STUDIES ON TUMOUR ACTIVE COMPOUNDS WITH MULTIPLE METAL CENTRES

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A thesis submitted in fulfillment
of the requirements for the degree of
Doctor of Philosophy

SCHOOL OF BIOMEDICAL SCIENCES,
FACULTY OF HEALTH SCIENCES,
University of Sydney, AUSTRALIA

December, 2003

DECLARATION

I, the author of the thesis, declare that none of the material in this thesis has been previously submitted by me or any other candidate for any degree to this or any other university.

ACKNOWLEDGEMENTS

First of all, I thank God Almighty for giving me the courage and ability to start and finish this thesis.

There are a number of people who have helped me during the course of my research; however, it is impossible to record all of them here. I hope that they accept my sincere thanks and appreciation. I find myself deeply indebted to some people that I have to mention their names.

I would like to express my deep gratitude and thanks to my supervisor Dr. Fazlul Huq, whose encouragement, scholastic guidance, valuable advice, constructive comments and suggestions for the improvement of the thesis have all been of inestimable value for the preparation of the thesis in its present form. I am greatly indebted to him.

I am indebted to my associate supervisor Dr. Philip Beale, whose comments and valuable advice were of great help to the development of the thesis.

My special thanks and appreciation go to my government for granting me a scholarship to carry on my higher studies and in return I'm so grateful for their help, support and concern during my studies.

I am indebted to Ms. Mei Zhang of RPAH, Sydney and Mr. David Lockwood of School Biomedical Sciences for their assistance. Special thanks are also due to my colleagues Dr. Zahed Hossain, Ahmed Abdullah, Jung Qing Yu, Ashraf Chowdhury, Mariam Bibi, Mohammad Farhad, Hasan Tayyem, Howard Cheng and Ahmed Alharby for their company and encouragement.

Last but not least, my greatest debt of gratitude and appreciation is to my wife, whose help, courage and support are behind my achievement. The support I received from her made my life easier and happier; I cannot imagine my life without her. I appreciate her forbearance and patience during the course of my research. I feel obliged to mention my children Osamah, Hind, Ali, and Anas who have shown a great sense of responsibility, understanding and courage. I will never forget the prayers of my parents back home, which have been a constant source of encouragement throughout my life. Special thanks are due to them as well.

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ABSTRACT

Four tumour active trinuclear complexes: DH4Cl: [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_4NH_2)_2$]$Cl_4$, DH5Cl: [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_5NH_2)_2$]$Cl_4$, DH6Cl: [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_6NH_2)_2$]$Cl_4$, DH7Cl: [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2$-$(H_2N(CH_2)_7NH_2)_2$]$Cl_4$ and one dinuclear complex DHD: [{*trans*-$PtCl(NH_3)_2$}*m*-{$H_2N(CH_2)_6NH_2$}{*trans*-$PdCl(NH_3)_2$]$Cl(NO_3)$, have been prepared and characterised based on elemental analyses, IR, Raman, mass and ^{1}H NMR spectral measurements. For the trinuclear complexes, the synthesis has been carried out using a step-up method branching out from the central palladium unit. A purity of about 95% has been obtained by repeated dissolution and precipitation. The activity against human cancer cell lines including ovary cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, non small lung cell line: NCI-H640 and melanoma: Me-10538 have been determined based on MMT assay. Cell uptakes, DNA-binding have been determined for ovary cell lines: A2780, A2780^{cisR}. The nature of interaction with pBR322 plasmid DNA and ssDNA has been studied for trinuclear complexes DH4Cl, DH5Cl, DH6Cl and DH7Cl and the dinuclear complex DHD. Interaction of DH6Cl with adenine and guanine has also been studied by HPLC. The compounds are found to exhibit significant anticancer activity against cancer cell lines especially ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}. DH6Cl in which the linking diamine has six carbon atoms is found to be the most active compound. As the number of carbon atoms in the

linking diamine is changed from the optimum value of six, the activity is found to decrease, illustrating the structure-activity relationship. The increase in uptake of the trinuclear complexes in A2780 cell line with the increase in size of the linking diamine coupled with the low molar conductivity values found for the solutions of the compounds suggest that the compounds would remain in solution as undissociated 'molecules' and hence could cross the cell membrane by passive diffusion. Much lower resistance factors for the all the multinuclear compounds including DHD as applied to $A2780^{cisR}$ cell line, as compared to that for cisplatin, suggest that the compounds are able to overcome multiple mechanisms of resistance operating in the cell line. All of the multinuclear complexes are expected to form long-range interstrand GG adducts with DNA, causing irreversible global changes in the DNA conformation but unlike cisplatin do not cause sufficient DNA bending to be recognized by HMG 1 protein. Increasing prevention of BamH1 digestion with the increase in concentration of the multinuclear compounds also provide support to the idea that the compounds because of the formation of a plethora of interstrand GG adducts are able to cause irreversible changes in DNA conformation. The results of the study show that indeed new trinuclear tumour active compounds can be found by replacing the central platinum unit in BBR3464 with other suitable metal units.

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ABBREVIATIONS

TMN	TMN system, TMN stands for Tumour, Nodes and Metastases
DNA	Deoxyribonucleic acid
Cis-DDP	*cis*-diamminedichloroplatinum(II) also called cisplatin
Trans-DDP	*trans*-diamminedichloroplatinum(II) also called transplatin
GSH:	Glutathione
MT:	Metallothionine
HMG:	High mobility group
MMR:	Mismatch repair
DACH:	1,2-diaminocyclohexane
CBCD	*cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II), also called carboplatin
JM	Johnson Matthey
ICR	Institute of Cancer Research
JM216	bis(acetato)ammine-dichloro(cyclohexylamine)platinum(IV)
JM335	*trans*-ammine(cyclohexylaminedichloro-dihydroxo)platinum(IV)
ZD0473	*cis*-amminedichloro(2-methylpyridine)platinum(II)
BBR3464	$[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2\}]^{4+}$
DH4Cl	$[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pd(NH_3)_2\text{-}(H_2N(CH_2)_4NH_2)_2]Cl_4$
DH5Cl	$[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pd(NH_3)_2\text{-}(H_2N(CH_2)_5NH_2)_2]Cl_4$
DH6Cl	$[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pd(NH_3)_2\text{-}(H_2N(CH_2)_6NH_2)_2]Cl_4$
DH7Cl	$[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pd(NH_3)_2\text{-}(H_2N(CH_2)_7NH_2)_2]Cl_4$
DHD	$[\{trans\text{-}PtCl(NH_3)_2\}\{\mu\text{-}(H_2N(CH_2)_6NH_2)\}\{trans\text{-}PdCl(NH_3)_2]Cl(NO_3)$
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
Et_3N	Triethyl amine
EDTA	Ethylenediaminetetraacetic acid
dpzm	4,4'-dipyrazolylmethane

AAS	Atomic absorption spectrophotometry
IR	Infrared
NMR	Nuclear magnetic resonance
MTT	3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazoliume bromide
FCS	Fetal calf serum
PBS	Phosphate buffered saline
Triton X-100	t-Octylphenooxypolyethoxyethanol
ssDNA	Salmon sperm DNA
AMP	Adonosine-5'-monophosphate
ri	Molar ratio
NB	Nucleobase
HPLC	High performance liquid chromatography
IC_{50} or ID_{50}	Concentration required to inhibit cell growth by 50% in cell culture
IC_{90}	Concentration required to inhibit cell growth by 90% in cell culture
RF	Resistant factor

CHAPTER ONE

1. LITERATURE REVIEW

1.1. INTRODUCTION

1.1.1. *CANCER*

Cancer may be defined as a disease or a group of diseases in which the cells divide and multiply without control, have the capacity to metastasise in the body, destroy healthy tissue, and endanger life (Salmon and Santorlli 1987; Cooper 1992a). It is one of the major causes of death in many countries of the world (McGrew and McGrew 1985; Salmon and Santorelli 1987).

A tumour or a neoplasm is any group of cells, which may be either benign or malignant (Cooper 1993). Neoplasms that have only the characteristic of encapsulated localized growth are classified as benign. On the other hand neoplasms with characteristics of invasiveness and the capacity to metastasise are classified as malignant (Calman *et al.* 1980). The term malignancy should be limited to refer to malignant neoplasm only (Pitot 1986). Thus only malignant tumours are called cancer.

Diagnosis of cancer is achieved through the integration of clinical history, microscopic appearance of tumour including X-ray appearance in the case of bone and breast tumours and microscopic examination (Jass 1999).

Differential diagnosis of benign and malignant tumours usually involves obtaining a tissue specimen by biopsy surgical excision or exfoliative cytology. The commonly diagnosed cancers in men are prostate, lung, colon, urinary bladder and the most common cancers in women are the breast, colon, lung, and then the ovary and uterus.

Cancer can be classified histologically into one of three main groups:

Carcinomas: These are of endodermal or ectodermal origin and constitute approximately 90% of all human cancers such as the lung, stomach and intestine or from glands such as the breast and prostate.

Sarcomas: These are rare in human and begin in connective tissue such as muscle, bone, tendons and cartilage.

Leukaemias and lymphomas: These constitute about 8% of all human cancers and arise from the blood forming cells and cells of lymph system (Calman *et al.* 1980; Murphy *et al.* 1997).

Within cancers of the same organ there may be marked variations in the histological appearance. For example, lung cancer can be divided into adenocarcinomas, squamous carcinoma, small cell and large cell carcinomas. Skin cancers can be divided into basal cell carcinomas, squamous cell carcinomas and melanomas.

Also there can be marked variations in the degree of cellular differentiation. Leukaemia commonly is one of four types: acute myelocytic leukaemia (AML) and chronic melocytic leukaemia (CML), acute lymphocytic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL) (Ruddon 1995). These differences in histological classification and the degree of

cellular differentiation may determine the treatment protocol used, especially with chemotherapeutic agents.

Cancers are also classified based on the extent of tumour progression. The classification is based on a system called TMN system developed by the International Union Against Cancer and American Joint Committee on Cancer. In the abbreviation 'TMN', T defines the primary tumour, N indicates the involvement of regional lymph nodes and M indicates the absence or presence of metastases (Rubin 1973; Rubin and Cooper 1993). The system describes how far cancer has spread anatomically, what is important in planning treatment and in evaluating result of treatment.

An understanding of the normal cell cycle and behavior of cancerous cells is necessary in order to comprehend how chemotherapy destroys cells, in particular how compounds such as cisplatin effects the cancer cells in the dividing state.

Normal cell cycle consists of two overall phases: division and interphase. Division comprises both nuclear division (mitosis M) and cell fission. Before cell division can occur, the cell must double its mass and duplicate all its contents. This period of growth in the cell cycle is referred to as interphase. Interphase makes up ninety percent of the total cell cycle and consists of three phases: gap phase G1 which is between M phase and S phase, a synthesis phase S in which the DNA content of the cell is doubled and the chromosomes are replicated and a second gap phase G2 which is between S phase and mitosis (Alberts 2002).

1.1.2. *CAUSES OF CANCER*

Some forms of cancer may be inherited; the defective genes are passed down from parents to their children (Stoler 1991; Cavenee and White 1995). Family history has long been

recognized as an important risk factor for the majority of common cancer such as breast, colon, ovarian and prostate cancer (Phillips 1999).

Some other cancers are caused by outside factors eg cigarette smoking. Tobacco is estimated to be responsible for about 2.6% of total world burden of death (Bishop 1999a; Burton and Giles 1999) and a key factor in causing about 30% of all cancers (Pratt 1994). Smoking is a risk factor not only for lung cancer but also for cancers of mouth, pharynx, larynx, oesophagus, urinary bladder, pancreas, liver and kidney (Doll and Peto 1981; Doll 1996).

Some other risk factors include unhealthy dietary habits (especially high fat intake and alcohol use), viral infection, ultraviolet radiation, exposure to large doses of radiation from medical X-rays, hormone replacement therapy and exposure to some chemicals such as arsenic, asbestos, nickel, chromium, cadmium and vinyl chloride.

1.1.3. *TREATMENT OF CANCER*

The principle in cancer treatment is to cure the cancer patient with minimal functional and structural impairment (Rubin *et al.* 1993).

There are three main methods of cancer treatment, which are: (1) surgery, (2) radiation therapy and (3) chemotherapy. The actual choice of the method of treatment depends on the type of cancer and its developmental stage.

The rate of survival is better when the disease is localised to primary site. Once regional spread has occurred survival rate goes down, and decreases dramatically once distant metastasis has occurred (Pratt 1994).

Surgery in combination with radiation therapy and chemotherapy is still the best method of treatment in most of the cancer cases.

1.1.3.1. SURGERY

It is the first line treatment against cancer depending on its stage of development, location and type of the tumour. Surgical removal of the tumour at an early stage in the cancer often can cure it completely except for some tumour locations like brain tumour. Surgical resection of malignant primary tumour may cure or control the tumour depending on whether or not all of the cancer cells have been removed.

Unfortunately, about 70% of cancers have already metastasized by the time of diagnosis and therefore cannot be cured by surgery alone (Cooper 1992b). In spite of that, surgery remains the most effective method of treatment for patients with solid tumour.

1.1.3.2. RADIATION

Radiation therapy involves the use of ionising radiation to kill cancerous cells. It is usually used for the treatment of localized tumours, both benign and malignant. The primary target of radiation is deoxyribonucleic acid (DNA) (Dizdaroglu 1992).

This kind of therapy like chemotherapy does not have the ability to distinguish between the cancer cells and normal cells and because of that it shows significant toxicity.

Radiation therapy can be used alone in different kinds of tumour including early stage head and neck cancers (Lee *et al.* 1993), Hodgkin's disease (Farah *et al.* 1988) and cervical cancer (Perez *et al.* 1986).

Radiation therapy has also been used in combination with surgery and/or chemotherapy to eliminate cancer cells that have invaded normal tissue post-operative or during surgery.

The three treatment methods can be used together in many cases. A good example is locally advanced breast cancer (Bonadonna *et al.* 1990). In general, the effectiveness of radiation is enhanced when it is used in combination with either surgery or chemotherapy or both. A better example is cervix cancer where radiation plus cisplatin has improved survival for early stage cancer.

1.1.3.3. CHEMOTHERAPY

The modern chemotherapy treatment began in the late 1940s mainly with purine and pyrimidine analogues and the research in this area continues to provide the best action, more effective but less toxic drugs. Chemotherapy is different from surgery and radiation. It is a systemic treatment because the chemotherapy drugs enter the blood stream, are distributed throughout the body and can attack cancer cells wherever they are in the body. Numerous compounds have been developed, but at present there are only a limited number of anticancer drugs in clinical use. Cytotoxicity of these drugs is often due to the damage caused to DNA or interference with DNA synthesis. Most of the anticancer drugs that are in use today are non-selective in their mechanism of action (Ratain and Ewesuedo 1999). Often, they can have similar effects on both normal and cancer cells, thus producing a number of side-effects, which include nausea, vomiting, diarrhea, anaemia, ineffective blood clotting, suppression of immune system, hair loss and toxicity to specific organs such as kidney, bladder, liver and heart. Most of the side effects are dose dependant (Bishop 1999b). Notable examples of successes with chemotherapy include the treatment of Burkitt's lymphoma, Hodgkin's

disease, acute lymphocytic leukaemia, chorio carcinoma, ovarian cancer and testicular cancer (Cooper 1992b; Pratt 1994).

Chemotherapy is often administered before or after local therapy with surgery or radiation. This form of therapy shows a definite benefit in a subset of patients with breast cancer and colorectal cancer (O'Connell *et al.* 1988). The use of two or more drugs with different mechanisms of action in combination offers a more effective method of treatment as it reduces the chances of the development of drug resistance (Wittes and Goldin 1986) which is one of the major problems in anticancer chemotherapy. Drug resistance is usually mediated through more than one pathways including gene amplification, dcreased drug uptake, increased efflux, detoxification and decreased engagement of apoptosis. The selection of standard chemotherapy combination regimes to treat individual patients is based upon tumour histology and extent of disease (Chabner 1990).

Anticancer drugs can be classified based on whether they interfere with DNA synthesis or cause damage to DNA. Anti-metabolite drugs interfere with DNA synthesis. Examples are 5-fluorouracil, methotrexate and tomudex. Other drugs interfere with cell division. For example, L-asparaginase bacterial enzyme inhibits cell growth by depleting circulating L-asparagin, thus inhibiting protein synthesis. Some other drugs, which act by damaging DNA, include alkylating agents such as cyclophosphamide, melphalan, and platinum compounds such as cisplatin and carboplatin (Miller *et al.* 1992; Clarke and Rivory 1999).

Drugs that interact with DNA can be divided into several classes based on the nature of interaction. Some drugs interact with DNA non-covalently such as intercalation (e.g. daunorubicin) or groove binding (e.g. distamycin A). Some other drugs such as cisplatin and

mitomycin C form covalent adducts with DNA. A third class binds to DNA and subsequently causes the breakage of DNA backbone (e.g bleomycin) (Yang *et al.* 1999). Electrochemotherapy is a new method of treatment that consists of a combination of a chemotherapeutic agent and pulsed electric field. The electrical field destabilizes cell membranes, allowing increased movement of the molecules into the cell (Jaroszeski *et al.* 1997; Heller *et al.* 1999).

In addition to the three approaches to cancer treatment described above, there are a number of new treatment methods still under clinical trials. Two examples are immunotherapy and bone marrow transplants.

1.1.4. *STRACTURE OF DNA*

Since the subject of present research is platinum-based tumour active compounds that bind covalently with DNA (as a result of which DNA conformations are changed), it is appropriate to have a brief look at the structure of DNA. The word DNA stands for deoxyribonucleic acid. It is the molecule of heredity that stores the genetic information. It is a long molecule made by joining together of many deoxyribonucleotide subunits. Each nucleotide in DNA is composed of a molecule of 2'-deoxyribose (S), a nucleobase (NB), and an inorganic phosphate (P). Figure 1.1 gives a symbolic representation of the structure of a nucleotide.

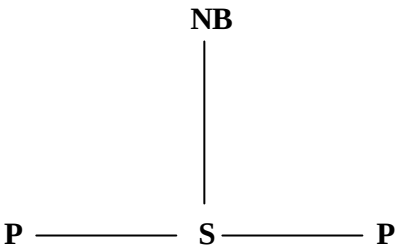
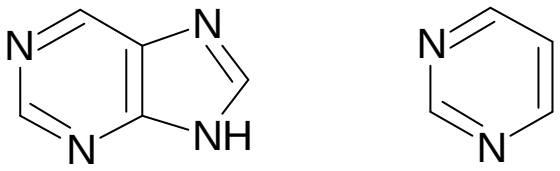


Figure 1. 1 Representation of a nucleotide

The four bases found in DNA are adenine (A), guanine (G), thymine (T) and cytosine (C). These are also known as nucleobases. Adenine and guanine are derivatives of purine whereas thymine and cytosine are derivatives of pyrimidine. The following figure gives the structures of purine and pyrimidine.



Purine Pyrimidine

Figure 1. 2 Structures of purine and pyrimidine

The following figure gives the structures of adenine, guanine, thymine and cytosine.

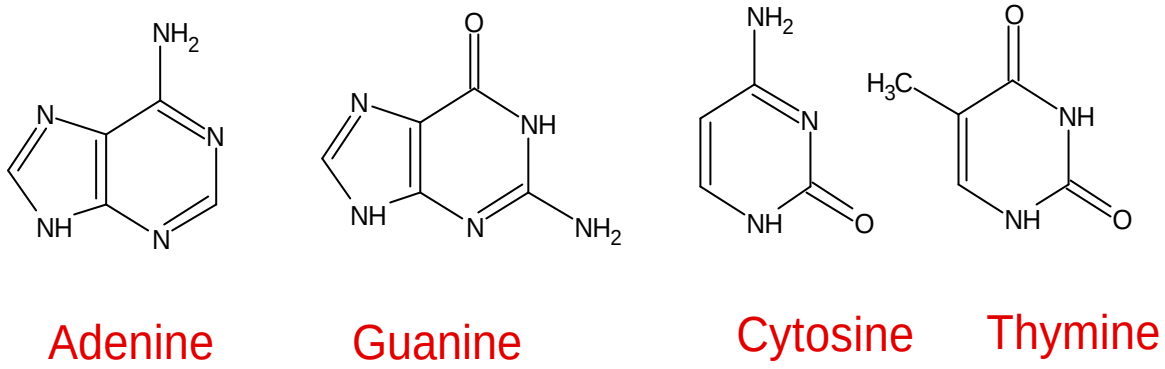


Figure 1. 3 The structures of adenine (A), guanine (G), thymine (T) and cytosine (C)

In DNA, C1 of a 2'-deoxyribose is linked to N1 of a pyrimidine base or N9 of a purine base. A phosphate group joins two adjacent 2'-deoxyribose units by phosphodiester bonds. Each 2'-deoxyribose in turn is joined to two phosphates through its C3' and C5' centres so that in each DNA strand the sugar at one end has 5' hydroxyl group free and that at the other end has 3' hydroxyl group free.

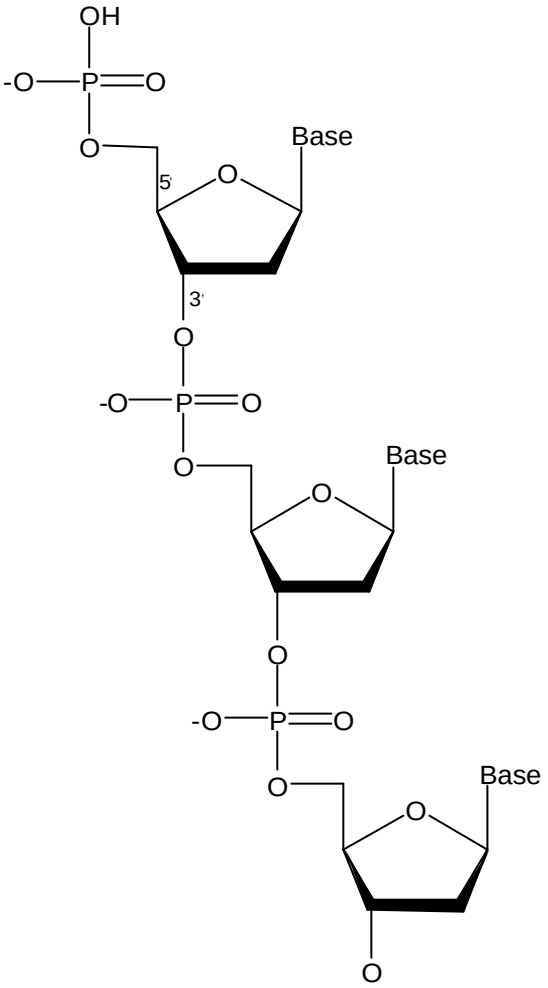


Figure 1. 4 Representation of a polynucleotide chain

DNA usually exists in the form of a doub le helix in which two polynucleotide chains run in opposite directions. The bases in the double helix are always linked in pairs: adenine with thymine by two hydrogen bonds and guanine with cytosine by three hydrogen bonds (Figure 1.5).

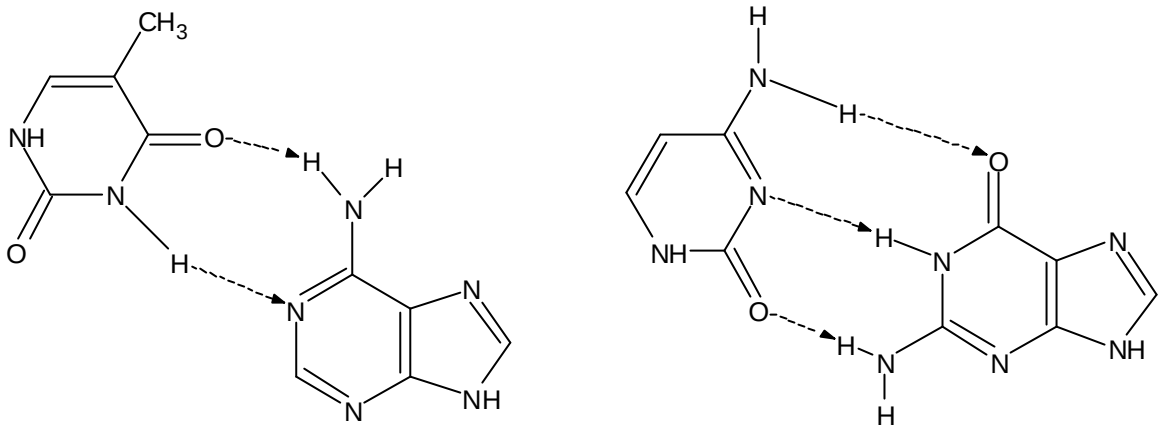


Figure 1. 5 The hydrogen bonding, between adenine and thymine, and between guanine and cytosine.

DNA has been found to exist in a number of forms (A to E and Z). These forms are distinguished by a number of parameters: (1) the number of base pairs that occupy each turn of the helix, (2) the pitch or angle between each base pair, (3) the helical diameter and (4)- the handedness of double helix (right or left).

B form of DNA is most common. It exists as a right-handed double helix. It has a length of 3.4 nm per turn and within a single turn 10 base pairs exist. The width is 2.0 nm.

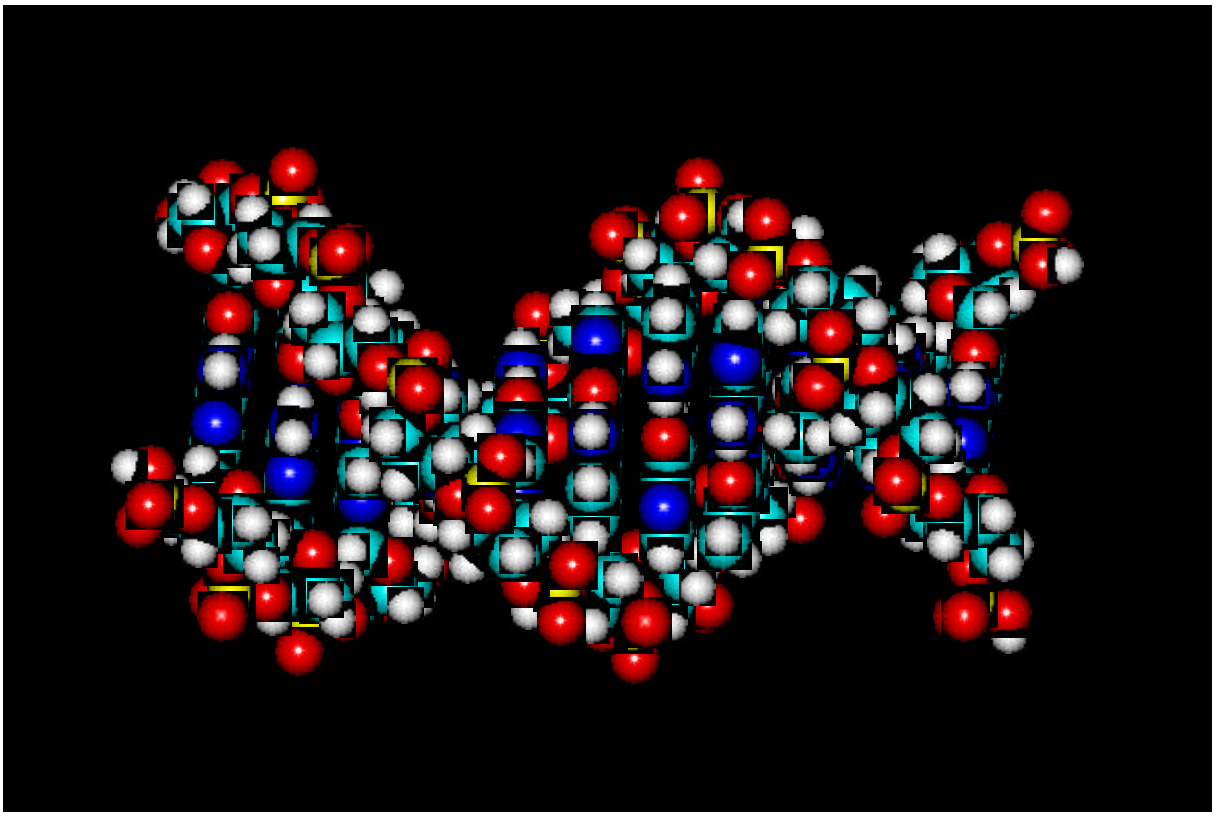


Figure 1. 6 Representation of double helical DNA.

B-DNA has two different kinds of grooves called major and minor grooves. These grooves have different width and depth. The major groove is deeper than the minor groove. These are a consequence of glycosyl bonds between the sugars and bases in a given base pair being not directly opposite to each other.

Proteins can interact with B DNA through its grooves - specifically with exposed atoms of nucleotides. This interaction is usually mediated through hydrogen bonds (Stryer 1988; Granner 1990).

1.1.5. *METALS IN CHEMOTHERAPY*

The history of medicine shows that metal compounds were used extensively in the medical therapy. For example, antimony compounds were used as emetics and in the treatment of tropical disease, whereas bismuth compounds were used as dermatological antiseptics (Asperger and Centina-Cizmek 1999).

It is well known that many metals are essential for physiology of the human body and compounds of the essential elements eg iron can be used as therapeutic drugs. Vanadate and vanadyl ions have potential as insulin mimetic agents in the treatment of human diabetes mellitus (Melchior *et al.* 1999; Thompson *et al.* 1999).

For several decades manic-depressive psychoses have been treated with lithium salt (Lithium carbonate). The Li^+ ion is therapeutically valuable because it counteracts both phases in the typically cyclic course of this disorder (Birch and Philips 1991).

Figure 1.7 highlights the thirty elements that are believed to be essential for life. A number of inorganic compounds, which are non-essential according to the present knowledge, are used in chemotherapy.

H																He
Li	Be											***B***	***C***	***N***	***O***	***F***	Nu
Na	***Mg***											Al	***Si***	***P***	***S***	***Cl***	Ar
K	***Ca***	Sc	Ti	***V***	***Cr***	***Mn***	***Fe***	***Co***	***Ni***	***Cu***	***Zn***	Ga	Ge	***As***	***Se***	***Br***	Kr
Rb	Sr	Y	Zr	Nb	***Mo***	Tc	Ru	Rh	Pd	Ag	***Cd***	In	***Sn***	Sb	Te	***I***	Xe
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Ti	Pb	Bi	Po	At	Rn
Fr	Ra																

Figure 1. 7 The thirty highlighted elements believed essential to life (Bowser 1993)

Chemotherapy involves the use of drugs to injure an invading organism ideally without causing any injury to the host. This definition covers antibacterial, antiviral and anticancer agents (Farrell 1999) and would cover a lot of the compounds based on nonessential elements that are used in chemotherapy. The greatest success of inorganic chemotherapy is the advent of cisplatin and its analogue carboplatin, their adoption into clinical use and the number of tumour active compounds based on platinum that are currently under clinical trials. The details of platinum based anticancer drugs are given in chapter 2.

Following the success of cisplatin and similar platinum-containing anticancer drugs, a large number of complexes of other metals also have been studied and in several cases subjected to clinical trial. The compounds that have reached the clinical trial include compounds of germanium such as carboxyethylgermanium, gallium salts such as gallium chloride and titanium compounds such as budotitane (Keppler and Vogel 1996)

Compounds of other metals that have been investigated as anticancer drugs include those of ruthenium, gold, copper, cobalt, palladium, rhodium and tin. In general palladium(II) compounds are far more labile than their platinum counterparts and therefore less tumour active and more toxic. However, some palladium complexes such as [Pd(meorot)(*trans*-dach)] have been reported to show activity close to that of cisplatin (Buckley 1994). In

another study of platinum and palladium compounds containing ligands: ß-carboline alkaloids, pyrazoles, DMSO and ferrocenylphosphines, it was found that: (1) *cis*-palladium compounds were more active than the corresponding platinum compounds and (2) palladium compounds were more active in the *trans*-form than in the *cis*-form (al-Allaf and Rashan 2001). Two dinuclear palladium(II) complexes, $\{[Pd(en)Cl]_2(bpse)\}(NO_3)_2$ and $\{[Pd(en)Cl]_2(bpsu)\}(NO_3)_2$ have also been reported to show greater activity than cisplatin against the HCT8 cell line (Zhao *et al.* 1998a).

It will be seen later than the antitumour properties of platinum compounds are attributable to a great extent to the slow kinetics of their ligand displacement reactions. In that respect, ruthenium complexes behave like platinum complexes although ruthenium compounds are generally found to be slightly less reactive than the corresponding platinum compounds. Thus, it is logical to expect that ruthenium compounds also should display antitumour activity (Van Houten *et al.* 1993). Ruthenium complexes are found mainly as ruthenium(II) and ruthenium(III) in aqueous solution. In both the oxidation states, the metal ion is found to be in an octahedral geometry. Like platinum(II), ruthenium ions have a high affinity for nitrogen and sulfur donor ligands. During the last two decades, ruthenium complexes with diverse coordination environments have shown promising results in the biological field not only as antitumour agents but also as NO scavengers and immunosuppressive agents. Ruthenium(III) complexes are generally more inert than the corresponding ruthenium(II) complexes. It is believed that the inert, and therefore inactive ruthenium(III) complexes act as pro-drugs which are activated by reduction in situ to the corresponding more active ruthenium(II) species. Ruthenium(III) species may be expected to be reduced more easily in tumour masses which are generally considered as reducing, hypoxic environments compared

to surrounding more aerated tissues. Therefore larger amounts of reactive ruthenium(II) species might be generated in tumour tissues so that selective cytotoxicity may be produced against solid tumours (Clarke 1993)

Ruthenium(III) and ruthenium(II) complexes such as *trans*-$RuCl_2(DMSO)_4$, $(DMSO)_2H[$*trans*-$RuCl_4(DMSO)_2$ and *trans*-$Him[RuCl_4(im)_2]$ were extensively studied for their anticancer activity. Some of the ruthenium complexes show activity against several cancer cell lines with a spectrum of activity different from that of cisplatin (Clarke 1993; Keppler *et al.* 1993; Mestroni *et al.* 1993; Sava 1994; Pieper *et al.* 1999). The Ru(III) complex salt Na[*trans*-$Ru(Im)Me_2SO)Cl_4$](NAMI) was found to inhibit selectively spontaneous lung metastases in a model of solid metastasing tumour MCa mammary carcinoma of CBA mice (Sava *et al.* 1992; Sava 1994). Compared to cisplatin, it was found to be less active in inhibiting primary tumour growth. It is currently under a clinical trial (Zhang and Lippard 2003).

Gold complexes are also known for their use in the treatment of primary chronic polyarthritis. The most important antirheumatics gold complexes are gold(I) sodium thiomalate (myochrysin), gold(I) thioglucose (solganol), trisodium bis(thiosulphato) aurate(I) and 2,3,4,6-tetrakis-O-acetyl-1-thio-β-D-glucopyranosato-S(triethylphosphine)gold(I) (auranofin) (Asperger and Centina-Cizmek 1999).

As stated earlier, some of gold complexes show antitumour activity e.g. triphenylphosphine(8-thiotheophyllinato)gold, which is found to be active against several tumour cell types (Show III 1999).

Since this project deals with new multinuclear tumour active compounds containing both platinum and palladium centres, it is appropriate to review first the current state of the development of platinum-based anticancer drugs.

CHAPTER TWO

2. PLATINUM BASED ANTICANCER DRUGS

Platinum is a third row transition metal that has eight electrons in the outer *d sub* shell. Palladium and nickel, which occupy the second and first transition series, have a similar configuration of outer electrons.

Platinum has two dominant oxidation states +2 or +4, designated as Pt(II) and Pt(IV) respectively. It can be found in the +5 and +6 oxidation states as well. In Pt(II) complexes, the coordination number of platinum is usually four and it has a square planar geometry.

The complexes of Pt(IV) have coordination number of six in an octahedral configuration. Both Pt(II) and Pt(IV) complexes can have *cis*- and *trans*-configurations. Platinum coordination complexes represent a unique and an important class of antitumour agents (Reed 1990) one of which namely cisplatin has been used in chemotherapy for more than 30 years. Cisplatin is considered to be the parent compound among tumour active platinum complexes.

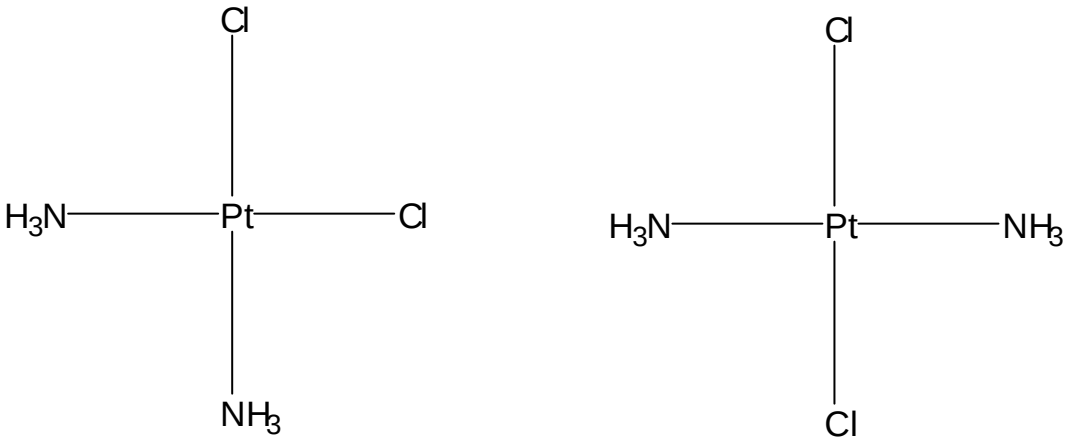
2.1. CISPLATIN

As stated in chapter 1, two commonly used platinum-based anticancer drugs are cisplatin and carboplatin. Cisplatin is *cis*-diamminedichloroplatinum(II), abbreviated also as *cis*-DDP. It is the first inorganic antitumour drug used in clinics (Drobnik 1983) and is one of the most widely used and successful drugs in cancer chemotherapy (Pil and Lippard 1997; Bierbach *et al.* 1999). It was first described by Michele Peyrone in 1895 (Kelland 1993). More than a century later, the compound was coincidentally discovered as an anticancer drug by Barnett Rosenberg in 1964 when he examined the effect of an electric field on bacterial growth (Rosenberg *et al.* 1965; Rosenberg *et al.* 1967). He found that there was a strong filamentous growth but no cell division. Platinum electrodes used in the experiment produced (in presence of ammonium chloride) a number of Pt(II) and Pt(IV) compounds including cisplatin, that inhibited cell division causing the bacteria to become long and filamentous (Rosenberg *et al.* 1969). This accidental discovery led him and his associates to synthesize some simple platinum complexes and examine their antitumour activity. In 1971 the first clinical use of cisplatin was initiated (Hill *et al.* 1971) and now it is widely used for cancer treatment (Loehrer and Einhorn 1984).

Today cisplatin is routinely used alone or in combination with other anticancer drugs for the treatment of cancers of lung, ovary, testes and bladder (Di Blasi *et al.* 1998). More about the development of cisplatin as anticancer drug will be considered later in section 2.1.2.

2.1.1. *CHEMISTRY OF CISPLATIN*

As stated earlier, cisplatin (also known as *cis*-DDP) is *cis*-diamminedichloroplatinum(II). It is a simple inorganic neutral, square planar complex containing platinum(II) bonded to two non-labile ammonia ligands and two labile chloro ligands oriented in a *cis*-configuration. The labile ligands are also known as the leaving groups whereas the non-labile ligands are also known as the carrier ligands. Transplatin (also known as *trans*-DDP) also has the same ligands but in a *trans*-geometry. The chemical structures of cisplatin and transplatin are shown in figure 2.1.



Cisplatin (*cis*-DDP) Transplatin (*trans*-DDP)

Figure 2. 1 Structures of cisplatin and transplatin

Whereas cisplatin is active compound against a wide variety of tumours, transplatin is inactive but toxic (Lippert 1996). This is believed to be associated with the difference in reactivity of the two compounds. Transplatin is found to be more reactive than cisplatin so that ligand displacement reactions would be faster for transplatin than for cisplatin. The two compounds may also differ in the nature of adducts formed with DNA. Whereas both cisplatin and transplatin can form monofunctional adducts

almost at the same rate, they differ in the rate of closure of the monofunctional adducts (faster for cisplatin than transplatin) and in the actual types of bifunctional adducts formed. Cisplatin monofunctional adducts can close to form 1,2-intrastrand bifunctional crosslinks (Milkevitch *et al.* 1997) and transplatin monofunctional adducts can close mainly to form interstrand bifunctional cross links. For this reason, a great deal of effort has been placed on discovering the specific proteins that recognize cisplatin-DNA complexes and examining how the interaction of these proteins with the complexes might lead to cell death (Pil and Lippard 1997). Figure 2.2 illustrates the formation of different bifunctional adducts by cisplatin and transplatin.

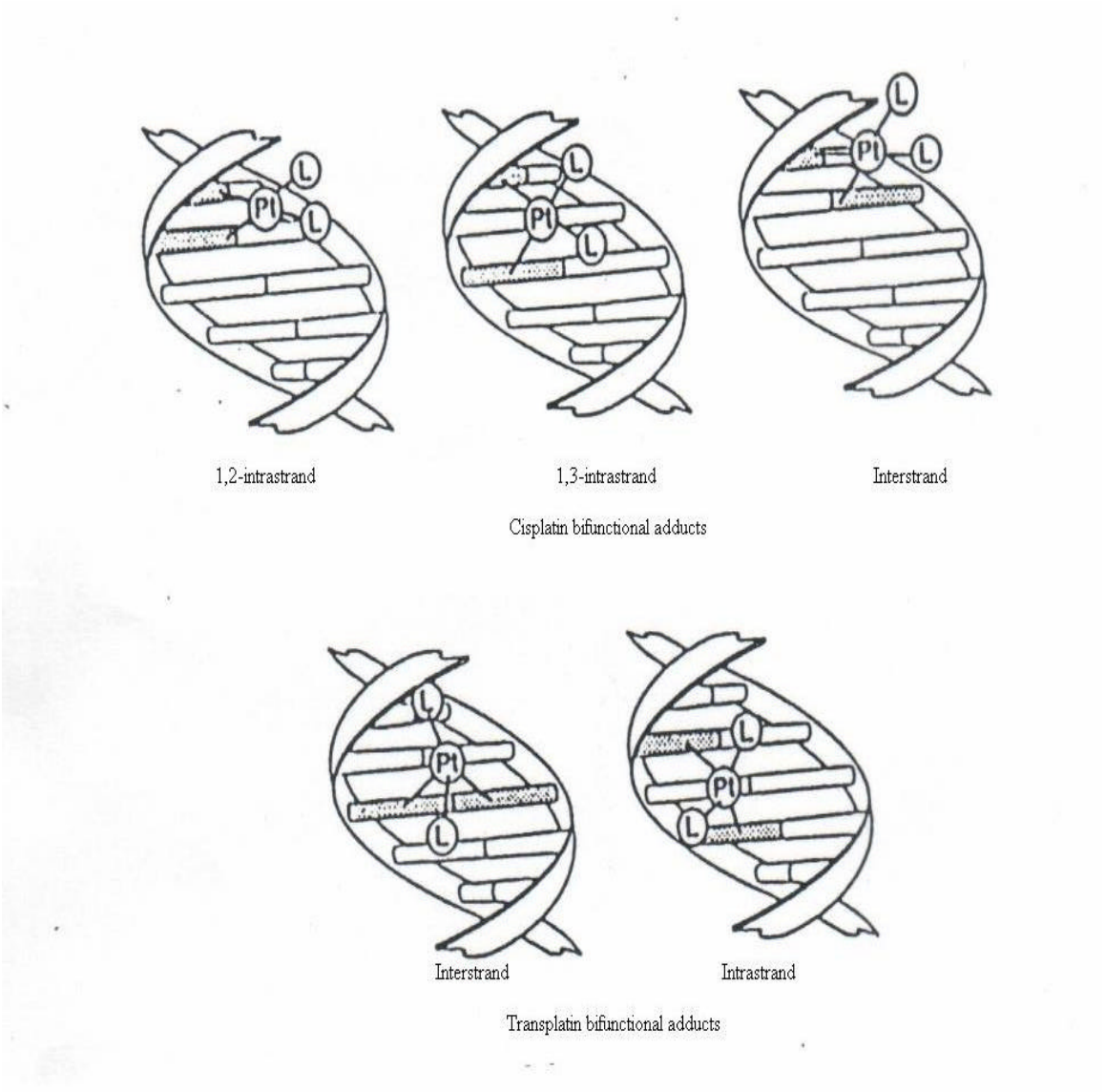


Figure 2. 2 Bifunctional adducts on DNA of cisplatin (top) and transplastin (bottom) [Based on (Farrell 1996)]

As stated earlier, the antitumour activity of cisplatin and other platinum-based anticancer drugs is attributed to a great extent to the kinetics of ligand displacement reactions (Berners-Price and Appleton 2000). The primary target of cisplatin is the nitrogen centres in the nucleobases of DNA. It is believed that the bonds between the metal ion and the nitrogen centres of nucleobases are sufficiently long-lived to

interfere with the process of cell division or to trigger other intracellular mechanisms that recognize irreparable damage to cells. On the other hand, the compounds should be sufficiently reactive for the injected drug to undergo in a relatively short period of time a sequence of reactions that allows the "leaving groups" present initially on the compound to be replaced by nitrogen centres in nucleobases.

Cisplatin is a relatively inert molecule that does not directly react with molecules in the biological systems that bind to platinum through nitrogen or oxygen donor centres. It enters the cell by both passive diffusion (Jamieson and Lippard 1999) and carrier-mediated transport (Andrews and Howell 1990; Gately and Howell 1993; Andrews 1994). Recently cisplatin has also been found to enter cells by active transport mediated by the copper transporter Ctr1P in yeast and mammals (Ishida *et al.* 2002). Cisplatin in aqueous solution is slowly hydrolyzed in which the two labile chloride ligands are progressively replaced by water molecules in a rather complicated manner (Orton *et al.* 1993) to produce *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ and *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ (Figure 2.3). Depending on the pH of the solution, the bound water molecules may deprotonate to produce hydroxo complexes. The first step of hydrolysis is the rate-determining step ($t_{1/2}$ = 1.9 h) in the reaction of cisplatin with DNA(Gelasco and Lippard 1999) as the positively charged aqua species are more reactive than the neutral molecule and react readily with donor nitrogen ligands such as nucleobases. Although the high chloride ion concentrations (> 100 mM) present in blood plasma and extracellular fluid would prevent the hydrolysis of cisplatin, once it crossed the cell membrane such reactions can occur easily since chloride concentration in the intracellular fluid is much lower (about 4 mM).

Thus, activated aqua species are produced within the cell, and these then react with nucleobases (Bloemink and Reedijk 1996).

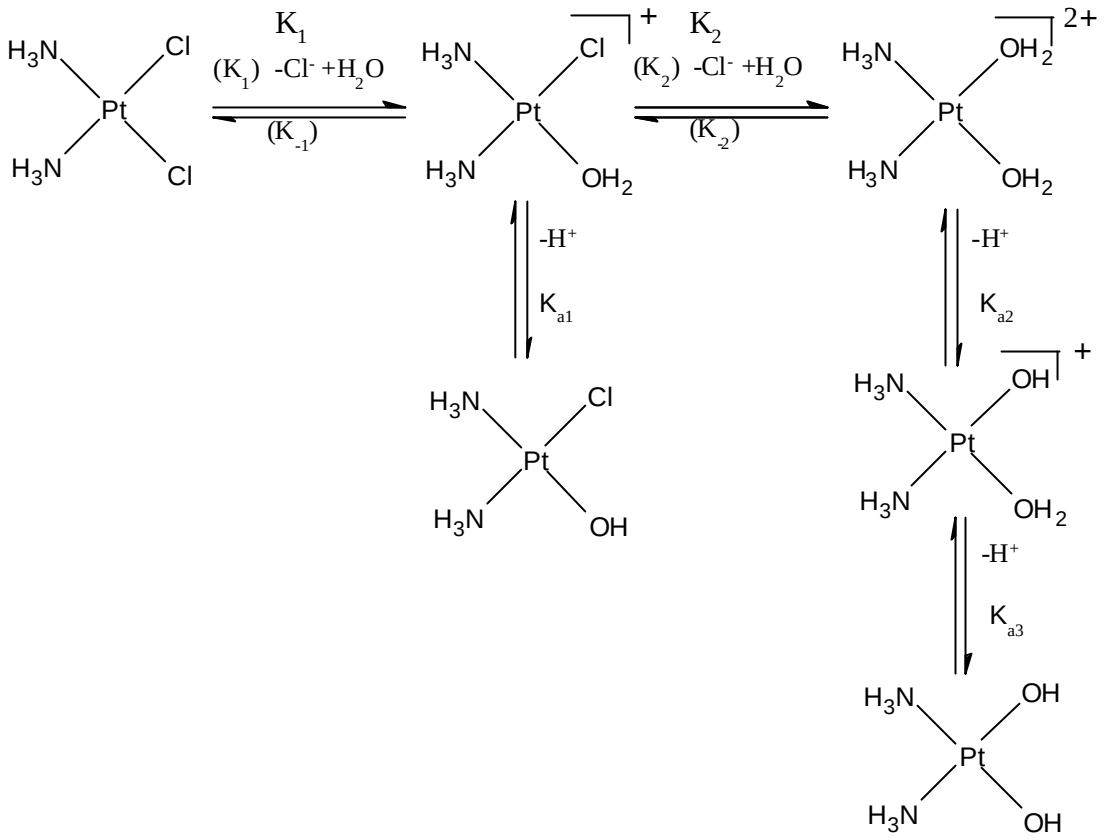


Figure 2. 3 Hydrolysis of cisplatin in aqueous solution.[Based on Berners-Price et al and El-Khateeb et al (El-Khateeb *et al.* 1999; Berners-Price and Appleton 2000)]

On a simple level, cisplatin causes apoptosis by covalent binding with nucleophilic sites on guanine present in all DNA in the cancer cells (Jamieson and Lippard 1999). Once *cis*-$[Pt(H_2O)(NH_3)_2]^+$ is formed, binding occurs mainly at N7 position of guanine followed by N7 position of adenine and then N3 position of cytosine (Mansy *et al.* 1973; Rahn 1984). It has been suggested that in vivo the positively charged *cis*-$[Pt(H_2O)(NH_3)_2]^+$ ion diffuses to the polynegatively charged DNA and then rapidly migrates along the helix to the preferred dGpdG binding sites (Dunham *et al.* 1998).

Binding of cisplatin to G-N7 leads to stability due to the large intrinsic basicity of G-N7 and the additional stablization produced by the formation of hydrogen bond interaction between the coordinated water molecule and G-O6 unlike that in the case of adenine where repulsive interaction is produced (Arpalahti 1996) figure 2.4.

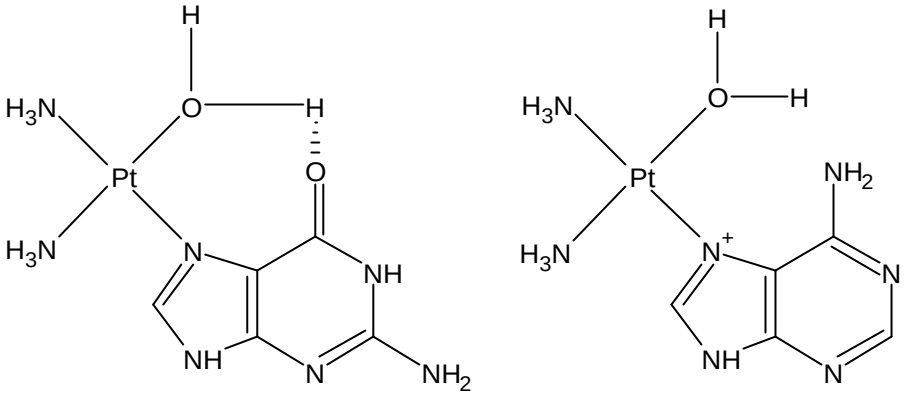


Figure 2. 4 Hydrogen bonding stabilization in guanine and destabilization in adenine

As stated earlier, cisplatin monofunctional adducts close mainly to form intrastrand bifunctional adducts. The actual adducts formed are intrastrand dGpdG (60-65%) and intrastrand dApdG (20-25%), intrastrand dGpXpdG (5-6%), where p stands for phosphate and X stands for a deoxyribonucleotide and interstrand GG (Fichtinger-Schepman *et al.* 1982; Fichtinger-Schepman *et al.* 1985; Eastman 1986, 1987). It can be seen that intrastrand dGpdG and dApdG adducts together account for about 90% of the total adducts.

The structure of platinated DNA is significantly distorted, resulting in a decrease in melting temperature (Hermann *et al.* 1979), shortening (Cohen *et al.* 1979), unwinding (Macquet and Butour 1978) and even some local denaturation of the DNA (Scovell and Capponi 1982).

Because cisplatin and its analogues were found to be tumour active but transplatin and its analogues were found to be inactive, it was suggested that to be tumour active platinum compounds need to satisfy the following requirements, which are known as the classical structure activity relationships (Connors *et al.* 1979).

- Complexes should be neutral
- A *cis* geometry is required with general formula *cis*-$PtX_2(amine)_2$ for Pt(II) compounds and *cis*-$PtX_2Y_2(amine)_2$ for Pt(IV) compounds.
- The complexes should have two anionic leaving groups of intermediate binding strength such as Cl^- and oxalate.
- The complexes should have two non-leaving groups (also called carrier ligands) that are usually primary or secondary amines (but not tertiary amines as they cannot form hydrogen bonds with nearby bases).

Even with such relatively broad requirements, it was found that at least four series of compounds that violated the above structure-activity relationship could be tumour active (Farrell 1993). For example, compounds with a *trans*-geometry and multiple metal centres (that have structures very different from that of cisplatin), have also been found to be tumour active. Because of a different nature of interaction with DNA, such compounds are expected to have a different spectrum of activity. These 'rule breaker' platinum compounds will be considered more fully later in the chapter.

2.1.2. *USES AND LIMITATIONS OF CISPLATIN*

As stated earlier, cisplatin is one of the most potent and widely used anticancer drugs that show activity against slow-growing as well as the rapidly-growing tumours.

However, it has a number of side effects and also cancer cells can have intrinsic resistance to the drug or develop resistance due to the continued use of cisplatin.

2.1.2.1. USES OF CISPLATIN

Cisplatin is administrated intravenously, diluted in physiological saline every 3-4 weeks at doses from 50-120 mg/m^2 (Kelland 2000b). The drug has been shown to be more effective when given regionally (Howell *et al.* 1983). As stated earlier, cisplatin is an effective drug against a wide variety of cancers when given alone or in combination with other anticancer drugs. It is highly effective against testicular and ovarian cancers (Hay and Miller 1998); it has greater than 90% cure rate in testicular cancer (Zhang and Lippard 2003). Cisplatin is also used to treat other kinds of cancers such as non small cell lung cancer, head, neck, cervix, bladder, oesophageal, breast as well as some paediatric malignancies (Hill *et al.* 1971; Higby *et al.* 1974; Von Hoff and Rozencweig 1979; Loehrer and Einhorn 1984; Highley and Hilary Calvert 2000).

Some of the drugs that can be given in combination with cisplatin are 5-fluorouracil (Esaki *et al.* 1992), arabinofuranosylcytosine (Swinnen *et al.* 1989) and aphicicolin andhydroxyurea (Masuda *et al.* 1990). There is a broad consensus that cisplatin together with paclitaxel provides a significant advantage in the treatment of advanced ovarian cancer (Adams *et al.* 1998) over the use of the drugs alone. As stated in chapter one, cisplatin as some other anticancer drugs can exert synergistic effect also, when used in combination with radiotherapy.

Both acquired and intrinsic resistances to cancer chemotherapeutic agents limit the cure rates (Scanlon *et al.* 1989).

2.1.2.2. LIMITATION OF CISPLATIN

As stated earlier, cisplatin is one of the most widely used and successful drugs in cancer chemotherapy, but it has a number of limitations including severe toxicity, development of resistance, poor and low activity against some kinds of cancers and poor solubility (Eastman 1991; Kim *et al.* 1994; Bloemink and Reedijk 1996).

Drug resistance

One of the most significant limitations toward the successful treatment of tumour with platinum compounds including cisplatin is the development of resistance in tumour cells (Johnson *et al.* 1993). Some cancer cells have intrinsic resistance to cisplatin and others develop resistance to its continued use (Scanlon *et al.* 1989). Cisplatin resistance can operate by a number of mechanisms. The three major mechanisms in cisplatin resistance are stated below. Two or more of these mechanisms operate together in most resistant cells.

- Decreased cellular accumulation of cisplatin, limiting the formation of drug-DNA adducts. The decrease in cellular accumulation is generally due to changes in membrane properties (Kawai *et al.* 1990) that may lead to changes in cisplatin transport and/or efflux. Decreased level of platinum accumulation has been reported in several different cell lines having acquired cisplatin resistance (Eastman and Schulte 1988; Gately and Howell 1993; Chu 1994). It has been suggested this was due to decreased drug uptake rather than enhanced drug efflux. It may be noted that cisplatin can cross the cell membrane by both passive diffusion and carrier-mediated transport (Gately and Howell 1993). Whereas the entry into the cell is more likely to be due to passive diffusion, the efflux out of the cell is more likely to be carrier-

mediated since the aquated forms of cisplatin produced inside the cell are positively charged. Increased efflux of cisplatin was observed in cisplatin-resistant cell line in vitro (Fujii *et al.* 1994). In multidrug-resistant cell lines it has been found that a P-glycoprotein acts as a pump and prevents the accumulation of drugs in the cell (Gottesman and Pastan 1993). Shen et al reported that the loss of folate binding protein was associated with decreased cellular accumulation of cisplatin (Shen *et al.* 1998).

- Cytosolic inactivation due to binding with various platinophiles including sulfur-containing molecules such as glutathione (GSH) and metallothionine (MT) (Reedijik and Teuben 1999), thus preventing binding of cisplatin with DNA. Increased glutathione level has been found in some cisplatin-resistant cells (Perez *et al.* 1990; Mistry *et al.* 1993). Cisplatin forms a 2:1 complex with GSH that can be eliminated from the cell by an ATP-dependent glutathione export pump (Ishikawa and Ali-Osman 1993). Like GSH, metallothionein (MT) is also involved in the deactivation of cisplatin. MT is believed to be involved in detoxification of heavy metal ions inside the cell (Chu 1994). Elevated levels of MT have been found in some cisplatin-resistant cells (Kelley and Rozencweig 1989). In fact, regulation of intracellular levels of sulfur-containing compounds appears to be important for eliminating some features of resistance of tumour cells to platinum drugs (Zhang *et al.* 2001).

- Increased DNA repair making the cell more resistant to cisplatin (Masuda *et al.* 1988; Masuda *et al.* 1990; Johnson *et al.* 1994). Cisplatin-DNA adducts may be removed due to hydrolysis of phosphdiester bonds on both sides of the lesion following which the DNA is repaired. Mismatch repair (MMR) plays an important role not only in drug resistance but also in maintaining the

integrity of the genome. Expression of MMR proteins is found to be associated with the sensitivity of mammalian cells to an ever increasing range of DNA-damaging agents including platinum-based anticancer drugs (Fink *et al.* 1998). Thus increased DNA repair may be due to the presence of certain proteins such as XPE-BF that recognizes many DNA lesions including those induced by UV radiation and cisplatin (Patterson and Chu 1989; Hwang and Chu 1993). Levels of XPE-BF were found to increase early in the development of cisplatin resistance (Chu 1994). Another example of DNA repair proteins is ERCC1-XPF, which is a heterodimer. It is believed to cut the strand on the 5' side of the damage and the XPG protein incises on the 3' side (Gibson 1997). On the other hand the high mobility group (HMG-domain) proteins, play an important role in cisplatin activity. Most of the HMG-domain proteins have the capacity to bind with cisplatin-DNA adducts (specifically to 1,2d(GPG)-cisplatin intrastrand cross-link) and prevent the repair mechanism (Zamble and Lippard 1999).

Toxicity

The second major limitation of cisplatin is its toxicity. These include renal dysfunction, nausea and vomiting, peripheral neuropathy, auditory impairment, mylosuppression, visual impairment and pancreatitis (Screnci and McKeage 1999; Highley and Hilary Calvert 2000). The dose-limiting nephrotoxicity of cisplatin almost prevented its development as anticancer drug until a solution in terms of forced hydration was suggested (Cvitkovic *et al.* 1977; Hayes *et al.* 1977). Hydration before and after, with or without mannitol is now routinely used to reduce nephrotoxicity (Weiss and Christian 1993). Even then glomerular filtration rate can decrease by 25% or more.

To reduce the toxic side effects of cisplatin and to widen the spectrum of activity, thousands of cisplatin analogues have been prepared by varying the nature of leaving groups and the carrier ligands. However, only a few drugs have reached the clinical trial and the rest of these have been discarded due to lack of advantage over cisplatin. When the nature of the leaving groups was modified, it was possible to make changes in toxicity profile of the compounds but no changes in the spectrum of activity could be achieved eg carboplatin which has 1,1-cyclobutanedicarboxylate as the leaving groups is found to be less toxic than cisplatin (van der Vijgh 1991). More about carboplatin will be considered in the next section. When the nature of the leaving groups was changed, it was possible to cause a limited change in the spectrum of activity eg oxaliplatin which has DACH (1,2-diaminocyclohexane) carrier ligand is found to be active against colorectal cancer whereas cisplatin and carboplatin are not (Misset *et al.* 2000). More about oxaliplatin will be considered in the next section.

Since all the cisplatin analogues generally form similar adducts with DNA that translate often into a similar spectrum of activity, it has been suggested that to have a spectrum of activity markedly different from that of cisplatin we need to develop compounds that would have novel chemical structures and biological properties. One such class of compounds are dinuclear and trinuclear platinum complexes. Before we consider polynuclear platinum compounds, we will review the development of cisplatin analogues and related compounds which are commonly known as second and third generation drugs.

2.2. SECOND AND THIRD GENERATION MONONUCLEAR PLATINUM BASED ANTICANCER DRUGS

As stated earlier, because of the limitations of cisplatin, many platinum compounds have been synthesized (Kelland 1993; Hay and Miller 1998) by changing the nature of the leaving groups and that of the carrier ligands. In the development of platinum compounds which are commonly known as second generation platinum drugs, the primary goal was to reduce the toxic side effects and to a lesser extent widen the spectrum of activity (Kelland *et al.* 1995). So far more than 23 cisplatin analogues have been tested in cancer patients but most were found to be unsuitable for further development because of poor aqueous solubility, formulation difficulties or severe toxicity (Kelland 1993). As noted earlier, the nature of the leaving groups plays a key role in determining the toxicity profile whereas that of the carrier ligand plays a dominant role in determining the spectrum of activity. Compounds with very labile ligands such as nitrate are found to be toxic rather than anticancer active as these may be deactivated before entry into cell. Compounds with strongly coordinating ligands such as SCN^- are also found to be inactive as these may not be replaced by nucleobases in DNA. Compounds with moderate labile ligands such as chloride and carboxylate are found to display higher antitumour activity (Bloemink and Reedijk 1996).

During the last thirty years, thousands of cisplatin analogues have been prepared by varying the nature of the leaving and the non-leaving groups. When the nature of the leaving groups was modulated it was possible to reduce toxicity but not the spectrum of activity. When the structure of carrier ligands was modified, it was possible to achieve a limited change in the spectrum of activity eg oxaliplatin which has a DACH

(1,2-diaminocyclohexane) carrier ligand is found to be active against colorectal cancer. The search for a less toxic drug based on the modification of the leaving groups at the Institute of Cancer Research in the U.K led to the development of carboplatin (Harrap 1995), which is [*cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II)]. It is now routinely used as alternative to cisplatin. It has a similar spectrum of activity to that of cisplatin but with reduced toxicity (van der Vijgh 1991).

In general cisplatin and its second generation analogues are administrated intravenously whereas third-generation compounds such as JM216 and ZD0473 can be administrated orally allowing a greater flexibility in dosing and increase in the potential use in palliative care (Barnard *et al.* 1999).

2.2.1. *CARBOPLATIN*

Carboplatin is *cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II) abbreviated as CBCD (figure 2.5).

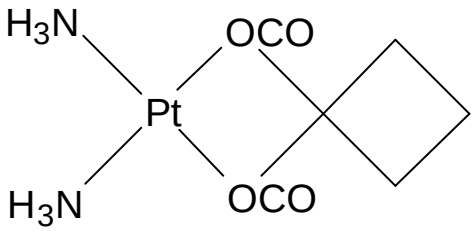


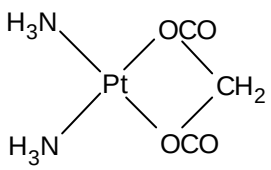
Figure 2. 5 Structure of carboplatin

As stated earlier, carboplatin was developed in the Institute of Cancer Research in the U.K. The development of the drug was actually a collaborative effort between Johnson Matthey (JM) and Institute of Cancer Research (ICR). It entered into clinical

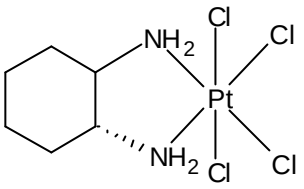
trial in 1981(Kelland 2000a). Carboplatin is the only analogue of cisplatin that is approved worldwide for clinical use (Ozols 1992). Carboplatin and cisplatin have been shown to form identical type of adducts with DNA (Perez 1998). The cyclobutanedicarboxylate ligand found in carboplatin is less labile than the chloride ligands present in cisplatin. This makes carboplatin less toxic but because of the presence of the same carrier ligands as in cisplatin activity is retained (Calvert *et al.* 1992; Lebwohl and Canetta 1998). Carboplatin has significantly less neurotoxicity and nephrotoxicity than cisplatin (Goddard *et al.* 1994 122) so that there is no need for hydration, facilitating its use in out-patients setting. Nausea and vomiting are also reduced compared to that in cisplatin (O'Dwyer *et al.* 1999). However myelosuppression is increased which is the dose-limiting toxicity of carboplatin (Blommaert *et al.* 1995). Carboplatin has now replaced cisplatin in many clinics, on the basis of its broadly similar spectrum of activity and reduced toxicity (Judson and Kelland 2000) and has emerged as the first-line treatment of choice for patients with advanced ovarian cancer (Kelland *et al.* 1999). Carboplatin can be given alone or in combination with other drugs. Combination of carboplatin with paclitaxel has been found to reduce carboplatin-induced thrombocytopenia and potentiate their activity against urothelial cancer (Lebwohl and Canetta 1998; Judson and Kelland 2000).

2.2.2. *CISPLATIN ANALOGUES*

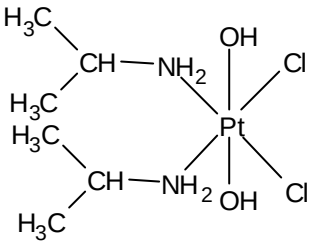
Several analogues of cisplatin or carboplatin that belong to the second and third generation of platinum compounds have been synthesized and studied in pre-clinical trial. Only a handful of these compounds entered the clinical trial and most of these have been discarded due to lack of advantage over cisplatin or caboplatin. The chemical structures of some of these compounds are shown in figure 2.6



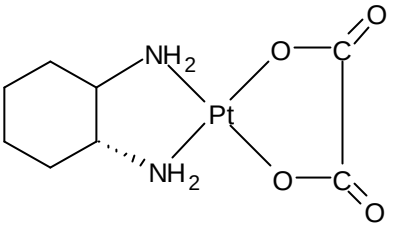
Nedaplatin



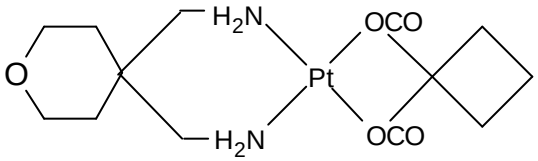
Tetraplatin (Ormaplatin)



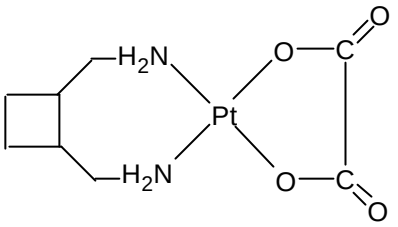
Iproplatin



Oxaliplatin



Enloplatin



Lobaplatin

Figure 2. 6 Chemical structures of cisplatin analogues in clinical trial

2.2.2.1. NEDAPLATIN (245-S)

Nedaplatin is *cis*-diammine(glycolato-*0,0'*)platinum(II). This is a water soluble platinum compound that was synthesized by Shionogi Pharmaceutical Company of Osaka, Japan in the mid 1980s and is registered for use in Japan (Judson and Kelland 2000). The compound has a novel structure involving a glycolate ring bound to platinum atom as a bidentate ligand. Phase II studies have shown that nedaplatin has a

spectrum of activity similar that of cisplatin but with lower nephrotoxcitiy (Akaza *et al.* 1992). Myelosuppression associated with thrombocytopenia is the dose-limiting toxicity of nedaplatin (Akaza *et al.* 1992; Kelland 1993).

2.2.2.2. TETRAPLATIN (ORMAPLATIN)

Tetraplatin also known as ormaplatin is tetrachloro-(*dl-trans*)-(1,2-diaminocyclohexane)platinum(IV). It is one of the DACH complexes that entered clinical trial. Like cisplatin and carboplatin, tetraplatin has to be administered intravenously. Tetraplatin was found to overcome cisplatin-resistance in murine L120 tumour (Goddard *et al.* 1991). In other studies on different human tumour cells with acquired resistance to cisplatin it was found that tetraplatin shared either partial or full cross-resistance with cisplatin (Hills *et al.* 1989; Kelland *et al.* 1992; Meijer *et al.* 1992; Mellish *et al.* 1993). The clinical trial of tetraplatin has been abandoned during phase I clinical trial due to its severe neurotoxicity (Figg *et al.* 1997).

2.2.2.3. OXALIPLATIN

Like tetraplatin, oxaliplatin is also a DACH containing platinum drug but unlike that in tetraplatin, platinum in oxaliplatin is in +2 oxidation state. Oxaliplatin is (1,2-*trans*-diaminocyclohexane)oxalatoplatinum(II). It was first synthesized by Kidani in 1978 (Kidani *et al.* 1980) and developed in Europe, primarily in France (Mathe *et al.* 1985). Several studies have shown that oxaliplatin with its slowly reacting leaving group (namely the oxalate) reduces the nephrotoxicity compared to that of cisplatin (Mathe *et al.* 1989). Neurotoxicity is the only major dose-limiting toxicity associated with oxaliplatin (Extra *et al.* 1990). The nature of binding of oxaliplatin with DNA is found to be similar to that of cisplatin but the rate of formation of intrastrand adducts is

lower (Saris *et al.* 1996). The actual number of DNA adducts formed by oxaliplatin is also found to less than that formed by cisplatin (Woynarowski *et al.* 1998).

Oxaliplatin has been found to be active against advanced colorectal cancer (Barefoot 2001) both as single agent (Becouarn *et al.* 1998) and in combination with flurouracil (Levi *et al.* 1994; Rixe *et al.* 1996). In contrast, cisplatin and carboplatin essentially have no activity against colorectal cancer (Misset *et al.* 2000). Oxaliplatin was also found to be active against a number of cisplatin- and carboplatin-resistant tumour cells (Rixe *et al.* 1996). Thus the development of oxaliplatin provides a clear example of change in toxicity profile and spectrum of activity due to a combination of changes in the leaving groups and the carrier ligands.

Recently it has been found that oxaliplatin is more potent than cisplatin in induction of apoptosis (Faivre *et al.* 2003). Oxaliplatin is currently registered for use in advanced colorectal cancer in United States, Europe, Asia and Latin America (Faivre *et al.* 2003).

2.2.2.4. IPROPLATIN

Iproplatin is *cis*-dichloro-*trans*-dihydroxo-bis(isopropylamine)platinum(IV), also known as CHIP and JM6. It was selected for clinical evaluation on the basis of its improved therapeutic index. In preclinical studies, it showed activity to cisplatin-responsive tumours (Foster *et al.* 1990). Myelosuppression is the dose-limiting toxicity of iproplatin similar to that for carboplatin (Lebwohl and Canetta 1998). However, in phase III clinical trials, it was found to be more toxic and less active than carboplatin. Further development of iproplatin was abandoned because of its lower activity as compared to carboplatin (Trask *et al.* 1991).

2.2.2.5. ENLOPLATIN

Enloplatin is 1,1-cyclobutanedicarboxylato-*0*',*0*'tetrahydro-4H-pyran-4,4-dimethyl amine-N,N'platinum(II). The compound is highly water soluble. It entered the phase I clinical trials. Myelosuppression and nephrotoxicity were found to be the dose-limiting toxicity of enloplatin. However further development of enloplatin was abandoned because of nephrotoxicity and low activity (Lebwohl and Canetta 1998).

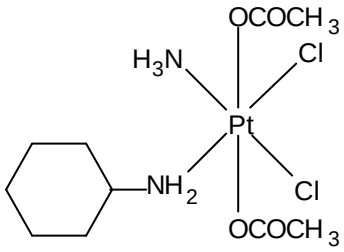
2.2.2.6. LOBAPLATIN

Lobaplatin is 1,2-diaminomethylcyclobutane-platinum(II)-lactate. It is a water soluble platinum(II) compound. Lobaplatin was developed in Germany and evaluated in other parts of the world and is currently used in China (Welink *et al.* 1999). In phase I trials it was found that thrombocytopenia was the dose-limiting toxicity of lobaplatin and no nephrotoxicity was noted (Kelland 1993). Lobaplatin has shown antitumour activity with incomplete cross-resistance in human gastric, testicular, lung and ovarian cancer xenografts (Gietema *et al.* 1993a; Gietema *et al.* 1993b). Both in phase I and phase II clinical trials lobaplatin demonstrated activity against esophageal, breast, head and neck, small lung and ovarian cancers (Welink *et al.* 1999).

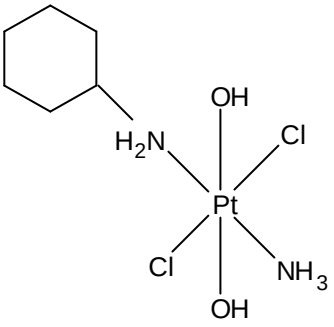
2.2.3. *ORALLY ACTIVE PLATINUM DRUGS*

Like cisplatin and carboplatin, all the second generation platinum based anticancer drugs are administered by intravenous infusion. When given orally, cisplatin and carboplatin are found to be less toxic but also less active (Kelland 2000a). It was stated earlier that oxaliplatin had a somewhat different spectrum of activity and toxicity profile as compared to that for cisplatin and carboplatin but like cisplatin and carboplatin the drug can only be given intravenously. Thus the structure of oxaliplatin was modified to obtain orally active compounds (Kizu *et al.* 1996). The aim of a

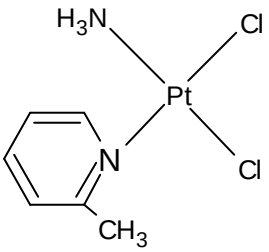
continued collaborative program between Johnson Matthey (JM) and Institute of Cancer Research (ICR) has also been to develop an orally active platinum drug that would have activity at least comparable to cisplatin and toxicity similar to that of carboplatin. Currently, a number of orally active platinum compounds are under clinical trials (Figure 2.7).



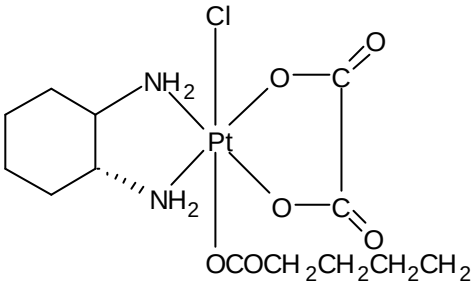
JM216



JM335



AMD473



C5-OH-Cl

Figure 2. 7 Chemical structures of MJ335 and the orally active compounds MJ216, AMD473 and C5-OHP-Cl

2.2.3.1. JM216

JM216 is bis(acetato)ammine-dichloro(cyclohexylamine)platinum(IV). It is given orally rather than by intravenous injection. The compound has been developed by

Bristol-Myer Company in collaboration with JM and ICR (Kelland 1993). It differs from cisplatin and carboplatin in the sense that it is a platinum(IV) compound rather than platinum(II). This feature makes JM216 more stable, which could be important in its passage through the gastrointestinal tract. JM216 is also found to be more lipophilic than other platinum drugs, a feature that may aid its absorption through the membranes of the gastrointestinal wall. It is however believed that as in the case of other platinum(IV) compounds, platinum is reduced from IV to II state before it interacts with DNA (Kelland 2000a).

JM216 entered clinical trial in 1992 (McKeage *et al.* 1995). In phase I trials, it was found that myelosuppression was the dose-limiting toxicity of the drug (McKeage *et al.* 1997). The antitumour activity of JM216 is equivalent to that of cisplatin and carboplatin (Barefoot 2001). JM216 could replace cisplatin for combined radio-chemotherapy treatment (Amorino *et al.* 1999).

2.2.3.2. JM335

JM335 is *trans*-ammine(dichlorocyclohexylamine-dihydroxo)platinum(IV). It provides an example of compounds that break the classical structure activity relationships. It is a rationally designed complex that forms a different spectrum of adducts with DNA (Kelland *et al.* 1999). It showed comparable activity to its *cis*-isomer and cisplatin against ADJ/PC6 plasmacytoma, L1210 and CH1 ovarian cancers (Perez *et al.* 2000) and was found to induce apopotosis in CH1 cell line (O'Neill *et al.* 1996). Early study with JM335 suggested that *in vitro* it succeeded in circumvention of acquired cisplatin resistance against some cell lines including $41M^{cisR}$ and $CH1^{cisR}$ (Kelland *et al.* 1994).

2.2.3.3. ZD0473

ZD0473 formerly known as AMD473 or JM473 is *cis*-amminedichloro(2-methylpyridine)platinum(II). It entered phase I clinical trials in 1997 at the Royal Marsden NHS Trust Hospital under the auspices of the UK Cancer Research Campaign. It is a novel platinum compound that has been designed to overcome acquired or intrinsic resistance to cisplatin (Judson and Kelland 2000). It has been suggested the bulkier methylpyridine ligand would increase steric hindrance so that the substitution reaction pathway is shifted more towards a dissociative rather associative mechanism. Thus the compound would have reduced susceptibility to inactivation by elevated intracellular thiol concentrations (Kelland 1999). As stated earlier, inactivation of cisplatin by GSH or MT is one of the common mechanisms of cisplatin resistance. ZD0473 was found to be less reactive than cisplatin towards thiourea and methionine (Holford *et al.* 1998a). However it was found that ZD0473 shares some mechanisms of resistance with cisplatin in A2780 cell line including reduced drug transport, increased GSH level and loss of MLH1 DNA mismatch repair (Holford *et al.* 2000). In vitro studies show that the antitumour activity of ZD0473 lies in between that of cisplatin and carboplatin (Holford *et al.* 1998b). ZD0473 has been found to show significant activity against four lung cell lines: PC-14/CDDP, PC-9/CDDP,SBC-3/CDDP and H69/CDDP that are resistant to cisplatin (Kawamura-Akiyama *et al.* 2002). The compound is also found to be quite active against $41M^{cisR}$ ovarian cell line that is resistant to cisplatin (Judson and Kelland 2000), indicating that ZD0473 has been able to overcome multiple mechanisms of resistance operating in the cell line. Preclinical studies showed that the dose-limiting toxicity of ZD0473 was myelosuppression similar to that of carboplatin (Raynaud *et al.* 1997). Phase I

clinical study also confirmed that the dose-limiting toxicity of ZD0473 was bone marrow suppression (Judson and Kelland 2000).

2.2.3.4. C5-OHP-Cl

With the aim of obtaining orally active derivatives of oxaliplatin, different compounds having the general formula: *trans,cis,cis*-[Pt(IV)($OCOC_nH_{n+1}$)$_2$(oxalate)(1R,2R cyclohexanediamine)] were prepared by Kidani *et al*, (Kizu *et al.* 1996). The *trans-bis*(n-valerato)(1R,2R-cyclohexanediamine)(oxalate)platinum(IV) code named C5-OHP-Cl was found to be the one that was most absorptive and most active (Kizu *et al.* 1998). It was found to be more effective than cisplatin in different tumour models (e.g L1210 leukemia and LMFS sarcoma) (Saegusa *et al.* 2001).

The following figure summarizes the development of different platinum-based tumour active compounds as a result of a cooperative program between Johnson Mathey and Institute of Cancer Research in the U.K.

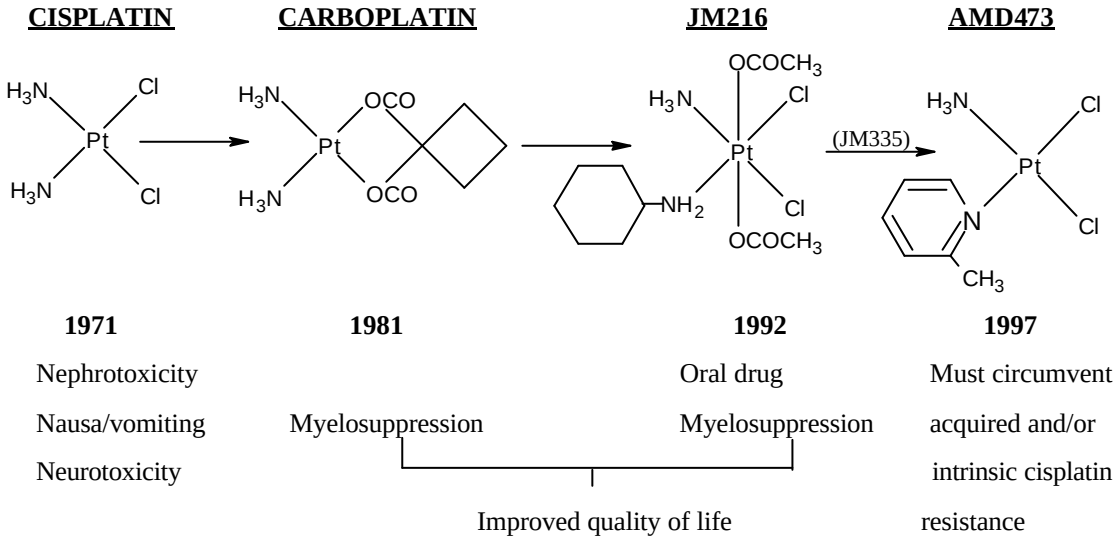


Figure 2. 8 Summary of the ICR/JM platinum drug program from carboplatin to ZD0473 (Based on Kelland (Kelland 2000a))

2.3. MULTINUCLEAR PLATINUM COMPOUNDS

In the previous section we have reviewed the development of second and third generation platinum-based anticancer drugs. It was found that changes in toxicity profile of the compounds could be achieved by changing the nature of the leaving groups whereas modulation of the carrier ligands could cause a limited change in the spectrum of activity. However, it is found that all cisplatin analogues generally form similar adducts with DNA that often translate into a similar spectrum of activity. Thus, it was suggested by Farrell that for platinum drugs to have a spectrum of activity very different from that of cisplatin, the compounds need to have structures distinctly different from that of cisplatin (Farrell 1993). Dinuclear and trinuclear platinum complexes represent a new class of tumour active compounds with structures and properties (including biological ones) distinctly different from those of cisplatin. The understanding that cisplatin acts by delivering the *cis*-$[Pt(NH_3)_2]$ moiety to DNA provided a rationale for systematically altering the structure of the platinum coordination sphere to arrive at altered activity/toxicity profiles (Farrell *et al.* 1999). As stated earlier, while only one analogue of cisplatin namely carboplatin entered full clinical use world wide, a number of other analogues such as oxaliplatin, ZD0473, JM216 and lobaplatin are being evaluated in Phase I and Phase II clinical trials. A number of other platinum-based compounds, such as tetraplatin and iproplatin, that previously entered clinical trials, are no longer under development, because of unacceptable toxic side effects, insufficient efficacy or a limited spectrum of activity. Attempts to design new platinum complexes with comparable antitumour activity but no cross-resistance with cisplatin and carboplatin led to development of a number of polynuclear platinum complexes (Perego *et al.* 1999a). In a recent review, Wheate et

al, summarise the development over the last 15 years of multinuclear platinum complexes as anticancer agents (Wheate and Collins 2003).

2.3.1. *DINUCLEAR PLATINUM COMPLEXES*

The dinuclear motif that was first reported in 1988 consisted of two *cis*-$[Pt(NH_3)_2]$ units linked together by a flexible diamine chain (Farrell *et al.* 1988; Farrell *et al.* 1990b). Systematic variation of the coordination sphere, chain length and steric effects within the linkers by Farrell and co-workers produced a wide range of possible structures some of which are given in figure 2.9. In terms of the adducts formed in DNA, the dinuclear complexes can be diverse ranging from being bifunctional to tetrafunctional binders to DNA. It was found that among the dinuclear complexes, the so-called 1,1/t,t series (where 1,1 indicate the presence of one chloro ligand on each platinum unit and the letter 't' stands for *trans*-geometry) gave consistently higher antitumour activity in cisplatin-resistant cells (Farrell and Spinelli 1999; Farrell 2000). It was also found that the length of diamine chain played an important role in the activity of the compounds. The 1,1/t,t compound with two platinum units linked by 1,6-diaminohexane was found to be much more active than the other dinuclear compounds (Menta *et al.* 1999). As the length of the linking diamine was increased above six or decreased below six, the activity of the compound was found to decrease.

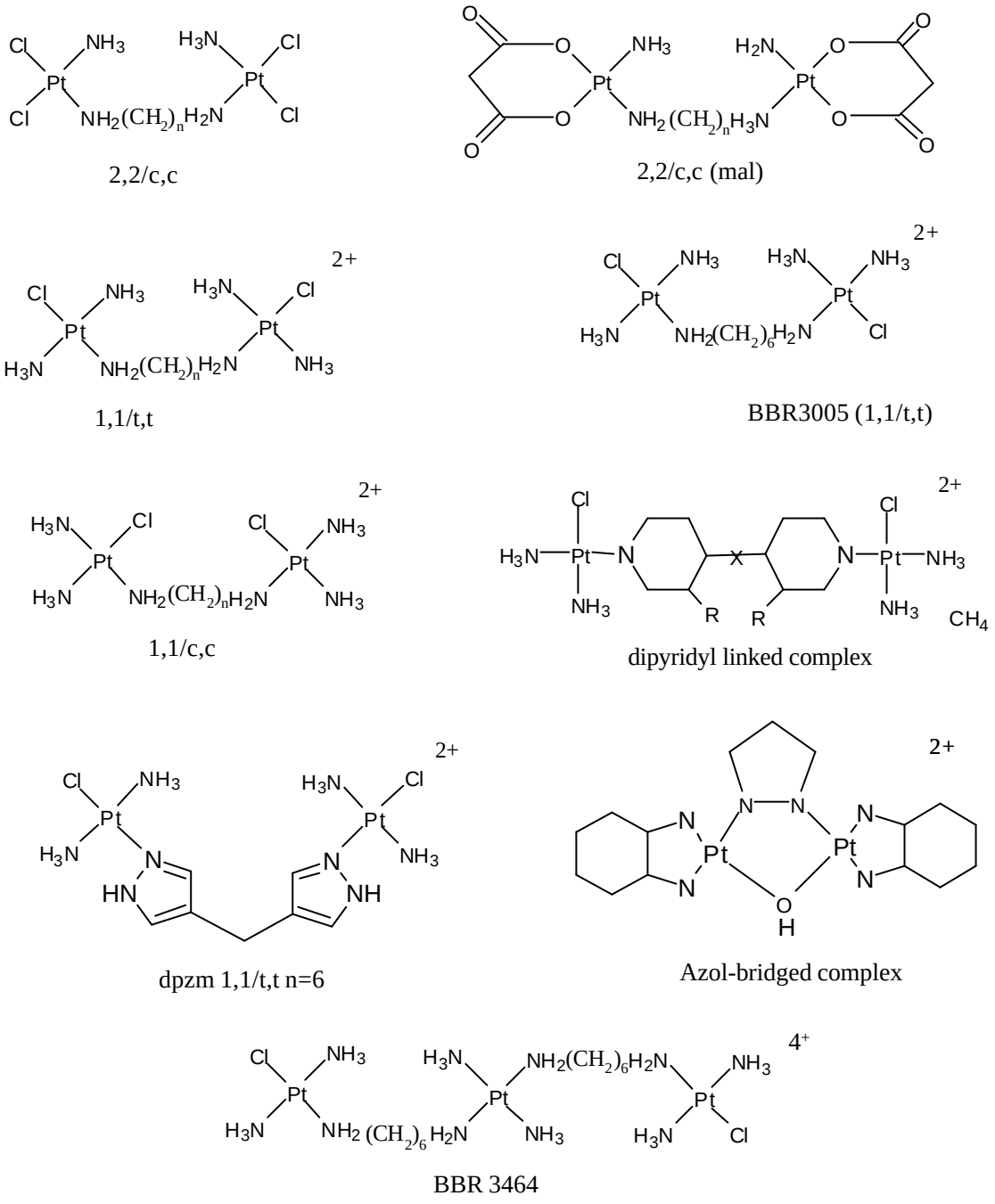


Figure 2. 9 Structures of different classes of dinuclear Pt(II) complexes and the trinuclear compound BBR3464

Cisplatin binds to DNA, forming mainly monofunctional adducts and bifunctional intrastrand adducts. DNA-DNA interstrand GG cross-links and DNA-protein crosslinks are produced to a much lesser extent with cisplatin. Inhibition of DNA synthesis and transcription is believed to be a consequence of Pt-DNA adducts

formation. In contrast, the predominant DNA binding mode of dinuclear platinum compounds is DNA-DNA interstrand cross-linking where one Pt coordination sphere binds to one DNA strand (Farrell 1995). The extent of interstrand cross-linking is dependent on the exact structure of the compound. At [drug] : DNA binding ratio (in terms of phosphate) ri > 0.025 the 1,1/t,t compounds were found to produce significantly more interstrand cross-links than their 2,2/c,c counterparts (where 2,2/c,c indicate the presence of two chloro ligands in *cis*-geometry on each platinum unit). The other difference between 1,1/t,t and 2,2/c,c compounds is that whereas for 2,2/c,c complexes the extent of overall binding with DNA increases linearly with the increase in Pt concentration, for 1,1/t,t complexes the extent of interstrand binding rises to a maximum and then falls off. It may be noted that for the dinuclear complexes, the formation of DNA-DNA cross-linking requires the presence of only one substitution-labile Pt-Cl bond on each Pt centre. Tetrafunctional 2,2/c,c dinuclear complexes have the ability to produce DNA-protein ternary cross-links and interhelical DNA-DNA cross-links, where two double helices are linked together (Buning *et al.* 1997).

Table 2.1 compares the in vitro cytoxicity of dinuclear and mononuclear platinum compounds in a human ovarian tumour panel (Farrell *et al.* 1999).

Table 2. 1 Comparison of in vitro cytotoxicity of dinuclear and mononuclear platinum compounds in a human ovarian tumour panel[a] (Farrell *et al.* 1999)

Compound	HX/62	SKVO-3	PXN/94	41M^{CisR}	41M	CH1^{CisR}	CH1
$[\{Pt(mal)(NH_3)\}_2$ $NH_2(CH_2)_4NH_2]$	44.5[a] ± 3.5	20.8 ± 3.7	9.9 ± 2.8	4.3 (3.1)[b] ± 1.4	1.4 ± 0.44	2.4(4.4) ± 0.44	0.54 ± 0.03
$[\{PtCl(NH_3)\}_2$ $NH_2(CH_2)_4NH_2]^{2+}$	46 ± 14.7	57 ± 8.8	14.3 ± 1.8	5.1(0.71) ± 0.78	7.2 ± 0.33	6.3 (2.4) ± 4.2	2.6 ± 1.4
Cisplatin	12.6 ± 1.5	4.4 ± 1.4	3 ± 0.57	1.4(6.1) ± 0.1	0.23 ± 0.03	0.67(6.7) ± 0.1	0.1 ± 0.014
Carboplatin	70 ± 7	38 ± 9	31 ± 5	10.4(2.8) ± 0.9	3.7 ± 0.4	4.2(4.2) ± 0.2	1 ± 0.2

a ID_{50} (μM) and b RF is resistance factor

Relative toxicity in hypoxic tissue provides an example of differences in biological activity that may exist among dinuclear complexes. It is known that tumours often have regions of hypoxia as a result of poor vascularization. As the cells are deprived of oxygen they may become resistant to radiotherapy and also to some forms of chemotherapy. As stated earlier, a common mode of treatment against cancer is to combine chemotherapy with radiation. Whereas radiosensitization and toxicity of the drug are found to be greater in hypoxic than aerobic cells as applied to cisplatin (Matthews *et al.* 1993) and 2,2/c,c dinuclear compounds in the form of malonates (Skov *et al.* 1998), the bifunctional 1,1/t,t and 1,1/c,c complexes do not display any such selectivity. According to Matthews et al, DNA-protein cross-linking may be an important determinant in hypoxic toxicity (Matthews *et al.* 1993). It can be seen that the hypothesis is supported by the above results. In vivo studies in human ovarian

carcinoma IGROV-1 show that the dinuclear compounds are more potent and less toxic than carboplatin at equivalent dose. No toxicity is observed at doses up to 80 mg/kg (Farrell *et al.* 1999). Elevated activity in cisplatin-resistant cells, and in some cases different spectrum of activity, suggest that the dinuclear platinum drugs have a potentially different mechanism than that proposed for cisplatin-analogues.

A commonly observed feature of cellular resistance to cisplatin is decreased cellular accumulation of the drug in the resistant cell line than in the parent one. The cellular accumulation of carboplatin and dinuclear complexes did not show change (Farrell *et al.* 1999).

In addition to the dinuclear platinum compounds described by Farrell and co-workers, other investigators such as Broomhead and co-workers also prepared dinuclear compounds but using 4,4'-dipyrazolylmethane as the linker instead of aliphatic diamines used by Farrell and co-workers (Broomhead *et al.* 1992; Broomhead *et al.* 1993). Zaho et al prepared dinuclear platinum complexes with dipyridyl linkers, which showed activity against L1210 cell line similar to or less than that of cisplatin. However, the compounds were found to be more active than cisplatin against cisplatin-resistant cell line HCT8. (Zhao *et al.* 1998a, 1998b). Azole-bridged dinuclear platinum(II) complexes $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)\text{-}(\mu\text{-}pz)][NO_3]_2$ and $[\{cis\text{-}Pt(NH_3)_2\}_2(\mu\text{-}OH)\text{-}(\mu\text{-}1,2,3\text{-}ta)][NO_3]_2$ were prepared by Kemedo et al. These compounds showed much higher activity than that of cisplatin against MCF7 and EVSA-T (breast cancer), WIDR (colon cancer), IGROV (ovarian cancer), M19 (melanoma), A978 (renal cancer) and H226 (non small cell lung cancer (Komeda *et al.* 2000). Wheate et al have prepared a number of multinuclear compounds linked with the 4,4'-dipyrazolylmethane (dpzm) ligand including dinuclear platinum complex (di-Pt) and trinuclear platinum complex (tri-Pt), which are analogues of

BBR3005 and BBR3464 (Wheate *et al.* 2001). Figure 2.9 shows the chemical structure for some of the dinuclear complexes.

2.3.2. *TRINUCLEAR Pt(II) COMPLEXES*

Trinuclear platinum complexes may be considered as a logical progression from the dinuclear compounds. Farrell and his associates as well as other researchers (Rauter *et al.* 1997; Farrell and Spinelli 1999; Wheate *et al.* 2001) have prepared and studied a number of trinuclear complexes based on platinum. A number of these complexes have been found to be highly active against both murine and human cancer cell lines. A notable example of trinuclear platinum complexes is BBR3464 that contains the trinuclear cation: $[\{trans\text{-}PtCl(NH_3)_2\}_2\{\mu\text{-}trans\text{-}Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2\}]^{4+}$ and for which the balancing anions can be nitrate or chloride. For simplicity of discussion, references to BBR3464 are commonly used to mean the trinuclear cation rather than the neutral molecule. Thus BBR3464 consists of three *trans*-platinum units joined together by two 1,6-diaminohexane chains (Figure 2.9). As described later, the compound showed a very high activity against a large number of cisplatin-resistant cancer cell lines. The compound entered clinical trials during 1998 (Pratesi *et al.* 1999; Judson and Kelland 2000). The following table summarizes the cytotoxicity and antitumour activity of a number of dinuclear compounds, the trinuclear compound BBR3464 and cisplatin in murine leukaemia LX-1 human tumour xenograft.

Table 2. 2 Cytotoxicity and antitumour activity of polynuclear platinum complexes in murine leukaemia sensitive (L1210) and resistant to cisplatin (L1210/*cis*-DDP) and in LX-1 human tumour xenograft (data adapted from Farrell (Farrell 2000))

	In vitro IC_{50} (µg/mL)		In vivo (L1210/*cis*-DDP only)		In vivo LX-1	
Compound	L1210	L1210/*cis*-DDP	Dose (mg/kg/d)	T/C%	Dose (mg/kg/d)	TWI%
BBR3537	0.76	4.40	2	100	_	_
BBR3571	0.042	0.0062	0.25	261	0.25	83
BBR3535	0.55	1.1	1	161,178	0.7	85
BBR3610	0.0012	0.0011	0.025	457	_	_
BBR3611	0.0005	0.00041	0.006	256	_	_
BBR3005	2.3	1.8	4.5	133	3	60
BBR3464	0.116	0.093	0.25	239,389	0.3	73
Cisplatin	0.9	8.3	6	110	4	38

IC_{50}: inhibiting concentration 50% of cellular growth after 48 h of drug exposure

TWI: tumour weight inhibition

T/C: median survival time of treated mice/median survival time of control X 100

As stated earlier, BBR3464 has been found to be highly active against a number of cisplatin-responsive and cisplatin-resistant cancer cell lines. The compound is able to circumvent inherent and acquired cisplatin-resistance in vitro and in vivo in a panel of human adult tumour models (Manzotti *et al.* 2000; Riccardi *et al.* 2001). It exhibits complete lack of cross-resistance in U2-OS/Pt (Perego *et al.* 1999a). The high level of antitumor activity in cisplatin-resistant cancer cell lines suggests that BBR3464 is able to overcome the multiple mechanisms of cisplatin resistance (Perego *et al.* 1999a; Pratesi *et al.* 1999). BBR3464 shows very high activity against cisplatin-

sensitive murine leukemia (L1210) and has complete lack of cross-resistance in (L1210/CDDP)(Di Blasi *et al.* 1998).

p53 is a tumour suppressor protein that is involved in the control of cell cycle, DNA repair and apoptosis (Brabec and Kasparkova 2002). According to Farrell a plausible explanation for the hypersensitivity of human tumours with mutant p53 to BBR3464 is that apoptosis induced by the drug is not mediated by p53. This is in contrast to apoptosis induced by cisplatin which is believed to be mediated by p53 (Kasparkova *et al.* 2001). The result supports the idea that antitumour activity of BBR3464 is mediated by a mechanism different from that of cisplatin and other classical platinum-based anticancer compounds (Farrell and Spinelli 1999; Pratesi *et al.* 1999; Farrell 2000). But the cellular determinants responsible for the activity of BBR3464 remain largely unknown (Colella *et al.* 2001).

Table 2.3 shows the cytotoxicity of cisplatin and BBR3464 in human tumour cell lines with different p53 status or expression based on (Pratesi *et al.* 1999).

Table 2. 3 Cytotoxicity of cisplatin and BBR3464 in human tumour cell lines with different p53 status or expression

		IC_{50} (μg/mL) (±SD)			
		Cisplatin		BBR3464	
Cell line (tumour type)	p53 status	1 h	72 h	1 h	72 h
A2780 (ovarian carcinoma)	Wild-type	1.2± 0.03	0.06±0.01	0.032±0.001	0.012±0.004
A2780/CP (ovarian carcinoma)	Wild-type	35±2.8	0.73±0.2	2.0±0.2	0.29±0.2
IGROV-1 (ovarian carcinoma)	Wild-type	4.3±1.3		8.0±2.8	
U2-OS (osteosarcoma)	Wild-type	2.4±0.1		1.7±0.01	
SW626 (ovarian carcinoma)	Mutant (codon273)	6.3±3.0		0.08±0.03	
SAOS (osteosarcoma)	Null	3.0		0.1±0.01	
POGB (SCLC)	Mutant (codon282)		0.25±0.07		0.009±0.006
A431 (cervical carcinoma)	Mutant (codon273)	10.4±3		3.1±1.8	

In general, the interaction with DNA of polynuclear platinum complexes is different from that of mononuclear platinum drugs (Farrell 2000). BBR3464 and other polynuclear complexes were designed to form long-range interstrand and intrastrand DNA crosslinks (Manzotti *et al.* 2000). The long-range interstrand crosslinks formed by BBR3464 are not recognized by HMG domain protein (Zehnulova *et al*. 2001). Also the rate of binding of the polynuclear complexes is found to be different from that of mononuclear complexes. For example, trinuclear compound BBR3464 binds to DNA more rapidly than cisplatin and the dinuclear compound BBR3005. BBR3005 forms more of shorter-range interstrand cross links than BBR3464. (Brabec *et al.* 1999).

From phase I and phase II clinical trials, it was found that the dose-limiting toxicity of BBR3464 was neutropenia and diarrhoea. However, the compound had no significant nephrotoxicity or neurotoxicity (Judson and Kelland 2000).

A number of dinuclear and trinuclear compounds using 4,4'-dipyrazolylmethane (dpzm) as the bridging ligand were prepared by Wheate et al (Wheate *et al.* 2001) (Figure 2.9). The compounds show lower activity than their analogues BBR3005 and BBR3464, which is attributed to the rigid nature of the dpzm ligand (Wheate *et al.* 2001). According to Wheate at al (Wheate and Collins 2003), the following structure-activity relationships applying to polynuclear platinum(II) complexes emerged from the systematic study of Farrell and his associates (Roberts *et al.* 1999a; Farrell 2000) and other researchers (Wheate *et al.* 2001).

1. 1,0,1/t,t,t complexes are more active than their 1,0,1/c,t,c or 1,0,1/t,c,t homologues (where the numerals 1,0,1 indicate the number of chloride

ligands bonded to platinum centres and the letters 't' and 'c' stand for *trans*- and *cis*- respectively).

2. The presence of one or two central -NH_2- groups appears to be essential to provide compounds with high toxicity and potency.

3. The length of linker chains also appears to play a key role in determining activity. Maximum activity is found for a certain length so that activity decreases when the length of the linker chain is either increased or decreased above or below the optimum value. This is found to be true for both spermine family of complexes where the central positive charge is provided by a protonated amine and trinuclear complexes where the central charge is provided by a metal centre.

It is generally accepted that *cis*-$[Pt(NH_3)_2(Cl)(H_2O)]^+$, formed by hydrolysis of one Pt-Cl, pre-associates with DNA (Wang *et al.* 2001; Wheate and Collins 2003) before binding to specific nucleobases in DNA. Wheate at al point out that since pre-association is stabilized largely by electrostatic forces, the pre-association of cationic multinuclear platinum complexes with DNA would be even stronger and therefore more important. It has been suggested that the pre-association of multinuclear platinum complexes with polyanionic DNA will significantly affect the rate and site of platination because an increased local concentration will increase the probability of a covalent interaction at these sites. Also, pre-association may induce a local conformation change in the DNA that may influence binding at a specific site.

As stated earlier, BBR3464 consists of three *trans*-platinum units connected together by two 1,6-diaminohexane chains. Only the two terminal platinum units in BBR3464 undergo covalent binding (mainly interstrand) with DNA whereas the central

platinum unit undergoes only noncovalent interactions such as hydrogen bonding and electrostatic interactions (Farrell and Spinelli 1999). It was hypothesized that although replacement of the central platinum unit with other suitable metal units may not significantly alter the covalent interactions of the terminal platinum units, it may have subtle effects on the noncovalent interactions such that anticancer active compounds with different spectrum of activity may result (Daghriri *et al.* 2001). The very high activity of BBR3464 means that the therapeutic window of the drug is likely to be very narrow. It may be noted that the clinical trials of BBR3464 have been stopped due to significant toxicity namely neutropenias, diarrhoea and nausea. It is quite possible that when the central platinum unit of BBR3464 is replaced by other suitable metal units, the resulting compounds may be active but not as potent as BBR3464 so that their therapeutic window would be wider. The present study deals with such heteronuclear compounds. The aims of the study are the following:

(1) To prepare and characterize polynuclear complexes containing both platinum and palladium centres.

(2) To determine the activity of the compounds against human cancer cells including cell uptake and binding with DNA.

(3) To study the nature of interaction with plasmid and genomic DNA and nucleobases.

(4) To investigate the structure-activity relationship in the designed trinuclear complexes relating to the length of the linker diamine.

The majority of the complexes are modeled on BBR3464 in which the central platinum unit has been replaced by the corresponding on palladium unit and the length of the linking diamine has been varied to contain from four to seven carbon

atoms. All of the compounds are found to be active (with different levels of activity) against a number of cancer cell lines.

CHAPTER THREE

3. EXPERIMENTAL

3.1. SYNTHESES OF COMPOUNDS

3.1.1. *MATERIALS*

Analytical grade concentrated ammonia solution, sodium hydroxide, potassium iodide; concentrated hydrochloric acid and methanol were purchased from Ajax chemicals, Auburn NSW, Australia. Ethanol, acetone, diethyl ether were purchased from APS chemicals, Australia. Potassiumtetrachloroplatinate(II), potassium tetrachloropalladate(II), cadaverine (1,5-diaminopentane) dihydrochloride, putrescine (tetramethylene diamine) dihydrochloride, 1,6-diaminohexane, 1,6-diaminohexane dihydrochloride, N-Boc-1,6-diaminohexane, 1,7-diminoheptane, 2,2' oxybis(ethylamine) dihydrochloride, N-(2-aminoethyl)-1,3-diaminopropane, silver nitrate, triethyl amine, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), dichloromethane, *trans*-diamminedichloropalladium(II) [transpalladin] and *trans*-diamminedichloroplatinum(II) [transplatin], were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia and used as received without further purification.

Transplatin and transpalladin were also prepared in our laboratory.

3.1.2. *PREPARATION OF TRANSPLATIN AND TRANSPALLADIN*

3.1.2.1. PREPARATION OF TRANSPLATIN

Transplatin was prepared based on the general methods of Kauffman (Kauffman and Cowan 1963) and Dhara (Dhara 1970). The Kauffman method was found to give a better yield. The following is a description of the synthesis of transplatin according to the Kauffman method.

2 mmol of potassium tetrachloroplatinate(II) dissolved in 15 mL of mQ water was mixed with 0.5 mL of concentrated HCl. The mixture was heated to boiling, to which 2 mL of concentrated ammonia was slowly added with stirring. It was then cautiously evaporated with stirring to reduce the volume to about 3 mL. 80 mL of 6 M hydrochloric acid was added to the resulting pale yellow solution. The volume of the mixture was reduced to about 6 mL by heating with stirring at 60^{o}C on a hot plate. During evaporation, the mixture first became turbid then clear. Yellow precipitate of transplatin was formed on standing. After being cooled to 0ºC for 15 min, the precipitate of transplatin was collected at the pump, washed first with ice cold water and then with acetone. It was recrystallized from 0.1 M HCl.

3.1.2.2. PREPARATION OF TRANSPALLADIN

Kauffman's method with slight modification was used to prepare transpalladin. Briefly potassium tetrachloropalladate(II) (1 mmol) was dissolved in 8 mL of mQ water and heated for 10 min at 60 ºC. 2 mL of concentrated aqueous ammonia was slowly added ɒ the solution with stirring. 40 mL of 6 M HCl was added to the resulting pink solution and the mixture was stirred for 2 h at 40-50 ºC. The orange

precipitate was filtered and washed twice with ice cold water followed by that with methanol.

3.1.3. *SYNTHESES OF TRINUCLEAR COMPOUNDS*

As stated earlier, in this project a number of trinuclear complexes based on BBR3464 have been prepared, which are [{*trans*-$PtCl(NH_3)_2$}$_2$ μ-{*trans*-$Pd(NH_3)_2$-($H_2N(CH_2)_4NH_2)_2$]Cl_4 (code named DH4Cl), [{*trans*-$PtCl(NH_3)_2$}$_2$ μ-{*trans*-$Pd(NH_3)_2$-($H_2N(CH_2)_5NH_2)_2$]Cl_4 (code named DH5Cl), [{*trans*-$PtCl(NH_3)_2$}$_2$ μ-{*trans*-$Pd(NH_3)_2$-($H_2N(CH_2)_6NH_2)_2$]Cl_4 (code named DH6Cl) and [{*trans*-$PtCl(NH_3)_2$}$_2$ μ-{*trans*-$Pd(NH_3)_2$-($H_2N(CH_2)_7NH_2)_2$]Cl_4 (code named DH7Cl). The complexes have been prepared by replacing the central platinum unit with the corresponding palladium unit. In addition, the dinuclear complex [{*trans*-$PtCl(NH_3)_2$}μ-{$H_2N(CH_2)_6NH_2$}{*trans*-$PdCl(NH_3)_2$]Cl(NO_3) (code named DHD), in which a transplatinum unit and a transpalladin unit are linked together by 1,6-diaminohexane, has also been synthesized.

In the case of trinuclear complexes, the size of linking diaminoalkyl chains have been varied to contain from 4 to 7 carbon atoms and the complexes have been prepared generally in the chloride form. The trinuclear complexes have been prepared by using step-up method of synthesis branching out from the central palladium unit. The procedures used were based on the method described by Farrell and co-workers for the synthesis of dinuclear complexes (Qu and Farrell 1992;Qu *et al.*1992).

However, the exact methods used have been varied to arrive at optimum yield and purity. This was required because the compounds in this project are trinuclear (rather than dinuclear) and also because palladium and platinum differ in their reactivities. It

should be noted that generally palladium complexes are much more labile than platinum complexes (Matilla *et al.* 1994).

3.1.3.1. PREPARATION OF DH6Cl

The method for the synthesis of DH6Cl is given first as it was the first compound prepared and studied. It will be seen later that it is also the most active compound.

In DH6Cl, two transplatinum units are linked to the central palladium unit by two 1, 6-diaminohexane chains. Thus, it is possible (in principle) to synthesize DH6Cl starting from one of the terminal units or the central unit. In actual fact, branching out from the central unit only gave reproducible results and the attempted syntheses starting with one of the terminal units always gave a mixture of the products. After many trials with different methods (Farrell *et al.* 1990a; Qu *et al.* 2000), the following procedure was used for the synthesis of DH6Cl.

1 mmol of transplatin (0.3 g) was dissolved in 20 mL of DMF to which was added 0.99 mmol of silver nitrate (0.1682 g). The mixture was stirred at room temperature for 24 h in the dark (Rauter *et al.* 1997; Zhao *et al.* 1998b). The mixture was then centrifuged at 5500 rpm for 30 min (Meroueh *et al.* 2000) to remove precipitate of AgCl. The supernatant was collected and kept at -16ºC.

A suspension of 0.5 mmol (0.106 g) of transpalladin in 10 mL of DMF was gently heated with stirring at 30-40 ºC for about 30 min.

1 mmol of 1,6-diaminohexane was dissolved in 4 mL of DMF to which 1 mL of 1 M HCl was added dropwise with stirring. The diamine solution was stirred for a further 15 min. It was then added to transpalladin suspension dropwise with stirring within 30 min of preparation. A yellow solution with some white precipitate was produced.

Stirring was continued for about 3 h following which 0.5 mL of 1 M NaOH was added with stirring to the mixture. The stirring was continued for a further 30 min to result into a clear light yellow solution.

The transplatin filtrate (1 mmol) that was prepared earlier and kept at -16°C, was then added to the light yellow solution with stirring at 40 ºC. Stirring was continued for a further 1 h. Then 70 µL of triethyl amine was added dropwise with stirring to the mixture. Stirring was continued for a further 1 h at the same temperature. The mixture was stirred for 48 h at room temperature.

The solution was filtered to remove any unreacted materials. The volume of the filtrate was reduced to 4 mL by using a vacuum concentrator consisting of Javac DD150 Double stage High Vacuum Pump Savant RVT 4104 Refrigerated Vapor Trap and Savant Speed Vac 110 Concentrator, and filtered again. 40 mL of dichloromethane was added to the concentrated solution. The mixture was left standing at 5 ºC for 6 h.

The light yellow precipitate produced was collected by filtration at the pump, washed first with ice cold water, then with methanol and finally with ethanol. It was then air dried. To improve purity, the crude product was recrystallized from DMF-methanol mixture. The weight of the final product was 0.258 g giving a yield of 49.4 %. Molar conductivity (?) at 0.0625 mM = 19.0 $ohm^{-1}cm^{2}mol^{-1}$. The steps in synthesis are shown in Figure 3.1

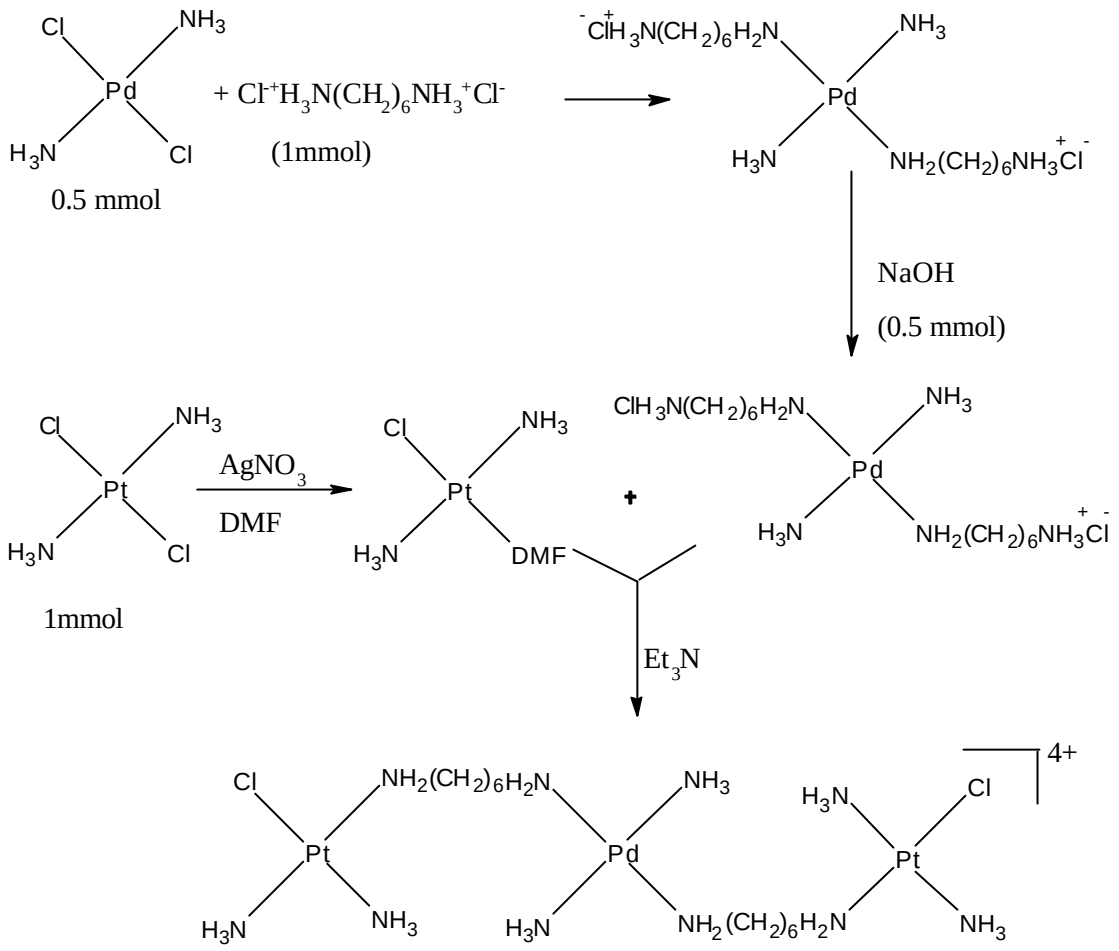


Figure 3. 1. Scheme for the synthesis of DH6Cl (Only the tetrapositive cation of DH6Cl is shown in the scheme, the valencing anions namely 4Cl⁻ are not shown).

3.1.3.2. PREPARATION OF DH4Cl

The method for the synthesis of DH4Cl was adopted from Farrell's procedures for the syntheses of dinuclear platinum(II) complexes (Farrell *et al.* 1990a; Farrell *et al.* 1990b; Qu and Farrell 1992). Essentially, the method is similar to that used for the synthesis of DH6Cl except that 1, 4-diaminobutane was used as the linking diamine instead of 1, 6- diaminohexane used in the preparation of DH6Cl.

1 mmol of transplatin (0.3 g) was dissolved in 20 mL of DMF to which was added 0.99 mmol of silver nitrate (0.1682 g). The mixture was stirred at room temperature for 24 h in the dark. The mixture was then centrifuged at 5500 rpm for 30 min at the end of which the supernatant was collected, and kept at -16ºC.

A suspension of 0.5 mmol (0.106 g) of transpalladin was made in 10 mL of mQ water and gently heated with stirring for 30 min at 30-40 ºC.

1 mmol (0.161 g) of tetramethylene diamine dihydrochloride (Putrescine dihydrochloride), dissolved in 3 mL of mQ water, was added to transpalladin suspension dropwise with stirring. The colour of the mixture changed first to light yellow and then cloudy yellow.

1 mmol of triethyl amine (140 µL) was added dropwise with mixing to the yellow solution, 0.5 mmol was added first followed by the addition of another 0.5 mmol after 30 min. The colour of the solution changed to light yellow. The mixture was stirred for 2 h at 40 ºC on a hot plate.

1 mmol (0.3 g) of transplatin supernatant (that was prepared earlier) was added to the above mixture to produce a yellow cloudy mixture. The mixture was stirred for 4 h at the same temperature (40 ºC).

It was stirred for further 48 h at room temperature. The mixture was filtered to remove any of the unreacted materials. The volume of the filtrate was reduced using the vacuum concentrator to about 8 mL. 40 mL of dichloromethane was then added to the concentrated solution. The mixture was then left standing at 5 ºC for 3 h. The light yellow precipitate was collected at the pump, washed first with dichloromethane, then with ice cold water and finally with methanol. DH4Cl was found to be soluble in warm water and hence ice cold water was used for washing. The resulting light

yellow solid was left in air to dry. The weight of the final product was 0.252 g giving a yield of 51.0 %. Molar conductivity (?) at 0.0625 mM = 16.0 $ohm^{-1}cm^2mol^{-1}$. The steps in synthesis are shown in Figure 3.2

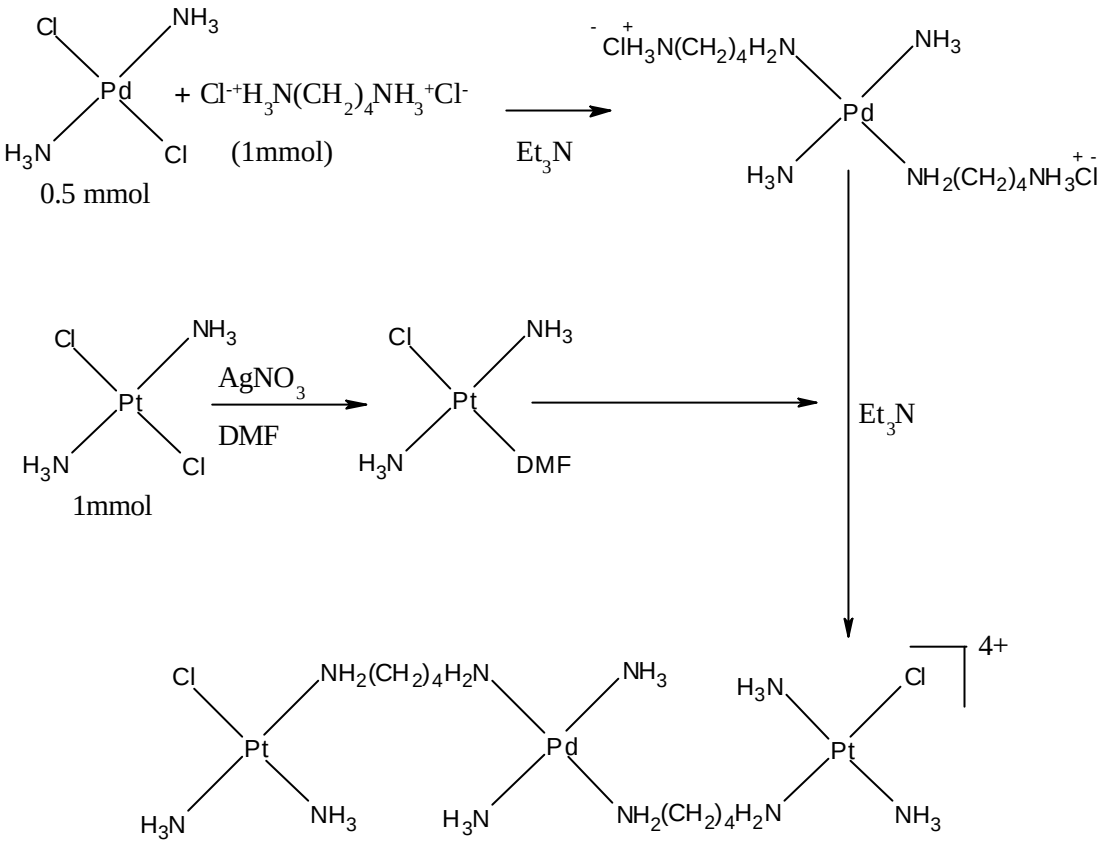


Figure 3. 2. Scheme for the synthesis of DH4Cl (Only the tetrapositive cation of DH4Cl is shown in the scheme, the valencing anions namely 4Cl- are not shown).

3.1.3.3. PREPARATION OF DH5Cl

1 mmol of transplatin (0.3 g) was dissolved in 20 mL of DMF to which was added 0.99 mmol of silver nitrate (0.1682 g). The mixture was stirred at room temperature for 24 h in the dark. The mixture was centrifuged at 5500 rpm for 30 min to separate

the precipitate of AgCl at the end of which the supernatant was collected and kept at -16ºC in two equal portions. A suspension of 0.5 mmol (0.106 g) of transpalladin, made in 10 mL of mQ water, was heated with gentle stirring at 40 ºC for about 30 min. 0.5 mmol of 1,5-diaminopentane dihydrochloride (cadaverine dihydrochloride) dissolved in 1 mL of mQ water was added to transpalladin suspension followed by the addition of 0.5 mmol of NaOH. The mixture was stirred for further 1 h at room temperature. Another 0.5 mmol of cadaverine dihydrochloride was dissolved in 1 mL of mQ water and mixed with 0.5 mmol of NaOH. The cadaverine-NaOH mixture was then added to the transpalladin diamine mixture. The resulting mixture was stirred for 6 h at room temperature. A clear light yellow solution was obtained, to which 0.5 mmol of transplatin filtrate was added and the mixture was stirred for 15 min. 0.5 mmol of NaOH was added to the mixture. Stirring was continued for 2 h at 40 ºC. Another 0.5 mmol of NaOH was added to the mixture. Then the second 0.5 mmol of transplatin filtrate was added. The mixture was stirred for 4 h at 40 ºC and then for 48 h at room temperature. It was then filtered to remove any unreacted materials. Then the volume of the filtrate was reduced to about 4 mL using the vacuum concentrator followed by filtration again. 20 mL of methanol was added to the filtrate. The mixture was left standing at 5 ºC for 12 h. The light yellow precipitate produced was collected at the pump. The precipitate was washed first with ice cold water, then with methanol and finally with ethanol. The precipitate was air dried. The weight of the final product was 0.163 g giving a yield of 32.1%. Molar conductivity (?) at 0.0625 mM = 8.0 $ohm^{-1}cm^{2}mol^{-1}$. The steps in synthesis are shown in Figure 3.3

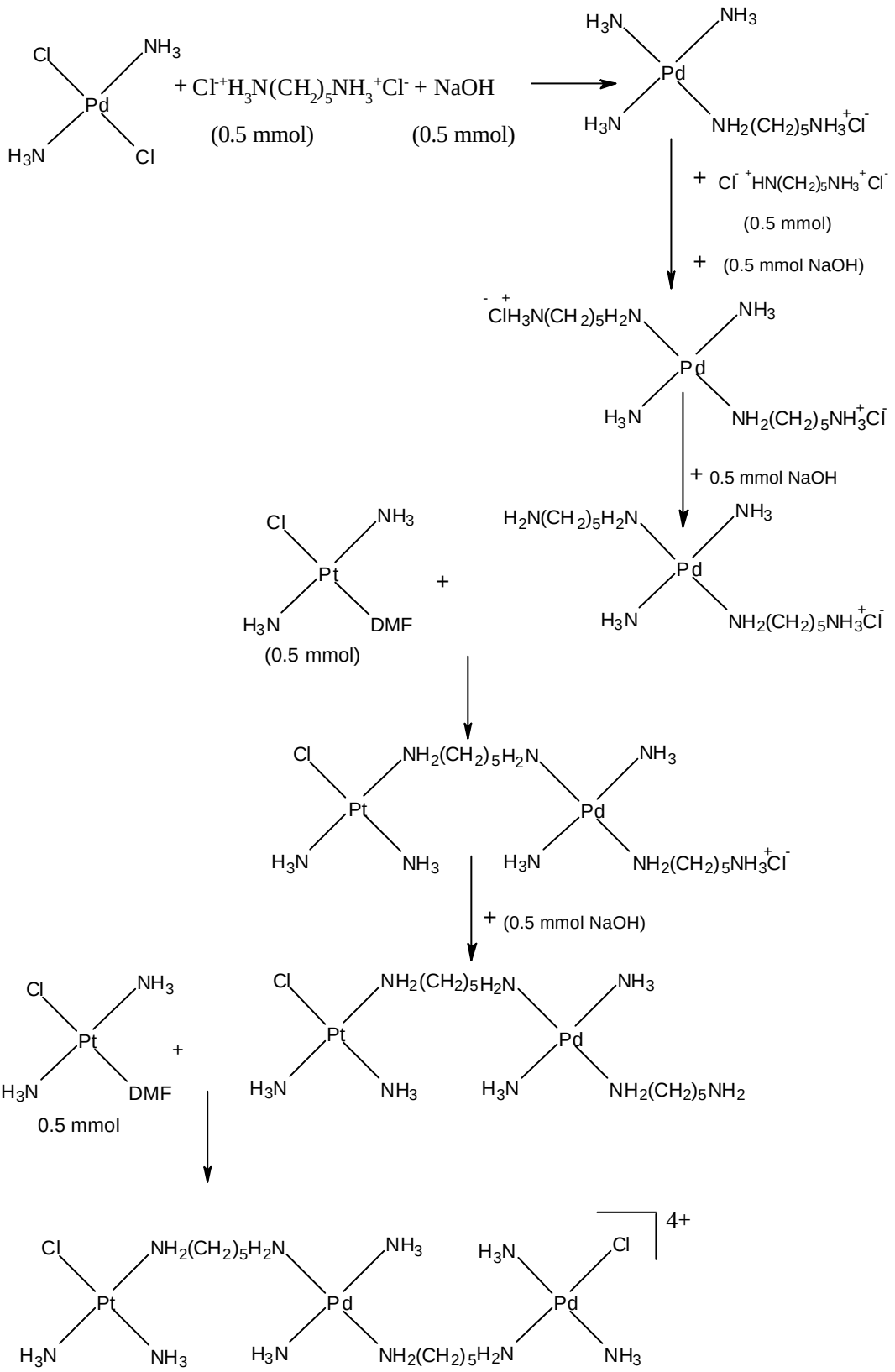


Figure 3. 3 Scheme showing the steps in the synthesis of DH5Cl (Only the tetrapositive cation of DH5Cl is shown in the scheme, the valencing anions namely $4Cl^-$ are not shown).

3.1.3.4. PREPARATION OF DH7Cl

The method used to prepare DH7Cl was essentially similar to that used to prepare DH6Cl, with slight modification.

0.5 mmol of transplatin (0.3 g) was dissolved in 10 mL of DMF to which was added 0.49 mmol of silver nitrate (0.0832 g). Another 0.5 mmol of transplatin (0.3 g) was dissolved separately in 10 mL of DMF and reacted with 0.49 mmol of silver nitrate. The mixtures were stirred at room temperature for 24 h in the dark.

The mixtures were centrifuged at 5500 rpm for 30 min, at the end of which the supernatants were collected and stored at 5ºC.

A suspension of 0.5 mmol (0.106 g) of transpalladin, made in 10 mL of DMF, was gently heated with stirring at 30-40 ºC for about 30 min.

0.5 mmol of 1,7-diaminoheptane (0.065 g) was mixed with 1.5 mL of mQ water and the mixture was acidified with 0.5 mL of 1 M HCl. It was stirred for 30 min. A second 0.5 mmol of 1,7-diaminoheptane was similarly treated.

The first acidified diamine portion (0.5 mmol) was added to transpalladin suspension to obtain a clear yellow solution. The mixture was stirred for 30 min and then the second acidified diamine portion (0.5 mmol) was added. The mixture was stirred for 2 h at room temperature.

0.5 mL of 1 M NaOH was added to the mixture to get a lighter clear yellow solution. It was then stirred for 15 min.

Then, first 0.5 mmol of transplatin filtrate was added with stirring at 50-60ºC. After 15 min the yellow solution became slightly cloudy and then clear. The mixture was stirred at 40-50C for 2 h.

At this point, another 0.5 mmol of transplatin filtrate was added while the mixture was stirred at 50ºC.

Then 70 µL of triethyl amine was added dropwise with stirring, following which the mixture was stirred first for 1 h at 50ºC and then for 48 h at room temperature.

The solution was filtered to remove any unreacted material. The volume of the filtrate was reduced to about 5 mL using the vacuum concentrator.

20 mL of methanol was added to the filtrate to form a yellow precipitate. The mixture was left standing at 5 ºC for 12 h to produce more of the precipitate. The light yellow precipitate was collected at the pump, washed first with ice cold water then with methanol and finally with ethanol. The precipitate was air dried. The weight of the final product was 0.158 g giving a yield of 39.5 %. Molar conductivity (?) at 0.0625 mM = 19.2 $ohm^{-1}cm^{2}mol^{-1}$.

3.1.3.5. PREPARATION OF DHD

As stated earlier, DHD is a dinuclear compound composed of a transplatin unit and a transpalladin unit connected together by 1,6-diaminohexane. It can be seen that cation: [{*trans*-$PtCl(NH_3)_2$}μ-{$H_2N(CH_2)_6NH_2$}{*trans*-$PdCl(NH_3)_2$] present in DHD is dipositively charged. In the following method used for the synthesis of DHD, the cation is found to be balanced by one Cl^- and one NO_3^- ion. Unlike DH4Cl, DH5Cl, DH6Cl and DH7Cl where only the terminal platinum centers bind covalently with DNA, both platinum and palladium centres in DHD are expected to bind covalently

with DNA and thus it was considered important to see whether anticancer activity was retained in DHD. The compound was prepared as follows:

0.75 mmol of transplatin (0.225 g) was dissolved in 18 mL of DMF to which was added 0.710 mmol of silver nitrate (0.121 g). The mixture was stirred at room temperature for 24 h in the dark. The mixture was then centrifuged at 5500 rpm for 30 min to separate precipitate of AgCl. The supernatant was collected and kept at -16ºC.

A suspension of 0.7 mmol (0.148 g) of transpalladin, made in 10 mL of DMF, was gently heated with stirring at 30-40 ºC for about 30 min.

0.7 mmol of 1, 6-diaminohexane dissolved in 3 mL DMF was added dropwise to transpalladin suspension to obtain first a clear yellow solution that afterwards turned slightly cloudy. The solution was stirred for 5 h at room temperature.

Transplatin filtrate (0.75 mmol) was added to the mixture followed by the addition of 90 µL of triethyl amine. The mixture was stirred for 3 h at 45 ºC to result into a clear yellow solution that contained tiny amounts of precipitate. Stirring was continued for further 45 h at room temperature.

The solution was filtered to remove any unreacted materials. The volume of the filtrate was reduced to 4 mL by using the vacuum concentrator. 40 mL of dichloromethane was added to the concentrated solution. The mixture was left standing at 5 ºC for 6 h.

The resulting yellow precipitate produced was collected by filtration at the pump, washed first with ice cold water, then with methanol and finally with ethanol. It was then air dried.

To improve purity, the crude product of DHD was dissolved in 25 mL of DMF and filtered. The volume of the filtrate was reduced to 4 mL by using the vacuum concentrator and 50 mL of dichloromethane was added to the concentrated solution. It was then left standing at room temperature for 3 h. The bright yellow precipitate of DHD was collected at the pump, washed with ice cold water, methanol and ethanol. The weight of the final product was 0.198 g giving a yield of 60.5 %. Molar conductivity (?) at 0.0625 mM = 16.0 $ohm^{-1}cm^2mol^{-1}$. The steps in synthesis are shown in Figure 3.4

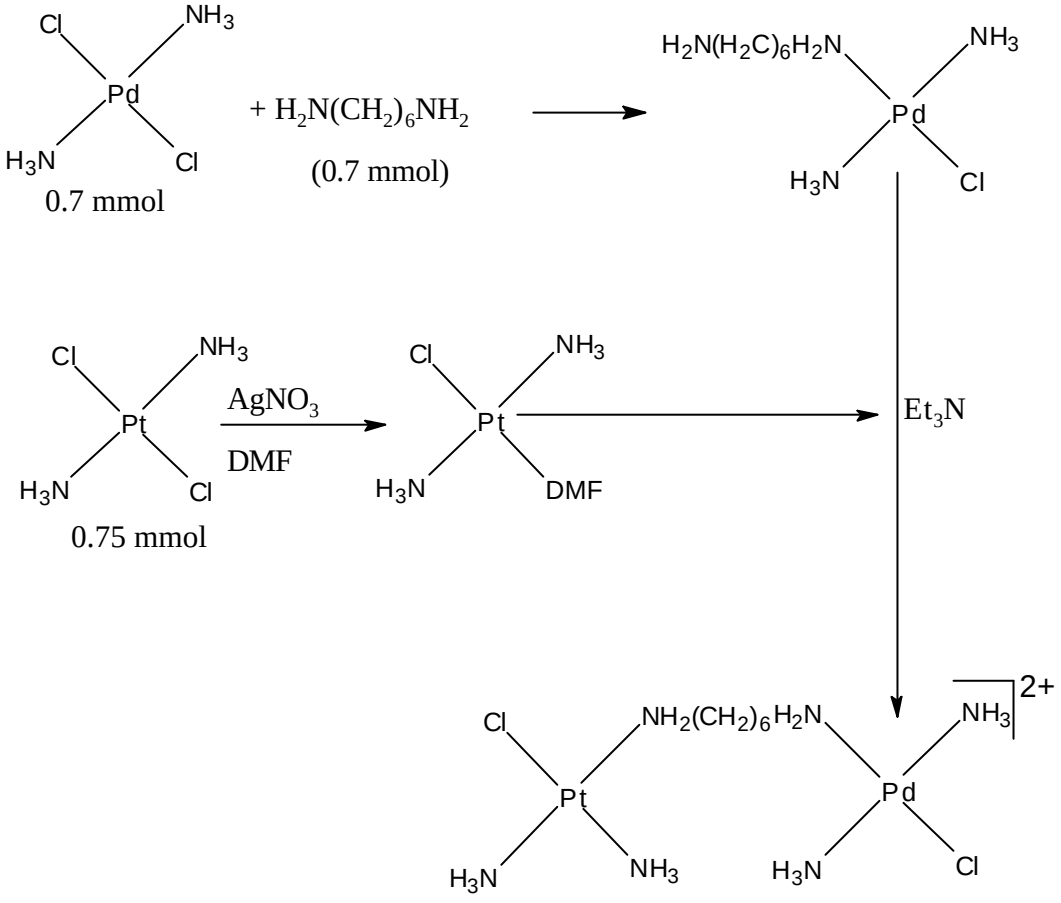


Figure 3. 4 Scheme showing the steps in the synthesis of DHD (Only the dipositive cation of DHD is shown in the scheme, the valencing anions namely Cl^- and NO_3^- are not shown).

[{*trans*-$PtCl(NH_3)_2$}$_2$*μ*-{*cis*-$PdCl_2(H_2N(CH_2)_6NH_2)_2$]$Cl_2$ (code named DH1Cl)

This was the first compound that was accidentally prepared because of the use of the wrong starting material namely potassium tetrachloropalladate instead of transpalladin. The compound was found to have activity similar to that of cisplatin. Briefly the method of preparation was as follows. 1 mmol (0.372 mg) of potassium tetrachloropalladate was dissolved in 7.5 mL mQ water to which 0.25 mL of concentrated HCl was added with stirring. The mixture was heated on a hot plate to about 90 °C for about 5 min after which the temperature was reduced to 60^{o}C. About 5 mmol of 1,6-diaminohexane was slowly added with stirring to the mixture. Stirring was continued for about 4 h. to obtain a clear yellow solution. The volume of the mixture was then reduced to about 6 mL by evaporation at 40^{o}C. 25 mL of 6 M HCl was then added. The volume of the mixture was reduced to about 10 mL by evaporation at 40^{o}C. A suspension of 1 mmol of transplatin in mQ water was added to the mixture with stirring. The mixture was then heated for one h at 50 °C following which another 1 mmol of transplatin suspension in mQ water was added. The mixture was stirred for 12 h at the same temperature. It was then left standing over night in the dark at room temperature. The precipitate of DH1Cl was collected at pump and washed with ice cold water. The impure product was recrystallized from 0.1 M HCl.

3.2. CHARACTERIZATION OF COMPOUNDS

3.2.1. *MICROANALYSIS*

Carbon, hydrogen, nitrogen and chloride were determined using the Micronalytical facility available at the School of Chemistry, Australian National University. Carlo Erba 1106 automatic analyzer was used for the determination of C, H and N contents and Cl was determined by titration with standardized mercuric nitrate.

Platinum and Palladium were determined by graphite furnace atomic absorption spectrophotometry (AAS) using the Varian SpectrAA20 plus Atomic Absorption Spectrophotometer with GTA-96 Graphite Furnace Tube Atomiser, available in the School of Biomedical Sciences, The University of Sydney. A variant of standard addition technique was used (Rothery 1991).

For the determination of platinum and palladium by AAS, 1 mg of sample was dissolved in 2 mL of DMF (or DMSO) and volume was made up to 100 mL with 0.1 M HCl in a volumetric flask. The solution was further diluted with 0.1 M HCl depending on the metal content. The furnace conditions were essentially the same as those given in Varian manual (Knowles 1988).

A diluted Pt standard (490 ppb) was prepared from the more concentrated platinum atomic absorption standard solution (980 μg Pt/mL in 5% HCl), obtained from Sigma. 0.5 mL of 980 ppm Pt standard solution was diluted to 100 mL with 0.1 M HCl to obtain 100 mL of 4900 ppb Pt solution. 20 mL of the 4900 ppb Pt solution was diluted to 200 mL with 0.1 M HCl to give 200 mL of 490 ppb Pt standard solution.

Likewise, a 60 ppb Pd standard was made by diluting the 1000 ppm palladium atomic absorption standard solution obtained from Mallinckrodt Specialty Chemicals Co. 0.5 mL of 1000 ppm Pd standard solution was diluted to 100 mL with 0.1 M HCl to obtain 100 mL of 5000 ppb Pd solution. 20 mL of the 5000 ppb Pd solution was further diluted to 200 mL with 0.1 M HCl to obtain 200 mL of 500 ppb Pd solution. 30 mL of 500 ppb Pd solution was diluted to 250 mL with 0.1 M HCl to obtain 250 mL of 60 ppb Pd standard solution.

To get ready for use in AAS analysis, all the glasswares were filled with 20% v/v HCl for 2 d, with 20% v/v HNO_3 for further 2 d and then rinsed with distilled water.

Tables 3.1 to 3.6 give the AAS conditions used for the determination of platinum and palladium contents.

Table 3. 1. Furnace operating conditions for the determination of platinum

Step No	Temperature (C^{o})	Time (sec)	Gas Flow (L/min)	Gas type	Read Command
1	85	20	3.0	Normal	No
2	90	30	3.0	Normal	No
3	95	20	3.0	Normal	No
4	120	30	3.0	Normal	No
5	400	2	3.0	Normal	No
6	800	2	3.0	Normal	No
7	1000	5	3.0	Normal	No
8	1200	1	3.0	Normal	No
9	1200	2	0	Normal	No
10	2700	1.3	0	Normal	Yes
11	2700	1	0	Normal	Yes
12	2700	2	3.0	Normal	No

Table 3. 2. Instrument parameters for the determination of platinum

Instrument mode	Absorbance
Calibration mode	Standard addition
Lamp position	1
Lamp current	8 mA
Slit width	0.2 nm
Wavelength	265.9 nm
Sample introduction	Sampler automixing
Time constant	0.05
Measurement time	1 (sec)
Replicates	2
Background correction	On

Table 3. 3. Sampler parameters used in AAS to determine platinum

	Standard (µL)	Sample (µL)	Blank (µL)	Total volume (µL)
Blank	---------	---------	30	30
Addition 1	2	2	26	30
Addition 2	4	2	24	30
Addition 3	6	2	22	30
Addition 4	8	2	20	30
Addition 5	10	2	18	30
Sample	-----	2	28	30

Table 3. 4. Furnace operating conditions for the determination of palladium

Step No	Temperature (C^{o})	Time (sec)	Gas Flow (L/min)	Gas type	Read Command
1	85	20	3.0	Normal	No
2	90	30	3.0	Normal	No
3	95	20	3.0	Normal	No
4	120	30	3.0	Normal	No
5	400	2	3.0	Normal	No
6	800	2	3.0	Normal	No
7	1000	5	3.0	Normal	No
8	1200	1	3.0	Normal	No
9	1200	2	0	Normal	No
10	2700	1.3	0	Normal	Yes
11	2700	1	0	Normal	Yes
12	2700	2	3.0	Normal	No

Table 3. 5. Instrument parameters for the determination of palladium

Instrument mode	Absorbance
Calibration mode	Standard additions
Measurement mode	Peak height
Lamp position	2
Lamp current	5 mA
Slit width	0.2 nm
Wavelength	244.8 nm
Sample introduction	Sampler automixing
Time constant	0.05
Measurement time	1 (sec)
Replicates	2
Background correction	On

Table 3. 6. Sampler parameters used in AAS to determine palladium

	Standard (µL)	Sample (µL)	Blank (µL)	Total volume (µL)
Blank	---------	---------	30	30
Addition 1	2	2	26	30
Addition 2	4	2	24	30
Addition 3	6	2	22	30
Addition 4	8	2	20	30
Addition 5	10	2	18	30
Sample	-----	2	28	30

Figures 3.5 and 3.6 give the typical standard-addition graphs applying to platinum and palladium respectively.

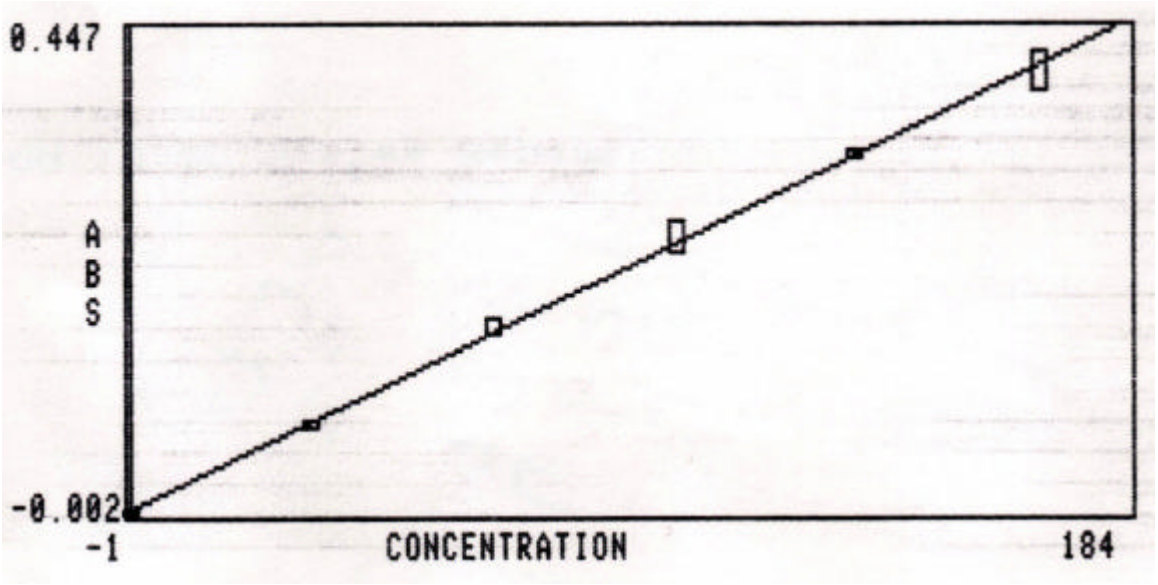


Figure 3. 5 Typical standards addition graph for platinum

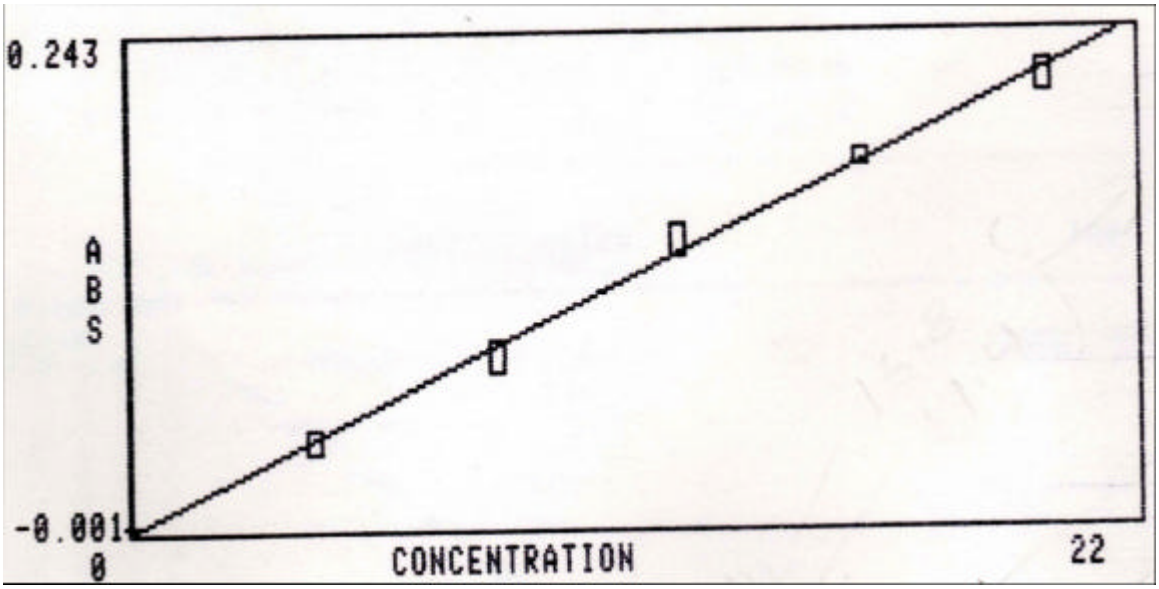


Figure 3. 6 Typical standards addition graph for palladium

Calculating percentage yield

Since 0.5 mmol of transpalladin at the maximum could produce 0.5 mmol of the compound, the theoretical yield = 0.5 x 10^{-3}x MW (compound).

Thus, % yield = (actual yield / theoretical yield) x 100

3.2.2. *MOLAR CONDUCTIVITY*

The molar conductivity values of DH4Cl, DH6Cl, DH7Cl and DHD in solution in 1:1 mixture of DMF and water were determined from the measurements of conductivities at concentrations: 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.0625 mM, using PW9506 digital conductivity meter. For DH5Cl, the solutions were made in DMF only since the compound although soluble in DMF, is insoluble in the mixture of DMF and water. The molar conductivity (Λ) was calculated as ? = k/c where k is the conductivity and c is the concentration (Atkins 1998).

3.2.3. *SPECTRAL STUDIES*

3.2.3.1. INFRARED SPECTRA

Infrared spectra were collected using a Bruker IFS66 spectrometer equipped with a Spectra-Tech Diffuse Reflectance Accessory (DRA). The spectrometer is equipped with the following: an air-cooled DTGS detector, a KBr beamsplitter with a spectral range of 4000 to 650 cm^{-1}. The instrument was run under vacuum during spectral acquisition. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of 128 scans and a Blackman-Harris 3-Term apodisation function was applied. Prior to analysis the samples were mixed, and lightly ground, with finely ground spectroscopic grade KBr. The spectra were then manipulated using the Kubelka-

Munk mathematical function in the OPUS™ software to convert the spectra from reflectance into absorbance.

3.2.3.2. RAMAN SPECTRA

Raman spectra were collected using a Bruker RFS100 Raman spectrometer equipped with the following: an air cooled Nd:YAG laser emitting at a wavelength of 1064 nm; and a liquid nitrogen cooled germanium detector with an extended spectral band range of 3500 to 50 cm^{-1}. 180° sampling geometry was employed. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of scans at a laser power of 0.130 mW for all the samples except for DH7Cl and DH6Cl was 0.065 mW, because these two samples were burned at 0.130 mW.

All the samples were scanned at 100 except the dinuclear compound DHD scanned at 20. A Blackman-Harris 4-Term apodisation function was applied and the spectra were not corrected for instrument response.

3.2.3.3. MASS SPECTROMETRY

Mass spectrometry is a highly useful technique to identify the structure of organic compounds. In this technique the sample is vaporised under high vacuum and the vapour is bombarded by a high energy electron beam to undergo fragmentation and producing ions of varying sizes. These ions are first accelerated by an electric field and then deflected by a magnetic field. The amount of deflection of an ion depends on it's mass/charge ratio (m/z) (Faust *et al.* 1992).

Solutions of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD, made in 10% DMF and 90% methanol, were sprayed into a Finnigan LCQ ion trap mass spectrometer. The flow

rate was 0.2 ml/min consisting of 50% methanol and 50% water. The ions observed were in positive ion mode.

3.2.3.4. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

Nuclear magnetic resonance spectroscopy (NMR spectroscopy) is one of the most widely used techniques that are used to identify compounds and to determine the structures of molecules. NMR spectroscopy is frequently divided into several categories which include: high resolution mode on homogenous mixture, high power mode on highly relaxing nuclei which exhibit broad liner or polymers, magic angle spinning and 3D NMR imaging of solution to a resolution of ~ 1 am.

Many functional groups can be identified by their ^{1}H chemical shift spectrum. The protons which are bonded to heteroatoms can be identified in ^{1}H NMR spectrum by using deuterium exchange (Silverstein *et al.* 1991).

^{1}H NMR was used in this project to identify the functional groups. Samples of the trinclear compounds DH4Cl, DH5Cl, DH6Cl, DH7Cl and dinuclear compound DHD were dissolved in deuterated DMSO except DH4Cl which was dissolved in deuterated DMF and prepared in 5 mm high precision Wilmad NMR tube. Bruker DPX400 spectrometer was used with frequency of 400.2 MHz. Spectra were referenced to internal solvent residues and all the spectra were recorded at 300 K (± 1 K). Temperatures quoted for acquisition were approximate and obtained from the uncalibrated variable temperature unit.

3.3. BIOLOGICAL ACTIVITY

3.3.1. *MATERIALS*

Three human ovarian carcinoma cell lines: A2780, A2780^{cisR} and A2780^{0ZD473R} were used in this study. A2780, A2780^{cisR} and A2780^{0ZD473R} were obtained from CAMR centre for Applied Microbiology & Research. The cell lines were produced as follows. Parent cisplatin-sensitive cell line A2780 was derived from an untreated ovarian cancer patient (Hamilton *et al.* 1984; Behrens *et al.* 1987). Cisplatin-resistant cell line A2780^{cisR} was developed by chronic exposure of parent cisplatin-sensitive A2780 cell to increasing concentrations of cisplatin (Behrens *et al.* 1987; Masuda *et al.* 1988). A2780^{ZD0473R} cell line was developed by in vitro exposure of parent cisplatin-sensitive A2780 cell to increasing concentrations of drug from 0.5 to 12.5 μM for a period of 7 months (Holford *et al.* 2000).

Large cell lung carcinoma cell line NCI-460 was obtained from the Global Bio-Source Centre ATCC in freeze medium. The NCI-H460 cell line was derived by A.F.Gazdar in 1982 from the pleural fluid of a patient with large cell cancer of lung (the cells express was easily detectable by p53 mRNA at levels comparable to normal lung tissue, and exhibited no gross structural DNA abnormalities) (Banks-Schlegel *et al.* 1985; Takahashi *et al.* 1989).

Melanoma cell line Me-10538, is a primary tumour cell line derived from human melanoma (Larizza *et al.* 1989).

All the above cell-lines were stored under liquid nitrogen at Royal Prince Alfred Hospital, Sydney.

Fetal calf serum, 5X RPMI 1640, 200 mM L-glutamine and 5.6% sodium bicarbonate were obtained from Thermo Trace Pty Ltd Melbourne, Australia. Trypsin, Hepes, Dulbecco's phosphate buffered saline powder, 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazoliume bromide (MTT) and dimethyl sulfoxide were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. 96 well culture clusters, flat bottom with lid were obtained from Edward Keller, and 25 cm^2 culture flasks were obtained from Crown Scientific. Microplate reader BIO-RAD Model 3550 was used to read the optical density of each well.

3.3.1.1. PREPARATION OF MEDIUM

The medium used for cell culture studies was mainly 10% fetal calf serum (FCS/RPMI 1640). One litre of the medium was prepared by mixing the following components together: 200 mL of (5X RPMI 1640), 100 mL of fetal calf serum (FCS), 20 mL of 20 mM hepes, 20 mL of 0.11% bicarbonate, 10 mL of 2 mM glutamine and 0.5 mL of saturated NaOH. Sterile water was used to make up the volume to one litre.

Table 3. 7 Constituents of the medium (FCS/RPMI 1640)

	2%	5%	10%
5x RPMI 1640	200 mL	200 mL	200 mL
FCS	20 mL	50 mL	100 mL
Hepes	20 mL	20 mL	20 mL
Bicarbonate	20 mL	20 mL	20 mL
Glutamine	10 mL	10 mL	10 mL
Sat. NaOH	0.5 mL	0.5 mL	0.5 mL
Volume of sterile water	729.5 mL	699.5 mL	649.5 mL

3.3.1.2. PHOSPHATE BUFFERED SALINE (PBS) PREPARATION

The materials required to prepare PBS were: PBS powder as supplied by Sigma, 1 M HCl, 1 M NaOH and mQ water. One litre of PBS was prepared according to the following procedure: 900 mL of mQ water at 15-20 $^{\circ}$C was transferred to 1 L volumetric flask followed by the addition of PBS powder while the mixture was gently stirred. The original package was rinsed with a small amount of mQ water to remove any traces of PBS powder. The pH of the medium was adjusted to 7.2- 7.5 and more mQ water was added to bring the volume of the solution up to the mark. The solution was immediately sterilized by filtration using a membrane with porosity of 0.22 microns. The final pH of the solution was found to be 7.2.

3.3.1.3. TRYPSIN PREPARATION

To prepare 100 mL of trypsin, the following procedure was followed:

0.02 gm of disodium salt of ethylene diamine tetraacetic acid (EDTA) was dissolved into 1 mL of sterilized water in a 100 mL sterilized bottle to which 10 mL of 2.5% trypsin was added and the volume was made up to 100 mL with PBS.

3.3.1.4. HEPES PREPARATION

1 M of hepes solution was prepared first which was then diluted to 20 mM by using sterile water. To make 100 mL of 1 M hepes, 23.83 g of hepes powder was dissolved in 90 mL sterile water in a 100 mL volumetric flask. Then the volume was made up to the mark by adding more sterile water.

3.3.1.5. CELLS RECOVERY FROM LIQUID NITROGEN

Before the cell was taken from liquid nitrogen tank, new flasks, tubes and reagent were placed in the Laminar flow hood after swabbing it with 70% alcohol. The vials containing the cell lines were taken from the liquid nitrogen tank and placed in a water bath at 37 ºC until half of the liquid thawed. The vials were swabbed with 70% alcohol then the cells were transferred into a labeled 10 mL sterile tube containing 9 mL of 10% RPMI 1640 culture medium. The tubes were then centrifuged for 3 min at 2500 rpm after which the medium was removed. 2 mL of fresh medium was added to the cells in each tube and then the cell suspensions were transferred into pre-labeled flasks each containing 8 mL of fresh medium. The flasks were incubated in an atmosphere of 5% CO_2 and 95% air at 37 ºC.

3.3.1.6. CELL SUBCULTURE

When the concentration of the cells exceeds the capacity of the containing medium then the cell growth ceases or is greatly reduced. When that happens, the medium

must be changed or we need to subculture. Subculture involves removal of the medium, dissociation of the cells in the monolayer with trypsin, dispersion of cells in medium, counting of cells and then dilution (Freshney 2000).

The following procedure was followed in subculture:

The hood was swabbed with 70% methanol. Then the required materials were placed in the hood. The flasks containing the cell lines were taken from the incubator and examined carefully under microscope for any sign of contamination or deterioration. The medium was removed from the flasks and discarded. The flasks were then washed with 2 mL of PBS. The PBS that was used for washing was removed and discarded. 1 mL of trypsin was added to each flask. The flasks were placed into an incubator at 37 ºC for 2-3 min. The flasks were removed from the incubator and the suspensions of the cells were examined on inverted microscope. 9 mL of 10% RPMI 1640 culture medium was added to each flask making the total volume 10 mL. 9 mL of suspension was removed and the cells contained in the suspension were counted by using hemocytometer. Depending on the cells count and condition, the 9 mL suspension could be used for seeding or was discarded if the cells were found to be unhealthy.

3.3.1.7. CELL COUNT

A hemocytometer was used for counting cells. The coverslip and the surface slide of the hemocytometer chambers were cleaned with 70% alcohol. The coverslip was then placed on the counting area. Cell suspension was mixed thoroughly and 20 µL of the cell suspension was collected and transferred immediately to the edge of each of the two chambers of the hemocytometer.

The hemocytometer was then placed on the microscope stage. A 10X objective was selected and focused on the grid lines in the chamber. The slide was moved to the central area of the grid which is bounded by three parallel lines and has an area of 1 mm^2. The cells lying in this area were counted. If fewer than 100 cells were found in the area, four squares surrounding the central square were counted and the results averaged.

3.3.1.8. STORAGE OF THE CELL LINES

When healthy cells entered the late log growth phase, cells were ready to be stored, for which the following procedure was used. Each of the monolayer cell lines was treated with trypsin and suspended in 9 mL of 10% RPMI 1640 culture medium. The suspension was removed and the cells contained in the suspension were counted. The cell suspension was centrifuged at 2500 rpm for 3 min. The medium was then tipped off and resuspended in 1 mL of 10% of DMSO. 1 mL of each cell suspension was transferred into a 2 mL pre-labeled NUNC vial. The vials were placed into polystyrene foam box surrounded by cotton wool and stored at -70 ºC for 48-72 h. After that the vials were removed from the foam box and transferred to the liquid nitrogen tank.

3.3.2. *MTT ASSAY*

MTT reduction assay is one of the best methods used to determine drug cytotoxicity, using unfixed cells for cell growth and viability. MTT assay shows a good correlation between spectrophotometric absorbance and the cell number (Mosmann 1983).

MTT is 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide. It is a yellow water soluble dye that is converted to insoluble purple formazen product by mitochondrial dehydrogenates of living cells only.

To make a stock solution of 5 mg/mL MTT, 0.02 g of MTT was dissolved in 2 mL sterile PBS (pH 7.2). This was stored in the dark at 4 $^{\circ}$C unless used as prepared. It was found to last for 3 weeks.

MTT assay protocol

The steps in MTT assay were as follows:

- Monolayer culture was trypsinized to produce cell suspension to which growth medium was added.
- The suspension was centrifuged for 5 min at 200 g to pellet the cells.
- The cells were suspended in growth medium and counted using hemocytometer.
- The cells were diluted to 2.5-50 x 10^3 cells/mL depending on the growth rate of the cell lines.
- The cell suspension was transferred to a petri dish. Using a multichannel pipette, 100 µL (200 µL in the case of adherent cells) was added to each well of the flat-bottomed 96-well plate in quadruplicate for each drug dose and control. The central 10 columns of the plate were used. 100 µL of growth medium was added to each well in column 1 and 12 to provide the blank for the plate reader, maintain the humidity and minimize the 'edge effect'
- The plate was incubated in humidified atmosphere at 37 $^{\circ}$C for 24 h.

- Serial dilutions of the cytotoxic drugs were prepared in growth medium.
- 100 µL of the cytotoxic drug was added to the cell. Four wells for each drug concentration and 100 µL of fresh growth medium was added to the cell controls.
- The mixtures of cells and drugs were then incubated in humidified atmosphere at 37 °C for 3 d.
- Before the assay, MTT stock solution was diluted in serum free RPMI-1640 medium to give a final concentration of 1 mg/mL. Then it was filtered through a 0.22 µm membrane to remove any blue formazan product.
- At the end of the drug exposure period, the medium was removed and 50 µL of 1 mg/mL MTT was placed in each well followed by incubation for a period of 4 h to 8 h at 37 ºC.
- MTT was aspirated and the remaining MTT-formazan crystals were dissolved by adding 150 µL of DMSO to each well.
- The plate was shaken gently for a few minutes until a purple colour appeared. Then the absorbance of each well was read in an ELISA plate reader set at 540 nm within 1 h of adding DMSO.
- The % of living cells was calculated as follows:

$$\% \text{ of living cells} = \frac{\text{Absorbance of sample - Absorbance of DMSO}}{\text{Absorbance of control - Absorbance of DMSO}} \times 100$$

(Carmichael *et al.* 1987; Freshney 2000)

3.3.3. *CYTOTOXICITY*

The cytotoxicity of designed complexes DH1Cl, DH6Cl, DH4Cl, DH5Cl, DH7Cl and DHD were evaluated in comparison to that of cisplatin on several human tumour cell lines including ovary cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, non small lung cell line: NCI-H640 and melanoma cell line (Me-10538) based on MTT assay. All cell lines were recovered from liquid nitrogen, grown as monolayers in RPMI 1640 medium containing 10% (v/v) fetal calf serum and 1% of L-glutamine. Growing cultures were maintained in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 ºC. Contamination of cell culture by mycoplasma is one of the major problems in tissue culture which can cause extensive changes (McGarrity *et al.* 1984). Accordingly Regular checks were carried out for mycoplasma by DNA fluorescent staining method, using fluorochrome DAPI (Hessling *et al.* 1980; Kotani and McGarrity 1985). The cell lines were sub-cultured two times every week.

As the compounds differed in activity, the actual concentrations used to test activity needed to be varied from one compound to another. For DH1Cl, DH6Cl and cisplatin, 50 µM stock solutions were serially diluted to give nine concentrations ranging from 0.25 to 50 µM in one set and from 0.05-25 µM in another set. Further dilution of solutions of DH6Cl, DHD and cisplatin was carried out to give five concentrations ranging from 0.02 to 12.5 µM (For DHD, activity was tested only for these concentrations).

For DH4Cl, DH5Cl and DH7Cl, activity was tested for the concentrations ranging from 0.1 to 62.5 µM. The compounds were dissolved in 1mL DMF, volume adjusted to 5 mL with culture medium which was then sterilized by filtration through a 0.22 µm membrane. DMSO was not used to make solution of compounds as it was found

that the activity of platinum compounds decreased when dissolved in DMSO (Tonew *et al.* 1984; Miller *et al.* 1999).

The cell lines grown in the culture flasks were trypsinized and 100 µL of medium containing between $5x10^3$and $1x10^5$ cells per well were seeded into 96 well microplate. Cells were incubated for 24 h to allow the cell to attach.

Solutions of compounds at various concentrations were added to the wells containing the cells (100 µL/well) in quadruplicate. The plates were incubated for three days at the end of which the medium was discarded and 50 µL of freshly prepared MTT was added to each well. The plates were incubated for 4-5 h at 37 ºC. Then MTT was removed and 150 µL of DMSO was added to each well. Within one hour of addition of DMSO, the absorbance was measured at 540 nm. The data were plotted as % of living cells against drug concentration, from which the IC_{50} value was determined. The IC_{50} value is defined as the inhibitory drug concentration causing a 50% reduction of absorbance and hence a 50% reduction of the surviving cell number as compared to that of untreated control (Perego *et al.* 1999b).

In the case of DH6Cl, DH4Cl, DH5Cl and DH7Cl, IC_{90} values (the drug concentrations causing a 90% cell kill) were also determined.

3.4. CELLULAR PLATINUM UPTAKE AND DNA BINDING

3.4.1. *MATERIALS*

For cell uptake and DNA binding studies, two human ovarian carcinoma cell lines: cisplatin-sensitive A2780 and cisplatin-resistant $A2780^{cisR}$ were used. These were described earlier (section 3.3.1). Triton X-100 (t-Octylphenooxypolyethoxyethanol)

was obtained from Sigma-Aldrich Pty Ltd, NSW, Australia and commercially available DNA purification kit (JETQUICK Blood DNA Spin kit/50) was obtained from Astral Scientific Pty Ltd, Australia.

3.4.2. *DRUG ADDITION AND CELL TREATMENT*

The cell uptake and DNA binding in 4 h were evaluated for trinuclear and dinuclear complexes: DH6Cl, DH5Cl, DH4Cl, DH7Cl, DHD in which cisplatin was used as reference. For cisplatin and DH6Cl, cell uptake and DNA binding in 2 h were also evaluated as applied to the cell lines: A2780 and A2780^{cisR}. For DH4Cl, whereas cell uptake in 2 h was determined for both cell lines: A2780 and A2780^{cisR}, DNA binding in 2 h was determined only for A2780 cell line. The method used for cell treatment was a modification of that described by Di Blasi et al (Di Blasi *et al.* 1998)

The cell lines: A2780 and A2780^{cisR} were subcultured at 10x10^5 cells/mL in 25 cm^2 tissue culture flasks each containing 10 mL of 10% FCS/RPMI 1640 medium. The flasks containing the cells (labeled to give the name of the compound and the time of the experiment) were incubated at 37°C for 24 h.

Solutions of compounds were added to the culture flasks containing exponentially growing cells in 10 mL of 10% FCS/RPMI 1640 culture medium at a cell density of 1x10^{6}cells/mL and to achieve a drug concentration of 50 µM.

At the end of drug exposure (2 or 4 h), the cell monolayers were washed immediately with 1 mL of PBS followed by trypsinization with 1 mL of trypsin. Then 9 mL of FCS/RPMI 1640 medium was added.

The cells were counted and then the cell suspensions were centrifuged at 3500 rpm for 2 min at 4 ºC. The cells were washed three times with ice-cold PBS. The pellets were

stored at -20 ºC until assayed for platinum and palladium contents. At least three independent experiments were preformed for the determination of both cell uptake and DNA binding.

3.4.3. *CELL UPTAKE*

The cell pellets were suspended in 0.5 mL of 1% triton X-100 (dissolved in mQ water), held on ice and then sonicated using Unisonics sonicater for 30 min in order to lyse the cells (Farrell *et al.* 1992; Roberts *et al.* 1999b) .

The total intercellular platinum and palladium contents were determined by graphite furnace AAS using the technique of standard addition. Because of a lower concentration, sample volume was increased to 4 µL or 8 µL instead of 2 µL used in microanalysis (section 3.2.1) and often multiple injections were required. Platinum and palladium contents were calculated as nmoL Pt per $2x10^6$ cells and nmoL Pd per $2x10^6$ cells respectively.

3.4.4. *PLATINUM DNA BINDING*

Because of a small sample volume and the need for multiple injections, generally samples were analyzed only for platinum. The procedure used was as follows. Following the incubation of cells with compounds for 2 and 4 h, the high molecular weight DNA was isolated and purified from cell pellet using commercially available kit JETQUICK Blood DNA Spin Kit/50. The protocol used was a modification of that used by Bowtell (Bowtell 1987)

The stored cell pellets were suspended in PBS to give a final volume of 200 µL, mixed with 10 µL of RNase A and incubated for 4 min at 37 ºC. After incubation, 25 µL of Proteinase K and 200 µL of Buffer K1 which contained guanidine

hydrochloride and a detergent were added and incubated for 10 min at 70 ºC. This was followed by addition of 200 mL of absolute ethanol and mixed very thoroughly to prevent any precipitation of nucleic acids due to the production of local high concentrations of ethanol.

The samples were each transferred to a JETQUIK micro-spin column with a 2 mL receiver tube and centrifuged for 1 min at 10,600 rpm to pass through the column silica membrane. The flowthrough was discarded. The samples in the column were washed by applying 500 µL buffer KX (containing high-salt buffer, capable of removing residual contaminations which may effect downstream applications supplied with the kit) and centrifuged for 1 min at 10,600 rpm. Again the flowthrough was discarded. The Micro-Spin unit with the receiver tube was re-assembled and washed with 500 µL of buffer K2 and centrifuged for 1 min at 10,600 rpm. Buffer K2 is a low-salt buffer that changes the high-salt conditions on the silica membrane. The flowthrough was discarded following which the sample columns and the empty receiver tube were centrifuged again for 2 min at the full speed of 13,000 rpm. The column receiver was replaced by 1.5 mL reaction tube and the DNA in the column was eluted from the membrane with 200 µL of 10 mM Tris-HCl buffer (pH 8.5).

The elution buffer was pre-warmed to 70 ºC and pipetted directly into the centre of the silica membrane. The spin columns were incubated for 5 min at room temperature after application of the elution buffer and centrifuged subsequently for 2 min at 10,600 rpm. DNA content and DNA purity was determined spectrophotometerically by using Varian Cary IE UV-Visible spectrophotometer. The purity of DNA was estimated by calculating the ratio between the absorbance values at 260 nm and 280 nm (A_{260}/ A_{280}) (Di Blasi *et al.* 1998) and the DNA concentration was calculated according to the following equation.

Concentration = Absorbance at 260 nm x dilution factor x 50 ng/µL

Platinum contents were determined by AAS using Varian SpectrAA-20 plus with GTA 96 facility. Because the amount of Pt that was bound to DNA was very small, sampler parameters again needed to be varied as compared to those used in microanalysis and cell uptake. For example, 16 µL samples were used instead of 2, 4 or 8 µL.

3.5. INTERACTION WITH DNA

Because the activity of platinum based anticancer drugs is believed to be associated with their interaction with DNA, the nature of interaction of the designed compounds, cisplatin and transplatin with pBR322 plasmid DNA and salmon sperm DNA was studied using gel electrophoresis. BamH1 digestion was also carried out to get further insight into the nature of conformational change of pBR322 plasmid DNA.

3.5.1. *MATERIALS*

Salmon sperm DNA (ssDNA) was obtained from Fluka, Switzerland and Sigma-Aldrich, NSW, Australia; pBR322 plasmid DNA (0.05 mg/mL in buffer consisting of 1 mM Tris-HCl at pH 7.5, 1 mM NaCl and 1 mM EDTA) was obtained from ICN Biomedicals, Ohio, USA. Trizma base, Trizma-HCl, disodium salt of ethylene diamine tetraacetic acid, boric acid, acetic acid and ethidium bromide were obtained from Sigma, USA. Agarose was obtained from ICN Australia. Restriction enzyme (BamH1), 10X digestion buffer and Polaroid black-and-white print film type 667 were obtained from Sigma, Australia.

3.5.1.1. PREPARATION OF 50X TAE STOCK SOLUTION AND TAE WORKING SOLUTION BUFFERS

TAE is one of the commonly used buffers for DNA gel electrophoresis. To make 250 mL of 50X TAE buffer solution, 4.65 g of Na_2EDTA, 60.55 g of Tris base and 14.275 mL of glacial acetic acid were required. The components were dissolved in a minimum volume of mQ water followed by the addition of more mQ water in order to bring the volume up to 250 mL. 40 mL of 50X TAE buffer solution was then diluted with mQ water to give 2000 mL of working TAE buffer solution.

3.5.1.2. GEL PREPARATION

To make 250 mL of 1% agrose gel, 2.5 g of agrose powder was added to abut 100 mL TAE buffer. More TAE buffer was added to make the volume 250 mL. The mixture was boiled in microwave for 1 min, swirled to mix and boiling was continued for another 4 min. Agrose solution was cooled for a few minutes. 60 µL of ethidium bromide was added to the gel and mixed (Stellwagen 1998). Then the gel was gently poured into the tray with comb placed in position, left at room temperature for 40 min to solidify.

3.5.1.3. SALMON SPERM DNA PREPARATION

A stock solution DNA was dissolved in 0.05 M Trizma buffer at pH 8.0. 250 mL of the buffer was prepared by dissolving 1.11 g of Trizma-HCl, and 0.663 g of Trizma base in mQ water. It was then sterilized by passing through 0.22 µm Millipore filter.

10 mg and 15 mg respectively of salmon sperm DNA were dissolved in Trizma buffer to give 10 mL stock solutions of the DNA at concentrations 1 mg/mL and 1.5 mg/mL respectively. DNA solutions were stored at -17ºC until used.

3.5.2. *INTERACTION BETWEEN pBR322 PLASMID DNA AND THE HETRONUCLEAR COMPLEXES.*

Plasmids are circular duplex, heavy molecular weight DNA ranging in size from two kilo bases to a few hundred kilo bases. Plasmid DNA is normally found in a supercoiled form commonly known as form I DNA. During isolation nicking by nucleases, chemical treatment, or mechanical shear, can cause strand breaks, producing singly-nicked open circular form (called form II DNA) and doubly-nicked open chain (called form III DNA) (Cantor and Schimmel 1980). Electrophoresis in agarose or polyacrylamide gels is a well established method used to separate the three forms of plasmid DNA (Mickel *et al.* 1977).

In an applied electric field, the DNA, being negatively charged due to the phosphate backbone, will migrate through the gel from the negative to positive electrodes. The three forms of plasmid DNA differ in the rate of migration through the gel. Form I being supercoiled and compacted, travels fastest. The flexible and relaxed circular Form II DNA has the lowest migration rate whereas the linear Form III DNA has the intermediate migration rate.

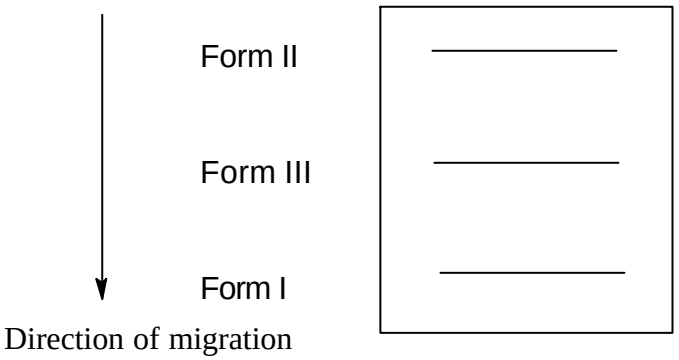


Figure 3. 7 Migration of the three forms of plasmid DNA in gel

Besides strand breakage, the unwinding of the supercoiled plasmid DNA can also be brought about by intercalation and covalent binding of compounds to nucleobases in DNA. For example, supercoiled form I plasmid DNA can change from negatively supercoiled form I through relaxed circular form I to positively supercoiled form I. This change in DNA conformation is reflected as a change in mobility through the gel. It will be considered later that like BBR3464, multinuclear Pt-Pd-Pt complexes form long range interstrand GG adducts with DNA, causing long range changes in DNA conformation.

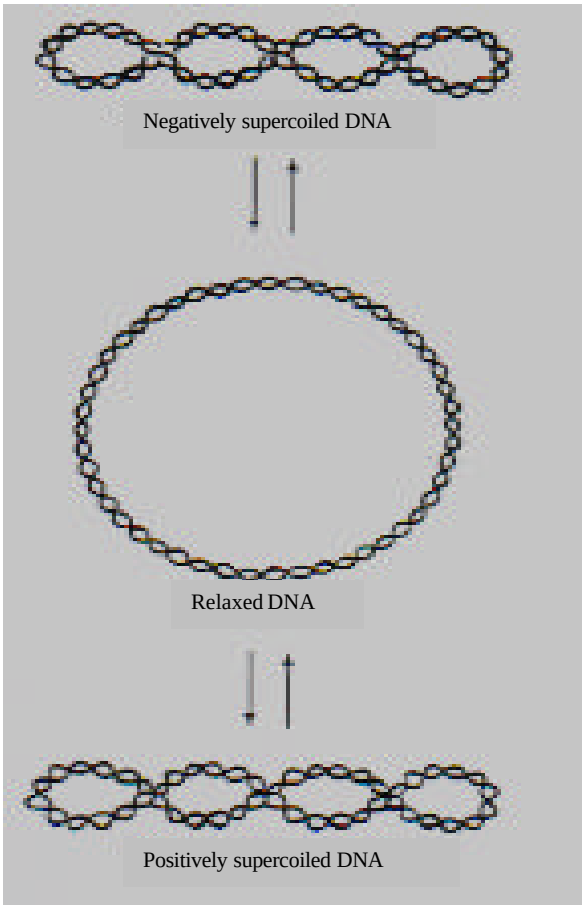


Figure 3. 8 Schematic representation of the unwinding of supercoiled plasmid DNA from negatively supercoiled form I through relaxed circular form to positively supercoiled form I

The plasmid-binding assay is a rapid in vitro technique that can be employed to study the interaction of platinum compounds with DNA. The assay involves the use of plasmid DNA and restriction enzymes. BamH1 is one of these restriction enzymes which hydrolyse the phosphodiester bond between adjacent guanines in DNA strands producing form II and ultimately form III DNA. When platinum compounds bind to the nucleotides adjacent to the recognition sequence, DNA may undergo conformational change or distortion such that cutting of the phosphodiester bond may be prevented. It will be seen later that as increasing concentrations of multinuclear complexes bind to DNA, BamH1digestion of plasmid DNA is increasingly prevented.

3.5.2.1. INTERACTION BETWEEN COMPOUNDS AND pBR322 PLASMID DNA: ELECTROPHORETIC ASSAY

1 mM stock solution of cisplatin, transplatin and multinuclear complexes (DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD) (5 mL in each case) were prepared by dissolving appropriate amounts of the compounds in 1 mL of DMF followed by addition of mQ water to give a total volume of 5 mL. To dissolve DH5Cl, pH was first increased to 10 with 0.1 M NaOH and then reduced to 7. The solutions of the compounds were serially diluted to give the concentrations: 0.025 mM, 0.035 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.15 mM, 0.2 mM and 0.3 mM.

To 1.2 µL of pBR322 plasmid DNA, was added 1 µL of solutions of the compounds (DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD, cisplatin and transplatin) at eight different concentrations. The total volume was made up to 20 µL by adding 17.8 µL of mQ water so that the concentrations of the compounds in the mixtures were: 1.25 µM, 1.875µM, 2.5 µM, 3.75 µM, 5.0 µM, 7.5 µM, 10 µM and 15 µM. The DNA blank was made by adding 18.8 µL mQ water to 1.2 µL of pBR322 plasmid DNA.

The samples including the DNA blank were incubated for 4 h on a shaking water bath at 37 ºC in the dark. At the end of incubation, the reaction was quenched by rapid cooling to 0 ºC. The samples were thawed then mixed with 4 µL of marker dye (0.25 % bromophenol blue and 40% of sucrose). 18 µL of each sample was loaded onto 1% agrose gel made in TAE buffer that contained ethidium bromide (0.5 mg/mL). The gel was stained in the same buffer (Onoa and Moreno 2002).

Electrophoresis was carried out in TAE buffer containing ethidium bromide at 100V for 3 h at room temperature. The bands of the plasmid DNA were viewed under short

wave UV light using the BIO-RAD Trans illuminatorIEC1010 and photographed with Polaroid camera (orange filter) using Polaroid black-and-white print film, type 667.

3.5.2.2. INTERACTION OF COMPOUNDS AND pBR322 PLASMID DNA IN PRESENCE OF BamH1 RESTRICTION ENZYME DIGESTION.

As stated earlier, BamH1 is a restriction endonuclease that hydrolyses the phosphodiester bonds. BamH1 is known to recognize the sequence G/GATCC and hydrolyse the phosphodiester bond between adjacent GG sites (Roberts *et al.* 1977). Supercoiled form I pBR322 plasmid DNA contains a single restriction site for BamH1 that converts the supercoiled form I and singly nicked circular form II to linear form III DNA (Sutcliffe 1979). The procedures used to make drug-plasmid DNA mixtures were identical to those described previously. However, only the following five drug concentrations: 1.875 µM, 2.5 µM, 5.0 µM, 10 µM and 15 µM were used for DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD. For cisplatin, in addition to these concentrations, 20 µM was also used. The mixtures were first incubated for 4 h on water bath at 37 ºC. The samples were then treated with BamH1 according to the procedure described by Sambrook (Sambrook *et al.* 1989) with some modifications as stated below.

Samples after incubation were treated with BamH1 (10 units μL^{-1}). To each 20 µL of the incubated drug-DNA mixtures was added 3 µL of 10X digestion buffer followed by the addition of 0.2 µL of BamH1 (2 units). The mixtures were left in shaking water bath at 37 ºC for 1 h at the end of which the reaction was stopped by rapid cooling. The samples were thawed and treated as described previously in section 3.5.2.1. The gel was subsequently stained with ethidium bromide and the bands of the plasmid DNA were viewed under short wave UV light and photographed with Polaroid camera (orange filter) using Polaroid black-and-white print film, type 667.

3.5.3. *INTERACTION WITH SALMON SPERM DNA*

Salmon sperm DNA (ssDNA) is a low molecular weight genomic DNA, ranging in size from 0.6 to 0.8 kilo base. Unlike plasmid DNA (which can exist in three forms), ssDNA can exist only in one form and thus its electrophoretogram has only one band.

The following procedure was used to investigate the interaction of multinuclear complexes with ssDNA by electrophoric assay. Whereas the concentration of the ssDNA was kept constant, that of the compounds was varied to have the values: 0.05 mM, 0.075 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.6 mM, and 0.8 mM.

To 2 µL of ssDNA (1 mg/mL), was added 2 µL of solutions of the compounds (DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD, cisplatin and transplatin) at nine different concentrations. The total volume was made up to 20 µL by adding 16 µL of mQ water so that the concentrations of the compounds in the mixtures were: 5 µM, 7.5 µM, 10 µM, 15 µM, 20 µM, 30 µM, 40 µM, 60 µM and 80 µM. DNA blank was made by adding 18 µL mQ water to 2 µL of ssDNA. The mixtures were first incubated for 4 h on a water bath at 37 ºC. The samples were thawed and treated as described previously in section 3.5.2.1. Electrophoresis was carried out also in TAE buffer containing ethidium bromide at 85V for 2.5 h at room temperature and ssDNA bands were viewed under short wave UV light and photographed with Polaroid camera (orange filter) using Polaroid black-and-white print film, type 667.

3.6. INTERACTION BETWEEN DH6Cl AND NUCLEOBASES

High pressure liquid chromatography also known as high performance liquid chromatography (HPLC) provides a highly sensitive and convenient method to investigate the binding between platinum-based anticancer drugs and nucleobases, nucleosides, nucleotides and DNA (Berners-Price and Appleton 2000). In a typical reverse phase HPLC experiment, appropriate choice of column, mobile phases, pH, flow rate, gradient, ion-pairing agent etc. would allow each adduct between a platinum drug and a nucleobase or nucleotide (eg guanine or AMP) to be eluted with a characteristic retention time. Determination of the platinum content of time peak fraction by graphite furnace AAS and nucleobase (or nucleotide) content by UV-visible spectrophotometry makes possible the calculation of the binding ratio between the drug and the nucleobase (or the nucleotide) applying to the fraction. Provided appropriate conditions are chosen, HPLC would offer an efficient method of purification of a drug or drug-nucleobase (or drug-nucleotide) adduct. Purified adducts can be structurally characterized by single crystal x-ray diffractometry (if suitable crystals can be grown) and/or by NMR spectroscopy. In this study, HPLC has been used to determine the binding ratio between the most active compound DH6Cl and the nucleobases guanine and adenine. As the multinuclear cations of DH4Cl, DH5Cl, DH6Cl and DH7Cl, have two labile chloro ligands, all of them are expected to form 1:2 (drug : NB) adducts where NB stands for guanine or adenine.

3.6.1. *MATERIALS*

Ammonium acetate and acetic acid were obtained from APS chemicals, Australia; HPLC grade methanol was obtained from Mallinckrodt, USA. All other chemicals including adenine and guanine were obtained from Sigma, USA.

3.6.2. *HPLC METHOD*

1 mL of 5 mM solution of DH6Cl dissolved in DMF was diluted to 5 mL with mQ water to give 1 mM DH6Cl solution. Equal volumes of 1 mM solution of DH6Cl and 2 mM solution of NB (guanine or adenine) were mixed together and incubated at 37°C in a shaking water bath for 24 h. To dissolve guanine in mQ water, pH was increased to about 10 by adding a tiny drop of 0.1 M NaOH. After incubation, 5-10 µL of each of the mixtures and appropriate components was injected separately into a Waters HPLC system, consisting of a Waters 600 controller, a Waters 600 pump, a Waters 746 data module, a Waters Dual ? absorbance detector and Waters C18 5 µm symmetry column with internal diameter of 3.9 mm and length of 150 mm. The wavelength was set at 260 nm.

The mobile phase consisted of 5 % methanol and 95% of ammonium acetate (0.1 M at pH 5.5) with a flow rate of 1 mL/min. The retention times of the peaks applying to the mixtures and the components were recorded and the peak fractions applying to the mixtures collected.

3.6.3. *BINDING RATIO*

As stated earlier, to determine the binding ratio between DH6Cl and nucleobases (guanine and adenine), the peak fractions of the mixtures were analyzed for nucleobase or nucleotide content by using Cary IE UV-visible spectrophotometer set at 260 nm and for platinum content by graphite furnace AAS. 0.025 mM solution of Guanine and adenine was used to determine molar absorptivity at 260 nm. Using the appropriate molar absorptivity value, the concentration of nucleobase in the fraction was calculated. From the values of platinum and nucleobase contents, the Pt : NB (where NB stands for nucleobase) ratio was calculated.

CHAPTER FOUR

4. RESULTS

4.1. CHARACTERIZATION OF COMPOUNDS

As stated before, in this project five trinuclear complexes (DH4Cl, DH5Cl, DH6Cl, DH7Cl and DH1Cl) and one dinuclear compound (DHD) were prepared and investigated for their antitumour activity and interaction with DNA.

4.1.1. *COMPOSITION*

The stoichiometries of the compounds were determined based on elemental analyses. C, H, N and Cl were determined using the facility of Micro-analytical Unit at Australian National University, School of Chemistry. Platinum and palladium were determined by graphite furnace AAS.

4.1.1.1. DH4Cl

DH4Cl is [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_4NH_2)_2$]$Cl_4$. It has the following structure.

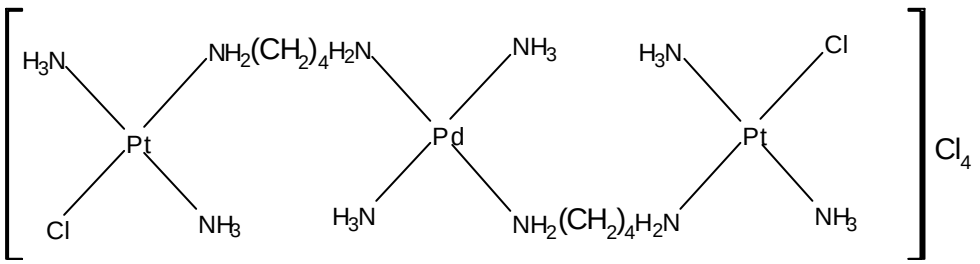


Figure 4. 1 Structure of DH4Cl

Formula: $C_8H_{42}N_{10}Cl_6Pt_2Pd$

Molar mass: 987.779 g mol^{-1}

Percentage yield: 51.0 %

The percentage composition of DH4Cl is given in Table 4.1.

Table 4. 1 The percentage composition of DH4Cl

Element	Calculated (%)	Found (%)
C	9.7	11.0
H	4.3	4.1
N	14.8	14.0
Cl	21.5	21.5
Pt	39.5	40.0
Pd	10.8	10.9

4.1.1.2. DH5Cl

DH5Cl is [{*trans*-PtCl(NH_3)$_2$}$_2$*m*-{*trans*-Pd(NH_3)$_2$($H_2N(CH_2)_5NH_2$)$_2$]Cl_4. It has the following structure.

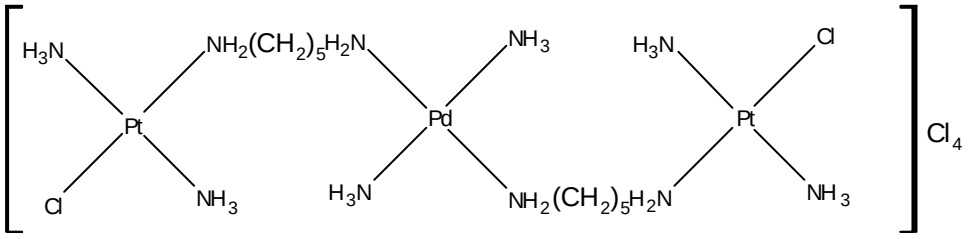


Figure 4. 2 Structure of DH5Cl

Formula: $C_{10}H_{46}N_{10}Cl_6Pt_2Pd$

Molar mass: 1015.832 g mol^{-1}

Percentage yield: 32.1 %

The percentage composition of DH5Cl is given in Table 4.2.

Table 4. 2 The percentage composition of DH5Cl

Element	Calculated (%)	Found (%)
C	11.8	12.0
H	4.6	3.9
N	13.8	11.8
Cl	20.9	23.3
Pt	38.4	40.0
Pd	10.5	10.2

4.1.1.3. DH6Cl

DH6Cl is [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_6NH_2)_2$]$Cl_4$. It has the following structure.

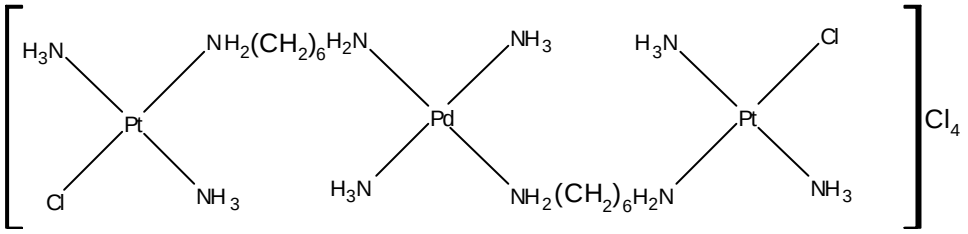


Figure 4. 3 Structure of DH6Cl

Formula: $C_{12}H_{50}N_{10}Cl_6Pt_2Pd$

Molar mass: 1043.885 g mol^{-1}

Percentage yield: 49.4 %

The percentage composition of DH6Cl is given in Table 4.3.

Table 4. 3 The percentage composition of DH6Cl

Element	Calculated (%)	Found (%)
C	13.8	12.6
H	4.8	4.2
N	13.4	13.0
Cl	20.4	20.8
Pt	37.4	36.0
Pd	10.2	10.8

4.1.1.4. DH7Cl

DH7Cl is [{*trans*-$PtCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2$-$(H_2N(CH_2)_7NH_2)_2$]Cl_4. It has the following structure.

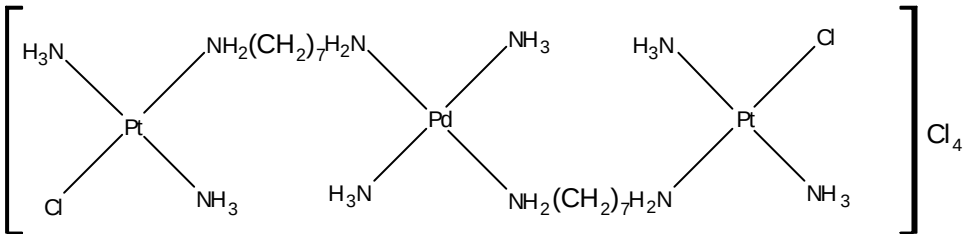


Figure 4. 4 Structure of DH7Cl

Formula: $C_{14}H_{54}N_{10}Cl_6Pt_2Pd$

Molar mass: 1071.938g mol^{-1}

Percentage yield: 39.5 %

The percentage composition of DH5Cl is given in Table 4.4.

Table 4. 4 The percentage composition of DH7Cl

Element	Calculated (%)	Found (%)
C	15.7	17.2
H	5.1	5.2
N	13.1	12.1
Cl	19.8	20.1
Pt	36.4	35.7
Pd	9.9	10.4

4.1.1.5. DHD

DHD is [{*trans*-$PtCl(NH_3)_2$}μ-{$H_2N(CH_2)_6NH_2$}{*trans*-$PdCl(NH_3)_2$]$Cl(NO_3)$. It is a dinuclear complex made by joining together of a transplatin unit and a transpalladin unit by a molecule of 1, 6- diaminohexane and in which the balancing negative ions are Cl^- and NO_3^-. It has the following structure.

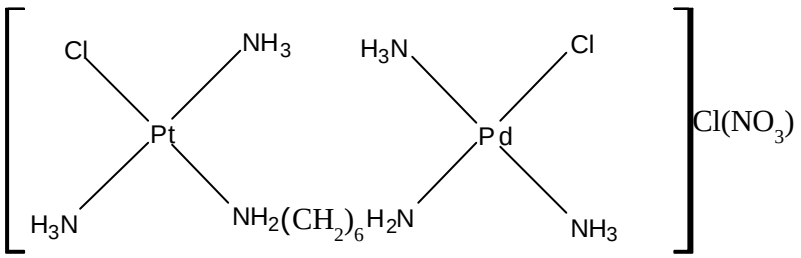


Figure 4. 5 Structure of DHD

Formula: $C_6H_{28}N_7Cl_3O_3PtPd$

Molar mass: 654.188 g mol^{-1}

Percentage yield: 60.5 %

The percentage composition of DHD is given in Table 4.5.

Table 4. 5 The percentage composition of DHD

Element	Calculated (%)	Found (%)
C	11.0	12.3
H	4.3	4.1
N	15.0	14.0
Cl	16.3	17.1
Pt	29.8	29.7
Pd	16.27	15.60

4.1.1.6. DH1Cl

DH1Cl is [{*trans*-$PtCl(NH_3)_2$}$_2$$\mu$-{*cis*-$PdCl_2(H_2N(CH_2)_6NH_2)_2$]$Cl_2$. It has the following structure.

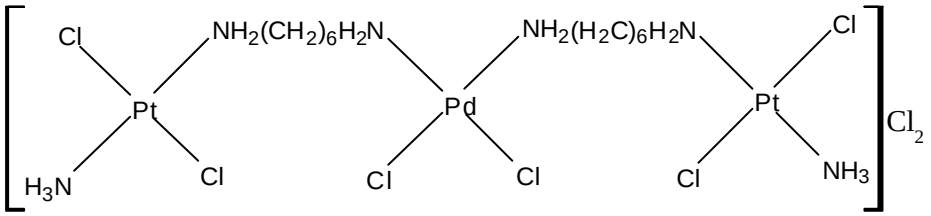


Figure 4. 6 Structure of DH1Cl

Formula: $C_{12}H_{44}N_8Cl_6Pt_2Pd$

Molar mass: 1009.8

Percentage yield: 50.5 %

The percentage composition of DH1Cl is given in Table 4.6.

Table 4. 6 The percentage composition of DH1Cl

Element	Calculated (%)	Found (%)
C	14.3	12.5
H	4.4	3.9
N	11.1	11.8
Cl	21.1	23.3
Pt	38.6	40.0
Pd	10.5	10.2

DH1Cl was the first compound that was accidentally prepared because of the use of the potassium tetrachloropalladate instead of transpalladin as the starting material for the central unit. Besides determination of stoichiometry of the compound by elemental analysis, only studies on its cytotoxicty were carried out. Thus, no other studies relating to cell uptake, binding with DNA were done for the compound.

4.1.2. *SPECTRAL STUDIES*

The IR, Raman, mass and ^{1}H NMR spectra of the compounds were taken in order to assist in the characterization of the compounds.

4.1.2.1. IR, RAMAN, MASS AND ^{1}H NMR SPECTRA OF DH4Cl

The IR and Raman spectra of DH4Cl are given in figure 4.7 and 48 followed by listings of selected bands where the letters ‘s’, ‘m’, ‘w’, ‘br’ and ‘d’ denote respectively strong, medium, weak, broad and doublet. The assignments of the bands are given in discussion (chapter 5).

The mass and ^{1}HNMR spectra of DH4Cl are given in figure 4.9 and 4.10. Selected peaks values of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD mass spectrum (m/z) and the values of ^{1}H NMR spectrum of these compounds are listed in table (4.7).

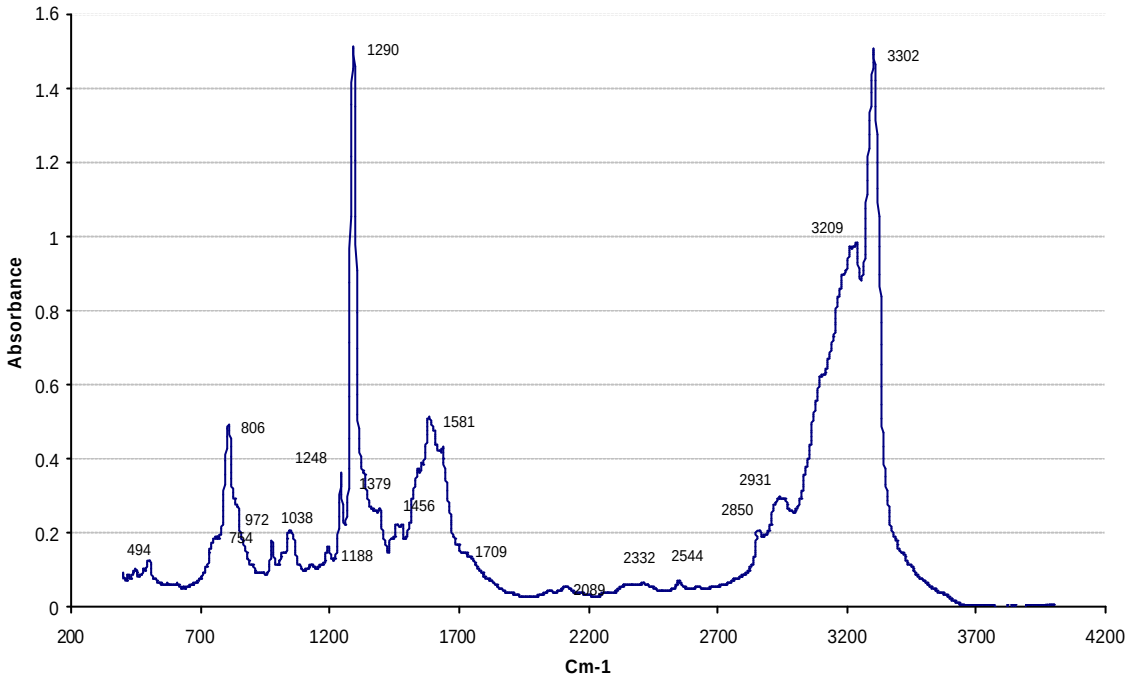


Figure 4. 7 IR spectrum of DH4Cl

Selected IR bands of DH4Cl:

3302s, 3209m, 2931w, 2850w, 2544w, 1709w, 1581m, 1456w, 1379w, 1290s, 1038w, 972w, 806m, 754w, 494w.

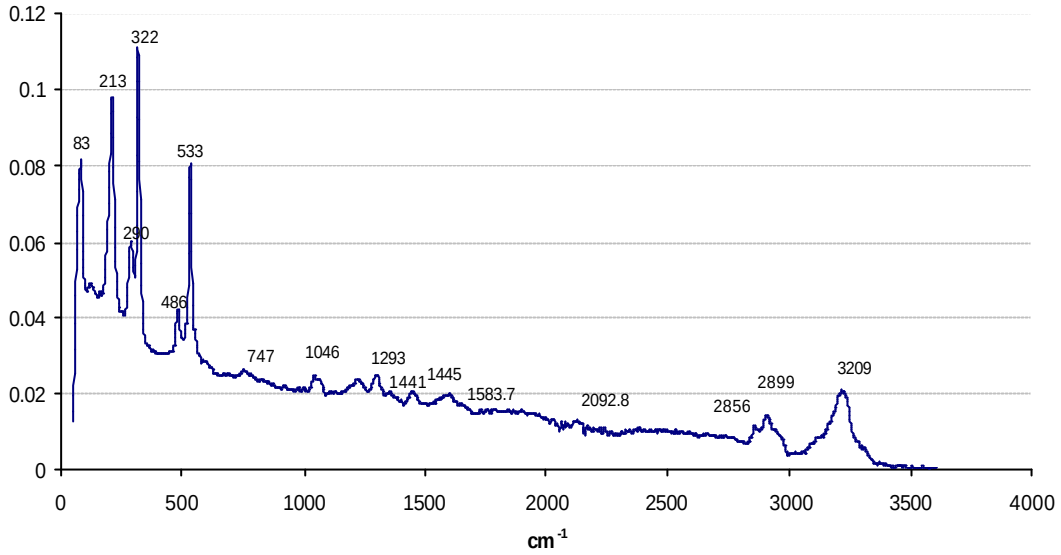


Figure 4. 8 Raman spectrum of DH4Cl

Selected Raman bands of DH4Cl:

3209w br, 2899w, 2856w, 2092w, 1445w, 1441w, 1293w, 1046w, 747w, 533s, 486w, 322s, 290w, 213s, 83s.

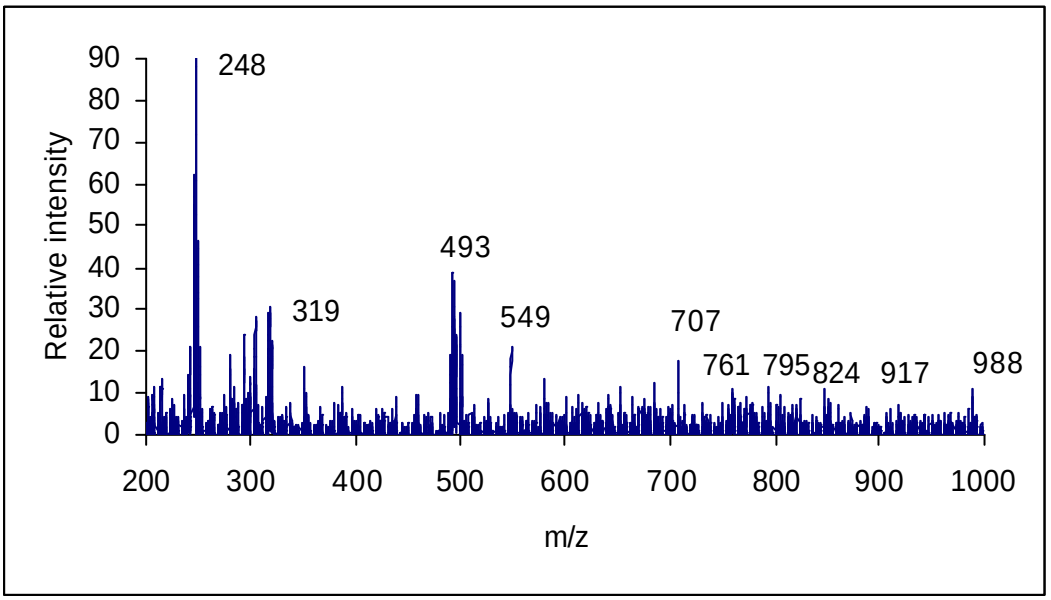


Figure 4. 9 Mass spectrum of DH4Cl

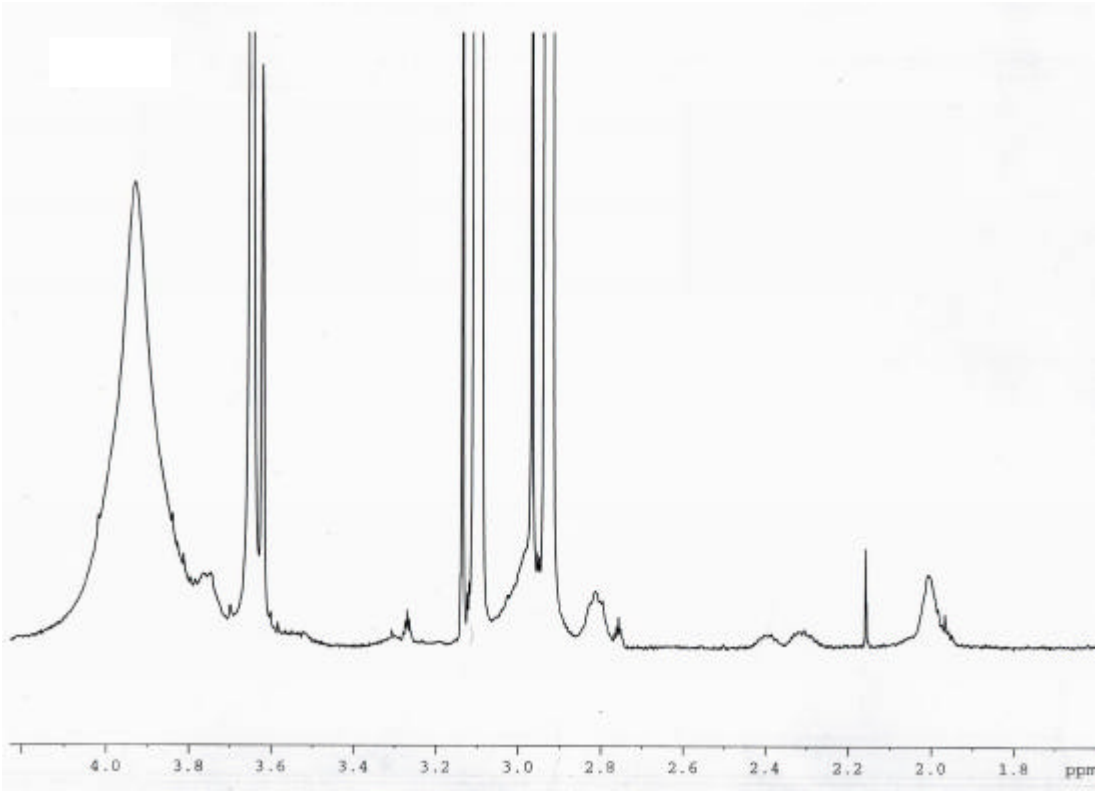


Figure 4. 10 ^{1}H NMR spectrum of DH4Cl

4.1.2.2. IR, RAMAN, MASS AND ^{1}H NMR SPECTRA OF DH5Cl

The IR and Raman spectra of DH5Cl are given in figures 4.11 and 4.12. Listings of selected bands of IR and Raman spectra are given below. The mass and ^{1}HNMR spectra of DH5Cl are given in figures 4.13 and 4.14.

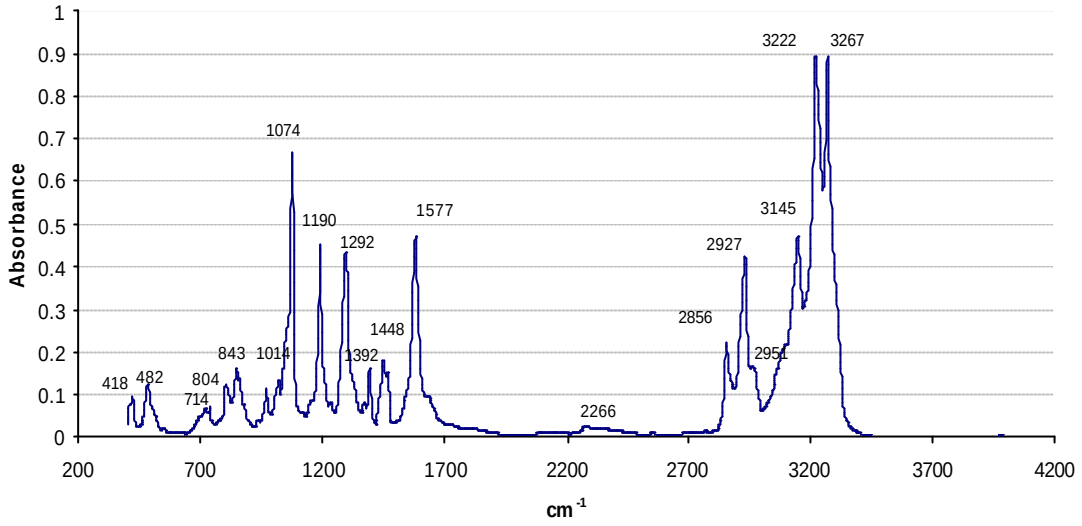


Figure 4. 11 IR spectrum of DH5Cl

Selected IR bands of DH5Cl:

3267s, 3222s, 3145m, 2951w, 2927m, 2856w, 2266w, 1577m, 1448w, 1392w, 1292m, 1190m, 1074s, 1014w, 966w, 843w, 804w, 714 w d, 482w, 418w.

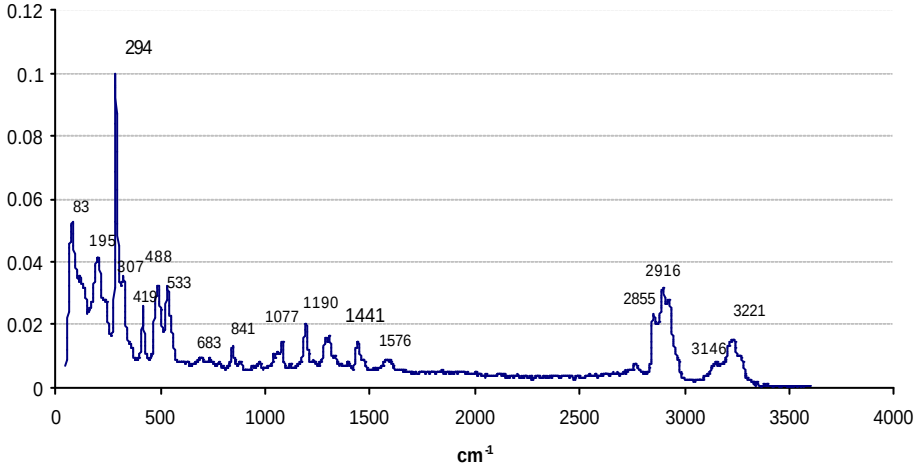


Figure 4. 12 Raman spectrum of DH5Cl

Selected Raman bands of DH5Cl

3221w, 3146w, 2916m, 2855w, 1576w, 1441w, 1190w, 1077w, 841w, 683w, 533m, 488m, 419w, 307w, 294s, 195m, 83m.

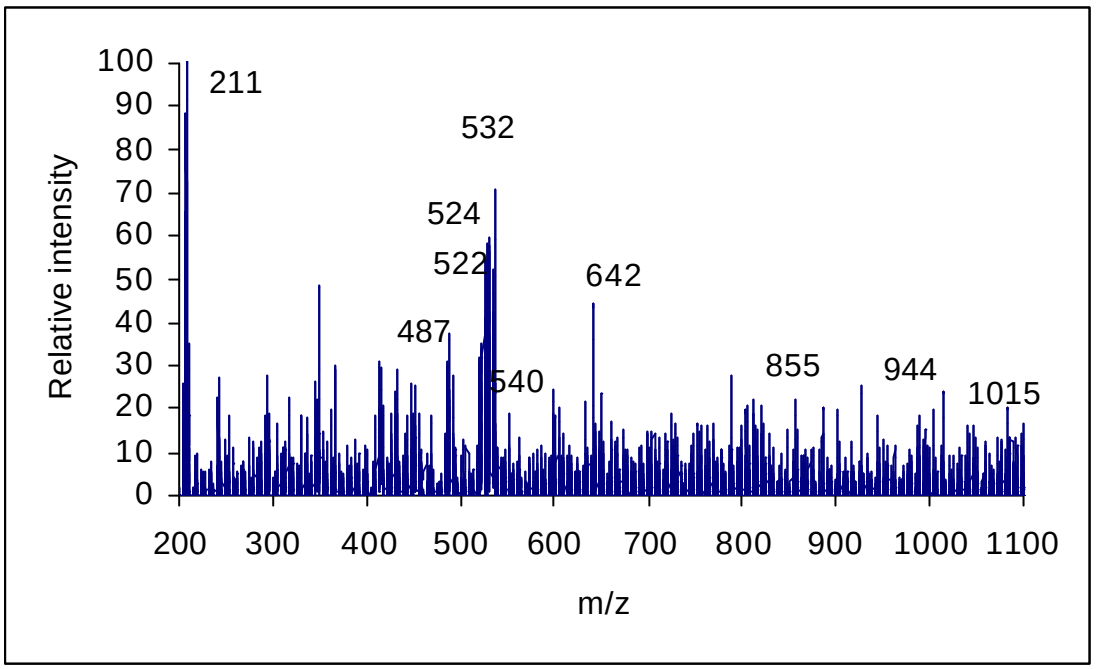


Figure 4. 13 Mass spectrum of DH5Cl

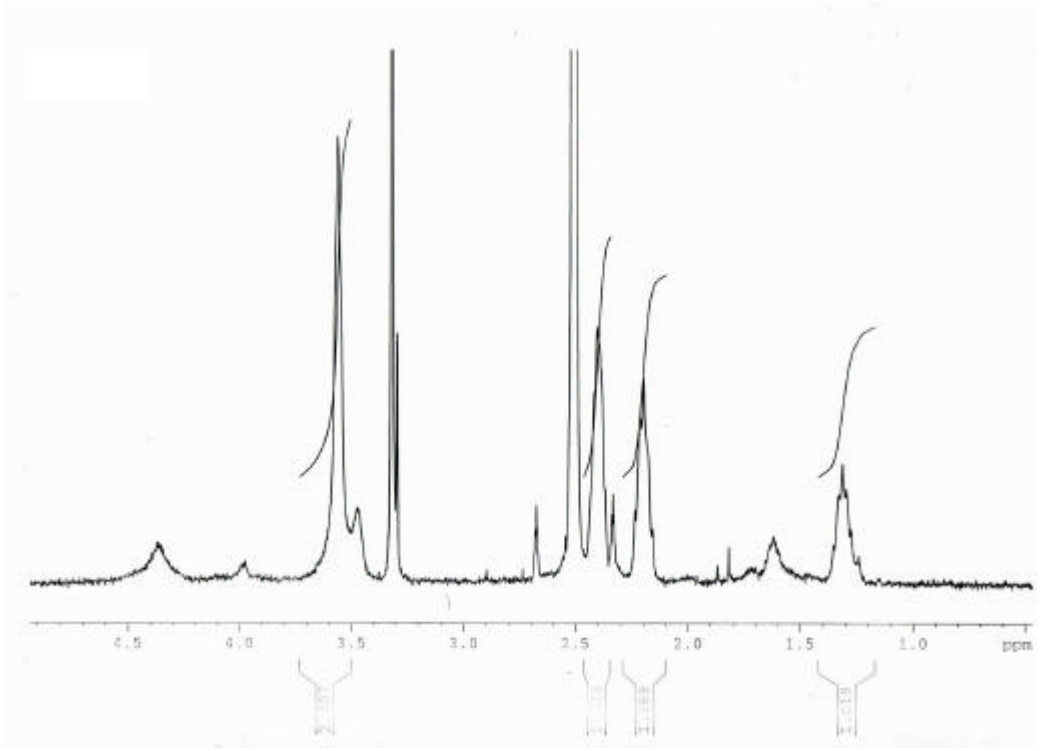


Figure 4. 14 ^{1}H NMR spectrum of DH5Cl

4.1.2.3. IR, RAMAN, MASS AND ^{1}H NMR SPECTRA OF DH6Cl

The IR and Raman spectra of DH6Cl are given in figures 4.15and 4.16. Listings of selected bands are given below. The mass and ^{1}HNMR spectra of DH6Cl are given in figures 4.17 and 4.18.

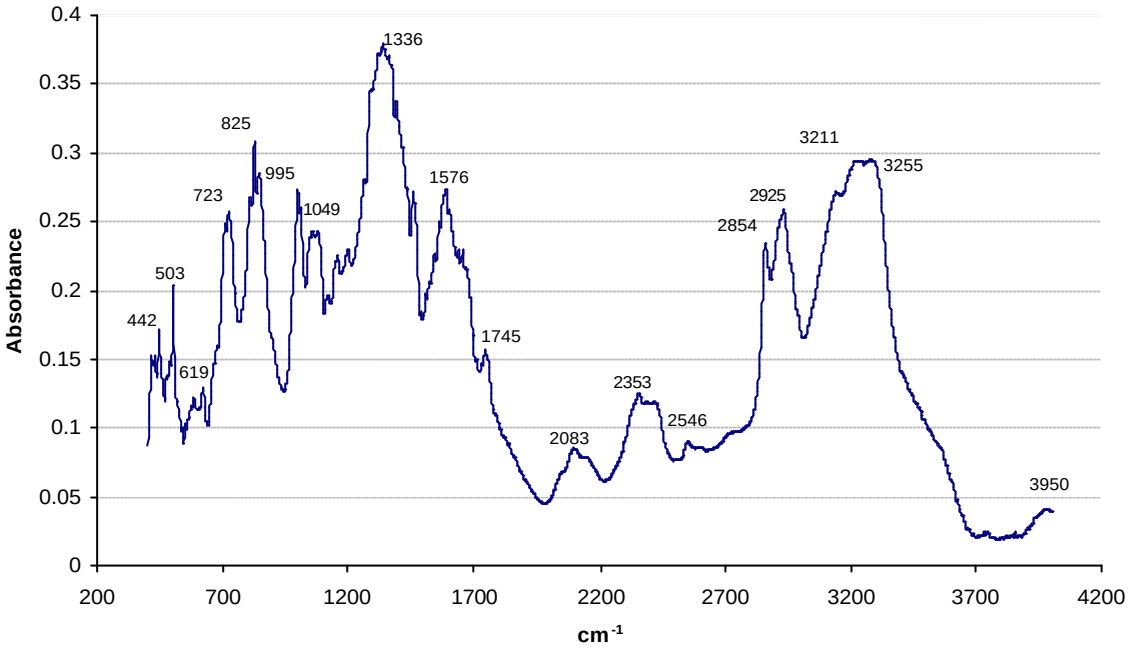


Figure 4. 15 IR spectrum of DH6Cl

Selected IR bands of DH6Cl

3950w br, 3255s br, 3211s, 2925s, 2854m, 1745m, 1576s, 1336s, 1049s d, 995s, 825s, 723m, 619w, 503m, 442w.

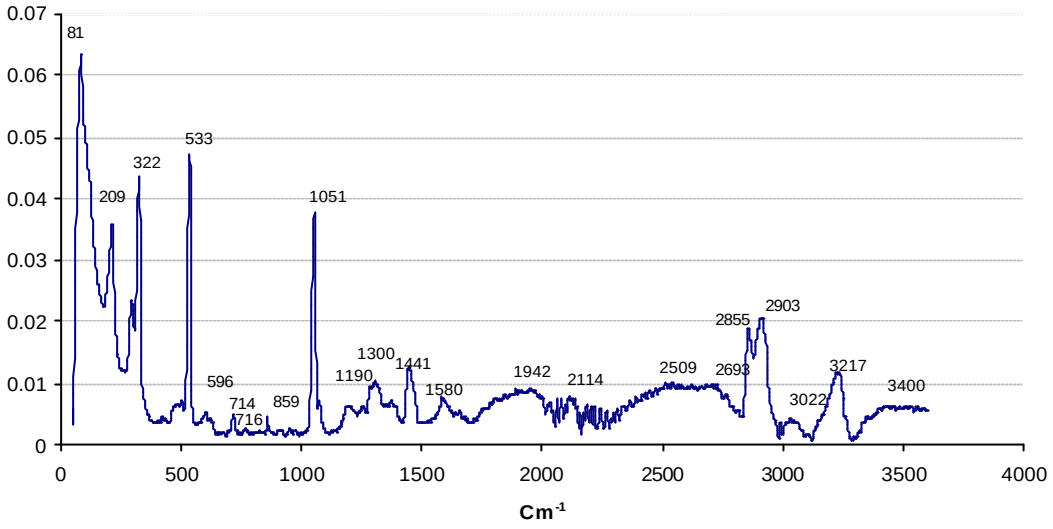


Figure 4. 16 Raman spectrum of DH6Cl

Selected Raman bands of DH6Cl:

3217w, 2903m, 2855m, 1441w, 1190w, 1051s, 859w, 716w, 596w, 533s, 322s, 295w, 209m, 81s

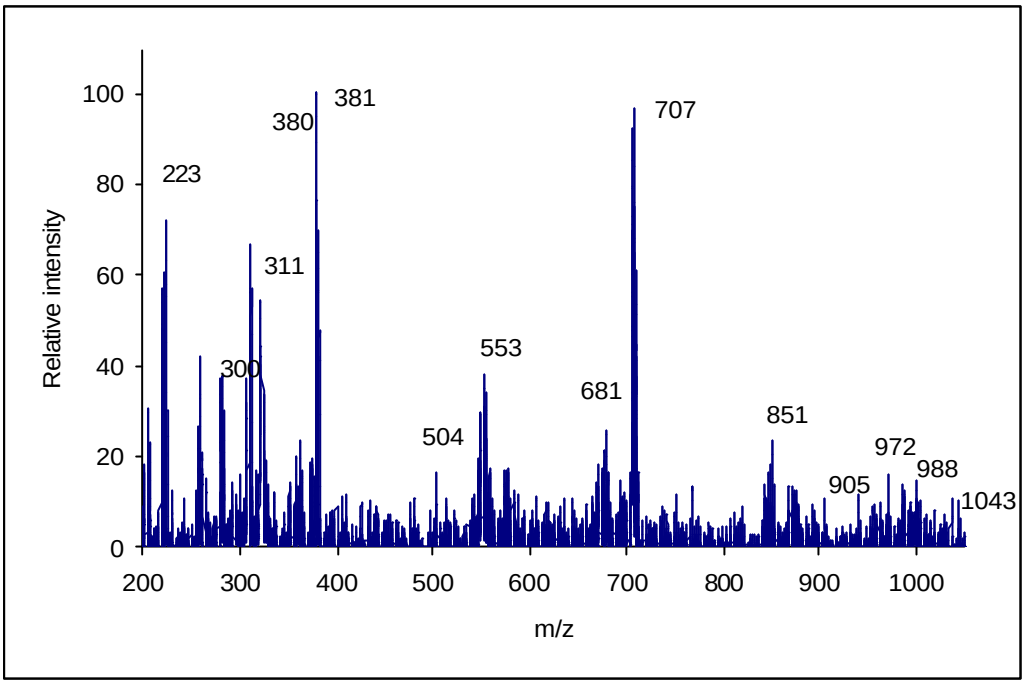


Figure 4. 17 Mass spectrum of DH6Cl

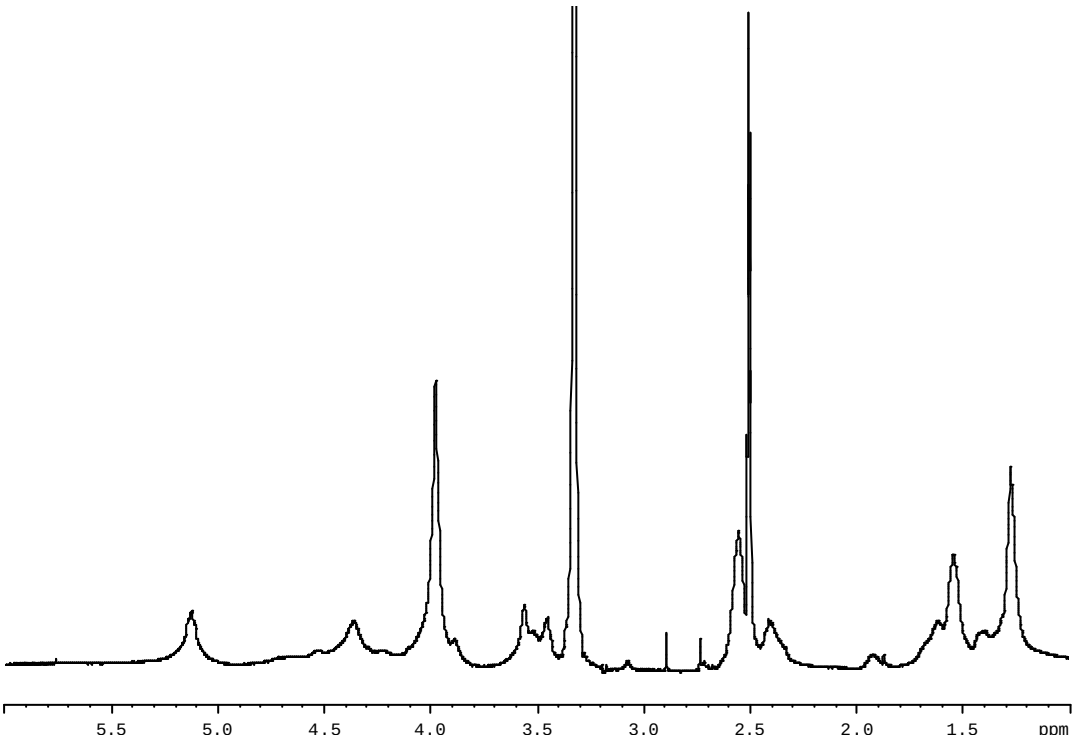


Figure 4. 18 ^{1}H NMR spectrum of DH6Cl

4.1.2.4. IR, RAMAN, MASS AND ^{1}H NMR SPECTRA OF DH7Cl

The IR and Raman spectra of DH7Cl are given in figure 4.19 and 4.20. Listings of selected bands of IR and Raman spectra are given below. The mass and ^{1}H NMR spectra of DH7Cl are given in figure 4.21 and 4.22.

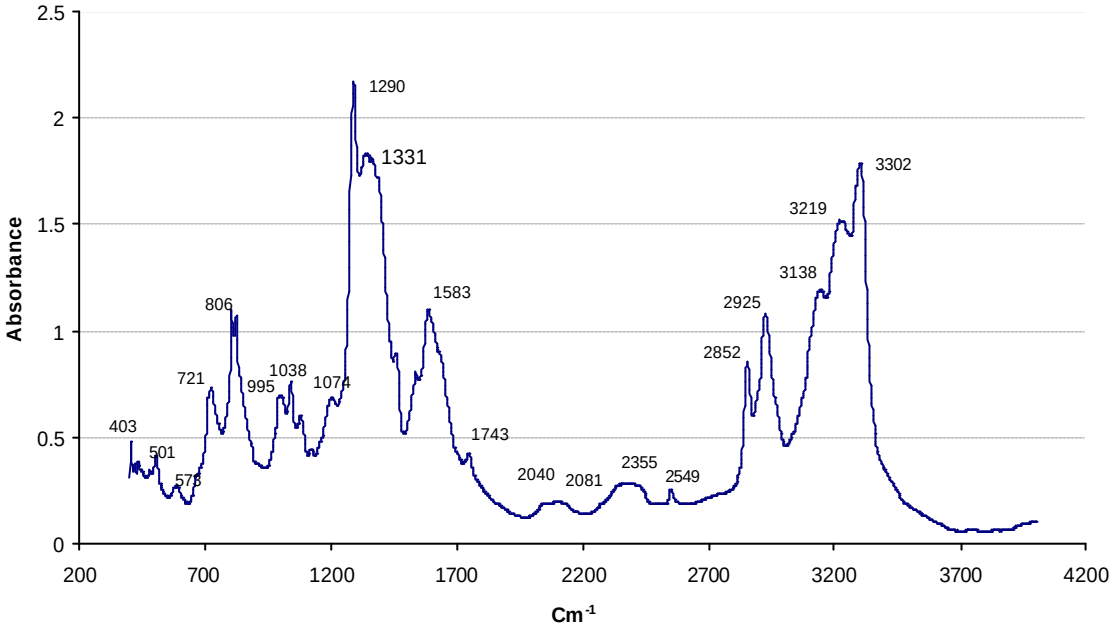


Figure 4. 19 IR spectrum of DH7Cl

Selected IR bands of DH7Cl:

3302s, 3219s, 3138m, 2925m, 2852w, 2355w, 2081w, 2040w, 1743w, 1583m, 1331s br, 1290s, 1074w, 1038w, 955w, 806m d, 721 w, 573w, 501w, 403w

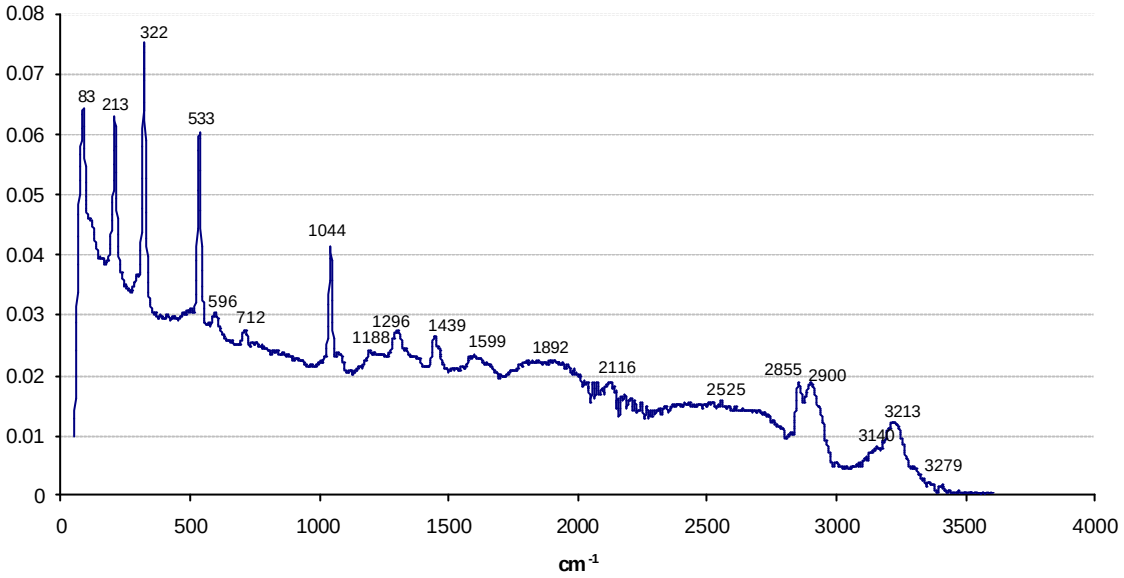


Figure 4. 20 Raman spectrum of DH7Cl

Selected Raman bands of DH7Cl

3213w br, 3140w, 2900w, 2855w, 1599w, 1439w, 1296w, 1188w, 1044m, 712w, 596w, 533s, 322s, 213s, 83s

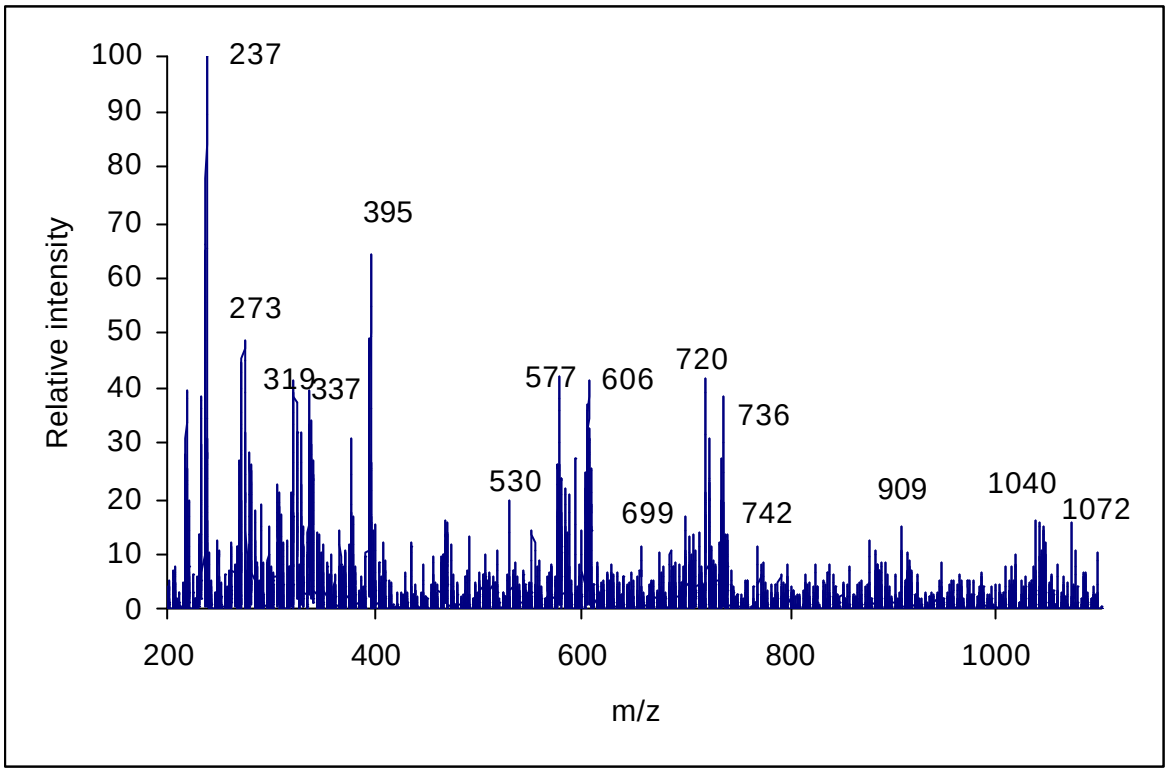


Figure 4. 21 Mass spectrum of DH7Cl

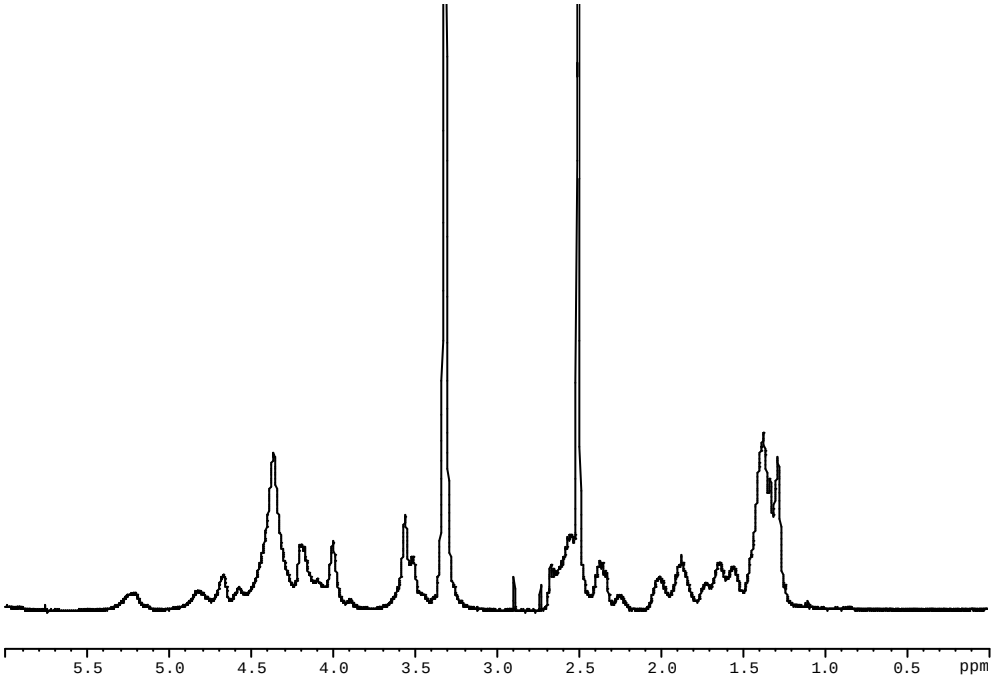


Figure 4. 22 ^{1}H NMR spectrum of DH7Cl

4.1.2.5. IR, RAMAN, MASS AND ^{1}H NMR SPECTRA OF DHD

The IR and Raman spectra of DHD are given in figures 4.23 and 4.24. Listings of selected bands of IR and Raman spectra are given below. The mass and ^{1}HNMR spectra of DHD are given in figures 4.25 and 4.26.

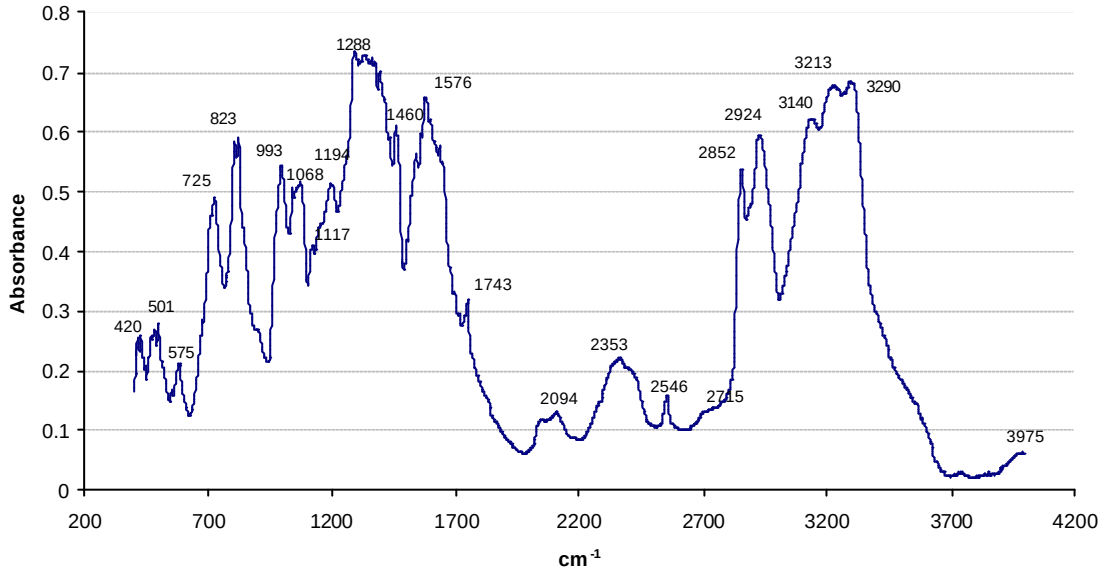


Figure 4. 23 IR spectrum of DHD

Selected bands of IR spectra of DHD

3975w br, 3290s, 3213s br, 3140s, 2924s, 2852m, 2353w, 1743m, 1576s, 1288s br, 1194m, 1068m d, 993s, 823s d, 725m, 575w, 501w d, 420w.

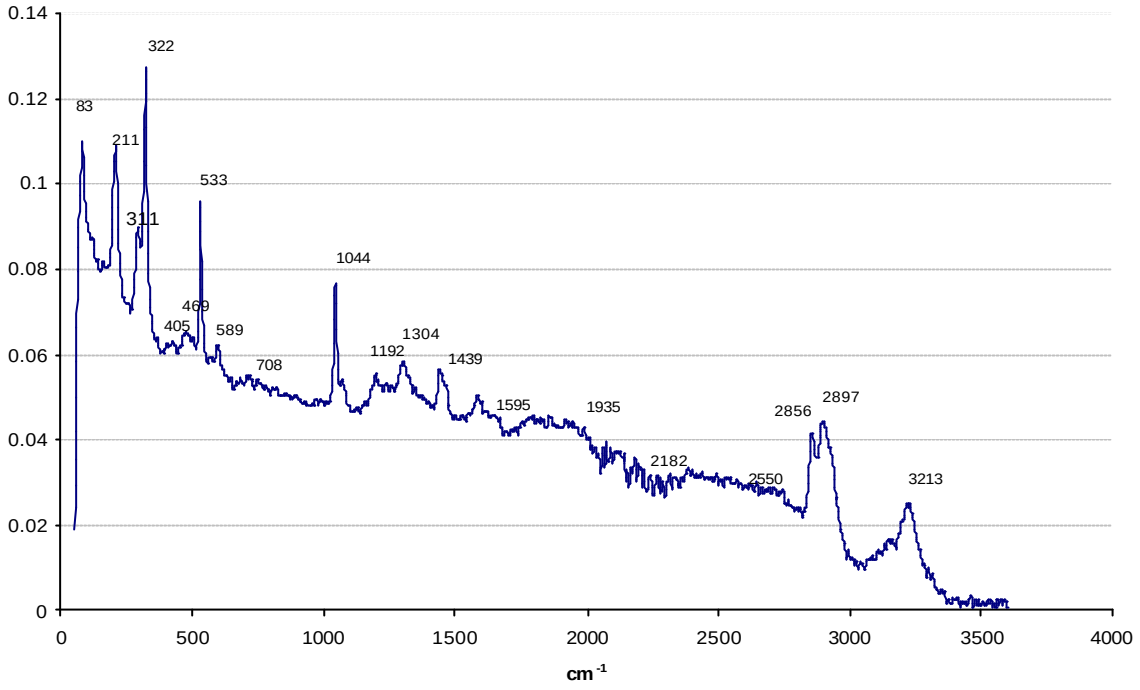


Figure 4. 24 Raman spectrum of DHD

Selected Raman bands of DHD:

3213w d, 2897m, 2856m, 1595w, 1439w, 1192w, 1044m, 708w, 589w d, 533s, 405w, 322s, 311w, 211s, 83s.

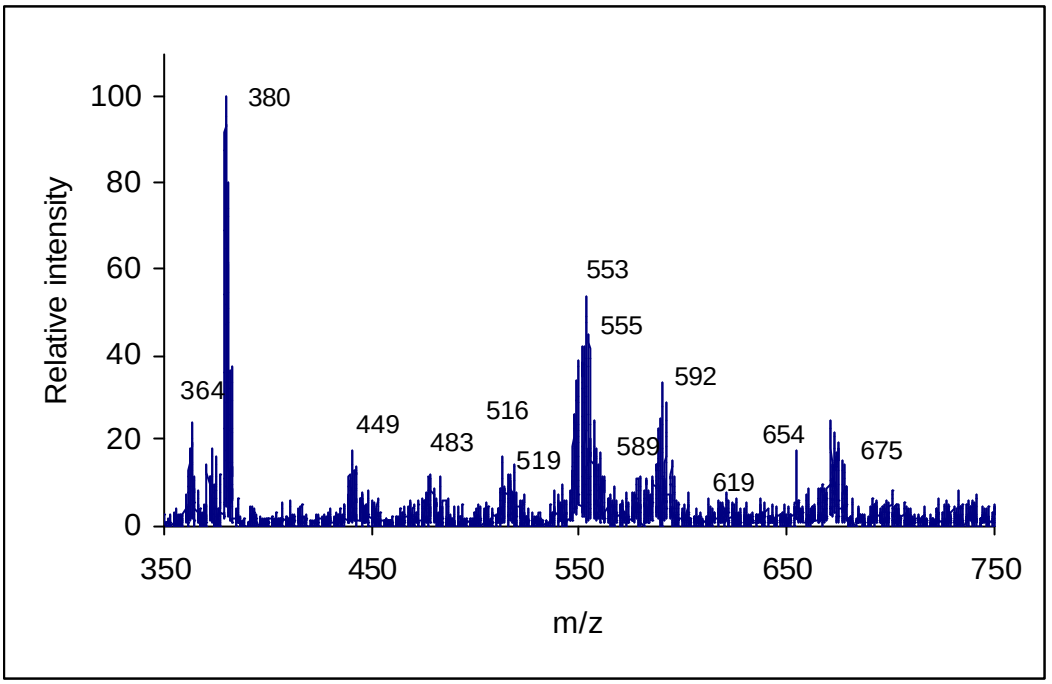


Figure 4. 25 Mass spectrum of DHD

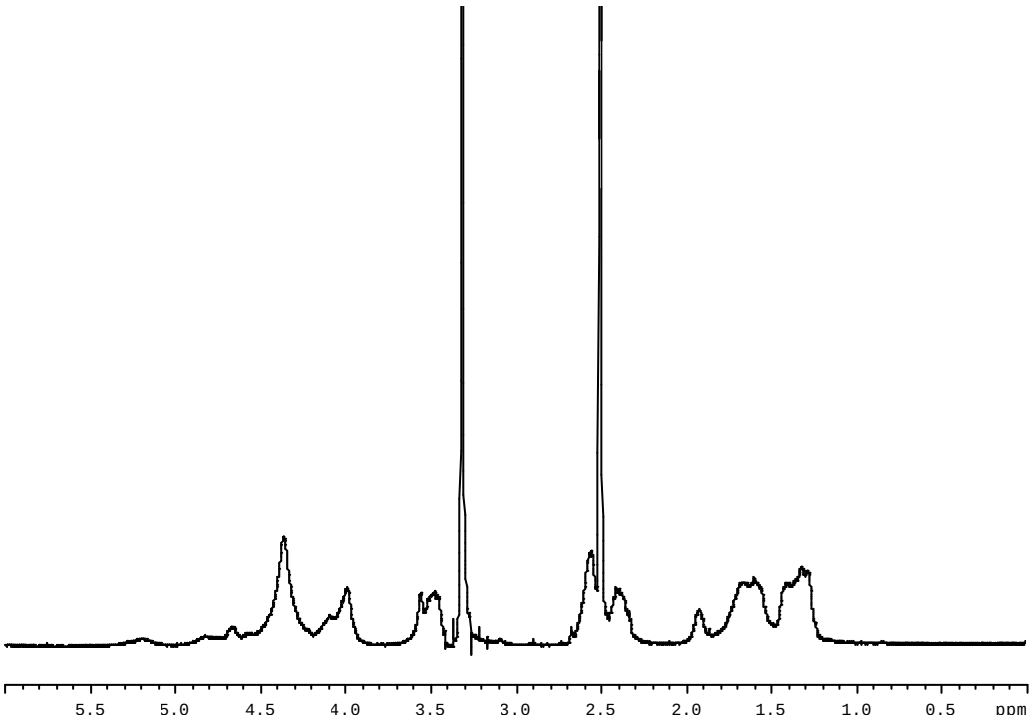


Figure 4. 26 ^{1}H NMR spectrum of DHD

Table 4. 7 Selected mass and ^{1}H NMR spectral peaks for DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Compound	Mass spectrum peaks values (m/z)	^{1}H NMR
DH4Cl	EIS-MS (DMF) (m/z: M = 988); (M – 2Cl + H = 917); (M - 4Cl - NH_3 - 5H = 824); (M - 4Cl -3NH_3 = 795); (M - 4Cl - 5NH_3 = 761); $Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_4NH_2\}Pd(NH_3)_2$ = 493; $ClPt\{NH_2(CH_2)_4NH_2\}$ = 319; $ClPd(NH_3)\{NH_2(CH_2)_4NH_2\}$ = 248.	^{1}H NMR DMF δ ppm: 3.9 (br, due to NH bonded to Pt and NH bonded to Pd).; 3.7 (due to CH_2) 2.8 (CH_2); 2.0 (due toCH_2).
DH5Cl	EIS-MS (DMF) (m/z: M = 1015); (M - 2Cl = 944); (M - 4Cl - NH_3 - H = 855); (M - Pt - 2NH_3 - 4Cl - 2H = 642); $(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pt(NH_2)$ = 540; $Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_3)_3$ = 524; $Cl(NH_2)Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_2)_2$ = 487; $(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_3)$ = 451; $(Pd\{NH_2(CH_2)_5NH_2\} + H)$ = 211.	^{1}H NMR DMSO δ ppm: 4.4 (br, due to NH_3); 4.0 (due to NH2); 3.6 (CH_2); 2.4 (CH_2); 2.2 (CH_2); 1.6 (?); 1.3 (CH_2).
DH6Cl	EIS-MS (DMF) (m/z: M = 1043); (M - 2Cl = 972); (M - 4Cl - 3NH_3 = 851); $Cl(NH_3)$-Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)(NH_2)\{NH_2(CH_2)_6NH_2\}$ = 707; $Cl(NH_2)_2$ Pt -μ-$\{NH_2(CH_2)_6NH_2\}$Pd Cl (NH_3) (NH_2) = 553; $(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}Pd(NH_3)Cl$ = 504; $Cl(NH_3)$ $(NH_2)Pt\{NH_2(CH_2)_6NH_2\}$ = 380; $Pt\{NH_2(CH_2)_6NH_2\}$= 311; $(Pd\{NH_2(CH_2)_6NH_2\} + H)$ = 223.	^{1}H NMR DMSO δ ppm: 5.1 (due to NH_3); 4.4 (due to NH_2); 4.0 (due to CH2); 3.6 (br, impurity ?); 2.4 (due to CH_2); 1.6 (due to CH2); 1.3 (due to CH_2).
DH7Cl	EIS-MS (DMF) (m/z: M = 1072); (M -Cl + 3H = 1040); (M - 4Cl - NH_3 - 4H = 909); $Cl(NH_3)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pt(NH_3)_2\{NH_2(CH_2)_7NH_2\}$ = 736; $(Cl(NH_3)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pt(NH_3)\{NH_2(CH_2)_7NH_2\} + H)$ = 720; $Cl_2(NH_2)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_2)Cl_2$ = 606; Pt-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_3)\{NH_2(CH_2)_7NH_2\}$= 577; $ClPt(NH_3)_2$-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_2)$ = 530; $ClPt(NH_3)_2\{NH_2(CH_2)_7NH_2\}$ = 395, $ClPd\{NH_2(CH_2)_7NH_2\}$ = 273; $(Pd\{NH_2(CH_2)_7NH_2\} + H)$ = 237.	^{1}H NMR DMSO δ ppm: 4.4 (due toNH_3); 4.2 (due toNH_2); 4.0 (NH_2); 3.6 (d, CH_2); 2.4 (CH_2); 1.9 (d, CH_2); 1.6 (t, due CH_2); 1.4 (d, impurity ?); 1.3 (CH_2).
DHD	EIS-MS (DMF) (m/z: (M = 654); Cl_2Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)Cl_2$ +2H) = 667; (M - NO_3 + H =592); $(Cl_3Pt$-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)_2(NH_2CH_2)$ + 4H) = 590; $(Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)_2$ - H) = 555; $Cl(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)(NH_2)$ = 553; $(Cl(NH_3)_2Pt\{NH_2(CH_2)_6NH_2\}$ - H) = 380, $Cl(NH_3)Pt\{NH_2(CH_2)_6NH_2\}$ = 364.	^{1}H NMR DMSO δ ppm: 4.4 (br, NH_3); 4.0 (d, NH_2); 3.6 (d,CH_2); 2.4 (CH_2); 1.9 (CH_2); 1.6 (d, CH_2); 1.3 (d, CH_2).

4.2. CYTOTOXICITY

The cytotoxicity of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DH1Cl, DHD and cisplatin against several human tumour cell lines including ovary cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, and non small lung cell line: NCI-H640 and melanoma: (Me-10538) has been determined using MTT reduction assay. The concentrations of the compounds were varied from 0 to the value required for ninety percent cell kill.

For DH1Cl, DH6Cl and cisplatin, 50 µM stock solution was serially diluted to give nine concentrations ranging from 0.25 to 50 µM in one set and from 0.125-25 µM in another set. Further dilution of solutions of DH6Cl, DHD and cisplatin was carried out to give five concentrations ranging from 0.02-12.5 µM.

From the three sets of results obtained the values at following concentrations: 0.01 µM, 0.05 µM, 0.125 µM, 0.25 µM, 1.25 µM, 6.25 µM, 12.25 µM, 25 µM, were plotted to give the survival curves from which the IC_{50} and IC_{90} values were determined. For DH4Cl, DH5Cl, and DH7Cl, activity was determined only at five concentrations ranging from 0.05 µM to 31.25 µM and for DHD it was determined for five concentrations ranging from 0.01 µM to 6.25 µM.

Tables 4.8 to 4.12 give percentage cell survival values as applied to A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538 cell lines when the cells were treated with increasing concentrations of the compounds DH6Cl, DH1Cl and cisplatin. The IC_{50} values are also given in the tables. In these tables (as in those for the other compounds), the mean and the standard deviations of at least three replicates are given. Figures 4.27 to 4.31 give the corresponding cell survival curves.

Table 4. 8 The cell survival rate as a percentage of the control for the ovary cell line A2780, when the cells were treated with DH6Cl, DH1Cl and cisplatin

Concentration of compound (μM)	% Cell Survival Rate		
	Cisplatin	DH6Cl	DH1Cl
0.01	101.1 ± 8.4	75.7 ± 6.0	96.5 ± 8.5
0.05	93.8 ± 8.1	49.9 ± 4.5	77.4 ± 9.9
0.125	80.8 ± 7.5	41.2 ± 2.3	68.9 ± 8.9
0.25	56.4 ± 4.8	26.5 ± 3.6	54.8 ± 5.8
1.25	20.8 ± 3.2	12.6 ± 4.1	35.1 ± 6.9
6.25	8.1 ± 1.0	7.7 ± 5.4	16.7 ± 5.6
12.5	5.6 ± 0.8	5.5 ± 1.1	8.2 ± 1.0
25.0	3.3 ± 0.3	2.0 ± 1.0	5.0 ± 1.0
IC_{50}	0.4 ± 0.08	0.05 ± 0.006	0.5 ± 0.1

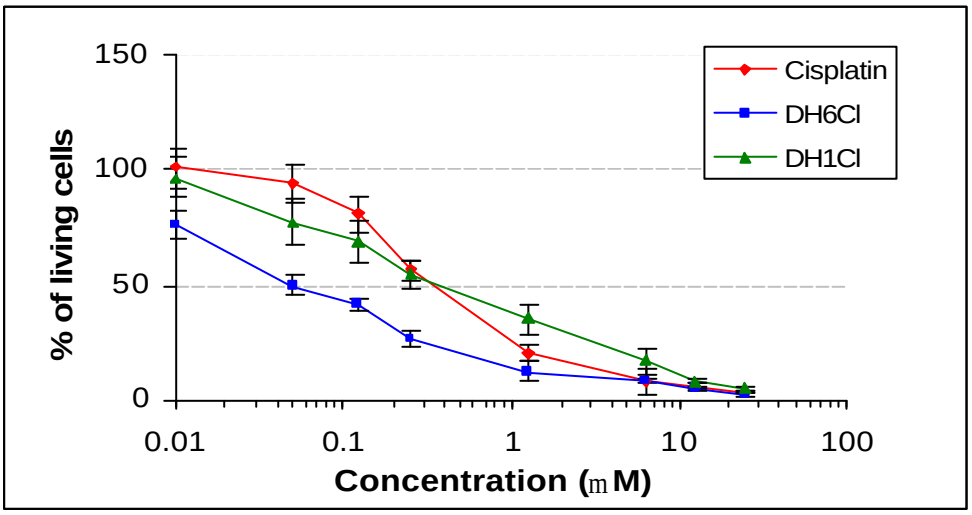


Figure 4. 27 Survival curve for the ovary cell line A2780 when treated with increasing concentrations of DH6Cl, DH1Cl and cisplatin.

Table 4. 9 The cell survival rate as a percentage of the control for the ovary cell line A2780^{cisR}, when the cells were treated with DH6Cl, DH1Cl and cisplatin

Concentration of compound (μM)	% Cell Survival Rate		
	Cisplatin	DH6Cl	DH1Cl
0.01	102.5 ± 8.9	89.4 ± 6.3	95.6 ± 8.4
0.05	98.5 ± 6.0	78.9 ± 1.8	86.9 ± 5.1
0.125	93.6 ± 6.9	70.1 ± 8.1	79.7 ± 4.6
0.25	85.6 ± 6.2	49.9 ± 2.2	67.1 ± 8.5
1.25	75.5 ± 4.1	37.9 ± 4.2	51.6 ± 2.7
6.25	33.8 ± 5.0	21.1 ± 3.0	29.5 ± 7.7
12.5	28.3 ± 3.7	14.3 ± 2.1	15.9 ± 4.8
25.0	25.3 ± 2.9	3.2 ± 0.9	4.7 ± 1.1
IC_{50}	4.4 ± 0.2	0.3 ± 0.01	1.8 ± 0.4

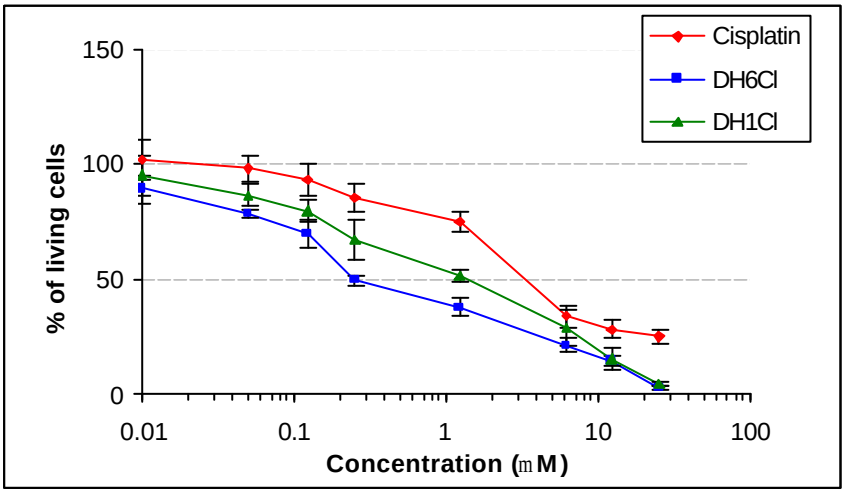


Figure 4. 28 Survival curve for the ovary cell line A2780^{cisR} when treated with increasing concentrations of DH6Cl, DH1Cl and cisplatin

Table 4. 10 The cell survival rate as a percentage of the control for the ovary cell line A2780^{ZD0473R} when the cells were treated with DH6Cl, DH1Cl and cisplatin

Concentration (μM)	% Cell Survival Rate		
	Cisplatin	DH6Cl	DH1Cl
0.01	101.8 ± 6.9	78.3 ± 7.8	94.6 ± 8.4
0.05	92.8 ± 7.9	71.6 ± 9.3	88.2 ± 7.7
0.125	85.5 ± 8.7	67.0 ± 0.6	72.1 ± 10.8
0.25	78.0 ± 7.3	47.4 ± 5.5	69.9 ± 9.8
1.25	40.8 ± 6.2	34.2 ± 4.2	53.5 ± 7.3
6.25	17.1 ± 3.9	19.3 ± 2.7	31.4 ± 6.5
12.5	11.6 ± 1.0	16.7 ± 1.4	17.7 ± 5.4
25.0	8.1 ± 1.1	5.4 ± 3.2	7.9 ± 2.8
IC_{50}	1.0 ± 0.1	0.2 ± 0.01	1.8 ± 0.3

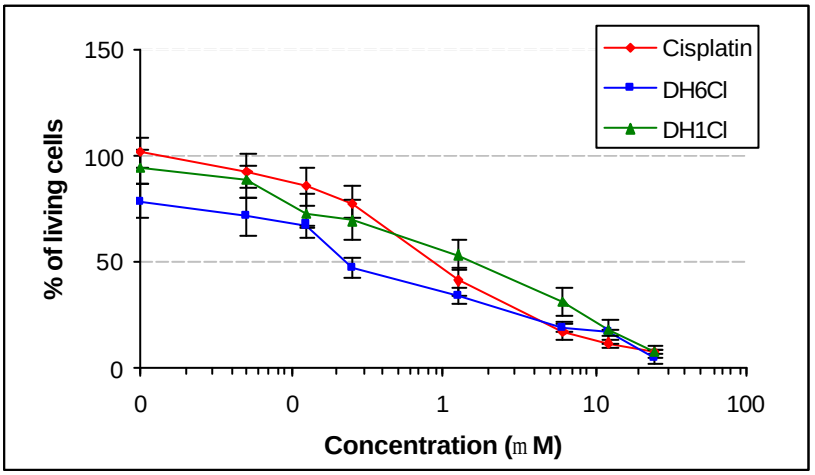


Figure 4. 29 Survival curve for the ovary cell line A2780 ^{ZD0473R} when treated with increasing concentrations of DH6Cl, DH1Cl and cisplatin

Table 4. 11 The cell survival rate as a percentage of the control for the melanoma cell line Me-10538, when the cells were treated with DH6Cl, DH1Cl and cisplatin

Concentration of compound (μM)	% Cell Survival Rate		
	Cisplatin	DH6Cl	DH1Cl
0.01	101.3 ± 6.5	92.5 ± 0.9	98.6 ± 8.8
0.05	98.1 ± 9.4	82.0 ± 5.6	90.0 ± 5.1
0.125	92.5 ± 5.2	76.3 ± 4.6	87.6 ± 5.9
0.25	85.2 ± 5.4	68.9 ± 5.5	80.0 ± 7.4
1.25	74.5 ± 7.4	38.3 ± 2.5	53.6 ± 4.2
6.25	44.7 ± 5.7	17.8 ± 1.3	26.4 ± 2.5
12.5	23.2 ± 4.5	13.8 ± 2.1	18.6 ± 3.0
25.0	13.8 ± 0.8	5.4 ± 0.7	9.2 ± 5.0
IC_{50}	5.0 ± 0.7	0.9 ± 0.04	2.0 ± 0.2

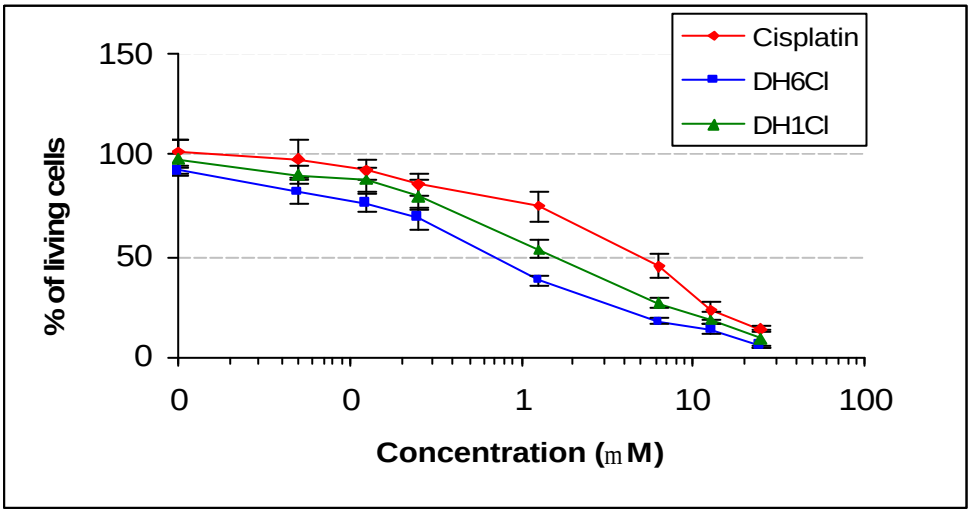


Figure 4. 30 Survival curve for melanoma cell line Me-10530 when treated with increasing concentrations of DH6Cl, DH1Cl and cisplatin

Table 4. 12 The cell survival rate as a percentage of the control for non small lung cell line NCI-H460, when the cells were treated with DH6Cl, DH1Cl and cisplatin

Concentration of compound (μM)	% Cell Survival Rate		
	Cisplatin	DH6Cl	DH1Cl
0.01	102.4 ± 4.8	92.6 ± 3.8	98.8 ± 1.0
0.05	92.8 ± 7.1	86.6 ± 3.8	95.4 ± 2.7
0.125	83.2 ± 9.6	74.5 ± 5.9	91.4 ± 6.9
0.25	75.1 ± 6.8	71.0 ± 3.1	85.2 ± 6.7
1.25	34.1 ± 3.8	45.1 ± 5.2	70.4 ± 7.6
6.25	17.9 ± 4.1	26.2 ± 7.9	45.7 ± 3.2
12.5	12.3 ± 1.7	21.1 ± 2.1	30.0 ± 6.5
25.0	8.0 ± 1.1	7.8 ± 1.4	8.0 ± 1.7
IC_{50}	0.9 ± 0.07	1.0 ± 0.07	5.1 ± 0.5

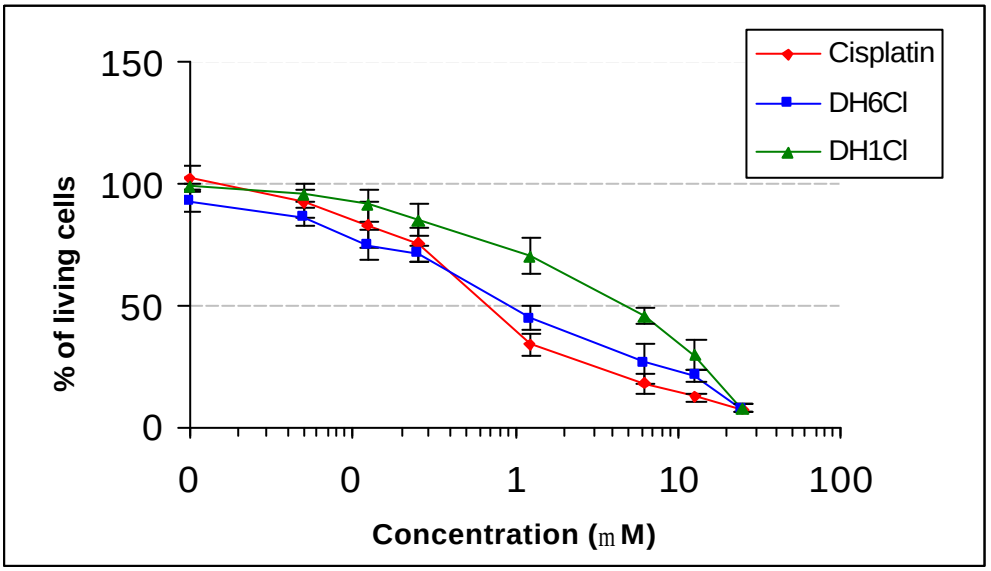


Figure 4. 31 Survival curve for the non small lung cell line NCI-H460 when treated with increasing concentrations of DH6Cl, DH1Cl and cisplatin

From the above results (Tables 4.8 to 4.12 and Figures 4.27 to 4.31), it appears that DH6Cl is more active than cisplatin against all of the cell lines except NCI-H460 against which it shows activity similar to that of cisplatin. It will be seen later that DH6Cl is in fact the most active compound among all the compounds synthesized and studied in this project. When activity of DH6Cl is compared with that of DH1Cl it is found that DH6Cl is more active than DH1Cl against all the cell lines. DH1Cl is found to be more active than cisplatin against the cell lines $A2780^{cisR}$ and Me-10538. It is found to have activity similar to that of cisplatin against A2780 and $A2780^{ZD0473R}$ cell lines and a lower activity than cisplatin against non small lung cell line NCI-H640.

Tables 4.13 to 4.17 give percentage cell survival values as applied to A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538 cell lines when the cells were treated with increasing concentrations of the compounds DH4Cl, DH5Cl and DH7Cl. The IC_{50} values are also given in the tables. Figures 4.32 to 4.36 give the corresponding cell survival curves.

Table 4. 13 The cell survival rate as a percentage of the control for the ovary cell line A2780, when the cells were treated with DH4Cl, DH5Cl and DH7Cl

Concentration of compound (μM)	% Cell Survival Rate		
	DH4Cl	DH5Cl	DH7Cl
0.05	95.9 ± 3.7	94.9 ± 5.6	89.1 ± 7.5
0.25	72.5 ± 5.8	89.3 ± 9.0	73.4 ± 7.0
1.25	69.0 ± 2.8	72.9 ± 8.6	43.4 ± 4.4
6.25	24.7 ± 6.8	21.6 ± 3.7	25.9 ± 2.7
31.25	1.6 ± 1.6	1.2 ± 0.5	1.9 ± 0.02
IC_{50}	3.2 ± 0.2	3.4 ± 0.4	1.0 ± 0.1

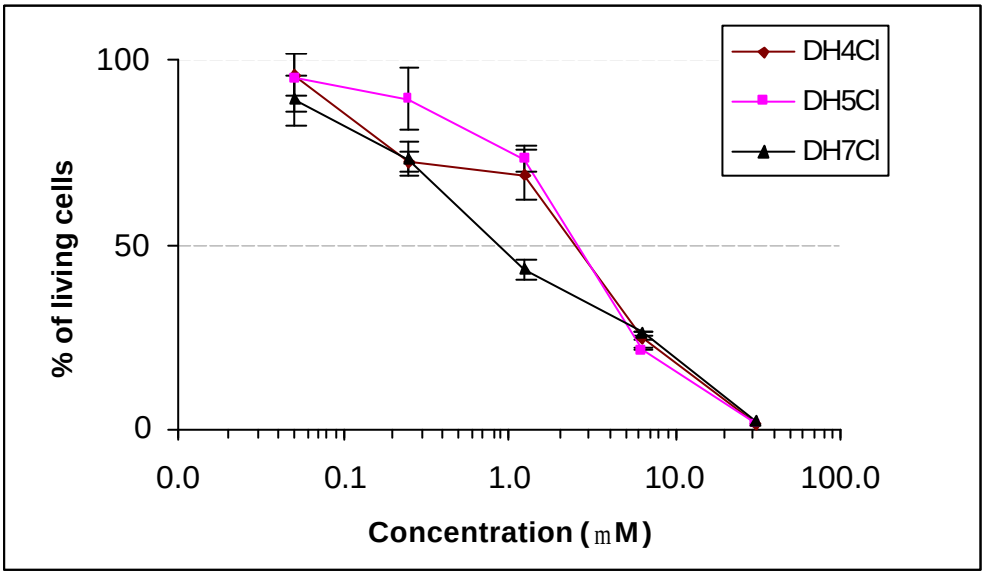


Figure 4. 32 Survival curve for the ovary cell line A2780 when treated with increasing concentrations of DH4Cl, DH5Cl and DH7Cl

Table 4. 14 The cell survival rate as a percentage of the control for the ovary cell line A2780^{cisR}, when the cells were treated with DH4Cl, DH5Cl and DH7Cl

Concentration of compound (μM)	% Cell Survival Rate		
	DH4Cl	DH5Cl	DH7Cl
0.05	89.4 ± 5.0	91.6 ± 8.9	81.3 ± 4.9
0.25	78.8 ± 3.2	78.7 ± 4.0	78.1 ± 1.5
1.25	70.3 ± 2.1	67.0 ± 6.6	65.3 ± 8.9
6.25	29.7 ± 5.2	37.8 ± 4.9	26.9 ± 3.9
31.25	1.0 ± 0.1	2.2 ± 0.9	2.5 ± 0.4
IC_{50}	3.7 ± 0.3	4.0 ± 0.3	1.8 ± 0.4

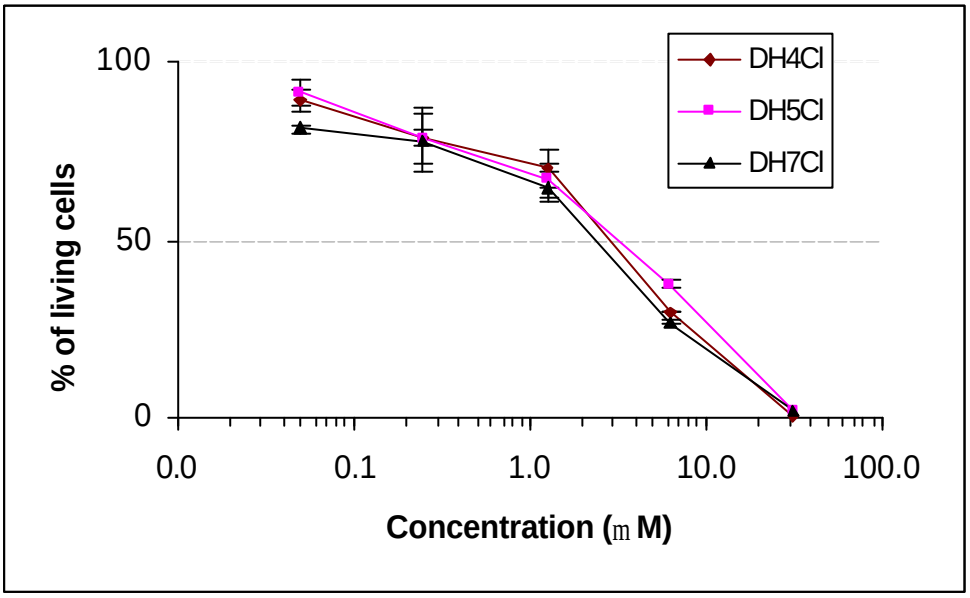


Figure 4. 33 Survival curve for the ovary cell line A2780^{cisR} when treated with increasing concentrations of DH4Cl, DH5Cl and DH7Cl

Table 4. 15 The cell survival rate as a percentage of the control for the ovary cell line A2780^{ZD0473R}, when the cells were treated with DH4Cl, DH5Cl and DH7Cl

Concentration of compound (μM)	% Cell Survival Rate		
	DH4Cl	DH5Cl	DH7Cl
0.05	98.0 ± 4.8	88.9 ± 10.2	86.3 ± 8.5
0.25	95.2 ± 6.0	82.2 ± 8.8	83.8 ± 8.4
1.25	84.2 ± 4.2	73.4 ± 2.8	74.6 ± 4.7
6.25	42.0 ± 4.7	37.3 ± 9.5	41.1 ± 0.9
31.25	1.9 ± 0.3	3.5 ± 2.4	2.4 ± 0.3
IC_{50}	5.3 ± 0.4	4.5 ± 0.7	4.9 ± 0.2

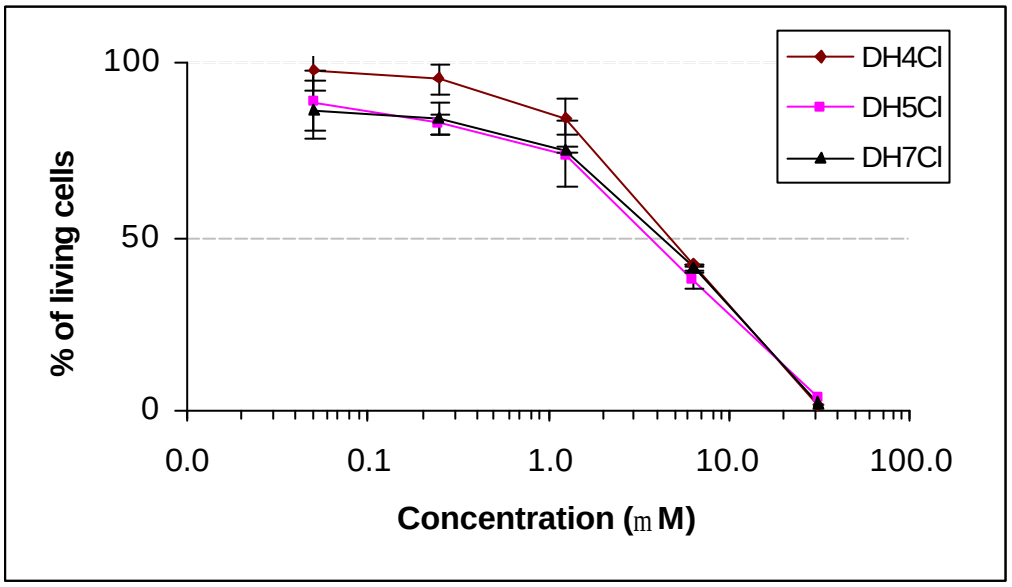


Figure 4. 34 Survival curve for the ovary cell line A2780^{ZD0473R} when treated with increasing concentrations of DH4Cl, DH5Cl and DH7Cl

Table 4. 16 The cell survival rate as a percentage of the control for the melanoma cell line Me-10538, when the cells were treated with DH4Cl, DH5Cl and DH7Cl

Concentration of compound (μM)	% Cell Survival Rate		
	DH4Cl	DH5Cl	DH7Cl
0.05	92.6 ± 3.8	96.4 ± 6.4	88.8 ± 4.6
0.25	90.8 ± 4.9	93.9 ± 4.6	77.7 ± 6.1
1.25	78.2 ± 5.9	78.1 ± 5.6	58.6 ± 8.6
6.25	47.9 ± 2.1	48.6 ± 7.7	26.7 ± 5.5
31.25	10.0 ± 2.2	1.7 ± 0.3	2.2 ± 0.6
IC_{50}	5.9 ± 0.3	5.4 ± 0.9	2.5 ± 0.8

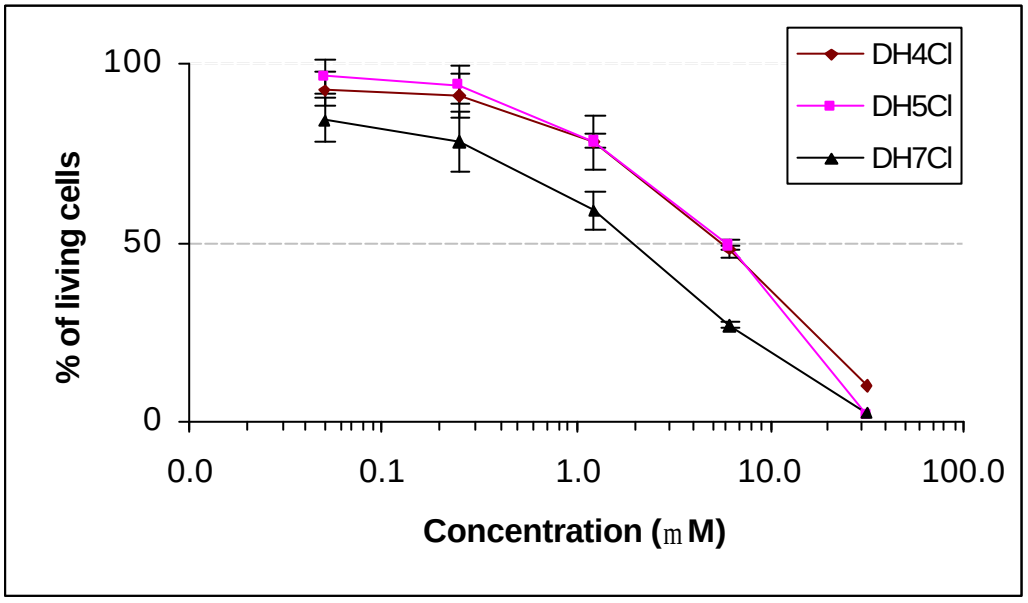


Figure 4. 35 Survival curve for the melanoma cell line Me-10538 when treated with increasing concentrations of DH4Cl, DH5Cl and DH7Cl

Table 4. 17 The cell survival rate as a percentage of the control for the non small lung cell line NCI-H460, when the cells were treated with DH4Cl, DH5Cl and DH7Cl

Concentration of compound (μM)	% Cell Survival Rate		
	DH4Cl	DH5Cl	DH7Cl
0.05	82.0 ± 3.9	87.5 ± 5.6	92.0 ± 4.7
0.25	77.2 ± 4.4	85.5 ± 5.4	80.9 ± 4.9
1.25	71.2 ± 6.4	73.3 ± 4.5	68.2 ± 6.0
6.25	66.5 ± 5.9	57.0 ± 8.9	38.6 ± 3.1
31.25	5.5 ± 2.4	5.0 ± 2.6	3.9 ± 1.2
IC_{50}	12.6 ±1.6	9.2 ± 2.6	4.3 ± 0.5

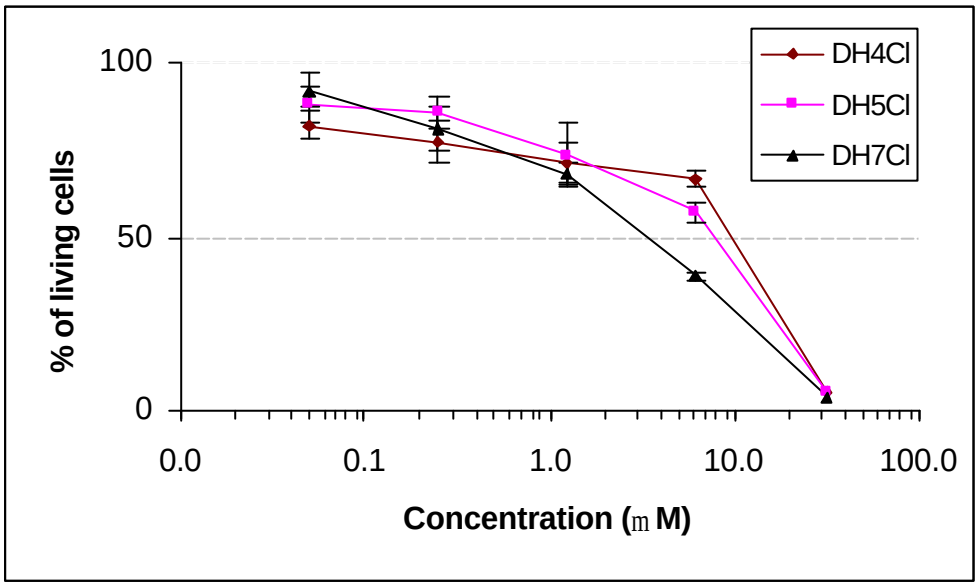


Figure 4. 36 Survival curve for the non small lung cell line NCI-H460 when treated with increasing concentrations of DH4Cl, DH5Cl and DH7Cl

The results given in tables 4.13 to 4.17 and figures 4.32 to 4.36 show that DH4Cl and DH5Cl are less active than cisplatin against all the cell lines. DH7Cl is found to be less active than cisplatin against A2780, A2780^{ZD0473R} and NCI-H640 cell lines but

more active than cisplatin against A2780^{cisR} and Me-10538 cell lines. DH7Cl is found to be more active than DH4Cl and DH5Cl against all the cell lines except A2780^{ZD0473R} against which it shows activity similar to that of DH4Cl and DH5Cl.

Tables 4.18 and 4.19 give percentage cell survival in A2780, A2780^{cisR}, A2780^{ZD0473R}, NCI-H640 and Me-10538 cells when the cells were treated with increasing concentrations of DHD and cisplatin. Figure 4.37 gives the corresponding cell survival curves.

Table 4. 18 The cell survival rate as a percentage of the control for the ovary cell lines A2780, A2780^{cisR} and A2780^{ZD0473R}, when the cells were treated with DHD and cisplatin

Concentration of compound (μM)	% Cell Survival Rate					
	A2780		A2780^{cisR}		A2780^{ZD0473R}	
	Cisplatin	DHD	Cisplatin	DHD	cisplatin	DHD
0.01	101.1±8.4	91.8 ± 6.0	102.5 ±8.9	95.9 ±1.0	101.8 ±6.9	96.9 ± 1.9
0.05	93.8 ± 8.1	76.6 ± 6.9	98.5 ± 6.0	95.9 ± 1.0	92.8 ± 7.9	78.2 ± 7.2
0.25	56.4 ± 4.8	44.9 ± 4.7	85.6 ± 6.2	62.2 ± 5.5	78.0 ± 7.3	54.9 ± 8.7
1.25	20.8 ± 3.2	31.2 ± 8.7	75.5 ± 4.1	45.4 ± 5.0	40.8 ± 6.2	34.3 ±4.9
6.25	8.1 ± 1.0	9.0 ± 5.3	33.8 ± 5.0	25.4 ± 5.4	17.1 ± 3.9	16.0 ± 6.5
IC_{50}	0.44±0.08	0.25±0.05	4.4 ± 0.2	0.96±0.09	1.0 ± 0.1	0.47 ± 0.08

Table 4. 19 The cell survival rate as a percentage of the control for the non small lung cell line NCI-H640 and melanoma Me-10538, when the cells were treated with DHD and cisplatin

Concentration of compound (μM)	% Cell Survival Rate			
	Me-10538		NCI-H460	
	Cisplatin	DHD	Cisplatin	DHD
0.01	101.3 ± 6.5	98.1 ± 1.3	102.4 ± 4.8	92.9 ± 2.0
0.05	98.1 ± 9.4	93.7 ± 4.0	92.8 ± 7.1	85.5 ± 3.5
0.25	85.2 ± 5.4	82.6 ± 4.4	75.1 ± 6.8	71.9 ± 5.3
1.25	74.5 ± 7.4	59.6 ± 6.9	34.1 ± 3.8	63.4 ± 6.0
6.25	44.7 ± 5.7	27.5 ± 4.6	17.9 ± 4.1	40.9 ± 6.5
IC_{50}	5.0 ± 0.7	2.7 ± 0.6	0.9 ± 0.07	4.2 ± 0.9

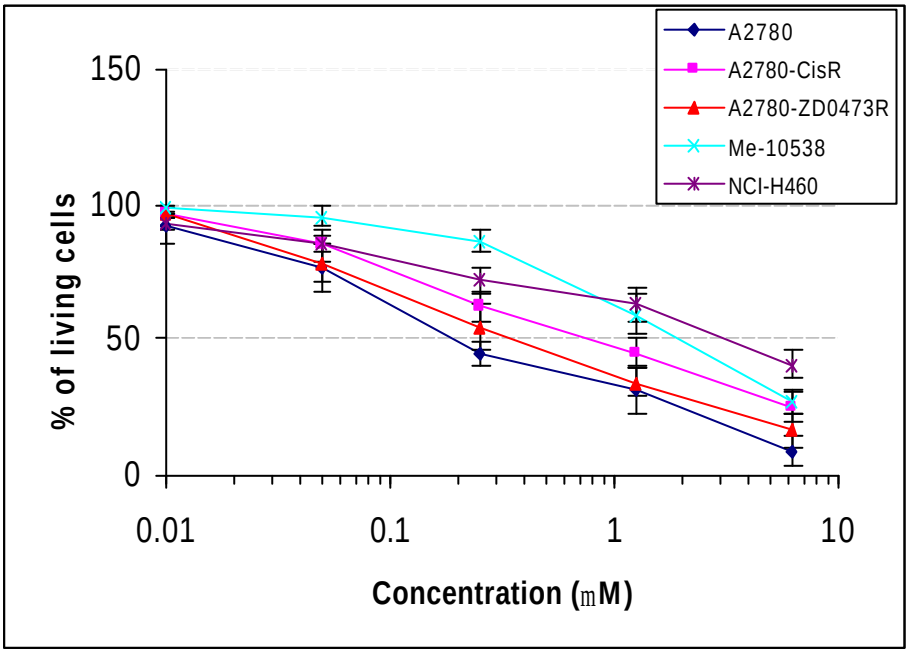


Figure 4. 37 Survival curve for the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538 treated with increasing concentrations of DHD.

Table 4.18 and 4.19 and the figure 4.37 show that DHD is more active than cisplatin against all of the cell lines except NCI-H460 against which it is found to be less active than cisplatin.

4.2.1. *ACTIVITY SUMMARY*

Table 4.20 summarizes the activity of all of the compounds (DH4Cl, DH5Cl, DH6Cl, DH7Cl, DH1Cl, DHD and cisplatin) in terms of their IC_{50} values and resistance factors (RF).

Table 4. 20 IC_{50} values and resistance factors (RF) for DH4Cl, DH5Cl, DH6Cl, DH7Cl, DH1Cl, DHD and cisplatin against the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, Me-10538 and NCI-H640

IC50 (μM) ± SD and resistant factors						
compound	A2780	$A2780^{cisR}$	RF	$A2780^{ZD0473R}$	Me-10538	NCI-H460
DH4Cl	3.2 ± 0.2	3.7 ± 0.3	1.1	5.3 ± 0.4	5.9 ± 0.3	12.6 ± 1.6
DH5Cl	3.4 ± 0.4	4.0 ± 0.3	1.2	4.5 ± 0.7	5.4 ± 0.9	9.2 ± 2.6
DH6Cl	0.048±0.006	0.25 ±0.01	5.2	0.23 ± 0.01	0.87 ± 0.04	1.0 ± 0.07
DH7Cl	1.03 ± 0.1	2.9 ± 0.5	2.8	4.9 ± 0.2	2.5 ± 0.8	4.3 ± 0.5
DHD	0.25 ± 0.05	0.96 ±0.09	3.8	0.47 ± 0.08	2.7 ± 0.6	4.2 ± 0.9
DH1Cl	0.49 ± 0.1	1.8 ± 0.4	3.7	1.8 ± 0.3	2.0 ± 0.2	5.1 ± 0.5
cisplatin	0.44 ± 0.08	4.4 ± 0.2	10.0	1.0 ± 0.1	5.0 ± 0.7	0.93 ± 0.07

It can be seen that among all the multinuclear compounds including DHD, DH6Cl has the lowest IC_{50} for all the cell lines indicating that DH6Cl is the most active compound. DH6Cl is found to be about nine times as active as cisplatin against the human ovary cell lines A2780. DHD is also found to be more active than cisplatin

against A2780 cell line whereas DH1Cl has activity similar to that of cisplatin. All other compounds are found to be less active than cisplatin. The actual order of activity of the compounds against A2780 cell lines is: DH6Cl > DHD > cisplatin = DH1Cl > DH7Cl > DH4Cl and DH5Cl.

For the cisplatin-resistant cell line $A2780^{cisR}$, it is found that DH6Cl is about seventeen times as active as cisplatin, DHD nearly five times as active as cisplatin and DH1Cl about two times as active as cisplatin. All other compounds DH4Cl, DH5Cl and DH7Cl are also found to be marginally more active than cisplatin. The actual order of activity of the compounds against $A2780^{cisR}$ cell line is: DH6Cl > DHD > DH1Cl > DH7Cl >DH4Cl = DH5Cl > cisplatin. The resistance factors for DH4Cl, DH5Cl, DH6Cl, DH5Cl, DH7Cl, DHD, DH1Cl and cisplatin as applied to the ovary cell lines A2780 and $A2780^{cisR}$ are respectively 1.1, 1.2, 5.2, 2.8, 3.8, 3.7 and 10.0. It appears that all of the multinuclear compounds have resistance factors lower than that of cisplatin.

For $A2780^{ZD0473R}$ cell line, DH6Cl is found to be about four times as active as cisplatin, and DHD about two times as active as cisplatin. All other compounds (DH4Cl, DH5Cl, DH7Cl and DH1Cl) are found to be less active than cisplatin. The actual order of activity of the compounds against $A2780^{ZD0473R}$ cell line is: DH6Cl > DHD > cisplatin > DH1Cl > DH5Cl = DH7Cl > DH4Cl. For the melanoma cell line Me-10538, DH6Cl is found to be six times as active as cislatin whereas DHD and DH7Cl are found to be nearly two times as active as cisplatin. DH1Cl is nearly 2.5 times as active as cisplatin. The other two compounds (DH4Cl and DH5Cl) are slightly less active than cisplatin against the cell line. For the non small lung cell line NCI-H460, DH6Cl is found to have activity similar to that of cisplatin. All the other

compounds (DH4Cl, DH5Cl, DH7Cl, DHD and DH1Cl) are found to be less active than cisplatin.

Table 4 .21 gives the IC_{90} values (i.e. drug concentration required for 90% cell kill) for the multinuclear compounds DH4Cl, DH5Cl, DH6Cl and DH7Cl as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538. The IC_{90} values for cisplatin are not given in the table 4.21 since the values of cisplatin could be determined only against three cell lines A2780, $A2780^{ZD0473R}$ and NCI-H640 and not for the other two cell lines $A2780^{cisR}$ and Me-10538 as the IC_{90} values were greater than the highest concentration used. For the cell lines A2780, $A2780^{ZD0473R}$ and NCI-H640, the IC_{90} values for cisplatin were 5.5 µM ± 0.3, 20.6 µM ± 0.6 and 19.3 µM ± 3.2 respectively.

Table 4. 21 IC_{90} for DH4Cl, DH5Cl, DH6Cl and DH7Cl against the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, Me-10538 and NCI-H640

IC90 (µM) ± SD, cell lines					
compound	A2780	$A2780^{cisR}$	$A2780^{ZD0473R}$	Me-10538	NCI-H460
DH4Cl	21.0 ± 2.9	22.9 ± 1.9	26.2 ± 0.5	30.9 ± 0.8	29.7 ± 0.6
DH5Cl	20.2 ± 1.9	25.9 ± 0.9	25.6 ± 1.8	26.6 ± 0.9	28.4 ± 1.5
DH6Cl	3.6 ± 0.8	17.2 ±1.3	20.1 ± 1.4	17.7 ± 2.6	23.2 ± 1.1
DH7Cl	22.1 ± 1.8	23.4 ± 0.9	26.3 ± 0.2	22.8 ± 2.2	26.8 ± 0.8

Based on the IC_{90} values also, it appears that DH6Cl is the most active compound. The variations in activities of the compounds from one cell line to another and due to

a change in the size of the linking diamine or due to whether the compound is dinuclear or trinuclear will be discussed in chapter 5.

4.3. CELL UPTAKE AND DNA BINDING

As stated before, the cell uptake and DNA binding in 4 h were determined for the multinuclear complexes: DH6Cl, DH5Cl, DH4Cl, DH7Cl, DHD in which cisplatin was used as the reference. For DH6Cl and cisplatin, cell uptake and DNA binding in 2 h were also evaluated as applied to the cell lines: A2780 and A2780^{cisR}. For DH4Cl, whereas cell uptake in 2 h was determined for both cell lines: A2780 and A2780^{cisR}, DNA binding in 2 h was determined only for A2780 cell line.

4.3.1. *CELL UPTAKE*

Generally, the cellular accumulation of platinum has been used as a measure of the cell uptake of compounds. Since the multinuclear complexes contain both platinum and palladium, the determination of palladium levels also should provide an equivalent measure of the drug uptake provided the compounds (more exactly the multinuclear cations) remain essentially intact before entry into the cell. A departure from the expected value for the molar ratio of Pt and Pd uptake (2 : 1 for DH4Cl, DH5Cl, DH6Cl and DH7Cl and 1 : 1 for DHD) can be taken as a measure of the extent of decomposition of the compounds. Thus in this study, cell uptake of palladium in 4 h was also determined to provide a measure of the extent of decomposition of the compounds.

Cellular uptakes of platinum and palladium were calculated as nmol Pt per $2x10^6$ cells and nmol Pd per $2x10^6$ cells respectively. Table 4.22 and figure 4.38 give the Pt cell uptake in the human ovary cell lines: A2780 and A2780^{cisR} in 4 h as applied to

DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin. Table 4.23 gives the Pt cell uptake in 2 h in A2780 and A2780^{cisR} cell lines as applied to DH6Cl, DH4Cl and cisplatin. Figure 4.39 gives the change in Pt cell uptake in 2 to 4 h in A2780 and A2780^{cisR} cell lines as applied to DH6Cl, DH4Cl and cisplatin. Table 4.24 and figure 4.40 give the Pd cell uptake in the human ovary cell lines: A2780 and A2780^{cisR} in 4 h as applied to DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD.

Table 4. 22 Pt cell uptake as nmol Pt per 2x10^6 cells in 4 h in A2780 and A2780^{cisR} as applied to cisplatin, DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Compound	A2780 nmol Pt/2x10^{6}cells	S.D.	A2780^{cisR} nmol Pt/2x10^{6}cells	S.D.
Cisplatin	0.048	± 0.004	0.028	± 0.005
DH4Cl	0.404	± 0.025	0.261	± 0.058
DH5Cl	0.499	± 0.058	0.250	± 0.027
DH6Cl	0.527	± 0.038	0.757	± 0.043
DH7Cl	0.676	± 0.040	0.286	± 0.036
DHD	0.160	± 0.036	0.272	± 0.010

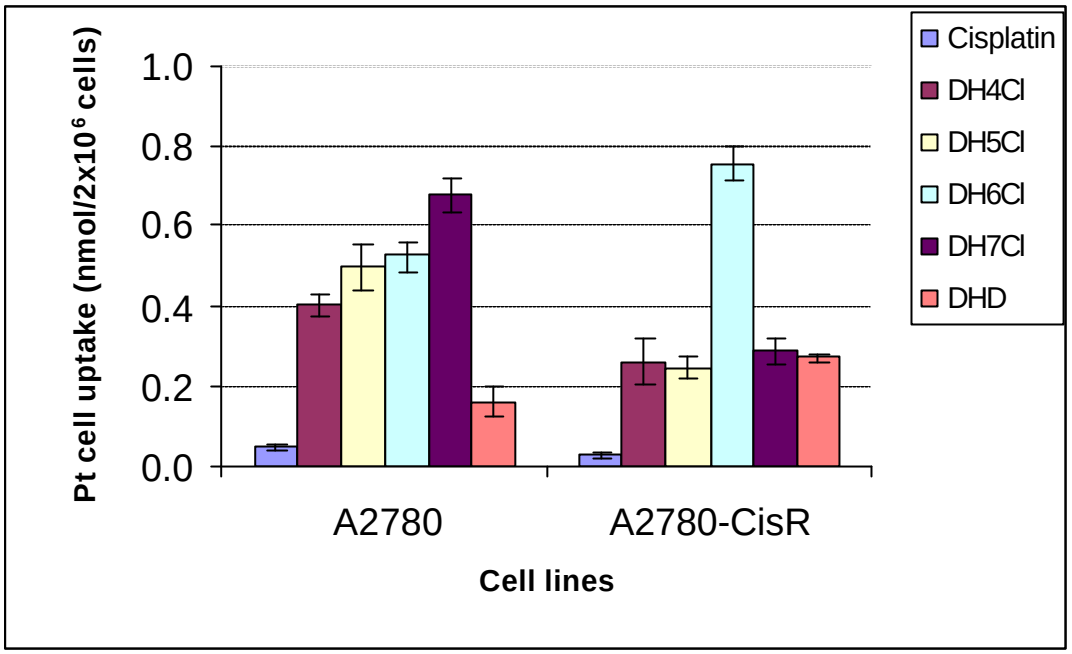


Figure 4. 38 Pt cell uptake as nmol Pt per $2x10^6$ cells in A2780 and $A2780^{cisR}$ cells in 4 h as applied to cisplatin, DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Table 4. 23 Pt cell uptake as nmol Pt per $2x10^6$ cells in A2780 and $A2780^{cisR}$ in 2 h as applied to cisplatin, DH4Cl and DH6Cl

Compound	A2780 nmol Pt/$2x10^6$cells	S.D.	$A2780^{cisR}$ nmol Pt/$2x10^6$cells	S.D.
Cisplatin	0.039	± 0.004	0.006	± 0.002
DH4Cl	0.222	± 0.008	0.150	± 0.010
DH6Cl	0.344	± 0.067	0.235	± 0.012

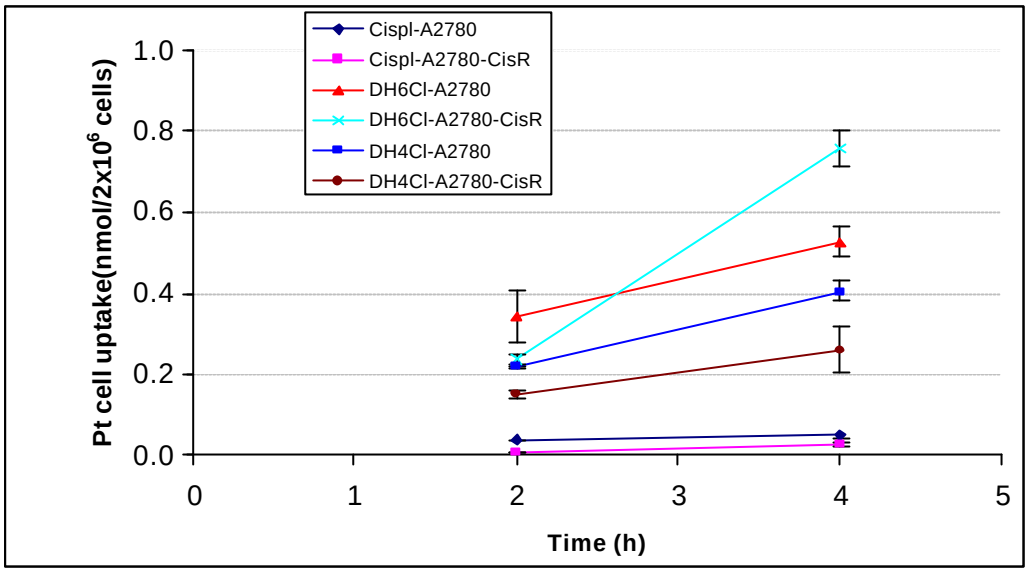


Figure 4. 39 Pt cell uptake in A2780 and A2780^{cisR} at 2 h and 4 h as applied to DH4Cl, DH6Cl and cisplatin

It is found that for all the multinuclear compounds DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD, the platinum cell uptake in A2780 and A2780^{cisR} cell lines is much greater than that for cisplatin. The actual order of the platinum cell uptake in A2780 cell line is: DH7Cl > DH6Cl > DH5Cl > DH4Cl > DHD > cisplatin.

In A2780^{cisR} cell line the order of the platinum cell uptake is: DH6Cl > DH7Cl > DHD > DH4Cl > DH5Cl > cisplatin. It should be noted that for DH6Cl and DHD, platinum cell uptake in the cisplatin–resistant cell line A2780^{cisR} is found to be greater than that in the cisplatin–responsive cell line A2780. For all other compounds, Pt cell uptake is found to be less in A2780^{cisR} cell line than in A2780 cell line. From tables 4.22, 4.23 and figure 4.39, it appears that whereas the platinum cell uptake of DH4Cl and cisplatin reaches saturation level in about 2 h, that of DH6Cl continues to increase with time and more so in A2780^{cisR} cell line than in A2780 cell line. It should be noted whereas cisplatin has one Pt per unit, all of the trinuclear compounds has two Pt per unit.

Table 4. 24 Pd cell uptake as nmol Pd per $2x10^6$ cells in 4 h in A2780 and $A2780^{cisR}$ as applied to DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Compound	A2780 nmol Pd /$2x10^6$cells	S.D.	A2780cisR A2780 Nmol Pd /$2x10^6$cells	S.D.
DH4Cl	0.183	± 0.016	0.156	± 0.047
DH5Cl	0.226	± 0.029	0.118	± 0.024
DH6Cl	0.309	± 0.061	0.402	± 0.071
DH7Cl	0.289	± 0.043	0.130	± 0.042
DHD	0.120	± 0.011	0.219	± 0.041

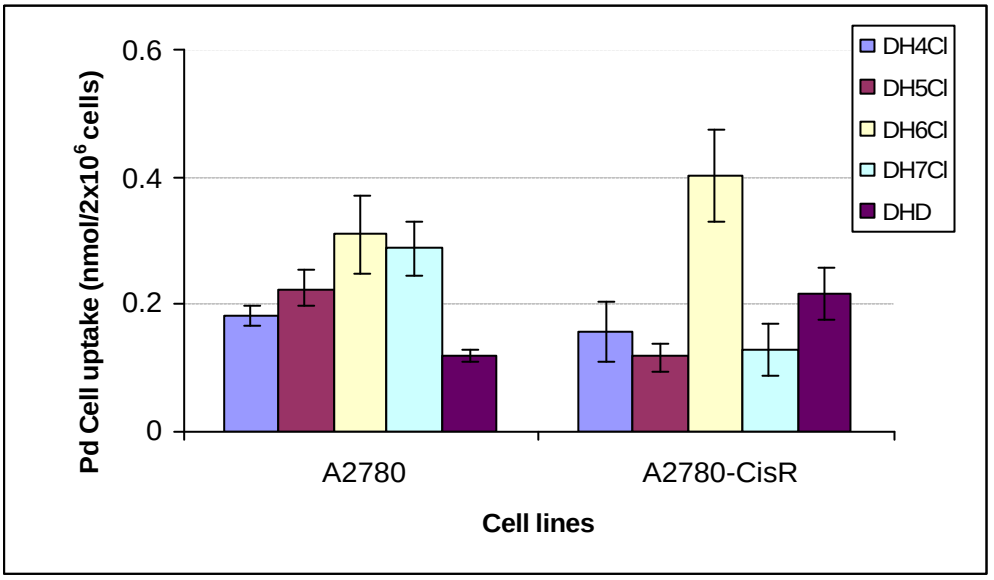


Figure 4. 40 Pd cell uptake as nmol Pd per $2x10^6$ cells in A2780 and $A2780^{cisR}$ cells in 4 h as applied to DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

It is found that the order of palladium cell uptake in A2780 cell line is: DH6Cl > DH7Cl > DH5Cl > DH4Cl >DHD indicating that the highest palladium uptake occurs in the case of DH6Cl. It has been noted earlier that the highest platinum uptake occurs

in the case of DH7Cl, which is slightly less than DH6Cl in case of palladium cell uptake.

In A2780^{cisR} cell, palladium uptake is highest for DH6Cl with the order of uptake of palladium in A2780^{cisR} cell line being DH6Cl > DHD > DH4Cl > DH7Cl >DH5Cl. When the uptake of palladium in A2780 cells is compared to that in A2780^{cisR} cells, it is also found that uptake of palladium in the cisplatin-resistant cell line is greater than that in the cisplatin-responsive cell line for DH6Cl and DHD and lower for DH4Cl, DH5Cl and DH7Cl. It has noted earlier that platinum uptake is also greater in A2780^{cisR} cell line than in A2780 cell line for DH6Cl and DHD but not for DH4Cl, DH5Cl and DH7Cl. The results will be discussed in chapter 5.

Table 4.25 gives the molar ratio of the platinum and palladium cell uptake as applied to DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD.

Table 4. 25 Molar ratio of the platinum and palladium cell uptakes in A2780 and A2780^{cisR} for DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Compounds	nmol Pt per $2x10^6$ /nmol Pd per $2x10^6$	
	A2780	A2780^{cisR}
DH4Cl	2.2	1.7
DH5Cl	2.2	2.1
DH6Cl	1.7	1.8
DH7Cl	2.3	2.2
DHD	1.3	1.2

It is found that the molar ratio of the cellular uptakes of platinum and palladium is close to the expected values of 2:1 in the case of DH4Cl, DH5Cl, DH6Cl and DH7Cl and 1:1 in the case of DHD. The result may also suggest the occurrence of a partial damage of the compounds. A more detailed discussion of the results will be given in chapter 5.

4.3.2. *DNA BINDING*

Table 4.26 and figure 4.41 give the level of platinum-DNA binding expressed as nanomoles of Pt per milligram of DNA in A2780 and A2780^{cisR} cells achieved in 4 h for the compounds DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin. Table 4.27 gives the platinum binding in 2 h for DH4Cl in A2780 cells and for DH6Cl and cisplatin in both A2780 and A2780^{cisR} cells. Figure 4.42 gives the change in platinum-DNA binding (expressed as nmol Pt per mg DNA) from 2 to 4 h in A2780 and A2780^{cisR} cell lines as applied to DH6Cl and cisplatin and in A2780 cell line only as applied to DH4Cl.

Table 4. 26 Level of Pt-DNA binding (expressed as nmol Pt per mg DNA) in 4 h in A2780 and A2780^{cisR} cells as applied to cisplatin, DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Compound	A2780 nmol Pt /mg DNA	S.D.	A2780cisR A2780 nmol Pt /mg DNA	S.D.
Cisplatin	0.195	± 0.068	0.110	± 0.011
DH4Cl	0.181	± 0.005	0.292	± 0.046
DH5Cl	0.867	± 0.197	0.831	± 0.127
DH6Cl	3.643	± 0.474	1.828	± 0.127
DH7Cl	1.427	± 0.330	0.580	± 0.046
DHD	0.418	± 0.104	0.302	± 0.033

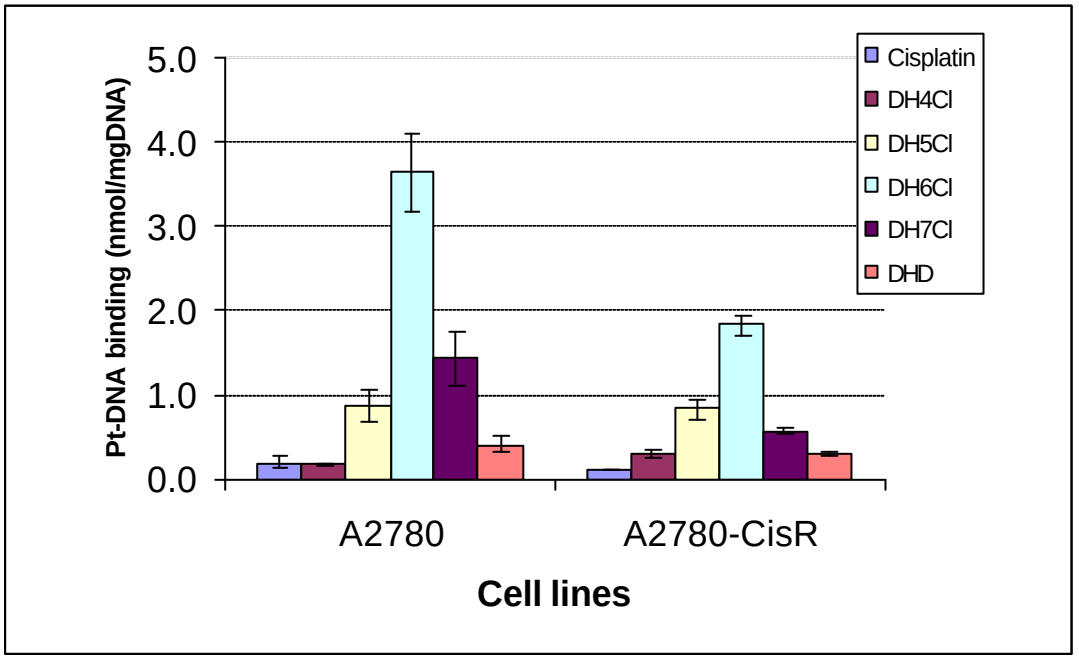


Figure 4. 41 Level of Pt-DNA binding expressed as nmol Pt per mg of DNA in A2780 and A2780^{cisR} cells in 4 h as applied to cisplatin, DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD

Table 4. 27 Level of Pt-DNA binding in 2 h (expressed as nmol Pt per mg of DNA) in A2780 and A2780^{cisR} cells as applied to DH6Cl and cisplatin and in A2780 cells only as applied to DH4Cl

Compound	A2780 nmol Pt/mg DNA	S.D	A2780^{cisR} nmol Pt/mg DNA	S.D
Cisplatin	0.106	± 0.014	0.000	± 0.000
DH4Cl	0.264	± 0.038		
DH6Cl	1.901	± 0.182	1.374	± 0.445

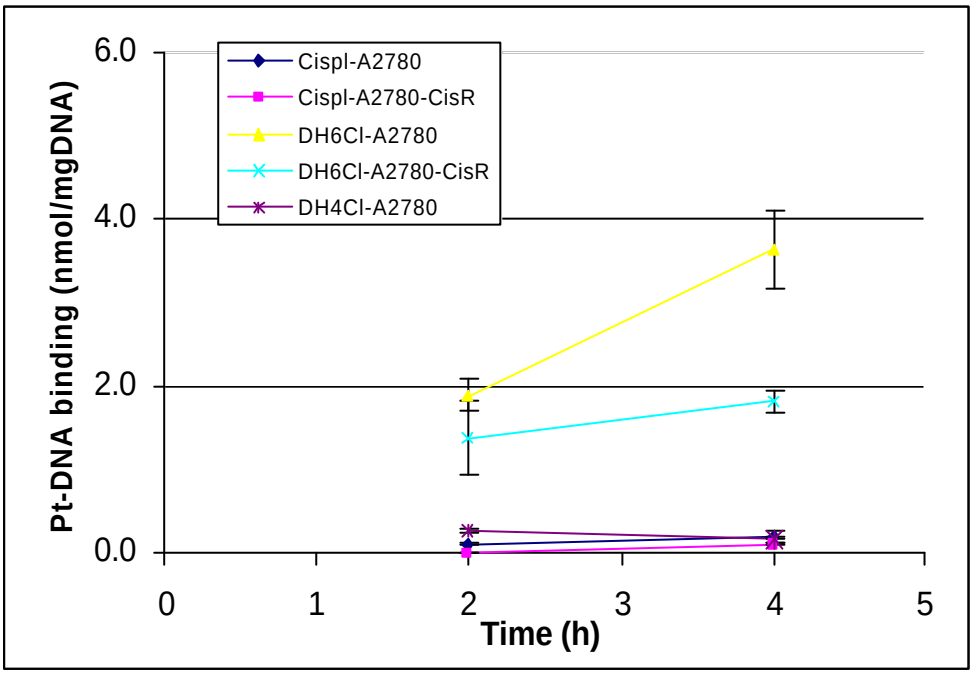


Figure 4. 42 Pt-DNA binding in A2780 and A2780^{cisR} in 2 h and 4 h as applied to cisplatin, DH6Cl and in A2780 as applied to DH4Cl

It is found that for all the compounds except DH4Cl, the level of platinum-DNA binding is less in the cisplain-resistant cell line A2780^{cisR} than in the cisplatin-responsive cell line A2780. It is also found that for all the multinuclear compounds except DH4Cl, the level of platinum-DNA binding is greater than that of cisplatin in

both A2780 and A2780^{cisR} cells. For DH4Cl, the value in A2780 cells is slightly less than that for cisplatin but significantly greater than that for cisplatin in A2780^{cisR} cells. The actual order of the level of platinum-DNA binding in A2780 cell line is: DH6Cl > DH7Cl >DH5Cl > DHD > cisplatin = DH4Cl and in A2780^{cisR} cell line it is: DH6Cl > DH5Cl >DH7Cl > DHD > DH4Cl > cisplatin.

It is found that for DH4Cl, the level of platinum-DNA binding decreases with the time from 2 to 4 h but that of DH6Cl continues to increase in both A2780 and A2780^{cisR} cells (more so in A2780 cells). For cisplatin also, the level of platinum-DNA binding is found to increase with the time but not as much as that for DH6Cl (especially applying to A2780 cells). It should be noted that the binding of cisplatin with DNA in A2780^{cisR} cells in 2 h could not be determined as it was below the detection threshold.

4.3.3. *GEL ELECTROPHORESIS*

Gel electrophoresis was used to investigate the conformational change and damage caused to both non-genomic DNA (pBR322 plasmid DNA) and genomic DNA (salmon sperm DNA) due to their covalent binding with the compounds.

4.3.3.1. INTERACTION WITH PBR322 PLASMID DNA

Figure 4.43 gives the electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin from 1.25 µM to 15 µM.

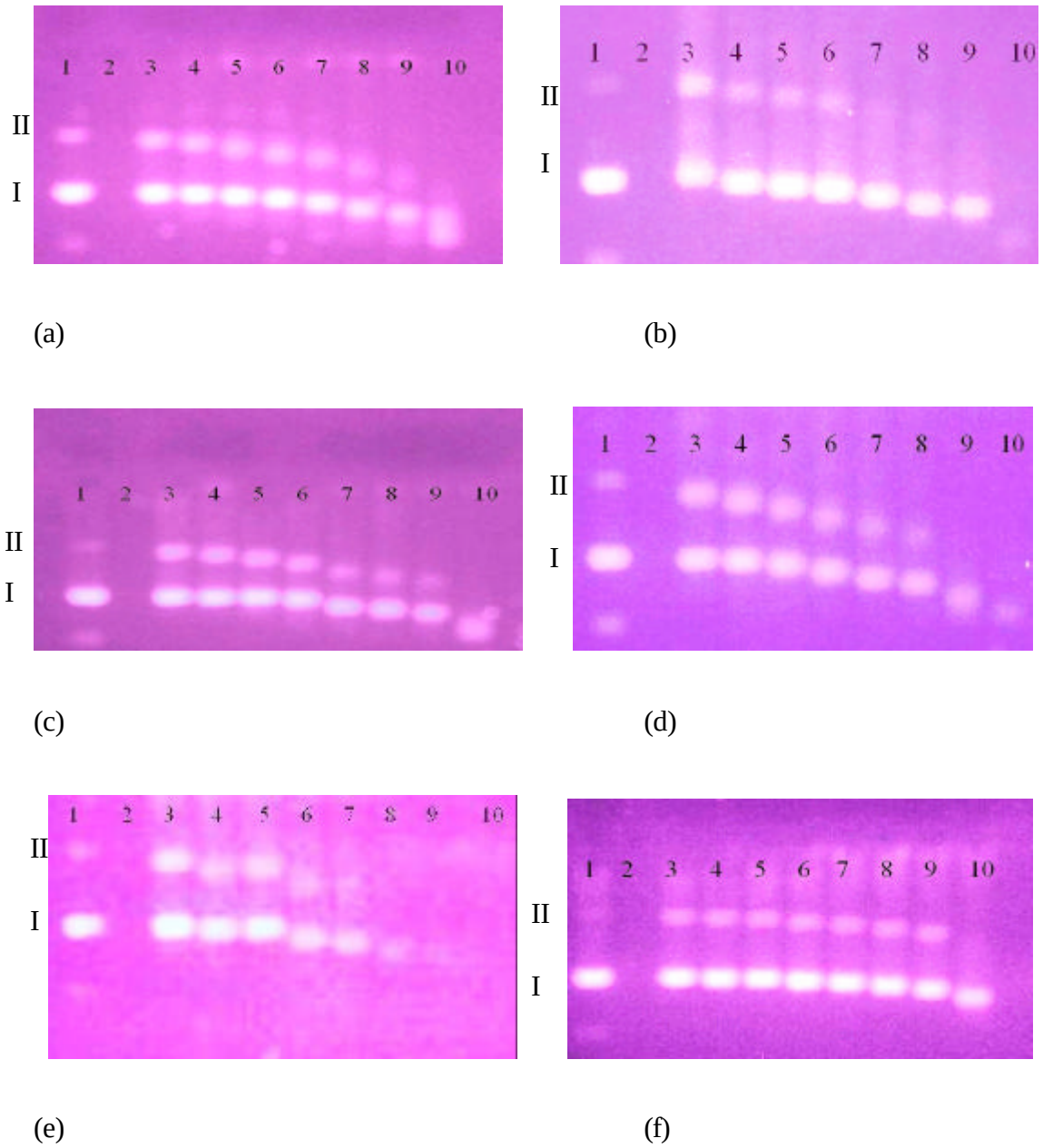


Figure 4. 43 Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of (a) DH4Cl, (b) DH5Cl, (c) DH6Cl, (d) DH7Cl, (e) DHD and (f) cisplatin

Lane 1 in the electrophoretograms applies to untreated pBR322 plasmid DNA to serve as a control, lane 2 blank, lanes 3 to 10 apply to plasmid DNA interacted with increasing concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin

(lane 3: 1.25 µM, lane 4: 1.88 µM, lane 5: 2.5 µM, lane 6: 3.75 µM, lane 7: 5 µM, lane 8: 7.5 µM, lane 9: 10 µM, lane 10: 15 µM). Cisplatin serves as a reference.

One highly prominent band corresponding to form I and a weak band were observed in unreacted pBR322 plasmid DNA. In addition, a weak frontal band was also observed in unreacted pBR322 plasmid DNA. As the plasmid DNA was allowed to interact with the increasing concentration of the compounds, the mobility of both the DNA bands increased but at different rates such that the separation between the two bands decreased. In fact, the two bands coalesced into one band at the concentration: 15 µM in the case of DH4Cl and DH6Cl and 10 µM in the case of DH7Cl. Coalescing of the two bands did not occur in the case of DH5Cl, DHD and cisplatin. As pBR322 plasmid DNA interacted with increasing concentrations of the compounds, besides the change in mobility changes in intensity of the DNA bands were also observed. At low concentrations of the compounds, the intensity of the weak band was found to be greater than in unreacted pBR322 DNA but at higher concentrations of the compounds there was a progressive decrease in intensity of the band such that it was not observable at concentration of DH5Cl= 10 µM, and concentration of DHD = 3.75 µM. Generally, the intensity of the prominent band found in unreacted pBR322 plasmid DNA also decreased with the increase in concentration of the compounds.

4.3.3.2. BamH1 DIGESTION

BamH1 digestion combined with gel electrophoresis was used to gain further insight into the binding of the compounds with pBR322 plasmid DNA. BamH1 is a restriction enzyme that is known to recognize the G/GATCC and hydrolyse phosphodiester bond between adjacent guanine sites (Roberts *et al.* 1977) pBR322 plasmid DNA contains a single restriction site for BamH1(Sutcliffe 1979) that

converts pBR322 plasmid DNA supercoiled form I and single nicked circular form II to linear form III DNA. However, when platinum compounds at increasing concentrations bind to guanines in the DNA, BamH1 digestion may be increasingly prevented.

Figure 4.44 gives the electrophoretograms applying to incubated mixtures of pBR322 plasmid DNA and varying concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD, and cisplatin that were digested with BamH1 for a period of 1 h at 37ºC before they were subjected to electrophoresis. The concentrations of the compounds were varied from 1.87 μM to 15 μM and in case of cisplatin a still higher concentration (namely 20 μM) was used, whilst that of the DNA was kept constant.

Lane 1 applies to the untreated pBR322 plasmid DNA digested with BamH1, lane 2: blank, lanes 3 to 7: apply to pBR322 plasmid DNA interacted with increasing concentrations of compounds (1.87 μM, 2.5 μM, 5 μM, 10 μM, 15 μM respectively) followed by BamH1 digestion, lane 8: blank and lane 9 applies to untreated and undigested pBR322 plasmid DNA. When unreacted pBR322 plasmid DNA was digested with BamH1, only one band corresponding to form III band was observed. In the untreated and undigested pBR322 plasmid DNA, generally two bands corresponding to form I and form II were observed.

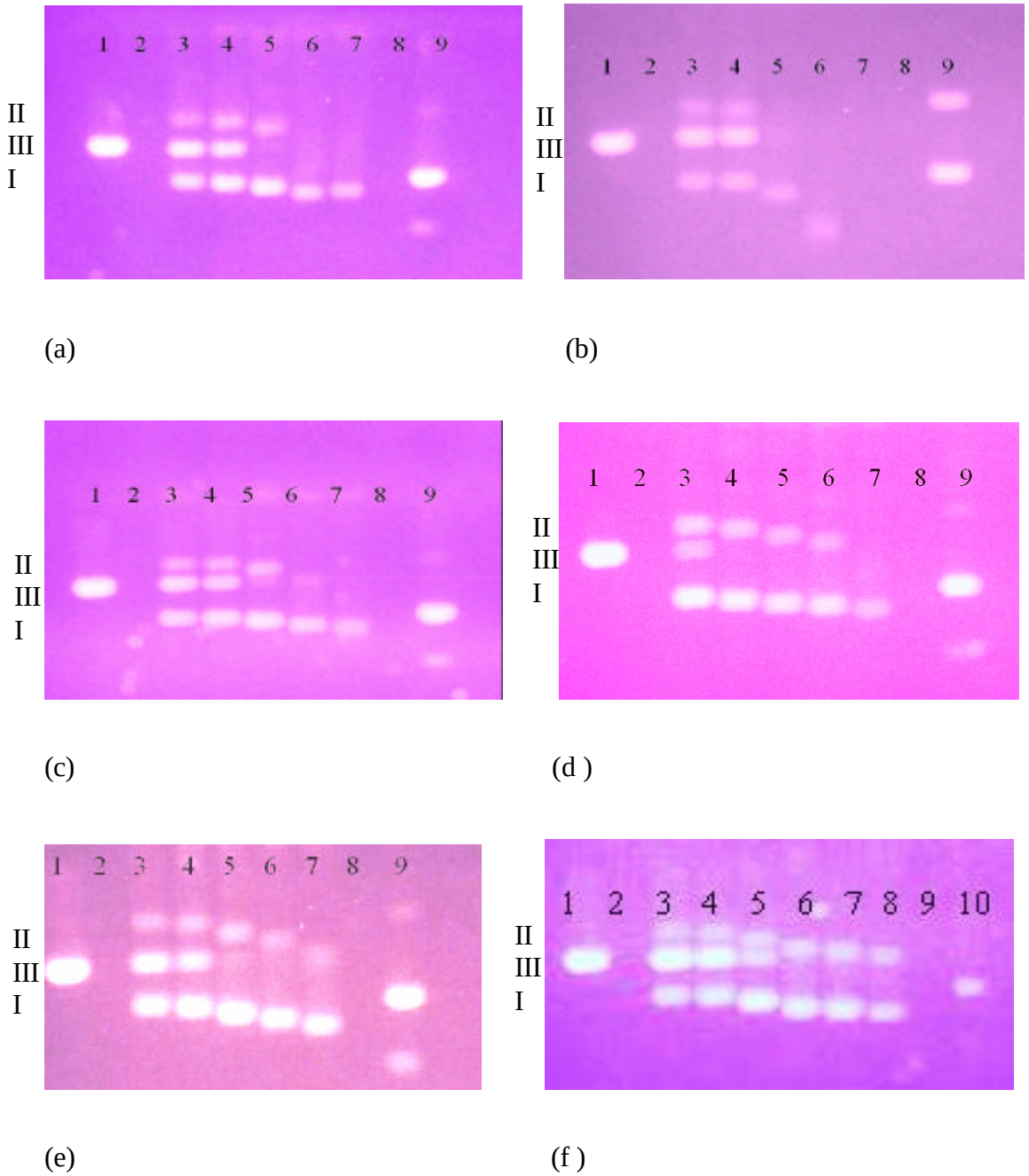


Figure 4. 44 Electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of compounds: (a) DH4Cl, (b) DH5Cl, (c) DH6Cl, (d) DH7Cl, (e) DHD and (f) cisplatin, followed by their digestion with BamH1

When the incubated mixtures of pBR322 plasmid DNA and increasing concentrations of DH4Cl ranging from 1.87 µM to 15 µM were digested with BamH1 (Figure 4.44 a), three bands corresponding to forms I, II and III were observed for concentrations of DH4Cl ranging from 1.87 µM to 2.5 µM, two bands corresponding to forms I and II were observed at concentration of DH4Cl = 5 µM, and only one band corresponding to form I was observed at concentration of DH4Cl = 10 µM.

In the case of DH5Cl (Figure 4.44 b), forms I, II and III bands were observed for concentrations of DH5Cl ranging from 1.87 µM to 2.5 µM above which only form I band was observed whose mobility increased sharply with the increase in DH5Cl concentration.

In the case of DH6Cl (Figure 4.44 c), three bands corresponding to forms I, II and III were observed for concentrations of DH6Cl ranging from 1.87 µM to 2.5 µM. Forms I and II bands were observed for concentrations of DH6Cl ranging from 5 µM to 10 µM and only form I band was observed at concentration of DH6Cl = 15 µM. In the case of DH7Cl (Figure 4.44 d), three bands corresponding to form I, II, III were observed at concentration of DH7Cl = 1.87 µM, two bands corresponding to forms I and II were observed for concentrations of DH7Cl ranging from 2.5 µM to 10 µM and only the form I band was observed at concentration of DH7Cl = 15 µM. In the case of DHD (Figure 4.44 e), three bands corresponding to form I, II, III were observed at concentrations of DHD ranging from 1.87 µM to 2.5 µM, and two bands corresponding to forms I and II were observed at concentrations of DHD ranging from 5 µM to 15 µM. In the case of cisplatin (Figure 4.44 f), three bands corresponding to forms I, II, III were observed for concentrations of cisplatin ranging from 1.87 µM to 5 µM, two bands corresponding to forms I and II were observed for

concentrations of cisplatin ranging from 10 µM to 20 µM as one more concentration of cisplatin was added. Table 4.28 summarises the above results.

Table 4. 28 Bands observed in the incubated mixtures of pBR322 plasmid DNA and varying concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin followed by BamH1 digestion

Drug	[Drug] in µM						
	0	1.87	2.5	5	10	15	20
DH4Cl	III	I, II, III	I, II, III	I, II	I	I	-
DH5Cl	III	I, II, III	I, II, III	I	I		-
DH6Cl	III	I, II, III	I, II, III	I, II	I	I	-
DH7Cl	III	I, II, III	I, II	I, II	I, II	I	-
DHD	III	I, II, III	I, II, III	I, II	I, II	I, II	-
cisplatin	III	I, II, III	I, II, III	I, II, III	I, II	I, II	I, II

4.3.3.3. INTERACTION BETWEEN THE COMPOUNDS AND SALMON SPERM DNA (ssDNA)

Salmon sperm DNA is a double-stranded genomic DNA having molecular mass ranging from 0.6 to 0.8 kilo base.

Figure 4.45 gives the electrophoretograms applying to the incubated mixtures of ssDNA and varying concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD, and cisplatin ranging from 5 µM to 60 µM.

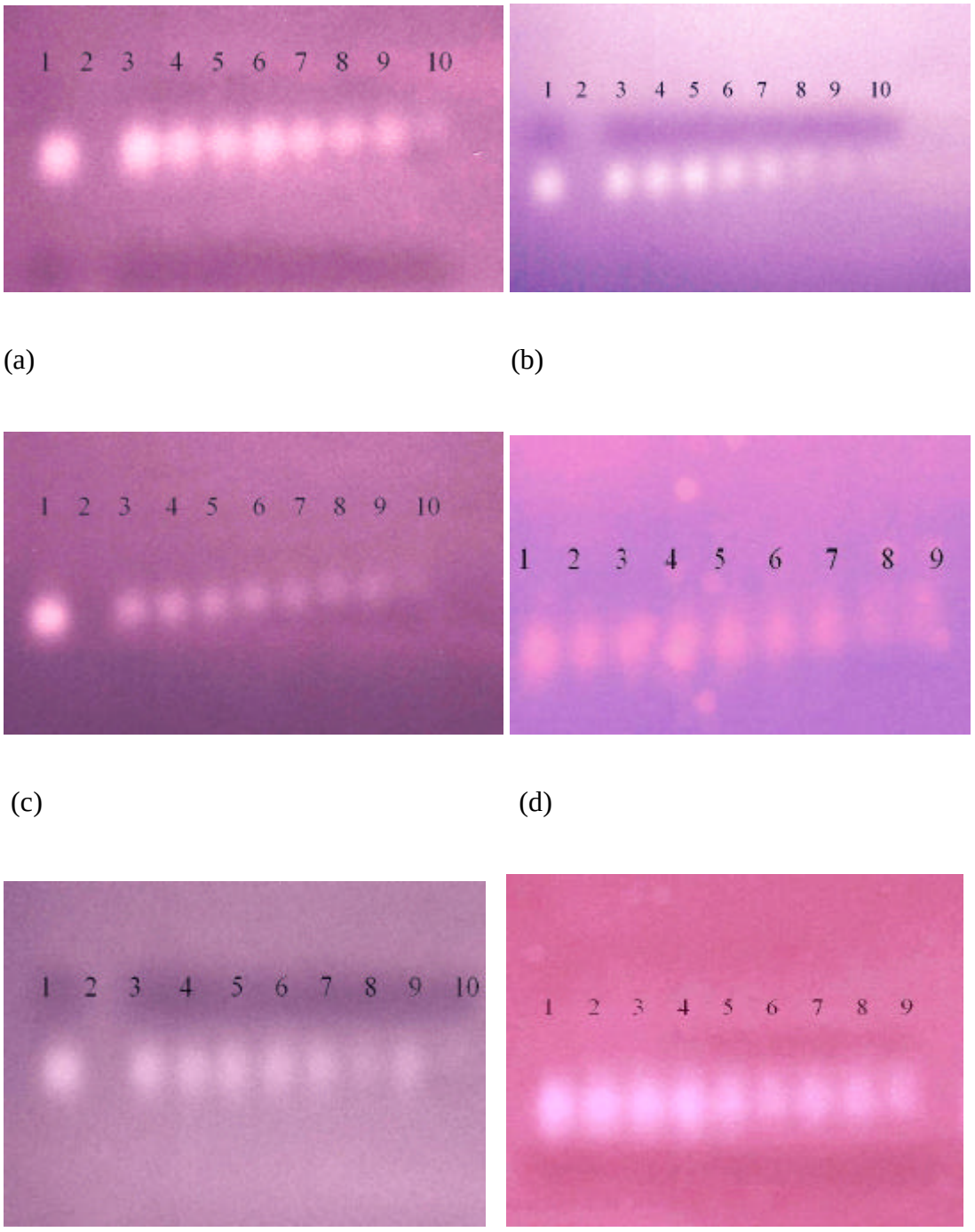


Figure 4. 45 Electrophoretograms applying to the interaction of ssDNA with increasing concentrations of (a) DH4Cl, (b) DH5Cl, (c) DH6Cl, (d) DH7Cl, (e) DHD and (f) cisplatin

In the electrophoretograms (a), (b), (c) and (e), lane 1 applies to untreated ssDNA as control, lane 2 blank, lanes 3 to 10 apply to ssDNA interacted with increasing concentrations of DH4Cl, DH5Cl, DH6Cl and DHD (lane 3: 5 µM, lane 4: 7.5 µM, lane 5: 10 µM, lane 6: 15 µM, lane 7: 20 µM, lane 8: 30 µM, lane 9: 40 µM, lane 10: 60 µM). In electrophoretograms (d) and (f), lane 1 applies to untreated ssDNA, lanes 2 to apply ss DNA interacted with increasing concentrations of DH7Cl and cisplatin (lane 2: 5 µM, lane 3: 7.5 µM, lane 4: 10 µM, lane 5: 15 µM, lane 6: 20 µM, lane 7: 30 µM, lane 8: 40 µM, lane 9: 60 µM).

A single band was observed in both untreated and reacted salmon sperm DNA. In general, as the concentration of the compounds was increased the intensity of the band was found to decrease. The mobility of the band also decreased slightly with the increase in concentration of the compounds. The change in mobility and intensity was found to be least in the case of DHD and cisplatin and most prominent in the case of DH6Cl.

4.4. INTERACTION BETWEEN DH6Cl AND NUCLEOBASES

4.4.1. *HPLC*

As stated in chapter four, 1 mM solution of DH6Cl was mixed with an equal volume of 2 mM solution of guanine or adenine following which the mixtures were incubated for 24 h at 37°C in a shaking water bath. 5 µL of each of the incubated mixtures was injected into the HPLC system as was done for the solutions of DH6Cl and the nucleobases.

The retention times of the main peaks with the detector set at ? = 260 nm for the mixtures and the components are shown in table 4.29.

Table 4. 29 The retention times of the main peaks with the detector set at ? = 260 nm for the mixtures and the components

Reactant A	Reactant B	Retention time (min), major peak 1	Retention time (min), major peak 2	Retention time (min), minor peak
Adenine	None	6.13	-	-
Guanine	None	3.10	-	-
None	DH6Cl	2.67	3.91	
Adenine	DH6Cl	2.83	3.72	2.98
Guanine	DH6Cl	2.69	3.65	2.99

4.4.2. *BINDING RATIO*

The two major peak fractions in the incubated mixtures of DH6Cl and adenine had the retention times of 2.83 and 3.72 min and those in the incubated mixtures of DH6Cl and guanine had the retention times of 2.69 and 3.65 min. To determine the Pt : NB binding ratio, the peak fractions were collected and their platinum and nucleobase contents were determined by graphite furnace AAS and UV-visible spectrophotometry respectively. The minor peak fraction at 2.98 min found in the

incubated mixture of DH6Cl and adenine and that at 2.99 min found in the incubated mixture of DH6Cl and guanine could not be collected because of a small separation from the neighbouring peaks.

Table 4. 30 Pt : NB binding ratios of the major peaks in the incubated mixtures of DH6Cl with adenine and guanine

Mixture / retention time	Absorbance of the fraction at 260 nm	Concentration of NB in the fraction (mol L^{-1})	Pt content in the fraction (ppb)	Pt concentration (mol L^{-1})	Pt : NB
DH6Cl + Adenine / 3.72 min	0.0308	0.000002265	22	0.00000169	0.75
DH6Cl + Adenine / 2.83 min	0.1844	0.00001356	5	0.00000038	0
DH6Cl + Guanine / 3.65 min	0.01186	0.000001467	17	0.000001307	0.90
DH6Cl + Guanine / 2.69 min	0.0868	0.00001074	6	0.00000046	0

The molar extinction coefficient (e) for guanine adenine at 260 nm were found to be 8080 and 13596 respectively.

CHAPTER FIVE

5. DISCUSSION

In this study, a number of multinuclear complexes containing platinum and palladium had been prepared, characterized based on elemental analyses and spectral measurements, and evaluated for their activity against cancer cell lines. The cell uptake, extent and nature of binding with DNA of the compounds had also been determined.

5.1. CHARACTERIZATION OF COMPOUNDS

The trinuclear complexes: DH4Cl, DH5Cl, DH6Cl and DH7Cl and the dinuclear compound: DHD were synthesized using step up method of syntheses (starting with transpalladin) and characterized by elemental analyses, IR, Raman, mass and ^{1}H NMR spectral studies.

In the case of trinuclear complexes, the size of the linking alkyl diamine was varied to contain from four to seven carbon atoms. In the case of DHD, the linking diamine was 1,6-diaminohexane. Although the methods of syntheses of the compounds (given in chapter 3) were essentially the same, the actual method used varied in detail depending on the size and state (i.e. whether used as a free base or in the form of hydrochloride) of the diamine and whether the compound was dinuclear or trinuclear. It was found that the size of the diamine influenced the solubility of the trinuclear compounds. Whereas DH4Cl in which the linking diamine was 1,4-diaminobutane is

soluble in water, DH6Cl (in which the linking diamine was 1,6-diaminohexane) and DH7Cl (in which the linking diamine was 1,7-diaminoheptane) are insoluble in water. DH6Cl is soluble in DMF and DH7Cl is almost soluble in DMF. DH5Cl (in which the linking diamine was 1,5-diaminopentane) is soluble in pure DMF but not in mixture of DMF and water. However, the solubility of DH5Cl in water increased significantly as the solution was made basic (pH = 10). DHD is insoluble in water but soluble in DMF. Although in both DHD and DH6Cl, the linking diamine was 1,6-diaminohexane, it is found that DHD was more soluble in DMF than DH6Cl. This could be due to a difference in size of the molecules and the number of metal centres. All of the compounds were found to be soluble in DMSO. It was observed by Farrell and co-workers (Farrell *et al.* 1990b; Farrell 1995) that the solubility in water of dinuclear compounds can be increased by replacing the chloride leaving group with water-solubilizing groups such as malonate. However the potency of malonate compounds was found to be lower than that of chloride containing compounds (Roberts *et al.* 1989; Kraker *et al.* 1992).

5.1.1. *ELEMENTAL ANALYSES*

The result of elemental analysis show that the purity attained for DH4Cl was about ninety five percent with a yield of fifty one percent. The purity of DH5Cl was about ninety three for which the yield was about thirty two percent. The purity of DH6Cl was about ninety five percent with a yield of about forty nine percent. The purity of DH7Cl was about ninety five percent with a yield about forty percent. For the dinuclear compound (DHD), the purity was about ninety five percent with a yield of about sixty percent. It can be seen that the yield was lowest for DH5Cl and DH7Cl. For the two compounds, repeated dissolution and precipitation were carried out to improve the purity. Even after repeated precipitation, none of the compounds could be

obtained in a state of extremely high purity. The major problem with repeated dissolution and precipitation to increase purity was that of progressively decreasing yield. Other investigators also found that it was difficult to obtain multinuclear complexes (especially trinuclear ones) in a state of high purity. One reason for the relatively low purity could be that some other compounds were formed at the same time. For example, in the synthesis of trinuclear complexes, some dinuclear and mononuclear complexes could also be formed. The linking diamine being a bidentate ligand, can easily form a chelate with a metal ion, thus producing mononuclear complexes (Qu and Farrell 1992; Farrell 1995). Another reason for relatively low purity could be due to co-precipitation of other molecules such as DMF or dichloromethane. Further purification of the compounds could have been attempted by using other physical methods such as high pressure liquid chromatography (Rauter *et al.* 1997) or by chromatography on a silica gel (Quiroga *et al.* 1999)

5.1.2. *SPECTRAL STUDIES*

5.1.2.1. INTERPRTATION OF IR AND RAMAN SPECTRAL DATA

The interpretation of the bands observed in IR and Raman spectra has been based mainly on published spectra (Silverstein *et al.* 1991; Nakamoto 1997)

DH4Cl

IR

The bands at 3302 and 3209 cm^{-1} are believed to be due to N-H stretch that are in agreement with previously published values (Broomhead *et al.* 1992; Zhao *et al.* 1998b). The bands at 2931 and 2850 cm^{-1} are due to asymmetrical and symmetrical

CH_2 stretching vibrations. The bands at 1709, 1581 and 1456 cm^{-1} are believed to be due to N-H bending vibrations whereas those at 1379 and 1290 cm^{-1} are due to C-H bending vibration. The band at 1038 cm^{-1} is due to CH_2 wagging. The band at 972 cm^{-1} is believed to be due to C-C stretch. The bands at 806 and 754 cm^{-1} are believed due to N-H wagging. The band at 494 cm^{-1} is due to Pt-N stretching vibration.

Raman

The band at 3209 cm^{-1} is due N-H stretching vibration whereas those at 2899 and 2856 cm^{-1} are believed to be due to C-H stretching vibrations. The bands at 1445, 1441 cm^{-1} are due to N-H bending vibrations. The band at 1293 cm^{-1} is believed to be due to C-H wagging vibration. The band at 1046 cm^{-1} is due to C-C stretch. The band at 747 cm^{-1} is due to the C-H out of plane bending vibration. The band at 533 cm^{-1} is due to Pt-N stretching vibration that is in agreement with the previously published value of 535 cm^{-1} (Ali *et al.* 1999) and that at 486 cm^{-1} is due to Pd-N stretching vibration. The band at 322 cm^{-1} is due to Pt-Cl stretching vibration, that is in agreement with previously published values ranging from 319 to 350 cm^{-1} (Farrell *et al.* 1990a; Broomhead *et al.* 1992; Qu *et al.* 1992; Schuhmann *et al.* 1995; Onoa *et al.* 1999). The band at 213 cm^{-1} is due to Pt-N and Pd-N bending vibrations. The band at 83 cm^{-1} is believed to be associated with lattice mode

DH5Cl

IR

The bands at 3267, 3222 and 3145 cm^{-1} are due to N-H stretch whereas those at 2951, 2927 and 2856 cm^{-1} are due to CH_2 stretching vibrations. The bands at 1577, 1448, 1392 and 1292 cm^{-1} are due to N-H bending vibrations whereas those at 1190, 1072 and 1014 cm^{-1} are due to CH_2 bending vibrations. The band at 966 cm^{-1} is believed to

be due to C-C stretch. The band at 804 cm^{-1} is believed due to N-H wagging. The band at 714 is due to C-H out of plane bending vibration. The bands at 482 and 418 cm^{-1} are due to Pt-N and Pd-N stretching vibrations.

Raman

The band at 3221 and 3146 cm^{-1} are due N-H stretching vibration whereas those at 2916 and 2855 cm^{-1} are believed to be due to C-H stretching vibrations. The band at 1441 cm^{-1} is due to N-H bending vibration. The band at 1077 cm^{-1} is due to C-C stretch. The band at 683 cm^{-1} is due to C-H out of plane bending vibration. The band at 533 cm^{-1} is due to Pt-N stretch and that at 488 cm^{-1} is due to Pd-N stretch. The bands at 307 and 294 cm^{-1} are due to Pt-Cl stretching vibrations whereas that at 195 cm^{-1} is due to Pt-N and Pd-N bending vibrations. The band at 83 cm^{-1} is believed to be associated with lattice mode.

DH6Cl

IR

The band at 3950 cm^{-1} is believed to be due to aliphatic C-H stretch combination band. The bands at 3255 and 3211 cm^{-1} are due to N-H stretch whereas those at 2925 and 2854 cm^{-1} are due to CH_2 stretching vibrations. The bands at 1745 and 1576 cm^{-1} are due to N-H bending vibrations whereas that at 1336 cm^{-1} is due to C-H bending vibration. The band at 1049 cm^{-1} is due to CH_2 wagging. The band at 995 cm^{-1} is believed to be due to C-C stretch. The bands at 825, 723 and 619 cm^{-1} are believed due to N-H wagging. The bands at 503 and 488 cm^{-1} are due to Pt-N and Pd-N stretching vibrations.

Raman

The band at 3217 cm^{-1} is due N-H stretching vibration whereas those at 2903 and 2855 cm^{-1} are believed to be due to C-H stretching vibrations. The band at 1441 cm^{-1} is due to N-H bending vibration. The band at 1190 cm^{-1} is believed to be due to C-H bending vibration whereas that at 1051 cm^{-1} is due to CH_2 wagging. The band at 716 cm^{-1} is due to N-H wagging. The bands at 596 and 533 cm^{-1} are due to Pt-N and Pd-N stretching vibrations. The band at 322 cm^{-1} is due to Pt-Cl stretching vibration whereas that at 209 cm^{-1} is due to Pt-N and Pd-N bending vibrations. The band at 81 cm^{-1} is believed to be associated with lattice mode

DH7Cl

IR

The bands at 3302, 3219 and 3138 cm^{-1} are due to N-H stretch whereas those at 2925 and 2852 cm^{-1} are due to CH_2 stretching vibrations. The bands at 1583, 1331 and 1290 cm^{-1} are due to N-H bending vibrations whereas those at 1074 and 1038 cm^{-1} are due to CH_2 bending vibrations. The band at 995 cm^{-1} is believed to be due to C-C stretch. The band at 806 cm^{-1} is believed due to N-H wagging. The band at 721 cm^{-1} is due to C-H out of plane bending vibration. The bands at 501 and 403 cm^{-1} are due to Pt-N and Pd-N stretching vibrations.

Raman

The bands at 3213 and 3140 cm^{-1} are due N-H stretching vibrations whereas those at 2900 and 2855 cm^{-1} are believed to be due to C-H stretching vibrations. The band at 1599 cm^{-1} is due to N-H bending vibration. The band at 1296 cm^{-1} is believed to be due to C-H wagging vibration. The band at 721 cm^{-1} is due to N-H wagging. The

bands at 596 and 533 cm^{-1} are due to Pt-N and Pd-N stretching vibrations. The band at 322 cm^{-1} is due to Pt-Cl stretching vibration whereas that at 213 cm^{-1} is due to Pt-N and Pd-N bending vibrations. The band at 83 cm^{-1} is believed to be associated with lattice mode.

DHD

IR

The band at 3975 cm^{-1} is believed to be due to aliphatic C-H stretch combination band. The bands at 3290 and 3213 cm^{-1} are due to N-H stretch whereas those at 2924 and 2852 cm^{-1} are due to CH_2 stretching vibrations. The bands at 1743 and 1576 cm^{-1} are due to N-H bending vibrations whereas that at 1288 cm^{-1} is due to C-H bending vibration. The band at 1068 cm^{-1} is due to CH_2 wagging. The band at 993 cm^{-1} is believed to be due to C-C stretch. The bands at 823 and 725 cm^{-1} are believed due to N-H wagging. The bands at 501 and 420 cm^{-1} are due to Pt-N and Pd-N stretching vibrations.

Raman

The band at 3213 cm^{-1} is due N-H stretching vibration whereas those at 2897 and 2856 cm^{-1} are believed to be due to C-H stretching vibrations. The band at 1439 cm^{-1} is due to N-H bending vibration. The band at 1192 cm^{-1} is believed to be due to C-H bending vibration whereas that at 1044 cm^{-1} is due to CH_2 wagging. The band at 708 cm^{-1} is due to N-H wagging. The bands at 589 and 533 cm^{-1} are due to Pt-N and Pd-N stretching vibrations. The band at 322 and 311 cm^{-1} are due to Pt-Cl and Pd-Cl stretching vibrations that are in agreement with previously published value for Pd-Cl stretching vibration ranging from 304 to 319 cm^{-1} (Zhao *et al.* 1998a; Zhao *et al.*

1999). The band at 211 cm^{-1} is due to Pt-N and Pd-N bending vibrations. The band at 83 cm^{-1} is believed to be associated with lattice mode

5.1.2.2. MASS AND ^{1}H NMR SPECTRAL ANALYSES

The mass and ^{1}H NMR spectra of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD were given in chapter 4 (Figures 4.9, 4.12, 4.15, 4.18, 4.21 and Table 4.7). The assignment of the peaks and related discussions are given in this section. It is found that the mass spectra of the compounds are characterized by the presence of a large number of peaks. This is due to the many ways in which the molecules (all of which are large and flexible) can fragment and also the fragments can rejoin in situ. Some peaks in the mass spectra may correspond to more than one species. The interpretation of bands observed in mass and ^{1}H NMR has been based on published spectra (Silverstein *et al.* 1991; Berners-Price and Sadler 1996; Gottlieb *et al.* 1997; Macomber 1998).

Mass spectrum of DH4Cl

It can be seen that the mass spectrum of DH4Cl has a small peak with m/z equal to 988 that corresponds to M^+. The presence of M^+ peak indicates that in solution in the mixture of 10% DMF and 90% methanol, DH4Cl can exist as undissociated ionic aggregates. The molar conductivity value of solution of DH4Cl in 1:1 mixture of DMF and water also shows that the compound remains largely undissociated in the solution. Other solutions of multinuclear complexes namely DH5Cl, DH6Cl, DH7Cl and DHD were also found to have low molar conductivity values again suggesting that the compounds remained largely undissociated in solution in 1:1 mixture of DMF and water (or in DMF in the case of DH5Cl).

The peak with m/z = 917 corresponds to (M – 2Cl + H), that at 824 corresponds to (M – 4Cl – NH_3 -5H), that at 795 corresponds to (M – 4Cl – $3NH_3$), that at 761 corresponds to (M – 4Cl – $5NH_3$), that at 493 corresponds to $Cl(NH_3)_2$Pt-*m*-$\{NH_2(CH_2)_4NH_2\}Pd(NH_3)_2$, that at 319 corresponds to $ClPt\{NH_2(CH_2)_4NH_2\}$ and that at 248 corresponds to $Cl(NH_3)Pd\{NH_2(CH_2)_6NH_2\}$. It can be seen that the peaks observed in the mass spectrum of DH4Cl provide support for its suggested structure.

^{1}H NMR spectrum of DH4Cl

It can be seen that ^{1}H NMR spectrum of DH4Cl gives a broad proton resonance with chemical shift value of 3.9 ppm. This is believed to be due to NH_2 and NH_3 bonded to Pt and Pd. The peaks at δ = 2.0 and 2.8 ppm are believed to be due to CH_2 protons away from NH_2 group whereas that at δ = 3.7 ppm is believed to be due CH_2 protons adjacent to NH_2 group. Two small broad peaks at δ = 2.3 and 2.4 ppm could be due to some kind of impurity that has not been characterized.

Mass spectrum of DH5Cl

A small peak observed in the mass spectrum of DH5Cl with m/z = 1015 corresponds to M^+, that at 944 corresponds to (M – 2Cl), that at 855 corresponds to (M – 4Cl – NH_3 – H), that at 624 corresponds to (M – Pt – $2NH_3$ – 5Cl – 2H), that at 540 does not directly correspond to any molecular fragment of DH5Cl. It may be due to $(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pt(NH_2)$ formed in situ. The peak with m/z = 524 corresponds to $Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_3)_3$. The peak with m/z = 487 may be due to $Cl(NH_2)Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_2)_2$, that at 451 corresponds to $(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_5NH_2\}Pd(NH_3)$. The peak with m/z = 211 may be due to ($Pd\{NH_2(CH_2)_5NH_2\}$ + H). It can be seen that the peaks observed in the mass spectrum of DH5Cl provide support for the suggested structure of DH5Cl. A more conclusive proof of the structure of the compound may be obtained when we combine the above results with ^{1}H NMR spectral data and molar conductivity value.

^{1}H NMR spectrum of DH5Cl

The ^{1}H NMR spectrum of DH5Cl gives a broad proton resonance with chemical shift value of 4.4 ppm. This is believed to be due to NH_3 protons. The other broad peak at δ = 4.0 ppm is believed to be due to NH_2 protons. The sizes of the peaks at δ = 4.4 and 4.0 ppm appear to be in the ratio of the number of NH_3 and NH_2 protons (18:8). The peak at δ = 3.6 ppm is believed to be due to CH_2 protons adjacent to NH_2 group whereas those at δ = 2.4 and 2.2 ppm are believed to be due CH_2 protons away from the NH_2 group. The peaks at δ = 1.3 ppm is due to the protons that lie in the middle of the carbon chain. The small peak at δ = 1.6 ppm may be due some impurity present.

Mass spectrum of DH6Cl

The mass spectrum of DH6Cl has a small peak with m/z = 1043 that corresponds to M^+. The peak with m/z = 972 corresponds to (M – 2Cl), that at 851 corresponds to (M – 4Cl – $3NH_3$). The peak at 707 corresponds to $Cl(NH_3)$-Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)(NH_2)\{NH_2(CH_2)_6NH_2\}$ which is believed to be formed in situ from joining of fragments. The peak with m/z = 553 corresponds to $Cl(NH_2)_2$ Pt -μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)(NH_2)$. The peak at 504 may be due to $(NH_3)_2$Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pd(NH_3)Cl$ and that at 380 corresponds to $Cl(NH_3)(NH_2)$Pt $\{NH_2(CH_2)_6NH_2\}$. The peak at m/z 311 may be due to $Pt\{NH_2(CH_2)_6NH_2\}$. The peak at 223 corresponds to ($Pd\{NH_2(CH_2)_6NH_2\}$ + H). It can be seen that the peaks observed in the mass spectrum of DH6Cl provide support for the suggested structure of the compound. Once again, a more conclusive proof for the structure may be obtained when the above results are combined with other ^{1}H NMR, IR and Raman spectral data, molar conductivity value and elemental composition.

^{1}H NMR spectrum of DH6Cl

The ^{1}H NMR spectrum of DH6Cl gives a broad proton resonance with chemical shift value of 5.1 ppm. This is believed to be due to NH_3. The other broad resonance at δ = 4.4 ppm is believed to be due to NH_2. The peak at δ = 4.0 ppm is believed to be due to protons of CH_2 group that is adjacent to NH_2. The peaks at δ = 2.4 and 1.6 ppm are believed to be due CH_2 protons away from the NH_2 group. The peak at δ = 1.3 ppm is due to the protons of CH_2 groups that lie in the middle of the carbon chain. The broad peak at δ = 3.6 ppm may be due to some impurity that has not been characterized. The ^{1}H NMR results of DH6Cl appear to be similar to those for DH5Cl.

Mass spectrum of DH7Cl

A small peak observed in the mass spectrum of DH7Cl with m/z = 1072 corresponds to M^+. The small peak with m/z = 1040 corresponds to (M – Cl + 3H), that at 909 may be due to (M – 4Cl – NH_3 – 4H), that at 736 may be due to $Cl(NH_3)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pt(NH_3)_2\{NH_2(CH_2)_7NH_2\}$ which is believed to be formed in situ from joining of fragments. The peak at 720 may be due to ($Cl(NH_3)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pt(NH_3)\{NH_2(CH_2)_7NH_2\}$ + H) (formed in situ from joining of fragments), that at 606 corresponds to $Cl_2(NH_2)Pt$-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_2)Cl_2$. The peak at 577 may be due to Pt-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_3)\{NH_2(CH_2)_7NH_2\}$, that at 530 may be due to $ClPt(NH_3)_2$-μ-$\{NH_2(CH_2)_7NH_2\}Pd(NH_2)$, that at 395 may be due to $ClPt(NH_3)_2\{NH_2(CH_2)_7NH_2\}$ and the peak at 273 is due to $ClPd\{NH_2(CH_2)_7NH_2\}$. The peak with m/z = 237 corresponds to ($Pd\{NH_2(CH_2)_7NH_2\}$ + H). It can be seen that the peaks observed in the mass spectrum of DH7Cl provide support for its suggested structure. Once again, a more conclusive proof for the structure may be obtained when the results are combined with other data such as those obtained from IR, Raman and ^{1}H NMR spectra and elemental composition.

^{1}H NMR spectrum of DH7Cl

The ^{1}H NMR spectrum of DH7Cl gives a broad proton resonance with chemical shift value of 4.4 ppm. This is believed to be due to NH_3 protons. The peak at 4.2 ppm is believed to be due to NH_2 that is bonded to Pt. The peak at δ = 4.0 ppm is believed to be due to NH_2 that is bonded to Pd. The peak at δ = 3.6 ppm is believed to be due to CH_2 protons adjacent to NH_2 group whereas those at δ = 2.4, 1.9 and 1.6 ppm are believed to be due CH_2 protons away from the NH_2 group. The peaks at δ = 1.3 ppm is due to protons of CH_2 group that lies in the middle of the carbon chain. The peak at δ = 1.4 ppm may be

due to some impurity that has not been characterized. The results are to some extent similar to those for DH5Cl and DH6Cl.

Mass spectrum of DHD

The peak observed in the mass spectrum of DHD with m/z = 654 corresponds M^+, that at 667 may be due to Cl_2Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)Cl_2$ + 2H) (a structure that can be formed in situ from joining of fragments), that at 592 may be due to (M - NO_3 + H), that at 590 due to (Cl_3Pt-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)_2(NH_2CH_2)$ + 4H), that at 555 corresponds to ($Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)_2$ – H), that at 553 corresponds to $Cl(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)(NH_2)$, that at 380 may be due to ($Cl(NH_3)_2Pt\{NH_2(CH_2)_6NH_2\}$ – H) and that at 364 may be due to $Cl(NH_3)Pt\{NH_2(CH_2)_6NH_2\}$. It can be seen that peaks observed in the mass spectrum of DHD provide support for the suggested structure for the compound. In particular, it clearly indicates the existence of multicentred cation.

^{1}H NMR spectrum of DHD

The ^{1}H NMR spectrum of DHD gives a broad proton resonance with chemical shift value of 4.4 ppm. This is believed to be due to NH_3 protons. The peak at δ = 4.0 ppm is believed to be due to NH_2 protons. The peak at δ = 3.6?ppm is believed to be due to CH_2 protons adjacent to NH_2 group whereas those at δ = 2.4, 1.9 and 1.6 ppm are believed to be due CH_2 protons away from the NH_2 group. The peak at δ = 1.3 ppm is due to the protons of CH_2 groups that lie in the middle of the carbon chain.

5.2. ACTIVITY OF COMPOUNDS

The multinuclear complexes code named: DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and DH1Cl were synthesized, characterized and evaluated for their activity against human ovary cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, melanoma cell lines: Me-10538 and non small lung cell line: NCI-H460, using the MTT reduction assay. The cell uptake and the extent of binding with DNA had also been determined.

All of the compounds were found to show significant antitumour activity, especially against cisplatin-resistant ovary cancer cell line A2780^{cisR}. Table 4.20 gives a summary of the activity of the compounds in terms of their IC_{50} values and resistant factors.

Among the multinuclear compounds, DH6Cl was found to be the most active compound – about nine times as active as cisplatin against the human ovary cell line A2780. DHD was also found to be more active than cisplatin against A2780 cell line whereas DH1Cl had activity similar to that of cisplatin. All other compounds were found to be less active than cisplatin against A2780 cell line. The actual order of activity of the compounds against A2780 cell line was: DH6Cl > DHD > cisplatin = DH1Cl > DH7Cl > DH4Cl ≈ DH5Cl. For the trinuclear compounds DH4Cl, DH5Cl, DH6Cl and DH7Cl, as the number of carbon atoms present in the linking diamine was decreased below six (eg in DH5Cl and DH4Cl) and increased above six (as in DH7Cl), activity was found to decrease. As stated in chapter 2, it was found that the 1,1/t,t compound with two platinum units linked by 1,6-diaminohexane was found to be much more active than the other dinuclear compounds (Menta *et al.* 1999) obtained by varying the chain length and/or the nature of the coordination geometry. The dinuclear compound DHD of the present study met both these requirements for optimum activity except that one of the platinum centres had been replaced by the corresponding palladium unit.

DHD was found be more active than cisplatin against A2780 cancer cell line. The two important factors that determine the activity of multinuclear platinum complexes are: (1) the size of the linking diamine and (2) the flexibility of the diamine linker that allows the formation of a plethora interstrand adducts of varying sizes. It has been reported that multinuclear platinum complexes with 4,4'-dipyrazolylmethane (dpzm) ligand had lower activity than BBR3464, although dpzm has approximately the same length as 1,6-diaminohexane (the linker in BBR3464). This was explained to be due to the lower flexibility of the dpzm ligand (Wheate *et al.* 2001). According to Qu et al the flexibility of the linking diamine allows the formation of a plethora of interstrand GG adducts dictated by the sequence of the bases in the DNA (Qu *et al.* 2003), thus providing an answer as to why the multinuclear complexes with flexible diamines are found to be more active than the ones with non-flexible linkers. This point will be further discussed later in the chapter.

As to why the activity of the trinuclear complexes changes with the change in length of the linking diamine, molecular modeling calculations have been carried out to investigate the effects of binding of multinuclear cations with the DNA. The results of the analysis will be presented later in the chapter.

When we compare the activity of DH6Cl with that of BBR3464 in A2780 cell line, it appears that BBR3464 is more active than DH6Cl (at least 2.6 times as active as DH6Cl) (Farrell 2000). It should however be noted that different exposure times used for the determination of IC_{50} values for the two compounds - 2 h in case of BBR3464 and 72 h in case of DH6Cl - leave some uncertainly in the indirect comparison made via cisplatin, since unlike BBR3464 and DH6Cl, cisplatin is expected to react at a much slower rate. The IC_{50} value will go down as the time of exposure is increased. This means that the difference in activity between cisplatin and trinuclear compounds would be more

pronounced when the period of exposure was shorter. The lower activity of DH6Cl as compared to that of BBR3464 means that the therapeutic window of DH6Cl may be greater than that for BBR3464. This could be highly desirable since the extremely high activity of BBR3464 means that the compound has a very narrow therapeutic window. As stated earlier, BBR3464 was in the phase II clinical trials (Davies *et al.* 2002) before it was stopped because of high toxicity. Neutropenia is found to be the dose-limiting toxicity of BBR3464 that leads to diarrhea and nausea (Judson and Kelland 2000; Wheate and Collins 2003).

For the cisplatin-resistant cell line $A2780^{cisR}$, DH6Cl was found to be about seventeen times as active as cisplatin, DHD nearly five times as active as cisplatin and the other trinuclear compounds were also found to be marginally more active than cisplatin. The actual order of activity of the compounds against $A2780^{cisR}$ cell line was: DH6Cl > DHD > DH1Cl > DH7Cl >DH4Cl = DH5Cl > cisplatin. Thus, all of the multinuclear compounds have resistance factors lower than that of cisplatin. Much higher activity of DH6Cl and DHD (as compared to cisplatin) in ovary cell lines (especially cisplatin-resistant cell line $A2780^{cisR}$) suggests that the two compounds had been able to overcome multiple mechanisms of cisplatin resistance that might be operating in $A2780^{CisR}$ cell line, including decreased uptake, elevated glutathione level and increased DNA repair (Perez 1998; Reedijik and Teuben 1999; Brown 2000). Although DH4Cl, DH5Cl and DH7Cl were found to be less active than DH6Cl and DHD (against A2780 and $A2780^{cisR}$ cell lines), the compounds had lower resistant factors than DH6Cl and DHD. This means that the decrease in activity in going from A2780 to $A2780^{cisR}$ cell lines is less pronounced in the case of DH4Cl, DH5Cl and DH7Cl than in the case of DH6Cl and DHD. The results suggest that DH4Cl, DH5Cl and DH7Cl can overcome the mechanisms of platinum drug resistance.

Although, DH6Cl was found to be less active than BBR3464 in A2780 cell line, it was found to be more active than BBR3464 in $A2780^{cisR}$ cell line. When the period of incubation was seventy two hours, BBR3464 was reported to be 2.52 times as active as cisplatin (Pratesi *et al.* 1999) whereas in the present study it was found that DH6Cl was seventeen times as active as cisplatin. The results suggest that DH6Cl is able to overcome resistance in $A2780^{cisR}$ cell better than BBR3464.

For $A2780^{ZD0473R}$ cell line, DH6Cl was found to be about four times as active as cisplatin and DHD about two times as active as cisplatin. All other compounds (DH4Cl, DH5Cl, DH7Cl and DH1Cl) were found to be less active than cisplatin. The high activity of DH6Cl and DHD in $A2780^{ZD0473R}$ cell line as compared to cisplatin also indicates the two compounds were better able to overcome multiple mechanisms of ZD0473 resistance operating in the cell, including decreased cell uptake and DNA binding, increased glutathione level, loss of DNA mismatch repair gene hMLH1 and increased expression of the anti-apoptotic protein Bc12 (Holford *et al.* 2000). It may be noted that the activity of DH6Cl and DHD in $A2780^{ZD0473R}$ cell line is less than that in $A2780^{cisR}$ cell line, which may be a consequence of different mechanisms of resistance operating in $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines. Determination of cell uptake and platinum-DNA binding in $A2780^{ZD0473R}$ cell line for DH6Cl and ZD0473 would provide useful information.

For the melanoma cell line Me-10538, DH6Cl was found to be about six times as active as cisplatin. DH1Cl, DHD, DH7Cl were found to be about two times as active as cisplatin. DH4Cl and DH5Cl were found to be less active than cisplatin. The higher activity of DH6Cl, DH1Cl, DHD and DH7Cl in Me-10538 cell line as compared to that for cisplatin may mean that the compounds are better able to overcome resistance that is operating in the cell line.

For the non small lung cell line NCI-H460, the most active multinuclear compound was once again DH6Cl whose activity was found to be comparable to that of cisplatin. All of other multinuclear compounds (DH4Cl, DH5Cl, DH7Cl, DHD and DH1Cl) were found to be less active than cisplatin, indicating that compounds were unable to overcome cisplatin resistance present in the cell line.

Besides IC_{50} value, IC_{90} value (which may be defined as the drug concentration required for ninety percent cell kill) may also be a relevant parameter in evaluating the use of a drug in cancer therapy (Table 4.21). When we consider the IC_{90} values also it is found that DH6Cl is the most active compound in all cell lines (especially in A2780 cell for which the IC_{90} value of DH6Cl was found to about one sixth of that of other trinuclear compounds). For the other cell lines namely $A2780^{cisR}$, $A2780^{ZD0473R}$, Me-10538 and NCI-H460, the difference in IC_{90} values of DH6Cl and the rest of the multinuclear compounds was less pronounced.

5.3. CELL UPTAKE AND BINDING WITH DNA

Since the anticancer activity of platinum drugs is believed to be due their binding with nucleobases in DNA, knowledge of the cell uptake of the compounds is considered to be important as the extent of DNA binding and cell uptake could be to be correlated. It should however be noted that the cell uptake per se may not give a quantitative measure of the extent of DNA binding and hence the activity, as platinum drugs may be deactivated due to their binding with cellular platinophiles such as glutathione (Gibson 2002; Najajreh *et al.* 2003) before they have a chance to bind with DNA. Nevertheless, it has been reported that there was a decrease in uptake in cells that were resistant to platinum drugs (Gately and Howell 1993; Mellish *et al.* 1995; Perego *et al.* 1999b).

5.3.1. *CELL UPTAKE*

As stated in chapters 3 and 4, cell uptake and DNA binding of the multinuclear compounds were investigated as applied to the human ovary cell lines A2780 and A2780^{cisR} only, in which the cellular accumulation of platinum was used as a measure of the cell uptake of compounds. However, the compounds being multinuclear containing both platinum and palladium centres, the determination of platinum level alone does not provide any information on the stability or the occurrence of decomposition of the compounds. Thus in this study, cell uptake of palladium in 4 h was also determined to provide a measure of the extent of decomposition of the compounds.

5.3.1.1. PLATINUM UPTAKE

Figures 4.38, 4.39 and tables 4.22, 4.23 give the Pt cell uptake in the human ovary cell lines: A2780 and A2780^{cisR} for DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD together with that for cisplatin. Figures 5.1 and 5.2 give a summary of the results (together with IC_{50} values and levels of DNA binding) on a relative scale as compared to cisplatin.

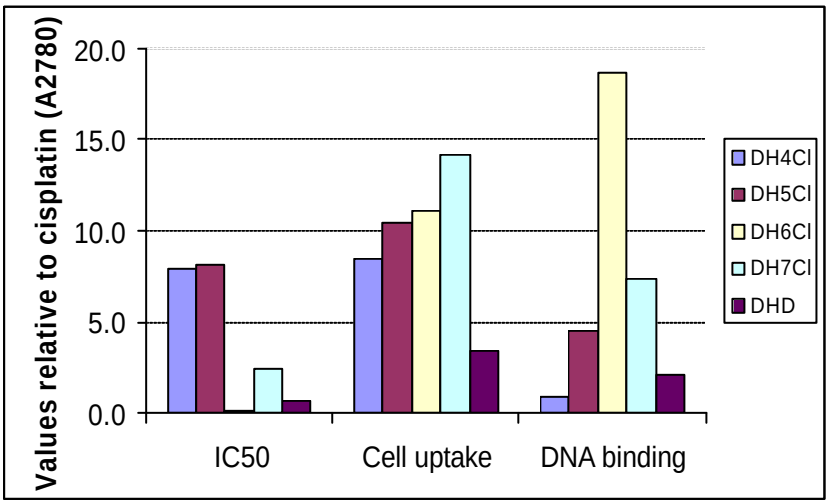


Figure 5. 1 The IC_{50} values, cell uptake and DNA-binding of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD relative to cisplatin as applied to the A2780 cell line

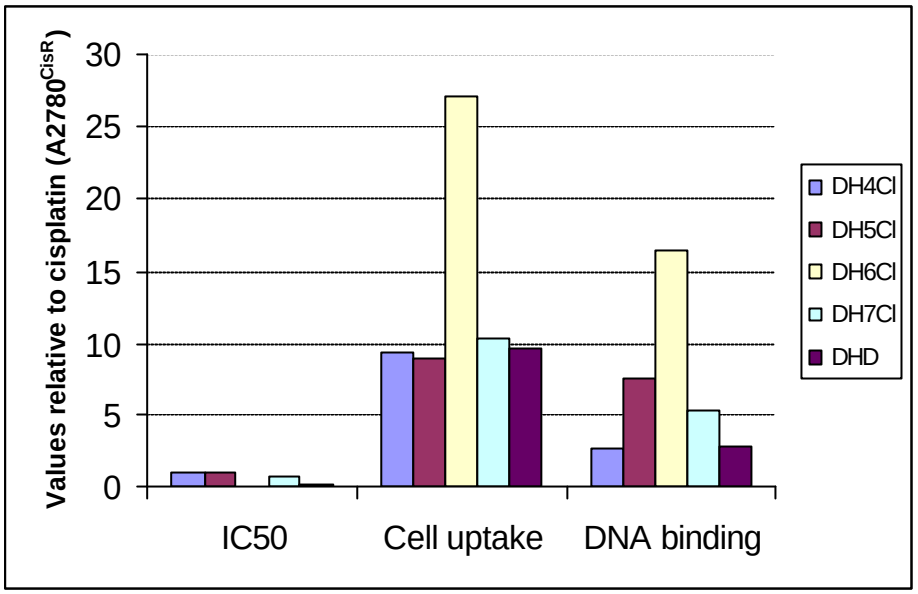


Figure 5. 2 The IC_{50} values, cell uptake and DNA-binding of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD relative to cisplatin as applied to the $A2780^{cisR}$ cell line

It was found that for all the multinuclear compounds DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD, the platinum uptake in A2780 and $A2780^{cisR}$ cell lines was much greater than that for cisplatin. It was reported that the platinum cell uptake in A2780 cell line was greater (than that for cisplatin) for both the dinuclear compound BBR3571 and the trinuclear compound BBR3464 (Farrell 2000).

Since as noted earlier, all of the trinuclear compounds except DH6Cl and DHD were found to be less active than cisplatin against A2780 cell line, the results illustrate that the cell uptake per se may not necessarily give an indication of the level of the antitumour activity of the compounds. As noted earlier, platinum compounds could be deactivated within the cell by a number of means (eg due to binding with sulfur containing ligands such as glutathione and metallothionein present in the cell) (Reedijik and Teuben 1999) before they had chance to bind with DNA. Thus a clearer picture about activity may result when the level of platinum-DNA is considered.

The actual order of the platinum cell uptake in A2780 cell line was: DH7Cl > DH6Cl > DH5Cl > DH4Cl > DHD > cisplatin, indicating that as the number of carbon atoms present in the linking diamine chain was increased, the uptake of the drug in A2780 cell also increased. Since the multinuclear cations are highly charged, one would expect their transport across the cell membrane to be carrier-mediated. On the other hand, that the cell uptake was found to increase with the increase in the size of the linking diamine (eg with the increase in the lipophilicity of the diamine), suggests that the multinuclear cations might be crossing the cell membrane by facilitated diffusion. It is possible that the multinuclear cations get bound to the hydrophobic pocket of the carrier molecules in which the interaction between linking diamine and the hydrophobic pocket plays a key role. Study of the cell uptake as a function of drug concentration may provide further light on the matter. Alternatively, the compounds might be crossing the cell membrane by passive diffusion provided the compounds remained undissociated before they crossed the cell membrane. Low molar conductivity values found for 0.0625 mM solutions of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD (16.0, 8.0, 19.0, 19.2 and 16.0 $ohm^{-1}cm^2mol^{-1}$ respectively) made in 1:1 mixture of DMF and water (or in DMF in the case of DH5Cl) suggest that the compounds persist largely as undissociated ionic aggregates in solution in DMF and water mixture. In the cell culture, cell uptake and DNA binding studies, the compounds were first dissolved in a small amount of DMF before being diluted with the culture media. If the compounds remained undissociated in such situations, it would provide a simple explanation as to why the cell uptake of the compounds increased with the increase in the size of the linking diamine. Thus, as the number of carbon atoms present in the linking diamine increase, the polarity of the molecule decreases and consequently the rate of passive diffusion and therefore cell uptake increase. It should be noted that the degree of dissociation of the compounds into polynuclear cations and

chloride anions (and nitrate in the case of DHD) and further hydrolysis of polynuclear cations is likely to increase with in the cell because of a lower chloride concentration and further dilution of the compound effectively with polar water molecules. A highly significant effect of the dissociation and the hydrolysis would be to enhance the attraction, pre-association and covalent binding between the polynuclear cations and the DNA. The increase in molar conductivity values with the decrease in concentration of the compounds (obtained by dilution even with 1:1 mixture of DMF and water or with DMF alone), as shown in the figure below provides support to the idea that degree of dissociation would increase with dilution.

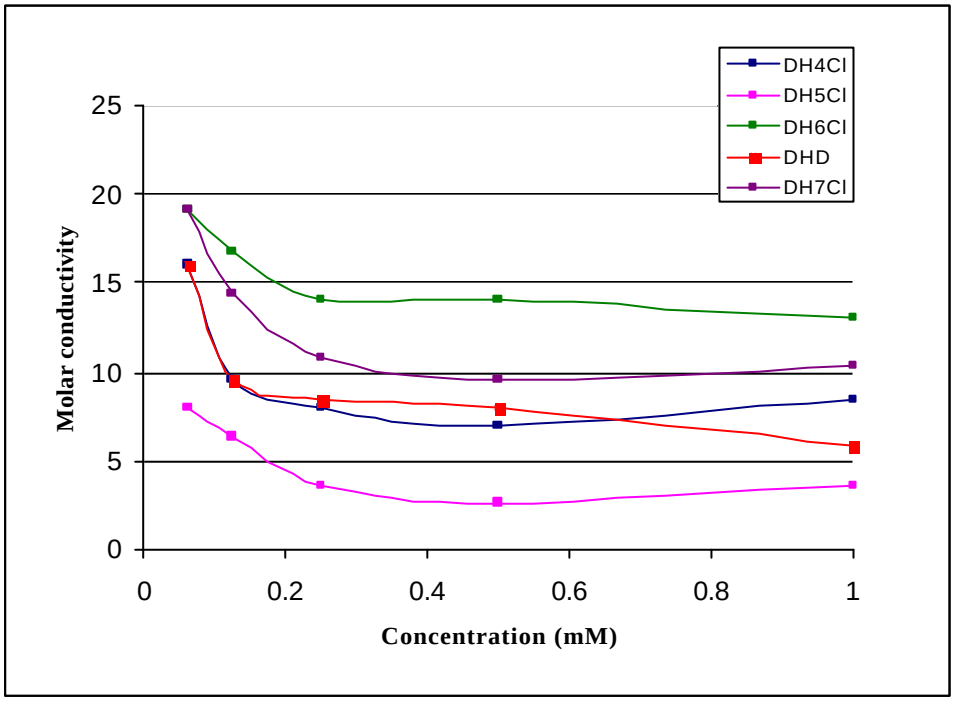


Figure 5. 3 The change in molar conductivity values for solutions of polynuclear compounds with the change in concentration.

The order of the platinum uptake in A2780^{cisR} cell was: DH6Cl > DH7Cl > DHD > DH4Cl > DH5Cl > cisplatin, which was in line with that of the activity of the compounds

against the cell line. It should be seen that for DH6Cl and DHD, platinum cell uptake in the cisplatin-resistant cell line A2780^{cisR} was greater than in the cisplatin-responsive cell line A2780. This finding is in conflict with previous studies applying to BBR3464 and other multinuclear platinum complexes where it was found that the accumulation of platinum was reduced in the resistant cell lines L1210/DDP and U_2-OS/Pt (Di Blasi *et al.* 1998; Perego *et al.* 1999b; Roberts *et al.* 1999a; Wheate *et al.* 2001). The increased uptake of DH6Cl and DHD in A2780^{cisR} cell line than in A2780 cell line might be due to reduced efflux. It was stated earlier that one of the possible mechanisms of resistance to cisplatin is associated with increased efflux of the drug from the cell. Thus increased accumulation of DH6Cl and DHD in A2780^{cisR} cells than in the parent cell line indicates that the compounds have been able to overcome the mechanism of resistance associated with increased efflux. On the other hand, the reduced uptake of DH4Cl, DH5Cl and DH7Cl in A2780^{cisR} cell line than in A2780 cell line could mean that the compounds have not been able to overcome such resistance.

Although the IC_{50} values (on a scale relative to cisplatin) of all the multinuclear compounds were found to be lower in A2780^{cisR} cell than in A2780 cell (Figures 5.1 and 5.2), on an absolute scale the values were higher in the resistant cell line than in the sensitive cell line (Table 4.20). This means that: (1) all the multinuclear compounds are less active against the resistant cell line: A2780^{cisR} than the parent cell line: A2780, and (2) the decrease in activity in going from the parent cell line to the resistant cell line is less pronounced in the case of multinuclear complexes than that in cisplatin (suggesting that the compounds have been to overcome partially the mechsnisms of resistance operating in A2780^{cisR} cell line).

The results suggest that other mechanisms of resistance besides reduced uptake (some of which may be increased deactivation of the drugs due to their binding with cellular platinophiles and increased DNA repair) might be operating in A2780^{cisR} cell as applied to the multinuclear compounds.

Time course experiments (Figure 4.39) showed that whereas the cell uptake in terms of platinum of DH4Cl and cisplatin reached saturation in about 2 h that of DH6Cl continued to increase with time and more so in A2780^{cisR} cell line compared to A2780 cell line. Further experiments involving longer periods of time would be needed to find out whether saturation could at all be achieved for DH6Cl. It should however be noted an increase in cellular uptake with the increase in time (in some cases over a period of up to 48 h) was reported for dinuclear and trinuclear platinum complexes for a number of cell lines including L1210 and U_2-OS (Di Blasi *et al.* 1998; Roberts *et al.* 1999b; Wheate *et al.* 2001).

5.3.1.2. PALLADIUM UPTAKE

Since the multinuclear complexes contain both platinum and palladium, the determination of palladium levels also should provide an equivalent measure of the drug uptake, provided the compounds remain essentially intact before their entry into the cell. A departure from the expected value for the molar ratio of Pt and Pd uptake (2:1 for DH4Cl, DH5Cl, DH6Cl and DH7Cl and 1:1 for DHD) can result only if the compounds break down in solution, separating platinum and palladium centres. It should however be noted that the agreement between the observed and the expected molar ratios of Pt and Pd does not eliminate the possibility of such breakdown. For example, if the breakdown occurred outside the cell but the components containing Pt and Pd accumulate wthin the cell at the same net rate, the observed ratio would match the expected one. Conversely, if the

compounds broke down within the cell and if the components escaped from the cell at the same rate, the observed molar ratio would again match the expected one.

Table 4.25 gives the observed molar ratios of Pt and Pd cell uptakes in 4 h in A2780 and $A2780^{cisR}$ cell lines. It is found that as applied to A2780 cell line, the molar ratios of Pt and Pd cell uptake for DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD were respectively 2.2 ± 0.3, 2.2 ± 0.5, 1.7 ± 0.5, 2.3 ± 0.5 and 1.3 ± 0.4. For the $A2780^{cisR}$ cell line, the corresponding values are 1.7 ± 0.9, 2.1 ± 0.6, 1.9 ± 0.4, 2.2 ± 1 and 1.2 ± 0.3. Although in most cases, the observed ratios are found to differ from the expected ones, the deviations are not considered to be significant as they lie in all cases within one standard deviation from the expected values. The random scatter (rather than a systematic trend) observed in the values of Pt : Pd molar ratios indicates that the departure is actually an artifact of the limitation of the measurements. As discussed earlier, solutions of all the compounds were found to have low molar conductivity values indicating that compounds remained essentially intact in solution.

5.3.2. *PLATINUM DNA BINDING*

As stated earlier, cell uptake per se may not give a true measure of the activity of a platinum drug. Since the anticancer activity of platinum-based drugs is believed to be associated with their binding with DNA, a better understanding of the activity of the drugs may be obtained when we consider the extent of binding of Pt with DNA.

Tables 4.26, 4.27 and figures 4.41, 4.42 give the extent of platinum-DNA binding in nanomoles Pt per mg DNA in A2780 and $A2780^{cisR}$ cell lines for the compounds: DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin.

It was found that for all the compounds including cisplatin, the level of platinum-DNA binding was less in the resistant cell A2780^{cisR} than in the responsive cell line A2780, in line with their lower activity in the resistant cell line. The results are in agreement with the previously published values in which it was found that the lower activity of BBR3464 in U2-OS/Pt cell line as compared to that in U2-OS cell line corresponded to a lower level of Pt-DNA binding in the former than in the latter (Perego *et al.* 1999a).

The actual order of Pt-DNA binding in A2780 cell line was DH6Cl > DH7Cl >DH5Cl > DHD > cisplatin = DH4Cl and that in A2780^{cisR} cell line was DH6Cl > DH5Cl > DH7Cl > DHD > DH4Cl > cisplatin. It should be seen that for the trinuclear complexes: DH4Cl, DH5Cl, DH6Cl and DH7Cl, the order of Pt-DNA binding in A2780 cell line was mostly in line with that of the activity of the compounds against the cell line except for DH5Cl (which although dissolved readily in DMF, produced cloudiness when water was added and became clear only when the mixture was made basic with NaOH). In the case of A2780^{cisR} cell line also, the order of activity of the trinuclear complexes DH4Cl, DH5Cl, DH6Cl and DH7Cl was found to be generally in line with that of the Pt-DNA binding level (except for DH5Cl). Since the trinuclear complexes differ in length between the two terminal centres (this point will be further considered later in the chapter), they all are expected to form long-range adducts and hence cause similar but not identical conformational changes in DNA. Thus, an increase in the level of Pt-DNA would mean a greater number of interstrand adducts and consequently a greater conformational change in DNA resulting into a greater activity.

For the dinuclear complex DHD, although the level of Pt-DNA binding was found to be lower than that for DH5Cl, DH6Cl and DH7Cl, it was found to have a greater activity than all the trinuclear complexes except DH6Cl against both A2780 and A2780^{cisR} cell

lines. This is believed to be due to a difference in the exact nature of binding of the compounds with DNA – whereas in the case of trinuclear complexes covalent binding with DNA involves the two terminal Pt centres with the central palladium unit undergoing only noncovalent interactions such as hydrogen bonding and electrostatic interaction, in the case of DHD both platinum and palladium centres are likely to be involved in covalent interactions. The other difference between the trinuclear complexes (eg DH6Cl) and the dinuclear complex DHD is expected to be in the percentage of long-range interstrand adducts because of a difference in their lengths. The trinuclear complexes (DH4Cl, DH5Cl, DH6Cl and DH7Cl) having longer lengths than the dinuclear complex DHD, are likely to form more of long-range interstrand adducts than DHD. This idea is in line with the observation made by Brabec et al while considering the DNA binding of dinuclear and trinuclear platinum complexes (Brabec *et al.* 1999). Dinuclear platinum complexes were reported to be more effective than BBR3464 at inhibiting ethanol induced B $\rightarrow$ A transition in CT DNA which was considered to be due a greater percentage of interstrand adducts formed by the dinuclear complexes (McGregor *et al.* 1999).

It was reported that dinuclear compound BBR3005 overall formed more interstrand adducts than the trinuclear compound BBR3464 (Brabec *et al.* 1999) although it had a lower activity than BBR3464. It should also be noted that Wheate et al (Wheate *et al.* 2001) designed two multinuclear complexes containing rigid linkers that were capable of forming long-range interstrand adducts and yet displayed significantly lower activity than BBR3464 that has flexible linkers. Flexible linkers would allow the formation of a plethora of interstrand adducts of different sizes that appear to translate into a higher activity.

That cisplatin did not fit the activity and platinum-DNA binding orders for the A2780 cell line (ie although DNA binding level for cisplatin was less it was found to be more active than DH7Cl, DH5Cl and DH4Cl), is not totally unexpected when we note that the nature of binding of cisplatin with DNA is likely to be different from that of multinuclear compounds. Cisplatin forms mainly intrastrand GG (Eastman 1987), AG (Fichtinger-Schepman *et al.* 1985) and a small amount of GXG adducts (Brabec 2000) but, as stated earlier, multinuclear complexes in the present study are expected to form mainly long-range interstrand GG adducts (similar to those formed by BBR3464) (Brabec *et al.* 1999). Whereas 1, 2-bifunctional binding of cisplatin would cause mainly local bending of DNA, the long-range interstrand adducts formed by multinuclear compounds would cause long-range distortion of DNA in which the conformations of the intervening bases not directly involved in the cross-link were also altered (Qu *et al.* 2003). According to Farrell and Spinelli (Farrell and Spinelli 1999), DNA binding of multinuclear platinum complexes is characterized by flexible, non-directional DNA adducts and a greater percentage of interstrand to intrastrand adducts and the ability to induce conformational changes in the DNA from B to A and from B to Z forms. Whereas B to A conformational changes induced by electrostatic interactions (eg those caused by $Co(NH_3)_6^{3+}$) were found to be reversible, B to A and B to Z conformational changes caused by multinuclear platinum compounds were found to be irreversible, as these were induced by covalent binding (McGregor *et al.* 2002).

The bending induced in DNA by binding of cisplatin is recognized by high-mobility group domain (HMG) proteins and this recognition is believed to be the pathway for processing and differential repair of cellular cisplatin-DNA adducts (Jamieson *et al.* 1999; Jamieson and Lippard 1999). However it is found that in the formation of long-range interstrand adducts by BBR3464, DNA is not sufficiently bent to be recognized by HMG

1 protein (Zehnulova *et al.* 2001). Instead, the long-range cross-links are very effective in inducing B to A or B to Z transformations. Cellular alkaline eluation studies have shown that the interstrand cross links formed by BBR3464 persist over time, suggesting a lack of DNA repair (Roberts *et al.* 1999a; Roberts *et al.* 1999b). It has been suggested that the induction of Z-DNA within the cell would have serious consequences with regard to transcription and DNA replication. Qu et al (Qu *et al.* 2003) reported that in the formation of 1,4-GG octamer, the *syn* conformation was induced in the adenine moieties not just within the strand bounded by the two platinum binding sites but also those at the end of the strand. They found that Waston-Crick pairing was essentially maintained and that the central linker was situated in the minor groove of the DNA. The authors point out the cooperative nature of the B to Z transformation lends itself easily to the delocalization of the lesions beyond the binding site. It was suggested that the factors that might contribute to the delocalization would include the linking of the two separated platinating sites and the presence of the charge and electrostatic interactions introduced after incorporation of BBR3464 into the oligonucleotide. The contacts between the lipophilic backone of BBR3464 and DNA may be especially effective in displacing water from within DNA and thus facilitating conformational transitions. All of the above discussion could equally apply to the multinuclear complexes of the present study. It has been suggested (McGregor 1999) that in canonical poly (dGdC).poly (dGdC), polynuclear platinum complexes can form a plethora of intra- and interstrand cross links. Molecular mechanics calculations using HyperChem 7 (Hypercube 2002) show that when fully stretched, the lengths of DH4Cl, DH5Cl, DH6Cl and DH7Cl but with the chlorides replaced by nitrogens are respectively 2.10 nm, 2.41 nm, 2.8 nm and 2.85 nm. It is found that in the double stranded B DNA, the interstrand N7 (guanine) to N7 (guanine) distances are: G (1) to G (6): 1.59 nm, G (1) to G (7): 1.93 nm, G (1) to G (8): 2.28 nm, G (1) to G (9): 2.68

nm, G (1) to G (10): 2.97 nm and G (1) to G (11): 3.29 nm. It can be seen that when fully stretched the best match between the length of the molecule and the interstrand N7 (guanine) to N7 (guanine) distance occurs for DH6Cl. This point will be further discussed in section 5.4.1.1 when we consider the interaction of the compounds with pBR322 plasmid DNA.

The higher activity of the multinuclear complexes (especially DH6Cl) as compared to cisplatin in $A2780^{cisR}$ may indicates the ability to overcome DNA repair mechanisms. It has been suggested that BBR3464 overcomes multiple mechanisms of cisplatin resistance including alterations in DNA mismatch repair (Perego *et al.* 1999a; Perego *et al.* 1999b).

BBR3464 induces a cellular response different from that of cisplatin resulting into somewhat different modes of cell death (Servidei *et al.* 2001). Flow cytometric experiments showed that BBR3464 was able to induce a persistent block of OAW42 and OAW42MER cells in the G_2M phase, whereas cisplatin caused an initial accumulation of cells in the S phase followed by an increase in the G_2M cell fraction in both cell lines (Orlandi *et al.* 2001).

Based on molar conductivity values it was concluded earlier that all of the multinuclear complexes in the present study remained essentially undissociated in 1:1 mixture of DMF and water. However, this may not be true in biological fluids where the presence of a large volume of water (which is much more polar and has a much larger dielectric constant) will serve to dilute the solutions of the compounds and reduce the influence of the organic solvent. This means that like BBR3464 the compounds DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD may be dissociated into ions in solution in biological fluids.

If this were so, the high charge on DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD cations as in the case of BBR3464 (+ 4 charge) and BBR3005 (+ 2 charge) would facilitate their

rapid binding with DNA. It was reported that $t_{1/2}$ (ie the half time of the binding reaction of the compound with DNA), was 40 min for BBR3464, 200-300 min for dinuclear BBR3005 and 4 h for cisplatin (Brabec *et al.* 1999), indicating that the trinuclear platinum complex was considerably faster than dinuclear complex or cisplatin in binding with DNA. It was suggested that the rapid binding of BBR3464 could affect sequence specificity - the high charge could lead to initial electrostatic interactions (very different from those found in small molecules such as cisplatin and the alkylating agents) that led to enhanced sequence specificity. It is generally accepted that *cis*-$[Pt(NH_3)_2(Cl)(H_2O)]^+$, formed by hydrolysis of one Pt-Cl bond, pre-associates with DNA (Wang *et al.* 2001; Wheate and Collins 2003) before binding to specific nucleobases in DNA. Wheate at al (Wheate and Collins 2003) point out that pre-association with DNA would be even stronger and therefore more important in the case of cationic multinuclear platinum complexes since it is stabilized largely by electrostatic forces. It has been suggested that the pre-association of multinuclear platinum complexes with polyanionic DNA will significantly affect the rate and site of platination because the increased local concentration achieved through pre-association will increase the probability of a covalent interaction at these sites. Also, pre-association may induce a local conformational change in DNA that may influence binding at a specific site.

The above idea may be equally applicable to the interaction of the multinuclear complexes: DH4Cl, DH5Cl, DH6Cl and DH7Cl with DNA.

5.4. INTERACTION WITH DNA AND NUCLEOBASES

5.4.1. *INTERACTION WITH DNA*

As stated in the previous section, multinuclear complexes DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD are expected to form long-range interstrand adducts with DNA, resulting into conformational changes in the DNA. When the conformation of DNA is changed, its mobility through a gel may also be altered. Thus the interaction between trinuclear compounds (DH4Cl, DH5Cl, DH6Cl, and DH7Cl), dinuclear compound (DHD) and cisplatin with non-genomic pBR322 plasmid DNA and genomic salmon sperm DNA were investigated using gel electrophoresis. BamH1 digestion was used to gain further insight into the binding of the compounds with pBR322 plasmid DNA. The results of the studies were given in Chapter 4.

5.4.1.1. INTERACTION WITH pBBR322 PLASMID DNA

Plasmid DNA is normally found in compact supercoiled form but during isolation, chemical treatment or mechanical shear, breaks can be introduced allowing the DNA to relax and form an open circular form (Cantor and Schimmel 1980).

Thus, pBR322 plasmid DNA can exist in three forms: supercoiled form I, singly nicked circular form II and doubly nicked liner form III. DNA being negatively charged due to the phosphate backbone will migrate through the gel from the negative electrode to positive electrode. The supercoiled form I migrates at the fastest rate; the singly nicked circular form II has the lowest migration rate whereas the doubly nicked linear form III DNA has the intermediate migration rate.

The electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin ranging

from 1.25 μM to 15 μM were given in figure 4.43. The molar ratios (ri) between pBR322 plasmid DNA (in terms of phosphate) and the compounds were: 0.0133, 0.02, 0.07, 0.040, 0.053, 0.080, 0.107, and 0.16.

Unreacted pBR322 plasmid DNA gave a highly prominent band corresponding to supercoiled form I and a weak band corresponding to singly-nicked form II. In some cases, a weak frontal band was also observed in untreated pBR322 plasmid DNA. When the DNA was allowed to interact with increasing concentrations of the compounds, changes in DNA bands in terms of both intensity and mobility took place.

The presence of a small amount of DH4Cl, DH5Cl, DH6Cl and DH7Cl was found to be sufficient to cause a large increase in the intensity of the form II band (as compared to that found in the untreated DNA). On the other hand, at high concentrations of compounds the intensity of both form I and form II bands was found to decrease. The initial sharp increase in the intensity of the form II band in the presence of a small amount of the drugs points to DNA damage in which form I plasmid DNA is changed to form II DNA. The decrease in intensity at high concentrations of compounds is due to a partial breakdown of DNA caused by the covalent binding of the compounds.

As pBR322 plasmid DNA was allowed to interact with the increasing concentration of the compounds, the mobility of both forms I and II plasmid DNA bands increased but at different rates such that the two forms co-migrated at the highest concentration (namely 15 μM; ri = 0.16) in the case of DH4Cl, DH6Cl and 10 μM in the case of DH7Cl. Coalescing of the form I and form II bands however did not occur in the case of DH5Cl, cisplatin and DHD, indicating that for the three compounds the difference in the increase in mobility of the two bands with the increase in concentration of the compounds was less marked as compared to that in DH4Cl, DH6Cl and DH7Cl. The separation between form

I and form II bands became less distinct in the interaction of pBR322 plasmid DNA with DH5Cl - a streaking band was observed in which form II band could not be seen at concentration of DH5Cl = 10 μM (ri = 0.107). At the highest concentration (namely 15 μM), the intensity of the form I band also was greatly reduced and its mobility increased.

The results suggest that DH5Cl had been able to cause a greater damage to the DNA than the other multinuclear compounds. It may be noted that unlike DH4Cl (which was found to be soluble in water) and DH6Cl and DH7Cl (which were found to be soluble in DMF and in mixture of water and DMF), DH5Cl dissolved neither in water nor in mixture of water and DMF unless the solution was made basic (pH 10) with NaOH.

The increase in mobility of the DNA bands with the increase in concentration of the compounds is believed to be due a change in conformation of the DNA (both supercoiled form I and singly nicked form II) brought about by covalent binding between the nucleobases in the DNA and the platinum centres of the compounds. It has been noted earlier that whereas cisplatin because of the formation of mainly intrastrand GG and AG adducts (Stehlikova *et al.* 2002) causes a local bending of the DNA, multinuclear complexes (because of the formation of long-range interstrand adducts that affect the conformation of the intervening bases as well as those outside of the binding sites) cause a long-range conformational change in DNA. Thus, irreversible A $\rightarrow$ Z and B $\rightarrow$ Z transitions were found to be introduced by multinuclear platinum complexes (Farrell *et al.* 1995; McGregor *et al.* 2002).

When pBR322 plasmid DNA was allowed to interact with increasing concentrations of the dinuclear complex DHD (that has a *trans*-platinum and a *trans*-palladium unit linked together by 1,6-diaminohexane), it was found that (as in the case of trinuclear complexes) the presence of a small amount of the compound caused a sharp increase in the intensity

of the form II band as compared to that in the unreacted DNA. However, as the concentration of DHD was increased above 3.75 µM, there was a progressive decrease in the intensity of the band such that it totally disappeared at concentration of DHD = 7.5 µM. A less marked decrease in intensity of the form I band also was observed with the increase in concentration of DHD above 3.75 µM, such that the form I band disappeared only at a much higher concentration, namely 15 µM. The results show that a greater damage to pBR322 plasmid DNA was caused by DHD than trinuclear complexes. As stated earlier, although both dincuclear and trinuclear platinum complexes form mainly interstrand adducts with DNA, Pt-DNA binding in the two cases differ significantly – whereas dincuclear complexes produce a greater percentage of shorter-range interstrand adducts, the trinuclear complexes produce a greater proportion of long-range adducts and that the total number of interstrand adducts would be greater in the case of dinuclear complexes than in the case of trinuclear complexes (Brabec *et al.* 1999; Wheate *et al.* 2001).

When pBR322 plasmid DNA was allowed to interact with increasing concentrations of cisplatin, although the mobility of both form I and form II bands increased slightly, the coalescing of the bands did not occur. At concentration of cisplatin = 15 µM (ri = 0.16), the form II band disappeared, indicating the occurrence of DNA damage. As discussed earlier, the nature of binding of cisplatin with DNA is different from that applying to its binding with multinuclear compounds – whereas cisplatin forms mainly intarstrand bifunctional Pt(GG) and Pt(AG) adducts (Stehlikova *et al.* 2002) multinuclear complexes form a range of interstrand adducts. It was also noted earlier that this difference in binding produces different conformational changes in the DNA and different extent of DNA damage at higher concentrations.

As stated earlier, the change in mobility of the pBR322 DNA bands as a result of its interaction with multinuclear compounds is believed to be due to interstrand ladder-like binding of multinuclear cations primarily with the GG sites of DNA. It was also noted that the high flexibility of the 'molecules' would allow the formation of a plethora of different interstrand adducts of varying lengths, dictated by the sequence of nucleobases. As stated earlier, Qu et al reported that in the formation of 1,4-GG interstrand adduct by BBR3464 with the self complementary 5'-d(ATG*TACAT)$_2$-3' octamer the *syn* conformation was induced in the adenine moieties not just within the strand bounded by the two platinum binding sites but also those at the end of the strand. They found that Watson-Crick pairing was essentially maintained and that the central linker was situated in the minor groove of the DNA. The authors pointed out that the cooperative nature of the B to Z transformation would lend itself easily to the delocalization of the lesions beyond the binding site. It was suggested that the factors that might contribute to the delocalization would include the linking of the two separated platinating sites and the presence of charge and electrostatic interactions introduced after incorporation of BBR3464 into the oligonucleotide. The contacts between the lipophilic backbone of BBR3464 and DNA might be especially effective in displacing water from within DNA and thus facilitating conformational transitions. It has been suggested that in canonical poly(dGdC).poly(dGdC), polynuclear platinum complexes can form a plethora of intra- and interstrand cross links (Qu *et al.* 2003).

Like BBR3464, DH6Cl as well the other trinuclear compounds (DH4Cl, DH5Cl and DH7Cl) may form a number of interstrand GG adducts of varying lengths. It was noted earlier that when adducts of maximum possible lengths were formed, a slight lengthening of DNA strands would occur in the case of DH6Cl and a more pronounced change (lengthening or shortening) in the case of DH5Cl, DH4Cl and DH7Cl. To explore

whether the difference in lengths of the multinuclear cations could also cause a difference in non-covalent interactions between the 'molecules' and DNA, the optimised multicentred cations of DH6Cl and DH5Cl were positioned so as to form interstrand bifunctional GG adducts with the double stranded DNA fragment d(GGGGGGCCCCCC) (Figures 5.4 and 5.5).

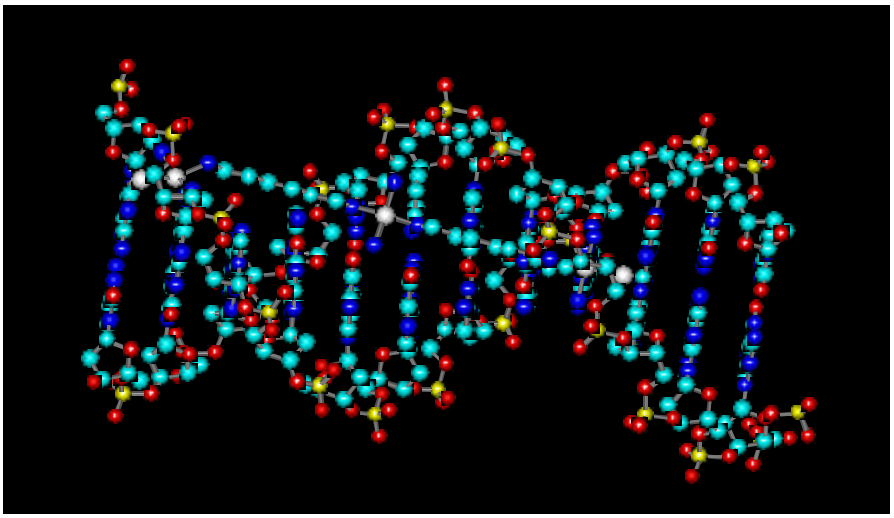


Figure 5. 4 Docking between DH6Cl and double-stranded DNA fragment d(GGGGGGCCCCCC) showing that DH6Cl cover a distance of over nine nucleotides

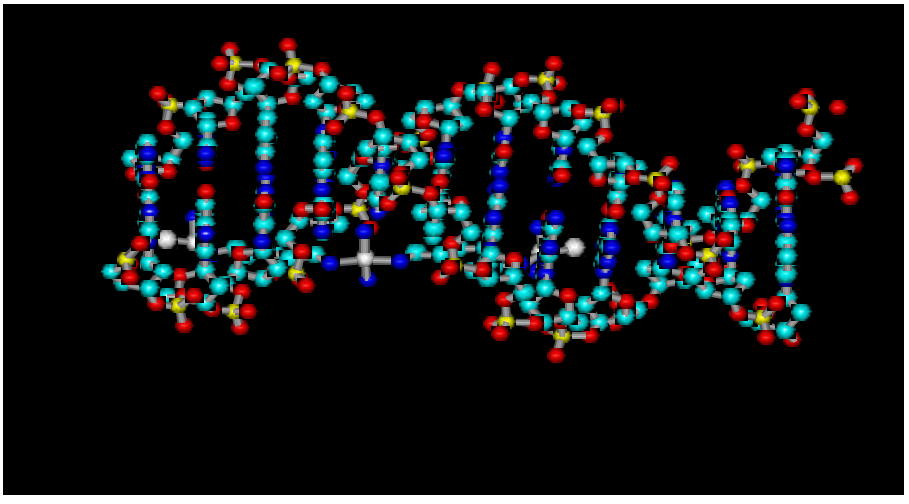


Figure 5. 5 Docking between DH5Cl and double-stranded DNA fragment d(GGGGGGCCCCCC) showing that DH5Cl cover a distance of over eight nucleotides

It is found that the central palladium unit is better positioned within helix (avoiding short repulsive contacts), in the case of DH6Cl than in the case of DH5Cl. The findings are similar to those observed for multinuclear palladium complexes [{*trans*-$PdCl(NH_3)_2$}$_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_6NH_2\}_2]Cl_4$ and [{*trans*-$PdCl(NH_3)_2\}_2$*m*-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_5NH_2\}_2]Cl_4$ (Huq *et al.* 2003).

5.4.1.2. BamH1 DIGESTION

As stated in chapter 4, BamH1 digestion combined with gel electrophoresis was used to gain further insight into the nature of binding of the polynuclear compounds with pBR322 plasmid DNA. BamH1 is a restriction enzyme that is known to recognize the G/GATCC and hydrolyse the phosphodiester bond between adjacent guanine sites (Roberts *et al.* 1977). pBR322 plasmid DNA contains a single restriction site for BamH1 (Sutcliffe 1979) that converts supercoiled form I and singly nicked circular form II pBR322 plasmid DNA to linear form III DNA. However, when platinum compounds at increasing concentrations bind to guanines in the DNA, BamH1 digestion may be increasingly prevented.

Figure 4.44 gives the electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD and cisplatin that were digested with BamH1 for 1 h before the mixtures were loaded onto the gel. Table 4.28 gives a summary of the bands observed.

When the incubated mixtures of pBR322 plasmid DNA and increasing concentrations of DH4Cl ranging from 1.87 µM to 15 µM (ri ranged from 0.02 to 0.16) were digested with BamH1, three bands corresponding to form I, II, III were observed for concentration of

DH4Cl ranging from 1.87 µM to 2.5 µM. When concentration of DH4Cl was 5 µM (ri = 0.053), forms I and II bands were observed and when concentration of DH4Cl was = 10 µM (r_i = 0.107), only form I band was observed. In the case of DH5Cl, form I, II, III bands were observed for concentrations of DH5Cl ranging from 1.87 µM to 2.52 µM above which only form I band was observed whose mobility increased sharply with the increase in concentration of DH5Cl. In the case of DH6Cl, three bands corresponding to form I, II, III were observed for concentrations of DH6Cl ranging from 1.87 µM to 2.50 µM. Forms I and II were observed for concentrations of DH6Cl ranging from 5 µM to 10 µM and only form I band was observed at concentration of DH6Cl = 15 µM. In the case of DH7Cl, three bands corresponding to forms I, II and III were observed at concentration of DH7Cl = 1.87 µM, two bands corresponding to form I and II were observed for concentrations of DH7Cl ranging from 2.5 µM to 10 µM and only the form I band was observed at concentration of DH7Cl = 15 µM. In the case of DHD three bands corresponding to form I, II, III were observed for concentrations of DHD ranging from 1.87 µM to 2.5 µM and two bands corresponding to forms I and II were observed for concentrations of DHD ranging from 5 µM to 15 µM. In the case of cisplatin, three bands corresponding to forms I, II and III were observed for concentrations of cisplatin ranging from 1.87 µM to 5 µM, two bands corresponding to forms I and II are observed for concentrations of cisplatin ranging from 10 µM to even 20 µM. In the case of pBR322 plasmid DNA that was not treated with the compounds but digested with BamH1, only form III band was observed.

When arranged in the decreasing order of prevention of BamH1 digestion, the compounds were:

DH7Cl > DH5Cl > DH6Cl > DH4Cl > DHD > cisplatin.

For untreated pBR322 plasmid DNA, BamH1 digestion at the specific GG site was not prevented thus producing only form III DNA. It is clear that in the presence of increasing concentrations of the compounds, there was a corresponding increase in prevention of BamH1 digestion. Thus, the observance of forms I, II and III at 1.87 μM of all the compounds indicates that BamH1 digestion has been partially but not totally prevented. The observe that only the supercoiled form I band at 10 μM in the case of DH4Cl and at 15 μM in the case of DH6Cl and DH7Cl indicate total prevention of BamH1 nicking at the specific GG site. It should be noted that the total prevention did not happen in case of DHD even at the highest concentration used (15 μM) although the form II band became much weaker in intensity. In the case of cisplatin, total prevention of BamH1 cutting did not occur even at 20 μM indicating that cisplatin was less efficient than the multinuclear complexes in preventing BamH1 digestion. These results also support the idea that whereas cisplatin-DNA binding causes a local distortion of the DNA, interstrand binding between multinuclear complexes and the DNA causes a more global change in the conformation of the DNA. Previously published results showed that dinuclear platinum compounds were more effective than cisplatin in inhibiting the cutting of DNA by restriction endonuclease (Farrell *et al.* 1988).

5.4.1.3. INTERACTION WITH ssDNA

Figure 4.40 gives the electrophoretograms applying to the incubated mixtures of ssDNA and varying concentrations of DH4Cl, DH5Cl, DH6Cl, DH7Cl, DHD, and cisplatin ranging from 5 μM to 60 μM.

A single band was observed in both unreacted and reacted salmon sperm DNA. As the concentration of the drugs was increased, it was found that both the intensity and the mobility of the band decreased. It was also found that the results were slightly different

from one compound to another. For example, it was found that the decrease in mobility with the increase in concentration was less pronounced in the case of DHD and cisplatin (least in the case of cisplatin). The decrease in mobility is believed to be due to covalent binding of the compounds with DNA, causing an increase in its molecular mass and a decrease in its overall negative charge. The decrease in intensity of the band indicates the occurrence of DNA damage brought about by the covalent binding. It may be noted that a decrease in intensity would also be observed if the binding of the compounds with DNA caused a decrease in DNA fluorescence unlike that in the binding of DNA with Ag^{+} where an increase in DNA fluorescence was reported (Hossain and Huq 2002). A smaller decrease in mobility with the increase in concentration in the case of DHD and cisplatin as compared to that in the case of DH4Cl, DH5Cl, DH6Cl and DH7Cl may also be seen to illustrate the difference in the nature of binding of the compounds (multinuclear compared to mononuclear cisplatin, trinuclear compounds compared to dinuclear DHD). As stated earlier, different types of binding may bring about different types of conformational change in the DNA that may manifest itself as a change in mobility through the gel. The change in mobility may also be a consequence (at least in part) of different molecular sizes of the compounds.

5.4.2. *INTERACTION BETWEEN DH6Cl AND NUCLEOBASES*

Like BBR3464, trinuclear complexes DH4Cl, DH5Cl, DH6Cl and DH7Cl all have one labile chloride ligand bonded to each of the two terminal platinum centres so that each of the two terminal platinum centres can form only one covalent bond with nuclebases in DNA. Based on the number of labile chloride ligands present, the trinuclear complexes would be expected to form 1 : 2 adducts with nucleobases for which the Pt : NB binding ratio would be 1 : 1. In this study HPLC has been used to determine the binding ratio

between the most active trinuclear complex DH6Cl and nucleobases adenine and guanine. It was noted earlier that HPLC provides a highly sensitive and convenient method to investigate binding between platinum-based anticancer drugs and nucleobases, nucleosides, nucleotides and DNA (Berners-Price and Appleton 2000). It is believed that the binding mode of the other trinuclear complexes DH4Cl, DH5Cl, DH7Cl with nucleobases would be similar to that of DH6Cl.

The retention time of adenine, guanine and DH6Cl were found to be 6.13, 3.10 and 3.91 min respectively. The second peak observed in the chromatogram of DH6Cl having the retention time of 2.67 min is believed to be due to DMF. When DH6Cl was allowed to interact with adenine, there were two major peaks in the chromatogram of the incubated mixture having the retention times of 2.83 and 3.72 min. In addition, there was a small peak that had the retention time of 2.98 min. The Pt : NB binding ratio of the peak having the retention time of 3.72 min was found to be 0.75. The peak at 2.83 min did not have any significant platinum. The observed UV absorbance at 260 nm for the fraction with retention time of 2.83 min might be due to DMF rather than adenine. The departure of the observed binding ratio (0.75) from the expected value of 1 : 1 may be considered to mean that the binding between DH6Cl and adenine had not gone to completion. It may be noted that like BBR3464, DH6Cl (and other trinuclear complexes of the present study) are more likely to bind to N7 centre of guanine rather than N7 centre of adenine. When DH6Cl was allowed to interact with guanine, again there were two major peaks in the chromatogram of the incubated mixture, having the retention times of 2.69 and 3.65 min. In addition, there was a small peak that had the retention time of 2.99 min as was found in the incubated mixture of DH6Cl and adenine. The Pt : NB binding ratio for the peak at 3.65 min was found to be 0.90 (a value that is much closer to 1 than 0.75). The results show that binding of DH6Cl with guanine to form 1 : 2 (DH6Cl : NB) adduct is more complete

than its binding with adenine. Further experiments on the interaction of DH6Cl with a mixture of adenine and guanine, would show whether the compound indeed has a preference to bind to guanine than adenine, as is considered to be the case in its reaction with DNA.

CHAPTER SIX

6. CONCLUSION

Cancer is one of the leading causes of death all over the world. Chemotherapy is one of the three methods of cancer treatment used alone or in combination with the other two methods namely surgery and radiation therapy. Although cisplatin and its analogue carboplatin are two of the most commonly used anticancer drugs, the compounds have a number of side effects and a limited spectrum of activity because of inherent or acquired resistance. In an attempt to reduce the side effects and widen the spectrum of activity, thousands of cisplatin analogues have been prepared by varying the nature of the leaving groups and that of the carrier ligands. Some of the designed complexes are indeed found to have reduced side effects and to some extent a different spectrum of activity. Unlike cisplatin and carboplatin (that have to be adminstreated intravenously), some of the designed complexes (e.g. ZD0473) can be taken orally. In spite of the tremendous progress made in altering toxicity profile, as stated earlier, only a limited advancement had been made in altering the spectrum of activity. Thus, presently attention is also given to 'rule breaker' platinum compounds and compounds of other metals such as those of ruthenium with the aim of arriving at tumour active compounds with very different spectra of activity. Dinuclear and trinuclear platinum complexes represent one such class of compounds. Although the exact mechanism of action of platinum-based anticancer drugs remains unknown, it is believed to be associated with their binding with DNA. Whereas cisplatin and its analogues form mainly monofunctional and instrastrand bifunctional adducts with guanine and adenine, dinuclear and trinuclear platinum(II)

complexes are expected to form a plethora of long-range interstrand adducts with guanine. Intrastrand bifunctional adducts cause a local bending of the DNA strand. Long-range interstrand adducts cause a global change in the conformation of DNA. Differences in the nature of binding with DNA of dinuclear and trinuclear platinum(II) complexes as compared to that of cisplatin and its analogues, mean that that the compounds may be able to overcome multiple mechanisms of resistance that apply to cisplatin. Thus, the dinuclear complexes have been found to be highly active against a number of cisplatin-resistant cell lines.

A notable example of trinuclear complexes is BBR3464 that is found to be highly active against a large number of both murine and human cancer cell lines. BBR3464 consists of three *trans*-platinum units joined together two by 1,6-diaminohexane chains (Figure 2.9). As described later, the compound showed very high activity against a large number of cisplatin-resistant cancer cell lines. Since only the two terminals platinum units in BBR3464 undergo covalent binding (mainly interstrand) with DNA whereas the central platinum unit undergoes only noncovalent interactions such as hydrogen bonding and electrostatic interactions (Farrell and Spinelli 1999), it was hypothesized that although replacement of the central platinum unit with other suitable metal units might not significantly alter the covalent interactions of the terminal platinum units, it might have subtle effects on the noncovalent interactions such that anticancer active compounds with different spectrum of activity could result (Daghriri *et al.* 2001). Hence the present project in which a number of polunuclear complexes based on platinum and palladium have been synthesized, characterized and quantified for their antitumour activity including cell uptake, level and nature of binding with DNA. Four of the compounds (DH4Cl, DH5Cl, DH6Cl and DH7Cl) were based on BBR3464 in which the central platinum unit had been replaced by a corresponding palladium unit and length of the linking diamine had been

varied to contain from four to seven carbon atoms. In addition, a dinuclear complex (DHD) in which a *trans*-platinum unit and a *trans*-palladium unit are connected together by 1,6-diaminohexane and another trinuclear complex (DH1Cl) in which two terminal *trans*-platinum units and a central *cis*-dicloropalladium unit are connected together by two 1,6-diaminohexane chains had been prepared. All of the compounds show significant activity against human ovarian cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$. DH6Cl is found to be the most active compound. It has the lowest IC_{50} for all the cell lines. DH6Cl is found to be about nine times as active as cisplatin against the human ovary cell lines A2780, about seventeen times as active as cisplatin against $A2780^{cisR}$ cell line, and about four times as active as cisplatin against $A2780^{ZD0473R}$ cell line. The next most active compound is the dinuclear complex DHD which is found to be about two times as active as cisplatin against A2780 cell line, about four times as active as cisplatin against $A2780^{cisR}$ cell line and about two times as active as cisplatin against $A2780^{ZD0473R}$ cell line. For the melanoma cell line Me-10538, DH6Cl is found to be six times as active as cislatin and DHD is found to be nearly two times as active as cisplatin. The resistance factors for DH4Cl, DH5Cl, DH6Cl, DH5Cl, DH7Cl, DHD, DH1Cl and cisplatin as applied to the ovary cell lines A2780 and $A2780^{cisR}$ are respectively 1.1, 1.2, 5.2, 2.8, 3.8, 3.7 and 10.0 indicating that all of the compounds are better able to overcome cisplatin resistance in $A2780^{cisR}$ cell line than cisplatin. The observation that among the four BBR3464-based trinuclear compounds (DH4Cl, DH5Cl, DH6Cl and DH7Cl), DH6Cl (in which the linking diamine has six carbon atoms) has the highest activity against all of the cell lines (A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538) is in agreement with the structure-activity relationship formulated for polynuclear platinum-based drugs.

When uptake of DH4Cl, DH5Cl, DH6Cl and DH7Cl in A2780 cell line is considered, it is found that the uptake increases with the increase in the number of carbon atoms present in

the linking diamine chains. This observation coupled with low molar conductivity values observed for the solutions of the compounds in 1:1 mixture of DMF and water or in pure DMF, provide support to the idea that the molecules of the compounds (more precisely undissociated ionic aggregates) are able to cross the cell membrane by passive diffusion. It may be noted that as the number of carbon atoms present in the linking diamine increases, the lipophilicity of the molecule increases and hence the increase in the rate of diffusion with the increase in the size of the molecules. Time course experiments on cell uptake for all the trinuclear compounds (done only for DH4Cl and DH6Cl in this study) would provide more light on the matter. When uptake of DH4Cl, DH5Cl, DH6Cl and DH7Cl in $A2780^{cisR}$ cell line is considered, it is found that the uptake is highest for DH6Cl (which is about three times that of the other trinuclear compounds). When uptakes in A2780 and $A2780^{cisR}$ cell lines are compared, it is found that for DH6Cl the uptake in the cisplatin-resistant cell $A2780^{cisR}$ is greater than that in the cisplatin-sensitive cell line A2780, whereas the converse is true for the other trinuclear compounds. That DH6Cl rather than DH7Cl has highest uptake in $A2780^{cisR}$ cell line, could be the result of reduced efflux of DH6Cl so that net accumulation of platinum is highest for the compound. The results suggest that DH6Cl had been better able to overcome mechanisms of resistance operating in $A2780^{cisR}$ cell line, especially the one that is associated with the increased efflux from the cell. For the dinculear compound DHD also, it was found that the uptake in $A2780^{cisR}$ cell line was greater than that in A2780 cell line. As in the case of DH6Cl, this may be the result of reduced efflux. Since the anticancer activity of polynuclear platinum complexes is believed to be associated with the formation of a plethora of interstrand GG adducts causing global changes in the conformation of DNA, the extent of binding of DH4Cl, DH5Cl, DH6Cl, DH7Cl and DHD with cellular DNA was determined as applied to A2780 and $A2780^{cisR}$ cell lines. It was found that for all the compounds

including cisplatin, the level of platinum-DNA binding was less in the resistant cell line $A2780^{cisR}$ than in the responsive cell line A2780, in line with their lower activity in the resistant cell line. The results are in agreement with the previously published values in which it was found that the lower activity of BBR3464 in U2-OS/Pt cell line as compared to that in U2-OS cell line corresponded to a lower level of Pt-DNA binding in the former than in the latter (Perego *et al.* 1999a). It should be seen that for the trinuclear complexes: DH4Cl, DH5Cl, DH6Cl and DH7Cl, the order of Pt-DNA binding in A2780 and $A2780^{cisR}$ cell lines was mostly in line with that of the Pt-DNA binding level. An increase in the level of Pt-DNA would mean a greater number of interstrand adducts and consequently a greater conformational change in DNA resulting into a greater activity.

For the dinuclear complex DHD, even though the level of Pt-DNA binding was found to be lower than that for DH5Cl, DH6Cl and DH7Cl, it was found to have a greater activity than all the trinuclear complexes except DH6Cl against both A2780 and $A2780^{cisR}$ cell lines. This may be due to a greater tendency to form interstrand adducts by dinuclear complexes than their trinuclear counterparts. It may be noted that dinuclear platinum complexes were reported to be more effective than the trinuclear complex BBR3464 at inhibiting ethanol induced B $\rightarrow$ A transition in CT DNA which was considered to be due a greater percentage of interstrand adducts (McGregor *et al.* 1999) formed by the dinuclear complexes.

Interaction between trinuclear compounds (DH4Cl, DH5Cl, DH6Cl, and DH7Cl), dinuclear compound (DHD) with pBR322 plasmid DNA and salmon sperm DNA combined with BamH1 digestion showed that all of the compounds were able to cause global changes in the conformation of DNA. As a result, mobility of DNA bands through the gel was found to change. Also an increase in prevention of cutting of pBR322 plasmid

DNA by BamH1 with the increase in concentration of the compounds was observed. These observations also, may be considered to support the idea of formation of a range of interstrand GG adducts (dictated by the sequence of nucleobases) between the polynuclear compounds and the DNA, thus inducing global changes in its conformation. Binding of the compounds with DNA was also found to cause DNA breakdown especially at high concentrations of compounds. This is believed to be the result of covalent binding.

Molecular modeling analyses show that the central palladium unit in DH6Cl is better positioned within helix (avoiding short repulsive contacts) than that in DH4Cl, DH5Cl and DH7Cl, indicating that DH6Cl would be able to bind to DNA more strongly than the other trinuclear complexes.

HPLC results show that DH6Cl binds more strongly with guanine than adenine forming

1 : 2 (drug : NB) adduct, in line with the idea that the polynuclear complexes are expected to bind to N7 centres of guanine in the DNA.

Finally, it can be seen that the present study gives support to the idea that new tumour active polynuclear compounds could be found by replacing the central platinum centre in BBR3464 with other suitable metal centres.

What is next?

It is clear that some of the compounds of the present study show significant activity against a number of cancer cell lines such that the most active compound namely DH6Cl has the potential to develop into an anticancer drug. The next step would be to determine the toxicity profile of the lead compound using suitable animal models eg human xenograft mouse models.

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