Studies on new platinum complexes with *trans*-geometry

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Declaration:

I, Jun Qing Yu, state that the work in the thesis entitled "Studies on new platinum complexes with *trans*-geometry" has not been submitted for any form of credit to any other University or institution.

The source of the information contained herein is original and is solely the work of the author, except as indicated in the text.

Jun Qing Yu

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Contents

I. Abstract:

Four *trans*-planaramineplatinum(II) complexes code named: YH9, YH10, YH11 and YH12 of the form *trans*-$PtL(NH_3)Cl_2$, where L = 2-hydroxypyridine, imidazole, 3-hydroxypyridine and imidazo(1,2-α)pyridine respectively, have been prepared and characterised based on elemental analyses, IR, Raman, mass and ^1H NMR spectral measurements. The activity of the compounds against a number of human cancer cell lines, cell uptake, DNA-binding and nature of interaction with pBR322 plasimd DNA and salmon sperm DNA have been determined. There is a wide variation in the activity of the compounds depending on the nature of the planaramine ligand illustrating structure-activity relationship. YH12, i.e. the compound having imidazo(1,2-α)pyridine ligand as one of the carrier ligands in the *trans*-configuration is found to be significantly more active than cisplatin against ovarian $A2780^{cisR}$ cancer cell line corresponding with higher Pt-DNA binding. The compound is more active in the cisplatin-resistant cell line $A2780^{cisR}$ than cisplatin-responsive cell line A2780, indicating that YH12 has been able to overcome multiple mechanisms of cisplatin resistance operating in $A2780^{cisR}$ cell line. All other compounds have resistance factors less than that for cisplatin in the A2780 and $A2780^{cisR}$ cell lines. A greater prevention of BamH1 digestion with increasing concentration of the compounds indicates that as the compounds bind with nucleobases in DNA, the DNA conformation is changed sufficiently so as to prevent BamH1 digestion at the specific GG site. Gel electrophoresis results also indicate that as the compounds bind to DNA, unwinding of supercoiled form I DNA takes place to change it from the negatively supercoiled form I through relaxed circular form I to the positively supercoiled form I.

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1 Chapter One: Introduction

1.1 Cancer and treatments

1.1.1 History of Cancer

The disease of cancer dates back as far as prehistoric times when it existed on animals long before humans arrived on earth (Brothwell 1967). The earliest cancer affecting humans was documented in Egyptian mummies some 5000 years ago where evidence of osteogenic sarcoma and nasopharyngeal carcinomas were found (Shimkin 1977). Although cancer is a disease of great antiquity, it is also pre-eminently a modern disease. For example, it was estimated that in the year 1985 world wide there were 7.6 million (Parkin, Pisani *et al.* 1993) cases of cancer excluding non-melanocytic skin cancers, which would be another 2.75 million cases (Armstrong and Kricker 1995). Among these, 5 million deaths from cancer (excluding non-melanoma skin cancer) were reported (Pisani, Parkin *et al.* 1993). It was also estimated that the number of new cancer cases in the year 2000 would be about 10.6 million (excluding non-melanocytic skin cancers) (Bishop 1999). The most recent report by Shibuya *et al* showed that cancer has accounted for over 7 million deaths (13% of total mortality) in 2000. Lung cancer was the most common type of cancers all over the world, followed by cancers of stomach, liver, colon and rectum, and breast (Shibuya, Mathers *et al.* 2002).

1.1.2 Cancer definition

Cancer may be defined as an abnormal cell different from the normal one (Katzung 1987), that divides and multiples uncontrolled and has the capacity to metastasise elsewhere in the body. Uncontrolled multiplication occurs because cell production is greater than cell loss, whereas in normal adult tissues these rates are equal (Buono and NA. 1995). Normal tissues may be classified as being of three types according to the proliferative capabilities. These are (1) continually renewing tissues, such as intestine, skin, and bone-marrow, (2) conditionally renewing tissues such as liver and lung, which renew their population in response to injury, and (3) non-renewing tissues, such as nerve and muscle (Leblond 1963).

Both normal and neoplastic cells go through cell cycle process for growing and replication. This involves five steps, which are designated by the letters and subscripts G_0, G_1, S, G_2 and M. Cells in G_0 phase is in resting stage which is considered to be out of the cell cycle (Bingham 1978). G_1 is referred to as the first gap or first growth phase, which prepares the cell for the synthesis of DNA [2'deoxyribonucleic acid] by producing RNA [ribonucleic acid] and protein (Bingham 1978). The synthesis of DNA is the major event occurring during the S phase. DNA codes the genetic information necessary for the growth, repair, and reproduction of the cell (Groenwald 1987). Cells in G_2 phase are in the second growth period or second gap, during which, mitotic spindle apparatus is prepared. Actual cell division, or mitosis, occurs during the M phase. The parent cell divides into two daughter cells, each one containing the same number and kind of chromosomes as the parent cell. At the completion of the M phase, the cell will either re-enter the cell cycle at G_1 to undergo further maturation

and replication or await activation by resting in G_0. Tumours grow by a progressive, steady expansion (Ingwersen 1996).

1.1.3 Treatments for cancer

Three commonly used methods for treatment of cancer are: surgery excision, radiation treatment and chemotherapy, used alone or in combination. Surgery can cure cancer that is localised but not metastasised. Radiation is only effective to tumour that is growing slowly and it is usually used in combination with surgery. The third mode of treatment is chemotherapy with cytotoxic drugs, the goal of which is to inhibit the replication of cells (McGrew and McGrew 1985).

According to cell cycle activity, chemotherapeutic agents can be classified into two major groups: cell cycle phase-specific and cell cycle phase-nonspecific. Cell cycle phase-specific agents kill those proliferating cells that are only in a specific phase of the cell cycle including phases G_1 through M. For example, vinblastine kills cell in the M phase whereas 5-fluorouracil is lethal only to cell in S phase. Cell in G_1 phase is vulnerable to prednisone whereas cell in G_2 phase is sensitive to bleomycin. Cell cycle phase-nonspecific agents affect cells in all phases from G_0 to M and their main target is DNA. These drugs include antibiotics (dactinomycin and daunorubicin), nitrosoureas (carmustine and lomustine), miscellaneous (decarbazine and procarbazine), and alkylating agents such as busulfan, cyclophosphamide and platinum-based drugs such as cisplatin and carboplatin (Ingwersen 1996).

As an effective cell cycle phase-nonspecific agent, cisplatin [*cis*-diamminedichloroplatinum (II)] is currently one of the most widely used anticancer drugs.

1.2 The anticancer drug cisplatin

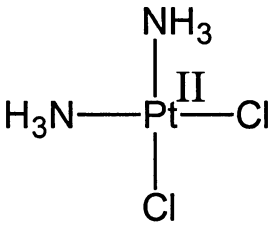
1.2.1 Historical introduction

The anticancer activity of cisplatin was accidentally discovered by Rosenberg in 1961 when he was attempting to study the effect of electric field on the growth of *E. coli.* He used platinum electrodes immersed in a solution of ammonium chloride (NH_4Cl). Under the conditions of the experiment platinum combined with NH_4^+ and Cl^- ions to form cisplatin in addition to other products. He found that cisplatin markedly inhibited cell division in bacteria (Rosenberg, VanCamp *et al.* 1965). Then the experiment of its anticancer activity was carried out in mice with sarcoma-180 and L1210 leukaemia. The results again confirmed the anticancer activity of cisplatin (Rosenberg, VanCamp *et al.* 1969). Later it was brought to the clinical trial in 1971 (Hill, Speer *et al.* 1971). The first evidence of substantial single-agent antitumour effect became available in 1974, both in testicular (Hill, Speer *et al.* 1971) and ovarian cancers. Eventually it was used as a routine treatment for cancer patients (Loehrer and Einhorn 1984). It is highly effective against testicular and ovarian cancers (success rate being over 90% in testicular cancer), and moderately effective for other cancers such as head, neck, lung, and bone tumours. (O'Dwyer, Hohnson *et al.* 1997). In addition, other antitumour drugs, such as 5-fluorouracil (Esaki, Nakano *et al.* 1992), arabinofuranosylcytosine (Swinnen, Barnes *et al.* 1989), or aphicicolin and hydroxyurea (Masuda, Tanaka *et al.* 1990) can enhance the cytotoxicity of

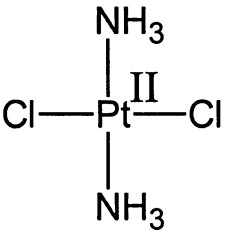
cisplatin. Also, cisplatin itself can exert synergistic effects when used together with radiotherapy (Bartelink, Begg *et al.* 1999). Although cisplatin is a highly effective anticancer drug, it has a number of side effects such as nausea, vomiting, hair loss etc. and cancer cells develop resistance to the continued use of cisplatin. The side effects of cisplatin will be considered in greater detail in sections 1.2.6 and 1.2.7.

1.2.2 The chemical structure of cisplatin

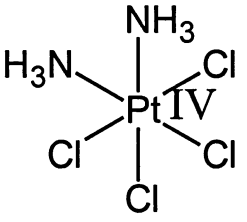
Platinum lies in the third row of transition metals in the Periodic Table and has eight electrons in the outer d sub-shell. Compared to nickel and palladium, which occupy analogous positions in the first and second transitions series, platinum atom has a greater total number of electrons, the orbitals of its outer electrons are more polarisable, and bonds that are formed from these orbitals have more covalent in character. As a result, platinum has two dominant valence states, +2 and +4, which are designated as Pt(II) and Pt(IV). It forms square planar complexes in the lower oxidation state and octahedral complexes in the higher one. Structures with two similar chemical groups (ligands) on the same side of the molecule are said to be in a *cis*-geometry while those with like groups on the opposite side are said to be in a *trans*-geometry. The basic structures of *cis*- and *trans*-platinum compounds are shown in Figure 1.2-1. Reactions can occur in Pt(II) complexes in which one or both ligands is displaced by competing nucleophile, in analogy to the reactions of alkylating agents.



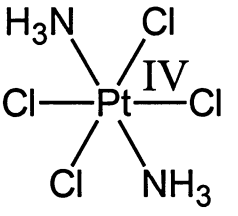
cis-dichlorodiammineplatinum (II)
(cisplatin)



trans-dichlorodiammineplatinum (II)
(tansplatin)



cis-tetrachlorodiammineplatinum(IV)



trans-tetrachlorodiammineplatinum(IV)

Figure 1.2-1: Structures of cisplatin, transplatin and their platinum(IV) analogues

Cisplatin is a neutral, square planar molecule containing two ammonia ligands and two chloride ligands in a *cis*-configuration. It was first synthesized by Michele Peyrone in 1844 and was known as *Peyrone's Chloride* (Peyrone 1845). The sterical configuration of molecule was not established until 1892 when Alfred Werner elucidated the differences between the *cis*- and *trans*-complexes (Werner 1893).

1.2.3 Transport across cell membrane

The exact mechanism by which cisplatin is taken up by the cells is not fully understood. However, it was suggested that (being a neutral molecule in the un-ionized form), the drug could be passively transported across the cell membrane without the need of any carrier (Hromas, North *et al.* 1987; Binks and Dobrota 1990; Mann, Andrews *et al.* 1991). It diffuses either directly through the lipid bilayer, or

through water-filled channels, or through both pathways at similar rates (Andrews 2000). When cisplatin is administered to the human body intravenously, it first enters into the blood plasma. The high chloride ion concentration in serum (150 mM) prevents hydrolysis of the chloride ligands of cisplatin. After diffusing into individual cells where the chloride concentration is much lower (4 mM), the neutral form of the drug hydrolyses to form aqua species. It first hydrolyses to mono- and then to di-aquadiammineplatinum(II) complexes. The Pt-OH_2 bond is much more reactive than Pt-Cl bond (Berners-Price and Appleton 2000). Thus the aqua species react more readily than cisplatin with a variety of intracellular macromolecules including DNA, RNA, and proteins; some other hydrolysis products are also formed inside the cell (eg. depending on the pH of the solution, the bound water molecules may deprotonate to give hydroxo complexes). These can be seen in Figure 1.2-2 (Gelasco and Lippard 1999). The rate of the first step in the hydrolysis of cisplatin is much less than the reaction of the mono-aqua species with cellular platinophiles including DNA. Thus the first step in hydrolysis of cisplatin is the rate-determining step in its reaction of DNA. The high reactivity of the aqua species is associated with the lability of the bound water molecules so that the aqua species are very toxic (Cleare and Hoeschele 1973).

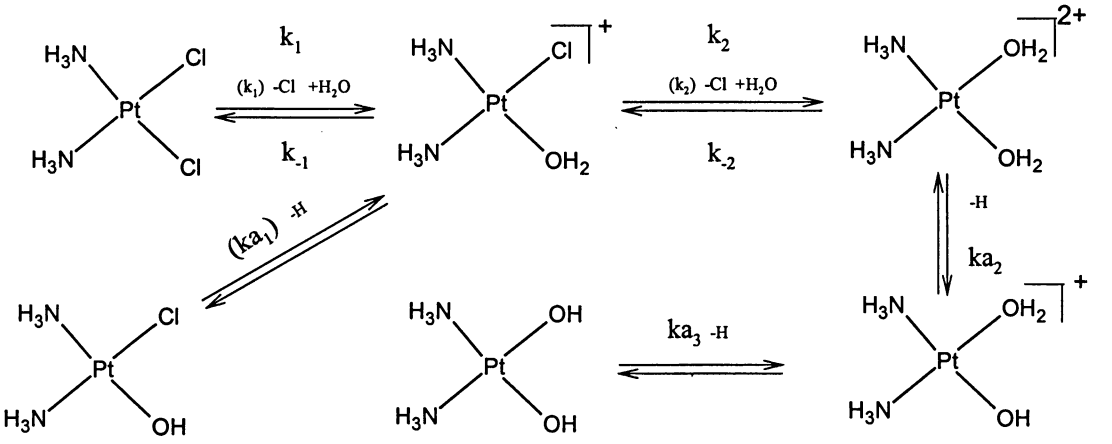


Figure 1.2-2: Kinetic behaviour of platinum-ammine compounds

Thus the term, leaving groups have been applied to the chloride ligands. In contrast, the ammino groups, as well as similar substituents that are not susceptible to displacement in physiological environments, are termed non-leaving groups or carrier ligands.

1.2.4 Mechanism of action of cisplatin

1.2.4.1 DNA as the target of cisplatin

The exact mechanism of how cisplatin interferes with cancer remains unknown. However, it is believed to be associated with its interaction DNA, which is its main intracellular target. This was first supported by Rosenberg's discovery that cisplatin inhibited cell division in bacteria (Rosenberg, VanCamp *et al.* 1965). Howle and Gale also found that cisplatin selectively inhibited DNA synthesis in Ehrlich ascites tumour cells (Howle and Gale 1970). Further more, Harder *et al* demonstrated that the depression of DNA synthesis caused by cisplatin was not due to inhibition of DNA

polymerase, but rather it was due to action of the drug on the DNA template (Harder, Smith *et al.* 1976).

1.2.4.2 Chemical structure of DNA

As stated earlier, the anticancer activity of cisplatin is believed to be associated with its binding with DNA. The word DNA stands for deoxyribonucleic acid. It is a long threadlike macromolecule that carries genetic information. DNA is a polymer of 2'-deoxyribonucleotides. Each 2'-deoxyribonucleotide has three essential components which are a heterocyclic nitrogen base, a molecule of 2'-deoxy-D-ribose and one or more phosphates joined together covalently (Stryer 1995). The four nitrogen bases (hence forth called nucleobases) that are found in DNA are the purine bases adenine and guanine and the pyrimidine bases cytosine and thymine. The variant part in a polynucleotide chain is the sequence of bases that carries the genetic information. The structures of the four nucleobases along with those of purine and pyrimidine are shown in Figure 1.2-3.

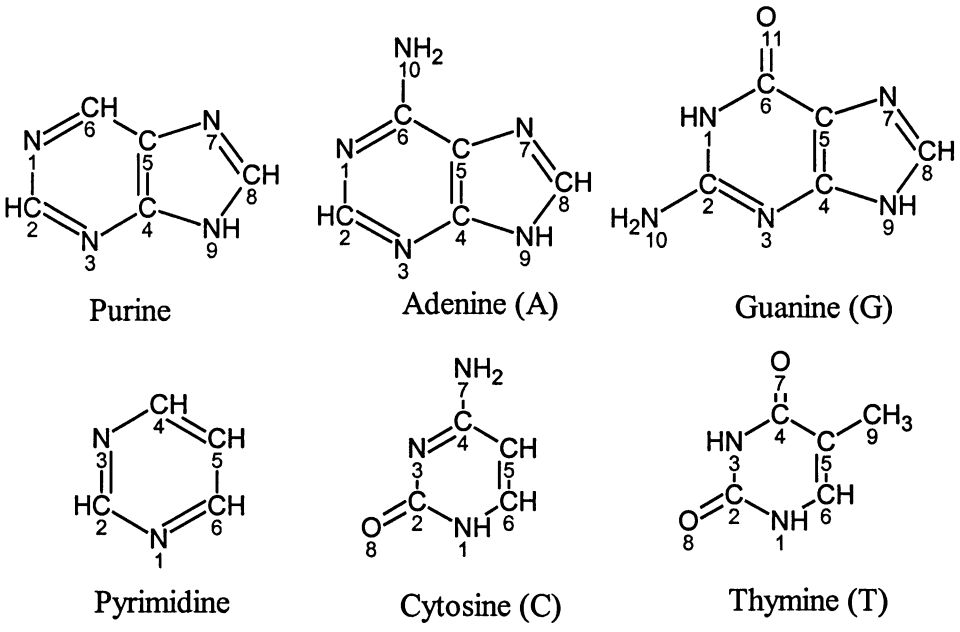


Figure 1.2-3: Nitrogen bases present in DNA

The nucleobases are bonded at C1' carbon atom of 2'-deoxy-D-ribose, which is a pentose sugar without 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 1.2-4.

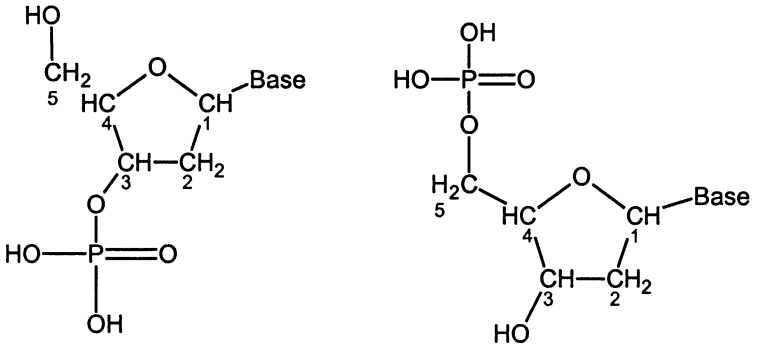


Figure 1.2-4: Structure of 2'nucleotide

When polynucleotide chain is formed, phosphate groups link to deoxyriboses at their 3' and 5' positions to form the backbone of DNA. Then two of these polynucleotide chains are joined together inside by hydrogen bonds between nucleobases. Thymine is

linked to adenine through two hydrogen bonds. Cytosine is connected with guanine through three hydrogen bonds. The pairing of the bases can be seen in Figure 1.2-5.

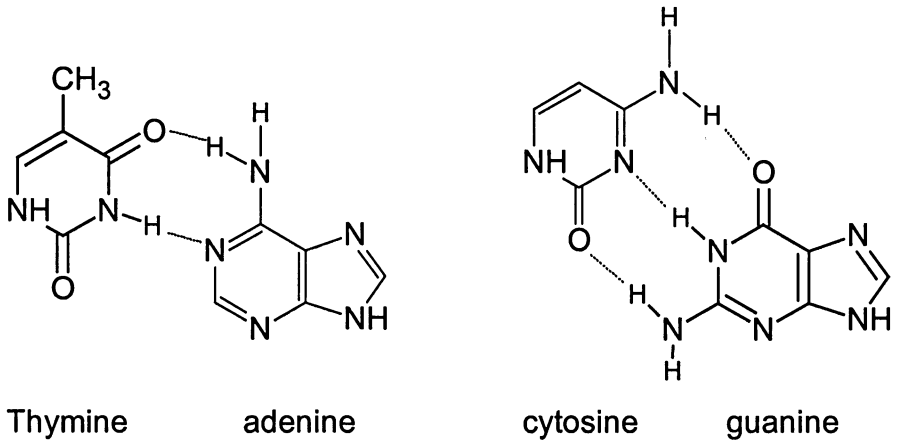


Figure 1.2-5: Pairing of bases in DNA through hydrogen bonds

1.2.4.3 Binding of cisplatin with DNA

It was stated earlier that the aqua species produced from cisplatin are much more reactive and they are the ones that combine with DNA inside the cell. The non-leaving groups eg. NH_3 (ammine) in cisplatin can provide steric hindrance and can undergo noncovalent interactions such as H-bonding with DNA (Bloemink and Reedijk 1996). Platinum(II) is a class B metal ion which binds preferentially with nitrogen and sulfur centres. The activated aqua species containing platinum(II) therefore bind to nitrogen centres in nucleobases of DNA (Mansy, Rosenberg *et al.* 1973). The binding sites in the order of decreasing occurrence are the N7 position of guanine (G) (Rahn 1984; van Hemelryck, Guittet *et al.* 1986), N7 position of adenine, N1 position of adenine, and N3 position of cytosine (Mansy, Chu *et al.* 1978). The reason for the large stability of the Pt-N7(G) bond is the large intrinsic basicity of the N7 in guanine and the additional stabilization by H-bond interaction with O6 in

guanine whereas repulsive interaction is found to occur in the case of adenine (Arpalahti 1996) (see Figure 1.2-6).

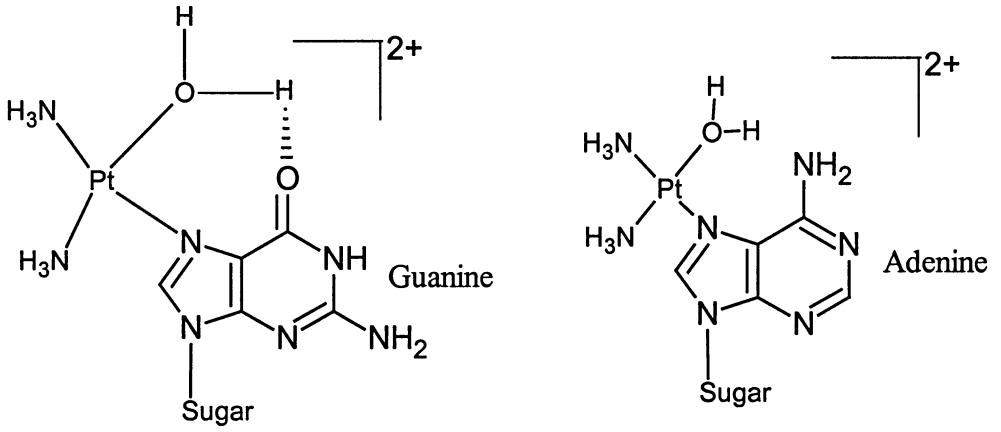


Figure 1.2-6: H-bonding formed in guanine but not in adenine.

Six major adducts were found to be formed between cisplatin and nucleobases in DNA. These include 1,2-intrastrand GG cross-links (about 65% of the DNA platination) and intrastrand 1,2-intrastrand AG cross-links (about 20%). Another 9% of the platination derives from a cross-link between two deoxyguanosines separated by a third nucleoside (GXG) (Eastman 1987). There are also interstrand cross-links formed between two deoxyguanosines occurring at less than 1% of the total platinaiton of DNA (Eastman 1985). Other adducts include DNA-glutathione cross-link (Eastman 1987) and DNA-protein cross-link (Kasparkova and Brabec 1995).

1.2.5 Cell apoptosis

It is believed that cisplatin and most other platinum-based anticancer drugs induce damage to tumours via induction of apoptosis (Boulikas and Vougiouka 2003). However, it is not exactly understood how drug-induced DNA damage results in the death of cells, particularly in patients. As stated earlier, cisplatin forms a variety of

stable covalent adducts with DNA; only stronger nucleophiles such as cyanide or thiourea can reverse the platinum-DNA bond (Filipski, Kohn *et al.* 1979). As cisplatin binds to DNA, the structure of DNA becomes significantly distorted. It was shown that when cisplatin forms 1,2-d(GpG) intrastrand cross-link, local bending of DNA strand takes place. In duplex DNA, platinum induces a roll of 26-50°C between the guanine bases involved in the cross-link, displacement of platinum from the planes of the guanine rings, and global bend of the helix axis towards the major groove. It also enhances solvent accessibility caused by hydrogen-bonding with the 5'-coordinated GC base pair. In addition, the minor groove in the vicinity of the adduct is affected by the cisplatin cross-link which unwinds the duplex and induces a widening and flattening of the minor groove (van Boom, Yang *et al.* 1996; Dunham, Dunham *et al.* 1998; Gelasco and Lippard 1998; Suo, Lippard *et al.* 1999). In some other studies it was found that binding of cisplatin to DNA resulted a decrease in melting temperature (Hermann, Houssier *et al.* 1979), shortening(Cohen, Bauer *et al.* 1979), unwiding (Maquet and Butour 1978), and even some local denaturation of DNA helix (Scovell and Capponi 1982). The platinated DNA that is distorted causes cells to arrest at either the G1, S or G2 phase of the cell cycle in an attempt to repair the damage.

In vitro studies revealed that 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links formed by cisplatin are refractory to excision repair (excision repair for the 1,2-d(GpG) adduct is 15-20 fold less efficient than the 1,3-d(GpTpG) adduct) (Moggs, Yarema *et al.* 1996). Moreover, low levels of the enzymes XPA and the ERCC1-XPF endonuclease complex, found in cisplatin sensitive testicular tumour, that recognize damaged DNA explain why tumour cells are unable to repair cisplatin induced damage (Lee, Parker *et al.* 1993). Consequently this distortion does not initiate

nucleotide excision repair. The cells eventually undergo an aberrant mitosis followed by apoptosis (Eastman 1999). Therefore cisplatin is generally considered to be cell-cycle phase non-specific drug, but it is still more toxic to dividing cells than to resting cells.

Although cisplatin can react with nucleophilic sites on other intracellular macromolecules eg. it can form adducts with protein and RNA; it has however been suggested that the levels of its binding with RNA and protein are too low to inactivate such molecules (Pascoe and Roberts 1974; Akaboshi, Kawai *et al.* 1992).

1.2.6 Side effects of cisplatin

As stated earlier, cisplatin is a widely used anticancer drug that is highly effective against testicular and ovarian cancers. However, it has a number of side effects such as nausea and vomiting, irreversible renal damage, persistent tinnitus, and occasional haematological toxicity (DeConti, Toftness *et al.* 1973; Higby, Wallace *et al.* 1973). The exact mechanisms of the toxic effects of cisplatin remain poorly understood but it is likely that the reaction of cisplatin with cellular constituents such as glutathione and metallothionein is involved (Calvert, Newell *et al.* 1995).

1.2.6.1 Toxicity to rapidly proliferating normal cell populations

It is believed that one of the causes of toxicity of cisplatin is its interference with cell replication that brings about tumour cell death. However, it is unable to target malignant cells exclusively and hence may not spare normal, rapidly dividing cells. Organs that that are constantly active responding to the human body's need (eg. bone

marrow that produces white blood cells, red blood cells, and platelets) can be affected. Cisplatin causes bone marrow depression with nadirs (the lowest point reached in the peripheral blood count after chemotherapy) occurring in 14 days and recovery in 21 days (Dorr and Fritz 1980).

1.2.6.2 Toxicity to other organ systems

Cisplatin might have a greater affinity for some organs and cause organ toxicity over time such as neurotoxicity. Cisplatin-induced neurotoxicity most commonly occurs in the form of a peripheral neuropathy, causing symptoms of numbness, tingling, and paresthesiae in the extremities, difficulty in walking. (Calvert, Newell *et al.* 1995). The exact mechanism of neurotoxicity is unknown but it was suggested that the toxic effect might be on cells in the dorsal root ganglia and its incidence seems to be related to the cumulative dose of cisplatin administered (Gregg, Molepo *et al.* 1992). Therefore, neuropathy is an increasingly more common side effect when higher doses of cisplatin are administered (Mollman, Glover *et al.* 1988). It is one of the major factors that limit the dose and number of repeated treatments of cisplatin that can be given.

Toxicity is also found in kidneys which is responsible for drug excretion (Daugaard and Abildgaard 1989). Cisplatin can cause proximal and distal renal tubular necrosis (Schilsky 1984) and produces focal degeneration of basement membrane, hyaline droplet deposits in renal tubules and decrease the ability of the renal tubules to reabsorb magnesium and calcium (Lydon 1986). The resulting hypomagnesaemia can cause convulsions (Schilsky and Anderson 1979). Simultaneous administration of

intravenous hydration, mannitol, chloride-containing vehicles and the avoidance of other nephrotoxic drugs are found to significantly reduce the incidence and severity of nephrotoxicity associated with cisplatin chemotherapy (Comis 1980; McKeage 2000). High levels of nephrotoxicity observed during drug administration almost prevented the further development of cisplatin as an anticancer drug. However, prehydration therapy with intravenous saline and controlled dosage levels of less than 120 mg/m^2 greatly reduced renal toxicity (Gelasco and Lippard 1999). The need for rigorous hydration therapy often restricts the use of cisplatin to in-patients.

Another side effect is ototoxicity which might be due to cochlear damage caused by cisplatin and the auditory sequelae including tinnitus and hearing loss (Moroso and Blair 1983). Tinnitus induced by cisplatin is typically high-pitched, intermittent, and not particularly bothersome to most patients (McKeage 2000). In addition, the drug produces pronounced nausea and vomiting due to the release of serotonin from enterochromaffin cells in the gut wall (Cubeddu, Hoffmann *et al.* 1990), and may make patients unable to comply with their cancer therapy regimen as prescribed. Ondansetron combined with dexamethasone is the most effective and least ototoxic antiemetic regimen currently available for the prevention of nausea and vomiting associated with cisplatin (Del Favero, Roila *et al.* 1993). Other side effects include retinal toxicity (Wilding, Caruso *et al.* 1985), various vascular disorders such as Raynaud's phenomenon and angina pectoris, ischaemia of the toes (Aass, Kaasa *et al.* 1990).

1.2.7 Resistance to cisplatin

Cisplatin has a limited spectrum of activity. This is because some cancer cells have intrinsic resistance to cisplatin and some others may develop resistance due to continued use (acquired resistance). The mechanisms of resistance may be multi-factorial including: (1) mechanisms that limit the formation of cytotoxic platinum-DNA adducts and (2) mechanisms that operate down-stream of the drug/target interaction to promote cell survival. Modulation of these factors might overcome intrinsic and acquired resistance of tumours, thereby expanding the therapeutic potential of cisplatin.

1.2.7.1 Decreased accumulation

Reduced intracellular accumulation of cisplatin is frequently observed in cisplatin-resistant cell lines (Andrews and Howell 1990; Parker, Eastman *et al.* 1991; Dempke, Shellard *et al.* 1992). The exact mechanism by which cisplatin is taken up by the cells is not fully understood. It has been suggested that cisplatin enters the cells by passive diffusion (Hromas, Andrews *et al.* 1987; Binks and Dobrota 1990; Andrews, Mann *et al.* 1991). However, in the study of cisplatin resistant cells it was found that the sodium-potassium ATPase inhibitor ouabain inhibits drug uptake, suggesting that cisplatin uptake is mediated by cell membrane proteins (Andrews, Velury *et al.* 1988). Some other observations on carrier-mediated transport supported the idea that uptake is partially energy-dependent (Dornish, Pettersen *et al.* 1984; Dornish and Pettersen 1985; Dornish, Melvik *et al.* 1986; Dornish and Pettersen 1989; Dornish, Pettersen *et al.* 1989). It was proposed by Gately and Howell that approximately one-half of drug

uptake is by passive diffusion and the other half by facilitated diffusion through an as yet unidentified pump (Gately and Howell 1993).

In addition, increased efflux of the drug from cisplatin-resistant cells had also been observed (Bergmann 1990; Fujii, Mutoh *et al.* 1994). Some studies suggested that a 200 kDa protein (Kawai, Kamatani *et al.* 1990) and ATP-dependent glutathione S-conjugate export pump (Ishikawa 1992) might be involved in reducing intracellular cisplatin concentration in cisplatin-resistant cells.

1.2.7.2 Inactivation of cisplatin

Intracellular inactivation of platinum drugs may occur prior to DNA binding. It is often found that the levels of glutathione (GSH) are high in cisplatin-resistant cells (Hosking, Whelan *et al.* 1990; Mistry, Kelland *et al.* 1991; Godwin, Meister *et al.* 1992). DL-buthionine-S,R-sulfoximine (BSO) has been used to inhibit GSH synthetase that reduces GSH levels and therefore increases cisplatin sensitivity in some cancer cells (Hromas, Andrews *et al.* 1987; Hamilton, Lai *et al.* 1989; Chen and Zeller 1990). Cisplatin can be covalently linked to GSH to form a GS-Pt complex that can be transported out of the cell by the ATP-dependent pump (Ishikawa and Ali-Osman 1993). In vitro studies showed that conjugation with GSH quenched platinum-DNA mono adducts, preventing them from being converted to potentially cytotoxic crosslinks, thereby reducing the cytotoxic potential of the adducts (Eastman 1987; Ishikawa and Ali-Osman 1993; Mistry, Loh *et al.* 1993).

Cisplatin can also bind to metallothioneins (MT's) as a result of which its anticancer activity may be affected. The MT's are a family of cysteine rich proteins involved in Zn^{2+} homeostasis and in the detoxification of heavy metals such as cadmium. Cisplatin was found to bind to metallothionein in a ratio of one molecule of protein to 10 molecules of cisplatin (Pattanaik, Bachowski *et al.* 1992). Modulating MT levels in some cases can alter cisplatin sensitivity (Kelley, Basu *et al.* 1988; Kondo, Woo *et al.* 1995)

1.2.7.3 Increased DNA repair

Damaged DNA lesions can be excised as a small, single-stranded oligonucleotide fragment, and new DNA is synthesized to fill the resulting gap by the process of nucleotide excision repair (NER) and this function is found throughout the natural world in organisms ranging from mycoplasma to mammals (Lehmann 1995). Mammalian NER process requires the factors XPA, RPA, XPB, XPD, TFIIH, XPG, and ERCC1-XPF. XPA containing zinc-finger domain that recognizes and binds to the damaged part of DNA in association with the single-strand-binding protein RPA. TFIIH can be recruited by the XPA-RPA complex and performs its local DNA opening function around a site of damage by the subunits XPB and XPD, which have helicase activities. XPG and ERCC1-XPF are both structure-specific nucleases. The first one can cleave the opened up damaged DNA site on the strand that leads off from the junction in the 3'-5' direction while the other one can cut on the strand leading off in the 5'-3' direction. The incision part of DNA then initiates DNA repair synthesis reaction by filling the gap with DNA polymerase ε (POL ε) together with accessory proteins replication factor C (PFC) and proliferating-cell nuclear antigen (PCNA).

The process is completed by joining the new DNA to the old with a DNA ligase. The following figure illustrates the proposed steps of NER associated with cisplatin lesion.

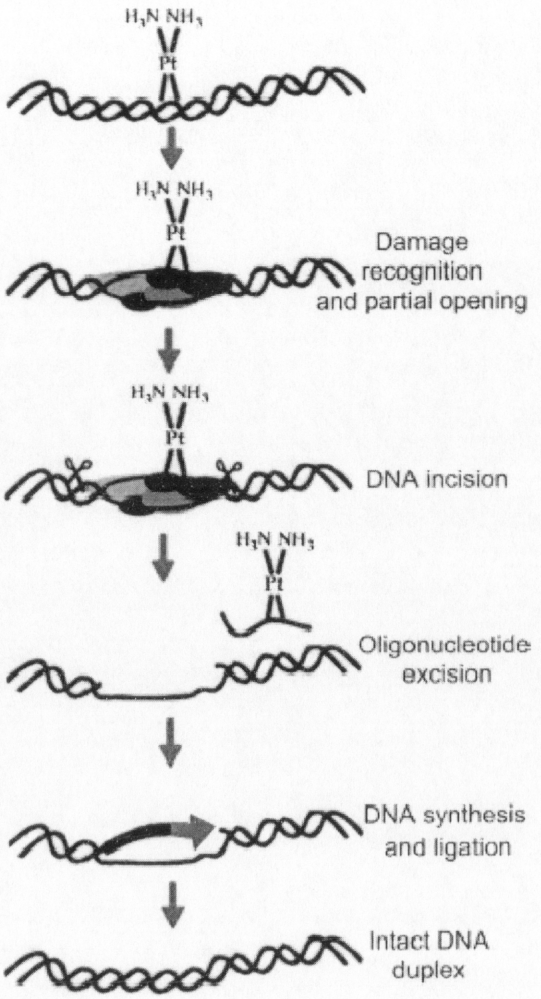


Figure 1.2-7: Scheme illustrating the proposed steps of NER associated with cisplatin lesion (Brabec and Kasparkova 2002)

The capacity to rapidly and efficiently repair DNA damage clearly plays a role in determining a tumour cell's sensitivity to platinum drugs. An elevated level of RNA for ERRCC1 and XPA were detected in tumours of ovarian cancer patients who were clinically resistant to cisplatin chemotherapy (Dabholkar, Bostick-Bruton *et al.* 1992; Dabholkar, Christian *et al.* 1994). Other evidence was also found in the report that gene complementation with XPA and XPD in NER-deficient cells [xeroderma

pigmentosum group A (XP-A), XP-D, XP-F, XP-G, Cockayne syndrome group A (CS-A), and CS-B] increases resistance to cisplatin (Furuta, Ueda *et al.* 2002).

1.2.7.4 Increased DNA damage tolerance

Cell apoptosis might also be caused by mismatch repair (MMR) after the formation of cisplatin adducts. MMR is a post-replicative DNA repair process that corrects single-base mismatches and small mismatched loops in DNA by removing such errors from the newly synthesized strand (Parsons, Li *et al.* 1993). However, it is believed that MMR is not as important as NER for the removal of cisplatin adducts from DNA but serve as a detector of adducts. Weak DNA errors can be easily detected by the MMR system and manage to repair this damage. However, if such damage resulting from cisplatin reaches a certain threshold, the cellular repair systems are overwhelmed with too much damage that would lead the DNA-damaged cell into programmed cell death. The presence of 1,2-d(GpG) and 1,2-d(ApG) intrastrand adducts as unrepaired lesion in DNA may be recognized by the MMR system and triggers apoptosis to cause cell death by as yet unknown mechanisms (Fink, Aebi *et al.* 1998).

Loss of MMR may result in the survival of a cell with mutagenic adducts in its DNA that would have otherwise died and thus increase cellular tolerance for cisplatin-DNA adducts. Such mutagenic effects of cisplatin were demonstrated in the studies by Lin *et al* that increasing the concentration of cisplatin produced progressively higher mutant frequencies in MMR-deficient cells than in MMR-proficient cells (Ali, Whitmire *et al.* 1999). Further more complementary studies revealed that cancer cell lines with inactivation of MMR, such as the colorectal cancer cell HCT116 deficient in hMLH1, the colorectal cancer cell line DLD1 deficient in hMSH6, and the

endometrial cancer cell line HEC59 deficient in hMSH2, were up to four-fold more resistant to cisplatin than the corresponding sublines that have not been corrected for the repair defect by chromosomal transfer (Aebi, Kurdi-Haidar *et al.* 1996; Fink, Aebi *et al.* 1998; Vaisman, Varchenko *et al.* 1998). Studies with the isolated cisplatin resistant cell lines after exposure to the drug in vitro frequently found the mutator phenotype associated with MMR defects with mutated in the hMLH1 genes. It is unclear, however, whether cisplatin treatment causes inactivation of the mismatch repair genes, or it selects for cells that are already deficient in mismatch repair (Aebi, Kurdi-Haidar *et al.* 1996; Anthoney, McIlwrath *et al.* 1996; Drummond, Anthoney *et al.* 1996; Brown, Hirst *et al.* 1997).

The following figure gives a pictorial representation of various processes associated with cisplatin resistance.

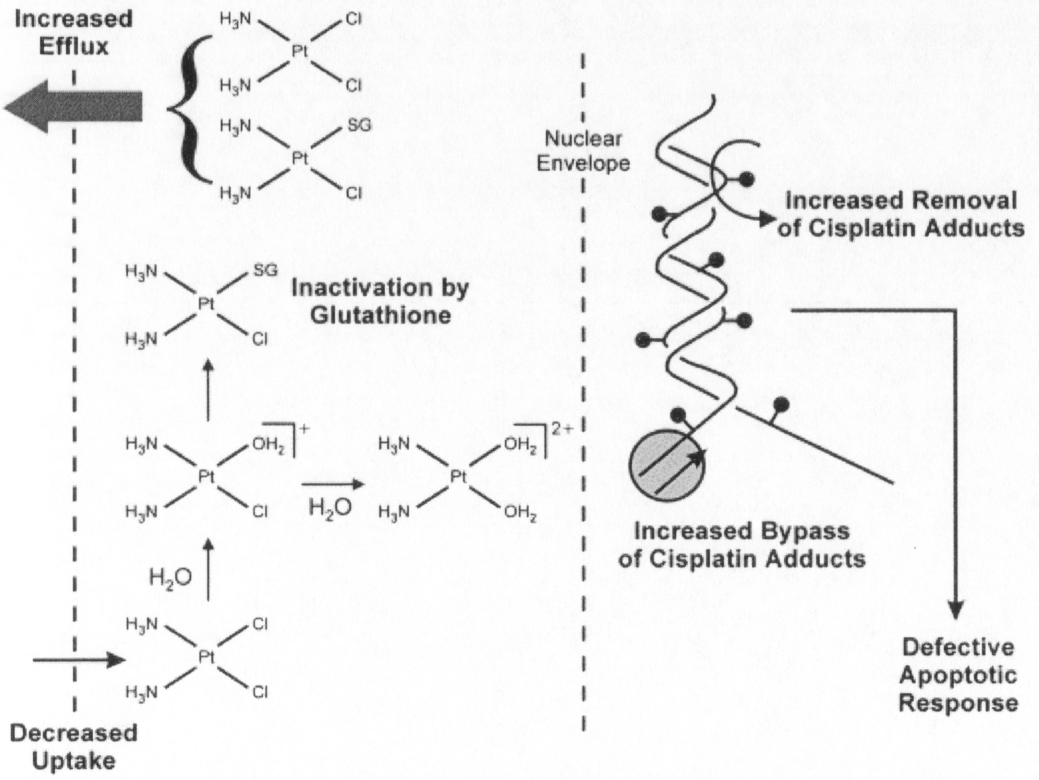


Figure 1.2-8: Pictorial representation of proposed processes associated with cisplatin resistance (Kartalou and Essigmann 2001)

1.2.8 Structure-Activity Relationships (SAR)

Due to the severe side effects of cisplatin and its intrinsic resistance against some more common tumour types, a great deal of effort has been expended into structural modifications of cisplatin. From an intensive study of a large number of platinum complexes, the classical structure-activity relationships (SAR) have been formulated (Connors, Cleare *et al.* 1979). These may be stated as follows.

1. The complexes should be electrically neutral so that they are able to cross cell membrane by passive diffusion (although the active forms may be charged and are produced due to ligand exchange inside the cell).
2. The complexes should have a pair of leaving groups with intermediate binding strength (such as chloride, bromide, citrate, oxalate, and other carboxylic acid residues) in a *cis* geometry.
3. The complexes should contain tightly bound ligands that are neutral and inert to substitution such as amine systems. The amine ligands should possess at least one NH moiety necessary for hydrogen bonding with DNA (Bloemink and Reedijk 1996). The tightly bound ligands are often termed as carrier ligands.

It may be noted that an increasing number of platinum compounds are being reported in the literature which appear to violate the above structure activity relationships (Coluccia, Nassi *et al.* 1993; Goddard, Valenti *et al.* 1994; Natile and Couluccia 1999). It will be seen later that the platinum compounds in the present project also violate the classical structure activity relationships.

1.2.8.1 Cisplatin Analogues

As stated earlier, in order to combat the numerous side effects of cisplatin and to widen the spectrum of activity, thousands of cisplatin analogues have been prepared by varying the nature of the leaving groups and the carrier ligands. In the development of compounds which are now known as second generation drugs, the aim was to reduce toxic side effects of the compounds whereas in the development of the third generation drugs, the aim was mainly to widen the spectrum of activity. When the nature of the leaving groups was modified it was possible to reduce toxic side effects but no significant change in the spectrum of activity could be achieved (Cleare and Hoeschele 1973). For example, carboplatin which has different leaving groups than cisplatin (1,1-cyclobutanedicarboxylate in carboplatin and chlorides in cisplatin) but the same carrier ligands as those for cisplatin, is found to be less toxic than cisplatin. A more detailed discussion on carboplatin is given in section 1.2.8.1.1. When the nature of the carrier ligands was changed, it was possible to some extent widen the spectrum of activity. For example oxaliplatin which has 1,2-diaminocyclohexane as the carrier ligand as against ammonia ligands in cisplatin, is found to be active against colorectal cancer whereas cisplatin is not (Cvitkovic and Bekradda 1999). More information about oxaliplatin will be given later in the chapter. As stated earlier, an example of a second generation drug is carboplatin. Another example is JM216 (which is also known as a third generation drug) (Gelasco and Lippard 1999). Some examples of third generation drugs are oxaliplatin, ZD0473 and Kplatin.

1.2.8.1.1 Second generation drugs

Carboplatin

As stated earlier, the aim in the development of second-generation drug has been to reduce the side effects of cisplatin. This was achieved to a great extent by changing the nature of the leaving groups. Cisplatin is a neutral molecule that can pass through the cell membrane by passive diffusion and is activated inside the cell when the chloride ligands are replaced by water molecules. The positive ion produced is highly electrophilic and capable of binding with DNA (Highley and Calvert 2000). If the leaving groups were more reactive than chloride ligands (eg. nitrate) a more toxic compound would result. On the other hand, if the leaving groups are slightly less reactive than chloride ligands, a less toxic compound may be produced. An example of such a compound is carboplatin in which the chloride ligands of cisplatin are replaced by 1,1-cyclobutanedicarboxylate group while the carrier ligands remain the same as that in cisplatin. Thus carboplatin is *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (van der Vijgh 1991). The structure of carboplatin is shown in the Figure 1.2-9.

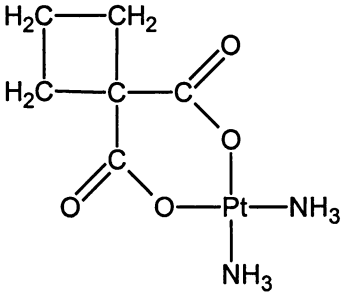


Figure 1.2-9: Structure of Carboplatin

Carboplatin is associated with little or no renal toxicity so there is no need for hydration (Highley and Calvert 2000), allowing its use in the out-patient setting. Peripheral neurotoxicity is rare with carboplatin (Harrap 1985). Nausea and vomiting

are reduced compared to cisplatin. Hearing loss and tinnitus are also lower. It causes less hypomagnesemia and hypocalcemia than cisplatin. In phase II study, carboplatin was found to have a more favourable therapeutic index than cisplatin, particularly with regard to non-hematologic toxicities (Belani and Group 2002). However carboplatin causes more myelosuppression, particularly thrombocytopenia, which is dose limiting and usually manifests about 14 d after treatment (Calvert, Newell *et al.* 1989). Like cisplatin, carboplatin also can cause allergic reactions, most often in the form of facial flushing, erythema, and pruritus which usually occur during a rechallenge with platinum compounds (sometimes years after the initial exposure) (Highley and Calvert 2000). There is a significantly higher chance of recovery of gonadal function in carboplatin-treated patients than in cisplatin-treated patients. Because of lower toxicity profile of carboplatin, it is now routinely used in cancer treatment (Ali, Whitmire *et al.* 1999).

However, in vitro studies have shown that carboplatin and cisplatin exert very similar cytotoxic effects and phase III clinical trials have demonstrated their equivalency in the treatment of ovarian cancer (Alberts, Green *et al.* 1992). Carboplatin has an elimination half-life of approximately 2.5 to 6 h. Carboplatin crosses the blood-brain barrier. Carboplatin is a safe and effective first-line of treatment for women with advanced cancer (Sandercock, Parmar *et al.* 2002).

Nedaplatin

Nedaplatin is another second generation platinum drug which is also known as 254-S. Nedaplatin is *cis*-diammineglycolatoplatinum(II) (Figure 1.2-10). It was developed by the Shionogi Pharmaceutical Company of Osaka, Japan. The compound showed greater antitumour activity than cisplatin in preclinical studies involving rodent

models (P388 leukaemia, B16 melanoma, Lewis lung carcinoma) and human xenografts (MX-1 breast carcinoma, Daudi lymphoma) (Shiratori, Kasai *et al.* 1985). However, nedaplatin was found to be cross-resistant with cisplatin in the L1210/CDDP leukaemia model (Suzumura, Kato *et al.* 1989). In phase II studies, the compound has been found to be active against non-small cell lung cancer, small cell lung cancer, testicular cancer, transitional-cell carcinoma of the urinary tract, hormone-refractory prostate cancer, cancers of head and neck, ovary and cervix (Fukuda, Shinkai *et al.* 1990; Ota, Miyake *et al.* 1991; Akaza, Togashi *et al.* 1992). It has reduced nephrotoxicity, gastrointestinal toxicity but more thrombocytopaenia (Shiratori, Kasai *et al.* 1985; Sasaki, Amano *et al.* 1991).

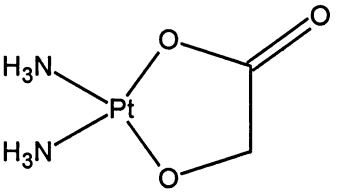


Figure 1.2-10: Structure of Nedaplatin

JM 216

JM 216 is a new platinum compound that can be given orally rather than by intravenous injection. Oral administration is more convenient for patients, for which the cost of delivery of treatment is also lower. Chemically, it is *cis,trans,cis*-$[PtCl_2(OAc)_2NH_3(c\text{-}C_6H_{11}NH_2)]$ ie bis(acetato)amminedichloro(cyclohexylamine)platinum(IV) (Figure 1.2-11). It should be noted that unlike cisplatin and carboplatin which are platinum(II) complexes, JM216 is a platinum(IV) complex. The higher oxidation state for platinum makes JM216 more stable, which could be important as it moves through the contents of the gastrointestinal tract (McKeage 2000). The compound is more lipophilic than

cisplatin and carboplatin, a feature that may aid its absorption through the membranes of the gastrointestinal wall. Octanol-water partition coefficient for JM216 was reported to be 0.1 (Kelland 2000). JM216 was one of the first 30 platinum(IV) mixed amine dicarboxylates to be evaluated against mice bearing ADJ/PC6 tumour which had been widely used in defining the structure-activity relationships for platinum based anticancer drugs (Connors, Cleare *et al.* 1979). A clear gain in therapeutic index (TI) was observed for the compound for oral over ip dosing. However, JM216 like other dicarboxylate compounds were found to be less active against SKOV-3 (an ovarian tumour that does not respond to cisplatin) (Kelland 2000). The toxicity of JM216 includes thrombocytopenia, neutropenia, nausea, vomiting, diarrhoea, and constipation. Toxicity to hearing, kidney, and peripheral nerves may sometimes occur (McKeage 2000). JM216 is also classified as a third generation drug. Overall JM216 represents a good orally active anticancer drug with tumour activity at least comparable to iv carboplatin in most models, low emetic properties, and favourable physicochemical properties. The solubility of JM216 is in water: about 0.3 mg/mL and in saline: about 0.4 mg/mL. The compound has good stability in acid (half-life in 1 M HCl is of the order of several hours). Currently JM216 is undergoing phase III clinical trials.

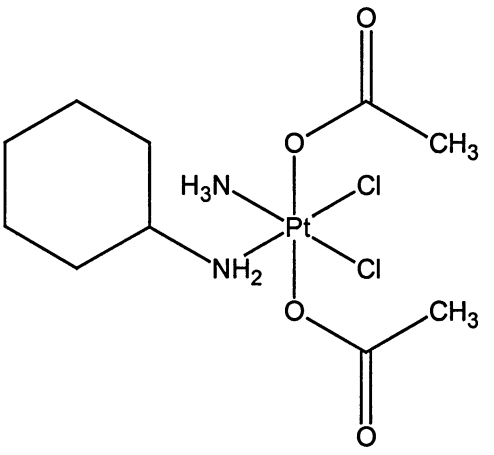


Figure 1.2-11: Structure of JM216

1.2.8.1.2 Third generation drugs

As stated earlier, intrinsic and acquired resistance limit the response of cisplatin and carboplatin therapy. Thus a great deal of effort has been expended to identify platinum compounds which lack cross-resistance with cisplatin/carboplatin. Whereas modulation of leaving groups had made possible to alter the toxicity profile of compounds, when the carrier ligands were altered it was possible to cause a limited change in the spectrum of activity. For example, oxaliplatin is found to be active against colorectal cancer whereas cisplatin is not.

Oxaliplatin

Oxaliplatin is (*trans*-R,R-1,2-diaminocyclohexane)oxalatoplatinum(II). Its structure is shown in the figure 1.2-12. It can be seen that the DACH ligand (1,2-diaminocyclohexane) in oxaliplatin is in the form of *trans-R,R-* which is different from its other two isomers *cis-R,S*-DACH and *trans-S,S*-DACH. In vitro and in vivo studies showed that the compounds containing *trans-R,R*-DACH were the most active ones. Oxaliplatin was first synthesised in 1978 (Kidani, Noji *et al.* 1978) and was the first platinum drug to show clinical activity in murine leukaemia that are resistant to

cisplatin (Kraker and Moore 1988). The DNA adducts of oxaliplatin are predominantly intra-strand cross-links (Jennerwein, Eastman *et al.* 1989; Saris, van de Vaart *et al.* 1996) and evidence for non-cross-resistance has been obtained in vivo (Tashiro, Kawada *et al.* 1989). As stated earlier, oxaliplatin has shown activity in colorectal cancer (Extra, Espie *et al.* 1990). Also, the compound has become a treatment option after failure of cisplatin or carboplatin therapy both in relapsed or refractory ovarian cancer (Chollet, Bensmaine *et al.* 1996; Piccart, Green *et al.* 2000) and in refractory germ-cell tumours (Kollmannsberger, Mayer *et al.* 2001). Oxaliplatin lacks nephrotoxicity and ototoxicity. However, its dose-limiting toxicity is myelosuppression affecting three blood cell lineages (Kelland 1993). It has gastrointestinal side effects similar to cisplatin (Mathe, Kidani *et al.* 1986).

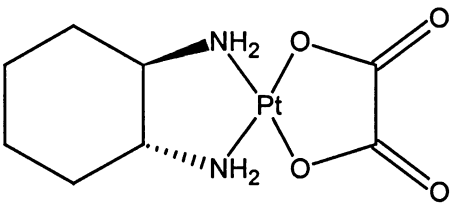


Figure 1.2-12: Structure of Oxaliplatin

The DNA adducts of oxaliplatin are believed to be repaired mainly by nucleotide excision repair mechanism. The drug is detoxified by glutathione-related enzymes (Arnould, Hennebelle *et al.* 2003). Oxaliplatin has shown a wide antitumour effect both in vitro and in vivo and lack cross-resistance with cisplatin and carboplatin. In combination with fluorouracil, the drug has been approved in Europe, Asia, and Latin America for treatment of metastatic colorectal cancer.

ZD0473 (formerly known as JM473 or AMD473) is one of the third generation drugs that can be administered orally. Chemically it is *cis*-amminedichloro(2-methylpyridine)platinum(II). The structure of the compound is shown in the Figure 1.2-13. The rationale for the synthesis of this drug was to find a platinum compound that would have reduced susceptibility to inactivation by elevated levels of intracellular thiols. As mentioned earlier that drug inactivation by ctyoplasmic thiol-containing species, principally glutathione (GHS) and metallothionein (MT), contribute to resistance of platinum drug. It was suggested that the introduction of sterically hindered group would cause ligand exchange at the platinum centre to follow dissociative rather associative pathway thereby reducing the susceptibility of platinum to sulfur containing platinophiles (Holford, Raynaud *et al.* 1998; Holford, Sharp *et al.* 1998). It may be noted that a common mechanism of resistance of platinum-based anticancer drugs is deactivation of drugs due to their binding with sulfur-containing platinophiles.

Holford *et al* found that ZD0473 was less reactive than cisplatin to the thiol-containing molecules: methionine and GSH and that the rate of aquation for the compound was much lower than that for cisplatin (Holford, Raynaud *et al.* 1998; Holford, Sharp *et al.* 1998). It showed in vitro antitumour activity against acquired cisplatin-resistant cell lines: $41M^{cisR}$, $CH1^{cisR}$ and $A2780^{cisR}$ and marked in vivo activity against a variety of murine (ADJ/PC6 plasmacytoma, L1210 leukaemia) and human ovarian carcinoma xenograft models, including several that possessed acquired resistance to cisplatin ($ADJ/PC6^{cisR}$, $L1210^{cisR}$ and $CH1^{cisR}$) and HX110 that is resistant to carboplatin) (Raynaud, Boxall *et al.* 1997). In phase II studies, the drug

gave a moderate response in platinum-pretreated patients with refractory or relapsed ovarian (Gore, Atkinson *et al.* 2001) and small cell lung cancer (Bonomi, MOdiano *et al.* 2001). ZD0473 had no signs of nephrotoxicity and neurotoxicity but myelosuppression (predominantly thrombocytopaenia) was found to be its dose-limiting toxicity with maximum tolerated dose (MTD) identified as 150 mg/m^2 (Trigo, Beale *et al.* 1999; Stevenson, Blair *et al.* 2001). It should be however noted the results of clinical studies did not fully support the idea that the drug should be much superior to carboplatin in overcoming thiol-mediated toxicity, a conclusion that could be drawn from pre-clinical studies (Baird and Kaye 2003). It appears that there is always likely to be uncertainty in drawing conclusions from in vitro models especially in the case of platinum-refractory tumours, where multiple mechanisms of drug resistance are likely to operate at the same time.

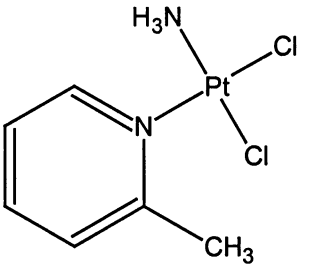


Figure 1.2-13: Structure of ZD0473

The following figure summarises the development of a number of platinum-based tumour active compounds as a result of a cooperative interaction between Institute of Cancer Research (ICR) in the U.K. and Johnson Matthey (JM).

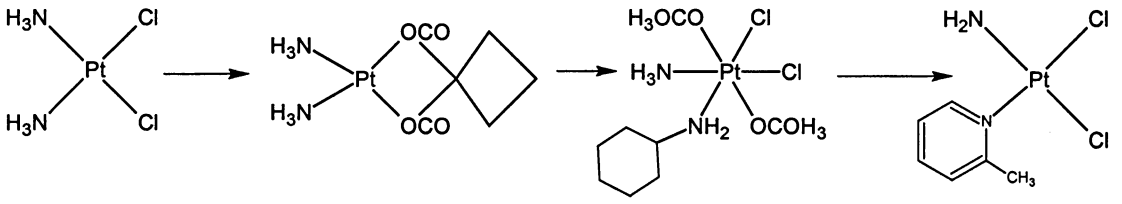


Figure 1.2-14: Summary of the platinum drug discovery program

From the above review the following points can be made:

(1) It has been possible to alter the toxicity profile of platinum drugs by modulating the nature of the leaving groups.

(2) When the nature of the carrier ligands was modulated, it has been possible to cause a limited change in the spectrum of activity and also to alter the toxicity profile.

(3) Proper choice of leaving groups and carrier ligands have produced compounds that can be taken orally rather by intravenous injection.

But since all cisplatin analogues form similar adducts with DNA that often translate into a similar spectrum of activity, we need to develop compounds with structures very different from that of cisplatin. The compounds will interact with DNA differently that is expected to translate into a different of activity (O'Dwyer, Stevenson *et al.* 1999). Tumour active *trans*-planaramineplatinum(II) complexes constitute one such class of compounds. The aim of the present study is in fact to design and study new tumour active *trans*-planaramineplatinum(II) complexes. In the section we shall review the current literature on *trans*-platinum complexes.

1.3 Tumour active platinum compounds with *trans*-geometry

The reason why transplatin is toxic rather than tumour active is believed to be due to its higher reactivity so that the compound is deactivated before it has a chance to interact with DNA. This means that the *trans*-geometry could be activated for anticancer activity if the reactivity of *trans*-platinum compounds could be slowed down by the introduction of sterically hindered ligands (Farrell, Ha *et al.* 1989; Farrell, Kelland *et al.* 1992). In general, it was found that the cytotoxicity of the *trans* complexes containing sterically hindered ligands was one-order of magnitude greater than that of transplatin and that the *trans* complexes were at least as cytotoxic as the corresponding *cis*-isomers (Farrell, Kelland *et al.* 1992). In vitro studies showed that many *trans* Pt(IV) complexes had activity comparable to that of cisplatin and were able to overcome acquired resistance associated with either reduced uptake or increased removal of platinum-DNA adduct (Kelland, Barnard *et al.* 1995). Some of the *trans* Pt(IV) complexes were found to display significant in vivo activity against murine ADJ/PC6 plasmacytoma model, most of which had axial hydroxide ligands. Before we consider in detail tumour active platinum complexes with *trans*-geometry, it is appropriate that we should have a more detailed look at transplatin.

1.3.1 Transplatin

Transplatin is *trans*-diamminedichloroplatinum(II) (see Figure 1.3-1). It is the geometrical isomer of cisplatin. As stated earlier, transplatin was found to be toxic rather than tumour active. It was found to be much less efficient than cisplatin in

inhibiting DNA replication and transcription (Johnson, Hoeschele *et al.* 1980; Mello, Lippard *et al.* 1995). Transplatin-DNA adducts are more rapidly repaired than cisplatin-DNA adducts, consistent with the observation that high-mobility group protein HMG1 is unable to recognize transplatin adducts (Ciccarelli, Solomon *et al.* 1985; Heiger-Bernays, Essigmann *et al.* 1990; Pil and Lippard 1992). The most obvious difference between cisplatin and transplatin in their binding with DNA is that unlike cisplatin, transplatin is unable to produce 1,2-intrastrand crosslinks (Kasparkova and Brabec 1995). Instead it forms interstrand crosslinks between complementary G and C residues (Brabec and Leng 1993). The first binding step both results in the formation of monofunctional adducts in the case of both cisplatin and transplatin. Whereas the cisplatin monofunctional adducts close to form mainly 1,2-intrastrand bifunctional GG and AG adducts, monofunctional adducts of transplatin and other *trans*-platinum complexes close mainly to form interstrand GC adducts. However, as stated earlier, the lack of anticancer activity of transplatin is believed to be associated to the higher reactivity of transplatin. Transplatin is kinetically more reactive than cisplatin and hence more susceptible to inactivation by cellular platinophiles such as glutathione. As a result the delivery of the compound to the tumour side is prevented (Farrell 1996; Wong and Giandomenico 1999; Natile and Coluccia 2001).

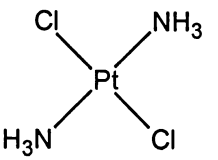


Figure 1.3-1: Structure of Transplatin

1.3.2 Exceptions to the SAR

Recently, several exceptions to the SAR (considered earlier) have been reported. It was found that *trans*-platinum complexes with NH_3 carrier ligands replaced by more sterically hindered groups could be tumour active. Ligand substitution in square-planar complexes usually takes place through an associative mechanism in which a five-coordinate trigonal bipyramidal transition state is formed (Natile and Coluccia 2001). As the ammonia ligands are replaced by the more sterically demanding carrier ligands, axial access to platinum is reduced and therefore the formation of the five-coordinate intermediate is also reduced. The increase in stability retards the formation of undesirable intermediates and hence increases the delivery of the drug to the binding sites in DNA (Farrell, Ha *et al.* 1989).

1.3.3 Mechanism of action of *trans*-platinum compounds

The preparation of tumour active platinum compounds with *trans*-geometry broke the paradigm of classical structure activity relationships (Coluccia, Nassi *et al.* 1993; Goddard, Valenti *et al.* 1994). Bulky amine ligands instead of the simple NH_3 bonded to platinum in *trans* geometry can slow down the reactivity of the *trans* geometry so that the kinetics of its reaction become similar to that of cisplatin (Farrell 1996). This was first reported by Farrell *et al* in 1989 (Farrell, Ha *et al.* 1989). One such class of compounds are the *trans*-platinum complexes with planar amine ligands eg *trans*-$[PtCl_2(pyridine)_2]$.

A possible mechanism for the higher cytotoxicity of the *trans*-platinum complexes with planar amine ligands may be due to a higher cell uptake as compared to that for

the *cis* isomer (Farrell, Kelland *et al.* 1992). A greater inhibition of DNA synthesis as compared to cisplatin and transplatin was also observed for the *trans*-planaramineplatinum(II) complexes. The complexes can form monofunctional adducts, interstrand crosslinks and protein-associated strand breaks with the proportions being dependent on the structure of the compounds (Farrell, Appleton *et al.* 1995). So far, the mechanism of protein-associated strand breaks is unknown but some studies suggest the involvement of topoisomerase inhibitors (Farrell 1996).

1.3.4 Tumour active *trans*-platinum complexes

Four different structural types of *trans*-platinum complexes have been reported to be anticancer active:

1) *Trans*-planaramineplatinum(II) complexes having the general formula: *trans*-Pt(II)Cl_2(L)(L') where either L or L' or both stand for planaramines. Three distinct classes of complexes were examined (see Figure 1.3-2): (a) L = L' = pyridine, N-methylimidazole, thiazole, (b) L = quinoline and L' = RR'SO (R = Me, R' = Me, Bz, or Ph), and (c) L = NH_3 and L' = quinoline or thiazole (Van Beusichem and Farrell 1992)(see Figure 1.3-2). It was reported that in general the cytotoxicity of *trans*-planaramineplatinum(II) complexes was about one order of magnitude greater than that of transplatin and at least equivalent to that of their *cis* isomers and cisplatin (Farrell, Kelland *et al.* 1992). The compounds were found to be active against cisplatin-resistant cell line L1210/DDP (Farrell, Ha *et al.* 1989; Farrell, Kelland *et al.* 1992; Goddard, Valenti *et al.* 1994). Studies on cellular and molecular pharmacology showed that there was a higher uptake of *trans*-$PtCl_2$(pyridine)$_2$ than cisplatin in L1210/0 cells(Farrell, Kelland *et al.* 1992). Also *trans*-[$PtCl_2(NH_3)$(quinoline)] was

found to accumulate in CHO cells more efficiently than cisplatin (Skov, Adomat *et al.* 1994). The main features of binding of *trans*-pyridine complexes with DNA are: a greater propensity to form inter-strand crosslinks (about 18% as compared to < 5% for *cis*-isomers) and the ability to cause a large unwinding angle (17° as compared to 13° for cisplatin and 9° for transplatin). It has been suggested that both features may lead to differential repair in biological systems and to antitumour activity and there may not be a correlation between levels of total bound platinum and cytotoxicity (Mellish, Barnard *et al.* 1995). Several *tans*-platinum (IV) compounds act as prodrugs for which the active compounds are believed to be the reduced *trans*-Pt(II) compounds (Natile and Couluccia 1999).

Another feature of binding of *trans*-planaramineplatinum(II) complexes with cellular DNA was (besides the formation of interstrand crosslink) the occurrence of protein-associated DNA breaks (Farrell 1996). RNA polymerase mapping experiments suggested that the DNA adducts of *trans*-[$PtCl_2(NH_3)$(quinoline)] were preferentially formed at guanine residues (Zakovska, Novakova *et al.* 1998). A greater interstrand crosslinking ability or the formation of crosslinks structurally different form those of cisplatin could explain why *trans*-$PtCl_2(pyridine)_2$ was found to be toxic against cisplatin-resistant cell lines.

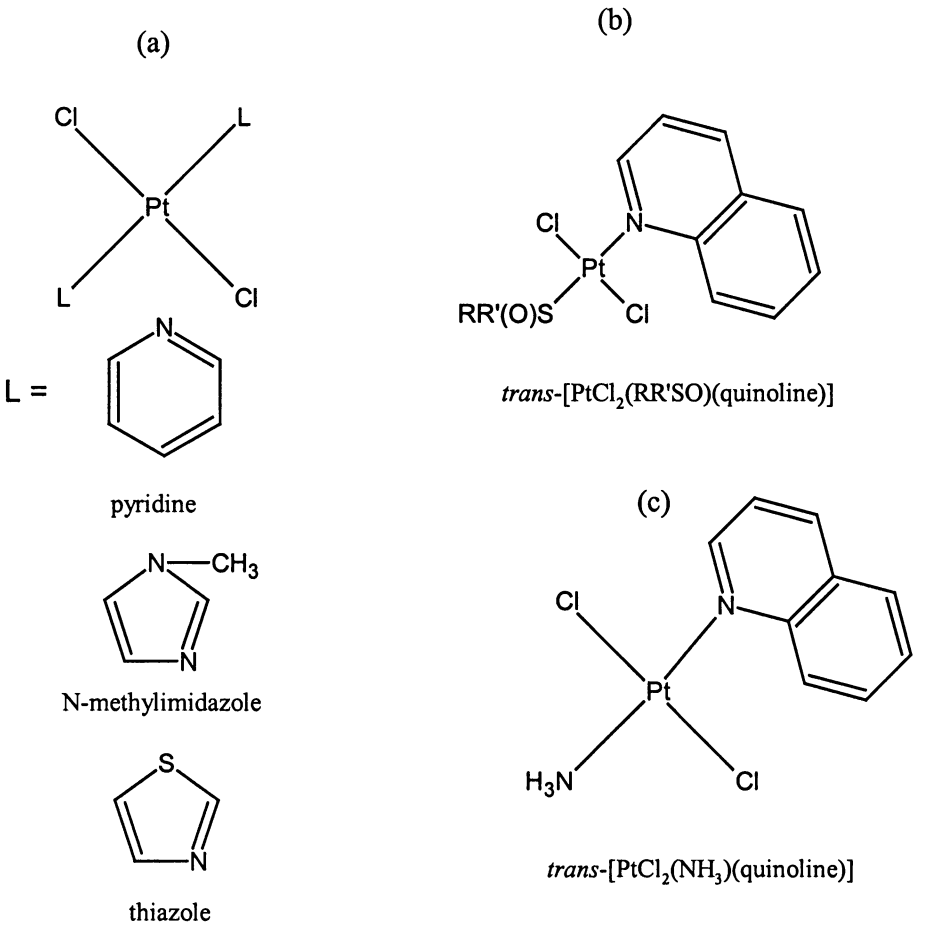


Figure 1.3-2: *Trans*-platinum complexes with planar amines

Although high in vivo activity was not achieved with *trans*-planaramineplatinum(II) complexes (Van Beusichem and Farrell 1992), the development of the compounds was important from the mechanistic point and also clearly demonstrated that tumour active compounds with *trans*-platinum geometry could be found. As stated earlier, the planar amine ligands hinder the approach of incoming nucleophiles to the axial positions of platinum centres, providing an explanation as to why *trans*-planaramineplatinum(II) complexes are found to be less reactive than transplatin not only with DNA but also with glutathione.

Although *trans*-planaramineplatinum(II) complexes are found to be less reactive and hence generally more active than transplatin, the current literature indicates that depending on the actual nature of the planar amine ligands present, the compounds could vary widely in their antitumour activity and other properties such as the extent of damage and unwinding caused to DNA, the level of cell uptake, extent of binding with DNA and other platinophiles (Kelland, Barnard *et al.* 1995; Najajreh, Peleg-Shulman *et al.* 2003). For example, Farrell found that the compounds: *trans*-$[PtCl_2(NH_3)(quinoline)]$, *trans*-$[PtCl_2(NH_3)(thiazole)]$ and *trans*-$[PtCl_2(pyridine)_2$ *trans*-planaramineplatinum(II) differed in their ability to cause protein-associated DNA strand breaks, the actual order being *trans*-$[PtCl_2(NH_3)(quinoline)]$ < *trans*-$[PtCl_2(NH_3)(thiazole)]$ < *trans*-$[PtCl_2(pyridine)_2$ (Farrell 1996).

Trans-planaramineplatinum(II) compounds containing pyridine-based ligands such as 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine could differ in their characteristic properties including activity, possibly due to differences in the extent of both covalent and noncovalent interactions such as hydrogen bonding and steric hindrance. To our knowledge so far no study has been carried out on *trans*-planaramineplatinum(II) compounds containing such substituted pyridine ligands. The present study involves four *trans*-planaramineplatinum(II) compounds, three of which contains pyridine based ligands. It will be seen later that there is indeed a wide variation in the properties of the compounds including their activity against human cancer cell lines. It is appropriate to note that although a great deal of knowledge has been gathered on structure-activity relationship and the situation is getting better, serendipity still plays an important role in the discovery of new tumour active compounds.

2) *Trans*-Pt(II)Cl_2 complexes with iminoether ligands such as *trans*-[Pt(II)Cl_2(iminoether)$_2$] (see Figure 1.3-3.). Like aromatic amines, iminoethers are planar ligands containing nitrogen donor centres and like aliphatic amines they have at least one hydrogen (atom bonded to nitrogen) that can form hydrogen bond.

The cytotoxicity of these complexes against P388 leukaemia cells were reported to be similar to that of cisplatin but greater than that of transplatin (Coluccia, Nassi *et al.* 1993). The compounds were found to overcome cisplatin resistance of A2780/cp8 ovarian cancer cells (Boccarelli, Coluccia *et al.* 1999). In vivo studies showed the compounds to have activity against P388 leukaemia in mice and cisplatin-resistant subline P388/DDP (Coluccia, Nassi *et al.* 1993). It also showed remarkable antitumor activity in mice bearing Lewis lung carcinoma (Coluccia, Boccarelli *et al.* 1995). Cellular accumulation of *trans*-platinum-iminoether complexes was found to be greater than that of cisplatin, indicating the compounds were able to overcome cisplatin resistance associated with decreased cellular uptake (Coluccia, Nassi *et al.* 1999; Coluccia, Nassi *et al.* 1999). The prevalent adducts formed between double-stranded DNA and *trans*-platinum-iminoether complexes are monofunctional adducts at G residues, which do not evolve into DNA crosslinks (Zaludova, Zakovska *et al.* 1997; Boccarelli, Coluccia *et al.* 1999). Under analogous experimental conditions, *trans*-iminoether complexes were found to form reduced inter-strand crosslinks with DNA than transplatin, cisplatin and *cis*-iminoether complexes (Comess and Lippard 1993; Mc A'Nulty and Lippard 1995). It terminates the transcription of DNA a factor that was believed to responsible for the cytotoxic activity of this class of compounds (Natile and Couluccia 1999).

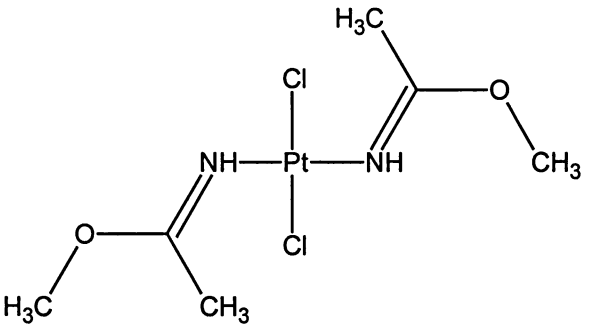


Figure 1.3-3: *Trans*-$PtCl_2$(iminoether)$_2$

3) *Trans*-Pt(II)Cl_2 complexes with asymmetric aliphatic amines such as *trans*-[$PtCl_2$(amine)(isopropylamine)] (where amine = dimethylamine, isopropylamine and propylamine) (see Figure 1.3-4) (Montero, Diaz *et al.* 1999). This class of complexes was found to be active against cisplatin-sensitive cell lines and more active than cisplatin against HL-60 leukaemia cells (Murray, Cunningham *et al.* 1983). Significant activity was also observed against cisplatin-resistant murine keratinocytes transformed by H-ras oncogene (Pam 212-ras cells) (Montero, Diaz *et al.* 1999). *Trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] was found to readily form interstrand cross links in double-strand DNA (Perez, Montero *et al.* 2000). At a similar level of platinum binding to DNA, *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] was found to produce more stop sites of T4 DNA polymerase than cisplatin. Also, the compound produced unique stretches of DNA polymerase stop sites in alternating purine-pyrimidine sequences. This result is considered to be a good indicator of DNA interstrand crosslinks (Brabec and Leng 1993).

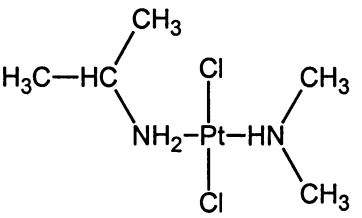


Figure 1.3-4: *Trans*-$PtCl_2$(dimethylamine)(isopropylamine)

4) *Trans*-Pt(IV) complexes such as *trans*-[ammine(cyclohexylamine)dichlorodihydroxoplatinum(IV)], JM335 (see Figure 1.3-5) (Drees, Dengler *et al.* 1995; Goddard, Orr *et al.* 1996). *Trans*-[ammine(cyclohexylamine)dichlorodihydroxoplatinum(IV)] showed cytotoxicity comparable to its *cis* counterpart and cisplatin against ADJ/PC6 plasmacytoma, L1210 leukaemic cells and CH1 ovarian carcinoma cells (Goddard, Orr *et al.* 1996; O'Neill, Ormerod *et al.* 1996). It was also found that the complex was able to circumvent cisplatin-acquired resistance in a variety of resistant human tumour cell lines (Goddard, Valenti *et al.* 1994). JM335, was found to exhibit antitumour activity in human ovarian carcinoma xenografts implanted in mice (Kelland, Barnard *et al.* 1994). It was also found that some of this class compounds lacked cross-resistance to cisplatin in vitro and even retained activity in cisplatin-resistant murine tumours. As stated earlier, Pt(IV) complexes are considered to act as prodrugs which are reduced to form active Pt(II) compounds both in tissue culture and within the cell (Kelland, Barnard *et al.* 1995; Goddard, Orr *et al.* 1996). Increased cellular accumulation of *trans*-Pt(IV) complexes was found in $41M^{cisR}$ cells, a cell line that is resistant to cisplatin because of reduced drug uptake. The increased cellular uptake is considered to provide an explanation for the higher cytotoxic activity of the *trans*-Pt(IV) complexes. In DNA binding studies it was found that JM335 formed interstrand crosslinks and induced single strand breaks (Mellish, Barnard *et al.* 1995).

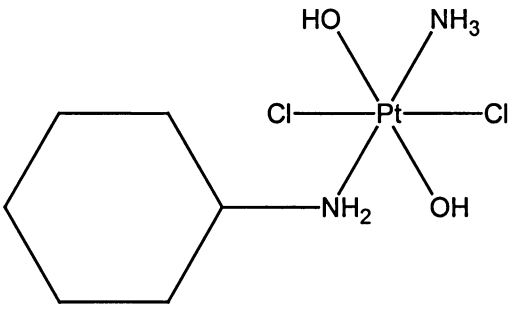


Figure 1.3-5: *Trans*-ammine(cyclohexylamine)dicholorodihydroxoplatinum(IV)

The above results indicate that the nature of interaction of the four types of *trans*-platinum complexes is different from that of cisplatin and their *cis* isomers. *Trans*-platinum complexes generally have a greater propensity to form interstrand adducts whereas cisplatin and other *cis*-platinum complexes have a greater tendency to form intrastrand bifunctional adducts. The compounds could also differ in other factors such as cellular accumulation.

Although a number of *trans*-planaramineplatinum(II) complexes have been prepared and investigated, there is still a need for further study involving other planaramine and nonplanaramine ligands to provide useful information on mechanism of their action, drug resistance and to arrive at more active and less toxic drugs. At the very least, such information is considered to provide vital information in the rational design of new drugs.

1.4 Project aims

The aims of this project were:

1. To synthesize new platinum-based anticancer drugs with *trans*-geometry by variation of the heterocyclic rings.
2. To quantify the activity of designed platinum complexes against cancer cell lines and the nature of their interaction with DNA.
3. To investigate structure-activity relation in the designed complexes.

The next chapter describes the methods and procedures used in the synthesis and characterisation of the compounds, their activity, cell uptake and binding with DNA.

2 Chapter Two: Materials and Methods

2.1 Synthesis of *trans*-platinum complexes

2.1.1 Introduction

As stated earlier, in this project a number of *trans*-planaramineplatinum(II) complexes have been prepared and investigated for their antitumour activity and nature of binding with DNA and its constituents. First cisplatin is prepared from potassium tetrachloroplatinate, which is then used as the starting material for the synthesis of *trans*-planaramineplatinum(II) complexes.

The preparation of cisplatin and transplatin was first described by Peyrone (Peyrone 1845). Later, the method used was modified by Kauffman and Cowan (Kauffman and Cowan 1963). They prepared the cisplatin by the action of aqueous ammonia on potassium tetrachloroplatinate(II). However, the method was time-consuming and had low yield (due to the formation of by-products such as magnus salt). A new and rapid method was later described by Dhara (Dhara 1970) in which the formation of magnus salt and other by-products could be avoided. The method involves the interaction of potassium tetrachloroplatinate (K_2PtCl_4) (Amo-Ochoa, Gonzalez *et al.* 1996) with potassium iodide and ammonia (Figure 2.1-1). The method gave quantitative yield.

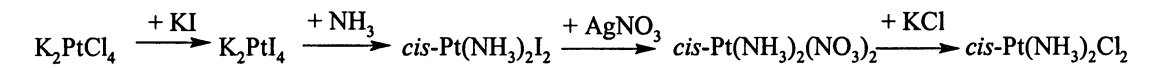
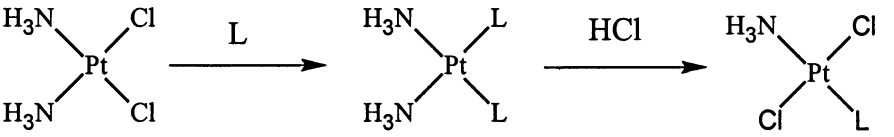


Figure 2.1-1: Synthesis of cisplatin by Dhara method

The method used for the synthesis of *trans*-planaramineplatinum(II) complexes from cisplatin is based on the difference in the *trans* effect of halide and amine ligands in

platinum(II) complexes, allowing selective substitution and hence control of the stereochemistry (Kauffman and Cowan 1963) (Figure 2.1-2).



L = ligand

Figure 2.1-2: Synthesis of *trans*-platinum complexes

2.1.2 Preparation of cisplatin

Materials:

Potassium tetrachloroplatinate(II), $K_2[PtCl_4]$ (Sigma Chemical Company St. Louise USA); potassium iodide (KI) (BDH Chemicals, Australia Pty. Ltd.) ; 28% ammonia solution (UnivaR Asia Pacific Speciality Chemicals Ltd. Auckland new Zealand); silver nitrate ($AgNO_3$); potassium Chloride (KCl) (BDH Chemicals, Australia Pty. Ltd.)

Method:

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

Potassium chloride (0.11 g, 1.5 mmol) was added to the solution. The mixture was left on a water bath at 37°C for 30 min for crystals of cisplatin to form. The crystals were filtered and washed with ice cold water and ethanol and left for air drying.

2.1.3 Preparation of YH9 [*trans*-$PtCl_2(NH_3)$(2-hydroxypyridine)]

As stated earlier, *trans*-$PtCl_2(NH_3)(L)$ complexes where L stands for a planaramine (such as 2-hydroxypyridine and 3-hydroxypyridine) were prepared from cisplatin utilizing the difference in the *trans* effect of halide and planaramine ligands allowing selective substitution and hence control of stereochemistry (Kauffman and Cowan 1963).

Materials:

Cisplatin [$PtCl_2(NH_3)_2$] (prepared from potassium tetrachloroplatinate); 2-hydroxypyridine (Sigma Chemical Company St. Louise USA); 10 M HCl (UnivaR Asia Pacific Speciality Chemicals Lty Auckland new Zealand)

Method:

Cisplatin (0.3 g, 1 mmol) was added to 8 mL of mQ water. 2-hydroxypyridine (0.19 g, 2 mmol), dissolved in a minimum amount of water, was added to cisplatin suspension. The mixture was heated at 70°C with reflux for 1 h to cause 2-hydroxypyridine ligand replace the chloro ligands. The product was cooled to room temperature to stop the reaction. Concentrated HCl (1.22 mL, 12.2 mmol) was added to the mixture followed by heating to 70°C with reflux to produce yellow needle shaped crystals of YH9. The crystals were collected at the pump, washed with mQ water and ice cold ethanol.

2.1.4 Preparation of YH10 [*trans*-$PtCl_2(NH_3)$(imidazole)]

Materials:

Cisplatin [$PtCl_2(NH_3)_2$] (prepared from potassium tetrachloroplatinate); imidazole (Sigma Chemical Company St. Louise USA); 10 M HCl (UnivaR Asia Pacific Speciality Chemicals Lty Auckland new Zealand)

Method:

Cisplatin (0.3 g, 1 mmol) was dissolved in 8 mL of mQ water. Imidazole (0.136 g, 2 mmol), dissolved in a minimum amount of water, was added to the solution of cisplatin. The mixture was heated at 70°C with reflux for 1 h to cause imidazole ligand replace the chloro ligands. The product was cooled to room temperature to stop the reaction. Concentrated HCl (1.22 mL, 12.2mmol) was added followed by heating to 70°C with reflux to produce yellow thin needle-shaped crystals of YH10. The crystals were collected at the pump, washed with mQ water and ice cold ethanol.

2.1.5 Preparation of YH11 [*trans*-$PtCl_2(NH_3)$(3-hydroxypyridine)]

Materials:

Cisplatin [$PtCl_2(NH_3)_2$] (prepared from potassium tetrachloroplatinate); 3-hydroxypyridine (Sigma Chemical Company St. Louise USA); 10 M HCl (UnivaR Asia Pacific Speciality Chemicals Lty Auckland new Zealand)

Method:

Cisplatin (0.3 g, 1mmol) was added to 8 mL of mQ water. 3-hydroxypyridine (0.19 g, 2 mmol), dissolved in 2 mL of ethanol (Huq and Yu 2002), was added to the cisplatin suspension. The resulting mixture was heated at 70°C with reflux for 1 h to cause 3-hydroxypyridine ligand replace the chloro ligands. The product was cooled to room temperature to stop the reaction. Concentrated HCl (1.22 mL, 12.2mmol) was added followed by heating to 70°C with reflux to produce thin yellow platy crystals of YH11. The crystals were collected at the pump, washed with mQ water and ice cold ethanol.

2.1.6 Preparation of YH12 {*trans*-$PtCl_2(NH_3)$[imidazo(1,2-α)pyridine]}

Materials:

Cisplatin [$PtCl_2(NH_3)_2$] (prepared from potassium tetrachloroplatinate); imidazo(1,2-α)pyridine (Sigma Chemical Company St. Louise USA); 10 M HCl (UnivaR Asia Pacific Speciality Chemicals Lty Auckland new Zealand)

Method:

Cisplatin (0.3 g, 1 mmol) was added to 4 mL of mQ water. Imidazo(1,2-α)pyridine (0.2 mL, 2 mmol) was added to the cisplatin suspension. The mixture was heated at 70°C with reflux for 1 h to cause the imidazo(1,2-α)pyridine ligand replace the chloro ligands. The product was cooled to room temperature to stop the reaction. Concentrated HCl (1.22 mL, 12.2 mmol) was added followed by heating to 70°C with reflux to produce thin yellow platy crystals of YH12. The crystals were collected at the pump, washed with mQ water and ice cold ethanol.

2.2 Characterization of Compounds

2.2.1 Microanalysis

2.2.1.1 Brief description of the methods and instruments

Microanalysis was carried out to determine the elemental composition of the complexes. C, H and N were determined using Carlo Erba 1106 automatic analyzer available at Australian National University. Cl was also determined at Australian National University by titration with standardised mercuric nitrate.

2.2.2 Atomic Absorption Spectroscopy

2.2.2.1 Basic principle

Atomic absorption spectroscopy (AAS) is a commonly used technique for determining metal (and metalloid) contents in samples. The technique is based on the principle that absorption of a photon at a specific wavelength can cause an electronic transition of an atom from the stable ground state to an excited state. Accordingly, it is necessary that the sample should be first vaporized, atomized and desolvated by means of either a flame or a graphite furnace. In this project, graphite furnace AAS has been used to determine platinum contents of compounds. The technique has also been used to determine cellular accumulation of platinum and the extent of binding of platinum with DNA, to provide a measure of cellular drug uptake and the level of drug-DNA binding respectively.

Method:

As stated earlier, platinum contents were determined by graphite furnace atomic absorption spectroscopy (AAS). A Varian SpectrAA-20 Atomic Absorption Spectrophotometer with GRA-96 Graphite Furnace Tube Atomiser, available in the School of Biomedical Sciences, The University of Sydney was used.

All the glasswares were cleaned first by detergent, then soaked into 20% v/v HCl solution for 2 day and 20% v/v HNO_3 solution for further 2 days. Then glasswares were then rinsed with distilled water.

A standard platinum solution 490 ppb was prepared by diluting the concentrated 980 μg /mL platinum atomic absorption standard solution (Sigma Chemical Company St. Louise USA) with 0.1 M HCl. This was done firstly by diluting 0.5 mL of 980 ppm Pt solution with 0.1 M HCl to give 100 mL of 4900 ppb Pt standard solution. 20 mL of 4900 ppb Pt standard solution was further diluted with 0.1 M HCl to give 200 mL of 490 ppb Pt standard solution.

The AAS conditions used for the determination of platinum are given in Tables 2.2-1, 2.2-2 and 2.2-3.

Table 2.2-1: Furnace operating conditions for the determination of platinum

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

Table 2.2-2: Instrument parameters for the determination of platinum

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

Table 2.2-3: Sampler parameters used in AAS to determine platinum

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

The typical standard-addition graphs applying to platinum is shown in the Figure 2.2-1.

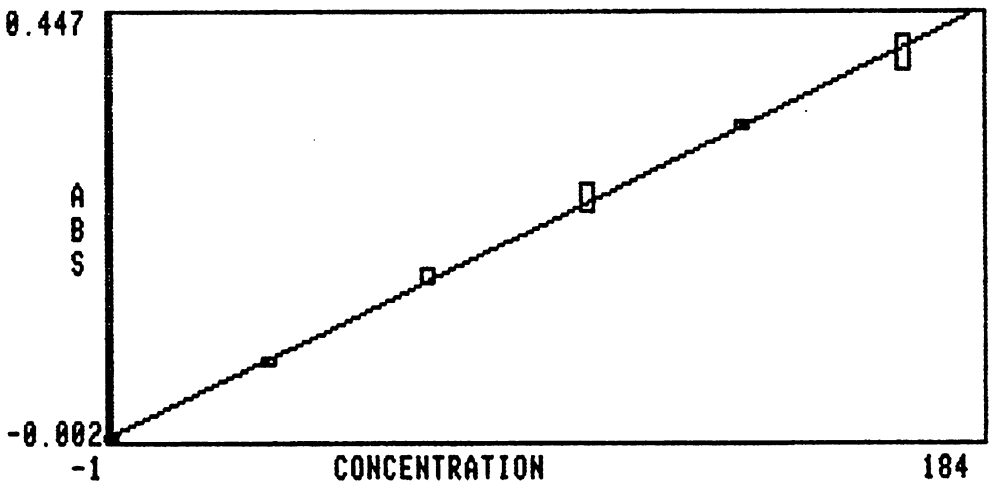


Figure 2.2-1: Typical standard addition graph for platinum

2.2.3 Percentage yield calculation

Since 1 mmol of cisplatin would produce at the maximum 1 mmol of the compound, the theoretically yield = 1 x 10^{-3} x MW (compound).

$$\text{Thus, percentage yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100$$

2.2.4 Molar conductivity

The molar conductivity values of YH9, YH10, YH11 and YH12 in solution in 1: 4 mixture of DMF and water at 298 K were determined using PW9506 digital conductivity meter. The conductivity values were measured at the concentrations: 0.5 mM, 0.25 mM, 0.1 mM and 0.01 mM. The molar conductivity (Λ) was calculated as $\Lambda = k/c$ where k is the conductivity and c is the concentration (Atkins 1998). The molar conductivity values obtained were then plotted against concentration to determine the limiting values.

2.2.5 Infrared (IR) Spectroscopy

2.2.5.1 Introduction to IR Spectroscopy

As a result of absorption of infrared (IR) radiation a molecule can undergo vibrational excitations. Because each molecule has its own characteristic modes of vibration, the infrared spectrum of a molecule is like the fingerprint of the molecule. In this project the IR spectroscopy has been used to identify the presence of functional groups and hence to aid in the structural characterization of the compounds (Twardowski, Anzenbacher *et al.* 1992).

Method

Infrared spectra were collected using a Bruker IFS66 spectrometer equipped with a Spectra-Tech Diffuse Reflectance Accessory (DRA), an air-cooled DTGS detector, a KBr beam splitter with a spectral range of 4000 to 650 cm^{-1}. The instrument was run under a vacuum during spectral acquisition. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of 128 scans and a Blackman-Haris 3-Term apodisation function applied. Prior to analysis the samples were mixed, and lightly ground, with finely ground spectroscopic grade KBr. The spectra were then manipulated using the Kubelka-Munk mathematical function in the OPUS™ software to convert the spectra from reflectance into absorbance.

2.2.6 Raman Spectroscopy

2.2.6.1 Introduction to Raman spectroscopy

Although Raman spectroscopy is based on scattering of suitable electromagnetic radiation (Twardowski, Anzenbacher *et al.* 1992) and IR spectroscopy is based on

absorption of IR radiation, like IR spectrum, the Raman spectrum provides information about the vibrational frequencies of molecule. The IR and Raman spectra are considered to be complementary as together they provide more complete information on vibrational modes than that provided by either the IR or the Raman spectrum alone. In this project, the Raman spectrum has been used to identify the presence of various functional groups and hence to aid in the structural characterization of the compounds (Nakamoto 1997).

Method

Raman spectra were collected using a Bruker RFS100 Raman spectrometer equipped with an air cooled Nd:YAG laser emitting at a wavelength of 1064 nm, and a liquid nitrogen cooled germanium detector with an extended spectral band range of 3500 to 50 cm^{-1}. 180° sampling geometry was employed. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of 100 scans at a laser power of 0.065 mW. A Blackman-Harris 4-Term apodisation function was applied and the spectra were not corrected for instrument response.

2.2.7 Mass spectrometry

2.2.7.1 Basic principle

A mass spectrometer is an instrument used for measuring molecular mass and mass of fragments and hence to provide structural information of a sample. It produces charged particles (ions) from chemical substances and uses electric and magnetic fields to measure the mass of the charge particles. It consists essentially of three parts: (1) an ion source in which the introduced sample molecules are ionized, (2) an analyzer where ions are extracted and separated according to their mass-to-charged

ratios (m/z), and (3) a detector which collects the signals generated from the separated ions then stores and processes the information in the format of an intensity versus m/z spectrum. The entire system is maintained under high vacuum to give the ions free movement (devoid of hindrance from air molecules) (Siuzdak 1996).

2.2.7.2 Obtaining the mass spectrum of YH9, YH10, YH11 and YH12

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

ESI is a very soft ionization technique. It is most used for analyzing large or labile molecules such as peptides, proteins, organometallics and polymers. Samples are introduced as solutions using the infusion syringe pump, liquid chromatograph (LC) or through the loop injection port with a LC pump. Water or organic solvents such as methanol, acetone and $CHCl_3$ can be used to dissolve the sample. If the sample is not ionic, some source of protons (acetic acid or formic acid) is added to promote the formation of cations.

A typical experimental condition of this instrument is: ESI spray voltage 5 kV; nitrogen sheath gas pressure 60 psi; heated capillary temperature 200 °C; full scan 50 to 2000 m/z. For loop injection, the typical mobile flow phase is 50% methanol/50%

water with 1% acetic acid and the flow rate is 100 microliter per minute. The exact experimental condition varies to optimize the signal.

To obtain mass spectra, solutions of YH9, YH10, YH11 and YH12, made in 10% DMF and 90% methanol, were sprayed into a Finnigan LCQ 10 N trap mass spectrometer in which fragmentation was produced by eletrospray ionization (ESI).

2.2.7.3 Interpretation of the mass spectrum of the compounds

In confirming the structure of YH9, YH10, YH11, and YH12, the ratio of isotopes of the atoms would be taken into the consideration. Isotope splitting patterns for molecules and fragments were calculated using the program Sheffield ChemPuter.

2.2.8 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (in short NMR) is one of the most widely used techniques employed for the identification of compounds and to determine structures of molecules. In this study, ^{1}H NMR was used to identify functional groups. ^{1}H NMR spectra of YH9, YH10, YH11 and YH12 were recorded in dimethylsulfoxide-d_6 (DMSO-d_6) solution in a Bruker AVANCE DPX 400 spectrometer. Spectra were referenced to internal solvent residues and were recorded at 300 K (±1 K).

2.3 Interaction with pBR322 plasmid DNA and ssDNA

2.3.1 Basic principle of gel electrophoresis

Gel electrophoresis uses an electric field to move nucleic acid 'molecules'. Being negatively charged due to phosphate groups present in the backbone, nucleic acid molecules will be attracted to the positive electrode. The rate of migration is

dependent upon the size and the conformation of the DNA, concentration of the supporting gel matrix, electric field strength, and composition of the electrophoresis buffer and the presence of intercalating dyes.

DNA migrates through gel matrices at rates that are inversely proportional to the log of the number of base pairs (Helling, Goodman *et al.* 1974). Thus large molecules of DNA migrate more slowly through the gel than smaller molecules. Plasmids are small circular, double-stranded DNA molecules ranging in size from two to several hundred kilobases found in some bacteria. They are extrachromosomal genetic elements and can replicate independently of chromosomal DNA. Plasmids carry genes for the inactivation of antibiotics, metabolism of natural products, production of toxins, and synthesis of enzymes. (Stryer 1981). Plasmid DNA can exist in three forms: supercoiled circular (form I), singly nicked circular (form II), and doubly nicked linear (form III) (see Figure2.3-1). The rate of migration through the gel is different for the three forms (Thorne 1966; Thorne 1967). Form I, being supercoiled and compact, will migrate at the fastest rate and the flexible and relaxed Form II DNA travels at the slowest rate. The linear Form III is able to snake through the gel and thus will migrate faster than the Form II DNA (see Figure 2.3-2).

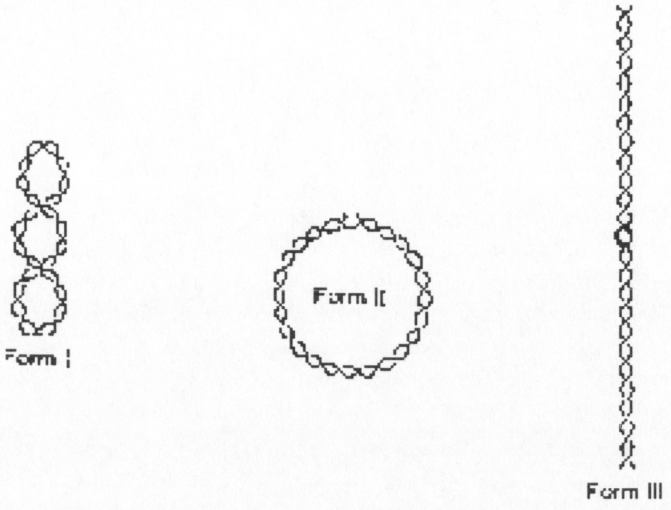


Figure 2.3-1: The three conformational forms of plasmid DNA

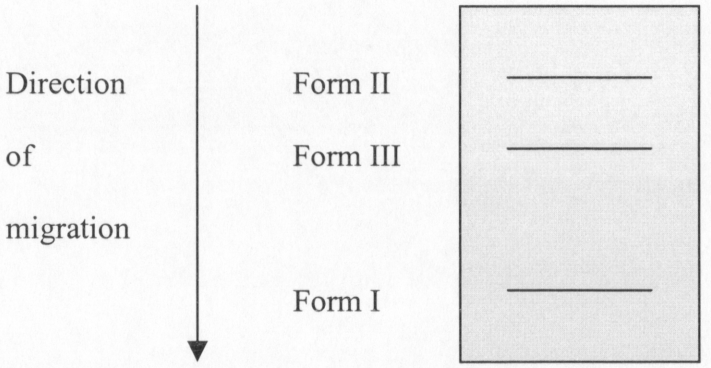


Figure 2.3-2: Migration pattern of Form I, Form II and Form III plasmid DNA in an electric field

Agarose is a high molecular weight polysaccharide extracted from the walls of certain marine red algae and is insoluble in cold water but dissolves readily in boiling water. When used as supporting running matrix for DNA, agarose gel has the advantages of being non-toxic gel medium, easy to cast and quick to run. It forms side-by-side aggregates which 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. Thus DNA would take a longer time

to run the same distance through a higher percent gel than a lower percent 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, the electrophoretic mobility of DNA also depends on the electric field strength. The rate of migration of DNA is proportional to the voltage applied. However, as the electric field strength is increased, the temperature also increases due to the resistive heating of the gel by the electric field. In the worst case, the gel melts and the DNA denatures. Thus, electric strength should be controlled so that the resistive heating can be approximately balanced by evaporative cooling. To obtain maximum resolution of DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5 V/cm between the electrodes (Sambrook, Maniatis *et al.* 1989).

The composition and ionic strength of the electrophoresis buffer also affects the migration rate of DNA. In ions-free electrophoresis buffer, there is lack of electrical conductance and DNA migrates very slowly. In contrast, in high ionic strength electrophoresis buffer although electrical conductance would be high, significant amounts of heat may be generated that can cause gel melting and DNA denaturation. There are several different buffers commonly used in the gel electrophoresis such as TAE buffer containing EDTA (pH 8.0) and tris-acetate (TAE), or TBE (Tris-borate) with EDTA, or TPE (Tris-phosphate) with EDTA at a concentration of approximately 50 mM. DNA fragments migrate faster through TAE than through TBE and TPE and the resolution of supercoiled 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 visualised by ultraviolet radiation using a transilluminator (Stryer 1981). It is used to detect DNA in agarose. However, it reduces the electrophoretic mobility of linear DNA by about 15% by extending the length of linear and nicked circular DNA molecules and making them more rigid. It is usually used to monitor the mobility of DNA during the electrophoresis. However, it is better to run the gel in the absence of the dye and stain the gel after the electrophoresis in order to give sharper bands.

2.3.2 Materials and Methods

Interaction between YH9, YH10, YH11 and YH12 and pBR322 plasmid DNA and Salmon sperm DNA (ssDNA) was studied by agarose gel electrophoresis. The method used was a modification of that described by Stellwagen (Stellwagen 1998).

Materials:

pBR322 plasmid DNA was purchased from ICN Biomedicals, Ohio, USA.

ssDNA was obtained from Fluka, Switzerland and Sigma-Aldrich, NSW, Australia.

Preparation of 10 mL of 0.05 M Trizma buffer:

0.0788 g of Trizma hydrochloride (Sigma, USA) was dissolved in 9 mL of mQ water and the pH was adjusted to 8 by adding 1 M NaOH. The volume was then made up to 10 mL with mQ water. The solution was sterilized immediately by filtration.

Preparation of 1 mg/mL ssDNA:

10 mg of ssDNA was dissolved in 0.05 M Trizma buffer and stored at –17°C until used.

Method:

pBR322 plasmid DNA aliquots (0.5 mg mL^{-1}) or ssDNA (1 mg mL^{-1}) were incubated in the presence of the increasing concentrations of compounds ranging from 1 μM to 160 μM. Incubation was carried out in a shaking water bath at 37°C for 4 h. 16 μL aliquots of drug-DNA mixtures containing 0.5 μg of pBR322 plasmid DNA or 2 μg of ssDNA was loaded onto the 1% agarose gel and electrophoresis was carried under TAE buffer for 2 h at 5 V cm^{-1}. At the end of electrophoresis, the gel was stained in the same buffer containing ethidium bromide (0.5 mg mL^{-1}). The gel was visualised under UV light using the Bio-Rad Trans illuminator IEC 1010. The illuminated gel was photographed with a Polaroid camera (a red filter and Polaroid type of film were used).

2.4 BamH1 restriction enzyme digestion

2.4.1 Restriction enzymes

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

2.4.2 Materials and Methods

Materials:

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

Method:

In this series of experiments, an identical set of drug-DNA mixtures as that described previously, was first incubated for 4 h on a shaking water bath at 37°C and then subjected to BamH1 (10 units μL^{-1}) digestion. To each 20 μL of incubated drug-DNA mixtures were added 2 μL of 10x digestion buffer SB first and then 0.1 μL BamH1 (1 unit). The mixtures were left in a shaking water bath at 37°C for 1 h at the end of which the reaction was terminated by rapid cooling. The gel was subsequently stained by ethidium bromide and visualised under UV light and photographed as described previously.

2.5 Biological Activity

2.5.1 Introduction of tissue culture

Tissue culture is a method for studying the behaviour of animal cells free of systemic variations. The advantages of tissue culture are control of the physiochemical environment in terms of pH, temperature, osmotic pressure, O_2 and CO_2 tension, and control of the physiological conditions including hormone and nutrient concentrations. It also produces identical cell line by subculture technique that can maintain a homogeneous culture of the most vigorous cell type. Reagent at lower, and defined, concentration may have direct access to the cell, making possible a lower cost.

Cytotoxicity and screening of pharmaceutics, cosmetics, etc can be performed with tissue culture that will avoid the legal, moral, and ethical questions of animal experimentation. New developments in multiwell plates and robotics have further introduced significant economies in time and scale (Freshney 1994).

2.5.2 Basic technique of cell culture

There are three main types of tissue cultures. The first one is organ culture that retains the architecture characteristic of the tissue in vivo. The second one is primary explant culture which is a fragment of tissue promoted to attach, migrate in the plane of the solid substrate. The last one is cell culture which is the outgrowth tissue from the primary explant and is dispersed into a cell suspension, which can be further cultured as an adherent monolayer on a solid substrate or as a suspension in the culture medium. (Schaeffer 1990)

To maintain a cell culture a suitable medium should be supplied. Its pH value should be stabilized around 7.4 by buffering with sodium bicarbonate in solution and carbon dioxide in the gas phase in equilibrium to regulate the pH value. HEPES is another much stronger buffer in the pH range: 7.2 – 7.6. A complete medium should contain a larger number of different amino acids including nonessential amino acids and additional vitamins such as the B-group, plus choline, folic acid, inositol, and nicotinamide, salts (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO^{2-}, PO_4^{3-}, and HCO^{3-}), glucose, extra metabolites (e.g., nucleosides, tricarboxylic acid cycle intermediates, and lipids), hormones and growth factors. Commercially available serum are commonly made to fulfil this requirement and contains 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 to supply for respiration in vivo. (Freshney 1994)

Most cells from solid tissues grow as adherent monolayers in vitro. They need to attach and spread out on the substrate before they will start to proliferate. This is associated with the transmembrane proteins that cause cell-cell- and cell-substrate adhesion (Klagsbrun and Baird 1991; Rosenman and Gallatin 1991; Yamada 1991). Then they 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 (Todaro and Green 1963; Brouty-Boye, Gresser *et al.* 1979; Brouty-Boye, Tucker *et al.* 1980). Harvesting cells for study, processing or subculture requires dissociation and detachment of the monolayer. Enzyme trypsin, a pancreatic serine protease, is the substance most frequently applied in the treatment of the cell layer (Scherer, Syverton *et al.* 1953).

2.5.3 Materials and Methods

2.5.3.1 Cell lines

The human ovarian cancer A2780 cell line, the human ovarian A2780 cisplatin-resistant cell line, and the human ovarian A2780 AMD473-resistant cell line were gifts from Dr. Philip Beale, Sydney, Australia. The A2780 human ovarian cancer cell line was originally established from tumour tissue from an untreated patient (Hamilton, Young *et al.* 1984). The human ovarian A2780 cisplatin-resistant cell line has been developed by chronic exposure of the parent cisplatin-sensitive A2780 cell

line to increasing concentrations of cisplatin (Behrens, Hamilton *et al.* 1987). Acquired resistant ZD0473 cell line was established using the A2780 ovarian carcinoma cell line by in vitro exposure over a 7-month period to increasing concentrations of drug ZD0473 (Holford, Beale *et al.* 2000). The melanoma cell line Me10538 and the non-small lung cancer cell line NCI-H460 were gifts from Ms. Mei Zhang, RPAH, Sydney, Australia. The melanoma cell line Me10538 was originally established from human melanomas primary tumours (Larizza, Doneda *et al.* 1989). NCI-H460 cell line was derived from the pleural fluid of a patient with large cell cancer of the lung (Banks-Schlegel, Gazdar *et al.* 1985).

2.5.3.2 Maintenance of the cell lines

All the cell lines were grown in tissue culture flasks in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2mM glutamine without antibiotics.

Materials:

Foetal calf serum, 5 x RPMI 1640, 200 mM L-glutamine and 5.6% sodium, bicarbonate, 0.25% trypsin were obtained from Trace Biosciences Pty Ltd. Other reagents were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia

1 M Hepes (Sigma-Aldrich Pty Ltd, NSW, Australia)

Preparation of 100 mL of 1 M Hepes

23.83 g of Hepes was dissolved in mQ water in a 100 mL volume metric flask. The solution was sterilized immediately by filtration and stored at 4°C.

Preparation of 1 L of 10% fetal calf serum/RPMI1640 (10%FCS)

To make 1 L of 10% fetal calf serum/RPMI1640, the following reagents are required:

5xRPMI 1640:	200 mL
10% Fetal calf serum;	100 mL
1M Hepes:	20 mL
200mM Glutamine:	10 mL
5.6% sodium bicarbonate:	20 mL
Saturated NaOH:	1 mL

The above components were mixed together and the volume is made up to 1 litre with sterile distilled water.

Phosphate buffered saline (PBS) (Sigma-Aldrich Pty Ltd, NSW, Australia) (1000 mL)

To make 1000 mL of phosphate buffered saline (PBS), all of the PBS powder present in a bottle (9.6 g) was dissolved in 900 mL mQ water followed by the addition of more mQ water to make the final volume 1000 mL. The solution was sterilized immediately by filtration.

0.25% trypsin (100 mL)

To make 100 mL of 0.25% trypsin, the following reagents are required:

2.5% trypsin:	10 mL
2% (g/mL) EDTA:	1 mL

The above components were mixed together and the volume was made up to 100 mL by PBS (prepared earlier). The solution was then filtered to sterilize.

Method of Maintenance of the cell lines:

Medium in the cell culture flask was first withdrawn and discarded. The flask was then rinsed with 1 mL of PBS to remove traces of serum that would inhibit the action of the trypsin. 1 mL of 0.25% trypsin was added to the flask which was rotated to cover all the cell layer by trypsin. It was then left for 2 min in the incubator at 37°C until all the cells rounded up. Cell detachment was checked by tilting the flask. If the cells are detached, the monolayer should slide down the surface. 9 mL of 10% FCS was then added to the flask to inhibit the action of typsin. The cells in the medium was counted and appropriate dilution of cells was set up. To reduce the cell concentration at subculture by 10 fold, 9 mL of this cell suspension was withdrawn and discarded. Then 9 mL of fresh 10% FCS was added. The flask was capped and returned to the incubator at 37°C with a humidified atmosphere of 5% CO_2 and 95% air. This procedure was repeated every three to four days to maintain in logarithmic growth of the cells.

2.5.3.3 Storage of the cell lines

All the cell lines in late log phase, which were healthy in growth and freedom from contamination, were chosen for storage. Dimethyl sulfoxide (DMSO) was chosen as cryoprotectants which could permeate cell membranes rapidly to increase the intracellular concentration so as to inhibit intracellular ice formation which is the main cause of freezing damage to cell (Lovelock and Bishop 1959). The cell suspension should be frozen slowly, at –1°C per minute which can be achieved by

laying the cell vial on cotton wool in a box at –70°C in a regular deep freezer (Harris and Griffiths 1977).

Materials:

10% FCS/RPMI 1640

Preparation of 20% Dimethyl sulfoxide (DMSO) 10mL:

2 mL of DMSO was added to 8 mL of 10% FCS/RPMI 1640 and allowed to cool to below 37°C.

Method:

The late log phase growth cells free from contamination were first gently harvested by spinning then resuspended in 10% FCS/RPMI 1640 to a concentration of $2x10^6$ cells/mL. 20% of DMSO equal in volume to that of the cell suspension, was slowly added to the cells to give a final DMSO concentration of 10%. 1 mL of this mixture of cells and cryoprotectant was then transferred to prelabelled NUNC vials and stored overnight in a box surrounded by cotton-wool at –70°C and then transferred to liquid nitrogen tank.

2.5.4 MTT-Based cytotoxicity Assay

2.5.4.1 Introduction to cytotoxicity

For anticancer agents, cytotoxicity may be crucial to their action. Cellular models of in vitro toxicology is simple and highly reproducible, and less costly and time consuming than in vivo experiments. Molecular mechanisms of toxicity can be easily explored and detailed and early identification of cellular damage can be made. However, it has a number of disadvantages that systemic toxic effects cannot by

studied and it is difficult to correlate the in vitro situation to the in vivo one both in terms of experimental design and results. The system is overly simplified compared to the complexity of the organism and not much toxic mechanisms can be studied in each test. Nevertheless, the broad aim of in vitro toxicity testing is to screen compounds in order to get an indication of their general cytotoxicity.

2.5.4.2 MTT cell viability assay

MTT reduction assay is one of the most commonly used methods to determine the cytotoxicity of drugs. It is a rapid and sensitive colorimetric assay based on the formation of a coloured insoluble formazan salt. It is an indirect method to measure the proportion of viable cells following a drug exposure. (Jansen, Perez *et al.* 2001; Baruah, Rector *et al.* 2002; Woolley, Tetlow *et al.* 2002)

MTT, Methylthiazoletetrazolium [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], is a yellow water-soluble tetrazolium dye that is reduced by mitochondrial enzyme, succinate dehydrogenase in live cells to a purple formazan product (Carmichael, DeGraff *et al.* 1987). Dead cells or tissue culture medium cannot cause such reduction (Mosmann 1983). The formazan crystals is insoluble in aqueous solutions but can be dissolved in DMSO. The absorbance as a measure of formazan production can be analysed on a scanning multiwell spectrophotometer and the percentage of viable cells quantified. However, cell contamination may cause false negative results (Denecke, Becker *et al.* 1999) and it is impossible to distinguish between cytostatic and cytocidal effects of the drugs on cells.

2.5.4.3 Materials and Method

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

Materials

10% FCS/RPMI 1640

0.25% trypsin

1 mg/mL MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma):

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

Dimethyl sulfoxide (DMSO)

Method:

The human ovarian cancer cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, the melanoma cell line: Me10538 and the non-small lung cancer cell line: NCI-H460 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). 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 then incubated for 24 h at 37°C in a humidified atmosphere to allow them to attach. Platinum complexes were first dissolved in a minimum volume of DMF, then diluted to the required concentrations by adding mQ water and finally filtered to sterilize. A serial fivefold dilutions of the

drugs ranging from 0.02 μM to 62.5 μM in 10% FCS/RPMI 1640 medium were prepared and added to equal volumes of cell culture in quadruplicate wells, then left to incubate under normal growth conditions for 72 h. The inhibition of the cell growth was determined using the MTT reduction assay (Mosmann 1983). 4 h after the addition of MTT (50 μL per well of 1 mg mL^{-1} MTT solution), the yellow formazan crystals produced from the reduction of MTT was dissolved in 150 mL of DMSO and the absorbance of the resulting solution was read with a plate reader (Bio-Rad Model 3550 Microplate Reader) set at 540 nm.

The percentage of living cells was calculated as follows:

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

For each drug concentration, at least three independent experiments were done.

Dosage response curves were constructed by plotting the percentage of viable cells against drug concentration. The IC_{50} value (i.e. the drug concentration required to cause 50% cell kill) was then calculated from curve.

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

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

2.5.5 Determination of platinum uptake and DNA binding in Cells

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

2.5.5.1 Cell uptake

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

Following incubation with compounds, cell pellets were suspended in 0.5 mL 1% triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace AAS using a Varian SpectrAA-20 plus with GTA 96 atomic absorption spectrophotometer using a variant of standard addition technique (Roberts, Peroutka *et al.* 1999).

2.5.5.2 Binding of platinum to DNA

Materials:

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

Method:

Following drug incubation high molecular weight DNA was isolated from cell pellet using JETQUICK Blood DNA Spin Kit/50 and the modified protocol of Bowtell (1987) (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 then added to the mixture followed by incubation for 10 min at 70°C. Then 200 μL of absolute ethanol was added and mixed thoroughly to prevent any precipitation of nucleic acids due to high local alcohol concentrations. The samples were then centrifuged for 1 min at 10600 rpm through the silica membrane using JETQUICK micro-spin column. The columns containing the samples were then washed with 500 μL of buffer KX (containing high-salt buffer to remove residual contaminations) and centrifuged for 1 min at 10,600 rpm. These were again washed with 500 μL buffer K2 (containing low-salt buffer to change the high-salt conditions on the silica membrane to low-salt) and centrifuged for 1 min at 10,600 rpm. To further clear the silica membrane from residual liquid, the sample columns were centrifuged again for 2 min at full speed (13,000 rpm). The column receivers were changed and the purified DNA in the column was eluted from the membrane with 200 μL of 10 mM Tris-HCl buffer (pH 8.5). DNA content was determined by UV spectrophotometry (260 nm) (Varian Cary 1E UV-Visible with Varian Cary Temperature Controller) and platinum levels were determined by graphite furnace AAS. A_{260}/A_{280} ratios were found to be between 1.75 and 1.8 for all samples.

2.6 Interaction between YH9, YH10, YH11 and YH12, and nucleobases

High performance liquid chromatography (HPLC) provides a convenient method to investigate the binding between platinum-based anticancer drugs and nucleobases,

nucleosides, nucleotides and DNA (Berners-Price and Appleton 2000). Appropriate choice of column, mobile phases, pH, flow rate, gradient, ion-pairing agent etc. allows allow each adduct between a platinum drug and a nucleobase (guanine and adenine) to be eluted with a characteristic retention time. Determination of the platinum content of time peak fraction by graphite furnace AAS and nucleobase content by UV-visible spectrophotometry makes possible the calculation of the binding ratio between the drug and the nucleobase applying to the fraction. Provided appropriate conditions are chosen, HPLC would offer an efficient method of purification of a drug or drug-nucleobase adduct. Purified adducts can be structurally characterized by single crystal x-ray diffractometry (if suitable crystals can be found) and also by NMR spectroscopy. In this study, HPLC has been used to determine the binding ratio between YH9, YH10, YH11 and the nucleobases guanine and adenine. As YH9, YH10, YH11 and YH12 have two labile chloro ligands, all of them are expected to form 1:2 (drug : NB) adducts where NB stands for guanine or adenine.

2.6.1 Materials

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

2.6.2 HPLC method

1 mL of 5 mM solution of YH9, YH10, YH11 and YH12 dissolved in DMF was diluted to 5 mL with mQ water to give 1 mM solution of the compounds. Equal volumes of 1 mM solution of the drug and 2 mM solution of NB (guanine or adenine) were mixed together and incubated at 37°C in a shaking water bath for 24 h. To dissolve guanine in mQ water, pH was increased to about 10 by adding a tiny drop of

0.1 M NaOH. After incubation, 5-10 μL of each of the mixtures and appropriate components was injected separately into a Waters HPLC system, consisting of a Waters 600 controller, a Waters 600 pump, a Waters 746 data module, a Waters Dual λ absorbance detector and Waters Nova-Pak C18 column consisting of Waters RCM 8 x 10 Module and Resolve™ Cartridge set at a pressure of 17 Mpa. The wavelength was set at 260 nm.

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

2.6.3 Binding ratio

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

3 Chapter Three: Results

As stated earlier, in this project four new *trans*-planaraminieplatinum(II) have been synthesized, characterized and investigated for their antitumour activity against human cancer cell lines and the nature of binding with DNA. This chapter describes the results of the studies.

3.1 Synthesis of *trans*-planaramineplatinum(II) complexes

3.1.1 Preparation of YH9 [*trans*-$PtCl_2(NH_3)$(2-hydroxypyridine)]

From the reaction of 1 mmol (0.3 g) of cisplatin with 2 mmol (0.19 g) of 2-hydroxypyridine, 0.113 g (0.30 mmol) of YH9 (Figure 3.1-1) was obtained giving a yield of 30%.

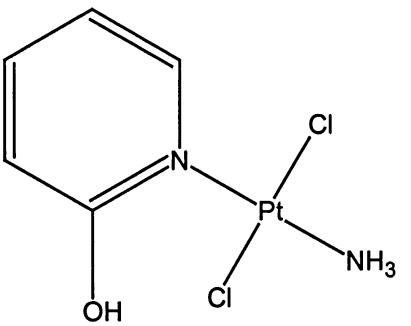


Figure 3.1-1: Structure of YH9

3.1.2 Preparation of YH10 [*trans*-$PtCl_2(NH_3)$(imidazole)]

From the reaction of 1 mmol (0.3 g) of cisplatin with 2 mmol (0.136 g) of imidazole, 0.081 g (0.23 mmol) of YH10 (Figure 3.1-2) was obtained giving a yield of 23%.

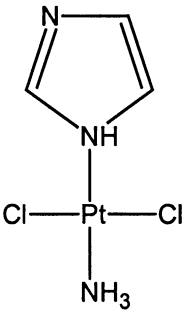


Figure 3.1-2: Structure of YH10

3.1.3 Preparation of YH11 [*trans*-$PtCl_2(NH_3)$(3-hydroxypyridine)]

From the reaction of 1 mmol (0.3 g) of cisplatin with 2 mmol (0.19 g) of 3-hydroxypyridine, 0.193 g (0.51 mmol) of YH11 (Figure 3.1-3) was obtained giving a yield of 51%.

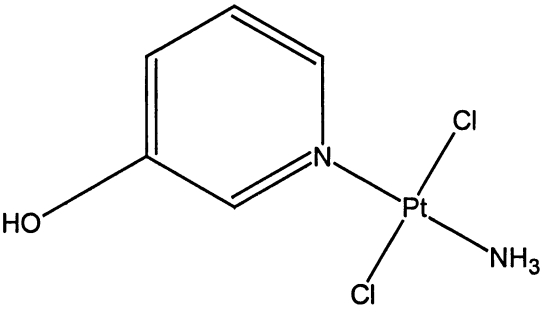


Figure 3.1-3: Structure of YH11

3.1.4 Preparation of YH12 {*trans*-$PtCl_2(NH_3)$[imidazo(1,2-α)pyridine]}

From the reaction of 1 mmol (0.3 g) of cisplatin with 2 mmol (0.2 mL) of imidazo(1,2-α)pyridine, 0.1 g (0.25 mmol) of YH12 (Figure 3.1-4) was obtained giving a yield of 50% .

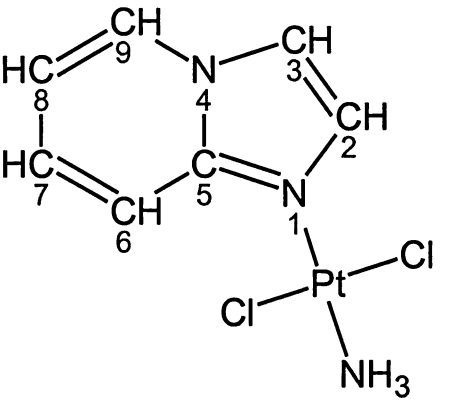


Figure 3.1-4: Structure of YH12

3.2 Elemental compositions of YH9, YH10, YH11 and YH12

As stated earlier, microanalyses were carried out to determine the elemental compositions of YH9, YH10, YH11 and YH12. Table 3.2-1 gives the calculated and the observed values for %C, %H, %N, %Cl and %Pt.

Table 3.2-1: Elemental compositions of YH9, YH10, YH11 and YH12

	YH9		YH10	
	Calculated (%)	Observed (%)	Calculated (%)	Observed (%)
C	15.88	16.03	10.26	10.39
H	2.13	2.27	2.01	1.89
N	7.41	7.27	11.97	12.09
Cl	18.75	18.55	20.20	20.15
Pt	51.59	51.35	55.56	55.12
	YH11		YH12	
C	15.88	15.93	20.96	21.32
H	2.13	2.03	2.26	1.99
N	7.41	7.32	10.47	10.37
Cl	18.75	19.20	17.68	16.88
Pt	51.59	52.0	48.63	48.11

3.3 Molar conductivity

Table 3.3-1 and Figure 3.3-1 give the molar conductivity values (in ohm^{-1} cm^2 mol^{-1}) of YH9, YH10, YH11 and YH12. Figure 3.3-1 gives the plot of molar conductivity values against concentration of the compounds.

Table 3.3-1: Molar conductivity values (in ohm^{-1} cm^2 mol^{-1}) of YH9, YH10, YH11 and YH12

Concentration (mM)	YH9	YH10	YH11	YH12
0.5	10.4	7.2	3.6	11.8
0.25	14.4	7.2	5.2	14.8
0.1	18	10	11	19
0.01	70	70	90	120
0	78	78	102	132

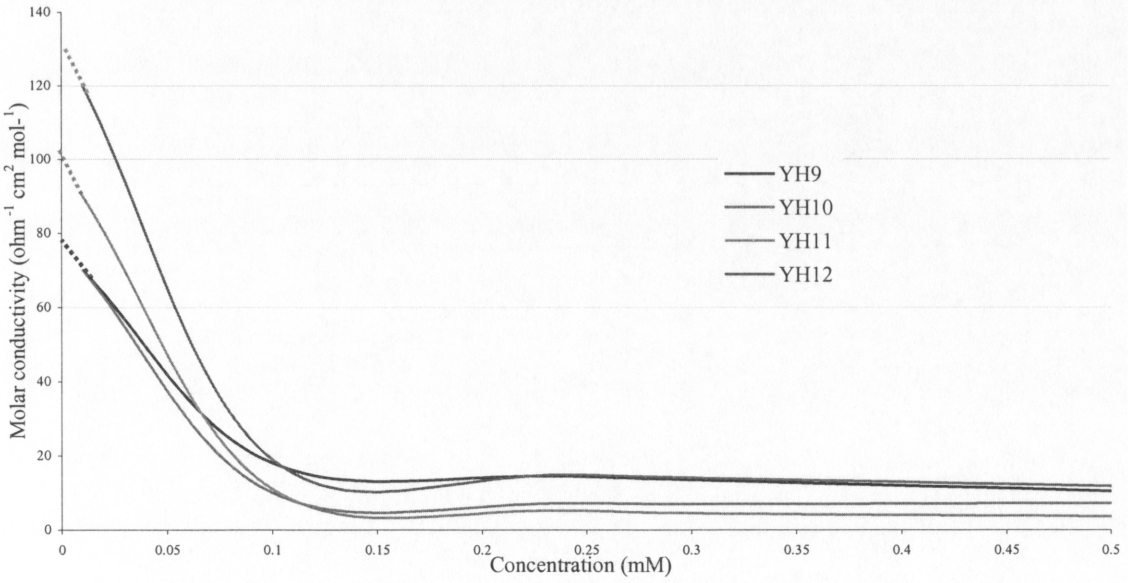


Figure 3.3-1: Molar conductivity (in ohm^{-1} cm^2 mol^{-1}) of YH9, YH10, YH11 and YH12

The extrapolated values of molar conductivity values in ohm^{-1} cm^2 mol^{-1} at zero concentration (Λ_0) were found to be 78, 78, 102 and 132 for YH9, YH10, YH11 and YH12 respectively.

3.4 Spectral analyses

IR, Raman, mass and ^1H NMR spectral analyses were carried out to aid in the structural characterization of YH9, YH10, YH11 and YH12.

3.4.1 The IR and Raman spectra of YH9, YH10, YH11 and YH12

Figures 3.4-1, 3.4-2, 3.4-3 and 3.4-4 give the IR spectra of YH9, YH10, YH11 and YH12 respectively and Figures 3.4-5, 3.4-6, 3.4-7 and 3.4-8 give the corresponding Raman spectra. The major peaks observed in IR and Raman spectra of YH9, YH10, YH11 and YH12 are listed in Table 3.4-1. More detailed discussions on the bands are given in Chapter 4.

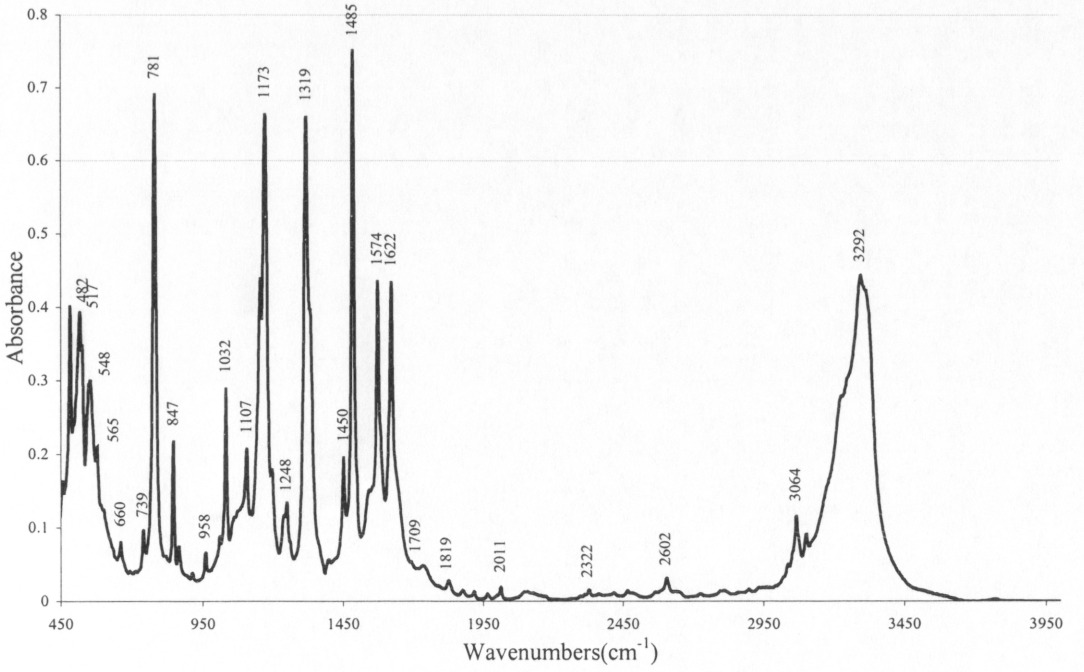


Figure 3.4-1: IR spectrum of YH9

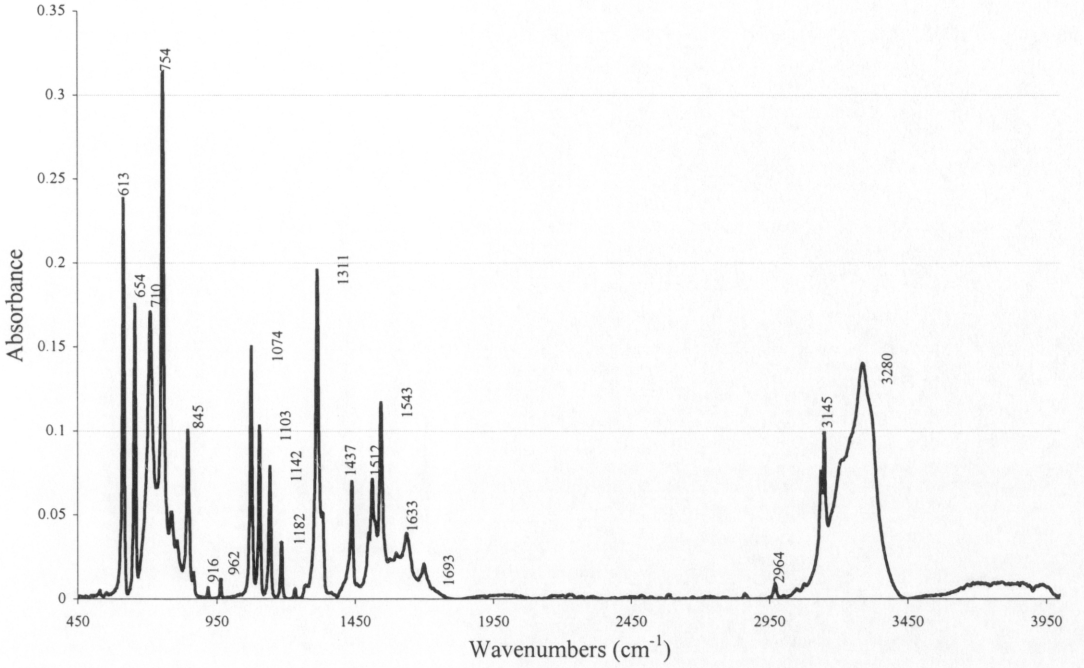


Figure 3.4-2: IR spectrum of YH10

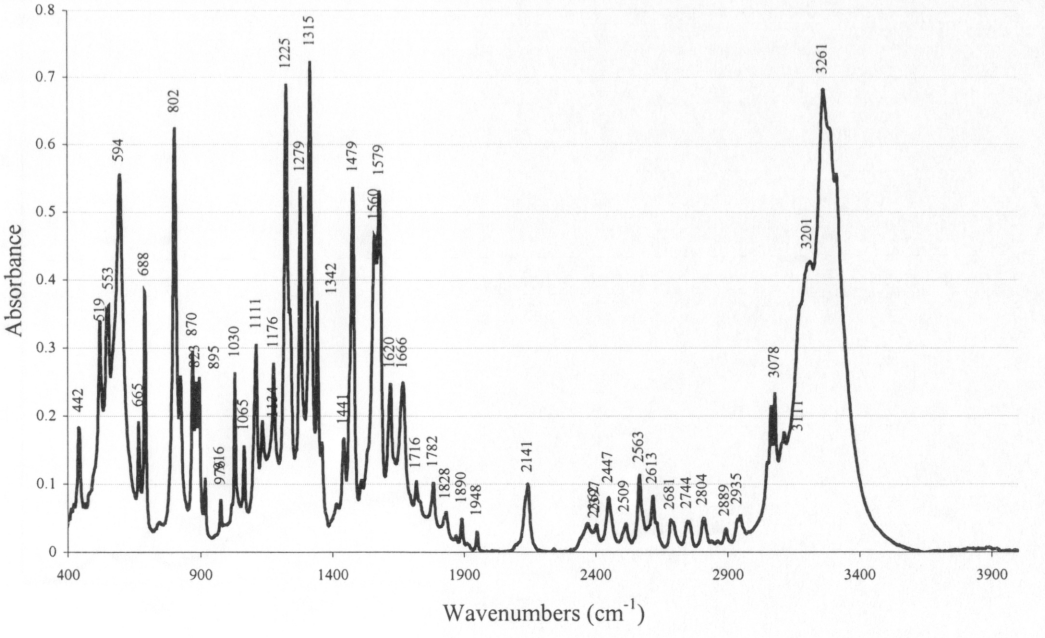


Figure 3.4-3: IR spectrum of YH11

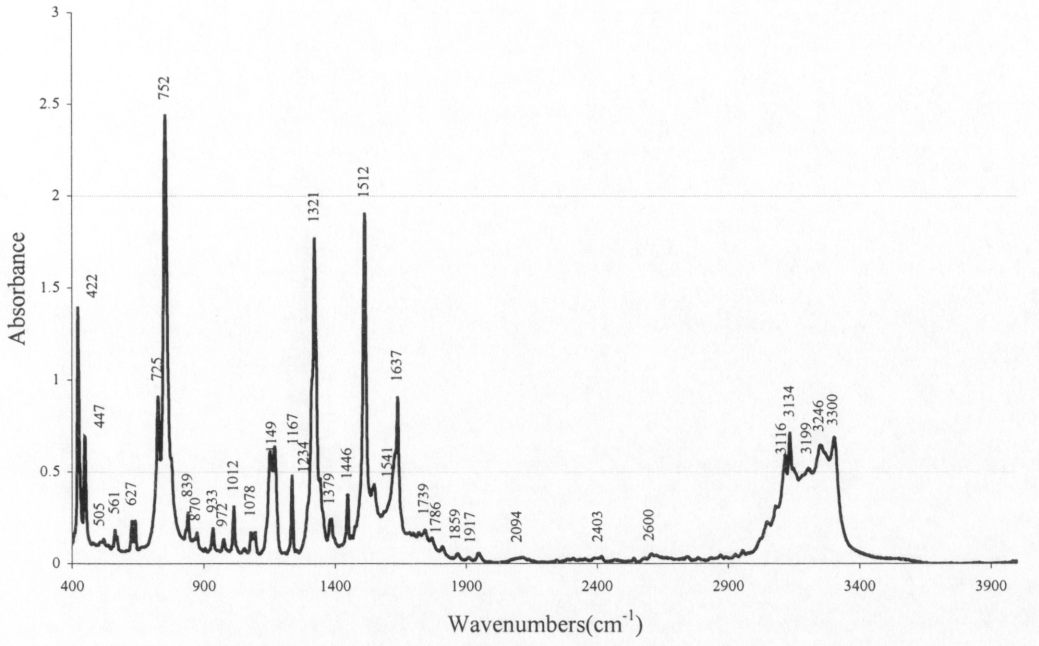


Figure 3.4-4: IR spectrum of YH12

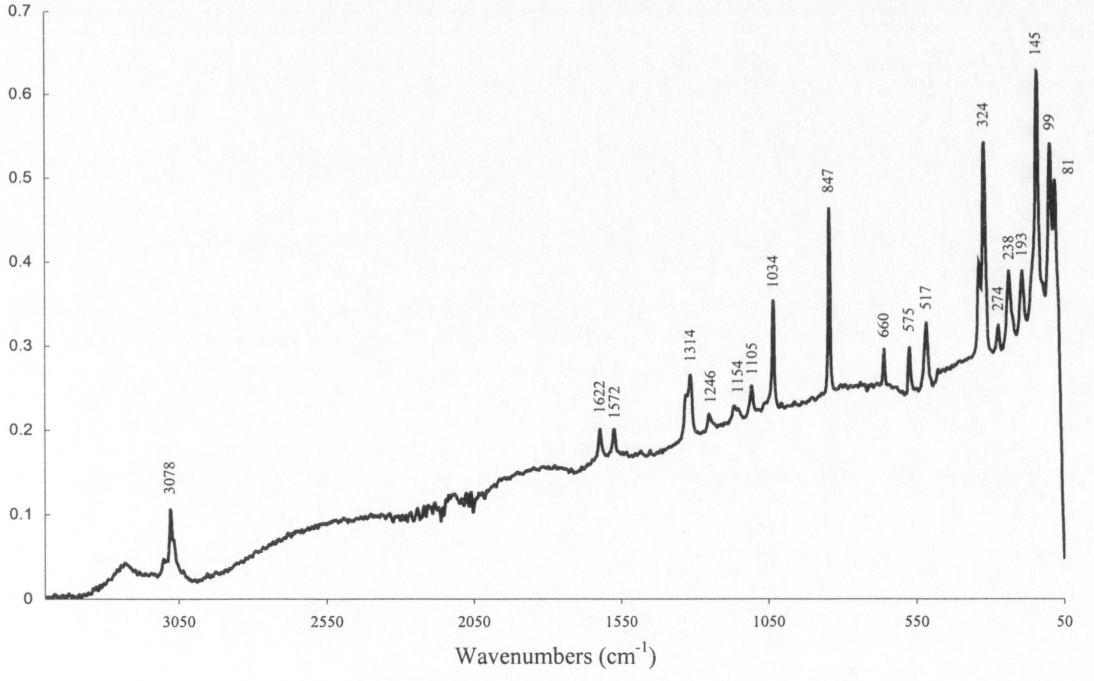


Figure 3.4-5: Raman spectrum of YH9

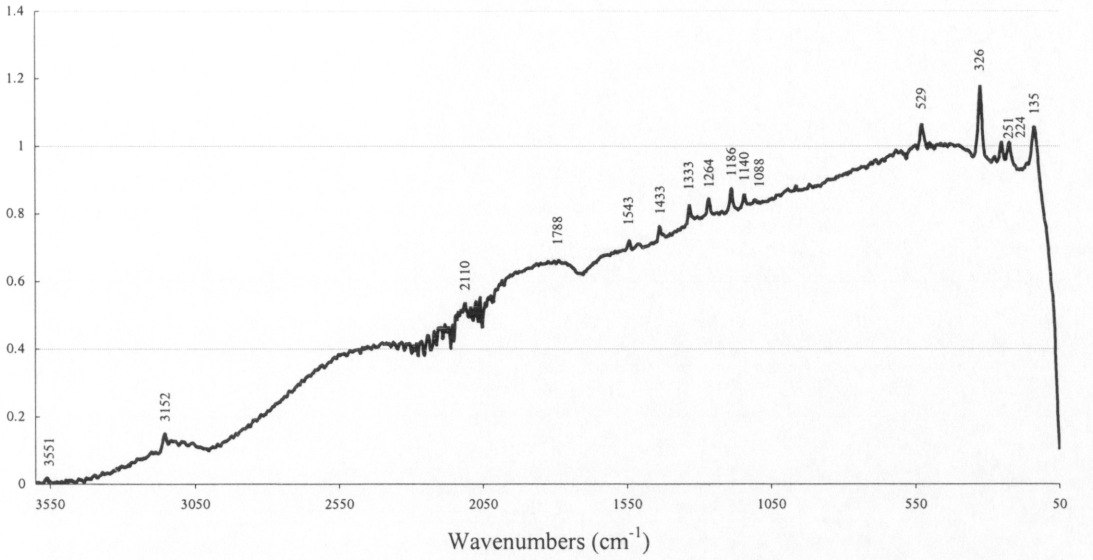


Figure 3.4-6: Raman spectrum of YH10

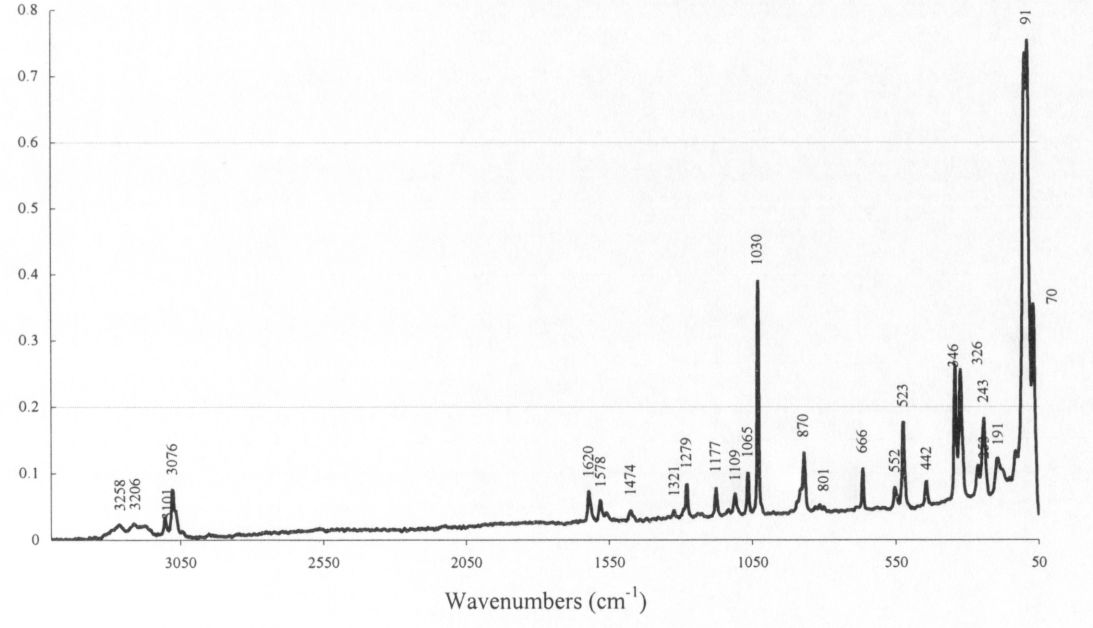


Figure 3.4-7: Raman spectrum of YH11

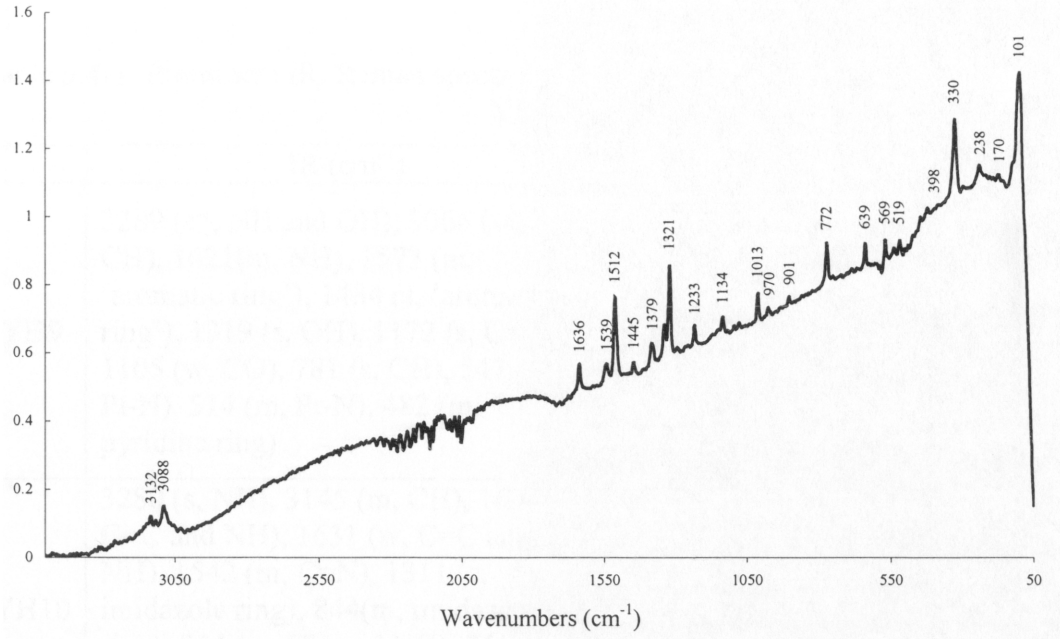


Figure 3.4-8: Raman spectrum of YH12

Table 3.4-1: Prominent IR, Raman spectral bands observed in YH9, YH10, YH11 and YH12.

	IR (cm^{-1})	Raman (cm^{-1})
YH9	3289 (s*, NH and OH), 3064 (w, CH), 1621(m, NH), 1573 (m, 'aromatic ring'), 1484 (s, 'aromatic ring'), 1319 (s, OH), 1172 (s, CO), 1105 (w, CO), 781 (s, CH), 547 (m, Pt-N), 514 (m, Pt-N), 482 (m, pyridine ring)	3160 (w, NH and CH), 1572 (w, NH), 1311 (w d, CH), 1240 (w, CH), 1153 (w, CH), 1034 (m, CH), 847 (s, CH), 659 (w, pyridine ring), 573 (w, Pt-N(NH_3)), 517 (w, Pt-N(NH_3)), 237 (w, Pt-N(2-hydroxypyridine)), 193(w, Pt-N(2-hydroxypyridine)), 143 (s, Pt-N), 81 (m, lattice)
YH10	3280 (s, NH), 3145 (m, CH), 1697 (w, C=C and NH), 1631 (w, C=C and NH), 1542 (m, C=N), 1311 (s, imidazole ring), 844(m, imidazole ring), 844 (m, CH and NH), 755 (s, CH and NH), 709 (m, CH), 653 (m, CH), 614 (s, CH and NH)	3151 (w, NH), 1782 (w br, 'aromatic' CH overtone), 1431 (w, CN), 1331 (w, CH), 1263 (w, NH), 1186 (w, NH), 1138 (w, NH), 524 (w, Pt-N), 326 (m, Pt-Cl), 249 (w, Pt-N(imidazole)), 222 (w, imidazole), 133 (Pt-N)
YH11	3261 (s br, NH and OH), 3077 (w, CH), 2562 (w, pyridine ring), 2447 (w, pyridine ring), 2141 (w, pyridine ring), 1887 (w, pyridine ring), 1610 (w, NH), 1579 (m, NH), 1315 (s, OH), 1224 (s, CO), 869 (m, CH), 802 (s, CH), 688 (m, pyridine ring), 593 (s, Pt-N(NH_3)), 545 (m, Pt-N(NH_3)), 518(m, Pt-N(NH_3)), 441 (w, pyridine ring)	3253 (w br, NH and OH), 3190 (w br, NH and OH), 3099 (w, CH), 3074 (w, CH), 1618 (w, pyridine ring), 1577 (w, NH), 1469 (w, NH), 1321 (w, OH), 1279 (w, CH), 1176 (w, CH), 1109 (w, CO), 1030 (s, CH), 870 (m, CH), 655 (w, CH), 523 (m, Pt-N), 442 (w, Pt-N), 345 (m, Pt-Cl), 316 (m, Pt-Cl), 243 (m, Pt-N(3-hydroxypyridine)), 187 (w, Pt-N), 91 (s, Pt-N), 67 (s, lattice)
YH12	3243 (m br, NH), 3243 (m br, NH), 3133 (m br, CH), 1637 (m, ring stretch), 1511 (s, ring stretch), 1446 (w, ring stretch), 1320 (s, ring), 1234 (w, C-N), 1149 (w br, C-N), 838 (w, ring), 752 (s, CH), 725 (m, CH), 447 (m, Pt-N), 422 (s, Pt-N)	3120 (w, NH), 3082 (w, CH), 1635 (w, ring), 1512 (m, ring), 1377 (w, ring), 1321 (m, NH), 1234 (w, NH), 1132 (w, NH), 1012 (w, CH), 887 (w, CH), 771 (w, CH), 638 (w, CH), 569 (w, P-N), 328 (m, Pt-Cl), 239 (w, Pt-N(ring), 100 (Pt-N)

* The letters 's', 'm', 'w' and 'br' stand for 'strong', 'medium', 'weak' and 'broad' respectively

3.4.2 The Mass Spectra of YH9, YH10, YH11 and YH12

Figures 3.4-9, 3.4-10, 3.4-11, 3.4-12, 3.4-13, 3.4-14, 3.4-16 and 3.4-16 give the mass spectra of YH9, YH10, YH11 and YH12 respectively. The major peaks observed in

the mass spectra of YH9, YH10, YH11 and YH12 are listed in Table 3.4-2. More detailed discussions of the peaks are given in Chapter 4.

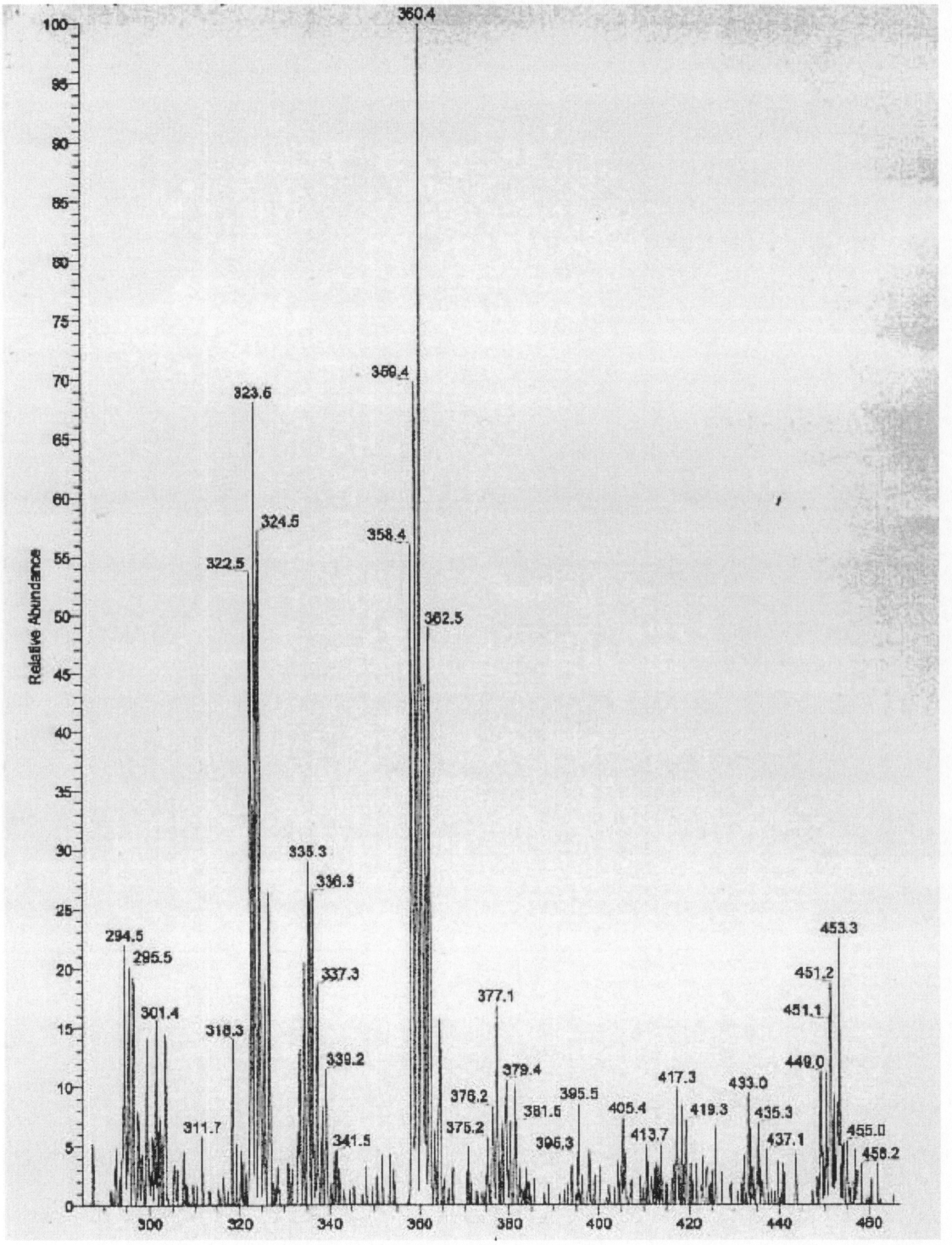


Figure 3.4-9: The mass spectrum of YH9 – spectrum 1

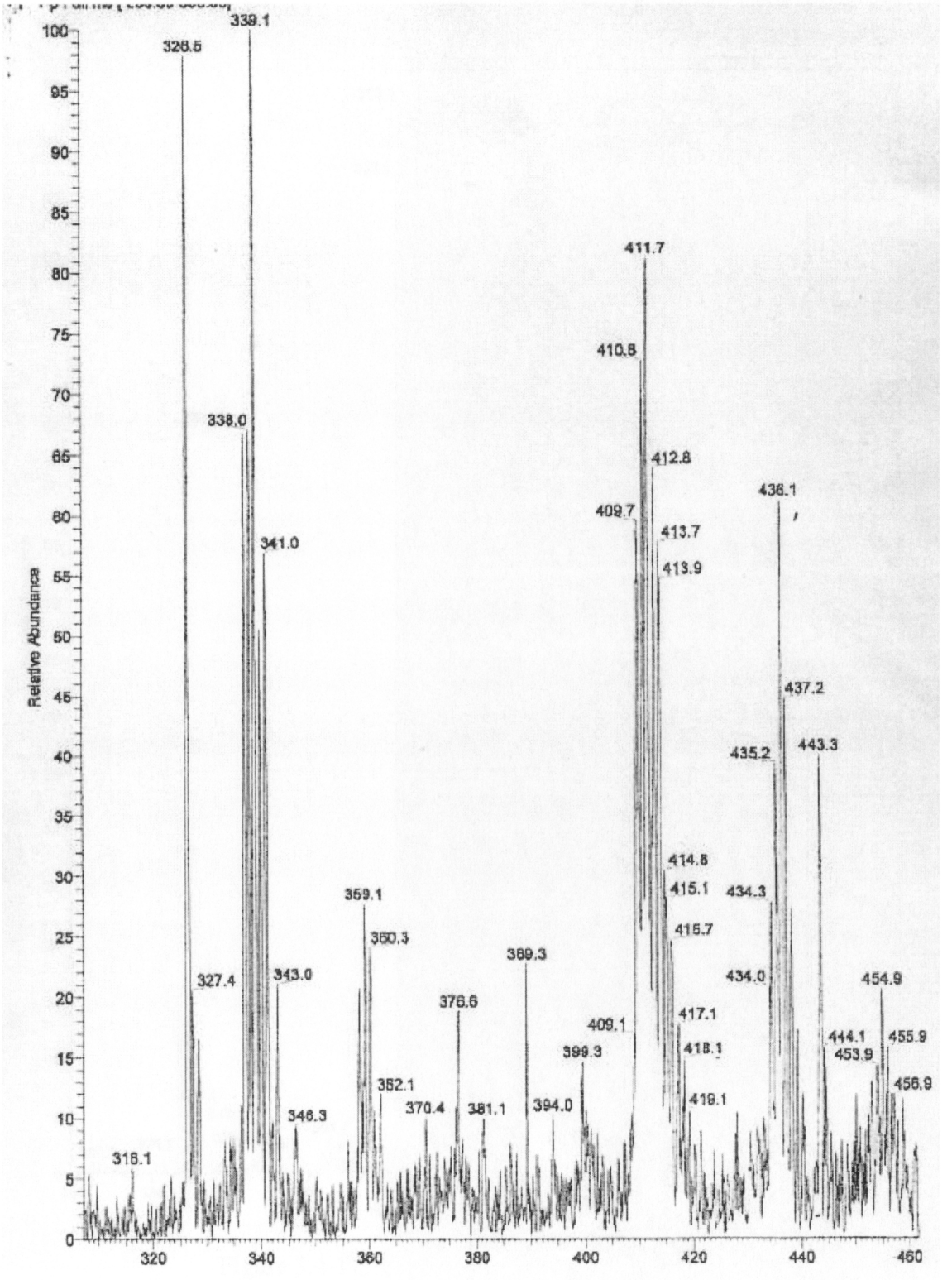


Figure 3.4-10: The mass spectrum of YH9 – spectrum 2

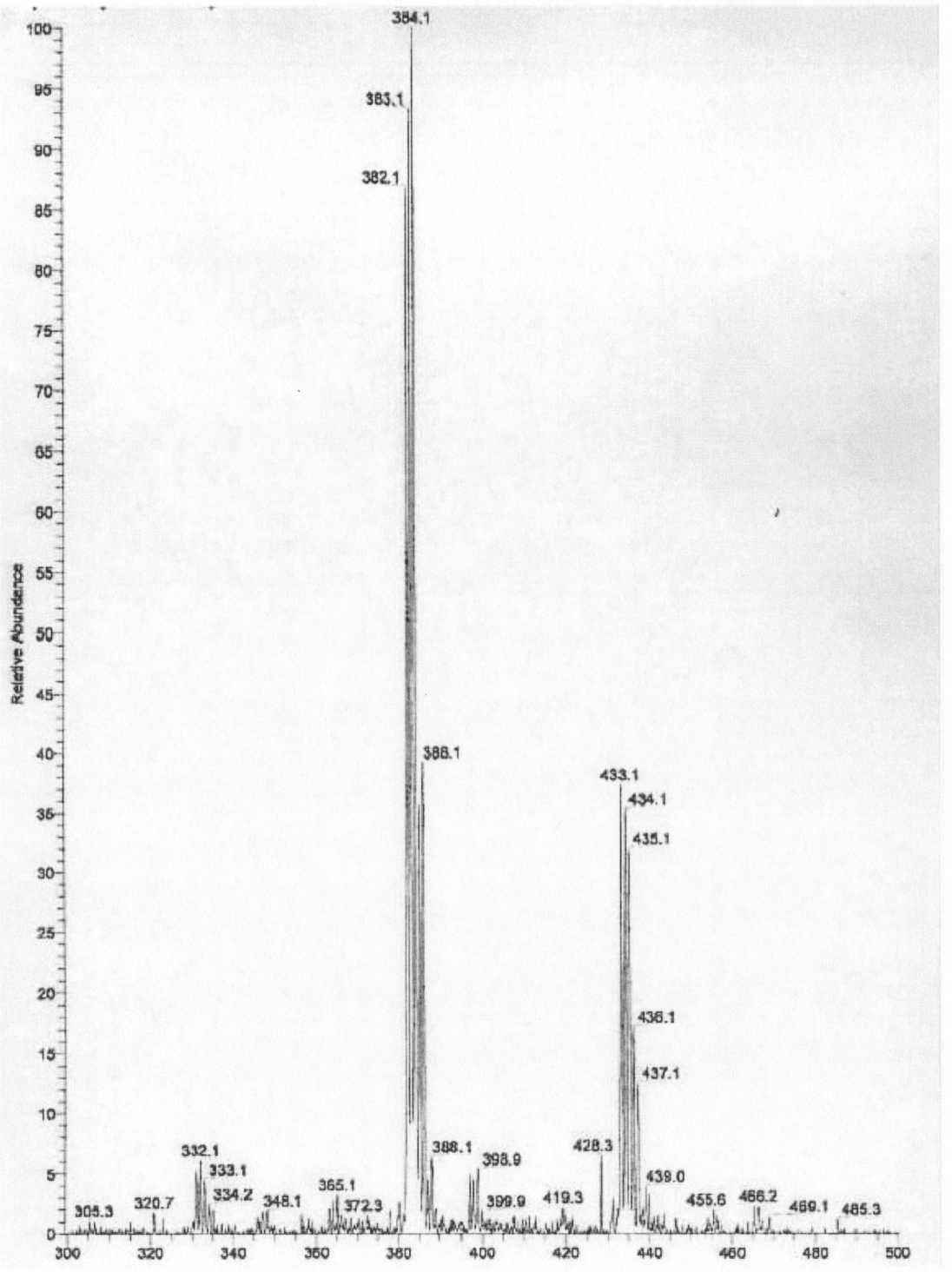


Figure 3.4-11: The mass spectrum of YH10

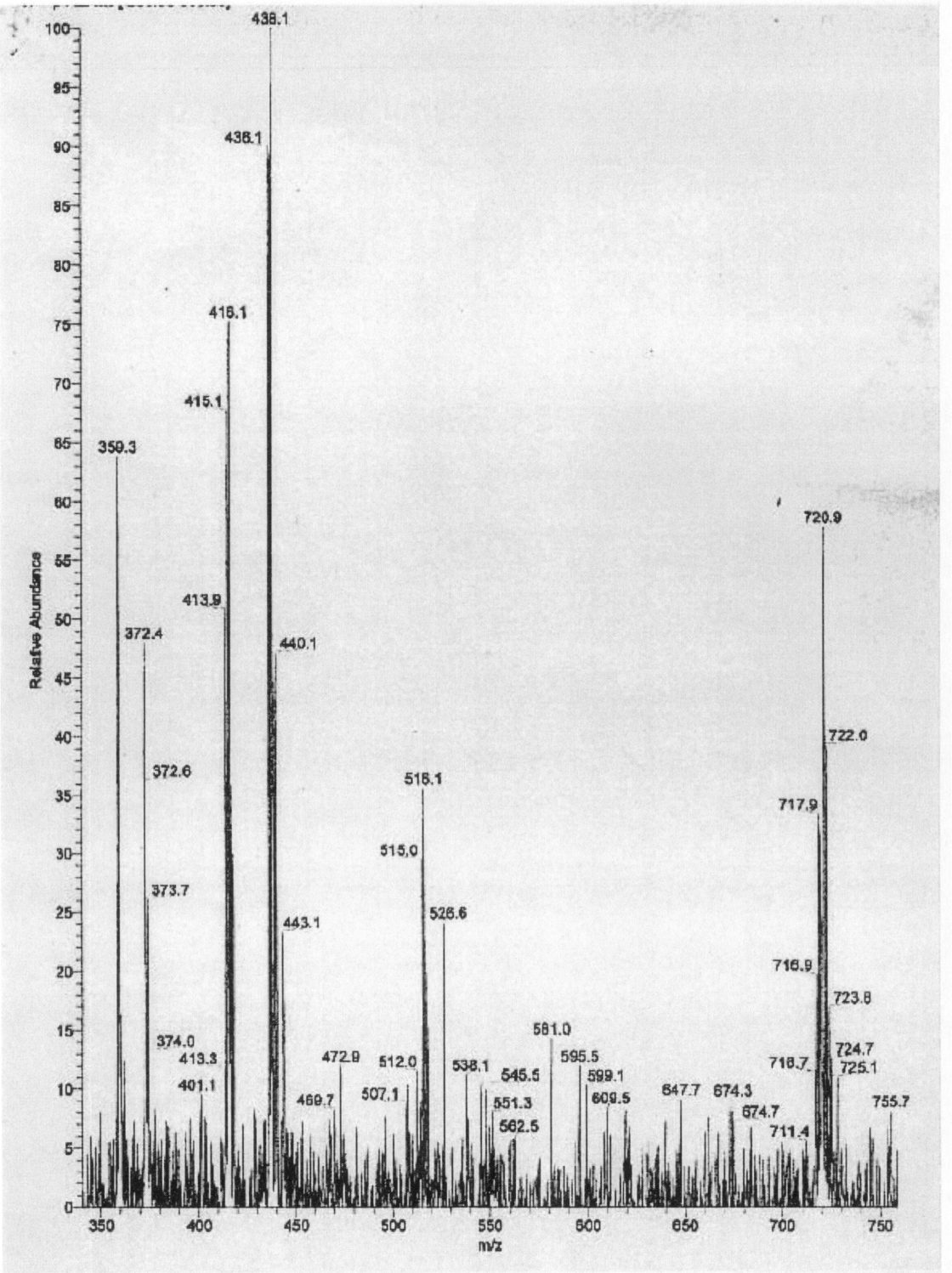


Figure 3.4-12: The Mass spectrum of YH11 – spectrum 1

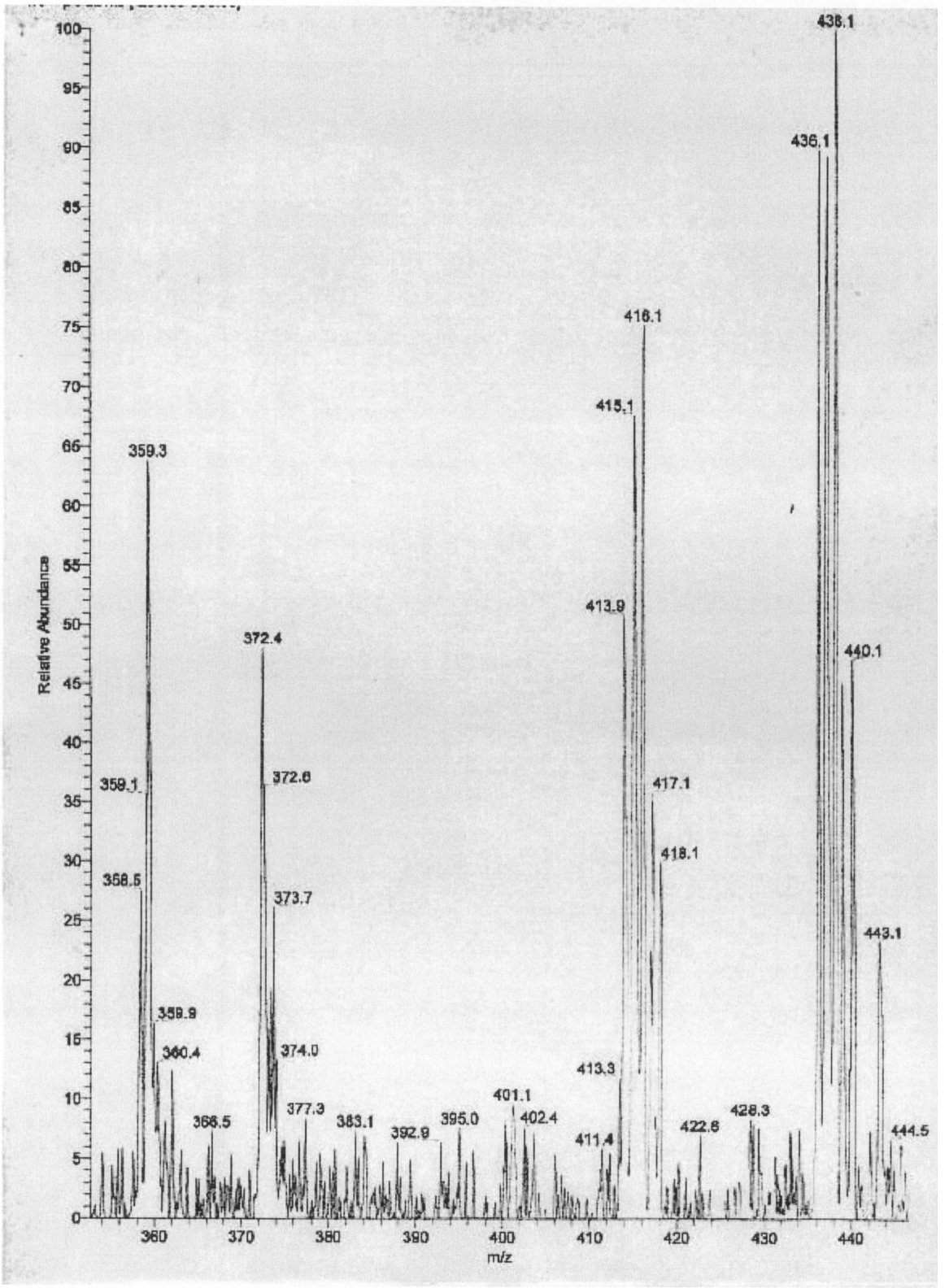


Figure 3.4-13: The mass spectrum of YH11 – spectrum 2

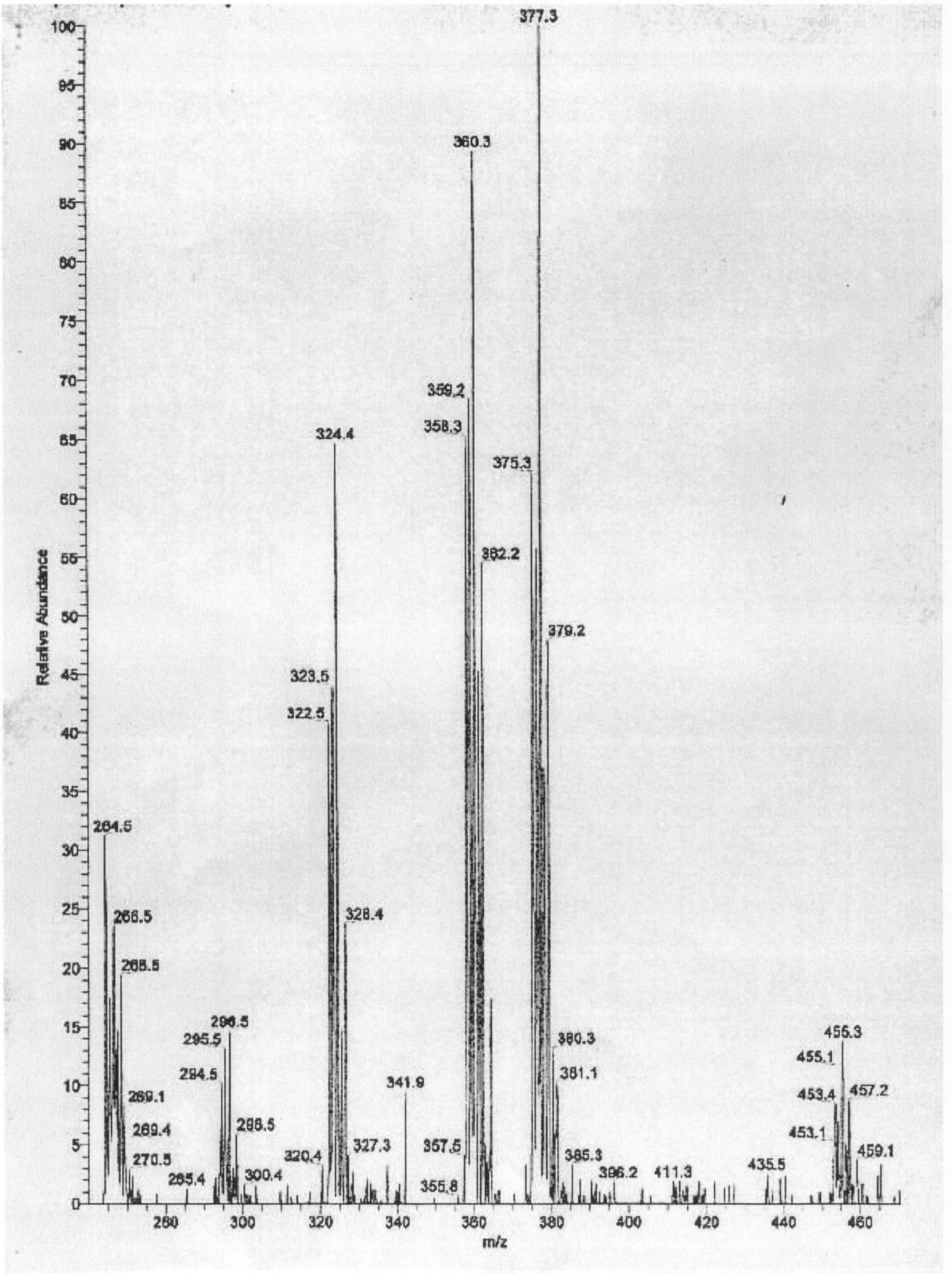


Figure 3.4-14: The mass spectrum of YH11 – spectrum 3

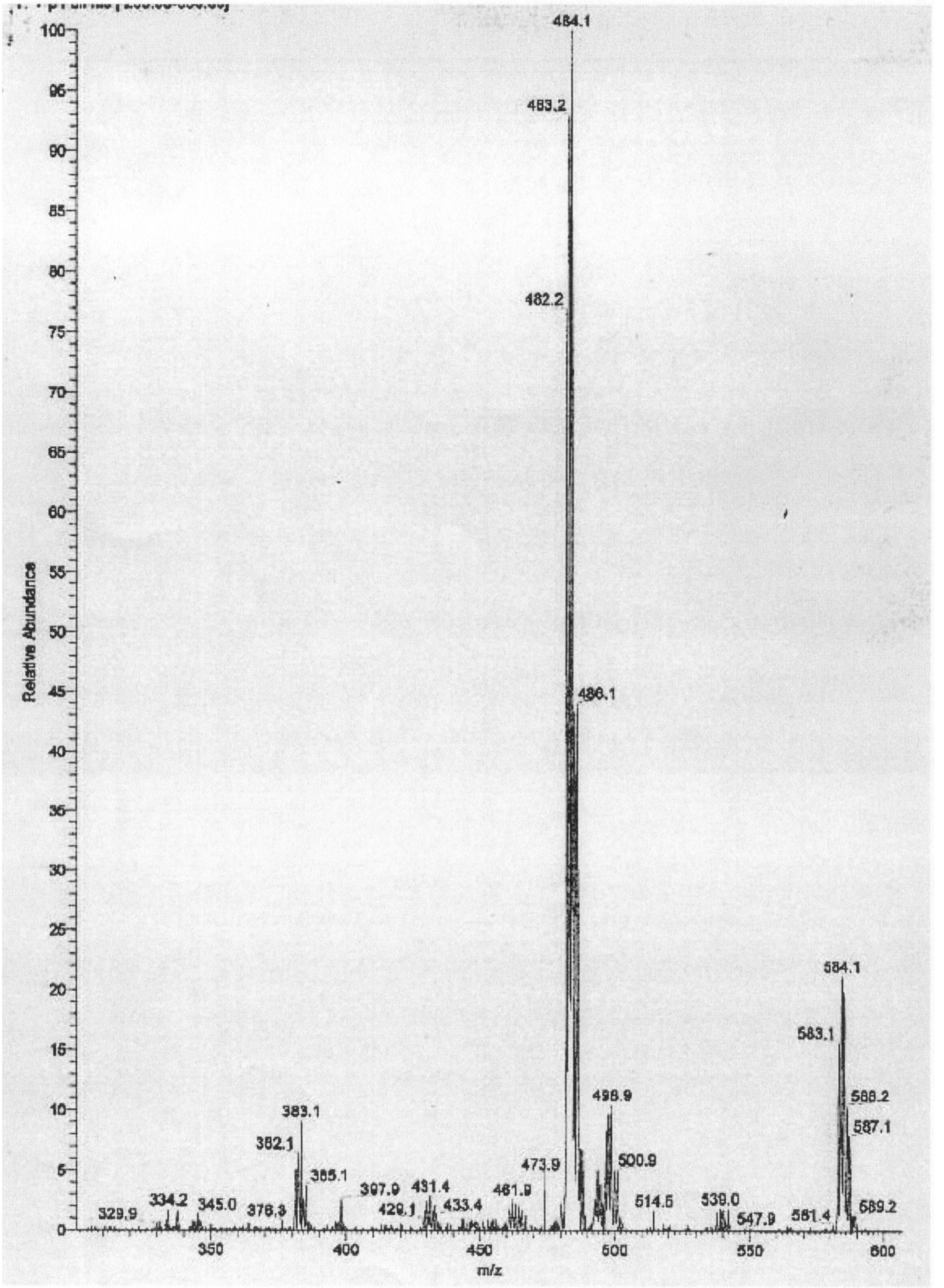


Figure 3.4-15: The spectrum of YH12 – spectrum 1

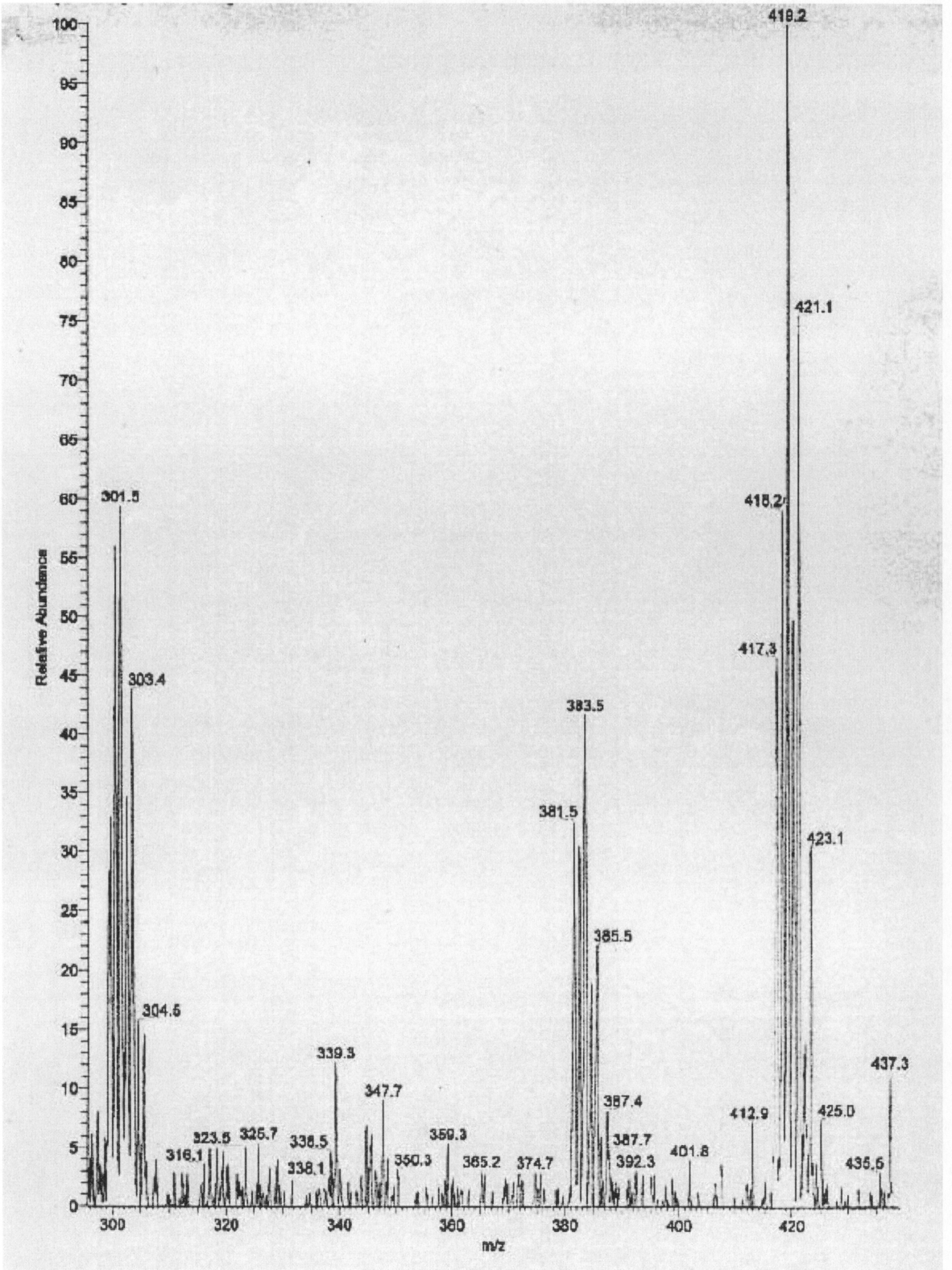


Figure 3.4-16: The mass spectrum of YH12 – spectrum 2

Table 3.4-2: The Mass spectra of YH9, YH10, YH11, and YH12 where the number in parentheses after each m/z value indicates the relative intensity.

	ESI Mass (m/z)
YH9	ESI-MS (DMF) (M - H) = 377 (0.17); (M – H – NH_3) = 360; (M – Cl – 4H) = 339 (1.00); (M – Cl – NH_3 – 2H) = 324 (0.69)).
YH10	ESI-MS (DMF) (Pt(imidazole)$_3$Cl = 433 (0.38); (M – Cl + imidazole) = 384; (M – NH_3 – 2H) = 332 (0.07)).
YH11	EIS-MS (DMF) (PtCl(3-hydroxypyridine)$_3$ = 516 (0.36); (M – Cl + 3-hydroxypyridine) = 438 (1.00) ; (M – H) = 377 (0.09); (M – H – NH_3) = 360; (M – Cl – NH_3 – H) = 324 (0.66); (M – Cl – 3-hydroxypyridine + NH_3) = 265 (0.31)).
YH12	ESI-MS (DMF) ((PtCl{imidazo(1,2-α)pyridine}$_3$ – H) = 584 (0.22); Pt(NH_3)(imidazo(1,2-α)pyridine)$_2$Cl = 484 (1.00); Pt (imidazo(1,2-α)pyridine)$_2$ = 431 (0.04); M – NH_3 – H = 383 (0.09); $PtCl_3$ = 301 (0.59)).

3.4.3 The nuclear magnetic resonance spectra (NMR) of YH9, YH10, YH11 and YH12

Figures 3.4-17, 3.4-18, 3.4-19 and 3.4-20 give the ^{1}H NMR spectra of YH9, YH10, YH11 and YH12 respectively. The prominent peaks observed in ^{1}H NMR spectra of YH9, YH10, YH11, and YH12 are listed in Table 3.4-3. More detailed discussions of the peaks are given in Chapter 4.

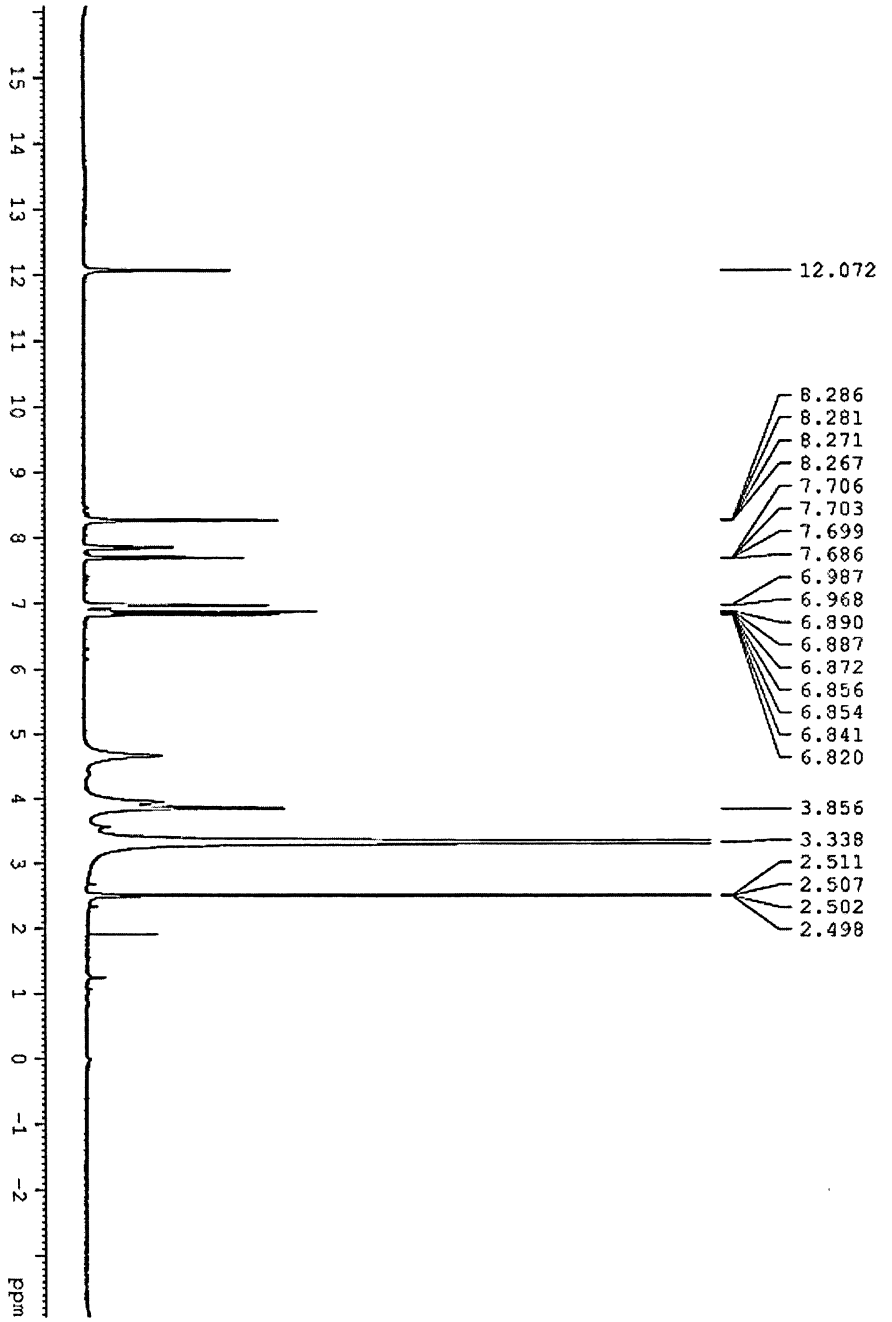


Figure 3.4-17: ^{1}H NMR spectrum of YH9

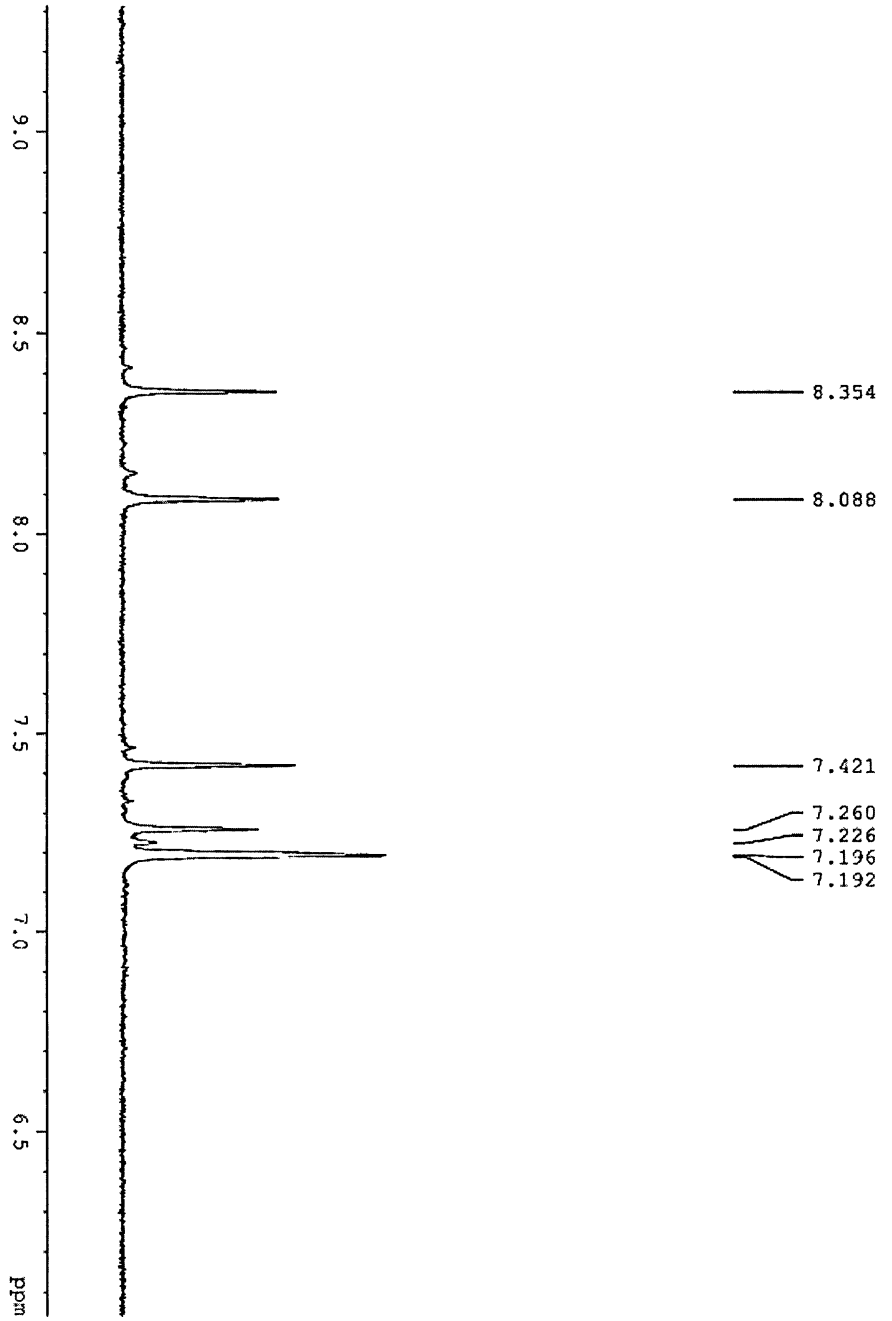


Figure 3.4-18: ^{1}H NMR spectrum of YH10

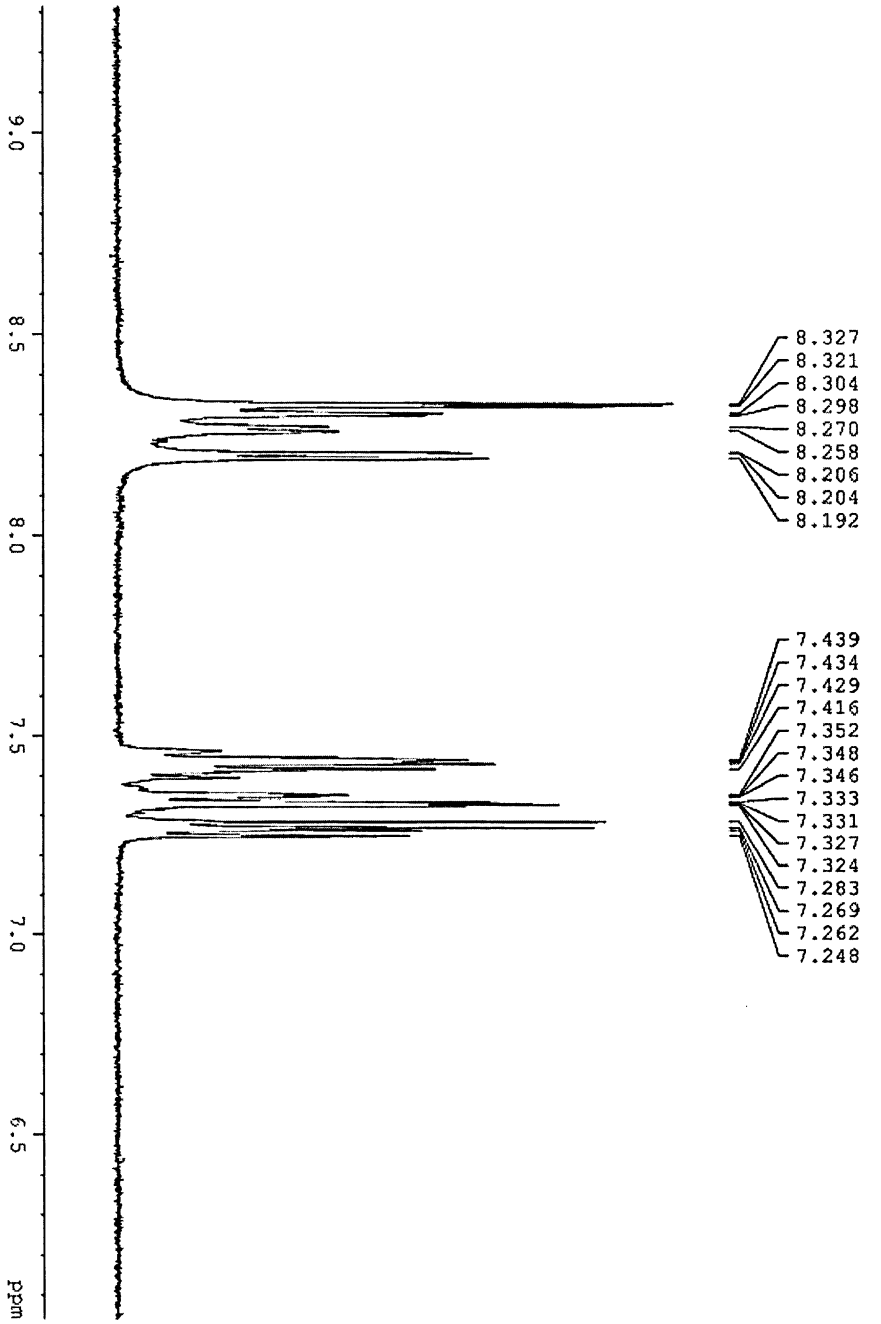


Figure 3.4-19: ^{1}H NMR spectrum of YH11

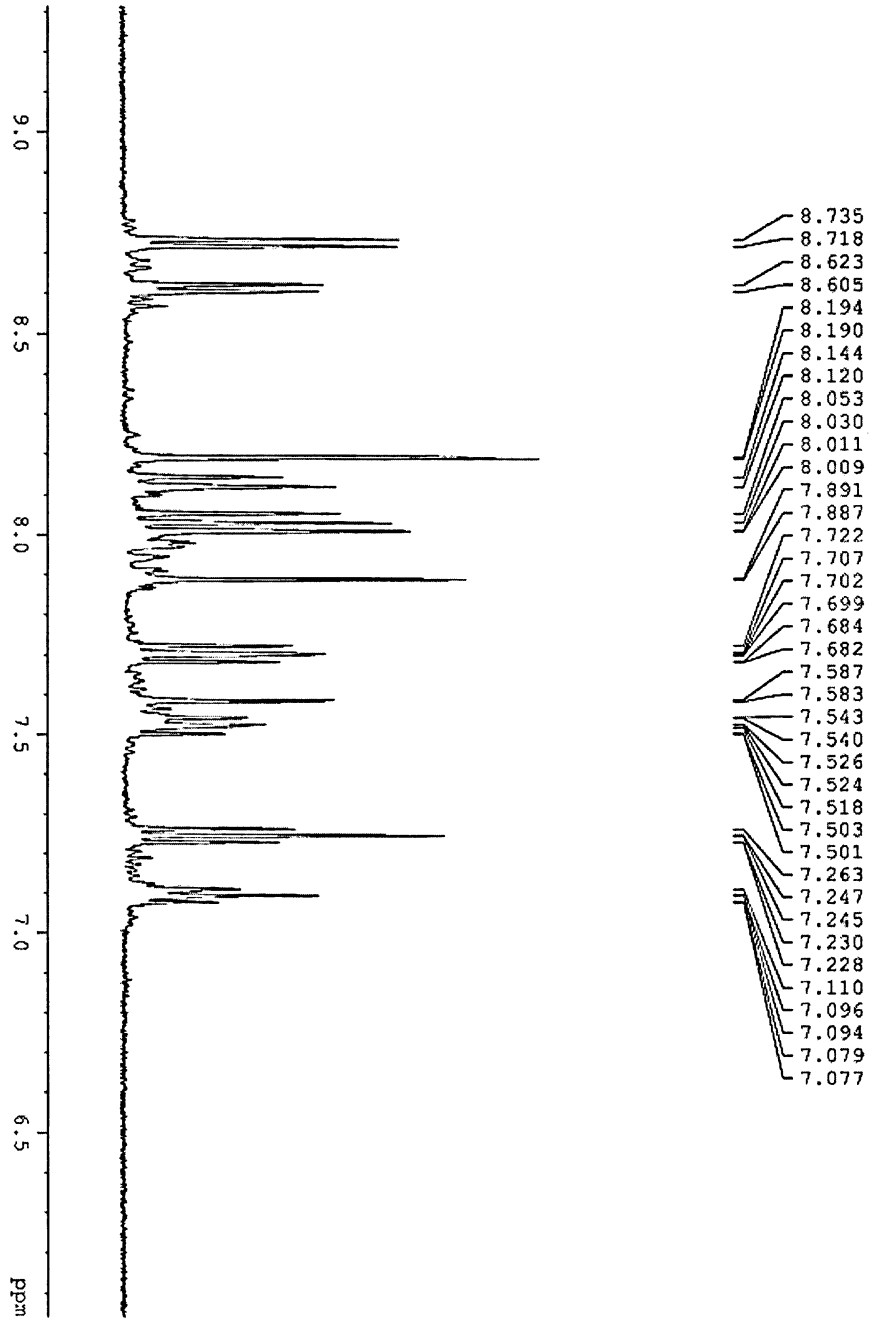


Figure 3.4-20: ^{1}H NMR spectrum of YH12

Table 3.4-3: Prominent peaks observed in ^{1}H NMR spectra of YH9, YH10, YH11, and YH12

	^{1}H NMR
YH9	^{1} H NMR DMSO δ ppm: 12.07 (s*, due to OH); 8.28; 7.85 (t, ??); 7.70 (quartet, due to CH Para); 6.85 (quartet, CH meta); 4.60 (s, due to NH-Pt); 3.86 (d; NH-Pt); 3.34 (s, due to water); 2.50 (due to DMSO)
YH10	^{1}H NMR DMSO δ ppm: 8.35 (s, due to NH); 8.09 (s, due to CH); 7.42 (s, due to CH); 7.23 (q, due to CH); 4.60 (s, due to NH-Pt); 3.76 (d, due to NH-Pt); 3.32 (s, due to water); 2.50 (due to DMSO).
YH11	^{1}H NMR DMSO δ ppm: 10.80 (s, due to OH); 8.32 (s, due to CH Ortho); 8.30 (d, due to CH); 8.26 (d, due to CH); 7.33 (q, due to CH); 6.85 (q, due to CH meta); 4.60 (s, due to NH-Pt); 3.90 (d; due to NH-Pt); 3.32 (s, due to water); 2.50 (due to DMSO)
YH12	^{1}H NMR DMSO δ ppm: 8.73 (d, due to CH); 8.61 (d, due to CH); 8.19 (d, due to CH); 8.03 (q, due to CH); 7.70 (q, due to CH); 4.60 (s, due to NH-Pt); 3.96 (d; due to NH-Pt); 3.34 (s, due to water); 2.50 (due to DMSO)

* The letters 's', 'd', 't' and 'q' stand for 'singlet', 'doublet', 'triplet' and 'quartet' respectively.

3.5 Interaction with pBR322 plasmid DNA and ssDNA

Figure 3.5-1 gives the electrophoretograms applying to the interaction of pBR322 plasmid DNA with cisplatin, transplatin, YH9, YH10, YH11 and YH12 at concentrations of compounds ranging from 1 μM to 60 μM. Lane 1 applies to pBR322 plasmid DNA only, lanes 2 to 6 apply to plasmid DNA interacted with increasing concentrations of the compounds. Cisplatin and transplatin serve as references. As pBR322 plasmid DNA, which is initially found to be a mixture of supercoiled form I and singly-nicked circular form II, is allowed to interact with

increasing concentrations of compounds, the mobility of the form I band decreases while that of the form II band remains essentially unchanged so that the two bands co-migrate at concentrations: 5 μM for cisplatin, YH11 and YH12, 10 μM for transplatin and YH10, 20 μM for YH9, beyond which the bands separate again. The changes in mobility of the bands are believed to be due to covalent binding of the compounds with DNA. More detailed discussions on the actual nature of binding of the compounds with pBR322 plasmid DNA are given in Chapter 4.

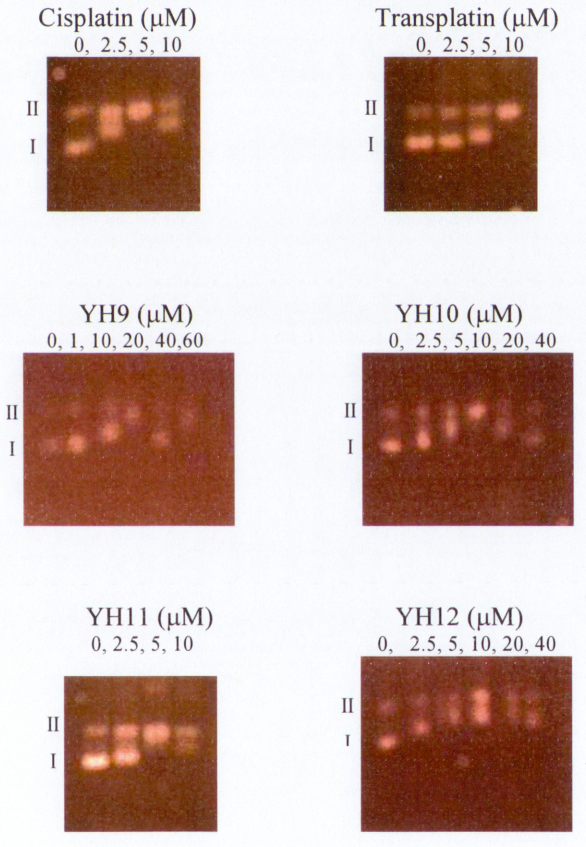
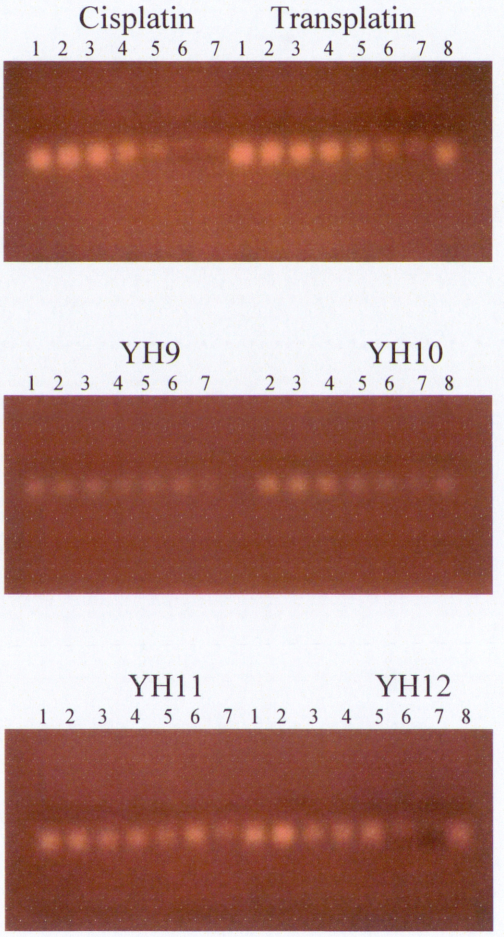


Figure 3.5-1: Interaction between pBR322 and platinum complexes

Figure 3.5-2 gives the electrophoretograms applying to the interaction of ssDNA with increasing concentrations of cisplatin, transplatin, YH9, YH10, YH11 and YH12 ranging from 1 μM to 160 μM. Lane 1 and 8 applies to ssDNA only, lanes 2 to 6

apply to ssDNA interacted with increasing concentrations of the compounds. Cisplatin and transplatin, again, serve as references. As ssDNA is allowed to interact with increasing concentrations of compounds, the intensity of the platinated DNA band decreases. The decrease in intensity is more pronounced at concentration ≥ 40 μM for cisplatin, 80 YH12 and 160 μM for transplatin, YH9, YH10, and YH11. There is no significant change in the mobility of the band with the change in concentration of the compounds.



line	1	2	3	4	5	6	7	8
Compounds (μM)	0	5	10	20	40	80	160	0

Figure 3.5-2: Interaction between ssDNA and platinum complexes

3.6 BamH1 restriction enzyme digestion

As stated earlier, BamH1 digestion was carried out to provide information on changes in DNA conformation due to its binding with the platinum compounds. Figure 3.6-1 gives the electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and cisplatin, transplatin, YH9, YH10, YH11 and YH12 following their digestion with BamH1 for a period of 1 h at 37°C. The concentrations of compounds were varied from 2.5 μM to 10 μM. Lane 1 applies to pBR322 plasmid DNA only, lanes 2 to 6 apply to plasmid DNA incubated with increasing concentrations of compounds.

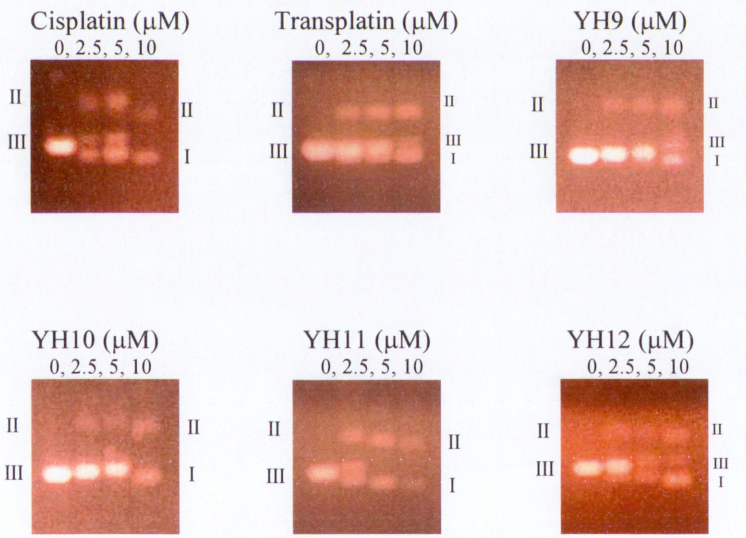


Figure 3.6-1: Gel electrophoresis of BamH1 treated drug-pBR322 DNA

In the absence of the platinum compounds, only the linear form III band was observed, indicating that all of the pBR322 plasmid DNA was doubly nicked by BamH1 at the specific GG site. As the concentration of the compounds was increased, it was found that BamH1 digestion was increasingly prevented producing a mixture of forms I, II

and III at low concentrations of compounds and a mixture of forms I and II only at higher concentrations (10 μM for cisplatin and YH10, 5 μM for YH11). Table 3.6-1 lists the bands observed at different concentrations of the compounds.

Table 3.6-1: Bands observed after BamH1 digestion of incubated mixtures of drug-pBR322 plasmid DNA

Drug	[Drug] in μM			
	0	2.5	5	10
Cisplatin	III	I, II, III	I, II, III	I, II
Transplatin	III	II, III	I, II, III	I, II, III
YH9	III	II, III	II, III	I, II, III
YH10	III	II, III	II, III	I, II
YH11	III	I, II, III	I, II	I, II
YH12	III	I, II, III	I, II, III	I, II, III

3.7 Cytotoxicity

The antitumour activity of the four *trans*-planaramineplatinum(II) compounds YH9, YH10, YH11 and YH12 along with that for cisplatin, against human cancer cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538, was determined using MTT reduction assay. A serial fivefold dilution of the compounds namely 31.25 μM, 6.25 μM, 1.25 μM, 0.25 μM, and 0.05 μM was used.

3.7.1 Cytotoxicity of YH9

Table 3.7-1 gives the percentages of viable cells at various concentrations of YH9 (from 0.05 μM to 31.25 μM) along with IC_{50} values, as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Figure 3.7-1 gives the corresponding cell survival curves. The IC_{50} values for YH9 are: A2780: 14.9 ± 8.1 μM, $A2780^{cisR}$: 18.5 ± 13.5 μM, $A2780^{ZD0473R}$: 10.9 ± 1.1 μM, NCI-H460: 44.7 ± 6.8 μM and Me-10538: 26.3 ± 2.3 μM, indicating that the compound is most active against cell line: $A2780^{ZD0473R}$ and least active against the cell line: NCI-H460.

Table 3.7-1: Viable cells with YH9 as a percentage of control as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

YH9	%	%	%	%	%
Concentration (μM)	A2780	$A2780^{cisR}$	$A2780^{ZD0473R}$	NCI-H460	Me-10538
31.25	37.7 ± 10.1	36.5 ± 14.6	14.7 ± 2.5	61.26 ± 3.4	47.4 ± 1.8
6.25	61.5 ± 7.5	62.7 ± 6.2	68.7 ± 4.2	71.2 ± 4.1	69.0 ± 7.9
1.25	72.5 ± 9.0	70.7 ± 4.8	80.3 ± 3.4	78.28 ± 2.5	75.4 ± 8.1
0.25	80.3 ± 5.9	78.3 ± 5.5	89.7 ± 2.0	83.68 ± 1.2	82.1 ± 7.7
0.05	88.4 ± 3.3	85.3 ± 5.1	94.3 ± 3.6	91.8 ± 0.7	89.2 ± 5.2
IC_{50} (μM)	14.9 ± 8.1	18.5 ± 13.5	10.9 ± 1.1	44.7 ± 6.8	26.3 ± 2.3

Where %: percentage of viable cells

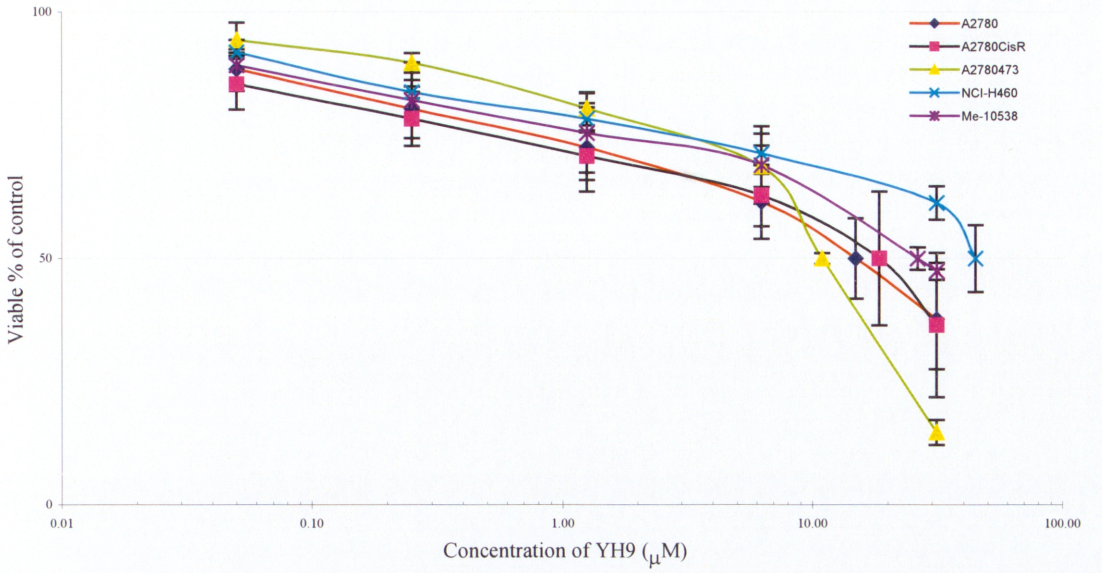


Figure 3.7-1: Cell survival curves for YH9 as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

3.7.2 Cytotoxicity of YH10

Table 3.7-2 gives the percentages of viable cells at various concentrations of YH10 (from 0.05 μM to 31.25 μM) along with IC_{50} values, as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Figure 3.7-2 gives the corresponding cell survival curves. The IC_{50} values for YH10 are: A2780: 13.2 ± 1.5 μM, $A2780^{cisR}$: 16.5 ± 3.4 μM, $A2780^{ZD0473R}$: 17.9 ± 3.5 μM, NCI-H460: 39.0 ± 4.4 μM, and Me-10538: 23.0 ± 2.2 μM, indicating that the compound is most active against cell line: A2780 and least active against the cell line: NCI-H460.

Table 3.7-2: A2780, A2780^{cisR}, A2780^{ZD0473R}, NCI-H460, and Me-10538 Viable cells as a percentage of control with YH10

YH10	%	%	%	%	%
Concentration (μM)	A2780	A2780^{cisR}	A2780^{ZD0473R}	NCI-H460	Me-10538
31.25	20.9 ± 5.1	39.8 ± 8.1	39.0 ± 2.6	58.4 ± 3.0	44.3 ± 2.5
6.25	75.1 ± 4.1	63.2 ± 5.8	70.9 ± 6.5	66.2 ± 3.9	73.2 ± 5.1
1.25	81.6 ± 4.8	75.0 ± 8.8	76.1 ± 7.3	71.2 ± 0.6	78.0 ± 3.8
0.25	87.5 ± 3.2	80.7 ± 8.8	82.0 ± 5.3	79.6 ± 4.5	83.4 ± 2.6
0.05	93.0 ± 1.2	88.0 ± 6.0	90.3 ± 2.8	88.6 ± 5.1	89.3 ± 1.6
IC_{50} (μM)	13.2 ± 1.5	16.5 ± 3.4	17.9 ± 3.5	39.0 ± 4.4	23.0 ± 2.2

Where %: percentage of viable cells

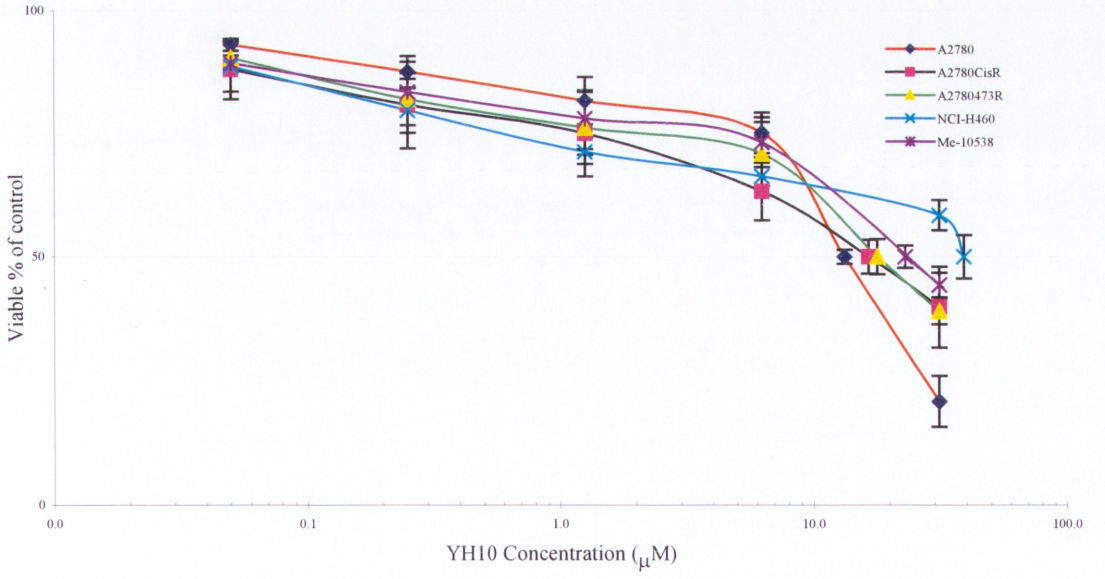


Figure 3.7-2: Cell survival curves for YH10 as applied to the cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, NCI-H460, and Me-10538

3.7.3 Cytotoxicity of YH11

Table 3.7-3 gives the percentages of viable cells at various concentrations of YH11 (from 0.05 μM to 31.25 μM) along with IC_{50} values, as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Figure 3.7-3 gives the corresponding cell survival curves. The IC_{50} values for YH11 are: A2780: 10.8 ± 1.2 μM, $A2780^{cisR}$: 15.0 ± 2.1 μM, $A2780^{ZD0473R}$: 16.2 ± 1.6 μM, NCI-H460: 33.8 ± 4.6 μM, and Me-10538: 20.0 ± 2.7 μM, indicating that the compound is most active against cell line: A2780 and least active against the cell line: NCI-H460.

Table 3.7-3: Viable cells with YH11 as a percentage of control as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

YH11	%	%	%	%	%
Concentration (μM)	A2780	$A2780^{cisR}$	$A2780^{ZD0473R}$	NCI-H460	Me-10538
31.25	23.5 ± 1.8	40.9 ± 2.3	33.3 ± 5.5	53.5 ± 3.0	43.8 ± 1.9
6.25	64.0 ± 4.7	60.8 ± 3.7	73.2 ± 4.4	61.7 ± 3.5	67.6 ± 8.9
1.25	79.9 ± 3.9	70.6 ± 2.9	78.5 ± 2.7	69.6 ± 1.4	76.6 ± 8.8
0.25	84.3 ± 2.5	76.7 ± 2.7	82.5 ± 0.6	76.9 ± 2.4	83.0 ± 6.7
0.05	89.7 ± 3.9	85.6 ± 4.1	91.1 ± 3.1	85.6 ± 4.1	91.5 ± 1.9
IC_{50} (μM)	10.8 ± 1.2	15.0 ± 2.1	16.2 ± 1.6	33.8 ± 4.6	20.0 ± 2.7

Where %: percentage of viable cells

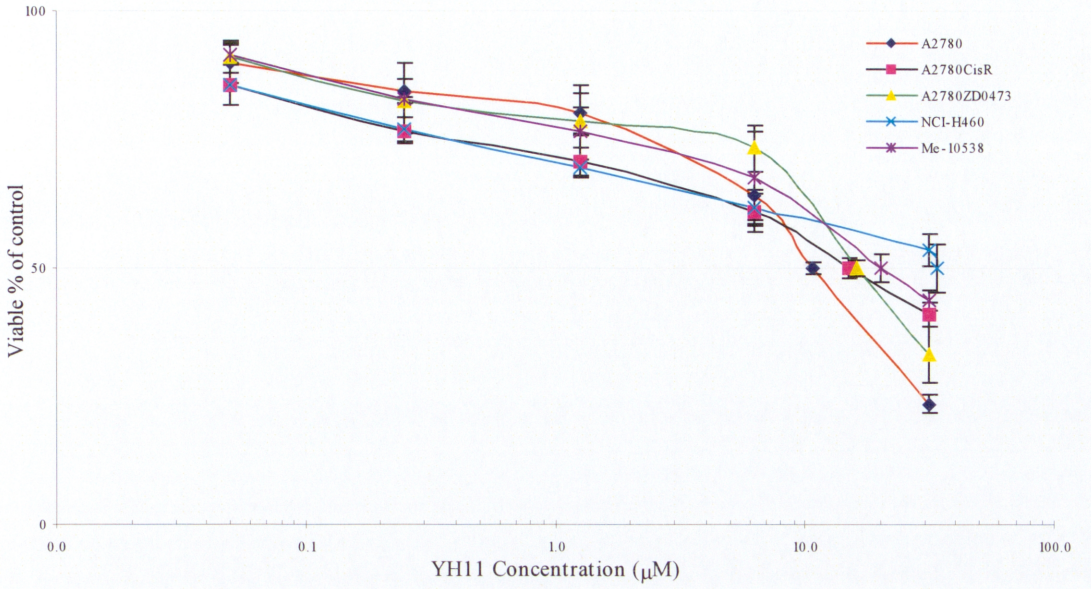


Figure 3.7-3: Cell survival curves for YH11 as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

3.7.4 Cytotoxicity of YH12

Table 3.7-4 gives the percentages of viable cells at various concentrations of YH12 (from 0.05 μM to 31.25 μM) along with IC_{50} values, as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Figure 3.7-4 gives the corresponding cell survival curves. The IC_{50} values for YH12 are: A2780: 4.4 ± 1.6 μM, $A2780^{cisR}$: 2.3 ± 0.5 μM, $A2780^{ZD0473R}$: 4.9 ± 0.3 μM, NCI-H460: 9.2 ± 2.0 μM, and Me-10538: 8.1 ± 1.3 μM, indicating that the compound is most active against cell line: $A2780^{cisR}$ and least active against the cell line: NCI-H460.

Table 3.7-4: A2780, A2780^{cisR}, A2780^{ZD0473R}, NCI-H460, and Me-10538 Viable cells as a percentage of control with YH12

YH12	%	%	%	%	%
Concentration (μM)	A2780	A2780^{cisR}	A2780^{ZD0473R}	NCI-H460	Me-10538
31.25	5.9 ± 2.9	3.1 ± 2.5	1.6 ± 0.8	19.2 ± 5.5	11.2 ± 1.7
6.25	44.8 ± 3.8	38.9 ± 1.1	45.8 ± 1.5	59.1 ± 5.7	57.6 ± 5.2
1.25	69.2 ± 11.4	57.0 ± 3.1	72.5 ± 4.9	72.2 ± 5.1	71.2 ± 2.6
0.25	80.8 ± 9.7	73.0 ± 2.3	82.3 ± 1.3	80.3 ± 4.4	79.8 ± 1.2
0.05	87.4 ± 8.2	82.9 ± 0.8	89.0 ± 1.9	87.3 ± 4.5	86.8 ± 0.4
IC_{50} (μM)	4.4 ± 1.6	2.3 ± 0.5	4.9 ± 0.3	9.2 ± 2.0	8.1 ± 1.3

Where %: percentage of viable cells

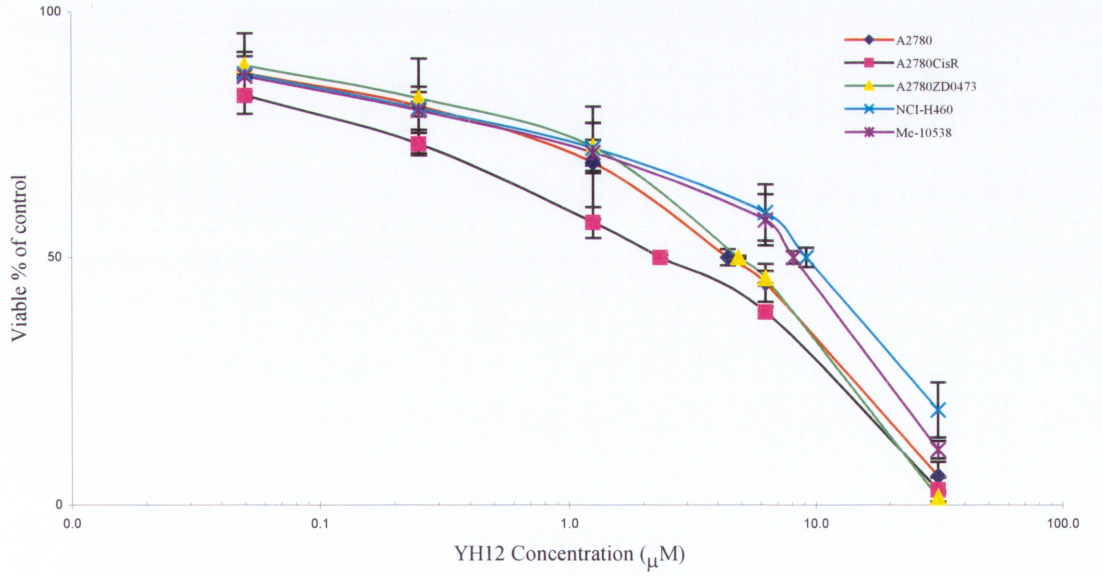


Figure 3.7-4: Cell survival curves for YH12 as applied to the cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, NCI-H460, and Me-10538

3.7.5 Cytotoxicity of cisplatin

Table 3.7-5 gives the percentages of viable cells at various concentrations of cisplatin (from 0.01 μM to 6.25 μM) along with IC_{50} values, as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Figure 3.7-5 gives the corresponding cell survival curves. The IC_{50} values for cisplatin are: A2780: 0.4 ± 0.1 μM, $A2780^{cisR}$: 4.4 ± 0.1 μM, $A2780^{ZD0473R}$: 2.1 ± 0.6 μM, NCI-H: 1.1 ± 0.2 μM and Me-10538: 3.4 ± 0.6 μM, indicating that the compound is most active against cell line: A2780 and least active against the cell line: $A2780^{cisR}$.

Table 3.7-5: Viable cells with cisplatin as a percentage of control as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

Cisplatin	%	%	%	%	%
Concentration (μM)	A2780	$A2780^{cisR}$	$A2780^{ZD0473R}$	NCI-H460	Me-10538
6.25	8.2 ± 0.9	42.2 ± 0.7	25.7 ± 5.5	26.9 ± 1.1	33.9 ± 4.7
1.25	23.9 ± 6.4	78.6 ± 0.7	61.7 ± 6.9	47.4 ± 3.8	76.6 ± 9.1
0.25	64.5 ± 3.9	97.8 ± 3.8	94.9 ± 4.6	84.7 ± 6.4	96.7 ± 3.5
0.05	84.1 ± 1.7	99.9 ± 5.9	103.3 ± 4.8	90.6 ± 4.5	116.8 ± 6.3
0.01	89.3 ± 0.2	99.4 ± 3.5	107.5 ± 2.8	94.3 ± 7.7	97.9 ± 8.7
IC_{50} (μM)	0.4 ± 0.1	4.4 ± 0.1	2.1 ± 0.6	1.1 ± 0.2	3.4 ± 0.6

Where %: percentage of viable cells

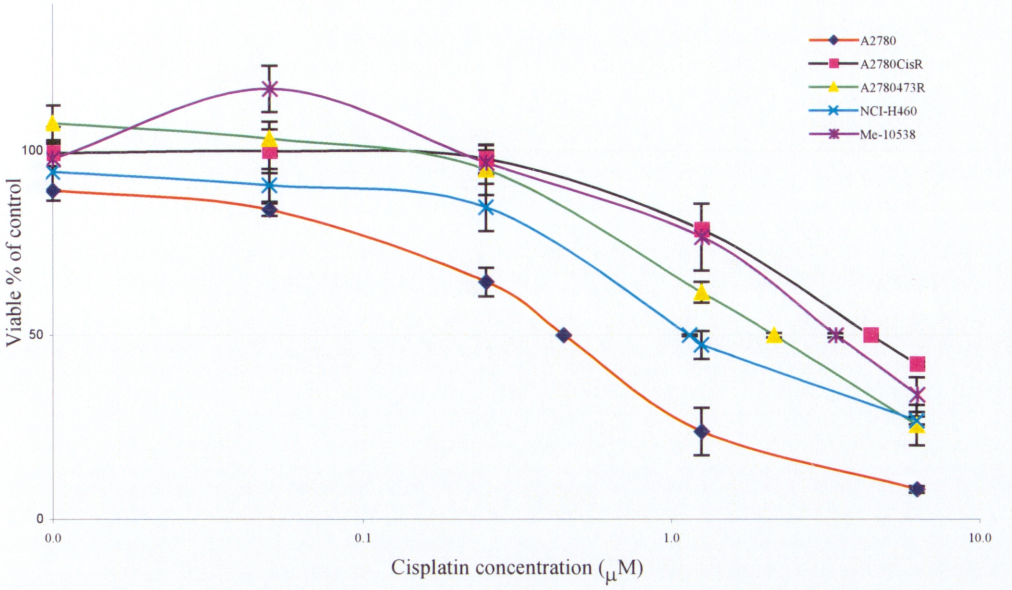


Figure 3.7-5: Cell survival curves for cisplatin as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538

3.7.6 Resistance factors

Table 3.7-6 and Figure 3.7-6 give the IC_{50} values for YH9, YH10, YH11, YH12 and cisplatin as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538. Table 3.7-6 also gives the resistance factors of YH9, YH10, YH11, YH12 and cisplatin as applied to the cell line combinations: A2780 and $A2780^{cisR}$, A2780 and $A2780^{ZD0473}$. The resistance factors are also shown in Figure 3.7-7.

Table 3.7-6: IC_{50} values and resistance factors for cisplatin, YH9, YH10, YH11, and YH12 as applied cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H460, and Me-10538.

	A2780	$A2780^{cisR}$	$\frac{IC_{50}A2780^{cisR}}{IC_{50}A2780}$	$A2780^{ZD0473R}$	$\frac{IC_{50}A2780^{ZD0473R}}{IC_{50}A2780}$	NCI-H460	Me10538
	IC_{50}	IC_{50}	RF	IC_{50}	RF	IC_{50}	IC_{50}
Cisplatin	0.45 ± 0.1	4.4 ± 0.1	9.9	2.2 ± 0.6	4.8	1.2 ± 0.2	3.4 ± 0.6
YH9	15 ± 8	19 ± 14	1.2	11 ± 1	0.7	45 ± 7	26 ± 2
YH10	13.2 ± 1.5	16.5 ± 3.4	1.3	17.9 ± 3.5	1.3	39.0 ± 4.4	23.0 ± 2.3
YH11	11.7 ± 1.9	15.0 ± 2.1	1.3	16.2 ± 1.6	1.4	33.8 ± 4.6	20.0 ± 2.7
YH12	4.4 ± 1.6	2.3 ± 0.5	0.5	4.9 ± 0.3	1.1	9.2 ± 2.0	8.1 ± 1.3

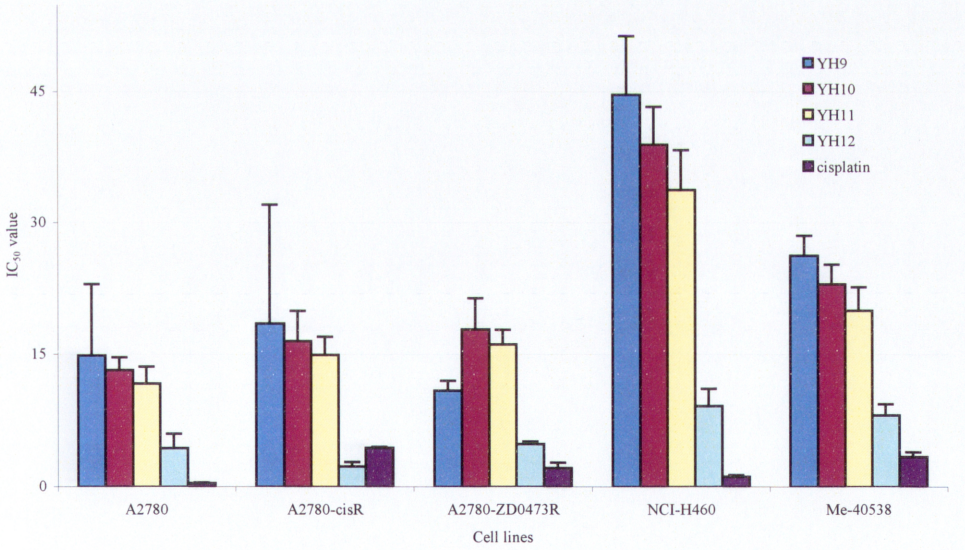


Figure 3.7-6: IC_{50} values for YH9, YH19, YH11, YH12 and cisplatin as applied to the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473}$, NCI-H460 and Me-10538

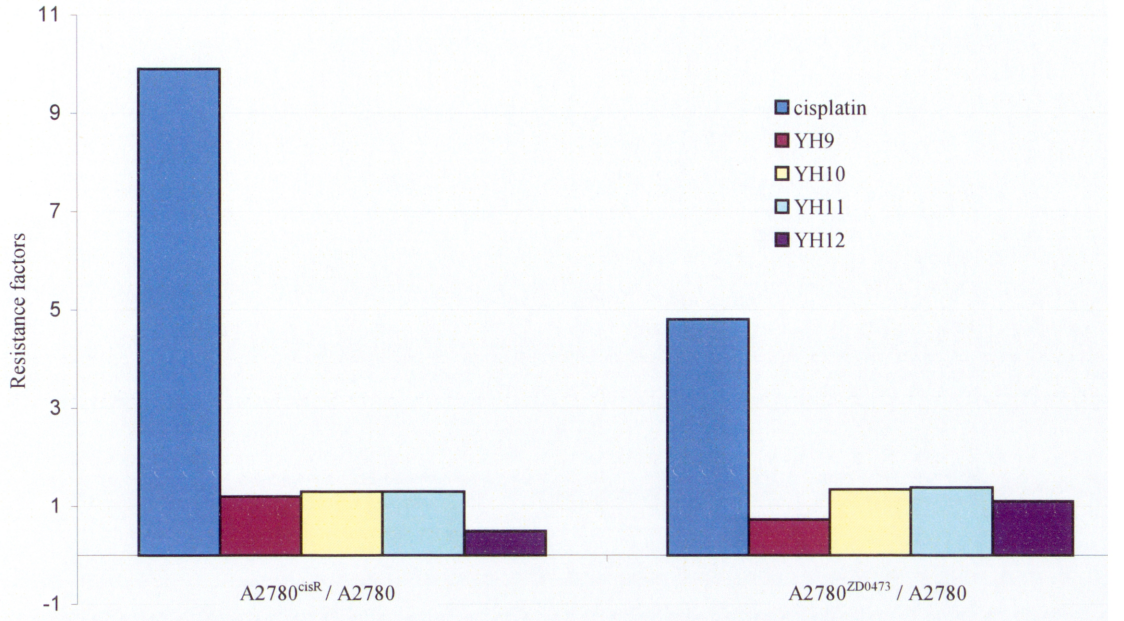


Figure 3.7-7: Resistance factors of YH9, YH10, YH11, YH12, and cisplatin as applied to the cell line combinations: A2780 and A2780^{cisR}, A2780 and A2780^{ZD0473}

It can be seen that YH9, YH10, YH11 and YH12 are less active than cisplatin against the cell lines: A2780, A2780^{ZD0473}, NCI-H460 and Me-10538. YH12 is however found to be more active than cisplatin against the cell line A2780^{cisR}. When the activity of the compound in the A2780 cell line is compared with that in A2780^{cisR} cell line, it is found that the compound is more active against the resistant cell line than the parent cell line. As applied to the cell lines: A2780 and A2780^{cisR}, YH9, YH10, YH11 and YH12 all have resistance factors significantly less than that of cisplatin – whereas for cisplatin RF is about 10, for YH9, YH10 and YH11, it is about 1 and for YH12 it is 0.5. YH12 is found to be the most active *trans*-planaramineplatinum(II) compound against all the cell lines. As applied to the cell lines: A2780 and A2780^{ZD0473} also, all the four *trans*-planaramineplatinum(II) compounds (YH9, YH10, YH11 and YH12) have resistance factors less than that of cisplatin. Whereas for cisplatin RF is about 5, for YH10, YH11 and YH12, it is about 1 and for YH9 it is 0.7, indicating that YH9 is more active against the resistant cell

line A2780^{ZD0473} than the parent cell line A2780. The order of activity of the compounds including cisplatin against the cell line: A2780 is: cisplatin > YH12 > YH11 > YH10 > YH9, that against the cell line: A2780^{cisR} is: YH12> cisplatin > YH11 > YH10 > YH9, that against the cell line: A2780 ^{ZD0473R} is: cisplatin > YH12 > YH9 >YH11 > YH10, that against the cell line: NCI-H460 is: cisplatin > YH12 > YH11 > YH10 > YH9 and that against the cell line: Me-10538 is: cisplatin > YH12 > YH11 > YH10 > YH9.

3.8 Cell uptake and DNA binding

3.8.1 Platinum cell uptake

Table 3.8-1 and Figure 3.8-1 give the total intracellular platinum levels found in the cisplatin responsive carcinoma ovarian cell line A2780 and cisplatin resistant cell line A2780^{cisR} after exposure to 50 μM of YH9, YH10, YH11, YH12, and cisplatin for 4 h. In A2780 cell line, the levels of platinum cell uptake (in nmol/2x10^6 cells) are: for YH9 0.68 ± 0.03, for YH10: 0.14 ± 0.02, for YH11: 0.89 ± 0.06, for YH12: 0.32 ± 0.10, and for cisplatin: 0.05 ± 0.004. In A2780^{cisR} cell line, the values are: for YH9 0.40 ± 0.02, for YH10: 0.20 ± 0.01, for YH11: 0.16 ± 0.04, for YH12: 0.12 ± 0.05 and for cisplatin: 0.03 ± 0.02.

Table 3.8-1: Pt accumulation in A2780 and A2780^{cisR} cells as applied to cisplatin, YH9, YH10, YH11 and YH12

	A2780 Pt (nmol/2x10^6 cells)	A2780^{cisR} Pt (nmol/2x10^6 cells)
cisplatin	0.05 ± 0.004	0.03 ± 0.02
YH9	0.68 ± 0.03	0.40 ± 0.02
YH10	0.14 ± 0.02	0.20 ± 0.01
YH11	0.89 ± 0.06	0.16 ± 0.04
YH12	0.32 ± 0.10	0.12 ± 0.05

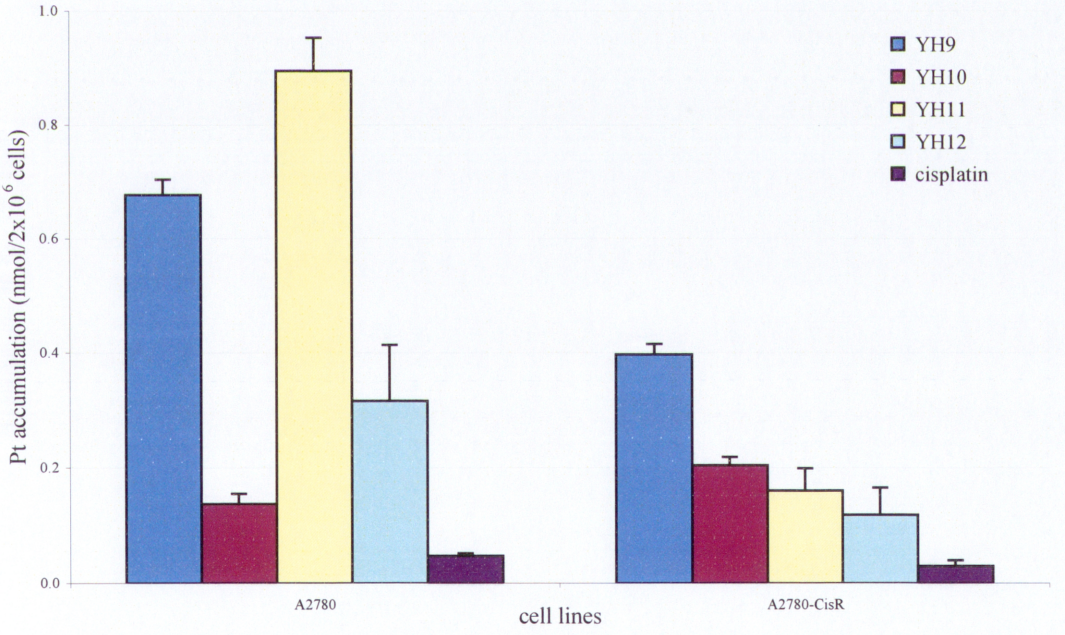


Figure 3.8-1: Pt accumulation in A2780 and A2780^{cisR} cells as applied to cisplatin, YH9, YH10, YH11 and YH12

3.8.2 DNA binding

Table 3.8-2 and Figure 3.8-2 give the levels of Pt-DNA binding in cisplatin responsive carcinoma ovarian cell line A2780 and cisplatin resistant cell line $A2780^{cisR}$ after exposure to 50 μM of YH9, YH10, YH11, YH12, and cisplatin for 4 h. Figure 3.8-3 gives the same values on a relative scale. In A2780 cell line, the levels of Pt-DNA binding (in nmol Pt/mg DNA) are: for YH9: 0.49 ± 0.15, for YH10: 0.50 ± 0.08, for YH11: 0.63 ± 0.12, for YH12: 0.74 ± 0.05 and for cisplatin: 0.29 ± 0.22. In $A2780^{cisR}$ cell line, the values are: for YH9: 0.16 ± 0.05, for YH10: 0.53 ± 0.18, for YH11: 0.70 ± 0.24, for YH12: 1.90 ± 0.15 and for cisplatin: 0.19 ± 0.10.

Table 3.8-2: Levels of Pt-DNA binding in A2780 and $A2780^{cisR}$ cells

	A2780 nmol Pt/mg DNA	$A2780^{cisR}$ nmol Pt/mg DNA
Cisplatin	0.29 ± 0.22	0.19 ± 0.10
YH9	0.49 ± 0.15	0.16 ± 0.05
YH10	0.50 ± 0.08	0.53 ± 0.18
YH11	0.63 ± 0.12	0.70 ± 0.24
YH12	0.74 ± 0.05	1.90 ± 0.15

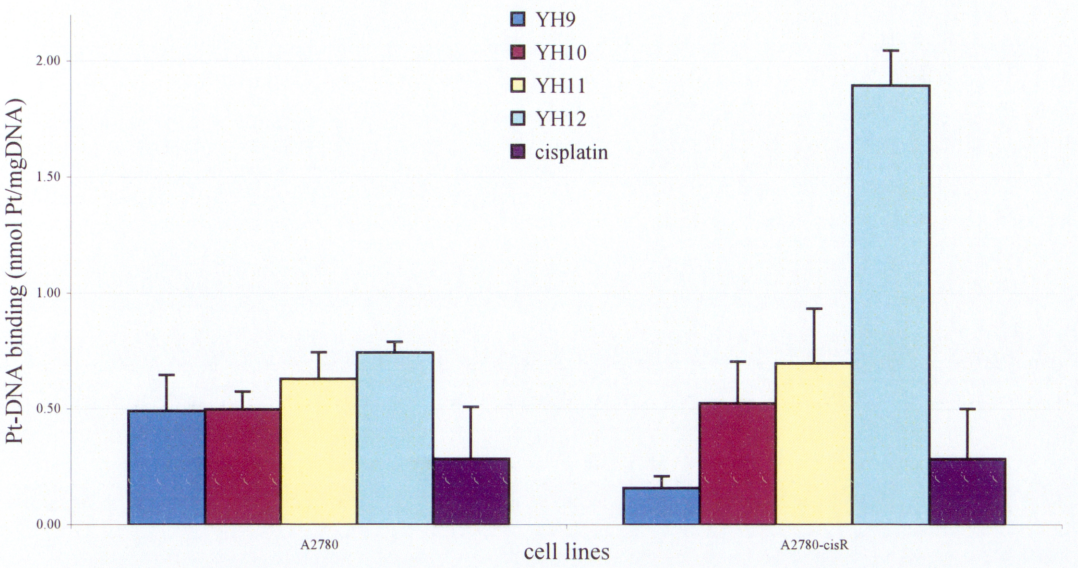
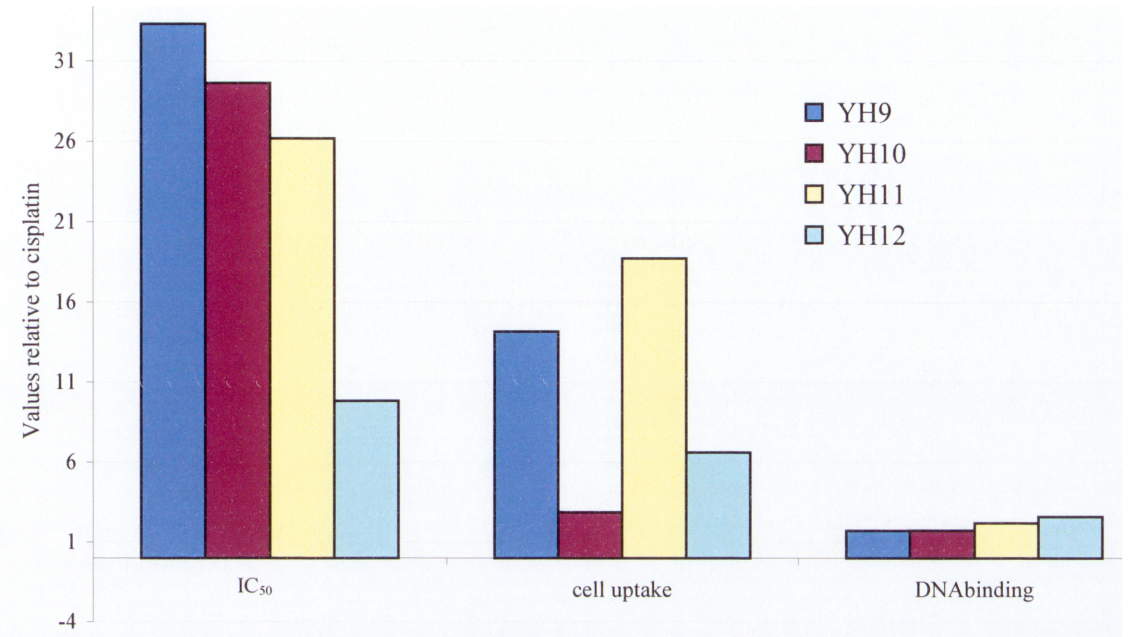
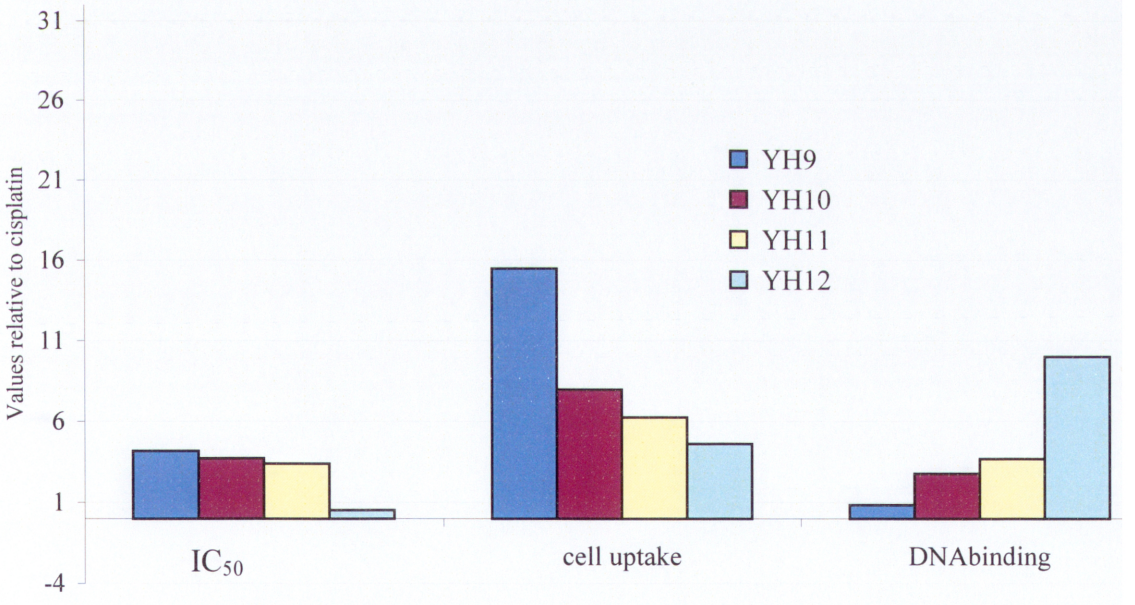


Figure 3.8-2: Levels of Pt-DNA binding in A2780 and A2780^{cisR} cells



(a)



(b)

Figure 3.8-3: Relative values of IC_{50}, cell uptake, and levels of Pt-DNA binding in: (a) A2780 cell line and (b) $A2780^{cisR}$ cell line

3.9 Interaction between YH9, YH10, YH11 and YH12, and nucleobases

YH9, YH10, YH11 and YH12, all have two labile chloride ligands per molecule. Thus each of the compounds is expected to form 1 : 2 adducts with nucleobases.

The retention time of adenine and guanine were found to be 24.61 and 5.96 min respectively. The retention times for the major peak fractions in incubated the mixtures of YH9, YH11, YH11 and YH12 with adenine were found to be 25.20, 24.51, 25.40 and 25.19 min respectively and those in the major peak fractions in the incubated mixtures of YH9, YH10, YH11 and YH12, and guanine were found to be 6.14, 5.87, 5.73 and 5.86 min respectively. Examples of chromatograms are provided by Figure 3.9-1 that gives the chromatograms applying to: (a) guanine and (b) 1 : 1 incubated mixture of guanine and YH12.

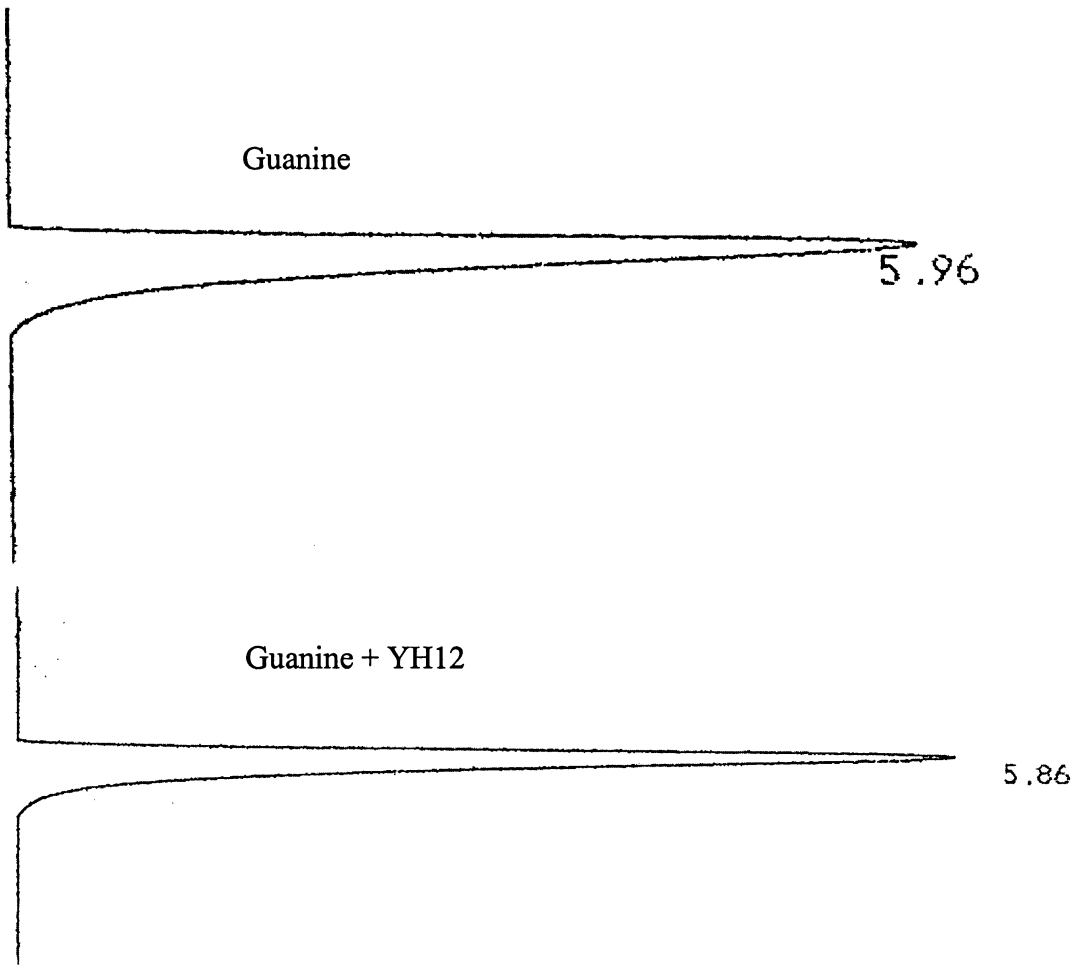


Figure 3.9-1: Chromatograms applying to: (a) guanine and (b) 1 : 1 incubated mixture of guanine and YH12

The Pt : NB binding ratios of the major peak in the incubated mixtures of YH9, YH10, YH11 and YH12, and adenine were all found to be close 1 and those applying to the major peaks in the incubated mixtures of YH9, YH10, YH11 and YH12, and guanine were all found to be close to the expected value of 2.

4 Chapter Four: Discussion

4.1 Characterization

As stated earlier, elemental and spectral analyses were carried out to characterize the designed complexes.

4.1.1 Elemental compositions of YH9, YH10, YH11 and YH12

Table 3.2-1 gives the elemental compositions of YH9, YH10, YH11 and YH12. The purity of each of the compounds is about 99%. As stated earlier, the compounds were synthesized from cisplatin utilizing the difference in the *trans* effect of halide and amine ligands in platinum(II) complexes (Kauffman and Cowan 1963). Generally, the yield was low (30% for YH9, 23% for YH10, 51% for YH11 and 50% for YH12) although the method used for the synthesis was the same in all cases except that whereas 2-hydroxypyridine and imidazole were soluble in water, 3-hydroxyyridine was not (which had to be dissolved in ethanol). Imidazo(1,2-α)pyridine being a liquid was not dissolved in any solvent. It may be noted that other investigators also found wide variation in the yield of *trans*-planaramineplatinum(II) complexes (Kelland, Barnard *et al.* 1995).

4.1.2 Molar conductivity

The limiting values of molar conductivity (in ohm^{-1} cm^2 mol^{-1}) at zero concentration of YH9, YH10, YH11 and YH12 at 298 K were found to be 78, 78, 102 and 132 respectively. These values are significantly lower than the expected values of about 280 for 1:2 electrolyte (even though the measurements were done 24 h after dissolution), indicating that the degree of dissociation is of the order of about 30% in the case of YH9, YH10 and YH11 and about 50% in the case of YH12. These results

suggest that the compounds may be crossing the cell membrane by both passive diffusion and carrier-mediated transport.

4.1.3 Spectral analyses

4.1.3.1 IR and Raman analyses

Most of the peak assignments are based on published spectra of the ligands and metal complexes of pyridine, substituted pyridines, imidazole and substituted imidazoles (Goodgame, Goodgame *et al.* 1968; Salama and Spiro 1977; Hodgson, Percy *et al.* 1980; Silverstein, Bassler *et al.* 1991; Kelland, Barnard *et al.* 1995; Nakamoto 1997). Although IR and Raman spectral measurements alone do not usually provide clear proof of the structures of compounds, it can be seen from the following analyses that the IR and Raman spectral data of YH9, YH10, YH11 and YH12 provide support for the suggested structures of the compounds. Likewise, it will be shown that mass and ^{1}H NMR spectral data also provide support for the suggested structures.

4.1.3.1.1 YH9 [*trans*-(ammino)(2-hydroxypyridinato)dichloroplatinum(II)]

IR

The broad band at 3292 cm^{-1} is believed due to N-H and O-H stretching vibrations whereas that at 3064 cm^{-1} is due to C-H stretch. The band at 1622 cm^{-1} is believed to be due to N-H bending vibration whereas those at 1574 and 1485 cm^{-1} are believed due to the pyridine ring stretching vibrations. The band at 1319 cm^{-1} is due to O-H bending vibration. The band at 1173 cm^{-1} is believed due to C-O stretch. The band at 1107 cm^{-1} is believed to be due to C-H in plane bending related to the heterocyclic ring. The band at 1032 cm^{-1} is due to C-N stretching vibration. The bands at 844 and 781 cm^{-1} are due to out of plane C-H bending vibrations. The bands at 548 and 517

cm^{-1} are believed to be due to Pt-N(NH_3) stretching vibrations whereas that at 482 cm^{-1} is associated with the vibration of pyridine ring.

Raman

The band at 3160 cm^{-1} is due to N-H and C-H stretching vibrations. The bands at 1622 and 1572 cm^{-1} are due to N-H bending vibrations. The bands at 1314, 1246 and 1154 cm^{-1} are believed to be due to C-H in-plane bending vibrations relating to the pyridine ring. The band at 1034 cm^{-1} is due to pyridine ring stretch. The band at 1105 cm^{-1} is due to C-O stretch. The band at 847 cm^{-1} is due to C-H out-of-plane bending. The band at 660 cm^{-1} is associated with the vibration of the pyridine ring. The bands at 517 and 575 cm^{-1} are due to Pt-N(NH_3) stretching vibrations. The band at 324 cm^{-1} is due to Pt-Cl stretching vibration. This frequency is similar to the reported values for Pt-Cl vibration applying to *trans*-planaraminedicholoroplatinum(II) complexes and slightly lower than the values reported for Pt(IV) complexes indicating that the Pt-Cl bond would be stronger in the Pt(IV) complexes than in the Pt(II) complexes (Kelland, Barnard *et al.* 1995).The bands at 193 and 238 cm^{-1} are due to Pt-N(2-hydroxypyridine) stretching vibrations which are in agreement with the reported values (Nakamoto 1997) . The band at 145 cm^{-1} is due to Pt-N bending vibration. The band at 81 cm^{-1} is believed to be associated with the lattice mode.

4.1.3.1.2 YH10 [*trans*-(ammino)(imidazolato)dichloroplatinum(II)]

IR

The broad band at 3280 cm^{-1} is believed to be due to N-H stretching vibration whereas that at 3145 cm^{-1} is believed to be due to C-H stretch. The bands at 1633 cm^{-1} and 1693 cm^{-1} are believed to be due to C=C stretching and N-H bending vibrations. The band at 1543 cm^{-1} is believed to be due to C= N stretch. The bands at 1512 and 1437

cm^{-1} are associated with the stretching vibrations of the imidazole ring. The band at 1314 cm^{-1} is due to in plane deformation of the imidazole ring. The bands at 1182 and 1142 cm^{-1} are due to C-H in plane bending vibrations whereas those at 845 and 754 cm^{-1} are due to C-H out of plane bending vibrations. The bands at 710, 654 and 613 cm^{-1} are believed to be due to out of plane bending of the ring N-H bond.

Raman

The band at 3152 cm^{-1} is due to N-H stretching vibration. The band at 1788 cm^{-1} is believed to be an overtone band associated with 'aromatic' C-H stretch. The band at 1543 cm^{-1} is believed to be due to C=C and C=N stretching vibrations. The band at 1433 cm^{-1} is believed to be due to C-N stretch. The band at 1333 and 1140 cm^{-1} are due to imidazole ring stretch. The band at 1264 cm^{-1} is due to C-H bending vibration. The band at 1186 cm^{-1} is believed to be due to N-H bending vibration. The band at 529 cm^{-1} is due to Pt-N stretching vibration whereas that at 326 cm^{-1} is due to Pt-Cl stretching vibration. The bands at 224 and 251 cm^{-1} are due Pt-N(imidazole) stretching vibrations whereas that at 135 cm^{-1} is due to Pt-N bending vibration.

4.1.3.1.3 YH11 [*trans*-(ammino)(3-hydroxypyridinato)dichloroplatinum(II)]

IR

The broad band at 3261 cm^{-1} is believed due to N-H and O-H stretching vibrations whereas that at 3078 cm^{-1} is due to C-H stretch. The bands at 2563, 2447, 2141 and 1890 cm^{-1} are associated with the vibrations of 3-hydroxypyridine ring. The bands at 1620 and 1579 cm^{-1} are due to N-H bending vibrations. The band at 1225 cm^{-1} is believed to be due to C-O stretching vibration. The band at 1315 cm^{-1} is believed to be due to in plane O-H bending. The band at 1030 cm^{-1} is due to pyridine ring stretch. The bands at 870, 802 and 688 cm^{-1} are believed to be associated with the out of plane

bending of the ring C-H bonds. The band at 688 cm^{-1} applies to the in plane deformation of the pyridine ring whereas that at 442 cm^{-1} applies to out of plane deformation of the ring. The bands at 519, 553 and 594 cm^{-1} are believed to be due to Pt-N(NH_3) stretching vibrations.

Raman

The bands at 3258 and 3206 cm^{-1} are due to O-H and N-H stretching vibrations. The bands at 3101 and 3076 cm^{-1} are due to C-H stretch. The band at 1620 cm^{-1} and is associated with the vibration of 3-hydroxypyridine ring. The bands at 1578 and 1474 cm^{-1} are believed to be due to N-H bending vibrations. The band at 1321 cm^{-1} is due to O-H bending vibration. The bands at 1279 and 1177 cm^{-1} are believed to be due to C-H in plane bending vibrations. The band at 1109 cm^{-1} is due to C-O stretch. The band at 1030 cm^{-1} is due to C-H in plane bending of the hetereocyclic ring. The bands at 870 and 666 cm^{-1} are believed to be due to C-H out of plane bending. The bands at 523 and 442 cm^{-1} due to Pt-N stretching (NH_3) vibrations whereas that at 346 cm^{-1} is due to Pt-Cl stretching vibration. The band at 243 cm^{-1} is due Pt-N(3-hydroxypyridine) stretching vibration. The bands at 191 and 91 cm^{-1} are believed to be due to Pt-N bending vibrations. The band at 70 cm^{-1} is associated with the lattice mode.

When the frequencies of Pt-Cl vibration in YH9, YH10, YH11 and YH12 are compared, it is found that whereas the values in YH9, YH11 and YH12 are similar, the value for YH11 is significantly greater. This means that the strength of the Pt-Cl bond would be strongest in YH11. It was stated in section 3.7.1 that the uptake in A2780 cells was highest for YH11. A greater strength of Pt-Cl bond would mean a smaller extent of pre-dissociation due to hydrolysis and therefore a larger rate of cell uptake provided the compounds enter the cells by passive diffusion.

4.1.3.1.4 YH12 [*trans*-(ammino)(imidazo(1,2-α)pyridinato)dichloroplatinum(II)]

IR

The bands at 3300 and 3246 cm^{-1} are believed to be due to symmetric and asymmetric NH_3 stretch and that at 3134 cm^{-1} is due to C-H stretch. The bands 1637, 1512, 1446 and 1321 cm^{-1} are believed to be due to ring stretch. The bands at 1512, 1321 and 1149 cm^{-1} are due to ring stretch. The band at 1234 cm^{-1} is believed to be due to C-N stretching vibration. The band at 933 cm^{-1} is due to bending vibration of the imidazole ring. The bands at 839, 752 and 725 cm^{-1} are believed to be due to C-H bending vibrations. The bands at 422 and 447 cm^{-1} are believed to be due to Pt-N(NH_3) stretching vibrations.

Raman

The band at 3130 cm^{-1} is believed to be due to N-H stretching vibration whereas that at 3082 cm^{-1} is due to C-H stretch. The bands at 1512, 1445, 1379 and 1321 cm^{-1} are believed to be due to ring stretch. The band at 1233 and 1134 cm^{-1} are believed to be due to N-H bending vibrations. The band at 1013 cm^{-1} is due to C-H in plane bending of the hetereocyclic ring. The bands at 901, 772 and 639 cm^{-1} are believed to be due to C-H out of plane bending. The band at 569 cm^{-1} is due to Pt-N(NH_3) stretching vibration whereas that at 330 is due to Pt-Cl stretching vibration. The band at 238 cm^{-1} is due Pt-N(ring) stretching vibration whereas that at 101 cm^{-1} is due to Pt-N(NH_3) bending vibration.

4.1.3.2 Mass spectrum

4.1.3.2.1 The mass spectrum of YH9

The mass spectrum of YH9 has a peak with m/z = 377 that corresponds to (M – H), that at m/z = 360 corresponds to (M – H – NH_3), that at m/z = 339 corresponds to (M – Cl – 4H) and that at m/z = 324 may be due to (M – Cl – NH_3 – 2H). It can be seen

that the mass spectral data of YH9 provide support for the suggested structure of the compound.

4.1.3.2.2 The mass spectrum YH10

The mass spectrum of YH10 has a large peak with m/z = 384 due to Pt(imidazole)$_3$(NH_3)Cl that is believed to be formed in situ from the joining of the fragments produced from YH10. The peak at m/z = 332 corresponds to (M – NH_3 - 2H).

4.1.3.2.3 Mass spectrum of YH11

The mass spectrum of YH11 has a peak with m/z = 516 due to PtCl{3-hydroxypyridine}$_3$ that is believed to be formed in situ from the joining of the fragments produced from YH11. The peak at m/z = 438 is believed to be due to (M – Cl + 3-hydroxypyridine), that at m/z = 360 corresponds to (M – H – NH_3), that at m/z = 324 corresponds to (M – Cl – NH_3 – H) and that at m/z = 265 corresponds to (M – Cl – 3-hydroxypyridine + NH_3). It can be seen that the mass spectral data of YH11 provide support for the suggested structure of the compound.

4.1.3.2.4 Mass spectrum of YH12

The mass spectrum of YH12 has a peak with m/z = 584 due to PtCl{imidazo(1,2-α)pyridine}$_3$ – H) that is believed to be formed in situ from the joining of the fragments produced from YH12. The peak at m/z = 484 is believed to be due to PtCl(NH_3){imidazo(1,2-α)pyridine}$_2$ that is believed to be formed in situ from the joining of the fragments produced from YH12. The peak at m/z = 383 corresponds to

(M – NH_3 – H), that at m/z = 324 corresponds to (M – Cl – NH_3 – H) and that at m/z = 265 corresponds to (M – Cl – 3-hydroxypyridine + NH_3) and that at m/z = 301 is believed to be due to $PtCl_3$. It can be seen that the mass spectral data of YH12 provide support for the suggested structure of the compound.

4.1.3.3 Nuclear magnetic resonance spectroscopy (NMR)

4.1.3.3.1 ^{1}H NMR spectrum of YH9

The resonance at δ = 12.07 ppm is believed to be due to OH proton. The resonance at δ = 8.28 ppm is due to ortho CH proton and that at δ = 8.27 ppm is due to CH proton adjacent to the carbon to which OH group is attached. The resonance at δ = 7.70 ppm is due to CH proton at the para position and that at δ = 6.85 ppm is due to CH proton at the second meta position. The resonance at δ = 4.60 ppm is due to NH bonded to Pt. Likewise, the resonance at δ = 3.86 ppm is believed to be due to NH bonded to Pt. The resonance at δ = 3.34 ppm is due to water and that at δ = 2.50 ppm is due to DMSO.

4.1.3.3.2 ^{1}H NMR spectrum of YH10

The resonance at δ = 8.35 ppm is due to ortho NH proton and that at δ = 8.09 ppm is due to ortho CH proton that lies in between the two nitrogens. The resonance at δ = 7.42 ppm is due to the other ortho CH proton and that at δ = 7.23 ppm is due to CH proton at the meta position. It is difficult to explain why there is an additional proton resonance at 7.24 pm unless it is assumed that the nitrogen at the third position is protonated. It is interesting to note that the NH resonance has shifted from 13.4 ppm

in pure imidazole to 8.35 ppm in the Pt-complex. The resonance at δ = 4.60 ppm is due to NH bonded to Pt. Likewise, the resonance at δ = 3.76 ppm is believed to be due to NH bonded to Pt. The resonance at δ = 3.32 ppm is due to water and that at δ = 2.50 ppm is due to DMSO.

4.1.3.3.3 ^{1}H NMR spectrum of YH11

The resonance at δ = 10.90 ppm is believed to be due to OH proton. The resonance at δ= 8.32 ppm is due to H attached to the second carbon and that at δ = 8.30 ppm is due to H attached to the sixth carbon. The resonance at δ = 8.26 ppm is due to H attached to the fourth carbon and that at δ = 7.33 ppm is due to H attached to the fifth carbon. The resonance at δ = 4.60 ppm is due to NH bonded to Pt. Likewise, the resonance at δ = 3.90 ppm is believed to be due to NH bonded to Pt. The resonance at δ = 3.31 ppm is due to water and that at δ = 2.50 ppm is due to DMSO.

4.1.3.3.4 ^{1}H NMR spectrum of YH12

The resonance at δ = 8.73 ppm is due to H attached to the second carbon (see Figure 3.1-4 for the numbering scheme used) and that at δ = 8.61 ppm is due to H attached to the third carbon. The resonance at δ = 8.19 ppm is due to H attached to the ninth carbon and that at δ = 8.03 ppm is due to H attached to the eighth carbon. The resonance at δ = 7.70 ppm is due to H attached to the seventh carbon. The resonance at δ = 4.60 ppm is due to NH bonded to Pt. Likewise, the resonance at δ = 3.96 ppm is believed to be due to NH bonded to Pt. The resonance at δ = 3.34 ppm is due to water and that at δ = 2.50 ppm is due to DMSO.

Finally, it is appropriate to note that although the method of synthesis of compounds (that utilizes the *trans*-effect of chloride ligand), the achieved purity and the spectral results provide support for their suggested structures, it would be more confirming if the structures were confirmed by x-ray diffractometry.

4.2 Interaction with pBR322 plasmid DNA and ssDNA

The interaction of YH9, YH10, YH11, YH12, cisplatin and transplatin with pBR322 plasmid DNA and ssDNA was studied by using gel electrophoresis in order to obtain information on changes in DNA as a result of the binding of the drugs. The change in mobility of the form I band (first decrease and then increase) with the increase in concentration of compounds is due to unwinding of the supercoiled form I DNA to change from negatively supercoiled form I through the relaxed circular form I to finally positively supercoiled form I (see Figure 4.2-1) (Lippard 1994). When two forms comigrate, form I is fully relaxed. The conformational changes in form I DNA are caused by the covalent binding of platinum compounds with nucleobases in DNA. As stated earlier, forms I and II bands coalesce at 5 μM concentration in the case of cisplatin and 10 μM in the case of transplatin indicating that binding of cisplatin causes a greater unwinding of the supercoiled form I DNA than the binding of transplatin. It was reported that the unwinding angle for cisplatin was 13°, that for transplatin was 9° and that for *trans*-[$PtCl_2$(pyridine)$_2$] was 17° (Zou, Van Houten *et al.* 1993). None of the *trans*-planaramineplatinum(II) complexes in the present study (YH9, YH10, YH11 and YH12) caused a greater unwinding of pBR322 plasmid DNA than cisplatin. Whereas cisplatin forms mainly intrastrand bifunctional GG and AG adducts with duplex DNA (Fichtinger-Schepman, van der Veer *et al.* 1985) (and

hence causes a local bending of a DNA strand), like transplatin *trans*-planaramineplatinum(II) complexes are more likely to form mofunctional with guanine and bifunctional intrastrand GC and GG adducts (Brabec and Leng 1993). It will be seen later that bifunctional interstrand binding (because of the mismatch between appropriate interstrand distance and that between the two *trans* 'arms' of platinum), brings the DNA strands closer together so that more global changes in DNA conformation are introduced. Positioning of the planaramine and ammino ligands along the helix also adds to the distortion by pushing the neighbouring base pairs away.

As compared to that due to transplatin, the unwinding of supercoiled form I DNA is greater in the case of YH11 and YH12, smaller in the case of YH9 and similar in the case of YH10. The results for YH9, YH10, YH11 and YH12 clearly demonstrate that the extent of unwinding is a function of the nature of the planaramine ligand and for substituted pyridines, it is a function of the nature of substitution. For example, 3-hydroxypyridine bonded to platinum(II) in a *trans*-geometry is found to cause a greater unwinding than 2-hydroxypyridine.

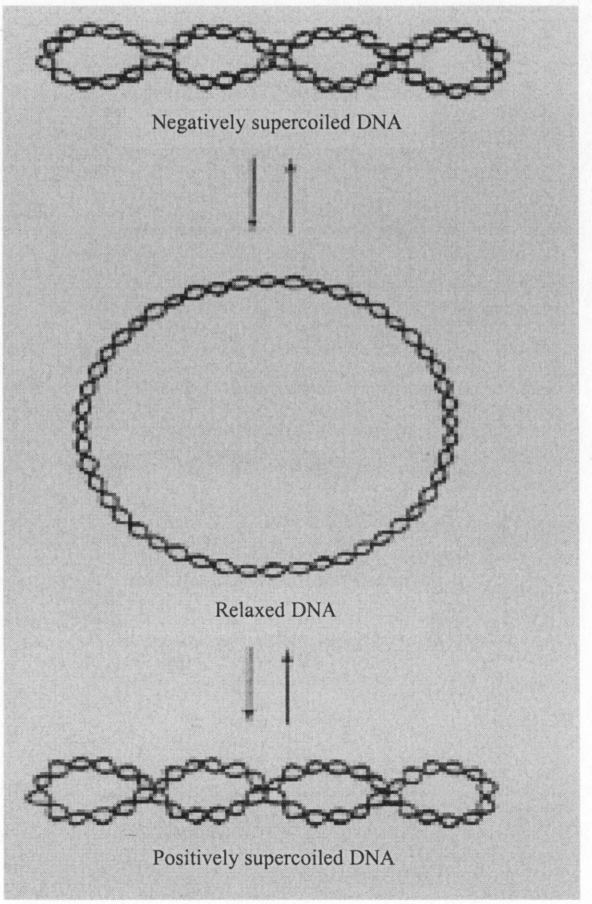


Figure 4.2-1: Schematic presentation of the unwinding of supercoiled plasmid DNA from negatively supercoiled form I through relaxed circular form I to positively supercoiled form I (Lippard 1994)

Figure 3.5-2 gives the electrophoretograms applying to the interaction of ssDNA with cisplatin, transplatin, YH9, YH10, YH11 and YH12. As the concentrations of the compounds are increased, the intensities of the mixture of drug-DNA decrease indicating an increase in damage to DNA due to the binding of Pt with DNA.

4.3 BamH1 restriction enzyme digestion

BamH1 is a restriction enzyme that hydrolyses the phosphodiester bond between adjacent guanine sites in a DNA strand (Kessler and Manta 1990). The prevention of BamH1 digestion indicates changes in DNA conformation such that the enzyme is unable to recognize the site. Arranged in the order of increasing prevention of BamH1 digestion (from lowest to highest), the compounds were: YH9 < transplatin < YH10 < YH12 < cisplatin < YH11. Thus within the concentration range: 1 to 10 μM, YH9 was found to be least efficient in preventing BamH1 digestion whilst YH11 was found to be the most efficient compound. This result is in line with the observation that YH9 caused the smallest unwinding of form I DNA whereas YH11 caused the greatest unwinding. It can therefore be seen that both the unwinding and the BamH1 digestion results support the idea that among all the compounds studied, YH9 caused the least conformational change of the pBR322 form I DNA.

YH10, YH11 and YH12 were found to be more efficient than transplatin in preventing BamH1 digestion. As stated earlier, YH11 was found to be more efficient than cisplatin in preventing BamH1 digestion.

There are known differences between cisplatin and transplatin. (1) Transplatin is more reactive than cisplatin and the *trans*-configuration is more favourable for a crosslinking reaction with proteins than the *cis*-configuration. (2) Whereas cisplatin forms mainly intrastrand bifunctional GG and AG adducts, *trans*-platinum complexes form mainly interstrand GC (50%), GG (40%) and GA (10%) adducts (Eastman, Jennerwein *et al.* 1988). A recent study has shown that the closure of the monofunctional *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts results in the formation of first interstrand *trans*-$[Pt(NH_3)_2(dGdC)]^{2+}$ crosslinks and eventually into other lesions such as interstrand dGdG adduct (Bernalmendez, Boudvillain *et al.* 1997). An interstrand crosslink between G and A residues separated by 4-5 base pairs was even detected. Although both cisplatin and transplatin form monofunctional adducts at approximately the same rate, it has been reported that the rate of closure of monofunctional adducts into bifunctional ones is faster by at least one order of magnitude in the case of cisplatin than transplatin (Eastman and Barry 1987; Eastman, Jennerwein *et al.* 1988). The long lifetime of monofunctional adducts and their strong reactivity with sulfur-containing nucleophiles could explain at least in part the clinical inefficiency of transplatin (Eastman and Barry 1987; Eastman, Jennerwein *et al.* 1988). As noted earlier, the *trans* configuration is more favourable for a crosslinking reaction with proteins than the *cis* configuration.

The closure of monofunctional adducts into bifunctional interstand GC adducts has been reported to be faster for *trans*-(pyridine)(ammine)dichloroplatinum(II) than for transplatin although a significant reduction in reactivity occurs when NH_3 is replaced by pyridine or quinoline (Kasparkova, Marini *et al.* 2003). This means that for the *trans*-pyridineplatinum(II) complexes, although the rate of formation of

monofunctional adducts may be lower than that for transplatin, once the monofunctional adducts are formed, reactivity towards the formation of bifunctional lesions is increased. According to Kasparkova *et al* (Kasparkova, Marini *et al.* 2003), the high rate of closure of monofunctional adducts to form interstrand bifunctional adducts in the case of *trans*-planaramineplatinum(II) complexes, may be due to conformational changes induced in double-stranded DNA. Thus, although 4 h incubation may be not be enough for any significant closure of the monofunctional adducts into bifunctional legions in the case of transplatin (for transplatin, the closure was found to be 80% complete after 48 h of incubation) (Eastman and Barry 1987; Eastman, Jennerwein *et al.* 1988), significant closure may occur in the same period of time (4 h) in the case of *trans*-planaramineplatinum(II) complexes e.g. in those containing pyridine or substituted pyridine ligands. However, the occurrence of significant unwinding even after 4 h of incubation with transplatin and *trans*-planaramineplatinum(II) complexes (so that form I and form II bands comigrate) suggest that the formation of monofunctional adducts may be sufficient to cause enough conformational change in DNA such that BamH1 digestion is prevented. Steric crowding due to bulky ligands as in the case of *trans*-planaramineplatinum(II) complexes may also be playing a prominent role in distorting the DNA.

4.4 Cytotoxicity

The cytotoxicity of YH9, YH10, YH11, YH12 and cisplatin has been determined against the human ovarian carcinoma cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, the melanoma cell line Me10538 and non-small lung cancer cell line NCI-H460 after a treatment period of 72 h based on MTT reduction assay.

In this study, the IC_{50} value for cisplatin against A2780 was found to be 0.45 μM as compared to the reported values of 0.36 μM (Shamsuddin, Takahashi *et al.* 1996), 0.17 μM (Ali, Whitmire *et al.* 1999) and 3.6 μM (Perez, Montero *et al.* 2001). Whereas in the present study, the exposure time was 72 h, in the studies of Shamsuddin *et al*, Ali *et al* and Perez *et al* the exposure times were respectively 120 h, 120 h and 24 h. It may be noted that all of the studies were also based on MTT reduction assay. Noting that the IC_{50} value is expected to decrease with the increase in exposure time (and vice versa), the value found in the present study can be seen to fit with the reported values.

For A2780, the IC_{50} values for YH9, YH10, YH11 and YH12 are all found to be greater than that for cisplatin indicating all of the compounds are less active than cisplatin against the cell line. The order of the IC_{50} values from highest to lowest is YH9 > YH10 > YH11 > YH12 > cisplatin, indicating that among the *trans*-planaramineplatinum(II) complexes YH12 is most active and YH9 is least active against the cell line. It will be seen later that for YH9, which is found to be marginally less active than YH10 against A2780 cell line, the cell uptake is greater than that for YH10 but the extent Pt-DNA is similar to that for YH10. The results show that it is the level of Pt-DNA binding (rather than the level of cell uptake per se) that can give a better indication of the activity of the compounds.

Table 3.7-6 outlines the resistance factors for YH9, YH10, YH11 and YH12. The values range from 0.5 to 1.3 compared to 9.9 for cisplatin. A smaller decrease in activities for YH9, YH10 and YH11 than for cisplatin and an increase in activity for YH12 in going from A2780 and $A2780^{cisR}$ cell lines indicate that on a relative scale

the compounds are better able to overcome resistance in $A2780^{cisR}$ cells than cisplatin. For YH12, the resistance factor is 0.50, indicating that YH12 is more active (twice as much) against $A2780^{cisR}$ cell line than against A2780 cell line. The results suggest that the compound has been able to overcome better the mechanisms of resistance operating in $A2780^{cisR}$ cell line than in A2780 cell.

All of the four *trans*-planaramineplatinum(II) complexes are less active than cisplatin against the $A2780^{ZD0473R}$ cell line. The most active compound is YH12 as it was the case with the other cell lines. YH9 was found to have resistance factor of 0.7 indicating that the compound is more active against the resistant cell line: $A2780^{ZD0473R}$ than the parent cell line: A2780, suggesting that the compound is better able to overcome resistance in $A2780^{ZD0473R}$ cell than in A2780 cell.

Based on sulphorhodamine B growth inhibition assay, it was found that the IC_{50} values for the drug ZD0473 against $A2780^{ZD0473R}$ and A2780 cell lines were respectively 3.6 ± 0.2 and 1.32 ± 0.2 μM (Holford, Beale *et al.* 2000) giving a resistance factor of 2.7. Although the period of drug exposure in the study was 96 h as against 72 h in the present study, it is clear that all of the compounds YH9, YH10, YH11 and YH12 are less active than ZD0473 against the cell lines. However, when the resistance factors are considered, it is found that the compounds have significantly lower resistance factors than ZD0473. This means that the decrease in activity in going from A2780 cell line to $A2780^{ZD0473R}$ cell is less pronounced in the case of the designed complexes than in the case of ZD0473 and (as noted earlier) there was an increase in activity in the case of YH9. The results suggest that on a scale relative to the values in A2780 cell line, the designed complexes are better able to overcome

resistance in A2780^{ZD0473R} cell line than the drug ZD0473. It may be noted that ZD0473 like cisplatin is more likely to form mainly intrastrand bifunctional adducts whereas YH9, YH10, YH11 and YH12 are more likely to form bifunctional intrastrand adducts. This difference is further explored later in the chapter.

The same pattern of activity was observed for NCI-H460 and Me10538 cell lines with YH12 being the most active compound and YH9 the least active.

The reason why YH9, YH10, YH11 and YH12 are less active than cisplatin against A2780 cell line even though Pt-DNA binding in the cell line is higher than for cisplatin, appears to be related to the difference in the nature of the adducts formed. Whereas cisplatin ultimately forms mainly intrastrand bifunctional adducts, given sufficient time YH11 and YH12 as well as YH9 and YH10 can be expected to form mainly interstrand bifunctional adducts. Recent findings suggest that Pt(GNG) 1,3-intrastrand adduct is also likely to be formed with *trans*-planaramineplatinum(II) complexes (Kasparkova, Novakova *et al.* 2003). It is possible that the repair enzymes present in A2780 cells are able to repair better YH11 and YH12 induced lesions than cisplatin induced lesions. It was stated earlier that both cisplatin and transplatin form monofunctional adducts at the same rate but the two compounds differ in the closure of monofunctional adducts into bifunctional lesions. However, 48 h of exposure in the cell culture study may be enough to cause closure of majority of the monofunctional adducts in the case of cisplatin, transplatin and *trans*-planaramineplatinum(II) complexes.

The results of two dimensional NMR study (Paquet, Boudvillain *et al.* 1999) show that when transplatin forms interstrand GC crosslink with DNA, the platinated G residue adopts the *syn* conformation and the bases adjacent to the crosslink remain paired. It is also seen that the NH_3 molecules of transplatin are on either side of the plane of the crosslinked bases and push away the adjacent paired bases along the axis of the double helix. The model provides an explanation as to why the closure of the transplatin-GN7 monofunctional adduct into interstrand bifunctional GN7-CN3 adduct is slow. Two events namely rotation of the platinated G residue from the *anti* to *syn* conformation and vertical displacement of the adjacent base pairs have to take place concomitantly in order to bring the platinum unit near the N3 atom of cytosine residue. That the rate of closure is faster for the *trans*-pyridineplatinum(II) complexes than for transplatin can be explained in terms of a greater vertical displacement of the adjacent base pairs caused by the bulkier planaramine ligands.

The higher activity of YH12 could be due to a greater steric constrain introduced by the bulkier imidazo(1,2-α)pyridine ligand causing a much greater distortion of the DNA including a greater vertical displacement of the adjacent base pairs. The other reason why DNA gets distorted when interstrand bifunctional adducts are formed is due to a mismatch between binding sites distance and that between the two '*trans*-arms' of platinum(II). For transplatin and *trans*-planaramineplatinum(II) complexes, the distance between binding sites in DNA needs to be about 0.36 nm in order to form interstrand bifunctional adducts. However, the distance between opposite N7-guanine and N3-cytosine is 0.62 nm. Thus the DNA interstrand crosslinking by cisplatin requires a distortion of the canonical B-DNA conformation (Sherman, Gibson *et al.* 1988; Lippert 1989). The degree of distortion would be greater for the *cis*-isomer than

the *trans*-isomer. It was shown that interstrand GC adducts bend DNA more than intrastrand 1,2-GG adduct. Thus, when *trans*-planaramineplatinum(II) complexes form interstrand adducts, there will be a conformational change in DNA.

4.5 Platinum accumulation in A2780 and A2780^{cisR}

Figure 3.8-1 gives amount of platinum accumulated in A2780 cells expressed as nanomoles Pt per 10^6 cells. For all the compounds: YH9, YH10, YH11 and YH12, amount of platinum accumulated in A2780 and A2780^{cisR} cells, is found to be greater than cisplatin although the compounds were found to be less active than cisplatin. This is in line with the reported observations that the uptake of pyridine complexes by L1210 cells was greater for the *trans*-isomer over the *cis*-isomer by a factor of about 4 (Boccarelli, Coluccia *et al.* 1999).

4.6 Platinum-DNA binding

Figure 3.8-2 gives the amount of platinum in nanomoles bound to per milligram of DNA in A2780 and A2780^{cisR} cells. For all the compounds: YH9, YH10, YH11 and YH12, the levels of platinum-DNA binding in A2780 and A2780^{cisR} cells were found to be greater than that for cisplatin even though all of the compounds were less active than cisplatin against A2780 cell line and all except YH12 were less active than cisplatin against A2780^{cisR} cell line as well. This suggests that the level of platinum bound to DNA per se does not provide an explanation for variation in cytotoxicity of the compounds. The levels of Pt-DNA binding in A2780^{cisR} cell line for YH10, YH11 and YH12 are found to be three, four and ten times respectively as compared to that

for cisplatin. It appears that whereas a ten-fold increase in Pt-DNA binding has translated into an increase in cytotoxicity, three- and four-fold increase has not.

It should be noted that cytotoxicity, cell uptake and level of binding with DNA of transplatin was not determined as it is well known that transplatin is not cytotoxic. In a comparative study of the binding properties of five compounds with *cis*-configuration (cisplatin, transplatin, JM118, JM216 and JM149) and two compounds with *trans*-configuration (transplatin and JM335) in two human ovarian carcinoma cell lines (SKOV-3 and CHI), no correlation was found between levels of total platinum bound to DNA and cytotoxicity (Mellish, Barnard *et al.* 1995).

A much greater amount of YH12 is found to bind to DNA in $A2780^{cisR}$ cell line than in A2780 cell line. It appears that YH12 has been able to overcome to some extent cisplatin resistance in $A2780^{cisR}$ cells. Although the exact mechanism of cisplatin resistance remains largely unclear, as stated earlier one or more of the following may operate together in most of the resistant cells: (1) decreased cellular accumulation of cisplatin, (2) increased levels of glutathione or glutathione-S-transferase activity, (3) increased levels of intracellular metallothioneins, (4) enhanced DNA repair, and (5) loss of DNA mismatch repair (MMR) activity (Epstein 1990; Johnson, Ozols *et al.* 1993; Bates and Vousden 1996). The absence of MMR in human ovarian tumour models has been found to correlate with the loss of p53-dependent apoptosis (Anthoney, McIlwrath *et al.* 1996). The most clearly understood function of MMR is its role in the correction of mismatches that may occur during DNA replication or DNA recombination. That the cellular accumulation of the most active *trans*-planaramineplatinum(II) complex YH12 in $A2780^{cisR}$ cells is less than in A2780 cells

and yet the level of platinum-DNA is greater in A2780^{cisR} cells than in A2780 cells, may be taken to indicate that the degree of deactivation of the drug by cellular platinophiles is less in the cisplatin-resistant cell than in the cisplatin-responsive cell line. In any case, the results clearly show that the compound has been able to overcome some mechanisms of cisplatin resistance operating in A2780 cells.

For the compound YH10, although the level of platinum accumulation in A2780^{cisR} cells is found greater than that in A2780 cells, the levels of platinum-DNA in the two cell lines are similar, in line with the similar activity of the drug against the two cell lines. For YH11, there is a marked decrease in cell uptake in going from A2780 cells to A2780^{cisR} cells. But the level of platinum-DNA remains essentially unchanged in line with similar activity.

The high levels of platinum-DNA binding observed for both YH11 and YH12 in A2780 and A2780^{cisR} cell lines appear to be in line with the idea that a greater conformational change is induced in pBR322 plasmid DNA by the compounds (as evident from a greater prevention of BamH1 digestion). (Although pBR322 plasmid DNA and the cellular DNA would differ in many respects, it is important to note that both are duplex DNA and that the conformational change in DNA and the tumour activity of the platinum drugs are both associated with the drug-DNA binding). It should however be noted the actual level of platinum-DNA binding in A2780^{cisR} cell line is significantly greater for YH12 than for YH11, in line with much higher activity of YH12 than YH11 against the cell line. The results support the idea that a greater level of similar type of binding (intrastrand bifunctional) with DNA may translate into a higher level of activity.

It is believed that the interaction of platinum drugs with DNA involves a number of stages including aquation of the complex, preassociation with DNA, formation of monofunctional adduct, closure to a bifunctional adduct, distortion of the DNA and recognition of the distortion (Hambley 2001). Higher molar conductivity values observed for YH11 and YH12 (highest for YH12) than for YH9 and YH10 (Table 3.3-1) indicate that the degree of dissociation is greater for the compounds. That the level of platinum-DNA binding in A2780 and $A2780^{cisR}$ cells is greater for YH11 and YH12 than for YH9 and YH10, provides support to the idea that the intracellular aquation of the complexes precedes their binding with DNA.

The activity of YH10 and YH11 in $A2780^{ZD0473R}$ and $A2780^{cisR}$ cell lines are found to be similar. YH9 is found to be more active in $A2780^{ZD0473R}$ cell line than in $A2780^{cisR}$ cell line and the converse is true for YH12, although YH12 is the most active compound against $A2780^{ZD0473R}$ cell line. The results point out that the mechanisms of drug resistance are complex. The determination of uptake of the drugs in $A2780^{ZD0473R}$ cells and the level of platinum-DNA binding would provide more light on the matter.

4.7 Interaction between YH9, YH10, YH11 and YH12, and nucleobases

As stated earlier, YH9, YH10, YH11 and YH12 all have two labile chloride ligands per molecule so that the compounds are expected to form 1 : 2 adducts with adenine and guanine. In this study HPLC has been used to determine the binding ratio between the complexes and nucleobases adenine and guanine.

When YH9 was allowed to interact with adenine, there was one major peak in the chromatogram of the incubated mixture having the retention time of 25.20 min as against the retention time of 24.61 min for adenine. The Pt : NB binding ratio of the peak was found to be close to one indicating that YH9 combined with adenine forming 1 : 1 adduct rather than the expected 1 : 2 adduct. The retention times of the major peaks in the incubated mixtures of YH10, YH11 and YH12 with adenine were respectively 24.51, 25.40 and 25.19 min respectively. Again, it was found that the Pt : NB binding ratio in all cases was close to one. However, the Pt : NB binding ratios of the major peaks in the incubated mixtures of YH9, YH10, YH11 and YH12, and guanine were all found to be close to 2. The results show that whereas the binding of YH9, YH10, YH11 and YH12 with guanine has gone to completion after 24 h of incubation that with adenine has not. Since acetic acid is a good ligand for platinum(II) that can readily displace coordinated water (Appleton, Berry *et al.* 1984), it is possible that the observed 1 : 1 ratio is due to the formation of a mixed complex in which platinum(II) is coordinated to adenine and acetate.

It is difficult to decide whether the difference in reactivity of the compounds with adenine and guanine is due to kinetic and/or thermodynamic factors. Further experiments with longer periods of incubation and the use of mobile phase that does not contain any components that will bind to platinum(II) under elution conditions may provide light on the matter.

It may be noted that the *trans*-planaramineplatinum(II) complexes in the present study (like other such compounds) are believed to bind with duplex DNA, forming monofunctional adducts, and bifunctional interstrand GC adducts that may ultimately

evolve into interstrand GG adducts (Brabec and Leng 1993). The results of the HPLC studies may be taken to provide support to the idea that the compounds have a preference to bind with guanine over adenine.

Finally it should be noted that the results of the present study support the idea that the replacement of one ammonia ligand of the inactive transplatin by the aromatic planaramine ligand can significantly increase the cytotoxicity of the compounds and clearly show that it is possible to alter the level of activity and thus the spectrum of activity of *trans*-planaramineplatinum(II) complexes by altering the nature of the planaramine ligand. It is quite likely that we are yet to see the full potential of tumour active platinum and other metal compounds.

5 Chapter Five: Conclusion

Three commonly used methods for treatment of cancer are: surgery excision, radiation treatment and chemotherapy, used alone or in combination. Surgery can cure cancer that is localised but not metastasised. Radiation is only effective to tumour that is growing slowly and it is usually used in combination with surgery. The third mode of treatment is chemotherapy with cytotoxic drugs, the goal of which is to inhibit the replication of cells. Although cisplatin and its analogue carboplatin are two of the most widely used anticancer drugs, the compounds have a number of side effects and a limited spectrum of activity. Some cancer cells have inherent resistance to the drugs and others develop resistance due to continued use. Thus thousands of cisplatin analogues have been prepared by varying the nature of the leaving groups and the carrier ligands with the aim of reducing toxic side effects and widening the spectrum of activity. Alteration in the nature of the leaving groups has made possible a reduction in side effects (eg carboplatin which has carboxylate leaving group in stead of chloride in cisplatin is found to be less toxic than cisplatin) and alteration in the structure of the carrier ligands has made possible to achieve a limited change in the spectrum of activity (eg unlike cisplatin oxaliplatin is found to be active against colorectal cancer). Some orally active compounds (eg ZD0473) have also been designed. In spite of the limited success in altering the spectrum of activity, it may be true to say that generally all cisplatin analogues have a similar spectrum of activity and often develop cross resistance with cisplatin.

Thus currently attention is given to the rule breaker platinum compounds (and compounds of other metals) with the idea that the different nature of binding of the compounds with DNA would translate into a different spectrum of activity. One such

class of compounds are the *trans*-planaramineplatinum(II) complexes which have shown significant activity against both cisplatin-responsive and cisplatin-resistant cancer cell lines. The present study deals with the synthesis, characterization, tumour activity of four *trans*-planaramineplatinum(II) complexes, code named YH9, YH10, YH11 and YH12, of the form *trans*-$PtL(NH_3)Cl_2$ where L stands for 2-hydroxypyridine, imidazole, 3-hydroxypyridine and imidazo(1,2-α)pyridine respectively. The compounds have been characterized by elemental analyses, IR, Raman, mass and ^1H NMR spectral studies, and molar conductivity measurement.

The activity of the compounds against human cancer cell lines including ovarian: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, melanoma cell line Me-10538, cell uptake, DNA-binding and nature of interaction with pBR322 plasmid DNA and salmon sperm DNA have been studied.

All of the compounds are found to be less active than cisplatin against the ovary cell line A2780, the actual order of activity being cisplatin > YH12 > YH11 > YH10 > YH9. Although for YH9 the uptake in A2780 cell was greater than that for YH10, the extent of Pt-DNA binding was similar for both the compounds in line with their similar activity, indicating that it is the level of drug-DNA binding (rather than cell uptake per se) that is a better indicator of the activity of compounds.

For $A2780^{cisR}$ cell line, all of the compounds except YH12 are found to be less active than cisplatin, the actual order of activity being YH12> cisplatin > YH11 > YH10 > YH9. YH12 is found to be twice as active against $A2780^{cisR}$ cell line than the parent cell line A2780 suggesting that the compound has been able to overcome better the

mechanisms of resistance operating in $A2780^{cisR}$ cell line than those operating in the parent cell line.

For $A2780^{ZD0473}$ cell line, all of the compounds are found to be less active than cisplatin, the actual order of activity being cisplatin > YH12 > YH9 > YH11 > YH10. YH9 was found to be more active against the resistant cell $A2780^{ZD0473R}$ than the parent cell line A2780. The results suggest that the compound has been better able to overcome resistance in the $A2780^{ZD0473}$ cell line than that in the parent cell line.

The same pattern of activity is observed for NCI-H460 and Me10538 cell lines with YH12 the most active compound and YH9 the least active.

For all the compounds: YH9, YH10, YH11 and YH12, amount of platinum accumulated in A2780 and $A2780^{cisR}$ cells, was found to be greater than cisplatin although the compounds were found to be less active than cisplatin. The levels of platinum-DNA binding in A2780 and $A2780^{cisR}$ cells were also found to be greater than cisplatin. The mismatch in the level of activity of the compounds as compared to cisplatin and that of platinum-DNA binding is not difficult to explain when we note that whereas cisplatin is more likely to form bifunctional intrastrand adducts with duplex DNA, YH9, YH10, YH11 and YH12 are more likely to form bifunctional interstrand adducts.

It appears that whereas a ten-fold increase in Pt-DNA binding for YH12 has translated in an increase in cytotoxicity, three- and four-fold increase has not; rather YH10 and YH11 are found to be less active than cisplatin against $A2780^{cisR}$ cell line.

Gel electrophoresis results show that as the compounds bind with pBR322 plasmid DNA, unwinding of supercoiled form I DNA takes place to change from negatively supercoiled form I through the relaxed circular form I to positively supercoiled form I. As the DNA conformation is changed mobility of the form I band changes. The changes in DNA conformation are believed to be due to binding of the compounds with the DNA. As stated earlier, unlike cisplatin, which forms mainly 1,2-intrastrand adducts, *trans*-planaramineplatinum(II) complexes are believed to bifunctional interstrand adducts in addition to monofunctional adducts. Formation of interstrand adducts induces more of a global change in the conformation of the DNA whereas formation of 1,2-intrastrand adduct induces a local kink in the DNA strand. Increasing prevention of BamH1 digestion with the increase in concentration of cisplatin and *trans*-planaramineplatinum(II) complexes indicates both global and local conformational changes in DNA may be sufficient to prevent recognition by the restriction enzyme.

Finally, the above results show that it is possible to design *trans*-planaramineplatinum(II) complexes with activity much greater than cisplatin and that the activity of the designed complexes can be modulated by changing the nature of the carrier ligand. Thus, the results of the present study support the hypothesis that platinum compounds that bind to DNA in a manner different from that of cisplatin may have altered pharmacological properties (Kasparkova, Marini *et al.* 2003). In particular, the variation in activity of YH9, YH10, YH11 and YH12 against the cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R} illustrate structure-activity relationship.

What is next?

Although the method of synthesis, the achieved purity and the spectral results all provide support for the structures of the compounds, it would be better to confirm the structures by x-ray diffraction.

It is clear that one of the designed complexes namely YH12 is significantly more active than cisplatin against the cisplatin-resistant cell line $A2780^{cisR}$ indicating that the compound is able to overcome resistance in the cell line better than cisplatin. Further experiments need to be carried out to explore the possible mechanisms of resistance including the increase in concentration of cellular platinophiles (eg glutathione) and nucleotide excision repair.

To understand why YH9, YH10, YH11 and YH12 display very low activity against the cell lines: NCI-H460 and Me10538, it would be useful to determine the cell uptake and the level of drug-DNA binding of the compounds applying to the cell lines.

YH12 appears to be sufficiently active against $A2780^{cisR}$ cell line to warrant studies on the toxicity of the compound using suitable human xenograft mouse models.

The journey in the discovery of newer and better metal-based anticancer drugs appears to be far from complete.

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7 Appendix: List of Abbreviations:

AAS	Atomic absorption spectroscopy
CS	Cockayne syndrome
DMA	Deoxyribonucleic acid
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Dimethylsulfoxide-d_6
DRA	Diffuse Reflectance Accessory
EDTA	Disodiumethylenediaminetetraacetate
ESI	Eletrospray ionization
GHS	Glutathione
ICR	Institute of Cancer Research
IR	Infrared
JM	Johnson Matthey
LC	Liquid chromatograph
MMR	Mismatch repair
MT	Metallothionein
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PCNA	Proliferating-cell nuclear antigen
PFC	Proteins replication factor C
POL	Polymerase
RF	Resistance factors

SAR	Structure-Activity Relationships
SIM	Selected Ion Monitoring
ssDNA	Salmon sperm DNA
TAE	Tris-acetate
TBE	Tris-borate
XP	Xeroderma pigmentosum group
YH9	*Trans*-(ammino)(2-hydroxypyridinato)dichloroplatinum(II)
YH10	*Trans*-(ammino)(imidazolato)dichloroplatinum(II)
YH11	*Trans*-(ammino)(3-hydroxypyridinato)dichloroplatinum(II)
YH12	*Trans*-(ammino)(imidazo(1,2-□)pyridinato)dichloroplatinum(II)