# Biological Evaluation of *N*-Ferrocenyl Amino Acid and Dipeptide Ester Derivatives as Potential Anti-Cancer Agents and investigation into their mode of action in preparation of *in vivo* evaluation.

by

James Murphy B.Sc.

A thesis presented for the degree of M.Sc

at

Dublin City University

Under the supervision of Dr. Peter T. M. Kenny

and

Dr. Norma O'Donovan


*OllScoil Chathair Bhaile Átha Cliath*

*School of Chemical Sciences*

*January 2014*

*I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of MSc, is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.*

**Signed:** 
**ID No.: 56472761**
**Date: 29-09-2013**

# Acknowledgements

First and foremost, I would like to give my utmost gratitude to Dr. Norma O'Donovan. Without your help this thesis would surely have fallen at many hurdles along the way. Your patience, knowledge and ability to be on top of 5 or more projects at any one time, while the rest of struggle to keep their head above board on their own will never fail to astound me.

To the rest of the group under Norma's tutelage, Alex, Denis, Martina, Karen, Trish, Alexandra, it was a pleasure to work with you all and hope I haven't forgotten anyone. Lab meeting and and lab clean-ups didn't seem all that bad in hind sight.

To Dr. Peter Kenny I would like to thank you for giving me the opportunity to undertake this work and for introducing me to all of the PKRG, both past and present. Christmas "lunch" is something to look forward to every year and although I might miss the first one or two after finishing up, I promise i'll be back to pitch in at some stage.

I would like to thank the HRB (Health Research Board) for providing funding for this endeavour. It could not have been done without it.

As this is acknowledgments, I must acknowledge what some might call an "addiction" to diet coke. When it gets to 4 in the morning and you don't drink coffee, it's always handy to have a pit of a pick-me-up

To Jess, although we may have been continents apart for much of this work, and time difference for skype may have kept me from work now and then. To hear and see you can make it all better. I love you very much.

To my cousins, Ciara, Aoife and Siobhán for stepping in at the last minute and doing some trans-atlantic delivery work, I can't thank you enough. I wish you all great success and your education and futures.

To my family, thanks for the support along the way. Brian, always good to talk shop but I think we both know who is the driving force behind much of our "success".
Mam, I can honestly say that it wasn't the nagging that got this finished, but rather the hope to make your proud. I love you very much and wish to dedicate this thesis to you.

# Table of Contents

## Abstract

The aim of this research thesis was to explore the structure-activity relationship (SAR) of ferrocenyl-peptide bioconjugates and to explore methods by which these compounds exert their anti-proliferative on cancer cells. Furthermore, the applicability across different cell types including cancer cells which have acquired resistance to chemotherapeutics and non-cancerous cells was investigated. Finally, we attempted to overcome the insolubility of the ferrocene compounds in a liquid which is suitable for delivery *in vivo*.

We evaluated a panel of novel ferrocenyl-peptide bioconjugates for their anti-cancer potential in lung and melanoma cells. Utilizing structure-activity relationship (SAR) studies we found the anti-proliferative activity of the compounds tested varies greatly depending on the configuration of the compound. Two of the most active compounds *N*-(6-ferrocenyl-2-naphthoyl)-glycine-glycine ethyl and methyl ester showed $IC_{50}$ values in the nanomolar range. Cisplatin was used as a comparison and was found to have higher $IC_{50}$ values.

From these SAR studies the following trends were observed:

1. 1-alkyl-1'- *N-ortho*- derivatives exhibited lower growth inhibition compared to the *meta* and *para* derivatives for the dipeptides employed
2. Cytotoxicity of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters, decreases with the increase of the size of the alkyl group incorporated (propyl < ethyl < methyl) on the ferrocene moiety
3. The order of the amino acids in the dipeptide chain is crucial for activity, the trend being Gly Gly > Gly L-Ala > Gly L-Leu > Gly L-Phe.
4. The presence of the ethynyl moiety does not result in any significant enhancement of the anti-proliferative effect of the *N*-(ferrocenyl) benzoyl and naphthoyl dipeptide derivatives.
5. Replacing the ethyl ester in the previously most active, *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester, with a methyl ester further increases its activity.

Two of the biggest factors which affect the use of chemotherapeutic drugs in a clinical setting are the side-effects caused by the drug and also resistance of the

cancer cells to the drug. We have addressed this issue by testing the most active compounds from the SAR studies in cell lines which have been made resistant to two common chemotherapeutics, cisplatin and temozolomide. We also tested the two most active compounds in normal human dermal fibroblast (NHDF) cells as an indicator of the effect which the compounds would have on healthy tissue. Results were favourable as one of the compounds was able to overcome the resistance and both compounds showed less cytotoxicity against NHDF than cisplatin.

We analysed the effect the most active compound had on the cell cycle in lung cancer and melanoma cells. We observed an increase in the sub-$G_0$ fraction which is indicative of apoptosis. Induction of apoptosis was confirmed using the TUNEL assay which detects late stage apoptotic cells.

Due to the insolubility it was not possible to test the compounds *in vivo*. However, we tested a range of approaches to improve their solubility for future *in vivo* testing.

From the results gathered, a number of conclusions can be made. It is clear that chemical structure of the ferrocenyl compounds plays an important role in their activity levels. A key aspect in the mode of action of the compound tested is the induction of apoptosis/necorsis. With resistance to chemotherapeutics being an ongoing problem in cancer treatment, the ability of a compound to overcome resitance to important chemotherapeutics while having a significantly lower activity level in normal human cells is an appealing characterstic of the tested compound. Finally, the issue with solubility has, at the time of writing this research thesis, been unsolved. However, with promising research being undertaken in the field of improving solubility of compounds, the future looks hopeful.

# Chapter 1

# Introduction

## 1.1 Cancer

The term “cancer” is used to describe a disease distinguishable by the abnormal growth of cells which ultimately evolves into a population of cells that can invade tissues and spread to other parts of the body. At a molecular level, cancer cells are characterised by numerous alterations in multiple gene expression which leads to deviation from the normal program for cell division and cell differentiation [1]. The growth of the tumour cell population is promoted by an imbalance between cell replication and cell death. Over time, this aberrant growth of cancer cells progresses to form a malignant tumour capable of invading locally, spreading to regional lymph nodes and metastasising to distant organs within the body. Thus, if cancer cell growth is allowed to continue unchecked over a prolonged period, this disease can prove to be life threatening and even fatal to the patient.

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [2]. Cancer is a major worldwide health problem that results in a huge loss of life every year. About 12.7 new million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 world-wide [3], which despite major advances is cancer treatment is a rise on the 2002 figures of 10.9 million new cancer cases and 6.7 million cancer associated deaths [2]. By 2020, the world population is expected to have increased to 7.5 billion; of this number, approximately 15 million new cancer cases will be diagnosed, and 12 million cancer patients will die [4].

The possibility of developing cancer is dependent on a number of risk factors which can vary according to the tumour location, for example, there is an increased risk of developing melanoma following over-exposure to ultraviolet (UV) radiation. Tobacco is the greatest risk factor in the development of many cancers (lung, larynx, oesophagus, stomach, pancreas, kidney, liver and bladder). Other factors include genetic predisposition, lack of balanced diet and physical activity, obesity, alcohol consumption, exposure to certain chemicals and gases (asbestos, benzene and radon gas), exposure to ultraviolet or ionising radiation, infections such as the human papillomavirus (HPV) and treatments such as exposure to estrogen through Hormone Replacement Therapy (HRT) [5].

In Ireland, the most common forms of cancer in males are cancers of the prostate, colon, lung and lymphoma whereas among women cancers of the breast, colon, lung and ovaries are the most common [6].

Five-year survival rates can be used to gain a more accurate picture of the most lethal forms of cancer that affect the Irish population. Survival rates are primarily dependent on the location of the tumour, since this influences the ease with which the tumour can be detected, which in turns aids detection and treatment at an early stage. As a consequence, survival rates for the many distinct forms of cancer show a large variation. Perhaps the most striking example is the survival rate for males diagnosed with pancreatic cancer (5.4 %) compared to that observed for males diagnosed with testicular cancer (96.9 %) [6].

### 1.1.1 Lung Cancer

Lung cancer accounted for 13 % (1.6 million) of the cancer cases and 18 % (1.4 million) of cancer deaths in 2008. Smoking accounts for 80 % of the worldwide lung cancer burden in males and at least 50 % of the burden in females [7, 8]. Cigarette smoke exposure is a major causative factor with approximately 87 % of lung-cancer cases resulting from this single cause [9]. The majority of cases present with advanced disease and are typically associated with a less than 5 year survival duration. The disease can progress significantly before symptoms develop. However, there is generally an increase in occurrence of the common symptoms of expectoration and cough over time in clinical cases [10]. Lung cancers can be histologically classified into two main groups, non small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) with 80 % of cases falling into the first group and the remaining 20 % into the second [11]. The staging of lung cancers is carried out in order to determine treatment regimes and to compare efficacies of new treatments across clinical trials. This is generally based on the (TNM) classification, where T represents the scale of the primary tumour, N represents the lymph node involvement and M represents the presence metastasis [12, 13].

Chemotherapy is the mainstay treatment for small cell lung cancer as surgery is often not an option due to most patients presenting with metastasis. Cisplatin, carboplatin, doxorubicin, vincristine, paclitaxel and docetaxel are among the active

chemotherapy drugs approved in SCLC treatment. Single agent therapy produces a more short lived response and so combination therapy is the standard care. Although response rates are high (75-80 %), most patients will suffer from relapsed disease [14-16]. Many single anti-cancer agents have activity against NSCLC and these include cisplatin, carboplatin, paclitaxel, docetaxel, vinorelbine and gemcitabine; however, a modest increase in response rates is seen with combinations and so platinum-based combinations form the standard care [17,18]. Paclitaxel or docetaxel in combination with cisplatin has been recommended as an option in first-line treatment of advanced NSCLC. Surgical resection is more commonly employed in non small cell lung cancer treatment and carried out where possible [18].

### 1.1.2 Melanoma

Melanoma is a sub-type of skin cancer. One in every three cancers diagnosed worldwide is a skin cancer [19]. Although melanoma accounts for less than 5 % of all skin cancers, it is responsible for the majority of skin cancer-related mortality [20]. It is estimated that 76,690 will be diagnosed with, and 9,480 people will die of melanoma of the skin in 2013. The overall 5-year relative survival for 2003-2009 from 18 SEER (Surveillance, Epidemiology, and End Results) geographic areas was 91.3 %. Fortunately, the overall death rate attributed to melanoma has not significantly increased since 1990 [21]. Approximately 87 % of all patients with melanoma are ultimately cured of their cancer, a number that is even higher with early detection [22]. Exposure to UV radiation is the most significant environmental factor contributing to melanoma [23]. Multiple studies now clearly demonstrate a significant correlation between tanning bed use and melanoma [24-27].

There are number of prognostic factors that are an indicator of the severity of the melanoma. The status of the regional lymph nodes is the single most important prognostic factor for overall survival [28, 29]. Metastasis to regional lymph nodes increases the chance of mortality from melanoma substantially. Other prognostic factors, in order of importance, are Breslow thickness, ulceration, mitotic rate, age, anatomical location of the primary tumour, and gender [30-47].

Breslow thickness describes a system of stratification that measured the depth of invasion as vertical thickness of the melanoma in millimetres [48]. As one would

predict, the prognosis worsens as the thickness of the melanoma increases. Using Breslow thickness, melanomas are commonly considered to be thin (≤1 mm), of intermediate thickness (>1-4 mm), or thick (>4 mm).

Ulceration is defined pathologically as the absence of an intact epithelium overlying the melanoma and has emerged as a very robust predictor of prognosis. Patients with ulcerated melanomas have a worse prognosis than those with nonulcerated melanomas [31-37]

Several other factors have minor effects on prognosis. Age affects prognosis: older patients have a greater risk of melanoma mortality than do younger patients [47]. Patients with axial (trunk, head, and neck) melanomas have a worse prognosis than patients with extremity tumours [43]. Women have a better prognosis than men, for reasons that are not clear [44].

If identified early, surgical excision is an effective treatment of melanoma. Among patients diagnosed with malignant melanoma in SEER registries, wide-excision surgery is the primary treatment for 31 % of patients with stage I disease, 46 % of patients with stage II disease, 53 % of patients with stage III disease, and 9 % of patients with stage IV disease [49]. The importance of early detection cannot be underestimated. This can be seen by the 5-year survival rates. For those with localized melanoma, the 5-year survival rate is 98.2 %; 5-year survival rates for individuals with regional and distant stage disease are 61.7 % and 15.2 %, respectively [49].

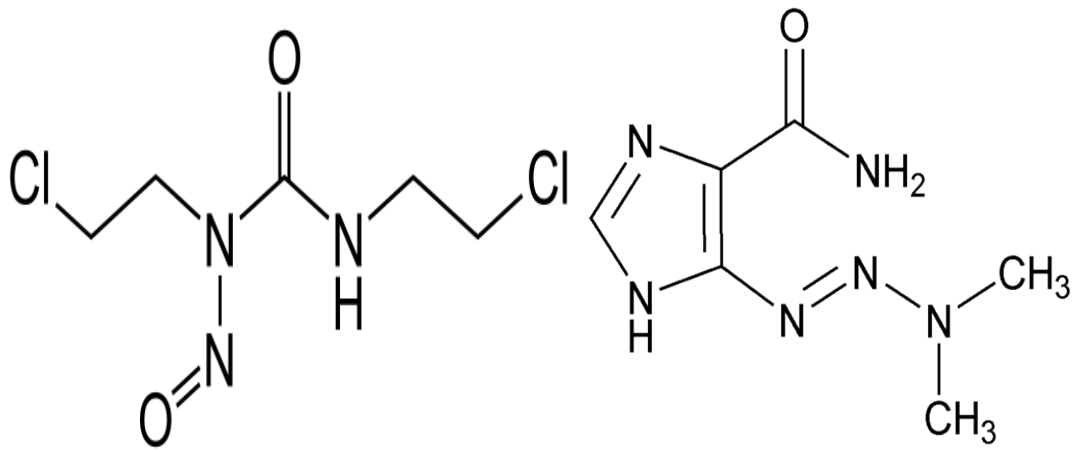
The reason for the poor prognosis to melanoma which has metastasized is the low sensitivity to systemic treatments. These treatments include chemotherapy, immunotherapy and radiation therapy.

The most widely used single chemotherapeutic agent is dacarbazine (DTIC). DTIC is a non classical alkylating agent whose metabolite methylates DNA to exert its cytotoxic activity [50]. Overall response rates are low at 10 – 20 %, with complete remission observed in < 5 % of patients [51]. Such poor responses to single agent chemotherapy led to investigations into combinations of chemotherapeutics. The most common combined chemotherapy regimens used as standard for the treatment of metastatic melanoma are the Dartmouth regimen and CVD regimen. The

Darthmouth regimen is a four-drug combination comprising cisplatin (3a), carmustine (fig. 1a), dacarbazine (fig. 1b) and Tamoxifen (fig. 5a). The CVD regimen is a combination of cisplatin, vinblastine and dacarbazine [52]. In the randomized experiments to date, it has been reported that no drug or combination of therapies is superior to dacarbazine alone [52]. Several randomised, controlled trials have compared single-agent dacarbazine with combined chemotherapy. The results from these studies demonstrated that dacarbazine is as effective as combined chemotherapy regimens, with an advantage that it is better tolerated and with fewer side-effects [53-55]. Dacarbazine is also convenient to administer and inexpensive [53]. Further testing and randomised trials have shown that the Dartmouth regime has actually little or no benefit compared to the single agent dacarbazine treatment. The associated toxicity with the treatment is so severe that high fatality rates were recorded. The response rate of the regimen was short and long-term remissions from the disease were rare [56]. Although the benefits of dacarbazine are modest, it remains the reference treatment for Stage IV melanoma and is the only chemotherapeutic agent approved by the US FDA for the therapy of metastatic melanoma.

Over the last decade, significant progress has been made in the systemic treatment of stage IV melanoma. It has long been suspected that defects in the host immune system play a major role in the pathway for melanoma tumorigenesis and metastasis. Regulatory pathways that limit the host's immune response have become a particularly interesting molecular target for treatment of metastatic melanoma. Cytotoxic T-lymphocyte-associated antigen (CTLA-4) has been described as an immune checkpoint molecule that downregulates the pathways of T cell activation [57,58]. Ipilimumab is a monoclonal antibody that blocks CTLA-4 to promote antitumor immunity [58]. In 2004, a randomized, controlled, multicenter phase III trial was initiated for patients with unresectable stage III or stage IV melanoma whose disease had progressed while receiving standard systemic therapy [59]. This was the first randomized, controlled trial to show a significant improvement in overall survival for patients with metastatic melanoma treated with systemic therapy, increasing the median overall survival of 6.4 months for the gp100 (a synthetic peptide cancer vaccine) vaccine alone to 10.0 months when given in combination with the Ipilimumab [59].

Another molecular pathway that has been shown to play a role in the progression of melanoma is the mitogen-activated protein kinase pathway, which is regulated by the protein, V-raf murine sarcoma viral oncogene homolog B1 (BRAF). Mutations in BRAF are found in 40 % - 60 % of cutaneous melanomas and result in constitutive activation of the mitogen-activated protein kinase pathway [60,61]. Approximately 90 % of these mutations are BRAF V600E, which is effectively inhibited by the monoclonal antibody vemurafenib [62]. At 6-month interim analysis, vemurafenib showed a statistically significant improvement in overall survival compared with DTIC (84 % vs 64 %). In addition to an improvement in overall survival, vemurafenib also showed a significant improvement in tumor response rate. Forty-eight percent of patients had a confirmed, objective response to vemurafenib compared with only a 5 % response rate for patients treated with DTIC [63]. In 2012, the FDA approved ipilimumab and vemurafenib for the treatment of stage IV melanoma.




(a) (b)

Figure 1: Chemical structure of 2 of the chemotherapeutics administered as part of the dartmouth regimen, carmustine (a) and dacarbazine (b) [52]. Cisplatin (fig 3a) and tamoxifen are the remaining chemotherpautic agents which are also administered.

## 1.2 Bioorganometallic Anticancer Drugs

Bioorganometallics is the study of biologically active organometallic compounds. Organometallic compounds are defined as a metal complex containing at least one direct, covalent metal-carbon bond [64]. Bioorganometallic chemistry developed as a rapidly growing and maturing area which links classical organometallic chemistry to biology, medicine, and molecular biotechnology. The advantages of organometallics are that they have great structural variety and have more diverse stereochemistry than organic compounds. Furthermore, they are kinetically stable, usually uncharged, and relatively lipophilic and their metal atom is in a low oxidation state. Because of these properties they offer opportunities for the design of novel classes of anticancer drugs [64].

### 1.2.1 Metallocenes

In 1973, Ernst Otto Fischer and Geoffrey Wilkinson were awarded the Nobel Prize in Chemistry for their pioneering work performed independently on the chemistry of the organometallic, so called "sandwich compounds". Metallocenes are subset of the larger group of compounds known as sandwich compounds [65]. Metallocenes are defined, by the Internation Union of Pure and Applied Chemistry IUPAC, as "Organometallic coordination compounds in which one atom of a transition metal such as iron, ruthenium or osmium is bonded to and only to the face of two cyclopentadienyl [$\eta^5$-($C_5H_5$)] ligands which lie in parallel planes"[66]. The term metallocene was coined after the discovery of the structure of ferrocene and so led to the work on other similar, metal-based compounds. As soon as the metallocenes were discovered, the medical potential offered by this new family of compounds rapidly came under study and became the subject of a number of publications [67-69]. In the mid-1970s, Kopf-Maier was the first to study the effect of various metallocenes, including those of ferrocene, on the growth of ascites in mice, the model available at that time for *in vivo* testing of the effectiveness of molecules on the growth of cancer cells. The results obtained showed a moderate effect for the salts of ferriciniums but only in high dose [68,70,71].

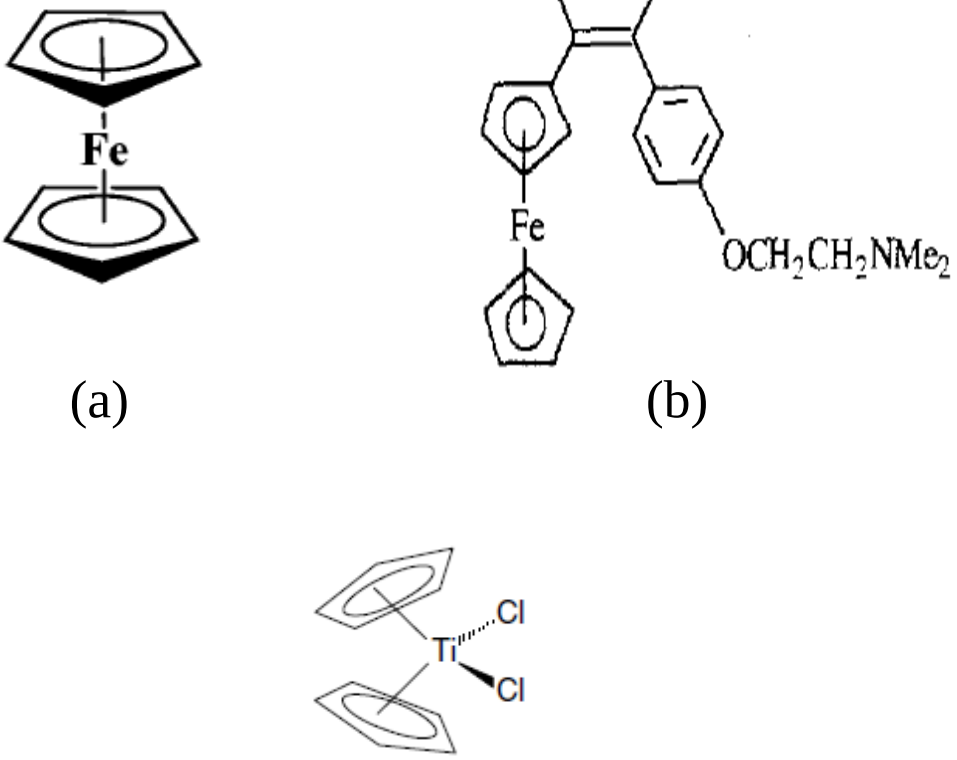



Figure 2: Chemical structures of examples of metallocenes. The archetypal metallocene, ferrocene (a) [46]. A derivative of tamoxifen which has ferrocene incorporated in its structure, ferrocifen (b) [131]. Titanocene dichloride (c) [181].

## 1.3 Chemotherapy

Chemotherapy has become one of the cornerstones of modern cancer treatment. The link between chemical compounds and their anti-cancer effects was initially found in the autopsies of allied soldiers who were exposed to mustard gas after a ship holding 30-35 kg of mustard gas bombs was blown up after an air raid in Italy, dispersing the gas [72]. Autopsy results showed a suppression of growth of lymphoid and myeloid tissue. Trials were then set up using a suitable pharmaceutical derivative which showed a reduction in tumour mass for the patient; however the effect only lasted for only a few weeks [72]. The next step, and first non-accidental step, to using chemicals to treat cancer was the antifolates. Antifolates were used in the treatment of acute lymphoblastic leukemia, and are still used in the form of clinically approved methotrexate. The antifolates function by interfering with the production of purines and pyrimidines and therefore with DNA synthesis [73]. Since those early experiments, interest in chemopharmaceuticals as a cancer treatment has come on in leaps and bounds. In ~65 years whole families of chemotherapeutics have been developed and the search for more improved drugs is ongoing. The latest generation of chemotherapeutic agents are those involved in targeted therapy. Up until this point most chemotherapeutics were purely cytotoxic agents which attempt to find a significant balance in favour of being toxic to cancer cells over healthy cells.

### 1.3.1 Cisplatin

Platinum drugs are included in approximately 50–70 % of therapeutic schemes used to treat cancer patients [74,75]. Cisplatin (fig. 3a) is highly effective in the treatment of testicular and ovarian cancers and is also widely employed for treating bladder, cervical, head and neck, oesophageal and small cell lung cancer [76]. The biochemical mechanisms of cisplatin cytotoxicity involve the binding of the drug to DNA and non-DNA targets and further induction of cell death through apoptosis, necrosis or both within the heterogeneous population of cells that forms a tumoural mass [77]. Although cytotoxicity of cisplatin is primarily attributed to its reactivity against DNA and subsequent lesions, more than 80 % binds to nucleophilic sites of intracellular constituents like thiol containing peptides, proteins, replication enzymes, and RNA [78,79-81].

Cisplatin induces cell cycle arrest followed by apoptosis. There are two main checkpoints ($G_1$/S and $G_2$/M) in the cell cycle that screen the proper evolution of the process in order to avoid the acquisition and accumulation of mutations [82]. In the event of DNA damage, the cell cycle will be arrested to help the function of the repair machinery. The $G_1$/S checkpoint allows DNA restoration before replication, and $G_2$/M facilitates the repair of DNA damaged during S or $G_2$ steps to prevent its segregation into daughter cells [83]. G2 arrest seems to be essential to trigger cisplatin-induced cell death [84]. It is commonly accepted that futile attempts to repair cisplatin-DNA adducts induce cytotoxic processes that finally end up in the triggering of apoptosis [85]. It has also been reported that, in other cell lines, particularly those with resistance to the drug, cisplatin produces characteristic features of necrosis, which is considered a mode of cell death due to general cellular machinery failure [86-88]. Moreover, it has been found that necrotic and apoptotic cell death may take place together in the same population of cisplatin-treated cells [89].

Resistance to cisplatin is generally considered a multifactorial phenomenon, which is mainly due to (i) reduced drug accumulation, (ii) inactivation by thiol containing species, (iii) increased repair of platinum-DNA adducts, and (iv) increase of cis-diamminedichloroplatinum (cis-DDP) adducts tolerance and failure of cell death pathways. These resistance mechanisms are cell line-dependent so that a particular tumour may exhibit one, two or even all the above-mentioned mechanisms [90,91]. Some tumours such as colorectal and non small lung cancers are initially resistant to cisplatin while other like ovarian and small cell lung cancers easily acquire resistance to the drug [92]

#### 1.3.1.1 Cisplatin Derivatives

With cisplatin resistance becoming a problem and in an effort to reduce toxicity a lot of research was dedicated to finding derivatives to overcome these problems. In the mid 1980s, after many failed derivatives, carboplatin showed promising results in clinical trials [93].

#### 1.3.1.2 Carboplatin

Carboplatin (Fig. 3b) contains the {*cis*-Pt($NH_3$)$_2$} active fragment of cisplatin, but the two chloride leaving groups are replaced by a bidentate dicarboxylate. The biological mechanism of action of carboplatin appears to be analogous to that of cisplatin with the induction of DNA adducts. The spectrum of cancers that can be treated is also identical. The change of leaving group reduces the activity of the agent somewhat, however. While it is as effective in ovarian cancer, it is less potent against testicular, head, and neck cancers. Correspondingly, the side effects are less severe. Consequently, cisplatin has tended to remain the agent of choice, with carboplatin used when there is a clinical need to minimize the platinum drug side effects because of other medical conditions [94].

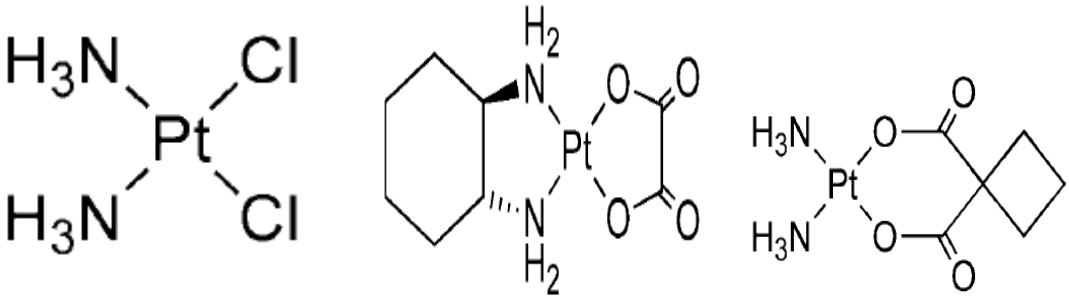
A major advantage of carboplatin over cisplatin was the reduction of nephrotoxicity, neurotoxicity and gastrointestinal toxicity. However, it does have its own toxicities, mainly myelosupression [95]. For that reason carboplatin is not given to patients with an already weakened immune system. The primary treatment regime which utilizes carboplatin is for ovarian cancer, and was approved for such in 1989 [96]. Despite overcoming many of the toxicity issues associated with cisplatin, resistance remains an issue due to both drugs having the same mode of action.

#### 1.3.1.3 Oxaliplatin

Another platinum drug, oxaliplatin (Fig. 3c) was the third to achieve clinical acceptance. The clinical advantage of oxaliplatin is that it has a different spectrum of activity: in particular, it is effective against colorectal cancer, a disease not treatable using cisplatin or carboplatin [96]. Moreover, oxaliplatin is active against some cisplatin-resistant cancers. The precise biological reasons for the difference in spectrum of activity and the ability of this agent to circumvent some cisplatin resistance mechanisms remain to be fully elucidated.

Oxaliplatin was developed in the late 1970s as a further attempt to overcome some of the problems associated with cisplatin, namely nephrotoxicity and resistance. It is a member of a family of derivatives known as the “dach” family, named because they were constructed by replacing the amine radicals of cisplatin with a 1,2-

diaminocyclohexane radical [97]. Though many showed significant anti-tumour activity, oxaliplatin was the only one which was sufficiently water soluble [98]. Currently, oxaliplatin is used in conjunction with 5-fluorouracil and often folinic acid in the treatment of colorectal cancer [99,100]. The toxicities of cisplatin and carboplatin are overcome using oxaliplatin; however there is still a dose-limiting toxicity for cumulative sensory peripheral neuropathy that is exacerbated by exposure to cold, although this is more easily reversed compared to cisplatin [101,102].




(a) (b) (c)

Figure 3: Clinically established anti-cancer platinum complexes cisplatin (a), carboplatin (b) and oxaliplatin (c) [75]

### 1.3.2 Ferrocene

Ferrocene (fig. 4) is perhaps the quintessential organometallic molecule, indeed the elucidation of this sandwich compound gave rise to the field of organometallic chemistry. First discovered in 1951, ferrocene is an organometallic compound owing its name to its similarity in reactivity to benzene [103]. The elucidation of the structure also gave rise to the terms “metallocene” and “sandwich compound” [104]. Some of the properties of ferrocene have lent themselves to its incorporation into a variety of applications. Its stability and reversible redox properties make it an ideal constituent of many biologically active compounds, amongst them being antimalarial, anti-fungal, antibacterial and of particular interest, anti-cancer agents. The anticancer potential of ferrocene derivatives was first studied in the late 1970s, when Brynes and co-workers reported the antitumour activity of ferrocenyl compounds bearing amine or amide groups against lymphocytic leukemia [105]. Since then, several types of ferrocenyl compounds have been synthesised and evaluated in terms of anticancer properties. The discovery of the cytotoxic properties of ferricinium salts on Ehrlich ascite tumours by Köpf and Köpf-Maier [106,107] was an early breakthrough for the subsequent development of novel preparations of this class of anticancer agent.

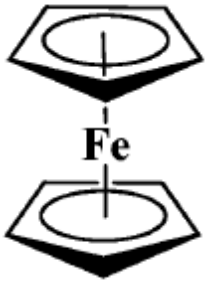



Figure 4: Chemical structure of ferrocene [46].

#### 1.3.2.1 Mode of Action

Although ferrocene itself is not cytotoxic, its derivatives, including ferrocinium species, are cytotoxic. Their potential to induce the formation of reactive oxygen species (ROS), such as hydroxyl radicals, which are capable of damaging DNA and other biomolecules is considered to be important in their mode of action.

The biological processes controlling carcinogenicity and cancer progression are diverse in the extreme and only partially understood even at this present time. ROS and associated free-radical reactions are significant contributors to the overall interplay of these processes [108–110].

Reduction of $O_2$ in the respiring aerobic cell produces $O_2^{\bullet -}$ derivatives which in turn produces hydrogen peroxide and the highly destructive hydroxyl free radical ($^{\bullet}OH$). In the healthy organism these species will be modulated by the detoxifying action of a variety of cell protective enzymes, such as superoxide dismutase (SOD), catalase, glutathione (GSH) and peroxidase, mobilised by the host's defence mechanisms [111,112]. Under normal physiologic conditions, cells control ROS levels by balancing the generation of ROS with their elimination by a scavenging system [113]. However, under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids, and DNA, giving rise to fatal lesions in cells that, in turn, contribute to many human diseases, including cancer [114-118].

If kept under control, reactive oxygen species, such as hydrogen peroxide, may in fact perform a vital role in sustaining homeostasis, and thus healthy equilibrium, through induction of apoptosis of detrimental cells. If left unchecked, on the other hand, the very same species can be found to operate against the host's well being. Strongly reduced SOD activity, for example, was observed in many cancer cells [112], and the generation of superoxide and secondary free radicals in elevated concentrations may initiate pathological reactions, as these species bind to the cell's DNA with resultant replication errors during the mitotic stage [110]. Carcinogenesis may thus be triggered.

Most cancer cells both in isolated form and in tissue exhibit enhanced ROS production [119]. Since they function with a heightened basal level of ROS, cancer

cells are more vulnerable to oxidative stress than healthy cells [3]. Exogenous compounds inducing production of ROS or other radicals can potentially be used as anticancer drugs.

Jaouen and co-workers have developed an anticancer drug, hydroxyferrocifen, a ferrocenyl analogue of 4-hydroxytamoxifen [120]. Among all the ferrocene derived cancer therapeutics, the ferrocifens are probably the most widely studied, showing very promising results for breast cancer [121,122]. This compound is converted in cells into toxic quinone methide [123,124] which alkylates GSH, thus inhibiting the antioxidative system of cells. The ferrocene fragment in this prodrug facilitates formation of the quinone methide rather than acts itself as a catalyst of ROS generation. It appears that the reversible and mild oxidation chemistry of ferrocene allows it to act as an intramolecular oxidant of a distant phenol group, when linked by a conjugated system, [125] thereby lowering the oxidation potential necessary for toxic quinine methide formation. These compounds are selectively active on cancer cells, with approximately 100 times less activity on normal cells, [123] probably due to their prodrug features.

#### 1.3.2.2 Previously synthesized ferrocene-based compounds

The quest for new and improved chemotherapeutic agents is constant. With the early work of *Kenny et al.,* N-ferrocene peptide derivatives containing amide NH groups there has been an ongoing search for improvements which can be made structurally to enchance the anti-cancer activity of the compound. The aim of this research is to examine the latest generation of derivatives and see if any improvements have been made by way of enhanced anti-proliferative activity across a panel of cell lines and to identify potential modes of action that account for this activity. The natural progression for chemotherpautics which show high levels of activity *in vitro* is to test it in a live animal study, *in vivo*. For this reason, an attempt to make a solution which would be acceptable to be given intravenously was attempted.

Previous studies within the group have been carried to evaluate the *in vitro* activity of novel ferrocenyl benzoyl amino acid and dipeptide esters. *N*-{*ortho*-(ferrocenyl) benzoyl} glycine ethyl ester was initially tested for its *in vitro* anti-proliferative activity. This compound was found to be cytotoxic, whereas the starting material,

*ortho*-ferrocenyl ethyl benzoate, was completely inactive against the cell line. Therefore, other derivatives were prepared and evaluated for their anticancer activity against H1299 lung cancer cells. The dipeptide derivative *N* {*ortho*-(ferrocenyl) benzoyl} glycine glycine ethyl ester was shown to have an improvement in activity, while *N*-{*ortho*-(ferrocenyl) benzoyl} glycine L-alanine had an even greater improvement in activity. The *N*- {*meta*-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester and *N*-{para-(ferrocenyl)- benzoyl} glycine L-alanine ethyl ester were also tested and gave similarly high levels of activity [126-130]. As an extension of this study we now report the synthesis and structural characterization of novel 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters. These novel derivatives differ from the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters by having an alkyl moiety on the previously unsubstituted cyclopentadiene ring. a series of novel 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters **64–99** were prepared by coupling alkyl ferrocenyl benzoic acids to the dipeptide ethyl esters using the conventional *N*-(3-dimethylaminopropyl)-*N*'- ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) coupling protocol.

Incorporation of electron donating groups is a well known strategy to lower oxidation potentials of ferrocene moieties [131]. Thus, the incorporation of the various alkyl chain groups (electron donating groups) to the unsubstituted cyclopentadiene ring of the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters should improve the cytotoxicity of these derivatives. In addition, the alkyl chain should also increase the lipophilicity of the reported derivatives [126-130].

To date we have shown that the replacement of the conjugated linker of *N*-(ferrocenyl) benzoyl dipeptide esters with a naphthoyl linker leads to an improvement in antiproliferative of the compound [132-134]. From the series of *N*-(ferrocenyl) naphthoyl amino acid and dipeptide ethyl esters reported, the *N*-{6-(ferrocenyl)-2-napthoyl} glycine glycine ethyl ester displayed an IC50 value of 0.13 ± 0.01 μM in the H1299 lung cancer cell line [134]. From the promising results obtained from the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*- (ferrocenyl) benzoyl dipeptide esters **64–99.** It has been clearly demonstrated in the SAR study of the 1-alkyl-1' derivatives that employing the incorporation of a methyl derivative to the

unsubstituted cyclopentadiene ring enhances the anti-proliferative effect of the ferrocenyl dipeptide bioconjugates relative to the *N*-(ferrocenyl) benzoyl dipeptide derivatives in the H1299 lung cancer cell line [126-130]. This SAR study involves the biological evaluation of the incorporation of an ethynyl moiety between the ferrocene moiety and the conjugate linker. Furthermore, the incorporation of L-leucine and L-phenylalanine into the dipeptide chains have been shown in the preliminary screen of the 1-alkyl-1' derivatives (**64-69**) to display low percentage growth inhibition values in H1299 lung cancer cell line. The synthesis of the new ferrocenyl conjugates involved Sonogashira coupling of an ethynyl ferrocene to three bromo acylated acids to generate *para*-(ferrocenyl) ethynyl benzoic acid, naphthoic acid and 2-furanoic acid.

In a further extension to ferrocenyl benzoyl and naphthoyl bioconjugates reported, a new library of ferrocenyl based bioconjugates **105-138,** which consist of four key moieties (i) an electroactive core (ii) a -C≡C- moiety (iii) three different aromatic linkers and (iv) and a series of amino acid and dipeptide esters are now reported here. The incorporation of the -C≡C- moiety between the ferrocene moiety and the aromatic spacer and the use of three different aromatic rings are attempts to further improve the cytotoxicity of the previously prepared *N*-*para*, *N*-*meta* and *N*-*ortho*-(ferrocenyl) benzoyl dipeptide esters.

A series of *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} and *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} amino acid and dipeptide esters were prepared and structurally characterized. Each novel compound incorporated an electroactive ferrocene core, a -C≡C-, a conjugated aromatic linker and a dipeptide chain with each part exerting a specific effect on biological activity. The ferrocene moiety is required for the possible production of hydroxyl radicals, the -C≡C- and conjugated aromatic linkers facilitates this process by lowering the redox potential of the ferrocene and the peptide fragment can interact with other molecules *via* hydrogen bonding.

For the synthesis of *para*, *meta* and *ortho*-(ferrocenyl) benzoic acid the synthetic protocol involved the diazonium coupling of ferrocene to 2, 3, 4-ethylamino benzoate followed by basic hydrolysis [126-130]. The use of the diazonium coupling reaction in the preparation of the monoarylferrocenes derivatives proceeds with a

low degree of regiocontrol and produces intractable mixtures of mono-, di- and poly-arylferrocene derivatives with low yields of less than 8% for the desired monoarylferrocene derivatives. For the synthesis of the ethynyl analogues **105-138,** the synthetic protocol involved the use of Sonogashira cross coupling of ethynyl ferrocene to three bromo acylated acids to generate *para*-(ferrocenyl) ethynyl benzoic acid, 6-(ferrocenyl) ethynyl-2-naphthoic acid, and 5-(ferrocenyl) ethynyl-2-furanoic acids. In this coupling reaction, yields greater than 60% were obtained. These high yields are largely due to the fact that Sonogashira cross coupling is a substrate specific reaction between an alkyne and an aryl halides employed in the coupling reaction which involves the use of palladium catalyst and copper iodine co catalyst.

The acid phosphatase assay was chosen as the method to determine cell growth. The assay is based on the hydrolysis of the *p*-nitrophenol phosphate by intra-cellular acid phosphatises in viable cells to produce *p*-nitrophenol. The assay shows higher sensitivity and reproducibility in comparison to cell proliferation assays based on the reduction of tetrazolium salts.

Previous work within the group have identified ferrocenyl compounds have an effect on the cellular cycle of cancer cells. For this reason a cell cycle assay was used utilizing propidium iodide as a stain which can measure DNA content which changes at each phase of the cell cycle. Results from cell cycle analysis have suggested that apoptosis/necrosis plays a role in the activity of these ferrocenyl compounds. The TUNEL assay was therefore used to confirm or deny this as it is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades.

#### 1.3.2.3 Ferrocifen

One of the big successes of ferrocene-based anticancer drugs has been a series of ferrocene derivatives of Tamoxifen (fig. 5a) which have emerged from the laboratories of Jaouen [124]. Tamoxifen is an organic drug that acts on the estrogen receptor and has had a very significant impact in the treatment of breast cancer. Approximately 30 % of breast cancer patients fail to respond to tamoxifen treatment. In part, this is due to the presence of two different estrogen receptors (ERα and ERβ) in humans; tamoxifen is effective only against breast cancers which are ERα

positive. The ferrocifens (5b) are agents in which one phenyl ring of tamoxifen is replaced with ferrocene. These agents are highly active against breast cancer cells that are ERα negative (ERβ positive) as well as breast cancers that are ERα positive. Ferrocifens are the first molecules shown to be active against both hormone-dependent and hormone-independent breast cancer cells [125].

The role of the ferrocene is believed not to be solely restricted to that of a structural unit. Rather, it appears that the ferrocene could be oxidised to ferrocenium in the cell and subsequently cause oxidative damage through Fenton-type mechanisms [135]. The activity of ferrocenyl compounds depends on the oxidation state of iron in the ferrocene moiety. The mechanism of ferrocifen as one of the Fe(II) compounds has been studied and the results indicate that the ferrocifens act by changing the conformation of the receptor protein [136].

In addition, when ferrocifen binds to ERβ, an 'oxidant/antioxidant' mechanism may occur. The ferrocifen–ERβ complex is thought to dimerize and attach itself to a particular region of DNA, and $Fe^{2+}$ complexes are known to be oxidized to $Fe^{3+}$ by $O_2$, leading to the generation of highly reactive •OH radicals [137]. These radicals could damage the DNA strand close to the binding site, thus explaining the observed antiproliferative effect in connection with the ERβ receptor [138]. DNA damage produced by exposure to ferrocifens was observed [139]. Currently, there is a library of around 300 ferrocene complexes which have been tested for their antiproliferative effect mainly on breast cancer and prostate cancer cells. [140-146]. Recently, Jaouen's group have developed a method of effectively solubilising the most active compounds in the library and *in vivo* testing has shown promising results [147].

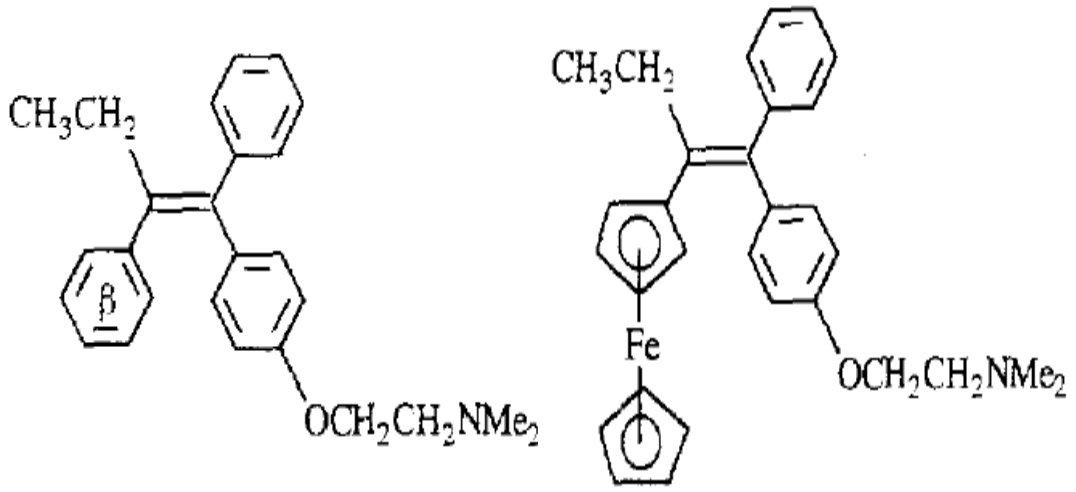



Figure 5: Chemical structure of Tamoxifen (a) dervived anti-cancer compound, ferrocifen (b) in which the aromatic β ring of Tamoxifen is replaced by a ferrocene moiety [140].

### 1.3.3 Other Metal-Based Chemotherapeutics

#### 1.3.3.1 Gallium

Toxicities and anti-tumour activities of gallium salts were described as early as 1971 [148,149]. Gallium nitrate, a simple gallium salt, can be considered a 'first generation' gallium compound that was investigated for its anticancer activity in humans [150]. This drug is, therefore, the standard against which newer gallium compounds are compared. In Phase II clinical trials conducted to evaluate the spectrum of its antineoplastic activity, gallium nitrate was administered to patients with a variety of different cancers, of which it was found to have antineoplastic activity primarily against advanced bladder cancer and non-Hodgkin's lymphoma [151-154]. In these malignancies, significant responses to gallium nitrate were seen in patients whose tumours had relapsed or failed to respond to conventional chemotherapy. Encouraged by these findings, a limited number of clinical trials ensued in which gallium nitrate was administered in combination with other chemotherapeutic agents (vinblastine, ifosfamide, 5-fluorouracil, mitoguazone, etoposide and hydroxyurea) to patients with bladder cancer or non-Hodgkin's lymphoma. These trials demonstrated that gallium nitrate could be safely combined with other drugs with a good clinical outcome [151-153].

A phase II trial yielded a disappointing objective response rate of only 3 % in patients with advanced malignant melanoma, discouraging further evaluation [154]. Gallium nitrate showed notable anticancer activity in phase II trials in lymphoma and bladder cancer [155]. However, the applicability of gallium nitrate as an anticancer drug was questioned by nephrotoxicity (in the case of short infusions) and occasional severe optical neuropathy (in the case of continuous infusions). An improved therapeutic index was expected to result from prolonged exposure to low steady-state plasma gallium concentrations, but attempts to accomplish this with oral administration of gallium chloride yielded unsatisfactory results because of insufficient bioavailability [156]. Complexing gallium with suitable chelators has been pursued to stabilise gallium against hydrolysis, which is the major impediment for intestinal absorption, and to facilitate membrane permeation, namely tris(8-

quinolinolato)gallium(III) (KP46) and tris(3-hydroxy-2-methyl-4H-pyran-4-onato)gallium(III) (gallium maltolate).

The concentration required to inhibit 50 % of growth ($IC_{50}$ value) of KP46 in a variety of melanoma, ovary, breast, colon and lung cancer cell lines *in vitro* ranged from 0.85 to 10.4 μM [157], which is much lower than that reported for gallium nitrate. In addition, the combination of KP46 and platinum drugs acts synergistically to inhibit the proliferation of ovarian and colon cancer cell lines in vitro [158]. In a Phase I/II clinical trial of oral KP46 in patients with solid tumours (renal, ovarian, stomach and parotid gland), the drug was found to be well-tolerated over a dose range of 30 to 480 mg/$m^2$ daily for 14 days; no significant dose-limiting toxicities were observed [159,160]. Responses to treatment were seen in three out of four patients with renal cancer [159,160]. The treatment of renal cancer is challenging since this disease does not respond to conventional chemotherapy or to gallium nitrate; hence, the initial activity of KP64 in this malignancy is noteworthy and should be further explored. Clinically achievable concentrations of KP46 proved to be highly effective in melanoma cells from primary explants of cutaneous and lymph node metastases. For KP46, there is preliminary evidence for activity in renal cell carcinoma, with one partial response and two disease stabilisations for up to 11 months [161]. Clinical activity in this malignancy, which is otherwise largely chemoresistant probably due to effective detoxification mechanisms, is unprecedented in the development of anticancer metal compounds. KP46 has yet to progress to phase II trials, in which it could qualify for combinations with other drugs (such as cisplatin) for increased effect. There are ongoing investigations on the anti-tumor mechanism of action and key targets of KP46, to make the clinical development of KP46 more straightforward. Antitumour activity is thought to be by interaction with ribonucleotide reductase followed by impairment of the catalytic activity of the enzyme [162]

Gallium maltolate is a gallium compound that has been developed for oral use. Allamneni et al. reported that following oral administration of gallium maltolate to normal subjects, gallium in the circulation is found bound to transferrin, the iron transport protein, with very little of it unbound [163]. In another study utilizing a panel of lymphoma cell lines, gallium maltolate was shown to induce apoptosis at earlier time points and at significantly lower concentrations than gallium nitrate

[164]. Evidence that gallium maltolate has antineoplastic activity in humans was recently provided by Bernstein et al. [165]. These investigators reported a patient with advanced hepatocellular carcinoma that had failed to respond to treatment with the drug sorafenib. Treatment of this patient with 1500 mg/day of oral gallium maltolate resulted in marked clinical improvement of the patient's symptoms and a significant reduction in the size of the hepatic mass, as measured by imaging studies of the liver [166].

Both complexes show a high bioavailability in animal models when administered via the oral route [167,168] and are more potent inhibitors of tumour cell proliferation *in vitro* than gallium salts [169,170]. No dose-limiting toxicities were encountered in phase I studies with either gallium maltolate or with KP46 [169, 170], reflecting the higher than expected tolerability of these compounds.

#### 1.3.3.2 Titanium

The pioneering work of Köpf and Köpf-Maier in the early 1980s showed the antiproliferative properties of titanocene dichloride, [$TiCp_2Cl_2$], and the compound was studied in phase I clinical trials in 1993 [171–173]. Although the compound advanced as far as stage II clinical trials in patients with breast metastatic carcinoma [174] and advanced renal cell carcinoma [175], low levels of activity discouraged further trials. The compound was found to bind more weakly to DNA bases than cisplatin with the hard $Ti^{IV}$ ion showing higher affinity to the phosphate backbone of DNA [176]. The main disadvantages of titanocenium compounds, which may have led to the low activities in the clinical phase II trials, are their poor solubility in aqueous media and their hydrolytic instability under physiological conditions [174].

Since that time there has been a renewed interest in titanocene with research groups developing derivatives with amino acids [177,178], amide functionalized titanocenyls [179], titanocene derivatives with alkylammonium substituents on the cyclopentadienyl rings [180–182], steroid-functionalized titanocenes [183], and alkenyl-substituted titanocene or *ansa*-titanocene derivatives [184–186]. One such agent is Titanocene Y, which contains methoxyphenyl substituents of the Cp rings which confer the agent with higher potency [187]. The anti-proliferative activity of titanocene Y and other titanocenes has been studied in 36 human tumour cell lines

[188] *in vitro* and *ex vivo* showing that renal cancer is a major target for this novel class of titanocenes. They also showed significant activity against ovarian, prostate, cervix, lung, colon and breast tumours [189].

*Ex vivo* experiments demonstrated that titanocene Y has significant cytotoxic effects comparable to cisplatin when tested on explanted renal cell, ovarian, non-small cell lung and colon tumours [190]. Titanocene Y induced cell death in freshly explanted human breast cancer cells and led to reduction of tumour volume in a MCF-7 human breast cancer xenograft mouse model. During exposure, no mouse was lost, indicating low toxicity of the agent [191]. In human epidermoid cancer cells treatment with titanocene Y induced caspase-3 and -7 dependent apoptosis *in vitro* and led to reduction of tumour growth in an A431 xenograft model of epidermoid cancer *in vivo* [192]. In the same studies, A431 cancer cells were stained with Annexin V and propidium iodide to detect early and late apoptotic cells, confirming that cell death occurs via apoptosis. Further *in vivo* tests with xenografted Caki-1 tumours in mice revealed a significant and dose dependent inhibition of tumour growth with no severe side effects at non lethal doses [193]. Presently titanocene Y is under investigation for its potential ability to overcome resistance to conventional anti-cancer drugs and to identify the main mechanisms of action, focusing on the apoptotic pathways through which titanocene Y causes apoptosis.

#### 1.3.3.3 Ruthenium

Ruthenium-based compounds have attracted the interest of many researchers who envisaged the possibility of using this metal to develop innovative characteristics in new compounds to treat tumours with a selective cytotoxicity for cancer cells [194, 195]. Ruthenium complexes, including some organometallics, have shown interesting anticancer properties *in vivo* and may represent new and efficient therapeutic agents to be used as alternative to platinum drugs [196]. The successful clinical phase I trials of indazoliumtrans-[tetrachlorobis(1H-indazole)-ruthenate(III)], KP1019 (fig 6b) and imidazolium trans-[tetrachloro-(S-dimethyl sulfoxide)(1H-imidazole)ruthenate(III)], NAMI-A (Fig. 6a) two coordination complexes based on ruthenium, has led to considerable interest in anticancer drugs based on this element [197,198].

In general, compared to platinum-based drugs, ruthenium drugs are less toxic and are also active in tumours that do not respond well to the platinum drugs. Ruthenium is particularly attractive as the ligand exchange kinetics in its complexes can be similar to those of platinum complexes.

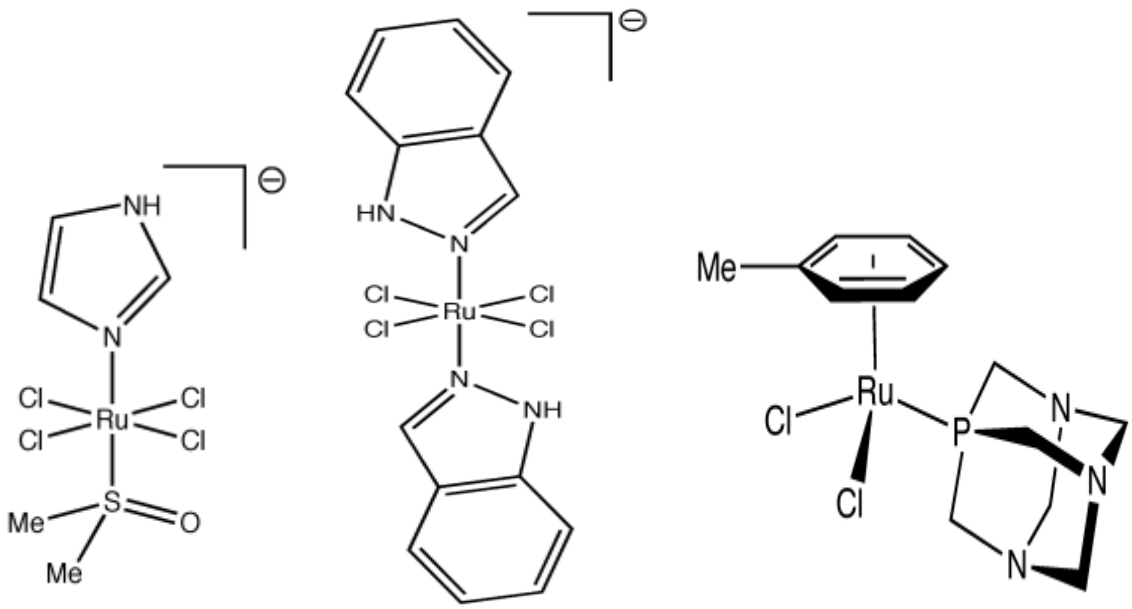
The first ruthenium agent to enter clinical trials was NAMI-A [199,200]. That this drug has made it to clinical trials is quite remarkable because it is not very active against cancer cell lines, which are the usual first screen for activity (failed the NCI cell line panel screen) [201]. While NAMI-A can bind to DNA, this is not believed to be the source of its biological action. Rather, NAMI-A seems to act as an anti-angiogenic and anti-invasive agent. Its anti-angiogenic properties may relate to the ability of NAMI-A to scavenge Nitric Oxide (NO) produced by epithelial cells. The anti-invasive properties appear to be a result of its interaction with extracellular or external cell membrane receptor proteins. Today NAMI-A is studied for the use as a second line therapy in the metastatic NSCLC in combination with gemcitabine. Although the study is still ongoing, preliminary data of dose finding show the possibility to treat these patients with the combination NAMI-A and gemcitabine. The main toxicities are flebitis at the injection site, for which reason the drug is infused through a central venous catheter, general malaise and cutaneous blister formation [202].

The second of the ruthenium agents is indazolium trans- [tetrachloro-bis(1H-indazole)ruthenate(III)] (KP1019) (fig.6b) [197]. Despite its structural similarity to NAMI-A, this agent is a cytotoxin which is active against primary tumours, and is being investigated for activity against colorectal cancers. The agent causes apoptosis via the mitochondrial pathway and is believed to act as a pro-drug with the actual active species as yet not identified. Transferrin is normally used to transport iron centres into the cell and is over-expressed in cancer cells. It is believed that the ruthenium complex is transported into cells by transferrin, which can bind two ruthenium centres. The complex is released from transferrin at acidic pH’s such as those found in endosomes, which is also used to release iron from transferrin inside the cell. Thus, it appears that KP1019 can very effectively use the natural iron transport systems to enter the cell. Once inside the cell, the agent can bind to DNA. KP1019, and its more soluble sodium salt KP1339, are undergoing clinical studies in humans. KP1019 has already completed a phase I clinical trial with 8 patients

showing 5 disease stabilizations out of the 6 patients evaluable, in some cases up to 10 weeks even if only one cycle of drug administration was performed [197]. KP1019 was given as flat dose to patients starting from 25 mg/patient and stopping dose escalation at 600 mg/patient because of solubility problems of the drug [197].

Like NAMI-A and KP1019, (RAPTA) (Fig. 6c) agents are aryl ruthenium piano-stool complexes [202]. RAPTA-T, similarly to almost all the other derivatives of this series [203] is only weakly cytotoxic, although its cytotoxicity is better against tumour cells than non-tumorigenic cells while showing a significant ability to modify cell behaviour in terms of invasion and metastasis [204]. The more recent RAPTA compounds [205], although conceived to mimic conventional platinum drugs, are generally free of *in vitro* cytotoxicity and have properties reminiscent of NAMI-A on invasion and metastasis *in vitro*, although *in vivo* they have a markedly different general chemistry to NAMI-A [206]. The compounds developed out of studies to create pH-dependent DNA-binding agents, but were shown to be of only very low toxicity toward cancer cell lines. Like NAMI-A, these agents are inactive against primary tumours but found *in vivo* to have activity against metastases. The RAPTA complexes are slightly less potent antimetastatic agents than NAMI-A, but (in mice) less toxic and thus can be administered in higher doses. As for NAMI-A, the indications are that proteins, rather than DNA, are the biomolecular targets for action of the drug.

Like the gallium-based anti-cancer compound, KP46, the progress (section 1.3.1.3.1) of the ruthenium-based compounds are halted by a lack of information on their mode of action. At time of writing, NAMI-A, KP1019 and RAPTA-T are at clinical phase I/II, clinical phase I and preclinical evaluation respectively. For example, the strong and unique property to target cancer metastasis of NAMI-A should prompt the search for more potent and selective molecules, a process that today seems not possible unless we know why metastases and not the cells of the corresponding primary tumours are so sensitive to this drug [206].




(a) (b) (c)

Figure 6: Chemical structure of ruthenium based, anti-cancer compounds NAMI-A (a) [194], KP1019 (b) [197] and RAPTA (c) [203].

## 1.4 Multiple Drug Resistance (MDR)

Drug resistance, that is, the appearance of reduced or missing response of cancer cells to applied chemotherapeutic agents, is a serious problem for the treatment of cancer [207]. This phenomenon can be divided into intrinsic drug resistance, where the application of drugs has no effect at all, and acquired drug resistance, where a normal response is observed at the beginning of the therapy, which then diminishes quickly and often disappears completely after a certain period of time [208,209]. Multiple Drug Resistance (MDR) corresponds to a particular form of drug resistance, characterized by the simultaneous appearance of resistance to the applied chemotherapeutic agent and cross-resistance to a number of functionally and structurally diverse hydrophobic drugs, with different mechanisms of action [210.211].

It has been shown that MDR cells overexpress certain efflux proteins, which leads to a significantly lower intracellular level of chemotherapeutical agents [212]. The most prominent examples of this superfamily of proteins, for which a similar mechanism of action is assumed, are P-glycoprotein (Pgp) and MDR protein (MRP1) [213]. While Pgp mainly transports neutral and charged molecules in unmodified form, MRP1 is also able to accept metabolized substrates such as GSH, glucuronide, or sulfate conjugates [214-216].

There has been limited research on the applicability of metal-based chemotherapeutics to overcome MDR, however the group of Prokop et al. have investigated the potential of a number of metal-based compounds including yttrium [217], iron [218,219], titanium [220] and gold [221].

HUNI 068 (fig. 7), an F-moc protected amino acid ferrocenyl compound [222], is active against cancer cells and overcomes different mechanisms of MDR, a major problem in the treatment of relapsed malignant diseases [223]. Tumour cells develop MDR by various cellular based mechanisms, for example by decreasing the pro-/anti-apoptotic protein ratio (for example, overexpression of Bcl-2) or by excretion of the compound applied through membrane drug transporters, such as, *p*-glycoprotein [224]. The leukemia cell line Nalm-6 was made resistant to vincristine (Nalm-6/Vcr) and daunorubicine (Nalm-6/Dauno). Additionally, a vincristine-

resistant lymphoma cell line (BJAB/Vcr) was established. The Nalm-6/Vcr cells were tested with different common chemotherapeutic drugs, and the cells were tolerant to fludarabine and paclitaxel in addition, thereby fulfilling criteria of MDR [219].

The multiple drug resistant Nalm-6/Vcr cells were more sensitive to treatment with HUNI 068 than the parental Nalm-6 cells. At 30 µM HUNI 068 induced apoptosis in 20 % of the BJAB/Vcr population as well as the parental BJAB cells. At a higher concentration of 50 µM, HUNI 068 induced apoptosis in 63 % of the BJAB/Vcr population, while the same concentration affected 79 % of the non-resistant BJAB cells which suggests at a higher concentration, some mechanism of resistance may have an effect on apoptotic induction.

To determine a mechanism of overcoming resistance, the expression of 84 apoptosis relevant genes was examined in BJAB cells treated with HUNI 068. Cells showed a 50-fold upregulation of the pro-apoptotic harakiri gene [219]. Harakiri is an apoptosis-promoting protein, which interacts with Bcl-2, but not with the death-promoting homologs, Bax or Bak [225]. It is assumed that the overexpression of harakiri induced by HUNI 068 neutralized the apoptosis-suppressing effects of Bcl-2 in the BJAB/Vcr cells.

Since the discovery of antiproliferative effects of Auranofin, an established clinical drug for the treatment of rheumatoid arthritis with relatively mild side effects [226], a huge variety of Au(I) complexes derived from Auranofin have been synthesized and tested for anticancer activity. Although many of these compounds showed promising results against a panel of cancer cell lines *in vitro*, their *in vivo* efficacy was limited [227].

A number of gold azide compounds were synthesized. Of particular interest were diethylphenylphosphine (4-(4-Boc-L-phenylalanine-L-leucine methyl ester)-1H,2,3-triazolyl)gold(I) **6b** and diethylphenylphosphine (4-(4-L-phenylalanine-D-arginine-L-phenylalanine-L-lysine-amide)-1H,2,3-triazolyl)gold(I) **8b** and the effect of the gold-peptide conjugates on p53-mutant MDA-MB231 breast cancer cells was evaluated. Besides high aggressiveness and poor prognosis, mutant-p53 expressing tumours also display increased resistance against various anticancer treatments [228].

A Bionas sensor chip system was employed, which is used for the continuous online measurement of essential cellular parameters, such as respiration rate and extracellular acidification rate [229, 230]. The respiration rate is a direct measure of ATP production within functional mitochondria, while the acidification rate mainly indicates the presence of lactic acid, a product from the anaerobic oxidation of NADH to NAD+ during glycolysis. Alterations in both of these cellular functions play a role in apoptosis.

As expected, incubation of p53-mutant MDA-MB-231 cells with 20 µM cisplatin did not significantly alter the respiration and acidification rate compared to the non-treated control. In contrast, exposure to 20 µM 6b and 8b strongly affects both cellular parameters. The standard respiration rate drops to 20 % within just 3–7 hours after the addition of 6b, 8b. The strong decrease in cellular respiration is mirrored by an initially steep increase of the standard acidification rate for 6b and 8b which can be explained by an enhanced compensatory glycolysis. Ultimately, as the cells perish, all parameters irreversibly drop to basal levels. The results show that the (phosphine)gold(I) conjugates are able to break the resistance of the p53-mutant MDA-MB231 cells against cisplatin, which also points to a different mode of action for these gold drugs compared to the former. It has been shown that unlike cisplatin, DNA can be excluded as a relevant target for these gold peptide conjugates [231]. Instead it has been proposed that inhibition of thioredoxin reductase (TrxR), an important enzyme within the cellular and mitochondrial redox system, is the most relevant factor for the antiproliferative effects displayed by gold compounds [232].

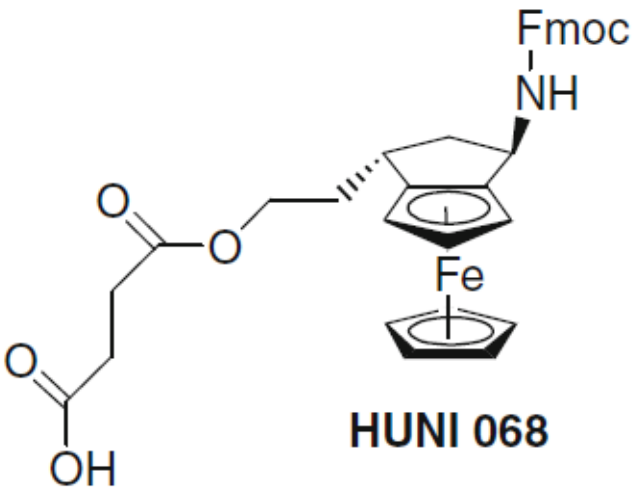



Figure 7: Chemical structure of HUNI 068 [222]

## 1.5 Drug Solubility

Organometallic complexes are not normally soluble in aqueous media, however *in vitro* experiments can be carried out in the presence of a low percentage of alcohol or DMSO (0.5-1 %) without causing any damage to cells. To conduct *in vivo* evaluations, it is necessary to address the water solubility issue of these lipophilic molecules. DMSO is one of the most common solvents used experimentally to dissolve hydrophobic substances for *in vivo* and *in vitro* purposes. The toxicity of DMSO is low: $LD_{50}$ in mice is 6.2 mL/kg (when applied intraperitoneally (i.p)) or 3.7 mL/kg (applied intravenously (i.v.)), $LD_{50}$ in rats is 9.9 mL/kg (i.p) or 7.4 mL/kg (i.v.) [233]. However, DMSO by itself has a variety of biological actions which can confound the effects of drugs when DMSO is used as a vehicle. A substantial effect has been found even with doses as low as 0.1 mL/kg [234]. For this reason it is favourable to find alternative vehicles for drug delivery *in vivo.*

Following the discovery of the strong antiproliferative effects of the ferrocifens *in vitro*, *in vivo* studies were performed. These experiments were carried out in nude mice on xenografts of different cancers (breast, ovarian, prostate). In these first tests Fc-OH-Tam and Fc-diOH, were solubilized in a mixture of PEG300 and carboxymethylcellulose and administered by intraperitoneal injection or orally. None of these experiments led to significant regression of the tumours in the treated mice and very rapid elimination of the complexes from the circulatory system was noted. The solution was found in the newly available option of working with lipid nanocapsules, which provided some very interesting approaches for the formulation of this family of molecules. The effect of Fc-diOH was tested in rats, implanted with brain tumours (9L glioma cells). This cell line is classically used as a model for human glioblastoma, a particularly aggressive cancer that remains incurable to date. The injection of Lipid Nanocapsules (LNC) containing Fc-diOH induced a significant reduction in the growth of the tumours by approximately 75-80 %. The use of this formulation allowed, for the first time, observation of an *in vivo* effect on tumour growth [235].

After this a new generation of LNCs were developed. A PEG moiety with a long chain (DSPE-mPEG2000) is added to the surface of the first-generation LNC to produce a stealth effect, that is, to make the LNC undetectable by the animal's

immune system and thus increase the time they remain in the animal's circulatory system. This time the LNC were administered intravenously and the results obtained were spectacular, since in this case an almost-total disappearance of the tumours was observed in the treated animals after 21 days [236].

Nanomedicines have appeared as a compelling system to develop an injectable formulation of Fc-O-HTAM offering great benefits such as increased bioavailability, protection against *in vivo* degradation and enhancement of pharmacokinetic properties [237].

The method used to load the LNCs with Fc-OH-TAM is a 2 step process. Step one involves mixing all the excipients (Solutol® HS15 (17.2 % w/w), Lipoid® (1.5 % w/w), Labrafac® (20.8 % w/w), NaCl (0.5 % w/w) and water (60 % w/w) under magnetic stirring and heating from room temperature to 90 °C. Three cycles of progressive cooling and heating between 90 and 60 °C were then carried out. Step two is an irreversible shock induced by sudden dilution with water (28.5 % (v/v)) applied to the mixture at 70-72 °C. Slow magnetic stirring was then applied to the suspension for 5 minutes. Fc-OH-TAM loaded LNCs were prepared according to this process with drug added as a solid powder at step one to the other excipients. The drug was solubilized during the heating step and ultimately encapsulated over the last cooling step [238].

This method allowed successful delivery of Fc-OH-TAM to mice bearing TNBC (Triple Negative Breast Cancer) xenografted tumours in severe combined immunodeficiency (SCID) mice. Fc-OH-TAM-LNC treatment markedly delayed the tumour growth compared to the untreated control group and the unloaded LNC treated group, which had similar tumor evolutions. As a result, the hampered tumour growth rate led to an impressive low tumour volume at day 38 with 36 % tumour volume reduction compared to that of control groups [239].

# Chapter 2

# Materials and Methods

### 2.1. Cells and Reagents

Growth Media and Foetal Calf Serum (FCS) were obtained from Sigma-Aldrich. Cell lines were maintained at 37 °C with 5 % $CO_2$.

Table 2.1: Details of cell lines and growth conditions used for this study.

| Cell Name | Source | Cell Type | Media + Additives |
|---|---|---|---|
| **HT144** | ATCC | Melanoma | RPMI + 10 % FCS |
| **Lox-IMVI** | NCI | Melanoma | RPMI + 10 % FCS |
| **Malme-3M** | NCI | Melanoma | RPMI + 10 % FCS |
| **Malme-3M-TMZ** | NICB Cell Bank | Temozolomide Resistant Melanoma | RPMI + 10 % FCS |
| **Sk-Mel-28** | NCI | Melanoma | RPMI + 10 % FCS |
| **A549** | ATCC | Adenocarcinoma | DMEM/Ham's F12 + 10 % FCS |
| **A549-Cpt** | NICB Cell Bank | Carboplatin resistant Adenocarcinoma | DMEM/Ham's F12 + 10 % FCS |
| **H1299** | ATCC | Non-small Cell Lung Carcinoma | RPMI + 10 % FCS + 10 mM L-Glutamine |
| **SKBR3** | ATCC | Breast Cancer | RPMI + 10 % FCS |
| **MDA-MB-361** | ATCC | Breast Cancer | RPMI + 10 % FCS |
| **NHDF** | Clonetics | Normal human dermal fibroblast | DMEM + 10 % FCS |

### 2.2 Compound Preparation

All ferrocene-based compounds were synthesised by Dr Peter Kenny's research group, School of Chemical Sciences, Dublin City University. Compounds were weighed using an analytical balance and dissolved in DMSO (Sigma) to a concentration of 10 mM. 1 mL was aliquoted and was used as a working stock. The remainder was stored at -20 °C. The synthesis of tested compounds is described in Section 1.3.2.2.

## 2.3. Proliferation Assays

Proliferation was measured using an acid phosphatase assay. 1 x $10^3$ cells/well were seeded in 96-well plates, except for HT144 and Malme-3M which were seeded at 2 x $10^3$ cells/well. Plates were incubated overnight at 37 °C followed by addition of drug at the appropriate concentrations and incubated for a further 5 days until wells were 80 % to 90 % confluent. All media was removed and the wells were washed once with PBS (Sigma)**.** 10 mM paranitrophenol phosphate substrate (Sigma-Aldrich) in 0.1 M sodium acetate buffer (Sigma) with 0.1 % Triton X (Sigma) pH 5.5 was added to each well and incubated at 37 °C for 2 hours. 50 µL of 1 M NaOH (Sigma) was added and the absorbance was read at 405 nM (reference – 620 nM).

### 2.3.1 Screening of Compounds

Due to the large volume of compounds which were to be tested, the amount of compound to be evaluated was reduced by conducting preliminary screenings. This involved treating the cell with 10 µM of each compound and depending on the percentage growth inhibition, compounds were selected for $IC_{50}$ analysis. Compounds which showed very high levels of anti proliferative activity were further screened at 1 µM

## 2.4. *Mycoplasma* analysis of cell lines

Cell lines were tested for possible Mycoplasma contamination every three months approximately. Mycoplasma negative NRK (normal rat kidney fibroblast) cells were used as indicator cells for this analysis. The cells were incubated with a sample volume of supernatant from the cell lines in question and then examined for *Mycoplasma* contamination. A fluorescent Hoechst stain (Sigma) was used in this analysis. The stain binds specifically to DNA and stains the nucleus of the cell in addition to any *Mycoplasma* present. *Mycoplasma* infection was indicated by fluorescent bodies in the cytoplasm of the NRK cells.

## 2.5. Cell Cycle Analysis

The Guava Cell Cycle Assay uses the nuclear DNA stain, propidium iodide (PI), to measure cell cycle. Resting cells ($G_0/G_1$) contain two copies of each chromosome. Cells undergoing mitosis synthesize chromosomal DNA (S phase), which results in increased fluorescence intensity. When all chromosomal DNA has doubled ($G_2$/M phase), cells fluoresce with twice the intensity of the initial population. 2.5 x $10^4$ cells were seeded per well in 24-well plates and incubated overnight at 37 °C. After 24 hours appropriate concentrations of test compound were added to the wells. Plates were then incubated at 37 °C for a further 72 hours. Media was collected and the wells washed once with PBS (Sigma)**.** Cells were trypsinised and added to the media collected for each sample. Cells were centrifuged at 300 x g for 5 minutes and the media was aspirated. The cell pellet was re-suspended in 150 µL PBS and transferred to a round bottomed 96 well plate. The plate was centrifuged at 300 x g for 5 minutes and the supernatant aspirated leaving approximately 15 µL in each well. The remaining volume was used to resuspend the cells and 200 µL of ice cold 70 % ethanol (Fluka) was added. The plates were then stored at -20 °C for 2 hours. After fixing, the cells were centrifuged at 450 x g for 5 minutes, the supernatant removed, washed with 200 µL of PBS and centrifuged again at 450 x g. The PBS was then removed and 200 µL of Guava Cell Cycle reagent (Millipore) was added to each well. The cells were mixed by pipetting and stored at room temperature shielded from light for 30 minutes. Cells were analysed on the Guava EasyCyte (Guava Technologies).

## 2.6. Apoptosis Assay using Terminal DNA Transferase-mediated dUTP Nick End Labelling (TUNEL).

The Guava® TUNEL Assay detects apoptosis-induced DNA fragmentation through a quantitative fluorescence assay. Terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of bromo-deoxyuridine (BrdU) residues into the fragmenting nuclear DNA at the 3'-hydroxyl ends by nicked end labelling. A TRITC conjugated anti-BrdU antibody can then label the 3'-hydroxyl ends for detection by a Guava System. 2.0 x $10^4$ cells were seeded per well in 24-well plates and incubated overnight at 37 °C, followed by addition of test compound at the appropriate

concentrations. After 72 hours, media was removed and the wells washed once with PBS (Sigma)**.** Cells were trypsinised and resuspended in 1 mL of media before being transferred to an eppendorf. Cells were centrifuged at 300 x g for 5 minutes and the medium was aspirated. The pellet was re-suspended in 150 μL of PBS and transferred to a round bottomed 96 well plate. 50 μL of 4 % para-formaldehyde (Sigma) prepared in PBS was added to the wells and mixed. Cells were incubated at 4 °C for 60 minutes. The plate was centrifuged at 300 x g for 5 minutes and the supernatant aspirated leaving approximately 15 μL in each well. The remaining volume was used to resuspend the cells and 200 μL of ice cold 70 % ethanol (Fluka) was added to the cells. The plates were then stored at -20 °C for at least 2 hours (maximum 2 weeks). After fixation, the cells including positive and negative controls (Millipore) were centrifuged at 300 x g for 5 minutes. The supernatant was aspirated, and the cells washed with 200 μL of wash buffer (Millipore) and then centrifuged again at 300 x g for 5 minutes. The wash buffer was aspirated and 25 μL of DNA labelling mix (Millipore) was added to each well and the cells mixed. The plates were covered with parafilm and incubated for 60 minutes at 37 °C. 200 μL of rinsing buffer (Millipore) was then added to each well and the plates centrifuged at 300 x g for 5 minutes. The supernatant was aspirated and 50 μL of anti-BrdU staining mix (Millipore) added to each well, with the plate stored in the dark at room temperature for 30 minutes. At the end of the incubation 150 μL of rinsing buffer was added to each well. Cells were analysed on the Guava EasyCyte (Guava Technologies). Positive and negative controls were performed with each assay.

## 2.7. Drug Solubility

DMSO (Sigma) was used to prepare stock solutions of ferrocene-based compounds and was suitable for use *in vitro* as the low levels needed do not have a significant effect on cells used.

A number of vehicles were used in an attempt to solubilise *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester to a concentration of 2 mg/mL. The vehicles used are listed below (table 2.2).

Table 2.2: List of vehicles used as an alternative to DMSO as a vehicle for compound delivery *in vivo*.

| Vehicle | Source |
|---|---|
| 80 mM citrate buffer | Sigma |
| 5 % Carboxymethyl Cellulose | Sigma |
| 1 % Carboxymethyl Cellulose | Sigma |
| 50 % Propylene Glycol solution | Sigma |
| Intralipid® 20 % | Fresenius Kabi |

*N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester was insoluble in the vehicles alone, as it was first dissolved in DMSO at a concentration of 8 mg/mL and then diluted 1 in 4 with 80 mM citrate buffer/5 % Carboxymethyl Cellulose/1 % Carboxymethyl Cellulose/50 % Propylene Glycol solution to give a 2 mg/mL solution.

Intralipid, which is used in some preparations of the anaesthetic drug, propfol, was tested. *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester compound was initially dissolved in chloroform at a concentration of 2 mg/mL. Once in solution, it was transferred to a round-bottom flask followed by evaporation using a rotary vacuum apparatus (Buchi Rotavapor R-210 with a B-491 heating bath, V-700 vacuum pump) until the chloroform (Sigma) had fully evaporated and a thin layer of the compound was left on the inside wall of the flask. Intralipid was then added to resolubilise the compound at 2 mg/mL however the compound did not solubilise.

A PEGylated derivative of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(61)** was synthesized and dissolved in ultrapure water (UHP). This water was purified to a standard of 12-18 MΩ / cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP). The solution was then filter sterilised through a 0.22 µm sterile filters (Millipore).

### 2.8 Statistical Analysis

$IC_{50}$ values were calculated using CalcuSyn Software (BioSoft). Student t-test (two-tailed with unequal variance) was used to compare the activity of test compounds to their corresponding control group.

# Chapter 3

# Results

## 3.1. Cytotoxicological evaluation of 1-alkyl-1'-*N-para, N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters

### 3.1.1 Introduction

Organometallic compounds containing transition metals, such as cobalt, copper, iron and platinum are known to have antiproliferative (*in vitro*) and antineoplastic (*in vivo*) activities. Platinum coordination compounds, such as cisplatin and carboplatin are currently being used in the treatment of a variety of tumours [240]. However, problems with toxicity, harsh side effects during administration and acquired drug resistance have lead to increased research to find alternatives to cisplatin and its analogues.

Ferrocene is a particularly useful organometallic compound for biological applications due to its electrochemical properties, its stability and its aromatic nature which allows for derivatization. As a result of these factors, ferrocene research has received an increased level of interest over the past decade [240]. Ferricenium salts are known to inhibit tumour growth through the formation of hydroxyl radicals under physiological conditions, leading to the oxidative damage of DNA [241]. The aim of this research is to develop novel ferrocenyl dipeptide bioconjugates for use as potential anticancer agents and to elucidate potential modes of action which cause their antiproliferative activity.

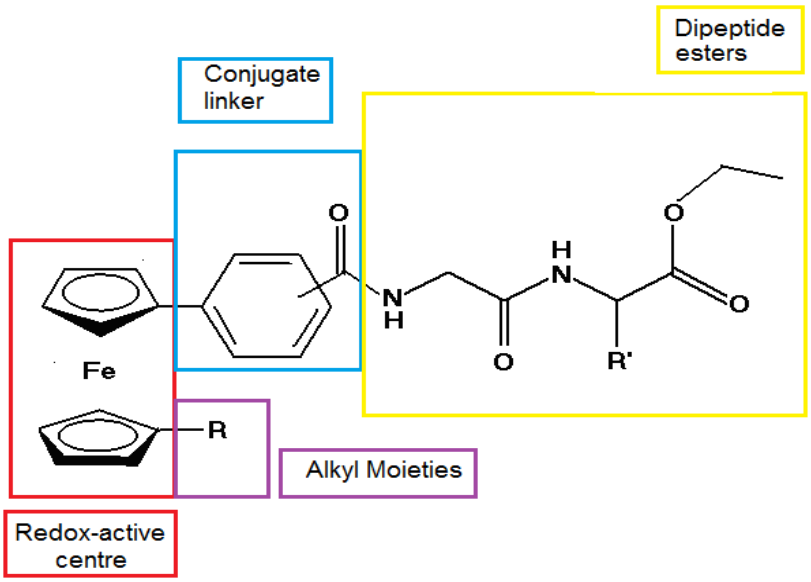
Previous studies within the group have been carried out on the non small cell lung cancer (NSCLC) cell line, H1299 to evaluate the *in vitro* activity of novel ferrocenyl benzoyl amino acid and dipeptide esters. The effect of these compounds on H1299 cell growth was expressed as $IC_{50}$ values. The $IC_{50}$ value is the concentration of a drug required for 50 % inhibition of cell growth. *N*-{*ortho*-(ferrocenyl) benzoyl} glycine ethyl ester was initially tested for its *in vitro* anti-proliferative activity against H1299 lung cancer cells. This compound was found to be cytotoxic and had an $IC_{50}$ value of 48 ± 1.0 µM, whereas the starting material, *ortho*-ferrocenyl ethyl benzoate, was completely inactive against the cell line. Therefore, other derivatives were prepared and evaluated for their anticancer activity against H1299 lung cancer cells. The dipeptide derivative *N*-{*ortho*-(ferrocenyl) benzoyl} glycine glycine ethyl ester was shown to have an $IC_{50}$ value of approximately 20 ± 1.7 µM, while *N*-

{*ortho*-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester had an $IC_{50}$ value of 5.3 ± 1.2 µM. The *N*-{*meta*-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester and *N*-{para-(ferrocenyl)-benzoyl} glycine L-alanine ethyl ester were also tested and gave results of 4.0 ± 0.7 µM and 6.6 ± 1.0 µM respectively [126-130].

As an extension of this study the cytotoxicological evaluation is reported of novel 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters synthesised by Dr Peter Kenny's research group, School of Chemical Sciences, Dublin City University. These compounds (Figure 3.1) consist of four key moieties:

(i) A redox active centre
(ii) A conjugate linker
(iii) A alkyl chain (further lowers the oxidation potential of the ferrocene moiety)
(iv) A dipeptide chain

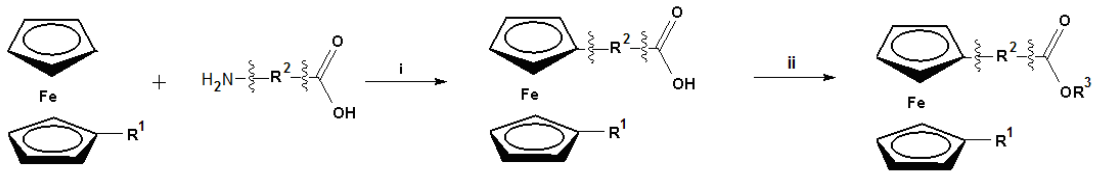
These novel derivatives differ from the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters by having an alkyl moiety on the previously unsubstituted cyclopentadiene ring (Figure 3.1). The primary objective of this research is to explore a structure-activity relationship (SAR) study of the incorporation of alkyl chains moieties on the unsubstituted ring of the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters [126-130]. The dipeptides employed in this investigation were Glycine Glycine (Gly Gly), Glycine L-Alanine (Gly L-Ala), Glycine L-Leucine (Gly L-Leu) and Glycine L-Phenylalanine (Gly L-Phe) ethyl esters. A tripeptide or tetrapeptide chain was shown to have a negative effect on biological activity [129]. Thus, a series of novel 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters **64–99** were prepared by coupling alkyl ferrocenyl benzoic acids to the dipeptide ethyl esters (Table 3.1).




**64–99**

R = $CH_3$ , $CH_2CH_3$ , $CH_2CH_2CH_3$
R' = H, $CH_3$ , $C_4H_9$, $C_7H_7$,

**Figure 3.1:** General structure of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters **64–99.**

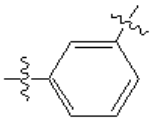
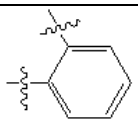
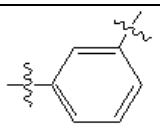
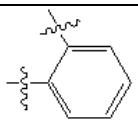
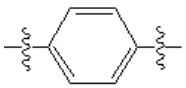
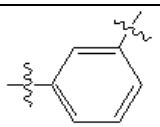
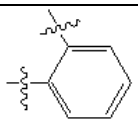



(i) $NaNO_2$, HCl

(ii) EDC, HOBt, Triethylamine and dipeptides ethyl esters ($R^3$)

**Figure 3.2:** The synthesis of 1-alkyl-1'-N-para, N-meta and N-ortho-(ferrocenyl) benzoyl dipeptide esters **64–99**.

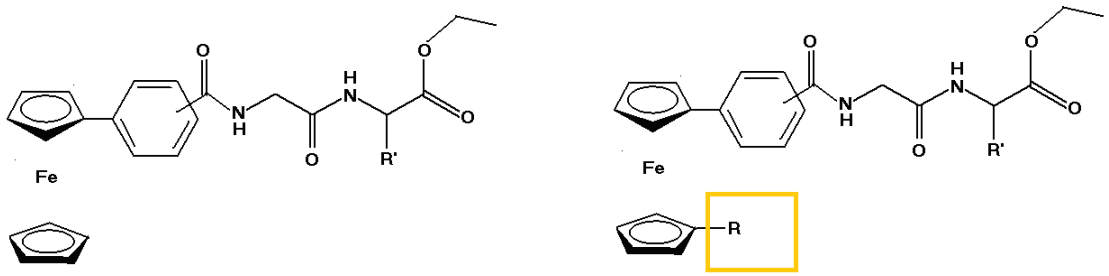
**Table 3.1:** The 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters **64–99** evaluated in this study.

| $R^1$ | $R^2$ | $R^3$ | Compound no. |
|---|---|---|---|
| -$CH_3$ (1-methyl-1') |  *N-para* | Gly Gly(OEt) | **64** |
| | | Gly L-Ala(OEt) | **65** |
| | | Gly L-Leu(OEt) | **66** |
| | | Gly L-Phe(OEt) | **67** |
| |  *N-meta* | Gly Gly(OEt) | **68** |
| | | Gly L-Ala(OEt) | **69** |
| | | Gly L-Leu(OEt) | **70** |
| | | Gly L-Phe(OEt) | **71** |
| |  *N-ortho* | Gly Gly(OEt) | **72** |
| | | Gly L-Ala(OEt) | **73** |
| | | Gly L-Leu(OEt) | **74** |
| | | Gly L-Phe(OEt) | **75** |
| -$CH_2CH_3$ (1-ethyl-1') |  *N-para* | Gly Gly(OEt) | **76** |
| | | Gly L-Ala(OEt) | **77** |
| | | Gly L-Leu(OEt) | **78** |
| | | Gly L-Phe(OEt) | **79** |
| |  *N-meta* | Gly Gly(OEt) | **80** |
| | | Gly L-Ala(OEt) | **81** |
| | | Gly L-Leu(OEt) | **82** |
| | | Gly L-Phe(OEt) | **83** |
| |  *N-ortho* | Gly Gly(OEt) | **84** |
| | | Gly L-Ala(OEt) | **85** |
| | | Gly L-Leu(OEt) | **86** |
| | | Gly L-Phe(OEt) | **87** |
| -$CH_2CH_2CH_3$ (1-propyl-1') |  *N-para* | Gly Gly(OEt) | **88** |
| | | Gly L-Ala(OEt) | **89** |
| | | Gly L-Leu(OEt) | **90** |
| | | Gly L-Phe(OEt) | **91** |
| |  *N-meta* | Gly Gly(OEt) | **92** |
| | | Gly L-Ala(OEt) | **93** |
| | | Gly L-Leu(OEt) | **94** |
| | | Gly L-Phe(OEt) | **95** |
| |  *N-ortho* | Gly Gly(OEt) | **96** |
| | | Gly L-Ala(OEt) | **97** |
| | | Gly L-Leu(OEt) | **98** |
| | | Gly L-Phe(OEt) | **99** |

### 3.1.2 Cytotoxicological evaluation of 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters

In previous studies carried out to evaluate the *in vitro* anticancer activity of *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters (figure 3.3) in non small cell lung cancer cell line, H1299, the general trend in cytotoxicity was *para* < *ortho* < *meta*. The dipeptide derivative *N*-{ortho-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester was shown to have an $IC_{50}$ value of 5.3 ± 1.2 µM. The *N*-{para-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester and the *N*-{meta-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester had $IC_{50}$ values of 6.6 ± 1.0 µM and 4.0 ± 0.7 µM respectively [126-130].

In a further SAR study a series of 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters were synthesised, structurally characterised and biologically evaluated. In total, 36 compounds were tested for their anti-proliferative effect on the non small cell lung cancer cell line, H1299. These novel derivatives differ from the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters by having an alkyl moiety on the previously unsubstituted ferrocene ring.




alkyl moiety (R) = $CH_3$; $CH_2CH_3$; $CH_2CH_2CH_3$
R' = H; $CH_3$; $C_4H_9$; $C_7H_7$

**Figure 3.3**: General structure of the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters and the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters.

### 3.1.3. *In vitro* cytotoxicity of 1-alkyl-1'-*N*-*para*, *N*-*meta* and *N*-*ortho*-(ferrocenyl) benzoyl dipeptide esters

The *in vitro* cytotoxicity of the 1-alkyl-1' derivatives **64–99** was evaluated by performing a comprehensive screen of every compound at a single dose (10 µM) in the H1299 cell line. The comprehensive screen was performed in triplicate by treating individual wells of a 96-well plate containing H1299 cells with a 10 µM solution of each test compound prepared in DMSO. A DMSO control and a control for normal (untreated) cell growth were included in the assays. The cells were then incubated for 5 days, until cell confluency was reached. At this point, cell survival was established through determination of the acid phosphatase activity of surviving cells. The results of the comprehensive screens are expressed as the percentage growth inhibition ± standard deviation (relative to the DMSO controls). Standard deviations have been calculated using data obtained from three independent experiments. The results for the preliminary screen of compounds **64–99** are depicted in figures 3.4-3.6.

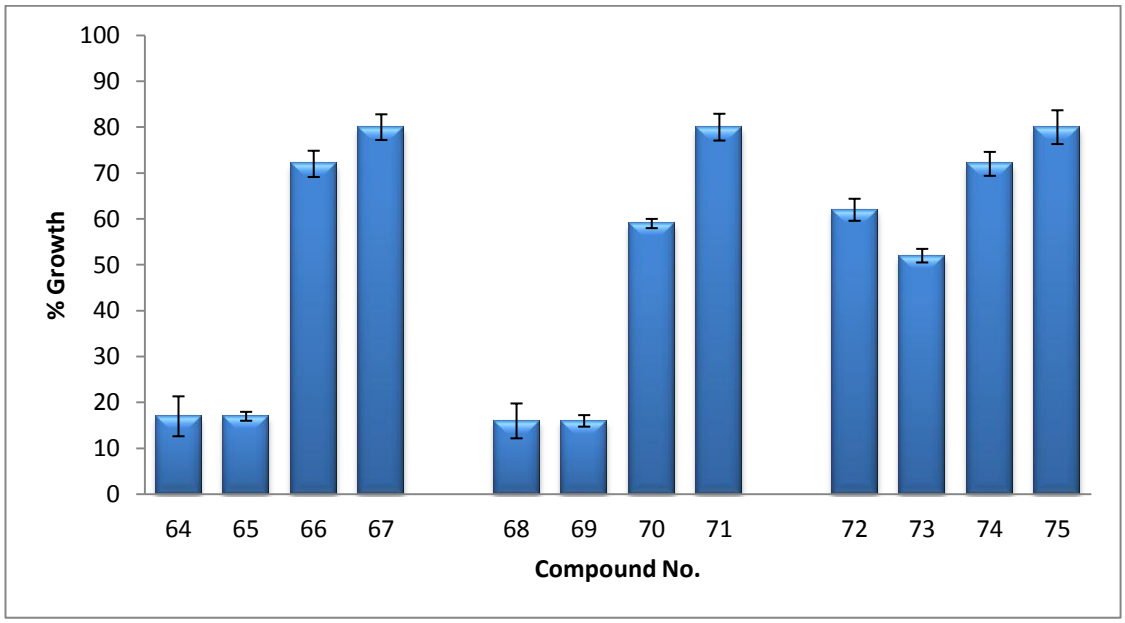
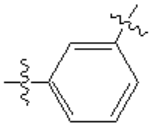
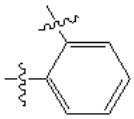



Figure 3.4: Percentage growth of H1299 cells after treatment of 10 µM of compounds **64-75**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments

**Table 3.2:** Percentage growth of H1299 lung cancer cells treated with 10 µM 1-methyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters **64–75**.

| **Alkyl** | **Benzoyl** | **Dipeptide ethyl ester** | **Compound No.** | **% growth at 10 µM** |
|---|---|---|---|---|
| -$CH_3$ (1-methyl-1') |  *N-para* | Gly Gly(OEt) | **64** | 17 ± 4.3 |
| | | Gly L-Ala(OEt) | **65** | 17 ± 1.0 |
| | | Gly L-Leu(OEt) | **66** | 72 ± 2.9 |
| | | Gly L-Phe(OEt) | **67** | 80 ± 2.8 |
| |  *N-meta* | Gly Gly(OEt) | **68** | 16 ± 3.8 |
| | | Gly L-Ala(OEt) | **69** | 16 ± 1.3 |
| | | Gly L-Leu(OEt) | **70** | 59 ± 1.0 |
| | | Gly L-Phe(OEt) | **71** | 80 ± 2.9 |
| |  *N-ortho* | Gly Gly(OEt) | **72** | 62 ± 2.4 |
| | | Gly L-Ala(OEt) | **73** | 52 ± 1.5 |
| | | Gly L-Leu(OEt) | **74** | 72 ± 2.6 |
| | | Gly L-Phe(OEt) | **75** | 80 ± 3.7 |

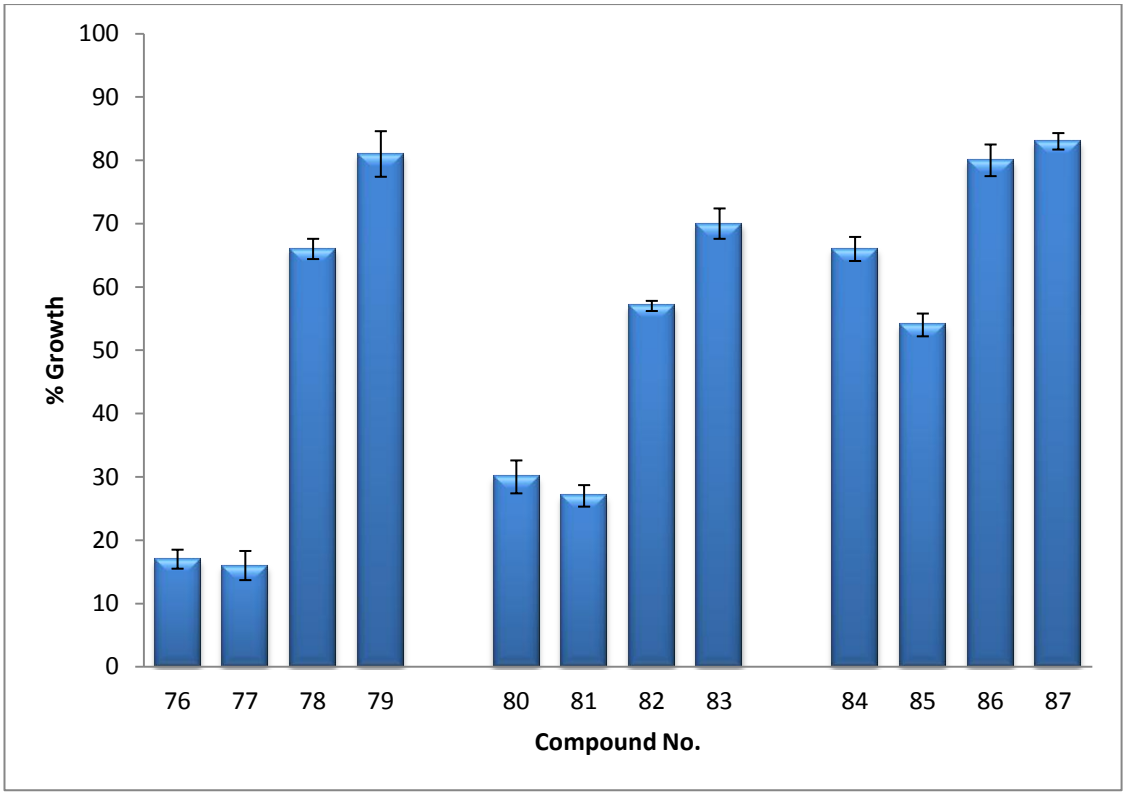
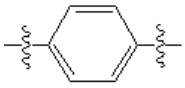
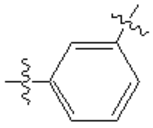
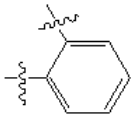



Figure 3.5: Percentage growth of H1299 cells after treatment of 10 µM of compounds **76-87**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

**Table 3.3:** Percentage growth of H1299 cells after treatment of 10 µM of compounds **76-87**.

| Alkyl | Benzoyl | Dipeptide ethyl ester | Compound No. | % growth at 10 µM |
|---|---|---|---|---|
| -$CH_2CH_3$ (1-ethyl-1') |  *N-para* | Gly Gly(OEt) | **76** | 17 ± 1.5 |
| | | Gly L-Ala(OEt) | **77** | 16 ± 2.3 |
| | | Gly L-Leu(OEt) | **78** | 66 ± 1.5 |
| | | Gly L-Phe(OEt) | **79** | 81 ± 3.6 |
| |  *N-meta* | Gly Gly(OEt) | **80** | 30 ± 2.6 |
| | | Gly L-Ala(OEt) | **81** | 27 ± 1.7 |
| | | Gly L-Leu(OEt) | **82** | 57 ± 0.8 |
| | | Gly L-Phe(OEt) | **83** | 70 ± 2.4 |
| |  *N-ortho* | Gly Gly(OEt) | **84** | 66 ± 1.9 |
| | | Gly L-Ala(OEt) | **85** | 54 ± 1.8 |
| | | Gly L-Leu(OEt) | **86** | 80 ± 2.5 |
| | | Gly L-Phe(OEt) | **87** | 83 ± 1.3 |

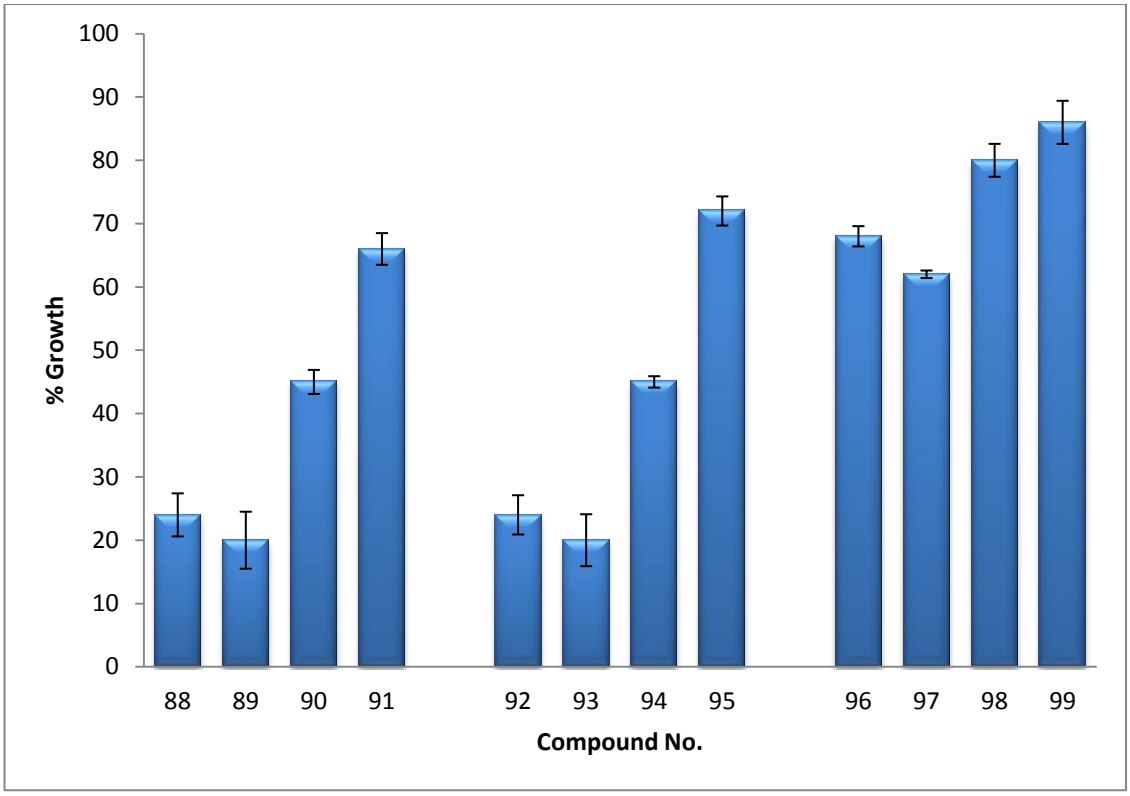
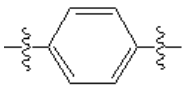
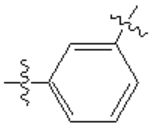
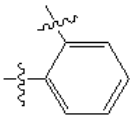



Figure 3.6: Percentage growth of H1299 cells after treatment of 10 µM of compounds **88-99**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

**Table 3.4:** Percentage growth of H1299 cells after treatment of 10 μM of compounds **88-99.**

| Alkyl | Benzoyl | Dipeptide ethyl ester | Compound No. | % growth at 10 μM |
|---|---|---|---|---|
| - $CH_2CH_2CH_3$ (1-propyl-1') |  *N-para* | Gly Gly(OEt) | **88** | 24 ± 3.4 |
| | | Gly L-Ala(OEt) | **89** | 20 ± 4.5 |
| | | Gly L-Leu(OEt) | **90** | 45 ± 1.9 |
| | | Gly L-Phe(OEt) | **91** | 66 ± 2.5 |
| |  *N-meta* | Gly Gly(OEt) | **92** | 24 ± 3.1 |
| | | Gly L-Ala(OEt) | **93** | 20 ± 4.1 |
| | | Gly L-Leu(OEt) | **94** | 45 ± 0.9 |
| | | Gly L-Phe(OEt) | **95** | 72 ± 2.3 |
| |  *N-ortho* | Gly Gly(OEt) | **96** | 68 ± 1.6 |
| | | Gly L-Ala(OEt) | **97** | 62 ± 0.6 |
| | | Gly L-Leu(OEt) | **98** | 80 ± 2.6 |
| | | Gly L-Phe(OEt) | **99** | 86 ± 3.4 |

From the preliminary screen at 10 μM a general trend can be observed, the 1-alkyl-1'- *N-ortho*- derivatives exhibited lower growth inhibition compared to the *meta* and *para* derivatives for the dipeptides employed in the SAR study. For instance, the 1-methyl-1'-*N*-{*ortho*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **72** inhibited growth by 38 ± 2.4 % whilst the 1-methyl-1'-*N-para* and *meta*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **(64** and **68)** inhibited growth by 83 ± 4.3 % and 84 ± 3.8 % respectively. The *ortho* derivatives were not investigated further.

A general trend was also observed in these compounds, that is, the Gly Gly (OEt) and Gly L-Ala (OEt) derivatives are more active than the Gly L-Leu and Gly L-Phe ethyl esters which inhibit growth by greater than 45 %. For instance the 1-ethyl-1'-*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **76** and glycine L-alanine ethyl ester **77** inhibit growth by 83 ± 1.5 % and 84 ± 2.3 % respectively, whilst the

Gly L-Leu(OEt) **78** and Gly L-Phe(OEt) **79** inhibited growth by 34 ± 1.6 % and 19 ± 3.6 respectively. Thus, the Gly L-Leu and Gly L-Phe ethyl ester derivatives were not investigated further. As a result, it can be concluded that when chiral α-amino acids with bulky side chains are used as the second amino acid in the dipeptide moiety a loss of anti-proliferative activity is observed for this particular group of compounds.

For the 1-alkyl-1'-*N*-*para*, *N*-*meta*-(ferrocenyl) benzoyl dipeptide esters, 12 lead compounds were identified which inhibited growth greater than 70 % and therefore, $IC_{50}$ values were determined for these compounds.

**Table 3.5.** $IC_{50}$ values for selected compounds, carboplatin and cisplatin against human lung carcinoma cell line H1299.

| **Compound Name** | **No.** | **$IC_{50}$(μM)** |
|---|---|---|
| Cisplatin | **8** | 1.5 ± 0.1 |
| Carboplatin | **9** | 10.0 ± 1.6 |
| 1-methyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **64** | 2.8 ± 1.2 |
| 1-methyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl} Gly L-Ala(OEt) | **65** | 4.5 ± 0.4 |
| 1-methyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **68** | 2.6 ± 0.6 |
| 1-methyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly L-Ala(OEt) | **69** | 6.7 ± 0.3 |
| 1-ethyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **76** | 3.5 ± 0.8 |
| 1-ethyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl} Gly L-Ala(OEt) | **77** | 5.6 ± 1.6 |
| 1-ethyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **80** | 6.1 ± 1.1 |
| 1-ethyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly L-Ala(OEt) | **81** | 13.3 ± 1.1 |
| 1-propyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **88** | 5.4 ± 1.2 |
| 1-propyl-1' -*N*- {*para*-(ferrocenyl)-benzoyl}Gly L-Ala(OEt) | **89** | 6.6 ± 2.1 |
| 1-propyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly Gly(OEt) | **92** | 11.3 ± 2.1 |
| 1-propyl-1' -*N*- {*meta*-(ferrocenyl)-benzoyl} Gly L-Ala(OEt) | **93** | 20.1 ± 2.5 |

From the $IC_{50}$ values (Table 3.5) of the 1-alkyl-1'-*N-para*, *N-meta* -(ferrocenyl) benzoyl Gly Gly and Gly L-Ala ethyl esters **64, 65, 68, 69, 76, 77, 80, 81, 88, 89, 92** and **93,** it can be seen that the cytotoxicity of the derivatives decreases as the size of the alkyl moiety incorporated increases (methyl < ethyl < propyl). Indeed, all the methyl and ethyl derivatives display $IC_{50}$ values that are lower than 15 µM. The *in vitro* cytotoxicity of the platinum (II) based anticancer drug carboplatin was also evaluated against the H1299 cell line and the carboplatin $IC_{50}$ value was determined to be 10.0 ± 1.6 µM (Table 3.5). Thus, compounds **64, 65, 68, 69, 76, 77, 80, 88** and **89** are more cytotoxic *in vitro* than the clinically employed anticancer drug carboplatin. In addition, the 1-methyl-1'-*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **64** displays an $IC_{50}$ value of 2.8 ± 1.2 µM, the 1-ethyl-1'–*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **76** displays an $IC_{50}$ value of 3.5 ± 0.8 µM and the 1-methyl- 1' -*N*- {*meta*-(ferrocenyl)-benzoyl} glycine glycine ethyl ester **68** shows an $IC_{50}$ value of 2.6 ± 0.6 µM. These compounds display improved bioactivity in comparison to the most active ferrocenyl benzoyl analogues previously studied. The *N*-{meta-(ferrocenyl) benzoyl} glycine L-alanine ethyl ester displayed an $IC_{50}$ value of 4.0 ± 0.7 µM. [129]

From these results, incorporation of methyl and ethyl alkyl groups results in an improved cytotoxicity of the *para* derivatives of the glycine glycine ethyl esters and an improved cytotoxicity of the methyl *meta* glycine glycine derivative. However, when the size of the chain length increased to ethyl, the 1-ethyl-1'-*N*-{meta-(ferrocenyl)-benzoyl} glycine L-alanine ethyl ester **81** displayed an $IC_{50}$ of 13.3 ± 1.1 µM. Thus, the increase in alkyl chain length resulted in a drastic decrease in the cytotoxicity of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters. Although compounds **64** and **68** are the most active derivatives of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters, they are less potent than cisplatin which has an $IC_{50}$ value of 1.5 ± 0.1 µM against human H1299 lung cancer cells.

### 3.1.4 Conclusion

Cytotoxicity of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters, decreases with the increase of the size of the alkyl group

incorporated (propyl < ethyl < methyl) on the ferrocene moiety. All the methyl and ethyl derivatives have $IC_{50}$ values that are lower than 15 μM. The order of the amino acids in the dipeptide chain is crucial for activity, the trend being Gly Gly > Gly L-Ala > Gly L-Leu > Gly L-Phe. The most active derivatives of the 1-alkyl-1'-*N*-*para*, *N*-*meta* and *N*-*ortho*-(ferrocenyl) benzoyl dipeptide esters are the 1-methyl-1'-*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **64** and the 1-methyl-1'-*N*-{*meta*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **68** which display $IC_{50}$ values of 2.8 ± 1.23 μM and 2.6 ± 0.62 μM respectively. These compounds are more active than carboplatin but are less effective compared to cisplatin which are clinically employed anticancer drugs.

### 3.2. Cytotoxicological evaluation of *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} and *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} amino acid and dipeptide esters

#### 3.2.1. Introduction

To date we have shown that the replacement of the conjugated linker of *N*-(ferrocenyl) benzoyl dipeptide esters with a naphthoyl linker leads to an improvement in antiproliferative effect in the non small cell lung cancer (NSCLC) cell line, H1299 [132-134]. From the series of *N*-(ferrocenyl) naphthoyl amino acid and dipeptide ethyl esters reported, the *N*-{6-(ferrocenyl)-2-napthoyl} glycine glycine ethyl ester **31** displayed an $IC_{50}$ value of 0.13 ± 0.01 μM in the H1299 lung cancer cell line [134].

We have been clearly demonstrated in the SAR study of the 1-alkyl-1' derivatives that employing the incorporation of a methyl derivative to the unsubstituted cyclopentadiene ring enhances the anti-proliferative effect of the ferrocenyl dipeptide bioconjugates. The most active derivative was the 1-methyl-1'-*N*-{*meta*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **68** which displayed an $IC_{50}$ value of 2.6 ± 0.6 μM.

In a continuation to this SAR study in search for novel compounds with increased anti-proliferative effect, a new library of ferrocenyl based bioconjugates **105-138**, which consist of four key moieties was synthesised (figure 3.8):

(i) An electroactive core
(ii) An ethynyl moiety
(iii) Three different aromatic linkers
(iv) A series of amino acid and dipeptide esters

This SAR study involves the cytotoxicological evaluation of the incorporation of an ethynyl moiety between the ferrocene moiety and the conjugate linker. Furthermore, the incorporation of L-leucine and L-phenylalanine into the dipeptide chains have been shown in the preliminary screen of the 1-alkyl-1' derivatives (**64-69**) to display low activity against the H1299 lung cancer cell line. The dipeptides and the amino acid employed were Glycine Glycine (Gly Gly), Glycine L-Alanine (Gly L-Ala), Glycine D-Alanine (Gly D-Ala), Sarcosine Glycine (Sar Gly), Sarcosine L-Alanine (Sar L-Ala), L-Proline L-Alanine (L-Pro L-Ala), L-Proline Glycine (L-Pro Gly), Glycine L-Leucine (Gly L-Leu) and Glycine L-Phenylalanine (Gly L-Phe) and γ-aminobutyric acid **(**GABA).

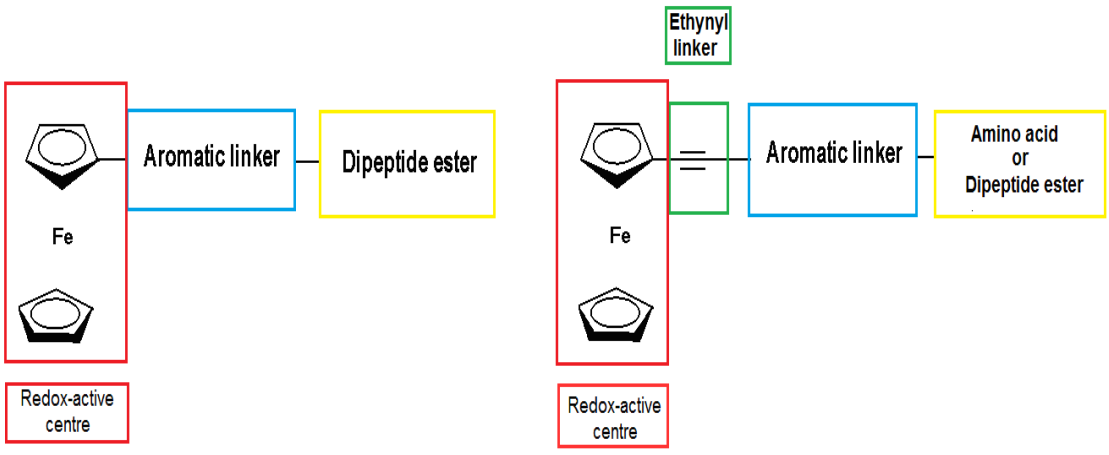
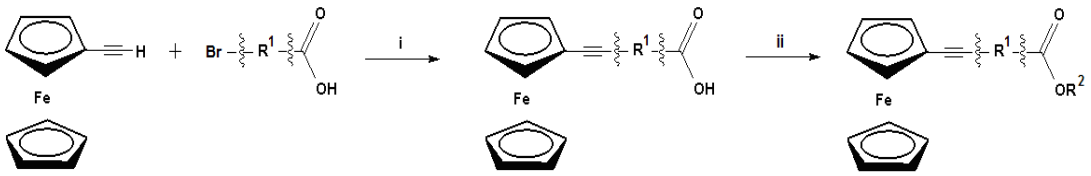



**Figure 3.7:** General structure of the *N*-(ferrocenyl) benzoyl and naphthoyl bioconjugates and *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} and *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} amino acid and dipeptide esters.

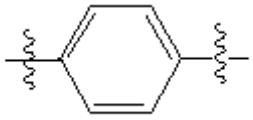
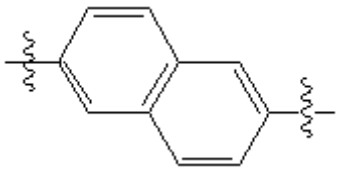
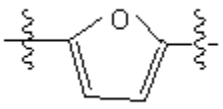



(i) TEA, $PPh_3$, *Bis*(triphenylphosphine)palladium(II) dichloride, THF, Cu(I)

(ii) EDC, HOBt, triethylamine, dipeptide esters and free *N*-terminal γ-Amino butyric acid esters

**Figure 3.8**: Synthesis of *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl}, *N*-{5-ferrocenyl ethynyl-2-furanoyl}amino acid and dipeptide esters **105-138**.

**Table 3.6:** *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl}, *N*-{5-ferrocenyl ethynyl-2-furanoyl} amino acid and dipeptide esters **105-138**.

| $R^1$ | $R^2$ | Compound no. |
|---|---|---|
|  | Gly Gly(OMe) | **105** |
| | GlyGly(OEt) | **106** |
| | Gly L-Ala(OMe) | **107** |
| | Gly L-Ala(OEt) | **108** |
| | Gly D-Ala(OMe) | **109** |
| | Gly D-Ala(OEt) | **110** |
| | GABA(OMe) | **111** |
| | GABA(OEt) | **112** |
| | Gly L-Phe(OEt) | **113** |
| | Gly L-Leu(OEt) | **114** |
| | Sar Gly(OEt) | **115** |
| | Sar Gly(OMe) | **116** |
| | Sar L-Ala(OEt) | **117** |
| | L-Pro Gly(OEt) | **118** |
| | L-Pro L-Ala(Oet) | **119** |
|  | Gly Gly(OEt) | **120** |
| | Gly L-Ala(OEt) | **121** |
| | Sar L-Ala(OEt) | **122** |
| | L-Pro Gly(OEt) | **123** |
| | GABA(OEt) | **124** |
| | L-Pro L-Ala(Oet) | **125** |
| | Gly L-Leu(OEt) | **126** |
| | Sar Gly(OEt) | **127** |
| | Gly L-Phe(OEt) | **128** |
|  | Gly Gly(OEt) | **129** |
| | Gly L-Ala(OEt) | **130** |
| | Gly D-Ala(OEt) | **131** |
| | Gly L-Phe(OEt) | **132** |
| | Gly L-Leu(OEt) | **133** |
| | Sar Gly(OEt) | **134** |
| | L-Pro Gly(OEt) | **135** |
| | L-Pro L-ala(OEt) | **136** |
| | Sar L-ala(OEt) | **137** |
| | GABA(OEt) | **138** |

### 3.2.2. Preliminary cytotoxicological evaluation of the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} amino acid and dipeptide esters

The *in vitro* cytotoxicity of the derivatives **105-119** was initially tested at 10 µM in the H1299 lung cancer cell line. The results for the percentage growth of the H1299 cell line compared to a DMSO control are depicted in Figure 3.9 and table 3.7.

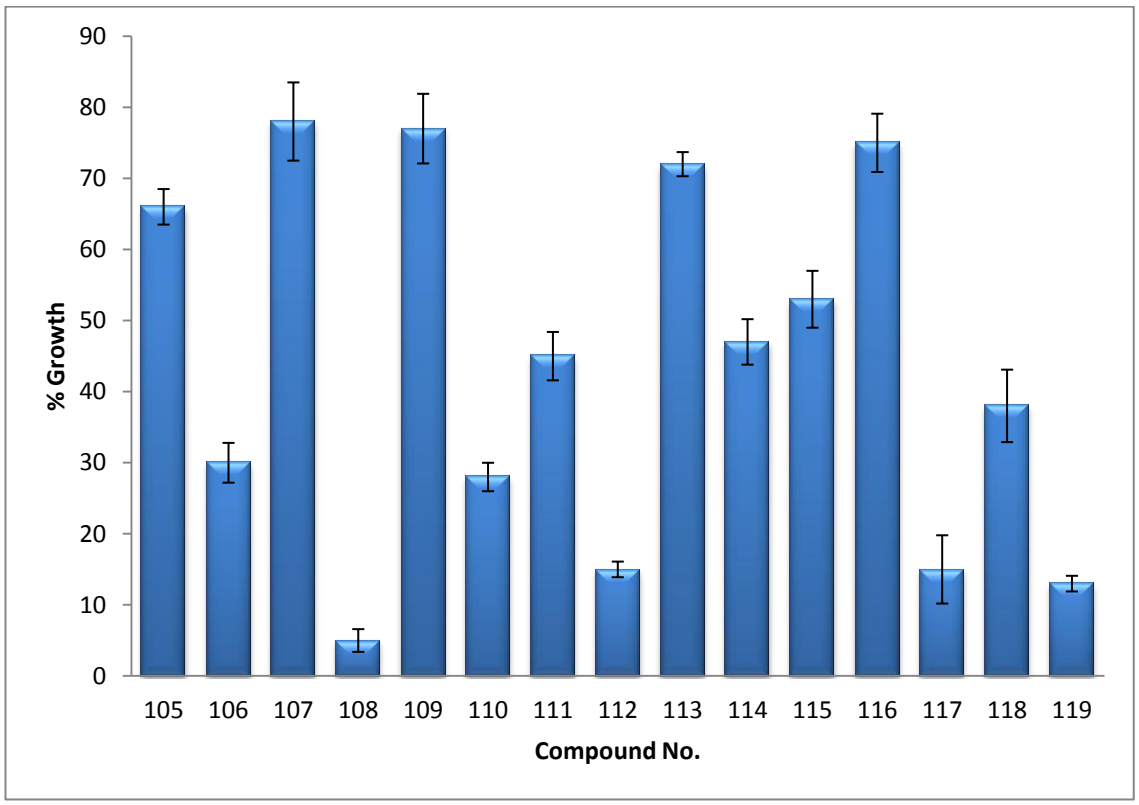
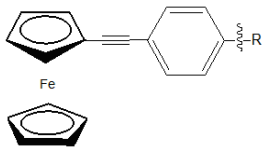



**Figure 3.9:** Percentage growth of H1299 cells after treatment of 10 µM of compounds **105-119**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

**Table 3.7:** Percentage growth at 10 μM on H1299 lung cancer cells for *N*-{*para*-(ferrocenyl) ethynyl benzoyl} amino acid and dipeptide esters **105-119**

| **Ferrocenyl bioconjugate** | **R** | **Compound no.** | **% growth at 10 μM** |
|---|---|---|---|
|  | Gly Gly(OMe) | **105** | 66 ± 2.5 |
| | Gly Gly(OEt) | **106** | 30 ± 2.8 |
| | Gly L-Ala(OMe) | **107** | 78 ± 5.5 |
| | Gly L-Ala(OEt) | **108** | 5 ± 1.6 |
| | Gly D-Ala(OMe) | **109** | 77 ± 4.9 |
| | Gly D-Ala(OEt) | **110** | 28 ± 2.0 |
| | GABA(OMe) | **111** | 45 ± 3.4 |
| | GABA(OEt) | **112** | 15 ± 1.1 |
| | Gly L-Phe(OEt) | **113** | 72 ± 1.7 |
| | Gly L-Leu(OEt) | **114** | 47 ± 3.2 |
| | Sar Gly(OEt) | **115** | 53 ± 4.0 |
| | Sar Gly(OMe) | **116** | 75 ± 4.1 |
| | Sar L-Ala(OEt) | **117** | 15 ± 4.8 |
| | L-Pro Gly(OEt) | **118** | 38 ± 5.1 |
| | L-Pro L-Ala(OEt) | **119** | 13 ± 1.1 |

From the preliminary screen at 10 μM a general trend can be observed, the methyl ester derivatives **105, 107, 109, 111, 116** inhibited cell growth by 22 ± 5.5 to 55 ± 3.4 % compared to the ethyl ester derivatives **106, 108, 110, 112, 113, 114, 115, 117, 118, 119** which inhibited growth by 47± 4.0 to 95 ± 1.6 %. Thus, the methyl ester derivatives were not investigated further.

A general trend was also observed in these derivatives, that is, the Gly L-Leu and Gly L-Phe ethyl esters inhibit growth by less that 53 %. For instance, the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} glycine L-phenylalanine ethyl ester **113** inhibited growth by 28 ± 1.7 % whilst the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} glycine L-leucine ethyl ester **114** inhibited growth by 53 ± 3.2 %. Thus, the Gly L-Leu and Gly L-Phe ethyl ester derivatives were not investigated further. As a result, it can be concluded that when chiral α-amino acids with bulky side chains are used as the second amino acid in the dipeptide moiety, a loss of anti-proliferative activity is

observed for this particular group of compounds. Compounds **106, 108, 110, 112, 117, 118** and **119** inhibited growth by greater than 60 %. Therefore, $IC_{50}$ values were determined for these compounds.

### 3.2.3 Preliminary cytotoxicological evaluation of the *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} amino acid and dipeptide esters

The *in vitro* anti-proliferative effect of compounds **120-128** were studied at a concentration of 10 µM in the H1299 lung cancer cells. The results of this biological study are shown in figure 3.10 and table 3.8. From the preliminary screen a general trend was observed, the Gly L-Leu **126** and Gly L-Phe **128** ethyl esters inhibited growth by less than 34 %. Thus, the Gly L-Leu and Gly L-Phe ethyl ester derivatives were not investigated further. Compounds **120, 121, 122, 123, 124, 125** and **127** inhibited growth by greater than 67 %. Therefore, $IC_{50}$ values were determined for these compounds.

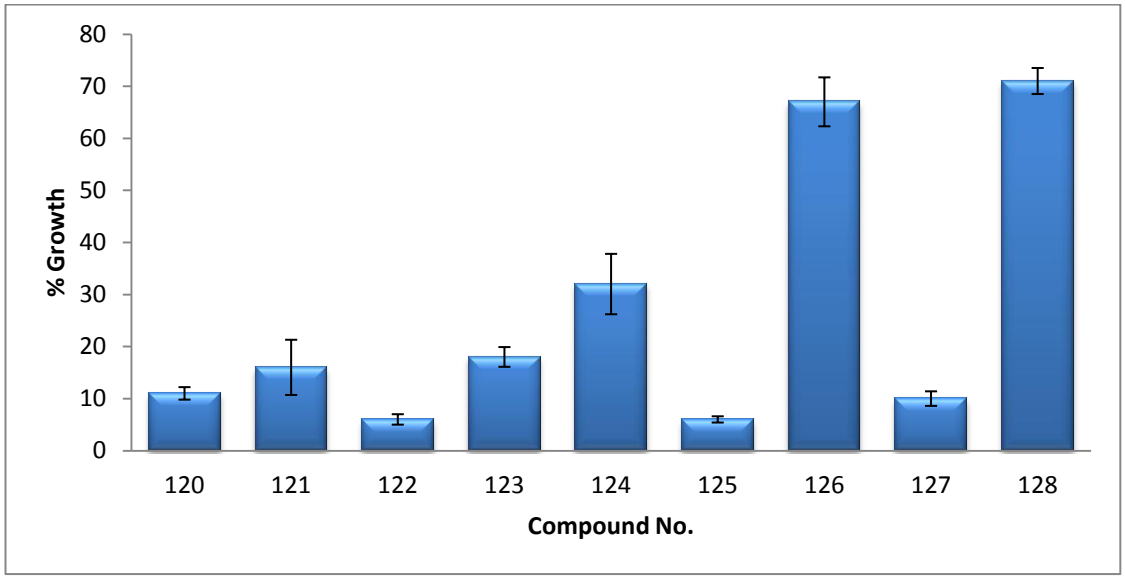
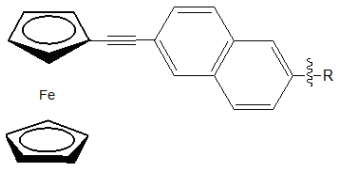



**Figure 3.10:** Percentage growth of H1299 cells after treatment of 10 µM of compounds **120-128**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

**Table 3.8:** Percentage growth at 10 µM on H1299 lung cancer cells for *N*-{*para*-(ferrocenyl) ethynyl naphthoyl} amino acid and dipeptide esters **120-128.**

| **Ferrocenyl bioconjugate** | **R** | **Compound no.** | **% growth at 10 µM** |
|---|---|---|---|
|  | Gly Gly(OEt) | **120** | 11 ± 1.2 |
| | Gly L-Ala(OEt) | **121** | 16 ± 5.3 |
| | Sar L-Ala(OEt) | **122** | 6 ± 1.0 |
| | L-Pro Gly(OEt) | **123** | 18 ± 1.92 |
| | GABA(OEt) | **124** | 32 ± 5.8 |
| | L-Pro L-Ala(Oet) | **125** | 6 ± 0.6 |
| | Gly L-Leu(OEt) | **126** | 67 ± 4.7 |
| | Sar Gly(OEt) | **127** | 10 ± 1.4 |
| | Gly L-Phe(OEt) | **128** | 71 ± 2.53 |

### 3.2.4 Preliminary cytotoxicological evaluation of the *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} amino acid and dipeptide esters

The *in vitro* cytotoxicity of the derivatives **129-138** against the human lung carcinoma cell line H1299 was evaluated by the acid phosphatase assay and the % growth inhibition achieved by the derivatives are depicted in figure 3.11 and table 3.9.

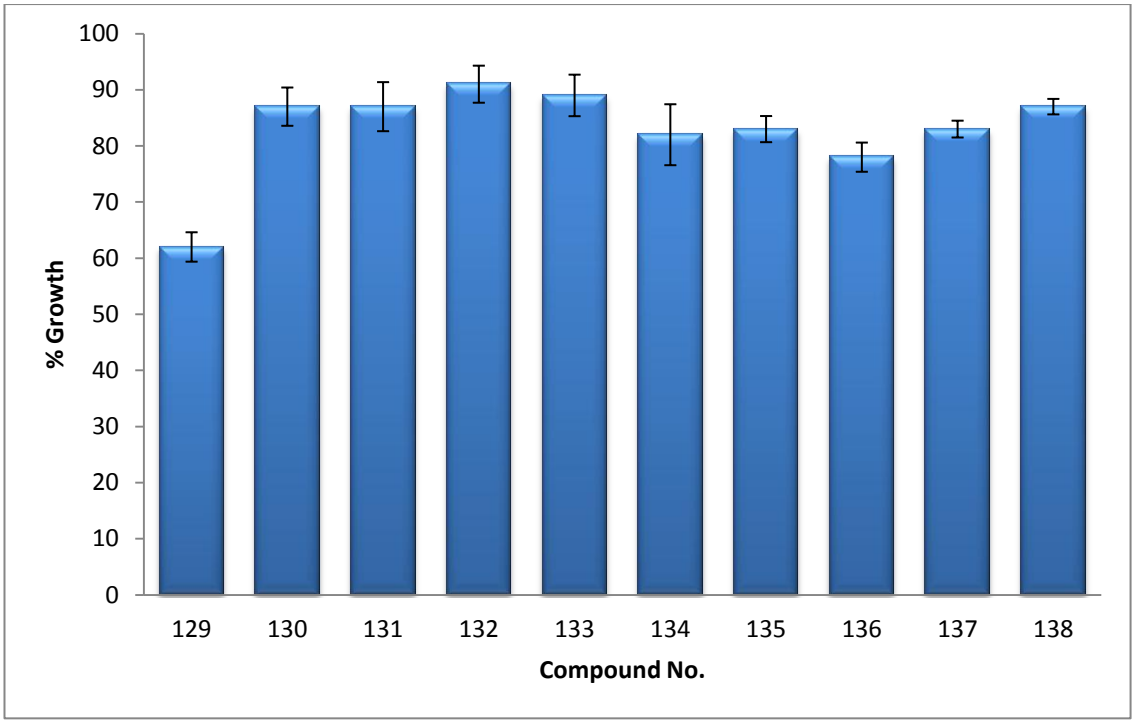
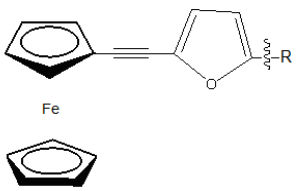



**Figure 3.11:** Percentage growth of H1299 cells after treatment of 10 µM of compounds **129-138**. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

**Table 3.9:** Percentage growth of H1299 lung cancer cells treated with 10 µM *N*-{*para*-(ferrocenyl) furanoyl naphthoyl} amino acid and dipeptide esters **129-138.**

| **Ferrocenyl bioconjugate** | **R** | **Compound no.** | **% growth at 10 µM** |
|---|---|---|---|
|  | Gly Gly(OEt) | **129** | 62 ± 2.6 |
| | Gly L-Ala(OEt) | **130** | 87 ± 3.4 |
| | Gly D-Ala(OEt) | **131** | 87 ± 4.4 |
| | Gly L-Phe(OEt) | **132** | 91 ± 3.3 |
| | Gly L-Leu(OEt) | **133** | 89 ± 3.7 |
| | Sar Gly(OEt) | **134** | 82 ± 5.4 |
| | L-Pro Gly(OEt) | **135** | 83 ± 2.3 |
| | L-Pro L-ala(Oet) | **136** | 78 ± 2.6 |
| | Sar L-ala(OEt) | **137** | 83 ± 1.5 |
| | GABA(OEt) | **138** | 87 ± 1.4 |



From the preliminary screen at 10 µM, all derivatives showed little effect on growth, with growth inhibition between 9 ± 3.3 and 38 ± 2.6 %. In the case of the *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} glycine glycine ethyl ester **129,** it achieved the

highest inhibition of growth of 38 ± 2.6 % and thus, an $IC_{50}$ value for **129** was determined. For compounds **130-138** no further investigation were carried out.

### 3.2.5 $IC_{50}$ value determination of *N*-{*para*-(ferrocenyl) ethynyl benzoyl} amino acid and dipeptide esters

Further studies were conducted as described in Section 3.1.3 to determine $IC_{50}$ values for the H1299 cell line. The $IC_{50}$ value for each compound was calculated using Calcusyn software, and standard deviations were calculated using data obtained from three independent experiments. The values obtained are listed in table 3.10.

**Table 3.10:** $IC_{50}$ values for selected compounds, carboplatin and cisplatin H1299 cells.

| **Compound Name** | **No.** | **$IC_{50}$ (μM)** |
|---|---|---|
| Cisplatin | **8** | 1.5 ± 0.1 |
| Carboplatin | **9** | 10.0 ± 1.6 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} Gly Gly(OEt) | **106** | 6.9 ± 2.1 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} Gly L-Ala(OEt) | **108** | 3.8 ± 1.9 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} Gly D-Ala(OEt) | **110** | 6.1 ± 3.4 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} GABA(OEt) | **112** | 4.9 ± 4.1 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} Sar L-Ala(OEt) | **117** | 7.1 ± 2.5 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} L-Pro Gly(OEt) | **118** | 8.3 ± 3.1 |
| *N*-{*para*-(ferrocenyl) ethynyl benzoyl} L-Pro L-Ala(OEt) | **119** | 5.7 ± 2.9 |

From the $IC_{50}$ values of compounds **106, 108, 110, 112, 117, 118** and **119** all exert a cytotoxic effect on the H1299 lung cancer cell line. All seven derivatives have an $IC_{50}$ value that is lower than 9 μM and more cytotoxic *in vitro* than carboplatin. The

most active compound was *N*-{*para*-(ferrocenyl) ethynyl benzoyl} glycine L-alanine ethyl ester **108** which had an $IC_{50}$ value of 3.8 ± 1.9 µM.

The *in vitro* cytotoxicity of cisplatin was also evaluated in the H1299 cell line and the $IC_{50}$ value was determined to be 1.5 ± 0.1 µM. Thus, compounds **106, 108, 110, 112, 117, 118** and **119** are less cytotoxic *in vitro* than cisplatin.

Previously, *in vitro* cytotoxic assays have shown the *N*-(ferrocenyl) naphthoyl derivatives to be more active than the *N*-(ferrocenyl) benzoyl derivatives with the most active derivative, *N*-{6-(ferrocenyl)-2-napthoyl} glycine glycine ethyl ester displaying an $IC_{50}$ value of 0.13 ± 0.01 µM [131]. For the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} glycine glycine ethyl ester, an $IC_{50}$ value of 6.9 ± 5.1 µM was obtained. Thus, it can be concluded that the presence of the ethynyl moiety did not result in an increase in the cytoxicity of the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} amino acid and dipeptide esters, when compared to the *N*-(ferrocenyl) benzoyl and naphthoyl analogues previously reported [126-130].

### 3.2.6 $IC_{50}$ value determination of *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} amino acid and dipeptide esters

The $IC_{50}$ values for derivatives **120-128** were determined by the acid phosphatase assay as previously described in section 3.2.3. The $IC_{50}$ values for derivatives **120-127** relative to cisplatin and carboplatin are depicted in table 3.11.

**Table 3.11:** $IC_{50}$ values for selected compounds, carboplatin and cisplatin in H1299 cells.

| **Compound Name** | **No.** | **$IC_{50}$ (µM)** |
|---|---|---|
| Cisplatin | **8** | 1.5 ± 0.1 |
| Carboplatin | **9** | 10.0 ± 1.6 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} Gly Gly(OEt) | **120** | 5.0 ± 4.1 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} Gly L-Ala(OEt) | **121** | 5.0 ± 3.6 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} Sar L-Ala(OEt) | **122** | 3.2 ± 2.6 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} L-Pro Gly(OEt) | **123** | 5.1 ± 1.4 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} GABA(OEt) | **124** | 7.2 ± 1.5 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} L-Pro L-Ala(OEt) | **125** | 3.8 ± 2.0 |
| *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} Sar Gly(OEt) | **127** | 4.7 ± 3.7 |

From the $IC_{50}$ values of the *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} amino acid and dipeptide ethyl esters **120, 121, 122, 123, 124, 125** and **127,** all exert a cytotoxic effect on the H1299 lung cancer cell line. All seven derivatives have an $IC_{50}$ value that is lower than 7.3 µM and more cytotoxic *in vitro* than carboplatin. The most active compound was *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} sarcosine L-alanine ethyl ester **122** which had an $IC_{50}$ value of 3.2 ± 2.6 µM.

### 3.2.7 $IC_{50}$ value determination of *N*-{5-(ferrocenyl) ethynyl-2-furanoyl}-glycine-glycine ethyl ester 129.

The *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} glycine glycine ethyl ester **129** was the only derivative of the N- {para-(ferrocenyl) furanoyl naphthoyl} amino acid and dipeptide esters series to be tested to determine the $IC_{50}$ value (table 3.12).

**Table 3.12:** $IC_{50}$ values for **129**, carboplatin and cisplatin in H1299 cells.

| **Compound Name** | **No.** | **$IC_{50}$(µM)** |
|---|---|---|
| Cisplatin | **8** | 1.5 ± 0.1 |
| Carboplatin | **9** | 10 ± 1.6 |
| *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} Gly Gly(OEt) | **129** | 23 ± 5.1 |

From the results obtained, compound **129** displayed an $IC_{50}$ value of 23 ± 5.1 µM and was less cytotoxic *in vitro* than cisplatin or carboplatin. In addition, when compared to the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} **106** and *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} glycine glycine ethyl esters **120** with $IC_{50}$ values of 6.9 ± 2.1 µM and 5.0 ± 4.1 µM respectively, compound **129** was also less cytotoxic. As a result it can be concluded that the furanoyl ring is not a bioactive aromatic conjugate linker that promotes anti-proliferative activity in the H1299 lung cancer line.

### 3.2.8 Conclusion

The cytotoxicological evaluation of *N*-{*para*-(ferrocenyl) ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} and *N*-{5-(ferrocenyl) ethynyl-2-furanoyl} amino acid and dipeptide esters was carried out in the H1299 lung cancer cell line. The most active derivative *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} sarcosine L-alanine ethyl ester **122** displayed an $IC_{50}$ value of 3.2 ± 2.6 µM. This compound is more active than the clinically used carboplatin but is less effective than cisplatin. A general trend can be seen between the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} and *N*-

{6-(ferrocenyl) ethynyl-2-naphthoyl} amino acid and dipeptide esters. The Gly Gly, Sar L-Ala, L-Pro Gly, L-Pro L-Ala ethyl esters of the *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} derivatives are more active than the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} derivatives whilst the Gly L-Ala and GABA esters of the *N*-{*para*-(ferrocenyl) ethynyl benzoyl} derivatives are more active than the *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} derivatives.

From the SAR studies carried out it has been clearly demonstrated that the presence of the ethynyl moiety does not result in any significant enhancement of the anti-proliferative effect of the *N*-(ferrocenyl) benzoyl and naphthoyl dipeptide derivatives. However, the incorporation of a methyl derivative to the unsubstituted cyclopentadiene ring of the *N*-(ferrocenyl) benzoyl dipeptide derivatives showed an enhancement of the anti-proliferative effect. Thus, the incorporation of a methyl group in the most active *N*-{6-(ferrocenyl)-2-napthoyl} glycine glycine ethyl ester **31** ($IC_{50}$ value of 0.13 ± 0.01 μM) could result in the formulation of a potent novel bioorganometallic anticancer agent.

## 3.3 Cytotoxicological evaluation of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with altered ester moiety

### 3.3.1 Introduction

To date Dr Peter Kenny's group have shown that the replacement of the conjugated linker of *N*-(ferrocenyl) benzoyl dipeptide esters with a naphthoyl linker leads to an improvement in antiproliferative effect in the non small cell lung cancer (NSCLC) cell line, H1299. They synthesized a number of novel compounds with varying lengths of ester residues with marked variety in activity.

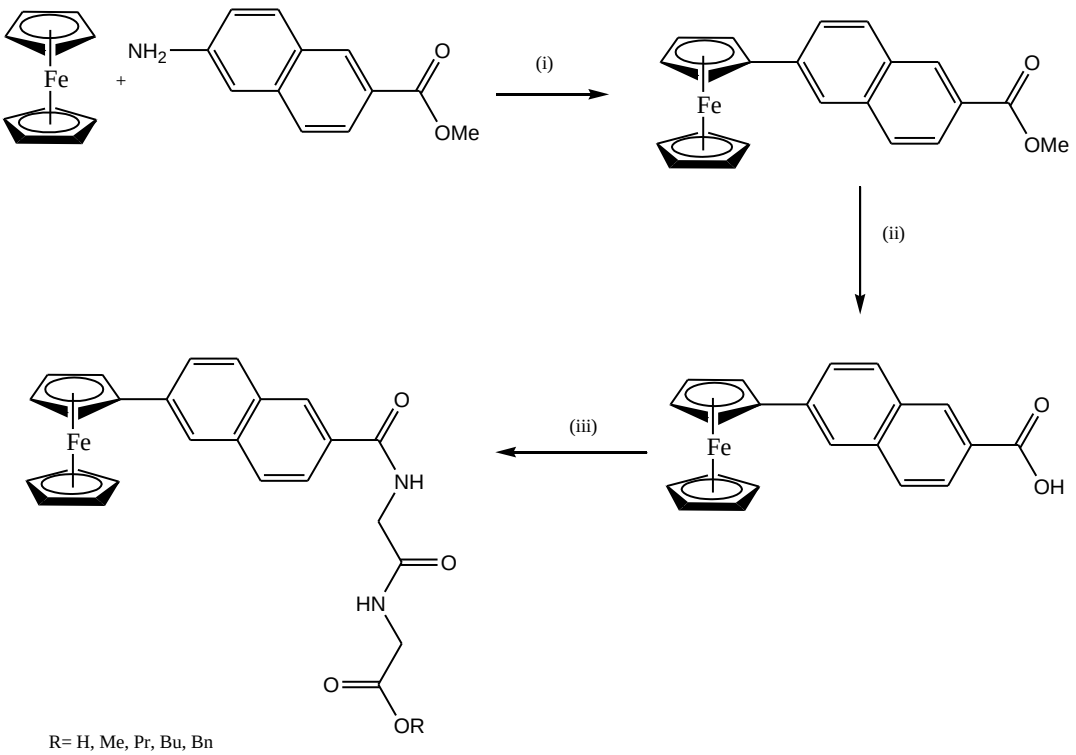
**Ferrocene:** Ferrocene acts as an electroactive core. It has a low oxidation potential and reversible redox potential which are believed to be important for anti-proliferative effect. In the SAR study of the 1-alkyl-1' derivatives, incorporation of a methyl derivative to the unsubstituted cyclopentadiene ring of the ferrocene molecule enhanced the anti-proliferative effect of the ferrocenyl dipeptide bioconjugates. The most active derivative was the 1-methyl-1'-*N*-{*meta*-(ferrocenyl)

benzoyl} glycine glycine ethyl ester **68** which displayed an $IC_{50}$ value of 2.6 ± 0.6 μM.

**Conjugated linker:** The conjugated linker was considered to be the most fundamental region that may be altered, since the conjugating ability of the linker is important in determining the redox potential of the derivative. Previous studies have shown that the replacement of the conjugated linker of *N*-(ferrocenyl) benzoyl dipeptide esters with a naphthoyl linker leads to an improvement in antiproliferative effect in the non small cell lung cancer (NSCLC) cell line, H1299 [132-134]. From the series of *N*-(ferrocenyl) naphthoyl amino acid and dipeptide ethyl esters reported, the *N*-{6-(ferrocenyl)-2-napthoyl} glycine glycine ethyl ester **31** displayed an $IC_{50}$ value of 0.13 ± 0.01 μM in the H1299 lung cancer cell line.

**Peptide chain:** In relation to the peptide chain, the 20 essential α-amino acids provide a great assortment of side chain functional groups. The previous study indicated that anti-proliferative effect is best when the small, neutral α-amino acid glycine is present in the peptide chain.

**Ester group:** Previously it was thought that the ester portion of the compound did not play a role in the activity of the compound but instead may have downstream applications such as oral absorption and biodistribution. The objective of this SAR study was to determine if there is any relationship between the ester moiety and biological activity.




(i) $NaNO_2$, HCl, 5 °C; (ii) NaOH/MeOH, $H_2O$; (iii) EDC, NHS, $Et_3N$, amino acid/dipeptide ester.

**Figure 3.12:** The general reaction scheme for the synthesis of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with altered ester moiety.

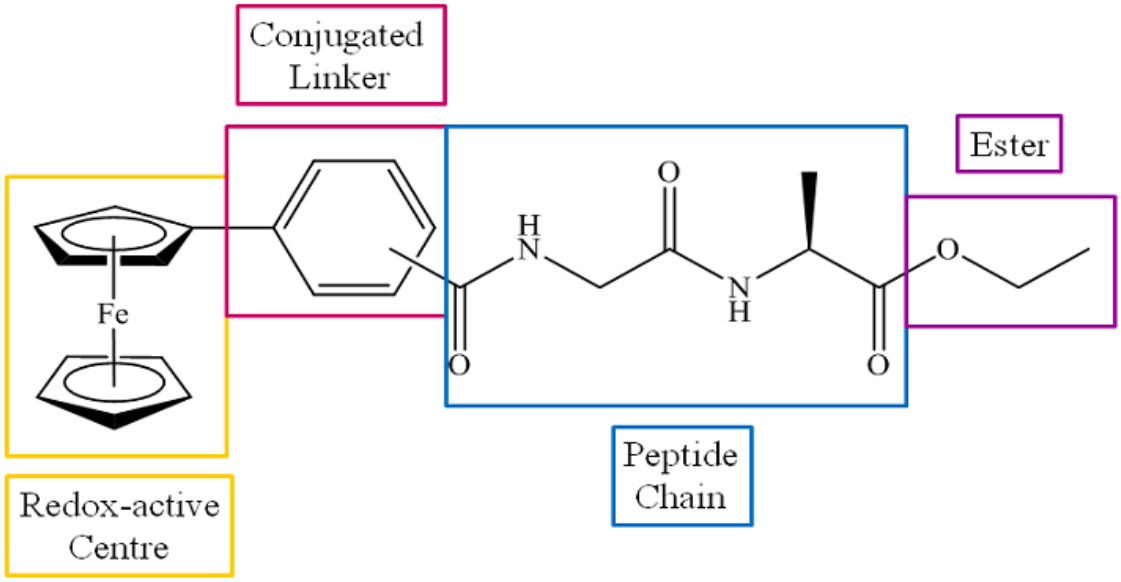



**Figure 3.13:** General structure of constituent regions of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with altered ester moiety.

### 3.3.2 Preliminary screening of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-compounds with altered ester moiety

The compound *N*-(6-ferrocenyl-2-naphthoyl)-glycine-glycine ethyl ester **(31)** has previously been shown to be the most active bioconjugate synthesised by this group to date with $IC_{50}$ values of 0.13 ± 0.02 and 1.10 ± 0.13 in H1299 and Sk-Mel-28 respectively. A series of derivatives were synthesized based on **31** with a modified ester moiety. To assess if any of these compounds were biologically active, a comprehensive screen of every compound at a single dose (10 μM) was performed in a panel of cell lines which included 3 melanoma cell lines; HT-144, Lox-IMVI and Malme-3M and 2 lung cancer call lines; A549 and H1299. The results for the preliminary screen of *N*-(6-ferrocenyl-2-naphthoyl)-glycine-glycine methyl, ethyl, propyl, butyl, benzyl **30–34** are depicted in figure 3.14 and table 3.13.

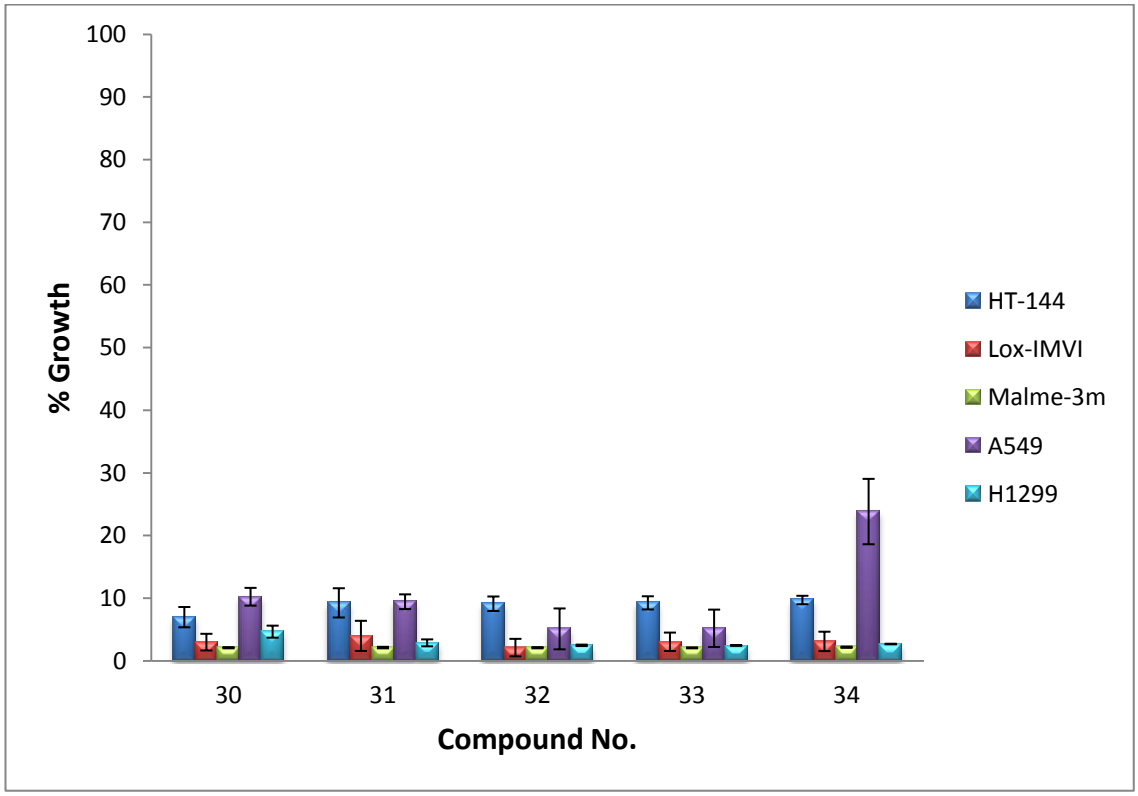



**Figure 3.14:** Effect of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with modified ester group **(30-34)** at 10 μM. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate assays.

Table 3.13: Percentage growth of cell lines, HT-144, Lox-IMVI, Malme-3M, A549 and H1299, when treated with 10 μM *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with modified ester group **30-34.**

| **Compound** | **Compound No.** | **Cell Line** | **% growth at 10 μM** |
|---|---|---|---|
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OMe | **30** | HT-144 | 7.0 ± 1.6 |
| | | Lox-IMVI | 3.0 ± 1.3 |
| | | Malme-3M | 2.1 ± 0.1 |
| | | A549 | 10.2 ± 1.4 |
| | | H1299 | 4.7 ± 1.0 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OEt | **31** | HT-144 | 9.2 ± 2.3 |
| | | Lox-IMVI | 4.0 ± 2.4 |
| | | Malme-3M | 2.1 ± 0.1 |
| | | A549 | 9.4 ± 1.2 |
| | | H1299 | 2.9 ± 0.6 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OPr | **32** | HT-144 | 9.1 ± 1.2 |
| | | Lox-IMVI | 2.1 ± 1.4 |
| | | Malme-3M | 2.1 ± 0.1 |
| | | A549 | 5.1 ± 3.3 |
| | | H1299 | 2.5 ± 0.1 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBu | **33** | HT-144 | 9.2 ± 1.1 |
| | | Lox-IMVI | 3.0 ± 1.5 |
| | | Malme-3M | 2.1 ± 0.1 |
| | | A549 | 5.2 ± 3.0 |
| | | H1299 | 2.5 ± 0.1 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBn | **34** | HT-144 | 9.7 ± 0.7 |
| | | Lox-IMVI | 3.1 ± 1.5 |
| | | Malme-3M | 2.2 ± 0.1 |
| | | A549 | 23.8 ± 5.2 |
| | | H1299 | 2.7 ± 0.0 |

The results of the preliminary screen at 10 μM (table 3.13) show that, with the exception of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBn **34** and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OMe **30** in A549 cells, all derivatives exhibit growth inhibitions levels > 90 %.

It can also be seen that for every compound **30-34**, cell lines HT-144 and A549 exhibit a lower sensitivity in each case when compared to Lox-IMVI, Malme-3M and H1299. For example, the *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBu **33** show a % growth inhibition at 10 μM between 97.0 – 97.9 % in cell lines Lox-IMVI, Malme-3M and H1299 but showed a lower % growth inhibition at 10 μM in A549 at 94.8 ± 3.0 % and even lower % growth inhibition in HT-144 cells at 90.8 ± 1.1 %.

The 10 μM preliminary screen confirmed activity for each compound with the ester moiety exhibiting little difference in activity. A secondary screening was performed at 1 μM with all other conditions remaining the same in an attempt to identify any difference in activity at a lower concentration.

From the results of the 1 μM screen a clearer idea of the difference in activity levels of compounds **30-34** can be seen. Compounds **32-34** show no appreciable effect on the growth of any of the cell lines at 1 μM. Compounds **30** and **31** reduce cell growth to below 40 % across all cell lines with **30** having the greatest antiproliferative effect at 1 μM.

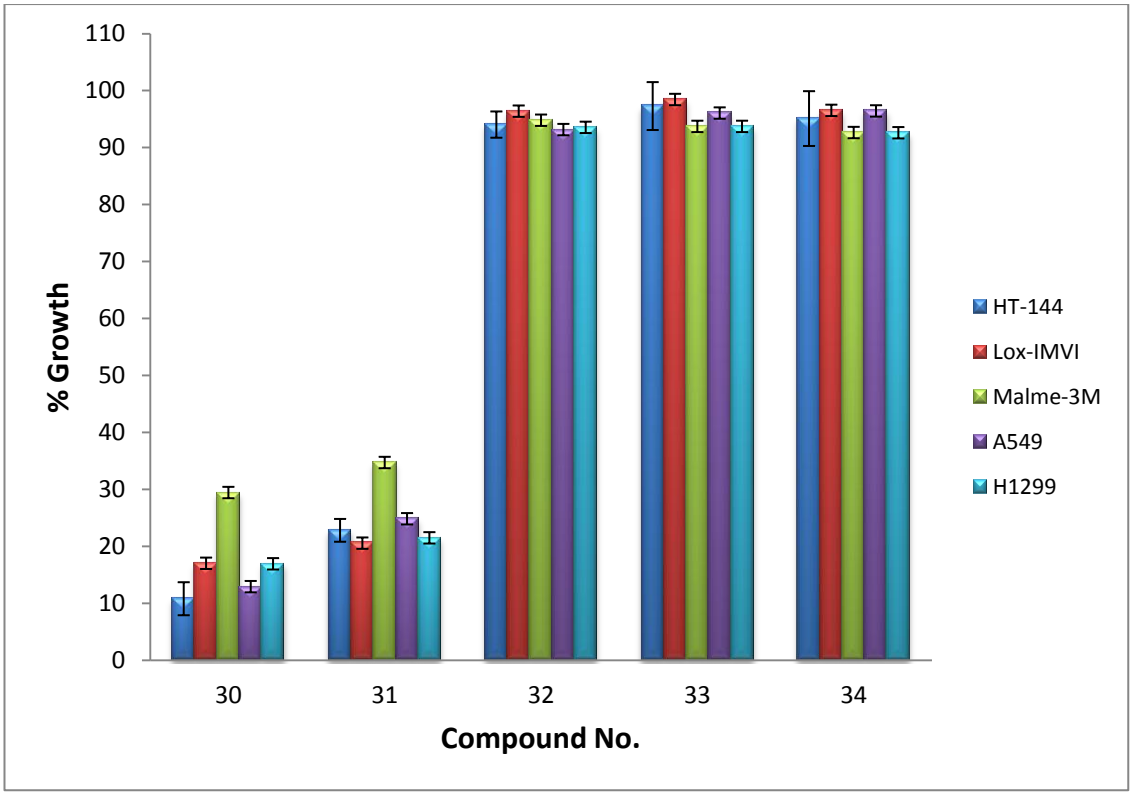



**Figure 3.15:** Effect of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with modified ester group at 1 µM. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate assays.

Table 3.14: Percentage growth of cell lines, HT-144, Lox-IMVI, Malme-3M, A549 and H1299, with 1 μM *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with modified ester group **30-34**

| Compound | Compound No. | Cell Line | % growth at 10 μM |
|---|---|---|---|
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OMe | **30** | HT-144 | 10.8 ± 2.9 |
| | | Lox-IMVI | 17.0 ± 2.6 |
| | | Malme-3M | 29.5 ± 3.2 |
| | | A549 | 12.9 ± 0.4 |
| | | H1299 | 16.9 ± 2.0 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OEt | **31** | HT-144 | 22.8 ± 2.0 |
| | | Lox-IMVI | 20.6 ± 4.7 |
| | | Malme-3M | 34.7 ± 3.5 |
| | | A549 | 24.8 ± 1.9 |
| | | H1299 | 21.5 ± 3.3 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OPr | **32** | HT-144 | 94.0 ± 2.3 |
| | | Lox-IMVI | 96.4 ± 3.4 |
| | | Malme-3M | 94.8 ± 6.3 |
| | | A549 | 93.2 ± 1.8 |
| | | H1299 | 93.6 ± 4.0 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBu | **33** | HT-144 | 97.3 ± 4.2 |
| | | Lox-IMVI | 98.4 ± 3.6 |
| | | Malme-3M | 93.7 ± 6.5 |
| | | A549 | 96.1 ± 3.4 |
| | | H1299 | 93.7 ± 5.2 |
| *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine-OBn | **34** | HT-144 | 95.1 ± 4.8 |
| | | Lox-IMVI | 96.5 ± 2.3 |
| | | Malme-3M | 92.6 ± 11.7 |
| | | A549 | 96.4 ± 7.9 |
| | | H1299 | 92.6 ± 6.6 |

### 3.3.3 Cytotoxicological evaluation of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (30) and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester (31)

To get a better insight into the efficacy of these compounds, the two most active, *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **(30)** and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(31)** were selected for further analysis. *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl **31** has previously been evaluated for anti-proliferative activity in H1299 cells and achieved an $IC_{50}$ of 0.13 ± 0.01 µM. The cell line panel has been expanded to include melanoma cell lines; Malme-3M, Lox-IMVI and HT-144 as well as lung cancer cell line, A549, and tested sensitivity to **30** and **31** (Figure 3.16 and 3.17).

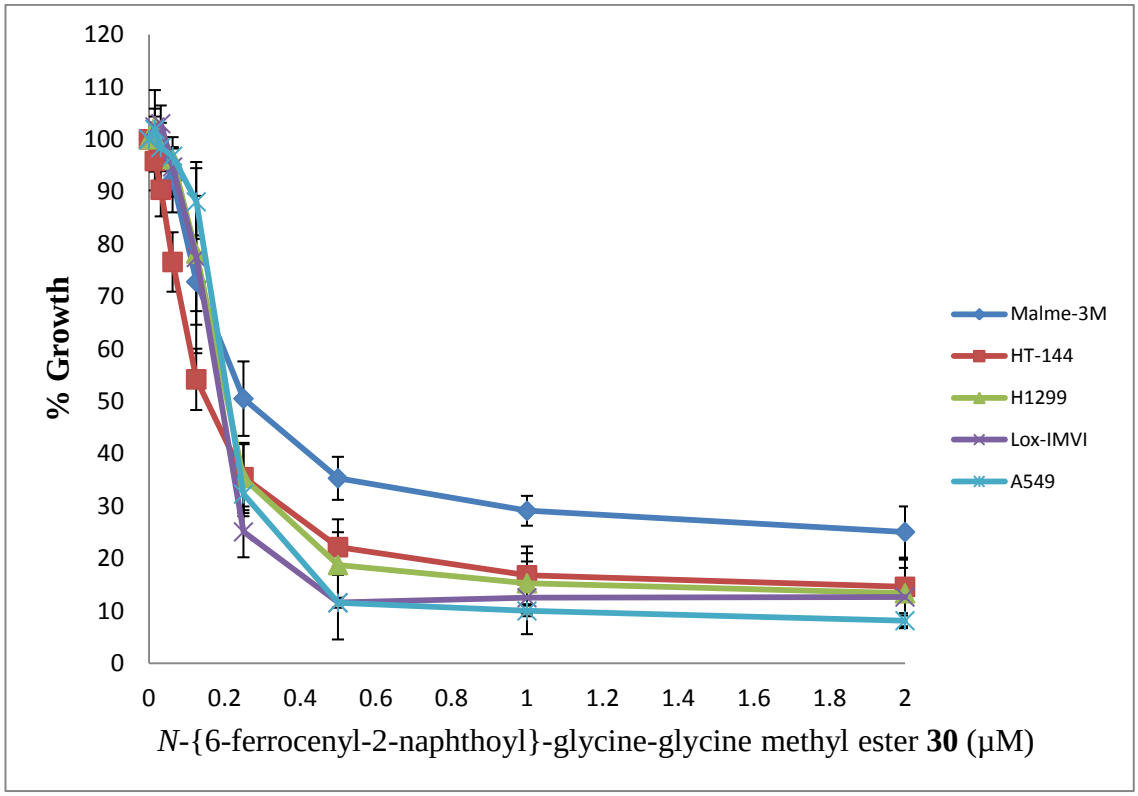



**Figure 3.16:** Inhibition curve of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (**30)** in cell lines H1299, Malme-3M, Lox-IMVI, HT-144 and A549. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

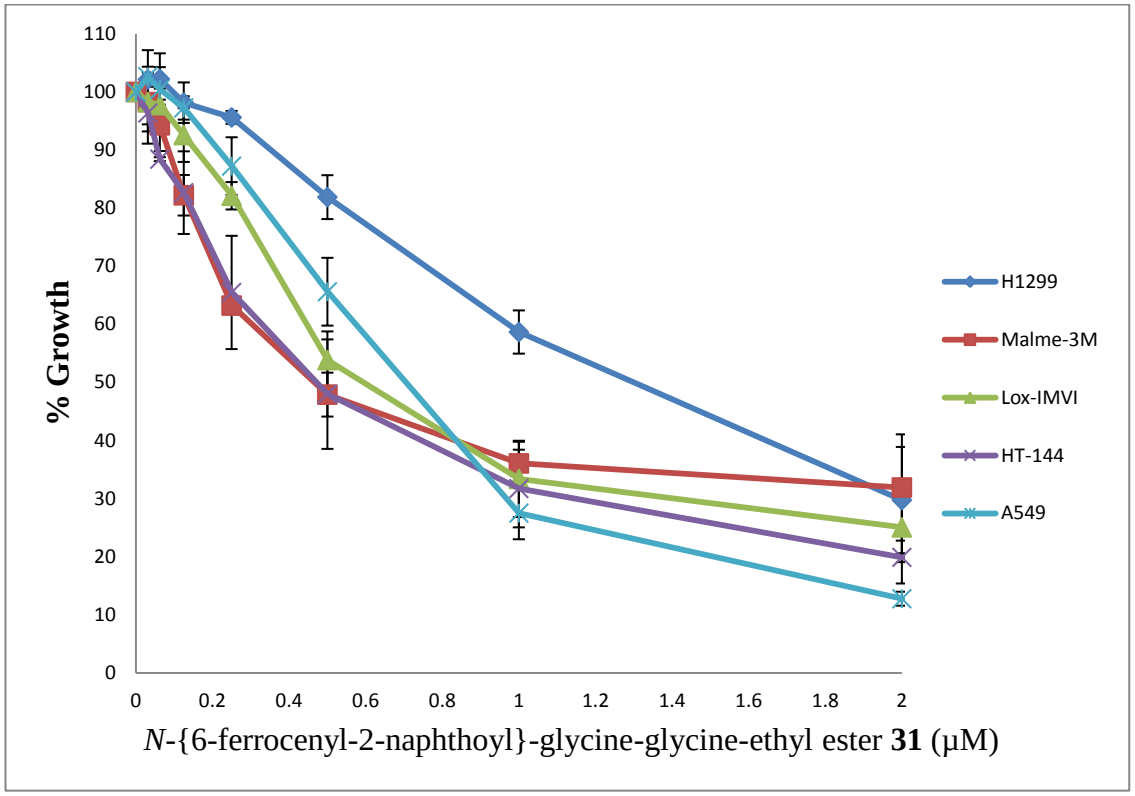



**Figure 3.17:** Dose response curve for *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester (**31)** in H1299, Malme-3M, Lox-IMVI, HT-144 and A549. Growth is expressed relative to cells treated with an equivalent volume of DMSO. Error bars represent the standard deviation of triplicate experiments.

$IC_{50}$ values were determined in the panel of cell lines H1299, Malme-3M, Lox-IMVI, HT-144 and A549 for compounds **30** and **31,** as well as cisplatin which is used as a reference compound to assess relative levels of anti-proliferative activity (table 3.15). Both **30** and **31** show lower $IC_{50}$ values (which is indicative of higher anti-proliferative activity) when compared to cisplatin. Also, in all cell lines, **30** shows lower $IC_{50}$ values than **31**. The $IC_{50}$ values of **30** are consistent across cell types, ranging from 0.2 ± 0.02 to 0.3 ± 0.05 µM. In comparison, $IC_{50}$ values for **31** range from 0.5 ± 0.02 to 1.4 ± 0.21 µM. It is note-worthy that the $IC_{50}$ value for **31** in H1299 is recorded at 1.4 ± 0.21 µM but was previously reported as 0.13 ± 0.01 µM in the same cell line, a > 10 fold lowering of growth inhibition activity. Potential reasons for this are discussed in section 4.1.3.

Table 3.15: $IC_{50}$ values for *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **(31)** and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(30)** in H1299, Malme-3M, Lox-IMVI, HT-144 and A549. Cisplatin is used as a reference. Standard deviations for triplicate assays are presented.

| | A549 (µM) | H1299 (µM) | HT-144 (µM) | Lox-IMVI (µM) | Malme-3M (µM) |
|---|---|---|---|---|---|
| **Cisplatin** | 2.6 ± 0.1 | 2.1 ± 0.2 | 3.0 ± 0.4 | 2.9 ± 0.9 | 2.7 ± 0.5 |
| **30** | 0.7 ± 0.07 | 1.4 ± 0.21 | 0.5 ± 0.06 | 0.5 ± 0.04 | 0.5 ± 0.02 |
| **31** | 0.2 ± 0.03 | 0.3 ± 0.04 | 0.2 ± 0.02 | 0.2 ± 0.06 | 0.3 ± 0.05 |

### 3.3.4 Activity of 6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (30) and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl (31) in cisplatin and temozolomide resistant cell lines

Intrinsic and acquired resistance are a common problem with chemotherapeutics and are a major hurdle to effective cancer treatment. Often resistance to one chemotherapeutic agent can also play a role in resistance to others due to similarities in modes of action, this is referred to as cross-resistance. *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl and ethyl ester **(30, 31)** were evaluated in a melanoma cell line model of acquired temozolomide (TMZ) resistance (Malme-3M/TMZ) and a lung cancer cell line model of cisplatin resistance (A549/Cpt) (figures 3.18 and 3.19).

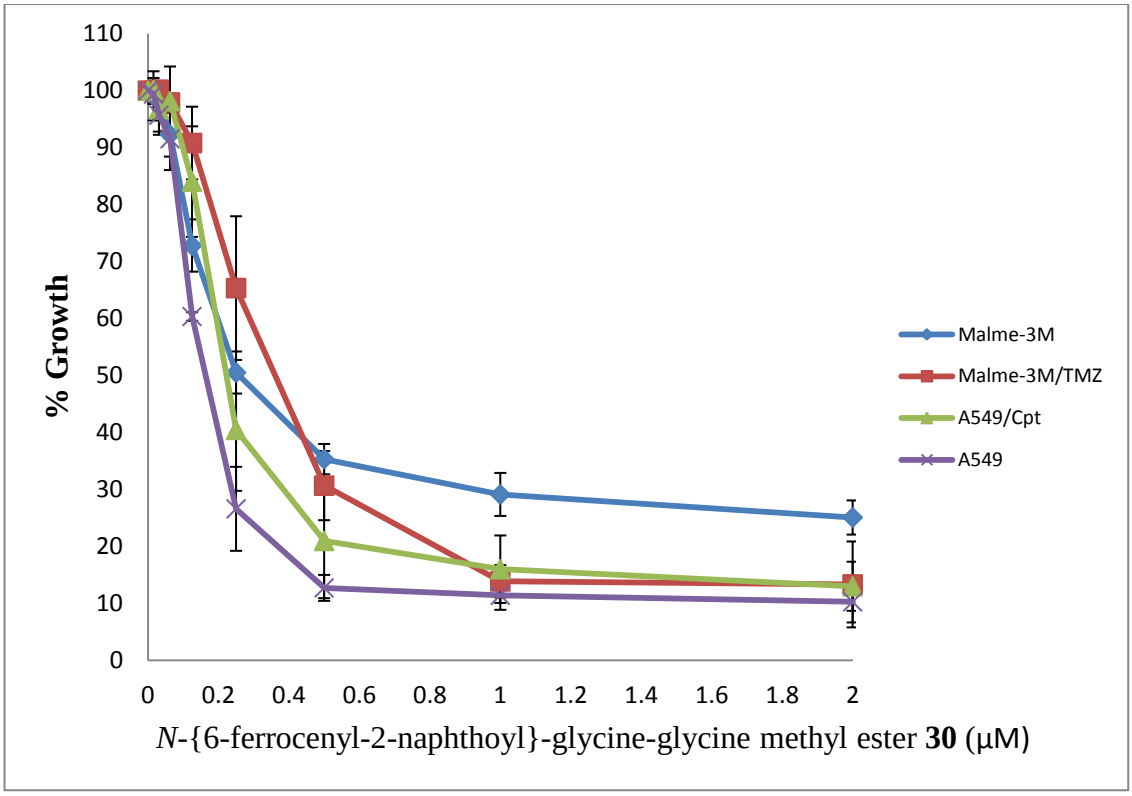



**Figure 3.18:** Effect of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester on growth of A549 and Malme-3M cells compared to their resistant variants A549/Cpt and Malme-3M/TMZ, respectively Error bars represent the standard deviation of triplicate assays.

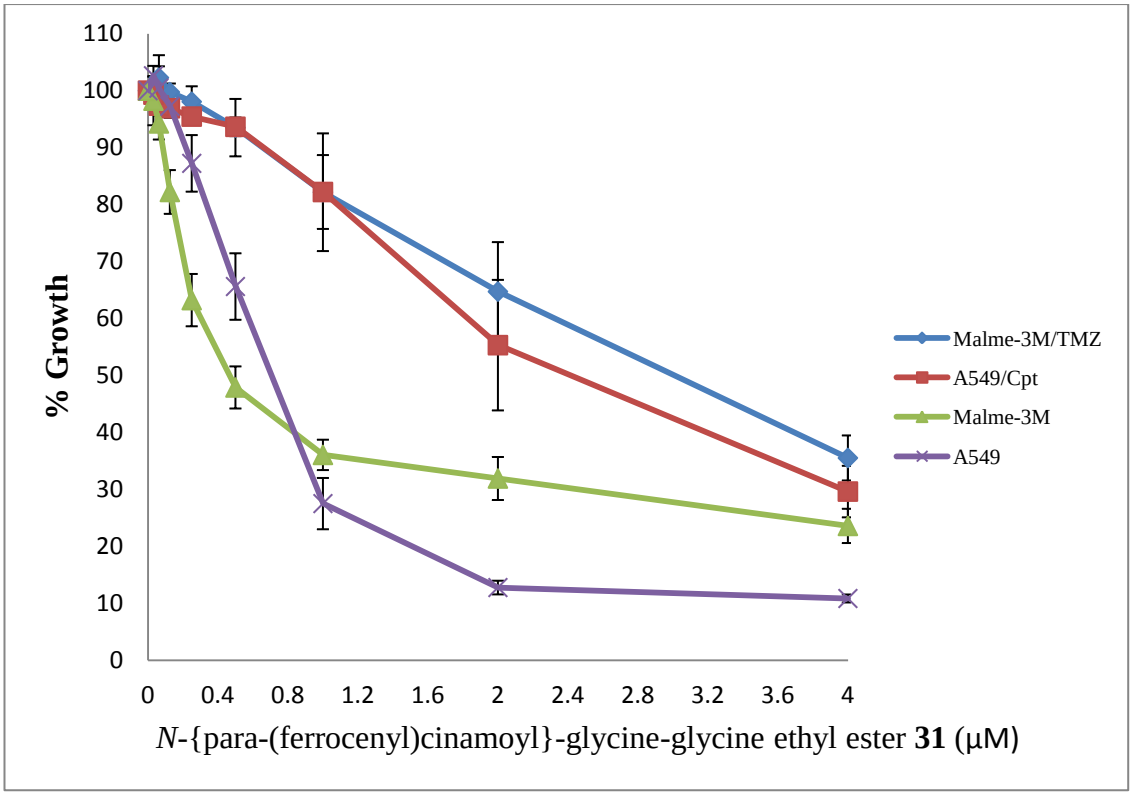



**Figure 3.19:** Effect *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl **31** on growth of A549 and Malme-3M cells compared to their resistant variants A549/Cpt and Malme-3M/TMZ, respectively Error bars represent the standard deviation of triplicate assays.

The $IC_{50}$ values of **30** and **31** were evaluated in A549 and its cisplatin resistant derivative and Malme-3M and its temozolomide resistant derivative (table 3.16). $IC_{50}$ values of cisplatin and temozolomide were also determined to confirm the resistant phenotype of the cells. The A549/Cpt cell line showed 1.72 fold resistance to cisplatin compared to the parental A549 cells. The Malme-3M/TMZ showed 2.3 fold ressistance to temozolomide compared to the parental Malme-3M cells. There was cross resistance in both resistant cell lines to compound **30** with $IC_{50}$ values increasing from 0.7 ± 0.04 µM in A549 cells to 2.4 ± 0.6 µM in A549/Cpt cells (3.4 fold resistance) and from 0.5 ± 0.1 µM in Malme-3M cell to 2.8 ± 0.5 µM in Malme-3M/TMZ cells (5.6 fold resistance). A 2 fold increase in $IC_{50}$ value was observed for **31** in A549/Cpt cells compared to A549 cells, from 0.2 ± 0.04 µM to 0.4 ± 0.1 µM but no difference in $IC_{50}$ value was observed between Malme-3M/TMZ and Malme-3M, suggesting that 31 may overcome the mechanisms of acquired temozolomide resistance in this cell line model.

Table 3.16: $IC_{50}$ values for *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **(30)** and *N*-{para-(ferrocenyl)cinamoyl}-glycine-glycine ethyl ester **(31)** in A549 and its cisplatin resistant derivative (A549/Cpt), and Malme-3M and its temozolomide resistant derivative (Malme-3M/TMZ). $IC_{50}$ values are also given for cisplatin and temozolomide where appropriate as a reference. Standard deviations for triplicate assays are presented.

| | Cisplatin (μM) | **30** (μM) | **31** (μM) | Temozolomide (μM) |
|---|---|---|---|---|
| **A549** | 3.6 ± 0.7 | 0.2 ± 0.04 | 0.7 ± 0.04 | |
| **A549/Cpt** | 6.2 ± 0.9 | 0.4 ± 0.1 | 2.4 ± 0.6 | |
| **Malme-3M** | | 0.3 ± 0.05 | 0.5 ± 0.1 | 209 ± 27 |
| **Malme-3M/TMZ** | | 0.3 ± 0.1 | 2.8 ± 0.5 | 483 ± 32 |

### 3.3.5 Effect of 6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (30) on the cell cycle in A549 and HT-144 cells

The growth and division of cells proceeds *via* the cell cycle, which is typically divided into four distinct stages: the synthesis (S), mitosis (M) and two gap ($G_1$ and $G_2$) phases. Most normal cells, unless they have received a stimulus to proliferate or differentiate, exist in a non-dividing, quiescent state known as $G_0$. [269] Cancer cells, on the other hand, grow at an uncontrolled rate. As the cell progresses from the G1 to the S phase the cell must decide whether to divide into two daughter cells or die, as the cell can only exist in subsequent phases for a short period. Many chemotherapeutic agents, including ciplatin, function by blocking cell cycle progression after this restriction point [270].

The progression of cells through the cell cycle can be tracked using a propidium iodide stain. The stain is a fluorescent molecule that intercalates with DNA in the cell nucleus. Discrimination between the various stages of the cell cycle is possible due to changes in the concentrations of DNA in the cells as the cycle progresses. Resting cells (G0/G1 phase) contain two copies of each chromosome. As the cells

progress towards mitosis, they synthesise DNA (S phase), which allows for more intercalation and increases the fluorescence intensity. When all chromosomes have been replicated, the DNA content has doubled (G2/M phase), and thus, the cells fluoresce with twice the intensity of the G0/G1 phase. Cells in which the DNA has become fragmented or degraded (such as apoptotic cells) will exhibit a decrease in fluorescence intensity, compared to the G0/G1 phase. These cells will appear as a broad "sub-G0/G1" peak, and can represent, in addition to apoptotic cells, nuclear fragments, clumps of chromosomes, micronuclei or cells undergoing differentiation [271]. A flow cytometer is then used to analyse the DNA content of the cell to determine what phase the cells are at.

In both A549 (Figure 3.20) and HT-144 (Figure 3.21) cell lines, compound **30** most notably caused a significant increase of cells in the sub $G_0$ fraction of the cell cycle. This is most evident in the HT-144 cell line where there is an increase from 1.7 % of cells in the sub $G_0$ fraction of control cells to 55.4 % of cells treated with 2µM of **30**. This suggests that the primary mode of action of **30** is induction of cell apoptosis and/or necrosis. A small increase in $G_1$ arrest was also observed in A549 cells but not in HT-144 cells. In both cell lines, the percentage of cells in the S and $G_2$/M phases decreases with increasing concentrations of compound **30**. In both cell lines the most noticeable aspect is the strongly significant correlation between compound concentration and cells in the Sub $G_0$ fraction from concentration of 0.125 µM and above. This is an indication of cells undergoing apoptosis/necrosis.

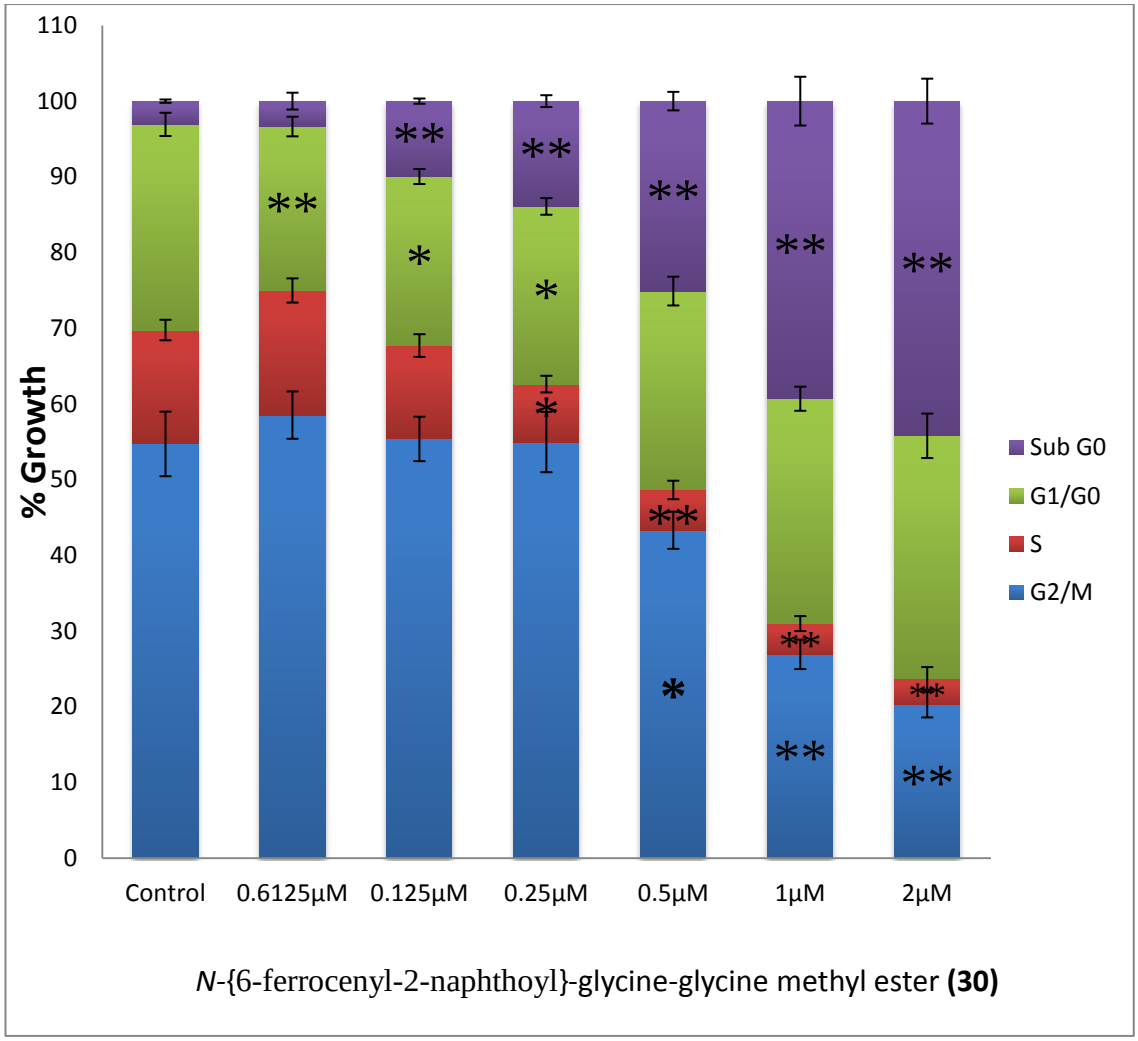



**Figure 3.20:** Percentage of A549 cells in the Sub $G_0$, $G_1/G_0$, S and $G_2/M$ phases of cell cycle after 72 hours treatment with *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **30**. Error bars represent the standard deviation of triplicate assays. "*" indicates $p < 0.05$, "**" indicates $p < 0.01$ for treated cells compared to controls, using the Student's T-test

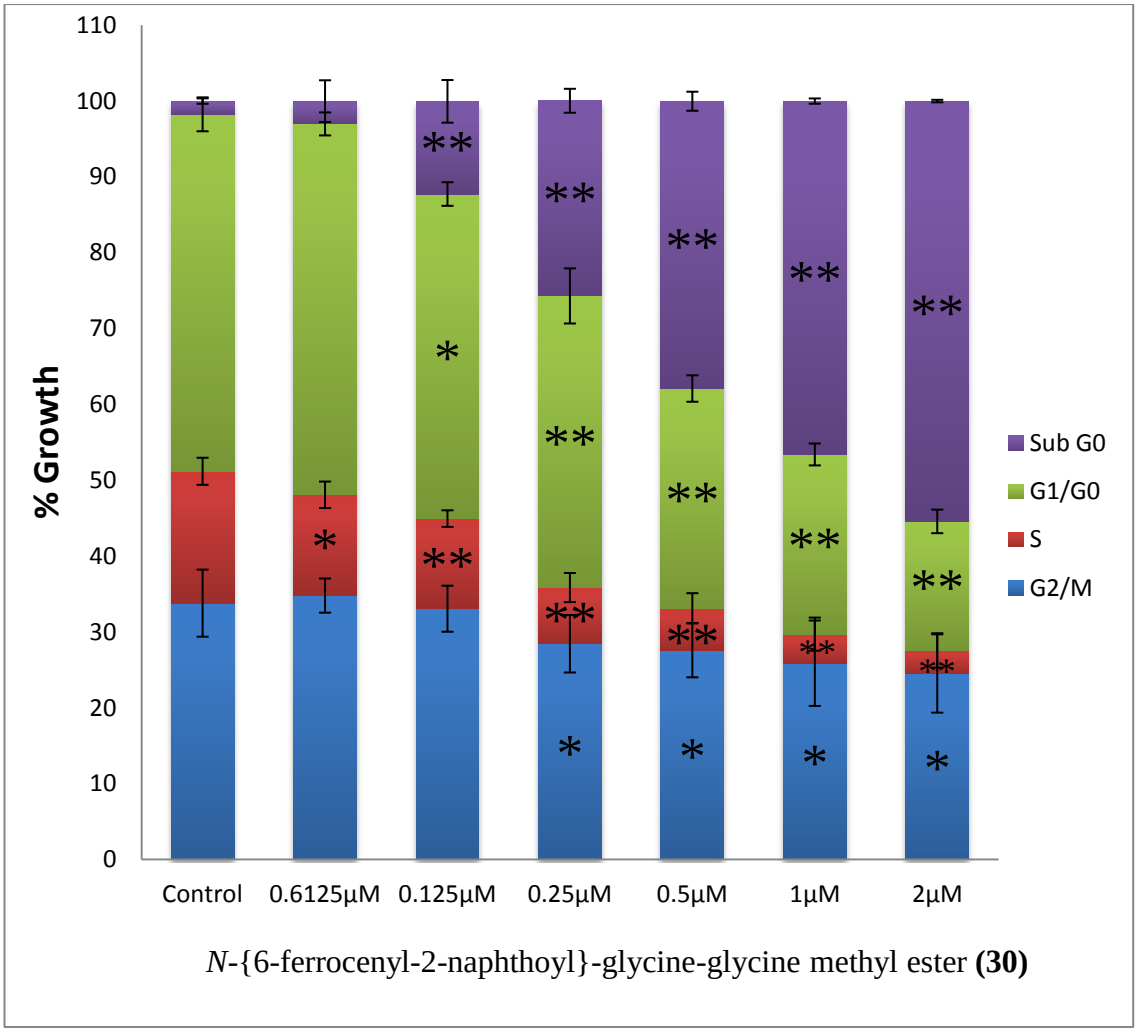



**Figure 3.21:** Percentage of HT-144 cells in the Sub $G_0$, $G_1/G_0$, S and $G_2$/M phases of cell cycle after 72 hours treatment with *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **30**. Error bars represent the standard deviation of triplicate assays. . "*" indicates $p < 0.05$, "**" indicates $p < 0.01$ for treated cells compared to controls, using the Student's T-test

### 3.3.6 Effect of 6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (30) on apoptosis of A549 and HT-144 cells

To determine if the sub-$G_0$ increases observed in the cell cycle assay were associated with increased apoptosis, theTUNEL apoptosis assay was performed. One of the hallmarks of late-stage apoptosis, or programmed cell death, is the fragmentation of nuclear chromatin. This generates DNA strands with exposed 3'-hydroxyl ends, which are enzymatically labeled in the classic TUNEL assay. The Guava® TUNEL

assay determines mid- to late- stage apoptosis when DNA fragmentation is occurring in cells. The DNA degradation generates DNA strands with exposed 3'-hydroxyl ends and terminal deoxynucleotidyl transferase (TdT) catalyses the incorporation of bromodeoxyuridine (BrdU) residues into the fragmenting nuclear DNA at the 3'-hydroxyl ends by nicked end labeling. A TRITC-conjugated anti-BrdU antibody can then label the 3'-hydroxyl ends for detection by a Guava System.

For both A549 (Figure 3.22) and HT-144 (Figure 3.23) cell lines, increasing concentrations of compound **30** caused an increase in apoptosis induction. A dramatic increase in apoptosis induction was observed when the concentration was increased from 0.25 μM to 0.5 μM, 15.0 ± 9.7 % to 66.8 ± 8.8 % for A549 cells and 12.7 ± 3.2 % to 59.8 ± 4.5 % in HT-144, suggesting that a threshold concentration between 0.25 and 0.5 μM is required for efficient induction of cell death. In both cell lines, there is a strongly significant correlation between compound concentration and % of apopototic cells with p-values of $< 0.01$.

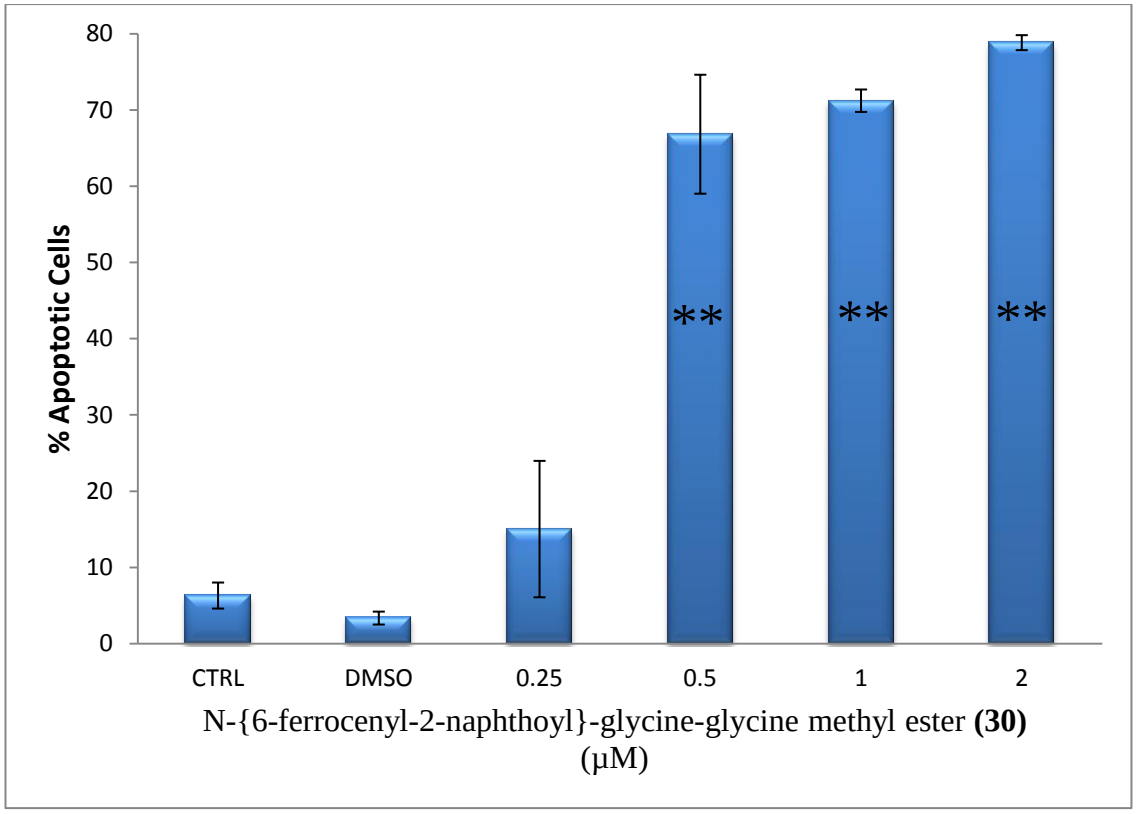



**Figure 3.22:** Measurement of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester induced apoptosis in A549 after 72 hours of treatment using the TUNEL assay. A control of equivalent DMSO concentration is used to ensure that DMSO does not contribute to apoptosis levels. Error bars represent the standard deviation of triplicate assays. . "*" indicates $p < 0.05$, "**" indicates $p < 0.01$ for treated cells compared to controls, using the Student's T-test

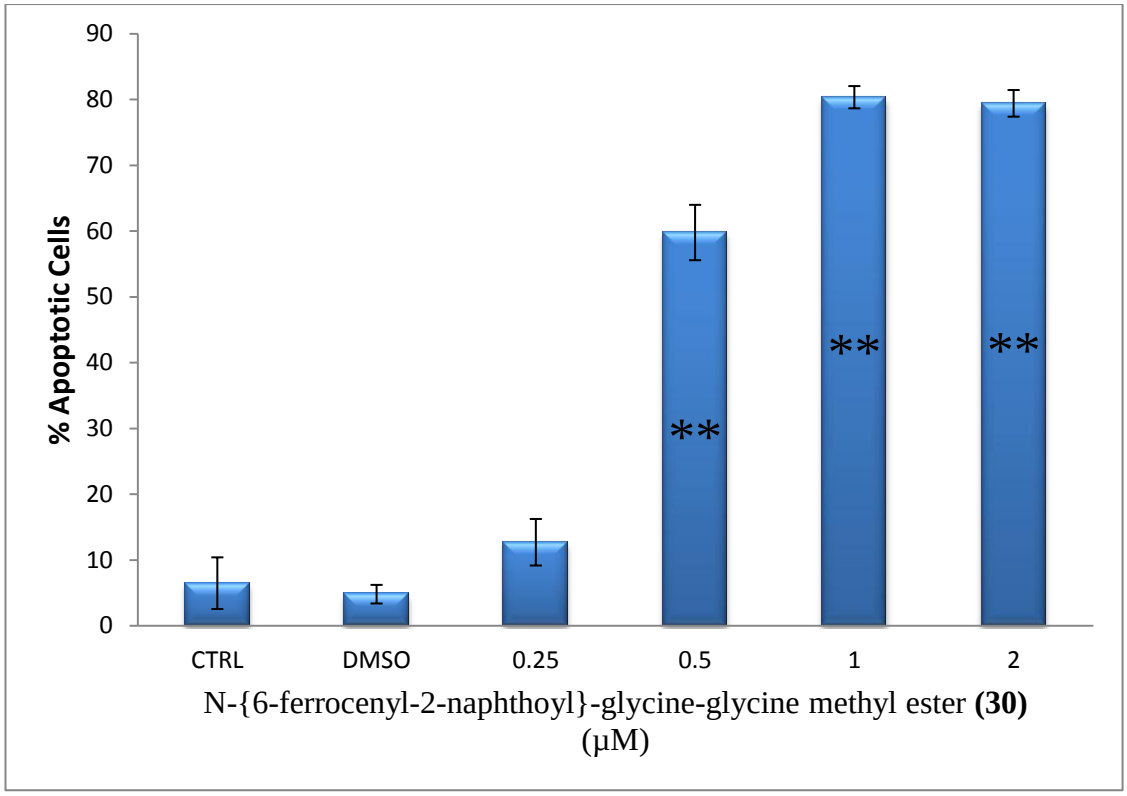



**Figure 3.23:** Measurement of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester induced apoptosis in HT-144 after 72 hours of treatment using the TUNEL assay. A control of equivalent DMSO concentration is used to ensure that DMSO does not contribute to apoptosis levels. Error bars represent the standard deviation of triplicate assays. . "*" indicates $p < 0.05$, "**" indicates $p < 0.01$ for treated cells compared to controls, using the Student's T-test

### 3.3.7 Effect of 6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester (30) and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl (31) on Normal Human Dermal Fibroblast (NHDF) cells.

Normal Human Dermal Fibroblast (NHDF) cells are isolated from juvenile foreskin. They are often used in wound healing studies and dermatological research however they also have a role in cancer research, as control normal cells. Most chemotherapy regimens rely, at least partially, on the cytotoxic properties that eliminate cancerous cells. The desirable anti-proliferative effects of chemotherapeutic drugs also have an effect on healthy cells of the body, which often result in side-effects. It is a trade-off between the anti-proliferative effects in cancerous cells and in healthy cells. For that reason the effect of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **(30)** and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(31)** on NHDF cells (Table 3.17) was comapred. Cisplatin was used as a reference.

Table 3.17: $IC_{50}$ values for *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine methyl ester **(30)** and *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(31)** and reference compound cisplatin, in Normal Human Dermal Fibroblast (NHDF) cells. Standard deviations for triplicate assays are presented. Student's t-test was performed to determine significant differences between treatments and cell that were treated with cisplatin. * denotes $p \leq 0.05$

| | **30** (μM) | **31** (μM) | Cisplatin (μM) |
|---|---|---|---|
| **NHDF** | 12.0 ± 3.8 | 14.2 ± 2.4* | 10.0 ± 1.8 |

The cisplatin $IC_{50}$ value was determined to be 10.0 ± 1.8 μM. In contrast, the $IC_{50}$ values for compounds **30** and **31** of 12.0 ± 3.8 and 14.2 ± 2.5 μM, respectively. Due to overlapping standard deviations, **31** is significantly less cytotoxic than cisplatin however it cannot be shown that **30** is significantly less or more cytotoxic than cisplatin.

### 3.3.8 Conclusions

After selecting the two most active compounds from the initial screen in table 3.14 for further analysis a number of properties associated with the mechanism of action of these compounds was determined. After expanding the cell line panel, it can be seen that the activity is not confined to a single cancer type but is effective across, but not limited to, melanoma and lung. Across all cell lines compound **(30)** is more effective than **(31)**. It is worth noting that both of the lead compounds surpass the level of activity of cisplatin in all cell lines, excluding NHDF.

To investigate the potential of these compounds to overcome resistance to DNA damaging chemotherapy drugs, the $IC_{50}$ values for the two compounds in A549 and Malme-3M cells was comapred and their cisplatin and temozolomide resistant derivatives, respectively. Compound **30** shows susceptibility to cross resistance with both cisplatin and temozolomide, with greater than three-fold resistance in the cisplatin resistant A549 cells and greater than five-fold resistance in the temozolomide resistant Malme-3M cells. Compound **31** may exhibit slight cross-resistance in A549/Cpt cells but Malme-3M/TMZ appears to retain sensitivity to **31**.

Cell cycle analysis suggests that compound 30 induces slight $G_1$ arrest in A549 cells but the predominant effect observed was an increase in the sub-$G_0$ fraction, in both cell lines tested.

The results of the cell cycle assay were reinforced by the results of the apoptosis assay. Late stage apoptosis is shown in a dose dependent manner using the TUNEL assay. In both A549 and HT-144 cell lines there is a significant increase in apoptosis induction particularly at concentrations higher than 0.5 μM.

The cytotoxicity of **30** and **31** was analysed in normal cells and compared against cisplatin as a reference for the levels of toxicity observed with chemotherapy drugs used in clinical practice. Similar $IC_{50}$ values were observed for compounds **30** and **31** but the values were higher than observed in the cancer cell lines (table 3.15), suggesting selective anti-cancer activity. This analysis would suggest that the ferrocenyl compounds are potential anti-cancer compounds with greatly reduced toxicity to normal cells.

## 3.4 Solubility

### 3.4.1 Biological solvents/excipients/vehicles/carriers

The potential of a number of solvents to be used to reduce the level of DMSO used for the administration of ferrocenyl compounds was tested by first dissolving *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **31** in DMSO to a concentration of 8 mg/mL. This was then diluted with the relevant vehicle to a 1 in 4 solution which would give a working stock solution of 2 mg/mL. This solution was then analysed in A549 cells at a concentration of 1 µM as the % growth inhibition achieved with 1 µM of **31** when dissolved in 100 % DMSO is already known to be 75.2 ± 1.9 % (table 3.14). A control was used for each vehicle, in which a 25 % DMSO/ 75 % vehicle solution was tested to ensure that this had no anti-proliferative effect on the cells.

Table 3.18: Percentage growth of A549 cell after treatment with 1 µM *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **31** using a variety of vehicles for delivery. A control group was also tested which used an equivalent concentration of vehicle without **31**.

| Vehicle (25 % DMSO) | **% growth** | |
|---|---|---|
| | Control | 31 (1 µM) |
| 80 mM citrate buffer | 98.8 % | 83.8 % |
| 5 % Carboxymethyl Cellulose | 98.9 % | 86.0 % |
| 1 % Carboxymethyl Cellulose | 96.8 % | 60.2 % |
| 50 % Propylene Glycol Solution | 103.1 % | 96.1 % |
| Kolliphor® EL | 4.2 % | 15.0 % |

Compound **31** in Kolliphor® EL showed the greatest anti-proliferative activity (table 3.18) with a growth inhibition of 85 %. However, the control group showed a 95.8 % growth inhibition in A549 cells with. Kolliphor® EL was not pursued as a viable delivery method due to the high anti-proliferative effect of the control.

The control groups of the remaining vehicles tested had no appreciable effect on the growth of the cells. From previous results we know that *N*-{6-ferrocenyl-2-

naphthoyl}-glycine-glycine ethyl ester **31**, when dissolved in 100 % DMSO and tested at a concentration of 1 µM, has a growth inhibition level of 75.2 ± 1.9 %. With the exception of Kolliphor® EL, the vehicle that allowed for the greatest activity of **31** was 1 % carboxymethyl cellulose with a growth inhibition level of 39.8 %. This is a 1.9 fold reduction in activity. 80 mM citrate buffer, 5 % Ccarboxymethyl cellulose and 50 % propylene glycol all showed lower level of activity < 20 %. These activity levels are too low and so other avenues must be explored as a viable delivery system for these ferrocenyl compounds *in vivo*.

### 3.4.2 PEGylation

PEGylation is the covalent attachment of polythelene glycol (PEG) to another molecule, often a drug. Some of the desirable properties that make PEGylation an attractive option are the ability to avoid detection by the immune system, increasing hydrodynamic size to lengthen circulatory time and increasing solubility of hydrophobic drugs and proteins.

PEGylation improves the pharmacokinetic and pharmacodynamic properties of polypeptide drugs by increasing water solubility, reducing renal clearance and limiting toxicity [250,251]

A PEGylated derivative of for *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(31)** was synthesized (by Andy Garry Harry, School of Chemical Sciences, DCU) and was water soluble to at least 10 mM. However, the structural alteration had an effect on the cytotoxic activity of the compound as an $IC_{50}$ value was not achieved using concentration up to 20 µM in A549 cells.

### 3.4.3 Conclusion

Our attempts to find a viable method for a delivery method *in vivo* of *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **(31)** were ultimately unsuccessful. Additional avenues must be explored if the compound is to progress into animal studies and give a better assessment for the potential of these ferrocenyl derivatives as an anti-cancer agent. As discussed (section 1.5), lipid nanocapsules have recently already been shown as an effective delivery system for ferrocenyl compounds and as such would be worth exploring [225].

# Chapter 4

# Discussion

## 4.1 Structure-Activity Relationship (SAR) studies

### 4.1.1 1-alkyl-1'-*N-para, N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters

The primary objective of this research was to explore a structure-activity relationship (SAR) study of the incorporation of alkyl chains moieties on the unsubstituted ring of the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters. In previous studies carried out to evaluate the *in vitro* anticancer activity of *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters, the general trend in cytotoxicity was *para* < *ortho* < *meta* [126-130]. The most active derivatives of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters are 1-methyl-1'-*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **64**, 1-ethyl-1'–*N*-{*para*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **76** with and 1-methyl 1'-*N*-{*meta*-(ferrocenyl) benzoyl} glycine glycine ethyl ester **68**. These compounds display improved bioactivity in comparison to the corresponding most active benzoyl analogues.

1-alkyl-1'-*N-ortho*- derivatives exhibited lower percentage growth inhibition values compared to the *meta* and *para* derivatives for the dipeptides employed in the SAR study. Gly Gly (OEt) and Gly L-Ala (OEt) derivatives are more active than the Gly L-Leu and Gly L-Phe ethyl esters. It can be concluded that when chiral α-amino acids with bulky side chains are used as the second amino acid in the dipeptide moiety a loss of anti-proliferative activity is observed for this particular group of compounds. Cytotoxicity of the 1-alkyl-1'-*N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters, decreases with the increase of the size of the alkyl group incorporated (propyl < ethyl < methyl) on the ferrocene moiety. The order of the amino acids in the dipeptide chain is crucial for activity, the trend being Gly Gly > Gly L-Ala > Gly L-Leu > Gly L-Phe.

Incorporation of electron donating groups is a well know strategy to lower oxidation potentials of ferrocene moieties [131]. Thus, the incorporation of the various alkyl chain groups (electron donating groups) to the un-substituted cyclopentadiene ring of the *N-para*, *N-meta* and *N-ortho*-(ferrocenyl) benzoyl dipeptide esters should improve the cytotoxicity of these derivatives. [126-130]. Incorporation of methyl and

ethyl alkyl groups results in an improved cytotoxicity of the *para* derivatives of the glycine glycine ethyl esters and an improved cytotoxicity of the methyl *meta* glycine glycine derivative. However, when the size of the chain length increased to ethyl the $IC_{50}$ value was *ca.* three times higher than the unsubstituted analogue [129]. Thus, the increase in alkyl chain length resulted in a drastic decrease in the cytotoxicity of the 1-alkyl-1'-*N*-*para*, *N*-*meta* and *N*-*ortho*-(ferrocenyl) benzoyl dipeptide esters.

### 4.1.2 *N*-{*para*-(ferrocenyl)ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} and *N*-{5-(ferrocenyl)ethynyl-2-furanoyl} amino acid and dipeptide esters

This SAR study involves the cytotoxicological evaluation of the incorporation of an ethynyl moiety between the ferrocene moiety and the conjugate linker. The incorporation of the -C≡C- moiety between the ferrocene moiety and the aromatic spacer and the use of three different aromatic rings are attempts to further improve the cytotoxicity of the previously prepared *N*-*para*, *N*-*meta* and *N*-*ortho*-(ferrocenyl)benzoyl dipeptide esters. The ferrocene moiety is required for the possible production of hydroxyl radicals, the -C≡C- and conjugated aromatic linkers facilitates this process by lowering the redox potential of the ferrocene and the peptide fragment can interact with other molecules *via* hydrogen bonding.

The incorporation of a methyl derivative to the unsubstituted cyclopentadiene ring of the *N*-(ferrocenyl) benzoyl dipeptide derivatives showed an enhancement of the anti-proliferative effect, atlthough methyl ester derivatives exhibited lower percentage growth inhibition values compared to the ethyl ester derivatives.

Gly L-Leu and Gly L-Phe ethyl esters displayed low growth inhibition activity. Thus, the Gly L-Leu and Gly L-Phe ethyl ester derivatives were not investigated further. As a result, it can be concluded that when chiral α-amino acids with bulky side chains are used as the second amino acid in the dipeptide moiety, a loss of anti-proliferative activity is observed for this particular group of compounds.

The presence of the ethynyl moiety had a negative effect of anti-proliferative effect compared to analogous compounds prepared previously lacking the ethynyl group

and the the furanoyl ring is not a bioactive aromatic conjugate linker that promotes anti-proliferative effect.

We have previously postulated the potential of these ferrocenyl dipeptide derivative compounds to interact with DNA and that this interaction could play a role in the anti proliferative effects of the compounds [132]. It has been seen in polyaromatic drugs, such as anthracyclines, that they can inhibit DNA and RNA synthesis by intercalating between base pairs and prevent cancer cells from replicating. If these polyaromatic ferrocenyl derivatives also have the ability to interact with DNA/RNA then the hydrogen bond donor and acceptor atoms present in the peptide side chain, could then interact with the nucleotide bases positioned in the centre of the helix. The presence of the ethynyl group may disrupt this interaction. There is also a possiblility that ferrocene, in its oxidized $Fe^{3+}$ state could interact with the negatively charged phosphate backbone of the DNA double helix. Thus, it is possible for the naphthoyl ferrocenyl bioconjugates to possess two distinct modes of action: the ability to cause oxidative damage to DNA through ROS production and the ability to intercalate with DNA, both of which would result in the disruption of cancer cell replication. [132]

### 4.1.3. Conclusion

From the results of the SAR studies of the two groups; **1**-alkyl-1'-*N*-*para*, *N*-*meta*, *N*-*ortho*-(ferrocenyl) benzoyl dipeptide esters and *N*-{*para*-(ferrocenyl)ethynyl benzoyl}, *N*-{6-(ferrocenyl) ethynyl-2-naphthoyl} *N*-{5-(ferrocenyl)ethynyl-2-furanoyl} amino acid and dipeptide esters, there was a large range of $IC_{50}$ which ranged from the most active, 2.6 ± 0.6 µM **(68)** to the compound with the highest $IC_{50}$ value, 23 ± 5.1 µM **(129)** to many that were not active enough to pass the 10 µM screen. $IC_{50}$ values of $< 5$ µM are among the lowest this group has produced, however we have synthesized and biologically evauluated compounds in the nanomolar range. The trends we have seen in these compounds can be investigated to improve biological activity of some of the most active compounds in the continual effort to produce an improved anti-cancer compound.

## 4.2. *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine- compounds with altered ester moiety

*N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31** has previously been evaluated by this group and was the most active compound tested, with an $IC_{50}$ of 0.13 ± 0.02 and 1.10 ± 0.13 in H1299 and Sk-Mel-28 respectively.

For this reason it was selected to perform a SAR to evaluate the role of the ester moiety on biological activity. *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester was used as a reference as well as derivatives that replaced the ethyl ester with a methyl, propyl, butyl and benzyl ester. An initial screening at 10 µM showed there to be no correlation between ester moiety and activity. There was a trend that showed a slightly lower activity level of each compound in HT-144 and A549 cells when compared to Lox-IMVI, MalMe-3M and H-1299 cells. The compounds were then tested at 1 µM, at which point the biological activity of propyl, butyl and benzyl derivatives had all dropped to < 10 % growth inhibition. At 1 µM, the methyl derivative **30** showed an improvement in activity to the reference compound **31**. It appears that the ester moiety plays a crucial role in the biological activity of these compounds and a shorter chain is favoured. This is confirmed when the $IC_{50}$ values are taken into account. Across all cell lines *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30** has a lower $IC_{50}$ when compared to *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31**. It is also worth noting that both compounds have a lower $IC_{50}$ than cisplatin. As cisplatin is commonly used as a single agent, and as part of a chemotherapy regimen, this is a promising early result for the potential of these ferrocenyl compounds.

The $IC_{50}$ value for *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31** was found to be markedly higher in H1299 cells than in any of the other cell lines tested. Also, from previous results the $IC_{50}$ of **31** was found to be 0.13 ± 0.02 µM in H1299 cells, however when tested as a reference compound concurrently with **30** it was found to be 1.4 ± 0.21 µM, a > 10 fold decrease in activity. Potential reasons for this are discussed in section 4.3

## 4.3 Further cytotoxicological evaluation of *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester (30) and *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester (31)

Given the strong anti-proliferative effects of *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester (**30**) and *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester (**31**), additional cytotoxicological evaluation was warranted.

Resistance to chemotherapeutics is a major concern when trying to treat any cancer type. Cancer cells exhibit resistantance to chemotherapeutics for a number of reasons. Broadly, some of these reason are i) intrinsic resistance, where a cancer cell is naturally resistant to a certain chemotherapy treatment, ii) acquired resistance, where a cancer cell develops resistance to a chemotherapy treatment that was once effective and iii) multiple drug resistance, where a cancer cell may have developed a mechanism of resistance that can affect the activity of more than one chemotherapy treatment. An example of multiple drug resistance can be seen between cisplatin and temozolomide, both of which share a common resistance to mismatch repair. HCT 116 (*MLH1* mutant) and DLD1 (*MSH6* mutant) MMR deficient colon carcinoma cells are resistant to TMZ treatment. MMR-deficient cells are reported to be up to 100-fold less sensitive to methylating agents compared with their MMR proficient counterparts. MLH1 deficiency is also associated with cisplatin resistance [255-260]. Low MSH2 (which forms a heterodimer with MSH6) levels predict cisplatin benefits in patients with resected lung cancer and high MSH2 levels are a positive prognostic factor for untreated lung cancer patients [253,257,258,260].

We tested *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30** and *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31** in two cell line models of acquired resistance to cisplatin and temozolomide, to determine if either of these compounds could overcome the cisplatin/temozolomide resistance mechanisms. The specific resistance mechanisms to cisplatin and temozolomide in these cells have not yet been elucidated.

From the $IC_{50}$ values, Compound **31** shows no significant variation in activity between the parental and resistant cell lines whereas the cells show resistance to

compound **30** which indicates that **30** is susceptible to similar mechanisms of resistance to that of cisplatin and temozolomide.

Cell cycle analysis in both A549 and HT-144 cell lines has shown *N*-(6-ferrocenyl-2-naphthoyl)-glycine-glycine methyl ester **30** causes a significant increase in the sub-$G_0/G_1$ peak, which is suggestive of apoptosis and/or necrosis.

Due to apoptosis being heavily implicated as a primary mode of action of *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30** and *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31**, TUNEL analysis was undertaken in A549 and HT-144 cells with *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30**. A dose dependent relationship was observed between the concentration of **30** and the induction of apoptosis. This further implicates apoptotic induction as a primary mode of action of *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30**. When we compare the results of the cell cycle and TUNEL assay we can see that levels of apoptosis appear higher in the TUNEL analysis when compared to the sub $G_0$ fraction, indicative of apoptotic/necrotic cells, of the cell cycle analysis. A reason for this may be that in order for cells to be seen in the Sub $G_0/G_1$ area, they must have lost enough DNA to appear there; so if cells enter apoptosis from the S or $G_2$/M phase of the cell cycle or if there is an aneuploid population undergoing apoptosis, they may not appear in the Sub $G_0/G_1$ peak [82]. Also cells that have lost DNA for any other reason, for example, death by some other form of oncosis, will appear in the Sub$G_0/G_1$ region; so we have to be careful about how we define the sub $G_0/G_1$ peak [84]. As with all methods that are used to detect apoptosis, the more processing steps involved, the greater the likelihood of losing cells and apoptotic and necrotic cells are sometimes preferentially lost [84]. Although late-stage apoptosis has been implicated as a mode of action of **30**, like many other chemotherpaeutics, this may not be the only method whichis in effect. We know that ROS production has an effect on many cellular processes and as such, other modes of action are worthy of further investigation.

Another major problem with chemotherapeutic regimens is the side effects that generally accompany them. As chemotherapy treatment is generally systemic, side effects arise from cytotoxic agents affecting healthy cells as well as cancer cells. As such, we attempted to address this issue by testing *N*-(6-ferrocenyl-2-naphthoyl)

glycine glycine methyl ester **30** and *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31** in NHDF cells, using cisplatin as a reference. The $IC_{50}$ results showed that **30** and **31** are less cytotoxic than cisplatin in NHDF cells. As ROS production is thought to play an important role in the anti proliferative effect of ferrocenyl compound, the reason why the compounds tested have such high activity levels in cancer cells but not NHDF cells may be due to the increased regulation of ROS in healthy cells compared to cancer cells. The difference in activity between cancer cells and normal cells would suggest that healthy cells tolerate the compounds better than cancer cells. Selectivity of chemotherapeutics to cancer cellsis a highly desirable quality as it may suggest reduced side effects to the drug.

## 4.4 Future work

Previous studies of *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **31** have shown an $IC_{50}$ 0.13 ± 0.02 μM in H1299 cells, whereas in the SAR study reported here, the $IC_{50}$ value achieved was increased by greater than 10 fold (1.4 ± 0.21 μM). Although precautions were taken to ensure reproducibility in results there are a number of factors which may account for the difference in activity. The compound used was of the same batch, however the compound was stored for many months before further testing. The compound is thought to be stable and was evaluated by TLC and NMR before testing and showed no degradation, when variations in $IC_{50}$ values were first notices, TLC analysis was carried out again and showed no degradation. Before testing, **31** was weighed using an analytical balance, however some discrepancies in making stock solutions may contribute to the difference in activity. The H1299 cells were obtained from an internal cell repository and may contribute to a potential difference in activity levels. Differences in FCS (Foetal Calf Serum) batches, which is added to the growth medium, play a role in the characteristics of the cells and can effect responsiveness to drugs. Some or all of these factors may play a role in the discrepancies in activity from those previously reported. This issue should be addressed before further analysis is undertaken.

The natural progression of compounds which have shown strong anti-cancer properties is an evaluation *in vivo*. DMSO is one of the most common solvents used experimentally to dissolve hydrophobic substances for *in vitro* testing. All of the

compounds tested were dissolved in DMSO and an equivalent volume was used to ensure that DMSO alone had no anti-proliferative activity. Although DMSO can be used as a delivery method of compounds *in vitro*, it is not well tolerated for *in vivo* models. Due to this reason we explored a number of alternative delivery methods and used *N*-{6-ferrocenyl-2-naphthoyl}-glycine-glycine ethyl ester **31** to test the efficacy of the drug delivery vehicles. Suspension of **31** in Kolliphor EL was the only drug delivery method which demonstrated a similar anti-proliferative effect *in vitro* when compared to DMSO delivery; however the control group showed an even greater affect. For this reason it cannot be concluded that the biological activity is due to **31**. 80 mM citrate buffer, 5 % carboxymethyl cellulose, 1 % carboxymethyl cellulose and 50 % propylene glycol solution all significantly reduced the activity of **31**. Alternative methods of drug delivery are necessary before these ferrocenyl compounds can be tested in an *in vivo* model.

PEGylation and Intralipid have both been used as a method of drug delivery for hydrophobic compounds [253,254]. Attempts to use PEGylation as an effective method of drug delivery for ferrocenyl compound **31** was unsuccessful as it removed all anti-proliferative activity of the compound, which further strengthens the hypothesis that the structural composition of the compound plays a pivotal role in its activity. **31** was also completely insoluble in intralipid.

Nanocapsules show great potential in overcoming the water solubility issue of these molecules as they offer benefits such as increased bioavailability, protection against *in vivo* degradation and enhancement of pharmacokinetic properties as well as greatly increasing solubility in water [255]. LNCs (lipid nanocapsules) are a promising alternative as they have already been tested successfully *in vivo* with the ferrocenyl compound, FcOHTAM. FcOHTAM was encapsulated in an LNC and tested *in vivo* in xenografted triple negative breast cancer mice. As a consequence, a significantly lower tumor volume was obtained at the end of the experiment with a difference of 36 % at day 38 compared to the untreated group. The LNC was also shown to have no effect on the $IC_{50}$ value [132]. For these reasons, LNC's make a great candidate for the advancement of compounds **30** and **31** into *in vivo* testing [256].

As ROS is implicated as being a factor in the mode of action of ferrocenyl compounds (section 1.3.1.2.1). This is possible *via* a Fenton-type reaction, in which $HO^{\bullet}$ radicals are generated from the superoxide dismutation product, hydrogen peroxide ($H_2O_2$). To investigate this, the rate of Fenton-reaction mediated 8-oxo-7,8-dihydroguanine (8-oxoGua) [257] formation from guanine was monitored. Guanine was chosen as it has the lowest oxidation potential of all the DNA bases and is considered the clinical biomarker for oxidatively damaged DNA [258] We have previously reported on the ability of these derivatives to utilize this as a mode of action and studies confirm that **31** is capable of causing oxidative damage to guanine, and it does so by the generation of $HO^{\bullet}$ radicals from $H_2O_2$ The hydroxyl radical (HO•) generated oxidises guanine to oxo-8-guanine thus altering the base pairing sequence to adenine which results in DNA damage leading to cell apoptosis [259].

It would be of interest to investigate the levels of ROS produced by some of the most active ferrocenyl compounds. The production of quinone methide has also been shown to play a role in the activity of ferrocenyl compounds. The oxidation of ferrocene promotes the oxidation of one phenol group into a quinone methide through a proton transfer coupled to an intramolecular electron-transfer mechanism [123]. This is expected to damage target cells by forming adducts with biological bases, such as DNA or proteins [124–125]. As DNA damage is also thought to play a role in the activity of the compounds tested, identification of quinine methide production would be worth exploring.

Further investigation into the specific mode(s) of action involved in the anti-proliferative activity of these compounds would be valuable. Protemomic profiling would be of interest, particularly in relation to the method *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **31** uses to overcome cisplatin and temozolomide resistance which could be compared against *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine ethyl ester **30** which was sensitive to the resistant cells. As seen in section 1.4, ferrocenyl compounds have shown to have an effect on apoptosis relevant protein harikiri [260]. As apoptosis is shown to play an important role in the activity of **30** and **31**, proteomic analysis could shed new light on the cellular interactions of these ferrocenyl compounds.

## 4.5. Summary and Conclusion

In summation, we have investigated a number of structure-activity relationships that have shown us the importance of the structural composition to the biological activity of these ferrocenyl compounds. We have also identified our most active compound to date, *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30** with $IC_{50}$ values in a nanomolar range. This compound has a number of characteristics that make it a viable option for further investigation, such as:

(i) Strong anti-proliferative activity in cancer cells with nanomolar concentrations, greater than that of cisplatin.
(ii) Low cytotoxity in NHDF cells, of a similar level as cisplatin.
(iii) Selectivity of activity to cancer cells.
(iv) The ability to overcome multiple drug resistance (MDR).

Further investigation into the mode of action of these compounds would be of interest, particularly protemomic work to identify target proteins involved in multiple drug resistance, quinine methide production as well as internal ROS production. LNC's may hold the key to overcoming the solubility issues associated with these ferrocenyl comounds and if this is the case then *in vivo* is an important next step to evaluating the most active compound, *N*-(6-ferrocenyl-2-naphthoyl) glycine glycine methyl ester **30**.

## Abbreviations

| | | |
|---|---|---|
| $IC_{50}$ | - | Inhibitory concentration 50 % |
| TUNEL | - | Terminal DNA transferase-mediated dUTP nick end labelling |
| UV | - | Ultra Violet |
| HPV | - | Human papillomavirus |
| HRT | - | Hormone Replacement Therapy |
| NSCLC | - | Non-Small-Cell Lung Cancer |
| SCLC | - | Small-Cell Lung Cancer |
| SEER | - | Surveillance, Epidemiology, and End Results |
| DTIC | - | Dacarbazine |
| CVD | - | cisplatin, vinblastine and dacarbazine |
| CTLA-4 | - | Cytotoxic T-lymphocyte associated antigen 4 receptors |
| gp100 | - | glycoprotein 100 |
| BRAF | - | V-raf murine sarcoma viral oncogene homolog B1 |
| FDA | - | Food and Drug Administration |
| IUPCA | - | Internation Union of Pure and Applied Chemistry |
| DNA | - | Deoxyribonucleic Acid |
| RNA | - | Ribonucleic Acid |
| cis-DDP | - | cis-diamminedichloroplatinum |
| ROS | - | Reactive Oxygen Species |
| SOD | - | Superoxide Dismutase |
| GSH | - | Glutathione |
| ER | - | Estrogen Receptor |
| KP46 | - | tris(8-quinolinolato)gallium(III) |
| KP1019 | - | indazoliumtrans-[tetrachlorobis(1H-indazole)-ruthenate(III)] |
| NAMI-A | - | imidazolium trans-[tetrachloro-(S-dimethyl sulfoxide)(1H-imidazole)ruthenate(III)] |
| NO | - | Nitrous Oxide |
| RAPTA | - | Ruthenium Arene 1,3,5-Triaza-7-Phosphaadamantane |
| MDR | - | Multiple Drug Resistance |
| Pgp | - | Permeability Glycoprotein |
| Vcr | - | Vincristine |
| Bcl-2 | - | B-cell lymphoma 2 |

| | | |
|---|---|---|
| Bax | - | Bcl-2-associated X protein |
| Bak | - | Bcl-2 homologous antagonist killer |
| ATP | - | Adenosine triphosphate |
| NADH | - | Nicotinamide adenine dinucleotide |
| TrxR | - | Thioredoxin reductase |
| $LD_{50}$ | - | Median Lethal Dose |
| DMSO | - | Dimethyl sulfoxide |
| PEG | - | Polyethylene glycol |
| LNC | - | Lipid Nanocapsule |
| TNBC | - | Triple-Negative Breast Cancer |
| FCS | - | Fetal Calf Serum |
| DMEM | - | Dulbecco's modified Eagle's medium |
| RPMI | - | Roswell Park Memorial Institute medium |
| Cpt | - | Cisplatin |
| ATCC | - | American Type Culture Collection |
| NICB | - | National Institute of Cellular Biotechnology |
| PBS | - | Phosphate Buffered Saline |
| Fc-OH-TAM | - | Hydroxy-Ferrocifen |
| PI | - | Propidium Iodide |
| SAR | - | Structure-Activity Relathionship |
| OEt | - | Ethyl ester |
| OMe | - | Methyl ester |
| OBu | - | Butyl ester |
| OBn | - | Benzyl ester |
| OPr | - | Propyl ester |
| GABA | - | gamma-Aminobutyric acid |
| TMZ | - | Temozolomide |
| OH• | - | Hydroxide Ion |

# References


[1] R. W. Ruddon, Cancer Biology, Oxford University Press, 2007.
[2] World Health Organization. The Global Burden of Disease: 2004 Update. Geneva: World Health Organization; 2008.
[3] Ahmedin, J., *Global Cancer Statistics*
[4]. F. Brayand, and B. Moller. Predicting the future burden of cancer. *Nat. Rev. Cancer*. 6:63–74 (2006)
[5]. D. W. Donnelly, Gavin, A. T., Comber, H. "Cancer in Ireland: A summary report,"
2009.
[6] D. W. Donnelly, Gavin, A. T., Comber, H. "Cancer in Ireland 1994-2004: A comprehensive report," 2009.
[7] Bray FI, Weiderpass E. *Lung cancer mortality trends in 36 European countries: secular trends and birth cohort patterns by sex and region 1970-2007*. Int J Cancer. 2010; **126**:1454-66.
[8] Ezzati M, Henley SJ, Lopez AD, Thun MJ. *Role of smoking in global and regional cancer epidemiology: current patterns and data needs*. Int J Cancer. 2005; **116**: 963-71.
[9] Hecht, S.S., *Cigarette smoking and lung cancer: chemical mechanisms and approaches to prevention.* Lancet Oncol, 2002. **3**(8): p. 461-9.
[10] Rom, W.N., et al., *Molecular and genetic aspects of lung cancer.* Am J Respir Crit Care Med, 2000. **161**(4 Pt 1): p. 1355-67.
[11] Travis, W.D., L.B. Travis, and S.S. Devesa, *Lung cancer.* Cancer, 1995. **75**(1 Suppl): p. 191-202.
[12] Mountain, C.F., *Staging classification of lung cancer. A critical evaluation.* Clin Chest Med, 2002. **23**(1): p. 103-21.
[13] Beadsmoore, C.J. and N.J. Screaton, *Classification, staging and prognosis of lung cancer.* Eur J Radiol, 2003. **45**(1): p. 8-17.
1345-89.
[14] Chua, Y.J., C. Steer, and D. Yip, *Recent advances in management of smallcell lung cancer.* Cancer Treat Rev, 2004. **30**(6): p. 521-43.
[15] Hoffman, P.C., A.M. Mauer, and E.E. Vokes, *Lung cancer.* Lancet, 2000. **355**(9202): p. 479-85.
[16] Riedel, R.F. and J. Crawford, *Small-cell lung cancer: a review of clinical trials.* Semin Thorac Cardiovasc Surg, 2003. **15**(4): p. 448-56.
[17] Socinski, M.A., *Cytotoxic chemotherapy in advanced non-small cell lung cancer: a review of standard treatment paradigms.* Clin Cancer Res, 2004. **10**(12 Pt 2): p. 4210s-4214s.
[18] Zarogoulidis, K., Zarogoulidis, P., Darwiche, K., et. al. *Treatment of non-small cell lung cancer (NSCLC).* J Thorac Dis, 2013. **5(**S4): p. 389-396.
[19] World Health Organization; 2010. WHO, Sunbeds, tanning and UV exposure, in Fact sheet N°287.
[20] Miller AJ, Mihm Jr. MC. Melanoma. N *Engl J Med*. 2006;355: 51–65.
[21] Howlader N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975-2010*, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site, 2013. Accessed 30.08.13
[22] Weinstock MA. Cutaneous melanoma: public health approach to early detection. *Dermatol Ther*. 2006; 19: 26–31.

[23]. Kamino H. Spitzoid melanoma. Clin Dermatol. 2009;27:545–555.
[24]. Levine JA, Sorace M, Spencer J, Seigel DM. The indoor UV tanning industry: a review of skin cancer risk, health benefit claims, and regulation. J Am Acad Dermatol. 2005; 53: 1038–1044.
[25]. Swerdlow AJ, Weinstock MA. Do tanning lamps cause melanoma? An epidemiologic assessment. J Am Acad Dermatol. 1998; 38: 89–98.
[26] Westerdahl J, Ingvar C, Masback A, Jonsson N, Olsson H. Risk of cutaneous malignant melanoma in relation to use of sunbeds: further evidence for UV-A carcinogenicity. Br J Cancer. 2000; 82: 1593–1599.
[27] Ting W, Schultz K, Cac NN, Peterson M, Walling HW. Tanning bed exposure increases the risk of malignant melanoma. Int J Dermatol. 2007; 46: 1253–1257.
[28] Scolyer RA, Murali R, McCarthy SW, Thompson JF. Pathologic examination of sentinel lymph nodes from melanoma patients. Semin Diagn Pathol. 2008; 25:100–111.
[29] Gershenwald JE, Thompson W, Mansfield PF, et al. Multi-institutional melanoma lymphatic mapping experience: the prognostic value of sentinel lymph node status in 612 stage I or II melanoma patients. J Clin Oncol. 1999; 17: 976–983.
[30] Scolyer RA, Thompson JF, Stretch JR, Sharma R, McCarthy SW. Pathology of melanocytic lesions: new, controversial, and clinically important issues. J Surg Oncol. 2004; 86: 200–211.
[29] Egger ME, Tabler BL, Dunki-Jacobs EM, et al.Clinicopathologic and survival differences between upper and lower extremity melanomas. Am Surg. 2012; 78: 779–787.
[30] Mervic L. Prognostic factors in patients with localized primary cutaneous melanoma. Acta Dermatovenerol Alp Panonica Adriat. 2012; 21: 27–31.
[31] Allen AC, Spitz S. Malignant melanoma; a clinicopathological analysis of the criteria for diagnosis and prognosis. Cancer. 1953; 6: 1–45.
[32] Tompkins VN. Cutaneous melanoma: ulceration as a prognostic sign. Cancer. 1953; 6: 1215–1218.
[33] Cochran AJ. Histology and prognosis in malignant melanoma. J Pathol. 1969; 97: 459–468.
[34] Franklin JD, Reynolds VH, Page DL. Cutaneous melanoma: a twenty-year retrospective study with clinicopathologic correlation. Plast Reconstr Surg. 1975; 56: 277–285.
[35] Huvos AG, Shah AP, Mike V. Prognostic factors in cutaneous malignant melanoma. A comparative study of long term and short term survivors. Hum Pathol. 1974; 5: 347–357.
[36] Balch CM, Wilkerson JA, MuradTM, Soong SJ, Ingalls AL, Maddox WA. The prognostic significance of ulceration of cutaneous melanoma. Cancer. 1980; 45: 3012–3017.
[37] Balch CM, Murad TM, Soong SJ, Ingalls AL, Halpern NB, Maddox WA. A multifactorial analysis of melanoma: prognostic histopathological features comparing Clark's and Breslow's staging methods. Ann Surg. 1978; 188: 732–742.
[38] Grande Sarpa H, Reinke K, Shaikh L, et al. Prognostic significance of extent of ulceration in primary cutaneous melanoma. Am J Surg Pathol. 2006; 30: 1396–1400.

[39] Eigentler TK, Buettner PG, Leiter U, Garbe C. Impact of ulceration in stages I to III cutaneous melanoma as staged by the American Joint Committee on Cancer Staging System: ananalys is of the German Central Malignant Melanoma Registry. J Clin Oncol. 2004; 22: 4376–4383.
[40] Edge SB, Byrd DR, Compton CC, eds. AJCC Cancer Staging Manual. 7th ed. New York: Springer; 2010.
[41] Balch CM, GershenwaldJE, SoongSJ, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009; 27:6199–6206.
[42] Roach BA, Burton AL, Mays MP, et al. Does mitotic rate predict sentinel lymph node metastasis or survival in patients with intermediate and thick melanoma? Am J Surg. 2010; 200:759–763. [discussion63-4].
[43] Callender GG, Egger ME, Burton AL, et al. Prognostic implications of anatomic location of primary cutaneous melanoma of 1mm or thicker. Am J Surg. 2011; 202:659–664. [discussion64-5].
[44] Balch CM, Soong SJ, Gershenwald JE, et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol. 2001; 19:3622–3634.
[45] Burton AL, Gilbert J, Farmer RW, et al. Regression does not predict nodal metastasis or survival in patients with cutaneous melanoma. Am Surg. 2011; 77:1009–1013.
[46] Clark Jr.WH, From L, Bernardino EA, Mihm MC. The histogenesis and biologic behaviour of primary human malignant melanomas of the skin. Cancer Res. 1969; 29:705–727.
[47] Page AJ, Li A, Hestley A, Murray D, Carlson GW, Delman KA. Increasing age is associated with worse prognostic factors and increased distant recurrences despite fewer sentinel lymph node positives in melanoma. Int J Surg Oncol. 2012;2012: 456987.
[48] Breslow A. Thickness, cross-sectional areas and depth ofinvasion in the prognosis of cutaneous melanoma. Ann Surg. 1970; 172:902–908.
[49] Surveillance, Epidemiology, and End Results (SEER) Program. SEER*Stat Database:
Incidence-SEER 17 Regs Public Use, Nov. 2010 Sub (2000-2008)-Linked to County Attributes-Total US, 1969-2008 Counties. Bethesda, MD: National Cancer Institute, Division of Cancer Control and Population Sciences, Surveillance Research Program, Cancer Statistics Branch; 2011.
[50] Lee SM, Margison GP, Thatcher N, et al., Formation and loss of O6-methyldeoxyguanosine in human leucocyte DNA following sequential DTIC and fotemustine chemotherapy. *Br. J. Cancer* (1994) **69**(5): 853-7
[51] Anderson CM, Buzaid AC, Legha SS, Systemic treatments for advanced cutaneous melanoma. *Oncology (Huntingt)* (1995) **9**(11): 1149-58
[52] Anderson CM, Buzaid AC, Legha SS. Systemic treatments for advanced cutaneous melanoma. Oncology (Williston Park). 1995; 9:1149–1158. discussion 1163–1144, 1167–1148.
[53] Chapman PB, Einhorn LH, eyers ML, Saxman S, Destro AN, Panageas KS et al. Phase III multicenter randomized trial of the Dartmouth regimen versus

dacarbazine in patients with metastatic melanoma. *J. Clin. Oncol*. (1999) **17**(9): 2745-2751
[54] Luikart SD, Kennealey GT, Kirkwood JM. Randomized Phase III trial of vinblastine, bleomycin, and cisdichlorodiammine – platinum versus dacarbazine in malignant melanoma. *J. Clin. Oncol.* (1984) **2**(3): 164-168
[55] Lopez M, Perno CF, Di Lauro I, Papaldo P, Ganzina F, Barduagni A. Controlled study of DTIC versus DTIC plus epirubicin in metastatic malignant melanoma. *Invest. New Drugs* (1984) **2**(3): 319-322
[56]. Becker, J.C., E. Kampgen, and E. Brocker, *Classical chemotherapy for metastatic melanoma.* Clin Exp Dermatol, 2000. **25**(6): p. 503-8.
[57]. O'Day SJ, Hamid O, Urba WJ. Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): a novel strategy for the treatment of melanoma and other malignancies. Cancer. 2007; 110: 2614–2627.
[58] Fong L, Small EJ. Anti-cytotoxic T-lymphocyte antigen-4 antibody: the first in an emerging class of immunomodulatory antibodies for cancer treatment. J Clin Oncol. 2008; 26: 5275–5283.
[59]. Hodi F. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010; 363: 711–723.
[60] Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417: 949–954.
[61] Curtin JA, Fridly and J,Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005; 353: 2135–2147.
[62] Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature. 2010; 467: 596–599.
[63] Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutations. New Engl J Med. 2011; 364: 2507–2516.
[64]Gilles Gasser, Ingo Ott, and Nils Metzler-Nolte, Organometallic Anticancer Compounds. 2011. J. Med. Chem. 2011, 54, 3–25.
[65] "Press Release: The 1973 Nobel Prize in Chemistry". Nobelprize.org. Acessed 20 Sep 2013 http://nobelprize.org/nobel_prizes/chemistry/laureates/1973/press.html
[66] Moss, GP; Smith, PAS; Tavernier, D; *Pure & App. Chem*; **67**, 1307-1375. (1995).
[67] P. Köpf-Maier, H. Köpf, Chem. Rev. 87 (1987) 1137-1152.
[68] P. Köpf-Maier, Eur. J. Clin. Pharmacol. 47 (1994) 1-16.
[69] M.M. Harding, G. Mokdsi, Curr. Med. Chem. 7 (2000) 1289-1303.
[70] P. Köpf-Maier, H. Köpf, E.W. Neuse, Angew. Chem. Int. Ed. Engl. 23 (1984) 456-457.
[71] P. Köpf-Maier, Antitumor Bis(Cyclopentadienyl)Metal Complexes, in: B.K. Keppler (Ed.), Metal Complexes in Cancer Chemotherapy, VCH, Weinheim, 1993, pp. 259-296.
[72] Goodman, LS; Wintrobe, MM; Damshek, W; *JAMA*; **3**, 126-132. (1946).
[73] McGuire, JJ; *Curr. Pharm. Des.*; **9**, 2593-2613. (2003).
[74] P. J. Dyson and G. Sava,Dalton Trans., 2006, 1929–1933.
[75] M. A. Jakupec, M. Galanski, V. B. Arion, C. G. Hartinger and B. K. Keppler,Dalton Trans., 2008, 183–194.
[76] Giaccone, G., *Clinical Perspectives on Platinum Resistance*. Drugs, 2000. **59**(6): p. 9-17
[77] Cvitkovic, E., *A historical perspective on oxaliplatin: Rethinking the role of platinum compounds and learning from near misses.* Semin Oncol, 1998. **25**: p. 1-3

[78] M.A.Fuertes, C.Alonso, and J.M.Pérez, *Biochemical modulation of cisplatin mechanisms of action: enhancement of antitumor activity and circumvention of drug resistance*, Chemical Reviews, 2003. **103**(3), p. 645–62.
[79] A.R.Timerbaev, C.G.Hartinger, S.S.Aleksenko, andB. K. Keppler, *Interactions of antitumor metallodrugs with serum proteins: advances in characterization using modern analytical methodology*, Chemical Reviews, 2006. **106**(6), p. 2224–48.
[80] E. Volckova, F. Evanics, W. W. Yang, and R. N. Bose, *Un-winding of DNA polymerases by the antitumor drug, cis-diamminedichloroplatinum(II),* Chemical Communications, 2003, **9**(10), p. 1128-29.
[81] S. Ahmad, *Platinum-DNA interactions and subsequent cellular processes controlling sensitivity to anticancer platinum complexes*, Chemistry and Biodiversity, 2010. **7**(3), no. 3, p. 543-66.
[82] Dasika, G.K., et al., *DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis*. Oncogene, 1999. **18**: 7883-99
[83] Attardi, L.D.; de Vries, A.; Jacks, T. *Activation of the p53-dependent G1 checkpoint response in mouse embryo fibroblasts depends on the specific DNA damage inducer* Oncogene, 2004, **23**: 973.
[84] Sorenson, C.M.; Eastman, A. *Influence of cis-Diamminedichloroplatinum(II) on DNA Synthesis and Cell Cycle Progression in Excision Repair Proficient and Deficient Chinese Hamster Ovary Cells*. Cancer Res, 1988, **48**: 6703.
[85] González, V.M., et al, *Is cisplatin-induced cell death always produced by apoptosis?* Mol Pharmacol, 2001, **59**(4): p. 657-63.
[86] Guchelaar, H.J., et al, *Apoptosis- and necrosis-inducing potential of cladribine, cytarabine, cisplatin, and 5-fluorouracil in vitro: a quantitative pharmacodynamic model* Cancer Chem Pharmacol, 1998. **42**(1): p. 77-83.
[87] Matsumoto, M., Tsuchida, T. and Kawamoto, K., *Cisplatin-induced cell death in human glioma: Apoptosis or necrosis?* Int J Oncol, 1997. 11(6): p. 1209-1212.
[88] Pérez, J.M., et al, *A cycloplatinated compound of p-isopropylbenzaldehyde thiosemicarbazone and its chloro-bridged derivative induce apoptosis in cis-DDP resistant cells which overexpress the H-ras oncogene* C. J Inorg Biochem, 1999, **73**(4), p. 235-43.
[89] Montero, E.I., et al, *Apoptosis Induction and DNA Interstrand Cross-Link Formation by Cytotoxictrans-[PtCl2(NH(CH3)2)(NHCH(CH3)2)]: Cross-Linking between d(G) and Complementary d(C) within Oligonucleotide Duplexes*. Chembiochem, 2002, **3**(1), 61-67.
[90] Chu, G., *Cellular Responses to Cisplatin*. J Biol Chem, 1994. **269**, p. 787-90.
[91] Kelland, L.R., *Preclinical perspectives on platinum resistance*. Drugs, 2000. **59**: p. 1-8.
[92] M.A.Fuertes,C.Alonso,andJ.M.P´erez,"*Biochemical modulation of cisplatin mechanisms of action: enhancement of antitumor activity and circumvention of drug resistance*," Chemical Reviews, vol. 103, no. 3, pp. 645–662, 2003.
[93]. Harrap, KR; *Cancer Treat. Rev.* **12**, 21–33 (1985).
[94] B. Lippert (Ed.). Cisplatin, Chemistry and Biochemistry of a Leading Anticancer Drug, Wiley-VCH, Weinheim (1999).
[95]. Knox, RJ; Friedlos, F; Lydall, DA; Roberts, JJ; *Cancer Res.* **46**, 1972–1979 (1986).
[96]. Aabo, K. *et al.*. *Br. J. Cancer* **78**, 1479–1487 (1998).
[96] B. Lippert (Ed.). Cisplatin, Chemistry and Biochemistry of a Leading Anticancer Drug, Wiley-VCH, Weinheim (1999).

[97] Chaney SG, Campbell SL, Temple B, Bassett E, Wu Y, Faldu M. *J Inorg Biochem*; **98**:1551–1559. (2004).
[98] Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, et al.. *Biochem Pharmacol.*; **52**, 1855–65. (1996)
[99] Ibrahim, A; Hirschfeld, S; Cohen, MH; Griebel, DJ; Williams, GA; Pazdur, R; *Oncologist*; **9**, 8–12. (2004).
[100]. Mechetner, E; Brünner, N; Parker, RJ; *Scand J Gastroenterol*; **46**: 70–78. (2011).
[101]. Machover, D; Diaz-Rubio, E; de Gramont, A; Schilf, A; Gastiaburu, JJ; Brienza, S; Itzhaki, M; Metzger, G; N'Daw, D; Vignoud, J; Abad, A; Francois, E; Gamelin, E; Marty, M; Sastre, J; Seitz JE; Ychou, M; *Ann. Oncol;* **7**, 95-98, (1996)
[102]. Grothey, A; *Sem Oncol*; **30**: 5–13. (2003).
[103] Woodward, R.B., Rosenblum, M., Whiting, M.C. *A New Aromatic System*. J. Am. Chem. Soc. 1952. **74**: p. 3458-59.
[104] M. Rosenblum,Chemistry of the Iron-Group Metallocenes, Part I, Interscience, New York, 1965
[105] V. J. Fiorina, R. J. Dubois and S. Brynes, *Ferrocenyl polyamines as agents for the chemoimmunotherapy of cancer*, J. Med. Chem., 1978, **21**(4), p. 393-395.
[106] P. Köpf-Maier, H. K¨ opf, and E.W.Neuse,"Ferrocenium salts—the first antineoplastic iron compounds," Angewandte Chemie—International Edition in English,vol. 23, no.6,pp. 456–457, 1984.
[107] P. Köpf-Maier, H. Kopf, and E. W. Neuse, "Ferriceniumcom-plexes: a new type of water-soluble antitumour agent,"Journal of Cancer Research and Clinical Oncology, vol. 108, no. 3, pp. 336–340, 1984.
[108] W. Troll, G. Witz, B. Goldstein, D. Stone, T. Sugimura, in Carcinogenesis, Vol. 7, E. Hecker et al. eds., (Raven Press, New York, 1982) p. 593.
[109] T. W. Kensler, D. M. Bush, and W. J. Kozumbo,Science.221, 75 (1983).
[110] P. J. O'Brien,Environ. Health Persp.64, 219 (1985).
[111] I. Fridovich,Annu. Rev. Biochem.44, 147 (1976).
[112] L. W. Oberley and G. R. Buettner, Cancer Res.39, 1141 (1979).
[113] Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol 4: 278–286, 2008
[114] Alexander A, Cai SL, Kim J, Nanez A, Sahin M, MacLean KH, Inoki K, Guan KL, Shen J, Person MD, Kusewitt D, Mills GB, Kastan MB, and Walker CL. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 107: 4153–4158, 2010.
[115] Benhar M, Engelberg D, and Levitzki A. ROS, stress activated kinases and stress signaling in cancer. EMBO Rep 3: 420–425, 2002.
[116] D'Autreaux B and Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 8: 813–824, 2007.
[117] Fruehauf JP and Meyskens FL, Jr. Reactive oxygen species: a breath of life or death? Clin Cancer Res 13: 789–794, 2007.
[118] Veal EA, Day AM, and Morgan BA. Hydrogen peroxide sensing and signaling. Mol Cell 26: 1–14, 2007.
[119] (a) Halliwell, B. Oxidative stress and cancer: have we moved forward? Biochem. J.2007, 401,1−11. (b) Engel, R. H.; Evens, A. M. Oxidative stress and apoptosis: a new treatment paradigm in cancer. Front. Biosci. 2006, 11, 300−312. (c)

Finkel, T. Oxidant signals and oxidative stress. Curr. Opin. Cell Biol. 2003, 15,247−254. (d) Schumacker, P. T. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 2006, 10, 175−176. (d) Antunes, F.; Cadenas, R. Estimation of H2O2 gradients across biomembranes.FEBS Lett.2000, 475, 121−126. (e) Szatrowski, T. P.; Nathan, C. F. Production of large amounts of hydrogen peroxide by human tumour cells.Cancer Res.1991, 51, 794−798. (f) O'Donnell-Tormey, J.; DeBoer, C. J.; Nathan, C. F. Resistance of human tumour cells in vitro to oxidative cytolysis. J. Clin. Invest. 1985, 76,80−86. (g) Stone, J. R. An assessment of proposed mechanisms for sensing hydrogen peroxide in mammalian systems. Arch. Biochem. Biophys. 2004, 422, 119−124

[120] (a) Hamels, D.; Dansette, P. M.; Hillard, E. A.; Top, S.; Vessieres, A.; Herson, P.; Jaouen, G.; Mansuy, D. Ferrocenyl quinine methides as strong antiproliferative agents: formation by metabolic and chemical oxidation of ferrocenyl phenols.Angew. Chem., Int. Ed. 2009, 48,9124−9126. (b) Jaouen, G.; Top, S.; Vessieres, A. Organometallics targeted to specific biological sites: the development of new therapies. InBioorganometallics; Jaouen, G., Ed.; Wiley-VCH: Weinheim, Germany, 2006; p 65. (c) Hillard, E. A.; Vessieres, A.; Thouin, L.; Jaouen, G.; Amatore, C. Ferrocene-mediated proton-coupled electron transfer in a series of ferrocifen-type breast-cancer drug candidates.Angew. Chem., Int. Ed.2006, 45, 285−290.

[121] A. Nguyen, A. Vessières, E. A. Hillard, S. Top, P. Pigeon and G. Jaouen, *Ferrocifens and ferrocifenols as new potential weapons against breast cancer.* Chimia, 2007, **61**: p. 716–24.

[122] E. A. Hillard, A. Vessières and G. Jaouen, *Ferrocene Functionalized Endocrine Modulators as Anticancer Agents*. Top. Organomet. Chem., 2010, **32**, 81–117.

[123] Allard, E.; Passirani, C.; Garcion, E.; Pigeon, P.; Vessières, A.; Jaouen, G.; Benoit, J. P.J. *Lipid nanocapsules loaded with an organometallic tamoxifen derivative as a novel drug-carrier system for experimental malignant gliomas* Contr. Release. 2008. **130**(2), 146 - 53.

[124] Khosrow, K.; Rigas, B. The mechanism of action of nitric oxide-donating aspirin. Biochem. Biophys. Res. Commun.2007, 358, 1096−1101.

[125] Hillard, E. A.; Vessières, A.; Thouin, L.; Jaouen, G.; Amatore, C.Angew. Chem., Int. Ed.2006, **45**, 285 - 90.

[124]. A. Vessiéres, S. Top, W. Beck, E. Hillard, G. Jaouen. *Metal complex SERMs (selective oestrogen receptor modulators). The influence of different metal units on breast cancer cell antiproliferative effects.* Dalton Trans. 2006. **4,** p. 529-41.

[125] Vessieres A, Top S, BeckW, Hillarda E, Jaouen G. *Dalton Trans.* 2006; 529.

[126] Goel, A., Savage, D., Alley, S.R., Kelly, P.N., O'Sullivan, D., Mueller-Bunz, H., and Kenny, P.T.M., *J. Organomet. Chem.*, **2007**, *692*, 1292-1299.

[127] Goel, A., Savage, D., Alley, S.R., Hogan, T., Kelly, P.N., Draper, S., Fitchett C.M., and Kenny P.T.M, *J. Organomet. Chem.*, **2006**, *691*, 4686-4693.

[128] Corry, A.J., Goel, A., Alley, S.R., Kelly, P.N., O'Sullivan, D., Savage, D., and Kenny, P.T.M., *J. Organomet. Chem.*, **2007** *692*, 1405-1410

[129] Corry, A.J., Mooney, A., O'Sullivan, D., and Kenny, P.T.M., *Inorganica Chimica Acta.*, **2009**, *362*, 2957-2961.

[130] Corry, A.J., O'Donovan, N., Mooney, A., O'Sullivan, D., Rai, D.K., Kenny, P.T.M., *J. Organomet. Chem.*, **2009**, *694*, 880-885.

[131] Vogel , M., Rausch, M.D., Rosenberg, H., *J. Organomet. Chem.*, **1957**, *22*, 1016-1019

[132] Mooney, A., Corry, A.J., O'Sullivan, D., Rai, D.K., and Kenny, P.T.M., J. Organomet. Chem., 2009, 694, 886-894
[133] Mooney, A., Corry, A.J., N'i Ruairc, C., Mahgoub, T., O'Sullivan, D., O'Donovan, N., Crown, J., Varughese, S., Draper, S.M., Rai, D.K., and Kenny, P.T.M., Dalton. Trans., 2010, 39, 8228–8239
[134] Mooney, A., Tiedt, R., Maghoub, T., O'Sullivan, D., O'Donovan, N., Crown, J., Varughese, S., White, B., and Kenny, P.T.M., J. Med. Chem., 2012, 55, 5455−5466
[135] C. Boit, Curr Med Chem Anti-Infect Agents, 2004. **3**, 135. 76 S. Top, J. Tang, A. Vessie`res, D. Carrez, C. Provot and G. Jaouen,Chem. Commun., 1996, 955–956.
[136] Osella D, Mahboobi H, Colangelo D, Cavigiolio G, Vessieres A, Jaouen G. *Inorg. Chim. Acta* 2005; **358**: 1993.
[137]. Top S, Vessieres A, Cabestaing C, Laios I, Leclercq G, Provot C, Jaouen G. *J. Organometal. Chem.* 2001; **637**: 500.
[138] Tamura H, Miwa M. *Chem. Lett.* 1997; **11**: 1177.
[139] Top S, Vessieres A, Leclercq G, Quivy J, Tang J, Vaissermann J, Huche M, Jaouen G. *Chem. Eur. J.* 2003; **9**: 5223.
[140] S. Top, B. Dauer, J. Vaissermann and G. Jaouen,J. Organomet. Chem., 1997, 541, 355–361.
78 S. Top, A. Vessie`res, G. Leclercq, J. Quivy, J. Tang, J. Vaissermann, M. Huche´ and G. Jaouen,Chem.–Eur. J., 2003, 9, 5223–5236.
[141] O. Payen, S. Top, A. Vessières, E. Brule, A. Lauzier, M.A.Plamont, M.J. McGlinchey, H. Muller-Bunz, G. Jaouen, J. Organomet. Chem. 696 (2011) 1049-1056.
[142] A. Nguyen, S. Top, P. Pigeon, A. Vessières, E.A. Hillard, M.A. Plamont, M. Huché, C. Rigamonti, G. Jaouen, Chem. Eur. J. 15 (2009) 684-696.
[143] O. Payen, S. Top, A. Vessières, E. Brulé, M.-A. Plamont, M.J. McGlinchey, H. Müller-Bunz, G. Jaouen, J. Med. Chem. 51 (2008) 1791-1799.
[144] G. Jaouen, A. Vessières, D. Plazuk, Ferrocene Derivatives with Anticancer Activity, CNRS, Patent FR20080054533.
[145] D. Plazuk, A. Vessières, E.A. Hillard, O. Buriez, E. Labbé, P. Pigeon, M.A. Plamont, C. Amatore, J. Zakrzewski, G. Jaouen, J. Med. Chem. 52 (2009) 4964-4967.
[146] M. Görmen, D. Plazuk, P. Pigeon, E.A. Hillard, M.A. Plamont, S. Top, A. Vessières, G. Jaouen, Tetrahedron Lett. 51 (2010) 118-120.
[147] Vessières A, Metal carbonyl tracers and the ferrocifen family: Two facets of bioorganometallic chemistry. Journal of Organometallic Chemistry 734 (2013) 3-16
[148] Hart M.M., Adamson R.H., *Antitumor activity and toxicity of salts of inorganic group 3a metals: aluminum, gallium, indium, and thallium*. Proc. Natl. Acad. Sci. USA. 1971. **68**(7): 1623–26.
[149] Hart M.M., Smith C.F., Yancey S.T. and Adamson R.H., Toxicity and antitumor activity of gallium nitrate and periodically related metal salts J. NatI. Cancer Inst. 1971. **47**(5): 1121–27.
[150] Foster B.J., Clagett-Carr K., Hoth D. and Leyland-Jones B., *Gallium nitrate. The second metal with clinical activity*. Cancer Treat Rep. 1986. **70**(11):1311–19.
[151] Einhorn L., *Gallium nitrate in the treatment of bladder cancer*. Semin Oncol. 2003. **30**(2 Suppl 5): 34–41.
[152] Straus D.J., *Gallium nitrate in the treatment of lymphoma*. Semin Oncol. 2003. 30(2 Suppl 5): 25– 33.

[153] Chitambar CR. *Gallium nitrate for the treatment of non-Hodgkin's lymphoma*. Expert Opin Investig Drugs. 2004. **13**(5): 531–41.
[154] Casper E.S., Stanton G.F., Sordillo P.P., et al., *Phase II trial of gallium nitrate in patients with advanced malignant melanoma*. Cancer Treat Rep. 1985. **69**(9): 1019–20.
[155] Chitambar C.R., *Gallium compounds as antineoplastic agents*. Curr Opin Oncol. 2004. **16**(6): 547-52.
[156] Collery P., Keppler B., Madoulet C. and Desoize B., *Gallium in Cancer Treatment* Crit Rev Oncol Hematol. 2002. **42**(3): 283-96.
[157] Valiahdi, SM.; Jakupec, MA.; Marculescu, R.; Keppler, BK. *Tris(8 quinolinolato)gallium(III) exerts strong antiproliferative effects in melanoma cells*. In: Alpoim, MC.; Morais, PV.; Santos, MA.; Cristovao, L.; Centeno, JA.; Collery, P., editors. Metal Ions in Biology and Medicine. John Libbey Eurotext; Paris, France: 2006. 282-86.
[158] Jakupec, MA.; Collery, P.; Keppler, BK. *Synergistic antiproliferative effects of tris(8- quinolinolato)gallium(III) (KP46) in combination with platinum drugs in ovarian and colon carcinoma cells*. In: Collery, P.; Maymard, I.; Theophanides, T.; Khassanova, L.; Collery, T., editors. Metal Ions in Biology and Medicine. John Libbey Eurotext; Paris, France: 2008. 110-15.
[159] Hofheinz RD, Dittrich C, Jakupec MA, et al. Early results *from a Phase I study on orally administered tris(8-quinolinolato)gallium(III) (FFC11, KP46) in patients with solid tumors – a CESAR study (Central European Society for Anticancer Drug Research [EWIV])*. Int J Clin Pharmacol Ther. 2005; **43**(12): 590–91.
[160] Collery, P.; Jakupec, MA.; Kynast, B.; Keppler, BK. *Preclinical and early clinical development of the oral Gallium complex KP46 (FFC11)*. In: Alpoim, MC.; Morais, PV.; Santos, MA.; Cristovao, L.; Centeno, JA.; Collery, P., editors. Metal Ions in Biology and Medicine. John Libbey Eurotext; Paris, France: 2006. 521-24.
[161] R.-D. Hofheinz, C. Dittrich, M. A. Jakupec, Aet al., *Early results from a phase I study on orally administered tris(8-quinolinolato)gallium(III) (FFC11, KP46) in patients with solid tumors--a CESAR study (Central European Society for Anticancer Drug Research--EWIV)* Int. J. Clin. Pharmacol. Ther., 2005, **43**, 590.
[162] Valiahdi SM, Heffeter P, Jakupec MA, et. al. *The gallium complex KP46 exerts strong activity against primary explanted melanoma cells and induces apoptosis in melanoma cell lines*. Melanoma Research, 2009, **19**(5): 283-93.
[163] Allamneni K.P., Burns R.B., Gray D.J., et al., *Gallium maltolate treatment results in transferrin-bound gallium in patient serum*. Proc Am Assoc Cancer Res. 2004. **45**: 230, Abstract 1013.
[164] Chitambar C.R., Purpi D.P., Woodliff J., et al., *Development of gallium compounds for treatment of lymphoma. Gallium maltolate, a novel hydroxypyrone gallium compound induces apoptosis and circumvents lymphoma cell resistance to gallium nitrate*. J Pharmacol Exp Ther. 2007. **322**(3):1228–36.
[165] Bernstein L.R., van der Hoeven J.J.M., Boer R.O., *Hepatocellular cancer detection by gallium scan and subsequent treatment by gallium maltolate: rationale and case study*. Anti-Cancer Agents Med Chem. 2011. **11**(6): 585–90
[166] M. Thiel, T. Schilling, D. C. Gey, et al., *Relevance of Tumour Models for Anticancer Drug Development*, ed. H. H. Fiebig and A. M. Burger, in Contributions to Oncology, ed. W. Queisser and W. Scheithauer, Karger, Basel, 1999,
vol. 54, p. 439.

[167] Bernstein L.R., Tanner T., Godfrey C. and Noll B., *Chemistry and Pharmacokinetics of Gallium Maltolate, a Compound With High Oral Gallium Bioavailability*. Met-Based Drugs, 2000, **7**(1), 33-47
[168] P. Collery, F. Lechenault, A. Cazabat, et al., *Inhibitory effects of gallium chloride and tris (8-quinolinolato) gallium III on A549 human malignant cell line*. Anticancer Res. 2000. **20**(2a): 955-58.
[169] Chua M.S., Bernstein L.R. and So S.K.S., *Gallium maltolate is a promising chemotherapeutic agent for the treatment of hepatocellular carcinoma*. Anticancer Res. 2006. **26**(3a): 1739-43.
[170] Lum B.L., Srivanas S., Beck J.T., et al., *Phase I study of oral gallium maltolate in patients with hematologic malignancies*, Blood. 2002, **100**(11): 224B.
[171] A. Korfel, M.E. Scheulen, H.J. Schmoll et al., "Phase I clinical and pharmacokinetic study of titanocene dichloride in adults with advanced solid tumors," *Clinical Cancer Research*, vol. 4, no. 11, pp. 2701–2708, 1998.
[172] C V. Christodoulou, D.R. Ferry, D.W. Fyfe et al., "Phase I trial of weekly scheduling and pharmacokinetics of titanocene dichloride in patients with advanced cancer," *Journal of Clinical Oncology*, vol. 16, no. 8, pp. 2761–2769, 1998.
[173] K. Mross, P. Robben-Bathe, L. Edler et al., "Phase I clinical trial of a day-1, -3, -5 every 3 weeks schedule with titanocene dichloride (MKT 5) in patients with advanced cancer: a study of the phase I study group of the association for medical oncology (AIO) of the German Cancer Society," *Onkologie*, vol. 23, no. 6, pp. 576–579, 2000.
[174] N. Kröger, U. R. Kleeberg, K. Mross, et al., "Phase II clinical trial of titanocene dichloride in patients with metastatic breast cancer," *Onkologie*, vol. 23, no. 1, pp. 60–62, 2000.
[175] G. Lümmen, H. Sperling, H. Luboldt, et al., "Phase II trial of titanocene dichloride in advanced renal-cell carcinoma," *Cancer Chemotherapy and Pharmacology*, vol. 42, no. 5, pp. 415–417, 1998.
26 M. Guo, Z. Guo and P. J. Sadler,JBIC, J. Biol. Inorg. Chem., 2001,6, 698–707.
M. Singh,Curr. Pharm. Des., 1999, 5, 443–451.
[176] R. Hernández, J. Lamboy, L. M. Gao, et al., "Structure-activity studies of Ti(IV) complexes: aqueous stability and cytotoxic properties in colon cancer HT-29 cells," *Journal of Biological Inorganic Chemistry*, vol. 13, no. 5, pp. 685–692, 2008.
[177] R. Hernández, J.M´endez, J. Lamboy, et al., "Titanium(IV) complexes: cytotoxicity and cellular uptake of titanium(IV) complexes on caco-2 cell line," *Toxicology in Vitro*, vol. 24, no. 1, pp. 178–183, 2010.
[178] L. M. Gao, J. Matta, A. L. Rheingold, and E. Meléndez, "Synthesis, structure and biological activity of amide-functionalized titanocenyls: improving their cytotoxic properties," *Journal of Organometallic Chemistry*, vol. 694, no. 26, pp. 4134–4139, 2009.
[179] A. Gansäuer, I. Winkler, D. Worgull et al., "Carbonyl-substituted titanocenes: a novel class of cytostatic compounds with high antitumor and antileukemic activity," *Chemistry*, vol. 14, no. 14, pp. 4160–4163, 2008.
[180] O. R. Allen, L. Croll, A. L. Gott, et al., "Functionalized cyclopentadienyl titanium organometallic compounds as new antitumor drugs," *Organometallics*, vol. 23, no. 2, pp. 288–292, 2004.
[181] O. R. Allen, A. L. Gott, J. A. Hartley, et al., "Functionalised cyclopentadienyl titanium compounds as potential anticancer drugs," *Dalton Transactions*, no. 43, pp. 5082–5090, 2007.

[182] G. D. Potter, M. C. Baird, and S. P. C. Cole, “A new series of titanocene dichloride derivatives bearing chiral alkylammonium groups; Assessment of their cytotoxic properties,” *Inorganica Chimica Acta*, vol. 364, no. 1, pp. 16–22, 2010.
[183] L. M. Gao, J. L. Vera, J. Matta et al., “Synthesis and cytotoxicity studies of steroid-functionalized titanocenes as potential anticancer drugs: sex steroids as potential vectors for titanocenes,” *Journal of Biological Inorganic Chemistry*, vol. 15, no. 6, pp. 851–859, 2010.
[184] S. Gómez-Ruiz, G. N. Kaluđerović, S. Prashar et al., “Cytotoxic studies of substituted titanocene and ansa-titanocene anticancer drugs,” *Journal of Inorganic Biochemistry*, vol. 102, no. 8, pp. 1558–1570, 2008.
[185] S. Gómez-Ruiz, G.N. Kaluđerović Ž . Žižak et al., “Anticancer drugs based on alkenyl and boryl substituted titanocene complexes,” *Journal of Organometallic Chemistry*, vol. 694, no. 13, pp. 1981–1987, 2009.
[186] G. N. Kaluđerović , V. Tayurskaya, R. Paschke, et al, “Synthesis, characterization and biological studies of alkenyl-substituted titanocene(IV) carboxylate complexes,” *Applied Organometallic Chemistry*, vol. 24, no. 9, pp. 656–662, 2010.
[187] O. Oberschmidt, A. R. Hanauske, C. Pampillon, N. J. Sweeney, K. Strohfeldt, M. Tacke. Anticancer Drugs18, 317 (2007).
[188] Kelter, G.; Sweeney, N. J.; Strohfeldt, K et al., In-vitro anti-tumor activity studies of bridged and unbridged benzyl-substituted titanocenes. Anti-Cancer Drug 2005, **16**(10), 1091-98.
[189] Claffey, J.; Gleeson, B.; Hogan, M.;et al., Fluorinated Derivatives of Titanocene Y: Synthesis and Cytotoxicity Studies Eur. J. Inorg. Chem. 2008, **2008**(26), 4074-82.
[190] Oberschmidt, O., Hanauske, A.R., Pampillon, C., et al., 2007. Antiproliferative activity of Titanocene Y against tumor colony-forming units. Anticancer Drugs 18, 317– 321.
[191] Beckhove, P., Oberschmidt, O., Hanauske, A.R., et al., 2007. Antitumor activity of Titanocene Y against freshly explanted human breast tumor cells and in xenografted MCF-7 tumors in mice. Anticancer Drugs 18, 311–315.
[192] Bannon, J.H., Fichtner, I., O’Neill, A., et al., 2007. Substituted titanocenes induce caspase-dependent apoptosis in human epidermoid carcinoma cells in vitro and exhibit antitumour activity *in vivo*. Br. J. Cancer 97, 1234–1241.
[193] Fichtner, I., Pampillon, C., Sweeney, N.J., et al., 2006. Anti-tumor activity of Titanocene Y in xenografted Caki-1 tumors in mice. Anticancer Drugs 17, 333–336
[194] V. Brabec, O. Nováková, Drug Resistance Updates 9 (2006) 111–122.
[195] A. Levina, A. Mitra, P.A. Lay, Metallomics 1 (2009) 458–470.
[196] E.S. Antonarakis, A. Emadi, Cancer Chemotherapy and Pharmacology 66 (2010) 1–9.
[197] C.G. Hartinger, S. Zorbas-Seifried, M.A. Jakupec, B. Kynast, H. Zorbas, B.K. Keppler, *J. Inorg. Biochem.* 100 (2006) 891–904
[198] C. G. Hartinger, M. A. Jakupec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P. J. Dyson and B. K. Keppler, Chem. Biodiversity, 2008, 5, 2140–2155.
[199] (a) Z. Guo, P. J. Sadler. Angew. Chem., Int. Ed.38, 1512 (1999); (b) T. Storr, K. H. Thompson, C. Orvig. Chem. Soc. Rev.35, 534 (2006).
[200] (a) E. Alessio, G. Mestroni, A. Bergamo, G. Sava. Curr. Top. Med. Chem.4, 1525 (2004); (b) E. Alessio, G. Mestroni, A. Bergamo, G. Sava. Metal Ions Biol. Sys.42, 323 (2004).

[201] J. M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J. H. Beijnen and J. H. M. Schellens, Clin. Cancer Res., 2004, 10, 3717–3727.
[202] W. H. Ang, P. J. Dyson. Eur. J. Inorg. Chem.4003 (2006).
[203] C. Scolaro, A. Bergamo, L. Brescacin, R. Delfino, M. Cocchietto, G. Laurenczy, T.J. Gledbach, G. Sava, P.J. Dyson, Journal of Medicinal Chemistry 48 (2005) 4161–4171.
[204] A. Bergamo, A. Masi, P.J. Dyson, G. Sava, International Journal of Oncology 33 (2008) 1281–1289.
[205] P. J. Dyson, Chimia, 2007, 61, 698–703.
[206] Bergamo A , Gaiddon C , Schellens JHM et. al., Approaching tumour therapy beyond platinum drugs Status of the art and perspectives of ruthenium drug candidates. *J. Inorg. Biochem.* **106** (2012) 90–99
[207] Teodori, E.; Dei, S.; Scapecchi, S.; Gualtieri, F. The medicinal chemistry of multidrug resistance (MDR) reversing drugs. *Farmaco* **2002**, *57*, 385-415.
[208] Hill, B. Drug resistance: An overview of the current state of the art. *Int. J. Oncol.* **1996**, *9*, 197-203.
[209] Lacombe, P. S.; Vicente, J. A. G.; Page`s, J. G.; Morselli, P. L. Causes and problems of nonresponse or poor response to drugs. *Drugs* **1996**, *51*, 552-570.
[210] Endicott, J. A.; Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Re*V. *Biochem.* **1989**, *58*, 137-171.
[211] Mitscher, L. A.; Pillai, S. P.; Gentry, E. J.; Shankel, D. M. Multiple drug resistance. *Med. Res. Re*V. **1999**, *19*, 477-496.
[212] Kessel, D.; Bottenrill, V.; Wodinsky, I. Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. *Cancer Res.* **1968**, *28*, 938-941.
[213] Aszalos, A.; Ross, D. D. Biochemical and clinical aspects of efflux pump related resistance to anti-cancer drugs. *Anticancer Res.* **1998**, *18*, 2937-2944.
[214] Renes, J.; De Vries, E. G. E.; Nienhuis, E. F.; Jansen, P. L. M.; Muller, M. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. *Br. J. Pharmacol.* **1999**, *126*, 681-688.
[215] Rappa, G.; Lorico, A.; Flavell, R. A.; Sartorelli, A. C. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. *Cancer Res.* **1997**, *57*, 5232-5237.
[216] Manciu, L.; Chang, X.-B.; Riordan, J. R.; Ruysschaert, J. M. Multidrug resistance protein MRP1 reconstituted into lipid vesicles: Secondary structure and nucleotide-induced tertiary structure changes. *Biochemistry* **2000**, *39*, 13026-13033
[217] Lee S., Peckermann I., Abinet E., et. al., The rare-earth yttrium complex [YR(mtbmp)(thf)] triggers apoptosis via the extrinsic pathway and overcomes multiple drug resistance in leukemic cells. Med Oncol (2012) 29:235–242.
[218] Lee S., Hille A., Kitanovic I., et. al., [FeIII(salophene)Cl], a potent iron salophene complex overcomes multiple drug resistance in lymphoma and leukemia cells. Leukemia Research 35 (2011) 387–393.
[219] Kater B., Hunold A., Schmalz H.G., et. al., Iron containing anti-tumoral agents: unexpected apoptosis-inducing activity of a ferrocene amino acid derivative. J Cancer Res Clin Oncol (2011) 137:639–649
[220] Kater L., Claffey J., Hogan M., et. al., The role of the intrinsic FAS pathway in Titanocene Y apoptosis: The mechanism of overcoming multiple drug resistance in malignant leukemia cells. Toxicology in Vitro 26 (2012) 119–124.
[221] Köster S.D., Alborzinia H., Can S., et. al., A spontaneous gold(I)-azide alkyne cycloaddition reaction yields gold-peptide bioconjugates which overcome cisplatin resistance in a p53-mutant cancer cell line. Chem. Sci., 2012, 3, 2062

[222] (b). Hunold A, Neundorf I, James P, NeudörX J, Schmalz HG (2009) Stereoselective synthesis of new ferrocene-derived amino acid building blocks. Eur J Org Chem 4429–4440
[223] Uderzo C, Conter V, Dini G, Locatelli F, Miniero R, Tamaro P (2001) Treatment of childhood acute lymphoblastic leukemia after the first relapse: curative strategies. Haematologica 86:1–7
[224] Krishna R, Mayer LD (2000) Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 11:265–283
[225] Inohara N, Ding L, Chen S, Nunez G (1997) Harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). EMBO J 16:1686–1694
[226] C. K. Mirabelli, R. K. Johnson, C. M. Sung, L. Faucette, K. Muirhead and S. T. Crooke, Cancer Res., 1985, 45, 32–39.
[227] C. F. Shaw III, Chem. Rev., 1999, 99, 2589–2600.
[228]. Adorno M., Cordenonsi M., Montagner M., et al., *A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis*. Cell, 2009. **137**(1): 87–98.
[229] Harlos M., Ott I., Gust R., et al., *Synthesis, biological activity, and structure-activity relationships for potent cytotoxic rhodium(III) polypyridyl complexes*. J. Med. Chem., 2008. **51**(13): 3924–33.
[230] Rubbiani R., Kitanovic I., Alborzinia H., et al., *Benzimidazol-2-ylidene gold(I) complexes are thioredoxin reductase inhibitors with multiple antitumor properties*. J. Med. Chem., 2010. **53**(24): 8608–18.
[231] Köster S.D., Alborzinia H., Can S., et al., *A spontaneous gold(i)-azide alkyne cycloaddition reaction yields gold-peptide bioconjugates which overcome cisplatin resistance in a p53-mutant cancer cell line*. Chem. Sci., 2012. **3**(6): 2062-72
[232] Bindoli A., Rigobello M.P., Scutari G., et al., *Thioredoxin reductase: A target for gold compounds acting as potential anticancer drugs*. Coord. Chem. Rev., 2009. **253**(11-12): 1692–1707.
[233] Bartsch W, Sponer G, Dietmann K, Fuchs G 1976 Acute toxicity of various solvents in the mouse and rat. LD50 of ethanol, diethylacetamide, dimethylformamide, dimethylsulfoxide, glycerine, N-methylpyrrolidone, polyethylene glycol 400, 1,2-propanediol and Tween 20. *Arzneimittelforschung 26:* 1581–3.
[234] Jaeschke H, Cover C, Bajt M L 2006 Role of caspases in acetaminophen-induced liver injury. *Life Sci 78:* 1670–6.
[235] Allard E., Passirani C., Garcion E., et al., *Lipid nanocapsules loaded with an organometallic tamoxifen derivative as a novel drug-carrier system for experimental malignant gliomas*. J. Control. Release., 2008. **130**(2): 146-53.
[236] Huynh N.T., Morille M., Bejaud J., et al., *Treatment of 9L gliosarcoma in rats by ferrociphenol-loaded lipid nanocapsules based on a passive targeting strategy via the EPR effect*. Pharm. Res., 2011. **28**(12): 3189-98
[237] Huynh N.T., Passirani C., Saulnier P. and Benoit JP., *Lipid nanocapsules: a new platform for nanomedicine*. Int. J. Pharm., 2009. **379**(2): 201-9.
[238] Lehmann B.D., Bauer J.A., Chen X., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies*., J. Clin. Invest., 2011. **121**(7): 2750-67.

[239] Lainé, A.L., Adriaenssens, E., Vessieres, A., et al. *The in vivo performance of ferrocenyl tamoxifen lipid nanocapsules in xenografted triple negative breast cancer*. Biomaterials., 2013. **34**(28): 6949-56.
[240] Togni, A., Hayashi, T., *Ferrocenes*, **1994**, Wiley, VCH publications.
[241] Savage, D., Gallagher, J. F., Ida, Y., Kenny, P. T. M., *Inorg. Chem. Comm.*, **2002**, *5*, 1034-1040.
[242] Davis, F. F. et al. In Peptide and Protein Drug Delivery (ed Lee, V. H. L.) 226–231 (Marcel Dekker, New York, 1990).
[243] Nucci, M. L., Schorr, R. & Abuchowski, A. The therapeutic value of polyethylene glycol modified protein. Adv. Drug Deliv. Rev. 6, 133–151 (1991).
[244] Lainé, A.L., Adriaenssens, E., Vessieres, A., et al. *The in vivo performance of ferrocenyl tamoxifen lipid nanocapsules in xenografted triple negative breast cancer*. Biomaterials., 2013. **34**(28): 6949-56.
[247] Aebi S, Kurdi-Haidar B, Gordon R, Cenni B, Zheng H, Fink D et al. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. Cancer Res 56: 3087–3090.
[248] Drummond JT, Anthoney A, Brown R, Modrich P. (1996). Cisplatin and adriamycin resistance are associated with MutLalpha and mismatch repair deficiency in an ovarian tumor cell line. J Biol Chem 271: 19645–19648.
[249] Brown R, Hirst GL, Gallagher WM, McIlwrath AJ, Margison GP, van der Zee AG et al. (1997). hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents. Oncogene 15: 45–52.
[250] Fink D, Aebi S, Howell SB. (1998). The role of DNA mismatch repair in drug resistance. Clin Cancer Res 4: 1–6.
[251] Gifford G, Paul J, Vasey PA, Kaye SB, Brown R. (2004). The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 10: 4420–4426.
[252] Kamal NS, Soria JC, Mendiboure J, Planchard D, Olaussen KA, Rousseau V et al. (2010). MutS homologue 2 and the long-term benefit of adjuvant chemotherapy in lung cancer. Clin Cancer Res 16: 1206–1215.
[253] Veronese, F.M., Pasut G., *PEGylation, successful approach to drug delivery*. Drug Discovery Today. **10**(21), 2005, 1451–58
[254] Demiryurek A.T., Cinel I., Kahraman S., et al., *Propofol and Intralipid interact with reactive oxygen species: a chemiluminescence study*. 1998. Br. J. Anaesth. **80**(5), 649-54
[255] Buriez O., Heldt J.M., Labb E., et al., *Reactivity and Antiproliferative Activity of Ferrocenyl–Tamoxifen Adducts with Cyclodextrins against Hormone-Independent Breast-Cancer Cell Lines*. Chem. Eur. J. 2008, **14**, 8195–8203
[256] Huynh NT, Passirani C, Saulnier P, Benoit JP. Lipid nanocapsules: a new platform for nanomedicine. Int J Pharm 2009;379:201-9.
[257] Cooke, M. S.; Loft, S.; Olinski, R.; Evans, M. D.; Bialkowski, K.; Wagner, J. R.; Dedon, P. C.; Moller, P.; Greenberg, M. M.; Cadet, J., Recommendations for Standardized Description of and Nomenclature Concerning Oxidatively Damaged Nucleobases in DNA. *Chemical Research in Toxicology* 2010, *23* (4), 705-707.
[258] (a) van Loon, B.; Markkanen, E.; Hubscher, U., Oxygen as a friend and enemy: How to combat the mutational potential of 8-oxo-guanine. *DNA Repair* 2010, *9* (6), 604-616; (b) Peoples, M. C.; Karnes, H. T., Recent developments in analytical methodology for 8-hydroxy-2 '-deoxyguanosine and related compounds. *J. Chromatogr. B* 2005, *827* (1), 5-15.

[259] Kirshner, J. R.; He, S. Q.; Balasubramanyam, V.; Kepros, J.; Yang, C. Y.; Zhang, M.; Du, Z. J.; Barsoum, J.; Bertin, J. *Elesclomol Induces Cancer Cell Apoptosis Through Oxidative Stress*. Mol. Cancer Ther. 2008, **7**, 2319−2327.
[260] Kater B., Hunold A., Schmalz H.G., et. al., Iron containing anti-tumoral agents: unexpected apoptosis-inducing activity of a ferrocene amino acid derivative. J Cancer Res Clin Oncol (2011) 137:639–649