Mechanism of Action and Gold Nanoparticle Delivery of Pt(IV) Prodrugs of Cisplatin

Yi Shi
Syracuse University

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Abstract

Mechanism of Action and Gold Nanoparticle Delivery of Pt(IV) Prodrugs of Cisplatin

Yi Shi

Syracuse University, December 2012

The antitumor effects of platinum(IV) complexes, considered prodrugs for cisplatin, are believed to be due to biological reduction of Pt(IV) to Pt(II), with the reduction products binding to DNA and other cellular targets. In this work we used pBR322 DNA to capture the products of reduction of oxoplatin, $c,t,c$-$[\text{PtCl}_2(\text{OH})_2(\text{NH}_3)_2]$, 2, and a carboxylate-modified analog, $c,t,c$-$[\text{PtCl}_2(\text{OH})(\text{O}_2\text{CCH}_2\text{CH}_2\text{CO}_2\text{H})(\text{NH}_3)_2]$, 3, by ascorbic acid (AsA) or glutathione (GSH). Since carbonate plays a significant role in the speciation of platinum complexes in solution, we also investigated the effects of carbonate on the reduction/DNA-binding process. In pH 7.4 buffer in the absence of carbonate, both 2 and 3 are reduced by AsA to cisplatin (confirmed using $^{195}\text{Pt}$ NMR), which binds to and unwinds closed circular DNA in a manner consistent with the formation of the well-known 1, 2 intrastrand DNA crosslink. However, when GSH is used as the reducing agent for 2 and 3, $^{195}\text{Pt}$ NMR shows that cisplatin is not produced in the reaction medium. Although the Pt(II) products bind to closed circular DNA, their effect on the mobility of Form I DNA is different from that produced by cisplatin. When physiological carbonate is present in the reduction medium, $^{13}\text{C}$ NMR shows that Pt(II) carbonato complexes form which block or impede platinum binding to DNA. Another Pt(IV) complex, 1-adamantanemethylamine-Pt(IV), 4, which is also a potential prodrug of cisplatin was synthesized by tethering 1-
 adamantanemethylamine to oxoplatin using a dicarboxylate linker. To achieve higher anticancer activity and better targeting, gold nanoparticle (AuNP) coated with the host ligand per-6-thio-β-cyclodextrin, was synthesized as a drug delivery vehicle for complex 4. The results of cytotoxicity studies show that the loaded delivery vehicle inhibits the growth of human neuroblastoma cells and optical microscopy indicates that the nanoparticles localize in the nucleus of the cell. The binding of complex 4 and β-cyclodextrin is also investigated using 1H NMR, and a model with a 1:1 stoichiometry with formation of adamantane dimers is proposed. To achieve better understanding of the effect of recovery time on cell cytotoxicity, the toxic effects of three metal complexes containing the 2,2'-bpyridine ligand, Cu(bpy)(NCS)₂, 8, [Cu(bpy)₂(H₂O)](PF₆)₂, 9, and Zn(bpy)₂(NCS)₃, 10, were investigated toward human neuroblastoma cells (SK-N-SH) and ovarian cancer cells (OVCAR-3) using two different cytotoxicity assays. The cells were exposed to various concentrations of the compounds for 1 h and the percent inhibition of cell growth, I, measured for various times after exposure, i.e., as a function of the recovery time t. After developing the theory showing the relationship between I and t, the cytotoxicity data were analyzed to reveal that the two copper complexes, 8 and 9, cause the cells to divide at a slower rate than the controls during the recovery period, but the zinc complex, 10, had little or no effect on cell division during the recovery period. The usual metric for reporting cytotoxicity is IC₅₀, which is the concentration of agent required to inhibit cell growth to 50% of the control population. However, since IC₅₀ can depend on the recovery time, t, as is the case for 8 and 9, reporting IC₅₀ for a single recovery time can hide important information about the long-time effects of a cytotoxic agent on the health of the cell population.
Mechanism of Action and Gold Nanoparticle Delivery of Pt(IV) Prodrugs of Cisplatin

by

Yi Shi

B.S. Jilin University, Changchun, Jilin, China 2007

Dissertation
Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Chemistry

Syracuse University
December 2012
Dedicated to my parents,
for nourishing my soul,

and my best friend, Taotao Chen,
for always being there for me
Acknowledgments

Working on the Ph.D. has been a wonderful and eye opening experience. In this journey, I am indebted to many people for making my life in Syracuse unforgettable. First of all, my deepest appreciation goes to my advisor Prof. James C. Dabrowiak. Working with him has been a real pleasure and honor to me. He has oriented and supported me with patience and care throughout my Ph.D career. I would like to thank him for sharing his wealth of wisdom and knowledge. His encouragement strengthened me and it was his help that made this work possible.

Furthermore, I would like to thank Prof. Jerry Goodisman for all the beneficial discussions and generous help and support on data analysis; Prof. Yan-Yeung Luk and his former postdoctoral associate Dr. Dawei Cui, former graduate student Dr. Deepali Prashar, graduate student Sijie Yang for their help on my experiments and for sharing their lab facilities; Ms. Bonnie Toms for meticulous instruction on cell culture studies; Prof. Mathew M. Maye for advice on my research in gold nanoparticles. Also, I would like to thank Prof. Lallan Mishra research group for providing the metal complexes used in my cell studies; Dr. Deborah. J. Kerwood for her contributions to NMR studies; Ms. Shu-An Liu for her contributions to gel studies and my lab mate Colleen Alexander for sharing lab space and the helpful discussions.

In addition, I appreciate that Prof. Dabrowiak, Prof. James T. Spencer and Prof. Goodisman served on my graduate research committee for five years and Prof. Joseph Chaiken, Prof. Luk, Prof. Maye, Prof. Dacheng Ren, who along with Prof. Goodisman and Prof. Dabrowiak made time to serve on my doctoral defense committee. I am thankful for the Department of Chemistry at Syracuse University, and the Department of Pediatrics at SUNY
Upstate Medical University, for their financial support of this research. I also want to thank all the staff in the Department of Chemistry who always work hard to help the graduate students.

Lastly, a special thank you goes out to my family and friends. Nobody knows how grateful I am for receiving infinite love and support from my family and friends from home, despite we are thousands of miles apart. I also feel very lucky to get to know my friends in Syracuse, especially my former roommate Qianqian Cao, current roommates Bin Deng and Sijie Yang. I will never forget the laughs and tears we shared during the past five years.
## Table of Contents

Acknowledgments ........................................................................................................ vi

Table of contents ........................................................................................................ viii

List of symbols and acronyms ...................................................................................... xi

List of figures ................................................................................................................ xiii

List of tables .................................................................................................................. xvi

1. Introduction .............................................................................................................. 1

2. Pt (IV) complexes as prodrugs for cisplatin .............................................................. 11

2.1 Introduction .......................................................................................................... 11

2.2 Materials and methods ....................................................................................... 13

2.2.1 Instrumentation ............................................................................................. 13

2.2.2 Synthesis of oxoplatin (2) ............................................................................ 13

2.2.3 Synthesis of c,t,c-[PtCl₂(OH)(O₂CCH₂CH₂CO₂H)(NH₃)₂] (3) ......................... 14

2.2.4 DNA binding studies of Pt(IV) complexes to pBR322 DNA ......................... 14

2.2.5 $^{195}$Pt NMR of Pt(IV) complexes in the presence of ascorbic acid or glutathione in pH 7.4 buffer with or without NaHCO₃ ......................................................... 15

2.2.6 $^{13}$C NMR of Pt(IV) complexes in the presence of ascorbic acid in pH 7.4 buffer with NaH$^{13}$CO₃ ............................................................................................................. 16

2.3. Results ................................................................................................................. 17

2.3.1 DNA capture-gel electrophoresis experiments .............................................. 17
3.2.11 Cytotoxicity study of 4-βCD inclusion complex toward neuroblastoma cells (compared with cisplatin)..................................................................................................................46
3.2.12 DNA capture-gel electrophoresis experiments of 4-βCD inclusion complex ........47
3.2.13 Cytotoxicity study of 6 toward neuroblastoma cells (compared with cisplatin)......48
3.3 Results ..................................................................................................................................................................................50
3.4 Discussion ..................................................................................................................................................................................63
3.5 Conclusions ..................................................................................................................................................................................68
4. Cytotoxicity of Cu(II) And Zn(II) 2,2’-Bipyridyl Complexes: Dependence of IC₅₀ on Recovery Time ..................................................................................................................................................69
4.1. Introduction ..................................................................................................................................................................................69
4.2. Materials and methods ...............................................................................................................................................................72
4.2.1 Materials ..................................................................................................................................................................................72
4.2.2 Cytotoxicity studies ...............................................................................................................................................................72
4.2.3 DNA cleavage studies by agarose gel electrophoresis .........................................................................................................74
4.3 Results and Analysis ...............................................................................................................................................................76
4. 4 Discussion ..................................................................................................................................................................................91
4. 5 Conclusions ..................................................................................................................................................................................94
5. Appendix .....................................................................................................................................................................................96
References ..................................................................................................................................................................................108
Vita .....................................................................................................................................................................................122
**List of Symbols and Acronyms**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Acronym / Description</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<tr>
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<td>gold nanoparticles</td>
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<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>TBE</td>
<td>tris-base/borate acid/ ethylenediaminetetraacetic acid</td>
</tr>
<tr>
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<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
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<td>thermogravimetric analysis</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible spectroscopy</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1-1. Structures of Pt(II) and Pt(IV) anticancer drugs ................................. 2

Figure 2-1. Scheme for the synthesis of complexes 2 and 3 .................................. 12

Figure 2-2. Ethidium bromide stained agarose gels of pBR322 DNA ......................... 17

Figure 2-3. Distance of migration of the band from the loading well in the gel in arbitrary units as a function of \( r \), where \( r = [\text{Pt}]/[\text{DNA-bp}] \) ................................................................. 20

Figure 2-4. \(^{195}\text{Pt}\) NMR spectra. (a) Reduction of oxoplatin, 2, with AsA. (b) Reduction of oxoplatin, 2, with GSH. (c) Reduction of the oxoplatin analog, 3, with AsA. (d) Reduction of the oxoplatin analog, 3, with GSH................................................................. 26

Figure 2-5. \(^{13}\text{C}\) NMR spectra of the products of reduction of 2 and 3 with ascorbic acid in the presence of NaH\(^{13}\text{CO}_3\). .......................................................................................... 29

Figure 2-6. Scheme showing the reaction of a Pt(II) aqua species with molecular carbon dioxide and associated equilibria. ................................................................. 34

Figure 3-1. Synthetic scheme of thiolated \( \beta \)-CD (3) functionalized AuNP loaded with Pt(IV) complex 4 ................................................................. 39

Figure 3-2. UV–vis absorption spectra of AuNP 5 and AuNP 6 aqueous solution ........... 50

Figure 3-3. TEM images and diameter histogram of AuNPs 5 and 6 ............................. 51

Figure 3-4. TGA curve of AuNP 5 ............................................................................. 52

Figure 3-5. \(^1\text{H}\) NMR of 4 and \( \beta \)CD .................................................................... 53

Figure 3-6. Chemical shift of \(^1\text{H}\) NMR of 4 and \( \beta \)CD vs \( r \) .............................. 54

Figure 3-7. \(^1\text{H}\) NMR of 4 in \( d_6\)-DMSO/\( D_2\)O 4:1 (v/v) ........................................... 55
Figure 3-8. Chemical shift of $^1$H NMR of 4 as a function of concentration.................................56

Figure 3-9. Chemical shift of $^1$H NMR of 1-adamantanemethylamine as a function of concentration.........................................................................................................................57

Figure 3-10. Chemical shift of $^1$H NMR of 1-adamantanemethanol as a function of concentration...........................................................................................................................................58

Figure 3-11. Percent inhibition vs. the concentration of Pt of 4-βCD and cisplatin toward SK-N-SH cells ......................................................................................................................................................60

Figure 3-12. Ethidium bromide stained agarose gel of pBR322 DNA in the presence of the inclusion complex of 4-βCD, in pH 7.4 HEPES buffer with AsA as the reducing agent ...........60

Figure 3-13. Microscope images of untreated SK-N-SH cells (a and d) and cells treated with 2 (20 μM Pt) for 24 h (b and c) and 72 h (e and f) .........................................................................................................................................61

Figure 3-14. Percent inhibition vs concentration of Pt of 2 and cisplatin after 72 h incubation for different concentrations of 2 and cisplatin........................................................................................................62

Figure 4-1. Structures of the metal complexes 8-10 ..............................................................................70

Figure 4-2. Percent inhibition of cell growth vs. compound concentration toward SK-N-SH cells .........................................................................................................................................................70

Figure 4-3. Percent inhibition of cell growth vs. compound concentration toward OVCAR-3 cells .........................................................................................................................................................78

Figure 4-4. The ratio $N_t/N_u$ plotted against recovery time $t$ for fixed concentration of agent using the CCK-F assay ........................................................................................................................86
Figure 4-5. The ratio $N_t/N_u$ plotted against recovery time $t$ for fixed concentration of agent using the CCK-8 assay ..................................................................................................................87

Figure 4-6. Agarose gel electrophoresis images of 8 (a), 9 (b) and 10 (c) cleavage of pBR322 DNA ...........................................................................................................................................89

Figure 5.1 IR spectrum of oxoplatin (2) .................................................................................................................96

Figure 5.2 $^{195}$Pt NMR spectrum of 3 in H$_2$O/D$_2$O (95/5) ..........................................................................................97

Figure 5.3 $^1$H NMR spectrum of 3 in d6-DMSO...........................................................................................................98

Figure 5.4 $^{13}$C NMR spectrum of 3 in d6-DMSO.........................................................................................................99

Figure 5.5 DEPT135 NMR spectrum of 3 in d6-DMSO .................................................................................................100

Figure 5.6 $^1$H NMR spectrum of 4 in d6-DMSO ..............................................................................................................101

Figure 5.7 $^1$H NMR spectrum of per-6-iodo-β-cyclodextrin in d6-DMSO .................................................................102

Figure 5.8 $^1$H NMR spectrum of 7 in d6-DMSO ..............................................................................................................103

Figure 5.9 Mass spectrum of 3 .......................................................................................................................................104

Figure 5.10 Mass spectrum of 4 .....................................................................................................................................104

Figure 5.11 Mass spectrum of 7 .....................................................................................................................................105

Figure 5.12 Microscope images of untreated SK-N-SH cells and cells treated with 6 (20 μM Pt) for 0 h (a and b), 24 h (c and d), 48 h (e and f), 72 h (before washing, g and h) and 72 h (after washing, i and j) .................................................................106
List of Tables

Table 2-1. Relative slopes (slope divided by the y-intercept) from the mobility plots shown in Figure 2-3, with statistical errors. ..................................................................................................................22

Table 3-1. Binding constants of 4 and βCD in d6-DMSO/D2O 4/1 at room temperature in 1:1 stoichiometry without and with formation of AD dimers.................................................................66

Table 4-1. Molar inhibition parameter, α, and r-squared values for all complexes and recovery times, derived from the results plotted in Figures 4-2 and 4-3 .............................................................83

Table 4-2. Slopes with errors of plots of Nt/N0 vs. time for a fixed concentration of complex ...85
1. Introduction

Metallic elements play essential roles in biological systems due to their unique properties. Metal atoms lose electrons easily to form cations which are soluble in biological fluids, and can act as charge carriers, e.g. sodium-potassium pump, and bind with electron rich biological molecules such as proteins and DNA along with small molecules crucial to life such as O$_2$. The positively charged metal ions are able to control catalysis processes by modifying electron flow in a substrate or enzyme and multivalent metal ions can provide sites for redox activities. Although inorganic compounds with metals have been used in the medical field since up to 5000 years ago when Egyptians used copper to sterilize water (3000 BC) and gold was used in a variety of medicines in China (2500 BC), medicinal inorganic chemistry emerged as a discipline just about 40 years ago, since the discovery of the antitumor activity of cisplatin.\(^1\)

The small molecule drug *cis*-diamminedichloroplatinum(II), cisplatin (Figure 1-1), is renowned for a high level and broad spectrum of anticancer activity. Cisplatin was first synthesized by Michele Peyrone in 1845 and known as Peyrone’s chloride thereafter.\(^2\) In 1890s, Alfred Werner proposed the square planer configuration of cisplatin, distinguishing cisplatin from its *trans* stereoisomer transplatin, *trans*-\([\text{PtCl}_2(\text{NH}_3)_2]\), and the study was a foundation of Alfred Werner’s theory of coordination chemistry for which he won the Nobel Prize in 1913.\(^2\) To improve the purity of cisplatin and reaction yield, Dhar published “A rapid method for the synthesis of cis-\([\text{PtCl}_2(\text{NH}_3)_2]\)”, which many subsequent cisplatin synthesis methods were based on. The procedure was dependent on the *trans* effect, first introduced by Chernyaev in 1926 which elucidate that the rate of substitution of a ligand in a square planar or octahedral metal complex is dependent on the nature of group opposite to it.\(^2\)
The antitumor activity of cisplatin was discovered by Rosenberg and coworkers in 1960s, when they were examining whether electrical currents affect cellular division. The researchers found that the inhibition of cellular division observed in the study was not due to electrical current but platinum hydrolysis products formed from platinum electrodes. After testing a group of various Pt(II) and Pt(IV) compounds, they reported that cis-tetrachlorodiammineplatinum(IV), cis- [PtCl$_4$(NH$_3$)$_2$] (Figure 1-1), was the potent agent responsible for the inhibition while the trans form of the Pt(IV) complex was found to be ineffective. The researchers further tested cis- [PtCl$_4$(NH$_3$)$_2$], its Pt(II) analog, later to be named cisplatin and other neutral platinum complexes against Sarcoma 180 tumors in Swiss white mice to find again that the cis-configurations were active while the corresponding trans-configurations were not active. The large solid tumors were shrunk by the cis forms of the complexes and the mice survived the cancer and became healthy. Cisplatin has been tested against a large number of various animal tumors and was the first platinum drug chosen by National Cancer Institute to be...
entered into clinical trials. The complex ultimately succeeded in clinical trials and was approved by the U.S. Food and Drug Administration (FDA) in 1978 under the name of Platinol® for use in humans to treat conditions such as testicular and ovarian cancers and ultimately obtained worldwide approval.\(^5\)

It is widely accepted that nuclear DNA is a crucial biological target of cisplatin along with other targets such as RNA, enzymes containing sulfur and mitochondria\(^6,7\). Cisplatin enters cancer cells through passive diffusion and active transport mechanisms.\(^8,9\) Inside cancer cells the chloride concentration is relatively low so that one of the chloride ligands is substituted by the oxygen on a water molecule, forming a positive charged species of which the water ligand can be further replaced by N-7 of a guanine or adenine on DNA to form a monofunctional DNA adduct and the second chloride ion on cisplatin can either be replaced directly by N-7 of an adjacent guanine/adenine or firstly be aquated and then replaced by N-7 of an adjacent guanine or adenine. Approximately 65% of the bifunctional adducts are GpG, and 25% are ApG.\(^10\) The intrastrand coordination bonds between Pt(II) and DNA bases cause distortions of DNA strand and thus inhibit DNA replications and lead to cell death.

The research of producing platinum-based anticancer compounds is continuing after the discovery of anticancer activity of cisplatin. The second-generation drug, carboplatin (diamine[1,1-cyclobutandicarboxylato(2-)]-O,O’-platinum(II)) (Figure 1-1) with fewer side effects was the second to enter US market as Paraplatin® in 1989 and became approved worldwide to be used in cancer chemotherapy.\(^5\) The reduction of side effects was achieved by replacing dichloride ligands with 1,1-cyclobutandicarboxylate, which has a lower rate constant of aquation, \(10^{-8} \text{ s}^{-1}\), compared with that of cisplatin \(\left(10^{-5} \text{ s}^{-1}\right)\).\(^5,11-12\) After aquation, carboplatin produces the same active species as cisplatin to form DNA adducts and is therefore effective in
treat the same cancers as cisplatin, and the lower reactivity allows higher doses (300-450 mg m\(^{-2}\)) than cisplatin (20-120 mg m\(^{-2}\)) in administration. The third platinum-based anticancer drug succeeded in clinical trials and approved by FDA is another Pt(II) complex, oxaliplatin ([oxalate(2-)-O,O'][(1R,2R)-cyclohexanediamine-\(N,N'\)] platinum(II)) (Figure 1-1), under the name of Eloxatin\(^\circ\). It was the first drug approved to be able to overcome cisplatin resistance and also the first platinum-based drug that is active against metastatic colorectal cancer in combination with fluorouracil and folinic acid.\(^5,13\) In structure, two ammine ligands are replaced by a single bidentate ligand (1\(R,2R\))-cyclohexane-1,2-diamine (\(R, R\)-dach).\(^14\) This complex is proposed to overcome cisplatin resistance by forming different adducts with DNA, that is, it chiefly forms GpG intrastrand adducts. The bulky hydrophobic dach ligand points into the DNA major groove therefore inhibits binding of DNA repair proteins.\(^15\)

Since the discovery of cisplatin, although a large number of Pt(II) drugs which exhibit anticancer activity have been discovered,\(^16\) only a few of them can be used in clinical therapy.\(^17\) Cisplatin, carboplatin and oxaliplatin are the only platinum-based drugs approved by FDA for clinical use.\(^18\) Cisplatin and other Pt(II) complexes are vulnerable in the plasma as Pt(II) is easily attacked by proteins containing thiol groups and this protein bonding reaction is blamed for deactivation of cisplatin and some severe side effects.\(^16\) Moreover, cisplatin is also highly cytotoxic toward rapidly dividing healthy cells hence its application is limited. As severe dose-limiting side effects and acquired or intrinsic resistance to cisplatin are frequently observed,\(^19,20\) a new generation of platinum drugs, Pt(IV) octahedral compounds, e.g. satraplatin (Figure 1-1), is being developed to overcome these drawbacks. Platinum(IV) complexes, which have relatively lower ligand exchange rates than Pt(II) are considered to be promising prodrugs of biologically active Pt(II) complexes. Tobe and Khokhar and later Cleare et al.,\(^21\) suggested that Pt(IV)
complexes may be reduced to Pt(II) complexes in vivo and later exert their cytotoxic effects in a manner analogous to cisplatin and its analogs. The hypothesis was reinforced by the fact that in clinical trials of a Pt(IV) complex, iproplatin, \( c.t.c-\left[\text{PtCl}_2(\text{OH})_2((\text{CH}_3)_2\text{CHNH}_2)_2\right] \) (Figure 1-1), \( \text{cis-}[\text{PtCl}_2((\text{CH}_3)_2\text{CHNH}_2)_2] \) was detected in the plasma and urine, indicating that iproplatin was reduced in the body to a Pt(II) complex.\(^{22-23}\) The octahedral structure of Pt(IV) allows more modifications through introducing functional groups to axial positions without altering the property of their active reducing product, because the Pt(IV) complexes will be metabolized and lose the axial groups in blood. For instance, satraplatin (JM216), a Pt(IV) complex, is the first orally bioavailable platinum compound investigated in active clinical trials. By introducing two axial acetate groups, satraplain attains more lipophilicity, thus its oral bioavailability is increased.

Ascorbic acid (AsA) and glutathione (GSH) are biological reducing agents frequently cited in connection with the reduction of Pt(IV) complexes to Pt(II).\(^{24-26}\) Ascorbic acid, present in blood plasma with a concentration of 50-150 \( \mu \text{M} \) and the cytosol at ~1 mM,\(^{27-28}\) is a two electron reducing agent capable of reducing Pt(IV) to Pt(II). The study of Blatter et al.\(^{26}\) showed that the AsA reduced species of iproplatin (Figure 1-1) and the related complex oxoplatin, \( c.t.c-\left[\text{PtCl}_2(\text{OH})_2(\text{NH}_3)_2\right] \), can bind to and unwind closed circular PM2 DNA. Green and Evans\(^ {29}\) and Choi \textit{et al}.\(^ {30}\) measured the second order rate constant for the ascorbate reduction of iproplatin at 40 °C obtaining \( k = 0.33 \) and 0.103 M\(^{-1}\) s\(^{-1}\), respectively, while Weaver and Bose\(^ {25}\) investigated the mechanism of reduction of oxoplain by ascorbate. The tripeptide, glutathione (GSH), \( \gamma\)-glutamylcysteinylglycine, contains a cysteine thiol residue and is ~2 mM in the cytosol and ~850 \( \mu \text{M} \) in blood.\(^ {31-32}\) Reduced GSH is a potent one-electron reducing agent which is capable of reducing Pt(IV) to Pt(II). However, the nature of the GSH-produced reduction products has been
the subject of inquiry. Recently, Volckova, et al.\textsuperscript{33} show that iproplatin is readily reduced by GSH to produce \textit{cis}-di(isopropylamine)chloro-glutathionatoplatinum(II) which has the thiolate of a GSH molecule bound to Pt(II). Since this type of complex could not form a 1,2 intrastrand crosslink with DNA,\textsuperscript{34} it was suggested that GSH-reduced iproplatin may exhibit its cytotoxic effects by modifying biological targets other than DNA in the cell. Although the Pt(IV) complex \textit{cis}-[PtCl\(_4\)(NH\(_3\))\(_2\)] is considered a prodrug of cisplatin, Nakai, et al.\textsuperscript{35} have recently revealed that the reduction by GSH of \textit{cis}-[PtCl\(_4\)(NH\(_3\))\(_2\)] actually does not produce cisplatin. The study also showed that in the presence of calf thymus DNA in the reduction medium, the Pt(II) product produced which is captured by DNA does not form the 1, 2 intrastrand DNA crosslink. Gibson and coworkers\textsuperscript{36-37} monitored the rate of reduction of Pt(IV) complexes in extracts obtained from whole cells and they found that reductive elimination of \textit{cis}-diam(m)inedichloridoplatinum(IV) complexes with axial acetato ligands by whole cell extracts does exclusively produce a Pt(II) product with the expected, \textit{cis}-PtCl\(_2\)N\(_2\) geometry. To investigate activation of Pt(IV) complexes, Platts et al.\textsuperscript{38} monitored and correlated the electrochemical peak potentials and octanol-water partition coefficients of 31 Pt(IV) complexes with the potential to produce cisplatin or cisplatin-type complexes through reductive elimination to Pt(II) compounds. The study showed a good correlation between the aforementioned properties and the calculated surface area, orbital energies, dipole moments and atomic partial charges of the compounds and these investigators developed models in the study which have the potential to predict the antiproliferative properties of the compounds from calculated data.

Carbonate, in equilibrium with bicarbonate and dissolved CO\(_2\), is a crucial component of the pH buffering system in human body and exists at high concentrations extensively in the blood, interstitial fluid and cytosol. Van Eldik and coworkers revealed that metal hydroxo
species with cobalt, chromium, iridium, rhodium or zinc can react with dissolved CO$_2$ and form carbonato complexes with second order rate constants of k = 37-590 M$^{-1}$S$^{-1}$ at 25 °C.$^{39-41}$ Centerwall et al. revealed that cisplatin forms carbonato complexes $cis$-[PtCl(CO$_3$)(NH$_3$)$_2$]$^-$ and $cis$-[Pt(CO$_3$)$_2$(NH$_3$)$_2$]$^{2-}$ in carbonate buffer using HSQC NMR$^{42}$ and research by Di Pasqua et al. has shown that carboplatin can also form carbonato complexes.$^{43}$ Later, Hong and coworkers reported cisplatin-DNA monofunctional adducts are formed in the presence of carbonate and carbonate prevents them from converting to bifunctional adducts.$^{44}$ In this work, the influence of carbonate in the binding of reduced species of oxoplatin and one of its Pt(IV) analogues $c,t,c$-[PtCl$_2$(OH)(O$_2$CCH$_2$CH$_2$CO$_2$H)(NH$_3$)$_2$], 3 (Figure 2-1), is studied to investigate the role of carbonate in mechanism of action of platinum-based anticancer drugs.

To explore new possibilities of rapidly and selectively “arming” targeting vectors with cytotoxic platinum complexes, we modified the prodrug of cisplatin, complex 3, by attaching 1-adamantanemethylamine to the carboxylate functional group of the complex though EDC/NHS crosslinking reaction to form the Pt-adamantane conjugate 4 (Figure 2-1). We also examined the potential of host-guest interactions between $\beta$-cyclodextrin and adamantane for delivery applications involving the adamantane conjugate. Beta-cyclodextrin ($\beta$CD), which is a cyclic oligosaccharides composed of seven D(+) glucopyranosyl units joined by $\alpha$-1,4-glycosidic bonds, is widely used as a host in pharmaceutical formulations for increasing the solubility, stability and availability of drugs$^{45-47}$ The hydrophilic outer surface of $\beta$CD provides water solubility through hydrogen bonds and the hydrophobic inner surface allows inclusion of small molecules through a host-guest interaction driven by van der Waals forces. Adamantane (AD) is a well-studied diamondoid hydrophobic guest molecule that binds to $\beta$CD by rapid inclusion with a binding constant, $K \sim 10^2$-10$^3$ M$^{-1}$, $^{48-49}$ resulting in stable products. When this inclusion
complex is combined with a targeting vector equipped with βCD, a conjugate having the potential to direct platinum complexes to tumor sites is yielded. Halamikova et al. showed that two adamantane-tethered platinum based complexes, \( t,t,t-[\text{PtCl}_2(\text{CH}_3\text{CO}_2)_2(\text{NH}_3)(1\text{-adamantylamine})] \) and its reduced analog \( t-[\text{PtCl}_2(\text{NH}_3)(1\text{-adamantylamine})] \) are able to circumvent both acquired and intrinsic cisplatin resistance and have higher accumulation in A2780 cells due to their lipophilic property.\(^5\) Earlier, Prashar et al. conjugated AD to an analog of the Pt(II) anticancer drug carboplatin, and showed that the inclusion complex with βCD is more toxic toward human neuroblastoma SK-N-SH cells and delivers more platinum to closed circular pBR322 DNA than carboplatin.\(^5\)

In this study, to increase cancer targeting effect of the Pt(IV)-adamantane conjugate, a cyclodextrin coated gold nanoparticle delivery system was constructed. Gold nanoparticles (AuNPs), or gold colloids, were found to be used for therapeutic and decorative purposes in ancient China and Egypt and was first reported by Faraday in scientific article in 1857.\(^52-53\) Gold nanoparticles are synthesized conventionally by reduction of gold (III) derivatives. The simplest and most prevalent method is reduction of \( \text{HAuCl}_4 \) using trisodium citrate in aqueous solution, firstly reported by Turkevitch in 1951,\(^54\) and refined by Frens in 1973 to obtain tunable size between 16 and 147 nm by changing the ratio between the reducing agents to gold.\(^55-56\) In 2006, Kimling et al. further modified the method and produced AuNPs with better controlled size distribution within the range 9-120 nm.\(^57\) Citrate is a weak field ligand which stabilizes AuNPs in aqueous solution; the AuNPs are usually subsequently functionalized using ligands of biological interest by replacing citrate. A large variety of stabilizers such as ligands, surfactants, polymers, dendrimers, biomolecules can be used to stabilize AuNPs, of which the most stable AuNPs reported are capped with thiolates because of the soft acid-soft base interaction between gold

\[ \text{AuNPs} \]
and sulfur. Giersig and Mulvaney disclosed the synthesis of AuNPs stabilized by alkanethiols of various chain lengths in 1993 while the most popular synthetic scheme of AuNPs is the Brust-Schiffrin method published in 1994 using thiol as a stabilizer. Inspired by Faraday’s two-phase system, Brust-Schiffrin biphasic synthesis is a convenient one-step method that comprises growth of the metallic nuclei and simultaneous attachment of self-assembled thiol monolayer on the nuclei, using HAuCl₄, a thiol as a capping ligand, tetraoctylammonium bromide as the phase-transfer reagent, NaBH₄ as a reducing agent in water-toluene to produce thiolate-AuNPs.

Gold nanoparticles are reported to have biocompatible properties such as nontoxicity, nonimmunogenicity and high tissue permeability and are widely investigated as drug delivery vehicles to improve the biodistribution of drugs and achieve better targeting. Due to their suitable size, nanoparticles are accumulated and entrapped in tumors through passive targeting and will not be eliminated due to defense mechanisms of body therefore they keep circulating in the blood stream and allow themselves to be taken to the target receptor. General features of tumors include leaky blood vessels with gap sizes of 100 nm to 2 μm and poor lymphatic drainage which result in enhanced permeability and retention (EPR) effect of AuNPs. Gold nanoparticles have become attractive drug delivery vehicles also because they can be readily functionalized by ligands containing functional groups such as thiol, phospine, phosphine oxide, amine and carboxylate, therefore allow delivery of various payloads to their targets. In this work, AuNPs coated with per-6-thio-β-cyclodextrin, a hepta-thiol ligand, were synthesized as a drug delivery vehicle for a Pt(IV) prodrug of the widely used anticancer drug cisplatin. The cytotoxicity of the Pt(IV) complexed loaded AuNP delivery vehicle and the of the system was tested toward human neuroblastoma (SK-N-SH).
In vitro, cytotoxicity testing is widely used in basic research and clinical diagnostics. These measurements of cellular sensitivity of cytotoxicity are dependent on a large number of variables, one of which is recovery time that has been noted to be important but not well explored. It is reported that when Murine leukemia L1210 cells is treated with cisplatin, low concentrations of the drug causes the cells to be transiently arrested in the G\(_2\) phase for about 3 days before this phase is bypassed and growth is resumed.\(^63\) In another study, Bogdanovic et al. reported that cisplatin analogs containing sulfur carrier ligands affected the growth of MCF7 human breast cancer cells during the recovery period and both inhibitory and stimulatory effects on cell division were observed, depending on the platinum complex being investigated.\(^64\) This makes the reported value of IC\(_{50}\) strongly dependent on the time (recovery period) at which inhibition is measured. As the last part of this work, the dependence of IC\(_{50}\) of three metal complexes on recovery time was investigated using human neuroblastoma cell line (SK-N-SH) and ovarian cancer cell line (OVCAR-3).
2. Pt (IV) Complexes as Prodrugs for Cisplatin

2.1 Introduction

It is widely accepted that Pt(IV) exhibit anticancer activity by being reduced to its Pt(II) analogs in vivo\(^\text{21-23}\) and ascorbic acid (AsA) and glutathione (GSH) are biological reducing agents frequently cited in connection with the reduction of Pt(IV) complexes to Pt(II).

![Diagram showing the synthesis of complexes 1, 2, and 3.]

Figure 2-1. Scheme for the synthesis of complexes 2 and 3.

In this chapter we first examined the ability of a Pt(IV) complex oxoplatin, 2, and one of its carboxylate modified analogs, 3 (Figure 2-1), to produce cisplatin in the presence of the biologically common reducing agents, ascorbic acid (AsA) and glutathione (GSH). Oxoplatin exhibits potent oral anticancer activity\(^\text{65-66}\) and its analogs has been used in a host of drug delivery applications involving nanotechnology.\(^\text{67-69}\) The synthesis of oxoplatin was first reported by Chugaev and Khlopin in 1927.\(^\text{70}\) Later studies of Presnov et al. showed that oxoplatin has a wide spectrum of antineoplastic action therefore inhibits growth of solid and ascetic forms of transplantable tumors.\(^\text{71}\) Olszewski et al. reported that oxoplatin induced S-phase arrest and cell death in 38 cancer cell lines and the IC\(_{50}\) values are 2.5-fold higher than its Pt(II) analog
cisplatin (1, Fig.1a). Since Pt(II) complexes bind to DNA, we used pBR322 DNA as a capture agent for the reduction products of 2 and 3 and measured the mobility of the platinated DNA forms in an agarose gel. It is known that physiological carbonate plays a significant role in the speciation of cisplatin in solution and thus affects the kinetics of the platinum-DNA interaction and possibly the type of the DNA adduct formed. Therefore, we also investigated platinum binding to pBR322 DNA in the presence and absence of carbonate buffer. We used $^{195}$Pt NMR to identify the nature of the platinum species formed in the reduction and $^{13}$C NMR to detect Pt(II) carbonato complexes when the reduction is carried out in carbonate media.
2.2 Materials and Methods

2.2.1 Instrumentation

One dimensional $^1$H NMR spectra were obtained using a Bruker Avance 300 MHz spectrometer with chemical shifts determined by setting the hydrogen impurity in DMSO-$d_6$ to 2.50 ppm. One dimensional $^{195}$Pt NMR spectra were recorded on a Bruker Avance 500 MHz NMR instrument utilizing either a Nalorac 5 mm indirect detection gradient probe or a Nalorac 10 mm broadband observe probe. A solution of 10 mM potassium hexachloroplatinate in 95% H$_2$O/5% D$_2$O was used as an external standard (0 ppm). All experiments were done at 25 °C using a 2 s relaxation delay time and a spectral width of 600 ppm with 32K points. One dimensional carbon-13 NMR spectra were recorded at 25 °C on a Bruker Avance 500 MHz NMR instrument equipped with a 5 mm Bruker QXI gradient probe. The standard $^{13}$C pulse sequence was used with proton broad-band decoupling and a spectral window of 250 ppm. The relaxation delay was set to 2 s, the total number of points to 16K, and the total number of scans set to 6000. The internal chemical shift standard, set at 126.1 ppm, was $^{13}$CO$_2$(aq). Infrared spectra were obtained on nujol mulls using a Nicolet 200 FT-IR spectrometer and the mass spectral data were obtained using positive electrospray ionization with a Bruker 12 Tesla APEX-Qe FTICR-MS with an Apollo II ion source (COSMIC Lab, Norfolk, VA).

2.2.2 Synthesis of Oxoplatin (2)

This compound was synthesized in a manner analogous to that earlier reported by Brandon and Dabrowiak.$^{76}$ Hydrogen peroxide (30 wt %, 20 mL) was added dropwise to a
suspension of cisplatin, 1, (0.4 g, 1.33 mmol) in H_{2}O (12 mL) at 65 °C. After 4 h, the bright yellow solution was cooled at room temperature overnight to afford yellow crystals. The crystals were recovered by filtration, washed with ice cold water and dried in vacuo. Yield: 65.6% (0.291 g, 0.872 mmol). IR 3458 (s, OH stretch), 1072 (m, Pt-OH bend), 536 (m, Pt-N(O) stretch). $^{195}\text{Pt}$ NMR (500 MHz, D$_2$O), δ (ppm) = 853.

2.2.3 Synthesis of c,t,c-[PtCl$_2$(OH)(O$_2$CCH$_2$CH$_2$CO$_2$H)(NH$_3$)$_2$] (3)

This compound was synthesized in a manner analogous to that described by Dhar et al.$^{68}$ To a suspension of compound 2 (0.2 g, 0.6 mmol) in DMSO (16 mL) was added succinic anhydride (0.06 g, 0.6 mmol) and the mixture was stirred overnight at RT to afford a bright yellow solution. After reducing the volume of the solution in vacuo to ~ 0.5 mL, addition of ~10 mL of ice cold acetone caused precipitation of a pale yellow solid, which was collected via filtration, washed with acetone and dried in vacuo. Yield: 97.2% (0.253 g, 0.583 mmol). $^1$H NMR (300 MHz, DMSO-d$_6$), δ (ppm) = 5.93 m, 6H (NH$_3$); 2.39 m, 4H (CH$_2$). $J(^1\text{H}-^{195}\text{Pt}) = 103.2$ Hz, $J(^1\text{H}-^{14}\text{N}) = 25.5$ Hz. $^{13}$C NMR (75 MHz, DMSO-d$_6$): δ = 179.73 (C=O), 174.19 (C=O), 30.21 (CH$_2$), 31.24(CH$_2$) ppm. MS (1:1 THF:MeOH with NaCl) calculated m/e of (C$_4$H$_{12}$N$_2$O$_5$Cl$_2$Pt)Na$^+$ = 456.0, observed m/e = 456.0. $^{195}$Pt NMR, δ (ppm) = 944.

2.2.4 DNA Binding Studies of Pt(IV) Complexes to pBR322 DNA
The DNA binding/capture studies were done in a total volume of 20 μl containing 38.5 μM (bp) pBR322 DNA (New England BioLabs Inc.), in 12 mM HEPES, N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonicacid (pH 7.4) buffer or, when sodium bicarbonate was present, in 12 mM HEPES buffer plus 12 mM sodium bicarbonate (pH 7.4). The two reducing agents used were glutathione (GSH, 2 mM) and ascorbic acid (AsA, 50 μM). Cisplatin, 1, was used as purchased (Sigma-Aldrich, St. Louis, MO). Stock solutions of the platinum compounds in distilled water, 1 and 3, or DPBS (Dulbecco’s phosphate buffered saline) buffer, 4, were in the range of 40–650 μM. Appropriate volumes of the stock solutions were added to solutions containing the buffer and DNA to give the final concentrations given above and values of $r$, where $r = [\text{platinum compound}]/[\text{DNA-bp}]$, given in the captions to figures. The samples were incubated at 37 °C for 24 h in sealed Eppendorf tubes after which time 2.5 μl of a loading dye containing 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. An 8 μl volume of each sample containing the loading dye was loaded in the wells of a 1% agarose gel. Electrophoresis was carried out for a period of ~4 h, at 100 V. After electrophoresis, the gel was immersed in 300 ml of deionized water containing 300 μl of a 0.5 mg/ml solution of ethidium bromide for 30 min to stain DNA, and then soaked in water alone for 15 min to de-stain the background of the gel. A digital image of the stained gel was captured using a Kodak Gel Logic 100 imaging system equipped with Fisher Biotech IT-88A transilluminator. This DNA binding study was performed by Shu-An Liu (University of Technology Graz) under this author’s direction.

2.2.5 $^{195}$Pt NMR of Pt(IV) Complexes in the Presence of Ascorbic Acid or Glutathione in pH 7.4 Buffer with or without NaHCO$_3$
Compound 3 (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid or 20 mM GSH in 12 mM HEPES buffer with or without 12 mM NaHCO$_3$, pH 7.4. Compound 4 (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid or 20 mM GSH in DPBS buffer with or without 12 mM NaHCO$_3$, pH 7.4. The solvent of the above samples was H$_2$O/D$_2$O (95/5) and the concentrations given are the final concentrations.

2.2.6 $^{13}$C NMR of Pt(IV) Complexes in the Presence of Ascorbic Acid in pH 7.4 Buffer with NaH$^{13}$CO$_3$

Compound 3 (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid in 12 mM HEPES buffer with 0.5 M NaH$^{13}$CO$_3$, pH 7.4. Compound 4 (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid in DPBS buffer with 0.5 mM NaH$^{13}$CO$_3$, pH 7.4. The solvent of the above samples was H$_2$O/D$_2$O (90/10); the final concentrations in the reaction mixture are given.
2.3. Results

2.3.1 DNA Capture-Gel Electrophoresis Experiments
Figure 2-2. (a)-(f) Ethidium bromide stained agarose gels of pBR322 DNA (38.5 μM). (a)-(e), pBR322 DNA in the presence of either 2, or 3, in the absence or presence of 12 mM sodium bicarbonate with either ascorbic acid, AsA (50 μM) or glutathione, GSH (2 mM) as the reducing agent. The fastest migrating band (bottom band) is covalently closed circular form I DNA while the slowest migrating band (top band) is nicked circular form II DNA. (a) Compound 2, AsA, sodium bicarbonate, lanes 5-11; no bicarbonate, lanes 12-18. For this and the subsequent gels,
lane, $r$, i.e., the lane number followed by $r$ where, $r = [\text{compound}]/[\text{DNA-bp}]$, are given. Lane, $r$: 5, 0.26; 6, 0.52; 7, 1.04; 8, 1.17; 9, 1.30; 10, 1.43; 11, 1.56; 1, 0 (DNA); 2, 0 (DNA + AsA); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + AsA + bicarbonate). The values of $r$ for lanes 12-18 are the same as for lanes 5-11. (b) Compound 3, AsA, sodium bicarbonate, lanes 5-11; no bicarbonate, lanes 12-18. Lane, $r$: 5, 0.26; 6, 0.52; 7, 1.04; 8, 1.17; 9, 1.30; 10, 1.43; 11, 1.56; 1, 0 (DNA); 2, 0 (DNA + ascorbic acid); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + ascorbic acid + bicarbonate). The values of $r$ for lanes 12-18 are the same as for lanes 5-11. (c) Compound 2, GSH, sodium bicarbonate, lanes 5-11; no bicarbonate, lanes 12-18. Lane, $r$: 5, 1.69; 6, 3.38; 7, 5.06; 8, 6.75; 9, 7.60; 10, 8.44; 11, 10.13; 1, 0 (DNA); 2, 0 (DNA + GHS); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + GSH + bicarbonate). The values of $r$ for lanes 12-18 are the same as for lanes 5-11. (d). Compound 3, GSH, sodium bicarbonate, lanes 5-9; no bicarbonate, lanes 12-18. Lane, $r$: 5, 1.30; 6, 2.60; 7, 3.90; 8, 5.19; 9, 5.84; 10, 6.49; 11, 7.79; 1, 0 (DNA); 2, 0 (DNA + GSH); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + GSH + bicarbonate). The values of $r$ for lanes 12-18 are the same as for lanes 5-11. (e). Compound 3, GSH. Lane, $r$: 3, 1.30; 4, 1.49; 5, 1.69; 6, 1.88; 7, 2.08; 8, 2.27; 9, 2.60; 10, 2.92; 11, 3.25; 12, 3.57; 13, 3.90; 14, 4.22; 15, 4.55; 16, 4.87; 17, 5.19; 18, 5.52; 1, 0 (DNA); 2, 0 (DNA + GSH). (f) Compound 1, cisplatin, 2 mM GSH in 12 mM HEPES buffer, pH 7.4, lanes 3-9; no GSH, lanes 11-17, the values of $r$ for lanes 3-9 and 11-17 are the same (condition error, lane 13). Lane, $r$: 3 (11), 0.08; 4 (12), 0.09; 5 (13), 0.10; 6 (14), 0.11; 7 (15), 0.12; 8 (16), 0.13; 9 (17), 0.14; 1 (10), 0 (DNA), 2, 0 (DNA + GSH).
Figure 2-3. Distance of migration of the band from the loading well in the gel in arbitrary units as a function of $r$, where $r = [Pt]/[DNA-\text{bp}]$, referred to as mobility plots, for the agarose gels shown in Figure 2. The lines shown are linear fits to the data. Square is closed circular Form I pBR322 DNA in the presence of carbonate; triangle is Form I DNA without carbonate; circle is
nicked circular Form II DNA in the presence of carbonate; diamond is Form II DNA in the absence of carbonate.

Figure 2-2(a)-(e) show images of agarose gels of pBR322 DNA in the presence of reducing agents and either 2 or 3 in aqueous solutions with or without carbonate while Figure 2-2(f) shows the binding of cisplatin, 1 to pBR322 DNA in the presence and absence of GSH. In Figure 2-2(a)-(f), the distance of migration of the DNA form from the loading well in arbitrary units is plotted versus \( r \), where \( r = [\text{Pt}]/[\text{DNA-bp}] \), with linear fits to the data. As the initial concentrations of DNA, the reducing agent, carbonate (when present) and time are kept constant in the experiments, the plots show changes in DNA mobility as a function of the total initial concentration of Pt(IV) complex. In these experiments pBR322 DNA which has both closed and nicked circular forms, was used as capture agent for the reduction products, Pt(II) compounds, which bind to the DNA forms therefore affect mobilities of DNA in the gel. Since electrophoresis conditions varied slightly from experiment to experiment, and these affect the distance of migration in the gel, the slope from the linear fits to the data in Figure 2-3 were divided by the y-intercept to give relative slopes, which are presented in Table 1 with statistical errors.

The agarose gel image of pBR322 DNA with oxoplatin, 2 in the presence of reducing agent ascorbic acid (AsA) is shown in Figure 2-2 (a) and Figure 2-3(a) and the mobility plots and their relative slopes given in Table 2-1. Two bands of major forms of pBR322 Form I (closed circular), the fast migrating form and Form II ( nicked circular), the slow migrating form, are shown in each lane. When carbonate is present, the relative slopes of Form I and II are close to zero which means that mobility is neither changed nor dependent on \( r \), suggesting that the Pt(II) species produced in the reduction are not binding to DNA. When carbonate is absent from the
Refers to the reducing agent and the Pt(IV) compound involved in the reduction reaction; the letter in parenthesis refers to the gel image in Figure 2 and the mobility plot in Figure 3. The values given in the table are relative slopes of the linear fits to the mobility plots shown in Figure 3 in units of r⁻¹ multiplied by 10³, followed by the sign of the slope in parentheses, r = [Pt]/[DNA-bp]. Positive and negative slopes indicate that the DNA form migrates faster and slower, respectively, with increasing r, than the same form in the absence of added platinum compound. Form I and Form II are closed and nicked circular pBR322 DNA, respectively. For (e), a much better fit (sum of square deviations = 60 instead of 377) is obtained by using one line for r < 3.3 and a second for r > 3.3).

Table 2-1. Relative slopes (slope divided by the y-intercept) from the mobility plots shown in Figure 2-3, with statistical errors.

<table>
<thead>
<tr>
<th>Systema</th>
<th>Form Ib</th>
<th>Form II</th>
<th>Form I + HCO₃⁻</th>
<th>Form II + HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsA and 2 (a)</td>
<td>no data</td>
<td>45 ± 15 (+)</td>
<td>7.8 ± 5.2 (0)</td>
<td>3.6 ± 4.4 (0)</td>
</tr>
<tr>
<td>AsA and 3 (b)</td>
<td>13.2 ± 7.5 (+)</td>
<td>6.1 ± 12.4 (0)</td>
<td>−9.4 ± 10.4 (0)</td>
<td>11.4 ± 7.3 (+)</td>
</tr>
<tr>
<td>GSH and 2 (c)</td>
<td>−3.3 ± 1.2 (−)</td>
<td>6.2 ± 0.6 (+)</td>
<td>0.9 ± 1.2 (0)</td>
<td>3.0 ± 1.5 (+)</td>
</tr>
<tr>
<td>GSH and 3 (d)</td>
<td>18.0 ± 3.5 (+)</td>
<td>−11.1 ± 4.0 (−)</td>
<td>1.6 ± 0.7 (+)</td>
<td>1.9 ± 0.5 (+)</td>
</tr>
<tr>
<td>GSH and 3 (e)</td>
<td>15.5 ± 2.4 (c) (+)</td>
<td>−9.2 ± 3.4 (c) (−)</td>
<td>no data</td>
<td>no data</td>
</tr>
<tr>
<td>cisplatin (f)</td>
<td>(1.92 ± 0.96)×10³ (+)</td>
<td>−260 ± 110 (−)</td>
<td>−960 ± 340 (−)</td>
<td>630 ± 100 (+)</td>
</tr>
</tbody>
</table>

a Refers to the reducing agent and the Pt(IV) compound involved in the reduction reaction; the letter in parenthesis refers to the gel image in Figure 2 and the mobility plot in Figure 3. The values given in the table are relative slopes of the linear fits to the mobility plots shown in Figure 3 in units of r⁻¹ multiplied by 10³, followed by the sign of the slope in parentheses, r = [Pt]/[DNA-bp]. Positive and negative slopes indicate that the DNA form migrates faster and slower, respectively, with increasing r, than the same form in the absence of added platinum compound. 
bForm I and Form II are closed and nicked circular pBR322 DNA, respectively. 
cFor (e), a much better fit (sum of square deviations = 60 instead of 377) is obtained by using one line for r < 3.3 and a second for r > 3.3).

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Table 2-1. Relative slopes (slope divided by the y-intercept) from the mobility plots shown in Figure 2-3, with statistical errors.

medium Form I is hard to observe while Form II runs slightly faster than its counterpart without carbonate and its mobility plot has a positive relative slope (the mobility of DNA increases with r), Table 1. The difficulty in detecting Form I (only a weak band for this form is observed in lane 12) suggests that Form I is co-migrating with Form II at some values of r and/or the crosslink blocks ethidium bromide binding to DNA leading to reduced staining. The increase in mobility and positive slope of Form II is consistent with the formation of the well-known cisplatin 1, 2 intrastrand crosslink. ¹⁹⁵Pt NMR shows that the reduction of 2 by AsA produces cisplatin and its aquated/substituted products and ¹³C NMR indicates that in carbonate media Pt(II) carbonato complexes have formed.
Figures 2-2(b), 2-3(b) and Table 1 show the results of the DNA capture experiment when the oxoplatin analog, 3, is reduced by AsA in the presence and absence of carbonate. In the presence of carbonate, mobilities are like those of the controls with the slope of Form I zero and the slope of Form II small and positive with a substantial uncertainty indicating no DNA binding formed. In the absence of carbonate, Figure 2-2(b) and 2-3(b) show that the mobilities of both DNA forms are evidently different than the controls indicating that binding of Pt(II) to has occurred. As shown in Table 2-1, the slope for Form II is zero while the slope for Form I is likely positive. Dulbecco's phosphate-buffered saline (DPBS), which contains chloride ion, is used as the stock solution of 3. Since the chloride ion suppressed the aquation of cisplatin, the crosslink appears to be formed but shows not much change in the mobility of Form I with $r$. $^{195}$Pt NMR shows that the reduction of 3 with AsA produces cisplatin without its aquated/substituted products and $^{13}$C NMR indicates that in carbonate media Pt(II) carbonato complexes are formed.

Figures 2-2(c), 2-3(c) and Table 2-1 give the results of the DNA capture experiment when 2 is reduced by GSH. In the presence of carbonate, the mobilities of both forms are similar to controls with the relative slope of Form I zero and the slope of Form II slightly positive with a significant error showing that carbonate efficiently blocks the binding of Pt(II) products to DNA. However, in the absence of carbonate, the mobility of Form I is decreased comparing to the control and its relative slope is negative (mobility decreases with $r$) while the slope of Form II is positive (mobility increases with $r$). $^{195}$Pt NMR analysis shows that reduction of 3 with GSH does not produce cisplatin.

Figures 2-2(d) and 2-3(d) show the effects on pBR322 DNA mobility when oxoplatin analog, 3, is reduced by GSH. When carbonate is present, the mobilities of both forms are similar to those of the controls as predicted and the slopes of both forms are only slightly positive.
showing little platinum binding to DNA, Table 1. When carbonate is absent, the mobilities of both forms decrease compared to controls and the relative slopes of both forms are significant with Form I having a positive slope (mobility increases with $r$) and Form II a negative slope, and the results are opposite to what is observed for 2 being reduced by GSH Figures 2-2(c) and 2-3(c), Table 2-1.

To further explore the DNA capture experiment involving the reduction of 3 by GSH in the absence of carbonate, a larger number of values of $r$ in the range of 1.3-5.52 with smaller intervals were investigated, (Figures 2-2(e) and 2-3(e) and Table 2-1). It is evident that both forms are shifted from the controls and their mobilities change systematically with $r$. From linear fits of the mobilities to $r$, Form I has a positive slope while Form II a negative slope with signs and magnitudes comparable to those observed in Figures 2-2 (d) and 2-3 (d) and Table 2-1. However closer inspection reveals that the mobility is weakly bimodal in nature with much better fits to the data being obtained by two lines, one for $r < 3.3$ and a second for $r > 3.3$ (see Table 2-1). While the mobility of Form I is closer to that of Form II for higher $r$, the two forms never co-migrate. $^{195}$Pt NMR analysis shows that reduction of 3 with GSH does not produce cisplatin.

Figures 2-2(f) and 2-3(f) show cisplatin binding to pBR322 DNA in the presence and absence of GSH with the relative slopes of the mobility plots given in Table 2-1. In the presence of GSH Forms I and II have large negative and positive slopes, respectively which is the same slope pattern observed for the reduction of 2 with GSH in the absence of carbonate. This suggest that some cisplatin may also be produced in the reduction of 2 by GSH and that possibly a cisplatin-GSH complex is interacting with DNA. In the absence of GSH, the mobility of Form I is much lower than the control and Forms I and II nearly co-migrate at $r = 0.9$. In Table 1, the magnitudes of the relative slopes of both forms are much larger than any other slope revealing
that the effect of the drug on the mobility of the form is significant due to the formation of the 1, 2 intrastrand crosslink. Since the slope of Form I is positive, the writhe is positive indicating a high loading of platinum on DNA.\textsuperscript{77-78} The negative slope for Form II is also consistent with a high loading of platinum on DNA.
2.3.2 $^{195}$Pt NMR

Figure 2-4. $^{195}$Pt NMR spectra. (a) Reduction of oxoplatin, 2, with AsA. (b) Reduction of oxoplatin, 2, with GSH. (c) Reduction of the oxoplatin analog, 3, with AsA. (d) Reduction of the oxoplatin analog, 3, with GSH.
$^{195}$Pt NMR was used to identify the nature of the platinum species in solution when 2 and 3 are reduced by either AsA or GSH. The incubation time of the reduction solution was 24 h, after which time NMR spectra were obtained; the solutions contained no DNA. Also, the ratio of reducing agent to platinum complex was 1:1 for AsA reducing 2 and 3 and 2:1 for GSH reducing 2 and 3, much different than the ratios employed in the DNA capture-gel studies. The choice of ratios of reducing agent to platinum was largely influenced by the relatively low sensitivity of $^{195}$Pt NMR which required millimolar concentrations of the platinum complex in the NMR tube. The concentration of the reducing agent, if the ratios used in the gel studies were to be maintained, would be unrealistically large. In these reactions, ascorbic acid acts as a two electron reducing agent to convert one molecule of a Pt(IV) complex to a Pt(II) product and the AsA is oxidized to dehydroascorbic acid.$^{25}$ In the case of the reduction of the Pt(IV) complex with iproplatin, three molecules of GSH are required to completely reduce one molecule of 2. Two GSH molecules supply the two electrons necessary to convert Pt(IV) to Pt(II) (producing the disulfide, GSSG), and one molecule of GSH, in its deprotonated form, becomes bound to Pt(II) to give as the final product, cis-di(isopropylamine)chloro-glutathionatoplatinum(II), cis-$[\text{PtCl}(\text{GS})(\text{NH}_2\text{CH(CH}_3)_2\text{)})_2$.\textsuperscript{33}

The $^{195}$Pt NMR spectra of the platinum species observed in the reduction reactions are shown in Figure 2-4. Figure 2-4(a) shows that the reaction of 2 with AsA in 12 mM HEPES buffer produces a strong signal for cisplatin at -2140 ppm, a number of weak signals in the region -1670 to -1830 ppm for Pt(II) species having a PtN$_2$O$_2$ coordination environment,$^{79}$ and a weak signal for unreacted 2, at 853 ppm. Interestingly, when two equivalents of GSH are used as the reducing agent for oxoplatin, only a signal of reduced intensity for 3 is observed in the $^{195}$Pt NMR spectrum at 853 ppm, i.e., no Pt(II) products are observed, Figure 2-4(d). Although an
exhaustive search in the chemical shift range -4000 to 1500 ppm failed to find additional $^{195}$Pt NMR resonances, the electrophoresis results clearly show that some Pt(II) complexes are produced in the reduction and that they bind to DNA, Figures 2-2(c), 2-3(c) and Table 2-1.

Figure 2-4(c) shows the $^{195}$Pt spectrum of the oxoplatin analog, 3, 24 h after exposure to one equivalent of AsA in DPBS buffer. As is evident from the figure, the only detectable $^{195}$Pt NMR signal is the strong peak at -2140 ppm which is due to cisplatin. The absence of the Pt(IV) starting material at 943 ppm shows that the reaction of 3 with AsA went to completion. In addition, the lack of Pt(II) aqua species and/or cisplatin reaction products with ascorbate or dehydroascorbic acid, as in Figure 2-4(a), shows that the presence of a high concentration of chloride ion, ~140 mM, in DPBS, suppresses the reaction of cisplatin with components in solution keeping the drug in its dichloro form.

The $^{195}$Pt NMR spectrum of the reaction of 3 with two equivalents of GSH for 24 h in DPBS buffer only exhibits a resonance (of reduced intensity) for the unreacted complex, 3, at 943 ppm, i.e. no resonances for cisplatin/other Pt(II) complexes are observed, Figure 2-4(d).
2.3.3 $^{13}$C NMR

Figure 2-5. $^{13}$C NMR spectra of the products of reduction of 2 (top spectrum) and 3 (bottom spectrum) with ascorbic acid in the presence of NaH$^{13}$CO$_3$
In addition to $^{195}$Pt NMR, the reduction of 2 and 3 with AsA, which produces cisplatin, was monitored with $^{13}$C NMR in the presence of H$^{13}$CO$_3^{-}$. Figure 2-5 shows that the reduction of either compound with AsA produces a number of Pt(II) carbonato complexes which have resonances on the low field side of free bicarbonate ion at 162.0 ppm. $^{42-43}$ Reduction of 2 in carbonate produces carbonato resonances at 168.1 – 168.7 ppm, while reduction of 3 with AsA gives a group of carbonato resonances in the region 167.2 – 169.7 ppm. Since carbonato complexes reduce the binding of cisplatin to DNA, $^{73-75}$ the $^{13}$C NMR results explain the observed decrease in binding of Pt(II) compound to DNA when carbonate is present in the reduction medium, Figures 2-2, 2-3 and Table 2-1.
2.4. Discussion

The study of Bose and coworkers\textsuperscript{25} include the kinetics and mechanism of reduction of oxoplatin, 2, with AsA and iproplatin, with GSH, showing that AsA reduces 2 with a second order rate constant of \( \sim 2 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.3 at 22 \( ^\circ \text{C} \) and that cisplatin is produced by the reduction. As shown in Figure 2-5, in this work cisplatin is produced in the reduction and \(^{195}\text{Pt} \) NMR spectra (Figure 2-4) further indicate that the drug reacts during the 24 h reaction period to produce Pt(II)N\(_2\)O\(_2\) type products. These products appear to be aquated and/or carboxyl substituted products of the drug, the carboxylate ligand most likely being supplied by dehydroascorbic acid.\textsuperscript{79} Although the reduction reaction of 2 by AsA would be expected to have an approximate half-life of \( \sim 1 \text{ min} \) under the conditions of the \(^{195}\text{Pt} \) NMR experiment ([AsA], [3] =10 mM, 37 \( ^\circ \text{C} \)), interestingly, not all 2 has been reduced to Pt(II) products as revealed by the remaining relatively weak resonance at 853 ppm (Figure 2-5). One possible cause is the stoichiometry of the reduction is not 1:1. Since aquated and/or carboxyl substituted products of cisplatin should react with DNA to form the 1, 2 intrastrand crosslink, the \(^{195}\text{Pt} \) NMR results corroborated the analyses of the gel DNA mobility studies which suggest that the reduction product of 2 with AsA produces the crosslink, Figures 2-3(a), 2-4(a) and Table 2-1.

In Figure 2-4 (b), it shows that when oxoplatin is reduced by GSH at a 1:2 ratio of reactants, only a weakened signal for 2 at 853 ppm is observed in the \(^{195}\text{Pt} \) NMR spectrum. Although it is evident that a reaction has taken place, no Pt(II) products have been detected by the \(^{195}\text{Pt} \) NMR spectrum. In the case of the related complex, iproplatin, reduction with GSH occurs with a second order rate constant of 0.49 M\(^{-1}\)s\(^{-1}\) at 22 \( ^\circ \text{C} \) at pH 7.0 with three molecules of GSH being required to reduce one equivalent of iproplatin to produce, \textit{cis-}di(isopropylamine)chloro-glutathionatoplatinum(II).\textsuperscript{33} Since iproplatin and oxoplatin have
similar structures, it is reasonable to assume that 2 would also react with GSH in a 1:3 stoichiometry which explains the peak at 853 ppm as due to remaining 3 in the $^{195}$Pt NMR sample. Moreover, if the rates of reaction for iproplatin and 2 with GSH are similar, the $t_{1/2}$ of the reaction of 2 with GSH under the conditions of $^{195}$Pt NMR experiment ([GSH], [2] =10 mM, 37 °C) is ~3 h, so that the reaction is complete after 24 h incubation. Volckova et al. 33 show that reduction of iproplatin with GSH produces cis-di(isopropylamine)chloro-glutathionatochloroplatinum(II), the corresponding Pt(II) complex for the reduction of iproplatin, but cis-diamminechloro-glutathionatochloroplatinum(II), cis-[PtCl(GS)(NH$_3$)$_2$], is not detected in Figure 2-4(b) in which resonance would be expected at ~3000 ppm. 79 Failure to observe the Pt(II) product (s) in the $^{195}$ Pt NMR spectrum highly suggests that although the Pt(II)-GSH adduct is formed, due to the polydentate nature of GSH and the long reaction period, 24 h, it converts to other species, each of which has too low intensity to be detected in the NMR experiment. The DNA capture experiments evidently show that a Pt(II) complex has been produced in the reaction, and is captured by DNA. However, its effect on the supercoiling of DNA is different from that of cisplatin, i.e., the 1, 2 intrastrand crosslink is not formed, Figures 2-2(f) and 2-3(f), Table 2-1. 31, 73-75 If the initial reduction product in the reaction is cis-[PtCl(GS)(NH$_3$)$_2$], it could react with DNA through the loss of the chloride ligand to produce a monofunctional adduct, but due to the presence of the thiolate ligand, the monofunctional adduct would not be likely to produce an intrastrand crosslink.

The reduction of 3 by AsA is similar to that of oxoplatin except that only cisplatin is observed in the $^{195}$Pt NMR spectrum, Figure 2-4(c), indicating that the reaction is complete. The absence of hydrolysis products and/or reaction products of cisplatin is explained by the high chloride concentration in the DPBS buffer (~140 mM) in the $^{195}$Pt NMR sample. Since the
reduction of 3 with AsA in the presence of DNA (no carbonate) was carried out in DPBS buffer, some reactive Pt(II) species should be present which explains the mobility difference of Form I DNA relative from its control, Figures 2-2(b) and 2-3(b). However, in this case mobilities are essentially independent of $r$. This is because $[\text{Cl}^-]$ is relatively high and, as chloride suppresses DNA binding, mobilities are not very sensitive to $r$, Table 2-1.

The $^{195}$Pt NMR spectrum of the reduction of 3 with GSH is similar to that of 2 with GSH in that only some of unreacted Pt(IV) complex remains after 24 h in the NMR solution. While this agrees with the reaction stoichiometry of iproplatin with GSH, Volckova et al. \textsuperscript{33}, i.e., some Pt(IV) should remain unreacted, Pt(II) products are not detected in the NMR spectrum. However, in the DNA gel study of this reduction in the absence of carbonate, DNA captures a Pt(II) complex. The bound platinum does not affect mobility in the same way as the intrastrand crosslink which means Forms I and II never co-migrate at any value of $r$, Figures 2-2(d,e) and 2-3(d,e), Table 1. Comparing to the reaction of 2 reduced by GSH, the Pt(II) product is likely cis-$[\text{PtCl(GS)(NH}_3)_2]$ which would be expected to bind to DNA in a mono-functional manner (loss of Cl$^-$) and not produce the intrastrand crosslink.

Figure 2-5 shows $^{13}$C NMR spectra of reductions of 2 and 3 with AsA in the presence of $^{13}$C-labeled carbonate. As is observed from the spectra, $^{13}$C NMR resonances in the region 167.2 – 169.7 ppm indicate the formation of Pt(II) carbonato complexes. It is well known that carbonato complexes can form by two paths: the first is a conventional ligand substitution reaction at the metal center, in which carbonate is exchanged with a ligand bound to the metal ion; the second is by a unique and rapid route in which the oxygen atom of a metal-bound hydroxo ligand attacks the carbon atom of molecular carbon dioxide, to produce a bound carbonate (hydrogencarbonate) ligand, Figure 2-6.\textsuperscript{80} As the latter reaction does not involve a
metal-ligand bond breaking step, this reaction rate is much higher than conventional ligand exchange reactions, which is in the range of 50-600 M\(^{-1}\) s\(^{-1}\) depending on the metal ion\(^{80}\). The \(^{13}\)C NMR spectra (Figure 2-6) show that there are a larger number of distinct carbonato species formed in the reduction of 3 with AsA than that of 2. The additional carbonato species observed could be due to the liberated dicarboxylate ligand which could act as a ligand toward Pt(II) and therefore increase the number of carbonato products detected in the reaction.

![Scheme showing the reaction of a Pt(II) aqua species with molecular carbon dioxide and associated equilibria.](image)

After the initial report of the anticancer activity of cisplatin, Rosenberg proposed that the anticancer properties of the drug are due to its ability to form an intrastrand crosslink at two adjacent purine bases of DNA\(^{81}\). Subsequent work by many research groups confirmed the existence of this type of lesion, finding that an adduct at the sequence GG is most common\(^{31}\). The mechanism for cisplatin binding to DNA starts with the formation of a mono-aqua complex with positive charge, cis-[PtCl(H\(_2\)O)(NH\(_3\))\(_2\)]\(^+\), which further reacts with N-7 of guanine through the loss of a water molecule to form the monofunctional adduct cis-[PtCl(G)(NH\(_3\))\(_2\)]\(^+\).\(^{82}\) This DNA adduct then converts to the 1, 2 intrastrand crosslink, cis-[Pt(GG)(NH\(_3\))\(_2\)]\(^+\), directly through the loss of the chloride ligand from Pt(II) or through an intermediate step also involving the aqua complex. When carbonate is present, the bound water molecule of cis-
[PtCl(H₂O)(NH₃)₂]⁺ may be partially or fully converted into a carbonate form, as shown in Figure 2-6. Moreover, as carbonate/hydrogencarbonate is a poorer leaving ligand than water, binding of the complex to DNA would be reduced which is corroborated by the gel studies that show that binding is insignificant in the presence of carbonate.73-75

Todd et al.75 argued that carbonate in the medium does not change the type of adduct formed on DNA, i.e. the 1, 2 intrastrand crosslink is still formed under this condition, with reduced amount of the crosslink that forms. However, characterization of the DNA adduct in these studies required techniques (HPLC, mass spectrometry, etc.) that would allow CO₂ gas to escape from the system. This could shift the equilibria given in Figure 2-6 to the left and, the cisplatin-DNA adduct that initially forms in carbonate could have been destroyed without being noticed in the workup thus producing and leaving only the 1, 2 intrastrand crosslink. Binter et al.73 on the other hand measured mobilities and band intensities of platinated DNA in gel electrophoresis experiments revealing that the presence of carbonate causes cisplatin to form a monofunctional adduct on DNA. While these studies had none of the structural characterization rigor of the study by Todd et al.,75 they were much less prone to shifts in the chemical equilibrium in Fig.2-6. In a later study, Sorokanich et al.74 were unable to reproduce the band intensity changes observed by Binter et al.73 but the investigators found that at large values of r, Forms I and II never co-migrate in the gel. This again suggested that the adduct in the presence of carbonate is different from the 1, 2 intrastrand crosslink. The living cell maintains a cytosolic concentration of ~12 mM carbonate, of which dissolved CO₂ is an important component. Therefore cisplatin adducts that form on genomic DNA are exposed to a “carbonate pressure” that may be difficult to imitate in laboratory experiments. Since carbonate can change the
speciation of cisplatin and carboplatin, additional study on how carbonate influences the rate and mechanism of the formation as well as the structure of the DNA adduct is meaningful.

Using the published rate constants for the reduction of 2 with ascorbic acid and reduction of iroplatin with GSH,\textsuperscript{25, 33} it is interesting to speculate on the Pt(II) products formed if 2 and 3 are present in blood. While \( k_{\text{AsA}}/k_{\text{GSH}} \sim 4 \), the ratio \([\text{AsA}]/[\text{GSH}]\) in blood (plasma) is 100 mM/850 mM = \( \sim 0.12 \)\textsuperscript{27-28, 31-32} suggesting that only half (4 x 0.12) of the reduction product would be cisplatin, reduction product by AsA, with the remaining half being a non-cisplatin complex, formed by GSH reduction. Another aspect is that the reduction potentials of 2 and 3 are \(-0.82\) and \(-0.46\) V (NHE),\textsuperscript{68, 84} respectively. This shows that 3 is more easily reduced than 2. The reduction potentials for AsA and GSH are \(-0.065\)V and \(-0.16\)V respectively, so that GSH is a slightly stronger reducing agent than AsA.\textsuperscript{85-86} Since the circulation time of a particle in the blood is 1-10 h, there would be enough opportunity for a compound such as 3 to be reduced on the way to the tumor site.\textsuperscript{25, 33-35} The platinum “cargo” released from the carrier before the vehicle reaches its intended target could also reach and kill tumor cells, which could impart characteristics to the delivery system that are a combination of both the nano-carrier and the free drug.
2.5. Conclusions

In this work we show that oxoplatin, 2, and its analog, 3, can be reduced to by the biologically common reducing agents ascorbic acid and glutathione. The reduction product by AsA is cisplatin while treatment of the Pt(IV) compounds with GSH does not produce cisplatin. The gel analyses show that the Pt(II) product(s) produced by GSH bind to closed circular pBR322 DNA without formation of the well-known 1, 2 intrastrand DNA crosslink which is characteristic of cisplatin binding to DNA. It is also shown that in the presence of physiological carbonate, Pt(II) products from reduction of 2 and 3 are prevented from binding to pBR322 DNA.
3. Functionalized Gold Nanoparticle as a Delivery Vehicle for a Prodrug of Cisplatin

3.1 Introduction

Gold nanoparticles are reported to have biocompatible properties such as nontoxicity, nonimmunogenicity and high tissue permeability and are widely investigated as drug delivery vehicles to improve the biodistribution of drugs and achieve better targeting.\textsuperscript{58,61} In this work, AuNPs (5, Figure 3-1) coated with per-6-thio-\(\beta\)-cyclodextrin (7, Figure 3-1), a hepta-thiol ligand, were synthesized as a drug delivery vehicle for a Pt(IV) prodrug of the widely used anticancer drug cisplatin. Liu et al. disclosed a one-step synthetic scheme toward cyclodextrin-modified gold nanospheres (2-7 nm in diameter) by reduction of \(\text{HAuCl}_4\) using \(\text{NaBH}_4\) in the presence of capping ligand per-6-thio-\(\beta\)-cyclodextrin.\textsuperscript{87} To improve the stability of this functionalized AuNP, Li et al. modified Liu’s synthesis by replacing DMSO solvent with DMSO/H\(_2\)O solvent mixture.\textsuperscript{88} Beta-cyclodextrin (\(\beta\)CD) is a cyclic oligosaccharide consisting of seven connected glucose moieties that has a cone shape with a hydrophilic and a hydrophobic surface located on the outer and inner sides, respectively. The hydrophilic outer surface provides water solubility and the inner surface allows inclusion of small hydrophobic molecules through a host-guest interaction driven by van der Waals forces. Beta-cyclodextrin modified AuNPs can be used as multisite hosts for binding guest molecules of interest. The Kaifer group reported that \(\beta\)CD attached on the surface of AuNP can be used to control particle aggregation by accommodating ferrocene derivative or ferrocene dimer\textsuperscript{89} and their noncovalent binding with \(\beta\)CD was later confirmed by measuring
Figure 3-1. Synthetic scheme of thiolated β-CD (7) functionalized AuNP loaded with Pt(IV) complex 4.

Li et al. developed a fluorescent assay for detecting naphthol isomers using fluorescence dye-incorporated βCD functionalized gold nanoparticle though the host-guest interaction between βCD and naphthol. The Shen group constructed a nanoenzyme system, a
supramolecular assembly of the copper complex of triethyltetramine-adamantane and βCD covalently bound to the surface of AuNP, to catalyze hydrolysis of an active ester 4,4’-dinitrodiphenyl carbonate. Kim and colleagues reported on the therapeutic application of CD-AuNP for delivery of an anticancer drug β-lapachone by efficient noncovalent encapsulation of the drug into βCD.

In this work, a Pt(IV) complex, 1-adamantanemethylamine-Pt(IV) (4, Figure 3-1), which is a potential prodrug of the Pt(II) anticancer agent cisplatin is loaded onto βCD modified AuNP through host-guest interaction. The average number of complex 4 molecules loaded onto one AuNP has been estimated and the cytotoxicity studies of the loaded delivery vehicle 6 toward human neuroblastoma cell line (SK-N-SH) show inhibition to cell growth. The binding between βCD and 4 was investigated using 1H NMR titration. We also determined the cytotoxicity of the inclusion complex 4-βCD, toward SK-N-SH cells and, using agarose gel electrophoresis, studied the ability of the ascorbic acid reduction product of 4-βCD to bind to and unwind pBR322 DNA.
3.2. Materials and Methods

3.2.1 Instrumentation

Mass spectra were obtained using an FTICR-MS with an Apollo II ion source (COSMIC Lab, Norfolk, VA). Optical images of cells under white light illumination were obtained using a Leica DMI 4000 B microscope with a Leica DFC 340 FX digital firewire camera system (Syracuse Biomaterials Institute). Inductively coupled plasma mass spectrometry (ICP-MS) data were obtained using a Perkin Elmer Elan DRC-e (SUNY College of Environmental Science and Forestry). One dimensional $^1$H NMR spectra were obtained using a Bruker Avance 300 MHz spectrometer at room temperature using a 5 mm probe. The proton resonance at 2.50 ppm, due to a small amount of the protonated form of DMSO in d$_6$-DMSO, was used as the internal chemical shift standard.

3.2.2 Synthesis of 1-adamantanemethylamine-Pt(IV) Conjugate (4)

The conjugate Pt-AD, 4, was synthesized by adding 1-adamantanemethylamine (18.2 mg, 110 µmol), 3 (43.4 mg, 100 µmol)$^{92}$, N-hydroxysuccinimide (12.7 mg, 110 µmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 23.0 mg, 120 µmol) to anhydrous DMSO (0.6 mL) and stirring the resulting solution at room temperature for 24h. The volume of the solution was reduced in vacuo to produce bright yellow oil which was washed with ~ 10 ml diethyl ether. After removal of the diethyl ether, ~ 5 ml of water was added which produced a light yellow precipitate. The product was collected by filtration, washed with ice cold diethyl ether and dried in vacuo. Yield: 57.2%. Mass spectrum of Pt-AD, 4 (1:1 THF:MeOH with NaCl)
expected m/z of \((C_{15}H_{29}N_{3}O_{4}Cl_{12}Pt)\) \(Na^+ = 603.1\), observed m/e = 603.1. \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta \) 7.66 (t, 1H, NH), \(\delta \) 5.95 (m, 6H, 2 \(\times\) NH\(_3\)), \(\delta \) 2.75 (d, 2H, CH\(_2\)), \(\delta \) 2.35 (q, 4H, 2 \(\times\) CH\(_2\)), \(\delta \) 2.16 (s, 3H, 3 \(\times\) CH adamantane) \(\delta \) 1.63 (q, 6H, 3 \(\times\) CH\(_2\) adamantane), \(\delta \) 1.41 (s, 6H, 3 \(\times\) CH\(_2\) adamantane).

### 3.2.3 Synthesis of Per-6-iodo-\(\beta\)-cyclodextrin

This compound was prepared as previously reported by Defaye and Gadelle.\(^93\) To a stirred solution of triphenylphospane (21 g, 80 mmol) and iodine (2.02 g, 80 mmol) in 80 mL DMF was added \(\beta\)CD (4.32 g, 3.8 mmol). The solution mixture was stirred at 80 °C for 19 h and then concentrated to half of the volume under vacuum. The pH was adjusted by adding 3 M sodium methoxide in methanol to 9-10 with simultaneous cooling. The solution was then kept at room temperature for 30 min to destroy the formate esters formed. It was thereafter poured into 150 mL ice water to bring out a light brown precipitate which was collected by filtration. Yield: 73.1%. \(^1\)H NMR (300 MHz, \(d_6\)-DMSO) \(\delta \) = 3.24-3.47 (m, 21 H), 3.56-3.68 (m, 14 H), 3.80 (bd, 7 H), 4.99 (d, 7 H), 5.94 (d, 7 H), 6.06 (d, 7 H).

### 3.2.4 Synthesis of Per-6-thio-\(\beta\)-cyclodextrin (7)

This compound was prepared as previously reported by Rojas et al.\(^94\) To a solution of per-6-iodo-\(\beta\)-cyclodextrin (0.965 g, 0.48 mmol) dissolved in DMF (10 ml) was added thiourea (0.301 g, 3.95 mmol). The reaction mixture was heated to 70 °C and stirred overnight under an argon atmosphere. The DMF was then removed under reduced pressure to afford yellow oil and
was dissolved in 50 mL of water. Sodium hydroxide (0.26 g, 6.5 mmol) was added and the mixture was heated to a gentle reflux under an argon atmosphere for 1 h. The resulting suspension was acidified with aqueous NaHSO₄ to bring out a brown precipitate. The precipitate was filtered and washed with distilled water and dried under vacuum to yield 3 (81.0%) as an off-white powder. ¹H NMR (300 MHz, DMSO-d₆): δ = 2.14 (bd, 7 H), 2.73 (m, 7 H), 3.21 (bd, 7 H), 3.35 (m, 14 H), 3.64 (bd, 14 H), 4.93 (d, 7 H), 5.83 (s, 7 H), 5.94 (d, 7 H). HRMS (in 1:1 THF:MeOH 0.1% FA w/ NaCl): expected m/e of (C₄H₁₂N₂O₅C₁₂Pt)Na⁺ = 1269.2. Observed m/e = 1269.2.
3.2.5 Synthesis of Thiolated β-cyclodextrin Functionalized Gold Nanoparticles (5)

The functionalized AuNP was prepared as previously reported using perthiolated β-cyclodextrin as capping molecules.\(^8\) A solution of 48 mL DMSO containing 90 mg of NaBH\(_4\) (2.4 mmol) and 24.0 mg (0.019 mmol) of thiolated β-cyclodextrin was added to a solution of 60 mL DMSO/H\(_2\)O 4/1 containing 12.2 mg (0.036 mmol) of HAuCl\(_4\). The reaction solution was stirred at room temperature for 24 h. 96 mL of CH\(_3\)CN was added to bring out precipitate which was collected by centrifugation. The precipitate was first washed with 50 mL of mixed solvents CH\(_3\)CN and DMSO (1:1 v/v), the precipitate was isolated by centrifugation and washed again with and 50 mL of ethanol. The precipitate was isolated by centrifugation and dried in vacuo.

The dried solid was taken up in 0.2 mL of H\(_2\)O and solution was kept at room temperature for 30 min before adding 0.6 mL of CH\(_3\)CN which induced precipitation of purified 5. The βCD-capped AuNP was collected by centrifugation and dried in vacuo overnight to obtain 10.7 mg dried product. UV-Vis spectrum of the product in aqueous solution has an absorption peak at 515 nm.

3.2.6 Synthesis of Thiolated β-cyclodextrin Functionalized Gold Nanoparticles Loaded with 1-adamantanemethylamine-Pt(IV) Conjugate (6)

To a solution of 0.75 mg of 4 (1.29×10\(^{-3}\) mmol), dissolved in 0.4 mL DMSO was added a solution of 6.5 mg of 5 (1.29×10\(^{-3}\) mmol of gold-attached βCD), dissolved in 0.1 mL H\(_2\)O. The reaction mixture was stirred overnight at room temperature following which time, 1.5 mL of CH\(_3\)CN was added to induce precipitation of 4 which was collected by centrifugation. The precipitate was washed with 2 mL of CH\(_3\)CN and DMSO (3:1 v/v), washed a second time with 1
mL of ethanol, and the inclusion complex (4.0 mg) was isolated by centrifugation and dried overnight in vacuo. The weight percentage of Pt in 4 (2.50 %) was obtained through ICP-MS analysis. UV-Vis spectrum of the product in aqueous solution has an absorption peak at 515 nm.

3.2.7 UV-visible Absorption Spectra of 5 and 6

Cary 50 UV-vis spectrophotometer was utilized, with baseline correction in the region 200-800 nm. Aqueous solution of AuNP were prepared and tested in a cuvette with a 1 cm path length.

3.2.8 Thermogravimetric Analysis (TGA) of Thiolated β-cyclodextrin Functionalized Gold Nanoparticles

TGA was carried out on a TGAQ500 by raising the temperature of the sample at a rate of 5 °C/min from room temperature to 100 °C. The temperature is held at 100 °C for 30 min to eliminate water contained in the sample. Then the temperature was further increased to 200 °C at the same rate and held for 1 hour to eliminate DMSO followed by a temperature increase up to 800 °C at 5 °C/min.

3.2.9 Transmission Electron Microscopy (TEM) of 5 and 6
TEM measurements were performed on a JEOL 2000EX operated at 120 kV with a tungsten filament (SUNY College of Environmental Science and Forestry, Syracuse, NY). The diameters of AuNP 5 and 6 were calculated using ImageJ software on populations >100 particles (counts).

### 3.2.10 ¹H NMR Studies of Adamantane Derivatives and 4-βCD Inclusion Complex

One dimensional ¹H NMR was obtained using a Bruker Avance 300 MHz spectrometer at room temperature. A series of spectra with various \( r = [4]/[\beta CD] \) were collected in solvent mixture of d₆-DMSO/D₂O 4/1, and the chemical shift of protons on HA, HB₁, HB₂ and HC was plotted against \( r \) (Figure 3-6). Complex 4 was first dissolved in d₆-DMSO 0.48 mL to which 0.12 mL D₂O was added, the final concentration of 4 was 16.7 mM. To this solution, by repeatedly adding βCD, measurements with increasing \( r \) values of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 were made. A spectrum was collected 2 h after each addition to allow equilibrium of binding. ¹H NMR of 4 and two other adamantane derivatives, 1-adamantanemethylamine and 1-adamantanemethanol at various concentrations was tested in d₆-DMSO/D₂O 4/1 (Figures 3-8, 9 and 10). The d₆-DMSO proton impurity signal at 2.50 ppm is used as internal standard.

### 3.2.11 Cytotoxicity Study of 4-βCD Inclusion Complex toward Neuroblastoma Cells

(Compared with Cisplatin)

The studies involving human neuroblastoma (SK-N-SH) cells were carried out under standard conditions as previously reported. The studies involving human neuroblastoma (SK-
N-SH) cells were carried out under standard conditions in a humidified, 37 °C, 5 % CO₂ atmosphere in an incubator. The culture medium used for SK-N-SH cells was Eagle's minimum essential medium (EMEM) to which had been added 10 % Fetal Calf Serum (FCS), 100 µg/mL streptomycin, 100 IU/mL penicillin and 2.0 mM L-glutamine. Four solutions of Pt complex in medium having Pt concentrations 2, 5, 10 and 20 µM were prepared by dissolving a weighed amount of 4 or cisplatin. Complex 4 was first dissolved in 0.2 ml of DMSO which was diluted with an aqueous solution of βCD to give a final H₂O-DMSO solution with [4]/[βCD] = 0.5 and DMSO, 0.4% (v/v). A stoichiometric excess of βCD was used to drive the equilibrium in favor of the inclusion complex. To each of 60 wells (6 groups of 10 wells each) in the central portions of four 96-well microplates were added: medium alone containing no cells, Group 1, and medium containing 5×10⁴ cells/mL, Groups 2-6. The cells were allowed to grow for 24 h reaching ~ 30% confluency after which time the medium was removed and replaced with 100 µL of medium containing compound having concentrations of 0, 2, 5, 10, and 20 µM Pt for Groups 2-6, respectively. After a 72 h exposure time, the medium in the wells containing cells was removed and replaced and washed twice with fresh medium. The number of live cells was determined using the CCK-8 assay (Dojindo Molecular Technologies, Inc. Gaithersburg, MD) according to the specified protocol and percent inhibitions determined as previously described⁹⁵.

3.2.12 DNA Capture-Gel Electrophoresis Experiments of 4-βCD Inclusion Complex

The DNA binding studies were done in a total volume of 20 µl containing 38.5 µM base pairs pBR322 DNA and 12 mM pH 7.4 HEPES buffer with 0.4% DMSO v/v. The final concentration of 4 was in the range of 2.5-60 µM while the final concentrations of βCD was in the range 5-120 µM with, [4]/[βCD] = 0.5. Ascorbic acid (final concentration 50 µM) was used
as the reducing agent for the Pt(IV) complex. The inclusion complex 4-βCD, was first dissolved in 0.1 ml DMSO followed by the addition of 24.9 ml of HEPES buffer to give a final aqueous solution containing 0.4% DMSO v/v. Appropriate volumes of stock solutions were added to the solutions containing the buffer and DNA to give the final concentrations in the reaction solutions containing DNA stated above. The values of \( r \), where, \( r = \frac{[4]}{[\text{DNA-bp}]} \), in the reaction solutions are given in the captions of figures. The samples were incubated at 37 °C for 24 h in sealed Eppendorf tubes and electrophoresis/analysis carried out as previously reported. The percent inhibition of cell growth was calculated using the Equation (1), where \( \%I \) is the percent inhibition, \( A_t \) is the absorbance of wells with cells containing culture medium + Pt complex, \( A_c \) is the absorbance of wells with cells and culture medium, and \( A_m \) is the absorbance of wells without cells but with culture medium.

\[
\%I = 100 \times \left[ \frac{A_c - A_t}{A_c - A_m} \right] \tag{1}
\]

3.2.13 Cytotoxicity Study of 6 toward Neuroblastoma Cells (Compared with Cisplatin)

The studies involving human neuroblastoma (SK-N-SH) cells were carried out under the same conditions as stated in 3.2.12. Four solutions of the complex in medium having Pt concentrations, 2, 5, 10 and 20 µM were prepared by dissolving a weighed amount of delivery system 6 or cisplatin. To each of 60 wells, 6 groups of 10 wells each, in the central portions of four 96-well microplates were added, medium alone containing no cells, Group 1, and medium containing \( 5 \times 10^4 \) cells/mL, Groups 2-6. The cells were allowed to grow for 24 h reaching \(~30\%\) confluency after which time the medium was removed and replaced with 100 µL of medium.
containing compound having concentrations of 0, 2, 5, 10, and 20 µM Pt for Groups 2-6, respectively. After 72h exposure time, the medium in the wells containing cells was removed and replaced and washed twice with fresh medium. The number of live cells was determined using the CCK-8 assay according to the specified protocol. The percent inhibition of cell growth was calculated using the Equation (1).
3.3 Results

Thiolated βCD, 7, was synthesized following literature method\textsuperscript{94} and characterized using \textsuperscript{1}H NMR and mass spectrometry. Gold nanoparticles capped with 3 were synthesized through a one-step reaction by reducing HAuCl\textsubscript{4} using NaBH\textsubscript{4} in the presence of 7 in DMSO/H\textsubscript{2}O solvent mixture as reported by Li et al.\textsuperscript{88} UV-Vis spectrum of 5 shows a surface plasmon absorption peak located at 515 nm (Figure 3-2). The size of the AuNPs is 4.7 ± 1.1 nm in diameter which is determined based on transmission electron microscopy (TEM) image (Figure 3-3).

Thermogravimetric analysis (TGA) curve (Figure 3-4) of 5 shows a weight loss of 26.63% from 209.10 °C to 460.59 °C which is the weight percent of thiolated βCD, and the weight percent of Au is 64.67%.

Figure 3-2. UV–vis absorption spectra of AuNP 5 and AuNP 6 aqueous solution.
Figure 3-3. TEM images of 5 (a), 6 (b) and diameter histogram (c) and (d) of 1 and 2 based calculation of >100 particles in TEM images respectively.
Pt(IV) anticancer prodrug 4 is synthesized as previously reported and loaded onto functionalized AuNP 5 at room temperature. UV-Vis spectra (Figure 3-2) of the delivery vehicle before and after loading with 4 both have a peak at the wavelength of 515 nm, which indicates the presence of gold nanoparticles (AuNPs) and no significant change in diameter due to the loading. This expectation is confirmed by the results calculated based on TEM images (Figure 3-3) which shows insignificant change in diameter after the loading of 4. The weight percent of Pt (2.50%) in the 4 loaded AuNP is achieved through inductively coupled plasma-mass spectroscopy (ICP-MS).
Figure 3-5. $^1$H NMR of 4 and βCD ($r = [βCD]/[4]=0.2, [4] =16.7 \text{ mM}$) in d$_6$-DMSO/D$_2$O 4:1 (v/v). See Figure. 3-1 for chemical shift assignment.
Figure 3-6. Chemical shift of $^1$H NMR of 4 and βCD inclusion complex vs $r$, [4] = 16.7 mM. (a) HA, (b) The average of HB$_1$ and HB$_2$, (c) HC.
Figure 3-7. $^1$H NMR of 4 in $d_6$-DMSO/D$_2$O 4:1 (v/v). (a) 2 mM, (b) 40 mM.
Figure 3-8. Chemical shift of $^1$H NMR of 4 as a function of concentration. (a) HA, (b) The average of HB$_1$ and HB$_2$, (c) HC.
Figure 3-9. Chemical shift of $^1$H NMR of 1-adamantanemethylamine as a function of concentration. (a) HA, (b) The average of HB$_1$ and HB$_2$, (c) HC.
Figure 3-10. Chemical shift of $^1H$ NMR of 1-adamantanemethanol as a function of concentration. (a) HA, (b) The average of HB$_1$ and HB$_2$, (c) HC.
The binding between βCD and 4 is investigated through $^1$H NMR (Figure 3-5) in $d_6$-DMSO/D$_2$O 4:1 (v/v). The chemical shift of signals of protons HA, HB$_1$, HB$_2$ and HC of 4 is plotted against $r$, the concentration ratio of βCD to 4. The chemical shift of protons on adamantyl group (AD) increases with increasing $r$ value (Figure 3-6) which indicates a deshielding effect due to host-guest interaction. Analysis of these curves gives information about the stoichiometry. To further investigate the stoichiometry of the host-guest inclusion complex formed by βCD and 4, $^1$H NMR spectra of Pt(IV)-adamantane 4 complex (Figure 3-7) and two other molecules with adamantyl group, 1-adamantanemethylamine and 1-adamantanemethanol (Figure 3-9, 3-10) at various concentrations were collected at various concentrations were collected. In Figure 3-7, $^1$H NMR peak broadening is observed at relatively high concentration (Figure 3-7) and there is a general trend of chemical shift decreasing with increasing concentration for all three adamantyl molecules (Figures 3-8~10). This implies a change in the environment of protons at higher concentrations.
Figure 3-11. Percent inhibition vs. the concentration of Pt of 4-βCD (open bar) and cisplatin (black bar) toward SK-N-SH cells.

Figure 3-12. Ethidium bromide stained agarose gel of pBR322 DNA in the presence of the inclusion complex of 4-βCD, in pH 7.4 HEPES buffer with AsA as the reducing agent.

Figure 3-11 gives the results of the cytotoxicity of the inclusion complex, 4-βCD and, compared with cisplatin, toward human neuroblastoma (SK-N-SH) cell line. As shown in bar
graph, inclusion complex inhibits the growth of the cell population by ~70%, versus ~98% for cisplatin, at a Pt concentration of 20 μM. Therefore the inclusion complex is less toxic to the cells than is cisplatin. Using pBR322 DNA to capture the platinum Pt(II) product formed when the 4-βCD is reduced by AsA (Figure 3-12), closed circular Form I DNA shows a biphasic mobility pattern which is consistent with the formation of the 1, 2 intrastrand crosslink 31.

Figure 3-13. Microscope images of untreated SK-N-SH cells (a and d) and cells treated with 6 (20 μM Pt) for 24 h (b and c) and 72 h (e and f). The dark spots in (b) and (c) show the aggregation formed by internalized AuNPs.
Cytotoxicity of the constructed delivery system (6) is investigated toward human neuroblastoma cell line SK-N-SH. The cells were exposed to the delivery system and cisplatin respectively for 72 h. As shown in Figure 3-13, the areas in frames show an example of accumulations of AuNP in SK-N-SH cells with 24 and 48 h exposure time at 20 μM Pt concentration, and there are less cells in the delivery system treated group than the untreated control group with healthy cells. It is illustrated in Figure 3-14 that the delivery vehicle has a percent inhibition up to ~70% on the growth of SK-N-SH cells after 72 h exposure time at a Pt concentration of 20 μM and the percent inhibition is dose dependent. However, the percent inhibition is lower than cisplatin as the Pt(IV) conjugate might not be completely reduced.
3.4 Discussion

The ultimate goal of this work is to construct a delivery system to transport drugs like complex 4 through host-guest interaction; therefore it is important to investigate the amount of drug molecules that can be loaded onto the vehicle. To reveal the delivery capacity of the vehicle through host-guest interaction pertaining to βCD, we estimated the average number \( n \) of attached βCD molecules per AuNP using equation (2)\(^7\) and (3), where \( N \) is the average number of gold atoms per AuNP, \( \rho \) is the density for fcc gold (19.3 g/cm\(^3\)), \( D \) is the average core diameter of the particles (nm), \( M \) is the atomic weight of gold (197 g/mol) and \( M' \) is the molecular weight of thiolated βCD (1240 g/mol). Based on the experimental data from TEM (Figure 3-3) and TGA (Figure 3-4), \( D = 4.7 \) nm, wt\%(βCD) = 26.63\% and wt\%(Au) = 64.67\%, therefore the calculated value of \( n \) is ~210.

\[
N = \frac{\pi \rho \left( \frac{D}{10^7} \right)^3}{6 \left( \frac{M}{N_A} \right)} = 30.90 \ D^3 \tag{2}
\]

\[
n = \frac{NM \ \text{wt\%}(\beta CD)}{M' \ \text{wt\%}(\text{Au})} = 4.91 \ D^3 \times \frac{\text{wt\%}(\beta CD)}{\text{wt\%}(\text{Au})} \tag{3}
\]

The modified βCD (7) can be conjugated to AuNP surface by a maximum of seven S-Au covalent bonds. Assuming thiolated βCD are close packed and form a monolayer of coating on the surface of the AuNP, the theoretical maximum number of cyclodextrins per AuNP could be estimated using equation (4), where \( S_{Au} \) is the surface area of AuNP (nm\(^2\)), \( S_{CD} \) is the surface area occupied by the wider opening of close packed βCD (nm\(^2\)) and \( d \) is the van der Waals surface diameter of the larger opening of βCD which has a value of 1.54 nm. As \( n \) is roughly...
estimated in this work, calculating the number of βCD molecules forming a monolayer using the surface diameter of either the smaller or larger end, would not lead to significant variation. By inserting the experimental data, the result of this maximum number is ~34 which is much lower than the number calculated (~210) using the experimental data. As workup procedure in atmosphere is involved, we proposed a mechanism for binding: on the surface of AuNP, disulfide bonds are formed between thiol βCD molecules so that a portion of the βCD molecules are not directly attached to the surface of AuNP through Au-S bond as shown in Figure 3-1.

\[
\frac{S_{\text{Au}}}{S_{\text{CD}}} = \frac{\pi D^2}{\sqrt{3} d^2} = 3.62 \left[ \frac{D}{d} \right]^2
\]  
(4)

As it is possible that the βCD binding sites on the surface of AuNP are not fully occupied by 7, Equation (5) is used to estimate the average number (n’) of 4 loaded onto one AuNP, where M’’ is the atomic weight of Pt (195.08g/mol). Based on ICP-MS result, wt%(Pt) is 2.50%, therefore the value of n’ is ~125, which means ~60% of cyclodextrin sites on AuNP are loaded with complex 4.

\[
n’ = \frac{NM \ \text{wt}(\text{Pt})}{M’’ \ \text{wt}(\text{Au})} = 31.20 \ D^3 \times \frac{\text{wt}(\text{Pt})}{\text{wt}(\text{Au})}
\]  
(5)

To further study the binding between 4 and 7, 1H NMR titration of 4 with βCD has been conducted in d₆-DMSO/D₂O 4:1 (v/v). This part of work was done with great help from Dr. Jerry Goodisman. As shown in Figure 3-6, with fixed concentration of 4 (16.7 mM), chemical shift of protons on AD increases with higher r value (r = [βCD]/[4]) indicating a deshielding effect due to host-guest interaction. As it is commonly accepted that βCD and adamantane derivatives form inclusion complexes at a ratio of 1:1, 98 we calculated the binding constant of
βCD and 4 based on 1:1 stoichiometry. From the chemical shift data for the protons on HA, HB₁, HB₂ and HC, the calculated binding constants are 82.2±19.7 M⁻¹, 16.5±6.4 M⁻¹ and 5.09±1.71 M⁻¹, respectively, which are very inconsistent (Table 3-1). Therefore it appears that the 1:1 binding model does not fit in this case.

As observed in Figure 3-7(a), ¹H NMR signals of protons on HB₁ and HB₂ of complex 4 with a concentration of 2 mM is a clear quartet while at higher concentration (40 mM, Figure 3-7), the peak broadened and as shown in Figure 3-7(b), the chemical shift decreases with increasing concentration, which can be evidences of change in the environment of protons at higher concentrations. Therefore, we proposed a model of binding of 4 to βCD at 1:1 ratio with formation of complex 4 dimers, (4)₂, so that there are two equilibria in the system with K₁ and K₂ as equilibrium constants, shown in Equation (6) and (7).

\[
\beta CD + 4 \rightleftharpoons K_1 \beta CD-4 \quad (6)
\]

\[
2 \ 4 \rightleftharpoons K_2 \ (4)_2 \quad (7)
\]

The values of K₂ and the maximum chemical shift changes are varied to minimize the mean-square deviation between experimental and theoretical chemical shifts. It is found that when the value of K₂ is 0.0002 M⁻¹, the binding constants calculated based on protons on C₂, C₃ and C₄ are consistent. The values are 49.7±20.9 M⁻¹, 77.4±18.7 M⁻¹ and 38.3±17.5 M⁻¹, respectively. As the value of K₂ is low (0.0002 M⁻¹), the concentration of (4)₂ at equilibrium would also be low which is in accordance with the assumption that almost all the bound AD is a monomer.
Table 3-1. Binding constants of 4 and βCD in d6-DMSO/D2O 80/20 at room temperature in 1:1 stoichiometry without and with formation of AD dimers.

<table>
<thead>
<tr>
<th>Binding Constant (M⁻¹)</th>
<th>1:1</th>
<th>1:1 AD dimerization model K₁ (K₂ = 0.0002 M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>82.2±19.7</td>
<td>77.4±18.7</td>
</tr>
<tr>
<td>HB₁, HB₂</td>
<td>16.5±6.4</td>
<td>38.3±17.5</td>
</tr>
<tr>
<td>HC</td>
<td>5.09±1.71</td>
<td>49.7±20.9</td>
</tr>
</tbody>
</table>

To test the cytotoxicity of the constructed delivery system (6), human neuroblastoma cell line SK-N-SH was chosen as the test system since Pt based antitumor complexes such as cisplatin and carboplatin are used to treat childhood cancers including neuroblastoma.⁹⁹-¹⁰¹ As shown in Figure 3-13, accumulations of AuNP in SK-N-SH cells occurs within 24 h exposure time at 20 μM Pt concentration, and there are fewer cells in the delivery system treated group than the untreated control group with healthy cells. The dark spots in cancer cells in Figure 3-13(c) shows the accumulation of AuNP in cells after 24 h exposure, while after 72 h exposure shown in Figure 3-13(f), the cells have been destroyed and the obscure dark spots show the release of AuNP from broken cells. Figure 3-14 shows that the delivery vehicle has a percent inhibition up to ~70% on the growth of SK-N-SH cells after 72 h exposure time at a Pt concentration of 20 μM. The percent inhibition is dose dependent and lower than that of cisplatin indicating the Pt(IV) conjugate might not be completely reduced. Earlier study by us has shown than the two precursors of 4, c,t,c-[PtCl₂(OH)₂(NH₃)₂] and c,t,c-[PtCl₂(OH)(O₂CCH₂CH₂CO₂H)(NH₃)₂], can be reduced by bioavailable reducing agents ascorbic acid or glutathione and unwind plasmid circular DNA.¹⁰² The anticancer activity of 4 could be exerted by reducing agents in biological system; the reduction product Pt(II) complex then binds with DNA strands to induce cell death.
Using pBR322 DNA to capture the platinum Pt(II) product formed when the 4-βCD is reduced by AsA, closed circular Form I DNA shows a biphasic mobility pattern which is consistent with the formation of the 1, 2 intrastrand crosslink. When \( r \) increases, mobility of Form I DNA first decreases and then increases with co-migration of both Form I and Form II DNA at some value of \( r \), in this case occurring at \( r = 0.52 \), which is a hallmark of the gel pattern for the cisplatin-produced 1, 2 intrastrand crosslink. While the amount of platinum entering the cells for either agent is unknown, the cytotoxic effects of the Pt(IV) compound are very likely dependent on its ability to be reduced to a more reactive Pt(II) complex. As discussed above, 3, which has the same donor ligands as 4 and its inclusion complex, 4-βCD, can be reduced by either AsA or GSH, with the former reducing agent producing cisplatin and the latter producing a Pt(II) complex of unknown structure. Since there is no AsA and only a very small amount of GSH, \( \sim 3 \) μM, in the culture medium, it appears that the site of reduction of the inclusion complex is the cytosol of the neuroblastoma cells where the concentrations of AsA and GSH are, \( \sim 1 \) and \( \sim 2 \) mM, respectively. Once the Pt(IV) ion in the inclusion complex is reduced to Pt(II) in the cytosol, the product(s) could bind to proteins and/or DNA causing the cell to enter into apoptosis and die.
3.5 Conclusions

Gold nanoparticles (AuNPs) coated with per-6-thio-β-cyclodextrin, a hepta-thiol ligand, were synthesized as a drug delivery vehicle for a Pt(IV) complex, 1-adamantanemethylamine-Pt(IV) (4), which is a potential prodrug of the Pt(II) anticancer agent cisplatin. It is estimated that an average of 210 hepta-thiol ligands are bound to the surface of the particle. After loading the surface of the thiolated β-cyclodextrin modified AuNPs with 4 which binds through host-guest interaction, chemical analysis shows that ~125 Pt (IV) conjugates are bound to the particle. The results of cytotoxicity studies show that the loaded delivery vehicle inhibits the growth of human neuroblastoma cells. Beta cyclodextrin facilitates the aqueous solubility of complex 4 through a host-guest interaction and the inclusion complex formed shows inhibition on the growth of SK-N-SH cells but it is less toxic than cisplatin. Agarose gel electrophoresis indicates that reduction of 4-βCD by AsA in the presence of pBR322 DNA results in the formation of platinum-DNA, 1, 2 intrastrand crosslinks consistent with cisplatin being formed in the reaction.

The binding of complex 4 and β-cyclodextrin is also investigated using $^1$H NMR, and a model with a 1:1 stoichiometry with formation of adamantane dimers is proposed.
4.2 Cytotoxicity of Cu(II) And Zn(II) 2,2’-Bipyridyl Complexes: Dependence of IC$_{50}$ on Recovery Time

4.1. Introduction

Copper in its ionic forms plays an important role in various proteins and metallo enzymes in biological systems.$^{103}$ Since free aquated copper is toxic to cells, nature has developed a sophisticated mechanism for transporting the ion from its ingestion in the food supply to its ultimate incorporation in critical biological components in the cell. Imbalanced copper levels in the body lead to abnormal conditions which include Menkes disease, the neurodegenerative genetic disorder. This disease is a childhood disorder which is characterized by reduced absorption of copper and results in delayed development, mental retardation and early mortality.$^{31,104}$ In contrast, Wilson’s disease, which can be Parkinson-like in nature, is characterized by an excess of copper in the body and leads to reduced hepatic ability and/or neurological problems. Copper ions are also important for blood vessel development in tumors and the ion is believed to contribute to the growth, invasion and metastasis of cancer.$^{105}$

Dwyer and coworkers reported that the Cu$^{+2}$ complex of 3,4,7,8-tetramethyl-1, 10-phenanthroline inhibits the growth of Land-schuetz ascites tumor in 1965.$^{106}$ Thereafter, the anti-cancer properties of many copper complexes containing, 1, 10 phenanthroline, (o-phen) and related ligands have been actively investigated.$^{105,107-110}$ Although the mechanism of the anti-cancer activity of these complexes is not known, it is possible that the compounds bind directly to target molecules in the cell or participate in redox chemistry that produces radicals that damage biomolecules in the cell. In 2009, Dou, Verani, and their coworkers synthesized a group of Cu$^{+2}$ complexes containing asymmetric tridentate donor ligands that induce apoptosis in C4-
2B and PC-3 human prostate cancer cells. Studies of mechanism with the compounds show that they inhibit the chymotrypsin-like activity of the 20S proteasome. This mechanism of inhibition makes them similar to the anticancer drug bortezomib, which is used for treating multiple myeloma. The Casiopeínas are a group of Cu\(^{2+}\) mixed-ligand antineoplastic agents which contain 1, 10-phenanthroline or 2, 2’-bipyridine (bpy) and other bidentate ligands. It is reported that these compounds exhibit cytotoxicity, genotoxicity and antitumor effects but their mode of action is presently unknown. Earlier Koiri et al., reported that the complexes, M(bpy)(Ac)\(_2\)•H\(_2\)O, where M = Cu(II) and Zn(II), bpy = 2,2’-bipyridine and Ac = CH\(_3\)CO\(_2\) bind to purified lactate dehydrogenase, LDH. Moreover, when solutions of the compounds are perfused into mice, they are able to reduce the levels of LDH in tissues and organs. Since inhibition of LDH blocks the interconversion of NADH and NAD+, a reaction that is critical for cell survival, inhibiting the enzyme with small molecules could be a useful strategy for killing cancer cells in chemotherapy.

![Figure 4-1. Structures of metal complexes 8-10](image)

In this report we measured the percent inhibition, \(I\), of Cu(bpy)(NCS)\(_2\), 8,

\([\text{Cu(bpy)}_2(\text{H}_2\text{O})](\text{PF}_6)_2\), 9, and Zn(bpy)\(_2\)(NCS)\(_2\), 10, toward neuroblastoma (SK-N-SH) and ovarian cancer (OVCAR-3) cells as a function of time after the cells have been exposed to the
metal complex, i.e., as a function of the recovery time, $t$. The study revealed that while surviving cells previously exposed to the zinc complex divide normally during the recovery period, surviving cells previously exposed to the copper complexes are slowed from dividing during the recovery period. After deriving the relationship between $I$ and $t$ we show that the measured value of $IC_{50}$ for the two copper complexes strongly depends on $t$ while $IC_{50}$ for the zinc complex is relatively independent of $t$. Since earlier reports by Sigman et al. that copper-phenanthroline complexes can be activated to cleave DNA,$^{118}$ we also studied the ability of the complexes to cut closed circular pBR322 DNA in the presence of the biologically common reducing agent, ascorbic acid.
4.2. Materials and methods

4.2.1 Materials

Human neuroblastoma (SK-N-SH) cells and human ovarian carcinoma (OVCAR-3) cells were purchased from the American Type Culture Collection (ATCC Number HTB-11 and HTB-161). Minimum essential medium with Earle’s salts and L-glutamine (EMEM)(10-010), RPMI 1640, fetal calf serum (FCS), cell-stripper, and trypan blue stain, were from Mediatech (Herndon, VA). The cell counting kits, CCK-F and CCK-8, which determine the number of live cells in a population, were obtained from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). Plasmid pBR322 was purchased from Invitrogen Inc. (Carlsbad, CA). For the DNA cleavage studies, HEPES, agarose, Trizma base, boric acid, EDTA, ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO), the plasmid DNA (pBR322), 25 µg/µl, was from Invitrogen (Carlsbad, CA).

4.2.2 Cytotoxicity Studies

The studies involving human neuroblastoma (SK-N-SH) cells and human ovarian carcinoma (OVCAR-3) cells were carried out under standard conditions in a humidified, 37 °C, 5 % CO₂ atmosphere in an incubator. The culture medium used for SK-N-SH cells was Eagle's minimum essential medium (EMEM) to which had been added 10 % Fetal Calf Serum (FCS), 100 µg/mL streptomycin, 100 IU/mL penicillin and 2.0 mM L-glutamine. The culture medium used for OVCAR-3 was RPMI 1640 (Roswell Park Memorial Institute) to which had been added 20 % Fetal Calf Serum (FCS), 100 µg/mL streptomycin, 100 IU/mL penicillin, 2.0 mM L-
glutamine and 10 µg/mL insulin. Four solutions of the complex in medium having concentrations, 250, 500, 1000, and 2000 µM were prepared by dissolving a weighed amount of complex in 0.3 mL of DMSO followed by the addition 5.7 mL of culture medium to give the indicated final compound and DMSO (5%) concentrations. To each of 60 wells, 6 groups of 10 wells each, in the central portions of four 96-well microplates were added, medium alone containing no cells, Group 1, and medium containing 5×10^4 cells/mL, Groups 2-6. The cells were allowed to grow for 24 h reaching ~30% confluency after which time the medium was removed and replaced with 100 µL of fresh medium containing 5 % DMSO, Group 2, or 100 µL of medium containing compound having concentrations of, 250, 500, 1000, and 2000 µM for Groups 3-6, respectively. After an exposure time to copper/zinc compound of 1 h, the medium in the wells containing cells was removed and replaced with fresh medium and the cells were allowed to recover for 2, 26, 50, and 74 h at the end of which time the number of live cells was determined using the CCK-F assay according to the specified protocol. The CCK-F assay relies on esterases in live cells to convert a precursor compound into a calcein dye the amount of which can be quantitated by fluorescence. The viability of SK-N-SH cells was also determined using a second method, the CCK-8 assay, following the same procedure. The CCK-8 assay relies on dehydrogenases in live cells to convert a tetrazolium salt into formazan dye the amount of which can be quantitated by absorption spectroscopy. The concentrations of copper/zinc complex used were 25 µM, 250 µM, 2500 µM.

The percent inhibition of **8-10** was calculated using the equation (8), where \( I \) is the percent inhibition, \( A_D \) is the absorbance or fluorescence of wells containing cells + culture medium + 5 % DMSO, \( A_T \) is the absorbance or fluorescence of wells with cells containing culture medium + 5 % DMSO + the copper/zinc complex, \( A_C \) is the absorbance or fluorescence
of wells with cells and culture medium, and $A_M$ is the absorbance or fluorescence of wells without cells but with culture medium.

$$I = 100 \times \left[ \frac{A_r - A_{in}}{A_c - A_M} \right]$$

(8)

4.2.3 DNA Cleavage Studies by Agarose Gel Electrophoresis

Six solutions, 20 µl each, in 10 mM HEPES buffer (pH 7.4) each containing various final concentrations of 8 and 9 (1, 2, 4, 6, 8 and 10 µM) and 10 (10, 20, 30, 40, 50 and 60 µM) and plasmid DNA (33.3 µM base pairs) were prepared. Since stock solutions of the complex were prepared in a manner analogous to those described for the cell studies, the reaction solutions also contained 5 % DMSO. The reaction solutions, along with solutions containing DNA alone in 10 mM HEPES buffer with and without 5 % DMSO, were incubated at 37 °C for 30 min. After this time a stock solution of ascorbic acid (final concentration, 1.0 mM) was added to the complex-containing solutions and the resulting mixtures were incubated at 37 °C for an additional 30 min to produce DNA strand cleavage. As a control DNA in buffer and 5% DMSO was incubated with complex for 0.5 h in the absence of activator. Following incubation, the reaction mixtures were quenched by rapid cooling using dry ice and 2.5 µl of a loading buffer containing 50 % glycerol, 0.25 % bromphenol blue and 0.25 % xylene cyanol was added to each sample. An 8 µl volume of each solution was loaded onto a 1 % (w/v) agarose gel in 0.5X (0.045 M Tris-borate and 0.001 M EDTA) TBE buffer (pH 7.4) and electrophoresis was carried out at 100 V for 4 h. The gel was exposed to a $5 \times 10^{-5}$ % solution of ethidium bromide for 30 min and washed (de-
stained) in distilled water for 15 min. An image of the gel was captured using a Kodak Gel Logic 100 imaging system.
4.3 Results and Analysis
Figure 4-2. Percent inhibition of cell growth vs. compound concentration on SK-N-SH cells. The cells were exposed to a compound for 1 hour and incubated on separate plates for 2h, 26h, 50h and 74h before measurement. Data collected from CCK-8 and CCK-F assays were represented by squares and diamonds respectively. The curves were the best fits of equation (9) to the data from CCK-F assays. First four panels, results for Cu(bpy)(NCS)$_2$, 8, next four panels, results for [Cu(bpy)$_2$(H$_2$O)$_2$](PF$_6$)$_2$, 9, last four panels, results for Zn(bpy)$_2$(NCS)$_2$, 10.
Figure 4-3. Percent inhibition of cell growth vs. compound concentration on OVCAR-3 cells. The cells were exposed to a compound for 1 hour and incubated on separate plates for 2h, 26h, 50h and 74h before measurement. Data collected from CCK-F assays was represented by diamonds. The curves were the best fits of equation (9) to the data from CCK-F assays. First four panels, results for Cu(bpy)(NCS)$_2$, 8; next four panels, results for [Cu(bpy)(H$_2$O)$_2$](PF$_6$)$_2$, 9, last four panels, results for Zn(bpy)(NCS)$_2$, 10.

The data analysis of this work was done with generous help from Dr. Jerrey Goodisman.

Figures 4-2 and 4-3 show the percent inhibition of SK-N-SH neuroblastoma cells and OVCAR-3 ovarian cancer cells by Cu(bpy)(NCS)$_2$, 8, [Cu(bpy)$_2$(H$_2$O)](PF$_6$)$_2$, 9, and Zn(bpy)$_2$(NCS)$_2$, 10, as functions of concentration for various recovery times. Either cancer cell line was exposed to one of the metal complexes for one hour, after which the complex solution was removed and replaced by fresh medium. The recovery time is the period after the removal of metal complex, during which the cells were incubated. Cytotoxicity of complexes 8, 9 and 10 was investigated with SK-N-SH cell line using both CCK-F and CCK-8 assays, and OVCAR-3 cell line using CCK-F assay. CCK-8 is a colorimetric assay which is similar to but more sensitive than MTT assay. CCK-8 relies on the conversion of a colorless tetrazolium salt into an optically detectable formazan dye by dehydrogenases. Absorbance of controls and cell culture exposed to various concentrations of complexes 8, 9 and 10 after different lengths of recovery time were read. Since Cu(II) and Zn(II) bipyridyl complexes were reported to be inhibitors of lactate dehydrogenase (LDH), we also tested the cytotoxicity of the metal complexes using CCK-F assay which is not dependent on dehydrogenases. CCK-F is a fluorometric assay in which a fluorescent dye, calcein, produced from Calcein-AM (3’’,6’’-di(O-acetyl)-2’,7’’-bis[N,N-bis-(carboxymethyl)aminomethyl]-fluorescein, tetraacetoxymethyl ester) by esterases inside live cells is released to the culture medium and its concentration is proportional to the number of live cells.
cells. Therefore, the possible ability of complex 8, 9 and 10 to inhibit LDH would not influence the result of CCK-F. Fluorescence of controls and cell culture exposed to various concentrations of complex 8, 9 and 10 after different lengths of recovery time were read.

Based on data from CCK-8 assay, curves shown in Figures 4-2 and 4-3 are the best fits to the function

\[ I = 100(1 - e^{-\alpha c}) \]  

where \( I \) is the percent inhibition of cell growth, \( \alpha \) is the molar inhibition parameter and \( c \) is the concentration of metal complex. This function is selected based on the assumption that the number of live cells increases exponentially. When the cells are not treated with 8-10, no inhibition occurs while no matter how high concentration of 8-10 is applied, the percent inhibition does not exceed 100. In (9), \( I \) is an exponential function of \( c \) and when \( c = 0, I = 0 \); when \( c \to \infty, I \to 100 \). The measured percent inhibition \( I \) is equal to

\[ I = 100(1 - N_t/N_u) \]  

where \( N_t \) is the number of live cells in a microplate well treated with metal complex and \( N_u \) the number of live cells in a well not treated with 8-10; \( N_t/N_u \) is obtained from the absorbance or fluorescence according to (8). The dispersed data points represented by diamonds in Figures 1 and 2 were collected from CCK-F assay.

We suppose that without treating with metal complexes, cells in the control group reproduce with growth constant \( k \) so, if the number of live cells at time 0 is \( N_0 \), the number of live cells at time \( t \) (\( N_t \)) is \( N_0e^{kt} \). During the exposure period, if the metal complex only kills a fraction of the cells, \( f \), without affecting the health of the surviving cells, the number of live cells
at time 0 (just after the exposure period) is \((1 - f)N_0\) and the number of live cells at time \(t\) \((N_t)\) is \((1 - f)N_0e^{kt}\). It is evident from (10) that \(I\) is always equal to \(100f\), independent of the recovery time \(t\).

It is expected that \(f\) depends on the concentration of drug used, \(c\), becoming zero for \(c = 0\) and approaching 1 for \(c \to \infty\). A simple representation of this is: \(f = 1 - \exp(-ac)\), as used in equation (9). When the percent inhibition of 50\%, i.e. \(f\) is 0.5, the concentration \(c\) is equal to \(\ln(2)/\alpha\), which is the value of IC\(_{50}\). This gives a single number for IC\(_{50}\) only in the simplest case, when \(I\) depends on \(c\) but not on recovery time \(t\). It is evident from the calculated IC\(_{50}\) values, given in Table 4-1, as well as from the initial slopes in Figures 4-2 and 4-3, that this is not at all the case for the copper complexes considered here.

The initial slopes of the plots almost always increase with the recovery time. The reason is that the copper complexes have a long-lasting effect on the health of the surviving cells, besides killing a fraction \(f\) of the cells during the time that the cells are exposed to the agent. They have continual hindrance to division of the surviving cells after the agent is removed, i.e., during the recovery period. Therefore, simply measuring the IC\(_{50}\) by measuring \(f\) at one recovery time may hide important information about this additional effect. If the effects of the drug are long-lasting, so that the surviving cells are in less than perfect health, they will have a growth rate \(k'\) which is smaller than \(k\). Then, assuming a fraction \(f\) of the original cells were killed, the number of treated cells at time \(t\) is

\[N_t(t) = (1 - f) N_0 e^{k't}\]

with the percent inhibition
\[ I = 100 \frac{N_o e^{k't} - (1 - f)N_o e^{k't}}{N_o e^{k't}} = 100[1 - (1 - f)e^{(k' - k)t}] \]  

Since \( k' \) is smaller than \( k \) and \( f \) is not negative, the percent inhibition, \( I \), will increase with \( t \). For a fixed value of \( t \), we abbreviate \( e^{(k' - k)t} \) by \( C(t) \). Then, inserting the concentration-dependence of \( f \), \( f = 1 - \exp(-\alpha c) \), we have

\[ I = 100[1 - C(t) e^{-\alpha c}] \]  

There is possibility that \( C(t) \) depends on concentration \( c \) as well as on \( t \). However, the limited number of data points in the case of the metal complexes in this report (3 to 5 in each case) and the relatively large experimental errors do not justify using a two-parameter function to fit the data. Thus we have used (9) instead of (12) in Figures 4-2 and 4-3, corresponding to \( C = 1 \). If, however, \( C(t) \) really decreases with \( t \), the second term in (12) will be smaller for larger \( t \), which will appear as an increase in the value of \( \alpha \) (molar inhibition parameter) with \( t \).
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<th>IC$_{50,\mu}$M</th>
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$^a$ Cu(bpy)(NCS)$_2$, 8, [Cu(bpy)$_2$(H$_2$O)](PF$_6$)$_2$, 9, and Zn(bpy)$_2$(NCS)$_2$, 10.

Table 4-1. Molar inhibition parameter, $\alpha$, and r-squared values for all complexes and recovery times, derived from the results plotted in Figures 4-2 and 4-3.$^a$
It is apparent from the figures that the fits to (9) are good overall. This is shown by the values of \( r^2 \) (goodness of fit), which are calculated according to

\[
r^2 = 1 - \frac{\sum_{j} (I_j - I^\text{cal}_j)^2}{\sum_{j} (I_j - \overline{I})^2}
\]

where \( I_j \) is the measured inhibition for the \( j^{\text{th}} \) complex concentration, \( \overline{I} \) is the mean value of \( I_j \), and \( I^\text{cal}_j \) the corresponding inhibition calculated according to (9). Table 4-1 shows \( r^2 \) for all 24 conditions employed in this study, as well as the molar inhibition parameter \( \alpha \) for each.

As shown above, by considering how the molar inhibition parameter \( \alpha \) depends on recovery time \( t \), one can determine whether there are long-lasting, effects on the cells (in addition to the initial cell kill produced by exposure to the agent). If, for example, the agent hinders cell division but the cells remain competent to be detected as being alive with a standard cell assay, \( \alpha \) will increase with \( t \). If there are no effects of the agent on the cells after the initial kill, \( \alpha \) will be constant or independent of \( t \) during the recovery period. In order to determine whether \( \alpha \) depends on \( t \), we have performed linear fits on all six data sets given in Table 4-1. For 8 the slope is \((7.8 \pm 3.1) \times 10^{-6} \text{ µM}^{-1} \text{ h}^{-1}\) for SK-N-SH and \((5.7 \pm 2.0) \times 10^{-5} \text{ µM}^{-1} \text{ h}^{-1}\) for OVCAR-3, i.e. the slopes are definitely positive. This indicates that, besides killing some cells during the 1 h exposure time, compound 8 produces long-term effects, in that cells divide more slowly during the recovery period. For 9, the slope is \((1.6 \pm 0.4) \times 10^{-6} \text{ µM}^{-1} \text{ h}^{-1}\) for SK-N-SH and \((1.9 \pm 1.4) \times 10^{-5} \text{ µM}^{-1} \text{ h}^{-1}\) for OVCAR-3. Since these slopes are definitely positive, 9 also hinders cell division during the recovery period. For 10, the slope is \((3.8 \pm 6.6) \times 10^{-6} \text{ µM}^{-1} \text{ h}^{-1}\) for SK-N-SH and \((-1.7 \pm 1.8) \times 10^{-6} \text{ µM}^{-1} \text{ h}^{-1}\) for OVCAR-3, i.e., the slopes are essentially zero in both cases. Thus the zinc
complex differs from the copper complexes in that the copper complexes hinder cell division
during recovery but the zinc complex, which also kills cells during the exposure period, does not
affect cell division during the recovery period.

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Table 4-2. Slopes with errors of plots of \( N_t/N_u \) vs. time for a fixed concentration of complex.\(^a\)
Figure 4-4. The ratio $N_t/N_u$ is plotted against recovery time $t$ for fixed concentration of agent, where $N_t$ = number of cells in treated population and $N_u$ = number of cells in untreated population. $N_t$ and $N_u$ were measured using the CCK-F assay. First six plots are for compound 8, next six for compound 9, last six for compound 10. For the copper compounds 8 and 9, $N_t/N_u$ decreases with $t$, but this is not the case for the zinc compound 10.

Figure 4-5. The ratio $N_t/N_u$ is plotted against recovery time $t$ for fixed concentration of agent, where $N_t$ = number of cells in treated population and $N_u$ = number of cells in untreated population. $N_t$ and $N_u$ were measured using the CCK-8 assay. First three plots are for complex 8, next three for complex 9, last three for complex 10. For the copper compounds 8 and 9, $N_t/N_u$ decreases with $t$, but this is not the case for the zinc compound 10.

There is evidence that IC$_{50}$ may depend on recovery time if the agent being tested affects the division rate of cells after exposure to the agent. In Figures 4-4 and 4-5 and Table 4-2, we show directly the effect of recovery time $t$ on the ratio of treated to non-treated cells, $N_t/N_u$; for a fixed concentration of complex, $N_t/N_u$ is plotted against $t$. If there are no effects on division,
$N_t/N_u$ will be independent of $t$, but if division is hindered due to exposure to the agent, $N_t/N_u$ will decrease with $t$. It may also happen that $N_t/N_u$ increases with $t$, which means that the surviving cells divide more rapidly than the untreated cells after exposure to the agent. Table 4-2 shows the results of linear fits of $N_t/N_u$ vs. $t$ for fixed $c$ of metal complex. For the copper compounds, the slope is definitely negative (magnitude of slope greater than standard error) for both assays and both cell types except for one case. For SK-N-SH cells with the CCK-8 assay and 25 $\mu$M of 8, the slope is negative but its magnitude is less than the standard error, so that the slope is statistically zero. It is clear that the two copper complexes diminish the reproductive capacity of the cells which survive the initial exposure to the complex. For the zinc compound, 10, the situation is quite different in that the slope is statistically zero in three cases, statistically negative in only one case, and statistically positive in five cases. This shows that the reproductive capacity of the surviving cells which have been treated with the zinc complex is certainly not diminished. In contrast, the metal complex may even enhance division.

We also compared the results of the two assays, CCK-8 and CCK-F, for neuroblastoma, SK-N-SH cells. It is evident in Figure 4-2 that in 32 out of 36 points, the measured inhibition for the CCK-8 assay is within one standard deviation of the curve obtained by fitting the inhibitions measured using the CCK-F assay. The agreement suggests that both assays are detecting the same number of live cells.
Figure 4-6. Agarose gel electrophoresis images of 8 (a), 9 (b) and 10 (c) cleavage of pBR322 DNA in the presence of the activator, ascorbic acid (1 mM, 0.5 h), and a control group (d) showing the effects of the metal complexes alone on DNA in the absence of the activator. The studies were done in 1 mM HEPES buffer with 5% (v/v) DMSO, pH 7.4 at 37 °C. In (a)-(c), lane 1: DNA alone, lane 2: DNA plus 5% DMSO, lane 3: DNA with ascorbic acid, no complex. (a) For lanes 4-6, which contained ascorbic acid, the lane numbers and [8]/[DNA-base pairs] are: 4, 0.03; 5, 0.06; 6, 0.12; 7, 0.18; 8, 0.24; 9, 0.30. (b) For lanes 4-6, which contain ascorbic acid, the lane numbers and [9]/[DNA-base pairs] are: 4, 0.3; 5, 0.6; 6, 1.2; 7, 1.8; 8, 2.4; 9, 3.0. (c) For lanes 4-6, which contained ascorbic acid, the lane numbers and [10]/[DNA-base pairs] are: 4, 0.3; 5, 0.6; 6, 0.9; 7, 1.2; 8, 1.5; 9, 1.8. A control group without ascorbic acid is shown in (d). Lane 1: DNA alone, lane 2: DNA plus 5% DMSO. For lanes 3 and 4, [8]/[DNA-base pairs] are 0.15 and 0.30; for lanes 5 and 6, [9]/[DNA-base pairs] are 0.3 and 3.0; for lanes 7 and 8, [10]/[DNA-base pairs] are 0.3 and 3.0. After electrophoresis, the DNA was stained with ethidium bromide.

Since copper-o-phenanthroline complexes are known to cleave DNA in the presence of activating agents,118 the ability of the 2, 2'-bipyridine compounds 8-10 to cleave the closed circular pBR322 DNA in the presence and absence of ascorbate was also studied. As is evident from Figure 4-6, 8 and 9 can be activated by ascorbate to cut closed circular DNA, eventually reducing the plasmid to low molecular weight fragments which appear as an ethidium bromide-
stained streak in the gel. This behavior contrasts with that of Zn(bpy)$_2$(NCS)$_2$, Figure 4-6 (c), which produces no DNA cleavage in the presence of ascorbate. In the absence of ascorbic acid none of the complexes produced DNA cleavage, Figure 4-6 (d).
4. Discussion

In this work, the correlations of cytotoxic properties and recovery time, $t$, is investigated with three metal complexes: Cu(bpy)(NCS)$_2$, 8, [Cu(bpy)$_2$(H$_2$O)](PF$_6$)$_2$, 9, and Zn(bpy)$_2$(NCS)$_2$, 10. Structurally, 8 is a 4-coordinate approximately square planar Cu(II) complex with N-bonded thiocyanate ligands, 9 is a 5-coordinate, trigonal bipyramidal Cu(II) complex with a coordinated water molecule and 10 is a 6-coordinate approximately octahedral Zn(II) compound with two N-bonded thiocyanate ligands (Figure 4-1). The cytotoxocities of the metal complexes toward neuroblastoma, SK-N-SH and ovarian cancer, OVCAR-3, were measured using two standard assays to detect the number of live cells. To reveal long lasting effects that the complexes may have on the cells after 1 h exposure, the number of living cells was determined at different recovery times. The results show that the copper complexes, 8 and 9, hinder the cell growth, while the zinc complex, 10, has no, or possibly a weak stimulating, effect on cell division during the recovery period (Figures 4-2 and 4-3 and Table 4-1). As shown, $IC_{50}$ is equal to $ln(2)/\alpha$, and $\alpha$ is the molar inhibition parameter. Since $\alpha$ can depend on recovery time, reporting $IC_{50}$ for a single recovery time can hide important information concerning the health of the cell population after exposure to the toxin.

In this case the hidden information is that the copper complexes, 8 and 9, not only kill some of the SK-N-SH and OVCAR-3 cells during the 1 h exposure period, but also have a longer-lasting effect on the surviving cells, causing them to divide more slowly than healthy cells in a control group. This is shown in the slopes of plots of $N_t/N_u$ vs. recovery time $t$ (Figures 4-4 and 4-5 and Table 4-2). The larger the value of $t$, the greater the percent inhibition at a certain concentration of compound. Therefore, the apparent value of $IC_{50}$ decreases with larger $t$ values, i.e., the compounds appear more cytotoxic for larger $t$. As shown in Table 4-1, the $IC_{50}$ of the
two copper complexes 8 and 9 is lowered by more than an order of magnitude when the recovery time is increased from 2 h to 74 h for both cell lines studied. Structurally, 8 contains one 2,2’-bipyridine ligand and the compound is uncharged while 9 has two 2,2’-bipyridine ligands and the complex is a divalent cation (Figure 4-1). Interestingly, 9 is apparently more toxic than 8 toward neuroblastoma cells but there is no apparent difference in the toxicity of the compounds toward the ovarian cancer cell line (Table 4-1). However, additional study will be required to discover if this difference in cytotoxicity toward the two cell lines is related to the charge on the compounds and/or the number of 2,2’-bipyridine ligands attached to the metal ion. On the contrast, as shown in Table 4-1, the zinc complex 10 does not show a decrease in IC\textsubscript{50} with t and may even enhance the ability of cells to divide at certain concentrations.

Recovery time has been noted to be important in Murine leukemia L1210 cells treated with cisplatin, in that low concentrations of the drug caused the cells to be transiently arrested in the G\textsubscript{2} phase for about 3 days before this phase is bypassed and growth is resumed.\textsuperscript{63} In another study, Bogdanovic et al. reported that cisplatin analogs containing sulfur carrier ligands affected the growth of MCF7 human breast cancer cells during the recovery period\textsuperscript{64} and both inhibitory and stimulatory effects on cell division were observed, depending on the platinum complex being investigated. This makes the reported value of IC\textsubscript{50} strongly dependent on the time (recovery period) at which inhibition is measured.

Evidently, percent inhibition observed for cell growth depends not only on the concentration of cytotoxic agent but also on the length of time that the cells are in contact with the agent, i.e. the exposure time. It is believed that the relevant parameter is the AUC, the Area Under the Curve giving drug concentration vs. time.\textsuperscript{31} If this curve resembles a rectangle, AUC =
concentration $\times$ exposure time. In a study involving Jurkat cells and cisplatin, the percent viability of the cell population measured 24 h after exposure to the drug correlated inversely with AUC, where AUC was calculated as the product of exposure time and drug concentration. If the amount of cell death does depend only on AUC, one can obtain the same information about inhibition using any convenient exposure time. In this study, we have used 1 h in the experiments. In a very crude way this approximates a short i.v. administration of cisplatin and its rapid clearance from blood, $t_{1/2} \sim 0.5$ h.$^{31}$

While this study provides no detailed mechanistic information on how the 2, 2’-bipyridine complexes are able to kill neuroblastoma and ovarian cancer cells, the facts that (a) all of the complexes have the bpy ligand and (b) only the copper complexes have long-lasting effects during recovery period suggests that the coordinated copper ion is important in slowing the division of and killing cells. Since it is known that Cu(II) in the biological system can facilitate the production of hydroxyl radical, hydrogen peroxide, and superoxide, collectively referred to as ROS,$^{31,118,120}$ it is possible that 8 and 9 induce ROS which damage biological molecules in the cell. This mechanism, which may produce the damage observed to biological molecules in Alzheimer’s Disease, requires a reducing agent and molecular oxygen, and the copper ion must change its oxidation state.

Since DNA is well known as an important target for anticancer agents, we investigated the ability of 8-10 to cleave closed circular pBR322 DNA. Figure 5 (a) and (b) show that the two copper compounds, 8 and 9, induce breakage of closed circular pBR322 DNA in the presence of an excess of the common biological reducing agent ascorbic acid. The level of breakage is in a manner which is dependent on the concentration of complex present in the medium. Since
molecular oxygen was not excluded in the reaction, this observation is consistent with copper-mediated damage to DNA through the production of ROS. However, in the case of the zinc complex, 10, Figure 4-6 (c) shows that cannot break DNA under the same activating conditions. Since Zn(II) cannot easily change its oxidation state, this observation is also consistent with 8 and 9 damaging DNA via a ROS-generating type mechanism. In the absence of ascorbic acid, none of the complexes cut pBR322 DNA, Figure 4-6(d), which shows the reducing agent plays an important role in the cleavage process.

Earlier it was suggested that lactate dehydrogenase, LDH, may be a biological target for M(bpy)(Ac)\(_2\)•H\(_2\)O, where M = Cu(II) and Zn(II), bpy = 2,2’-bipyridine and Ac = CH\(_3\)CO\(_2\)\(^-\), compounds which are similar in structure to 8-10. The CCK-8 cell assay used in this investigation requires viable cellular dehydrogenases for reducing a tetrazolium salt to a formazan dye while the CCK-F assay requires viable esterases for producing a detectable fluorophore for quantitating the number of live cells. In view of the fact that both assays, within error, yield the same number of live cells, it would seem that the killing mechanism used by these compounds does not involve either esterases or dehydrogenases. Clearly additional work will be needed to uncover the molecular mechanism by which these interesting compounds kill cancer cells.

**4. 5 Conclusions**

In this work, the cytotoxicities of three compounds, Cu(bpy)(NCS)\(_2\), 8, [Cu(bpy)\(_2\)(H\(_2\)O)](PF\(_6\))\(_2\), 9, and Zn(bpy)\(_2\)(NCS)\(_2\), 10, toward neuroblastoma, SK-N-SH and ovarian cancer, OVCAR-3, cells as a function of recovery time, \(t\), was investigated using CCK-8 and CCK-F assays. Cells endured exposure to a metal complex for 1 h, after which the agent
was removed and the number of live cells measured using two standard cell counting assays. For the two copper complexes the results depend strongly on drug concentration and on the time allowed to elapse between removal of the metal complex and the measurement of the number of live cells in the population, i.e., the recovery time \( t \), showing that the measured value of \( IC_{50} \) strongly depends on the recovery time. We suggest that when measuring the cytotoxicity of an agent in a cell study it is important to measure the percent inhibition and \( IC_{50} \) at various recovery times which will uncover the long-term effects of the toxin on the health of the cell population. In contrast to the copper complexes, the zinc complex, 10, was only weakly cytotoxic and had little or no effect on cell division during the recovery period.

The study also showed that, in the presence of ascorbate, Cu(bpy)(NCS)$_2$, 8 and [Cu(bpy)$_2$(H$_2$O)](PF$_6$)$_2$, 9, cleave closed circular pBR322 DNA while Zn(bpy)$_2$(NCS)$_2$, 10, cannot induce DNA strand scission, an observation which is consistent with the redox inertness of the Zn(II) ion in 10. Additional work will be needed to determine if DNA and/or proteins are targets for these metal complexes in the cell.
Figure 5-1. IR spectrum of oxoplatin (2)
Figure 5-2. $^{5}$Pt NMR spectrum of 3 in H$_2$O/D$_2$O (95/5). A solution of 10 mM potassium hexachloroplatinate in H$_2$O/D$_2$O (95/5) was used as an external standard (0 ppm).
Figure 5-3 $^1$H NMR spectrum of $3$ in $d_6$-DMSO.
Figure 5-4. $^{13}$C NMR spectrum of 3 in d$_6$-DMSO.
Figure 5-5. DEPT135 NMR spectrum of 3 in d$_6$-DMSO.
Figure 5-6. $^1$H NMR spectrum of 4 in d$_6$-DMSO.
Figure 5-7. $^1$H NMR spectrum of per-6-iodo-β-cyclodextrin in $d_6$-DMSO.
Figure 5-8. $^1$H NMR spectrum of 7 d$_6$-DMSO.
Figure 5-9. Mass spectrum of 3

Figure 5-10. Mass spectrum of 4
Figure 5-11. Mass spectrum of 7.
Figure 5-12. Microscope images of untreated SK-N-SH cells and cells treated with 6 (20 μM Pt) for 0 h (a and b), 24 h (c and d), 48 h (e and f), 72 h (before washing, g and h) and 72 h (after washing, i and j)
References


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