Time-Resolved Femtosecond Laser Spectroscopic Study of the Reaction Mechanism of Cisplatin as the Most Important Chemotherapeutic Drug

by

Saeed Kalantari

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Physics

Waterloo, Ontario, Canada, 2007

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Being currently the second and potentially becoming the first cause of death in North America, cancer has been the focus of researchers from various areas of science. Chemotherapy is one of the leading treatment options for treating various types of cancer, particularly advanced cancers. Among different chemotherapeutic drugs currently used against cancer, cisplatin (CDDP) is considered as one of the most effective and widely used. Despite being the most successful drug in cancer chemotherapy, cisplatin has some shortcomings that restrict it from being more widely used. More than three decades of intensive research has not yet clearly explained why these shortcomings are associated with cisplatin treatment.

Using the advanced "Pump-Probe Femtosecond Time-Resolved Transient Absorption Laser Spectroscopy" technique to study the reaction dynamics of cisplatin at the molecular level, we discovered new findings that make a profound contribution to our understanding of the mechanism of activity of CDDP as a very widely used and effective anticancer drug. By observing the reaction dynamics of cisplatin both in its reaction with electrons in water, and with all four DNA bases, in real time, we shed new light on the issues that have long been elusive for the last three decades.

In addition to these two main research goals, we utilized the same methodology to examine the reaction dynamics of transplatin, which currently is not a clinically effective drug, despite being an isomer of cisplatin. In this part we mainly focused on examining the molecular reaction dynamics of transplatin photoactivation caused by UV irradiation. Here, we studied the transplatin reaction with electrons in water as well as with four DNA bases. Results obtained in this thesis work clearly reveal the mechanism underlying the cytotoxicity enhancement of this molecule after it is irradiated by UV radiation. The data obtained by our experiments provide a mechanistic understanding of this cytotoxicity enhancement at the molecular level.

As the last goal in our research, we compared the explored reaction dynamics of cisplatin with its clinically ineffective trans isomer, transplatin. We believe that our understanding of one of these isomers, would also contribute to understanding of the other. The methodology we used to examine transplatin was the same as what we used for cisplatin. First, we monitored the possible reactions of transplatin with prehydrated electrons in water. After this, the dynamics of transplatin's reactions with DNA bases were examined.

For the first time, we directly observed the reactions of cisplatin and discovered that its extremely high reactivity with prehydrated electrons happens in an ultrafast process. We also showed the order of reactivity of all four DNA bases with cisplatin and transplatin to be G > A > C > T. Finally, in comparing cis and trans isomers of DDP, we discovered cisplatin to be about 50% more reactive, suggesting why it is much more cytotoxic. In all of these

investigations we obtained promising results that revolutionize our understanding of the mechanism of action of cisplatin as a very important drug in current cancer chemotherapy.

Acknowledgements

First, I would like to thank my supervisor Professor Qing-Bin Lu for his guidance and support during my Masters program. I appreciate his efforts in explaining basic ideas and novel concepts of Biophotonics and Ultrafast Sciences. Successful completion of this Master's research work is due to his vast knowledge of Biophotonics and his extensive experience in research.

My whole hearted love goes to my family for their life long support. I would like to express my especial gratitude to my parents (Abbas & Akram) who taught me values of life. I would also like to thank my brother (Hamid) and my two sisters (Nafiseh & Marzieh) who were always a great blessing to me.

I thank all members of our group for their discussions and help.

I had the privilege of having many amazing friends during my Master's program at Waterloo. Here, I would like to express my appreciation of their friendship and my wish that our ties will remain forever.

I also thank the staff members of the Physics and Astronomy Department, especially Judy McDonnell for their kind help during my Master's program.

I also thank Somayyeh Rahimi and Andy Stumpf for their valuable discussions while writing this thesis.

Financial support for this Master's project from the CIHR, NSERC, CFI, and OIT are sincerely acknowledged.

Dedication

This thesis is dedicated to my family. My parents, Abbas & Akram, my brother Hamid, and my two sisters, Nafiseh & Marzieh, who are the most valuable things I have in the entire universe.

Contents

Chapter 1 Introduction 1

1.1 Cancer and its Current Treatments 1
1.2 Cisplatin 4
1.3 Cisplatin Derivative Drugs 8
1.4 Transplatin, an Isomer of Cisplatin 8
1.5 The Mechanism of Action of Cisplatin as an Anticancer Drug 9
1.6 The Major Objectives of this Thesis 12
1.7 Structure of this Thesis 12
References 13

Chapter 2 Experimental Technique 17

2.1 Transition State of a Chemical Reaction 17
2.2 Pump-Probe Femtosecond Transient Laser Spectroscopy 18
2.3 Laboratory for Ultrafast Biophotonics (Femtobiology) at the University of Waterloo 20
References 21

Chapter 3 Molecular Mechanism of Action of Cisplatin 22

3.1 Introduction 22
3.2 Transient Absorption Kinetic Trace of Water 23
3.3 Experiments on Electron-Transfer Reaction of Cisplatin with Prehydrated Electron in Water 26
3.4 Experiments on Electron-Transfer Reaction of Cisplatin with DNA Nucleotides 30
3.5 Conclusions 35
References 37

Chapter 4 Molecular Mechanism of Action of Transplatin 40

4.1 Introduction 40

4.2 Experiments on Electron-Transfer Reaction of Transplatin with Prehydrated Electron in Water 41
4.3 Experiments on Electron-Transfer Reaction of Transplatin with DNA Nucleotides 43
4.4 Conclusions.....46
References.....48

Chapter 5 Conclusions 49

List of Figures

Figure 1.1 Cisplatin Molecule 5

Figure 1.2 Transplatin Molecule 9

Figure 2.1 Free Energy Curve of a Chemical Reaction 18

Figure 2.2 Schematic diagram of a fs time-resolved laser spectroscopy 19

Figure 3.1 Femtosecond transient absorption spectrum of water, pumped at 318 nm and probed at 266 nm.....26

Figure 3.2 Static UV-Visible Absorption Spectrum of Cisplatin 27

Figure 3.3 Femtosecond Transient Absorption Spectra of Cisplatin at Various Pump Pulse Energies with Pump at 318 nm and Probe at 266 nm 28

Figure 3.4 Corrected Transient Absorption Spectrum of Cisplatin 30

Figure 3.5 All Four Types of DNA nucleotides (a) Purines, (b) Pyrimidines 31

Figure 3.6 Femtosecond transient absorption spectrum of water (black line) and also 2 mM CDDP in pure water (red line) pumped at 318 nm and probed at 333 nm 33

Figure 3.7 Femtosecond transient absorption spectrum of 2 mM Cisplatin with 2mM solution of all 4 DNA bases (Cytosine, Thymine, Guanine, and Adenine) in water, pumped at 318 nm and probed at 266 nm 34

Figure 4.1 Femtosecond Transient Absorption Spectra of 2 mM Transplatin at Various Pump Pulse Energies with Pump Wavelength at 318 nm and Probe Wavelength at 266 nm 42

Figure 4.2 Femtosecond Transient Absorption Spectra of pure water alone (black line) and also with transplatin (red line) with Pump at 318 nm and Probe at 333 nm. The pump power is 25 μW.....44

Figure 4.3 Femtosecond transient absorption spectrum of 2 mM transplatin with 2mM solution of all 4 DNA bases (Cytosine, Thymine, Guanine, and Adenine) in water, pumped at 318 nm and probed at 266 nm 45

Chapter 1

Introduction

Cisplatin is one of the most successful and widely used drugs in current cancer chemotherapy. The high rate of success in treating some types of cancer, such as testicular cancer, has established a key role for this drug in any cancer chemotherapeutic treatment plan. Despite being one of the most promising anticancer drugs ever discovered, cisplatin also has very severe shortcomings that have raised some concerns in regard to more widespread use of it in chemotherapy. The two main shortcomings of cisplatin are its toxic side effects and drug resistance. Extensive research that aimed to understand the exact mechanism of these severe drawbacks has not been very successful so far. Traditional methods that have been applied to understand, in detail, the precise mechanism of action of this important anticancer drug were also not able to offer mechanistic explanations of how this drug reacts at the molecular level and how it induces the toxic side effects and also how tumors develop resistance to it.

In this thesis work we applied the advanced Pump-Probe Femto Second Time Resolved Transient Absorption Laser Spectroscopy, which is the most powerful method for real-time observation of molecular reactions, to directly observe the reaction dynamics of cisplatin as well as its clinically ineffective trans isomer transplatin. With this cutting-edge technique, we were able to look at the reactions of cisplatin and transplatin with prehydrated electrons as well as with DNA bases in real time and at the molecular level.

This thesis research presents a first-ever-discovered mechanistic understanding of the reaction dynamics of cisplatin and transplatin at the molecular level. The experimental discoveries explained in this thesis may greatly contribute to the understanding and modification of the course of reactions involved in cisplatin activity.

1.1 Cancer and its Current Treatments

Cancer is the name of the disease in which cells divide in an uncontrolled way. This abnormal growth of cells may generate a mass of abnormal cells that is called a tumor [1].

1.1.1 Causes of Cancer

In search for the basic causes of cancer, many theories such as over activation of oncogenes, malfunction of cellular growth factors, immune system failure, and inactivation of tumor suppressor genes have been proposed. Some external stimuli

(carcinogens) like UV radiation, some special chemicals, retroviruses, smoke, and many others have also been proven to contribute to cancer [1-2].

1.1.2 Cancer Treatments

Currently, the major modalities of cancer treatment are **surgery**, **radiotherapy** and **chemotherapy**, while **photodynamic therapy** (PDT) is emerging as a relatively new cancer therapy. Besides these major treatment methods, other minor modalities such as immunotherapy and stem cell therapy are also contributing to the treatment of cancer [1,3].

1.1.2.1 Surgery

Surgery is one of the chief forms of treatment for malignant as well as benign tumors. After new developments of surgical methods, this modality is being widely used in cancer therapy not only as a means of cure, but also for biopsies, palliative treatment, laparotomy and removal of endocrine glands to modify the hormonal status and suppression of pain by neurosurgical procedures [1,3].

This treatment modality has some disadvantages as well. One of the biggest issues in cancer surgery is that if the disease has progressed to the systemic stage, surgery cannot offer a complete cure for it. The effectiveness of surgery is limited not as much by the size of the tumor as by its distribution. Even a relatively large tumor can be removed completely by surgery if it has not spread to another part of the body. Conversely, a small tumor that has dispersed even a few cells to other organs such as the lungs, liver or brain cannot be treated successfully by removing the primary tumor alone [1-2].

The high risk of a surgical operation in some parts of the body is also a huge concern in many cases [3].

1.1.2.2 Radiotherapy

Radiotherapy is the application of radiation in order to destroy cancer cells. The use of radiation in therapy began very quickly (within months) after the discovery of the X-ray by Roentgen [1,3]. Radiotherapy of cancer is being applied for both curative and palliative purposes.

Various types of radiation sources have been used for cancer radiotherapy such as X-rays, gamma rays, electron beams, neutron beams, proton beams, and ultrasound [3].

The main shortcoming of cancer radiation therapy is that it requires very complicated delivery methods in order to save the normal cells, while killing tumor cells. Radiation also damages the healthy tissues such as skin on their way to reach the tumor cells.

Another main concern raised by this method is that patients are usually scanned by an imaging method in order that their tumor contours, needed for radiotherapy delivery, be obtained. Then, the special treatment and radiation fractionation plan is tailored to them, but unfortunately, by the time they go to the clinic to receive the radiation, which is within weeks in the best cases, the tumor position and shape has changed and the contour is not as exact as it should be [3].

1.1.2.3 Chemotherapy

Chemotherapy, which is the treatment of cancer by the application of drugs, is another modality of cancer treatment, especially used for advanced cancer that has spread [1].
Chemotherapy's effectiveness depends on the nature of the cancer cell. In the process of becoming cancerous, the tumor cell is no longer subject to the principles that control the growth of a normal cell [2]. The cancer cell has changed some of its metabolic properties and may possess quantitatively different nutritional requirements or different enzymatic processes, compared to normal cells. In many respects, therefore, it acts in the same way as do foreign invaders such as bacteria, parasites, or viruses [2,4]. Although cancer cells differ somewhat from normal cells in their nutritional requirements, unfortunately they do not differ from other human cells as much as bacteria or fungi do [4]. In fighting parasitic or bacterial diseases, qualitative differences in metabolism between the parasite and the host will often allow the infecting organism to be killed by drug levels that are harmless to the patient [3-4].

Differences between the metabolism of cancer cells and healthy cells, on the other hand, appear to be quantitative rather than qualitative, so that the amount of therapy given is limited by the tolerance of the patient's most sensitive cells. Because there are indeed small quantitative differences, nutritional requirements that differ in degree, it is possible for just the right dose of a compound to kill the cancer cells without irreversibly damaging the normal cells [3].

The logical approach, then, to finding new chemical agents active against cancer would be to find differences between the normal cell and the cancer cell that could be exploited in designing compounds to kill specifically the cancer cell. This rational approach, however, has not so far been of great clinical benefit. In general, the active drugs have been discovered on an empirical or observational basis [4]. These compounds have demonstrated activity against tumors in tissue culture or in mice, and their actual mechanism of action has often been investigated years after discovery of their activity [4]. Cisplatin is a very good example of a compound that demonstrates this principle.

A certain amount of this empirical work, however, has had a rational basis. When certain compounds have shown beneficial effects, many closely related derivatives have been synthesized and tested [4].

The development of cells resistant to any single agent is a major problem in using chemotherapeutic agents, so combination chemotherapy, which is applying various chemotherapeutic anticancer drugs at the same time, has been developed to improve this problem [3-4]. Therefore in combination chemotherapy, the cancer cells resistant to one of the chemicals can still be killed by the other drugs, because the chance of a cell's developing resistance simultaneously to all drugs in a combination is very small.

There is usually no cross-resistance between different types of agents with different mechanisms of action. For example, a leukemic cell that develops resistance to mercaptopurine may still be responsive to methotrexate, cytaxan, vincristine, or adriamycin, and vice versa [3].

Treatment Limitations in Chemotherapy

Chemotherapy treatment is limited not as much by the spread of the disease, as by its total mass. Although anticancer drugs penetrate the body and search out any clumps of cells that may have lodged in other organs, chemotherapy has great difficulty in destroying all cells in a large tumor [1,3,4]. For example, a single course of therapy, pushed to the limit of the patient's ability to tolerate it, may destroy 99-99.9% of the tumor cells. In a large tumor weighing 1 kg and containing 10^{12} cells, a 99.9% kill would still leave a billion (10^9) living tumor cells. The same treatment, however, used against a relatively small clump of cells, 10^2 or 10^3, would leave essentially no living cancer cells [3-4].
Unfortunately chemotherapy destroys not only cancer cells, but also many rapidly dividing normal body cells such as cells lining the gastrointestinal tract, hair follicles, bone marrow cells, and lymphocytes involved in the immune defense system. This destruction of normal cells results in the common side effects of chemotherapy: nausea, vomiting, diarrhea, hair loss, and increased susceptibility to infection. The normal cells of the body, however, usually recover rapidly from these side effects after the course of treatment [3-4].

Each of the major cancer treatment methods has its own advantages and disadvantages, as mentioned above, so in order to tailor the best treatment plan for a specific patient, physicians try to exploit the advantages of each of these three main treatment modalities in order to minimize the shortcomings and side effects of treatment. This has given rise to treatment strategies that combine these methods to profit from very special strengths of each treatment method [3-4].

Cisplatin, one of the leading drugs in current cancer chemotherapy, will be discussed in greater detail in this chapter.

1.2 Cisplatin

Cisplatin [cis-diamminedichloroplatinum (II)] (also called CDDP) is undoubtedly one of the most successful and widely used anticancer drugs introduced into general oncology practice [5]. Very strong evidence for this claim is the number of patients who have been successfully treated by this drug. For example the overall cure rate for testicular cancer is currently more than 90% and is nearly 100% for tumors that are promptly diagnosed [6].

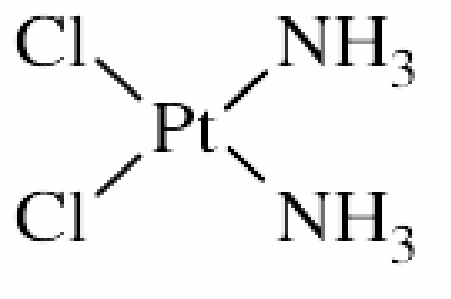


Fig. 1.1 Cisplatin

1.2.1 History

The discovery of cisplatin as an antitumor drug has a very interesting story. CDDP as the chemical complex that we know today was first synthesized by a chemist named M. Peyrone in 1845. Cisplatin used to be called Peyrone's chloride at the time [7,8].

Despite its synthesis, cisplatin's structure was not known until 1893 when a chemistry Nobel laureate, Alfred Werner explained its structure in detail. After this, cisplatin did not attract any special attention until in the 1960s a Michigan State University's Physics professor named Barnett Rosenberg accidentally discovered that cisplatin can induce antitumor effects. In the 1960s, Rosenberg was interested in studying the effects of electric fields and electric currents on the growth rate of E.coli (a type of bacteria). For this reason, he designed an experiment, in which he put positive and negative electrodes in the E.coli medium. Very unexpectedly, he noticed that the E.coli he was studying under these conditions kept growing in such a way that their lengths were a couple of hundred times longer than usual E.coli under natural circumstances. He then understood that his E.coli samples stopped dividing and that's why they were getting longer than usual, because they kept growing instead of dividing.

At first he thought this effect was merely due to the presence of electric fields in the E.coli medium, but more extensive investigations revealed that a platinum-containing compound produced by the reaction of the platinum electrode and NH_4Cl solution in the presence of light and an electrical current was responsible for inhibiting E.coli from division [9]. To confirm his hypothesis, Rosenberg and his colleagues applied their newly discovered platinum compounds on some types of tumors in mice [10-11]. These

compounds were shown to be very effective against selected tumors and cisplatin was the most effective against tumor cells among these platinum-containing compounds. This research, first published in *Nature* in 1969 [12], was a great breakthrough in the history of cancer chemotherapy drugs. Phase I of the human clinical trials of CDDP started in 1971 and cisplatin was approved by Food and Drug Administration (FDA) in 1978 under the name Platinol [13].

1.2.2 Types of Cancers Treated with CDDP

Cisplatin has been used against various types of cancers. Although it is especially effective against ovarian and testicular cancer, it has also been employed for treating cervical, head and neck, esophageal, bladder and small cell lung cancer [14].

The extensive range of cancer types against which cisplatin has showed promising results, has established the key role of this drug as a predominant part in almost all single / combinational chemotherapy treatments.

Typical doses of cisplatin are 100 mg/day for up to five consecutive days, and it is usually administered in a series of intravenous injections to cancer patients [15].

1.2.3 Shortcomings of Cisplatin

In spite of being one of the key drugs in cancer chemotherapy treatment, Cisplatin also has two important shortcomings that have caused some concern about its usage. These two drawbacks are its toxic side effects and resistance (both intrinsic and acquired) [16].

1.2.3.1 Toxicity

One of the two major problems that limit more widespread use of cisplatin is the severe toxic side effects that CDDP induces.

The toxic side effects of cisplatin treatment mainly includes nephrotoxicity, nausea and vomiting, ototoxicity, blood related issues, and neurotoxicity [5,16].

Nephrotoxicity

Nephrotoxicity is among the major dose-limiting effects in cisplatin treatment. It is very dose-dependent and unfortunately irreversible in some cases.

Renal damage caused by cisplatin includes tubular degeneration, loss of brush border and mineralization of tubular epithelial cells, all of which are cumulative. So, patients are put

on regimes of combined hydration and diuretics, which has proved to be substantially helpful in reducing the nephrotoxicity of cisplatin treatment [5].

Nephrotoxicity can also result in peripheral neuropathy, tinnitus and other severe health problems [17].

Nausea and Vomiting

The nausea and vomiting caused by cisplatin is very severe. It occurs within the first two hours after administration, and will last anywhere from ¼ day to 3 days. Standard antiemetic drugs are therefore prescribed to reduce the severity of the effect. It is the severity of this toxic side effect that causes some patients to desist from cisplatin treatment even with the addition of antiemetics.

In addition to severe nausea and vomiting, patients experience a loss of appetite and taste, and may find it difficult to eat [3].

Ototoxicity

Another dose-dependent effect of cisplatin is ototoxicity. Again, it is a cumulative and irreversible side effect that becomes more significant when treatment is combined with radiation therapy [3].

The ototoxicity of cisplatin also appears to be most significant in children, who may experience loss of balance along with hearing difficulties due to greater sensitivity to the drug. These effects usually decrease when the treatment ends, however many patients experience irreversible hearing loss in the high frequency range (>4kHz).

Blood related issues

Myelosuppression (reduction of bone marrow function) is almost the same as other anticancer drugs, however thrombocytopenia (reduction of platelets and thus ability for blood to clot) and granulocytopenia (reduction of a class of white cells and thus ability to fight off infections), may be observed by days 7-9 and 17-19 after injection, respectively [3]. Blood counts fall 1-2 weeks after treatment, therefore patients may become anaemic, and susceptible to infections, at least temporarily.
The extent of blood cell reduction is dependent on the size of the dose and whether other drugs are used in conjunction with cisplatin [9].

Neurotoxicity of cisplatin is also an acute toxic side effect of this drug [4].

1.2.3.2 Resistance

There are two kinds of tumors resistant to CDDP:

Some tumors such as colorectal and non-small-cell lung cancers have intrinsic resistance to cisplatin, while some other tumors develop resistance after the initial series of treatments. Examples of these types are ovarian and small cell lung cancers [18].

Extensive research has not yet explained the molecular bases of cisplatin resistance in any type of cell [19]. Although a biochemical modulation (manipulation of cellular biochemical pathways by chemical agents to produce selective enhancement of the efficacy of an antitumor drug) of the mechanism of activity of cisplatin was suggested by Fuertes et al, in order to overcome cisplatin resistance and also to improve the antitumor activity [20], lack of sufficient knowledge about the mechanism of action of cisplatin, which is required for this approach, has made this solution less accessible.

Expectedly, decreased apoptosis has been associated with drug resistance in very recent studies [21].

1.3 Cisplatin Derivative Drugs

Over 3000 cisplatin analogues have been tested as anticancer drug candidates [22-23], aimed to reduce either the tumors' resistance to cisplatin [14, 24] or the severe side effects [25], but after all these studies, only 28 platinum compounds could enter Phase I clinical trials and unfortunately, most of these candidate drugs have faced various difficulties in the clinic [26, 27].

Among these 28 candidates, only three (carboplatin, oxaliplatin and nedaplatin) are currently registered for clinical use [28]. Finally, only one has been approved by the FDA: oxaliplatin, for the treatment of colorectal cancer [28]. This means that the real mechanism underlying these clinically active anticancer drugs is unknown. *Thus, the search for novel anticancer drugs by traditional methods has proven to be a difficult and inefficient task.*

Altogether the importance of cisplatin-like drugs is emphasized by the fact that there is hardly any clinical regimen of combination chemotherapy today that does not contain cisplatin or another platinum drug [29-32].

1.4 Transplatin, an Isomer of Cisplatin

Transplatin (TDDP) is the trans isomer of cisplatin. Transplatin has exactly the same chemical formula ($Pt(NH_3)_2Cl_2$), but a slightly different ligand coordination. Since the chemical composition of these two isomers is similar, many of their physical and chemical characteristics are closely alike.

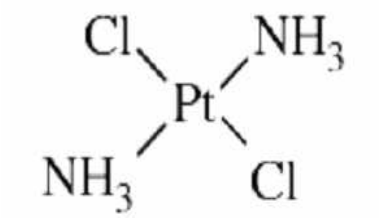


Fig. 1.2 Transplatin

What makes the story of cisplatin activity very puzzling is the fact that the trans isomer (transplatin) is not effective at all while the cis isomer is one of the most effective anticancer drugs ever discovered [33-34].

It has been observed that the cytotoxicity of transplatin in tumor cells is remarkably enhanced after irradiation by UVA (~320 nm to ~400 nm) light. The reason for this cytotoxicity enhancement has been demonstrated to be due to the promotion of the loss of the second chloride of transplatin, caused by UVA light. It was observed that upon photoactivation of transplatin by UVA, bifunctional (mainly interstrand) Cross Links (CL) are produced with a considerably higher frequency than in the dark [35].

1.5 The Mechanism of Action of Cisplatin as an Anticancer Drug

It is well known that binding of cisplatin to genomic DNA (gDNA) in the cell nucleus is the main reason for its antitumor properties [36].

The convincing evidence that proves that DNA is the main target for cisplatin is the early observation that cells deficient in DNA repair are hypersensitive to cisplatin [37-38].

The damage induced upon binding of cisplatin to DNA triggers structural rearrangements that may interfere with normal transcription and/or DNA replication mechanisms [39]. Eventually, these disruptions in DNA transcription and/or replication would trigger cytotoxic processes that lead to cancer cell death through apoptosis, necrosis, or both [36,40].

How Does Cisplatin Reach DNA in The Cell?

Besides the unanswered question about the mechanism of binding of cisplatin to DNA bases, the series of events that happen to CDDP before it reaches the DNA as its main target has also been the subject of controversy. These events include the possible interactions that cisplatin may encounter after it is administered to the body on its way to the cells, which may include its binding with enzymes, peptides, etc. After this stage, CDDP must penetrate through the cell membrane in order to enter the cell. This part is one of the most complicated stages of cisplatin's travel to the cell's DNA. Earlier studies suggested that the cellular uptake was just a passive diffusion [41-42], but recently this

idea has been challenged by researchers who believe they have discovered a direct link between the cellular management of copper and platinum concentrations [43].

The last barrier on cisplatin's way to reach the DNA is the nucleus membrane. Transport of cisplatin through the nuclear membrane is very poorly understood, and whether or not special nuclear peptides play a role remains uncertain [44].

How Does Platinum Attack DNA?

X-ray crystallography studies have shown that the N7 position of the imidazole rings of purine DNA bases (specifically guanine) are the most favored binding site in genomic DNA for binding with cisplatin [45].

Solution studies have approved this preferred binding site as well [46].

Cisplatin binding with DNA happens in two steps. Initially it binds to the N7 site of purine DNA bases (mostly guanine) in order to generate monofunctional DNA adducts. Subsequently, most of these monofunctional adducts further react with the N7 position of an adjacent purine base to make an intrastrand or interstrand cross-link [47-48].

No convincing explanation has so far been proposed to justify the fact that cisplatin happens to bind with this site of purines (guanine and adenine). The higher preference of cisplatin to bind with guanine over adenine has also remained a mystery.

DNA-Cisplatin Adducts Percentages

The reaction of cisplatin with DNA may lead to the formation of various structurally different adducts.

It has been found that in cisplatin reactions with DNA in vitro, 60-65% of adducts formed are 1,2-d(GpG) intrastrand cross-links and 20-25% are 1,2-d(ApG) intrastrand cross-links. Taken together, these account for ~90% of the total adducts formed.

Minor adducts, that account for the remaining 10%, include 1,3-intrastrand cross-links, interstrand cross-links [49], and protein-DNA cross-links [50].

Most Effective Cisplatin-DNA Adduct

There is still debate as to which types of cisplatin-DNA adducts are the most important in mediating the cytotoxicity of cis-DDP.

Support for the role of the major 1,2-intrastrand adducts in cisplatin-induced tumor cell killing, comes from the fact that the trans isomer of cisplatin, trans-DDP or transplatin

(which is clinically inactive), cannot form these adducts as it mainly forms 1,3-intrastrand and interstrand DNA cross-links [51]. Some researchers have reported that they have observed intrastrand adducts with guanine and adenine in cancer patients who were successfully treated with CDDP [52].

Further support for the importance of 1,2-intrastrand DNA adducts in cancer cell killing comes from the observation that the 1,2-intrastrand adducts are less effectively removed from DNA by repair enzymes than 1,3-intrastrand adducts [53-54].

Understanding Cisplatin's Mechanism of Action

Despite the established fact that the ultimate target of cisplatin is the DNA in cells [55-56], the exact processes that lead to this binding are not fully known [57]. It is well established that before cisplatin binds to DNA bases, at least one of the Pt-Chlorine bonds should be broken in order to produce more active species that can attack DNA.

Although hydrolysis of cisplatin has been proposed as the main mechanism that leads to the formation of reactive aqua complexes, mainly $[PtCl(H_2O)(NH_3)_2]^+$ that can bind with DNA bases [58], experimental results do not fully agree with it.

Firstly, the main product of hydrolysis of cisplatin which is $[PtCl(H_2O)(NH_3)_2]^+$ cannot readily bind with two DNA bases, and secondly the other hydrolysis product that might be able to bifunctionally bind with DNA bases is $[Pt(H_2O)_2(NH_3)_2]^{2+}$ which has been noted experimentally to have the least chances of being produced in the hydrolysis of cisplatin [59].

The rate constant that these experiments have arrived at for the production of $[Pt(H_2O)_2(NH_3)_2]^{2+}$ is in the order of only 10^{-4} $M^{-1}s^{-1}$. The real constant rate would be even lower than this as this number has been calculated under the higher temperature of (45°C) instead of body temperature (37°C) and in a solvent that is absent in the human body [60].

The very low rate constant of $[Pt(H_2O)_2(NH_3)_2]^{2+}$ which is the only hydrolysis product able to bind with two DNA bases seems to be inconsistent with the established data that bifunctional adducts of cisplatin-DNA are the main products of cisplatin's reaction with DNA bases.

Finding a new anticancer drug is a very costly and time consuming process. It is estimated that more than 10 000 compounds need to be screened in order to obtain a new, effective anticancer drug [61].

There is a strong need for a mechanistic understanding of the action of cisplatin-like anticancer drugs at the molecular level, which can, in turn, lead to the mechanism-based design of new drugs. Indeed, some researchers have recently predicted that in the next decade, improved antitumor drugs will be developed based on the knowledge of the

cisplatin-DNA interaction and the binding dynamics and kinetics of these Pt compounds to DNA and proteins.

1.6 The Major Objectives of this Thesis

The research project for this thesis is to directly address the desperate need for understanding the precise mechanism of action of cisplatin at the molecular level. The advanced "Femtosecond Pump-Probe Transient Absorption Laser Spectroscopy" technique enabled us to perform, for the first time, real-time direct observations of cisplatin reactions with electrons, either generated during radiolysis / photolysis of water or from an electron donor, the guanine or adenine bases in DNA.

By means of this methodology, we also examined, for the first time, the reaction dynamics of transplatin in pure water and also in its reactions with all four types of DNA bases, (adenine, guanine, thymine, and cytosine) at the molecular level. In this way we could observe the differences that exist between the reaction dynamics of these two isomers.

It has been claimed that transplatin becomes cytotoxic when photoactivated by UVA light [35]. Since our pump laser pulse is in the UVA region, we also had the privilege of directly observing the reaction dynamics of this cytotoxicity enhancement in order to offer a better mechanistic understanding of the basis of this photoactivation.

This research is the first of its type and has provided new promising insights into the molecular level mechanistic understanding of CDDP activity.

1.7 Structure of this Thesis

Following this introduction, the principle of time-resolved femtosecond (fs) laser spectroscopy is described in chapter 2.

In chapter 3, the results for the reaction dynamics of cisplatin as an anticancer drug will be presented and discussed: we first discuss the very strong transfer reaction of the prehydrated electron to cisplatin, and then examine the reaction dynamics of cisplatin in reaction with its final therapeutic targets, the DNA bases. In this chapter, we will also provide the first experimental evidence that clearly explains why cisplatin binds with guanine and adenine more than with thymine and cytosine and also how this reaction takes place in real time.

In chapter 4, we will examine the clinically ineffective trans isomer of cisplatin, i.e., transplatin. First, the results for the reaction of transplatin with prehydrated electrons in pure water are presented and discussed, followed by the dynamics of transplatin reactions with all four different DNA bases. The implication of the results for the observed photo-activation of transplatin will also be discussed.

Finally, the conclusions drawn from this work will be summarized in chapter 5.

References

1. Creasey, W.A. 1981 “Cancer An Introduction”. Oxford University Press, USA.
2. Neel, B.G., & Kumar, R. 1993 “The Molecular Basis of Human Cancer”. Blackwell/Futura.
3. Devita, V.T., Hellman, S., & Rosenberg, S.A. 2005 “Cancer Principles & Practice on Oncology”. 7th Edition, Lippincott Williams & Wilkins.
4. LaFond, R.E. (Ed.), 1988 “Cancer, The Outlaw Cell”. 2nd edition, American Chemical Society (ACS) Publication.
5. Wong, E., & Giandomenico, C.M. 1999 “Current Status of Platinum-Based Antitumor Drugs”. *Chem. Rev.* **99**, 2451-2466.
6. Bosl, G.J., Bajorin, D.F., & Sheinfeld, J. 2001 “Cancer of the Testis”. (DeVita, V. T. J., Hellman, S. & Rosenberg, S. A., Eds.), Lippincott Williams & Wilkins, Philadelphia.
7. Peyrone, M. 1844 *Liebigs Ann. Chem*. 51 1.
8. Pil, P., Lippard, S. J. 1997 “Encyclopedia of Cancer”. Bertino, J. R., Ed., Academic Press, San Diego, CA.
9. Lippert, B. 1999 “Cisplatin, Chemistry and Biochemistry of a Leading Anticancer Drug” Wiley, Weinheim, Germany.
10. Rosenberg, B., & Van Camp, L. 1970 “The Successful Regression of Large Solid Sarcoma 180 Tumors by Platinum Compounds”. *Cancer Res.* **3**0, 1799-1802.
11. Kociba, R.J., Sleight, S.D., & Rosenberg, B. *Cancer Chemother. Rep.*, **54**, 325-328.
12. Rosenberg B., Vancamp L., Trosko J.E. & Mansour V.H. 1969 “Platinum Compounds: a New Class of Potent Antitumour Agents”, *Nature* **222,** 385.
13. Higby, D.J., Wallace, H. J., Albert, D. J. & Holland, J.F. 1974 *Cancer* **33**, 1219-1228.
14. Giaccone, G. 2000 *Drugs* **59**, 9.
15. Reedijk, J. 1996 *Chem. Commun.* 801.
16. Siddik, Z.H. 2003 “Cisplatin: mode of cytotoxic action and molecular basis of resistance”. *Oncogene* **22**, 7265-7279.
17. Decatris, M.P., Sundar, S. & O'Byrne, K.J. 2004 “Platinum-based chemotherapy in metastatic breast cancer: current status”. *Cancer Treat. Rev*. **30**, 53−81.
18. Pérez, R.P. 1998 “Cellular and molecular determinants of cisplatin resistance” *Eur. J. Cancer* **34**, 1535-1542.
19. Fuertes, M.A., Alonso, C. & Pérez, J.M. 2003 “Biochemical Modulation of Cisplatin Mechanisms of Action: Enhancement of Antitumor Activity and Circumvention of Drug Resistance”. *Chem. Rev*. **103** (3), 645 -662.
20. Fuertes, M.A., Alonso, C., & Perez, J.M. 2003 “Biochemical Modulation of Cisplatin Mechanisms of Action: Enhancement of Antitumor Activity and Circumvention of Drug Resistance”. *Chem. Rev*. **103**, 645-662.

21. Borst, P., Borst, J. & Smets, L.A. 2001 “Does resistance to apoptosis affect clinical response to antitumor drugs?”. *Drug Resist. Updates* **4**, 129-131.
22. Weiss, R.B., Christian, M.C. 1993 *Drugs* **46**, 360-377.
23. Wang, D., & Lippard, S.J. 2005 “Cellular Processing of Platinum Anticancer Drugs”. *Nat. Rev. Drug Discovery* **4**, 307-320.
24. Kelland, L. R. 2000 *Drugs* **59**, 1.
25. Judson, I., & Kelland, L.R. 2000 “New Developments and Approaches in the Platinum Arena”. *Drugs* **59**, 29.
26. Lebwohl, D., & Canetta, R. 1998 “Clinical development of platinum complexes in cancer therapy: an historical perspective and an update”. *Eur. J. Cancer* **34**, 1522-1534.
27. Fuertes, M.A., Castilla, J., Alonso, C., & Pérez, J.M. 2002 *Curr. Med. Chem.-Anti Cancer Agents* **2**, 539.
28. Wang, D., & Lippard, S.J. 2005 “Cellular Processing of Platinum Anticancer Drugs”. *Nature Reviews: Drug Discovery*, **4**, 307.
29. Yang, D., van Boom, S.S.G.E., Reedijk, J., van Boom, J.H., Farrell, N. & Wang, A.H.J. 1995 “A novel DNA structure induced by the anticancer bisplatinum compound crosslinked to a GpC site in DNA”. *Nat. Struct. Biol.* **2**, 577-586.
30. Farrell, N., Kelland, L.R., Roberts, J.D. & Vanbeusichem, M. 1992 “Activation of the trans Geometry in Platinum Antitumor Complexes: A Survey of the Cytotoxicity of trans Complexes Containing Planar Ligands in Murine L1210 and Human Tumor Panels and Studies on Their Mechanism of Action”. *Cancer Res.* **52**, 5065-5072.
31. Brabec, V., Kasparkova, J., Vrana, O., Novakova, O., Cox, J.W., Qu, Y. & Farrell, N. 1999. “DNA Modifications by a Novel Bifunctional Trinuclear Platinum Phase I Anticancer Agent”. *Biochemistry* **38**, 6781-6790.
32. Heringova, P., Woods, J., Mackay, F.S., Kasparkova, J., Sadler, P.J., & Brabec, V. 2006 “Transplatin Is Cytotoxic When Photoactivated: Enhanced Formation of DNA Cross-Links”. *J. Med. Chem.*, **49** (26), 7792 -7798.
33. Lepre, C.A., & Lippard, S.J. 1990 “Nucleic Acids and Molecular Biology”. Vol 4, (Eckstein, F., & Lilley, D. M. J., Eds.), Springer-Verlag, Berlin.
34. Lippert, B. 1996 “Metal Ions in Biological Systems”. Vol 33, (Sigel, A., & Sigel, H., Eds.), Marcel Dekker, Inc., New York.
35. Heringova, P., Woods, J., Mackay, F.S., Kasparkova, J., Sadler, P.J., & Brabec, V. 2006 “Transplatin Is Cytotoxic When Photoactivated: Enhanced Formation of DNA Cross-Links”. *J. Med. Chem.*, **49** (26), 7792 -7798.
36. González, V.M., Fuertes, M.A., Alonso, C., Pérez, J.M. 2001 “Is Cisplatin-Induced Cell Death Always Produced by Apoptosis?”. *Mol. Pharmacol*. **59**, 657-663.
37. Brouwer, J., van de Putte, P., Fichtinger-Schepman, A.M.J. & Reedijk, J. 1981 “Base-Pair Substitution Hotspots in GAG and GCG Nucleotide Sequences in Escherichia coli K-12 Induced by cis-Diamminedichloroplatinum (II)”. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7010-7014.
38. Popoff, S., Beck, D.J., & Rupp, W.D. 1987 “Repair of plasmid DNA damaged in vitro with cis- or trans-diamminedichloroplatinum(II) in Escherichia coli”. *Mutat. Res*. **183**(2), 129-137.

39. Payet, D., Gaucheron, F., Sip, M., & Leng, M. 1993 *Nucleic Acids Res*., **21**, 5846.
40. Barry, M.A., Behnke, C.A. & Eastman, A. 1990 "Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia". *Biochem. Pharmacol*. **40**, 2353-2360.
41. Hromas, R.A., North, J.A. & Burns, C.P. 1987 "Decreased cisplatin uptake by resistant L1210 leukemia cells". *Cancer Lett*. **36**, 197−201.
42. Binks, S. P. & Dobrota, M. 1990 "Kinetics and mechanism of uptake of platinum-based pharmaceuticals by the rat small intestine". *Biochem. Pharmacol*. **40**, 1329−1336.
43. Ishida, S., Lee, J., Thiele, D.J. & Herskowitz, I. 2002 "Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals". *Proc. Natl Acad. Sci. U.S.A.* **99**, 14298−14302.
44. Nitiss, J.L. 2002 "A copper connection to the uptake of platinum anticancer drugs". *Proc. Natl. Acad. Sci. U.S.A.* **99**, 13963-13965.
45. Takahara, P.M., Rosenzweig, A.C., Frederick, C.A., & Lippard, S.J. 1995 "Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin". *Nature* **377**, 649 – 652.
46. Yang, D., van Boom, S.S.G.E., Reedijk, J., van Boom, J.H., & Wang, A.H.J. 1995 "Structure and Isomerization of an Intrastrand Cisplatin-Cross-Linked Octamer DNA Duplex by NMR Analysis". *Biochemistry* **34**, 12912-12921.
47. Sherman, S.E., & Lippard, S. 1987 "Structural aspects of platinum anticancer drug interactions with DNA". *J. Chem. Rev*. **87**, 1153.
48. Reedijk, J. 1992 "The relevance of hydrogen bonding in the mechanism of action of platinum antitumor compounds". *Inorg. Chim. Acta* **198**, 873-881.
49. Fichtinger-Schepman, A.M.J., van der Veer, J.L., den Hartog, J.H.J., Lohman, P.H.M., & Reedijk, 1985 "Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation". *Biochemistry* **24**, 707-713.
50. Auge, P., & Kozelka, J. 1997 "Transformations and recognition of platinum-DNA adducts: recent developments". *Transition Met. Chem. (N.Y.)* **22**, 91-96.
51. Eastman, A., & Barry, M.A. 1987 "Interaction of trans-diamminedichloroplatinum (II) with DNA: formation of mono functional adducts and their reaction with glutathione". *Biochemistry* **26**, 3303-3307.
52. Reed, E., Ozols, R.A., Tarone, R., Yuspa, S.H., & Poirier, M.C. 1987 "Platinum-DNA Adducts in Leukocyte DNA Correlate with Disease Response in Ovarian Cancer Patients Receiving Platinum-Based Chemotherapy". *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5024-5028.
53. Szymkowski, D.E., Yarema, K., Essigmann, J.M., Lippard, S.J., & Wood, R.D. 1992 "An Intrastrand d(GpG) Platinum Crosslink in Duplex M13 DNA is Refractory to Repair by Human Cell Extracts". *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10772.
54. Mu, D., Hsu, D.S., & Sancar, A. 1996 "Reaction Mechanism of Human DNA Repair Excision Nuclease". *J. Biol. Chem*. **271**, 8285-8294.
55. Zamble, D.B., & Lippard, S.J. 1995 "Cisplatin and DNA repair in cancer chemotherapy". *Trends Biochem. Sci.* **20**, 435-439.

56. Johnson, N.P., Butour, J.L., Villani, G., Wimmer, F.L., Defais, M., Pierson, V., & Brabec, V. 1989 “Metal antitumor compounds: The mechanism of action of platinum complexes”. *Prog. Clin. Biochem. Med*. **10**, 1-24.
57. Reedijk, J. 1999 “Why Does Cisplatin Reach Guanine-N7 with Competing S-Donor Ligands Available in the Cell?”. *Chem. Rev*. **99** (9), 2499 -2510.
58. Reedijk, J. 2003 “New clues for platinum antitumor chemistry: Kinetically controlled metal binding to DNA”. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 3611-3616.
59. Berners-Price, S.J., Frenkiel, T.A., Frey, U., Ranford, J.D., & Sadler, P.J. 1992 “Hydrolysis products of cisplatin: pK_a determination via [^{1}H, ^{15}N] NMR spectroscopy”. J. Chem. Soc., Chem. Commun. **10**,789-791.
60. Miller, S.E., & House, D.A. 1990 “The hydrolysis products of cis-dichlorodiammineplatinum(II) 3. Hydrolysis kinetics at physiological pH”. *Inorg. Chim. Acta* **173**, 53-60.
61. Hambley, T.W. 1997 “The influence of structure on the activity and toxicity of Pt anti-cancer drugs”. Coord. Chem. Rev. ***166***, 181-223.

Chapter 2

Experimental Technique

The advanced technique applied for my M. Sc. project is the Pump-Probe Femtosecond Time-Resolved Transient Absorption Laser Spectroscopy. This prestigious method of experimentation is known as the most powerful technique for real-time observation of molecular reactions in solutions [1].

2.1 Transition State of a Chemical Reaction

The underlying concept of this technique is simple and direct. Figure 1 below depicts the free energy curve of a typical chemical reaction versus time (progression of reaction). As one can find in a basic chemistry textbook, in order to start the reaction, the reactants (here AB and CD) require a specific amount of energy (activation energy, E_A). Reactants need this energy to overcome the potential barrier of the reaction in order to enter the next stage of the reaction which is called the transition state or intermediate state [2]. This phase of the reaction is very difficult to investigate as the species formed in this stage are extremely unstable and short-lived. The typical life time of transition states is in the time orders of picosecond (10^{-12} s) to femtosecond (10^{-15} s). After this phase the reaction will proceed and the products will be formed based on what have been made in the transition state.

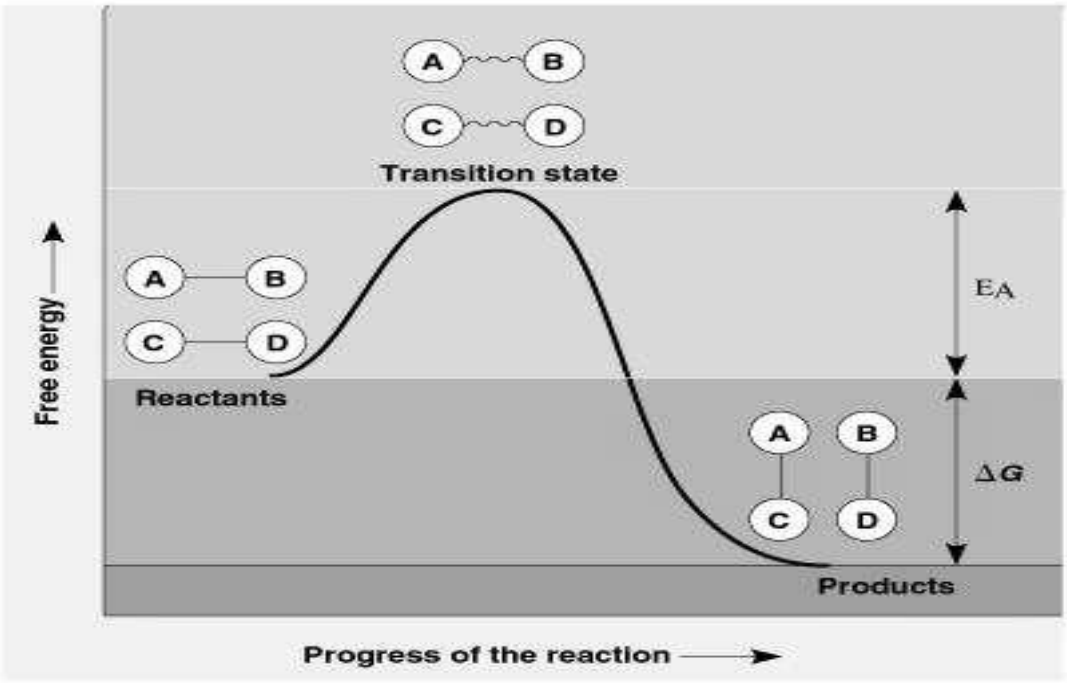


Figure 2.1 Free Energy Curve of a Chemical Reaction

Examining the characteristics of the transition state is crucial in understanding the dynamics of a chemical reaction. For example, precise knowledge about the complexes formed in the intermediate phase of a chemical reaction will give invaluable insight into why specific products are formed in that specific reaction. This will enable us to precisely modulate a chemical reaction in the way that we want it to proceed [2].

The variety of complexes formed in the transition state of a chemical reaction on one hand and the ultrafast nature of the formation and evolution of these complexes in the transition state on the other hand make it very complicated to directly study the properties of transition states. This was not possible until Professor Zewail invented a technique that made it possible to directly examine the dynamics of the transition states of chemical reactions. This discovery, for which he received the 1999 Nobel Prize in Chemistry, uses femtosecond laser spectroscopy to study the transition states of chemical reactions [3].

2.2 Pump-Probe Femtosecond Transient Laser Spectroscopy

Figure 2.2 schematically illustrates the principle of femtosecond laser spectroscopy. Two major components of this technique are pump and probe laser pulses.

The role of the pump laser pulse is to initiate the reaction or to create a reacting species such as an electron by giving the specific amount of energy required for a specific

reaction of interest in order to get started. By tuning the wavelength of the pump laser pulse we can precisely adjust the energy of the pump pulse to be exactly equal to the required energy for the reaction of interest to get started either by excitation of the specific reactant that absorbs the pump pulse energy or by generation of reactive species in the sample such as electrons or radicals.

After initiation of the reaction by a pump laser pulse, a second laser pulse (probe) irradiates the sample at specific time delays after the pump pulse. The probe laser detects the formation of complexes that are in the transition state and also follows their evolution with time. Like the pump laser pulse, the wavelength of the probe pulse can be tuned from IR to UV. The fact that every single complex in the universe has its own specific absorption or fluorescence characteristics (spectroscopic finger prints) enables us to precisely detect different species among the complexes formed in the transition state of the reaction.

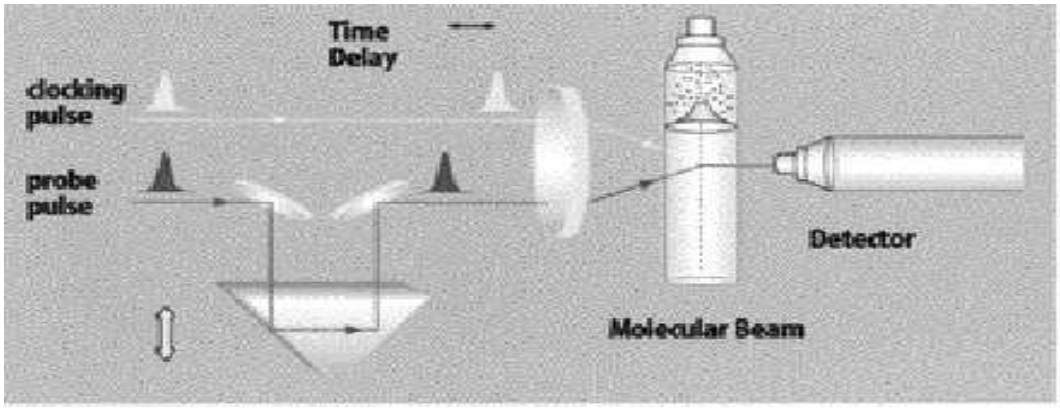


Figure 2.2 Schematic diagram of a fs time-resolved laser spectroscopy.

The time delay between pump and probe laser pulses starts from zero, where the pump and probe pulses arrive at the sample simultaneously, to time delays as long as one nanosecond.

The choice of the desired time resolution in pump-probe laser spectroscopy depends on the lifetime of the transition state of the chemical reaction under investigation. In our experiments, the time delay between pump and probe pulses were made possible by applying a micro stepping motor that could cause optical path differences in the order of micro meters (10^{-6} m) or submicrometer (10^{-7} m) between pump and probe pulses. According to the following calculations, a path difference of 1 μm can give rise to a time difference in the order of femtoseconds

$$\Delta t = \frac{\Delta X}{C\text{air}} \sim 1/3 \times 10^{-14} \sim 3.3 \times 10^{-15} = 3.3 \text{ fs}$$

(if we suppose the speed of light in air to be same as it is in the vacuum)

So we could record images with time differences in the order of femtoseconds if a laser pulse of such a short duration is used. In reality, the typical lifetime of the transition state

of a molecular reaction is in the order of picoseconds or longer. Thus, a time resolution of ~0.1 ps is adequate for observation of the transition state.

Our special interest in applying Pump-Probe Femtosecond Time-Resolved Transient Absorption Laser Spectroscopy as our methodology for this thesis project was to directly observe the transition state of the ultrafast electron transfer (UET) reactions of cisplatin and transplatin. This technique is known to be the most versatile and powerful method for these types of observations [1,4]. Real-time knowledge of the transition state of an ET reaction can help us in predicting, understanding, and changing the pathway of the reaction in the way we like.

2.3 Laboratory for Ultrafast Biophotonics (Femtobiology) at the University of Waterloo

The spectroscopic experiments of this thesis project were done at the Laboratory for Ultrafast Biophotonics (Femtobiology) in the Department of Physics and Astronomy, at the University of Waterloo. This laboratory was successfully built by my supervisor Dr. Qing-Bin Lu [5, 6].

In this laboratory, the standard methodology for femtosecond transient absorption experiments has been applied. A Ti:sapphire laser amplifier system is applied as a laser source producing pulses with a ~120 femtosecond band width and 1 mJ energy per pulse, centered at λ= 800 nm with a repetition rate of 1 kHz. Transient absorbance of as small as 10^{-4} of an intermediate reaction species at the very low pump energy of $\leq$50 nanojoules (nJ) and the very low probe energy of $\leq$1 nJ could be achieved in our spectroscopic studies.

Our laser source was a very sensitive device and having it working in perfect condition required a very special and stable laboratory situation, as any changes in the temperature or humidity of the laboratory could easily make the laser system unstable and accordingly increase the noise level.

In order to tune pump and probe pulses in wavelengths from UV to IR, two optical parametric amplifiers (OPA) were applied. OPA is an optical device that allows tuning of laser pulses based on the principles of nonlinear optics. In order to avoid the contribution from polarization anisotropy due to orientation motions of molecules, we set the polarization of pump and probe pulses at the magic angle of 54.7º.

We used pump pulse energies of around 0.1 μJ to 0.3 μJ for the first part of the experiments of cisplatin and transplatin (cisplatin and transplatin reactions with prehydrated electrons).

We used a 5 mm quartz cell as the container of all samples we studied. In order to avoid any photoproduct accumulation in the quartz cell, we used a magnetic stirring bar inside the cell.

All the instrumentation we used in our spectroscopic studies (including all the electronics, stepping motor, etc.) were connected to a personal computer and were controlled by a Labview program.

References

1. Polanyi, J.C., & Zewail, A.H. 1995 "Direct observation of the transition state". *Acc. Chem. Res.* **28**, 119-132.
2. Atkins, P.W. 2006 "Physical Chemistry". 8th edition, W.H. Freeman and Co., New York.
3. Nobel Prize Foundation Website (for chemistry prize in 1999).
4. Zewail, A.H. 2000 "Femtochemistry: Atomic-scale dynamics of the chemical bond using ultrafast lasers (Nobel lecture)". *Angew. Chem., Int. Ed.* **39**, 2587-2631.
5. Wang, C.R., Hu, A., & Lu, Q.B. 2006 "Direct Observation of the Transition State of Ultrafast Electron Transfer Reaction of a Radiosensitizing Drug Bromodeoxyuridine". *J. Chem. Phys. (Communication)* **124**, 241102-241102.
6. Lu, Q.B. 2007 "Molecular Reaction Mechanisms of Combination Treatments of Low-Dose Cisplatin with Radiotherapy and Photodynamic Therapy". *Journal of Medicinal Chemistry* **50**, 2601-2604.

Chapter 3

Molecular Mechanism of Action of Cisplatin

In this chapter we present the results of our femtosecond pump-probe laser spectroscopic studies of the reaction dynamics of cisplatin (CDDP). First we explain the spectra of pure water taken by our technique. After this we discuss the cisplatin spectra in pure water solution. Then, the reactions of cisplatin with different DNA bases are presented and discussed. This chapter ends with a conclusion section.

3.1 Introduction

Electron transfer (ET) is the basis of many reactions in molecular systems of chemical, biological, and biomedical significance [1-5]. For example "isotopic exchange reactions" were among the very early experiments in chemistry that were explained by electron transfer (ET) [1]. Another example is the discovery of the important role of ET in DNA damage and its repair mechanisms. For this reason, various biophysical and biochemical methods have been applied in order to understand electron transfer (ET) in DNA [2]. Photo-oxygenation is another example where ET plays an essential role. It had long been thought that photo-oxygenation proceeds via energy-transfer quenching, involving the sensitization of oxygen by the sensitizer triplet to form excited singlet oxygen, the reactive intermediate. However, several reactions could not be explained by this mechanism. Recent studies clearly support the claim that electron transfer (ET) is the basis of such reactions [6].

Real-time observation of the transition state in an ET reaction may lead to the prediction, understanding, and modification of the course of the reaction. Among available techniques, time-resolved femtosecond (1 fs = 10^{-15} s) laser spectroscopy is the most powerful technique for direct observation of the reaction transition state and reaction dynamics [7-8].

It has been known that halogen (Cl, Br, and I)-containing molecules have efficient dissociative attachment reactions with low energy electrons. These reactions produce a halogen anion and a neutral radical [9-12].

It was also discovered by Dr. Lu (my supervisor) and his colleagues that the existence of polar molecules such as NH_3 can cause large enhancements in dissociative electron attachment reactions of halogenated molecules. More specifically, for the induced reaction of a chlorine (halogen) containing molecule in the NH_3 liquid or solid, they proposed a dissociative ET mechanism for the enhancements:

$$e^- : (NH_3)_n + ABCl_x \rightarrow [ABCl_x]^{*-} \rightarrow Cl^- + ABCl_{x-1}^{\cdot} \qquad (1)$$

Here $e^-:(NH_3)_n$ represents a presolvated electron (e_{pre}^-) localized in the small polar molecular cluster $(NH_3)_n$ and $ABCl_x$ represents a given molecule that contains *x* chlorine atoms [13,14].

Based on the above findings about electron transfer reactions, it is expected that cisplatin ($Pt(NH_3)_2Cl_2$), will show efficient dissociative attachment reactions with weakly bound electrons, as this molecule itself has both two chlorine atoms and two NH_3 groups bound to a Pt atom. Knowing this, we initially focused our attention on the aqueous phase of CDDP to study the possibility of any electron transfer (ET) reactions related to this molecule.

It has long been known that free electrons can be trapped in polar solvents such as water and ammonia. Until the 1970s, much knowledge about solvated electrons had been obtained [15]. With the advent of the ultrafast laser spectroscopy, the solvation dynamics of electrons in liquid water has been investigated intensely over the past two decades [16,17]. It is now well known that electron solvation occurs through essentially two major stages after generation of the electrons by two-photon absorption of UV light in pure water [18-20].

It has been discovered that electrons pass through short-lived (<1 ps) transient states before becoming fully solvated. Prehydrated electrons are denoted collectively as e_{pre}^- hereafter. The precise physical nature of these ultrashort-lived transient states is still under investigation [21]. e_{pre}^- is considered to be highly reactive and to play a significant role in radiation-induced reactions of molecules in polar media such as water [21]. For example, it has been suggested that e_{pre}^- in ice is responsible for the strong enhancement of the dissociation of chlorofluorocarbons (CFCs) adsorbed on ice surfaces [22] which is greatly related to the formation of the ozone hole in earth's atmosphere [23].

However, it is very difficult to directly observe the reaction of e_{pre}^- due to its ultrashort lifetime, on the femtosecond time scale. This was not achieved until very recently. Our group was the first to observe the transition states XdU^{*-} of the ultrafast electron transfer (ET) reactions of important radiosensitizers, halo-deoxyuridine (XdU, X=Cl, Br and I), with the precursor to the hydrated electron, which is another example that shows the importance of e_{pre}^- in current research [5].

3.2 Transient Absorption Kinetic Trace of Water

Transient absorption kinetic trace of pure water is the starting part in our spectroscopic studies. Since all samples we have been working with were studied in the aqueous phase, the water transient absorption kinetic trace is required to provide the base trace from which any given kinetic trace can be subtracted in order to get its net kinetic trace. For our highly sensitive system, it is much better to use ultrapure water, because in this way one can make sure no other unexpected substances, existing in non-pure water, is having any effect on the system. In our experiments, we had the privilege of using a

BARNSTEAD ultrapure water supplier. Our ultrapure water had the electric resistivity of higher than 18 MΩ/cm and Total Organic Carbon (TOC) of less than 1 part per billion (ppb).

A typical water spectrum recorded with pump wavelength of 318 nm and probe wavelength of 266 nm is shown in Figure 3.1 below. The observed narrow peak at delay time zero in the water spectra is not the electron signal but simply the artificial coherence spike, which has been studied previously [24-27]. This non-resonant coherence spike has been known to be mainly due to conventional stimulated Raman scattering (SRS) or impulsive stimulated Raman scattering (ISRS) [26]. SRS gives a sharp peak in the transient signal of the solvent if the difference between pump and probe frequencies matches a vibrational mode in the electronic ground state while in ISRS, an ultrafast pump pulse excites low-frequency modes in the electronic ground state of the solvent, which leads to the scattering of the probe pulse and then gives rise to a signal at any probe wavelength. The intensity of this coherence spike depends linearly on the pump power at a certain pump power range.

Despite the complications that the coherence spike imposes on the extraction of reaction dynamics from the kinetic traces, measuring the position and the full width at half maximum (FWHM) of the peak can be used as a means for direct, in-situ determinations of not only the delay time zero but the instrument temporal response, which are otherwise difficult to obtain in pump-probe experiments [21]. Due to the fact that the coherence spike corresponds to the optimum temporal overlap between pump and probe pulses, the delay time zero and the instrument temporal response can be measured.

The procedure in our spectroscopic studies is as follows: we take the spectrum of a given complex (in this case cisplatin) in its solvent solution. Then, we take the spectrum of its solvent separately, at the same condition (baseline spectrum, in this case ultrapure water) and then subtract the complex spectrum from that of the solvent, (baseline) in order to get the net spectrum of the given complex.

Beside the exclusive need for the ultrapure water spectrum as the baseline for our spectroscopic studies, we used some other features of water spectra as important criteria for other issues that were of great value for us. One of these issues is the evaluation of the noise level in our experimentation setup every time we wanted to start taking our samples' spectra.

The laser system, the optical parametric amplifiers and the other optical instruments are very sensitive devices and tiny changes of any kind in any part of the system can increase the noise level of our scanning. Keeping the noise of the system at the lowest possible level was of great importance for us. For instance (as will be discussed later), for our studies of the reactions of the DNA bases with cisplatin and transplatin, we had to obtain a sufficient signal to noise ratio even with just one single scan of the sample. This is due to the drastic electron-induced reaction in the sample. For this reason, having even an intermediate noise level could greatly decrease the quality of our spectra, so working with the lowest possible noise level in our system was of extreme importance to us. To achieve

a perfect level of noise was not always very easy, as many factors such as lab temperature and humidity, laser system stability, pulse profile symmetry and alignment, sample type, extent and strength of the reaction under study, etc. can all have effects on the signal to noise ratio. Thanks to the experimental skills of my supervisor Dr. Lu, we could almost always benefit from the lowest possible noise level for such laser systems. As is shown below (i.e. Fig. 3.1) we were mostly working with a very low noise level ($\pm 10^{-4}$), which as far as we are concerned, is considered one of the best noise levels for such systems in the world.

Whenever the sample under our spectroscopic studies was undergoing a very strong reaction, rapid fluctuations were usually seen during the scanning of their spectra. These fluctuations are basically different in nature from the systemic noise of the instruments, because if you replace the sample by pure water and take the spectrum of water with the same setup of the instruments, you might see that the noise level is even perfect ($\pm 10^{-4}$). This means that these two concepts of instrumentation noise and fluctuations caused by strong reactions can easily be mixed up together. So if you evaluate the noise level of the system based on the fluctuations of the sample you are examining, you might be greatly misled. For this reason, looking at the transient absorption spectra of pure water in every experimental run is the best possible way to evaluate the level of noise in the system in that special condition.

The other important criterion for which water spectra were used is double checking the cleanness of the samples' containers (quartz cells). In particular, to clean the cell in which a cycle of the electron-induced drastic reaction of cisplatin has occurred is not a trivial, but a challenging task. The pump-probe femtosecond laser scanning system is a very sensitive technique, so we had to make sure all the equipment we were using was clean. In many cases, if the cell, in which we hold the samples, was not as clean as it should be, its water spectrum would change depending on how unclean it was. The water kinetic trace is very sensitive to the cleanness of the cell, thus we profited from this property as one of our cleanness assurance tests in addition to other checks we made for cleanness evaluations of our cell-washing procedures.

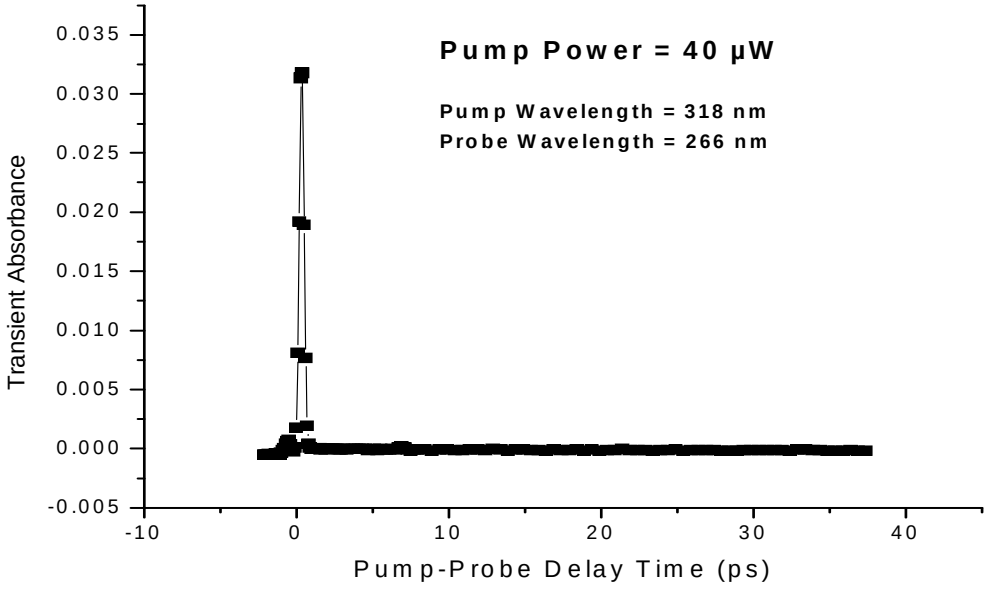


Figure 3.1 Femtosecond transient absorption spectrum of water, pumped at 318 nm and probed at 266 nm.

3.3 Experiments on Electron-Transfer Reaction of Cisplatin with Prehydrated Electron in Water

As mentioned earlier in the introduction of this chapter, cisplatin, a small molecule in which two NH_3 groups and two chlorine atoms are bound to a Pt atom, is expected to have very efficient dissociative attachment reactions with weakly bound electrons. In this thesis work, the results of the first time-resolved femtosecond laser spectroscopic study of the molecular reaction mechanism of cisplatin as an effective chemotherapeutic drug is reported. Our results discover the high reactivity of CDDP with weakly bound prehydrated electrons.

The existence of water in the cell medium where cisplatin becomes reactive and binds to DNA is another reason for our investigations of electron transfers to cisplatin in water.

3.3.1 Experimental Details

To examine the reactivity of cisplatin with electrons, we set up our femtosecond time-resolved laser spectroscopic system with the proper adjustments in order to see the real time reaction dynamics of cisplatin in aqueous phase. Pump-Probe Femtosecond Time-Resolved Transient Absorption Laser Spectroscopy is one of the most precise and powerful methods for direct examination of reaction transition states and dynamics [7, 8].

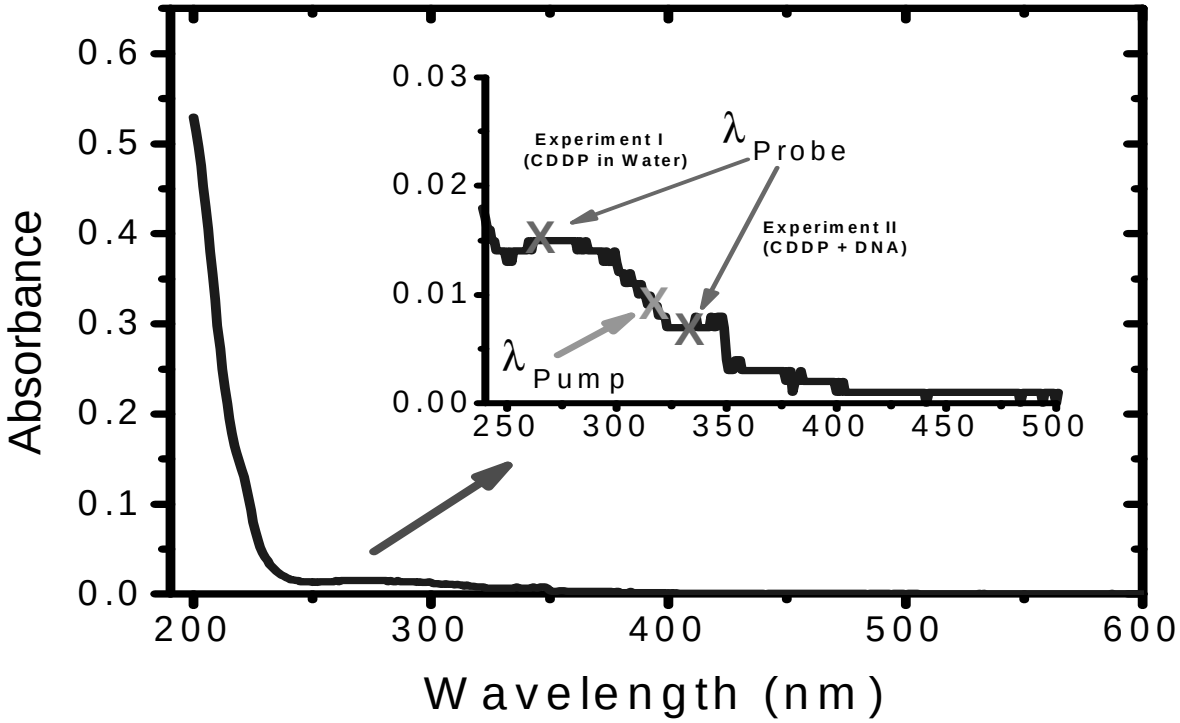


Figure 3.2 Static UV-Visible Absorption Spectrum of Cisplatin [28].

We tuned the pump laser to the wavelength of 318 nm and the probe laser was set at 266 nm. The choice of 318 nm for the pump laser comes from the fact that prehydrated electrons can be produced via two-photon excitation of water at this wavelength with pump pulse energies of 0.15 μJ to 0.3 μJ [5,21].

The best species we could probe during our cisplatin spectroscopic experiments was cisplatin in its ground state. Cisplatin has no functional group capable of absorbing UV. This is shown above in its "UV-Visible Static Absorption Spectrum" (Fig. 3.2). In fact, cisplatin has an absorption band extending up to 350 nm and the major absorption peak is located at below 200 nm [28].

In general, the absorption wavelengths of the transition anionic states of halogenated molecules (cisplatin in our case) are slightly larger than those of their ground-state parent molecules, but they have much weaker signals compared to their parent molecules [5].

In order to observe cisplatin in its ground state, we set the probe laser at 266 nm because cisplatin has a local absorption maximum at this probe wavelength (Fig. 3.2). The cisplatin molecule will leave its ground state either upon direct absorption of pump pulse

or upon attack of excited electrons, so the parent cisplatin molecules will be depleted in the solution and this will give rise to the observation of the negative transient absorption signals of cisplatin. This monitoring enabled us to precisely follow the amount of cisplatin in the sample.

3.3.2 Results and Discussion

Figure 3.3 shows the transient absorption kinetic trace of the cisplatin solution (2.0 mM) at various pump pulse energies from a 100 nJ/pulse to a 300 nJ/pulse.

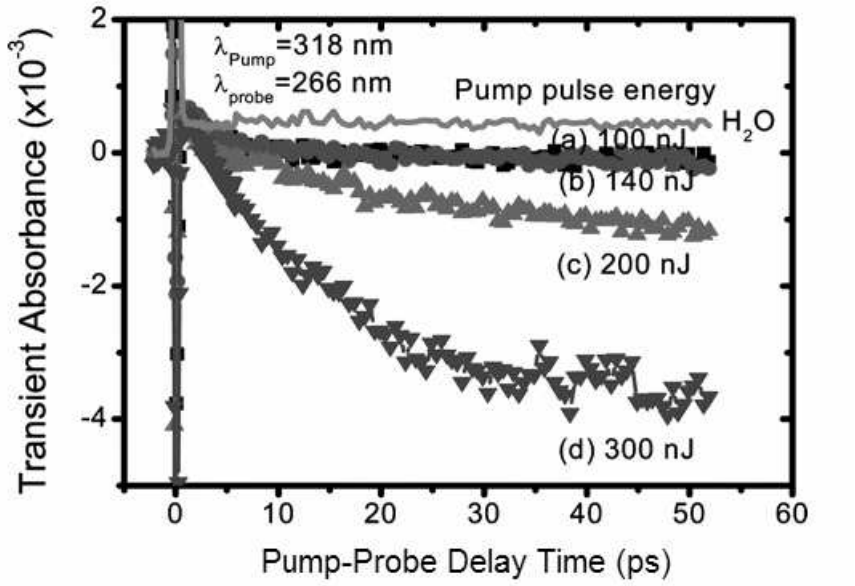


Figure 3.3 Femtosecond Transient Absorption Spectra of Cisplatin at Various Pump Pulse Energies with Pump at 318 nm and Probe at 266 nm. The solid line with a sharp spike at time zero is the spectrum for the pure water obtained at the highest pump pulse energy of 300 nJ [28].

As you can see from the above spectrum, the spectral lines of the 100 nJ/pulse and the 140 nJ/pulse are almost flat, indicating that the amount of cisplatin has remained constant for pump pulse energies below 140 nJ. Sudden excitation of cisplatin by the pump pulse at 318 nm causes the photobleaching that is seen in the vicinity of time zero and the small positive signal in the first few picoseconds is due to the CDDP excited state (CDDP*).

Interestingly, the depletion of cisplatin was observed as the pump pulse energy was increased to a certain level where a considerable amount of electrons could be generated. At the pump energy of 200 nJ per pulse, a clear depletion of cisplatin was observed and as we increased the pump pulse energy to higher values the transient absorption signal of the cisplatin became even more negative, indicating that more CDDP molecules are depleted with more electrons generated at higher pump energies.

The electrons that are generated by two-UV-photon excitation of water are believed to be captured by cisplatin. These electrons break one or both of the Pt–Cl bonds and produce cisplatin reactive radicals, either $Pt(NH_3)_2Cl^{\bullet}$ or $Pt(NH_3)^{\bullet}$. Chlorine ions (Cl^-) are also released in these reactions. We believe these reactive radicals are responsible for the cytotoxicity of cisplatin. There are two leaving Cl^- groups in cisplatin, so ET reactions happen in two steps. These steps are shown by the following chemical equations:

(2-a) $e_p^- + Pt(NH_3)_2Cl_2 \rightarrow [Pt(NH_3)_2Cl_2]^{*-} \rightarrow Cl^- + Pt(NH_3)_2Cl^{\bullet}$

(2-b) $e_p^- + Pt(NH_3)_2Cl \rightarrow [Pt(NH_3)_2Cl]^{*-} \rightarrow Cl^- + Pt(NH_3)^{\bullet}$

A cisplatin molecule can lose its chlorine atoms either by two-step attachments of two electrons to cisplatin that releases one Cl^- ion in each step (single-electron attachment process) or by a one-step two-electron process releasing two Cl^- ions simultaneously. The quadratic dependence of the CDDP depletion on the pump pulse energy is a characteristic of the first process while the second process will lead to a quadruple dependence. The results shown in Figure 3.3 reveal a relationship between quadratic and quadruple dependencies of the cisplatin depletion signal on the pump pulse energy, indicating that both single-electron and double-electron transfer processes take place.

To have a clearer view of the formation dynamics of $CDDP^{*-}$ as well as CDDP in its ground state, we can remove the CDDP* signals from the spectrum of figure 3.3. The fact that the CDDP* signal linearly depends on pump pulse energy enables us to remove its signals by subtracting the (d) curve (300 nJ/pulse) from the tripled of (a) curve (100 nJ/pulse) in the spectrum of figure 3.3. The result of this modification is shown in figure 3.4 below.

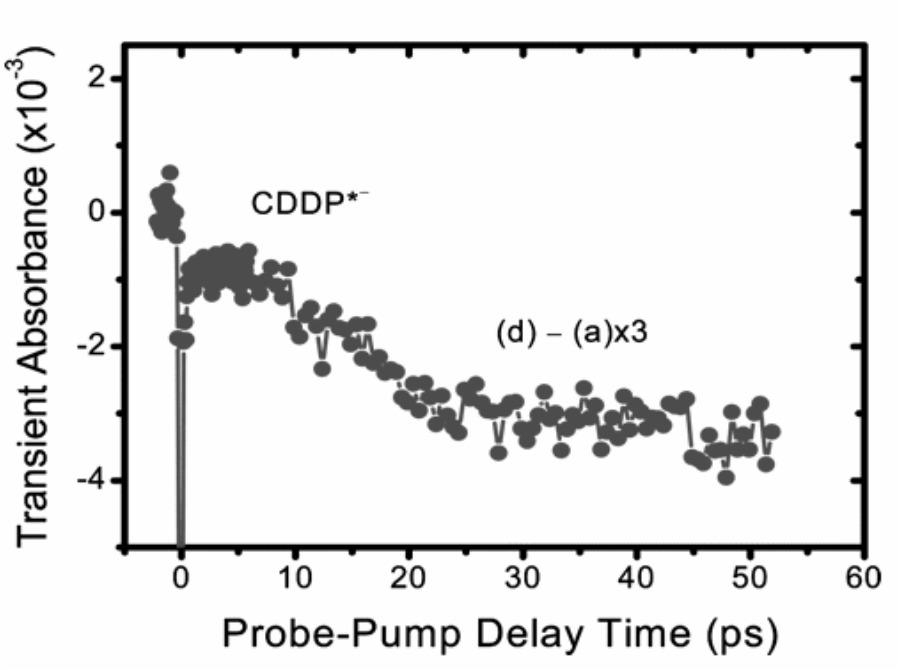


Figure 3.4 Corrected Transient Absorption Spectrum of Cisplatin [28].

The transition anionic state of cisplatin (CDDP*$^-$) is now clearly recognizable in the decay part of the negative signal in the first 20 ps. This decay overlaps with the negative depletion signal of the cisplatin molecules in their ground state.

Ultrafast Electron Transfer

It was observed (Figure 3.4) that the rise time of the signal (the formation time of CDDP*), is clearly within a picosecond (10^{-12} s) after two photon excitation of water by the pump pulse. This time interval is equal to the lifetime ($\leq$1 ps) of prehydrated electrons [21, 29]. This observation proves that cisplatin shows a high reactivity with weakly bound prehydrated electrons in water.

By running the experiments up to this point, our results experimentally showed the existence and dynamics of electron transfer reactions of presolvated electrons in water to cisplatin. In the next stage, we will proceed to study the reaction mechanisms of cisplatin with different DNA bases.

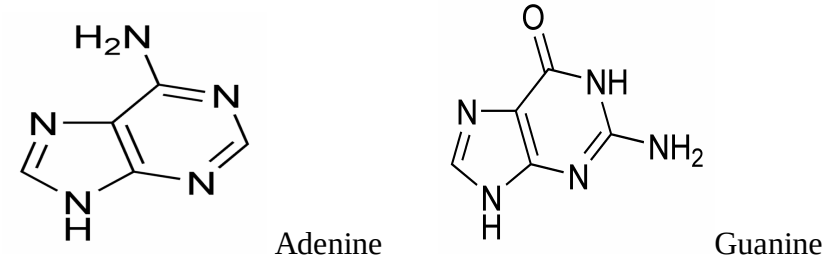
3.4 Experiments on Electron-Transfer Reactions of Cisplatin with DNA Nucleotides

In the second part of our experiments, we were interested in examining the dynamics of cisplatin, (and in the next chapter, of its clinically ineffective trans isomer, Transplatin) reactions with all four mononucleotides, namely, adenine (2'-deoxyadenosine- 5'-

monophosphate or dAMP), guanine (2'-deoxyguanosine 5'-monophosphate or dGMP), thymine (dTMP), and cytosine (dCMP). These nucleotides were purchased from Sigma-Aldrich.

The structure of the DNA bases adenine and guanine are very similar to each other and both are called purines. The structure of cytosine and thymine are also very similar to each other and then are called pyrimidines. Figure 3.5 shows the chemical structure of all four DNA nucleotides.

(a) Purines



(b) Pyrimidines

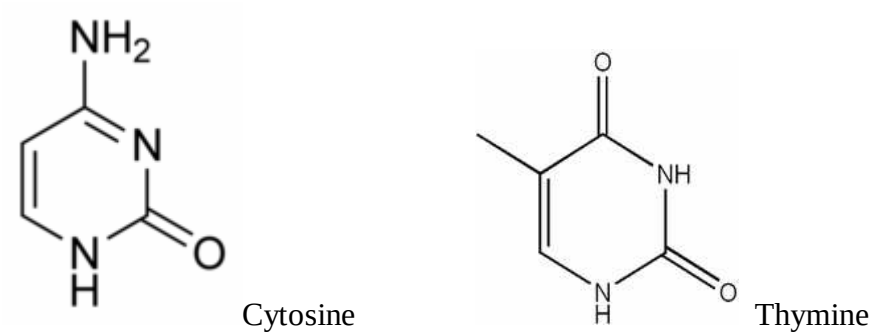


Fig. 3.5 All Four Types of DNA Nucleotides (a) Purines, (b) Pyrimidines

The importance of this part of our experiments comes from the established fact that DNA is the final target of cisplatin, and more importantly, this DNA binding is known to be the main reason for the cytotoxic effects of cisplatin.

3.4.1 Experimental Details

In order to complete this part of our experiments, we had to change the wavelength of the probe pulse as well as the energy of the pump pulse.

The probe wavelength had to be changed from 266 nm to 333 nm because the DNA bases have strong absorption in the 266 nm region and using this probe wavelength would have caused some undesired consequences such as

1. Damaging DNA bases while scanning the spectrum
2. Exciting DNA bases and possibly deviating the reaction path from the natural one and consequently misleading us from making rational judgments about the reaction characteristics
3. Preventing us from making precise conclusions about the cisplatin content in the sample during scanning. This is due to the overlapping absorption of cisplatin and DNA bases at 266 nm. As mentioned earlier in this chapter, we set the 266 nm probe to monitor the amount of cisplatin in the sample, so if DNA bases absorb some parts of the probe as well, making precise inferences concerning the probe signal would be very difficult as the absorption of these two species will be mixed up.

In order to avoid these problems, we chose 333 nm for the probe laser pulse. Cisplatin still has detectable absorption in this wavelength while DNA bases have no absorption at 333 nm.

The second change we made in our spectroscopic system was keeping the pump pulse energy at energy levels below 140 nJ/pulse in these experiments so that no electron could be produced via the two-UV-photon excitation of water, as seen in the spectrum for the pure CDDP without the presence of DNA nucleotides (Fig 3.3). This was because we were interested in monitoring the reactions of cisplatin with electrons that are *exclusively* from the ground-state of DNA bases. For this purpose, a low pump power was used and as Figure 3.6 shows, no prehydrated electron could be generated by this pump power.

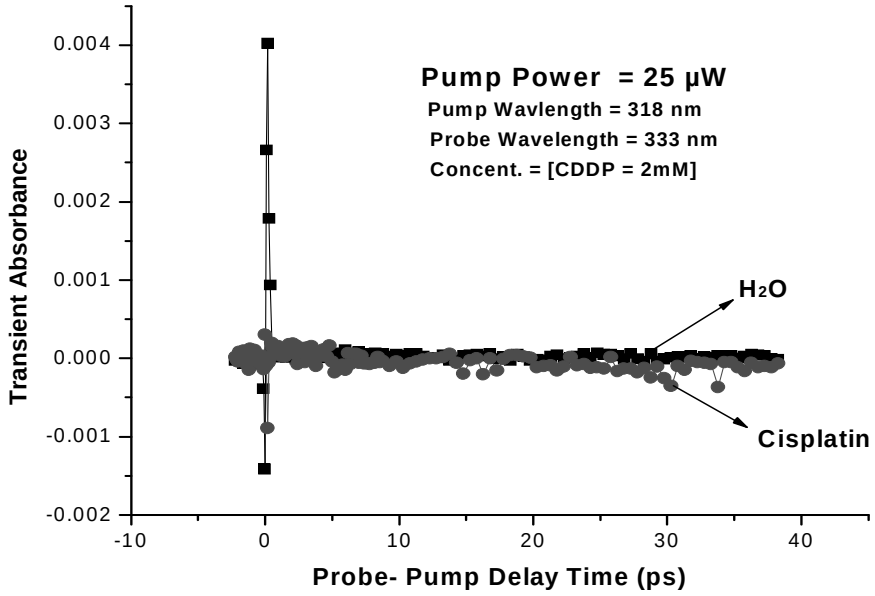


Fig. 3.6 Femtosecond transient absorption spectrum of water (black line) and also 2 mM CDDP in pure water (red line) pumped at 318 nm and probed at 333 nm.

A cisplatin solution with 2 mM concentration was prepared as before. According to the procedures of doing our experiments, we first obtained the transient absorption spectrum of the base line (in our case, ultrapure water). Then, we took the transient spectrum of cisplatin in pure water. After this, a fresh sample of cisplatin was added to the specific DNA nucleotides. Concentration of the cisplatin and the base was kept at 2 mM for all experiments. At the end, their spectra were subtracted from H_2O's spectrum in order to get their net spectra.

3.4.2 Results and Discussion

Guanine

Guanine was the first DNA nucleotide whose reactions with cisplatin were studied. This base is considered the most important base in reaction with cisplatin as previous studies such as x-ray crystallography proved dominant binding of cisplatin to it [30].

After adding guanine to cisplatin, the reaction started very readily and cisplatin was greatly depleted in the solution. Here, unlike the case for cisplatin, alone in the solution, we could see that guanine reacted greatly with the drug in such a way that after subtraction of its spectrum from the baseline, a sharply decreasing negative transient absorption (representing the cisplatin content) was observed. The cisplatin reaction with guanine proceeded so strongly that every successive scan that we recorded, was about 30% changed from the preceding one and this trend continued for more than 10 scans.

This issue forced us to suffice to one single scan in order to be more precise in our experiments of cisplatin with DNA bases. Thus, we scanned our samples one time and then continued the experiments with a fresh sample in a new cell.

The strong depletion of the transient absorption signal of this reaction (Fig. 3.7) clearly shows the strong reaction of cisplatin with guanine.

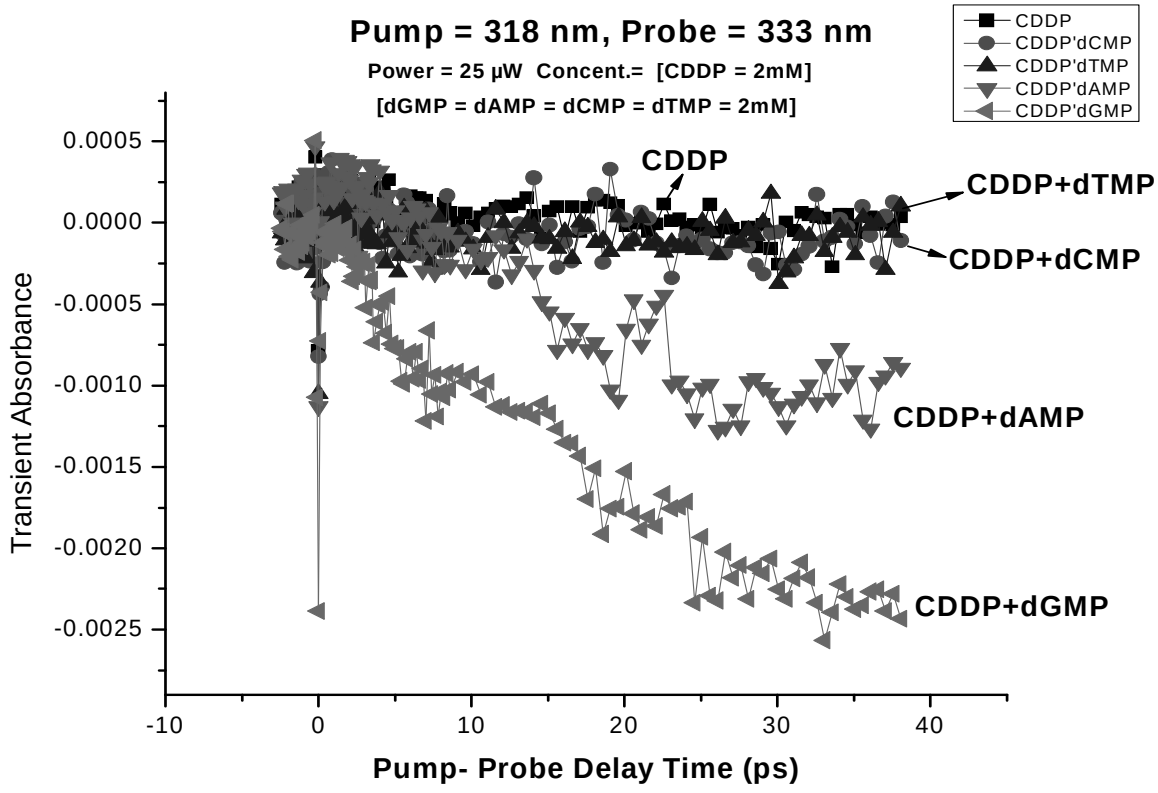


Fig. 3.7 Femtosecond transient absorption spectrum of 2 mM Cisplatin with 2mM solution of all 4 DNA bases (Cytosine, Thymine, Guanine, and Adenine) in water, pumped at 318 nm and probed at 266 nm.

The negative transient absorption signal (the amount of cisplatin depletion) was recorded to be about 3×10^{-3}, which is easily recognizable in the vertical axis of the spectrum (Fig. 3.7).

Cytosine

After guanine, we investigated the reaction of cisplatin with the DNA base cytosine. This base did not show any significant reaction with cisplatin and the amount of CDDP depletion was so small that we were persuaded that the reaction is negligible. The flat line of the cytosine + cisplatin spectrum in figure 3.7 clearly supports this conclusion.

Adenine

In our series of investigating all four DNA bases' reactions with cisplatin, we next looked at the reactions of the other purine base (adenine). The reaction of cisplatin with adenine was a strong reaction. Although it was not as strong as guanine's reaction with cisplatin, it was much stronger than with cytosine. The strength of the adenine reaction with cisplatin was observed as it was expected. Being a purine and structurally very similar to guanine, adenine was expected to show fairly high reactivity with cisplatin.

The amount of depletion of adenine was about 1.5×10^{-3} which is around 50% lower than that of guanine, meaning that guanine's reaction with cisplatin proceeds in a way that is almost two times stronger than cisplatin's reaction with adenine.

Each successive scan of the adenine spectrum sharply varied with respect to the previous one and the difference was observed to be more than 30% on average. This also supported our strategy of taking just one scan for our spectrum.

Thymine

Finally, we examined the combination of cisplatin with the last DNA base, thymine. We could hardly see any reaction happening between thymine and cisplatin, as a very tiny amount of cisplatin was used up and reacted with the thymine. The amount of thymine depletion was the lowest of all four DNA bases. It was even slightly lower than the cytosine depletion, although the spectral lines of these two bases overlapped in some regions of the spectrum. The mixture of cisplatin and thymine was so stable that, unlike the guanine and adenine cases, each successive scan of its spectrum was almost exactly the same as the previous one. As seen in figure 3.7, the thymine absorption signal is almost a flat line, slightly lower than the cisplatin spectral line. This characteristic clearly reveals there is no considerable electron transfer reaction taking place between CDDP and this DNA base.

Another characteristic of the thymine spectral line in the transient absorption spectrum is that it is very smooth and not many fluctuations can be seen in it. This can also give a qualitative measure of the weakness of this reaction compared with guanine and adenine under the same experimental conditions.

3.5 Conclusions

For the first time we explored new findings concerning the reaction dynamics of cisplatin, and shed light on some of the confusions that existed in the field regarding the mechanism of action of this leading drug at the molecular level and also the dynamics of its bindings with different DNA bases.

Our ultrafast high sensitivity real-time femtosecond laser spectroscopy technique is one of the most direct methods to look at the heart of the chemical reactions (transition states) in order to precisely examine the dynamics of the reaction in real time while the reaction is proceeding.

One of the first things that we observed during our experiments on cisplatin is that CDDP is a very strong electron capturer and strongly reacts with electrons, either generated during radiolysis / photolysis of water or from an electron donor such as guanine / adenine bases in DNA.

Second in our findings is that the electron transfer (ET) reaction between cisplatin and prehydrated electrons occurs in an ultrafast process. This electron transfer occurring in less than 1 picosecond (10^{-12} sec) is convincing evidence that the transferred electrons are prehydrated. This is very clearly seen in the spectra of cisplatin in pure water (Fig. 3.3).

One of the most important discoveries that our results clearly show is that among all four DNA bases, guanine shows the greatest preference for binding with cisplatin and its reaction with this drug is the strongest. The fact that cisplatin happens to bind mostly with guanine in the body after it is given to cancer patients, was already discovered by different methods, but our results provide the first and only experimental evidence that explains *why* this preference exists and also what happens, at the molecular level, that causes the guanine base to become the most favored binding site for cisplatin. We have now uncovered that this is because guanine is thermodynamically the best electron donor among the four DNA bases.

The reaction of cisplatin with guanine is so strong that the absorption signal in its dynamic transient absorption spectrum sharply decreases and significant depletion of cisplatin is observed. This finding was to some extent expected for us, but such a high reactivity of cisplatin with guanine was surprising.

Our results yielded further support by providing experimental proofs about the order of the reactivity of different DNA bases (all four types) with cisplatin. Our experimental results show the order of reactivity of different DNA bases with CDDP is guanine > adenine > cytosine > thymine. Very interestingly, this order is exactly the same as the order of the thermodynamical preference of electron donation among the four DNA bases, which was already known [31]. This fact offers a very persuasive confirmation and a means of double checking our findings. We found cisplatin to be a very strong electron capturer and the order that we obtained for the preference of all four DNA bases to react with cisplatin is exactly what was logically expected.

The real-time and ultrafast examinations of the reaction dynamics and spectroscopic properties of one of the most widely used anticancer drugs, cisplatin, at the molecular level, with and without its final target (DNA bases) that were accomplished in our laboratory enable us to make a very promising contribution to a better understanding of the mechanism of action of this most important anticancer drug. We believe that the important progress we made in discovering cisplatin's electron reactivity will play an important role in the universal efforts to improve the shortcomings of this drug.

The cutting-edge research done in our lab will have great influences in this area of pharmaceutical research and we are very optimistic that our findings represent a big step in the history of this drug.

References

1. Marcus, R.A. 1993 "Electron-transfer reactions in chemistry-theory and experiment" (Nobel lecture). *Angew. Chem., Int. Ed. Engl*. **32**, 1111-1121.
2. Wan, C., Fiebig, T., Kelley, S.O., Treadway, C.R., Barton, J.K., & Zewail, A.H. 1999 "Femtosecond dynamics of DNA-mediated electron transfer". *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6014-6019.
3. Jortner, J., Boxon, M. Eds. 1999 "Advances in Chemical Physics: Electron Transfer-From Isolated Molecules to Biomolecules". Vol. 106, Wiley, New York.
4. Qu, X.G., Wan, C.Z., Becker, H.C., Zhong, D.P., & Zewail, A.H. 2001 "The anticancer drug-DNA complex: Femtosecond primary dynamics for anthracycline antibiotics function". *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14212-14217.
5. Wang, C.R., Hu, A., & Lu, Q.B. 2006 "Direct Observation of the Transition State of Ultrafast Electron Transfer Reaction of a Radiosensitizing Drug Bromodeoxyuridine". *J. Chem. Phys.* **124**, 241102-241105. Wang, C.R., & Lu, Q.B. 2007 "Real-Time Observation of Molecular Reaction Mechanism of Aqueous 5-Halo-2'-deoxyuridines under UV/Ionizing Radiation". *Angew. Chem. Intl. Ed.* DOI: 10.1002/anie.200701559.
6. Kavarnos G.J., & Turro, N.J. 1986 "Photosensitization by reversible electron transfer: theories, experimental evidence, and examples". *Chem. Rev*. **86**, 401-449.
7. Polanyi, J.C., & Zewail, A.H. 1995 "Direct observation of the transition state". *Acc. Chem. Res.* **28**, 119-132.
8. Zewail, A.H. "Femtochemistry: Atomic-scale dynamics of the chemical bond using ultrafast lasers" (Nobel lecture). 2000 *Angew. Chem., Int. Ed.* **39**, 2587-2631.
9. Christophorou, L.G., McCorkle, D.L., & Christodoulides, A.A. 1984 "Electron-Molecule Interactions and Their Applications". Vol. 1, Academic Press, Orlando.
10. Dixon-Warren, S.J., Jensen, E.T., & Polanyi, J.C. 1991 "Direct evidence for charge-transfer photodissociation at a metal-surface-CCl4/Ag(111)". *Phys. Rev. Lett.* **67**, 2395-2398.
11. Illenberger, E. 1992 "Electron-attachment reactions in molecular clusters". *Chem. Rev*. **92**, 1589-1609.

12. Rowntree, P., Sanche, L., Parenteau, L., Meinke, M., Weik, F., & Illenberger, E. 1994 "Dissociative electron-attachment to condensed and adsorbed halomethanes". *J. Chem. Phys*. **101**, 4248-4259.
13. Lu, Q.B., & Madey, T.E. 1999 "Negative-ion enhancements in electron-stimulated desorption of CF_2Cl_2 coadsorbed with nonpolar and polar gases on Ru(0001)". *Phys. Rev. Lett.* **82**, 4122-4125. 1999 "Giant enhancement of electron-induced dissociation of chlorofluorocarbons coadsorbed with water or ammonia ices: Implications for the atmospheric ozone depletion". *J. Chem. Phys*. **111**, 2861-2864.
14. Lu, Q.B., & Sanche, L. 2001 "Enhanced Dissociative Electron Attachment to CF_2Cl_2 by Transfer of Electrons Localized in Preexisting Traps of Water and Ammonia Ice". *Phys. Rev*. **B63**, 153403-153406.
15. Hart, E.J., & Anbar, M. 1970 "The Hydrated Electron". Wiley-Interscience: New York.
16. Long, F.H., Lu, H., & Eisenthal, K.B. 1990 "Femtosecond studies of the presolvated electron: An excited state of the solvated electron?" Phys. Rev. Lett. ***64***, 1469-1472.
17. Silva, C., Walhout, P.K., Yokoyama, K., & Barbara, P.F. 1998 "Femtosecond Solvation Dynamics of the Hydrated Electron". *Phys. Rev. Lett.* **80**, 1086-1089.
18. Laenen, R., Roth, T., & Laubereau, A. 2000 "Novel Precursors of Solvated Electrons in Water: Evidence for a Charge Transfer Process". *Phys. Rev. Lett.* **85**, 50-53.
19. Laenen, R., & Roth, T. 2001 "Generation of solvated electrons in neat water: new results from femtosecond spectroscopy". *J. Mol. Struct*. **598**, 37-43.
20. Anderson, N.A., Hang, K., Asbury, J.B., & Lian, T. 2000 "Ultrafast mid-IR detection of the direct precursor to the presolvated electron following electron ejection from ferrocyanide". *Chem. Phys. Lett*. **329**, 386-392.
21. Lu, Q.B., Baskin, J.S. & Zewail, A.H. 2004, "The Presolvated Electron in Water: Can It Be Scavenged at Long Range?". *J. Phys. Chem*. **B108**, 10509-10514.
22. Lu, Q.B., & Sanche, L. 2001 "Enhanced dissociative electron attachment to CF2Cl2 by transfer of electrons in precursors to the solvated state in water and ammonia ice". *Phys. Rev*. **B63**, 153403-153406.
23. Lu, Q.B., & Sanche, L. 2001 "Effects of Cosmic Rays on Atmospheric Chlorofluorocarbon Dissociation and Ozone Depletion". *Phys. Rev. Lett.* **87**, 078501-07851.
24. Heinz, T.F., Palfrey, S.L., & Eisenthal, K.B. 1984 "Coherent coupling effects in pump-probe measurements with collinear, copropagating beams". *Optic Lett*. **9**,359-361.
25. Balk, M.W., & Fleming, G.R. 1985 "Dependence of the coherence spike on the material dephasing time in pump–probe experiments". *J. Chem. Phys*. **83**:4300-4307.
26. Kovalenko, S.A., Ernsting, N.P., & Ruthmann, J. 1996 "Femtosecond hole-burning spectroscopy of the dye DCM in solution: the transition from the locally excited to a charge-transfer state". *Chem. Phys. Lett*. **258**:445-454.

27. Yan, Y.X., Gamble E.B., & Nelson, K.A. 1985 “Impulsive stimulated scattering: General importance in femtosecond laser pulse interactions with matter, and spectroscopic applications”. *J. Chem. Phys*. **83**, 5391-5399.
28. Lu, Q.B., Kalantari, S., & Wang, C.R. 2007, “Electron Transfer Mechanism of Action of the Anticancer Drug Cisplatin with DNA at the Molecular Level”. *Molecular Pharmaceutics* **4**(4) Published on Web 06/09/07.
29. Laenen, R., Roth, T., & Laubereau, A. 2000 “Novel precursors of solvated electrons in water: Evidence for a charge transfer process”. *Phys. Rev. Lett*. **85**, 50-53.
30. Takahara, P.M., Rosenzweig, A.C., Frederick, C.A., & Lippard, S.J. 1995 “Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin”. *Nature* **377**, 649-652.
31. Seidel, C.A.M., Schulz, A., & Sauer, M.H.M. 1996 “Nucleobase-Specific Quenching of Fluorescent Dyes. 1. Nucleobase One-Electron Redox Potentials and Their Correlation with Static and Dynamic Quenching Efficiencies”. *J. Phys. Chem.* **100**, 5541-5553.

Chapter 4

Molecular Mechanism of Action of Transplatin

In chapter 3, the first-ever results of femtosecond pump-probe transient laser spectroscopic studies of the molecular reaction dynamics of cisplatin (CDDP) as one of the most widely used chemotherapeutic anticancer drugs were presented and discussed. In this chapter, we present the results obtained from the femtosecond time resolved laser spectroscopy experiments of CDDP's trans isomer, i.e. transplatin (TDDP). Here, the major objective was to directly observe the molecular mechanism of transplatin cytotoxicity enhancement upon UV photoactivation. These studies include the molecular reaction dynamics of transplatin in pure water as well as with DNA bases.

The methodology applied in these experiments is exactly the same as that applied to cisplatin (explained in chapter 3). The last section of this chapter contains the conclusions drawn from our results.

4.1 Introduction

As mentioned in chapter 1, transplatin does not show any cytotoxicity and is clinically an ineffective compound, but very recently it has been observed that the cytotoxicity of transplatin in tumor cells can be remarkably enhanced upon irradiation by UVA (~320 nm to ~400 nm) light [1]. The reason for this cytotoxicity enhancement has been demonstrated to be due to the promotion of the removal of the second chloride of transplatin by UVA light. It was observed that upon photoactivation of transplatin by UVA, bifunctional DNA adducts of transplatin (mainly interstrand cross links) are produced with a considerably higher frequency than in the dark [2]. The main research objective of this chapter is to reveal the mechanism underlying the enhancement of the cytotoxicity of transplatin once it is photoactivated.

Applying UV radiation as the pump laser pulse (318 nm) in our experiments, we were able to look at the photoactivation reactions of transplatin with DNA bases as its pharmaceutical target. If we understand the molecular dynamics of transplatin's reactions with DNA bases after it is photoactivated by UVA irradiation, there may be a hope for it to enter clinical trials and become an effective anticancer drug in the near future.

Our other research objective in conducting the transplatin experiments was to compare the reaction dynamics of transplatin with its clinically effective cis isomer i.e. Cisplatin (CDDP). Since these two molecules are isomers, our understanding of each one of them will greatly contribute to understanding of the other. We believe comparing the properties of these two isomers will lead to profound insights that may enable us to expand our knowledge of the shortcomings of cisplatin and consequently enable us to improve it. For

this reason, we first compare these two isomers in terms of their reactivity with prehydrated electrons in pure water.

The interest of research on properties of transplatin comes from the mysterious fact that the trans isomer does not show any antitumor effects while the cis isomer (cisplatin), is one of the strongest antitumor drugs ever known [3,4]. The puzzling fact that transplatin, despite having a very similar chemical composition, does not induce any cytotoxicity has attracted scientists' attention for many years. In the following sections we report the first ever results on transplatin molecular reactions obtained by means of femtosecond time resolved transient laser spectroscopy.

4.2 Experiments on Electron-Transfer Reaction of Transplatin with Prehydrated Electrons in Water

Transplatin has the same chemical formula as cisplatin and the difference in their chemical structure is that in transplatin the Cl atoms and NH_3 groups have a trans orientation. Based on these close similarities between cisplatin and transplatin one could speculate that transplatin may also show certain reactivity with the prehydrated electron excited by a pump pulse in pure water (just as we observed in the case of cisplatin in the last chapter). The obtained results of the femtosecond time-resolved laser spectroscopic examination of the molecular reaction mechanism of transplatin as the isomer of cisplatin are discussed in this chapter. Our experimental evidences reveal the reactivity of TDDP with weakly bound prehydrated electrons.

4.2.1 Experimental Details

In order to start our spectroscopic studies of transplatin, we used the same experimental setup as we did for cisplatin. Initially, we restricted ourselves to look at the reaction dynamics of transplatin in pure water to see any possible electron transfer reaction, as we had observed in the case of cisplatin.

Both cisplatin and transplatin were bought in solid phase from Sigma-Aldrich and was used as supplied. Both of these two compounds needed to be solved in water before spectroscopic studies. Since cisplatin has a much higher water solubility (0.253 gr per 100 gr water) than transplatin (0.036 gr per 100 gr water) [5], preparing its solution took much less time than the transplatin solution preparation; we had to put the transplatin solution in a heater at 60º C for at least 24 h before the experiments in order to get a well solved transplatin solution. Transplatin in stock solutions was kept at 2 mM.

We adjusted the wavelength of the pump laser to 318 nm and the probe wavelength to 266 nm. As described in the previous chapter, we took the transplatin solution transient absorption spectrum and subtracted it from the ultrapure water spectrum in order to get the net spectrum of transplatin.

Various pump pulse energies from 40 nJ to 320 nJ (powers from 10 μW to 80 μW) were used in order to see the changes of the reactions with respect to pump pulse energy.

4.2.2 Results and Discussion

Transient absorption kinetic trace of the transplatin solution, with 2.0 mM concentration, at various pump pulse energies (from 40 nJ to 320 nJ) is shown in Figure 4.1.

No negative transient signal is visible in the spectral lines of 40 nJ/pulse and 120 nJ/pulse revealing that for pump pulse energies below 120 nJ/pulse, the amount of transplatin in the sample has not changed. The photobleaching observed near the time zero is due the abrupt excitation of transplatin by the pump pulse at 318 nm. The small positive signal in the first few picoseconds is also caused by the TDDP excited state (TDDP*).

As the pump pulse energy was increased to a certain level, clear depletion of transplatin was observed. This is due to the considerable amount of electrons that are produced at high pump pulse energies. For example as you can see in the spectra of Figure 4.1 below, at the pump energy of 240 nJ per pulse, a significant depletion of transplatin is visible. As we increased the pump pulse energy, the transplatin depletion was seen to be increased accordingly. This phenomenon was similar to what we observed for cisplatin (described in chapter 3).

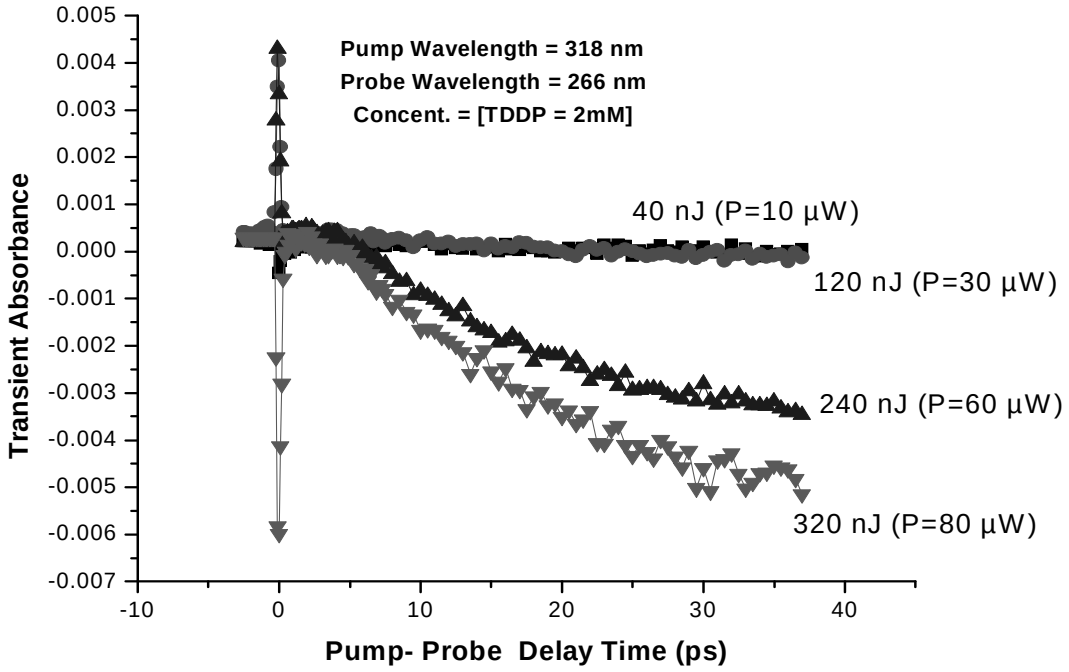


Fig. 4.1 Femtosecond Transient Absorption Spectra of 2 mM transplatin at Various Pump Pulse Energies with Pump wavelength at 318 nm and Probe wavelength at 266 nm.

Our experimental evidences revealed the dynamics of electron transfer reactions of presolvated electrons with transplatin in water. Our results concerning the reaction mechanisms of transplatin with different DNA nucleotides will be discussed in the following section.

4.3 Experiments on Electron-Transfer Reactions of Transplatin with DNA Nucleotides

In this section of our studies we look at the reaction characteristics of transplatin with four DNA nucleotides with the pump pulse laser in the UVA region. The details of the instrumentation are exactly what were applied in chapter 3 for examining cisplatin reactions with DNA nucleotides. The DNA nucleotides used for these experiments were exactly the same as those used in chapter three. The results of the reactions of each of the different nucleotides with transplatin are explained in the following section.

4.3.1 Experimental Details

For our studies of the reaction dynamics of transplatin with DNA bases, the optimum pump pulse energy had to be kept so low that no prehydrated electrons were generated and consequently no ET reactions could happen between the electrons and transplatin. Thus, the pump pulse energy of ≤ 0.1 μJ (pump power of 25 μW) was used to avoid the creation of prehydrated electrons. As shown in Figure 4.2 below, no ET reactions happen between the prehydrated electrons and transplatin at this pump pulse energy. The concentration of transplatin and DNA bases were all kept at 2 mM for all of the experiments.

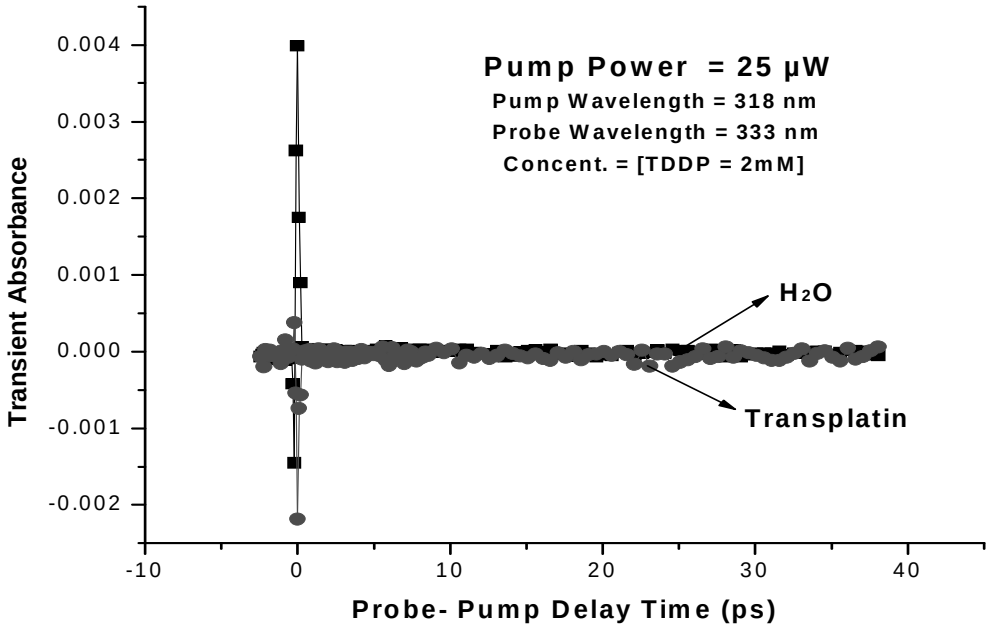


Fig. 4.2 Femtosecond Transient Absorption Spectra of pure water alone (black line) and also with transplatin (red line) with Pump at 318 nm and Probe at 333 nm. The pump power is 25 µW.

The four mononucleotides adenine (2'-deoxyadenosine- 5'-monophosphate or dAMP), guanine (2'-deoxyguanosine 5'-monophosphate or dGMP), thymine (dTMP), and cytosine (dCMP) were purchased from Sigma-Aldrich and used as supplied.

4.3.2 Results and Discussion

Guanine

In our investigation of transplatin reactions with all four DNA bases, we started with guanine. Interestingly the guanine seemed to react with photoactivated transplatin rather strongly. The amount of depletion of the transplatin after the addition of guanine is very close to the depletion amount of cisplatin in its reaction with adenine and almost half of the reactivity of cisplatin in its reaction with guanine.

This reaction seemed to have some fluctuations (as you see in the spectrum of Fig. 4.3 below), but they were less than the fluctuations of the reaction of cisplatin with guanine.

While taking the spectra, each scan changed with respect to the previous one, so just as in the cisplatin case, we decided to conduct our experiments by taking just a single scan for each spectrum of the transplatin in combination with each of the DNA bases. This constraint made our procedure more difficult as we had to repeat our scanning many times with fresh samples to make sure it was well reproducible. Due to the ultra high

sensitivity of our experimentation setup, we had to be exceptionally careful in taking the spectra, as any tiny change in the system could cause some detectable changes in our results.

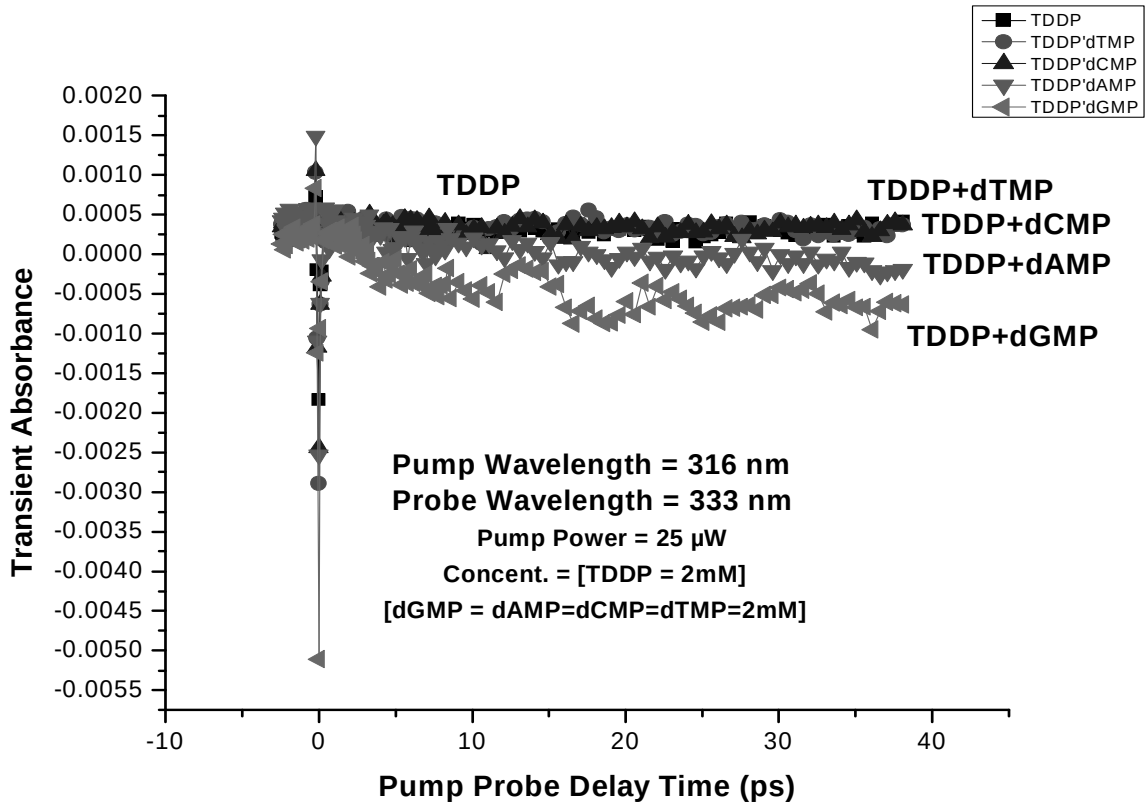


Fig. 4.3 Femtosecond transient absorption spectrum of 2 mM transplatin with 2mM solution of all 4 DNA bases (Cytosine, Thymine, Guanine, and Adenine) in water, pumped at 318 nm and probed at 266 nm.

Cytosine

The cytosine reactivity with photoactivated transplatin was very low. As observed in the spectrum of figure 4.3, the decrease in its depletion line is not very detectable. The cytosine depletion in its reaction with transplatin was again less than its depletion with cisplatin although both are very low.

Adenine

Adenine is another DNA base which has more importance than cytosine and thymine here, because it is expected to show more reactivity. This was exactly what we observed, as shown in Figure 4.3. The DNA base adenine showed more preference in binding with

transplatin than cytosine. Its depletion amount was almost half of the depletion of guanine with transplatin and also half of the depletion amount of adenine in its reaction with cisplatin. The reaction of adenine with photoactivated transplatin yielded a smoother absorption signal curve (less fluctuations) compared to the fluctuations that were seen in its reaction with cisplatin.

Thymine

As the final step in the study of the reactivity of different DNA bases with transplatin, we finally looked at thymine. This DNA base showed the least reactivity with photoactivated transplatin. Basically what we observed was a flat line in its transient absorption spectrum (fig. 4.3) representing almost no reaction taking place. This line is so flat that the amount of transplatin depletion it suggests is almost in the order of the errors of our experiments and one can hardly claim that any reaction is actually happening between thymine and transplatin. Thymine and cytosine are very similar in terms of their reactivity with transplatin.

4.4 Conclusions

The results of the reaction mechanism of transplatin have significance in that transplatin can become cytotoxic upon UV irradiation. Results obtained in our experiments clearly support this enhancement. Our experiments provide evidence for the molecular mechanism of this cytotoxicity enhancement. The reactions of UV-irradiated transplatin with DNA mono nucleotides clearly support the idea that photoactivation can turn the ineffective parent transplatin molecule to an effective and cytotoxic compound.

A further goal of this chapter is to discuss the similarities and dissimilarities of transplatin and cisplatin, based on our experiments. These will be further discussed in what follows.

In finding an answer to our questions regarding the possibility of electron transfers to transplatin, our data showed that this molecule is also an electron capturer and can react with the excited prehydrated electrons in water. The existence of the electron-attracting nature of transplatin makes this molecule similar to cisplatin, but they differ in that cisplatin is a stronger electron capturer than transplatin.

The ultrafast electron transfer happening at ≤ 1 ps clearly shows that the electrons reacting with transplatin are prehydrated electrons, as they have exactly the same life time [6].

Transplatin showed the same order of preference for binding with the different DNA bases as cisplatin, i.e. guanine > adenine > cytosine > thymine. This finding was expected as transplatin has also been proved to be an electron capturer and this order of the DNA bases is just the order of their thermodynamical preference for donating an electron [7]. In regard to this characteristic, both cisplatin and transplatin behaved similarly.

Comparing the reaction dynamics of transplatin and cisplatin based on their reactions with DNA bases may greatly extend our knowledge of the mechanism of activity of cisplatin.

In the case of guanine, the depletion of cisplatin was almost two times more than the depletion of transplatin in its reaction with guanine. Besides that, the depletion line (negative absorption signal) of the reaction of guanine with cisplatin seems to show more fluctuations which itself is a measure that qualitatively shows the strength of the reaction.

By comparing the dynamics of the reactions of the other purine DNA base (adenine) with transplatin and cisplatin, different parts of the scenario fit together better. Just like the case of guanine, the amount of transplatin depletion in its reaction with adenine is half of the depletion amount of cisplatin while reacting with adenine. The negative absorption signal in the spectrum of transplatin with adenine shows fewer fluctuations than what was observed in the reaction of adenine with cisplatin. It might be interesting that the depletion amount of adenine in its reaction with cisplatin is very close to the depletion amount of guanine in its reaction with transplatin, signifying that more reactivity of one DNA base (guanine) is compensated by less reactivity of the electron absorber (transplatin) in such a way that the total combination has almost the same depletion amount.

The two other DNA bases, thymine and cytosine (both pyrimidines), hardly show any reactivity with transplatin. As seen in the transient absorption spectrum of transplatin with DNA bases, (fig. 4.3) the spectral lines of thymine and cytosine largely overlap with the spectral line of transplatin in such a way that one can hardly assign any reactivity to them.

Here, both the cytosine and thymine spectral lines are very much the same and their subtraction from each other will give almost zero difference. The superiority of the reactivity of cytosine over thymine observed in their reaction with cisplatin (chapter three) is much less pronounced here in their reaction with transplatin.

Based on the results just mentioned, we conclude that cisplatin showed more than 50% higher reactivity than its trans isomer, transplatin, in their reactions with purine DNA bases, guanine and adenine.

The evidence discussed in this chapter indicates that transplatin has great potential to become a UV photoactivated anticancer drug candidate. The rather high reactivity of transplatin with DNA bases guanine and adenine upon UV photoactivation obtained by our femtosecond time resolved laser spectroscopy completely supports this hope.

This thesis study suggests that photoactivated transplatin may be explored as a promising anticancer drug candidate to enter general oncology practice.

References

1. Kohen, E., Santus, R., & Hirschberg, J.G. 1995 “Photobiology”. Academic Press.
2. Heringova, P., Woods, J., Mackay, F.S., Kasparkova, J., Sadler, P.J., & Brabec,V. 2006 “Transplatin Is Cytotoxic When Photoactivated: Enhanced Formation of DNA Cross-Links”. *J. Med. Chem*., **49** (26), 7792 -7798.
3. Lepre, C.A., & Lippard, S.J. 1990 “Nucleic Acids and Molecular Biology”. Vol. 4, (Eckstein, F., & Lilley, D.M.J., Eds.), Springer-Verlag, Berlin.
4. Lippert, B. 1996 “Metal Ions in Biological Systems”. Vol. 33, (Sigel, A., & Sigel, H., Eds.), Marcel Dekker, Inc., New York.
5. Lide, D.R. 2004 “CRC Handbook Chemistry and Physics”. 85th edition.
6. Lu, Q.B., Kalantari, S. & Wang, C.R. 2007, “Electron Transfer Mechanism of Action of the Anticancer Drug Cisplatin with DNA at the Molecular Level”. *Molecular Pharmaceutics* **4**(4), Published on Web 06/09/07.
7. Seidel, C.A.M., Schulz, A., & Sauer, M.H.M. 1996 “Nucleobase-Specific Quenching of Fluorescent Dyes. 1. Nucleobase One-Electron Redox Potentials and Their Correlation with Static and Dynamic Quenching Efficiencies”. *J. Phys. Chem.* **100**, 5541-5553.

Chapter 5

Conclusions

We have applied state-of-the-art Pump-Probe Femtosecond Time-Resolved Transient Absorption Laser Spectroscopy to investigate the dynamics of cisplatin reactions at the molecular level. For the first time, we revealed the mechanism of action at the true molecular level of CDDP, one of the most effective and widely used anticancer drugs. We examined the reaction dynamics of cisplatin in its reactions with prehydrated electrons in water, as well as with all four types of DNA bases i.e. guanine, adenine, thymine, and cytosine.

In the second part of this thesis research project we investigated the characteristics of the trans isomer of cisplatin, i.e. transplatin. In the transplatin case, we were especially interested in looking at the molecular mechanism of the photoactivation of this molecule upon UV irradiation. The data obtained by femtosecond time resolved laser spectroscopy clearly explains the dynamics of this cytotoxicity enhancement at the molecular level.

In our investigations of cisplatin, we initially proved that cisplatin is an extremely strong electron acceptor by looking at its reaction dynamics with prehydrated electrons in water, generated by the pump pulse energies greater than 140 nJ at 318 nm. These electron transfer reactions proceeded very rapidly and efficiently.

The electron transfer reaction between the electrons produced in water and cisplatin happened in an ultrafast process. The formation of cisplatin anionic transient states was seen to be within 1 picosecond (10^{-12} s) which is the same as the life times of prehydrated electrons ($\leq$ 1 ps) clearly proving the reacting electrons produced in water to be prehydrated electrons. The prehydrated electrons are believed to break Pt-Cl bonds that finally lead to the production of reactive species $Pt(NH_3)^{\cdot}$ or $Pt(NH_3)_2Cl^{\cdot}$ that contribute to the cytotoxicity of cisplatin.

By establishing the role of prehydrated electrons in the process of activity of cisplatin, we pushed forward the investigation of the reaction mechanisms of cisplatin with its clinical targets which are DNA bases.

In this thesis research work, we reported, for the first time, the dynamics of cisplatin's reaction with all four types of DNA bases and proved the order of their reactivity with cisplatin to be Guanine > Adenine > Cytosine > Thymine, which is in complete agreement with the known thermodynamic preference of electron donation among the four DNA bases.

This discovery was an expected consequence of our first observation that cisplatin is a strong electron acceptor. Thus, the stronger electron donor a molecule (DNA base) is, the

more efficient ET reaction would be expected. As guanine is the strongest electron donor with the lowest required energy among the four DNA bases, its reaction with cisplatin was observed to be the most effective.

These parts of our research were experimentally very sophisticated, as the reactions were observed to be very strong and drastic.

The reaction of cisplatin with adenine was not seen to be as strong as cisplatin reaction with guanine. To be more precise, adenine was observed to be half as reactive compared to guanine, but it is still the second reactive base among all four bases. This is again in complete accordance with the known statistics of cisplatin binding with DNA bases.

The extent of cisplatin's reactions with other two bases, cytosine and thymine, were not comparable to those of guanine and adenine. Although cytosine showed more reactivity than thymine, both of the reactions were so weak that one can hardly relate them to cisplatin's anticancer effects.

For the first time, we provided a mechanistic understanding at the molecular level as to why cisplatin binds with DNA bases in the very special order of G > A > C > T.

In our examinations of transplatin, we were focused on investigating the molecular reaction dynamics of the UV caused photoactivation. Results obtained in our experiments on transplatin well support the cytotoxicity enhancement of this molecule after it is irradiated by UV radiation. Our experiments provide evidence for the mechanistic understanding of this cytotoxicity enhancement at the molecular level.

The findings obtained concerning transplatin in this thesis study suggest that photoactivated transplatin may be explored as a very promising anticancer drug candidate for general oncology practice.

Our final research aim was to compare the explored reaction dynamics of cisplatin with that of transplatin in order to shed new light on the old mystery of why this isomer is not effective despite being so similar to cisplatin.

We obtained experimental evidence, for the first time, to support the order of reactivity of photoactivated transplatin with DNA bases being G > A > C > T just as we observed in the case of cisplatin. The higher reactivity of DNA bases guanine and adenine with cisplatin than with transplatin was the main difference that we recorded in comparing these two isomers.

According to our obtained data, transplatin showed 50% less reactivity than cisplatin in reactions with DNA bases.

Beside these mentioned discoveries, we believe this research also contributes to our knowledge of the effects of species like NH_3 on the enhancement of electron-induced reactions of halogenated molecules.

All these findings provide a molecular-level mechanistic understanding of cisplatin cytotoxicity. The very precise data we achieved by means of our cutting-edge experimental method provide the most profound understanding about the dynamics of cisplatin and transplatin reactions with DNA as its most important target. We shed new light on issues that have long been elusive in the history of cisplatin research, for the last four decades. We believe our discoveries will have significant effects in improving the therapeutic applications of this exceptional chemotherapeutic drug, such as the circumvention of drug resistance and the reduction of toxic side effects. Moreover, our findings will have significance for mechanism-based design and development of new anticancer drugs in the future.