Spring 2016

Binding of Oxaliplatin and its Analogs with DNA Nucleotides at Variable pH and Concentration Levels

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BINDING OF OXALIPLATIN AND ITS ANALOGS WITH DNA NUCLEOTIDES AT VARIABLE pH AND CONCENTRATION LEVELS

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

By
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May 2016

BINDING OF OXALIPHATIN AND ITS ANALOGS WITH DNA NUCLEOTIDES AT VARIABLE pH AND CONCENTRATION LEVELS

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I dedicate my thesis to three important persons in my life-

My parents Shobha Sehgal and Varinder Kumar Sehgal, who have always believed in my dreams and have struggled throughout their life to provide their children the best possible education.

My husband Sahil Rampal, who is my catalyst and has been consistently motivating me to bring out best in me.

ACKNOWLEDGEMENTS

Foremost, I would like to pay my deepest thanks to my research advisor Dr. Kevin Williams, who has offered me the opportunity to work under his direction. I sincerely acknowledge him for his immense guidance and continuous motivation, that he provided me to complete my work within stipulated time. I could not have imagined having a better mentor for my research study and thesis writing.

My sincere thanks goes to the committee members Dr. Eric Conte and Dr. Darwin Dahl, who have assisted me to complete my work without errors. I am grateful for their time and efforts they put to be on my thesis defense committee.

I acknowledge my sincere gratitude to Dr. Stuart Burris, Department Head for cooperation and financial support, he handed me to complete my program. I also thank Haley Smith, Graduate Associate for always solving my silly to serious queries with love and patience.

I would like to thank Chemistry Department and Graduate School, Western Kentucky University for accepting me as a master's student and offering me graduate assistantship throughout my program. I specially acknowledge the efforts of Dr. Cathleen Webb and Dr. Carl Fox for supporting me to continue my program by approving additional funding.

On my personal front, I would like to express my profound gratitude to my family and friends for their endless moral and emotional support. This work would not have been possible without the blessings of God and elders. I am indebted to my parents Shobha Sehgal and Varinder Kumar Sehgal; and my in-law parents Anita Rampal and Ravinderpal Rampal for their heavenly blessings and eternal incitement in my odd times. From my deep heart, I humbly thank my better half Sahil Rampal for his unconditional love and infinite

motivation. I am grateful to my siblings Monika Sehgal Arora, Neeraj Kumar and Naveen Kumar for their huge love, support and appreciation.

I specially acknowledge my life coach Karam Singh, who made me to believe in myself and filled me with positivity. I thank him for veering my life on the right path and guiding me with his great wisdom.

Last but not least, I am grateful to my students in India and United States who propelled me to improve my teaching abilities and helped me to pave my path for academic career in coming future.

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Rippa Sehgal May 2016 56 Pages

Directed By: Dr. Kevin Williams, Dr. Eric Conte and Dr. Darwin Dahl

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Oxaliplatin is one of the three FDA-approved platinum anticancer drugs and considered a third generation drug, discovered after the first generation drug cisplatin and second generation drug carboplatin. It is known to react with proteins and DNA nucleotides in the body. Reaction with DNA occurs primarily at guanosine residues and secondarily at adenine residues for oxaliplatin and other platinum drugs. We have previously studied oxaliplatin and an analog with additional steric hindrance in the amine ligand and found that the analog had different reactivity with methionine. Now, we have prepared oxaliplatin and its three analogs Pt(Me_2dach)(ox), Pt(en)(ox) and Pt(Me_4en)(ox) and have reacted each platinum compound with both guanine and adenine nucleotides at pH 4 and pH 7 at different molar ratios. These reactions have been characterized by Nuclear Magnetic Resonance (NMR) spectroscopy equipment over time to observe the formation of products and compare them on the basis of their kinetics and binding affinities. NMR has shown that even under the conditions of excess platinum, the dominant products are usually those with two nucleotides coordinated to one platinum center. Reactions are faster at pH 7 than pH 4 due to deprotonation of phosphate group. Reactions of GMP with a platinum center are faster than reaction with AMP because of the chelate formed by the oxalate ligand. The extra methyl groups on the oxaliplatin analogs do not appear to slow down the reactions

with nucleotides considerably. The pH generally affects the rate but does not substantially affect the product distribution.

CHAPTER 1

INTRODUCTION

The American Chemical Society has estimated about 1,658,370 new cancer cases and 589,430 new cancer deaths in 2015 in the United States from the cancer incidence, survival and mortality data collected by the National Cancer Institute, the Centers of Disease Control and Prevention, the North American Association of Central Cancer Registries and the National Center for Health Statistics. Cancer is ranked as the second most fatal disease in United States and it is estimated that this deadly disease will surpass the heart disease death rate within next 5 years. However, for the past two decades, cancer death rates have been declined by 22%.[1] Out of several treatments available for cancer, exploitation of platinum based drugs has created a vast pharmaceutical surge accounted for the availability of three different generations of platinum drugs.[2] Our research is basically focused on the third generation 'dach' (dach= diaminocyclohexane) class platinum products. We have studied the binding of Oxaliplatin and its various analogs with DNA nucleobases 5'-Adenosine Monophosphate and 5'-Guanosine Monophosphate at different pH and concentration levels. All the conclusions have been made by analyzing the data obtained from Nuclear Magnetic Resonance spectroscopy. ^{1}H NMR spectra have been collected to draw strong predictions and conclude the ideas of reactivity rates of different analogs. Reactions have been compared at different parameters that include duration, pH, ligands and molar ratios. To compare the rate kinetics of different platinum compounds, reactions have been monitored over the time and NMR signals have been collected. In our research, we have drawn conclusions regarding the reactivity of oxaliplatin and its analogs with DNA nucleobases that can lead to other significant ideas in drug formulation. The

understanding of binding of drugs with biological molecules can play a major role in identifying the efficacy and limitations of drugs. Our research has compared the binding affinities of oxaliplatin and its three analogs; [Pt(II)(Me_2dach)(ox)], [Pt(II)(en)(ox)] and [Pt(II)(Me_4en)(ox)] to determine their efficiency by variations of ligands, reacting environment and molar ratios.

1.1 Cancer

The main cause of cancer is alterations in genes caused mainly due to genetic mutations, environmental factors and unhealthy lifestyle that results in division and growth of cells in uncontrolled manner. The human body is made up of trillion of cells and these cells grow and divide orderly to carry on the metabolic activities in the body. In cancer conditions, cell growth becomes unsystematic, that propels unwanted grown cells to accumulate and spread in surrounding tissues in the form of malignant tumors.[3]

Cancer cells differ from normal cells on the basis of functional specialization. Cancer cells are found to be functionally less specialized as compared to normal cells. Moreover, cancer cells are lacking the signals that help them to stop dividing at the right time.[4] So, they overgrow and utilize the nutrition from normal cells, leaving the normal cells weary and less functional. Cancer cells attack the immune system and make the body easily prone to other dysfunctionalities.[2]

The main drivers of cancer are three types of genes- proto-oncogenes, tumor suppressor genes and DNA repair genes. Proto- oncogenes are functional to do normal growth and division but their alteration tends to make them over functional and they become oncogenes that are responsible for the unrequired and uncontrolled growth of cells. Alterations in

tumor suppressor genes can also cause the uncontrolled cell division and growth. DNA repair genes help in repairing the damaged DNA. Mutations in these genes can give rise to tumor cells.[5]

Cancer can spread from its primary location to the other parts of body and process is called metastasis. In metastatic cancer, cancer cells shed away from primary tumor and commute to other organs via the circulatory system. Metastatic cancer cells usually possess main features as primary cancer cells.[6] The studies suggest that patients suffering with metastatic cancer experience more pain than the patients with non- metastatic cancer. Further studies revealed that in the cases of metastatic carcinomas, patients who report pain in high proportion were diagnosed with breast and prostate cancer sites. An interesting fact is that, in several cases the cause of the pain was the treatment itself, which includes post-surgery complications and side effects of treatment.[3,6]

1.2 Types of Cancer

There are almost 200 types of cancer, each with different diagnostic features, growth and spread rates[1]. Here, I am mentioning some categories of cancers depending upon their origin cells.

Carcinoma: This is the most common type of tumor, originating from epithelial cells. Adenocarcinoma begins in epithelial cells containing fluid or mucus. For instance, breast, colon and prostrate are the types of adenocarcinomas. Basal cell carcinoma forms in outer layer of skin called epidermis. Squamous cell carcinoma develops in squamous cells that exist beneath the skin cells. These cells also form a line around internal organs such as stomach, kidneys, lungs and urinary tract.[7]

Sarcoma: Sarcomas are bone and soft tissue cancers that can infect the skeletal as well as circulatory system. Osteosarcoma, leiomyosarcoma, malignant fibrous histiocytoma, liposarcoma and dermatofibrosarcoma are the common types of sarcoma.[8]

Leukemia: Cancer originating in the bone-marrow and spreading in the blood circulatory system is called leukemia. It basically involves the abnormal growth of leukocytes (white blood cells) that hinders the growth of erythrocytes (red blood cells), thus making the body tissues oxygen deficient. Leukemia can be acute or chronic. Acute leukemia grows faster than chronic leukemia.[7]

Lymphoma: Cancer existing in lymphocytes (T cells and B cells), the fighter cells of the immune system, is known as lymphoma. It can be in specifically B cells that is called Hodgkin lymphoma or can be in both T cells and B cells and is called Non- Hodgkin lymphoma.[9]

Multiple Myeloma: It is the tumor growth in plasma cells of immune system. Cancerous plasma cells, called myeloma cells are the cause of infection in bone marrow and bones throughout the body. It is also known as Kahler disease.[9,10]

Melanoma: Melanocytes are the specialized cells to produce the skin color pigment melanin. The tumorous growth of melanocytes can happen in skin tissues and other pigmented tissues.[9]

1.3 Three generations of Platinum based anticancer drugs

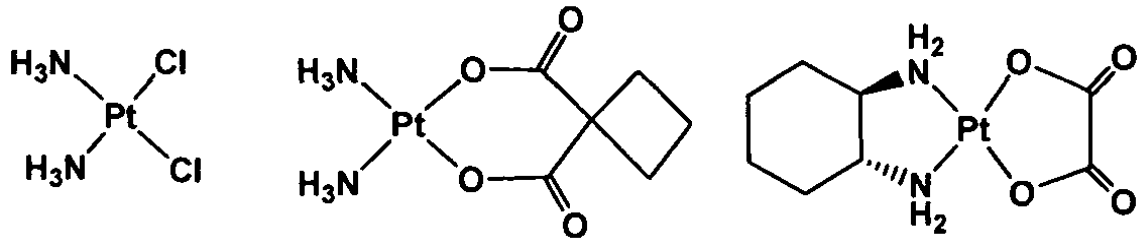
The involvement of platinum based anti-cancer drugs in cancer treatment was started with accidental discovery of cisplatin. Cisplatin, a square planar platinum(II) complex, was first synthesized by Michael Peyrone in 1845 but its anti-cancer activity was first reported by

biophysicist Barnett Rosenberg in 1968.[11] It all started when Rosenberg's research team at Michigan State University, was working on bacteria *Escherichia Coli* in electric field, with electrodes constituted of platinum metal. It was observed that *E. coli* elongated rather than dividing. It was manifested that platinum electrodes reacted with bacteria and caused this aberration. So, this serendipity gave an idea that platinum can interrupt with the cell division process and can serve as an anti- carcinogenic agent.[12] Since then, Cisplatin is considered as the first generation platinum based anti-cancer drug and is being used in the treatment of testicular and ovarian cancer. However, cisplatin efficacy was reduced due to cisplatin resistance.[13,14] To approach the alternative drugs, platinum compounds with different ligands were innovated which resulted into the discovery of carboplatin and vast class of 'dach' compounds.

Carboplatin, cis-Diammine(1,1-cyclobutandicarboxylato)platinum(II) was discovered in 1980s and got approval by FDA in 2003. It is known as a second generation drug,[15] because of its discovery after cisplatin in the search of better platinum drugs.[2] The trade name of carboplatin is Paraplatin.[16] It has shown its effectiveness to fight the lung and ovarian cancers.[17] It showed same side effects as parent drug cisplatin, but with less severity.

Although, cisplatin and carboplatin have shown accountable anti-tumor activity, the exhibition of intrinsic or acquired cross- resistance by the cells has limited their efficacy.[17] Moreover, treatment of cancer by using Cisplatin and Carboplatin comes with the side effects such as emetogenesis (vomiting), nephrotoxicity and neurotoxicity.[13] To overcome these limitations, several attempts have been made to synthesize the platinum analogues, but only some analogues are being approved by FDA.[18] Out of these, Oxaliplatin is approved for the treatment of colorectal cancer. It is basically the derivative of drug

tetraplatin and is comparatively more water soluble.[2] Figure 1 represents the skeletal structure of three generation of platinum drugs that differ from each in regard to different carrier and leaving ligands attached to the platinum center.



(A) (B) (C)

Figure 1: Skeletal presentation of (A) cisplatin, (B) carboplatin and (C) oxaliplatin

1.4 Mechanism of action of platinum based drugs

Platinum drugs act as alkylating agents that bind to DNA and cause a bend in its structure, therefore creating hindrance in transcription.[19] It is generally accepted that ultimate event in the mechanism of platinum drugs is DNA platination. But, platinum complexes also show interaction with other biomolecules such as methionine and cysteine residues.[20] All the three generation drugs possess the same mechanism of reaction. In the case of platinum drugs, drug activity is characterized by the inhibition of DNA synthesis by the binding of platinum with DNA strands to form platinated intrastrand DNA adducts and sometimes interstrand cross links. After entering the blood, these platinum compounds circulate in their inert forms- chlorides on cisplatin, dicarboxylate on carboplatin, and oxalate on oxaliplatin- which have affinity to bind with proteins and DNA nucleobases.[21] Figure 2 represents the DNA-Pt adducts formed by binding of the drug at N^7 position of 5'-GMP.

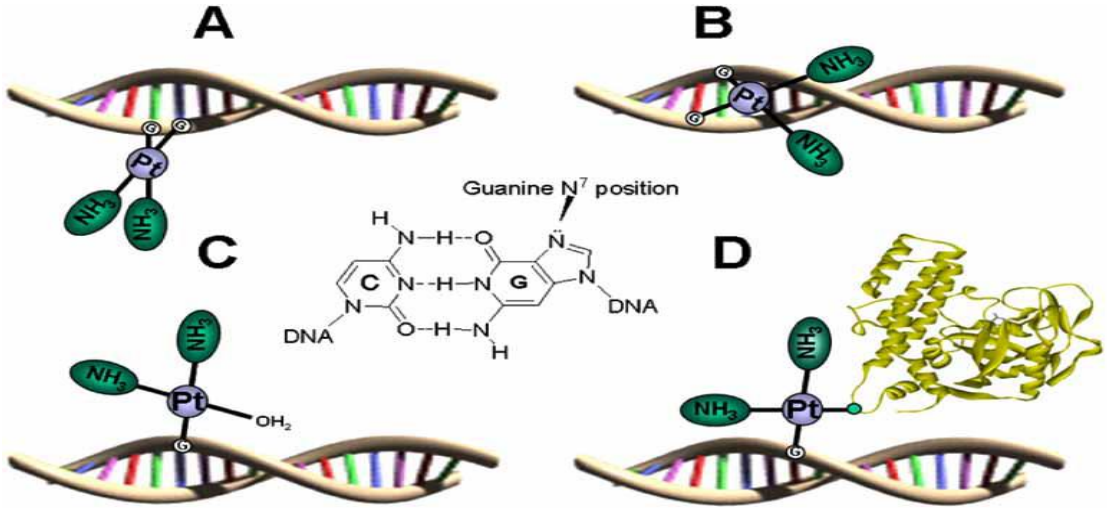


Figure 2: Platinum- guanine adducts formed by binding at N^7 position of 5'-GMP; (A)1,2-intrastrand cross-link, (B) Inter-strand cross-link (C) Mono-functional adduct (D) Protein-DNA cross-link.[22]

The major attribute of the antitumor properties of platinum based drugs is due to the ligand displacement reactions in the drug.[23] The primary target of the platinum containing drugs is nitrogen donor atoms in the nucleobases of DNA. The platinum atom of the drug binds covalently with the N7 positions of guanine bases primarily and adenine bases secondarily (fig. 2).[24] Recent results also depict that thermodynamically, platinum drug binding to guanine-N7 is more favored as compared to adenine. These linkages to the DNA basically occur by replacing chloride ions in case of cisplatin, or cyclobutane-1, 1 –dicarboxylate (CBDCA) and oxalate ligands in the case of carboplatin and oxaliplatin, respectively. The interference in the cell division process is caused by the strong bond between the platinum(II) and nucleobase nitrogen atoms.[25]

Previous studies suggested that cisplatin is absorbed by passive diffusion down the concentration gradient, as structural analogues are favorable for its entrance into the cells. Structure- activity relationships of cisplatin with biomolecules indicated that there is

necessity of cis geometry of amines and presence of at least one N-H group for entry in the blood. Other studies favoring facilitated transport mechanism suggest the involvement of protein CTR1, which is the efficient and high affinity copper transporter through the plasma membrane. It exists as homotrimer and possesses a pore composed of different amino acids such as methionine and histidine, so this pore acts as a passage for copper.[26] It is proposed that the uptake of platinum drugs might be following the same mechanism, and it is favored by the affinity between platinum drugs with nucleophilic sulfur sites of cysteine and methionine.[27] The link between copper and cisplatin transportation is interfering because they exhibit bi-directional cross resistance and reduce the uptake of each other. As the terminals of CTR1 contain methionine and histidine domains, it has been suggested that these domains may bind to cisplatin and its analogues and deactivate the platinum center by displacing the ammine ligands.[28] Figure 3 shows the passive diffusion of cisplatin from higher chloride ion concentration to lower chloride ion concentration.

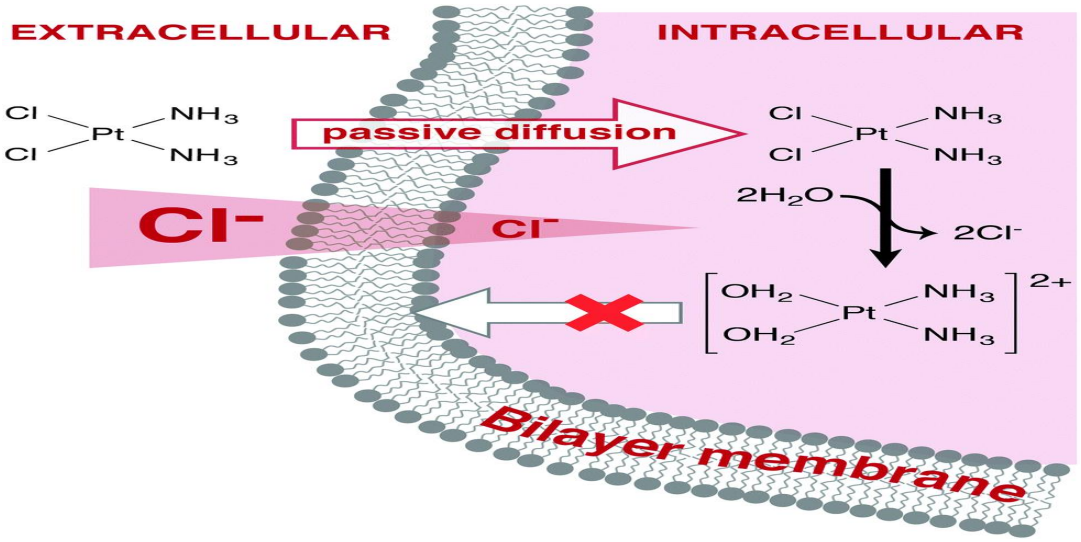


Figure 3: Passive diffusion of cisplatin inside the cell from higher chloride ion concentration to lower chloride ion concentration.[29]

Hydrolysis of platinum drug is considered mandatory because it reacts with biomolecules only in aqueous form.[30] For instance, the hydrolysis of cisplatin results into the formation of $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$. This process is facilitated by the replacement of chloride ligands by water.[31] The platinum- water adducts are usually more reactive than platinum-chloride adducts. Moreover, these adducts bear positive charge and being hydrophilic they cannot cross the hydrophobic membrane. The aqua complexes get trapped within the cell and after diffusing into nucleus through nuclear membrane they react with nitrogen containing DNA nucleobases. At the N^7 positions of guanine and adenine, aqua complexes form interstrand or intrastrand cross bridges that include 1,2-intrastrand, 1,3-intrastrand, 1,3-interstrand, 1,2-interstrand and DNA-protein adducts. The minor groove of DNA is exposed to form bonds with different proteins such as HMG domain proteins and TATA box binding proteins.[32,33] For cisplatin and its derivatives, intrastrand chelation takes place during the binding at two neighboring guanines which distorts the DNA while changing its interactions with proteins.[20]

There are basically two modes of cell death induced by platinum drug- necrosis and apoptosis.[4] In necrosis, cytosolic swelling and early shed of plasma membrane is exhibited. In contrast, apoptosis is characterized by condensation of chromatin, DNA fragmentation and shrinkage of cells. During cell death, the mode depends upon the concentration of platinum drug.[4] At high concentration, necrosis occurs whereas apoptosis occur if concentration is low. However, apoptosis is the main response given by cells to platinum drugs.[28]

1.5 Oxaliplatin: Discovery and mechanism of action

The first attempt to substitute the ammine ligands in cisplatin with 'dach' ligand was made by Connors in 1972.[34] Dach family compounds were taken into more consideration after its demonstrative activity in cisplatin resistant L- 1210 leukemia cells was noted by Burchenal. Further, Kidani differentiated the dach compounds into cis and trans geometric isomers and trans into trans-l and trans-d optical isomers, which resulted into the discovery of trans-l 'dach' oxalate platinum compound, Oxaliplatin,[35] which was found soluble in aqueous environment and showed its efficacy in cisplatin resistant malignancies.[16] Oxaliplatin can be used in the combination of other anti-cancer drugs and differentiated into first line therapy and second line therapy.[36] France was the first nation to approve it in 1996, however, USA approved it in 2002 with the trade name Eloxatin which is usually used in combination with 5-fluorouracil and leucovorin to treat colorectal cancer.[37]

Oxaliplatin is distinguished from its predecessors by a diaminocyclohexane moiety present in its structure[2]. In oxaliplatin, there is a single bidentate ligand at the place of two ammine ligands. Dach ligands are bulky and hydrophobic and target into DNA major groove, thus impairing the capability of DNA repair proteins. The bulky carrier ligand diaminocyclohexane (DACH) of oxaliplatin, also helps in the less cross resistance and increases drug efficiency.[38]

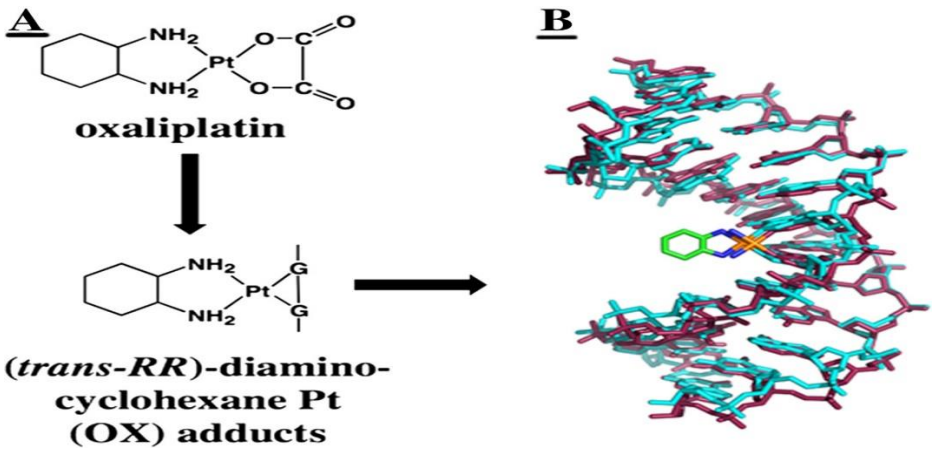


Figure 4: Oxaliplatin- DNA adduct formation (A) Conversion of oxaliplatin into adduct; (B) 3-D structure of adduct.[32]

For cisplatin, the affinity with nuclear DNA is accounted by the tendency of acquated chloride ligands to move from outer higher chloride ion concentration environment to the inner lower chloride ion concentration by the phenomena of passive diffusion. In the case of another platinum(II) drugs, toxicity and distribution of drug depends upon the nature of adducts formed as well as the nature of leaving groups.[39] The cytotoxic effect of oxaliplatin is characterized by the arresting of high dosed cells in G2/M phase of cell cycle coupled with the delay of S phase after 72 hours treatment.[38] It is also evident that resistance shown to cisplatin and carboplatin cannot be concluded as the state of cross-resistance for oxaliplatin. So, oxaliplatin may be a solution for the cisplatin-resistant tumors and its combination with other platinum compounds and alkylating agents can be very beneficial.[2]

Oxaliplatin demonstrates its cytotoxic effect basically by forming the platinated intrastrand and interstrand DNA adducts (fig. 4), which results into the inhibition of DNA replication process and thus the tumor cell dies due to cellular apoptosis.[40] The bulky dach moiety contributes to enhancement of cytotoxicity as it shows the different pattern of adduct

formation than cisplatin and carboplatin.[31] The different pattern may be attributed to the product formation due to bulky moiety. Basically, it shows cytotoxic effect in the cell lines of colon in the range of 0.5 to 240 um and in the range of 0.12 to 19.8 um in ovary.[37]

However, oxaliplatin does not show nephrotoxicity like cisplatin and myelosuppression like carboplatin but it exhibits neurological and gastrointestinal toxicities.[30] The main effects of oxaliplatin are G2/M cell cycle arrest and transient S phase delay. The recent clinical trials on HT29, MCF7 and Hela cell lines confirm that it is relevant to prolong the exposure time to enhance the efficacy of drug.[38]

The variances in competence and molecular mechanisms of cisplatin and oxaliplatin is estimated because of the differential binding affinity of damage recognition proteins with cisplatin and oxaliplatin adducts formed over adjacent guanines in genomic DNA. It was found that constraints imposed by cyclohexane ring of oxaliplatin is responsible for its negligible binding tendency to HMG-domain proteins, which may further influence the conformations for platinum-guanine adducts.[33,41]

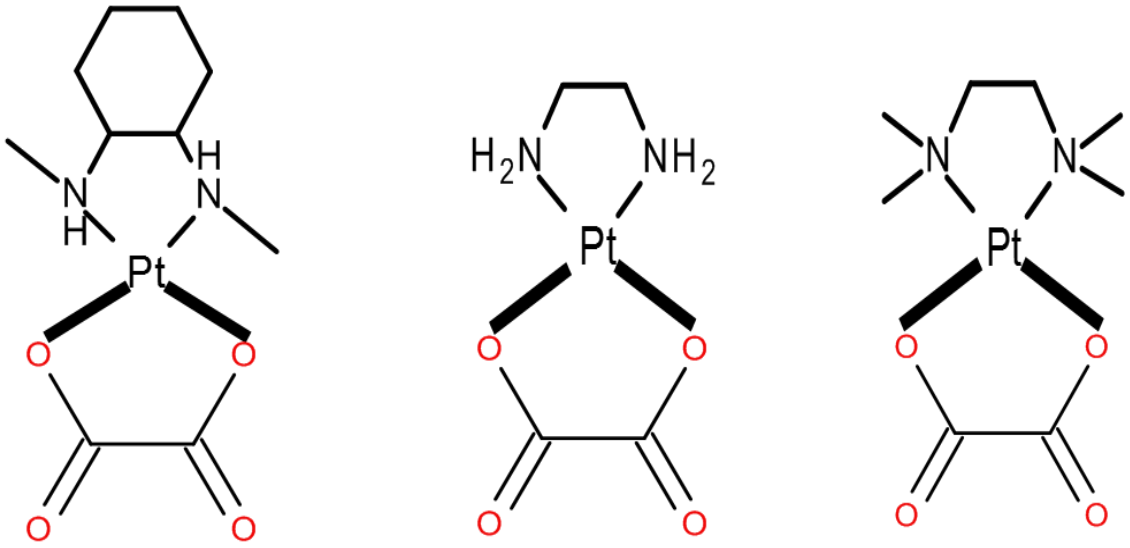
1.6 Analogs of Oxaliplatin

In addition to oxaliplatin, three other analogs of oxaliplatin (fig. 5) were studied in terms of their rate kinetics to bind with DNA nucleotides under different reacting conditions. Basically, all analogs are derived from oxaliplatin by changing the ‘dach’ carrier ligand. Their description is as follows:

a) Pt(Me_2dach)(ox) : This platinum compound differs from oxaliplatin, because it has Me_2dach (N,N-dimethyl-1,2-diaminocyclohexane) carrier ligand at the place of dach (diaminocyclohexane) carrier ligand.

b) Pt(en)(ox) : This compound contains ethylenediamine moiety as a carrier ligand.

c) Pt(Me_4en)(ox) : It has extra bulky tetramethyl ethylenediamine moiety at the place of dach moiety as a carrier ligand.



(A) (B) (C)

Figure 5: Skeletal presentation of (A) Pt(Me_2dach)(ox); (B) Pt(en)(ox) and (C)Pt(Me_4en)(ox)

1.7 Nuclear Magnetic Resonance Spectroscopy

We have used Nuclear Magnetic Spectroscopy to study the kinetics of reactions. Reactions of platinum drugs in aqueous solutions can be analyzed by the exploitation of various NMR nuclei. In our research project, we have used ^{1}H NMR spectroscopy to characterize the products and to monitor the kinetics of reactions with DNA nucleobases. ^{1}H NMR spectra provides a separate signal for each unique hydrogen atom in a compound, which potentially helps to locate each hydrogen atom with regard to the chemical shifts of proton interaction. We can obtain sharper lines by using the spin-1/2 nuclei. For ^{1}H NMR spectra, lowest detection limits are obtained at high magnetic field strengths, because sensitivity increases

at $B^{3/2}$, where B is the magnetic field strength. ^{1}H NMR studies can be utilized to study the binding of platinum based anti-cancer drug with DNA nucleotides and other biomolecules. In a spectrum the size of reactant signals and product signals are found proportional to the quantity of reactants and products. As the reaction proceeds with time, reactant signals start to become shorter and product signals start appearing until the completion of the reaction, noted by disappearance of reactant signals. So, we can predict that reaction is complete and there is no more reactant available to transform into product. We are able to determine the rate of reaction from the decrease of reactant signal and the increase of product signal.

1.8 Previous Research

In the Williams lab, research is mainly focused to study the binding patterns and affinities of platinum drug analogs with DNA nucleobases and proteins. The toxicity of platinum drugs is known to exist due to its capability to bind with proteins especially methionine and cysteine residues, but for most of the platinum compounds binding with methionine residues is more favorable. Platinum is a soft metal, so sulfur atoms present in methionine and cysteine are considered as the major target of platinum attack. However, 5'- GMP is also a strong competitor to be attacked by platinum at N^7 position. It was found that when both biomolecules compete with each other for one coordination site in platinum compound, it has been studied that for most of the platinum compounds the co-ordination with methionine occurs first that is followed by the co-ordination with 5'-GMP at N^7 position eventually.[20]

It was hypothesized that bulky amine ligands can show different levels of steric clashes for guanine and methionine. To predict the relative stabilities of methionine and guanine complexes molecular mechanic calculations were utilized. The predictions were cross-

checked by using NMR spectroscopy. The reactions were also observed with bulky (Me_4en) ligand, but they appear to be energetically unfavorable due to the steric clashes. It was also observed that (Me_4en) does not form 2:1 methionine: Pt complex for methionine but it has favored the same for guanine. So, 2:1 stoichiometry was favored for Guanosine monophosphate adducts but methionine adducts only favored the 1:1 stoichiometry.[42]

Further, interaction of N- acetylmethionine with a non-C_2-symmetrical platinum diamine complex $[Pt(Et_2en)(D_2O)_2]^{2+}$ was determined. NMR studies of the reaction indicated two set of resonances with intermediate chemical exchange. It was also suggested that sulfur chirality inversion is responsible for chemical exchange. Moreover, observation of sulfur containing complex in the initial stage of reaction indicated slow chelation of oxygen atom.[43]

In 2009, a bulky Pt(II) triamine complex $[Pt(Me_5dien)(NO_3)]$ NO_3 was reported that had found to react with 5'-GMP faster than with N- Acetylmethionine. The slower reactions with the latter could be due to the steric clashes between methyl groups of (Me_5dien) and N- Acetylmethionine.[44]

In another project, the reactions of Pt(II) diamine and triamine complexes were observed with selenomethionine, that indicated the appearance of two Se- CH_3 resonances, as Se exhibits different chirality after binding with platinum. It was suggested that, in comparison to methionine, selenomethionine reacted faster and both can displace each other in Pt(II) triamine complexes. In reactions with potassium tetrachloroplatinate trans complexes were observed in the case of methionine but not for selenomethionine.[45]

Oxaliplatin and its derivative Pt(Me_2dach)(ox) were synthesized and reacted with N-acetylmethionine, it was predicted that extra methyl groups in the derivative did not slow down the reaction that was in contrast to other bulky ligands such as me_4en and me_5dien which reduce the reactivity of methionine relative to DNA or protein targets. Moreover, (Me_2dach) was considered as an unique ligand, because extra methyl groups effected the affinity at second co-ordination site without effecting the coordination at first coordination site.[40]

1.9 Our Approach

To understand the reactivity of platinum drugs in biological systems, our lab has lead several projects under Dr. Kevin Williams's supervision. Our present study is dealing with the binding of oxaliplatin and its analogs with DNA nucleobases 5'-adenosine monophosphate and 5'-guanosine monophosphate (fig. 6). Along with oxaliplatin we have synthesized three another analogs of oxaliplatin by changing or making the carrier ligands bulky; [Pt(II)(Me_2dach)(ox)], [Pt(II)(en)(ox)] and [Pt(II)(Me_4en)(ox)]. We have studied the reactions of their binding with DNA nucleobases 5'-AMP and 5'-GMP under different reaction conditions. To monitor the effect of pH on binding affinity, most of the reactions are set at pH 4 or pH 7. Affinities and attaching capabilities of different ligands are studied through NMR spectroscopy. Our focus of research is to predict the nominal affinities of different ligands of platinum drugs which can contribute in discovering better drug with fewer side effects.

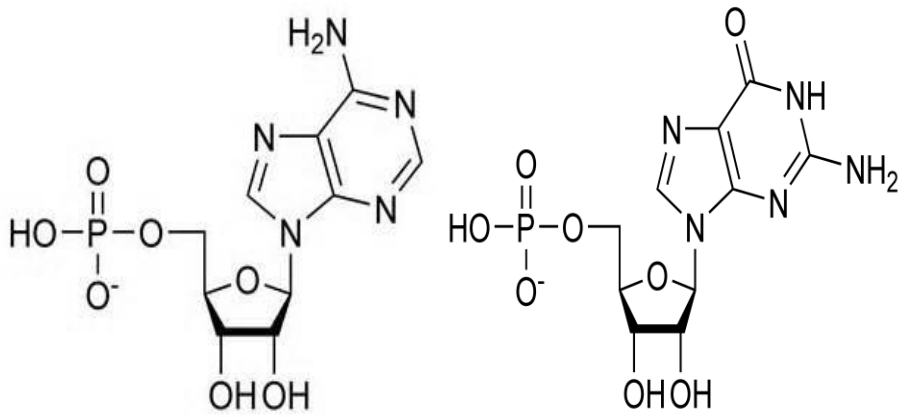


Figure 6: Structures of 5'-Adenosine Monophosphate (left) and 5'-Guanosine Monophosphate (right).

CHAPTER 2

METHODOLOGY

2.1 Reagents

Oxalic acid ($C_2H_2O_4$), silver nitrate ($AgNO_3$), deuterium oxide (D_2O), methanol (CH_3OH), potassium tetrachloroplatinate (K_2PtCl_4), ethanol (C_2H_5OH), were from Aldrich. 5' –adenosine monophosphate (5' –AMP), 5' –guanosine monophosphate (5' –GMP), diaminocyclohexane, dimethyldiaminocyclohexane, chloroethylenediamineplatinum(II), ethylenediamine and N,N,N',N'-tetramethylethylenediamine were from Acros Organics. All the reagents were utilized as received without any further purification.

2.2 Synthesis of Silver Oxalate

Silver oxalate is a raw material for the synthesis of oxaliplatin and its analogs. We prepared it by mixing 125 mg of oxalic acid and 325 mg of silver nitrate in 10 ml of DI water in a flask covered completely with aluminum foil. The mixture was then allowed to stir on stir plate of magnetic stirrer to provide considerable time for the completion of reaction. The precipitate was filtered using filter paper and washed with 20 ml of water. After drying, it was stored in dark to avoid the reaction with light.

2.3 Synthesis of Diaminocyclohexanedichloroplatinum(II)

For the synthesis of oxaliplatin, it is first necessary to synthesize Pt(dach)Cl_2, for that we made two solutions. First a solution consisting of 64 mg diaminocyclohexane ligand dissolved in 5 ml of methanol in a 10 ml beaker. A second solution containing 232 mg (equimolar amount) of potassium tetrachloroplatinate (K_2PtCl_4) in 5 ml of DI water in

another 10 ml beaker. A second solution was added drop by drop to the first solution while stirring. After thoroughly mixing the two solutions, the mixture was then put on magnetic stirrer to stir for 2-3 hours. A yellow precipitate was obtained by vacuum filtration followed by rinsing with wash solution made by mixing 7 mL of ethanol in 5 mL of water. The yield of $Pt(dach)Cl_2$ was 122 mg.

2.4 Synthesis of Diaminocyclohexaneoxalatoplatinum(II)

Pt(dach)(ox) or oxaliplatin was synthesized by dissolving equimolar amounts of $Pt(dach)Cl_2$ and silver oxalate in 35 ml of DI water in a 100 ml flask. The solution was then stirred for 2 days to maximize product. To remove the AgCl precipitate from the sample we exploited the process of micro- filtration. To filter the sample, a syringe and 0.2 micron non-disposable filter was used and filtrate is collected into 50 ml round bottom flask. The collected filtrate was dehydrated using a rotary evaporator and the resulting residue was collected. The yield of oxaliplatin was 26.4 mg.

2.5 Synthesis of N,N-dimethyl-1,2- diaminocyclohexanedichloroplatinum(II)

For the synthesis of this analog of oxaliplatin, first we needed to initially synthesize $Pt(Me_2dach)Cl_2$. For that we made two solutions. The first solution was made of 56.8 mg dimethyldiaminocyclohexane ligand dissolved in 5 ml of methanol in a 10 ml beaker. The second solution contained 166 mg (equimolar amount) of potassium tetrachloroplatinate (K_2PtCl_4) and 5 ml of DI water in another 10 ml beaker. The second solution was added drop-wise to first solution while stirring. After thoroughly mixing the two solutions, the mixture was put on magnetic stirrer to stir for 2-3 hours. We obtained a golden color

precipitate by vacuum filtration and rinsed it with the wash solution of ethanol in water. The yield of $Pt(Me_2dach)Cl_2$ was 135.4 mg.

2.6 Synthesis of N,N-dimethyl-1,2-diaminocyclohexaneoxalatoplatinum(II)

$Pt(Me_2dach)(ox)$ is synthesized by dissolving equimolar amounts of $Pt(Me_2dach)Cl_2$ and silver oxalate in 35 ml of DI water in a 100 ml flask. The solution was stirred for 2 days. The AgCl precipitate was removed by micro- filtration. To filter the sample, a syringe and 0.2 micron non-disposable filter was used and filtrate was collected into 50 ml round bottom flask. The collected filtrate was dehydrated using a rotary evaporator and dried product was collected. The yield of $Pt(Me_2dach)(ox)$ was 75.6 mg.

2.7 Synthesis of Ethylenediamineoxalatoplatinum(II)

Ethylenediamine oxalato platinum(II) was synthesized by dissolving equimolar amounts of ethylenediamine dichloro platinum(II) (used as received) and silver oxalate in 35 ml of DI water in a 100 ml flask. After the reaction was complete, AgCl precipitate was removed from the sample by the process of micro- filtration. The collected filtrate was dehydrated using a rotary evaporator and the solid material was collected. The yield of Ethylenediamine oxalato platinum(II) was 112 mg.

2.8 Synthesis of N,N,N',N'-tetramethylethylenediamineoxalatoplatinum(II)

N,N,N',N'-tetramethylethylenediamine oxalato platinum(II) was synthesized by dissolving equimolar amounts of dichlorotetramethylethylenediamineplatinum(II) (previously synthesized by other lab members) and silver oxalate in 35 ml of DI water in a 100 ml flask. The solution was then kept to stir for 2 days to synthesize the final product. To remove the AgCl precipitate from the sample, a syringe and 0.2 micron non-disposable

filter was used and filtrate is collected into 50 ml round bottom flask. The yield of tetramethylethylenediamineoxalatoplatinum(II) was 108 mg.

2.9 Preparation of stock solutions

Stock solutions were prepared by weighing platinum drug and nucleobases 5'-adenosine monophosphate and 5'-guanosine monophosphate according to their molar ratios and dissolving them in deuterium hydroxide in eppendorf tubes. Samples were then transferred into NMR tubes and their ^{1}H NMR spectra were collected.

Stock solution of Pt(dach)(ox) or oxaliplatin: 3.9 mg of oxaliplatin [Mol Wt- 390 g] was dissolved in 1mL of deuterium oxide to obtain 10 mM solution.

Stock solution of Pt(Me_2dach)(ox): 4.3 mg of Pt(Me_2dach)(ox) [Mol Wt- 430 g] was dissolved in 1mL of deuterium oxide to obtain 10 mM solution.

Stock solution of Pt(en)(ox): 3.4 mg of Pt(en)(ox) [Mol Wt- 340 g] was dissolved in 1 mL of deuterium oxide to obtain 10 mM solution.

Stock solution of Pt(Me_4en)(ox): 4.0 mg of Pt(Me_4en)(ox) [Mol Wt- 400 g] was dissolved in 1 mL of deuterium oxide to obtain 10 mM solution.

Stock solution of 5'-AMP: 3.5 mg of 5'-AMP [Mol Wt- 347.2 g] was dissolved in 1 mL of deuterium oxide to obtain 10 mM solution.

Stock solution of 5'-GMP: 4.1 mg of 5'-GMP [Mol Wt- 407.19 g] was dissolved in 1 mL of deuterium oxide to obtain 10 mM solution.

2.10 Methods

Our methods to set the experiment involved adjustment of required pH of stock solution prior to mixing. After the adjustment of pH, equal amount of stock solutions of platinum drug and DNA nucleobase were mixed in the required concentration to make it ready to run on NMR instrument.

Solutions used for pH adjustment

1% nitric acid in deuterium oxide or 10% nitric acid in deuterium oxide were used to lower the pH. 1% sodium hydroxide in deuterium oxide or 10% sodium hydroxide in deuterium oxide were used to raise the pH of stock solutions.

a) Reaction of AMP with oxaliplatin

The experiment was set by mixing the stock solutions of oxaliplatin and AMP. To prepare 1:1 ratio, 0.5 ml of each solution was mixed in 1.5 mL Eppendorf vial and required pH. Usually, we set the reactions to pH 4 or 7. The samples were also prepared in 1:2 and 2:1 ratios to observe the effect of concentration variations on the binding of platinum drug with DNA nucleobases.

b) Reaction of GMP with oxaliplatin

To set the reactions of guanosine monophosphate with oxaliplatin, we mixed 0.5 ml aliquot of stock solution of GMP and 0.5 ml aliquot of stock solution of oxaliplatin in 1.5 mL eppendorf vial to set the reaction in 1:1 ratio. The reactions were also set in 2:1 and 1:2 ratios by reducing the concentration of one reagent to the half and keeping the other at

same. The solutions were also prepared at pH 4 and pH 7 to observe the effects of variable pH on the kinetics of reaction.

c) Reaction of AMP with Pt(Me_2dach)(ox)

Pt(Me_2dach)(ox) is an analog of oxaliplatin prepared by replacing the (dach) ligand with (Me_2dach) ligand which results in the bulkiness of carrier ligand. In order to set the reactions of AMP with Pt(Me_2dach)(ox), 0.5 mL aliquot was taken from 10 mM 5'-GMP stock solution and was mixed with 0.5 ml of Pt(Me_2dach)(ox) stock solution.

We also set the reactions at 2:1 and 1:2 molar ratios as well as pH 4 and pH 7 to study the effect of variable pH and concentration levels on the binding of drug with AMP.

d) Reaction of GMP with Pt(Me_2dach)(ox)

The reactions of GMP with Pt(Me_2dach)(ox) are set by mixing 0.5 mL of stock solution of each reagent in 1.5 mL of eppendorf vial. Both sample preparations can be varied to set the reactions on different pH and concentration. To set the reaction at 2:1, concentration of drug was reduced to half and to set the reaction at 1:2 concentration of GMP was reduced to half or concentration of drug was enhanced to double.

e) Reaction of AMP with Pt(en)(ox)

To analyze the kinetics of binding of AMP with Pt(en)(ox), another analog of oxaliplatin, reactions were set by mixing 0.5 mL of stock solution of both reagents in 1.5 mL eppendorf vial.

f) Reaction of GMP with Pt(en)(ox)

Another reaction is set with same drug but different DNA nucleobase. First solution was made by dissolving 4.1 mg in 1.0 mL of D_2O and second solution is prepared by dissolving 3.4 mg of Pt(en)(ox) in 1.0 mL of D_2O. To set the reaction, 0.5 mL of each solution is mixed in 1.0 mL eppendorf tube at required pH.

g) Reaction of AMP with Pt(Me_4en)(ox)

To make the carrier ligand bulkier previously studied analog was attached with tetramethyl moiety and its reaction was set with AMP. Reaction of AMP with Pt(Me_4en)(ox) was set by mixing 0.5 ml of stock solution of 5'-AMP with 0.5 mL stock solution of Pt(Me_4en)(ox) in 1.5 mL eppendorf tube.

h) Reaction of GMP with Pt(Me_4en)(ox)

The reaction of GMP with Pt(Me_4en)(ox) was set to study the kinetics and draw the comparison with AMP and its reactions with another platinum compounds. To set the reaction we mixed 0.5 mL of stock solutions of both reagents in 1.5 mL eppendorf vial.

NMR Setup

After setting the reactions, at required pH and concentration, samples were transferred into NMR tubes to collect the ^1H NMR spectra of the samples. NMR equipment consists of NMR sample holder and processor, NMR software JOEL Delta v5.0.4 (installed on the computer) and nitrogen gas cylinder (fig. 7). NMR tubes filled with sample solutions are loaded in the sample holder and processed by giving the commands on the computer. To load the required sample, first blank or reference NMR tube is unloaded. After running the

samples, collected data can be printed out by using attached printer. Usually it takes 15- 20 min to run a sample. After the completion of process, sample is unloaded and blank or reference solution is reloaded over the NMR processor.

Figure 7: NMR software (JOEL Delta v5.0.4) installed on computer, NMR sample holder and processor (500 MHz), nitrogen gas cylinder connected to NMR and NMR tubes.

CHAPTER 3

RESULTS

Our experimental data has been analyzed over the time to approach the results verifying the impact of ligand substitution, variable pH and concentration levels on the kinetics of binding of platinum drugs with DNA nucleobases, 5'-adenosine monophosphate and 5'-guanosine monophosphate. NMR spectroscopy was performed to obtain the ^{1}H NMR spectra of the samples made by mixing platinum compound with 5'-AMP or 5'-GMP in specific molar ratio, where solvent was deuterium oxide instead of water, to discourage the interference of water molecules with the spectra signals. We focused to determine the product distribution and appearance of peaks for the reactions of oxaliplatin and its analogs with AMP and GMP at various molar ratios in different pH conditions. After the detailed analysis of signals we have collected and analyzed partial ^{1}H NMR spectra to sum up the following results:

3.1 ^{1}H NMR spectra for reactions of AMP with Oxaliplatin in 1:1 ratio at pH 4 and pH 7

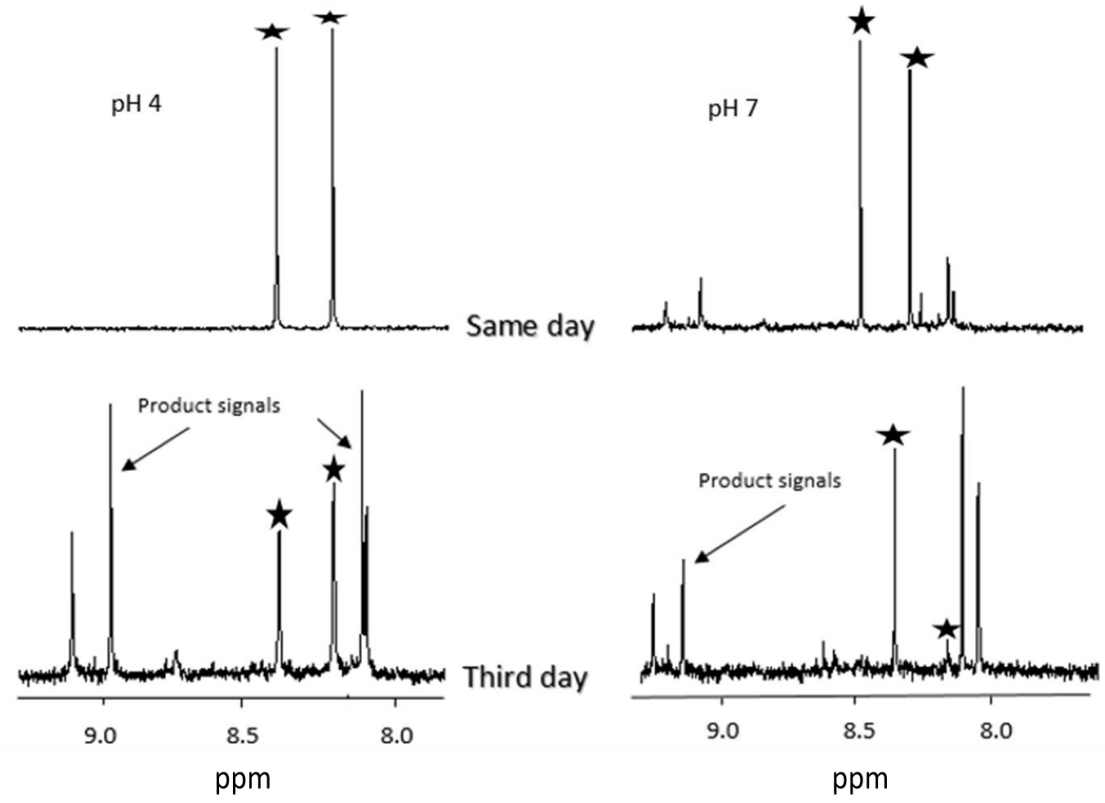


Figure 8: ^{1}H NMR spectra of reactions of AMP with oxaliplatin in 1:1 ratio at pH 4 and pH 7 obtained on same day and third day. * represents the unreacted nucleotide.

We set the reactions of AMP with oxaliplatin at pH 4 and pH 7 and monitored the ^{1}H NMR spectra of samples on the same day and third day (fig. 8). After 3 hours, at pH 4 and pH 7 there were two signals in the range of 8.1-8.3 ppm corresponding to unreacted AMP, which are represented by * in figure 8. It was found that on the same day of reaction setting, no product signals were visible at pH 4. At pH 7, multiple sets of small product signals started appearing after 8 hours at 8.1 ppm and at 9.0 ppm.

When we allowed the compounds to react for three days, prominent product signals at pH 4 and pH 7 were visible. For pH 4, product signals at 8.1 ppm were overlapping but for pH 7, two different signals corresponding to products were visible in 8.0- 8.1 ppm range.

3.2 ^{1}H NMR spectra for reactions of GMP with Oxaliplatin in 1:1 at pH 4 and pH 7

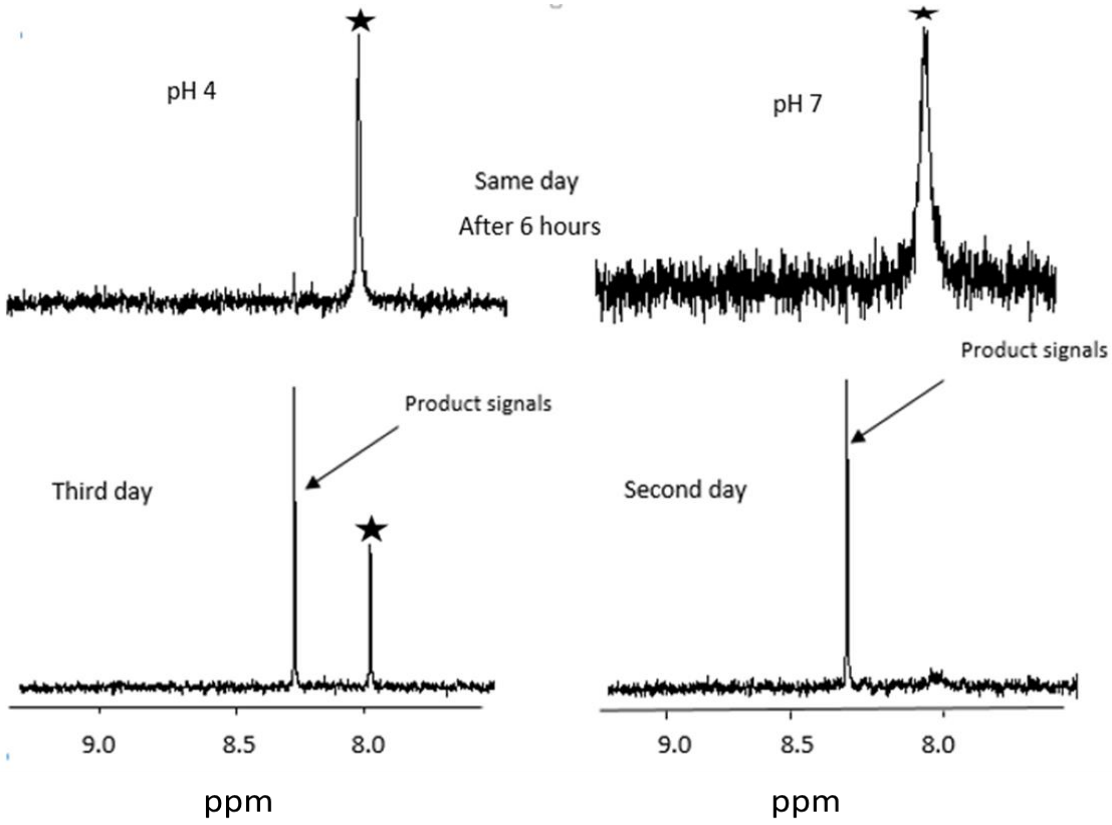


Figure 9: ^{1}H NMR spectra obtained by reacting GMP with oxaliplatin in 1:1 ratio at pH 4 and pH 7 obtained on same day, third day and second day. * represents the unreacted nucleotide.

In the next experiment, we have analyzed the reactions of another DNA nucleobase 5'-guanosine monophosphate with oxaliplatin at pH 4 and pH 7 (fig. 9). After 6 hours, very

small product signals started appearing at pH 4 and pH 7 at 8.2 ppm. The unreacted nucleotide signals are represented by * in the figure.

At pH 7, until the second day, all the reactant signals disappear and only product signal was visible. In contrast, at pH 4, even after three days, it appeared that the reactions achieved only 50% conversion.

NMR signal obtained for GMP and oxaliplatin at pH 4 on the third day had demostrated the continuity of reaction, whereas NMR signals obtained for GMP and dach at pH 7 on the second day had shown that reaction was complete. Thus, this reaction at pH 7 is faster than at pH 4.

3.3 ^{1}H NMR spectra for reactions of AMP with Pt(Me$_2$dach)(ox) in 1:1 at pH 4 and pH 7

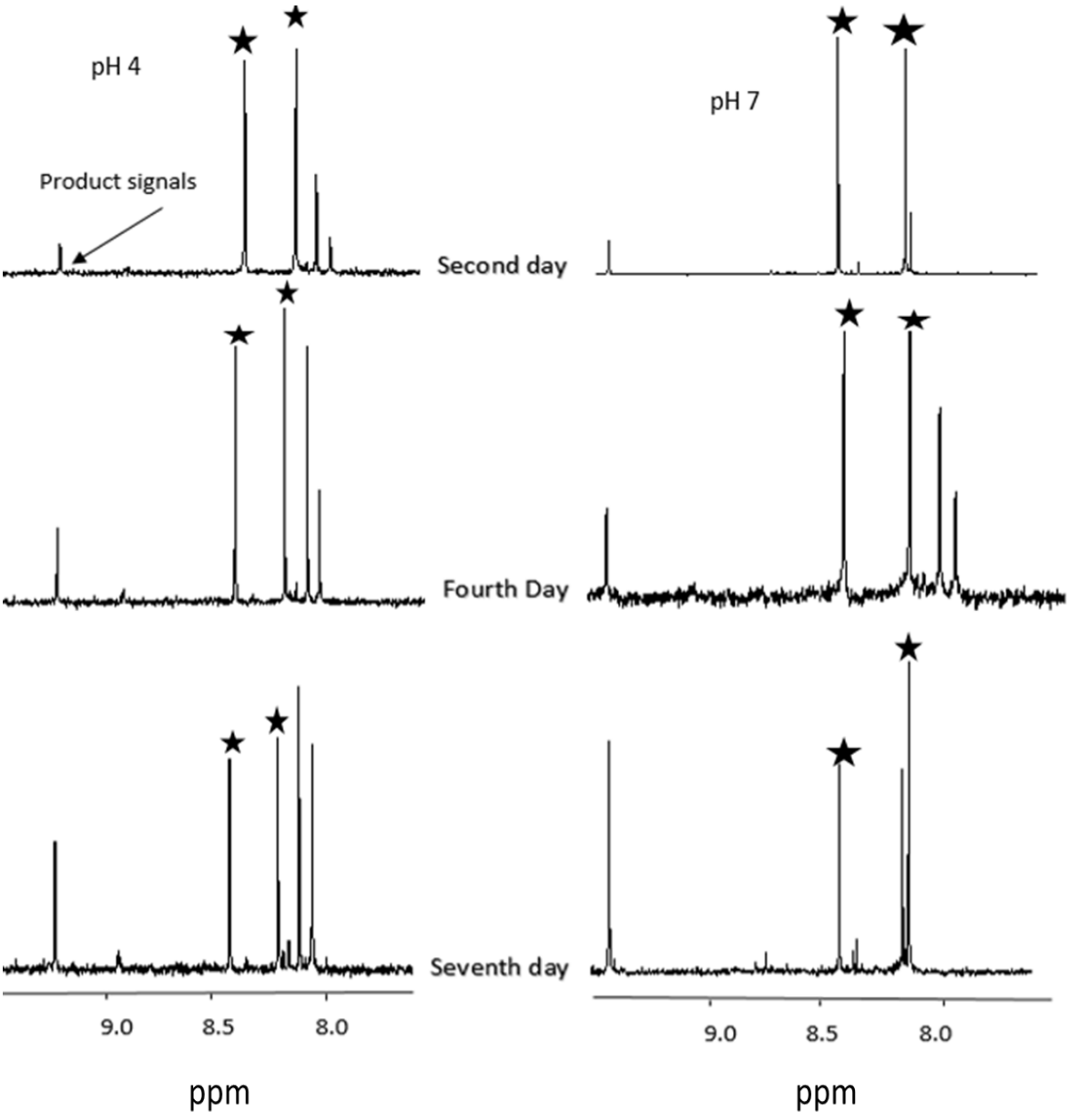


Figure 10: ^{1}H NMR spectra of AMP with Pt(Me$_2$dach)(ox) in 1:1 ratio at pH 4 and 7 on second day, fourth day and seventh day. * represents the unreacted nucleotide.

In the series of experiments, we set the reaction of AMP with Pt(Me_2dach)(ox) and monitored it over seven days. We have compared the NMR signals obtained at second, fourth and seventh day (fig. 10). In the figure * corresponds to signals of unreacted nucleobase. After montioring the reactions by obtaining their ^{1}H NMR spectra we did not observe any product signal after couple of hours. After 30 hours product signals started appearing beyond 9.0 ppm and near 8.0 ppm at the right side of reactant signals.

On the second day, product signals at pH 4 and pH 7 were quite similar. As the reaction proceeded till fourth day, bigger signals at pH 7 were distinguishable. On the fourth day two product signals were noticable beyond 8 ppm that seemed to be corresponding to intermediates because they disappear until seventh day of reaction. On the seventh day, product signals beyond 9 ppm at pH 7 were much bigger than at pH 4.

3.4 ^{1}H NMR spectra for reactions of AMP and GMP: Pt(en)(ox) in 2:1 ratio at pH 4

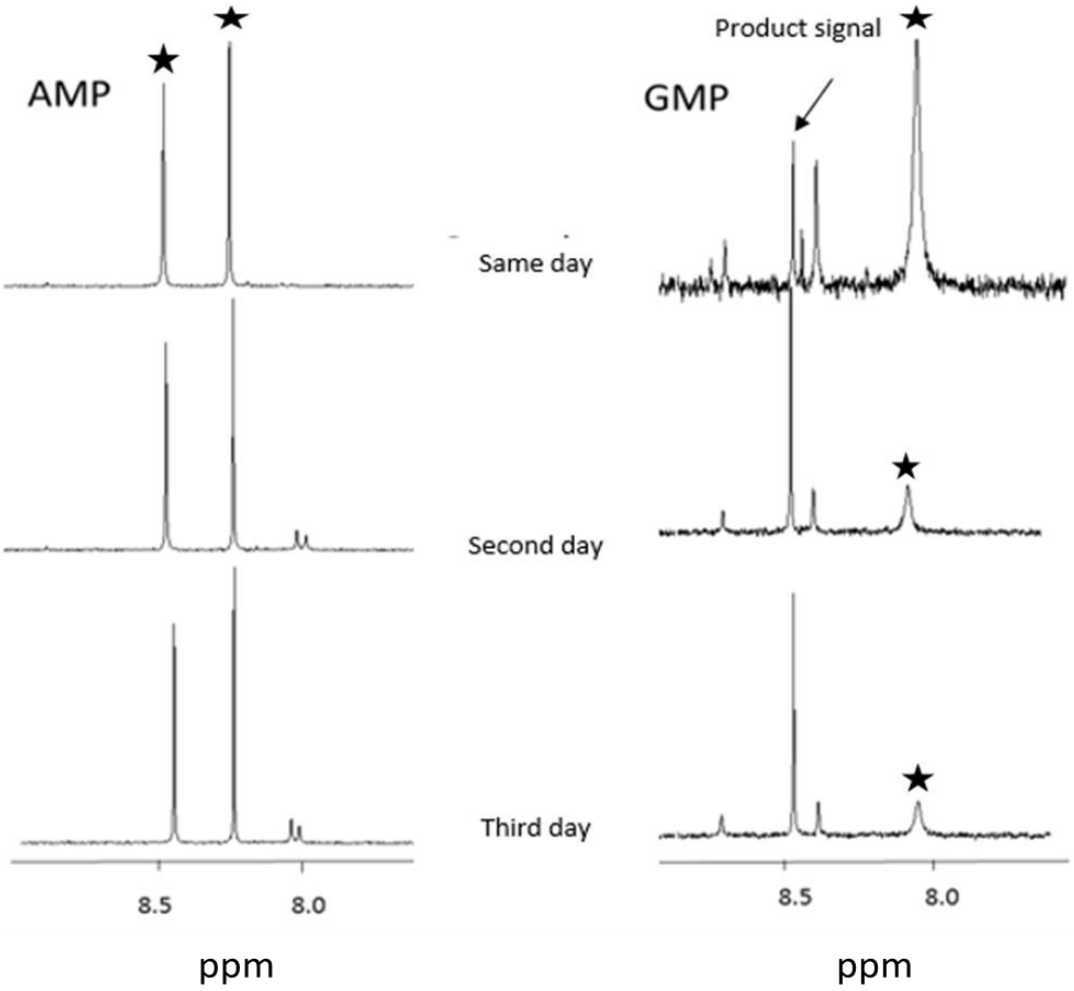


Figure 11: ^{1}H NMR spectra of AMP and GMP with Pt(en)(ox) at pH 4 and 2:1 ratio on same day, second day and third day. * represents the unreacted nucleotide.

We set the reaction of AMP and GMP with Pt(en)(ox) at pH 4 and 2:1 ratio, where concentration of DNA nucleobase is taken double as compared to concentration of platinum drug. We monitored the reaction on same day, second day and third day (fig. 11). For GMP, on the same day small product signal was visible at 8.5 ppm whereas one signal corresponding to intermediate was noticeable at 8.4 ppm, which became shorter on second

day. In case of GMP, till second day, reaction run very fast leaving small unreacted GMP signal that was further shortened till third day. In case of AMP, until fourth day product signals were not appeared that depicts that reaction of platinum center was faster with GMP. Unreacted nucleotide signals were represented with * in figure 11.

3.5 ^{1}H NMR spectra for reactions of AMP and GMP : Pt(Me$_2$dach)(ox) in 2:1 ratio at pH 4

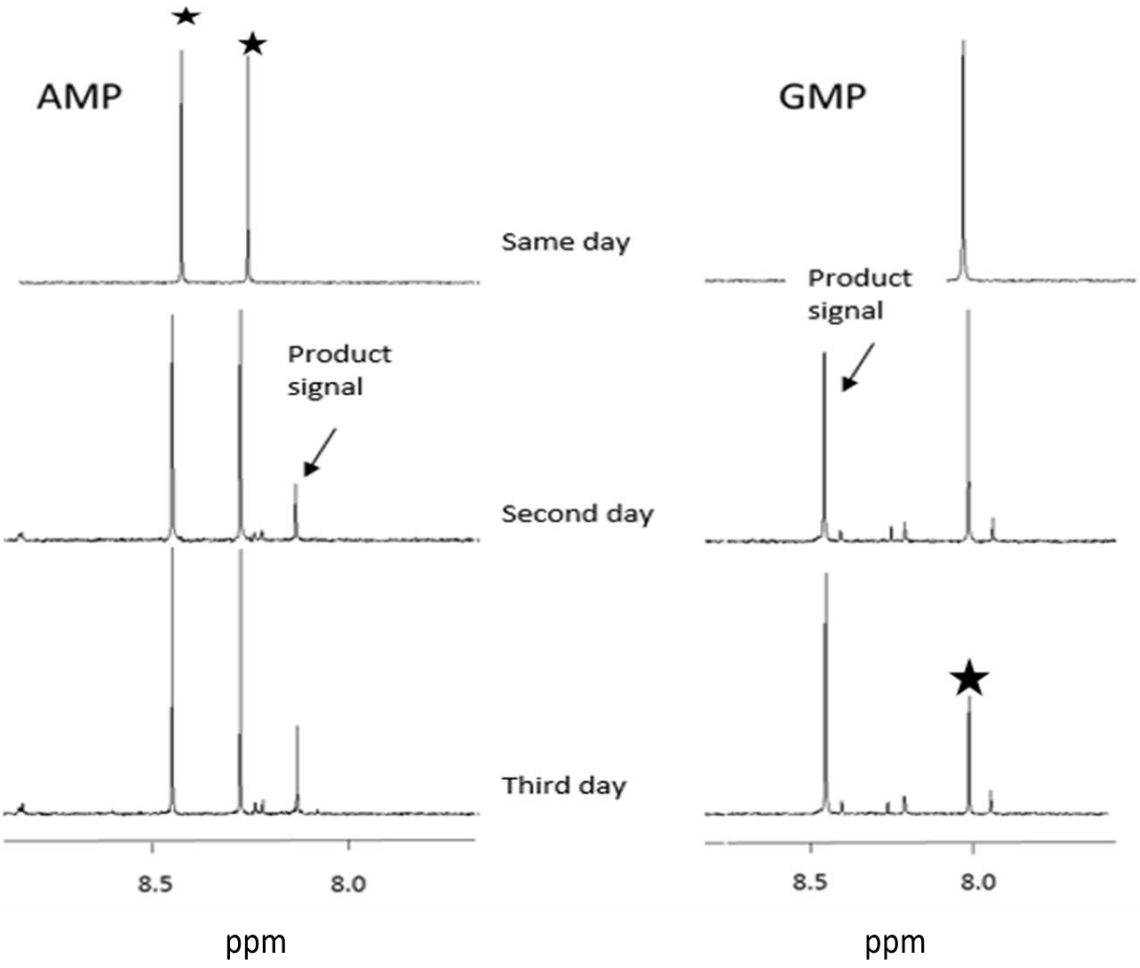


Figure 12: ^{1}H NMR spectra of AMP and GMP with Pt(Me$_2$dach)(ox) at pH 4 and 2:1 ratio on same day, second day and third day. * represents the unreacted nucleotide.

At 2:1 ratio, we set the another experiment in which we had allowed the AMP and GMP to react with Pt(Me_2dach)(ox) at pH 4. We have compared the NMR signals obtained at same day, second day and third day (fig. 12). ^{1}H NMR spectra obtained after setting the reactions, showed only reactant signals and reactions were allowed to proceed overnight.

On the second day, for AMP we noticed small signal at 8.1 ppm corresponding to products. For GMP large product signal was noticable at 8.4 ppm. Unreacted nucleobase signals were represented by * in the figure.

On the third day, spectra depicted bigger product signals and smaller reactant signals, but for GMP product signals were very much prominent as compared to signals for AMP.

3.6 ^{1}H NMR spectra for reactions of AMP and GMP : Pt(Me$_2$dach)(ox) in 1:2 ratio at pH 4

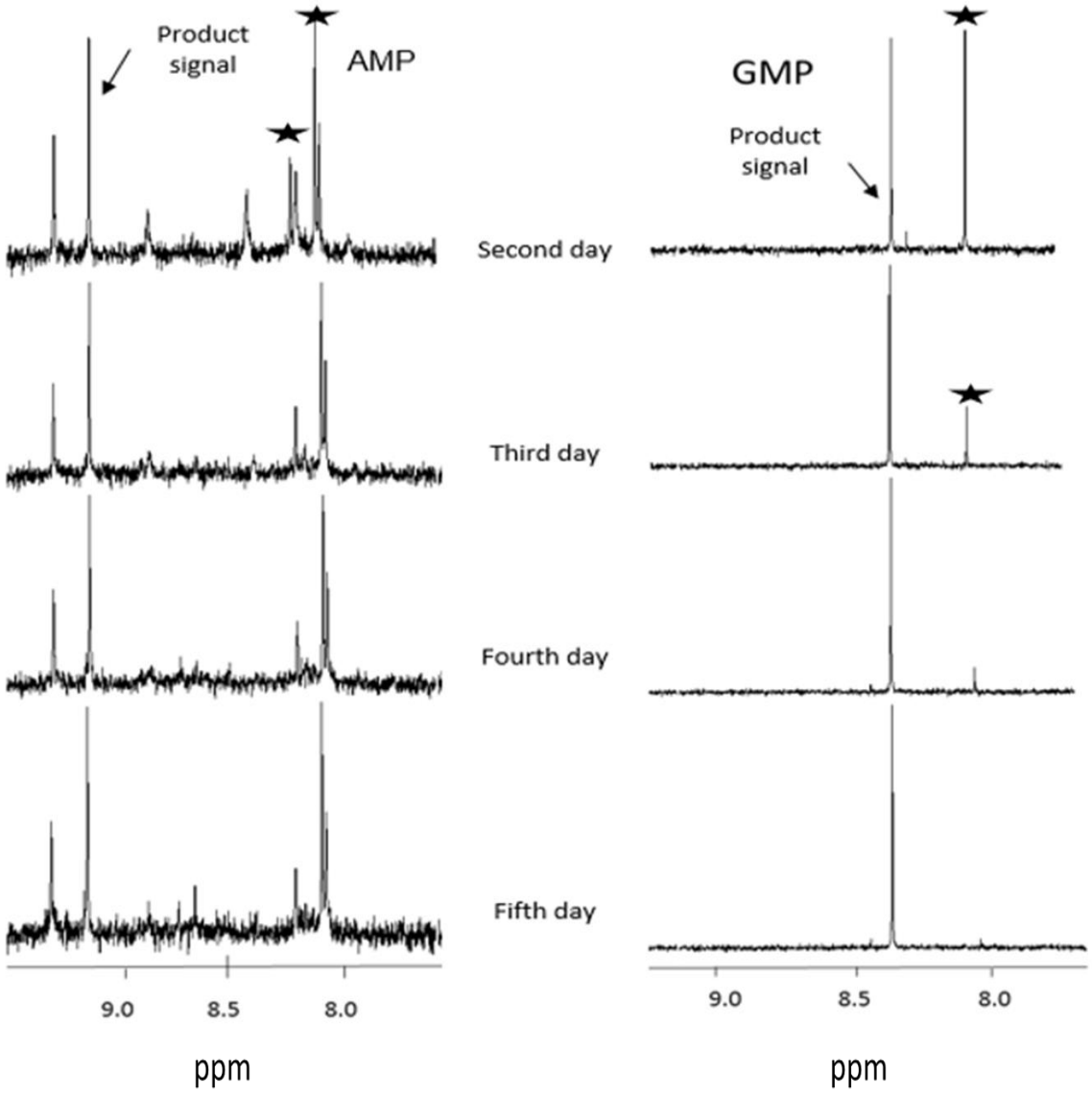


Figure 13: ^{1}H NMR spectra of reactions of AMP and GMP: Pt(Me$_2$dach)(ox) in 1:2 ratio at pH 4 obtained on second, third, fourth and fifth day. * represents the unreacted nucleotide.

Next, we set the reactions of AMP and GMP with Pt(Me_2dach)(ox) at 1:2 ratio and monitored the reactions for five days consecutively (fig. 13). In the first column of figure we have shown the signals obtained on second day. For AMP, there were two signals at 8.2-8.0 ppm range corresponding to unreacted AMP (represented by *), two signals were present beyond 9.0 ppm corrsponding to products and one small signal at 8.4 ppm corresponding to intermediate. For GMP, one signal was present at 8.0 ppm corresponding to unreacted GMP (represented by *) and one signal was visible at 8.4 ppm corresponding to products.

On the third and fourth day reactions for GMP were proceeding at nominal pace, that was noticable from shortened reactant signals and large product signals. For AMP, signals seemed unchanged depicting that reaction was completed on the second day. Intermediate signal at 8.4 ppm was also disappeared.

On the fifth day, for GMP reaction was proceeded further and very minute signal of unreacted GMP was noticed at 8.0 ppm.

3.7 ^{1}H NMR spectra for reactions of AMP and GMP with Pt(Me$_4$en)(ox) in 1:1 ratio

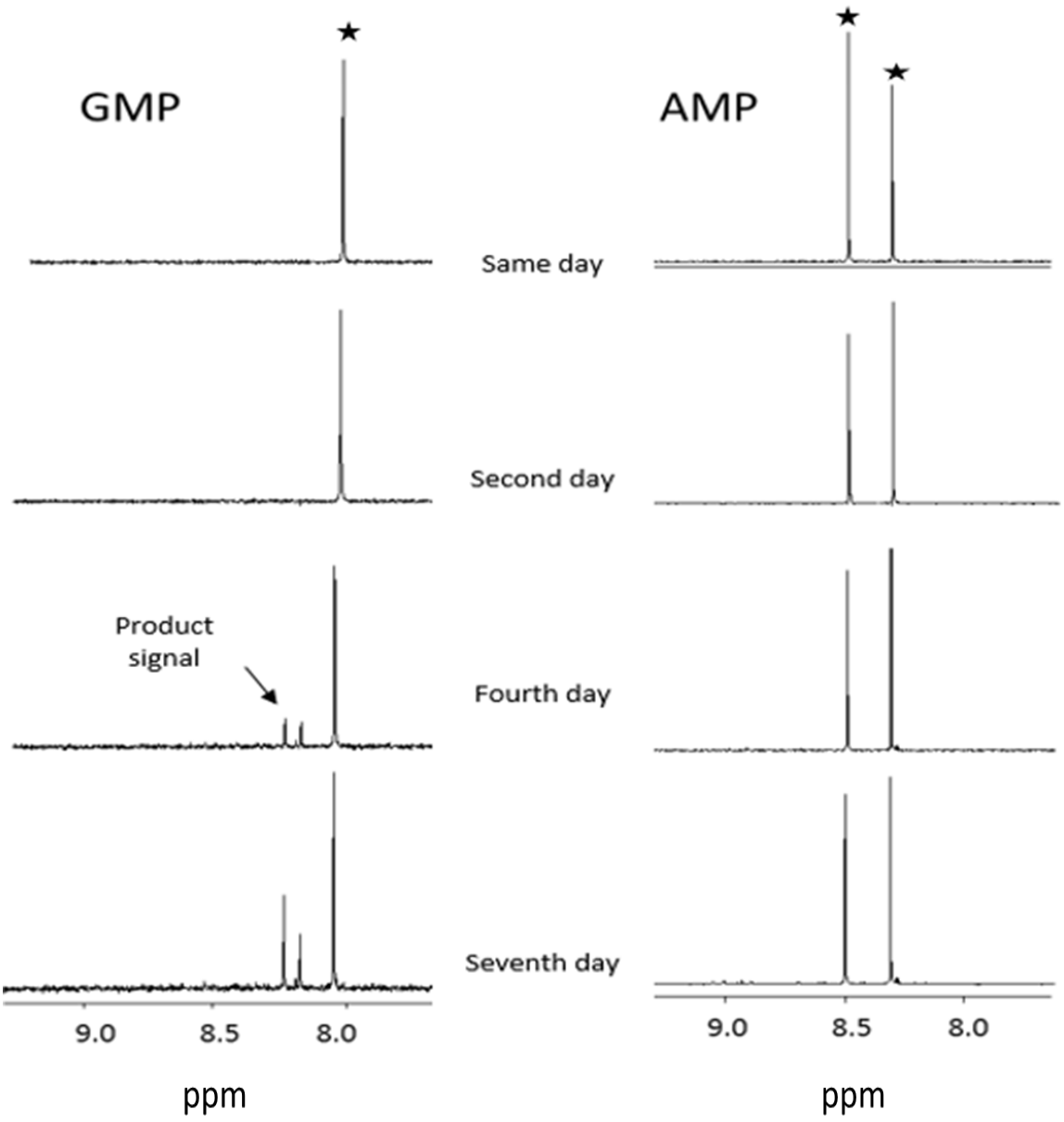


Figure 14: ^{1}H NMR spectra of reactions of AMP and GMP with Pt(Me$_4$en)(ox) in 1:1 ratio obtained on same, second, fourth and seventh day. * represents the unreacted nucleotide.

To observe the binding affinities of DNA nucleobases with another analog of oxaliplatin, Pt(Me_4en)(ox), we set the reactions at pH 4 in 1:1 ratio and monitored the reaction kinetics by obtaining ^{1}H NMR spectra for seven days (fig. 14).

In figure 11, we have compared the signals obtained on the same day, second day, fourth day and seventh day. We observed that the reactions with both nucleobases were extremely slow. Spectra collected on the same day depicted only the signals of unreacted nucleobases represented by * in the figure. Unreacted GMP signals were found at 8.0 ppm and unreacted AMP signals were found at 8.5-8.4 ppm range. Data collected on second day, showed the same signals as of the previous day.

On the third day, reaction of GMP was proceeded, spectra demonstrated the appearance of multiple small signals at 8.2-8.3 ppm range corresponding to products. Whereas, on the third day, AMP seemed unreacted, as only unreacted AMP signals were visible at 8.5-8.4 ppm range.

In the last column of the figure, we have shown the data obtained on the seventh day of reaction setting. Reaction with GMP seemed proceeded further as product signals appeared in 8.2-8.3 ppm range looked bigger. But, for AMP even on the seventh day, no product signals were noticed. The bulkier ligand attachment can be the reason for slow kinetics of the reaction.

3.8 ^{1}H NMR spectra for reactions of GMP with Pt(Me$_4$en)(ox) at pH 4 and pH 7

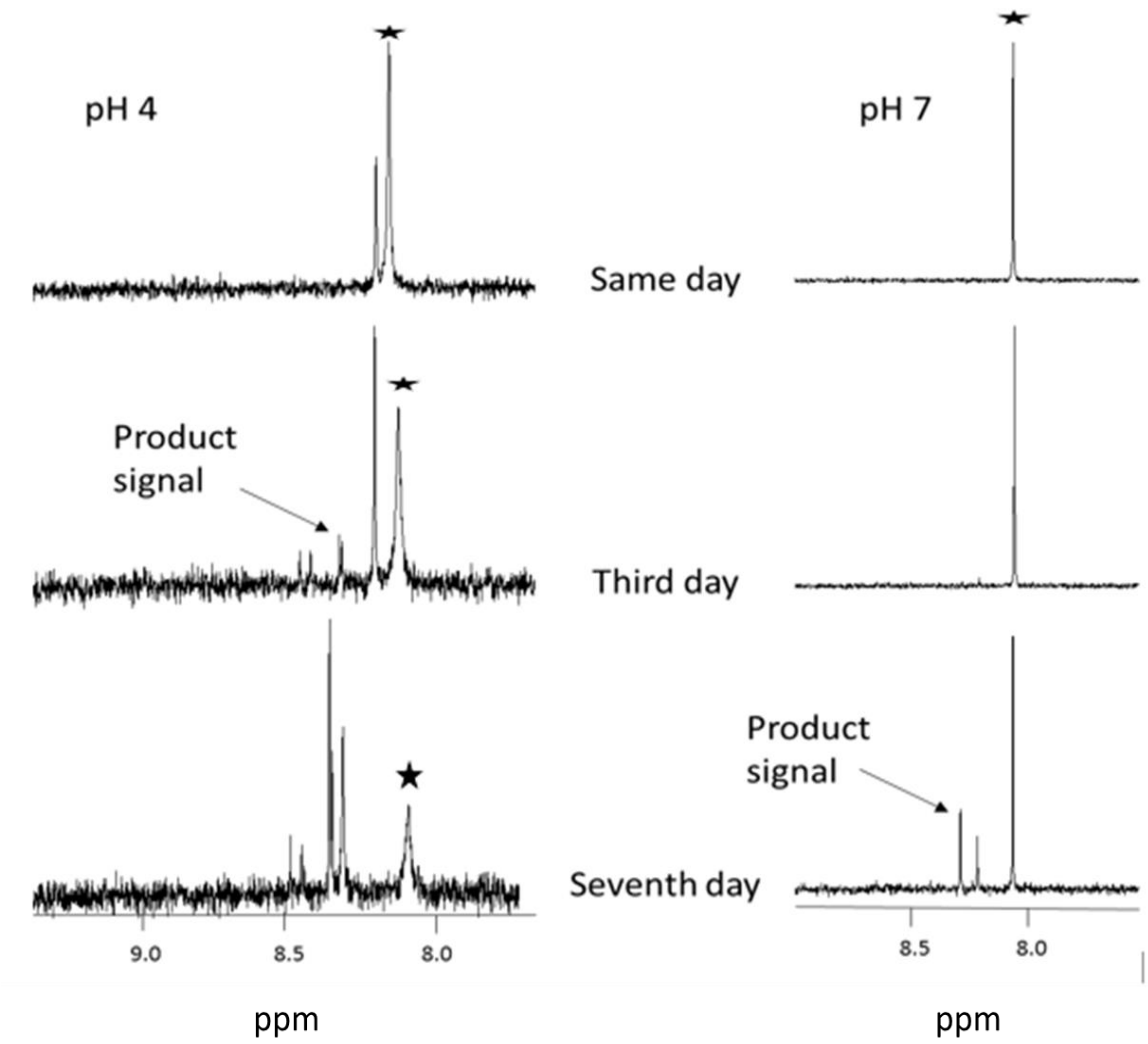


Figure 15: ^{1}H NMR spectra of reactions of GMP with Pt(Me$_4$en)(ox) in 1:1 ratio obtained on same day, third day and seventh day. * represents the unreacted nucleotide.

Further, to investigate the reaction patterns of Pt(Me$_4$en)(ox) with DNA nucleobases at different pH levels, we first set the reaction of GMP with this analog and monitored the rate kinetics by obtaining the ^{1}H NMR spectra until seven days (fig. 15). Here, in figure we have compared the signals obtained on same day, third day and seventh day.

On the same day, at pH 4 we observed two signals in 8.1-8.2 ppm range, bigger signal was corresponding to unreacted GMP and smaller one was depicted for an intermediate. At pH 7 only one signal for unreacted GMP was noticed at 8.1 ppm.

On the third day, for pH 4 unreacted GMP signal became smaller (represented by *) and intermediate signal became larger. At pH 4, multiple signals were noticed in the range of 8.3-8.5 ppm corresponding to products. At pH 7, spectra looked similar, depicting the slow pace of reaction.

The third column of the figure is showing the spectra collected on seventh day. At pH 4, unreacted GMP signal looked smaller and intermediate signal disappeared, indicating their conversion into products that was evident from bigger product signals in 8.3-8.5 ppm range. At pH 7, two product signals were visible in the range of 8.2-8.3 ppm, but still signal for unreacted GMP at 8.1 ppm was quite prominent.

At pH 4, we observed the appearance of product signals on the third day and till seventh day, reaction seemed almost complete, only leaving product signals and very small reactant signal on the spectra. On contrast, for pH 7 we had observed the product signal after third day. Even on the seventh day product signal was not very much prominent. This reaction pattern of Pt(Me_4en)(ox) was reported opposite to oxaliplatin and its other analogs.

CHAPTER 4

DISCUSSION

In our research, we reacted the oxaliplatin and its analogs Pt(Me_2dach)(ox), Pt(en)(ox) and Pt(Me_4en)(ox) with DNA nucleobases 5'-AMP and 5'-GMP. We used NMR equipment to study the reaction kinetics and signal patterns. We have studied the reactions at various pH and concentration levels and drew the predictions on the basis of their binding affinity and kinetics. We have drawn the following results to discuss:

Reaction kinetics at pH 4 and pH 7 varies with carrier ligand

After monitoring the reactions of various analogs of oxaliplatin with AMP and GMP over time at pH 4 and pH 7, we hypothesized that reactions at pH 7 are generally faster than the analogous reactions at pH 4. In all combinations of drugs and DNA nucleobases we have observed adduct signals appearing prior at pH 7 and as the reactions proceeded the exhibition of more prominent signals at pH 7 supported our hypothesis. But, analog Pt(Me_4en)(ox) had shown the reverse pattern of affinity as its reaction seemed somewhat faster at pH 4 than pH 7.

When we compared the reaction kinetics of oxaliplatin with AMP and GMP, it was utterly clear from figure 5 and figure 6 that appearance of product signals at pH 7 were much faster than at pH 4. We predicted that pH environment provided to the reaction is preferable when it is neutral or basic rather than acidic.

Reactions with Pt(Me_2dach)(ox) indicated the same pattern of reactions and pH favorability, but to less extent than its parent oxaliplatin. This behaviour can be attributed to comparitively bulkier carrier ligand and slow reaction mode for Pt(Me_2dach)(ox). So,

we estimated that steric clashes arised due to bulkiness, actually buffered the differences of pH and steric clashes seemed more strong at pH 7.

In the case of Pt(Me_4en)(ox), reactions at pH 4 were seemed faster than pH 7 (fig. 15). This probing is contradictory to our hypothesis and again the reason could be the steric clashes occurred at pH 7 due to bulkiness of ligands.

Bulkier carrier ligands considerably slow down the reactions

From our experiments we have observed that replacing the 'dach' carrier ligand with sterically hindered ones, has resulted into the time extension when we observed the product signals for the first time. Moreover, the rise in product signals observed over the days seems also slower in case of analogs as compared to oxaliplatin. In the case of cisplatin analogs, it was hypothesized that bulky amine ligands can show different levels of steric clashes for guanine and methionine[42]. In our research project, replacement of less sterically ligand with more sterically hindered one has shown the same effects that can also be attributed to the steric clashes between the ligand atoms and nucleobase elements.

In the case of oxaliplatin, DNA exhibits a non-polar region due to the presence of cyclohexane ring. Therefore, oxaliplatin-DNA adducts are processed in the different way by a cellular mechanism. Additionally, in contrast to diammineplatinum(II) complexes, oxaliplatin has lipophilic properties that helps the drug to diffuse through the plasma membrane more efficiently. This hypothesis can be useful to perform an attempt to develop better cytotoxic oxaliplatin derivatives by increasing the lipophilicity of the amine ligand[46].

Previously, in the synthesis process of new oxaliplatin derivatives, it was observed that when smaller substituents at 4^{th} position of dach ligand were replaced by bigger

substituents, cytotoxic properties were reduced due to steric hindrance. The structural-activity relationship was deduced by carrying the experiments in ovarian, colon, melanoma and leukemia cells. It was observed that only in the case of leukemia cells, introduction of bigger substituents did not affect the cytotoxicity. But, in all other cases cytotoxicity of oxaliplatin derivatives was inversely proportional to the size of substituent in the dach ligand[46].

Dependency of cytotoxicity on 4th position was further explained stereo-chemically, by comparing the oxaliplatin analogues on the basis of their equatorial or axial substitution. It is quite reasonable to predict that the tendency of oxaliplatin to inhibit the replication is directly or indirectly linked to the steric demand of cyclohexane ring. It was found that in ovarian cancer cells (CH1) and colon cancer cells (SW480) while observing the cytotoxic potency of oxaliplatin analogues substituted with 4-methyl and 4-ethyl , equatorial position of substituent was considered more preferable over axial position[47].

It was observed that platinum drugs with trans positioned amino groups exhibit better cytotoxic profile cis isomer. So, mono and dialkyl substituted trans-cyclohexane-1,2 diamine derivatives were studied and 4-methyl-, cis-4,5-dimethyl- and 4,4-dimethyl substituted derivatives were found to enhance the cytotoxic levels of oxaliplatin[48].

In our experiments, we have synthesized three derivatives of oxaliplatin by substituting dach ligand with (Me_2dach), (en) and (Me_4en) to study the effect of size of substituent on the cytotoxic properties of oxaliplatin drug by comparing the kinetics of the reactions. We have found that (Me_2dach) and (Me_4en) have shown slower reactivity than (Dach) and (en) ligands respectively. Moreover, when we compared the spectra of me_2dach (nucleobase: me_2dach = 2:1) obtained from the same day (fig. 11) with the spectra obtained for en

(nucleobase: en = 2: 1) on the same day (fig. 11). It is clearly observable that (en) is reacting faster than (Me_2dach).

In the comparison of ethylene ligands, we have deduced that for (Me_4en) ligand, in reactions with GMP at pH 7, product signals were not visible till sixth day after the setting of reaction (fig.12), but for en ligand product signals appeared on very first day of reaction (fig.11). It lucidly displays that bulkier ligand slows down the reaction.

However, reactions of nucleobases with dach and (Me_2dach) seemed comparable. The reaction of AMP with (dach) has provided sharp signal on the same day of reaction setting (fig. 8), whereas while reacting with (Me_2dach), AMP has generated product signals on the second day of reaction setting (fig. 9). So, it is apparent that (Me_2dach) reacts slower than dach but still it does not affect the reaction rate to greater extent.

Affinity for GMP is preferred over AMP

Formation of adducts is mainly attributed to the affinity of platinum drug for DNA nucleobases. Previous research in the Williams lab indicated that reactions of $[Pt(Me_5dien)(D_2O)]^{2+}$ were much faster with GMP as compared to N-AcMet.[44] Further studies were performed to probe the rates of reactions of $[Pt(en)(D_2O)_2]^{2+}$ and $[Pt(Me_4en)(D_2O)_2]^{2+}$ with GMP, guanosine, N-AcHis and N-AcMet. It was indicated that $[Pt(en)(D_2O)_2]^{2+}$ reacts usually faster than $[Pt(Me_4en)(D_2O)_2]^{2+}$, due to the possession of less bulkier ligand. It was also probed that reactions of both complexes with GMP were faster than N-AcMet, but $[Pt(en)(D_2O)_2]^{2+}$ had shown more affinity to GMP because former establishes hydrogen bonding with phosphate group of GMP. It was also suggested

that proteins that coordinate via His residues are not hindered for their reactivity by bulky ligands.[42]

Our series of experiments showing the reactivity patterns of platinum compounds with nucleobases have suggested that affinity for GMP is preferred over AMP. After monitoring the reactions of oxaliplatin with both nucleobases (fig. 8 and fig. 9), we noticed that in the case of GMP signals corresponding to products were arising much faster as for AMP.

When we observed the reactions with Pt(Me_2dach)(ox), where ratio of nucleobase:Pt(Me_2dach)(ox) was 2:1, still higher affinity for GMP was probed (fig 12). Similarly, for Pt(en)(ox) we noticed that at 2:1 ratio, reactions in the case of GMP were faster. So, we deduced that binding with GMP is preferable at all concentrations. This further suggests that first available target for the drug is guanosine site of nuclear DNA and AMP is not preferred for binding in the availability of GMP. It also suggests that barriers between binding of platinum drug and GMP are comparatively less as in the case of AMP. As, reaction with AMP requires the breaking of chelate.

pH and concentration variations does not affect the product distribution

We have monitored the reactions of oxaliplatin and its analogs with DNA nucleobases at different pH and concentration levels and noticed that variations in pH and molar ratios does not affect the product distribution.

Observation of similar sets of products also suggests the similar mechanism of reactions at variable molar ratios and pH levels. So, we were able to estimate that mechanism of reactions depends upon the structure of the reactants and not on the concentration or reacting environment.

Monitoring of reactions of oxaliplatin and its analogs with 5'-AMP and 5'-GMP in various combinations and at different pH exhibited the same set of products. Those products are most likely the ones with 2 nucleotides coordinated to the same platinum center. Thus, reaction of the second nucleotide is faster than the reaction of the first because the first reaction requires breaking of chelate. It indicates that pH and concentration variations can affect the reaction kinetics but it barely affect the product distribution.

CHAPTER 5

CONCLUSION

The designing and synthesis of platinum based anti-cancer drugs is an exclusive approach to more effective therapy development; however, there is vast room for improvement to account the optimization of tumor selectivity and target delivery of the drug. In the 48 years, since the discovery of first drug cisplatin by Rosenberg, only few drugs have been approved whereas several have been disapproved during clinical trials. The main reason of disapproval is severe side effects associated with them. We have performed the reactions of third generation platinum based anti-cancer drug oxaliplatin and its analogs with DNA nucleobases 5'-AMP and 5'-GMP to probe the pharmokinetics of parent drug and its derivatives synthesized by substituting its 'dach' ligand with another ligands. We monitored the reactions by collecting partial ^{1}H NMR spectra over the duration of several days after varying pH and molar ratios.

We have paid particular emphasis over the variations in ligands, pH environment and molar ratios and have found that bulkier ligands have a higher tendency to impose steric clashes and thus hinder the paced binding of drug with nucleobase. It has been observed that pH generally affects the rate but not the product distribution. It has also been noticed that the pH effect is more noticeable in the case of lighter ligands but shows little effect for bulkier ligand complexes.

Our experimental findings favor the ideas from previous studies that binding with GMP is preferred over AMP in the case of oxaliplatin and its analogs because of the chelate formed by oxalate bond with GMP. Our results indicate that extra methyl groups on

Pt(Me_2dach)(ox) and Pt(Me_4en)(ox) do not appear to hinder reaction with nucleotides considerably.

It is also significant to mention that reactions carried at different molar ratios elucidate the same product patterns, indicating that nature of products does not depend upon the concentration of either nucleobase or platinum complex.

In regard to practical applications, future direction of the project is to test the various oxaliplatin derivatives in vivo conditions. Like parent oxaliplatin, they can be employed in combination of other drugs to possibly enhance their efficacy.

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Abbreviations

NMR- Nuclear Magnetic Resonance

DNA- Deoxyribonucleic acid

5'- AMP- 5'- Adenosine Monophosphate

5'- GMP- 5'- Guanosine Monophosphate

D_2O- Deuterium Oxide

Pt(dach)(ox)- Diaminocyclohexaneoxalatoplatinum(II)

Pt(Me_2dach)(ox)- N,N-dimethyl-1,2- diaminocyclohexaneoxalatoplatinum(II)

Pt(en)(ox)- Ethylenediamineoxalatoplatinum(II)

Pt(Me_4en)(ox)- N,N,N',N'-tetramethylethylenediamineoxalatoplatinum(II)

mM- millimolar

mL- milliliter

mg- milligram

ppm- parts per million