An investigation of the \textit{in vitro} anticancer properties of selected platinum compounds

by
Debbie du Plessis-Stoman

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Supervisors: Dr M. van de Venter
Prof J. G. H. Du Preez
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SUMMARY

This dissertation mainly deals with some biochemical aspects regarding the efficacy of novel platinum anticancer compounds, as part of a broader study in which both chemistry and biochemistry are involved.

Various novel diamine and N-S donor chelate compounds of platinum II and IV have been developed in which factors such as stereochemistry, ligand exchange rate and biocompatibility were considered as additional parameters. In the first order testing, each of these compounds was tested with reference to their “killing” potential by comparing their rate of killing, over a period of 48 hours with those of cisplatin and oxaliplatin. Some 80 compounds were tested in this way. Although only a few could be regarded as equal to or even better than cisplatin and oxaliplatin, the testing of these compounds on cancer cells provided useful knowledge for the further development of novel compounds.

Four of the better compounds, namely Y9, Y14, Y16 and Lt16.2 were selected for further studies to obtain more detailed knowledge of their anticancer action, including some flow cytometric studies. In addition to the above, cisplatin resistant cells were produced for each of the three different cell lines tested, namely, HeLa, HT29 and MCF7 cancer cell lines, by intermittent and incremental exposure to cisplatin (all the cell lines tested became resistant to cisplatin). Each of the selected compounds were exposed to the cells in the same manner, in order to attempt the induction of resistance against these compounds in the three cell lines tested (i.e. whether these cells will become resistant to the various compounds). Each of these selected platinum containing compounds were subsequently tested against the “cisplatin resistant” cell lines in order to determine their efficacy against such cells. One such compound could be singled out, since cervical cancer cells (HeLa cells) do not become resistant to it. This behaviour is similar to that of oxaliplatin against cervical cancer and colon cancer (HT29) cells (oxaliplatin is the number one treatment for colon cancer at present). This compound also proved to be more active against cisplatin resistant cell lines. It was found that all the compounds induced apoptosis in the cell lines tested as well as inhibit the DNA cycle at one or more phase.
Finally, an effort was made to evaluate the different compounds by comparing them with respect to their properties relating to anticancer action.
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<th>Description</th>
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<tr>
<td>Au</td>
<td>gold</td>
</tr>
<tr>
<td>CBDCA</td>
<td>carboplatin</td>
</tr>
<tr>
<td>CDDP</td>
<td>cisplatin</td>
</tr>
<tr>
<td>CIN</td>
<td>cervical intra-epithelial neoplasia</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CN⁻</td>
<td>cyanide</td>
</tr>
<tr>
<td>Br⁻</td>
<td>bromide</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra acetate</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>GST</td>
<td>glutathione-S transferase</td>
</tr>
<tr>
<td>GS-X pump</td>
<td>glutathione S-conjugate export pump</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HMG-domain proteins</td>
<td>high mobility group-domain-proteins</td>
</tr>
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<td>human papilloma virus</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>I⁻</td>
<td>iodide</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>inhibiting concentration at which 50% of cells are non-viable</td>
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<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
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<td>IDC</td>
<td>invasive ductal carcinoma</td>
</tr>
<tr>
<td>LS</td>
<td>large subunit</td>
</tr>
<tr>
<td>MIN</td>
<td>microsatellite instability</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MT</td>
<td>metallocationin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>N</td>
<td>nitrogen</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>NMMU</td>
<td>Nelson Mandela Metropolitan University</td>
</tr>
<tr>
<td>NO²⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>PBSA</td>
<td>phosphate buffered saline excluding Ca²⁺ and Mg²⁺</td>
</tr>
<tr>
<td>Pd</td>
<td>palladium</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>Pt</td>
<td>platinum</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S</td>
<td>sulfur</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>thiocyanite</td>
</tr>
<tr>
<td>SC(NH₂)₂</td>
<td>thiourea</td>
</tr>
<tr>
<td>SS</td>
<td>small subunit</td>
</tr>
<tr>
<td>STD</td>
<td>sexually transmitted disease</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>Trans-DDP</td>
<td>transplatin</td>
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CHAPTER 1  LITERATURE REVIEW

1.1 INTRODUCTION

Cancer is an important public health concern around the world and is a significant cause of death in the human population (Parker *et al.* 1997; Walker and Walker, 1999). The application of inorganic chemistry to medicine (“Elemental Medicine”) is a fast developing field, and novel therapeutic and diagnostic metal complexes are now having an impact on medical practice. Advances in bio-inorganic chemistry are vital for improving the design of compounds to lessen toxic side effects and understand their mechanisms of action (Sadler and Guo, 1998).

The platinum drugs represent a unique and important class of anti-tumour agents. The discovery of the neutral, square planar, coordination complex *cis*-diamminedichloroplatinum (II) (cisplatin) in the 1970’s has revolutionized the chemotherapy of human cancer (Gonzalez *et al.* 2001). When cisplatin was used in combination chemotherapy with other drugs, it achieved cure rates of up to 90% against testicular cancer, and also displayed significant activity against other types of tumours (Gupta *et al.* 2004). The results of radiotherapy alone are unsatisfactory, since overall only 5-7% of the patients survive more than 5 years and Winterhalder *et al.* (2004) showed that there is a definite survival benefit of adding platinum based chemotherapy to radiation for the treatment of non-small-cell lung cancer. Unfortunately the full therapeutic potential of cisplatin has not been realized due to the serious side effects and emergence of cisplatin-resistant tumour cells related to treatment with cisplatin. Extensive hydration, improved schedules of administration, alternate routes of administration and use of protective agents against specific side effects are some of the protective methods employed that have allowed the use of higher doses of cisplatin against cisplatin-resistant tumours and has extended the list of tumour systems responsive to cisplatin chemotherapy (Walker and Walker, 1999).

The incorporation of cisplatin into a number of cisplatin-based anti-cancer drug combinations has improved its efficiency and allowed the use of lower doses of cisplatin, which reduces its toxic side effects. The availability of cisplatin analogues with reduced toxicity, but increased efficiency against cisplatin-resistant tumours, has
expanded the potential scope and therapeutic promise of the platinum anti-cancer agents. Cisplatin is widely used for the treatment of many malignancies, including testicular, ovarian, bladder, cervical, head and neck, and small-cell and non-small-cell lung cancers and the development of chemotherapy with the platinum anti-tumour compounds is still in progress. (Gonzalez et al. 2001; Walker and Walker, 1999).

1.2 METAL COMPLEXES IN MEDICINES

Most of the main classes of pharmaceutical agents contain examples of metal compounds that are currently used clinically; moreover, new areas of use are fast emerging. Targeting is vital, because of the toxicity often associated with metal compounds. If they can be delivered only to the tissues, cells and receptors where they are required, the toxicity may be reduced. The ease with which many metal complexes undergo ligand substitution and redox reactions might mean that the active species are biotransformation products of the administered complex. Identification of these active species may lead to the more effective use of metal compounds as drugs (Sadler and Guo, 1998).

A programme for the development of novel Pt anticancer agents has been initiated at this university. In view of the interdisciplinary nature of such a program the expertise of the chemistry and biochemistry departments have been joined in a combined effort, to cover both the chemical and biochemical aspects thereof.

The research unit for Platinum Group Chemistry, plan, synthesize and characterise the chemical aspects and the Biochemistry Department evaluates the anticancer action in terms of their efficacy in killing a number of types of cancer cells as well as their mode of anticancer action. The latter study is essentially in order to provide feedback, which enables a correlation of their anticancer properties with their structure and physical chemical characteristics. This dissertation deals with the Biochemical aspects of this project. It is however, advantageous to deal with some chemical background.
1.2.1 Platinum anticancer agents

1.2.1.1 Some basic chemistry

In order to obtain insight as to how the interaction of a metal, such as platinum, can achieve anticancer results, it is necessary to briefly review the most important properties of the metal.

(i) Ligand preferences

Pt is a typical later 5d transition metal, which forms highly covalent bonds with N and S donor atom ligands, like aliphatic and aromatic amines and thiols, thioethers, thiocarbamates, etc. Its compounds are kinetically stable (relatively slow ligand exchanges) and thermodynamically stable with the above-mentioned ligand types (Wilkinson, 1987).

(ii) Ligand exchange rate

Pt(II) has the potential to form a stable bond with N donor ligands in the DNA helix, thus preventing DNA replication and promoting cell death. The ligand exchange is controlled by the “trans effect” which is the effect of a coordinated ligand on the rate of replacement of a ligand trans to it. A sequence of trans effectiveness is: \( \text{H}_2\text{O} < \text{NH}_3 \sim \text{amines} < \text{Cl}^- < \text{Br}^- < \text{SCN}^- < \text{I}^- < \text{NO}_2^- < \text{SC(NH}_2)_2 < \text{thioethers} < \text{CN}^- \). This behaviour relates directly to the anticancer action of Pt(II) since neither too fast exchange nor too slow exchange is required for optimal action (Tobe, 1972).

(iii) Coordination chemistry

Platinum (II) is without exception found in a square planar symmetry. It can exist in either a cis or trans complex form.

![cis and trans complexes](image)

The anions are normally the “leaving groups” which implies that they can readily be replaced. Exchange of bidentate anions, like oxalate ions, are slower than e.g. monodentate ones, like Cl\(^-\). The stability of square planar complexes as compared to higher coordination, e.g. five-coordination is an important
parameter in controlling ligand exchange, since an important mechanism by which it occurs is via a five-coordinated intermediate. Ligand exchange can also be slowed down by stereochemical crowding of Pt(II) in the complex which will suppress the formation of the above mentioned intermediate form (Wilkinson, 1987; Tobe, 1972).

(iv) Hydrophobicity

Hydrophobicity of the Pt(II) complex is a further significant parameter, e.g. in the case of an organic amine donor with an alkyl or aryl “tail end”, cell membrane transport is promoted and thus uptake of the complex (Hambley, 1997).

(v) S donor ligands

The interaction, with the anticancer agent, of the S donor ligands present in the biological fluid, more specifically thiols (like glutathione and cysteine) is important. Thiols normally bond irreversibly to Pt(II) under the conditions obtaining in body fluids (pH ≅ neutral), thus rendering the Pt(II) agent inert and ineffective for anticancer action. Whereas an N7 atom of guanine can replace a thioethereal S atom from a Pt(II) complex, it cannot do so with an thiolate ion RS⁻.

Thioethers react as follows with Pt(II) compounds:

\[
\left[ \begin{array}{c} \text{Cl} \\
\text{Pt} \\
\text{Cl} \\
\text{Cl} \\
\end{array} \right]^{2-} + \text{RSR} \rightarrow \left[ \begin{array}{c} \text{Cl} \\
\text{Pt} \\
\text{SR}_2 \\
\text{Cl} \\
\end{array} \right]^- + \text{Cl}^-
\]

Through a neutral S donor action, thiols react as follows with Pt(II) compounds:

\[
\left[ \begin{array}{c} \text{Cl} \\
\text{Pt} \\
\text{Cl} \\
\text{Cl} \\
\end{array} \right]^{2-} + \text{HSR} \rightarrow \left[ \begin{array}{c} \text{Cl} \\
\text{Pt} \\
\text{SR} \\
\text{Cl} \\
\end{array} \right]^{2-} + \text{HCl}
\]

i.e. deprotonation of the thiols occurs due to the strong Pt-S interaction and simultaneous weakening of the S-H bond. Thus, amounting to changing of the neutral HSR compound into a thiolate ion (SR).
The very strong trans-directing capability of the –SR group does not allow strong bonding \textit{trans} to it, except by another \textit{~}SR group, thus reducing anticancer action.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{The space-filling models of two sulfur containing biomolecules (A) Cysteine and (B) Glutathione.}
\end{figure}

The space-filling models depicted in figure 1.1 shows the relatively large size of thiol sulfur which acts as a strong donor towards Pt(II) as described in the text. The above compounds can act as detoxifying agents against the platinum agents (Bierbach \textit{et al.} 1998; Reedijk, 1999).

\begin{enumerate}
\item[(vi)] Biocompatibility
The platinum compounds should have significant solubility in body fluid. Solubility should conveniently be between 10 and 100 \textmu M (Los \textit{et al.} 1996).
\item[(vii)] Mode of bonding
In general, the \textit{cis}-compounds are the most effective anticancer agents, e.g. cisplatin is much better than transplatin. It is interpreted that the \textit{cis} complex can form intramolecular adducts with N atoms in the helix much more readily than the \textit{trans} complex. These result in a kind of chelate adduct that is kinetically and thermodynamically more stable and can thus more readily cause cell death.
\end{enumerate}
The space-filling models of two important nucleotide bases, adenine and guanine (Figure 1.2), show that the N7 atom of the guanine is more “exposed” or lesser crowded than the corresponding N7 atom of the adenine. The result is that the former acts as a stronger ligand towards Pt(II) than the latter. For example, the guanine N7 atom can replace a thioethereal sulfur atom bonded to platinum, whereas that of adenine cannot (Jamieson and Lippard, 1999).

(viii) Stereochemistry
The influence of stereochemistry in relation to the control of kinetics of ligand exchange is not the only result of stereochemical crowding of these agents. The role of the “organic bulk” of the ligands bonded to the platinum has also been related to an interaction mode with the major groove of the helix, in a fashion that promotes the bonding of the complex to the helix. Evidence for such interaction came from 1,2-diaminocyclohexane compounds such as oxaliplatin (Scheeff et al. 1999).

1.2.1.2 Novel complexes
In the design of novel anticancer agents all the above factors had to be considered. It was decided to concentrate on novel diamines and N-S chelates (S=thioether groups) as “non-leaving” groups. The structures were varied to allow for different stereochemical interactions and hydrophobicity. For “leaving groups” chloride and dicarboxylate anions were used.
N-N and N-S signifies bidentate chelate ligands forming five-membered ring complexes with Pt(II) and X=monoanionic species. The latter could be replaced by dianionic species.

1.2.1.3 Important biochemical aspects

Platinum (II) complexes are amongst the most extensively used drugs for the treatment of cancer. Three injectable diammine compounds have been approved for clinical use. These include cisplatin, carboplatin and 254-S (Figure 1.3). Several others are on clinical trials. Studies on reducing the toxicity of platinum anticancer complexes towards normal cells, evading acquired resistance to cisplatin, and increasing the spectrum of activity of platinum complexes towards a wider range of types of cancer, are currently in progress (Sadler and Guo, 1998). Other cisplatin analogues include oxaliplatin, nedaplatin, loboplatin, ZD0473, oxoplatin and Se-Pt conjugate (Figure 1.3) (Wozniak and Blasiak, 2002).
The decisive target for Pt is DNA and certain platinated DNA adducts trigger DNA degradation and apoptosis (programmed cell death) (Sadler and Guo, 1998). Apoptosis is an actively performed cellular suicidal process, which is essential for development and maintenance of tissue homeostasis of multicellular organisms. Prominent morphological features such as membrane blebbing, nuclear condensation, DNA fragmentation and apoptotic body formation characterise it. Caspases, which belong to the family of cysteine proteases, play a crucial role in the implementation of apoptosis. Caspases are synthesized as inactive zymogens, which become activated by cleavage after a specific aspartate residue within a linker domain between a large subunit (LS) and a small subunit (SS), in addition to cleaving off of the NH₂-terminal pro-domain. The LS and SS subsequently combine to form active caspases. When cells receive death signals, a subset of caspases is activated in a structured fashion and directs the processing and activation of critical effector/executioner caspases (Araya...
et al. 2002). Apoptosis has three different stages (Figure 1.4). The first is an initiation phase, in which a stimulus is received, followed by engagement of any of several possible pathways that respond to the stimulus. The effector phase is the second stage. During this phase all the possible initiating signals are integrated and a “decision” to live or die is made. The last stage is the common irreversible execution phase, during which some proteins autodigest and DNA is cleaved. Bcl-2 is an oncogene that seems to be at the convergence of many apoptotic pathways and the ratio of Bcl-2 to Bax protein might be the final determinant of whether a cell enters the execution phase. Bax is a gene that encodes a dominant inhibitor of Bel-2. A conserved feature of the execution phase of apoptosis is the specific degradation of a series of proteins by the cysteine-aspartate-specific proteases, or caspases. Caspases are activated when an apoptotic stimulus induces the release of cytochrome c from mitochondria. However, little is known about what initiates activation of the first caspase and what constitute the critical substrates for caspase cleavage (Gonzalez et al. 2001).

Figure 1.4: Scheme of the converging pathways leading to apoptosis in mammalian cells. A great variety of stimuli, such as depletion of survival signals, death signals, physical and chemical agents, and loss of cell-cell contacts, can initiate apoptosis through numerous different means. The effector phase integrates these signals leading to the decision of life or death. (PDGF, platelet-derived growth factor; TNF, tumour necrosis factor; CDDP, cisplatin, bFGF, basic fibroblast growth factor; FasL, Fas ligand) (Gonzalez et al. 2001).
The efficacy of cisplatin in the clinic is restricted in several ways. These include the spectrum of its anticancer activity, since it is not active enough against several types of cancer, the development of resistance after continued treatment, and its high toxicity to some normal cells (Sadler and Guo, 1998).

1.2.1.4 Other complexes

Bismuth (III) compounds, such as bismuth subcitrate and subsalicylate, are commonly used for the treatment of diarrhoea, dyspepsia and gastric and duodenal ulcers. Bi (III) is active against the bacterium *Helicobacter pylori*, which is associated with the mucus layer of ulcers (Sadler and Guo, 1998).

Injectable Au (I) thiolate drugs such as aurothiomalate (Myocrisin®), aurothioglucose (Solganol®), and aurothiopropanol sulfonate (Allochrysin®), and the oral drug auranofin (Ridaura®), are widely used for the treatment of difficult cases of rheumatoid arthritis. Gold (I) has a much higher affinity for thiolate S compared to thio-ether S, and a much lower affinity for N and O ligands. Consequently Au (I) binds to DNA very weakly and is not usually carcinogenic or mutagenic. Thiolate exchange reactions on Au (I) occur effortlessly and therefore the administered drugs are probably not the pharmacologically active species (Sadler and Guo, 1998).

1.3 CANCER

1.3.1 Cervical cancer

Cervical cancer develops in the lining of the cervix, the lower part of the uterus that enters the vagina. This condition usually develops over time. Normal cervical cells may gradually undergo changes to become pre-cancerous and then cancerous. Cervical intra-epithelial neoplasia (CIN) is the term used to describe these abnormal changes. CIN is classified according to the degree of cell abnormality. Low-grade CIN indicates a minimal change in the cells and high-grade CIN indicates a greater degree of abnormality. Most (80-90%) invasive cervical cancer develops in flat, scaly surface cells that line the cervix (called squamous cell carcinomas). Approximately
10-15% of cases develop in glandular surface cells (called adenocarcinomas) (http://www.oncologychannel.com/cervicalcancer/).

Cancer of the cervix is the second most common cancer in women worldwide and is a leading cause of cancer-related death in women in under-developed countries. Worldwide, approximately 500 000 cases of cervical cancer are diagnosed each year, although routine screening has decreased the incidence of invasive cervical cancer. Invasive cervical cancer is more common in women middle aged and older and in women of poor socio-economic status, who are less likely to receive regular screening and early treatment. There is also a higher rate of incidence among African American, Hispanic, and Native American women (http://www.oncologychannel.com/cervicalcancer/).

The cause of cervical cancer is unknown. Infection with two types of human papilloma virus (HPV), which are transmitted sexually, is strongly associated with cervical and vulvar cancer and is the primary risk factor. Evidence of HPV is found in nearly 80% of cervical carcinomas. Human immunodeficiency virus (HIV) infection reduces the immune system’s ability to fight infection (including HPV infection) and increases the likelihood that pre-cancerous cells will progress to cancer. Women who smoke are twice as likely to develop cervical cancer, since chemicals in cigarette smoke may increase the risk by damaging cervical cells. Other risk factors include age (the condition is rare in women younger than age 15) and race (invasive cancer rates are higher in African Americans, Hispanics, and Native Americans) (http://www.oncologychannel.com/cervicalcancer/). Sexual activity that increases the risk for infection with HPV and HIV and for cervical cancer includes the following: (i) having multiple sexual partners or having sex with a promiscuous partner; (ii) history of sexually transmitted disease (STD) and (iii) sexual intercourse at a young age (http://www.oncologychannel.com/cervicalcancer/).

Cisplatin was found to be cytotoxic to the cervical cancer cell line, HeLa. A cisplatin-resistant HeLa variant cell line, which also exhibits cross-resistance to UV radiation and an enhancement in repair of UV-DNA adducts, has been found and therefore the development of novel drugs for the treatment of cervical cancer is necessary (Chao,
1994). The Hela cell line was first isolated from the cervix of a 31 year old black female and is an adenocarcinoma cell line (http://www.ACTT.org).

### 1.3.2 Breast cancer

Breast cancer is malignant abnormal cell growth in the breast. Cancer cells may spread to other areas of the body (called metastasis). Fibrocystic changes (e.g., formation of cysts, scar tissue) may cause benign lumps in the breast. In women, breast cancer is the second most common type of cancer and the second leading cause of cancer-related deaths. Approximately 200,000 women in the United States are diagnosed with breast cancer each year, and the disease causes about 40,000 deaths annually. The incidence of breast cancer rises after age 40. The highest incidence (approximately 80% of invasive cases) occurs in women over age 50. Most breast cancer develops in glandular tissue and is classified as adenocarcinoma. The earliest form of the disease, ductal carcinoma in situ (DCIS), develops solely in the milk ducts. The most common type of breast cancer, invasive ductal carcinoma (IDC), develops from DCIS, spreads through the duct walls, and invades the breast tissue (http://www.oncologychannel.com/breastcancer/).

Most women who develop breast cancer have no identifiable risk factors other than their gender. The condition is 100 times more common in women. The growth of breast cancer tumours is often affected by the presence of oestrogen and progesterone. The following risk factors result from exposure to these hormones: (i) age (over age 50); (ii) first pregnancy after age 30; (iii) long-term (more than 5 years) hormone replacement therapy (HRT); (iv) menstruation before age 12; (v) menopause after age 50; and (vi) nulliparity (never gave birth) (http://www.oncologychannel.com/breast-cancer/).

Other risk factors include the following: (i) family history of the disease; (ii) genetic link; (iii) history of breast biopsy or radiation to the chest; (iv) moderate alcohol use (2 to 5 drinks daily); (v) obesity; (iv) personal history of the disease (women with a history of breast cancer are 3 to 4 times more likely to have a recurrence); (v) race (slightly more common in Caucasians); and (vi) sedentary lifestyle (http://www.oncologychannel.com/breastcancer/).
Approximately 5% of breast cancer cases have a genetic link that results from an inherited mutation in genes identified as BRCA1 and BRCA2. Patients who inherit an altered BRCA1 or BRCA2 gene have an increased risk for developing pre-menopausal breast cancer and are more likely to have family members with the condition. The cause of breast cancer is unknown (http://www.oncologychannel.com/breastcancer/). The breast cancer cell line, MCF7, was isolated from the mammary gland (breast), of a 69-year-old Caucasian female and is an adenocarcinoma cell line (http://www.ATCC.org).

1.3.3 Colon cancer

The colon and rectum are part of the digestive tract. Together, they comprise the large intestine, which is located in the abdomen between the small intestine and the anus. Cancer that originates in the colon or rectum is called colorectal cancer. The colon absorbs water, electrolytes, and nutrients from food and transports them into the bloodstream. Most (over 95%) colorectal cancers are adenocarcinomas that develop when a mutation occurs in cells that line the wall of the colon or rectum. The disease often begins as an intestinal polyp, also called an adenoma, which is an abnormal growth of tissue. Polyps gradually can become pre-cancerous and then cancerous. Incidence of colorectal cancer is highest in developed countries such as the United States and Japan, and lowest in developing countries in Africa and Asia. According to the American Cancer Society, it is the third most common type of cancer in both men and women in the United States. Incidence is slightly higher in men than women, and is highest in African American men. The death rate from colorectal cancer has declined over the past 15 years due to improved screening methods and advances in treatment. However, the emergence of cisplatin-resistant colon carcinoma cell lines (eg. HT29) necessitates the development of novel drugs for the treatment of colon cancer (http://www.oncologychannel.com/coloncancer/).

The efficiency of cisplatin is low in colorectal cancer, with fewer than 20% clinical responses when used alone or in combination. Oxaliplatin is clinically active in metastatic colorectal cancer. However, an acquired resistance to oxaliplatin always develops after a few months of treatment. Hence, the development of novel drugs for the treatment of colorectal cancer is also imperative (Sergent et al. 2002). The HT29
cell line was isolated in 1964, is an adenocarcinoma cell line and the cells are distributed for research purposes only (http://www.ATCC.org).

1.4 CISPLATIN

Cisplatin belongs to the group of medicines known as alkylating agents (http://www.nlm.nih.gov/medlineplus/druginfo/uspdi/202143.html) and is chemotherapy that is given as a treatment for some types of cancer. It is predominantly used to treat testicular, bladder, lung, oesophagus, stomach, and ovarian cancers. It is a clear fluid, which is given as an infusion into the vein through a cannula as a sterile saline solution. It may be administered through a central line (inserted under the skin into a vein near the collarbone), or a line inserted into a vein in the crook of the arm (http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individualdrugs/Cisplatin and http://chemcases.com/cisplat/). The dose of cisplatin will be different for different patients, since it may depend on a number of things, including what the medicine is being used for, the patient’s size, and whether or not other medicines are also being taken. If a combination of medicines is administered to a patient, it is extremely important that the patient receives each one at the proper time (http://www.nlm.nih.gov/medlineplus/druginfo/uspdi/202143.html). Single Agent Therapy may include: 2 to 3 mg/kg every 3 to 4 weeks, 20 mg/m²/day for 5 days every 3 to 4 weeks, 100-120 mg/m² every 3 to 4 weeks or 80 mg/m² (24 hour infusion) every 3 to 4 weeks (http://www.intox.org/databank/documents/pharm/cisplat/cisplat.htm).

1.4.1 Mechanism of action

Cisplatin coordinates to DNA and this coordination complex not only inhibits replication and transcription of DNA, but also leads to programmed cell death (apoptosis). However, formation of any platinated coordination complex with DNA is not adequate for cytotoxic activity. The corresponding trans isomer of cisplatin (namely, trans-DDP) also forms a coordination complex with DNA but in contrast to cisplatin, trans-DDP is not an efficient chemotherapeutic agent. Due to the difference in geometry between cis- and trans-DDP, the types of coordination complexes formed by the two compounds with DNA are different. These differences are critically
important in determining the value of a particular compound for the treatment of cancer. Hence, a great deal of effort has been placed on discovering the specific cellular proteins that recognize cisplatin-DNA complexes and then examining how the interaction of these proteins with the complexes might lead to programmed cell death of cancer cells (http://chemcases.com/cisplat/; Jordan and Carmo-Fonseca, 2000).

The sterile saline solution in which cisplatin is administered contains sodium chloride. When cisplatin enters the bloodstream, it remains intact due to the relatively high concentration of chloride ions. The neutral cisplatin enters the cell by passive diffusion or alternatively via active uptake by the cell. Once inside the cell, the molecule undergoes hydrolysis, due to a much lower concentration of chloride ions, and a molecule of water replaces a chloride ligand. Hence a positively charged species is generated (Figure 1.5). The reaction occurring is as follows:

\[
\text{Pt(NH}_3\text{)}_2\text{Cl}_2 + \text{H}_2\text{O} \rightarrow [\text{Pt(NH}_3\text{)}_2\text{Cl(H}_2\text{O)}]^{\text{+}} + \text{Cl}^{-}
\]

\[
[\text{Pt(NH}_3\text{)}_2\text{Cl(H}_2\text{O)}]^{\text{+}} + \text{H}_2\text{O} \rightarrow [\text{Pt(NH}_3\text{)}_2\text{(H}_2\text{O)}_2]^{2\text{+}}
\]


Figure 1.5: The cellular uptake of cisplatin and its targets (http://chemcases.com/cisplat/)

Cisplatin has a number of possible targets once inside the cell (Figure 1.5). These include DNA, RNA, sulfur-containing enzymes (metallothionein and glutathione),
and mitochondria. The effects of cisplatin on mitochondrial DNA are not well understood, but there is a possibility that damage to mitochondrial DNA that results from treatment with cisplatin, contributes to cell death. The interaction of cisplatin with sulphur-containing enzymes is better understood and is thought to be involved in the resistance of cells to cisplatin. The effects of cisplatin on RNA and DNA have been studied extensively. Although cisplatin can coordinate to RNA, this interaction is not thought to play a significant role in cisplatin’s mechanism of action in the body. The reasons for this are (i) a single damaged RNA molecule can be replaced by newly synthesized material and studies have shown that cisplatin does not affect RNA synthesis (DNA synthesis is affected) and; (ii) when cisplatin was administered \textit{in vitro} at its lethal dose to a strain of cancer cells, only a small fraction (1% to 10%) of RNA molecules were damaged. Cisplatin coordinates to DNA primarily through certain nitrogen atoms of the DNA base pairs. These nitrogen atoms (in particular, the N7 atoms of purines) are free to coordinate to cisplatin, since they do not form hydrogen bonds with any other DNA bases (Figure 1.6) (http://chemcases.com/cisplat/; Gonzalez \textit{et al.} 2001; Perez, 1998).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{cisplat.png}
\caption{The DNA base pairs. Cisplatin coordinates to the N7 atoms of the purine (guanine and adenine) bases (http://chemcases.com/cisplat/).}
\end{figure}
Numerous types of cisplatin: DNA coordination complexes, or adducts, may be formed. The most significant of these appear to be the ones in which the two chlorine ligands of cisplatin are replaced by purine nitrogen atoms on adjacent bases on the same strand of DNA; these complexes are referred to as 1,2-intrastrand adducts. The purine bases most frequently involved in these adducts are guanines; however, adducts involving one guanine and one adenine are also found. The formation of these adducts causes the purines to become de-stacked and the DNA helix to become kinked (Figure 1.7) (http://chemcases.com/cisplat/).

**Figure 1.7:** (A) The structure of cisplatin coordinated to a dinucleotide containing two guanines. The guanine bases, which would normally be parallel to one another, are de-stacked. (B) The structure of cisplatin coordinated to two guanines in a DNA duplex (http://chemcases.com/cisplat/).
Cisplatin begins its interaction with cancer DNA by binding to the nitrogen atoms of the DNA bases, typically guanine. Approximately 90% of cisplatin-DNA adducts are 1,2 intrastrand cross-links, of which about 65% are two adjacent N7 guanine sites and about 25% are two adjacent N7 guanine-N7 adenine sites. The high occurrence of intrastrand adducts is due to the shortest distance between two N7 atoms. The remaining adducts are comprised of interstrand cross-links and monofunctional cisplatin adducts. Due to conformational constraints, bi-functional adducts are formed only in purines that are adjacent or directly across (not more than one base pair apart) from each other (Figure 1.8) (http://bio.chem.niu.edu/Resources/fall2000/Templates/DNA%20and%20cisplatin/adducts.htm; Siddik, 2003).

![Figure 1.8:](http://bio.chem.niu.edu/Resources/fall2000/Templates/DNA%20and%20cisplatin/adducts.htm)

**Figure 1.8:** (A) Schematic representation of cisplatin adducts. The platinated nucleosides are underlined (Wozniak and Blasiak, 2002). (B) The main adducts formed in the interaction of cisplatin with DNA. (a) interstrand cross-link; (b) 1,2-intrastrand cross-link; (c) 1,3-intrastrand cross-link and; (d) protein-DNA cross-link (Gonzalez et al. 2001).

Hence, these cisplatin adducts aid in destabilization of the DNA. Cisplatin binding severely distorts DNA by twisting, unwinding, and shortening the duplex. It causes significant bending of about 40° away from the site of attachment. Although the overall structure of the double helix remains intact, the distortion is absorbed by conformational changes in the sugar-phosphate backbone near the platinum lesion. This propagates steric constraints that lead to a loss in helix stability.
Trans-DDP is lesser active than cisplatin, due to its geometry, it cannot form a 1, 2-intrastrand adduct with DNA. Hence, the 1, 2-intrastrand adducts, formed between cisplatin and DNA, are believed to be vital for anticancer activity of cisplatin. It has been found that this binding affects both replication and transcription of DNA, as well as mechanisms of DNA repair. The effects of both cisplatin and trans-DDP on DNA replication were studied both in vitro and in vivo. In vitro studies on both prokaryotic and eukaryotic cells showed that DNA adducts of both cisplatin and trans-DDP blocked the action of DNA polymerase, which is necessary for replication (http://chemcases.com/cisplat/). Hence, the presence of an adduct alters the alignment of DNA thus altering the binding site for DNA polymerase, an important enzyme for DNA synthesis. This in turn slows down the protein conformational change necessary for polymerisation of that specific portion. The binding of the next correct nucleotide is therefore affected. Polymerases are able to bypass cisplatin adducts only approximately 10% of the time (http://bio.chem.niu.edu/Resources/fall2000/Templates/DNA%20and%20cisplatin/adducts.htm). Likewise, in vivo studies showed that cisplatin and trans-DDP inhibited replication equally well. Since studies have shown that cisplatin is an effective antitumour agent but trans-DDP is not, these results suggest that DNA replication is not the only factor important for the clinical activity of cisplatin in the destruction of cancer cells. The effects of cisplatin and trans-DDP on DNA transcription are more difficult to interpret than the effects on replication. However, cisplatin does not appear to inhibit transcription (http://chemcases.com/cisplat/).

The cytotoxic activity of cisplatin may arise from the cells’ inability to repair DNA damage caused by cisplatin. In vitro studies on cell extracts suggested that the most common 1,2-intrastrand cisplatin: DNA adducts are not readily repaired by the excision repair system. Researchers have conducted additional studies to address the likelihood that cisplatin’s cytotoxic activity may result from a failure of the excision repair system. This repair system relies on the fact that, before the damaged portion of DNA is even excised from the rest of the strand, the cell must recognize it. The cell detects DNA damage by the action of damage recognition proteins. Hence,
evidence of proteins attached to cisplatin: DNA adducts were looked for. Several proteins that bind to cisplatin: DNA adducts were isolated and these proteins all contain a portion of similar or even identical sequences of amino acids, called a high mobility group (HMG). The proteins in this class are called HMG-domain proteins. It has been shown that HMG-domain proteins bind cisplatin: DNA adducts \textit{in vitro}. \textit{In vivo} assays on yeast have also provided confirmation that HMG-domain proteins are important for the activity of cisplatin, since cells lacking the gene that codes for HMG-domain proteins are less sensitive to cisplatin than cells containing the gene. Hence, cisplatin is less effective in killing these cells (http://chemcases.com/cisplat/; Jordan and Carmo-Fonseca, 2000).

Two theories explain the possible role of HMG-domain proteins in cisplatin’s cytotoxic activity. Various HMG-domain proteins are transcription factors, and are thus required for the synthesis of RNA from a DNA template. The first theory claims that if HMG-domain-containing transcription factors bind preferentially to the cisplatin: DNA adducts, they could interfere with the transcriptional machinery, most probably leading to cell death. The second theory asserts that when HMG-domain proteins bind to the cisplatin: DNA adducts, these adducts would not be recognized by the repair machinery and DNA repair would then be slower than normal (Figure 1.9) (http://chemcases.com/cisplat/).

\textbf{Figure 1.9:} Model for the inhibition of cisplatin adduct repair in the presence of HMG-domain proteins (http://chemcases.com/cisplat/).
1.4.2 Toxic side-effects

Cisplatin interferes with the growth of cancer cells, which are ultimately destroyed. Since the growth of normal body cells may also be affected by cisplatin, other effects will inevitably also occur. Each person's reaction to chemotherapy is unique. Some people have very few side effects, while others may experience more. Some of the more common side effects include: (i) Nausea and vomiting, for which there are now very effective anti-sickness drugs to prevent or greatly reduce it. If they do occur they may begin a few hours after the treatment is given and last for up to a few days. (ii) The kidneys may be affected by cisplatin treatment. Usually this does not cause any symptoms, and the effect is mild, but if the effect is severe the kidneys can be permanently damaged unless the treatment is stopped. Therefore the kidneys will be checked by a blood test before each treatment. Plenty of fluid will be given intravenously before and after the treatment to support normal kidney functions (http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individualdrugs/Cisplatin).

The less common side effects include: (i) Temporary reduction in the production of blood cells by the bone marrow. This can result in anaemia leading to tiredness; an increased risk of bruising or bleeding and an increased risk of infection. This effect can begin from seven days after the treatment has been given and the number of blood cells may be at its lowest at 10-14 days after the chemotherapy. The bone marrow gradually returns to normal within 21-28 days. The extent to which blood cells are reduced depends on the dose of chemotherapy and which other chemotherapy drugs, if any, are given in combination. Hence, a patient’s blood should be monitored regularly. (ii) Numbness or tingling in hands or feet may occur. This is due to the effect of cisplatin on nerves and is known as peripheral neuropathy. A patient may notice that they have difficulty doing up buttons or performing similar tasks. This side effect usually disappears slowly a few months after the treatment ends (http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individualdrugs/Cisplatin). Peripheral sensory neuropathy develops in the majority of patients treated with cumulative doses of cisplatin higher than 300 mg/m². The pathology involves primary sensory neurons of dorsal root ganglia as well as peripheral glial cells. Gill and Windebank, (1998) believed that neuropathy may arise secondary to renal failure during treatment with cisplatin, but it was shown that it is unlikely that the
neuropathic findings in a study by Ozturk et al. (2005), were due to toxic side-effects, since none of the mice in this study showed signs of significant tissue damage in the kidney sections (Ozturk et al. 2005). (iii) Ototoxicity (changes in hearing) may occur. Cisplatin and carboplatin are highly effective chemotherapeutic agents with ototoxic side effects that limit their efficacy. Formation of reactive oxygen species (ROS) in CDDP-exposed cochlear tissues is involved in ototoxic action of the CDDP on hair cells (Eshraghi et al. 2006). A patient may experience tinnitus (ringing in the ears) and may lose the ability to hear some high-pitched sounds. Again, this effect usually decreases when the treatment ends, although, in a small number of cases, it may be permanent. (http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individualdrugs/Cisplatin). Animal models have suggested an important role of reactive oxygen species (ROS) in the pathophysiology of cisplatin-induced ototoxicity. The activity of anti-oxidative enzymes undergoes changes following cisplatin exposure, and various radical scavengers and antioxidants protect against hearing loss in animals in vivo. It has been shown that there is significant loss of outer hair cells in the region of the cochlea of mice after exposure to cisplatin and that cisplatin reduces the amount of total antioxidant in cochlear tissue (Minami et al. 2004). (iv) Temporary taste alterations may occur. (v) Patients may experience a loss of appetite. (vi) Diarrhoea may occur as a side-effect to cisplatin treatment and this can usually be easily controlled, but it is important to drink plenty of fluids if this side-effect occurs; and (vii) Patients may experience allergic reactions. Signs of an allergic reaction include skin rashes and itching, a high temperature, shivering, redness or darkening of the face, dizziness, a headache, breathlessness, anxiety and a diuretic effect. (http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individual-drugs/Cis-platin).

1.4.3 Cisplatin resistance

Cisplatin is one of the most widely used chemotherapeutic drugs for the treatment of cancer. Initially, platinum-based therapies are very efficient in treating a wide range of cancers; however, recurrence and resistance remain the foremost limitation to curative therapies. Various mechanisms of resistance have been proposed, including reduced drug concentration in the cell, drug inactivation, increased DNA repair, or failure to turn on cell death pathways (Min et al. 2004). Although cisplatin resistance
is clearly multifactorial in nature, increased levels of cellular thiols are often associated with the cisplatin-resistant phenotype. Cellular thiols, including glutathione (GSH), can sequester cisplatin, leading to a reduction in the levels of cisplatin–DNA damage. DNA is the therapeutic target of cisplatin, and efficacy is a function of cisplatin–DNA adducts inhibiting DNA replication and transcription, eventually resulting in apoptosis. Reduced efficacy of the cisplatin is often observed in cells with increased GSH levels. Modulating the levels of intracellular thiols has been demonstrated to influence cisplatin cytotoxicity in numerous studies (Turchi, 2006). In human ovarian carcinoma cell lines, it was found that resistance to cisplatin was accompanied by cross-resistance to copper. These results were consistent with the concept that cisplatin enters and exits the cell via transporters that normally mediate copper homeostasis (Katano et al. 2002). However, despite numerous studies, the understanding of resistance to this widely used drug remains poor, and the signaling pathways that activate the variety of proposed mechanisms of resistance are largely unknown (Min et al. 2004).

The ability of cancer cells to become cross-resistant to structurally and functionally unrelated anti-cancer drugs is an event known as multidrug resistance. The incidence of multidrug resistance depends on the type of tumour, treatment status and other factors. Factors that affect response to anti-cancer chemotherapy are cell kinetic, pharmokinetic and cellular drug resistance mechanisms (Table 1.1). Some mechanisms of cellular drug resistance that occur at a molecular level include: (i) reduction of the intracellular accumulation of anti-cancer drugs by either increasing drug efflux and/ or decreasing drug uptake; (ii) sequestration of drugs; (iii) alterations in drug targets or (iv) activation of detoxifying systems, such as glutathione/ glutathione-S transferases. Other factors that may play a role in multidrug resistance include increased repair of drug-induced DNA damage, blocked apoptosis, disruptions in signalling pathways and alterations of factors involved in cell cycle control (Filipits, 2004).
Table 1.1: Mechanisms of drug resistance (Filipits, 2004).

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Individual process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell kinetic resistance</td>
<td>Tumour growth</td>
</tr>
<tr>
<td>Pharmokinetic resistance</td>
<td>Poor absorption&lt;br&gt;Excessive metabolism&lt;br&gt;Poor penetration to certain sites&lt;br&gt;Blood supply of the tumour&lt;br&gt;Drug diffusion</td>
</tr>
<tr>
<td>Cellular drug resistance</td>
<td>Increased drug efflux&lt;br&gt;Decreased drug uptake&lt;br&gt;Sequestration of drugs&lt;br&gt;Alterations in drug targets&lt;br&gt;Activation of detoxifying systems&lt;br&gt;Increased repair of drug-induced DNA damage&lt;br&gt;Blocked apoptosis&lt;br&gt;Disruption in signalling pathways&lt;br&gt;Alterations of factors involved in cell cycle regulation</td>
</tr>
</tbody>
</table>

Cisplatin resistance might develop due to monthly acute exposure to the drug. This is generally associated with decreases in intracellular accumulation of the drug. The defect in drug accumulation is usually modest, even when the level of resistance is quite high. Studies of cisplatin-resistant cells with decreased drug accumulation have identified that membrane proteins may play a role, through either decreased or increased expression. Modulation of cisplatin accumulation has been achieved by treatments that are thought to have membrane-fluidising effects, such as hyperthermia, as well as by using drugs that are membrane-active and function as signal transduction modulators. Calcium channel blockers and calmodulin inhibitors are known for their capacity to circumvent multidrug resistance by reducing the increased drug efflux in cancer cells (Chao, 1996).

Long-term exposure to increasing concentrations of cisplatin may result in increased levels of glutathione (GSH) and metallothionein (MT). GSH functions in membrane transport, drug inactivation and elimination by conjugation with substances, such as platinum containing agents (Dunfield and Guernsey, 2002). GSH is the most abundant thiol in the cell, present at concentrations of 0.5 to 10 mM. It is a potent nucleophile and reacts with cisplatin and alkylating agents. GSH and cisplatin react in a 2:1 molar ratio and form a cisplatin complex that is then eliminated from the cell by an ATP-dependent GSH S-conjugate export pump; the GS-X pump. In combination with GSH, GSH S-transferase (GST) may play a role in cisplatin resistance, since it is responsible for the conjugation of chemicals to the thiol group.
Hence, GSH may protect cells by intercepting reactive platinum complexes before they can react with DNA as well as by supporting DNA repair, most probably by stabilization of repair enzymes, such as DNA polymerase \( \alpha \) or by promoting the formation of deoxyribonucleotides. This means that cisplatin-resistant cells usually express an improved radical scavenging system, such as GSH and the GS-X pump (Chao, 1996; Chen et al. 1998). It has been proposed that glutathione may protect cells by binding to or reacting with drugs, by interacting with reactive oxygen moieties or with other radicals produced by radiation, by preventing damage to proteins or DNA, or by participating in the repair process (Godwin et al. 1992).

MT is a small protein that contains 20 cysteine residues; hence it exhibits a high affinity for metals (Choi et al. 2004). Both transplatin and cisplatin bind to MT, with 10 platinum atoms per molecule. The binding rate constant for MT is much higher than for GSH. Upon binding to MT, cisplatin loses its amine ligands and displaces heavy metal ions. Mammalian MT is presumed to have a role in the detoxification of heavy metal ions. Selection of cell lines with cisplatin often has no effect on MT. However, cell lines selected with heavy metals show increases in MT and become cross-resistant to cisplatin (Chao, 1996; Perez, 1998).

Cisplatin adducts are primarily repaired by the nucleotide excision repair pathway. Poor repair of d(GpG) intrastrand cross-links may contribute to cisplatin cytotoxicity. Cisplatin-resistant cell lines show increased levels of DNA repair (measured by the loss of platinum adducts), reactivation of cisplatin-damaged plasmid DNA and DNA repair synthesis. Studies suggest that cisplatin resistance could be attributed to the elevation of various enzymes involved in DNA excision repair, such as polymerase and thymidine triphosphate synthesis inhibitors and agents affecting topoisomerase II and DNA accessibility. An important finding is that DNA repair is always increased in resistant cells, while drug uptake, efflux, GSH level or MT level may remain unchanged. Since increased DNA repair occurs early and consistently during cisplatin selection, it appears to be activated first, and then the cell may induce additional mechanisms affecting GSH, MT, drug accumulation and efflux in order to achieve higher degrees of resistance (Chao, 1996).
Genomic instability [characterised as microsatellite instability (MIN)] is associated with loss of DNA mismatch repair (MMR) protein. Several studies showed that loss of DNA MMR protein confers resistance to some interacting DNA chemotherapeutic drugs. This was mainly reported for cisplatin (Picard et al. 2002). It has been postulated that tumours that are defective in MMR become more resistant to cisplatin than their MMR-proficient counterparts (Rosell et al. 2003).

1.4.4 Cisplatin analogues

Once a successful drug has been discovered for the treatment of a particular disease or condition, researchers will often try to improve on that drug by synthesizing and studying related compounds, referred to as analogues or second-generation drugs. The reasons for manufacture of such analogues include (i) the analogues may be able to improve on the efficacy of the original drug, meaning that lower doses are able to produce the same beneficial effects; (ii) the toxicity profile of the analogues may be better than that of the original drug; hence, the analogues may have fewer toxic side effects than the original drug; (iii) the analogues may be used to treat cases that have become resistant to the original drug and; (iv) if the original drug can only be administered intravenously, the analogues may be able to be taken orally. After the efficacy and toxicity profiles of second-generation analogues are obtained, more analogues can be synthesized and studied; these analogues are called third-generation drugs. Many second-generation analogues of cisplatin have been made; some have been found to produce the same therapeutic effects as cisplatin; but with lower required doses and reduced side effects (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm). The second generation platinum drugs, carboplatin, nedaplatin and oxaliplatin have all exchanged the Cl⁻ leaving groups of cisplatin for kinetically less labile chelating dicarboxylate or glycolate ligands. As a result patients treated with these compounds suffer from less severe dose-limiting side effects (Zutphen et al. 2006).

Carboplatin [diammine{1,1-cyclobutane-dicarboxylato}platinum{II} or CBDCA for short (http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Analogues.html)] has proven to be the most useful of the cisplatin analogues, and was approved by the FDA for the treatment of ovarian cancers in 1989. Carboplatin and cisplatin have been shown to
form an identical type of adduct with DNA and have similar activities against ovarian and lung tumours. However, carboplatin is less toxic to the peripheral nervous system and the kidneys. The reduced toxicity of carboplatin compared to cisplatin is thought to be due to the structure of carboplatin (Figure 1.3B). The presence of the bidentate dicarboxylate ligand (Figure 1.10) in carboplatin slows down the degradation of carboplatin into potentially damaging derivatives (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm).

![Carboxylate and dicarboxylate group](http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm)

**Figure 1.10:** A carboxylate and dicarboxylate group. The dicarboxylate group is a bidentate ligand; hence it can bind a metal ion in two places (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm).

At 37°C the retention half-life of carboplatin in blood plasma is 30 hours, whereas that for cisplatin is only 1.5-3.6 hours. In addition to the lower toxicity of carboplatin, it has been shown to work in some cases when cisplatin has failed. The decreased toxicity of carboplatin and the activity of carboplatin against cisplatin-resistant tumours have led to greater use of carboplatin (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm). Unfortunately tumours resistant to carboplatin are becoming more common, and the cause of drug resistance is unclear (http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Analogues.html).

The third most widely available drug related to cisplatin is oxaliplatin. The dose-limiting factor is sensory neuropathy. Oxaliplatin is generally less toxic, with reduced myelosuppression, nephrotoxicity and ototoxicity; however, nausea and vomiting remain acute, but respond well to anti-emetics. The activity of oxaliplatin has been shown to have a more powerful pharmacological effect than cisplatin, which is due to its different mechanism of action. Studies indicate it attaches onto proteins, which are
vital for DNA transcription, thus preventing cell division and causing eventual cell death (http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Analogues.html). Oxaliplatin is active in the treatment of colorectal cancers, while cisplatin and carboplatin are inactive (Hector et al. 2001).

In addition to second-generation cisplatin analogues, several third-generation drugs have been synthesized and tested. One broad class of these drugs, the amine/amine platinum (IV) dicarboxylates (Figure 1.11), can be taken orally, which is a noteworthy advance over cisplatin, which must be administered intravenously. These complexes are stable enough to survive passage down the digestive tract. They are then transported across the gastrointestinal mucosa into the bloodstream. After absorption into the bloodstream, these compounds are metabolised to form four-coordinate, platinum (II) cisplatin analogues. These new four-coordinate complexes are assumed to be the active forms of the drug (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm). It has been found that bulky ligands destabilize the six-coordinate platinum (IV) state, which results in a faster rate of reduction to the four-coordinate platinum (II) state, and that a compound with a faster reduction time exhibits the highest cytotoxicity (Choi et al. 1998).

![Figure 1.11:](http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm)

During the last 30 years, thousands of cisplatin analogues have been prepared by varying the nature of the leaving groups and the carrier ligands. However, all cisplatin analogues in general form similar adducts with DNA that often translate into a similar spectrum of activity. Hence, recently attention has been given to platinum compounds with structures distinctly different from that of cisplatin, with the idea that their different nature of interaction with DNA would translate into different spectrums...
of activity and toxicity profile. One such class of compounds is the polynuclear platinum complexes that contain two or more platinum units linked together by di-amino-alkane chains. An important example is BBR3464 that consists of three trans-platinum units joined together by two 1,6-di-amino-hexane chains. BBR3464 has been found to evade the inherent or acquired cisplatin-resistance \textit{in vitro} and \textit{in vivo} in a panel of human adult tumour models. BBR3464 was in phase II stage of clinical trial before it was stopped due to significant toxicity namely neutropenias, diarrhoea and nausea (Huq \textit{et al.} 2004). These multinuclear complexes exhibit different levels of cellular uptake and form a higher proportion of interstrand cross-links than cisplatin or carboplatin, and it is thought that these complexes derive their improved activity primarily from the different adducts that they form with DNA (Wheate \textit{et al.} 2001). It has therapeutic doses approximately one-tenth that of cisplatin, activity in a broad spectrum of human tumours and is active against cisplatin resistant cell lines. Hence, BBR3464 represents the first genuinely new platinum-based structure to enter the clinic in 30 years (Roberts \textit{et al.} 1999).

Cancer is a universal problem, which affects many people. Cisplatin showed great promise in the treatment of cancer. However, due to the numerous toxic side effects and the emergence of cisplatin resistant cell lines, drugs with less toxic side-effects, improved cytotoxicity against cancer cells and activity against cisplatin resistant cell lines are urgently needed. Hence, the development and screening of novel anti-tumour drugs is imperative.
1.5 INTRODUCTION TO THE PRESENT STUDY

Cancer is an important public health concern around the world and the application of inorganic chemistry to medicine is a fast developing field. Novel therapeutic and diagnostic metal complexes are having a vast impact on medical practice. Advances in bio-coordination chemistry are crucial for improving the design of compounds to reduce toxic side effects and to aid in the understanding of their mechanisms of action.

The platinum drugs represent a unique and important class of anti-tumour agents. The discovery of cisplatin in the 1970’s revolutionized the chemotherapy of human cancer. Unfortunately the full therapeutic potential of cisplatin has not been realized due to its serious side effects and the emergence of cisplatin-resistant tumour cells.

A programme for the development of novel platinum containing anticancer agents has been initiated at this university. The expertise of both the chemistry and biochemistry departments has been used to develop novel platinum containing compounds.

As mentioned before, the Research Unit for Platinum Group Chemistry, plan, synthesize and characterise the chemical aspects and the Biochemistry Department evaluates the anticancer action in terms of their efficacy in killing a number of types of cancer cells as well as their mode of anticancer action. The latter study is essential to provide feedback, which enables a correlation of their anticancer properties with their structure and physical chemical characteristics.

This dissertation focuses mainly on the biochemical aspects of this project. This study assessed novel platinum containing compounds for possible enhanced cytotoxicity against cancer cells. The most active of these novel drugs were tested for activity against cisplatin-resistant tumour cell lines. In addition, it was verified whether some of these compounds were capable of inducing resistance in the cell lines tested.

The objectives of this study were: (i) to screen novel platinum containing compounds against MCF7, HeLa and HT29 cell lines, to compare their activities to that of
cisplatin and to select the four most promising compounds for further investigation; 
(ii) to develop cisplatin-resistant HT29, MCF7 and HeLa cell lines; (iii) to determine 
whether these platinum containing compounds are active against cisplatin-resistant 
cells; (iv) to verify whether the selected platinum containing compounds are capable 
of inducing resistance in the cancer cell lines tested and (v) to identify the mode of 
anticancer action of these compounds (i.e. apoptosis or necrosis).
CHAPTER 2  MATERIALS AND METHODS

2.1 ACQUIRING CANCER CELL LINES

Cancer cell lines (HeLa, MCF7 and HT29), purchased from Highveld Biological (Johannesburg, South Africa), were taken from liquid nitrogen stocks. The cells were thawed in a water-bath at 37°C for approximately one to two minutes and added to 10 mL of pre-warmed (37°C) antibiotic free RPMI 1640 (Sigma) growth medium containing 10% foetal calf serum (FCS) (Sigma), in order to dilute out the DMSO (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Key_Resources/ECACC_Handbook/Cell_Culture_Techniques_12.html). The cells were centrifuged (500 x g, 5 min, 25°C) in order to obtain a cell pellet (www.corning.com/lifesciences) and the supernatant discarded. The pellet was resuspended in 10 mL pre-warmed total growth medium and transferred to a cell culture dish. The cells were incubated in a humidified CO₂ incubator (5% CO₂) at 37°C until the monolayer was subconfluent.

2.2 ACQUIRING PLATINUM CONTAINING COMPOUNDS

Di-chloro and di-carboxylato Pt (II) and Pd (II) diamine derivatives were obtained from the Research Unit for Platinum Group Chemistry at the NMMU. A total of 84 compounds were screened for this study and the four with the best activity in terms of growth inhibition were selected for further investigation. The different compounds are distinguished by abbreviations, which were given to each compound in a completely random manner, since revealing the true names or structures of these compounds may compromise future patents.

2.3 SUBCULTURE OF CELLS

The cultures were viewed using an inverted phase contrast microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Standard trypsinisation procedures were performed (trypsin obtained from Roche) and a cell count was obtained by using 20 μL of the cell suspension and counting with an
improved Neubauer haemocytometer. The required number of cells were transferred to new labelled cell culture dishes containing pre-warmed antibiotic free RPMI 1640:10% FCS and these dishes were incubated as before (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Key_Resources/ECACC_Handbook/Cell_Culture_Techniques_12.html; Freshney, 2000; Celis, 1998).

### 2.4 CELL QUANTIFICATION

Under sterile conditions 20 μL of cell suspension was transferred to an eppendorf tube and an equal volume of 0.4% trypan blue in PBSA (dilution factor: 2) was added and mixed by gentle pipetting. An improved Neubauer haemocytometer was used to perform the count and the number of viable (colourless) and non-viable cells (blue) was counted and the percentage of viable cells was calculated (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Key_Resources/ECACC_Handbook/Cell_Culture_Techniques_12.html; Feshney, 2000; Celis, 1998).

### 2.5 CRYOPRESERVATION OF CELL LINES

The cells were brought into suspension using trypsin/EDTA (Roche) and re-suspended in 1 mL of fresh medium. A small aliquot of cells (20 μL) were used to perform a cell count. The remaining culture was centrifuged (500 x g, 5 min, 25°C) and the cells (pellet) re-suspended at a density of 2-4x10^6 cells per ml in freeze medium (RPMI 1640:10% FCS and 10% DMSO). One-millilitre aliquots were transferred into cryoprotective vials and standard cryopreservation methods were used to preserve the cells (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Key_Resources/ECACC_Handbook/Cell_Culture_Techniques_12.html; Freshney, 2000; Celis, 1998).
2.6 SOLUBILITY TEST

To determine the optimal solubility conditions of the platinum containing compounds, some of these compounds were solubilised in RPMI 1640:10% FCS or DMSO, respectively. When solubilised in RPMI 1640:10% FCS, the compound was weighed off to make up the desired amount of the stock solution at a concentration of 100 μM and dissolved in said amount of RPMI 1640:10% FCS. This solution was sonicated for 10 minutes and filter sterilized through a 0.22 μm syringe filter unit (Sigma); the desired dilution was prepared and these dilutions were applied to the cells. When solubilised in DMSO, the final DMSO concentration was 0.25%. Hence, 2.5 μL of DMSO was added for each millilitre of the stock solution that was prepared. The DMSO was added to the weighed compound first, vortexed until dissolved and the solution was made up to the desired volume with RPMI 1640:10% FCS. When dissolved in DMSO, no filter sterilization step was required and the dilutions needed were prepared under sterile conditions and then applied to the cells.

The MTT assay was used to determine the percentage of inhibition caused by these compounds each of three cancer cell lines (see Section 2.7.1), which was then compared to each other. One millilitre of these solutions was then sent for Inductively Coupled Plasma Mass Spectrometer (ICP-MS) analysis, where the exact concentration of platinum present in the solutions was determined and the ICP-MS value calculated. This was necessary because of the low solubility of some of the compounds and allowed for more accurate comparison of the cytotoxicity of different compounds.

2.7 CYTOXICITY ASSAYS

2.7.1 Compounds screened using the MTT assay

The reduction of tetrazolium salts is now widely accepted as a reliable way to determine cell proliferation and viability. The yellow tetrazolium salt MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes. The resulting
intracellular purple formazan can be solubilised and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to cell death, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells (Hagopian et al. 1999; Huq et al. 2004).

In this study, cytotoxicity was determined by a modified MTT assay. Cells in exponential growth were trypsinised, counted using a haemocytometer and diluted to a density of 30 000 cells per millilitre. Aliquots (200 μL) of cell suspensions were added to each well of a 96-well culture plate and incubated for 24 hours. The novel platinum containing compounds were screened for cytotoxicity using 10 and 100 μM concentrations. Cisplatin, also at 10 and 100 μM concentrations, served as a positive control. The spent medium was removed from the 96-well plate and 200 μL aliquots of the treatments were added to the wells in quadruplicate. The plate was incubated in a 37°C-5% CO₂ humidified incubator for 48 or 96 hours. A 5 mg/mL stock solution of MTT in PBSA was stored at 4°C and used to prepare a 1 mg/mL solution of MTT in growth medium on the day of the assay. This was applied to wells in 200 μL aliquots. The plate was incubated for 3 hours before the MTT was removed and replaced by 200 μL of DMSO to dissolve the MTT formazan. The plates were agitated on a shaker for 5 min, before the absorbance was read at 540 nm on a Labsystems Multiskan MS Plate Reader. The values obtained were used to determine the percentage inhibition of cell growth (Hagopian et al. 1999; Huq et al. 2004).

2.7.2 Preparation of novel platinum containing compounds prior to screening

The treatment solutions (100 μM) were made up in pre-warmed (37°C) RPMI 1640: 10% FCS. The samples were vortexed for approximately 1 min, sonicated for 10 min and briefly vortexed again before it was ready for use. The 100 μM solutions were filter sterilized using 0.22 μm syringe filter units. A 10x dilution was prepared in order to obtain a 10 μM solution of the treatment. These solutions were applied to cells in 200 μL aliquots for 48 or 96 hours (Section 2.7.1). A one-millilitre aliquot of each of these solutions was sent to the Research Unit for Platinum Group Chemistry at the NMMU for ICP-MS analysis to be performed. This enabled us to plot the
graphs using the actual concentrations of each solution, which renders the comparison of results more accurate.

2.7.3 Determination of the four best compounds used for further analysis

The results obtained from the cytotoxicity screening were compared and the four best compounds were chosen by taking into account the percentage inhibition obtained at the actual concentrations of each of these solutions (obtained via ICP-MS analysis). Two positive controls (cisplatin and oxaliplatin) were also included for comparison.

2.8 COMPARISON BETWEEN THE CELLTITER-BLUE\textsuperscript{TM} CELL VIABILITY ASSAY AND THE MTT ASSAY

Another cytotoxicity assay is the CellTiter-Blue\textsuperscript{TM} Cell Viability Assay (Promega). This method provides a homogenous, fluorescent method for monitoring cell viability. The assay is based on the ability of living cells to convert the redox dye, resazurin into resorufin, a fluorescent end product. Nonviable cells rapidly lose their metabolic capacity and thus do not generate a fluorescent signal. This procedure involves adding the single reagent (20 \(\mu\)L) directly to cells cultured in black culture 96-well culture plates (Nunc) in serum-supplemented medium and adding 100 \(\mu\)L of RPMI 1640: 10\% FCS. After incubation steps of 1, 2 and 3 hrs, data were recorded using a (ThermoLabsystems Fluoroskan AscentFL fluorescence microplate reader) at excitation and emission wavelengths of 560 and 590 nm, respectively (Promega Life Science Catalog, 2004). Three incubation times were used to select the optimal time.

This method was applied to the platinum containing compounds that showed the highest cytotoxicity on the HT29 cell line, as well as on cisplatin and oxaliplatin, and the results compared to that obtained via the MTT cell proliferation assay to verify the results.
2.9 DOSE RESPONSE CURVES FOR THE FOUR BEST COMPOUNDS

Dose response curves were prepared for each of the four best compounds, as well as the two positive controls, in order to obtain IC50 values. The concentrations used were 100, 50, 10, 5 and 1 μM. IC50 values were calculated from the log-dose response curves using GraphPad Prism 4.

Due to the very low solubility of compound Lt16.2, it had to be tested at lower concentrations. The compounds Lt16.2, cisplatin and oxaliplatin were applied to the cancer cell lines at concentrations of 5, 2.5, 1, 0.5, 0.25 and 1 μM, and the MTT assay (Section 2.7.1) performed, in order to determine whether Lt16.2 has a better anticancer action than the positive controls at lower concentrations.

2.10 THE EFFECT OF GSH ON THE FOUR BEST COMPOUNDS

The MTT method was used as described in section 2.7.1. In order to determine the effect that GSH has on the anticancer action of the four best platinum containing compounds and the two positive controls, these compounds were applied to the cancer cells at a concentration of 100 μM (or the highest concentration obtainable) in the presence of different amounts of GSH. The concentrations of GSH used were 0.003, 0.5, 5 and 10 mM, where the 0.003 mM GSH was actually normal medium, since RPMI 1640 contains 0.003 mM GSH.

2.11 INDUCTION OF CISPLATIN RESISTANCE IN CANCER CELL LINES

Cisplatin-resistant cell lines were developed by intermittent, incremental exposure of the cells to 10 and 100 μM concentrations of cisplatin (Refer to Table 2.1) (Godwin et al. 1992). The cells were exposed to cisplatin for different times and the time to reach confluence was noted. After exposure to cisplatin, the cells were subcultured again, left to grow without any interference until confluent. Then it was subcultured again, left to grow for 24 hours, exposed to cisplatin, the cisplatin was replaced with normal medium and the time to reach confluence was noted. When the time to reach confluence corresponded to that of the wild-type cells, indicating the development of
resistance, the cells were exposed for an additional two times and were frozen for further analysis (Harstrick et al. 1998).

**Table 2.1:** The conditions for inducing cisplatin resistance in cancer cell lines

<table>
<thead>
<tr>
<th>[Cisplatin] (μM)</th>
<th>Time exposed (hours)</th>
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</thead>
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<td>10</td>
<td>0.5</td>
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**2.12 VERIFYING WHETHER NOVEL PLATINUM CONTAINING COMPOUNDS INDUCE RESISTANCE IN CANCER CELL LINES**

Once the four best compounds were selected from screening results, an experiment was performed to establish if they would also induce resistance in the cancer cell lines. This was performed by intermittent, incremental exposure of the cells to 10 and 100 μM concentrations (or the highest concentrations obtainable) of the specific platinum containing compound (Table 2.2) as described in section 2.10 for induction of cisplatin-resistance (Godwin et al. 1992).

**Table 2.2:** Conditions for inducing resistance to platinum containing compounds in three cancer cell lines.

<table>
<thead>
<tr>
<th>[Treatment] (μM)</th>
<th>Time exposed (hours)</th>
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<td>10</td>
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2.13 SCREENING OF BEST COMPOUNDS AGAINST THEIR RESPECTIVE RESISTANT CELL LINES IN ORDER TO OBTAIN IC50 VALUES

The chosen compounds were screened against their respective resistant cell lines in order to obtain IC50 values. These IC50 values were compared to those obtained using the wild type cells, in order to verify whether resistance has been induced. The concentrations at which these compounds were screened were 100, 50, 10, 5 and 1 \( \mu \text{M} \). IC50 values were calculated from the log-dose response curves using GraphPad Prism 4.

2.14 SCREENING OF NOVEL PLATINUM CONTAINING COMPOUNDS FOR ACTIVITY AGAINST CISPLATIN-RESISTANT CELLS

The compounds that were established to have increased activity against cancer cells, compared to cisplatin, were screened as before, but this time the cisplatin-resistant cell lines were used. The IC50 values obtained were compared to those obtained with the normal cell lines.

2.15 DETECTION OF APOPTOSIS BY THE CASPASE-GLO\textsuperscript{TM} 3/7 ASSAY

The Caspase-Glo\textsuperscript{TM} 3/7 Assay (Promega) is a homogeneous, luminescent assay, which measures caspase-3 and -7 activities. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. The addition of a single Caspase-Glo\textsuperscript{TM} 3/7 Reagent format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present (Promega Technical Bulletin No. 323/ www.promega.com).

A 96-well plate was seeded with 200 \( \mu \text{L} \) of cell suspension at a density of 6000 cells per well, with a blank (culture medium only) and control (containing cells and culture
medium, but no compound) in quadruplicate, and incubated overnight. After 24 hrs, 100 μL of the platinum containing compounds was added to each well and incubated for 24 hrs according to the manufacturer's instructions. This incubation time was chosen because the small volume of growth medium would possibly not be able to sustain the cells for 48 hrs and removal of some of the medium prior to adding the reagent was not feasible, as that may have altered the results. After the incubation step, 100 μL of reagent were added to each well and the plate was incubated for 1 hour, after which the contents were transferred to a white-walled 96-well plate in order to read the luminescence on a ThermoLabsystems Fluoroskan AscentFL fluorescence/luminescence microplate reader.

2.16 FLOW CYTOMETRY

Flow cytometry was once considered to be the sole domain of immunologists, but has now evolved into a broadly applicable assay platform due to technological advances in accessory equipment and reagents as well as in cytometer changes. Due to a wide variety of dyes and assays, a flow cytometer can be used to gain multiplex information in a single assay or to perform a number of assays that will characterise compounds or elucidate end point mechanisms (Lem and Cappione, 2005).

2.16.1 Optimisation of conditions for Annexin V-FITC Assay

The Annexin V-FITC kit is an apoptosis detection kit based on the binding properties of Annexin V to phosphatidylserine and the DNA-intercalating capabilities of propidium iodide (PI). The appearance of phosphatidylserine (PS) residues on the surface of the cell can be used to detect and measure apoptosis. The presence of PS on the cell surface creates one of the specific signals for recognition and removal of apoptotic cells by macrophages. These PS changes can be detected with the anticoagulant, Annexin V, which has a high affinity for binding to PS. As the apoptotic process progresses, cell membrane integrity is lost. Using DNA specific viability dyes, such as propidium iodide (PI) it is possible to distinguish between early apoptotic, late apoptotic, and dead cells. Annexin V stains apoptotic cells, whereas PI
stains only necrotic or late apoptotic cells, which enabled us to distinguish between apoptotic and necrotic cells.

For this experiment, nine 10 cm culture dishes containing 30 000 cells/mL (equivalent to 1.19x10⁶ cells per plate) of each cell line were prepared (i.e. 27 plates). These plates were left to grow for 24 hours, before cisplatin was applied to the cells. The experiment was designed to have three time periods of exposure, 24, 48 and 96 hours. For each time period there was a control plate, to which only growth medium was added, and a plate to which a concentration of cisplatin equivalent to its IC25 value and its IC50 value respectively, was added. These plates were then left for the applicable time period before the Annexin V–FITC Apoptosis Detection Kit was applied to the cells. It was, however, found that after 96 hours the cell viability was very low, and therefore the 96 hour plates were discarded before applying the kit to these cells.

The reagents of the Annexin V–FITC Apoptosis Detection Kit (Beckman Coulter) were prepared as per kit instructions. After exposure to cisplatin, the monolayers were trypsinised and the cell samples were washed with ice-cold PBSA and centrifuged (500 x g, 5 min, 4°C). The supernatant was discarded and the pellet resuspended in ice-cold 1X binding buffer to 2x10⁶ cells/mL. The tubes were kept on ice. To 100 μL of the cell suspensions, 1 μL of Annexin V-FITC solution and 5 μL of dissolved PI was added and mixed gently. The tubes were incubated for 15 minutes on ice and in the dark before 400 μL of ice-cold 1x binding buffer were added. The samples were analysed on a Beckman Coulter FC500 flow cytometer. A minimum of 10000 events was recorded per sample.

2.16.2 Induction of apoptosis by platinum containing compounds

For this experiment, the cells were treated as described in section 2.16.1. From the results obtained from the above-mentioned section, it was decided to perform this experiment using the IC50 concentrations and incubation times of 12 and 48 hours. The two positive controls as well as the four best compounds were added to the cells at a concentration equivalent to their respective IC50 values and incubated for 12 and
48 hours before the Annexin V–FITC Apoptosis Detection Kit was applied to the cells.

2.16.3 Coulter®DNA Prep™ Reagents Kit

Growth and proliferation in eukaryotic cells is characterized by distinct phases of development known as the cell cycle. The sequence of cell cycle events can be identified as follows: Initiating from a quiescent or resting state (Phase G0), cell growth and preparation of chromosomes for replication takes place (Phase G1). The cycle continues with synthesis of DNA (S Phase) and is followed by preparations for cell division (Phase G2). The cycle completes with mitosis (M Phase) and is perpetuated with the newly divided cells. Flow cytometry offers a rapid method for measuring the DNA content of cells and provides a convenient research tool to monitor cell cycle status and regulation. An exponentially growing population of cells will have a DNA content distribution containing an initial peak of G0/G1 cells, a valley of S Phase cells, and a second peak containing G2/M cells. Measurements can be performed and are based on the ability of nuclear dyes, such as DAPI and PI, to bind selectively to DNA under appropriate staining conditions. Cells stained with such dyes emit fluorescence in direct proportion to their DNA content (Enten and Monson, 2005). Cells are fixed and exposed to PI. Since the cells are fixed, PI can bind to all cells, not only to necrotic or late apoptotic cells as in the Annexin V – FITC assay.

The Coulter®DNA Prep™ Reagents Kit (Beckman-Coulter) was used to determine during which stage in the cell cycle these compounds inhibit cell growth. The kit was applied to the same cells prepared in the previous experiment, which was incubated with the platinum containing compounds for 48 hours. The kit was ready-to-use, and the only “preparation” required was to bring the kit contents to room temperature before use. After trypsinisation, the cells were resuspended in sheath fluid (Beckman-Coulter) and centrifuged (500 x g, 5 min, 4°C). The supernatant was discarded and the pellet resuspended in sheath fluid to a cell density of 2x10^6 cells/ mL. To 100 μL of this cell suspension, 100 μL of binding buffer was added and the suspension left at room temperature for 5 minutes. One millilitre of PI was added and the samples
analysed on a Beckman Coulter FC500 flow cytometer, recording a minimum of 10000 events per sample.

2.17 STATISTICAL ANALYSIS

Results were compared using the two-tailed student’s t-test.
CHAPTER 3 RESULTS

3.1 PRESENTATION AND INTERPRETATION OF RESULTS

Initially the compounds were dissolved in growth medium using the molar mass of each pure compound to obtain a 100 µM solution for screening purposes. These results were depicted in the form of bar graphs (Figure 3.1). This method however proved to be inaccurate due to variations in solubility. This in turn can be related to the different degrees of hydrophobicity, which made it difficult to obtain even such dilute solutions. The results can therefore only be considered as approximate indications of the relative potential as anticancer agents. To overcome this problem, for all of these experiments an aliquot of each of the dissolved compounds was used for ICP-MS analysis. In many cases the actual concentrations of the solutions were not 100 µM; instead they comprised saturated solutions of variable concentrations. The result of this is that direct comparison of activity versus concentration was difficult. In an effort to have an improved mode of comparison, the percentage inhibition data were plotted against the actual concentrations (Figure 3.2). These results reflected a very similar picture to the previous with reference to activity. It can, however, be regarded as an improvement since accurate concentrations were used and the relative competitiveness of these reagents with cisplatin is reflected in a more reliable manner [eg. in the case of Y9, Y14 and cisplatin at about 75 µM (Figure 3.2A)]. In the case of Lt16.2 on HeLa cells (Figure 3.2A) a high activity is revealed at low concentrations. In this case the hydrophobicity of the non-leaving groups is the reason for its limited solubility.
Figure 3.1: Treatments screened against (A) HeLa; (B) HT29 and (C) MCF7 cells. Results were plotted assuming 100% solubility for all compounds. (Data points represent Mean±SD of quadruplicate values).
In this experiment it is very important to note the sample, Lt16.2. Although it had a fairly high percentage of inhibition, it had only 4.62 μM in solution as apposed to the 100 μM we believed it to have had. Figure 3.2 shows the actual effect of this compound on the cancer cells, in accordance to the amount of compound solubilised in the sample. If figure 3.1 was used to identify compounds with good activity, Lt16.2 would not have been selected.

**Figure 3.2:** Treatments screened against (A) HeLa; (B) HT29 and (C) MCF7 cells. Results were plotted using the actual concentrations as determined with ICP-MS analysis. (Data points represent Mean±SD of quadruplicate values. Data points joined via dotted lines for the sake of clarity and for reasons explained in section 3.1).
Figure 3.2 shows that Lt16.2’s anticancer action could compete well with that of cisplatin, Y9 and Y14, especially at the lower concentrations. From these results Y9 and Y14 were chosen as two of the best compounds on which further experiments would be performed. Lt16.2 seemed to have good anticancer action as well, so further experiments were performed on this sample to verify these results.

3.2 SOLUBILITY TEST

In an attempt to improve the solubility of the platinum containing compounds, stock solutions were prepared in DMSO before dilution with culture medium. These, as well as solutions prepared by direct solubilisation in culture medium, were compared with respect to their effect on cancer cell growth and true concentration as determined by ICP-MS analysis.

The ICP-MS values (Table 3.1) showed that most of the platinum containing compounds were slightly more soluble in the DMSO stock solution, (prepared as described in Section 2.6) than in RPMI 1640 supplemented with 10% FCS. When these compounds were screened on the cancer cell lines, it was found that the differences in the percentages of inhibition yielded were not significant, although slightly higher percentages of inhibition were reached when these compounds were solubilised in RPMI 1640:10% FCS (Figure 3.3). This might be because of a chemical reaction between the platinum containing compounds and the DMSO, which slightly inactivates the compounds. Hence in all further experiments the compounds were solubilised directly in RPMI 1640:10% FCS. The determination of ICP-MS values made it possible to plot the actual concentration of the compounds, and thus aided in the comparison of the results.
Table 3.1:  ICP-MS values (μM) of platinum containing compounds solubilised in DMSO or RPMI 1640:10% FCS.

<table>
<thead>
<tr>
<th>Platinum containing compound</th>
<th>RPMI 1640:10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin 1</td>
<td>77.01</td>
</tr>
<tr>
<td>Cisplatin 2</td>
<td>75.46</td>
</tr>
<tr>
<td>J5 1</td>
<td>60.71</td>
</tr>
<tr>
<td>J5 2</td>
<td>66.06</td>
</tr>
<tr>
<td>J7</td>
<td>67.28</td>
</tr>
<tr>
<td>J12</td>
<td>54.80</td>
</tr>
</tbody>
</table>

1, 2 Two preparations of the same compound.

Figure 3.3: Comparison of growth inhibitory activity of platinum containing compounds solubilised in DMSO and in RPMI 1640:10% FCS. (A) HeLa; (B) HT29 and (C) MCF7 cells. (Solid line – Solubilised in DMSO before dilution in RPMI:10% FCS; Broken line – Directly solubilised in RPMI 1640:10% FCS). (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons explained in section 3.1).
3.3 CYTOTOXICITY ASSAYS

3.3.1 Compound screening and selection of the four best compounds

A total of 84 compounds were screened against HeLa, HT29 and MCF7 cells and depicted as XY-scatter plots (Figures A.1 – A.3), for the reasons explained in section 3.1. These plots were used to select the four best compounds as compared to the positive controls, cisplatin and oxaliplatin. The results of all the compounds that were not selected are shown in Appendix A while that of the four best compounds as well as the two positive controls are shown in figures 3.4 – 3.6. Although all the platinum containing compounds were fully characterized and analysed, including C,H,N analysis to ensure an high degree of purity, Y9 and Lt16.2 were recrystallised and again tested in view of their suggested potential as an anticancer agent. These tests agreed well with those previously obtained.

The Y compounds were platinumdiaminedichloro species whereas the Lt complex was a platinum(II)neutralbidentateddicarboxylato species. In general these results indicated that these Y compounds compared well with cisplatin in all the cancer cell lines tested (cisplatin is also a platinumdiaminedichloro species). As all the D curves in figures 3.4 – 3.6 indicate, Lt16.2 revealed a comparatively high activity at low concentrations. Ligand exchange of Pt(II) dicarboxylato species has been shown to be ~100 times slower than their chloro analogues (Knox et al. 1986). Therefore it was expected that Lt16.2 should have a different anticancer action profile (Figures 3.4 – 3.6 D) to that of cisplatin.
Figure 3.4: The four best compounds screened against HeLa cells (A) Y9/Y9 recrystallized; (B) Y14; (C) Y16 recrystallized and (D) Lt16.2 (Showing only the low treatment concentrations for clarity). Some compounds were tested on more than one occasion, hence the different data sets depicted in the figures. Some of the compounds were recrystallized after their potential as anticancer agents were realised, therefore some of the figures contain data for recrystallized (rec) compounds as well. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons mentioned in sectioned 3.1).
Figure 3.5: The four best compounds screened against HT29 cells (A) Y9/Y9 recrystallized; (B) Y14; (C) Y16 recrystallized and (D) Lt16.2 (Showing only the low treatment concentrations for clarity). Some compounds were tested on more than one occasion, hence the different data sets depicted in the figures. Some of the compounds were recrystallized after their potential as anticancer agents were realised, therefore some of the figures contain data for recrystallized (rec) compounds as well. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons mentioned in section 3.1).
Figure 3.6: The four best compounds screened against MCF7 cells (A) Y9/Y9 recrystallized; (B) Y14; (C) Y16 recrystallized and (D) Lt16.2 (Showing only the low treatment concentrations for clarity). Some compounds were tested on more than one occasion, hence the different data sets depicted in the figures. Some of the compounds were recrystallized after their potential as anticancer agents were realised, therefore some of the figures contain data for recrystallized (rec) compounds as well. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons mentioned in section 3.1).

From the graphs in figures 3.4 – 3.6 and figures A.1 – A.3 the compounds, cisplatin and oxaliplatin (positive controls), Y9, Y14, Y16, and Lt16.2 were chosen as the best
compounds and these were accordingly used for further experiments. Other compounds showed promise as well, but were not as active as the selected ones. JM216 was another positive control and had a very good anticancer action against all three cancer cell lines [Figure A1(K), A2(L) and A3(L)], but was not chosen as a positive control for further analysis. From figures 3.4D, 3.5D and 3.6D, it was clear that Lt16.2 was active despite its low solubility and at low concentrations it seemed as if Lt16.2 had a better anticancer action than cisplatin. To verify this, cisplatin, oxaliplatin and Lt16.2 were also screened at lower concentrations.

3.3.2 Verification of Lt16.2’s anticancer action

In an attempt to verify that Lt16.2 had an increased anticancer action to that of the positive controls, at lower concentrations, these three compounds were screened at lower concentrations. It could be seen that Lt16.2 had a better anticancer action against HeLa and MCF7 cells than on HT29 cells (Figure 3.7 A and C). When cisplatin, oxaliplatin and Lt16.2 were screened at low concentrations (Figure 3.7), the pronounced levelling off of some of the curves could most probably be related to the low concentrations of the Pt (II). However, it was clear from these results that at lower concentrations Lt16.2’s anticancer action exceeded that of cisplatin and oxaliplatin.
Figure 3.7: A comparison between the anticancer action of Lt16.2, cisplatin and oxaliplatin when screened at lower concentrations against (A) HeLa; (B) HT29 and (C) MCF7 cells. (Data points represent Mean±SD of quadruplicate values).
If this compound were more soluble, it would most probably have a better anticancer action than cisplatin and oxaliplatin. These results led to the selection of Lt16.2 as one of the better samples, on which further experiments were performed.

### 3.4 COMPARISON BETWEEN THE CELLTITER-BLUE™CELL VIABILITY ASSAY AND THE MTT ASSAY

The CellTiter-Blue™Cell Viability Assay and the MTT assay are two alternative assays that measures cytotoxicity. They are fluorimetric and spectrophotometric assays respectively. The CellTiter-Blue™Cell Viability Assay was used to confirm the MTT results obtained during screening. Figure 3.8 showed that there were few significant differences between the results obtained from the MTT assay and the CellTiter-Blue™Cell Viability Assay. The only significant differences were between the results obtained for the lower concentrations of Lt16.2 and Y16 (p<0.01 for both), where the MTT assay gave a higher percentage inhibition than the CellTiter-Blue™ Assay. For the sake of consistency and personal preference, the MTT method was chosen as the preferred method of anticancer screening for further experiments.

![Figure 3.8: Comparison between results obtained using MTT and CellTiter-Blue™Cell Viability Assay (CTB), screened on the HT29 cell line. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons mentioned in section 3.1).](image-url)
3.5 DOSE RESPONSE CURVES FOR THE FOUR BEST COMPOUNDS

From the dose response curves (Figure 3.9), the IC50 values of each compound for the different cell lines were determined (Table 3.2). Dose response curves were also performed on Chang liver cells (Figure 3.9D), in order to determine the effects of the platinum containing compounds on the liver. It could further give an indication of the degree of detoxification that would occur when these drugs are administered and passed through the liver.

![Graphs showing dose response curves for different compounds on various cell lines.](image)

**Figure 3.9:** Dose response curves of the four best compounds on (A) HeLa; (B) HT29; (C) MCF7 and (D) Chang liver cells. (Data points represent Mean±SD of quadruplicate values).
Table 3.2: The IC50 values (μM) of the platinum-containing compounds for the three cancer cell lines and Chang liver cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin</th>
<th>Oxaliplatin</th>
<th>Y9</th>
<th>Y14</th>
<th>Y16</th>
<th>Lt16.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>1.5</td>
<td>12</td>
<td>22</td>
<td>18</td>
<td>18</td>
<td>≥7.21</td>
</tr>
<tr>
<td>HT29</td>
<td>2.7</td>
<td>35</td>
<td>35</td>
<td>18</td>
<td>35</td>
<td>≥7.21</td>
</tr>
<tr>
<td>MCF7</td>
<td>2.3</td>
<td>8</td>
<td>22</td>
<td>18</td>
<td>28</td>
<td>≥7.21</td>
</tr>
<tr>
<td>Chang liver</td>
<td>6</td>
<td>9</td>
<td>18</td>
<td>43</td>
<td>34</td>
<td>≥7.21</td>
</tr>
</tbody>
</table>

From the IC50 values (Table 3.2), it could be seen that none of the compounds had a lower IC50 value than cisplatin, but some of the compounds had lower IC50 values than oxaliplatin. Since Lt16.2 did not reach an inhibitory percentage of 50%, due to its low solubility, IC50 values could not be calculated for this compound. The dose response curves in figure 3.9B shows that in the concentration region lower than 1 μM, Lt16.2 had a higher percentage inhibition than the other compounds, except for oxaliplatin.

Oxaliplatin had higher IC50 values than cisplatin on all three cell lines. Interestingly, this was especially true for HT29 cells even though oxaliplatin is the preferred treatment for colorectal cancer. This shows that the compound with the lowest IC50 is not necessarily the best choice for treatment because many other factors should be considered at the same time. In the case of oxaliplatin and cisplatin, for example, the former has a slower action but fewer toxic side effects (http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Analogues.html; Zutphen et al. 2006).

As a general rule oxaliplatin and cisplatin exhibited a lower IC50 value and therefore higher cytotoxicity for all the cell lines as compared to the novel compounds. The dose response curves on the Chang liver cells were performed in order to determine the hepatotoxicity of the compounds to these cells. However, these results could also give some information as to the rate of detoxification of these compounds. A higher IC50 value on the Chang liver cells than for the cancer cell lines was obtained for cisplatin, Y14 and Y16. This may indicate that these compounds detoxify faster than the others.

Oxaliplatin and Y9 showed a lower IC50 on Chang liver than for the cancer cells, implying that they are more active against these cells and do not detoxify as fast. It is
known that oxaliplatin, which has fewer toxic side-effects, has a slower anticancer action than cisplatin and is detoxified more slowly. As mentioned, the IC50 values on the Chang liver cells were relatively high for some compounds, but Chang liver cells have a high amount of GSH present and GSH has been shown to inhibit the action of platinum containing anticancer agents, which might account for these high values (Zhang et al. 1999; Zhang et al. 2000). To verify this, the four best compounds were screened against the cancer cell lines in the presence of GSH.

### 3.6 THE EFFECT OF GSH ON THE FOUR BEST COMPOUNDS

Cellular thiols, including glutathione (GSH), can sequester cisplatin, leading to a reduction in the levels of cisplatin–DNA damage. Reduced efficacy of the cisplatin is often observed in cells with increased GSH levels. GSH functions in membrane transport, drug inactivation and elimination by conjugation with substances, such as platinum containing agents (Dunfield and Guernsey, 2002). GSH is the most abundant thiol in the cell, present at concentrations of 0.5 to 10 mM (Chao, 1996; Chen et al. 1998). It is a potent nucleophile and reacts with cisplatin and alkylating agents. GSH and cisplatin react in a 2:1 molar ratio and form a cisplatin complex that is then eliminated from the cell. Hence, GSH may protect cells by intercepting reactive platinum complexes before they can react with DNA as well as by supporting DNA repair, most probably by stabilization of repair enzymes, such as DNA polymerase α or by promoting the formation of deoxyribonucleotides (Chao, 1996; Chen et al. 1998).

This means that cisplatin-resistant cells usually express an improved radical scavenging system, such as GSH and the GS-X pump (Chao, 1996; Chen et al. 1998). It has been proposed that glutathione may protect cells by binding to or reacting with drugs, by interacting with reactive oxygen moieties or with other radicals produced by radiation, by preventing damage to proteins or DNA, or by participating in the repair process (Godwin et al. 1992).
The four best compounds were tested in the presence of varying concentrations of GSH. Each compound was tested at the highest concentration possible (i.e. as close to 100 μM as the solubility of each compound allowed).

Figure 3.10 clearly indicates that the presence of GSH inhibited the anticancer action of the platinum containing compounds, although to different extents. Cisplatin and Lt16.2 showed inhibition of their anticancer action in the presence of GSH, but to a lesser extent than the other compounds. Cisplatin exhibited a higher IC50 value in the Chang liver cells than in the cancer cells (previous section), which implies a fast rate of detoxification.

The results obtained in this section show that GSH does play a role in the detoxification of cisplatin, but there may be other mechanisms at work as well. The same applies for Lt16.2, especially on HeLa and MCF7 cell lines, although no IC50 value could be obtained for the Chang liver cells because of this compound’s low solubility. Y14’s anticancer action was inhibited dramatically by the presence of GSH, which is in line with it achieving the highest IC50 value on Chang liver cells in the previous section. Y16 was detoxified fast according to the data compiled by the dose response curves on Chang liver cells, and from the results obtained in this section it can be seen that GSH plays a major role in the detoxification of these compounds. Oxaliplatin and Y9 exhibited slow detoxification according to the Chang liver cell dose response curves, but the results obtained in this section also imply that GSH plays a major role in its detoxification. It seems that the effect of the compounds on the HT29 cell line was inhibited the most by the presence of GSH.
Figure 3.10: The effect of GSH on the anticancer activity of the four best compounds on (A) HeLa; (B) HT29 and (C) MCF7 cells. Each compound was tested at the highest achievable concentration. (Data points represent Mean±SD of quadruplicate values).
3.7 INDUCTION OF DRUG RESISTANCE IN CANCER CELL LINES

3.7.1 Induction of resistance to novel compounds

HeLa, HT29 and MCF7 cells were exposed to the four best compounds for different times and at different concentrations to establish whether these compounds would induce resistance in the cells. The concentrations and exposure times are given in Section 2.11. To verify that resistance has been induced, dose response curves were done on the resistant cells with the relevant compound. From these dose response curves, the IC50 values of each compound for the various treated cell lines were determined (Tables 3.3 and 3.4).

Table 3.3: IC50 values (µM) for platinum containing compounds on its respective “resistance induced” cell lines as well as on “normal” cancer cells.

<table>
<thead>
<tr>
<th>Platinum-containing compound</th>
<th>Cell type</th>
<th>HeLa</th>
<th>HT29</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>Cisplatin resistant</td>
<td>15 (10x)</td>
<td>22 (8.1x)</td>
<td>85 (37x)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>1.5</td>
<td>2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Oxaliplatin resistant</td>
<td>12 (1x)</td>
<td>40 (1.1x)</td>
<td>15 (1.9x)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>12</td>
<td>35</td>
<td>8.0</td>
</tr>
<tr>
<td>Y9</td>
<td>Y9 resistant</td>
<td>25 (1.1x)</td>
<td>74 (2.1x)</td>
<td>40 (1.8x)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>22</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>Y14</td>
<td>Y14 resistant</td>
<td>89 (4.7x)</td>
<td>38 (2.1x)</td>
<td>89 (4.9x)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>19</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Y16</td>
<td>Y16 resistant</td>
<td>45 (2.7x)</td>
<td>84 (2.4x)</td>
<td>84 (3x)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>18</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>Lt16.2 resistant</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not determined due to low solubility

Brackets: Fold increase in IC50 value relative to that of untreated cells of the same cell line (i.e. how many times less active)
Table 3.4: Comparison between the maximum percentage inhibition reached by Lt16.2 on its “resistance induced” cell lines and on “normal” cancer cell lines.

<table>
<thead>
<tr>
<th>μM in solution</th>
<th>Maximum % inhibition reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Normal cells</td>
<td>7.21</td>
</tr>
<tr>
<td>Lt16.2 resistant cells</td>
<td>9.8</td>
</tr>
<tr>
<td>T test value</td>
<td>NS</td>
</tr>
</tbody>
</table>

Brackets: Fold increase in maximum percentage inhibition relative to that of untreated cells of the same cell line.

NS: Not significant

According to the results in table 3.3, all of the compounds have are able to induce resistance in most of the cell lines, but not to the same degree as cisplatin in any of the cell lines. As could be expected from the literature, oxaliplatin also induced less resistance than cisplatin, supporting the use of this in vitro model for the investigation of resistance to anticancer agents. The HeLa cell line has proven to be less susceptible to become resistant to oxaliplatin and Y9. The IC50 of oxaliplatin stayed the same for both the normal and the resistant HeLa cells. Oxaliplatin hardly induced resistance in the HT29 cell line, since the IC50 value of the cell line in which it was attempted to induce resistance increased only slightly. It was also mentioned by Culy et al. (2000), that oxaliplatin induces less resistance than cisplatin. This result might influence its choice as preferred treatment for colon cancer, in addition to the fact that its toxic side effects are relatively low (http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Analogues.html).

The low solubility of Lt16.2 made it impossible to calculate IC50 values and therefore only the maximum percentage inhibition obtained at the highest possible concentration could be compared. Table 3.4 shows that no resistance to Lt16.2 has been induced in HeLa cells, since the maximum percentage of inhibition reached was not significantly higher for “Lt16.2 resistant” cells, even though the concentration of Lt16.2 was approximately 2 μM higher during that experiment. However, it appears as if resistance to Lt16.2 has been induced in HT29 (p<0.01).
3.7.2 Effect of novel compounds on cisplatin resistant cell lines

In order to determine whether the novel platinum-containing compounds are active against *cisplatin resistant* cell lines, the IC50 values for each of the compounds on cisplatin resistant HeLa, HT29 and MCF7 cell lines were determined from dose response curves (Tables 3.5 – 3.6).

**Table 3.5:** IC50 values (μM) of the compounds screened against cisplatin resistant cell lines.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>Cisplatin</th>
<th>Oxaliplatin</th>
<th>Y9</th>
<th>Y14</th>
<th>Y16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>HeLa</td>
<td>1.5</td>
<td>12</td>
<td>22</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>2.7</td>
<td>35</td>
<td>35</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>2.3</td>
<td>8.0</td>
<td>22</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Cisplatin resistant</td>
<td>HeLa</td>
<td>15 (10x)</td>
<td>18 (1.5x)</td>
<td>65 (3x)</td>
<td>45 (2.4x)</td>
<td>75 (4.2x)</td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>22 (8.1x)</td>
<td>6.2 (0.18x)</td>
<td>60 (1.7x)</td>
<td>42 (2.3x)</td>
<td>50 (1.4x)</td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>85 (37x)</td>
<td>25 (3.1x)</td>
<td>58 (2.6x)</td>
<td>40 (2.2x)</td>
<td>52 (1.9x)</td>
</tr>
</tbody>
</table>

Brackets: Fold increase in IC50 value relative to that of untreated cells of the same cell line.

**Table 3.6:** Comparison between the maximum percentage inhibition reached by Lt16.2 when screened on cisplatin resistant cell lines and on “normal” cell lines.

<table>
<thead>
<tr>
<th>µM in solution</th>
<th>Maximum % inhibition reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Normal cells</td>
<td>7.21</td>
</tr>
<tr>
<td>Cisplatin resistant cells</td>
<td>9.50</td>
</tr>
<tr>
<td>T test values</td>
<td>p=0.001</td>
</tr>
</tbody>
</table>

Brackets: Fold increase in maximum percentage inhibition relative to that of untreated cells of the same cell line.

NS: Not significant

Table 3.5 shows that most of the compounds had higher IC50 values for the cisplatin resistant cell lines and were therefore less active against the cisplatin resistant cell lines. Oxaliplatin, however, was more active against cisplatin resistant HT29 cells than normal cells. Again, this is a further positive aspect of the action of oxaliplatin for the treatment of colon cancer. Although there was a reduction in the efficacy of the novel compounds against cisplatin resistant cells, this reduction was markedly smaller than that of cisplatin itself.
Since Lt16.2 is not very soluble, an IC50 value could not be obtained and the highest percentage of inhibition obtained was therefore used for the purpose of comparison (Table 3.6). The data in table 3.6 for Lt16.2 indicate that in spite of the fact that a saturated solution is in the region of only 7 – 10 µM, the percentage inhibition for all three of the cisplatin resistant cell lines increased to varying degrees. These increases were found to be insignificant in the HT29 and MCF7 cell lines, but were quite significant in the HeLa cell line.

3.8 CASPASE-GLO™3/7 ASSAY

Caspases, which belong to the family of cysteine proteases, play a crucial role in the implementation of apoptosis. Caspases are synthesized as inactive zymogens, which become activated by cleavage after a specific aspartate residue within a linker domain between a large subunit (LS) and a small subunit (SS), in addition to cleaving off of the NH2-terminal pro-domain. The LS and SS subsequently combine to form active caspases. When cells receive death signals, a subset of caspases is activated in a structured fashion and directs the processing and activation of critical effector/executioner caspases (Araya et al. 2002).

The caspase assay showed that cisplatin significantly activated caspase 3/7 activities in HeLa cells and relatively highly in the other cell lines (p<0.001), as compared to oxaliplatin and the other compounds (Figure 3.11). This agrees with published data by Ludwig and Oberleither (2004), who found that cisplatin induced much more caspase 3 activity on MDCK-C7 cells after a 48 hour incubation period than oxaliplatin and carboplatin, which did not induce much more than the control. Fujie et al. (2005) found that oxaliplatin only induced a one-fold increase in the caspase 3 activity in HT29 cells after a 48 hour incubation period. In the present study, the only statistically significant increase for oxaliplatin was found to be in the HeLa cell line (p<0.05). All the other compounds had similar caspase 3/7 activity to that of control cells. The lower activities induced by oxaliplatin and the other compounds suggest that an alternative path of inducing apoptosis might have been activated. However, for the novel compounds, it could also indicate that these induce necrosis. To verify whether apoptosis or necrosis was the dominant pathway, the Annexin V-FITC assay was performed on a Beckman-Coulter Flow Cytometer.
Figure 3.11: Caspase 3/7 activity induced in cancer cells by platinum compounds after a 48-hour exposure to the compounds and a two-hour incubation period with the Caspase-Glo™3/7 Assay Kit. Values are expressed as percentage chemiluminescence relative to control cells. (Data points represent Mean±SD of quadruplicate values). The only significant increases above the control were found to be for cisplatin on all three cell lines (p<0.001) and oxaliplatin in the HeLa cell line (p<0.05).

3.9 FLOW CYTOMETRY

3.9.1 Optimisation of conditions for Annexin V-FITC Assay

The Annexin V-FITC kit is an apoptosis detection kit based on the binding properties of Annexin V to phosphatidylserine and the DNA-intercalating capabilities of propidium iodide (PI). The appearance of phosphatidylserine (PS) residues on the surface of the cell can be used to detect and measure apoptosis. The presence of PS on the cell surface creates one of the specific signals for recognition and removal of apoptotic cells by macrophages. These PS changes can be detected with the anticoagulant, Annexin V, which has a high affinity for binding to PS. As the apoptotic process progresses, cell membrane integrity is lost. Using DNA specific viability dyes, such as Propidium Iodide (PI) it is possible to distinguish between early apoptotic, late apoptotic, and dead cells. Annexin V stains apoptotic cells, whereas PI stains only necrotic or late apoptotic cells, which enabled us to distinguish between apoptotic and necrotic cells.
After acquisition of the results for this experiment, it was realised that, when running this experiment the voltage settings were too low, but with the help of an Applications Specialist from Beckman-Coulter the problem was rectified. The results are nevertheless reported, though with the caveat that they may not be precise.

To establish the optimal incubation time and treatment concentration for this assay, the three cancer cell lines were incubated with cisplatin at its IC25 and IC50 concentrations for 24 and 48 hours. The percentages of apoptotic, late apoptotic and necrotic cells for these experiments are shown in tables 3.7 and 3.8. For ease of comparison, only increases above the control are indicted.

**Table 3.7:** Comparison between the percentages of apoptosis and necrosis obtained in cells exposed to cisplatin for 24 hours at concentrations equivalent to the IC25 and IC50 values for each cell line. (Percentages expressed as percentage above control as obtained from dot plots after flow cytometry).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>[Cisplatin]</th>
<th>Apoptotic</th>
<th>Late Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>IC25</td>
<td>0.0</td>
<td>0.0</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>0.0</td>
<td>0.0</td>
<td>17.4</td>
</tr>
<tr>
<td>HT29</td>
<td>IC25</td>
<td>4.5</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>30.6</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MCF7</td>
<td>IC25</td>
<td>15.8</td>
<td>7.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>0.0</td>
<td>0.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Table 3.8: Comparison between the percentages of apoptosis and necrosis obtained in cells exposed to cisplatin for 48 hours at concentrations equivalent to the IC25 and IC50 values for each cell line. (Percentages expressed as percentage above control as obtained from dot plots after flow cytometry).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[Cisplatin]</th>
<th>Apoptotic</th>
<th>Late Apoptotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>IC25</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>15.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>HT29</td>
<td>IC25</td>
<td>0.0</td>
<td>0.0</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>1.3</td>
<td>0.0</td>
<td>10.9</td>
</tr>
<tr>
<td>MCF7</td>
<td>IC25</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>IC50</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Tables 3.7 and 3.8 show that more apoptosis occurred after 24 hours for the HT29 and MCF7 cell lines, but not for the HeLa cell line, where more apoptosis occurred after 48 hours. For all the cell lines, except MCF7, the IC50 concentrations induced more apoptosis than the IC25 concentrations; therefore the IC50 concentrations were chosen as optimal for this experiment. The varying results obtained for the incubation times led to the conclusion that the samples should also be tested at 12 and 48 hours in order to get a complete picture. It could be expected that induction of apoptosis would not follow the same time course in different cell types and with different apoptosis inducers and therefore a relatively short and a longer incubation time were included.

3.9.2 Induction of apoptosis by platinum containing compounds

Table 3.9 shows the percentages of apoptosis and necrosis obtained when the cells were exposed to the platinum containing compounds at a concentration equivalent to their IC50’s, for a period of 12 hours. Table 3.9 indicates that for most of the compounds an incubation period of 12 hours was too short to see sufficient apoptosis.
Table 3.9: Summary of results obtained from the Annexin V – FITC Assay after a 12 hour incubation with platinum containing compounds at their respective IC50’s (Percentages expressed as percentage above control as obtained from dot plots after flow cytometry).

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa</th>
<th>HT29</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>L A</td>
<td>N</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.6</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>13.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Y9</td>
<td>0.8</td>
<td>0.0</td>
<td>19.7</td>
</tr>
<tr>
<td>Y14</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Y16</td>
<td>16.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>4.0</td>
<td>0.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

A: Apoptotic  
L A: Late Apoptotic  
N: Necrotic

Table 3.10: Summary of results obtained from the Annexin V – FITC Assay after a 48 hour incubation with platinum containing compounds at their respective IC50’s (Percentages expressed as percentage above control as obtained from dot plots after flow cytometry).

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa</th>
<th>HT29</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>L A</td>
<td>N</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.0</td>
<td>0.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Y9</td>
<td>18.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Y14</td>
<td>27.1</td>
<td>0.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Y16</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>0.0</td>
<td>0.0</td>
<td>10.3</td>
</tr>
</tbody>
</table>

A: Apoptotic  
L A: Late Apoptotic  
N: Necrotic

All of the compounds induced apoptosis in HeLa cells after 12 hours, except for Y9, which induced very little and Y14, which induced no apoptosis (Table 3.9). Table 3.10 shows that after 48 hours only Y9 and Y14 showed some apoptosis in the HeLa cell line. These results would suggest that Y9 and Y14 have a slightly slower anticancer action on the HeLa cells.
The HT29 cell line only showed apoptosis for Lt16.2 and Y9. Y9 and Y14 did not induce apoptosis in the MCF7 cell line at all, but all of the other compounds did induce apoptosis after 48 hours. This suggests that these compounds have a slower anticancer action on the MCF7 cell line.

As explained previously, the accuracy of the results from the Annexin V-FITC assay cannot be guaranteed because of the problem with the voltage settings on the flow cytometer. However, the results certainly indicate the induction of apoptosis by the novel compounds. The next series of experiments was designed to confirm the observation of apoptosis induction and to investigate the effects of the compounds on cell cycle progression.

3.9.3 Coulter®DNA Prep™Reagents Kit

Normal cell cycle events are governed by complex control mechanisms. Cell cycle arrest most commonly occurs at the G1/S or G2/M boundaries. When cells transform, these control mechanisms are altered and cell growth becomes uncontrolled. Cell cycle analysis (or DNA cycle analysis) provides a useful tool in the search for new anticancer agents as it gives some insight into possible mechanisms of action of the drugs. Typically, cisplatin will cause a delay in the S phase because of its interaction with the DNA (Shapiro and Harper, 1999). The effects on the cell cycle of the compounds under investigation in this study were compared to that of cisplatin and oxaliplatin.

The Coulter®DNA Prep™Reagents Kit was applied to cells, which were exposed to a concentration equivalent to the IC50 value of each of the platinum containing compounds for 48 hours. This was done in order to determine which stage(s) of the DNA cycle (Figure 3.12) these compounds affect. It was possible to visualise the stages affected, by comparing the area of the peaks. A larger peak area indicated that the DNA cycle has been arrested or delayed at that particular stage, since the DNA could not move on to the next phase.
Figure 3.12: A diagram indicating where the different DNA phases are depicted on a histogram obtained with the Beckman-Coulter FC500 flow cytometer. (These results were obtained from HT29 control cells).

Figure 3.12 shows where the different phases of the DNA cycle could be seen on the histograms obtained from the Beckman-Coulter FC500 Flow Cytometer. The Sub-G1 phase peak provides evidence of apoptosis. Apoptotic cells can be seen to the left of the G0/G1 peak due to DNA fragmentation occurring during apoptosis, DNA is lost from the permeabilised cells thus leaving these cells with less DNA (Kwon et al. 2005; http://www.icms.qmul.ac.uk/flowcytometry/uses/apoptosis/dnafragmentation/). The G0/G1 phase is the initial phase in the DNA cell cycle, the S-phase is the synthesis phase, where different cells are in various stages of copying their DNA and the G2/M phase is the mitosis phase where the cells divide and after which the cycle is complete.

For each cell line, the cells were seeded into 10 cm dishes at 1.9x10⁶ cells per plate. These cells were exposed to each of the compounds separately and control cells were prepared, where no compound was applied to the cells. Each of these plates containing the cells was trypsinised after 48 hours and the cells were exposed to the Coulter® DNA Prep™ Reagents Kit. The results were read on a Beckman-Coulter FC500 Flow Cytometer. The plots obtained were similar to that shown in Figure.
3.12. For visual comparison, overlay plots of each of these compounds, compared to the control result were plotted for each cell line (Figures 3.13 – 3.15).

**Figure 3.13:** Visual comparison of HeLa cell cycle histograms of untreated cells (blue lines) and cells treated with platinum containing compounds for 48 hours (red lines). (A) Cisplatin, (B) Oxaliplatin; (C) Y9; (D) Y14; (E) Y16 and (F) Lt16.2.
**Figure 3.14:** Visual comparison of HT29 cell cycle histograms of untreated cells (blue lines) and cells treated with platinum containing compounds for 48 hours (red lines). (A) Cisplatin, (B) Oxaliplatin; (C) Y9; (D) Y14; (E) Y16 and (F) Lt16.2.
Figure 3.15: Visual comparison of MCF7 cell cycle histograms of untreated cells (blue lines) and cells treated with platinum containing compounds for 48 hours (red lines). (A) Cisplatin, (B) Oxaliplatin; (C) Y9; (D) Y14; (E) Y16 and (F) Lt16.2.

The area of the peaks in figures 3.13 – 3.15 show that the DNA was affected to different extents in the different cell lines. From these figures the percentages of cells in the different DNA phases were calculated by the Beckman-Coulter FC500 Flow Cytometer’s software and these are summarised in tables 3.11 – 3.13. An increase in the percentage of cells in a particular DNA phase, compared to the control value, showed that the compound has blocked or delayed that particular phase in the DNA cycle.

For the anticancer agents tested in this study, tables 3.11 – 3.13 show that the cell line within which the most alteration occurred to its DNA cycle was HT29. The cell line revealing the least alteration to its DNA cycle was MCF7. The graph in figure 3.14A and data in table 3.12 illustrate that cisplatin imposed an S-phase delay in all three cell lines, which was in agreement with previous reports (Shapiro and Harper, 1999).
None of the other compounds caused as significant a S-phase delay as cisplatin in the HT29 cell line.

The Sub-G1 phase peak depicts apoptotic cells (Kwon et al. 2005). In all of the cell lines, cisplatin induced more apoptosis than oxaliplatin, with the most significant difference between the two in the HT29 cell line.

Table 3.11: The percentages of cells in each of the DNA cycle phases after HeLa (cervical cancer) cells were exposed to the platinum containing compounds for 48 hours.

<table>
<thead>
<tr>
<th></th>
<th>Sub-G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2</td>
<td>78.9</td>
<td>3.9</td>
<td>16.0</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>6.1</td>
<td>40.6</td>
<td>7.7</td>
<td>41.3</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>4.6</td>
<td>52.4</td>
<td>3.8</td>
<td>39.2</td>
</tr>
<tr>
<td>Y9</td>
<td>3.3</td>
<td>52.8</td>
<td>4.5</td>
<td>39.1</td>
</tr>
<tr>
<td>Y14</td>
<td>1.7</td>
<td>74.7</td>
<td>7.3</td>
<td>16.2</td>
</tr>
<tr>
<td>Y16</td>
<td>2.1</td>
<td>67.1</td>
<td>7.0</td>
<td>23.8</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>1.4</td>
<td>72.1</td>
<td>1.3</td>
<td>25.2</td>
</tr>
</tbody>
</table>

Figure 3.16: The effect that the platinum containing compounds had on HeLa cells with reference to (A) apoptosis and (B) imposing delays in the different DNA phases. The values depicted in these graphs have been taken directly from the corresponding table above and are relative to the control.
Table 3.12: The percentages of cells in each of the DNA cycle phases after HT29 (colon cancer) cells were exposed to the platinum containing compounds for 48 hours.

<table>
<thead>
<tr>
<th></th>
<th>Sub-G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5</td>
<td>60.0</td>
<td>17.7</td>
<td>14.9</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>21.2</td>
<td>12.4</td>
<td>52.1</td>
<td>14.3</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>9.0</td>
<td>39.9</td>
<td>35.3</td>
<td>15.9</td>
</tr>
<tr>
<td>Y9</td>
<td>14.7</td>
<td>28.6</td>
<td>32.3</td>
<td>24.4</td>
</tr>
<tr>
<td>Y14</td>
<td>12.9</td>
<td>56.3</td>
<td>11.0</td>
<td>19.7</td>
</tr>
<tr>
<td>Y16</td>
<td>14.4</td>
<td>37.0</td>
<td>20.6</td>
<td>27.9</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>24.0</td>
<td>50.3</td>
<td>15.6</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Figure 3.17: The effect that the platinum containing compounds had on HT29 cells with reference to (A) apoptosis and (B) imposing delays in the different DNA phases. The values depicted in these graphs have been taken directly from the corresponding table above and are relative to the control.
Table 3.13: The percentages of cells in each of the DNA cycle phases after MCF7 (breast cancer) cells were exposed to the platinum containing compounds for 48 hours.

<table>
<thead>
<tr>
<th></th>
<th>Sub-G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5</td>
<td>79.8</td>
<td>4.5</td>
<td>14.2</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.3</td>
<td>29.3</td>
<td>10</td>
<td>57.4</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>0.97</td>
<td>66.6</td>
<td>2.9</td>
<td>29.5</td>
</tr>
<tr>
<td>Y9</td>
<td>4.1</td>
<td>67</td>
<td>11.6</td>
<td>17.2</td>
</tr>
<tr>
<td>Y14</td>
<td>0.98</td>
<td>66.5</td>
<td>10.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Y16</td>
<td>1.1</td>
<td>58</td>
<td>11.1</td>
<td>30.2</td>
</tr>
<tr>
<td>Lt16.2</td>
<td>0.67</td>
<td>77.6</td>
<td>8.7</td>
<td>13.1</td>
</tr>
</tbody>
</table>

Figure 3.18: The effect that the platinum containing compounds had on MCF7 cells with reference to (A) apoptosis and (B) imposing delays in the different DNA phases. The values depicted in these graphs have been taken directly from the corresponding table above and are relative to the control.

All the compounds induced apoptosis in HeLa cells (Table 3.11 and Figure 3.16A), but not to such an extent as cisplatin (6.1%) or oxaliplatin (4.6%). Y9 induced the most apoptosis (3.3%) of the novel platinum containing compounds as indicated by the percentages of cells in the sub-G1 phase.

In the HeLa cell line cisplatin imposed a delay in the S and G2/M phase (Table 3.11 and Figure 3.16B), since the percentage of cells in the S-phase increased from 3.9% in the control cells, to 7.7% in the cells exposed to cisplatin. The percentage of cells in
the G2M phase increased from 16% to 42.3% after exposure to cisplatin. In this manner, the rest of the results obtained were compared to these of cisplatin and oxaliplatin. Oxaliplatin only delayed the G2/M phase in the HeLa cell line. It was now possible to compare the phases affected by the compounds to the phases affected by cisplatin and oxaliplatin. Y9 and Y16 imposed delays in the same phases as cisplatin and Lt16.2 followed the trend of oxaliplatin. Y14 only caused an S-phase delay. The fact that Y9 and Y16 affected the DNA cycle in the same manner as capsulation is probably an undesired result, since cisplatin causes so many toxic side-effects. Hence, whenever a compound acts like oxaliplatin or in a completely different manner to cisplatin, it might have less toxic side effects. Lt16.2 and Y14 fall into this category for this cell line. The effect that Y14 has on the cell cycle will have to be investigated further, since neither oxaliplatin nor cisplatin displays such a result, but since cisplatin is known to impose a delay in the S-phase, this compound might well exhibit the same effects as cisplatin. The fact that Lt16.2 acts like oxaliplatin on the DNA cycle, may indicate less toxic side effects, similar to those caused by oxaliplatin.

The results obtained for the HT29 cell line (colon cancer cell line) are discussed below.

In the HT29 cell line all the novel platinum containing compounds induced more apoptosis than oxaliplatin (Table 3.12 and Figure 3.17A), however, Lt16.2 induced more apoptosis than cisplatin as well. This compound also had the highest percentage of apoptosis induced overall during this experiment. However, this compound induces resistance in HT29 cells.

Table 3.12 and Figure 3.17B show that in the HT29 cell line both cisplatin and oxaliplatin imposed a delay in the S-phase, but oxaliplatin imposed an additional delay in the G2/M phase as well. None of the novel platinum containing compounds followed the trend of cisplatin. Y9 delayed the S and the G2/M phase, whereas Y14 and Y16 delayed only the G2/M phase. Hence Y9 acted similarly to oxaliplatin, but again, the fact the Y9 induces resistance in HT29 cells might prove to render these results less significant. Lt16.2, however, did not delay any of the DNA phases in this
cell line, apparently through its rapid induction of apoptosis. Unfortunately, this compound induces resistance in HT29 cells.

Finally, the results obtained for the MCF7 cell line (breast cancer cell line) are discussed below.

In the MCF7 cell line oxaliplatin did not induce much apoptosis, since the percentage of cells in the Sub-G1 phase was even lower than that of the control cells (Table 3.13 and Figure 3.18A). Cisplatin did induce apoptosis, but not as much as on the HeLa and HT29 cell lines. Most of the compounds did not induce much apoptosis in this cell line, since the percentages of cells in the sub-G1 phase are all approximately the same as that of oxaliplatin. The only exception was Y9, which had a higher percentage of apoptosis than cisplatin.

Table 3.13 and Figure 3.18B show that, in the MCF7 cell line, cisplatin and oxaliplatin behaved the same as in the HeLa cell line, where cisplatin imposed a delay in the S and the G2/M phase and oxaliplatin impose a delay in the G2/M phase only. Y9, Y14 and Y16 followed the trend of cisplatin, which might imply that the same toxic side effects associated with cisplatin may be exhibited by these compounds. Lt16.2 only delayed the S-phase. This result need to be studied further, in order to see what toxic side effects may be associated with this compound. Since cisplatin is known to impose a delay in the S-phase, this compound might well exhibit the same effects as cisplatin.
CHAPTER 4  DISCUSSION AND CONCLUSION

Cancer is an important public health concern around the world, given that it is a most important disease entity and a cause of death in the human population (Parker et al. 1997 and Walker and Walker, 1999). The application of inorganic chemistry to medicine is a fast developing field, and novel therapeutic and diagnostic metal complexes are now having an impact on medical practice. Advances in bio-inorganic chemistry are vital for improving the design of compounds to lessen toxic side effects and understand their mechanisms of action (Sadler and Guo, 1998).

In this study, it was shown that it might be better to solubilise the platinum containing compounds in RPMI 1640 containing 10% foetal calf serum (Table 3.1 and Figure 3.3), since chemical reactions between these compounds and DMSO may alter the structure of the compounds before application to the cancer cells, which in turn may alter its anticancer action against these cells.

A major problem in the screening of these compounds was their differences in solubility. This made comparisons between the anticancer actions of these compounds extremely difficult. Fortunately the technique of ICP-MS analysis can accurately determine the amount of the compound in a solution. It was thus imperative to subject the samples to ICP-MS analysis and to depict the results as a function of the concentration of the platinum containing compound present. This is clearly visible in the results obtained from Lt16.2, where the growth inhibition was comparable and even higher than that of the positive control, cisplatin, when the true concentrations were compared (Figures 3.1 and 3.2).

Another important finding was that some of these compounds might be more active than cisplatin at lower concentrations, such as Lt16.2 (Figure 3.7). Therefore, if the solubility of such a compound could be improved, or if it could be encapsulated, to improve delivery and control the release, it might prove to be a very good alternative anticancer agent.

There were no significant differences between the results obtained from the MTT or the CellTiter-Blue™ cell viability assays, except for the lower concentrations of Y16
and Lt16.2, which could be a result of the extremely low concentrations in solution (Figure 3.8). Hence a choice was made mostly on personal preference and for the sake of consistency, since all previous samples were screened using the MTT cell viability assay.

Eighty-four novel platinum containing compounds were screened and from these, four of the best compounds were chosen. They included Y9 (containing an diamine chelate as non-leaving group and Cl as leaving groups), Y14 (differing from Y9 in the bulk of its non-leaving group), Y16 (structurally comparable to that of Y14) and Lt16.2 (containing an non-leaving group consisting of an NS donor chelate ligand, containing a thioethereal sulphur donor atom, and its leaving group is a dicarboxylate ion). Cisplatin and oxaliplatin were chosen as positive controls. None of these compounds exhibited IC50’s similar to cisplatin, but some did show similarity to that obtained for oxaliplatin. This is not necessarily a negative finding, as oxaliplatin, with its higher IC50’s as compared to cisplatin, is known to have much less toxic side effects than cisplatin.

The effect of GSH on the anticancer action of these platinum containing compounds was shown to be concentration dependent, since at high GSH concentrations the anticancer action was decreased. This was shown by the high IC50 values obtained for some of these compounds screened against Chang liver cells. Chang liver cells have a high amount of GSH present (Zhang et al. 1999 and Zhang et al. 2000), which would inhibit the action of the compounds. Another possible reason for some of these high IC50 values may be that the compounds are not as toxic to liver cells as cisplatin, which is a very important parameter that has to be present in any potential novel anticancer agent. When combining the results obtained from the dose response curves on Chang liver cells and the effect of GSH on the action of these compounds, it could be deducted that cisplatin detoxifies quickly and that GSH plays a role in its detoxification, but possibly other detoxification mechanisms operate as well, since GSH did not have such a great effect on the action of this compound. The same argument applies for Lt16.2, although no IC50 value could be obtained for this compound because of its low solubility. Y14 and Y16 were detoxified quickly, similar to cisplatin, which was expected since their structures are similar to cisplatin, but the GSH experiment showed that GSH may play a more important role in the
detoxification of Y14 and Y16 than cisplatin. Oxaliplatin and Y9 exhibited slow
detoxification, but the GSH experiment also showed that GSH plays a significant role
in their detoxification. Y9 also has a similar structure to cisplatin, and therefore a
similar detoxification pattern as cisplatin was expected. Lt16.2 does not have a
structure similar to any of the other novel compounds or the positive controls. These
results do not mean that no other detoxification mechanisms are operate for the novel
compounds, Y9, Y14 and Y16 or for oxaliplatin: they merely show that it is more
likely that, for cisplatin and Lt16.2, other mechanisms apply, since GSH did not have
such a great effect on their action.

A serious drawback of the novel compounds was that all of them were capable of
inducing resistance in the cancer cells (Table 3.3), with the exception of Lt16.2 on
HeLa cells (Table 3.4). This is one of the most important parameters in evaluation of
the potential novel anticancer agents, since one of the biggest problems with the
current agents is that resistant tumours frequently appear, which renders the
treatments unsuitable. It is noteworthy, however, that none of the novel compounds
induced resistance to the same degree as cisplatin in any of the cell lines. As
expected, oxaliplatin did not induce resistance to the same degree as cisplatin (Culy et
al. 2000). Furthermore, the increases in IC50 values caused by the novel compounds
compared much better with oxaliplatin than cisplatin, suggesting that they might still
be useful. These results further indicate that oxaliplatin was more active against
cisplatin resistant HT29 cells than against normal HT29 cells (Table 3.5). Oxaliplatin
is currently used to treat colon cancer and was found to be active against cisplatin
resistant colon cancer cells (Se et al. 2005). A very important finding is that Lt16.2
may be active against cisplatin resistant cells. As explained in section 3.6, an IC50
value could not be determined for this compound, since it has such a low solubility,
but the highest percentage of inhibition reached was used to determine whether it was
more active against the cisplatin resistant cell lines. These values (Table 3.6) showed
that Lt16.2 was definitely more active against cisplatin resistant HeLa cells, and most
probably active against cisplatin resistant HT29 cells, but as mentioned, the fact that it
induces resistance in the HT29 cell line may outweigh its positive aspects. However,
it appears to be a promising novel anticancer agent against cervical cancer.
The Caspase-Glo™ 3/7 Assay (Figure 3.11) showed no significant induction of caspase 3/7 activities by any of the novel platinum containing compounds. Oxaliplatin only increased the caspase 3/7 activity in HeLa cells (p<0.05), whereas cisplatin induced a high activity in all three cell lines (p<0.001). However, this does not mean that no apoptotic pathways were activated; it may merely indicate that another caspase might be activated or that the incubation time was not optimal for these compounds. Due to a limited number of wells that could be assayed with this kit, the assay was only performed after a 48 hour exposure to the treatments. It is quite possible that this incubation time was not optimal for the activation of caspases 3 and 7, since Budzisz et al. (2004) incubated their cells with the platinum compounds for 1, 2 and 5 hours and Mirowski et al. (2003) incubated their cells with the platinum compounds for 24 hours. However, others (Fujie et al. 2005; Ludwig and Oberleithner, 2004) incubated their cells for 48 hours and Ludwig and Oberleithner (2004) also found that caspase activity was only visible after a 8 hour incubation. These conflicting results suggest that the caspase assay should be performed after various incubation times, between 2 and 48 hours, before an opinion can be formed.

The results obtained by flow cytometry indicated that most of these compounds induced apoptosis to some extent and imposed a delay in one or more of the DNA phases, although the apoptosis data obtained via the two different methods showed no correlation. This might be because of the faulty settings used during the AnnexinV-FITC experiment and therefore it can only with be said with certainty that all these compounds did induce apoptosis. However, the most promising results were obtained for Lt16.2. These experiments showed that this compound induced apoptosis in the HeLa cell line and that it imposed a delay in the same phase as oxaliplatin in this cell line. Although some of the other compounds also showed promise, from the flow cytometry results, they have the drawback that they induce resistance in the cells.

In order to determine whether these compounds might be potential novel anticancer agents, the results of some previous experiments are required. Therefore a summary of the results obtained for each novel compound follows. Since cisplatin and oxaliplatin are used as references in this study, their typical effects on the three cancer cell lines will firstly be summarised.
*Cisplatin* imposed a significant delay in the S-phase of each of the three cell lines (See the A curves in each of figures 3.13 – 3.15; the B graphs in figures 3.16 – 3.18 and also data in the corresponding tables). This behaviour agrees with that reported in the literature (Shapiro and Harper, 1999). It also induced a relatively high degree of apoptosis (Figures 3.16 – 3.18A). Except in the case of HT29 cells, it caused prominent delays in the G2/M phase of the DNA. *However, cisplatin induced resistance to all the cancer cell lines tested in this study.*

*Oxaliplatin* behaved differently to cisplatin, since the extent of apoptosis was only relatively high in the case of cervical cancer cells, whereas for both colon and breast cancer cells it was low. It delayed the G2/M phases of all three cell lines tested, but only the S phase of HT29 cells. Oxaliplatin, although inducing resistance in MCF7 cells, *did not induce resistance in HeLa and HT29 (colon cancer) cells.* *Cisplatin resistant cells did not affect the behaviour of oxaliplatin in a significant manner.*

*Y9* can be regarded as related to cisplatin in that it has a diamine chelate as non-leaving group and Cl⁻ as leaving groups. In general, such compounds are characterized by relatively rapid ligand exchange of their leaving groups. The compound, Y9, showed a high percentage of inhibition in all the cell lines; however, *it imposed resistance against all the cell lines* after the cells were intermittently and incrementally exposed to the compound. It also did not show any significant differences in its effect on the treatment of cisplatin resistant cells. This compound mimicked cisplatin in the effects it has on the DNA cycle in both HeLa and MCF7 cells, while it acted similar to oxaliplatin in HT29 cells in delaying the different DNA phases. *Y9 was one of the compounds that induced apoptosis in all of the cell lines.* However, in light of the above results, specifically with reference to the resistance induced, this may not be regarded as a major improvement over cisplatin, judging from the present experimental data.

*Y14* differs from Y9 in the bulk of its non-leaving group. The compound, Y14 also induced resistance in all of the cell lines after the cells were intermittently and incrementally exposed to this compound. It was not more active against cisplatin resistant cell lines and did not induce apoptosis in the MCF7 cell line. Furthermore, in the MCF7 cell line, this compound acted similar on the DNA cycle as cisplatin. In
the HeLa cell line, it imposed a delay in the S-phase, which is a known effect of cisplatin as well. These results showed that the behaviour of this compound did not differ enough from cisplatin to be considered as an improvement, according to the present experimental data.

Y16’s structural aspects are again comparable to that of Y14. The compound, Y16 showed similar results to those obtained for Y9 and Y14. This compound also induced resistance in the cells after intermittent and incremental exposure of the cells to it. It did not have an increased anticancer action against cisplatin resistant cells, either. It induced apoptosis in HeLa and HT29 cells, but not in MCF7 cells. Its effects on the DNA cycle were similar to that of cisplatin in HeLa and MCF7 cells. Therefore, this compound would not be considered as an improved anticancer agent, judging from the present experimental data.

Lt16.2 differs structurally from all of the above compounds, since the non-leaving group consists of an NS donor chelate ligand, containing a thioethereal sulfur donor atom, and its leaving group is a dicarboxylate ion. Lt16.2 showed the same results as mentioned for the other compounds on HT29 and MCF7 cells. However, the results obtained for Lt16.2 on HeLa cells were of particular importance, since this compound did not induce resistance in HeLa cells and was even more active against cisplatin resistant HeLa cells as well. There were some apoptotic cells present in the Sub-G1 phase (Table 3.11 and Figure 3.16A); therefore, it destroyed the cells via apoptosis. This compound can be regarded as a potentially improved novel anticancer agent for use in the treatment of cervical cancer in light of the above-mentioned properties.
CHAPTER 5  FINAL PERSPECTIVE REGARDING THIS STUDY

This work was initiated in an effort to produce novel, improved anticancer agents, as stated in the introduction. It is however very difficult to determine what a good anticancer agent is. For example, is cisplatin a good anticancer agent? Although it has a high “killing rate”, it also has numerous toxic side effects. Its tendency to induce resistance in cells is again a negative effect. Yet, in spite of this, for the last approximately thirty years it has been rather commonly used.

Is oxaliplatin a good agent? It has a much lower “killing rate” than cisplatin, however it has lesser side effects and does not render certain cancer cells resistant. It is presently rated number one for the treatment of colon cancer.

The above-illustrated complexities however should not discourage the search for new improved anticancer agents, especially if the severity of the disease is taken into account. It is realised that the monitoring of the anticancer action of the compounds in this work can only be considered as first order to select those compounds that exhibited potential. Even those that are in the latter category should be tested further in biological systems.

A significant number of compounds have been tested, and as expected, a much smaller number proved to have potential according to the experiments performed in this study. A few diamine compounds were shown to be worth follow-up tests, namely Y9, Y14 and Y16. Furthermore, a new type of compound (Lt16.2) with an NS-donor chelate as non-leaving group and a dicarboxylate leaving group, although with limited solubility in the biological system, can be regarded as very promising for cervical cancer treatment, since it does not induce resistance in HeLa cells.

The work created some new concepts, which will be worthwhile pursuing for the further search of improved anticancer agents.
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APPENDIX

A SCREENING OF COMPOUNDS USING MTT ASSAY
Figure A.1: Treatments screened against HeLa cells. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons explained in section 3.1).
Figure A.2: Treatments screened against HT29 cells. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons explained in section 3.1).
Figure A.3: Treatments screened against MCF7 cells. (Data points represent Mean±SD of quadruplicate values. Data points joined for the sake of clarity and for reasons explained in section 3.1).
Figure B.1: Representative dot plots of Annexin V-FITC results. These results were obtained with HT29 cells treated with Cisplatin for 24 hrs [(A) Control; (B) IC25; and (C) IC50]