Studies on new trinuclear palladium compounds

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DEDICATION

This work is dedicated to my parents
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Abstract

The present study deals with the synthesis and characterization of six tri-palladium complexes code named MH3, MH4, MH5, MH6, MH7 and MH8 that contained two planaramine ligands bound to the central or each of the terminal metal ions. The activity of the compounds against human cancer cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}, cell uptake, levels of DNA-binding and nature of interaction with salmon sperm and pBR322 plasmid DNA have also been determined. Whereas cisplatin binds with DNA forming mainly intrastrand GG adduct that causes local bending of a DNA strand, the tri-palladium complexes are expected to bind with DNA forming a number of long-range interstrand GG adducts that would cause a global change in DNA conformation. Among the designed complexes, MH6 that has two 2-hydroxypyridine ligands bound to each of the two terminal palladium ions is found to be most active. The compound also has the highest cell uptake and Pd-DNA binding levels. In contrast, MH8 which has two 4-hydroxypyridine ligands bound to each of the two terminal palladium ions is found to be least active. The results indicate that, as applied to the terminal metal centres, 2-hydroxypyridine would be more activating than 4-hydroxypyridine perhaps because of greater protection provided to the terminal centres from coming in contact with the solvent molecules. In contrast, when bound to the central metal centre, 4-hydroxypyridine appears to play a slightly greater activating role than 2-hydroxypyridine or 3-hydroxypyridine, suggesting that non-covalent interactions such as hydrogen bonding associated with the ligand rather than its steric effect may be a more important determinant of antitumour property. The results illustrate structure-activity relationships.
and suggest that the tri-palladium complex containing two 2-hydroxypyridine ligands bound to each of the three metal centres or the compound that contains two 2-hydroxypyridine ligands bound to each of the two terminal metal centres and two 4-hydroxypyridine ligands bound to the central metal centre, may be much more active than any of the designed complexes.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3-related</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>BBR3464</td>
<td>$[^{\text{trans}}\text{PtCl(NH}_3)_2{\mu\text{-trans-Pt(NH}_3)_2(NH}_2(CH}_2)_6NH}_2}_2]^{4+}$</td>
</tr>
<tr>
<td>BD</td>
<td>Budotitane</td>
</tr>
<tr>
<td>BP</td>
<td>Benzopyrene (BP)</td>
</tr>
<tr>
<td>CBCD</td>
<td>cis-diammine-1,1-cyclobutane dicarboxylate platinum(II)</td>
</tr>
<tr>
<td>CDKs</td>
<td>Cyclin-dependent protein kinases</td>
</tr>
<tr>
<td>CH25</td>
<td>$[^{\text{trans}}\text{PtCl(NH}_3)_2{\text{trans-Pd(NH}_3)_2(2\text{-hydroxypyridine)-(H}_2\text{N(CH}_2)_6NH}_2}_2}_2\text{Cl}_4$</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>cis-diamminedichloroplatinum(II) or Cisplatin</td>
</tr>
<tr>
<td>CP</td>
<td>Cycloplatin</td>
</tr>
<tr>
<td>DACH</td>
<td>1,2-diaminocyclohexane</td>
</tr>
<tr>
<td>DH6Cl</td>
<td>$[^{\text{trans}}\text{PtCl(NH}_3)_2{\mu\text{trans-Pd(NH}_3)_2(\text{H}_2\text{N(CH}_2)_6NH}_2}_2}_2\text{Cl}_4$</td>
</tr>
<tr>
<td>DIEN</td>
<td>Diethylenetriamine</td>
</tr>
<tr>
<td>DMF</td>
<td>$N,N$-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double strand DNA breaks</td>
</tr>
<tr>
<td>EN</td>
<td>Ethylenediamine</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HAs</td>
<td>Heterocyclic amines</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group</td>
</tr>
</tbody>
</table>
HPLC High performance liquid chromatography
HRPC Hormone-refractory prostate cancer
IC$_{50}$ Concentration required to inhibit cell growth by 50%
IR Infrared
JM118 $cis$-amminedichloro(cyclohexylamine)platinum(II)
JM216 bis-aceto-amminedichloro-cyclohexylammine-platinum(IV)
MH1 $[trans$-PdCl(NH$_3$)$_2$]$_3$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH2 $[trans$-PdCl(NH$_3$)$_2$]$_3$ (H$_2$N(CH$_2$)$_5$NH$_2$)$_2$]Cl$_4$
MH3 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(4-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH4 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(3-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH5 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(2-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH6 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(2-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH7 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(3-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MH8 $[trans$-PdCl(NH$_3$)$_2$]$_2$ $[trans$-Pd(4-hydroxypyridine)]$_2$ (H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$
MM Multiple myeloma (cells)
MMR Mismatch repair
MT Metallothionein
MTD Maximum tolerated dose
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
PAH Polyhydrocarbons
PBS Phosphate buffered saline
PCD Programmed cell death
PGM Platinum group metals
RF Resistant factor
RNA Ribonucleic acid
RNS Reactive nitrogen species
RPMI Refers to media developed at Roswell Park Memorial Institute
ROS Reactive oxygen species
SCC  Squamous cell carcinoma  
SCLC  Small cell lung cancer  
SsDNA  Salmon Sperm DNA  
TAE  Tris-acetate EDTA  
TBE  Tris-borate EDTA  
TH5  \( trans-PdCl_2(3\text{-hydroxypyridine})_2 \)  
TH6  \( trans-PdCl_2(2\text{-hydroxypyridine})_2 \)  
TH7  \( trans-PdCl_2(4\text{-hydroxypyridine})_2 \)  
TPE  Tris-phosphate EDTA  
trans-DDP  Transplatin  
Triton X-100  t-Octylphenoxypolyethoxyethanol  
TS  Tumour suppressor  
UV  Ultraviolet  
ZD0473  \( cis\text{-amminedichloro(2-methylpyridine)platinum(II)} \)
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1. Chapter one: Introduction

1.1. Cancer:

Cancer is one of the leading causes of death all over the world. It is characterized by uncontrolled growth of malignant cells that have the capacity to metastasize (Cooper, 1992) and the ability to penetrate surrounding tissues and other parts of the body (Rees et al., 1993). Cancer can begin almost anywhere in the body. The word ‘cancer’ stands for ‘crab’ in Latin, meaning the creeping movements of cancer cells around the body (Lamb, 2002). Most cancers arise from a mutation in a single cell and therefore represent a monoclonal population, but some growths are polyclonal, indicating that they have developed following more than one mutation (Rees et al., 1993). Although the human life is highly complex, as with other life, it starts as a single cell. At first, the single cell divides to produce two identical cells. The division process continues to produce many millions of cells. This is a controlled and systematic process. But when cancer occurs, the control mechanism is lost so that cells multiply indefinitely to produce an ever-larger mass of cancer cells (Katzung, 1987). The mass of cancer cells together is called a tumour (Evans, 1991). Cancer cells from this primary tumour may then spread, or metastasize, to other parts of the body, where new tumours may begin to grow (Harpham, 2003). Tumours are named after the type of cell where the cancer starts. For example, carcinomas begin in the skin or tissue that covers the surface of internal organs and glands. Sarcomas begin in the connective tissue, such as muscle, fat, cartilage or bone. There are more than 200 types of cancer corresponding to a similar number of cell types in the body (Ellis, 2003). In general the size of cancer mass must reach up to 1 centimetre or made of 1 million cells, before it can be detected (Sekeres, 2004). Exceptions are cancers of blood and bone marrow, also
known as lymphomas. Lymphomas and leukemias are examples of tumours or cancers presenting in body fluids (the blood and bone marrow) and are detectable by laboratory tests of the blood (Cooper, 1993). Solid tumours including cancers of the lung, breast, prostate, colon, rectum, and bladder, are not present in large enough numbers in body fluids to be detected with a blood test. But they may release some chemicals that are detectable in body fluids (Cooper, 1993). Tumours can be benign (noncancerous) or malignant (cancerous) (Cooper, 1993). Benign tumours may grow, but they do not spread to other parts of the body and are not usually life threatening, but malignant tumours grow and invade other tissues in the body, destroying surrounding body tissues (Goyns, 1999).

1.2. Cell cycle:

The human body is essentially a collection of organized and differentiated cells so that cells may be defined as building blocks of different parts of the body that differ in size, shape and function. They have the ability to reproduce by repeated division (Lamb, 2002). Both normal and cancer cells are able to multiply through the process termed as cell cycle (Collins et al., 1997). Different steps or phases of the cell cycle are illustrated Figure 1.1.
Figure 1.1: The phases of Cell Cycle

Interphase is the resting stage between cell divisions that lasts for 12 to 24 h in mammalian cells. The interphase can be divided into 4 steps (Osiecki, 2003): Gap 0 (G0), Gap 1 (G1), S (synthesis) phase, Gap 2 (G2).

**Gap 0 (G0):**

G0 step is termed as resting step during which cells are not yet ready to divide. This may be a temporary resting period or more permanent one depending on the types of cells (Osiecki, 2003).

**Gap 1 (G1):**

During Gap 1, cells increase in size, produce RNA and synthesize protein. An important cell cycle control mechanism is activated during this period (G1 checkpoint) to ensure that everything is ready for DNA synthesis. This step lasts for 12 –30 h (Osiecki, 2003).
**S Phase:**

S phase is synthesis step during which DNA replication occurs - cell replicates its DNA such that the newly produced two cells have the right amount of DNA as the parent cell. This step lasts for 6 –20 h (Osiecki, 2003).

**Gap 2 (G2):**

During the gap 2 (G2) that lies in between S phase and M phase, the cell will continue to grow and produce new proteins. At the end of this phase there is another control checkpoint (denoted as G2 checkpoint) to determine if the cell can now proceed to enter M phase (mitosis) and divide. Gap 2 lasts for 2-10 h (Osiecki, 2003).

**1.2.1. M Phase (or Mitosis):**

During M phase (also called mitosis phase), the parent cell divides in two identical daughter cells. During this phase, cell growth and protein production stop. All of the cell's energy is focused to result into orderly division. All cells must accurately replicate and segregate their chromosomes during cell division. Mitosis is much shorter than interphase, lasting for only 1 to 2 h.

Throughout the cell cycle there are checkpoints that regulate progression through the cycle ensuring that each step takes place only once and in the right sequence (Collins et al., 1997). At the G1 checkpoint, the cell must make a commitment to continue into the S-phase, the DNA synthesis step or to stop at G1 and wait until conditions are more optimal for cell replication to occur. Once the commitment is made the cell automatically goes through S, G2 and M phases to return to G1 phase. If the cell is blocked at S, G2 or M check points it dies (Osiecki, 2003).
1.2.2. Stages in development of cancer

The induction of cancer (carcinogenesis) is a complex, multistage process that affects many genes and gene products and the whole process is critical for the regulation of numerous cellular functions (Bode and Dong, 2006; Walaszek et al., 2004). Although tumours originate from normal cells that can transform into cancer cells through a series of changes (Cooper, 1993), it is generally accepted that cancer develops in three distinct stages (Bode and Dong, 2006; Hennings et al., 1993) termed: (a) initiation, (b) promotion, and (c) progression. These are briefly described as follows:

1.2.3. Initiation

In this step the activation of oncogenes and the inactivation of tumour suppressor (TS) genes take place, initiating mutation of cellular DNA (Walaszek et al., 2004). This can be due to chemical exposure, radiation or viral infection. Oncogenes are mutated forms of genes that cause normal cells to grow out of control and later to be converted to cancer cells. However, oncogenes are present in all human cell nuclei known as proto-oncogenes. They are dormant or have only a low level of activity probably concerned with differentiation and growth control (Sutherland, 1999). There is strong evidence to suggest that oncogenes may be triggered into excessive activity, conferring malignant properties on the host cells (Rees et al., 1993). A quantitative internal change in the gene must occur resulting in nonresponsiveness to normal control mechanisms (Rees et al., 1993). The step is reversible and consists of a single gene mutation and is usually caused by environmental genotoxic agents such as chemicals, radiation, and viruses (Walaszek et al., 2004).
Cancer involves the formation of an altered cell that becomes the mutated initiated cell after a round of DNA synthesis (Klaunig et al., 1998). This initiated cell may clonally grow through either the induction of cell proliferation or the inhibition of apoptosis to form a focal lesion. Subsequent additional DNA damage and genetic instability may allow selective focal lesions to progress to the neoplastic stage.

1.2.4. Promotion

This step follows initiation and is associated with the propagation of the initiated cell population and requires continual exposure to the promoting agent. During this step, some major changes in the genome may occur and but unlike initiation this step is reversible (Hennings et al., 1993; Stoll, 2000).

PROGRESSION: This is the final step in the development of cancer in which benign tumours change in malignant ones that are capable of invading adjacent tissues and metastasizing to distant sites (Hennings et al., 1993; Osiecki, 2003; Walaszek et al., 2004). This step is normally irreversible (Hennings et al., 1993).

1.2.5. The malignant phase

During malignant phase tumour cells invade normal cells and implant at distant sites in a process known as metastasis (Osiecki, 2003). For this to take place, cancer cells...
must detach from the primary site to penetrate the surrounding vasculature (blood vessels) (Osiecki, 2003).

1.3. Causes of cancer:

As described below, both genetic and environmental factors may be responsible for the development of cancer.

1.3.1. Genetic causes of cancer:

Cancer is caused by abnormalities in the genetic mechanisms that control cellular growth and proliferation. These genetic abnormalities are usually acquired but some individuals because of their genetic makeup may be predisposed to develop cancer or may have a greater chance to develop cancer than others (Hodgson and Maher, 1993). Almost every human cancer has both sporadic and genetic counterparts (Hodgson and Maher, 1993). Genetic predisposition may result from inherited mutation in genes that are directly involved in normal cellular growth and proliferation (Hodgson and Maher, 1993). If the mutation is passed from one of the parents to the child, it is called a germline mutation. When a germline mutation is passed on from a parent to the child, it is present in every cell of the child's body, including the reproductive sperm and egg cells. Because the mutation affects reproductive cells, it is passed from generation to generation. On the other hand, mutations that occur by chance over the period of time in cells of the body are known as "acquired." Acquired mutations are not inherited, and hence are not present in all cells of the body and not passed down to the next generation. Acquired mutations are always involved in causing cancer. The following figure shows that a number of mutations may be involved in the development of cancer (Hodgson and Maher, 1993).
Mainly three types of genes are associated with cancer and these are oncogenes, TS genes and DNA repair genes (Boyer, 1998).

### 1.3.1.1. Oncogenes

Oncogenes are mutated forms of proto-oncogenes that cause normal cells to grow out of control and become cancer cells. Proto-oncogenes are a normal component of the genome necessary for cell function. Normally they control how often a cell divides and the degree to which it differentiates (or specializes). However, when altered or mutated, proto-oncogenes become oncogenes and promote development of cancer (Hodgson and Maher, 1993). When that happens, cells divide much more quickly.
leading to cancer. Proto-oncogenes may become activated to oncogenes by one or more of the following mechanisms (Barlow and Malkin, 2001)

(a) Fusion of proto-oncogenes with another gene by translocation or inversion: These events are frequently observed in leukemias, which is a translocation product involving the \textit{abl} oncogene with the \textit{bcr} gene.

(b) Juxtaposition with immunoglobulin regulatory elements or T-cell receptor genes in B- and T-cell lymphomas through chromosome breakage and reunion. Such rearrangement results in aberrant oncogene expression in immune cells that up-regulate lineage-specific genes necessary for their function.

(c) Amplification, for example, \textit{Myc} is amplified in the human leukaemia cell line HL-60.

(d) Point mutation. One such mutation occurs in \textit{Ras}, a member of a family of genes encoding proteins involved in signal transduction. When activated, these membrane-bound G-proteins transmit signals by phosphorylation to other molecules. The most common \textit{Ras} mutation results in a change from glycine to arginine at amino acid 12, which constitutively activates the protein and sends inappropriate growth signals to the nucleus.

1.3.1.2. Tumour-suppressor genes:

Present in normal cells, tumour suppressor (TS) genes inhibit cell proliferation by slowing down cell division and DNA repair, and telling cells when to die (through a process known as apoptosis or programmed cell death). Through these processes, they
can also suppress tumour development. However, when a TS gene is mutated, it can lead to tumour formation or growth (Boyer, 1998). Whereas oncogenes stimulate cell division and tumour development, TS genes normally act to inhibit these processes (Cooper, 1993). Therefore, formation of oncogenes and inactivation of TS genes represent complementary events in the development of cancer, both contributing to increased cell division and loss of normal cell growth. When TS genes do not work properly, cells can grow out of control, which can lead to cancer.

The properties of TS genes include the following:

(a) Both copies of a specific TS genes need to be mutated (both members of the gene pair) in order to cause a change in cell growth and tumour formation to occur (Cooper, 1993). For this reason, TS genes are said to be "recessive" at the cellular level.

(b) Mutations in TS genes are usually acquired. The two mutations in a TS gene pair may occur as the result of aging and/or environmental exposures (Boyer, 1998).

(c) A mutation in a TS gene can also be inherited. In these cases, a mutation in one copy of the TS gene pair is inherited from a parent, and is therefore present in all cells of a person (germline mutation) (Boyer, 1998). The mutation in the second copy of the gene (which is necessary for tumour formation and cell growth change) is acquired and usually occurs only in a single cell or a handful of cells. If the second "hit" or mutation occurs in a type of cell that needs this particular TS gene to control cell growth, the process of tumour formation will begin. This mechanism is also known as the "two-hit theory."
Most of the genes associated with hereditary cancer are TS genes and most mutations in TS genes are not inherited (Cooper, 1993).

About 30 TS genes have been identified, including p53, BRCA1, BRCA2, APC, and RB1. p53 is the most commonly mutated gene associated with cancer (Cooper, 1993).

1.3.1.3. p53 tumour suppressor gene

p53 TS gene, helps to regulate the cell cycle. p53, also known as tumour protein 53 (TP53), is a transcription factor that regulates the cell cycle and hence functions as a tumour suppressor (Cooper, 1993) as it is very important for cells in multicellular organisms to suppress cancer. Normally, if anything damages the DNA in the body's cells, protein p53 puts a brake on the cell cycle. When something goes wrong with the gene, such as a mutation, cell division can get out of control. Activation of the p53 pathway allows those affected cells to undergo DNA editing and repair while arrested in G1 phase, followed either by apoptosis or normal cell division (Lesser et al., 2001). Cellular repair systems set to work and, if necessary, get rid of the damaged cells by apoptosis. p53 plays a key role in ensuring that damaged cells are destroyed by apoptosis. But if p53 itself has mutated it no longer works properly so that proliferation of damaged cells goes on unchecked and cancer and its spread can occur. p53 plays a key role in many of the cell's anticancer mechanisms. It can induce growth arrest, apoptosis and cell senescence. Active p53 is induced after the effects of various cancer-causing agents such as UV radiation, oncogenes and some DNA-damaging drugs. DNA damage is sensed by 'checkpoints' in a cell's cycle that activate ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) which in turn can inhibit the cell cycle engine to go through either CHK1 or CHK2 (Ho et al.,
CHK1 and CHK2 are structurally unrelated but functionally overlapping serine/threonine kinases that are activated in response to many different genotoxic insults (Bartek et al., 2001). The activated check points then cause delay in cell cycle progression to facilitate either DNA repair or induce cell death (in order to eliminate damaged cells, and thus to protect the organism from cancer) (Zaugg et al., 2007).

CHK2 is activated mainly by ATM in response to double strand DNA breaks (DSBs) and its activation involves dimerization and autophosphorylation. In contrast, the labile CHK1 protein is largely restricted to S and G2 phases and it is active even in unperturbed cell cycle. However, CHK1 is further activated in response to DNA damage or stalled replication and this may not require dimerization or autophosphorylation of CHK1 (Bartek and Lukas, 2003). Oncogenes also stimulate p53 activation, mediated by the protein p14ARF. Some oncogenes can also stimulate the transcription of proteins which bind to MDM2 and inhibit its activity. Once activated p53 activates expression of several genes including one encoding for p21. p21 binds to the G1-S/CDK and S/CDK complexes (molecules important for the G1/S transition in the cell cycle) inhibiting their activity (Cooper, 1993). As outlined below, p53 is involved in anticancer mechanisms in a number of ways:

1. It can activate DNA repair proteins when DNA has sustained damage.
2. It can also hold the cell cycle at the G1/S regulation point on DNA damage recognition (if it holds the cell here for long enough, the DNA repair proteins will have time to fix the damage and the cell will be allowed to continue the cell cycle).
3. It can initiate apoptosis, if the DNA damage proves to be irreparable
1.3.1.4. DNA Repair genes

Cells are constantly subjected to DNA damage by being exposed to oxidative free radicals, gamma radiation, ultraviolet (UV) light, or endogenous and exogenous chemical agents (Barlow and Malkin, 2001). These agents induce single and double-strand DNA breaks, nucleotide base alterations and inter and intrastrand cross-links. Any of these changes ultimately may lead to development of cancer. When genes are altered or mutated, however, mismatches (mistakes) occur in the DNA. If these mistakes occur in TS genes or proto-oncogenes, eventually these will lead to uncontrolled cell growth and tumour formation (Boyer, 1998). Mutations in DNA repair genes can be inherited from a parent or acquired as a result of aging and environmental exposures. DNA repair genes require two mutations (both members of the gene pair) in order for the process of tumour formation to occur. It takes mutations in several of these genes for cancer to develop. In most cases of cancer, all the mutations are acquired. In inherited cancer, one mutation is passed down from the parent, but the remainder is acquired. Because it takes more than a single mutation to cause cancer, not all people who inherit a mutation in a TS gene, proto-oncogene, or DNA repair gene will develop cancer (Boyer, 1998).

1.3.2. Environmental causes of cancer:

The concept of environment is often used with a broad scope, including all non-genetic factors such as diet, lifestyle and infectious agents. In this broad sense, the environment is implicated in the causation of the majority of human cancers (Ellis, 2003: Boffetta, 2003). In a more specific sense, however, environmental factors include only the (natural or man-made) agents encountered by humans in their daily life, upon which they have no or limited personal control (Boffetta and Nyberg, 2003).
A number of causative factors related to environment are also responsible for the
growth of different types of cancer. Most cancers are caused by exposure to
carcinogens that can cause damage to DNA. As stated earlier, the majority of cancers
are the result of different environmental factors. A number of such factors are listed
below:

1.3.2.1. Alcohol Abuse

At least 3 percent of all cancers result from alcohol abuse (Ellis, 2003). It is believed
that alcohol acts as a promoter of carcinogens (Ellis, 2003). Women have a higher
chance of developing cancer of breast, liver, larynx and oesophagus and men that of
liver, larynx, oropharynx and oesophagus, due to higher consumption of alcohol
(Cooper, 1992). It is well known that excessive drinking of alcohol causes cirrhosis,
or scarring of the liver (Olver, 1998). This in turn can result in primary cancers of the
liver.

1.3.2.2. Emotional Factors

Emotional, psychological and behavioural factors such as long and unresolved
bitterness, loss of a loved one, acute depression may contribute to the development of
cancer (Ellis, 2003).

1.3.2.3. Indoor air pollution

Based on very high lung cancer rates observed in some regions of China and
elsewhere among women who spend much of their time at home, exposure to indoor
air pollution from combustion sources used for heating and cooking, as well as high levels of cooking oil vapours resulting from some cooking methods, have been identified as risk factors for lung cancer (Boffetta and Nyberg, 2003).

1.3.2.4. Inorganic arsenic in drinking water

Inorganic arsenic causes cancer at various sites in humans. The main source of environmental exposure to arsenic for the general population is through ingestion of contaminated water. A high level of arsenic in groundwater (up to 2–5000 µg/L) is found in areas of Argentina, Bangladesh, Bolivia, Chile, China (Xinjiang, Shanxi), India (West Bengal), Mexico, Mongolia, Taiwan, Thailand, the USA (Arizona, California, Nevada) and Vietnam (Boffetta and Nyberg, 2003). There is strong evidence of an increased risk of bladder, skin and lung cancers following consumption of water with high arsenic contamination (Boffetta and Nyberg, 2003). The evidence for an increased risk of other cancers, such as those of the liver, colon and kidney, are weaker but suggestive of a systemic effect.

1.3.2.5. Water chlorination by-products

Access to unpolluted water is one of the requirements of human health. Water quality is influenced by seasons, geology and discharges of agriculture and industry. Microbiological contamination of water is controlled by disinfection methods based on oxidants like chlorine, hypochlorite, chloramine, chlorine dioxine and ozone. Drinking water may contain a variety of potentially carcinogenic agents, including chlorination by-products (Boffetta and Nyberg, 2003). Showering and bathing represent another important source of exposure to chlorination by-products.
1.3.2.6. Aromatic hydrocarbons

Aromatic hydrocarbons such as benzene that is used as a starting material to make resins, rubbers, drugs, lubricants, dyes, detergents, nylon and synthetic fibres may be responsible for causing cancer of bladder and breast (Ellis, 2003; Rees et al., 1993). Often one or more of the metabolic products of aromatic hydrocarbons is more toxic than the parent compounds. For example, benzene epoxide produced from oxidation of benzene is more toxic and carcinogenic than the benzene itself (Bauer et al., 2003). These aromatic hydrocarbons more exactly their active metabolites that have electron-deficient regions on the molecular surface may induce oxidative stress due to glutathione depletion and cause DNA damage due to oxidation of nucleobases (Huq, 2006).

1.3.2.7. Heavy metals

Besides arsenic, a number of other heavy metals including cadmium, nickel, and chromium appear to be carcinogenic. These metals can cause cancer of lung, larynx and bladder (Souhami and Tobias, 2005). Even essential metal ions such as Cu$^{2+}$ and Fe$^{3+}$ that can undergo redox cycling can cause DNA damage due to oxidative stress that may result in genetic changes and ultimately cancer. Widely used platinum-based anticancer drugs such as cisplatin and carboplatin are also carcinogenic and may be responsible for the downstream production of cancer in a later part of life (Bauer and Brozoski, 2005).
1.3.2.8. Chloroform

Chloroform is a highly toxic chlorinated hydrocarbon that was used some time ago as an anaesthetic. It may be produced from chlorine vapours formed in hot showers. Studies showed that animals that drank chloroform containing water developed cancer of the liver and kidneys (Schoeny et al., 2006). Cough medicine high in chloroform content could be carcinogenic producing cancer of liver and kidneys (Ellis, 2003).

1.3.2.9. Industrial chemicals

Pesticides and other agricultural and industrial chemicals are also responsible for causing cancer of the bladder, breast and liver (Ellis, 2003; Rees et al., 1993). Formaldehyde is considered as a carcinogenic agent that causes cancer of bladder (Souhami and Tobias, 2005).

1.3.2.10. Food Processing Methods

Overheating, barbecuing or frying of dietary oils causes the production of carcinogenic benzopyrene (BP) and toxic polyhydrocarbons (PAH). These substances are strongly linked to cancer when ingested or inhaled in excessive amount. Carcinogenic PAHs are derived from phenanthrene. Additional rings and methyl groups of the three aromatic rings can convert inactive phenanthrene into an active carcinogen PAH (Pecorino, 2005). Heterocyclic amines (HAs) are also formed in protein-rich foods (meat and fish) during high temperature cooking such as frying and grilling. Since most HAs are potent mutagens and almost all are carcinogenic to laboratory animals, their formation in cooked foods is a health concern (Klassen et al., 2002).
1.3.2.11. Radiation

Excessive exposure to gamma and ultraviolet rays causes different types of cancers such as cancer of skin, sarcoma (soft tissue), ovary, leukaemia, breast and prostate (Rees et al., 1993). Overexposure to sunlight is also a major factor for the development of malignant melanomas on the skin. The risk is greater in fair-skinned people who have had periods of sunburn throughout their life. Ultraviolet (UV) radiation causes inflammation, gene mutation and immunosuppression in the skin (Halliday, 2005). These biological changes are responsible for photocarcinogenesis. UV radiation in sunlight is divided into two wavebands, UVB and UVA, both of which contribute to these biological changes, and therefore probably to skin cancer in humans and animal models (Halliday, 2005). Unprotected exposure to the sun exposes the skin to UVB radiation, which can directly alter the DNA by forming pyrimidine dimers and cause mutations (Pecorino, 2005). Exposure to UV radiation causes the production of reactive oxygen species (ROS) such as singlet oxygen [$^1\text{O}_2$], superoxide radicals [$\text{O}_2^-$], hydrogen peroxide [$\text{H}_2\text{O}_2$], and hydroxyl radicals [$\text{HO}^-$], which can also damage DNA (Lesser et al., 2001). Oxidative damage caused by UV radiation contributes to inflammation, gene mutation and immunosuppression (Halliday, 2005). Oxidative stress is also known to play an important role in apoptosis through $p53$. $p53$ expression has also been shown to upregulate the production of ROS, which then functions as a signal for the initiation of apoptotic pathways (Johnson et al., 1996). However, UV light is especially carcinogenic to the skin because it does not penetrate the body any deeper than skin. The skin is made up of squamous cells, basal cells and melanocytes. Skin cancers are classified by the cell type they affect: such as squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and melanoma respectively (Pecorino, 2005).
1.3.2.12. Poor Dietary Habits

Poor dietary habits are responsible for the 35% of cancer cases. Poor dietary habits may include (Ellis, 2003):

a) High intake of bacon, corned beef, maize oil, cottonseed oil, sassafras oil, champignon and mushrooms.
b) Excessive pepper may cause or exacerbate cancer.
c) Burnt foods like toast and fried pork may increase the risk of stomach cancer (Cooper, 1992).
d) Eating meats that contain antibiotics and other growth enhancing agents may provoke cancer.
e) Excessive fat intake, such as rancid, oxidized fat and altered vegetable oils may increase risk of colon and breast cancer (Cooper, 1992).
f) Excessive intake of caffeine (Cooper, 1992).
g) Excessive intake of simple sugars tends to feed cancer.
h) Food additives or contaminants that includes artificial additives, artificial colours, flavourings, artificial sweeteners (saccharin), propyl gallate, sulfites, BHT, BVO, BHA, MSG, sulphur dioxide etc.
i) High level of triglyceride and trans-fatty acids contributes towards cancer onset.
j) Inadequate antioxidant and immune system nutrients.
k) Low fibre intake may causes cancer of colon and rectum (Rees et al., 1993).
1.3.2.13. Recreational and Prescribed Drugs

Recreational drugs and prescribed drugs may also be responsible for causing cancer. Consumption of large amount of drugs like marijuana, LSD and valium may increase the growth of cancer cells (Ellis, 2003).

1.3.2.14. Tobacco Use

Cigarette smoke and other smoking accounts for 40% of all cancer deaths (Pecorino, 2005). About 81 carcinogens including PAHs have been identified in cigarette smoke. Consumption of two or more packets of cigarettes a day may increase the risk of developing cancer 20–25 fold. Cancer of lung, bladder, larynx and pancreas have been found to be associated with smoking (Rees et al., 1993).

1.3.2.15. Viruses

Sometimes viruses are also possible causes of cancer. Hepatitis B and hepatitis C viruses may be linked to liver cancer. A virus known as human papilloma may responsible for the cancer of cervix. Human immunodeficiency virus (HIV) and helicobacter may be other viruses that could increase one’s risk of developing cancer (Ellis, 2003).

1.4. Treatments of cancer

Although cancer is a dreaded disease, many types of cancer can be cured fully especially if they are detected early and for many others, the life span of the patients can be increased. The three most common modalities used for treatment of cancer are surgery, radiation therapy and chemotherapy (Parvez, 2005) that are often used in
combination. The choice of treatment varies depending upon the type of cancer and also the extent to which the disease has progressed (Cooper, 1992).

1.4.1. Surgery

Surgery involves the removal of cancerous tissue from the body. It is the primary treatment for many types of cancer. The early stage tumours that have not yet invaded surrounding normal tissue can be completely removed (Cooper, 1992). However, surgery may not be suitable for cancers that have already metastasized. Besides treatment, surgery can also confirm a diagnosis (Biopsy), determine how far a person’s cancer has advanced (stages), relieve side effects (such as obstruction), or ease pain (palliative surgery) (Lamb, 2002). One common side effect of surgery is pain. However, this can be successfully treated in most people (Goyns, 1999).

1.4.2. Radiation treatment

Almost half of all cancer patients receive some form of radiotherapy as part of their treatment (Goyns, 1999). In 2002 The Royal College of Radiologists reported that of those cured from cancer, 49% were cured by surgery, 40% by radiotherapy and 11% were by chemotherapy (Souhami and Tobias, 2005). Radiation therapy uses high-energy electromagnetic waves such as x-rays and gamma rays to kill cancer cells but they all have the same ability to kill the non-cancer cells as well (Goyns, 1999; Souhami and Tobias, 2005). Radiation therapy is a local treatment in which only a part of the body is exposed to the radiation. The goals of radiation therapy may include shrinking the tumour before surgery, keeping the tumour from returning after surgery, eliminating cancer cells in other parts of the body and relieving pain (palliative care). Radiation therapy can be given in two ways: internally and
externally. With internal radiation therapy, small tubes or implants (also called seeds) containing radioactive materials are placed in the body near the tumour (Cooper, 1992). With this therapy, the person does not need to come to the hospital every day to be treated, and the doctor can use a higher dose of radiation.

However, internal radiation therapy can only be used if the tumour is in a location where the doctor can place the implant. With external-beam radiation therapy, a machine directs high-energy rays at the tumour from outside the body. Before beginning external-beam radiation therapy, the doctor will plan where to aim the radiation. The goal is to hit as much of the tumour as possible, while minimizing the exposure of healthy tissue. Radiation therapy can damage normal cells, thus causing such side effects as tiredness (fatigue), swelling, redness or irritation of the skin, hair loss, diarrhoea, skin damage, sterility, cough or shortness of breath, mouth sores and digestive problems (Cooper, 1992). The side effects associated with radiation therapy may disappear once treatment is finished.

1.4.3. Chemotherapy

One of the most common ways to combat cancer is chemotherapy. As stated earlier, localized cancers can be treated effectively by surgery or radiotherapy, but the success of that treatment is limited by metastasis of cancer cells to distant body sites (Cooper, 1992). While use of surgery and radiotherapy can result in a cure in 40% (mainly smaller tumours) of all cancer patients, the remaining 60% of patients may still die as a result of metastasis (Meyskens, 1995; Verweij and de Jonge, 2000). One of the advantages of chemotherapy (which may also be its disadvantage) over surgery and radiotherapy is that it is a systemic treatment affecting the entire body so that it can be used to treat cancers that have metastasised (Galanski et al., 2003; Loehrer and
Einhorn, 1984). An ideal anticancer drug would cause injury only to cancerous cells without affecting the normal cells. In actual fact, all anticancer drugs cause some injury to normal cells as well. This is the reason why anticancer drugs often have various side effects including nausea, vomiting, and fatigue (Cooper, 1992). Depending on the drug, some people may also experience tingling or numbness in the arms and legs, hair loss, and mouth sores. Because drugs can damage blood cells, patients may experience anaemia (low red blood cell counts) or an increased risk of infection (low white blood cell counts) (Goyns, 1999). Side effects can usually be treated and often go away once the treatment is finished. During chemotherapy, a person may lose appetite or develop an aversion to the taste or smell of food. Drugs with different mechanisms are often used in combination to maximise the kill rate of cancer cells and minimise the side effects and development of resistance (Ho, 2004). More about combination therapy will follow later. Chemotherapy drugs use many different mechanisms to kill cancerous cells and work by blocking DNA replication, either by damaging the DNA directly or by interfering with other biomolecules (e.g. enzymes or proteins) necessary to make DNA or its building blocks and eventually causing the cells to die (Haskell, 1995; Ho, 2004).

1.4.4. Combination chemotherapy

In combination therapy, two or more treatment modalities are used to treat cancer patients. For example, chemotherapy can be given before or after radiotherapy. In combination chemotherapy, two or more drugs are given in combination to kill cancerous cells. Combination chemotherapy accomplishes the following three important objectives that may not be achieved with single-agent therapy (Olver, 1998):
1. Maximization of cell-kill within the tolerated range for each drug

2. A broader range of coverage of resistant cell lines in a heterogeneous tumour population

3. Prevention or slowing the development of new drug-resistant cell lines.

Selection of drugs for the combination regimens:

For the combination therapy, proper selection of drugs is a critical factor determining the outcomes. The following criteria serve as a guide for drug selection (Olver, 1998):

1. Only drugs known to be active as single agents should be selected for combinations and preferentially drugs that induce complete remissions should be included.

2. Drugs with different mechanisms of action should be combined in order to allow for additive or synergistic effects on tumours.

3. Drugs with differing dose-limiting toxicities should be combined to allow each drug to be given at full or nearly full therapeutic doses.

4. Drugs should be used in their optimal dose and schedule.

5. Drugs should be given at consistent intervals. The treatment-free interval between cycles should be the shortest possible time for recovery of the most sensitive normal tissue.

6. Drugs with different patterns of resistance should be combined to minimize cross-resistance.

1.5. DNA Binding

Since the discovery of cis-diamminedichloroplatinum(II) (or cisplatin) as an antitumour agent, much research has been focused on clarifying its properties and
biological target (Fichtinger-Schepman et al., 1984). There are many cellular components that can react with cisplatin e.g. RNA and glutathione. Therefore much efforts has been devoted to the investigation of interaction of cisplatin with DNA and its constituents, because DNA is likely to be the most relevant target in cells for anticancer drugs (Lippard, 1982). Thus many studies have been performed to establish the nature of platinum binding to the DNA (McA’Nulty et al., 1996). Through intensive research efforts over the past several years, there is now a fairly detailed understanding of how cisplatin and its analogues bind to DNA and how the DNA is structurally modified as a result of this interaction (Fichtinger-Schepman et al., 1984).

To better understand the binding of cisplatin and other metal-based anticancer drugs with DNA, it is essential to have a clear idea about the structure of the DNA, and its conformations and the binding sites that DNA provides for metal ions. For example, the primary target of platinum anticancer drugs in DNA is the N7 position of guanine bases in DNA (Baik et al., 2003).

1.5.1. DNA

The word ‘DNA’ is actually an acronym standing for deoxyribonucleic acid. It is a polynucleotide - a long polymer of nucleotides that encodes the genetic information in the sequence of nucleobases. Each nucleotide consists of a sugar (2’-deoxyribose), a nucleobase and a phosphate. The common form of DNA consists of two antiparallel polydeoxynucleotide strands intertwined with one another to form a right-handed double helix (Koolman and Rëohm, 2005). The backbone of DNA strands formed by 2’-deoxyribose and phosphate residues are linked by phosphodiester bonds (Koolman and Rëohm, 2005). The intertwined strands make two grooves of different widths,
referred to as the major groove and the minor groove, which may facilitate binding with specific proteins. It will be seen later that platinum and palladium complexes primarily bind to nucleobases in the major groove of the DNA. In the structure of DNA known as the B form, the helix makes a turn every 3.4 nm, and the distance between two neighbouring base pairs is 0.34 nm. More about DNA conformations will be considered later.

Figure 1.4: The normal right-handed double helix structure of DNA, also known as form B DNA (http://kedicikkopekcik.blogcu.com/3028629/)

There are about 10 base pairs per turn of the helix. The nucleobases found in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T) (Snustad and Simmons,
2006). Whereas adenine and guanine are purine bases, cytosine and thymine are pyrimidine bases.

The structures of the four nucleobases along with those of purine and pyrimidine are shown in Figure 1.5.

![Figure 1.5: Structures of nucleobases of the DNA](image)

When polynucleotide chain is formed, phosphate groups link to deoxyriboses at their 3’ and 5’ positions to form the backbone of DNA. In the double-stranded DNA, two of these polynucleotide chains are joined together inside by hydrogen bonds between nucleobases. Thymine is linked to adenine through two hydrogen bonds whereas cytosine is linked to guanine through three hydrogen bonds (Stryer et al., 1995). The pairing of the bases can be seen in Figure 1.6.
As stated earlier, DNA is a polynucleotide. More exactly, it is polymer of 2’-deoxyribonucleotide since the sugar present in DNA is 2’-deoxyribose (Nicklin et al., 2002). The variant part in a polynucleotide chain is the sequence of bases that carries the genetic information. Nucleotides are held together by phosphodiester bonds. More exactly the bond is between the 3’-carbon on one deoxyribose sugar and the 5’-carbon on the next to form the backbone of the molecule. The nucleobases are attached to the 1’-position of the sugar by glycosidic bond. The two strands of DNA run anti-parallel to one another, so the backbone of each strand of phosphate and ribose are identical to one another, retaining the 5’ to 3’ linkages. As shown below, in the DNA molecule, the sequence of bases in 5’ → 3’ direction is stated so that the complementary strand will run in the opposite direction (Nicklin et al., 2002).

5’–ACGTCTTAGCTAGC-3’
3’-TGCAGAATCGATCG-5’

Under physiological condition, the nature of DNA is important to consider due to its fundamental importance in metal DNA interactions. The low pKₐ value of the phosphate groups makes the DNA polynegatively charged in a large pH range. As a
result, the charge density is high, and the DNA attracts positively charged ions and repels negatively charged ones. In this respect, the nucleobases act as ligands and thus coordinate to positively charged metal ions (Lemma et al., 2000). The nucleobases are bonded at C1’ carbon atom of 2’-deoxy-D-ribose, which is a pentose sugar devoid of hydroxyl group at the 2’ position while phosphate is linked to either 3’ or 5’ position of 2’-deoxy-D-ribose. The illustration can be seen in Figure 1.7.

![Figure 1.7: Structure of 2’ nucleotide](image)

### 1.5.1.1. DNA Conformations

DNA being a flexible dynamic molecule can adopt several different conformations such as A-DNA, B-DNA, and Z-DNA (Koolman and Réohm, 2005; Snustad and Simmons, 2006). These conformations are dependent on the base sequence and/or bound proteins (Snustad and Simmons, 2006). B, A and Z DNAs are described as follows:

**B-DNA:** The Watson-Crick double helix DNA is called B-DNA (Montgomery et al., 1996). It is the most common form of DNA. Under the physiological condition (in aqueous solutions containing low concentrations of salts) DNA appears in the B-conformation. Intracellular B-DNA appears to have an average of 10.4 nucleotide
pairs per turn of the helix (Snustad and Simmons, 2006). It was stated earlier that between the two backbones of the two individual strands there are two grooves with different widths called the major and the minor grooves. The major groove in B-DNA is visible at the top and bottom, while the minor groove is in the middle. DNA binding proteins and transcription factors usually interact in the major groove, with its more easily accessible bases (Koolman and Rèohm, 2005). Both the grooves in B-DNA are of moderate depth and are well solvated by water molecules. The details about the major and minor grooves are described more fully in the next section. The following figure (Figure 1.8) gives a pictorial representation of A, B and Z-DNA.

![Comparison between A, B form and Z form](http://mol-biol4masters.org/Deoxy_Ribonucleic_Acid2-Structure.htm)

**A-DNA:** Like B-DNA, A-DNA is also right handed with 11 nucleotide pairs per turn. However, the bases are no longer arranged at right angles to the axis of the helix, as is
found in the B-form (Koolman and Réohm, 2005; Snustad and Simmons, 2006). In A-DNA base pairs are tilted because the deoxyribose moiety “puckers” differently (Montgomery et al., 1996). The minor groove almost disappear in A-DNA and the major groove is narrower than that in B-DNA (Koolman and Réohm, 2005). A-DNA is shorter and has a thicker double helix with a diameter of 2.3 nm. A-DNA almost never exists in vivo form (Snustad and Simmons, 2006). Under certain conditions, for example, in high concentration of salts or in a slightly dehydrated state, B-DNA can change to A-DNA (Koolman and Réohm, 2005; Montgomery et al., 1996; Snustad and Simmons, 2006).

**Z-DNA:** Z-DNA is left handed double helical form of DNA. The letter ‘Z’ stands for ‘zigzagged path’ applying to the sugar-phosphate backbone (Snustad and Simmons, 2006). Z-DNA can form within GC-rich regions of B-DNA (Koolman and Réohm, 2005). This structure results from the alternating anti and syn conformations of the purine bases relative to the deoxyribosyl unit (Montgomery et al., 1996). Z-DNA has a smaller pitch than B-DNA. The physiological significance of Z-DNA remains unknown (Koolman and Réohm, 2005).

The following table summarizes details of A-DNA, B-DNA and Z-DNA (Blackburn and Gait, 1996).
Table 1.1: Average helix parameters for the major DNA conformations.

<table>
<thead>
<tr>
<th>Structure type</th>
<th>Helix sense</th>
<th>Sugar pucker</th>
<th>Groove width/Å</th>
<th>Groove depth/Å</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Minor</td>
<td>Major</td>
</tr>
<tr>
<td>A-DNA</td>
<td>R</td>
<td>C3’-endo</td>
<td>11.0</td>
<td>2.7</td>
</tr>
<tr>
<td>B-DNA</td>
<td>R</td>
<td>C2’-endo</td>
<td>5.7</td>
<td>11.7</td>
</tr>
<tr>
<td>Z-DNA</td>
<td>L</td>
<td>C3’-endo(syn)</td>
<td>2.0</td>
<td>8.8</td>
</tr>
</tbody>
</table>

1.5.1.2. More on major and minor grooves of DNA

As stated earlier, two types of unequal grooves called major and minor grooves exist in A-DNA, B-DNA and Z-DNA. The grooves exist because the glycosidic bonds of a base pair are not diametrically opposite to each other. The N7 position of purine bases (both guanine and adenine) are exposed in the major groove of DNA double helix (Trimmer and Essigmann, 1999). The minor groove is narrow and shallow, locates between the two strands and contains the pyrimidine O2 and the purine N3 of the base pair. The major groove runs between the turns of helix, is slightly wider and deeper, exists on the opposite of the pair. Each groove has several hydrogen bond donor and acceptor centres. N3 of guanine and adenine, O2 of guanine are potential hydrogen acceptors. As applied to tumour active platinum complexes, grooves in DNA play a key role by providing binding sites for platinum. It is found that only the N7 positions of guanine and adenine in the major groove (being nucleophilic and most accessible) are capable of coordination to the platinum and out of these two sites the N7 position of guanine is more preferred (Yang and Wang, 1998).
The figure below shows the major and minor grooves of DNA including their relative positions. The N-9 and N-1 atoms of purines and pyrimidines bases respectively are connected to the DNA deoxyribose-phosphate backbone.

Figure 1.9: Watson–Crick base pairs, showing the N-7 atoms of purines, which represent the principal sites of platinum co-ordination.
2. Chapter two: Metal-based anticancer drugs

Although medicinal applications of metals can be traced back to the 16th century (Desoize, 2002), the discovery of antitumour activity of cisplatin in 1965 and its subsequent clinical development in 1971, was the catalyst for the major research effort being applied in investigating the potential of metal compounds in cancer therapy (Fricker, 1994). Metal ions, being positively charged, are favoured to bind to negatively charged biomolecules, and the constituents of proteins and nucleic acids provide excellent donor centres. Metal ions are known to exert an inductive effect by coordination to the site of reaction and serve as redox sites that function by either electron or atom transfer (Keppler, 1993).

The pharmaceutical use of metal complexes has excellent potential and a broad array of medicinal applications of metal complexes have been investigated (Zhang and Lippard, 2003). However, heavy metals and their complexes are also well known for their toxic effects. Acute heavy metal poisoning results in severe gastrointestinal symptoms, particularly nausea, vomiting, diarrhoea, and abdominal pain (Rademaker-Lakhai et al., 2004). Accumulation of metal ions in the body can lead to deleterious effects. The kidney is frequently affected by heavy metals, because they accumulate in and damage renal tubular cells resulting in disturbance of renal metabolic systems. Chronic low-level exposure to heavy metals can cause neuromuscular injury (McKeage, 1995). A number of factors such as bio-distribution and clearance of the metal complexes and its pharmacological specificity, and favourable physiological
responses *in vitro* study and *in vivo* investigation using xenografts and animal models need to be considered before the targeted compounds can enter clinical trials. Nevertheless a mechanistic understanding of how metal complexes achieve their activities is crucial to their clinical success, as well as to the rational design of new compounds with improved potency (Zhang and Lippard, 2003).

The current development of successful metallo-pharmaceuticals, which include the platinum anticancer drugs (Jamieson and Lippard, 1999) and radio-diagnostic agents, (Clarke and Podbielski, 1987) all indicate the utility of complexes as both therapeutic and diagnostic agents. The greatest success of inorganic chemistry is the discovery of cisplatin and its analogue carboplatin and their introduction in the clinic and also the numbers of tumour active platinum compounds that are currently under clinical trials.

### 2.1. Platinum-based anticancer drugs

Platinum, palladium, ruthenium, rhodium, osmium, and iridium together make up a group of elements known as platinum group metals (PGM) due to similarity in their chemical properties. Platinum is a transition metal found lying at group VIII in the periodic table. The principal oxidation states of platinum are +2 and +4. In the +2 state it forms mainly square planar complexes and in the +4 state it forms mainly octahedral complexes. The higher states +5 and +6 also occur in a few fluoro compounds. In general, platinum (II) has a high affinity for sulfur and nitrogen, and is expected to bind to S and N centres of peptides and proteins and to N centres of nucleobases in DNA. Although the exact mechanism of action of platinum-based anticancer drugs is not known, it is believed to be associated with their binding with nucleobases in DNA.
Platinum is the most important metal used in the treatment of cancer over the last decade (Guo and Sadler, 2000). The first platinum-based anticancer drug is cisplatin and as stated earlier it is one of the most widely used anti-cancer drugs. Cytotoxic drugs were first considered for therapy of some cancers around 1950 (Kennedy, 1991). A large jump in the cure rate for several cancer types was achieved between 1950 and 1990 (from 0% for all in 1950 to 75%, 80% and 90% respectively), especially for childhood, acute lymphoblastic leukaemia, Hodgkin’s disease, and testicular tumours (Krakoff, 1991). Other, relatively common, types of cancer (Boring et al., 1994), including head and neck, large bowel, stomach, pancreatic, liver, cervical and melanoma, remain mostly refractory to cytotoxic chemotherapy, with no demonstrable prolongation of life (Krakoff, 1991).

Out of thousands of synthesized and evaluated platinum complexes, only three namely cisplatin, carboplatin, and oxaliplatin, have been approved for worldwide clinical practice (in 1978, 1993, and 2002, respectively), while nedaplatin, lobaplatin and heptaplatin have been approved as anticancer agents only in Japan, China and South Korea, respectively (Hartinger et al., 2006). In the next section more detailed information about cisplatin will be given.

### 2.2. Cisplatin

Cisplatin, more exactly *cis*-diamminedichloroplatinum(II) and also known as *cis* DDP, is one of the most widely used anticancer drugs. It is a simple complex in which a Pt(II) ion is covalently bonded to two chlorine atoms and two ammonia molecules in a *cis*-geometry. It entered clinical trial in 1971 and became available in market in 1978 (Lebwohl and Canetta, 1998). Cisplatin has a broad spectrum of activity against different types of cancers. It can be used both as a single agent or in combination in
the treatment of many types of cancer including testicular and ovarian cancer, non-small cell lung cancer (NSCLC), esophagus, bladder, cervix, endometrium gastric and head and neck cancer (Baik et al., 2003; Hay and Miller, 1998). It is also included among the most effective cytotoxic agents in head and neck cancer, with single agent response rates ranging from 25% to 30% (Marcu et al., 2005). Besides its different spectrum of clinical efficacy, cisplatin causes many unwanted toxic side effects, including nausea and vomiting, neurotoxicity, nephrotoxicity, myelosuppression and ototoxicity (Cooley et al., 1994; Go and Adjei, 1999). Moreover, cancer cells become resistant due to the continued use of cisplatin. This resistance can be caused by a number of cellular adaptations, including reduced uptake, inactivation by glutathione and other anti-oxidants, and increased levels of DNA repair or DNA tolerance (Piccart et al., 2001; Rabik and Dolan, 2007). More about cisplatin toxicity and resistance factors will follow later on this section.

2.2.1. Historical background of cisplatin

Cisplatin was first synthesised in 1845 by Peyrone (Peyrone, 1845). Its biological activity was accidentally discovered by Barnett Rosenberg in 1965 (Rosenberg et al., 1967; Rosenberg et al., 1965; Trimmer and Essigmann, 1999). During an experiment designed to study the effect of electric fields on growth of *Escherichia coli* bacteria, Rosenberg observed that cells held between charged platinum electrodes grew in size but did not divide (Abu-Surrah, 2007; Trimmer and Essigmann, 1999). A number of compounds were used during electrolysis and later only cisplatin showed the ability to hinder cell division. Therefore, the inhibitory effect of cisplatin on cell division suggested that cisplatin might have potential characteristics as an anticancer agent (Rosenberg et al., 1967; Rosenberg et al., 1965; Trimmer and Essigmann, 1999).
Approval of cisplatin for treatment of testicular and ovarian cancer was given in 1978 (Weiss and Christian, 1993). Now cisplatin is one of the most commonly used chemotherapeutic agents for the treatment of different types of human cancer (Trimmer and Essigmann, 1999).

### 2.2.2. Chemistry of cisplatin

As stated earlier, cisplatin is a simple inorganic complex in which a Pt(II) ion is covalently bonded to two Cl⁻ ions and two ammonia molecules in a cis-geometry. It is a neutral, square planar and water-soluble molecule (Kelland, 1993). The trans isomer of cisplatin is called transplatin. In transplatin also, platinum (II) ion is covalently bonded to two Cl⁻ ions and two ammonia molecules but in a trans geometry (Figure 2.1).

![Figure 2.1: Structures of cisplatin and transplatin](image)

Cisplatin is anticancer active, but transplatin is inactive but toxic. The toxicity of transplatin is believed to be due to its higher reactivity so that it is increasingly deactivated before binding with DNA (Lippert, 1996). This idea is consistent with the observation that trans-geometry can be activated for antitumour activity by the introduction of one or two bulky planar amine ligands (Huq et al., 2007).

Both cisplatin and transplatin bind with nucleobases in DNA (mainly guanine and adenine). Whereas cisplatin forms mainly intrastrand 1, 2-d (GpG) and d(ApG)
adducts, transplatin is more likely to form interstrand d(GpG) adducts (Lippert, 1996). Transplatin cannot form 1,2-intrastrand adducts because of its geometrical limitation.

### 2.2.3. More about cisplatin and transplatin

Both cisplatin and transplatin have two chloride ligands that are less tightly bound (and hence are called the leaving groups) and two ammonia ligands that are more tightly bound (and hence are called the carrier ligands). As stated earlier, whereas cisplatin forms mainly intrastrand bifunctional adducts, transplatin forms mainly interstrand bifunctional adducts. Although both cis and trans adducts inhibit DNA replication, cisplatin displays much greater cytotoxicity than transplatin. As noted earlier, whereas cisplatin is highly tumour active transplatin is in fact considered to be inactive but toxic (Loehrer and Einhorn, 1984). Also, cisplatin is found to have greater mutagenic properties than transplatin (Fichtinger-Schepman et al., 1982; Lippert, 1996; Roberts and Thomson, 1979; Zlatanova et al., 1998). Studies with cells that are deficient and proficient in DNA repair fail to show any correlation between concentrations of cisplatin that inhibit DNA synthesis and cell death (Sorenson and Eastman, 1988). Thus, the effect of platinum compounds on DNA replication is not regarded as a major contributor to their cytotoxicity. As stated earlier, a fundamental reason for the difference in activity of cisplatin and transplatin is related to their reactivity. Transplatin being more reactive than cisplatin, ligand displacement reactions would be faster for transplatin than cisplatin as a result of which there would be greater deactivation of transplatin before binding with DNA and greater repair of transplatin-DNA adducts.
2.2.4. Administration of cisplatin

Cisplatin in solution is administered as an intravenous injection in a variety of dose schedules. The dosages range from 5 mg/m\(^2\)/day intravenously for 5 days (Lokich and Zipoli, 1984). Dosage schedules range from 15 min bolus every 3-4 weeks to daily infusions for 3-7 days. The length of the infusion will depend on the protocol used. The dose of cisplatin is based on clinical response and the renal, haematologic and otic tolerance of the patient (Cooley et al., 1994). To alleviate the problems of toxic side effects, cisplatin therapy is preceded by prehydration.

2.2.5. Clinical use of cisplatin

Cisplatin is one of the most frequently used anticancer drugs. It is one of the most active agents against testicular, lung, cervical, bladder and head and neck cancers. It is used as a single agent and in combination regimens to treat a variety of solid tumours (Cooley et al., 1994; Highley and Calvert, 2000). Cisplatin is also used to treat Hodgkin's and non-Hodgkin's lymphomas, neuroblastoma, sarcomas, multiple myeloma, melanoma, and mesothelioma (Highley and Calvert, 2000). Due to its successful contribution, testicular cancer has become a model for curable neoplasm. The survival rate of the patients has increased to 80% today as compared to 5% in the early 1970’s (Einhorn, 2002).

2.2.6. Cisplatin side effects

As stated earlier, although cisplatin is one of the most widely used anticancer drugs, cisplatin chemotherapy is associated with a number of side effects including nephrotoxicity, neurotoxicity, hepatotoxicity, bone marrow suppression, ototoxicity, nausea, vomiting and hair loss (Hofmann et al., 2006). These toxicities are dose-
dependent, limiting therapy and maximum tolerated dose (MTD). For cisplatin, MTD is considered to be 100 to 120 mg/m$^2$ per cycle and should be administered with adequate pre- and post-hydration (Hartmann and Lipp, 2003; Markman, 2003). However, accidental overdose of cisplatin may occur. Although reaction to chemotherapy is different from person to person, nearly all people who are treated with cisplatin experience gastrointestinal problems. Figure 2.2 summarizes toxic side effects of cisplatin and its analogues carboplatin and oxaliplatin, followed by a more detailed description.

Figure 2.2: Toxicities associated with treatment with cisplatin (Rabik and Dolan, 2007).
2.2.6.1. Nausea and vomiting

Nausea, vomiting and anorexia are common side effects of cisplatin (Go and Adjei, 1999), that may occur immediately after treatment (≤24 h) or may be delayed (>24 h). These can be devastating and so severe that it may be dose limiting (Cooley et al., 1994) resulting in interruption or termination of treatment (Morrow et al., 1998). It has been reported that patients (without pre-medication with antiemetics) experience on the average more than ten emetic episodes after a dose of 120 mg/m$^2$ (Go and Adjei, 1999). Although a range of antiemetic agents are available, use of a 5HT3 antagonist together with a glucocorticoid steroid is the current preferred regimens (Gralla, 1998; Piccart et al., 2001). It may be noted that antiemetic regimens carry their own risk of adverse events which include mild headache, constipation or diarrhoea with 5HT3 antagonists and sleep disturbances with corticosteroids (Piccart et al., 2001). It is also important to note that, despite the use of a 5HT3 antagonist, patients still rank nausea and vomiting among the top three most distressing chemotherapy-associated adverse events (de Boer-Dennert et al., 1997).

2.2.6.2. Neurotoxicity:

Neurotoxicity is one of the well-known dose limiting side effects of cisplatin. The incidence of neuropathy is found in about 49-92 % patients with peripheral neuropathies being most common (Cooley et al., 1994). Numbness of fingers and toes is the first symptom. This can progress to difficulty with fine motor skills such as buttoning shirts. The most severely affected patients can lose sensation to the hip, which often impairs balance and ambulation (Cooley et al., 1994). Neurotoxicity is commonly irreversible (Alberts and Noel, 1995; Quasthoff and Hartung, 2002).
2.2.6.3. Nephrotoxicity

Nephrotoxicity is another well-known dose-limiting toxicity of cisplatin, found in 30–50% of patients. It becomes more severe and prolonged with repeated courses of the drug (Cooley et al., 1994; Go and Adjei, 1999; Piccart et al., 2001). The severity of nephrotoxicity almost led to abandonment of the compound until its management was achieved through forced hydration (O'Dwyer et al., 2000). Pre- and post-treatment hydration with intravenous saline and mannitol diuretics effectively reduces cisplatin associated nephrotoxicity, although renal toxicity may still occur even after adequate hydration (Piccart et al., 2001). It can manifest as acute renal failure or as a more chronic disease characterized by electrolyte wasting (Hutchison et al., 1988). Morphological studies showed that mainly the proximal tubule is affected (Dobyan et al., 1980). This is a metabolically active region where most of the filtered NaCl is reabsorbed through active transport. Subsequent events, including impairment of distal tubular reabsorbance, renal blood flow and glomerular filtration, result in enhanced excretion of enzymes, protein and other electrolytes including magnesium and potassium (Daugaard and Abildgaard, 1989). Today nephrotoxicity is no longer a dose limiting side effect as it can effectively be ameliorated by prehydration and mannitol stimulated diuresis (Finley et al., 1985). Nephrotoxicity can range from mild and reversible to severe and irreversible renal failure. Additional manifestations include hyperuricemia and electrolyte imbalances (Cooley et al., 1994).
2.2.6.4. **Myelosuppression**

Cisplatin causes mild and transient myelosuppression specifically neutropenia and thrombocytopenia (Figure 2.2) (Rabik and Dolan, 2007). It is a dose-related side effect, reversible and possibly cumulative (Cooley et al., 1994; Go and Adjei, 1999). All three hematopoietic cell lines can be affected (Go and Adjei, 1999). Cisplatin causes myelosuppression in 25% to 30% of patients and is considered to have a mild hematological toxicity profile (McKeage, 1995). The incidence of severe thrombocytopenia or leukopenia is approximately 5% to 6% of patients (Cooley et al., 1994; Go and Adjei, 1999).

2.2.6.5. **Ototoxicity**

Ototoxicity is also another dose limiting side effect of cisplatin. It appears to be dose related and cumulative beginning at cumulative doses of 200 mg/m² (Schaefer et al., 1996). Risk factors that increase the development of ototoxicity include: (a) age of >40, (b) rapid infusion, (c) preexisting hearing loss and (d) concurrent use of ototoxic drugs (Cooley et al., 1994). Ototoxicity occurs in approximately 23-54% of adult patients and more than half of pediatric patients receiving cisplatin treatment (Figure 2.2) (Rybak and Whitworth, 2005). In adults, bolus higher doses of cisplatin are found to be more ototoxic and nephrotoxic than repeated infusions of lower doses (Reddel et al., 1982). For children, prolonged infusions are less nephrotoxic than bolus doses (Lanvers-Kaminsky C, 2006). Cisplatin damages the outer hair cells of the cochlea (inner ear) that results in functional deficits (Rybak and Whitworth, 2005). The mechanism involved underlying these side effects is most likely the production of ROS in the cochlea, which can trigger cell-death pathways. The most common
characteristics of cisplatin-induced ototoxicity is loss of the high-frequency ranges of hearing (Laurell et al., 1987). Prolonged use of cisplatin can affect the speech hearing range. The initial symptom is tinnitus, which may occur as early as 4 days after the treatment. The tinnitus may progress to unilateral or bilateral hearing loss (Cooley et al., 1994). In cases of severe hearing loss, patients may have difficulty hearing speech in the presence of background noise (Cooley et al., 1994). Not all patients with tinnitus may develop more severe ototoxicity (Reddel et al., 1982) e.g. one-third of patients with tinnitus do not develop audiometric abnormalities. Once hearing loss occurs, it appears to be irreversible in the majority of cases; 26% of patients may have a partial recovery (Aguilar-Markulis et al., 1981). Careful monitoring of audiometry should be performed prior to initiation of therapy and prior to subsequent doses of cisplatin.

2.2.7. Biochemical mechanisms of action of cisplatin

Although much progress has been made in understanding the mode of action of cisplatin and the mechanisms involved in its antitumour activity (Jamieson and Lippard, 1999), the knowledge available on the mechanisms of action of the drug remains incomplete. What is clear is that the primary target of cisplatin is genomic DNA, preferably the N7 position of guanine bases (Baik et al., 2003). Different steps that cisplatin undergoes after entry into the cell are described as follows:

2.2.7.1. Cellular accumulation of cisplatin

As stated earlier, cisplatin is administered to patients through intravenous injection. Being a neutral molecule it can diffuse rapidly into cells by passive diffusion (Gately and Howell, 1993). It is also transported into and out of the cells by active transport
(Bose, 2002; Cepeda et al., 2007). However, most of the cisplatin molecules (about 90%) are deactivated due to their binding with albumin and other plasma proteins containing thiol groups (Cepeda et al., 2007; Judson and Kelland, 2000). Loss of the chloride ligands from the cisplatin molecule facilitates its binding to genomic DNA. The steps involved in the binding of cisplatin with cellular DNA from entry into the cell, hydrolysis inside the cell to produce much more reactive aquated species followed by their binding with nucleobases in DNA are given in Figure 2.3 (Guminski et al., 2002; Lloyd and Kelland, 1999). A more detailed description of hydrolysis of cisplatin followed by binding of with DNA and other cellular targets is given in the following paragraph.

Figure 2.3: The pathway of the accumulation of cisplatin in the cell (Guminski et al., 2002; Lloyd and Kelland, 1999). A (entering the cell), B (in the cytoplasm) and C (in the nuclear).
2.2.7.2. **Hydrolysis of cisplatin**

As noted earlier whereas cisplatin is a relatively inert molecule, its aquated species are highly reactive (as water is a much weaker ligand than chloride ion) so that hydrolysis of cisplatin is believed to be the key step in the activation of the molecule for its binding with cellular targets including DNA (Miller and House, 1991). Whereas in the blood stream (plasma), hydrolysis of cisplatin would be suppressed because of high chloride concentration (100 mM), that within the cell would take place readily because of a much lower chloride ion concentration (4 mM) in the cytoplasm (Sedletska *et al.*, 2005). It should also be noted that even though cisplatin may remain neutral in the blood plasma, substantial loss of platinum still occurs due to the reaction of cisplatin with proteins and extracellular enzymes (Bose, 2002). Within the cell, the chloro ligands of cisplatin may be replaced by water in a step-wise manner to form \( \text{cis-}[\text{PtCl(H}_2\text{O})(\text{NH}_3)]^+ \) and \( \text{cis-}[\text{Pt(H}_2\text{O})_2(\text{NH}_3)]^{2+} \) cations. Figure 2.4 gives a schematic representation of the fate of cisplatin in aqueous solution (at physiological pH) (Bose, 2002; Pil and Lippard, 1996).
Figure 2.4: Schematic representation of the fate of cisplatin in aqueous solution at physiological pH.

It is believed that the formation of the mono aqua species cis-[PtCl(H₂O)(NH₃)]⁺ is sufficient to initiate further reaction with biomolecules including DNA (Hausheer et al., 1998). Thus, once cis-[PtCl(H₂O)(NH₃)]⁺ is formed, the second chloride is also replaced either via a second aquation process or direct reaction of the mono chloro species with cellular platinophiles such as DNA, RNA, proteins and thiols such as glutathione and metallothioneins (Bose, 2002; Trimmer and Essigmann, 1999).

2.2.7.3. Binding to DNA and other cellular targets

Even though only about 1% of cisplatin molecules bind with DNA (Ho et al., 2003; Perez, 1998), binding between cisplatin and DNA in the cell nucleus is believed to be largely responsible for its anticancer properties (Gonzalez et al., 2001). The N7 atoms
of guanine and adenine located in the major groove of the double helix are the most accessible and reactive nucleophilic sites for platinum coordination to DNA (Cepeda et al., 2007). Pt$^{2+}$ binds to the N7 centres to form both monofunctional and bifunctional DNA adducts. In the first DNA-binding step, monofunctional adducts are formed preferentially at the N7 atoms of guanine residues located in the major groove of DNA in B-conformation (Loskotova and Brabec, 1999). Binding to N7 position of guanine is preferred over that in adenine. This is because whilst stabilization is produced in the case of guanine due to the formation of hydrogen bond between coordinated water molecule and G-O6, binding with adenine is associated with repulsive interaction (Arpalahti, 1996). This is illustrated in Figure 2.5.

![Figure 2.5: Hydrogen bonding stabilization in guanine and destabilization in adenine](image)

It has been shown that monofunctional adducts of cisplatin distort DNA (Brabec et al., 1994; Brabec et al., 1992; Bursova et al., 2005), disturbing the stacking interactions in double-helical DNA due to its local unwinding (by about 6°). However, no intrinsic bending is induced in DNA as a result of the binding. Monofunctional adducts of cisplatin also reduce thermal stability of DNA in a sequence-dependent manner (Brabec et al., 1992; Van Garderen et al., 1989). Monofunctional platinum-DNA adducts further react to form bi-functional adducts (Figure 2.5) (Fichtinger-Schepman et al., 1982). The bifunctional adducts can be
grouped into six major categories: 1,2-intrastrand d(GpG) adduct between adjacent guanines; 1,2-intrastrand d(ApG) adduct between an adjacent adenine and guanine; intrastrand adducts between purines separated by one or more intervening bases [e.g. d(GNG) where N stands for a nucleotide]; interstrand adducts linking the two strands of the DNA double helix; monofunctional adduct coordinated to a single purine; and protein–DNA cross-link (where cisplatin coordinates to a protein molecule and a nucleobase) (Ho et al., 2003). Figure 2.6 illustrates different types of binding modes of cisplatin to DNA and proteins (Bose, 2002).
Formation of therapeutically inactive monofunctional platinum adducts also occurs at N3 position of dC, N7 position of dG, and N1 and N7 position of dA (Zlatanova et al., 1998).

Figure 2.6: Various binding modes of cisplatin to DNA and proteins (Bose, 2002).
As stated earlier, the most common adduct is 1,2-d(GpG) intrastrand cross-link between adjacent guanines (Perez, 1998) (Figure 2.6). It is generally assumed that the key binding mode for exhibiting cisplatin-induced cytotoxicity is also 1,2-d(GpG) intrastrand DNA adduct, which bends the double helix towards the major groove (Bose, 2002; Cepeda et al., 2007) (Figure 2.7).

Figure 2.7: A depiction of the effect of the major 1,2- GG intrastrand cross-link in a double-stranded oligonucleotide. The bending of the DNA backbone towards the major groove is clearly perceptible.

All bifunctional adducts cause major distortions of the local DNA structure, involving both bending and unwinding of the double helix (Trimmer and Essigmann, 1999). Both 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links unwind DNA by 13°, while the 1,3-d(GpXpG) intrastrand cross-links unwind DNA by 23°. Intra- and interstrand cross-links cause very different structural alterations in the double helix, which may be recognized and processed differently by different cellular factors. Nevertheless, the distortion creates a large kink in the B-form DNA as demonstrated by X-ray structural
and NMR studies (Admiraal et al., 1987; Sherman et al., 1988). There is still debate as to which types of cisplatin-DNA adducts are the most important in mediating the cytotoxicity of cisplatin. Due to the formation of intrastrand, interstrand and DNA-protein cross-links, the damaged DNA then becomes inactivated and thus unable to replicate (Cooley et al., 1994; Eastman, 1987). However, increasing evidence indicates that intrastrand adducts provide the strongest basis for the cytotoxic action of cisplatin (Siddik, 2002). Cisplatin-DNA adducts are suggested to exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis (Meyn et al., 1995; Moufarij et al., 2003; Sorenson et al., 1990). However, bending of the DNA double helix is similar for the three types of intrastrand adducts (Comess and Lippard, 1993). DNA then binds to the high mobility group (HMG) domains of proteins. Binding of HMG triggers structural rearrangements, preventing DNA transcription activity and inducing recognition by damage repair proteins, ultimately resulting in cell death through apoptosis, necrosis, or both (Robertazzi and Platts, 2005).

2.2.7.4. Cisplatin induced cell death pathways: apoptosis and necrosis

The major goal of cancer chemotherapy is to commit tumour cells to death following exposure to anticancer agents. It is believed that cisplatin induces cell death mainly by apoptosis (Gonzalez et al., 2001) and to some extent by necrosis. Apoptosis may be defined as programmed cell death (PCD). It is considered to be a controlled pathway that requires ATP (adenosine triphosphate) and de novo protein synthesis (Wang and Lippard, 2005). Futile attempts to repair cisplatin-DNA adducts are believed to induce
cytotoxic processes that finally end up in the triggering of apoptosis (Gonzalez et al., 2001). However, as noted earlier, the great majority of cisplatin molecules bind to proteins rather than DNA and such binding also plays a role in triggering apoptotic pathways (Gonzalez et al., 2001; Kruidering et al., 1998). It has been reported that some types of cancer cells when exposed to cisplatin show internucleosomal DNA degradation in approximately 180 base pair fragments, blebbing of the cell surface, and cell shrinkage, all of which is consistent with apoptosis as a mode of cell death (Henkels and Turchi, 1997). Besides this, it has also been reported that, in other cell lines, particularly those with resistance to the drug, cisplatin produces characteristic features of necrosis, which is considered a mode of cell death due to general cellular machinery failure (Matsumoto et al., 1997; Perez et al., 1999). The mode of cell death induced by cisplatin is also concentration dependent (Gonzalez et al., 2001). TG gene p53 that plays a key role in maintaining genomic integrity may be involved in the induction of apoptosis by cisplatin (Gonzalez et al., 2001). DNA damage by cisplatin may lead to over expression of p53 protein that subsequently induces both expression of downstream p21WAF protein and G1 phase cell cycle arrest (Reed, 1999). In the event of irreparable DNA damage, p53 protein induces apoptosis (Fisher, 1994). The p53 gene also directly affects expression of other downstream genes that regulate sensitivity to apoptosis, activating transcription of proapoptotic Bax and repressing transcription of antiapoptotic Bcl-2 proteins (Eastman, 1999). On the other hand, although cisplatin may initiate apoptosis through pathways modulated by p53, this TS gene is not always required for apoptosis (Gonzalez et al., 2001). In some ovarian tumour cell lines, cisplatin treatment decreases or even abolishes p53 protein levels. Interestingly, most of these cisplatin-treated cells have features characteristic of necrotic cell death (Gonzalez et al., 2000).
Apoptosis and necrosis have been traditionally considered as two separate modes of cell death from the consideration of both morphology and mechanism (Wyllie, 1987). In the 1980s, necrosis was considered to be the mode of cell-death induced by DNA-damaging anticancer agents because of the activity poly (ADP ribose) polymerase-1 (PARP-1) (Eguchi et al., 1997). PARP-1 is activated by the DNA strands breaks caused by anticancer agents and cleaves the glycolytic coenzyme NAD$^+$, provoking the formation of poly(ADP-ribose) moieties. Depletion of NAD$^+$ inhibits glycolytic production of ATP with subsequent ATP depletion that leads to necrotic cell death (Fuertes et al., 2003). However, by the 1990s, it was thought that most of the clinically effective anticancer agents that bind to DNA kill cancer cells by apoptosis.

An outstanding contribution to the study of the biochemical mechanisms of cell death was the discovery by the end of the 1990s that intracellular ATP levels dictate whether antitumour drugs like cisplatin as well as other chemical and physical agents induce cell death by necrosis or apoptosis and that both processes of cell death are linked (Cepeda et al., 2007). Figure 2.8 shows an outline of the interconnections between apoptotic and necrotic pathways within the cell in response to cisplatin assault (Cepeda et al., 2007).
Figure 2.8: Biochemical cell death pathways induced by cisplatin showing the interconnections between apoptosis and necrosis (discontinuous arrows) depending on the metabolic condition of the tumor cell.

It may be seen that activation of caspases, before PARP-1 induced cleavage of NAD\(^+\), induces depletion of ATP levels to cause cell death by necrosis, also leads to execution of apoptosis. Caspase blocking by inhibitors of apoptosis (IAPs) plus continued activity by PARP-1, and the decrease in ATP level from the loss of electron transport in broken mitochondria, lead the cell to necrosis because of continuation of PARP-induced ATP depletion (Fuertes et al., 2004). It has also been reported that failure of PARP-1 cleavage enhances apoptosis (Hercog and Wang, 1999).

The above discussions suggest that, rather than a functional opposition between necrosis and apoptosis, there is a functional cooperativity between the two processes. Some cells might die as a result of an unfinished apoptotic program. For example, in
L1210 murine leukemic cells, cisplatin-induced cell death might be the consequence of a defective apoptotic program that lacks some morphological and biochemical properties attributed to “classic” apoptosis (Segal-Bendirdjian and Jacquemin-Sablon, 1995).

2.2.8. Resistance

Drug resistance that is often responsible for the failure of the drugs presents a major problem in cancer chemotherapy (Ohmichi et al., 2005). Cisplatin is no exception - some cancer cells (e.g. melanoma cancer cells) are intrinsically resistant to cisplatin whilst others (e.g. ovarian cancer cells) develop the same due to repeated use (Fram, 1992). Drug resistance can occur at many different points from administration of the drug to the growth of the tumour cell (Links and Brown, 1999). Although, the exact mechanisms of resistance are not known, it is believed that resistance to cisplatin may be associated with one or more of the following: (1) reduced cell uptake, (2) increased deactivation within the cell before binding with DNA and (3) increased DNA repair. These are described more fully as follows.

2.2.8.1. Drug uptake/efflux

A major mechanism of resistance to cisplatin is decreased effective concentration of the drug in the cell (Rabik and Dolan, 2007). For example, reduction in cisplatin concentration of 20-70% has been observed in a number of cell lines resistant to cisplatin including ovarian, testicular, cervical, breast, head and neck (Kartalou and Essigmann, 2001; Kelland, 1993; Siddik, 2003). Reduced intracellular accumulation of cisplatin may be due to decreased uptake and/or increased efflux. To date the exact mechanism by which cisplatin is taken-up by the cells is not fully understood.
although both passive diffusion and carrier-mediated transport are believed to be involved (Safaei, 2006). The idea that cisplatin can cross the cell membrane by passive diffusion arises from the fact that cisplatin is a neutral molecule and remains so in a medium where chloride concentration is high (such as that found in blood plasma), and is supported by the observation that intracellular accumulation of cisplatin proceeds linearly with time over a period of 60 min and does not reach a plateau up to a drug concentration of 1 mM (Binks and Dobrota, 1990). It was therefore suggested that passive diffusion was the main mechanism by which cisplatin enters the cell (Cepeda et al., 2007). Moreover, recent observations point towards a direct connection between the cellular concentrations of copper and platinum leading to the proposition that cisplatin may cross the cell membrane by both carrier-mediated transport and passive diffusion. Gately and Howell proposed that approximately one-half of cisplatin uptake takes place by passive diffusion and the other half by facilitated diffusion (Gately and Howell, 1993; Kartalou and Essigmann, 2001). Ctr1 (a high-affinity copper transporter) is the main uptake transporter and experiments with the yeast protein Ctr1 and cisplatin, revealed that mutation or deletion of the Ctr1 gene leads to stronger cisplatin resistance and intracellular reduction of platinum levels in yeast and in mouse cells (Ishida et al., 2002; Safaei, 2006). In human cells, the main Cu uptake transporter is the 190 amino acid Cu transporter1 (hCtr1) (Moller et al., 2000), while results of studies with transgenic mice indicate that Ctr1 is essential for the survival of mammalian embryos (Lee et al., 2001). Ctr1 predominantly resides in the plasma membrane (Klomp et al., 2002). Boulikas and Vougiouka (Boulikas and Vougiouka, 2003) reported that deletion of Ctr1 resulted in a 16-fold reduction in the uptake of copper and 8-fold reduction in the uptake of cisplatin. Ctr1-deficient cells were 1.9-fold more resistant to the cytotoxic effect of
cisplatin than the Ctrl-replete cells (Boulikas and Vougiouka, 2003). Interestingly, cisplatin accumulation in cells increased when human Ctrl gene was overexpressed, although the ability of the drug to access its cytotoxic targets was unaltered (Holzer et al., 2004). The mechanism by which the Ctrl transports Cu across the plasma membrane remains to be determined. It has been suggested that Ctrl binds Cu via the methionine and histidine-rich amino terminal domain and transports it across the membrane through pores which it forms by oligomeric association (Klomp et al., 2002; Lee et al., 2001). Besides Ctrl there are other Cu influx transporters available in the cell, one of which is divalent metal transporter 1 (DMT1) (also known as natural resistance associated macrophage protein 2, Nramp2) (Puig and Thiele, 2002). However, the involvement of DMT1 transporter in Cu influx relative to Ctrl in human tumour cells remains to be determined.

On the other hand, mammalian cells have two structurally similar efflux transporter and these are P-type ATPases, ATP7A and ATP7B. These efflux transporters that mediate the efflux of Cu, are found to be associated with cisplatin resistance in vitro in various cancers (Safaei, 2006). Increased expression of ATP7A and ATP7B is linked to reduced level of binding with DNA thus reducing its cytotoxicity (Kelland, 2007a; Safaei, 2006). Defects in ATP7A and ATP7B produce Menkes and Wilson’s disease respectively. In both cases, impaired Cu efflux results in excessive accumulation of Cu in the brain, liver and kidney (Safaei et al., 2004). ATP7A is expressed in most tissues and intestinal epithelium except liver (Murata et al., 1997; Vulpe et al., 1993). ATP7B is more expressed in liver and kidney but less in brain of normal individuals (Tanzi et al., 1993).
2.2.8.2. Increase deactivation

Increased deactivation by binding with cellular platinophiles such as glutathione (GSH) (Figure 2.9) and metallothionein (MT) before binding with DNA is a major mechanism of resistance to platinum drugs. In the resistant cancer cell lines, concentrations of these thiol-containing platinophiles are often found to be elevated thus offering greater resistance due to increased deactivation (Siddik, 2003). Although cisplatin, given as intravenous injection, remains largely as an undissociated neutral molecule in the blood plasma, it becomes aquated in the cytoplasm to form positively charged species that react readily with GSH, MT and other thiol-containing platinophiles before they reach DNA in the nucleus of tumour cells (Brabec and Kasparkova, 2002; Rabik and Dolan, 2007).

Generally, sulfur-containing biomolecules, such as those containing thiols are known to be highly reactive towards cisplatin. On the other hand, biomolecules containing thioethers (e.g. methionine residue), including isolated methionine molecules, also form platinum–sulfur adducts. However, these adducts have been postulated to be a drug reservoir for DNA platination meaning they may function as intermediates that are transformed into platinum–DNA adducts. (Barnham et al., 1995; van Boom et al., 1999).

![Figure 2.9: Structure of glutathione](image)

Figure 2.9: Structure of glutathione
Oxidative stress, glutathione and other antioxidants:

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are damaging to proteins, DNA, cell membrane and other biomolecules, are a part of aerobic life so that cell has defense mechanisms in the form of antioxidants to remove these reactive species. Glutathione is an important cellular antioxidant that helps to maintain a balance between the production and removal of ROS and RNS. As stated earlier, cisplatin is detoxified by glutathione through adduct formation (Rudin et al., 2003). Several ovarian cell lines known to be resistant to cisplatin showed a correlation between the degree of resistance and the levels of GSH, likely due to increased d-glutamylcysteine (Godwin et al., 1992). An additional member of the antioxidant defense system is thioredoxin (Trx) which, like glutathione, regulates the oxidation-reduction environment of the cell (Arner et al., 2001). Thioredoxin is involved in the regulation of transcription factors, apoptosis, and DNA synthesis, among others (Rabik and Dolan, 2007). An MT is a very low molecular weight protein comprised of several cysteine and aromatic amino acid residues (Siegsmund et al., 1999). Interestingly, MTs are thought to be involved in controlling levels of copper and zinc, as well as protecting cells from oxidative stress and toxicities associated with heavy metals, including copper, cadmium, and zinc (Siegsmund et al., 1999; Toyoda et al., 2000). It may be noted that only a very small amount of platinum (<5%) is found to be coordinated to DNA, and most of the platinum was coordinated to the thiols. Similarly, DNA modification by cisplatin was totally abolished by thiol-blocking agents or thiol drugs (Sadowitz et al., 2002) and glutathione is known to protect bacteria by covalently trapping platinum before it can bind to DNA (Salles and Calsou, 1992).
2.2.8.3. Increased DNA repair

Increased DNA repair is a major contributor that leads to clinical drug resistance (Reed, 1998). DNA repair is believed to be one of the principal mechanisms of cisplatin resistance. Both intrinsic and acquired resistance have been found to be associated with DNA repair (Masuda et al., 1988; Zeng-Rong et al., 1995). Cell lines selected for resistance to cisplatin after prolonged culture in the presence of cisplatin have significantly higher levels of repair than the corresponding parental cell lines (Kartalou and Essigmann, 2001), indicating that DNA repair is an important determinant of cisplatin resistance. Moreover, differential capacity to repair cisplatin adducts is postulated to be responsible for part of the variability in clinical response to cisplatin based chemotherapy (Jones et al., 1994). A cell line established from the tumour of an ovarian cancer patient that was not responding to chemotherapy had a three-fold higher repair synthesis activity than the cell line established from the tumour of the patient prior to the onset of resistance (Lai et al., 1988).

2.2.8.4. Nucleotide excision repair (NER)

Nucleotide excision repair (NER) is one of the major pathways responsible for the removal of platinum adducts and repair of DNA damage (Siddik, 2003). The significance of NER is highlighted by the finding that a cellular defect in this pathway results in hypersensitivity to cisplatin, and that restoration of NER integrity re-establishes sensitivity to normal levels (Chaney and Sancar, 1996; Furuta et al., 2002). NER has broad specificity, and no differences are observed in the excision of adducts induced by cisplatin and structurally diverse platinum-based drugs (Chaney and Vaisman, 1999). Indeed, enhanced repair of adducts in resistant cells also applies
to platinum analogues that are effective against the resistant phenotype (Jennerwein et al., 1991), and this suggests that increased repair as a mechanism of resistance may be difficult to overcome through the platinum analogue drug development process. Although the NER complex is composed of at least 17 different proteins (Friedberg, 2001; Sancar, 1994), it appears that up regulation of only a few rate-limiting proteins is necessary to increase the excision repair capacity in resistant tumour cells (Reed, 1998). The following figure (Figure 2.10) illustrates the steps in NER (Brabec and Kasparkova, 2002).
Figure 2.10: Biochemical steps involved in NER.

Different intracellular proteins recognize the cisplatin-DNA adduct and induce NER. It is observed that the 1,3-GG intrastrand adduct is more efficiently repaired than the 1,2-GG intrastrand or 1,2-AG intrastrand adducts (Huang et al., 1994; Zamble et al.,
Certain proteins like XPA, XPE and ERCCI-XPF have a significant role in DNA repair such that an increased level of the proteins indicates increased DNA repair. Recent observation that cisplatin sensitive testicular cancer has a low level of XPA and ERCCI-XPF provides support to the idea (Koberle et al., 1999).

2.2.8.5. Mismatch repair

Mismatch repair is a post-replication repair system that corrects unpaired or mispaired nucleotides (Cepeda et al., 2007). The mismatch repair pathway (MMR) has also been proposed to be involved in cisplatin resistance (Fink et al., 1996; Rabik and Dolan, 2007). The presence of MMR is thought to mediate cisplatin- and carboplatin-induced apoptosis (Karran et al., 2003; Obmolova et al., 2000). Tumour cells deficient in MMR are 2-3 fold more resistant to cisplatin treatment compared to cells proficient in MMR (Stojic et al., 2004). Similarly, restoration of hMLH1 expression in a resistant derivative of the A2780 ovarian cell line that lacked hMLH1, restored cisplatin sensitivity to the level of the parent line (Durant et al., 1999). The mismatch repair system involves at least five proteins (MLH1, MSH2, MSH3, MSH6 and PMS2) and functions as a repair mechanism that needs ATP (Cepeda et al., 2007; Fishel, 2001). The relationship between DNA damage recognition by MMR proteins and cytotoxicity remains undefined. However, there is evidence that other cellular repair mechanisms, MMR can affect antitumour efficacy of cisplatin (Aebi et al., 1997; Fink et al., 1996) and that dysfunction of this type of DNA repair may result in cisplatin resistance or tolerance (Johnson et al., 1998). The function of the MMR is to scan newly synthesized DNA and remove mismatches that result from nucleotide incorporation errors made by DNA polymerases. To explain cisplatin tolerance, it is assumed that replication bypass of DNA adducts of cisplatin leads to mutations.
During MMR, the strand to be corrected is nicked, a short fragment containing the mismatch is excised, and new DNA fragment is synthesized (Brabec and Kasparkova, 2002). The MMR system always replaces the incorrect sequence in the daughter strand, which would leave the cisplatin adduct unrepaired. During DNA synthesis to replace the excised short fragment, the DNA polymerases would again incorporate mutations followed by attempts to remove them. The repeated nicks in DNA formed at each ineffective cycle of repair could trigger a cell death response (Brabec and Kasparkova, 2002). This activity initiates a futile cycle (Figure 2.11).

Figure 2.11: Hypothetical mismatch repair of 1,2-GG intrastrand cross-link of cisplatin
2.2.8.6. Evasion of apoptosis

Chemotherapy and radiotherapy kill proliferating cells mainly by apoptosis. Experimental models indicate that a critical balance exists between cell cycle arrest (that may allow DNA repair and drug resistance) and apoptosis (chemosensitivity) (Baird and Kaye, 2003). An intricate network of factors has evolved to control this balance. The response to chemotherapy may also be attenuated through the over expression of other anti-apoptotic proteins, or the reduced activity of pro-apoptotic proteins. Probably the exact mechanisms of chemotherapy-induced cell death depend on the cell type, the drug given, the tumour microenvironment and a host of other factors.
3. Chapter three: Mono and poly-nuclear platinum Complexes

3.1. Mononuclear platinum complexes

As stated earlier, cisplatin is a widely used, routinely employed drug for the treatment of ovarian cancer, testicular cancer, and head & neck tumours (Abu-Surrah, 2007). Since its introduction into the clinic in 1971, cisplatin has had a significant impact on the treatment of cancer (Roberts et al., 2005; Sadler and Guo, 1998). However, as noted earlier, the drug has several undesirable side effects including nausea, vomiting, nephrotoxicity and myelotoxicity. Also in some cases, tumour cells become resistant to further treatment following the initial treatment. The adverse effects of cisplatin and its limited spectrum of activity prompted a parallel synthesis effort to design more effective and less toxic analogues (O'Dwyer et al., 2000). Based on the successful results of cisplatin, Cleare and Hoeschele defined a set of “structure-activity relationships” rules that platinum compounds needed to follow to have activity similar to that of cisplatin (Wheate and Collins, 2005).

More about Structure-Activity relationships:

The criteria that together constitute the classical structure-activity relationships are the following:

(a) Must be neutral;

(b) Have two monodentate leaving groups or one bidentate leaving group with a lability in the “window of reactivity” centred on chloride;
(c) Have the leaving groups in the *cis*-configuration;

(d) Not contain hydroxy or hydroxo ligands that make the complexes highly toxic

(e) Have *non-leaving* or carrier ligands, preferably amine or ammine ligands that are inert.

This chapter describes the development of new platinum drugs. The chapter is divided into two sections namely second generation and third generation platinum drugs. It will be seen that the majority of the compounds obey the classical structure-activity relationships, however an increasing number of tumour active compounds are being synthesized that violate the rules in one way or another (e.g. there are now tumour active platinum compounds with *trans*-geometry and compounds that have two or more platinum centres). This chapter also gives a brief preview on the development of other metal-based tumour active compounds besides platinum.

### 3.2. Development of new platinum complexes

Since the introduction of cisplatin in the last forty years, thousands of mononuclear platinum complexes has been synthesized and evaluated for their anticancer activity (Abu-Surrah, 2007; Jakupec *et al.*, 2003; Kelland, 2007a; 2007b). The main aim of these investigations was to obtain drugs of similar anticancer activity but with reduced toxicity as compared to cisplatin (Abu-Surrah, 2007). Among them, only 33 platinum agents entered clinical trials of which only carboplatin has received worldwide approval so far and another four drugs have gained only regional limited approval include oxaliplatin (that has gained approval in a few countries), nedaplatin (that has gained approval in Japan) and lobaplatin (that has gained approval in China and SKI 2053R in South Korea) (Abu-Surrah and Kettunen, 2006; Galanski *et al.*, 2003; Jakupec *et al.*, 2003; Kauffman and Cowan, 1963). Cisplatin is considered to be a
parent drug or first generation platinum drug while carboplatin and other analogues of cisplatin that possess carboplatin-like structures are considered as second generation platinum drugs (Jakupec et al., 2003; Kelland, 1993). Other platinum drugs, particularly chelates containing 1,2-diaminocyclohexane (DACH), compounds containing sterically hindered ligands and the evolving field of non-classic (trans- and multinuclear) platinum complexes are considered to constitute third generation platinum drugs (Jakupec et al., 2003). The next three sections provide a review of second- and third-generation platinum drugs.

3.2.1. The second generation platinum drugs

As stated earlier, the analogues of cisplatin carrying chelating carboxylate leaving groups are known as the second generation platinum drugs (Jakupec et al., 2003). One such compound is carboplatin that has received worldwide approval to treat cancer. It has been found that modification of cisplatin to contain less labile leaving groups alters both the pharmacokinetics and the toxicity profile of the drug. Thus the replacement of the chloride leaving groups of cisplatin with a cyclobutanodicarboxylato ligand to produce carboplatin (Fig. 3.1) reduced the toxicity while retaining antitumour activity similar to that of cisplatin (Galanski et al., 2003; Harrap, 1985). At effective doses, carboplatin produced substantially less nausea, vomiting, nephrotoxicity and neurotoxicity than cisplatin, (Calvert et al., 1989). The adducts formed by cisplatin and carboplatin are largely identical, thus providing an explanation for their similar anti-tumour activity. Besides carboplatin, only another second generation platinum drug, namely nedaplatin, has been approved for clinical use (Jolley et al., 2001). The development of second generation platinum drugs is described in detail in this section.
3.2.1.1. Carboplatin

Carboplatin or Paraplatin is cis-diammine-1,1-cyclobutaneacarboxylateplatinum(II) abbreviated as CBDCA (Abu-Surrah, 2007; Jakupec et al., 2003; Kelland, 1993). It was developed by Johnson Matthey and displays a spectrum of activity similar to cisplatin (Coluccia et al., 1993). Carboplatin was approved by the US Food and Drug Administration (FDA) in 1988 (Kelland, 2002). To date, carboplatin is the only other platinum drug, besides cisplatin, to have obtained worldwide approval for clinical use (Galanski et al., 2003; Jolley et al., 2001; Perez et al., 1991). Figure 3.1 gives the structure of Carboplatin.

![Figure 3.1: Structure of carboplatin](image)

Carboplatin has gained popularity in clinical treatment due to its vastly reduced side-effects (Ho et al., 2003). Carboplatin exhibits cross-resistance with cisplatin so that the two drugs are only active against the same types of tumours (Barefoot, 2001). Phase III trials have demonstrated the equivalence of carboplatin and cisplatin in the treatment of ovarian cancer (Alberts et al., 1992) but in testicular and head and neck cancers, cisplatin appears to be superior (Bajorin et al., 1993). The tolerability of carboplatin is better than that of cisplatin and has been attributed to its higher stability but lower reactivity (Jakupec et al., 2003). Although carboplatin causes little or no nephrotoxicity and neurotoxicity as compared to cisplatin (Lippert, 1999), bone
marrow suppression remains the dose-limiting toxicity for the drug. The reduced toxicity of carboplatin is attributed to the slow nucleophilic substitution (by water) of the chelated dicarboxylato leaving group whereas the low reactivity of the compound is attributed to the slowness in ring opening (Barefoot, 2001; Neidle et al., 1980). As a result, carboplatin can be administered at much higher doses (900 mg/m²) than cisplatin (60-120 mg/m²). Because carboplatin has a spectrum activity the same as that of cisplatin, several analogues of carboplatin were developed in the hope of improving on the anticancer activity of carboplatin (Abu-Surrah, 2007).

3.2.1.2. Nedaplatin

Nedaplatin (cis-diammine-glycolate-O,O'-platinum(II), 254-S) is another successful second-generation platinum drug developed by Shionogi Pharmaceutical Co., Osaka, Japan (Hasegawa et al., 2004). It has the same ammine carrier ligands as cisplatin (Tsuda et al., 2004) but instead of two chloride leaving groups has a five-membered glycolate ring structure that acts as a bidentate ligand (Monk et al., 1998). It was first synthesized in mid 1980s and has been widely used in Japan for the past 15 years to treat various types of cancer such as ovarian cancer, head and neck cancer and lung cancer (Jakupec et al., 2003; Kelland, 2007a). The antitumour activity of nedaplatin is comparable to that of cisplatin (Kobayashi et al., 1991) but it is less toxic to the kidney, as seen in preclinical experiments (Kameyama et al., 1990). Figure 3.2 gives the structure of nedaplatin.
Nedaplatin has higher solubility in water (about ten times more) than cisplatin (Tsuda et al., 2004) and shows very limited binding to plasma protein (Sugeno et al., 1991). The pharmacokinetic profile of nedaplatin is similar to that of carboplatin (Sasaki et al., 1989). The dose limiting toxicity of nedaplatin appears to be myelosuppression, especially thrombocytopenia (Tsuda et al., 2004) Nedaplatin produced promising response rates in phase II trials for the treatment of SCC arising from the head and neck (Inuyama et al., 1992) lung (Yamamoto et al., 2000) oesophagus (Taguchi et al., 1992) and uterine cervix (Noda et al., 1992) Also in Phase II studies, nedaplatin monotherapy generated a response rate of 46.3% in cervical cancer and 53.1% in patients with squamous cell carcinoma (Kato et al., 1992)

### 3.2.1.3. Miboplatin (DWA2114R)

Miboplatin or DWA2114R is 2-aminomethyl-pyrrolidine (1,1-cyclobutanedicarboxylato)platinum(II) a water soluble analogue of carboplatin (Holford et al., 1998a; Holford et al., 1998b; Kelland, 1993). Preclinical studies showed that the antitumour activity of miboplatin is similar to that of cisplatin and carboplatin against a number of murine tumours (Kelland, 1993) In general, carboplatin and miboplatin are found to be less nephrotoxic than cisplatin in rodents (Matsumoto et al., 1991). Figure 3.3 gives the structure of miboplatin.
In K562 human leukemia cells, the cytotoxic activities of miboplatin are found to be more dependent on exposure time than cisplatin (Kobayashi et al., 1991). The DNA adducts formed by miboplatin are similar to those of cisplatin. It is believed that the bulky ligand of DWA2114R may inhibit nucleotide excision repair (Matsumoto et al., 1991). The phase II study revealed that the response rate in ovarian cancer cells is about 44% whereas that in breast, prostate and lung cancers ranges from 15% to 20% (Majima, 1991). The compound has been abandoned because of lack of any advantages over cisplatin (Jakupec et al., 2003).

### 3.2.1.4. Zeniplatin

Zeniplatin is \([\text{2,2-bis(aminomethyl)-1,3-propanediol-}N,N']\text{[1,2-cyclobutane dicarboxylato(2-)O,O']platinum(II)}\). It is another water-soluble analogue of carboplatin. In human tumour xenograft and animal tumour models, zeniplatin showed a spectrum of antitumour activity different from that of cisplatin and carboplatin (Aamdal et al., 1997; Kelland, 1993). It also showed a broader spectrum of antitumour activity without any significant nephrotoxicity in advanced renal cancer and advanced malignant melanoma (Aamdal et al., 1997). Figure 3.4 gives the structure of zeniplatin.
Zeniplatin showed anticancer activity in ovarian carcinoma, advanced non-small-cell lung cancer and metastatic breast cancer (Aamdal et al., 1997; Meijer et al., 1992). Zeniplatin showed cross-resistance in two cisplatin-resistant human tumour cell lines (GLC₄-small cell lung cancer and Tetra-embryonal carcinoma) (Meijer et al., 1992). However it is found that zeniplatin has nephrotoxicity similar to that of cisplatin and causes myelosuppression similar to carboplatin (Jakupec et al., 2003).

### 3.2.1.5. Enoplatin

Enoplatin is [1,1-cyclo-butanedi-carboxylato(2-)0,0][tetrahydro-4H-pyran-4,4-dimethanamine-N,N]platinum(II). It is also an analogue of carboplatin. In vivo the compound was found to be showed most active in cisplatin-resistant L1210CPR murine leukaemia. In breast MX-1 and ovarian H207 human tumour xenografts, it shows activity similar to that of cisplatin and carboplatin (Kelland, 1993). Figure 3.5 gives the structure of enoplatin.

![Figure 3.5: Structure of enoplatin (CL287, 110)](image)
In GLC₄-small cell lung cancer, and tetra-embryonal carcinoma, enoplatin showed partial lack of cross-resistance to cisplatin (Meijer et al., 1992). However, (like zeniplatin) enoplatin was found to have nephrotoxicity similar to that of cisplatin and to cause myelosupression similar to carboplatin stated earlier so that the compound was abandoned.

### 3.2.1.6. **CL973 (NK121)**

CL973 or [SP-4-3-(R)]-1,1-cyclobutane-dicarboxylato (2-) or (2-methyl-1,4-butanediamine-N, N')platinum is a water-soluble platinum diamine analogue of carboplatin. In some respect the compound may be regarded as a carboplatin–DACH hybrid platinum complex (Kelland, 1993). Figure 3.6 gives the structure of CI-973 (NK121).

![Figure 3.6: Structure of Cl-973 (NK121)](image)

It has shown activity against cisplatin-resistant tumours leukaemia (L1210, P388, K562) and human ovarian carcinoma sublines (Elliott et al., 1994; Kelland, 1993; Theriault et al., 1996). CI-973 has similar problems to that of miboplatin and has been abandoned because of the lack of any advantages over cisplatin (Jakupec et al., 2003).
3.2.1.7. Iproplatin

Iproplatin is cis-dichloro-trans-dihydroxo-bis(isopropylamine)platinum(IV), also known as JM9 or CHIP (Kelland, 1993). It was the first Pt(IV) complex possessing an octahedral configuration (rather than the square-planar configuration of cisplatin and carboplatin) that entered extensive clinical trials. Figure 3.7 gives the structure of iproplatin.

![Structure of Iproplatin](image)

Figure 3.7: Iproplatin (also known as CHIP and JM9)

Iproplatin was selected for clinical evaluation because of its favourable efficacy profile in preclinical studies (such as less nephrotoxicity). It shows activity in cisplatin-responsive tumours but shares cross-resistance with cisplatin and carboplatin (Foster et al., 1990). In randomised trials, iproplatin was found to be more toxic and significantly less active than carboplatin, thus limiting its clinical utility (Kelland, 1993). Myelosuppression is the dose-limiting toxicity of iproplatin. The compound failed to provide any advantage over carboplatin in terms of response and survival of patients with advanced ovarian cancer; this led to cessation of its further development (Jakupec et al., 2003).
3.2.1.8. Lobaplatin

Lobaplatin is (1,2-diamminomethylcyclobutane-platinum(II) lactate, code named D-19466). It is a diastereometric mixture of platinum(II) complexes, containing 1,2-bis(amminomethyl)cyclobutane as the carrier ligand and lactic acid as the leaving group (McKeage, 2001). Figure 3.8 gives the structure of lobaplatin.

![Structure of lobaplatin](image)

Figure 3.8: Structure of lobaplatin

Lobaplatin is water-soluble and is approved for the treatment of chronic myelogenous leukemia and inoperable metastatic breast and small-cell lung cancer in China. However, the drug is not under consideration for development in the Western world (Barefoot, 2001; Jakupec et al., 2003; Perabo and Muller, 2007; Welink et al., 1999). The dose limiting toxicity of lobaplatin is thrombocytopenia. Another side effect is mild gastrointestinal toxicity. No incidence of nephrotoxicity, neurotoxicity and ototoxicity has been reported (Fiebig et al., 1991; Jakupec et al., 2003; Saris et al., 1996). Lobaplatin forms mainly intra-strand GG and AG cross-links with DNA (McKeage, 2001). The compound is found to be less reactive than cisplatin towards isolated DNA.

3.2.1.9. Cycloplatin

Cycloplatin (CP) or amminecyclopentylamine-S-(−)-malatoplumum(II), is a novel antitumour platinum compound, synthesized in Russia (Drees et al., 1995; Nersesyan et al., 2003). Cycloplatin has shown antitumour activity in murine tumour models and
it is not nephrotoxic (Jakupec et al., 2003). Figure 3.9 gives the structure of cycloplatin.

![Figure 3.9: Structure of cycloplatin](image)

The literature on the activity and toxicity of CP appears to be conflicting. The compound is found to be more potent but less toxic than cisplatin and carboplatin (Kelland, 1993; Nersesyan et al., 2003).

### 3.2.2. The third generation platinum drugs

The second generation platinum drugs carboplatin and nedaplatin, although having approval for clinical use in many countries, did not provide any advantage over cisplatin in terms of spectrum of activity (Wong and Giandomenico, 1999). Thus, the search for better platinum drugs with much reduced side effects and wider spectrum of activity continues (Wong and Giandomenico, 1999). During the development of third generation platinum drugs, many different structural forms of drugs were investigated and most of them belonged to the following two different groups: (a) 1,2-diamminocyclohexane (DACH)-containing platinum complexes (e.g., oxaliplatin) and (b) sterically hindered platinum complexes (e.g., picoplatin or ZD0473).
3.2.2.1. 1,2-Diamminocyclohexane containing platinum complexes

The 1,2-diamminocyclohexane- or DACH-platinum compounds are a series of compounds containing a diaminocyclohexane carrier ligand (Connors et al., 1972; Kidani et al., 1976; Rixe et al., 1996). These compounds have a unique cytotoxicity profile as compared to cisplatin and exhibit collateral sensitivity in some cisplatin-resistant cell lines (Burchenal et al., 1980). The clinical development of DACH platinum drugs was initially hampered due to unacceptable toxicities; however, significant interest has been rekindled by the development of the less toxic analogue, oxaliplatin ((DACH-(oxalato)platinum(II)). Oxaliplatin has shown activity alone or in combination with 5 fluorouracil/leucovorin in colon cancer, a disease that was previously considered to be unresponsive to platinum drugs (Cvitkovic and Bekradda, 1999). Some of the DACH-containing compounds are described in the following paragraphs:

3.2.2.2. Oxaliplatin (Eloxatin)

Oxaliplatin or trans-L-diaminocyclohexaneoxalatoplatinum(II) is a novel water-soluble, recently developed third generation cisplatin analogue, characterized by the presence of a rigid diaminocyclohexane (DACH) moiety as the carrier ligand (Carrato et al., 2002; Kidani et al., 1978; Misset et al., 2000). Figure 3.10 gives the structure of oxaliplatin. It is active against several types of tumours including some cisplatin/carboplatin-refractory diseases such as colorectal cancer (Carrato et al., 2002; Raymond et al., 1998). First synthesised by Kidani and his co-workers in Japan in 1978 (Kidani et al., 1978), it has been developed successfully for the treatment of colorectal cancer in France (Barefoot, 2001; Extra et al., 1990). Currently it is registered for use in advanced colorectal cancer in the United States, Europe, Asia and
Latin America (Faivre et al., 2003). Furthermore, oxaliplatin in combination with 5FU–LV has been found to be active in metastatic gastric cancer (Di Lauro et al., 2007; Kelland, 2007a; Louvet et al., 2002).

Figure 3.10: Structure of oxaliplatin

Oxaliplatin is used to treat patients with colorectal cancer whose disease has recurred or become worse following initial therapy with a combination of other drugs (Rixe et al., 1996). In preclinical in vitro and in vivo models oxaliplatin has been shown to be synergistic with other anticancer drugs, including cisplatin, carboplatin, 5-FU, cyclophosphamide, irinotecan, topotecan and gemcitabine (Misset et al., 2000). The administration of oxaliplatin in combination with 5-Fluorouracil has been approved in Europe, Asia and Latin America for the treatment of metastatic colorectal cancer (Boulikas and Vougiouka, 2003). Unlike cisplatin, oxaliplatin is devoid of nephrotoxicity and ototoxicity. It is less myelosuppressant than carboplatin (Boulikas and Vougiouka, 2003). However, oxaliplatin induces a dose-limiting cumulative peripheral sensory neuropathy (Levi et al., 2000). In vivo, oxaliplatin undergoes extensive nonenzymatic biotransformation (Graham et al., 2000). Nucleophiles, such as endogenous bicarbonate and dihydrogen phosphate, have been suggested to displace the oxalato group forming unstable reactants that can be hydrolyzed to aqua complexes (Raymond et al., 1998). The aqua complexes may be important for the cytotoxic effect of oxaliplatin, possibly mediated by the formation of DNA adducts.
(Graham et al., 2000; Raymond et al., 1998; Videhult et al., 2002). Oxaliplatin is found to be more potent than cisplatin in induction of apoptosis (Faivre et al., 2003).

3.2.2.3. Aroplatin (DACH-L-NDDP)

Aroplatin is *cis*-bis-neodecanoato-*trans*-R,R-1,2-diaminocyclohexane platinum (II)(NDDP) and it was the first liposomal platinum drug to enter clinical trials (Dragovich et al., 2006; Jakupec et al., 2003). During the phase II trial in 20 patients with advanced colorectal cancer, the drug was found to be refractory to fluorouracil/leucovorin, capecitabine or irinotecan. Figure 3.11 gives the structure of DACH-L-NDDP.

![Figure 3.11: Structure of DACH-L-NDDP](image)

L-NDDP has been shown to be active in the human ovarian tumour cell line A2780 and in cisplatin-resistant cell line A2780/PPD. The drug does not share cross-resistance to cisplatin (Han et al., 1993). L-NDDP has also been found to be active in both cisplatin-sensitive human colon carcinoma LoVo cells and in cisplatin-resistant LoVo/PDD cells (Han et al., 1994). Rare neurotoxicity was found, but no nephrotoxicity was reported (Perez-Soler et al., 1990). Early-phase trials with L-NDDP, carried out during 1990s, focused on intraperitoneal and intrapleural routes of administration and demonstrated clinical activity in these settings (Perez-Soler et al., 1997). Toxicological properties and tumour-inhibiting activities of single isomers in
mice were found to be on the whole comparable to the isomeric mixture, though some isomers were somewhat more active in cisplatin-resistant murine leukaemia (Khokhar et al., 1991). The dose-limiting toxicity of L-NDDP is myelosuppression with a toxicity profile similar to that of carboplatin (Jakupec et al., 2003; Perez-Soler et al., 1990).

### 3.2.2.4. AP5346 (ProLindac)

AP5346 is a DACH platinum drug, bound to a water soluble biocompatible nanoparticle co-polymer of N-(2-hydroxypropyl)methacrylamide (HPMA) (Kelland, 2007b). Preclinical studies in mice showed that the drug was 20-fold less toxic than oxaliplatin and possessed greater antitumour activity than oxaliplatin in a mouse model of melanoma and a xenograft model of human ovarian cancer (Kelland, 2007b). A phase I clinical trial with AP5346 recommended a dose of 640 mg/m² for progressing to phase II, and a partial response was reported for two patients (in patients with metastatic melanoma and ovarian cancer) (Campone et al., 2007). Phase II trials are ongoing in patients with recurrent ovarian or head and neck cancers (Kelland, 2007b). Figure 3.12 gives the partial chemical structure of AP5346.
From the pharmacokinetic studies in the B16 melanoma it was found that a single
dose of AP5346 delivered 16-times more platinum to the tumour, and 14 times
more platinum to tumour DNA (Misset, 1998). *In vivo* preclinical models AP5346
exhibited a typical toxicity profile with the dose limiting toxicity being
impairment of renal function and myelosuppression (Markman *et al.*, 1999). So
far no adverse events were observed (Kelland, 2007b).

### 3.2.2.5. Ormaplatin

Ormaplatin or tetraplatin \([\text{trans-D,L-1,2-diaminocyclohexane-tetrachloro-}
\text{platinum(IV)}]\) is a DACH-containing stable platinum (IV) complex which has
exhibited activity against cisplatin-resistant cell lines (Tutsch *et al.*, 1999). Figure
3.13 gives the structure of ormaplatin.
Figure 3.13: Structure of ormaplatin (Tetraplatin)

For example, ormaplatin has been reported to be much more active than cisplatin in cisplatin-resistant KB and A2780 cells (Rixe et al., 1996). Ormaplatin and its hydrolysis products may be uniquely neurotoxic. After several phase I studies, the development of ormaplatin was halted due to severe and irreversible peripheral unpredictable neurotoxicity (Jakupec et al., 2003; Serenci and McKeage, 1999).

3.2.2.6. Sterically hindered platinum complexes

Although two platinum drugs cisplatin and carboplatin have played a major role in the chemotherapeutic treatment of a variety of cancers over the past 40 years (Kawamura-Akiyama et al., 2002; Kelland, 2007a), their antitumour activity is limited due to intrinsic and/or acquired resistance to the compounds. These limitations have driven intensive efforts to synthesize new platinum-based drugs that would overcome resistance in tumour cells. Sterically hindered platinum complexes were rationally designed to circumvent resistance by blocking cellular detoxification by GSH and other cellular thiols whilst the ability to form cytotoxic lesions with DNA remained (Ho et al., 2003; Kelland, 1999). The following provides a more detailed of sterically hindered platinum complexes:
3.2.2.7. ZD0473 or Picoplatin:

ZD0473 or picoplatin (formerly known as JM473 and AMD0473) is cis-aminedichloro(2-methylpyridine)platinum(II) is a new generation platinum agent that has been developed in an effort to circumvent cisplatin resistance (Kawamura-Akiyama et al., 2002). The drug contains a bulky methylpyridine ligand at its platinum centre, which is responsible for its ability to overcome platinum resistance (Kelland, 1999). Crystal structure analysis of ZD0473 has shown that the pyridine ring is tilted 102.7° with respect to the PtN₂Cl₂ square plane. The sterically hindered nonleaving group slows associative substitution reactions on the square planar platinum. This is important for biological nucleophiles that are able to bind platinum without prior hydrolysis of the chloro-ligand (e.g. thiol groups of proteins or peptides). ZD0473 that was formerly called AMD473 or JM473 is now undergoing worldwide phase II and III clinical trials (Michalska and Wysokinski, 2005). Figure 3.14 gives the structure of ZD0473.

![Figure 3.14: Structure of ZD0473 (Picoplatin)](image)

ZD0473 has been proved to be active, either by direct injection or by oral administration, toward both tumour cell lines intrinsically resistant to cisplatin and some human ovarian carcinoma xenograph sublines characterized by an acquired resistance to the reference drug after prolonged treatment (Raynaud et al., 1997).
ZD0473 hydrolyses more slowly (two fold) than cisplatin and also shows reduced rates of reaction with thiourea and methionine while maintaining reactivity towards DNA (Holford et al., 1998a). ZD0473 also displays \textit{(in vitro)} cytotoxicity intermediate between cisplatin and carboplatin. In a growth inhibition assay using 11 human ovarian cancer cell-lines, IC$_{50}$ of ZD0473 was found to be 8.1 µM, higher than the mean value for cisplatin (2.6 µM) but lower than the mean value for carboplatin (20.3 µM) (Holford \textit{et al.}, 1998b). It has antitumour activity equivalent to that of both cisplatin and carboplatin (Barefoot, 2001).

3.2.2.8. Satraplatin (JM216) and its main metabolite JM118

Satraplatin is a novel platinum(IV) complex, namely bis-acetato-amminedichlorocyclohexylamineplatinum(IV) (JM216), that has shown activity in patients with hormone-refractory prostate cancer (HRPC) in phase I and phase 2 trials (Sternberg \textit{et al.}, 2003). Satraplatin is orally active making it easier for outpatient administration and has antitumour activity similar to that of intravenously administered cisplatin and carboplatin (Barefoot, 2001; Kelland, 1993; 1999; Natile and Coluccia, 2004). Figure 3.15 gives the structure of satraplatin and its metabolite JM118 (Samimi and Howell, 2006).

![Structure of satraplatin (JM216) and its main metabolite JM118](image)

Figure 3.15: Structure of satraplatin (JM216), its main metabolite JM118.
Satraplatin has demonstrated activity in patients with advanced prostate cancer in a randomized Phase II trial and is currently being investigated in the same patient population in a Phase III trial (Samimi and Howell, 2006). After oral administration satraplatin is rapidly metabolized during absorption and at least six metabolites have been identified. The most abundant metabolite is JM118 which is sixteen times more potent than native satraplatin (Samimi et al., 2007). JM118 forms intra- and interstrand crosslinks in DNA in a manner similar to cisplatin (Boulikas and Vougiouka, 2003; Samimi et al., 2007).

### 3.2.2.9. JM335

*Trans* platinum(IV) dihydroxo complexes represent a new group of platinum(IV) drugs among which the most widely studied compound is *trans,trans,trans-[PtCl₂(OH)₂(c-C₆H₁₁NH₂)(NH₃)]*(JM335). It exhibits antitumour activity in both murine and human subcutaneous cell tumour models. Figure 3.16 gives the structure of JM335.

![Structure of JM335](image)

Figure 3.16: Structure of JM335

The mechanism for the antitumour activity is not entirely known, but JM335 is reported to be efficient in forming interstrand cross-links to DNA and to be able to cause single-strand breaks and represents a rationally designed complex with different
spectrum of adducts with DNA than cisplatin (Kelland et al., 1999; Lemma et al., 2000).

3.2.3. **Summary of 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generation platinum drugs**

The following figure (Figure 3.17) by Kelland (Kelland, 2007a) provides a brief summary on the development of 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generation platinum drugs, stating the important goals and achievements of each stage.
Figure 3.17: Platinum drug ‘family tree’ representing the development of 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generation of tumour active platinum compounds.

More about the development on platinum complexes (as multi-centred complexes) will be discussed in the following few sections:
3.3. Development of multi-centred complexes

It was noted earlier that thousands of cisplatin analogues had been prepared by varying the natures of the leaving groups and the carrier ligands, with the aim of reducing the side effects and widening the spectrum of activity. Although it has been possible to cause much reduction in toxic side effects, only a limited change in the spectrum of activity has been achieved. Thus, attention has also been directed at platinum compounds that violate the classical structure-activity relationships in one way or another (Daghriri et al., 2004). Dinuclear and trinuclear platinum complexes represent one such class of rule-breaker platinum compounds; (Farrell, 2000; Perego et al., 1999a; Perego et al., 1999b). Whereas cisplatin binds with DNA forming mainly intrastrand Pt(GG) and Pt(AG) adducts, multi-centred platinum complexes are expected to form a range of interstrand Pt(GG) adducts so that the compounds are expected to have spectrum of activity quite different from that of cisplatin (Kasparkova et al., 2004). Indeed a number of highly tumour active multi-centred platinum complexes have been prepared by Farrell and others (Daghriri et al., 2004; Farrell, 1993; Farrell et al., 1995; Wu et al., 1994). This section aims to provide a review of the development of di- and tri-nuclear platinum complexes.
3.3.1. Di-nuclear platinum complexes:

Dinuclear platinum complexes synthesized by Farrell and co-workers consist of two monofunctional [Pt(NH$_3$)$_2$Cl] moieties connected by a flexible diamine linker (Farrell et al., 1995; Wu et al., 1994). The dinuclear platinum compounds were found to be active against cisplatin-resistant cancers in vivo (Farrell, 1993). Among the 1,1/trans, trans series, for example BBR3005 (1, 1/t, t), was identified as having the most promising pattern of antitumour activity and DNA-binding.

![Figure 3.18: Dinuclear platinum complexes](image)

Recently, Reedijk et al., reported a new class of dinuclear platinum complexes containing an azine-bridge, such as [{cis-Pt(NH$_3$)$_2$Cl}$_2$(µ-phthalazine)](NO$_3$)$_2$ (Kalayda et al., 2006) that have been evaluated for their cytotoxicity against several human tumour cell lines including cisplatin-sensitive and cisplatin-resistant L1210 murine leukemia cell lines. The cytotoxicity of [{cis-Pt(NH$_3$)$_2$Cl}$_2$(µ-phthalazine)](NO$_3$)$_2$ in the L1210 cell lines is similar to that of cisplatin (Kalayda et al., 2006).
3.3.2. Tri-nuclear platinum complexes

Trinuclear platinum complexes, as the name suggests, have three platinum centres connected together by linking chains. A notable example of tri-nuclear platinum complex is BBR3464 that entered clinical trials in late 1997 (Jansen et al., 1999; Qu et al., 1997). BBR3464 consists of the trinuclear cation \([\text{\{trans-PtCl(NH}_3\}_2\{\mu-trans-Pt(NH}_3)_2(NH_2(CH_2)_6NH_2)_2\}]^{4+}\) and balancing anions which are usually Cl\(^-\) ions. Often the abbreviation BBR3464 is used to mean the trinuclear cation only (Figure: 3.19). Only the two terminal platinum ions bind covalently with DNA forming a number of long-range interstrand GG adducts whereas the central platinum ion is involved only in non-covalent interactions (Perego et al., 1999b).

![Figure 3.19: Structure of BBR3464](image)

BBR3464 is found to be more potent than cisplatin and significantly more active in the human tumour cell lines and xenografts that are refractory or poorly responsive to cisplatin (Kasparkova et al., 2004). The preclinical anticancer profile of BBR3464 is highlighted by exceptional potency with the therapeutic doses approximately 1/10th that of cisplatin and a broad range of activity in solid human tumours (Di Blasi et al., 1998). It has potential for development in the treatment of non-small cell lung cancer, gastric cancer, ovarian cancer, small-cell lung cancer and other solid tumours.
including pancreatic cancer (Wheate and Collins, 2003). Also, BBR3464 displays consistently high antitumour activity in human tumour xenografts that are characterized as p53 mutants. BBR3464 adducts inhibit binding of p53 to the DNA sequence with an efficiency similar to that exhibited by DNA adducts of cisplatin. No adduct of BBR3464 increases the binding affinity of active p53 to DNA lacking the consensus sequence. This is a fundamental contrast to cisplatin whose major 1,2-intrastrand cross links increase binding affinity of p53 to DNA lacking consensus sequence (Zehnulova et al., 2001). On average, cells with mutant p53 are more resistant to cisplatin (O'Connor et al., 1997). whereas BBR3464 maintains activity in several such tumours that have acquired or inherent resistance to cisplatin (Pratesi et al., 1999). However, after the initial phase II trials the development of BBR3464 was stopped because of high toxicity and relatively poor response rate (Daghriri et al., 2004; Kelland, 2007a).

**More about DNA binding mechanism of BBR3464**

The high charge on BBR3464 facilitates rapid binding to DNA significantly faster than the neutral cisplatin (Manzotti et al., 2000). It was however found that BBR3464 reacted faster with single stranded DNA and RNA than it did with double-stranded DNA. It has been suggested that this may have been due to differences in conformation of the double-stranded DNA, single strand DNA and RNA (Kasparkova et al., 2002). The flexibility of 1,6-diaminohexane linker groups enables BBR3464 to form a large number of DNA adducts including long-range intra- and interstrand cross-links (Zehnulova et al., 2001) in which the two terminal platinum atoms coordinate to the N7 positions of guanines in the major groove. Binding of BBR3464 with DNA causes a global change in DNA conformation different from that caused by cisplatin e.g. kink, bend or unwinding of the helices that are recognized by HMG-
domain proteins (Qu et al., 2003). As stated earlier, the central platinum unit in BBR3464 is involved in non-covalent interactions such as hydrogen bonding of ammines with centres in DNA such as the O6 of guanine or the O3 of thymine and electrostatic interaction because of the presence of positive charge. These interactions (electrostatic and hydrogen bonding) are believed to facilitate the specific recognition of target sites on DNA (Brabec et al., 1999). The typical DNA conformational changes induced by BBR3464 are from B to Z-type in poly(dG-dC).poly(dG-dC) DNA and from B to A-type in poly(dG).poly(dC) DNA (McGregor et al., 1999). These conformational changes can be induced at concentrations as low as 12.5 mM or at a complex to DNA ratio (rb) of 0.013 (Johnson et al., 1992).

3.4. Non-platinum anticancer drugs

The successful achievement of cisplatin and other platinum complexes as anticancer drugs has stimulated the search for other metal complexes as anticancer agents (Alessio et al., 2004), with the aim of overcoming clinical problems of platinum drugs (e.g., cisplatin and carboplatin) namely limited spectrum of activity, acquired resistance and side effects. Among them, compounds containing titanium (Ti), vanadium (V) and ruthenium (Ru) have shown good antitumour activity and remarkably lower toxicity (Keppler and Vogel, 1996; Yang and Guo, 1998). This section aims to provide a brief overview on the development of other metal complexes as anticancer drugs.
3.4.1. Titanium complexes

Two important representatives of titanium based tumour active compounds are budotitane (BD) and titanocene dichloride. BD is \(\text{[cis-diethoxybis(1-phenylbutane-1,3-dionato)titanium (IV)]}\). It is a structural analogue of cisplatin and was the first non-platinum compound that reached clinical trial (Keppler, 1993; Pil and Lippard, 1997).

![Figure 3.20: Structures of (a) Budotitane and (b) Titanocene](image)

In preclinical studies using established screening models, BD has shown considerable antitumour activity (Schilling et al., 1996). The main side effects include hepatotoxicity with liver necrosis and hemorrhagic pleural effusions, increase of reticulocytes and leukocytes and decrease in hemoglobin. The mechanism of action of BD is unknown, but appears to be different from that of cisplatin (Keppler and Schmahl, 1986). The clinical development of BD was stopped due to problems associated with drug formulation (Caruso and Rossi, 2004).

Titanocene dichloride, \((\text{Cp})_2\text{TiCl}_2\), where Cp stands for cyclopentadienyl, is another titanium-based antitumour agent. It has two Cl leaving groups (Lummen et al.,...
The compound (Fig. 3.20b) exhibits anti-proliferative activity in a wide spectrum of murine and human tumours including cisplatin-refractory colon 38 adenocarcinoma (Kopf-Maier and Kopf, 1987; Kopf-Maier et al., 1981) and xenografted human adenocarcinomas of the gastrointestinal tract (Kopf-Maier, 1989). From *in vitro* studies involving ovarian carcinoma cells it was found that there was a lack of cross-resistance between TiCp₂Cl₂ and cisplatin, suggesting that their mechanisms of action might be at least partially different (Harstrick *et al.*, 1993; Kurbacher *et al.*, 1994). The main toxic side effect of the compound is reversible hepatotoxicity (Kopf-Maier and Kopf, 1986). In phase II clinical trials with advanced renal-cell carcinoma and breast metastatic carcinoma it was found that TiCp₂Cl₂ offered no therapeutic advantage although the toxic side effects were low (Kröger *et al.*, 2000; Lummen *et al.*, 1998). The disappointing outcomes from the studies led to discontinuation of the clinical development of TiCp₂Cl₂ (Keppler, 2003).

### 3.4.2. Ruthenium complexes

Ruthenium complexes have attracted large interest for development as anticancer drugs because ruthenium has a large range of oxidation states accessible under physiological conditions and also because ruthenium compounds are known to be less toxic than their platinum counterparts (Alessio *et al.*, 2004; Ang and Dyson, 2006). The reason for lower toxicity is believed to be due to the ability of ruthenium complexes to mimic iron in the binding to biomolecules such as albumin and transferrin (Ang and Dyson, 2006; Dyson and Sava, 2006). Transferrin receptors are over expressed in rapidly dividing cancer cells (because they have a greater demand for iron) allowing ruthenium-based drugs to be more effectively delivered to the cancer cells (Dyson and Sava, 2006). In the last three decades a wide range of
ruthenium agents has been synthesized and tested for antitumour properties (Rademaker-Lakhai et al., 2004). Figure 3.21 lists some ruthenium complexes that have been found to be tumour active.

![Figure 3.21: Structures of some ruthenium-based metal complexes:](image)

(a) NAMI-A                           (b) NAMI                           (c) Ru-azpy complex

Figure 3.21: Structures of some ruthenium-based metal complexes:

(a) \(\text{trans-}[\text{Na}][\text{Ru(Im)}(\text{Me}_2\text{SO})\text{Cl}_4]\) (Im = Imidazole) or NAMI and (b) \(\text{trans-}[\text{ImH}][\text{Ru(Im)}(\text{Me}_2\text{SO})\text{Cl}_4]\) or NAMI-A (c) a Ru-azpy complex (azpy = 2-phenylazopyridine) (Alessio et al., 2004).

Among the ruthenium complexes, NAMI standing for \(\text{trans-}[\text{Na}][\text{Ru(Im)}(\text{Me}_2\text{SO})\text{Cl}_4]\) (Im = Imidazole) and its analogue, NAMI-A standing for \(\text{trans-}[\text{ImH}][\text{Ru(Im)}(\text{Me}_2\text{SO})\text{Cl}_4]\), (Figure 3.22a and 3.22b) were developed and thoroughly investigated and both compounds proved to have an in vivo activity different from that of platinum drugs (Alessio et al., 2004; Clarke, 2003). NAMI-A is a leading compound that shows selective effects for solid tumour metastases related to a mechanism of action involving the inhibition of the processes of tumour invasiveness (Bergamo and Sava, 2007) so that NAMI-A opens a new window to new perspectives in cancer chemotherapy. Both NAMI and NAMI-A have shown a specific antimetastatic activity against solid metastasising tumours in mice. In particular NAMI-A after extensive preclinical investigations, has recently and successfully completed a Phase 1 trial and NAMI-A is the first ruthenium antitumour complex that
has entered clinical testing (Alessio et al., 2004; Clarke, 2003; Rademaker-Lakhai et al., 2004). NAMI and NAMI-A could be of particular use in minimizing the growth of undetected micrometastases following surgery or radiotherapy (Clarke et al., 1999).

Recently, several Ru(II) arene compounds with the formula $[\text{Ru}^{\text{II}}(\eta^6\text{-arene})(\text{en})\text{X}]^+$ ($\text{X} = \text{Cl}$ or $\text{I}$, arene = $p$-cumene or biphenyl, en = ethylenediamine or $N$-ethylethylenediamine, (Figure 3.22c) were found to inhibit the proliferation of human ovarian cancer cells. Some of the IC$_{50}$ values are comparable with that of carboplatin (Morris et al., 2001). These complexes do not inhibit topoisomerase II activity (Zhang and Lippard, 2003).

3.4.3. Gold complexes

Gold complexes had been employed in medicine since ancient times to treat a variety of human ailments (Messori and Marcon, 2004; Tiekink, 2002). Although gold complexes are mainly used to treat rheumatoid arthritis, many gold complexes have been reported to show antitumour activity (McKeage et al., 2002). Extensive studies have been conducted on gold complexes in oxidation states $+1$ and $+3$, their solution behaviour and their reactivity with biomolecules. Under physiological condition it is possible to have relatively easy interchange between the oxidation states $+1$ and $+3$. This property may be relevant for the pharmacology of gold drugs (Messori and Marcon, 2004). At $+3$ oxidation state gold has the same electronic configuration and structural characteristics as the most widely used anti-cancer drug, cisplatin. Some of the gold-based compounds that have therapeutic potential as anticancer agents are described in the following paragraphs:
Tetrahedral gold(I) complexes with 1,2-bis(diphenylphosphino)ethane and 1,2-bis(dipyridylphosphino)ethane ligands (Figure 3.22a) display *in vitro* antitumour activity in a wide range of cancer cell lines including some cisplatin-resistant cell lines (Kostova, 2006; Zhang and Lippard, 2003). Mechanistic studies on gold complexes suggest that DNA is not the primary target. Rather, their cytotoxicity is mediated by their ability to alter mitochondrial function and inhibit protein synthesis (Zhang and Lippard, 2003). Recently, a hydrophilic tetrakis-(tris(hydroxy-methyl)phosphine)gold(I) complex (Figure 3.22b) has been reported to be cytotoxic against several tumour cell lines. Cell cycle studies with HCT-15 cells (derived from human colon carcinoma) revealed that inhibition of cell growth might result from elongation of the G1 phase of the cell cycle (Pillarsetty *et al.*, 2003).

![Figure 3.22: Structures of several Au(I) and Au(III) complexes:](image)

(a) Au(I) complexes with 1,2-bis(diphenylphosphino)ethane and 1,2-bis(dipyridylphosphino)ethane ligands. (b) Tetrakis((trishydroxymethyl)phosphine)gold(I) complex. (c) Chlorotriphenylphosphine-1,3
bis(diphenylphosphino)propanegold(I) complex. (d) [Au(bipy)(OH)$_2$]PF$_6$ and [Au(bipy$^2$-H)(OH)]PF$_6$.

Another Au(I) complex having both monophosphine and diphosphine ligands (Figure 3.22c) has recently been reported to be highly cytotoxic against several tumour cell lines with micromolar range IC$_{50}$ values (Caruso et al., 2003). Au(III) complexes, with their metal centres being isoelectronic and isostructural to Pt(II), are promising candidates as anticancer agents. Indeed, several Au(III) compounds with multidentate ligands such as ethylenediamine(en), diethylenetriamine(dien) and damp (N-benzyl-$N,N$-dimethylamine) are active against human cancer cell lines (Buckley et al., 1996; Messori et al., 2000). A recent in vitro cytotoxicity study demonstrated promising activity of two Au(III) complexes with bispyridyl ligands, [Au(bipy)(OH)$_2$]PF$_6$ and [Au(bipy-H)(OH)]PF$_6$ (Figure 3.22d) (Marcon et al., 2002). Both complexes are quite stable under physiological conditions, with [Au(bipy-H)(OH)][PF$_6$] being resistant to sodium ascorbate reduction. Mechanistic studies indicated that as for other gold complexes, DNA is not the primary cellular target of these complexes. The complexes showed significant cytotoxic effects towards A2780S, A2780cisR and SKOV3 tumour cell lines; lower effects were detected on the CCRF-CEM/S and CCRF-CEM/R lines.

3.4.4. Vanadium complexes

Vanadium is a physiologically essential element that can be found in both anionic and cationic forms, having oxidation states ranging from -1 to +5 (D'Cruz and Uckun, 2002). Vanadium complexes with oxidation states +4 (IV) and +5 (V) have been shown to modulate cellular redox potential, regulate enzymatic phosphorylation, and exert various other effects in multiple biological systems (Narla et al., 2001). A
number of vanadocene complexes were reported to possess promising antitumour activity (El-Naggar et al., 1998). Vanadocenes have been found to exhibit significant antitumour properties both in vitro and in vivo. Bis-(cyclopentadienyl)vanadium(II)chloride, \([\eta^5-C_5H_5]_2VCl_2\) (Figure 3.23a), which belongs to the metallocene class of antitumour agents, has turned out to be one of the most promising candidates for development as an anticancer drug among non-platinum compounds.

![Bis(cyclopentadienyl)vanadium(II)chloride](image1)

![Metvan](image2)

Figure 3.23: Bis(cyclopentadienyl)vanadium(II)chloride (vanadocene); (b) Metvan

Vanadocene dichloride has been shown to be a potent antitumour agent against mouse tumours. The antitumour effects of vanadocene dichloride against human colon and lung carcinomas were shown to be due to the vanadium accumulation in nucleic acid-rich regions and to the inhibition of DNA and RNA synthesis in tumour cells (Evangelou, 2002), suggesting the binding of this compound to the DNA.

Another tumour active vanadium compound is bis(4,7-dimethyl-1,10-phenanthroline)sulfatooxovanadium(IV) or metvan (Figure 3.23b). It was identified as a highly promising multitargeted anticancer vanadium complex with apoptosis-inducing
activity. The compound is highly active against cisplatin-resistant ovarian cancer and testicular cancer cell lines (D'Cruz and Uckun, 2002). Metvan induces apoptosis at nanomolar and low micromolar concentrations, in human leukaemia cells, multiple myeloma (MM) cells and solid tumour cells derived from breast cancer, glioblastoma, ovarian, prostate and testicular cancer patients (D'Cruz and Uckun, 2002; Dong et al., 2000; Narla et al., 2000).

3.4.5. Rhodium complexes

Rhodium belongs to the same group as platinum and ruthenium. Although rhodium compounds that are analogues of corresponding platinum and ruthenium compounds are found to possess significant antitumour properties, they are found to be less effective as anticancer agents, mainly due to toxic side effects (Katsaros and Anagnostopoulou, 2002). However dimeric µ-acetato dimers of rhodium(II) as well as monomeric square planar rhodium(I) and octahedral rhodium(III) complexes have shown interesting antitumour properties. The dirhodium tetraacetate complex, [(CH₃COO)₄(H₂O)₂Rh₂(II)], (Figure 3.25) has much more inhibitory effects towards Escherichia coli DNA polymerase I and exhibits excellent antitumour activity against P388 lymphocytic leukemia and sarcoma 180 but it showed little activity against L1210 and B16 melanoma (Howard et al., 1979).
Figure 3.24: Six-coordinate cage complex of rhodium(II) carboxylates, [(RCOO)₄L₂Rh₂(II)], R = alkyl group, L = H₂O or other donor solvent.

Recent structural studies suggest that the antitumour activity of di-rhodium(II) carboxylates may bear analogy to that of cisplatin as they bind to adjacent guanines on DNA (Dunbar et al., 1994). Antitumour activity has been found to increase in the series [(RCOO)₄(H₂O)₂Rh₂(II)] (R=alkyl group) with the increase in the lipophilicity of the R group (Howard RA et al., 1979). Thus rhodium(II) acetate (R = CH₃), propionate (R = CH₃CH₂), butyrate (R = CH₃CH₂CH₂) and pentanoate (R = CH₃CH₂CH₂CH₂) show a considerable variation in their antitumour activity against Ehrlich ascites tumour cells in mice, with the pentanoate complex being the most active (Katsaros and Anagnostopoulou, 2002). It should however be noted that lengthening the carboxylate alkyl (R) chain beyond the pentanoate reduces the drugs’ therapeutic efficacy (Katsaros and Anagnostopoulou, 2002).

### 3.4.6. Palladium complexes:

Palladium is a soft silver-white metal that is quite similar to platinum in its chemical and physical properties. It has the lowest density and the lowest melting point among the platinum group metals. Palladium is chemically attacked by sulfuric, nitric and hydrochloric acids in which it dissolves slowly. Common oxidation states of palladium are 0, +1, +2 and +4. The notable analogy between the coordination
chemistry of platinum(II) and palladium(II) compounds has advocated studies of Pd(II) complexes as antitumour drugs (Eastman and Schulte, 1988). A key factor that might explain why platinum complexes can be active whereas the corresponding palladium complexes are generally inactive but toxic, is the differences in the rates of their ligand exchange reactions. The hydrolysis of the leaving ligands in palladium complexes is about $10^5$ times faster than their corresponding platinum analogues (Butour et al., 1997). They dissociate readily in solution leading to very reactive species that are unable to reach their pharmacological targets (Abu-Surrah and Kettunen, 2006) because of rapid binding with other ligands. In addition, some of them undergo conversion to inactive trans-conformation. Considerably higher reactivity of palladium complexes implies that if an antitumour palladium drug is to be developed, it must somehow be stabilized by a strongly coordinated nitrogen ligand and a suitable leaving group. If this group is reasonably non-labile, the drug may maintain its structural integrity in vivo long enough to show antitumour activity.

A series of mononuclear and dinuclear palladium complexes has been investigated by different researchers including their cytotoxicity against a number of cancer cell lines. Some of the palladium compounds showed some anticancer activities but so far no promising candidate has emerged. In this section a review of the palladium complexes that have been investigated for antitumour activity will be provided. Palladium complexes based on 2-mercapto-pyridines (MP) were synthesized and tested for their anticancer activity by Carrara et al. (Carrara et al., 1997). It was reported that the palladium compound the $[(MP)_3Pd(Br)]Br$ (Figure 3.25a) had the potential for therapeutic use with IC$_{50}$ values lower than cisplatin for LoVo cell lines (Carrara et al., 1997). Another palladium complex which contains the bulky nitrogen ligand harmine (7-methoxy-1-methyl-9H-pyrido[3,4-b]indole, trans-[Pd(harmine)(DMSO)] $^{[3]}$)
Cl₂ (Figure 3.25.b) was found to exhibit a greater cytotoxic activity against P388, L₁₂₁₀ and K₅₆₂ cell lines than cisplatin (Al-Allaf and Rashan, 1998).

A different series of palladium compounds containing two chelating ligands: N-N and O-O (XO₃; selenite or tellurite) were prepared (Mansuri-Torshizi et al., 1991). In these complexes selenite and tellurite ligands coordinate to palladium as bidentate ligands through two oxygen atoms. The N-N ligand did not influence the activity but the oxygen coordinated leaving group did. Selenite complexes were found to be better cytotoxic agents than tellurite complexes and cisplatin. The complex (bipy)Pd(SeO₃) (Figure 3.25c) that inhibited the growth of P388 lymphocytic leukaemia cells, was found to bind to DNA through a coordinate covalent bond. Aromatic ligands such as 1,10-phenanthroline, which is one of the most used ligands in coordination chemistry, has been utilized in the field of antitumour-transition metal
chemistry. Its planar geometry enables its participation as a DNA intercalator. Several derivatives of 1,10-phenanthroline were prepared and used as tetradeinate ligands. The activities of [[(N,N-dialkyl-1,10-phenanthroline-2,9-dimathamine)Pd(II)] (Figure 3.25d) (alkyl: Me, Ethyl, propyl,cyclohexyl) are significantly dependent on the nature of the alkyl substituents. The complexes bearing the bulkiest groups have been tested against L1210 and Bel7402 tumour cell lines and showed lower IC\textsubscript{50} values than cisplatin (Zhao et al., 1998a).

![Di-palladium complexes](image)

Figure 3.26: Structures of some di-palladium complexes.

Navarro-Raninger et al. reported (Navarro-Raninger et al., 1993) the synthesis of putrescine and spermine-based dinuclear complexes: [PdCl\textsubscript{4}(Put)\textsubscript{2}] (Figure 3.26a) and [PdCl\textsubscript{4}(sperm)\textsubscript{2}] [PdCl\textsubscript{4}(Put)\textsubscript{2}] (Figure 3.26b). The putrescine complex is a coordination complex of dimeric nature. In the spermine complex, the 4 amino groups of the spermine coordinate to two \textit{cis}-Pd-centres. The cytotoxicity results showed that the putrescine complex is more active than cisplatin against MDA-MB 468 and HL-60 human cancer cell lines. Spermidine complex also showed significant antitumour activity (Navarro-Raninger et al., 1993). In another investigation, Zhao, et al.
reported dinuclear palladium complexes containing two functional [Pd(en)(pyridine)Cl]⁺ units bridged by Se or S (Zhao et al., 1998b) (Figure 3.26c) that are water soluble and have lower IC₅₀ values than cisplatin against the HCT8 cancer cell line.

### 3.5. Present study

The present study is a natural progression of the studies on tumour active metal compounds carried out in this laboratory and in particular on palladium complexes. In this laboratory, a number of mononuclear palladium complexes with sterically hindered ligands and trinuclear palladium analogues of BBR3464 code named MH1 and MH2 have been synthesized and tested for activity against human ovarian cancer cell lines (Huq et al., 2006; Huq et al., 2008). Although MH1 and MH2 show only slight activity, some of the mononuclear palladium complexes containing planaramine ligands have displayed much higher activity and more so against the resistant cell lines (much more than cisplatin), indicating that the reduction in reactivity of palladium complexes can indeed result in tumour active compounds. A trinuclear Pt-Pd-Pt complex code named DH6Cl (based on BBR3464) has also been found to be much more active than cisplatin against ovarian cancer cell lines, thus indicating that significant decrease in reactivity of the central palladium ion occurs due to what has been termed as the cage-in effect (Daghriri et al., 2004). Another trinuclear Pt-Pd-Pt complex code named CH25 (synthesized in this laboratory) in which the central palladium ion is bound to one 2-hydroxypyridine ligand was found to be highly active against human ovarian cancer cell lines (Cheng et al., 2005).
Figure 3.27: Structures of MH1 (a), MH2 (b), DH6Cl (c) and CH25 (d).

This project aims to synthesize and investigate for activity against human ovarian cancer cell lines trinuclear palladium complexes in which the central or the terminal palladium ions are bound to two planaramine ligands such as 2-hydroxypyridine or 3-hydroxypyridine or 4-hydroxypyridine. Our working hypothesis is that the cage-in effect due to the linker chains and the steric hindrance provided by the planaramine ligands may reduce the rates of ligand exchange reactions sufficiently so as to endow significant antitumour property to the compounds. It is also believed that variations in activity of the designed complexes based on the positions of the planaramine ligands (central or terminal) and the actual nature of the planaramine ligand (2-
hydroxypyridine, 3-hydroxypyridine or 4-hydroxypyridine) may also provide information on structure-activity relationships. The project also aims to investigate the nature of interaction of the designed complexes with DNA in addition to the determination of cell uptake and level of binding with nuclear DNA.. Thus, the aims of my project are:

1) To design, synthesise and characterize six new trinuclear palladium (Pd-Pd-Pd) complexes containing two planaramine ligands (2-hydroxypyridine or 3-hydroxypyridine or 4-hydroxypyridine) bonded to central or terminal palladium ions.

2) To determine the activity of designed complexes against ovarian cancer cell lines A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} including cell uptake and level of binding of with cellular DNA.

3) To determine the nature of interaction of the compounds with DNA

Precisely, the following compounds will be synthesized and investigated for anticancer activity and binding with DNA.
Figure 3.28: Structures of the targeted trans-planaraminepalladium(II) complexes, bonded planaramine ligand in central position.

MH3: \( \{\text{trans-PdCl(NH}_3\}_2 \} \{\text{trans-Pd(4-hydroxypyridine)}\}_2 \{\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\}_2 \text{Cl}_4 \)

MH4: \( \{\text{trans-PdCl(NH}_3\}_2 \} \{\text{trans-Pd(3-hydroxypyridine)}\}_2 \{\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\}_2 \text{Cl}_4 \)

MH5: \( \{\text{trans-PdCl(NH}_3\}_2 \} \{\text{trans-Pd(2-hydroxypyridine)}\}_2 \{\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\}_2 \text{Cl}_4 \)
MH6: \(\text{trans}-\text{PdCl}(\text{NH}_3)_2\)[\{\text{trans}-\text{Pd}(2\text{-hydroxypyridine})\}_2\{\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2\}_2\]_2\text{Cl}_4

MH7: \(\text{trans}-\text{PdCl}(\text{NH}_3)_2\)[\{\text{trans}-\text{Pd}(3\text{-hydroxypyridine})\}_2\{\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2\}_2\]_2\text{Cl}_4

MH8: \(\text{trans}-\text{PdCl}(\text{NH}_3)_2\)[\{\text{trans}-\text{Pd}(4\text{-hydroxypyridine})\}_2\{\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2\}_2\]_2\text{C}

Figure 3.29: Structures of the targeted \textit{trans}-planaraminepalladium(II) complexes, bonded planaramine ligand in two terminal positions.
4. Chapter four: Materials and Methods

4.1. Introduction

As stated chapter 3, this project deals with the synthesis, characterization, activity in human ovarian cancer cell lines including cell uptake and level of binding with nuclear DNA, and the nature of binding with pBR322 plasmid DNA of a number of trinuclear palladium complexes containing planaramine ligands bonded to the central or the terminal metal ions. The experimental methods and procedures used in this study are described in this chapter. The method of preparation of cisplatin has also been included as the compound is used as a reference in activity results.

4.2. Materials

Potassium tetrachloropalladate \([\text{K}_2\text{PdCl}_4]\), 1,6-diaminohexane \([\text{C}_6\text{H}_{16}\text{N}_2]\) and N,N-dimethylformamide [DMF] \([\text{C}_3\text{H}_7\text{NO}]\) were obtained from Sigma Chemical Company St. Louis USA; dichloromethane \([\text{CH}_2\text{Cl}_2]\), 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine and potassium tetrachloroplatinate \([\text{K}_2\text{PtCl}_4]\) were obtained from Aldrich Chemical Company Milwaukee USA; acetone \([((\text{CH}_3)_2\text{CO})\), dichloromethane \([\text{CH}_2\text{Cl}_2]\) and silver nitrate (AgNO\(_3\)) were obtained from Ajax Chemicals Auburn NSW Australia; methanol \([\text{CH}_3\text{OH}]\) and ethanol \([\text{C}_2\text{H}_5\text{OH}]\) were obtained from Merck Pty. Limited Kilsyth VIC Australia; hydrochloric acid (HCl) was obtained from Asia Pacific Specialty Chemicals Ltd. NSW, Australia. Dimethyl sulfoxide (DMSO), dichloromethane, trans-diaminedichloropalladium(II) [transpalladin], salmon sperm DNA were purchased from Sigma-Aldrich, NSW,
Australia and pBR322 plasmid DNA was purchased from ICN Biomedicals, Ohio, USA. Foetal calf serum, 5 x RPMI 1640, 200 mM L-glutamine and 5.6% sodium bicarbonate were obtained from Trace Biosciences Pty Ltd. Trypsin, Hepes and Dulbecco's phosphate buffered saline powder were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. 96 well culture cluster plates (flat bottom with lid) were obtained from Edward Keller, and 25 cm² culture flasks were obtained from Crown Scientific. 3-[4,5-dimethylthiazol-2-yl]-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. Micro-plate reader BIO-RAD Model 3550 was used to read the optical density of each well.

4.3. Methods

4.3.1. Preparation of cisplatin

The preparation of cisplatin was first described by Peyrone (Peyrone, 1845), which was modified by Kauffman and Cowan in 1963 (Kauffman and Cowan, 1963). The method involved the action of aqueous ammonia on potassium tetrachloroplatinate(II) and was found to be time-consuming and of low yield (due to the formation of by-products such as magnus salt). A new and rapid method in which the formation of magnus salt and other by-products could be avoided and gave quantitative yield, was described by Dhara in 1970 (Dhara, 1970). It involves the interaction of potassium tetrachloroplatinate (K₂PtCl₄) with potassium iodide followed by reaction with ammonia, silver nitrate and potassium chloride (Figure 4.1).
Figure 4.1: Synthesis of cisplatin by Dhara method.

One mmol (415 mg) of potassium tetrachloroplatinate was dissolved in 10 mL of milli Q (mQ) water followed by the addition of 12 mmol (2.0 g) of potassium iodide. The mixture was stirred on a hot plate for 5 min at 37°C to produce $\text{K}_2\text{PtI}_4$ which was reacted with 2 mmol (280 µL of concentrated ammonia) of aqueous ammonia with stirring at 37°C for one hour to form precipitate of cis-$\text{Pt(NH}_3)_2\text{I}_2$.

The dark yellow precipitate of cis-$\text{Pt(NH}_3)_2\text{I}_2$ was washed in succession with ice-cold water and ethanol. The precipitate was dried for 24 h. Two mmol of silver nitrate (339.8 mg), and one mmol of cis-$\text{Pt(NH}_3)_2\text{I}_2$ were mixed together followed by the addition of 4 mL of mQ water to the mixture. The mixture was stirred at 37°C for 30 min to produce cis-$\text{Pt(NH}_3)_2(\text{NO}_3)_2$ in solution and precipitate of silver iodide. The mixture was centrifuged at 5500 rpm for 15 min. The supernatant consisting of cis-$\text{Pt(NH}_3)_2(\text{NO}_3)_2$ in solution was collected. Two mmol of potassium chloride (0.148 g) was added to the solution. The mixture was stirred for 30 min at 37°C to form the crystals of cisplatin. The crystals were filtered and washed with ice-cold water and ethanol and air-dried.

### 4.3.2. Preparation of trinuclear palladium compounds

A step-up method of synthesis branching out from the central unit was employed to prepare the trinuclear palladium compounds MH3, MH4, MH5, MH6, MH7 and MH8. The procedures used were a modification of that described by Daghriri for the
synthesis of trinuclear Pt-Pd-Pt complexes (Daghriri, 2003). MH3, MH4 and MH5 have the general formula: 

\[
\{\text{trans-PdCl(NH}_3)\}_2\{\mu\text{-trans-Pd(C}_5\text{H}_5\text{NO})\}_2\{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\}_2]\text{Cl}_4
\]

whereas MH6, MH7 and MH8 have the general formula: 

\[
\{\text{trans-PdCl(C}_5\text{H}_5\text{NO})\}_2\{\mu\text{-trans-Pd(NH}_3)\}_2\{(\text{NH}_2(\text{CH}_2)_6\text{NH}_2\}_2\}\text{Cl}_4.
\]

It can be noted that the linking chain in all the compounds is 1,6-diaminohexane.

First transpalladin and mononuclear transpalladium compounds code named TH5, TH6 and TH7 were prepared based on the Kauffman method (Kauffman and Cowan, 1963), as described in sections 4.2.3 and 4.2.5. The procedures used for the synthesis of TH5, TH6 and TH7 were actually described previously by Huq et al. (Huq et al., 2007). TH5, TH6 and TH7 have the general formula \text{trans-PdL}_2\text{Cl}_2 \text{ (where L = 3-hydroxypyridine for TH5, 2-hydroxypyridine for TH6 and 4-hydroxypyridine for TH7). In MH3, MH4 and MH5, trans-PdL}_2\text{Cl}_2 \text{ served as the central unit and transpalladin served as the terminal units, and vice versa in MH6, MH7 and MH8.}

Figure 4.2 gives the general structure for MH3, MH4 and MH5 and figure 4.3 gives the same for MH6, MH7 and MH8.

Figure 4.2: General structure for MH3, MH4 and MH5

\[
\begin{align*}
\text{NH}_3 & \quad \text{L} & \quad \text{NH}_3 \\
\text{Cl-Pd} & \quad \text{H}_2\text{N(\text{CH}_2)_6\text{NH}_2} & \quad \text{L} & \quad \text{H}_2\text{N(\text{CH}_2)_6\text{NH}_2-Pd-Cl} \\
\text{NH}_3 & & \text{L} & & \text{NH}_3
\end{align*}
\]

MH3, MH4 and MH5 \quad (L = 2, 3 or 4-hydroxy pyridine)
MH6, MH7 and MH8 (L = 2, 3 or 4-hydroxy pyridine)

Figure 4.3: General structure for MH6, MH7 and MH8

4.3.3. Preparation of \(\textit{trans-}\text{PdCl}_2(\text{NH}_3)_2\)

1 mmol (326.5 mg) of potassium tetrachloropalladate (II) \([\text{K}_2\text{PdCl}_4]\) was dissolved in 7.5 mL of mQ water to produce a dark brown solution. 0.25 mL of concentrated HCl was added to the solution with stirring; the temperature was increased to about 60\(^\circ\)C. 1.2 mL of concentrated aqueous ammonia was slowly added (with stirring) to the solution. The solution was kept at 50-60\(^\circ\)C until the colour changed through dark pink to light pink. Then 40 mL of 6 M HCl was added. The mixture then became turbid with yellow-orange colour. The suspension was stirred for further 2 h at 40-50\(^\circ\)C. The volume of the solution was reduced to about 3 mL by slow heating at 40-50\(^\circ\)C in a fume hood while it was constantly stirred followed by cooling in an ice-bath at 0\(^\circ\)C for about 40 min. The precipitate of \(\textit{trans-}\text{PdCl}_2(\text{NH}_3)_2\) was collected at the pump and washed with acetone and ice-cold mQ water. The filtrate was evaporated to reduce the volume and cooled in an ice bath to obtain a further crop of \(\textit{trans-}\text{PdCl}_2(\text{NH}_3)_2\). The impure \(\textit{trans-}\text{PdCl}_2(\text{NH}_3)_2\) was dissolved in a minimum quantity of hot 0.01 M HCl and was cooled in an ice-bath for 2 h for crystals to form. The crystals of \(\textit{trans-}\text{PdCl}_2(\text{NH}_3)_2\) (light brown in colour) were collected at the pump and air-dried. Yield = 60\%.
4.3.4. Preparation of transpalladin-DMF filtrate

Transpalladin-DMF filtrate in which chloro ligands of transpalladin are replaced by DMF and which is required for the synthesis of trinuclear complexes was prepared as follows. 0.212 gm (1.0 mmol) of transpalladin was dissolved in 16 mL of DMF to produce a yellow-orange solution. 0.98 mmol of silver nitrate (0.1664 gm) was dissolved in 4 mL of mQ water. The silver nitrate solution was added to transpalladin solution to produce white precipitate of AgCl. The mixture was stirred in the dark for 20 h at room temperature. It was then centrifuged at 5500 rpm for 10 min to remove the AgCl precipitate. The supernatant (yellow orange in colour) was collected by filtration at the pump to remove any particles of AgCl. The transpalladin-DMF solution (brown-red) was then kept in the refrigerator until used.

4.3.5. Preparation of TH5, TH6 and TH7

As noted earlier, TH5, TH6 and TH7 were used as starting materials for the synthesis of trinuclear palladium compounds. Figure 4.4 gives the schemata for the synthesis of TH5, TH6 and TH7 following the method described by Huq F and co-workers (Huq et al., 2007).

\[
\begin{align*}
K_2Cl & \xrightarrow{4L} PdClLClLHCl & PdClLClL
\end{align*}
\]

(L = 2, 3 or 4-hydroxypyridine)

Figure 4.4: Schematic representation for the synthesis of TH6, TH5 and TH7
4.3.6. *Trans*-PdCl$_2$(3-hydroxypyridine)$_2$ [TH5]):

One mmol (0.326 g) of potassium tetrachloropalladate was dissolved in 7.5 mL of milliQ water to which 0.25 mL concentrated hydrochloric acid was added. The solution was heated to 50 °C for 15 min. 10 mmol (0.951 g) of 3-hydroxypyridine, dissolved in 2 mL of DMF, was slowly added to the potassium tetrachloropalladate solution. The mixture was kept on stirring at around 50 °C for 30 min. After 30 min the volume of the mixture was reduced to about 5 mL resulting in the formation of white precipitate. 40 mL of 6 M hydrochloric acid was added which caused the formation of some yellow precipitate. The mixture was heated at 50 °C with reflux for 5 h following which the flask was brought to room temperature and then kept on ice for 1 h. Yellow precipitate of TH5 was collected at the pump, washed with mQ water and ethanol and air-dried. The weight of the final product was 0.252 g giving a yield of 69.0 %. The steps in synthesis are shown in Figure 4.5.

![Figure 4.5: Schematic for the synthesis of TH5](image)

4.3.7. *Trans*-PdCl$_2$(2-hydroxypyridine)$_2$ [TH6]

One mmol (0.326 g) of potassium tetrachloropalladate was dissolved in 7.5 mL of mQ water and 0.25 mL of concentrated hydrochloric acid was added. The solution was heated to 45 °C for 15 min. 10 mmol (0.951 g) of 2-hydroxypyridine, dissolved in 2
mL DMF, was slowly added to the potassium tetrachloropalladate solution. The colour turned red after 1 min then to orange after 4 min and there was some orange precipitate. The solution was continued to be stirred for 25 min at about 30 °C. 40 mL 6 M hydrochloric acid was added, the mixture was heated at 50 °C with reflux for 5 h following which the flask was cooled to room temperature. It was then kept on ice for 1 h. The yellow precipitate of TH6 was collected at the pump, washed with mQ water and ethanol and air-dried. The weight of the final product was 0.271 g giving a yield of 74.0 %. The steps in synthesis are shown in Figure 4.6.

![Figure 4.6: Schematic for the synthesis of TH6](image)

**4.3.8. Trans-PdCl₂(4-hydroxypyridine)₂ [TH7]:**

One mmol (0.326 g) of potassium tetrachloropalladate was dissolved in 7.5 mL of mQ water and 0.25 mL of concentrated hydrochloric acid was added. The solution was heated to 45 °C. Ten mmol (0.951 g) of 4-hydroxypyridine, dissolved in 2 mL of mQ water, was slowly added to the potassium tetrachloropalladate solution. The colour turned yellow that was followed by the formation of some yellow precipitate. The solution was stirred for about 25 min at 45 °C, after which the volume was reduced to about 10 mL. Then, 40 mL 6 M hydrochloric acid was added. Stirring was continued at room temperature for 8 h. Some yellow precipitate appeared and the flask was kept on ice for 1 h for more precipitate to form. The yellow precipitate of TH7 was
collected at the pump, washed with mQ water and ethanol and air-dried. The weight of the final product was 0.289 g giving a yield of 79.0%. The steps in synthesis are shown in Figure 4.7.

![Figure 4.7: Schematic for the synthesis of TH7](image)

**4.3.9. Preparation of \[\{\textit{trans}\text{-PdCl(NH}_3\text{)}_2\}\text{\{\mu\text{-trans-}}\text{Pd(C}_5\text{H}_5\text{NO)}_2\text{\{NH}_2\text{(CH}_2\text{)}_6\text{NH}_2\text{)}_2\}\text{Cl}_4 \text{[MH3]}\)

As stated earlier, trinuclear complexes were prepared using essentially the same step-up method of synthesis branching out from the central unit. For MH3, TH7 served as the central unit. 0.091 g (0.25 mmol) of TH7 was dissolved in 3 mL of DMF by stirring at 30-40°C for about 30 min. 0.058 g (0.5 mmol) of 1,6-diaminohexane was added to the 1.5 mL of mQ water followed by the addition of 500 µL of 1 M HCl. The solution was stirred at 40°C for 15 min. The solution of 1,6-diaminohexane was added to the solution of TH7 (within 30 min of its preparation) at 40°C resulting in the formation of some yellow precipitate. The mixture was stirred at 40°C for 15 min. 250 µL of 1 M NaOH was added to the mixture and stirred for a further 5 min at 40°C. 0.053 g (0.25 mmol) of transpalladin-DMF filtrate (prepared previously as described in section 4.3.4) was added to the mixture followed by stirring for 5 min at 40°C.
Then, a further 250 µL of 1 M NaOH was added to the mixture that was stirred for 5 min at 35°C followed by the addition of a further 0.25 mmol of transpalladin-DMF filtrate at 40°C. The mixture was then stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 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. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in ice for 2 h to produce yellow precipitate of MH3. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and methanol, and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was used to purify the crude product of MH3. The steps involved in synthesis are shown in Figure 4.8.
Figure 4.8: Schematic representation for the synthesis of MH3
4.3.10. Preparation of $[\text{trans-PdCl(NH}_3]_2\{\text{µ-\textit{trans-}}$

$\text{Pd(C}_5\text{H}_5\text{NO})]_2\{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\}_2\text{Cl}_4 [\text{MH}_4]$

For the trinuclear complex MH4, TH5 served as the central unit. 0.091 g (0.25 mmol) of TH5 was dissolved in 5 mL of DMF by stirring at 30-40°C for about 30 min. 0.058 g (0.5 mmol) of 1,6-diaminohexane was dissolved in 500 µL of 1 M HCl followed by the addition of 1.5 mL of DMF. The 1,6-diaminohaxane solution was stirred at 40°C for 15 min following which it was added to the solution of TH7 also at 40°C resulting in the formation of some yellow precipitate. The mixture was stirred for 15 min. 250 µL of 1 M NaOH was added to the mixture and stirred for a further 5 min at 40°C. 0.053 g (0.25 mmol) of transpalladin-DMF filtrate (prepared previously as described in section 4.3.4) was added to the mixture followed by stirring for a further 5 min. Then, a further 250 µL of 1 M NaOH was added to the mixture that was stirred for 5 min at 40°C. This was followed by the addition of a further 0.25 mmol of transpalladin-DMF filtrate at 40°C. The mixture was then stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapour Trap and Savant Speed Vac 110 Concentrator. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in ice for 2 h to produce yellow precipitate of MH4. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and methanol, and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was carried out to purify the crude product of MH4. The steps involved in synthesis are shown in Figure 4.9.
4.3.11. Preparation of \( \{\{\text{trans-PdCl(NH}_3\}_2\} \mu\{\text{trans-Pd(C}_5\text{H}_5\text{NO})\}_2\{\text{NH}_2\{\text{CH}_2\}_6\text{NH}_2\}_2\}\text{Cl}_4 \) [MH5]

For the trinuclear complex MH5, TH6 served as the central unit. 0.091 g (0.25 mmol) of TH6 was dissolved in 5 mL of DMF by stirring at 30-40°C for about 30 min. 0.058
g (0.5 mmol) of 1,6-diaminohexane was dissolved in 500 µL of 1 M HCl followed by the addition of 2 mL of DMF. The 1,6-diaminohexane solution was stirred at 40°C for 10 min following which it was added to the solution of TH6 also maintained at 40°C resulting in the formation of some yellow precipitate. The mixture was stirred at 40°C for 15 min. 250 µL of 1 M of NaOH was added to the mixture following which it was stirred for a further 5 min at 40°C. 0.053g (0.25 mmol) of transpalladin-DMF filtrate (prepared previously as described in section 4.3.4) was added to the mixture that was stirred for 5 min. Then, a further 250 µL of 1 M NaOH was added to the mixture that was stirred for another 5 min at 40°C followed by the addition of a further 0.053 g (0.25 mmol) of transpalladin-DMF filtrate at the same temperature. The mixture was then stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapour Trap and Savant Speed Vac 110 Concentrator. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in ice for 2 h to produce yellow precipitate of MH5. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and methanol, and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was used to purify the crude product of MH5. The steps involved in synthesis are shown in Figure 4.10.
4.3.12. Preparation of \([\{\textit{trans}-\text{PdCl}(C_5H_5NO)\}_2\}\{\mu-\textit{trans-}
\text{Pd(NH}_3\}_2\{\textit{(NH}_2\text{CH}_2\text{NH}_2\}_2\}])_4\text{Cl}_4\) \([\text{MH6}]\)

For the trinuclear complex MH6, transpalladin-DMF filtrate served central unit and
TH6 served as the terminal units. To 0.053 g (0.25 mmol) of transpalladin-DMF
filtrate (prepared as described in section 4.3.4) was added 0.058 g (0.5 mmol) of 1,6-diaminohexane in solution that was prepared by dissolving the diamine first in 500 µL of 1 M HCl followed by the addition of 2 mL of DMF. The mixture was stirred at 40°C for 15 min. 250 µL of 1 M NaOH was added to the mixture following which it was stirred for a further 5 min at the same temperature. 0.091 g (0.25 mmol) of TH6 dissolved in 5 mL of DMF at 30-40°C was added to the mixture that was stirred for a further 5 min. 250 µL of 1 M NaOH was added to the mixture followed by 5 min stirring. This was followed by the addition of a further 0.091 g (0.25 mmol) of TH6 dissolved in DMF as described previously. The mixture was stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapour Trap and Savant Speed Vac 110 Concentrator. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in ice for 2 h to produce yellow precipitate of MH6. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was used to purify the crude product of MH6. The steps involved in synthesis are shown in Figure 4.11.
Figure 4.11: Schematic representation for the synthesis of MH6
4.3.13. Preparation of \([\{\text{trans-PdCl(C}_5\text{H}_5\text{NO)}_2\}\}_2 \{\text{µ-trans-Pd(NH}_3\}_2\{(\text{NH}_2\text{(CH}_2\text{)}_6\text{NH}_2\}_2])\text{Cl}_4\) [MH7]

For the trinuclear complex MH7, transpalladin-DMF filtrate served central unit and TH5 served as the terminal units. To 0.053 g (0.25 mmol) of transpalladin-DMF filtrate (prepared as described in section 4.3.4) was added 0.058 g (0.5 mmol) of 1,6-diaminohexane in solution that was prepared by dissolving the diamine first in 500 µL of 1 M HCl followed by the addition of 2 mL of DMF. The mixture was stirred at 40°C for 15 min. 250 µL of 1 M NaOH was added to the mixture following which it was stirred for a further 5 min at the same temperature. 0.091 g (0.25 mmol) of TH5 dissolved in 5 mL of DMF at 30-40°C was added to the mixture followed by 5 min stirring. 250 µL of 1 M NaOH was added to the mixture that was stirred for a further 5 min at 40°C. This was followed by the addition of a further 0.091 g (0.25 mmol) of TH5 dissolved in DMF as described previously. The mixture was stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapour Trap and Savant Speed Vac 110 Concentrator. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in an ice for 2 h to produce yellow precipitate of MH7. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was used to purify the crude product of MH7. The steps involved in synthesis are shown in Figure 4.12.
Figure 4.12: Schematic representation for the synthesis of MH7
4.3.14. Preparation of $\{\text{trans-PdCl(C}_5\text{H}_5\text{NO)}_2\}_2\{\mu\text{-trans-Pd(NH}_3\}_2\{(\text{NH}_2\text{(CH}_2\text{)}_6\text{(NH}_2\}_2\}_\text{Cl}_4 \ [\text{MH8}]

For the trinuclear complex MH8 also, transpalladin-DMF filtrate served central unit (as in the case of MH6 and MH7), and TH7 served as the terminal units. To 0.053 g (0.25 mmol) of transpalladin-DMF filtrate (prepared as described in section 4.3.4) was added 0.058 g (0.5 mmol) of 1,6-diaminohexane in solution that was prepared by dissolving the diamine first in 500 µL of 1 M HCl followed by the addition of 2 mL of DMF. The mixture was stirred at 40°C for 15 min. 250 µL of 1 M NaOH was added to the mixture followed by stirring for a further 5 min at the same temperature.

0.091 g (0.25 mmol) of TH7 dissolved in 5 mL of DMF at 30-40°C was added to the mixture that was stirred for a further 5 min. 250 µL of 1 M NaOH was added to the mixture that was stirred for a further 5 min at 40°C. This was followed by the addition of a further 0.091 g (0.25 mmol) of TH7 dissolved in DMF as described previously. The mixture was stirred at 40°C for 3 h. The solution was filtered to remove any suspended particle. The volume of the filtrate was reduced to 3 mL using a vacuum concentrator consisting of a Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapour Trap and Savant Speed Vac 110 Concentrator. 20 mL of dichloromethane was added to the concentrated solution. The mixture was placed in ice for 2 h to produce yellow precipitate of MH6. The precipitate was collected by filtration at the pump, washed with ice-cold mQ water and air-dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding dichloromethane was used to purify the crude product of MH8. The steps involved in synthesis are shown in Figure 4.13.
Figure 4.13: Schematic representation for the synthesis of MH8
4.4. Characterization

**Microanalysis:** Elemental composition of the compounds was determined by microanalysis. Carbon, hydrogen and nitrogen contents were determined using the facility at the Australian National University whereas the palladium content was determined by graphite furnace atomic absorption spectroscopy (AAS) using the Varian AA240 Atomic Absorption Spectrophotometer with GTA120 Graphite Furnace Tube Atomizer, available in the Discipline of Biomedical Science, Faculty of Medicine, The University of Sydney (Rothery, 1991).

For the determination of the palladium content of MH3, MH4, MH5, MH6 MH7 and MH8, a serial dilution of compound solutions ranging from 0.01 mM to 0.0025 mM with 0.1 M HCl was carried out. To make ready for use in AAS analysis, all glassware was first washed with detergent, then soaked in 20% v/v HCl for 48 h, then in 20% v/v HNO₃ for another 48 h and finally rinsed with mQ water.

**Other analysis:** Infrared, mass and $^1$H NMR spectra were recorded using the facilities available in the School of Chemistry, The University of Sydney.

4.4.1. AAS analysis:

4.4.1.1. **Preparation palladium standard solution for AAS analysis:**

A standard palladium solution (500 ppb) was made by diluting 1020 ppm atomic absorption standard palladium solution, obtained from Mallinckrodt Specialty Chemicals Company. Precisely, to obtain 100 mL of 500 ppb Pd, 0.049 mL of 1020 µg Pd/mL Pd was diluted to 100 mL with 0.1 M HCl.
4.4.1.1. AAS conditions and furnace parameters

Tables 4.1, 4.2 and 4.3 summarize the conditions for AAS used for the determination of palladium. The furnace parameters were essentially the same as those given in the Varian manual (Knowles, 1988). Figure 4.14 gives the typical absorbance versus concentration graph.

Table 4.1: Furnace operating conditions for the determination of palladium content

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Gas Flow (L/min)</th>
<th>Gas type</th>
<th>Read Command</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>85</td>
<td>5</td>
<td>0.3</td>
<td>Normal</td>
<td>--------------</td>
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<tr>
<td>2</td>
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<td>0.3</td>
<td>Normal</td>
<td>--------------</td>
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<td>120</td>
<td>10</td>
<td>0.3</td>
<td>Normal</td>
<td>--------------</td>
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<td>5</td>
<td>0.3</td>
<td>Normal</td>
<td>--------------</td>
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<td>--------------</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>2</td>
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</tr>
<tr>
<td>7</td>
<td>2500</td>
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<td>Read steps, Start signal</td>
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Table 4.2: Furnace parameters used for the determination of palladium content

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<td></td>
<td>Calibration mode: Standard addition</td>
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<td></td>
<td>Smoothing: 7 point</td>
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<td>Replicates standard and sample: 2</td>
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<td></td>
<td>Precision % standard and sample: 5</td>
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<td>Monochromator: Wavelength [nm] 244.8</td>
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<td></td>
<td>Slit width [nm] 0.5</td>
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<td></td>
<td>Back ground correction: on</td>
</tr>
<tr>
<td>Standard</td>
<td>Upper valid concentration: 500</td>
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</tr>
<tr>
<td></td>
<td>Reslop rate: 10</td>
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<tr>
<td></td>
<td>Reslop standard no.: 2</td>
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<tr>
<td></td>
<td>Expansion factor: 1</td>
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<tr>
<td></td>
<td>Internal standard: 0.5</td>
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Table 4.3: Sample parameters used in AAS analysis to determine the palladium contents.

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<th>Method Conc.</th>
<th>Mix Conc.</th>
<th>Blank (µL)</th>
<th>Bulk (standard) (µL)</th>
<th>Sample (µL)</th>
<th>Total volume (µL)</th>
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</thead>
<tbody>
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<td>-----</td>
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<td>16</td>
<td>4</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

Volume reduction factor: 2
Sample injection rate: 1

Figure 4.14: Typical absorbance versus concentration graph for palladium
4.4.2. Calculating percentage of yield for each compound

To obtain percentage yield for a compound, the ratio of actual yield over the theoretical yield was multiplied by 100.

4.4.3. Spectral analysis:

4.4.3.1. Infrared Spectra:

Infrared (IR) spectra are routinely used to aid in the characterization of compounds. The IR spectrum of the molecule is considered as a finger-print of a molecule, because each molecule has its own characteristic modes of vibration. It is often difficult if not impossible to determine the structure of a molecule based on its IR spectrum alone. However, the spectrum can provide valuable information related to its structure e.g. it can indicate the presence of functional groups such as C-H, -OH, -NH, C=C, etc (Silverstein et al., 1991).

Infrared spectra were collected using a Bruker IFS66 spectrometer. The spectrometer is equipped with an air-cooled DTGS detector, a KBr beamsplitter with a spectral range of 4000 to 400 cm\(^{-1}\) and polyethylene reflectance between 250 – 500 cm\(^{-1}\). The instrument was run under vacuum during spectral acquisition. Spectra were recorded at a resolution of 4 cm\(^{-1}\), with the co-addition of 128 scans and a Blackman-Harris 3-Term apodisation function was applied. Prior to analysis the samples were mixed, and lightly ground, with finely ground spectroscopic grade KBr. The spectra were then manipulated using the Kubelka-Munk mathematical function in the OPUS™ software to convert the spectra from reflectance into absorbance.
4.4.3.2. **Mass spectra:**

Mass spectrometry also aids in the characterization of a compound by providing molecular mass and masses of molecular fragments (more exactly mass to charge ratios from which masses can be determined). This technique is more sensitive than IR or nuclear magnetic resonance spectroscopy (\textsuperscript{1}H NMR); only a microgram or less is required to record a mass spectrum.

To obtain mass spectra of MH3, MH4, MH5, MH6, MH7 and MH8, solutions of compounds were made in 10\% DMF and 90\% methanol and then sprayed into a Finnigan LCQ ion trap mass spectrometer available in the School of Chemistry, The University of Sydney. The flow rate was 0.2 mL/min consisting of 50\% methanol and 50\% water. The ions observed were in positive ion mode.

4.4.3.3. **\textsuperscript{1}H NMR spectra:**

Nuclear magnetic resonance or \textsuperscript{1}H NMR spectroscopy is one of the most widely used methods for determining the structures of molecules and to quantify nature of interaction between molecules. \textsuperscript{1}H NMR spectroscopy 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, 1998). \textsuperscript{1}H NMR can be applied to the study of three states of matter: solids, liquids and gases. Some nuclei such as \textsuperscript{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 into 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 transform methods; 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., 1991).

All compounds (MH3, MH4, MH5, MH6, MH7 and MH8) were dissolved in deuterated DMSO and $^1$H NMR spectra of the compounds were recorded in a Bruker DPX400 spectrometer using a 5 mm high precision Wilmad NMR tube. Spectra were referenced to internal solvent residues and all the spectra were recorded at 300 K ($\pm 1$ K).

### 4.4.4. Molar conductivity

As the conductivity of a solution depends on the number of ions present, it is normal to express it in terms of molar conductivity, $\Lambda_m$, which is defined as $\Lambda_m = k/c$ where $k$ is the conductivity and $c$ is the molar concentration. The SI unit of molar conductivity is siemens meter-squared per mole (Sm$^{-2}$mol$^{-1}$). Whereas neutral non-polar compounds can cross the cell membrane by passive diffusion, polar molecules and charged species are more likely to cross the cell membrane by carrier mediated transport. Since molar conductivity values relate to degree of dissociation, the values can provide valuable information on likely mode(s) of transport across the cell membrane.
In the case of MH3, MH4, MH5, MH6, MH7 and MH8, molar conductivity values were determined at concentrations ranging from 0.5 mM to 1.0 mM. The compounds were first dissolved in 1:1 mixture of DMF to obtain 1 mM solutions, which were then progressively diluted with mQ water to obtain solutions at concentrations ranging from 0.5 mM to 1.0 mM. The conductivity values were measured using a PW9506 digital conductivity meter and a 2100-Digital conductivity meter respectively. Molar conductivity values were then plotted against concentration to obtain the limiting value i.e. the value at zero concentration or infinite dilution (Atkins, 1998).

4.5. Biological activity

4.5.1. Interaction with DNA

The activity of platinum-based anticancer drugs is believed to be associated with their binding with nucleobases in DNA. As a result, conformational changes and damage to DNA may take place so that the rate of migration of DNA bands in an electric field may also change. In this project, the interaction of the designed palladium complexes with pBR322 plasmid DNA and salmon sperm DNA (ssDNA) was studied using gel electrophoresis. Further experiments in which pBR322 plasmid DNA was first interacted with varying concentrations of compounds followed by BamH1 digestion were carried out to provide more information on DNA conformation change. Cisplatin was used as a reference. More about plasmid and ssDNAs and BamH1 digestion are considered later.
4.5.1.1. Basic principle of gel electrophoresis

The term electrophoresis is used to describe the migration of charged particle under the influence of an electric field. Gel electrophoresis refers to the technique in which molecules are forced across a span of gel by the application of an electrical field. Activated electrodes at either end of the gel provide the driving force. DNA being poly-negatively charged due to the phosphate backbone, it will migrate towards the positive electrode. A number of factors including the size of the DNA and its conformation, concentration of the supporting gel matrix, electric field strength, composition of the electrophoresis buffer, the presence of intercalating dyes and temperature would influence the rate of migration through the gel (Helling et al., 1974). pBR322 plasmid DNA is circular, duplex, high molecular weight DNA ranging in size from two kilo bases to a few hundred kilo bases. It can exist in three forms: supercoiled circular form I, singly nicked relaxed circular form II, and doubly nicked linear form III (Cantor and Schimmel, 1980) (Figure 415). Supercoiled form I is the most common form, form II or form III are produced by chemical or physical alteration (Barton, 1994).
The supercoiled form I being compacted migrates at the fastest rate whereas the relaxed circular form II migrates at the slowest rate. The linear form III DNA has the intermediate rate of migration (Figure 4.16).

As stated earlier, palladium and platinum compounds can cause changes in DNA conformation as a result of covalent binding. One of the changes that may occur is unwinding of the DNA. For example, supercoiled form I pBR322 plasmid DNA can change from negatively supercoiled form I to positively supercoiled form I through relaxed circular form II (Figure 4.17). This change can cause a change in mobility of DNA through the gel.
Figure 4.17: Topological forms of closed circular DNA, negatively supercoiled form I through relaxed circular form to positively supercoiled form I
4.5.1.2. Factors affecting electrophoresis

As stated earlier, in this project, electrophoresis using agarose gel was carried out to investigate conformational and other changes in pBR322 plasmid DNA and salmon sperm DNA (ssDNA) as a result of their interaction with the designed palladium complexes. Agarose is a high molecular weight polysaccharide extracted from the walls of certain marine red algae that is insoluble in cold water but dissolves readily in boiling water. When used as supporting running matrix for DNA, agarose gel has the advantage of providing a non-toxic gel medium that is easy to cast and quick to run. It forms side-by-side aggregates that condense into a three-dimensional, interlocking network held together by noncovalent hydrogen bonds. The electrophoretic mobility of DNA is inversely related to the concentration of agarose so that DNA would take a longer time to run the same distance through the gel at a higher concentration than a lower one. In addition, use of different concentrations of gel makes possible the resolution of a wide range of DNA molecules in terms of molecular size (Ogston, 1958; Rodbard and Chrambach, 1970).

As stated earlier, electric field strength is one of the factors that determine the electrophoretic mobility of DNA. The higher the voltage the higher will be the rate of migration of DNA. However, as the electric field strength is increased, temperature would also increase due to the resistive heating of the gel by the electric field. In the worst case, the gel would melt resulting in DNA denaturation. 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., 1987).
The composition and ionic strength of the electrophoresis buffer also affect the rate of migration of DNA. In ions-free electrophoresis buffer, there is lack of electrical conductance and DNA would migrate 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. Several different buffers including TAE buffer containing EDTA (pH 8.0) and tris-acetate (TAE), TBE buffer (Tris-borate) with EDTA and TPE buffer (Tris-phosphate) with EDTA at a concentration of approximately 50 mM are commonly used in gel electrophoresis. DNA fragments migrate faster through TAE than through TBE and TPE and the resolution of supercoiled DNAs 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 visualized 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 gel in the absence of the dye and stain the gel after the electrophoresis in order to give sharper bands.

4.5.1.3. 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 (BamHI), 10X digestion buffer and Polaroid black-and-white print film type 667 were obtained from Sigma, Australia.

4.5.1.4. 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 50 X TAE buffer solution, 4.66 g of 50 mM Na₂EDTA, 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 to bring the volume up to 250 mL. 40 mL of 50 X TAE buffer solution was then diluted with mQ water to give 2000 mL of TAE working buffer solution.

4.5.1.5. 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 oven for 1 min, swirled to mix and boiling was continued for a further 4 min. Agarose solution was cooled for a few minutes. 60 µL of ethidium bromide was added to the gel that was swirled gently to disperse the dye (Stellwagen, 1998). The gel was then gently poured into the tray with a well comb placed in position and left at room temperature for 30 min to solidify.
4.5.1.6. Preparation of ssDNA

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 -17ºC 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 0.22 µm Millipore filter.

4.5.2. Interaction with ssDNA

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

4.5.2.1. Method

To 4 µL aliquots of solutions of ssDNA (at 1.5 mg/mL) were added varied amounts of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin in solution and the total volume was made up to 20 µL by adding mQ water so that the concentrations of compounds ranged from 1.25 µM to 60 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 37ºC for 4 h. 16 µL aliquots of drug-DNA mixtures were loaded onto the 1% agarose gel and electrophoresis was run in 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 (Polaroid type (black-and-white) film was used).

### 4.5.3. Interaction with pBR322 plasmid DNA

In the interaction of compounds with pBR322 plasmid DNA, as in their interaction with ssDNA, the amount of DNA was kept constant while the concentrations of compounds were varied. Exactly, 1.5 µL of supplied pBR322 plasmid DNA in solution was added to varied amounts of solutions of the compounds at different concentrations ranging from: 1.25 µM to 60 µM. 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 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.
4.5.4. Interaction with pBR322 plasmid DNA combined with BamH1

BamH1 is a restriction endonuclease that hydrolyses the phosphodiester bonds and isolated from *Bacillus amyloliquefaciens* H. BamH1 is known to recognize the sequence G/GATCC and hydrolyze the phosphodiester bond between adjacent GG sites (Smith and Chirikjian, 1979). pBR322 plasmid DNA Supercoiled form I contains a single restriction site for BamH1 (Sutcliffe, 1979) that converts the supercoiled form I and also singly nicked circular form II to linear form III DNA. As conformational changes are induced in pBR322 plasmid DNA due to its interaction with varying concentrations of compounds, prevention of BamH1 digestion may occur so that the results of BamH1 digestion may provide information on drug-DNA interaction. pBR322 plasmid DNA was first interacted with varying concentrations of compounds followed by BamH1 digestion. Then the electrophoresis of digested DNA was carried out as described below.

4.5.4.1. Methods

In this series of experiments, an identical set of drug-DNA mixtures as that described previously in (4.5.3), was first incubated with varying concentrations of compounds 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 mixtures were added 2 µL of 10X digestion buffer SB first and then 0.3 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.
4.5.5. Introduction to tissue culture

Animal or plant cells, removed from tissues, continue to grow if supplied with the appropriate nutrients and conditions. When carried out in a laboratory, the process is called “cell culture”. It occurs in vitro (‘in glass’) as opposed to in vivo (‘in life’). The culture process allows single cells to act as independent units, much like a microorganism such as a bacterium or fungus. The cells are capable of dividing, increasing in size and also in a batch culture, can continue to grow until limited by culture variables such as nutrient depletion. Cell culture offers a method for studying the behaviour of animal cells free of systemic variations and allowing careful control of the physiochemical and physiological environments in terms of pH, temperature, osmotic pressure, concentrations of O₂ and CO₂, hormone, nutrient concentrations, etc. Another advantage of cell culture is that it produces identical cell lines using subculture techniques to maintain a homogeneous culture of the most vigorous cell type (Freshney, 1994).

To maintain a cell by using cell subculture, a suitable medium is needed whose pH should be maintained around 7.4 by buffering with sodium bicarbonate solution. A complete medium must contain all essential nutrients including different amino acids (both essential and nonessential ones), vitamins such as the B-group, plus choline, folic acid, inositol, and nicotinamide, salts (Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, SO₄²⁻, PO₄³⁻, and HCO₃⁻), glucose, extra metabolites (e.g., nucleosides, tricarboxylic acid cycle intermediates, and lipids), hormones and growth factors. There are commercially available serums that fulfil this requirement and also contain growth factors, which promote cell proliferation, and adhesion factors and antitrypsin activity, which promote cell attachment. The optimal temperature for most human and warm-blooded
animal cell lines is 37 °C and it should have oxygen in the gas phase for respiration in vivo (Freshney, 1994).

Most cells from solid tissues grow in vitro as an adherent monolayer. Cells need to attach and spread out on the substrate before they will start to proliferate. Trans-membrane proteins are involved in causing cell-cell and cell-substrate adhesion (Klagsbrun and Baird, 1991; Rosenman and Gallatin, 1991). Cells then move about and divide until they form a one cell thick monolayer, completely covering the surfaces of the culture vessel. Movement and proliferation normally cease when confluence is reached meaning all the available growth area is utilized and the cells make close contact to each other (Brouty-Boye et al., 1979; Brouty-Boye et al., 1980; Todaro and Green, 1963). This is followed by dissociation and detachment of the monolayer when cells are to be harvested for study, processing or subculture. Enzyme trypsin, a pancreatic serine protease, is most frequently applied in the treatment of the cell layer.

4.5.5.1. Materials

The three human ovarian cancer cell lines, A2780, its cisplatin-resistant form A2780<sup>cisR</sup> and ZD0473-resistant form A2780<sup>ZD0473R</sup> 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 (Behrens et al., 1987; Hamilton et al., 1984). Cisplatin-resistant cell line A2780<sup>cisR</sup> has been developed by chronic exposure of parent cisplatin-sensitive A2780 cells to increasing concentrations of cisplatin (Behrens et al., 1987; Masuda et al., 1988) and the cell line A2780<sup>ZD0473R</sup> was developed by in vitro exposure of parent cisplatin-sensitive A2780
cells to increasing concentrations from 0.5 to 12.5 µM of drug ZD0473 for a period of 7 months (Holford et al., 2000). All the above cell-lines were stored under liquid nitrogen at Royal Prince Alfred Hospital, Sydney.

Fetal calf serum, 5X RPMI 1640, 200 mM L-glutamine and 5.6% sodium bicarbonate were obtained from Thermo Trace Pty Ltd Melbourne, Australia. Trypsin, Heps, Dulbecco's phosphate buffered saline powder(PBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-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 cm² culture flasks were obtained from Crown Scientific. Microplate reader BIO-RAD Model 3550 was used to read the optical density of each well.

4.5.5.2. Maintenance of the cell lines

All the cell lines were grown in tissue culture flasks kept in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2 mM glutamine without antibiotics.

4.5.5.3. Preparation of the medium

The medium used for cell culture was mainly 10% fetal calf serum (FCS/RPMI 1640). As listed in table 4.4, one litre of the medium was prepared by mixing the following components together: 200 mL of (5X RPMI 1640), 100 mL of 10% fetal 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 of saturated NaOH. Sterile water was used to make up the volume to one litre.

Table 4.4: Constituents of the medium (FCS/RPMI 1640) containing different percentages of FCS.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>2% FCS</th>
<th>5% FCS</th>
<th>10% FCS</th>
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<tr>
<td>5 x RPMI 1640</td>
<td>200 mL</td>
<td>200 mL</td>
<td>200 mL</td>
</tr>
<tr>
<td>FCS</td>
<td>20 mL</td>
<td>50 mL</td>
<td>100 mL</td>
</tr>
<tr>
<td>Hepes</td>
<td>20 mL</td>
<td>20 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>20 mL</td>
<td>20 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10 mL</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
<tr>
<td>Sat. NaOH</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Volume of sterile water</td>
<td>729.5 mL</td>
<td>699.5 mL</td>
<td>649.5 mL</td>
</tr>
</tbody>
</table>

4.5.5.4. Phosphate buffered saline (PBS)

4.5.5.4.1. Materials

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

4.5.5.4.2. 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 added 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.

4.5.5.5. Trypsin

To prepare 100 mL of trypsin, 0.02 g 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 kept at 4°C in the fridge) was added and the volume was made up to 100 mL with PBS (89 mL PBS). It was then filtered using a 0.22 micron filter and kept in fridge for later use.

4.5.5.6. 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.

4.5.5.7. 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\(^{\text{cisR}}\) and A2780\(^{\text{ZD0473R}}\) 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 labeled 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-labeled flasks each containing 8 mL of fresh medium. The flasks were incubated in an atmosphere of 5% CO$_2$ and 95% air at 37 °C.

4.5.5.8. 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, 1994).

4.5.5.9. Procedure

The hood was swabbed with cavacide first then with 70% ethanol. Then the required materials were placed in the hood. The flasks containing the cell lines were taken from the incubator and examined carefully under microscope for any sign of contamination or deterioration. The medium was removed from the flasks and discarded. The flasks were then washed with 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\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}, and 1 min for A2780. The flasks were removed from the incubator and the suspensions of the cells were examined on 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, and 1 mL of the suspension left in each flask was treated 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.

4.5.5.10. Cell count

A hemocytometer was used for counting cells. The coverslip and the surface slide of the hemocytometer chambers were cleaned with 70% alcohol. The coverslip was then placed on the counting area 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\textsuperscript{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.
4.5.5.11. Storage of the cell lines

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

4.5.5.12. MTT-Based cytotoxicity assay

4.5.5.12.1. Introduction to cytotoxicity

Cellular model of *in vitro* toxicology offers a simple and highly reproducible method for the determination of activity of anticancer drugs. It is less costly and less time consuming than *in vivo* experiments. It allows easy exploration and detailed analyses of the molecular mechanisms of toxicity and early identification of cellular damage. However, it is not possible to study systemic toxic effects using the cellular model. It is also difficult to correlate the *in vitro* situation to the *in vivo* one both in terms of experimental design and results as the cellular system is highly simplified compared to the complexity of the organism. Nevertheless, it plays a key role in drug
development as the broad aim of in vitro toxicity testing is to screen compounds in order to get an indication of their general cytotoxicity.

4.5.5.12.2. 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. The method is based on the reduction of a yellow tetrazolium component (MTT) into an insoluble purple formazan product by the mitochondria of viable cells (Carmichael et al., 1987). After incubation of the cells with the MTT for several hours, DMSO is added to lyse the cells and solubilize the coloured crystals. Samples are read using an ELISA plate reader at a wavelength of 570 nm. The amount of colours produced is directly proportional to the number of viable cells. IC$_{50}$ refers to the amount of drug required to reduce tissue viability to 50%. It should be noted MTT reduction assay is an indirect method to measure the proportion of viable cells following a drug exposure (Baruah et al., 2002; Jansen et al., 2001; Woolley et al., 2002). It may be noted that in MTT reduction assay, cell contamination may cause false negative results (Denecke et al., 1999) and that it is impossible to distinguish between cytostatic and cytocidal effects of the drugs on cells.

4.5.5.12.3. Methods

The human ovarian cancer cell lines: A2780, A2780$^{vis}$ 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 fetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2 mM glutamine (Freshney, 1994). As
noted earlier, cytotoxicity was determined using 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 incubated for 24 h at 37°C in a humidified atmosphere to allow cells to attach. Platinum and palladium complexes were first dissolved in a minimum volume of DMF, then diluted to the required concentrations by adding mQ water and filtered to sterilize. A serial dilution of the drugs ranging from 0.02 µM to 40 µM in 10% FCS/RPMI 1640 medium was made and added to equal volumes of cell culture in quadruplicate wells. The plates containing cells and drugs were left to incubate under normal growth conditions for 72 h. At the end of 72 h of incubation, MTT (50 µL per well of 1 mg mL⁻¹ MTT solution) was added to each plate. Four hours after the addition of MTT, the yellow formazan crystals produced from the reduction of MTT. Then the MTT in the solution was discarded. 150 µL of DMSO was added to each well to dissolve the MTT-formazan and the absorbance of the resulting solution was read with Bio-Rad Model 3550 Microplate Reader set at 570 nm.

The percentage of living cells was calculated according to the following equation:

\[
\% \text{ Of living cells} = \frac{\text{Absorbance of sample} - \text{Absorbance of DMSO (Blank)}}{\text{Absorbance of control} - \text{Absorbance of DMSO (Blank)}} \times 100
\]

At least three independent experiments were done for each drug concentration. Dosage response curves were constructed by plotting the percentage of viable cells against drug concentration. The IC₅₀ value was then determined from curve. The resistance factors (RF) applying to the cell lines: A2780 and A2780\text{cisR} were
calculated from the IC$_{50}$ values according to the following equation (Carmichael et al., 1987; Freshney, 2000; Mosmann, 1983).

$$\text{Resistance Factor (RF)} = \frac{\text{IC}_{50} \text{ value for } A2780^{\text{cisR}}}{\text{IC}_{50} \text{ value for } A2780}$$

4.5.6. Cellular platinum uptakes and DNA binding

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

4.5.6.1. Materials

Three human ovarian cancer cell lines A2780 (named as the parent cell line), its cisplatin-resistant form A2780$^{\text{cisR}}$ and ZD0473-resistant form A2780$^{\text{ZD0473R}}$. Triton X-100 (t-Octylphenoxypolyethoxyethanol) was obtained from Sigma-Aldrich Pty Ltd, NSW, Australia and commercially available DNA purification kit (JETQUICK Blood & Cell Culture DNA Spin kit/50) was obtained from Astral Scientific Pty Ltd, Australia.

4.5.6.2. Cell uptake

Designed palladium complexes and cisplatin (used as a reference) (at 50 µM final concentration) were added to culture plates containing exponentially growing A2780, A2780$^{\text{cisR}}$ and A2780$^{\text{ZD0473R}}$ 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 the cell monolayers were trypsinized and cell suspension (5 mL) was transferred to a 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 sonicated (5 min). Total intracellular palladium or platinum contents were determined by graphite furnace AAS using a Varian SpectrAA-240 plus with GTA 120 atomic absorption spectrophotometer using a variant of standard addition technique (Roberts et al., 1999).

4.5.6.3. Palladium and Platinum (cisplatin only) DNA binding

Following incubation of cells with drugs, high molecular weight DNA was isolated from cell pellet using JETQUICK Blood DNA Spin Kit/50 according to 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 RNase 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 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 column. The columns containing the samples were washed with 500 µL of buffer KX (containing high-salt buffer to remove residual contamination) and centrifuged for 1 min at 10,600 rpm. The columns 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 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). Palladium 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 ensuring its high purity (Holford et al., 2000), and the DNA concentration was calculated according to the following equation:

Concentration = Absorbance at 260 nm X 50 ng/µL
5. Chapter Five: Results

5.1. Characterization of compounds:

As stated earlier, in this project we synthesized six trinuclear palladium compounds code named MH3, MH4, MH5, MH6, MH7 and MH8 (Figures 5.2 to 5.7) containing two or four hydroxypyridine ligands bonded to central or terminal palladium ions, with the aim of exploring structure-activity relationships. In all the compounds, palladium ions are linked together by 1,6-diaminohexane chains. In MH3, MH4 and MH5 the central Pd\(^{2+}\) ion is bound to two planaramine ligands whereas in MH6, MH7 and MH8, each terminal Pd\(^{2+}\) ion is bonded to two planaramine ligands. Mononuclear \textit{trans}-planaraminepalladium(II) complexes TH5, TH6 and TH7 served as starting materials for the synthesis of the trinuclear palladium complexes. MH3, MH4, MH5, MH6, MH7 and MH8 have been characterized by elemental analyses, spectral studies and molar conductivity measurements.

5.2. Characterization of MH3, MH4, MH5, MH6, MH7 and MH8

\textbf{MH3:} \{\textit{trans}-PdCl(NH\(_3\))\}_2\{\textit{trans}-Pd(4-hydroxypyridine)\}_2\{H\(_2\)N\}(CH\(_2\)_6NH\(_2\))\_2Cl\(_4\)

Formula: C\(_{22}\)H\(_{54}\)Cl\(_6\)N\(_{10}\)O\(_2\)Pd\(_3\) Molar mass: 1022.704 g mol\(^{-1}\)
Percentage of yield: 74 % (Figure: 5.1)
**Figure 5.1: Structure of MH3**

**MH4:** \{trans-PdCl(NH₃)₂\}_2 \{trans-Pd(3-hydroxypyridine)\}_2 \{H₂N(CH₂)₆NH₂\}_2Cl₄

Formula: C₂₂H₅₄Cl₆N₁₀O₂Pd₃  Molar mass: 1022.704 g mol⁻¹  
Percentage of yield: 69 % (Figure: 5.2)

**Figure 5.2: Structure of MH4**

**MH5:** \{trans-PdCl(NH₃)₂\}_2 \{trans-Pd(2-hydroxypyridine)\}_2 \{H₂N(CH₂)₆NH₂\}_2Cl₄

Formula: C₂₂H₅₄Cl₆N₁₀O₂Pd₃  Molar mass: 1022.704 g mol⁻¹  
Percentage of yield: 72 % (Figure: 5.3)
Figure 5.3: Structure of MH5

**MH6:** \{trans-PdCl(NH₃)₂\}\{trans-Pd(2-hydroxypyridine)\}_2\{H₂N(CH₂)₆NH₂\}_2Cl₄

Formula: C₃₂H₅₈Cl₆N₁₀O₄Pd₃  Molar mass: 1178.8414 g mol⁻¹
Percentage of yield: 80 % (Figure: 5.4)

Figure 5.4: Structure of MH6

**MH7:** \{trans-PdCl(NH₃)₂\}\{trans-Pd(3-hydroxypyridine)\}_2\{H₂N(CH₂)₆NH₂\}_2Cl₄

Formula: C₃₂H₅₈Cl₆N₁₀O₄Pd₃  Molar mass: 1178.8414 g mol⁻¹
Percentage of yield: 63 % (Figure: 5.5)
Figure 5.5: Structure of MH7

MH8: \(\{\text{trans-Pd} \text{Cl(NH}_3\}_2\} [\{\text{trans-Pd}(4\text{-hydroxypyridine})\}_2 \{\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\}_2\} \text{Cl}_4\)

Formula: C\(_{32}\)H\(_{58}\)Cl\(_6\)N\(_{10}\)O\(_4\)Pd\(_3\)  Molar mass: 1178.8414 g mol\(^{-1}\)
Percentage of yield: 81 % (Figure: 5.6)

Figure 5.6: Structure of MH8
5.3. Elemental Compositions of MH3, MH4, MH5, MH6, MH7 and MH8

Microanalyses were carried out to determine the elemental compositions of MH3, MH4, MH5, MH6, MH7 and MH8. Table 4.1 gives the calculated and the observed values for %C, %H, %N and %Pd.

Table 5.1: Elemental compositions of MH3, MH4, MH5, MH6, MH7 and MH8.

<table>
<thead>
<tr>
<th>Element %</th>
<th>MH3 $\text{C}<em>{22}\text{H}</em>{54}\text{Cl}<em>6\text{N}</em>{10}\text{O}_2\text{Pd}_3$</th>
<th>MH4 $\text{C}<em>{22}\text{H}</em>{54}\text{Cl}<em>6\text{N}</em>{10}\text{O}_2\text{Pd}_3$</th>
<th>MH5 $\text{C}<em>{22}\text{H}</em>{54}\text{Cl}<em>6\text{N}</em>{10}\text{O}_2\text{Pd}_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>25.8 ± 0.4 24.7 ± 0.4 25.8 ± 0.4 25.8 ± 0.4 25.8 ± 0.4</td>
<td>5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4</td>
<td>13.7 ± 0.4 10.5 ± 0.4 13.7 ± 0.4 13.7 ± 0.4 13.7 ± 0.4</td>
</tr>
<tr>
<td>H</td>
<td>5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4</td>
<td>5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4 5.3 ± 0.4</td>
<td>10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4</td>
</tr>
<tr>
<td>N</td>
<td>13.7 ± 0.4 10.5 ± 0.4 13.7 ± 0.4 13.7 ± 0.4 13.7 ± 0.4</td>
<td>13.7 ± 0.4 11.7 ± 0.4 13.7 ± 0.4 13.7 ± 0.4 13.7 ± 0.4</td>
<td>10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4 10.5 ± 0.4</td>
</tr>
<tr>
<td>Pd</td>
<td>31.2 ± 0.4 31.1 ± 0.4 31.2 ± 0.4 31.2 ± 0.4 31.2 ± 0.4</td>
<td>31.2 ± 0.4 30.6 ± 0.4 31.2 ± 0.4 31.2 ± 0.4 31.2 ± 0.4</td>
<td>31.1 ± 0.4 31.1 ± 0.4 31.1 ± 0.4 31.1 ± 0.4 31.1 ± 0.4</td>
</tr>
</tbody>
</table>

5.4. Spectral Analyses

IR, mass and $^1$H NMR spectral analyses were carried out to aid in the structural characterization of MH3, MH4, MH5, MH6, MH7 and MH8. Most of the peak
assignments are based on published spectra of the ligands and metal complexes of pyridine, substituted pyridines (Nakamoto, 1997; Silverstein et al., 1991).

5.4.1. IR

Figures 5.7 to 5.12 give the IR spectra of MH3, MH4, MH5, MH6, MH7 and MH8. The major peaks observed in IR spectra of MH3, MH4, MH5, MH6, MH7 and MH8 are listed in Table 5.2, followed by descriptions on peak assignment. The letters ‘s’, ‘m’ and ‘w’, and denote strong, medium and weak respectively. More detailed descriptions of the bands relating to the structures of the compounds are given in Chapter seven.
Figure 5.7: IR spectrum of MH3
Figure 5.8: IR spectrum of MH4
Figure 5.9: IR spectrum of MH5
Figure 5.10: IR spectrum of MH6
Figure 5.11: IR spectrum of MH7
Figure 5.12: IR spectrum of MH8
Table 5.2 Prominent IR spectral bands observed for MH3, MH4, MH5, MH6, MH7 and MH8

<table>
<thead>
<tr>
<th></th>
<th>IR (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH3</td>
<td>3268 (s, O-H stretching vibration), 3216 (s, O-H stretching vibration), 3135 (m, N-H stretching vibration), 2924 (s, N-H stretching vibration), 2854 (m, C-H stretching vibration), 1616 (s, N-H bending vibration), 1585 (m, pyridine ring stretching vibration), 1509 (m, C-N stretching vibration), 1449 (m, O-H bending vibration), 1384 (s, OH and CH bending vibrations), 1309 (m, ring in-plane deformation), 1247 (m, C-O stretching vibration), 1201 (m, C-O stretching vibration), 1060 (m, C-C bending vibration), 1019 (m, C-N bending vibration), 835 (m, C-C bending vibration), 536 (m, Pd-N stretching vibration).</td>
</tr>
<tr>
<td>MH4</td>
<td>3268 (m, O-H stretching vibration); 3133 (w, N-H stretching vibration); 2923 (m, C-H stretching vibration); 2854 (w, C-H stretching vibration); 1585 (m, N-H bending vibration) 1385 (s, C-O stretching vibration); 1291 (w, pyridine ring stretching vibration); 1220 (m, C-O stretching vibration); 1177 (w, C-N stretching vibration); 1054 (w, C-C bending vibration); 1016 (w, C-N bending vibration); 872 (w, C-H out of plane bending); 825 (w, C-H out of plane bending); 798 (m, ring out of plane deformation); 693 (w, m, aromatic C=C bending); 574 (w, Pd-N stretching vibration).</td>
</tr>
<tr>
<td></td>
<td>MH5</td>
</tr>
<tr>
<td>---</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>3517 (m, O–H stretching vibration); 3236 (s, N–H stretching vibration); 3136 (m, N–H stretching vibration); 2929 (m, C–H stretching vibration); 2855 (m, C–H stretching vibration); 1655 (m, N–H bending vibration); 1610 (s, C-N stretching vibration); 1540 (m, pyridine ring stretching vibration); 1491 (s, C-N stretching vibration); 1438 (s, O–H bending vibration); 1384 (s, ring in-plane deformation); 1280 (m, C-O stretching vibration); 1151 (m, C–C stretching vibration); 1114 (m, C-N stretching vibration); 1076 (m, C-H bending vibration); 862 (m, C–H wagging vibration); 777 (m, C–H out of plane bending vibration); 738 (m, C-H out of plane bending vibration); 582 (m, Pd-N stretching vibration); 531 (m, Pd-N stretching vibration); 491 (m, Pd-N stretching vibration).</td>
</tr>
<tr>
<td></td>
<td>3531 (w, O–H stretching vibration); 3319 (w, O–H stretching vibration); 3223 (w, N–H stretching vibration); 3136 (w, C–H stretching vibration); 2930 (w, C–H stretching vibration); 1856 (w, N–H bending vibration); 1649 (m, N–H bending vibration); 1605 (m, pyridine ring stretching vibration); 1493 (m, pyridine ring stretching vibration); 1384 (m, C–H bending vibration); 1324 (m, C-O stretching vibration); 1248 (m, OH and CH bending vibrations); 1156 (m, C–C stretching vibration); 1114 (m, C–N stretching vibration); 1028 (m, C-H bending vibration); 858 (m, C–H wagging vibration); 792 (s, C–H out of plane bending vibration); 597 (w, Pd-N stretching vibration); 565 (w, Pd-N stretching vibration); 504 (m, Pd-N stretching vibration).</td>
</tr>
<tr>
<td></td>
<td>3503 (w, O–H stretching vibration); 3229 (m, N-H stretching vibration); 3129 (m, N-H stretching vibration); 3065 (m, C-H stretching vibration); 2933 (m, C-H stretching vibration); 2358 (w, ring stretching vibration); 1655 (w, N–H bending vibration); 1562 (s, C=C stretching vibration); 1479 (s, ring stretching vibration); 1413 (m, C-O stretching vibration); 1384 (m, C–H bending vibration); 1296 (s, OH and CH bending vibrations); 1253 (m, OH and CH bending vibrations); 1185 (m, C–C stretching vibration); 1108 (m, C–N stretching vibration); 1018 (m, C-N stretching vibration); 880 (m, N-H wagging vibration); 800 (m, C–C bending vibration); 694 (m, aromatic C=C bending); 660 (m, C-H out of plane bending); 575 (w, Pd-N stretching vibration); 510 (w, Pd-N stretching vibration).</td>
</tr>
<tr>
<td></td>
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<tr>
<td>---</td>
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</tr>
<tr>
<td>MH8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3882 (w, O–H stretching vibration); 3313 (s, N-H stretching vibration); 2926 (s, C-H stretching vibration); 2855 (s, C-H stretching vibration); 2650 (m, C-H stretching vibration); 2595 (m, C-H stretching vibration); 2419 (m, ring stretching vibration); 2357 (m, ring stretching vibration); 1923 (m, N–H bending vibration); 1615 (s, pyridine ring stretching vibration); 1587 (s, C=C stretching vibration); 1507 (s, pyridine ring stretching vibration); 1450 (s, C-H bending vibration); 1390 (s, O-H bending vibration); 1290 (s, O-H bending vibration); 1247 (s, OH and CH bending vibrations); 1099 (s, C-O stretching vibration); 1060 (s, C-H in plane bending); 1036 (m, C-N stretching vibration); 954 (m, C–C bending vibration); 839 (s, aromatic ring stretching vibration); 662 (m, C-H out of plane bending); 566 (s, Pd-N stretching vibration); 535 (s, Pd-N stretching vibration).</td>
</tr>
</tbody>
</table>

**5.4.2. Mass spectra**

Mass spectra of MH3, MH4, MH5, MH6, MH7 and MH8 are given in the figures (5.13 to 5.18) respectively. The major peaks observed are listed in table 5.3. Detailed discussions of the peaks are given in Chapter six.
Figure 5.13: The mass spectrum of MH3
Figure 5.14: The mass spectrum of MH4
Figure 5.15: The mass spectrum of MH5
Figure 5.16: The mass spectrum of MH6
Figure 5.17: The mass spectrum of MH7
Figure 5.18: The mass spectrum of MH8
Table 5.3: The Mass spectra of MH3, MH4, MH5, MH6, MH7 and MH8 where the number in parentheses after each m/z value indicates the relative intensity.

<table>
<thead>
<tr>
<th>MH3</th>
<th>ESI Mass (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI-MS (DMSO) (m/z: M = 1023); {NH2(CH2)6NH2}Pd(C3H3NO)</td>
<td></td>
</tr>
<tr>
<td>{NH2(CH2)6NH2}PdNH3Cl – H+ = 899 (0.13); Pd{((NH3)2} {NH2(CH2)6NH2}</td>
<td></td>
</tr>
<tr>
<td>Pd(C3H3NO)2 {NH2(CH2)6NH2}Pd{(NH3)2}Cl – H+ = 844 (0.60); Pd{(NH3)2}</td>
<td></td>
</tr>
<tr>
<td>{NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd{(NH3)2}Cl3 = 727 (0.30); Pd{NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd - H+ = 551 (0.70);</td>
<td></td>
</tr>
<tr>
<td>{NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Cl2 + H+ = 411 (0.56);</td>
<td></td>
</tr>
<tr>
<td>Pd(C3H3NO)(NH3){NH2(CH2)6NH2}Cl + 2H+ = 373 (0.50).</td>
<td></td>
</tr>
</tbody>
</table>

| MH4 | ESI Mass (m/z: M = 1023); Pd(C3H5NO)2{NH2(CH2)6NH2} + H+ = 1024 (0.15); Pd{((NH3)2} {NH2(CH2)6NH2}Pd(C3H3NO)2 {NH2(CH2)6NH2}Pd{(NH3)2}Cl + 2H+ = 847 (0.33); Pd{((NH3)2} {NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd{(NH3)2}Cl3 = 727 (0.34); Pd{NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd - 2H+ = 550 (1.00); PdCl{NH2(CH2)6NH2}Pd - 4H+ = 361 (0.27); Pd(C3H4N)Cl + H+ = 223 (1.00). |

| MH5 | ESI Mass (m/z: M = 1023); Pd{((NH3)2} {NH2(CH2)6NH2}Pd(C3H5NO)2 {NH2(CH2)6NH2}Pd{(NH3)2}Cl – H+ = 844 (0.33); ClPd{NH2(CH2)6NH2}Pd(C3H3NO) | |
|-----|----------------|
| {NH2(CH2)6NH2}Pd{(NH3)2}Cl – 2H+ = 790 (0.32); Pd{(NH3)2} | |
| {NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd{(NH3)2}Cl + 2H+ = 729 (0.58); Pd | |
| {NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd – H+ = 551 (0.51); Pd{(NH3)2} | |
| {NH2(CH2)6NH2}Pd{NH2(CH2)6NH2}Pd – 3H+ = 471 (0.23); Pd{NH2(CH2)6NH2} | |
| H+ = 221 (1.00) | |

| MH6 | ESI Mass (m/z: M = 1180); ClPd(C3H5NO){NH2(CH2)6NH2}Pd(NH3){NH2(CH2)6NH2}Pd(C3H5NO) + 2H+ = 903 (0.13); PdCl2(C3H5NO){NH2(CH2)6NH2}Pd | |
|-----|----------------|
| = 532 (0.65); Pd(C3H5NO){NH2(CH2)6NH2}Pd + H+ = 426 (0.36); Pd{NH2(CH2)6NH2}PdCl + | |
| | | |

185
3H⁺ = 368 (0.57); PdCl₃{NH₂(CH₂)₆NH₂} –2H⁺ = 327 (1.00); Pd{NH₂(CH₂)₆NH₂} – H⁺ = 221 (1.00).

**MH7**

ESI-MS (DMSO)(m/z: M = 1180); Cl₆Pd(C₅H₅NO)₂{NH₂(CH₂)₆NH₂}Pd(C₅H₅NO)(NH₃) – H⁺ = 843 (0.14); Cl₂Pd(C₅H₅NO)₂{NH₂(CH₂)₆NH₂}Pd(NH₃) + 3H⁺ = 610 (0.17); Pd{NH₂(CH₂)₆NH₂}Pd(C₅H₅NO) + H⁺ = 428 (0.67); PdCl₂(C₅H₅NO)₂ – 2OH + H⁺ = 370 (1.00); PdCl₂{NH₂(CH₂)₆NH₂} + 2H⁺ = 296 (0.44); PdCl(C₅H₅NO) – OH = 221 (0.29).

**MH8**

ESI-MS (DMSO)(m/z: M = 1180); Cl₃Pd(C₅H₅NO){NH₂(CH₂)₆NH₂}Pd(NH₃){NH₂(CH₂)₆NH₂}Pd(C₅H₅NO)₂ – 3H⁺ = 1028 (0.15); Cl₂Pd(C₅H₅NO)₂{NH₂(CH₂)₆NH₂}Pd(NH₃)(C₅H₅NO) – 3H = 699 (0.63); Pd{NH₂(CH₂)₆NH₂}(NH₃)(C₅H₅NO)₂PdCl₂ – 3H⁺ = 604 (1.00); Pd(C₅H₅NO){NH₂(CH₂)₆NH₂}Pd + H⁺ = 426 (0.38); Pd {NH₂(CH₂)₆NH₂}PdCl = 365 (0.83); Pd₂{NH₂(CH₂)₆NH₂} + H⁺ = 331 (0.75); Pd(C₅H₅NO)₂ – H⁺ = 295 (0.66); Pd{NH₂(CH₂)₆NH₂} – H⁺ = 221 (0.29).

**5.4.3. ¹H NMR Spectra**

Figures (5.19 to 5.24) give the ¹H NMR spectra of MH3, MH4, MH5, MH6, MH7 and MH8 respectively. The prominent resonances are listed in Table 5.4 (for MH3, MH4 and MH5) and 5.9 (MH6, MH7 and MH8). More detailed discussions are given in Chapter seven. The letters ‘s’, ‘d’, ‘t’, ‘q’ and ‘br’ stand for ‘singlet’, ‘doublet’, ‘triplet’, ‘quartet’ and ‘broad’ respectively.
Figure 5.19: $^1$H NMR spectrum of MH3
Figure 5.20: $^1$H NMR spectrum of MH4
Figure 5.21: $^1$H NMR spectrum of MH5
Figure 5.22: $^1$H NMR spectrum of MH6
Figure 5.23: $^1$H NMR spectrum of MH7
Figure 5.24: $^1$H NMR spectrum of MH8
Table 5.4: Prominent peaks observed in $^1$H NMR spectra of MH3, MH4, MH5, MH6, MH7 and MH8

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH3</td>
<td>$^1$H NMR DMSO δ ppm: 11.4 (br s, due to OH); 9.76 (t, due to CH ortho); 7.26 (s, due to CH meta); 4.82 (t, due to NH$_2$); 2.45 (t, due to DMSO); 2.35 (t, due to NH); 1.67 (d, due to CH$_2$); 1.62 (q, due to CH$_2$); 1.37 (t, due to CH$_2$); 1.30 (s, due to CH$_2$); 1.25 (s, due to CH$_2$); 0.88 (t, due to CH$_2$).</td>
</tr>
<tr>
<td>MH4</td>
<td>$^1$H NMR DMSO δ ppm: 8.48 (s, due to OH); 8.33 (q, CH ortho); 8.04 (s, due to CH ortho); 7.48 (q, CH meta); 7.42 (d, CH para); 3.53 (s, due to NH$_3$); 2.93 (p, due to CH$_2$); 2.76 (p, due to CH$_2$); 2.65 (br, due to CH$_2$); 1.4 (br, due to CH$_2$).</td>
</tr>
<tr>
<td>MH5</td>
<td>$^1$H NMR DMSO δ ppm: 11.6 (s, due to OH), 7.94 (s, due to CH ortho); 7.57 (d, due to CH meta); 7.36 (t, due to CH meta), 6.32 (d, due to CH para); 5.96 (d, due to NH); 3.51 (t, due to NH$_3$); 2.97 (s, due to NH$_2$); 2.88 (s, due to NH$_2$); 2.72 (s, due to CH$_2$); 2.58 (s, due to DMSO); 2.39 (s, due to CH$_2$); 2.22 (t, due to CH$_2$); 1.56 (t, due to CH$_2$); 1.26 (t, due to CH$_2$).</td>
</tr>
<tr>
<td>MH6</td>
<td>$^1$H NMR CDCl$_3$ δ ppm: 11.5 (br s, due to OH); 8.54 (d, due to CH ortho); 8.04 (s, due to CH ortho); 7.50 (t, due to CH meta); 6.94 (d, due to CH meta); 6.39 (t, due to CH para); 3.57 (d, due to NH$_3$); 3.46 (d, due to water dissolved in DMF); 3.14 (s, due to CH$_2$); 2.97 (d, due to CH$_2$) 2.93 (p, due to CH$_2$); 2.88 (s, due to CH$_2$); 2.81 (t, due to CH$_2$); 2.62 (d, due to DMSO); 1.99 (s, due to CH$_2$); 1.78 (s, due to CH$_2$); 1.50 (s, due to CH$_2$) 1.39 (s, due to CH$_2$); 1.29 (s, due to CH$_2$).</td>
</tr>
<tr>
<td>MH7</td>
<td>$^1$H NMR DMSO δ ppm: 8.3 (s, due to CH ortho); 8.3 (s, ?); 8.19 (d, due to CH ortho); 8.01 (d, CH para); 7.32 (q, due to CH meta); 4.26 (br s, due to</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NH; 3.32 (br s, believed to be due to water dissolved in DMF); 2.50 (p, due to DMSO); 2.4 (s br, due to CH₂); 1.90 (s, due to CH₂); 1.70 (t, due to CH₂); 1.30 (d, due to CH₂).</td>
<td>¹H NMR</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>MH8</td>
<td>¹H NMR DMSO δ ppm: 12.2 (s br, due to OH); 8.45 (d, due to CH ortho); 8.03 (s, ?); 6.97 (d, due to CH meta); 3.90 (s, due to NH); 3.60 (p, due to water dissolved in DMSO); 3.50 (s, due to NH₂); to 2.95 (s, due to NH₂); 2.91 (p, due to NH₂); 2.77 (d, due to CH₂); 2.74 (p, due to CH₂); 2.61 (q, due to DMSO); 1.75 (d, due to CH₂); 1.57 (s, due to CH₂); 1.45 (p, due to CH₂).</td>
</tr>
</tbody>
</table>
5.5. Molar Conductivity

Table 5.5 gives the conductivity values (in ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\)) for MH3, MH4, MH5, MH6, MH7 and MH8 at concentrations of the compounds and figure 5.25 gives the corresponding molar conductivity versus concentration plots. The limiting molar conductivity values (in ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\)) at zero concentration (\(\Lambda_0\)) for MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin are 690, 820, 730, 970, 580, 1010 and 270 respectively.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>0.00 mM</th>
<th>0.05 mM</th>
<th>0.1 mM</th>
<th>0.2 mM</th>
<th>0.4 mM</th>
<th>1.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH3</td>
<td>690</td>
<td>600</td>
<td>510</td>
<td>425</td>
<td>362.5</td>
<td>239</td>
</tr>
<tr>
<td>MH4</td>
<td>820</td>
<td>720</td>
<td>620</td>
<td>570</td>
<td>495</td>
<td>303</td>
</tr>
<tr>
<td>MH5</td>
<td>730</td>
<td>680</td>
<td>630</td>
<td>595</td>
<td>495</td>
<td>310</td>
</tr>
<tr>
<td>MH6</td>
<td>970</td>
<td>800</td>
<td>630</td>
<td>570</td>
<td>485</td>
<td>288</td>
</tr>
<tr>
<td>MH7</td>
<td>580</td>
<td>460</td>
<td>340</td>
<td>285</td>
<td>240</td>
<td>153</td>
</tr>
<tr>
<td>MH8</td>
<td>1010</td>
<td>780</td>
<td>550</td>
<td>435</td>
<td>327.5</td>
<td>181</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>270</td>
<td>220</td>
<td>170</td>
<td>140</td>
<td>130</td>
<td>84</td>
</tr>
</tbody>
</table>
Figure 5.25: Molar conductivity (in ohm$^{-1}$ cm$^2$ mol$^{-1}$) for MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin.
5.6. Interaction between the compounds and ssDNA

Figure 5.26 shows the electrophoretograms applying to interaction (for 5 h at 37°C) of ssDNA with varying concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin ranging from 2.5 to 60 µM.

Figure 5.26: Electrophoretograms applying to the interaction for 5 h at 37°C of ssDNA with increasing concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin
(lane 1: untreated ssDNA, lane 2: 2.5 µM, lane 3: 5 µM, lane 4: 7.5 µM, lane 5: 10 µM, lane 6: 15 µM, lane 7: 20 µM, lane 8: 30 µM, lane 9: 40 µM and lane 10: 60 µM lane 11: untreated ssDNA). The experiments were performed in triplicate and similar results were found.

In the electrophoretograms, lane 1 and lane 11 apply to untreated ssDNA as control, lanes 2 to 10 apply to ssDNA interacted with increasing concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. For all compounds (including cisplatin) concentrations were: lane 2: 2.5 µM, lane 3: 5 µM, lane 4: 7.5 µM, lane 5: 10 µM, lane 6: 15 µM, lane 7: 20 µM, lane 8: 30 µM, lane 9: 40 µM and lane 10: 60 µM.

A single band was observed in both untreated and reacted salmon sperm DNA. In general, as the concentration of the compounds was increased, the intensity of the band was found to decrease (especially at high concentrations) except in the case of MH8 for which the intensity of the band remained essentially unchanged. There was no significant change in mobility of the band with the increase in concentration.
5.7. Interaction with pBR322 plasmid DNA

Figure 5.27 shows the electrophoretograms applying to interaction (for 5 h at 37°C) of pBR322 plasmid DNA with varying concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin ranging from 2.5 to 60 µM.

Figure 5.27: Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin (lane 1: untreated pBR322 plasmid DNA, lane 2: 2.5 µM, lane 3: 5 µM, lane 4: 7.5 µM, lane 5: 10 µM, lane 6: 15 µM, lane 7: 20 µM, lane 8: 30 µM, lane 9: 40 µM and lane 10: 60 µM lane 11: untreated pBR322 plasmid DNA). The experiments were performed in triplicate and similar results were found.
In the electrophoretograms, lane 1 and lane 11 apply to untreated pBR322 plasmid DNA to serve as control, lanes 2 to 10 apply to pBR322 plasmid DNA interacted with increasing concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. The concentrations were, lane 2: 2.5 µM, lane 3: 5 µM, lane 4: 7.5 µM, lane 5: 10 µM, lane 6: 15 µM, lane 7: 20 µM, lane 8: 30 µM, lane 9: 40 µM and lane 10: 60 µM.

In general two DNA bands were observed in both untreated and treated pBR322 plasmid DNA. As the concentrations of the compounds were increased, there was significant increase in the mobility of the frontal band (form I band) in the case of MH3, MH4, MH6 and MH7. No noticeable change in mobility of bands was observed in the case of MH5 and MH8. Also as the concentrations of the compounds were changed, significant decrease in intensity of the DNA bands was observed in the case of MH4, MH6, MH7 and MH8.

### 5.8. BamH1 Digestion

BamH1 digestion was carried out to obtain information on changes in DNA conformation as a result of interaction with varying concentrations of compounds. Figure 5.28 shows the electrophoretograms applying to the interaction of pBR322 plasmid DNA with varying concentrations (from 2.5 µM to 60 µM) of MH3, MH4, MH5, MH6, MH7, MH8 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 5.28: Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin followed by their digestion with BamH1. (lane A: untreated and undigested pBR322 plasmid DNA, lane B: untreated and digested pBR322 plasmid DNA, lane 1: 2.5 µM, lane 2: 5 µM, lane 3: 7.5 µM, lane 4: 10 µM, lane 5: 15 µM, lane 6: 20 µM, lane 7: 30 µM, lane 8: 40 µM and lane 9: 60 µM). The experiments were performed in triplicate and similar results were found.

In the electrophoretograms, lane A applies to the untreated pBR322 plasmid DNA and undigested with BamH1, lane B applies to untreated but digested with BamH1. Lanes 1 to 9 apply to pBR322 plasmid DNA interacted with increasing concentrations of the
compounds followed by BamH1 digestion. The concentrations were, lane 1: 2.5 µM, lane 2: 5 µM, lane 3: 7.5 µM, lane 4: 10 µM, lane 5: 15 µM, lane 6: 20 µM, lane 7: 30 µM, lane 8: 40 µM and lane 9: 60 µM.

When untreated pBR322 plasmid band was digested with BamH1, only one band corresponding to form III band was observed whereas in the untreated and undigested pBR322 plasmid DNA, generally two bands corresponding to form I and form II were observed. When pBR322 plasmid DNA was interacted with increasing concentrations of MH3 followed by BamH1 digestion, generally three bands corresponding to forms I, II and III were observed at all concentrations of 2.5 µM to 60 µM. For the concentration range 2.5 to 10 µM and also for the concentration 60 µM, form III band was most prominent whilst forms I and II bands were weak. At 15 µM concentration of MH3, all the three bands were almost equally bright. In case of MH4, at higher concentrations ranging from 7.5 µM to 60 µM three bands corresponding to forms I, II and III were observed whereas at lower concentrations of 2.5 µM and 5 µM two bands corresponding to forms II and III were observed. In the case of MH5, three bands corresponding to forms I, II and III were observed for concentrations ranging from 2.5 µM to 10 µM and one band corresponding to form III was observed at concentrations ranging from 15 µM to 60 µM. In the case of MH6, three bands corresponding to forms I, II and III were observed for concentrations ranging from 15 µM to 60 µM and two bands corresponding to forms I and III were observed at 10 µM concentration. One band corresponding to form III was observed at concentrations ranging from 2.5 µM to 7.5 µM. In case of MH7, three bands corresponding to forms I, II and III were observed for concentrations of 30 µM and 60 µM, two bands corresponding to forms I and III were observed at concentrations ranging from 2.5
µM to 20 µM. As pBR322 plasmid DNA was interacted with increasing concentrations of MH8 followed by BamH1 digestion, three bands corresponding to forms I, II and III were observed for concentrations ranging from 2.5 µM to 15 µM, two bands corresponding to forms I and III were observed at concentrations: 20 µM, 30 µM and 40 µM and no band observed at 60 µM concentration. It was also found that the bands observed at concentrations ranging from 20 µM to 40 µM increased in mobility and became more streaky as the concentration was increased. The streakiness of the band implies the occurrence of DNA damage whereas the change in mobility may be due to both DNA damage and change in DNA conformation.

When pBR322 plasmid DNA was interacted with increasing concentrations of cisplatin followed by BamH1 digestion, three bands corresponding to forms I, II and III were observed at concentrations: 2.5 µM, 5 µM, 7.5 µM, 10 µM, 20 µM and 40 µM, two bands corresponding to forms I and III were observed at 15 µM concentration, and two bands corresponding to forms I and II were observed at 30 µM concentration. No band was found at highest concentration namely 60 µM. Table 5.6 gives a summary of the observed bands.
Table 5.6: Bands observed after BamH1 digestion of incubated mixtures of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin with pBR322 plasmid DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration indicator for the bands #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

[For all compounds, 1: 2.5 µM, 2: 5 µM, 3: 7.5 µM, 4: 10 µM, 5: 15 µM, 6: 20 µM, 7: 30 µM, 8: 40 µM, and 9: 60 µM.]

5.9. Cytotoxicity studies

The cytotoxicity of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin against human ovarian cancer cell lines: A2780, A2780cisR and A2780ZD6473R have been determined using MTT reduction assay. Experiments on activities of MH3, MH4, MH5 along with those for cisplatin were done as one set and those for MH6, MH7 and MH8 along with those for cisplatin were carried out as another set. This was because 96-well plate could accommodate a maximum of four drugs only according to the design of the experiment. Values are means from three independent experiments to give % survival at each concentration. The % cell survival values were plotted against concentration of the drugs to give the survival curves. The IC50 values were determined from the cell survival curves.
5.9.1. Cytotoxicity study for MH3, MH4, MH5 and cisplatin

Tables 5.7 to 5.9 give the percentage cell survival values as applied to the ovarian cancer cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}, when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin. The IC\textsubscript{50} values are also given in the tables. Figures 5.29 to 5.31 give the corresponding cell survival curves.

Table 5.7: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780, when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin.

<table>
<thead>
<tr>
<th>Concentration of compounds (µM)</th>
<th>% Cell Survival Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH3</td>
</tr>
<tr>
<td>50</td>
<td>24.2 ± 4.1</td>
</tr>
<tr>
<td>10</td>
<td>55.5 ± 3.7</td>
</tr>
<tr>
<td>2</td>
<td>63.6 ± 5.8</td>
</tr>
<tr>
<td>0.4</td>
<td>84.8 ± 0.3</td>
</tr>
<tr>
<td>0.08</td>
<td>88.8 ± 2.1</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>16.5 ± 3.0</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.
Figure 5.29: Cell survival curves for the human ovarian cancer cell line A2780 when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin.
Table 5.8: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780\textsuperscript{cisR}, when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin.

<table>
<thead>
<tr>
<th>Concentration of the compounds (µM)</th>
<th>% Cell Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH3</td>
</tr>
<tr>
<td>50</td>
<td>15.9 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>71.0 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>75.2 ± 1.7</td>
</tr>
<tr>
<td>0.4</td>
<td>85.7 ± 2.5</td>
</tr>
<tr>
<td>0.08</td>
<td>90.8 ± 0.4</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>24.7 ± 0.4</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.

IC\textsubscript{50} represents the concentration of a drug that is required for 50% inhibition \textit{in vitro}.
Figure 5.30: Cell survival curves for the human ovarian cancer cell line A2780^cisR when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin.
Table 5.9: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780<sup>ZD0473R</sup>, when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin

<table>
<thead>
<tr>
<th>Concentration of the compounds (µM)</th>
<th>% Cell Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH3</td>
</tr>
<tr>
<td>50</td>
<td>14.2 ± 3.1</td>
</tr>
<tr>
<td>10</td>
<td>67.0 ± 4.7</td>
</tr>
<tr>
<td>2</td>
<td>72.0 ± 5.0</td>
</tr>
<tr>
<td>0.4</td>
<td>82.2 ± 3.2</td>
</tr>
<tr>
<td>0.08</td>
<td>90.4 ± 4.5</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>28.9 ± 1.2</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.
Figure 5.31: Cell survival curves for the human ovarian cancer cell line A2780\textsuperscript{ZD0473R} when the cells were treated with increasing concentrations of MH3, MH4, MH5 and cisplatin.

5.9.2. Cytotoxicity of MH6, MH7, MH8 and cisplatin

Tables 5.10 to 5.12 give the percentage cell survival values as applied to the cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}, when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin. The IC\textsubscript{50} values are also given in the tables. Figures 5.29 to 5.31 give the corresponding cell survival curves.
Table 5.10: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780, when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin

<table>
<thead>
<tr>
<th>Concentration of the compounds (µM)</th>
<th>% Cell Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH6</td>
</tr>
<tr>
<td>50</td>
<td>8.8 ± 3.4</td>
</tr>
<tr>
<td>10</td>
<td>49.4 ± 4.9</td>
</tr>
<tr>
<td>2</td>
<td>67.6 ± 3.6</td>
</tr>
<tr>
<td>0.4</td>
<td>90.1 ± 3.9</td>
</tr>
<tr>
<td>0.08</td>
<td>92.8 ± 4.1</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>9.0 ± 0.3</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.
Figure 5.32: Cell survival curve for the human ovarian cancer cell line A2780 when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin.
Table 5.11: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780\textsuperscript{cisR}, when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin.

<table>
<thead>
<tr>
<th>Concentration of the compounds (µM)</th>
<th>% Cell Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH6</td>
</tr>
<tr>
<td>50</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>34.4 ± 2.3</td>
</tr>
<tr>
<td>2</td>
<td>68.9 ± 2.9</td>
</tr>
<tr>
<td>0.4</td>
<td>93.8 ± 1.6</td>
</tr>
<tr>
<td>0.08</td>
<td>95.0 ± 4.5</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>6.3 ± 0.5</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.
Figure 5.33: Cell survival curve for the human ovarian cancer cell line A2780\textsuperscript{cisR} when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin.
Table 5.12: Cell survival values as percentages of controls for the human ovarian cancer cell line A2780ZD0473R, when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin.

<table>
<thead>
<tr>
<th>Concentration of the compounds (µM)</th>
<th>% Cell Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH6</td>
</tr>
<tr>
<td>50</td>
<td>12.1 ± 2.8</td>
</tr>
<tr>
<td>10</td>
<td>69.8 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>80.6 ± 3.6</td>
</tr>
<tr>
<td>0.4</td>
<td>89.5 ± 1.8</td>
</tr>
<tr>
<td>0.08</td>
<td>93.4 ± 0.5</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>22.6 ± 0.4</td>
</tr>
</tbody>
</table>

The values given are the average of three independent measurements with each measurement being the average of results obtained from 4 identical wells with 6000 to 9000 cells per well.
Figure 5.34: Cell survival curve for the human ovarian cancer cell line A2780<sup>ZD0473R</sup> when the cells were treated with increasing concentrations of MH6, MH7, MH8 and cisplatin.

5.9.3. Activity summary

The IC<sub>50</sub> values and resistance factors (RF) of all of the compounds MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin are listed in table 5.13.
Table 5.13: IC₅₀ values and resistance factors (RF) for MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin as applied to the human ovarian cancer cell lines: A2780, A2780\(^{\text{cisR}}\) and A2780\(^{\text{ZD0473R}}\).

<table>
<thead>
<tr>
<th>Compound</th>
<th>A2780 (µM) ± SD</th>
<th>A2780(^{\text{cisR}}) (µM) ± SD</th>
<th>RF</th>
<th>A2780(^{\text{ZD0473R}}) (µM) ± SD</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH3</td>
<td>16.5 ± 3.0</td>
<td>24.7 ± 0.4</td>
<td>1.50</td>
<td>28.9 ± 1.2</td>
<td>1.75</td>
</tr>
<tr>
<td>MH4</td>
<td>32.1 ± 2.0</td>
<td>24.5 ± 2.4</td>
<td>0.76</td>
<td>24.9 ± 2.1</td>
<td>0.77</td>
</tr>
<tr>
<td>MH5</td>
<td>18.2 ± 1.5</td>
<td>21.9 ± 4.5</td>
<td>1.20</td>
<td>25.1 ± 2.6</td>
<td>1.38</td>
</tr>
<tr>
<td>MH6</td>
<td>9.0 ± 0.3</td>
<td>6.3 ± 0.5</td>
<td>0.70</td>
<td>22.6 ± 0.4</td>
<td>2.51</td>
</tr>
<tr>
<td>MH7</td>
<td>24.0 ± 4.5</td>
<td>23.7 ± 2.1</td>
<td>0.99</td>
<td>23.2 ± 4.4</td>
<td>1.03</td>
</tr>
<tr>
<td>MH8</td>
<td>32.5 ± 0.9</td>
<td>46.0 ± 2.1</td>
<td>1.42</td>
<td>45.9 ± 2.2</td>
<td>1.41</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.6 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>6.1</td>
<td>3.6 ± 1.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The results are averages of those obtained from 4 identical wells with 6000 to 9000 cells per well.
5.10. Cell uptake and DNA binding

5.10.1. Cell uptake

The cellular accumulation of palladium was used as a measure of the cell uptake for all new trinuclear palladium compounds (MH3, MH4, MH5, MH6, MH7 and MH8). Also the cellular accumulation of platinum was used as a measure of the cell uptake for cisplatin which was used as a reference. Tables 5.14 to 5.16 and figures 5.35 to 5.37 give the total intracellular palladium and platinum levels (expressed as nanomoles Pt or Pd per 2 x 10^6 cells) found in the cell lines: A2780, A2780^cisR and A2780^ZD0473R after exposure to 50 µM concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin for 2, 4 and 24 h. Among the trinuclear palladium compounds, MH3, MH6 and MH8 are found to have the highest cell uptake in the ovarian cancer cell lines A2780 and A2780^cisR. As applied to the cell line A2780^ZD0473R the results appear to be more complicated although MH6 and MH8 still have higher uptakes. Generally, the reference compound cisplatin is found to have a smaller Pt uptake than Pd uptake of the trinuclear palladium compounds. The results will be discussed in chapter six.
Table 5.14: Pd or Pt accumulation in A2780 cells in 2 h, 4 h and 24 h as applied to cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>2 h (mol Pd or Pt per 2x10^6 cells)</th>
<th>4 h (mol Pd or Pt per 2x10^6 cells)</th>
<th>24 h (mol Pd or Pt per 2x10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin (Pt)</td>
<td>0.57 ± 0.04</td>
<td>0.45 ± 0.00</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>1.24 ± 0.15</td>
<td>1.72 ± 0.20</td>
<td>1.17 ± 0.16</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>0.38 ± 0.02</td>
<td>0.83 ± 0.14</td>
<td>0.71 ± 0.05</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>0.98 ± 0.07</td>
<td>0.32 ± 0.04</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>1.75 ± 0.04</td>
<td>1.75 ± 0.14</td>
<td>2.52 ± 0.15</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>0.53 ± 0.04</td>
<td>0.89 ± 0.06</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>1.14 ± 0.09</td>
<td>1.85 ± 0.12</td>
<td>0.99 ± 0.09</td>
</tr>
</tbody>
</table>
Figure 5.35: Pd or Pt cell uptake as nmol Pd or Pt per $2 \times 10^6$ cells in 2 h, 4 h and 24 h in A2780 cell line as applied to cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8.
Table 5.15: Pd or Pt accumulation in A2780\textsuperscript{cisR} cells in 2 h, 4 h and 24 h as applied to cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>2 h (mol Pd or Pt per 2x10\textsuperscript{6} cells)</th>
<th>4 h (mol Pd or Pt per 2x10\textsuperscript{6} cells)</th>
<th>24 h (mol Pd or Pt per 2x10\textsuperscript{6} cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin (Pt)</td>
<td>0.16 ± 0.02</td>
<td>0.40 ± 0.03</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>1.44 ± 0.13</td>
<td>1.80 ± 0.17</td>
<td>1.59 ± 0.30</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>0.73 ± 0.06</td>
<td>1.13 ± 0.03</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>0.72 ± 0.08</td>
<td>0.27 ± 0.04</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>1.62 ± 0.12</td>
<td>1.74 ± 0.03</td>
<td>2.31 ± 0.16</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>0.76 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>1.43 ± 0.12</td>
<td>1.77 ± 0.11</td>
<td>0.94 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 5.36: Pd or Pt cell uptake as nmol Pd or Pt per 2x10^6 cells in 2 h, 4 h and 24 h in A2780^{cisR} cell line as applied to cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8.
Table 5.16: Pd and Pt accumulation in A2780<sup>ZD0473R</sup> cells in 2 h, 4 h and 24 h as applied to cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>2 h</th>
<th>4 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mol Pd or Pt per 2×10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
<td>(mol Pd or Pt per 2×10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
<td>(mol Pd or Pt per 2×10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
</tr>
<tr>
<td>Cisplatin (Pt)</td>
<td>0.19 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>1.48 ± 0.15</td>
<td>1.76 ± 0.14</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>0.83 ± 0.04</td>
<td>0.72 ± 0.03</td>
<td>0.53 ± 0.01</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>1.45 ± 0.08</td>
<td>0.95 ± 0.04</td>
<td>1.60 ± 0.07</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>3.10 ± 0.07</td>
<td>2.25 ± 0.09</td>
<td>1.56 ± 0.12</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>0.70 ± 0.01</td>
<td>0.94 ± 0.08</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>1.20 ± 0.07</td>
<td>2.93 ± 0.13</td>
<td>2.31 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 5.37: Pd or Pt cell uptake as nmol Pd or Pt per 2x10^6 cells in 2 h, 4 h and 24 h in A2780^{ZD0473R} cells as applied to Cisplatin, MH3, MH4, MH5, MH6, MH7 and MH8.
5.10.2. DNA binding

Tables 5.17 to 5.19 and figures 5.38 to 5.40 give the levels of palladium-DNA binding for MH3, MH4, MH5, MH6, MH7, MH8 and platinum-DNA binding for cisplatin, expressed as nanomol of Pd or Pt per milligram of DNA, in A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} cells in 2, 4 and 24 h. It can be seen that, for the three cell lines, the lowest level of metal-DNA binding applies to cisplatin at 2, 4 and 24 h. The levels of Pd-DNA binding are found to vary significantly among the trinuclear compounds. At 2 h, the highest levels of metal-DNA binding apply to MH6 in A2780 and A2780\textsuperscript{cisR} cell lines whereas in A2780\textsuperscript{ZD0473R} the highest level applies to MH4. In 4 h, the highest levels of metal-DNA binding apply to MH8 in A2780 and A2780\textsuperscript{cisR} cell lines whereas in A2780\textsuperscript{ZD0473R} the highest level applies to MH4 as was the case in 2 h. At 24 h, the highest level of metal-DNA binding applies to MH6 in A2780 cell lines, to MH3 in A2780\textsuperscript{cisR} cell line and to MH8 in A2780\textsuperscript{ZD0473R} cell line. The actual orders (from highest to lowest) of metal-DNA binding at 2, 4 and 24 h for all the compounds including cisplatin, in A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} cells are given below:

**At 2 h:**

A2780: MH6 > MH8 > MH3 > MH4 > MH5>MH7>cisplatin.

A2780\textsuperscript{cisR}: MH6 > MH3 > MH7 > MH8 > MH4>MH5>cisplatin.

A2780\textsuperscript{ZD0473R}: MH4 > MH6 > MH5 > MH8 > MH7>MH3>cisplatin
At 4 h:

A2780: MH8 > MH6 > MH4 > MH7 > MH5 > MH3 > cisplatin

A2780\textsuperscript{cisR}: MH8 > MH4 > MH5 > MH6 > MH3 > MH7 > cisplatin

A2780\textsuperscript{ZD0473R}: MH4 and MH5 > MH6 > MH7 > MH8 > MH3 > cisplatin.

At 24 h:

A2780: MH6 > MH8 > MH7 > MH4 > MH5 > MH3 > cisplatin

A2780\textsuperscript{cisR}: MH3 > MH4 > MH6 > MH7 > MH5 > MH8 > cisplatin

A2780\textsuperscript{ZD0473R}: MH8 > MH5 > MH6 > MH3 > MH7 > MH4 > cisplatin
Table 5.17: Levels of Pd binding with DNA in 2 h in A2780, A2780\textsuperscript{GisR} and A2780\textsuperscript{ZD0473R} cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>A2780</th>
<th>A2780\textsuperscript{GisR}</th>
<th>A2780\textsuperscript{ZD0473R}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>85.3 ± 3.9</td>
<td>77.9 ± 2.4</td>
<td>33.7 ± 23</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>68.2 ± 2.4</td>
<td>41.5 ± 7.5</td>
<td>60.7 ± 2.0</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>44.0 ± 2.4</td>
<td>36.3 ± 3.2</td>
<td>53.2 ± 2.5</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>136.1 ± 2.8</td>
<td>84.7 ± 3.6</td>
<td>54.1 ± 2.1</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>41.4 ± 0.8</td>
<td>55.6 ± 2.8</td>
<td>39.6 ± 2.7</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>130.7 ± 1.6</td>
<td>46.4 ± 1.9</td>
<td>41.4 ± 2.8</td>
</tr>
<tr>
<td>Cisplatin (Pt)</td>
<td>16.2 ± 3.0</td>
<td>15.4 ± 1.4</td>
<td>12.0 ± 3.1</td>
</tr>
</tbody>
</table>
Figure 5.38: Levels of Pd and Pt binding with DNA in 2 h in A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin.
Table 5.18: Levels of Pd and Pt binding with DNA in 4 h in A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>A2780</th>
<th>A2780\textsuperscript{cisR}</th>
<th>A2780\textsuperscript{ZD0473R}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>27.4 ± 1.0</td>
<td>29.9 ± 2.5</td>
<td>47.6 ± 3.5</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>46.6 ± 2.9</td>
<td>120.4 ± 1.2</td>
<td>67.9 ± 2.0</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>33.5 ± 1.6</td>
<td>45.7 ± 2.5</td>
<td>62.8 ± 2.0</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>50.8 ± 1.0</td>
<td>42.1 ± 1.0</td>
<td>56.2 ± 2.8</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>38.7 ± 0.4</td>
<td>23.0 ± 2.5</td>
<td>53.0 ± 3.6</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>54.7 ± 0.3</td>
<td>122.8 ± 1.0</td>
<td>52.1 ± 1.7</td>
</tr>
<tr>
<td>Cisplatin (Pt)</td>
<td>13.3 ± 1.4</td>
<td>13.1 ± 1.2</td>
<td>15.0 ± 2.4</td>
</tr>
</tbody>
</table>
Figure 5.39: Levels of Pd and Pt binding with DNA in 4 h in A2780, A2780cisR and A2780ZD0473R cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin.
Table 5.19: Levels of Pd and Pt binding with DNA in 24 h in A2780, A2780<sup>cisR</sup> and A2780<sup>ZD0473R</sup> cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. – the symbol within parentheses in column 1 indicates the metal to which it applies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>A2780</th>
<th>A2780&lt;sup&gt;cisR&lt;/sup&gt;</th>
<th>A2780&lt;sup&gt;ZD0473R&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
<td>nmol Pd or Pt per mg DNA</td>
</tr>
<tr>
<td>MH3 (Pd)</td>
<td>32.2 ± 0.8</td>
<td>79.2 ± 3.8</td>
<td>63.4 ± 0.1</td>
</tr>
<tr>
<td>MH4 (Pd)</td>
<td>39.3 ± 4.2</td>
<td>50.8 ± 0.2</td>
<td>49.3 ± 0.5</td>
</tr>
<tr>
<td>MH5 (Pd)</td>
<td>38.2 ± 2.0</td>
<td>29.3 ± 0.7</td>
<td>73.1 ± 1.9</td>
</tr>
<tr>
<td>MH6 (Pd)</td>
<td>102.4 ± 0.3</td>
<td>50.2 ± 2.6</td>
<td>65.9 ± 2.2</td>
</tr>
<tr>
<td>MH7 (Pd)</td>
<td>39.7 ± 2.9</td>
<td>36.1 ± 2.3</td>
<td>56.8 ± 2.9</td>
</tr>
<tr>
<td>MH8 (Pd)</td>
<td>66.0 ± 0.3</td>
<td>22.2 ± 0.8</td>
<td>117.9 ± 2.6</td>
</tr>
<tr>
<td>Cisplatin (Pt)</td>
<td>18.5 ± 2.5</td>
<td>12.1 ± 4.5</td>
<td>14.4 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 5.40: Levels of Pd and Pt binding with DNA in 24 h in A2780, A2780\(^{\text{cisR}}\) and A2780\(^{\text{ZD0473R}}\) cells as applied to MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin.


6. Chapter Six: Discussion

In this project, six new trinuclear palladium complexes containing planar amine ligands (2-hydroxy, 3-hydroxy and 4-hydroxy pyridine ligands) bound to central or terminal palladium ions, code named MH3, MH4, MH5, MH6, MH7 and MH8, have been prepared, characterized and investigated for antitumour activity against human ovarian cancer cell lines. The nature of binding of the compounds with salmon sperm DNA and pBR322 plasmid DNA has also been investigated. In MH3, MH4 and MH5, the central palladium ion is bound to two molecules of 2-hydroxypyridine, 3-hydroxypyridine and 4-hydroxypyridine ligands respectively whereas in MH6, MH7 and MH8, each of the two terminal palladium ions is bound to two molecules of 4-hydroxypyridine, 3-hydroxypyridine and 2-hydroxypyridine ligands respectively. The compounds have been characterized based on elemental analyses and a number of spectral measurements. The antitumour activity has been determined based on MTT reduction assay. The interaction of the compounds with DNA has been investigated using agarose gel electrophoresis.

6.1. Characterization of compounds

As stated earlier, trinuclear palladium compounds code named MH3, MH4, MH5, MH6, MH7 and MH8, have been synthesized using a step-up method of synthesis (branching out from the central unit), and characterized by elemental analyses, IR, mass and $^1$H NMR spectral studies.
6.2. Elemental analysis

Table 5.1 gave the elemental compositions of MH3, MH4, MH5, MH6, MH7 and MH8. It can be seen that none of the compounds could be obtained in a state of very high purity (observed purity was about 90%) although attempts were made to purify the compounds by repeated dissolution in DMF and precipitation by adding dichloromethane. This was found to be the case with trinuclear Pt-Pt-Pt and Pt-Pd-Pt complexes as well (Cheng et al., 2005; Daghriri, 2003; Farrell, 2000; Huq et al., 2008). A likely reason for the low purity is that some dinuclear complexes are formed along with the trinuclear complexes. This was also observed by Farrell and co-workers in the synthesis of BBR3464 and related compounds (Farrell et al., 1999). Some dissociation of trinuclear cations with trans-geometry for the metal centres is always expected because of the trans-labilizing effect due to chloride ligands. Palladium being much more reactive than platinum, the dissociation of trinuclear cations is likely to be more significant in the case of trinuclear Pd-Pd-Pd complexes than the trinuclear Pt-Pt-Pt and Pt-Pd-Pt complexes. As suggested by Huq et al. (Huq et al., 2008) another possible reason for the low purity may be due to co-precipitation of solvent molecules such as DMF or dichloromethane. Other methods of purification such as high pressure liquid chromatography (which in principle should produce purer products) were not attempted. The percentage of yields were: 74% for MH3, 69% for MH4, 72% for MH5, 80% for MH6, 63% for MH7 and 81% for MH8.
6.3. Spectral analyses

In the absence of confirmation of structures by single crystal x-ray diffractometry (as no suitable crystals could be grown), the results of IR, mass and $^1$H NMR spectral studies were used to aid in the structural characterization of the compounds. Although none of these methods on its own could fully characterize the compounds, the combined results can be seen to provide a more convincing support for the suggested structures.

6.3.1. IR Spectra

IR spectra for MH3, MH4, MH5, MH6, MH7 and MH8 were given in chapter four (Figures 5.7 to 5.12). The lists of the major peaks were given in Table 5.2. Most of the peak assignments were based on published spectra of the hydroxy pyridine ligands and hydroxy pyridine ligands of the metal complexes and substituted hydroxy pyridine ligands (Kelland et al., 1995; Silverstein et al., 1991). It can be seen that the peaks listed in Table 5.2 and the description given conform to the suggested structures. In particular, the listed frequencies indicate the presence of such functional groups as CH, CO, NH and ‘aromatic ring’ and Pd-N bonds.

MH3

The strong bands at 3268 and 3216 cm$^{-1}$ are believed to be due to O–H stretching vibrations whereas the band at 1449 cm$^{-1}$ is due to O-H bending vibration. The bands at 3135 and 2924 cm$^{-1}$ are believed to be due to N–H stretching vibrations whereas the band at 1616 cm$^{-1}$ is believed to be due to N–H bending vibration. The bands at 2854 and 1384 cm$^{-1}$ are due to C–H stretching and bending vibrations respectively. The
bands at 1509 and 1019 cm$^{-1}$ are due to C–N stretching and bending vibrations respectively. The band at 1585 cm$^{-1}$ is due to pyridine ring stretching vibration whereas the band at 1384 cm$^{-1}$ is due to OH and CH bending vibrations. The band at 1309 cm$^{-1}$ is due to a ring in-plane deformation whereas the bands at 1247 and 1201 cm$^{-1}$ are believed to be due to C-O stretching vibrations. The band at 536 cm$^{-1}$ is due to Pd–N stretching vibration. Finally the bands at 1060 and 835 cm$^{-1}$ are due to C-C bending vibrations.

**MH4**

The weak band at 3268 cm$^{-1}$ is believed to be due to O–H stretching vibration. The bands at 3133 and 1585 cm$^{-1}$ are believed to be due to N–H stretching and bending vibrations respectively. The bands at 2923 and 2854 cm$^{-1}$ are believed to be due to C-H stretching vibrations. The bands at 1385 and 1220 cm$^{-1}$ are due to C–O stretching vibrations whereas those at 1177 and 1016 cm$^{-1}$ are believed to be C–N stretching and bending vibrations respectively. The band at 1291 cm$^{-1}$ is due to pyridine ring stretching vibration; the band at 1054 cm$^{-1}$ is due to C–C bending vibration; the bands at 872 and 825 cm$^{-1}$ are due to C-H out of plane bending vibrations. The band at 798 cm$^{-1}$ is due to ring out of plane deformation whereas the band at 693 cm$^{-1}$ is due to aromatic C=C bending. The band at 574 cm$^{-1}$ is due to Pd–N (pyridine) stretching vibration.

**MH5**

The band at 3517 cm$^{-1}$ is believed to be due to O–H stretching vibration whereas that at 1438 cm$^{-1}$ is considered to be O–H bending vibration. The bands at 3236 and 3136
cm\(^{-1}\) are due to N–H stretching vibrations whereas that at 1655 cm\(^{-1}\) is believed to be due to N–H bending vibration. The bands at 2929 and 2855 cm\(^{-1}\) are believed to be due to C–H stretching vibrations whereas that at 1076 cm\(^{-1}\) is considered to be due to C-H bending vibration. The bands at 1610, 1491 and 1114 cm\(^{-1}\) are due to C–N stretching vibrations whereas the medium band at 1540 cm\(^{-1}\) is due to pyridine ring stretching vibration. The band at 1384 is believed to be due to ring in-plane deformation whereas that at 1280 cm\(^{-1}\) is due to C–O stretching vibration. The band at 1151 cm\(^{-1}\) is due to C–C stretching vibration whereas that at 862 cm\(^{-1}\) is due to C–H wagging vibration. The band at 777 cm\(^{-1}\) is due to C–H out of plane bending vibration whereas that at 738 cm\(^{-1}\) is due to C-H out of plane bending vibration. Finally the bands at 582, 531 and 491 cm\(^{-1}\) are due to Pd–N stretching vibrations.

**MH6**

The bands at 3531 and 3319 cm\(^{-1}\) are believed to be due to O–H stretching vibrations whereas that at 3223 cm\(^{-1}\) is believed to be due to N–H stretching vibration. The bands at 1856 and 1649 cm\(^{-1}\) are believed to be due to N–H bending vibrations. The bands at 3136 and 2930 cm\(^{-1}\) are due to C–H stretching vibrations whereas those at 1384 and 1028 cm\(^{-1}\) are believed to be due to C–H bending vibrations. The bands at 1605 and 1493 cm\(^{-1}\) is due to pyridine ring stretching vibrations whereas that at 1324 cm\(^{-1}\) is due to C-O stretching vibration. The band at 1248 cm\(^{-1}\) is due to OH or CH bending vibration. The band 1156 cm\(^{-1}\) is due to C–C stretching vibration and whereas that at 1114 cm\(^{-1}\) is due to C–N stretching vibration. The band at 858 cm\(^{-1}\) is due to C–H wagging vibration whereas that at 792 cm\(^{-1}\) is due to C–H out of plane bending vibration. Finally the bands at 597, 565 and 504 cm\(^{-1}\) are due to Pt–N stretching vibrations.
The weak band at 3503 cm$^{-1}$ is believed to be due to O–H stretching vibration; the bands at 3229 and 3129 cm$^{-1}$ are believed to be due to N–H stretching vibration. The band at 1655 cm$^{-1}$ is believed to be due to N–H bending vibration. The bands at 3065 and 2933 cm$^{-1}$ are due to C–H stretching vibrations whereas that at 1384 cm$^{-1}$ is believed to be due to C–H bending vibration. The bands at 1296 and 1253 cm$^{-1}$ are due to OH or CH bending vibrations. The bands at 2358 and 1479 cm$^{-1}$ are believed to be due to pyridine ring stretching vibrations; the band at 1562 cm$^{-1}$ is due to C=C stretching vibration. The band at 1413 cm$^{-1}$ is due to C–O stretching vibration. The bands at 1108 and 1018 cm$^{-1}$ are believed to be due to C–N stretching vibrations; the band at 1185 cm$^{-1}$ is due to C–C stretching vibration. The band at 800 cm$^{-1}$ is due to C–C bending vibration whereas the band at 880 cm$^{-1}$ is believed to be due to N-H wagging vibration. The band at 694 cm$^{-1}$ is due to aromatic C=C bending and the band at 660 cm$^{-1}$ is believed to be due to C–H out of plane bending vibration. Finally the bands at 575 and 510 cm$^{-1}$ are due to Pt–N stretching vibrations.

The weak band at 3882 cm$^{-1}$ is believed to be due to O–H stretching vibration. The bands at 3313 and 1923 cm$^{-1}$ are believed to be due to N–H stretching and bending vibrations respectively. The bands at 2926, 2855 and 2650 cm$^{-1}$ are believed to be due to C–H stretching vibrations. The band at 1450 cm$^{-1}$ is believed to be due to C–H bending vibration. The bands at 2419 and 2357 cm$^{-1}$ are believed to be due to ring stretching vibrations. The bands at 1615 and 1507 cm$^{-1}$ are also believed to be due to pyridine ring stretching vibrations. The band at 1587 cm$^{-1}$ is believed to be due to
C=C stretching vibration. The bands at 1390 and 1290 cm\(^{-1}\) are believed to be due to O–H bending vibrations whereas the band at 1247 cm\(^{-1}\) is believed to be due to O–H or C–H bending vibration. The band at 1099 cm\(^{-1}\) is believed to be due to C-O stretching vibration whereas that at 1060 cm\(^{-1}\) is due to C-H in plane bending. The band at 1036 cm\(^{-1}\) is due to C–N stretching vibration whereas the band at 954 cm\(^{-1}\) is believed to be due to C–C bending vibration. The band at 839 cm\(^{-1}\) is believed to be due to aromatic ring stretching vibration whereas the bands at 662 cm\(^{-1}\) are due to C–H out of plane bending vibrations. Finally the bands at 566 and 535 cm\(^{-1}\) are due to Pt–N stretching vibrations.

6.3.2. Mass spectra

The mass spectra of the compounds are characterized by the presence of numerous peaks (Tables 5.13-5.18). Among them only a few are believed to correspond to molecules or molecular fragments while other peaks correspond to species that are believed to be formed in situ or could be due to impurities of the compounds.

**MH3**

ESI-MS (DMSO)(m/z: M = 1023). No peak is observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The large peak at m/z = 899 corresponds to \([M – (NH_3) – 3Cl^– + H^+]\), that at m/z = 844 corresponds to \(M – 5Cl^– – H^+\), that at m/z = 727 corresponds to \([M – 2(C_5H_5NO) – 3Cl^–]\), that at m/z = 551 corresponds to \([M – 2(C_5H_5NO) – 4(NH_3) – 6Cl^– – H^+]\). The peak at m/z = 411 corresponds to \([M – 2Pd – 2(C_5H_5NO) – 4(NH_3) – 4Cl^– + H^+]\) where M stands for the molecule MH3 and that at m/z = 373 corresponds to \([(M – 2Pd – NH_2(CH_2)_6NH_2 – (C_5H_5NO) – 3(NH_3) – 5Cl^– + 2H^+]\).
MH4

ESI-MS (DMSO)(m/z: M = 1023). The large peak at m/z = 1024 corresponds to M + H⁺, where M stands for MH4. The peak at m/z = 847 corresponds to (M – 5Cl⁻ + 2H⁺) and that at m/z = 727 corresponds to [M – 2(C₅H₅NO) – 3Cl⁻] where M stands for the molecule MH4. The peak at m/z = 550 corresponds to [M – 2(C₅H₅NO) – 4(NH₃) – 6Cl⁻ – 2H⁺], that at m/z = 361 corresponds to [M – Pd – 2(C₅H₅NO) – 4(NH₃) – NH₂(CH₂)₆NH₂ – 5Cl⁻ – 4H⁺] and that at m/z = 223 corresponds to [M – 2Pd – OH – (C₅H₅NO) – 4(NH₃) – 2{NH₂(CH₂)₆NH₂} – 5Cl⁻ + H⁺].

MH5

ESI-MS (DMSO)(m/z: M = 1023). No peak was observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The peak at m/z = 844 corresponds to (M – 5Cl⁻ – H⁺) and that at m/z = 790 corresponds to [M – 3Cl⁻ – (C₅H₅NO) – 2(NH₃) + 2H⁺], where M stands for the molecule MH5. The peak at m/z = 729 corresponds to [M – 3Cl⁻ – 2(C₅H₅NO) + 2H⁺], that at m/z = 551 corresponds to [M – 4(NH₃) – 2(C₅H₅NO) – 6Cl⁻ – H⁺] and that at m/z = 471 corresponds to [M – Pd – 2(C₅H₅NO) – 4(NH₃) – NH₂(CH₂)₆NH₂ – 4Cl⁻ + 3H⁺]. Finally, the peak at m/z = 221 corresponds to [M – 2Pd – 2(C₅H₅NO) – 4(NH₃) – NH₂(CH₂)₆NH₂ – 6Cl⁻ – H⁺].

MH6

ESI-MS (DMSO)(m/z: M = 1180). No peak was observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The peak
at m/z = 903 corresponds to \([M – 2\text{Cl}^– – 2(\text{C}_5\text{H}_5\text{NO}) – \text{NH}_3 + 2\text{H}^+]\) and that at m/z = 532 corresponds to \([M – 3\text{Cl}^– – \text{Pd} – 3(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \text{NH}_2(\text{CH}_2)_6\text{NH}_2],\) where M stands for the molecule MH6. The peak at m/z = 426 corresponds to \([M – 6\text{Cl}^– – \text{Pd} – 3(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} + \text{H}^+]\), that at m/z = 368 corresponds to \([M – 5\text{Cl}^– – \text{Pd} – 4(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} + 3\text{H}^+]\) and that at m/z = 327 corresponds to \([M – 3\text{Cl}^– – 2\text{Pd} – 4(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} – 2\text{H}^+]\). Finally, the peak at m/z = 221 corresponds to \([M – 2\text{Pd} – 2(\text{C}_5\text{H}_5\text{NO}) – 4(\text{NH}_3) – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} – 6\text{Cl}^– – \text{H}^+]\).

**MH7**

EIS-MS (DMSO) (m/z: M = 1180). No peak was observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The peak at m/z = 843 corresponds to \([M – \text{Pd} – \text{C}_5\text{H}_5\text{NO} – \text{NH}_3 – \text{NH}_2(\text{CH}_2)_6\text{NH}_2 – \text{H}^+]\), that at m/z = 610 corresponds to \([M – \text{Pd} – 4\text{Cl}^– – 2(\text{C}_5\text{H}_5\text{NO}) – \text{NH}_3 – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} + 3\text{H}^+]\) and that at m/z = 428 corresponds to \([M – \text{Pd} – 6\text{Cl}^– – 3(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} + \text{H}^+]\). The peak at m/z = 370 corresponds to \([M – 2\text{Pd} – 3\text{Cl}^– – 2(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – 2\{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} – 2(\text{OH}) + \text{H}^+]\), that at m/z = 296 corresponds to \([M – 2\text{Pd} – 4\text{Cl}^– – 4(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – \text{NH}_2(\text{CH}_2)_6\text{NH}_2 + 2\text{H}^+]\) and that at m/z = 221 corresponds to \([M – 2\text{Pd} – 5\text{Cl}^– – 3(\text{C}_5\text{H}_5\text{NO}) – 2(\text{NH}_3) – 2\{\text{NH}_2(\text{CH}_2)_6\text{NH}_2\} – \text{OH}]\).

**MH8**

EIS-MS (DMSO) (m/z: M = 1180). The peak at m/z = 1028 corresponds to \([M – \text{NH}_3 – \text{C}_5\text{H}_5\text{NO} – \text{Cl}^– – 3\text{H}^+]\), that at m/z = 699 corresponds to \([M – \text{Pd} – \text{NH}_3 – \text{C}_5\text{H}_5\text{NO} – \text{Cl}^– – 3\text{H}^+]\)
4Cl⁻ – 3H⁺] and that at m/z = 604 corresponds to [M – Pd – NH₃ – NH₂(CH₂)₆NH₂ – 2(C₅H₅NO) – 4Cl⁻ – 3H⁺]. The peak at m/z = 426 corresponds to [M – 6Cl⁻ – Pd – 3(C₅H₅NO) – 2(NH₃) – NH₂(CH₂)₆NH₂ + H⁺], that at m/z = 365 corresponds to [M – 5Cl⁻ – 2Pd – 4(C₅H₅NO) – 2(NH₃) – NH₂(CH₂)₆NH₂], and that at m/z = 331 corresponds to [M – 6Cl⁻ – 2Pd – 4(C₅H₅NO) – 2(NH₃) – NH₂(CH₂)₆NH₂ + H⁺]. The peak at m/z = 295 corresponds to [M – 6Cl⁻ – 2Pd – 4(C₅H₅NO) – 2(NH₃) – 2{NH₂(CH₂)₆NH₂} – H⁺], that at m/z = 221 corresponds to [M – 6Cl⁻ – 2Pd – 4(C₅H₅NO) – 2(NH₃) – NH₂(CH₂)₆NH₂ – H⁺].

6.3.3. ¹H NMR Spectra

The ¹H NMR spectra of compounds can also been seen to provide support to the suggested structures for the compounds as the proton resonances indicate the presence of various functional groups. It should however be noted that in some cases the observed ¹H NMR spectra (Tables 5.19 to 5.24) are found to be more complex than the ones expected from suggested structures e.g. often the splitting of the lines is found to be more complex. It is believed that the complexity of the spectra may be partly due to the presence of impurities and some dissociation of the complexes.

MH3

The weak broad resonance at δ = 11.4 ppm (singlet) is believed to be due to OH proton. The resonance at δ = 9.76 ppm (triplet) is due to CH ortho proton, that at δ = 7.26 ppm (singlet) is due to CH meta proton, that at δ = 4.82 ppm (triplet) is due to NH₂ proton, that at δ = 2.45 ppm (triplet) is due to DMSO and that at δ = 2.35 ppm (triplet) is due to NH proton. The resonance at δ = 1.67 ppm (doublet) is believed to
be due to CH₂ and that at δ = 1.62 ppm (quartet) is due to CH₂. The resonance at δ = 1.37 ppm (triplet) is believed to be due to CH₂ and that at δ = 1.30 ppm (singlet) is due to CH₂. The resonance at δ = 1.25 ppm (singlet) is believed to be due to CH₂ and that at δ = 0.88 ppm (triplet) is also due to CH₂.

MH4

The resonance at δ = 8.48 ppm (singlet) is believed to be due to OH proton, that at δ = 8.33 ppm (doublet) is believed to be due to CH ortho proton and that at δ = 8.3 ppm (singlet) is also due to CH ortho proton. The resonance at δ = 7.48 ppm (quartet) is due to CH meta and that δ = 7.42 ppm (doublet) is due to CH para proton. The resonance at δ = 3.53 ppm (singlet) is due to NH₃ and that at δ = 293 ppm (pentet) is due to CH₂. The resonance at δ = 2.76 ppm (pentet) is due to is due to CH₂. Broad but weak resonances at δ = 2.65 and 1.4 ppm are also believed to be due to CH₂.

MH5

The broad resonance at δ = 11.6 ppm (singlet) is believed to be due to OH proton, that at δ = 7.94 ppm (singlet) is believed to be due to CH ortho proton and that at δ = 7.57 ppm (doublet) is due to CH meta proton. The resonance at δ = 7.36 ppm (triplet) is believed to be due to CH meta proton and that at δ = 6.32 ppm (doublet) is due to CH para proton. The resonance at δ = 5.96 ppm (doublet) is due to NH. The resonance at δ = 3.51 ppm (triplet) is believed to be due to NH₃ and that at δ = 297 ppm (quartet) is due to due to NH₂. The resonance at δ = 2.88 ppm (singlet) is believed to be due to NH₂ and that at δ = 2.72 ppm (singlet) is due to due to CH₂. The resonance at δ = 2.58
ppm (singlet) is believed to be due to DMSO and that at \( \delta = 2.39 \) ppm (singlet) is due to due to CH\(_2\). Finally, the resonances at \( \delta = 2.39 \) ppm (singlet), 2.22 ppm (triplet), 1.56 ppm (triplet) and 1.26 ppm (triplet) are believed to be due to CH\(_2\).

**MH6**

The broad but weak resonance at \( \delta = 11.5 \) ppm (singlet) is believed to be due to OH proton, that at \( \delta = 8.54 \) ppm (doublet) is believed to be due to CH ortho and that at \( \delta = 8.04 \) ppm (singlet) is believed to be due to CH ortho. The resonance at \( \delta = 7.50 \) ppm (triplet) is believed to be due to CH meta proton and that at \( \delta = 6.94 \) ppm (doublet) is believed to be due to CH meta proton. The resonance at \( \delta = 6.39 \) ppm (triplet) is believed to be due to CH para. The resonance at \( \delta = 3.57 \) ppm (doublet) is believed to be due to NH\(_3\) and the resonance at \( \delta = 3.46 \) ppm (doublet) is believed to be due to due to water dissolved in DMF and that at \( \delta = 3.14 \) ppm (singlet) is believed to be due to CH\(_2\) meta. The resonances at \( \delta = 2.97 \) ppm (doublet), 2.93 ppm (pentet), 2.88 ppm (singlet) and 2.81 ppm (triplet) are believed to be due to CH\(_2\). The resonance at \( \delta = 2.62 \) ppm (doublet) is is believed to be due to DMSO and that at \( \delta = 1.99 \) ppm (singlet) is due to due to CH\(_2\). Finally, the resonances at \( \delta = 1.78 \) ppm (singlet), 1.50 ppm (singlet), 1.39 ppm (singlet) and 1.29 ppm (singlet) are believed to be due to CH\(_2\).

**MH7**

The resonance at \( \delta = 8.3 \) ppm (singlet) is believed to be due to CH ortho proton. Second resonance at \( \delta = 8.3 \) ppm (singlet) may also be due to CH ortho proton but
applying to the free ligand. If this is so, the results indicate that some dissociation of
the compound has taken place in solution so there is a mixture of bound and unbound
3-hydroxypyridine. The resonance at $\delta = 8.19$ ppm (doublet) is believed to be due to
CH ortho proton and that at $\delta = 8.01$ ppm (doublet) is believed to be due to CH para
proton. The resonance at $\delta = 7.32$ ppm (quartet) is believed to be due to CH meta
proton. The broad resonance at $\delta = 4.26$ ppm (singlet) is believed to be due to NH$_2$
and that at $\delta = 3.32$ ppm (singlet) is believed to be due to due to water dissolved in
DMF. The resonance at $\delta = 2.50$ ppm (pentet) is believed to be due to DMSO. The
resonance at $\delta = 2.4$ ppm is believed to be due to CH$_2$ and that at $\delta = 1.90$ ppm
(singlet) is due to due to CH$_2$. Finally, the resonances at $\delta = 1.70$ ppm (triplet) and
1.30 ppm (doublet) are believed to be due to due to CH$_2$.

**MH8**

The broad resonance at $\delta = 12.2$ ppm (singlet) is believed to be due to OH proton. The
resonance at $\delta = 8.45$ ppm (doublet) is believed to be due to CH ortho proton. The
resonance at $\delta = 8.03$ ppm (singlet) remains unidentified. It may due to CH ortho
proton of the free 4-hydroxypyridine ligand and if so, the results indicate some
dissociation of MH8 in solution. The resonance at $\delta = 6.97$ ppm (doublet) is believed
to be due to CH meta proton. The broad resonance at $\delta = 3.60$ ppm (pentet) is due to
water dissolved in DMSO and that at $\delta = 3.50$ ppm (singlet) is due to NH$_3$. The
resonance at $\delta = 2.91$ ppm (pentet) NH$_2$. The resonances at $\delta = 2.77$ ppm (doublet)
and 2.74 ppm (pentet) are believed to be due to CH$_2$. The resonance at $\delta = 2.61$ ppm
(quartet) is due to DMSO. Finally, the resonances at $\delta = 1.75$ ppm (doublet), 1.57
ppm (singlet) and 1.45 ppm (pentet) are believed to be due to CH$_2$. 
6.4. Molar conductivity

The molar conductivity values provide information on the extent of dissociation of the compounds, which in turn may provide information about the possible modes of transport across the cell membrane. It may be noted non-polar molecules can cross the cell membrane by passive diffusion whereas the highly charged and polar molecules may require carriers to cross the cell membrane. The molar conductivity values may also provide information on the binding process with DNA. As positively charged species are likely to be attracted to the negatively charged DNA because of the phosphate backbone, the covalent binding of metal-based drugs such as aquated cisplatin may be preceded by associative interaction. Assuming that the synthesized compounds MH3, MH4, MH5, MH6, MH7 and MH8 remain undissociated, in solution in water the compounds should provide poly-positively charged trinuclear cations and the balancing chloride ions.

The limiting molar conductivity values in ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) at zero concentration of MH3, MH4, MH5, MH6, MH7 MH8 and cisplatin at 298 K were found to be 690, 820, 730, 970, 580, 1010 and 270 respectively. It can be seen that the values for the designed trinuclear Pd-Pd-Pd complexes MH3, MH4, MH5, MH6, MH7 and MH8 are significantly higher than that for the mononuclear platinum drug cisplatin. The reason for the large differences in molar conductivity values between the designed complexes and cisplatin becomes evident when we note the difference in nature of the compounds. Whereas cisplatin is a neutral molecule which on hydrolysis can ultimately produce a 1:2 electrolyte (composed of a dipositive cation and two uninegative chloride ions), MH3, MH4, MH5, MH6, MH7 and MH8 are ionic compounds each composed of trinuclear tetrapositive cation and 4 uninegative
chloride ions. The trinuclear tetrapositive Pd-Pd-Pd cation can further ionize to produce hexapositive trinuclear cation and two uninegative chloride ions. When we compare the limiting molar conductivity values of MH3, MH4, MH5, MH6, MH7 and MH8 with those of trinuclear Pt-Pt-Pt and Pt-Pd-Pt compounds such as TH1 and TH8 that are reported to have values of 384 and 270 ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) respectively (Huq \textit{et al.}, 2007; Huq \textit{et al.} 2008), it is evident that the Pd-Pd-Pd complexes have significantly larger values. The main reason for this difference is believed to be due to greater reactivity of the Pd\(^{2+}\) ion than the Pt\(^{2+}\) ion so that the Pd-Cl bond would be more labile than the Pt-Cl bond and the trinuclear Pd-Pd-Pd cation is more likely to breakdown in solution than the corresponding Pt-Pt-Pt and Pt-Pd-Pt cations. Another reason for the difference may be associated with the solvent in which the compounds were dissolved. Whereas TH1 and TH8 were first dissolved in minimum volumes of DMF and then diluted with mQ water, the designed complexes in this study were first dissolved in minimum volumes of DMSO and then diluted with mQ water.

Since cisplatin is given as an intravenous injection, it is expected that the molecule would remain undissociated in blood serum that has a high chloride concentration. This means that cisplatin can cross the cell membrane by passive diffusion. In actual fact, the molecule is known to be transported across the cell membrane cisplatin by both passive diffusion and carrier-mediated transport (Wang and Lippard, 2005). In contrast, MH3, MH4, MH5, MH6, MH7 and MH8 are expected to cross the cell membrane by carrier-mediated transport only as unlike cisplatin (which is expected to remain un-ionized in blood serum where chloride concentration is high), MH3, MH4, MH5, MH6, MH7 and MH8 being ionic compounds would produce cations of at least four unit charge even in the presence of high chloride concentrations. Recent observations suggest that cisplatin can also cross the cell membrane by pinocytosis.
This mechanism may also apply to BBR3464 and tri palladium complexes of the present study. Studies on cell uptake as a function of change in concentration may provide further light on the matter.

6.5. Interaction with ssDNA

Figure 5.26 gave the electrophoretograms applying to the incubated mixtures of ssDNA and varying concentrations of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin. A single band was observed in both untreated and treated ssDNAs. As the concentration of the drugs was increased the intensity of the band decreased but there was no observable change in mobility of the band. The decrease in intensity of the band with the increase in concentration of the compounds indicates the occurrence of DNA damage caused by interaction of the compounds with DNA. All the tri palladium complexes are believed to bind covalently with ssDNA via the terminal metal centres forming a range of interstrand GG adducts (dictated by the sequence of nucleobases) that cause a global change in DNA conformation (Di Blasi et al., 1998; Farrell, 2000). The planaramaine ligands are likely to be involved in non-covalent interactions including hydrogen bonding and stacking interaction. It was noted earlier that among the tri palladium complexes, DNA damage was more significant in the case of MH5, MH6 and MH7. MH5 has two 3-hydroxypyridine ligands bound to central palladium ion whereas MH6 has two 2-hydroxypyridine ligands bound to each of the two terminal palladium ions and MH7 has two 3-hydroxypyridine ligands bound to each of the two terminal palladium ions. The least damaging compound MH8 has two 4-hydroxypyriine ligands bound to each of the two terminal palladium ions. The differences in DNA damage suggest that non-covalent interactions involving planaramine ligands may be playing a key role in inducing DNA damage. That 2-
hydroxypyrindine and 3-hydroxypyrindine in a *trans*-geometry are found to be more damaging than 4-hydroxypridine indicates that the position of the hydroxyl group may be a key determinant of the extent of DNA damage (and the level of activity that will be considered later), thus illustrating structure-reactivity (and structure-activity) relationships. From steric considerations it can be seen that trinuclear palladium ions may be better protected from the onslaught of solvent molecules and other palladinophiles when the metal ions are bound to 2-hydropyridine and 3-hydroxypyrindine than 4-hydroxypyrindine in a *trans*-geometry.

### 6.6. Interaction with pBR322 plasmid DNA

Figure 5.27 gave the electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of MH3, MH4, MH5, MH6, MH7 and MH8 and cisplatin.

As stated earlier, pBR322 plasmid DNA can exist in three forms namely supercoiled form I, singly nicked relaxed circular form II and doubly nicked liner form III. DNA being poly negatively charged due to the phosphate backbone will migrate through the gel from the negative to positive electrode. The supercoiled form I migrates at the fastest rate, the singly nicked circular form II has the lowest migration rate whereas the doubly nicked linear form III DNA has the intermediate migration rate. As PBR322 plasmid DNA interacted with increasing concentrations of compounds, significant DNA damage (indicated by streaking of the band and/or absence of the DNA bands) was observed in the case of MH4, MH6, MH7 and MH8. No DNA band was observed when the DNA was interacted with high concentrations of MH8 unlike that in the case of ssDNA for which the intensity of the single DNA band essentially showed no observable change with the change in concentration of MH8. The results
indicate that pBR322 plasmid DNA is more susceptible to damage than ssDNA. It is not however clear why there is a greater damage to pBR322 plasmid DNA with high concentrations of MH8 than with other tri palladium compounds even though some of them were found to be more damaging than MH8 to ssDNA. Noticeable change in mobility of the bands was observed at higher concentrations of MH6 and MH7, indicating the occurrence of change in DNA conformation.

When pBR322 plasmid DNA interacted with the trinuclear palladium compounds, in general two DNA bands corresponding to forms I and II were observed in both treated and untreated DNA. As the concentrations of the compounds were increased, there was significant increase in the mobility of the bands (especially at higher concentrations) in the case of MH3, MH4, MH6 and MH7. The change in mobility of bands was less significant in the case of MH5 and MH8. Also, as the concentrations of the compounds were increased, significant changes in intensity of the DNA bands were observed in the case of MH4, MH6, MH7 and MH8. The change in mobility of the bands is believed to be primarily due to changes in DNA conformation as a result of covalent binding of the compounds with the DNA. Also, the binding of the polynuclear metal cations with the DNA will serve to increase its molecular mass and reduce its overall negative charge, once again causing a decrease in the rate of migration of the DNA bands towards the positive electrode. The decrease in intensity of the DNA bands (and streaking of the bands observed in the case of MH4, MH6 and MH7) indicates the occurrence of DNA damage. This is also believed to be caused by the binding of the palladium compounds with the DNA. The position of the hydroxyl group on the planaramine ligand appears to be a key determinant of the extent of DNA damage; generally the presence of 3-hydroxypyridine (and 2-hydroxypyridine) is found to cause greater damage than that of 4-hydroxypyridine. It was noted
earlier that 2-hydroxypyridine and 3-hydroxypyridine would provide greater protection to the metal ion from the onslaught of solvent molecules than 4-hydroxypyridine so that more trinuclear cations may prevail in solutions of MH4, MH5, MH6 and MH7 than of MH3 and MH8. However, the extent of DNA damage does not appear to be simply the result of the frequency of the long-range interstrand GG adducts as no significant damage is observed in the case of MH5 (that has two 2-hydroxypyridine ligands bound to central palladium ion). Besides steric effect, other non-covalent interactions including hydrogen bonding and stacking interaction may be playing key roles in inducing both DNA damage and conformational change in DNA.

6.7. BamH1 restriction enzyme digestion

Figure 5.28 gave the electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of MH3, MH4, MH5, MH6, MH7 and MH8 and cisplatin that were digested with BamH1 for 1 h. BamH1 is a restriction endonuclease that recognizes the sequence G/GATCC and hydrolyses the phosphodiester bond between adjacent GG sites. It was also noted that pBR322 plasmid DNA 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. Thus when untreated 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, generally two bands corresponding to forms I and II were observed. When pBR322 plasmid DNA was interacted with increasing concentrations of MH3 followed by BamH1 digestion, generally three bands corresponding to forms I, II and III were observed at all concentrations of 2.5 µM to
60 µM. The absence of form II band at 20 µM is believed to be an artefact. The results indicate MH3 on binding with pBR322 plasmid DNA has been able to partially prevent digestion of the DNA by BamH1. This is believed to be due to change in DNA conformation brought about by covalent binding of the compound with DNA.

In the case of MH4, two bands corresponding to forms II and III were observed at concentrations ranging from 2.5 µM to 5 µM and three bands corresponding to forms I, II and III were observed for concentrations ranging from 7.5 µM to 60 µM. The results indicate that MH4 has also been able to partially prevent BamH1 digestion of pBR322 plasmid DNA that is also believed to be due to changes in DNA conformation brought about by covalent binding of the compound with the DNA.

When the BamH1 digestion results in MH3 and MH4 are compared, it can be seen that MH3 has been slightly more efficient in preventing enzyme digestion than MH4. Noting that MH3 has two 4-hydroxypyridine ligands bound to the central metal ion whereas has MH4 has two 3-hydroxypyridine ligands bound to the same metal ion, it appears that non-covalent interactions involving the planaramine ligands may be playing a key role in inducing changes in DNA conformation and consequent prevention of BamH1 digestion. In the case of MH5, three bands corresponding to forms I, II and III were observed for concentrations ranging from 2.5 µM to 10 µM, one band corresponding to form III was observed at the concentration 15 µM and 60 µM and. The presence of forms I, II and III bands at low concentrations of the compound indicates partial prevention of BamH1 digestion which is also believed to be due to covalent binding of the compound with the DNA. The absence of forms I and II bands at higher concentrations of MH5, indicate that besides BamH1, the compound is able to cause DNA damage. Noting that MH5 has two 2-hydroxypyridine ligands bound to the central metal ion, it appears that non-covalent
interactions associated with the planaramine ligand may be involved in inducing damage to the DNA at higher concentrations. In the case of MH6, one band corresponding to form III was observed at the concentrations ranging from 2.5 µM to 10 µM, three bands corresponding to forms I, II and III were observed at concentrations ranging from 15 µM to 60 µM. The results indicate that MH6 also has some ability to prevent BamH1 digestion but much less than that of MH3, MH4 or MH5. It may be noted that among the trinuclear palladium complexes, MH6 (which has two 2-hydroxypyridine ligands bound to each of the terminal metal centres) is found to be most active against the ovarian cancer cell lines even though it is much less able to prevent BamH1 digestion. It will be discussed later that the trinuclear cation of MH6 is most likely to persist in solution because of the steric hindrance provided by 2-hydroxypyridine ligands for solvent molecules from coming in contact with the metal ions. The above results may be seen to indicate that the biological activity is much more complex where both covalent and non-covalent interactions may be playing key roles. In the case of MH7, two bands corresponding to forms I and III were observed at concentrations ranging from 2.5 µM to 20 µM, three bands corresponding to forms I, II and III were observed at the concentrations ranging from 30 µM to 60 µM. In terms of ability to prevent BamH1 digestion, MH7 (which has two 3-hydroxypyridine ligands bound to each of the terminal metal centres) is found to be similar to MH6. Finally for MH8, three bands corresponding to forms I, II and III were observed at concentrations ranging from 2.5 µM to 10 µM and, 15 µM and 40 µM; and two bands corresponding to forms I and III were observed at the concentrations 20 µM and 30 µM and no band was observed at 60 µM concentration. Although MH8 is found to be better able to prevent BamH1 digestion than MH6 and MH7, it is in fact the least active compound, once again giving support to the idea that
biological activity can be a consequence of many different processes. As noted earlier, the bands observed at higher concentrations of MH8 were streakier and had greater mobility. The streakiness of the bands may indicate the occurrence of DNA damage whereas the change in mobility may be due to both DNA damage and change in DNA conformation.

6.8. Activity of the compounds

Table 5.13 gave the IC\textsubscript{50} values and resistance factors (RF) for MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin as applied to the human ovarian cancer cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}. It can be seen that although all the six compounds MH3, MH4, MH5, MH6, MH7 and MH8 display some activity against the cancer cell lines, the actual level of activity is lower than that of cisplatin. However, the trinuclear palladium compounds are found to have much lower resistance factors than cisplatin. The most active compound MH6 is found to be significantly more active against the resistant cell line A2780\textsuperscript{cisR} than the parent cell line A2780 with an RF value of 0.7 for the combination whereas the corresponding value for cisplatin is 6.1. This means that at the level of its activity MH6 has been better able to overcome mechanisms of resistance operating in A2780\textsuperscript{cisR} cell line than cisplatin. Noting that each of the two terminal palladium ions in MH6 is bound to two 2-hydroxypyridine ligands whereas that in the least active compound MH8 it is bound to two 4-hydroxypyridine ligands, it can be concluded that the presence of 2-hydroxypyridine ligands in the terminal positions serves to enhance antitumour activity and that of 4-hydroxypyridine ligands serves to reduce the activity. The results can be seen to illustrate structure-activity relationship associated with the nature of the planaramine ligands. The activating effect of 2-hydroxypyridine towards
antitumour activity of the trinuclear palladium compounds is believed to be associated
(at least in part) to the steric effect. More exactly, the hydroxyl group bound to the
second ring atom would provide steric hindrance against the approach of solvent
molecules to the metal ion. In contrast, the steric effect would be much less in the case
of 4-hydroxypyridine ligand. This means that the kinetic stability rendered to the
trinuclear cation by 2-hydroxypyridine ligand would be much greater than that due to
4-hydroxypyridine ligand. As a result, the number of long-range interstrand GG
adducts that can be formed by MH6 would be significantly greater than that formed
by MH8. Besides the steric effect, non-covalent interactions such as hydrogen
bonding and stacking interaction (involving substituted pyridine ligands) may also be
playing a significant role in activity of the compounds. It was concluded earlier that
the least active compound MH8 also caused least conformational change in DNA
giving support to the idea that both the conformational change induced in DNA and
the displayed antitumour activity are a consequence of covalent binding of the
trinuclear cations with the DNA. Another compound of the present study containing
2-hydroxypyridine ligand is MH5 that has two 2-hydroxypyridine ligands bound to
the central palladium ion. Although the compound is less active than MH6, it is found
to be significantly more active than MH8, once again illustrating the activating role
played by 2-hydroxypyridine ligand. From above, it follows that a trinuclear Pd-Pd-
Pd complex in which each of the three palladium ions is bound to two 2-
hydroxypyridine ligands may be found to be much more active as its trinuclear cation
would be expected to be much more stable. However, since the compound has not
been prepared, this remains to be confirmed.

Besides MH8, another trinuclear compound which has very low activity against
A2780 cell line is MH4 that has two 3-hydroxypyridine ligands bound to central
palladium ion. Unlike MH8 which has much lower activity in resistant cell lines A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}, MH4 is found to be slightly more active against the resistant cell lines than the parent cell line. The results suggest that MH4 has been better able to overcome mechanisms of resistance operating in the ovarian cancer cell lines than MH8. The second most active compound namely MH3 has two 4-hydroxypyridine ligands bound to the central palladium ion. However, MH3 is found to be less active against the resistant cell lines A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} than the parent cell line A2780. It was noted earlier that the presence of 4-hydroxypyridine ligands at the terminal metal centres serves to lower activity and that of 2-hydroxypyridine and 3-hydroxypyridine ligands at the positions serves to enhance activity. The converse appears to be true as applied to the central position. This means that the roles played by the pyridine ligands may be position-dependent. Whereas at the terminal positions, stabilizing effect would be more critical (since the metal ions at such positions are more likely to be exposed to solvent molecules), it is less so at the central position (because of the stabilization already provided by the caged-in effect on the metal ion). In other words, other interactions involving planaramine ligands such as hydrogen bonding and stacking interaction may be more important in activity when they are bound to the central metal ion. That is, the activating effect of a planaramine ligand is position dependent.
6.9. Cell uptake

Tables 5.14 to 5.16 gave the cell uptakes of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin in 2 h, 4 h, and 24 h as applied to the ovarian cancer cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}. MH6 is generally found to have the highest cell uptakes in all the three ovarian cancer cell lines in line with the highest activity of the compound. However, the least active compound MH8 is also found to have higher cell uptakes (in some cases) than MH3, MH5 and MH7 which are more active than MH8. For example, MH8 has higher cell uptakes than MH4 and MH7 at 2, 4 and 24 h in A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} cell lines. The results illustrate that cell uptakes do not necessarily correlate with activity as palladium compounds may be deactivated before binding with DNA. It has been noted in a number of platinum drugs only about 1% of the compound entering the cell actually binds with DNA (Ghezzi \textit{et al.}, 2004; Ho \textit{et al.}, 2003). The fraction may be even smaller in the case of palladium complexes as they are more likely to be deactivated before binding with DNA. It is the level of binding of the compounds with the DNA (more exactly the level of critical drug-DNA adducts) that is expected to be a better indicator of activity or lack of it.

When the cell uptakes of trinuclear palladium compounds in the three ovarian cancer cell lines are compared with those of cisplatin, trinuclear palladium compounds are often found to have much higher cell uptakes than cisplatin especially at 2 h. The results illustrate that tri-palladium compounds like their tri-platinum analogues (Wheate and Collins, 2005) can cross the cell membrane much faster than cisplatin. Whereas cisplatin is found to cross the cell membrane by both passive diffusion and carrier-mediated transport (Ghezzi \textit{et al.}, 2004), positively charged trinuclear
palladium and platinum ions are expected to cross the same by carrier-mediated transport only (Daghriri et al., 2004). It may be noted that one of the advantages of trinuclear platinum drugs such as BBR3464 is their fast cell uptake in addition to the advantages provided by high positive charge and increased flexibility resulting in faster association with DNA and formation of a number of interstrand GG adducts (Liu et al., 2006).

6.10. DNA binding

Tables 5.17 to 5.19 gave the levels of palladium-DNA binding of MH3, MH4, MH5, MH6, MH7, MH8 and cisplatin in 2 h, 4 h, and 24 h as applied to the ovarian cancer cell lines: A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}. MH6 is found to have the highest level of Pd-DNA binding at 2 and 24 h in A2780 cell in line with the highest activity of the compound. However, the least active compound MH8 is also found to have higher levels of Pd-DNA binding (in some cases) than other trinuclear palladium compounds such as MH3, MH4, MH5 and MH7. More perplexing is the level of Pd-DNA binding at 24 h in A2780\textsuperscript{ZD0473R} cell line which is found to be the highest for MH8. Although it is difficult to explain these apparently conflicting results, it appears that even the levels of Pd-DNA binding per se may not always provide a true measure of activity of the compounds as non-critical adducts may predominate (and non-covalent interactions involving planaramine ligands may also be playing critical roles). It was noted earlier that as applied to the terminal metal centres, whereas 2-hydroxypyridine would be an activating planaramine ligand (providing protection more to terminal trinuclear palladium ions from attack due to solvent molecules), relatively speaking, 4-hydroxypyridine would be a deactivating one (providing much lower protection to the terminal metal centres. Because the trinuclear cation of MH8
is expected to be much less stable than that of other tripalladium complexes, the observed higher levels of Pd-DNA binding of MH8 are less likely to provide a true or meaningful measure of the levels of long-range interstrand GG adducts (Farrel, 2000). Antitumour activity of tripalladium complexes (however small it may be) like that of corresponding platinum complexes, is believed to be associated with global changes in DNA conformation brought about by long-range interstrand GG adducts.

When the levels of Pt-DNA binding of cisplatin are compared with the levels of Pd-DNA binding of tripalladium complexes, it is found that the Pt-DNA binding levels are much lower than the Pd-DNA binding levels even though cisplatin is much more active than all the tripalladium complexes. Whereas cisplatin is expected to form mainly intrastrand Pt(GG) and Pt(AG) adducts that cause local bending of a DNA strand, trinuclear Pd cations (like their platinum analogues such as BBR3464 and TH1) are expected to form a plethora of long-range interstrand GG adducts. However, the Pd-DNA adducts, being much more labile than the corresponding Pt-DNA adducts, in general they may not persist long enough in solution to translate into significant antitumour activity. In fact, one of the disadvantages of even the trinuclear platinum drugs such as BBR3464 is their progressive biotransformation and degradation in solution in the cellular matrix (Summa et al., 2007).

When the levels of Pd-DNA binding applying to MH6 in A2780, A2780\(^{\text{cisR}}\) and A2780\(^{\text{ZD0473R}}\) cell lines, at 24 h are compared, it is found that the higher level of binding does not necessarily result in higher activity. For example, for MH6, the lowest level of Pd-DNA binding is observed in A2780\(^{\text{cisR}}\) cell line although it is the most active against this cell line. The above results indicate that no clearer conclusions regarding activity of the designed tripalladium complexes can be made
perhaps because of their high reactivity and hence low biological half-life) except to say that the presence of 2-hydroxypyridine ligand at the terminal positions would serve to enhance activity (by providing protection to the trinuclear cation from the onslaught of solvent molecules). However, as applied to the central position, 4-hydroxypyridine is found to be slightly more activating than 2-hydroxypyridine and 3-hydroxypyridine at least in the parent cell line. This means that the tri palladium complex in which each of the two terminal palladium ions is bound to two 2-hydroxypyridine ligands and the central metal ion is bound to two 4-hydroxypyridine ligands may be significantly more active. As noted earlier, another tri palladium complex in which each of the three palladium ions is bound to two 2-hydroxypyridine ligands, may also be significantly more active. However, the ideas remain to be confirmed as the suggested compounds have not been prepared.

When the levels of Pd-DNA binding at 2 and 24 h of tri palladium complexes are compared with the corresponding values for cisplatin, it is generally found that whereas for the tri palladium complexes the values at 24 h are lower than those at 2 h, for cisplatin the converse is generally true. The results indicate that the tri palladium complexes bind with DNA much faster than cisplatin. Triplatinum complexes such as BBR3464, TH1 and CH9 have also been reported to bind faster with cellular DNA than cisplatin (Cheng et al., 2005; Farrell, 2000; Huq et al., 2008). The decrease in the level of Pd-DNA binding with an increase in time may indicate the occurrence of increased DNA repair. It is reasonable to assume that the Pd-DNA adducts may be repaired by excision repair, which is known to be one of the dominant mechanisms of resistance applying to the platinum-based anticancer drugs (Siddik, 2003).
7. Chapter Seven: Conclusion

Cancer is one of the leading causes of death all over the world and it is one of the most dreaded diseases of modern times. Widespread use in the clinic and increasing volume of sale indicate that even in the post-genomic age, there is a need for shotgun chemotherapy such as that based on platinum drugs cisplatin, carboplatin and oxaliplatin. Currently attention is given to designing rule-breaker platinum compounds and compounds of other metals such as ruthenium with the aim of widening the spectrum of activity and reducing the side effects. Although palladium and platinum belong to the same group in the periodic table, whereas platinum compounds can be tumour active, the corresponding palladium compounds are often found to be inactive and toxic. The inactivity and high toxicity of palladium compounds are believed to be due to their much higher reactivity so that very few palladium-DNA adducts can be formed within the cell and the few that are formed may not persist long enough for the manifestation of antitumour activity. Following the idea that the trans-geometry in platinum can be activated for antitumour activity by the introduction of bulky planaramine ligands (that reduces reactivity), it was thought the reactivity of palladium compounds could also be decreased by the introduction of sterically hindered ligands so that palladium compounds with observable antitumour activity might be produced. Thus, this project aimed to synthesize and investigate for anticancer activity against human ovarian cancer cell lines a number of trinuclear palladium complexes in which the central or the terminal palladium ions are bound to two planaramine ligands such as 2-hydroxypyridine, 3-hydroxypyridine or 4-hydroxypyridine. It was hypothesised that the compounds would display significant antitumour activity. Also, the activity of the compounds
may exhibit significant variations depending on the positions of the planaramine ligands (i.e. whether bound to central or terminal metal ions) and the actual nature of the planaramine ligand (2-hydroxypyridine, 3-hydroxypyridine or 4-hydroxypyridine), thus providing information on structure-activity relationships. Six trinuclear complexes code named MH3, MH4, MH5, MH6, MH7 and MH8 were prepared and evaluated for activity against ovarian cancer cell lines A2780, A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R}. Although all the palladium compounds synthesised were found to be less active than cisplatin against the cell lines, MH6 was found to be much more active than the other palladium complexes whereas MH8 is found to be least active. MH6 has two 2-hydroxypyridine ligands bound to each of the two terminal palladium ions whereas MH8 has two 4-hydroxypyridine ligands bound to each of the two terminal palladium ions. It was suggested that whereas 2-hydroxypyridine ligand would provide significant protection to palladium ions from the onslaught of solvent molecules or other ligands, 4-hydroxypyridine ligand would provide much lower protection. This means that the polynuclear cation of MH6 would be much more stable than that of MH8. Greater activity of MH6 in resistant cell lines A2780\textsuperscript{cisR} and A2780\textsuperscript{ZD0473R} than in parent cell line A2780 (the converse is true for cisplatin), indicates that at the level of its activity MH6 has been better able to overcome mechanisms of resistance operating in the cell lines than cisplatin. Whereas trinuclear palladium compounds like their platinum counterparts can form long-range interstrand GG adducts, cisplatin binds with DNA to form mainly intrastrand GG adduct.

When the cell uptakes and levels of binding with cellular DNA of designed trinuclear palladium complexes are compared, the results appear to be confusing as the least active compound is found to have much higher cell uptakes and Pd-DNA binding levels than some of the other tri-palladium complexes, perhaps pointing to the fact
that the antitumour activity is simply not a consequence of drug-DNA binding. The generally observed decrease in the level of Pd-DNA with the increase in time may be an indicator of the lability of the adducts

**What is next?**

As suggested earlier, tripalladium complex in which each of the three palladium ions is bound to two 2-hydroxypyridine ligands may be found to be significantly more active than the six designed complexes because of greater stabilization of the trinuclear metal ion provided by the 2-hydroxypyridine ligand. It was also suggested that the tripalladium complex in which the central metal ion is bound to two 4-hydroxypyridine ligands whereas each of the terminal metal ions is bound to two 2-hydroxypyridine ligands may also be significantly more active than any of the designed complexes. However, the ideas remain to be confirmed as the suggested compounds have not been prepared. The tripalladium complex in which each of the metal centres is bound to two 2-methylpyridine ligands may also be found to be significantly more active than the any of the designed complexes in the present study. Since in determining IC$_{50}$ values, cancer cells were incubated with drugs for a period of 72 hours, it would be meaningful to determine cell uptake and level of drug-DNA binding as applied to 72 h incubation, especially to determine the changes in uptake and binding levels over the period 24 to 72 h that may shed more light on the activity of the designed complexes.
References:


Cheng, H., Huq, F., Beale, P. and Fisher, K. (2005). "Synthesis, characterization, activities, cell uptake and DNA binding of trinuclear complex: \{trans-PtCl(NH\textsubscript{3})\textsubscript{2}\textsubscript{2}μ-trans-Pt(NH\textsubscript{3})(2-hydroxypyridine)\}²\{H\textsubscript{2}N(CH\textsubscript{2})\textsubscript{6}NH\textsubscript{2}\}²Cl\textsubscript{4}." European Journal of Medicinal Chemistry 40(8): 772-81.


Hausheer, F. H., Kanter, P., Cao, S., Haridas, K., Seetharamulu, P., Reddy, D., Petluru, P., Zhao, M., Murali, D., Saxe, J. D., Yao, S., Martinez, N.,


hydroxypyridine)$_2$(H$_2$N(CH$_2$)$_6$NH$_2$)$_2$]Cl$_4$ in Ovarian Cancer Cell Lines." ChemMedChem(Accepted).


matrix proteins precede renal tubular cell detachment and apoptosis in vitro."


Biochemical Pharmacology 37(24): 4597-600.


pharmacokinetic study of an one-hour infusion of ormaplatin (NSC 363812)." Investigational New Drugs 17(1): 63-72.


http://kedicikkopekcik.blogcu.com/3028629/

http://mol-biol4masters.org/Deoxy_Ribonucleic_Acid2-Structure.htm