A combination of platinum anticancer drugs and mangiferin causes increased efficacy in cancer cell lines.

by

Debbie du Plessis-Stoman

Submitted in fulfilment of the requirements for
the degree of
PHILOSOPHIAE DOCTORAE

in the
Faculty of Science
at the

Nelson Mandela Metropolitan University
2010

Promoter: Prof M. van de Venter

DECLARATION

In accordance with Rule G4.6.3, I, Debbie du Plessis-Stoman, hereby declare that this thesis represents my own, unaided work and where necessary due credit has been given. This thesis has not previously been submitted for assessment to another University or for another qualification.

27 March 2010

D. DU PLESSIS-STOMAN

DATE

CONTENTS

SUMMARY

This thesis mainly deals with some biochemical aspects regarding the efficacy of novel platinum anticancer compounds alone and in combination with mangiferin, 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. Numerous novel compounds were tested in this way, using the MTT cell viability assay and the three cancer cell lines MCF7, HT29 and HeLa. Although only a few could be regarded as equal to or even better than cisplatin, CPA7 and oxaliplatin, the testing of these compounds on cancer cells provided useful knowledge for the further development of novel compounds.

Three of the better compounds, namely Yol 25, Yol 29.1 and Mar 4.1.4 were selected for further studies, together with oxaliplatin and CPA7 as positive controls, to obtain more detailed knowledge of their anticancer action, both alone and when applied in combination with mangiferin. In addition to the above, resistant cells were produced for each of the three different cell lines tested and all the selected compounds, both in the presence and absence of mangiferin. The effects of these treatments on the activation of NFκB when applied to normal and resistant cell lines were also investigated.

All the compounds induced apoptosis in the cell lines tested as well as alter the DNA cycle at one or more phase. Additionally, combination of these compounds with mangiferin enhanced the above-mentioned effects. Mangiferin decreases the IC50 values of the platinum drugs by up to 3.4 times and, although mangiferin alone did not induce cell cycle arrest, the presence of mangiferin in combination with oxaliplatin and Yol 25 shows an earlier and greatly enhanced delay in the S-phase, while cells treated with CPA7, Yol 29.1 and Mar 4.1.4 in combination with mangiferin showed a later, but greatly enhanced delay in the S-phase. It was also found that mangiferin acts as an NFκB inhibitor when applied in combination with these drugs, which, in

turn, reduces the occurrence of resistance in the cell lines. Resistance to oxaliplatin was counteracted by the combination with mangiferin in HeLa and HT29, but not in MCF7 cells, while resistance to CPA7 was only counteracted in the MCF7 cell line. Yol 25 and Mar 4.1.4 did not seem to induce resistance in HeLa and MCF7 cells, but did in HT29 cells, whereas Yol 29.1 caused resistance in HeLa and HT29 cells, but not in MCF7 cells.

Finally, an effort was made to evaluate the different compounds by comparing them with respect to their properties relating to anticancer action with and without the addition of mangiferin.

Keywords: apoptosis, platinum anticancer compounds, mangiferin, resistance, NFκB, cancer cell lines.

ACKNOWLEDGEMENTS

The author records her appreciation to:

Prof. M. van de Venter, Department of Biochemistry and Microbiology, for her invaluable supervision and encouragement during the course of this study.

Professor J.G.H. du Preez, Research Unit for Platinum Group Chemistry, for his continual support and guidance throughout the study.

The Research Unit for Platinum Group Chemistry, for supplying novel platinum containing compounds for screening.

All staff and postgraduate students in the Department of Biochemistry and Microbiology, as well as the Department of Chemistry, Nelson Mandela Metropolitan University, for their continual support and encouragement.

Platco, Shimoda Biotech Pty (Ltd) and the Technology and Human Resources for Industry Program (THRIP) for financial support.

My family and friends for their support and encouragement.

LIST OF ABBREVIATIONS

APAF1	apoptotic protease activating factor 1
BID	*Bcl-2* Interacting Domain
bFGF	basic fibroblast growth factor
CBDCA	carboplatin
CDDP	cisplatin
CDK	cyclin dependent kinase
CIN	cervical intra-epithelial neoplasia
DCIS	ductal carcinoma *in situ*
DISC	death-inducing signalling complex
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
FasL	Fas ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GSH/GSSG	glutathione (reduced/oxidised)
GST	glutathione-S transferase
GS-X pump	glutathione S-conjugate export pump
HIV	human immunodeficiency virus
HMG-domain proteins	high mobility group-domain-proteins
HPV	human papilloma virus
HRT	hormone replacement therapy
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
IC50	inhibiting concentration at which 50% of cells are non-viable
IDC	invasive ductal carcinoma
IKK	IκB (inhibitor proteins) kinase complex
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
LDH	lactate dehydrogenase
LS	large subunit
MIN	microsatellite instability
MMR	mismatch repair
MRP	multidrug resistant protein
MT	metallothionein
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NFκB	nuclear factor κ B
NMMU	Nelson Mandela Metropolitan University
NS	not significant
PBSA	phosphate buffered saline excluding Ca^{2+} and Mg^{2+}
PDGF	platelet-derived growth factor
PI	propidium iodide
PS	phosphatidylserine
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
SS	small subunit
STAT	signal transducer and activator of transcription
STD	sexually transmitted disease
TNF	tumour necrosis factor
Trans-DDP	transplatin

LIST OF FIGURES

Page

LIST OF TABLES

Page

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 and 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). Cisplatin begins its interaction with cancer DNA by binding to the nitrogen atoms of the DNA bases, typically guanine. 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 (Siddik, 2003, Scheeff *et al*. 1999).

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 and Walker and Walker, 1999).

At the same time traditional medicine is an integral part of South African cultural life, a position that is unlikely to change to any significant degree in years to come. It is estimated that between 12 and 15 million South Africans depend on traditional herbal medicines from as many as 700 indigenous plant species (Duncan *et al*. 1999).

The anticancer properties of tea are well known, and the tumour inhibition potential of certain polyphenolic compounds from green and black teas has been well documented (Marnewick *et al*. 2000 and Marnewick *et al.* 2004). Honeybush tea (*Cyclopia intermedia*) is a South African herbal beverage that is currently receiving prominent attention. Honeybush tea is prepared from the leaves, stems and flowers of several *Cyclopia* spp. The major phenolic components of the unprocessed South African honeybush tea are the xanthone, mangiferin and the flavanone, hesperidin (Marnewick *et al*. 2005).

In a study at the University of Dundee (Scotland) it has been found that the development of anti-cancer drugs that can “switch” the function of NF-κB (nuclear

factor κ B) from anti-apoptotic to pro-apoptotic may prove to be beneficial in the fight against cancer. Supranormal activation of NF-κB is connected to cancer. Active NF-κB promotes tumour growth by increasing the transcription of genes that are: anti-apoptotic; inducers of cell proliferation; pro-angiogenic; pro-metastatic and responsible for other cellular mechanisms related to tumour growth. Hence, tumour prevention may be achieved through inhibition of abnormally active NF-κB (Perkins, 2000 and Perkins, 2004).

Mangiferin is a natural polyphenol known to exhibit anti-inflammatory, antioxidant, and antiviral effects, though the molecular mechanism underlying these effects has not been well characterized. NF-κB plays an important role in these processes; hence it may be possible that mangiferin modulates NF-κB activation. Sarkar *et al.* (2004) showed that mangiferin blocks tumour necrosis factor (TNF)-induced NF-κB activation and NF-κB-dependent genes like *ICAM1* and *COX2*. The effect was mediated through inhibition κB I(inhibitor proteins) -kinase complex (IKK) activation and subsequent blocking of phosphorylation and degradation of IκB-α (inhibitor protein α). In addition, mangiferin inhibits TNF-induced p65 phosphorylation as well as translocation to the nucleus and also inhibits NF-κB activation induced by other inflammatory agents.

Mangiferin, similar to other known antioxidants, inhibits TNF-induced reactive oxygen intermediate (ROI) generation. It enhances glutathione (GSH) levels by almost 2-fold more than other antioxidants, and at the same time it decreases the levels of oxidized glutathione (GSSG) and increases the activity of catalase. Depletion of GSH by buthionine sulfoximine led to a significant reversal of mangiferin effect. Hence, mangiferin, with its ability to inhibit NF-κB and increase the intracellular GSH levels, may prove to be a potent drug for anti-inflammatory and antioxidant therapy. Mangiferin-mediated down-regulation of NF-κB also shows potential for chemotherapeutic agent-mediated cell death, suggesting a role in combination therapy for cancer (Sarkar *et al.* 2004). However, it is known that high GSH levels inhibits the anticancer action of platinum drugs (via detoxification) as well as plays a role in the induction of resistance towards these compounds in cancer cells (Turchi, 2006). Therefore it will be necessary to investigate the combined effects of mangiferin and platinum anticancer drugs, as an increased intake of

mangiferin may not necessarily lead to increased action in the chemotherapeutic action of platinum drugs.

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 coupled in a joint effort to cover both the chemical and biochemical aspects of the development of such novel compounds. The Research Unit for Platinum Group Chemistry, plan, synthesize and characterise the chemical aspects and the Department of Biochemistry and Microbiology 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.

The combined anticancer effect of platinum drugs and mangiferin, which have different modes of action, might prove to be very useful in the treatment of cancer. Sarkar *et al.* (2004) has also shown that when cells were pre-treated with mangiferin for 3 hours prior to exposure to cisplatin, an enhancement of approximately 20% in the anticancer action of cisplatin could be observed. Therefore the combined mode of action of these two treatments needs to be investigated further.

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. If mangiferin, a natural polyphenol found in *Cyclopia* spp., aids in the enhanced anticancer action of some novel platinum containing complexes, a new combination therapy against cancer can be proposed. Furthermore, the use of traditional herbal medicines in these treatments might render it more acceptable to many South Africans. Hence, the development and screening of novel anti-tumour drugs and the discovery of the effects that mangiferin might have on the anti-cancer action of these drugs is imperative.

1.2 CANCER

1.2.1 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) (Bosch *et al*. 2002 and Garland, 2003).

Cancer of the cervix is common 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 (Bosch *et al*. 2002 and Garland, 2003).

The cause of cervical cancer is unknown. Infection with two types of human papilloma virus (HPV), which is 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). 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 (Bosch *et al*. 2002 and Garland, 2003).

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.

1.2.2 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. Breast cancer is the second most common type of cancer and the fifth 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 (Tryggvadottir *et al*. 2003 and Rogozinska-Szczepka *et al*. 2004).

.

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. 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). 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 (Tryggvadottir *et al.* 2003 and Rogozinska-Szczepka *et al*. 2004).

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 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 (Rogozinska-Szczepka *et al*. 2004).

1.2.3 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 (Venook, 2005).

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.

1.3 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).

1.3.1 Platinum anticancer agents

1.3.1.1 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).

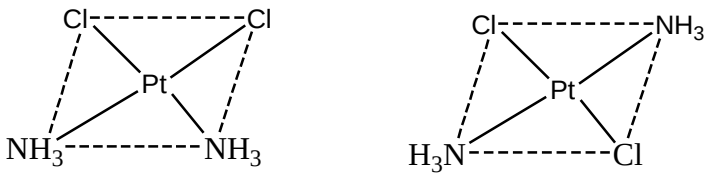
(ii) Ligand exchange rate

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: H_2O <NH_3 ~ amines < Cl^- ~ Br^- < SCN^- ~ I^- ~ NO_2^- < $SC(NH_2)_2$ < thioethers < 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 almost without exception found in a square planar symmetry. It can exist in either a *cis* or *trans* complex form.



cis *trans*

The anions are normally the "leaving groups" which implies that they can readily be replaced. Exchange of bidentate anions, like dicarboxylate ions, are slower than e.g. oxalate and malonate, like Cl^-, as a result of an entropy contribution to their stability. Ligand exchange occurs via two mechanisms; firstly by an S_N2 mechanism, i.e. through the formation of a five coordinated activated intermediate species (either square-pyramidal or trigonal bipyramidal) or, secondly, by an S_N1 mechanism, i.e. through bond-breaking and thus the formation of a three coordinated intermediate species. The first mechanism (S_N2) is the most likely to occur since it is normally energetically easier. Complexation of the Pt(II) with ligands which are bulky in the vicinity of the donor atom can avoid the formation of a five-coordinated species and thus force the mechanism to an S_N1 mechanism. This will lead to slower ligand exchange. 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 and Tobe, 1972).

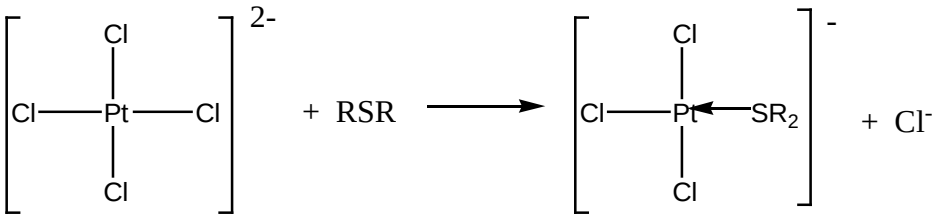
(iv) Hydrophobicity

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).

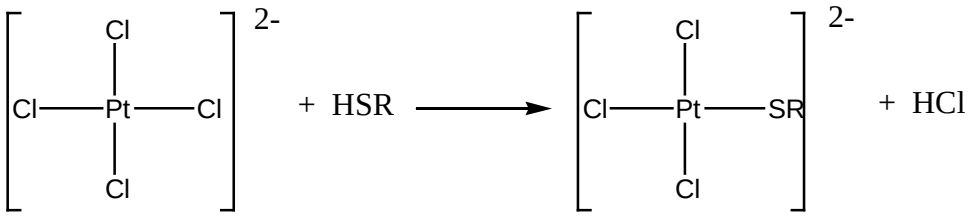
(v) S donor ligands

The interaction, with the anticancer agent, of 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 prevailing 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 a thiolate ion RS^-.

Thioethers react as follows with Pt(II) compounds:

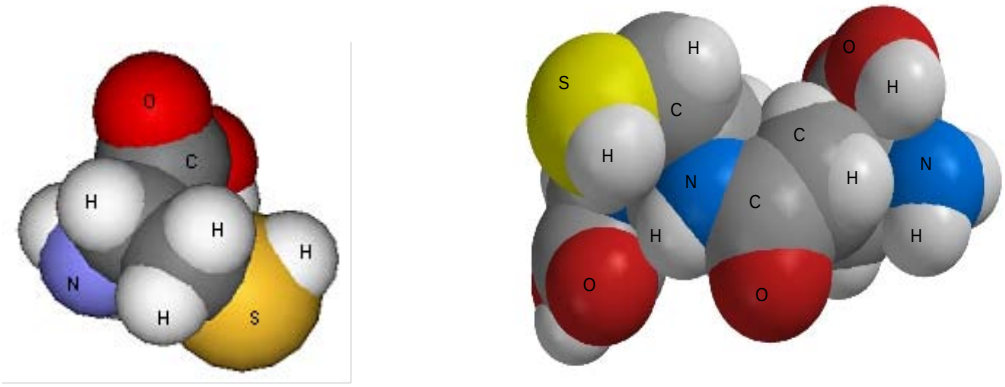


namely through a neutral sulphur. The reaction is however reversible. Thiols react as follows with Pt(II) compounds:



The rate of the thiol reaction is much slower than that of the thioether (like methionine) 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

trans to it, except by another ¯SR group, thus reducing anticancer action. 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 *et al*. 1998 and Reedijk, 1999).



A B

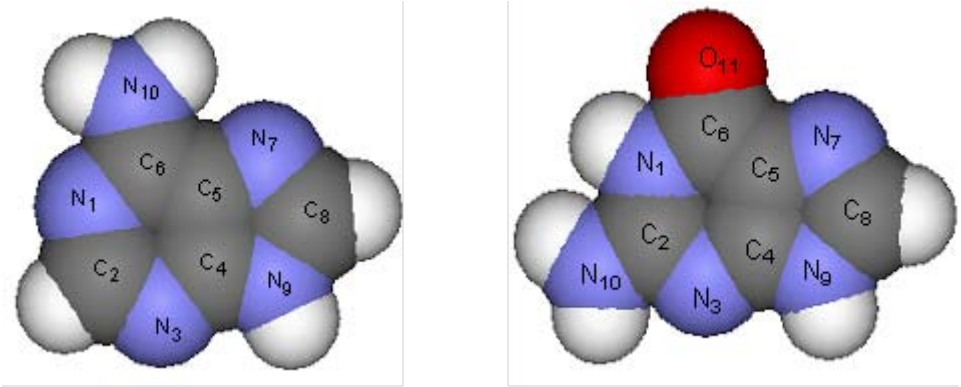
Figure 1.1: The space-filling models of two sulfur containing biomolecules (A) Cysteine and (B) Glutathione.

(vi) Biocompatibility

The platinum compounds should have significant solubility in body fluid. Solubility should conveniently be between 10 and 100 μM (Los *et al*. 1996).

(vii) Mode of bonding

In general, the *cis*-compounds are the most effective anticancer agents, e.g. cisplatin is much better than transplatin. It is interpreted that the *cis* complex can form intramolecular adducts with N atoms in the helix much more readily than the *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. 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.



A B

Figure 1.2: The space-filling models of two important nucleotide bases (A) Adenine and (B) Guanine.

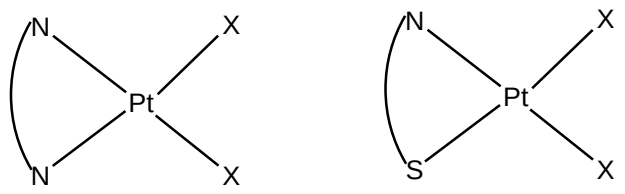
The result is that the former acts as a stronger ligand towards Pt(II) than the latter. For example, the guanine N7 atom can replace a thioethereal sulfur atom bonded to platinum, whereas that of adenine cannot (Jamieson and Lippard, 1999).

(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).

1.3.1.2 <u>Novel complexes</u>

In the design of novel anticancer agents all the above factors had to be considered. It was decided by The Research Unit for Platinum Group Chemistry 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.



X=anion

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.

1.3.1.3 <u>Important biochemical aspects</u>

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 undergoing 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 important research fields. Other cisplatin analogues include oxaliplatin, nedaplatin, loboplatin, ZD0473, oxoplatin and Se-Pt conjugate (Figure 1.3) (Sadler and Guo, 1998 and Wozniak and Blasiak, 2002).

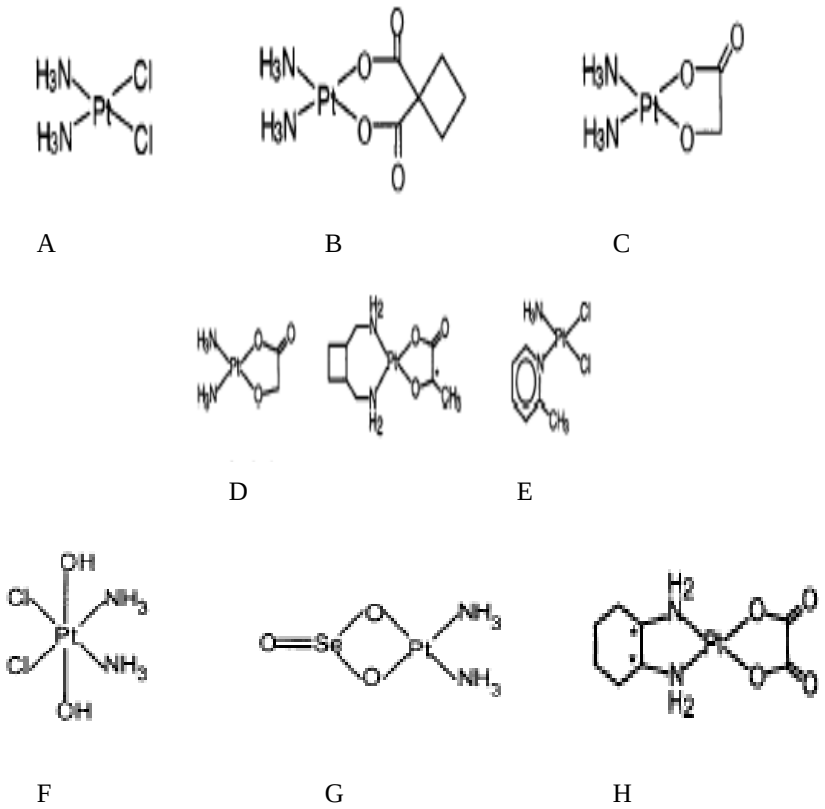


Figure 1.3: The three injectable diammine compounds that have been approved for clinical use; (A) Cisplatin, (B) Carboplatin, (C) 254-S (nedaplatin) (Sadler and Guo, 1998) and the structures of the cisplatin analogues; (D) lobaplatin, (E) ZD0473, (F) oxoplatin, (G) Se-Pt conjugate and (H) oxaliplatin (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).

1.3.2 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).

1.4 CISPLATIN

Cisplatin belongs to the group of medicines known as alkylating agents 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. 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. Single Agent Therapy may include: 2 to 3 mg/kg every 3 to 4 weeks, 20 mg/m^2/day for 5 days every 3 to 4 weeks, 100-120 mg/m^2 every 3 to 4 weeks or 80 mg/m^2 (24 hour infusion) every 3 to 4 weeks (Lidor *et al*. 1993).

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). 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 NH_2-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 Bcl-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. (Gonzalez *et al*. 2001). The caspases most often coupled with programmed cell death are caspases 2, 3, 6, 7, 8, 9 and 10. Caspase cascades contain upstream (initiator) caspases (caspase 2, 8 and 10), which are activated by death receptor signalosomes; caspase 9, which is activated by the mitochondrial cytochrome *c*/Apaf-1 derived apoptosome and downstream (effector) caspases (caspase 3, 6 and 7) that cleave the proteins involved in apoptosis. Caspase 2, 8 and 10 are activated by death receptor signalosomes (death-inducing signaling complex (DISC)). In addition caspases 8 or 10 activates pro-apoptotic factors such as BID that induce the release of cytochrome *c* from the mitochondria or activates caspase-3, 6 or 7 directly. The mitochondrial release of cytochrome *c* activates Caspase 9 and the cytosolic cytochrome *c* binds and induces oligomerization of apoptotic protease activating factor 1 (APAF-1) followed by recruitment of procaspase-9 to form an apoptosome. Apoptosome-associated procaspase-9 self-activates and then activates the downstream

caspase 3 and/ or 7. These downstream executioner caspases (3, 6, and 7) in turn cleave numerous proteins, enzymes and signalling molecules (Khosravi-Far and Esposti, 2004 and Wajant, 2003).

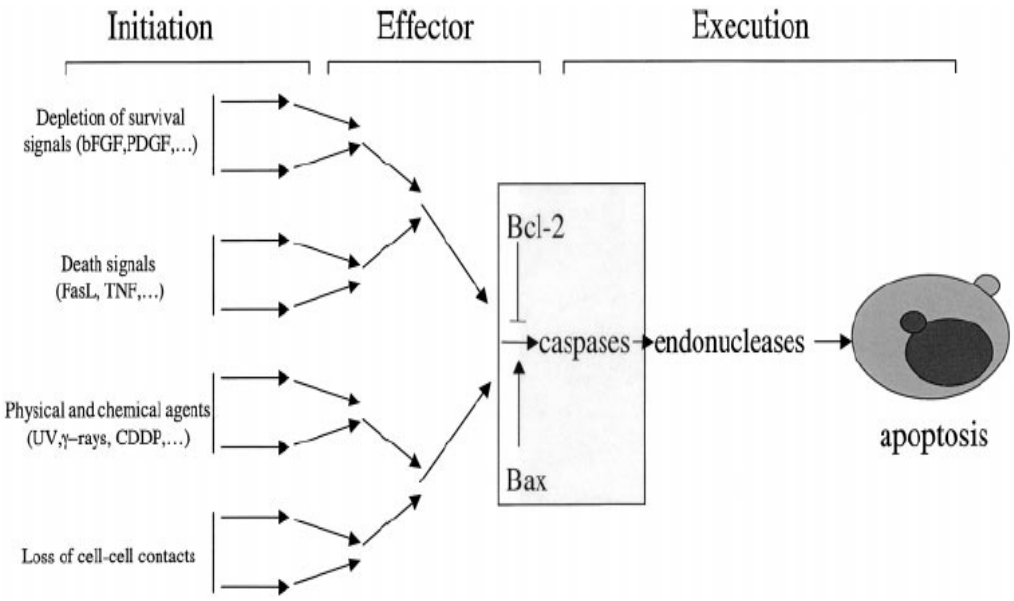


Figure 1.4: Scheme of the converging pathways leading to apoptosis in mammalian cells. A great variety of stimuli, such as depletion of survival signals, death signals, physical and chemical agents, and loss of cell-cell contacts, can initiate apoptosis through numerous different means. The effector 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).

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. Cisplatin also binds to plasma and cellular proteins which is responsible for the difficulty in clearing it from the circulation and tissue. It binds irreversibly to sulfhydryl groups of low- and high-molecular- weight molecules, which correlates with a fall in the concentration of sulfhydryl moieties in the kidney, especially in the mitochondrial and cytosolic fractions. This causes the inhibition of a number of sulfhydryl-containing enzymes (including ATPases, thymidylate synthetase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, γ-glutamylcysteine synthetase, and ribonucleotide reductase). The depletion of sulfhydryl groups, which include glutathione, may alter the redox state and contribute to cisplatin-induced cytotoxicity. Thus the toxicity of cisplatin may be due to a combination of factors that include, but may not be restricted to, DNA cross-linking, inhibition of enzymatic activity, and depletion of the antioxidant pool (Jordan and Carmo-Fonseca, 2000 and Sheihk-Hamad, 2008).

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:

$$Pt(NH_3)_2Cl_2 + H_2O \rightarrow [Pt(NH_3)_2Cl(H_2O)]^+ + Cl^-$$
$$[Pt(NH_3)_2Cl(H_2O)]^+ + H_2O \rightarrow [Pt(NH_3)_2(H_2O)_2]^{2+} + Cl^-$$

(Jordan and Carmo-Fonseca, 2000 and Helm and States, 2009).

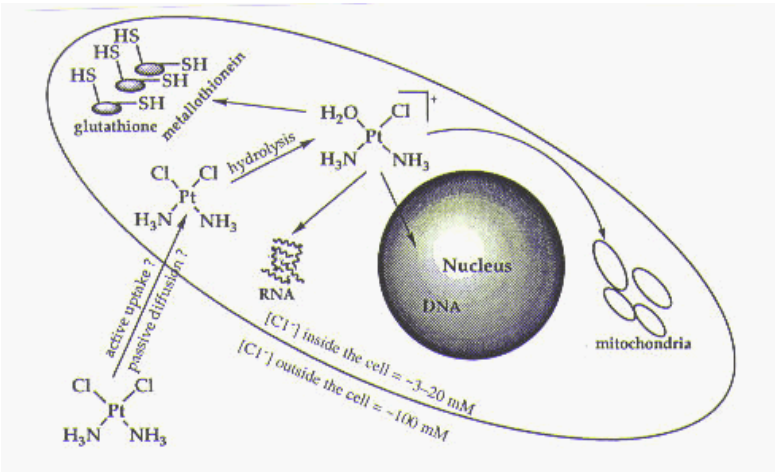


Figure 1.5: The cellular uptake of cisplatin and its targets (http://chemcases.com/cisplat/)

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 sulfur-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 *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) (Perez, 1998 and Helm and States, 2009).

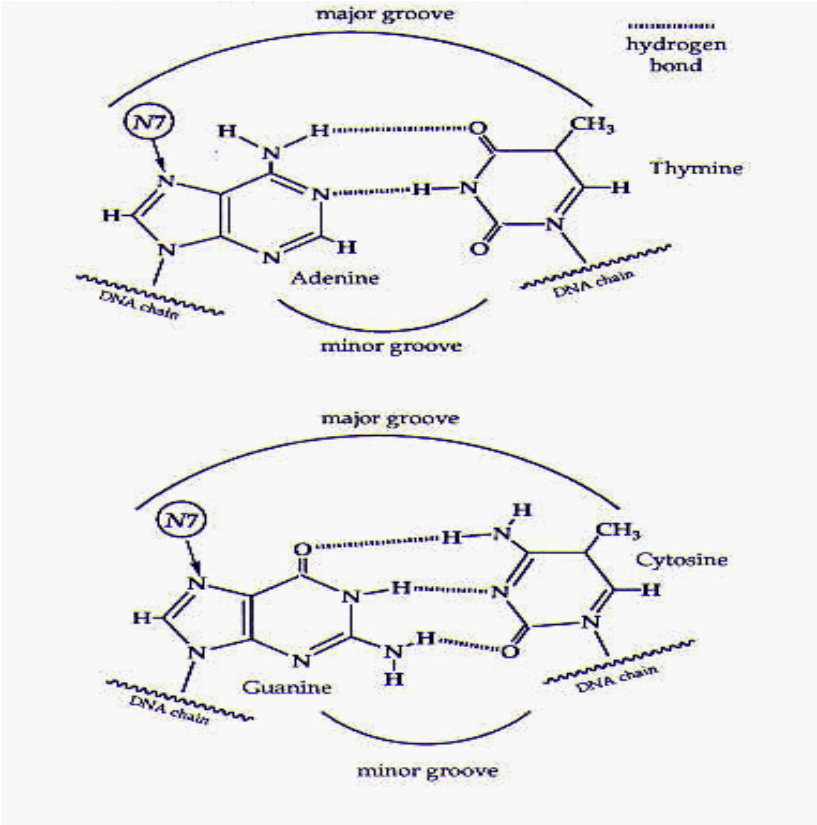
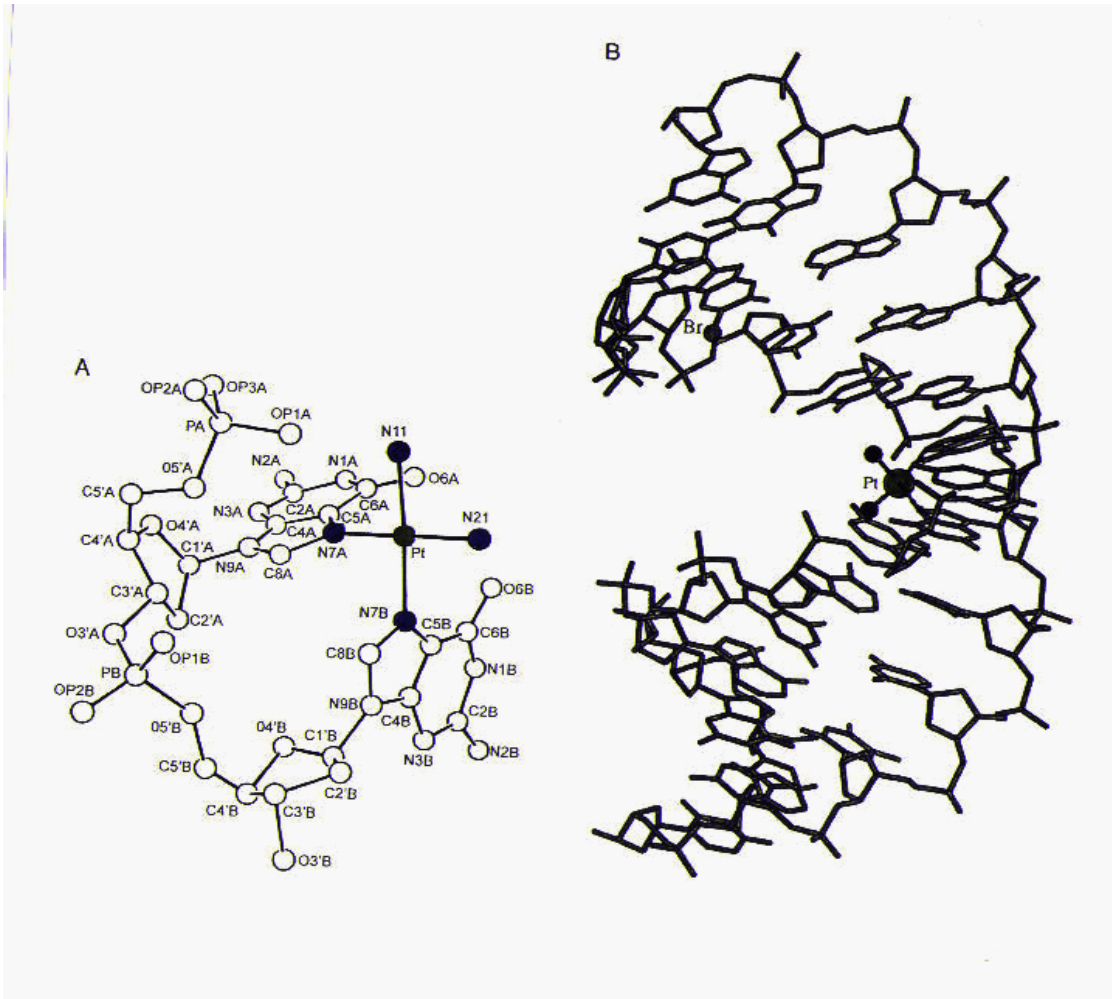


Figure 1.6: The DNA base pairs. Cisplatin coordinates to the N7 atoms of the purine (guanine and adenine) bases (http://chemcases.com/cisplat/).

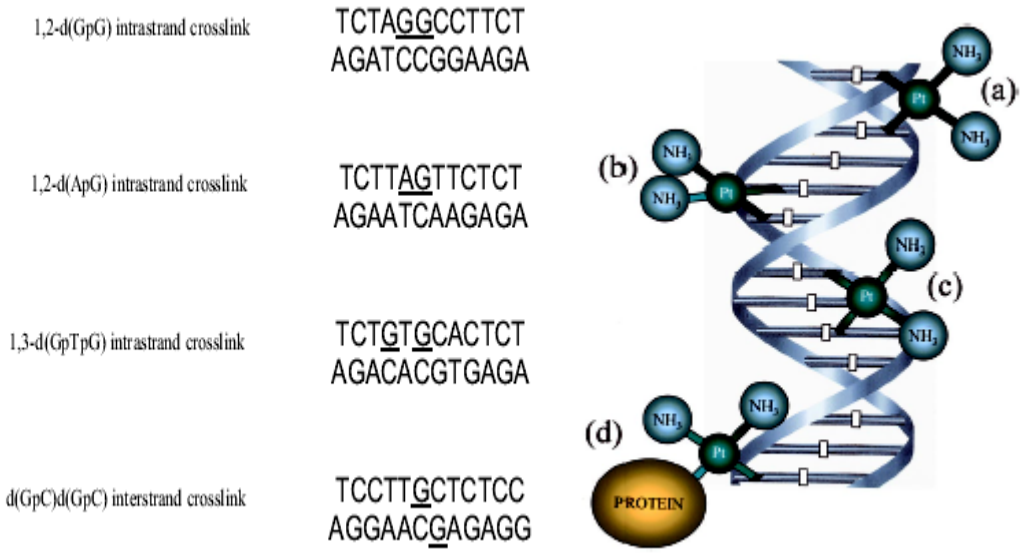
Numerous types of cisplatin: DNA coordination complexes, or adducts, may be formed. The most significant of these appear to be the ones in which the two chlorine ligands of cisplatin are replaced by purine nitrogen atoms on adjacent bases on the same strand of DNA; these complexes are referred to as 1,2-intrastrand adducts. The purine bases most frequently involved in these adducts are guanines; however, adducts involving one guanine and one adenine is also found. The formation of these adducts causes the purines to become de-stacked and the DNA helix to become kinked (Figure 1.7) (Petit *et al*. 2003 and Voland *et al*. 2006).



A B

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) (Wozniak and Blasiak, 2002; Gonzalez *et al*. 2001 and Siddik, 2003).



A B

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).

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. (Siddik, 2003; Scheeff *et al*. 1999).

Trans-DDP is less 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 cisplatin and *trans*-DDP on DNA replication were studied both *in vitro* and *in vivo*. *In vitro* studies on 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. 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. 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 (Scheeff *et al*. 1999).

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 *in vitro*. *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 (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) (Huang *et al.* 1994).

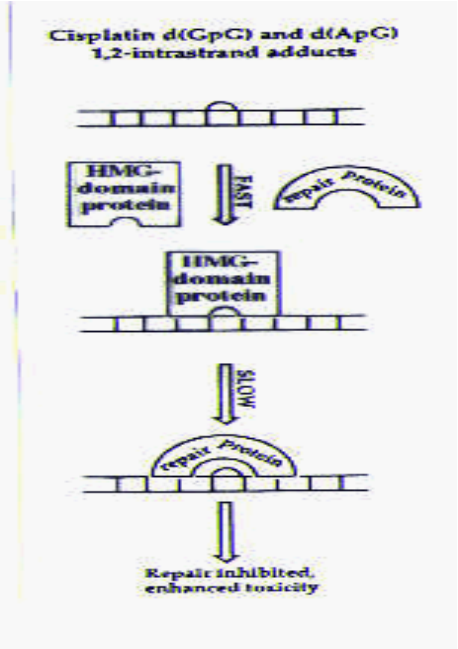


Figure 1.9: Model for the inhibition of cisplatin adduct repair in the presence of HMG-domain proteins (http://chemcases.com/cisplat/).

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.

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 (Ozturk *et al*. 2005).

(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. Peripheral sensory neuropathy develops in the majority of patients treated with cumulative doses of cisplatin higher than 300 mg/m^2. 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 renal 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. 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 (www.chemocare.com).

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 signalling pathways that activate the variety of proposed mechanisms of resistance are largely unknown (Min *et al*. 2004).

Platinum resistance mechanisms can be grouped into two main groups. The first is those that limit the formation of cytotoxic platinum-DNA adducts and the second those that prevent cell death from occurring after platinum-DNA adduct formation. Group I includes decreased drug accumulation and increased drug inactivation by cellular protein and non-protein thiols. Group II includes increased platinum-DNA adduct repair and increased platinum-DNA damage tolerance. Multidrug resistance protein (MRP) is a member of a family of transport proteins that facilitates the transport of a variety of glutathione-coupled and unmodified drugs out of cells. However, the over-expression of MRP alone does not confer resistance. (Helm and States, 2009).

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).

Mechanism	**Individual process**
Cell kinetic resistance	Tumour growth
Pharmacokinetic resistance	Poor absorption Excessive metabolism Poor penetration to certain sites Blood supply of the tumour Drug diffusion
Cellular drug resistance	Increased drug efflux Decreased drug uptake Sequestration of drugs Alterations in drug targets Activation of detoxifying systems Increased repair of drug-induced DNA damage Blocked apoptosis Disruption in signalling pathways Alterations of factors involved in cell cycle regulation

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 and Helm and States, 2009).

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 10mM. 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. 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. This means that cisplatin-resistant cells usually express an improved radical scavenging system, such as GSH and the GS-X pump (Chao, 1996 and 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 and Helm and States, 2009).

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, at a ratio of 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. Exposure of cell lines to cisplatin often has no effect on MT. However, cell lines exposed to heavy metals show increases in MT and become cross-resistant to cisplatin (Chao, 1996; Perez, 1998 and Helm and States, 2009).

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 by Chao (1996) 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 and Helm and States, 2009).

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

Although cisplatin has been used since 1978 it has many disadvantages and research is ongoing towards improved anticancer agents. In order to understand the reasons for its very moderate performance it is necessary to investigate its chemical and structural properties. Very recently an article appeared in Inorganic Chemistry which stated that the unstable tetraaqua Pt(II) complex consists of a distorted pentacoordinated structure in perchloric acid solution with four equidistant oxygen atoms of H_2O in the xy plane and a longer distant oxygen atom on the z axis. It was also stated that the cis-diamminediaquaplatinum(II) ion is very similar. This sheds some light on the reactivity of cisplatin. It seems to suggest that the Pt(II), when surrounded by such small donors, is very “open” towards attack for formation of relatively low activation energies for ligand exchange (Galilehvand and Laffin, 2008).

This provides useful information for the search for 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. 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 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 (Atsushi *et al.* 1994; Go and Adjei, 1999 and Katsuyuki and Keitara, 2007).

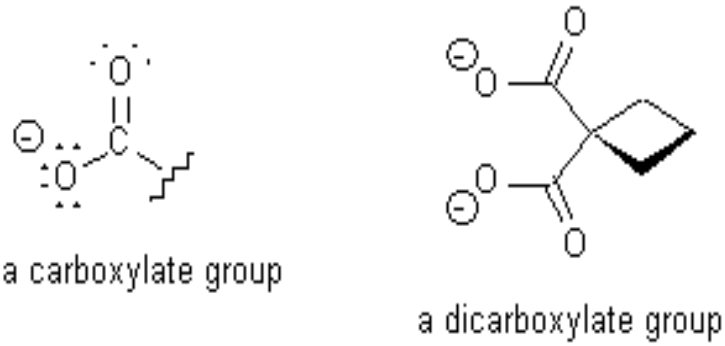


Figure 1.10: A carboxylate and dicarboxylate group. The dicarboxylate group is a bidentate ligand; hence it can bind a metal ion in two places (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm).

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 (Atsushi *et al.* 1994; Go and Adjei, 1999 and Katsuyuki and Keitara, 2007). Unfortunately tumours resistant to carboplatin are becoming more common, and the cause of drug resistance is unclear.

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 (Ibrahim *et al*. 2004 and Helberg *et al*. 2009). Oxaliplatin is active in the treatment of colorectal cancers, while cisplatin and carboplatin are inactive (Hector *et al*. 2001). Oxaliplatin disrupts DNA replication and transcription by the formation of intrastrand DNA adducts, but the downstream molecular events underlying the cytotoxic effects of this chemotherapeutic agent have not been well characterised. Arango *et al.* (2004) showed that exposure of proliferating colorectal cancer cells to oxaliplatin induces a G2/M phase arrest in the DNA cell cycle and a molecular cascade of events consistent with an intrinsic mechanism of apoptosis. They also discovered that the apoptotic cascade initiated by oxaliplatin is characterised by translocation of Bax to the mitochondria and cytochrome *c* release into the cytosol. Oxaliplatin treatment resulted in caspase 3 activation and oxaliplatin-induced apoptosis was counteracted by inhibition of caspase activity, but was independent of Fas/FasL association (Arango *et al.* 2004).

In addition to second-generation cisplatin analogues, several third-generation drugs have been synthesized and tested. One broad class of these drugs, the amine/ammine 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 (Atsushi *et al.* 1994; Go and Adjei, 1997 and Katsuyuki and Keitara, 2007). 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).

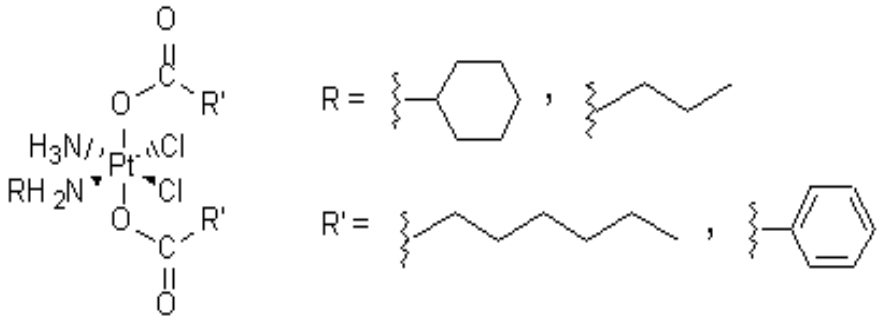


Figure 1.11: The general structure of amine/ ammine platinum (IV) dicarboxylates. Many different R and R' groups were tried; two examples for each are shown here (http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm).

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 *in vitro* and *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 *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 *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 clinical trials in 30 years (Roberts *et al*. 1999).

Among the signal transducer and activator of transcription (STAT) proteins, Stat3 activity is frequently up-regulated in many human tumours. Thus, targeting Stat3 as novel cancer therapeutics has become an important research topic. Turkson *et al.* (2004) reports that certain platinum-containing compounds disrupt Stat3 signalling and suppress its biological functions. They showed that the novel platinum (IV) compound, CPA7 ($Pt(NH_3)_2Cl_3(NO_2)$), blocks Stat3 activity *in vitro* at low micromolar concentrations. In malignant cells that harbor constitutively activated Stat3, CPA7 inhibits cell growth and induces apoptosis in a manner that reflects the decrease of persistent Stat3 activity. Cells that do not contain persistent Stat3 activity are marginally affected or are not affected by these compounds at all. Thus, the modulation of oncogenic signal transduction pathways, such as Stat3, may be one of the key molecular mechanisms for the antitumour effects of platinum (IV)–containing complexes. CPA7 interferes with Stat3 and disrupts its ability to bind to DNA *in vitro*. (Turkson *et al.* 2004)

1.5 MANGIFERIN

As stated earlier, combination therapy of Pt(II) anticancer agents with other molecules, having similar properties, was shown to have synergistic effects. Phenolic antioxidants have been found to exhibit anti-inflammatory, anticarcinogenic, and antidiabetic activities in animals. Humans consume these antioxidants from dietary sources, either as natural components or as synthetic food additives. This broad spectrum of the function of phenolic antioxidants suggests that there may be multiple targets through which they interfere with various cellular functions and protect against pathological lesions such as cancer and inflammatory diseases. There is an increasing interest in the therapeutic use of antioxidants to prevent tissue damage induced by overproduction of reactive oxygen intermediates (ROI), by reducing free radical

formation or by scavenging or promoting the breakdown of these species. Recently, the polyphenol mangiferin, a C-glucosylxanthone, specifically 1,3,6,7-hydroxyxanthone-C2-β-D-glucoside (figure 1.12), has attracted considerable interest in view of its numerous pharmacological activities, which includes antitumour, antiviral, antidiabetic, anti bone resorption, and antioxidant activity (Sarkar *et al*. 2004). Mangiferin is a major component of Honeybush tea, prepared from Cyclopia *spp*. (Marnewick *et a*l. 2000).

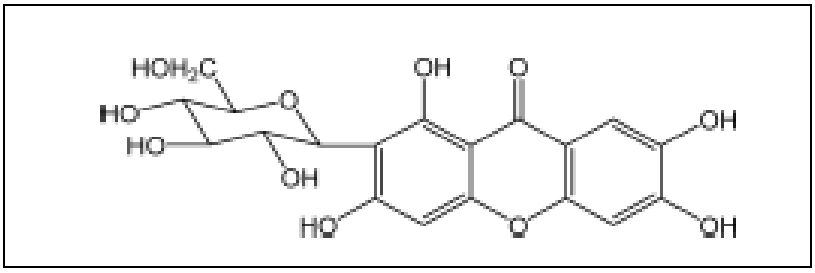


Figure 1.12 The structure of mangiferin (1,3,6,7-hydroxyxanthone-C2-β-D-glucoside) (Jung *et al*., 2009).

Honeybush (*Cyclopia spp*.) is indigenous to the cape of South Africa and is used to make a beverage and a medicinal tea. It has become internationally known as a substitute for ordinary tea (*Camellia sinensis*) and with the dramatic growth in the use of honeybush during the past years, export of honeybush tea products is now a major industry, following up on the success of rooibos. The honeybush plant is easily recognized by its trifoliate leaves, single-flowered inflorescences, and sweetly scented, bright yellow flowers. The most sought-after components for the tea are the leaves and flowers but the stems are also included. Honeybush tea is made as a simple herbal infusion and one of its recognized benefits as a tea substitute is its lack of caffeine, which makes it particularly suited for nighttime consumption and for those who experience nervousness and want to avoid ordinary tea. Hence, it has a reputation as a calming beverage, though it may not have any specific sedative properties. It also has low tannin content. The tea can be consumed daily, or rotated with other teas, such as rooibos and ordinary tea (Marnewick *et al.* 2004).

As carcinogenic, inflammatory, and growth modulatory effects of many chemicals are mediated by NFκB, it has been hypothesized by Sarkar *et al*. (2004) that the

suppression of the NFκB activation pathway accounts for mangiferin activity. They have shown that mangiferin inhibits NFκB by preventing the activation of IKK followed by phosphorylation and degradation of its inhibitory subunit IκBα. They have demonstrated that mangiferin increases glutathione level and inhibits TNF-activated ROI and proved that downregulation of NFκB by mangiferin leads to potentiation of chemotherapeutic agent-mediated apoptosis.

There is a growing interest in substances exhibiting antioxidant properties that are supplied to humans as food components or as specific preventive pharmaceuticals. The plant kingdom offers a broad range of natural antioxidants and phenolics have been shown to provide a defense against oxidative stress from oxidizing agents and free radicals. Many herbal infusions have antioxidative and pharmacological properties related to the presence of phenolic compounds (Osama *et al*. 2009).

1.6 INTRODUCTION TO THE PRESENT STUDY

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. If mangiferin, a natural polyphenol found in *Cyclopia* spp., aids in the enhanced anticancer action of some novel platinum containing complexes, a new combination therapy against cancer can be proposed. Furthermore, the use of traditional herbal medicines in these treatments might render it more acceptable to many South Africans. Hence, the development and screening of novel anti-tumour drugs and the discovery of the effects that mangiferin might have on the anti-cancer action of these drugs is imperative.

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 thesis focuses mainly on the biochemical aspects of this project. This study assessed novel platinum containing compounds for possible enhanced cytotoxicity against cancer cells. In addition, the effects of mangiferin on the anticancer action of these compounds were also investigated.

CHAPTER 2

A COMBINATION OF PLATINUM ANTICANCER DRUGS AND MANGIFERIN CAUSES INCREASED EFFICACY IN CANCER CELL LINES

2.1 INTRODUCTION

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.

The Research Unit for Platinum Group Chemistry plan, synthesize and characterize 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 thesis focuses mainly on the biochemical aspects of this project. This study assessed novel platinum containing compounds for possible enhanced cytotoxicity against cancer cells. The first order screening involved the testing of the anticancer efficacy of approximately 400 novel complexes (synthesized by the Research Unit for Platinum Group Chemistry), by performing the MTT assay on HeLa, HT29 and MCF7 cancer cells after they were treated with 100 and 10µM solutions of these complexes for 48 hours. The results obtained were compared to that of cisplatin (positive control) and accordingly the most active of these complexes were chosen for further studies. Of these novel complexes, approximately 2% showed activity similar or better than cisplatin; approximately 40% showed activity that was less than that of cisplatin and approximately 58% showed very slight or no activity. Since these complexes were screened without any knowledge of their structures, no conclusions relevant to this study could be made from the screening results obtained and therefore these results were not included. These chosen drugs were then tested for increased efficacy when applied in combination with 10µg/ml mangiferin. Additionally, it was

verified whether some of these compounds were capable of inducing resistance in the cell lines tested and whether the combination with mangiferin would affect the degree of resistance induced. The mechanism of action of these drugs, alone, and in combination with mangiferin was also studied.

The complexes selected for this study were $Pt(dach)C_2O_4$, i.e. oxaliplatin (dach=trans-R,R-1,2-diaminocyclohexane);
$Pt(hme)Cl_2$ (Bouwer, 2008) (hme=L-histidinemethylester) – abbreviated as (Yol25);
$Pt(ama)Cl_2$ (Bouwer, 2008) (ama=2-aminobenzylamine) – abbreviated as (Yol29.1);
$Pt(dach)(C_2O_4)NO_2)Cl$ (Louw, 2008) – abbreviated as (Mar 4.1.4);
and $Pt(NH_3)_2Cl_3(NO_2)$ referred to as CPA_7 (Anagnostopoulou *et al*. 2006))
The above complexes were all produced in high degree of purity (>98.5%)

The objectives of this study were: (1) To screen novel platinum containing compounds against selected cancer cell lines and compare their activities to that of cisplatin; (2) To confirm whether mangiferin enhances the cytotoxicity of some of these compounds; (3) To determine the mode of anticancer action of these compounds and; (4) To determine the mode of anticancer action of these compounds when applied in combination with mangiferin; (5) To determine whether these compounds, with and without the addition of mangiferin, cause the development of resistant cancer cell lines.

2.2 MATERIALS AND METHODS

2.2.1 Drugs. Cisplatin, oxaliplatin, Yol 25, Yol 29.1, Mar 4.1.4 and CPA7 (described above) were synthesized by the Research Unit for Platinum Group Chemistry at the Nelson Mandela Metropolitan University. The drug solutions were prepared fresh on the day of use by dissolving the required concentration in RPMI1640 (Sigma) containing 10% foetal calf serum (Delta Bioproducts, South Africa) by vortexing (1 minute) followed by sonication (15 minutes). 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 cytotoxicity of different compounds. Mangiferin was purchased from Sigma and the required concentrations were also prepared fresh in RPMI1640 containing 10% foetal calf serum on the day of use.

2.2.2 Cell Lines. Three human cancer cell lines, HeLa (cervical cancer), HT29 (colon cancer) and MCF7 (breast cancer) (Highveld Biological; Johannesburg), were maintained in 10 cm culture dishes (Nunc) at 37ºC in a humidified incubator containing 5% CO_2 in RPMI1640 supplemented with 10% foetal calf serum. The cells were found to be mycoplasma free via screening with the MycoProbe™ Mycoplasma Detection Kit (R&D Systems).

2.2.3 Cytotoxicity and dose response curves. Cytotoxicity screening of approximately 400 novel compounds and dose response curves for selected compounds were performed by using the MTT assay (Sigma). The IC50 values were calculated using the GraphPad Prism4 software package. The reduction of tetrazolium salts is widely accepted as a reliable way to examine cell proliferation. 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 solubilized and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells (Hagopian *et al*. 1999 and Huq *et al*. 2004).

Cells (200µl per well) were seeded in flat-bottom 96 well culture plates (Nunc) at 30 000 cells per milliliter and incubated overnight at 37ºC in a humidified incubator containing 5% CO_2. Cells were allowed to attach and recover for 24 hours before the platinum containing complexes were added to the wells at concentrations of 100 and 10µM (for screening) or 100, 50, 25, 10, 5 and 1µM (for dose response curves), with and without the addition of 10µg/ml mangiferin, and incubated for 48 hours before the MTT assay was performed. A stock solution of MTT was prepared in PBSA (5mg/ml) and further diluted to 0.5mg/ml with RPMI1640 containing 10% foetal calf serum. The medium was removed from the wells and replaced by this MTT solution

(200µL aliquots) before the plates were incubated for another 3 hours. After incubation the MTT solution was removed from the wells and replaced by 200µL DMSO before the plates were analyzed on a Labsystems Multiskan MS Plate Reader at 540nm.

2.2.4 Active caspase 3 levels. Caspases (cysteine aspartic acid-specific proteases) play a key initiator role in the intrinsic apoptotic pathway of mammalian cells. Caspase 3 is one of the key executioners of apoptosis. It is either partially or totally responsible for the proteolytic cleavage of many key proteins. Proteolytic processing of its inactive zymogen into activated p17 and p12 fragments causes its activation. Active caspase 3 levels were determined using the Cleaved Caspase 3 (Asp 175) Alexa Fluor® 488 Antibody (Beckman Coulter). The anti-Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment of activated caspase 3, which results from cleavage adjacent to aspartic acid 175. The antibody does not recognize full length caspase 3 or other cleaved caspases.

Cells were seeded into 10cm cell culture dishes (Nunc) at 1.15×10^5 cells per ml and incubated in a 37ºC incubator supplemented with 5% CO_2. Cells were allowed to attach and recover for 24 hours before 10µM of the platinum containing complexes were added to cells, with and without the addition of 10µg/ml mangiferin, and incubated for 15 hours. The assay was performed, as per kit instructions, and analyzed on a Beckman Coulter FC500 flow cytometer. An appropriate isotypic control antibody was used to ensure that antibody binding was specific.

2.2.5 LDH release. Lactate dehydrogenase is a cytosolic enzyme that catalyses the transformation of pyruvate to lactate. It is released from the cell upon loss of plasma membrane integrity or necrosis. LDH release was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). This kit quantitatively measures LDH in culture supernatants with a 30-minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. The reaction is as follows:

$$NAD^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{Pyruvate} + NADH$$

$$NADH + INT \xrightarrow{\text{Diaphorase}} NAD^+ + \text{formazan (red)}$$

Cells (200µl per well) were seeded in flat-bottom 96 well culture plates (Nunc) at 30 000 cells per milliliter and incubated overnight at 37ºC in a humidified incubator containing 5% CO_2. Cells were allowed to attach and recover for 24 hours before they were incubated for 48 hours with 100µM of the platinum containing complexes, with and without the addition of 10µg/ml mangiferin. The assay was performed as per kit instructions and the results analyzed on a Labsystems Multiskan MS Plate Reader at 492nm.

2.2.6 Trypan blue assay. Trypan blue stains non-viable cells a bright blue colour, since it is able to enter the "leaking" cell membranes of these cells and is not able to enter the intact membranes of viable cells. The trypan blue assay was performed using a standard 0.4% trypan blue solution (Sigma) in phosphate buffered saline without calcium or magnesium. Cells (1ml per well) were seeded in flat-bottom 24 well culture plates (Nunc) at 500 000 cells per milliliter and incubated overnight at 37ºC in a humidified incubator containing 5% CO_2. Cells were allowed to attach and recover for 24 hours before they were exposed to 10µM of the platinum containing complexes, with and without the addition of 10µg/ml mangiferin for 48 hours. The cells were trypsinized and counted with a heamocytometer in a 1:1 dilution of cells in a 0.4% trypan blue solution and the percentage of non-viable cells was calculated.

2.2.7 DNA cell cycle analysis. 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). 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 propidium iodide (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). 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. It is possible to visualise the stages affected, by comparing the area of the peaks (Figure 2.1). A larger peak area indicates that the DNA cycle has been arrested or delayed at that particular stage, since the DNA could not move on to the next phase.

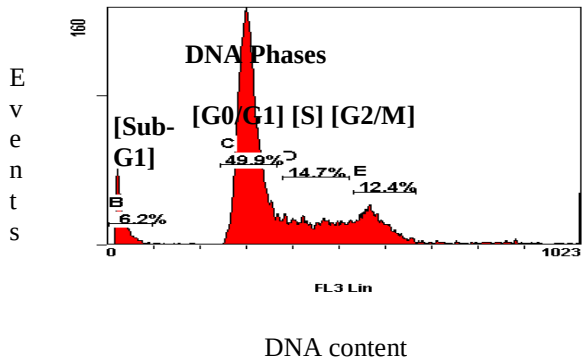


Figure 2.1 A diagram indicating where the different DNA phases are depicted on a histogram obtained with the Beckman-Coulter FC500 flow cytometer. (These results were obtained from HT29 control cells).

Figure 2.1 shows where the different phases of the DNA cycle can be seen on the histograms that will be obtained from the Beckman-Coulter FC500 Flow Cytometer.

The Sub-G1 phase refers evidence for 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 permeabilized cells thus rendering these cells with less DNA (Kwon *et al*. 2005).

DNA cell cycle analysis was performed using the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter). This kit is used in the preparation of intact cells and isolated cell nuclei for the quantitative measurement of cellular DNA content via flow cytometry. This measurement is based upon the ability of PI to bind to double stranded nucleic acids under appropriate staining conditions. PI binds to DNA by intercalating between the bases with little or no sequence preference; however, it also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. PI cannot enter intact membranes and are generally excluded from viable cells, therefore a premeabilisation reagent (in this case sodium azide) is used to allow dye penetration into the nucleus during cell cycle analysis. DNA stained in this manner will emit fluorescence in direct proportion to their DNA content. The flow cytometer measures the fluorescence from each stained cell as it passes through the laser beam. Flow cytometric analysis provides quantitative data and the ability to measure large numbers of cells rapidly.

Cells were seeded into 10cm cell culture dishes (Nunc) at 1.15×10^5 cells per ml and incubated in a 37ºC incubator supplemented with 5% CO_2. Cells were allowed to attach and recover for 24 hours before they were incubated for 48 hours with 10µM of the platinum containing complexes, with and without the addition of 10µg/ml mangiferin. The assay was performed as per kit instructions and the results analyzed on a Beckman Coulter FC500 flow cytometer.

2.2.8 DNA fragmentation assay. DNA fragmentation assays were performed using the Apo-BRDU™ kit (Phoenix Flow Systems). This kit is a two colour TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labelling assay) useful for labeling DNA breaks and total cellular DNA to detect apoptotic cells. DNA in apoptotic cells is broken up by cellular nucleases, whereas DNA in non-apoptotic cells remains largely intact. The DNA fragments cause a multitude of 3'-hydroxyl ends in the DNA. This kit utilizes this feature by labeling the 3'-hydroxyl ends with

bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template independent addition of the 3'-hydroxyl ends of double or single stranded DNA. Non-apoptotic cells do not incorporate such a high amount of Br-dUTP, since they lack these 3-hydroxyl ends, and the Br-dUTP sites are identified by a fluorescein labeled antiBrdU monoclonal antibody.

HT29 cells (5mL aliquots) were seeded into 50ml non-adherent cell culture flasks (Nunc) at a density of 100 000 cells/mL and were incubated for 48 hours with 10µM of the platinum containing complexes, with and without the addition of 10µg/ml mangiferin, before the assay was performed as per kit instructions and the results analyzed on a Beckman Coulter FC500 flow cytometer.

2.2.9 Mitochondrial transmembrane potential assay. Mitochondria produce ATP through oxidative phosphorylation, which involves a series of redox reactions that transfer electrons through multiple protein complexes in the inner mitochondrial membrane. Protons are then pumped out of the mitochondrial matrix, generating the mitochondrial membrane potential ($\Delta\Psi_m$), which is not only harnessed to generate ATP, but is also responsible for mitochondrial Ca^{2+} uptake, metabolite and protein transport, production of reactive oxygen species, and has also been related to the process of apoptosis. Hence, the health and bioenergetic function of the mitochondria depend on its membrane potential. Preservation of the $\Delta\Psi_m$ is essential during normal conditions, and especially, during conditions of stress and disease. Depolarization of the membrane results in a reduction of ATP production and is also thought to precipitate the release of pro-apoptotic factors in some cell systems. Thus, there are good reasons to search for therapeutic agents that decrease $\Delta\Psi_m$ as possible pro-apoptotic drugs, for example, in the case of cancer (Wong and Cortopassi, 2002).

Mitochondrial transmembrane potential studies were performed using the Guava® EasyCyte™ MitoPotential™ Kit (Guava Technologies). This kit is a mix and read multiparametric assay to measure mitochondrial membrane potential and apoptosis. JC-1, a cationic dye that fluoresces either green or orange depending upon mitochondrial membrane potential, is used to evaluate the mitochondrial membrane potential changes. A second dye, 7-Aminoactinomycin (7-AAD) is used to measure

apoptosis. Using both these dyes simultaneously will measure both the mitochondrial membrane potential changes and apoptosis. Since apoptosis has already been measured previously, only JC-1 was used to stain the cells during this experiment. Healthy cells stained with JC-1 will fluoresce mostly orange as JC-aggregrates are formed in the mitochondria due to increases in the dye concentration. As cells become apoptotic, holes form in the mitochondrial membranes, which allow the dye to leak into the cytoplasm where the dye exists as monomers. This will result in the fluorescence of the dye to shift from mostly orange (FL2) to mostly green (FL1). HT29 cells were seeded into 50ml non-adherent cell culture flasks (Nunc) and were incubated for 48 hours with 10µM of the platinum containing complexes, with and without the addition of 10µg/ml mangiferin, before the assay was performed as per kit instructions (excluding the addition of 7-AAD) and the results analyzed on a Beckman Coulter FC500 flow cytometer.

2.2.10 Induction of drug resistance. This section of the work was done in an effort to determine whether the chosen compounds induce resistance in the cell lines (a big draw-back of existing anticancer drugs, such as cisplatin) as well as the effect mangiferin will have on this induction of resistance. These resistant cells were developed according to the method of Godwin *et al*. (1992). HeLa, HT29 and MCF7 cancer cells were exposed intermittently and incrementally to the complexes, with and without the addition of 10µg/ml mangiferin. Table 2.1 illustrates the conditions for inducing resistance in these cells. The cells were maintained as described in section 2.2.2 and subcultured via trypsinization as soon as 80% confluency was reached (monitored microscopically). After trypsinization cells were maintained in RPMI1640 containing 10% foetal calf serum for at least one more subculture, depending on the general appearance of these cells, microscopically, before the next exposure to the treatments. If the cells did not grow optimally during this period or looked unhealthy, an additional subculture and growing period without the addition of the treatments were allowed in an attempt to avoid the untimely demise of these cells. In the event of such demise, the experiment had to be repeated from the beginning on normal control cells, as shown in table 2.1. This procedure typically extended over a period of 12 to 16 weeks. The degree of resistance was determined by performing dose response curves on these cells as described in section 2.2.3.

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

[Cisplatin] (μM)	Time exposed (hours)
10	0.5
10	1.0
10	1.5
100	0.5
100	1.0
100	1.5
100	1.0
100	1.0

2.2.11 NFκB Assay. Members of the Rel/NFκB family of transcription factors form one of the first lines of defense against infectious diseases and cellular stress. They initiate a highly coordinated response in multiple cell types that efficiently counteracts the threat to the health of the organism. Activation of NFκB occurs in response to a wide variety of stimuli, i.e. cytokines, growth factors, physiological, physical and oxidative stress and certain pharmaceutical drugs and chemicals. These stimuli generally target the protein IκB, to which the two major subunits of NFκB, p50 and p65, are bound to form inactive IκB/NFκB complexes. Phosphorylation and ubiquitination of IκB protein releases NFκB, allowing it to translocate into the nucleus as the activated form. The Phospho-NFκB p65 (Ser536) (93H1) Rabbit mAb (Alexa Fluor®488 Conjugate) (Cell Signalling Technology) was used to study the effect of drug resistance, with and without the addition of 10μg/ml mangiferin, on the expression of NFκB. This antibody recognises Phospho-NFκB p65 (Ser536) from human, mouse and rat only when phosphorylated at serine 536. It does not cross-react with the p50 subunit or other related proteins. HT29 cells (5 mL aliquots) were seeded into 50ml non-adherent cell culture flasks (Nunc) at a density of 100 000 cells/mL and were incubated for 48 hours with 10μM of the respective test compounds, with and without the addition of 10μg/ml mangiferin, before the assay was performed as per kit instructions and the results analyzed on a Beckman Coulter FC500 flow cytometer with a minimum of 10 000 events being recorded. Rabbit IgG isotype control was used.

2.2.12 Statistical analysis. Statistical analysis was performed via the Student's t-test. Certain experiments (trypan blue assay and caspase 3 assay) were performed in

duplicate and therefore the average deviation of the values is depicted on the graphs. For flow cytometric analysis a minimum of 10 000 gated events were recorded. IC50 values were determined from dose-response curves using the GraphPad Prism4 software package

2.3 RESULTS

2.3.1 Cell viability

From the approximately 400 novel compounds that were screened for toxicity at 10 and 100µM against HeLa, HT29 and MCF7 cells (results not shown), three were selected for further studies together with oxaliplatin and CPA7 as positive controls. The molecular formulae for these compounds were given in the introduction and for simplicity they will be referred to as Yol 25, Yol 29.1 and Mar 4.1.4.

Dose response curves were obtained for the selected platinum compounds against HeLa, HT29 and MCF7 cells after a 48 hour exposure. IC50 values were calculated from these curves. From table 2.2 it can be seen that the IC50 values for Mar 4.1.4 were lower than those of the positive controls against all three cell lines. The other two selected compounds were not as effective in killing the cancer cells as Mar 4.1.4 but they seemed to be more selective for the breast (MCF7) and cervical (HeLa) cancer cells. The results in table 2.2 further indicate that mangiferin enhances the anticancer activity of the tested platinum containing anticancer drugs, as lower IC50 values were obtained in combination with mangiferin in all samples except for oxaliplatin in MCF7 cells. The approximate concentrations of oxaliplatin and cisplatin in the blood of an average (70kg) male after treatment are 52 and 97µM respectively. Therefore, the decrease in the IC50 values caused by combination treatment may have a very significant effect in a patient. It may also allow that a lower concentration of these drugs be administered, thus potentially decreasing the toxic side effects.

Table 2.2: The IC50 values obtained for the platinum drugs with and without the addition mangiferin.

Compound	Mangiferin added 10µg/ml	Cell line HeLa	HT29	MCF7
Cisplatin	No	10.66	8.15	14.26
	Yes	8.28 (1.3)	5.61 (1.5)	11.48 (1.2)
Oxaliplatin	No	11.92	12.30	6.21
	Yes	6.86 (1.7)	3.63 (3.4)	8.31 (0.75)
Yol 25	No	8.70	21.11	6.79
	Yes	5.89 (1.5)	12.53 (1.7)	4.96 (1.4)
Yol 29.1	No	9.26	12.66	8.31
	Yes	5.95 (1.6)	9.20 (1.4)	6.98 (1.2)
CPA7	No	5.14	9.81	9.29
	Yes	2.93 (1.8)	6.89 (1.4)	3.59 (2.6)
Mar 4.1.4	No	4.81	3.07	5.86
	Yes	2.53 (1.9)	1.94 (1.6)	1.89 (3.1)

Brackets: Fold decrease in IC50 value compared to that of the same compound in the absence of mangiferin.

During apoptosis, the plasma membrane remains intact to prevent "leakage" of cellular content into the surrounding tissue. This prevents inflammation commonly associated with necrosis and is therefore the preferred method of cell death induced by chemotherapy. By measuring trypan blue uptake or LDH release, one can obtain an indication of membrane integrity and therefore the type of cell death. The trypan blue assay (table 2.3), showed a general decrease in the percentage of non-viable cells when treated with mangiferin. Yol 29.1 and CPA7 were the exceptions and showed slight increases in the percentage of non-viable cells in MCF7 and HT29 cells.

The trypan blue results correlated to most of the LDH assay results (figure 2.2), which showed a general decrease in the LDH release in combination with mangiferin. These results might indicate that mangiferin favours cell death as a result of apoptosis, rather than necrosis. Yol 25 was the only sample to show a significant increase in the amount of LDH release in the presence of mangiferin (this occurred in HeLa cells). Interestingly, both Yol 29.1 and CPA7 showed a significant decrease in the amount of LDH release in the presence of mangiferin in the cell lines in which an increase in viable cells were seen from the trypan blue assay (MCF7 and HT29 cells respectively). Yol 25 also showed a significant decrease in LDH release by MCF7 cells in the presence of mangiferin. The fact that contradictory results were obtained

between the trypan blue and LDH assays for some compounds might also be attributed to the incubation times. Some of these compounds are bound to act faster or slower than others. The phenomenon of secondary necrosis, seen in *in vitro* studies but not *in vivo*, may also play a role here. Interestingly, mangiferin, when applied alone, caused an increase in the percentage of LDH released. Further studies were required to investigate the effect of mangiferin in the mode of cell death induced by the platinum compounds.

Table 2.3: The percentage of non-viable cells (according to the trypan blue assay) with and without the addition of 10µg/ml mangiferin.

Treatment (10µM)	**Mangiferin added (10µg/ml)**	**HeLa**	**HT29**	**MCF7**
Oxaliplatin	No	80.7±1.1	55.6±8.3	54.2±4.2
	Yes	45.7±2.6 (1.8)	42.6±3.4 (1.3)	33.5±5.7 (1.6)
Yol 25	No	63.7±4.5	36.1±11.3	68.8±10.4
	Yes	51.5±1.9 (1.2)	29.0±3.0 (1.2)	56.6±8.7 (1.2)
Yol 29.1	No	81.6±0.3	57.1±9.8	47.9±6.3
	Yes	65.7±3.1 (1.2)	72.3±7.4 (0.8)	65.8±0.5 (0.7)
CPA7	No	91.4±0.5	80.9±1.0	80.0±5.0
	Yes	83.1±0.1 (1.1)	85.4±1.1 (0.9)	84.3±0.1 (0.9)
Mar 4.1.4	No	80.7±1.1	68.7±1.3	62.1±7.9
	Yes	63.8±5.2 (1.3)	62.6±5.9 (1.1)	68.2±13.3 (0.9)

Values represent mean ± average deviation, n=2; Brackets = fold decrease due to addition of mangiferin

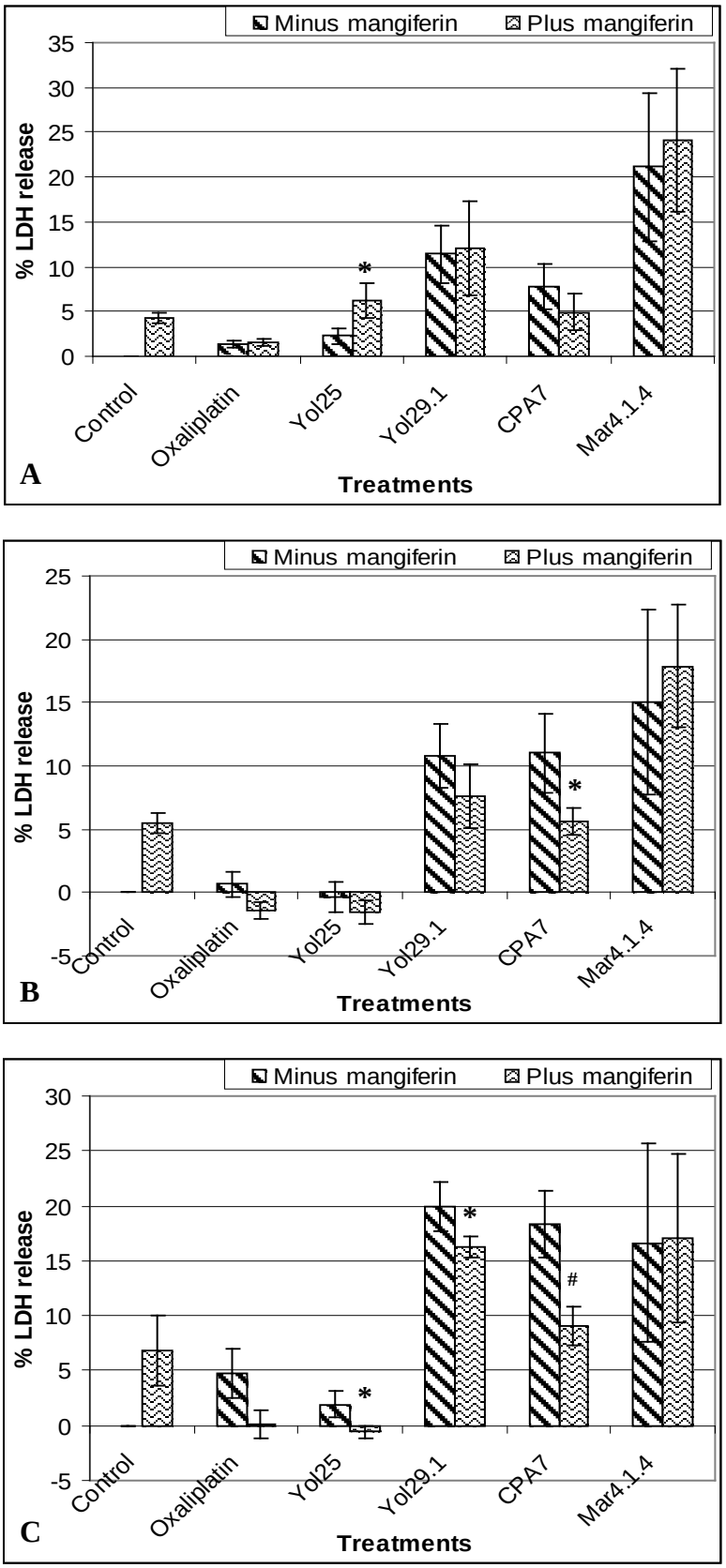


Figure 2.2: The percentage LDH released with and without the addition of 10µg/ml mangiferin (values represent mean ± SEM; n = 8). (A) HeLa; (B) HT29; and (C) MCF7 cell line. * $p \leq 0.05$; # $p < 0.01$ as compared to corresponding treatment without mangiferin.

2.3.2 Cell cycle analysis

The results obtained from the DNA cell cycle analysis of HT29 cells (figure 2.3A) indicates that mangiferin alone had no effect on the cell cycle of HT29 cells, but increased the amount of cells in the sub-G1 phase (apoptotic cells) from 2.6% to 6.2%. In the absence of mangiferin, oxaliplatin did not have any pronounced effect except for an increase in the sub-G1 peak to 6.4%. Yol 25 and Yol 29.1 caused a slight increase in the G1/G0 peak, whereas CPA7 and Mar 4.1.4 both caused an accumulation of the cells in S phase and an increase in the sub-G1 peak. CPA7 clearly had the most pronounced effect on the cell cycle.

It was found that mangiferin causes an *earlier*, enhanced delay in the S-phase in combination with oxaliplatin and Yol 25 and a *later*, enhanced delay in the S-phase in combination with CPA7. It seems that Yol 29.1 and Mar 4.1.4 act similarly to CPA7, but a late S or early G2/M phase delay occur, as opposed to a strictly S phase delay seen in CPA7. The percentage apoptosis (cells in the sub-G1 peak) was also increased in combination with mangiferin. Cell cycle analysis was also performed on MCF7 and HeLa cells (Figure 2.3B and 2.3C respectively)

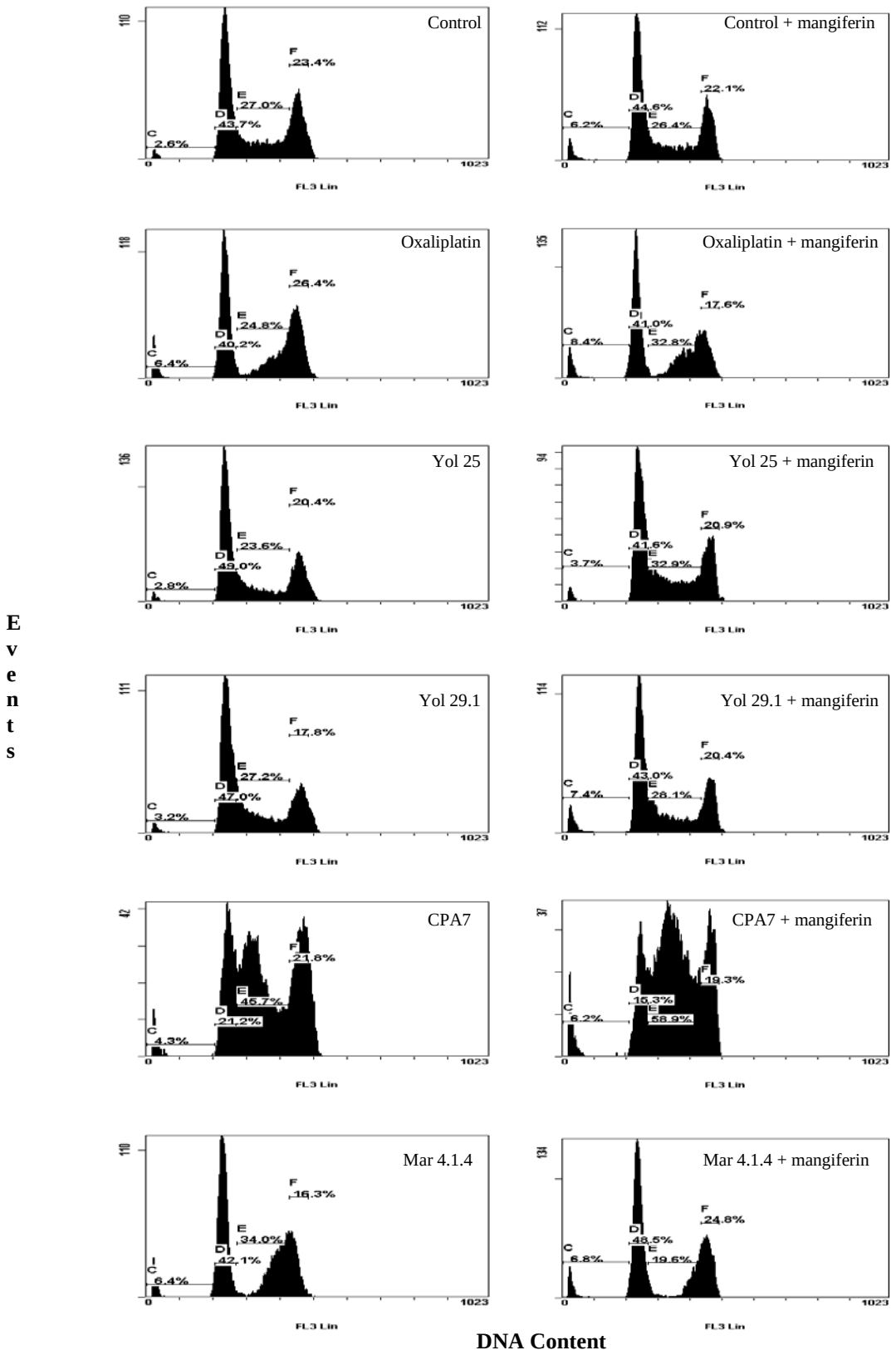


Figure 2.3A: DNA cell cycle analysis with and without addition of 10µg/ml mangiferin on HT29 cells. Regions indicate cell cycle phases: C = Sub-G1; D = G0/G1; E = S and F = G2/M phase.

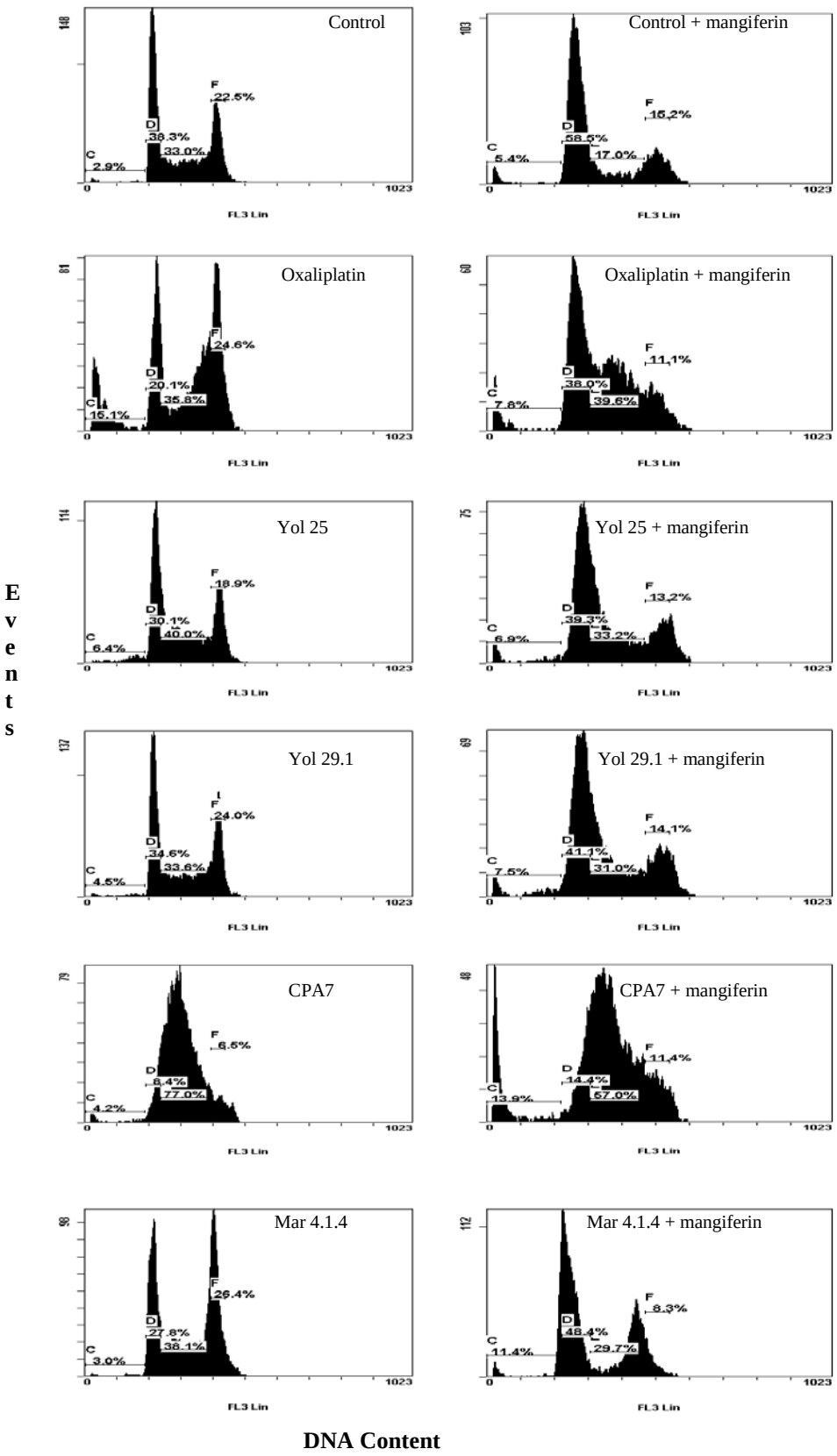


Figure 2.3B: DNA cell cycle analysis with and without addition of 10µg/ml mangiferin on MCF7 cells. Regions indicate cell cycle phases: C = Sub-G1; D = G0/G1; E = S and F = G2/M phase.

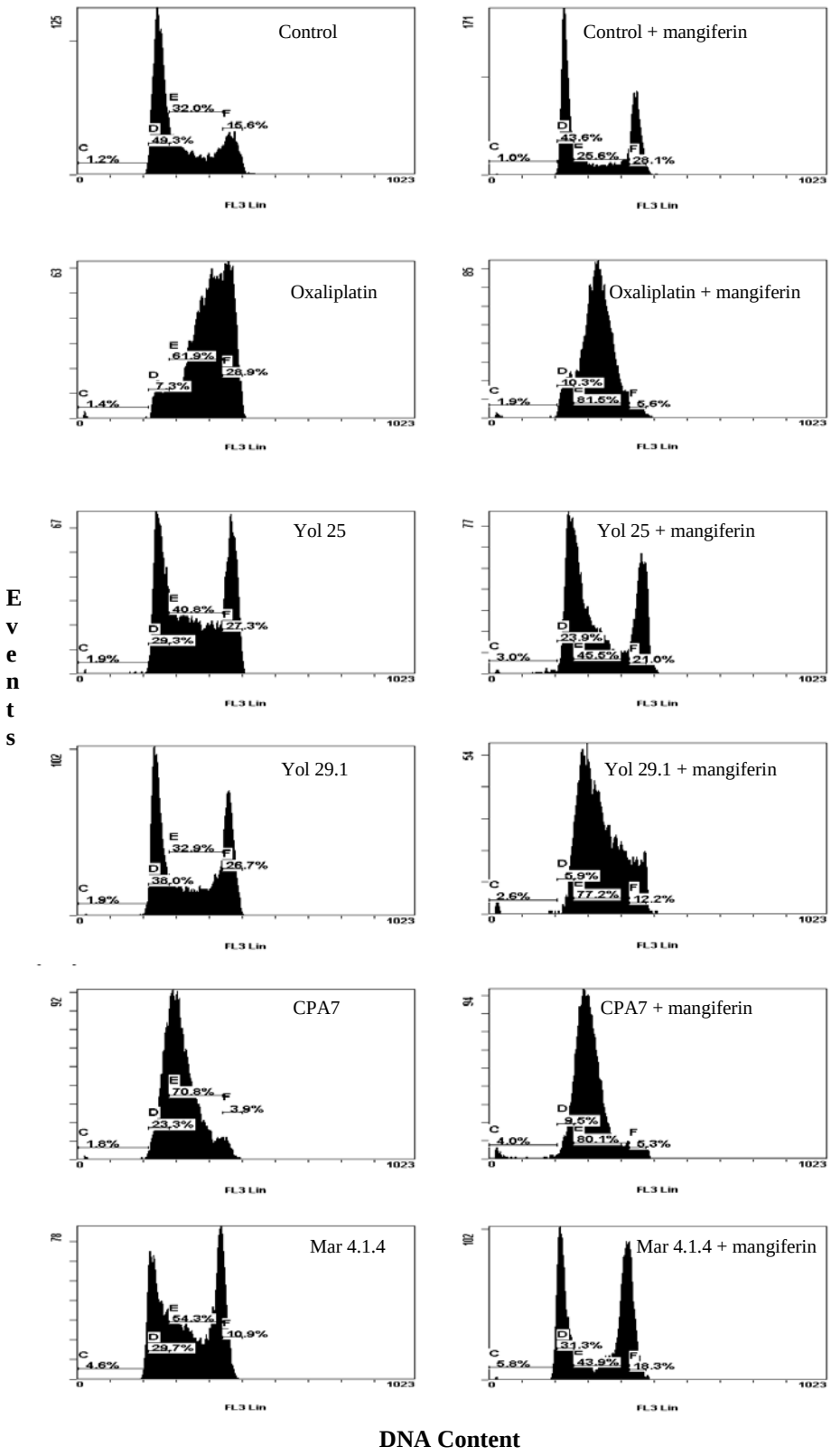


Figure 2.3C: DNA cell cycle analysis with and without addition of 10µg/ml mangiferin on HeLa cells. Regions indicate cell cycle phases: C = Sub-G1; D = G0/G1; E = S and F = G2/M phase.

Figure 2.3B shows that mangiferin alone causes a G0/G1 or very early S phase delay as well as increased apoptotic cells (sub-G1 phase) in MCF7 cells. Oxaliplatin and Mar 4.1.4 showed a late S/ early G2/M phase delay as well as increased apoptosis for oxaliplatin, from 2.9% to 15.1%. Yol 25 and CPA7 showed an S-phase delay with increased cells in the sub-G1 phase and Yol 29.1 showed only a slight increase in the sub-G1 phase with no other effects.

In the presence of mangiferin, oxaliplatin, Yol 25, Yol 29.1 and Mar 4.1.4 showed an earlier, enhanced S-phase/ early G0/G1 phase delay, in MCF7 cells, with increased apoptosis for Yol 25, Yol 29.1 and Mar 4.1.4 (Figure 2.3B). Oxaliplatin showed a decrease in cells in the sub-G1 phase. CPA7 showed an increase in the sub-G1 phase, but the effect on the cell cycle cannot be differentiated because of the already pronounced S phase arrest seen even in the absence of mangiferin.

Figure 2.3C shows that mangiferin alone caused a delay in the G2/M phase in HeLa cells. There was no effect on the cells in the sub-G1 phase. Oxaliplatin and Yol 25 caused an S/ early G2/M phase delay, Yol 29.1 delayed the cell cycle in the G2/M phase whereas CPA7 and Mar 4.1.4 caused an early S phase delay. There was a small increase in the amount of cells in the sub-G1 phase for all of the complexes, with a more significant increase occurring in the cells treated with Mar 4.1.4. In the presence of mangiferin, oxaliplatin, CPA7 and Yol 25 treated MCF7 cells showed an earlier, enhanced S-phase delay with an increase the amount of cells in the sub-G1 phase. Yol 29.1 showed an earlier, delay in the S-phase. There was also an increase in the sub-G1 peak. Mar 4.1.4 showed G2/M phase delay but this might even have been a later, enhanced S-phase delay as well as more cells in the sub-G1 phase. Unfortunately, it cannot be said with absolute certainty during which phase the cell cycle is affected, because of a certain amount of overlap between the peaks of different phases. Therefore, the broader terms such as early S/ late G0/G1 phase etc. are being used. It was attempted to analyze the data with the Multicycle software package, but since these complexes induced such dramatic effects on the DNA cell cycle, the data could not be analyzed accurately with this software.

Since similar results were obtained in all the cell lines, and oxaliplatin is currently being used clinically to treat colon cancer, only the HT29 cell line was tested for

DNA fragmentation and mitochondrial transmembrane potential to verify this increased apoptosis. The DNA fragmentation assay (figure 2.5) showed that all samples had more DNA fragmentation in the presence of mangiferin except for CPA7, and the mitochondrial transmembrane potential assay (figure 2.6) showed more depolarization in the presence of mangiferin in all samples except for oxaliplatin and Mar 4.1.4.

2.3.3 Apoptosis induction

Caspase 3 is an executioner caspase commonly used as an indicator of apoptosis induction. A monoclonal antibody raised specifically against the activated enzyme was used in conjunction with flow cytometry to compare the levels of active caspase 3 in control cells with that in cells treated with the selected compounds and/or mangiferin. Histograms of events versus fluorescence intensity (active caspase 3) were used to obtain the percentages of cells that stained positive for active caspase 3 (see Figure 2.4A as an example) and these values were plotted as bar graphs (Figure 2.4B and C). Non-specific binding of the antibody was corrected for by using an isotype control antibody.

All the platinum compounds increased the levels of activated caspase 3 in HeLa and HT29 cells, except for oxaliplatin in HT29 cells. The most pronounced effect was seen with Yol 25 (figure 2.4A and B). A further increase in the percentage of activated caspase 3 (figure 2.4) in oxaliplatin treated HeLa and HT29 cells, as well as in CPA7 treated HeLa cells, indicates that mangiferin may also aid in the caspase 3 activation of some of these compounds. However, a decrease in the activated caspase 3 when exposed to the compounds plus mangiferin could also be seen in some of these compounds, especially in the HT29 cell line. In both HeLa and HT29 cells mangiferin caused an increase in the amount of caspase 3 when applied alone, but a more pronounced increase was seen in the HT29 cell line. The MCF7 cell line does not express caspase 3 (Yang *et al*. 2001), therefore only HeLa and HT29 cell lines were tested for activated caspase 3 activities.

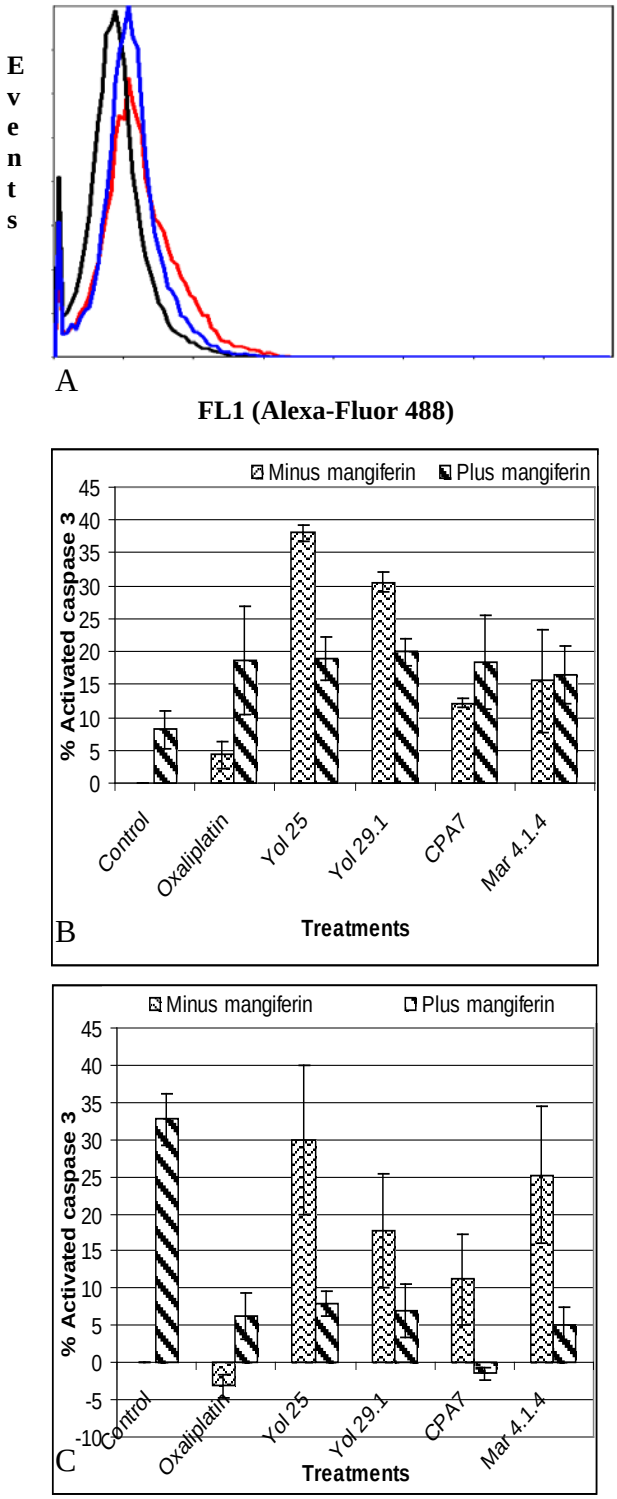


Figure 2.4: Caspase 3 activated by platinum compounds with and without addition of 10µg/ml mangiferin. (A) Histogram overlay as an example of the results obtained in HeLa cells: Black = Control; Red = Yol 25 and Blue = Yol 25 + mangiferin treated cells; (B) A summary of the results obtained in HeLa cells, expressed as percentage cells that stained positive for active caspase 3; and (C) A summary of the results obtained in HT29 cells, expressed as percentage cells that stained positive for active caspase 3. Values in (B) and (C) represent mean ± average deviation, n=2.

The DNA fragmentation assay (figure 2.5) showed that all the samples had more DNA fragmentation in the presence of mangiferin except for CPA7. Interestingly CPA7, when applied alone, showed the most DNA fragmentation of all the compounds tested, with and without mangiferin. Even after the reduction in DNA fragmentation occurred in combination with mangiferin, it still exhibited the highest percentage of DNA fragmentation except for Mar 4.1.4 in the presence of mangiferin. This might suggest that CPA7 causes apoptosis via mechanisms involving DNA fragmentation. The most pronounced increase in DNA fragmentation in the presence of mangiferin also occurred in Mar 4.1.4 treated cells.

The mitochondrial transmembrane potential assay (figure 2.6) showed more depolarization in the presence of mangiferin in all samples except for oxaliplatin and Mar 4.1.4. Mangiferin alone, as well as Yol 25 in combination with mangiferin showed the most significant increases in depolarized cells.

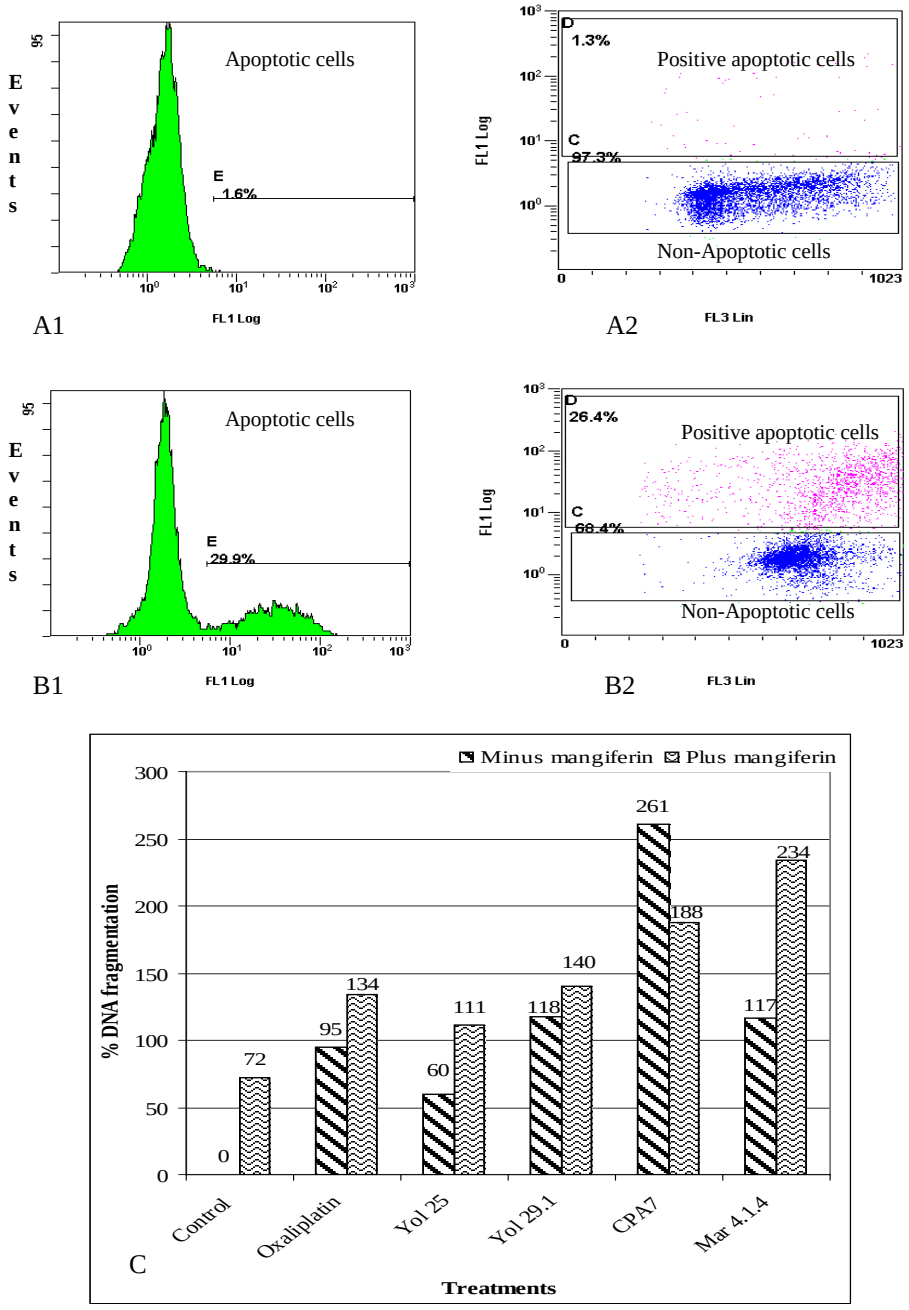


Figure 2.5: DNA fragmentation induced by platinum compounds in HT29 cells with and without the addition of 10µg/ml mangiferin. (A1) A Histogram of Events vs cells stained with fluorescein labeled antiBrdU monoclonal antibody (FL1 log) for negative control cells and (A2) its corresponding density plot of fluorescein labeled antiBrdU monoclonal antibody bound cells (FL1 log) vs DNA area (FL3 lin). (B1) A Histogram of Events vs cells stained with fluorescein labeled antiBrdU monoclonal antibody (FL1 log) for oxaliplatin treated cells and (B2) its corresponding density plot of fluorescein labeled antiBrdU monoclonal antibody bound cells (FL1 log) vs DNA area (FL3 lin). (C) A summary of the mean fluorescence intensity (FL1) results obtained calculated as a percentage of control (Bars indicate values from single experiment; a repeat of this experiment showed similar trends).

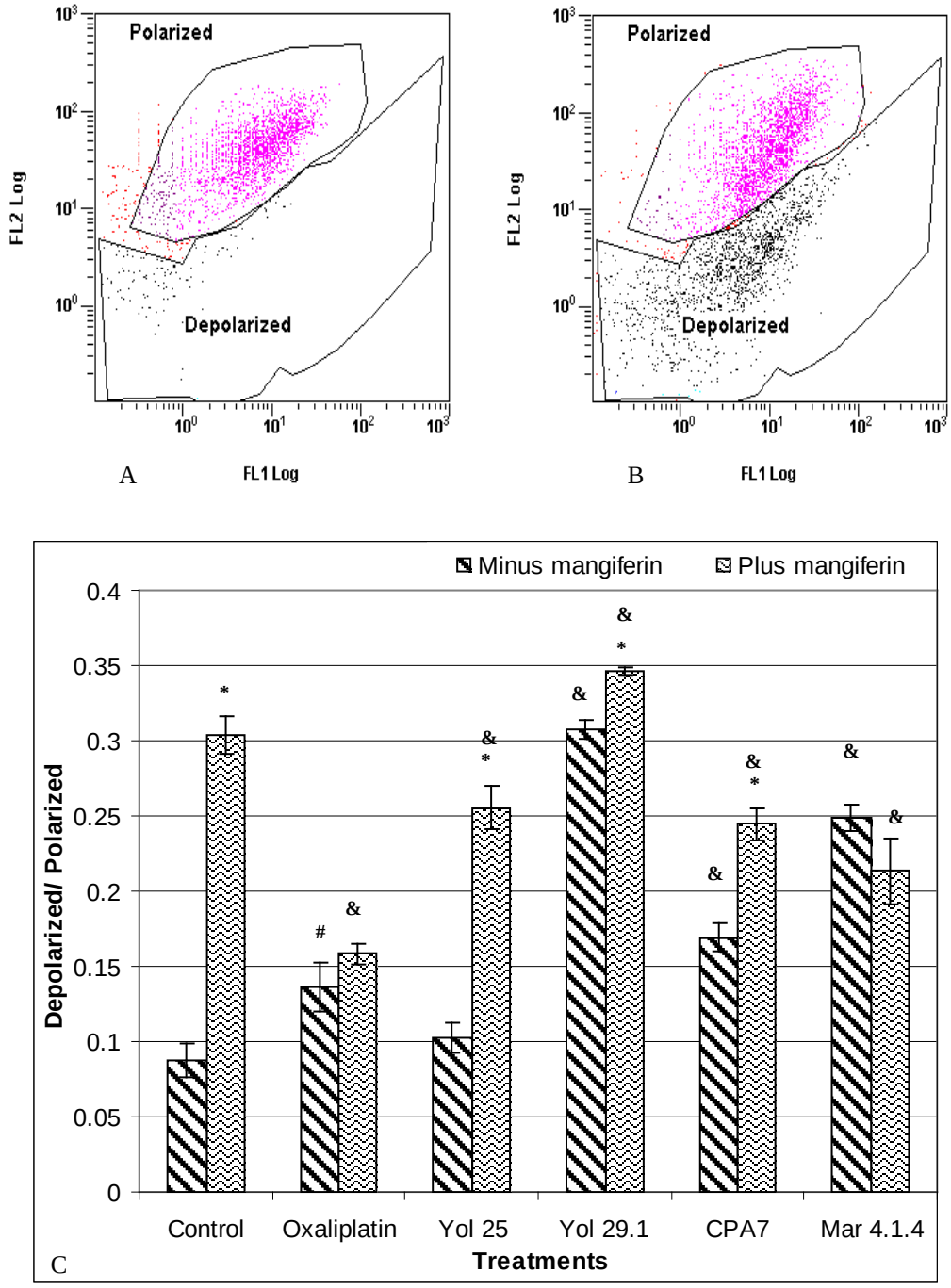


Figure 2.6: Mitchondrial membrane potential measured with JC1in HT29 cells exposed to platinum compounds with and without the addition of 10µg/ml mangiferin. (A) A density plot of FL1 log vs FL2 log for negative control cells. (B) A density plot of FL1 log vs FL2 log for oxaliplatin treated cells. (C) A summary of the results obtained for the platinum compounds expressed as the ratio of depolarized (FL1) to polarized (FL2) membranes via the mean fluorescence intensity values obtained for each (values represent mean±standard deviation; n=3)

* $p<0.001$ as compared to corresponding treatment without mangiferin; # $p<0.05$ and & $p<0.001$ as compared to the control.

2.3.4 Drug resistance

Cancer cells develop resistance to most chemotherapeutic drugs over time and the search is ongoing for one that does not have this disadvantage. The three cell lines used in this study were exposed to the compounds under investigation over long periods to establish whether or not resistance would develop. Mangiferin has previously been shown to increase GSH levels. Since increased GSH levels have also been associated with cisplatin resistance in tumours, drug resistance was also induced in the presence of mangiferin (Dunfield and Guernsey, 2002; Sarkar *et al*. 2004 and Turchi, 2006). After exposure to the compounds, IC50 values were determined as a measure of the cells' sensitivity to each compound. The levels of activated NFκB were used as a further measure of drug resistance.

The IC50 values depicted in table 2.4 generally indicate that resistant cells had a higher IC50 value than normal cells and that the presence of 10µg/ml mangiferin lowered the IC50 value in both normal and resistant cells. The HT29 cell line was most prone to become resistant against the drugs, whereas the MCF7 cell line was the least prone to become resistant. The presence of mangiferin had the most promising results in terms of lowering the IC50s in Yol 25 and oxaliplatin treatment, especially in the resistant cells. Yol 29.1 showed the least promising results. Oxaliplatin showed the most resistance induced, of all the complexes tested, in the HeLa and the HT29 cell lines. It also showed a reduction in the IC50 values when combination treatment was applied. This is very promising, since oxaliplatin is currently used to treat colon cancer and resistance induction is a negative feature of this drug. Yol 25 exposure did not influence the IC50 values in HeLa and MCF7 cells, but did cause an increase in the HT29 cells. Combination treatment caused a decrease in the IC50 values for all cell lines. Yol 29.1 exposure did not influence the IC50 values in MCF7 cells with and without combination treatment, but did cause increased IC50s in the other cell lines. However, combination treatment with mangiferin caused a further increase in the IC50s instead of the expected decrease. CPA7 exposure did not influence the IC50 values in HT29 cells and a decrease in the value was seen in resistant HeLa cells. Combination treatment with CPA7 and mangiferin caused a decrease in the IC50 value in MCF7 cells, but an increase in both HeLa and HT29 cells. Mar 4.1.4 did not seem to induce resistance in HeLa and MCF7 cells, but

combination treatment with mangiferin caused a decrease in the IC50s, especially in the MCF7 cell line. However, in the cells that were resistance induced in the presence of mangiferin, an increase in the IC50s was seen.

Table 2.4: The IC50 values for the platinum drugs against normal* as well as resistant** cells, with and without the addition of 10µg/ml mangiferin.

Compound	Mangiferin added (10µg/ml)	Normal HeLa	Resistant HeLa	Normal HT29	Resistant HT29	Normal MCF7	Resistant MCF7
Oxaliplatin	No	3	**12**	6	**12**	8	6
	Yes	4	***7***	*3*	***4***	*5*	**8**
Yol 25	No	9	9	21	**26**	7	7
	Yes	*6*	*5*	*13*	***18***	*5*	***6***
Yol 29.1	No	3	**6**	5	**11**	5	5
	Yes	4	**9**	7	**13**	11	11
CPA 7	No	5	4	10	10	9	5
	Yes	*3*	**4**	*7*	**11**	*4*	*3*
Mar 4.1.4	No	5	5	3	**6**	6	6
	Yes	*3*	**5**	*2*	**6**	*2*	**8**

Bold = Increase in IC50 value as compared to normal cells

Italics = Decrease in IC50 values in presence of mangiferin

* Cells not previously exposed to any platinum compound

** Cells exposed intermittently and incrementally over a long period of time to the respective platinum compound.

The NFκB assay (Figure 2.7) indicates that resistant HT29 cells had a higher amount of activated NFκB than normal HT29 cells for all complexes except Mar 4.1.4. This amount of activated NFκB was reduced when resistance was induced in the presence of mangiferin in oxaliplatin, CPA7 and Yol 29.1 resistant cells. Therefore, mangiferin has been shown to reduce the amount of activated NFκB associated with resistant tumour cells and may prove beneficial in the prevention of resistance to platinum anticancer drugs in tumour cells. Yol 25 and Mar 4.1.4 showed an increase in the NFκB levels when mangiferin is present. However, the presence of mangiferin did decrease the IC50 values; quite significantly, in Yol 25 treated HT29 cells (table 2.4). This suggests an alternative mechanism of resistance induction for this complex in HT29 cells (possibly increased GSH or MT levels, enhancing detoxification). Mar 4.1.4 might also exhibit such an alternative mode of resistance induction, although the

degree of resistance induced by this drug was not as significant (according to the IC50s) as for Yol 25.

Normal cells treated with mangiferin alone showed an unexpected, increased amount of NFκB activation. This suggests that mangiferin in combination with platinum containing complexes might have a synergistic effect and does not act as an NFκB inhibitor/ anticancer agent on its own. It has been shown previously that the development of anti-cancer drugs that can "switch" the function of NFκB from anti-apoptotic to pro-apoptotic may prove to be beneficial in the fight against cancer and that tumour prevention may be achieved through inhibition of abnormally active NFκB (Perkins, 2000; Perkins, 2004).

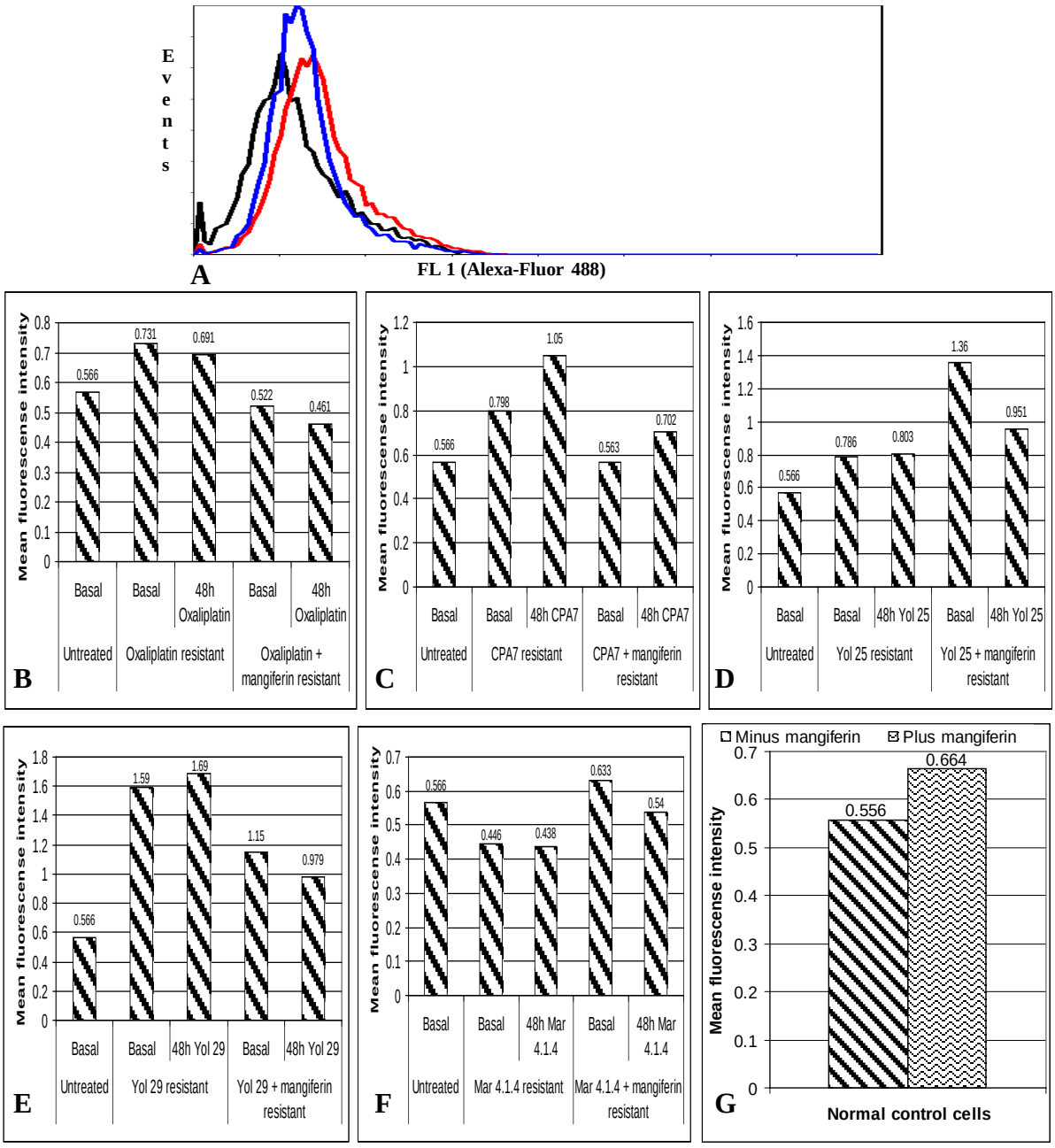


Figure 2.7: (A) Histogram overlay of the results obtained for the NFκB assay (Events vs FL1) in HT29 cells. Black = Control; Red = oxaliplatin resistant and Blue = oxaliplatin + mangiferin resistant cells. (B-G) Activated NFκB (expressed as mean fluorescence intensity) in untreated and resistance-induced HT29 cells. Resistance was induced through intermittent, incremental exposure to the respective complex or complex + 10μg/ml mangiferin. These cells were finally seeded into culture flasks and levels of activated NF-κB measured in cells that received no further treatment (basal) or 10μM of the respective complex for a further 48 hours. [(B) Oxaliplatin; (C) CPA7; (D) Yol 25; (E) Yol 29.1; (F) Mar 4.1.4.; (G) Mangiferin alone] Bars indicate values from single experiment, a repeat of this experiment showed similar trends.

2.4 DISCUSSION

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. In this study it was shown that mangiferin, a natural polyphenol found in *Cyclopia spp*., *Canscora decussata* (Bhattacharya *et al.* 1972) and *Mangifera indica* (Muruganandan *et al*. 2005) aids in the enhanced anticancer action of oxaliplatin, CPA7 and three novel platinum containing anticancer drugs. Hence a new combination therapy against cancer can be proposed. Furthermore, Honeybush tea that is a rich source of antioxidants may be used in conjunction with chemotherapy to reduce the side-effects associated with it (although some controversy still exists around this) (Drisko *et al.* 2003a, Drisko *et al*. 2003b and Lawenda *et al*. 2008). The use of traditional herbal medicines in these treatments might also render it more acceptable to many South Africans.

The discovery that mangiferin decreases the IC50 values (table 2.2) of the platinum drugs by up to 3.4 times is very promising. Caspases (cysteine aspartic acid-specific proteases) play a key initiator role in the intrinsic apoptotic pathway of mammalian cells. The fact that an increase in the caspase 3 activity (figure 2.4) is observed during combination treatment with oxaliplatin and mangiferin in both HeLa and HT29 cells indicates that the decreased IC50 values may be due to increased apoptosis. The concentration of oxaliplatin in the bloodstream of a patient after treatment is approximately 52μM (85mg/m^2 administered). Therefore, a 3.4 times decrease in the IC50 may implicate a much higher activity *in vivo*. It may also mean that a much lower dose can be applied, thereby lessening the toxic side-effects associated with the treatment at such high doses.

Lactate dehydrogenase is a cytosolic enzyme that catalyses the transformation of pyruvate to lactate. It is released from the cell upon loss of plasma membrane integrity or necrosis (Tang *et al.* 2004). The results obtained in this study (figure 2.2) indicate that some of the platinum drugs cause less LDH release when administered in combination with mangiferin. Taken together with the lower IC50 values in the

presence of mangiferin, it can be assumed that less necrosis occurs in the cells when mangiferin is present. These results correlated to the results obtained from the trypan blue assay (table 2.3). A decrease in the amount of non-viable cells was observed in this assay for most compounds when applied in combination with mangiferin. Trypan blue stains dead/ necrotic cells with "leaky" cell membranes, hence a decrease in the percentage of non-viable cells from this assay indicates less necrosis has taken place. These results therefore show strong evidence that mangiferin favours apoptotic cell death instead of necrosis. CPA7 and Mar 4.1.4 in combination with mangiferin showed an increase in the caspase 3 activity in HeLa cells, but not in HT29 cells, suggesting different modes of action in these two cell lines. Alternatively, it may also implicate that CPA7 and Mar 4.1.4 act much faster than the other complexes and that a shorter incubation time would have shown the desired results for these two complexes. Interestingly, both CPA7 and Mar 4.1.4 are Pt(IV) complexes, which may also play a role in this observation and the reaction times mentioned above. The MCF7 cell line was not tested for caspase 3 activity since this cell line does not express functional caspase 3.

The results obtained from the DNA cell cycle analysis (figure 2.3) clearly shows an S-phase arrest occurs in both oxaliplatin and CPA7 treated cells. Fujie *et al* (2005) also showed that oxaliplatin causes an S or G2M phase delay in the cell cycle of HT29 cells. Although mangiferin alone did not induce cell cycle arrest, the presence of mangiferin in combination with oxaliplatin shows an earlier and greatly enhanced delay in the S-phase, while cells treated with CPA7 in combination with mangiferin showed a later, but greatly enhanced delay in the S-phase. Yol 25 acts similarly to oxaliplatin, whereas Yol 29.1 and Mar 4.1.4 seem to act similar to CPA7, although, in the latter, the delay in the S phase is very late and can be classified as a late S, early G2/M phase delay. It would only be possible to identify the exact nature of the cell cycle arrest by investigating the presence of specific cyclins and cyclin-dependent kinases (CDKs). DNA replication in eukaryotic cells takes place during the S phase. The transition from G1 into S phase in mammalian cells is regulated by at least two CDKs, cyclin E/Cdk2 and cyclin A/Cdk2. Cyclin E/Cdk2 activity appears in late G1, while cyclin A/Cdk2 activity appears at the onset of DNA synthesis. Cyclin A is also required later for the S/G2 transition (Frouin *et al*. 2002).

The Sub-G1 phase refers evidence for 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 rendering these cells with less DNA (Kwon *et al*. 2005). The percentage of apoptotic cells were also increased in the cells subjected to a combination treatment. This was verified by a DNA fragmentation assay (figure 2.5) that showed an increase in the amount of DNA fragmentation in HT29 cells subjected to a combination treatment in contrast to cells treated with the platinum containing complexes alone. CPA7 however showed a decrease in the amount of DNA fragmentation when cells were treated in combination with mangiferin. This finding, combined with the decreased caspase 3 activity in combination treatment of CPA7 and mangiferin on HT29 cells, might indicate that mitochondrial induced paraptotic cell death occurs in these cells (Tang *et al*. 2004). A process that would be a '*continuum*' between apoptosis and necrosis is referred to as '*parapoptosis*' (De Souza Pagnussat *et al*. 2007). Features characteristic of necrotic and apoptotic cell death are not only occurring in the same tissues but simultaneously in the same cells. Parapoptosis (also known as necrapoptosis), is a process that begins with a common death signal or toxic stress but that culminates in either necrosis or apoptosis, depending on other modifying factors. Cell death mediated by the mitochondrial pathway illustrates this idea. When onset of the mitochondrial membrane depolarization is rapid and cellular ATP levels drop dramatically, then early cell lysis ensues. If this progression is slower, or if other sources of ATP generation are available, then profound ATP depletion is avoided, allowing apoptotic signalling to proceed. Later if ATP levels finally collapse, cell lysis supervenes in a pattern of secondary necrosis. Pure apoptosis and pure necrosis represent extremes in the spectrum of necrapoptotic responses, but the more typical response of tissues and cells to injurious stresses and other death signals is a mixture of events associated with apoptotic and necrotic cell death (Lemasters, 1999).

Mitochondria produce ATP through oxidative phosphorylation, which involves a series of redox reactions that transfer electrons through multiple protein complexes in the inner mitochondrial membrane. Protons are then pumped out of the mitochondrial matrix, generating the mitochondrial membrane potential ($\Delta\Psi_m$), which is not only harnessed to generate ATP, but is also responsible for mitochondrial Ca^{2+} uptake,

metabolite and protein transport, production of reactive oxygen species, and has also been related to the process of apoptosis. Hence, the health and bioenergetic function of the mitochondria depend on its membrane potential. Preservation of the $\Delta\Psi_m$ is essential during normal conditions and, especially, during conditions of stress and disease. Depolarization of the membrane results in a reduction of ATP production and is also thought to precipitate the release of pro-apoptotic factors in some cell systems. Thus, there are good reasons to search for therapeutic agents to decrease $\Delta\Psi_m$ as possible pro-apoptotic drugs, for example, in the case of cancer (Wong and Cortopassi, 2002). This assay was performed to verify whether some platinum containing complexes use other mechanisms of action in combination with mangiferin, causing apoptosis. The results obtained (figure 2.6) shows that the amount of depolarization does not increase in cells treated with oxaliplatin or Mar 4.1.4 in combination with mangiferin, but does in cells treated with Yol 25, Yol 29.1 and CPA7 in combination with mangiferin. This may, again, be indicative of mitochondrial induced parapoptotic cell death in CPA7 treated cells.

NFκB is a transcription factor that exerts anti-apoptotic effects and is often activated in cancer cells in response to chemotherapeutic agents. This promotes cancer therapy resistance in tumours (Baldwin, 2001). The improper activation of NFκB in diseases such as tumourigenesis (Lee *et al*. 2008 and Paul *et al*. 2006) has been linked to TNFα and other members of its superfamily. Through the activation of NFκB, TNFα induces the expression of various genes that can be involved in tissue invasion and metastasis. In addition, activation of NFκB can suppress apoptosis, which is likely to enhance tumourigenesis. Many natural compounds, including the phenolics, have been found to inhibit upstream signalling molecules that are involved in TNFα expression (Paul *et al*. 2006). Leiro *et al.* (2004) illustrated that mangiferin modulates the expression of a large number of genes that are critical for the regulation of apoptosis and tumourigenesis, and raised the possibility that it may be of value in the treatment of cancer. Platinum drugs continue to be major chemotherapy drugs for cancer treatment. Nevertheless, acquired or intrinsic resistance to these compounds is common in human tumours. One mechanism of resistance induction is the avoidance of cells entering the apoptotic pathway. NFκB is important in the final response of cells to platinum drugs, and it has been shown that inhibition of NFκB sensitizes cancer cells to the effects of these drugs. New approaches focusing on the inhibition

of NFκB could help to minimize or even eliminate resistance to platinum drugs. In order to increase the benefit of current platinum-based drugs and to direct effort to obtain improved agents, it is of great importance to understand the molecular basis of acquired and intrinsic resistance. Focusing on the inhibition of this factor could help to reduce or even eliminate resistance to platinum drugs or to provide drugs with less toxic side effects (Lagunas and Melendez-Zajgla, 2008).

The NFκB assay (Fig. 2.7 A - E) indicates that all the resistant HT29 cells had a higher basal level of activated NFκB than normal HT29 cells, except for Mar 4.1.4 resistant HT29 cells. This was also true for CPA7 despite the fact that there is no increase in IC50 after resistance induction. This amount of activated NFκB was reduced to below that of normal control cells when resistance to oxaliplatin and CPA7 (fig. 2.7 A and B) was induced in the presence of mangiferin, while Yol 29 (Fig. 2.7 D) also lead to a decrease in the NFκB levels, although not to below that of the normal (basal) level. Therefore, for these three complexes, mangiferin has been shown to reduce the amount of activated NFκB associated with resistant tumour cells and may prove beneficial in the prevention of resistance to these platinum anticancer drugs in tumour cells. After an additional treatment of the oxaliplatin plus mangiferin resistant cells with oxaliplatin, there is a further decrease in the amount of NFκB activated (Fig. 2.7 A). These results indicate that mangiferin in combination with oxaliplatin, CPA7 and Yol 29 acts as an NFκB inhibitor. Mangiferin alone does not act as an NFκB inhibitor, therefore the synergistic action of mangiferin and these complexes are very important. It has been shown previously that the development of anti-cancer drugs that can “switch” the function of NFκB from anti-apoptotic to pro-apoptotic may prove to be beneficial in the fight against cancer and that tumour prevention may be achieved through inhibition of abnormally active NFκB (Perkins, 2000; Perkins, 2004).

An additional treatment of the CPA7 + mangiferin resistant HT29 cells (Fig. 2.7 B) resulted in an increase in the activated NFκB levels. Yol 25 and Mar 4.1.4 showed opposite behaviour to the previously discussed complexes. Both these complexes show increased NFκB levels when resistance was induced in the presence of mangiferin, however an additional treatment with the respective complexes resulted in

lowered activated NFκB levels. These results indicate a different mode of action for these two complexes.

Mabuchi *et al*. (2004) showed that combination therapy of cisplatin with an NFκB inhibitor would increase the therapeutic efficacy of cisplatin. Our results indicate that mangiferin is an NFκB inhibitor (Fig. 2.7) when applied in combination with platinum anticancer drugs and that it increases the therapeutic efficacy of platinum anticancer drugs and decreases the amount of resistance induced by these complexes (Table 2.4). Furthermore, we have shown evidence that mangiferin may counteract the development of resistance to oxaliplatin, CPA7 and Yol29.1 in HT29 colon cancer cells by reducing the activation of NFκB (Fig. 2.7 A, B and D).

Mangiferin inhibits NF-κB and increases the intracellular GSH levels (Sarkar *et al.* 2004). It is known that high GSH levels reduce the anticancer action of platinum drugs via detoxification. It also plays a role in the induction of resistance towards these compounds in cancer cells (Turchi, 2006). However, although GSH depletion could be useful to increase the therapeutic efficacy of cancer treatment by anticancer drug combinations (Meurette *et al*. 2005), the fact that mangiferin *increases* GSH levels does not seem to influence the efficacy of these combination treatments and further study into this finding needs to be conducted.

In order to determine whether these compounds alone or in combination with mangiferin might be potential novel anticancer therapies, a summary of the results obtained for each novel compound follows. Since oxaliplatin and CPA7 are used as references in this study, their typical effects on the three cancer cell lines will firstly be summarised.

Oxaliplatin (Pt(dach)C_2O_4) exhibited the highest fold decrease (3.4x) of IC50 in the HT29 cells treated with oxaliplatin in combination with mangiferin. This is very significant, since oxaliplatin is clinically used to treat colon cancer (Martin *et al*. 2000). There was a 1.7 fold decrease in the IC50 values in the HeLa cell line and a slight increase in the IC50 values was observed in the MCF7 cell line (0.75 fold decrease) (table 2.2). Caspase 3 activation was increased in HeLa and HT29 cells when combination treatment was applied (figure 2.4). This could indicate that the

decreased IC50 values may be due to increased apoptosis. The trypan blue assay show strong evidence that mangiferin favours apoptotic cell death instead of necrosis when applied in combination with oxaliplatin. No significant differences were seen between the amount of LDH released with and without the addition of mangiferin to oxaliplatin (figure 2.2). The DNA cell cycle analysis revealed that oxaliplatin causes an S-phase delay in the DNA cycle and that a combination with mangiferin caused an earlier, enhanced delay in this phase (figure 2.3). It was previously found that oxaliplatin causes an S-phase delay in HT29 cells (Rakitina *et al.* 2007). There was also an increase in the amount of cells in the sub-G1 (apoptotic) phase. The DNA fragmentation assay revealed that more DNA fragmentation occurred when oxaliplatin was applied in combination with mangiferin (figure 2.5), but no significant difference was seen in the mitochondrial membrane potential (figure 2.6). Table 2.4 shows that resistance to oxaliplatin was counteracted by the combination with mangiferin in HeLa and HT29 cells, but not in MCF7 cells and the NFκB assay indicates that this may be due to a decrease in the activated NFκB levels when oxaliplatin is applied in combination with mangiferin (figure 2.7). These results show great promise in improving the efficacy of oxaliplatin by combination treatment with mangiferin. It has previously been shown that reversal of oxaliplatin chemo-resistance can be achieved by NF-kB inhibition (Almendro *et al*. 2006) and that downregulation of NF-*κ*B transactivation by pharmacological inhibitors enhances oxaliplatin cytotoxicity in several colon adenocarcinoma cell lines (Lagunas and Melendez-Zajgla, 2008).

CPA7 ($Pt(NH_3)_2Cl_3(NO_2)$) showed a decrease in the IC50 for all cell lines when applied in combination with mangiferin (table 2.2). A slight increase in the percentage of activated caspase 3 occurred in the HeLa cells, but a decrease occurred in the HT29 cells; suggesting an alternative mode of action (parapoptosis) or a faster action in this cell line (figure 2.4). The trypan blue assay showed that a decrease in the percentage of non-viable cells occurred in the HeLa cell line when CPA7 was applied in combination with mangiferin, but slight increases occurred in the HT29 and MCF7 cells (table 2.3), which may also be accounted for by an faster action of this drug. There were no significant differences in the percentage LDH released in the HeLa cell line, but the HT29 and MCF7 cells showed a significant decrease in the amount of LDH released when applied in combination with mangiferin (figure 2.2).

When CPA7 was applied in combination with mangiferin, a later, enhanced S-phase delay occurred (as opposed to the earlier delay seen in the oxaliplatin combination treatment) (figure 2.3). There was a decreased amount of DNA fragmentation when combined with mangiferin (figure 2.5), but an increase in the amount of depolarized cells (figure 2.6), indicating that apoptosis is occurring. Table 2.4 indicated that the IC50 values decreased when applied in combination with mangiferin in all cell lines, but resistance was only counteracted in the MCF7 cell line. The activated NFκB levels were increased in CPA7 resistant HT29 cells, but the addition of mangiferin decreased this amount (figure 2.7). Not much has been published on CPA7 in the scientific literature before. The biological effects of CPA7 were shown to include growth inhibition and apoptosis of malignant cells that harbour constitutively active Stat3. These effects were found to be stronger and more selective against malignant cells that contain persistently active Stat3 than those that do not. CPA7 is a platinum (IV) complex, in contrast to oxaliplatin, which is a platinum (II) complex (Turkson *et al*. 2004).

Yol25 ($Pt(hme)Cl_2$) showed lower IC50 values and thus better *in vitro* efficacy than cisplatin and oxaliplatin in all cell lines except in HT29 cells. There was also a slight increase in the IC50 for the MCF7 cell line as compared to oxaliplatin (table 2.2). Therefore Yol 25 had a reduced anticancer action on HT29 cells compared to cisplatin and oxaliplatin. Similarly it exhibited a higher IC50, when compared to CPA7, in HT29 cells. In contrast to this, Yol 25 showed the most activated caspase 3 of all complexes tested (figure 2.4), indicating that apoptosis is occurring in the cells. It also showed a slight decrease in LDH release in HT29 cells (figure 2.2). This indicates decreased necrosis which will lead to less inflammation. It caused an early S-phase delay in the DNA cell cycle when applied alone, as did CPA7, but not to such a great extent as CPA7. No effect was seen on the subG1 phase (figure 2.3). Less DNA fragmentation and mitochondrial membrane depolarization (figures 2.5 and 2.6) occurred when Yol 25 was applied alone as compared to oxaliplatin and CPA7. This complex did not seem to induce resistance in HeLa and MCF7 cells, but did in HT29 cells (table 2.4), which was probably caused by an increased activation of NFκB (figure 2.7).

In combination with mangiferin, Yol 25 showed a decrease in the IC50s (table 2.2). The activated caspase 3 levels decreased in both HeLa and HT29 cells when a combination treatment was applied (figure 2.4). This corresponds to the results obtained for CPA7 in the HT29 cell line. This compound alone caused the most activation of caspase 3 of all the compounds tested. This suggests that Yol 25 alone induces apoptosis via a caspase 3 activation pathway, but when applied in combination with mangiferin, this pathway does not play such a significant role any more and an alternate path is used. This alternate path may very well be parapoptosis, as discussed above. The trypan blue assay showed a decrease in the amount of non-viable cells when applied in combination with mangiferin (table 2.3) and a significant increase in the LDH levels was seen in the HeLa cells, whereas a significant decrease occurred in the MCF7 cell line. No effect was seen in the HT29 cell line (figure 2.2). The increase in the LDH levels seen in the HeLa cells might suggest that a certain amount of necrosis takes place when a combination is applied. The DNA cell cycle results corresponds with those obtained for oxaliplatin in combination with mangiferin, since an earlier, enhanced delay in the S-phase was seen (figure 2.2). There was an increased amount of DNA fragmentation (figure 2.5) and an increased amount of depolarized cells (figure 2.6). However, Yol 25 alone did not have a significantly higher amount of depolarized cells than the control cells; suggesting that Yol 25 does not rely on a mitochondrial induced apoptotic pathway when applied alone, again suggesting parapoptosis. A decrease in the IC50 value was seen in the HT29 cells when combination treatment was applied. It was also interesting to note that the HeLa and MCF7 cell lines did not seem to become resistant to this compound whether it was applied alone or in combination (table 2.4). Interestingly, there was an increase in the amount of activated NFκB when applied in combination; however, after an additional 48 hour treatment with Yol 25 alone, this value decreased for the cells rendered resistant in the presence of mangiferin whereas those rendered resistant in the absence of mangiferin did not seem to affect the NFκB levels (figure 2.7).

In conclusion it seems that Yol 25 might be a worthy novel anticancer drug, even though the IC50s were higher than that of the positive controls. This, unfortunately, will necessitate the administration of a higher dose of this drug, which might cause more severe toxic side effects. However, the application of this drug in combination with mangiferin might be the answer to this, since this combination lowered the IC50s

significantly and might cause parapoptosis to occur. Further testing of this drug, alone and in combination with mangiferin is therefore recommended.

Yol 29.1 (Pt(ama)Cl_2) showed decreased IC50s in HeLa cells, compared to cisplatin and oxaliplatin, and in MCF7 cells, compared to oxaliplatin and CPA7. In HT29 cells it had a slightly lower IC50 than oxaliplatin, cisplatin and CPA7. This drug alone is more effective in terms of IC50 than Yol 25 alone (table 2.2). Yol 29.1 also induced more caspase 3 than either oxaliplatin or CPA7 (figure 2.4). No effect on LDH release was seen in HeLa and HT29 cells, but a significant decrease was observed in the MCF7 cell line (figure 2.2). Its effect on the DNA cell cycle was similar to that of CPA7 and Yol 25, causing an early S phase delay. There were slightly more cells in the subG1 phase than for Yol 25 (figure 2.3). Yol 29.1 caused more DNA fragmentation than oxaliplatin, but less than CPA7 (figure 2.5) and exhibited the most depolarization of the mitochondrial membrane of all treatments, including the positive controls (figure 2.6). This suggests that a mitochondrial induced apoptotic pathway is activated by this drug. Yol 29.1 caused resistance in HeLa and HT29 cells, but not in MCF7 cells (table 2.4). The resistance in HeLa and HT29 was most probably as a result of the much higher levels of activated NFκB (figure 2.7).

When Yol 29.1 was applied in combination with mangiferin it showed a decrease in the IC50s (table 2.2) as well as decreases in the amount of activated caspase 3, similar to Yol 25 (figure 2.4). A decrease in the amount of trypan blue positive (non-viable) cells was seen in the HeLa cell line, but in both the HT29 and MCF7 cell lines an increase occurred (table 2.3). There was a significant decrease in the amount of LDH release in the MCF7 cells, but no effect was seen in the HeLa and HT29 cell lines (figure 2.2). Yol 29.1 in combination with mangiferin acts similar to CPA7 on the DNA cell cycle and a later, enhanced S-phase delay was seen. However this delay could be interpreted as a late S/ early G2/M phase delay as opposed to a strict S-phase delay as observed for CPA7 (figure 2.3). An increase in the amount of DNA fragmentation (figure 2.5) and the amount of depolarized cells (figure 2.6) was observed for combination treatments, but the addition of mangiferin caused increased IC50s in the cell lines. It also seems that resistance was not induced in the MCF7 cell line (table 2.4). The resistance-induced HT29 cells showed a decreased level of activated NFκB when applied in combination with mangiferin (figure 2.7).

In conclusion, Yol 29.1 seems to be more efficient that Yol 25 in terms of IC50 values, but causes resistance in both HeLa and HT29 cells. Furthermore, the addition of mangiferin does not decrease the IC50 values, but rather increases it. However, NFκB levels decrease in the presence of mangiferin, suggesting that NFκB does not play a role in resistance induction by this compound. Yol 29.1 alone would be more efficient than Yol 25, but the mechanism of resistance should be studied and other combination therapies investigated.

Mar 4.1.4 (Pt(dach)(C_2O_4)NO_2)Cl) shows lower IC50 values than cisplatin, oxaliplatin and CPA7, proving to be more efficient than any of the positive controls in terms of IC50s (table 2.2). It also showed increased caspase 3 levels compared to the positive controls (figure 2.4). However, much higher LDH levels were obtained than for the positive controls in the HeLa cell line, with lower levels obtained in HT29 and MCF7 cells compared to CPA7, but not to oxaliplatin (figure 2.2). Mar 4.1.4 acted similar to oxaliplatin by causing a late S-phase delay in the DNA cell cycle as well as resulting in a similar amount of cells in the subG1 phase (figure 2.3). An increased amount of DNA fragmentation was seen compared to oxaliplatin, but not to CPA7 (figure 2.5) and more depolarization of the mitochondrial membrane occurred compared to the positive controls (figure 2.6). This drug did not induce resistance in HeLa and MCF7 cells, but did in HT29 cells (table 2.4). Interestingly, the amount of activated NFκB in the HT29 cell line decreased, suggesting that NFκB does not play a significant role in the induction of resistance by this drug (figure 2.7).

In combination with mangiferin a decrease in the IC50 values was observed, especially for the MCF7 cell line (3.1 fold decrease as opposed to a 3.4 fold decrease for oxaliplatin) (table 2.2). Combination treatment did not affect the caspase 3 levels in the HeLa cell line, but a decrease in the level of activated caspase 3 was seen in the HT29 cell line. This again suggests that an alternate apoptotic path is activated (as for CPA7, Yol 25 and Yol 29.1) (figure 2.4). Using the trypan blue assay, a decrease in the amount of non-viable cells was found in the HeLa and HT29 cells, but not in the MCF7 cells (table 2.3). No significant differences in the LDH levels were observed (figure 2.2) but similarly to Yol 29.1 it caused a later, enhanced delay in the late S/ early G2/M phase of the DNA cycle (figure 2.3). There was an increase in the

amount of DNA fragmentation when applied in combination with mangiferin (figure 2.5), but no effect on the mitochondrial membrane potential was seen (figure 2.6). This was also true for oxaliplatin. This compound did not induce resistance in the HeLa and MCF7 cell lines and the presence of mangiferin did not affect the IC50s of the resistance-induced cells, but it did lower the IC50s for the normal cells. Resistance was induced in the HT29 cell line but a decrease in the NFκB levels was seen in resistant cells, contradicting the belief that resistance can be linked to increased NFκB levels. When resistance was induced in the presence of mangiferin, the level of NFκB increased to above that of the control cells, as was expected (figure 2.7).

In conclusion, Mar 4.1.4 seems to be the most effective drug tested in this study, including the positive controls. The addition of mangiferin further lowers the IC50 values of this drug, rendering it even more effective. The fact that no resistance was induced in HeLa and MCF7 cells shows even more promise for this drug. Unfortunately the addition of mangiferin does not seem to counteract the resistance induced in the HT29 cells, and further studies into the mechanisms applied to induce resistance is required. Mar 4.1.4 is a "pro-drug" for oxaliplatin and is reduced, amongst others, to oxaliplatin in the body. This might explain why it acts similar to oxaliplatin in some of these experiments. The slower breakdown of this drug will probably enhance its efficacy and eliminate/ reduce some of the toxic side-effects. In light of the increased efficacy and apoptosis that occurs in combination with mangiferin, further investigation into such a combination treatment is strongly recommended.

In this study the combined mechanism of the anticancer action of platinum containing complexes in combination with mangiferin was investigated. The present study indicates that mangiferin in combination with platinum anticancer drugs favours apoptotic cell death and thereby improves their efficacy *in vitro*. In addition, combination therapy with mangiferin may also counteract the development of resistance in cancer cell lines.

Unfortunately no published literature could be found to indicate how much mangiferin is present in a cup of tea such as Honeybush, nor how much mangiferin is

absorbed through the gastro-intestinal tract. Such information would assist in extrapolating our *in vitro* results to the *in vivo* situation. However, the most significant decrease in the IC50 values was seen in colon cancer cells treated with oxaliplatin in combination with mangiferin. In this instance, where the tea would be taken orally and the epithelial cells of the gastro-intestinal tract would be directly exposed to the mangiferin, the absorption of the tea is not that relevant, which renders these results even more promising.

CHAPTER 3
FINAL PERSPECTIVE ON THE PRESENT STUDY

This work was initiated in an effort to identify novel platinum compounds with improved anticancer activity, 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 should however not discourage the search for new improved anticancer agents, especially if the severity of the disease is taken into account. It is realized 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 compounds were shown to be worthy of follow-up tests, namely Yol 25 ($Pt(hme)Cl_2$), Yol 29.1 ($Pt(ama)Cl_2$) and Mar 4.1.4 ($Pt(dach)(C_2O_4)NO_2)Cl$). Furthermore, some of these complexes can be regarded as very promising for cancer treatment, since it does not induce resistance in some cancer cell lines. It was also found that the addition of mangiferin can improve the efficacy of these platinum drugs as well as reduce the degree of resistance induced by these complexes. This highlights the importance of investigating novel combination treatments with platinum drugs, in order to improve its efficacy, lessen toxic side effects as well as reduce resistance.

It would be interesting to identify the exact nature of cell cycle arrest by investigating the presence of specific cyclins and cyclin-dependent kinases. Another interesting aspect would be to investigate the effect that these compounds, alone and in combination with mangiferin, have on STAT3 activation, p53 status, and/or Bcl/ Bax family expression activation. There is thought to be a positive link between the simultaneous deactivation of STAT3 and NFκB during targeting cancer cells. These above-mentioned experiments can be performed using flow cytometry.

There is also a possibility that the toxicity of these drugs can be less than that of the drugs currently used to treat cancer. In addition, the combination of these drugs with mangiferin may further decrease these toxic side-effects. Therefore, the toxicity of these drugs (particularly nephrotoxicity and neurotoxicity), on the internal organs, alone and as a combination therapy, should be investigated. This could be performed by using, for example, Chang liver (non-malignant) and HepG2 (cancerous) cells to perform dose response curves. This will indicate whether these compounds are cyto-specific or toxic to normal liver cells.

A neuronal cell line, such as the neuron-like rat pheochromocytoma cell line, PC-12, can be used to determine the effect of these compounds, alone as well as in combination with mangiferin, on the neurons. After treatment the cells can be fixed and permeabilized before being stained with a fluorescent dye, and the neuron lengths can be observed microscopically. This may also aid in determining whether mangiferin could rescue the neurites from the damage induced by the compounds. However, since certain limitations exist for *in vitro* studies, ultimately these experiments would have to be performed *in vivo*, before proceeding to clinical trials.

The work created some new concepts, which will be worthwhile pursuing for the further search of improved anticancer agents. Based on the results obtained in this study it can be concluded that mangiferin might be used as a combination therapy with platinum anticancer drugs in order to increase the efficacy of these drugs as well as counteract the development of drug-resistant cells.

REFERENCES

Almendro, V., J. Maurel, J. Augé, G. Laus, J. Domingo-Domenech, E. Fernández and P. Gascon. 2006. Role of metalloproteinase-7 in oxaliplatin acquired resistance in colorectal cancer cell lines. *Journal of Clinical Oncology* **24**: 20042.

Anagnostopoulou, A., A. Vultair, R. Arulanandam, J. Lukson, R. Jove, J.S. Kim and A.D. Hamilton. 2006. Differential effects of Stat 3 inhibition in sparse vs confluent normal and breast cancer cells. *Cancer Letters* **242**: 120-132.

Arango, D., A. J. Wilson, Q. Shi, G. A. Corner, M. J. Aranes, C. Nicholas, M. Lesser, J. M. Mariadason and L. H. Augenlicht. 2004. Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. *British Journal of Cancer* **91:** 1931-1946.

Araya, R., R. Takahashi and Y. Nomura. 2002. Yeast two-hybrid screening using constitutive-active caspase-7 as bait in the identification of PA28γ as an effector caspase substrate. *Cell Death and Differentiation* **9**: 322-328.

Atsushi, H., S. Shuji, A. Kosuke and K. Takafumi. 1994. A comparison of *in vitro* platinum-DNA adduct formation between carboplatin and cisplatin. *International Journal of Biochemistry* **26:** 1009-1016.

Baldwin A. S. 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *Journal of Clinical Investigation* **107:** 241-246.

Bhattacharya, S. K., A. K. Sanyal and S. Ghosal. 1972. Monoamine oxidase-inhibiting activity of mangiferin isolated from Canscora decussate. *Naturwissenschaften* **59**: 651.

Bierbach, U., J. D. Roberts and N. Farrell. 1998. Modification of Pt(II) Antitumor Complexes with Sulfur Ligands. 2. Reactivity and Nucleotide Binding Properties of Cationic Complexes of the Types [PtCl(diamine)(L)]NO_3 and [{PtCl(diamine}$_2$(L-

L)]$(NO_3)_2$ (L=Monofunctional Thiourea Derivative); (L-L= Bifunctional Thiourea Derivative) in Relation to Their Cytotoxicity. *Inorganic Chemistry* **37**: 717-723.

Bosch, F. X., A. Lorincz, N. Muñoz, C. J. L. M. Meijer, K.V. Shah. 2002. The causal relation between human papillomavirus and cervical cancer. *Journal of Clinical Pathology* **55**: 244-265.

Bouwer, Y. December 2008. Nelson Mandela Metropolitan University. PhD Thesis.

Chao C. C. K. 1994. Decreased Accumulation as a Mechanism of Resistance to *cis*-diamminedichloroplatinum (II) in Cervix Carcinoma HeLa Cells: Relation to DNA Repair. *Molecular Pharmacology* **45**: 1137-1144.

Chao, C. C. K. 1996. Molecular Basis of *cis*-Diamminedichloroplatinum (II) Resistance: A Review. *Journal of the Formosan Medical Association* **95**: 893-900.

Chen, Y, Z. Guo, J. A. Parkinson and P. J. Sadler. 1998. Kinetic control of reactions of a sterically hindered platinum picoline anticancer complex with guanosine 5'-monophosphate and glutathione. *Journal of the Chemical Society, Dalton Transactions*: 3577-3585.

Choi, C., Y. Cha, C. An, K. Kyung-Jong, K. Kweon-Cheon, S. Moon, Z. H. Lee and Y. Min. 2004. Molecular mechanisms of heptaplatin effective against cisplatin-resistant cancer cell lines: less involvement of metallothionein. *Cancer Cell International* **4** (Online: http://www.canceri.com/content/4/1/6).

Choi, S., C. Filotto, M. Bisanzo, S. Delaney, D. Lagasee, J. L. Whitworth, A. Jusko, C. Li, N. A. Wood, J. Willingham, A. Schwenker and K. Spaulding. 1998. Reduction and Anticancer Activity of Platinum (IV) Complexes. *Inorganic Chemistry* **37**: 2500-2504.

De Souza Pagnussat, A., M. C. Faccioni-Heuser, C. A. Netto and M. Achava. 2007. An ultrastructural study of cell death in the CA1 pyramidal field of the

hippocapmus in rats submitted to transient global ischemia followed by reperfusion. *Journal of Anatomy* **211**: 589-599.

Drisko, J.A., J. Chapman and V.J. Hunter. 2003a. The use of antioxidants with first-line chemotherapy in two cases of ovarian cancer. *Journal of the American College of Nutrition* **22**: 118-123.

Drisko, J.A., J. Chapman and V.J. Hunter. 2003b. The use of antioxidant therapies during chemotherapy. *Gynecologic Oncology* **88**: 434-439.

Duncan, A. C., A. K. Jager, J. van Staden. 1999. Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. *Journal of Ethnopharmacology* **68:** 63–70

Dunfield, L. D. and D. L. Guernsey. 2002. Platinum Resistance in Ovarian Cancer. *Dalhousie Medical Journal*, Fall 2002 Issue.

Enten, J. and M. Monson. 2005. Beckman-Coulter Application Information. DNA Cell cycle analysis with 4′, 6-Diamidino-2-Phenylindole (DAPI) or Propidium Iodide (PI) nuclear stains, (A-2012A).

Eshraghi, A. A., M. Bublik and T. R. Van De Water. 2006. Mechanisms of chemotherapeutic-induced hearing loss and otoprotection. *Drug Discovery Today: Disease Mechanisms* **10**: 1016.

Filipits, M. 2004. Mechanisms of cancer: multidrug resistance. *Drug Discovery Today: Disease Mechanisms* **1**: 229-234.

Frouin, I., A. Montecucco, G. Biamonti, U. HuÈbscher, S. Spadari and G. Maga. 2002. Cell cycle-dependent dynamic association of cyclin/Cdk complexes with human DNA replication proteins. *The EMBO Journal* **21:** 2485-2495.

Fujie, Y., H. Yamamoto, C. Y. Ngan, A. Takagi, T. Hayashi, R. Suzuki, K. Ezumi, I. Takemasa, M. Ikeda, M. Sekimoto, N. Matsuura and M. Monden.

2005. Oxaliplatin, a Potent Inhibitor of Survivin, Enhances Paclitaxel-induced Apoptosis and Mitotic Catastrophe in Colon Cancer Cells. *Japanese Journal of Clinical Oncology* **35**: 453–463.

Galilehvand, F. and L. Laffin. 2008. Structure of the Hydrated Platinum(II) Ion and the cis-Diammineplatinum(II) Complex in Acidic Aqueous Solution : An EXAFS Study. *Inorganic Chemistry* **47**: 3248-3254.

Garland, S. M. 2003. Cancer Screening. Can we really beat cervical cancer? *Medical Journal of Australia* **178:** 647-650.

Gill, J. S. and A. J. Windebank. 1998. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. *Journal of Clinical Investigation* **101**: 2842-2850.

Go, R. S. and A. A. Adjei. 1999. Review of the Comparative Pharmacology and Clinical Activity of Cisplatin and Carboplatin. *Journal of Clinical Oncology* **17:** 409-422.

Godwin, A. K., A. Meister, P. J. Dwyer, C. S. Huang, T. C. Hamilton and M. E. Anderson. 1992. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proceedings of the National Academy of Sciences, USA* **89**: 3070-3074.

Gonzalez, V. M., M. A. Fuertes, C. Alonso and J. M. Perez. 2001. Is Cisplatin-Induced Cell Death Always Produced by Apoptosis. *Molecular Pharmacology* **59**: 657-663.

Gupta, R., J. L. Beck, M. M. Sheil and S. F. Ralph. 2004. Identification of binuclear GA and AG intrastrand crosslinks formed between cisplatin and DNA. *Journal of Inorganic Biochemistry*. (Online: www.elsevier.com/locate/jinorgbio).

Hagopian, G. S., G. B. Mills, A. R. Khokhar, R. C. Bast, Jr., and Z. H. Siddik. 1999. Expression of *p53* in Cisplatin-resistant Ovarian Cancer Cell Lines:

Modulation with the Novel Platinum Analogue (*1R, 2R*-Diaminocyclohexane)(*trans*-diacetato)(dichloro)-platinum (IV). *Clinical Cancer Research* **5**: 655-663.

Hambley, T. W. 1997. The Influence of Structure on the Activity and Toxicity of Platinum Anticancer Drugs. *Coordination Chemistry Reviews* **166**: 181-223.

Hector, S., W. Bolanowska-Higdon, J. Zdanowict, S. Hitt and L. Pendyala. 2001. In vitro studies on the mechanisms of oxaliplatin resistance. *Cancer Chemotherapy Pharmacology* **48**: 398-406.

Helm, C. W. and J. C. States. 2009. Enhancing the efficacy of cisplatin in ovarian cancer treatment – could arsenic have a role. *Journal of Ovarian Research* **2:** (doi:10.1186/1757-2215-2-2). Online: http://www.ovarianresearch.com/content/2/1/2

Helberg, V., I. Wallin, S. Eriksson, E. Hernlund, E. Jerremalm, M. Berndtsson, S. Eksborg, E. S. J. Arnér, M. Shoshan, H. Ehrsson and G. Laurell. 2009. Cisplatin and Oxaliplatin Toxicity: Importance of Cochlear Kinetics as a Determinant for Ototoxicity. *Journal of the National Cancer Institute* **101**: 37-47.

http://chemcases.com/cisplat/).

http://science.kennesaw.edu/~mhermes/cisplat/cisplat13.htm

Huang, J. C., D. B. Zamble, J. T. Reardon, S. J. Lippard and A. Sancar. 1994. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proceedings of the National Academy of Sciences USA* **91:** 10394-10398.

Huq, F., H. Daghriri, J. Q. Yu, H. Tayyem, P. Beale and M. Zhang. 2004. Synthesis, characterization, activities, cell uptake and DNA binding of [{*trans*-$PtCl(NH_3)_2$} {μ-($H_2N(CH_2)_6NH2$)}{*trans*-$PdCl(NH_3)_2$](NO_3)Cl. *European Journal of Medicinal Chemistry* **39**: 947-958.

Ibrahim, A., S. Hirschfeld, M. H. Cohen, D. J. Griebel, G. A. Williams and R. Pazdur. 2004. FDA Drug approval summaries: Oxaliplatin. *The Oncologist* **9**: 8-12.

Jamieson, E. R. and S. J. Lippard. 1999. Structure, Recognition and Processing of Cisplatin-DNA Adducts. *Chemistry Reviews* **99**: 2467-2498.

Jordan, P. and M. Carmo-Fonseca. 2000. Molecular mechanisms involved in cisplatin cytotoxicity. *Cellular and Molecular Life Sciences* **57**: 1229-1235.

Jung, K., L. Bomi, J. H. Sang, H. R. Jong and K. Dong-Hyun. 2009. Mangiferin Ameliorates Scopolamine-Induced Learning Deficits in Mice. *Biological and Pharmaceutical Bulletin* **32:** 242-246.

Katano, K., A. Kondo, R. Safaei, A. Holzer, G. Samimi, M. Mishima,Y. Kuo, M. Rochdi and S. B. Howell. 2002. Acquisition of Resistance to Cisplatin Is Accompanied by Changes in the Cellular Pharmacology of Copper. *Cancer Research* **62**: 6559-6565.

Katsuyuki, H. and Keitaro, M. 2007. Long-Standing Debate on Cisplatin- Versus Carboplatin-Based Chemotherapy in the Treatment of Advanced Non-small Cell Lung Cancer. *Journal of Thoracic Oncology* **2:** 96.

Khosravi-Far, R. and Esposti, M. D. 2004. Death receptor signals to mitochondria. *Cancer Biology and Therapy*. **3:** 1051-1057.

Kwon, H. J., Y. K. Hong, K. H. Kim, C. H. Han, S. H. Cho, J. S. Choi, Byung-Woo Kim. 2005. Methanolic extracts of *Pterocarpus santalinus* induces apoptisis in HeLa cells. *Journal of Ethnopharmacology* **105**: 229-234.

Lagunas, V. M. and J. Melendez-Zajgla. 2008. Nuclear Factor-kappa B as a Resistance Factor to Platinum-Based Antineoplasic Drugs. *Metal-Based Drugs* **2008:** Article ID 576104 (doi:10.1155/2008/576104).

Lawenda, B. D., K. M. Kelly, E. J. Ladas, S. M. Sagar, A. Vickers and J. B. Blumberg. 2008. Should supplemental antioxidant administration be avoided during chemotherapy and radiation therapy? *Journal of the national cancer institute* **100:** 773-783.

Lee, T. L., J. Yeh, J. Friedman, B. Yan, X. Yang, N. T. Yeh, C. van Waes and Z. Chen. 2008. A signal network involving coactivated NF-kappaB and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas. *International Journal of Cancer* **122:** 1987-1998.

Leiro J., J. A. Arranza, M. Yanez, F. M. Ubeira, M. L. Sanmartın, F. Orallo. 2004. Expression profiles of genes involved in the mouse nuclear factor-kappa B signal transduction pathway are modulated by mangiferin. *International Immunopharmacology* **4:** 763-778.

Lem, J. J. and A. Cappione. 2005. Flow cytometry: A Mainstream Drug Discovery Tool. *Innovations in Pharmaceutical Technology* **18:** 32-36.

Lemasters, J. J. 1999. Mechanisms of Hepatic Toxicity vs Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *American Journal of Physiology: Gastrointestinal and Liver Physiology* **276**: 1-6.

Lidor, Y. J., K. C. O'Briant, F. J. Xu, T. C. Hamilton, R. F. Ozols and R. C. Bast, Jr. 1993. Alkylating Agents and Immunotoxins Exert Synergistic Cytotoxic Activity against Ovarian Cancer Cells. Mechanism of Action. *Journal of Clinical Investigation*: **92**: 2440-2447.

Los, G., D. Gately, M. L. Costello, F. Thiebaut, P. Naredi and S. B. Howell. 1996. Membrane transport of Platinum Compounds. Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy, Vol. 2., Plenum Press, New York.

Louw, M. December 2008. Nelson Mandela Metropolitan University. MSc Dissertation.

Mabuchi S., M. Ohmichi, Y. Nishio, T. Hayasaka, A. Kimura, T. Ohta, M. Saito, J. Kawagoe, K. Takahashi, N. Yada-Hashimoto, M. Sakata, T. Motoyama, H. Kurachi, K. Tasaka and Y. Murata. 2004. Inhibition of NFkB increases the efficacy of Cisplatin in in vitro and in vivo ovarian cancer models. *The Journal of Biological Chemistry* **10:** 1-39.

Marnewick, J., W. C. A. Gelderblom and E. Joubert. 2000. An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research* **471**: 157–166.

Marnewick, J. L., W. Batenburg, P. Swart, E. Joubert, S. Swanevelder, P. Swart and W. C. A. Gelderblom. 2004. *Ex vivo* modulation of chemical-induced mutagenesis by subcellular liver fractions of rats treated with rooibos (*Aspalathus linearis*) tea, honeybush (*Cyclopia intermedia*) tea, as well as green and black (*Camellia sinensis*) teas. *Mutation Research* **558**: 145–154

Marnewick, J., E. Joubert, S. Josepha, S. Swanevelder, P. Swart and W. Gelderblom. 2005. Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas. *Cancer Letters* **224**: 193–202.

Martin, A. G., G. F. Lockwood, D. Greenslade, S. Brienza, M. Bayssas and E. Gamelin. 2000. Clinical Pharmacokinetics of Oxaliplatin: A Critical Review. *Clinical Cancer Research* **6**: 1205–1218.

Meurette, O., L. Lefeuvre-Orfila, A. Rebillard, D. Lagadic-Gossmann and M. Dimanche-Boitrel. 2005. Role of Intracellular Glutathione in Cell Sensitivity to the Apoptosis Induced by Tumor Necrosis Factor α - Related Apoptosis-Inducing Ligand/Anticancer Drug Combinations. *Clinical Cancer Research* **11:** 3075-3083.

Min, J., A. L. Stegner, H. Alexander and S. Alexander. 2004. Overexpression of Sphingosine-1-Phosphate Lyase or Inhibition of Sphingosine Kinase in *Dictyostelium discoideum* Results in a Selective Increase in Sensitivity to Platinum-Based Chemotherapy Drugs. *Eukaryotic cell* **3**: 795-805.

Minami, S. B., S. Sha and J. Schacht. 2004. Antioxidant protection in a new animal model of cisplatin-induced ototoxicity. *Hearing Research* **198**: 137-143.

Muruganandan S, Srinivasan K, Gupta S, Gupta PK and Lal J. 2005. Effect of mangiferin on hyperglycemia and atherogenicity in streptozotocin diabetic rats. *Journal of Ethnopharmacology* **97**: 497-501.

Osama, K., A., Gaber, M. G. Shehab and R. M. Romeilah. 2009. Effects of *Rosmarinus Officinalis* Extract on Induced Nitric Oxide and Stimulated ProinXammatory Mediators Via- Bacterial Lipopolysaccharide (LPS). *Australian Journal of Basic and Applied Sciences* **3**: 1223-1233.

Ozturk, G., E. Erdogan, O. Anlar, M. Kosem and M. Taspinar. 2005. Effect of leukaemia inhibitory factor in experimental cisplatin neuropathy in mice. *Cytokine* **29:** 31-41.

Parker, S. H., T. Tong, S. Bolden and P. A. Wingo. 1997. Cancer Statistics, 1997. *CA: A Cancer Journal for Clinicians* **47**: 5-27.

Paul, A. T., V. M. Gohil and K.K. Bhutani. 2006. Modulating TNF-α signaling with natural products. *Drug Discovery Today* **11:** 725-732.

Perez, R. P. 1998. Cellular and Molecular Determinants of Cisplatin Resistance. *European Journal of Cancer* **34**: 1535-1542.

Perkins, N. D. 2000. The Rel/NF-kB family: friend and foe. *Trends In Biochemical Sciences* **25:** 434-440.

Perkins, N. D. 2004. NF-kB: tumor promoter or suppressor? *TRENDS in Cell Biology* **14:** 64-69.

Petit, T., D. J. Bearss, D. A. Troyer, R. M. Munoz and J. J. Windle. 2003. p53-independent Response to Cisplatin and Oxaliplatin in MMTV-*ras* Mouse Salivary Tumors. *Molecular Cancer Therapeutics* **2**: 165-171.

Picard, S., N. Franco, C. Sergent, B. Chauffert and S. Lizard-Nacol. 2002. Analysis of microsatellite instability in acquired drug-resistance human tumor cell lines. *Oncology Reports* **9**: 971-976.

Rakitina T. V., I. A. Vasilevskaya and P. J. O'Dwyer. 2007. Inhibition of G1/S transition potentiates oxaliplatin-induced cell death in colon cancer cell lines. *Biochemical pharmacology* **73:** 1715-1726.

Reedijk, J. 1999. Why does Cisplatin Reach Guanine-N7 with Competing S-Donor Ligands Available in the Cell? *Chemistry Reviews* **99**: 2499-2510.

Roberts, J. D., J. Peroutka and N. Farrell. 1999. Cellular pharmacology of polynuclear platinum anti-cancer agents. *Journal of Inorganic Biochemistry* **77**: 51-57.

Rogozinska-Szczepka, J., B. Utracka-Hutka, E. Grzybowska, B. Maka, E. Nowicka, A. Smok-Ragankiewicz, H. Zientek, J. Steffen and A. Wojciechowska-Łacka. 2004. BRCA1 and BRCA2 mutations as prognostic factors in bilateral breast cancer patients. *Annals of Oncology* **15:** 1373-1376.

Rosell, R., M. Taron, A. Barnadas, G. Scagliotti, C. Sarries and B. Roig. 2003. Nucleotide Excision Repair Pathways Involved in Cisplatin Resistance in Non-Small-Cell Lung Cancer. *Cancer Control* **10**: 297-304.

Sadler, P. J. and Z. Guo. 1998. Metal complexes in medicine: Design and mechanism of action. *Pure and Applied Chemistry* **70**: 863-971.

Sarkar, A., Y. Sreenivasan, G. T. Ramesh and S. K. Manna. 2004. β-D-Glucoside Suppresses Tumor Necrosis Factor-induced Activation of Nuclear Transcription Factor κB but Potentiates Apoptosis. *Journal of Biological Chemistry* **279**: 33768-33781.

Scheeff, E. D., J. M. Briggs and S. B. Howell. 1999. Molecular Modelling of the Intrastrand Guanine-guanine DNA Adducts Produced by Cisplatin and Oxaliplatin. *Molecular Pharmacology* **56**: 633-643.

Sheikh-Hamad, D. 2008. Cisplatin-induced cytoxicity: is the nucleus relevant? *American Journal of Physiology: Renal Physiology* **295**: F42-F43.

Sergent, C., N. Franco, C. Chapusot, S. Lizard-Nacol, N. Isambert, M. Correia and B. Chauffert. 2002. Human colon cancer cells surviving high doses of cisplatin or oxaliplatin *in vitro* are not defective in DNA mismatch repair proteins. *Cancer Chemotherapy Pharmacology* **49**: 445-452.

Siddik, Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22:** 7265-7279.

Tang, S. Y., M. Whiteman, A. Jenner, Z. F. Peng and B. Halliwell. 2004. Mechanism of cell death induced by an antioxidant extract of *Cratoxylum cochinchinense* (YCT) in Jurkat T cells: The role of reactive oxygen species and calcium. *Free Radical Biology and Medicine* **36**: 1588-1611.

Tobe, M. L. 1972. **Inorganic reaction mechanisms,** Thomas Nelson and Sons Ltd, London, pp 42-68.

Tryggvadottir, L., E. J. Olafsdottir, S. Gudlaugsdottir, S. Thorlacius, J. G. Jonasson, H. Tulinius and J. E. Eyfjord. 2003. BRCA2 mutation carriers, reproductive factors and breast cancer risk. *Breast Cancer Research* **5:** R121-R128.

Turchi, J. J. 2006. Nitric oxide and cisplatin resistance: NO easy answers. *Proceeding of the National Academy of Sciences* **103:** 4337-4338.

Turkson, J., S. Zhang, J. Palmer, H. Kay, J. Stanko, L. B. Mora, S. Sebti, H. Yu and R. Jove. 2004. Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent antitumor activity. *Molecular Cancer Therapeutics* **3**: 1533-1542.

Venook, A. 2005. Critical Evaluation of Current Treatments in Metastatic Colorectal Cancer**.** *The Oncologist* **10:** 250-261.

Voland, C., A. Bord, A. Peleraux, G. Penarier, D. Carriere, S. Galiegue, E. Cvitkovic, O. Jbilo and P. Casellas. 2006. Repression of cell cycle–related proteins by oxaliplatin but not cisplatin in human colon cancer cells. *Molecular Cancer Therapeutics* **5**: 2149-2157.

Wajant, H. 2003. Death receptors. *Essays in Biochemistry* **39**: 53-71.

Walker, E. M. and S. M. Walker. 1999. Evolution of chemotherapy with platinum compounds. *Annals of Clinical and Laboratory Science* **29**: 263-274.

Wheate, N. J., C. Cullinane, L. K. Webster and J. G. Collins. 2001. Synthesis, cytotoxicity, cell uptake and DNA interstrand cross-linking of 4'4'-dipyrazolylmethane-linked multinuclear platinum anti-cancer complexes. *Anti-Cancer Drug Design* **16**: 91-98.

Wilkinson, G. 1987. Comprehensive coordination chemistry, Late Transition Elements, Vol 5, Pergamon Press, Oxford.

Winterhalder, R., S. Deschler-Marini, C. Landmann, R. Kann, J. Passweg, R. Herrmann and M. Pless. 2004. Vinorelbine plus low-dose cisplatinum with concomitant radiotherapy for the treatment of locally advanced or inoperable non-metastasized non-small-cell lung cancer (stage I-IIIB): a phase II study. *Radiotherapy and Oncology* **73:** 321-324.

Wong, A. and J. A. Cortopassi. 2002. High-throughput measurement of mitochondrial membrane potential in a neural cell line using a fluorescence plate reader. *Biochemical and Biophysical Research Communications* **298**: 750-754.

Wozniak, K. and J. Blasiak. 2002. Recognition and repair of DNA-cisplatin adducts. *Acta Biochimica Polonica* **49**: 583-596.

www.chemocare.com

Yang, X., T. L. Sladek, X. Liu, B. R. Butler, C. J. Froelich and A. D. Thor. 2001. Reconstitution of Caspase 3 Sensitizes MCF-7 Breast Cancer Cells to Doxorubicin- and Etoposide-induced Apoptosis. *Cancer Research* **61**: 348-354.

Zutphen, S., E. Pantoja, R. Soriano, C. Soro, D.M. Tooke, A.L. Spek, H. den Dulk, J. Brouwer and J. Reedijk. 2006. New antitumour active platinum compounds containing carboxylate ligands in *trans* geometry: synthesis, crystal structure and biological activity. *Dalton Transactions:* 1020-1023.