Influence of Cisplatin and Vinblastine on Radiation-Induced Cellular Damage

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Abstract

It is widely accepted that the combination of radiotherapy and chemotherapy with different cytotoxic drugs can increase the effectiveness of cancer treatment. Despite many experimental and clinical attempts on combined treatment of cancer with chemotherapeutic drugs and radiation, there is still no general consensus for optimal dose and time sequence of radiation and drug administration. The main rationale for the combination of chemotherapeutic drugs and radiation is to enhance tumour control while limiting normal tissue toxicity.

In this study, Chinese hamster ovarian cancer cells (CHO-K1) were used as an in vitro model for assessing the effects of cisplatin and vinblastine on the toxicity of ionizing radiation. CHO-K1 cells were treated with neutrons and Co-60 γ-rays in the presence and absence of cisplatin and vinblastine, and the induced damage and cell survival using the micronuclei and colony forming assays, were determined respectively. Cisplatin uptake in the CHO-K1 cells was also assessed using particle induced X-ray emission (PIXE).

The drug concentrations corresponding to 30% cell survival (EC_{30}) were 1.88±0.56 μg/ml and 7.13±0.82 ng/ml for cisplatin (1 h exposure) and vinblastine (24 h exposure), respectively. These concentrations were then used in subsequent experiments to assess the influence of the drugs on cellular radiosensitivity. Cultures were irradiated immediately after drug addition for cisplatin and 16 h later for vinblastine as preliminary investigations resulted in no vinblastine toxicity for 2 h exposure. The radiation dose modifying factors for neutrons and photons treated with cisplatin were 1.08±0.04 and

0.71±0.09, respectively. The dose modifying factor for the γ-ray/vinblastine treatment was found to be 0.86±0.06. Cisplatin uptake was found to be concentration dependent, and the drug was exclusively localized in cellular nuclei.

The results show that cisplatin, at the EC_{30} level, has no influence on gamma ray and neutron toxicity. Similarly, at the same dose level, vinblastine does not increase of gamma ray toxicity. In general, cisplatin and vinblastine appear to protect CHO-K1 cells against the effects of γ-irradiation. Further studies involving the timing of irradiation and drug exposure, may shed more light on any distinct influence of these drugs on cellular sensitivity to different radiation modalities. Radiation appeared to inhibit cisplatin uptake when cultures were exposed to γ-rays immediately after drug treatment, indicating that drug uptake may be influenced by certain radiation-induced processes.

DECLARATION

I hereby declare that the work contained in this dissertation is my own work and has not been submitted for any degree or other qualification in any other university or institution.

Signed ..... Date August 17, 2002

Santswere Gaopalelwe Abell

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"Nobody should seek his own good, but the good of others"

1 Corinthians 10 v 24

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List of Abbreviations

µg	:	microgram
α-MEM	:	alpha minimum essential medium
BN	:	binucleation
BNC	:	binucleated cells
C	:	carbon
CHO	:	Chinese hamster ovary
Cis-Pt	:	cisplatin/cis-diamminedichloroplatinum (II)
cm	:	centimeter
cm^3	:	cubic centimeter
D-bar	:	mean inactivation dose
FBS	:	foetal bovine serum
Gy	:	gray
h	:	hour
H	:	hydrogen
LET	:	linear energy transfer
L-Q	:	linear-quadratic
MeV	:	mega electron volt
mg	:	milligram
min	:	minute
ml	:	milliliter
mm	:	millimeter

MN	:	micronuclei
MNF	:	micronuclei frequency
N	:	nitrogen
ng	:	nanogram
NMP	:	nuclear microprobe
O	:	oxygen
Ppm	:	parts per million
PIXE	:	particle induced X-ray emission
RBE	:	relative biological effectiveness
SD	:	standard deviation

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Chapter1

1. Introduction to the study and research problem

1.1. Radiotherapy

The field of radiation oncology involves the treatment of cancerous and neoplastic tissue with ionizing radiation. Depending on the type of tumour and clinical circumstances, radiation therapy may be used alone or in combination with other modalities such as surgery and chemotherapy. Radiation therapy either alone or in combination with surgery and/or chemotherapeutics has become an important aspect in the treatment of solid tumours (Peckham 1982).

In the 1950s it was demonstrated that lethally irradiated animals could recover if given fresh bone marrow cells (Lorenz *et al.*, 1951). This initiated the treatment of patients with end-stage leukemia using whole body irradiation and marrow infusion (Thomas and Ferrebee 1962). The management of acute leukemia with total body irradiation and bone marrow transplantation has demonstrated that radiotherapy has a useful systemic role, although its value is likely to be restricted to the minority of human tumours that are clinically radioresponsive (Blume *et al.*, 1980; Powles *et al.*, 1980; Keane *et al.*, 1981; Thomas et al., 1982).

Although ionizing radiation is of great interest, it is clear that the major role of radiotherapy will continue to be the control of primary and loco-regional disease. In essence, radiotherapy research is focused on identifying factors that determine tumour response in relation to normal tissue damage and to develop methods for overcoming the

problem of local treatment failure (Peckham 1982; Arriagada *et al.*, 1991; Cuzick *et al.*, 1994).

The basic aim of radiotherapy is to destroy all cells of a malignant tumour while keeping the damage to the surrounding normal tissues as low as possible. The detrimental effects of radiation exposure have typically been attributed to its ability to cause DNA damage (Obe *et al.*, 1992). In some cases, this objective can be reached by physical means (Steel *et al.*, 1983). Alternatively, metabolic targeting of radio-iodine to treat well-differentiated thyroid carcinoma using a radiolabelled monoclonal antibody demonstrates the potential value of the selective delivery of ionizing irradiation (Rao *et al.*, 1989; Howell *et al.*, 1991).

Radiotherapy may be delivered by external beam, brachytherapy or a combination of the two. External beam radiation therapy entails exposure to high energy particles at some distance from the patient, while brachytherapy involves the interstial implantation of radioactive sources near or within the tumour. Radiant energy is deposited in biologic material in a discrete yet random fashion, and the biologic effects occur as a result of the transfer of energy to atoms or molecules within the cell.

1.1.1. Radiation quality and radiotherapy

The basis of radiotherapy is the interaction of ionizing radiation (e.g. x-rays, γ-rays, neutrons and electrons) with tissue at the molecular level. This interaction depends on the energy of secondary charged particles induced by the incident radiation. These can break

chemical bonds and inflict cellular injury. The efficiency with which different types of ionizing radiation cause biological damage depends on the average energy lost along the path of the particles often referred to as the linear energy transfer (LET). It has also been shown that ionizing radiation with high-LET (e.g. α-particles, protons and neutrons) are generally more potent per unit dose than low LET radiation (e.g. x- and γ-rays) in inducing DNA double-strand breaks (dsb) (Prise *et al.*, 1990). This is because the DNA dsb are considered as the most critical molecular damage induced by ionizing radiation in mammalian cells (Radford, 1985; 1986). Due to the clustering of ionizations induced by high LET particles, the extent of dsb induced by such radiation is thought to be more severe and more difficult to restore by the repair mechanisms compared to those induced by low LET radiation (Ward, 1985, Goodhead, 1989).

Ionizing radiation deposits energy that injures or destroys cells in the treated volume (the target tissue) by damaging the genetic component (DNA) in the individual cells. The effectiveness of ionizing radiation in inducing biological damage that results in endpoints such cell death depends strongly on radiation quality which is a direct reflection of LET (Elkind 1991, Schwartz *et al.*, 1991, Lett, 1992). This is taken as indirect evidence that different radiation qualities do induce different types of dsb (Obe *et al.*, 1992).

Energy deposition may produce DNA damage either directly or indirectly. While the direct mode of damage induction involves the ionization of the DNA itself, products of water radiolysis react with the DNA molecules to cause damage in the case of indirect effects (Hall 1988). Both routes contribute significantly to DNA damage *in vivo*

(Chapman *et al.*, 1973). High LET radiation (e.g. neutrons and α-particles) predominantly cause damage through the direct effects. A high ionization density raises the likelihood for direct effects as energy deposition occurs in close proximity to the radiation track, and the resulting short ranged secondary electrons have a high probability of interacting with any biological target in the vicinity. Alternatively, radiation may interact with other molecules (particularly water) in the cell to produce free radicals that are able to diffuse through short ranges to damage critical targets within the nucleus. These radicals, atoms or molecules with unpaired electrons in the outer shell, are highly reactive and can interact with DNA molecules to cause bond breakage by electron displacement. This mode of action is predominant in the case of low-LET irradiation.

When cells are irradiated, a possible immediate response to any cellular DNA damage is the initiation of numerous biochemical pathways to repair the damage (Li *et al.*, 2001). Mammalian cells possess complex and efficient DNA repair processes capable of recognizing and repairing most genomic damage (Sancar and Sancar 1988). This is beneficial in the case of normal tissue but disadvantageous when the target tissue is cancerous, as efficient repair implies that the cells will regain their normal function. In the event of inadequate repair, two possibilities may arise. Firstly, the cells may accumulate high levels of damage to result in cell death or loss of reproductive integrity. This is most beneficial in tumour control, but can adversely affect organ function if significant volumes of normal tissue are involved. In the second scenario, inefficient repair may result in residual damage (mutations) (ICRP 1990). This may not affect tumor control but can lead to the transformation of normal cells to cancerous cells. In certain

cell types, such as resting lymphocytes, the persistence of DNA damage may lead to apoptotic cell death (Sellins and Cohen 1987, Payne *et al.*, 1992). The induction of apoptosis in response to DNA damage has been proposed to serves as a protective mechanism by eliminating genetically damaged cells (Kondo 1988). Factors of ionizing radiation that influence the level of biological damage are the quality, dose rate and the dose of radiation, with dose being the most significant for predicting the effect on tissue (Dainiak 2002). The biological response to radiation exposure may however not be evident immediately after exposure and may appear years later.

High-LET radiation is biologically much more affective than low-LET radiation such as γ- and X-rays (Prise *et al.*, 1998). The higher radiation-induced interaction probability in DNA fragments following high-LET irradiation has been attributed to the high ionization density (Schöllnberger *et al.*, 2002). The high toxicity of high-LET is thought to be a result of the elevated proportions of damage per unit dose that remain unrepaired compared to that of low-LET irradiation (Fertil *et al.*, 1982, Fertil *et al.*, 1984, Böhm *et al.*, 1992; Britten *et al.*, 1992; Slabbert *et al.*, 1996). High-LET radiation also seems to yield higher fractions of more long-lived DNA breaks compared with γ-rays (Ritter *et al.*, 1977; Weber and Flentje 1993). It has been suggested that such differences in reparability reflect a rise in the complexity of DNA damage with increasing ionization density, which is more deleterious to cells (Goodhead 1994, deLara *et al.*, 1995). Empirical clinical observations have also shown that high-LET irradiation is beneficial for the treatment of certain radioresistant tumours (Fertil *et al.*, 1984; Wambersie and Gueulette, 1984; Schmitt and Wambersie, 1990,). In an in vitro model using eight human tumour cell lines

with a wide spectrum of photon sensitivities, it was shown that p(66)/Be neutrons are on average 3.85 times more effective in inducing micronuclei when compared to ^{60}Co γ-rays (Akudugu *et al.*, 2003). This indicates a potential therapeutic gain for the neutrons and is consistent with earlier findings (Slabbert *et al.*, 1996).

The biological effectiveness of different types of radiation can be characterized by a parameter called the relative biological effectiveness (RBE) (Tubiana 1990). The relative biological effectiveness for a given test radiation, is calculated as the ratio of dose of a reference radiation, usually x rays, required to produce the same biological effect as was seen with a test dose, D_T, of another radiation. Many scientific investigations have been conducted to study the differing effectiveness of radiations under different experimental conditions. Analysis of the RBE is a useful way to compare and contrast the results observed in these studies. RBE is dose and endpoint dependent. In this study, the RBE is defined for a given endpoint as follows:

$$RBE = D_{ref}/D_{test} \qquad (1)$$

Where, D_{ref} and D_{test} are the reference and test doses, respectively.

1.2. Chemotherapy

Chemotherapy uses drugs to treat cancer cells (Calvagna 2003) and is sometimes the first choice for treating many cancers. It differs from surgery and radiotherapy in that it is almost always used as a systemic treatment and drugs travel throughout the whole body rather than being confined to diseased tissue. This is important because chemotherapy can reach cancer cells that may have metastasized or spread to other parts of the body.

Chemotherapy protocols strive to maximize the elimination of cancer cells while minimizing the negative effects that these drugs have on healthy tissues (Calvagna 2003). Chemotherapy drugs are divided into several groups based on how they affect specific chemical substances within cancer cells, cellular activities or processes, and phases of the cell cycle. Optimum dosage of chemotherapeutics can be difficult to determine since too low concentrations are expected to be ineffective against the tumour, and excessive doses may be highly toxic to the patient. The side effects of chemotherapy are due to the fact that cancer cells are not the only rapidly dividing cells. Blood cells and epithelial cells (mouth, intestinal tract, nose, nails, vagina, and hair) are also rapidly dividing and are adversely affected during chemotherapy. A wide range of chemotherapeutic drugs are currently in use in various combinations (Lagrange *et al.*, 1993; Long III *et al.*, 2005; von der Maase *et al.*, 2005). In this investigation, two drugs, namely cis-diamminedichloroplatinum (II) (commonly known as cisplatin) and vinblastine will be used.

1.2.1. Cisplatin

The use of cisplatin (cis-Pt) as an effective anti-cancer drug in chemotherapy has been documented (Loehrer *et al.*, 1984; Benedetti *et al.*, 2002). Several studies have suggested that the therapeutic effect of cisplatin is related to its ability to cross-link cellular DNA (Scherman *et al.*, 1985; Bruhn *et al.*, 1991; Gonzalez *et al.*, 2001). Although neutral in plasma, the platinum complex becomes aquated intracellularly to form a positively charged platinum species that interacts with nucleophilic sites of the cellular DNA (Zwelling *et al.*, 1979). This mechanism of anticancer activity which results in the

formation of platinum-DNA adducts through intercalation and DNA binding and is generally thought to be responsible for the inhibition DNA and RNA synthesis, inhibition of polymerase and the induction of apoptosis or programmed cell death (Barry *et al.*, 1990; Lepre *et al.*, 1990; Fisher 1994; Takahara *et al.*, 1995; Ormerod *et al.*, 1996; Schröder *et al.*, 1996; Schröder *et al.*, 1997).

1.2.2. Vinblastine

Vinblastine is an antimitotic drug and has been used for the treatment of various tumours (Rowinsky *et al.*, 1991; Beck *et al.*, 1997; Gidding *et al.*, 1999). It belongs to the group of medicines known as antineoplastic agents, commonly referred to as vinca alkaloids (Beck *et al.*, 1997). Vinca alkaloids destabilize polymerized tubulin by blocking the region involved in tubulin dimmer attachment, therefore, preventing polymerization of microtubules (Himes *et al.*, 1991; Jordan *et al.*, 1991). Vinblastine interferes with the growth of cancer cells by inhibiting mitosis (cell division) in metaphase. This is achieved by binding to tubulin and preventing the cell from making the spindles that are necessary for chromosome separation during cell division (Jordan *et al.*, 1991; Beck *et al.*, 1997). Vinca alkaloids also possess other biochemical effects such as inhibiting the synthesis of proteins and nucleic acids, and altering the lipid metabolism (Jordan *et al.*, 1991; Beck *et al.*, 1997).

1.3. Motivation for doing the study

Neutrons and other high-LET particles (e.g. α-particles and heavy ions like Ne, Ar and C), induce more severe biological damage than sparsely ionizing low-LET radiation (e.g. x- and γ-rays) for the same absorbed dose (Hall *et al.*, 1979; Spotheim-Maurizot *et al.*, 1990; Wambersie *et al.*, 1992). The therapeutic gain of neutrons when compared to conventional radiation (e.g. γ-rays) has been illustrated by the fact that the neutron-induced a much higher micronuclei frequency (MNF) per unit dose differs than that induced by photons. It has been found that high-LET neutrons are on average about 1.65 more toxic than low-LET radiation (Jones, 1982; Slabbert *et al.*, 1985; Tates *et al.*, 1989; Böhm *et al.*, 1990, 1992; Darroudi *et al.*, 1992; Huber *et al.*, 1994; Britten *et al.*, 1997; Heimers *et al.*, 1999; Akudugu *et al.*, 2003). Similar findings have been documented for peripheral blood lymphocytes irradiated with neutrons and α-particles (Chen *et al.*, 1984; Vral *et al.*, 1994; Greinert *et al.*, 1999).

The introduction of a neutron therapy facility at iThemba Laboratory for Accelerator Based Science (LABS) (Faure, South Africa), has made possible thorough analysis of the physical and biological characteristics of the p(66)/Be neutron beam. A general description of the neutron therapy facility, its physical characteristics, dosimetry and γ-contamination has been detailed (Jones *et al.*, 1988, 1992; Jones 1989). A basic requirement for the medical application of fast neutron beams is the determination of the RBE for clinically relevant effects. Relative biological effectiveness varies with the biological system and endpoint (early and late effects) and with the absorbed dose level (Hall *et al.*, 1979; Wambersie *et al.*, 1984).

The RBE for reproductive cell death and other biological endpoints has been shown to depend on ionization density, with maximum values significantly higher than unity for low-LET (Wulf *et al.*, 1985; Raju *et al.*, 1991; Stenerlöw, 1995).

Although neutron therapy has been shown to be an effective form of radiation therapy, more frequent normal tissue complications have been observed following treatment with d(15)/Be neutrons compared with megavoltage x-ray therapy (Scalliet, 1991). Elsewhere, a high complication rate for late reacting tissue has been reported for patients treated with a high-LET p(42)/Be neutron beam (Halpern *et al.*, 1990). For conventional low-LET radiation therapy, dose fractionation has the advantage that late reacting tissue is preferentially spared compared to early responding tissue. This is supported by the fact that slow growing normal tissues have lower α/β ratio (2 – 5) than acutely responding tissue (10 – 20) (Withers *et al.*, 1982; Halpern *et al.*, 1990). In neutron therapy, α/β ratios are approximately the same for both normal tissue and tumours from which the differential tissue sparing is lost (Withers *et al.*, 1982). Thus, complications often arise in late-responding tissue as the relative biological effectiveness (RBE) is underestimated with fractionation. It has been reported that the RBE for the central nervous system can be as high as 5.3 compared to 4.3 for early-reacting skin damage (Halnan and Hornsey 1981). Both these values are significantly higher than an RBE value of 3 usually assumed for normal tissue in neutron therapy (Slabbert *et al.*, 2000).

The combination of radiotherapy and chemotherapy using different cytotoxic drugs has been proposed as an alternative protocol to increase the effectiveness of the treatment of

different malignancies (Hill, 1991). The result of a combined modality depends on the mechanism of action of the drugs used. The main rationale in the combination of chemotherapy with radiation is to eliminate the population of tumour cells that may be relatively resistant to one of the modalities, assuming that the exposure of the cells to one cytotoxic agent reduces their ability to confront and resist an additional insult (Hill, 1991; Schilsky, 1992; Vokes, 1993). The tactic of damage induction by chemotherapy followed by irradiation proved very effective in achieving a response, but did not cause additive toxicity for patients with advanced regional disease (Blum *et al.*, 1986).

The combination of cisplatin and ionizing radiation may be a promising approach in the treatment of several malignant tumours, based on the suggestion that the drug is an effective radiosensitizer (Douple and Richmond, 1980; Nias, 1985, Dewit, 1987). In a number of studies a clear cisplatin-induced radiosensitization via repair perturbation has been described (Carde and Laval, 1987; Herman and Teicher, 1988; Korbelk and Teicher, 1989; Herman *et al.*, 1990; Pfeffer *et al.*, 1990; Van Rongen *et al.*, 1991; Yan and Durand, 1991; Nguyen *et al.*, 1993). An additive (individual interaction) effect has also been observed (Basham *et al.*, 1989). A synergistic (working together) cell killing effect of combined cisplatin and ionizing radiation treatment has been observed in both prokaryotic and eukaryotic systems, and this effect has been used in cancer therapy (Richmond and Powers, 1976; Skov and MacPhail, 1991; Raaphorst *et al.*, 1996). Some of the effects observed for this combination fitted the classification of a sub-additive (antagonistic or opposite interaction) response (Steel, 1993). Other studies have reported a cross-resistance (one agent causing ineffectiveness of the other) in several cisplatin-

resistance human tumour cell lines only to neutron but not to photon irradiation (Wallner and Li, 1987; Schwartz *et al.*, 1988).

Vinblastine has been used in cases of Hodgkin's disease in human (Harker *et al.*, 1993) and mystocytoma in dogs (Crow, 1977; Thamm *et al.*, 1999; Davies *et al.*, 2004). The potential myelosuppressive (reduction in the ability of the bone marrow to produce blood cells) effect of vinblastine is based on investigations of the mechanisms and kinetics of chemotherapy-induced neutropenia (Boggs *et al.*, 1963, 1996a, b). Also, joint administration of vinblastine and prenisolone has been reported to be effective in the treatment of canine mast cell tumours with only very moderate side effects (Thamm *et al.*, 1999, Davies *et al.*, 2004). On the other hand, it has been demonstrated that the treatment of Sticker's sarcoma with low dosages of vinblastine does not show evidence of toxicity (Waseoki and Mazur, 1977; Singh *et al.*, 1996). An in vitro study using a combination of γ-radiation and vinblastine showed only subtle effects (Sui and Fan, 2005). With the proposed combination of radiotherapy and chemotherapy as an alternative protocol in the management of cancer, it is expected that several drawbacks of radiotherapy and chemotherapy may be overcome: specifically, that the effect of chemotherapeutics are not generally tumour specific, the inability to target occult or overt distant metastases at presentation, cell resistances and/or normal tissue toxicity to both modalities.

1.4. Hypothesis

All types of radiation have biological effects, but some are more effective in producing biological damage per radiation dose than others. As stated earlier, high-LET radiation (e.g. α-particles and neutrons) are biologically more effective and toxic compared to low-LET radiation (e.g. x-rays and γ-rays). Neutrons are known to induce low levels of sublethal reparable damage than ^{60}Co γ-rays (Ward, 1985, Goodhead, 1989, Prise *et al.*, 1990).

Cisplatin inhibits DNA synthesis and causes cell death. Therefore, cisplatin should induce micronuclei as micronuclei formation reflects DNA loss and non-reparable damage. The micronucleus assay should be able to detect cisplatin-induced damage. Cisplatin is expected to cause low-reparable damage. It is expected that radiation will change the membrane characteristics of the cells and influence cisplatin uptake. Cisplatin is expected to produce very stable bifunctional adducts with DNA and induce different types of inter- and intra-strand cross-links responsible for inhibition of replication and transcription leading to cell death (Barry *et al.*, 1990; Lepre *et al.*, 1990; Fisher 1994; Takahara *et al.*, 1995; Ormerod *et al.*, 1996; Schröder *et al.*, 1996; Schröder *et al.*, 1997). Hence, less interaction is expected between cisplatin and neutrons when compared with conventional radiation.

Vinblastine inhibits cell division, destabilizes polymerized microtubulin and blocks polymerization of microtubules, and hence leads to cell inactivation via programmed cell death (Himes *et al.*, 1991; Jordan *et al.*, 1991). As the CHO-K1 cells are rapidly diving,

vinblastine alone will induce cell death. The addition of radiation is expected to further potentiate the induction of cell inactivation by vinblastine. Vinblastine suppresses the dynamic stability of microtubules by binding to the ends of the microtubules, thus inhibiting mitosis (Jordan *et al.*, 1991; Beck *et al.*, 1997). It should therefore be expected that the adverse effect of neutrons would be higher than those following gamma irradiation when the drug is presented.

1.5. Purpose of the study

Toxicity of chemotherapeutic agents to normal tissue causes mobility in the form of unpleasant side effects (Rosenoer and Curby, 1975). The normal tissue tolerance or toxicity to radiation and/or chemotherapy is a major factor which limits the efficacy of tumour control (Keifer *et al.*, 1990, Tramer *et al.*, 2001).

Chemotherapeutic drugs given in combination with ionizing radiation would increase the therapeutic effect above that achieved by single agent modality. The combination of cisplatin with ionization radiation is well established but may be further developed for the treatment of malignant tumours other than cervix. Cisplatin cytotoxicity depends on the drug concentration and the exposure time (Nias 1985; Dewit 1987; van Rongen *et al.*, 1991). However, the relative timing of the two modalities has not been studied in detail. Despite many experimental and clinical attempts to combine cisplatin and radiation, there is no general consensus on optimal doses and time sequence of radiation and drug administration (van Rongen *et al.*, 1991; Kallman, 1994; Nakamoto *et al.*, 1996). The promising clinical profile of vinblastine and its involvement in mitotic arrest by binding

to tubulin have promoted considerable interest in its combination with radiation therapy to a treat variety of solid tumours (Beck *et al.*, 1997; Cunningham *et al.*, 1998; Mikhak *et al.*, 1999; Kimmick *et al.*, 2002). However, the efficacy and the interaction between the vinca alkaloids and ionizing radiation have not been studied in sufficient detail (Grau *et al.*, 1994; Rajagopalan *et al.*, 2003; Zhang *et al.*, 2004).

It was therefore proposed to use different concentrations of cisplatin and vinblastine in combination with ionizing radiation (neutrons and γ-rays) in cultured CHO-K1 cells to examine the outcome of combined effects of each drug with irradiation. For the first part of the study, I will investigate the cytotoxicity of the drugs alone. The biological endpoints will be mitotic cell survival (colony assay) and micronucleus yield after blocking cytokinesis with cytochalasin-B. To test the efficacy of the combination of radiation and drug, early damage (micronuclei) and permanent mitotic damage (clonogenic survival) will be examined. Another aspect of the study will address the influence of ionizing radiation on cisplatin uptake. Nuclear microprobe analysis using particle induced X-ray emission (PIXE) techniques will be employed.

Chapter 2

2. Materials and methods

2.1 Cell line and maintaince

The Chinese hamster ovary (CHO-K1) used were from a stock culture of the Radiobiology Laboratory of iThemba LABS (Slabbert *et al.*, 1999; Ntuane 2000; Nkuna 2001; Sebeela 2003). The cells are easily maintained in cultures and have a doubling time of 11 h with a high plating efficiency of greater than 70%. These cells grow as monolayers and are suitable for cell damage and survival studies based on the formation of micronuclei (MN) and colony formation, respectively.

CHO-K1 cells previously frozen in liquid nitrogen were thawed and maintained as monolayers in T-25 culture flasks in complete culture medium at 37°C in an atmosphere of 5% CO_2. The medium consisted of Eagle's minimum essential medium (α-MEM) was supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Confluent cultures were trypsinized and sub-cultured for subsequent experiments. For drug and radiation toxicity studies, cells were plated and incubated for ~ 3 h to allow for cell attachment prior to use.

2.2. Cisplatin

Cisplatin or *cis*-diamminedichloroplatinum (II) ($Cl_2H_6N_2Pt$ or $Pt^{II}(NH_3)_2Cl_2$, MW = 300.1; Sigma, South Africa; structure in figure 2.1). Cisplatin has an initial half-life in plasma of 25 – 50 min. The stock solution of cisplatin, 0.5 mg/ml, is inactive when stored in a cool place below 25°C away from light.

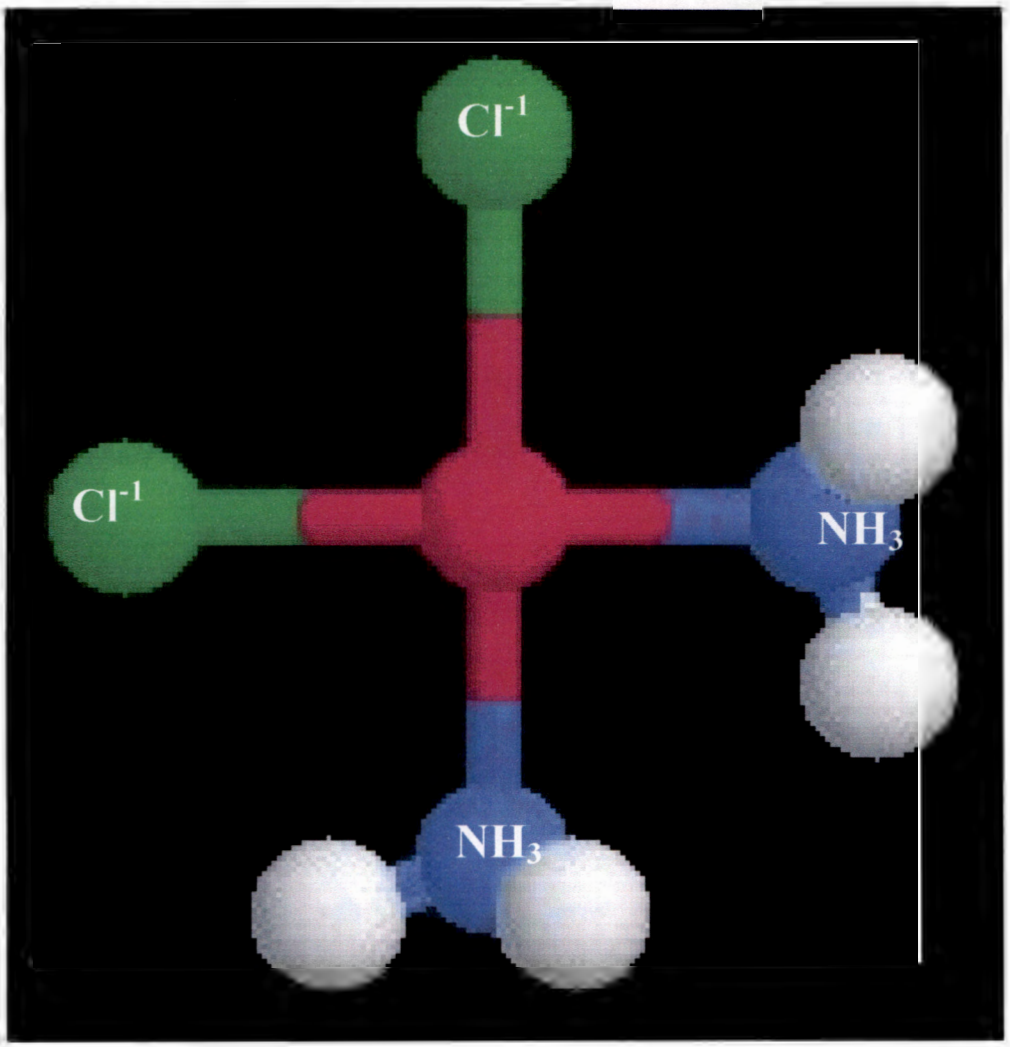


Figure 2.1. The chemical structure of cis-dichlorodiammineplatinum (II)

2.3. Vinblastine

Vinblastine ($C_{46}H_{58}N_4O_9$; MW = 810.974; Sigma, South Africa; structure in figure 2.2) is a vinca alkaloid antineoplastic agent, found in the Madagascar periwinkle, Catharanthus roseus. Vinblastine has a half-life in the bloodstream of 24 h. The stock solution of vinblastine, 1 mg/ml, is stored below 20°C.

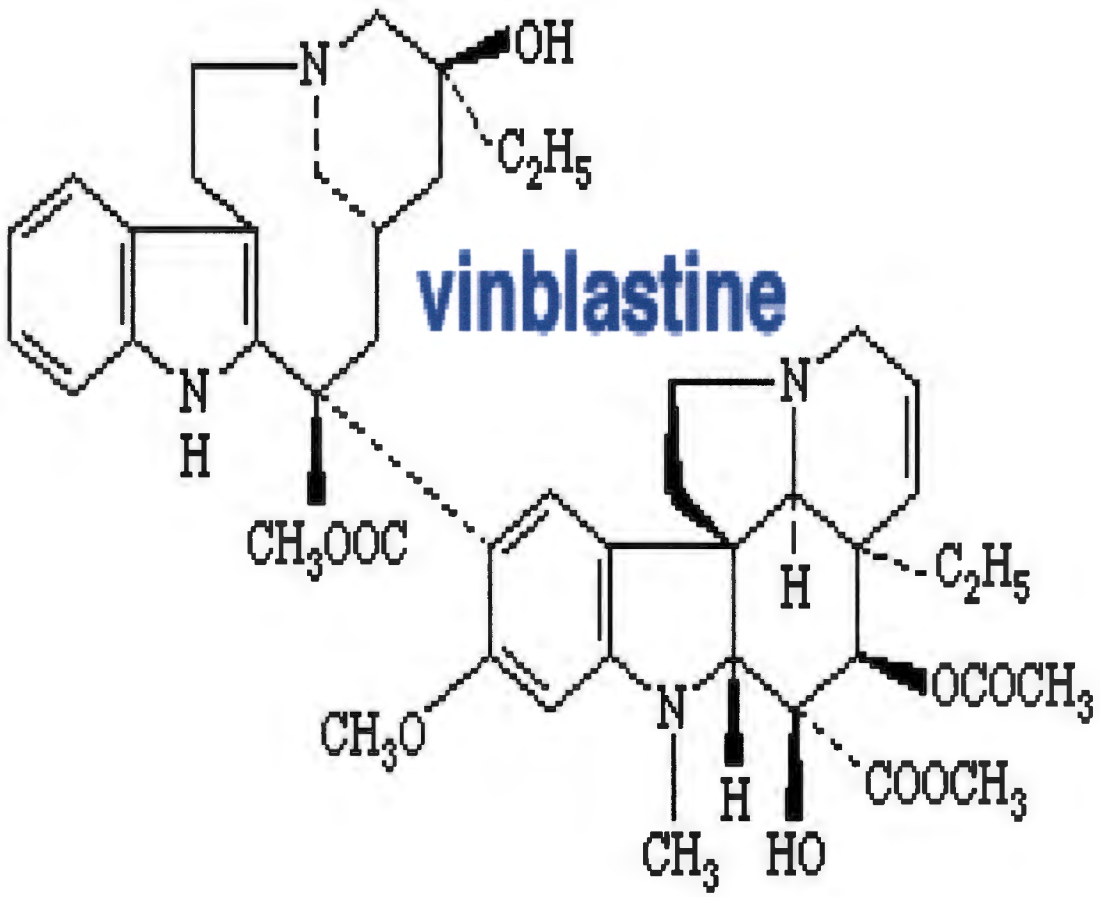


Figure 2.2. The chemical structure of Vinblastine

2.4. Irradiation of cultures

2.4.1. ^{60}Co γ-irradiation

Photon irradiation was from a ^{60}Co γ-ray source, orientated upwards in a vertical position, directed upwards. The build-up material was a 6 mm thick perspex table on which samples where placed in a 30 x 30 cm^2 field with a 50 mm thick backscatter perspex block placed directly above. Samples were given doses of 0 – 10 Gy were delivered to the samples at a dose rate of 0.3 Gy per min. Dose calibration was performed with a 0.6 cm^3 Farmer ionization chamber.

2.4.2. p(66)/Be neutron irradiation

Neutron exposures were conducted using a vertical beam directed downwards. Samples were placed on a 15 cm thick backscatter perspex block. Build-up material consisted of a 20 mm thick polyethylene. Under these conditions the γ-component of the beam was 6.9% and the total dose rate to the samples was 0.4 Gy per min (Jones et al., 1992). Cultures were irradiated to doses of 0-5 Gy.

2.5. Drug treatment

To establish optimum doses of cisplatin for subsequent experiments, newly plated cells were treated with 0 – 10 μg/ml doses of the drug for 30 and 60 min. For vinblastine experiments, the drug were administered at concentrations of 0 – 18 ng/ml for 2 or 24 h. After each drug exposure period, cultures were washed and re-incubated in fresh medium.

2.6. Micronucleus assay for cisplatin exposure

Exponentially growing cells were trypsinized into single-cell suspensions and plated (approximately 50 000-60 000 cells per dish) in 35 mm plastic petri dishes containing a 22 mm x 22 mm glass coverslips to a final medium volume of 2 ml. Cells were allowed to attach for 4 h before being treated with cisplatin (0 - 10 μg per ml). The cell cultures were then re-incubated for periods of 30 and 60 min.

Immediately after drug treatment, the cultures were washed and the growth medium was changed. The cultures with drug-free medium were treated with cytochalasin B, previously dissolved in dimethylsulphoxide, to a final concentration of 2 μg per ml and incubated for 24 h. Cytochalasin B is a drug that yields binucleated cells by permitting karyokinesis (the normal duplication of nuclei in cellular division) whilst inhibiting cytokinesis (normal cytoplasmic division) (Fenech and Morley 1985). Binucleated cells serve as a diagnostic tool to assess the impact of toxins after one cell division when loss of chromosome fragments (micronuclei) is being scored. Binucleation stage clearly underlines that micronucleus formation as early damage event. For the CHO-K1 cell line, the percentage of binucleated cells has been found to be greater than 50% in unirradiated cultures and shown to occur within 24 h (Sebeela 2003; Moruri 2005).

The cells were fixed with methanol:acetic acid (3:1 v/v) after discarding the growth medium and rinsing the coverslips with PBS. Samples were air dried for subsequent acridine orange staining. After staining the coverslips were mounted on glass microscope slides for fluorescence microscopy. Micronuclei scoring was restricted to binucleated

cells (see figure 2.1), according to criteria outlined elsewhere (Ono *et al.*, 1994). The level of micronuclei induction was found to be independent of exposure time (3.2). Therefore, subsequent cisplatin exposures lasted for 1 h.

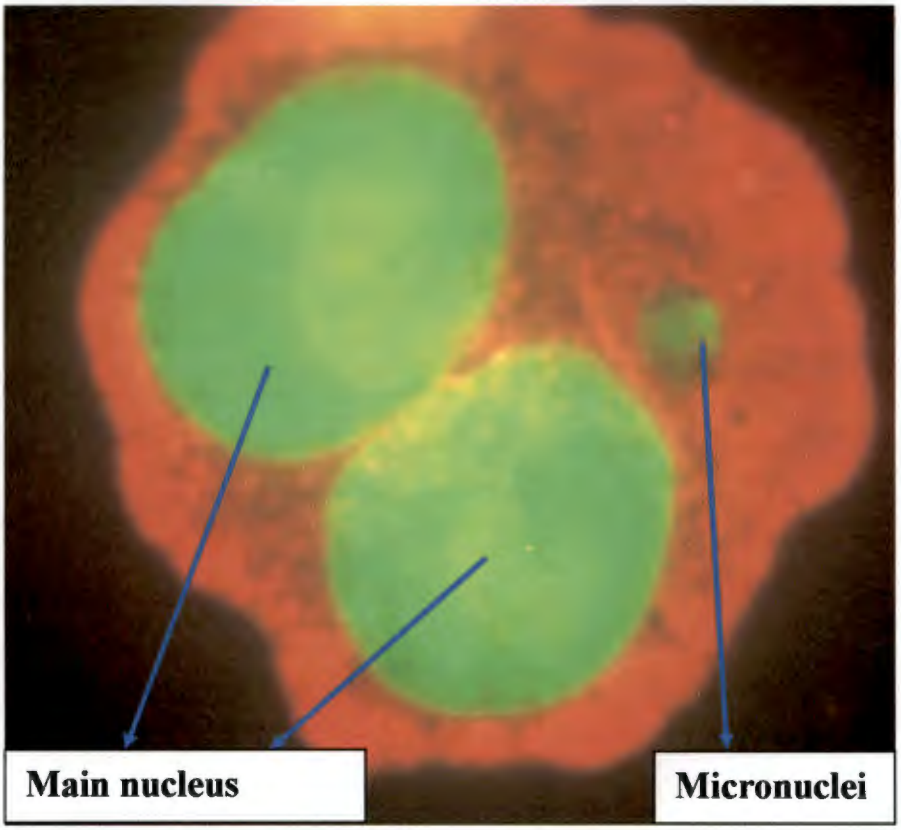


Figure 2.3. An example of a bimucleated cell with a micronucleus after cisplatin exposure.

2.7. Cell survival assay

Cell survival was assessed using the colony forming assay. Between 200- 20 000 cells were seeded in 60 mm petri dishes and allowed to attach of 4 h. Cultures were then exposed to either ^{60}Co γ-rays, p(66)/Be neutrons, cisplatin or vinblastine. The drug treated, irradiated and control cell cultures were then incubated for 7 days and colonies were fixed with methanol:acetic acid 1:1, v/v), stained with 0.01% Amido black in fixative, air-dried and counted. Colonies containing at least 50 cells were scored. Three independent experiments were performed for each treatment and the mean (±SD) surviving fraction was determined. Data for the radiation experiments were fitted to the linear-quadratic (L-Q) model of the form: $S = \exp(-\alpha D + \beta D^2)$ to generate survival curves. The mean inactivation doses (D-bar) were calculated as the areas under survival-dose response curves plotted on a linear-linear scale.

Drug toxicity data were fitted to a four parameter logistic equation of the form: where, the X-axis is the logarithm of concentration and Y the sigmoid response. Y starts at the bottom and goes to the top with a sigmoid shape. This is identical to the "four parameter logistic equation.

2.8. Drug-radiation interaction assay

Cisplatin and vinblastine were administered before radiation treatment. For cisplatin-radiation interaction, cells were treated with 0 – 10 μg/ml cisplatin and irradiation either with ^{60}Co γ-rays or neutrons was given immediately for both micronuclei and colony forming assays. In the case of vinblastine, cells were exposed to the drug for 24 h, but

were irradiated 16 h after drug administration. Preliminary experiments indicated that vinblastine exposure required over 2 h to inflict a measurable level of cytotoxicity.

2.9. Modifying factor

The dependence of dose-modifying factor on the doses of ionizing radiation and drugs concentration as well as the duration of its application was studied for CHO-K1 cells. The results obtained were described and interpreted by means of the mathematical model of linear regression in accordance with which the lower reparable is expected to result from the additional lethal damage arising from the interaction of sublesions induced by both agents.

2.10. Cellular uptake of cisplatin.

2.10.1. Cisplatin exposure for microanalysis

Thin Formvar films were prepared and used as support for cell culture. As the cells were cultured directly on these supports, the preparation of Formvar foils was carried under aseptic conditions. In order to facilitate cell attachment, the Formvar was precoated with an attachment factor, gelatin type B (Sigma). The specimen holders were placed into 35 mm plastic Petri dishes and covered with culture medium containing cells. The cultures were then incubated for ~ 4 h to allow the cells to attach to the Formvar film. The cultures were then incubated with cisplatin at concentrations of 100 and 200 μg/ml for 1 h. Some of these cell samples were irradiated to 2 Gy with γ-rays immediately after drug addition.

2.11.2. Sample preparation

After cisplatin exposure, the growth medium was removed and the samples were freeze dried using Leica CFD cryosorption freeze-dryer (figure 3.1 and 3.2) and then placed under vacuum in an irradiation chamber. A freeze-drying technique was performed to prevent elemental redistribution within cells as described previously (Przybylowicz *et al.*, 1999). Briefly, sample holders were dipped into propane cooled with liquid nitrogen, allowing cell cryofixation at -196° C. Preservation of cell structure integrity was checked using a scanning electron microscope. Microscopic examination enabled the selection of individual cells for elemental microanalysis and their morphological identification.

Figure 2.4. The Leica EM CFC-Cryo-workstation that is used for Plunge Freezing (Immersion Cryofixation)

Figure 2.5 Leica EM CFD cryostation freeze dryer.

(A) (B) (C)

Figure 2.6. The cell samples on a Formvar foil after freeze-drying and their micrographs (B and C, exposed at different cisplatin concentrations).

2.10.3. Nuclear Microprobe analysis

Microanalyses were performed using the Nuclear Microprobe at the Materials Research Group, (iThemba LABS). The features of this facility and its capabilities for biological sample analysis have been previously reported (Prozesky *et al.*, 1995; Przybylowicz *et al.*, 2004). A 3.0 MeV energy proton beam, focused to ~ 3 x 3 μm and a scan size of 128 x 128 pixels was used for the irradiation of cell samples mounted on a ladder which is controlled by stepper motors to accurately position the samples in the path of the proton beam. Simultaneous measurements of particle induced X-ray emission (PIXE) and proton backscattering (BS) were performed. Characteristic X-rays emitted were detected with a Si(Li) X-ray detector located 35 mm from the target at 135° shielded with a 125 μm thick Be filter to absorb scattered protons (Mesjasz-Przybylowicz *et al.*, 2002). Backscattered protons were detected using an annular Si surface barrier detector which is 100 mm thick positioned at 176° (Prozesky *et al.*, 1995; Przybylowicz *et al.*, 1998, 1999).

Data were collected in list-mode and processed using a PC version of GeoPIXE (Ryan *et al.*, 2000). This included the generation of true elemental maps using the dynamic analysis method refined after the experiment by using matrix composition matching of selected parts of scanned areas rather than using average matrix composition from the whole scanned area, the extraction of PIXE and BS spectra from selected regions of interest and fitting these PIXE spectra.

The matrix composition (C, H, O and N) matching selected parts of scanned areas and the aerial density were obtained from analysis of corresponding BS spectra using the RUMP

simulation package, with non-Rutherford cross-sections for isotopically natural C and O at a laboratory angle of 170° (Przybylowicz *et al.*, 2003). This involves the viewing of synthesized spectral overlays for experiments and modification of parameters until an adequate fit is achieved (Doolittle *et al.*, 1986). Simulation of BS spectra provided cellular and Formvar foil thickness (equation 3.1) and matrix elemental composition. Simulated parameters were used for correcting for the yields of the PIXE spectra which were initially fitted using cellulose ($C_6H_{10}O_5$) (Pineda *et al.*, 1988).

$$\text{Aerial density (mg/cm}^2) = \frac{\text{Thickness (/cm}^2)\left(\frac{\text{W(g)}}{\text{Natoms(mol)}}\right)}{\text{NA}\left(\frac{\text{atoms}}{\text{cm}^2}\right)} \times 10000 \qquad (2)$$

Where by

Thickness (/cm^2) – the thickness simulated by RUMP

W (g) – weight in grams

N atoms (mol) – the number of atoms

N_A (atoms/cm^2) – Avogadro's constant

2.11. Statistical analysis

The data for micronuclei frequency (MNF) and binucleation index were best fitted to non-linear regression of second order polynomial, while data for cell survival fractions were best fitted to the linear quadratic model of the form:

$$S = \exp(-\alpha D - \beta D^2), \qquad (3),$$

where S is the surviving fraction, D is the radiation dose in Gy, α is the linear coefficient (in Gy^{-1}) and β is the quadratic coefficient (in Gy^{-2}).

The cytotoxicity data of both drugs were best fitted the four parameter logistic equation in the form:

$$Y = B + \{(T - B)/(1 + 10^{(\log EC50 - X)*Hillslope})\} \quad (4),$$

where B is the bottom or minimum survival achievable and T is the maximum survival achievable. X is the logarithm of concentration. Y is the sigmoid dose-response; Y start at the bottom and goes to the top with a sigmoid shape.

Chapter 3

3. Results

3.1. Cisplatin toxicity in CHO-K1 cells

3.1.1. Binucleation

The proportion of binucleated cells (BNC) was found to decrease with increasing drug concentration for both exposure times (figure 3.1). For the 30 and 60 min treatments, the frequency of binucleated cells in the treated cultures did not show a significant difference. An overall reduction in cell proliferation to about 30% at 10 µg/ml was observed for both exposure periods (figure 3.1). Cisplatin treatment did not have a negative impact on the kinetics of the CHO-K1 cells as indicated by the significantly high numbers of binucleated cells that were scored for at all doses (Table 3.1 and 3.2), and

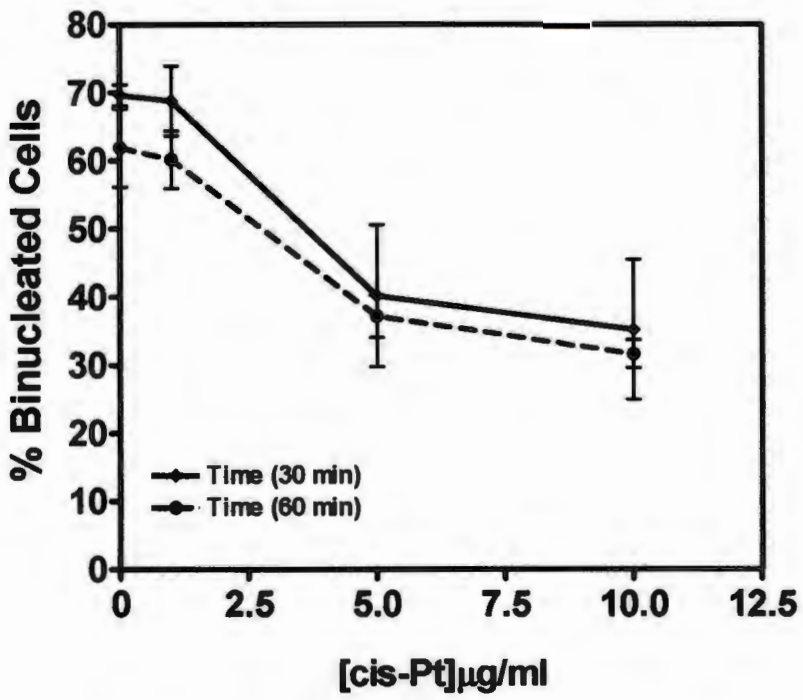


Figure 3.1. Percentage binucleation in CHO-K1 cell cultures treated with varying concentrations of cisplatin for 30 (dashed line) and 60 (solid line) min, respectively.

3.1.2. Micronucleation

The ability of cisplatin to induce damage in the form of micronuclei was assessed. The micronuclei frequency distribution corresponding to the binucleation discussed in the previous section are summarized in tables 3.1 and 3.2.

Table 3.1. Micronuclei frequency distribution in binucleated CHO-K1 cells following culture exposure to cisplatin (cis-Pt) for 30 min. Data pooled from three independent experiments.

Cis-Pt (µg/ml)	Total # of BNC scored	0 MN	1 MN	2 MN	Total # of MN
0	4985	4912	68	5	78
1.0	5970	5780	157	26	215
5.0	5304	4809	387	87	594
10.0	5288	4882	329	85	466

Table 3.2. Micronuclei frequency distribution in binucleated CHO-K1 cells following culture exposure to cisplatin (cis-Pt) for 60 min. Data pooled from three independent experiments.

cis-Pt (µg/ml)	Total # of BNC scored	0 MN	1 MN	2 MN	Total # of MN
0	5706	5623	73	10	93
1.0	4780	4626	129	23	181
5.0	3626	3361	216	53	367
10.0	2636	2441	144	39	266

The effect of the drug on CHO-K1 cells based on micronucleus formation was expressed in terms of micronucleus yield (derived from the data in tables 3.1 and 3.2) as a function of drug concentration and is illustrated in figure 3.2. Control values of micronuclei frequency for 30 and 60 min experiments were found to be 0.032 and 0.060 MN per BNC, respectively, indicating an insignificant inter-experimental variation in background micronuclei expression.

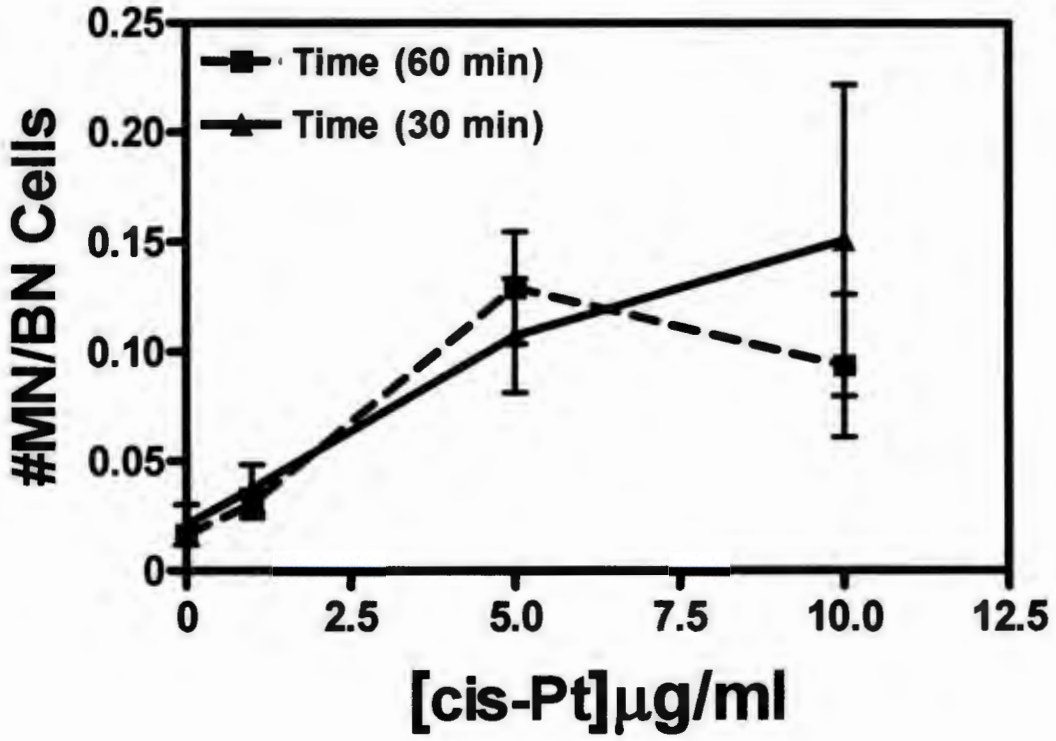


Figure 3.2. Dose-response curve of micronuclei frequency observed per binucleated CHO-K1 cells as a function of cisplatin concentration. Times of exposure were 30 (solid line) and 60 (dashed line) min.

Cisplatin exposure resulted in a dose-dependent increase in micronuclei frequency in cultures of CHO-K1 cells at all doses for 30-min exposure, and only up to 5 μg/ml for the 60-min treatment. Beyond this concentration, there was a reduction in MN yield.

3.2. Effects of cisplatin on CHO-K1 cell response to ^{60}Co γ-ray irradiation

3.2.1. Binucleation

Similar to the dose-response profiles observed for cisplatin treatment alone, (minimum binucleation of ~ 30% at 10 μg/ml), the binucleation index decreased continuously with increasing cisplatin concentration reaching a minimum value of ~16% at 10 μg/ml (figure 3.3).

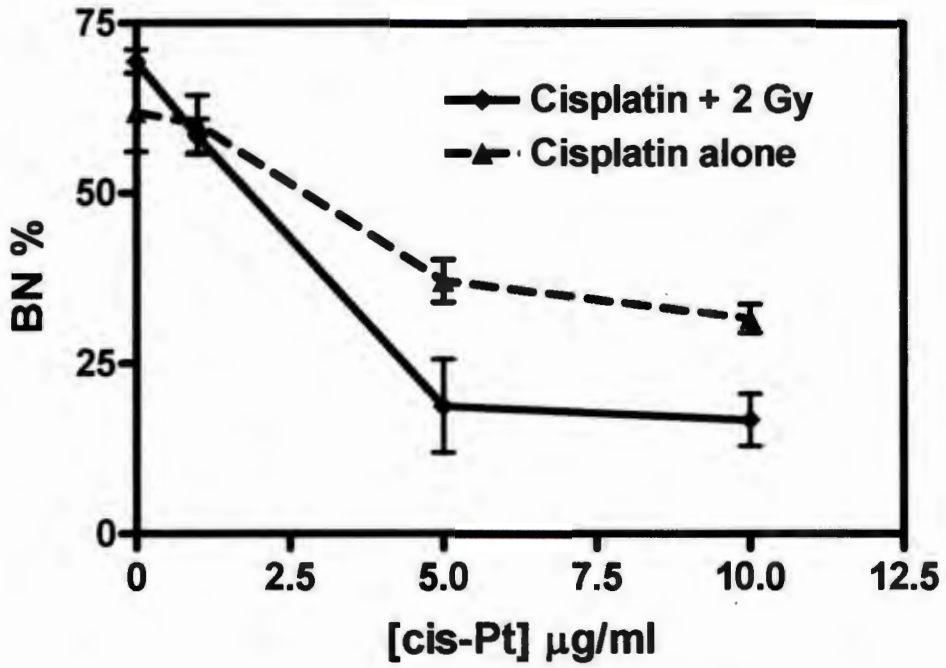


Figure 3.3. Percentage binucleation for CHO-K1 cell in cultures irradiation to 2 Gy ^{60}Co γ-rays as a function of cisplatin concentration (solid line). Cells were exposed to cisplatin for 60 min. Dashed line represents the response to cisplatin only (from figure 3.1 for comparison).

3.2.2. Micronucleation

The micronuclei frequencies resulting from the combined 1 h exposure of cells to increasing concentration of cisplatin and 2 Gy ^{60}Co γ-irradiation are presented in table 3.3. The background (no cis-Pt, no radiation) micronucleus yield was observed to be 0.07 MN per BNC.

Table 3.3. Micronuclei frequency distribution in binucleated CHO-K1 cells exposed to cisplatin for 60 min and 2 Gy γ-irradiation.

cis-Pt (μg/ml) + 2 Gy	Total # of BNC	0 MN	1 MN	2 MN	Total # of MN
0	5699	5196	461	42	545
1.0	7393	6952	608	41	693
5.0	2380	2239	250	45	364
10.0	2141	1951	242	46	355

The micronuclei frequency-dose response curve determined from the data in table 3.3 is illustrated in figure 3.4. As the concentration of cisplatin increases, the micronucleus rises until beyond 5 μg/ml when the micronucleus yield falls.

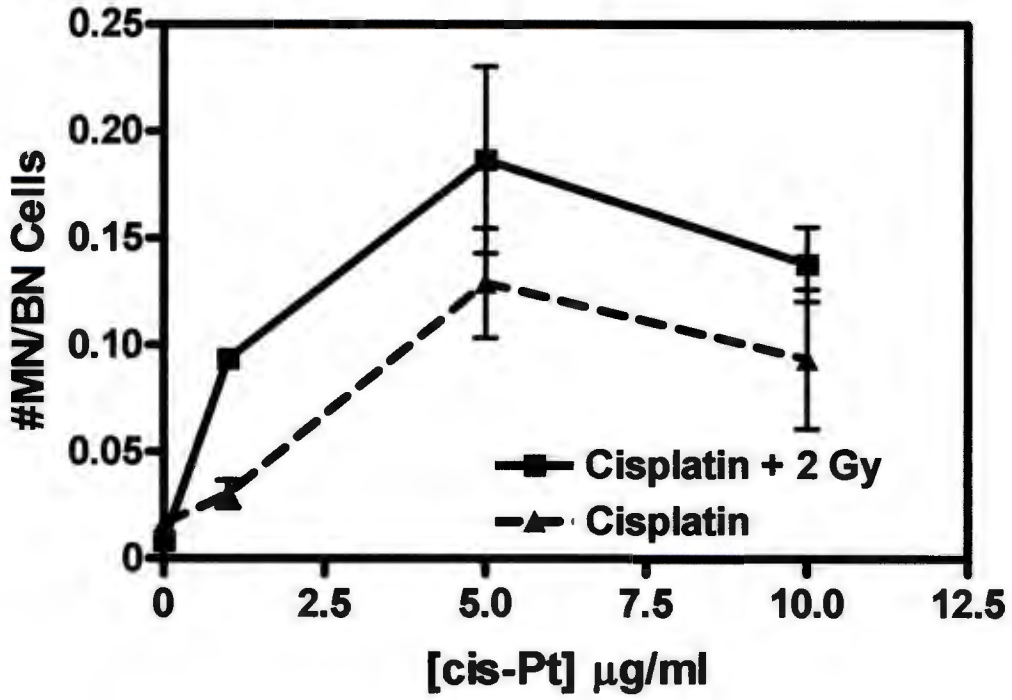


Figure 3.4. Dose-response curve of micronuclei frequency observed per binucleated CHO-K1 cell in cultures irradiated to 2 Gy with ^{60}Co γ-rays as a function of cisplatin concentration (solid curve). Cells were exposed to cisplatin for 60 min. Dashed curve represents the response to cisplatin only for (from figure 3.2 for comparison).

At 5 µg/ml of cisplatin, the MN yields with and without 2 Gy of γ-irradiation were 0.19 and 0.11 MN per BN cell, respectively. For exposure to 10 µg/ml of cisplatin alone and with 2 Gy, the corresponding MN yields were 0.14 and 0.09 MN per BNC, respectively.

3.3. CHO-K1 cell survival following cisplatin and radiation treatment.

The cytotoxicity of cisplatin was alone and in combination with γ-irradiation in CHO-K1 cells was assessed using the colony forming assay. Following exposure to varying concentrations of cisplatin, with and without 2 Gy of radiation, the surviving fractions were determined. The surviving fractions as a function of cisplatin concentration are plotted in figure 3.5. The drug concentrations corresponding to 30% cell survival (EC_{30}) were then established.

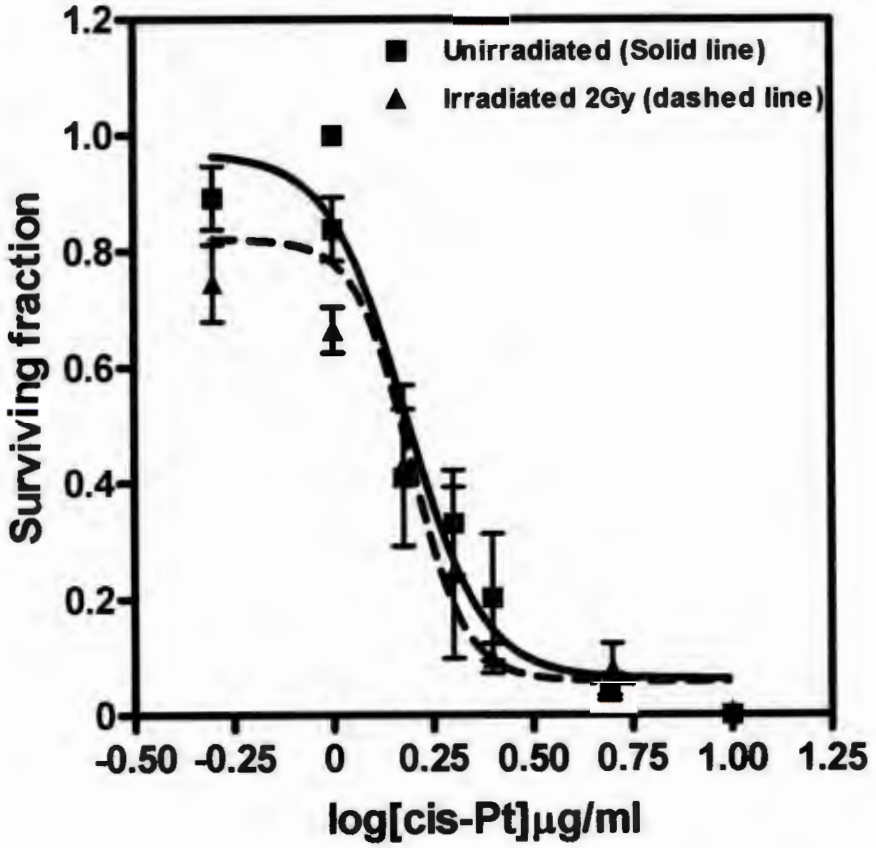


Figure 3.5. Cisplatin dose-response curve for CHO-K1 cells for 1 h exposure, with and without 2 Gy γ-irradiation.

The cytotoxicity of cisplatin was found to be dependent upon the cisplatin concentration. The mean (± SD) EC_{30} values for cisplatin treatment alone and when the cultures were also exposed to 2 Gy γ-rays found to be 1.88 ± 0.56 and 1.76 ± 0.36 μg/ml, respectively (figure 3.5). This difference was no significant. However, at cisplatin concentrations below 1μg/ml, a dose of 2 Gy of γ-irradiation produced a lower survival than cisplatin alone. For instance, at 0.6 μg/ml of cisplatin (i.e. at log[cis-Pt] = -0.22), the mean (± SD) surviving fractions for cisplatin alone was 0.94 ± 0.06 μg/ml and when 2 Gy of radiation were added, the survival was 0.80 ± 0.05 μg/ml, indicating a possible radiosensitization by cis-Pt.

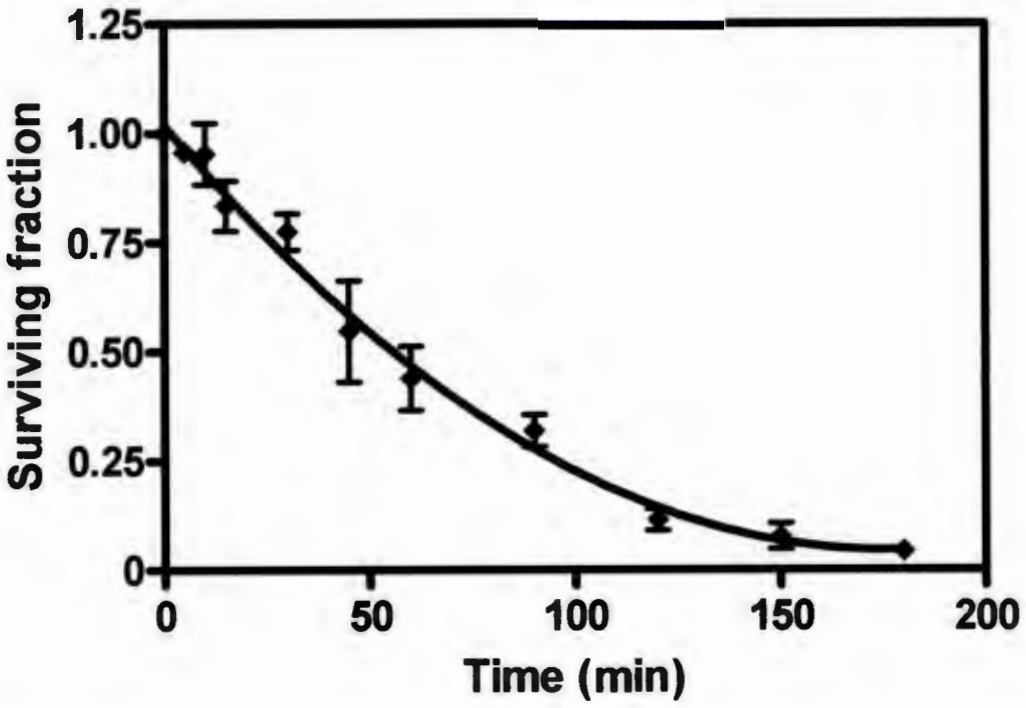


Figure 3.6. Surviving fraction of CHO-K1 cells treated with 1.88 μg/ml (EC_{30}) of cisplatin plotted as a function time.

The survival data of CHO-K1 cells when exposed to cisplatin over varying periods of time are plotted in figure 3.6. Survival was found to decrease exponential with exposure time. The surviving fractions for 60 and 180 min exposures were 30 and 5%, respectively. The high cell loss for the 180 min exposure indicates that the influence of added irradiation on cytotoxicity would be difficult to assess. Therefore, cultures were exposed to cisplatin for 1 h in subsequent radiation experiment.

3.4 Radiation modifying factor

The radiation dose-response curves for CHO-K1 cells with and without cisplatin at the level of EC30 are presented in figure 3.7. The mean inactivation doses for γ-irradiation alone and when cisplatin was present were found to be 4.38±0.65 and 3.08±0.04 Gy, respectively. The modifying factor emerged as 0.71±0.09.

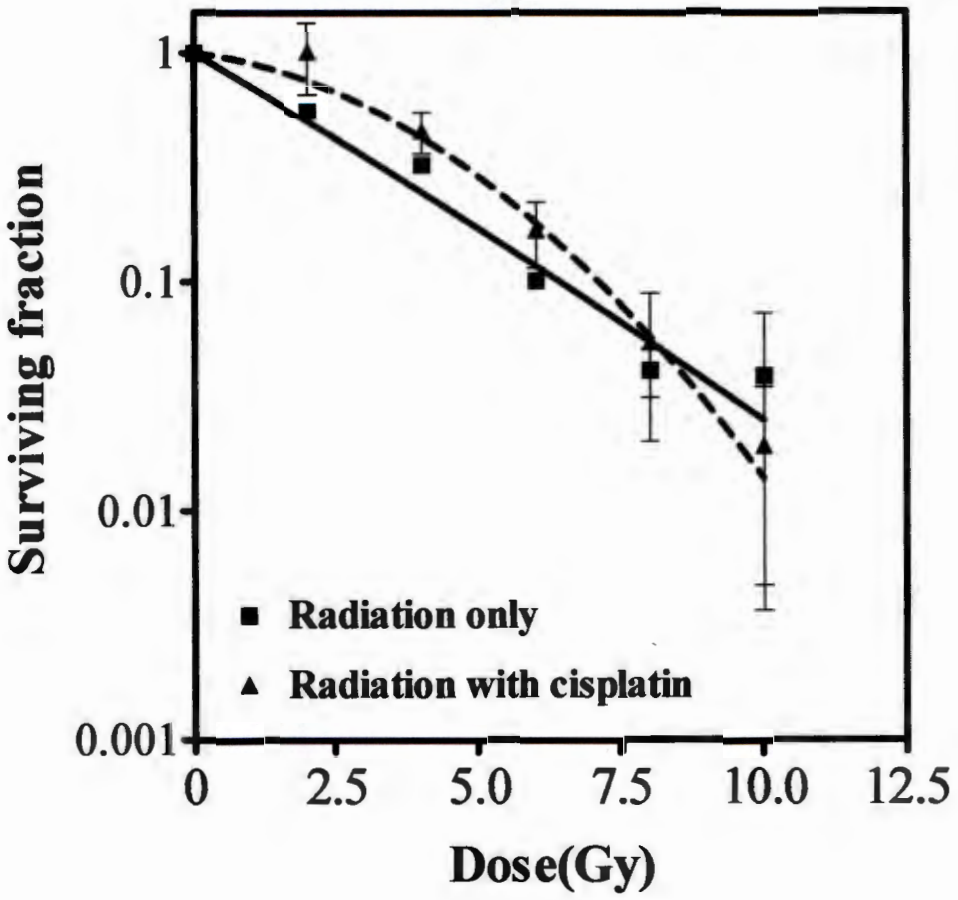


Figure 3.7. Dose response curves for CHO-K1 cells following γ-irradiation alone (solid line) and when cell irradiated in the presence of cisplatin (dashed line). Irradiation was performed immediately after drug administration.

To assess the role of radiation quality in cisplatin modification of the radiosensitivity, a similar study was performed using p(66/Be) neutrons. The survival-dose response to neutron irradiation following exposure to cisplatin is presented in figure 3.8. The mean inactivation doses for neutron irradiation alone and in the presence of cisplatin were 1.58±0.13 and 1.70±0.07 Gy, respectively. The modifying factor was 1.08±0.04.

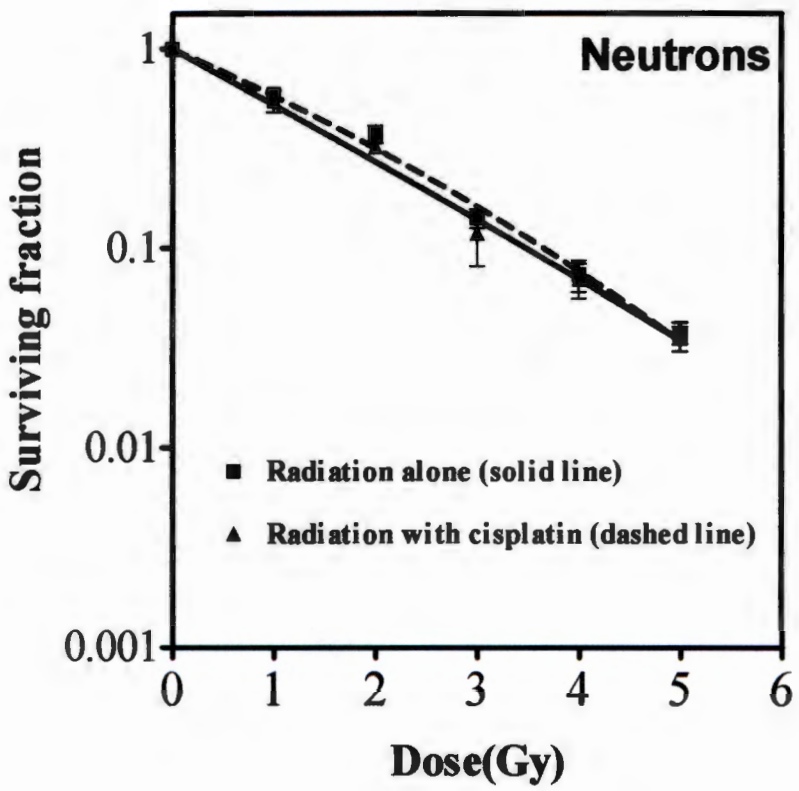


Figure 3.8. Dose response curves for CHO-K1 cells following neutron irradiation alone (dashed line) and when cells were irradiated in the presence of cisplatin (solid line) immediately after drug administration.

3.5. Vinblastine toxicity in CHO-K1 cells

The cytotoxicity of vinblastine in CHO-K1 cells was assessed using the colony forming assay. This set of experiments was performed to establish an optimum period for vinblastine exposure, as well as to determine the drug dose corresponding to 30% cell survival (EC_{30}). The survival-dose response curves are shown in figure 3.9. Vinblastine concentrations as high as 18 ng/ml did not result in a reduction of cell survival to below

80% when cultures were exposed to the drug for only 2 hours. The EC30 concentration for vinblastine in these cells following a 24 h exposure was found to be 7.13±0.82 ng/ml.

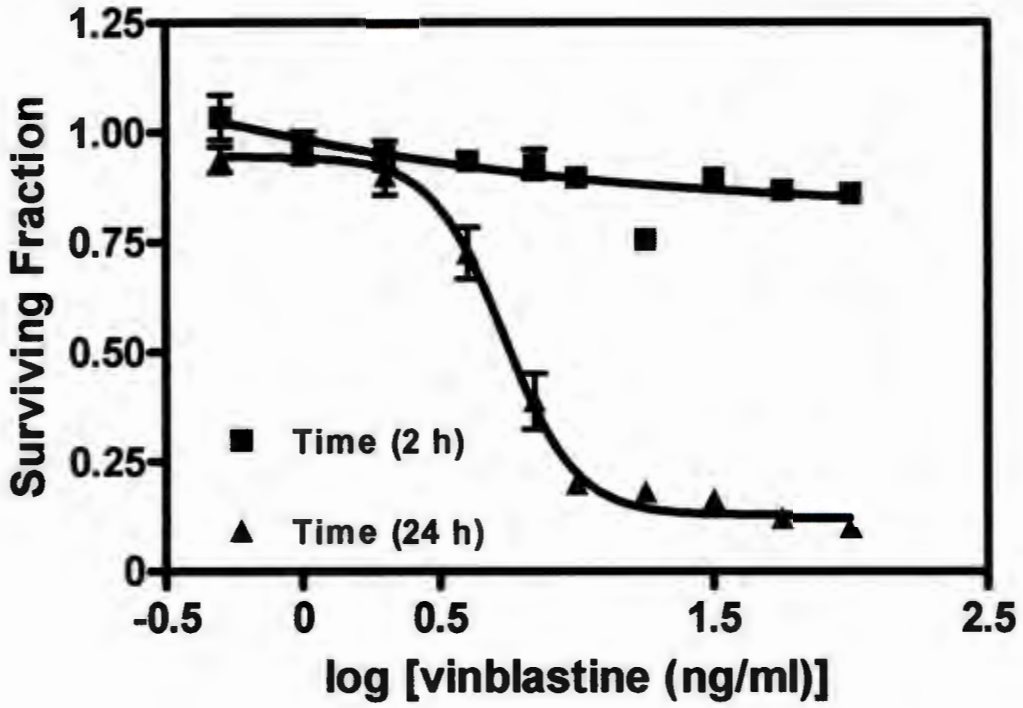


Figure 3.9. Dose response curve for CHO-K1 cells after 2 and 24 hours vinblastine exposure

To establish whether 24 h is optimum for vinblastine exposure and irradiation, cultures were treated with the EC30 concentration (7.13 ng/ml) for periods ranging from 0 – 24 h. The surviving fractions were plotted as a function of time as illustrated in figure 3.10.

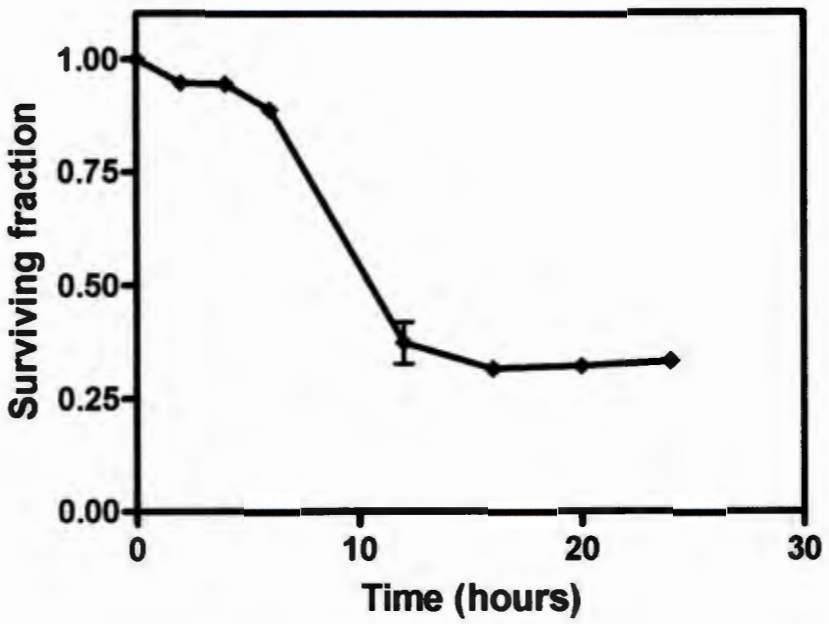


Figure 3.10. Surviving fraction of CHO-K1 cells treated with 7.13 ng/ml vinblastine concentration plotted as a function time (min).

It is shown that the toxicity of vinblastine increases exponentially with time and plateaus at 30% survival after 16 h of exposure. This indicates that for the EC30 concentration, exposure times beyond 16 h do not improve toxicity. A vinblastine concentration of 7.13 ng/ml and an exposure time of 16 h were used for subsequent experiments.

3.5.1. Gamma radiation modifying factor.

Figure 3.11 shows the cell survival curves when CHO-K1 cells were irradiated with γ-rays in the presence and absence of vinblastine. Vinblastine was administered to the cultures at EC30 (7.13 ng/ml) for 16 h prior to the irradiation.

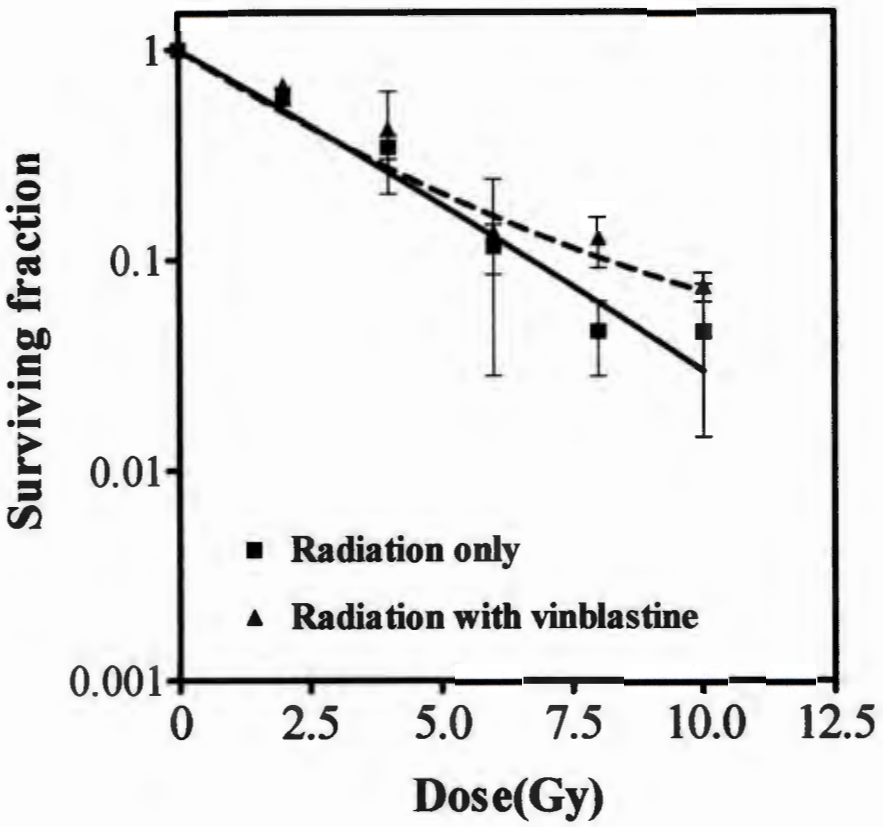


Figure 3.11 Cell survival curves for CHO-K1 cells when irradiated with γ-rays in the absence (solid line) and presence (dashed line) of vinblastine.

The mean inactivation doses for radiation alone and in the presence of vinblastine were 3.79±0.27 and 3.27±0.29 Gy, respectively. Based on the mean inactivation doses, the modifying factor was found to be 0.86±0.06.

3.6. Quantitative mapping of platinum and essential trace metals in cisplatin treated CHO-K1 cells

The aim of this study was to locate the cell nucleus in order to assess the drug spatial repartition between the cell nucleus and cytoplasm of CHO-K1 cells. Table 4.1 displays the PIXE elemental distributions in CHO-K1 cells exposed to different cisplatin concentration for 1 h with or without γ-irradiation. The respective drug concentrations in the incubation medium were 100 and 200 μg/ml.

The conditions of cell growth on Formvar foils supports were not modified in comparison to normal growth on plastic culture dishes. Figure 4.1 shows typical X-ray spectra from CHO-K1 cells samples after micro-PIXE analysis. Sodium (Na), aluminum (Al), phosphorus (P), chlorine (Cl), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), zinc (Zn), bromine (Br), and platinum (Pt) were detected in the samples. Elements were identified by their characteristic X-rays K-lines, except Pt by its L- and M-lines. Potassium is the major intracellular metal ion in living cells. The spatial distribution of this element depicts the cell boundaries, especially the nucleus (Fig 4.2).

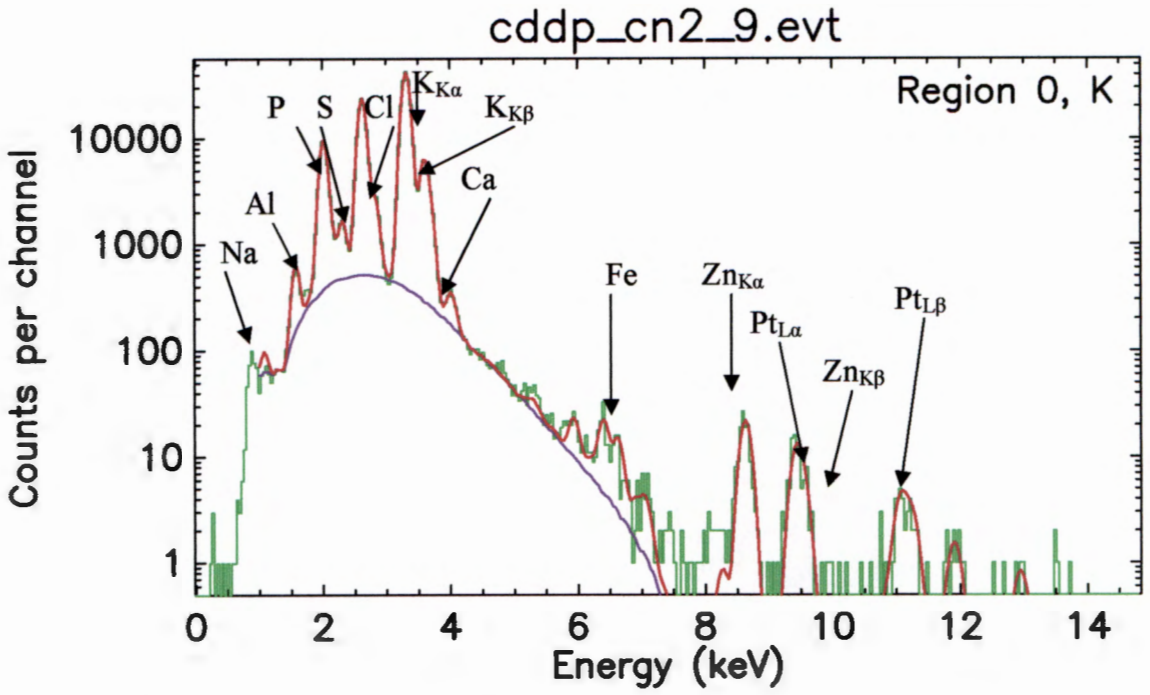
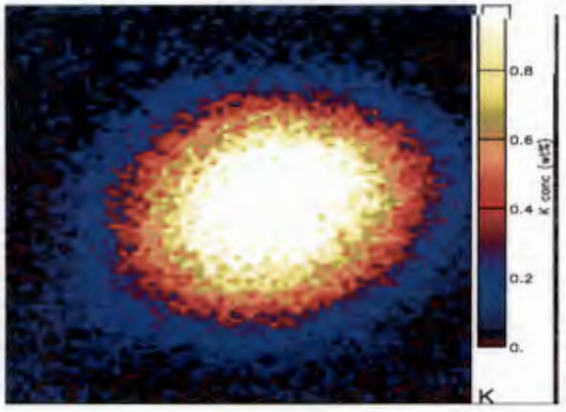
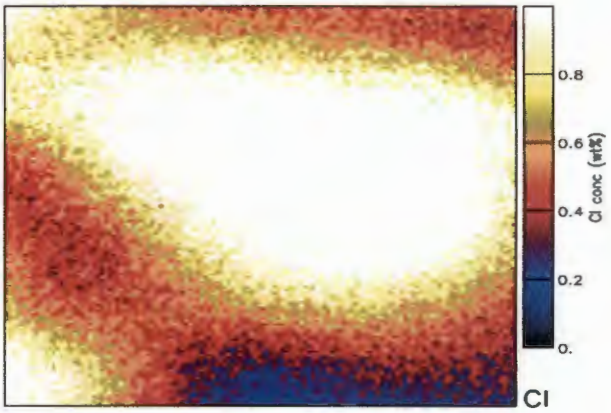


Figure 3.12. PIXE spectra from nuclear microprobe analysis of Chinese hamster ovarian cells (CHO-K1) incubated with 200 μg/ml of cisplatin, irradiated immediately to 2 Gy of γ-rays and incubated for 1 h.



A



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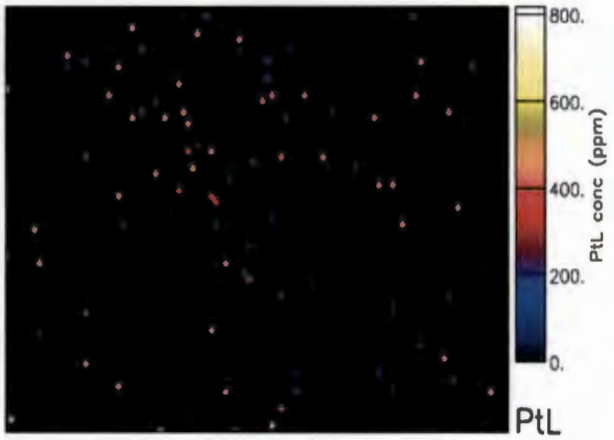

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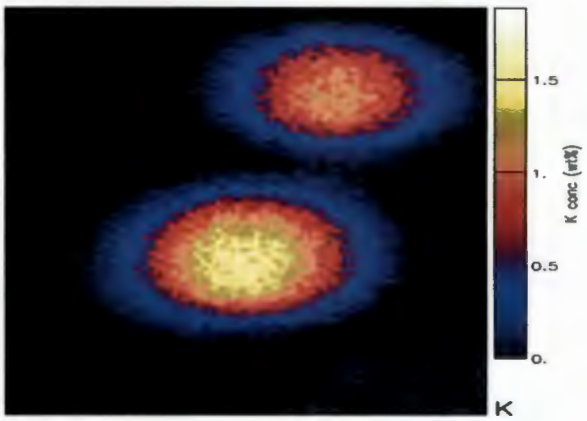

D

Figure 3.13. Qualitative elemental mappings of various elemental distributions within the CHO-K1 cells. A and D show the spatial distribution of potassium, B depicts chlorine distribution and C shows platinum distribution.

Table 3.4. Elemental distribution between different cell samples exposed to different cisplatin concentrations with or without 2 Gy γ-irradiation. A is a Formvar Foil, B cell samples treated with 100 μg/ml cis-Pt, C is cell samples treated with 100 μg/ml cis-Pt and 2 Gy γ-rays, D is cell samples treated with 200 μg/ml cis-Pt and E is cell samples treated with 200 μg/ml cis-Pt and 2 Gy γ-rays.

Concentration (ppm) ± Uncertainty (ppm) (Detection limits {99% confidence}											
File	**Si**	**P**	**S**	**Cl**	**K**	**Ca**	**Fe**	**Cu**	**Zn**	**Br**	**Pt L**
O	1405 ± 168 (263)	2032 ± 160 (104)	876 ± 47 (62)	35169 ± 278 (44)	2332 ± 58 (50)	581 ± 26 (32)	24 ± 8 (12)		<45 ± 18 (45)	<155 ± 63 (155)	<144 ± (144)
A	4693 ± 219 (207)	31281 ± 458 (82)	3536 ± 70 (49)	24765 ± 192 (35)	13116 ± 123 (24)	426 ± 25 (26)	125 ± 9 (6.9)	15 ± 6 (13)	197 ± 30 (16)	<55 ± 24 (55)	<52 ± 26 (52)
A	2853 ± 144 (209)	22309 ± 300 (82)	1788 ± 48 (50)	39470 ± 292 (35)	5262 ± 99 (24)	1786 ± 26 (26)	51 ± 7 (7.7)	<15 ± 6 (15)	166 ± 26 (19)	<66 ± 29 (66)	<62 ± 32 (62)
A	3790 ± 178 (147)	22301 ± 475 (58)	1754 ± 30 (35)	32418 ± 207 (25)	4568 ± 73 (17)	1183 ± 17 (19)	104 ± 7 (6.1)	13 ± 4 (8.3)	157 ± 14 (10)	70 ± 19 (36)	<34 ± 17 (104)
B	1247 ± 160 (266)	14305 ± 303 (104)	1820 ± 52 (62)	26078 ± 239 (44)	17032 ± 123 (30)	1290 ± 58 (33)	62 ± 10 (10)	<26 ± 10 (26)	124 ± 21 (32)	<111 ± 47 (111)	575 ± 78 (104)
B	470 ± 285 (276)	20738 ± 308 (103)	2150 ± 46 (60)	55496 ± 507 (41)	30269 ± 264 (27)	718 ± 41 (29)	35 ± 7 (9.8)	21 ± 7 (14)	118 ± 15 (18)	92 ± 32 (62)	582 ± 55 (57)
C	37 ± 14 (12)	850 ± 16 (4.6)	79 ± 3 (2.7)	783 ± 10 (1.9)	1326 ± 9 (1.2)	9 ± 3 (1.1)	1.5 ± 0.4 (0.5)	<1.2 ± 0.5 (1.2)	7 ± 1 (1.5)	<5 ± 2 (5.2)	18 ± 4 (4.7)
D	2915 ± 218 (234)	34791 ± 385 (94)	4579 ± 52 (57)	28842 ± 254 (26)	50187 ± 334 (29)	220 ± 29 (32)	72 ± 8 (8.8)	24 ± 6 (12)	218 ± 20 (14)	70 ± 24 (50)	404 ± 56 (47)
D	2910 ± 351 (303)	33975 ± 941 (120)	5093 ± 70 (73)	30267 ± 309 (53)	46470 ± 258 (36)	333 ± 42 (40)	87 ± 10 (11)	68 ± 12 (19)	224 ± 37 (24)	<83 ± 36 (83)	414 ± 59 (78)
D	3141 ± 573 (498)	52200 ± 1388 (193)	7754 ± 108 (113)	40263 ± 452 (80)	71119 ± 607 (53)	687 ± 54 (49)	91 ± 21 (16)	45 ± 20 (30)	309 ± 35 (38)	<131 ± 53 (131)	750 ± 138 (122)
E	<84 ± 168 (84)	15858 ± 332 (31)	1505 ± 22 (18)	26777 ± 235 (13)	23321 ± 182 (806)	328 ± 19 (9.6)	27 ± 3 (3.4)		103 ± 5 (3.2)	37 ± 8 (10)	190 ± 15 (9.5)
E	<151 ± 168 (84)	23784 ± 579 (57)	2328 ± 46 (33)	59401 ± 593 (23)	34675 ± 195 (15)	2925 ± 41 (17)	58 ± 5 (6.8)	14 ± 3 (5.4)	153 ± 9 (5.9)	85 ± 15 (20)	259 ± 21 (19)
E	431 ± 276 (267)	20186 ± 259 (100)	2116 ± 44 (58)	56403 ± 597 (40)	29844 ± 254 (26)	734 ± 40 (29)	35 ± 7 (9.5)	21 ± 7 (14)	117 ± 14 (17)	88 ± 31 (59)	570 ± 53 (55)

3.7. Cellular accumulation of cisplatin.

Backscattering (BS) spectrometry was carried out simultaneously for quantitative normalization of PIXE results (figure 4.3). After BS simulation, the matrix composition is compared with that of the assumed cellulose. This is done to determine accurately the matrix composition of the cell sample.

The major elements measured with this method (C, N and O) were representative of the organic mass of the cell samples (including the Formvar film). The accurate mass of the analyzed cells were calculated by subtracting the Formvar contribution. The average thickness, the aerial density and matrix composition of blank Formvar foils were measured and found to be 0.7 mg/cm^2, 0.09 mg/cm^2 and $C_{34.722}H_{62.5}O_{2.778}$, respectively.

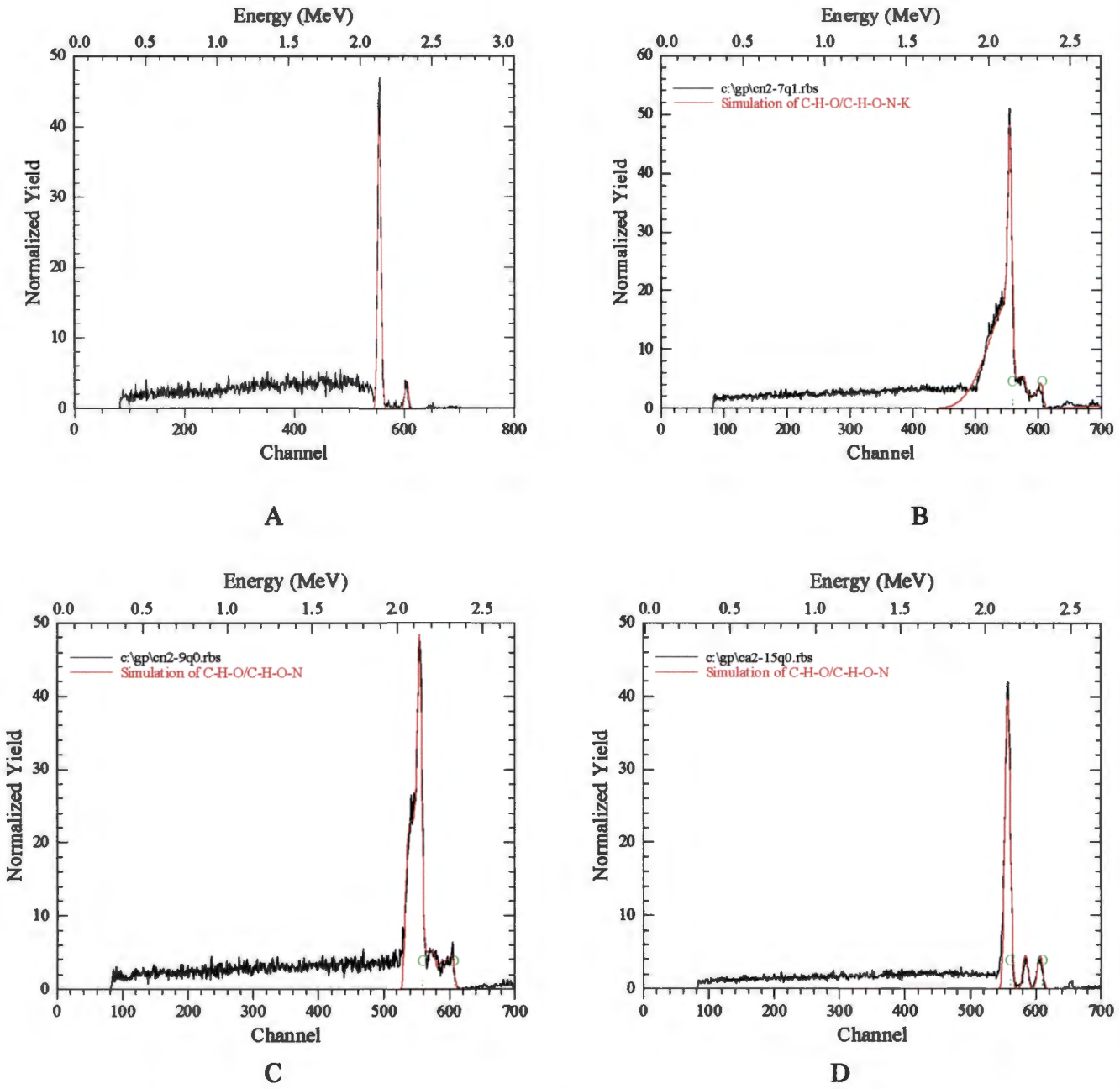

Figure 3.14. Backscattered spectra from nuclear microprobe analysis of CHO-K1 cells and Formvar foil. (A) Formvar thickness 0.7 mg/cm^2 and composition $C_{34.722}H_{62.5}O_{2.778}$, (B) cell thickness 3.2 mg/cm^2 and composition $C_7H_{110}O_1N_4K_{0.3}$, (C) cell thickness 1.7 mg/cm^2 and composition $C_4H_{40}O_{0.6}N_2$ and (D) cell thickness 0.9 mg/cm^2 and composition $C_{12}H_{40}O_{0.9}N_{3.5}$

Platinum accumulated less in irradiated cell samples when compared to the unirradiated samples (figure 3.15). The difference was significant for the sample treated with 100 µg/ml compared with 200 samples.

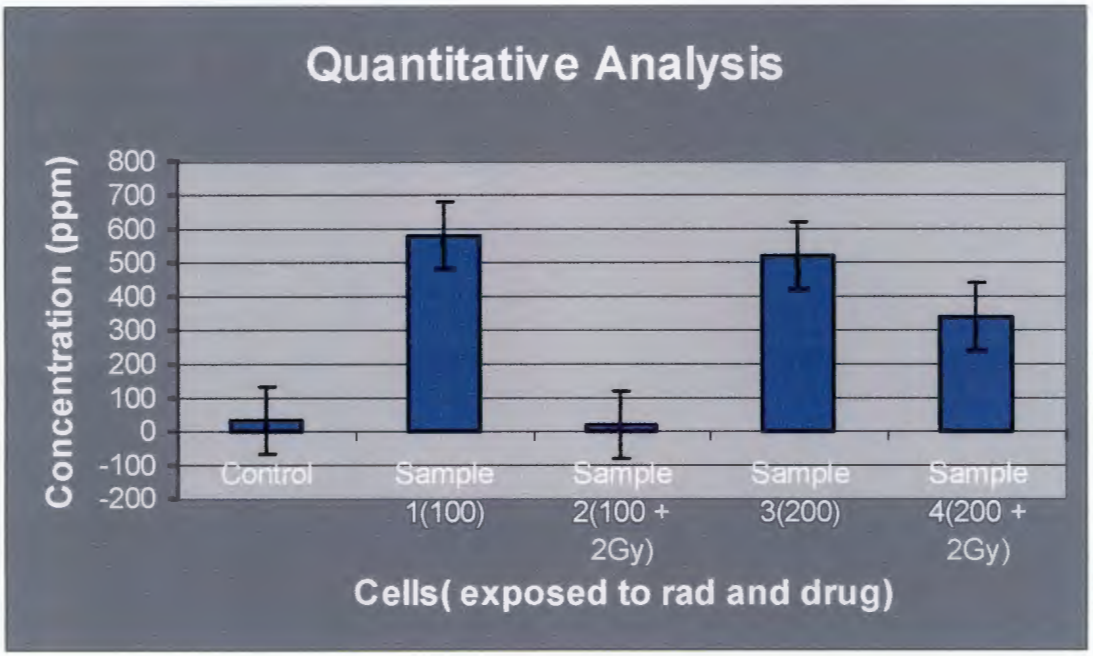

Figure 3.15. PIXE quantitative analysis for five samples CHO-K1 cells treated with cisplatin and γ-radiation as follows: control (no drug, 0 Gy), sample 1 (100 µg/ml, 0 Gy), sample 2 (100 µg/ml, 2 Gy), sample 3 (200 µg/ml, 0 Gy) and sample 4 (200 µg/ml, 2 Gy).

Chapter 4

4. Discussion

4.1. Cisplatin toxicity in CHO-K1 cells

4.1.1. Binucleation

The data in figure 3.1 show that cisplatin inhibits the proliferation of the CHO-K1 cells. These time intervals are based on the 1 h half-life of free cisplatin in culture medium is (Drobnik, 1981; Daley-Yates and MacBrien, 1984). It may be concluded that the reduction in proliferation was a direct indication of its cytotoxicity. Hence, the use of MN assay to assess cisplatin damage was feasible.

Cisplatin interacts with cellular DNA and this is the main molecular mechanism of its cytotoxicity. The majority (about 90%) of DNA lesions are intrastrand cross-links between the neighbouring DNA bases (Plooy *et al.*, 1984; Eastman, 1990; Vogel *et al.*, 1991). Searle and Bloom suggested that masses of nuclear material left in the equatorial plain during cell division are responsible for inhibition of cytokinesis and formation of binucleated cells (Searle and Bloom, 1979). This phenomenon has been thought to result in micronuclei induction when cells are exposed to cisplatin (Rodilla *et al.*, 1990, 1991; Rodilla, 1993). Although cisplatin has been shown to induce binucleation and micronuclei in CHO-K1 cells (Rodilla *et al.*, 1993), the levels of proliferation were extremely low and of the order of 50 binucleated cells per 1000 cells. Therefore, the cytokinesis-block MN assay is more superior in assessing cisplatin induced damage in cells that have undergone a single nuclear division. Since cell proliferation for cisplatin

exposure of 30 and 60 min was nearly identical, no significant difference was observed. Therefore, an exposure period of 60 min was choosing for subsequent experiments.

4.1.2. Micronucleation

The data in figure 3.2 represent the relationship between cisplatin-induced damage based on micronucleus formation in CHO-K1 cells and cisplatin concentration for 30 and 60 min drug exposures. In general, an increase in cis-Pt concentration resulted in a rise in micronucleus yield for time exposures. However, at concentration above 5 μg/ml for a 60 min exposure a reduction micronuclei frequency was observed. This is likely due to an increased toxicity corresponding to the longer exposure period. Hence, relatively fewer cells would be expected to survive the micronucleus burden for subsequent scoring as suggested elsewhere (Akudugu *et al.*, 2000). This observation is supported by the significant reduction in total binucleation as shown in tables 3.1 and 3.2.

4.2. Effects of cisplatin on CHO-K1 cell response to ^{60}Co γ-ray irradiation

4.2.1. Binucleation

In figure 3.3, the proportion of cells that have undergone one cell division following a 60 min exposure to varying concentration of cis-Pt with and without 2 Gy of γ-irradiation is plotted as a function cis-Pt concentration. The proliferative capacity of the CHO-K1 cells was found to decrease but reached a plateau at doses of 5 μg/ml and beyond. The binucleation indexes at the plateau stage for unirradiated and irradiated cultures were found to be 30 and 16%, respectively. The difference in binucleation of 14%, which represents the effect of radiation alone, is similar to that induce by 2 Gy of γ-irradiation in

the same cell culture in a previous study (Moruri, 2005). This is an indication that the effects of cisplatin and γ-irradiation on cell proliferation are independent and additive.

4.2.2. Micronucleation

Figure 3.4 illustrates the relationship between micronucleus yield and cisplatin concentrations with and without 2 Gy gamma irradiation. Micronucleus yield increased with increasing concentration of cisplatin to the maximum at about 5 μg/ml and then decreased at higher drug concentrations. This reduction in micronucleus yield may be due to the high cytotoxicity at these doses which result in lower proportions of cells surviving to become binucleated to express micronuclei. The differences of 0.08 and 0.05 MN per BNC in MN yield with and without irradiation for 5 and 10 μg/ml of cisplatin, respectively, compare well with a depression of 0.07 MN per BNC from 2 Gy γ-rays obtained previously (Moruri, 2005). This clearly indicates that the combined effect of the two modalities, based on early cellular damage, is additive by nature and this shows that the addition of γ-irradiation enhances cisplatin damage. This effect may be attributed to the fact that cisplatin induces micronuclei by interacting with cellular DNA (Rodilla *et al.*, 1990, 1991; Rodilla, 1993), which is the critical target for MN induction following radiation exposure (Akudugu *et al.*, 2003).

4.3. CHO-K1 cell survival following cisplatin and radiation treatment.

From the data in figure 3.5, doses of cisplatin below 1 μg/ml produced lower survival when combined with 2 Gy of γ-rays than the survival corresponding to 2 Gy alone. A concentration of 0.6 μg/ml (2 μM) of the drug decreases the cell survival at SF2 from 94

to 80%. This implies that a cisplatin dose lower than EC30 is radiosensitizing. It has been shown that a much lower cisplatin concentration of 0.1 μM is radioprotective at doses less than 2 Gy (Caney *et al*, 2004). This may be explained by the fact that Caney *et al.* exposed cultures to cisplatin only 1 h post-irradiation. In the study cells were exposed to radiation in the presence of cisplatin. It seems likely that the kinetics of cisplatin uptake may be different when the drug is present during irradiation, and this could affect the net response of cells to the two agents. Despite considerable effort in establish the optimal time sequences for cisplatin and radiation exposure, published findings have been inconclusive (Kallman, 1994; Lagrange *et al.*, 1993; van Rongen *et al.*, 1991; Nakamoto *et al.*, 1996).

To assess whether 1 h cisplatin exposure is optimal for clonogenic survival as previously demonstrated for MN formation, given that MN yield does not always correlate with cell survival (Akudugu *et al.*, 2003), cultures were exposed to 1.88 μg/ml of cisplatin for up to 180 min. This resulted in a decrease in cell survival from 30% (for 60-min exposure) to 5% (figure 3.6). Hence, the 60 min exposure was used in subsequent experiments since the 5% survival after 180 min is too narrow a window for adequate detection of any added effects of irradiation. These findings are in agreement with previous observations on the time-dependence of cisplatin cytotoxicity in other cell lines (Zwelling and Kohn, 1982; Wallner and Li, 1987; Vexler *et al.*, 1995).

4.4. Radiation modifying factor

From the data presented in figure 3.7, a modifying factor of 0.71±0.09 emerged indicating that cisplatin inhibits the CHO-K1 cell radiosensitivity. This modifying factor demonstrates a sub-additive response for combined cisplatin and radiation treatment, and agrees with experimental and clinical observations reported elsewhere (Basham *et al.*, 1989; Twentyman *et al.*, 1991, 1992; Britten *et al.*, 1993).

On the other hand, these data contrast with a number of studies in which a clear cisplatin-induced radiosensitization was observed (Pfeffer *et al.*, 1990; van Roogen *et al.*, 1991; Nguyen *et al.*, 1993; Kallman, 1994; Gorodetsky *et al.*, 1995; Nakamoto *et al.*, 1996). The discrepancy between the various studies may be attributed to differences in the biological systems examined or the conditions under which the cells were irradiated or exposed to the drug. Of critical importance to the outcome of each exposure are the duration of the drug exposure investigation, the modality of radiation given, and the timing of radiation following cisplatin treatment (Dewit, 1987; van Rongen *et al.*, 1991; Kallman, 1994).

The influence of radiation quality on cisplatin radiosensitization was assessed using p(66/Be) neutrons. The survival-dose response curves presented in figure 3.8 give a modifying of 1.08±0.04 for neutron irradiation. This indicates that the presence of cisplatin did not affect the response of the CHO-K1 cells to neutron exposure. This is consistent with the expectation that cisplatin would not enhance neutron damage because neutrons are known to induce low levels of sublethal and predominantly non-reparable

damage (Ward, 1985, Goodhead, 1989, Prise *et al.*, 1990). A very small effect of cisplatin on neutron sensitivity has been previously recorded (Britten *et al.*, 1992), but an enhanced sensitivity to neutrons by addition of cisplatin has also been reported (Lambin *et al.*, 1993; Kim *et al.*, 2000). These contradictions may be due to the experimental design and possibly exposure to cisplatin prior to irradiation. If cisplatin is added prior to irradiation cells may have to process the additional neutron damage and indeed show less survival than in the absence of cisplatin.

4.5. Vinblastine toxicity in CHO-K1 cells

Figure 3.9 shows the dependence of cell survival on vinblastine concentration for 2 and 24 h exposure. It is evident that vinblastine action is time-dependent and that a 24 h exposure is required to harness the toxicity of this drug. The EC30 was found to be 7.13±0.82 ng/ml, while the EC50 was 5.35±0.78 ng/ml. This EC50 is significantly higher than the 0.41 ng/ml reported for HeLa cells (Jordan et al., 1991) and for a variety of human prostate cell lines (Serafin *et al.*, 2001, 2002; Serafin and Böhm, 2005). This indicates that vinblastine toxicity is cell type-dependent. It is also possible that the differences in vinblastine toxicity in the two studies are due to the differing experimental designs.

4.5.1. Gamma radiation modifying factor for vinblastine.

Figure 3.11 shows the radiation dose survival curves for CHO-K1 cells in the absence or presence (EC30 concentration) of vinblastine. Based on the mean inactivation doses the modifying factor was found to be 0.86±0.06, suggesting radiationprotection and that

pretreatment with vinblastine may inhibit gamma sensitivity. This is consistent with previous reports showing that vinblastine counteract the toxicity of irradiation (Rajagopalan *et al.*, 2003; Sui *et al.*, 2005).

4.6. Cellular accumulation of cisplatin

Figure 3.15 represents the influence of radiation and cis-Pt concentration on the cellular uptake of the drug. A significant and concentration independent level of drug uptake was observed in unirradiated samples. No drug uptake was seen in cultures treated with a 100 μg/ml and 2 Gy of γ-irradiation. On the other hand, the uptake in samples treated to 200 μg/ml and irradiated was significant by not as high as that found in the drug only samples. The lack of cis-Pt uptake in the 100 μg/ml and radiation samples may be due to a number of factors including decreased platinum accumulation, increased DNA damage recognition and repair, and increased synthesis of biological sulfur-containing nucleophiles (Andrews and Howell, 1990; Muggia and Los, 1993; Perez *et al.*, 1990; Timer-Bosscha *et al.*, 1992). The apparent significant uptake seen in the samples exposed to 200 μg/ml and irradiated may be attributed to the high drug burden. A high drug burden may lead to a cis-Pt binding to nuclear DNA that is independent of radiation-induced damage recognition and repair. The individual cell analysis of CHO-K1 cells demonstrated a homogenous distribution of cisplatin within nuclei. This is in agreement with the fact that cisplatin binds to numerous nucleophiles in nuclei (Zwelling and Kohn, 1976; Bernges and Holler, 1991; Kelland *et al.*, 1992). Radiation-induced biochemical changes may be underlying factor for the decreased drug uptake. This finding suggests the existence, at least *in vitro*, of biological changes in irradiated cells leading to a

decreased drug accumulation. The reason for the high drug uptake in the irradiated cells exposed to 200 μg/ml is not clear, but it may be speculated that at certain high concentrations of cisplatin the inhibitive effect of radiation becomes insignificant. If radiation had an effect on cisplatin, its short fall was no detection of cisplatin at very low concentration. Radiation tended to inhibit cisplatin uptake, but only when high concentration where added.

Chapter 5

5. Conclusions and recommendations

The combination of chemotherapeutic drugs and ionizing radiation was expected to increase radiotoxicity. It was also hypothesized that the influence of drugs on neutron cytotoxicity will be less when compared to their influence on the toxicity of conventional radiation, since neutrons are high-LET and induce low levels of reparable damage.

The cytotoxicity of the two drugs was investigated to establish optimum concentrations and exposure times for combination with ionizing radiation. In CHO-K1 cells, the cisplatin treatment was found to inhibit gamma radiotoxicity by a factor of 30% and hence display a radioprotective effect. In the case of neutron irradiation and cisplatin treatment, no effect on radiotoxicity could be demonstrated and thus is consistent with the hypothesis. Similar findings were observed for vinblastine and gamma radiation contrasting the hypothesis. No clear cut results were obtained for the combination of vinblastine and neutron radiation. These findings led to the following conclusions:

1. The influence of the two drugs on radiotoxicity is dependent on the time of drug administration in relation to irradiation and the duration of drug exposure. The combined effect of the drug treatment and ionizing radiation with reference to the time sequence of the treatments as well as to the duration of the drug response should be further investigated.

2. The use of cell lines with different doubling time and p53 studies might also add some useful information. A faster doubling cell line might be more sensitized by chemotherapeutics than its slow dividing counterpart. P53 is involved in a variety of cellular mechanisms including DNA repair, cell cycle blocks and apoptosis and would be expected to influence the effects of chemotherapeutic drugs on radiosensitivity.

3. The foregoing results demonstrate that these factors are crucial for optimum use of ionizing radiation and chemotherapeutic drugs to achieve a toxicity enhancement. The distinction between additive and synergistic effects also needs further analysis.

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