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Studies on new tumour active platinum compounds

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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.

Jehad Moh'd Al-Shuneigat

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Contents

Abstract

Four mononuclear *trans*-planaramineplatinum complexes code named JH1, JH2, JH3, and JH4, and two trinuclear complexes code named JH5 and JH6 have been prepared and characterised based on elemental analyses, IR, mass and ^{1}H NMR spectral measurements. The activity of the compounds against human ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R}, cell uptake, level of binding with DNA and nature of interaction with pBR322 plasmid DNA and salmon sperm DNA have been determined.

Among the mononuclear complexes JH1 is found to be most active: four times more active than cisplatin in the resistant cell line A2780^{cisR} and six times more active in the resistant cell line A2780^{ZD0473R}. Between the two trinuclear compounds, JH6 is found to be more active than JH5 in all three ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R}. JH6 is also more active than cisplatin in all the ovarian cell lines A2780, A2780^{cisR} and A2780^{ZD0473R}: two times more active than cisplatin in the parent cell line A2780, three times more active in the resistant cell line A2780^{cisR} and about two times more active in the resistant cell line A2780^{ZD0473R}. All of the designed complexes have lower resistance factors than cisplatin as applied to the resistant cell lines, indicating that at the level of their activity the compounds have been better able to overcome mechanisms of resistance operating in A2780^{cisR} and A2780^{ZD0473R} cell lines.

Among the mononuclear complexes, JH1 has the highest level of binding with DNA in all three ovarian cancer cell lines following 24 h of incubation, in line with the highest activity of the compound. The higher cytotoxicity of JH6 over JH5 is not found to be in line with the corresponding platinum-DNA binding levels. However, binding of JH6 to

DNA is believed to be more sequence dependent than that of JH5, influencing downstream processes such as protein recognition, cell cycle arrest, DNA repair and other cellular events; this may explain why JH6 is more cytotoxic than JH5. While JH1, JH2, JH3 and JH4 are expected to form mainly monofunctional adducts, 1,2-interstrand adduct, and 1,3-intrastrand adducts with DNA, JH5 and JH6 are expected to form long-range intra- and inter-strand adducts. Covalent and non-covalent interactions associated with the formation of the adducts would cause more of a global change in DNA conformation and also DNA damage as is observed in the interaction of increasing concentrations of the compounds with pBR322 plasmid DNA.

Abbreviations

AAS	Atomic absorption spectrophotometry
CBCD	*Cis*-diamine-1,1-cyclobutanedicarboxylateplatinum
CDKs	Cyclin dependant kinase
CDKIs	Cyclin dependant kinase inhibitor
Cis-DDP	Cisplatin
C5-OHP-Cl	*Trans*-bis(n-valerato)(1R,2R-cyclohexanediamine)(oxalate) platinum(IV)
DACH	1,2-diamminocyclohexane
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FACT	facilitates chromatin transcription.
FCS	Foetal calf serum
FDA	Food and Drug Authority (USA)
G0	Gape phase 0
G1	Gape phase 1
G2	Gape phase 2
GF	Growth factors
GS	Growth signal
GSH	Glutathione
IC_{50}	Concentration required to inhibit cell growth by 50%
IR	Infrared spectrum
JH1	*Trans*-(thiazole)(amine)dichloroplatinum
JH2	*Trans*-bis(thiazole)dichloroplatinum
JH3	*Trans*-{(imidazo)(thiazole)dichloroplatinum
JH4	*Trans*-bis-{(3-hydroxypyridine)(thiazole)dichloroplatinum

JH5	[{*Trans*-$PtCl(NH_3)_2$}$_2$-{*trans*-Pt(thiazole)$_2$}-{$H_2N(CH_2)_6NH_2$}$_2$]$Cl_3(NO_3)$
JH6	[{*Trans*-$PtCl(NH_3)_2$}$_2$-{*trans*-Pt(thiazole)NH_3}-{$H_2N(CH_2)_6NH_2$}$_2$]$Cl_3(NO_3)$
JM118	*Cis*-aminedichloro(cyclohexaylamine)platinum
JM216	Bis-aceto-aminedichloro-cyclohexylamine-platinum(IV)
JM335	*Trans*-amine(cyclohexylaminedichloro-dihydroxo)platinum(IV)
M	Mitosis phase
Mdm2	Murine Double Minute-2
MDR	Multidrug drug resistance proteins
MMR	Mismatch repair
MT	Metallothionein
MTT	3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide
NER	Nucleotide excision repair
NMR	Nuclear Magnetic Resonance
NSCLC	Non-small cell lung cancer
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPA	Replication Protein A
S	Synthesis phase
SAR	Structure activity relationship
SCLC	Small cell lung cancer
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
Trans-DDP	Transplatin
TWI	Tumour weight inhibition
UV	Ultraviolet
XPA	Xeroderma pigmentosum group A
XPC	Xeroderma pigmentosum group C
XPF	Xeroderma pigmentosum group F

XPG	Xeroderma pigmentosum group G
ZD0473	C*is*-aminedichloro(2-methylpyridine)platinum

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1. Chapter One

1.1. Cancer: Introduction to cancer

Cancer is one of the deadliest diseases for humans. In the USA, it is the leading cause of death among children (accidents exempted) and is expected to be so for adults in the near future (Collins and Workman, 2006). There are more than 100 types of cancer that differ in many characteristics including growth rate, causes, invasiveness and response to treatment (Pecorino, 2005; Ruddon, 2007). Among cancer deaths in the USA, the leading cause of death in both males and females is lung cancer followed by that of the digestive tract (Oh et al., 2007). In males the next most prevalent cancer is that of the prostrate whereas in females it is breast cancer.

Cancer is a genetic disease characterized by uncontrolled cell division and metastasis (Pecorino, 2005). Mutations to genes that control cell growth and replication including: apoptosis genes, repair genes, oncogenes, suppressor genes, or genes that regulate them may lead to the transformation of normal cells to tumour cells. As the tumour cells continue to divide bypassing apoptosis, balance between cell multiplication and cell death is lost resulting in an increase in tumour cell population. The mass of cells with mutated genes in an organ or tissue is known as a tumour (Ross, 1998; Hanahan and Weinberg, 2000; Bertram, 2001; Hill, 2001; Mautner and Huang, 2003; Hoogervorst et al., 2005; Kohno et al., 2005; Collins and Workman, 2006; Weinberg, 2007).

There are two types of tumours. The first type known as malignant tumour or cancer is characterised by invasiveness and metastasis. The second type known as benign or non-cancerous tumour is localised (Pecorino, 2005) .

The development of cancer takes place in three distinct stages termed: initiation, promotion, and progression. In the initiation stage, the DNA of the cell undergoes substantial permanent change due to the effect of an initiation agent. In the promotion stage, the cells with damaged DNA start dividing through the effect of promoters. In the progression stage, the number of the cells with damaged DNA increases dramatically; this increases genetic changes and instability associated with increased invasions (Pitot, 1986; Hill, 2001; Pecorino, 2005). Even though cancer may originate in one organ, in time it may spread to other parts of the body. However, cancers are named according to the tissue from which the cancerous cells originate. For example lung cancer may spread to the brain or kidney or other parts of the body but still is called lung cancer.

1.1.1 Transformation and development of cancer cells

It is believed that between 2-6 genes mutations are required for a cell to be transformed to malignant ones (Hill, 2001). It is generally accepted that tumours are monoclonal growth rather than polyclonal. Biochemical and genetic markers studies on tumour populations show that cells in a tumour populations are identical, thus providing support to the idea that tumours originate from a single cell (Weinberg, 2007).

It is believed that normal human cells undergo the following functional alterations to be transformed into malignant cancer cells: (1) generation of own growth signal, (2) resistance to growth inhibition, (3) ability to escape apoptosis, (4) uncontrolled proliferation, (5) induction and sustainment of angiogenesis, (6) metastasis and invasiveness (Figure 1.1) (Hanahan and Weinberg, 2000).

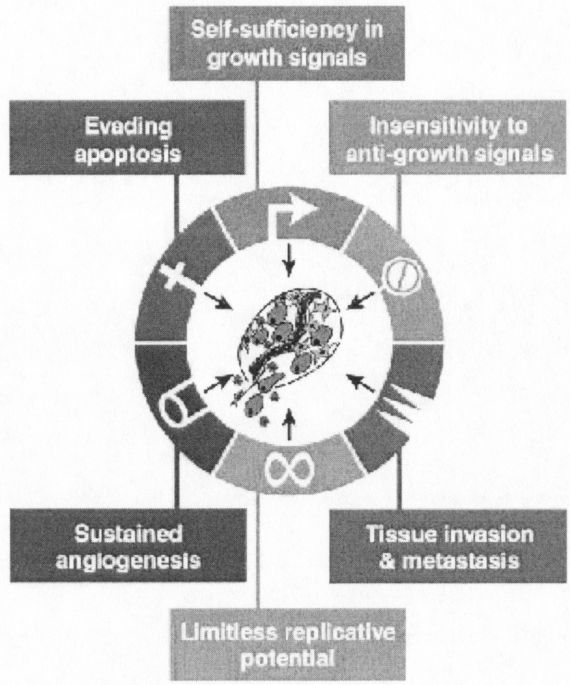


Figure 1-1 Acquired Capabilities of Cancer

1.1.1.1 Generation of own growth signal

Cell signalling circuits transmit growth, antigrowth, differentiation, and live or die signals. It is expected that within the next decade or so it will be possible to graph the full cellular signalling network (Figure 1.2), (Hanahan and Weinberg, 2000). Once the full cellular signalling pathway is known, it will be possible to test the effect of each genetic defect and also that of anticancer drugs on cell signalling. Undoubtedly, this would be a great step forward towards understanding the mechanisms of cancer and the means for cancer treatment (Hanahan and Weinberg, 2000).

Growth factors are essential for the survival of cells. When they bind to their transmembrane receptors, growth signals are transmitted across the cell membrane through activation of different signalling pathways; without these signals cells would

die (Hanahan and Weinberg, 2000; Talapatra and Thompson, 2001; Bianco et al., 2006).

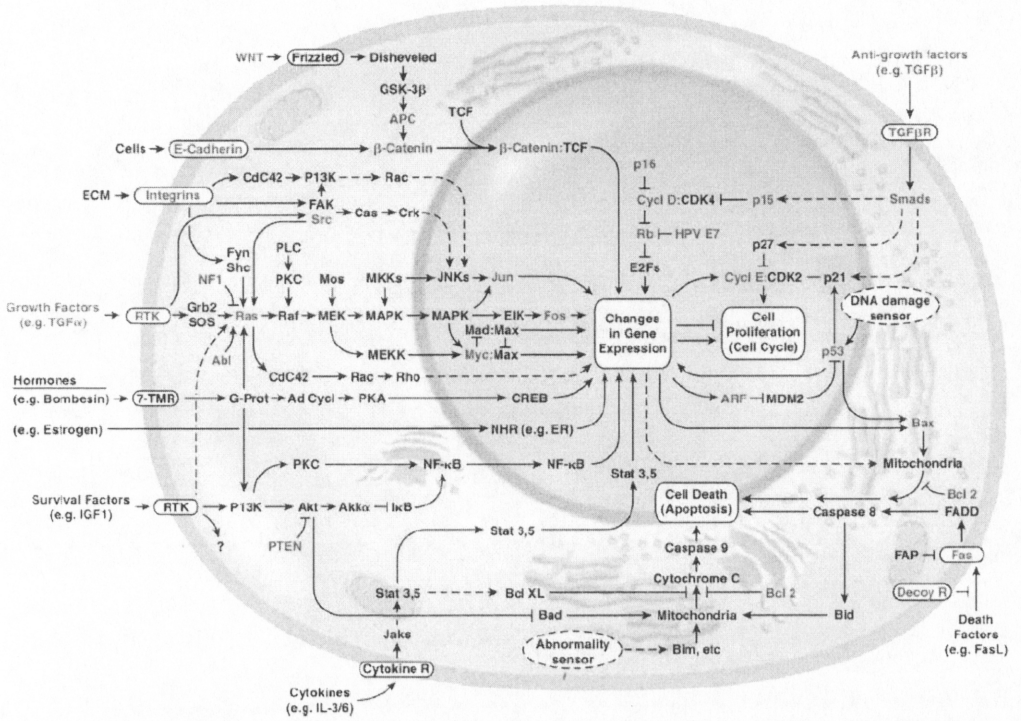


Figure 1-2 Cellular signalling networks

Cells in the human body also require mitogenic growth signals (Scheeff et al., 1999) for proliferation; no cell can proliferate without these signals. Mitogenic growth factors produced in some cells, stimulate proliferation of some other cells. As tumour cells can produce many of their own mitogenic growth factors (GFs), their reliance on exogenous growth stimulation is lowered. For example, glioblastomas and sarcomas produce PDGF (platelet-derived growth factor) and TGFa (tumour growth factor a) respectively (Weinberg, 1994; Hanahan and Weinberg, 2000; Gale, 2003; Mautner and Huang, 2003; Hooper, 2005; Bianco et al., 2006).

It is believed that tumour cells achieve self-sufficiency in mitogenic growth signals (Scheeff et al., 1999) through molecular alterations to signalling pathways, by employing one or more of the following strategies:

(a) Deregulation of cell surface growth receptors: As many of cell surface growth receptors are over-expressed in cancer cells, the cells become oversensitive to very low levels of GFs that would not trigger proliferation in normal cells. For example, the epidermal GF receptor (EGF-R/*erbB*) is up-regulated in stomach, brain, and breast tumours (Hanahan and Weinberg, 2000; Bianco et al., 2006).

(b) Over-expression of extracellular matrix receptors: In tumour cells extracellular matrix receptors may be over-expressed. The receptors link cells to extracellular matrix (ECM) that transmits pro-growth signals, resistance to apoptosis, and cell proliferation. The signals can also activate the SOS-Ras-Raf-MAP kinase pathway (Hanahan and Weinberg, 2000; Gale, 2003).

(c) Alteration to growth signalling pathways: Alteration to growth signalling pathways in the cytoplasm circuitry, especially the SOS-Ras-Raf-MAP cascade (that receives and processes the signals produced by GF receptors) results in release of mitogenic signals, without stimulation from the usual sources.

1.1.1.2 Resistance to growth inhibitor

Inhibitors that are able to block cell proliferation are found in the extracellular matrix and on the surface of neighbouring cell. In cancers, cells can become resistant to growth inhibitors due to loss of tumour suppressor genes (Nebert, 2002; Hooper, 2005).

1.1.1.3 Ability to escape apoptosis

Cancer cells multiply due to increased rate of proliferation bypassing apoptosis (that may be defined as the programmed cell death). The apoptotic pathway is present in each cell of our body; once activated by different signals it leads to cell death. The life or death of a cell relies on signals received through survival receptors such as IGF-1R and IL-3 present on the surface of the cell and the death receptors FAS and TNFα. Disruption of the FAS death receptors and mutation of p53 and other tumour suppressor genes play a crucial role in the cancer cell avoiding apoptosis (Hanahan and Weinberg, 2000; Nebert, 2002; Hooper, 2005).

1.1.1.4 Uncontrolled proliferation

Resistance to growth inhibitor, the ability to generate their own growth signal and escape of apoptosis, all lead to uncontrolled proliferation of cancer cells (Hanahan and Weinberg, 2000; Nebert, 2002; Hooper, 2005).

1.1.1.5 Induction and sustainment of angiogenesis

The process of formation of new blood vessels in a tumour is known as angiogenesis (Cao, 2004; Bianco et al., 2006; Bhat and Singh, 2008). Angiogenesis is important for delivery of nutrients to tumour cells and progression of a pre-malignant lesion to a fully invasive tumour (Bianco et al., 2006; Bhat and Singh, 2008). The process of angiogenesis, shown in Figure 1.3 (Van Horssen et al., 2006), can be divided into four stages. In stage 1, the latent tumour makes the angiogenic switch.

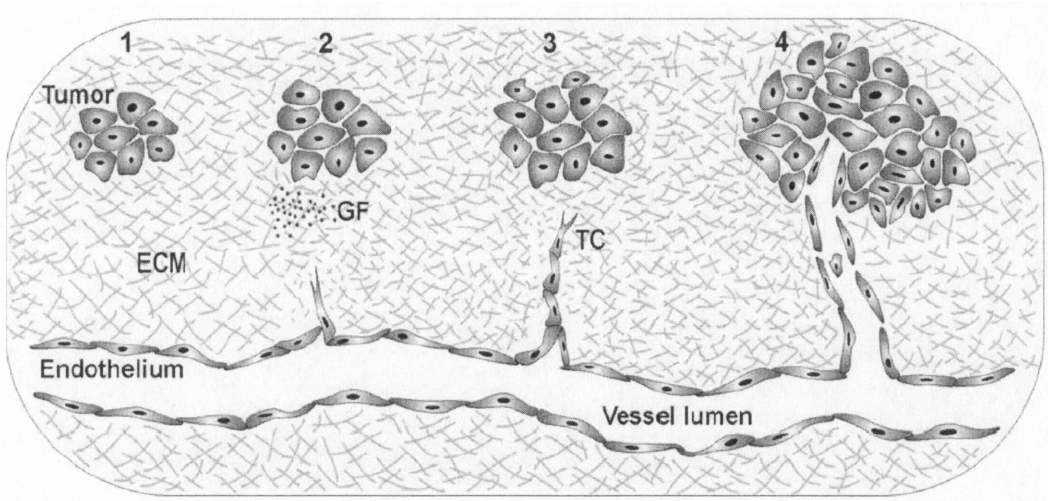


Figure 1-3 The sequential steps during tumour angiogenesis

In stage 2, when the size of the tumour reaches 0.5 to 1.0 mm in diameter it starts producing growth factors such as vascular endothelial growth factor which activates the endothelial cells living adjacent to blood vessels. Once activated the endothelial cells start to proliferate and migrate towards the tumour. In stage 3, endothelial cells sprout to develop into endothelial tip cells. Finally in stage 4, the sprout forms a lumen and the tumour becomes connected to the vascular system (Hanahan and Weinberg, 2000; Hill, 2001; Van Horssen et al., 2006).

The blood vessels formed in a tumour are different to those in normal blood vessels. They are leaky, their capillaries are longer, there are no differences between arterioles and venules, and the associated lymphatic vessels do not function properly. These characteristics lead to increased fluid pressure and restrict the flow of fresh blood within the tumour which could affect the outcome of chemotherapy as a result of altered delivery (Hill, 2001; Cao, 2004).

1.1.1.6 Metastasis and invasiveness

Metastasis is the main cause (90%) of human cancer death. It is the last stage in the development of cancer. This refers to the ability of the cancer cells to spread away from the primary tumour and invade other parts of the body (Hooper, 2005; Stafford et al., 2008). The main way of transport for metastatic spread is through blood vessel and lymphatic channels. Although the exact mechanisms of invasion and metastasis remain unclear, it is known that there are two main events in metastasis. The first involves changes in attachment of cells and the second includes activation of extracellular proteases (Hill, 2001; Mehlen and Puisieux, 2006). In normal tissue, cells interact with each other and with the extracellular matrix (ECM). Cells produce adhesion substances known as integrins and cadherins. The former is involved in cell to ECM interaction whereas the latter is involved in cell to cell interaction (Evan and Vousden, 2001; Hill, 2001; Brunton et al., 2004; Mehlen and Puisieux, 2006). Cadherins are mutated in most of the epithelial cancers and this plays a crucial rule in enhancing invasiveness and metastasis. Conversely, restoration of cadherins has been found to weaken the invasion and metastatic process (Hanahan and Weinberg, 2000; Hill, 2001; Brunton et al., 2004). The activation of extracellular protease facilitates the invasion of cancer cells by promoting degradation of ECM.

Cancer cells characteristics:

Tumour cells differ from normal cells in a number of characteristics including:

(1) Cell size: Tumour cells are either abnormally large or abnormally small compared to normal cells (Martini, 2004),

(2) Nuclear size: Nuclear size in tumour cells is much larger than that in normal cells and it has irregular chromatin distribution (Hill, 2001; Drescher and Lynch, 2005),

(3) Ability to grow in suspension: Unlike normal cells tumour cells are able to grow in suspension (Hill, 2001) and

(4) Specialization: Cancer cells are less differentiated than normal cells (Mautner and Huang, 2003).

1.1.2 Genes and cancer

Cancer is predominantly a disease stemming from genetic changes. In the past, viruses were thought to be responsible for cancer (as many common infections are caused by viruses). Currently it is believed that cervical carcinoma, hepatomas, some head and neck cancer, kaposi's sarcoma, some anal cancers, and Burkitts lymphoma are caused by viruses. 99.7% of cervical tumours have fragments of papillomavirus genome integrated to the host cell genome (Weinberg, 2007).

It has been shown that the mutagenic function of carcinogens is related to their ability to cause genetic changes including translocation, amplification, mutations, deletions and abnormal gene regulation to proto-oncogene and tumour suppressor genes (Diamandis, 1997).

The risk factors for cancer may be classified under the headings environment and heredity. It is estimated that environmental factors are responsible for 50%-80% of cancer while about 5-10% of all solid tumours and a smaller portion of leukaemia and lymphoma can be related to inherited genes (Hill, 2001; Rubin et al., 2001; Rieger, 2004). Environmental factors include smoking (30%), diet (30%), sexual and reproductive patterns (7%), industrial products (5%), alcohol consumption (3%), sunlight exposure (3%), and drugs and medical procedures (especially X-rays) (1%) (the numbers in parentheses indicate the weight of the factors) (Ruddon, 2007).

The growth of normal cells is regulated by a very fine balance between growth promoting proto-oncogenes and growth suppressor genes. When suppressor genes are lost through mutations or some other means, the negative effect on cell growth ceases to exist resulting in uncontrolled cell proliferation. This is often facilitated by inactivation of DNA repair genes that leads to genetic instability (Weinberg, 1994; Collins and Workman, 2006) so that proto-oncogenes and tumour suppressor genes are the most prominent genes that are involved in cancer.

1.1.2.1 Proto oncogene and oncogene

Proto-oncogenes normally promote cellular growth and differentiation (including gene expression, DNA synthesis, cytoskeleton membrane changes and cell-to-cell metabolism), encode and regulate response to external signals, kinase signal, transduction and transcription factors that control other genes. When cell growth is completed, proto-oncogenes are turned off (Diamandis, 1997; Hill, 2001; Perry, 2001; Ponder, 2001; Mautner and Huang, 2003; Hoogervorst et al., 2005).

Oncogenes are the mutated forms of proto-oncogenes (Ponder, 2001) that promote overproduction of growth factors or increase their activity (Beer and Pitot, 1989; Weinberg, 1994; Diamandis, 1997; Mautner and Huang, 2003; Sherr, 2004; Pecorino, 2005). Oncogenes also disrupt the activity of cyclins and cyclin-dependent kinases (CDKs); disruption of cyclin-dependent kinases distracts the cell cycle checkpoint leading to uncontrolled cell division of the mutated cells (Mautner and Huang, 2003).

Proto-oncogenes get converted to oncogenes through mutation and gene amplification and translocation. The most common inducers of mutation are viral infections and chemicals (Perry, 2001). Gene amplification occurs when a certain region of the chromosome undergoes several rounds of DNA synthesis in the same cell cycle.

Cancer has always been found to be associated with amplifications of oncogenes. For example, ERBB2 amplification is always found in breast and ovarian cancers (Hill, 2001).

Translocation is the movement of a fragment of chromosome from one chromosome to another. As applied to some genes, it may lead to changes or lack of control of expression. For example, in Burkitt's lymphoma MYC oncogene initially found at chromosome 8 is translocated to chromosome 2, 14, or 22.

1.1.2.2 Tumour suppressor genes

When proto-oncogenes were discovered, it was thought that cells must have some other genes that would counter the effect of proto-oncogenes (Sherr, 2004). Tumour suppressor genes regulate many functions in cells including cell cycle, recognition of DNA damage and its repair and protein degradation. It is not clear why tumour suppressor genes are associated with some cancer but not with others (Sherr, 2004).

In tumour suppressor genes, only one allele of the gene pair is enough to function as a suppressor. This means that to lose suppressor function both alleles of a tumour suppressor gene pair have to be mutated (Sherr, 2004).

There are three characteristics of tumour suppressor genes: (1) they are all recessive, (2) they play a major role in inherited tumour and (3) they are mostly mutated and inactivated (Sherr, 2004). The most known and studied tumour suppressor gene is p53. As applied to tumour formation, whereas oncogenes may be considered as accelerators, tumour suppressor would be like brakes.

1.1.2.2.1 Cellular role of p53

p53, often called the guardian of the genome, is the most studied tumour suppressor gene. p53 protein is made up of 393 amino acids and is not important for cell growth or development (Prives and Hall, 1999; Cadwell and Zambetti, 2001; Hill, 2001; Vousden, 2001; Soussi and Lozano, 2005). However, p53 is a transcriptional factor that regulates the expression of more than one hundred genes (Adimoolam and Ford, 2003; Oren, 2003; Ford, 2005; Yu and Zhang, 2005). A transcription factor can be defined as a molecule that binds to the promoter or enhancer part of a gene to regulate the expression of that gene (Nebert, 2002). There are many internal and external stress signals that activate p53 including DNA damage, heat shock, hypoxia, hyperoxia, growth factors, metabolic change, anchorage, cell-cell contact, and activated oncogenes (Prives and Hall, 1999; Haupt et al., 2003; Ford, 2005; Harris and Levine, 2005; Schuler and Green, 2005).

The main role of p53 in the cell, as shown in Figure 1.4 (Brabec and Kasparkova, 2005), is to regulate the cellular response to internal and external stress signals through regulation of cell cycle arrest and apoptosis (Prives and Hall, 1999; Ford, 2005; Harris and Levine, 2005; Schuler and Green, 2005; Zamzami, 2005). The regulations include cell cycle checkpoints, differentiation, and DNA repair including NER proteins (Schwartz and Rotter, 1998; Lowe, 1999; Adimoolam and Ford, 2003; Oren, 2003; Eastman, 2004; Ford, 2005). p53 is the most mutated gene in human cancer (Hoogervorst et al., 2005). It has been found that p53 has 75%-80% mutations or abnormalities in colon tumours alone (DeVita et al., 1993; Reles et al., 2001; Adimoolam and Ford, 2003) and between 50-60% of all human cancers (Chang et al., 2000; Cadwell and Zambetti, 2001; Hofseth et al., 2004; Soussi and Lozano, 2005).

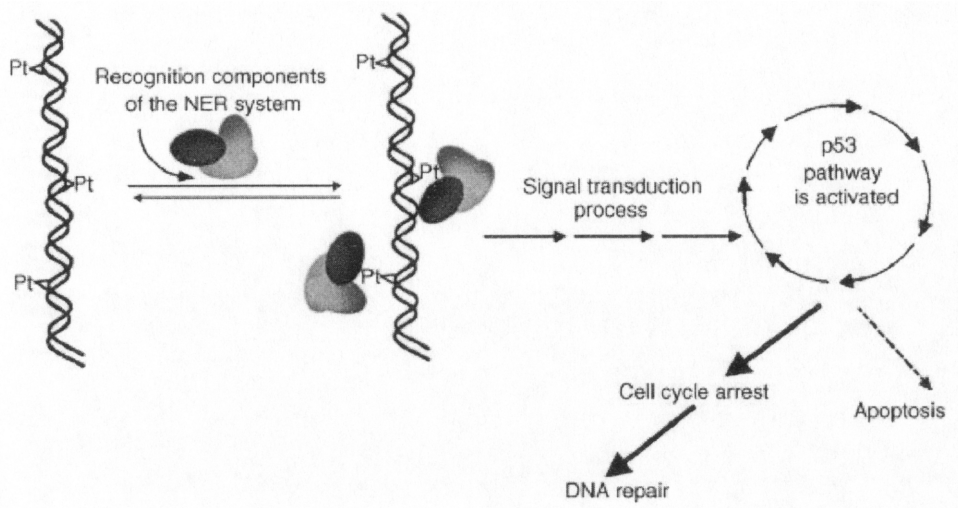


Figure 1-4 Role of p53 in the mechanism of resistance/sensitivity of tumours to cisplatin

Mutations in p53 lead to survival of cells with damaged DNA that may lead malignant transformation (Xu and El-Gewely, 2001). Around 90% of p53 mutations are point mutations resulting from DNA replication errors and environmental mutagens (Michalak et al., 2005).

p53 plays a crucial role in cellular response to chemotherapy and radiotherapy (Lowe, 1999). Chemotherapy and radiation cause DNA damage which could lead to p53 activation and apoptosis (Lowe, 1999). For this reason cells with mutated p53 are more resistant to chemotherapeutic agents than cells with wild type p53 (Cadwell and Zambetti, 2001; Mashima and Tsuruo, 2005). The loss of p53 function in mouse cell fibroblasts is found to reduce apoptosis and produce multi-drug resistant cells (Lowe, 1999). In addition, Reles *et al* found that p53 alterations caused resistance to platinum-based chemotherapy, early relapse, and shortened survival in ovarian cancer patients (Reles et al., 2001).

Beside p53, Bcl-2 has been found to play a role in radiotherapy and chemotherapy response. Mackey *et al* found that prostate cancer patients with an elevated Bcl-2/Bax ratio are at great risk of radiotherapy failure and suggested that Bcl-2/Bax ratio could be used as a marker of therapeutic response in prostate cancer patients (Mackey et al., 1998).

1.1.2.2.2 Regulation of p53 protein production

Unlike other cell cycle proteins, p53 protein is expressed at very low levels in normal cells because it is extremely unstable and rapidly degraded (Vousden, 2001). Murine Double Minute-2 (Mdm2) protein which is a product of proto-oncogenes causes the degradation of p53 by the ubiquitin-proteasome system. In addition to that, Mdm2 binds directly to p53 causing its inactivation (Prives and Hall, 1999; Vogt Sionov and Haupt, 1999; Oren, 2003).

p53 has a half-life between 10-20 minutes. Under stress conditions stabilizations and translation of p53 increase through post-translational modification. Post-translational modification, like phosphorylation, modifies p53 so that it is no more recognised by Mdm2 (Vogt Sionov and Haupt, 1999; Chang et al., 2000; Haupt et al., 2003; Lukas et al., 2004). Phosphorylation of p53 protein by JNK (c-jun kinase) and ATM kinase, greatly inhibits the interaction between p53 and Mdm2, thus increasing the half life of p53 and its accumulation in cells (Prives and Hall, 1999; Cadwell and Zambetti, 2001; Harris and Levine, 2005).

1.1.2.2.3 p53, life or death decision

Once activated, stabilized p53 accumulates in the nucleus and causes cell cycle arrest at G1 or G2 phase to permit DNA repair. If DNA damage is beyond repair, p53 will activate the expression of numerous pro-apoptotic genes, for example BAX, Bak, PUMA, BID, CD95, APAF-1, p53AIP1 that leads to apoptosis (Schwartz and Rotter, 1998; Lowe, 1999; Prives and Hall, 1999; Xu and El-Gewely, 2001; Chipuk and Green, 2006). In addition, p53 binds directly to anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) causing its inactivation (Zamzami, 2005; Chipuk and Green, 2006).

p53 controls G1 arrest through the activation of p21 which inhibits the activity of cyclin-dependent kinase (cdk). p53 also binds and inactivates kinase cyclin H/CDK7/MAT1. Both of these processes inhibit the phosphorylation of retinoblastoma (pRb) protein that causes the release of E2F-1 leading to cell arrest at G1 (Xu and El-Gewely, 2001; Ding and Fisher, 2002; Michalak et al., 2005).

It is believed that the activation of p21 causes cell cycle arrest at G1 phase, thus protecting the cell from apoptosis (Gartel and Tyner, 2002). It has been found that cells with mutated p21 have a defect in p53-induced cell cycle arrest (Michalak et al., 2005). On the other hand p53 controls G2 arrest through the activation of 14-3-3σ. Active 14-3-3σ binds with phosphorylated Cdc2-cyclin B1 in the cytoplasm resulting in the inhibition of nuclear importation of Cdc2-cyclin B1 and consequently cellular arrest at G2 phase. p53 can also arrest cells at G2 through the activation of Gadd45, and down regulating of Cdc2 (Xu and El-Gewely, 2001). When p21 or 14-3-3σ is removed, cells choose apoptosis over cell cycle arrest (Yu and Zhang, 2005). When DNA damage is beyond repair, p53 gets the cell out of the arrest state through up regulation of cell cycle progression factors like Cdc25C or through down regulating

cell cycle inhibitors such as weel. p53 inhibits the transcription of Bcl-2 and at the same time activates the transcription of Bax leading to the release of cytochrome C from mitochondria and causing apoptosis (Xu and El-Gewely, 2001). However, the mechanism by which p53 chooses between growth arrest and apoptosis is not known. Extensive research efforts show that several factors including the following may influence the choice:

(1) Cell type, cell response to Fas-Fas ligand signalling pathway, different ability to induce Bax, and the efficiency of DNA repair.

(2) Oncogenic composition of the cell, the Rb/E2F1 balance, Bax/Bcl-2 ratio, and growth and survival factors.

(3) The intensity of the stress conditions.

(4) The level of p53 expression and its interaction with specific proteins. Low level of p53 expression has been found to have anti-apoptotic activity, while high level of p53 induces apoptosis (Vogt Sionov and Haupt, 1999).

The Bcl-2 family plays a major role in regulating apoptosis pathway either in promoting or suppressing apoptosis (survival proteins). Bcl-2 family consists of cell survival proteins including: Bcl-2, Bcl-x_L, Bcl-w, A1, Mcl-1, and apoptosis proteins including: Bax/Bak, and BH3 proteins (Coultas and Strasser, 2003; Rapp et al., 2004). It is believed that the interaction between survival promoting and apoptosis promoting members of Bcl-2 regulate cell death (Coultas and Strasser, 2003).

In addition to the above many researchers believe that cell environment plays a crucial role in the life or death decision. When intracellular and extracellular survival signals are present, normally in the form of molecules involved in cell-to-cell signalling and cell-matrix interaction, cells will be resistant to p53 apoptosis. On the other hand in the absence of survival signals it is more likely that p53 will initiate

apoptosis (Oren, 2003; Pulido and Parrish, 2003; Vermeulen et al., 2003; Ohmichi et al., 2005).

1.1.3 Cancer treatment

Currently cancer is curable and preventable in many cases. It is estimated that about two third of cancer can be prevented through changes in life style including avoidance of smoking and drinking, and dietary alterations.

Cancer is treated by surgery, radiation, and chemotherapy, alone or in combination. The choice of treatment depends on cancer stage, type, treatment success, and patient's age and health (Rubin et al., 2001).

Staging is a system that defines the extent of cancer spread. It is an important step in the management of cancer as it will determine the type of treatment needed (Rubin et al., 2001). The TNM staging system is the approved system for cancer staging and classification by the International Union Against Cancer. According to this system the tumour is classified into three categories T, N, and M:

(1) T represents the extent of primary tumour. It is divided into T1, T2, T3 and T4 depending on the extent of cancer. T4 is considered a massive lesion that has invaded other organs and in general it is hard to treat.

(2) N represents the effect of cancer on lymph nodes. It is sub-classified in to N0, N1, N2 and N3. In N3 the lymph nodes are fixed by the tumour to surrounding structures and the size of lymph nodes is usually 6 cm or greater.

(3) M stands for metastasis. Presence of metastases is indicated by M+ and its absence is indicated by M- (Rubin et al., 2001).

1.1.3.1 Surgery

Surgery is the cornerstone of treatment for most solid cancers that are confined to the organ of origin. However, it may not deal with micrometastatic disease and therefore chemotherapy, radiotherapy or hormonal therapy is often given before or after definitive surgery (Cosaert and Quoix, 2002; Drescher and Lynch, 2005).

1.1.3.2 Ionizing Radiation

Ionizing radiation is very effective in killing cancer cells. When a molecule absorbs electromagnetic radiation of sufficient energy, it may undergo electronic excitation or ionization (Williams et al., 2001). In the case of ionization, the excited electrons may be considered to be shifted to infinitely high energy levels. When molecules lose electrons, molecular ions or free radicals are formed that are very reactive. DNA (more precisely, electron-rich nucleobases in DNA) is the main target of ionizing radiation. Ionizing radiation can also damage water molecules in cells leading to the formation of reactive oxygen species (ROS). ROS produced cause DNA damage including strand breakage and loss of nucleobases. DNA damage leads to cell death through apoptosis or necrosis (Dewey et al., 1995; Williams et al., 2001; Pecorino, 2005). One of the disadvantages of ionizing radiation is that it may kill both normal and cancer cells. However, the physiological differences between cancer cell and normal cell and advances in radiation therapy (such as Intensity Modulation Radiotherapy Technique abbreviated as IMRT's) have made it possible to specifically target tumour cells while leaving normal cells largely unaffected.

Clinical radiation is divided into two categories: curative therapy and palliative therapy.

(1) **Curative therapy** is high dose radiotherapy for a select number of cancers which can lead to complete eradication of the tumour cells leading to cure. It is applied to patients who have a chance of making full recovery. In most cases curative therapy is associated with acute side effects (Rubin et al., 2001).

(2) **Palliative therapy** is applied to patients with incurable disease. The main aim is to minimize cancer symptoms and improve quality of life. Normally there are very low rates of side effects (Rubin et al., 2001).

1.1.3.3 Chemotherapy

Chemotherapy involves the use of drugs to kill malignant cells. Chemotherapy works in many different ways including interacting with DNA, binding to cellular proteins such as tubulin, antimetabolites and interacting with DNA associated proteins such as topoismerase 1 and II. However the main target is to cause damage to DNA of cancer cells leading to apoptosis (Pecorino, 2005; Kostova, 2006). The effectiveness of chemotherapy can vary widely among patients due to differences in genetic make-up (Rickardson et al., 2005). The first antitumour chemotherapy involved the use of alkylating agents to kill cancerous cells in 1940s. Examples of alkylating agents include chlorambucil, cyclophosphoamide and nitrogen mustard (Chabner and Roberts Jr, 2005; Pecorino, 2005). Nitrogen mustard that was developed in the USA in 1922 to be used as a chemical weapon was found to possess antitumour activity shortly after that. The mechanism of action of mustard gas is believed to be similar to that of platinum drugs. It is believed that mustard gas forms inter- and intrastrand adducts with DNA through binding with N7 positions of guanines (Thurston, 2007). These adducts distort DNA structure, and inhibit DNA replication and transcription (Thurston, 2007).

Antimetabolites such as 6-mercaptopurine (6-MP) were developed to kill cancerous cells in 1952; the drugs are still in use (Ho et al., 2003; Chabner and Roberts Jr, 2005; Pecorino, 2005). The compounds mimic some crucial compounds needed by cells for example nitrogen bases to build DNA and RNA molecules. This would inhibit the early steps of DNA and RNA synthesis and as a result will stop or slow down the growth of tumour cells (Chabner and Roberts Jr, 2005).

In the 1960s scientists started scanning natural products from terrestrial and marine environments in search of antitumour drugs. This led to the discovery of taxanes in 1964 and camptothecins in 1966. Taxanes were obtained from the bark of the Pacific Yew tree. In 1971 the active ingredient of taxanes which is known as Paclitaxel was isolated and since then has been very effective in the treatment of ovarian cancer. Paclitaxel is an antimitotic that stabilizes microtubule assembly and thus inhibits cell division. On the other hand camptothecin, derived from a Chinese ornamental tree, inhibits topoisomerase I, an enzyme that allows DNA unwinding and strand breakage. Camptothecin (irinotecan) is approved for treatment against colon cancer (Chabner and Roberts Jr, 2005).

1.1.3.4 Survival and mortality

The curable rate for localized cancer is 50-80%, for cancer involving lymph node it is less than 50% while for cancers that have spread to other parts of the body it is 5-20% (Rubin et al., 2001). The decrease in mortality rate by age group in the latest data by Surveillance Epidemiology and End Results (SEER) is given in Table 1.1 below (Rubin et al., 2001):

Table 1.1 Decrease in mortality rate by age group

Age groups in years	Decrease in mortality rate in percentage
0 to 4	48%
15 to 34	28%
35 to 44	26%
45 to 54	18%
55 to 64	2%

The incidence of cancer is different in different groups. There is a decrease in mortality in white compared with black people and in men compared with women (Rubin et al., 2001).

In the state of New South Wales (NSW), Australia, "cancer caused 12,513 deaths (7,038 males and 5,475 females) in 2005. The leading causes of death in males were cancers of the lung (21%), prostate (14%) and bowel (13%). While in females the leading causes of deaths were breast and lung (16% each) and bowel (13%). Men were 1.5 times more likely to get cancer than women and the incidence of cancer increased with age" (Cancer Institute of NSW www.cancerinstitute.org.au) (Tracey et al., 2007).

1.2. Structure of DNA

In cells, there are two types of nucleic acids: deoxyribonucleic acid (DNA) (Figure 1.5) (Yang and Wang, 1999), and ribonucleic acid (RNA). DNA is believed to be the hereditary material in most of livings cells (DeVita et al., 1993; Yang and Wang, 1999). DNA is found mainly in the nucleus and little in the cytoplasm (Emery and Malcolm, 1995). The chromosomes are made up of DNA and some proteins. The number of chromosomes is fixed in each species, for example in humans it is 46.

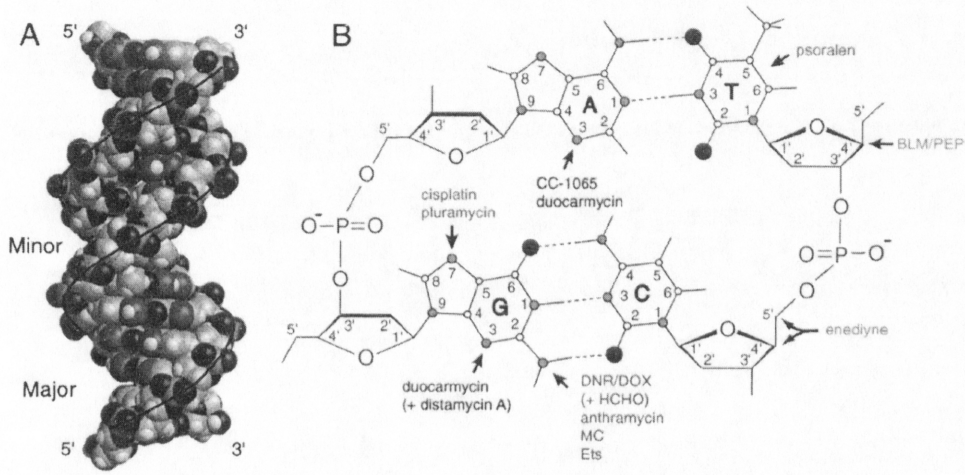


Figure 1-5 DNA structure

According to Watson and Crick model, shown in Figure 1.5 (Yang and Wang, 1999), DNA has a double-helical structure. The helical morphology of DNA is a result of one strand running from 5′ end to 3′ end while the second strand running parallel from 3′ end to 5′ end. The building blocks of DNA are known as nucleotides. Nucleotides are made up of a deoxyribose sugar attached to a phosphate group and to one nitrogenous base (Figure 1.6) (DeVita et al., 1993; Alcamo, 2001). There are four

different nitrogenous bases (also called nucleobases) in DNA: adenine (A), thymine (T), cytosine (C), and guanine (G). Therefore, DNA is made up of four different nucleotides blocks A, T, C, and G. The sugar-phosphate backbone forms the outside of the strands while the nucleobases form the inside as shown in Figure 1.5 (Rieger, 2004; Muller, 2006).

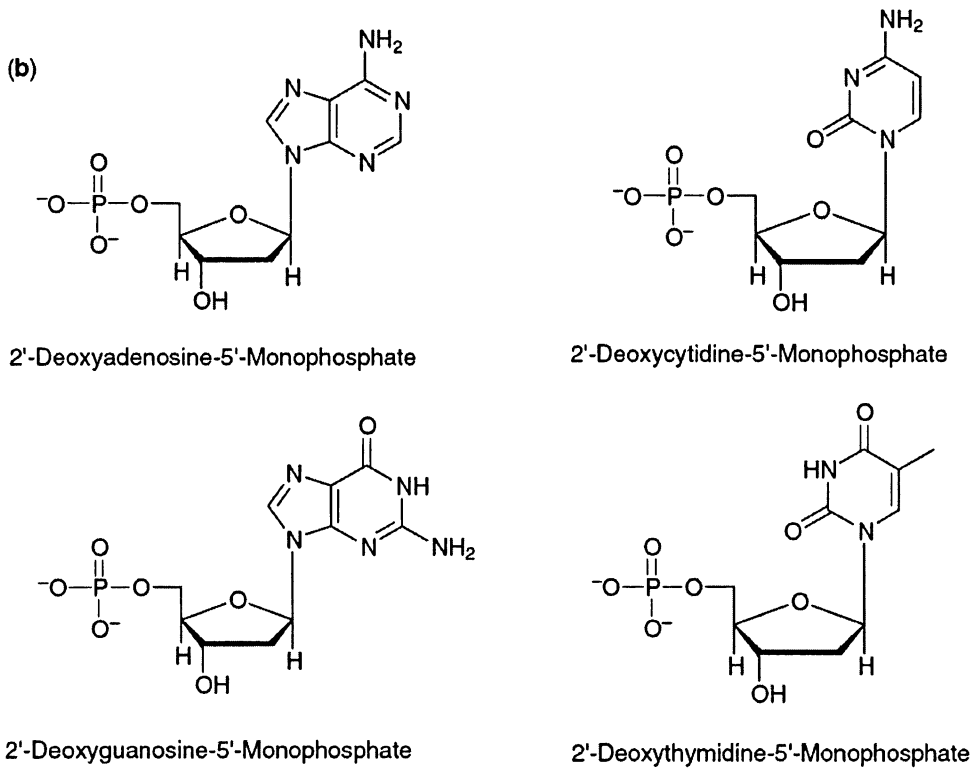


Figure 1-6 Structure of DNA nucleotides

Genes are defined regions of DNA. Each gene codes for a single polypeptide. It is estimated that each human somatic cell contains between 50,000 to 100,000 genes (DeVita et al., 1993). Genes control the inherited traits by controlling the synthesis of polypeptides (Alcamo, 2001).

The mechanism of protein production in human cell is summarized in Figure 1.7 (Chapman, 2007). The sequence of nitrogen bases in DNA provides the code of heredity. Any change in the sequence leads to mutation. Mutations may lead to the production of different proteins or in complete blockage of the protein production that may lead to alteration of the cell's ability to function normally (DeVita et al., 1993).

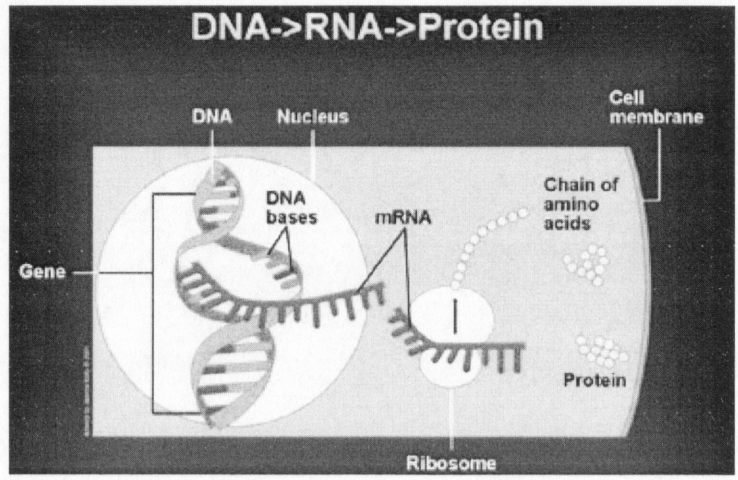


Figure 1-7 The process of protein synthesis

Only 3.5% of the total human genome carries codes for proteins while the rest is called junk DNA. Junk DNA does not code for any protein and this could explain the small number of harmful mutations in humans (Weinberg, 2007). It is also interesting to know that '99.9% of all human DNA is identical between individuals, thus all diversity among people comes from the remaining 0.1% (Rieger, 2004).

DNA can adopt different conformations depending on its particular nucleotide sequence, ionic strength, type of ions, and solvents (Daune, 1999). DNA conformations include A-DNA, B-DNA, and Z-DNA. The B-form of DNA is considered to be the one present under physiological conditions. As shown in Figure 1.5, B-DNA has two distinct helical grooves: the major and the minor groove. The

major groove is wide, while the minor groove is narrow with both grooves having almost equivalent depth.

There are many reactive sites on DNA surface including N2 amino group of guanine in the minor groove, N3 of guanine and adenine in the minor groove, N7 of guanine in the major groove, and C1, C4, and C5 of deoxyribose in B-DNA backbone (Yang and Wang, 1999).

1.3. Cell cycle

Cells in human body divide at different speed. Some cells like stomach lining divide every 2-3 days, while others like neurons and adult striated muscle cells do not divide (Mautner and Huang, 2003; Martini, 2004). The time needed for one cell cycle in tumour cells varies but is believed to be between 2 to 3 days (Hill, 2001).

Normally non-growing non-proliferating cells are in a resting state known as gap phase 0 (G0). When a cell receives an extracellular proliferation signal, it then enters the cell cycle. The cell cycle consists of four stages: gap phase 1 (G1), synthesis phase (S), gap phase 2 (G2), and mitosis phase (M) as shown in Figure 1.8 (Fischer et al., 2004).

In G1 phase, the cell doubles most of its cytoplasm organelles like ribosomes and mitochondria. In addition, the cell grows and prepares for DNA duplication through the production of RNA and proteins. In S phase, DNA is duplicated; therefore the cell at this stage contains double the quantity of DNA. The next phase is called G2 phase in which the cell prepares for mitosis through protein synthesis and completion of centriole replication (Martini, 2004). Finally, in M phase the cell divides into two identical daughter cells with same number of chromosomes.

If the daughter cells receive the proliferation signal they will re-enter the cell cycle through G1, if not they will enter the resting state G0 (Fischer et al., 2004; Ivanchuk and Rutka, 2004; Rieger, 2004).

The cell cycle is regulated by different check points. Check points are part of a signalling network that monitors molecular activity of each stage for any error. In the case of error it causes cell cycle arrests to permit DNA repair, and if the DNA damage is beyond repair, it promotes apoptosis. The best examples of these check point regulators are p53 gene and cyclin-dependant kinase (CDKs).

Both p53 and the retinoblastoma protein (pRB) act as checkpoints. Cell cycle progression is regulated by cyclin dependent kinases (Cdks) (Lowe, 1999; Prives and Hall, 1999; Xu and El-Gewely, 2001; Mautner and Huang, 2003; Eastman, 2004; Lukas et al., 2004; Rieger, 2004).

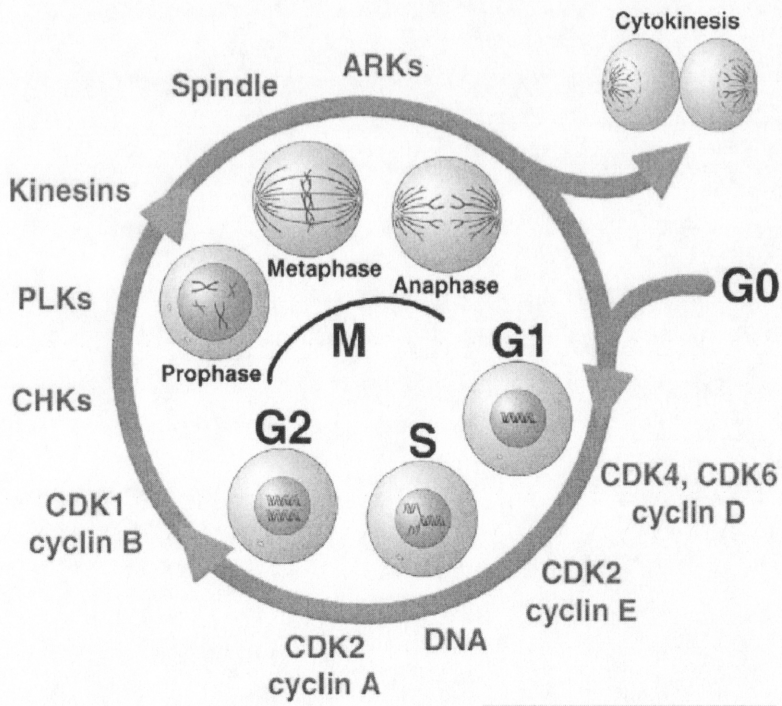


Figure 1-8 The mammalian cell cycle

Cyclin-dependant kinase (CDKs) and cyclin-dependant kinase inhibitor (CDKIs) activate and deactivate (respectively) phosphorylation of certain important proteins (Hill, 2001; Mautner and Huang, 2003; Eastman, 2004; Lukas et al., 2004). For example, cyclin D phosphorylates retinoblastoma protein pRB that is essential for regulation of cell cycle (Whyatt and Grosveld, 2002) and for transition of cell from G1 to S phase. When pRB is not phosphorylated, it binds to transcription factor E2F and inhibits the transcription of some important proteins necessary for transition to S phase. Phosphorylation of pRB releases E2F resulting in activation of transcription (Hill, 2001; Vermeulen et al., 2003). In cancer cells, some cell cycle check points are mutated. For example about 50% of tumour has mutation to p53 gene, and about 45% of breast cancer cases has mutation to cyclin D1 (Hill, 2001).

1.4. Apoptosis and necrosis

Apoptosis is defined as "programmed cell death". It is a genetically controlled process that requires adenosine triphosphate (ATP) and some proteins (Evan, 1994; Dewey et al., 1995; Abatado, 1996; Yonehara, 2002; Chipuk and Green, 2006). Apoptosis is a natural process that allows the body to get rid of aged and defective cells, remodel embryonic tissue, immune regulation, and tumour regression (Ashkenazi and Dixit, 1998; Schulze-Osthoff et al., 1998; Ding and Fisher, 2002; Yonehara, 2002; Mautner and Huang, 2003; Letai, 2006). It should however be noted that the initiation of an apoptotic signal does not commit a cell to apoptosis. Cells are committed to apoptosis only upon the activation of the following: BAX or BAK oligomerization, mitochondrial outer membrane permeabilization and caspase activation (Letai, 2006).

Apoptosis is regulated by a large number of genes that can be classified into three categories: suppressor genes like Bcl-2, Bcl-X_L, promoter genes like: Bax, Bak, Bad, and finally upstream genes like p53, Fas/Fas ligand (Wang et al., 1999).

On the other hand, necrosis is cell death due to injury including cell puncturing, respiratory poisons, extreme pH, temperature, or toxic condition. The main difference between apoptosis and necrosis is in the steps of cell death. The first step in necrosis is the swelling of the cytoplasm and organelles, the lysis of the plasma membrane and the unsystematic degradation of DNA followed by the release of cell contents into the surroundings, which may lead to inflammation. On the other hand apoptosis starts with the cell membrane developing bubble-like blebs, followed by shrinking of the nucleus, chromatin condensation near nuclear envelop, reduction in the cell size, degradation of genomic DNA and the loss of plasma membrane. The phosphatidylserine gets exposed on the external side of the plasma membrane. This is considered to be a signal to macrophage cells that then engulf these cells. Whereas in necrotic cell death the contents are released to the extracellular surrounds and that could lead to inflammation, this does not happen in apoptosis (Dewey et al., 1995; Abatado, 1996; Schulze-Osthoff et al., 1998; Robertson and Orrenius, 2000; Power et al., 2002; Yonehara, 2002; Fuertes et al., 2003). The time needed for apoptosis is between 30-120 minutes (Hanahan and Weinberg, 2000).

1.5. Telomeres and cancer

Telomeres are a repetitive sequence of six base pairs (TTAGGG) in DNA. It is about 6-12 kb long, arranged into a 'T-loop' structure, and is present at the end of chromosomes. Telomeres have many functions including protection from degradation,

end-to-end fusions and rearrangements of chromosomes, and maintenance of nuclear structure (Rodiera et al., 2005; Cepeda et al., 2007).

Normal cells in culture produce from 50-100 generations (Bertram, 2001) following which they acquire chromosomal abnormalities and die. On the other hand tumour cells in culture are capable of producing unlimited proliferation. The main reason between normal and tumour cells as applied to difference in abilities to proliferate is believed to be associated with telomeres. In normal cells telomeres become shorter by 50-200 bp with every cell division (Pecorino, 2005). The shortening of telomeres to a certain critical length leads to cellular permanent growth arrest phase known as cellular senescence or apoptosis through activation of p53. In addition, telomere shortening leads to telomere dysfunction as shown in Figure 1.9 (Ju and Rudolph, 2006) resulting in chromosomal instability which is a hallmark of carcinogenesis. Moreover, telomere dysfunction limits the regenerative capacity of organs during ageing leading to diseases (Figure 1.9) (Ju and Rudolph, 2006). In addition, telomere shortening explains why cells removed from older individuals divide fewer times in culture compared to cells obtained from younger ones (Granger et al., 2002). The shortening of DNA in every cell division can be explained as follows: DNA polymerase cannot initiate DNA replication; it needs RNA primer. After the completion of DNA replication, RNA primer at the 3′ end of the DNA is not replicated. As a consequence the chromosome gets shorter with each cell division (Bertram, 2001; Granger et al., 2002; Rodiera et al., 2005). In contrast, tumour cells maintain the length of telomers through activation of all enzymes known as telomerase. Telomerase is a reverse transcriptase enzyme. It uses its RNA component as a template to synthesize the telomere repeats at the end of chromosomes (Granger

et al., 2002; Cepeda et al., 2007). A direct relationship has been found between the aggressiveness of a tumour and the extent of activation of telomerase.

Due to the crucial role played by telomerase in the cancer cell, it has become a target for newly design treatment. Antisense and ribozyme technologies are the most popular mechanisms to target human telomerase. Clinical trials are needed to determine the effectiveness of this therapy (Pecorino, 2005).

In regards to the effect of the chemotherapeutic agent cisplatin on telomeres, it has been found that exposing HeLa human cervix carcinoma cells to low concentrations of cisplatin causes shortening and degradation of telomeres leading to cell death (Cepeda et al., 2007). This can be explained on the bases that telomeres are not transcribed so that they will not be repaired by the nucleotide excision repair (NER) system. Therefore low levels of telomere damage caused by cisplatin could be enough to induce cell death (Cepeda et al., 2007).

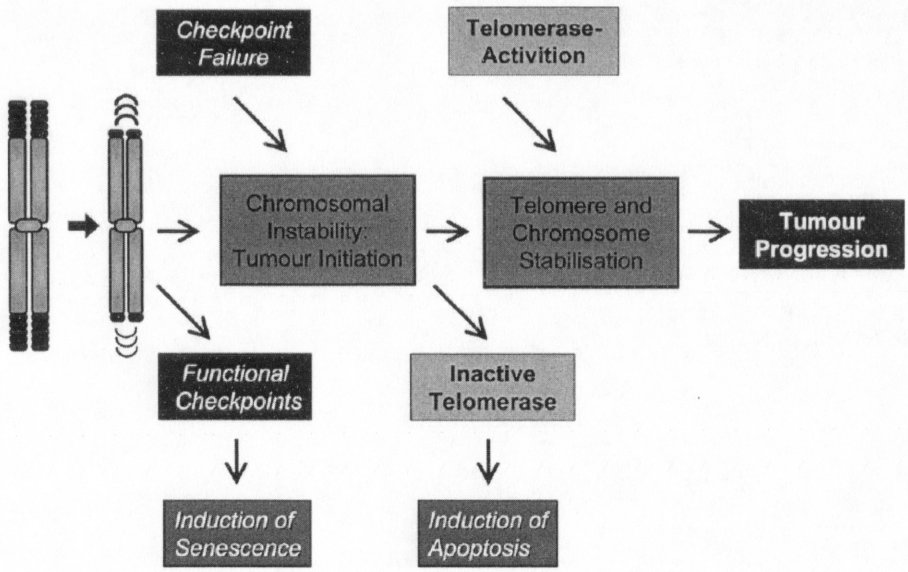


Figure 1-9 Consequences of telomere shortening

1.6. Mitochondrial Contribution to Cancer and Apoptosis

The main role of mitochondria in the cell is energy production. The number of mitochondria present in a single cell is estimated to be around 1000 copies. Although this number may vary depending on the need of the cell to energy (Preston et al., 2001; Kang and Hamasaki, 2005). A mitochondrion is surrounded by two membranes, the outer and inner-membrane. The outer membrane is highly permeable. The inner membrane is nearly impermeable and is folded into many cristae to increase the area needed for the production of adenosine tri-phosphate (ATP). The inner-membrane is called the matrix where the mitochondrial DNA and ribosomes are located (Preston et al., 2001). Mitochondrial DNA is a circular double strand, that represents less than 1% of cellular DNA (Modica-Napolitano and Singh, 2004; Kang and Hamasaki, 2005). Mitochondrial DNA is not covered with histone, lacks introns and its repairing system is less efficient than that of nuclear DNA. Therefore mutation accumulation in mitochondria is ten fold greater than nuclear DNA (Preston et al., 2001; Copeland et al., 2002; Modica-Napolitano and Singh, 2004). Mitochondrial DNA is continuously under oxidative stress. Exposure to reactive oxygen species (ROS) could lead to mitochondrial DNA damage which could be associated with the initial stage of neoplasia (Preston et al., 2001; Copeland et al., 2002). Moreover, chemotherapeutic agents like cisplatin have been found to accumulate in mitochondria and cause damage to DNA (Copeland et al., 2002). Therefore mitochondrial dysfunction due to toxicity is the key dose-limiting factor of many drugs (Preston et al., 2001). Tumour development in most cases is associated with mutation to mitochondrial DNA. The

first to notice the association between cancer and mitochondria was Otto Warburg in 1930. He noticed the association between cancers and cellular respiratory dysfunction (Modica-Napolitano and Singh, 2004). It has subsequently been reported that mutated forms of mitochondria were found in tumour tissue but not in the surrounding tissues (Kang and Hamasaki, 2005). In addition, mutated mitochondria were found to be contributing to chemotherapy resistance (Dias and Bailly, 2005). Mutation in mitochondrial DNA has been found in cancer of the bladder, breast, colon, head and neck, kidney, liver, lung, stomach, hematologic malignancies leukaemia and lymph (Copeland et al., 2002).

1.6.1 Mitochondrial control of apoptosis

There are two general signalling pathways that trigger apoptosis: firstly, cell death initiated from death receptors on the surface of the cell (extrinsic pathway), secondly, cell death initiated from mitochondrial dysfunction (intrinsic pathway). Although the two pathways operate independently there is a cross link between them (Ashkenazi and Dixit, 1998; Talapatra and Thompson, 2001; Ding and Fisher, 2002; Power et al., 2002; De Thonel and Eriksson, 2005; Yu and Zhang, 2005; Chipuk and Green, 2006; Dejean et al., 2006; Mehlen and Puisieux, 2006). The extrinsic pathway includes members of the tumour necrosis factor receptors family (TNFR) like CD95 (known as Fas) and TNFR-1. The binding of inducers to these receptors lead to activation of caspases that leads to apoptosis. Mitochondria have a central role in intrinsic pathway apoptosis as a mediator between Bcl-2 protein and caspases. Caspases are a family of cysteine proteases that play a key role in apoptosis through degradation of cell

components (Budd, 2002; Power et al., 2002; Rapp et al., 2004; Dias and Bailly, 2005; Kim, 2005).

Bcl-2 proteins are localized to mitochondria and are divided, as stated before, into pro-apoptotic proteins (like Bax and Bid) and anti-apoptotic proteins (like Bcl-2, Bcl-$_{XL}$). Bcl-2 plays a crucial role in apoptosis through controlling the release of cytochrome C from the inner membrane of mitochondria. When cytochrome C is released into cell cytosol, it reacts with apoptotic protease-activating factor (Apaf-1) and procaspase-9 to form a complex known as apoptosome. Apoptosome then activates caspase-9 which in turn activates caspase-3. Caspase-3 stimulates the fragmentation of DNA (Robertson and Orrenius, 2000; Preston et al., 2001; Budd, 2002; Mashima and Tsuruo, 2005; Dejean et al., 2006). In addition, mitochondria contribute to apoptosis by releasing procaspase-3, Smac/Diablo, and apoptosis inducing factor (AIF), that are believed to induce apoptosis (Preston et al., 2001; Dejean et al., 2006). There are many structural and functional differences between normal and cancer cell mitochondria that could serve as an anticancer drug target. Currently, attempts are being made to synthesise anticancer drugs targeting mitochondria (Modica-Napolitano and Singh, 2004).

2. Chapter Two

2.1. Platinum drug: Cisplatin discovery

Although cisplatin was first synthesised in 1845, its antitumour activity was discovered much later and accidentally in 1965 by Rosenberg. During his study on the effect of electrical current on the growth of *Escherichia coli* (*E.coli*), Rosenberg noticed the filamentous growth and elongation of the bacterial cells of up to 300 times (Hodes et al., 1992; Reedijk, 1992; Rosenberg, 1999; Jordan and Carmo-Fonseca, 2000; Kelland, 2000; Desoize and Madoulet, 2002; Reedijk, 2003). He later found that cisplatin, that had resulted from the reaction of platinum electrodes with ammonium chloride from the buffer solution, was the reason behind the *E.coli* elongation (Rosenberg, 1999; Kartalou and Essigmann, 2001b). It was later revealed that cisplatin prevented cell division that led to bacterial cell elongation (Rosenberg, 1999; Tannock et al., 2005).

In 1971 cisplatin was used for the first time in humans followed, in 1978, by its approval by the United States of America Food and Drug Authority (FDA) as an anticancer drug (Jamieson and Lippard, 1999; Desoize and Madoulet, 2002; Wozniak and Blasiak, 2002). Currently, cisplatin is one of the most widely used antitumour drug with more than 50% of cancer cases being treated with the drug (Hall et al., 2006).

2.1.1 Cisplatin treatment success

Although cisplatin is a very simple inorganic compound, as noted earlier, it is considered to be one of the most successful and widely used anticancer drugs in chemotherapy (Perego et al., 1999; Pratesi et al., 1999; Wozniak and Blasiak, 2002; Baik et al., 2003; Boulikas and Vougiouka, 2003; Fuertes et al., 2003; Rauterkus et al., 2003; Siddik, 2003; Wang and Lippard, 2005). It is highly effective against ovarian and testicular cancer. The success rate for testicular cancer is more than 90% and could reach 100% if treated during the early stages (Hiorns et al., 1999; Ho et al., 2003; Wang and Lippard, 2005). The drug is widely used in the treatment of many other tumours including bladder, cervical, head and neck, and small-cell and non-small-cell lung cancer (Chu, 1994; Hiorns et al., 1999; Ho et al., 2003; Wang and Lippard, 2005; Rabik and Dolan, 2007). It is used in combination with paclitaxel, vinorelbine or docetaxel for the treatment of small and non-small-cell lung cancers. Cisplatin is also used in combination with bleomycin and etoposide to improve the cure rate in testicular cancer (Natile and Coluccia, 2001).

There are many pharmacological factors affecting the cytotoxicity of platinum drugs including:

(1) Structure, nature, and frequency of the DNA-platinum adduct. The majority of research findings suggest that the nature of adduct structure plays a major role in the cytotoxicity of the platinum complexes. On the other hand, less correlation is found between the frequency of platinum binding to DNA and cytotoxicity (Natile and Coluccia, 2001; Ma et al., 2005).

(2) Cellular uptake and efflux (Ma et al., 2005): Cellular uptake may determine the level of Pt-DNA binding whereas Pt efflux may be associated with drug resistance. This will be discussed in more detail later in the thesis.

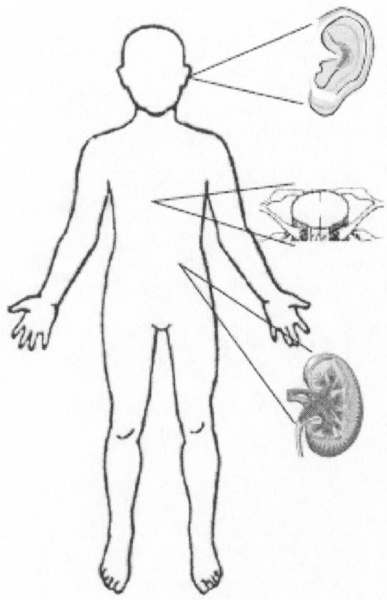
(3) Deactivation of platinum drugs by sulfur containing proteins will serve to reduce Pt-DNA binding (Harris et al., 2006; Liu et al., 2006). This will be discussed in more detail later in the thesis.

2.1.1.1 Side effects of cisplatin

Although cisplatin has been successful in treatment of different types of cancer, there are limitations in its use including inherent (natural) and acquired resistance and toxic side effects (Hiorns et al., 1999; Reedijk, 1999; Wang and Lippard, 2005). The exact mechanisms of drug resistance remain poorly understood although it is believed that more than one mechanism may operate concurrently.

It is believed that toxic effects due to cisplatin are mainly due to its reaction with cellular protein and extracellular enzymes (Bose, 2002). Cisplatin reacts with many biomolecules that contain sulfur and nitrogen through ligand exchange reactions. While reacting with nitrogen donors molecules require aquation, it is believed that cisplatin can react with sulfur donors such as amino acids methionine or cysteine without aquation (Jakupec et al., 2003). The major toxicities associated with cisplatin treatment (shown in Figure 2.1) include (1) nephrotoxicity, (2) ototoxicity and (3) neurotoxicity (Rabik and Dolan, 2007).

(1) Nephrotoxicity (kidney damage): Cisplatin is excreted through the kidney. Therefore, cisplatin will accumulate in kidney in high concentrations. This accumulation affects the terminal proximal tubule and the distal nephron. This may cause apoptosis or necrosis in these cells that may lead to kidney damage (Rabik and Dolan, 2007).



Toxicity	*Cisplatin*	*Carboplatin*	*Oxaliplatin*
Ototoxicity	Adult: 23-50% Children: >50%	~ 1%	Rare
Peripheral Neuropathy	Adult: 30-86% (avg 62%) Children: ~10% lower	Conventional dose: 6% High dose: 25%	Acute: 95% Cumulative: 15%
Nephrotoxicity	~20-41%	Patients with irregular kidney function	Rare
Myelosuppression	<5%	Conventional dose: 20-40% High dose: >90%	Rare

Figure 2-1 Toxicities associated with treatment with platinum agents

(2) Ototoxicity (hearing loss): It is estimated that 23-45% of patients treated with cisplatin experience ototoxicity. Treatment with platinum complexes may damage the outer hair cells of the cochlea in the inner ear. It is believed that the production of reactive oxygen species (ROS) in the cochlea leads to cell death in that area resulting in hearing loss (Rabik and Dolan, 2007).

(3) Neurotoxicity (nervous system damage): Cisplatin treatment may cause damage to central nervous system, in particular to the dorsal root ganglia of the spinal cord. Cisplatin causes primary sensory neuropathy, which is characterised by decreased sensor nerve conduction velocity due to calcium channel blockage (Rabik and Dolan, 2007).

Other side effects that could result from cisplatin treatment include severe nausea, vomiting, diarrhoea and elevated blood pressure (Kelland, 1993; Hiorns et al., 1999;

Cosaert and Quoix, 2002; Wheate and Collins, 2003; Wang and Lippard, 2005; Martınez et al., 2007).

To overcome nephrotoxicity intravenous hydration has been used. To protect patients from ototoxicity pre-treatment with thiol-containing drugs is used (Rabik and Dolan, 2007). Moreover, serotonin receptors antagonists and NK1 antagonists are used to reduce vomiting and nausea (Reedijk, 1999; Wong and Giandomenico, 1999).

In addition to the above problems, return or appearance of a second tumour in patients previously treated with cisplatin was also a major set back. This led many scientists to believe that cisplatin may be the cause behind the appearance of the second tumours (Bradley et al., 1993).

2.1.1.1.1 Structure of cisplatin

The structure of cisplatin, *cis*-diaminedichloroplatinum, is shown in Figure 2.2. The chloride ligands are on the same side of the molecule and the same is true for the ammonia ligands. This structure or distribution of ligands is called *cis*-geometry.

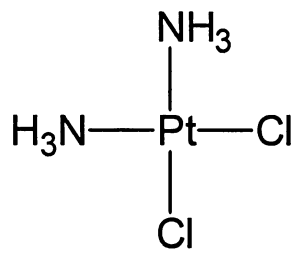


Figure 2-2 Structure of cisplatin

Ammonia ligands are tightly bound to platinum and therefore are called non-labile or carrier ligands whereas the chloride ligands are loosely bound to platinum and therefore are called labile ligands or leaving groups.

2.1.2 Mechanism of action of platinum drugs

2.1.2.1 Cisplatin uptake:

The exact mechanisms by which cisplatin enters the cell remain poorly understood. However, it is believed that cisplatin enters the cell by both passive diffusion and facilitated diffusion whereas its efflux out of the cell may be more due to active transport (Jamieson and Lippard, 1999; Fuertes et al., 2003; Ghezzi et al., 2004; Wang and Lippard, 2005). Given sufficient time, all molecules will naturally diffuse across cell membrane down their concentration gradient. The time needed depends on size and hydrophobicity of molecules (Los et al., 1996). Since cisplatin is a neutral small molecule, it is believed that it should readily cross the membrane through passive diffusion (Kartalou and Essigmann, 2001b). Moreover, it is believed that facilitated diffusion plays a role in the accumulation of cisplatin (Jamieson and Lippard, 1999; Fuertes et al., 2003; Wang and Lippard, 2005; Liu et al., 2006). According to Gately and Howell, 50% of cisplatin uptake is through passive diffusion and 50% through facilitated diffusion (Gately and Howell, 1993). This hypothesis agrees with the findings that accumulation cannot be inhibited by more than 50% (Los et al., 1996). In addition, cisplatin accumulation has been found to be energy and Na^+ dependent, affected by pH and intracellular signalling, and inhibited by aldehydes (Los et al., 1996).

2.1.2.2 Cisplatin hydrolysis and mechanism of cisplatin-DNA adducts formation

The high concentration of chloride in blood serum (about 100 mM) inhibits the hydrolysis of cisplatin (Fuertes et al., 2003). However, once cisplatin is inside the cell, where the concentration of chloride drops dramatically to about 5 mM, hydrolysis (the replacement of chloride with water) of cisplatin to form monoaquachloroplatinum $\{Pt(NH_3)_2Cl(H_2O)\}^+$ would be speeded up Figure 2.3 (Kelland, 2007). Cisplatin hydrolyses is a very important step. It produces the aquated active form of cisplatin that reacts with DNA (Pil and Lippard, 1997; Jamieson and Lippard, 1999; Berners-Price and Appleton, 2000; Bose, 2002; Wang and Lippard, 2005).

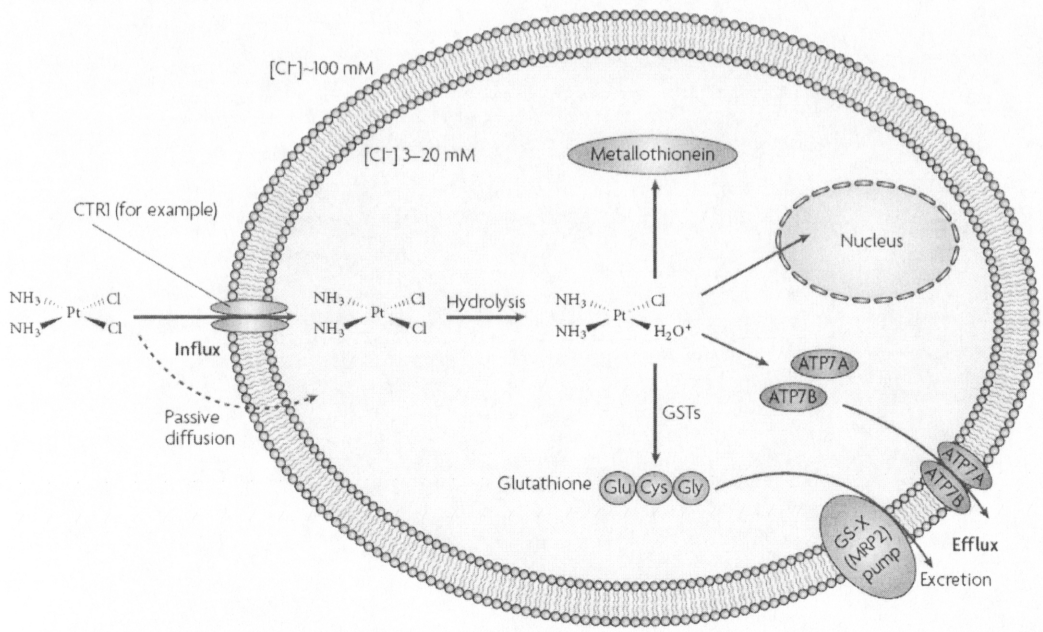


Figure 2-3 Mechanism of activation and resistance of cisplatin

The positively charged aquated form of cisplatin binds with purine bases (guanine and adenine) to form monofunctional adducts $\{Pt(NH_3)_2Cl(N7\text{-}pGp)$ and $\{Pt(NH_3)_2Cl(N7\text{-}pAp)\}$ (Jamieson and Lippard, 1999; Kartalou and Essigmann, 2001b; Ali et al., 2005).

N1, N3 and N7 are all possible binding sites for cisplatin in purine bases. However, cisplatin prefers N7 in guanine over other possible binding sites due to the following reasons: sites in the major groove are exposed and easily accessed, N7 is not involved in base pairing, possibility of strong hydrogen bond between the amine-hydrogen of cisplatin and the O=C6 moity of guanine, high basicity and finally N7 of guanine is the richest electron site (Gelasco and Lippard, 1999; Jamieson and Lippard, 1999; Lippert, 2000; Baik et al., 2003; Fuertes et al., 2003; Ho et al., 2003)

The next step involves the closure of the chelate by forming bifunctional adduct $\{Pt(NH_3)_2(d(GpG\text{-}N7(1)N7(2))$. This step requires the hydrolysis of the second chloride ligand followed by binding to N7 site in an adjacent purine base, as shown in Figure 2.4.

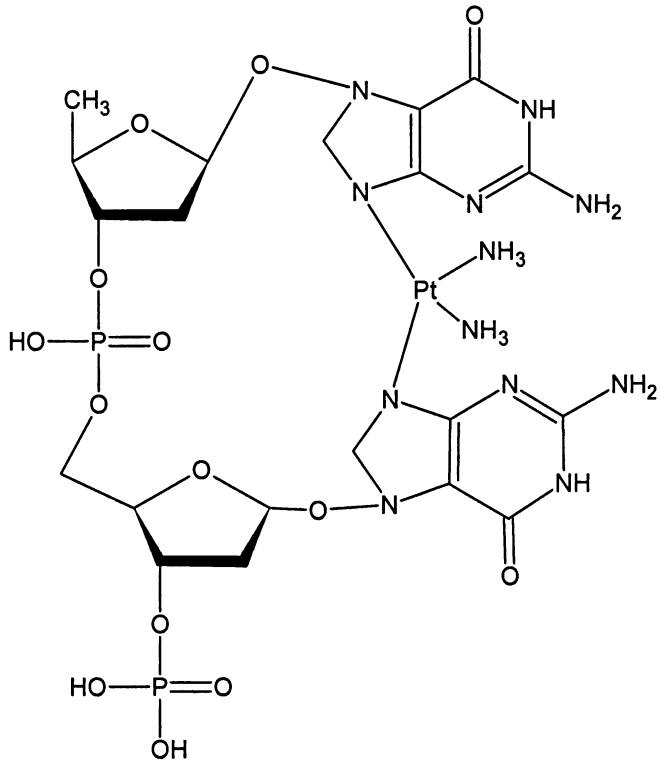


Figure 2-4 Binding of cisplatin to N7 of two adjacent guanine bases in DNA showing head to head conformation of nucleotides

Cisplatin binds with DNA forming a number of adducts as shown in Figure 2.5 (Kelland, 2000) 60-65% of adducts formed are 1,2-d(GpG) intrastrand crosslinks, while 20-25% are d(ApG) intrastrand crosslinks (Kelland, 2000; Crul et al., 2002; Fuertes et al., 2003). In addition, cisplatin forms around 2% 1,3 intrastrand crosslinks (GpXpG), around 2% mono adducts, around 2% interstrand crosslinks, and small percentage of DNA-protein crosslinks (Kelland, 2000b; Fuertes et al., 2003; Farrell, 2004). Therefore, cisplatin forms predominantly intrastrand crosslinks between neighbouring purine bases (Bernges and Holler, 1991; Wozniak and Blasiak, 2002).

It has been established that there is a direct correlation between the positive treatment response and the level of adducts formation specially 1,2 intrastrand adduct (Jamieson and Lippard, 1999). 1,2 intrastrand adduct is believed to be behind the anticancer

activity of cisplatin (Jamieson and Lippard, 1999; Stehlikova et al., 2002; Siddik, 2003).

The role of DNA-protein crosslinks is not well understood. It should however be noted that cisplatin forms more of these adducts than transplatin and the adducts are not removed by NER so that they could play a role in the cytotoxicity of cisplatin (Chvalova et al., 2007). Besides binding with DNA, cisplatin also reacts with many other cellular targets including: RNA, proteins, membrane phospholipids, and sulfur containing ligands (Jamieson and Lippard, 1999; Berners-Price and Appleton, 2000). It is estimated that only about 1% of the total platinum that enter the cell binds to DNA (Perez, 1998; Ho et al., 2003; Ghezzi et al., 2004).

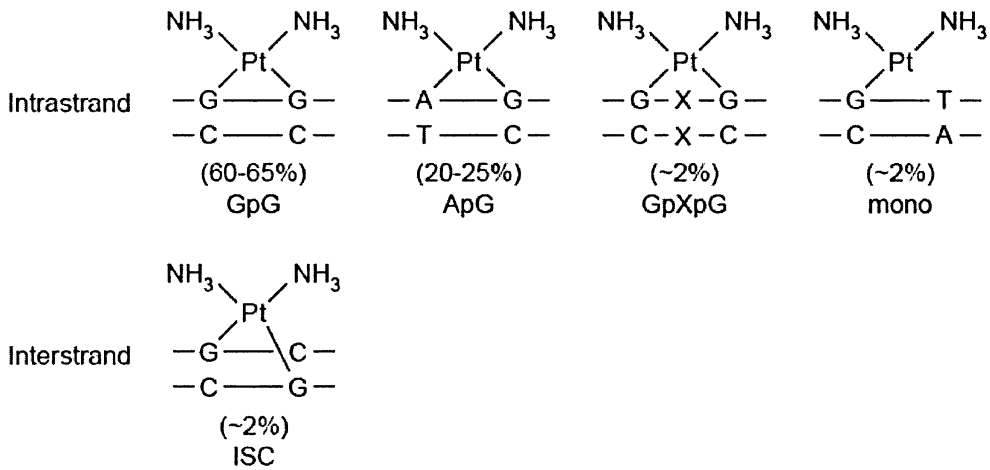


Figure 2-5 Binding of cisplatin to DNA: bifunctional types of cisplatin adduct

2.1.2.3 Cytotoxicity of cisplatin adducts

The binding of cisplatin with DNA distorts its structure (Brabec and Kasparkova, 2005). Cisplatin-DNA adducts cause DNA bending, unwinding, and loss of stability (Wheate and Collins, 2003). The 1,2-intrastrand adducts cause a rigid bending of

DNA by 34^0 (32^0-34^0) towards the major groove and unwind it by 13^0 (Kasparkova et al., 2002; Wozniak and Blasiak, 2002). In addition, cisplatin causes great distortion to hydrogen bonding in the 5'- coordinated GC base pair and flattening of the minor groove (Stehlikova et al., 2002). The 1,2-intrastrand Pt-d(GpG) adduct also causes guanine bases to roll towards one another by 49^0 (Roat-Malone, 2002).

The formation of cisplatin DNA adducts inhibits cellular replication and transcription by inhibiting DNA replication (Pinto and Lippard, 1985; Aupeix-Scheidler et al., 2000; Kartalou and Essigmann, 2001b; Bose, 2002; Ghezzi et al., 2004; Srivastava et al., 2004; Brabec and Kasparkova, 2005; Danford et al., 2005; Wang and Lippard, 2005). Initially, it was suggested as being the way by which cisplatin kills cancer cells (Pinto and Lippard, 1985; Jamieson and Lippard, 1999; Polak et al., 1999).

Since cisplatin is found to inhibit tumour cell growth at doses less than those needed for DNA synthesis inhibition, the idea is no longer in favour (Brabec and Kasparkova, 2005).

There are two pathways by which cisplatin could trigger apoptosis. The first mechanism is "repair shielding". High mobility group (HMG) proteins are believed to play a major role in cisplatin cytotoxicity. HMG proteins recognize the bending sites of DNA caused by cisplatin intrastrand adducts GpG or ApG, and binds to these sites as shown in Figure 2.6 (Jordan and Carmo-Fonseca, 2000). The binding blocks the way of DNA-repair proteins thus preventing adduct repair.

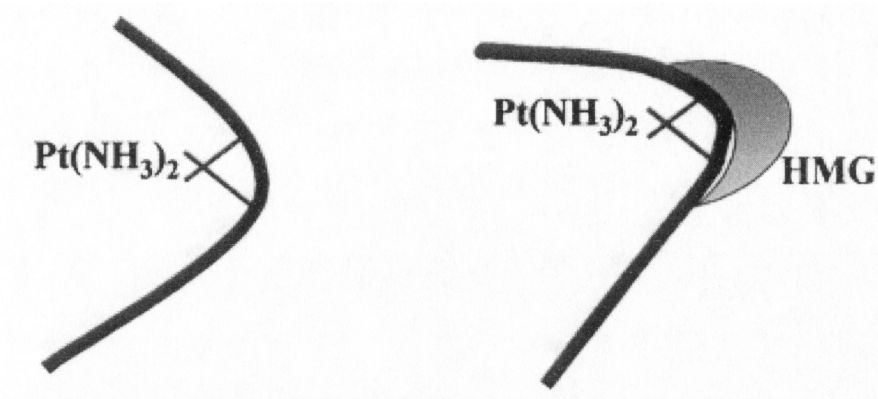


Figure 2-6 Binding of HMG proteins to cisplatin-DNA adduct

The persistence of these adducts causes blockage to transcription and DNA replication. In addition, it causes the activation of multiple signalling pathways including p53, Bcl-2 family, caspases, cyclins, CDKs, pRb, MAPK and other pathways that trigger apoptosis (Cohen and Lippard, 2001; Roat-Malone, 2002; Pasetto et al., 2006). The second hypothesis is through "hijacking" of nuclear factors. The binding of nuclear proteins and enzymes essential for cellular function to cisplatin-DNA adducts diverts these essential proteins from their crucial normal function. For example the SSRP1 protein is part of a protein complex that facilitates chromatin transcription (FACT) through RNA polymerase II. SSRP1 recognises and binds to the damaged DNA site. Allowing the FACT complex to bind to the damaged site of the DNA consequently result in inhibition of transcription (Cohen and Lippard, 2001).

2.1.2.4 Cisplatin therapeutic target

DNA molecules are poly-negatively charged due to the phosphate groups (Reedijk, 1992). Therefore they need positively charged cations to balance their negative charges (Reedijk, 1992). These can be protonated amines (spermine, spermidine) or protonated amino acid side chains (lysine, arginine), or metal ions (Lippert, 2000). Non-coordinating alkali metal ions condense in a cylinder shape around DNA that leads to partial charge neutralisation. Despite this, DNA still has great affinity toward cationic metals (Lippert, 2000). Cations prefer DNA over other biomolecules due to the ability of DNA to attract these cations to its surface (Rauterkus et al., 2003; Snygg et al., 2005; Kostova, 2006). Jamieson and Lippard conducted a study to measure the cellular target of cisplatin. In this study they measured the number of radiolabelled platinum atoms that got bound to DNA, RNA, and protein. The result of this experiment showed that 1 out of 3×10^4 to 3×10^5 protein molecules contained a platinum atom, for RNA between 1 to 1000 molecules contained a platinum atom and for DNA every molecule attracted 9 platinum atoms (Jamieson and Lippard, 1999).

Within 24 h of cisplatin administration, 65-98% was found to be bound to blood plasma proteins. As noted earlier, the role of cisplatin-plasma protein adducts is not yet known. However, the adducts are thought to cause drug inactivation and toxic side effects (Peleg-Shulman et al., 2002) although some researchers believe that some plasma proteins e.g. human serum albumin play a role in the activity of cisplatin. It is believed that cisplatin-methionine thioether adduct, but not cysteine thiolate adduct, is transformed to N7 adduct with guanine. Therefore, methionine proteins could work as a mediator to deliver cisplatin to DNA (Peleg-Shulman et al., 2002).

2.1.3 Mechanism of Cisplatin resistance

Many cancers have natural resistance to cisplatin, while others develop resistance later in the treatment (Desoize and Madoulet, 2002; Wheate and Collins, 2003). For example, about 50% of women with ovarian cancer have intrinsic resistance to cisplatin and a big percentage of the responsive ones acquire resistance in later stages of treatment (Wernyj and Morin, 2004). Acquired resistance is the main reason behind the failure of cisplatin treatment (Wheate and Collins, 2003). Tumours that acquire resistance during treatment become resistant not only to cisplatin but to other drugs even to those with a different mechanism of action (Brabec and Kasparkova, 2005; Ozben, 2006).

Fundamentally, there are two mechanisms by which a tumour develops resistance to chemotherapy namely selection and induction. In selection, cells deep in the bulk of a large tumour may receive low doses of drug due to poor tumour blood supply so they may last longer. Due to genetic instability and expression of drug-resistance genes, these cells may develop drug resistance. In induction, the anticancer agents may trigger signal transduction pathways that could alter the expression of genes. Therefore some anticancer agents may trigger and activate anti-apoptotic genes that may lead to drug resistance (Kohno et al., 2005).

The mechanisms of resistance to cisplatin shown in Figure 2.7 (Cepeda et al., 2007) include the following: (1) decreased drug accumulation, (2) increased level of DNA repair mainly by nucleotide excision repair (NER), (3) increased intracellular level of glutathione and metalothioneins, (4) loss of DNA mismatch repair (MMR) and (5) DNA tolerance by pass replication, and decreased apoptotic proteins. It is not yet

known whether these mechanisms work together or if there is any dominant one (Kelland, 1993; Fink and Howell, 2000; Wozniak and Blasiak, 2002; Vasey, 2003; Wheate and Collins, 2003; Brabec and Kasparkova, 2005; Ohmichi et al., 2005; Alderden et al., 2006).

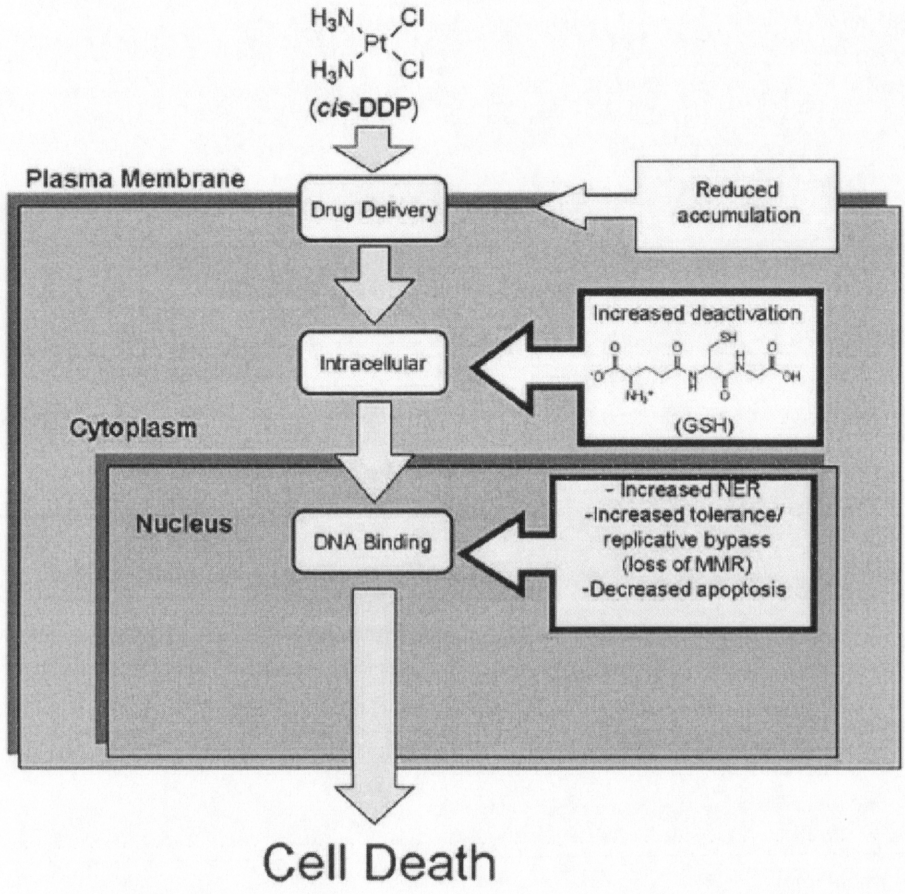


Figure 2-7 Schematic drawing showing the main biochemical mechanisms of resistance to cisplatin

2.1.3.1 Decreased drug accumulation

The decreased level of accumulation of cisplatin inside the cell could occur due to decreased uptake or increased efflux. The human multidrug resistance proteins

(MDR), which include seven members, are able to lower the intracellular drug concentration by pumping out the drug (Borst et al., 2000).

Efflux has been found to play a role in decreased level of cisplatin accumulation. An efflux protein, 200 kDa, is found to be over expressed in cisplatin resistant cells accompanied by decreased cellular accumulation of cisplatin, thus providing support to the idea that the protein may be playing a role in efflux (Kartalou and Essigmann, 2001b).

Copper main influx transport protein (CTR1), has been reported to play a role in the uptake of cisplatin. CTR1 binds to copper using methionine and histidine rich amino terminal and transports it across the cell plasma membrane. It may be noted that cells resistant to cisplatin have shown reduced accumulation of both cisplatin and copper (Holzer et al., 2004; Safaei et al., 2004; Ohmichi et al., 2005).

2.1.3.2 Inactivation of cisplatin by increasing the intracellular level of glutathione and metalothioneins

As noted earlier, cisplatin reacts with sulfur containing ligands including glutathione and metalothioneins without even the need for hydrolysis (Berners-Price and Appleton, 2000; Roat-Malone, 2002; Reedijk, 2003). Glutathione (γ-glutamylcysteinylglycin, GSH) is the most abundant sulfur containing molecule in the cell. It is a tripeptide thiol produced from cysteine, glycine and glutamine. Its function in the cell includes membrane transport and protecting cells from reactive oxygen species, reactive nitrogen species and free radicals. It binds with cisplatin inside the cell to form an inactive end product and then moves the product out of the cell. GSH also enhances DNA repair and increases cellular resistance to Pt-DNA adducts (Wong and Giandomenico, 1999; Boulikas and Vougiouka, 2003; Ho et al., 2003). In

addition, GSH binds to monofunctional adducts formed by platinum drugs with DNA, thus preventing its conversion to the effective bifunctional adducts (Kartalou and Essigmann, 2001b). Clinical studies show that there is a correlation between glutathione concentration and resistance to cisplatin in human ovarian carcinoma as well as in head and neck cancer (Wong and Giandomenico, 1999). Metalothionein (MT) is a sulfur containing low molecular weight intracellular protein that contains a high level of cysteine. It has a high affinity for binding with heavy metals (Stewart, 2007). MT is found in tissues of the liver, the intestines, and kidneys. Its main role in the cells is in detoxification of some heavy metals and in the regulation of coper and zinc metabolism (Lehne et al., 1998). A high level of MT has been linked to poor response to therapy in breast cancer and esophageal cancer (Lehne et al., 1998).

2.1.3.3 Increased level of DNA repair, nucleotide excision repair

Environmental factors such as ionizing radiations, toxicants, endogenous ROS, RNS and free radicals can cause damage to DNA. Nucleotide excision repair (NER) is considered to be the most important defence mechanism against DNA damage and in maintaining its integrity. NER is also considered to be one of the most important factors in tumour resistance to cisplatin. As shown in Figure 2.8 (Cepeda et al., 2007) NER recognises and removes cisplatin-DNA adducts (Zamble and Lippard, 1999; Ho et al., 2003; Farrell, 2004; Brabec and Kasparkova, 2005; Zorbas and Keppler, 2005; Cepeda et al., 2007; Stewart, 2007). NER involves many proteins including XPA, RPA, XPC, XPG, and XPF. The xeroderma pigmentosum group A (XPA) protein recognises a damaged area of DNA, like a cisplatin DNA adduct, and binds to it. This stimulates the binding of another protein known as RPA (Replication Protein A). The binding of XPA and RPA along with other proteins results in the formation of a

complex that unwinds DNA (De Laat et al., 1999; Zamble and Lippard, 1999; Crul et al., 2002; Hoogervorst et al., 2005). Then, with the help of other proteins XPG, XPF/ERCC1 and PCNA, along with the action of DNA polymerase, the damaged section of DNA is removed and the newly synthesised one is attached (Zamble and Lippard, 1999).

The XPC protein is believed to play a crucial role in NER. Cell cycle and cell proliferation genes are reported to be the most sensitive for XPC suggesting that it could have a role in triggering cell cycle arrest (Brabec and Kasparkova, 2005).

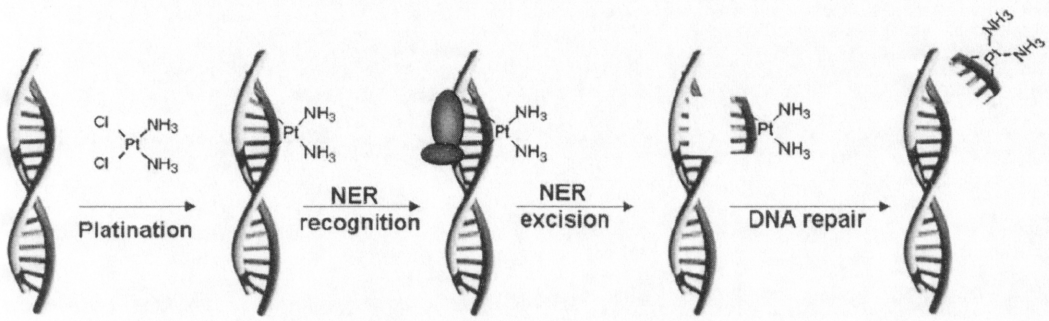


Figure 2-8 Nucleotide excision repair system (NER) steps involved in the repair of the major cisplatin 1,2-intrastrand crosslink

NER is more efficient in removing cisplatin 1,3-d(GpTpG) than cisplatin 1,2-d(GpG) or 1,2-d(ApG) intrastrand adducts (Zamble and Lippard, 1999). The 1,2-intrastrand adducts are not recognised by NER due to the fact that a number of proteins, mainly HMG proteins, bind and provide shielding for these adducts (Brabec and Kasparkova, 2005).

2.1.3.4 Mismatch repair (MMR)

The main role of mismatch repair (MMR) is to scan and correct mispaired and/or unpaired nucleotides that may result from DNA polymerase mistakes during replication. In other words, it corrects DNA replication errors. In addition, MMR also plays a role in cell cycle checkpoints and apoptosis (Perez, 1998; Aquilina et al., 1999; Buermeyer et al., 1999; Fuertes et al., 2003; Slupphaug et al., 2003; Brabec and Kasparkova, 2005). MMR scans and repairs only one strand of the newly synthesised DNA. Cells deficient in mismatch repair proteins (hMLH1, hMSH2, hPMS2, MutS) are less sensitive or resistant to cisplatin due to the failure to recognise the adducts and initiate an apoptotic signal (Aquilina et al., 1999; Boulikas and Vougiouka, 2003; Vasey, 2003; Stewart, 2007). The bypass of cisplatin-DNA adducts by DNA polymerase during DNA replication leads to mutation. Therefore to correct this mutation MMR proteins bind to the DNA daughter strand and synthesize a new DNA fragment to replace the mutated fragment. Because MMR works only on the daughter strand this will leave the cisplatin-DNA adduct on the other strand unrepaired. The repeated unsuccessful attempts by MMR to repair cisplatin-DNA adducts (futile cycling) is thought to trigger apoptosis (Perez, 1998; Brabec and Kasparkova, 2005; Zorbas and Keppler, 2005; Rabik and Dolan, 2007). Currently, intensive research is ongoing to find the relationship between defective MMR and some cancer types. This has started after the discovery that nonpolyposis colorectal cancer (HNPCC) is associated with mutation in mismatch repair genes (Buermeyer et al., 1999).

2.1.3.5 Overcoming cisplatin resistance:

There may be many ways that cisplatin resistance can be overcome. These may include:

(1) Designing new platinum compounds (possibly with structures different from that of cisplatin) that are able to overcome platinum resistance.

(2) Inactivating oncogenes and other genes involved in chemosensitivity by targeting its molecular mechanism by using ribozymes or antisense technology (Rickardson et al., 2005; Ozben, 2006).

(3) Identifying the mechanism of multi drug resistance (MDR) and identifying inhibitors (Ozben, 2006)

(5) Combination therapy with non cross-resistant agents (Vasey, 2003).

(6) Sequential therapy, through using an initial drug, then switching to another drug to eliminate cells that have developed resistance to the initial therapy, then continue use of initial drug (Vasey, 2003).

2.1.4 HMG Proteins

The HMG proteins are believed to play key roles in cellular processes including: transcription, chromatin and nucleosome remodelling, chromosomal changes during cell cycle, DNA repair, apoptosis, and DNA replication (Reeves and Adair, 2005). The most studied of the HMG proteins is HMG1, that is believed to be involved in transcription and chromatin remodelling (Cohen and Lippard, 2001).

Structurally there are two families of HMG proteins. The first family contains two or more HMG domains. Each domain has around 80 amino acids. This family of HMG

proteins includes HMG1, HMG2, the nucleolar RNA polymerase I transcription factor UBF, and mitochondrial transcription factor mtTF. The second family of HMG protein contain only one HMG domain, for example the tissue-specific transcription factors (Wozniak and Blasiak, 2002). HMG1, HMG2, and UBF proteins recognise and specifically bind to 1, 2-intrastrand adducts formed by cisplatin but not 1, 3-intrastrand adducts formed mainly by transplatin. The binding of these proteins blocks and thus inhibits the repair of 1,2-intrastrand adducts and causes further bending to DNA. All HMG domain proteins are able to bend DNA (Jamieson et al., 1999; Wozniak and Blasiak, 2002; Farrell, 2004). Therefore, it has been found that cells missing HMG proteins are less sensitive to cisplatin than the wild cells (Huang et al., 1994). On the other hand over expression of some HMG proteins sensitized cells to cisplatin not normally responding to cisplatin treatment (Rabik and Dolan, 2007).

2.2. Second and third generation platinum drugs

The synthesis of new cisplatin analogues is still carried out in many research centres around the world mainly with the aim of reducing toxicity and expanding the spectrum of activity. The nature of the ligands and geometry can affect hydrolysis of the Pt-Cl bond and the reactivity of the designed complexes with other molecules (Farrell et al., 1999). Thus new cisplatin analogues have been produced by changing either the nature of the carrier ligand or that of the leaving groups or both. It has been possible to alter intracellular distribution of the drug and reduce toxicity by changing the nature of the leaving groups. On the other hand, by changing the nature of the carrier ligands, it has been possible to achieve limited changes in spectrum of activity by altering the mix and structure of drug-DNA adducts (Ho et al., 2003).

An important and desirable feature of newly designed platinum complexes would be their water solubility that can be achieved by replacing the chloride ligands in cisplatin with chelating carboxylates (like cyclobutane dicarboxylate), oxalate and glycolate; oxidation of platinum to platinum (IV); and creation of anionic phosphono carboxylates complexes (Wong and Giandomenico, 1999; Pasetto et al., 2006).

The main idea behind the development of second-generation platinum drugs was to reduce the toxic side effects and to a lesser extent widen the spectrum of activity. The third generation platinum drugs extended the second generation work and included a number of platinum compounds with different spectra of activity and different modes of delivery, for example the orally administered drugs. Here we discuss some of second and third generation platinum drugs:

2.2.1 Carboplatin

The structure of carboplatin, $[Pt(C_6H_6O_4)(NH_3)_2]$, diamine [1,1-cyclobutanedicarboxylate (2-)]-O,σ-platinum, is shown in Figure 2.9.

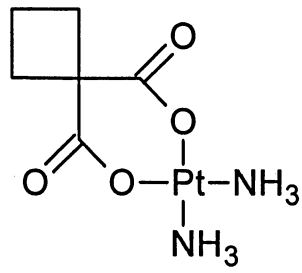


Figure 2-9 Structure of carboplatin

Carboplatin is a widely used anticancer drug. It was first approved in the United Kingdom and Canada in 1985. Carboplatin is given intravenously and shows cross resistance with cisplatin (Wong and Giandomenico, 1999; Reedijk, 2003). In carboplatin, the chloride ligands of cisplatin are replaced with chelating cyclobutanedicarboxylate ligands that enhances its water solubility. The hydrolysis rate of carboplatin is very slow because of the slow opening of the chelate ring so that its nucleophilic substitution by water molecules would occur at a slower rate. This enhances the stability of carboplatin, decreases its binding to proteins and hence decreases its toxic side effects (Chu, 1994; Wong and Giandomenico, 1999; Berners-Price and Appleton, 2000; Fuertes et al., 2003; Pasetto et al., 2006).

Carboplatin shows less antitumour activity than cisplatin and did not prove to be active in cisplatin resistant cancer in equal doses (Berners-Price and Appleton, 2000; Pasetto et al., 2006). For example cisplatin is more effective than carboplatin in treatment of germ cell tumours, head and neck, and bladder tumour. However, the main advantage of carboplatin over cisplatin is that it is less toxic than cisplatin so that it can be given in much higher doses than cisplatin. In addition, carboplatin is easier to use in combination therapy than cisplatin (Wong and Giandomenico, 1999; Reedijk, 2003; Wheate and Collins, 2003; Kostova, 2006).

2.2.2 Oxaliplatin

The structure of oxaliplatin [1,2-diaminocyclohexaneoxalatoplatinum] is shown in Figure 2.10. In oxaliplatin, the amine ligands present in cisplatin and carboplatin are replaced by 1,2-diaminocylohexane ligands (DACH) and the chloride leaving groups

are replaced by a chelating oxalate leaving group (Scheeff et al., 1999; Cohen and Lippard, 2001).

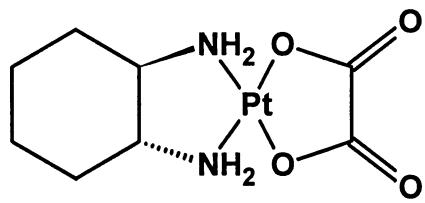


Figure 2-10 Structure of oxaliplatin

Oxaliplatin had been approved for clinical use by the FDA in August 2002 under the name Eloxatin™ (Wheate and Collins, 2003). Oxaliplatin is the only anticancer drug approved by the FDA that does not show cross-resistance with cisplatin. It has greater efficiency than cisplatin for the treatment of colorectal cancer when given with 5-fluorouracil (5FU) and folinic acid (Raymond et al., 2002; Pasetto et al., 2006; Thurston, 2007). The presence of 1,2-diaminocylohexan ligand (DACH) in oxaliplatin is believed to be the reason behind the absence of cross-resistance with cisplatin. The DACH ligand interacts with DNA distinctly differently than amino ligands (Wong and Giandomenico, 1999). Although both oxaliplatin and cisplatin form the same type of adducts with DNA, oxaliplatin-DNA adducts are more cytotoxic than those of cisplatin. It is believed that mismatch proteins along with other repair proteins such as HMG box protein recognize them differently (Scheeff et al., 1999; Wong and Giandomenico, 1999; Raymond et al., 2002; Wang and Lippard, 2005; Alderden et al., 2006; Pasetto et al., 2006). In addition, DNA polymerase is more efficient at error-free bypass of oxaliplatin compared to cisplatin. These differences of recognition and repair are thought to play a major role in the cytotoxicity difference between cisplatin and oxaliplatin (Wang and Lippard, 2005).

The side effects of oxaliplatin differ from cisplatin and carboplatin. It does not show nephrotoxicity; hearing loss is minimal and it is moderately emetogenic. The major toxicity of oxaliplatin is neurotoxicity which can be either acute transient cold induced dysaethesia or a dose dependent peripheral neuropathy which is usually reversible (Wong and Giandomenico, 1999; Brabec and Kasparkova, 2005).

2.2.3 Orally active drug Satraplatin (JM216)

JM216, [bis-aceto-aminedichloro-cyclohexylamine platinum(IV)] (Figure 2.11), is an orally active drug (Wong and Giandomenico, 1999). Oral treatment of cancer is of great benefit for patients as it is more convenient, less painful, and less costly. JM216 belongs to the platinum(IV) compounds making it more lipophilic and easing its absorption and passage through the gastrointestinal tract (McKeage, 2000). It is assumed that Pt(IV) is reduced to Pt to gain a cytotoxic effect (Ali et al., 2005).

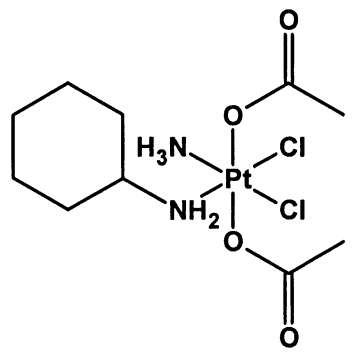


Figure 2-11 Structure of JM216

The results of an experiment carried out on mice to determine the absorption level and side effects of JM216, cisplatin, and carboplatin when taken orally, shows that the absorption level for JM216 was 71% of the administrated dose, for cisplatin it was

37%, and for carboplatin it was 22% (Wong and Giandomenico, 1999). While carboplatin caused severe gastrointestinal side effects, JM216 was more tolerable (Wong and Giandomenico, 1999). The phase I trial recommended doses of JM216 of 100-120 mg/m^2. In the phase II trial, although there was no response in non-small cell lung cancer, a good response of about 33% was achieved in hormone-refractory prostate cancer and the phase II trial is still going on (Wong and Giandomenico, 1999).

The toxicological profile of TJM216 is similar to that of carboplatin while its activity is similar to cisplatin. JM216 is even able to overcome cisplatin resistance related to reduced drug transport (Natile and Coluccia, 2001).

The phase III trial was aimed at examining the efficacy of satraplatin and prednisone on hormone-refractory prostate cancer (HRPC) patients who had no response to other chemotherapy agents. The results showed that satraplatin significantly reduced the risk of disease progression in these patients. In addition, satraplatin was well tolerated and showed clinical potential in combination therapy (Wosikowski et al., 2007; Choy et al., 2008).

2.2.4 JM335

The structure of JM335 {*trans*-amine(dichlorocyclohexylaminedihydroxo) platinum(IV)} is shown in Figure 2.12.

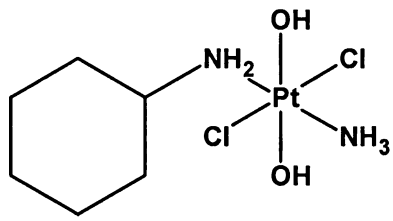


Figure 2-12 Structure of JM335

The cytotoxicity of JM335 is comparable to that of cisplatin and it is 50 fold more active than transplatin. The DNA binding of JM335 is distinct from cisplatin; it induces DNA single strand breakage. JM335 was the first transplatinum complex to show activity against many human ovarian carcinoma xenografts. However it was less than that of cisplatin and this is why it was not selected for clinical trial (Kelland, 2000). The lack of activity of JM335 was shown to be associated with a high level of GSH. This has provided a vital point for designing new platinum complexes that have reduced affinity towards thiols including ZD0473 (Kelland, 2000).

2.2.5 ZD0473 (formerly known as JM473 and AMD0473)

The structure of ZD0473 [*cis*-aminedichloro(2-methylpyridine) platinum] is shown in Figure 2.13.

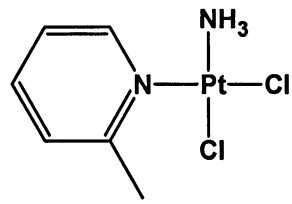


Figure 2-13 Structure of ZD0473

The main aim behind the design of ZD0437 was to overcome the platinum drug resistance caused by thiol-containing compounds including glutathione and metallothionein (Raynaud et al., 1997; Kelland et al., 1999; Holford et al., 2000; Kawamura-Akiyama et al., 2002; Gelmon et al., 2003; Twelves et al., 2003).

ZD0473 has 2-methylpyridine as its non-leaving ligand. This bulky ligand provides steric hindrance to the platinum centre and thus the compound become less susceptible and less reactive to thiol-containing molecules (Kelland et al., 1999).

A phase I clinical trial which started in 1997 has now been completed. Myelosuppression has been found to be the dose-limiting toxicity. The dose for phase II studies was 130 mg/m^2 every three weeks. As expected, and due to steric hindrance ZD0473 was found to be less reactive to glutathione and metallothionein than Cisplatin. The aquation rate of oxaliplatin was found to be two fold slower than that of Cisplatin. Worldwide, phase II and III clinical trials are now underway (Raynaud et al., 1997; Kelland et al., 1999; Rogers et al., 2002; Sharp et al., 2002; Gelmon et al., 2003). It was also reported that ZD0473 shows promising activity against some cisplatin resistant cell lines. ZD0437 has been found to overcome cisplatin resistant due to impaired drug transport and enhanced DNA repair (Holford et al., 2000; Kawamura-Akiyama et al., 2002; Rogers et al., 2002; Sharp et al., 2002; Gelmon et al., 2003; Oh et al., 2007). In ovarian carcinoma xenografts, ZD0473 showed much more growth delay than cisplatin and carboplatin (Ho et al., 2003). Furthermore Kawamura-Akiyama *et al* has found ZD0473 to be potent in cisplatin resistance human lung cancer cells (Kawamura-Akiyama et al., 2002).

A very interesting feature about ZD0473 is its oral activity. ZD0473 preserves its therapeutic profile when administered orally due to its reduced toxicity (Ho et al., 2003). ZD0473 has been found to form unique adducts including interstrand crosslinks (Kelland et al., 1999).

Rogers *et al* found synergy between ZD0473 and gemcitabine in two human ovarian carcinoma cell lines (A2780^{CisR} and CH1) (Rogers et al., 2002). The synergism appears to increase platinum–DNA adduct formation and increase gemcitabine

incorporation into DNA and RNA. Paclitaxel is one of the most widely used cytotoxics to be used in combination therapy with platinum agents to increase treatment efficacy. ZD0473 combined with paclitaxel has a manageable tolerability profile in a range of solid tumours including non-small-cell lung cancer. ZD0473 combination has a more manageable toxicity profile than other platinum-based combinations, particularly those containing cisplatin (Twelves et al., 2003).

2.2.6 Nedaplatin

The structure of nedaplatin, *cis*-diamine(glycolato-*O,O'*)platinum, is shown in Figure 2.14. Nedaplatin was approved in Japan in 1995 (Piccart et al., 2001; Jakupec et al., 2003).

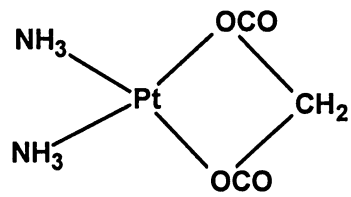


Figure 2-14 Structure of Nedaplatin

Nedaplatin has activity similar to that of cisplatin but it is less toxic. The dose-limiting toxicity of nedaplatin is myelosuppression, specifically thrombocytopenia, and low platelet count four weeks after drug administration (Piccart et al., 2001; Desoize and Madoulet, 2002). However nedaplatin has shown no superior response and overall survival advantage over cisplatin (Desoize and Madoulet, 2002; Brabec and Kasparkova, 2005). In phase II studies nedaplatin has shown activity in small and non-small lung cancer, testicular cancer, transitional-cell carcinoma of the urinary

tract and hormone-refractory prostate cancer, head and neck, ovarian, and cervical cancer (Jakupec et al., 2003). Nedaplatin and carboplatin have similar structures, therefore it's expected that they form similar types of DNA adducts (Brabec and Kasparkova, 2005).

2.2.7 Iproplatin

The structure of iproplatin {*cis*-dichloro-*trans*-dihydroxo-bis(isopropylamine) platinum(IV)}, also known as CHIP and JM6, is shown in Figure 2.15.

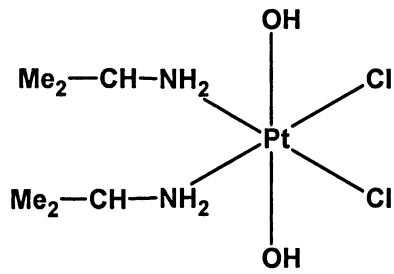


Figure 2-15 Structure of Iproplatin

Iproplatin showed activity in cisplatin responsive tumours. It was the first platinum (IV) complex to enter clinical trials. The dose limiting toxicity for iproplatin was myelosuppression (Lebwohl and Canetta, 1998). In phase III clinical trials, iproplatin showed less activity and more toxicity than carboplatin. Therefore further developments of this complex were abandoned (Trask et al., 1991).

2.2.8 Lobaplatin

The structure of lobaplatin {1,2-diaminomethylcyclobutaneplatinumlactate} is shown in Figure 2.16.

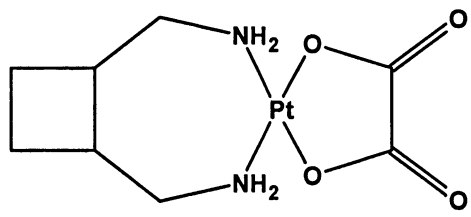


Figure 2-16 Structure of Lobaplatin

Lobaplatin is a water soluble platinum complex (Welink et al., 1999). Lobaplatin showed significant activity against cisplatin-resistant ovarian and testicular carcinoma xenografts and a complete lack of cross-resistance to cisplatin (Gietema et al., 1993). In phase I and II clinical trials, lobaplatin showed activity against esophageal, breast, head and neck, small lung and ovarian cancers (Welink et al., 1999). In comparison with carboplatin, thrombocytopenia is the dose-limiting toxicity for both drugs. Moreover, lobaplatin binds less with serum protein and is more rapidly excreted through urine (Kelland, 1993). Lobaplatin is an approved drug in China.

2.2.9 C5-OHP-Cl

The structure of C5-OHP-Cl: *trans*-bis(n-valerato)(1R,2R-cyclohexanediamine) (oxalate) platinum(IV) is shown in Figure 2.17.

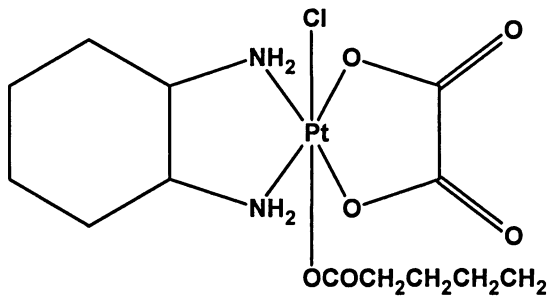


Figure 2-17 Structure of C5-OHP-Cl

The main aim behind C5-OHP-Cl preparation was to obtain an orally active oxaliplatin derivative (Kizu et al., 1996). C5-OHP-Cl has been found to have high absorption and activity levels. It has been found to be more active than cisplatin in many different tumours including L1210 leukaemia and LMFS sarcoma (Saegusa et al., 2001).

2.3. Transplatin

The structure of transplatin, *trans-diaminedichloroplatinum*, is shown in Figure 2.18. Transplatin is *trans*-isomer of cisplatin. It blocks transcription and replication of DNA, binds to the N7 atom of guanine and needs hydrolysis to bind to DNA, but still does not show any clinical antitumour activity (Boudvillain et al., 1995; Jamieson et al., 1999; Sartori et al., 2000).

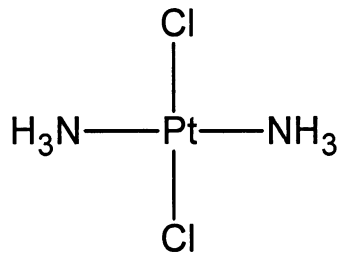


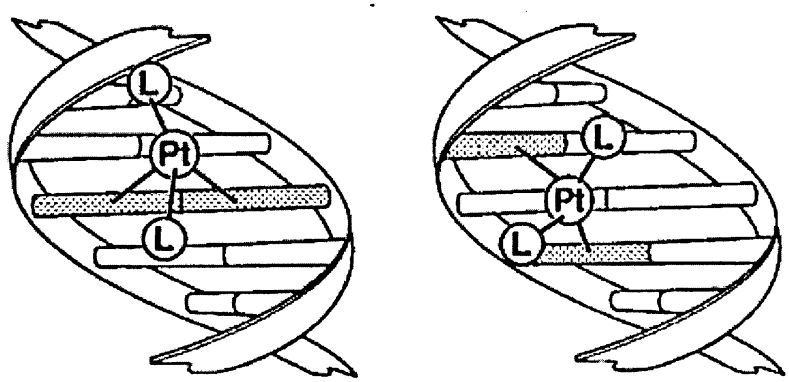
Figure 2-18 Structure of Transplatin

There are different theories that elucidate the antitumour inactivity of transplatin including: (1) inability of transplatin to form 1,2 intrastrand adducts, (2) deactivation of transplatin monofunctional adducts, (3) HMG protein recognition, and (4) high reactivity of transplatin.

(1) Inability of transplatin to form 1,2 intrastrand adducts: It is generally accepted that 1,2 intrastrand adducts are the lesion behind cisplatin cytotoxicity. Transplatin mainly forms monofunctional adducts, 1,3 intrastrand adduct (G1,G3), and 1,2 interstrand adduct as shown in Figure 2.19 but not 1,2 intrastrand adducts. The stereochemistry of transplatin does not allow the formation of 1,2 intrastrand adducts (Eastman et al., 1988; Brabec and Leng, 1993; Kelland, 1993; Boudvillain et al., 1995; Sigel and Lippert, 1998; Paquet et al., 1999; Giraud-Panis and Leng, 2000; Sartori et al., 2000; Cohen and Lippard, 2001; Natile and Coluccia, 2001; Peleg-Shulman et al., 2002; Wheate and Collins, 2003; Ma et al., 2005; Rabik and Dolan, 2007).

(2) Deactivation of transplatin monofunctional adducts: The closure of transplatin monofunctional adduct to form bifunctional adducts is very slow. It is been reported that after 1 hour of transplatin incubation with DNA 85% of adducts were monofunctional. After 24 hour of incubation only 50% of monofunctional adducts were transformed to bifunctional adducts. Transplatin monofunctional adducts are

susceptible to deactivation through binding of glutathione thus inhibiting the formation of active toxic bifunctional adducts. On the other hand cisplatin monofunctional adduct closure is faster so that glutathione has less effect on cisplatin toxicity than transplatin (Eastman et al., 1988; Dalbies et al., 1996).



Interstrand adduct 1,3 intrastrand adduct

Figure 2-19 Bifunctional types of transplatin adduct

(3) HMG proteins: HMG proteins as stated before recognise and bind to 1,2 intrastrand but not 1,3 intrastrand adducts. HMG proteins prevent repairing proteins from reaching this adduct (Natile and Coluccia, 2001).

(4) High reactivity of transplatin: Transplatin is more reactive than cisplatin. Therefore, it is more liable to deactivation than cisplatin (Wong and Giandomenico, 1999; Huq et al., 2004; Ma et al., 2005).

2.3.1 Activation of *trans*-platinum complexes

Based on intense research effort following the discovery of the antitumour activity of cisplatin, the following structure-activity relationship requirements were suggested: (1) the complex should to be neutral; (2) it should have a *cis* geometry; (3) it should have two leaving groups that are neither too loosely bound or too lightly held; (4) it should have two tightly bound non-leaving groups (Hodes et al., 1992; Bose, 2002; Radulovic et al., 2002). The requirement of *cis*-configuration is no longer considered to be essential as *trans* geometry can be activated for antitumour activity by replacement of the amine ligand with sterically hindered ligands such as planar amines (Ma et al., 2005). The use of bulky ligands could also alter the DNA binding mode of transplatinums and minimize its interaction with glutathione (Kasparkova et al., 2003; Huq et al., 2004b; Quiroga et al., 2007).

As discussed before, one suggested reason behind the inactivity of transplatin is its high reactivity so that it is more liable to deactivation. The replacement of amine ligands by bulky ligands in the *trans*-complexes increases the time needed to replace the chloride ligands. Bulky ligands such as pyridine block access to the platinum atom and therefore inhibit ligand substitution. It has been found that *trans* pyridine complexes are less reactive with glutathione and other sulfur containing molecules (Natile and Coluccia, 2001; Huq et al., 2004). In addition, the requirement of intrastrand bifunctional adducts with DNA can no longer be met. It is found that the activated form of *trans*-platinum complexes that form only monofunctional adducts still exhibits significant antitumour activity (Bose, 2002). It has been suggested that active transplatinum complexes that form only monofunctional adducts may

overcome cisplatin resistance (Radulovic et al., 2002). It is believed that the formation of monofunctional adducts along with stacking interaction with DNA bases can produce a similar effect to that of cisplatin (Martınez et al., 2007). It is interesting to note that the transplatinum adducts formed are recognised by cisplatin antibodies but not transplatin antibodies. Moreover, transplatinum complexes with planar amine ligand are found to form more of interstrand adducts than transplatin. After 48 h 30-40% of adducts are interstrand adducts and 30-40% of adducts are monofunctional (Martınez et al., 2007).

An important feature of *trans*-complexes with bulky ligands is that they do not show cross resistance with cisplatin in most of the cases examined such as murine L1210 leukaemia and human ovarian resistant tumour cells. It has been suggested that the *trans* complexes would have distinctly different cellular cytotoxicity to cisplatin (Natile and Coluccia, 2001). Cellular accumulation of *trans* pyridine complexes (carried out on L1210/0) is found to be greater than that of cisplatin (Natile and Coluccia, 2001). The introduction of pyridine ligands to transplatinum complexes is believed to make the complexes more lipophilic and hence the greater cellular uptake (Ghezzi et al., 2004). From above it appears that *trans*platinum complexes with bulky ligands such as pyridine may be able to overcome cisplatin resistance (Perez et al., 2000; Natile and Coluccia, 2001). Huq *et al* have synthesised several transplatinum complexes in the form *trans*-$PtL(NH_3)Cl_2$ and *trans*-PtL_2Cl_2. Some of these compounds are found to be more active than cisplatin and in some cases show greater activity in cisplatin-resistant cell lines than in cisplatin-responsive cell lines (Huq et al., 2004; Huq et al., 2004b).

2.4. Multinuclear platinum compounds

Polynuclear platinum complexes are a new class of platinum compounds that are unique in their structure, activity profile, and DNA binding. They contain two or more linked platinum ions that bind covalently with DNA. They are mostly charged and highly active thus proving that neutrality is not an essential requirement for activity. On the contrary, the best antitumour activity was achieved when the total charge of the cation responsible for activity is 3+ or 4+ (Farrell et al., 1999).

2.4.1 Dinuclear platinum compounds

The first dinuclear platinum compound, termed as 2,2/c,c (Figure 2.20) was reported in 1988. It was made up of two cisplatin units linked by a flexible diamine chain (Farrell et al., 1999; Farrell, 2004). This compound was tetrafunctional, neutral not charged, formed mainly interstrand adducts and proved to be very active even in cisplatin-resistant cells (Farrell, 2000).

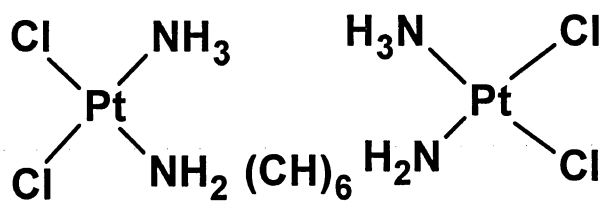
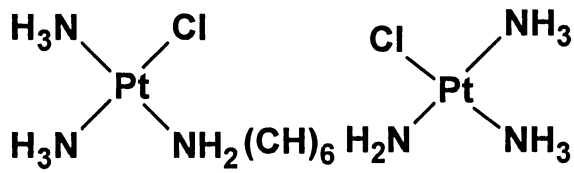


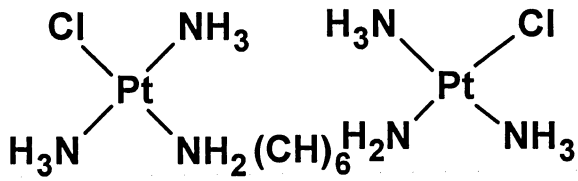
Figure 2-20 Structure of the tetrafunctional dinuclear platinum complex 2,2/c,c

In polynuclear complexes only two Pt-Cl (bifunctional) ligands are needed to produce the inter- or intra-strand adducts and this is why bifunctional compounds were preferred over tri or tetra-functional or other functional compounds (Farrell et al., 1999).

The synthesis of bifunctional platinum compounds was the most important development, representing a new class of polynuclear platinum compounds. These new dinuclear compounds such as 1,1/t,t and 1,1/c,c (Figure 2.21) were found to be very active even in cisplatin-resistant cell lines (Farrell et al., 1999; Farrell, 2000; Wheate and Collins, 2003).



(1,1/c,c)



(1,1/t,t)

Figure 2-21 Structure of bifunctional dinuclear platinum complexes (1,1/c,c) and (1,1/t,t)

In a study on the resistant murine leukaemia cell line L1210, the cellular uptake of both 1,1/t,t and 1,1/c,c was similar but their binding to DNA was different. The

complex 1,1/t,t bound more to DNA, while 1,1/c,c binding to DNA was less, due to steric effect (Farrell et al., 1999). The 1,1/t,t complex forms both types of adducts intra- and inter-strand, while 1,1/c,c forms only interstrand adducts. These results are very important in proving that 1,1/c,c complex forms only interstrand adducts and is still very toxic to tumour cell. The results suggest that the interstrand adduct formed by 1,1/c,c is different from the one formed by cisplatin or transplatin (Farrell et al., 1999).

The reason behind the superior activity of the *trans* form is believed to be due to the type of adducts formed by *trans* but not by *cis* configuration. Moreover, the rate of hydrolysis is also believed to play a role (Wheate and Collins, 2003)

In addition, a new dinuclear compound with a heterocycle bridging group has been synthesised. The presence of such a bridge permits the formation of van der Waal's interaction and hydrogen bonding with the DNA minor groove (Farrell, 2004).

The synthesis of dinuclear platinum compounds opened the window for the synthesis of trinuclear platinum compounds.

2.4.2 Structure activity relationship in polynuclear platinum complexes:

The antitumour activity of both di- and tri-nuclear platinum complexes is decided by many factors that have to be considered when designing new polynuclear platinum complexes including: (1) chain length and flexibility; (2) steric effect, hydrogen bonding and charges in the linker chain; (3) geometry of chloro ligands to the linking chain (Brabec et al., 1999; Wheate and Collins, 2003).

To increase water solubility and improve affinity towards DNA, H-bonding has to be increased in the backbone (Farrell et al., 1999; Wheate and Collins, 2003). In

addition, chain length plays a very important role in the antitumour activity of the compound (Farrell, 2000). The best length of the linking chain was found to be eight atoms (two amine and six-methylene groups) and the best example representing this case is the trinuclear BBR3464 (Wheate and Collins, 2003). The flexibility of the linker chain is found to play a major role in the cytotoxicity of the multinuclear compounds. When a rigid ligand like 4,4-dipyrazolylmethan (abbreviated as dpzm) was used, the di- and tri-nuclear compounds were found to be less active. The rigid dpzm, is believed to prevent these compounds from forming a sub-set of DNA adducts that are formed when a flexible ligand is used. Steric hindrance in the diamine linker was found to reduce cytotoxicity and DNA binding affinity (Farrell, 2004). The multinuclear compounds containing aromatic ligands were found to be less cytotoxic. This clearly indicates that straight chain linking ligands produce much more cytotoxicity than the aromatic ones (Wheate and Collins, 2003). In addition, the best cytotoxicity results were found when the linking ligand contained positive charges and hydrogen bonding involving either a charged platinum centre and/or charged amine groups (Wheate and Collins, 2003).

The central unit in di- and tri-nuclear platinum complexes does not form a covalent bond with DNA. It contributes to charge and hydrogen bonding of the complex and therefore increases the affinity of the complex to DNA, affects the charge-to-lipophilicity balance, and increases the length of the complex (Brabec et al., 1999; Hegmans et al., 2004).

The geometry of the chloro ligand is also believed to play an important role. It's been demonstrated in many studies that *trans* geometry is more active than the *cis* ones especially in cisplatin resistant cell lines. This has been noticed in both di- and trinuclear platinum compounds, including BBR3464. The BBR3464 (1,0,1/t,t,t,) is

more active than BBR3499 (1,0,1/t,c,t) hence the only difference between the two compounds is the central unit. Therefore *cis* configuration reduced the cytotoxicity of the compound in this case (Farrell et al., 1999; Kasparkova et al., 2003; Kasparkova et al., 2004).

Although the types of adducts formed by both BBR3464 and BBR3499 are nearly same, BBR3499 forms more interstrand adducts than BBR3464. It is believed that interstrand adducts are more cytotoxic than intrastrand adducts in polynuclear complexes. The reason why BBR3499 forms more interstrand adducts than BBR3464 and is still less cytotoxic than BBR3464, is believed to be due to differences in the structure of interstrand adducts formed by the two compounds (Roberts et al., 1999; Kasparkova et al., 2004). The adducts formed by BBR3499 are found to be more efficiently removed by cellular repair mechanisms than those formed by BBR3464.

In addition, the binding of BBR3464 was found to be faster than that of BBR3499 although both complexes have the same charge. This resulted in reduced sequence specificity of BBR3499 than BBR3464 (Kasparkova et al., 2004; Kasparkova, 2004b; Brabec and Kasparkova, 2005).

2.4.3 More on BBR3464

Although the first trinuclear compound was reported in 1993, the best-studied trinuclear compound and the first polynuclear complex to enter clinical trial was BBR3464 (Figure 2.22) (Farrell, 2000).

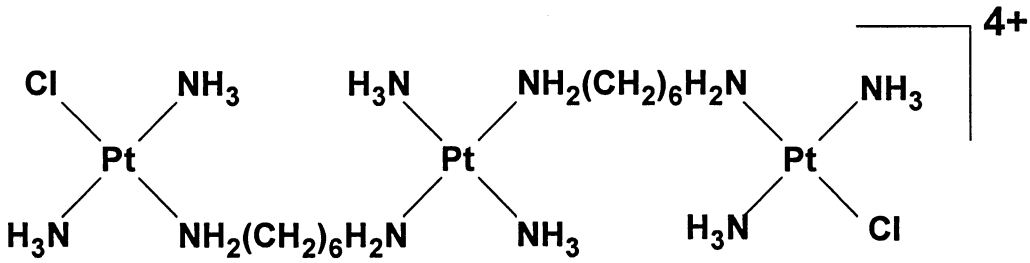


Figure 2-22 Structure of BBR3464

The cytotoxicity of trinuclear complexes represented by BBR3464 has been found to be much more than that of dinuclear compounds. Therefore, trinuclear complexes have been preferred over dinuclear complexes. There are many factors that contribute to the higher cytotoxicity of trinuclear complexes over dinuclear complexes including: the longer chain length of trinuclear complexes that covers both the major and minor groove and its greater flexibility. In contrast, the shorter chain length of dinuclear complexes is less flexible and covers only the major groove. In addition, the increased chain length of the trinuclear platinum complexes provides greater charge along the backbone and thus greater affinity towards DNA (Brabec et al., 1999). However, both di- and tri-nuclear compounds have been found to undergo degradation in the presence of detoxifying agents such as glutathione (Qu et al., 2004).

Tetranuclear and pentanuclear platinum compounds have also been synthesised. The antitumour activities of the compounds were found to be inconsistent showing remarkable cytotoxicity in some cell lines and weak activity in others (Farrell, 2004).

The unique structure of BBR3464 (1,0,1/t,t,t) is not based on cisplatin, and is made up of two *trans*-$[PtCl(NH_3)_2]^+$ linked by tetra amine $[Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2]^{2+}$. The central platinum coordination is different from the other terminal ones.

Only the two terminal Pt's bind covalently to DNA while the central Pt contributes to DNA affinity through electrostatic and hydrogen bonding with O6 in guanine or O3 in thymine and the overall 4+ charge of the molecule (Farrell, 2000; Wheate and Collins, 2003).

BBR3464 is ten fold more active than cisplatin and does not show cross resistance with cisplatin (Pratesi et al., 1999). In addition, BBR3464 has a broader spectrum of activity including that in p53 mutant cells (Brabec et al., 1999; Pratesi et al., 1999; Roberts et al., 1999; Manzotti et al., 2000; Servidei et al., 2001; Jodrell et al., 2004; Hensing et al., 2006).

IC_{50} values for BBR3464 and cisplatin after 1 hour exposure of human osteosarcoma U2-OS/ Pt resistant cell were 13.6 ± 2.7 and 0.89 ± 0.57 μM respectively. The ability of BBR3464 to overcome cisplatin resistant in osteosarcoma is believed to be due to a different interaction mechanism with DNA rather than overcoming a specific resistance mechanism (Perego et al., 1999). In a preclinical evaluation conducted by Manzotti *et al* in a number of human tumour cell lines including xenografts, BBR3464 was able to achieve tumour weight inhibition (TWI) of more than 80% in seven out of eighteen tested tumours, and to fully overcome cisplatin acquired resistance in three cell systems (Manzotti et al., 2000). The results clearly demonstrate that BBR3464 is much more active than cisplatin in many tumours including cisplatin resistant ones (Farrell et al., 1999).

The interaction of trinuclear compounds with DNA is characterised by: fast DNA binding, unwinding to DNA by 14^0, irreversible DNA conformational change from B→A and from B→Z DNA (Wheate and Collins, 2003; Farrell, 2004; Jodrell et al., 2004). The function of Z DNA is not well known but it is thought to have a great effect on DNA function (Farrell, 2004).

A phase I clinical trial conducted on BBR3464 demonstrated a significant response in cancers which are resistant to cisplatin including melanoma, pancreatic and lung cancer. The side effects were found to be dose related (Farrell, 2004; Jodrell et al., 2004). Diarrhoea and neutropenia were the main side effects when dosage reaches 1.1 mg m^{-2}. On the other hand none of other side effects such as nephrotoxicity, neurotoxicity, pulmonary toxicity or severe emesis have been reported (Kasparkova et al., 2002).

In the phase II clinical trial, which began in 2000, considerable declines indicating partial response in cisplatin resistant ovarian cancer and non-small cell lung cancer were recorded (Kasparkova et al., 2002; Ma et al., 2005).

2.4.3.1 Cellular uptake of BBR3464

The accumulation of BBR3464 in tumour cells was found to be four to five fold higher than that of cisplatin (Farrell et al., 1999). The cellular uptake and the interaction of trinuclear BBR3464 with the cell membrane are poorly understood. Liu *et al* suggested that the interaction of negatively charged lipids in the cell membrane with positively charged BBR 3464 could assist in the transport of these positively charged complexes (Liu et al., 2006). This agrees with the fact that the higher the charge of the polynuclear platinum complexes the faster is the uptake.

As discussed earlier, cisplatin is believed to enter cells through both passive diffusion and energy dependent process. Polynuclear charged platinum complexes are unlikely to cross the cell membrane by passive diffusion. Therefore, the mechanism of transport for polynuclear complexes is more likely to include active transport and/or facilitated diffusion (Roberts et al., 1999).

2.4.3.2 Pre-association and DNA binding of BBR3464 and other trinuclear complexes

The BBR3464 has 4^+ units of charges (after the hydrolysis of two Cl^- the total charge becomes 6^+). This means BBR3464 would have high affinity towards the negatively charged DNA (Kloster et al., 1999; Farrell, 2000; Wheate and Collins, 2003; Kasparkova, 2004b).

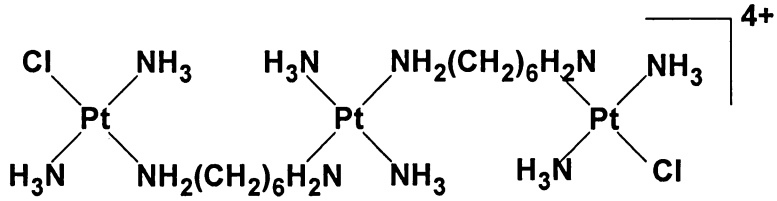
The $t_{½}$ i.e. time needed for binding of BBR3464 to DNA to be half completed is 40 min while that for cisplatin is around 240 min; the difference in values supports the idea that the charged BBR3464 binds faster with DNA than neutral cisplatin. In an experiment in which calf thymus DNA was incubated with 20 μM BBR3464 and cisplatin, the amount of BBR3464 that got bound to the DNA after 2 h was found to be seven times higher than that for cisplatin.

The rapid binding of BBR3464 to DNA suggests that it may be reacting with DNA in a different manner than cisplatin. For example, unlike cisplatin hydrolysis may not be a crucial step for binding of BBR3464 to DNA (Farrell et al., 1999). The aquation rate constant for BBR3464 is similar to that for cisplatin. Although the rate of N7G binding to form a monofunctional adduct is found to be similar for both BBR3464 and cisplatin being about 0.47 ± 0.06 M^{-1} s^{-1}, the rate of closure to form an interstrand bifunctional adduct in the case of BBR3464 is 10-55 fold faster than that in the case of cisplatin to form 1,2 GG intrastrand crosslink (Farrell, 2004). BBR3464 undergoes significant pre-association (non-covalent in nature) with DNA before forming the covalent adducts. The pre-association of the central linker unit of BBR3464 has a significant effect over the binding kinetics, the structure of the final adducts and the site of platination (Brabec et al., 1999; Wheate and Collins, 2003; Hegmans et al.,

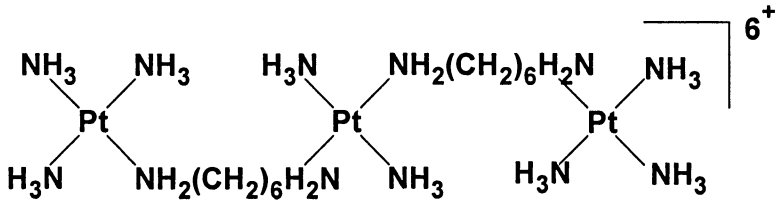
2004; Harris et al., 2006). In turn, the structure of adduct is important as it determines repair, protein recognition, and other cellular events in which non-covalent interactions are also expected to play a key role (Harris et al., 2006).

The pre-association induces reversible conformational change in DNA that becomes irreversible when the covalent binding is complete (Wheate and Collins, 2003). The multi-nuclear platinum compounds were found to prefer binding to the DNA minor groove despite the high charge, shape, steric effect, or the ligand used. The reason is due to favourable electrostatic interaction and van der Weals forces, moreover, it is very rich in adenine and thymine. On the other hand a guanine/cytosine rich area is unfavourable because of the steric effect of the guanine amino group (Wheate and Collins, 2003).

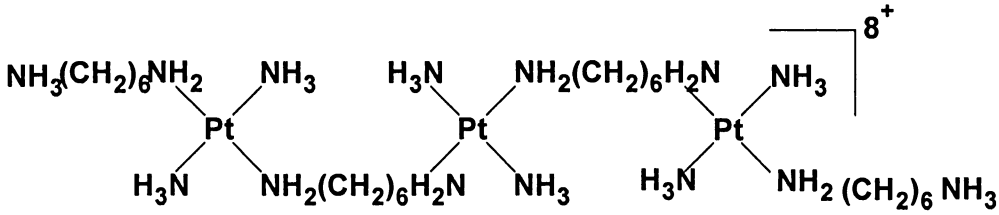
Qu *et al* synthesised two new trinuclear platinum compounds similar to BBR3464 (Figure 2.24) but which differed from BBR3464 in the sense that the two terminal platinums were bound to an inert ammonia or amine group instead of the chloride (Qu et al., 2004) so that the compounds couldn't bind covalently. The charge of these compounds was 6^+ and 8^+ respectively as shown in Figure 2.23.



BBR3464



6^+ trinuclear complex



8^+ trinuclear complex

Figure 2-23 Structure of BBR3464, 6^+ and 8^+ trinuclear complexes

When tested against cancer cell lines, these new compounds showed high ability to associate with DNA and to produce DNA conformational change as well as enhanced cellular uptake over cisplatin. Although the cytotoxicity of these compounds was less than that of BBR3464, they were able to trigger apoptosis even at micromolar concentration ranges similar to cisplatin. This experiment clearly demonstrates the importance of non-covalent binding (pre-association) to trigger apoptosis. Polynuclear non-covalent binders induced partially irreversible conformational changes in DNA

from B→A and B→Z. The stability of these conformational changes is related to the charge of the molecule - the complex with 8^+ charges produced more permanent change than that with 6^+ charges (Qu et al., 2004). In fact, the complex with 8^+ charges produced similar cytotoxicity to that of cisplatin (Harris et al., 2006). The increased charge of the complexes clearly enhanced their cellular uptake and cytotoxicity. Thus for further drug development it is essential to comprehend the role of non-covalent interactions on the activity of these drugs.

2.4.3.3 DNA binding and the formation of inter- and intra-strand adducts of polynuclear complexes

Polynuclear platinum complexes form different types of adducts than cisplatin (Chvalova et al., 2006). In general, polynuclear complexes form more interstrand adducts than cisplatin. The geometry of polynuclear complexes plays a major role in determining the type of adducts formed. For example, as stated before, the di-nuclear complex 1,1/c,c forms only interstrand adducts; the steric hindrance of the complex prevents the formation of any intrastrand adduct. On the other hand, its analogue 1,1/t,t forms 70-90% interstrand adducts (Wheate and Collins, 2003).

BBR3464 forms different types of inter- and intra-strand adducts. Intrastrand adducts includes 1,2-, 1,3-, and 1,5-intrastrand cross links (Farrell, 2004), however due to its length, formation of 1,2-intrastrand crosslinks is not favourable. BBR3464 adducts does not cause steric stress including bending of DNA as compared to cisplatin 1,2-intrastrand adducts (Zehnulova et al., 2001). In general, the intrastrand adducts formed by BBR3464 are flexible, do not cause stable bending (directional bending) of DNA, and block DNA or RNA polymerase. However, the adducts are not recognised

by HMG proteins so that they are easily removed by NER (Zehnulova et al., 2001; Kasparkova et al., 2002; Wheate and Collins, 2003; Farrell, 2004).

BBR 3464 forms a number of interstrand adducts including 1,2-, 1,4-, and 1,6-interstrand adducts. However, the most frequent one is the 1,4-interstrand adduct, while the least frequent one is 1,6-interstrand adduct (Kasparkova et al., 2002). Interstrand adducts comprise around 20% of the total adducts formed by BBR3464. They are formed between guanine residues in both 5′→5′and 3′→3′ direction. Interstrand adducts cause DNA bending towards the major groove of 15^0-21^0 (less than that caused by cisplatin which is around 32^0-34^0), and unwinding by 14^0. However, the interstrand adducts are hardly recognized by HMG proteins (Farrell, 2004). It has been suggested that the percentage of interstrand adduct depends on the charge of the complex rather than chain length (Farrell, 2004).

The interstrand adducts formed by BBR3464 are far more difficult to repair than the intrastrand adducts because they involve both strands of DNA and the complementary strand cannot be used for resynthesis (Farrell, 2004). It is found that the 1,4-interstrand (the major interstrand adduct formed by BBR3464) is not easily removed by NER (Boulikas and Vougiouka, 2003). Thus, it has been suggested that the toxicity of BBR3464 may be due to interstrand adducts rather than intrastrand adducts which are effectively removed by NER (Zehnulova et al., 2001; Kasparkova et al., 2002).

2.4.3.4 Mechanism of toxicity of polynuclear complexes

The cytotoxicity of platinum drugs does not depend on the number of Pt-DNA adducts formed; rather it depends on the effect of these adducts on downstream processes like protein recognition, cell cycle arrest, and DNA repair.

The conformational flexibility of the long range adducts formed by polynuclear complexes allows free rotation around the backbone and thus eliminates the steric demand of adducts as in the case of cisplatin. In addition, the conformational flexibility ensures that adducts are not recognised by proteins such as HMG that binds only to rigidly bent DNA. Therefore, unlike cisplatin, neither the hijacking nor shielding mechanism is likely as a mediator of toxicity of polynuclear complexes (Wheate and Collins, 2003; Farrell, 2004; Brabec and Kasparkova, 2005). However, it is suggested that long range adducts generally escape repair recognition making them very difficult to repair (Farrell, 2004; Brabec and Kasparkova, 2005). In addition, it is suggested that adducts like the 1, 4-interstrand adduct could cause blockage of DNA and RNA polymerases (Zehnulova et al., 2001; Kasparkova et al., 2002; Farrell, 2004). It has been hypothesised that the antitumour efficiency of the trinuclear complexes can be explained as the collective and "cooperative" effect of the adducts formed especially the interstrand adducts that cause a DNA conformational change affecting not only the binding site but much beyond (Farrell, 2004).

2.4.3.5 Binding of BBR3464 to single strand DNA and RNA

BBR3464 binds more favourably to single strand DNA or RNA than the double strand DNA. In addition, BBR3464 reacts faster with single strand DNA or RNA and produces more adducts than with the double strand DNA (Kloster et al., 1999; Wheate and Collins, 2003). It has been suggested that the fast binding of BBR3464 to single strand DNA may be due to the more defined conformation of the double strand DNA as compared to the single strand DNA (Wheate and Collins, 2003).

In cells, single strand DNA is found during transcription, replication, recombination and repair. Binding of BBR3464 to these essential cellular components deprives the

cell of their vital cellular role and therefore could play a role in the antitumour efficiency of the complex (Farrell, 2004). The strong attraction and binding of polynuclear complexes towards single strand DNA has many biological applications including telomerase inhibition and antisense gene therapy. The main aim of antisense technique is to inhibit protein expression by binding small molecules to messenger RNA so that the translation process is physically blocked (Farrell, 2004).

2.4.3.6 Effect of p53 on cytotoxicity of trinuclear complexes

The cytotoxicity of BBR3464 in both wild type p53 cells and p53 mutant cells are found to be the same suggesting that BBR3464 has a different DNA binding profile than cisplatin. Therefore, the apoptosis induced by BBR3464 is not mediated by p53 (Farrell et al., 1999; Pratesi et al., 1999; Farrell, 2000; Manzotti et al., 2000; Servidei et al., 2001; Perego et al., 2003). However, p53 could affect the sensitivity of tumour cells to BBR3464 (Kasparkova et al., 2004). It has been found that p53 binds less efficiently to the adducts formed by BBR3464 than those formed by cisplatin. Thus it is been hypothesized that the structural modifications induced in DNA by BBR3464 and cisplatin adducts produce different responses to p53 activation and recognition (Kasparkova et al., 2004; Brabec and Kasparkova, 2005). As discussed earlier, cisplatin is ineffective in treating tumour characterised as p53 mutant. Therefore, BBR3464 could be a very useful drug in treating over 50% of tumour cases that are characterised as p53 mutant.

2.5. Justification of the research

Although over 3000 cisplatin analogues has been synthesised in the past few years, only 28 of these have been evaluated in clinical trials and still fewer continue to be investigated (Pasetto et al., 2006). Most compounds were abandoned due to their unacceptably high toxicity or low activity (Perego et al., 1999; Farrell, 2004). Since the approval of cisplatin in 1978, only carboplatin has been approved in full clinical use and most recently oxaliplatin (Farrell, 2004).

The general view is that although some cisplatin analogues may overcome some cisplatin resistance, it is believed that the compounds may interact with DNA in a similar manner to that of cisplatin so the main issue of resistance will resurface sooner or later (Collins and Wheate, 2004; Huq et al., 2004b). Hence there is need for a novel approach to overcome cisplatin resistance that could lead to new platinum drugs that are structurally and functionally different from cisplatin. An ideal drug would be less toxic than cisplatin while maintaining similar cytotoxicity, overcome cisplatin resistance, have a wider spectrum of activity and a better method of administration than intravenous infusion. Currently, attention is given to rule-breaker platinum complexes with the idea that their different nature of interaction with DNA may change the spectrum of activity. Two such classes of compounds are the *trans*-planaramineplatinum complexes and platinum compounds with multiple metal centres. Both these classes of compounds are found to overcome cisplatin-resistance in a number of cancer cell lines. This thesis employs both the approaches with the aim of arriving at new tumour active platinum compounds. As stated earlier, although cisplatin is tumour active its *trans*-isomer transplatin is inactive but toxic mainly due

to its higher reactivity. Thus, it was suggested by Farrell (Farrell et al., 1992) that the *trans*-geometry could be activated for antitumour activity through the introduction of bulky carrier ligands such as planaramines. Indeed a number of such tumour active compounds have been designed by Huq laboratory and elsewhere (Farrell et al., 1992; Huq et al., 2004). *Trans* platinum complexes with sterically hindered ligands are believed to bind to DNA in a different mode than cisplatin; this may be the reason why the compounds may show activity in cisplatin resistant cell lines.

Polynuclear platinum complexes represent a new class of anticancer drugs with unique structure and DNA binding profile. The best studied polynuclear platinum complex is BBR3464. In Phase I trial, the compound demonstrated a clear response in cancers normally resistant to cisplatin while phase II studies demonstrated partial response in relapsed ovarian cancer and non-small cell lung cancer. Only the two terminal platinum ions in BBR3464 bind covalently with DNA whereas the central platinum ion is involved only in non-covalent interactions such as electrostatic interaction and hydrogen bonding via ammonia ligands. In the Huq laboratory also, a number of highly tumour active polynuclear complexes based on BBR3464 have been designed by replacing the central metal ion with other metal ions such as Pd^{2+} and/or by the introduction of sterically hindered ligands bound to the central metal ion (Daghriri et al., 2004; Huq et al., 2006). Sterically hindered ligands such as 3-hydroxypyridine bound to the central metal ion may be involved in non-covalent interactions with DNA bases and such interactions may serve to alter the spectrum of activity. Although a number of new trinuclear complexes have been prepared in this laboratory and elsewhere, the field is still relatively new and offers the possibility of designing new compounds with a better activity and toxicity profile and mode of delivery. Thus the aims of the present study were to:

(1) To synthesize and characterize new mono-nuclear and multinuclear platinum-based tumour active compounds with tans-geometry.

(2) To determine the activity of the designed platinum complexes against human cancer cells and their interaction with DNA, including cell uptake and DNA binding.

(3) To investigate structure-activity relationships in the designed complexes.

The present project is a part of the study being carried out in this laboratory to explore the activity and the nature of binding with DNA of new *trans*-planaramineplatinum complexes and polynuclear complexes. The synthesis of two of the mononuclear complexes namely JH1 and JH1 has been reported earlier (Van Beusichem and Farrell, 1992). However, as detailed studies on the activity of the compounds are lacking and since the compounds served as the starting materials for the new trinuclear complexes prepared in the present study, JH1 and JH2 have been investigated for activity and nature of binding with DNA.

3. Chapter Three

3.1. Materials and Methods: Introduction

In this project two new mononuclear *trans*-planaramineplatinum complexes code named JH3 and JH4 (containing thiazole, imidazole and 3-hydroxypyridine) and two new trinuclear transplatinum complexes code named JH5 and JH6 have been synthesised and investigated for activity against ovarian cancer cell lines and nature of binding with DNA. The two other mononuclear complexes JH1 and JH2 (containing thiazole ligand) whose synthesis has been described previously, have also been synthesized, and investigated for activity against ovarian cancer cell lines and the nature of binding with DNA (Weaver et al., 1970; Muir et al., 1988; Farrell et al., 1992; Van Beusichem and Farrell, 1992; Bierbach et al., 1999; Brabec et al., 2000; Kasparkova et al., 2003b; Farrell et al., 2004b; Kalinowska-Lisa et al., 2008). JH2 and JH1 served as the starting materials for the synthesis of JH5 and JH6 respectively. Although some results on the activity of JH1 and JH2 have also been reported, detailed studies on activity are lacking. In the present study, in addition to activity in A2780 cell line, that in resistant cell lines $A2780^{cisR}$ and $A2780^{ZD047R}$ have also been determined. We also determined cell uptake, level of binding with cellular DNA and nature of interaction with naked DNA, to provide a better understanding of structure-activity relationships.

The starting material for JH1 is cisplatin while the starting material for JH2, JH3, and JH4 is potassiumtetrachloroplatinate ($K_2[PtCl_4]$). Cisplatin was prepared using Dhara's method, Figure 3.1 (Dhara, 1970). The method used for the synthesis of *trans*-planaramineplatinum complexes from cisplatin is based on the difference in the

trans effect of halide and amine ligands in platinum complexes, allowing selective substitution and hence control of the stereochemistry (Kauffman and Cowan, 1963).

Figure 3-1 Synthesis of cisplatin by Dhara's method

3.2. Synthesis of compounds

3.2.1 Materials:

Imidazole, 3-hydroxy pyridine was obtained from Sigma Chemical Company St. Louise USA; transplatin was obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. N,N-dimethylformamide [DMF] [C_3H_7NO], dimethyl sulfoxide (DMSO), 1,6-diaminohexane dihydrochloride, tetramethylene diamine dihydrochloride, sodium hydroxide, silver nitrate ($AgNO_3$), potassium chloride (KCl) and potassium iodide (KI) were obtained from BDH Chemicals, Australia Pty. Ltd., concentrated hydrochloric acid (HCl), potassiumtetrachloroplatinate ($K_2[PtCl_4]$), ethanol, methanol, acetone, diethyl ether were purchased from APS chemicals, Australia, concentrated ammonia solution, triethyl amine, dichloromethane and 28% ammonia solution were obtained from Asia Pacific Speciality Chemicals Ltd. Auckland New Zealand. Thiazole was obtained from Sigma-Aldrich Chemie GmbH Germany.

3.2.2 Preparation of cisplatin and transplatin

3.2.2.1 Preparation of cisplatin

1 mmol (0.415 g) of potassium tetrachloroplatinate was dissolved in 5 mL of mQ water. Potassium iodide (1.327 g, 8 mmol), dissolved in 1 mL of mQ water, was added to the solution of potassium tetrachloroplatinate. The mixture was left on a shaking water bath at 37°C for 5 min to produce K_2PtI_4 which was then reacted with 2 mmol of aqueous ammonia at 37°C for 1 h to form a precipitate of *cis*-$Pt(NH_3)_2I_2$. The dark yellow precipitate was collected, washed in succession with water and ice cold ethanol and air dried. Silver nitrate (2 mmol) and *cis*-$Pt(NH_3)_2I_2$ were mixed together followed by the addition of 4 mL of mQ water to the mixture. The mixture was left on a shaking water bath at 37°C for 30 min to produce *cis*-$Pt(NH_3)_2(NO_3)_2$ and solid silver iodide. The mixture was centrifuged at 5500 rpm. The supernatant consisting of *cis*-$Pt(NH_3)_2(NO_3)_2$ in solution was collected. Potassium chloride (0.15 g, 2.0mmol) was added to the solution. The mixture was left on a water bath at 37°C for 30 min for crystals of cisplatin to form. The crystals were filtered and washed with ice cold water and ethanol and left for air drying (Dhara, 1970). Identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.2.2 Preparation of transplatin

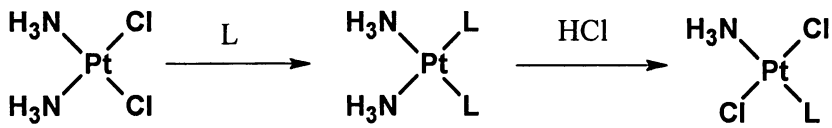
Two millimol of potassium tetrachloroplatinate (0.6 g) was dissolved in 15 mL of mQ water to which was added 0.5 mL of concentrated HCl. The mixture was heated to boiling following which 2 mL of concentrated ammonia was added drop wise with stirring to the mixture. It was then cautiously evaporated with stirring to reduce the volume to about 3 mL. About 80 mL of 6 M HCl was added to the resulting pale yellow solution. The volume of the mixture was reduced to about 6 mL by heating at 60°C on a hot plate with stirring. During evaporation, the mixture first became turbid then clear. Yellow precipitate of transplatin formed on standing. The mixture was then cooled to 0°C and kept at this temperature for 15 min for more precipitate of transplatin to form. The precipitate was collected at the pump, washed first with ice-cold water and then with acetone. It was recrystallized from 0.1 M HCl (Kauffman and Cowan, 1963). The identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.3 Syntheses of mononuclear compounds

3.2.3.1 Preparation of [*trans*-$PtCl_2(NH_3)$(Thiazole)] (JH1)

As stated earlier, *trans*-$PtCl_2(NH_3)$(thiazole) was prepared from cisplatin, utilizing the difference in the *trans* effect of halide and planaramine ligands allowing selective substitution and hence control of stereochemistry (Kauffman and Cowan, 1963) (Figure 3.2). As stated earlier, JH1 was originally prepared by Van Beusichem and

Farrell (Van Beusichem and Farrell, 1992). However we report here a new method of synthesis with better yield.



L = ligand

Figure 3-2 Synthesis of *trans*-platinum complexes

Method:

Cisplatin (0.3 g, 1 mmol) was added to 8 mL of mQ water. Thiazole (2 mmol, 0.142 mL), was dissolved in a minimum amount of water, and then added to cisplatin suspension. The mixture was heated for 1 h at 70°C with reflux which caused thiazole ligands to replace the chloro ligands. The product was cooled to room temperature to stop the reaction. Concentrated HCl (1.22 mL, 12.2 mmol) was added to the mixture followed by heating to 70°C with reflux to produce yellow needle shaped crystals of JH1. The crystals were collected at the pump, washed with mQ water and ice-cold ethanol. The identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.3.2 Preparation of *trans*-$PtCl_2L_2$(where L= thiazole)] (JH2)

The compound trans-$PtCl_2L_2$ (where L= thiazole) [code named JH2] was synthesized from potassium tetrachloroplatinate, according to the procedure described by Van Beusichem and Farrell (Van Beusichem and Farrell, 1992). As stated earlier, synthesis

of JH2 was described previously by Beusichem and Farrell (Van Beusichem and Farrell, 1992).

One mmol of K_2PtCl_4 (0.415g) was dissolved in 8 mL of mQ water and 0.25 mL of concentrated HCl was added. The solution was heated to 70^0C to which was added slowly 4 mmol of thiazole (0.284 g) dissolved in a minimum amount of water.

The temperature of the solution was maintained for 1 h at 70^0C with stirring. 40 mL of 6 M HCl was added and the mixture was heated under reflux for 7 h at 70^0C.
The mixture was quickly cooled to room temperature and then placed in ice for 1 h resulting in the formation of a precipitate of trans-$PtCl_2$(thiazole)$_2$, designated as JH2. The precipitate was collected by filtration at the pump, washed with ice-cold water and air-dried. The identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.3.3 Preparation of *trans*-$PtCl_2LL'$ (where L= thiazole, L' = imidazole) (JH3)

One mmol of K_2PtCl_4 (0.415 g) was dissolved in 10 mL of mQ water and 0.25 mL of concentrated HCl was added. The solution was heated to 70^0C to which was first added slowly with stirring 2 mmol of imidazole (0.136 g). The solution was kept at 70^0C for 1 h then 2 mmol of thiazole (0.142 mL) was added with stirring. The solution was then kept at 70^0C for 2 h. 40 mL of 6 M HCl was added and the mixture was heated under reflux for 7 h at 70^0C. The mixture was quickly cooled to room temperature and then placed in ice for 1 h resulting in the formation of a precipitate of *trans*-dichloro(thiazole) (imidazole)platinum. The precipitate was collected by filtration at the pump, washed with ice-cold water and air-dried. The volume of the filtrate was reduced to about 5 mL 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. Then allowed to stand for more precipitate to form. The crude product was purified by recrystallization from 0.100 M HCl. The identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.3.4 Preparation of *trans*-$PtCl_2LL'$ (where L= thiazole, L' = 3-hydroxypyridine) (JH4)

One mmol of K_2PtCl_4 (0.415 g) was dissolved in 10 mL of mQ water and 0.25 mL of concentrated HCl was added. The solution was heated to 70^0C to which was added slowly with stirring 2 mmol of thiazole (0.142 mL). The solution was kept at 70^0C for 1 h then 2 mmol 3-hydroxypyridine (0.19 g), dissolved in 2 mL of ethanol was added with stirring and kept at the same temperature (70^0C) for 2 h.

40 mL of 6 M HCl was added and the mixture was heated under reflux for 7 h at 70^0C. The mixture was quickly cooled to room temperature and then placed in ice for 1 h resulting in the formation of a precipitate of *trans*-dichloro(thiazole) (imidazole)platinum. The precipitate was collected by filtration at the pump, washed with ice-cold water and air-dried. The volume of the filtrate was reduced to about 5 mL using a vacuum concentrator and allowed to stand for more precipitate to form. The crude product was purified by recrystallization from 0.100 M HCl. The identity of the compound was confirmed by elemental analyses and spectral studies.

3.2.4 Syntheses of trinuclear platinum complexes

Two trinuclear complexes based on BBR3464 with one or two thiazole ligands bound to central platinum have been prepared. The three transplatinum units were linked

together by 1,6-diaminohexane. The trinuclear complexes have been prepared using a step-up method of synthesis branching out from the central platinum unit. The procedures used were based on the method described by Farrell and co-workers for the synthesis of dinuclear complexes (Qu et al., 1992; Qu and Farrell, 1992). However, the methods used in this project have been modified to give optimum yield and purity. Mononuclear complexes JH2 and JH1 prepared earlier served as the central platinum unit in JH5 and JH6 respectively. Branching out from the central unit was used because it gave more reproducible results than the attempted syntheses starting with one of the terminal units (that always gave a mixture of the products).

3.2.4.1 Preparation of [{*trans*-$PtCl(NH_3)_2$}$_2$$\mu$-{*trans*-Pt (thiazole)$_2$-$(H_2N(CH_2)_6NH_2)_2$}]$Cl_4$ (JH5)

Figure 3.3 gives the schematic of the steps used in the synthesis of JH5. Details of the procedure are as follows:

1 mmoL of transplatin (0.3 g) was dissolved in 20 mL of DMF to which was added 0.99 mmol of silver nitrate (0.1698 g). The mixture was stirred at room temperature for 24 h in the dark. The mixture was then centrifuged at 4800 rpm for 30 min, to remove the precipitate of AgCl. The supernatant was collected and kept at -16°C.

A suspension of 0.25 mmoL (0.0920g) of JH2 in 10 mL of DMF was gently heated with stirring at 60°C for about 20 min. 1 mmoL of 1, 6-diaminohexane (0.116 g) 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 dropwise with stirring to the JH2 suspension within 30 min of its preparation. Stirring was continued for 1 h at 60°C followed by 4h at 39°C. Then 200 µL of 1 M NaOH

was added with stirring. Stirring was continued for a further 20 min to result into a clear light brown solution and some brown precipitate.

The transplatin filtrate (0.25 mmol) was then added dropwise to the light yellow solution with stirring at 50 °C over a period of 25 min. Then 150 μL of 1 M NaOH was added drop wise with stirring to the solution. Stirring was continued for a further 5 min. at the same temperature (50°C). Then 0.25 mmol of transplatin was added. Stirring was continued for 50 min at 55°C, then for 15 days at room temperature.

The volume of the filtrate was reduced to 4 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump Savant RVT 4104 Refrigerated Vapor Trap and Savant Speed Vac 110 Concentrator. 40 mL of dichloromethane was added to the concentrated solution following which the mixture was left standing at 5°C for 6 h.

The light yellow precipitate produced was collected by filtration at the pump, washed first with ice-cold mQ water and then with ethanol. It was then air-dried. The vacuum concentrator was used to precipitate more of the compound in the liquid part. The identity of the compound was confirmed by microanalyses and spectral studies.

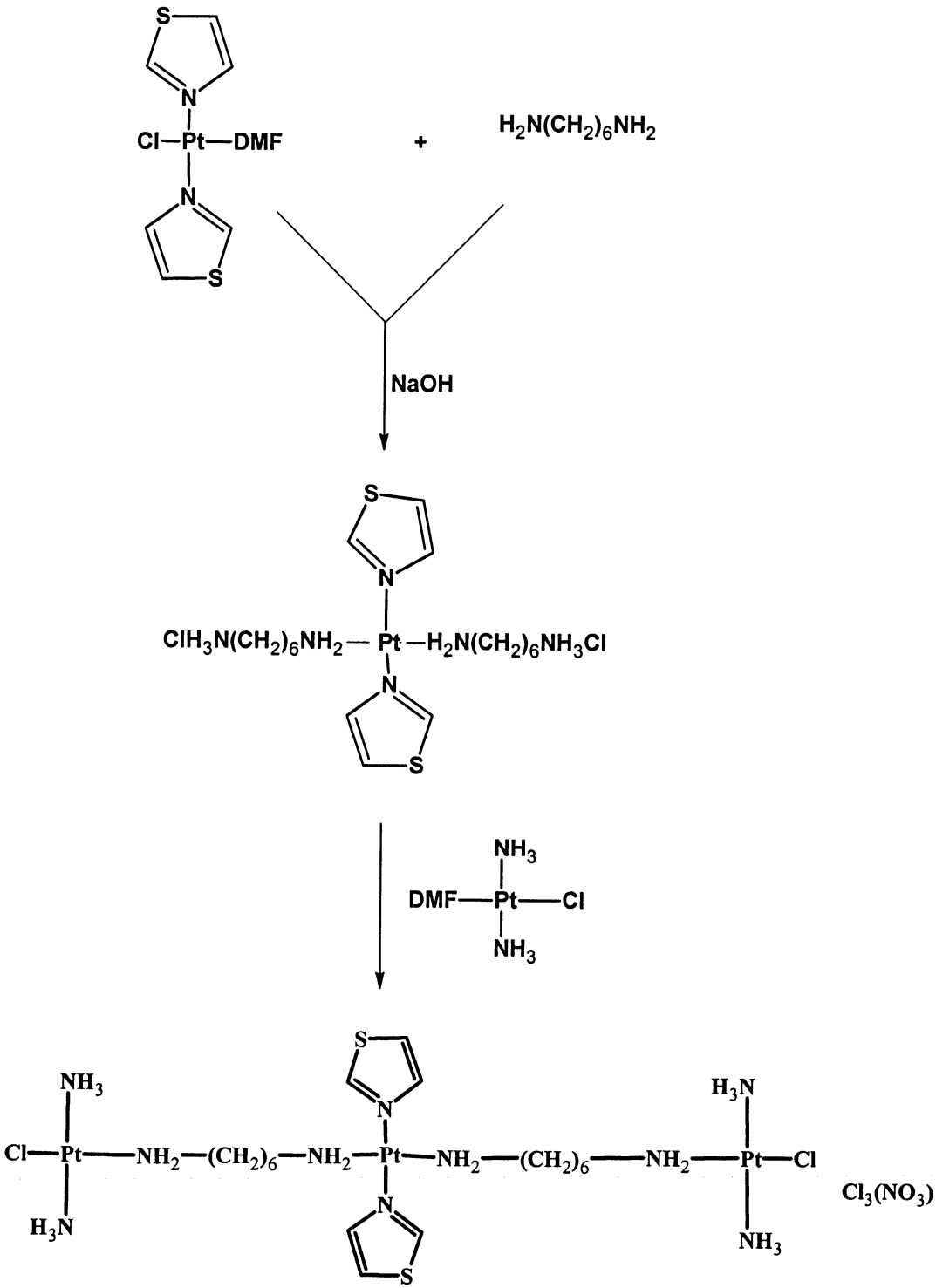


Figure 3-3 Schematic for the synthesis of JH5

3.2.4.2 Preparation of [{*trans*-$PtCl(NH_3)_2$}$_2\mu$-{*trans*-Pt (thiazole) NH_3-$(H_2N(CH_2)_6NH_2)_2$}]Cl_4 (JH6)

Figure 3.4 shows the schematic for the synthesis of JH5. Briefly, the steps were as follows:

1 mmoL of transplatin (0.3 g) was dissolved in 20 mL of DMF to which was added 0.99 mmol of silver nitrate (0.1698 g). The mixture was stirred at room temperature for 24 h in the dark. The mixture was then centrifuged at 4800 rpm for 30 min, to remove the AgCl precipitate. The supernatant was collected and kept at -16°C.

0.25 mmol (0.109g) of JH1 was dissolved in 5 mL of DMF. Silver nitrate (0.5 mmol, 0.085 g) was added to the mixture with stirring. Stirring was continued overnight at room temperature. The mixture was centrifuged at 4800 rpm for 15 min to collect the light yellow supernatant (All steps were carried out in the darks).

1 mmoL of 1, 6-diaminohexane (0.116 g) was dissolved in 4 mL of DMF to which 1 mL of 1 M HCl (100 μ HCl+ 900 μ mQ) was added drop wise with stirring. The diamine solution was stirred for a further 15 min. It was then added to the $PtCl_2$(Thiazole)NH_3 suspension drop wise with stirring within 30 min of its preparation. Stirring was continued at 60°C for about 1 h, then at 39°C for 4 h. Then, 200 μL of 1 M NaOH (0.4 g NaOH + 10 mL mQ) was added with stirring. Stirring was continued for a further 20 min to resulting in a clear light brown solution and some brown precipitate.

The transplatin filtrate (0.25 mmol) was then added drop wise to the light yellow solution with stirring at 50°C over a period of 25 min. Then 150 μL of 1 M NaOH was added drop wise with stirring to the solution. Stirring was continued for a further 5

min. at the same temperature (50°C). Then 0.25 mmol of transplatin was added. Stirring was continued for 50 min at 55°C, then for 15 days at room temperature.

The volume of the filtrate was reduced to 4 mL by using a vacuum concentrator as described previously. 40 mL of dichloromethane was added to the concentrated solution following which the mixture was left standing at 5°C for 6 h.

The light yellow precipitate produced was collected by filtration at the pump, washed first with ice-cold mQ water and then with ethanol. It was then air-dried. The vacuum concentrator was used to precipitate more of the compound in the liquid part. The identity of the compound was confirmed by microanalyses and spectral studies.

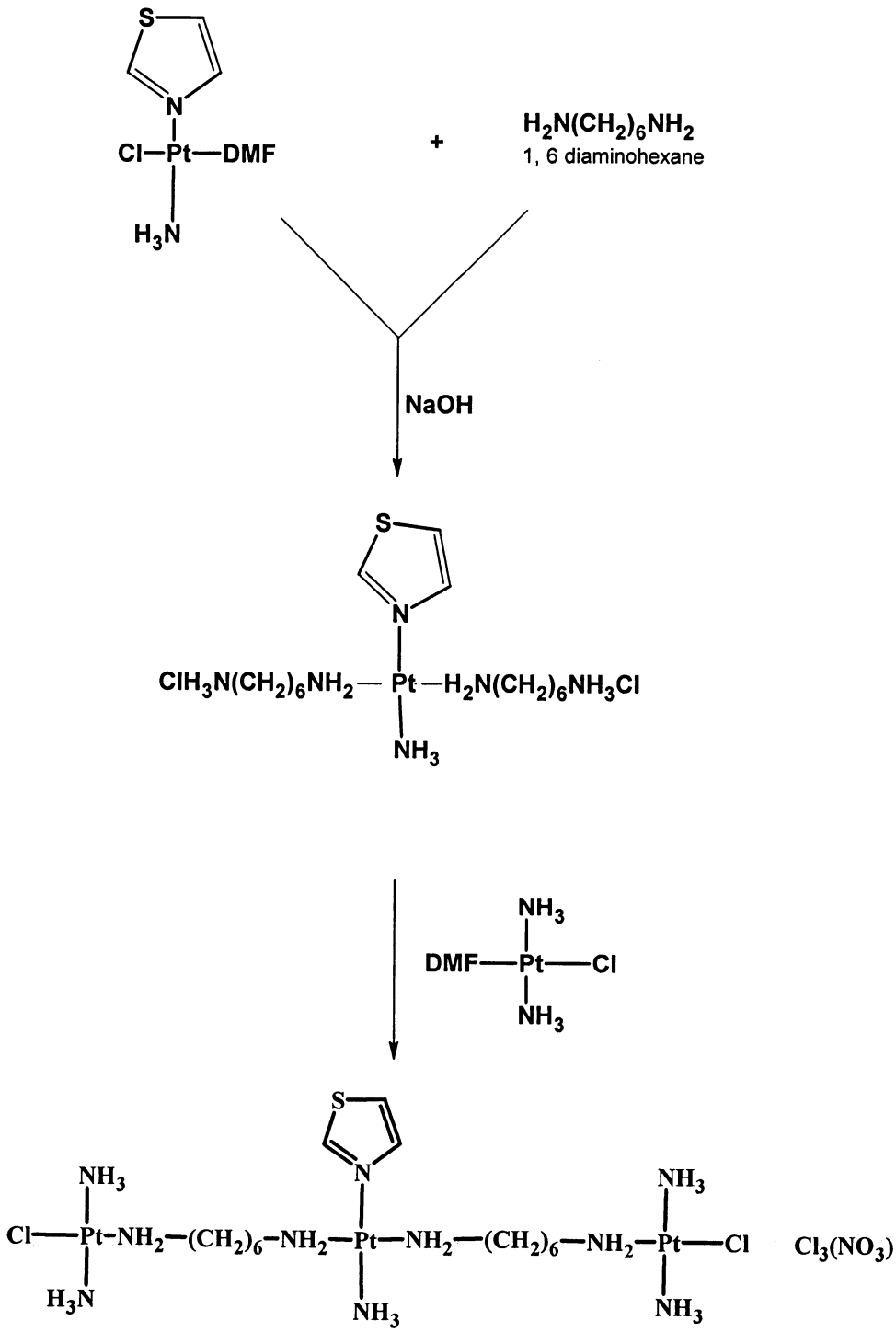


Figure 3-4 Schematic for the synthesis of JH6

3.3. Characterization of compounds

3.3.1 Microanalysis

Microanalysis was carried out to determine elemental composition of the compounds. Carbon, hydrogen, nitrogen and chlorine were determined using the micronalytical facility available at the School of Chemistry, Australian National University. A Carlo Erba 1106 automatic analyser was used for the determination of C, H and N contents and Cl was determined by titration with standardized mercuric nitrate.

Platinum was determined by graphite furnace atomic absorption spectroscopy (AAS) using a Varian SpectrAA240 plus Atomic Absorption Spectrophotometer with GTA-120 Graphite Furnace Tube Atomizer, available in School of Biomedical Sciences, The University of Sydney.

3.3.2 Atomic Absorption Spectroscopy (AAS)

3.3.2.1 Basic principle

In this project, graphite furnace atomic absorption spectroscopy (AAS) was used to determine the platinum content of compounds, platinum uptake by cells and levels of platinum-DNA binding.

3.3.2.2 Method

A Varian SpectrAA-20 Atomic Absorption Spectrophotometer with GRA-96 Graphite Furnace Tube Atomiser, available in the Discipline of Biomedical Science, School of Medical Sciences, The University of Sydney was used to determine platinum.

All glassware was cleaned first by detergent, and then soaked in 20% v/v HCl solution for 2 days followed by 20% v/v HNO_3 solution for further 2 days. The glassware was rinsed with distilled water.

Preparation of platinum standard solution

A diluted Pt standard (500 ppb) was prepared from the more concentrated platinum atomic absorption standard solution (970 ppm in 5% HCl), obtained from Sigma. 0.0128 mL of 970 ppm Pt standard solution was diluted to 25 mL with 0.1 M HCl to obtain 25 mL of 500 ppb Pt standard solution.

The AAS conditions used for the determination of platinum are given in Tables 3.1, 3.2, and 3.3. Figure 3.5 gives a typical absorbance versus concentration calibration graph.

Table 3.1 Furnace operating conditions for the determination of platinum

Step No.	Temperature (C°)	Time (s)	Gas Flow (L/min)	Gas type	Read Command
1	85	5	0.3	Normal	----------
2	95	40	0.3	Normal	----------
3	120	10	0.3	Normal	----------
4	1200	5	0.3	Normal	----------
5	1200	1	0.3	Normal	----------
6	1200	2	0	Normal	Start signal
7	2700	1.3	0	Normal	Read steps, Start signal
8	2700	2	0	Normal	Read steps, Start signal
9	2700	2	0.3	Normal	Start signal

Table 3.2 Instrument parameters for the determination of platinum

Type / mode	Method type: Furnace
	Sampling mode: Automixing
Measurement	Measurement mode: Peak Height
	Calibration mode: Standard addition
	Smoothing: 7 point
	Replicates standard and sample: 2
	Precision % standard and sample: 5
Optical	HC lamp, Lamp position: 2
	Lamp current [mA]: 10
	Monochromator: Wavelength [nm] 265.9
	Slit width [nm] 0.5
	Back ground correction: on
Standard	Upper valid concentration: 500
	Concentration decimal places: 2
Calibration	Calibration algorithm: Linear
	Recalibrate rate: 58
	Reslop rate: 10
	Reslop standard no.: 2
	Expansion factor: 1
	Internal standard: 0.5

Table 3.3 Sampler parameters used in AAS to determine platinum

Rate based solutions	Vial No.	Method Conc.	Mix Conc.	Blank (µL)	Bulk(standard) (µL)	Sample (µL)	Total volume (µL)
Reagent Blank	53	0	------	------	------	------	------
ADDITION 1	------	125	125	20	2	8	30
ADDITION 2	------	250	250	18	4	8	30
ADDITION 3	------	500	500	14	8	8	30
Volume reduction factor: 2				Sample injection rate: 1			

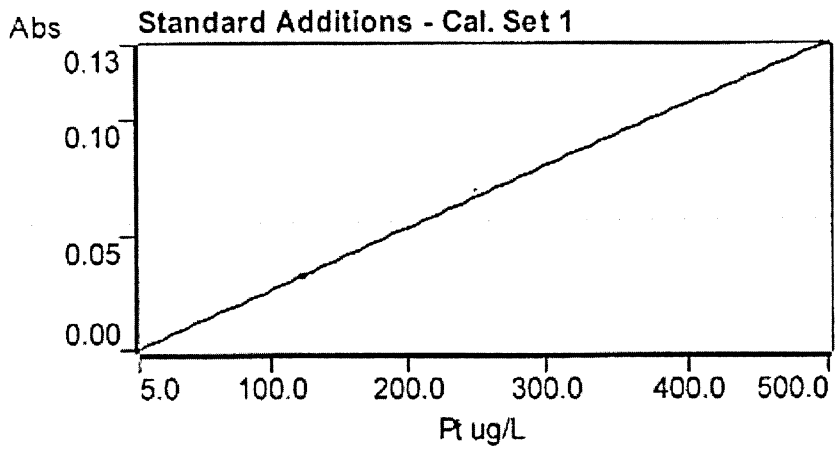


Figure 3-5 Typical standards addition graph for platinum

3.3.3 Calculating percentage yield of the compound

Percentage yield is calculated according to the following equation:

$$\text{Percentage yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100$$

3.3.4 Molar conductivity

The molar conductivities of the compounds solutions were determined as these would give an indication of the degree of ionization and therefore their likely modes of transport across the cell membrane. The molar conductivity (Λ) was calculated as $\Lambda = k/c$ where k is the specific conductivity and c is the concentration in mol L^{-1} (Atkins, 1998). The molar conductivity values were then plotted against concentration to determine the limiting values (i.e. the values at zero concentration). In this study, the molar conductivity values of solutions of JH1, JH2, JH3, JH4, JH5, and JH6 made initially in 1: 2 mixture of DMF and mQ water at 298 K (1 mM solution) and then serially diluted with mQ water, were determined using a PW9506 digital conductivity meter (EC 215 conductivity meter, Hanna instruments). The conductivity values were measured at the concentrations: 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM and 0.03125 mM.

3.3.5 Spectral studies

3.3.5.1 Infrared (IR) Spectroscopy

Infrared spectroscopy deals with interaction of infrared light with substances. As a result of absorption of infrared photons, molecules can undergo vibrational excitations. Because each molecule has its own characteristic patterns vibration, the infrared spectrum of a molecule is like the fingerprint of the molecule. In this project IR spectroscopy has been used to identify the presence of functional groups and hence to aid in the structural characterization of the compounds (Twardowski et al., 1992).

Method

Infrared spectra were collected using a Bruker IFS66 spectrometer equipped with a Spectra-Tech Diffuse Reflectance Accessory (DRA), an air-cooled DTGS detector and a KBr beam splitter with a spectral range of 4000 to 650 cm^{-1}. The instrument was run under a 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 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.

3.3.5.2 Mass spectrometry

Basic principle

The mass spectrometer is used to measure the molecular mass of a given sample. The mass spectrometer is made up of three main parts: ion source, analyser, and detector. In the ionisation process the sample is first ionized using an electron beam which also results in breaking down the molecule. The analyser then takes out the ions and separates them according to mass-to-charge ratio. The detector then collects the signals generated from the separated ions then stores and processes the information in the format of an intensity versus m/z spectrum The entire system is maintained under high vacuum to give the ions free movement from one part to another without any obstacles from the air in addition to that the ions path is controlled using a magnetic field (Silverstein et al., 1991a; Siuzdak, 1996).

Obtaining the mass spectrum of JH1, JH2, JH3, JH4, JH5, and JH6

A Finnigan LCQ ion trap mass spectrometer was used to collect mass spectra. The LCQ has an atmospheric pressure ionisation source that supports both ESI (Electrospray Ionization) and APCI (Atmospheric Pressure Chemical Ionization) probes. It uses a quadrupole ion trap mass analyser and is capable of different scan functions such as Full Scan (from 50 to 2000 m/z), Selected Ion Monitoring (SIM), MS/MS or MS^n (n = 1 to 10) and Zoom Scan. The data system of the LCQ uses Microsoft Windows NT operating environment and Finnigan LCQ data processing and instrument control software. This instrument is normally set at ESI mode.

ESI is a very soft ionization technique. It is most often used for analysing large or labile molecules such as peptides, proteins, organometallics and polymers. Samples are introduced as solutions using the infusion syringe pump, liquid chromatograph

(LC) or through the loop injection port with a LC pump. Water or organic solvents such as methanol, acetone and $CHCl_3$ can be used to dissolve the sample. If the sample is not ionic, some source of protons (acetic acid or formic acid) is added to promote the formation of cations.

A typical experimental condition of this instrument is: ESI spray voltage 5 kV; nitrogen sheath gas pressure 60 psi; heated capillary temperature 200°C; full scan 50 to 2000 m/z. For loop injection, the typical mobile flow phase is 50% methanol/50% water with 1% acetic acid and the flow rate is 100 microlitre per minute. The exact experimental condition varies to optimise the signal.

To obtain mass spectra, solutions of JH1, JH2, JH3, JH4, JH5, and JH6, made in 10% DMF and 90% methanol, were sprayed into a Finnigan LCQ 10 N trap mass spectrometer in which fragmentation was produced by eletrospray ionization (ESI).

Interpretation of the mass spectrum of the compounds

In confirming the structure of JH1, JH2, JH3, JH4, JH5, and JH6, the ratio of isotopes of the atoms was taken into consideration. Isotope splitting patterns for molecules and fragments were calculated using the program Sheffield ChemPuter.

3.3.5.3 Nuclear magnetic resonance spectroscopy (NMR)

Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) is a powerful technique used for identification and structural characterization of compounds. It provides vital information about molecular structure through measurement of the interaction of radio-frequency electromagnetic radiation with a collection of nuclei immersed in a strong magnetic field (Macomber, 1988). ^{1}H NMR can be applied to the study of three states of matter: solids, liquids and gases. Some nuclei such as ^{1}H (i.e. protons) possess spin and behave like tiny magnets as spinning charge has an associated

magnetic field. In the presence of an external magnetic field a nucleus possessing a spin can align itself either with the external field or against the field resulting splitting of energy levels. Absorption of appropriate radio frequency radiation can cause transition between the two energy levels. An NMR spectrum is essentially a plot of the intensity of absorption of radio frequency (RF) radiation along the y-axis against the frequency of the radiation along the x-axis. In modern pulse Fourier transformation method, radio frequency radiation is applied to the sample, the spectrum obtained by Fourier transformation is the response of the nuclear spins to these pulse trains. In this project, ^{1}H NMR has been used to aid in the structural characterization of the compounds along with IR and mass spectral studies. Specifically, ^{1}H NMR spectrum was used to indicate the presence of protons in different chemical environments and hence different functional groups (Silverstein et al., 1991b). In this project, ^{1}H NMR was used to identify functional groups. ^{1}H NMR spectra of JH1, JH2, JH3, JH4, JH5, and JH6 were recorded in dimethylsulfoxide-d_6 (DMSO-d_6) solution in a Bruker AVANCE DPX 400 spectrometer. Spectra were referenced to internal solvent residues and were recorded at 300 K (±1 K).

3.4. Biological Activity

3.4.1 Introduction to tissue Culture

Tissue culture was first introduced at the beginning of 1900. The term tissue culture refers to growing of tissue or cells (taken from their original environment) in an artificial environment (Freshney, 2000). There are a number of advantages of tissue culture over *in vivo* tests including control of physiochemical environment (pH,

temperature, and osmolarity), control of physiological conditions (hormones, nutrient concentration), lower cost and being faster. Moreover, with tissue culture, the legal, moral, and ethical issues of using animals for testing can be avoided. The majority of cytotoxicity tests for new drugs, cosmetics, food additives that were used to be carried out on animals, nowadays are done using tissue culture (Freshney, 2000).

However, tissue culture has many problems and issues including the need for strict aseptic conditions (mainly because of the slow growth of animal cells compared to bacteria, yeast or fungi). Cells in tissue culture need a complex environment so that a great care and experience are needed in dealing with cells. Often instability of cell lines may result in continuous culture due to chromosomal instability.

There are three types of tissue culture:

(1) Organ culture: In this type of tissue culture an organ or part of an organ is grown *in vitro* while maintaining the architecture or function of the organ. In organ culture, cell interaction and signalling are maintained unlike other types of tissue culture.

(2) Primary explant culture: The culture that follows from the isolation of a tissue and before the first subculture is called primary culture. There are two ways of getting the primary culture, the first through tissues adhering to a substrate (glass or plastic), then after few days cells start to migrate to form the primary culture. The second is the faster way by using enzymes (like trypsin) or mechanical methods to break down the substrate that holds the cells together and producing a suspension (known as the primary culture). Whichever method is used, primary culture is the first step in producing a uniform cell line.

(3) Cell culture: Cells dissociated from primary subculture or parent tissues into a cell suspension to form a monolayer on substrate or just remaining as a cell suspension is called cell culture. Most cells will adhere to a substrate before they start proliferation

to form a monolayer. The subculture of the primary culture forms a passage and the cells produced from the passage subculture produce cell lines.

Normal cells can grow and divide for a limited number times after which they lose their ability to divide and die. This phenomenon is genetically controlled and is known as senescence. The phenomenon gave rise to continuous culture which has the ability to divide indefinitely. This ability of continuous proliferation is due to mutation, especially to genes responsible for cell cycle check points like p53 (Freshney, 2000).

A suitable medium is an essential part of tissue culture; for cells to survive and proliferate in an artificial environment they need a very complex medium. Therefore, at the beginning of tissue culture, natural tissue extracts and body fluid were used as medium to grow cells. The need for large amounts of medium and consistent quality led to the introduction of chemically defined media.

Currently there are many different types of media, depending on the type of cell lines and the specific requirements of the tissue culture. However the cell culture medium should contain essential and non-essential amino acids, vitamins, salts, glucose, bases nucleoside etc, lipids and precursors, and may contain growth factors and hormones. In addition, the pH of the medium should be maintained between 7.4-7.7. This can be achieved through equilibrium buffering with sodium bicarbonate in solution and carbon dioxide in the gas phase. HEPES is a strong buffer that is used frequently with tissue culture medium to maintain the pH value between 7.2-7.6 (Freshney, 2000).

Contamination remains the major problem in tissue culture mainly due to the richness of cell culture medium in nutrients that attract different types of microorganisms. Therefore aseptic conditions have to be maintained all the times. Although experience and alertness minimize the problem, poor sterilization of equipment, faults in laminar

hood, poor maintenance of incubators and refrigerators, use of contaminated cell lines or biopsies, contribute to contamination.

The effect of contamination can be minimized through (1) regular checking of culture for any contamination using microscope and naked eye, and for Mycoplasma by empl; (2) initial checking of cells without antibiotics for any minor contamination; (3) making sure that all reagents to be used are sterile; (4) media, bottles, pipettes used exclusively for tissue culture; (5) sterile conditions have to be maintained at all times (Freshney, 2000).

3.4.2 Materials

The three human ovarian cancer cell lines, A2780, its cisplatin-resistant form $A2780^{cisR}$ and ZD0473-resistant form $A2780^{ZD0473R}$ used in this study were gifts from Dr. Philip Beale, NSW Cancer Centre, Sydney, Australia. The cell lines were produced as follows: Parent cisplatin-sensitive cell line A2780 was established from tissue obtained from an untreated ovarian cancer patient (Hamilton et al., 1984; Behrens et al., 1987). The cisplatin-resistant cell line $A2780^{cisR}$ was developed by chronic exposure of A2780 cells to increasing concentrations of cisplatin (Behrens et al., 1987; Masuda et al., 1988) and the cell line $A2780^{ZD0473R}$ was developed by *in vitro* exposure of A2780 cells to increasing concentrations from 0.5 to 12.5 μM of the drug ZD0473 for a period of 7 months (Holford et al., 2000). All the above cell-lines were stored in liquid nitrogen.

Foetal 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(PBS), 3-[4,5-dimethylthiazol-

2yl]-diphenyl tetrazoliume bromide (MTT) and dimethyl sulfoxide (DMSO) 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.

3.4.3 Maintenance of the cell lines

The cell lines were grown in tissue culture flasks (Falcon pour culture cellulaire, Bacto Laboratories Pty Ltd, NSW, 2170 Australia) in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated foetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2mM glutamine without antibiotics.

3.4.4 Preparation of FCS/RPMI 1640 complete tissue culture medium

The medium most often used for cell culture studies was mainly 10% foetal calf serum (FCS/RPMI 1640). As listed in Table 3.4, one litre of the medium was prepared by mixing the following components together: 200 mL of (5X RPMI 1640), 100 mL of 10% foetal calf serum (FCS), 20 mL of 1 M hepes, 20 mL of 5.6% sodium bicarbonate, 10 mL of 200 mM glutamine and 0.5 mL saturated NaOH. Sterile water was used to make up the volume to one litre.

Table 3.4 Constituents of the medium (FCS/RPMI 1640)

	2%	5%	10%
5 x 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

3.4.5 Phosphate buffered saline (PBS)

3.4.5.1 Materials

PBS powder as supplied by Sigma, 1 M HCl, 1 M NaOH and mQ water.

3.4.5.2 Procedure

Dulbecco's phosphate buffered saline powder was obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. To prepare one litre of PBS solution, 900 mL of mQ water was transferred to one litre volumetric flask followed by the addition of PBS powder (9.6 g) 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 by using 1 M HCl and 1 M NaOH, 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.

3.4.6 Trypsin

To prepare 100 mL of trypsin, 0.02 gm of disodium salt of ethylene diamine tetra-acetic acid (EDTA) was dissolved into 1 mL of sterilized water in a 100 mL sterilized bottle to which was added 10 mL of 2.5% trypsin (trypsin EDTA 1:250 which was prepared earlier and stored at 4°C) and the volume made up to 100 mL with PBS (89 mL PBS). It was then filtered using a 0.22 micron filter and stored at 4 ^{0}C.

3.4.7 Hepes

Hepes powder was obtained from Sigma-Aldrich, Australia. 100 mL of 1 M hepes solution was prepared by dissolving 23.83 g of hepes powder in 90 mL of mQ water in a 100 mL volumetric flask. Then the volume was made up to 100 mL by adding more water. The solution was sterilized immediately by filtration and stored at 4°C.

3.4.8 Cells recovery from liquid nitrogen

New flasks, tubes and media were placed in the hood after swabbing it with Cavacide for 2 min then with 70% alcohol for 3 min. The vials containing the three ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R} were taken from the liquid nitrogen tank and placed in a water bath at 37 °C until half of the liquid thawed. The

vials were swabbed with 70% alcohol. Then the cells from each vial were transferred into a labelled 10 mL sterile tube containing 9 mL of 10% RPMI 1640 culture medium. The tubes containing the three cell lines 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-labelled flasks each containing 8 mL of fresh medium. The flasks were incubated in an atmosphere of 5% CO_2 and 95% air at 37°C.

3.4.9 Cell subculture

Subculture involves removal of the medium, followed by washing the flask with PBS solution and dissociation of the cells in the monolayer with trypsin. Some loosely adherent cells may be subcultured by shaking the flask, collecting the cells in the medium and diluting as appropriate in fresh medium in a new flask. Subculture is done when the cell density (cell/cm^2 substrate) reaches a level such that all of the available substrate is occupied, or when the cell concentration (cells/mL medium) exceeds the capacity of the medium so that growth ceases or is greatly reduced. Then either the medium must be changed more frequently or the culture must be divided (Freshney, 2000).

3.4.9.1 Procedure

The hood was swabbed with cavacide first then 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 a microscope for any sign of contamination or deterioration. The old medium was removed from the flasks and

discarded. The flasks were then washed with 1 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°C for: 2-3 min for $A2780^{cisR}$ and $A2780^{ZD0473R}$, and 1 min for A2780 respectively. The flasks were removed from the incubator and the suspensions of the cells were examined using inverted microscope after shaking the flasks. 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 using a 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 in terms of morphology and growth. The 1 mL of healthy suspension left in each flask was diluted with 9 mL of 10% RPMI 1640 culture medium making the total volume 10 mL. The cells were then incubated at 37 °C in the incubator. The procedure was repeated every three to four days to maintain in logarithmic growth of the cells.

3.4.10 Cell count

A hemocytometer was used for counting cells. The cover slip and the surface slide of the hemocytometer chambers were cleaned with 70% alcohol. The cover slip was then placed on the counting area and the cell suspension was mixed thoroughly. 30 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 that is bounded by three parallel lines and has an area of 1 mm^2 (25 square). 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.

3.4.11 Storage of the cell lines

All the cell lines in late log phase, which were healthy in growth and free from contamination, were chosen for storage. Dimethyl sulfoxide (DMSO) was chosen to act as a cryoprotectant. DMSO could permeate cell membranes rapidly to increase the intracellular concentration so as to inhibit intracellular ice formation which is the main cause of freezing damage to cell (Lovelock and Bishop, 1959). The cell suspension should be frozen slowly, at the rate of -1°C per minute which can be achieved by laying the cell vial on cotton wool in a box at –70 ^{0}C in a deep freezer (Harris and Griffiths, 1977).

3.4.11.1 Materials:

10% FCS/RPMI 1640

20% Dimethyl sulfoxide (DMSO) 10mL:

To make 10 mL of 20% DMSO, two mL of DMSO was added to 8 mL of 10% FCS/RPMI 1640 and allowed to cool to below 37°C.

3.4.11.2 Method:

The late log phase growth cells free from contamination were first gently harvested by spinning then resuspended in 10% FCS/RPMI 1640 to a concentration of $2x10^6$ cells/mL. 20% of DMSO equal in volume to that of the cell suspension was slowly added to the cells to give a final DMSO concentration of 10%. One mL of this mixture of cells and cryoprotectant was then transferred to prelabeled NUNC vials and stored overnight in a box surrounded by cotton-wool at –70°C and then transferred to liquid nitrogen tank.

3.4.12 MTT-Based cytotoxicity assay

3.4.12.1 MTT cell viability assay

MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay is one of the most commonly used methods to determine the cytotoxicity of drugs. It is a rapid and sensitive colorimetric assay based on the formation of a coloured insoluble formazan salt. It is an indirect method to measure the proportion of viable cells following a drug exposure (Freshney, 2000; Jansen et al., 2001; Baruah et al., 2002; Woolley et al., 2002).

MTT is a yellow water-soluble tetrazolium dye that is reduced by the mitochondrial enzyme, succinate dehydrogenase, to a purple formazan product in live cells (Carmichael et al., 1987). Dead cells or tissue culture medium cannot cause such reduction of MTT (Mosmann, 1983). The formazan crystals are insoluble in aqueous solutions but can be dissolved in dimethyl sulfoxide (DMSO). Absorbance as a

measure of formazan production can be determined using a scanning multiwell spectrophotometer leading to the determination of percentage of viable cells. However, cell contamination may cause false negative results (Denecke et al., 1999) and it is impossible to distinguish between cytostatic and cytocidal effects of the drugs on cells.

3.4.12.2 Materials and Method

The method used was modified from that described by Plumb *et al* (Plumb et al., 1989).

Materials:

10% FCS/RPMI 1640

0.25% trypsin

1 mg/mL MTT (Sigma):

10 mg of MTT was dissolved in 10 mL of serum free RPMI-1640 medium then filtered through a 0.22 μM filter to remove any blue formazan product.

Dimethyl sulfoxide (DMSO)

Method:

The human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ were grown in 25 cm^2 tissue culture flasks in an incubator at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated foetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2 mM glutamine (Freshney, 2000). Cytotoxicity was determined using the MTT growth inhibition assay (Mosmann,

1983). Between 5000 to 9000 cells, depending on the growth characteristics of the cell line, were seeded into the wells of the flat-bottomed 96-well culture plate in 10% FCS/RPMI 1640 culture medium. The plate was then incubated for 24 h at 37°C in a humidified atmosphere to allow them to attach. Platinum complexes were first dissolved in a minimum volume of DMF, then diluted to the required concentrations by adding mQ water and finally filtered to sterilize. Serial fivefold dilutions of each of the drugs ranging from 0.625 μM to 30.0 μM in 10% FCS/RPMI 1640 medium were prepared and added to equal volumes of cell culture in quadruplicate wells, then left to incubate under normal growth conditions for 72 h. Inhibition of cell growth was determined using the MTT reduction assay (Mosmann, 1983). Four hours after the addition of MTT (50 μL per well of 1 mg mL^{-1} MTT solution), the yellow formazan crystals produced from the reduction of MTT was dissolved in 150 mL of DMSO and the absorbance of the resulting solution was read with a plate reader (Bio-Rad Model 3550 Microplate Reader) set at 540 nm.

The percentage of living cells was calculated as follows:

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

For each drug concentration, at least three independent experiments were done. Dosage response curves were constructed by plotting the percentage of viable cells against added drug concentration. The IC_{50} value (i.e. the drug concentration required to cause 50% cell kill) was then determined from the curve.

The resistance factors (RF) applying to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ were calculated from the IC_{50} values according to the equation:

$$\text{Resistance Factor (RF)} = \frac{IC_{50} \text{ value for resistant cell line}}{IC_{50} \text{ value for responsive cell line}}$$

3.5. Determination of cellular platinum uptake and DNA binding in Cells

The method used for the determination of drug uptake and binding with DNA was a modification of that described by Di Blasi *et al* (Di Blasi et al., 1998).

3.5.1 Cell uptake

The platinum complexes (at 50 μM final concentration) were added to culture plates containing exponentially growing A2780, A2780^{cisR} and A2780^{ZD0473R} cells in 10 mL 10% FCS/RPMI 1640 culture medium (cell density = 1 x 10^6 cells mL^{-1}). The cells containing the drugs were incubated for 2 h, 4 h and 24 h at the end of which cell monolayers were trypsinized and cell suspension (10 mL) was transferred to centrifuge tube and spun at 3500 rpm for 2 min at 4°C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at -20°C until assayed. At least three independent experiments were performed.

Following incubation with compounds, cell pellets were suspended in 0.5 mL 1% triton-X, held on ice then placed in an ultrasonic bath for 30 minutes. Total intracellular platinum contents were determined by graphite furnace AAS using a

Varian SpectrAA-20 plus with GTA 96 atomic absorption spectrophotometer (Roberts et al., 1999).

3.5.2 Binding of platinum to DNA

Materials:

A commercially available JETQUICK Blood DNA Spin Kit/50 used to isolate high molecular weight DNA from cell pellets was obtained from Astral Scientific.

Method:

Following incubation of cells with drugs, high molecular weight DNA was isolated from cell pellets using JETQUICK Blood DNA Spin Kit/50 and the modified protocol of Bowtell (Bowtell, 1987). The cell pellets were resuspended in PBS to a final volume of 200 μL and mixed with 10 μL of RNAase A, then incubated for 4 min at 37°C. 25 μL Proteinase K and 200 μL Buffer K1 (containing guanidine hydrochloride and a detergent) were then added to the mixture followed by incubation for 10 min at 70°C. Then 200 μL of absolute ethanol was added and mixed thoroughly to prevent any precipitation of nucleic acids due to high local alcohol concentrations. The samples were then centrifuged for 1 min at 10600 rpm through the silica membrane using JETQUICK micro-spin columns. The columns containing the samples were then washed with 500 μL of buffer KX (containing high-salt buffer to remove residual contaminants) and centrifuged for 1 min at 10,600 rpm. These were again washed with 500 μL buffer K2 (containing low-salt buffer to change the high-salt conditions on the silica membrane to low-salt) and centrifuged for 1 min at 10,600 rpm. To further clear the silica membrane from residual liquid the sample columns were centrifuged again for 2 min at full speed (13,000 rpm). The column receivers were

changed and the purified DNA in the column was eluted from the membrane with 200 μL of 10 mM Tris-HCl buffer (pH 8.5). DNA content was determined by UV spectrophotometry (260 nm) (Varian Cary 1E UV-Visible with Varian Cary Temperature Controller) and platinum levels were determined by graphite furnace. AAS. A_{260}/A_{280} ratios were found to be between 1.75 and 1.8 for all samples.

3.6. Interaction with DNA

3.6.1 Introduction

It is believed that much of platinum anticancer activity is due to its binding with DNA. The formation of platinum DNA adducts causes changes in DNA conformation and/or damage to the DNA. Changes in DNA conformation due to binding and DNA damage may be reflected as changes in rate of migration of DNA through a gel in an electric field. In this project, the interaction of the synthesized complexes with pBR322 plasmid DNA alone, with pBR322 plasmid DNA combined with BamH1 restriction enzyme digestion, and salmon sperm DNA (ssDNA) was investigated using gel electrophoresis.

3.6.2 Basic principle of gel electrophoresis

Agarose is a polysaccharide extracted from red algae that is non-toxic and insoluble in cold water but dissolves readily in boiling water. When agarose is mixed with buffer and heated to boiling it makes a homogeneous solution and pores are formed in the agarose gel through which DNA fragment are forced to travel when placed in an

electric field. The pore size depends on agarose concentration. DNA has a negative charge due to the phosphate groups so when an electric field is applied, DNA moves towards the positive electrode. The larger the DNA fragment the more difficult it is to migrate through the pores in the gel and the more slowly it will travel. Smaller fragment will travel faster and more easily so an inverse relationship exists between the size of the DNA fragment and the distance it migrate in the agarose gel. Therefore DNA fragments of different size get separated from one another (Helling et al., 1974). Plasmids are small circular, double-stranded DNA molecules ranging in size from two to several hundred kilo bases found in some bacteria. They are extra chromosomal genetic elements and can replicate independently of chromosomal DNA. Plasmids carry genes for the inactivation of antibiotics, metabolism of natural products, production of toxins, and synthesis of enzymes (Stryer, 1981). Plasmid DNA can exist in three forms: supercoiled circular (form I), singly nicked circular (form II), and doubly nicked linear (form III) (Figure 3.6). The rate of migration through the gel is different for the three forms. Form I, being supercoiled and compact will migrate at the fastest rate and the flexible and relaxed Form II DNA travels at the slowest rate. The linear Form III is able to snake through the gel and thus will migrate faster than the Form II DNA (Figure 3.7).

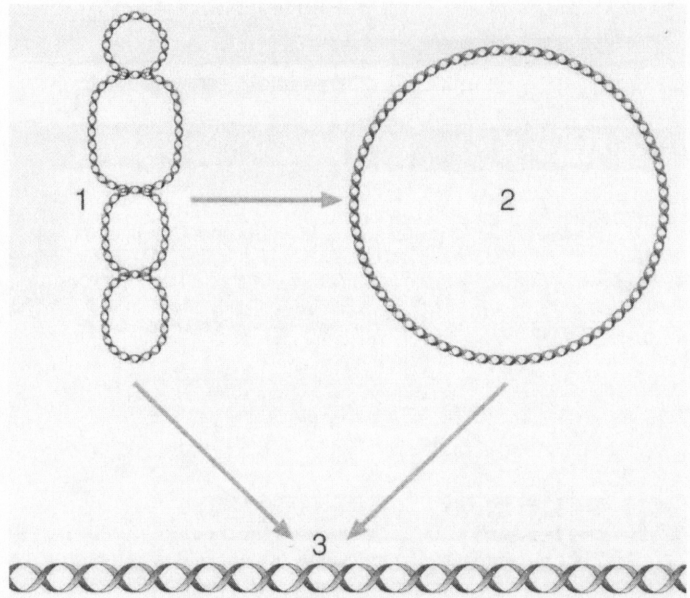


Figure 3-6 The three forms of plasmid DNA: (1) supercoiled form I, (2) circular form II, and (3) linear form III

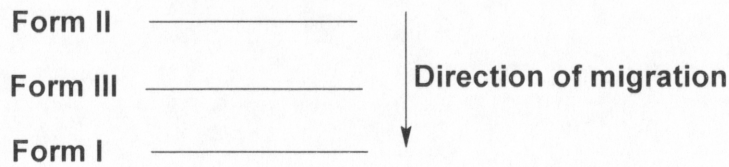


Figure 3-7 Migration pattern of Form I, Form II and Form III plasmid DNA in an electric field

The electrophoretic mobility of DNA also depends on the electric field strength. The rate of migration of DNA is proportional to the voltage applied. However, as the electric field strength is increased, the temperature also increases due to the resistive heating of the gel by the electric field. In the worst case, the gel melts and the DNA denatures. Thus, electric strength should be controlled so that the resistive heating can be approximately balanced by evaporative cooling. To obtain maximum resolution of

DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5 V/cm between the electrodes (Sambrook et al., 1989).

The composition and ionic strength of the electrophoresis buffer also affects the migration rate of DNA. In ion-free electrophoresis buffer, there is lack of electrical conductance and DNA migrates very slowly. In contrast, in high ionic strength electrophoresis buffer although electrical conductance would be high, significant amounts of heat may be generated that can cause gel melting and DNA denaturation. There are several different buffers commonly used in the gel electrophoresis such as TAE buffer containing EDTA (pH 8.0) and tris-acetate(Vermeulen et al., 2003), or TBE (Tris-borate) with EDTA, or TPE (Tris-phosphate) with EDTA at a concentration of approximately 50 mM. DNA fragments migrate faster through TAE than through TBE and TPE and the resolution of supercoiled DNA is better in TAE than in TBE.

Ethidium bromide is a fluorescent dye that will intercalate between stacked base pairs and exhibits an intense orange fluorescence when visualised by ultraviolet radiation using a transilluminator (Stryer, 1981). It is used to detect DNA in agarose. However, it reduces the electrophoretic mobility of linear DNA by about 15% by extending the length of linear and nicked circular DNA molecules and making them more rigid. It is usually used to monitor the mobility of DNA during the electrophoresis. However, it is better to run the gel in the absence of the dye and stain the gel after the electrophoresis in order to give sharper bands.

Restriction enzymes are proteins produced naturally from bacteria for protection against bacteriophage infection. Restriction enzymes cleave DNA molecules at specific palindromic nucleotides sequences (from 4 base pair to 8). To protect its own DNA from the action of its restriction enzyme, bacteria simultaneously produce

methylase an enzyme that adds methyl (CH_3) groups to the site-specific DNA sequence recognised by the restriction enzyme so the restriction enzymes cannot recognise the modified binding sites of the bacterial DNA and by this way its own DNA is protected. There are three types of restriction enzymes type I, type II, and type III. The most wildly used is type II because it recognizes a specific DNA sequence and cuts at that sequence in a predictable and specific manner.

3.6.3 Materials and Methods

Interaction between JH1, JH2, JH3, JH4, JH5, and JH6 with pBR322 plasmid DNA and Salmon sperm DNA (ssDNA) was studied using agarose gel electrophoresis. The method used was a modification of that described by Stellwagen (Stellwagen, 1998).

Materials

Agarose was obtained from ICN 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. Salmon sperm DNA (ssDNA) was obtained from Fluka, Switzerland and Sigma-Aldrich, NSW, Australia. Trizma-HCl, Trizma base disodium salt of ethylene diamine tetraacetic acid, boric acid, acetic acid and ethidium bromide were obtained from Sigma, USA. Restriction enzyme (BamH1), 10X digestion buffer and Polaroid black-and-white print film type 667 were obtained from Sigma, Australia.

3.6.4 Preparation of 50X TAE stock solution and working buffers

As stated earlier, TAE is one of the commonly used buffers for DNA gel electrophoresis. To make 250 mL of 50X TAE buffer solution, 4.66 g of 50 mM Na_2EDTA, 60.55 g of 2 M Tris base (Tris[hydroxymethyl]aminomethane) and 14.28 mL of 2 M glacial acetic 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 TAE working buffer solution.

3.6.5 Gel preparation

To make 250 mL of 1% agarose gel, 2.5 g of agarose powder was added to about 100 mL of TAE working buffer solution following which more TAE working buffer was added to make the volume 250 mL. The mixture was boiled in a microwave for 1 min, swirled to mix and boiling was continued for another 4 min. the agarose solution was cooled for a few minutes. 60 μL of ethidium bromide was added to the gel and swirl gently to disperse the dye (Stellwagen, 1998). Then the gel was gently poured into the tray with comb placed in position, left at room temperature for 30 min to solidify.

3.6.6 Preparation of Salmon Sperm DNA

Stock solutions of ssDNA were prepared by dissolving 10 to 15 mg of ssDNA in 10 mL of 0.05 M Trizma buffer at pH 8 to give DNA concentrations ranging from 1 mg/mL to 1.5 mg/mL. DNA solutions were stored at -17°C until used. To make 250 mL of Trizma buffer, 1.11 g of Trizma-HCl and 0.663 g of Trizma base were dissolved in minimum volume of mQ water and then the volume was made up to the mark by adding more mQ water. The buffer was sterilized by passing through a 0.22 μm Millipore filter.

3.6.7 Interaction with ssDNA

ssDNA is a low molecular weight genomic DNA, ranging in size from 0.6 to 0.8 kilo base. As it exists only in the linear form, its electrophoretogram would give only one band. In the interaction of drugs with ssDNA by electrophoretic assay, the amount of the ssDNA was kept constant (at 4 μL) while the concentration of the compound was varied.

3.6.7.1 Method

To 4 μL aliquots of solutions of ssDNA (at 1.5 mg/mL) were added varied amounts of solutions of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin, the total volume made up to 20 μL by adding mQ water so that the concentrations of compounds ranged from 12.5 μM to 150 μM. A DNA blank was prepared by adding 16 μL mQ water to 4 μL of

ssDNA. The mixtures were incubated in a shaking water bath at 37°C for 4 h. 16 μL aliquots of drug-DNA mixtures were loaded onto the 1% agarose gel and electrophoresis was carried out under TAE buffer for 2 h at 80 V cm^{-1} at room temperature. At the end of electrophoresis, the gel was stained in the same buffer containing ethidium bromide (0.5 mg mL^{-1}). The gel was visualized under UV light using the Bio-Rad Trans illuminator IEC 1010. The illuminated gel was photographed with a Polaroid camera.

3.6.8 Interaction with pBR322 plasmid DNA

In the interaction of synthesised platinum complexes with pBR322 plasmid DNA, the DNA quantity was kept constant while the concentrations of compounds were varied. 1.5 μL of pBR322 plasmid DNA in solution was added to solutions of the platinum complexes at different concentrations ranging from: 30 μM to 80 μM for TH5, TH6 and TH7, 6.25 μM to 60 μM; for TH8, 0.25 μM to 4.5 μM; for TH1 and 1 μM to 9 μM; for TH14. The total volume was made up to 20 μL by adding mQ water. The DNA blank was prepared by adding 18.5 μL mQ water to 1.5 μL of pBR322 plasmid DNA.

The samples including the DNA blank were incubated for 4 h on a shaking water bath at 37 °C in the dark. At the end of incubation, the reaction was quenched by rapid cooling to 0 °C for 20 min. The samples were thawed then mixed with 4 μL of marker dye (0.25% bromophenol blue and 40% of sucrose). 17 μL of each sample was loaded onto 1% agarose gel made in TAE buffer that contained ethidium bromide (1 mg/mL). The gel was stained in same buffer (Onoa and Moreno, 2002). Electrophoresis was carried out also in TAE buffer containing ethidium bromide at 80 V for 3 h at room

temperature. The bands of the plasmid DNA were viewed under short wave UV light using the BIO-RAD Trans illuminator IEC1010 and photographed with Polaroid camera (orange filter) using Polaroid black-and-white print film, type 667.

3.6.9 Interaction with pBR322 plasmid DNA combined with BamH1 restriction enzyme

3.6.9.1 Introduction

BamH1 is a restriction endonuclease, isolated from *Bacillus amyloliquefaciens* H (Wilson and Young, 1975) that hydrolyses the phosphodiester bond between adjacent guanine sites in each strand of the DNA (Kessler and Manta, 1990). It recognizes the sequence G/GATCC and hydrolyses the phosphodiester bond between adjacent guanine sites (Roberts et al., 1977). pBR322 plasmid DNA contains a single restriction site for BamH1 (Sutcliffe, 1979) which converts pBR322 plasmid DNA from supercoiled form I and singly nicked circular form II to linear form III DNA.

3.6.9.2 Materials and Methods

Materials:

BamH1 (Sigma-Aldrich Pty Ltd, NSW, Australia)

Method:

In this series of experiments, an identical set of drug-DNA mixtures, described previously, was first incubated for 4 h on a shaking water bath at 37°C and then subjected to BamH1 (10 units μL^{-1}) digestion. To each 20 μL of incubated drug-DNA mixture was added 2 μL of 10x digestion buffer SB first and then 0.1 μL BamH1 (1

unit). The mixtures were left in a shaking water bath at 37°C for 1 h at the end of which the reaction was terminated by rapid cooling. The gel was subsequently stained by ethidium bromide and visualised under UV light and photographed as described previously.

4. Chapter Four

4.1. Results

4.1.1 Characterization of compounds

The aim of this project was to synthesize new tumour active platinum compounds with one and three metal centres and determine their activity against human ovarian cancer cell lines. Four mononuclear *trans*-planaramineplatinum complexes code named JH1, JH2, JH3, and JH4 were synthesised in addition to two trinuclear platinum complexes code named JH5 and JH6.

The compounds have been characterized based on elemental analyses, spectral studies and molar conductivity measurements. Elemental analysis for C, H, N and Cl were carried out using the facilities of the Micro-analytical Unit at the Australian National University, School of Chemistry. The person who carried out the analyses was Viki Withers of the Micronalytical Unit in the Research School of Chemistry in the ANU. Platinum was determined using Varian AA240 graphite furnace Atomic Absorption spectrometer with GTA 120 graphite tube atomizer available in the Discipline of Biomedical Science, The University of Sydney.

4.1.1.1 Characterization of mononuclear complexes

4.1.1.1.1 JH1: *trans*-$PtCl_2(NH_3)$(thiazole)

Molecular formula = $C_3H_6Cl_2N_2PtS$, formula weight = 368.14.

Percentage yield = 43%, (Figure 4.1)

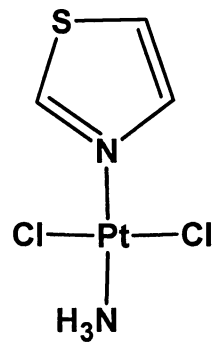


Figure 4-1 Structure of JH1

1.1.1.1.1. JH2: *trans*-$PtCl_2(thiazole)_2$

Molecular formula = $C_6H_6Cl_2N_2PtS_2$, formula weight = 436.23.

Percentage yield = 56%, (Figure 4.2)

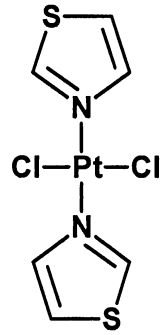


Figure 4-2 Structure of JH2

4.1.1.1.2 JH3: *trans*-$PtCl_2(thiazole)(imidazole)$

Molecular formula = $C_6H_7Cl_2N_3PtS$, formula weight = 419.18.

Percentage yield = 59%, (Figure 4.3)

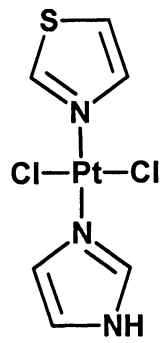


Figure 4-3 Structure of JH3

4.1.1.1.3 JH4: *trans*-$PtCl_2$(thiazole)(3-hydoxypyridine)

Molecular formula = $C_8H_8Cl_2N_2OPtS$, formula weight = 446.21.

Percentage yield = 67%, (Figure 4.4)

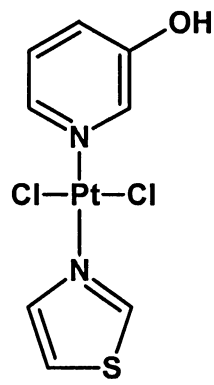


Figure 4-4 Structure of JH4

4.1.1.1.4 Elemental compositions of JH1, JH2, JH3, and JH4

The elemental compositions of JH1, JH2, JH3, and JH4 are given in Table 4.1 below. The starting material for JH1 was cisplatin while JH2, JH3, and JH4 were synthesized

using potassium tetrachloroplatinate as the starting material and utilizing the difference in the *trans* effect of halide and amine ligands in platinum complexes (Kauffman and Cowan, 1963).

Table 4.1 Elemental compositions of JH1, JH2, JH3, and JH4

	JH1		**JH2**	
	Calc.%	***Obs.%***	***Calc.%***	***Obs.%***
C	9.8	9.8 ± 0.4	16.5	16.6 ± 0.4
H	1.6	1.6 ± 0.4	1.4	1.5 ± 0.4
N	7.6	7.9 ± 0.4	6.4	6.5 ± 0.4
Cl	19.3	19.1 ± 0.4	16.3	16.2 ± 0.4
Pt	53.0	53.7 ± 1.2	44.7	44.4 ± 1.2
	JH3		**JH4**	
	Calc.%	***Obs.%***	***Calc.%***	***Obs.%***
C	17.2	17.2 ± 0.4	21.83	22.3 ± 0.4
H	1.7	1.7 ± 0.4	1.81	1.85 ± 0.4
N	10.0	9.7 ± 0.4	6.28	6.25 ± 0.4
Cl	16.9	16.7 ± 0.4	15.89	15.79 ± 0.4
Pt	46.5	45.3 ± 1.2	43.9	43.72 ± 1.2

4.1.2 Characterization of the trinuclear compounds

4.1.2.1 JH5: [{*trans*-$PtCl(NH_3)_2$}$_2$-{*trans*-$Pt(thiazole)_2$}-{$H_2N(CH_2)_6NH_2$}$_2$]Cl_3NO_3

Molecular Formula = $C_{18}H_{50}Cl_5N_{11}Pt_3S_2O_3$, Formula Weight = 1294.64.

Percentage yield = 62%, (Figure 4.5)

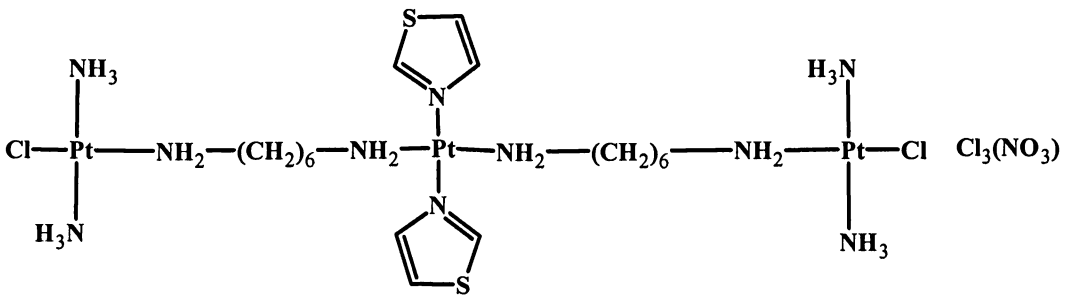


Figure 4-5 Structure of JH5

4.1.2.2 JH6: [{*trans*-$PtCl(NH_3)_2$}$_2$-{*trans*-$Pt(thiazole)NH_3$}{$H_2N(CH_2)_6NH_2$}$_2$]$Cl_3(NO_3)$

Molecular Formula = $C_{15}H_{50}Cl_5N_{11}Pt_3SO_3$, Formula Weight = 1227.18.

Percentage yield = 68%, (Figure 4.6)

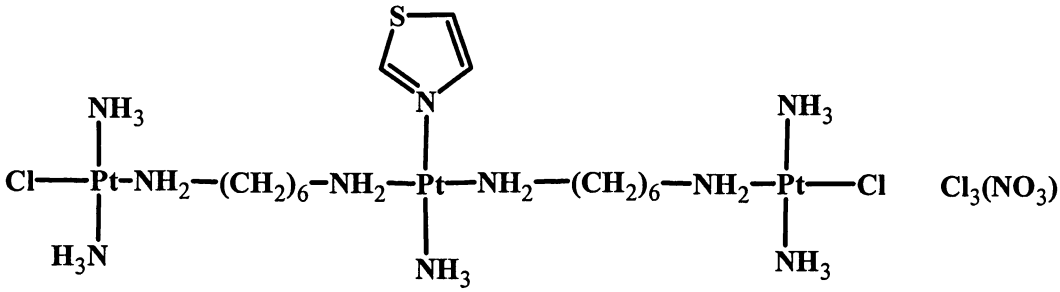


Figure 4-6 Structure of JH6

4.1.2.3 Elemental compositions of trinuclear complexes JH5 and JH6

The elemental composition of trinuclear JH5 and JH6 are shown in Table 4.2 below. The mononuclear JH1 was used as the central unit for synthesis of JH6 and JH2 for JH5.

Table 4.2 Elemental composition of JH5 and JH6

	JH5		JH6	
	Calc. %	*Obs. %*	*Calc. %*	*Obs. %*
C	16.7	17.2 ± 0.4	14.7	14.0 ± 0.4
H	3.9	4.4 ± 0.4	4.1	4.2 ± 0.4
N	11.9	11.8 ± 0.4	12.6	11.7 ± 0.4
Cl	13.7	12.8 ± 0.4	14.4	15.1 ± 0.4
Pt	45.2	46.1 ± 1.2	47.7	48.0 ± 1.2

4.2. Spectral studies

IR, mass spectral, and ^{1}H NMR analyses were carried out to assist in the structural characterization of JH1, JH2, JH3, JH4, JH5, and JH6. Most of the peak assignments are based on published spectra of the ligands and metal complexes of pyridine (Silverstein et al., 1991a; Nakamoto, 1997).

4.2.1 IR spectra of JH1, JH2, JH3, JH4, JH5, and JH6

The IR spectra of JH1, JH2, JH3, JH4, JH5, and JH6 are given in Figures 4.7 to 4.12 respectively. The major peaks of JH1, JH2, JH3, JH4, JH5, and JH6 are listed in Tables 4.3 and 4.4. The letters 's', 'm', 'w', 'br' and 'd' denote strong, medium, weak, broad and doublet respectively. The descriptions of the bands relating to the structures of the compounds are given in Chapter five.

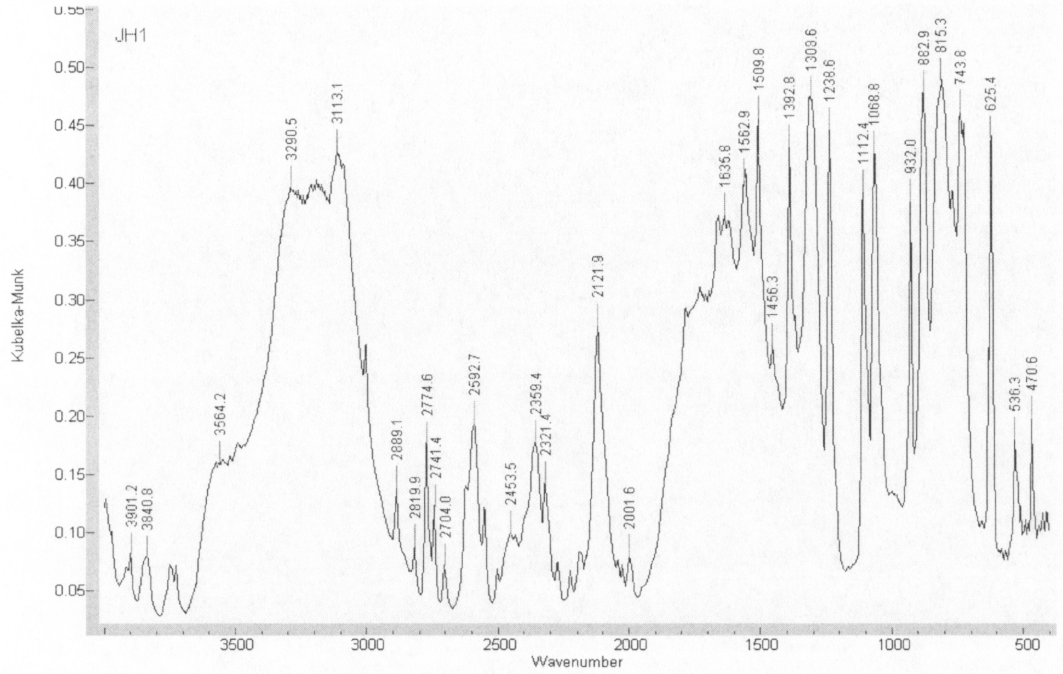


Figure 4-7 IR spectrum result for JH1

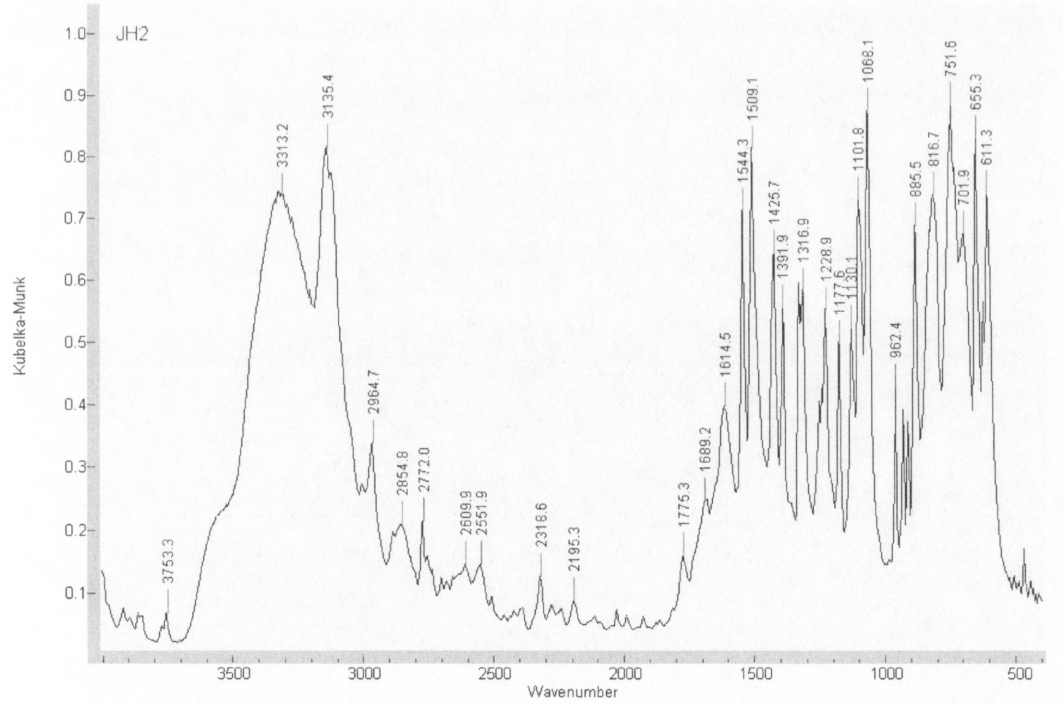


Figure 4-8 IR spectrum result for JH2

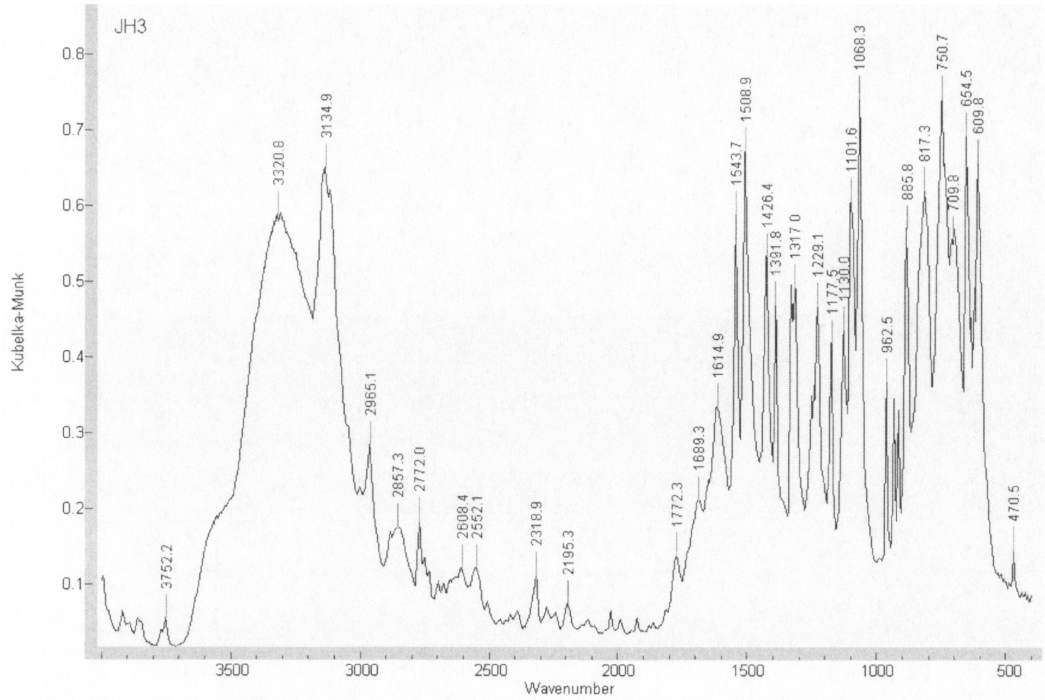


Figure 4-9 IR spectrum result for JH3

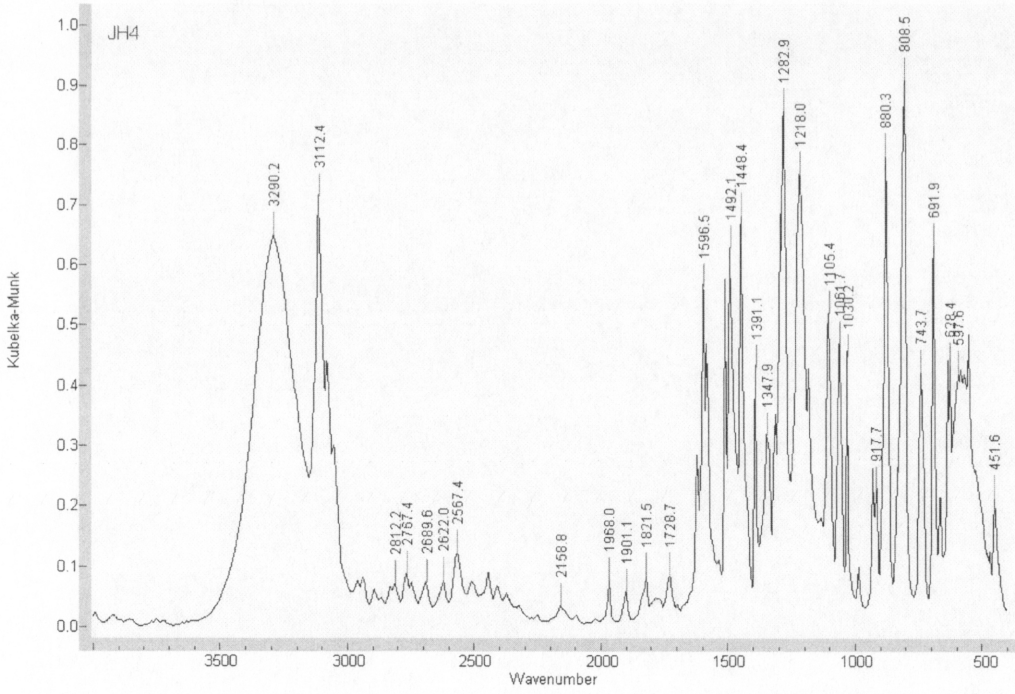


Figure 4-10 IR spectrum result for JH4

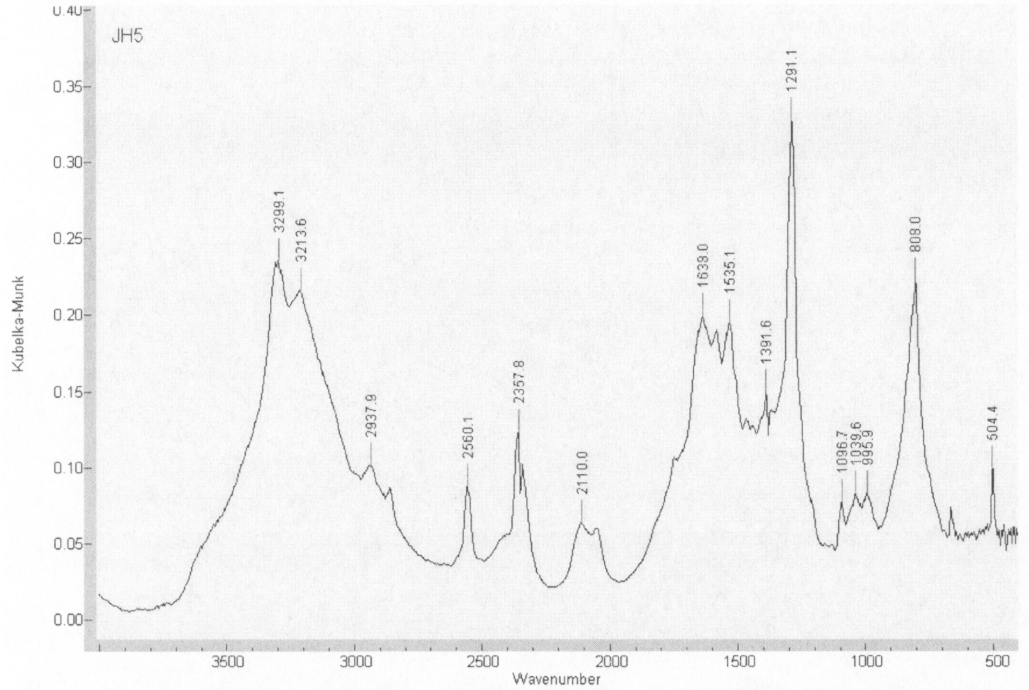


Figure 4-11 IR spectrum result for JH5

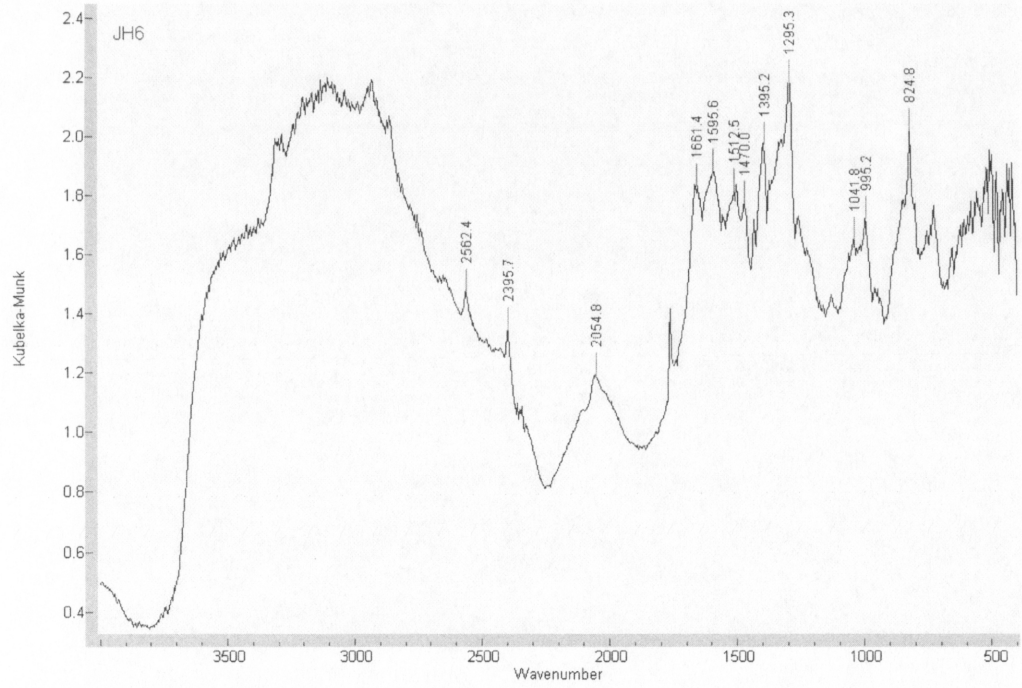


Figure 4-12 IR spectrum result for JH6

Table 4.3 Prominent IR spectral bands observed for mononuclear JH1, JH2, JH3, and JH4

	IR (CM^{-1})
JH1	3290.5 (s, N-H stretching vibration); 3113.1 (s, N-H stretching vibration); 2889.1 (w, C-H stretching vibration); 2819.9 (w, C-H stretching vibration); 1635.8 (s, N-H bending vibration); 1562.9 (s, thiazole ring stretching vibration); 1509.8 (s, C-N stretching vibration); 1456.3 (m, C-H bending vibration); 1392.8 (s, C-H bending vibration); 1308.6 (s, ring in-plane deformation); 1112.4 (s, C-S stretching vibrations); 1068.8 (s, C-C bending vibration); 882.9 (s, C-H out of plane bending); 815.3 (s, C-H out of plane bending); 743.8 (s, ring out-of-plane deformation); 625.4 (s, C=N bending); 536.3 (w, Pt-N stretching vibration); 470.6 (m, Pt-N stretching vibration).
JH2	3313.2 (s, N-H stretching vibration); 3135.4 (s, N-H stretching vibration); 2964.7 (m, C-H stretching vibration); 2854.8 (m, C-H stretching vibration); 1689.2 (m, N-H bending vibration); 1614.5 (m, N-H bending vibration); 1544.3 (s, thiazole ring stretching vibration); 1509.1 (s, C-N stretching vibration); 1425.7 (s, C-H bending vibration); 1391.9 (s, C-H bending vibration); 1316.9 (s, ring in-plane deformation); 1177.6 (s, C-S stretching vibration); 1130.1 (s, C-S stretching vibration); 1101.8 (s, C-N bending vibration); 1068.1 (s, C-C bending vibration); 885.5 (s, C-H out of plane bending); 816.7 (s, C-H out of plane bending); 751.6 (s, ring out-of-plane deformation); 701.9 (s, Pt-N thiazole); 611.3 (s, C=N bending); 531.3 (m, Pt-N stretching vibration); 470.5 (m, Pt-N stretching vibration).

JH3	3320.8 (s N-H stretching vibration); 3134.9 (s N-H stretching vibration); 2965.1 (m C-H stretching vibration); 2857.3 (w C-H stretching vibration); 1689.3 (m, N-H bending vibration); 1614.9 (m, C-C stretching vibration); 1543.7 (s, thiazole ring stretching vibration); 1508.9 (s, C-N stretching vibration); 1426.4 (s, C-H bending vibration); 1391.8 (s, C-H bending vibration); 1317 (s, ring in-plane deformation); 1177.5 (s, C-S stretching vibration); 1130 (s, C-N bending vibration); 1101.6 (s, C-C bending vibration); 1068.3 (s, C-C bending vibration); 885.8 (s, C-H out of plane bending); 750.7 (s, ring out-of-plane deformation); 709.8 (s, C-H out-of-plane bending); 470.5 (w, Pt-N).
JH4	3290.2 (s, O-H stretching vibration); 3112.4 (s, N-H stretching vibration); 2812.2 (w, C-H stretching vibration); 1596.5 (s, C=C stretching vibration); 1492.1 (s, N-H bending vibration); 1448.4 (s, C-H bending vibration); 1391.1 (s, C-H bending vibration); 1347.9 (m, ring stretch); 1282.9 (s, O-H and C-H bending vibrations); 1218 (s, C-O stretching vibration); 1105.4 (s, C-S stretching vibration); 1061.7 (s, C-N bending vibration); 1030.2 (s, C-C bending vibration); 917.7 (m, N-H wagging); 880.3 (s, N-H wagging); 808.5 (s, C-H out of plane bending); 628.4 (s, C=N bending); 597.6 (s, C=N bending); 451.6 (m, Pt-N stretching vibration).

Table 4.4 Prominent IR spectral bands observed for trinuclear JH5 and JH6

	IR (CM^{-1})
JH5	3299.1 (s, N-H stretching vibration); 3213.6 (s, N-H stretching vibration); 2937.9 (m, C-H stretching vibration); 2560.1 (w, C-H stretching vibration); 1638 (s, N-H bending vibration); 1535.1 (s, ring stretching vibration); 1391.6 (m, C-H bending vibration); 1291.1 (s, ring in-plane deformation); 1096.7 (w, C-C bending vibration); 1039.6 (w, C-C bending vibration); 995.9 (w, C-C bending vibration); 808 (s, C-H out of plane bending vibration); 504.4 (m, Pt-N stretching vibration).
JH6	3137.9 (m, N-H stretching vibration), 3087.2 (m, N-H stretching vibration), 2928.2 (s, C-H stretching vibration), 2562.4 (m, C-H stretching vibration); 1661.4 (s, N-H bending vibration); 1595.6 (s, N-H bending vibration); 1512.5 (s, ring stretching vibration); 1470 (s, ring stretching vibration); 1395.2 (s, C-H bending vibration); 1295.3 (s, ring in-plane deformation); 1041.8 (m, C-C bending vibration); 995.2 (s, C-C bending vibration); 824.8 (s, C-H out of plane bending vibration), 729.2 (ring out-of-plane deformation), 526.1 (m, Pt-N stretching vibration).

4.2.2 Mass spectra of JH1, JH2, JH3, JH4, JH5 and JH6

Mass spectra of JH1, JH2, JH3, JH4, JH5 and JH6 are given in the Figures 4.13 to 4.18 respectively. The major peaks observed are listed in Tables 4.5 and 4.6. More detailed discussion of the peaks is given in Chapter five.

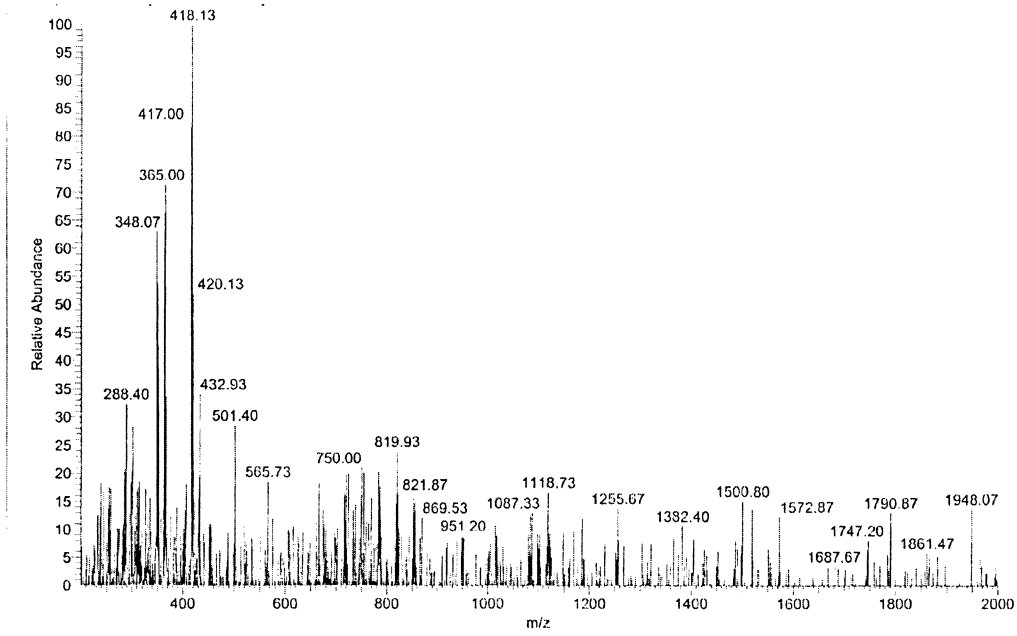


Figure 4-13 Mass spectrum of JH1

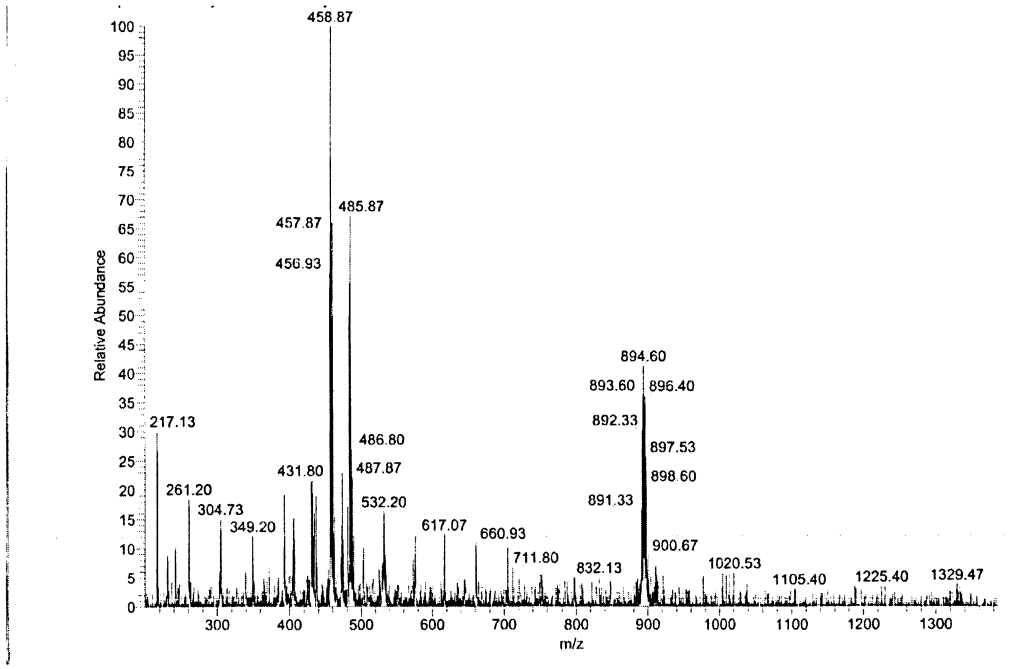


Figure 4-14 Mass spectrum of JH2

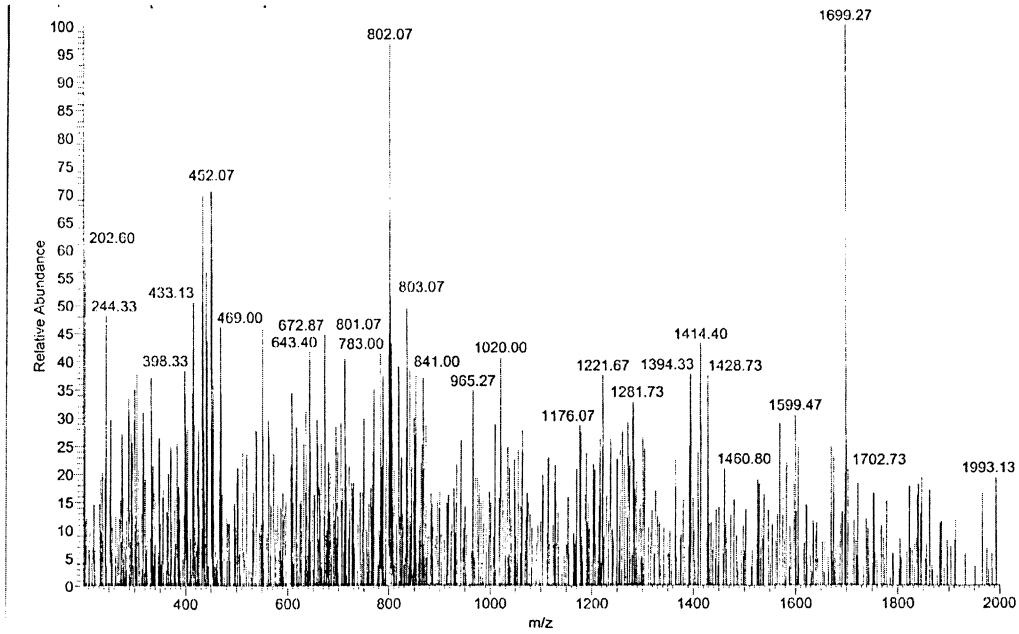


Figure 4-15 Mass spectrum of JH3

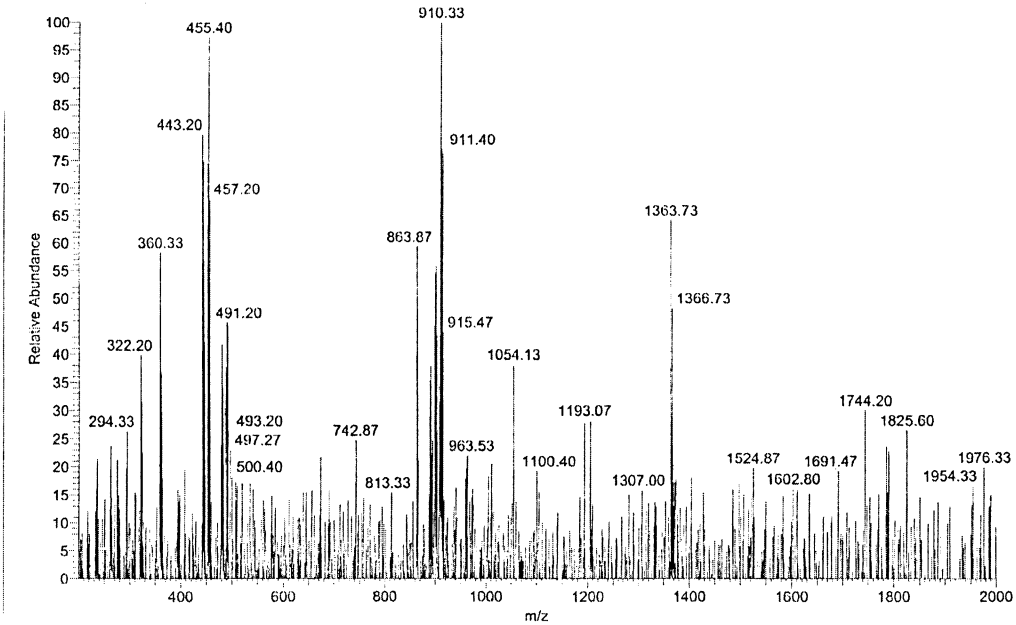


Figure 4-16 Mass spectrum of JH4

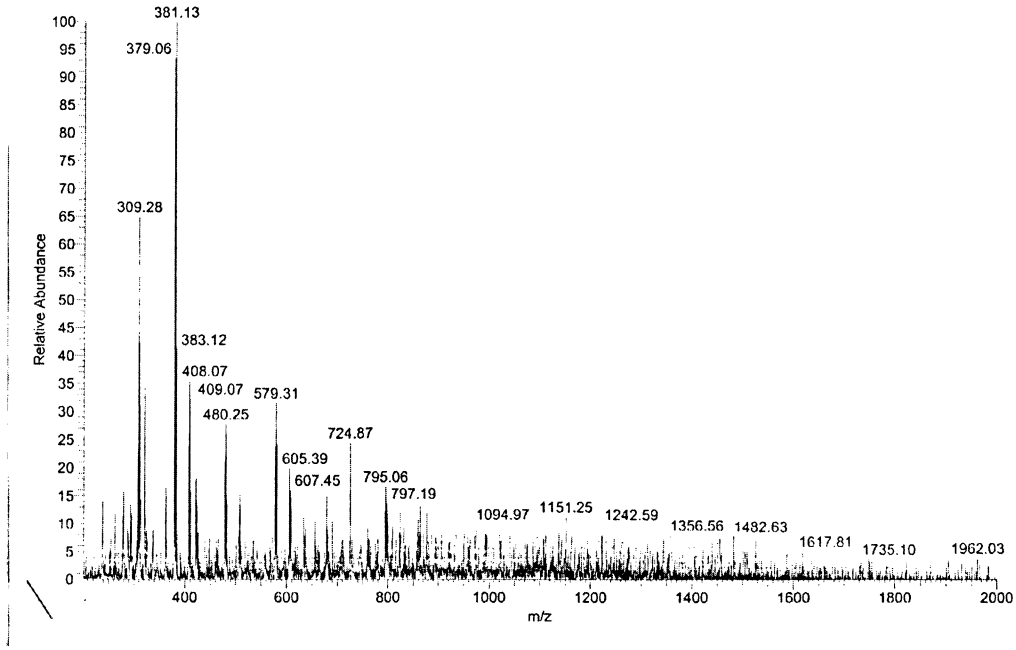


Figure 4-17 Mass spectrum of JH5

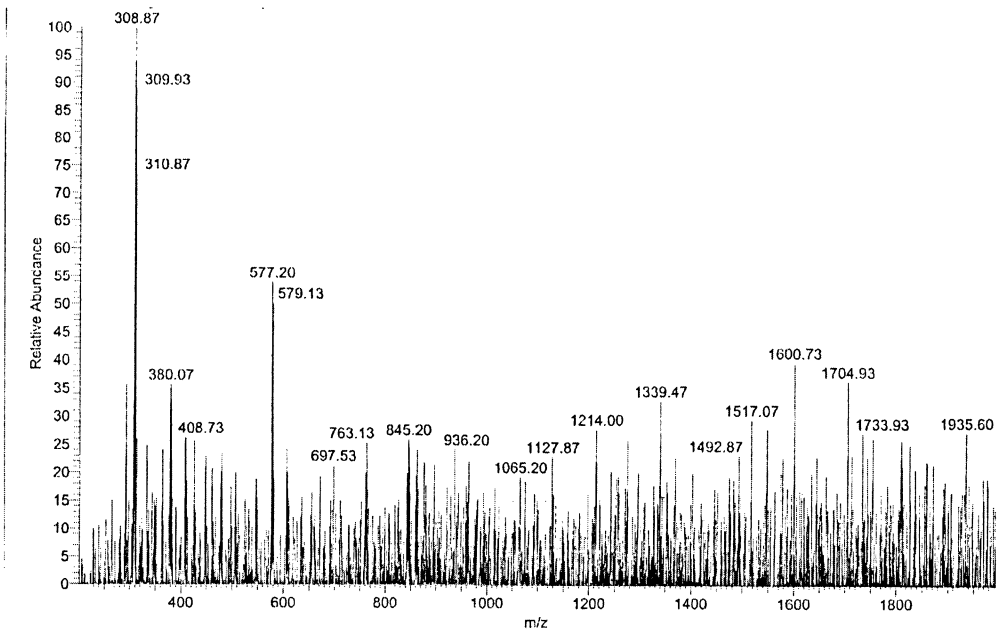


Figure 4-18 Mass spectrum of JH6

Table 4.5 Mass spectra of mononuclears JH1, JH2, JH3, and JH4 where the number in parentheses after each m/z value indicates the relative intensity

	ESI MASS (M/Z)
JH1	ESI-MS (DMF)(m/z: M = 368); $Pt(C_3H_3NS)(NH_3)_2Cl$ = 350 (0.54), (M – 3H = 365 (0.72)); $Pt(NH_3)(C_3H_3NS)_2Cl$ = 418 (1.00).
JH2	ESI-MS (DMF)(m/z: M = 436); M - 3H = 433 (0.21); $PtCl(C_3H_3NS)_3$ = 486 (0.27); $PtCl_2(C_3H_3NS)_5$ +7 H = 894 (0.43).
JH3	ESI-MS (DMF)(m/z: M = 419); $Pt(C_3H_3NS)_2(C_3H_4N_2)$ = 433 (0.51); $Pt(C_3H_3NS)(C_3H_4N_2)_2Cl$ = 452 (0.73); $Pt(C_3H_3NS)_2(C_3H_4N_2)Cl$ = 469 (0.47).
JH4	ESI-MS (DMF)(m/z: M = 446); (M – H) = 445.3 (0.76); $(Pt(C_5H_5NO)_2Cl_2)$ = 455 (0.98); $Pt_2(C_3H_3NS)(C_5H_5NO)_3Cl_4$ = 902 (0.56).

Table 4.6 Mass spectra of trinuclear JH5, and JH6 where the number in parentheses after each m/z value indicates the relative intensity

	ESI MASS (M/Z)
JH5	ESI-MS (DMF)(m/z: M = 1294.6); $PtCl(NH_3)_2(NH_2)_2(CH_2)_6$ = 381 (100); $PtCl(C_3H_3NS)(NH_2)_3(CH_2)_8$ + 4H = 480 (0.28);. $PtCl(C_3H_3NS)_2(NH_2)_3(CH_2)_9$ + 4H = 579 (0.33); $Pt(C_3H_3NS)_2(NH_2)_3(CH_2)_{11}Cl$ + 4H = 607 (0.17); $Pt_2Cl_5(NH_2)_4(CH_2)_{12}$ - 4H = 795 (0.18).
JH6	ESI-MS (DMF)(m/z: M = 1227.18); $PtCl(NH_3)_2NH_2(CH_2)_6NH_2$ = 381 (0.36); $Pt(NH_2)(CH_2)_6(NH_2)Pt(NH_3)_2(NH_2)(CH_2)_6(NH_2)Cl_3$ =762 (0.26); $PtCl\{(NH_2(CH_2)_6NH_2)\}Pt(NH_3)Cl\{NH_2(CH_2)_6NH_2)\}Pt(NH_3)Cl$ + 3H = 960 (0.25).

4.2.3 Nuclear magnetic resonance spectra (^{1}H NMR) of JH1, JH2, JH3, JH4, JH5, and JH6

The ^{1}H NMR spectra results of JH1, JH2, JH3, JH4, JH5, and JH6 respectively are shown in Figures 4.19 to 4.24. The prominent resonances are listed in Tables 4.7 and 4.8. The letters 's', 'd', 't' and 'q' stand for 'singlet', 'doublet', 'triplet' and 'quartet' respectively.

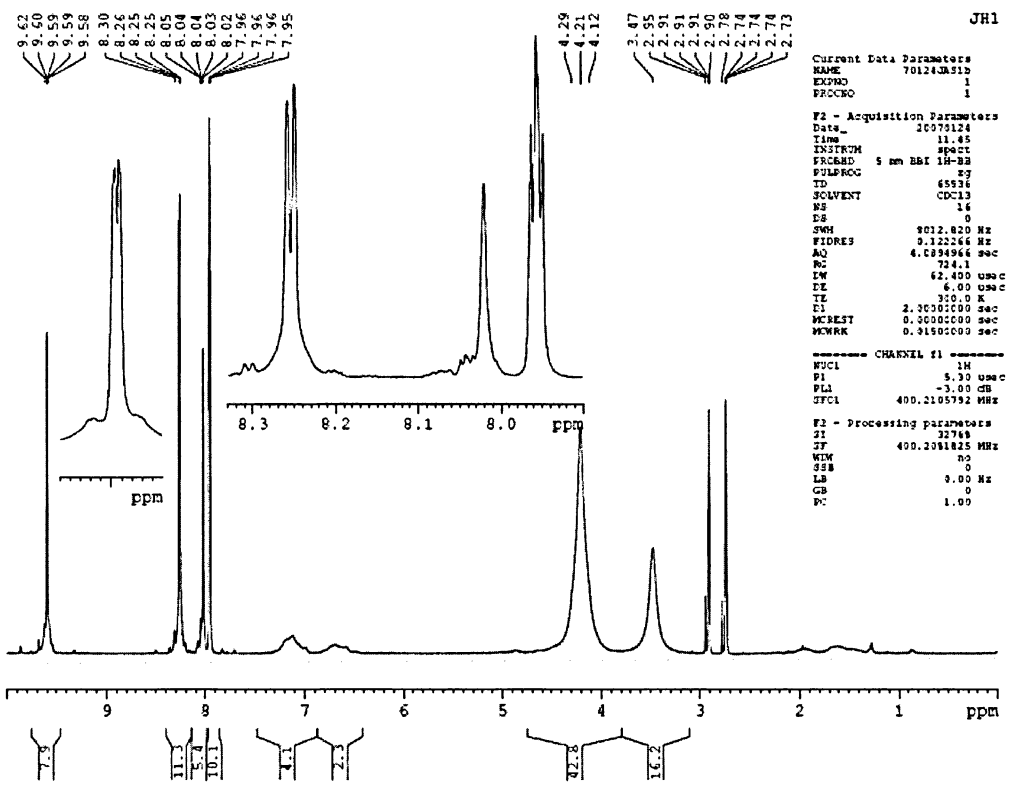


Figure 4-19 ^{1}H NMR spectrum of JH1

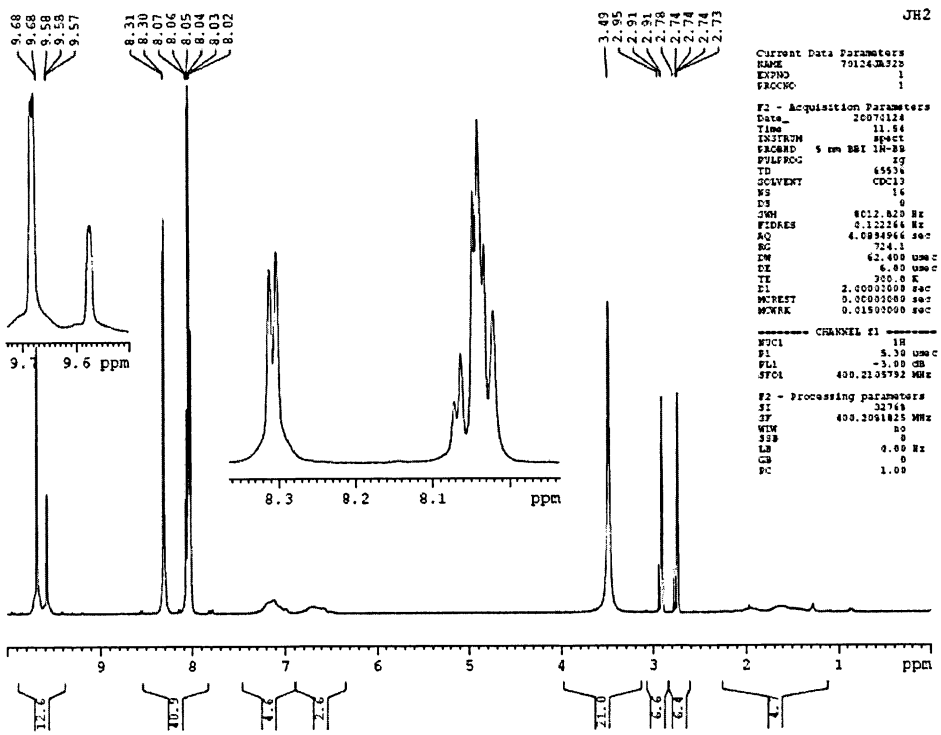


Figure 4-20 ^{1}H NMR spectrum of JH2

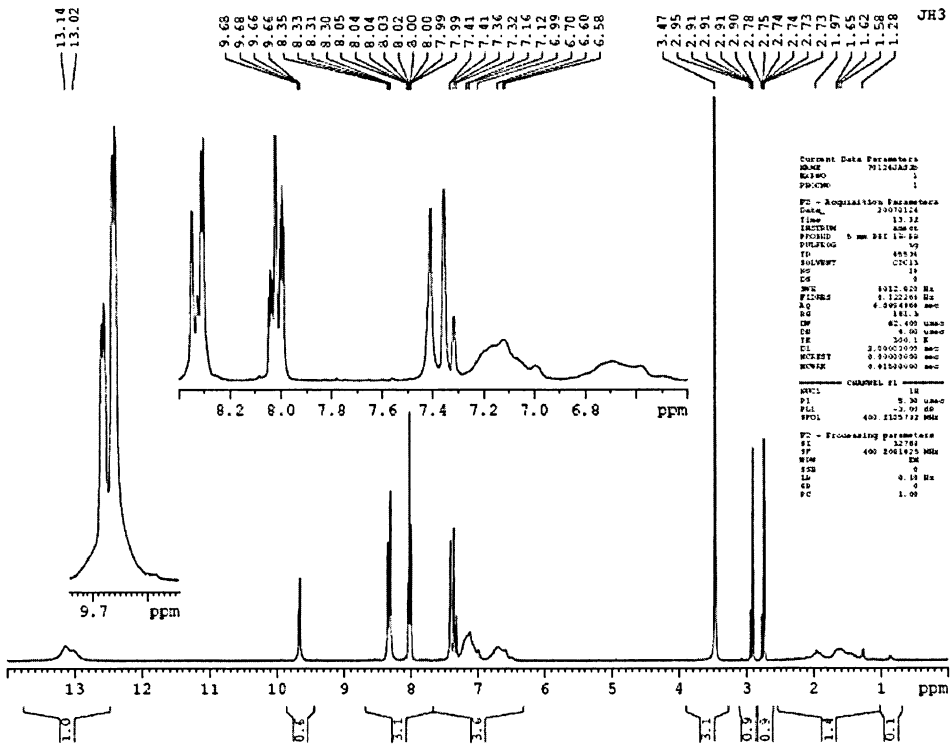


Figure 4-21 ^{1}H NMR spectrum of JH3

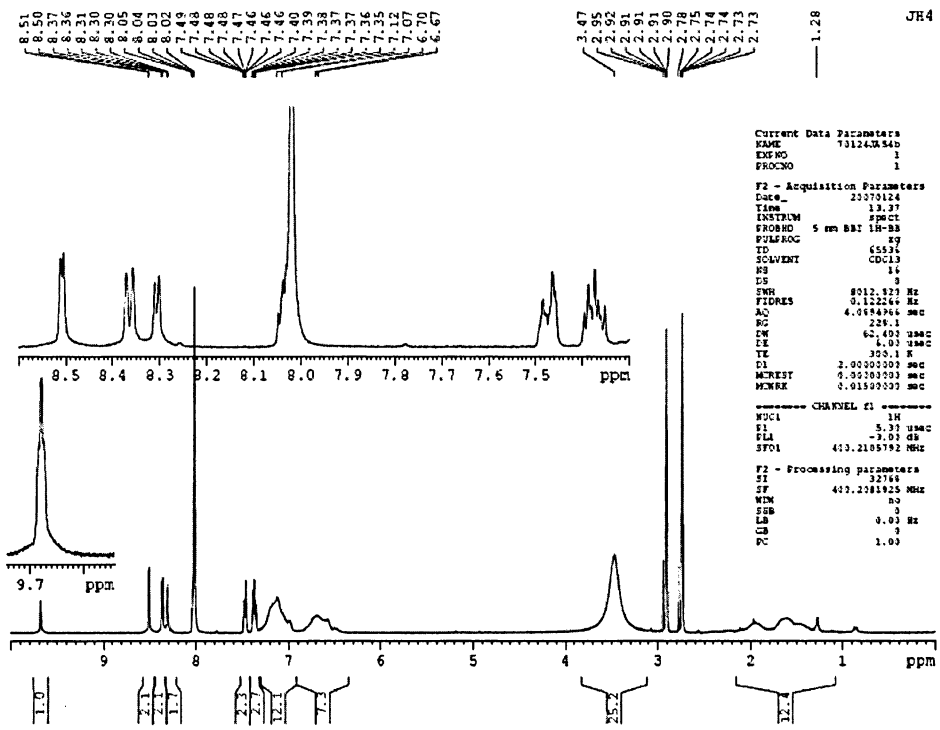


Figure 4-22 ^{1}H NMR spectrum of JH4

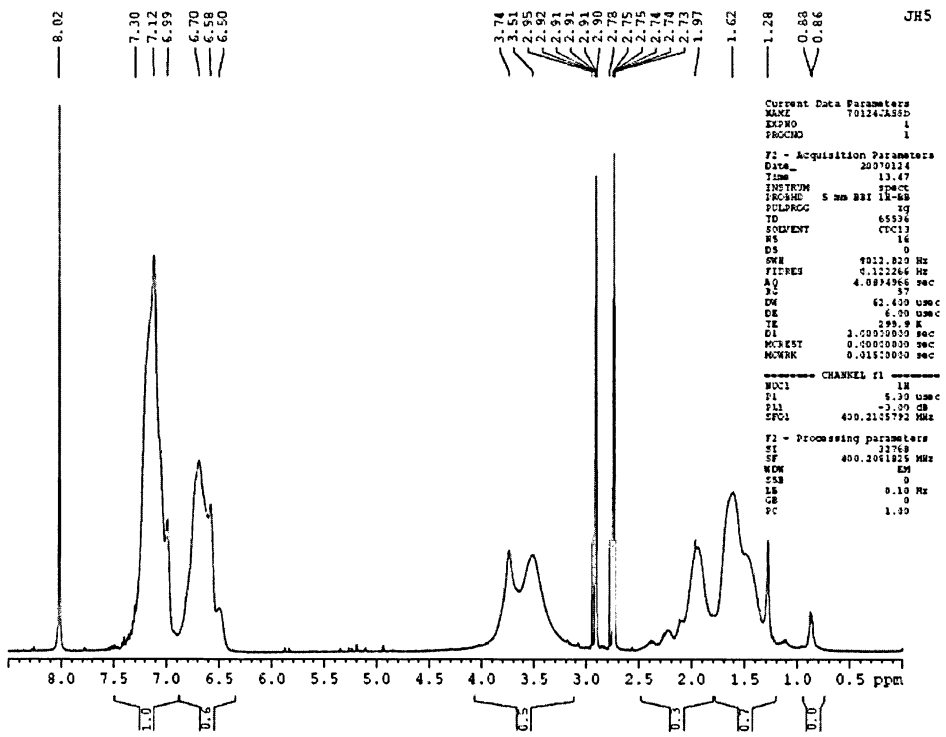


Figure 4-23 ^{1}H NMR spectrum of JH5

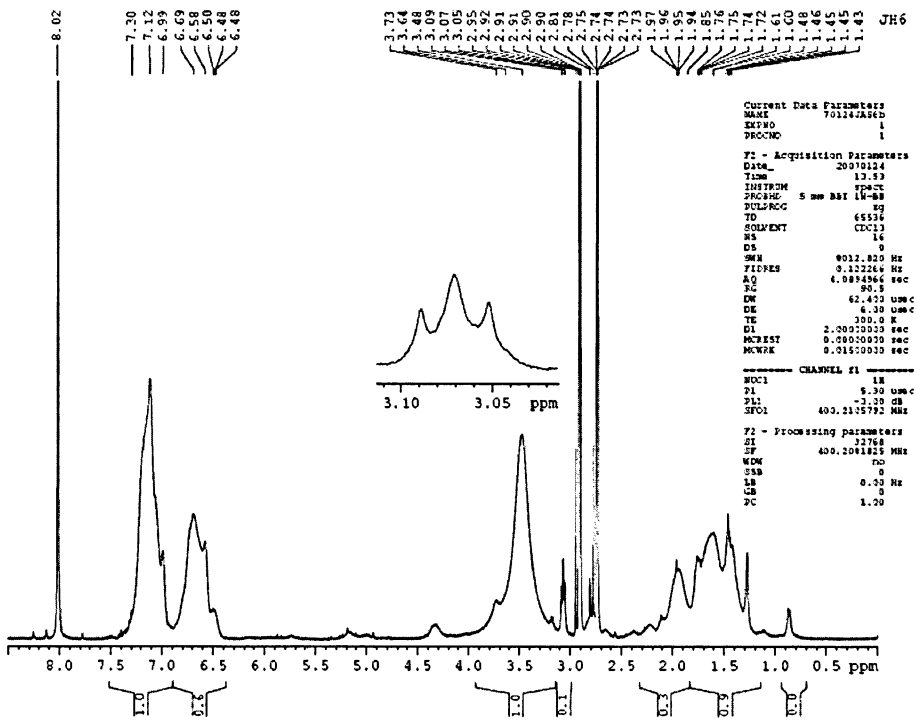
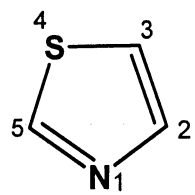


Figure 4-24 ^{1}H NMR spectrum of JH6

Table 4.7 Prominent peaks observed in ^1H NMR spectra of mononuclears JH1, JH2, JH3, and JH4

	^1H NMR
JH1	^1H NMR DMSO δ ppm: 9.6 (due to C_5H), 8.28 (due to C_2H), 8.02 (due to C_3H), 4.21 (due to NH-Pt), 3.47 (due to water dissolved in DMF), 2.91 (due to water), 2.74 (due to DMSO).
JH2	^1H NMR DMSO δ ppm: 9.68 (due to C_5H), 9.58 (due to impurity), 8.30 (due to C_2H), 8.05 (due to C_3H), 3.49 (due to NH-Pt), 2.95 (due to H_2O), 2.91 (due to water), 2.74 (due to DMSO).
JH3	^1H NMR DMSO δ ppm: 9.67 (due to C_5H - T), 8.32 (due to C_2H - T), 8.02 (due to C_3H - T), 8.00 (due to CH - I), 7.41 (due to CH - I) 7.36 (due to CH), 7.32 (due to CH - I), 3.47 (due to water dissolved in DMF), 2.93 (due to water), 2.74 (due to DMSO).
JH4	^1H NMR DMSO δ ppm: 9.68 (due to C_5H - T), 8.50 (due to CH ortho HP?), 8.37 (due to CH ortho - HP), 8.30 (due to C_2H -T), 8.03 (due to C_3H -T), 7.47 (due to CH para - HP), 7.37 (due to CH meta – HP), 3.47 (due to NH Pt), 2.91 (due to H_2O), 2.74 (due to DMSO).

Keys: T for thiazole; HP for 3-hydroxypyridine, I for imidazole. The Figure below shows the numbering of atoms in thiazole ring



Thiazole ring

Table 4.8 Prominent peaks observed in ^{1}H NMR spectra of trinuclear JH5 and JH6

	^{1}H NMR
JH5	^{1}H NMR DMSO δ ppm: 8.28 (due to C_2H-T), 8.02 (due to C_3H-T), 7.12 (due to C_2H-T), 6.7 (due to impurity), 3.74 (due to NH_3-Pt terminal), 3.51 (d, due to water dissolved in DMF), 2.91 (due to NH_2-Pt), 2.74 (due to DMSO), 2.2 (due to CH_2), 1.97 (t, due to CH_2), 1.62 (d, due to CH_2), 1.5 (t, due to CH_2), 1.28 (t, due to CH_2), 0.88 (t, due to CH_2).
JH6	^{1}H NMR DMSO δ ppm: 8.28 (due to C_2H-T), 8.02 (due to C_3H-T), 7.12 (due to C_2H-T), 6.69 (impurity), 4.32 (NH_3-Pt), 3.48 (due to H_2O dissolved in DMF), 3.07 (d, due to NH_2 terminal), 2.74 (due to DMSO), 2.2 (due to CH_2), 1.96 (due to CH_2), 1.75 (due to CH_2), 1.62 (due to CH_2), 1.45 (due to CH_2), 0.88 (due to CH_2).

Key: T for thiazole.

4.3. Molar conductivity

The molar conductivity values for JH1, JH2, JH3, JH4, JH5 and JH6 at different concentrations are given in Table 4.9. Figure 4.25 gives the molar conductivity versus concentration plots.

Table 4.9 Molar conductivity values for JH1, JH2, JH3, JH4, JH5 and JH6

Concentration	0	0.0625 mM	0.125 mM	0.25 mM	0.5 mM
JH1	110	65.6	30.4	19.2	17.6
JH2	58	49.6	32	19.2	14.8
JH3	95	54.4	27.2	26.8	26.2
JH4	115	72	26.4	18.8	12.6
JH5	175	120	76.8	45.6	30.6
JH6	395	344	302.4	274	201
Cisplatin	135	97.6	71.2	54.4	33.4

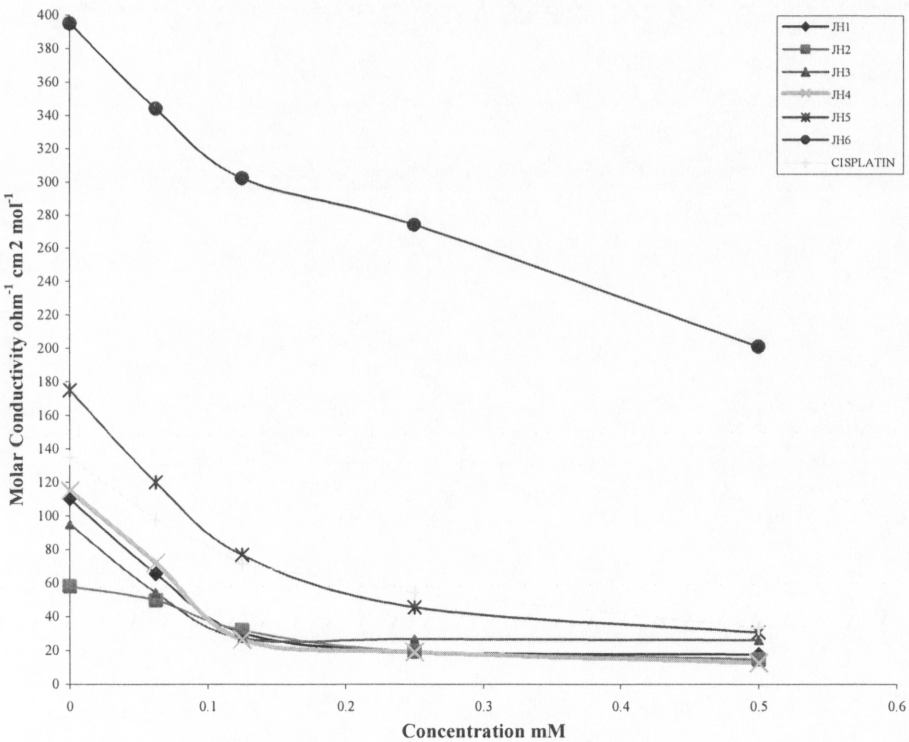


Figure 4-25 Molar conductivity (in ohm^{-1} cm^2 mol^{-1}) for JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin

The limiting molar conductivity values in ohm-1 cm^2 mol^{-1} at zero concentration (Λ_0) were found to be 110, 58, 95, 115, 175, 395, and 135 for JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin respectively.

4.4. Cytotoxicity

The cytotoxicity of the synthesized complexes JH1, JH2, JH3, JH4, JH5, and JH6 along with cisplatin against ovarian cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ have been determined using MTT reduction assay. The concentrations of the compounds were made from 1 mM stock solution and serially diluted to give five concentrations. The results obtained from at least four independent experiments were averaged to give % survival at each concentration. The percentage (%) cell survival values were plotted against concentration of the drugs to give the survival curves. The IC_{50} values were determined from the cell survival curves.

4.4.1 Cytotoxicity of JH1

The percentages of viable cells at various concentrations of JH1 and the IC_{50} values are given in Table 4.10, as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, and Figure 4.26 gives the corresponding cell survival curves.

Table 4.10 A2780, A2780^{cisR}, and A2780^{ZD0473R} viable cells as a percentage of control with JH1

JH1	% Cell Survival Values		
Concentration (μM)	**A2780**	**A2780^{cisR}**	**A2780^{ZD0473R}**
0.125	0.79 ± 0.15	0.94 ± 0.05	0.76 ± 0.08
0.50	0.54 ± 0.05	0.76 ± 0.05	0.62 ± 0.06
2.00	0.19 ± 0.05	0.41 ± 0.06	0.22 ± 0.09
8.00	0.15 ± 0.01	0.06 ± 0.00	0.12 ± 0.02
32.00	0.06 ± 0.01	0.03 ± 0.01	0.09 ± 0.02
IC50 value	0.55 ± 0.06	1.56 ± 0.05	0.78 ± 0.04

Where %: percentage of viable cells

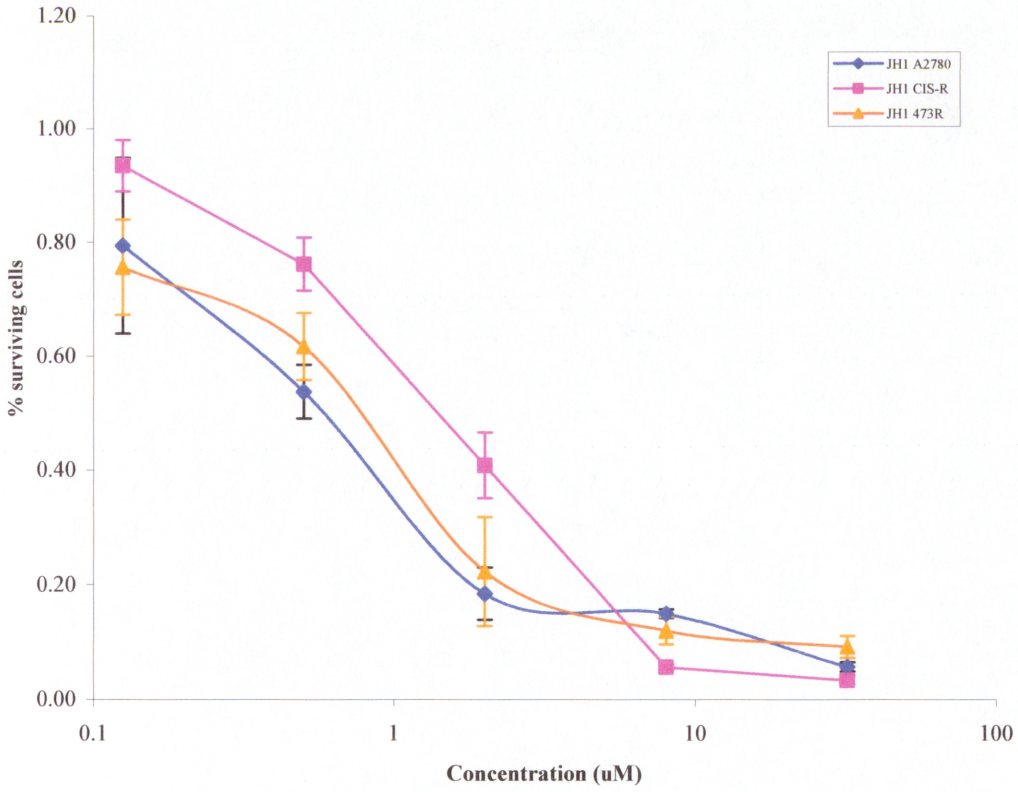


Figure 4-26 Cell survival curve for JH1 as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for JH1 are: A2780: 0.554±0.056 μM, $A2780^{cisR}$: 1.563±0.052 μM and $A2780^{ZD0473R}$: 0.782±0.035 μM. It is found that JH1 is more active against the parent cell line A2780 than the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

4.4.2 Cytotoxicity of JH2

The percentages of viable cells at various concentrations of JH2 and the IC_{50} values are given in Table 4.11, as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, and Figure 4.27 gives the corresponding cell survival curves.

Table 4.11 A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ viable cells as a percentage of control with JH2

JH2	**% Cell Survival Values**		
Concentration (μM)	**A2780**	**$A2780^{cisR}$**	**$A2780^{ZD0473R}$**
0.375	0.90 ± 0.07	0.96 ± 0.03	0.93 ± 0.03
1.50	0.69 ± 0.11	0.86 ± 0.04	0.83 ± 0.10
6.00	0.34 ± 0.08	0.59 ± 0.08	0.58 ± 0.00
24.00	0.14 ± 0.01	0.11 ± 0.03	0.10 ± 0.01
96.00	0.06 ± 0.01	0.05 ± 0.02	0.03 ± 0.00
IC50 value	3.18 ± 0.18	7.32 ± 0.57	7.60 ± 0.07

Where %: percentage of viable cells

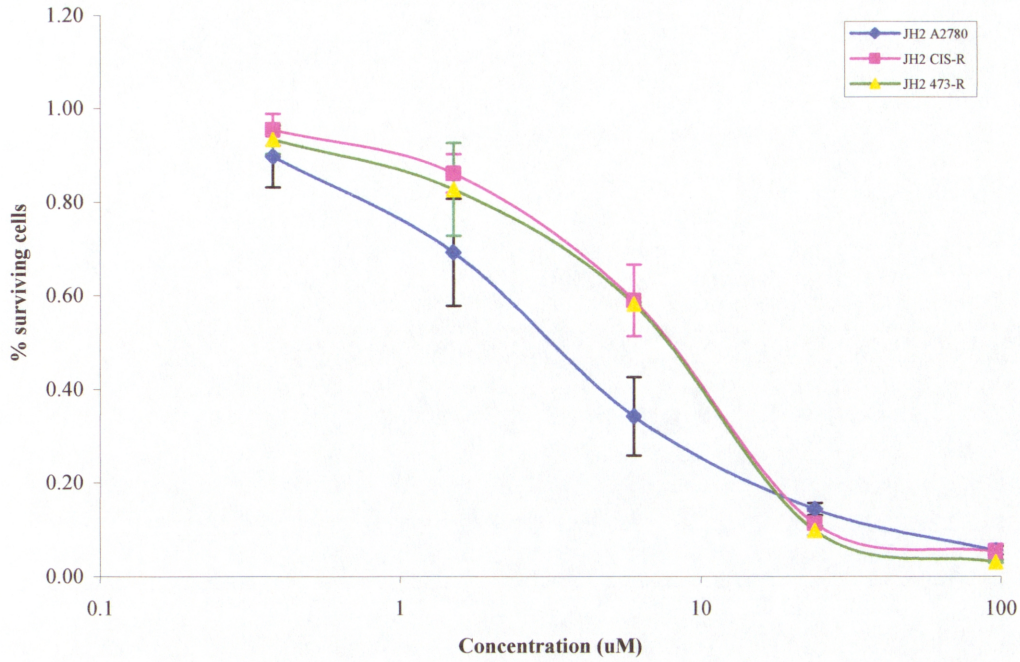


Figure 4-27 Cell survival curve for JH2 as applied to the cell lines: A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$

The IC_{50} values for JH2 are: A2780: 3.177±0.176 μM, $A2780^{cisR}$: 7.323±0.573 μM and $A2780^{ZD0473R}$: 7.599±0.071 μM. It is found that JH2 is more active against the parent cell line A2780 than the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

4.4.3 Cytotoxicity of JH3

The percentages of viable cells at various concentrations of JH3 and the IC_{50} values are given in Table 4.12, as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, and Figure 4.28 gives the corresponding cell survival curves.

Table 4.12 A2780, A2780^{cisR}, and A2780^{ZD0473R} viable cells as a percentage of control with JH3

JH3	% Cell Survival Values		
Concentration (μM)	**A2780**	**A2780^{cisR}**	**A2780^{ZD0473R}**
1.25	0.93 ± 0.08	0.97 ± 0.02	0.89 ± 0.10
3.125	0.70 ± 0.09	0.90 ± 0.03	0.74 ± 0.12
5.00	0.30 ± 0.08	0.25 ± 0.08	0.30 ± 0.12
20.00	0.15 ± 0.02	0.12 ± 0.02	0.04 ± 0.01
80.00	0.08 ± 0.03	0.06 ± 0.02	0.03 ± 0.01
IC_{50} value	3.91 ± 0.09	4.23 ± 0.14	4.10 ± 0.28

Where %: percentage of viable cells

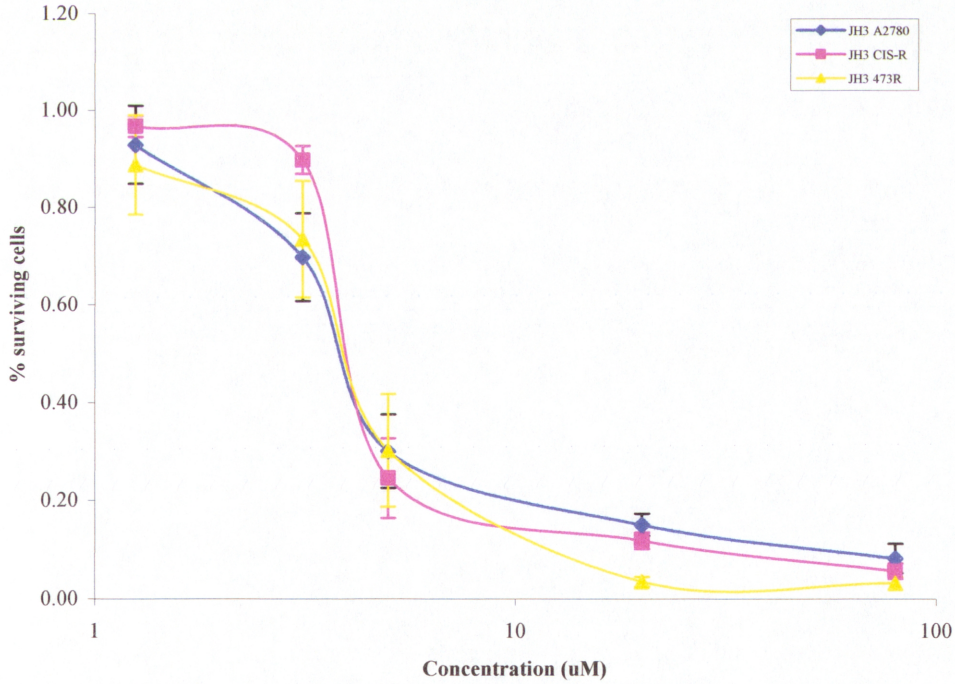


Figure 4-28 Cell survival curve for JH3 as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for JH3 are: A2780: 3.91±0.09 μM, $A2780^{cisR}$: 4.23±0.14 μM and $A2780^{ZD0473R}$: 4.10±0.28 μM. It is found that JH3 is more active against the parent cell line A2780 than the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

4.4.4 Cytotoxicity of JH4

The percentages of viable cells at various concentrations of JH4 and the IC_{50} values are given in Table 4.13, as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, and Figure 4.29 gives the corresponding cell survival curves.

Table 4.13 A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ viable cells as a percentage of control with JH4

JH4	**% Cell Survival Values**		
Concentration (μM)	**A2780**	**$A2780^{cisR}$**	**$A2780^{ZD0473R}$**
1.25	0.92 ± 0.05	0.96 ± 0.03	0.93 ± 0.04
3.125	0.80 ± 0.12	0.90 ± 0.06	0.68 ± 0.03
5.00	0.38 ± 0.11	0.45 ± 0.16	0.38 ± 0.14
20.00	0.07 ± 0.01	0.34 ± 0.05	0.10 ± 0.01
80.00	0.04 ± 0.01	0.12 ± 0.01	0.05 ± 0.01
IC_{50} value	4.48 ± 0.32	4.63 ± 0.44	3.90 ± 0.35

Where %: percentage of viable cells

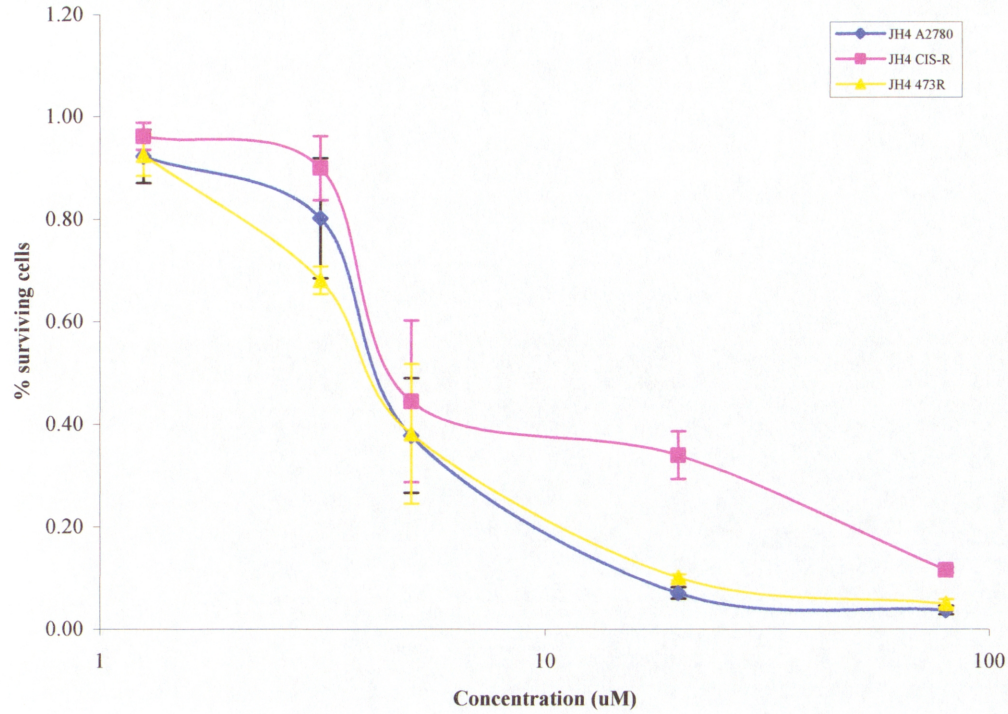


Figure 4-29 Cell survival curve for JH4 as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for JH4 are: A2780: 4.48±0.32 μM, A2780^{cisR}: 4.63±0.44 μM and A2780^{ZD0473R}: 3.90±0.35 μM. It is found that JH4 is more active against the parent cell line A2780 than the resistant cell lines: A2780^{cisR}. On the other hand JH4 has been found to be more active in A2780^{ZD0473R} cell line than that in parents cell line A2780.

4.4.5 Cytotoxicity of JH5

The percentages of viable cells at various concentrations of JH5 and the IC_{50} values are given in Table 4.14, as applied to the cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}, and Figure 4.30, gives the corresponding cell survival curves.

Table 4.14 A2780, A2780^{cisR}, and A2780^{ZD0473R} viable cells as a percentage of control with JH5

JH5	% Cell Survival Values		
Concentration (μM)	**A2780**	**A2780^{cisR}**	**A2780^{ZD0473R}**
0.125	0.92 ± 0.06	0.94 ± 0.02	0.93 ± 0.04
0.50	0.80 ± 0.09	0.90 ± 0.04	0.80 ± 0.10
2	0.55 ± 0.04	0.82 ± 0.06	0.69 ± 0.05
8	0.27 ± 0.06	0.44 ± 0.01	0.38 ± 0.03
32	0.08 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
IC_{50} value	2.74 ± 0.25	6.35 ± 0.20	4.64 ± 0.07

Where %: percentage of viable cells

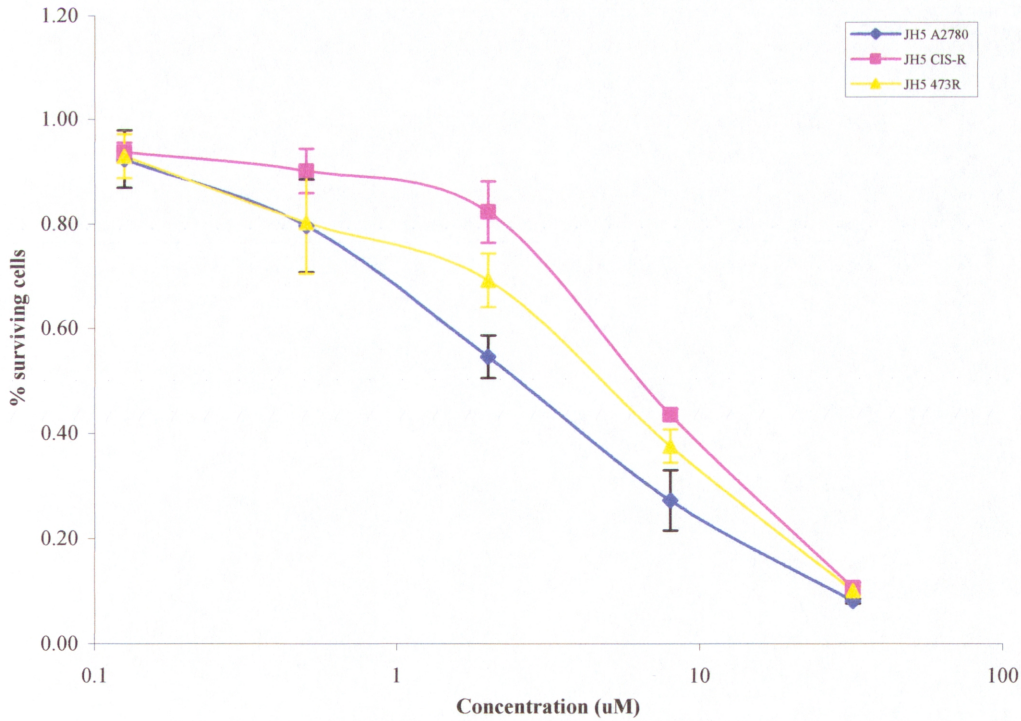


Figure 4-30 Cell survival curve for JH5 as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for JH5 are: A2780: 2.74±0.25 μM, $A2780^{cisR}$: 6.35±0.20 μM and $A2780^{ZD0473R}$: 4.64±0.07 μM. It is found that JH5 is more active against the parent cell line A2780 than the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

4.4.6 Cytotoxicity of JH6

The percentages of viable cells at various concentrations of JH6 and the IC_{50} values of JH6 as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ are given in Table 4.15 and Figure 4.31 gives the corresponding cell survival curves.

Table 4.15 A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ viable cells as a percentage of control with JH6

JH6	**% Cell Survival Values**		
Concentration (μM)	**A2780**	**$A2780^{cisR}$**	**$A2780^{ZD0473R}$**
0.125	0.63 ± 0.05	0.84 ± 0.04	0.89 ± 0.04
0.50	0.38 ± 0.04	0.77 ± 0.04	0.77 ± 0.03
2.00	0.24 ± 0.04	0.55 ± 0.07	0.57 ± 0.10
8.00	0.17 ± 0.01	0.07 ± 0.01	0.08 ± 0.02
32.00	0.14 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
IC_{50} value	0.25 ± 0.04	2.34 ± 0.35	2.62 ± 0.16

Where %: percentage of viable cells

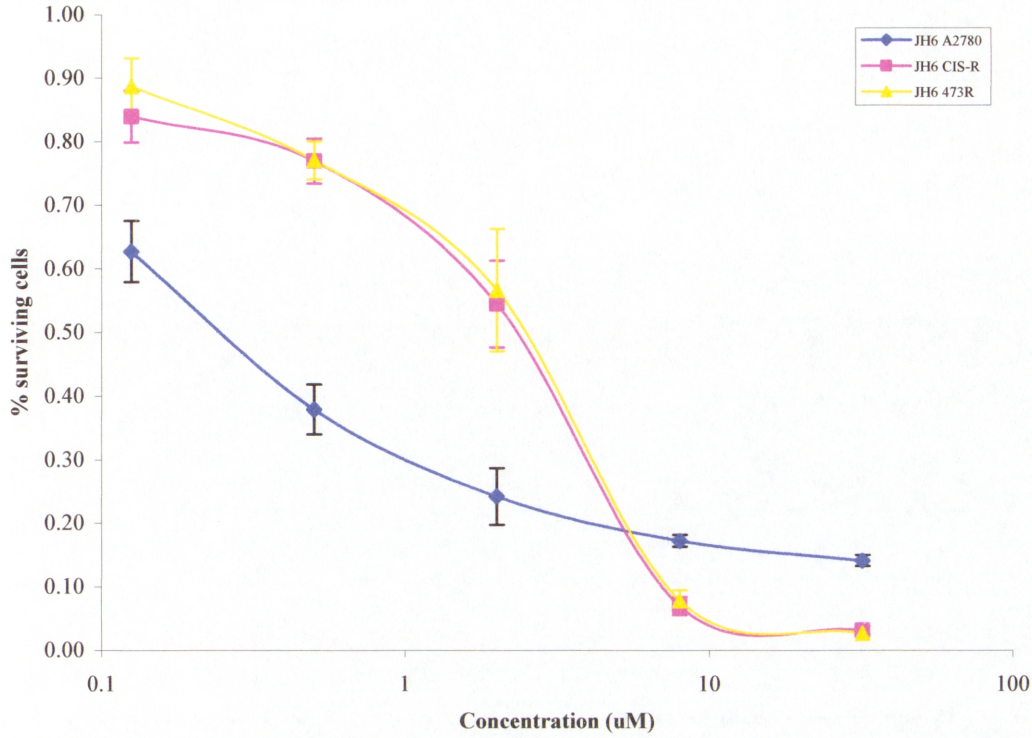


Figure 4-31 Cell survival curve for JH6 as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for JH6 are: A2780: 0.253 ± 0.035 μM, A2780^{cisR}: 2.336 ± 0.350 μM and A2780^{ZD0473R}: 2.623 ± 0.157 μM. It is found that JH6 is more active against the parent cell line A2780 than the resistant cell lines: A2780^{cisR} and A2780^{ZD0473R}.

4.4.7 Cytotoxicity of Cisplatin

Table 4.16 gives the percentages of viable cells at various concentrations of cisplatin and the IC_{50} values, as applied to the cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R} and Figure 4.32 gives the corresponding cell survival curves.

Table 4.16 A2780, A2780^{cisR}, and A2780^{ZD0473R} viable cells as a percentage of control with cisplatin

Cisplatin	% Cell Survival Values		
Concentration (μM)	**A2780**	**A2780^{cisR}**	**A2780^{ZD0473R}**
0.125	0.84 ± 0.10	0.95 ± 0.02	0.94 ± 0.03
0.50	0.46 ± 0.01	0.93 ± 0.01	0.89 ± 0.06
2.00	0.28 ± 0.05	0.81 ± 0.08	0.75 ± 0.08
8.00	0.24 ± 0.04	0.44 ± 0.02	0.35 ± 0.06
32.00	0.11 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
IC_{50} value	0.43 ± 0.02	6.41 ± 0.37	4.73 ± 0.22

Where %: percentage of viable cells

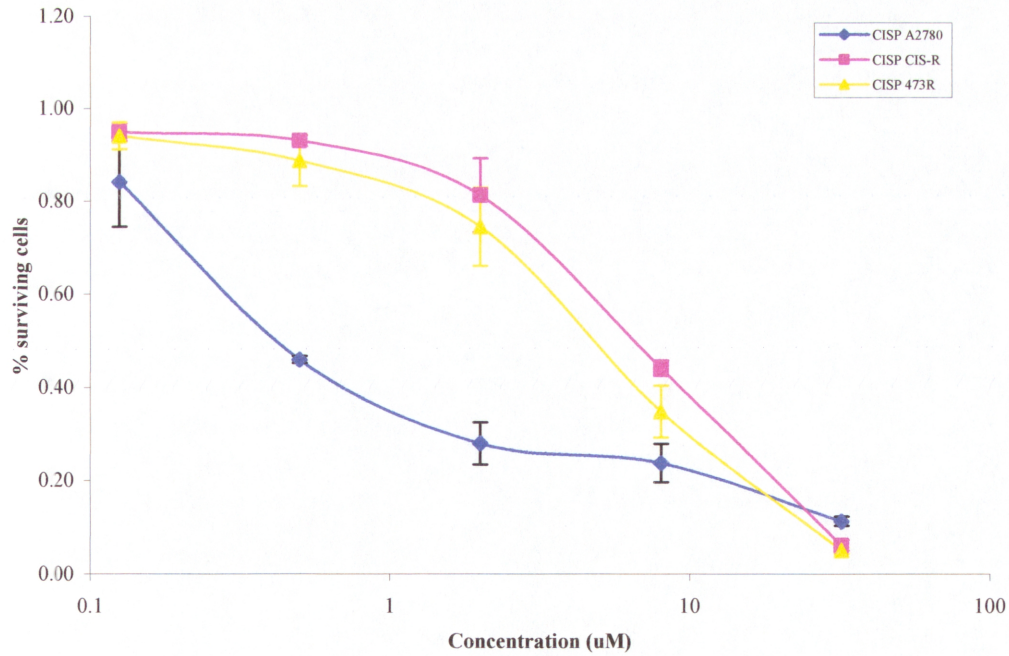


Figure 4-32 Cell survival curve for cisplatin as applied to the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}

The IC_{50} values for cisplatin are: A2780: 0.43± 0.02 μM, $A2780^{cisR}$: 6.41 ± 0. 37 μM and $A2780^{ZD0473R}$: 4.73 ± 0.22 μM. It is found that cisplatin is more active against the parent cell line A2780 than the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

4.4.8 Activity summary and resistance factor:

Table 4.17 and Figure 4.33 below show the IC_{50} values for all synthesised platinum complexes JH1, JH2, JH3, JH4, JH5, and JH6 along with those for cisplatin as applied to the ovarian cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$. Figure 4.34 gives the resistance factors for the synthesised platinum complexes.

Table 4.17 IC_{50} values and resistance factors (RF) for JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin

IC_{50} (μM) ± SD and Resistant Factors					
	A2780	**$A2780^{cisR}$**	**RF**	**$A2780^{ZD0473R}$**	**RF**
JH1	0.55 ± 0.06	1.56 ±0.05	2.82	0.78 ± 0.04	1.41
JH2	3.17 ± 0.17	7.32 ± 0.57	2.30	7.59 ± 0.07	2.39
JH3	3.90 ±0.09	4.22 ± 0.14	1.08	4.09 ± 0.28	1.00
JH4	4.47 ± 0.32	4.63 ± 0.43	1.03	3.90 ± 0.35	0.87
JH5	2.73 ± 0.25	6.35± 0.20	2.31	4.64 ± 0.07	1.69
JH6	0.25 ± .04	2.33 ± 0.35	9.22	2.62 ± 0.15	10.35
Cisplatin	0.42 ± 0.02	6.41 ± 0.36	14.96	4.72 ± 0.21	11.03

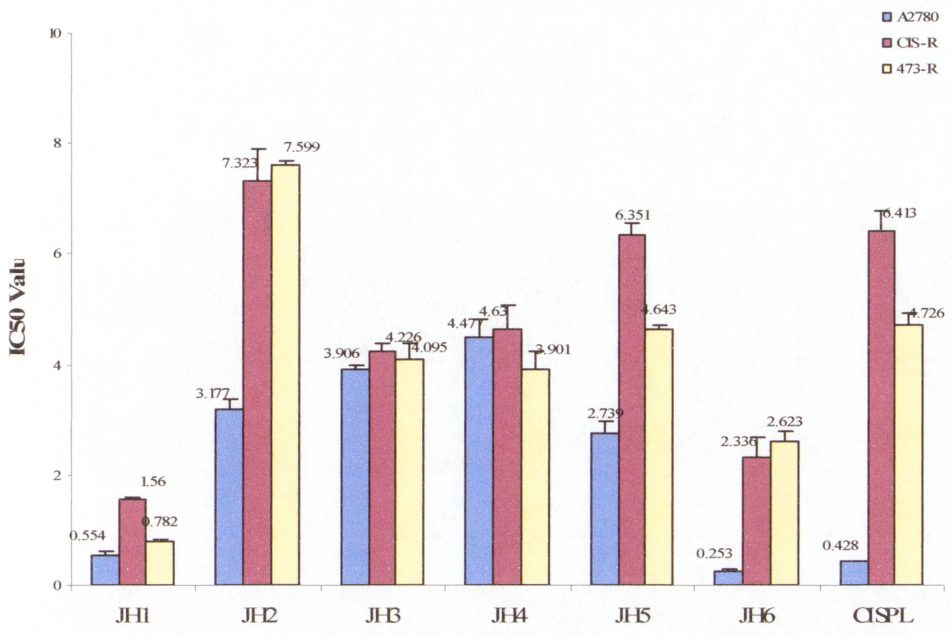


Figure 4-33 IC_{50} values for JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin as applied to the human ovary cancer cell lines: A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$.

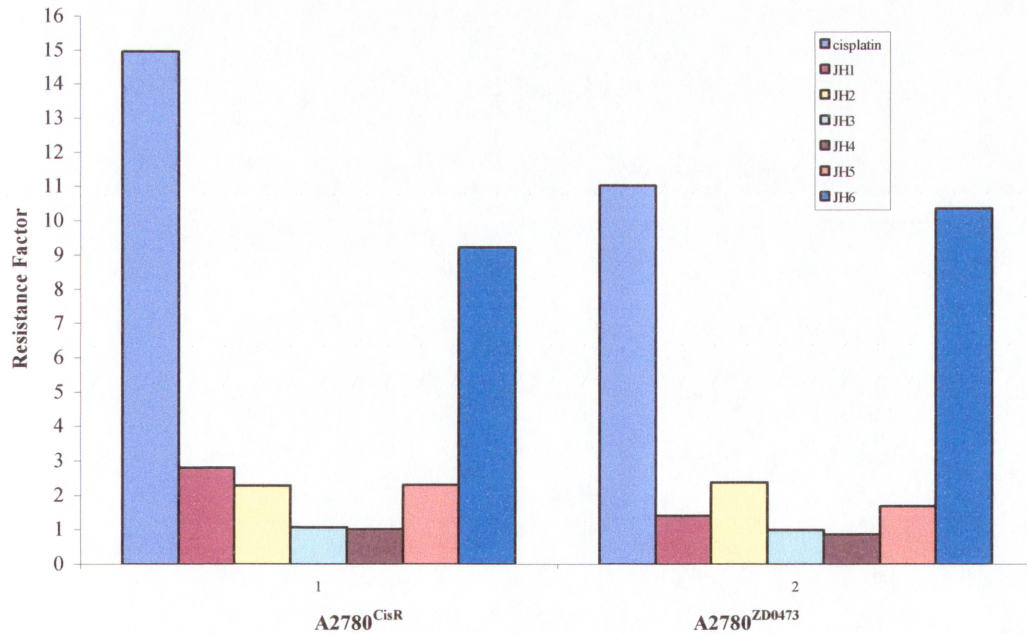


Figure 4-34 Resistance factors (RF) for JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin as applied to the human ovary resistant cancer cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

It can be seen that JH1, JH2, JH3, JH4, JH5, and JH6 differ in their activity against the ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$. Among the synthesised complexes JH6 are found to be more active than cisplatin in all the three cell lines. JH1was found to be the most active in resistance cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$. JH3 and JH4 were also found to be more active than cisplatin in the resistant cell lines. JH4 was found to be more active in the resistant cell line $A2780^{ZD0473R}$ than the parent cell line A2780.

4.5. Platinum Cell uptake

The cellular accumulation of platinum has been used as a measure of cell uptake of the compounds. Tables 4.18 to 4.20 and Figures 4.35 to 4.37 give the total intracellular platinum levels (expressed as nanomole Pt per 5 $x10^6$ cells) found in ovarian cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ after exposure to 25 μM concentration of JH1, JH2, JH4, JH5, JH6 and cisplatin for 2 h, 4 h and 24 h respectively.

Among the mononuclear complexes JH1, JH2, JH4 and cisplatin, the cell uptake is highest for JH4 in all cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ in 2 hours, 4 h, and 24 h. Among the trinuclear compounds cell uptake is higher for JH6 in all cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ in 2, 4, and 24 h except in the cell line $A2780^{cisR}$ in 4 h for which JH5 has higher uptake.

Table 4.18 Pt accumulation in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 2 h as applied to JH1, JH2, JH4, JH5, JH6, and cisplatin

Compound	A2780	A2780^{cisR}	A2780^{ZD0473R}
	nmol Pt per 2x10^{6}cells	nmol Pt per 2x10^{6}cells	nmol Pt per 2x10^{6}cells
JH1	1.33 ± 0.14	1.87 ± 0.62	1.57 ± 0.37
JH2	1.35 ± 0.17	1.43 ± 0.04	1.18 ± 0.07
JH4	3.49 ± 0.41	3.87 ± 0.30	3.90 ± 0.41
JH5	1.05 ± 0.05	1.25 ± 0.11	0.60 ± 0.14
JH6	3.21 ± 0.24	2.15 ± 0.08	1.04 ± 0.16
Cisplatin	0.19 ± 0.04	0.22 ± 0.01	0.14 ± 0.03

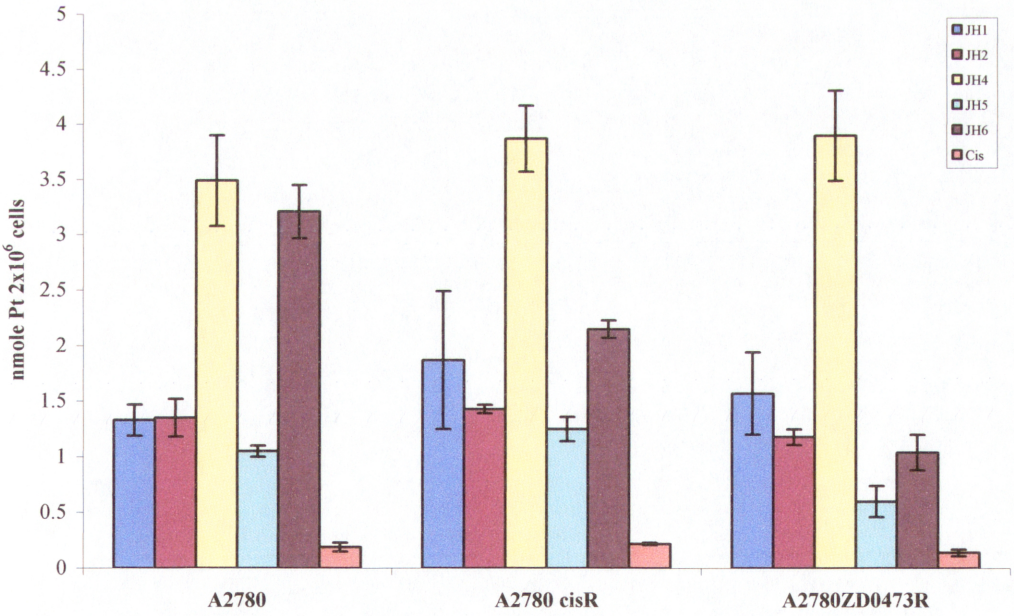


Figure 4-35 Pt accumulation in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 2 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Table 4.19 Pt accumulation in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 4 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Compound	A2780	A2780^{cisR}	A2780^{ZD0473R}
	nmol Pt per $2x10^6$cells	nmol Pt per $2x10^6$cells	nmol Pt per $2x10^6$cells
JH1	1.65 ± 0.16	1.94 ± 0.38	1.83 ± 0.20
JH2	1.79 ± 0.03	1.60 ± 0.07	1.80 ± 0.42
JH4	3.42 ± 0.12	3.64 ± 0.32	2.08 ± 0.02
JH5	1.33 ± 0.29	1.77 ± 0.12	1.05 ± 0.15
JH6	2.04 ± 0.16	1.43 ± 0.01	3.57 ± 0.49
Cisplatin	0.37 ± 0.01	0.41 ± 0.00	0.40 ± 0.07

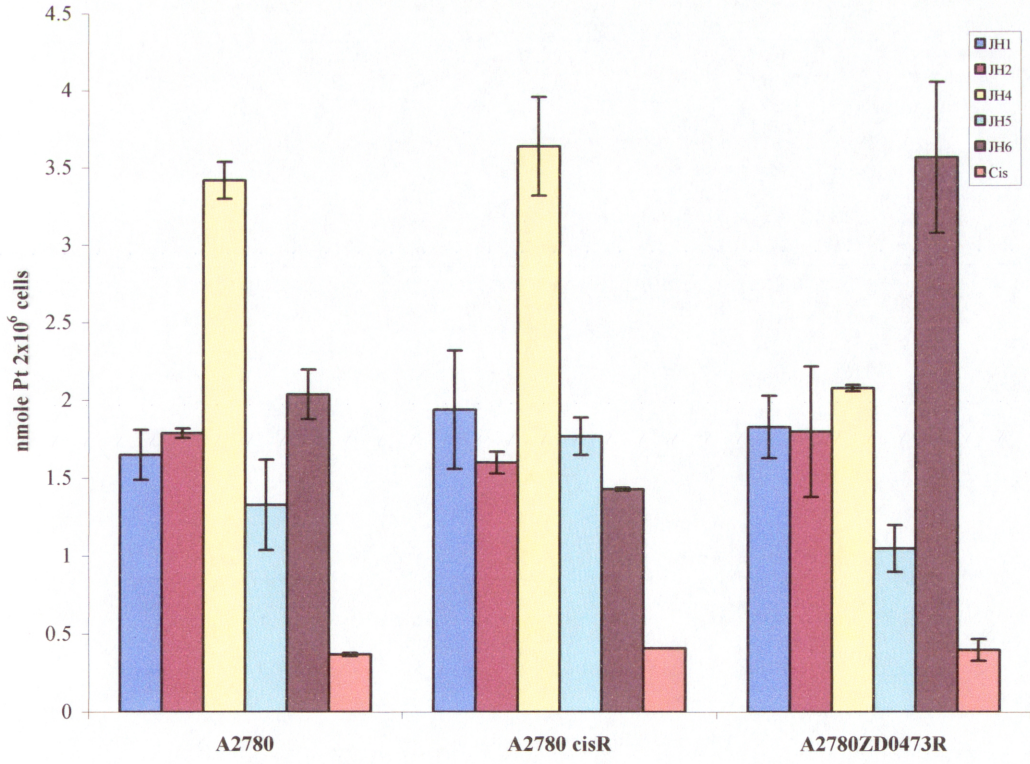


Figure 4-36 Pt accumulation in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 4 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Table 4.20 Pt accumulation in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Compound	A2780	A2780^{cisR}	A2780^{ZD0473R}
	nmol Pt per $2x10^6$cells	nmol Pt per $2x10^6$cells	nmol Pt per $2x10^6$cells
JH1	1.95 ± 0.09	1.95 ± 0.20	1.90 ± 0.36
JH2	2.16 ± 0.35	1.26 ± 0.19	1.53 ± 0.08
JH4	3.14 ± 0.39	2.96 ± 0.32	1.98 ± 0.13
JH5	0.74 ± 0.03	3.80 ± 0.10	2.03 ± 0.29
JH6	1.77 ± 0.33	4.25 ± 0.20	2.09 ± 0.27
Cisplatin	0.96 ± 0.15	0.93 ± 0.03	1.13 ± 0.23

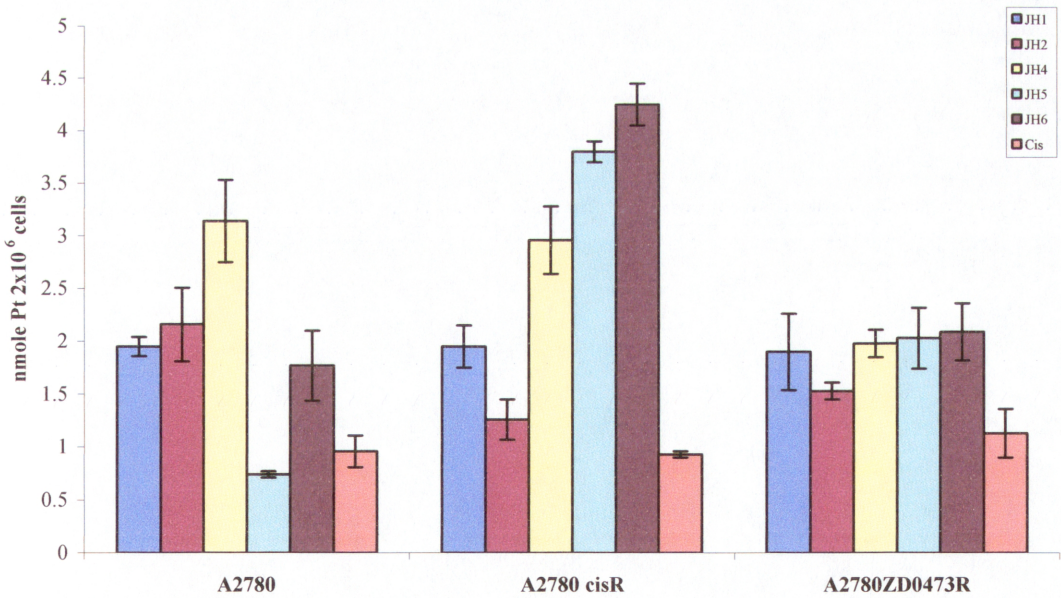


Figure 4-37 Pt accumulation in 24 h in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

4.5.1 Changes in cell uptake with time

Figures 4.38 to 4.40 show the changes in cell uptake for JH1, JH2, JH4, JH5, JH6, and cisplatin in 2, 4, and 24 h in A2780 A2780^{cisR} A2780^{ZD0473} ovarian cancer cell lines.

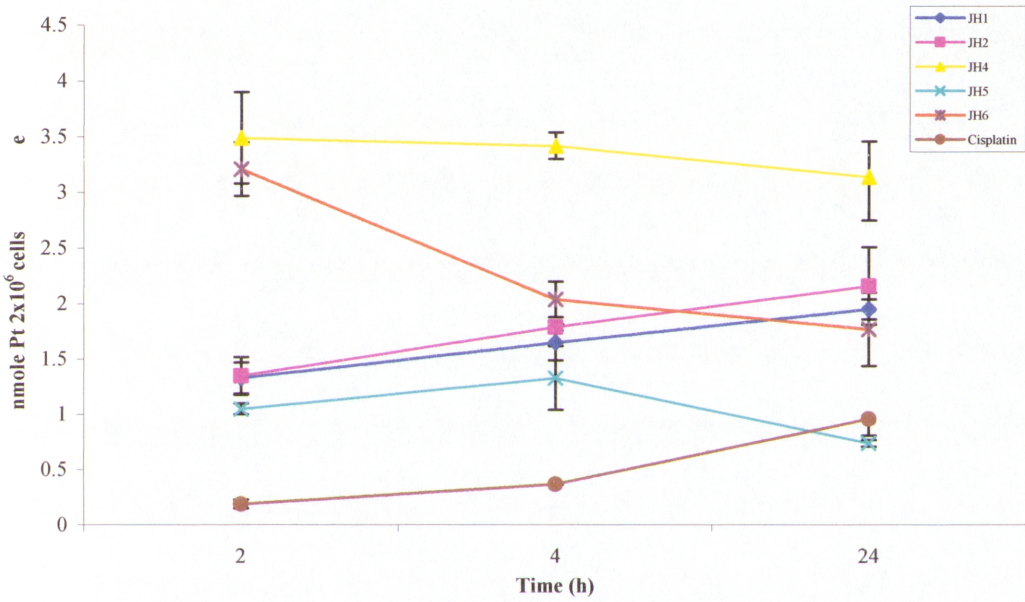


Figure 4-38: Pt accumulation in A2780 cells in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

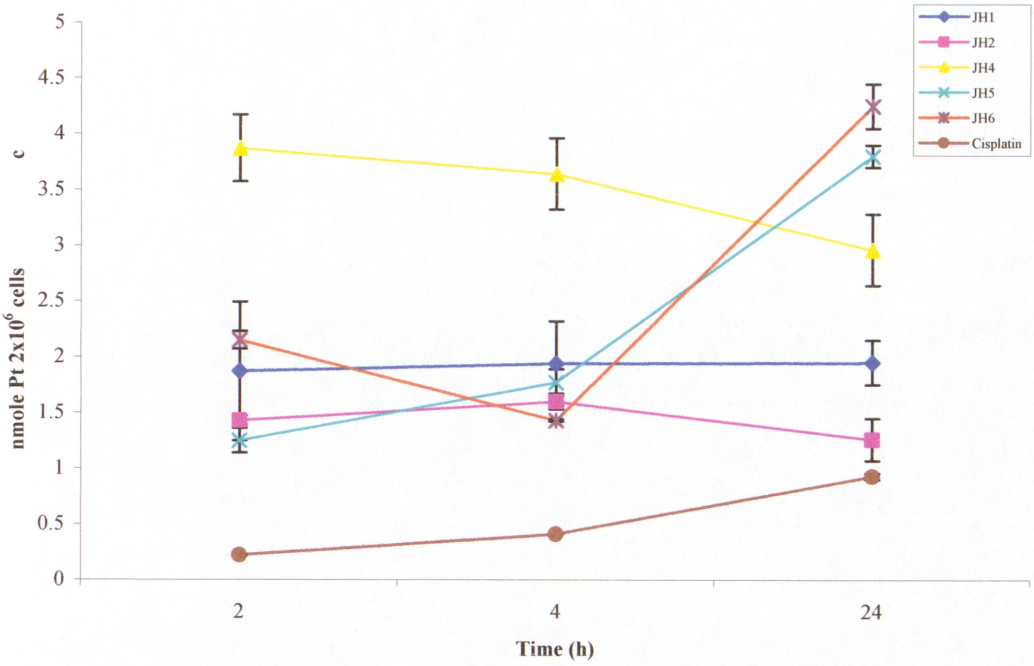


Figure 4-39: Pt accumulation in A2780^{cisR} cells in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

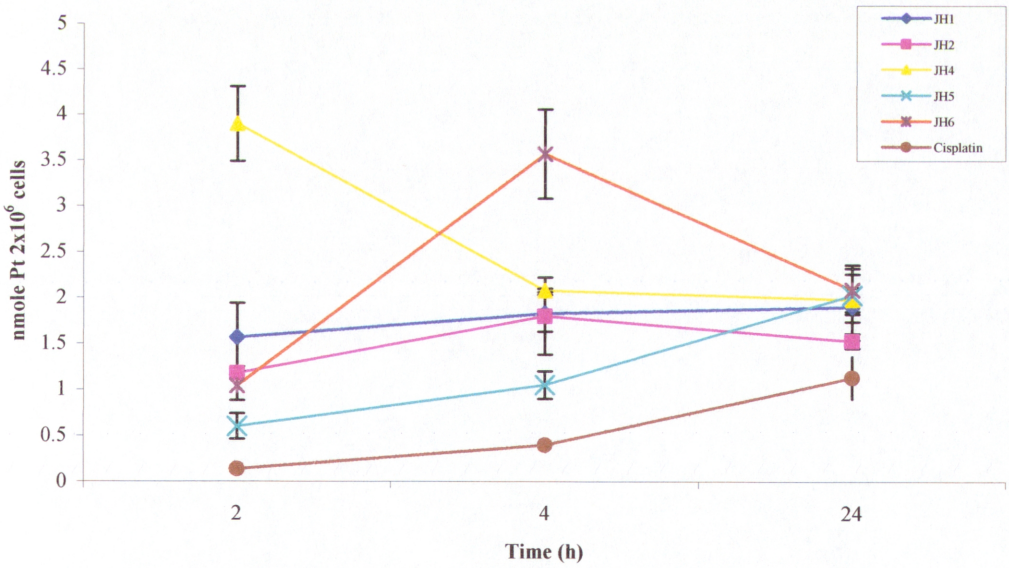


Figure 4-40: Pt accumulation in A2780^{ZD0473R} cells in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

4.6. DNA binding

Since the activity of platinum-based anticancer drugs is believed to be associated with their binding with cellular DNA, the levels of Pt-DNA binding for the synthesised platinum complexes have been determined. Tables 4.21 to 4.23 and Figures 4.41 to 4.43 give the levels of Pt-DNA binding (expressed as nanomole Pt per mg of DNA) for JH1, JH2, JH4, JH5, JH6 and cisplatin in ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{Z0473R}$ after exposure to 25 μM of the compounds for 2 h, 4 h and 24 h respectively.

For the mononuclear compounds JH1, JH2, JH4 and cisplatin, the levels of Pt-DNA binding in 2 h (Tables 4.21 to 4.23 and Figures 4.41 to 4.43) are as follows:

For the cell lines A2780 and $A2780^{ZD0473R}$, JH4 has highest levels of Pt-DNA binding while cisplatin the lowest levels. Among the *trans*-planaramineplatinum, JH2 has the lowest level of Pt-DNA binding. For the cell line $A2780^{cisR}$, JH1 has the highest level of Pt-DNA binding while JH2 has the lowest level.

In 4 h, the level of Pt-DNA binding is highest for JH1 as applied to the cell lines A2780 and $A2780^{cisR}$ whereas JH4 has the highest Pt-DNA binding level as applied to the cell line $A2780^{ZD0473R}$. In 4 h, cisplatin has the lowest Pt-DNA binding in the cell line A2780, whereas JH2 has the lowest levels as applied to the cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$.

In 24 h, the level of Pt-DNA binding is highest for cisplatin as applied to the cell line A2780 while JH1 has the highest level in both the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$. Among the mononuclear compounds, the lowest level of binding with DNA in 24 h is observed for JH2 in both $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines while

JH4 has the lowest level of drug-DNA binding in A2780 cell line.. Between the two trinuclear complexes JH5 and JH6, the level of Pt-DNA binding is higher for JH6 in all the three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ in both 2 and 4 h. In 24 h DNA binding is higher for JH5 in A2780 and $A2780^{ZD0473R}$ cell lines, while JH6 is higher for $A2780^{cisR}$. The results will be discussed more fully in chapter 5.

Table 4.21 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 2 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin.

Compound	A2780	A2780^{cisR}	A2780^{ZD0473}
	nmol Pt per mg DNA	nmol Pt per mg DNA	nmol Pt per mg DNA
JH1	3.02 ± 0.08	2.44 ± 0.13	1.27 ± 0.11
JH2	2.52 ± 0.26	1.21 ± 0.02	1.15 ± 0.01
JH4	3.07 ± 0.06	1.39 ± 0.01	2.32 ± 0.32
JH5	1.31 ± 0.21	1.20 ± 0.00	1.48 ± 0.00
JH6	1.72± 0.04	1.57 ± 0.05	2.18 ± 0.31
Cisplatin	1.23 ± 0.06	1.33 ± 0.04	1.44 ± 0.01

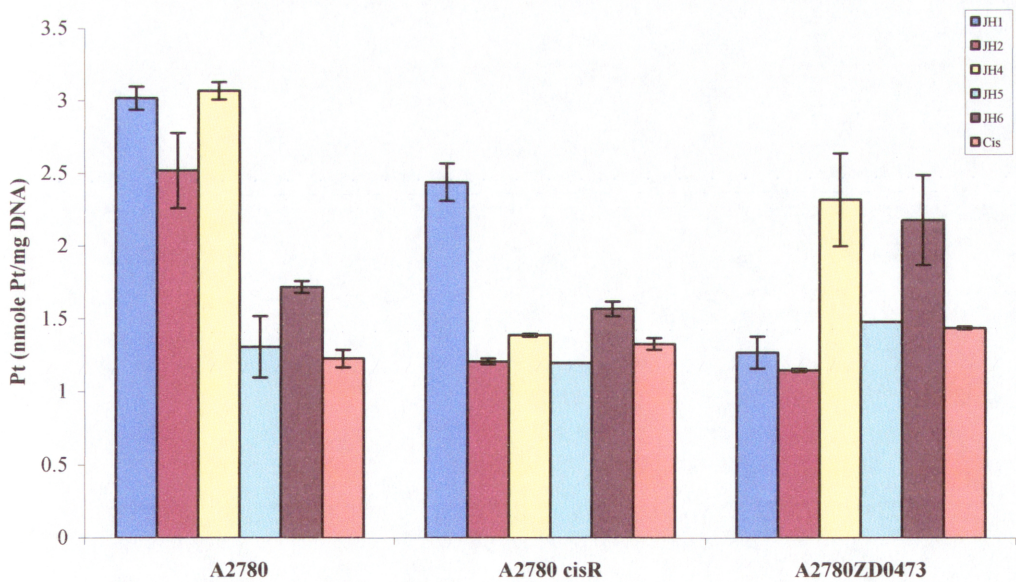


Figure 4-41 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 2 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Table 4.22 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 4 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Compound	A2780	A2780^{cisR}	A2780^{ZD0473}
	nmol Pt per mg DNA	nmol Pt per mg DNA	nmol Pt per mg DNA
JH1	3.74 ± 0.00	2.52 ± 0.02	1.27 ± 0.11
JH2	2.54 ± 0.30	1.14 ± 0.08	1.21 ± 0.10
JH4	1.48 ± 0.01	1.45 ± 0.04	2.05 ± 0.12
JH5	1.33 ± 0.01	1.14 ± 0.02	1.46 ± 0.07
JH6	1.55 ± 0.27	1.93 ± 0.20	2.29 ± 0.12
Cisplatin	1.43 ± 0.11	1.55 ± 0.06	1.50 ± 0.13

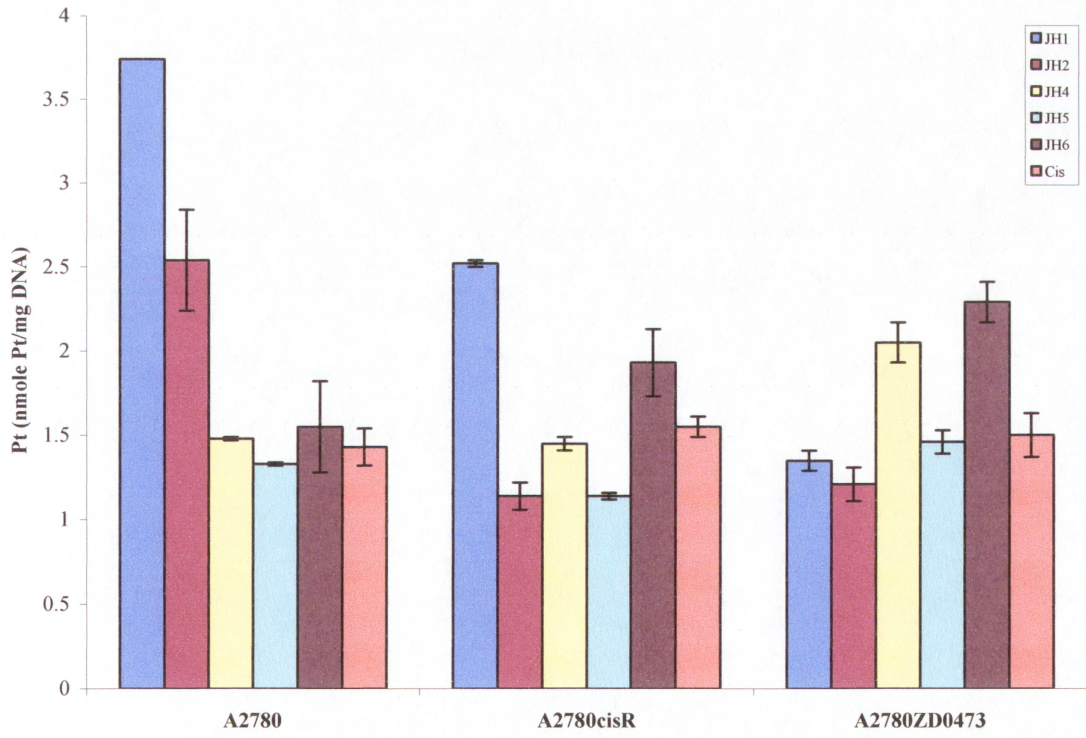


Figure 4-42 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 4 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Table 4.23 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

Compound	A2780	A2780^{cisR}	A2780^{ZD0473}
	nmol Pt per mg DNA	nmol Pt per mg DNA	nmol Pt per mg DNA
JH1	6.37 ± 0.01	4.88 ± 0.45	4.29 ± 0.22
JH2	1.71 ± 0.07	1.44 ± 0.10	1.17 ± 0.12
JH4	1.29 ± 0.06	1.50 ± 0.12	2.34 ± 0.01
JH5	5.30 ± 0.44	1.98 ± 0.14	4.21 ± 0.39
JH6	4.28 ± 0.64	2.43 ± 0.18	2.82 ± 0.26
Cisplatin	7.48 ± 0.68	2.75 ± 0.32	3.02 ± 0.08

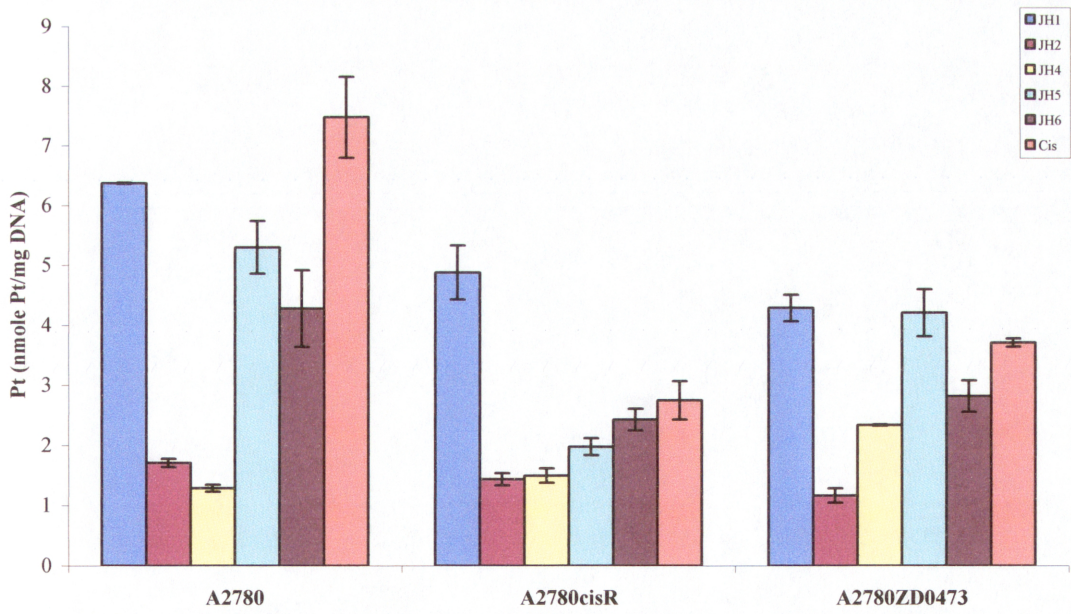


Figure 4-43 Levels of Pt binding with DNA in A2780, A2780^{cisR}, and A2780^{ZD0473R} cell lines in 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

4.6.1 DNA binding getting it together

Figures 4.44 to 4.46 give the levels of Pt binding with DNA in 2 h, 4 h and 24 h in A2780 A2780^{cisR} A2780^{ZD0473} cell line as applied to JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin. The figures illustrate changes in levels of Pt binding with DNA binding as a function of time.

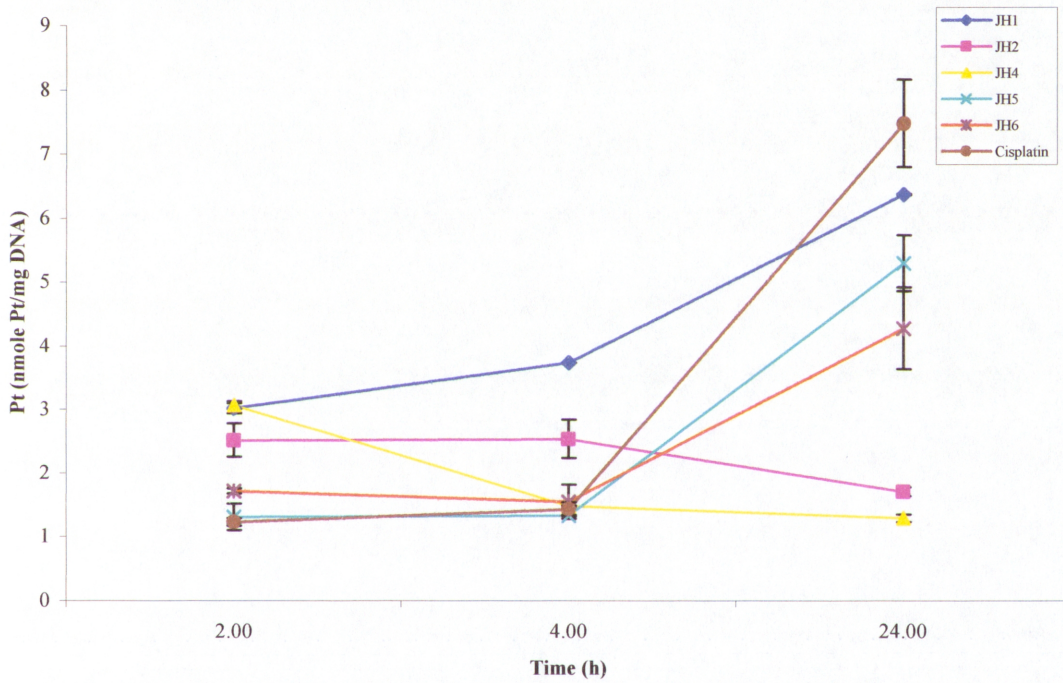


Figure 4-44: Levels of Pt binding with DNA in A2780 cell line in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

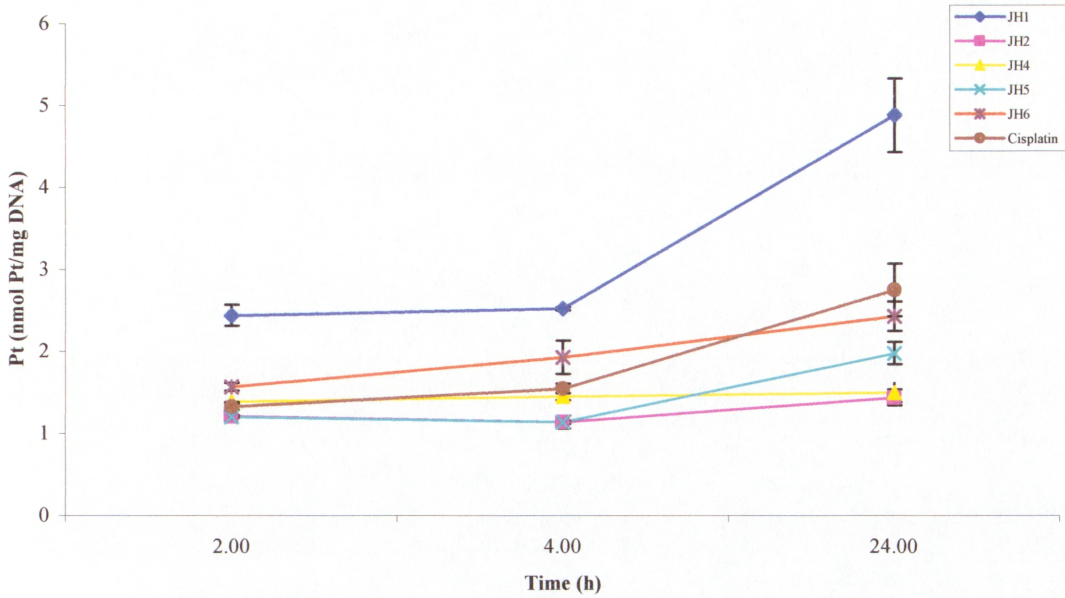


Figure 4-45: Levels of Pt binding with DNA in A2780^{cisR} cell line in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

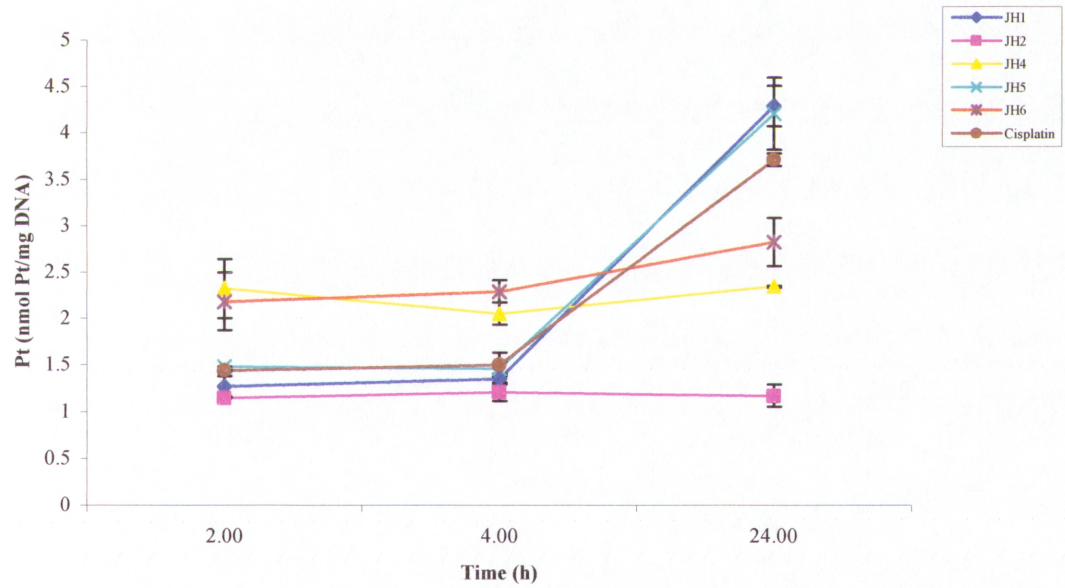


Figure 4-46: Levels of Pt binding with DNA in A2780^{ZD0473R} cell line in 2, 4, and 24 h as applied to JH1, JH2, JH4, JH5, JH6 and cisplatin

4.7. Gel electrophoresis

In this project, gel electrophoresis was used to obtain qualitative information on conformational changes in DNA and DNA damage, as applied to both genomic DNA (ssDNA) and non-genomic DNA (pBR322 plasmid DNA), due to their interaction with the compounds.

4.7.1 Interaction with salmon sperm DNA (ssDNA)

The covalent binding of platinum complexes with DNA can cause irreversible conformational changes including winding and bending of DNA. These conformational changes are believed to be reflected in changes to DNA mobility and visibility. Figure 4.47 gives the electrophoretograms applying to the incubated mixtures of ssDNA with JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin at a concentrations ranging from 5 to 200 μM for a period of 4 h at 37°C.

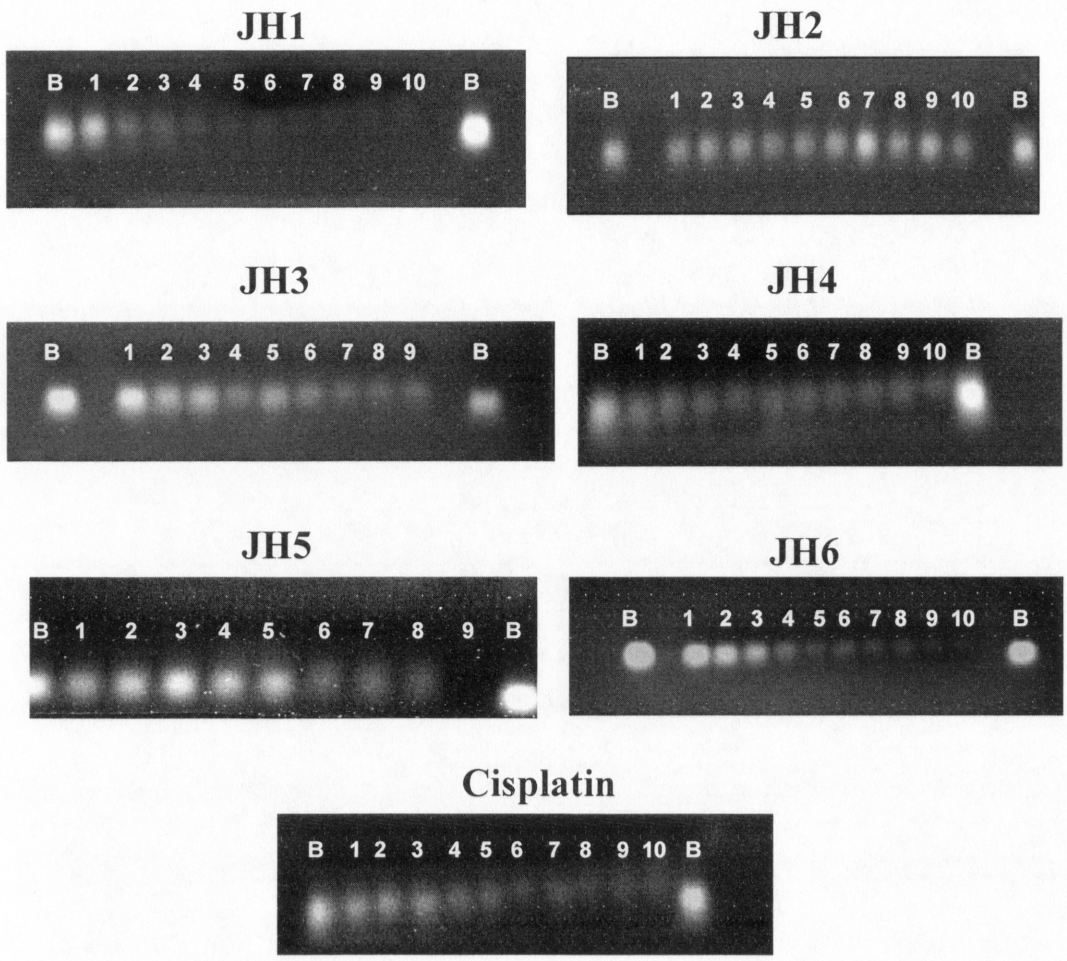


Figure 4-47 Electrophoretograms applying to the interaction of ssDNA with increasing concentrations of JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin

In the electrophoretograms, lane B applies to untreated ssDNA as control. Lanes 1 to 10 apply to ssDNA interacted with increasing concentrations of the synthesised platinum complexes JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin.

In all samples untreated and treated salmon sperm DNA, a single band was observed. In general, as the concentration of the platinum complexes was increased the intensity and generally the mobility of the band were found to decrease. The decrease in

intensity was most pronounced for JH1 and JH6 which were found to be most cytotoxic as well.

4.7.2 Interaction with pBR322 plasmid DNA

Figure 4.48 gives the electrophoretograms applying to incubated mixtures of pBR322 plasmid DNA and varying concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin for a period of 4 h at 37°C.

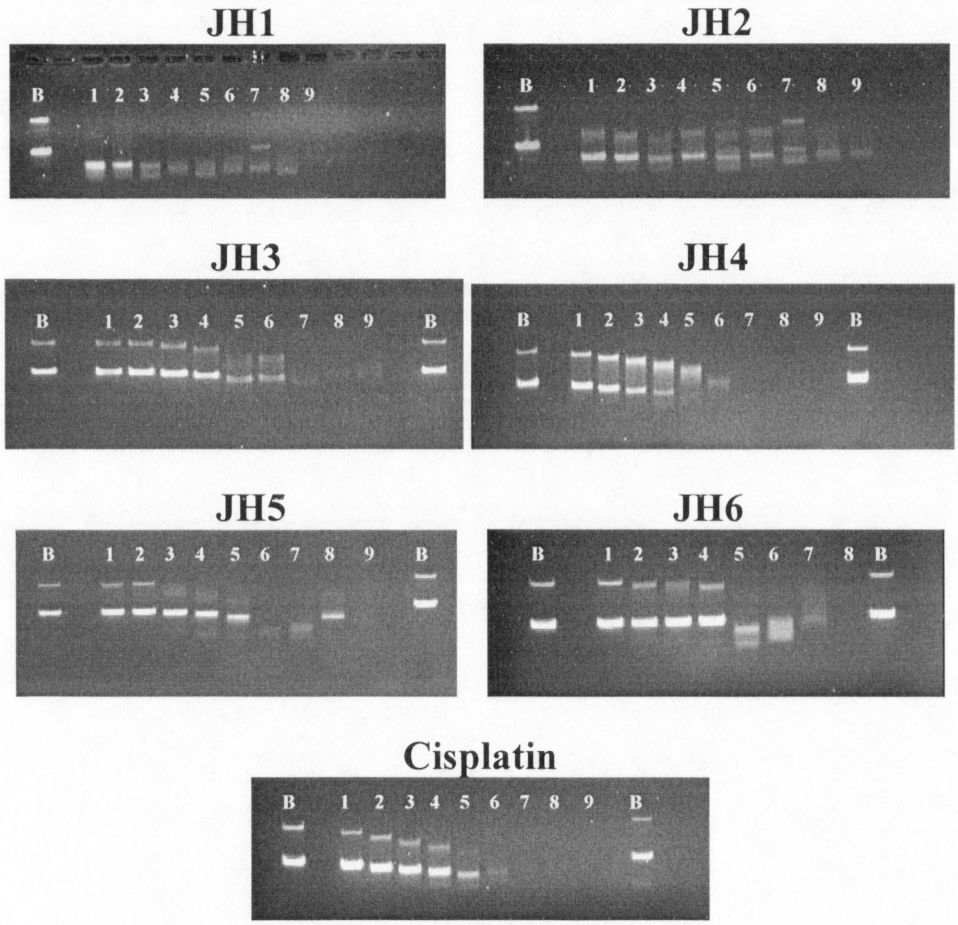


Figure 4-48 Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin

In the electrophoretograms, lane B applies to untreated pBR322 plasmid DNA to serve as a control. For JH1, JH2, JH3, JH4, JH5 and cisplatin, the concentrations are: lane 1: 0.488 μM, lane 2: 0.977 μM, lane 3: 1.953 μM, lane 4: 3.90 μM, lane 5: 7.81 μM, lane 6: 15.62 μM, lane 7: 31.25 μM, lane 8: 62.50 μM, and lane 9: 125 μM while for JH6 the values are: lane 1: 0.078 μM, lane 2: 0.156 μM, lane 3: 0.313 μM, lane 4: 0.625 μM, lane 5: 1.25 μM, lane 6: 2.50 μM, Lane 7: 5.00 μM, and lane 8: 10.00 μM.

In all cases untreated (lane B) pBR322 plasmid DNA gave two bands corresponding to supercoiled form I and singly-nicked form II.

From Figure 4.42, we can see that in general that as the concentrations of the compounds increases the intensity of the bands decreases and the mobility of the bands increases – more so for form I than form II so that the distance of separation between the two bands decreases. For JH1, two closely spaced bands are observed for concentrations ranging from 0.488 μM 1.953 μM. At the next higher concentration namely 3.90 μM, essentially one coalesced band is observed. At the next four higher concentrations (7.81 μM, 15.62 μM, 31.25 μM and 62.5 μM), separation between the bands is found to increase although intensity gets progressively diminished. At the highest concentration namely 125 μM no DNA band is observed. In the case of JH2, two bands corresponding to forms I and II are observed essentially at all concentrations. At the highest concentration namely 125 μM only one clear but faint band can be seen. In lanes 5 and 7 corresponding to concentrations 7.81 μM and 31.25 μM respectively three bands can be seen.

For the interaction of pBR322 plasmid DNA with increasing concentrations of JH3 two bands corresponding to forms I and II are observed for all concentrations up to

15.62 μM (lane 6) above which (lanes 7-9) a single faint DNA band can be seen. There is a gradual increase in mobility of the DNA bands with the increase in concentration of JH3, especially at lower concentrations.

In the interaction of pBR322 plasmid DNA with increasing concentrations of JH4, two bands corresponding to forms I and II are observed for concentrations ranging from 0.488 μM to 7.81 μM. At the next higher concentration namely 15.62 μM one coalesced band is observed and at still higher concentrations no DNA band can be seen.

In the interaction of pBR322 plasmid DNA with increasing concentrations of JH5, two bands corresponding to forms I and II can be seen for concentrations of the compound ranging from 0.488 μM to 7.81 μM. At the next two higher concentrations namely 15.62 μM and 31.25 μM essentially one coalesced band is observed. At the next higher concentration (62.50 μM) also one band is observed. However, it is found to be more intense than the band observed at 15.62 μM and 31.25 μM. No band is observed at the highest concentration (125 μM).

In the interaction of pBR322 plasmid DNA with increasing concentrations of JH6, two distinct bands corresponding to forms I and II can be seen for all concentrations of the compound ranging from 0.078 μM to 1.25 μM (lanes 1-5). The separation between the bands is found to decrease markedly in lane 6 (2.5 μM) and at the next higher concentration 5.0 μM essentially one coalesced band is observed. No band is observed at the highest concentration namely 10.0 μM. DNA bands in lane 5 are found to have the highest mobility.

In the interaction of pBR322 plasmid DNA with increasing concentrations of cisplatin, two bands corresponding to forms I and II are observed for all concentrations of the compound ranging from 0.488 μM to 7.81 μM (lanes 1-5) and at the next higher concentration namely 15.62 μM (lane 6) essentially one coalesced band is observed. At higher concentrations no clear DNA band can be seen. The mobility of the DNA bands is found to increase with the increase in concentration of cisplatin.

4.7.3 Interaction with pBR322 plasmid DNA combined with BamH1

Figure 4.49 gives the electrophoretograms applying to incubated mixtures of pBR322 plasmid DNA and varying concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin for a period of 4 h at 37°C followed by BamH1 digestion for a further period of 1 h at the same temperature.

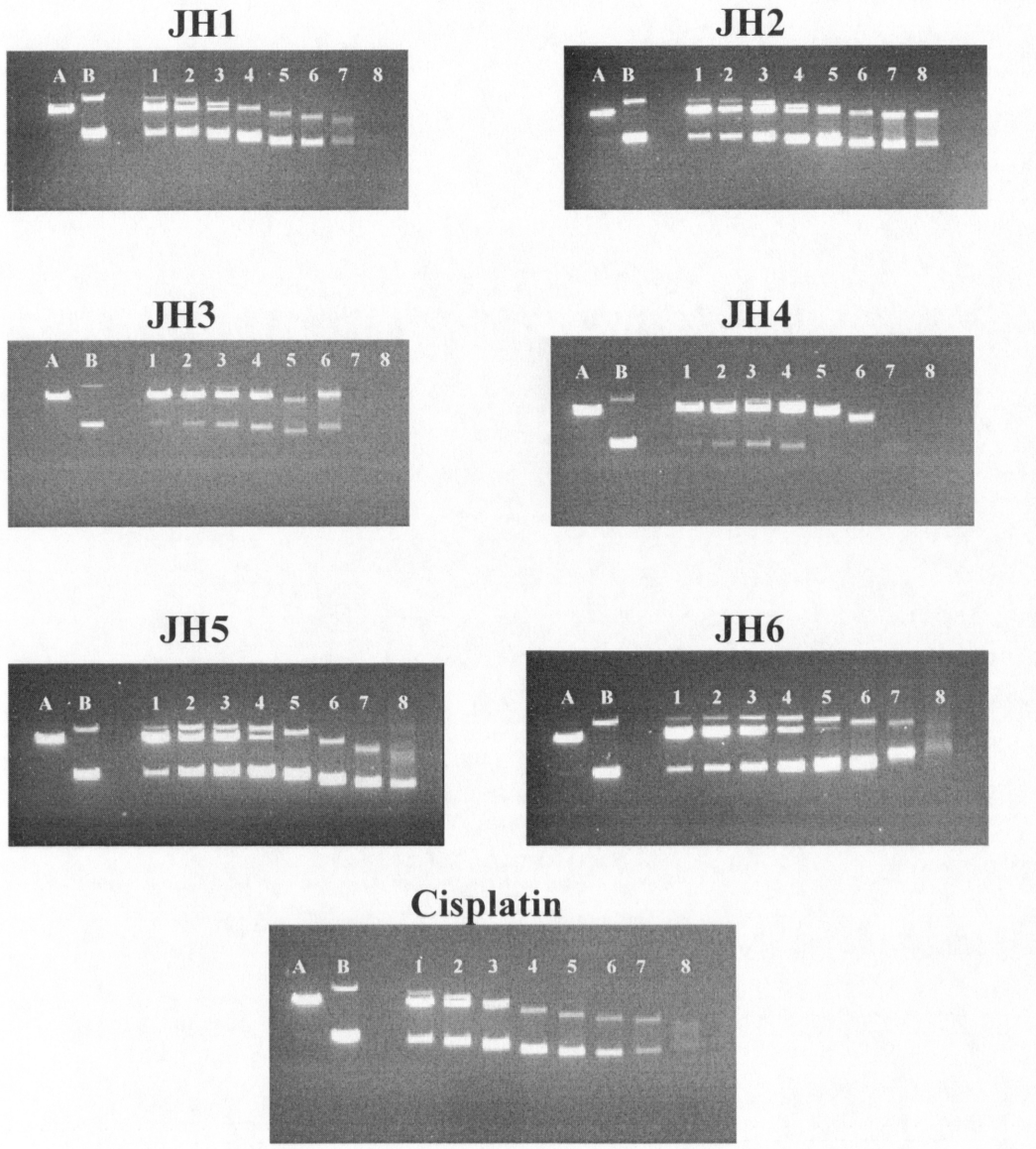


Figure 4-49 Electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of compounds: JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin followed by their digestion with BamH1

Lane A applies to untreated but digested (with BamH1) pBR322 plasmid DNA, lane B applies to the untreated and undigested pBR322 plasmid DNA. Lanes 1 to 8: apply to pBR322 plasmid DNA interacted with increasing concentrations of the compounds followed by BamH1 digestion. The concentrations of JH1, JH2, JH3, JH4, JH5, and

cisplatin used are as follows: lane 1: 0.469 μM, lane 2: 0.938 μM, lane 3: 1.87 μM, lane 4: 3.75 μM, lane 5: 7.5 μM, lane 6: 15.00 μM, lane 7: 30.00 μM and lane 8: 60.00 μM. Concentrations of JH6 are: lane 1: 0.078 μM, lane 2: 0.156 μM, lane 3: 0.313 μM, lane 4: 0.625 μM, lane 5: 1.25 μM, lane 6: 2.50 μM, Lane 7: 5.00 μM, and Lane 8: 10.00 μM.

When untreated pBR322 plasmid band is digested with BamH1, only one band corresponding to form III is observed whereas in the untreated and undigested pBR322 plasmid DNA, generally two bands corresponding to forms I and II can be seen.

When the DNA is interacted with increasing concentrations of JH1 followed by BamH1 digestion, three bands corresponding to forms I, II and III are observed in lanes 1, 2 and 3, for concentrations ranging from 0.469 μM to 1.875 μM. Two bands corresponding to forms I and II are observed in lanes 4, 5, 6, 7 at concentrations ranging from 3.75 μM to 30 μM while in lane 8 corresponding to 60 μM concentration a very faint band can be seen.

In the case of JH2, three bands corresponding to forms I, II and III are observed in lanes 1, 2, 3, and 4 for concentrations ranging from 0.469 μM to 3.75 μM and two bands corresponding to forms I and II are observed in lanes 5, 6, 7 and 8 at concentration ranging from 7.50 μM to 60 μM.

In the case of JH3, three bands corresponding to forms I, II and III (with forms II and III being close together and more intense than the form I band) are observed in lanes 1, 2, 3 and 4 for concentrations ranging from 0.469 μM to 3.75 μM. Two bands corresponding to forms I and II are observed in lanes 5 and 6 at concentrations

ranging from 7.5 μM to 15 μM. At still higher concentrations no distinct band can be seen.

In the case of JH4, three bands corresponding to forms I, II and III are observed in lanes 1, 2, 3 and 4 for concentrations ranging from 0.469 μM to 3.75 μM. Two bands corresponding to forms I and II are observed in lane 5 at 7.50 μM concentration. At the next higher concentration (lane 6, 15.00 μM) a single DNA band is observed. At still higher concentrations no clear DNA band can be seen.

In the case of JH5, three bands corresponding to forms I, II and III are observed in lanes 1, 2, 3, and 4 for concentrations ranging from 0.469 μM to 3.75 μM. Two bands corresponding to forms I and II are observed in lanes 5, 6, 7, and 8 at concentrations ranging from 7.50 μM to 60.00 μM. The bands observed at the highest concentration (in lane 8) sow some streakiness.

In the case of JH6, three bands corresponding to forms I, II and III are observed in lanes 1, 2, 3, 4 and 5 for concentrations ranging from 0.078 μM to 1.25 μM while two bands corresponding to forms I and II are observed in lanes 6, 7 and 8 at concentrations ranging from 2.50 μM to 10.00 μM. The bands observed at the highest concentration (lane 8) appear to be more streaky.

In the case of cisplatin, three bands corresponding to forms I, II and III are observed in lanes 1, 2 and 3 for concentrations ranging from 0.469 μM to 1.875 μM. Two bands corresponding to forms I and II are observed in lanes 4, 5, 6, and 7 at concentrations ranging from 3.75 μM to 30 μM. In lane 8 corresponding to 60 μM concentration one faint band can be seen.

Table 4.24 gives a summary of the bands observed in the interaction of pBR322 plasmid DNA with varying concentrations of the compounds followed by BamH1 digestion.

Table 4.24 Bands observed after BamH1 digestion of incubated mixtures of JH1, JH2, JH3, JH4, JH5, JH6, and cisplatin with pBR322 plasmid DNA

Compound	Concentration indicator for the bands #							
	1	2	3	4	5	6	7	8
Cisplatin	I,II,III	I,II,III	I,II,III	I,II	I,II	I,II	I,II	I
JH1	I,II,III	I,II,III	I,II,III	I,II	I,II	I,II	I,II	
JH2	I,II,III	I,II,III	I,II,III	I,II,III	I,II	I,II	I,II	I,II
JH3	I,II,III	I,II,III	I,II,III	I,II,III	I,II	I,II		
JH4	I,II,III	I,II,III	I,II,III	I,II,III	I,II	I		
JH5	I,II,III	I,II,III	I,II,III	I,II,III	I,II	I,II	I,II	I,II
JH6	I,II,III	I,II,III	I,II,III	I,II,III	I,II,III	I,II	I,II	I,II

5. Chapter Five

5.1. Discussion

5.1.1 Characterization of compounds

In this study, four mononuclear *trans*-planaramineplatinum complexes and two trinuclear platinum complexes with *trans*-geometry for the metal centres have been prepared and characterized using elemental analyses and spectral measurements. The activity of the compounds against three ovarian cancer cell lines, cell uptake, extent and nature of binding with DNA have also been determined.

The starting material for the mononuclear compound JH1 was cisplatin whereas that for JH2, JH3, and JH4 was potassium tetrachloroplatinate. The compounds were prepared according to modified Kauffman method (Kauffman and Cowan, 1963). JH1 has one planaramine ligand while the rest of the mononuclear compounds have two planaramine ligands per molecule. The trinuclear compounds JH5 and JH6 were synthesized using a step up method of syntheses branching out from the central unit. JH1 and JH2 have served as the central unit for JH6 and JH5 respectively. The linking diamine in both JH5 and JH6 is 1,6-diaminohexane.

5.2. Elemental analysis

The elemental compositions of JH1, JH2, JH3, JH4, JH5, and JH6 are given in Tables 4.1 and 4.2. The achieved purity of JH1, JH2 and JH3 is over 99% whereas that for

JH4 is close to 98%, for JH5 it is more than 93%, and for JH6 the purity is greater than 97%.

Since JH1, JH2 and JH3 have been obtained in a state of high purity; no further purification of the compounds has been attempted. It is difficult to obtain trinuclear complexes in a state of high purity (because of the formation of some dinuclear complexes at the same time (Farrell et al., 1999) and the possibility of breakdown in the purification process (Farrell, 2000; Daghriri, 2003; Cheng, 2005). Another possible reason behind the low purity of the trinuclear as suggested by Huq *et al*, is the co-precipitation of other molecules such as DMF or dichloromethane (Huq et al., 2008). Considering the above confounding factors, the purity of JH6 is considered to be acceptable. For JH4 and JH5, repeated dissolution in DMF followed by precipitation using dichloromethane has been carried out in order to increase the purity rate of the compounds.

The percentage yield for the synthesised complexes is 43% for JH1, 56% for JH2, 59% for JH3, 67% for JH4, 62% for JH5 and 68% for JH6.

5.3. Spectral analyses

The structures of the synthesised compounds could not be confirmed by single crystal x-ray diffractometry as no suitable crystals were available. Therefore spectral studies including IR, mass spectrum and ^{1}H NMR have been used to aid in the structural characterization of the compounds. None of the methods used on its own can completely characterize the compounds; however, combined results are believed to provide a greater support for the suggested structures.

5.3.1 IR

The IR results for JH1, JH2, JH3, JH4, JH5, and JH6 are given in Figures 4.7 to 4.12 in chapter four. Most of the peak assignments, Table 4.3 and 4.4, are based on published spectra of the ligands and metal complexes of pyridine and substituted pyridines (Silverstein et al., 1991a; Kelland et al., 1995; Nakamoto, 1997). The peaks listed in Table 4.3 and 4.4, and the descriptions given provide support to the suggested structures. In particular, it indicates the presence of groups such as CH, CO, NH and 'aromatic ring', and Pt-N bonds.

IR of JH1

The bands at 3290.5 and 3113.1 cm^{-1} are believed to be due to N-H stretching vibration; the bands at 2889.1 and 2819.9 cm^{-1} are believed to be due to C-H stretching vibration; the band at 1635.8 cm^{-1} is believed to be due to N-H bending vibration; the band at 1562.9 cm^{-1} is believed to be due to thiazole ring stretching vibration); the band at 1509.8 cm^{-1} is believed to be due to C-N stretching vibration; the bands at 1456.3 and 1392.8 cm^{-1} are believed to be due to C-H bending vibration; the band at 1308.6 cm^{-1} is believed to be due to ring in-plane deformation); the band at 1112.4 cm^{-1} is believed to be due to C-S stretching vibrations; the band at 1068.8 cm^{-1} is believed to be due to C-C bending vibration); the bands at 882.9 and 815.3 cm^{-1} are believed to be due to C-H out of plane bending; the band at 743.8 cm^{-1} is believed to be due to ring out-of-plane deformation; the band at 625.4 cm^{-1} is believed to be due to C=N bending; the bands at 536.3 and 470.6 cm^{-1} are believed to be due to Pt-N stretching vibration.

IR of JH2

The bands at 3313.2 and 3135.4 cm^{-1} are believed to be due to N-H stretching vibration; the bands at 2964.7 and 2854.8 cm^{-1} are believed to be due to C-H stretching vibration; the bands at 1689.2 and 1614.5 cm^{-1} are believed to be due to N-H bending vibration; the band at 1544.3 cm^{-1} is believed to be due to thiazole ring stretching vibration; the band at 1509.1 cm^{-1} is believed to be due to C-N stretching vibration. The bands at 1425.7 and 1391.9 cm^{-1} are believed to be due to C-H bending vibration; the band at 1316.9 cm^{-1} is believed to be due to ring in-plane deformation; the bands at 1177.6 and 1130.1 cm^{-1} are believed to be due to C-S stretching vibration; the band at 1101.8 cm^{-1} is believed to be due to C-N bending vibration; the band at 1068.1 cm^{-1} is believed to be due to C-C bending vibration; the bands at 885.5 and 816.7 cm^{-1} are believed to be due to C-H out of plane bending; the band at 751.6 cm^{-1} is believed to be due to ring out-of-plane deformation; the band at 701.9 cm^{-1} is believed to be due to Pt-N thiazole; the band at 611.3 cm^{-1} is believed to be due to C=N bending; the bands at 531.3 and 470.5 cm^{-1} are believed to be due to Pt-N stretching vibration).

IR of JH3

The bands at 3320.8 and 3134.9 cm^{-1} are believed to be due to N-H stretching vibration; the bands at 2965.1 and 2857.3 cm^{-1} are believed to be due to C-H stretching vibration; the band at 1689.3 cm^{-1} is believed to be due to N-H bending vibration; the band at 1614.9 cm^{-1} is believed to be due to C-C stretching vibration; the band at 1543.7 cm^{-1} is believed to be due to thiazole ring stretching vibration; the

band at 1508.9 cm^{-1} is believed to be due to C-N stretching vibration; the bands at 1426.4 and 1391.8 cm^{-1} are believed to be due to C-H bending vibration; the band at 1317 cm^{-1} is believed to be due to ring in-plane deformation; the band at 1177.5 cm^{-1} is believed to be due to C-S stretching vibration; the band at 1130 cm^{-1} is believed to be due to C-N bending vibration; the bands at 1101.6 and 1068.3 cm^{-1} are believed to be due to C-C bending vibration; the band at 885.8 cm^{-1} is believed to be due to C-H out of plane bending; the band at 750.7 cm^{-1} is believed to be due to ring out-of-plane deformation; the band at 709.8 cm^{-1} is believed to be due to C-H out-of-plane bending; the band at 470.5 cm^{-1} is believed to be due to Pt-N.

IR of JH4

The band at 3290.2 cm^{-1} is believed to be due to O-H stretching vibration; the band at 3112.4 cm^{-1} is believed to be due to N-H stretching vibration; the band at 2812.2 cm^{-1} is believed to be due to C-H stretching vibration; the band at 1596.5 cm^{-1} is believed to be due to C=C stretching vibration; the band at 1492.1 cm^{-1} is believed to be due to N-H bending vibration; those at 1448.4 and 1391.1 cm^{-1} are believed to be due to C-H bending vibration. The band at 1347.9 cm^{-1} is believed to be due to ring stretch; the band at 1282.9 cm^{-1} is believed to be due to O-H and C-H bending vibrations; the band at 1218 cm^{-1} is believed to be due to C-O stretching vibration; the band at 1105.4 cm^{-1} is believed to be due to C-S stretching vibration; the band at 1061.7 cm^{-1} is believed to be due to C-N bending vibration; the band at 1030.2 cm^{-1} is believed to be due to C-C bending vibration. The bands at 917.7 and 880.3 cm^{-1} are believed to be due to N-H wagging; the band at 808.5 cm^{-1} is believed to be due to C-H out of plane bending; the bands at 628.4 and 597.6 cm^{-1} are believed to be due to C=N bending; the band at 451.6 cm^{-1} is believed to be due to Pt-N stretching vibration.

IR of JH5

The bands at 3299.1 and 3213.6 cm^{-1} are believed to be due to N-H stretching vibration; the bands at 2937.9 and 2560.1 cm^{-1} are believed to be due to C-H stretching vibration; the band at 1638 cm^{-1} is believed to be due to N-H bending vibration; the band at 1535.1 cm^{-1} is believed to be due to ring stretching vibration; the band at 1391.6 cm^{-1} is believed to be due to C-H bending vibration; the band at 1291.1 cm^{-1} is believed to be due to ring in-plane deformation; the bands at 1096.7 and 1039.6 cm^{-1} are believed to be due to C-C bending vibration; the band at 995.9 cm^{-1} is believed to be due to C-C bending vibration; the band at 808 cm^{-1} is believed to be due to C-H out of plane bending vibration; the band at 504.4 cm^{-1} is believed to be due to Pt-N stretching vibration.

IR of JH6

The bands at 3137.9 and 3087.2 cm^{-1} are believed to be due to N-H stretching vibration; the bands at 2928.2 and 2562.4 cm^{-1} are believed to be due to C-H stretching vibration; the bands at 1661.4 and 1595.6 cm^{-1} are believed to be due to N-H bending vibration; the bands at 1512.5 and 1470 cm^{-1} are believed to be due to ring stretching vibration; the band at 1395.2 cm^{-1} is believed to be due to C-H bending vibration); the band at 1295.3 cm^{-1} is believed to be due to ring in-plane deformation; the bands at 1041.8 and 995.2 cm^{-1} are believed to be due to C-C bending vibration;

the band at 824.8 (s, C-H out of plane bending vibration); the band at 729.2 cm^{-1} is believed to be due to ring out-of-plane deformation; the band at 526.1 cm^{-1} is believed to be due to Pt-N stretching vibration).

5.3.2 Mass spectrum

The mass spectra results are shown in Figures 4.13 to 4.18 and the major peaks observed are listed in Table 4.5 and 4.6. Mass spectra of the compounds are characterized by the presence of numerous peaks. Some of the peaks correspond to molecules or molecular fragments while other peaks correspond to complexes that are believed to be formed in situ or could be due to impurities.

Mass spectrum of JH1

ESI-MS (DMF)(m/z: M = 368). The peak at m/z = 350 corresponds to $Pt(C_3H_3NS)(NH_3)_2Cl$. The peak at m/z = 365 corresponds to (M – 3H). The peak at m/z = 418 corresponds to $Pt(NH_3)(C_3H_3NS)_2Cl$.

Mass spectrum of JH2

ESI-MS (DMF)(m/z: M = 436). The peak at m/z = 433 corresponds to (M - 3H). The peak at m/z = 486 corresponds to $PtCl(C_3H_3NS)_3$. The peak at m/z = 894 corresponds to $[PtCl_2(C_3H_3NS)_5 +7H]$.

Mass spectrum of JH3

ESI-MS (DMF)(m/z: M = 419). The large peak at m/z = 433 corresponds to $Pt(C_3H_3NS)_2(C_3H_4N_2)$. The peak at m/z = 452 corresponds to $Pt(C_3H_3NS)(C_3H_4N_2)_2Cl$. The peak at m/z = 469 corresponds to $Pt(C_3H_3NS)_2(C_3H_4N_2)Cl$.

Mass spectrum of JH4

ESI-MS (DMF)(m/z: M = 446). The peak at m/z = 445.3 corresponds to (M – H). The peak at m/z = 455 corresponds to $(Pt(C_5H_5NO)_2Cl_2)$. The peak at m/z =902 corresponds to $Pt_2(C_3H_3NS)(C_5H_5NO)_3Cl_4$.

Mass spectrum of JH5

ESI-MS (DMF)(m/z: M = 1294.6). The peak at m/z = 381 corresponds to $PtCl(NH_3)_2(NH_2)_2(CH_2)_6$. The peak at m/z = 480 corresponds to $[PtCl(C_3H_3NS)(NH_2)_3(CH_2)_8 + 4H]$. The peak at m/z = 579 corresponds to $[PtCl(C_3H_3NS)_2(NH_2)_3(CH_2)_9 + 4H]$. The peak at m/z = 607 corresponds to $Pt(C_3H_3NS)_2(NH_2)_3(CH_2)_{11}ClH_4$. The peak at m/z = 795 corresponds to $[Pt_2Cl_5(NH_2)_4(CH_2)_{12} -4H]$.

Mass spectrum of JH6

ESI-MS (DMF)(m/z: M = 1058). The peak at m/z = 381 corresponds to $PtCl(NH_3)_2NH_2(CH_2)_6NH_2$. The peak at m/z = 762 corresponds to $Pt(NH_2)(CH_2)_6(NH_2)Pt(NH_3)_2(NH_2)(CH_2)_6$ $(NH_2)Cl_3$. The peak at m/z = 960 corresponds to $[PtCl\{(NH_2(CH_2)_6NH_2)\}Pt(NH_3)Cl\{NH_2(CH_2)_6NH_2)\}Pt(NH_3)Cl$ + 3H].

5.3.3 Nuclear magnetic resonance spectroscopy (NMR)

^1H NMR spectra of the synthesised compounds would also provide support to the suggested structures by the presence of proton resonances that indicate the presence of various functional groups. The ^1H NMR results are shown in Figures 4.19 to 4.24 and the major peaks observed are listed in Table 4.7 and 4.8.

^1H NMR spectrum of JH1

The resonance at δ = 9.6 ppm is believed to be due to C_5H proton. The resonance at δ =8.28 ppm is believed to be due to C_2H proton, that at δ = 8.02 ppm is believed to be due C_3H proton, that at δ = 4.21 ppm is believed to be due NH-Pt proton.

The resonance at δ = 3.47 ppm is believed to be due to water dissolved in DMF that at δ = 2.74 ppm is due to DMSO. The resonance at δ = 2.91 ppm is believed to be due to water.

^{1}H NMR spectrum of JH2

The resonance at δ = 9.68 ppm is believed to be due to C_5H. The resonance at δ = 9.58 ppm is believed to be due to impurity; that at δ = 8.30 ppm is believed to be due to C_2H; that at δ = 8.05 ppm is believed to be due to C_3H. The resonance at δ = 3.49 ppm is believed to be due to NH-Pt; that at δ = 2.95 ppm is believed to be due to H_2O and that at δ = 2.74 ppm is believed to be due to DMSO. The resonance at δ = 2.91 ppm is believed to be due to water.

^{1}H NMR spectrum of JH3

The resonance at δ = 9.67 ppm is believed to be due to C_5H – T. The resonance at δ = 8.32 ppm is believed to be due to C_2H – T; that at δ = 8.02 ppm is believed to be due to C_3H – T; that at δ = 8.00 ppm is believed to be due to CH – I. The resonance at δ = 7.41 ppm is believed to be due to CH – I; that at δ = 7.36 ppm is believed to be due to CH; that at δ = 7.32 ppm is believed to be due to CH – I. The resonance at δ = 3.47 ppm is believed to be due to water dissolved in DMF and that at δ = 2.74 ppm is believed to be due to DMSO. The resonance at δ = 2.91 ppm is believed to be due to water.

^{1}H NMR spectrum of JH4

The resonance at δ = 9.68 6 ppm is believed to be due to C_5H – T. The resonance at δ = 8.50 is believed to be due to CH ortho HP?; that at δ = 8.37 is believed to be due to CH ortho – HP; that at δ = 8.30 is believed to be due to C_2H -T. The resonance at δ = 8.03 ppm is believed to be due C_3H –T; that at δ = 7.47 ppm is believed to be due CH para – HP; that at δ = 7.37 ppm is believed to be due CH meta – HP; that at δ = 3.47 ppm is believed to be due NH Pt; that at δ = 2.91 ppm is believed to be due H_2O. Finally, the resonance at δ = 2.74 ppm is believed to be due DMSO.

^{1}H NMR spectrum of JH5

The resonance at δ = 8.28 ppm is believed to be due to C_2H-T. The resonance at δ = 8.02 ppm is believed to be due to C_3H – T. The resonance at δ = 7.12 (b) ppm is believed to be due to C_2H - T, that at δ = 6.7 (b) ppm is believed to be due to impurity?; that at δ = 3.74 ppm is believed to be due to NH_3-Pt; that at δ = 3.51 (b) ppm is believed to be due to H_2O dissolved in DMF. The resonance at δ = 2.91 ppm is believed to be due to NH_2-Pt; that at δ = 2.74 ppm is believed to be due to DMSO; that at δ = 2.2 (b) ppm is believed to be due to CH_2; that at δ = 1.97 (b) ppm is believed to be due to CH_2. The resonance at δ = 1.62 (b) ppm is believed to be due to CH_2; that at δ = 1.5 (b) ppm is believed to be due to CH_2; that at δ = 1.28 ppm is believed to be due to CH_2. Finally, the resonance at δ = 0.88 ppm is believed to be due to CH_2.

^{1}H NMR spectrum of JH6

The resonance at δ = 8.28 ppm is believed to be due to C_2H-T. The resonance at δ = 8.02 ppm is believed to be due to C_3H – T. The resonance at δ = 7.12 (b) ppm is believed to be due to C_2H – T; that at δ = 6.69 (b) ppm is believed to be due to impurity; that at δ = 4.32 (b) ppm is believed to be due to NH_3-Pt; and that at δ = 3.48 ppm is believed to be due to H_2O dissolved in DMF. The resonance at δ = 3.07 ppm is believed to be due to NH_2; that at δ =2.74 ppm is believed to be due to DMSO; that at δ =2.2 (b) ppm is believed to be due to CH_2; and that at δ =1.96 (b) ppm is believed to be due to CH_2. The resonance at δ = 1.75 (b) ppm is believed to be due to CH_2; that at δ = 1.62 (b) ppm is believed to be due to CH_2; and that at δ = 1.45 ppm is believed to be due to CH_2. Finally, the resonance at δ = 0.88 ppm is believed to be due to CH_2).

5.4. Molar conductivity

The main idea behind determining molar conductivity values is to provide information on the extent of dissociation of the compounds. This in turn would provide information on the likely mode(s) of transport of the compounds across the cell membrane. While the uncharged non-polar molecules can cross the cell membrane by passive diffusion and facilitated or active transport (Fuertes et al., 2003; Wang and Lippard, 2005), the polar molecules and charged species are more expected to cross the cell membrane through facilitated diffusion and active transport. Thus the neutral mononuclear complexes such as JH1, JH2, JH3, and JH4 are expected to cross

the cell membrane by both passive diffusion, and carrier mediated transports such as facilitated diffusion and active transport. On the other hand poly-positively charged cations produced from trinuclear compounds JH5 and JH6 are expected to cross the cell membrane by carrier mediated transport only. The molar conductivity values may also provide information on binding of the compounds with DNA. Positively charged species are more strongly attracted to the negatively charged DNA than the neutral molecules.

The limiting molar conductivity values in ohm^{-1} cm^2 mol^{-1} at zero concentration of JH1, JH2, JH3, JH4, JH5, and JH6 at 298 K are found to be 110, 58, 95,115, 175, and 395 respectively. It can be seen that the mononuclear compounds JH1, JH2, JH3, and JH4 have lower molar conductivity values than the trinuclear compounds JH5 and JH6. The reason for low molar conductivity of JH1, JH2, JH3 and JH4 may be clear when we note that the compounds are neutral and develop charge only when they are hydrolysed in solution in water in which chloride ions are replaced by water molecules. It may be noted that to determine molar conductivity values, the compounds were first dissolved in DMF then diluted with mQ water; the extent of hydrolysis would increase to reach the limiting values at infinite dilution. Thus, the limiting molar conductivity values for JH1, JH2, JH3, and JH4 may be considered to indicate the extent of dissociation of the compounds, being of about 39% in the case of JH1, 21% in the case of JH2, 34% in the case of JH3 and 41% in the case of JH4. As stated earlier, trinuclear compounds JH5 and JH6 have higher molar conductivity values. This is because JH5 and JH6 would dissociate in solution to produce a tetrapositive cation and four chloride ions. The tetrapositive cation may undergo further hydrolysis to develop at the most six units of positive charge.

Because of the high concentration of chloride ions in blood serum it is expected that mononuclear compounds JH1, JH2, JH3, and JH4 will remain undissociated in blood serum so that the compounds may enter the cell by both passive diffusion and mediated transport using copper transporters (Wang and Lippard, 2005). Carrier-mediated transport is expected to be more significant for the efflux of the compounds as they would develop positive charge inside the cell due to hydrolysis.

The results for JH1 and JH2 clearly show that as the number of planaramine ligands present in a molecule increases the degree of dissociation decreases. The only difference between JH1 and JH2 is that whereas JH1 has one thiazole ligand JH2 has two thiazole ligands. Therefore, platinum in JH2 would face a greater steric hindrance than in JH1 so that JH2 is expected to be less susceptible to ligand substitution reactions (Kelland et al., 1999).

Although the limiting molar conductivity values of trinuclear compounds JH5 and JH6 (175 and 395 respectively) are higher than that of cisplatin (135), it is found that cell uptake values for the trinuclear complexes are greater than those of cisplatin in the three ovarian cancer cell lines. It should be noted that higher cell uptakes have been reported for other multinuclear complexes as well. These are discussed more fully later in the chapter.

5.5. Activity against cancer cell lines

As stated earlier, the cytotoxicity of the synthesized compounds against three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ has been determined using the

MTT reduction assay. In addition, cell uptake, and the extent and nature of binding with DNA have also been determined.

5.5.1 Mononuclear compounds JH1, JH2, JH3 and JH4

The IC_{50} values and resistance factors (RF) for JH1, JH2, JH3, JH4 and cisplatin as applied to the human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ are given in Table 4.17. It can be seen that all the mononuclear compounds JH1, JH2, JH3, and JH4 are less active than cisplatin against the parent cell line A2780. The order of the IC_{50} values from highest to lowest is JH4 >JH3 >JH2> JH1 >cisplatin. The results indicate that among JH1, JH2, JH3 and JH4, JH1 is most active and JH4 is least active against the cell line. In actual fact, the IC_{50} value of JH1 0.55 ± 0.06 μM is quite close to that of cisplatin 0.42 ± 0.02 μM. As stated earlier, some results on activity of JH1 and JH2 have been reported previously (Farrell et al., 1992; Van Beusichem and Farrell, 1992; Bierbach et al., 1999; Farrell et al., 2004b). In particular, the activity of JH1 in the human ovarian cancer cell line A2780 and that of JH2 in leukemia cell line L1210 have been reported. The reported IC_{50} value for JH1 in A2780 was 4 μM. This is much greater (7.3 times as large) than the value found in the present study (0.55 ± 0.06 μM). Similarly, the reported IC_{50} value for cisplatin (Farrell et al., 2004b) was found to be greater (3.8 times as large) than that found in the present study (1.6 μM versus 0.42 ± 0.02 μM). The variations in the results can be seen to highlight the importance of reference in cell culture study. This is particularly relevant when we seek to compare results obtained in one laboratory with those found in another. Since reported activity results for JH2 applied only to leukemia cells, these cannot be compared with the present study.

As stated earlier, in the present study besides activity, cell uptake and level of binding with cellular DNA have also been determined. It will be seen later that the order of activity of JH1, JH2, JH3 and JH4 is in line with that of the level of binding with DNA resulting from 24 h incubation. As applied to the cell line $A2780^{cisR}$, again JH1 is found to be most active whereas the least active compound is JH2. The order of the IC_{50} values from highest to lowest is JH2 > cisplatin >JH4> JH3 > JH1. Once again, in general the cytotoxicity results are found to be in line with the DNA binding levels produced from 24 h incubation. As applied to $A2780^{ZD0473R}$ cell line also, JH1 is found to be the most active compound whereas JH2 has the lowest activity. The order of the IC_{50} values from highest to lowest is JH2 > cisplatin >JH3> JH4 > JH1. Generally, the activity values are found to be in line with the level of binding with DNA resulting from 24 h incubation. From above it is clear that among the mononuclear compounds JH1, JH2, JH3 and JH4, the most active compound in all three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ is JH1.

Although our understanding about the molecular mechanisms of activity of platinum complexes remains incomplete, the antitumour activity of platinum drugs is believed to be associated with their binding to DNA. As platinum drugs interact with DNA forming a number of covalent adducts, conformational changes in DNA may be induced. In addition, the interaction may also cause damage to the DNA such as strand breakage. As noted earlier, after 24 h incubation, the most active mononuclear compound JH1 is found to have the highest level of binding with DNA in all the three ovarian cancer cell lines. The results can be seen to provide support to the idea that the activity of *trans*-planaramineplatinum complexes in ovarian cancer cell lines is associated with their binding with DNA so that a higher binding level translates into a higher activity value. All the *trans*-planaramineplatinum complexes of the present

study, like other *trans*-planaramineplatinum complexes, are expected to form monofunctional Pt(G) adduct and interstrand bifunctional Pt(GG) adducts; the latter adducts may cause global distortion in DNA conformation as the distance of separation between the two DNA strands need to be decreased for the formation of G-Pt-G adduct. Although JH1, JH2, JH3 and JH4 all belong to the class of compounds termed: *trans*-planaramineplatinum complexes, JH1 differs from the rest in the sense that it contains only one planaramine ligand, namely a thiazole ligand, whereas JH2, JH3 and JH4 contain two planaramine ligands per molecule (two thiazole ligands in JH2, one thiazole and one imidazole ligand in JH3, one ligand and one 3-hydroxypyridine ligand in JH4). It has been reported that transplatinum complexes with one planaramine ligand cause a greater unwinding of DNA than those with two planaramine ligands. This enhanced unwinding caused by *trans*-planaramineplatinum complexes with one planaramine ligand allows positioning of the planaramine ligand in the adduct so it is more favourably placed to interact with DNA (Najajreh et al., 2005). It has also been suggested that *trans*-planaramineplatinum complexes containing two planaramine ligands cause 'unproductive' DNA distortions that are more recognized and repaired by different repair mechanisms (Brabec and Kasparkova, 2005). Moreover, transplatinum complexes with one planaramine ligand produce more interstrand DNA adduct than those with two planaramine ligands (Najajreh et al., 2005). Interstrand adducts are believed to play a more critical role in the cytotoxicity of transplatinum complexes; they are more difficult to repair than intrastrand adducts because they involve both strands of DNA so that the complementary strand cannot be used for resynthesis (Farrell, 2004).

JH1 is also found to be more active than cisplatin in the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$ although the activity is slightly less than that in the parent cell line

A2780. When DNA binding levels of cisplatin and JH1 are compared, it is found that JH1 has higher levels of DNA binding after 24 h incubation than cisplatin in both $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines in line with higher activity of JH1. It should however be noted that the higher level of DNA binding of platinum complexes may not necessarily result in higher activity. For example, JH3 and JH4 have lower levels of binding with DNA than cisplatin in $A2780^{cisR}$ cell line although both the complexes exhibit higher cytotoxicity than cisplatin against the cell line. Whereas cytotoxicity of cisplatin is believed to be associated mainly with intrastrand 1,2-Pt(GG) adduct, JH3 and JH4 cannot form such an adduct; the likely adducts for JH3 and JH4 are monofunctional adducts such as Pt(G) and bifunctional interstrand adducts such as G-Pt-G. It may be true to say that the activity of platinum drugs (e.g. higher activity of JH1, JH3 and JH4 than cisplatin in the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$) depends on the structure of adducts with DNA and their effect on downstream processes like protein recognition, cell cycle arrest, DNA repair and other cellular events (Harris et al., 2006) (Natile and Coluccia, 2001). As stated earlier, *trans*-platinum complexes including JH1, JH2, JH3, and JH4 are expected to form mainly monofunctional adducts, bifunctional interstrand G-Pt-G adduct, and 1,3 intrastrand adduct (G1,G3) (Eastman et al., 1988; Ma et al., 2005). The distinctive cytotoxicity profile of these adducts is as follows: (1) The monofunctional adducts which compromise 30-40% of the total adducts formed by *trans*-planaramine compounds are able to inhibit DNA synthesis, terminate RNA synthesis and create a DNA conformational distortion similar to that produced by cisplatin (Kasparkova et al., 2003; Martınez et al., 2007). The monofunctional adducts formed by *trans*-planaramine compounds are recognised by HMG proteins, similar to that of cisplatin 1,2-GG intrastrand adduct (Kasparkova et al., 2003b). Therefore the downstream

biological effects of the monofunctional adduct is expected to be similar to that 1,2 GG intrastrand adduct of cisplatin; (2) The planaramine ligand of *trans*-planaramineplatinum complexes is well positioned to interact with DNA through stacking interaction e.g. in the case of JH1 (Marini et al., 2005; Najajreh et al., 2005). The combination of monofunctional covalent binding and stacking interaction between the planar ligand and the DNA bases can produce a kink in the DNA (Kasparkova et al., 2003b; Martınez et al., 2007); (3) The *trans*-planaramineplatinum complexes form more interstrand adducts (about 30-40% of total adducts) than transplatin and cisplatin (Marini et al., 2005; Martınez et al., 2007). The interstrand adducts are not repaired by NER and they cause more of a global change in DNA conformation because of difference in distance between N7(G)-N7(G) in DNA (bout 0.62 nm) and that the two *trans* arms of transplatinum (about 0.36 nm); (4) *Trans*-planaramine complexes form 1,3-intrastrand adducts that compromise about 20-40% of the total adducts (Marini et al., 2005). The research conducted by Kasparkova *et al* found that the replacement of amine ligand in transplatin with one of piperidine, piperazine, or 4-picoline largely enhanced the stability of 1,3-GNG-intrastrand crosslink and at the same time the activity of these compounds is greatly enhanced (Kasparkova et al., 2003b). The intrastrand adducts formed by *trans*-planaramine complexes are much more stable than those formed by transplatin so they persist longer to cause the antitumour effect (Brabec and Kasparkova, 2005). In addition, *trans*-planaramine intrastrand adduct is harder to be removed by NER than cisplatin intrastrand adducts and is found to have bigger impact on DNA repair and replication (Marini et al., 2005).

It is believed that all of the above factors together determine the overall cytotoxicity of *trans*-planaramine complexes. While the monofunctional adduct produces an effect

similar to that of cisplatin, bifunctional adducts (including both interstrand and intrastrand adducts) together with stacking interaction between planaramine ligands and nucleobases in DNA produce unique cellular effect responsible for superior cytotoxicity of JH1, JH3, and JH4 over cisplatin especially in the resistant cell lines.

As stated in the introduction, two possible pathways by which cisplatin could trigger apoptosis are "repair shielding" and "hijacking" of nuclear factors (Jordan and Carmo-Fonseca, 2000). Both the mechanisms rely on cellular proteins recognition and binding. While "hijacking" of nuclear proteins and enzymes deprives cells from their crucial role, "repair shielding" prevents adducts from being repaired thus helping in the persistence of adduct and hence induction of cytotoxicity. As noted earlier, HMG and other nuclear factors recognise and bind to *trans*-planaramine adducts so that hijacking and shielding mechanisms can be mediators of cytotoxicity of *trans*-planaramine complexes.

All the mononuclear compounds JH1, JH2, JH3, and JH4 are found to have lower resistance factors than cisplatin as applied to the cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$ indicating that JH1, JH2, JH3, and JH4 have been better able to overcome the mechanisms of resistance operating in the cell lines. The resistance factor of JH4 as applied to the combination: A2780 and $A2780^{ZD0473R}$ is found to be 0.87, indicating that JH4 is slightly more active in the resistant cell line $A2780^{ZD0473R}$ than the parent cell line A2780. JH4 is a mixed ligand compound containing both a thiazole molecule and a 4-hydroxypyridine molecule as carrier ligands. The other mixed ligand complex is JH3 that has a thiazole ligand and an imidazole ligand as carrier ligands. In addition, JH3 and JH4 are found to be more active than JH2 in the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$ (but not in the parent cell line A2780), indicating that the compounds are better able to overcome mechanisms of resistance

operating in A2780^{cisR} and A2780^{ZD0473R}. The greater activity of JH3 and JH4 may be due to a greater steric hindrance provided by ligands in JH3 and JH4 than in JH2 so that JH3 and JH4 are less subject to deactivation.

5.5.2 Activity of trinuclear compounds (JH5 and JH6)

The IC_{50} values and resistance factors (RF) for trinuclear compounds JH5 and JH6 as applied to ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R} are given in Table 4.17. It can be seen that JH6 is more active than JH5 in all three ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R} - 10 times more in A2780, three times more in A2780^{cisR} and two times more in A2780^{ZD0473R}. The other trinuclear compound JH5 is less active than cisplatin in A2780 cell line but is more active than cisplatin in the resistant cell lines: A2780^{cisR} and A2780^{ZD0473R}. The more active compound JH6 is also more active than cisplatin in all the three cell lines A2780, A2780^{cisR} and A2780^{ZD0473R} - two times more active in A2780, three times more active in A2780^{cisR} and about two times more active in A2780^{ZD0473R}. The results suggest that JH6 has been better able to overcome mechanisms of resistance operating in A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines. As noted earlier, the less active trinuclear compound JH5 is also found to be more active than cisplatin against resistant cell lines A2780^{cisR} and A2780^{ZD0473R} although it is less active than cisplatin against the parent cell line A2780. Whereas cisplatin forms mainly intrastrand bifunctional 1,2-GG adduct with DNA, JH5 and JH6 are expected to form a number of long range inter- and intra-strand adducts with DNA, made possible due to the flexible nature of the linker chains. Interstrand adducts persist longer than intrastrand adducts and can cause a global change in DNA conformation e.g. from B to Z or from

B to A (Farrell, 2000). It is hypothesised that the antitumour efficiency of the trinuclear complexes can be explained as collective and "cooperative" effect of adducts (Farrell, 2004). Thus, much higher activity of JH6 in all three ovarian cell lines over cisplatin is thought to be due to difference in their binding with DNA and repair enzymes recognition.

JH5 and JH6 has three platinum centres in which the two terminal centres bind covalently with DNA whereas the central platinum ion undergoes only non-covalent interactions including electrostatic interaction and hydrogen bonding. The thiazole ligands bound to the central platinum ion may also undergo additional types of non-covalent interactions such as stacking interaction. JH5 has two thiazole ligands bounded to the central platinum, while JH6 has only one thiazole ligand bound to the central platinum. The greater steric crowding around the central metal ion in JH5 may make it more difficult to fit in within the helical DNA than JH6. Thus, it is found that the level of Pt-DNA binding after 2 h of incubation is less for JH5 than JH6. Therefore, the binding of JH5 with DNA is expected to be less sequence specific than that of JH6 resulting into different cytotoxicity (Kasparkova, 2004b). It may be true to say that cytotoxicity of platinum drugs does not depend merely on the number of Pt-DNA adducts formed – rather it depends on the nature of the adducts formed and the non-covalent interactions that the compound may undergo with DNA (Kasparkova et al., 2003b); (Brabec and Kasparkova, 2005; Harris et al., 2006). Steric hindrance provided by the diamine linker was found to reduce cytotoxicity and DNA binding affinity (Farrell, 2004) so that the presence of straight chain linking ligands would translate into greater cytotoxicity than that of the aromatic linker chains (Wheate and Collins, 2003). It is thus possible that the steric hindrance caused by the two thiazole ligands bound to the central platinum ion in JH5 reduces its cytotoxicity and ability

for DNA binding much more than JH6 which has only one thiazole ligand bound to central Pt. Similar observations were made in the case of two other trinuclear compounds namely DH6Cl and TH1 prepared in this laboratory. The central unit in DH6Cl is bound to two ammonia ligands while that in TH1 is bound to two 3-hydroxypyridine ligands. DH6Cl has been found to be 11 times more active than TH1 in A2780 cell line and 4 times more active than TH1 in the resistant cell line $A2780^{Cis\text{-}R}$. BBR3464 which also has two ammonia ligands bound to the central platinum unit is found to be more active than TH1. Therefore it may be concluded the greater steric hindrance around the central metal unit reduces cytotoxicity and the DNA binding affinity. The results can be seen to illustrate structure activity relationship in JH5 and JH6 and may explain the higher activity of JH6 over JH5.

Although JH6 is much more active than JH5 in all three cell lines, JH5 in fact found to have a lower resistance factor than JH6 and cisplatin. The resistance factors for JH5, JH6, and cisplatin as applied to $A2780^{cisR}$ are 2.3, 9.2, and 15.0 respectively and the corresponding values for $A2780^{ZD0473R}$ are 1.7, 10.4, and 11.0 respectively. The results suggest that at the level of its activity, JH5 has been better able to overcome mechanisms of resistance operating in $A2780^{cisR}$ $A2780^{ZD0473R}$ cell line.

5.6. Cell uptake

5.6.1 Mononuclear compounds (JH1, JH2, JH3 and JH4)

It is generally believed that antitumour activity of platinum drugs is associated with their binding with DNA which in turn is dependent on the uptake of drug in the cell. Tables 4.18 to 4.20 and Figures 4.35 to 4.40 gave the cell uptakes of JH1, JH2, JH3,

JH4 and cisplatin in 2 h, 4 h, and 24 h as applied to the ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}.

In A2780 cell line, in general an increase in cell uptake with the increase of duration of incubation is observed in the case of JH1, JH2 and cisplatin while generally there is decrease in cell uptake in the case of JH4. In A2780^{cisR} cell line, in general there is increase in cell uptake with increase in time in the case of JH1 and cisplatin while in the case of JH2 and JH4 there is a decrease in cell uptake with increase in time. For the cell line A2780$^{ZD0473-R}$, there is increase in cell uptake with increase in time for JH1 and cisplatin while generally there is a decrease in cell uptake with the increase in duration of incubation for JH2 and JH4.

The decrease in uptake of platinum complexes with increase in incubation time indicates increased efflux of the drugs out of the cell suggesting that the increased efflux may be a likely mechanism of resistance operating in the cell lines.

It can be seen from the results of mononuclear complexes that JH4 has the highest cell uptake in 2 h, 4 h, and 24 h in all the three cell lines A2780, A2780^{cisR} and A2780^{ZD0473R}, while cisplatin in general has the least cell uptake values in 2 h, 4 h, and 24 h in all three cell lines. No correlation was found between the activity of mononuclear complexes and their cellular accumulation. For example, JH4 is much less active than JH1 in all three cell lines but exhibits a higher cellular uptake. It has been reported earlier that increased drug uptake may not be indicator of activity (Kasparkova et al., 2003b). In addition, all synthesised *trans*-planaramine complexes exhibit higher cell uptakes than cisplatin in all three cell lines. The results are in agreement with the reported observations that *trans*-planaramine complexes exhibit higher cell uptakes than cisplatin (Kasparkova et al., 2003b).

5.6.2 Trinuclear compounds (JH5 and JH6)

Tables 4.18 to 4.20 and Figures 4.35 to 4.40 gave the cell uptake of JH5 and JH6 along with those of mononuclear compounds and cisplatin in 2 h, 4 h, and 24 h as applied to the ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$.

It can be seen that in general the more active compound JH6 has higher cell uptake values than JH5 in all three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ so that the cell uptake levels are in line with the activity values.

When the cell uptake values of JH6 at 2 h, 4 h, and 24 h are compared, it is found that in the cell line A2780 the highest uptake is observed at 2 h and the lowest is found at 24 h. For $A2780^{cisR}$ the highest uptake is observed at 24 h and the lowest is found at 4 h. For $A2780^{ZD0473R}$ cell line, the highest uptake is observed at 4 h and the lowest is found at 2 h. It appears that the rates of uptake of JH6 in the three ovarian cancer cell lines are different – fastest in A2780 cell line and slowest in $A2780^{cisR}$.

The decrease in uptake of platinum complexes (for example JH6 in A2780 cell line), with the increase in duration of incubation indicates that increased efflux may be a likely mechanism of resistance.

When the cell uptakes of trinuclear compounds JH5 and JH6 is compared with those for cisplatin, it is found that trinuclear compounds have much higher cell uptake values, meaning that the trinuclear platinum compounds are able to cross the cellular membrane faster than cisplatin.. These results are in agreement with the reported observations (Brabec and Kasparkova, 2005).

As stated earlier, mononuclear platinum complexes including cisplatin are expected to cross the cell membrane by both passive diffusion and carrier-mediated transport

(Fuertes et al., 2003; Wang and Lippard, 2005), while positively charged trinuclear platinum complexes are expected to cross the membrane by carrier-mediated transport only (Roberts et al., 1999).

5.7. DNA binding

5.7.1 Mononuclear compounds (JH1, JH2, JH3, and JH4)

Tables 4.21 to 4.23, and Figures 4.41 to 4.46 gave the levels of platinum DNA binding expressed as nanomoles of Pt per milligram of DNA for the mononuclear compounds JH1, JH2, JH3, JH4 and cisplatin in A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines in 2 h, 4 h, and 24 h. In general for the mononuclear complexes JH1, JH2, JH4 and cisplatin there is an increase in DNA binding level with the increase in duration of incubation in all three cell lines A2780 A2780^{cisR} A2780^{ZD0473R} except for JH2 and JH4 applying to the cell line A2780 where the level of Pt-DNA binding decreases with time. When levels of Pt-DNA binding of the compounds are compared, it is found that the level in 2 h is highest for JH4 and least for cisplatin; the level in 4 h is highest for JH1 and least for cisplatin. In 24 h the highest level of Pt-DNA binding is observed for cisplatin followed by JH1, JH2 and JH4. It is found that the levels of Pt-DNA binding in 24 h match with the Cytotoxicity of the compounds.

The highest level of Pt-DNA binding as applied to A2780^{cisR} cell line in 2 h, 4 h, and 24 h is observed for JH1 whereas the lowest level of Pt-DNA binding is found for JH2, again in line with the cytotoxicity of the compounds. The results can be seen to provide support to the idea that the activity of platinum drugs is associated with their binding with DNA.

The highest levels of Pt-DNA binding as applied to $A2780^{ZD0473R}$ cell line in 2 h and 4 h are observed for JH4 whereas the least levels are found for JH2. However, in 24 h the highest Pt-DNA level is observed for JH1 although the lowest level is found once again for JH2. Again the levels of Pt-DNA binding in 24 h can be seen to better match with the cytotoxicity of the compounds.

The above results generally show that the levels of Pt-DNA binding of the mononuclear complexes observed after 24 h (rather than 2 or 4 h) of incubation are in line with the toxicity of compounds in all three cell lines A2780 $A2780^{cisR}$ $A2780^{ZD0473R}$. As stated earlier, the activity of platinum based anticancer drugs is believed to be associated with their binding with DNA resulting in the formation of a variety of adducts that block replication and transcription and induce cell death (Brabec and Kasparkova, 2005). It should however be noted that platinum-DNA binding levels may not always correspond to activity and more so in the case of trinuclear compounds that will be considered later.

It was noted earlier that JH4 had the highest cell uptakes in 24 h in all the three lines A2780 $A2780^{cisR}$ $A2780^{ZD0473R}$. On the other hand JH4 is found to have the least level of Pt-DNA binding in A2780 and in $A2780^{cisR}$ cell lines. It was also noted for another compound namely JH2, the level of Pt-DNA binding decreases with the increase in duration of activation. It is believed that one or more of the following mechanisms of resistance may serve to decrease the level of Pt-DNA binding for platinum complexes: (1) decreased level of cellular accumulation through increased efflux out of the cell, (2) increased deactivation due to binding with increased intracellular levels of glutathione and metalothioneins; (3) enhanced level of DNA repair.

Mononuclear complexes including JH1, JH2, JH3, and JH4 are expected to form mainly monofunctional adducts, 1,2 -interstrand adducts, and 1,3-ntrastrand adducts (G1,G3) (Eastman et al., 1988; Ma et al., 2005). It is believed that the combination of all these adducts produce unique cellular effects that give cytotoxic superiority for *trans*-planaramine over cisplatin especially in the resistant cell lines.

The results show that the level of Pt-DNA binding rather than the level of cell uptake per se can give a more meaningful indication of the activity of the compounds.

5.7.2 Trinuclear compounds (JH5 and JH6)

Tables 4.21 to 4.23, and Figures 4.41 to 4.46 gave the levels of platinum DNA binding for JH4 and JH6 along with those for mononuclear compounds and cisplatin expressed as nanomoles of Pt per milligram of DNA in A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines in 2 h, 4 h, and 24 h.

JH6 is found to have higher level of Pt-DNA binding than JH5 in all the three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ in 2 h and 4 h, while in 24 h JH5 has the higher Pt-DNA binding levels in A2780 and $A2780^{ZD0473R}$ cell lines and JH6 has the higher level in $A2780^{cisR}$ cell line.

The decrease in the level of platinum binding to DNA with the increase in time indicates the occurrence of increased DNA repair. As stated before, intrastrand adducts formed by trinuclear cations are much easier to repair than their interstrand adducts as the latter adducts are not easily removed by NER or NER may not be involved in their removal (Boulikas and Vougiouka, 2003).

JH5 and JH6 are expected to form different types of inter- and intra-strand adducts including:

(1) Intrastrand adducts including 1,2-, 1,3-, and 1,5-cross links (Farrell, 2004). However, due to the long lengths of the trinuclear cations, formation of 1,2-intrastrand is not favourable (Zehnulova et al., 2001). In general, the intrastrand adducts formed by trinuclear platinum complexes like JH5 and JH6 are flexible, do not cause stable bending (directional bending) to DNA, or block DNA and RNA polymerase. However these adducts are not recognised by HMG proteins so that they are easily removed by NER (Zehnulova et al., 2001; Kasparkova et al., 2002; Wheate and Collins, 2003; Farrell, 2004; Brabec and Kasparkova, 2005).

(2) JH5 and JH6 are expected to form a range of interstrand adducts including 1,2-, 1,4-, and 1,6- interstrand adducts. The most frequent interstrand adduct is the 1,4-interstrand while the least frequent one is 1,6-interstrand (Kasparkova et al., 2002). The interstrand adducts are expected to compromise about 20% of the total adducts formed by JH5 and JH6 as in the case of BBR3464. The adducts are formed between guanine residues. Interstrand adducts cause DNA unwinding by 14^0 and bending towards the major groove of 15^0 and 21^0 respectively (much less than that caused by cisplatin which is between $32\text{-}34^0$). The interstrand adducts formed by trinuclear cations are far more difficult to repair than intrastrand adducts because they involve both strands of DNA and the complementary strand cannot be used for resynthesis (Farrell, 2004). It is been revealed that the 1,4-interstrand adduct (the major adduct formed by BBR3464) is not easily removed by NER (Boulikas and Vougiouka, 2003). Therefore it has been suggested that the toxicity of BBR3464 may be related to interstrand adducts rather than intrastrand adducts which are effectively removed by NER (Zehnulova et al., 2001; Kasparkova et al., 2002; Brabec and Kasparkova,

2005). Moreover, it has been suggested that 1,4-interstrand adduct formed by BBR3464 causes blockage to DNA and RNA polymerase (Zehnulova et al., 2001; Kasparkova et al., 2002; Farrell, 2004). In contrast, cisplatin is known to form short range intrastrand adduct mainly 1,2-intrastrand Pt(GG) adduct that causes a local bending of a DNA strand. Since the long range adducts formed by polynuclear cations are not recognised by proteins such as HMG proteins that bind only to rigidly bent DNA, the hijacking or shielding mechanism is unlikely to be a mediator for cytotoxicity of polynuclear complexes (Wheate and Collins, 2003; Farrell, 2004; Brabec and Kasparkova, 2005).

5.8. Interaction with ssDNA

The interaction of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin with salmon sperm DNA (ssDNA) was studied using gel electrophoresis in order to obtain qualitative information on changes in DNA in terms of conformation change and damage as a result of the interaction with the drugs.

Figure 4.47 gave the electrophoretograms applying to the incubated mixtures of ssDNA and varying concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin. A single DNA band was observed in both untreated ssDNA and the one that was interacted with increasing concentrations of the compounds. As the concentration of the compounds was increased both the intensity and the mobility of the band decreased. The decrease in intensity with the increase in concentration of the compounds indicates DNA damage caused by interaction of the compounds with DNA and decrease in mobility 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

As stated earlier, JH1 was found to be the most active compound among mononuclear complexes. It was also found that the levels of binding with DNA were higher for JH1 than other mononuclear compounds JH2, JH3, and JH4. It can be seen from electrophoretograms in Figure 4.41 that JH1 caused most DNA damage indicated (by greatest reduction in intensity of the DNA band) than JH2, JH3, JH4 and cisplatin. As stated earlier, 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. In addition, non-covalent interactions involving planaramine ligand may also be playing a role in causing the DNA damage. Although JH2, JH3, and JH4 have bulkier ligands than JH1, it has been found that *trans*-planaramineplatinum complexes with one planaramine ligand (such as JH1) is well positioned to interact with DNA through stacking interaction and therefore more efficient in inducing DNA damage than the rest of *trans*-planaramineplatinum complexes that two planaramine ligands per molecule (Marini et al., 2005; Najajreh et al., 2005).

It should be noted here that the concentrations used for JH6 were 10 fold lower than those used for other complexes JH1, JH2, JH3, JH4, JH5 and cisplatin. When similar concentrations were used for JH6 as for other compounds no band was visible indicating a more significant damage to DNA. Therefore damage to ssDNA caused by the trinuclear cation of JH6 was much more pronounced than all other compounds namely JH1, JH2, JH3, JH4, cisplatin and JH5.

JH6 is found to be the most active compound among all synthesised compounds and cisplatin in the parent cell line A2780. The effective length of JH6 and JH5 molecules can be altered due to the presence of flexible diamine chains, therefore the compounds are expected to form a number of long range inter and intra-strand GG adducts that cause a global change in DNA conformation (Farrell, 2000). On the other hand mononuclear compounds JH1, JH2, JH3, JH4 are expected to form short range interstrand and intrastrand adducts in addition to monofunctional adducts.

The decrease in band intensity and mobility caused by JH5 and JH6 are due to DNA damage, change in conformation, increase in molecular mass and reduction in negative charged caused by the covalent binding of the compounds with ssDNA. Non-covalent interactions involving planaramine ligands including hydrogen bonding and stacking interaction may also be playing a role in inducing DNA damage.

5.9. Interaction with pBR322 plasmid DNA

Figure 4.48 gave the electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin.

Plasmids are small circular, double-stranded DNA molecules that can exist in three forms: supercoiled circular (form I), singly nicked circular (form II) and doubly nicked linear (form III) (Figure 3.6). As noted earlier, DNA is polynegatively charged due to the phosphate backbone so that it will migrate through the gel towards the positive electrode. The rate of migration through the gel is different for the three forms. Form I, being supercoiled and compact will migrate at the fastest rate and the flexible and relaxed form II DNA travels at the slowest rate. The linear form III is able to snake through the gel and thus will migrate faster than the form II DNA (Figure 3.7).

As the plasmid DNA was interacted with the increasing concentrations of the compounds, in general, the mobility of DNA bands increased and their intensity decreased. The change in mobility is believed to be mainly due to changes in DNA conformation brought about due to covalent binding of the compounds with plasmid DNA. The decrease in intensity of the bands indicates the occurrence of DNA damage again is believed to be caused by the binding of the platinum compounds with the DNA.

As noted earlier, when pBR322 plasmid DNA was interacted with increasing concentrations of JH1, two closely spaced bands are observed for concentrations

ranging from 0.488 μM and 1.953 μM. At the next higher concentration namely 3.90 μM, essentially one coalesced band is observed. At the next four higher concentrations (7.81 μM, 15.62 μM, 31.25 μM and 62.5 μM), separation between the bands is found to increase although intensity gets progressively diminished. At the highest concentration namely 125 μM no DNA band is observed. When pBR322 plasmid DNA was interacted with increasing concentrations of JH2, two bands corresponding to forms I and II are observed essentially at all concentrations. At the highest concentration namely 125 μM only one clear but faint band can be seen. In lanes 5 and 7 corresponding to concentrations 7.81 μM and 31.25 μM respectively three bands can be seen. When pBR322 plasmid DNA was interacted with increasing concentrations of JH3 two bands corresponding to forms I and II are observed for all concentrations up to 15.62 μM (lane 6) above which (lanes 7-9) a single faint DNA band can be seen. There is a gradual increase in mobility of the DNA bands with the increase in concentration of JH3, especially at lower concentrations. In the interaction of pBR322 plasmid DNA with increasing concentrations of JH4, two bands corresponding to forms I and II are observed for concentrations ranging from 0.488 μM to 7.81 μM. At the next two higher concentrations namely 15.62 μM and 31.25 μM essentially one coalesced band is observed and at still higher concentrations (62.50 μM) one band is observed and at higher concentration no DNA band can be seen. Among JH1, JH2, JH3 and JH4, JH4 is found to be most damaging to DNA whereas JH2 is found to cause least damage to pBR322 plasmid DNA.

JH1, JH2, JH3 and JH4 are expected to form monofunctional adducts such as Pt-G, interstrand adducts such as G-Pt-G and to lesser quantity 1,3-intrastrand adduct such

as Pt(GNG) where N stands for a nucleotide. These adducts are believed to cause conformational changes in DNA including bending and unwinding that would serve to alter the mobility and the intensity of the bands. Non-covalent interactions such as hydrogen bonding and stacking in between nucleobases may also play a role. The disappearance of DNA bands at high concentration of JH1 indicates the occurrence of severe damage to DNA. Streaking of DNA bands also indicates DNA damage. Whereas JH1 has one thiazole ligand, JH2, JH3 and JH4 have two planaramine ligands per molecule. It is not clear why JH4 is most damaging to DNA and JH2 is least so. It should however be noted that whereas JH2 has two thiazole ligands per molecule, JH4 is a mixed ligand complex containing one thiazole ligand and one 3-hydroxypyridine ligand per molecule. The differences in DNA conformational change and DNA damage caused by JH1, JH2, JH3 and JH4 can be seen to illustrate structure-activity relationships and that non-covalent interactions also play a key role in DNA damage and conformational changes in DNA as it was found to be true in antitumour activity of the compounds.

When pBR322 plasmid DNA was interacted with increasing concentrations of JH5, two bands corresponding to forms I and II can be seen for concentrations of the compound ranging from 0.488 μM to 7.81 μM. At the next two higher concentrations namely 15.62 μM and 31.25 μM essentially one coalesced band is observed. At the next higher concentration (62.50 μM) also one band is observed. However, it is found to be more intense than the band observed 15.62 μM and 31.25 μM. No band is observed at the highest concentration (125 μM). When pBR322 plasmid DNA was interacted with increasing concentrations of JH6, two distinct bands corresponding to forms I and II can be seen for all concentrations of the compound from 0.078 μM to 1.25 μM (lanes 1-5). The separation between the bands is found to decrease markedly

in lane 6 (2.5 μM) and at the next higher concentration 5.0 μM essentially one coalesced band is observed. No band is observed at the highest concentration namely 10.0 μM. DNA band in lane 5 is found to have the highest mobility.

Among JH1, JH2, JH3, JH4, JH5 and JH6, JH6 is found to be most damaging to the plasmid DNA (a much lower concentration was used for JH6) in line with the highest activity observed for JH6. As noted earlier, whereas JH1, JH2, JH3 and JH4, can form mainly monofunctional Pt(G) and 1,2-bifunctional interstrand Pt(GG) adducts, JH5 and JH6 are more likely to form a number of long-range interstrand GPtPtPtG adducts that bring about more of a global change in DNA conformation. Whereas JH5 has two thiazole ligands bonded to the central platinum ion, JH6 has one thiazole ligand bonded to the central platinum ion. It was noted earlier that the higher activity of JH6 compared to JH5 did not show any correlation with the levels of Pt-DNA binding observed in the ovarian cancer cell lines. Once again, the results can be seen to illustrate that non-covalent interactions can play a significant role in causing changes in DNA and also in cytotoxicity.

5.10. BamH1 restriction enzyme digestion

Figure 4.49 gave the electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin that were digested with BamH1 for 1 h before the mixtures were loaded onto the gel.

Restriction enzymes are proteins produced naturally from bacteria for protection against bacteriophage infection. Restriction enzymes cleave DNA molecules at

specific palindromic nucleotides sequences (from 4 base pair to 8). BamH1 is a restriction endonuclease that recognizes the sequence of G/GATCC and hydrolyses the phosphodiester bond between adjacent GG sites. The plasmid used in this study, pBR322, contains a single restriction site for BamH1 that converts the supercoiled form I and also singly nicked circular form II to linear form III DNA. Therefore, as the results show in lane A when unreacted pBR322 plasmid DNA was digested with BamH1, only one band corresponding to form III band was observed whereas in the untreated and undigested pBR322 plasmid DNA, in lane B, generally two bands corresponding to form I and form II were observed.

When pBR322 plasmid DNA is interacted with increasing concentrations of JH1, JH2, JH3, JH4, JH5, JH6 and cisplatin,followed by BamH1 digestion, generally three bands corresponding to forms I, II and III are observed at low concentrations whereas two bands corresponding to forms I and II are observed at higher concentrations. The results indicate increasing prevention of BamH1 digestion with the increase in concentration of the compounds. The increasing prevention of BamH1 digestion is believed to be due to conformational changes in the DNA brought by covalent and non-covalent interactions of the compounds with the DNA. Among the *trans*-planaramineplatinum complexes, JH3 is found to have least ability to prevent BamH1 digestion whereas JH1 and JH2 are found to be more efficient suggesting that the latter two compounds cause a greater conformational change to DNA. JH4 is found to be most damaging to the DNA. Between two trinuclear complexes, the more active compound JH6 is found to prevent BamH1 digestion at lower concentrations.

6. Chapter Six: Conclusion

Cancer is the most dreaded disease of the present century. Although great advancement has been made in its treatment, cure for cancer remains an on-going challenge. Cancer is actually a common name for many diseases that are characterized with uncontrolled cell division and metastasis (Pecorino, 2005). Currently cancer is treated alone or in combination by surgery, radiation, and chemotherapy. The choice of treatment depends on cancer stage, type, treatment success, and patient's age and health. Although cisplatin and its analogues carboplatin and oxaliplatin are three of the most widely used anticancer drugs even in the postgenomic age, the drugs have a number of side effects and a limited spectrum of activity because of inherent or acquired resistance. Thus thousands of cisplatin analogues have been prepared with the aim of reducing side effects and widening spectrum of activity. It has been possible to reduce the side effects by changing the nature of the leaving group and a limited change in the spectrum of activity has also been achieved by changing the nature of the carrier ligands (Pasetto et al., 2006). The general view is that although some new cisplatin analogues may overcome some resistance mechanism of cisplatin, it is believed that these *cis* mononuclear compounds will interact with DNA in a similar manner as that of cisplatin so that the main issue of resistance will surface again sooner or later.

Currently attention is given to rule-breaker platinum complexes with the idea that different nature of interaction with DNA may change the spectrum of activity. Two such classes of compounds are the *trans*-planaramineplatinum complexes and

platinum compounds with multiple metal centres. Both of the classes of compounds are found to overcome cisplatin-resistance in a number of cancer cell lines.

In this study four *trans*-planaramineplatinum mononuclear complexes code named JH1, JH2, JH3, and JH4, and two trinuclear platinum complexes code named JH5 and JH6 had been prepared and characterized by elemental analyses, IR, mass spectrum and ^{1}H NMR spectral measurements. The activity of the synthesized compounds against three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ has been determined. Cell uptake, extent and nature of binding with DNA have also been determined.

Although JH1, JH2, JH3, and JH4 are less active than cisplatin against the parent cell line A2780, the compounds are found to be more active than cisplatin against the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$, with JH1 being most cytotoxic. JH1 that is only marginally less active than cisplatin in A2780 cell line is four times more active than cisplatin in the resistant cell line $A2780^{cisR}$ and six times more active than cisplatin in the resistant cell line $A2780^{ZD0473R}$.

Between the two trinuclear compounds, JH6 is more active than JH5 in all three ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ – it is 10 times more active than JH5 in the parent cell line A2780, three times more active in the resistant cell line $A2780^{cisR}$ and two times more active in $A2780^{ZD0473R}$ cell line. JH6 is also found to be more active than cisplatin in the ovarian cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ – two times more active than cisplatin in the parent cell line A2780, three times more active than cisplatin in the cisplatin-resistant cell line $A2780^{cisR}$ and about two times more active in the resistant cell line $A2780^{ZD0473R}$.

In the mononuclear complexes, the order of activity of the compounds is found to be in line with the level of binding with cellular DNA in A2780, A2780^{cisR}, A2780^{ZD0473R} cell lines after 24 h of incubation. In the trinuclear compounds, the order of activity of the compounds is found to be in line with the platinum-DNA binding level in A2780^{cisR} cell line but not in A2780 and A2780^{ZD0473R} cell lines. The results indicate that interaction between multinuclear complexes and DNA is likely to be more complex than that between mononuclear complexes and DNA.

The change in gel mobility and intensity of the DNA bands and increasing prevention of BamH1 digestion with the increase in concentration of the compounds provide support to the idea that binding of the platinum complexes with DNA brings about changes in DNA conformation and causes DNA damage. Both covalent and non-covalent interactions are expected to play a role in inducing DNA damage. A direct correlation is found between the levels of DNA damage and prevention of BamH1 digestion and the activity of the compounds.

The synthesised mononuclear *trans*-planaramineplatinum compounds are expected to form mainly monofunctional adducts, 1,2-interstrand adduct, and 1,3-intrastrand adducts (G1,G3). The higher activity of JH1, JH3, and JH4 as compared to cisplatin in the resistant cell lines A2780^{cisR} and A2780^{ZD0473R} can be attributed to the nature and/or quantity of adducts formed by these *trans*-complexes (Natile and Coluccia, 2001). While the monofunctional adducts of mononuclear *trans*-complexes produce similar effects as that of cisplatin, bifunctional adducts including interstrand and intrastrand together with stacking interaction between planaramine ligands and nucleobases in DNA may produce unique cellular effects that give cytotoxic superiority of *trans*-planaramine complexes over cisplatin especially in the resistant cell lines.

Besides the higher binding level with DNA, another factor responsible for higher activity of JH1 over JH2, JH3 and JH4 in all the three cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, is the difference in its structure – whereas JH1 has one planaramine ligand per molecule, JH2, JH3 and JH4 have two planaramine ligands per molecule. It has been reported that *trans*-planaramineplatinum compounds with one planaramine ligand produce greater unwinding of DNA than those containing two planaramine ligands. The enhanced unwinding of transplatinum complexes with one planaramine ligand allows positioning of the planaramine moieties in the adduct so it is more favourable to interact with DNA. Transplatinum complexes with two planaramine ligands do not allow this positioning. On the contrary unproductive distortion of DNA that is more easily recognized and deactivated or repaired by different repair mechanisms may be produced (Brabec and Kasparkova, 2005; Najajreh et al., 2005). Moreover, transplatinum complexes with one planaramine ligand produce more interstrand DNA adducts than those with two planaramine ligands (Najajreh et al., 2005). Interstrand adducts are believed to play a crucial role in the cytotoxicity of *trans*-planaramineplatinum compounds as they may be more difficult to repair than intrastrand adducts (Farrell, 2004).

The order of cytotoxicity of JH5 and JH6 is not in line with the level of platinum bound to DNA. However, JH6 is found to bind faster with cellular DNA than JH5 so that more sequence specific binding with DNA will eventually be produced by JH6 than JH5 and this difference may affect downstream processes such as protein recognition, cell cycle arrest, DNA repair and other cellular events (Brabec and Kasparkova, 2005; Harris et al., 2006). The flexibility of the linker chain is also found to play a major role in the cytotoxicity of the multinuclear compounds. Steric hindrance in the diamine linker was found to reduce cytotoxicity and DNA binding

affinity (Farrell 2004). The multinuclear compounds containing aromatic ligands were found to be less cytotoxic whereas straight chain linking ligands were found to produce greater cytotoxicity than the aromatic ones (Wheate and Collins, 2003). Therefore, it is possible that the greater steric hindrance provided by the two thiazole ligands bound to the central platinum unit in JH5 reduces its cytotoxicity and DNA affinity much more than that in JH6 which has only one planaramine ligand. This observation is in line with the reported results.

When activity of all synthesised complexes is compared, it is found that the trinuclear compound JH6 is the most active compound in the A2780 cell line while the mononuclear compound JH1 is the most active compound in $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines. It appears that JH6 is better able to overcome the mechanisms of resistance operating in A2780 cell line whereas JH1 is better able to overcome the mechanisms of resistance operating in $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines.

The variations in activity of mononuclear complexes JH1, JH2, JH3, JH4, and those of the trinuclear complexes JH5 and JH6 can be seen to illustrate structure-activity relationships.

What is next?

JH6 is found to be significantly more active than cisplatin against all three ovarian cancer cell lines A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$ while JH1 is so against the ovarian cancer cell lines $A2780^{cisR}$, $A2780^{ZD0473R}$, indicating that the compounds are better able to overcome resistance in the cell lines. Further experiments are needed to determine whether JH1 and JH6 show significant activity in other ovarian and non-ovarian cancer cell lines. The toxicity profile of the compounds using suitable animal

models needs to be explored before they can be considered for development as anticancer drugs.

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