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Inhibition of Cysteine Protease by Platinum (II) Diamine Complexes

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INHIBITION OF CYSTEINE PROTEASE BY PLATINUM (II) DIAMINE COMPLEXES

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Chaitanya Rapolu

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INHIBITION OF CYSTEINE PROTEASE BY PLATINUM (II) DIAMINE COMPLEXES

Date Recommended 11|22|2011

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30-JAN-2012

Dean, Graduate Studies and Research Date

I dedicate this thesis to my parents, without whom I would not be standing in this position where I am now and they have been great inspiration to me. Also**,** I would dedicate my thesis to my work to Dr. Kevin Williams, who has guided me throughout my research and helped greatly in acquiring the data.

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I would like to thank all of my friends for encouraging me every time I was down. Special thanks to my best friend Mr. Ananth, without whom I would not have travelled all the way from India and pursued my Masters degree, for building up self confidence in me, boosting up the courage in me and for being there with me every time I needed him. I would thank my research committee and my friend Ravipati Dhatri, who has helped me a lot in completion of my research successfully.

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Directed By: Dr. Kevin Williams, Dr. Stuart Burris and Dr. Bangbo Yan

Department of Chemistry Western Kentucky University

Chemotherapy is the first line of treatment used in cancer. Chemotherapy drugs such as cisplatin, carboplatin and oxaliplatin are used in treatment. Cisplatin enters the cell through copper transporter CTR1 by passive diffusion and bind to DNA and proteins. Cisplatin is found to inhibit several enzymes targeting cysteine, histidine and methionine residues, which are expected to be responsible for its anticancer activity. A better understanding of how the size and shape and leaving ligands of platinum complexes affect cysteine protease, papain enzyme are studied. This could give new ways to optimize anticancer activity. The activity of papain enzyme was measured on UV-Visible spectroscopy. The inhibition profile of papain with different platinum (II) complexes, and with different combinations was studied.

I. INTRODUCTION

A. HISTORY

Cancer is a large, heterogeneous class of diseases caused by uncontrolled growth of cells that destroy adjacent tissues, spreading throughout the body. Cancer is caused when cells are irresponsive to normal tissue controls due to genetic damage. These cells rapidly multiply and spread to form tumors.

Cancers are of two types- malignant tumors and benign tumors. Malignant tumors are differentiated from benign tumors as they show uncontrollable growth, invade locally, and metastasize to distant body parts. Benign tumors grow in one place and lack the ability to metastasize. Cancer is the second most common cause of death in the US, exceeded only by heart disease. In the US, cancer accounts for nearly 1 of every 4 deaths (American Chemical Society, Cancer Facts and figures, 2009). About-one half of all men and one-third of all women in US will develop cancer during their lifetimes.

Cancer is caused by both internal factors (such as hormones, inherited mutations, immune conditions) and acquired/environmental factors (such as radiation, diet, tobacco, infectious organisms).Various therapies can be used to treat cancer. They include surgery, biological therapy, hormone therapy, radiation, targeted therapy and chemotherapy. Cancer can also be treated by nanoparticles.[1] Chemotherapy is done by treating with different chemicals, such as platinum complexes. Another type of chemotherapy is the introduction of selenium.

Several studies have shown that selenium has strong anticancer activity on malignant tumors and has marginal effects on benign and normal cells.[2] Selenium has an antioxidant property that is responsible for cancer prevention. Selenium targets transcription factors

and enzymes. Selenium increases activity of p53 resulting in DNA repair induction.[3] In a double placebo study conducted by Clark and coworkers in 1996 on yeast cells, the researchers concluded that low doses of selenium concentration led to cancer prevention and moderate to high doses of selenium concentration led to cancer treatment.[4]

Paul Ehrich in 1909 published the first book on 'chemotherapy'.[5] In the beginning of 20th century the era of chemotherapy began. Soon after the first World War the potential therapeutic application of a chemical warfare agent, nitrogen mustard gas, was investigated by Louis Goodman and Alfred Gilman.[6] Their findings launched the use of chemical molecules to destroy cancer cells called chemotherapy. The first line of treatment is chemotherapy, though it has side effects.

Over the past 30 years, cancer has been treated by platinum-based drugs with metal complexes, notably cisplatin, oxaliplatin and carboplatin.[7] These are considered as potential anticancer agents. Structural activity relationships for platinum coordination compounds class has confirmed that compounds having cis-geometry only can block cell growth.[8] Cisplatin is a platinum based chemotherapy drug[15] used in treatment of various types of cancers including sarcomas and carcinomas.[12, 16] Cisplatin was approved for use in testicular and ovarian cancer by the U.S FDA in 1978. Cisplatin, the most active complex, was found to have antitumor activity whereas trans geometry compounds has not shown such activity.[9, 10]

In 1845, M.Peyrone first described the compound cisplatin; it was known as Peyrone salt and the structure was deduced in 1906.[11] Barnett Rosenberg, a professor of Michigan State University in 1965, discovered the antitumor activity of cisplatin while conducting an experiment on bacterial cell growth inhibition.[12]

The second generation platinum drugs include carboplatin.[13] Carboplatin has fewer side effects than cisplatin but is less active than cisplatin and thus carboplatin requires higher dose to be administered. Carboplatin is used to treat ovarian cancer. Third generation platinum compounds include oxaliplatin, which is more effective in treating colorectal cancer.[14] It has been approved in China and Europe for its clinical use. Cisplatin, carboplatin and oxaliplatin are administered intravenously rather than orally due to its solubility problems. Platinum based drugs are used as anti cancer agents with a broad range of anti-tumor activities.

B. CHEMISTRY OF CISPLATIN:

Most cisplatin derivatives inhibit the growth of cancer cells, having at least one N-H group that is responsible for hydrogen bond donor properties.[12]

The general formula of platinum anticancer complexes is[12]:

$$\text{Cis-}\ [PtX_2(NHR_2)_2]$$

R = organic fragment

X= leaving group, such as chloride or (chelating bis) carboxylate

Platinum has slow ligand-exchange kinetics.[7] Pt coordination compounds form Pt-ligand bonds having thermodynamic strengths as those of typical coordination bonds, but weaker than C-N, C-O, C-C double and single bonds.[7] The ligand-exchange of Pt compounds are slow, which leads to high kinetic stability with ligand exchange reactions in a minute to days rather than microseconds to seconds.[7]

C. COPPER TRANSPORTER:

Cisplatin, carboplatin and oxaliplatin are administered by i.v. due to their insoluble nature in aqueous systems. Previous studies indicate cisplatin uptake proceeds linearly with time for 60 minutes. Cisplatin enters the cell by passive diffusion due to differences in chloride concentration inside and outside the cell.[12] Recently it was found that platinum concentration inside the cell is directly linked to cellular management of copper.

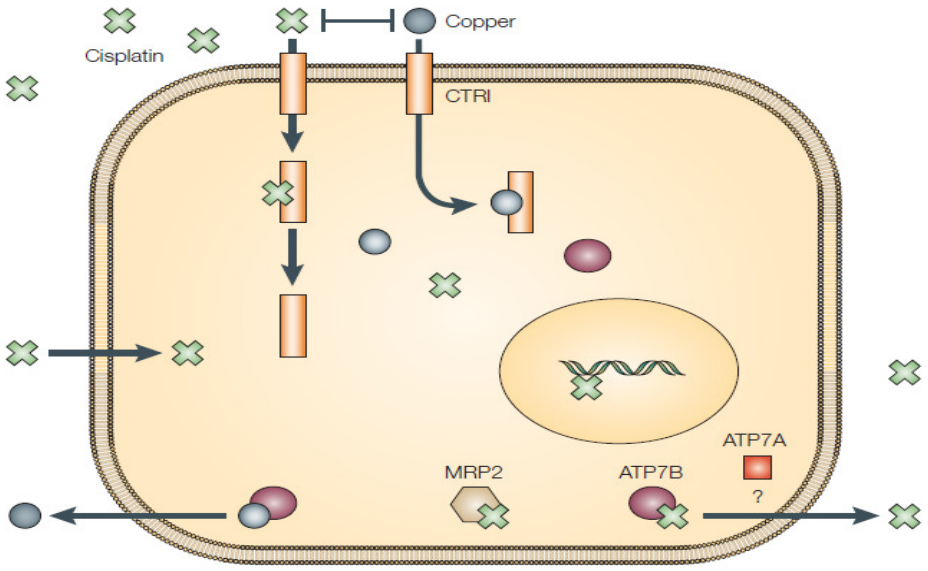

Figure 3.1 Mechanism of cisplatin uptake and efflux.[21]

In an experiment conducted with yeast protein, CTR1, a high affinity copper transporter, and cisplatin, it was found that deletion or mutation of CTR1 gene resulted in decreased levels of platinum levels and increased resistance to cisplatin.[20] Overexpression of human CTR1 caused accumulation of cisplatin in both yeast and mouse cells.

Cisplatin and copper might interfere with each other's mutual transport and trigger delocalization and degradation of CTR1.[21] This protein helps in active influx of platinum complexes. It has also been found that efflux proteins are also present for

excretion of platinum complexes. The exporter proteins that are involved in the efflux of platinum complexes are ATP-binding cassette transporter family C2 (ABCC2, also known as Mrp2/AB or ATP7B). Mrp2 is a multi drug resistant protein.[22]

Cisplatin enters the cell by passive diffusion. The chloride group of cisplatin is replaced by a water molecule due to which it gets activated.[12] This activated cisplatin enters the nucleus and reacts with DNA, though reaction with proteins is also possible.

D. MECHANISM OF CISPLATIN WITH DNA:

Platinum complexes enter the cell through diffusion and form $[Pt\ (NH)_3(OH_2)_2]^{2+}$ and $[Pt\ (NH)_3Cl\ (OH)_2]^{+}$ as they undergo aquation due to low cellular concentration of chloride ions. These aquated forms are more reactive to cellular targets. These cellular targets include DNA or amino acids present in the cell.[24] Thus the primary biological target for a platinum complex is DNA. The platinum atom of cisplatin interacts covalently with the guanine N7 position of DNA and forms 1, 3-intrastand and 1, 2-intrastrand crosslinks. These intrastrand crosslinks lead to cellular processes such as replication arrest, transcription inhibition, cell-cycle arrest, DNA repair or cell death.

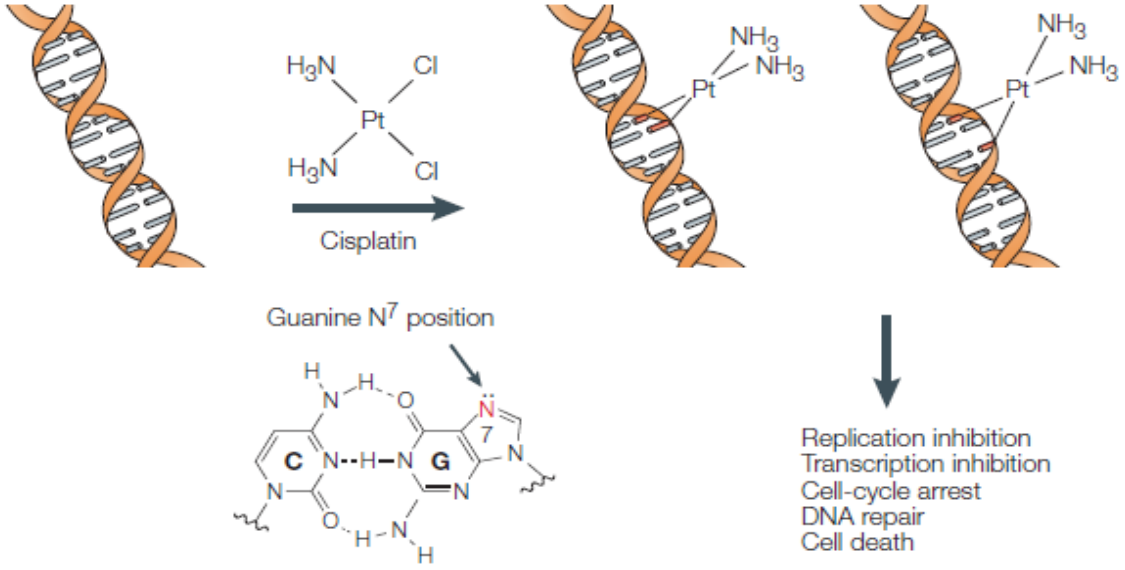

Figure 3.2 Schematic representation of formation and effects of cisplatin adducts.[15]

In the case of carboplatin and oxaliplatin, these interactions with DNA occur at the positions where cyclobutane-1, 1-dicarboxylate (CBDCA) and the oxalate ligands are attached to platinum respectively.[25] Pt-DNA adducts along with the proteins that bind to them promote cytotoxicity.

Binding to DNA leads to distortion of the double helical structure of DNA, thus impairing its processing. The 1, 2-intra strand cross links bend DNA significantly towards the major groove, exposing a wide shallow minor groove surface, to which several proteins bind.

These proteins include high-mobility group (HMG) proteins, repair proteins, transcription factors and Histone H1 proteins.[24] Binding of these proteins will lead to replication inhibition, transcription inhibition, cell cycle arrest and cell death.

E. MECHANISM OF CISPLATIN WITH PROTEINS:

Disturbance in the natural equilibrium of enzymatic activity of cysteine proteases results in protein degradation, which leads to pathological conditions such as cancer, neurological disorders and rheumatoid arthritis.[26] The normal balance of these enzymes can be restored through regulation by protein inhibitors, leading to a drug development opportunity.

Cisplatin is a potential electrophile and is known to react with any nucleophiles, including nucleophilic groups on nucleic acids and sulfhydryl groups on proteins. Reedjik and co-workers suggested that cisplatin reaction with proteins leads to inactivation of a few essential enzymes or proteins contributing to the biological activity of cisplatin.[27]

Platinum complexes are known to react with many cell components. Chemical preference for 'soft' metal platinum for 'soft' ligands, such as sulfur-donating ligands leads to drug binding to S-containing biomolecules and sulfur donating ligands that are present in high concentration inside the cell.[28]

Protein binding is known to contribute to platinum anticancer drug toxicity[37], however it was suggested that platinum reaction with biological thiols is a pathway for resistance and detoxification.[12] It was found that binding of platinum complexes to methionine residues could result in C-terminal cleavage at the residue, resulting in regioselective chemical cleavage of proteins under nonbiological conditions.[36] Hence Pt complexes interaction with protein may be is important.

Platinum complexes interact with many biomolecules. Amino acids present metal ions with a variety of potential donor atoms. The sulfur donor residue in amino acids is one of the primary targets owing to its relative softness for platinum.[29] In the absence of

S-donor groups, platinum can also bind to lone pair of nitrogen atoms of the amino acid histidine. Hence platinum complexes mainly target S-containing cysteine, N-containing histidine and S-containing methionine.

Cisplatin also interacts and inhibits proteins like thioredoxin reductase that contain a selenocysteine residue that is essential for the activity of the enzyme. This inhibition of the enzyme is irreversibile.[17] Thioredoxin reductase (TRXR) is a selenoprotein, mostly studied in cancer, present in mytochondria and cytoplasm.[18] TRXR plays a major role in neoplastic growth. It was reported that TRXR is induced in tumor and pre-neoplastic cells. High levels of TRXR were found in brain tumor and hepatocellular carcinoma cells. The propensity of cancer cells to over express TRXR selenoprotein makes it an apparent target in research directed at improved diagnostic and effectual drug targets.[2] The importance of TRXR expression for tumor cell development is exemplified by inhibition experiments. Inhibiting TRXR could possibly prevent cancer. Structure of human TRXR[2]:

Figure 3.1 Three dimensional structure of human TRXR.[35]

Active site of TRXR is the selenium (Sec) in the C-terminal redox centre -Gly-Cys-Sec-Gly-COOH residue.[2]TRXR is known to be inhibited via reaction with redox active residues by electrophilic compounds that have been proposed as a target for anticancer therapy. These include drugs such as platinum containing compounds, nitrosoureas and arsenicals.[19]

In a theoretical study done on reacting platinum derivatives with cysteine and methionine residues it was found that platinum formed stronger Pt-S dative bond with cysteine residue than that of methionine residue.[30] Cysteine formed irreversible platinum adducts. Hence it was concluded by T. Zimmermann that platinum targets cysteine and cysteine plays a major role in platinum metabolism.[30]

E. PAPAIN

Papain is also known as papaya protease I or cysteine protease enzyme.[31] It is obtained from *Carica papaya* and it is rich in four cysteine endopeptidases including papain, chymopapain, glycyl endopeptidase and carcain. Papain has broad specificity.[31] It has endopeptidase, amidase and esterase activities. The three dimensional structure of papain was first identified in1980 by Drenth.

Structure:

Papain is a single-chained polypeptide with three disulfide bridges. The sulfhyrdryl group is necessary for the activity of the enzyme. Papain has 345 amino acid residues.[31] Its three dimensional structure consists of two distinct structural domains with a cleft between them. This cleft contains the active site, which contains a catalytic diad that has been linked to the catalytic triad of chymotrypsin. The catalytic diad is made up of the amino acids - cysteine-25 (from which it gets its classification) and histidine-159. The mechanism by which it breaks peptide bonds involves deprotonation of Cys-25 by His-159.

The active site of the enzyme include cysteine (C) at 25th position and histidine (H) at 159th position. These active site residues provide unique functions to the enzyme. Remaining sulfides of the enzyme are found on three distinct disulfide bridges. Cysteine

is an α-amino acid. The side chain on cysteine is thiol, which is non-polar. This thiol side chain participates in enzymatic reactions, serving as a nucleophile. The thiol group of cysteine 25 in papain possesses nucleophilic reactivity in its un-ionized form. This results from its interaction with the histidine 159-asparagine system.[32]

Papain Structure:

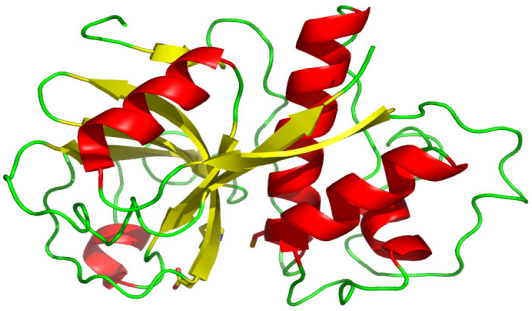

Figure 3.4 Three dimensional structure of papain

Sequence:

IPEYVDWRQKGAVTPVKNQGS*C*GSCWAFSAVVTIEGIIKIRTGNLNEYSEQELL
DCDRRSYGCNGGYPWSALQLVAQYGIHYRNTYPYEGVQRYCRSREKGPYAAKT
DGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGIFVGPCGNKV*DH*
AVAAVGYGPNYILIKNSWGTGWGENGYIRIKRGTGNSYGVCGLYTSSFYPVK

Fig 3.5 Sequence papain structure indicating its active site residues.

The present research studies the effect of size and shape of amine ligand, and leaving groups on platinum complexes with cysteine containing proteins. The investigation on papain enzymes will help us to discern trends in inhibition as the platinum complexes vary. Platinum complexes interact with papain enzyme by targeting cysteine active site.

II. EXPERIMENTS

A. MATERIALS USED:

Dimethyl sulfoxide (DMSO), Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride (Substrate), L-Cysteine, cis-diamine dichloroplatinum (II)-99%, potassium tetrachloroplatinate (K_2PtCl_4), papain enzyme were purchased from Sigma Aldrich.

Ethylenediamine platinum (II) dinitrate- $Pt(en)(NO_3)_2$, N, N, N', N'-tetrametylethylenediamine platinum (II)-dinitrate- $Pt(Me_4en)(NO_3)_2$, ethylenediamine platinum(II) oxalate- Pt(en)(ox), N, N, N', N'- tetrametylethylenediamine platinum (II)-oxalate- $Pt(Me_4en)(ox)$ were synthesized previously in our lab.

Distilled water is used for dialysis and buffer preparation.

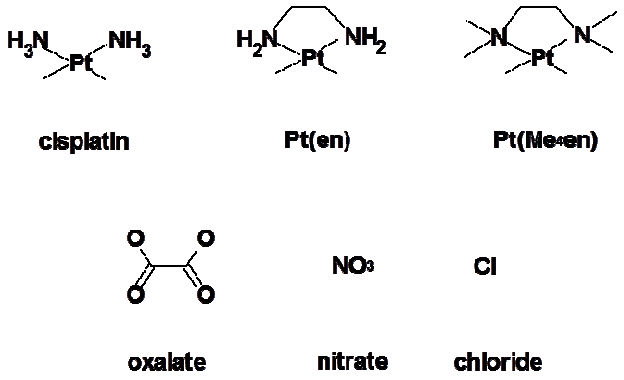

Figure 3.6 Structure of platinum complexes and leaving ligands.

B. UV-VISIBLE SPECTROMETER:

The uv-visible spectrophotometer model we used is uv-1201, and uv-1601 manufactured from SHIMADZU in year 2000. Parameters we used on the uv-visible spectrometer are wavelength: 410 nm, measuring time: 120 seconds (2 minutes) or 900 seconds (15 minutes).

C. PREPARATION OF BUFFER SOLUTIONS:

The buffer solutions we used are storage buffer (sodium acetate buffer) and phosphate buffer. 50m M sodium acetate buffer was prepared by mixing 18 ml of 0.2 M acetic acid to 32 ml of 0.2 M sodium acetate and diluted to 200 ml with distilled water. The pH was 5.

Phosphate buffer was prepared mixing dibasic potassium phosphate to phosphoric acid and made up to 1000ml with distilled water. Then the pH was adjusted to 7.6 with phosphoric acid.

D. METHODS:

For each platinum (II) complex, three runs of each combination were done.

a. Without cysteine, without dialysis-

20 mg of substrate (Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride) was weighed out and made to solution with 230 microliters of DMSO.

For the stock enzyme:

10 mg of papain enzyme was weighed. To this we added 1.0 ml of storage buffer and gently tilted the vial to get everything in solution. The solution set for 25-30 minutes.

For control papain:

Prepared 400 microliters of stock papain and added 400 microliters of storage buffer.

For inhibited papain:

Prepared 400 microliters of stock papain and to this added 400 microliters of platinum compound that is in storage buffer.

b. With cysteine, with dialysis:

In the commercial papain, cysteine is mostly inactive. Addition of L-cysteine activates inactive papain. L-cysteine breaks the disulphide bridge s of the papain enzyme and activates it. This activated enzyme readily reacts with platinum derivatives.

The unreactive cysteine was removed by membrane dialysis. Membrane dialysis is done in membrane tubing. The papain cysteine solution is transferred into membrane tubing and suspended in distilled water for 20 minutes. The buffer present in the solution and unreactive cysteine penetrated through this membrane tube. After dialysis storage buffer is added to the papain cysteine solution.

Stock sample, control papain and inhibited papain are prepared as discussed by above method, except that in stock sample 10 mg of papain is weighed, 1 mg of cysteine is added to it and made to solution by dissolving it in storage buffer.

E. ASSAY:

Assays will allow us to assess both the rate and the extent of inhibition as a function of bulk and leaving ligand.

The assay of prepared control and inhibited papain samples are done on a uv-visible spectrometer.

For control assay: In a clean vial or microfuge tube, 900 microliters of assay buffer, 5 microliters of Substrate, and 100 microliters of control papain sample were combined. The sample was mixed briefly and gently, and most of the sample was transferred to a clean cuvvette. The ΔA/min was recorded for a period of one hour at 410 nm wavelength.

For inhibition assay: In a clean vial or microfuge tube, 900 microliters of assay buffer, 5 microliters of Substrate, and 100 microliters of inhibited papain sample. The sample was mixed briefly and gently, and most of the sample was transferred to a clean cuvvette. The ΔA/min was recorded for a period of one hour at 410 nm wavelength.

The assay of the samples was carried out for every 15 minutes for about one hour, and the activity of the control and inhibited sample was measured. The control assay is compared with that of the inhibited sample. Each run of platinum complexes were done for three times for precision and reproducibility. The activity of the control sample and inhibited sample at a particular time was done simultaneously on a two slot spectrometer. The ΔA/min was recorded for both the control and the inhibited sample. The assay was done for each platinum complex for two times: three runs for without addition of cysteine to the enzyme and without dialysis, the other three runs for with addition of cysteine to the enzyme and with dialysis.

III. RESULTS

Platinum complexes with varying coordination sites and leaving ligand bulk have been studies for their interaction with enzyme papain.

A. OBSERVATION WITH UV- VISIBLE SPECTROSCOPY:

UV spectroscopy was carried out at fixed intervals by reacting platinum complexes with papain. The UV- parameters used were mentioned above. For without cysteine, without dialysis- ΔA/min value for the control sample was more when compared to the ΔA/min value for the inhibited sample for all the platinum complexes.

For with cysteine, with dialysis- ΔA/min value for the control sample was less for cisplatin, $K_2Pt(Cl)_4$, $Pt(en)(NO_3)_2$, Pt(en)(ox) when compared to the ΔA/min value for the inhibited sample.

B. OBSERVATION WITHOUT CYSTEINE, WITHOUT DIALYSIS:

Commercial papain is inactive.[31] When papain is reacted with platinum complexes, it was found that there was no significant inhibition of the enzyme.

a.Cisplatin- no cysteine, no dialysis:

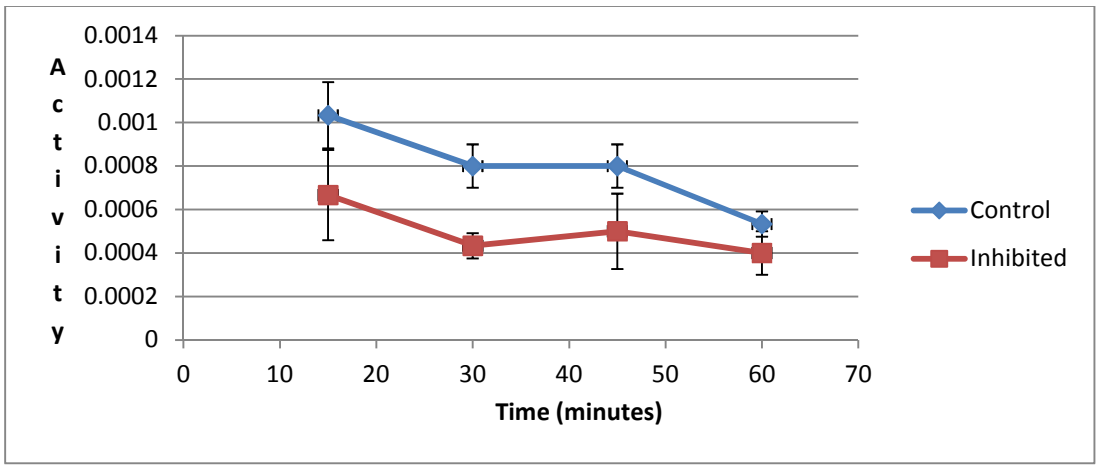

Figure 3.7 Enzymatic Assay of commercial papain with cisplatin.

The assay is done for every 15 minutes for about one hour. The graph is plotted for the activity versus time. Enzymatic activity is defined as ΔA/minute. The control line indicates the control papain sample and the inhibited line indicates the activity of inhibited cisplatin sample over a period of time. The black line present on the control and inhibited samples indicates the error bars for both the assays. Error bars represent the standard deviation of each control and inhibited papain.

In the above graph it is seen that the control line is above the inhibited line. The error bars of the control and inhibited samples overlap at 15 and 60 minutes. The error bars at 30, 45 minutes are not overlapped, indicating that cisplatin has significant inhibition at these times.

a.k_2PtCl_4- no cysteine, no dialysis:

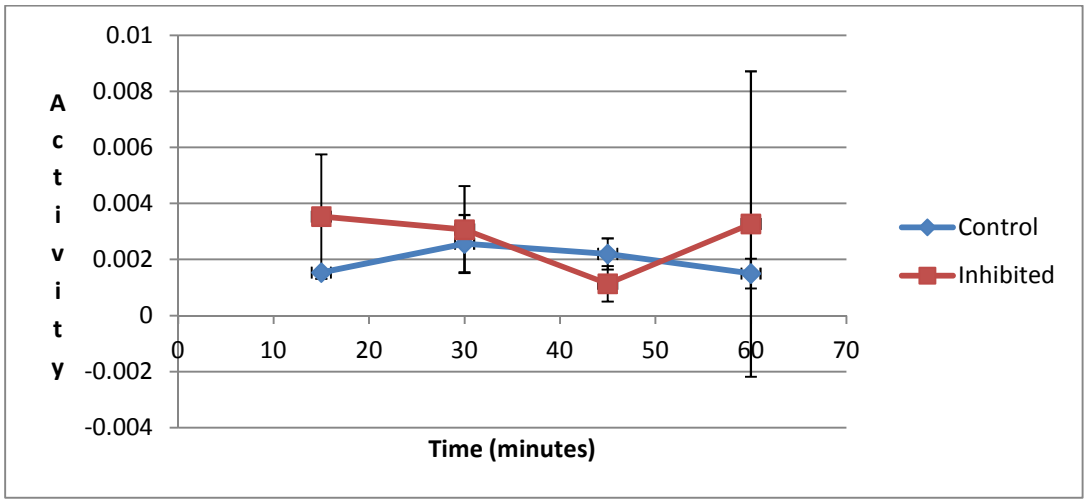

Figure 3.8 Enzymatic assay of commercial papain with K_2PtCl_4

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited K_2PtCl_4 sample.

Here the activity of the control is below the inhibited papain at 15, 30 and 60 minutes interval. At 45 minutes the inhibited line is below the control.

The error bars of the control and inhibited papain overlapped at every interval of time. Indicating that K_2PtCl_4 has no significant inhibition.

a.$Pt(en)(NO_3)_2$- no cysteine, no dialysis:

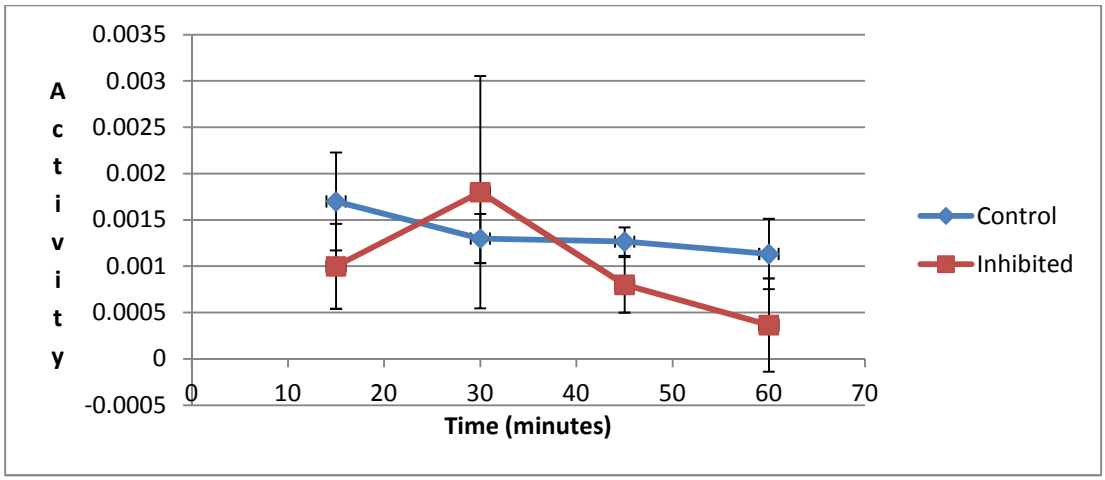

Figure 3.9 Enzymatic assay of commercial papain with $Pt(en)(NO_3)_2$.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample $Pt(en)(NO_3)_2$.

Here the activity of the control line is above the inhibited papain at 15, 45 and 60 minutes interval. At 30 minutes the control line is below the inhibited.

The error bars of the control and inhibited papain overlapped at every interval of time**,** which indicates this platinum complex does not show significant inhibition.

a.$Pt(Me_4en)(NO_3)_2$ -no cysteine, no dialysis:

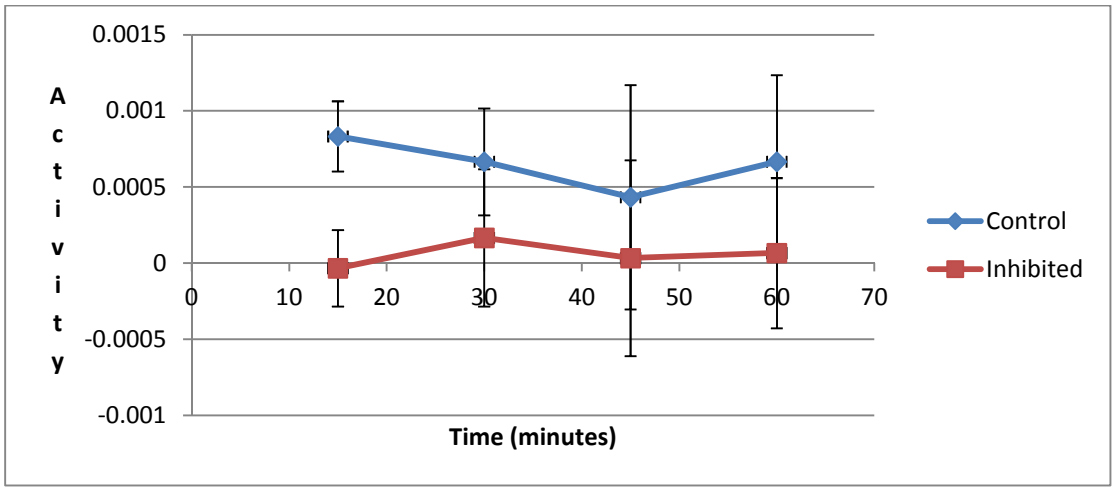

Figure 3.10 Enzymatic assay of commercial papain with $Pt(Me_4en)(NO_3)_2$.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample $Pt(Me_4en)(NO_3)_2$.

Here the activity of the control line is below the inhibited line at 15, 30, 45 and 60 minutes interval.

The error bars of the control and inhibited papain overlapped at 30, 45 and 60 minutes but at 15 minutes the error bars did not overlap. Thus, this platinum complex does not show significant inhibition.

a.Pt(en)(ox)-no cysteine, no dialysis:

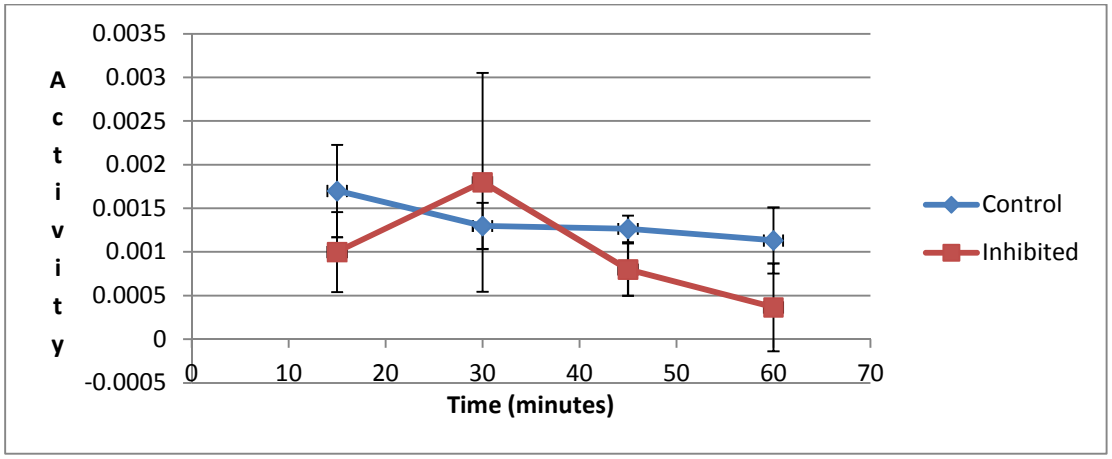

Figure 3.11 Enzymatic assay of commercial papain with Pt(en)(ox).

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample Pt(en)(ox).

Here the activity of the control line is on top of the inhibited line at 15, 45 and 60 minutes interval. At 30 minutes the control line is below the inhibited sample.

The error bars of the control and inhibited papain overlapped at 15, 30, 45 and 60 minutes. Thus, this platinum complex does not show significant inhibition.

C. OBSERVATION WITH CYSTEINE AND WITH DIALYSIS

a.Cisplatin- with cysteine, with dialysis:

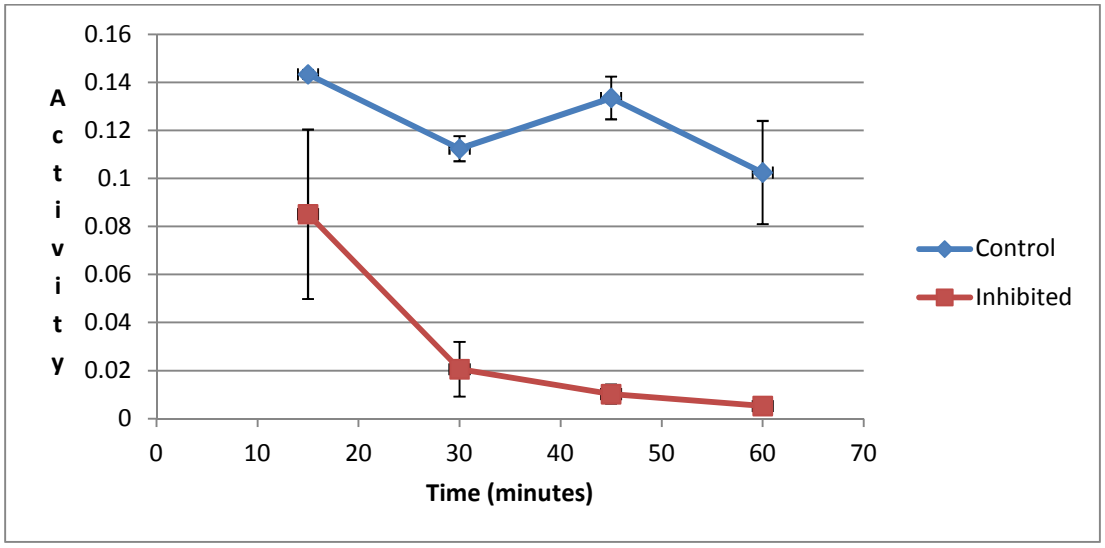

Figure 3.12 Enzymatic assay of activated papain with cisplatin.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample cisplatin.

Here the activity of the control line is on top of the inhibited line at 15, 30, 45 and 60 minutes interval. The error bars of the control and inhibited papain are not overlapped at 15, 30, 45 and 60 minutes. It indicates that cisplatin shows inhibition of papain enzyme.

a.K_2PtCl_4-with cysteine, with dialysis:

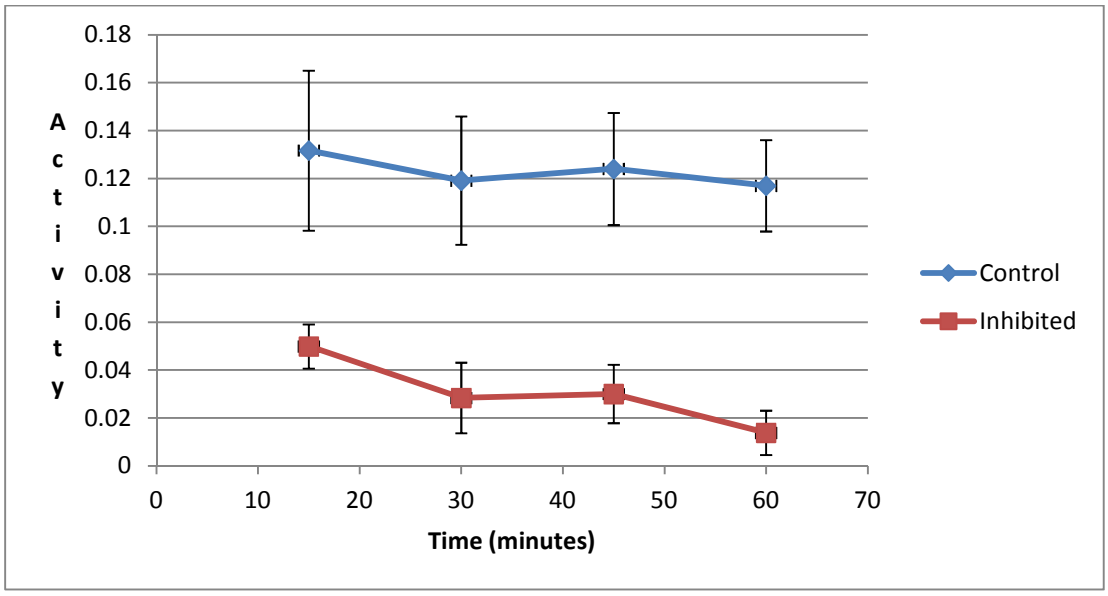

Figure 3.13 Enzymatic assay of activated papain with K_2PtCl_4.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample K_2PtCl_4.

Here the activity of the control line is on top of the inhibited line at 15, 30, 45 and 60 minutes interval. The error bars of the control and inhibited papain are not overlapped at 15, 30, 45 and 60 minutes. It indicates that K_2PtCl_4 shows inhibition on papain enzyme.

a.$Pt(en)(NO_3)_2$- with cysteine, with dialysis:

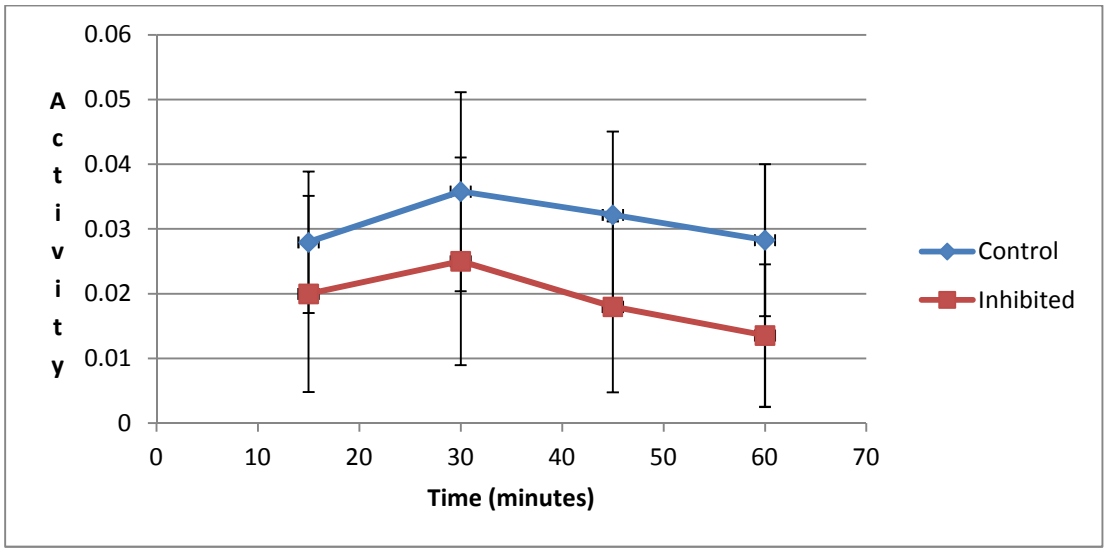

Figure 3.14 Enzymatic assay of activated papain with $Pt(en)(NO_3)_2$.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample $Pt(en)(NO_3)_2$.

Here the activity of the control line is on top of the inhibited line at 15, 30, 45 and 60 minutes interval. The error bars of the control and inhibited papain are overlapped at 15, 30, 45 and 60 minutes. It indicates that $Pt(en)(NO_3)_2$ could not show significant inhibition of the papain enzyme.

a.$Pt(Me_4en)(NO_3)_2$- with cysteine, with dialysis:

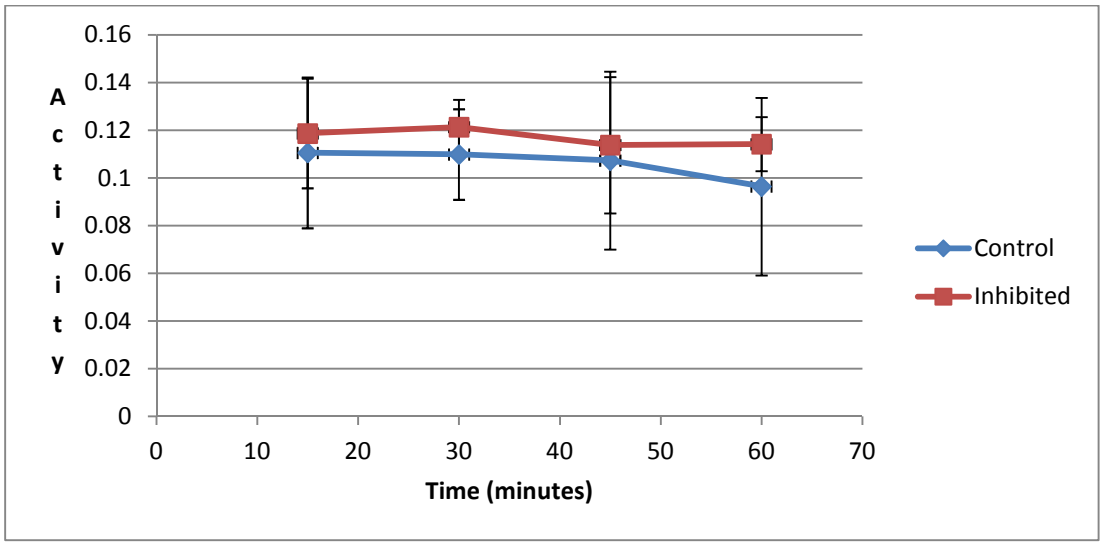

Figure 3.15 Enzymatic assay of activated papain with $Pt(Me_4en)(NO_3)_2$.

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample $Pt(Me_4en)(NO_3)_2$.

Here the activity of the control line is on top of the inhibited line at 15, 30, 45 and 60 minutes interval. The error bars of the control and inhibited papain are overlapped at 15, 30, 45 and 60 minutes. It indicates that could not show $Pt(Me_4en)(NO_3)_2$ significant inhibition of the papain enzyme.

a.Pt(en)(ox) with cysteine, with dialysis:

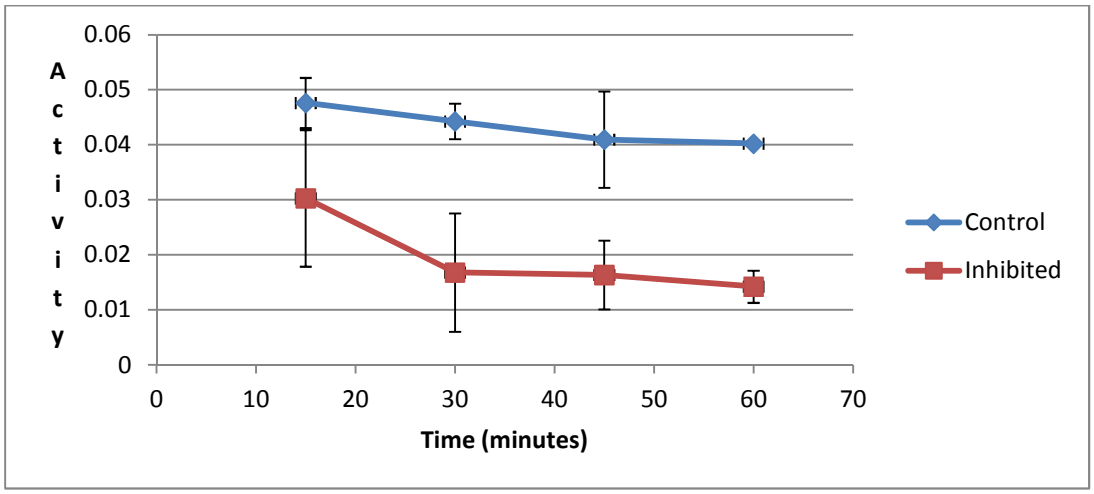

Figure 3.16 Enzymatic assay of activated papain with Pt(en)(ox).

The above figure represents a graph plotted for activity versus time for every 15 minutes for about one hour. The control line represents the activity of control papain sample and the inhibited line indicates the inhibited sample Pt(en)(ox).

Here the activity of the control line is on top of the inhibited line at 15, 30, 45 and 60 minutes interval. The error bars of the control and inhibited papain are overlapped at 15 minutes but did not overlap at 30, 45 and 60 minutes. It indicates that Pt(en)(ox) could show significant inhibition of the papain enzyme.

D. **OBSERVATIONS WITH LEAVING LIGANDS AND BULKY GROUPS:**

Assays were done with different platinum complexes containing different leaving ligands. The leaving ligands include chloro, nitrate, and oxalate groups.

- The leaving ligands play a major role in the inhibition of the papain enzyme.

The platinum complexes containing leaving ligands as chloro groups such as potassium tetrachloroplatinate and cisplatin showed greater inhibition than other platinum complexes.

- The platinum complexes containing oxalates as leaving ligand showed inhibition.

It was found the research that bulkiness of the group shows an effect on the inhibition profile of the papain enzyme.

- The platinum complex $Pt(Me_4en)(NO_3)_2$, having a bulkier group did not show significant inhibition on the enzyme.

IV. DISCUSSION

Platinum based drugs such as cisplatin, carboplatin and oxaliplatin have been used extensively for the treatment of cancer.[25] Drug resistance and nephrotoxicity are the major limitations of these anticancer drugs.

This research was an attempt to better understand of how the size and shape of the amine ligands affect the reactions of the platinum (II) complexes with papain, chymotrypsin and subtilisin enzymes, which could lead to insights about ways to improve efficacy of platinum-based drugs in cancer chemotherapy.

The platinum complexes differ in leaving groups and amount of amine ligand bulk. The activity of samples of each complex was measured by UV-Visible spectroscopy. Inhibition assays of papain by platinum complexes of 1mg/ml concentration were performed for every 15 minutes, for about an hour. The error bar at each point represents the mean of three trials, measuring the SD of three trials. The activity of the papain enzyme when without cysteine, without dialysis by cisplatin, showed significant inhibition as the error bars of control and inhibited were not overlapped throughout one hour.

For other platinum complexes such as $Pt(en)(NO_3)_2$, K_2PtCl_4, Pt(en)(ox) and $Pt(Me_4en)(NO_3)_2$, the errors bars of the control and inhibited samples overlapped throughout one hour. Hence it was concluded that these platinum complexes did not show significant inhibition on papain when papain was inactivated.

Inhibition assays were also performed after activating papain by addition of cysteine and subsequent dialysis. Cisplatin has shown significant inhibition of the enzyme, as the error bars of control and inhibited were far apart. The additional cysteine

breaks the disulfide bonds of papain, due to which cisplatin could target the cysteine residue leading to its inhibition.

Platinum complexes $Pt(en)(NO_3)_2$, K_2PtCl_4, Pt(en)(ox) showed relative inhibition of papain enzyme upon its activation by cysteine as these complexes are of equal size but with different leaving ligands. No noticeable difference in reactivity was observed as a function of leaving ligand.

N, N, N', N'- tetramethyl ethylene diamine Pt (II)-dinitrate, being a bulkier size, could not inhibit papain even upon its activation. Thus, that the platinum complex being bulkier did not allow the enzyme to react with the platinum complex. Previously, it was found that bulk slows reaction with methionine and guanine residues. [34] These results further suggest that reaction with the cysteine in the active site is also showed in separate results from our lab.

The activity of platinum compounds was also seen on subtilisin enzyme and chymotrypsin. Subtilisin and chymotrypsin are serine proteases with histidine residues in their active sites. Subtilisin and chymotrypsin exhibit different inhibition pattern than papain as histidine residue is targeted. Relatively similar methodology was used. The activity of the enzymes was measured with varying concentrations of the platinum complexes. It was found that the error bars for all of the platinum complexes except K_2PtCl_4 overlapped at each point. Hence the platinum complexes were not able to target histidine residues.

Previous research was done with inhibition assays on different peptides and enzymes containing different active sites such as selenocysteine, histidine and cysteine residues if platinum derivatives would target and lead to their inhibition.

Inhibition of TRXR by cisplatin was studied by Tetsuro and co-workers on HeLa cells, to see if the cisplatin targetetted selenium residue of TRXR.[32]The activity of TRXR was measured by insulin-reducing assay, and found that cisplatin targeted selenocysteine residue of TRXR leading to its inhibition. From our research it was found that cisplatin derivatives targeted cysteine residue of papain enzyme leading to its inhibition. In the study done on TRXR, researchers concluded that cisplatin showed time and dose dependent inhibition on TRXR.[32] Our research on papain, we have seen for time dependent inhibition, with mg of cisplatin. Inhibition assays on papain are done at 15, 30, 45, 60 minutes as that done with TRXR. TRXR showed inhibition at 45 minutes, with cisplatin concentration being 10, 20 and 30μM. Papain showed inhibition at 15 minutes with cisplatin. Thus, we conclude that cisplatin targets cysteine residue over selenocysteine, leading to papain inhibition in less time than that of TRXR. However, we note that higher concentrations of platinum complexes are utilized in our study.

Similar research was done by Mohammad and co-workers to see if cis-diamminediaquaplatinum(II) (PDC) and observed if cisplatin targets histidine residue of acetyl choline esterase enzyme (AChE). [33] AChE activity was measured by spectrophotometric method of Ellman et al with varying concentrations of cisplatin for a period of 30 minutes. They concluded that at 30 minutes PDC showed significant inhibition of AChE, and found that histidine residues were targeted by PDC leading to its inhibition. Our assay done on papain showed inhibition at 15 minutes by cisplatin. Thus, both cysteine and histidine are targeted by cisplatin.

Similar research was done to see if platinum derivatives target cysteine residue and would lead to inhibition of cysteine protease enzyme Cathepsin-B,[27] a thiol

dependent enzyme. At the active site of cathepsin-B, the conserved cysteine residue catalyzes the hydrolysis of amide bonds in proteins and peptides.[27] The assay was done by treating samples with DCG-0N active labels.[27] Researchers concluded that platinum derivatives could not inhibit cathepsin-B. We performed a similar assay except for treating with active label and SDS- page gel. In our research, it was found that cisplatin derivatives targeted cysteine residue leading to significant inhibition of papain enzyme.

The main reason for lack of cathepsin-B inhibition by platinum derivatives was assumed to be that the active site cathepsin-B was large enough to accommodate Pt (II) moiety and the binding kinetics of Pt (II) was too slow for efficient inhibition of cathepsin-B.[27] Pt (II) might have reacted with different residue other than the active site.It was found that a free cysteine amino acid has the ability to replace all or at least three of four amine ligand groups that surrounded the platinum atom, suggesting that the platinum compound was highly reactive to bind free cysteine residues.[27]

V. CONCLUSION

The reactions of platinum complexes with enzymes were possible to monitor by the use of UV-visible spectroscopy, and enzymatic assays.

This research was conducted to study the effects of leaving groups and bulk of protein active sites on the reaction of platinum complexes with cysteine and histidine containing proteins. In order to evaluate effects of increased bulk on protein in a platinum complex, the reaction of less bulky $Pt(en)(NO_3)_2$ shown in figure 3.14 was compared with $Pt(Me_4en)(NO_3)_2$, figure 3.15 and concluded that more bulky ligands slows down the reaction with cysteine residue. To understand how the change in leaving groups affect the reactions of platinum complex with cysteine containing enzymes, platinum with almost same bulk but with different leaving groups were studied. As K_2PtCl_4 has same amount of bulk as cisplatin, the results from the reactions of these two platinum complexes were used to understand the effects of leaving group.

We saw that chloride, nitrate and oxalate are excellent leaving groups, as the error bars for these platinum complexes did not overlap resulting in inhibition of cysteine protease enzyme papain.

The main target for the platinum complexes is the cysteine residues over the histidine residues. Papain was significantly inhibited by platinum complexes when it was activated by addition of cysteine and subsequent dialysis.

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