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The Interaction of Glutathione with Platinum, and the Role of pH in Altering Ligand Formation between Platinum and N-Acetylcysteine

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THE INTERACTION OF GLUTATHIONE WITH PLATINUM, AND THE ROLE OF PH IN ALTERING LIGAND FORMATION BETWEEN PLATINUM AND N-ACETYLCYSTEINE

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2015

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ABSTRACT

Cisplatin is an important anti-cancer drug. Structurally, cisplatin is a coordination complex, which means that it has a central metallic atom: platinum. Side interactions prevent cisplatin from effectively treating cancerous cells by reaction with DNA. Cisplatin has a high rate of forming protein adducts, primarily with sulfur containing amino acids. To better understand how cisplatin interacts with sulfur containing amino acids in the body, a platinum compound with an ethylenediamine ligand attached was reacted with N-acetylcysteine and glutathione. The reactions of cysteine with platinum were performed at three pH values: 7.0, 8.5, and 10. NMR spectroscopy was used to examine these reaction over time. A COSY and HMQC spectrum of glutathione's reaction with platinum were collected to better identify the reaction products. All ^{1}H NMR spectra were taken on a JOEL Eclipse 500 MHz NMR instrument. The appearance of a free ethylenediamine ligand signal, in many cases, suggests that the amino acids react to form products in which the ethylenediamine ligand is displaced as the amino acids react via both sulfur and nitrogen atoms.

Keywords: Cisplatin, ethylenediamine ligand, N-acetylcysteine, glutathione, NMR spectroscopy

Dedicated to my parents Gary and Brenda,
and siblings Brandi and Michael
for all of their love and support.

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people passionate about learning is a tremendous gift. Lastly, I would like to thank God, my parents, former teachers, and my friends for always believing in me.

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INTRODUCTION

The importance of cisplatin as an anti-cancer drug justifies it as a prime research topic. It is particularly effective in treating testicular and ovarian cancers. Although cisplatin is effective in treating specific types of cancer, it causes many serious side effects. The main side effects cause by cisplatin include nephrotoxicity, neurotoxicity, ototoxicity, nausea, and vomiting (O'Dwyer 1999).

Because cisplatin has a high rate of forming protein adducts, it is not only ineffectively absorbed into cells, but it is also less effective at actually targeting cancerous DNA. Curtailing protein adduct formation will make cisplatin much more effective at targeting cancerous DNA; however, this will require a thorough understanding of how cisplatin behaves in the body. Hopefully my research will be a small piece to this puzzle, and allow the scientific community to better understand cisplatin's overall behavior.

Cisplatin is a coordination complex, meaning that it has a central metallic atom. The central metallic atom in cisplatin is platinum.

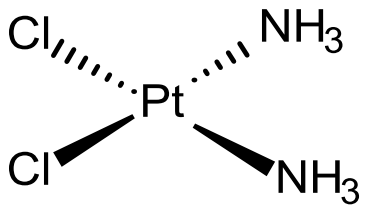


Figure 1.1: Cisplatin

Cisplatin works by cross-linking with cancerous DNA. When cisplatin cross-links with DNA it prevents the DNA from being replicated and results in cell apoptosis. Although cisplatin most commonly forms either intra-strand cross-links or inter-strand cross-links DNA adducts, intra-strand cross-links are more effective at damaging cancerous cells. This is because the DNA is poorly repaired (Coluccia and Natile 2007). Fortunately, the preferred DNA adduct formed by cisplatin is intra-stranded cross-links, which occurs about 65% of the time (Eastman 1999).

Cross-links occur particularly at guanine residues. Because other macromolecules in the body, and within individual cells can interact with cisplatin, its effectiveness at strictly targeting cancerous DNA is limited. Studies have shown that within 1 day of administering cisplatin intravenously, a shocking 65-98% of cisplatin is bound to proteins (DeConti et al. 1973).

Cisplatin is prone to interacting with proteins, particularly sulfur containing amino acids. Cisplatin has an especially high affinity towards proteins with cysteine and methionine residues because they are the two amino acids that contain sulfur. Cisplatin's interactions with proteins interferes with the drug's ability to cross-link with DNA, and this affects its ability to cause apoptosis in cancerous cells. It has been suggested that the

protein adducts are what causes the drug's harsh side effects and resistance to the drug (Kartalou and Essigmann 2001; Reedijk 1999).

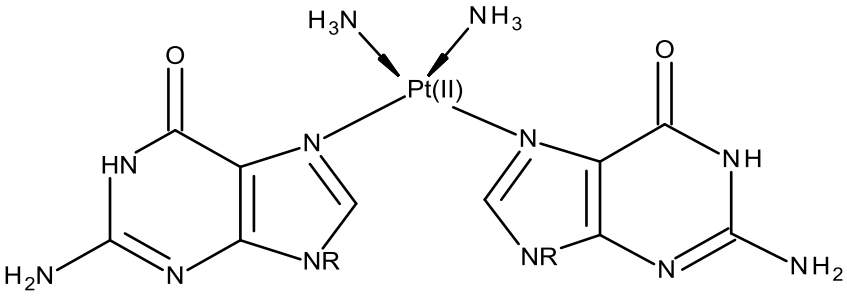


Figure 1.2: Cisplatin reacts with N(7) of guanine

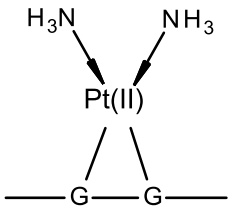


Figure 1.3: Cisplatin forms intra-strand cross-links: DNA poorly repaired

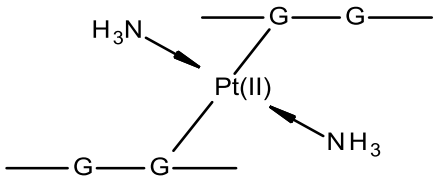


Figure 1.4: Trans-platinum forms inter-stand cross-links:

DNA repaired more efficiently

Initially, my research looked at how platinum interacted with N-acetylcysteine (N-AcCys) over time. Because cisplatin has a central metallic atom of platinum that is primarily involved in interactions with other atoms, I specifically studied how platinum interacted with N-AcCys. I also looked at how N-AcCys's interaction with platinum was

affected by altering the pH of the environment in which the reaction took place. The pH was altered to 7, 8.5, and 10 because the thiol group of N-AcCys has a pKa of ~8.5.

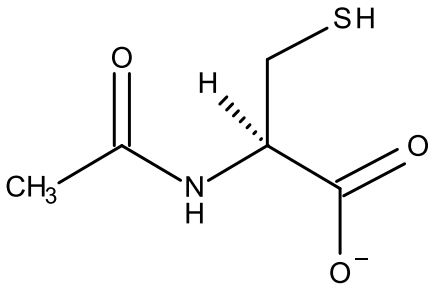


Figure 1.5: N-acetylcysteine

Next, I transitioned to looking at how platinum interacted with glutathione (GSH). GSH is synthesized in most cells within the body, and is a tripeptide, which contains the amino acids glutamate, cysteine and glycine. Larger molecules such as GSH have NMR spectra with many peaks that are harder to decode. It was necessary to perform correlation spectroscopy (COSY) and heteronuclear single quantum coherence spectroscopy (HMQC) to help clarify molecular changes in the GSH samples.

In conclusion, because cisplatin has a central metallic atom of platinum that is primarily involved in interactions with other atoms, I specifically studied how platinum interacted with cysteine residues, both free cysteine resides and cysteine in GSH. My work with platinum interacting with GSH can be compared to my results from cysteine's interaction with platinum. Studying these reactions will further advance the scientific communities' understanding of how cisplatin potentially reacts within the body.

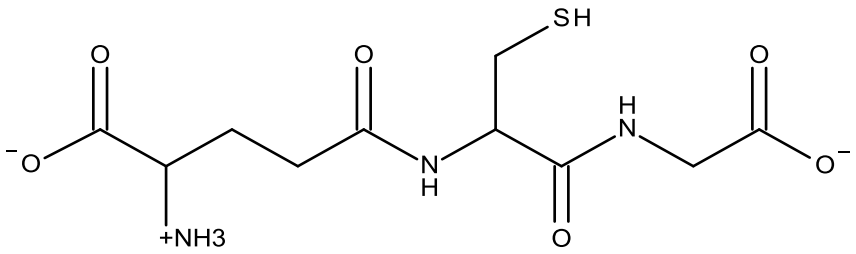


Figure 1.6: Reduced Glutathione

CHAPTER 2

METHODS

Synthesis of Platinum Compounds

Silver oxalate was prepared by mixing equal molar amounts of oxalic acid and silver nitrate in water. The reaction was stirred for several hours in an amber vial, and the insoluble silver oxalate was collected by vacuum filtration and rinsed with water.

Dichloro(ethylenediamine)platinum(II) (Pt(en)Cl2) was reacted with an equimolar amount of either silver nitrate or silver oxalate and the reaction was stirred in an amber vial overnight. The sample was filtered to remove the insoluble silver chloride, and the water was removed using a rotary evaporator (rotavap) with heating to leave either Pt(en)(NO3)2 or Pt(en)(ox), respectively.

NMR Spectroscopy

My research project relied on nuclear magnetic resonance (NMR). NMR utilizes a powerful magnet to analyze a sample and it produces a spectrum with peaks. All ^{1}H NMR spectra were taken on a JOEL Eclipse 500 MHz NMR instrument. The chemical shift of these peaks was determined in reference to deuterium oxide as a secondary reference. These spectrums are also used to identify what interactions are occurring within a given sample.

In general, samples were prepared by weighing out a specific amount of a sample in milligrams, and then adding 1 milliliter of deuterium oxide to that sample in a NMR

tube. The samples with only N-AcCys were prepared with 1.6 mg of N-AcCys. All three samples of N-AcCys and Pt at ~ pH of 7, 8.5, and 10 were made with 1.6 mg of N-AcCys, 1.7 mg of Pt(en)(ox), and 1 milliliter of deuterium oxide. A vortex was used to help dissolve the samples into the solution of deuterium oxide. The desired pH of each sample was achieved by adding either sodium deuteroxide to raise the pH, or nitric acid in deuterium oxide to lower the pH. These tubes were then labeled, and their contents are analyzed by NMR spectroscopy. To follow up, I checked samples again either after 24 hours and/or after 1 week to track a sample's reaction progress.

Both the reduced and oxidized samples of GSH were made with 3.0 mg of GSH dissolved in 1 milliliter of deuterium oxide. Another sample of reduced GSH was made with 20 mg for COSY and HMQC analysis. To emulate a 1 to 1 molar ratio a sample of 1.9 mg of $Pt(en)(NO_3)_2$ and 3.1 mg of reduced GSH was created. A sample of 6.2 mg of reduced GSH and 3.8 mg of $Pt(en)(NO_3)_2$ was also created.

COSY

COSY is a form of 2-dimensional spectroscopy. Peaks that appear on the diagonal are the connected peaks seen in the 1-dimensional NMR. Cross-peaks are the peaks that appear to the sides of the diagonal peaks. Cross-peaks represent protons that are connected with other protons at least one carbon bond apart. A COSY was taken on the sample of 20 mg of reduced GSH and on the sample of 1.9 mg of $Pt(en)(NO_3)_2$ and 3.1 mg of GSH.

HMQC

HMQC is also a 2-dimensional spectroscopy that plots molecules against a carbon and hydrogen axis. The produced spectrum has a peak for every unique proton that is

attached to a molecule's carbons. Magnetization is transferred from a molecule's hydrogens and carbons in a back-and-forth manner and signals are recorded at incremental times. Hydrogen signals are measured and recorded directly, and the chemical shift of carbon is measured and recorded secondarily. The series of experiments from multiple time increments established the dimensions formed by carbon atoms. A HMQC was taken on the sample of 20 mg of reduced GSH and on the sample of 1.9 mg of $Pt(en)(NO_3)_2$ and 3.1 mg of GSH.

CHAPTER 3

RESULTS

Part 1: N-acetylcysteine and Pt at ~ pH of 7, 8.5, and 10

Figure 3.1 shows the NMR of N-AcCys at pH ~ 7 with no Pt(en)(ox). The peak at 1.9 ppm represents the methyl H's of the acetyl group, the peak at 2.75 ppm represents the CH_2 group, and the peak at 4.2 ppm represents the α-H. After 24 hours the sample was analyzed once again by NMR. The two NMR figures show very few differences; thus, very little air oxidation of N-AcCys was seen. This suggests that air oxidation of N-AcCys is a slow process, and should not be a major factor in altering the chemical structure of N-AcCys at a pH of 7. The two NMR readings of N-AcCys at pH ~ 7 can be used as a baseline to better understand what occurs when N-AcCys reacts with Pt(en)(ox).

Following the same procedures, another sample was made with a pH of 7. Pt(en)(ox) was added to this sample of N-AcCys. Figure 3.2 shows N-AcCys and Pt(en)(ox) at pH ~ 7. Comparing figure 3.2 to figure 3.1, both at 30 minutes, reveals that the only new peak is at 2.35 and that this new peak must be from the ethylenediamine (en) ligand attached to platinum. As previously indicated the other peaks at 1.9 ppm, 2.75 ppm, and 4.2 ppm represents the methyl H's of the acetyl group, the CH_2 group, and the α-H respectively.

After 24 hours the reaction between N-AcCys and platinum produces a new product. After 24 hours the peak at 2.35 ppm, which represented the sample of unreacted platinum (ox) has significantly diminished and a new peak at 3.2 ppm has appeared. This new peak represents the ethylenediamine (en) ligand when it is not connected to platinum (ox).

The mechanisim for the reaction that results in the en ligand being discociated can be explained by previous research. When Pt(en)(ox) is reacted with N-AcCys, the sulfur atom of a N-AcCys groups binds to platinum. This process can eventually result in two N-AcCys groups being bound to Pt(ox) when enough N-AcCys is available. Next, to gain stability, Pt(ox) undergoes chelation, and in this process the en ligand is dissociated (Williams et al. 2011).

After one week, the peak at 2.35 ppm has completely disappeared, and the peak at 3.2 ppm has become even more noticeable. This signals that after one week the reaction of N-AcCys and platinum is complete. It appears that all of the en ligand has dissociated from platinum (ox). New peaks at 3.1 ppm signal that oxidation has begun to occur.

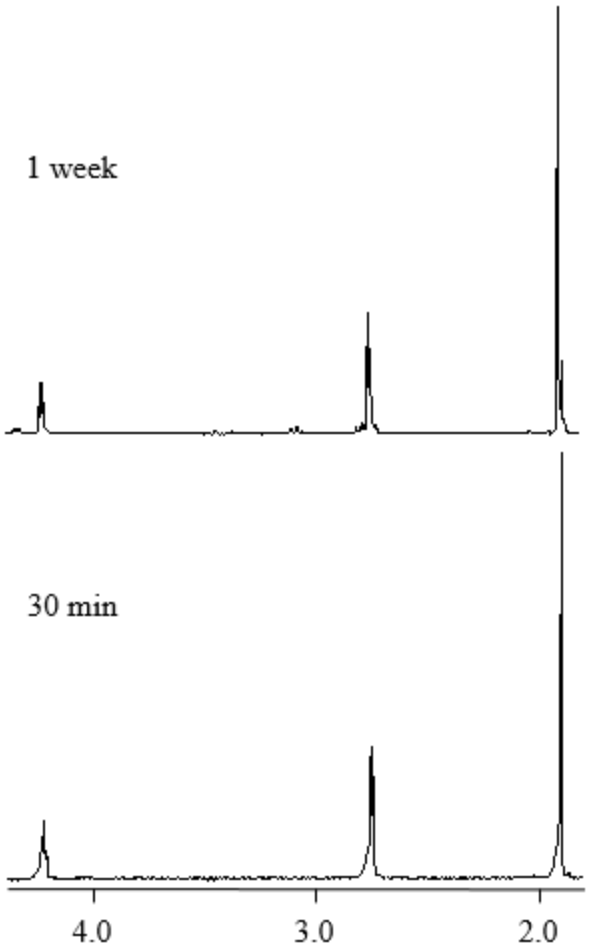


Figure 3.1: N-acetylcysteine at pH ~ 7

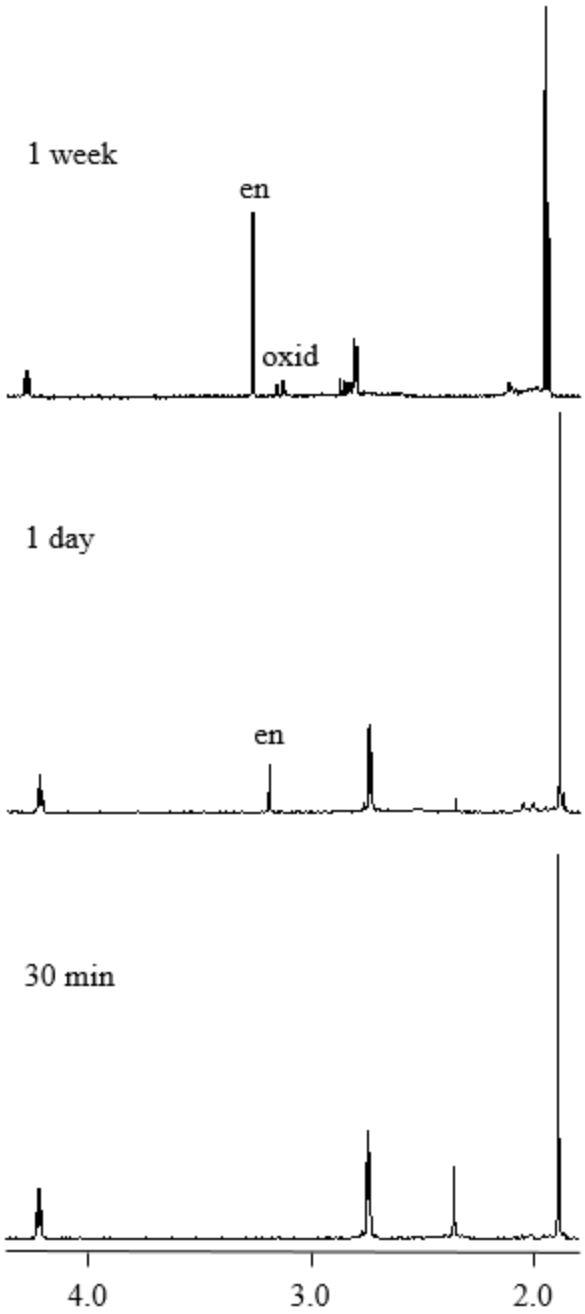


Figure 3.2: N-acetylcysteine and platinum at pH ~ 7

In figure 3.3 a sample of N-AcCys and platinum (ox) at pH ~ 8.5 was used. The figures were similar to those of N-AcCys and platinum (ox) at pH ~ 7. The only notable difference was that the peaks were smaller and chelation of platinum (ox) by cysteine occurred at a slower rate.

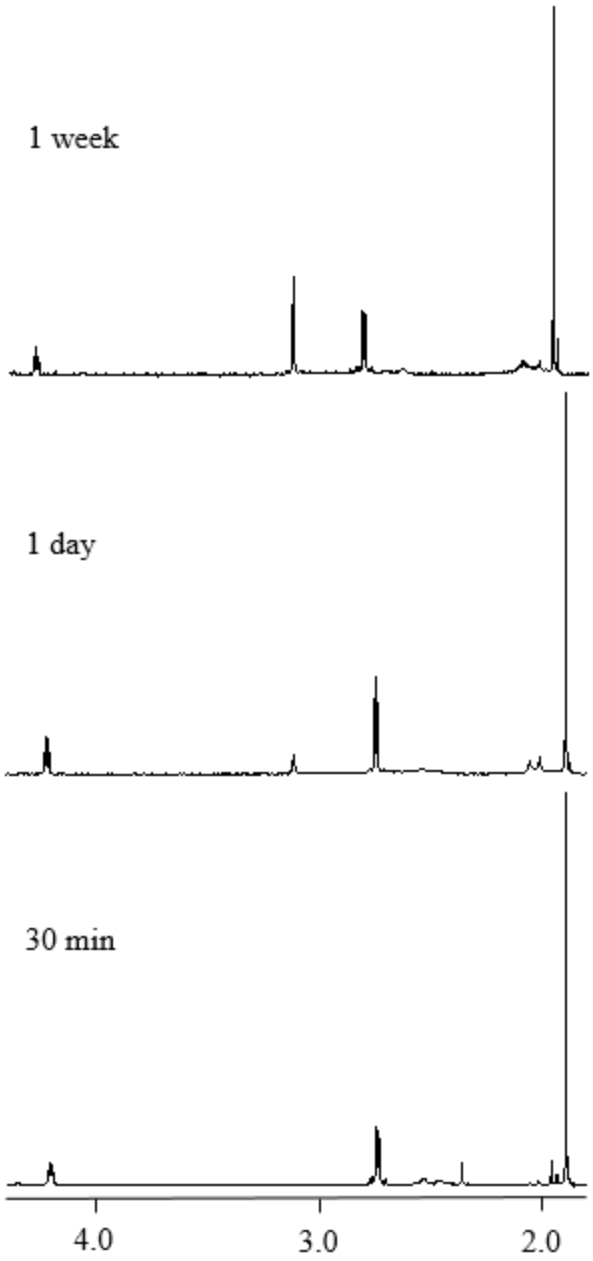


Figure 3.3: N-acetylcysteine at pH ~ 8.5

The third group of samples involved N-AcCyss and platinum at pH ~ 10. Figure 3.4 shows N-AcCys without platinum (ox) at pH ~ 10. Comparing figure 3.4 to figure 3.1 shows that the peak at 4.2 ppm did not materialize.

This is the peak that represents the α-H of N-AcCys. Like in figure 3.1, the peak at 1.9 ppm represents the methyl H's of the acetyl group, and the peak at 2.75 ppm

represents the CH_2 group. After one week significant oxidation was seen in the same sample that initially produced figure 3.3.

Figure 3.5 shows N-AcCys and platinum (ox) at pH ~ 10 at time intervals of 30 minutes, 24 hours, and 1 week. At pH of ~ 10 the continued trend of smaller, less defined peaks is observed. The chelation of platinum is still seen, but it is accompanied by significant oxidation of the sample. The graphs prove that the reaction between N-AcCys and platinum (ox) is the least efficient at pH ~ 10.

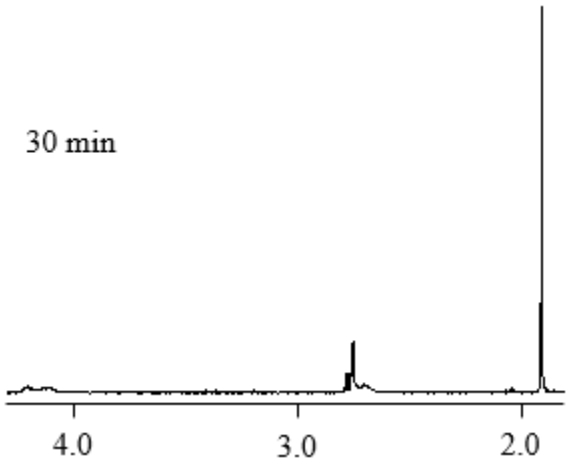


Figure 3.4: N-acetylcysteine at pH ~ 10

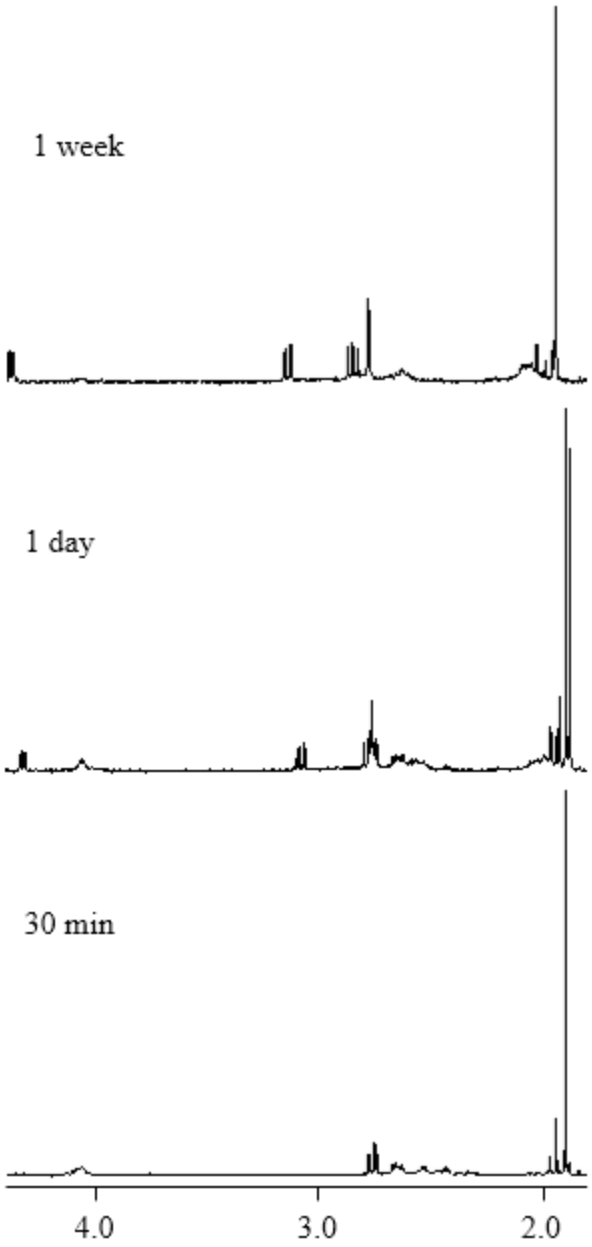


Figure 3.5: N-acetylcysteine and platinum at pH ~ 10

Part II: Glutathione's interaction with platinum

Before GSH's NMRs could be analyzed, a COSY and HMQC was required to help assign GSH's NMR signals. The first COSY and HMQC was run on a sample of 20 mg of reduced GSH. This was done after an NMR of the sample was recorded. The peak on the COSY at 2.0 ppm was assigned to glutamate's β hydrogens, because it was the

only group to be coupled with two cross-peaks at 2.4 and 3.6 ppm. This indicated that glutamate's β hydrogens were in the middle of the two groups it was coupled with: glutamte's α and γ hydrogens.

The γ hydrogens of glutamate were downfield by cysteine's thiol group and appeared on the COSY at 2.4 ppm. The β hydrogens of cysteine were further downfield because of their connection to a thiol group. This peak appeared at 2.75 ppm on the COSY. The α hydrogen of glutamate appeared on the COSY at 3.65 ppm. The α hydrogens of glycine appeared at 3.8 ppm on the COSY and were the only signals to leave no cross-peaks. The α hydrogen of cysteine appeared on the COSY the furthest downfield at 4.4 ppm because of its proximity to thiol.

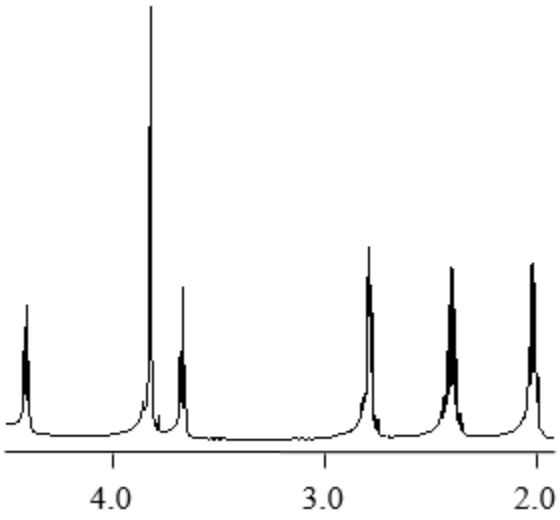


Figure 3.6: 20 mg reduced glutathione

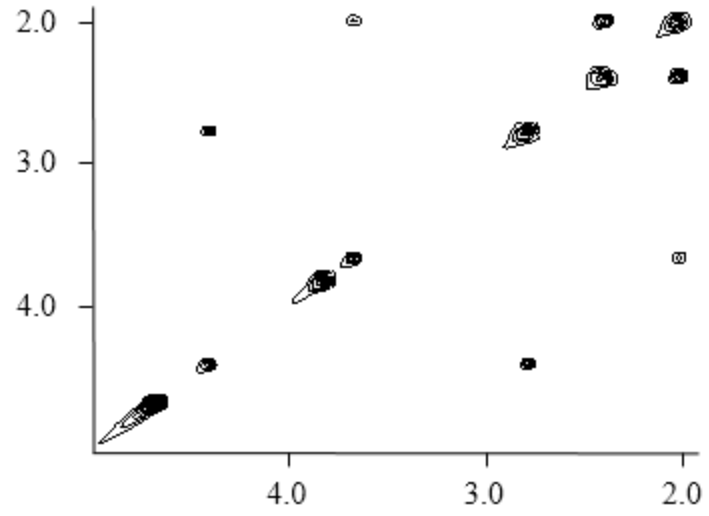


Figure 3.7: COSY 20 mg reduced glutathione

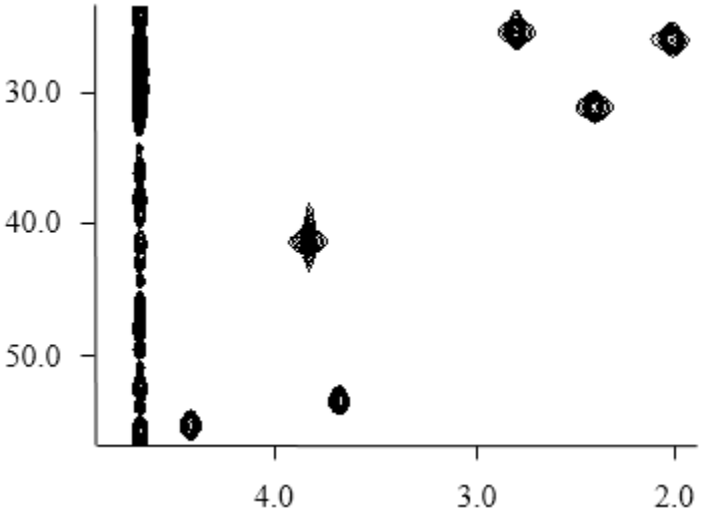


Figure 3.8: HMQC 20 mg reduced glutathione

After reduced GSH's peaks had been identified, the NMRs produced for both reduced and oxidized GSH could be analyzed. The purpose of these two NMRs was to have an idea of what reduced GSH and oxidized GSH looked like before they reacted with platinum. In addition, it was important to examine the two samples after two weeks to see how air oxidation altered their appearances. Oxidized and reduced GSH's NMRs

are shown in figure 3.9. The results from the COSY and HMQC helped identify the peaks seen on reduced and oxidized GSH's NMRs.

In oxidized GSH the peak at 4.55 ppm represents glutamate's β-H, the peak at 4.9 ppm represents γ-H of glutamate, the peak at 5.3 ppm represents β-H of cysteine, the peak at 5.65 ppm represents the α-H of cysteine, the peak at 6.2 ppm represents α-H of glutamate, and the peak at 6.35 ppm represents α-H of glycine.

In reduced GSH the peak at 4.55 ppm represents glutamate's β-H, the peak at 4.9 ppm represents the γ-H of glutamate, the peak at 5.3 ppm represents the β-H of cysteine, the peak at 6.2 ppm represents the α-H of glutamate, the peak at 6.4 ppm represents the α-H of glycine, and the peak at 6.95 ppm represents the α-H of cysteine.

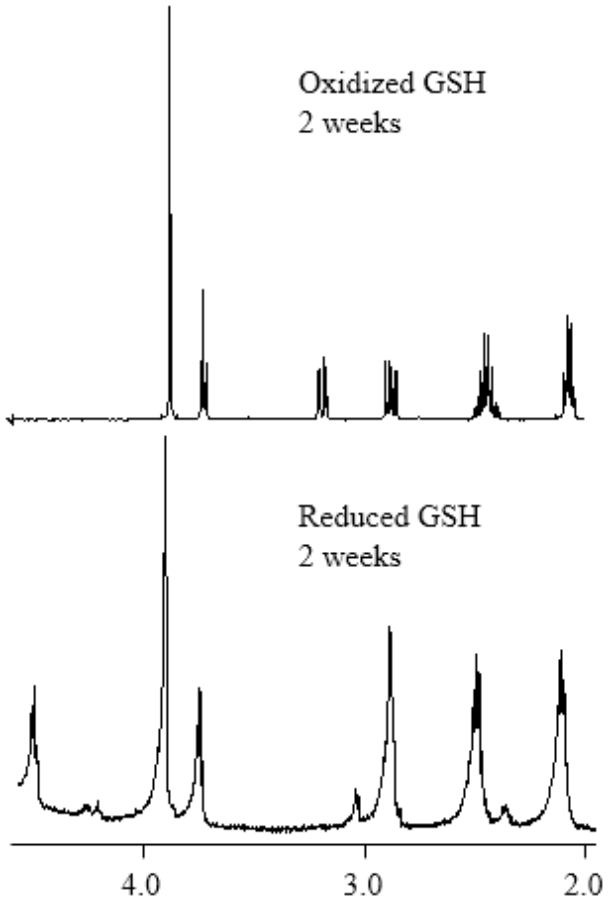


Figure 3.9: 3 mg reduced and oxidized glutathione

When $Pt(en)(NO_3)_2$ was added to reduced GSH, the NMR initially looked relatively similar to the NMR with only GSH. After one week the peak at 6.4 ppm, signaling α-H of glycine, was reduced and a new peak emerged at 3.2 ppm. The signals at 4.55 ppm, 4.9 ppm, and 5.3 ppm also diminished in signal strength. This new peak represented a slow, but immediate start to the dissociation of the en ligand.

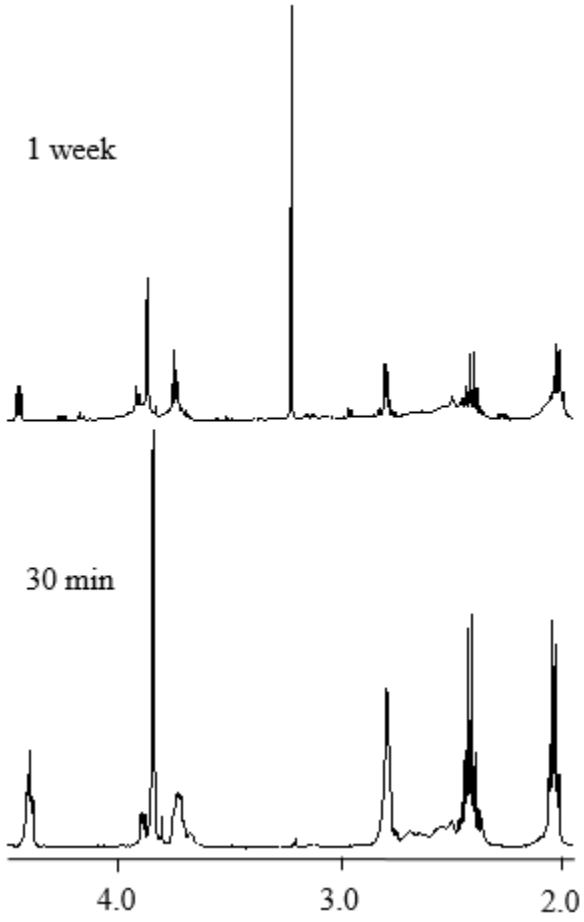


Figure 3.10: 6.2 mg GSH and 3.8 mg Pt

When 1.9 mg of $Pt(en)(NO_3)_2$ was added to 3.1 mg of reduced GSH the NMR-produced, broad-based peaks (figure 3.11). This, combined with the complexity of GSH's structure, required the second COSY and HMQC to clarify the reaction taking place between GSH and $Pt(en)(NO_3)_2$.

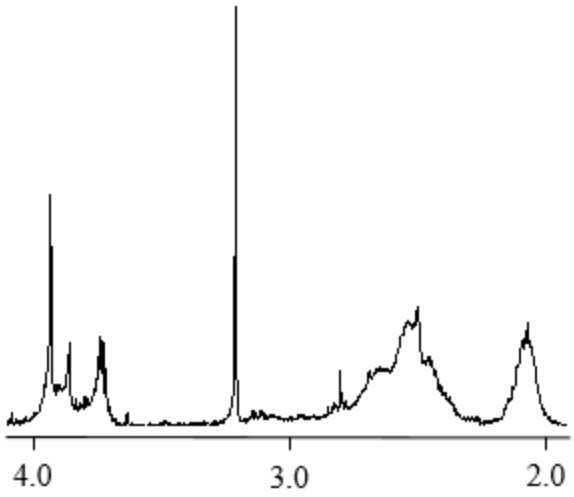


Figure 3.11: 1.9 mg Pt(en)$(NO_3)_2$ and 3.1mg GSH

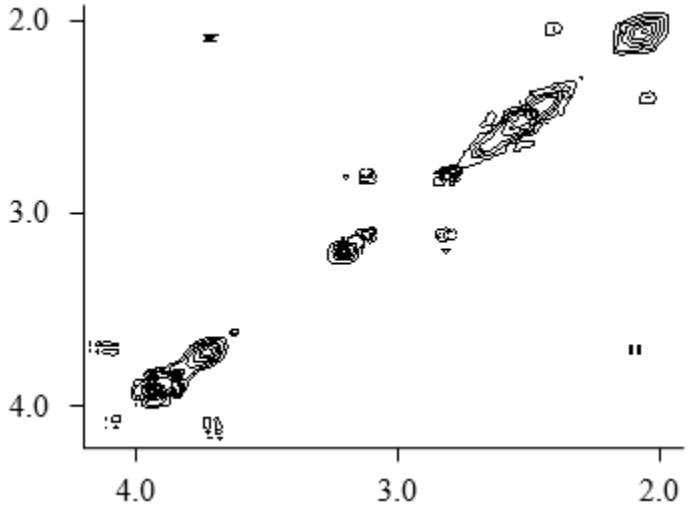


Figure 3.12: COSY 1.9 mg Pt(en)$(NO_3)_2$ and 3.1 mg GSH

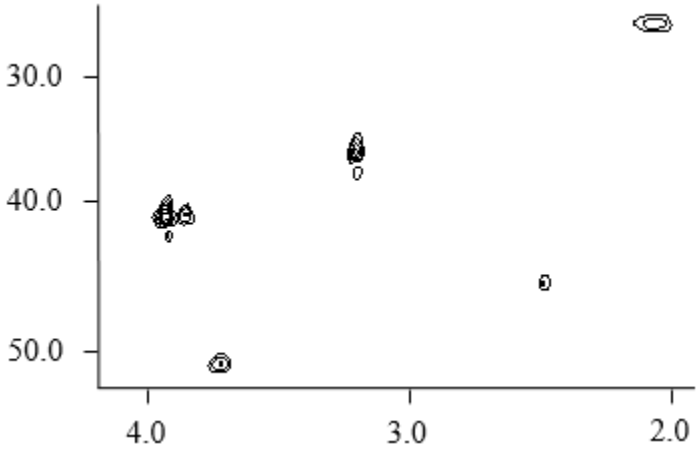


Figure 3.13: HMQC 1.9 mg Pt(en)$(NO_3)_2$ and 3.1 mg GSH

CHAPTER 4

DISCUSSION

Cysteine's thiol group has a typical pKa value of about 8.5. As the pH of cysteine approaches 8.5, the thiol group is deprotonated. The pKa of cysteine's carboxylic acid group is around 2 and the pKa of cysteine's amino group is around 9. At a pH below 2 both the amino and carboxylic groups are protonated, giving the carboxylic group a neutral charge and the amino group a positive charge. At a pH of 9 or above both groups are deprotonated and this gives the amino group a neutral charge and the carboxylic group a negative charge.

When the pH is between 2 and 9, cysteine exists in its zwitterionic form. In this form, most similar to human physiological pH, the molecule has a neutral charge; the amino group's positive charge balances the carboxylic group's negative charge. The attachment of the acetyl group slightly alters the pKa characteristics of cysteine. Since the experiments with N-acetyl cysteine at pH 7 and 8.5 yield virtually the same results, this suggests that none of the molecule's pKa values have been reached. Comparing the results from the reaction of N-acetyl cysteine and platinum at a pH of 7 and 8.5 show that a slightly higher pH causes platinum to react with N-acetyl cysteine's thiol group more slowly.

At pH 10 we see changes in the NMR spectrum. The peak at 4.2 ppm is not present as in the previous two spectrums. The signal could have either moved under the

water signal or else air oxidation could have occurred. The reaction at pH 10 shows that the chelation of N-acetyl cysteine occurs at a much slower pace.

GSH is a molecule abundant in most cells and it contains cysteine. Because of GSH's abundance in cells, it plays a big role in limiting cisplatin's ability to bind to DNA. The results show that the chelation of GSH's cisplatin group occurs at no noticeably slower speed than that of free cisplatin groups. This suggests that GSH's larger structure does not cause steric hindrance in this reaction.

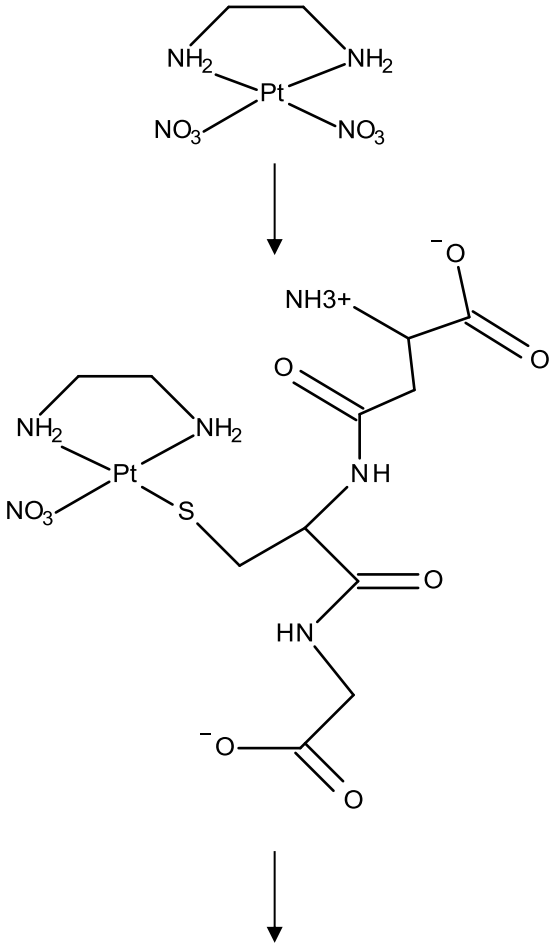
Figure 4.1 shows the proposed reaction in which glutathione binds to platinum. At the conclusion of this reaction the en ligand is displaced so the adduct can form an additional chelate ring; having 2 chelate rings makes the adduct more stable.

In a previous study (Lempers et al. 1987) altering the pH of GSH and a platinum complex showed that the ^{1}H chemical shifts of cysteine are independent of pH ranging from 4 through 10; however all other ^{1}H peaks from glutamate and glycine were found to be pH dependent. This suggests that the first ethylenediamine complex coordinates with GSH's sulfhydryl group across pH differences. Further experimentation revealed that the second ethylenediamine complex also coordinates to the sulfhydryl group.

The study also found that at a pH below 7, the adduct moves to completion at a very fast rate, as the second Pt-binding step occurs much faster than the first Pt-binding step. This is perhaps why the GSH sample's NMR only showed the complete product of the reaction and why figures 3.10 and 3.11 show no intermediate products (Lempers et al. 1987).

The primary result of a 1:1 ratio of GSH and a platinum complex has previously been found to produce 1 new peak within 1 hour. Additionally, this ratio produces a

predictable sulfur-bridged product (Oehlsen et al. 2005). In figures 3.10 and 3.11, 1 week resulted in 1 new peak, which suggests that only one sulfur-bridged product was produced.



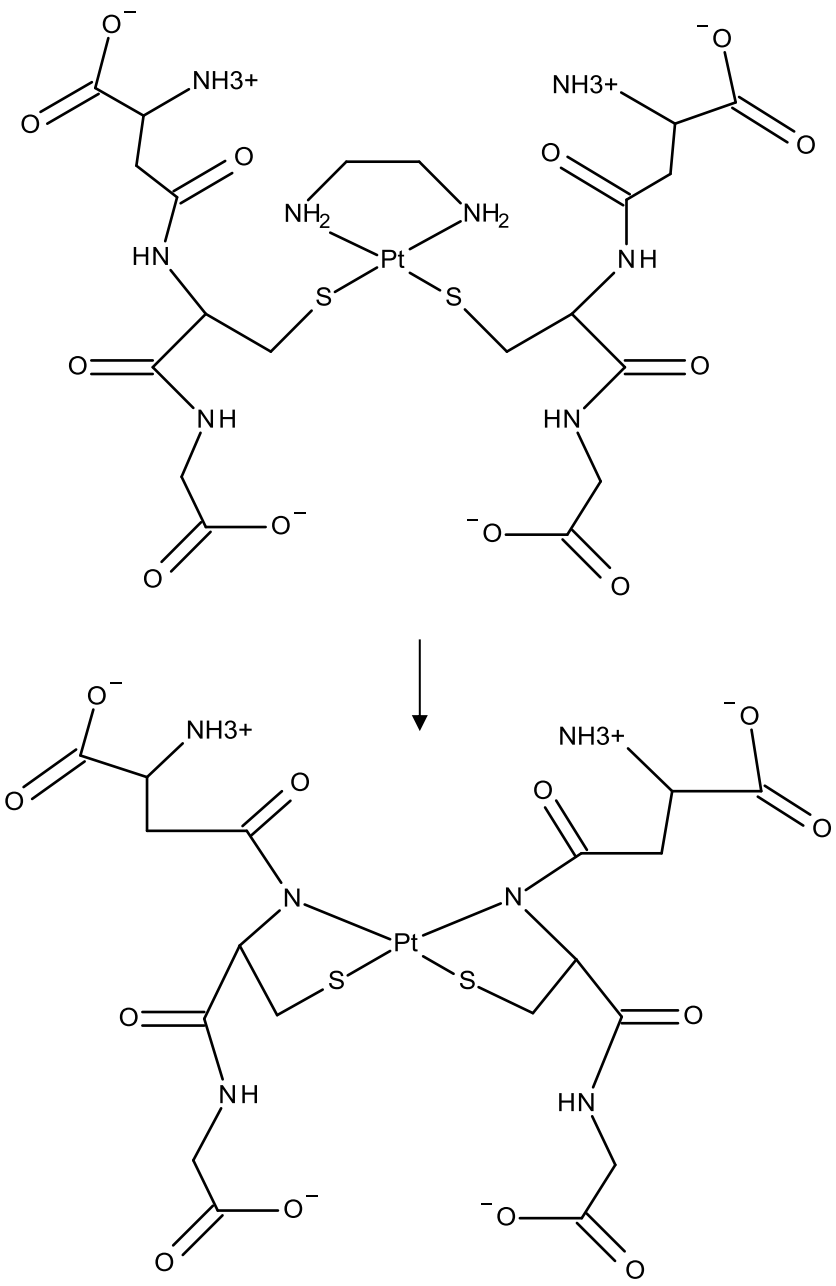


Figure 4.1: Proposed reaction scheme of $Pt(en)(NO_3)_2$ with GSH

REFERENCES

Berg J, Tymoczko J, and Stryer L. Biochemistry. Sixth ed. New York (NY): W. H. Freeman and Company; 2007.

Coluccia M, Natile G. Trans-platinum complexes in cancer therapy. Anti-Cancer Agents in Medicinal Chemistry. 2007;7(1):111-123.

DeConti RC, Toftness BR, Lange RC, Creasey WA. Clinical and pharmacological studies with cis-Diamminedichloroplatinum(II). Cancer Research. 1973;33:1310-1315.

Eastman A. The mechanism of action of cisplatin: from adducts to apoptosis. In: Bernhard Lippert, editor. Cisplatin: chemistry and biochemistry of a leading anticancer drug. Weinheim (Germany): Wiley; 1999. p. 111-134.

Kartalou M, Essigmann JM. Mechanisms of resistance to cisplatin. Mutation Research. 2001;478:23–43.

Lempers E, Inagaki K, Reedijk J. Reactions of [PtCl(dien)]Cl with glutathione, oxidized glutathione and S-methyl glutathione. Formation of an S-bridged dinuclear unit. Inorganica Chimica Acta. 1988;152:201-207.

O'Dwyer P, Stevenson J, and Johnson S. Clinical status of cisplatin, carboplatin, and other platinum-based antitumor drugs. In: Bernhard lippert, editor. Cisplatin: chemistry and biochemistry of a leading anticancer drug. Weinheim (Germany): Wiley; 1999. p. 29-69.

Oehlsen M, Hegmans A, Qu Y, and Farrell N. A surprisingly stable macrochelate formed from the reaction of cis dinuclear platinum antitumor compounds with reduced glutathione. Inorganic Chemistry. 2005;44:3004-3006.

Reedijk. Why does cisplatin reach guanine-N7 with competing S-donor ligands available in the cell? Chemical Reviews. 1999;99:2499-2510.

Williams K, Dudgeon R, Chmely S, Robey S. Reaction of platinum(II) diamine and triamine complexes with selenomethionine. Inorganica Chimica Acta. 2011;368(1):187-193.