Comparison of Phenanthriplatin, A Novel Monofunctional Platinum Based Anticancer Drug Candidate, with Cisplatin, A Classic Bifunctional Anticancer Drug

by

Meiyi Li

B.S., Chemistry
Fudan University, 2010

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Inorganic Chemistry
At the
Massachusetts Institute of Technology
September 2012

Signature of Author: _____

Department of Chemistry
July 20, 2012

Certified by: _____

Stephen J. Lippard
Arthur Amos Noyes Professor of Chemistry
Thesis Supervisor

Accepted by: _____

Robert W. Field
Haslam and Dewey Professor of Chemistry
Chairman, Departmental Committee for Graduate Students

Comparison of Phenanthriplatin, A Novel Monofunctional Platinum Based Anticancer Drug Candidate, with Cisplatin, A Classic Bifunctional Anticancer Drug

by

Meiyi Li

B.S., Chemistry
Fudan University, 2010

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Inorganic Chemistry

at the

Massachusetts Institute of Technology

July 2012

Comparison of Phenanthriplatin, A Novel Monofunctional Platinum Based Anticancer Drug Candidate, with Cisplatin, A Classic Bifunctional Anticancer Drug

by

Meiyi Li

Submitted to the Department of Chemistry
on 20th July, 2012, in Partial
Fulfillment of the Requirements for the Degree of
Master of Science in Inorganic Chemistry

Abstract

Nucleotide excision repair, a DNA repair mechanism, is the major repair pathway responsible for removal of platinum-based anticancer drugs. In this study, 146 bp duplexes were prepared containing either a site-specific cis-diammineplatinum(II)-DNA intrastrand d(GpG) cross-link or a *cis*-diamminephenanthridinechloroplatinum(II)-DNA dG adduct. Comparison of the repair efficiencies of the two adducts reveals that the diamminephenanthridinechloroplatinum(II)-DNA dG lesion eludes the nucleotide excision repair pathway better than diammineplatinum(II)-DNA intrastrand d(GpG) cross-link. A factor that may be relevant to the difference is the influence of platination on DNA-mediated charge transfer. Atomic force microscopy is a method by which we can explore the possibility that phenanthriplatin influences charge transfer properties of DNA. Long DNA duplexes site-specifically modified with cisplatin or phenthanriplatin were prepared for AFM studies.

Thesis supervisor: Stephen J. Lippard

Title: Arthur Amos Noyes Professor of Chemistry

For Shifu/Shangshi and my parents, who showed me the path in life and taught me to improve myself and help others.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Stephen J. Lippard, for all his help, support and guidance during my time in graduate school. I am very appreciative of his patience and understanding as I decided to change my career path.

Thank you to the Lippard lab, especially to members of Platinum subgroup. Thanks to Dr. Guangyu Zhu and Semi Park for their help in teaching me all the skills related to DNA work, as well as answering my many questions; thanks to Dr. Seung Jae Lee and Shawn Lu for their help and patience in showing me how to express, purify, and characterize proteins; thanks to Dr. Ying Song, Dr. Robert Radford, and Dr. Ga Young Park for their advice and helpful discussion; thanks to Timothy Johnstone for his help in all my written work; thanks to Alexandria Liang and Mik Minier for their friendship; many thanks to lab manager, Richard C. Girardi, for all his help during the time I stayed in the Lippard lab.

Over the past two years, my parents have endured a lot of complaining, and I thank both of them for their patience, support, and unconditional love. I also would like to thank my grandparents for their encouragement and inspiration.

Many thanks go to my dear friends: Brother Ying-Cai Tan for his help in learning *The Great Treatise on the Stages of the Path to Enlightenment* and finding the next step in my life; Sister Peggy Lin, Brother Barry Lin, Sister Daiyi Lee, and Brother Suwei Lee for their concern and help whenever I was in need; Sister Ruqi Tang and Hao Wang for their discussions, and all the rest members in the Great Treatise class. I also would like to thank the friends I met at Fudan University and MIT: Yang Tang, Jin Xie, Ziyao Tang, Hongfan Chen, Xijun Piao, Yang Li, Linshen Li, Yi Liu, Yuran Wang, Yongbao Sun, Jingnan Lu, Mingjuan Su, and Lei Wang for their friendship and willing assistance. Special thanks to Yue Guan for spending time on long distance phone calls with me, understanding my worries and doubts, and ordering presents for my parents on their birthdays when I was far away from home.

Finally, and most importantly, I would like to thank Master Tsong-kha-pa who wrote *The Great Treatise on the Stages of the Path to Enlightenment*,

and Shifu/Shangshi for teaching me the Great Treatise and showing me the direction of life. Without you, I would be continuously lost in various sufferings, but with you, I am able to find the right direction to make myself a better person and help others eventually.

TABLE OF CONTENTS

Chapter 1.

Comparison of Nucleotide Excision Repair Efficiency between Cisplatin and Phenanthriplatin Modified DNA

INTRODUCTION

Cisplatin, *cis*-diamminedichloroplatinum(II) was approved for clinical use in 1978 and remains one of the most effective and widely used anticancer drugs. It is used to treat a variety of human cancers including testicular, ovarian, and non-small cell lung cancers.[1-3] Cisplatin is worthy of the title 'the penicillin of cancer' but it still leaves something to be desired as there are a number of negative side-effects associated with its administration, including nephrotoxicity, emetogenesis, and neurotoxicity.[4] Acquired and inherent resistance to the drug must also be considered.[5] To overcome these limitations, thousands of cisplatin analogues have been synthesized and tested for anticancer activity. Among all these complexes, only carboplatin (*cis*-diammine(1,1'-cyclobutanedicarboxylato)platinum(II)) and oxaliplatin ((*trans*-L-diaminocyclohexane)oxalatoplatinum(II)) (Figure 1.1), have been approved by the FDA for the treatment of cancer.[4, 6]

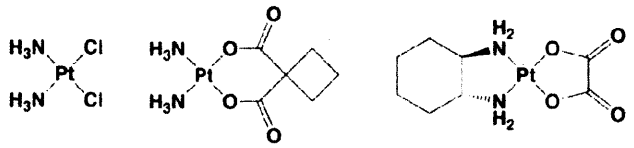


Figure 1.1. Chemical structures of FDA-approved platinum anticancer agents.

The mechanism of how platinum(II) anticancer drugs work has been studied extensively. Taking cisplatin as an example, the N7 positions of guanine bases in DNA form covalent bonds to the platinum atom after the drug has been activated by losing two chloride ligands.[7] The major products are 1,2-intrastrand and 1,3-intrastrand adducts comprising 50%-90% and 10%-25%, respectively, of the total adducts formed by cisplatin.[6, 8] The DNA

damage leads to transcription inhibition, which is believed to contribute to the cytotoxicity of cisplatin.[9, 10] After becoming a road block on DNA, the platinum lesion is either removed by nucleotide excision repair (NER) or apoptosis is triggered, the latter of which is the goal in platinum-based chemotherapy.[11, 12]

Based on the study of cisplatin, various compounds with non-classical structures have been designed in order to make new drugs that have fewer side effects or lower resistance than conventional platinum based agents. Among them, monofunctional platinum complexes caught researchers' attention by displaying an impressive antitumour spectrum and activites different from those of current platinum drugs.[13] Rather than forming DNA crosslinks, these compounds attack the N7 positions of guanine residues to form monofunctional platinum-DNA lesions, which significantly inhibit transcription, both in vitro and in live cells. Among the new monofunctional platinum compounds, *cis*-diamminephenanthridinechloroplatinum(II) nitrate (phenanthriplatin) (Figure 1.2) shows significant antitumor potential and is seven to forty times more cytotoxic than cisplatin in cancer cells from different origins.[14, 15]

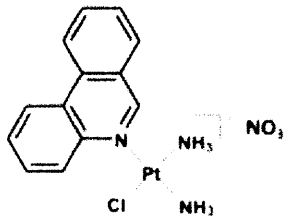


Figure 1.2. Structure of phenanthriplatin.

To date, compared with bifunctional anticancer agents, little work has been done to explain the mechanism of monofunctional platinum complexes. Previous research has shown that, when attached to the N7 position of a

guanine residue in DNA, monofunctional platinum compounds distort the DNA helix less than bifunctional platinum compunds do, allowing monofunctional lesions to elude the nucleotide excision repair pathway.[13] A direct NER study of phenanthriplatin has not previously been carried out, so in this thesis we studied the nucleotide excision repair efficiency of a phenanthriplatin platinated DNA duplex compared with that of a cisplatin platinated one.

EXPERIMENTAL PROCEDURES

Materials. Cisplatin was obtained from Strem Chemicals. Phosphoramidites and other reagents for DNA synthesis were obtained from Glen Research. All enzymes and enzyme buffers were purchased from New England Biolabs unless otherwise noted. All other reagents and solvents were purchased from commercial sources and used without further purification. DNA syntheses were performed on an Applied Biosystems Model 392 DNA/RNA Synthesizer at a 1.0 μmole scale. Radioactive gels were visualized using a Storm 840 phosphorimager and sample radioactivity was quantitated with a Beckman LS 6500 scintillation counter. HPLC was performed using an Agilent 1100 series system. Atomic absorption spectroscopy was performed on a Perkin-Elmer AAnalyst 600 system. UV-VIS spectra were obtained on a HP 8453 UV-visible spectrometer. Protein purifications were performed on a Biorad system having an automated fraction collector. Cell free extract from CHO AA8 cells was obtained from Joyce Reardon in Sancar lab at the University of North Carolina.

Synthesis, Purification, and Characterization of Oligonucleotides. To make platination site-specific on DNA, the target 146 bp DNA duplex was divided into several short strands that were synthesized separately. The final platinated 146 bp duplex was prepared by annealing and ligating five short strands (see Figure 1.3 for complete sequences). The cisplatin platination site is on the 14-mer and phenanthriplatin platination is on the 16-mer. The strategy for synthesizing a site-specifically platinated DNA probe is shown in Figure 1.4.

63-mer: 5'-ATC AAT ATC CAC CTG CAG ATT CTA CCA AAA GTG TAT TTG GAA ACT GCT CCA TCA AAA GGC ATG
14-mer: 5'-TTC ACC **GG**A ATT CC
69-mer: 5'-CCT CAA CAT CGG AAA ACT ACC TCG TCA AAG GTT TAT GTG AAA ACC ATC TTA GAC GTC CAC CTA TAA CTA
86-mer: 5'-ATG TTG AGG GGA ATT CCG GTG AAC ATG CCT TTT GAT GGA GCA GTT TCC AAA TAC ACT TTT GGT AGA ATC TGC AGG TGG ATA TTG AT
60-mer: 5'-TAG TTA TAG GTG GAC GTC TAA GAT GGT TTT CAC ATA AAC CTT TGA CGA GGT AGT TTT CCG

(A)

61-mer: 5'-ATC AAT ATC CAC CTG CAG ATT CTA CCA AAA GTG TAT TTG GAA ACT GCT CCA TCA AAA GGC A
16-mer: 5'-CCT CCT C**G**T CTC TTC C
69-mer: 5'-CCT CAA CAT CGG AAA ACT ACC TCG TCA AAG GTT TAT GTG AAA ACC ATC TTA GAC GTC CAC CTA TAA CTA
86-mer: 5'-ATG TTG AGG GGA AGA GAC GAG GAG GTG CCT TTT GAT GGA GCA GTT TCC AAA TAC ACT TTT GGT AGA ATC TGC AGG TGG ATA TTG AT
60-mer: 5'-TAG TTA TAG GTG GAC GTC TAA GAT GGT TTT CAC ATA AAC CTT TGA CGA GGT AGT TTT CCG

(B)

Figure 1.3. (A) Sequence of the five oligonucleotide starting materials for constructing the cisplatin platinated 146 bp duplex. (B) Sequence of the five oligonucleotide starting materials for constructing the phenanthriplatin platinated 146 bp duplex.The platination sites are highlighted in bold.

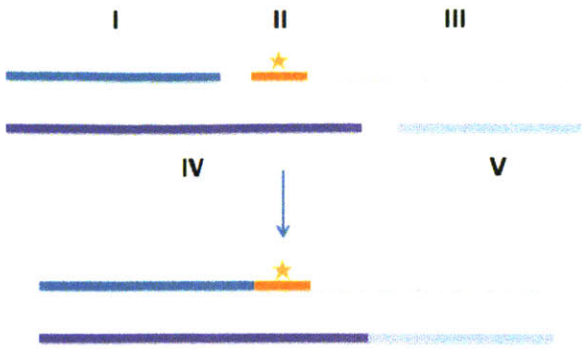


Figure 1.4. Strategy for synthesizing a site-specifically platinated DNA probe. For site-specifically cisplatin modified 146 bp, fragment I is 69-mer, fragment II is 14-mer, fragment III is 63-mer, fragment IV is 86-mer, and fragment V is 60-mer. For site-specifically phenanthriplatin modified 146 bp, fragment I is 69-mer, fragment II is 16-mer, fragment III is 61-mer, fragment IV is 86-mer, and fragment V is 60-mer. The platination sites are highlighted with stars.

All oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer on a 1 μmol scale and purified by 6% denaturing PAGE, except the 14-mer and 16-mer, which were purified by HPLC. To prepare the cisplatin platinated 14-mer oligonucleotide, cisplatin was allowed to react with $AgNO_3$ (2.0 equiv) for 5 hours at room temperature in the dark. The AgCl precipitate was removed by centrifugation. The 14-mer DNA was then combined with 2 equiv of activated cisplatin in 10 mM sodium phosphate buffer pH 6.8, and the solution was incubated at 37°C overnight. The product was purified by ion exchange HPLC and characterized by UV spectroscopy, atomic absorption spectroscopy (AAS), and nuclease S1 digestion. DNA concentrations were determined by quantifying the UV absorption at 260 nm. A purified phenanthriplatin platinated 16-mer was obtained from Dr. Ga Young Park.

Preparation of Radio-Labeled Full-Length Site-Specifically Modified Platinum-DNA Probes. Single stranded 14-mer oligonucleotides were 5'-end

labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. Single stranded 63-mer and 60-mer DNAs were 5'-end labeled (phosphorylated) with cold ATP and T4 polynucleotide kinase. The three phosphorylated short DNA strands were mixed with the other two, followed by ethanol precipitation. DNA pellets were re-dissolved in 1 X annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA), heated to 90 °C for 3 min and slowly cooled down to room temperature over 90 min. Subsequently, the annealed sample was treated with DNA ligase (New England Biolabs) to afford radiolabeled 146-bp duplex. Ligated DNA was ethanol precipitated and purified with 6% preparative denaturing polyacrylamide gels. Gel pieces containing single stranded 146-mers were excised with the aid of autoradiography and soaked in annealing buffer to extract DNA from the gel. Radioabelled DNA was isolated as a dry pellet by ethanol precipitation. Platinated duplexes were obtained by reannealing (DNA single strands were heated at 90 °C for 3 min and cooled to room temperature slowly over 3 hours) and characterized with 6% denaturing and 5% native polyacrylamide gels. The final products were quantitated by scintillation counting.

Nucleotide Excision Repair Assay. Both radiolabeled cisplatin and phenanthriplatin modified DNA samples (5-20 fmol) were incubated with 60 μg of cell free extract from CHO AA8 cells, which was obtained from Joyce Reardon in the Sancar lab, at 30 °C for 90 min in 25 μL of excision repair buffer (32 mM HEPES-KOH, pH 7.9, 64 mM KCl, 6.4 mM MgCl2 , 0.24 mM EDTA, 0.8 mM DTT, 2 mM ATP, 0.2 mg/mL BSA, 5.5% glycerol, 4.8% sucrose). Then the reaction mixtures were treated with proteinase K, phenol-chloroform extracted, and analyzed on an 8% denaturing PAGE gel. A

phosphorimager and the ImageQuant system were used to measure the levels of radioactivity in the bands arising from excised oligonucleotides (20-30 nucleotides).

RESULTS AND DISCUSSION

Synthesis, Purification, and Characterization of Oligonucleotides. Highly pure oligonucleotides were obtained by the methods described above. For short (< 20 bp) oligos, leaving the DMT group on during synthesis and purifying by reverse phase HPLC proved to be an easy way to separate the desired product from failure sequences, because the hydrophobic DMT group drastically increases the retention time on a C18 column. In a longer oligo synthesis, the lack of 100% capping efficiency leads to a greater percentage of strands being continued instead of acetylated. As a result, more products will be synthesized that are missing nucleotides from the middle of the strand, still having a DMT group attached at the end. These shorter sequences cannot be sufficiently resolved from the desired product by HPLC. PAGE is a more efficient method of separating oligos differing by only 1 nucleotide in length; longer strands are therefore purified by gel electrophoresis. A denaturing gel showing the purified 63-mer, 69-mer, 86-mer, and 60-mer DNA strands is depicted in Figure 1.5 and yields of purified oligonucleotides are shown in Table 1.1.

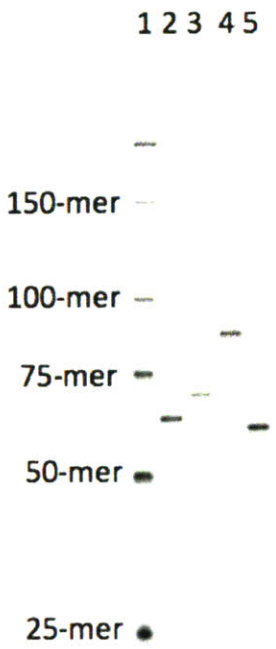


Figure 1.5. Characterization of purified oligonucleotides (see Figure 1.3): lane 1: DNA ladder; lane 2: 63-mer; lane 3: 69-mer; lane 4: 86-mer; lane 5: 60-mer.

Oligonucleotides	Yield (%)
63-mer	44
69-mer	36
86-mer	35
60-mer	69

Table 1.1. Yields of four oligonucleotides after denaturing PAGE purification. All DNA strands were synthesized on a 1 μmol scale and the yields were calculated based on UV-Vis absorption.

Platination of Short DNA Strands. The 14-mer was ordered from Integrated DNA Technologies and purified by HPLC; the methods of characterization are shown in Table 1.2 and purification results are given in Figure 1.6.

Column:	DNAPac PA-100, 9 x 250 mm,
Column Temp:	ambient
Flow rate:	4.0 mL/min
Detection:	UV, 260 nm
Mobile Phase	A: 25 mM Tris-HCl pH 7.4 5% acetonitrile
	B: 25 mM Tris-HCl pH 7.4 5% acetonitrile 1M NaCl

Time (min):	0	5	18	19
%B	10	30	40	10

(A)

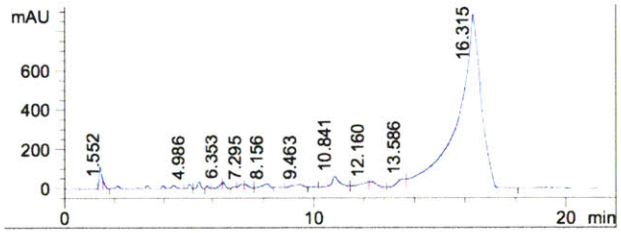
Column:	DNAPac PA-100, 9 x 250 mm,
Column Temp:	ambient
Flow rate:	4.0 mL/min
Detection:	UV, 260 nm
Mobile Phase	A: 25 mM Tris-HCl pH 7.4 5% acetonitrile
	B: 25 mM Tris-HCl pH 7.4 5% acetonitrile 1M NaCl

Time (min):	0	5	17	20
%B	10	50	80	10

(B)

Table 1.2. (A). HPLC conditions for purification of crude 14-mer. (B). HPLC conditions for characterization of the purified 14-mer.

(A)



(B)

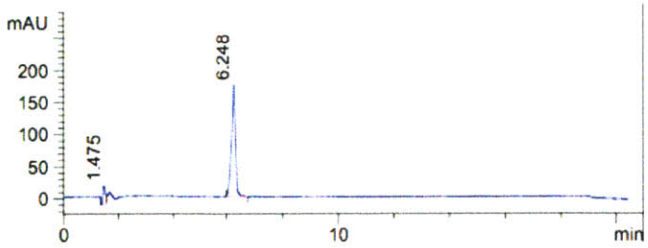


Figure 1.6. (A). HPLC chromatogram of the purification of the 14-mer from failure sequences. The peak at 16.315 min is the desired product; the failure sequences elute earlier, between 4–13 min. (B). Characterization of the purified 14-mer. *Note: purified DNA in (A) shown different location with the major peak in (B) was due to the different gradients (see Table 1.2).*

After treatment of the 14-mer with cisplatin, the platinated sample was purified by HPLC; the purification methods are shown in Table 1.3 and purification results are given in Figure 1.7.

Column:	DNAPac PA-100, 9 x 250 mm,
Column Temp:	ambient
Flow rate:	4.0 mL/min
Detection:	UV, 260 nm
Mobile Phase	A: 25 mM Tris-HCl pH 7.4 5% acetonitrile
	B: 25 mM Tris-HCl pH 7.4 5% acetonitrile 1M NaCl

Time (min):	0	3	11	11.5	12
%B	15	37	42	15	15

Table 1.3. HPLC conditions for isolation of the cisplatin modified 14-mer from the reaction mixture.

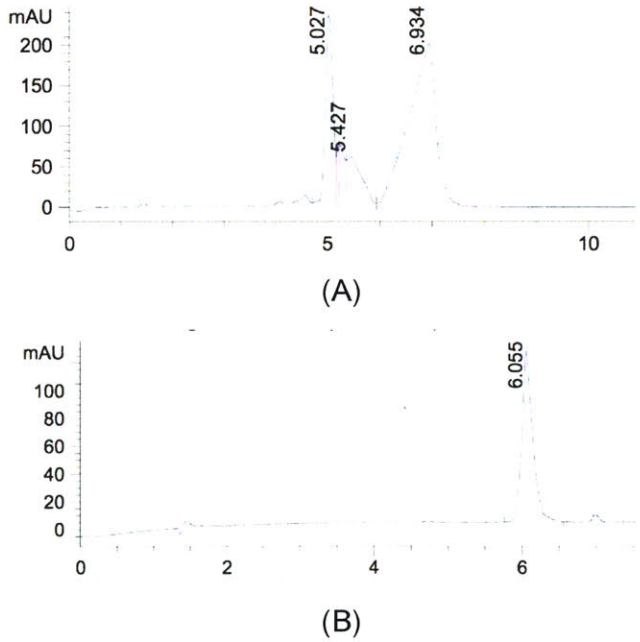


Figure 1.7. (A). HPLC chromatogram of the purification of cisplatin-14-mer. Peak 2 (6.934 min) is the starting material; peak 1 (5.027 min) is the platinated product. (B). Characterization of the purified cisplatin-14-mer.

The final product was tested by AA and UV-Vis spectroscopy and, as indicated by the r_b value (0.99 ± 0.01), the bound platinum to nucleotide ratio,

the isolated product was a pure mono-platinated 14-mer. To analyze the platinated 14-mer, S1 digestion was carried out and compared to results for the original 14-mer. The unplatinated (2 nmol) and platinated (2 nmol) 14-mer were incubated with 5 µL of S1 nuclease, separately, in 100 µL of enzyme buffer (50 mM sodium acetate pH 4.5, 280 mM NaCl, 4.5 mM $ZnSO_4$) at 37 °C overnight. Then 5 µL of 1.5 M Tris-HCl pH 8.8 and 1 µL calf intestinal phosphatase were added and the mixture was incubated for another 4 h at 37 °C. After addition of 6 µL of 0.1 M HCl and centrifugation, both samples were analyzed by HPLC. The results are given in Figure 1.8 and Table 1.4. The shift of the band at the G positions in the platinated sample provides clear evidence of the 1,2 GG-crosslink, because the platinum adduct occupies the N7 positions and blocks the reaction.

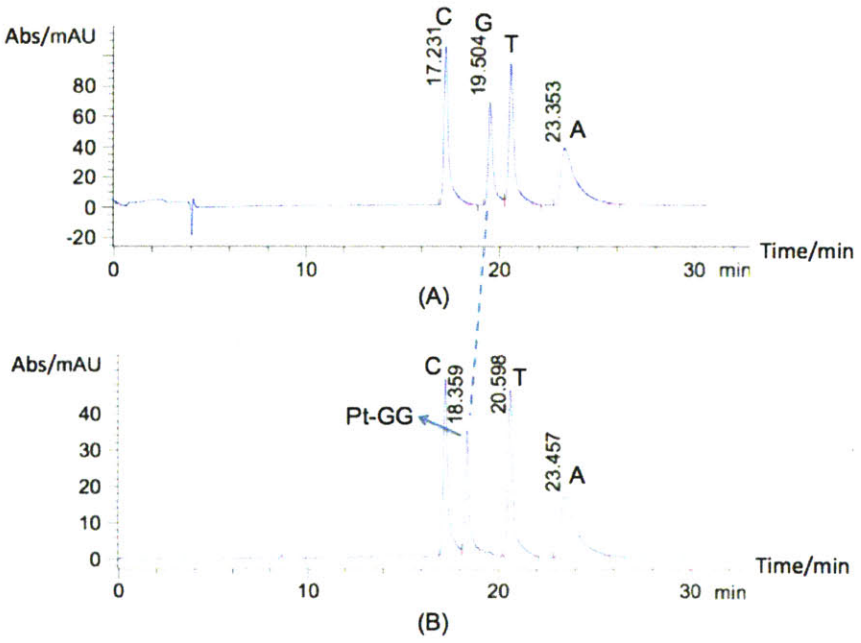


Figure 1.8. (A). HPLC chromatogram of digested 14-mer. (B). HPLC chromatogram of digested platinated 14-mer.

	Relative response factor	Theoretical value	14-mer	Pt-14-mer
C	1.0	5	5.0	5.0
G	1.6	2	2.0	1.8
T	1.2	4	4.1	4.4
A	2.0	3	2.8	2.8

Table 1.4. Characterization of 14-mer and cisplatin platinated 14-mer by nucleotide composition analysis. Relative response factors, which are due to the differences in the extinction coefficient of C, G, T, and A bases, were applied in calculations to integrate signals from different nucleobases.

Preparation of Radiolabeled Full-Length Site-Specifically Modified Platinum-DNA Probes. Ligated cis-Pt-146 bp and phenanthri-Pt-146 bp DNA duplexes were first purified by 6% denaturing gel electrophoresis to remove unligated short DNA strands as shown in Figures 1.9 and 1.10. The isolated samples were extracted from gel pieces and re-annealed followed by characterization with 5% native PAGE as shown in Figure 1.11.

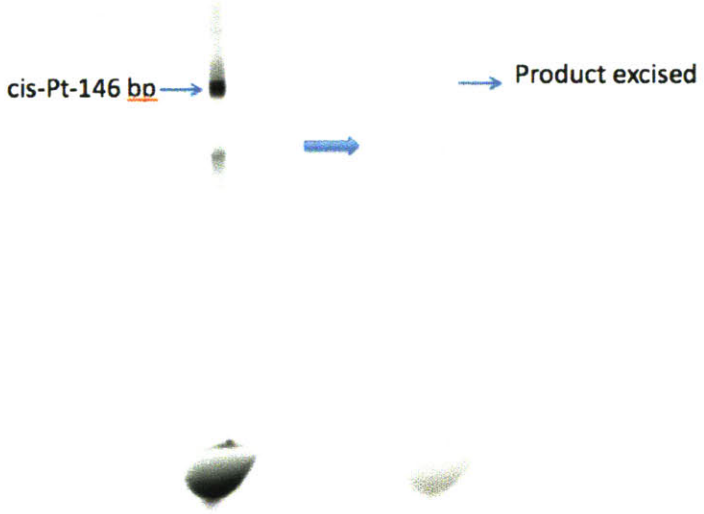


Figure 1.9. Purification of ligated site-specifically cisplatin modified 146 bp with 6% denaturing PAGE (70 V, 1800 W, 110 min).

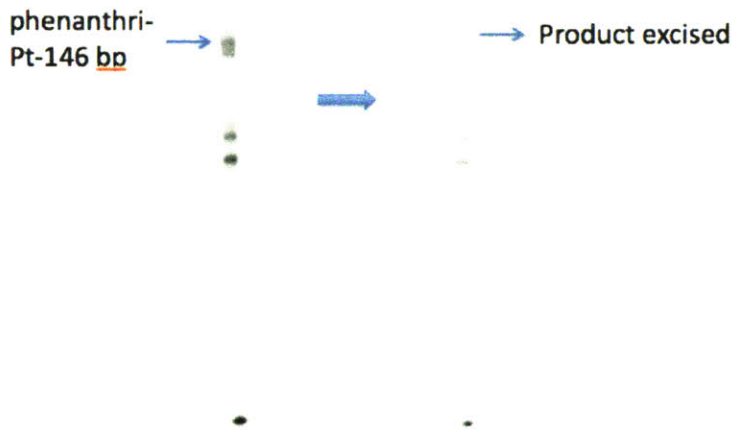


Figure 1.10. Purification of ligated site-specifically phenanthriplatin platinated 146 bp DNA with 6% denaturing PAGE.

Figure 1.11. Native PAGE gel characterization of purified site-specifically platinated 146 bp duplexes: lane 1: DNA ladder; lane 2: site-specifically cisplatin platinated 146 bp; lane 3: site-specifically phenanthriplatin platinated 146 bp.

Restriction Enzyme Digestion of the Cisplatin Modified 146 bp. Platination of the 146 bp duplex was checked by digestion with the restriction enzyme

EcoRI and comparison to digestion of the unplatinated 146 bp duplex. The modified DNA duplex is cut completely by the enzyme, but the platinated DNA duplex should not be cut at all. A 2.2% agarose gel run for the experiment, as shown in Figure 1.12, reveals that the platination cross-link blocks digestion (lane 5).

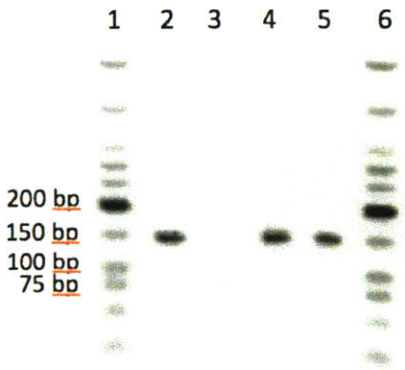


Figure 1.12. Agarose gel result of restriction digestion of unplatinated and cisplatin platinated 146 bp. Lane 1: DNA ladder; lane 2: unplatinated 146 bp; lane 3: unplatinated 146 bp incubated with EcoRI; the restriction site is in the middle of the duplex so the digestion products were ~75-mers; lane 4: cisPt-146 bp; lane 5: cisPt-146 bp incubated with EcoRI; Lane 6: DNA ladder.

Comparison of Nucleotide Excision Repair from Platinated 146 bp with Cisplatin versus Phenanthriplatin. The substrates used in this study were cisplatin and phenanthriplatin platinated 146 bp duplexes. After incubation with repair proteins, the reaction products were analyzed with 8% denaturing PAGE (Figure 1.13). Comparison of excision signals for cisplatin and phenanthriplatin platinated DNA indicates that the efficiency of NER of site-specifically cisplatin platinated DNA is 3-fold higher than that of site-specifically phenanthriplatin platinated DNA.

Figure 1.13. Excision assay with site-specifically platinated 146 bp duplexes. Lane 1: site-specifically cisplatin platinated 146 bp; lane 2: site-specifically phenanthriplatin platinated 146 bp.

SUMMARY AND CONCLUSION

Site-specifically platinated 146 bp duplexes were synthesized from cisplatin and phenanthriplatin, purified, and characterized for use in a nucleotide excision repair assay. The excision efficiency of the cisplatin modified DNA is 2.45%, which is greater than that of the phenanthriplatin modified sample, 0.52%. These results indicate that phenanthriplatin, a monofunctional anticancer drug candidate, can elude the nucleotide excision

repair pathway better than cisplatin. Other pathways for repairing this lesion remain to be investigated.

ACKNOWLEDGEMENTS

Thanks to Dr. Ga Young Park for making purified phenanthriplatin platinated 16-mer for ligation of phen-Pt-146 bp duplex, Joyce Reardon in the Sancar lab at the University of North Carolina for providing repair proteins, Dr. Guangyu Zhu and Semi Park for technical and experimental expertise, and members of the Lippard group for helpful discussion and suggestions.

Chapter 2.

Site-specifically Platinated Long DNA Strands from pCMV-GLuc Plasmids for Atomic Force Microscopy (AFM) Studies

INTRODUCTION

Various platinum compounds with non-classical structures have been designed in order to lower side effects or circumvent the resistance that arises in treatment with conventional platinum based agents.[16] Previous research revealed that some monofunctional platinum complexes are able to lead to apoptosis as well.[17] The structure of modified DNA reveals that bending, unwinding, and distortions of the double helix are critical determinants of cisplatin cytotoxicity in vivo.[4] Based on the monofunctional nature of phenanthriplatin-dG lesions and the expected lack of canonical DNA distortion, other factors such as its ability to stall RNA Pol II are likely to account for its activity. Another factor that may be relevant is the influence of platination on DNA-mediated charge transfer (CT). If the DNA contains a mismatched base pair or lesion that distorts the structure of the DNA base stack, charge transfer along the DNA strand will be cut off.[18] In electrochemical studies, this would be manifest as an attenuated signal compared to control DNA.[19]

Atomic force microscopy (AFM) is another method by which we can explore the possibility that phenanthriplatin influences charge transfer properties of DNA. Briefly, AFM is used to ascertain whether proteins that are able to send and receive charge redistribute onto strands of DNA that contain damage. This redistribution would result from an inability of the proteins to transfer charge through the DNA. When proteins collect around the damage, signal traveling through DNA stops.[20] AFM can be used to directly image the distribution of proteins on the DNA strand and provide some clues as to the

manner by which platinatum lesions are detected before excision. Long DNA strands with and without site-specific platination and with a mismatched base pair were prepared by the 'gapping' strategy, previously developed in the Lippard lab.[21] In addition to a strand containing the monofunctional drug phenanthriplatin, DNA strands containing either a site-specific cisplatin lesion or a mismatched base pair were also synthesized to be used as controls for the AFM experiments carried out by the Barton group at Caltech.

Experimental Procedures

Materials. All chemical reagents were obtained from Sigma-Aldrich unless otherwise stated. All enzymes and enzyme buffers were purchased from New England Biolabs unless otherwise noted. DNA strands were bought from IDT and DH5α cells were obtained from Invitrogen. DNA concentrations were measured by UV/vis spectroscopy at 260 nm using a Varian Cary 1E-spectrometer fitted with a microprobe and using an extinction coefficient of 50 µg/mL/OD. Agarose electrophoreses through gels (0.8-1.0% w/v) containing 0.5 µg/ml EtdBr were imaged using the BioRad Fluor-S MultiImager.

Vector Construction and Preparation. Gaussia luciferase expression vectors for incorporation of site-specific cisplatin-dGG and phenanthriplatin-dG lesions, pGLuc4temGG and pGLuc8temG, respectively, were prepared by following protocols reported previously.[21]

Preparation of Platinated Insertion Strand. A 16-mer oligonucleotide containing a site-specific *cis*-$\{Pt(NH_3)_2\}^{2+}$-dGG lesion and a 16-mer

oligonucleotide containing a site specific *cis*-$\{Pt(NH_3)_2(phen)\}^{2+}$-dG (phen=phenanthridine) lesion were made and purified by Dr. Ga Young Park.

Preparation of Long DNA Duplexes (3,982 bp, 2,230 bp, and 1,752 bp) from pGLuc Plasmids. Both pGLuc4temGG and pGLuc8temG plasmids were transformed into DH5α cells on LB agar plates separately with 100 mg/L of ampicillin. Colonies were randomly picked for larger scale inoculation. The plasmids were purified with a Qiagen maxiprep kit. With purified plasmids in hand, BstZ17I and ScaI restriction enzymes were used to obtain 3,982 bp, 2,230 bp, and 1,752 bp duplexes as shown in Figure 2.1.

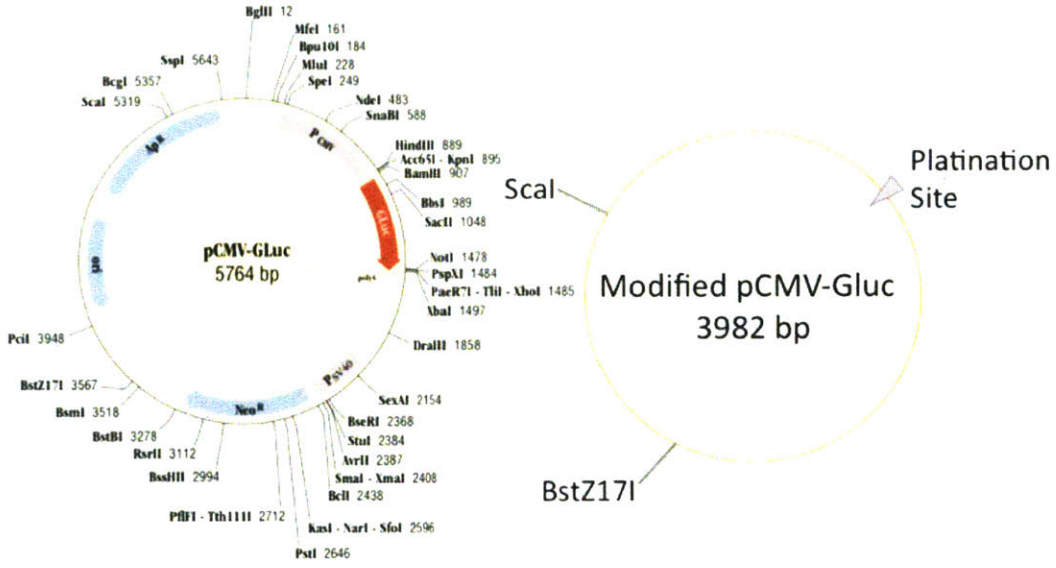


Figure 2.1. Left. Restriction map of pCMV-GLuc Vector from BioLabs Inc. website. Only unique restriction sites are shown. Rigth. Modified pGLuc vector with platination site and restriction sites related to preparation of long DNA strands for AFM studies.

The plasmid was incubated with a four-fold excess of restriction enzymes (BstZI17I and ScaI) at 37 °C for 80 min. Following incubation, two phenol/chloroform/isoamyl alcohol extractions were performed to remove the enzymes. An 0.8% agarose gel was run at 112 V for 100 min to separate the

DNA strands. Different bands were cut under UV light and separately put into 3,500 MWCO tubes with 5 mL of 1X TAE buffer. The tubing was sealed, and a voltage of 150 V was applied to the sample for another 60 min to elute the DNA from the gel resin into the buffer. The solution was concentrated and precipitated with ethanol. The DNA pellets were dissolved in water, and the DNA concentrations were measured by UV/Vis spectroscopy at 260 nm. Sample purity was verified by 1.2% analytical agarose gel as shown in Figures 2.2 and 2.3.

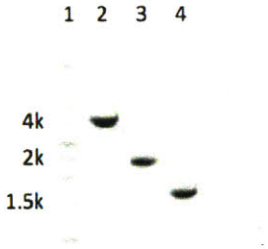


Figure 2.2. Agarose gel characterization of long DNA strands from pGLuc 4temGG plasmid. Lane 1: 1kb DNA ladder; lane 2: 3982 bp from pGLuc4temGG; lane 3: 2230 bp from pGLuc4temGG; lane 4: 1752 bp from pGLuc4temGG.

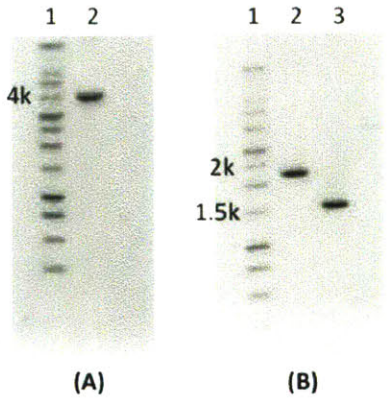


Figure 2.3. Agarose gel characterization of long DNA strands from pGLuc 8temG plasmid. (A) Lane 1: 1kb DNA ladder; lane 2: 3982 bp from pGLuc8temG. (B) Lane 1: 1kb DNA ladder; lane 2: 2230 bp from pGLuc8temG; lane 3: 1952 bp pGLuc8temG.

Preparation of Site-Specifically Platinated Long DNA Duplexes from pGLuc Plasmids. Site-specifically platinated pGLuc4temGG, 3,982 bp containing a *cis*-$\{Pt(NH_3)_2\}^{2+}$-dGG lesion between the CMV promoter and luciferase expression gene, was prepared following the strategy published previously[21] as shown in Figure 2.4.

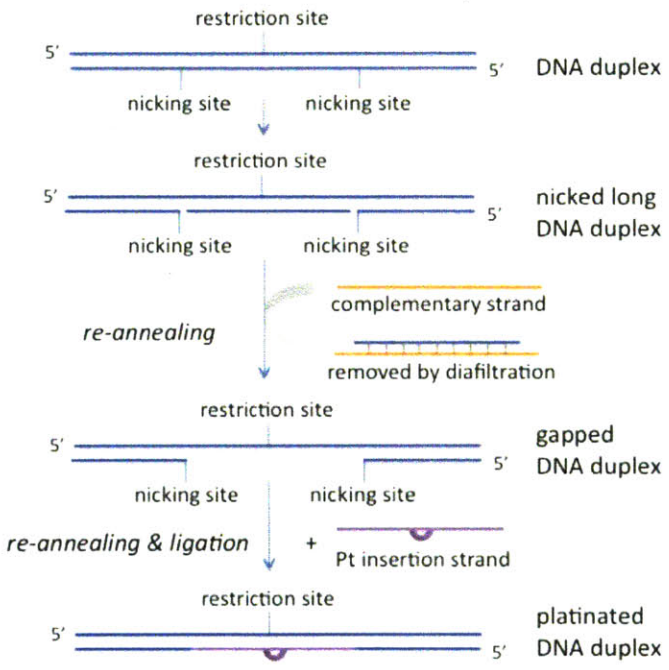


Figure 2.4. Overall scheme for preparing platinated DNA strands.

Briefly, a 90 µg quantity of pGLuc4temGG 3,982 bp was digested with 190 U Nt.BspQI at 37 °C for 1 h. The reaction mixture was heated at 80 °C for 20 min to deactivate the enzyme, followed by two phenol/chloroform/isoamyl alcohol extractions to remove the enzyme. The mixture was ethanol precipitated at -20 °C. The nicked plasmid was mixed with 1,000 equiv of complementary DNA strand, 5'-TTTTGGAAGAGACGAGGAGGTTTT, in a buffer of 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.4 M NaCl, pH 7.4, heated at 80 °C for 5 min, and subsequently cooled at 4 °C over 4 hr. The gapped plasmid

was purified by centrifugation through a 50 k MWCO ultrafiltration tube at 3,000 rpm, 4 °C for 30 min, and quantitated by UV-vis spectroscopy. A 55 μg quantity of the gapped plasmid was annealed with 100 equiv of the insertion strand in a buffer of 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.4 M NaCl, pH 7.4 from 90 °C to 4 °C over 4 hr.

Restriction Analysis of Site-Specifically Platinated Plasmids. To carry out a restriction analysis on ligated platinated or unplatinated plasmids, a 0.2 μg quantity of pGLuc4temGG 3,982 bp, 2230 bp, and mismatched 3,982 were incubated with 0.8 U PflMI at 37 °C for 70 min. The DNA samples were analyzed using 0.8% agarose gel electrophoresis. The gels containing 0.5 μg/mL ethidium bromide were analyzed with a BioRad Fluor-S MultiImager. For pGLuc8temG DNA samples, BsmBI was used for restriction analysis.

Atomic Force Microscopy (AFM) Images (These experiments were carried out by Pam Sontz in the Barton group). Three sets of samples were studied with AFM. In the first set, 6 mM $MgCl_2$ and 100 ng of mismatched-long strands 3982 bp with C:A mismatched were mixed with 100 ng of matched short strands 2230 bp and the mixture was incubated in Tris-EDTA buffer overnight at 4 °C, followed by incubation with 0.6 μM portion of wild-type Endo III overnight again. In the second set, 6 mM $MgCl_2$ and 100 ng of cisplatin 4temGG 3982 bp were mixed with 100 ng of matched control short strands 2230 bp and the mixture was incubated in Tris-EDTA buffer overnight at 4 °C, followed by incubation with 0.6 μM portion of wild-type Endo III overnight again. In the third set, 6 mM $MgCl_2$ and 100 ng of phenanthriplatin 8temG 3982 bp were mixed with 100 ng of matched control short strands 2230 bp

and the mixture was incubated in Tris-EDTA buffer overnight at 4 °C, followed by incubation with 0.6 μM portion of wild-type Endo III overnight again.

RESULTS AND DISCUSSION

Construction of pGLuc4temGG 3,982 bp and 2,230 bp Containing a Site-Specific Cisplatin Lesion, pGLuc4temGG 3,982 bp Containing a Mismatched Base Pair and pGLuc8temG 3,982 bp and 2,230 bp Containing Site-Specific Phenanthriplatin Lesion. A 16-mer synthetic oligonucleotide containing a site-specific cisplatin lesion, pGLuc4temGG-IS-Pt (5'-ACCTTCTG*G*CTCTTCC, where the asterisks denote the platinated bases) and a 16-mer containing a site-specific phenanthriplatin lesion, pGLuc8temG-IS-Pt (5'-ACTCCTCG*TCTCTTCC), were prepared by Dr. Ga Young. The positions of the modified sites in plasmids are shown in Figure 2.5.

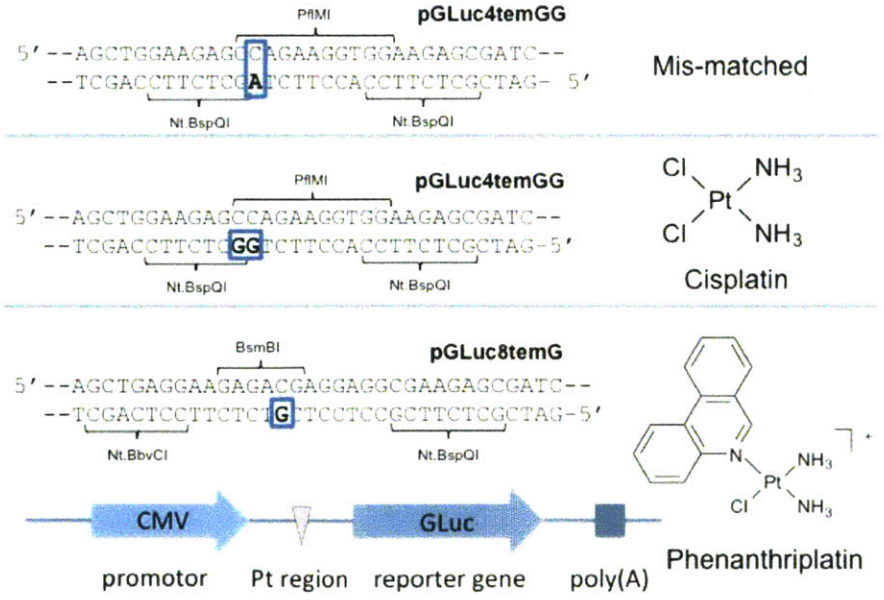


Figure 2.5. DNA sequence of the platination regions in pGLuc4temGG and pGLuc8temG vector; mismatched base pair and platination sites are highlighted in the blue boxes.

Two unique Nt.BbvCI restriction sites are 16 nucleotides away from each other. The designed 16-mer synthetic oligonucleotide containing a site-specific cisplatin-dGG lesion was inserted into the strand between the two nicking restriction sites by the "gapping" strategy that we published previously.[21] Restriction analysis of unplatinated and platinated DNA strands was carried out using PflMI. Platination of the DNA by cisplatin blocked restriction digestion, showing that incorporation of cisplatin was successful. Similar restriction enzyme digestions were carried out for all the DNA samples either modified by platination or mismatched base pair, as shown in Figures 2.6 – 2.10.

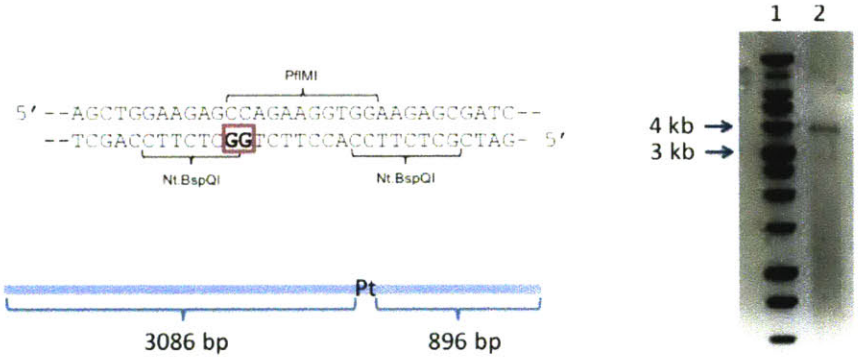


Figure 2.6. Agarose gel electrophoresis characterization of cisPt-4temGG 3982 bp. Lane 1: 1 kb DNA ladder;lane 2: cisPt-4temGG3982 bp after incubation with restriction enzyme PflMI.

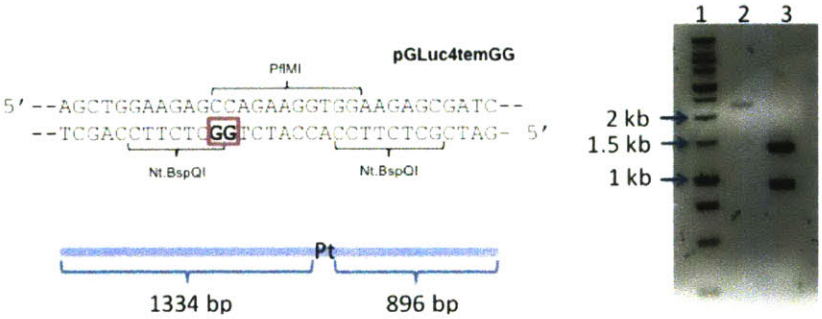


Figure 2.7. Agarose gel electrophoresis characterization of cisPt-4temGG 2230 bp. Lane 1: 1 kb DNA ladder; lane 2: cisPt-4temGG2230 bp after incubation with restriction enzyme PflMI. The platination blocked the digestion site so only one band was observed; lane 3: 4temGG2230 bp after incubation with restriction enzyme PflMI.

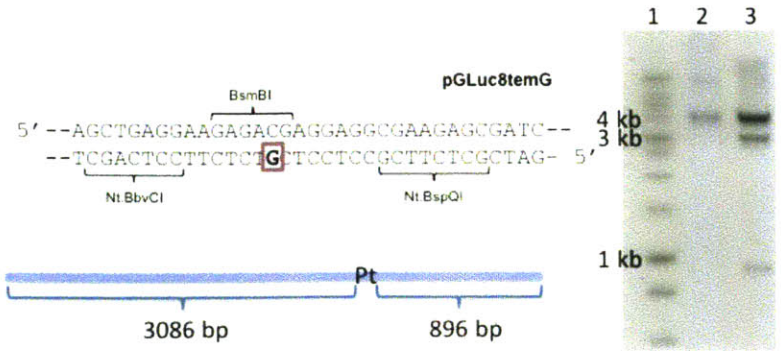


Figure 2.8. Agarose gel electrophoresis characterization of phenPt-8temG 3982 bp. Lane 1: 1 kb DNA ladder; lane 2: phenPt-8temG3982 bp after incubation with restriction enzyme BsmBI; lane 3: 8temG3982 bp after incubation with restriction enzyme BsmBI.

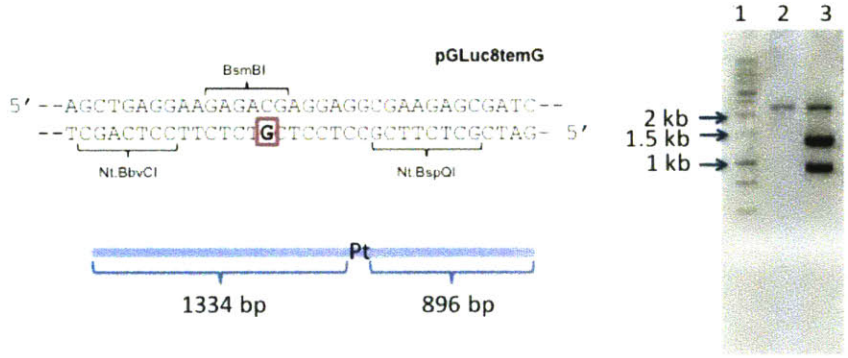


Figure 2.9. Agarose gel electrophoresis characterization of phenPt-8temG 2230 bp. Lane 1: 1 kb DNA ladder; lane 2: phenPt-8temG2230 bp after incubation with restriction enzyme BsmBI; lane 3: 8temG2230 bp after incubation with restriction enzyme BsmBI.

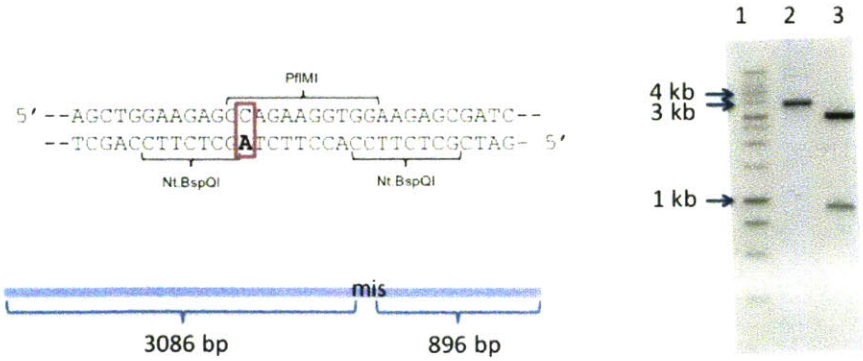


Figure 2.10. Agarose gel electrophoresis characterization of mismatched 4temGG3982 bp. Lane 1: 1 kb DNA ladder; lane 2: Mismatched 4temGG3982 bp after incubation with restriction enzyme PflMI; lane 3: 4temGG3982 bp after incubation with restriction enzyme PflMI.

Atomic Force Microscopy Images. An aliquot of mismatched 3982 bp and matched control short strands 2230 bp was imaged, and the results are shown in Figure 2.11. An aliquot of cisplatin 4temGG 3982 bp matched control short strands 2230 bp was imaged, and the results are shown in Figure 2.12. An aliquot of phenanthriplatin 8temG 3982 bp matched control short strands 2230 bp was imaged, and the results are shown in Figure 2.13.

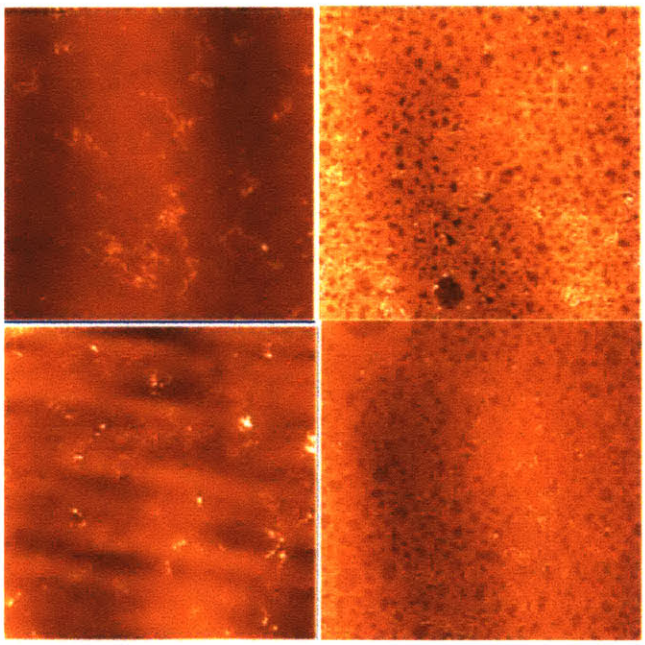


Figure 2.11. AFM images of mismatched mixtures (mismatched-long strands 3982 bp with C:A mismatched and matched short strands 2230 bp DNA) with 0.6 μM Endo III. The bright dots in the images are proteins.

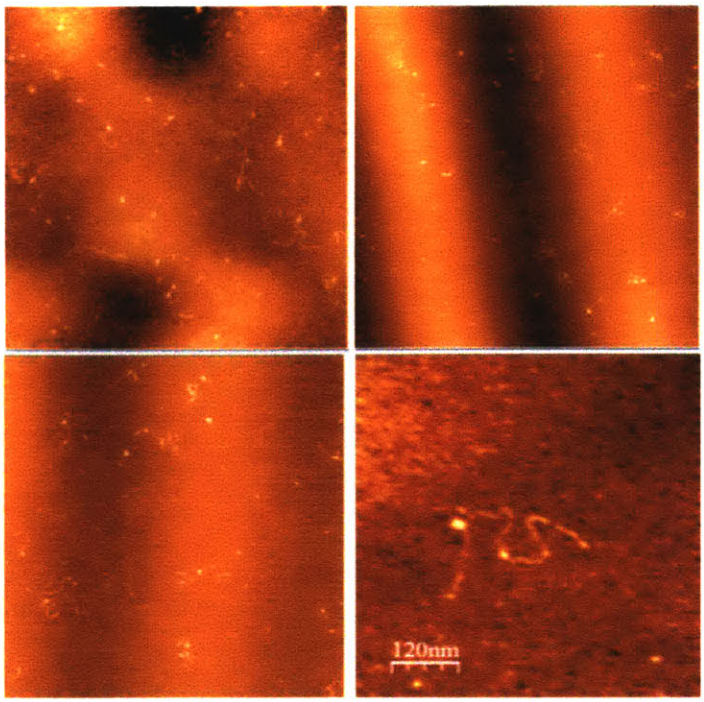


Figure 2.12. AFM images of mixture (cisplatin-long strands 3982 bp and matched short strands 2230 bp) DNA with 0.6 μM Endo III. The last image was zoomed in and it shows DNA strand bound with proteins.

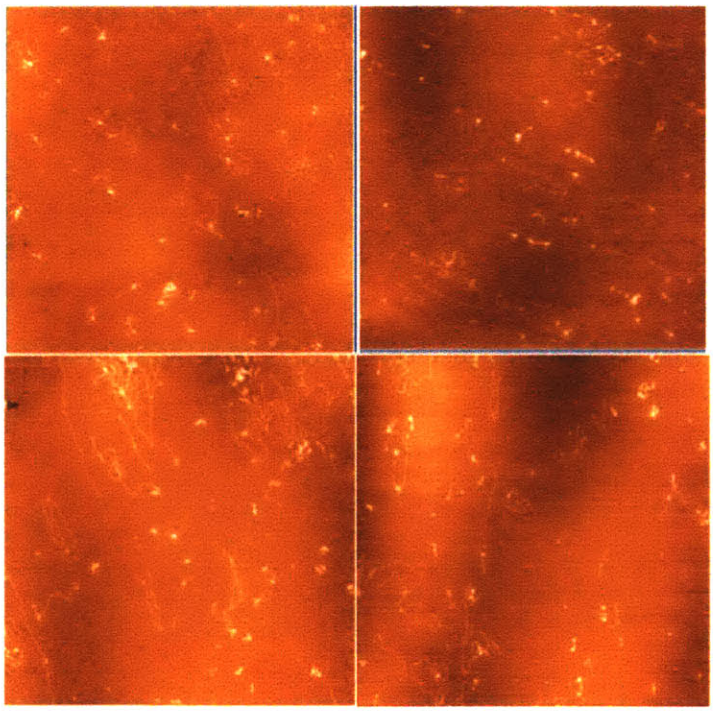


Figure 2.13. Mixture (Phenanthriplatin-Long strands 3982 bp and Matched Short Strands 2230 bp) DNA with 0.6 μM Endo III.

Examination of AFM images from our collaborator revealed a slight redistribution of Endonuclease III onto strands that contain a cisplatin 1,2-GG crosslink with a binding density ratio (long/short) of r = 1.23 ± 0.08. As a control, we examined the redistribution of Endonuclease III in the presence of mismatched 4temGG plasmid, where one guanine residue is replaced with an A base to form a C:A mismatch. This phenanthriplatin inhibits transcription but may evade repair machinery more than cisplatin. We observed that Endonuclease III does not redistribute onto the phenanthriplatin 8temG 3982 bp, r=1.04 ± 0.06. The binding ratios of three samples were summarized in Figure 2.14.

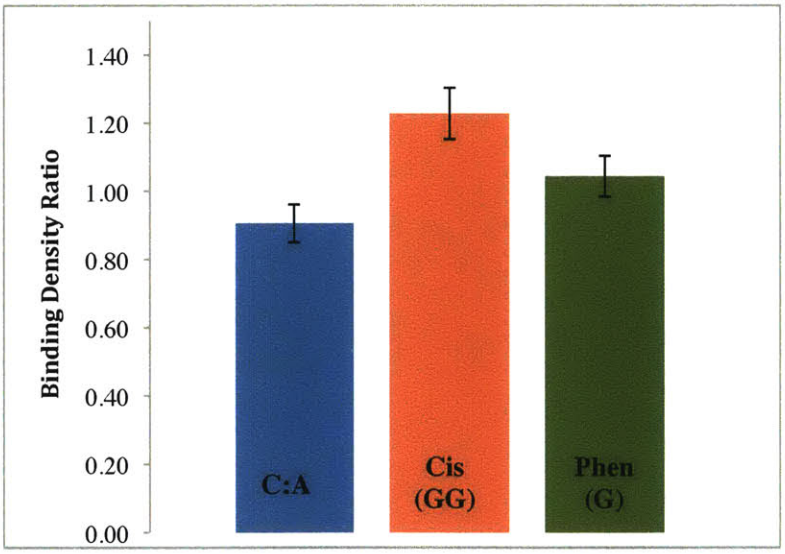


Figure 2.14. Binding Density Ratios of mismatched DNA, cisplatin platinated DNA and phenanthriplatin platinated DNA.

SUMMARY AND CONCLUSION

Eleven DNA strands were made, purified, characterized and summarized in Table 2.1. The cisplatin platinated 3982 bp, cisplatin platinated 2230 bp,

mismatched (C:A) 3982 bp, fully matched 3982 bp, 2230 bp, and 1752 bp from 4temGG plasmid, phenanthriplatin platinated 3982 bp, phenanthriplatin platinated 2230 bp, and fully matched 3982 bp, 2230 bp, and 1752 bp from 8temG plasmid. Both platinated DNA strands show higher binding density ratios than mismatched ones, indicating that platination could block the DNA mediated charge transport more effectively than mismatched base pairs. Compared with cisplatin modification, phenanthriplatin had a lower binding density ratio, which indicated that phenanthriplatin could evade the repair proteins' detection and this could be a possible reason for why phenanthriplatin showed a lower efficiency of nucleotide excision repair in Chapter 1.

DNA Strands for AFM Studies
1. Cisplatin (platinated) long strand from plasmid insertion (3982 bp)
2. Cisplatin (platinated) short strand from plasmid insertion (2230 bp)
3. Mismatched (C:A) long strand from plasmid insertion (3982 bp)
4. Long strand fully matched from plasmid insertion (3982 bp GG)
5. Short strands from plasmid restriction GG (2230 bp)
6. Short strands from plasmid restriction GG (1752 bp)
7. Phenanthriplatin (platinated) long strand from plasmid insertion (3982 bp)
8. Phenanthriplatin (platinated) short strand from plasmid insertion (2230 bp)
9. Long strand fully matched from plasmid insertion (3982 bp G)
10. Short strands from plasmid restriction G (2230 bp)
11. Short strands from plasmid restriction G (1752 bp)

Table 2.1. All the DNA strands prepared for AFM studies.

REFERENCES:

1. Lippard, S.J. New Chemistry of an Old Molecule - *cis*-[$Pt(NH_3)_2Cl_2$]. *Science* **218**, 1075-1082 (1982).
2. Keys, H.M. *et al.* Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. *New Engl. J. Med.* **340**, 1154-1161 (1999).
3. Morris, M. *et al.* Pelvic radiation with concurrent chemotherapy compared with pelvic and para-aortic radiation for high-risk cervical cancer. *New Engl. J. Med.* **340**, 1137-1143 (1999).
4. Wang, D. & Lippard, S.J. Cellular processing of platinum anticancer drugs. *Nat. Rev. Drug Discov.* **4**, 307-320 (2005).
5. Wong, E. & Giandomenico, C.M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **99**, 2451-2466 (1999).
6. Blommaert, F.A. *et al.* Formation of DNA-Adducts by the Anticancer Drug Carboplatin - Different Nucleotide-Sequence Preferences in-Vitro and in Cells. *Biochemistry* **34**, 8474-8480 (1995).
7. Fichtingerschepman, A.M.J., Vanderveer, J.L., Denhartog, J.H.J., Lohman, P.H.M. & Reedijk, J. Adducts of the Antitumor Drug *cis*-diamminedichloroplatinum(Ii) with DNA - Formation, Identification, and Quantitation. *Biochemistry* **24**, 707-713 (1985).
8. Jamieson, E.R. & Lippard, S.J. Structure, recognition, and processing of cisplatin-DNA adducts. *Chem. Rev.* **99**, 2467-2498 (1999).

9. Ang, W.H., Myint, M. & Lippard, S.J. Transcription Inhibition by Platinum-DNA Cross-Links in Live Mammalian Cells. *J. Am. Chem. Soc.* **132**, 7429-7435 (2010).

10. Shimizu, M. & Rosenber.B Similar Action to Uv-Irradiation and a Preferential Inhibition of DNA-Synthesis in Escherichia-Coli by Antitumor Platinum Compounds. *J. Antibiot* **26**, 243-245 (1973).

11. Lippard, S.J. & Hoeschele, J.D. Binding of *cis*-dichlorodiammineplatinum(II) and *trans*-dichlorodiammineplatinum(II) to the Nucleosome Core. *P. Natl. Acad. Sci. USA* **76**, 6091-6095 (1979).

12. Millard, J.T. & Wilkes, E.E. *cis*- and *trans*-Diamminedichloroplatinum(II) interstrand cross-linking of a defined sequence nucleosomal core particle. *Biochemistry* **39**, 16046-16055 (2000).

13. Wang, D., Zhu, G.Y., Huang, X.H. & Lippard, S.J. X-ray structure and mechanism of RNA polymerase II stalled by a monofunctional antineoplastic agent. *Abstr. Pap. Am. Chem. S.* **240** (2010).

14. Jaramillo, D. Wheate, N.J., Ralph, S.F., Howard, W.A., Tor, Y., and Aldrich-Wright, J.R. Polyamide platinum anticancer complexes designed to target specific DNA sequences. *Inorg. Chemistry* **45**, 6004-6013 (2006).

15. Hollis, L.S., Amundsen, A.R. & Stern, E.W. Chemical and Biological Properties of a New Series of *cis*-diammineplatinum(II) Antitumor Agents Containing 3 Nitrogen Donors - *cis*-$[Pt(NH_3)_2(N\text{-Donor})Cl]^+$. *J. Med. Chem.* **32**, 128-136 (1989).

16. Todd, R.C., Lovejoy, K.S. & Lippard, S.J. Understanding the effect of carbonate ion on cisplatin binding to DNA. *J. Am Chem. Soc.* **129**, 6370-6371 (2007).

17. Graf, N., Ang, W.H., Zhu, G.Y., Myint, M. & Lippard, S.J. Role of Endonucleases XPF and XPG in Nucleotide Excision Repair of Platinated DNA and Cisplatin/Oxaliplatin Cytotoxicity. *Chembiochem* **12**, 1115-1123 (2011).

18. Boal, A.K. *et al.* Redox signaling between DNA repair proteins for efficient lesion detection. *P. Natl. Acad. Sci. USA* **106**, 15237-15242 (2009).

19. Boon, E.M. & Barton, J.K. Charge transport in DNA. *Curr. Opin. Struct. Biol.* **12**, 320-329 (2002).

20. Sontz, P.A., Mui, T.P., Fuss, J.O., Tainer, J.A. & Barton, J.K. DNA charge transport as a first step in coordinating the detection of lesions by repair proteins. *P. Natl. Acad. Sci. USA* **109**, 1856-1861 (2012).

21. Ang, W.H., Brown, W.W. & Lippard, S.J. Preparation of Mammalian Expression Vectors Incorporating Site-Specifically Platinated-DNA Lesions. *Bioconjugate Chem.* **20**, 1058-1063 (2009).

BIOGRAPHICAL NOTE

The author was born on March 10th, 1988 in Luoyang, Henan, China, the daughter of Zhengang Li and Chunli Li. She started to learn piano and Chinese dancing before elementary school and bel canto when she was 11 years old. After graduated from Xingzhi Senior High school in Shanghai, she attended Fudan University in Shanghai, China in 2006 and joined Professor Fuyou Li's group in 2007. She went to the University of Hong Kong in 2008 as an exchange student and worked in the laboratory of Professor Chi-Ming Che. In 2009, she went to University of California, Los Angeles, for a summer research program and worked in the laboratory of Professor Carla Koehler. During her time at Fudan University, she earned Scholarship in Fudan University and Basic Science Scholarship several times. After she graduated from Fudan with a B.S. in chemistry in 2010, she attended the Massachusetts Institute of Technology to work in the laboratory of Professor Stephen J. Lippard. At the beginning of 2012, she started to learn *The Great Treatise on the Stages of the Path to Enlightenment (Lamrim Chenmo)*, which was written by Master Tsong-kha-pa. She is focused on learning *Lamrim Chenmo* taught by Shifu and is volunteering in Bliss and Wisdom group.