SYNTHESIS, CHARACTERIZATION, ELECTRONIC STRUCTURE AND DNA BINDING ABILITIES OF PLATINUM(II) COMPLEXES CONTINING 2,3-DI(2-THIENYL)-QUINOXALINE LIGAND

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WADAD S.S. FADEL

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Approval of the Graduate School of Natural andAppliedSciences, Atılım University.

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ABSTRACT

SYNTHESIS, CHARACTERIZATION, ELECTRONIC STRUCTURE AND DNA BINDING ABILITIES OF PLATINUM(II) COMPLEXES CONTAINING 2,3-DI(2-THIENYL)-QUINOXALINE LIGAND

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During the last decades, a number of transition-metal complexes have been utilized to probe nucleic acid structures and in the development of DNA-cleaving agents. Among these complexes platinum(II) ion containing ones are renown. For this purpose, platinum complexes of 2,3-di(2-thienyl)-quinoxaline were synthesized in *cis*, asymmetric and *bis* geometry in this work. Identification of the complexes was performed by using elemental analysis and various spectroscopic techniques.

The binding mode and extent of interaction of complexes was determined by UV titration, thermal decomposition, viscometric and fluorometric measurements. Thermodynamic parameters were also determined from the UV titrations which are repeatedly carried out at different temperaturesCytotoxicity tests of the complexes were completed at neutral medium and IC_{50} values were calculated for each complex.

The DNA binding studies indicated that all the complexes interact with DNA through groove binding or electrostatically. Cytotoxicity experiments performed on MDA231

cell line gave very promising results and revealed that the complex in cis geometry is the most active agent against to breast cancer.

Keywords: Antitumor drugs, platinum complexes, quinoxalin ligands, DNA binding ability, cytotoxicity.

ÖZ

2,3-Dİ(2-TİYENİL)-KUİNOKSALİN LİGANTI İÇEREN PLATİN(II) KOMPLEKSLERİNİN SENTEZİ, KARAKTERİZASYONU, ELEKTRONİK YAPILARI VE DNA'YA BAĞLANMA KABİLİYETLERİ

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Son dönemlerde, birçok geçiş metal kompleksi nükleik asit yapılarında değişiklik meydana getirerek, DNA sarmal yapısını kırmak için kullanılmaktadır. Bu komplekslerden platin(II) iyonu içeren yapılar en çok bilinen bileşiklerdir. Bu çalışmada, cis, bis ve asimetrik geometrilerde 2,3-di(2-tiyenil)-kuinoksalin içeren platin kompleksleri sentezlenmiştir. Hazırlanan komplekslerin kimyasal yapıları elemental analiz ve çeşitli spektroskopik teknikler kullanarak çözümlenmiştir.

Komplekslerin DNA'ya bağlanma modları ve bağlanma dereceleri UV-titrasyon, termal bozunma, vizkometrik ve florometrik ölçümlerle belirlenmiştir. Termodinamik parametreler de yine UV-titrasyon metodunun farklı sıcaklıklardaki tekrarıyla hesaplanmıştır. Komplekslerin sitetoksisite deneyleri yapılmış ve IC_{50} değerleri her bir kompleks için hesaplanmıştır.

DNA bağlanma çalışmaları tüm komplekslerin DNA'nın boşluklarına tutunduğunu ya da elektrostatik bir etkileşim yaptığını göstermiştir. MDA231 hücre hatlarında yapılan sitotoksisite deneylerinden oldukça umut verici sonuçlar elde edilmiştir. Tüm

kompleksler içinde cis-geometrisinde bulunan kompleksin meme kanserine karşı en etkin ajan olduğu gözlenmiştir.

Anahtar Kelimeler: Antitümör ilaçlar, platin kompleksleri, kuinoksalin, DNA bağlanma kabiliyeti, sitotoksisite.

DEDICATION

I dedicate this thesis to

My parents SAED AL KHSHIPE and SHARIFAH, Allah saves them

My sister SARA and all my brothers and sisters

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First and foremost I am grateful to Allah almighty for giving me strength, knowledge, health and wisdom to undertake this work

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LIST OF ABBREVIATIONS

A - Adenine

AA - Ascorbic acid

AN - Acetonitrile

Bis(ethylenediamine)platinum(II)chloride

Carboplatin - $[Pt(NH_3)_2(O,O\text{-cyclobutane-1,1-dicarboxylate})]$

Cis - DDP-cis-diamminedichloroplatinum(II)

Cisplatin - $[cis\text{-}Pt(NH_3)_2Cl_2]$

CTR - Copper transporters

CT - DNA-Calf-Thymus DNA

DMF - Dimethylformamide

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

EB - Ethidium bromide

EtOH - Ethanol

G - Guanine

G2 - Second stage of cell cycle

GSH - γ-glutamylcysteinylglycine (glutathione)

MeOH – Methanol

Met - Methionine

mRNA - Messenger Ribonucleic acid

MS - Mass Spectrometry

N - Nitrogen

Naddtc - Sodium diethyldithiocarbamate

NER - Nucleotide excision repair

NMR - Nuclear Magnetic Resonance

OCT - Organic cation transporters

Oxaliplatin - [Pt(1,2-trans-R,R-diaminocyclohexane)(O-O-oxalato)]

RNA - Ribonucleic acid

S - Sulfur

SAR - Structure–Activity Relationships

Satraplatin - [c,c,t ammine(cyclohexylamine)-dichlorodiacetatoplatinum(IV)]

STS - Sodium thiosulfate

UV–vis - Ultraviolet–visible Spectroscopy

ΔG^o - Standard Gibbs Free Energy

ΔH^o - Standard Enthalpy Change

ΔS^o - Standard Entropy Change

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CHAPTER 1

INTRODUCTION

Platinum coordination compounds are among the most utilized anticancer agents, even though platinum has not been determined to be an essential trace element in any living organism. The antitumor activities of recently reported platinum(II) complexes indicate that further modification of platinum coordination compounds will lead to the development of anticancer agents with higher efficient against chemotherapy-insensitive tumors [1].

Some platinum coordination complexes are active anticancer drugs in animals and human. This new class of chemotherapeutics was discovered during the course of investigation of the electric field effects on bacterial growth. The platinum electrodes electrolyzed during the experiment, releasing a platinum complex which caused complete cessation of cell division in the bacterial rods. With this filamentation assay system, able to identify the specific chemical as cis dichlorodiammineplatinum, a complex known since 1845 [2].

Since its introduction into clinical trials, cisplatin (cisdiammine- dichloro-platinumII) has had a major impact in cancer medicine, changing the course of therapeutic management of several tumors, such as those of the ovary, testes, and the head and neck [3]. Almost 30 years after its clinical benefits were first recognized, studies still continue in an effort to understand exactly how cisplatin works. There is no doubt, however, that DNA is the primary target of cisplatin, but still there are wide gaps in our fuller appreciation of the process that translates cisplatin-induced DNA damage into its characteristic drug-mediated cellular effects, namely, inhibition of DNA synthesis, suppression of RNA transcription, effects on the cell cycle, and the therapeutically beneficial process of apoptosis [4].

The early studies of cisplatin revealed it to be clinically challenging for patients and physicians alike. The initial studies were characterized by toxicity of a degree

hitherto unprecedented. Severe nausea and vomiting and nephrotoxicity in the form of renal failure almost led to studies being discontinued. The demonstration by Cvitkovic and co-workers [5], first in an animal model, then in a clinical trial, that aggressive diuresis could prevent the severe renal damage permitted the further investigation of the drug. These methods are still in standard use today, and the nausea and vomiting were ameliorated largely as a result of the investigation of intensive antiemetic regimens [6].

The anticancer drug cisplatin forms a variety of covalent DNA adducts. The consequences of this DNA damage are mediated by proteins which either bind to the cisplatin-DNA crosslinks or influence cellular pathways in response to the genotoxic stress. In either case, these proteins can regulate the processing of the cisplatin lesions and thereby affect cellular sensitivity to the drug. Identification of these proteins and exploration of their cellular functions has implicated multiple systems including several classes of DNA repair, transcription, cell cycle and cell death responses [7].

Cisplatin is believed to exert its cytotoxic effects by interacting with DNA, where it inhibits both replication and transcription and induces programmed cell-death. However, much data has been accumulated in recent years indicating that the replication machinery can elongate past cisplatin-DNA lesions in a mutagenic way [8]. Intervention of specific DNA polymerases and protein-protein interactions between replicative enzymes and DNA-damage-recognition proteins may lead to occasional mutagenic translesion synthesis. The consequences of cisplatin-induced mutations may severely alter the fate of the cell. When occurring in proto oncogenes, they can result in their activation leading to a key step in the process of tumorigenicity, or in the acquisition of a cisplatin-resistant phenotype [9].

An understanding of the mode of action is indeed desirable in refining therapeutic approaches that further enhance the antitumor activity of the platinum drug. This understanding is also critical for elucidating mechanisms underlying the drug-resistant phenotype, which radically limits the clinical utility of cisplatin. For example, ovarian cancer, which generally responds well to cisplatin-based therapy. The initial response rate of up to 70% is not durable, and results in a 5-year patient survival rate of only 15–20%, primarily as tumors become resistant to therapy [10].

In an alternative example with small cell lung cancer, the relapse rate can be as high as 95% [11]. The onset of resistance creates a further therapeutic complication in that tumors failing to respond to cisplatin are crossresistant to diverse unrelated antitumor drugs [12].

1.1. Objective of this study

The major objectives of this research:

- Modified structure of cisplatin, this can be associated with the displacement of ligand.
- To design new active platinum complexes against resistance cell lines, and larger group of antitumor activity produces less toxicity than cisplatin.
- To synthesize a new platinum-bound DNA binding complex as anti-cancer drug leads provide better efficiency with low resistance compared to cisplatin.
- To investigate from reduce the toxicity of platinum complex and to modify its activity through its association with metallic complexes 2,3-di(2-thienyl)-quinoxaline ligand.

CHAPTER 2

LITERATURE REVIEW

2.1. Platinum II Complexes for the Treatment of Cancer

Platinum compounds represent an unique class of DNA damaging anticancer agents Which are now used widely. Globally approved platinum drug is cisplatin, have a major role in medical oncology [13]. It was licensed for medical use in 1978/1979 [14]. Cisplatin is a chemotherapy medication used to treat various types of cancers, including sarcomas, some carcinomas (small cell lung cancer, squamous cell carcinoma of the head and neck and ovarian cancer), lymphomas, bladder cancer, breast cancer, cervical cancer and germ cell tumor. Cisplatin is particularly effective against testicular cancer [14].

Cisplatin, or *cis*-diamminedichloroplatinum(II), *cis*-$Pt(NH_3)_2Cl_2$ is the first of a series of square planar platinum(II), which described by Michele Peyrone in 1845, and known for a long time as Peyrone's salt [15]. The structure was deduced by Alfred Werner in 1893 [16]. In 1965, (Rosenberg, et al.) discovered that electrolysis of platinum electrodes generated a soluble platinum complex which inhibited binary fission in Escherichia coli bacteria. Although bacterial cell growth continued, cell division was arrested, the bacteria growing as filaments up to 300 times their normal length [17].

2.2. Geometric Structure of Platinum(II) Complexes

Geometric structure plays a crucial role in enhancing the catalytic activity of a material towards reactions such as oxygen reduction, methanol oxidation etc. Platinum has two dominant valence states, +2 and +4. The lower state forms square planar complexes, and the latter forms octahedral complexes [18].

2.2.1. A Square Planar Complex

All clinically used platinum drugs contain the element in the +2 oxidation state having almost exclusively square-planar coordination geometries. Square planar complexes that contain symmetrical bidentate ligands, such as cisplatin is a neutral complex, $Pt(NH_3)_2Cl_2$. It is neutral because the +2 charge of the original platinum(II) ion is exactly cancelled by the two negative charges supplied by the chloride ions. The platinum, the two chlorines, and the two nitrogens are all in the same plane [18] (Fig. 2.1.).

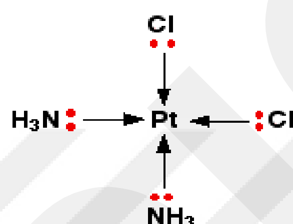


Figure 2.1: Neutral cisplatin[14], $Pt(NH_3)_2Cl_2$

2.2.2. Octahedral Geometry

Platinum(IV) complexes are compounds have shape containing six atoms or groups of atoms or ligands symmetrically arranged around a central atom, defining the vertices of an octahedron. The concept of octahedral coordination geometry was developed by Alfred Werner to explain the stoichiometries and isomerism in coordination compounds [16]. However, the electronic factors contributing to enhanced catalytic activity of particular structure/shape/morphology have not been understood so far [18].

2.2.3. Geometric Isomerism

The existence of coordination compounds with the same formula but different arrangements of the ligands was crucial in the development of coordination chemistry. Two compounds with the same formula but different arrangements of the atoms are called isomers [19]. This occurs in planar complexes like the $Pt(NH_3)_2Cl_2$. There are two completely different ways in which the ammonias and chloride ions could arrange themselves around the central platinum ion (Fig. 2.2.) .The terms *cis* and *trans* are used in the same way as they are in organic chemistry. *Trans*, that the

ammonias are arranged opposite each other in that version, and so are the chlorines. *Cis* in this instance, that just means that the ammonias and the chlorines are next door to each other [20].

Figure 2.2: Geometric isomers of anticancer active and inactive platinum(II) complexes[20]; (a) cis-diammine platinum dichloride (cis-platin); (b) trans-diammine platinum dichloride (trans-platin)

Because isomers usually have different physical and chemical properties, it is important to know which isomer we are dealing with if more than one isomer is possible [20]. The *trans* isomer is more soluble than *cis*-platin in water and most stable while, cisplatin has clinical importance in the treatment of anticancer. it is able to bind with DNA. By the two ammines in cisplatin can be replaced by nitrogen donors from a DNA strand [21].

2.3. Synthesis of Platinum (II) Complexes

The major reaction pathways involved in the synthesis of platinum(II) and other square-planar d^8 complexes involve associative ligand substitution. These reactions proceed through five-coordinate trigonal-bipyramidal intermediates. The stereochemistry of the resulting products is dictated by the relative trans effect of the ligands within the complex [22].

2.3.1. Synthesis of Cisplatin

Cis- and *trans*-diamminedichloro–platinum(II), are stereoisomers, representative members of the class of complexes having the general formula $[PtL_2X_2]$, where L is an am(m)ine or N-heterocycle and X is a halide or other labile ligand. Both cisplatin

and its trans isomer were first prepared over 100 years ago by Peyrone and Reiset, respectively, and were commonly known as Peyrone's chloride [23].

The synthesis of cisplatin starts from potassium tetrachloroplatinate. The tetraiodide is formed by reaction with an excess of potassium iodide. Reaction with ammonia forms $K_2[PtI_2(NH_3)_2]$ which is isolated as a yellow compound. When silver nitrate in water is added insoluble silver iodide precipitates and $K_2[Pt(OH_2)_2(NH_3)_2]$ remains in solution (Fig. 2.3.). Addition of potassium chloride will form the final product which precipitates In the triiodo intermediate the addition of the second ammonia ligand is governed by the trans effect [24].

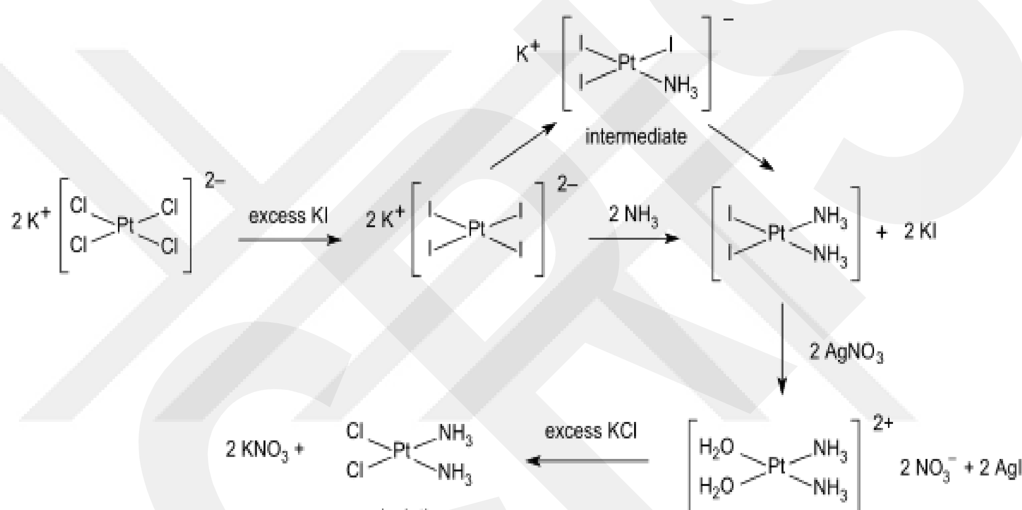


Figure 2.3: Synthesis of cisplatin[14]

2.4. Cisplatin and Other Platinum-Analogues

The platinum drugs represent a unique and important class of antitumor agents. The initial discovery of the anti-tumor properties of cisplatin by Rosenberg was quickly followed by clinical trials demonstrating its efficacy in a variety of solid tumors [25]. However, that nephrotoxicity and the emergence of drug-resistant tumor cells limited the overall efficacy of cisplatin [26]. The search for new platinum analogues that could circumvent the deleterious aspects of cisplatin therapy soon followed.

Carboplatin is a cisplatin analogue that is more easily administered and is less toxic at standard doses. This is due to a different pharmacokinetic profile resulting from

the substitution of a more stable leaving group [27]. Carboplatin and cisplatin form similar DNA adducts, which may explain, in part, the similar efficacy observed with the drugs in most solid tumors. The search for platinum analogues that do not exhibit cross-resistance with cisplatin and carboplatin has led to the synthesis of the DACH platinum compounds. The DACH platinum drug, oxaliplatin, has been shown to be active in combination with 5-fluorouracil and leucovorin for the treatment of colorectal cancer, a disease in which cisplatin and carboplatin show little activity [28].

2.4.1. The First Drug – Cisplatin

The application of cisplatinum compounds in cancer therapy was one of the most unexpected developments in pharmaceuticals in the last 50 years. Over many years much effort was put into studying the detail of this lesion and its interactions with proteins which might mediate the cell death process [29]. Early clinical results were variable, with occasional patients showing very positive results while others encountered marked toxic effects such as kidney and nerve damage. While these side effects remained uncontrolled there seemed little likelihood of cisplatin achieving regulatory approval, but fortunately a new administration procedure involving pre- and post-hydration significantly reduced the kidney toxicity (but did not eliminate it). This allowed somewhat higher doses to be given to patients with acceptable toxic risk and pronounced activity in genitourinary cancers (particularly testicular and ovarian cancer) was observed [30].

Cisplatin is used in 90% of cases of testicular cancer, ovarian cancer and bladder cancer. We also note a specific use in mesothelioma and lung cancer. Side effects were, it is a very nephrotoxic, and therefore must take into account the possibility of weakness in the treatment of the renal patient, in addition to the presence of strong ear toxicity, nausea and vomiting in addition to the classical model of the treatment of antineoplastic determined by the release of serotonin, Neuropathy by the appearance of hand and foot syndrome, lack of sense [31].

2.4.2. Second Generation – Carboplatin

Research continued to find a compound showing less toxicity than cisplatin, maintaining an effective anti-tumor, leading to the development of carboplatin in 1972 and approval of medical use in 1986 [32]. Carboplatin (Fig. 2.4) A planar complex of the second-generation platinum (II) coordination, that has bidentate dicarboxylate (the ligand is cyclobutane dicarboxylic acid, CBDCA) in place of the two chloride ligand, which are the leaving groups in cisplatin. The mechanism of action of carboplatin is similar to cisplatin, but less strong, perhaps because it is more stable and has a lower reaction rate of substitution in aqueous solution [33].

Carboplatin feature is certainly one of the most important reaction to determine cellular toxicity of platinum derivatives. As for cisplatin, the drug breakdown process provides coupling with reduced glutathione through transverse glutathione reaction. Carboplatin is eliminated through total filtration. For this reason, too this time, it is necessary to modify the dosage of the drug based on the kidney function of the patient. This feature is estimated by renal clearance of creatinine [34]. Also, low secretion rate of carboplatin means that the body is retained more, thus longer effects (longer half-life of 30 hours for carboplatin compared to 1.5-3.6 hours for cisplatin) [35].

The majer indications were found to be used as an antitumor for ovarian cancer, and also in combination with paclitaxel for non-small cell lung cancer. Its negative effects are less than cis-platinum, but nausea and vomiting persist but less severe. Neurotoxicity has seen with hands and feet parenthesis and we can see myelosuppression [36].

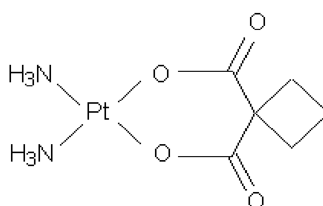


Figure 2.4: Structure of carboplatin[33]

2.4.3. Third Generation – Oxaliplatin

Oxaliplatin is a third-generation of platinum(II) complex synthesizer that was discovered in 1976 by Professor Yoshinori Kidani, it is the first of the compounds with a 1,2-diaminocyclohexane (DACH) core to achieve full clinical development [37]. The interest of DACH complexes has been generated by the need to develop and modify functional groups, a platinum derivative that would produce a response in cisplatin/carboplatin resistance tumors [38]. The molecular structure of oxaliplatin (Fig. 2.5) contains a central platinum atom surrounded by a DACH group and ligand represented by oxalate bidentate [39]. Oxaliplatin has a working mechanism and chemical behavior similar to other platinum derivatives. First, pro-drug oxaliplatin must be activated by converting it to Monocluro, Diclora and Diaco forms. This conversion was done with non-enzymatic degradation with exhalation group exfoliation [38].

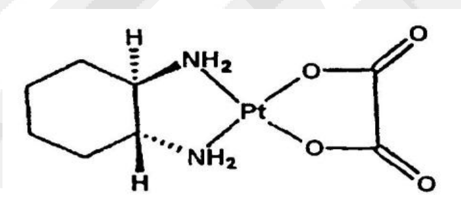


Figure 2.5: Structure of oxaliplatin[38]

The oxidation of oxaliplatin is slower than cisplatin. The intermediate step, which is associated with a DACH-platinum for two water molecules, is rather reactive and goes into the interaction with sulfhydride and amino groups of proteins, RNA and DNA [40]. The anti-drug efficacy is propably due to the formation of DACH-pt-DNA adducts. Other possible irreversible binding interactions with biomolecules such as albumin, cysteine (Cys), methionine (met) and low glutathione (GSH), specifically conjugation with GSH, are induced by the glutathione-S-transferase enzyme, to disrupt drugs and get cell detoxification [41]. The different products are then excreted from the coupling of the cell and are eliminated from the body primarily by renal excretion [42]. Oxaliplatin was later licensed as an advanced colorectal cancer treatment in 1994 [43].

Main indications this drug is used against metastatic colorectal cancer, especially at an advanced stage, against non Hodgkin lymphoma and ovarian cancer. Has less negative effects than the previous compound. The main toxicity, occurred in 90% of patients, is neurological, consisting of acute phase, showing a few hours after treatment, chronic stage. A few neutrophils have been shown, limiting the dose that can be used, in addition to classic nausea and vomiti in correlation with the release of serotonin [44].

2.4.4. Bis-platinum Derivatives

Bis(ethylenediamine)platinum(II) chloride, with formula $C_4H_{16}C_{l2}N_4Pt$. Is predicated upon the formation of bifunctional interastrand and interstrand crosslinks, Farrell and clusters have synthesized a series of DNA-binding drugs based on a binuclear platinum structure(Fig. 2.6). These drugs form DNA complexes that differ markedly in structure, sequence specificity, and formation kinetics from those of cisplatin [45] . The incorporation of more than one platinum molecule, each capable of adduct formation, together with a variable linker region, results in novel, structurally distinct interstrand crosslinks [46].

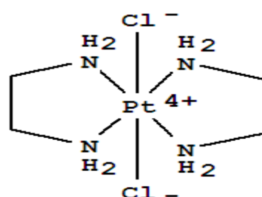


Figure 2.6: Structure of bis-platinum[45]

Reverse the case with cisplatin, interstrand croslinks are more common than interstrand lesions with bis-platinum derivatives. Furthermore, these lesions have more fundamental effects on DNA replication and gene transcription, and there is evidence that because of the conformational changes they exert on DNA, they are detected less efficiently by DNA recognition proteins damage. As a result, pest repair may be easier to implement. Some of these complexes themselves may bind and deactivate the repair proteins. The preclinical assessment of bisplatinum drugs proves

that they have in vivo activity in a variety of cisplatin resistance models, including rat leukemia and human ovarian cancer. Clinical trials have begun with these drugs [45].

2.4.5. Asymmetric Platinum(IV)

Platinum(IV) anticancer prodrugs have attracted much attention due to their relative inertness under physiological conditions. The oxidation state of the platinum atom in platinum coordination compounds determines the steric configuration of the molecule platinum(II) structures are planar molecules, while platinum(IV) derivatives assume an octahedral shape (Fig. 2.7). Though it was hoped that these differences could be used to circumvent platinum resistance, the two compounds developed in the clinic, iproplatin and ormaplatin, have not proven useful. In the case of the former, testing in Phase-II trials failed to reveal activity. In the case of ormaplatin, the platinum(IV) configuration is not maintained under biological conditions: conversion to a platinum(II) metabolite occurs within minutes [47].

Satraplatin [c,c,t-ammine(cyclohexylamine)-dichlorodiacetatoplatinum(IV)] represents the most successful Pt(IV) prodrug design-by virtue of its improved stability and exhibits an antiproliferative profile distinct from cisplatin (Fig. 2.7). It is being evaluated in several clinical trials against hormone-refractory prostate cancer as well as advanced solid tumors [48]. The resulting X-ray crystal structure provided the first geometric information about an antitumor active monofunctional platinum-DNA adduct. This complex inhibits transcription at a level comparable to that of cisplatin as revealed by in vitro studies [49]. Like oxaliplatin, it is selectively taken up by cells bearing organic cation transporters. There has been growing interest in the development of asymmetric Pt(IV) complexes as a means to engineer multiple functionalities, while circumventing the constraints of limited conjugation sites. Asymmetric Pt(IV) complexes have different axial ligands, which can be exploited independently or co-operatively to introduce specific properties to the pharmacophore. They are distinct from classical symmetric Pt(IV) complexes, which are less versatile and limited because both functional groups are identical [47].

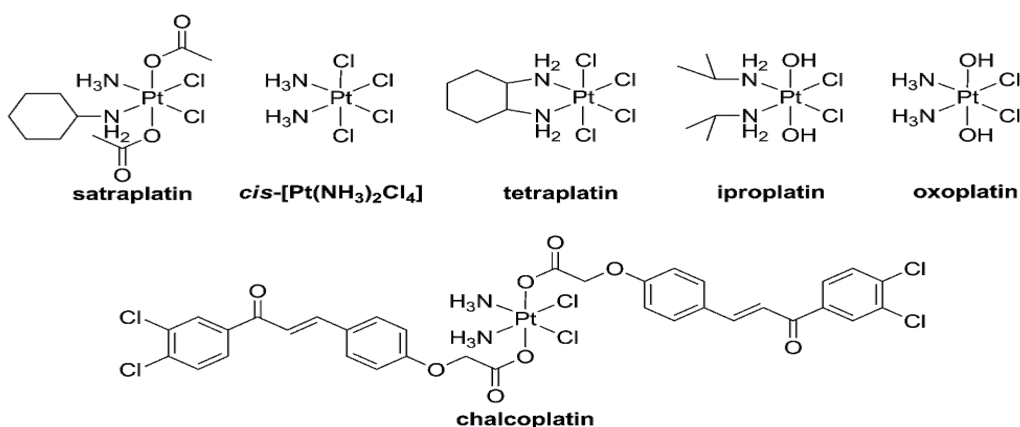


Figure 2.7: Asymmetric platinum(IV)[21]

2.5. Mechanism of action of platinum complex as an anti-cancer

The mechanism of action of platinum drugs (cisplatin, carboplatin and oxaliplatin), as well as structurally relevant platinum(II) complexes, approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), can be divided into four mechanisms: (i) cellular uptake, (ii) aquation/activation, (iii) DNA binding and (iv) cellular processing leading to apoptosis. Both of these processes will be treated in turn (Figure 2.2).

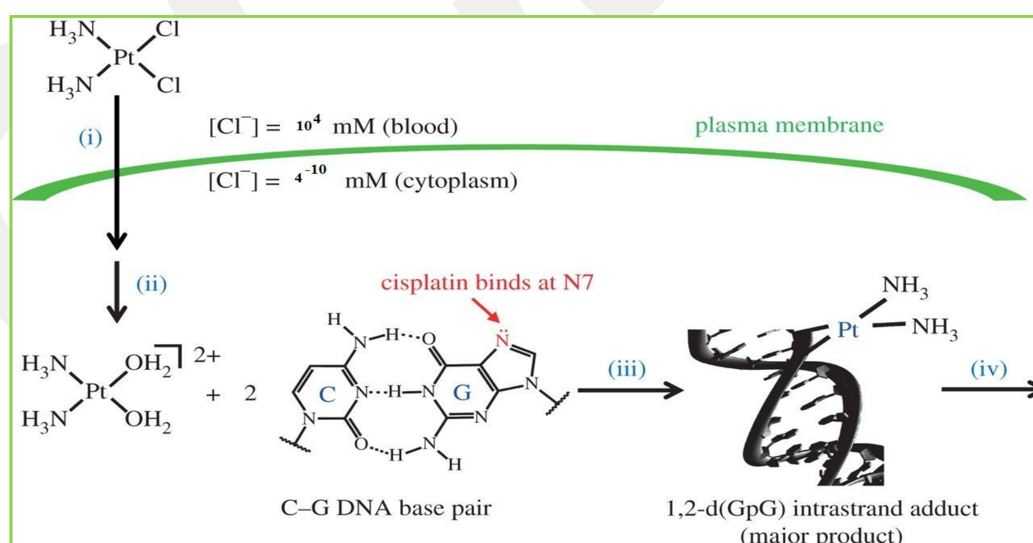


Figure 2.8: Mechanism of action of platinum complexes; (i) cellular uptake, (ii) aquation-activation, (iii) DNA binding and (iv) apoptosis cell[50]

2.5.1 Cellular Uptake

Platinum intravenous chemotherapy is administered as a saline solution to the mineral complex. The dissolved complex must pass through the cell membrane in order to interact with the target, DNA. Early reports provided conflicting data on cisplatin uptake on whether mediation was affected by passive or active transmission. It is currently accepted that some combination of the two most likely occurs [50].

Lately, much work has focused on the importance of copper carriers, such as CTR1, which can mediate absorption of cisplatin by yeast [51]. Although some evidence suggests that in human cells CTR1 cisplatin can be taken and delivered in a form capable of binding DNA and the release of apoptosis [52]. Other membrane proteins may also play a role in the absorption of platinum drugs and showed that overexpressing cells of OCT1 and OCT2 organic cation carriers were more sensitive to oxaliplatin than cisplatin [53]. This work with organic cation transporters led to the rediscovery and extension of anticancer activity of cation monofunctional compounds.

2.5.2. Aquation-Activation

Inside the cell, the platinum complex undergoes ligand substitution. For cisplatin, cytosolic chloride ion concentration that lowers (approx. 4 mM), compared with the extracellular matrix (approx. 100 mM). Inside the cell, the neutral cisplatin molecule undergoes hydrolysis, in which a chlorine ligand is replaced by a molecule of water, generating a positively charged species. Hydrolysis occurs inside the cell due to a much lower concentration of chloride ion (~3-20 mM) and therefore a higher concentration of water [54].

Inside the cell: $Pt^{II}(NH_3)_2Cl_2 + H_2O \rightarrow [\,Pt^{II}(NH_3)_2Cl(H_2O)]^+ + Cl^-$

$[Pt^{II}(NH_3)_2Cl(H_2O)]^+ + H_2O \rightarrow [Pt^{II}(NH_3)_2(H_2O)_2]^{2+}$

The aquation of cisplatin in the presence of DNA was measured by ^{195}Pt-NMR spectroscopy at 37°C. The half-life for aquation and DNA binding is therefore approximately 2 h [55]. Monofunctional and bifunctional are the number of covalent bonds formed between the platinum centre and DNA [56]. Carboplatin and

oxaliplatin are significantly more stable to aquation, as expected because of the chelating nature of the leaving group ligand. Carboplatin is stable for up to 60 days in water, but direct substitution of one of the carboxylates by nucleobases is possible without the need for an aquation intermediate [57]. It has been proposed that carbonate can activate carboplatin, but this mechanism is not operative with cisplatin. The aquation of oxaliplatin is also much slower than that of cisplatin [58].

2.5.3. DNA Binding

Inside the cell, cisplatin has a number of possible targets: DNA, RNA, sulfur-containing enzymes such as metallothionein and glutathione and mitochondria [59] (Fig. 2.9).

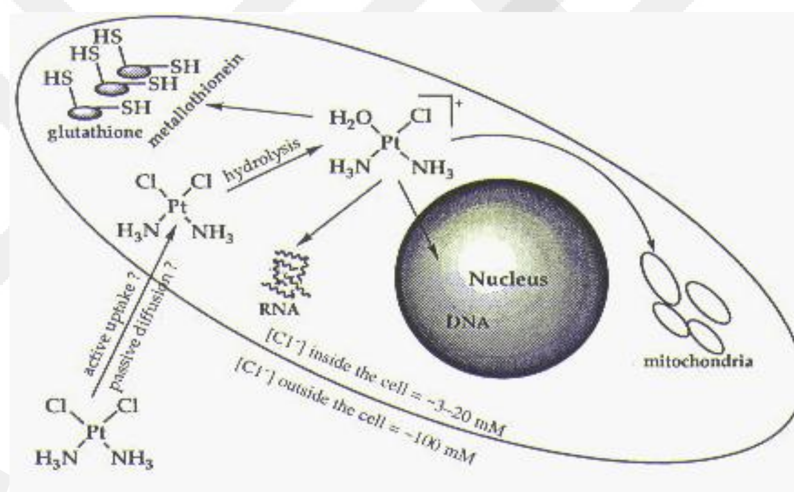


Figure 2.9: The targets of cisplatin binding inside the cell.

Generality, nucleophilic site on DNA is the N7 atoms of the purine, which is exposed in the major groove, and these sites are preferentially platinated [59] (Fig. 2.10).

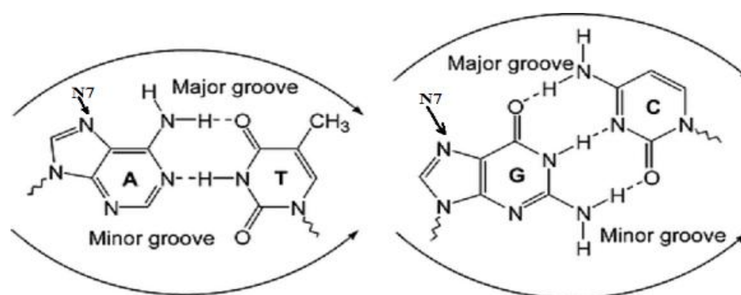


Figure 2.10: The DNA base pairs. Cisplatin coordinates to the N7 atoms of the purine (guanine and adenine) bases.

Many types of cisplatin–DNA coordination complexes, or adducts, can be formed. The most important of these appear to be the ones in which the two chlorine ligands of cisplatin are replaced by purine nitrogen atoms on adjacent bases on the same strand of DNA; these complexes are referred to 1,2-intrastrand adducts. The purine bases most commonly involved in these adducts are guanines; however, adducts involving one guanine and one adenine are also found. The formation of these adducts causes the purines to become destacked and the DNA helix to become kinked [60].

This feature arises from the propensity of cisplatin to form bifunctional cross-links on DNA. In vivo and in vitro, the major adducts are 1,2-intrastrand cross-links at the d(GpG) and d(ApG) sites (*cis*-{$Pt(NH_3)_2$[d(GpG)-N7(1),N7(2)]} and *cis*-{$Pt(NH_3)_2$[d(ApG)-N7(1), N7(2)]}) and they represent about 65 and 25% of the bound platinum respectively. monofunctional adducts coordinated to a single purine [61] (Fig. 2.11).

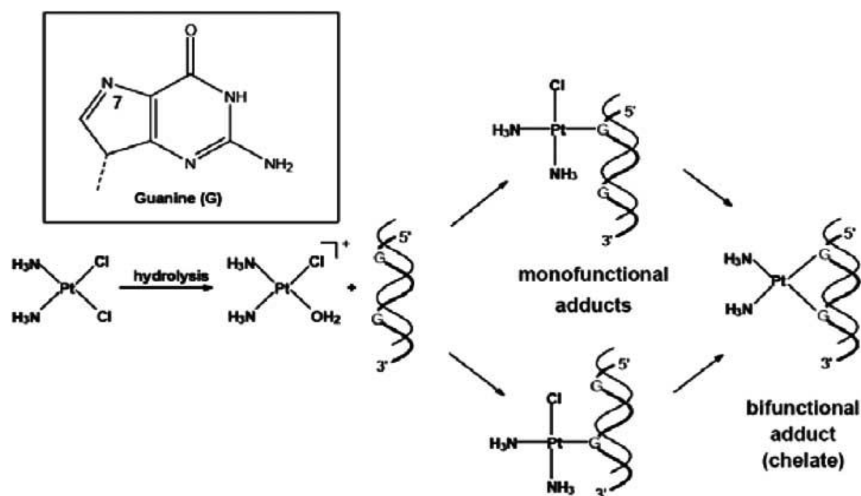


Figure 2.11: Pathways for GG intrastrand crosslinking of DNA by cisplatin. The insert shows the structure of guanine and the position of N7, the major Pt binding site[60].

2.5.4. Cellular Processing by DNA Cleavage

When damage occurs to DNA in cells, the common response is to stop the evolution of the cell cycle so that it can repair the damage before it passes to the daughter's cells, preventing the transmission of dangerous mutagenic lesions. [62] The DNA damage that results when cells are treated with cisplatin drives the cell cycle arrest at the G2 stage. Studies indicate that G2 arrest is necessary to induce apoptosis after cisplatin treatment [63]. Apoptosis is a controlled type of cell death which is energy-dependent leading to cell shrinkage, chromatin condensation, membrane budding, phosphatidylserine externalization, and activation of a family of cysteine proteases called caspases [64].

The main form of DNA repair used to remove cisplatin bifoncitoneal lesions is the repair of nucleotide excision (NER) [59]. This diagram uses for repair many proteins to remove a 30-mer oligonucleotide containing the platinated site and uses the complementary, now one stranded, DNA template for the new oligonucleotide synthesis to replace the excised piece [59-62]. If the damage can not be repaired, the cells initiate the process of apoptosis. P53 expression of pro-death genes including BAX increase after DNA damage, leading to release cytochrome C of mitochondria,

subsequent division of procaspase 9, activation of Caspases 3, 6, and 7. These Caspases decompose the cell components that are necessary to survive [64] (Figure 2.12).

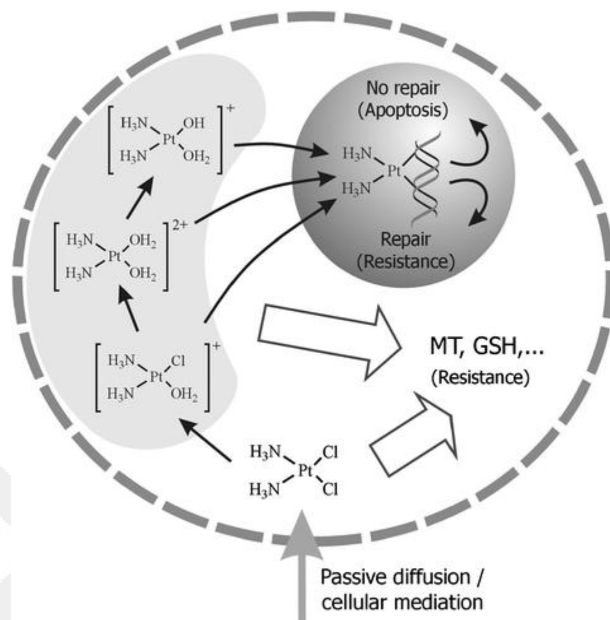


Figure 2.12: Cisplatin and DNA repair or apoptosis

2.6. Structural Aspects of Pt-Purine Interactions

The interaction of the widely used anticancer drug cisplatin with DNA bases was studied by extended X-ray absorption fine structure (EXAFS) and vibrational spectroscopy (FTIR, Raman and INS) both in the cisplatin–purine (adenine and guanine) and cisplatin–glutathione adducts [65]. The vibrational experimental spectra were fully assigned in the light of the calculated pattern for the most favoured geometry of each drug–purine adduct, and cisplatin's preference for guanine (G) relative to adenine (A) within the DNA double helix was experimentally verified: a complete N by S substitution in the metal coordination sphere was only observed for [cDDP–A2], reflecting a somewhat weaker Pt–A binding relative to Pt–G [66] (Fig. 2.13). The role of glutathione on the drug's pharmacokinetics, as well as on the stability of platinated DNA adducts, was evaluated as this is the basis for glutathione-mediated intracellular drug scavenging and in vivo resistance to Pt-based anticancer drugs. Spectroscopic evidence of the metal's preference for glutathione's

sulfur over purine's nitrogen binding sites was gathered, at least two sulfur atoms being detected in platinum's first coordination sphere [66].

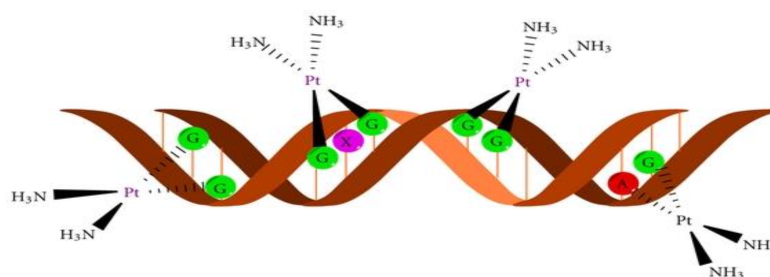


Figure 2.13: Pt-purine interactions

2.7. Effects of Platinum Binding on Nucleosome Structure

In eukaryotic organisms, ~80% of genomic DNA is wrapped in nucleosomes, which consists of 146 base pairs of DNA wrapped in a left-handed superhelix around a core of eight histone proteins [67]. It is therefore necessary to consider this component of the cellular environment when studying the interactions of platinum compounds with their biological target. chemical methods were used to study the structural effects of cisplatin binding on nucleosome structure and positioning [67] (Fig. 2.14).

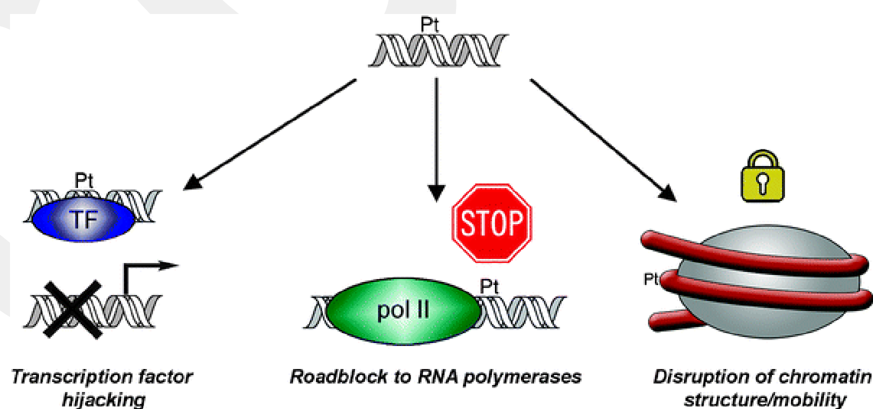


Figure 2.14: Effects of cisplatin binding on nucleosome structure[66]

Double-stranded DNAs containing a centralized site-specific 1,2-d(GpG) or 1,3-d(GpTpG) cross-link of cisplatin were reconstituted into nucleosome core particles

and analyzed by hydroxyl radical and exonuclease footprinting [68]. These investigations revealed that cisplatin intrastrand cross-links direct nucleosome positioning to a preferred rotational and translational setting, with the $\{Pt(NH_3)_2\}^{2+}$ moiety directed inwards toward the histone octamer protein core. This preferred position overrides that of strong native DNA positioning sequences and occurs in nucleosomes prepared from both native, containing a variety of post-translational modifications, and recombinant histones. Studies from another group demonstrated that cisplatin or oxaliplatin adducts inhibit ATP-independent nucleosome mobility in samples of nucleosome core particles treated with either drug [68]. These data demonstrate that platinum complexes influence not only the structure of the DNA double helix, but also that of nucleosomes.

2.8. Inhibition of Transcription by Platinum Anticancer Compounds

A key indication that cisplatin-DNA adducts inhibit transcription was uncovered when it was reported that G2 arrest of leukemia cells was required for apoptosis and that loss of DNA replication viability did not correlate with cell death. Prior to these results, inhibition of DNA replication had been widely considered to be a key to the mechanism of cisplatin cytotoxicity. These new data suggested that cells arrested in G2 phase because they could not synthesize mRNA necessary to pass into mitosis, implicating transcription inhibition as a critical determinant in the pathway of apoptosis triggered by cisplatin [69]. Numerous systems employing both site-specifically and globally platinated DNA templates, with both recombinant proteins and living cells, have been designed to study inhibition of transcription by cisplatin and other platinum anticancer agents. Taken together, the data clearly demonstrate that the ability of a platinum complex to block RNA synthesis correlates directly with its efficacy as an antitumor agent [70] (Fig. 2.15).

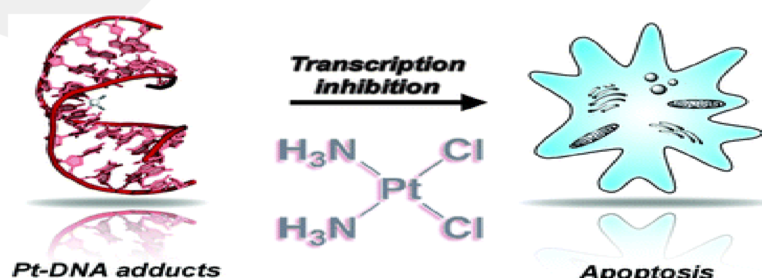


Figure 2.15: Inhibition of transcription[66]

2.9. Modification of Platinum Anticancer Complexes

During the last decades, a number of transition-metal complexes have been utilized to probe nucleic acid structures and in the development of DNA-cleaving agents, DNA photoprobes, DNA-molecular light switches and so forth. It has also been well documented that metal complexes can bind to DNA covalently as well noncovalently. Lippard pioneered work of the square-planar Pt(II) complexes with DNA [71], implied that non covalent interactions between transition-metal complexes and DNA can occur by intercalation, groove binding, or external electrostatic binding. As for cisplatin interaction with DNA bases, a great deal of theoretical calculations has also been published [72].

Conducting polymers have received considerable attention in recent years due to their attractive practical applications and numerous of them have been created to decrease the toxicity of platinum anticancer complexes. In this context, numerous investigations have focused on the synthesis of polymers with low bandgap. Polymers containing both thiophene rings and nitrogen containing heterocycles have recently been reported [73]. In particular, Kauffmann, et al. [74] reported the synthesis of 2,3-di(2-thienyl)quinoxaline, with 49% yield. Thienylquionoxalines 2 and 3 afford the opportunity to bind in either a metal-bridging and/or a bidentate (N, S) fashion by using the thiophene ring sulfur as well as the quinoxaline nitrogen. This (N, S) binding is not far-fetched; a few ligands similar in structure have shown to utilize both the N-containing and S-containing hetereocycle fragments when bound with a variety of metals [75] (Fig. 2.16).

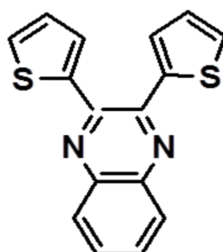


Figure 2.16: 2,3-di(2-thienyl)quinoxaline ligand

Many biological molecules may be targets for platinum compounds. Basic coordination chemistry knowledge predicts that S-donor ligands in proteins would rapidly bind and generate the most stable bonds. Also binding to lone-pairs of nitrogen atoms is known to be strong in the absence of S-ligands [76]. Consequently, these types of binding would involve amino-acid side chains from cysteine, methionine, histidine, and also the solvent exposed N(7) atoms of adenine and guanine in double-stranded DNA. There is a very strong (and rapid) interaction of Pt ions with S-donor ligands would leave no reactivity for N-donor ligands, with so many S-donors around in vivo. Nevertheless, the Pt-antitumor drugs do end up at N(7)-atoms of guanine [77].

The activity of sulfur towards platinum complexes has led to ameliorate the side effects of platinum therapy, without compromising its antitumor activity. These nucleophilic sulfur compounds include sodium thiosulfate (STS), sodium diethyldithiocarbamate (Naddtc), glutathione (GSH), methionine, thiourea, cysteine and sulfathiazole. The protective effect of these compounds is either due to prevention, or reversal of Pt-S adducts in proteins [78] (Fig. 2.17).

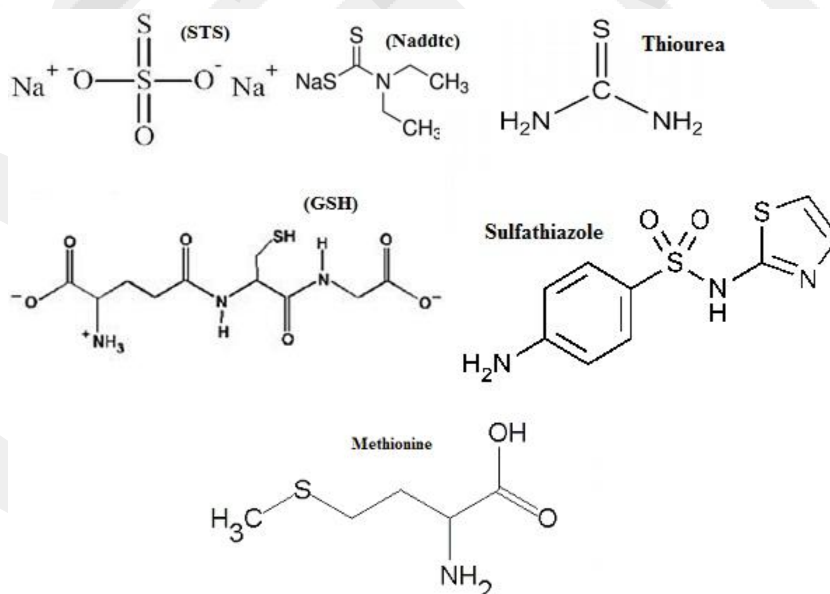


Figure 2.17: Structure of the nucleophilic sulfur compounds sodium thiosulfate (STS), sodium diethyldithiocarbamate (Naddtc), thiourea, glutathione (GSH), sulfathiazole and methionine[21].

Molecules sulfur-containing Endogenous and exogenous play an important role in the metabolism of platinum antitumor complexes. The binding of cisplatin to the intracellular thiol groups is known to be the cause of kidney toxicity and other side effects. Moreover, these interactions (platinum–sulfur) can have effects in medical application of Platinum-based drugs. It is now possible to remove sulfur compounds as chemo-protective agents to relieve serious toxic side effects [79].

STS was shown to provide protection from nephrotoxicity when administered in a period between 1 h prior to and 0.5 h after cisplatin injection. It has been shown that protein-bound cisplatin cannot be released by STS, although STS is able to break the Pt-thioether bond in methionine model systems. A likely explanation for its protecting effect is that STS is known to be concentrated extensively in the kidney, where it has been proven to react rapidly with cisplatin, thereby inactivating the drug locally [80].

Naddtc is effective in reducing several kinds of nephrotoxicity, as well as bone marrow toxicity, and, when administered 1 h to 4 h after cisplatin, it does not interfere with antitumor properties of cis-Pt. Naddtc is capable of reversing the Pt-methionine bond, yet incapable of reversing the Pt-cysteine bond [81].

The most important intracellular thiol GSH (glutathione) is present in varying concentrations (0.5 to 10 mM) in cells and has numerous cellular functions, including the detoxification of chemotherapeutic agents, and may play a role in modulating cisplatin cell sensitivity. GSH has recently also been shown to protect against cisplatin-induced toxicity in animal models. GSH was administered before cisplatin, and another study applied GSH injections prior to and after cisplatin. A clinical phase I study of cisplatin and GSH has shown that toxicity is significantly reduced in the case of ovarian cancer treatment with cisplatin. No significant changes in anti-tumor efficiency were observed, but renal toxicity was markedly reduced [82].

Together with thiourea, Naddtc is the only agent that results in protection against nephrotoxicity when administered after cisplatin treatment, at a time when most of

the reactive platinum species has already been taken up by cells or has been excreted through the urinary tract [83].

In this study, platinum complexes containing 2,3-di(2-thienyl)-quinoxaline in cis, asymmetric and bis geometry (Fig. 2.18) were synthesized, identified and their DNA binding ability are studied. The mode and extent of interaction of complexes have been determined by means of various spectroscopic as well as gel electrophoresis techniques.

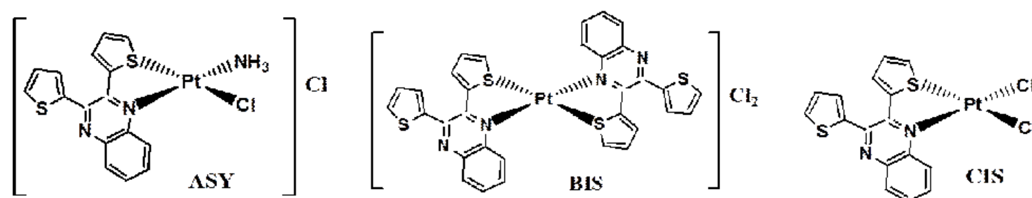


Figure 2.18: Structure of ligand containing platinum(II)complexes in asymmetric; bis and *cis*-geometry

CHAPTER 3

EXPERIMENTAL PART

3.1. Material

3.1. Instruments and Apparatus

The glassware (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes) were autoclaved at 121°C for 20 min, and instruments and apparatus which used throughout the experiments was carefully sterilized.

3.1.2. Chemicals

The pure and analytical grade chemicals were used as purchased in all experiments

3.1.3. Complexes Preparation

3.1.3.1. 2,3-di(2-thienyl)-quinoxaline ligand (L)

The ligand was prepared using the method of researcher Dr. Hüseyin Karaca and his group at the University of Sakarya, [84] and identified by 1H-NMR spectrum (Fig.B1).

^{1}H-NMR spectrum: δ: 7.06 (dd, 2H, H-4,4′); 7.17 (dd, 2H, H-3,3′); 7.75 (dd, 2H, H-5,5′); 7.79 (m, 2H, C_6H_4); 8.00 (m, 2H, C_6H_4)

3.1.3.2. [Aminochloro(dithienylquinoxalino)]platinum(II)chloride. monohydrate $[Pt(NH_3)Cl(L)]Cl.H_2O$ (*Asy*)

100 mg (0.241 mmol) K_2PtCl_4 was dissolved in 5 mL double distilled water. 40.7 mg (0.241 mmol) $AgNO_3$ was added directly to this aqueous platinum solution as shown

in (Fig.3.1). This solution was stirred at room temperature in the dark at night. Then, a precipitate AgCl was removed by filtration. 17.34 µl NH_3 (0.241mmol) was added to the clear yellow-color filtrate and stirred at room temperature for 1 hour. 10 mL DMF solution of 0.071 g (0.241mmol) ligand was added dropwise into the mixture above with stirring. After that, the resultant solution was refluxed at 40°C for 24 hours. During reflux, the solution color was changed from yellow to brown and a brown deposit was observed. The brown precipitate was collected under vacuum and dried at room temperature. The yield of complex is 61.66%.

Elemental Analysis: $[PtC_{16}H_{13}N_3S_2Cl_2].H_2O$: C, 32.67; H, 2.033; N, 6.18; S,10.38. Found: C, 32.32; H, 2.53 ; N, 7. 05; S, 10.77. MS(EI) m/z= 595 [M]; HRMS (EI) calcd: 595.4374, found: 595.3842.

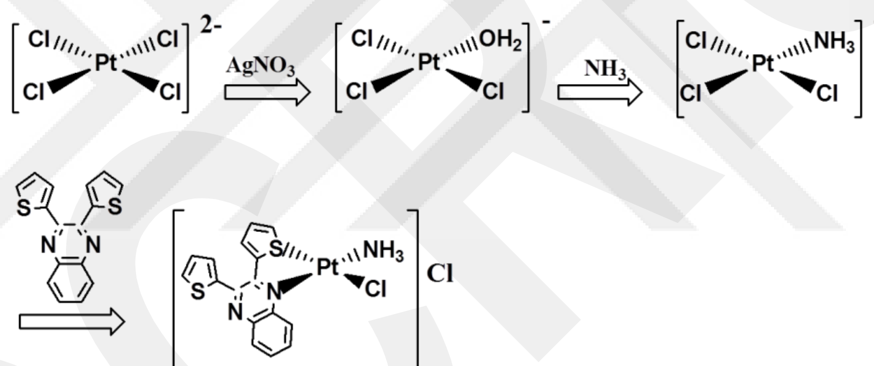


Figure 3.1: Asymmetric complex

3.1.3.3 *Bis*-[dithienylquinoxalino)]platinum(II)chloride:$[Pt(L_2)]Cl_2$. (*Bis*)

100 mg (0.241 mmol) K_2PtCl_4 was dissolved in 5 mL double distilled water. 163.7 mg (0.964 mmol) $AgNO_3$ was added directly to this aqueous platinum solution. This solution was stirred at room temperature in the dark at night. Then, precipitate AgCl was removed by filtration. 5 mL DMF solution of 0.1418 g (0.482 mmol) ligand was added dropwise into this mixture above with stirring. Then the solution was linked to a reflux condenser and refluxed at 40°C for 24 hours. The composition of the brown deposit was observed. The brown precipitate was collected under vacuum and dried at room temperature. The diagram is shown in (Fig.3.2) for these synthesis steps. The yield of complex is 31.93%.

Elemental Analysis: $PtC_{32}H_{20}S_4N_4Cl_2$: C, 44.36; H, 2.36; N, 6.55; S, 15.01. Found: C, 44.38; H, 2.701; N, 7.07; S, 15.15. MS(EI) m/z= 855 [M]; HRMS (EI) calcd: 854.7878, found: 854.6965.

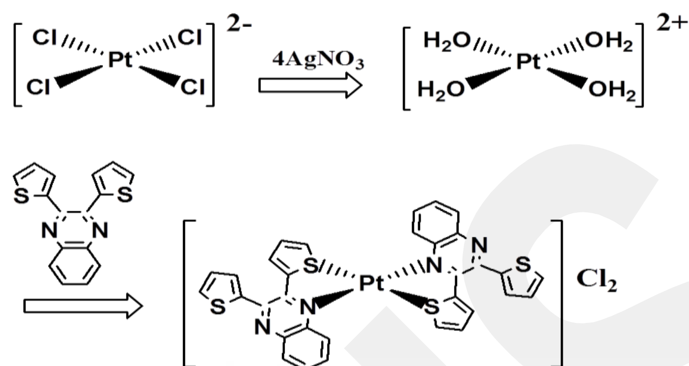


Figure 3.2: ***Bis*** **complex**

3.1.3.4. *Cis*-[Dichloro(dithienyl quinoxalino)]platinum(II):[$PtCl_2$(L)] (*Cis*)

As clearly observed in (Fig. 3.3), to the 3 mL DMF solution of 0.0694 g (0.241 mmol) ligand and 5 mL aqueous solution of 100 mg K_2PtCl_4 (0.241 mmol) was added dropwise. An immediate yellow precipitation was observed. The suspension was refluxed for 24 h at 40ºC, and the color of the precipitated turned to brown. Then, the dark brown precipitate was collected under vacuum and dried at room temperature. Yield of complex is 49.2%.

Elemental Analysis: $PtC_{16}H_{10}N_2S_2Cl_2$: C, 34.30; H, 1.799; N, 4.99; S, 11.44. Found: C, 34.38; H, 1.89; N, 4.91; S, 11.96. MS(EI) m/z= 561 [M+1]; HRMS (EI) calcd: 561.3208, found: 561.0386.

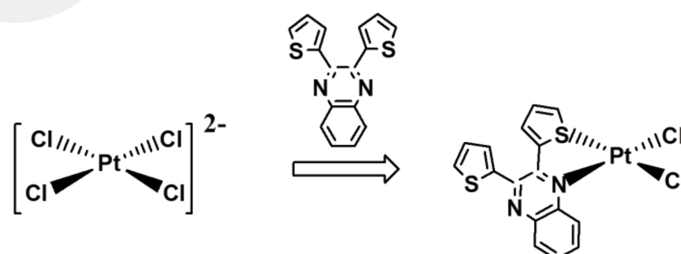


Figure 3.3: ***Cis*** **complex**

3.1.4. Solubility

Platinum(II) complexes "Asy, Bis and Cis" are soluble in acetone, while all of them slightly soluble in acetonitrile, and very very soluble in DMF. And the platinum(II) complex in Cis geometry is slightly soluble in ethanol, whereas platinum(II) complexes in Bis and Asy geometry are non soluble in ethanol. Lastly, All the platinum(II) complexes are nonsoluble in methanol and H_2O.

Table 3.1: Solubility of the complexes

Sample	AN	H_2O	EtOH	MeOH	Acetone	DMF
Asy	SS	NS	NS	NS	S	VVS
Bis	SS	NS	NS	NS	S	VVS
Cis	SS	NS	SS	NS	S	VVS
S: Soluble; SS: Slightly Soluble; VS: Very Soluble; VVS: Very Very Soluble and NS: Non Soluble						

3.2. Methods

3.2.1. UV-Tiration

The complexes were dissolved in a solvent mixture of DMF and Tris–HCl buffer (5 mM Tris–HCl; 50 mM NaCl, pH 7.11). Electronic absorption spectra were observed using a HP Agilent®8453 Spectrophotometer. This was followed by a change in the electronic absorption spectra of the complexes by maintaining the concentration of constant complexes with increased Calf Thymus DNA concentration (CT-DNA) (R=[DNA]/[Complex]=0-10).

Table 3.2.: Concentration of the platinum complexes and CT-DNA

Complexes	Concentration (M)		Incubation Time (min)
	Complexes	Calf thymus DNA	
Asy	3.0134×10^{-3}	$0\text{-}9 \times 10^{-4}$	45
Bis	3.6266×10^{-4}	$0\text{-}8.0 \times 10^{-4}$	30
Cis	2.0×10^{-3}	$0\text{-}5.35 \times 10^{-4}$	45

Better incubation time for the DNA-Drug interaction was observed in (Table 3.4) at 37°C spectroscopically for each Pt(II) complexes.

The intrinsic binding constant (K_b) of the three complexes with CT-DNA was determined at 430 nm using the following equation [85].

$$[DNA]/(\varepsilon_a-\varepsilon_f)=[DNA]/(\varepsilon_b-\varepsilon_f)+1/K_b[(\varepsilon_a-\varepsilon_f)] \quad (1)$$

Where:

ε_a = the apparent extinction coefficient, is expressed as $A_{obs}/[Pt]$.

ε_b and ε_f represent the extinction coefficients of free and the bound complex, respectively.

K_b = calculated from the ratio of the slope to the intercept obtained in a plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ *vs* [DNA].

For the purpose of understanding the nature of interaction between the three complexes and CT-DNA, thermodynamic experiments were performed and the temperature dependent binding constant Kb was determined at 310, 320, 330 and 340 K.

3.2.2. Measurement of Viscosity

Viscosity measurements were made using a viscometer (AND® SV-10 VIBRO Viscometer) at room temperature. The concentration of the complexes were varying between 0-70 µM while ct-DNA concentration was maintained constant at 50 µM in 1:1 (V/V) 5mM Tris HCl:50 mM NaCl buffer mixture at pH=7.11. Viscosity of the CT-DNA in presence (η) and the absence (η_o) of the complex was measured automatically. Data displayed $(\eta/\eta_o)^{1/3}$ versus 1/R.

3.2.3. Fluorescence Titration

Fluorometric measurements were performed with Thermo Scientific® Lumina Fluorescence Spectrometer by maintaining the concentration ethidium bromide (EB) pretreated CT-DNA concentration constant by varying the complexes concentration at 1:1 (V/V) 5mM Tris HCl:50 mM NaCl buffer mixture at pH=7.11.The extent of fluorescence quenching of EB bound to CT-DNA can be used to determine the extent of binding between the second molecule and CT-DNA. The fluorescence spectra of the ethidium bromide were measured using an excitation wavelength of 478 nm and the emission range was set between 500 and 800 nm by luminous software wave scan. The spectra were analyzed according to Stern-Volmer Eq Classic. (2) [86].

$$\mathbf{I_0 / I = 1 + K_{sv} . r} \qquad \mathbf{(2)}$$

Where

I_0 and **I** = are the fluorescence intensities at 608 nm in the absence and presence of the quencher, respectively.

K_{sv} = is the linear Stern–Volmer quenching constant.

r = is the concentration of the quencher.

2.2.4. Thermal Denaturation

Thermal denaturation studies were conducted with Agilent®8453 Spectrophotometer equipped with HAAKE temperature-controlled circular bath. Samples were prepared using 1:1 (V/V) 5mM Tris HCl:50 mM NaCl buffer mixture at pH=7.11.

Absorbance was measured at 260 nm in the presence and absence of the complexes. During the measurements the CT-DNA concentration was kept constant at 11.1 μM and the concentration of the complexes ranged between 10-160.1μM. The solution temperatur was increased by 2°C min^{-1}.

2.2.5. Cytotoxicity of the Platinum complexes

The cells were classified on 96-well plates and cover slips and incubated for 24 hours, under cell culture conditions. The platinum complex solutions were freshly prepared at concentrations 3 to 100 μM and added on to the cells, which were incubated again for 24, 48, 72 hours. Cell viability was determined using WST-1 agent(4-[3-[4-iodophenyl]-2–4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate]) (Roche, Cat # 11644807001) as described in [87].

CHAPTER 4

RESULTS AND DISCUSSION

4.1. The Spectral Identification of the Complexes

4.1.1. Electronic Absorption Spectrum

The electronic spectra of the platinum(II) complexes "Asy, Bis and Cis" were taken in DMF and display several bands which were assigned to various transitions on the basis of their absorption wavelength and molar absorption coefficient were given in (Figure 4.1 and Table 4.1).

Table 4.1: Electronic absorption spectral data for the platinum(II) complexes in DMF

Band No.	$\overline{\nu}$ (cm^{-1})	$\lambda_{(nm)}$	$\varepsilon_{(M^{-1}cm^{-1})}$
Asy complex			
I	2617.80	382	16059.45
II	3448.28	290(sh)	5903.17
Bis complex			
I	2645.50	380	3360
II	3448.28	290(sh)	5170
Cis complex			
I	2325.58	430	5903.71
II	3278.69	305 (sh)	9343.678
sh: shoulder			

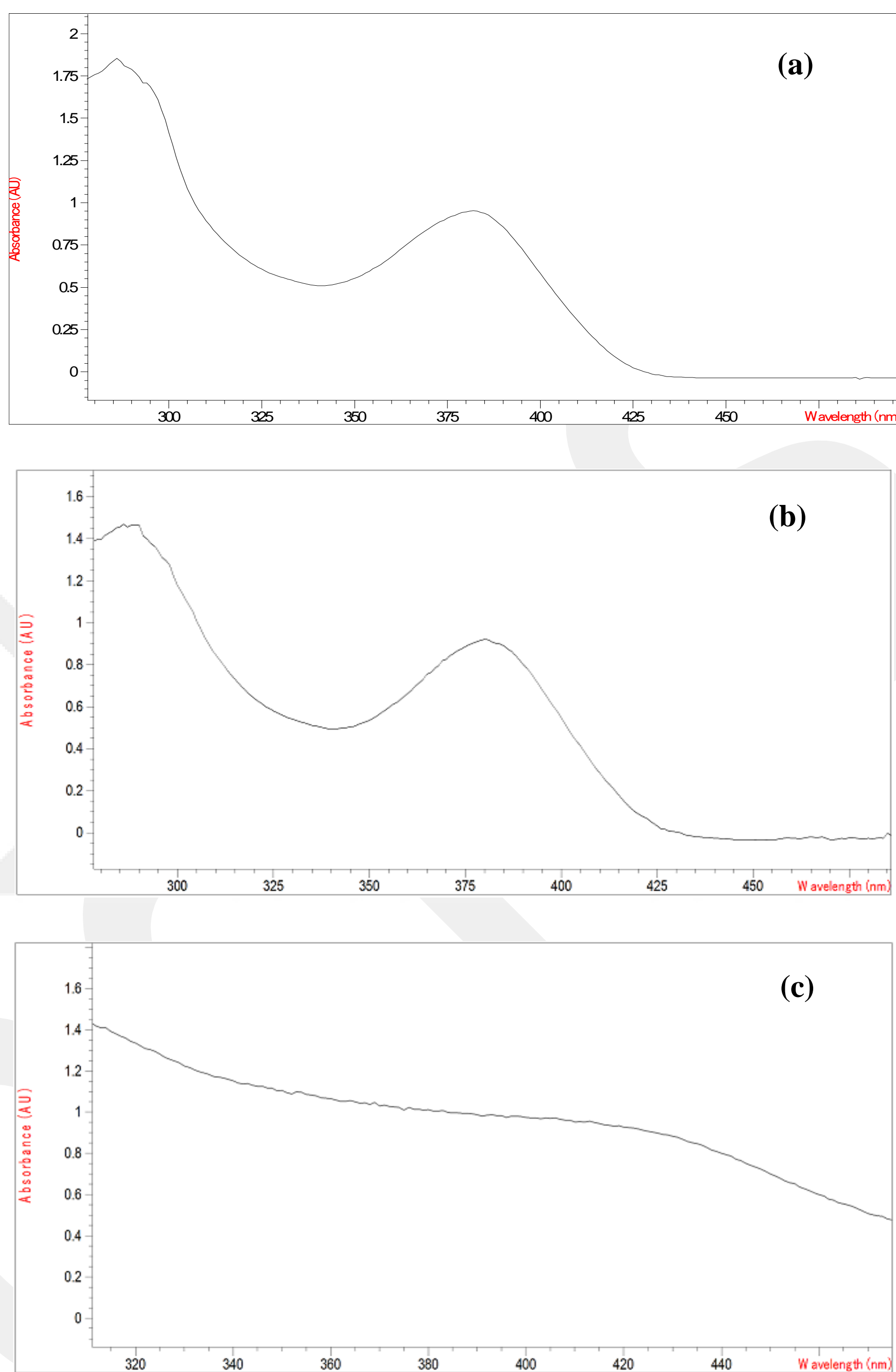


Figure 4.1.: Electronic absorption spectrum of the Asy (a), Bis (b) and *Cis (c)* complexes in DMF

The electronic absorption spectrum displays high intensity peaks in the UV regions at 290- 305 nm which are assigned to ligand field n→π* transition of C=N, C=C and C=S chromophores [88, 89]. The transitions observed at 380 and 382 nm in the spectra of Asy and Bis complexes (Fig.4.3) can be assigned to metal to ligand charge transfer (MLCT)transitions [89, 90]. The band observed at 430 nm in the electronic absorption spectrum of Cis complex, on the other hand, is assigned as the combination of MLCT and d-d transition [90, 91].

4.1.2. FTIR Spectrum

Infrared spectra were recorded at room temperature and selected vibrational frequencies are given in Table 4.2 for the 2,3-di(2-thienyl)-quinoxaline ligand and its *Asy*, *Bis* and *Cis*-platinum(II) complexes.

The main absorption peak of quinoxaline ligand is obtained in the range of 1660-1129 cm^{-1} as shown Figure A1. The aromatic $\nu_{C=N}$ vibration frequency of the ligand is captured at around 1600 cm^{-1} and the $\nu_{C=C}$ stretching vibration frequency of the aromatic group in the quinoxaline structure is obtained at around 1517 cm^{-1} [88, 90, 92]. Similarly, the aromatic ν_{C-N} vibration appears at 1120 cm^{-1} (Table 4.2). The bands appeared at around 3048 and 3080 cm^{-1} in the spectrum of the ligand considered as the aromatic ring $\nu_{(C-H)}$ vibration frequency. The $\nu_{(ph)}$ stretching vibration frequency of the quinoxaline structure is observed at around 1474 cm^{-1} [92-93]. $\delta_{(C\text{-}H\ out\ of\ plane\ ring)}$, $\delta_{(C\text{-}C\ out\ of\ plane)}$ and $\delta_{(C\text{-}C\ in\ plane)}$ stretching frequencies are observed at around 1061-981 cm^{-1}, 704-760 cm^{-1} and 1338 cm^{-1}, respectively. The absorption peak at about 840 is due to the $\nu_{(C\text{-}S\text{-}C\ ring)}$ vibration and the band at 1422 cm^{-1} is attributed to the $\delta_{(C\text{-}CH\ in\ plane)}$ vibrations [93].

In the IR spectrum of the *Asy*, *Bis* and Cis complexes (Table 4.2, Figure A2-A4), the multiple weak bands observed at around 3100-3000 cm^{-1} is concerned as aromatic C-H stretching vibrations. The aromatic C-H bending modes of benzene and its derivatives are generally captured in the range of 1300 cm^{-1} [93, 94] as observed in the ligand IR spectrum. On the other hand, the aromatic C-H vibration of all the platinum complexes is shifted to the region of 1230 cm^{-1}. The C-H out of plane bending vibrations is

generally in medium intensity and absorbs in the range of 900-600 cm^{-1} [94]. In the spectrum of the platinum complexes, such absorption bands are obtained in the region of 1063-938 cm^{-1}. The $\nu_{C=N}$ stretching vibration frequency of the aromatic group is observed at around 1650-1640 cm^{-1} for the platinum complexes. The observed 50 cm^{-1} deviation in the $\nu_{C=N}$ stretching vibration frequency of the aromatic group suggest the coordination of the ligand to the platinum(II) metal ions clearly. Typically, the shift occurred in ν(C-S-C) vibration frequency of thienyl group is other verification for the formation of the complex. The ν(C-S-C) vibration bands are observed at around 845-847 cm^{-1} with a 5-7 cm^{-1} deviancy for all the platinum complexes. The aromatic $\nu_{(C=C)}$ vibration frequencies, on the other hand appears at around 1521 and 1518 cm^{-1}. The absorption bands at around 3100-3000 cm^{-1} are related to the aromatic ν(C-H) vibration frequencies of the complexes [93-94]. The presence of the crystal water in the structure of *Asy* complex is confirmed by means of the broad $\nu_{(HOH)}$ vibration observed at around 3441 cm^{-1}. Unfortunately, this broad band obscures the $\nu_{(N-H)}$ vibration in the FTIR spectrum of the *Asy* complex.

As a conclusion; careful inspection of the FTIR spectra of the complexes and the ligand clearly reveals that the vibration frequencies of aromatic C=N and C-S-C groups deviates at least 10 cm^{-1} with the coordination of the ligand to the platinum(II) ion through its donor atoms, nitrogen and the sulphur, as expected.

Table 4.2. Selected infrared vibration frequencies (cm^{-1}) for the ligand and the platinum complexes.

Frequencies	*L* (cm^{-1})	*Asy* (cm^{-1})	*Bis* (cm^{-1})	*Cis* (cm^{-1})
$\nu_{(C=N)}$ aromatic	1600	1637	1658	1636
$\nu_{(C=C)}$ aromatic	1517	1521	1518	1560
$\nu_{(C-N)}$ aromatic	1129	1133	1125	1131
$\nu_{(Ar-H)}$	3080 3048	3093 3055	3105 3081 3064	3074 2925
$\nu_{(ph)}$	1474	1476	1474	1486
$\nu_{(C-H)}$ aromatic	1297	1230	1232	1230
$\delta_{(C-CH)}$ in plane	1422	1423	1420	1428
$\delta_{(C-H)}$ in plane bending	1338	1331	1331	1325
$\delta_{(C-C)}$ out of plane	760 704	762 711	760 728	759 717
$\delta_{(C-H)}$ out of plane ring	1061 1013 981	1061 938	1063 1044 982	1056 1033 954
$\nu_{(C-S-C)}$ ring	840	845	847	844
$\nu_{(HOH)}$		3441		

4.1.3. ^{1}H-NMR Spectrum

Proton NMR of 2,3-di(2-thienyl)-quinoxaline [95], and its *Asy*, *Cis* and *Bis* complexes is taken in d-DMSO. The chemical shifts of the complexes are shown in the Table 4.3 and 1H-NMR spectrum of complexes and the ligand is given in Appendix B. A slight deviation in the chemical shifts of the quinoxaline ligand refers to the coordination of the ligand to the platinum(II) ion observed through S and N donor groups [88].

3.1.4. Mass Spectrum

The concentration of the molecular ions in the platinum complexes determines the molecular weights; *Asy* complex; m/z = 595 [$M^+.H_2O$]; *Bis* complex m/z=855 [M^+] and *Cis* complex m/z=561 [M^++1]. The mass spectrum of the complexes are given in the Appendix D and Table (4.3).

Table 4.3. ^{1}H-NMR and Mass Spectral data for the ligand and the complexes

	^{1}H-NMR (d-DMSO)	Mass (m/z)
L	δ: 7.06 (dd, 2H, H-4,4′); 7.17 (dd, 2H, H-3,3′); 7.75 (dd, 2H, H-5,5′); 7.79 (m, 2H, C_6H_4); 8.00 (m, 2H, C_6H_4)	-
Asy	δ: 7.13 (2H, H-4,4′); 7.24 (dd, 2H, H-3,3′); 7.83 (dd, 2H, H-5,5′); 7.89 (m, 2H, C_6H_4);; 8.06 (2H, C_6H_4); 2.51 (H-NH_2)	595 [$M^+.H_2O$]
Bis	δ: 7.19 (2H, H-4,4′); 7.24 (dd, 2H, H-3,3′); 7.83 (dd, 2H, H-5,5′); 7.89 (m, 2H, C_6H_4);; 8.06 (2H, C_6H_4)	855 [M^+]
Cis	δ: 7.29 (2H, H-4,4′); 7.62 (dd, 2H, H-3,3′); 7.78 (dd, 2H, H-5,5′); 7.84 (m, 2H, C_6H_4); 7.97 (2H, C_6H_4)	561 [M^++1]

4.2. DNA – Binding Activity

4.2.1. Electronic Absorption Spectroscopy

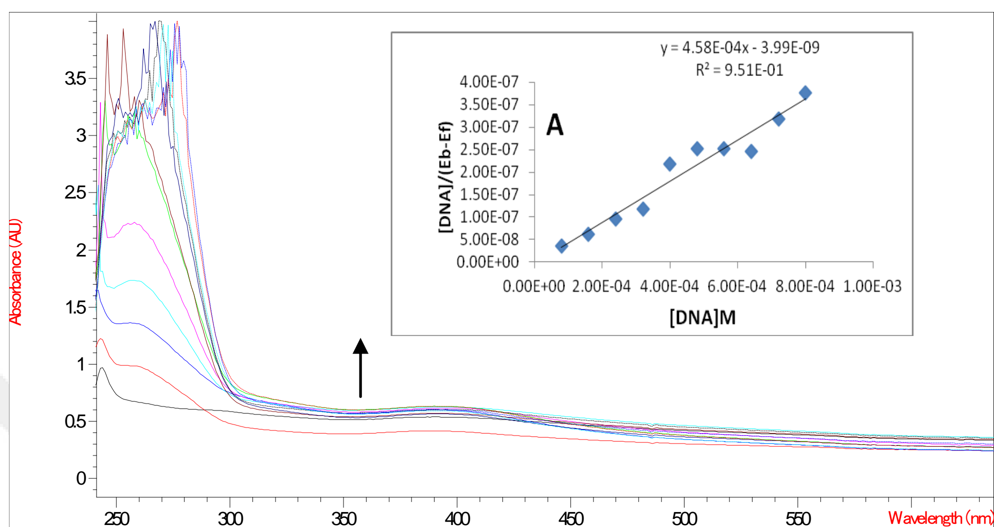
The interactions of metal complexes with DNA have been the subject of interest for the development of effective chemotherapeutic agents. Transition metal centers are particularly attractive moieties for such research since they exhibit well-defined coordination geometries and also often possess distinctive electrochemical or photophysical properties, thus enhancing the functionality of the binding agent [96].

The electronic spectra of the 2,3-di(2-thienyl)quinoxalin coordinated platinum(II) complexes upon the addition of DNA are shown in (Fig.4.4). The titrations results showed clearly that with increasing concentration of DNA added to the complexes, significant hyperchromism with red shift was observed for the Asy and Cis complexes. This can be attributed to a strong interaction between DNA and complexes [97].

Based on the results obtained from the UV-vis titration, it is inferred that the complexes underwent a non-intercalative mode of binding with DNA. Generally, hypochromism and hyperchromism are the two spectral features which are closely connected with the double helix structure of DNA. The observation of hypochromism is indicative of an intercalative mode of binding of DNA to the complexes along with the stabilization of the DNA double helix structure. On the other hand, the observation of hyperchromism is indicative of an electrostatic interaction. Hence, the observation of hyperchromism with red shift for our complexes showed that the new complexes interact with the secondary structure of CT-DNA electrostaticly [97].

UV titration experiments cannot be performed with the *Bis*–complex as a result of the solubility problem in tris-HCl buffer.

The intrinsic binding constant (K_b) were calculated (from the ratio of slope to intercept by using the plots of [DNA]/(ε_a–ε_f) versus [DNA]) (Fig. 4.4) for platinum(II) complexes are varies in the range of 4.27 x10^4 and 2.50x10^3 for Asy and *Cis* complexes respectively (Table 4.4).



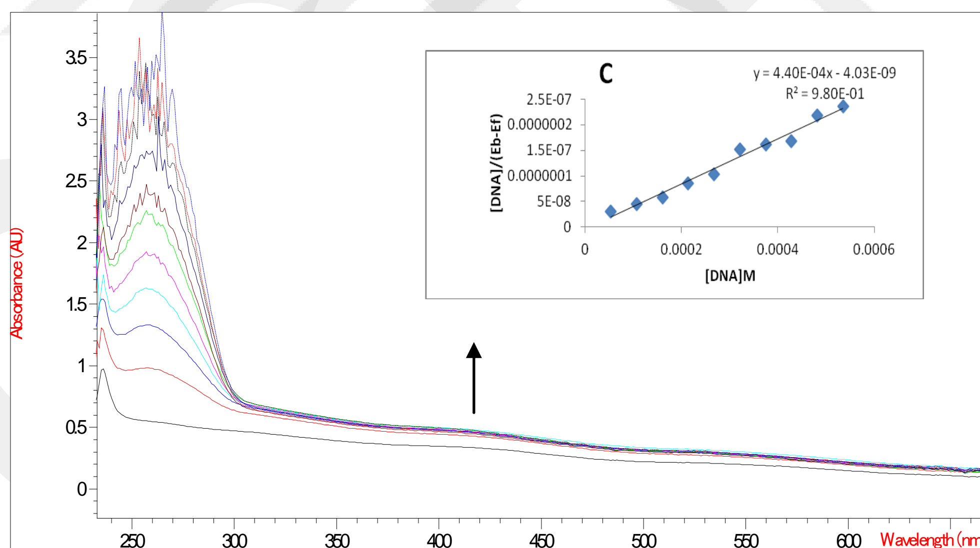


Figure 4.2: Electronic absorption spectra of *Asy* (A) and *Cis* (C) complexes respectively in Tris-HCl buffer (pH = 7.11) in the absence and in the presence of increasing amounts of CT-DNA

The significant difference in DNA-binding affinity of the platinum(II) complexes can be understood as a result of the fact that the complex with different ligands shows stronger binding affinity with [98]. Interestingly, these values are lower than the values of K_b mentioned in the classica literature or assays such as ethidium bromide (K_b =7x10^7 M^{-1}) [99]. On the other hand, the K_b value of the complexes is close to that of proflavin (K_b=4.1x10^5 M^{-1}) which is cited as intercalator [100] and at least ten times higher than K_b values or same groove binder complexes [101]. However, these results clearly indicate that Asy and Cis complexes are bind to DNA by an electrostatic or groove binding method.

To obtain a detailed view of the interaction between the platinum complex and DNA, a powerful approach is to parse the free energy of the binding reaction into its component terms. The four classes of noncovalent interactions that can play a role in the binding of drug molecules to biomolecules are hydrogen bonds, van der Waals forces, electrostatic interactions, and hydrophobic bond interactions [102].

In the intercalation process, a planar aromatic chromophore is inserted between two adjacent base pairs in a DNA helix. Alternatively, in minor groove binding, an isohelical drug molecule binds in the minor groove of DNA without inducing significant structural changes in the DNA [101]. While the complex formed from intercalation is stabilized by hydrophobic interactions and van der Waals forces, the complex formed from minor groove binding is stabilized mainly by hydrophobic interactions [103].

Consequently, in this study, the formation constant for complex-DNA adduct formation is evaluated allowing for the determination of thermodynamic parameters such as enthalpy (ΔH^o) and entropy (ΔS^o) by the Van’t Hoff equation.

$$\mathbf{Ln(K'_b) = (-\Delta H^o/RT) + (\Delta S^o/R)}$$

Binding constant (K'_b) values was calculated and data is depicted in (Table 4.4). The standard Gibbs Free energy change (ΔG^o) was calculated by using the equation:

$$\mathbf{\Delta G^o = -R\ T\ Ln(K_b')}$$

Where:

R and T represent the gas constant (8.314 J/mol.K) and the temperature (K)

The thermodynamic parameters (ΔH^o) and (ΔS^o) were calculated from these binding constants. Ross and coworkers [102] reported that when (ΔH^o) and (ΔS^o), the electrostatic force dominates the interaction; when (ΔH^o) and (ΔS^o), van der Waals interactions or hydrogen bonds dominate the reaction; and when (ΔH^o) and (ΔS^o), hydrophobic interactions dominate the binding process.

Table 4.4. $\Delta G°$, ΔH^o and ΔS^o data for *Asy* and *Cis* complexes

	Temperature(°C)	K'_b (M^{-1})	ΔG°(kJ)	ΔH° (kJ)	ΔS° (J/K)
Asy Complex	37	4.27E+4	-3290.6574		
	47	6.80E+4	-4362.2565	-3.82444	21.2007
	57	1.15E+5	-55387.816		
	67	1.53E+5	-6672.9624		
Cis Complex	37	2.50E+03	-20165.2	-103.526	402.3228
	47	1.60E+04	-25. 7544		
	57	1.00E+05	-31.5871		
	67	6.67E+04	-31.3981		

The negative value of ΔG^o revealed the interaction process is a spontaneous process. The (ΔH^o) and (ΔS^o) values of the complex-DNA adduct were -3.8244 to -103.526 and 21.2007 to 402.3228 J/molK for *Cis* and *Asy* complexes, respectively. From the thermodynamic data, it is quite clear that the DNA-complex adduct formation is both enthalpy and entropy favored [104].

4.2.2. Thermal Denaturation

The consequences of adduct formation on the stability of the double helix in CT-DNA were assayed by recording the DNA melting profiles. Thermal behavior of DNA in the presence of complexes can give insight into their conformational changes when temperature is raised and offer information about the interaction

strength of complexes with DNA. The stabilization of CT-DNA through the hydrogen-bonding and electrostatic interactions of the noncovalent complexes was further assessed by measuring the melting temperature [105].

We measured the changes in the absorbance at 260 nm as a function of temperature for DNA in absence and presence of complex. Our experiments were carried out for CT-DNA in the absence and presence of different amounts of platinum complex. the temperature was scanned from 30 to 90ºC. The denaturation temperatures were measured in 5mM Tris-HCl buffer solutions pH 7.11 containing (3.33 µM DNA) almost equal 82℃ in the absence of complex (Fig.4.5).

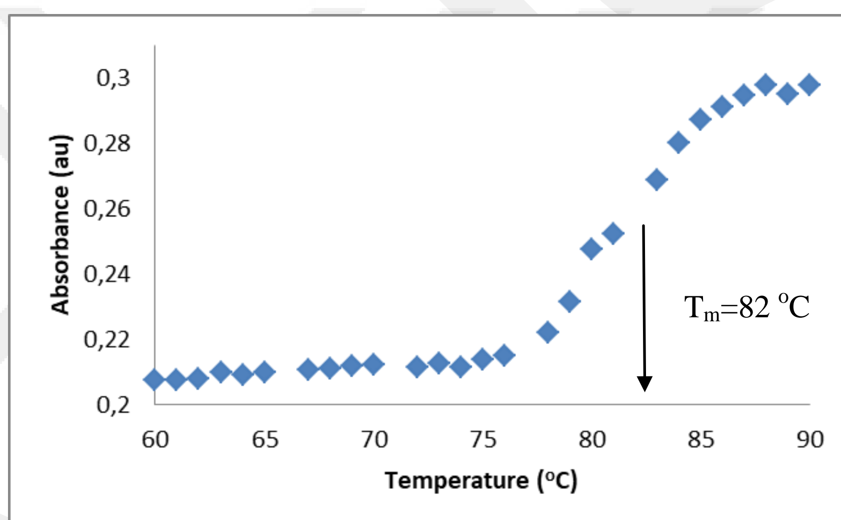


Figure 4.3: Thermal denaturation of DNA

Decrease of (1℃) was observed in the T_m profile of *Asy* complex whilst a same temperature (82 ℃) for *Cis* complex as compare to that of free DNA (Fig.4.6). Usually, the change in a melting point of a few degrees Celsius is considered as an interaction involving groove binding and/or electrostatic binding to the phosphate group [106-107]. Therefore, it can be suggested that the pre-melting effect our complexes is weak and they reacted with the phosphate groups electrostatically or performs groove binding through the double helix.

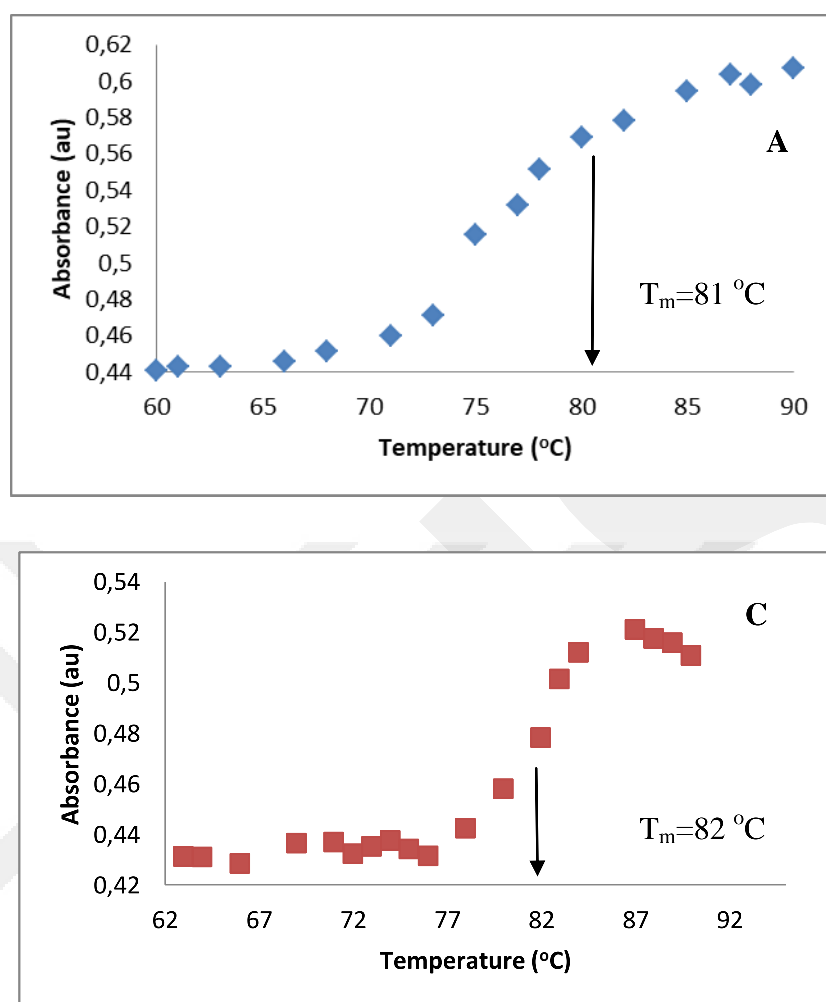


Figure 4.4: Thermal denaturation for *Asy* (A) and *Cis* (C) complexes

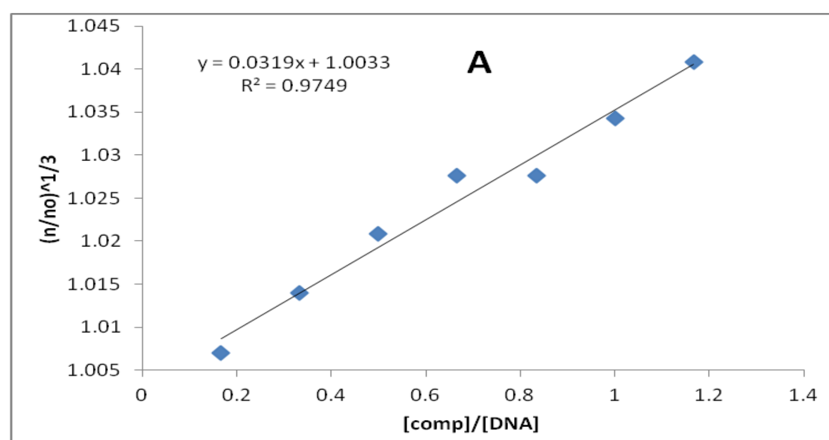
3.2.3. Viscosity Measurements

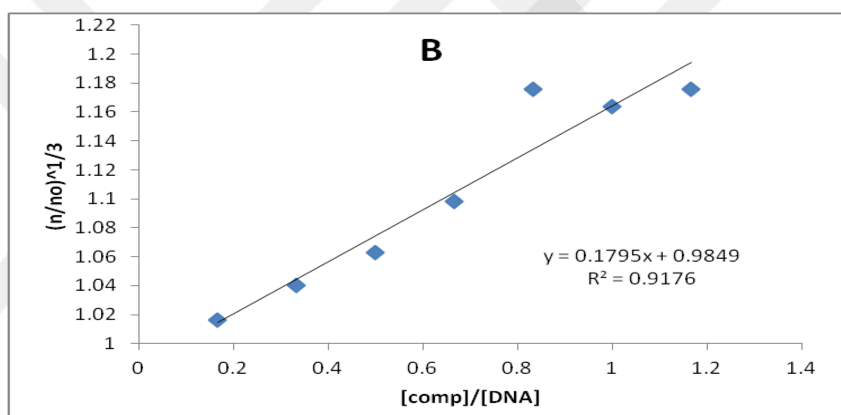
Optical photophysical techniques are widely used to study the binding model of the ligand, metal complexes, and DNA but not to give sufficient clues to support a binding model. Therefore, viscosity measurements were carried out to further clarify the interaction of metal complexes and DNA. Hydrodynamic measurements that are sensitive to the length change (i.e., viscosity and sedimentation) The least ambiguities and the most important tests of the binding model are considered in the solution in the absence of crystallographic structural data [108].

A classical intercalative mode causes a significant increase in viscosity of DNA solution due to increase in separation of base pairs at intercalation sites and hence an increase in overall DNA length. By contrast, complexes that bind exclusively in the DNA grooves by partial and/or nonclassical intercalation, under the same conditions, typically cause less pronounced (positive or negative) or no change in DNA solution viscosity [109].

The values of $(\eta/\eta_0)^{1/3}$ were plotted against [complex]/[DNA] concentration ratio, where η is the viscosity of the DNA in the presence and η_0 in the absence of the complexes (Fig.4.7).

Figure. 4.7 shows that the specific viscosity of the DNA sample clearly increases with the addition of the complex with a slope of 0.032-0.041 for *Cis* and *Asy*, while *Bis* for 0.18. Viscosity studies offer a strong argument for non-intercalation [110]. It was observed in classical organic intercalators such as ethidium bromide increasing the axial length of DNA and becoming rigid resulting in increase in the relative viscosity [111]. The results confirm the sensitivity of viscosity measurements for the different modes of DNA binding. In our study, it was observed that increasing the platinum complex concentration leads to a slight increase in DNA viscosity. Thus, we may conclude that the platinum(II) complex, certainly is the DNA groove binder.





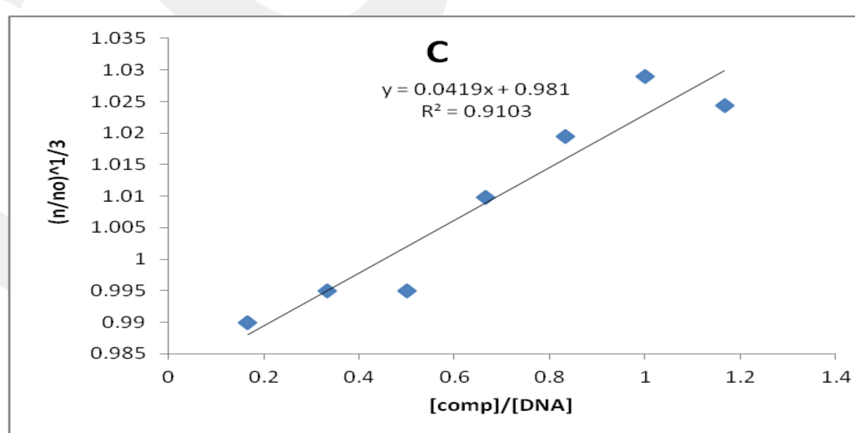


Figure 4.5: The changes in the relative viscosity of the CT-DNA in the presence of *Asy* (A), *Bis* (B) and *Cis* (C) complexes.

4.2.4. Fluorescence Quenching

Ethidium bromide (EB) emits intense fluorescence light in the presence of DNA, due to the strong intercalation between adjacent DNA base pairs. It has been previously mentioned that the enhanced fluorescence can be quenched by the adding a second molecule [106-107]. The extent of fluorescence of EB is used to determine the DNA binding range between the metal complex and DNA [112].

The emission spectra of Ethidium bromide (EB) bound to DNA in presence of complexes are given in Figure. 4.8. The fluorescence quenching curves of EB bound to DNA by Asy and Cis are given in the inset of Figure. 4.8. The quenching plots show that the quenching of Ethidium bromide (EB) bound to DNA by the complexes is in good agreement with the Stern–Volmer linear equation, which proves that the complexes bind to DNA. .

The classical Stern–Volmer equation [86] is,

$$I_0/ I = 1 + K_{sv} .r$$

where I_0 and I are the fluorescence intensities in the absence and the presence of complex, respectively, K_{sv} is a linear Stern–Volmer quenching constant dependent on the ratio of EB (the ratio of the bound concentration of EB to the concentration of DNA) and r is the ratio of total concentration of complex to that of DNA.

The addition of the platinum complexes to DNA pretreated with EB causes obvious reduction in emission intensity. In the linear fit plot of I_0/I versus [complex]/[DNA], the K_{sv} values for *Asy* and *Cis* are 0.249 and 0.27 respectively. These results confirmed that the interaction of the complex with DNA is rather weak although a partial intercalation may also be possible through the aromatic chromophore of the ligand. This weak binding ability of *Asy* and *Cis* complexex to ct-DNA are in agreement with the electrostatic interaction or groove binding [113-114] interactions as observed in the spectroscopic and viscometric results before.

The decresing trend in the fluorescence spectra in Figure 4.8 may due to the concealing of the EB emission as a result of the surface binding of the platinum(II) complexes to ct-DNA [115].

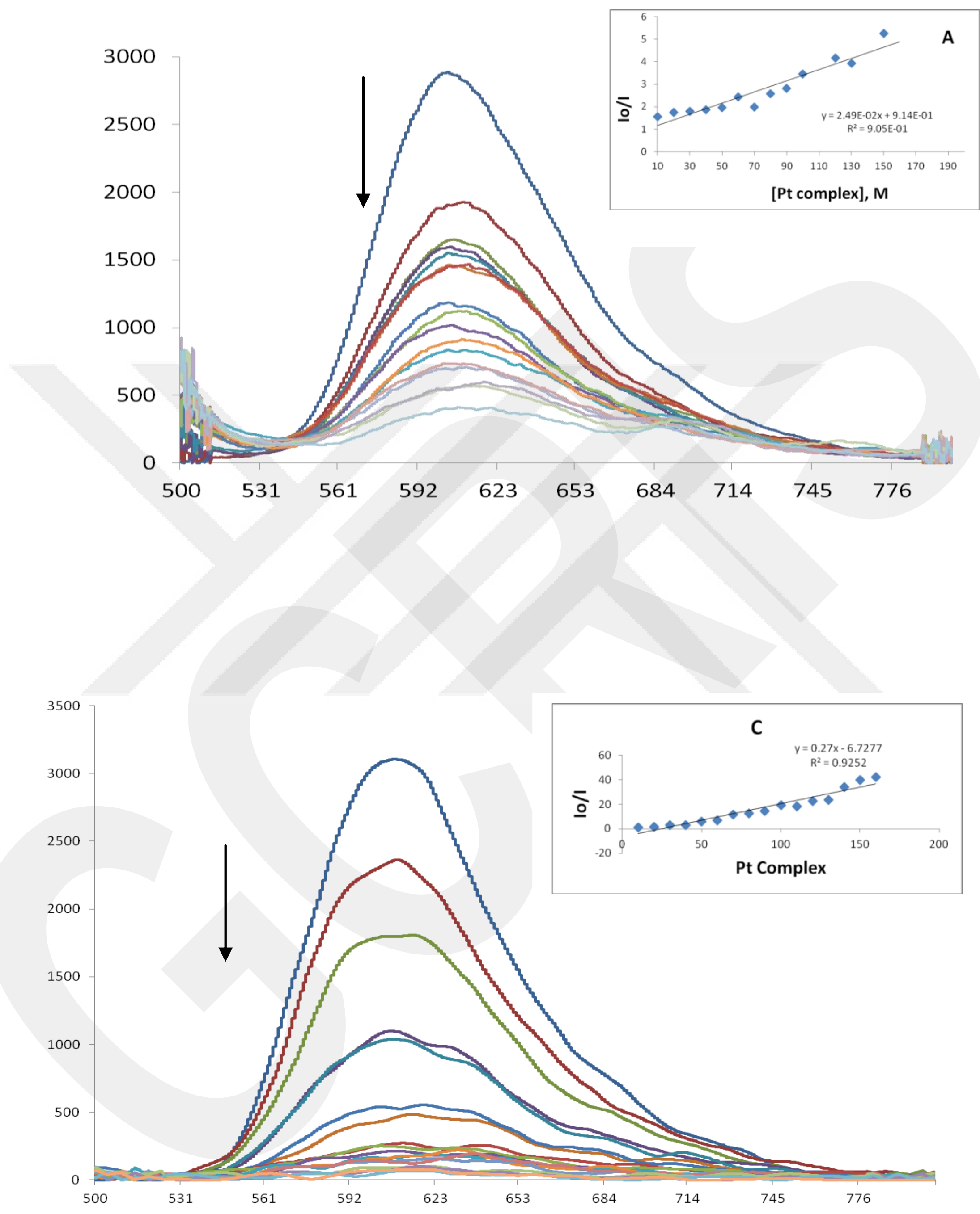


Figure 4.6: Fluorescence quenching observed upon addition of the *Asy* (A) and the *Cis* (C) complexes to the EB bound CT-DNA.

4.3. Cytotoxicity

Cytotoxicity of the platinum complexes *Asy*, *Bis* and *Cis* were examined in the MDA231 cell line to determine and compare their anticancer activity with Cisplatin. A seven point dose response curve was produced from which the IC_{50} value was calculated. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% cell growth inhibition. Therefore, the lower the IC_{50}, the fewer drugs is needed to achieve the desired effect and the less toxic effect [116].

Table 4.5 presents IC_{50} values of the complexes after 24, 48 and 72 hours. In vitro cytotoxicity studies of our complexes indicated that all the complexes more active towards to breast cancer tumor MDA 231 compared to Cisplatin.

Table 4.5. IC_{50} values of the platinum complexes in MDA321 cell line.

Compounds	IC_{50} (µM, 24 h)	IC_{50} (µM, 48h)	IC_{50} (µM, 72 h)
Cisplatin	78.96	47.16	47.23
Asy	46.09	39.08	33.19
Bis	36.25	91.66	91.69
Cis	18.22	12.75	16.25

Overall *Cis* complex is the more active agent among the platinum complexes in neutral medium with an IC_{50} of 12.95 µM in 48 h. In general trend in activity for the first 24 h was *Cis*>>*Bis*>*Asy*. It is well known that the platinum drugs containing one or two amine group in their structure is most active towards tumors [117]. Hence it is very surprising that Asy complex has the lowest IC_{50} value among all the three complexes although contains an amine group in its structure according to the results obtained for the first 24 hour measurements. Contrary to these results, for the 48 and 72 h measurements reveal that the *Asy* complex is much more active that Bis complex, even Cisplatin also much more active than the *Bis* complex (Fig.4.9) . This behavior of *Bis* and *Asy* complexes can be explained the hydrophobicity of them for the first 24 h. The more hydrophobic complex *Bis* is more active. But after 24 h, the

presence of amine group in the structure of the Asy complex clearly increases its activity.

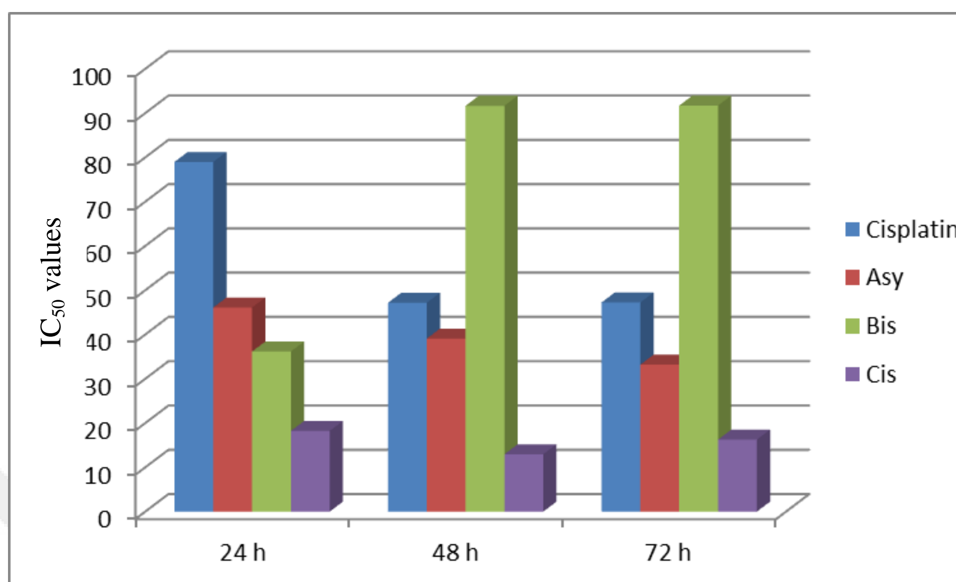


Figure 4.7. Plot of IC_{50} values for the platinum(II) complexes.

GCRIS

CHAPTER 5

CONCLUSIONS

In the experiments of this research, the platinum complexes containing 2,3-di(2-thienyl)-quinoxaline (L), $[Pt(NH_3)Cl(L)]Cl.H_2O$ (Asy), $[Pt(L)_2]Cl_2$ (Cis) and $[PtCl_2(L)]$(Bis) and completed their analyzes

The DNA binding capacity of the complexes was determined by using UV titration, thermal decomposition, viscometric and fluorometric techniques. The binding constants, K_b, of the complexes to DNA were found to be $4.27x10^4$ and $2.50x10^3$ for Asy and Cis complexes respectively. UV titration experiments clearly demonstrate that complexes bind to DNA by groove binding or electrostatic interaction. K_b value for the Bis complex con not be calculated due to the solubility problem of the complex in solution Tris-HCl buffer.

The temperature depended binding constant (K'_b) was calculated at 310, 320, 330 and 340 K for the *Asy* and *Cis* complexes. Afterwards, the standard Gibbs free energy change (ΔG^o) was calculated by using these K'_b values. The negative value of ΔG^o referred to the spontaneous interaction process between the complexes and DNA. The ΔH^o values of the complexes ranged between -3.82 and -103.5 kJ/mol while the ΔS^o values ranged between 21.2 to 402 J/K. The negative enthalpy and the positive entropy changes indicate that the binding is essentially entropy driven. The thermal denaturation and viscosity profiles of the DNA and the complex-DNA adduct also confirm the grove binding nature of the complexes. Fluorescence quenching experiments, conducted using the Asy, Bis and Cis complexes in the presence of EB show a decrease in the emission intensity. These quenching can be combined with the covering of the EB intercalating sides with our groove binder platinum complexes. the K_{sv} values for Asy and Cis are 0.249 and 0.27 respectively, indicating that the complexes bind the DNA almost on the same extent. The low K_{sv} values also

indicate weak binding methods of complexes. These results are also supporting the results of the electronic absorption titration studies.

In vitro cytotoxicity experiments were performed in MDA231 cell lines in neutral medium. The *Cis* complex has a significant effect on the survival rate of breast cancer tumors. In general, the cytotoxicity of the complexes changes gradually from *Cis* to *Bis* complex within 72 hours. Activity order of the complexes was observed as *Cis>>Asy> Bis*.

Asy and Cis complexes are also expected to show significant cellular toxicity in a reduced presence such as ascorbic acid and therefore cytotoxicity in the presence/absence of a reduction agent against other tumor types is on the agenda for further studies.

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APPENDIX A

INFRARED SPECTRA

Figure A1. Infrared spectrum of 2,3-di(2-Thienyl)-Quinoxaline Ligand in the range of 4000-400 cm^{-1}.

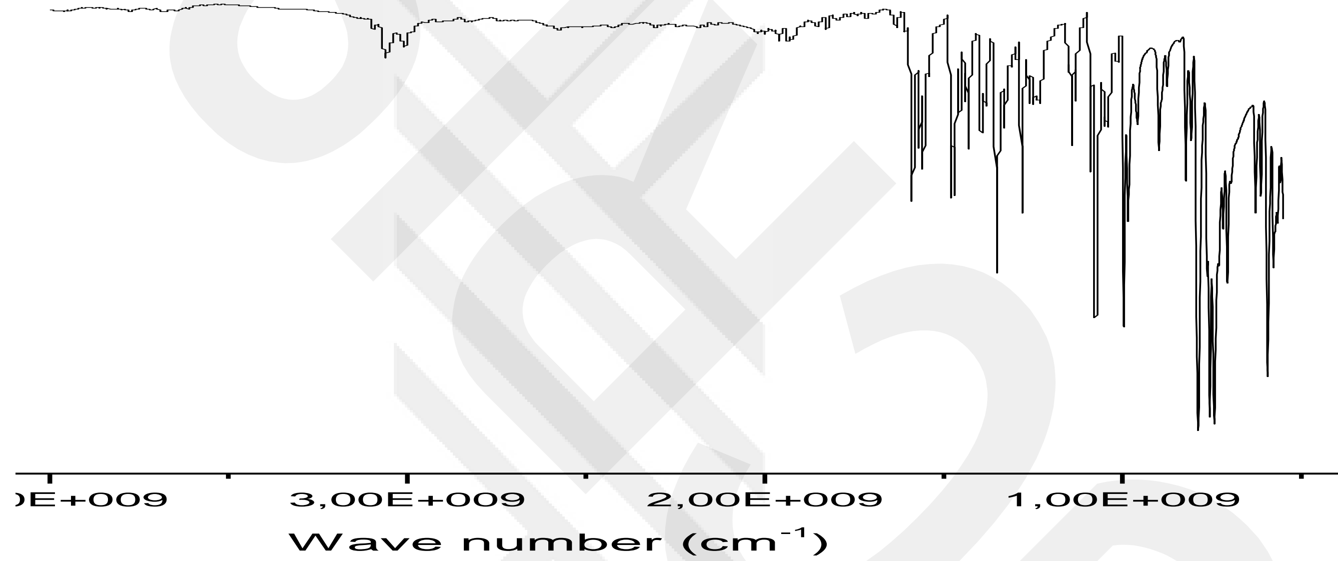


Figure A2. Infrared Spectrum of Asy complex in the range of 4000-400

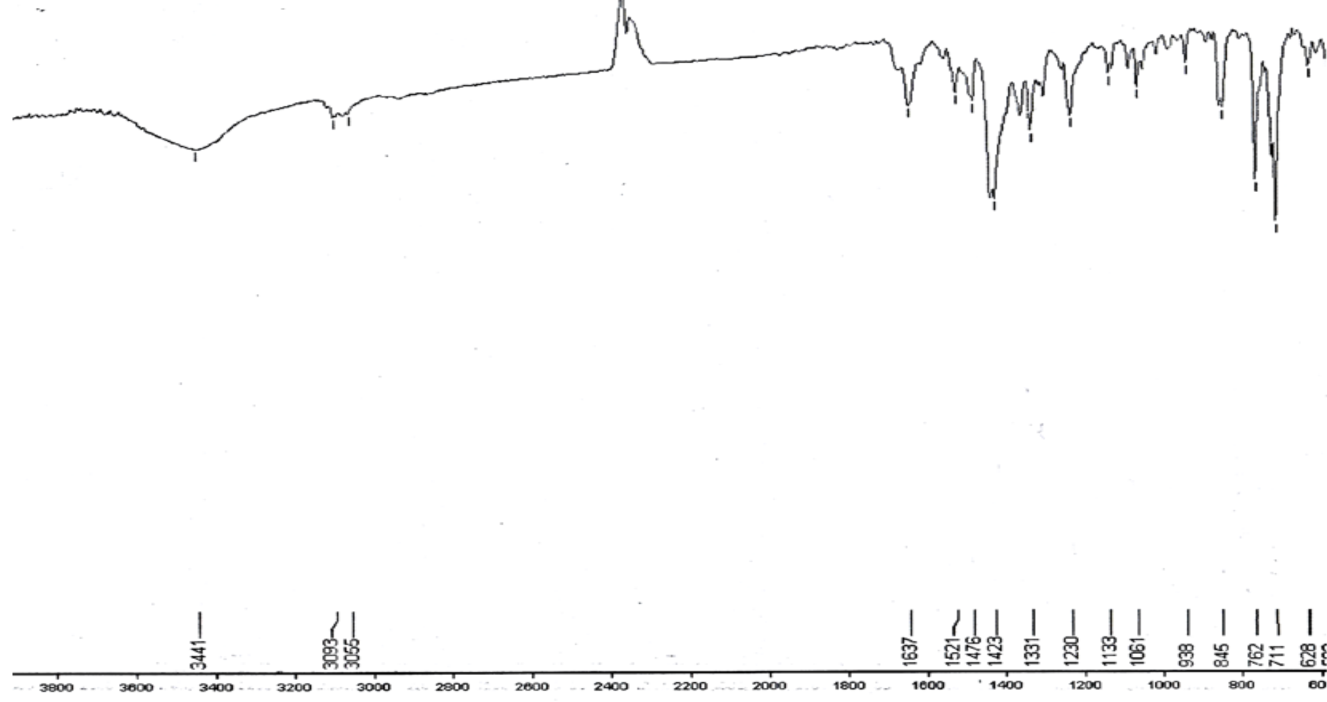


Figure A3. Infrared spectrum of Bis complex in the range of 4000-400 cm^{-1}.

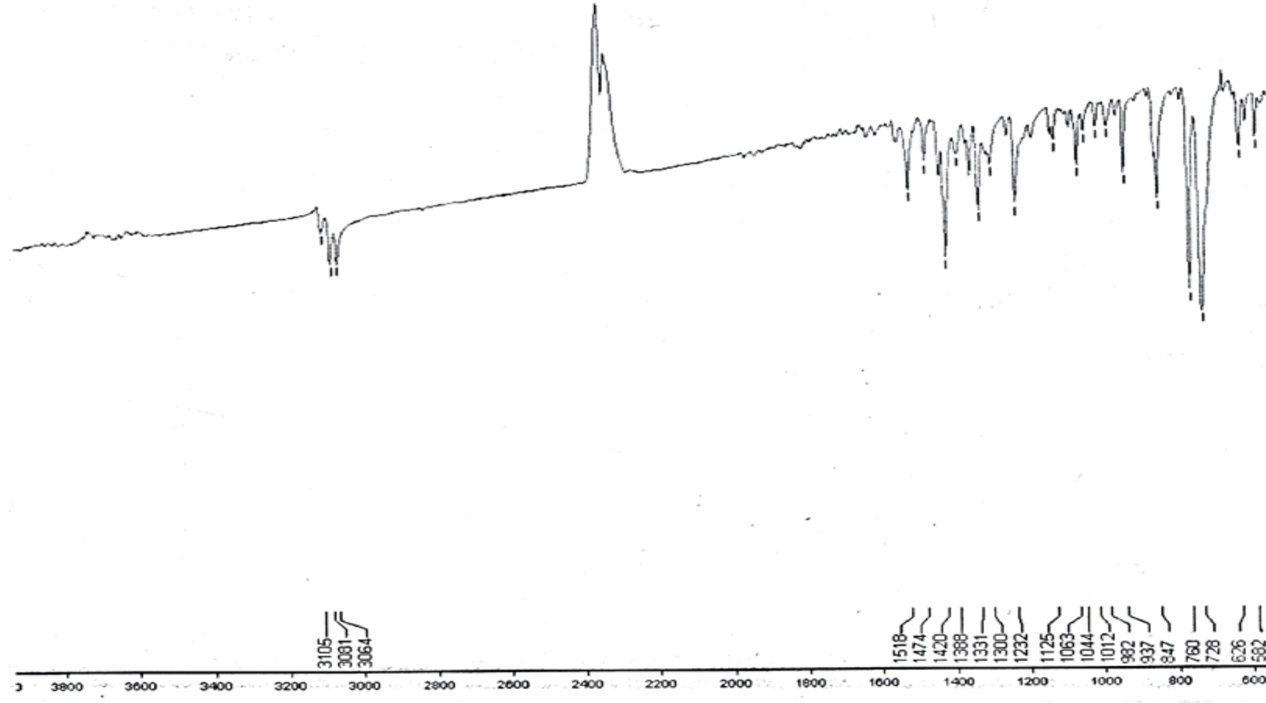
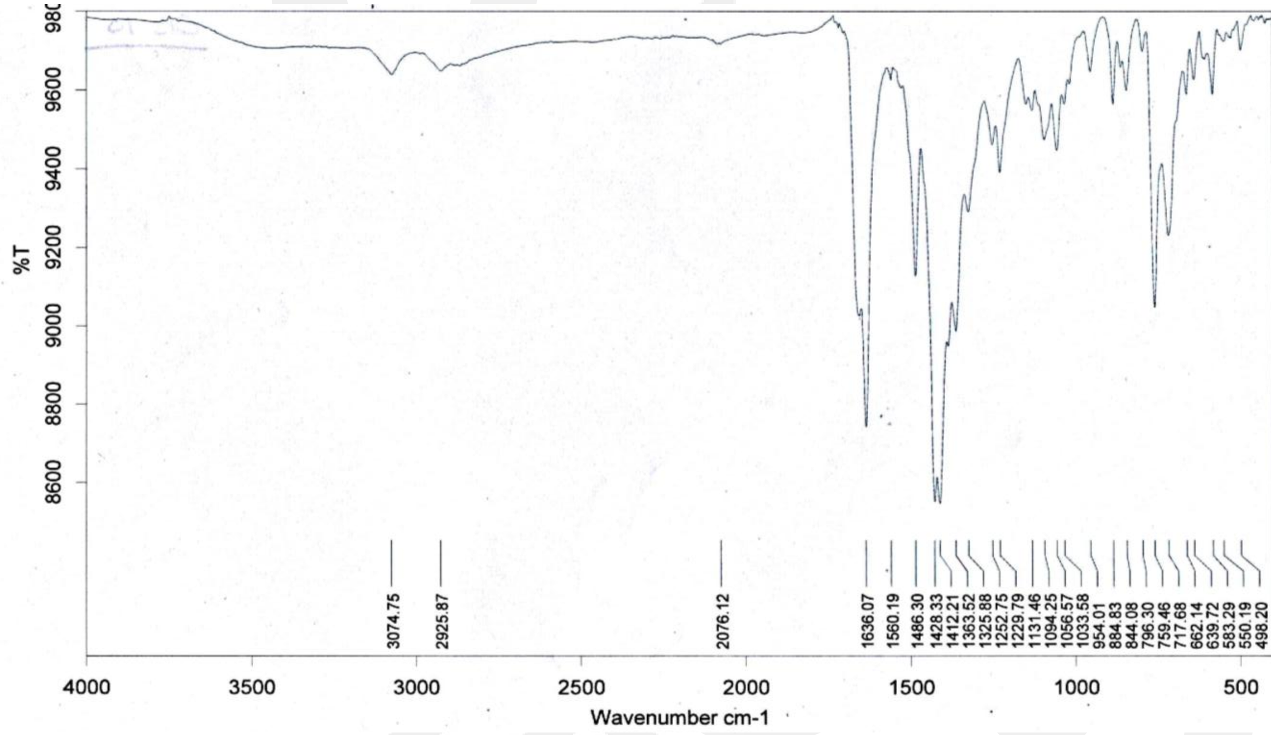


Figure A4.Infrared Spectrum of Cis complex in the range of 4000-400



APPENDIX B

^{1}H-NMR SPECTRA

Figure B1. ^{1}H-NMR spectrum of L in d-DMSO.

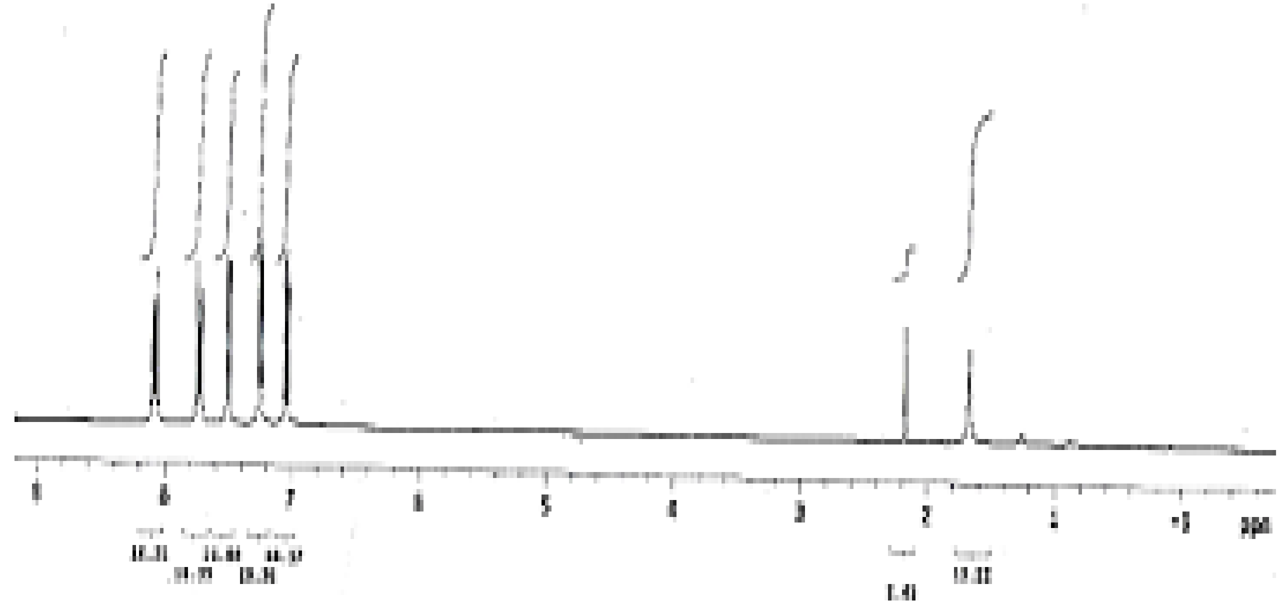


Figure B2. ^{1}H-NMR spectrum of *Asy* complex in d-DMSO.

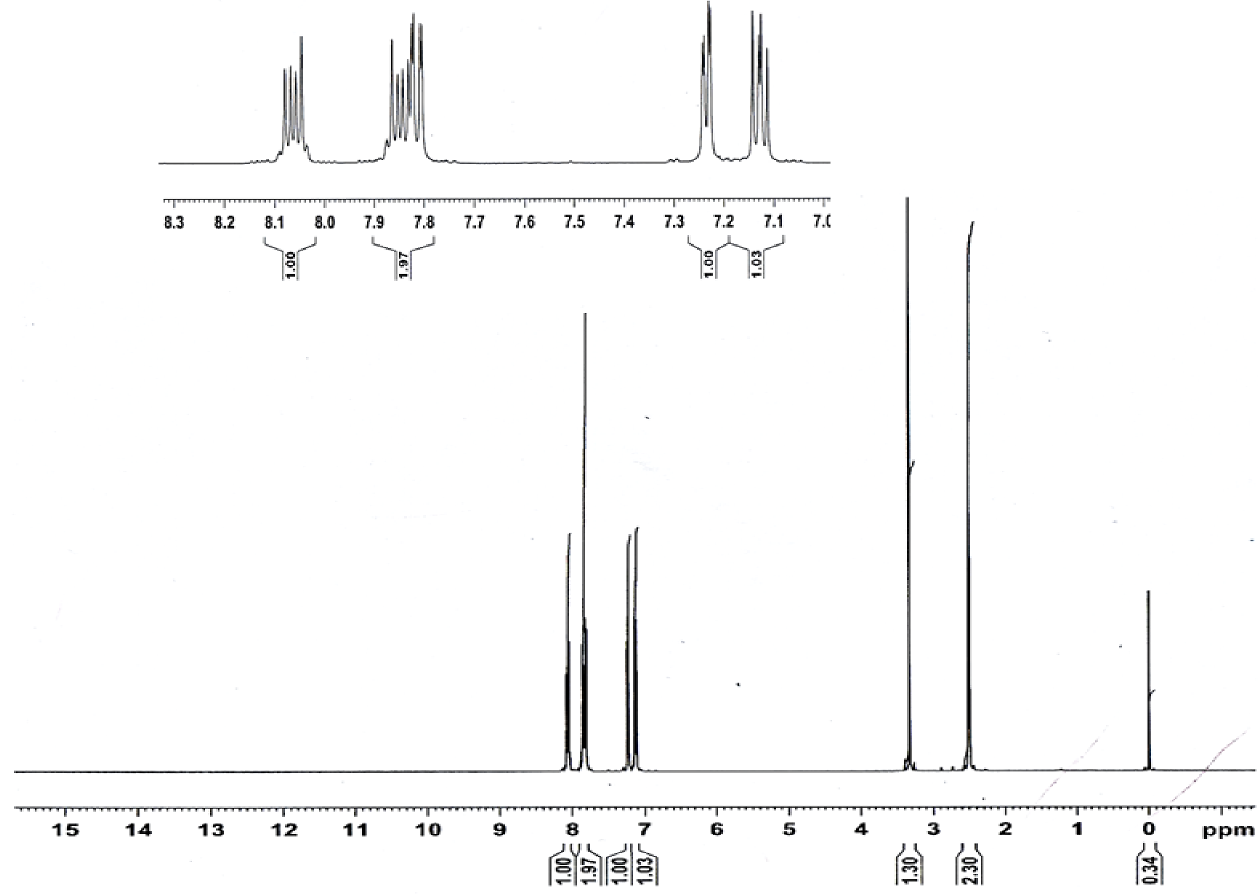


Figure B3. ^{1}H-NMR spectrum of *Bis* complex in d-DMSO.

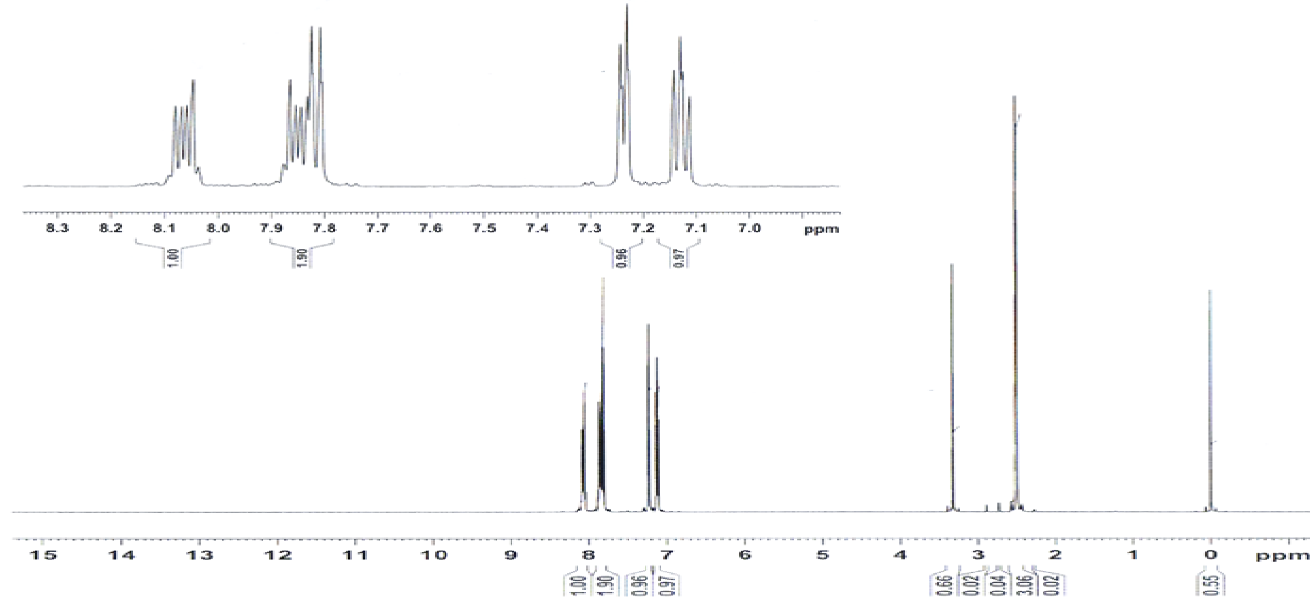
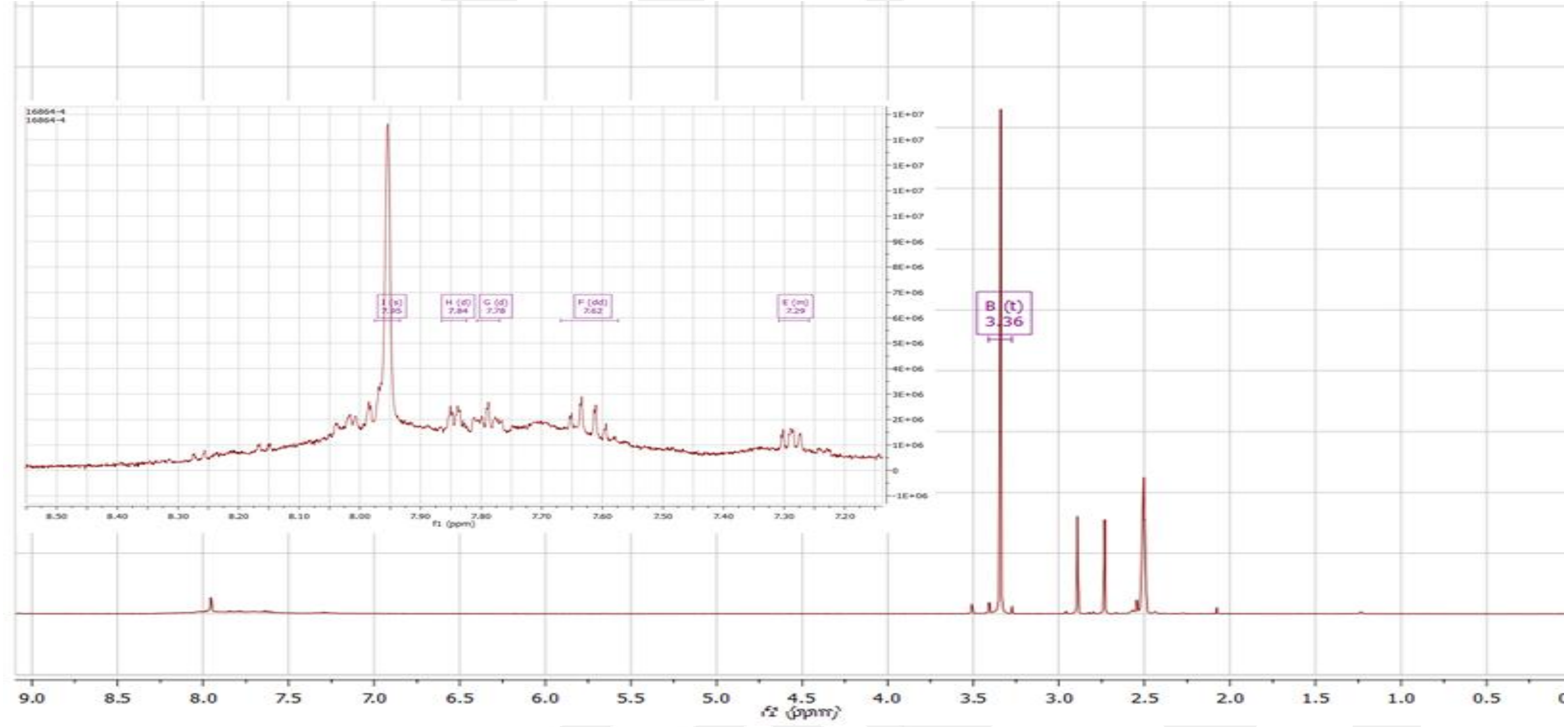


Figure B4. ^{1}H-NMR spectrum of *Cis* complex in d-DMSO.



APPENDIX C

MASS SPECTRA

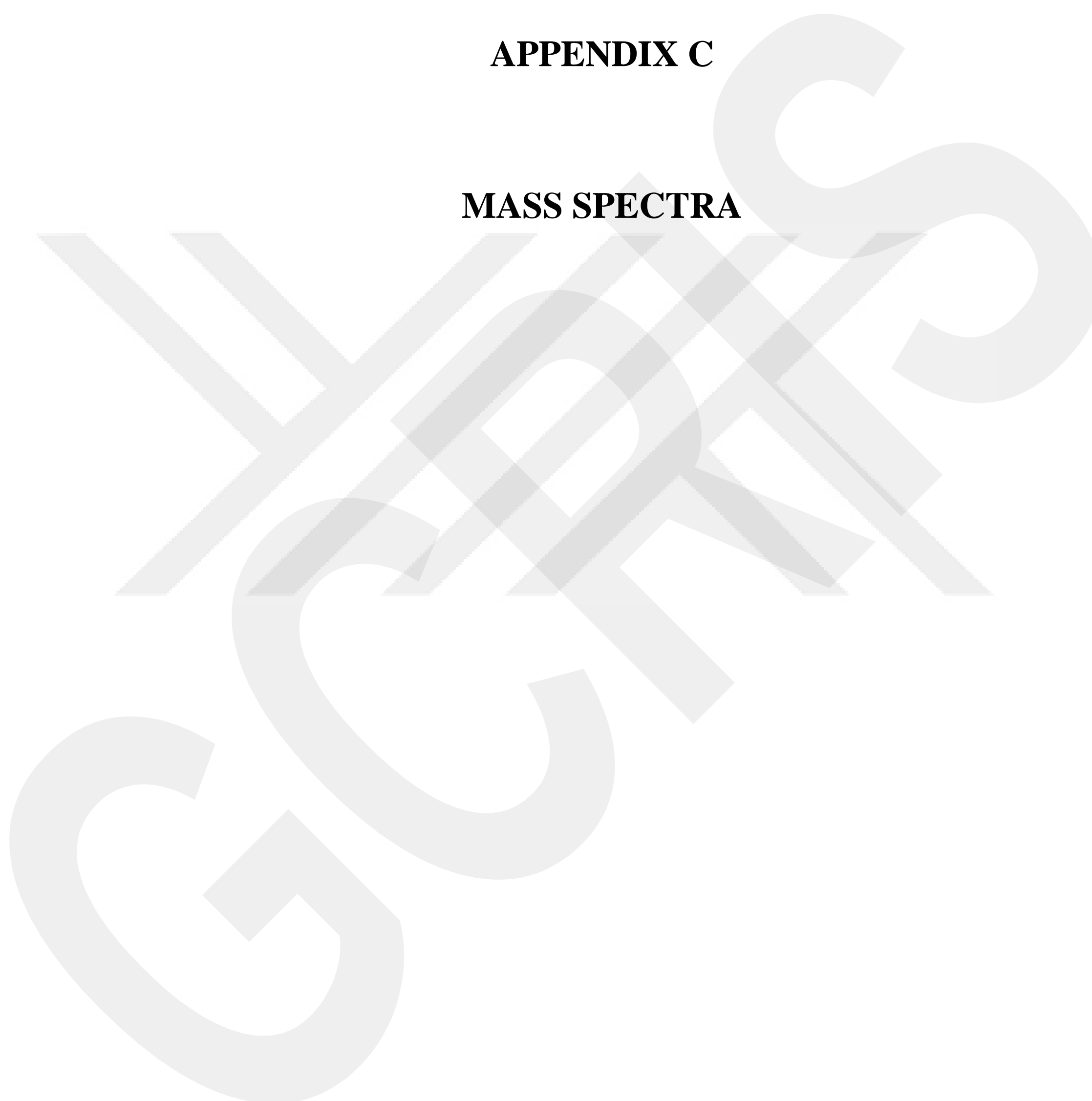


Figure C1. Mass spectrum of *Asy* complex

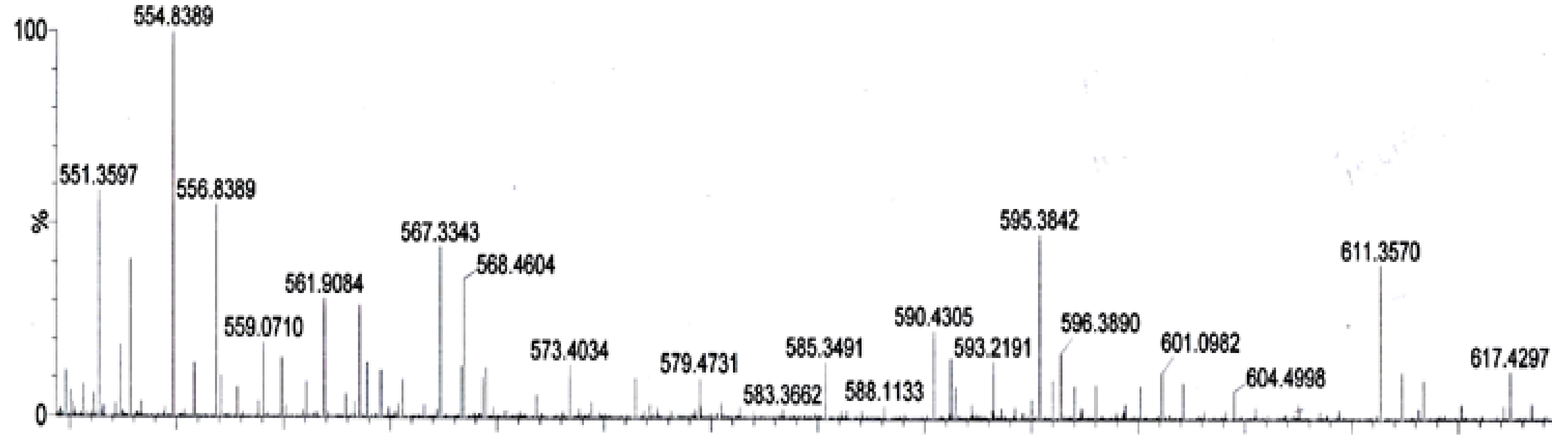


Figure C2. Mass spectrum of *Bis* complex

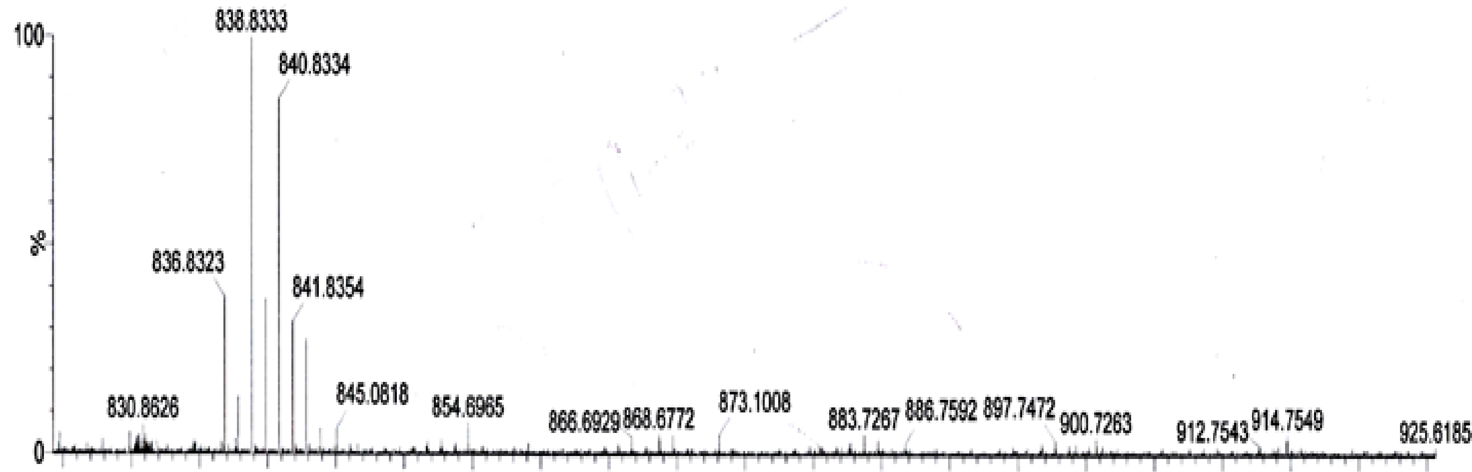


Figure C3. Mass spectrum of *Cis* complex.