THE EFFECT OF METAL BASED COMPLEXES ON THE SURVIVAL OF AEROBIC AND HYPOXIC CHINESE HAMSTER OVARY CELLS, *IN VITRO*.

by

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In dedication to my husband Paolo, my parents Smilie and Uvan Uys and in loving memory of my sister Sonja Uys.

SUMMARY

It is well established that many solid tumours are heterogeneous with respect to oxygenation, and contain regions of hypoxic cells, which due to their inherent resistance to ionizing radiation, limit the success of radiotherapy. Numerous chemicals and drugs have been investigated over the past few decades as potential radiosensitizers. The most notable of these being the organometallic compound, *cis*-diammine dichloroplatinum(II). The clinical success of this drug led to the synthesis of other types of organic cytotoxic metal–containing drugs. Prof. J.C. Swart from the University of the Orange Free State supplied seventeen novel iridium, ferrecenium and rhodium complexes, which I screened for cytotoxic activity against the CHO cell line.

The two most cytotoxic complexes namely, [Rh(fctca)(cod)] and [Rh(fctfa)(cod)], were tested for radiosensitizing potential against aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam by the MTT assay adapted to our laboratory conditions. The ferrocene betadiketones co-ordinated to them, Hfctca and Hfctfa and the Ir compliment of [Rh(fctfa)(cod)] namely, [Ir(fctfa)(cod)] were also assessed by the MTT assay. Interestingly, neither the ferrocene nor the iridium complexes showed noteworthy sensitization, which suggests that the rhodium is responsible for the efficacy observed. The radiosensitizing potential of the most active complex, [Rh(fctfa)(cod)] and cisplatin were also confirmed by the use of the more traditional clonogenic assay. Not only did the MTT assay deliver results comparable to the clonogenic technique, but one of the complexes [Rh(fctfa)(cod)] showed radiosensitizing potential against hypoxic CHO cells, equal to that of cisplatin.

The rhodium complex, [Rh(fctfa)(cod)] was also tested for radiosensitization properties against the CHO cell line in the presence of a

p(66)/Be neutron beam. Results indicated that [Rh(fctfa)(cod)] sensitizes cells to radiation possibly by inhibition of cell inactivation mechanisms that are normally associated with repairable damage.

Consequent work done on the flow cytometer where direct DNA damage after irradiation (8 MV photon beam) and drug treatment, was assessed on aerobic CHO cells by a technique adapted to our laboratory showed no significant increase in the forward angle scattered light (FSC) parameter which is an indication of radiation induced strand breaks. Furthermore, [Rh(fctfa)(cod)] showed a significantly greater increase in the side angle scattered light (SSC) parameter, which is an indication of the binding ability of the complex, compared to cisplatin, after treatment with different concentrations of the drugs. Results obtained from enumerating micronuclei frequencies after drug treatment and radiation confirmed that both cisplatin and [Rh(fctfa)(cod)] are more active under hypoxic conditions, with [Rh(fctfa)(cod)] responsible for more micronuclei per binucleated cell.

In conclusion, I have established that [Rh(fctfa)(cod)] has a cytotoxic activity comparable to that of cisplatin and that it sensitizes preferentially hypoxic CHO cells to radiation in the clinically relevant dose range. I have also identified the probable action by which [Rh(fctfa)(cod)] sensitizes CHO cells to radiation as being inhibition of repair capacity. Furthermore, results suggest that this complex binds covalently to DNA base pairs. The complex [Rh(fctfa)(cod)], has so far proven to possess interesting radiosensitizing potential which must be exploited for eventual therapeutic benefit.

OPSOMMING

Dit is alom bekend dat baie soliede gewasse hetrogeen ten opsigte van oksiegenasie is en dat dit streke van hipoksiese selle bevat wat weens hul inherente bestandheid teen ioniserende straling, die sukses van konvensionele radioterapie beperk. Verskeie chemiese geneesmiddels is die afgelope dekades vir potentiële radiosensitiserings eienskappe ondersoek. Die kliniese sukses wat met een organo-metaliese middel naamlik, sis-diamminedicloro platinum(II) behaal is, het daartoe gelei dat ander organiese sititoksiese metaalbevattende middels ontwerp is. Prof. J.C. Swart, verbonde aan die Universiteit van die Oranje Vrystaat het sewentien nuwe iridium, ferrisenium en rodium komplekse verskaf met wat deur my vir sititoksiese aktiewiteit teen die CHO sellyn ondersoek is.

Die twee aktiefste komplekse naamlik, [Rh(fctca)(cod)] en [Rh(fctfa)(cod)], is vir moontlike radiosensitieseerings eienskappe teen aerobiese en hipoksiese CHO selle, in die teenwoordigheid van 'n 8 MV foton bundel deur middel van die MTT tegniek wat vir ons laboratorium aangepas is, getoets. Die ferroseen betadiketone ge-koördineer met dié komplekse naamlik, Hfctca and Hfctfa asook die Ir kompliment van [Rh(fctfa)(cod)] naamlik, [Ir(fctfa)(cod)] is ook deur die MTT toetsing bepaal. Nie die ferroseen of die iridium komplekse het betekenisvolle sensitiseering getoon nie. Dit wil dus blyk dat die rodium vir die effektiwiteit van die kompleks verantwoordelik is. Die radiosensitiseerings potentiaal van die aktiefste kompleks, [Rh(fctfa)(cod)], en sisplatin, is deur die meer tradisionele kolonogeniese bepaling bevestig. Verder was die resultate van die MTT toetsing ook vergelykbaar met die kolonogeniese tegniek. Een van die komplekse, [Rh(fctfa)(cod)], het 'n radiosensitiseerings potentiaal teen hipoksiese CHO selle gelykstaande aan die van sisplatin getoon.

Die rodium kompleks [Rh(fctfa)(cod)] is ook vir radiosensitiseerings eienskappe in die teenwoordigheid van 'n p(66)/Be neutron bundel getoets. Die resultate toon dat die sensitiseerings aktiewitiet van [Rh(fctfa)(cod)], moontlik is weens inhibeering van die selinaktiverings meganismes wat gewoonlik met herstelbare skade geassosieër word.

Opeenvolgende studies, waar daar gebruik gemaak is van die vloeisitometer is gedoen. Daar is vasgestel dat daar DNA skade na straling (8 MV foton bundel) en behandeling met die kompleks, op aerobiese CHO selle is, deur middel van 'n tegniek wat vir ons laboratorium aangepas is. Die resultate dui daarop dat daar geen noemenswaardige toename in die voorwaartse-hoek verstrooide lig (FSC) parameter, wat 'n indikasie van stralings geinduseerde strand-breuke, is nie. Behandeling met verskillende konsentrasies van [Rh(fctfa)(cod)] het, in vergelyking met sisplatin, 'n noemenswaardige toename in die sywaartse-hoek verstrooide lig (SSC) parameter, wat 'n indikasie van die bindingsvermoeë van die kompleks is, getoon. Resultate wat verkry is met die mikrokerne tegniek, na afloop van kompleks behandeling en bestraling, het bevestig dat beide cisplatin en [Rh(fctfa)(cod)] aktiewer onder hipoksiese toestande is en dat [Rh(fctfa)(cod)] meer mikrokerne per twee-kernige sel veroorsaak.

In kort, het ek vasgestel dat [Rh(fctfa)(cod)] sititoksiese aktiwiteit besit vergelykbaar met dié van sisplatin. Die kompleks sensitiseer by voorkeur hipoksiese CHO selle vir straling in die klinies relevante dosis gebied. Die moontlike effektiwiteit waardeur [Rh(fctfa)(cod)] CHO selle sensitiseer vir straling is geidentifiseer as inhibisie van herstel kapasiteit. Verdere resultate wys daarop dat die kompleks kovalent met die DNA-basispare bind. Die kompleks, [Rh(fctfa)(cod)] besit interessante radiosensitiseerings eienskappe wat uitgebuit moet word vir toekomstige terapeutiese gebruik.

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LIST OF ABBREVIATIONS:

Be	-	Beryllium
Ca	-	Cancer
Ca^{++}	-	Calcium ion
CHO	-	Chinese Hamster Ovary
CO_2	-	Carbondioxide
^{60}Co	-	Cobalt – 60
^{51}Cr	-	Chrome – 51
d_{max}	-	depth of maximum dose
DMF	-	Dose modyfing factor
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
ER	-	Enhancement ratio
EB	-	Ethidium bromide
Fe	-	Iron
FIGO	-	Federation Internationale de Gynecologie et d'Obstetrique
FS	-	Forward scatter
Gy/MU	-	Gray per monitor unit
Ir	-	Iridium
LET	-	Linear energy transfer
LQ	-	Linear quadratic
MTT	-	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)
Mg^{++}	-	Magnesium ion
MID	-	Mean inactivation dose
N_2	-	Nitrogen
NAC	-	National Accelerator Centre
O_2	-	Oxygen
OER	-	Oxygen enhancement ratio

OD	-	Optical Density
p(66)Be	-	66 MV protons impinging on a beryllium target producing a neutron beam
PBS	-	Phosphate buffered saline
PCE	-	Polychromatic erytrocytes
PE	-	Plating efficiency
PI	-	Propedium iodide
RBE	-	Relative biological effectiveness
Rh	-	Rhodium
SEM	-	Standard error of the mean
SF	-	Survival fraction
SS	-	Side scatter
SSD	-	Source to surface distance
TRIS	-	(Hydroxymethyl)aminomethane – Biochemical buffer
V79	-	Murine fibroblast cell line

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

The presence of hypoxic cells, resistant to radiotherapy as a consequence of the rapid metabolism of oxygen in tumour tissue, is a limiting factor in the curability of tumours by radiation. Suggested methods used to overcome this problem are treatment in hyperbaric oxygen chambers and the introduction of high linear energy transfer (LET) radiation such as neutrons and heavy ions (Hall, 1994). Specific modification of tumour radiosensitivity can also be achieved by the use of chemical radiosensitizers in combination with conventional radiotherapy. These radiosensitizers have the capacity to increase the lethal effects of radiation when administered in combination with it (Skov, 1987a; Brown, 1989; Shenoy and Singh, 1992; Köpf-Maier, 1994 and Hall, 1994).

During the past 20 – 25 years, numerous chemicals and drugs have been investigated and added to the ever-increasing list of radiosensitizers (Shenoy and Singh, 1992). The therapeutic use of metals or metal-containing compounds, in the treatment of cancer was reported as early as the sixteenth century. However it was not until the 1960's that interest in the anti-tumour properties of metal containing compounds was reawakened by the discovery of the anti-tumour activity of the inorganic complex *cis*-diammine-dichloroplatinum(II) (cisplatin). This led to the development of other types of non-organic cytostatic metal-containing drugs.

Because the nucleus of a cell is far more radiosensitive than the surrounding cytoplasm it is important that the radiosensitizing drug is incorporated into the DNA. Targeting hypoxic cell radiosensitizers to DNA is done either by taking advantage of the DNA binding properties of certain metals, such as platinum

(Chibber *et al*, 1984; Teicher *et al*, 1984; Skov, 1987a), or by complexing it with a group that intercalates into DNA (Giraldi *et al*, 1977).

The therapeutic use of cisplatin is usually limited by nephrotoxicity and gastrointestinal irritation (Hacker *et al,* 1984; Nicolini, 1988). Rhodium (Rh), despite its heavy-metal character would appear to exhibit no nephrotoxicity (Craciunescu *et al*, 1991; Köpf-Maier, 1994). Giraldi *et al* (1977) reported that rhodium(acetylacccetonato)(cyclooctadiene) performs better against Erlich ascites tumours than cisplatin. Furthermore, both cisplatin (Douple and Richmond, 1979a; Geldof and Slotman, 1996) and Rh (II) complexes (Chibber *et al*, 1985) have been shown to specifically radiosensitize hypoxic cells.

It has been demonstrated that some ferricenium complexes can act as radiosensitizers of hypoxic cells *in vitro* and *in vivo* (Joy *et al*, 1989). The rationale behind evaluating such complexes as sensitizers is based on their redox properties and on the observation that these complexes have substantial cytotoxic activity towards Ehrlich ascites tumour cells both *in vitro* and *in vivo.* Ferrocene derivatives have further been shown to have antineoplastic activity (Neuse *et al*, 1984).

Prof. J.C. Swart, Department of Chemistry, University of the Orange Free State, South Africa, has developed unique Rhodium – Ferrocene complexes that have not been previously tested in any biological system. New ferrocene-containing betadiketones have been created from different ferrocenoic acids. The betadiketones are complexed to Rhodium(I) to generate Rh(betadiketone) (cyclooctadiene). Another transition metal with possible anti-tumour activity and radiosensitization ability is iridium (Ir), however, very little has so far been published with respect to these properties of Iridium(I)

complexes. Ir(I) analogs having the same chemical structure as their Rh(I) counterparts were also investigated.

In this thesis, I have undertaken to evaluate these-metal based complexes for radiosensitizing potential.

1.2 METAL COMPOUNDS WITH ANTI-TUMOUR PROPERTIES

1.2.1 Anti-tumour activity of cisplatin and other metal based complexes:

Rosenberg and co-workers in 1969 accidentally discovered the anti-tumour properties of the inorganic complex *cis*-diamminedichloroplatinum(II) (cisplatin). Since then, cisplatin and other related platinum compounds have become of the most frequently used cytostatic drugs world-wide in regimens of combination therapy for the treatment of solid carcinomas (Köpf-Maier, 1994). Cisplatin and carboplatin exhibit high efficacy against human ovarian, bladder, head and neck carcinomas (De Vita *et al*, 1985; Nicolini, 1988) and can effect a cure in most cases of testicular carcinoma.

With the discovery of the anti-tumour activity of cisplatin, other metal containing complexes have been investigated for possible anti-tumour efficacy. These compounds comprise main-group metallic compounds of gallium, germanium, tin and bismuth, early-transition metal complexes of titanium, vanadium, niobium, molybdenum and rhenium, and late-transition metal complexes of ruthenium, rhodium, iridium, platinum, copper and gold. In Figure 1.1 the periodic table is shown with the metals and metalloids, shaded in grey, that have so far been confirmed to function as a central metal in anti-tumour compounds. Many other inorganic and organometallic compounds containing metals and metalloids of the main groups 13 to 15 of the periodic

table and certain transition metals of groups 4 to 11 have been found to be cytostatically active (Köpf-Maier, 1994).

Apart from cisplatin, four other non-platinum metal anti-tumour compounds have so far been introduced into early clinical trials (Köpf-Maier, 1994). These four compounds, gallium nitrate, spirogermanium, budotitane and titanocene dichloride, have different mechanisms to cisplatin in effecting their cytostatic and tumour-inhibiting actions in leukaemias and solid carcinomas.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
H																	He
Li	Be											B	C	N	O	F	Ne
Na	Mg											Al	Si	P	S	Cl	Ar
K	Ca	Sc	**Ti**	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	**Ga**	**Ge**	As	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	J	Xe
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	**Pt**	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	Ac															

Transition metals

Early

Late

Figure 1.1. Early-transition, late-transition and main-group metals and metalloids that have so far been confirmed to function as a central metal in anti-tumour compounds, are shaded in grey in the periodic table of the elements (Köpf-Maier, 1994).

The anti-tumour activity of various gallium(III) salts against experimental animal tumours was discovered during the 1970's (Hart *et al*, 1971; Adamson *et al*, 1975), and clinical trials with gallium(III) nitrate soon proceeded. As a result of these trials, gallium(III) nitrate shows promise of becoming the superior therapy for cancer-related hypercalcaemia, since it was at that stage

more effective than all other therapeutic strategies (Warrel *et al*, 1988; Warrel, 1991; Todd and Fitton, 1991).

Two organnometallic compounds of germanium, carboxyethyl-germaniumsesquioxide (^{132}Ge) (Kumano *et al*, 1978, 1985) and spirogermanium (Rice *et al*, 1977), have also been shown to have antineoplastic activity.

The first transition metal compound containing a metal other than platinum to enter into clinical studies in 1986 was the inorganic compound cis-diethoxybis(1–phenylbutane-1,3-dionato)titanium(IV), (budotitane) (Keppler and Heim, 1988; Heim *et al*, 1990; Keppler *et al*, 1991). Budotitane was since recommended for phase II clinical studies (Heim *et al*, 1990).

The most active organometallic anti-tumour bis(cyclopentadienyl)metal diacido complex investigated so far is titanocene dichloride. This complex is also one of the first to be extensively tested against a broad spectrum of human carcinomas in the pre-clinical phase, and not only against common experimental animal tumour systems. These pre-clinical studies pointed to obvious anti-tumour activity of titanocene dichloride, especially against colorectal, lung and breast carcinomas (Keppler and Schmähl, 1986; Köpf-Maier, 1989; Berdel *et al*, 1994; Köpf-Maier and Chares, 1994).

Platinum is a late-transition metal, as is iron(Fe), rhodium(Rh) and iridium(Ir). The well known anti-tumour properties of platinum related compounds (most notably that of cisplatin) has strongly hinted at the possibility that other late-transition metal compounds may also be active as anti-tumour agents.

(i) Anti-tumour activity of Fe complexes:

Of all the organometallic iron complexes that have been tested *in vitro* for anti-tumour activity, only ionic ferricenium complexes of the type $[Fe^{III}(C_5H_5)_2]^+ X^-$, have shown anti-tumour activity against Ehrlich ascites tumour *in vivo* (Köpf-Maier *et al*, 1984). Very few other *in vivo* experiments have been done on ferricenium compounds due to their hydrolytic instability and limited activity against xenografted human carcinomas (Köpf-Maier and Köpf, 1988).

(ii) Anti-tumour activity of Rh complexes:

In 1975, Bear *et al* discovered Rh(II) compounds of the type; inorganic, binuclear, and caged rhodium carboxylates as anti-tumour agents. The most potent inhibitors of tumour growth were found to be the carboxylate derivatives, butyrate($R = CH_2CH_2CH_3$) and propionate($R = CH_2CH_3$). These compounds had pronounced activity in the P388 leukaemia and the Ehrlich ascites carcinoma in mice. Rao *et al* (1980) indicated that the inhibition of the synthesis of DNA by these complexes seemed to be the crucial mechanism.

Other rhodium(I) compounds with *in vivo* anti-tumour activity were the organometallic, neutral and square planar rhodium(I) cyclo-octadiene complexes [Rh(I)Cl(cod)(NH_3)] and [Rh(I)Cl(cod)(piperidine)], (cod = *cis,cis*-1,5-cyclo-octadiene), which had anti-tumour activity against the Ehrlich ascites tumour (Giraldi *et al*, 1974), and the acetylacetonato (acac) derivate [Rh(I)(acac)(cod)], which inhibited the growth of the leukaemia L1210, sarcoma 180, and Ehrlich ascites carcinoma (Giraldi, 1978) and had anti-tumour and antimetastatic activity in the metastasizing Lewis lung carcinoma similar to that of cisplatin (Sava *et al*, 1983). In 1989, Sava *et al* showed that the ionic and square planar Rh(I) complexes cyclo-octadiene-(2-pyridinalmethylimine) rhodium chloride [Rh(I)-(cod)($NC_5H_4CH{=}NCH_3$]$^+Cl^-$ and cyclo-octadiene-(2-pyridinalisopropylimine)rhodium chloride [Rh(I)-

(cod)(NC_5H_4CH)=N-i-C_3H_7]$^+Cl^-$ have similar activity and prolonged life in mice with the P388 leukaemia and to have antineoplastic and antimetastatic effects in the Lewis lung and the MCa mammary carcinoma systems. Sava *et al* (1989) suggested that both of these compounds have cytotoxic and immunomodulating effects, depending on the ligands present within the rhodium(I) complexes.

Some rhodium(I) carbonyl complexes of the type [Rh(I)(CO_2)L] (L= sulphonamide derivatives) have recently been described by Craciunescu *et al*, (1989) as anti-tumourals. These complexes had anti-proliferative activity against the ascites leukaemia P388, the Ehrich ascites tumour and advanced B16 melanoma, with the [Rh(I)$(CO)_2$ (sulphamethoxydiazine)] complex found to be the most active derivative both *in vitro* and *in vivo*. None of these compounds showed any nephrotoxicity, despite their heavy metal character.

(iii) Anti-tumour activity of Ir complexes:

The same group (Giraldi *et al*, 1974) that described the anti-tumour activity of Rh(I) complexes also investigated the anti-tumour activity of Ir(I) complexes *in vivo*. They found that the acetylacetonato(acac) derivate, [Ir(I)(acac)(cod)] inhibited the growth of the leukaemia L1210, sarcoma 180, and Ehrlich ascites carcinoma (Giraldi, 1978) and had anti-tumour activity in the metastasizing Lewis lung carcinoma, similar to that of cisplatin (Sava *et al*, 1983).

Sava *et al* (1987) studied Ir(I) complexes: [Ir(I)(acac)(cod)] and [Ir(I)(cod)Cl]^2 to compare their anti-tumour and antimetastatic properties in order to compare them with the Rh(I) analogs having the same chemical structures. It was found that the organometallic complexes of Rh(I) are more active than those of Ir(I) (Sava *et al*, 1987).

1.3 HYPOXIC RADIOSENSITIZERS

1.3.1 Hypoxic Tumours:

Powers and Tolmach (1963) investigated the radiation response of a solid subcutaneous lymphosarcoma in the mouse. This was the first unequivocal demonstration that a solid tumour could contain cells sufficiently hypoxic to be protected from cell killing by x-rays but still clonogenic and capable of providing a focus for tumour regrowth.

Hypoxia in tumours is the result from two entirely different mechanisms. Chronic hypoxia results from the limited diffusion distance of oxygen through respiring tissue (Thomlinson and Gray, 1955), whereas acute hypoxia is a result of the temporary closing of a tumour blood vessel and is therefore transient (Brown, 1979; Chaplin *et al*, 1986). An illustration of the difference between chronic and acute hypoxia is given in Figure 1.2.

There is supporting evidence that some human tumours contain a significant proportion of hypoxic cells (Denekamp *et al*, 1977; Groebe and Vaupel, 1988). During a normal course of radiotherapy, reoxygenation (the process by which cells that are hypoxic at the time of irradiation become oxygenated afterwards) of hypoxic tumour cells will occur. The extent and rapidity of reoxygenation differs widely for different tumour types. If the reoxygenation process is rapid and complete, the presence of hypoxic cells would have little influence on the outcome of a radiation schedule. The contrary is suggested by the limited tumour control, of certain human tumours, after a full course of radiotherapy (Bush *et al*, 1978; Brown, 1984; Chaplin, 1986). This prompts the need to discover an agent that will influence the way in which the presence of hypoxic cells limits tumour control.

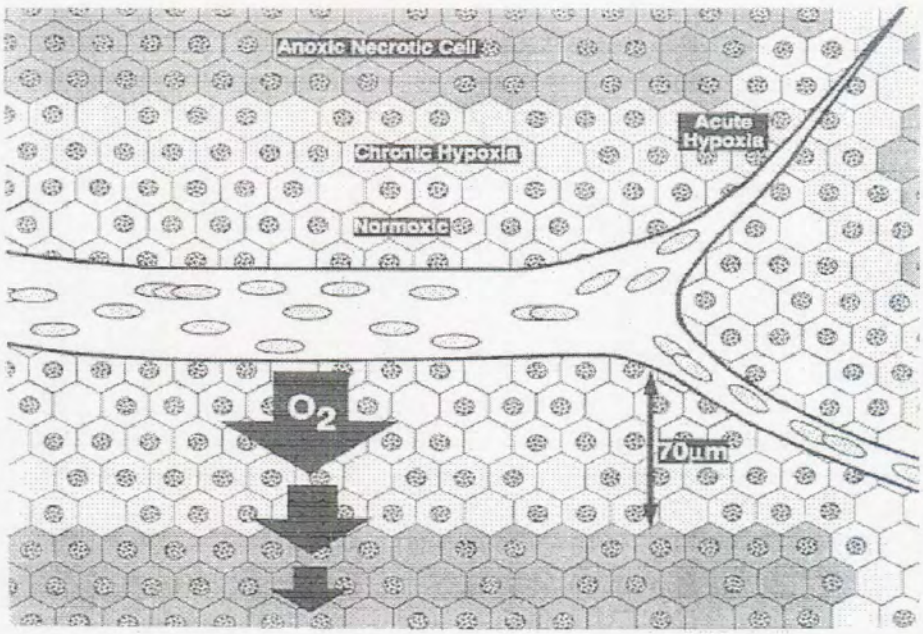


Figure 1.2. Diagram illustrating the difference between chronic and acute hypoxia. Chronic hypoxia results from the limited diffusion distance of oxygen in respiring tissue that is actively consuming oxygen. Cells that become hypoxic in this way remain hypoxic for long periods of time until they die and become necrotic. Acute hypoxia results from the temporary closing of tumour blood vessels. The cells are intermittently hypoxic since normoxia is restored each time the blood vessel opens up again. (Brown, 1990).

1.3.2 The Oxygen Effect:

No chemical or pharmacological agent can modify the biological effect of ionizing radiation to the extent that oxygen can. The ratio of aerated and hypoxic doses required to achieve the same biological effect is called the oxygen enhancement ratio (OER). Oxygen sensitizes living cells to sparsely ionizing particles such as x- and γ-rays by a factor of 2.5 - 3.0. This was first demonstrated in the roots of *Vicia faba* and later in mammalian cells (Read *et al*, 1952a; Read *et al*, 1952b; Read *et al*, 1952c; Gray *et al*, 1953; Dewey, 1960). For radiation of intermediate ionizing density, such as neutrons, the oxygen effect is apparent but not as pronounced as is the case with x-rays (Barendsen *et al*, 1966; Broerse *et al*, 1967).

The oxygen effect is apparent when oxygen is present during, or milliseconds after radiation. There is a general agreement that oxygen acts on the level of free radicals and it is these radicals that break chemical bonds, produce chemical changes, and initiate the chain of events that result in the final expression of biological damage. In a sense, oxygen is said to fix the radiation damage, this phenomenon is illustrated in Figure 1.3.

The dependence of the radiosensitivity of hypoxic tumours on oxygen, spurred the efforts of many researchers to create compounds that would mimic oxygen in its ability to sensitize biological materials to the effect of x-rays.

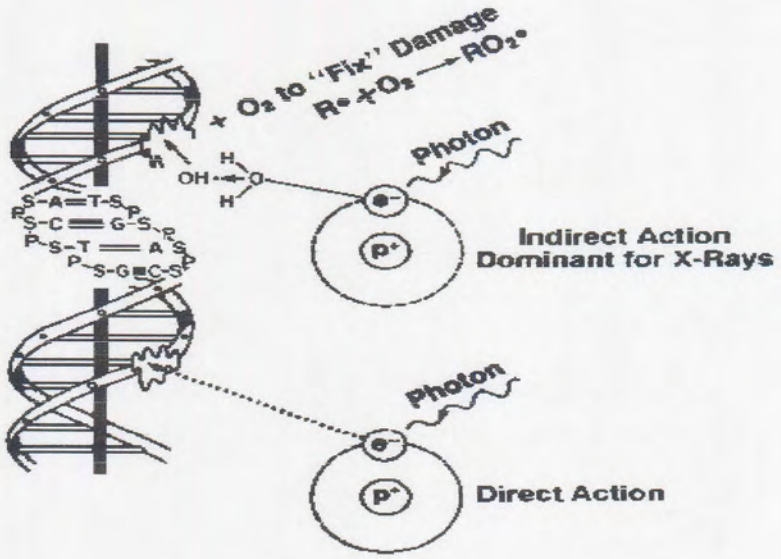


Figure 1.3. The oxygen fixation hypothesis. About two thirds of the biological damage produced by x-rays is by indirect action, mediated by free radicals. The damage produced by free radicals in DNA can be repaired under hypoxia but may be "fixed" (made permanent and irreparable) if molecular oxygen is available (Hall ed, 1994).

1.3.3 Hypoxic Cell Radiosensitizers:

Adams and his colleagues (1973) listed properties of hypoxic cell radiosensitizers of clinical relevance that still has bearing today (Hall, 1994):

- Radiosensitizers must selectively sensitize hypoxic cells at a non-toxic concentration to normal cells.
- They must be chemically stable and not subject to rapid metabolic breakdown.

- An elevated partition coefficient (a measure of the solubility of the drug in water or lipids) is an advantage, but the drug must still be capable of diffusing a considerable distance through a solid tumour.
- The drug must not be affected by the cell cycle, as it is likely that hypoxic cells in the tumour will be arrested in the G_1 phase of the cell cycle.
- It should be effective at the low doses (a few Gy) used in conventional fractionated radiotherapy.

Numerous drugs have been tested for their ability to sensitize hypoxic cells to radiation. Due to their different mechanisms of action categorization of radiosensitizers is not an easy task but they can roughly be classified as: (i) DNA-based analogs, (ii) agents of electron affinity, (iii) membrane active drugs, and (iv) miscellaneous compounds (Shenoy and Singh, 1992).

(i) DNA-based analogs:

Compounds that modify the radiation response of living cells must show a differential effect between tumours and normal tissues, to offer any gain. Some radiosensitizer's differential effect is based on the premise that tumour cells cycle faster and, therefore, incorporate more of the drug than the surrounding normal tissue. These agents, including the halogenated pyrimidines, directly alter the molecular apparatus determining the radiosensitivity (Djodjevic and Szybalski, 1960; Shipley *et al*, 1971). Due to the fact that the size of a halogen atom such as chlorine, bromine or iodine is very similar to that of a methyl group, they are actually incorporated into the DNA chain in place of thymine. In doing so they render the DNA more susceptible to radiation, by mechanisms not yet fully understood (Shenoy and Bam, 1992).

The only disadvantage of this type of sensitizer is that they are not hypoxic cell specific, in that they are just as easily incorporated into actively proliferating normal tissues.

(ii) Agents of electron affinity:

Hypoxic cell radiosensitizer's differential effect is based on the premise that hypoxic cells are endemic to tumours and not normal tissues, and will therefore increase the radiosensitivity of oxygen deficient cells, without any effect on normal aerated tissues. Because of the electron affinity of oxygen, it can therefore be expected that the electron affinity of compounds are directly related to their sensitizing efficiency.

The main difference between drugs that mimic the effect of oxygen and oxygen, per se, lies therein that they are not rapidly metabolized by the cells in the tumour through which they diffuse. Due to this, they can penetrate further than oxygen and reach all the hypoxic cells, even those most removed from the blood supply in the tumour.

A group of electron affinic sensitizers, the organic compounds, nitroimidazoles, have been subjected to intense investigation, *in vitro* and *in vivo*. Several 2-nitroimidazoles have been synthesized, which include the compounds misonidazole and etanidazole, both have been proven to be very potent radiosensitizers (Adams *et al*, 1976). Etanidazole is hydrophilic, consequently it does not cross the blood-brain barrier and is less neurotoxic.

Interest in the use of nitroimidazoles has been rekindled with the recent approach to take advantage of the DNA-binding properties of certain metals to direct known electron affinic sensitizers (e.g. nitroimidazoles), to the target of the radiation damage (i.e. the DNA) with the specific intent of decreasing

the overall concentration of sensitizer, and thereby decreasing the side-effects in metal/radiation interactions (Farrel and Skov, 1982).

(iii) Membrane active drugs:

The molecular basis of cellular damage due to ionizing radiation can be attributed to DNA damage. Apart from DNA, the cell membrane is also a critical site for radiation induced cell lethality (Shenoy and Singh, 1992). The role of the cell membrane in cellular lethality was first reported by Shenoy *et al* (1968) in their work with [^{131}I] iodoacetic acid as a radiosensitizer. They observed that most of the radioactivity was incorporated in the membrane proteins *E.coli* B/r, resulting in the inhibition of post-irradiation DNA and protein synthesis (Shenoy *et al*, 1970).

Since this first observation the effects of radiation and known membrane active drugs which include anaesthetics, analgesics and tranquillisers have been examined. Some drugs that have been found to sensitize the membrane proteins (*E.coli* B/r) to radiation under hypoxia are, procaine hydrochloride (a local anaesthetic) (Shenoy *et al*, 1974) and chlorpromazine (CPZ) (part of the phenothiazines, which include tranquillisers, anti-histaminics, anti-pruritics and anti-emetics)

(iv) Miscellaneous compounds:

There are various miscellaneous chemicals and drugs that affect cellular radiosensitivity, these compounds act by a variety of mechanisms which include, thiol depletion, modifiers of cellular metabolism, DNA intercalation and modifiers of DNA damage (Shenoy and Singh, 1992). The compounds that are being regarded with renewed interest are the metal-based compounds.

The use of metal-based complexes to modify sensitizer action is considerable because of the wide variety of factors that can be exploited. Some of these factors include: the identity of the metal, its ability to interact with DNA, its formal oxidation states *in vitro* and *in vivo*, and the nature of the accompanying ligand(s) (Joy *et al*, 1989).

By far the most successful metal-based complex currently in clinical use is the complex *cis*-diamminedichloro platinum(II) (*cis*-DDP) or cisplatin. Figure 1.4 presents the molecular structure of cisplatin.

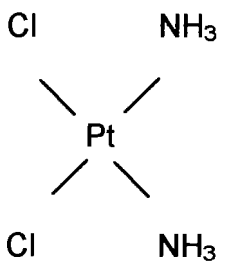


Figure 1.4: The molecular structure of the metal based complex *cis*-diamminedichloro platinum(II) (cisplatin)

1.3.4 Radiosensitization properties of cisplatin and other metal based complexes:

(i) Radiosensitization properties of cisplatin:

Many platinum related complexes have been studied for radiosensitizing properties, including a less toxic isomer of cisplatin, *trans*-DDP which proved to be a very effective radiosensitizer at low doses (Skov *et al,* 1989). Cisplatin has also been used in combination with nitroimidazoles. One such complex cis-$Pt(II)Cl_2$ $(metronidazole)_2$ or "flap", at 50 μM was reported by Bales *et al*

(1982) to give an enhancement factor of 2.4 when sensitizing hypoxic CHO cells.

Metal complexes, other than cisplatin that have so far been identified to be radiosensitizers of mammalian cells and/or bacterial cells, include complexes of Silver(I) [Ag(I)], Copper(I) [Cu(I)], Copper(II) [Cu(II)], Zinc(II) [Zn(II)], Lead(II) [Pb(II)], Rhodium(II) [Rh(II)], Ruthenium(II) [Ru(II)], Ruthenium(III) [Ru(III)], Cobalt(III) [Co(III)], and Iron(III) [Fe(III)] (Richmond and Powers, 1974; Hesselwood *et al*, 1978; Kirschiner *et al*, 1970; Cramp, 1967; Kiortsis, 1977; Ho and Ho, 1975)

(ii) Radiosensitization properties of Fe complexes:

Moroson and Tenney (1968) reported hypoxic radiosensitization enhancement ratios (ERs) of 2.4 for radioresistant strain B/r and 1.2 for radiosensitive strain B_{S-1} by ferricyanide, $[Fe(CN)_6]^{3-}$; 1 mmol dm^{-3}, in an investigation on thiol-binding agents in *Escherchia coli*. Ferricyanide did not sensitize oxic cells (Moroson and Tenney, 1968). Furthermore at a concentration of 0.1 mmol dm^{-3} the complex showed no enhancement in hypoxic CHO cells (Skov, 1987a).

Enhancement in both hypoxic and oxic conditions (1.2-1.3) was seen for Chinese hamster fibroblast (V79) cells by Douple *et al* (1980b) using nitroprusside ($[Fe(CN)^5NO]^{2-}$) at 10 μmol dm^{-3}.

Of the different ferricenium complexes that have so far been investigated for possible radiosensitization, the ferricenium salt, [Fe(cyclopentadienide)$_2$] showed radiosensitization with a dose modifying factor (DMF) of 2 in hypoxia against EMT6 and V79 cells (Teicher *et al*, 1987; Joy *et al*, 1989). Two ferricenium salts namely, trichloroacetate (FcTCA) at 10 μmol dm^{-3} and hexafluorophosphate ($FcPF_6$) at 10 μmol dm^{-3} showed DMF's of 1.6 and 2 for

hypoxic radiosensitization against V79 cells (Joy *et al*, 1989). Trichloroacetate (FcTCA) has also been shown to sensitize KHT sarcoma in mice, *in vivo*; an enhancement ratio of 1.3 was attained (Joy *et al*, 1989).

(iii) Radiosensitization properties of Rh complexes:

The interaction of Rh(II) carboxylate complexes with radiation has been described by Chibber and co-workers (1985). Sensitization under hypoxic conditions (ERs 1.9 - 2.1) was generally found to be moderately higher than under aerobic conditions (ERs 1.4 - 1.8) in V79 cells. Furthermore, Chibber *et al* (1985) suggested that thiol depletion was a more likely mechanism than an electron-affinic mechanism for these complexes.

Chibber *et al* (1984) and Goodgame *et al* (1986) have also used Rh(II) complexes to target misonidazole and analogs. They found the Rh complex better than the corresponding platinum complex and the RSU-1111 ligand alone, in sensitizing hypoxic V79 cells. The misonidazole complex of rhodium (at 20 μmol dm^{-3}) showed a better sensitization in hypoxia (ER of 1.8) than in air (ER ~1.2) (Goodgame *et al*, 1986).

The Rh(III) inorganic complex $[Rh(NH_3)_3Cl_3]$ did not sensitize bacterial spores but gave extremely high sensitivity in anoxia (100×) and in air (4×) when *Staphylococcus aureus* was irradiated in buffer (PBS) (Richmond *et al*, 1986a&b). Other Rh(III) complexes (ethylenediamine, nitrate) also demonstrated a large potentiation of radiation kill in bacteria, this effect was not seen when radiation took place in medium rather than buffer (Richmond *et al*, 1986b). It was also found (Richmond *et al*, 1986b) that mammalian cells may not be sensitized by these complexes.

One encouraging aspect in the development of hypoxic cell radiosensitizers is that radiosensitization of hypoxic cells occurs at a physico-chemical level, and is therefore mostly independent of cellular biochemistry. Consequently, radiosensitization is similar for mouse and human cells, which implies that radiosensitization in a mouse tumour with a specific drug concentration, is likely to produce a similar radiosensitization in a human tumour (Brown, 1989).

For the most part, the *in vitro* testing of radiosensitizers tend to over-predict (i.e. produce false positives rather than false negatives) for *in vivo* activity. There have been reports (Horsman *et al,* 1987) that some radiosensitizers, sensitize hypoxic tumour cells *in vivo*, but produce no effect *in vitro.* This illustrates the fact that an efficient tumour radiosensitizer can be missed with a purely *in vitro* scan.

1.3.5 Mechanism by which cisplatin and other metal complexes sensitize tumours to radiation:

(i) Mechanism by which cisplatin sensitizes tumours to radiation:

The mechanism(s) by which platinum complexes alter the effects of radiation is not clearly understood. Douple and Richmond (1979a; 1980) investigated the binding properties of platinum, while others (Richmond and Simic, 1978) related the sensitization of cisplatin to reduction of the metal centre. Recent studies on the radiation chemistry of *cis*- and *trans*-DDP seem to support the DNA binding mechanism (Butler *et al*, 1985). It has since been found that the guanine N7 position is a favourable site for metal ion binding, including platinum compounds (Gao *et al*, 1993). In 1996, Yang and Wang provided much insight into the structural interactions of platinum anticancer compounds with DNA. DNA structural distortion is associated with the intrastrand cisplatin adduct formation at the **G*G*** site (Yang *et al*, 1995; Gelasco and Lippard, 1998). The biological activity of cisplatin may be related to the interactions of

certain proteins with cisplatin-lesioned DNA (Chu, 1994; Zambel and Lippard, 1995). Platinum complexes are generally electrophilic and react preferentially with the hydrated electron in aqueous solution (Teicher *et al*, 1987).

(ii) Mechanism by which Fe complexes sensitize tumours to radiation:

It is well reported that there is a definite correlation between electron affinity and sensitizing ability (Adams *et al*, 1976). It can therefore be expected that some metal complexes with redox potentials comparable to that of nitroimidazoles will sensitize by this mechanism (Skov, 1987a). Adams (1987) found that Fe(III) sensitization could be due to reduction in the ferrous complex by the removal of an electron from the target radical (Bhattacharyya and Mandal, 1983).

Another method by which "oxygen"-mimic sensitizers can enhance radiation damage to the bases and sugars of the DNA is by the addition of the compound to a radical site on the DNA (Skov, 1987a). Rotlevi *et al* (1973) reported that certain metals, which include Fe(II) and Fe(III) quench radicals formed on solid DNA, which can then be interpreted as protection. If, however the enhancement of base/sugar damage is due to adduct formation between metals and radicals in the solid state, and if this occurs in solution, the induced DNA damage will be more difficult to repair (Skov, 1987a).

By making use of the binding properties of some metals to various states of sulphur, radiosensitization can take place by the intercellular depletion of thiols such as glutathione, a known radiation protector (Jocelyn, 1972). Ferricyanide has been described as a sensitizer (ER of 2.4 in hypoxic *E.coli*) because of its thiol-binding properties (Moroson and Tenney, 1968).

The release of toxic ligands due to the reduction of the metal upon reaction with e^-_{aq} (hydrated electron) led to the study of vitamin B_{12} (cyanocobalamine) and nitroprusside ($[Fe(CN)^5NO]^{2-}$), which contains cyanide as a ligand. Both were found to enhance radiation damage in V79 cells (Douple *et al*, 1980).

The mechanism by which ferricenium complexes sensitize cells to radiation damage is as yet not certain. There is evidence to support that glutathione depletion plays a role (Joy, 1988), but it is unlikely that all the observed biological effects can be attributed to this.

(iii) Mechanism by which Rh complexes sensitize tumours to radiation:
Giraldi, (1977) has suggested that Rh complexes may have a different mode of action to that of cisplatin. It has further been reported (Chibber *et al*, 1985) that Rh(II) and Pt(II) complexes do not operate by mechanisms similar to those occurring with electron affinic or stable free radical sensitizers. It should be clear however that if a metal was coupled to nitroimidazoles, an electron-affinic mechanism might be expected (Farrell and Skov, 1982; Butler *et al*, 1985).

Neither Pt(II) nor Rh(II) complexes have been found to act by addition to radicals due to DNA radiation damage (Chibber *et al*, 1985). It was proposed that sensitization by cisplatin is due to thiol depletion (Alvarez *et al*, 1978) but this does not appear to be generally accepted (Chibber *et al*, 1985). The ability of Rh(II) carboxylate complexes to increase radiation sensitivity of cells, lies in the fact that they deplete intracellular thiols, this correlates with their *in vitro* radiosensitization ability (Chibber *et al*, 1985).

Although drugs enhance radiosensitivity *in vitro*, they may fail *in vivo* because of insufficient drug uptake in solid tumours. By increasing the lipophilicity of a complex, a greater cellular uptake is observed as was seen in a series of

rhodium carboxylates in V79 cells (Chibber *et al*, 1985). This could be in contradiction to what is desired, because a greater cellular uptake of the more lipophilic complex would necessarily increase the toxicity of the complex. Thus a radiosensitizer with a high tumour affinity and less toxicity is essential for obtaining a high sensitizer enhancement ratio (SER) in clinical use.

In summary, metal complexes alter the effects of radiation in many ways. Certain metal complexes act as true radiosensitizers (i.e. nontoxic levels by free radical mechanisms), other complexes may interact with radiation due to slower chemical reactions (e.g. thiol depletion, DNA binding), while some act even slower at the biochemical level (e.g. inhibition of repair of radiation damage) (Skov, 1987).

1.4. MOLECULAR STRUCTURES OF METAL-BASED COMPLEXES

All the drugs in this study, excluding cisplatin which was obtained from Sigma Chemical Co., St Louis, USA, were supplied by Dr. J.C. Swarts, University of the Free State. In Figures 1.5 - 1.21 the molecular structures of all the Rh, Fe and Ir complexes with their chemical denominations and abbreviations are illustrated. All the betadiketones shown are always in equilibrium with the enol form, for simplicity in this thesis only the keto form will consistently be shown.

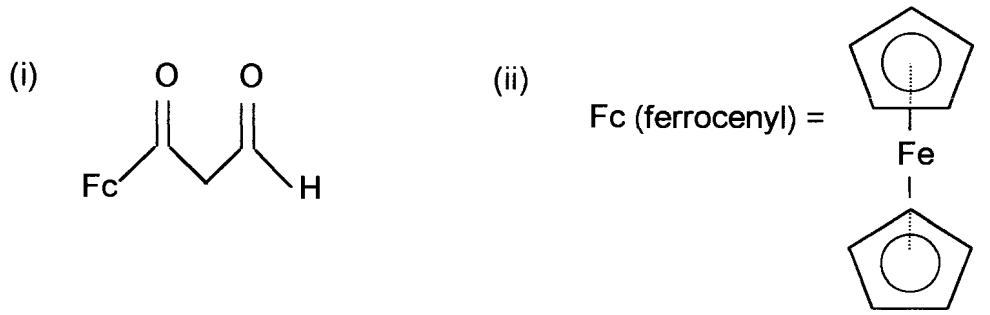


Figure 1.5: (i) The molecular structure of Ferrocenoylacetaldehyde, (Hfch).
(ii) Fc refers to the ferrocenyl – $FeC_{10}H_9 = Fe(C_5H_5)(C_5H_4)$, a dicyclopentadienyl moiety.

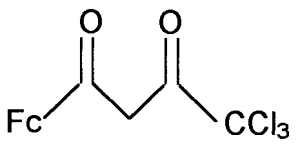


Figure 1.6: The molecular structure of Ferrocenoyltrichloroacetone, (Hfctca).

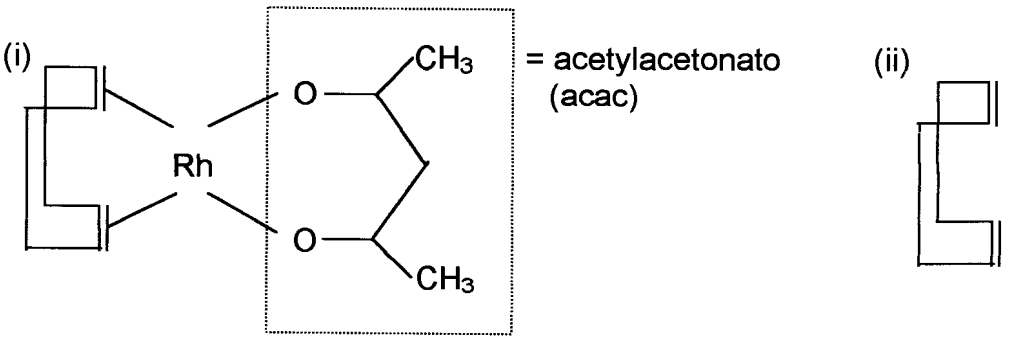
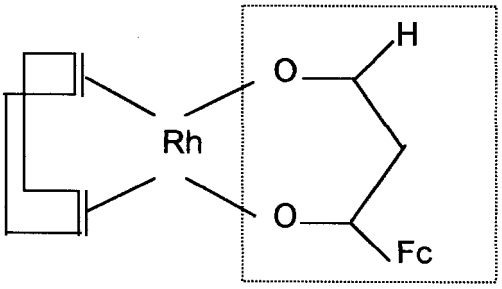
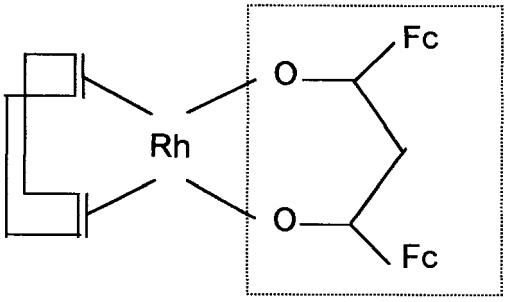


Figure 1.7: The molecular structures of (i) (η^4-1,5-cyclooctadiene) (1,3-pentanedionato-κ^2O,O')rhodium(I) [Rh(acac)(cod)] and (ii) cyclooctadiene (cod) – shown in the boat conformation.



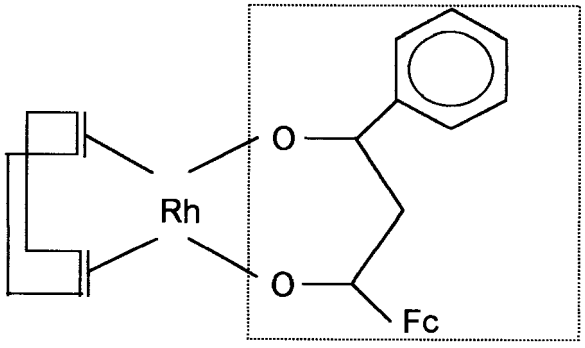
= 1-ferrocenyl-1,3 - propanedionato (FcH)

Figure 1.8: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-1, 3-propanedionato-κ^2O,O') rhodium(I), [Rh(fch)(cod)].



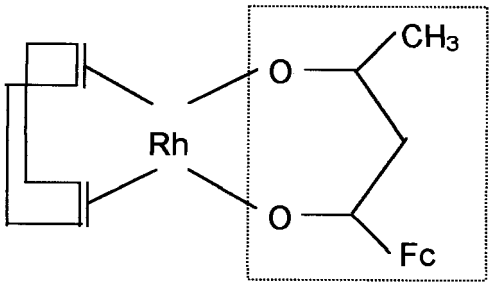
= 1,3 - diferrocenyl-1,3 - propanedionato (dfcm)

Figure 1.9: The molecular structure of (η^4-1,5-cyclooctadiene)(1,3-diferrocenyl-1,3-propanedionato-κ^2O,O')rhodium(I), [Rh(dfcm)(cod)].



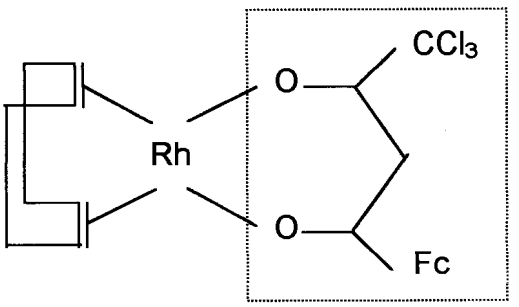
= 1-ferrocenyl-3-phenyl-1,3-propanedionato (bfcm)

Figure 1.10: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-3-phenyl-1,3-propanedionato-κ^2O,O')rhodium(I), [Rh(bfcm)(cod)].



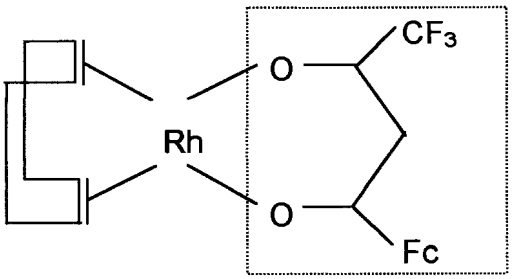
= ferrocenoylacetonato (fca)

Figure 1.11: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-1,3-butanedionato-κ^2O,O')rhodium(I), [Rh(fca)(cod)].



= ferrocenoyltrichloro-acetonato (fctca)

Figure 1.12: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-4,4,4-trichloro-1,3-butanedionato-κ^2O,O') rhodium(I), [Rh(fctca)(cod)].



= ferrocenoyltrifluoroacetonato (fctfa)

Figure 1.13: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-4,4,4-trifluoro-1,3-butanedionato-κ^2O,O') rhodium(I), [Rh(fctfa)(cod)].

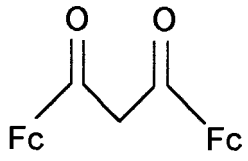


Figure 1.14: The molecular structure of diferrocenoylmethane, (Hdfcm).

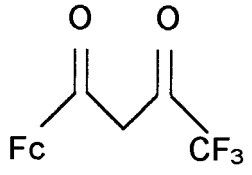


Figure 1.15: The molecular structure of ferrocenoyltrifluoroacetone, (Hfctfa).

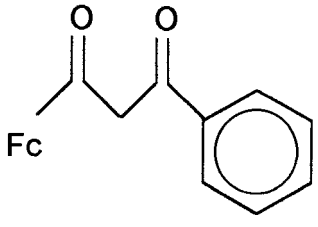


Figure 1.16: The molecular structure of benzoylferrocenoylmethane, (Hbfcm).

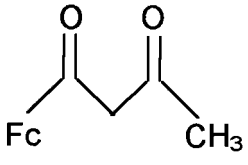


Figure 1.17: The molecular structure of ferrocenoylacetone, (Hfca).

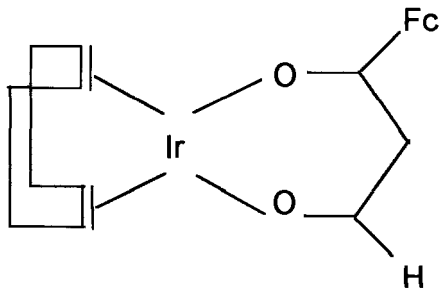


Figure 1.18: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl- 1,3-propanedionato-κ^2O,O') iridium(I), [Ir(fch)(cod)].

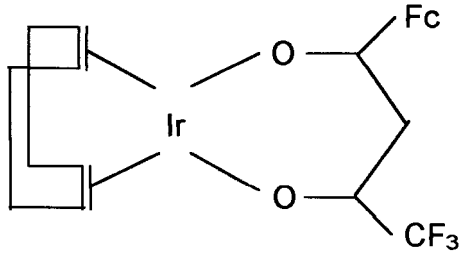


Figure 1.19: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-4,4,4-trifluoro-1,3-butanedionato-κ^2O,O')iridium(I), [Ir(fctfa)(cod)].

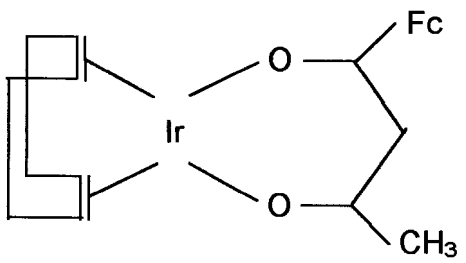


Figure 1.20: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-1,3-butanedionato-κ^2O,O')iridium(I), [Ir(fca)(cod)].

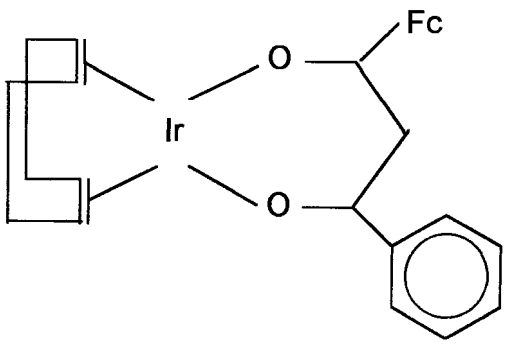


Figure 1.21: The molecular structure of (η^4-1,5-cyclooctadiene)(1-ferrocenyl-3-phenyl-1,3-propanedionato-κ^2O,O')iridium(I), [Ir(bfcm)(cod)].

1.4 OBJECTIVES

The primary objectives of the laboratory research described in this thesis were as follows:

1. To examine novel complexes of the middle to late transition metals iron (Fe), rhodium (Rh) and iridium (Ir) for cytotoxic activity.

2. To develop a procedure using the modular incubator chamber to create a reproducible aerobic and hypoxic environment in which to test for possible radiosensitization of different metal based complexes.

3. To investigate the radiosensitizing properties of ferrocene-containing betadiketonato cyclooctadiene rhodium(I) and iridium(I) complexes as well as the free betadiketone ligands.

4. To determine the mechanism by which these metallo complexes potentiate radiation damage.

CHAPTER 2: CYTOTOXIC EVALUATION OF METAL BASED COMPLEXES AGAINST THE CHINESE HAMSTER OVARY (CHO) CELL LINE, *IN VITRO*.

2.1 INTRODUCTION

There are several methods available for measuring the survival and/or proliferation of cells, eg. enumerating cells using dyes, measuring the release of ^{51}Cr-labelled protein after cell lysis and measuring incorporation of radioactive nucleotides during cell proliferation (Mosmann, 1983). Most of these methods are quite time consuming and not suited to rapidly quantify large numbers of samples.

A quantitative colourimetric assay for cell survival and proliferation has been developed. This assay makes use of a multiwell scanning spectrophotometer which measures a large number of samples with a high degree of precision (Mosmann, 1983). Furthermore, a colourimetric assay for living cells should ideally utilise a colourless substrate that is modified to a coloured product by any living cell. Tetrazolium salts have been chosen for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater *et al*, 1963). The tetrazolium ring is cleaved in active mitochondria hence the reaction only occurs in living cells thus the assay detects living and not dead cells, thereby the degree of activation of the cells is measured. By this method, the assay can be used to measure cytotoxicity, proliferation and activation (Mosmann, 1983).

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay is a rapid and quantitative assay with a high degree of precision capable of handling large numbers of samples. The assay is based on the ability of viable cells to convert MTT into a water-insoluble formazan product (Mosmann, 1983). By the addition of dimethyl sulphoxide (DMSO)

the stain can be solubilized and the optical density of the coloured product can be measured using a multiwell spectrophotometer.

The MTT colourimetric assay has since been used with success to measure cell growth and chemo-sensitivity to rapidly evaluate large numbers of compounds for their anti-cancer properties (Carmichael *et al*, 1987a; Alley *et al*, 1988).

In the present study, unless otherwise stated, all experiments were conducted using the CHO cell line. This cell line has been chosen by most radiobiologists due to its colony forming capability.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of the CHO cell line:

CHO cells, kindly supplied by the Department of Veterinary Sciences, Onderstepoort, South Africa, were maintained as monolayers in a mixture of Ham's F-12 medium (Bio Whittaker, Walkersville, Maryland, USA) supplemented with 10% heat inactivated foetal bovine serum (FCS, Delta Bioproducts, Kempton Park, RSA) and 0.1 mg/ml of penicillin and streptomycin, supplied as penstrep by Highveld Biological, Kelvin, RSA. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and all procedures were carried out in a laminar flow hood using aseptic techniques.

2.2.2 MTT Assay:

The MTT assay was performed in experiments conducted in both microtitre plates and in 5 ml glass test tubes. MTT (Sigma Chemicals Co., St Louis, USA) was dissolved in PBS (phosphate-buffered saline), at a concentration of 5 mg/ml and filter sterilized. After the required incubation period as stipulated by the study performed, either 20 µl of the MTT

solution was added to the wells of the microtitre plate (for the cytotoxicity assay) or 50 µl of the solution was added to the test tubes (for the radiosensitization assay). The plates / tubes were then incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 to allow the reduction of the tetrazolium. PBS was added to all of the wells / tubes and the plates / tubes were then centrifuged at 80 g (2000 rpm) for 10 min, after which the PBS-MTT solution was removed by gentle aspiration leaving the precipitate undisturbed. The formazin crystals were then dissolved by the addition of 100 µl of DMSO to each well of the microtite plate. In the case of the test tubes, 200 µl of DMSO was added to each test tube and 100 µl of this solution was then transferred to each well of the microtite plate. The plates were read in a multiwell spectrophotometer (CERES 900 EIA Workstation, BioTek Instruments INC., Winooski, USA) using a wavelength of 540 nm.

2.2.3 Evaluation of optimal cell concentration for cytotoxicity and radiosensitivity studies:

To establish the optimal cell concentration to be used in cytotoxicity and radiosensitivity studies, a cell proliferation study was done. Cells were grown in 200 ml growth flasks to confluency, trypsinated (Trypsin-Versene, Bio Whittaker, Walkersville, Maryland, USA) and the number of cells per ml enumerated with a haemocytometer. Different concentrations of cells, made up to a volume of 250 µl, were then seeded in triplicate into 5 ml glass test tubes, to a total volume of 0.5 ml complete medium and incubated over a period of seven days. The number of actively metabolizing cells was then determined by an assay based on the reactivity of MTT with viable cells (Mosmann, 1983).

2.2.4 Experimental drugs:

The different metal-based complexes were solubilized in sterile DMSO at a stock concentration of 2 mg / ml and stored as aliquots at - 20°C and diluted in complete medium to the required concentration immediately

before use. Appropriate solvent controls were included in the various assays described.

2.2.5 Cytotoxic evaluation of cisplatin, Rh, Fe and Ir Complexes:

One hundred microlitrers of an exponentially growing culture (600 cells / ml) were added to each well of a round-bottom 96 well plate containing 80 µl of medium. Different concentrations of the test drug, in 20 µl volumes, were added in triplicate to the respective wells. The plates were then incubated over a period of seven days at 37°C in a humidified atmosphere of 5% CO_2.

Growth inhibition by different concentrations of the drugs was measured with the MTT assay (Twentyman *et al*, 1992) and calculated as the IC_{50} value (defined as the molar drug concentration required to inhibit cell growth by 50%) for each drug, using linear regression analysis.

2.2.6 Statistical analysis:

The results are expressed as the mean ± standard error of the mean (SEM) for between 3 and 5 experiments, with at least 3 replicates for each concentration of the test agents or control systems in each experiment. Levels of statistical significance were calculated using the Student's paired t-test. Differences were considered significant if the probability value was less than 0.05.

2.3 RESULTS

The results of the evaluation of the optimal cell concentration to be used in the different studies are shown in Figure 2.1. In Figures 2.2 and 2.3 the cytotoxic evaluation of the different metal-based complexes are shown. Metal based complexes with similar cytotoxicities compared to that of cisplatin are presented in Table 2.1, and a comparison of the IC_{50} values

of the rhodium and the iridium complexes that have the same chemical structure is given in Table 2.2.

2.3.1 Evaluation of optimal cell concentration for cytotoxicity and radiosensitivity studies:

Optical density (OD) readings taken from the spectrophotometer were in the range 0.35– 0.8, for all concentrations between 100 and 600 cells / 0.5 ml, with a reading of 0.8 representing a confluent cell population. An exponentially growing cell culture was achieved with a cell concentration of 300 cells / 0.5 ml complete medium incubated over a period of seven days (Figure 2.1).

2.3.2 Cytotoxicities of cisplatin, Rh, Fe and Ir complexes:

The cytotoxic activity of cisplatin, the ferrocene betadiketones and the rhodium-ferrocene complexes using the MTT assay is shown in Figure 2.2. Of all the complexes tested, only [Rh(fctca)(cod)] and [Rh(fctfa)(cod)] compared favourably with cisplatin. The free betadiketone ligands coupled to these two Rh(I) complexes are Hfctca and Hfctfa. Hfctfa per se also showed significant cytotoxicity (Table 2.1.).

The cytotoxic activities of all the iridium complexes are shown in Figure 2.3. Table 2.2 summarizes the IC_{50} values of [Rh(fch)(cod)], [Rh(fctfa)(cod)], [Rh(fca)(cod)] and [Rh(bfcm)(cod)] compared to their iridium counterparts. All the iridium complexes showed similar cytotoxicities when compared to their rhodium compliments with the most active rhodium complex [Rh(fctfa)(cod)] (IC_{50} =1.38 ± 0.04) exhibiting a greater toxicity than its iridium counterpart [Ir(fctfa)(cod)] (IC_{50} =4.18 ± 0.01).

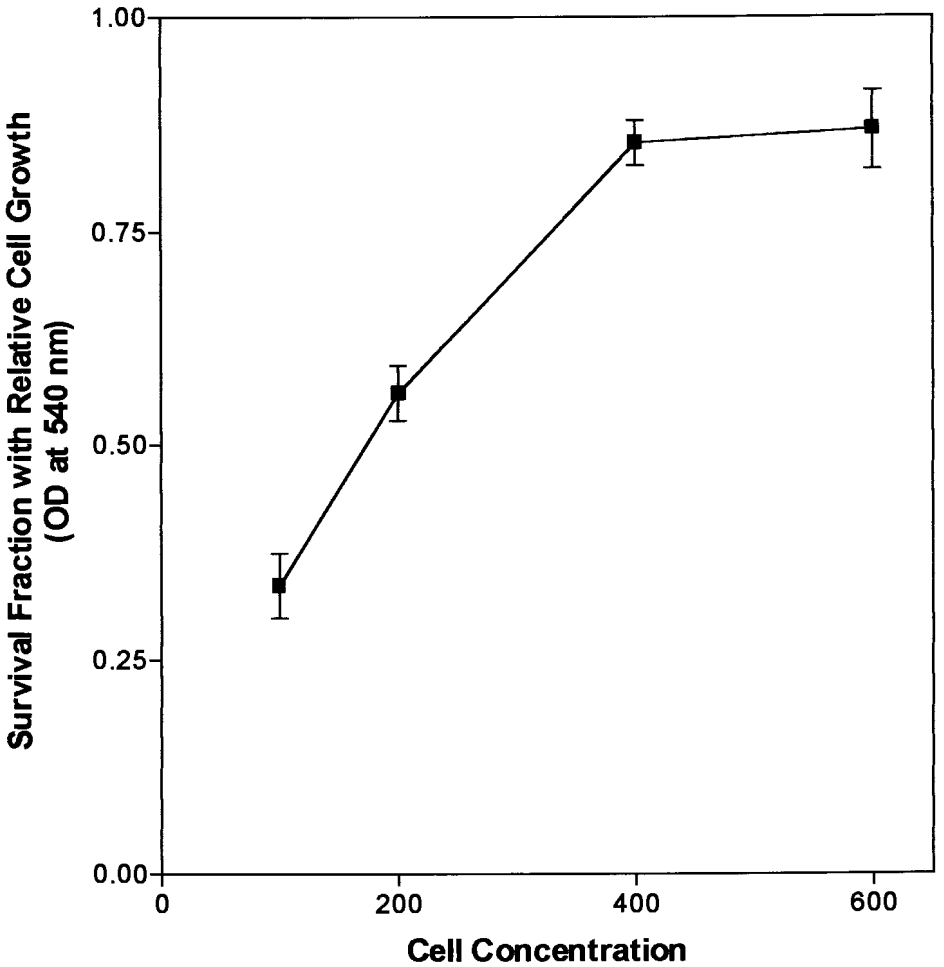


Figure 2.1: Exponential growth curve of the CHO cell line. Cell concentrations are given as the number of cells / 0.5 ml medium. Each end point represents the mean of three experiments ± standard error of the mean (SEM).

Table 2.1. The cytotoxic effects of cisplatin, Hfctca, [Rh(fctca)(cod)], Hfctfa and [Rh(fctfa)(cod)] on Chinese hamster ovary (CHO) cells.

Metal Complex	**IC_{50} (μM)**
Cisplatin	1.21 ± 0.25
Hfctca	8.64 ± 0.14*
[Rh(fctca)(cod)]	0.79 ± 0.14
Hfctfa	2.72 ± 0.25*
[Rh(fctfa)(cod)]	1.38 ± 0.04

The results are expressed as the mean drug concentration (μM) ± SEM causing 50% inhibition in growth (IC_{50}) of 3-5 experiments done in triplicate.

* Significantly different from treatment with cisplatin; $p \leq 0.01$

Table 2.2. The cytotoxic effects of [Rh(fch)(cod)], [Rh(fctfa)(cod)], [Rh(fca)(cod)], [Rh(bfcm)(cod)], and their irridium counterparts on Chinese hamster ovary (CHO) cells.

Metal Complex	IC_{50} (μM)
[Rh(fch)(cod)]	8.53 ± 2.75
[Ir(fch)(cod)]	2.40 ± 0.24
[Rh(fctfa)(cod)]	1.38 ± 0.04
[Ir(fctfa)(cod)]	4.18 ± 0.01
[Rh(fca)(cod)]	14.74 ± 4.4
[Ir(fca)(cod)]	4.87 ± 0.29
[Rh(bfcm)(cod)]	15.32 ± 2.6
[Ir(bfcm)(cod)]	6.13 ± 0.49

The results are expressed as the mean drug concentration (μM) ± SEM causing 50% inhibition in growth (IC_{50}) of 3-5 experiments done in triplicate.

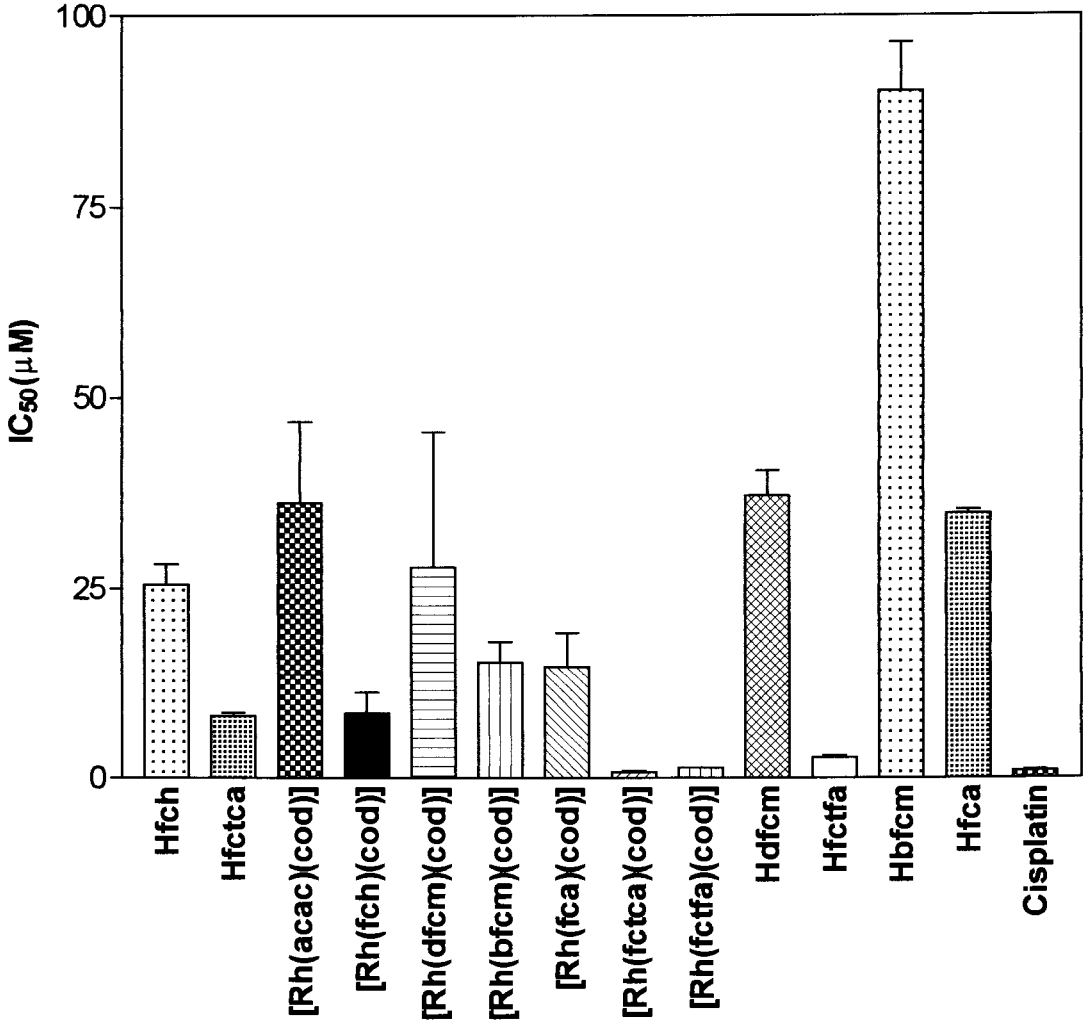


Figure 2.2: The effects of cisplatin, the ferrocene betadiketones and the rhodium - ferrocene complexes on the growth of CHO cells. The cytotoxic activity is expressed as the concentration (μM) at which cells showed a 50% inhibition in growth (IC_{50}). Each end point represents the mean of 3-6 experiments ± standard error of the mean (SEM).

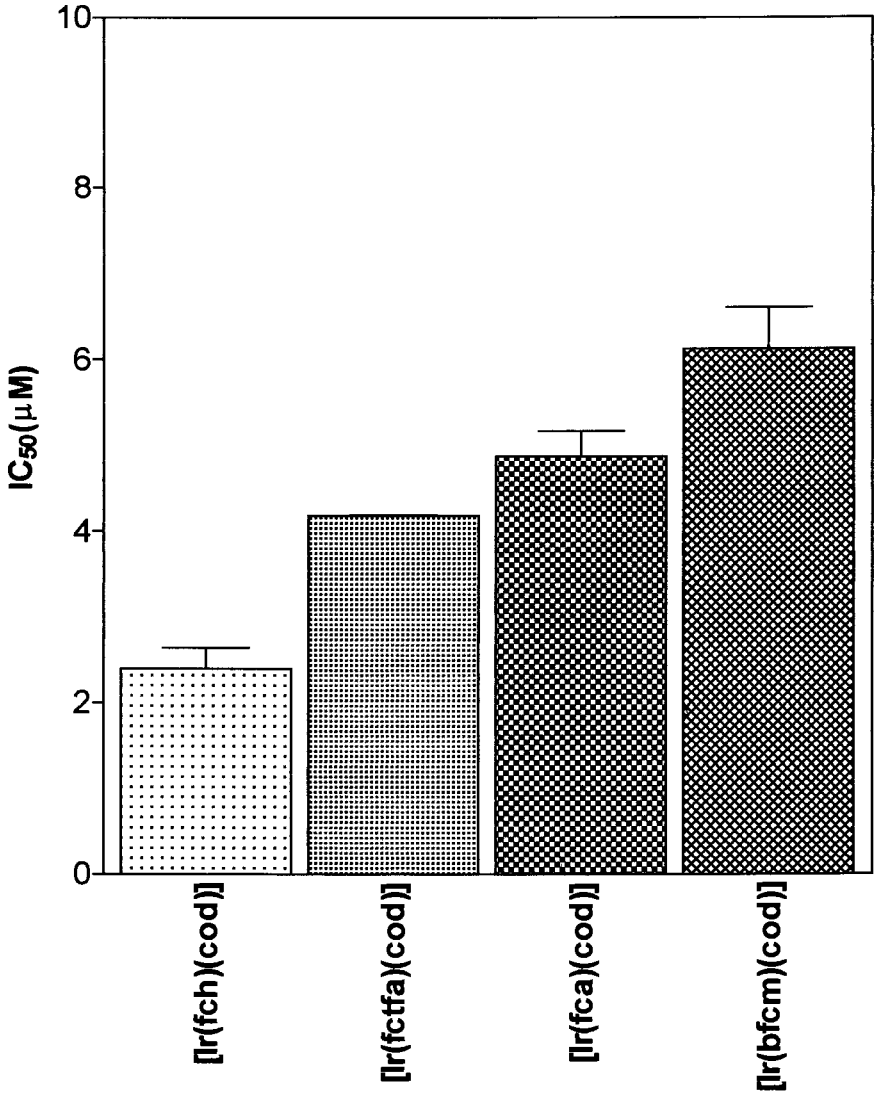


Figure 2.3: The effects of the iridium complexes on the growth of CHO cells. The cytotoxic activity is expressed as a concentration (μM) at which cells showed a 50% inhibition in growth (IC_{50}). Each end point represents the mean of 3-6 experiments ± standard error of the mean (SEM).

2.4 DISCUSSION

With the use of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide, a rapid and quantitative colourimetric assay of cell survival and proliferation is possible. The results can be read on a multiwell scanning spectrophotometer (ELISA plate reader) and show a high degree of precision (Mosmann, 1983). This method has also been successfully used by others (Mosmann, 1983; Alley *et al*, 1988 and Heo *et al*, 1990) in screening large numbers of drugs for cytotoxic activity against different murine and human cancer cell lines.

Since most of the experimental work done in this chapter entailed procedures with incubation periods of 6 days or more, it was essential that an optimal cell concentration was chosen to ensure that confluency was not reached before the completion of the incubation period. By making use of MTT, cell proliferation of the CHO cell line was assayed and it was found that a cell concentration of 600 cells / ml guaranteed exponential cell growth in attached cultures.

Furthermore, the MTT assay was used to screen cisplatin and the novel iridium, rhodium and ferrocene complexes for cytotoxic activity against the CHO cell line. Published IC_{50} concentrations of cisplatin used in the survival response assay of different cell lines, have values of 0.7 μM for the murine RIF1 tumour cell line after a 2 h incubation (Van de Vaart *et al*, 1997) and 2.1 μM (2h incubation) for the L1210 murine leukaemia cell line (Ormerod *et al*, 1994). Values of 5 μM after a 1 h exposure to cisplatin for V79 cells (Korbelik and Skov, 1989) and 1.5 μM for the same cells were noted after a 5h exposure (unpublished data, courtesy of the NAC, see Chapter 4). A direct comparison between our results and that published is not possible, since it is quite clear that drug toxicity is cell-type dependant and a function of exposure time, but our data (IC_{50} of 1.2 ± 0.25 for cisplatin remaining in the system for the total duration of the experiment) is in agreement with that found by the NAC using the V79 cell line.

Further results obtained from the cytotoxicity analyses, show that some of the rhodium complexes, namely [Rh(fctca)(cod)] and [Rh(fctfa)(cod)] compared favourably to that of cisplatin. This is indeed a promising finding, since in the literature very few notable cytotoxic effects for other rhodium complexes have been reported (Giraldi *et al*, 1974; Craciunescu *et al*, 1985; Craciunescu *et al*, 1989; Craciunescu *et al*, 1991).

The cytotoxicity of the iridium complexes tested in this study were not significantly different to the rhodium analogs having the same chemical structures. Sava *et al* (1987) on the other hand reported that Rh(I) complexes of the type $[Rh(cod)(N\text{-}N\text{-}R)]^{+}Cl^{-}$ ($R=CH_3, C_2H_5, iC_3H_7$) are more active than the Ir(I) analogs.

These studies so far indicate that [Rh(fctfa)(cod)] and [Rh(fctca)(cod] have similar cytotoxic activity to that of cisplatin and that the IC_{50} value obtained for cisplatin in this study is well within those of other published data. Furthermore, the ferrocene complexes, Hfctca and Hfctfa co-ordinated to the above mentioned rhodium complexes themselves showed considerable cytotoxic activity. The iridium complex having the same chemical structure as [Rh(fctfa)(cod)] also showed marked cytotoxicity.

In conclusion, the MTT assay is versatile and quantitative and is a significant advance over traditional techniques used for proliferation and cytotoxicity assays (Mosmann, 1983, Carmichael *et al*, 1987a). Furthermore, this assay has also been successfully used in the assessment of radiosensitivity of both murine and human cell lines in the presence of a range of cytotoxic drugs (Carmichael *et al*, 1987b; Stratford and Stephens, 1989 and Price and McMillan, 1990).

CHAPTER 3: THE EFFECT OF CISPLATIN AND METAL COMPLEXES OF Fe, Rh AND Ir ON AEROBIC AND HYPOXIC CHO CELLS IN THE PRESENCE OF AN 8MV PHOTON BEAM.

3.1. INTRODUCTION

The clonogenic assay is the traditionally established method of choice in measuring radiation cell survival, the MTT assay has however, found a new application in this regard (Carmichael *et al*, 1987b and Price and McMillan, 1990). In order for radiation cell survival to be comparable to MTT absorbency, the MTT assay has to be optimized for each cell line (Price and McMillan, 1990). This involves the use of low cell numbers to ensure exponential growth and optimal MTT concentrations and incubation times for a particular cell number. Optical density (OD) readings thus obtained will be proportional to the number of surviving, metabolizing cells (OD values are in the range 0.15–1.5) (Stratford and Stephens, 1989). Therefore, in cytotoxicity experiments, with no significant cytotostatic effect, OD is proportional to the surviving fraction over the first order of magnitude of cell survival (Carmichael *et al*, 1987a; Carmichael *et al*, 1987b).

When complying with all of these prerequisites, the MTT assay has been found to generate comparable results to those achieved using a clonogenic assay (Hall *et al*, 1975; Mohindra and Rauth, 1976; Moore *et al*, 1976; Adams *et al*, 1980; Stratford *et al*, 1986; Zeman *et al*, 1986). Carmichael *et al* (1987b), Stratford and Stephens (1989) and Price and McMillan (1990) all concluded from their investigation into the use of the MTT assay in the response of cells to ionizing radiation, that it is of great value in screening large numbers of potential radiosensitizers or protectors.

Results from both the MTT and conventional clonogenic assay were used to generate cell survival curves with different drug concentrations at relevant therapeutic doses (1 - 10 Gy). All the drugs tested for their radiosensitizing abilities were used at concentrations that did not inhibit more than 30% of the cell growth.

By making use of a known hypoxic cell sensitizer such as cisplatin, it is possible to compare results obtained in the present study with those published previously. Only metal complexes with cytotoxic properties comparable to cisplatin (see Chapter 2, Table 2.1) were tested for possible radiosensitization.

Previous studies (Nais, 1985; Dewitt, 1987; Douple, 1988) have found that cisplatin exhibits moderate improvement in the kill-rate of irradiated hypoxic cells (maximum enhancement ratios – ERs, of the order 1.3). These studies however, were based on data obtained using conventional clonogenic techniques at large radiation doses which have been shown to be inaccurate at therapeutically relevant doses (Bedford and Griggs, 1975). Korbelik and Skov (1989) reported ERs for cisplatin (at 2.5 and 1.0 μmol dm^{-3} for hypoxic V79 cells) of 2.2 and 1.7 respectively at a therapeutically relevant radiation dose range (1-3 Gy). Korbelik and Skov (1989) also reported that the oxic interaction of cisplatin disappears at low doses (ERs of 1.1 – 1.0). Cisplatin is therefore a more effective radiosensitizer at low radiation doses in hypoxic cells. Whereas electron affinic sensitizers such as the nitroimidazoles (Palcic *et al*, 1984; Skarsgard *et al*, 1986; Watts *et al*, 1986) and even oxygen itself are less effective in the clinically relevant radiation dose range (Korbelik and Skov, 1989; Skov and MacPhail, 1991).

3.2. MATERIALS AND METHODS

3.2.1 Experimental Equipment and Procedures:

The CHO cells were irradiated at room temperature (20°C) with an 8 MV photon beam using a Linear Accelerator (Phillips Linac) at a dose rate of approximately 1.0 Gy/MU at the depth of maximum dose (d_{max}).

A unique method using a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, California, USA) to establish an aerobic or hypoxic environment in which the CHO cells were irradiated, was developed. The modular incubator chamber was adapted by adding a tissue equivalent wax buildup of 2cm to the bottom. This was done to simplify the dosimetry, in that the depth of maximum dose for an 8 MV photon beam, occurs at 2cm.

A polystyrene disk was placed on top of the buildup material with five cut-outs, each with a diameter of 5cm, in which the glass test tubes were bunched together. A field size of 6 x 6 cm^2 was selected to cover the area of each cut-out. The bunched glass test tubes were then in turn radiated with doses ranging from 1 – 10 Gy. The procedure is illustrated in Figures 3.1 and 3.2.

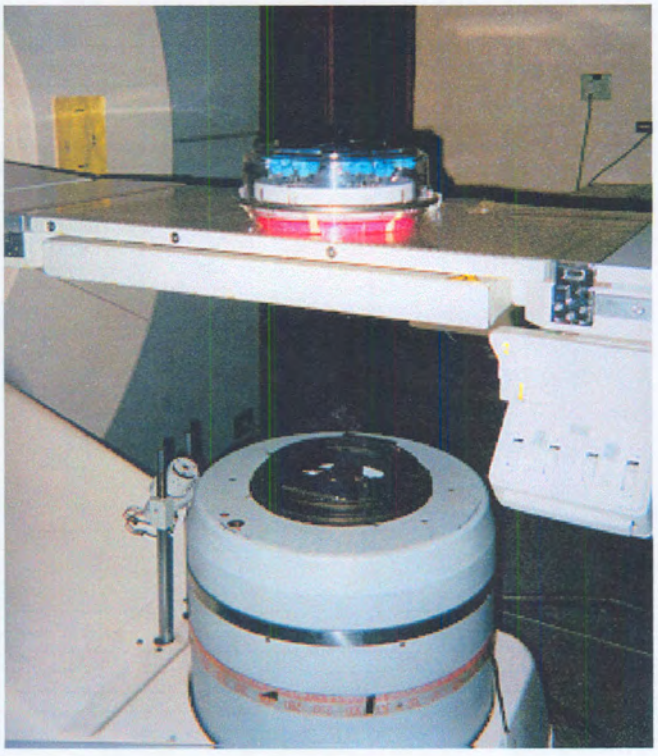


Figure 3.1. The modular incubator chamber was placed on the radio-lucent section of the table, the gantry positioned from below at a SSD (source-to-surface distance) of 100cm with d_{max} at 2cm. The roof-mounted laser was used to centre each cut-out.

Figure 3.2. The top view of the modular incubator chamber shows the glass test tubes bunched together in each of the five cut-outs. Each cut-out corresponds to a specific dose point, with dose points ranging from 1-10 Gy.

The accuracy of this setup was confirmed by an x-ray taken. The position of the five cut-outs was marked on the x-ray film and each in turn irradiated. The coincidence of the marked field and the radiation field ensured that the radiation field amply covers, and does not overlap, the groups of glass tubes bunched together in the cut-outs.

3.2.2 Determination of the OER using the modular incubator chamber:

The OER ratio for the CHO cells using a modular incubator chamber was determined as follows:

CHO cells were harvested from confluent tissue culture flasks using a trypsin/ versene mixture. The cells were seeded into glass test tubes at a concentration of 600 cells/ml, in a total volume of 0.5 ml complete medium. It is important to note at this point that only glass tubes can be used when conducting cytotoxicity experiments with drugs that are activated under hypoxic conditions. This is because oxygen is soluble in tissue culture plastic and is slowly released into the surrounding environment when the external O_2 tension decreases (Chapman, 1980).

The test tubes were left overnight in an incubator at 37°C in a humidified atmosphere of 5% CO_2 to allow the cells to adhere to the glass surface. After the incubation period the test tubes were placed inside the modulator incubator chamber.

To achieve an aerobic environment the unsealed modular incubator chamber was placed inside the incubator and gently shaken on an orbital shaker for 35 min. The shaker was switched off and the chamber left inside the incubator for a further hour before being sealed and taken to be radiated. Each group of bunched glass test tubes received one of the following doses; 1, 2, 4, 6 or 10 Gy, while a separate modular incubator chamber containing tubes treated in a similar fashion but left un -irradiated was used as a control. After irradiation the glass test tubes were removed from the chamber and placed in an incubator for a period of 6 days.

Hypoxia was achieved by following the same procedure as described above except that during the first 35 min incubation period a mixture of 5% CO_2 and 95% N_2 gas was passed through the chamber after which it was sealed.

After the 6 day incubation period, cell survival was ascertained using the MTT assay method described in Chapter 2, paragraph 2.2.2. The survival data thus generated can be represented by the linear-quadratic relationship

$$S = e^{-\alpha D - \beta D^2}$$

Where S is the fraction of cells surviving at a dose D and α and β are constants representing the linear and quadratic components of cell killing (Hall, 1994). All consequent survival data, unless otherwise stated, have been fit to the linear quadratic (LQ) model.

3.2.3 The effect of cisplatin and metal complexes of Fe, Rh and Ir on the survival of aerobic and hypoxic CHO cells in the presence of an 8MV photon beam determined by the MTT assay:

Drug aliquots were diluted in complete medium directly before being added to the test tubes. None of the final concentration of any of the drugs used showed an inhibition of cell growth greater than 30%. In all the experiments conducted, aerobic and hypoxic cells were incubated in the presence of the drug for a period of 1 hour and 35 min prior to radiation and the drug remained in the system until the end point was determined by the MTT assay as previously described.

3.2.4 The effect of cisplatin and [Rh(fctfa)(cod)] on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam determined by the clonogenic assay:

Cells were harvested as described in paragraph 3.2.2 but in this case glass petri dishes (6 cm, Anumbra) instead of glass test tubes were used. In each petri dish already containing 3.5 ml complete medium, 300 cells / ml per dose

point were seeded. A total volume of 0.50 ml of the drug or solvent control was added to each petri dish directly prior to the dishes being made aerobic or hypoxic by the method described in paragraph 3.2.2. A concentration of 0.78 μM of either cisplatin or [Rh(fctfa)(cod)] was added to the relevant petri dishes. To accommodate the petri dishes, the polystyrene disk was removed from the modular incubator chamber and the petri dishes placed directly on top of the build-up material with each dish representing a different dose point. The petri dishes were then irradiated as previously described and left to incubate for a period of six days.

On the sixth day, the remaining medium from the petri dishes was removed and the dishes rinsed with PBS. Fixative (methanol:acetic acid, 3:1) was added and left for ± 7 min, before being poured off and the crystal violet stain (0.02%) added and then left for ± 30 min before it was also poured off. The crystal violet was then gently washed from the cells with PBS, which left all viable colonies stained a dark blue colour. The petri dishes were then turned upside down to dry and only colonies of 50 or more cells on day 6 were scored. The results were corrected for plating efficiency (PE), with PE defined as the percentage of untreated cells seeded that grow into macroscopic colonies (Hall, 1994).

3.2.5 Statistical analysis:

The results are expressed as the mean ± standard error of the mean (SEM) for between 3 and 6 experiments, with at least 3 replicates for each concentration of the test agents or control systems in each. Levels of statistical significance were calculated using the Student's paired t-test. Differences were considered significant if the probability value was less than 0.05.

3.3. RESULTS

The OER calculated for CHO cells under hypoxia and normal aerated conditions is shown in Figure 3.3. The effects of cisplatin, Hfctca, [Rh(fctca)(cod)], Hfctfa, [Rh(fctfa)(cod)] and [Ir(fctfa)(cod)] on aerobic and hypoxic CHO cells, using the MTT assay method are respectively shown in Figures 3.4 - 3.15. A summary of each drug's fit parameters and dose modifying factors (DMF's) are given in Tables 3.1 – 3.12. The effect of cisplatin and [Rh(fctfa)(cod)] on aerobic and hypoxic CHO cells using the clonogenic assay method are respectively shown in Figures 3.16 - 3.19. The survival fit parameters for the clonogenic data are tabulated in Table 3.12 and the DMF's are summarised in Table 3.13.

3.3.1 Determination of the OER using the modular incubator chamber:

The data from the MTT assay was analyzed using the software program, Graph Pad Prism (Version 2). The cell survival fraction (S), relative to that of the control samples, for cells treated with a dose (D) of radiation, was fitted to a linear – quadratic equation as $\ln (S) = -\alpha D - \beta D^2$, where α and β are inactivation parameters estimated from a least squared fit. The β value determined for the aerobic curve proved to be negative but not significantly different from zero, it was consequently set to zero as a negative β coefficient has no biophysical meaning, and the α value recalculated. An α value of 0.3095 ± 0.01 Gy $^{-1}$ was calculated (R^2 of 0.91) for the aerobic curve and α and β values calculated for the hypoxic curve were 0.059 ± 0.02 Gy $^{-1}$ and 0.001 ± 0.002 Gy $^{-1}$ respectively (R^2 of 0.66). An OER of 2.34 was obtained, calculated as the ratio of the mean inactivation dose ($\bar{D}$) of the two curves. Kellerer and Hug (1972) first introduced the concept of $\bar{D}$ as a measure of radiosensitivity where $\bar{D}$ is proportional to the area under the survival curve plotted in linear coördinates. For the definition and calculation of the mean

inactivation dose refer to Appendix 1. The aerobic and hypoxic survival curves are shown in Figure 3.3.

3.3.2 The effect of cisplatin and metal complexes of Fe, Rh and Ir on the survival of aerobic and hypoxic CHO cells in the presence of an 8MV photon beam determined by the MTT assay:

(i) The effect of cisplatin on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam:

Cisplatin did not exhibit any radiosensitization effect for aerobic CHO cells (Figure 3.4) while at the same concentrations (0.39 and 0.78 μM) cisplatin sensitized hypoxic CHO cells to radiation (Figure 3.5). The LQ model fit parameters and dose modifying factors (DMF's) measured are given in Table 3.1 and Table 3.2 respectively.

For the treatment of hypoxic CHO cells with cisplatin, a DMF of 1.99 ± 0.02 at a very low non-toxic concentration of 0.78 μM was obtained, while at an even lower concentration of 0.39 μM a still noteworthy DMF of 1.41 ± 0.02 was seen. This is in agreement with what was observed by Skov and MacPhail (1991), using the clonogenic technique as endpoint, who reported enhancement ratios (ERs) up to 2.2, for hypoxic V79 cells at concentrations of 1 – 2.5 μM for low doses (in the therapeutic range ≤ 3 Gy). Skov and MacPhail (1991) reported very moderate to no sensitization of aerobic V79 cells (ER' s 1.1 – 1.0) however, we found that cisplatin showed moderate radioprotective qualities with DMF's of 1.06 ± 0.04 and 1.12 06 ± 0.04 seen for very low concentrations of 0.39 and 0.78 μM respectively. This is not the first time however that it has been reported that cisplatin exhibits radioprotecting characteristics (Zak and Drobnik, 1971).

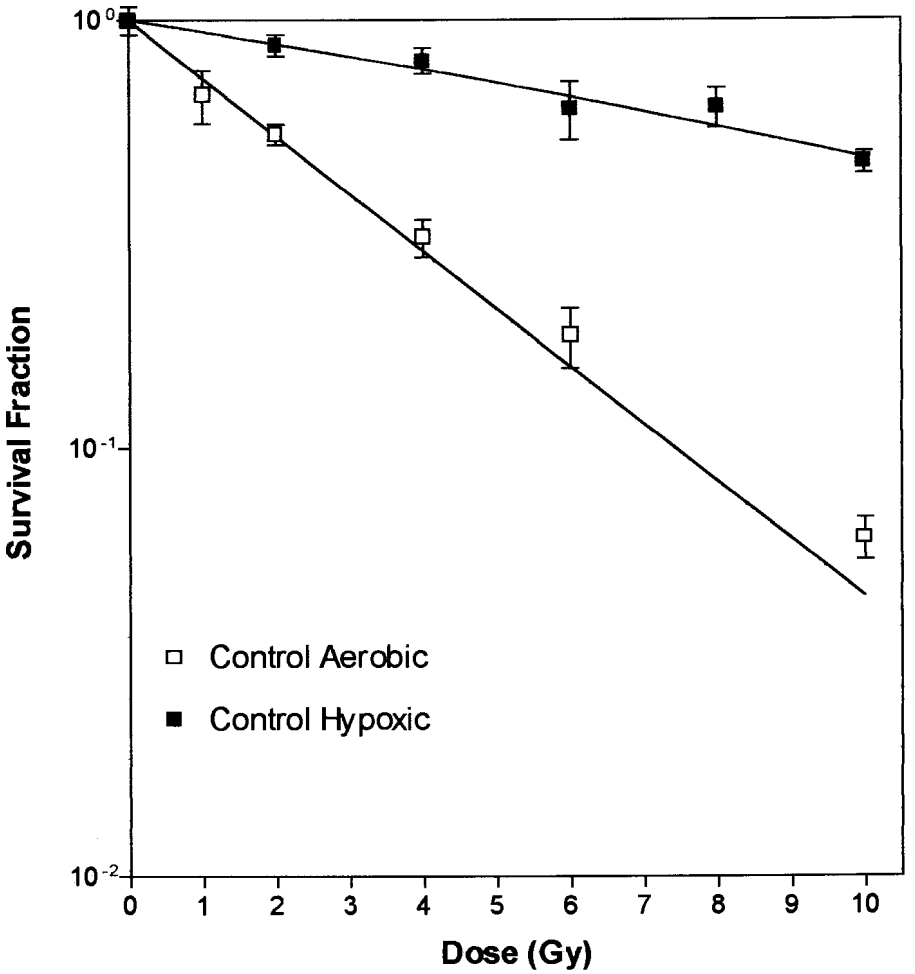


Figure 3.3. The cell survival fraction (S) was fitted to a linear quadratic equation as ln (S) = $-\alpha D-\beta D^2$. The OER was calculated as the ratio of the mean inactivation doses ($\overline{D}$) in Gy . Each end point represents the mean of three experiments ± standard error of the mean.

Table 3.1. MTT survival data of the effect of cisplatin on aerobic and hypoxic CHO cells represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.24 ± 0.04	0.004 ± 0.008	0.88
Aerobic cells + 0.39 μM Cisplatin	0.22 ± 0.01	-	0.87
Aerobic cells + 0.78 μM Cisplatin	0.21 ± 0.01	-	0.95
Hypoxic cells	0.06 ± 0.02	0.001 ± 0.002	0.66
Hypoxic cells + 0.39 μM Cisplatin	0.15 ± 0.01	-	0.75
Hypoxic cells + 0.78 μM Cisplatin	0.26 ± 0.01	-	0.82

The results represent the mean $\pm$ SEM of three to six experiments done in triplicate.

Table 3.2. Mean inactivation doses calculated from the response of CHO cells following radiation and treatment with different concentrations of cisplatin under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors (DMF's) are stated as the ratio of mean inactivation doses.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifying Factor (DMF)
Aerobic cells	3.79 ± 0.04	1.90 ± 0.04†
Aerobic cells + 0.39 μM Cisplatin	4.02 ± 0.01	1.06 ± 0.04*
Aerobic cells + 0.78 μM Cisplatin	4.25 ± 0.01	1.12 ± 0.04*
Hypoxic cells	7.24 ± 0.02	-
Hypoxic cells + 0.39 μM Cisplatin	5.14 ± 0.01	1.41 ± 0.02
Hypoxic cells + 0.78 μM Cisplatin	3.63 ± 0.01	1.99 ± 0.02

The results represent the mean ± SEM of three to five experiments done in triplicate.

†Oxygen Enhancement Ratio (OER)

*DMFs showing radioprotection

(ii) The effect of Hfctca, [Rh(fctca)(cod)], Hfctfa and [Rh(fctfa)(cod) on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon:

Hfctfa (1.56 and 3.125 μM) does not show significant sensitization of either aerobic (DMFs of 1.23 and 1.16) or hypoxic (DMFs of 1.10 and 1.18) CHO cells to radiation (Figures 3.6 and 3.7). The survival curve parameters and dose modifying factors (DMF's) measured are given in Tables 3.3 and 3.4.

Table 3.3. MTT survival data of the effect of Hfctca on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.29 ± 0.04	-	0.74
Aerobic cells + 1.56 μM Hfctca	0.36 ± 0.04	-	0.62
Aerobic cells + 3.125 μM Hfctca	0.34 ± 0.04	-	0.68
Hypoxic cells	0.17 ± 0.05	0.0004 ± 0.006	0.58
Hypoxic cells + 1.56 μM Hfctca	0.17 ± 0.02	-	0.91
Hypoxic cells + 3.125 μM Hfctca	0.22 ± 0.01	-	0.85

The results represent the mean ± SEM of three to six experiments done in triplicate.

Table 3.4. Mean inactivation doses calculated from the response of CHO cells following radiation and treatment with different concentrations of Hfctca under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors (DMF's) are stated as the ratio of mean inactivation doses.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifying Factor (DMF)
Aerobic cells	3.29 ± 0.04	1.50 ± 0.06[†]
Aerobic cells + 1.56 μM Hfctca	2.68 ± 0.04	1.23 ± 0.06
Aerobic cells + 3.125 μM Hfctca	2.84 ± 0.04	1.16 ± 0.06
Hypoxic cells	4.81 ± 0.05	-
Hypoxic cells + 1.56 μM Hfctca	4.39 ± 0.02	1.10 ± 0.05
Hypoxic cells + 3.125 μM Hfctca	4.06 ± 0.01	1.18 ± 0.05

The results represent the mean ± SEM of three to six experiments done in triplicate.

[†]Oxygen Enhancement Ratio (OER)

[Rh(fctca)(cod)] (0.19 and 0.39 μM) exhibited very moderate radioprotection properties for aerobic CHO cells (Figure 3.8) with DMFs of 1.06 and 1.22 measured respectively, it also showed very moderate sensitization of hypoxic CHO cells to radiation with both concentrations producing a DMF of 1.16 (Figure 3.9). At the same concentrations [Rh(fctca)(cod)] The survival fit parameters and dose modifying factors (DMF's) measured are given in Tables 3.5 and 3.6.

Table 3.5. MTT survival data of the effect of [Rh(fctca)(cod)] on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.29 ± 0.01	-	0.89
Aerobic cells + 0.19 μM [Rh(fctca)(cod)]	0.27 ± 0.01	-	0.81
Aerobic cells + 0.39 μM [Rh(fctca)(cod)]	0.23 ± 0.01	-	0.93
Hypoxic cells	0.09 ± 0.03	0.006 ± 0.004	0.65
Hypoxic cells + 0.19 μM [Rh(fctca)(cod)]	0.16 ± 0.01	-	0.54
Hypoxic cells + 0.39 μM [Rh(fctca)(cod)]	0.16 ± 0.01	-	0.62

The results represent the mean ± SEM of three to six experiments done in triplicate.

Table 3.6. Mean inactivation doses calculated from the response of CHO cells following radiation and treatment with different concentrations of [Rh(fctca)(cod)] under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors are stated as the ratio of mean inactivation doses.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifying Factor (DMF)
Aerobic cells	3.26 ± 0.01	1.75 ± 0.03†
Aerobic cells + 0.19 μM [Rh(fctca)(cod)]	3.47 ± 0.01	1.06 ± 0.01*
Aerobic cells + 0.39 μM [Rh(fctca)(cod)]	3.97 ± 0.01	1.22 ± 0.01*
Hypoxic cells	5.72 ± 0.03	-
Hypoxic cells + 0.19 μM [Rh(fctca)(cod)]	4.95 ± 0.01	1.16 ± 0.03
Hypoxic cells + 0.39 μM [Rh(fctca)(cod)]	4.92 ± 0.01	1.16 ± 0.03

The results represent the mean ± SEM of three to six experiments done in triplicate.

†Oxygen Enhancement Ratio (OER)

*DMFs showing radioprotection

Hfctfa (0.78 μM) did not sensitize aerobic cells to radiation with a DMF (1.01) not much greater than unity (Figure 3.10) while at the same concentration Hfctfa exhibited very moderate sensitization (DMF of 1.17) of hypoxic CHO cells to radiation (Figure 3.11). The dose LQ fit parameters and modifying factors (DMF's) measured are given in Tables 3.7 and 3.8.

Table 3.7. MTT survival data of the effect of Hfctfa on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.27 ± 0.01	-	0.78
Aerobic cells + 0.78 μM Hfctfa	0.26 ± 0.01	-	0.80
Hypoxic cells	0.13 ± 0.03	0.001 ± 0.004	0.58
Hypoxic cells + 0.78 μM Hfctfa	0.17 ± 0.01	-	0.68

The results represent the mean ± SEM of three to six experiments done in triplicate.

Table 3.8. Mean inactivation doses calculated from the response of aerobic and hypoxic CHO cells following radiation and treatment with different concentrations of Hfctfa under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors (DMF's) are stated as the ratio of mean inactivation doses.

Treatment	**Mean Inactivation Dose (Gy)**	**Dose Modifying Factor (DMF)**
Aerobic cells	3.46 ± 0.01	1.65 ± 0.03[†]
Aerobic cells + 0.78 μM Hfctfa	3.51 ± 0.01	1.01 ± 0.01
Hypoxic cells	5.68 ± 0.03	-
Hypoxic cells + 0.78 μM Hfctfa	4.84 ± 0.01	1.17 ± 0.03

The results represent the mean ± SEM of three to six experiments done in triplicate.

[†]Oxygen Enhancement Ratio (OER)

[Rh(fctfa)(cod)] exhibits very moderate (DMFs of 1.09 and 1.10 for 0.39 and 0.78 μM respectively) radioprotecting ability of aerobic CHO cells (Figure 3.12) while sensitizing hypoxic CHO cells to radiation (Figure 3.13) while. A very significant DMF of 1.93 was obtained for the hypoxic treated (0.78 μM) cells while at an even lower concentration (0.39 μM) a still noteworthy DMF of 1.53 was observed. The fit parameters of the survival curves generated and the dose modifying factors (DMF's) measured are given in Tables 3.9 and 3.10.

Table 3.9. MTT survival data of the effect of [Rh(fctfa)(cod)] on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.30 ± 0.01	-	0.91
Aerobic cells + 0.39 μM [Rh(fctfa)(cod)]	0.27 ± 0.01	-	0.91
Aerobic cells + 0.78 μM [Rh(fctfa)(cod)]	0.27 ± 0.02	-	0.63
Hypoxic cells	0.04 ± 0.02	0.004 ± 0.003	0.74
Hypoxic cells + 0.39 μM [Rh(fctfa)(cod)]	0.16 ± 0.01	-	0.63
Hypoxic cells + 0.78 μM [Rh(fctfa)(cod)]	0.22 ± 0.01	-	0.71

The results represent the mean ± SEM of three to six experiments done in triplicate.

Table 3.10. Mean inactivation doses calculated from the response of CHO cells following radiation and treatment with different concentrations of [Rh(fctfa)(cod)] under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors are stated as the ratio of mean inactivation doses.

Treatment	**Mean Inactivation Dose (Gy)**	**Dose Modifying Factor (DMF)**
Aerobic cells	3.13 ± 0.01	2.40 ± 0.02[†]
Aerobic cells + 0.39 μM [Rh(fctfa)(cod)]	3.44 ± 0.01	1.09 ± 0.01*
Aerobic cells + 0.78 μM [Rh(fctfa)(cod)]	3.45 ± 0.02	1.10 ± 0.02*
Hypoxic cells	7.52 ± 0.02	-
Hypoxic cells + 0.39 μM [Rh(fctfa)(cod)]	4.93 ± 0.01	1.53 ± 0.02
Hypoxic cells + 0.78 μM [Rh(fctfa)(cod)]	3.89 ± 0.01	1.93 ± 0.02

The results represent the mean ± SEM of three to six experiments done in triplicate.

[†]Oxygen Enhancement Ratio (OER)

*DMFs showing radioprotection

(iii) The effect of [Ir(fctfa)(cod)] on the survival of aerobic and hypoxic CHO cells in the presence of an 8MV photon beam:

Virtually no enhancement at a concentration of 0.78 μM of either aerobic (DMF of 1.04) or hypoxic (DMF of 1.06) cells were seen (Figures 3.14 and 3.15). The LQ fit parameters and DMFs measured are given in Table 3.11 and 3.12.

Table 3.11. MTT survival data of the effect of [Ir(fctfa)(cod)] on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.32 ± 0.01	-	0.93
Aerobic cells + 0.39 μM [Ir(fctfa)(cod)]	±	-	
Aerobic cells + 0.78 μM [Ir(fctfa)(cod)]	0.34 ± 0.01	-	0.93
Hypoxic cells	0.13 ± 0.01	-	0.63
Hypoxic cells + 0.39 μM [Ir(fctfa)(cod)]	±	-	
Hypoxic cells + 0.78 μM [Ir(fctfa)(cod)]	0.15 ± 0.07	-	0.60

The results represent the mean ± SEM of three to six experiments done in triplicate.

Table 3.12. Mean inactivation doses calculated from the response of CHO cells following radiation and treatment with different concentrations of [Ir(fctfa)(cod)] under aerobic or hypoxic conditions determined by the MTT assay. Dose modifying factors (DMF's) are stated as the ratio of mean inactivation doses.

Treatment	**Mean Inactivation Dose (Gy)**	**Dose Modifying Factor (DMF)**
Aerobic cells	2.98 ± 0.01	1.85 ± 0.01[†]
Aerobic cells + 0.39 μM [Ir(fctfa)(cod)]		
Aerobic cells + 0.78 μM [Ir(fctfa)(cod)]	2.86 ± 0.01	1.04 ± 0.01
Hypoxic cells	5.50 ± 0.01	-
Hypoxic cells + 0.39 μM [Ir(fctfa)(cod)]		
Hypoxic cells + 0.78 μM [Ir(fctfa)(cod)]	5.21 ± 0.07	1.06 ± 0.07

The results represent the mean ± SEM of three to six experiments done in triplicate.

[†]Oxygen Enhancement Ratio (OER)

3.3.3 *The effect of cisplatin and [Rh(fctfa)(cod)] on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam determined by the clonogenic assay:*

Traditionally radiation cell survival is measured using a clonogenic assay. Both cisplatin and the Rh complex, [Rh(fctfa)(cod)] showed promising radiation sensitizing potential using the MTT assay, and these results were confirmed with the clonogenic assay (Figures 3.16 to 3.19). In all the clonogenic experiments a single concentration of 0.78 μM was used for both cisplatin and [Rh(fctfa)(cod)] to confirm their radiosensitizing potential of aerobic and hypoxic CHO cells.

(i) The effect of cisplatin on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam:

Plating efficiencies taken as the average of each experiment ranged from 81% to 91% for aerobic and hypoxic CHO cells. The survival fraction (SF) at each dose point is determined by counting the number of macroscopic colonies as a fraction of the cells seeded, corrected for PE. The SF's were also corrected for toxicity. The fit parameters of the generated survival curves are summarised in Table 3.13, while the dose modifying effect of cisplatin is tabulated in Table 3.14.

Cisplatin showed very moderate sensitization of aerobic cells (Figure 3.16) with a DMF of 1.17 noted, however for hypoxic CHO cells a noteworthy DMF of 1.46 ± 0.07 (Figure 3.17) was observed. This agrees well with other published dose modification factors (1.2 - 2) measured for similar concentrations of cisplatin (Chibber et al, 1985, Van De Vaart et al, 1997).

Table 3.13. Clonogenic survival data of the effect of cisplatin on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.15 ± 0.06	0.035 ± 0.006	0.96
Aerobic cells + 0.78 μM Cisplatin	0.25 ± 0.07	0.028 ± 0.007	0.95
Hypoxic cells	0.005 ± 0.04	0.012 ± 0.004	0.85
Hypoxic cells + 0.78 μM Cisplatin	0.03 ± 0.07	0.025 ± 0.008	0.94

The results represent the mean ± SEM of three to six experiments.

Table 3.14. Dose modifying factors, expressed as the ratio of mean inactivation doses obtained from aerobic and hypoxic CHO cells irradiated with an 8 MV photon beam in the absence and presence of 0.78 μM cisplatin, determined by the clonogenic assay.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifyinng Factor (DMF)
Aerobic cells	3.17 ± 0.06	2.19 ± 0.06[†]
Aerobic cells + 0.78 μM Cisplatin	2.71 ± 0.07	1.17 ± 0.09
Hypoxic cells	6.95 ± 0.00	-
Hypoxic cells + 0.78 μM Cisplatin	4.75 ± 0.07	1.46 ± 0.07

The results represent the mean ± SEM of three to five experiments.

[†]Oxygen Enhancement Ratio (OER)

(ii) The effect of [Rh(fctfa)(cod)] on the survival of aerobic and hypoxic CHO cells in the presence of an 8 MV photon beam:

Plating efficiencies taken as the average of each experiment ranged from 87% to 92% for aerobic and hypoxic CHO cells. The survival levels were corrected for toxicity of the drugs. The fit parameters of the generated survival curves are summarised in Table 3.15, while the dose modifying effect of [Rhfctfa)(cod)] is tabulated in Table 3.16.

[Rhfctfa)(cod)] at a very low non-toxic concentration of 0.78 μM produced noteworthy DMFs for both aerobic (1.55) and hypoxic (1.80) treated cells (Figures 3.18 and 3.19). A similar result has been noted by Chibber *et al* (1985) where the complex Rh(II)methoxyacetate increased the radiation sensitivity of cells under both gaseous conditions with the greater effect being observed in hypoxia.

Table 3.15. Clonogenic survival data of the effect of [Rh(fctfa)(cod)] on aerobic and hypoxic CHO cells, represented by the LQ model, where α and β are constants representing the linear and quadratic components of cell killing and goodness of fit is given by the R^2 parameter.

Treatment	α	β	R^2
Aerobic cells	0.27 ± 0.07	0.03 ± 0.01	0.91
Aerobic cells + 0.78 μM [Rh(fctfa)(cod)]	0.19 ± 0.11	0.02 ± 0.02	0.85
Hypoxic cells	0.04 ± 0.05	0.01 ± 0.01	0.78
Hypoxic cells + 0.78 μM [Rh(fctfa)(cod)]	0.20 ± 0.11	0.02 ± 0.01	0.96

The results represent the mean ± SEM of three to five experiments.

Table 3.16. Dose modifying factors, expressed as the ratio of mean inactivation doses obtained from aerobic and hypoxic CHO cells irradiated with an 8 MV photon beam in the absence and presence of 0.78 μM [Rh(fctfa)(cod)], determined by the clonogenic assay.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifyinng Factor (DMF)
Aerobic cells	2.55 ± 0.06	2.42 ± 0.08[†]
Aerobic cells + 0.78 μM [Rh(fctfa)(cod)]	1.65 ± 0.11	1.55 ± 0.13
Hypoxic cells	6.17 ± 0.05	-
Hypoxic cells + 0.78 μM [Rh(fctfa)(cod)]	3.42 ± 0.11	1.80 ± 0.12

The results represent the mean ± SEM of to five experiments.

[†]Oxygen Enhancement Ratio (OER)

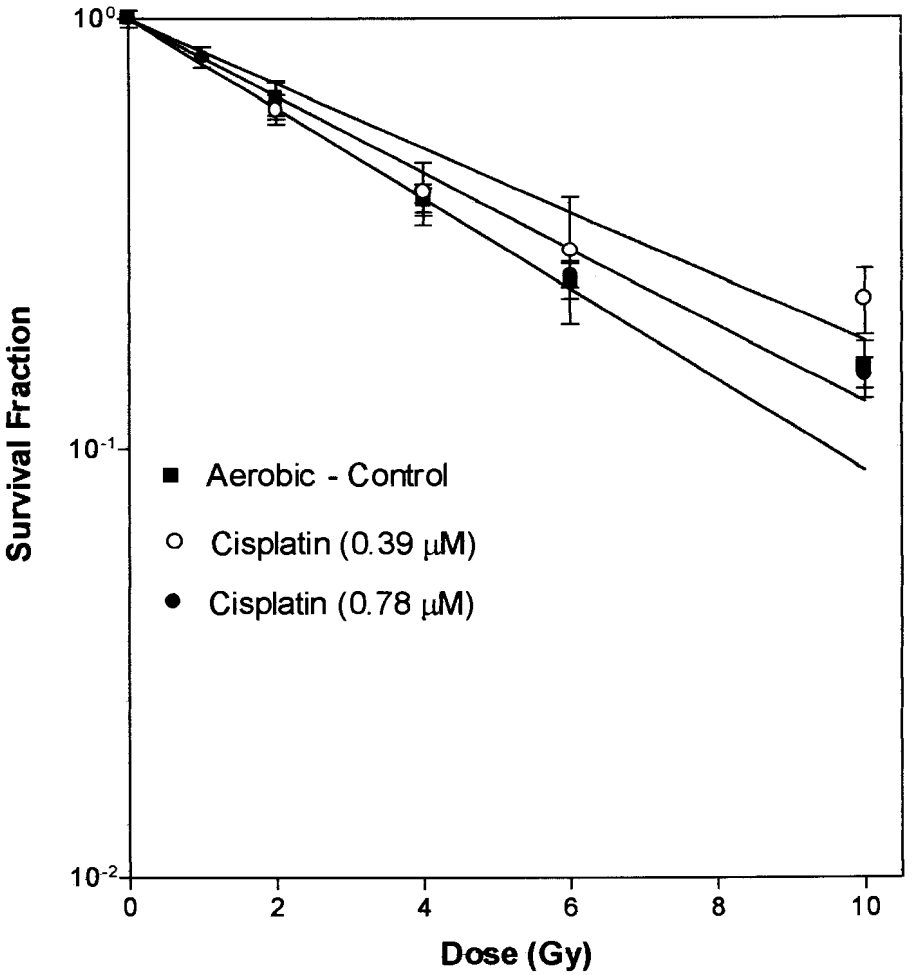


Figure 3.4 Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 0.39μM (○) and 0.78μM (●) cisplatin. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of six experiments ± standard error of the mean.

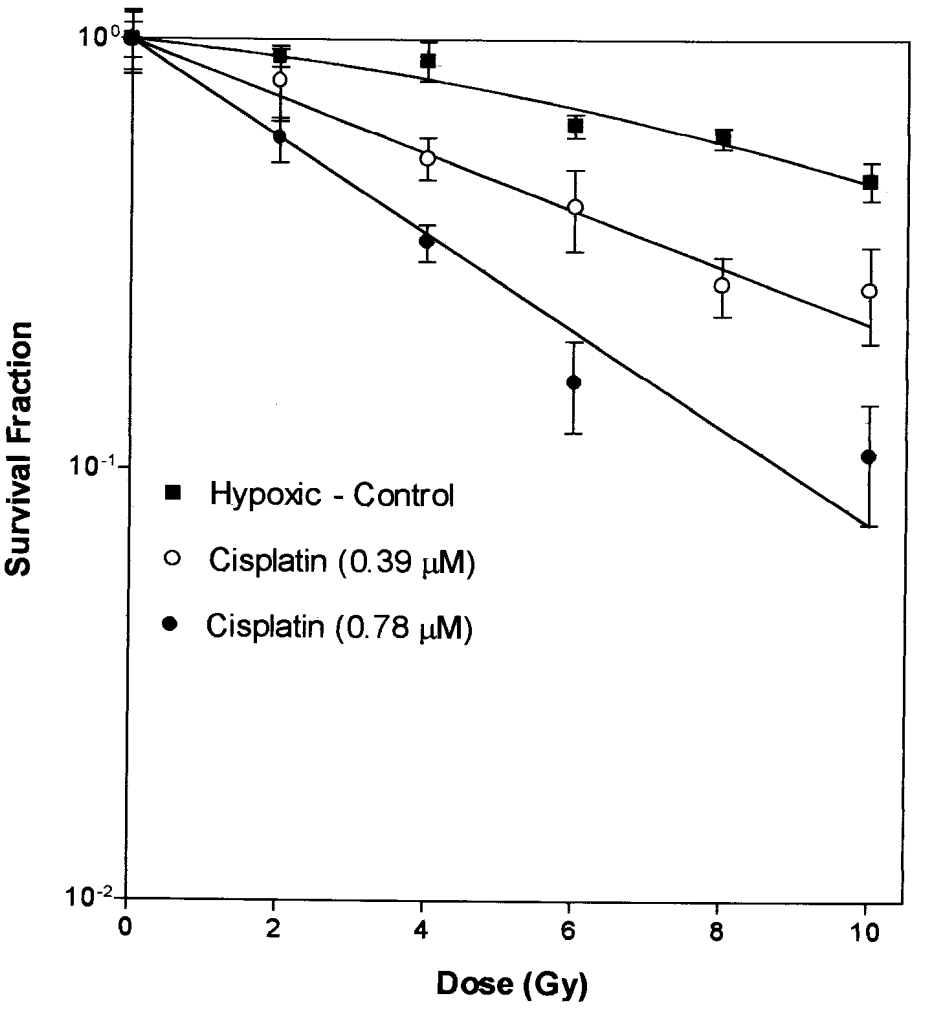


Figure 3.5 Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence of 0.39μM (○) and 0.78μM (●) cisplatin. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of six experiments ± standard error of the mean.

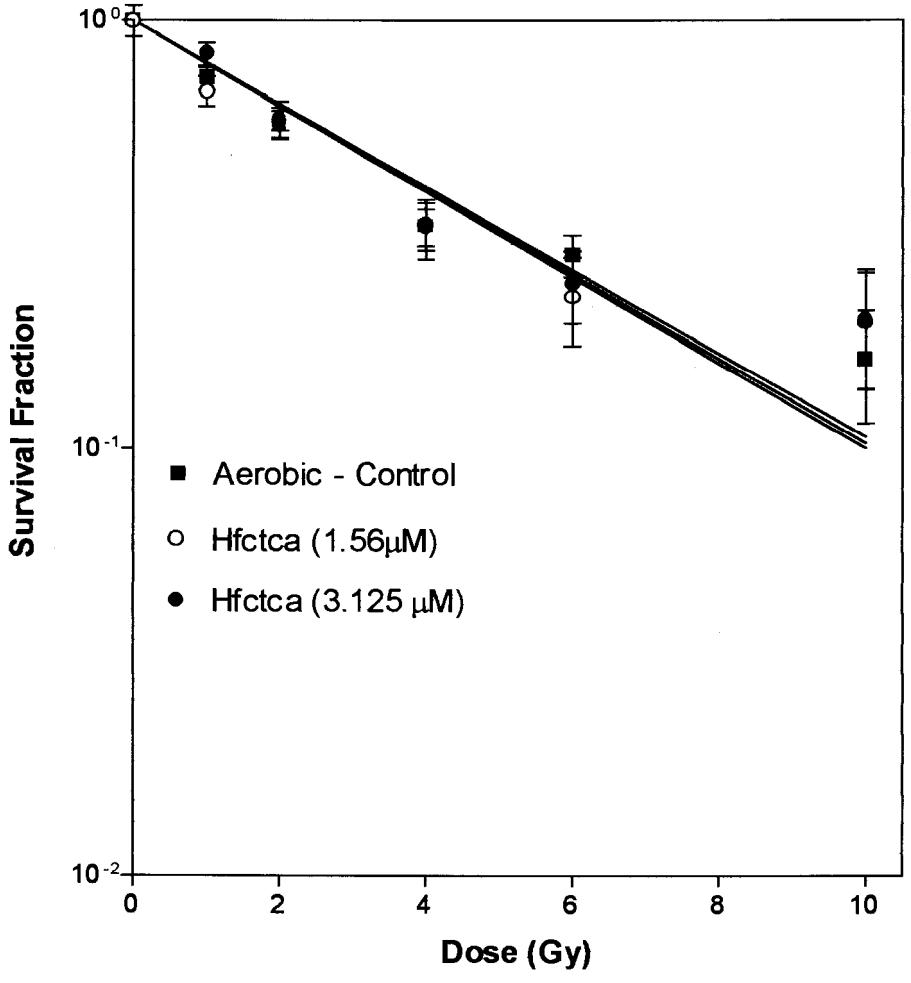


Figure 3.6. Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 1.56 μM (○) and 3.125 μM (●) Hfctca. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

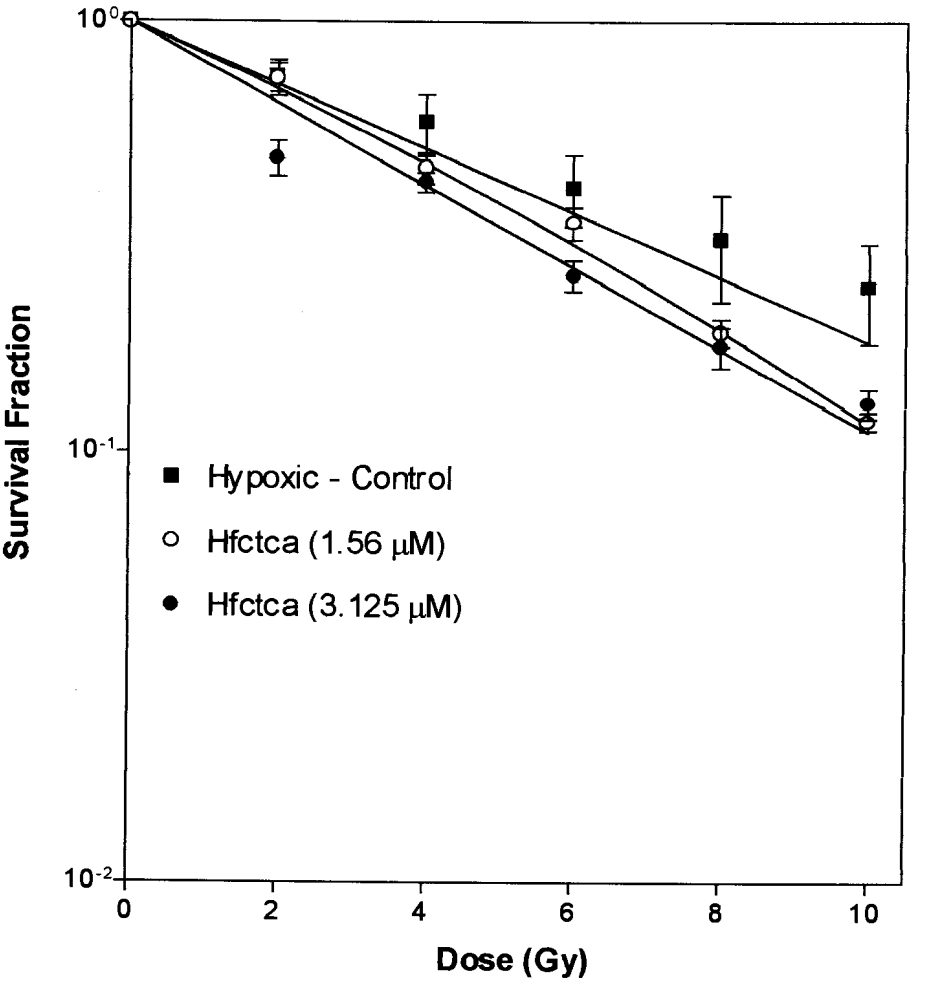


Figure 3.7 Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence of 1.56 μM (○) and 3.125 μM (●) Hfctca. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

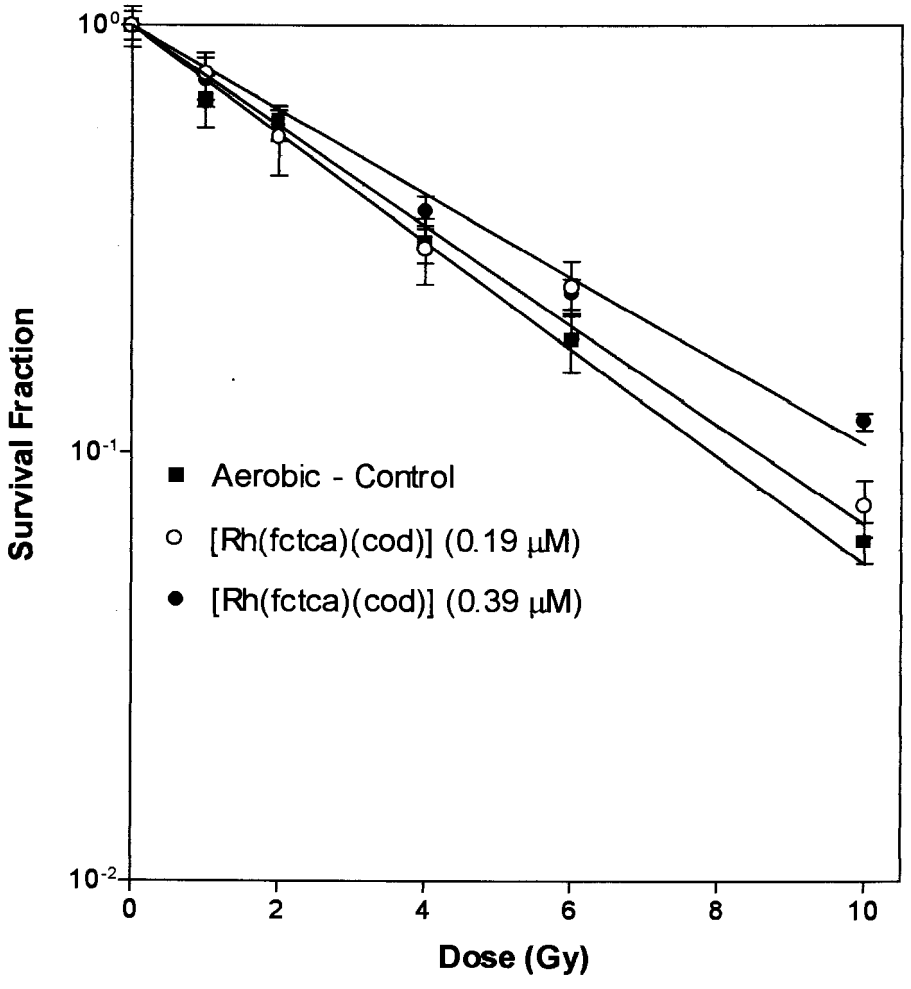


Figure 3.8. Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 0.19 μM (○) and 0.39 μM (●) [Rh(fctca)(cod)]. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

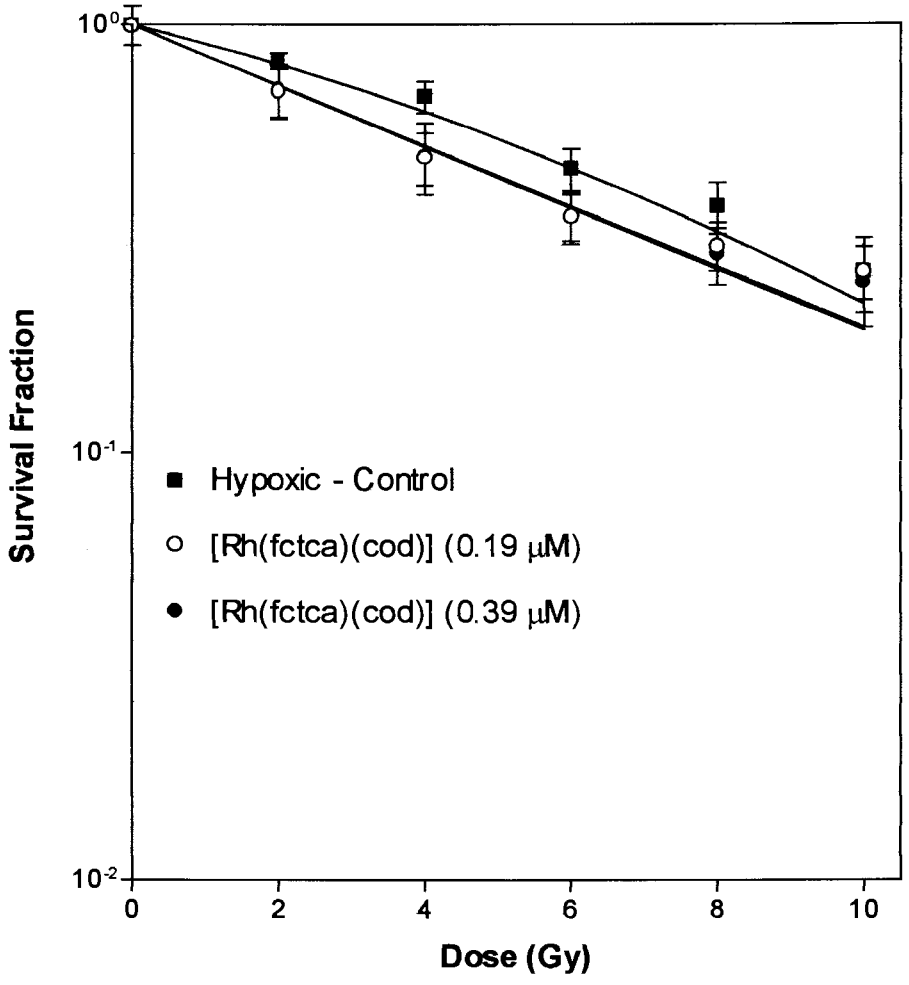


Figure 3.9. Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence of 0.19 μM (○) and 0.39 μM (●) [Rh(fctca)(cod)]. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

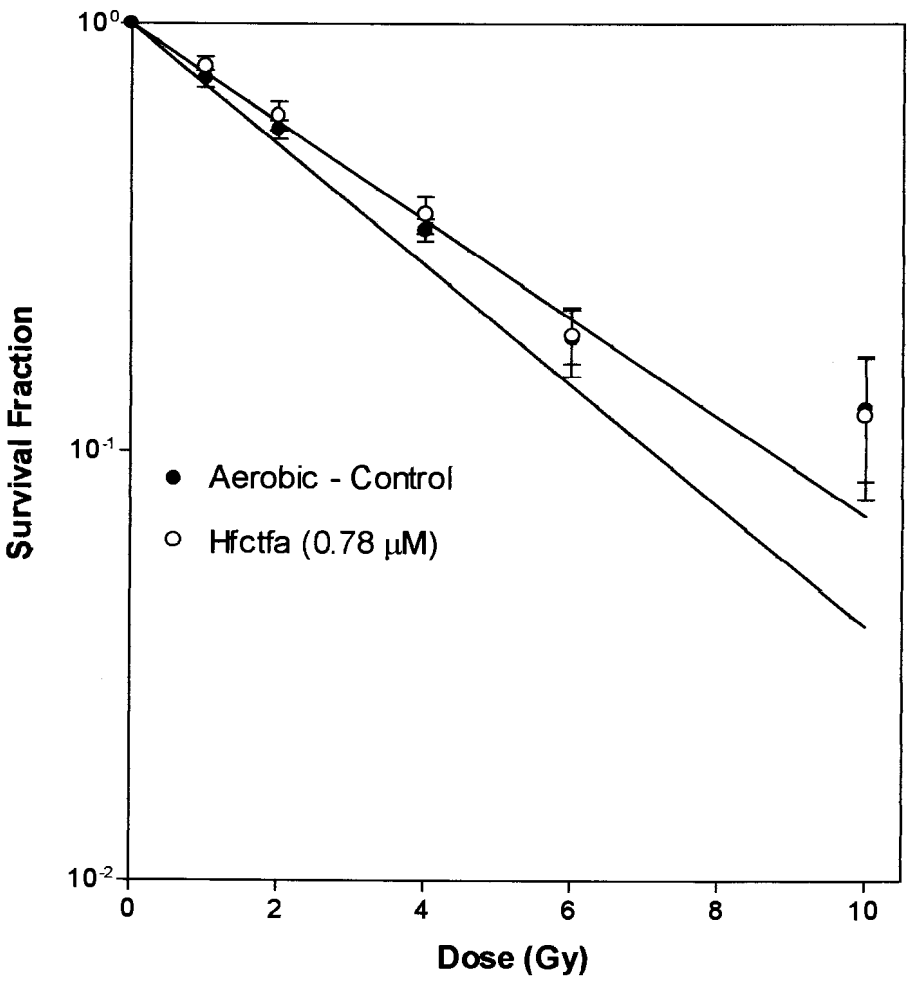


Figure 3.10. Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 0.78µM (○) Hfctfa. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

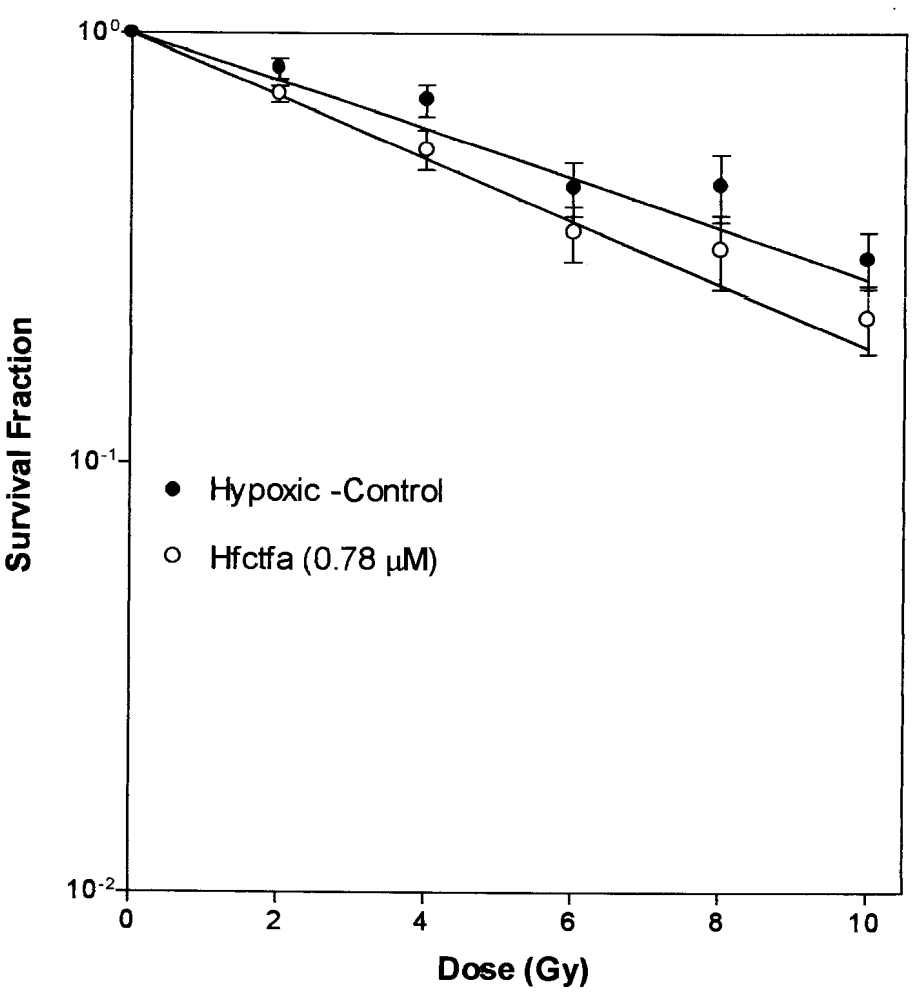


Figure 3.11. Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence of 0.78μM (○) Hfctfa. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

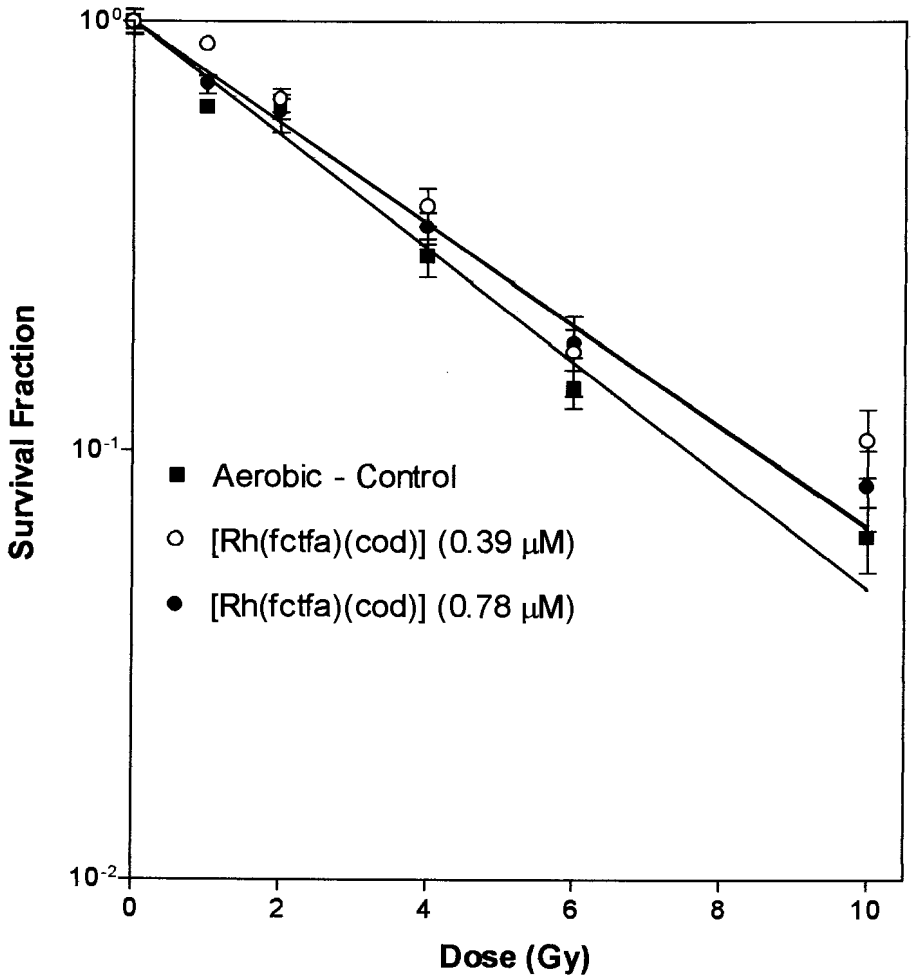


Figure 3.12. Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 0.39μM (○) and 0.78μM (●) [Rh(fctfa)(cod)]. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of six experiments ± standard error of the mean.

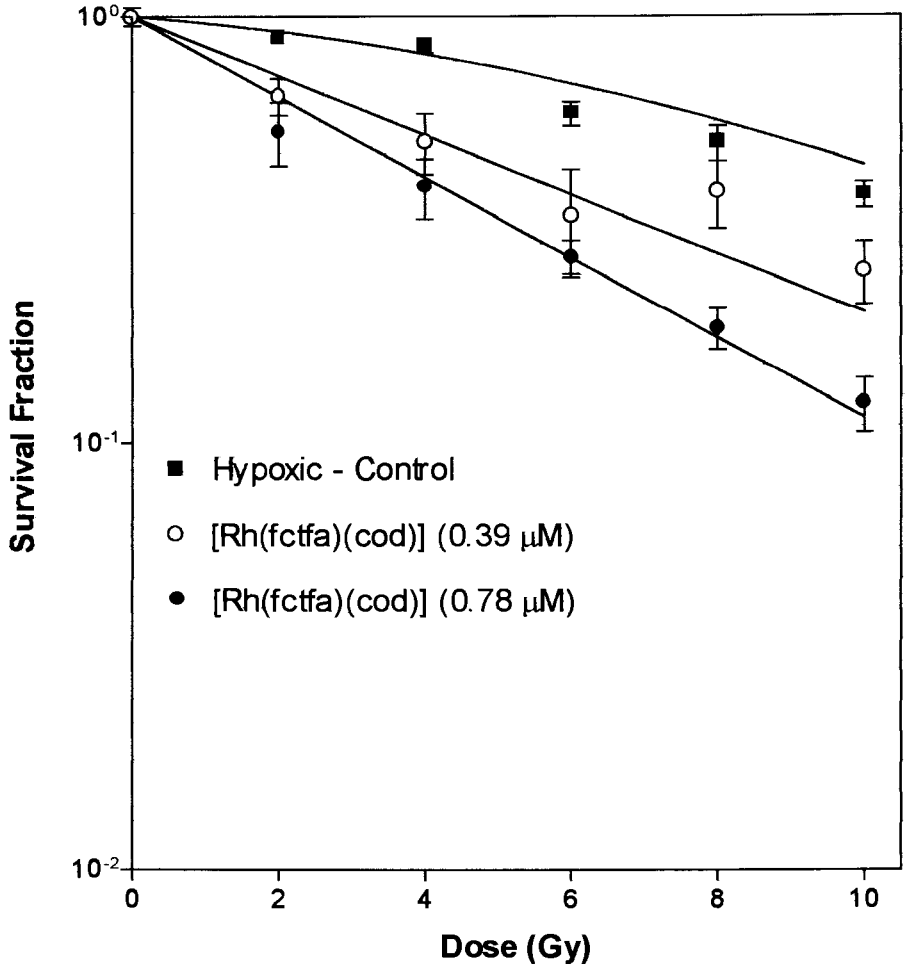


Figure 3.13. Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence of 0.39μM (○) and 0.78μM (●) [Rh(fctfa)(cod)]. The response to radiation of the untreated controls (■) under the same conditions are shown for comparison. Each end point represents the mean of six experiments ± standard error of the mean.

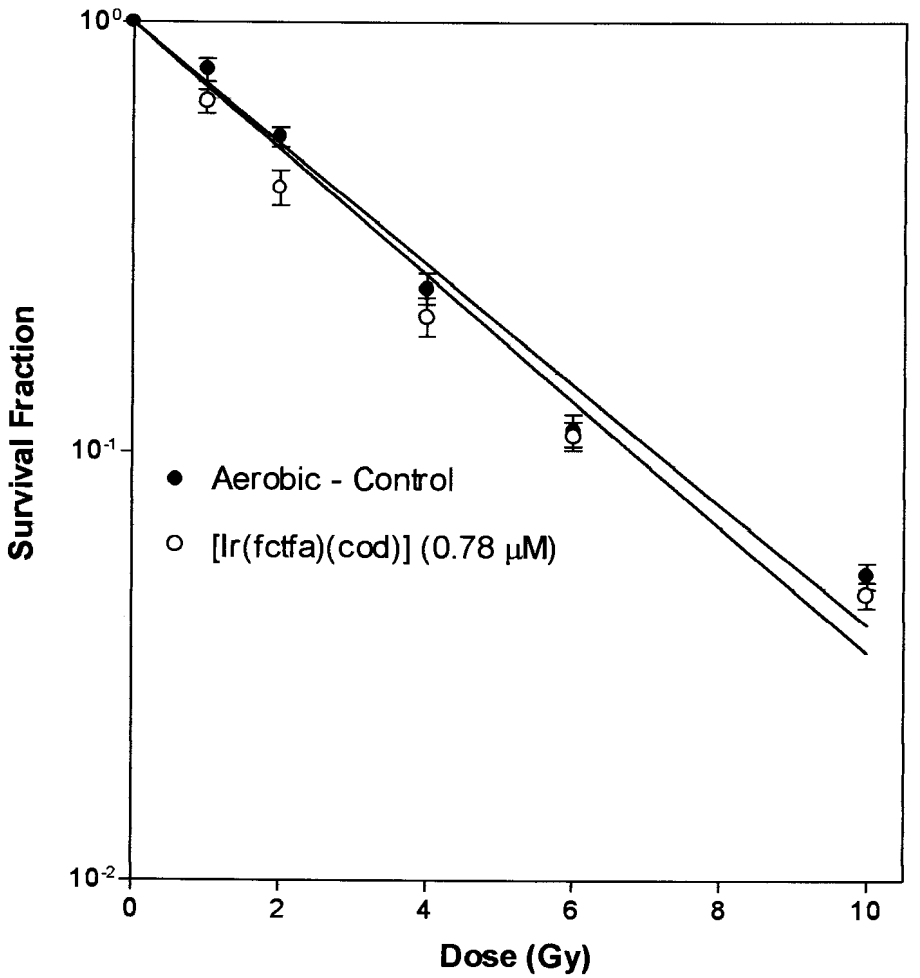


Figure 3.14. Growth fractions of CHO cells following irradiation under aerobic conditions in the presence of 0.78μM (○) [Ir(fctfa)(cod)]. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

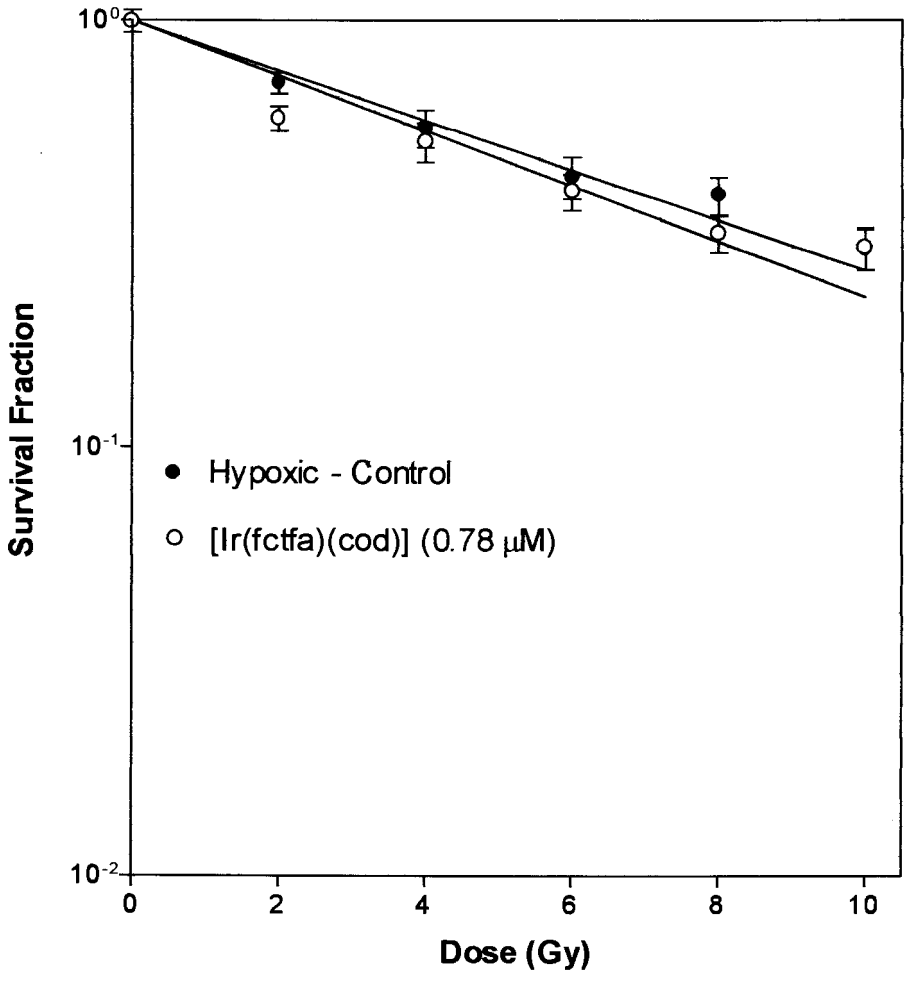


Figure 3.15. Growth fractions of CHO cells following irradiation under hypoxic conditions in the presence 0.78μM (○) [Ir(fctfa)(cod)]. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

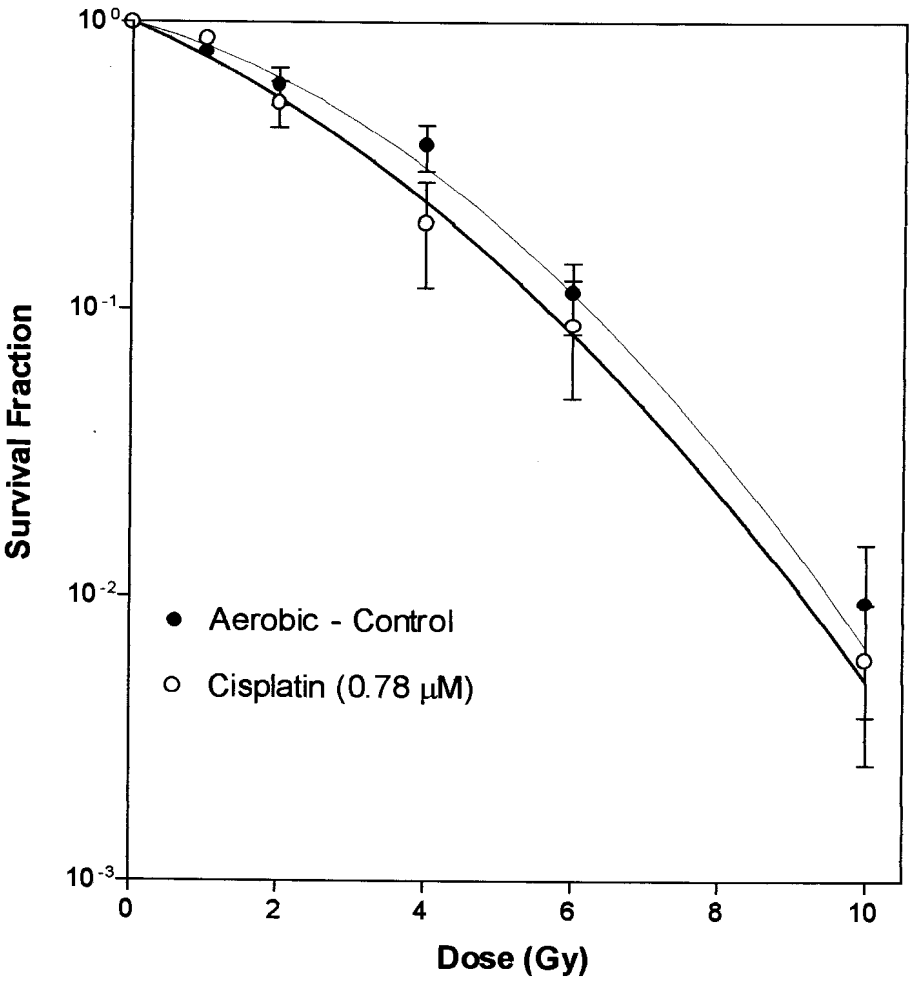


Figure 3.16. Growth fractions of CHO cells measured with the clonogenic assay, following irradiation under aerobic conditions in the presence of 0.78μM (o) cisplatin. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

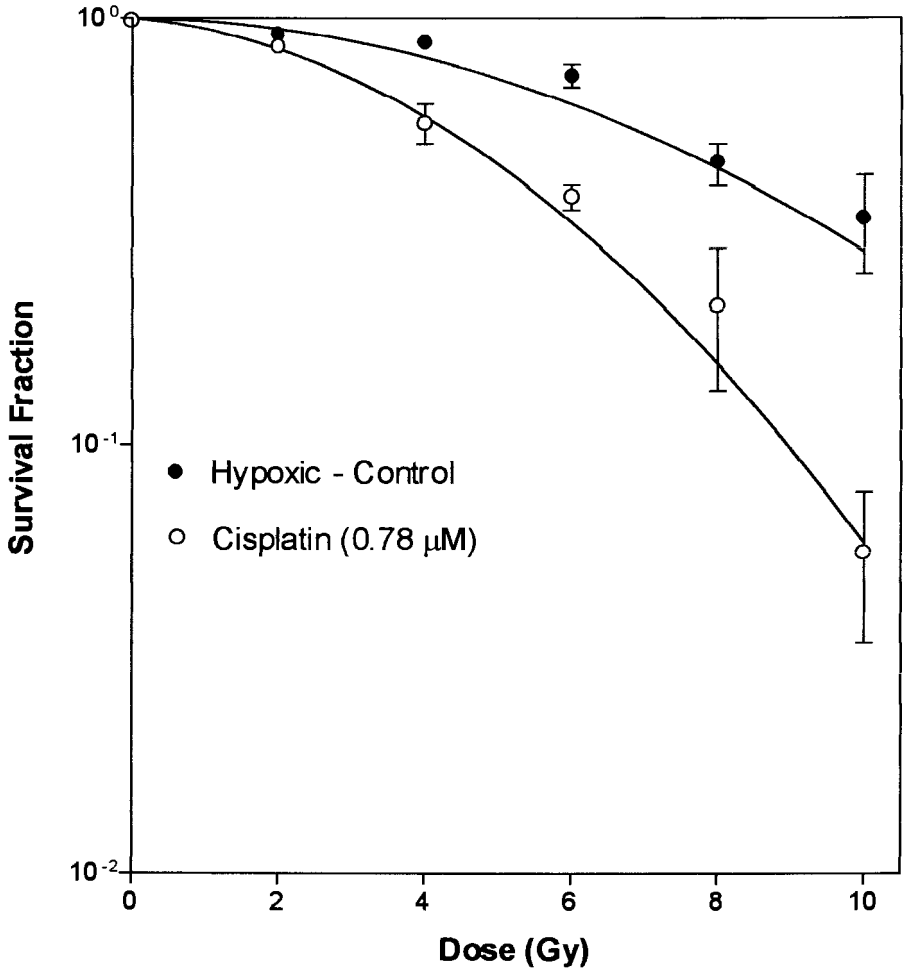


Figure 3.17. Growth fractions of CHO cells measured with the clonogenic assay, following irradiation under hypoxic conditions in the presence of 0.78μM (○) cisplatin. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

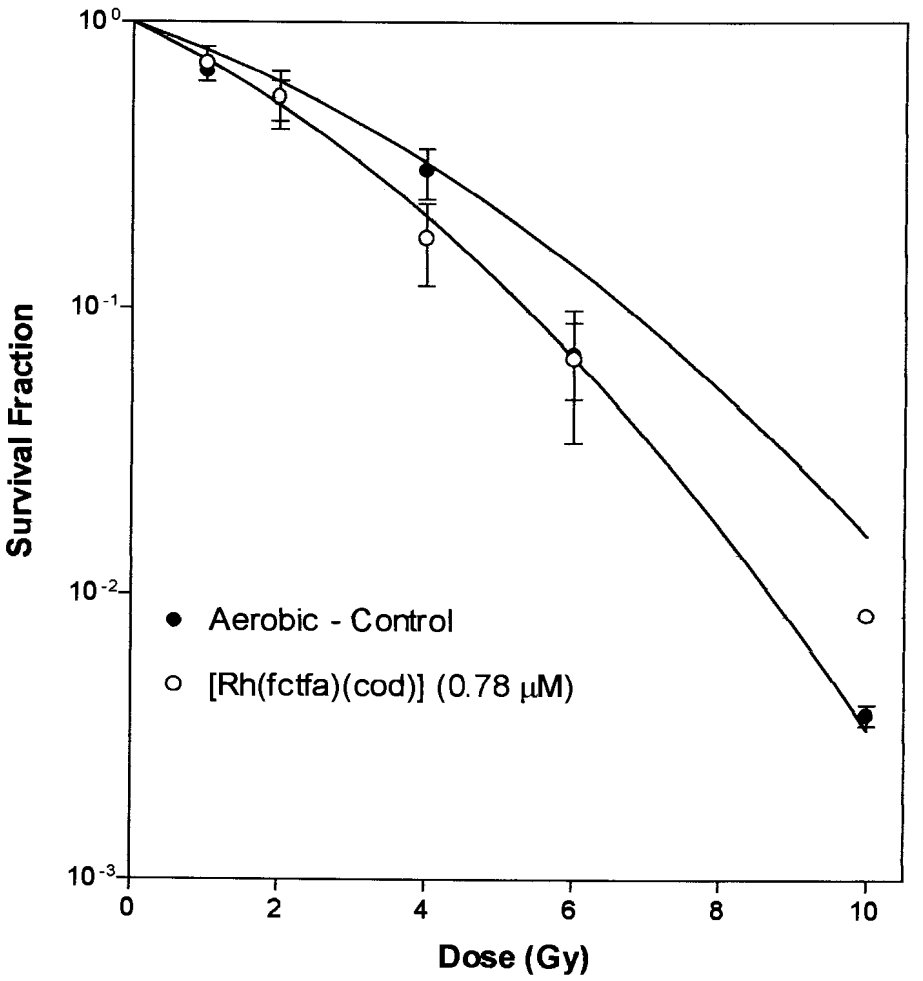


Figure 3.18. Growth fractions of CHO cells measured with the clonogenic assay, following irradiation under aerobic conditions in the presence of 0.78μM (○) [Rh(fctfa)(cod)]. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

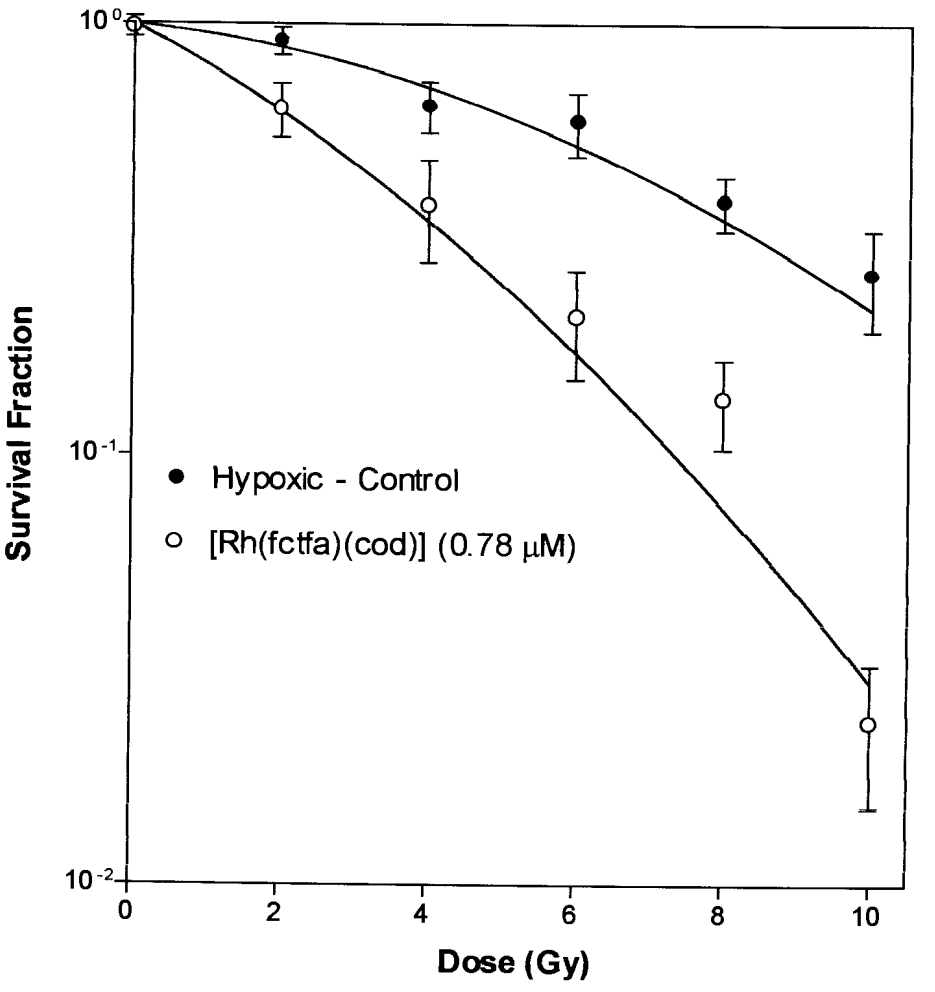


Figure 3.19. Growth fractions of CHO cells measured with the clonogenic assay, following irradiation under hypoxic conditions in the presence of 0.78μM (○) [Rh(fctfa)(cod)]. The response to radiation of the untreated controls (●) under the same conditions are shown for comparison. Each end point represents the mean of three experiments ± standard error of the mean.

3.4 DISCUSSION

Published Oxygen Enhancement Ratio's (OERs) for x-rays at high doses (1-30 Gy) have a value of between 2.5 and 3 (Read et al, 1952 a; Read et al, 1952 b; Read et al, 1952 c; Gray et al, 1953). With our system OERs ranging from 1.5 to 2.4 were obtained. This difference can be due to the different dose ranges used in the experiments. Skov *et al* (1994b) has noted a lower OER at low doses (1-3Gy) and although this has been discounted by some (Freyer *et al*, 1991) it has always been seen in all of their studies (OERs of 2.3 – 2.4) (Skov *et al*, 1989; Korbelik and Skov, 1989 and Skov and MacPhail, 1991). Our results thus seem to confirm what Skov and co-workers have noted.

To make this study clinically relevant, very low sub lethal concentrations (affecting a 70 – 90% cell survival) of all the drugs tested were used in the determination of cell survival of hypoxic and aerobic CHO cells. This choice has been influenced by observations from previous studies where very low concentrations of cisplatin (1 – 2.5 μM and even 0.1 μM) still produced measurable effects (ERs of 1.7 - 2.2 and 1.05) (Skov and Macphail, 1991, Skov *et al*, 1994a,b). Furthermore, the drugs tested remained in the system for the total duration of the assay, as again it was thought to be a truer simulation of the eventual use of the drugs.

Both the MTT and clonogenic assay techniques gave similar results for cisplatin treated hypoxic cells. Published dose modifying factors measured for cisplatin, range from 1.2 – 2 (Chibber et al, 1985, Van De Vaart et al, 1997). These values are in agreement with that obtained by our investigation using similar radiation doses and drug concentrations. However, cisplatin treated aerobic cells showed modest radioprotection ability making use of data obtained from the MTT assay while modest radiosensitization was seen at the

same concentration determined from clonogenic assay data. It has been reported that cisplatin exhibits radioprotecting properties (Zak and Drobnik, 1971) on the other hand Skov and MacPhail (1991) reported very moderate to no sensitization of aerobic V79 cells after treatment with cisplatin.

Very little has been published in the literature about the radiosensitization ability of Rh complexes. In one article by Chibber *et al*, (1985), DMFs of 2.0 were noted for some Rh(II) complexes against hypoxic V79 cells. This compound (Rh(II)methoxyacetate) increased the radiation sensitivity of cells under both gaseous conditions with the greater effect being observed in hypoxia. In their system however, cisplatin in comparison showed small levels of sensitization with DMFs of no more that 1.2 observed for hypoxic treated cells. When comparing our results obtained from aerobic and hypoxic treated [Rhfctfa)(cod)] cells, similar radiosensitization ability was seen for hypoxic treated cells determined by both the MTT and clonogenic assays. However [Rhfctfa)(cod)] exhibited radioprotecting properties determined from MTT data while on the other hand exhibiting noteworthy radiosensitization determined from clonogenic data. From these observations one could reason that the mechanism that sensitizes [Rh(fctfa)(cod)] treated cells to radiation is more effective under hypoxia.

Chibber *et al*, (1985) also proposed that toxicity is indicative of sensitization ability, this was however not seen by us. Comparing IC_{50} values, [Rh(fctca)(cod)] is more toxic than cisplatin and [Rh(fctfa)(cod)] but it gave very moderate enhancement of hypoxic cells. This complex, like cisplatin and its rhodium counterpart, [Rh(fctfa)(cod)] also showed moderate radioprotection against drug treated aerobic cells determined from MTT data. The ferrocene derivative of the rhodium complex [Rh(fctfa)(cod)] - Hfctca, was not only less toxic than its rhodium counterpart but also did not prove to be an

active radiosensitizer under either hypoxic and aerobic conditions. The other ferrocene complex tested, Hfctfa, did not show significant sensitization either.

Teicher *et al,* 1987 and Joy *et al,* 1989 reported that ferricenium salts are active radiosensitizers of hypoxic cells *in vitro* and *in vivo,* giving dose modifying factors ranging from 1.4 – 2. However I found no significant enhancement of either of the two ferrocene complexes, Hfctca and Hfctfa.

The iridium complex, [Ir(fctfa)(cod)] tested here also did not prove to be an active radiosensitizer of either hypoxic or aerobic cells.

In conclusion, the rhodium-ferrocene complex [Rh(fctfa)(cod)] at a very low non-toxic concentration of 0.78 μM, showed similar radiosensitizing activity compared to that of cisplatin. The ferrocene derivative, Hfctfa of the rhodium complex, [Rh(fctfa)(cod)] on its own showed only moderate sensitization, while the iridium analogue of this rhodium complex failed to show any radiosensitizing properties. It would therefore appear that the presence of the rhodium in this particular complex is responsible for its radiosensitization properties.

CHAPTER 4: THE EFFECT OF *cis*-DIAMMINEDICHLORO PLATINUM(II) AND [Rh(fctfa)(cod)] ON AEROBIC AND HYPOXIC CHO CELLS IN THE PRESENSE OF A p(66)/Be NEUTRON BEAM.

4.1. INTRODUCTION

At the National Accelerator Center (NAC) Faure, 66 MeV protons are applied to a beryllium target to produce a fast neutron beam for therapy. The average neutron energy in this beam is about 30 MeV. Neutron therapy is an effective way of treating radio-resistant hypoxic tumours. This is because the oxygen enhancement ratio (OER) for neutrons is about 1.6 and thus much less than that of conventional photon radiation (OER $\approx$ 3). Neutron damage is also less influenced by the chemical or biological state of a cell.

High LET radiation, such as neutrons, interact directly with critical targets within the cell, thus initiating the chain of events that lead to biological damage. This mode of direct action is responsible for more lethal damage, while for low LET radiation, such as photons the indirect action of radiation (i.e. radical formation) is the dominant process. As the role of repair phenomenon with neutrons is much less marked than that for photons, one can test the hypothesis that the drugs investigated interact with radiation through a reduction of cellular repair capacity (Lambin *et al*, 1993).

The neutron energy of the NAC beam is such that the depth dose characteristics is very similar to those of about 8 MV x-rays (Jones *et al*, 1994). However, a disadvantage of this is that the potential for therapeutic gain is limited compared to that associated with beams of lower neutron energy. This was seen in the reduction by 10 to 15% of local control rates of salivary gland tumors achieved at the NAC compared to the lower neutron

energy facility at Essen, Germany (Schmitt and Wambersie, 1990). In a recent study in Essen to compare the radiosensitivities of different cell lines for x-rays and a d(14)/Be neutron beam a direct comparison between the potential for therapeutic gain for p(66)/Be and d(14)/Be neutrons was done (Slabbert *et al*, 2000). A very strong correlation between radiosensitivities of human cancer cell types exposed to p(66)/Be neutrons and ^{60}Co γ-rays was seen. This was however not seen for cells exposed to d(14)/Be neutrons and x-rays. There is thus enough evidence to suggest that the neutron beam at the NAC shows limited relative biological effectiveness (RBE) and this motivates the search for a potential neutron sensitizer.

The most promising rhodium-ferrocene complex, [Rh(fctfa)(cod)] and cisplatin were selected for this trial with the neutron beam. In this chapter, data kindly provided by the NAC is also presented.

4.2. MATERIALS AND METHODS

4.2.1 Experimental Equipment and Procedures:

The neutron beam at the NAC was designed to have a similar dosimetry to that of an 8 MV photon beam, thus the 2 cm tissue equivalent buildup used in the previous set-up (Chapter 3, paragraph 3.2.1) was sufficient. Furthermore a calibration factor for each position in the cut-out was calculated to ensure a correct dose to that point. This was done by measuring the output of the cyclotron for a set dose at each position with a calibrated 0.5 cm^3 tissue equivalent ionization chamber (Mijnheer *et al*, 1987), the calibration factor was then calculated as the quotient of the set dose and the measured dose.

The neutron treatment facility has an isocentric treatment head, which made it possible to irradiate the incubator chamber from below, as was the case with the photon beam. Again the radio-lucent section of the table was used, thus

no further correction factors were required. A SSD of 150 cm was used compared to that of 100 cm for the photon beam. For the rest of the set-up the same procedure as described in Chapter 3, paragraph 3.2.1 was followed.

4.2.2.1 Evaluation of the cytotoxic effect of cisplatin on V79 cells determined by the clonogenic assay:

V79 cells were grown as monolayers in αMEM complete medium (Highveld Biological, SA) supplemented with 10% FCS, and maintained at 37°C in a humidified atmosphere of 5% CO_2. The cytotoxic activity of cisplatin on V79 cells was determined by a clonogenic assay. Cells were seeded at a known concentration into petri dishes (35 mm Tissue Culture Dish, Corning Glass works, NY), the cisplatin was added and then left for approximately 5 h to plate. Afterwards, the medium was removed and the dishes rinsed with PBS before adding new medium to the dishes. Cells were allowed to grow for several days until a sufficient number of colonies were noted in the control plates. The number of colonies formed at each cisplatin concentration was determined by staining the colonies as described in Chapter 3, paragraph 3.2.4.

4.2.2.2 The effect of cisplatin on the survival of V79 cells under aerobic conditions in the presence of a ^{60}Co photon beam and a p(66)/Be neutron beam determined by the clonogenic assay:

From the results of the toxicity test for cisplatin (Figure 4.3), 1.5 μM was determined as the concentration that inhibited the V79 cell growth by 50%. Using this concentration the effects of cisplatin on the survival of V79 cells after irradiation by a ^{60}Co photon beam were determined by colony formation.

Preparation for the experiment done with both photon and neutron beams, was the same. Cells were seeded into petri dishes approximately 5 hours

prior to radiation. Cisplatin was then added to each dish (except control dishes). Preceding irradiation, all the dishes were rinsed twice with PBS and new medium was added. All the experiments were done in triplicate. The ^{60}Co leg of the experiment received graded photon doses ranging from 2 to 10 Gy while the neutron leg received doses ranging from 1 to 5 Gy. After irradiation, cells were incubated for approximately 7 days, before staining the colonies. Only colonies of 50 or more cells on day 7 were scored.

4.2.3 *The effect of [Rh(fctfa)(cod)] on the survival of CHO cells under aerobic and hypoxic conditions in the presence of a p(66)/Be neutron beam determined by the MTT assay:*

CHO cells were grown in Ham's F-12 (Highveld Biological, SA) medium supplemented with 10% foetal bovine serum (Highveld Biological, SA). This experiment was done in triplicate on two different days, i.e. each end point being the mean of six data points. A total of 72 glass test tubes were prepared as described in paragraph 3.2.2, Chapter 3. Of these, 60 tubes were bunched together in groups (12 per group, 6 with and 6 without drugs) and placed into the modular incubator chamber to be irradiated, while a total of 12 test tubes, 6 with and 6 without drugs remained as a control. [Rh(fctfa)(cod)] at a concentration of 0.78 μM was used. The same procedure as described in Chapter 3 was followed to attain an aerobic and hypoxic environment inside the modular incubator chamber. In both the aerobic and hypoxic arm of the experiment, each group of bunched test tubes received doses ranging from 1 – 5 Gy. The MTT assay was performed as described previously and the 96 well plates were read using a manual spectrophotometer (EL 307C Microplate Reader, Bio-Tek Instruments, USA) at a wavelength of 590 nm.

4.2.4 Statistical Analysis:

The results are expressed as the mean ± standard error of the mean (SEM). Levels of statistical significance were calculated using the Student's paired t-test. Differences were considered significant if the probability value was less than 0.05.

4.3. RESULTS

4.3.1 Evaluation of the cytotoxic effect of cisplatin on V79 cells determined by the clonogenic assay:

The results, presented in Figure 4.1 was supplied by the NAC. The IC_{50} value for this data set was determined by point-to-point regression analysis from which a value of 1.36 μM was derived. A concentration of 1.5 μM cisplatin inhibiting 44 ± 0.04% growth of V79, was used in the following experiment.

4.3.2 The effect of ciplatin on the survival of V79 cells under aerobic conditions in the presence of a ^{60}Co photon beam and a p(66)/Be neutron beam determined by the clonogenic assay:

The following results were supplied by the NAC. For untreated V79 cells a mean plating efficiency of 76% was observed. A plating efficiency of 81% was attained for untreated control cells exposed to the neutron beam. The data was fitted as described in paragraph 3.3.1.

An α value of 0.20 ± 0.03 and β value of 0.02 ± 0.003 was calculated from the fit (R^2 = 0.98) of the survival curve for untreated V79 cells exposed to the ^{60}Co beam (Figure 4.2). From the fit (R^2 = 0.98) of the survival curve for treated cells, an α and β value of 0.33 ± 0.04 and 0.01 ± 0.01 respectively were observed (Figure 4.2). Although Slabbert *et al* (1996) noted that β values from survival curves of high LET sources for some cell types do not

significantly differ from zero, work done by the same author (1989) suggested the contrary for V79 cells irradiated with the clinical neutron beam at the NAC.

The survival curves, Figure 4.3, generated from the V79 data exposed to the neutron beam gave an α value of 0.54 ± 0.04 and a β value of 0.04 ± 0.01 ($R^2 = 0.99$). From the fit ($R^2 = 0.98$) of the survival curve for treated fibroblasts an α value of 0.65 ± 0.06 and a β value of 0.03 ± 0.02 was obtained. For a comparison between photon sensitivity and neutron RBE, the observed dose effect relationship was expressed in terms of mean inactivation dose ($\bar{D}$). A summary of the dose modifying factors is given in Table 4.1.

Table 4.1. Mean inactivation doses calculated from the response of V79 cells following treatment with radiation (^{60}Co and p(66)/Be) and/or a concentration of 1.5μM cisplatin under aerobic conditions. Dose modifying factors are stated as the ratio of mean inactivation doses.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifying Factor (DMF)
Aerobic cells + ^{60}Co	3.14 ± 0.03	
Aerobic cells + Cisplatin (^{60}Co)	2.54 ± 0.04	1.24 ± 0.05
Aerobic cells + (p(66)/Be)	1.54 ± 0.04	
Aerobic cells + Cisplatin (p(66)/Be)	1.39 ± 0.06	1.11 ± 0.07

The results represent the mean ± SEM of one experiment done in triplicate.

4.3.3 *The effect of [Rh(fctfa)(cod)] on the survival of CHO cells under aerobic and hypoxic conditions in the presence of a p(66)/Be neutron beam determined by the MTT assay:*

The purpose of this study was to see if [Rh(fctfa)(cod)] had any effect on the biological damage induced by the clinical neutron beam. Dose response curves for hypoxic and aerobic CHO cells when exposed to [Rh(fctfa)(cod)] and the neutron beam are shown in Figures 4.4 and 4.5 respectively. The survival curves were fitted to the linear quadratic equation as $\log_e S = -\alpha D - \beta D^2$. In cases were one of the inactivation parameters were found to be negative, it was set to zero and the other parameter re-estimated. The survival levels were corrected for toxicity of the drugs, with control concentrations of [Rh(fctfa)(cod)] responsible for ± 10% inhibition.

The β value in all of the survival curves generated was found to be negative and therefore set to zero, as a negative β value has no biophysical meaning. This is however not unusual as irradiation with high LET sources in general and the clinical neutron beam in particular is known to result in curves of some cell types with β values not significantly different from zero (Slabbert *et al*, 1996). An α value of 0.42 ± 0.01 was calculated for the untreated, irradiated aerobic CHO cells and these parameters increased to 0.44 ± 0.02 for CHO cells treated with 0.78 μM [Rh(fctfa)(cod)] prior to radiation. Goodness of fit criteria (R^2 values) were respectively 0.85 and 0.70, which is not significantly different from fits previously attained in this study for the CHO cell line using the MTT assay (See Chapter 3). When hypoxic CHO cells were exposed to the neutron beam, the α value increased from 0.35 ± 0.01 for the untreated, irradiated control to 0.39 ± 0.01 for cells treated with 0.78 μM [Rh(fctfa)(cod)]. Again the fit of the dose response curves to the data (R^2 values of 0.80 and 0.87 respectively) is in agreement with that previously

observed. The radiosensitivities are expressed in terms of mean inactivation dose ($\bar{D}$). A summary of the dose modifying factors is given in Table 4.2.

Table 4.2. Mean inactivation doses calculated from the response of aerobic and hypoxic CHO cells following treatment with radiation (p(66)/Be neutron beam) and/or a concentration of 0.78 μM [Rh(fctfa)(cod)]. Dose modifying factors are stated as the ratio of mean inactivation doses.

Treatment	Mean Inactivation Dose (Gy)	Dose Modifying Factor (DMF)
Aerobic cells	2.39 ± 0.01	1.21 ± 0.01[†]
Aerobic cell + [Rh(fctfa)(cod)]	2.29 ± 0.02	1.04 ± 0.02
Hypoxic cells	2.90 ± 0.01	
Hypoxic cells + [Rh(fctfa)(cod)]	2.57 ± 0.01	1.13 ± 0.01

The results represent the mean ± SEM of one experiment done in triplicate.

[†]Oxygen Enhancement Ratio (OER)

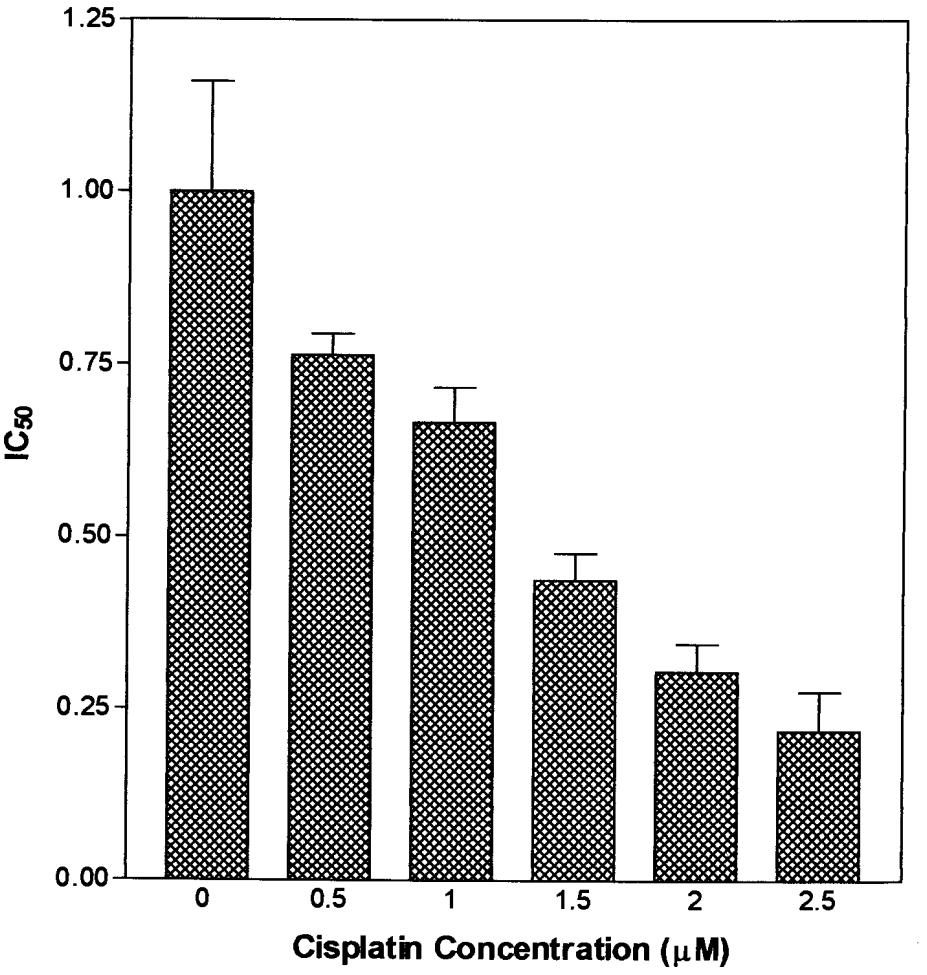


Figure 4.1. The effect of cisplatin, corrected for plating efficiency, on V79 cells. The cytotoxic activity is expressed as a concentration (μM) at which cells showed a 50% growth inhibition (IC_{50}). Each end point represents the mean ± standard error of the mean.

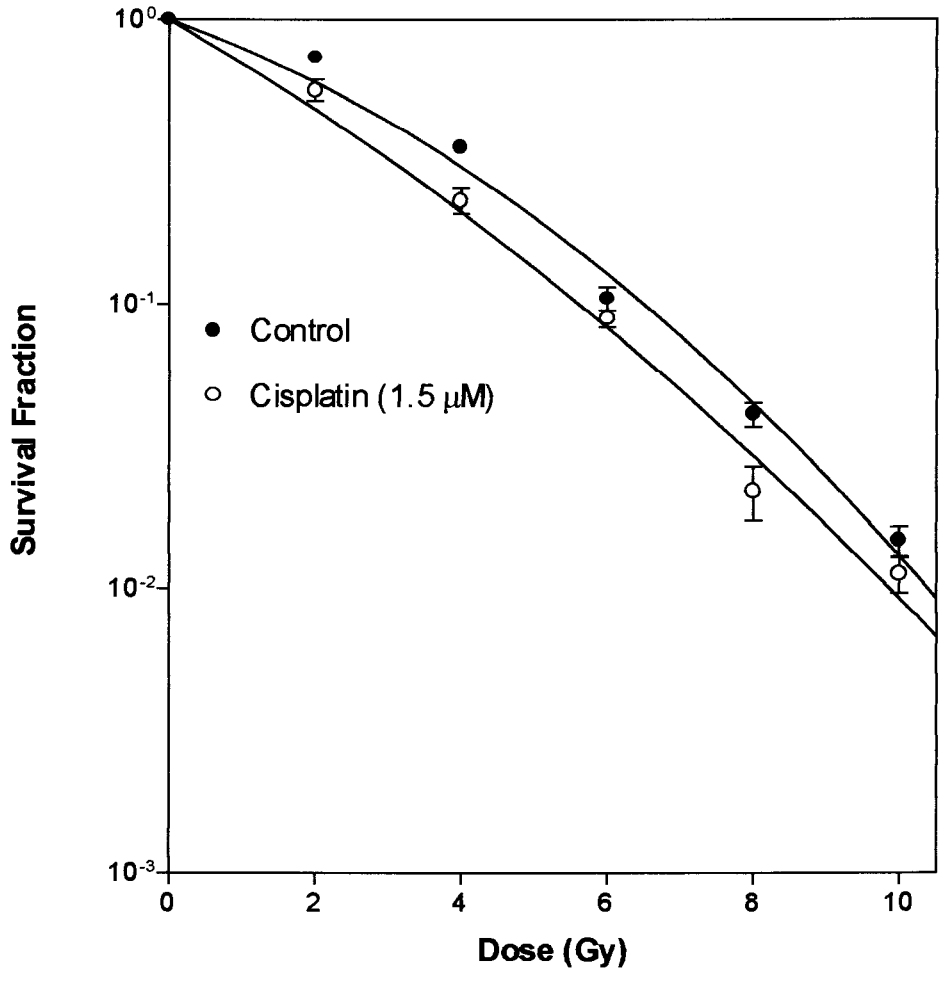


Figure 4.2. Growth fractions of aerobic V79 cells following irradiation (^{60}Co photon beam) after 5 h exposure to Cisplatin (○) at a concentration of 1.5 μM. The untreated control (●) irradiation response under aerobic conditions is shown for comparison. Each end point represents the mean ± standard error of the mean.

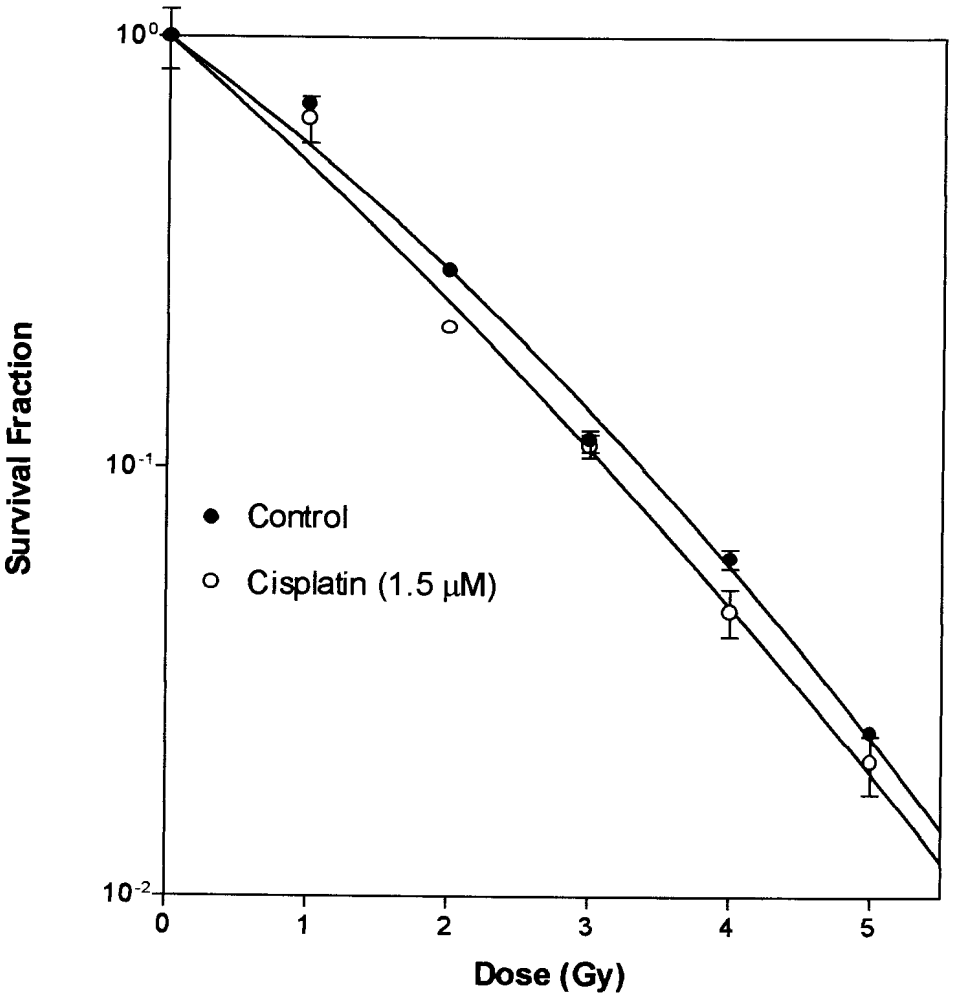


Figure 4.3. Growth fractions of hypoxic V79 cells following irradiation (p(66)/Be neutron beam) after 5 h exposure to Cisplatin (○) at a concentration of 1.5 µM. The untreated control irradiation response under normal aerobic conditions (●) is shown for comparison. Each end point represents the mean ± standard error of the mean.

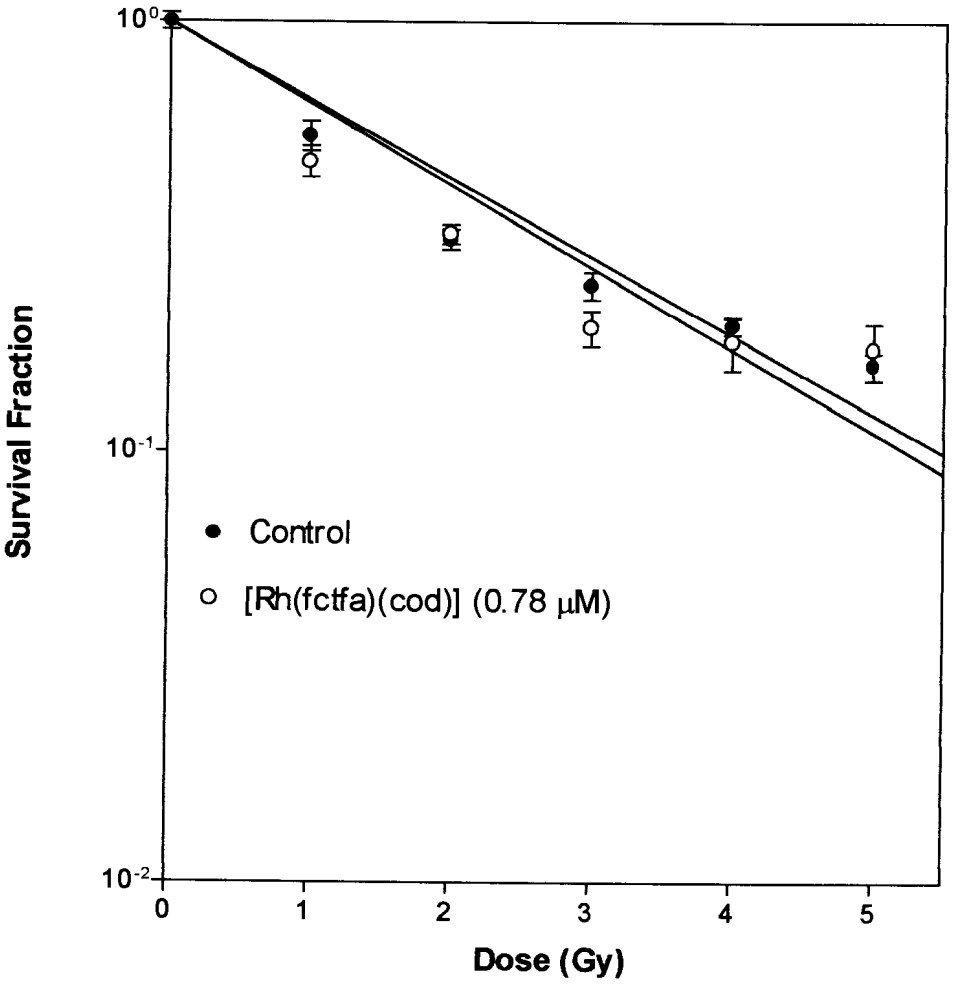


Figure 4.4. Growth fractions of aerobic CHO cells following irradiation (p(66)/Be neutron beam) in the presence of 0.78μM (○) [Rh(fctfa)(cod)]. The untreated control (●) irradiation response under aerobic conditions is shown for comparison. Each end point represents the mean ± standard error of the mean.

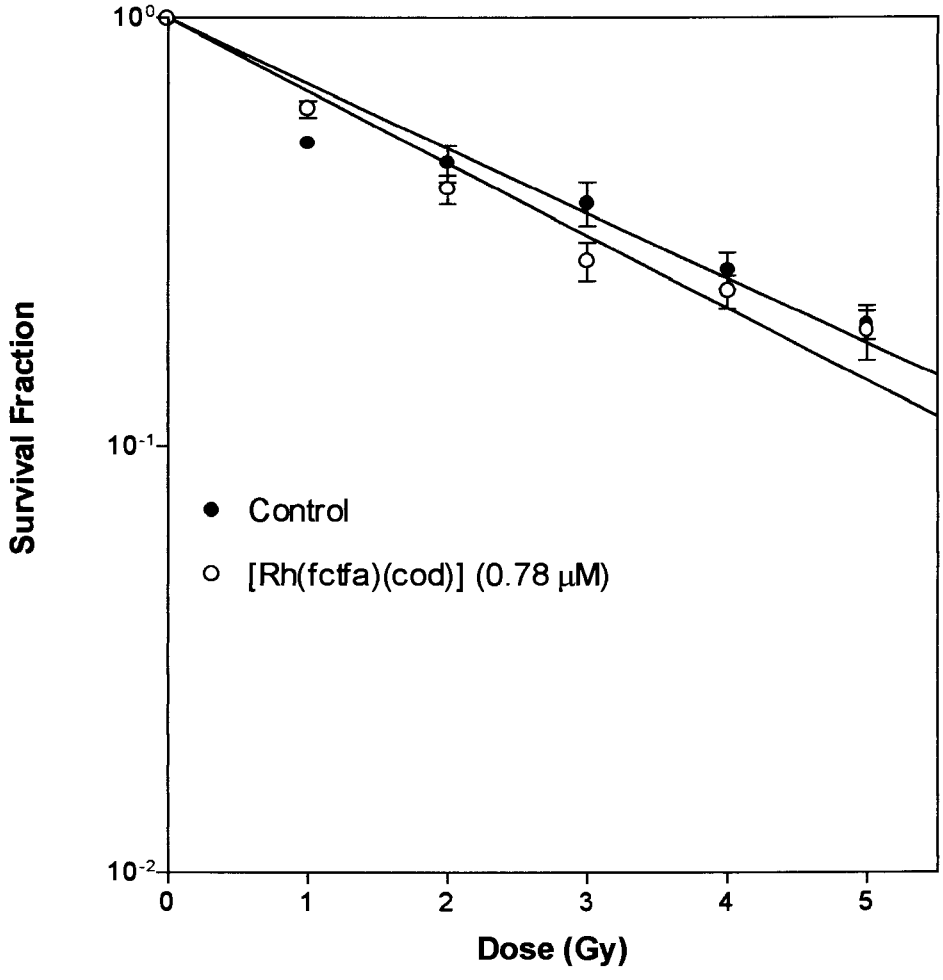


Figure 4.5. Growth fractions of hypoxic CHO cells following irradiation (p(66)/Be neutron beam) in the presence of 0.78μM (○) [Rh(fctfa)(cod)]. The untreated control (●) irradiation response under hypoxia is shown for comparison. Each end point represents the mean ± standard error of the mean.

4.4 DISCUSSION

The IC_{50} value of cisplatin obtained for V79 cells (± 1.5 μM) is in close agreement to that noted of cisplatin for CHO cells (1.21 ± 0.25) as was seen in Chapter 3. These values are also similar to those recorded in previous studies (Korbelik and Skov ,1989 and Van de Vaart *et al,* 1997). This again serves as confirmation that the MTT technique used as an end point in cytotoxic evaluations, show comparable results to that obtained from the clonogenic assay used by these researchers.

Cisplatin does not show marked enhancement in normal aerated cells with low LET radiation (Korbelik and Skov, 1989; Melvik and Pettersen, 1988), which corroborates with our result (DMF of 1.24 ± 0.05) after a 5h incubation with cisplatin in aerated V79 cells exposed to the ^{60}Co photon beam. A reduced dose modifying of 1.11 ± 0.07 is seen with neutrons compared to ^{60}Co γ-rays (Table 4.1) and this result would suggest that indirect actions involving radicals is in part responsible for sensitization by cisplatin.

An OER of 1.21 ± 0.01 was achieved using the modular incubator chamber in the experiment with rhodium treated cells. As the OER ratio for neutrons are in the region of about 1.6 (Hall, 1994), it is reasonable to conclude that optimal hypoxic conditions were not achieved in this single experiment. The OER ratio might be improved by increasing the number of experiments, thereby improving the statistical variance of data points. Nonetheless, even at this low OER, the survival curve data showed observable DMFs.

The MTT assay demonstrated by way of mean inactivation dose, a DMF of 1.13 ± 0.01 for [Rh(fctfa)(cod)] treated hypoxic cells at a very low non-toxic concentration of 0.78 μM, whereas no enhancement was seen under aerobic conditions with a DMF (1.04 ± 0.02) not much greater than unity. One can

thus conclude that [Rh(fctfa)(cod)] shows very moderate enhancement under hypoxia with a DMF of 1.13, while no enhancement was seen under oxia with a DMF of 1.04. In comparison a large DMF of 1.93 was achieved for hypoxic treated CHO cells with a low LET 8 MV photon beam. If one considers that neutrons are responsible for more direct damage (less repairable damage), whereas damage incurred by photons are by more indirect means (i.e. radical formation), it would seem to confirm the lack of sensitization of [Rh(fctfa)(cod)] experienced with the neutron beam. This lack of induction of indirect action translates in lack of sensitization. It further suggests that [Rh(fctfa)(cod)] in part sensitizes by inhibition of repair capacity which would have a more pronounced effect with photons compared to neutrons.

In conclusion, although I was unable to compare the radiosensitizing ability of cisplatin and [Rh(fctfa)(cod)] on hypoxic cells in combination with the p(66)/Be neutron beam, I have been successful to, in part, establish the action whereby [Rhfctfa)(cod)] sensitizes hypoxic cells to radiation, i.e. inhibition of cell inactivation mechanisms that is normally associated with repairable damage.

CHAPTER 5: THE EEFECTS OF *cis*-DIAMMINEDICHLORO PLATINUM(II) AND [Rh(fctfa)(cod)] ON DNA DAMAGE, INTERCALATION AND CELL KINETICS IN CHO CELLS, *IN VITRO.*

5.1. INTRODUCTION

There are various techniques available for the detection of damage in cells following radiation exposure. One of these techniques uses the single cell clonogenic assay as an endpoint. In Chapter 3, results obtained using this technique were compared to a more rapid and quantitative assay method, namely the MTT assay. As was seen in this chapter, similar results were obtained with the drugs tested. The major limitations of these types of assays are the long completion times and its irrelevance to cells that were incapable of further divisions and repair (Milner *et al*, 1987). Radiation insult can also be determined by scoring chromosomal aberrations by microscopic analyses (Evans, 1962), however this procedure is extremely time consuming and operator dependent. Therefore flow cytometry was investigated as an alternative by some (Milner *et al*, 1987; Wang *et al*, 1996 and Djuric *et al*, 1998) as an automated method of rapid detection of DNA damage. In this chapter the DNA damaging properties and possible intercalation of the drugs in DNA will be assessed by a method adapted from the work of Milner *et al* (1987) in which radiation induced DNA damage in mammalian cells were measured with the use of a flow cytometer. For comparison, DNA damage will also be assessed by the established method of determining micronuclei frequencies as a direct result of radiation induced chromosomal aberrations (Schmid, 1975; Countryman and Heddle, 1976 and MacGregor *et al*, 1980).

Flow cytometry is a laser-based technology that is used to measure characteristics of biological particles. Flow cytometers scan cells or particles

that flow in single file in a liquid stream past a laser beam. As the laser beam strikes the individual cells, the optical and electronics system of the flow cytometer is responsible for collecting and quantitating at least five parameters from the scattered light and the emitted fluorescence. The light scattering that occurs is directly related to structural and morphological cell features of the cells or particles measured.

Light that is scattered in a forward direction (in the direction of the laser beam) is analyzed as one parameter and is referred to as forward angle light scatter (FSC). FSC provides basic morphological information such as relative cell/particle size. Light scatter at 90° relative to the laser beam, is referred to as side angle light scatter (SSC). SSC is an indicator of granularity within the cytoplasm of cells as well as surface/membrane irregularities or topographies.

The flow cytometric nucleoid assay measures changes in the state of DNA supercoiling and integrity of the attachment of DNA loops to the nuclear matrix in individual eukaryotic cells (Wang *et al*, 1996). Nucleoids are isolated by exposing cells to a high salt buffer which releases the histone-depleted DNA still attached to the nuclear matrix and with its supercoiled organisation intact (Cook and Brazell, 1975 and Wilks *et al*, 1996). These nucleoids can be detected by light scatter from the laser beam within the flow cytometer (Wilks *et al*, 1996). As froward angle light scatter is related to particle size, alterations to the DNA configuration as a result of specific interaction of agents as well as changes due to irradiation can be monitored (Wilks *et al*, 1996). The size of the nucleoid increases with an increase in concentration of an intercalating agent, like propedium iodide, up to the maximum point where the supercoils are fully unwound (Wang *et al*, 1996). As the supercoils unwind with increase in intercalating agent concentration, the size of the nucleoids increases and are measured as an increase in forward angle light scatter (Wilks *et* al, 1996). Further increases in the concentration of the intercalating

agent after the nucleoids are fully unwound, cause the DNA loops to condense leading to a decrease in size while on the other hand, if the DNA is damaged, as in the case when irradiated, this rewinding cannot occur (Wilks *et al,* 1996).

Another method of quantitating radiation insult is by the micronuclei assay. Micronuclei are found in the cytoplasm outside the main nucleus and resemble the main nucleus in shape, structure, and staining properties. Micronuclei are formed due to the failure of acentric fragments (because of the lack of a centromere) to incorporate into the daughter nuclei during cell division (Sasaki and Norman, 1966; Heddle and Carrano, 1977). Micronuclei also occur when entire chromosomes lag behind during mitosis due to a failure of mitotic spindle formation (Schmid, 1975, 1976). Micronuclei induced by ionizing radiation are quite small in size (6 – 12 μm) and are mainly the cause of acentric fragments, however there are lower and upper extremes with these micronuclei arising mainly as a result of spindle effects (Högstedt and Karlsson, 1985).

The earliest observations on cytoplasmic bodies containing nuclear material believed to be the result of radiation-induced chromosome damage were made in murine and rat sperm (Brenneke, 1937) and in plant root meristematic cells (Thoday, 1951). Since then the usefulness of micronuclei for mutagenicity testing has been realised. This method has been used with success in numerous cell types, e.g. polychromatic erythrocytes (PCE) (Schmid, 1975 and MacGregor, 1980) and human peripheral blood lymphocytes (Countryman and Heddle, 1976). It has also been successfully used in a variety of cells in culture, e.g. in human fibroblasts and lymphoblastoid cells and in Chinese hamster ovary cells (Countryman and Heddle, 1976).

The detection of micronuclei is made more accurate and easier if performed on cells with preserved cytoplasm (Huber *et al*, 1983 and Högstedt, 1984). A method developed by Fenech and Morley (1985) using cytochalasin-B, produces binucleated cells with intact cytoplasm. Cytochalasin-B is a cytoplasmic cleavage inhibitor inducing binucleated cells (i.e. cells that divided only once) in the culture, which can be observed using phase-contrast microscopy.

5.2. MATERIALS AND METHODS

5.2.1 Flow cytometric analysis of the effect of cisplatin and [Rh(fctfa)(cod)] in the presence or absence of radiation on nucleoid size:

CHO cells were harvested from a confluent tissue culture flask, using a trypsin / versene mixture. The cells were washed once with medium and then twice with Hanks balanced salt solution (with phenol red, Ca^{++}, Mg^{++}, and HEPES 1g/L, Highveld Biological (PTY) LTD, RSA). The cells were made up in Hanks balanced salt solution to a concentration of 1 x 10^6 cells/ml. In all the flow cytometric experiments performed only the response of CHO cells under aerobic conditions were investigated. For radiation experiments, without drugs, one hundred microliters (100 µl) of the cell suspension was added to the Falcon test tubes. In experiments where the effect of the drugs on the DNA configuration or the sensitivity of DNA for radiation damage was investigated, 90 µl of the cell suspension was added to the Falcon test tubes. In these experiments 10 µl of the appropriate drug concentration (final concentration was 0.78 µM) was added to the cells and incubated at 37°C in a 5% CO_2 incubator for a total period of 95 minutes during which time the cells remained on a shaker incubator for the first 35 min. Immediately after incubation the tubes were placed on ice and irradiated with an 8 MV photon beam. Two to three falcon tubes per dose point held in place by a test tube

holder were placed on the radiolucent section of the table with appropriate build-up material as described elsewhere. After each dose ranging from 2 to 10 Gy the tubes were placed on ice. One millilitre (1 ml) of a high-salt lysing buffer (See Appendix 2) (10 mM disodium EDTA, 10 mM TRIS base, 2 M sodium chloride, 0.1 % Triton X-100 and 0.01% propedium iodide (PI); pH 8.0) was then added to the cells and the cells incubated for a further 45 minutes on ice, before the released nucleoids were analyzed on a flow cytometer (Coulter XL-MCL, Miami, Florida, USA). Only nucleoids that stained positive for the intercalating fluorescent dye, propedium iodide, were analyzed. The propedium iodide concentration used did not effect the light scattering profiles of the nucleoids.

Calibration and alignment was performed using Coulter Flow-Set fluorescent beads and Coulter DNA-Check fluorescent beads. Untreated intact cells were analyzed initially to establish normal forward and 90° light-scatter conditions. All subsequent analyses were done using the PI fluorescence signal as the data trigger parameter, allowing exclusion of non-DNA (i.e. non-nucleoid) light-scatter signals. Markers encompassing the entire range of signal were then established to determine the overall mean of the nucleoid light scatter signal.

5.2.2 Flow cytometric analysis of the intercalating potential of cisplatin and [Rh(fctfa)(cod)]:

The procedure as described in 5.2.1 was used except that the cells were not radiated and the lysing solution was added to the cells directly after incubation with the drug. The final drug concentration ranged from 0.4625 μM to 2000 μM.

5.2.3 The effect of cisplatin and [Rh(fctfa)(cod)] on nucleoids using different incubation times:

The procedure as described in 5.2.1 was used. The final drug concentration ranged from 0.78 μM to 100 μM. The cells were incubated in the presence of the drug for periods of 30, 60, 90 and 120 min respectively, before the lysis buffer was added.

5.2.4 DNA damage determined by the micronuclei assay:

Samples for irradiation were prepared according to a method described by Slabbert *et al* (1996), by seeding 10^5 cells in small petri dishes (diameter of 30 mm) containing coverslips. The petri dishes were left for a period of 4 to 5 hours to allow the cells to adhere to the coverslips. Drugs were then added at a final concentration of 0.78 μM directly before making the petri dishes aerobic or hypoxic by the method as discussed in Chapter 3, paragraph 3.2.2. The cells were then irradiated by an 8 MV photon beam and directly after irradiation, cytochalasin-B was added at a final concentration of 2 μg/ml. The samples were incubated further for approximately 24 hours or to a stage where 70% of the cells appeared to be binucleated. The slips were fixed with a methanol:acetic acid mixture (3:1) and stained with a nuclear specific stain (acridine orange). Cells were then examined using fluorescence microscopy and micronuclei scored using the criteria of Almássy *et al* (1987) and Ono *et al* (1994). For each sample, micronuclei were enumerated in at least 500 binucleated cells.

5.2.5 Statistical analysis:

The results are expressed as the mean ± standard error of the mean (SEM) for between 3 and 5 experiments, with at least 2 replicates for each concentration of the test agents or control systems in each experiment. Levels of statistical significance were calculated using the Student's paired t-

test. Differences were considered significant if the probability value was less than 0.05.

5.3. RESULTS

The effect of cisplatin (0.78 μM) and [Rh(fctfa)(cod)] (0.78 μM) on the FSC and SSC parameters after radiation, measured by flow cytometery is shown in Figures 5.1 and 5.2. Figures 5.3 and 5.4 show the scatter profiles for a range of different concentrations for both cisplatin and [Rh(fctfa)(cod)]. In Figures 5.5 to 5.8 the effect on the FSC and SSC parameters after different incubation times in the presence of cisplatin and [Rh(fctfa)(cod)] is shown. A summary of the micronuclei frequency as a measurement of direct DNA damage is given in Tables 5.1 and 5.2. A photograph taken by the fluorescence microscope of a binucleated cell with micronuclei is shown in Figure 5.9.

5.3.1 Flow cytometric analysis of the effect of cisplatin and [Rh(fctfa)(cod)] in the presence and absence of radiation on nucleoid size:

The effect of radiation on cells prior to nucleoid production and PI treatment is seen in the increase in laser scatter as shown in Figures 5.1 and 5.2. The FSC parameter for a concentration of 0.78 μM for both drugs increased dose dependently and as Wang *et al*, 1996 found that an increase in nucleoid size (i.e. FSC parameter) is an indication of DNA damage we can conclude that both drugs cause DNA damage. Furthermore, it is interesting to note that even at such a low non-toxic concentration an observable difference between control irradiated samples and drug treated samples was observed. The fact that this difference is not significant can partly be due to the relative short incubation time of the cells in the presence of the drugs. The SSC parameters of both drugs (Figures 5.1 and 5.2) did not significantly differ from the

untreated control response and remained mostly unchanged over the whole dose range.

5.3.2 *Flow cytometric analysis of the intercalating potential of cisplatin and [Rh(fctfa)(cod)]:*

Flow cytometry was used to measure the winding characteristics of nucleoids. Durandt *et al*, (2000) described a method whereby the change in FSC and SSC parameters after exposure to different known intercalating agents were used to indicate intercalation or binding to DNA of experimental titanium complexes. It was found that an increase followed by a decrease in FSC possibly indicates intercalation, whereas an increase in SSC is possibly indicative of the degree to which compounds are able to bind to DNA (Durandt *et al*, 2000). Considering these findings, it is interesting to note that the FSC profile for both cisplatin (Figure 5.3) and [Rh(fctfa)(cod)] (Figure 5.4) shows a increase and then a decrease in FSC over more or less the same range of concentrations (15.6 μM to 125 μM).

There is however a big difference in the SSC parameter of [Rh(fctfa)(cod)] (Figure 5.4) compared to cisplatin (Figure 5.3), with the SSC parameter of [Rh(fctfa)(cod)] at a concentration of 2000 μM reaching a value five times in magnitude compared to cisplatin at the same concentration.

5.3.3 *The effect of cisplatin and [Rh(fctfa)(cod)] on nucleoids using different incubation times:*

The time dependant incorporation of cisplatin and [Rh(fctfa)(cod)] are shown in Figures 5.5 to 5.8. It is interesting to note that the FSC parameters of the cisplatin treated cells (Figure 5.5) did not vary much with time but the SSC parameter of 100 μM cisplatin increased significantly ($p \leq 0.05$) more so with time than either the control or 0.78 μM cisplatin treated cells (Figure 5.6). In contrast the FSC parameter of the 100 μM [Rh(fctfa)(cod)] treated cells

(Figure 5.7) remained almost constant while for the control cells and 0.78 μM [Rh(fctfa)(cod)] treated cells this parameter decreased with time (Figure 5.7). Furthermore the SSC parameter of the 100 μM [Rh(fctfa)(cod)] treated cells increased drastically ($p \leq 0.001$) compared to that of both controls and 0.78μM [Rh(fctfa)(cod)] treated cells (Figure 5.8).

5.3.4 DNA damage determined by the micronuclei assay:

The results of the micronuclei assay are summarised in Tables 5.1 and 5.2. The number of micronuclei for control samples under aerobic conditions, (Table 5.1) increased from 11.33 ± 0.88 to 75 ± 5.00 after a clinically relevant dose of 4 Gy. Both cisplatin and [Rh(fctfa)(cod)] produced similar numbers (21 ± 0.00 and 23 ± 3.00 respectively) of micronuclei after incubation (24 h) of the cells in the presence of these drugs. At a dose of 4 Gy however, both drugs produced significantly ($p \leq 0.05$) more micronuclei per binucleated cell (122.5 ± 1.50 and 158.5 ± 8.50 respectively) compared to what was observed with the control samples (75 ± 5.00). The effect of radiation alone cannot account for these differences observed, synergism is thus observed between radiation and drug treatment with the greater effect seen with the rhodium treated cells.

From Table 5.2 it can be seen that a concentration of 0.78 μM cisplatin and [Rh(fctfa)(cod)] under hypoxic conditions produce similar numbers (30 and 33 respectively) of micronuclei per 500 binucleated cells. This number however increased to 143 ± 20.5 micronuclei for cisplatin treated hypoxic cells and 171 ± 8.5 micronuclei for [Rh(fctfa)(cod)] treated hypoxic cells at a dose of 4Gy. Again the significant ($p \leq 0.05$) differences in micronuclei observed after a dose of 4 Gy for drug treated and control samples showed a synergistic effect. [Rh(fctfa)(cod)] also produced more higher numbers (4.5 ± 2.50 compared to 0.50 ± 0.50) of micronuclei per binucleated cell.

Table 5.1. Micronuclei frequency distribution observed per 500 binucleated aerobic CHO cells following irradiation (8 MV photon beam) in the absence and presence of 0.78 μM cisplatin and [Rh(fctfa)(cod)].

Treatment	Number of cells containing *n* number of micronuclei					Total Number of Micronuclei Observed
	n = 0	*n = 1*	*n = 2*	*n = 3*	*n = 4*	
Control	490.0 ± 0.58	10.0 ± 0.58	1.5 ± 0.05	0	0	11.33 ± 0.88
Control + 4Gy	425.0 ± 5.00	57.5 ± 3.50	17.5 ± 1.50	0	0	75.00 ± 5.00
Cisplatin	478.0 ± 0.00	19.0 ± 0.00	2.00 ± 0.00	0	0	21.0 ± 0.00
Cisplatin + 4Gy	377.5 ± 1.50	91.5 ± 2.50	26.5 ± 4.50	4.0 ± 0.00	0.50 ± 0.50	122.5 ± 1.50
[Rh(fctfa)(cod)]	477.0 ± 3.00	18.0 ± 4.00	4.50 ± 0.50	1.0 ± 0.00	0	23.0 ± 3.00
[Rh(fctfa)(cod)] + 4Gy	341.5 ± 8.50	111 ± 1.00	33.5 ± 5.50	9.5 ± 0.50	4.50 ± 2.50	158.5 ± 8.50

The results are expressed as the mean number of micronuclei detected ± SEM of two determinations.

Table 5.2. Micronuclei frequency distribution observed per 500 binucleated hypoxic CHO cells following irradiation (8 MV photon beam) in the absence and presence of 0.78 μM cisplatin and [Rh(fctfa)(cod)].

Treatment	Number of cells containing *n* number of micronuclei					Total Number of Micronuclei Observed
	n = 0	*n = 1*	*n = 2*	*n = 3*	*n = 4*	
Control	479.0 ± 2.00	18.0 ± 1.00	2.50 ± 0.50	0.5 ± 0.50	0	21.0 ± 2.00
Control + 4Gy	375.5 ± 7.50	74.5 ± 7.50	22.0 ± 0.00	6.0 ± 0.00	0	102.5 ± 7.50
Cisplatin	470.0 ± 1.00	27.0 ± 1.00	2.00 ± 1.00	1.0 ± 1.00	0	30.00 ± 1.00
Cisplatin + 4Gy	356.5 ± 5.5	104.5 ± 2.50	33.0 ± 1.0	6.0 ± 2.00	0	143.5 ± 5.5
[Rh(fctfa)(cod)]	470.0 ± 0.00	30.0 ± 0.00	2.00 ± 0.00	1.0 ± 0.00	0	33.00 ± 0.00
[Rh(fctfa)(cod)] + 4Gy	329.0 ± 8.50	130.0 ± 2.00	29.0 ± 4.0	9.5 ± 1.50	2.0 ± 1.00	171.0 ± 8.50

The results are expressed as the mean number of micronuclei detected ± SEM of two determinations.

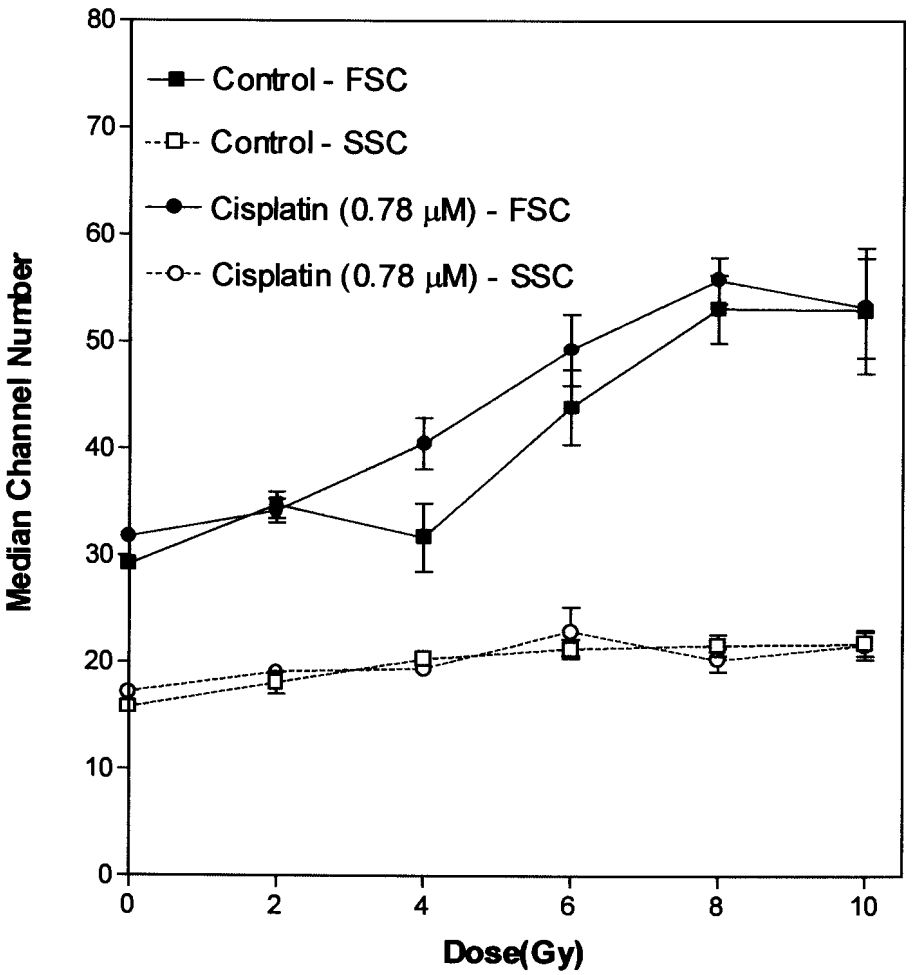


Figure 5.1. Forward angle scattered light (FSC) and side angle scattered light (SSC) parameters measured by flow cytometry after exposure to graded doses (0 – 10 Gy) in the presence or absence of cisplatin (0.78μM). Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

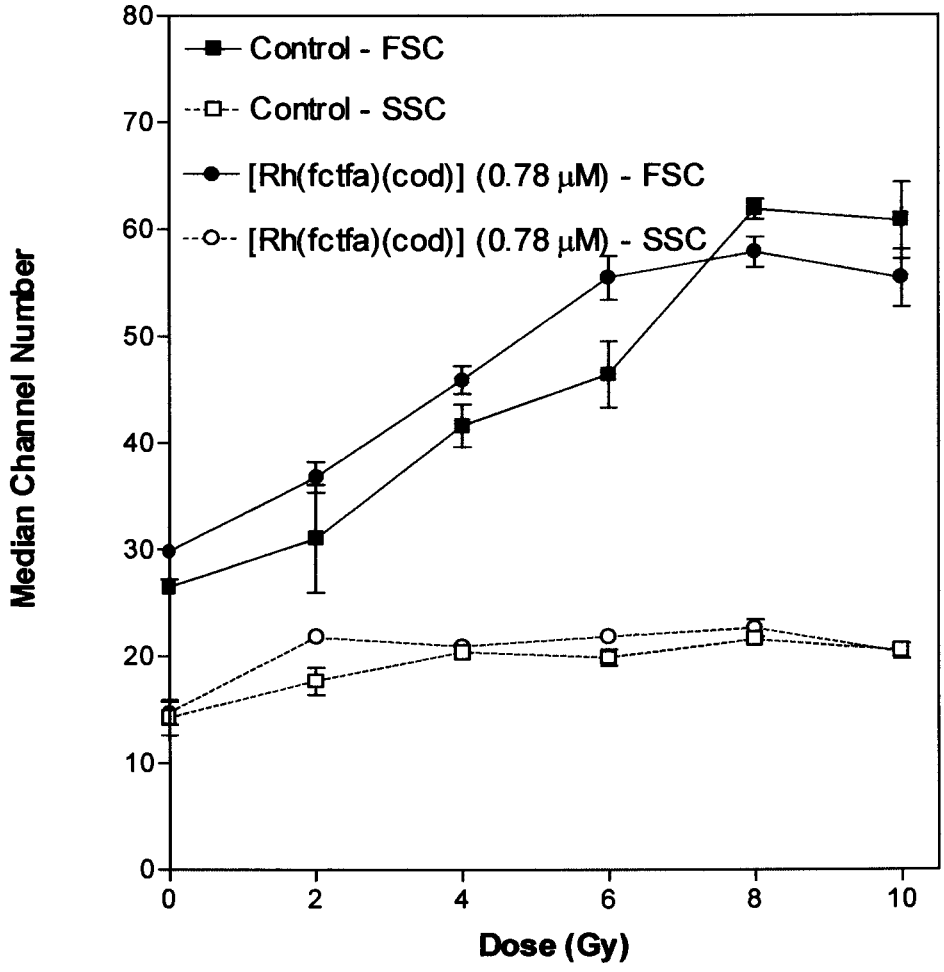


Figure 5.2. Forward angle scattered light (FSC) and side angle scattered light (SSC) parameters measured by flow cytometry after exposure to graded doses (0 – 10 Gy) in the presence or absence of [Rh(fctfa)(cod)] (0.78µM). Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

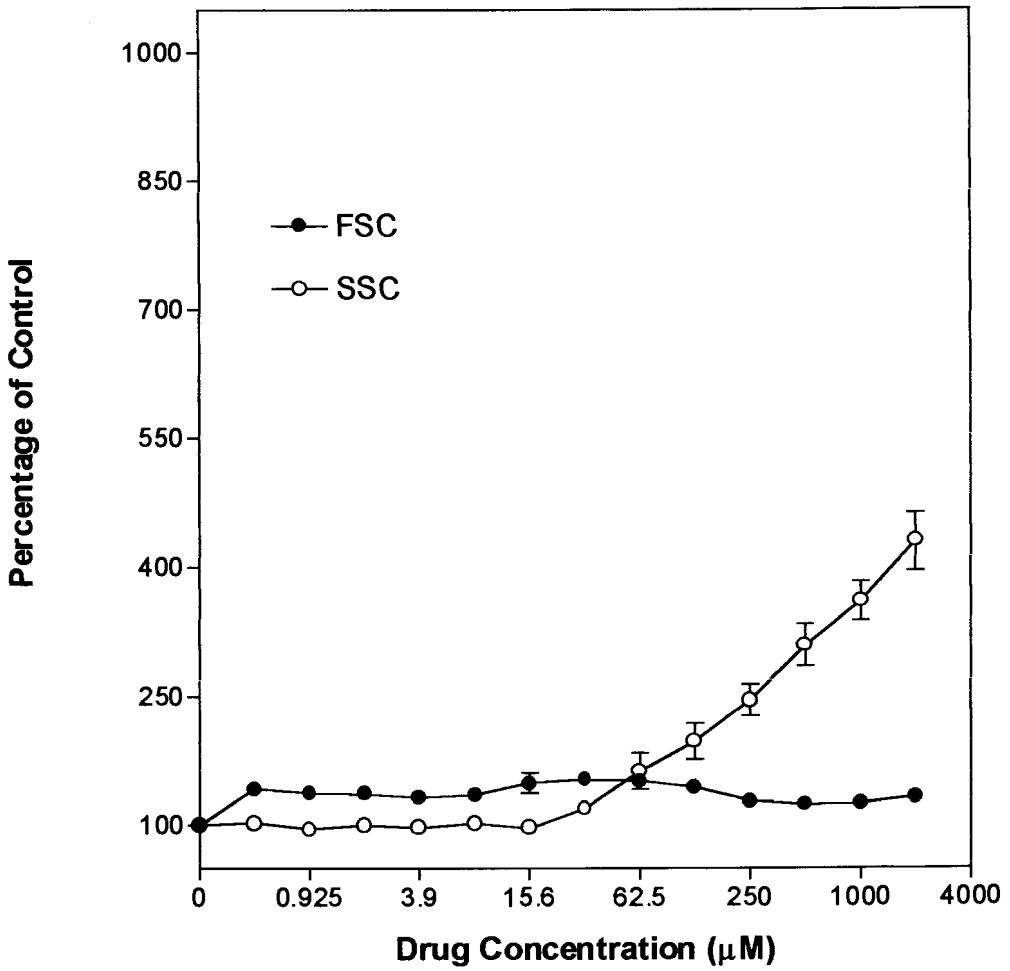


Figure 5.3. Forward angle scattered light (FSC) and side angle scattered light (SSC) parameters measured by flow cytometry in the presence of increasing concentrations of cisplatin. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

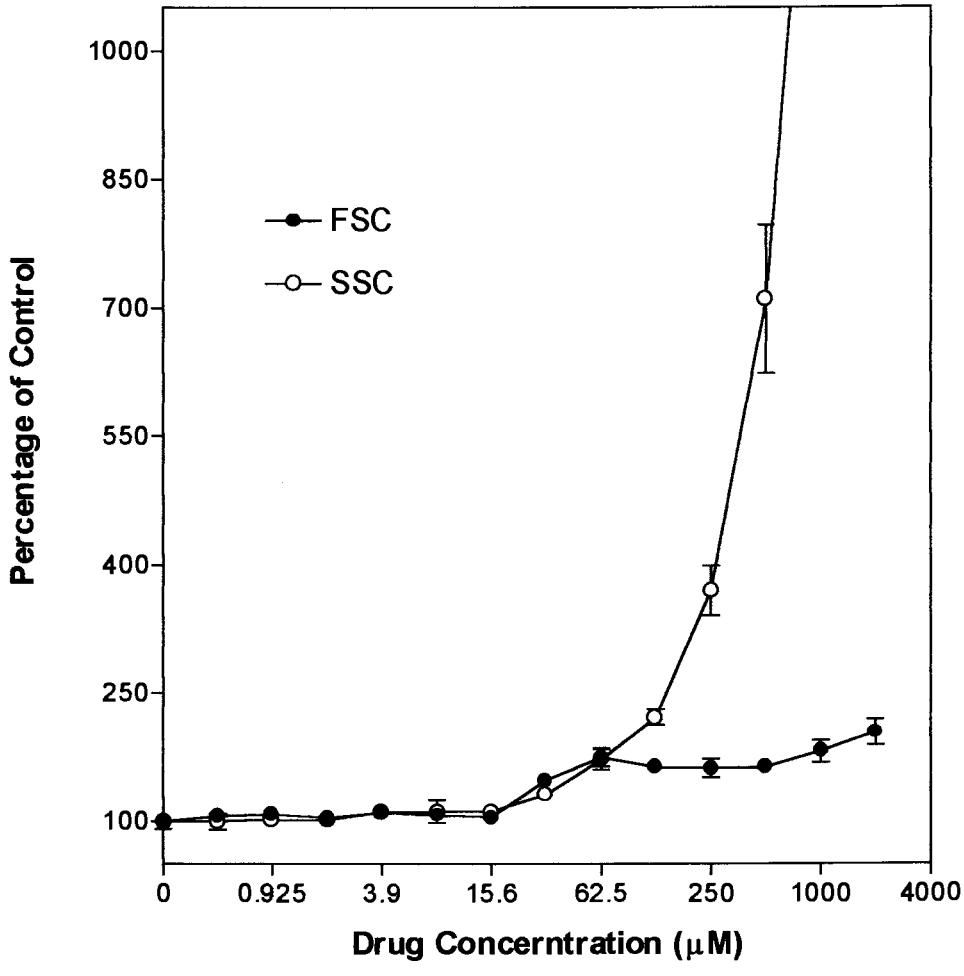


Figure 5.4. Forward angle scattered light (FSC) and side angle scattered light (SSC) parameters measured by flow cytometry in the presence of increasing concentrations of [Rh(fctfa)(cod)]. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

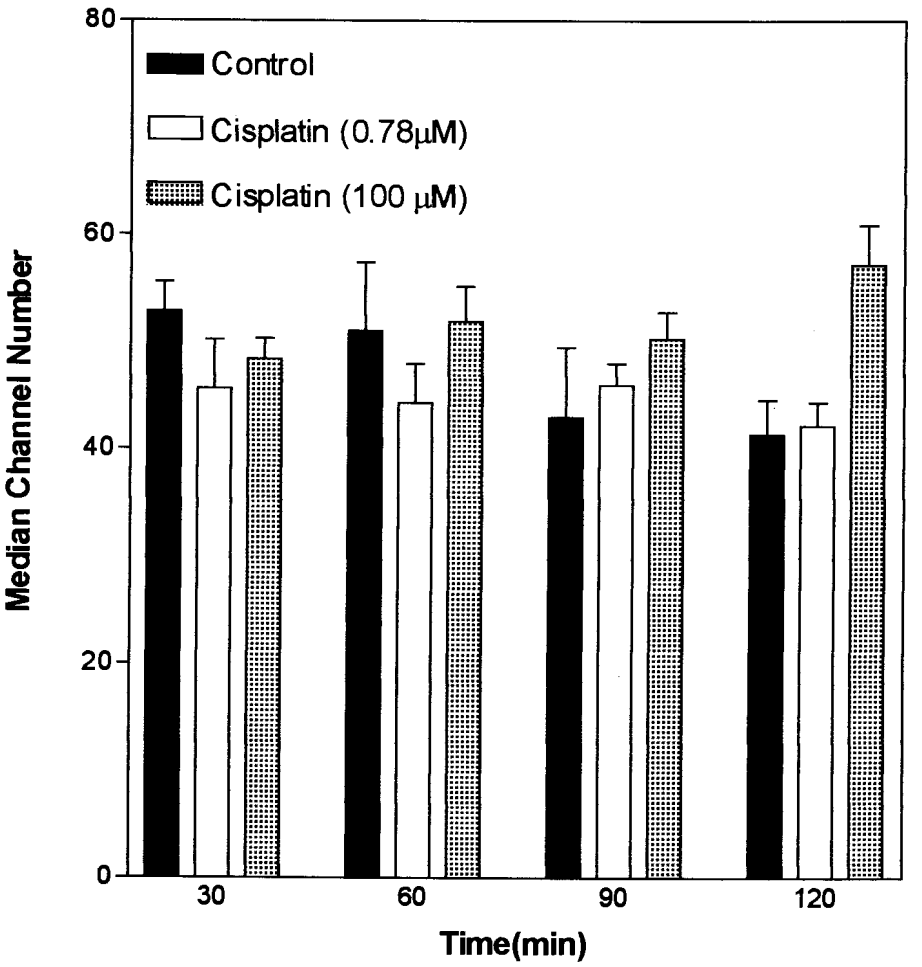


Figure 5.5. The effect of 0.78 μM and 100 μM cisplatin on the forward angle scattered light (FSC) parameter as a function of time. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

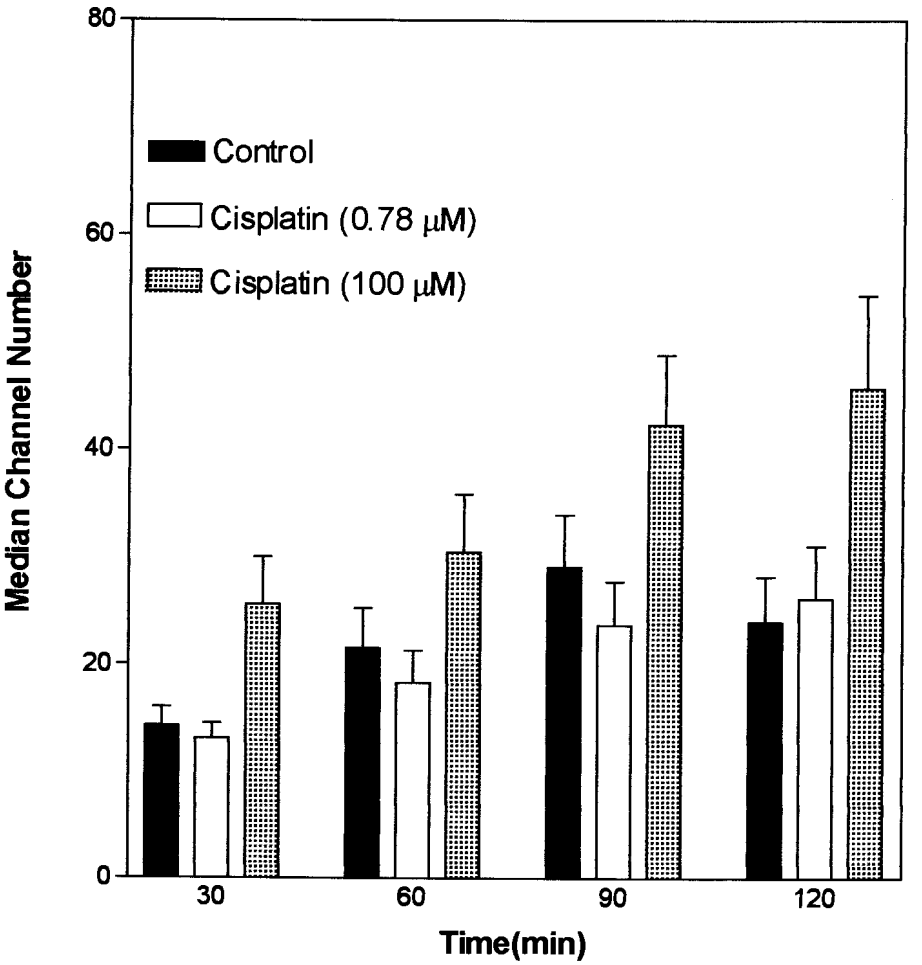


Figure 5.6. The effect of 0.78 μM and 100 μM cisplatin on the side angle scattered light (SSC) parameter as a function of time. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

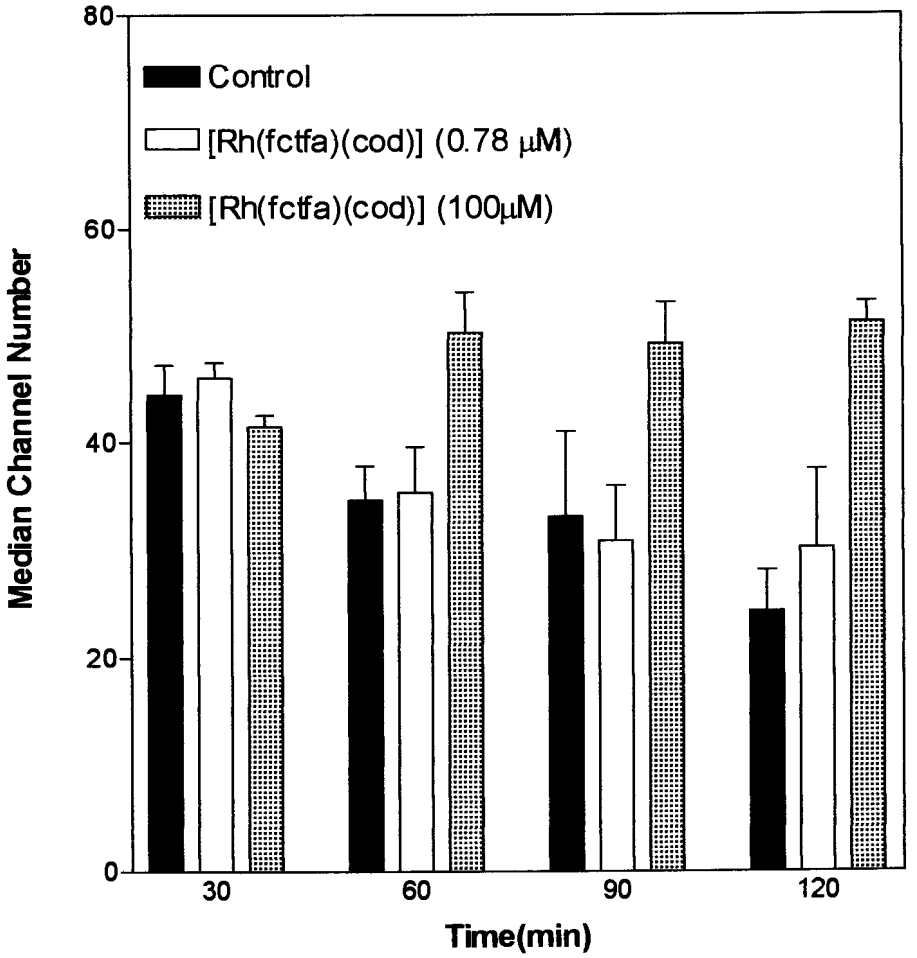


Figure 5.7. The effect of 0.78 μM and 100 μM [Rh(fctfa)(cod)] on the forward angle scattered light (FSC) parameter as a function of time. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

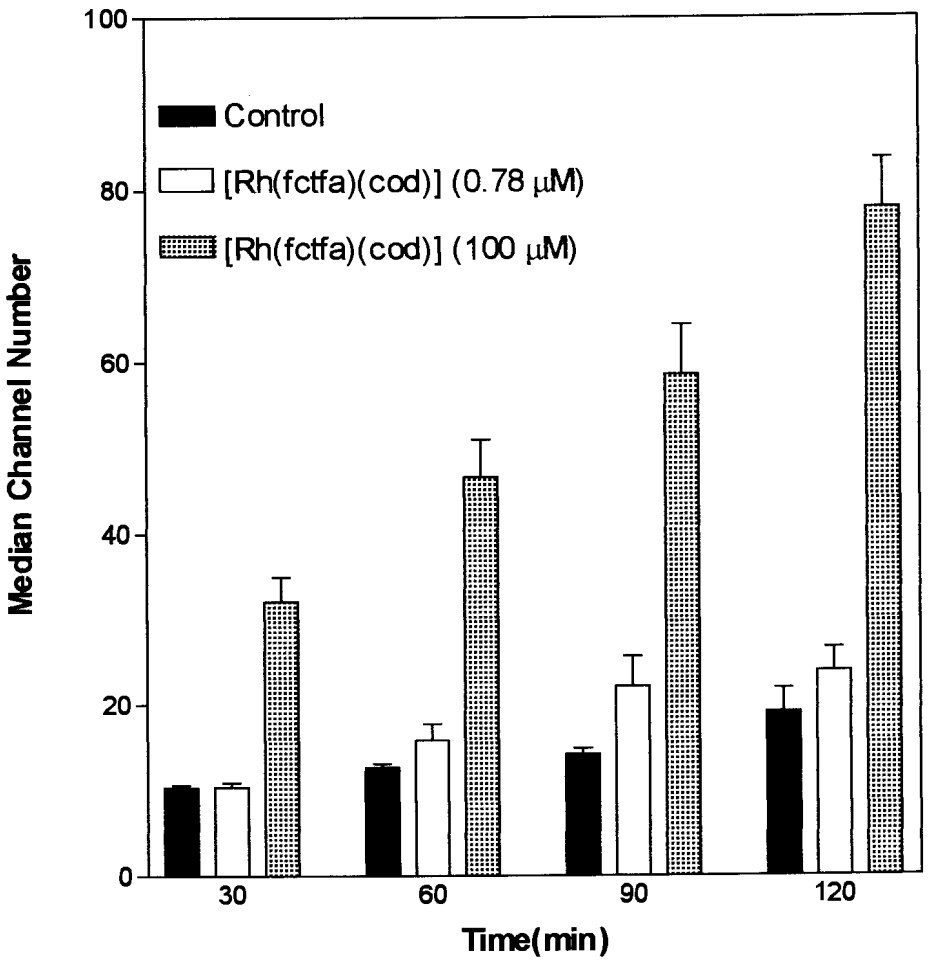


Figure 5.8. The effect of 0.78 μM and 100 μM [Rh(fctfa)(cod)] on the side angle scattered light (SSC) parameter as a function of time. Each result is expressed as the mean ± SEM of at least three experiments done in triplicate.

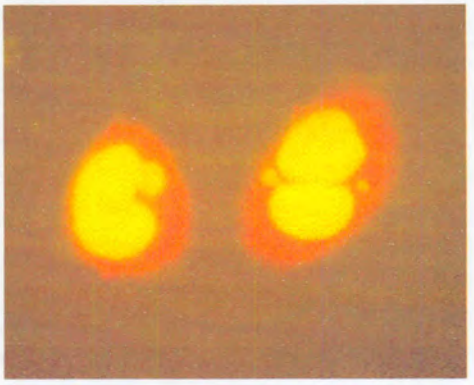


Figure 5.9. Micronuclei, detected by fluorescence microscopy, of CHO cells treated with [Rh(fctfa)(cod)] and exposed to 4 Gy.

5.4. DISCUSSION

Recent research on the action of some anticancer drugs on DNA, determined by high resolution x-ray diffraction and NMR spectroscopy indicates that cisplatin forms covalent linkages to DNA (Yang and Wang, 1999). It was found that the guanine N7 position is a favourable site for metal ion binding, including platinum compounds (Gao *et al*, 1993). Structural studies of rhodium complexes on DNA is not well reported however, there is a general sentiment that rhodium complexes and platinum complexes do not operate by similar mechanisms (Giraldi, 1977 and Chibber, 1985). Flow cytometric measurement of DNA supercoil changes have been used by some to indicate intercalation and binding (Wang *et al*, 1996 and Durandt *et al*, 2000) as well as radiation induced damage (Wang *et* al, 1996 and Wilks *et al*, 1996).

Damage incurred to cells exposed to radiation can be measured by the nucleoid assay using a flow cytometer. The rewinding of the DNA supercoils are inhibited due to radiation induced strand breaks (Roti Roti and Wright, 1987; Thomas and Thomas, 1989 and Wright *et al*, 1990) and results in the increase in nucleoid size (Wang *et al*, 1996). The increase in nucleoid size is measured as an increase in the FSC parameter. Both cisplatin and [Rh(fctfa)(cod)] at a concentration of 0.78 μM showed a dose dependant increase in FSC. The FSC and SSC parameters for the drug treated aerobic CHO cells however, did not differ significantly from the control radiation response. This is in agreement to what we found in Chapter 3, where a negligible sensitization effect determined by both MTT and clonogenic assay was seen for drug treated aerobic cells.

The determination of direct DNA damage by detection of micronuclei frequency seemed to confirm the results of treated CHO cells assessed by the clonogenic assay. At a clinically relevant dose of 4 Gy synergism was observed for both aerobic and hypoxic drug treated cells. A pronounced effect was however seen with hypoxic cells treated with cisplatin and [Rh(fctfa)(cod)]. This seems to strengthen our observation that [Rh(fctfa)(cod)] preferentially sensitizes hypoxic cells to radiation, a known property of cisplatin.

If we consider the effect of different concentrations of cisplatin on the nucleoids of CHO cells determined by flow cytometry we observe similar patterns of FSC and SSC for cisplatin and known intercalators (propedium iodide, ethedium bromide and doxorubicin – data not shown) (Durandt *et al*, 2000). The only possible explanation for the FSC pattern observed might be that because cisplatin binds covalently to adjacent guanine basis (Yang *et al*, 1995 and Yang and Wang, 1999) it may cause conformational changes of DNA as was observed by the increase followed by the decrease in FSC. The

increase in SSC is possibly an indication of the degree to which compounds are able to bind to DNA as was proposed by Durandt *et al* (2000). It is interesting to note that increasing concentrations of the rhodium complex, [Rh(fctfa)(cod)], showed similar FSC and SSC patterns compared to that of cisplatin. Following the same reasoning as that described above for cisplatin, it follows that [Rh(fctfa)(cod)] exhibits the same property of covalent bonding to DNA base pairs. This seems to confirm what was reported by others (Sartori *et al*, 1997) that Rh(I) complexes present covalent bonding with DNA bases. Furthermore, if an increase in SSC is an indicator of the binding ability of a complex to DNA, as was suggested by Durandt *et al* (2000), the rhodium complex, [Rh(fctfa)(cod)] seems to bind more effectively to DNA than cisplatin as the significant differences ($p \leq 0.05$) between the SSC parameters of these two complexes suggests.

Furthermore, from a direct comparison between the light scatter parameters after increasing incubation times in the presence of the drugs, we noted that the FSC parameter of the two drugs (100 μM) do not differ significantly. However the SSC parameter, which is an indication of the granularity/complexity of the DNA, for [Rh(fctfa)(cod)] treated cells differed significantly ($p \leq 0.05$) from that observed from the cisplatin treated cells. This can in part be ascribed to the physical size of the [Rh(fctfa)(cod)] molecule (M_r = 534.0), being bigger than that of the cisplatin molecule (M_r = 300.0). Thus as the rhodium complex binds to the DNA the nucleoids unwind as more of the drug is incorporated, the SSC parameter will thus dually increase.

At a more clinically relevant concentration (0.78 μM), the SSC parameters of both drugs do not differ significantly, but we see an interesting phenomenon in the FSC parameter of these drugs observed at incubation times greater than 60 min where a significant difference ($p \leq 0.005$) is noted. The FSC of

the rhodium treated cells decreases dramatically with increase in incubation time while the cisplatin treated cells FSC parameter remained unchanged with time. If we consider that FSC is an indication of size of the DNA, a decrease in size would indicate that the rhodium complex causes condensation of the nucleoid translating as a reduction in size of the DNA. The fact that we did not observe this behaviour at the higher concentration of 100μM, seems to shed some light on the mechanism that sensitises the [Rh(fctfa)(cod)] treated cells to radiation. As Giraldi *et al* (1977) noted the mechanism of rhodium(I) complexes and cisplatin are indeed not the same as this experiment so clearly indicates.

In conclusion, the use of flow cytometry compared to other methods allows for a rapid analysis of nuclear damage within individual cells. It could be a useful tool in the modelling of new and more effective anti-tumour metallo complexes regarding the experimental complexes presently examined.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION.

If we reconsider the list of requirements of a radiosensitizer as proposed by Adams in the early 1960s (Hall, 1994), we note that the novel complex [Rh(fctfa)(cod)] complies to almost all the prerequisites. Not only does [Rh(fctfa)(cod)] have a cytotoxic activity comparable to that of cisplatin, a known radiosensitizer, but at a very low, sub-lethal concentration, the drug produced a marked dose modifying factor (DMF) against hypoxic CHO cells in the clinically relavant dose range. Furthermore, it was also shown that [Rh(fctfa)(cod)] preferentially sensitizes hypoxic cells to radiation, with negligible dose modifying ability experienced under oxia.

Another encouraging finding was that the MTT assay used in this study produced similar results compared to that obtained from the more traditional clonogenic assay. Not only did this serve as confirmation that our novel system using the modular incubator chamber to attain aerobic and hypoxic conditions in conjunction with the MTT assay produces results comparable to that published for cisplatin, it also proved to be a more rapid and quantitative method of screening large numbers of potential drugs for radiosensitizing properties.

Nais (1985) reported that dose modifying factors produced by metal complexes as sensitizers are often small (<2) using cells in tissue culture however, as was seen for cisplatin, a relatively low effect still results in a noteworthy therapeutic gain in the clinic (Coughlin and Richmond, 1984). Although the results reported here cannot illustrate the activity of the tested complex against human tumours *in vivo*, they undoubtedly indicate that the complex exhibits interesting cancer cell inhibiting properties associated with remarkably low cytotoxic activity. Furthermore, it is extremely encouraging for future clinical exploitation of the complex, [Rh(fctfa)(cod)], considering that no evident nephrotoxcicity has been

reported (Giraldi *et al*, 1977; Sava *et al*, 1983; Craciunescu *et al*, 1989; Craciunescu *et al*, 1991) for rhodium(I) complexes compared to the severe side-effects experienced with cisplatin (Hacker *et al,* 1984; Nicolini, 1988).

The radiosensitization ability of [Rh(fctfa)(cod)] was not only tested using a photon beam (8 MV) but also using a p(66)/Be neutron beam. This was done to determine if the drug sensitizes by direct action, causing less repairable damage due to the induction of double strand breaks which would result in synergism observed with the neutron beam, or by more indirect action (e.g. radical formation – more repairable damage) to which neutrons per se are insensitive. From the lack of sensitazation of [Rh(fctfa)(cod)] treated hypoxic CHO cells with the neutron beam I am able to speculate that the radiosensitization action of the complex possibly targets the repair mechanisms of the cell.

Results obtained from the flow cytometric measurement of DNA damage incurred by irradiation of aerobic CHO cells in the presence of both cisplatin and [Rh(fctfa)(cod)] did not produce a marked increase in the forward angle scattered light (FSC) parameter which indicates DNA strand breaks (Wilks *et al*, 1996), this seems to confirm my finding that [Rh(fctfa)((cod)] is not effective under aerobic conditions. However, DNA damage assessed by the micronuclei assay showed an increase in micronuclei in aerobic and hypoxic cells treated with cisplatin and [Rh(fctfa)(cod)] with the greater effect observed with [Rh(fctfa)(cod)] treated hypoxic cells. Further light scatter measurements done by flow cytometry, where the effect of increasing concentrations of both drugs on the forward angle scattered light (FSC) and side angle scattered light (SSC) parameters were assessed, seems to indicate that both drugs bind covalently to DNA base pairs, with [Rh(fctfa)(cod)] exhibiting a greater increase in the SSC parameter which possibly indicates its ability to bind to DNA (Durandt *et al,* 2000).

Although there is still research to be done in order to fully understand the cytotoxic mechanism of the complex, [Rh(fctfa)(cod)] in interaction with radiation and biological systems, it is hoped that further structure-activity work and the use of animal model systems predictive of activity against human malignancies may lead to the eventual introduction of this novel complex into clinical trails.

In conclusion, this research shows that [Rh(fctfa)(cod)], regardless of the mechanism operating during the inhibition of tumour cells, possesses the right structural and chemical properties to interact with biological molecules to produce the desired effect.

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APPENDIX 1:

DEFINITION AND CALCULATION OF THE MEAN INACTIVATION DOSE:

The concept of mean inactivation dose $\overline{D}$, first introduced by Kellerer and Hug (1972), has subsequently been used to analyze mammalian survival curves (Fertil *et al*, 1984).

$$\overline{D} = -\int_0^\infty D \times \frac{d(S(D))}{dD} \times dD = \int_0^\infty D \times s(D) \times dD$$

After partial integration, we have the formula

$$\overline{D} = \int_0^\infty S(D)dD$$

$\overline{D}$ is thus equal to the area under the survival curve plotted in linear coördinates.

Where; S = survival fraction;
D = dose

and

$$S(D) = e^{-(\alpha D + \beta D^2)}$$

The parameters α and $\beta$are determined by linear regression analysis of the natural logarithm of S.

APPENDIX 2:

Lysis Buffer:

Lysis buffer (pH 8) (modified from Milner *et al,* 1987) is employed in all flow cytometry studies to release the DNA in the supercoiled form, the nucleiod.

Lysis buffer consists of:
- 10 mM diSodium EDTA
- 10 mM TRIS Buffer
- 2 M NaCl
- 0.1% Triton X
- 0.01% PI