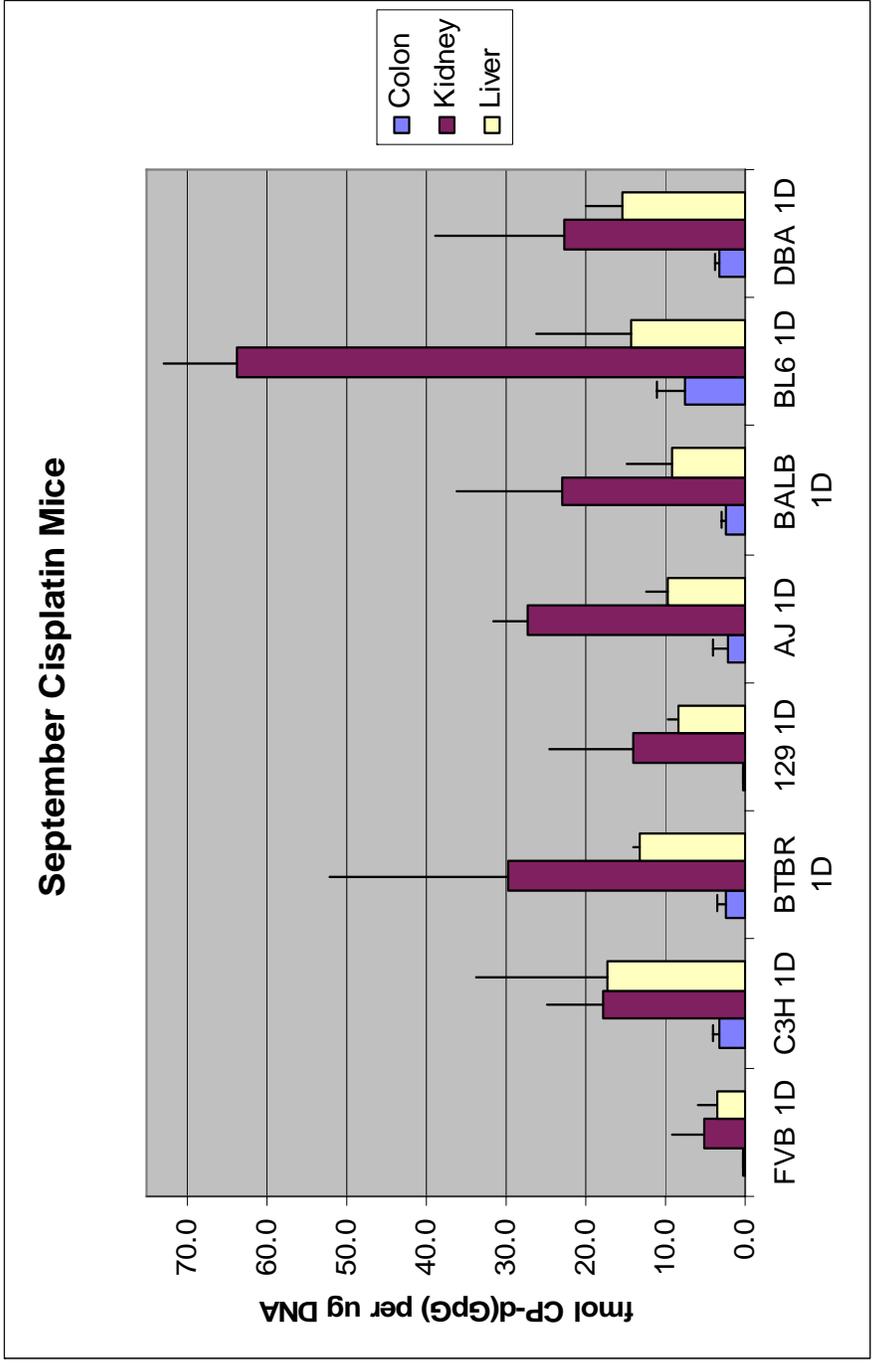


Figure 3.1.2 First experiment of Molecular Dosimetry of CP-d(GpG) with data from one day trend Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of eight inbred mouse strains after an *i.p.* injection of 7mg/kg cisplatin.

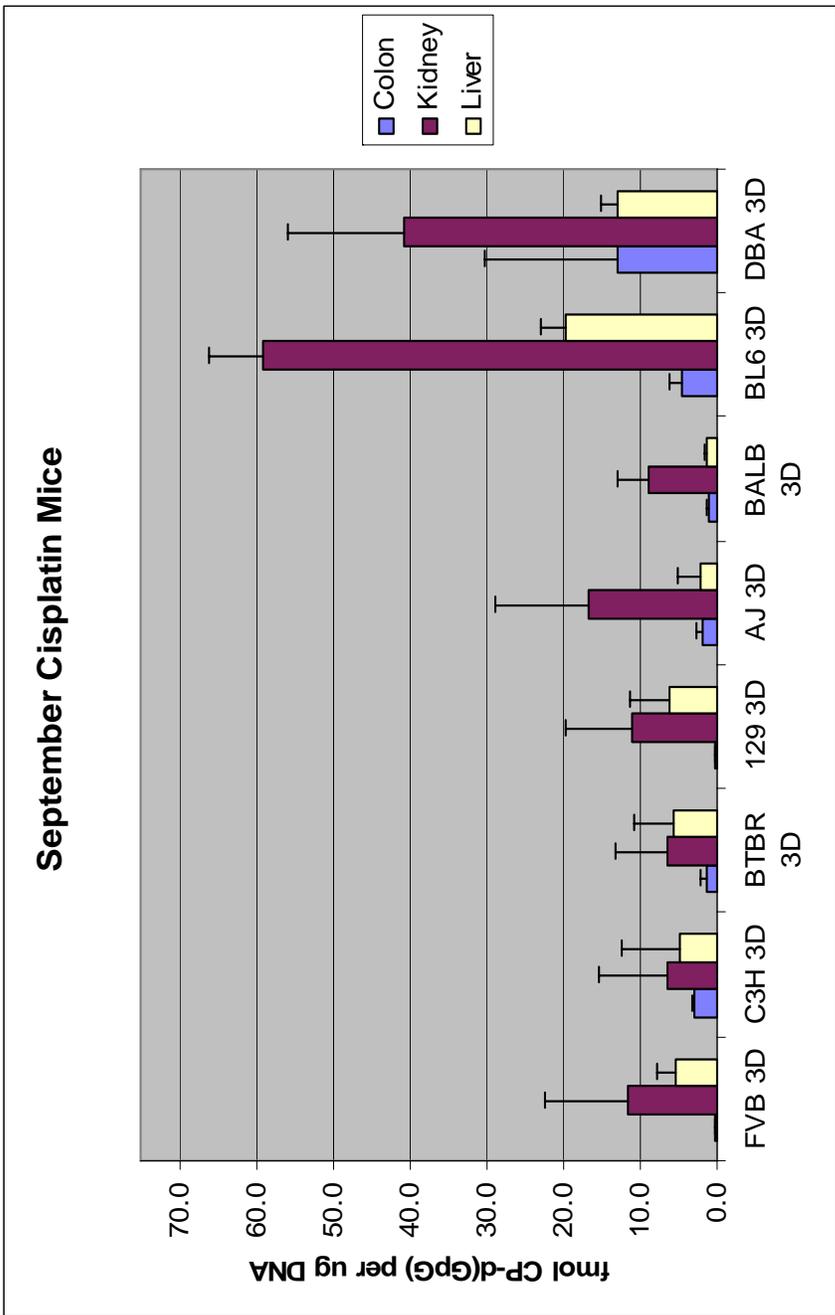


Figure 3.1.3.3 First experiment of Molecular Dosimetry of CP-d(GpG) with data from three day trend

Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of eight inbred mouse strains after an *i.p.* injection of 7mg/kg cisplatin.

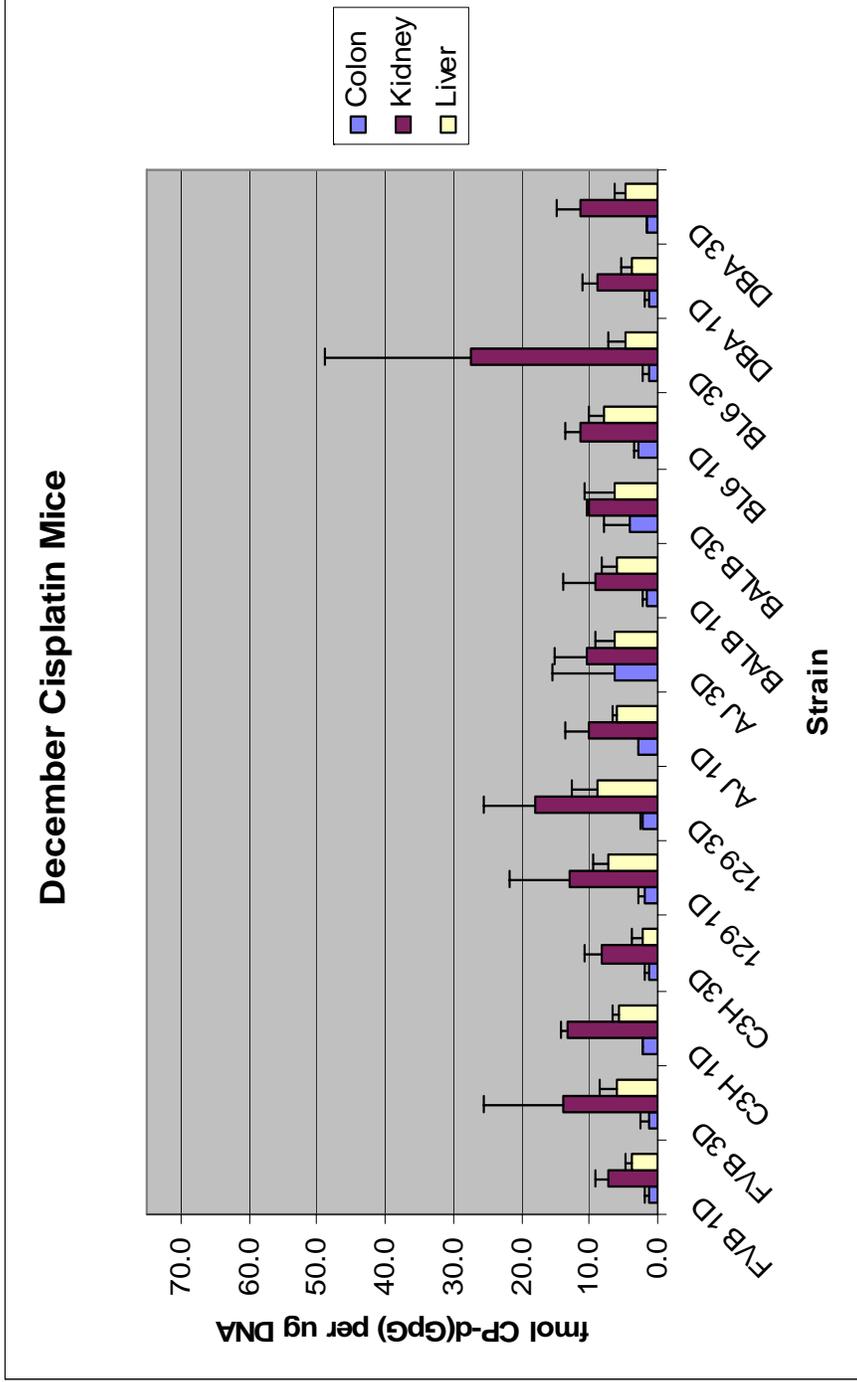


Figure 3.2.1 Second experiment of Molecular Dosimetry of CP-d(GpG) with one and three day trends

Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of seven inbred mouse strains one and three days post *i.p.* injection of 7mg/kg cisplatin. BTBR was

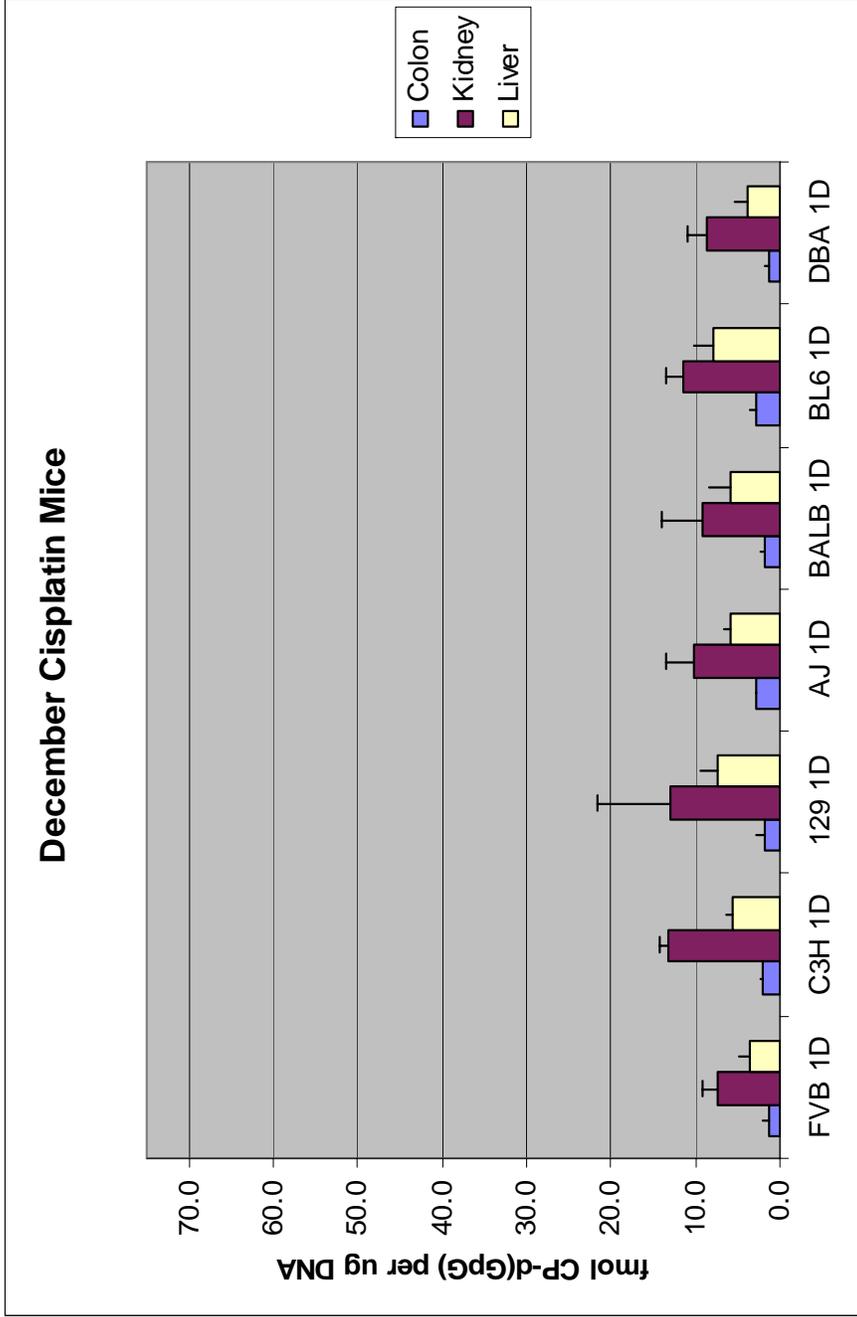


Figure 3.2.2 Second experiment of Molecular Dosimetry of CP-d(GpG) with data from one day trend Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of seven inbred mouse strains one and three days post *i.p.* injection of 7mg/kg cisplatin. BTBR was not included in this analysis.

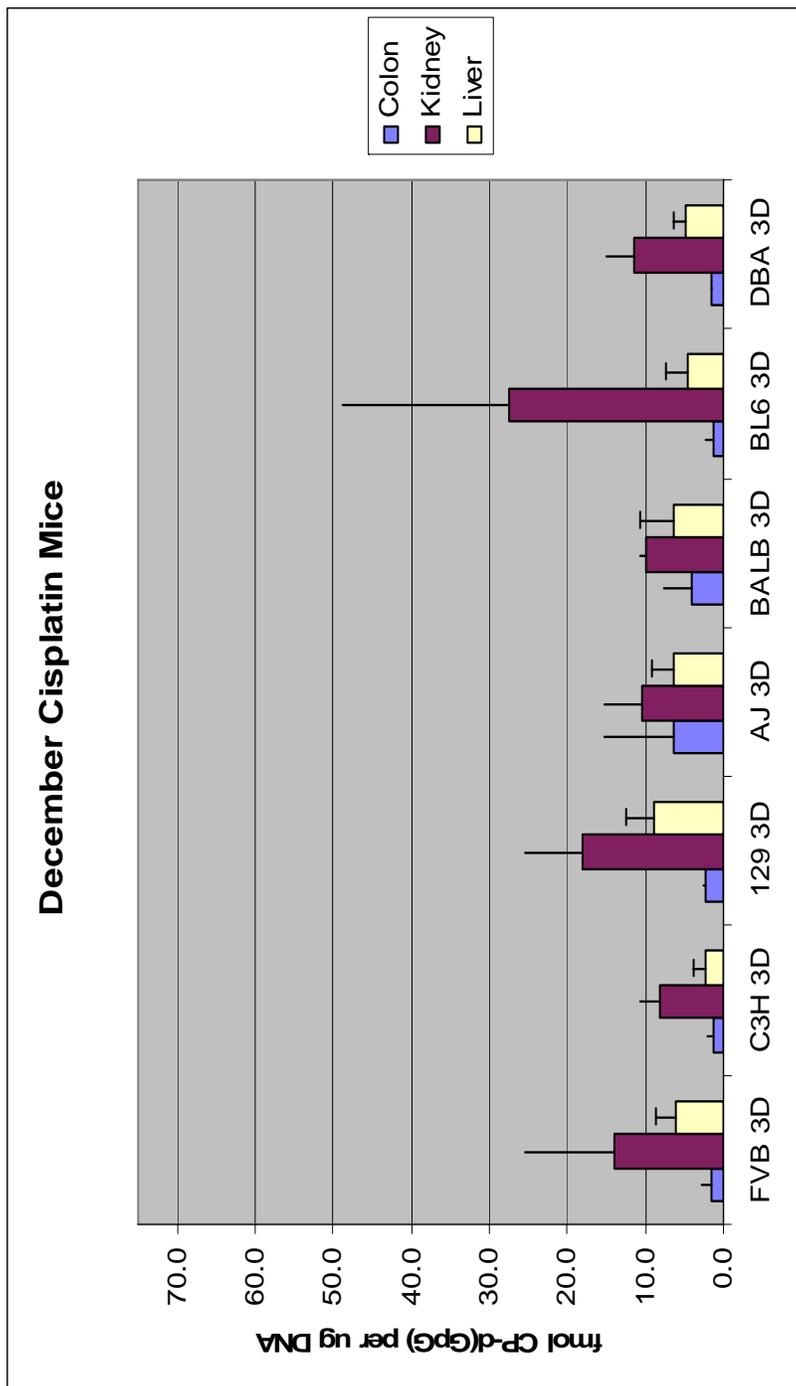


Figure 3.2.3 Second experiment of Molecular Dosimetry of CP-d(GpG) with data from three day trend

Molecular Dosimetry of CP-d(GpG) adducts in colon, liver and kidney of seven inbred mouse strains one and three days post *i.p.* injection of 7mg/kg cisplatin.

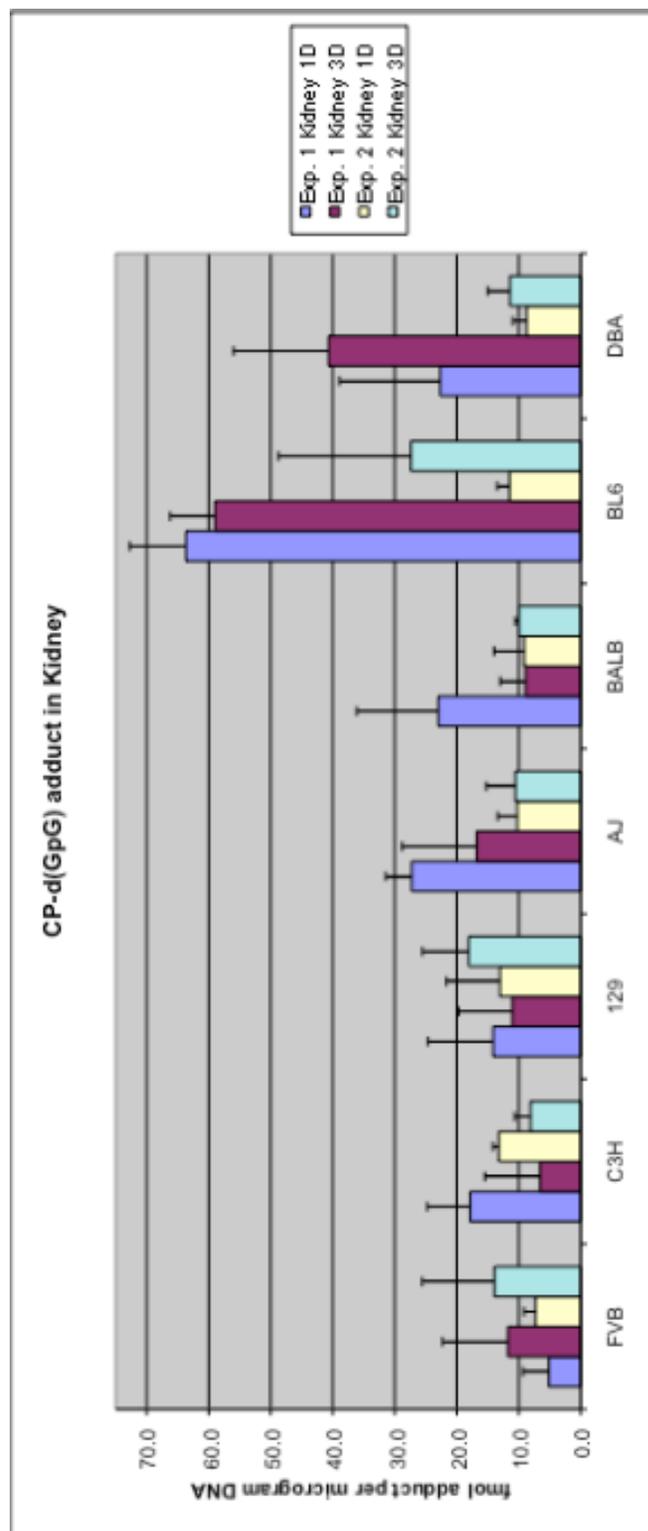


Figure 3.3.1 Distribution of CP-d(GpG) in the Kidney for both days and experiments

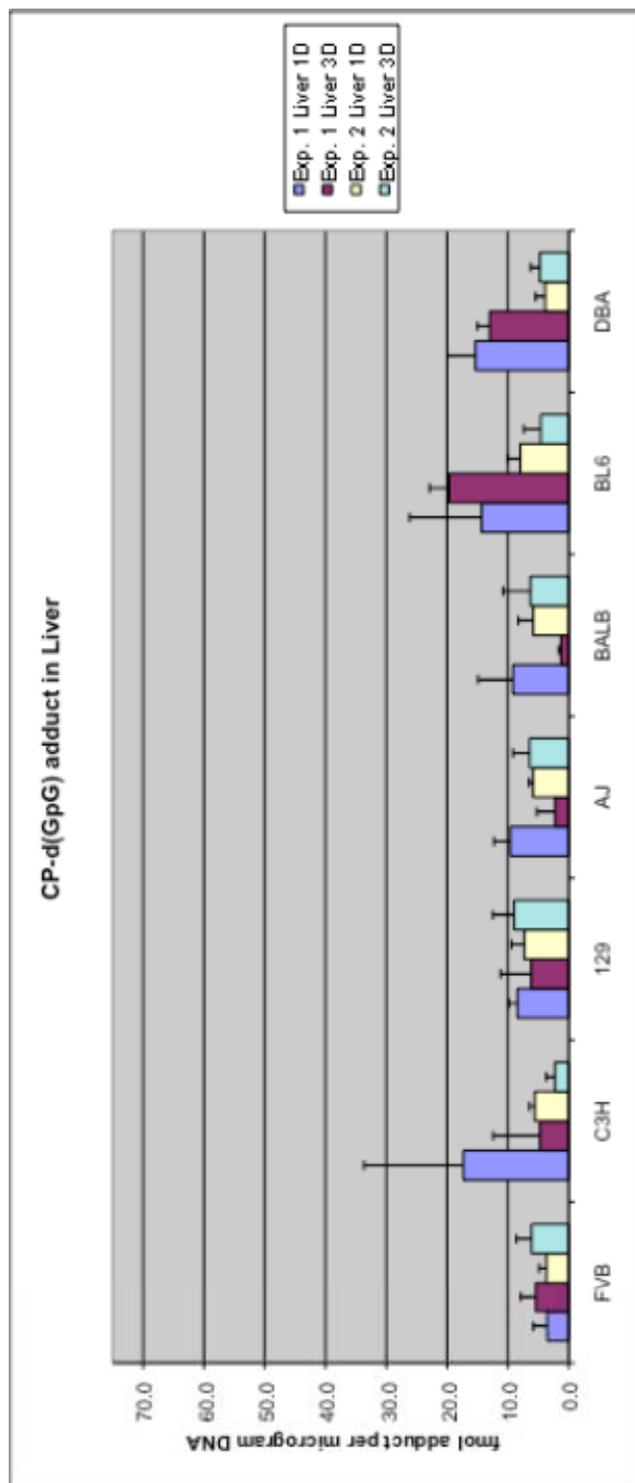


Figure 3.3.2 Distribution of CP-d(GpG) in the Liver for both days and experiments

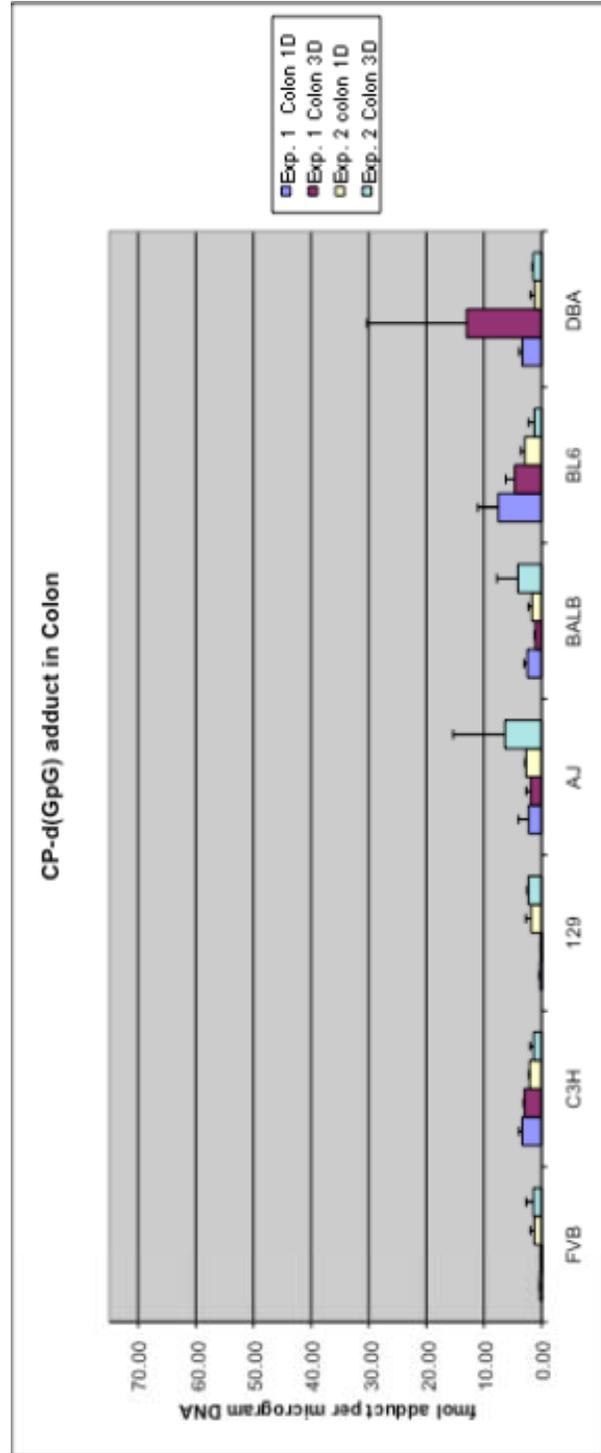


Figure 3.3.3 Distribution of CP-d(GpG) in the Colon for both days and experiments

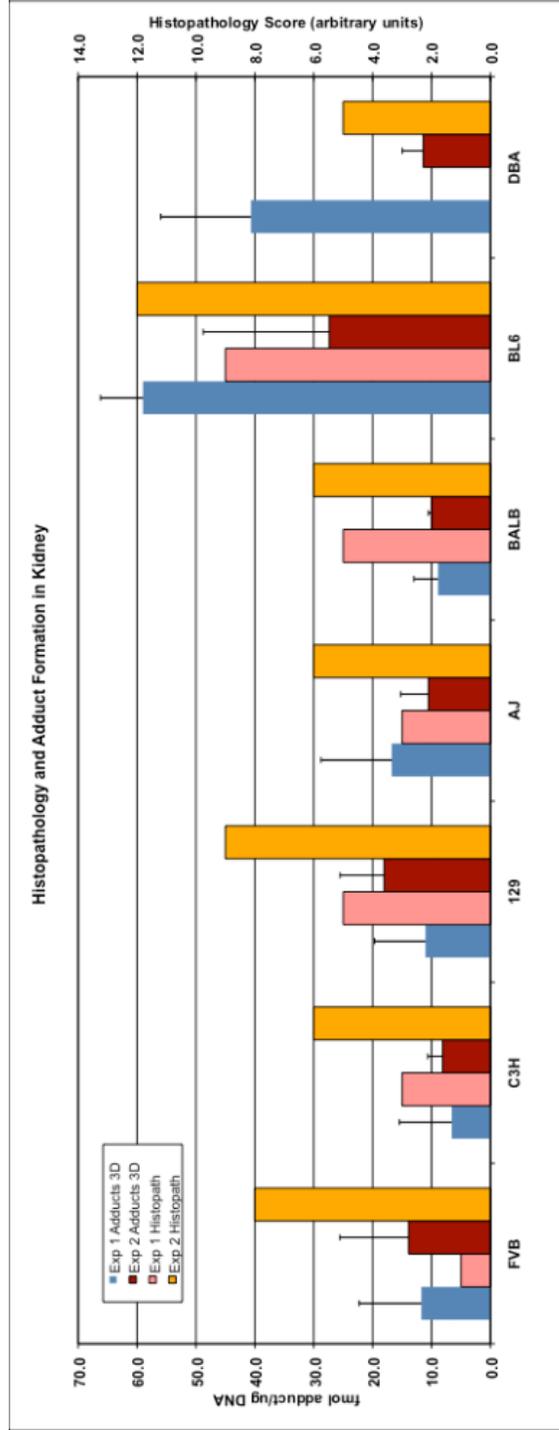


Figure 3.4 Comparison of Histology Scores to CP-d(GpG) Adducts collected on Day 3 of both experiments

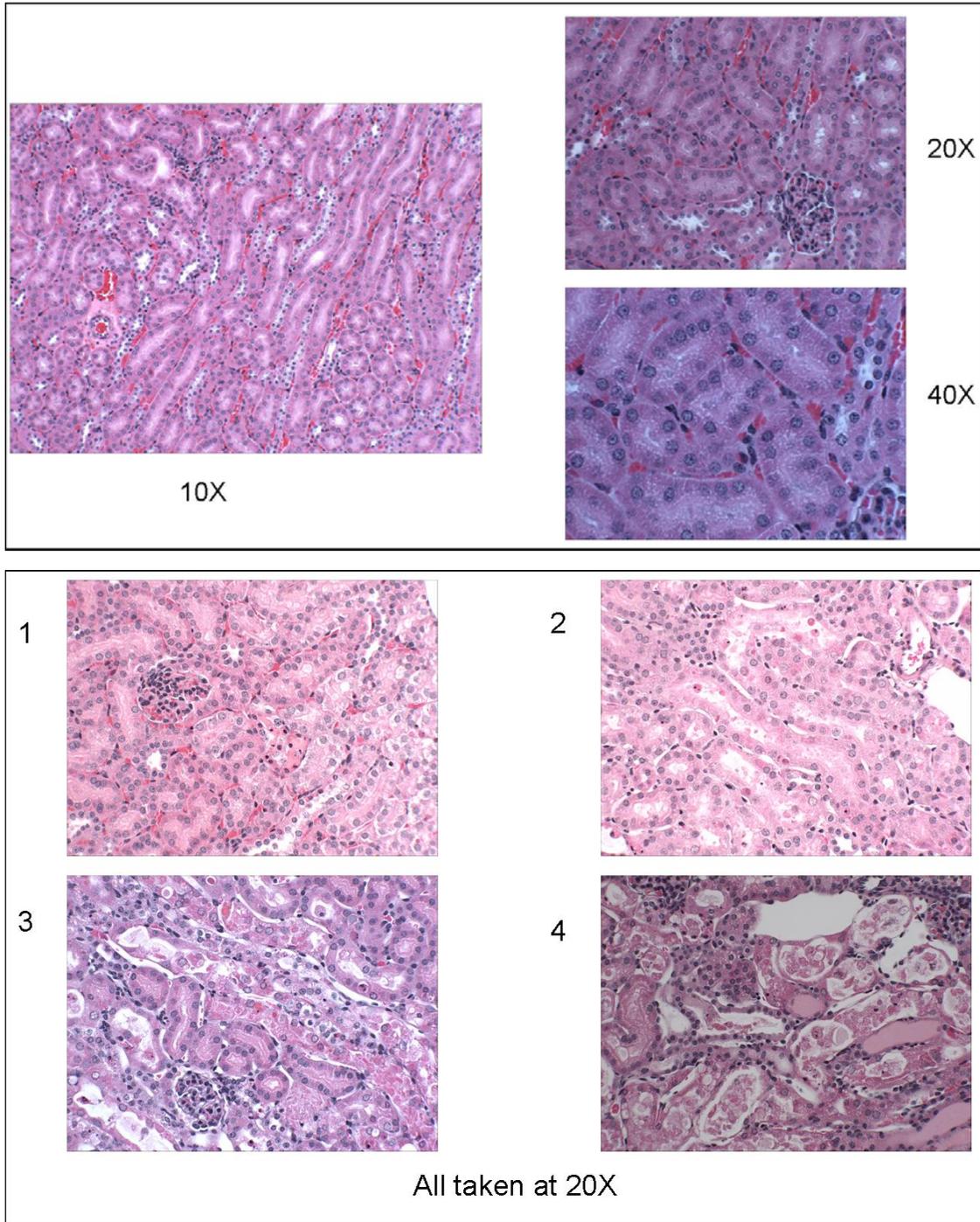


Figure 3.5 Kidney histology slide pictures.

The top panel contains an example of control kidney shown at multiple magnifications. The bottom panel contains representative slides showing each level of damage observed in our study. 1=minimal lesions; 2=mild lesions; 3=moderate lesions; 4= marked lesions

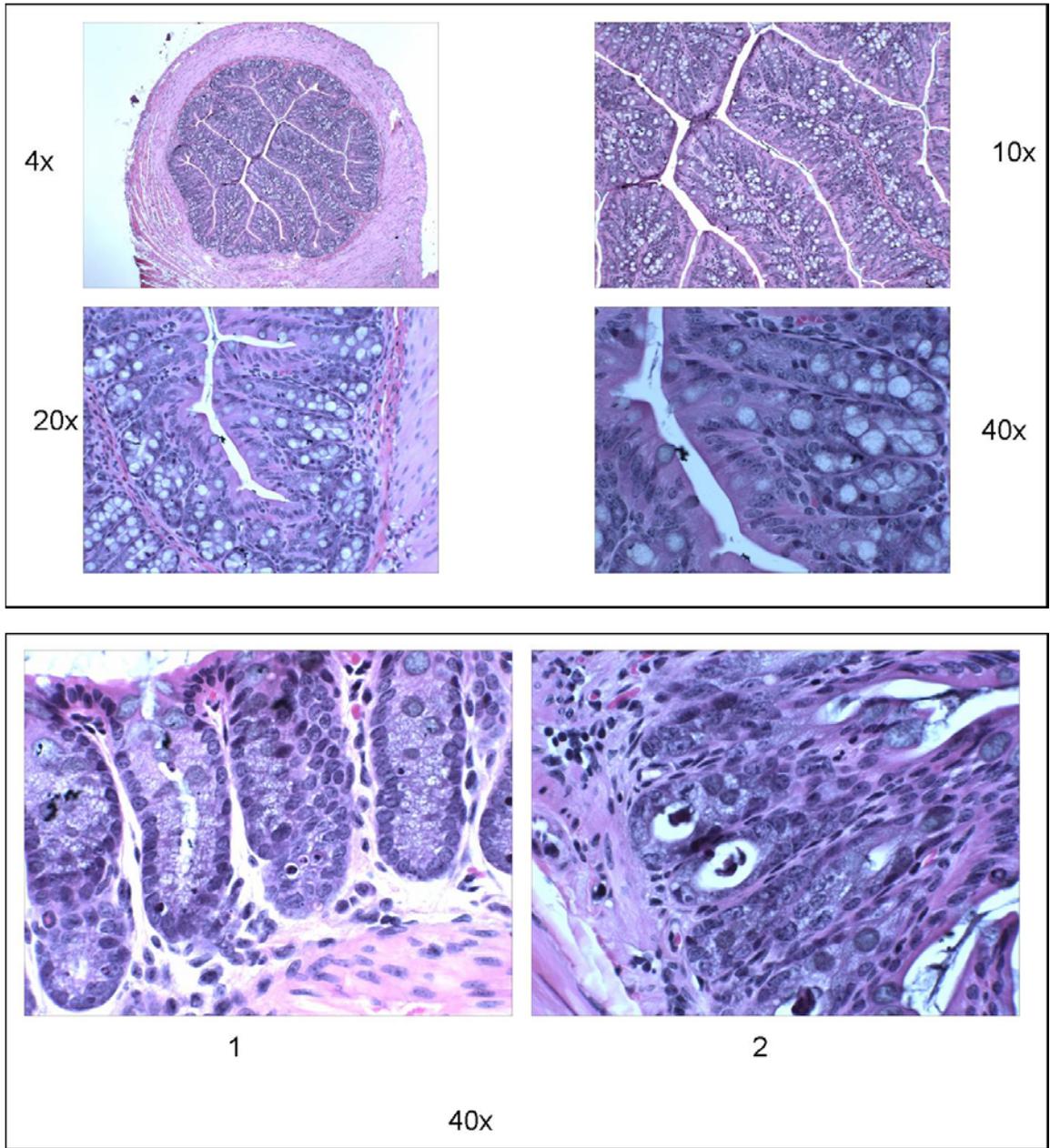


Figure 3.6 Colon histology slide pictures

The top panel shows an example of control colon at multiple magnifications. The bottom panel contains representative slides showing each level of damage observed in our study. 1=minimal lesions; 2=mild lesions

TABLES

September Histology Scores	Tissue	Time in days	129S1/SvImJ	A/J	BALB/cJ	C57BL/6J	BTBR T+TF/J	C3H/HEJ	DBA/2J	FVB/NJ
	Kidney	1	0,1 ^c	0	0	0,2 ^a ,3 ^c	1	0	0,6 ^c	0
		3	5	3	5	9,2 ^c ,4 ^d	9	3	3,8 ^c	1,2 ^c
	Liver	1	0	1 [*]	0	0, 2 ^a	2 [*]	1	0	0
		3	0	0	0	0, 8 ^b	6 [*]	0	2 [*]	2 [*]
	Colon	1	3	4	2	6	3	4	3	4
		3	3	5	3	3	3	3	3	4

n=3; *= time of death or unrelated to treatment; a= lymphocytes/plasma cells chronic lesion not related to treatment; b=centrilobular fatty change; c= tubular vacuolation/degeneration; d= tubular mineralization

Table 3.1 Histopathology scoring of September (1st) cisplatin mouse experiment.

Scores are reported here as the composite of three animals individual histology scores. Description of numerical score is as follows: 0= absent of no significant lesions, 1-3=minimal lesions (<5%), 4-6=mild lesions (5-25%), 7-9=moderate lesions (25-50%), 10-12=marked/severe lesions (>50%). In the above table, the first number refers to necrosis/apoptosis in tissue and following number(s) indicate other damage as indicated by superscript letters.

December Histology Scores	Tissue	Time in days	129S1/SvImJ	A/J	BALB/cJ	C57BL/6J	BTBR T+ TF/J	C3H/HEJ	DBA/2J	FVB/NJ
	<i>Kidney</i>	0	0	0	0	0	Not Available	0	0	0
		1	0	0	0	0		0	0,1 ^c	0
		3	9,5 ^c ,3 _d	6,2 _d	6,1 ^d	12		6	5	5
	<i>Liver</i>	0	0	0	0	0	Not Available	0	0	1
		1	0	0	0	1 [*]		0	2 [*]	0
		3	0	0	0	0		0	2 [*]	1 [*]
	<i>Colon</i>	0	0	0	0	0	Not Available	0	0	1
		1	2	1	3	4 [±]		3	3	3
		3	3	5	3	2 [#]		3	3 [±]	6

n=3; * = time of death or unrelated to treatment; a= lymphocytes/plasma cells chronic lesion not related to treatment; b=centrilobular fatty change; c= tubular vacuolation/degeneration; d= tubular mineralization; ± = only one section of colon on histology slide for one animal; # = autolysis of one animal's tissue

Table 3.2 Histopathology scoring of December (2nd) cisplatin mouse experiment.

Scores are reported here as the composite of three animals' individual histology scores. Time in days of 0 is for control animals. Description of numerical score is as follows: 0= absent of no significant lesions, 1-3=minimal lesions (<5%), 4-6=mild lesions (5-25%), 7-9=moderate lesions (25-50%), 10-12=marked/severe lesions (>50%). In the above table, the first number refers to necrosis/apoptosis in tissue and following number(s) indicate other damage as indicated by superscript letters.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

We have developed a sensitive and specific method to quantify cisplatin 1,2 guanine-guanine intrastrand cross links by tandem mass spectrometry. The sensitivity of this method is comparable to the ^{32}P postlabeling method, which was the most sensitive method for the quantification of individual cisplatin derived adducts. None of the previously published methods included an internal standard. 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 has the benefit of an internal standard, provides structural confirmation of adducts, and does not require the use of radioactivity. Both cisplatin and carboplatin form chemically identical platinum adducts, therefore this assay can be used to detect the Pt-d(GpG) adduct for both agents. Important advances and conclusions gained during the development of our method are summarized in the following list.

1. Our method is the first to use a stable isotope-labeled internal standard during mass spectrometric quantification of cisplatin-DNA adducts.
2. Our method has 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.

3. We have shown that we are able to measure adducts formed *in vitro* and *in vivo* at biologically relevant doses of cisplatin.

As discussed in Chapter 2, studies using peripheral blood cells of humans treated with cisplatin detected up to 0.25 fmol cisplatin intrastrand adducts per μg DNA in patients with testicular cancer and 0.16-14.1 fmol CP-d(GpG) per μg DNA in patients with gastric cancer (1,2). CP-d(GpG) adduct levels have been reported using ^{32}P postlabeling to be 4-5 times higher in primary head and neck squamous cell carcinoma (HNSCC) tumors than in peripheral blood cells or buccal cells obtained after intravenous or intraarterial administration of 100-150 mg/m^2 cisplatin (3). Therefore, our limit of quantification should be sufficient to measure cisplatin guanine-guanine adducts as a surrogate marker of dose using peripheral blood cells or directly in biopsied samples in a clinical setting. This highly sensitive and specific method could then be utilized in clinical studies to monitor adduct levels to provide a better understanding of cisplatin detoxification prior to Pt-DNA adduct formation and cisplatin-DNA adduct repair. Clinical samples that could be measured by this method include lymphocytes, buccal cells, and other tissues obtained during biopsy from patients which have received either cisplatin or carboplatin therapy.

Furthermore, with some modifications this method could be used also to quantify the Pt-d(ApG) adduct, allowing the detection of ~95% of all formed platinum adducts.

In our mouse study using ~21 mg/m^2 cisplatin, we were able to measure adducts one and three days after dosing. A normal clinical dose of cisplatin is 50-100 mg/m^2 , which suggests that we would be able to detect adducts several days after treatment in tissues of patients treated with cisplatin or carboplatin therapy.

Using our newly developed UPLC-MS/MS method, we determined the molecular dosimetry of cisplatin intrastrand guanine-guanine adducts after 24 and 72 hours in the kidney, liver and colon. The literature shows that platinum levels are the highest in the tumor, kidney, and liver following treatment. Since these studies measured total platinum, we were interested in measuring the CP-d(GpG) to see whether the kidney or the liver was the most affected site.

As we show in chapter 3, the molecular dosimetry data obtained were analyzed to determine if there was a strain, tissue or time point at which there was a significant increase or decrease in CP-d(GpG) adduct concentration. Using these characteristics, our initial plans were to identify cisplatin sensitive and resistant phenotypes which would ultimately be used to search the microarray data for affected genes. The correlation of the aforementioned cisplatin dosimetry with gene expression data could provide insight about genes or polymorphisms in the human population that may cause a cancer patient to be sensitive or resistant to cisplatin treatment. In the following paragraphs, we will use our experiences from this research to suggest ways to appropriately design and implement combined dosimetry and gene expression studies, which will provide better data to allow these correlations. This knowledge one day could allow patients undergo genetic testing prior to treatment that would determine the optimal therapies for that individual.

Because our first molecular dosimetry study had a small sample size ($n=3$) another study of the same size was performed to strengthen our data. However, there was considerable variability between the two molecular dosimetry studies. Therefore, the data from the studies could not be combined to increase our sampling

size. Because of confounding factors such as, mice in the 1st study undergoing treatment for a pinworm infestation and fresh cisplatin used for the 2nd but not the 1st study, it is difficult to make direct comparisons of Pt-DNA adduct levels and histology data between the studies. The range of overall adduct levels in the kidney, the most affected tissue, of the different strains was 5-60 fmol CP-d(GpG) per μg DNA in the 1st study and 8-27 fmol CP-d(GpG) per μg DNA in the 2nd study. Furthermore, the levels of organ toxicity observed through histopathology analysis were different within strains between studies. These factors taken together invalidate the use of the current dosimetry data set for correlation with the microarray data, in which pooled RNA was obtained from a different set of animals.

Another study with a larger number of animals of similar age and weight, and the use of verified dosing solutions would provide a more consistent and reliable data set for analysis. Likewise, the optimal design for a study involving both microarray and molecular dosimetry data collection would include the use of the same set of animals; this would ensure that increases or decreases observed in the array data would be directly comparable to cisplatin adduct molecular dosimetry. Also, since it is common practice for those employing microarray techniques to pool RNA to ensure an appropriate amount for analysis, for this type of study DNA from the same animals should also be pooled to ensure data will later be directly comparable. It should be noted that we have shown that the UPLC-MS/MS method has enough sensitivity to quantitate adducts from individual mice in tissues such as the colon from which a limited amount (<100 μg) of DNA can be isolated. The time points in the microarray study of 1, 3 and 7 days after dosing were selected based

on a colon cancer model and the time it takes cells to go from “the crypt to the tip”. By the 7th day of treatment, the colon crypt cells would have been shed from the villi and the repair of CP-d(GpG) adducts in the kidney and liver was expected to be complete. Also, preliminary data from the microarray study were available which suggested tissues would be destroyed or the animals would be dead from treatment by the 7th day. Therefore, the studies we performed only collected data at the 1 and 3 day time points. However, in a study that combines these techniques measuring adducts on the 7th day should be considered. This would allow to the study to follow intended the “tip to crypt” study design and determine adduct levels on day 7, providing a more complete analysis using the corresponding gene expression data.

Because of the necessity of pooling three animals per sample for the microarray portion of the study and to use enough animals for optimal statistical analysis, an $n=9 \times 3$ (21) would be required for each time point (controls, 1, 3, 7 day) and each strain (8) for a total of 672 animals. Even more animals may be required so that sufficient RNA and DNA can be isolated from the same tissues for analysis by both methods, making this a very large and expensive study. Alternatively, one could narrow the study to only include those strains most likely sensitive (BL6) and resistant (FVB) to cisplatin treatment, as determined during our molecular dosimetry studies.

Next, to more accurately mimic the manner in which patients are treated with cisplatin, a mini-pump allowing infusion of cisplatin over a longer time period and hydration pre- and post- dosing would be preferable to bolus *i.p.* injections. Using this mini-pump infusion of cisplatin with adequate hydration, it would be interesting to

see if there is a shift from the kidney having the highest concentration of CP-d(GpG) adducts. In our study design, the liver may develop an increased concentration of CP-d(GpG) adducts, if the kidney toxicity was reduced in this manner. Also, using that method of drug delivery combined with hydration, one would not expect the mice to be as sick (or in some cases dead) on day 3, since these outcomes were believed to be due to kidney failure. Conclusions from our animal study are summarized in the following list.

1. In both animal studies, the trend for distribution of CP-d(GpG) across all strain tissues was kidney>liver>colon.
2. While adducts were observed to be highest in the kidney, no overall correlation could be found between increased/decreased lesions in tissues and factors such as time after dosing or mouse strain. This may be due to the variability between our two studies.
3. Therefore, another study with a larger number of animals is needed to identify a sensitive or resistant phenotype using molecular dosimetry and microarray data obtained from the same set of animals.

As discussed in the introduction, several studies have compared platinum based DNA adducts obtained from normal vs. tumor tissue. These studies are not consistent in whether or not there is a correlation between adduct levels in normal tissues and survival or response to treatment. However, in each of these studies the “normal” tissues utilized are lymphocytes or buccal cells and the tumor tissue is obtained from biopsied tissue. This is generally done because there are limitations

to the human tissues one can obtain for research. However, it would be of interest to see if there is a correlation between normal and tumor adduct concentrations when the same tissue type is used. Such studies could be performed in animals. However as we learned during our animal study, great care must be taken when designing an animal study to ensure that the data obtained is consistent and of use when performing data analysis.

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