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Interaction Between Cisplatin and Radiation in the Treatment of Ovarian Cancer Cells

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Interaction between Cisplatin and Radiation in the Treatment of Ovarian Cancer Cells

Submitted by

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ABSTRACT

The A2780s is a cisplatin and radiation-sensitive human ovarian carcinoma cell line from which the more resistant variant, A2780cp, was derived. *In vitro* experiments on the radiosensitivity of these cells with different treatments may be representative of the *in vivo* radiosensitivity, and maybe of use to patients receiving radiotherapy. Therefore, many studies have been directed at evaluation of the inhibition of DNA repair in order to improve radiotherapy. Previous studies showed that drugs and hyperthermia are repair inhibitors increasing the effectiveness of radiation. Cisplatin can also radiosensitize the A2780s and A2780cp and inhibit the sublethal damage repair (SLDR) (Raaphorst *et al.*, 1995a). In this study we found no radiosensitization in the A2780s and A2780cp cells when cisplatin is added immediately before or after radiation. When cisplatin was added 24 hours prior to radiation, no increase in cellular resistance (adaptive response) to radiation treatment was observed. On the other hand cisplatin induced resistance when added during a pulse dose rate (PDR of 1Gy/h) radiation treatment.

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LIST OF ABBREVIATIONS

(A) : Number of adducts

(A2780s) : The parental human ovarian cancer cell line

(A2780cp) : Derived cisplatin resistant cell line

(α) : Alpha

(AovC-O) : The parental human ovarian cancer cell line

(AovC-DDP/Ro) : Derived cisplatin resistant cell line

(AP) : Apurinic/ Apyrimidinic

(ATM) : Ataxia telangiectasia mutated protein

(ATR) : Ataxia telangiectasia related protein

(B) : Number of single strand breaks

(β) : Beta

(BER) : Base excision repair

(Caski) : Human squamous carcinoma of the cervix cell line

(CHO) : Chinese hamster ovaries

(D) : Dose

(DMEM/F12) : Dulbecco's modified Eagle's medium with the 12 non-essential amino acids

(DMF) : Dose modifying factor

(DNA) : Deoxyribonucleic acid

(DNA-PKcs) : Deoxyribonucleic acid dependent protein kinase catalytic subunit

(dsb) : Double strand break

(dsbs) : Double strand breaks

(ER) : Enhancement ratio

(ERCC1) : Human excision repair gene cross-complementing Chinese hamster ovary mutant cell lines of complementation group1

(GSH) : Glutathione

(Gy) : Gray

(HDR) : High dose rate

(HCT116) : Human colorectal adenocarcinoma cell line

(Hela-S3) : Human adenocarcinoma of the cervix cell line

(HMG) : High mobility group

(HR) : Homologous repair

(IGROV-1) : The parental human ovarian cancer cell line

(IGROV-1/Pt) : Derived cisplatin resistant cell line

(LDR) : Low dose rate

(LX-1) : Human adenocarcinoma of the lung cell line

(μg) : Microgram

(μM) : Micromolar

(MDR) : Medium dose rate

(MMR) : Mismatch repair

(MRI-186) : Adenocarcinoma of the cervix

(n) : number of base pairs

(NER) : Nucleotide excision repair

(NHEJ) : Non-homologous end-joining pathway

(PDR) : Pulse dose rate

(PE) : Plating effeciency

(P_{int}) : Probability of interaction

(PLDR) : Potentially lethal damage repair

(Pt) : Cis-diamminedichloroplatinum II

(RIF1) : Mouse tumor model

(RNA) : Ribonucleic acid

(S) : Fraction of cells surviving

(SCID) : Severe combined immune- deficient

(SEM) : Standard error of the mean

(SF) : Suriving Fraction

(SLDR) : Sublethal damage repair

(SSB) : Single strand break

(SSBS) : Single strand breaks

(UV) : Ultraviolet

(U-87MG) : Human glioma cell line

(WAR) : Whole abdomen irradiation

(XP) : Xeroderma pigmentosum

(XPAC) : Human excision repair gene that correct the defect in XP group A cells

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To the beloved memory of my mother who died of ovarian cancer in the full blossom of her life.

1.0 INTRODUCTION

Cisplatin is a widely used chemotherapeutic agent. Unfortunately tumour cisplatin resistance often develops and causes failure of further cisplatin treatment. To circumvent this problem, a multimodal therapy is now used. A combination of different chemotherapeutic drugs with and without radiotherapy is now studied in clinical trials.

1.1 Overview of clinical outcome of cisplatin-based radio-chemotherapy

As we indicate below, studies demonstrate the positive effect of cisplatin-based radio-chemotherapy in some types of cancer. But it is not the case with every cancer, as we will see in the course of the present overview.

1.1.1 Cisplatin and radiation treatment of cervical, head and neck, and non-small cell lung cancer

Radiotherapy with concurrent cisplatin-based chemotherapy regimen has been viewed as the most decisive breakthrough in the treatment of cervical cancer, showing approximately a 50 percent reduction in risk of recurrence (Rose *et al.*, 1999;Keys *et al.*, 1999;Morris *et al.*, 1999). Concurrent, but not adjuvant, cisplatin based radio-chemotherapy has also been effective in head and neck

cancers (Brizel *et al.* 1998, Wendt *et al.* 1998, Fountzilas *et al.* 1997, Sharma & Wilson 1999, Harrison *et al.* 1998, Robbins *et al.* 1997, Chougule *et al.* 1994).

In a 2-year study of stage III non-small cell lung cancer, there was a 21% survival rate for cisplatin-based chemotherapy followed by radiotherapy compared to 14 % for the radiotherapy only group. Distant metastases were lower in the group that received cisplatin followed by radiation treatment and local control was poor in both groups (Le Chevalier *et al.*, 1991).

It was demonstrated in a 5-year study that concurrent cisplatin-based chemotherapy with radiotherapy showed a better response rate compared to the sequential approach in stage III non-small cell lung cancer. The survival rate for concurrent radio-chemotherapy was 15.8% compared to 8.9% for the sequential approach. However, there was no difference in the development of distant metastases (Furuse *et al.*, 1999). The results suggest that higher doses of chemotherapy, used in the sequential approach, are believed to eradicate the distant metastases and the smaller chemotherapy doses, used in the concurrent treatment, improve local control by acting as a radiosensitizer (Gandara *et al.*, 2000).

An important improvement of treatment for lung, cervical and head and neck cancers was obtained by the utilization of cisplatin in conjunction with radiotherapy. According to those clinical studies, sequential treatments, with cisplatin added before radiotherapy showed a lower or no therapeutic gain compared to the concurrent treatment. Therefore, for an effective chemoradiation treatment, cisplatin should be added simultaneously with

radiation.

1.1.2 Cisplatin and radiation in the treatment of ovarian cancer

Ovarian cancer, the type of cancer upon which we experimented, is the leading cause of death from gynecologic malignancies. It is also the fifth leading cause of cancer death in women (Landis *et al.*, 1998). Chemotherapy is often used to treat ovarian cancer and radiotherapy has also proven to play a curative role for patients with small volume disease after initial debulking surgery (Cardenes & Randall, 2000).

The introduction of platinum compounds and paclitaxel treatments was revolutionary in oncology. They were found to be successful for ovarian cancer patients immediately after surgery. There remains the problem, however, that there is often a relapse and progression of the disease, even if the initial treatment is effective. When relapse occurs, drug resistance will render further treatment ineffective and the cancer fatal (Cardenes & Randall, 2000).

The use of radiotherapy to overcome drug resistance also has its limitations. Pelvic irradiation cures early stage disease, but whole abdomen irradiation (WAR) has a limited role in the treatment of peritoneal metastases (Thomas & Dembo, 1993).

Since cisplatin is a well-known radiation sensitizer, concurrent chemotherapy and radiation therapy is now studied in the clinic. However, there is no evidence of a favorable outcome for concurrent WAR and intraperitoneal

cisplatin in ovarian cancer patients in stages III and IV (King *et al.*, 1991) or abdominopelvic radiotherapy with cisplatin for the early ovarian cancer (Wong *et al.*, 1999).

Unlike other types of cancer, the above references indicate that combined cisplatin and radiation has not been conclusively shown to be superior to either cisplatin or radiation alone in ovarian cancers.

1.2 Radiotherapy

Radiotherapy is one of the most common ways to fight cancer. It has a local action on the tumour itself with limited damage to surrounding tissue. Unlike chemotherapy it is well tolerated by the body. The radiotherapy weaponry is killing the targeted cancer cells through the emission of X-rays.

1.2.1 X-rays

When absorbed in the matter through which they pass, the X-rays release their energy to produce fast-moving charged particles (fast recoil electrons). These recoil electrons can ionize target molecules or indirectly interact with water to produce hydroxyl radicals, which interact with the target molecule. The hydroxyl radicals are responsible for two thirds of the biological damage caused by X-rays (Hall, 1994).

1.2.2 Single strand breaks (ssbs) and double strand breaks (dsbs)

The main effective target of ionizing radiation is DNA. Many single strand breaks are produced in DNA by ionizing radiation but are readily repaired, using the opposite strand as a template. Breaks in both strands, if well separated, are also readily repaired, since they are handled individually by the repair mechanism. It is largely accepted that breaks in both strands that are opposite, or separated by only a few base pairs, may lead to a double strand break. Thus, radiation-induced breakage and incorrect rejoining lead to chromosome and chromatid aberrations (Hall, 1994).

1.2.3 Potentially lethal damage repair (PLDR) and sublethal damage repair (SLDR)

The component of radiation damage that can be modified by manipulation of the post-irradiation conditions is known as potentially lethal damage. Potentially lethal damage repair (PLDR) can occur if cells are prevented from dividing after irradiation. Such prevention is viewed as an increase in survival.

Sublethal damage repair (SLDR) is an operational term that describes the increase in survival when a dose of radiation is split into two fractions separated by incubation time at 37°C (Hall., 1994). Very few ssbs fail to rejoin, so the vast majority are sublethal. Any such sublethal ssb may become a potentially lethal break pair, if another sublethal ssb occurs within some range where it is possible

for both of them to interact and cause a dsb (Bedford, 1991).

1.2.4 Linear Quadratic Model

Our purpose was, in part, to shed some light on the cell survival after radiation. The cell survival curve (Linear Quadratic Model) is used to assess the relationship between the radiation dose and the survival of the cells. The colony forming assay, or clonogenic assay, is often used to determine cell survival after giving different doses of radiation. When a cell is unable to divide to produce a large colony, it undergoes a reproductive death and it is no longer considered as a survivor. After irradiation treatment the cells are plated and the number of colonies counted. Survival is plotted against the radiation dose to give the survival curve. The survival curve has a specific shape. The linear quadratic model is used to describe the shape of the survival curve. According to this model, the expression for the cell survival is

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

Where S is the fraction of cells surviving a dose D, and α and β are constants. Normal or cancer cell lines derived from mammals or humans exhibit irradiation survival curves. In those cell lines, at the lower doses, the survival curve shows a decrease in survival proportional to the dose. At higher doses, the decrease in survival is proportional to the square of the dose. The linear component (α) accounts for dsbs from a single ionizing event. The quadratic component (β) accounts for dsbs from two distinct ionizing events (Hall., 1994).

The Linear Quadratic Model was used to fit all of our survival curves, even if it is not normally applied to the combined cisplatin and radiation treatment.

1.3 Repair of radiation damage

The DNA repair of radiation damage, the very mechanism that insures the survival of the species from exogenous and endogenous onslaughts, is the same mechanism that interferes with the radiation treatment. The relationship between cell viability and repair of dsbs was shown in yeast (Frankenberg-Schwager & Frankenberg, 1990). The study of various mammalian mutant cell lines indicated as well that cell survival did not correlate with the number of dsbs inflicted, but depended solely on the proficiency of particular cells to repair dsbs (Eguchi-Kasai *et al.*, 1991).

The types of DNA damage produced by radiation are base damage, a single strand break, a double strand break and cross-links. An excision repair process restores the base damage and ssb. A ssb can be restored by the repair mechanism through using information present on the remaining intact strand (Frankenberg-Schwager, 1989).

DNA double strand breaks are repaired by at least two repair pathways: The homologous repair (HR) pathway and the non-homologous end-joining pathway (NHEJ). Both systems are conserved throughout eukaryotic organisms, but while the NHEJ is a major repair pathway for DNA dsb repair in higher eukaryotes, unicellular organisms, such as yeast, rely most importantly on

HR {Jeggo 1998 86 /id}.

We will see now how the repair process of the double strand breaks and the damaged base operates.

1.3.1 The homologous repair (HR) pathway

The HR pathway uses an undamaged homologous strand as a template to repair the broken DNA strands. This is a high-fidelity repair mechanism since the information loss at the break is repaired using information from the undamaged homologous chromosome or sister chromatid.

In yeast, the set of genes, whose products play an important role in HR pathway, are the Rad series, Rad 50,51,52,54,55,57,59, the Mre11 and the Xrs2 genes. There are known mammalian homologues of these factors. The Rad 50, Mre11 and Xrs2 are involved in nuclease activity operating in the resection of the DNA dsb in the 5` to 3` direction. These resulting single strand tails are then bound by Rad 54 and 52. The Rad 51 finds an homologous sequence on an undamaged chromosome, or sister chromatid, bring it in close proximity of the damaged area and catalyses an exchange of DNA strands. The DNA polymerase copies information from the undamaged homologue strand to the damaged DNA strand. DNA ligase finally joins the DNA ends, the branch then migrates and the cross-over (Holliday junction) is resolved (Cromie *et al.*, 2001).

1.3.2 Non homologous end joining (NHEJ) pathway

The NHEJ repair pathway predominates in the restoration of mammalian double strand breaks. It ligates the two ends of DNA together at regions of little or no homology. The NHEJ in yeast and vertebrate cells involves essentially the same proteins: Ku70, Ku80, DNA ligase IV, and XRCC4 dimer. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is also required in mammalian cells, but its yeast homologue is still to be found. Direct ligation of the DNA ends is possible, but in most cases, the ends require some processing, leading to sequence deletions of various length. (Leskov *et al.*, 2001;Jeggo, 1998).

There is evidence that the activities of these repair pathways may vary in different phases of the cell cycle. After radiation, the HR pathway plays a dominant role in the S-G_2 phase, while the NHEJ pathway may play a greater role in the G_1-S phase of the cell cycle (Takata *et al.*, 1998;Lee *et al.*, 1997).

1.3.3 Base excision repair (BER) pathway

The base excision repair (BER) pathway involves the majority of the base and sugar phosphate lesions generated by ionizing radiation (Wallace, 1998). The enzymes involved in the BER pathway are the DNA glycosylases, with an associated AP (apurinic/apyrimidinic) lyase activity, and the AP endonuclease.

The DNA glycosylase recognizes the damaged bases. It releases the damaged bases and cleaves the AP site. The AP endonucleases hydrolyze the phosphodiester bonds at AP sites and remove blocking groups, such as 3`-phosphate and 3`-phosphoglycolate, produced during strand break formation by free radical reactions on the deoxyribose. If the base lesions are on the opposite strand, or if a ssb is in the vicinity of the base lesion, the BER may lead to a dsb (Weinfeld *et al.*, 2001). Now that we have reviewed the repair mechanism of the cell after radiation, let us review those mechanisms that detect damage and trigger a cascade of events that lead to repair of the damaged DNA or the death of the cell.

1.4 Cellular signaling of radiation damage

Once the DNA is damaged by radiation, multiple events lead to its repair and may also lead to the death of the cell when the damage is too severe. Cell suicide or apoptosis occurs when there is a risk for the damaged DNA to induce mutations that would compromise the integrity of the entire organism. The phosphoprotein P53, is involved in signaling for either apoptosis, cell cycle arrest or DNA repair. It is the key element in the protection of the genome. Before P53 is triggered there are different models for DNA damage detection.

There are various models for detecting and signaling DNA damage, but we have limited ourselves to those that appear more promising and more generally accepted.

1.4.1 Ataxia telangiectasia mutated protein (ATM)

The ATM (ataxia telangiectasia mutated protein) and the ATR (ATM related protein) are protein serine-threonine kinases which are involved in the signaling of the DNA dsbs in mammalian cells (Abraham, 2001). The ATM patients are hypersensitive to irradiation and are predisposed to cancer and neuro-degenerative disorders (Shiloh, 2001). When the ATM gene is disrupted in mouse cells, the ATM-/- deficient cells become hypersensitive to irradiation. The cells are also defective in cell cycle arrest and P53 up-regulation following irradiation (Xu & Baltimore, 1996). The *in vivo* experiments in ATM deficient mice also showed defective induction of P53 in the thymus of the animal after whole body irradiation (Barlow *et al.*, 1997).

1.4.2 DNA protein kinase (DNA-PK)

It is also believed that DNA-PK, involved in the NHEJ pathway, has a similar sequence and function to the ATM and ATR (ATM related) proteins and acts as a sensor for DNA dsbs. (Rosen *et al.*, 1999) (Smith & Jackson, 1999). It is also known to phosphorylate P53 (Smith & Jackson, 1999). The severe combined immune-deficient (SCID) mouse has a mutated DNA-PK and is extremely sensitive to radiation (Kirchgessner *et al.*, 1995). Once the radiation damage is sensed by the cells, P53 is activated, its half-life is increased and a nuclear signal is triggered, that leads to apoptosis or repair (Rosen *et al.*, 1999).

1.5 Different radiation regimens used in the clinic

After the above-overview of the effects of radiation on the cell, let us look at how they translate in the clinical treatment of cancer.

There is variation in the dose and the frequency of the irradiation treatment of cancer in general. The goal to achieve is always to have both a maximum tumour damage and a minimum normal tissue damage.

1.5.1 High dose rate (HDR), pulse dose rate (PDR) and low dose rate (LDR) irradiation

In the clinic, irradiation treatment can be given as HDR (high dose rate) for external beam therapy, or as LDR (low dose rate) and PDR (pulse dose rate) regimens for interstitial (into the tumour) and intracavitary therapy (proximity to the tumour). With the HDR treatment, patients receive, in general, 2 Gy per day. The radiation dose given cannot exceed the maximum dose that can be tolerated by the surrounding normal tissues.

The optimum fractionation can be defined as a schedule providing maximum tumour suppression and minimal normal tissue complications. It critically depends on the proliferative state of the tumour cells relative to that of the normal tissue at risk.

There are biological factors that influence the optimum total dose and the fraction size of fractionated radiotherapy. Fast proliferating tumours need shorter

irradiation schedules and for slow proliferating tumours, where there is large intracellular repair, larger doses per fraction are necessary (Fowler, 2001).

1.5.2 Brachytherapy treatment

Brachytherapy, a therapeutic modality currently used in the clinic, employs radioactive sources placed in proximity or directly into the tumour. The administered dose rates range between 0.6-0.8 Gy per hour. The malignant tissue receives very high doses compared to the normal tissues.

A good alternative to LDR brachytherapy is PDR brachytherapy. Instead of giving a constant low dose of irradiation, the dose is given as a pulse of radiation with an hour or more between each pulse.

The clear advantages of PDR treatment over LDR treatment in clinic are as follow:

1. The iridium source used can move stepwise through the implant and there is accurate control of the source position.
2. All the implants can be handled with one source.
3. The dose and dose rate can be prescribed for each point along the implant.
4. The source can deliver the pulse dose in an implant in 10 minutes or less.
5. There is no radiation exposure to the staff between the pulses, allowing visits between pulses of radiation.

6. No treatment interruption is necessary
7. The treatment time remains the same as LDR (de Pree *et al.*, 1999).

The dose rate in PDR brachytherapy is a very important component of the treatment. A study on human radiation sensitive and resistant cell lines showed that two different pulse dose rates, 4.25 Gy/h (a medium dose rate (MDR)), and 63 Gy/h (an HDR), given during each pulse of radiation, demonstrated different biological effectiveness. If less than 1 Gy is used every hour, the survival is equal to the LDR continuous irradiation treatment. Higher doses given during each pulse will largely decrease the survival of tumour cells and will locally act as an HDR irradiation treatment. (Pomp *et al.*, 1999).

Sensitization of PDR with hyperthermia (a known radiation sensitizer) is demonstrated in the glioma cells (U-87MG) at the plateau phase using 41°C hyperthermia treatment. The thermosensitizing effect is equal in both LDR (0.5 Gy/h) and PDR (1 Gy/h, 1 Gy/2h) (Raaphorst *et al.*, 1999). 40 °C hyperthermia also sensitizes the A2780s parental and A2780cp cisplatin resistant ovarian carcinoma cell lines to a PDR irradiation treatment of 1.6 Gy given every 3 hours. No sensitization was seen in the 0.53 Gy/h regimen (Niedbala *et al.*, 2001b).

What we have seen so far is a short summary of state of science on research and application of radiation in the treatment of cancer.

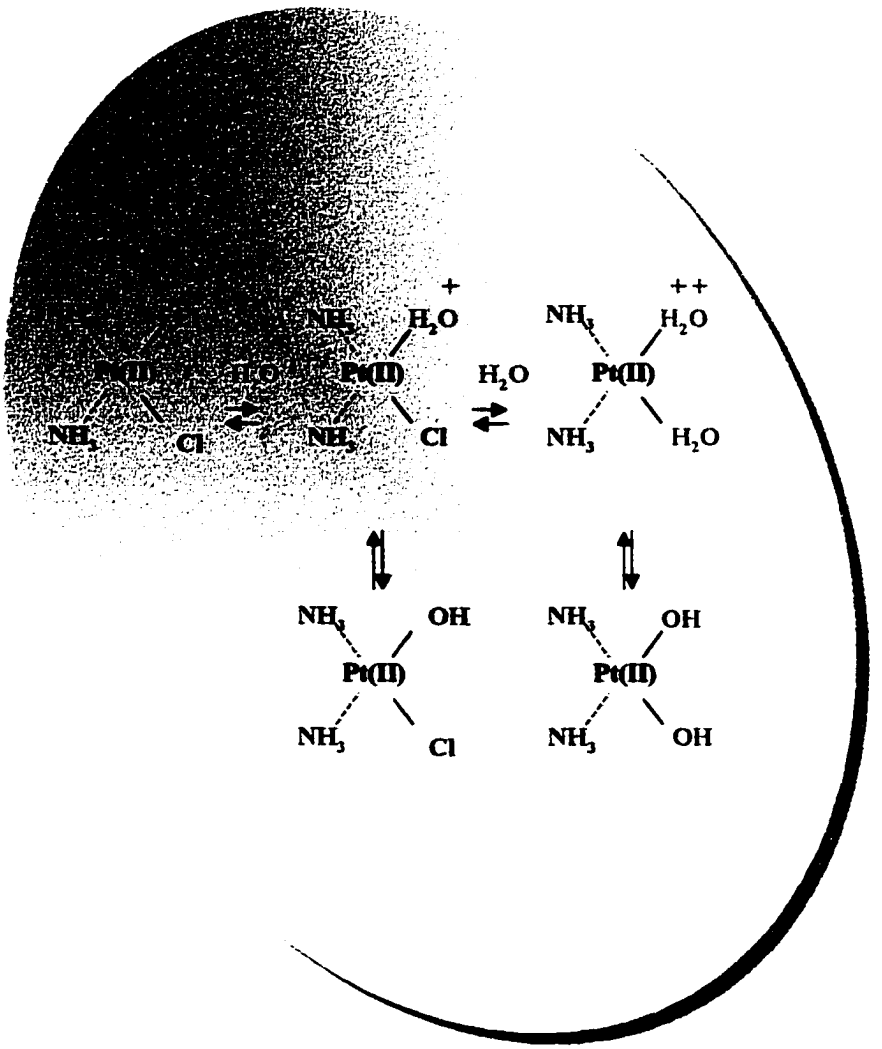
1.6 Cisplatin treatment

Cisplatin (cis-diamminedichloroplatinum II) is one of the most potent chemotherapeutic anti-tumour drugs. Its activity has been demonstrated against several types of tumours, such as testicular, ovarian, head and neck, and small cell lung cancers. Cisplatin follows a definite biochemical pattern in the treatment of cancers as we will see hereafter.

1.6.1 Activation of the cisplatin molecules (Fig.1.1.1)

Cisplatin is less reactive in blood and extra-cellular fluids where chloride concentration is high. The cisplatin reactivity increases when it enters the cell in a low chloride environment, where it undergoes hydration. It is the aquated cisplatin species that forms the reactive compound. The exchange of an anionic chloride for water molecules gives a platinum complex with a positive charge. The aqua group is a good leaving group and the positively charged complex is thought to be electrostatically attracted to the negatively charged DNA helix and allows the cisplatin molecule to react with nucleophilic groups containing oxygen, nitrogen or sulphur atoms with unpaired electrons. The nucleophilic groups are present in amino acid side chains, as well as purine bases of DNA and RNA (Jordan & Carmo-Fonseca, 2000;Cohen & Lippard, 2001a).

Figure 1.1.2. The cisplatin-DNA adduct complexes. Formation of the monofunctional and bifunctional cisplatin-DNA adducts.

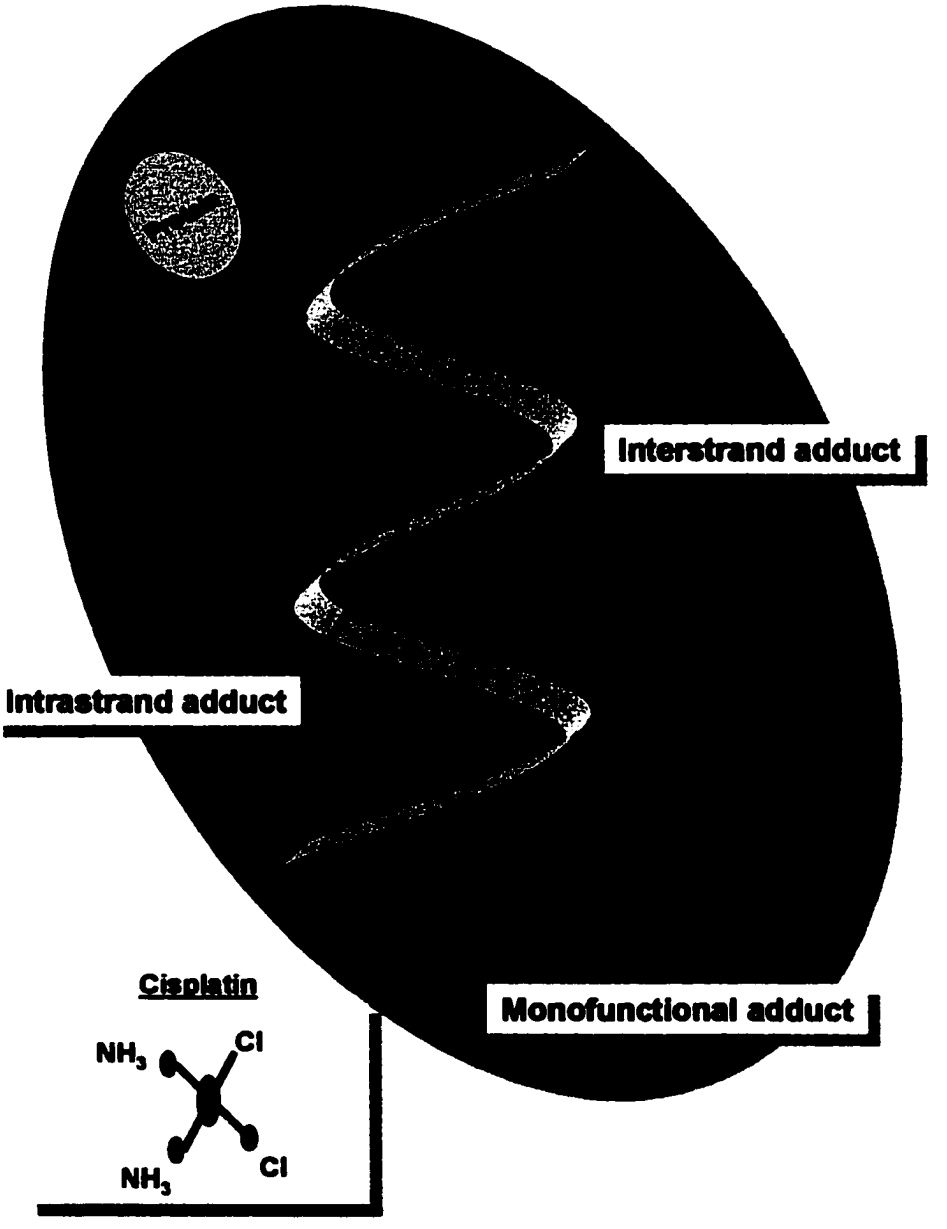

NH3
H2O
Pt(II)
Cl
NH3
H2O
Pt(II)
H2O
NH3
H2O
Pt(II)
NH3
H2O
NH3
OH
Pt(II)
NH3
Cl
NH3
OH
Pt(II)
NH3
OH

1.6.2 Formation of the cisplatin adducts (Fig 1.1.2)

The cisplatin cytotoxic effect is mostly mediated through damaging DNA by the formation of inter- and intrastrand cross-links, monofunctional adducts or DNA-protein cross-links. DNA repair deficient cells are more sensitive to cisplatin toxicity (Dijt *et al.*, 1988). The binding of cisplatin to RNA and protein is minimal and thus does not inactivate them (Akaboshi *et al.*, 1992). Cisplatin binds almost exclusively to the N7 atom of the purine bases with a preference for guanine over adenine.

Cisplatin forms approximately 65% 1,2-d(GpG), 25% 1,2-d(ApG) and 5-10% 1,3-d(GpNpG) intrastrand cross-links and a small percentage of interstrand cross-links and monofunctional adducts. The trans-DDP (trans-diamminedichloroplatinum II) has demonstrated very low anti-tumour activity. It forms predominantly the 1,3 intrastrand and interstrand cross-links. Since the configuration of the trans-isomer does not allow the formation of the 1,2 intrastrand cross-links, it suggests that the toxicity of cis-DDP originates from the 1,2-intrastrand cross-links (Cohen & Lippard, 2001b).

Figure 1.1.1. Biochemistry of cisplatin. Formation of the reactive aquated cisplatin species.


Interstrand adduct
Intrastrand adduct
Cisplatin
NH3
Cl
NH3
Cl
Monofunctional adduct

1.6.3 Cisplatin resistance

The potential mechanisms responsible for the acquired cisplatin-resistance fall into two categories. The first category is comprised of mechanisms which lead to a reduction in the formation of cytotoxic DNA lesions, such as a decrease in drug accumulation; an increase in drug efflux; and an enhanced intracellular drug inactivation by thiol-containing proteins or by non-protein sulphydryl molecules. The second category includes mechanisms that minimize the impact of cisplatin DNA lesions such as enhanced DNA repair; induced alterations in topoisomerase II; and an increase in tolerance for DNA damage (increased replicative bypass and alteration of p53-levels) (Crul *et al.*, 1997).

Studies involving three radiation-sensitive mutant cells and their more radiation-resistant counterparts showed no cross-sensitivity to cisplatin treatment (Raaphorst *et al.*, 1994). According to those studies, mechanisms causing radiation sensitivity may not be involved in cisplatin sensitivity.

1.7 Repair of the cisplatin adducts

The repair of cisplatin adducts is somewhat complex as we will see below. The nucleotide excision repair (NER) pathway plays a major role in its repair. Cisplatin resistance and increased repair often go together. Proteins binding to the cisplatin adducts as well as other repair mechanisms highly influence

cisplatin toxicity.

1.7.1 Nucleotide excision repair (NER) pathway

Cisplatin adducts are primarily repaired by the nucleotide excision repair (NER) pathway. Cell lines cultured from patients with xeroderma pigmentosum (XP), a disease caused by genetic deficiencies in the NER pathway, show a loss of the fast initial repair component and thus are highly sensitive to cisplatin (Dijt *et al.*, 1988).

It has been demonstrated that the three major adducts, the 1,2-d(GpG), 1,2-d(ApG), and the 1,3-d(GpTpG) intrastrand cross-links are repaired by the mammalian NER system. The 1,3-d(GpTpG) intrastrand adduct is the most efficiently repaired adduct and the level of repair in the 1,2-d(GpG) intrastrand adduct is similar to the 1,2-d(ApG) intrastrand adduct (Huang *et al.*, 1994b;Zamble *et al.*, 1996b).

In a study involving 28 patients with ovarian cancer, the level of mRNA in tumour tissues of two genes involved in the NER [the ERCC1 gene (human excision repair gene cross-complementing Chinese hamster ovary mutant cell lines of complementation group1) and XPAC (human excision repair gene that corrects the defect in XP group A cells)] were quantified before treatment. Sensitive tumours to platinum-based chemotherapy showed low levels of these NER factors. High levels of the ERCC1 and XPAC were found in non-responding tumours (Dabholkar *et al.*, 1994).

Enhancement of the repair pathway often resulted in cisplatin resistance. The cisplatin-resistant ovarian carcinoma A2780cp cell line shows an increase in DNA excision repair after exposure to cisplatin compared to its homologue parental cell line A2780s. The DNA excision repair was measured in these cell lines using bromodeoxyuridine DNA incorporation after cisplatin treatment (Lai *et al.*, 1988b).

1.7.2 High mobility group (HMG) proteins

Several proteins containing a high mobility group (HMG) domain bind cisplatin in a structured-specific manner. The HMG-domain proteins recognize the typical distortion of the 1,2-d(GpG) and 1,2-d(ApG) cisplatin-DNA intrastrand adducts but not the 1,3-d(GpTpG) intrastrand adducts. The HMG-domain proteins inhibit NER of the 1,2-d(GpG) and 1,2-d(ApG) intrastrand cisplatin adducts but not the 1,3-intrastand adducts (Huang *et al.*, 1994a;Zamble *et al.*, 1996a;Zamble *et al.*, 1996b). No HMG-specific protein known to date binds transplatin (Crul *et al.*, 1997).

In yeast, the IXR1 gene encodes a structured-specific recognition protein (SSRP1). This protein contains two HMG domains, which are binding specifically to DNA modified by the cisplatin adduct but not DNA modified by the transplatin adduct. Disruption of the IXR1 gene results in increased cisplatin resistance in yeast (Brown *et al.*, 1993).

There are two models of modulation of cisplatin toxicity by the HMG

proteins. They could either shield the cisplatin-DNA intrastrand adducts from the repaired enzymes, or they might be drawn away from their natural binding sites by binding to cisplatin-DNA intrastrand adducts. These mechanisms could also work in concert (Crul *et al.*, 1997).

1.7.3 Mismatch repair (MMR) proteins

Cisplatin adducts are also recognized by mismatch repair (MMR) proteins. The MMR system eliminates mistakes made during replication from newly synthesized strands. It also participates in the cell cycle checkpoint control system. Loss of MMR often results in resistance to cisplatin, carboplatin, ectoposide and other cytotoxic agents, but not transplatin. Recent observations support the hypothesis of a direct role for MMR in coupling cisplatin damage to an apoptotic response whereas a lack of MMR leads to cisplatin resistance (Fink *et al.*, 1998).

Loss of MMR can also result in an increased mutation rate throughout the genome and can lead to oncogenesis or drug resistance. The A2780s parental cell line is cisplatin sensitive and expresses the mismatch, mRNA and proteins (human MLH1, PMS2, MSH2 and MSH6). The A2780cp cisplatin-resistant cell line derived from the A2780s and 9 out of 10 cisplatin-resistant clones, derived independently from the A2780s after multiple exposures to cisplatin, lack hMLH1 mRNA and proteins. These hMLH1 deficient cell lines also show a loss of ability to engage G1 and G2 cell cycle arrest after cisplatin exposure. It was also found

that patients with ovarian cancer show an increased level of hMLH1-negative tumours after chemotherapy treatment (Brown *et al.*, 1997).

The hMLH1-deficient human colorectal adenocarcinoma HCT116 cell line is a known MMR deficient cell line. It lacks the hMLH1 MMR protein. The integration of chromosome 3 restores the expression of the hMLH1 gene. For example when a mixture of 5% of the original HCT116 cells with 95% chromosome 3-complemented HCT116 (HCT116 + chromosome 3) cells are treated with increasing levels of cisplatin, this results in an enrichment of the HCT116 MMR deficient cells (Fink *et al.*, 1997). The cisplatin treatment selected the MMR deficient cells and made the remaining population of cells more cisplatin-resistant.

Embryonic stem cells, which are deficient in the MSH2 MMR protein, also show resistance to cisplatin. The injection of these stem cells into nude mice showed that, after cisplatin treatment, the MMR deficient tumours were significantly bigger. The loss of MMR, which results in a higher level of resistance, could lead to failure of treatment *in vivo* (Fink *et al.*, 1997).

The level of P53 in MMR deficient HCT116 cells is higher after 72-96 hours post-cisplatin treatment, compared to the HCT116 +chromosome 3 cells. HCT116 cells become as sensitive to cisplatin (similar to the HCT116 +chromosome 3 cells) following disruption of the P53 gene. This indicates that the lack of the hMLH1 proteins in the HCT116 cells would enhance the protective role of p53 from the cisplatin-induced DNA damage (Vikhanskaya *et al.*, 1999).

1.8 Cisplatin cytotoxicity

Similarly to radiation cisplatin induced its cytotoxicity through activation of P53 and triggering of apoptosis. It could also interfere with DNA transcription and replication.

1.8.1 Cisplatin induction of apoptosis

The specific mechanisms that trigger apoptosis in response to cisplatin stresses have not yet been defined. These mechanisms have to include ways to detect DNA damage and determine whether it is strong enough to be lethal. The apoptosis pathway had been extensively studied in the ovarian carcinoma A2780 cell lines. In the case of both the sensitive and the cisplatin-resistant derivatives of A2780s cells, cisplatin DNA damage results in cell death via apoptosis (Henkels & Turchi, 1997).

We know that the P53 tumour suppressor gene is critical in regulating cell proliferation following DNA damage. P53 stops the cell cycle progression of damaged cells and in many cases it even causes apoptosis of the cells in a last attempt to protect the organism (Vogelstein *et al.*, 2000).

A model of two different pathways was proposed for the induction of P53. The first pathway involves the blockage of RNA polymerase II by its inhibitors (actinomycin D, α-amanitin, DRB, H7), UV (ultraviolet) radiation or cisplatin which appears to be sufficient for the induction of P53 in normal fibroblasts. In the

second pathway, the P53 response can be triggered by DNA strand breaks without the requirement of RNA polymerase II inhibition following exposure to ionizing radiation (Ljungman *et al.*, 1999).

Mutations in P53 were found to be in IGROV-1/Pt cisplatin-resistant ovarian cancer cell lines. As opposed to the parental cell line (IGROV-1), these mutations impaired the induction of apoptotic regulator genes, P21 and bax (Perego *et al.*, 1996). The A2780cp cisplatin-resistant cell line showed higher levels of P53 and Bcl-2 than the parental cell line A2780s. The A2780s Bcl-2 transfected cell line becomes cisplatin-resistant, showing the protective role of Bcl–2 against cisplatin. Bcl-2 expression delays P53 accumulation and induction of Bax mRNA. Also the A2780s mutant that over-expresses P53 becomes resistant to cisplatin. Bcl-2 and P53 mutant A2780s cells are even more resistant. Thus, the data confirms the importance of Bcl-2 and P53 genes in cisplatin-induced apoptosis and resistance (Eliopoulos *et al.*, 1995).

Incidentally, loss of P53 function is associated with the acquisition of cisplatin resistance. P53 genetic suppressor elements, which confer resistance to cisplatin, were found in the A2780 cell lines (Gallagher *et al.*, 1997). In both the cisplatin-resistant and the sensitive A2780 cell lines, cisplatin-induced apoptosis proceeds via a caspase-3 dependent and independent pathway (Henkels & Turchi, 1999). Using the DNA microarray, it was found that P53 related gene P73 activates P53 targets such as P21 and Bax in the A2780 cells. Overexpression of P73 was found to confer resistance to cisplatin and increase expression of DNA repair genes involved in MMR and NER (Vikhanskaya *et al.*,

2001).

In the various array of experiments done on cisplatin-induced apoptosis, we only reported those related to ovarian cancer cells in general or ovarian A2780 cell lines used for our own experiment.

1.8.2 Cisplatin inhibition of transcription and replication

Cisplatin is a known transcription inhibitor (Mymryk *et al.*, 1995). A cisplatin or transplatin-modified β-galactosidase reporter gene was transfected into hamster and human cell lines. The level of transcription was two to three times higher in the transplatin-modified reporter gene compared to the cisplatin-modified reporter gene. This difference was not due to increased repair of the transplatin adducts since the NER deficient cell lines still showed the same differences in the level of transcription. However, the RNA polymerase II bypass was significantly higher in the transplatin-treated reporter gene (Mello *et al.*, 1995). Since transplatin is largely less toxic than cisplatin, we can infer that the lower transcription activity in the presence of the cisplatin would account for part of its toxicity.

The use of a simian virus in an African green monkey cell line indicates that for the same amount of adducts bound to the viral DNA, both transplatin and cisplatin show the same inhibition level of replication. However, at the same drug concentration, the level of adducts was lower for the transplatin, due to an increase in repair, resulting in a decrease in inhibition of replication (Ciccarelli *et*

al., 1985). Therefore, we cannot conclude that cisplatin toxicity is due to inhibition of DNA replication.

1.9 Cisplatin radiosensitization

Cisplatin is increasingly used in combination with irradiation for experimental studies and is also used in clinic. Cisplatin is found to be a radiosensitizer in bacteria and some mammalian cells in culture. It can also inhibit both sublethal damage repair (SLDR) and potentially lethal damage repair (PLDR). Most studies find that cisplatin causes the largest supra-additive effect when added immediately or a short time period before radiation (Dewit, 1987). So far, it is not clearly established how, when it does, cisplatin acts as a radiation sensitizer. But, different models have been made to tentatively explain the potentiation of radiation by cisplatin.

1.9.1 Mathematical model of cisplatin and radiation interactions

Chadwick's model implies that when a cisplatin intrastrand adduct is present in the vicinity of an ssb in the opposite strand, the ssb cannot be repaired. Replication of the ssb will lead to a double strand break and becomes lethal to the cells (Chadwick *et al.*, 1976).

The probability of interaction (P_{int}) is described by the following equation: $P_{int}=(-n.A.B.10^{-10})$, where A is the number of adducts in the cell, B is the number

of single strand breaks in the cell and n is the number of base pairs required for the interaction to occur.

The enhancement ratio (ER) is described as follows: $ER_{dsb}=(dsb_x+dsb_{inter})/dsb_x$. The dsb_x are the number of double strand breaks induced by radiation and the dsb_{inter} are the number of double strand breaks induced by the interaction of cisplatin and radiation. The ER will increase with closer interaction distance and the dose of radiation or drug. It decreases when repair is taking place. An ER of 1.2 is often seen. The ER increases if the repair of the interacting lesion is severely compromised. The evaluation of the increase in dsb, when cisplatin is combined with radiation has been difficult. The cross-links reduce the elution rate of DNA in the electrophoresis gel, opposing any increase in elution caused by more breaks (Begg, 1990).

1.9.2 Mouse model for the use of cisplatin as a radiosensitizer

Intratumoural delivery of cisplatin with a polymer implant potentiates the effect of fractionated radiation treatment in the RIF1 mouse tumour model. Cisplatin given with fractionated regimens of 5 Gy per day for 5 days (Begg, 1990) or 5, 8 and 12 fractions of 9 Gy per day (Yapp *et al.*, 1998) significantly increases tumour growth delay in the RIF1 tumour in mice.

1.9.3 Cisplatin used as a radiosensitizer in cell cultures

Well-oxygenated cells are more sensitive to ionizing radiation than hypoxic cells (Douple, 1985). Oxygen as a radiosensitizer has been intensively studied, since there is strong evidence that hypoxia exists in rapidly dividing tumours, limiting the success of standard radiotherapy treatment.

Radiosensitization by oxygen and cisplatin at a low dose of radiation was studied in Chinese hamster V79 cells. Below 0.5 Gy, there was evidence of hypersensitivity followed by a resistant portion in the shape of the survival curves (Marples & Joiner, 1993). This resistance could be explained by an induction of a repair mechanism or a cell cycle delay, allowing more time for repair. Non-toxic cisplatin doses of 1 μM, for 1 hour prior to irradiation in hypoxic CHO cells sensitized the cells. The region of increased resistance is no longer seen. The question remains: Are the cells more radiation-sensitive in low dose regions or do they become resistant at higher radiation doses? (Skov *et al.*, 1994).

1.9.4 Cisplatin radiosensitization in the A2780cp and A2780s

In vitro experiments on the radiosensitivity of cancer cells with different treatments may be representative of *in vivo* radiosensitivity and may be useful for characterizing and optimizing responses to radiotherapy and/or chemotherapy. The present study investigates the response of the A2780s (cisplatin-sensitive) and the A2780cp (cisplatin-resistant) ovarian cell lines to both radiation and

cisplatin treatment.

Previous studies have also shown that simultaneous treatment with cisplatin, mild hyperthermia and low dose rate (LDR) irradiation of the A2780s and A2780cp cell lines induces a synergistic interaction resulting in the inhibition of sublethal damage repair (SLDR). For the A2780s, cisplatin and mild hyperthermia was effective for radiosensitization when, given before or after LDR irradiation. However, when submitted to the same process, the A2780cp resistant cell line showed less radiosensitization on the LDR irradiation response curve (Raaphorst *et al.*, 1997).

For the A2780s and A2780cp, cisplatin can also cause radiosensitization and inhibit SLDR, when given in combination with high dose rate (HDR) irradiation. Cisplatin-induced radioresistance may also occur in these cell lines. This would need further investigation.

1.10 Induced-radioresistance

Recent studies of *Saccharomyces cervisia* reported that the radiosensitizing effect of cisplatin was due to the inhibition of DNA repair processes involving error-free RAD-52-dependent recombinational repair. In contrast, sublethal doses of cisplatin given 2 hours before radiation increase the resistance to cell killing (Dolling *et al.*, 1999). However, the mechanisms of resistance are not yet known.

Previous studies on A2780s and A2780cp show that there was no obvious

difference in their level of radiation-induced DNA dsb or their dsb-rejoining rate. However, in the A2780cp cell line, there was a significantly lower level of dsb mis-rejoining events involving deletions between 13 to 444bp that arose through illegitimate recombination of short repetitive sequences such as those that appear through non-homologous repair. This demonstrates that the acquired resistance to radiation in the A2780cp cell line may be the result of an up-regulation of the homologous repair pathway (Britten *et al.*, 1999a).

Some research has shown that low doses of ionizing radiation and low doses of cisplatin induced nuclear activity in the A2780cp cell line that catalyses homologous double strand recombination. These studies have indicated a possible pathway for development of induced-resistance in the A2780cp cell line (Lehnert & Chow, 1997).

1.11 Specific aims of our study

The literature review has lead us to conclude that cisplatin interaction with radiation is complex and may not always lead to radiosensitization. Radiosensitization and possibly induction of radioresistance may be dependent on radiation application such as dose rate, pulsed irradiation, as well as the sequence of application. Therefore we set out to further characterized cisplatin-induced radiosensitization and radioresistance in an homologous pair of cell lines, the cisplatin-sensitive A2780s and cisplatin-resistant A2780cp lines. As previously indicated, there is an actual cisplatin radiosensitization in A2780s and

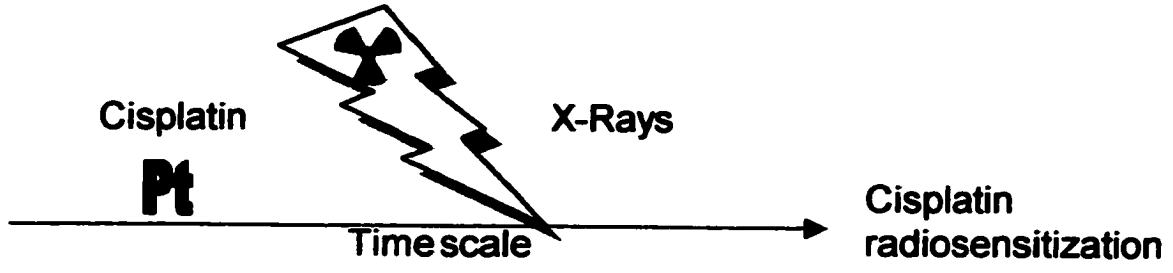
A2780cp, but no investigation has been undertaken to study radioresistance in these cell lines. We gave different doses of cisplatin immediately before and after irradiation to radiosensitize the cells. The previously published data could not be reproduced due to the use of different experimental procedures.

To test the radioresistance theory, small doses of cisplatin were given at different times before irradiation to check for an increase in cell survival after irradiation. No radioresistance was observed. To complete the study, pulse dose rate (PDR) experiments were conducted to test the effect of cisplatin on radiosensitization using pulsed irradiation. Our experiments on the ovarian tumour cell lines should lead to a better understanding of the effectiveness of cisplatin as a radiosensitizer and may help define optimal clinical protocols.

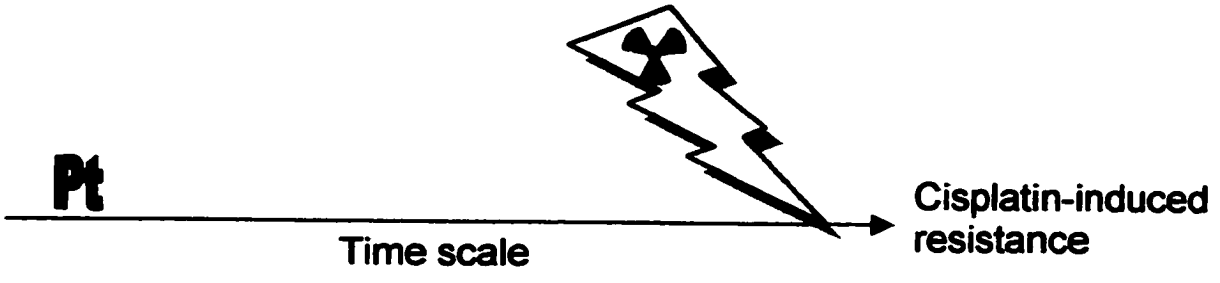
1.12 Hypothesis

1.12.1 Cisplatin added before a radiation dose

When cisplatin is added immediately before a radiation dose it could sensitize the cell to radiation.

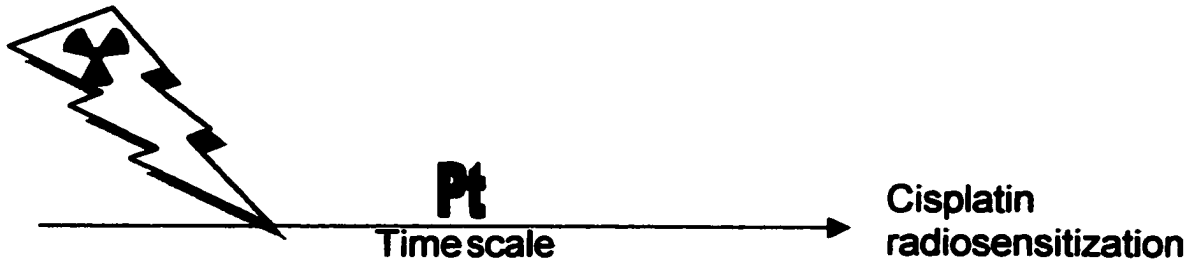


If cisplatin is added 24 hours before a radiation dose it could lead to an increase in repair and allow the cells to be more resistant to the subsequent dose of radiation.



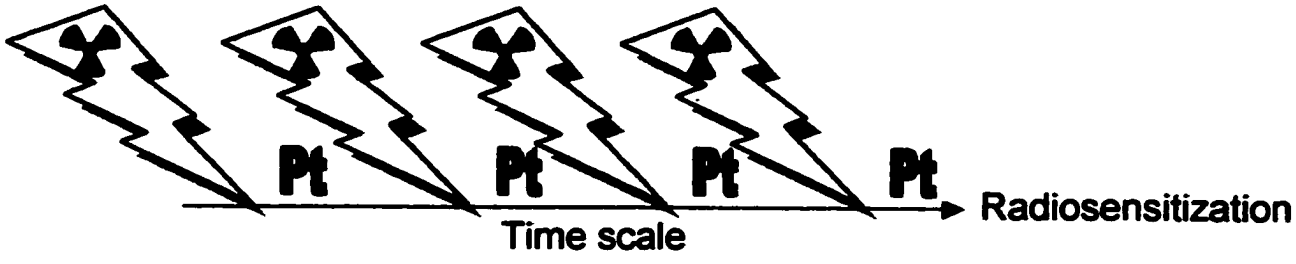
1.12.2 Cisplatin added after a radiation dose

Cisplatin added immediately after a radiation dose would radiosensitize the cell through inhibition of potentially lethal damage repair (PLDR) :



1.12.3 Cisplatin added during pulsed dose rate of radiation (PDR)

Cisplatin added during and between pulses of radiation would radiosensitize the cells through inhibition of sublethal damage repair:



2.0 MATERIALS AND METHODS

The A2780s cell line was derived from an untreated patient and provided by Dr. S. Aaronson of the National Cancer Institute. The A2780cp phenotype was established by progressively increasing concentrations of cisplatin up to 70 μM. By doing this, the cells were rendered more resistant to cisplatin and showed cross-resistance to radiation (Behrens *et al.*, 1987a;Lai *et al.*, 1988b).

All cell lines were cultured in DMEM/F12 media (GIBCO) and supplemented with 10% fetal bovine serum (Wisent). The powder form of DMEM/F12 (Dulbecco's modified Eagle's medium containing 12 non-essential amino acids and L-glutamine) was dissolved in ddH_20. After the media was dissolved, 10 mM bicarbonate (Boehringer Mannhein) and 20 mM Hepes (Sigma) were added. Finally, the pH was adjusted to 7.3 with 0.1 M HCl. Cells were incubated in T-75 flasks as confluent monolayers at 37°C in a humidified atmosphere containing 3% of CO_2.

Every flask was initially seeded with 10^5 cells. The media was replaced after 5 days and the experiment was performed on day 8. All the experiments used cells in plateau phase for better representation of tumour growth conditions and to minimize cell cycle redistribution during protracted experiments.

2.1 HDR and PDR

For both high dose rate (HDR) and pulse dose rate (PDR), cells were

irradiated in 25 cm^2 flasks at room temperature with a 250KVp Pantak industrial X-ray unit (bipolar series 2 HF-320) at a dose rate of 1.683 Gy/min. The PDR experiments were given as fractions of 1 Gy/h or 2 Gy/h. The cells were incubated for one hour at 37°C after each pulse of radiation.

2.2 Cisplatin treatment

The 1 mg/ml cisplatin injection solution (Faulding Canada Inc.) was provided to us by the pharmacy of the Ottawa Regional Cancer Centre. New stocks of cisplatin were brought in every two weeks. The cisplatin was stored in the dark since it undergoes partial degradation when exposed to light. Cisplatin was added to the depleted medium covering the cells in the plateau phase. The depleted media is acidic and cisplatin was always added to depleted media since it has been shown that cisplatin cytotoxicity varies with pH. Cisplatin was added to depleted media also to avoid stimulation of cell growth. Over time, cisplatin binds to proteins and nucleic acids both in cells and in media and subsequently its cytotoxicity is diminished (Prestayko A.W. *et al.*, 1980).

Cisplatin was refreshed when drug exposure exceeded 5 hours. After the cisplatin treatment, the cells were washed twice with 1ml of citrate saline solution (134 mM KCl (BDH), 17.6 mM citric acid (Sigma)). For sequential treatments, the cisplatin was either terminated 10 minutes before or started 10 minutes after the radiation treatment.

2.3 Clonogenic Assay

Puck *et al.* first described the clonogenic assay in 1957. This assay is used to determine the reproductive viability of cells. Even if a small population of cells survived a particular treatment, some of them may acquire severe genetic instability that prevents them from reproducing and forming colonies. Cancer cells that cannot reproduce are successfully eliminated by a chemotherapeutic or a radiotherapeutic regimen.

After cisplatin and/or radiation treatments, the medium bathing the cells was removed. Cells were then washed in 1 ml of citrate saline solution to eliminate calcium and magnesium ions present in the serum since they are known to inhibit trypsin's enzymatic activity (Freshney R.I., 1994).

The cells were then trypsinized with 0.5 ml of 0.2% trypsin in citrate saline solution. They were incubated in trypsin (0.25% w/v in citrate saline) for 5 minutes at 37°C until they began detaching from the plastic substrate of the flask. Media was then added to the cells to resuspend them and to also further inhibit trypsin activity. The cells in suspension were then added to an isotonic solution and counted using a Particle Data Elzone 80 electronic cell counter (Particule Data Inc). Then, the cells were diluted to a concentration that allows plating of a number of cells that limit the survival to 50 colonies per plate. Cells were then plated in 60mm culture dishes, each containing 4 ml of media. When more than 10^5 cells needed to be plated, 100 mm dishes with 10 ml of media were used. After 14 days of incubation, the plates were fixed with methanol and stained with

methylene blue solution (0.2% w/v in 70% ethanol) and colonies were counted using a Bantex model 900A colony counter. Colonies having greater than 50 cells were scored as survivors.

For every dose point, the cells were plated in triplicate. The plating efficiency (PE) was the untreated control. The PE determines the proportion of non-treated cells that are able to attach to the plate and form a colony. The plating efficiency is calculated as follows:

PE = Colonies counted / non-treated cells seeded

The surviving fraction (SF) was calculated according to the plating efficiency:

SF = Colonies counted / (cells seeded after a treatment dose x PE)

Typical plating efficiencies were ~65% for the A2780s and ~75% for the A2780cp. Surviving fraction curves were fitted using Sigma Plot 4 software (SPSS, Richmond, CA).

Every experiment was done three times and the mean and the standard error of the mean (SEM) were calculated for each set of experiments. The surviving fraction points were plotted as function of the dose. Differences in surviving fractions were tested with the one-tailed students unpaired t-test and a value of less than 0.05 was considered significant.

2.4 Normalization of the survival curves

Calculation of the unnormalized surviving fraction (SF):

$$SF_{\text{X-ray + cisplatin}} = \text{Colonies counted}_{\text{X-rays + cisplatin}} / (\text{cells seeded}_{\text{X-rays + cisplatin}} \times PE)$$

$$SF_{\text{cisplatin-alone}} = \text{Colonies counted}_{\text{cisplatin-alone}} / (\text{cells seeded}_{\text{cisplatin-alone}} \times PE)$$

Calculation of the normalized surviving fraction ($SF_{normalized}$):

$$SF_{normalized} = SF_{\text{X-ray + cisplatin}} / SF_{\text{cisplatin}}$$

$$SF_{normalized} = \frac{\text{Colonies counted}_{\text{X-rays + cisplatin}} \times \text{cells seeded}_{\text{cisplatin-alone}}}{\text{Colonies counted}_{\text{cisplatin-alone}} \times \text{cells seeded}_{\text{X-rays + cisplatin}}}$$

The normalized data are calculated for each of the individual experiments and henceforth the mean and the standard error of the mean are determined.

3.0 RESULTS

3.1 Characterization of the cell lines

In all the performed experiments, we used the ovarian carcinoma cell lines. The parental cell line A2780s was sensitive to both radiation and cisplatin treatment, unlike its resistant variant, the A2780cp.

Figs. 3.1.1 and 3.1.2 show the growth of ovarian carcinoma parental cell line (A2780s) and the resistant cell line (A2780cp). Both cell lines grew at a similar rate in DMEM/F12 medium, with a doubling time of approximately 24 hours. For each cell line, $1x10^5$ cells were plated on day 0. On day 5, the media was replaced and the cells continued to grow until reaching the plateau phase. For both cell lines, this occurred by day 8, the day when the experiment was performed.

In the plateau phase, cell proliferation is normally halted due to contact inhibition. All experiments were conducted with the cells at the plateau phase in order to minimize cell cycle effects (Wilkins *et al.*, 1997). From Figs. 3.1.1 and 3.1.2 it is evident that cells which were not fed on day 5 showed an earlier plateau phase.

Figs. 3.1.3, 3.1.4 and 3.1.5 show the responses of the A2780cp and the A2780s cells to cisplatin. The doses and the times of drug exposure chosen for

Figure 3.1.1. Growth curve for the A2780s cells. The cells that are fed at day 5 reached plateau by day 8. Each point represents one experiment.

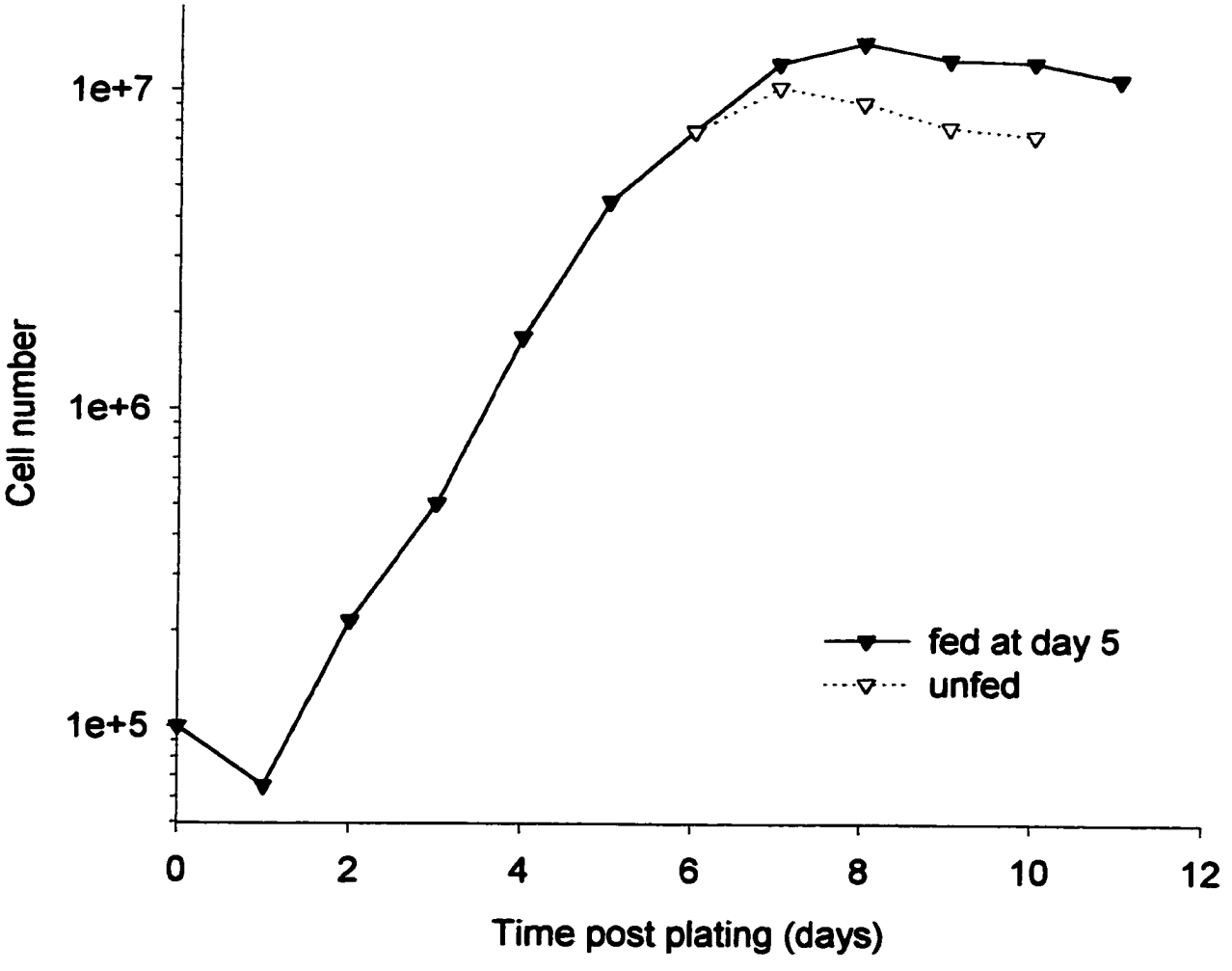
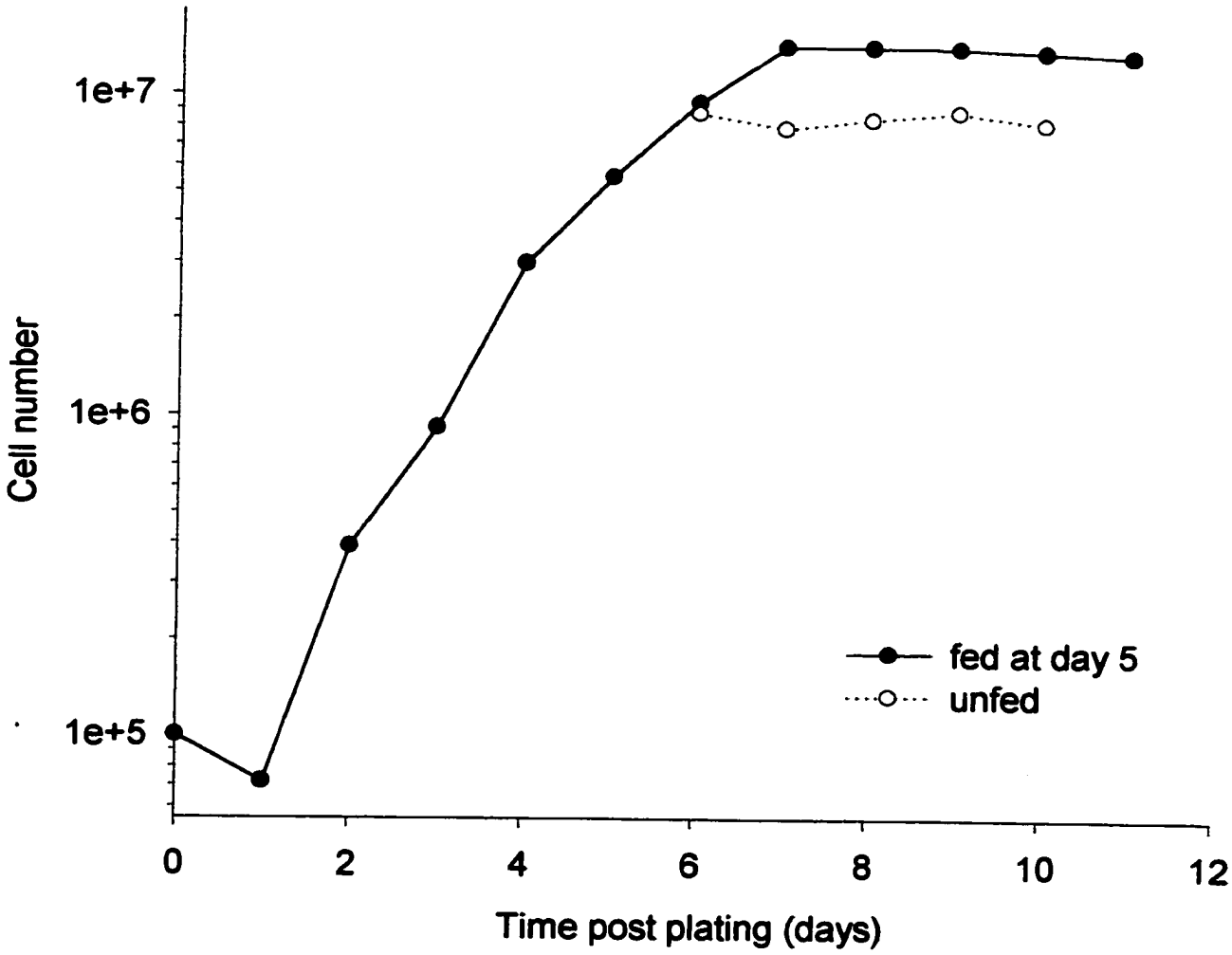

1e+7
1e+6
1e+5
Cell number
0
2
4
6
8
10
12
Time post plating (days)
fed at day 5
unfed

Figure 3.1.2. Growth curve for the A2780cp cells. The cells that are fed at day 5 reached plateau phase by day 8. Each point represents one experiment.


1e+7
1e+6
1e+5
Cell number
0
2
4
6
8
10
12
Time post plating (days)
fed at day 5
unfed

the cisplatin survival curves were related to the cisplatin doses used with radiation in the combination treatment experiments. Fig. 3.1.3 shows the survival of the A2780s and A2780cp after a one-hour treatment with different concentrations of cisplatin ranging from 2 μg/ml to 10 μg/ml. The A2780cp was approximately 5 times more resistant at 2 μg/ml cisplatin than the A2780s. As the cisplatin dose increased, the difference was even larger. The A2780cp was very resistant to cisplatin, reaching ~40% survival after a dose of 10 μg/ml cisplatin for 1 hour. For the A2780s, less than 0.1% survival was observed at the same cisplatin dose.

Cisplatin exposure for 1 to 3 hours at 2 μg/ml for the A2780s and 1 to 4 hours at 3 μg/ml for the A2780cp is shown in Fig. 3.1.4. Here, the time of exposure was varied while the concentration remained constant. The A2780cp exposed to 3 μg/ml cisplatin for 3 hours and the A2780s exposed to 2 μg/ml cisplatin for 1 hour showed a survival level of about 40%, confirming the large difference in cisplatin sensitivity for both cell lines.

Cisplatin exposure for 2 to 10 hours at 1 μg/ml and 3 μg/ml for the A2780cp and 0.5 μg/ml for the A2780s is shown in Fig. 3.1.5. Here, longer times of exposure were used and the different cisplatin concentrations were kept constant. For the A2780cp, the survival at 1 μg/ml for 10 hours was around 40%, about the same level as the one-hour cisplatin treatment with 10 μg/ml (Fig. 3.1.3). After a 6 hours treatment with 3 μg/ml, the A2780cp survival was lower than 10%. The A2780s was again much more sensitive to cisplatin exposure. When cultured with 0.5 μg/ml cisplatin for more than 8 hours, the survival was

Figure 3.1.3. Cisplatin survival curves for the A2780s and the A2780cp cells. Each point represents the average of three experiments and the error bars represent the standard error of the mean (SEM) between experiments.

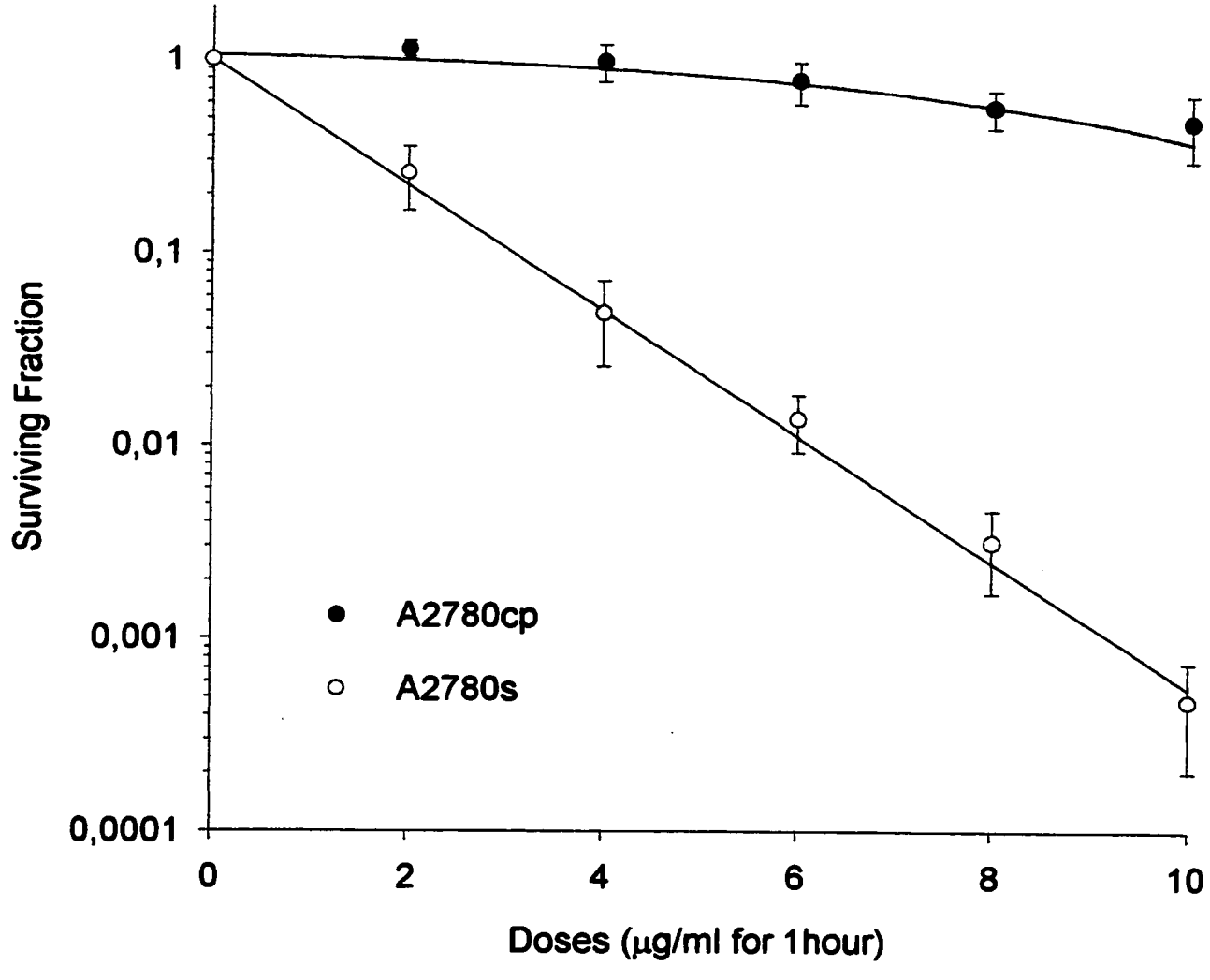

Surviving Fraction
1
0,1
0,01
0,001
0,0001
0
2
4
6
8
10
Doses (µg/ml for 1hour)
A2780cp
A2780s

Figure 3.1.4. Cisplatin survival curves with 3 μg/ml added for 1 to 4 hours to the A2780cp cells and 2 μg/ml added for 1 to 3 hours to the A2780s cells. Each point represents the average of three experiments, and the error bars represent the SEM between experiments.

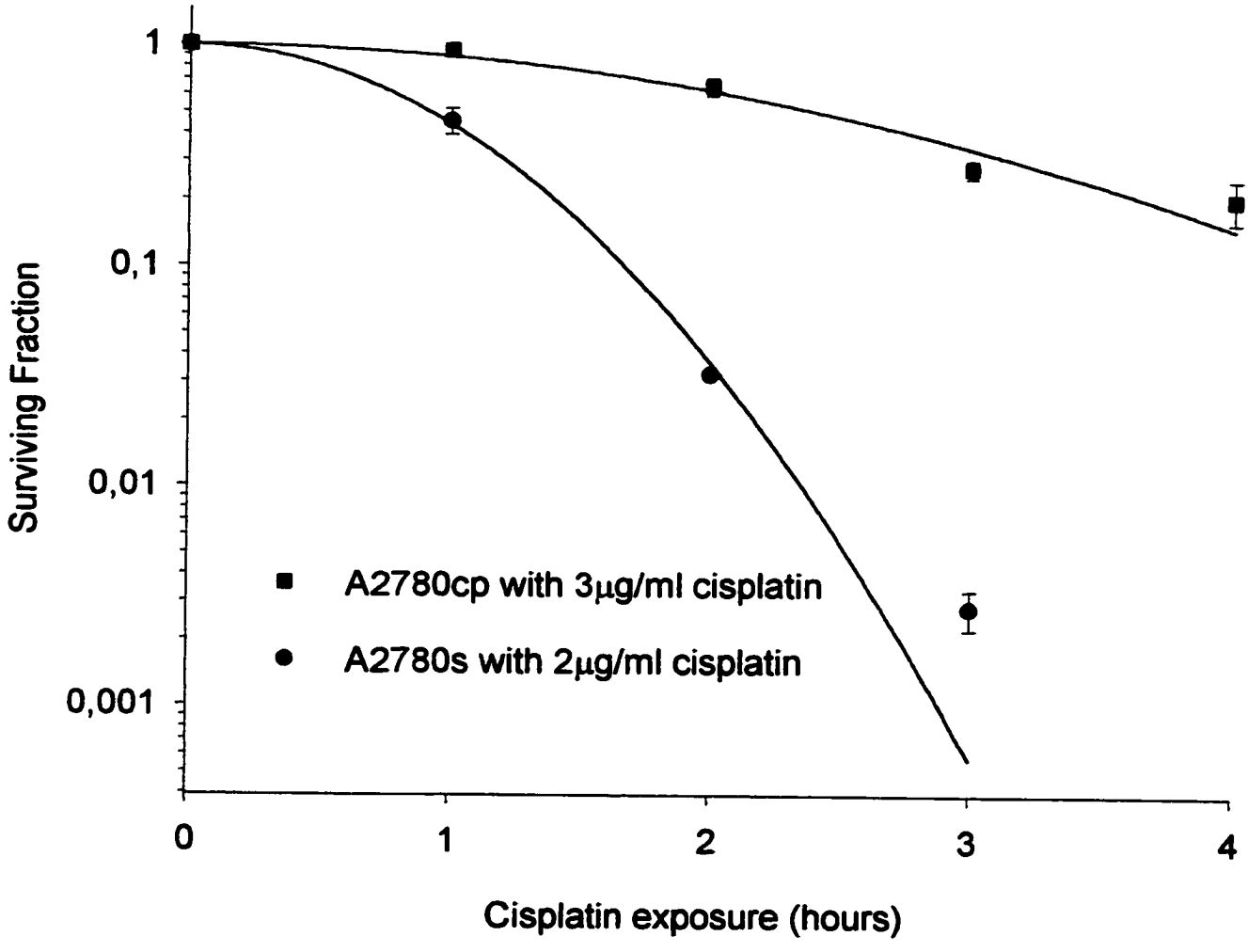
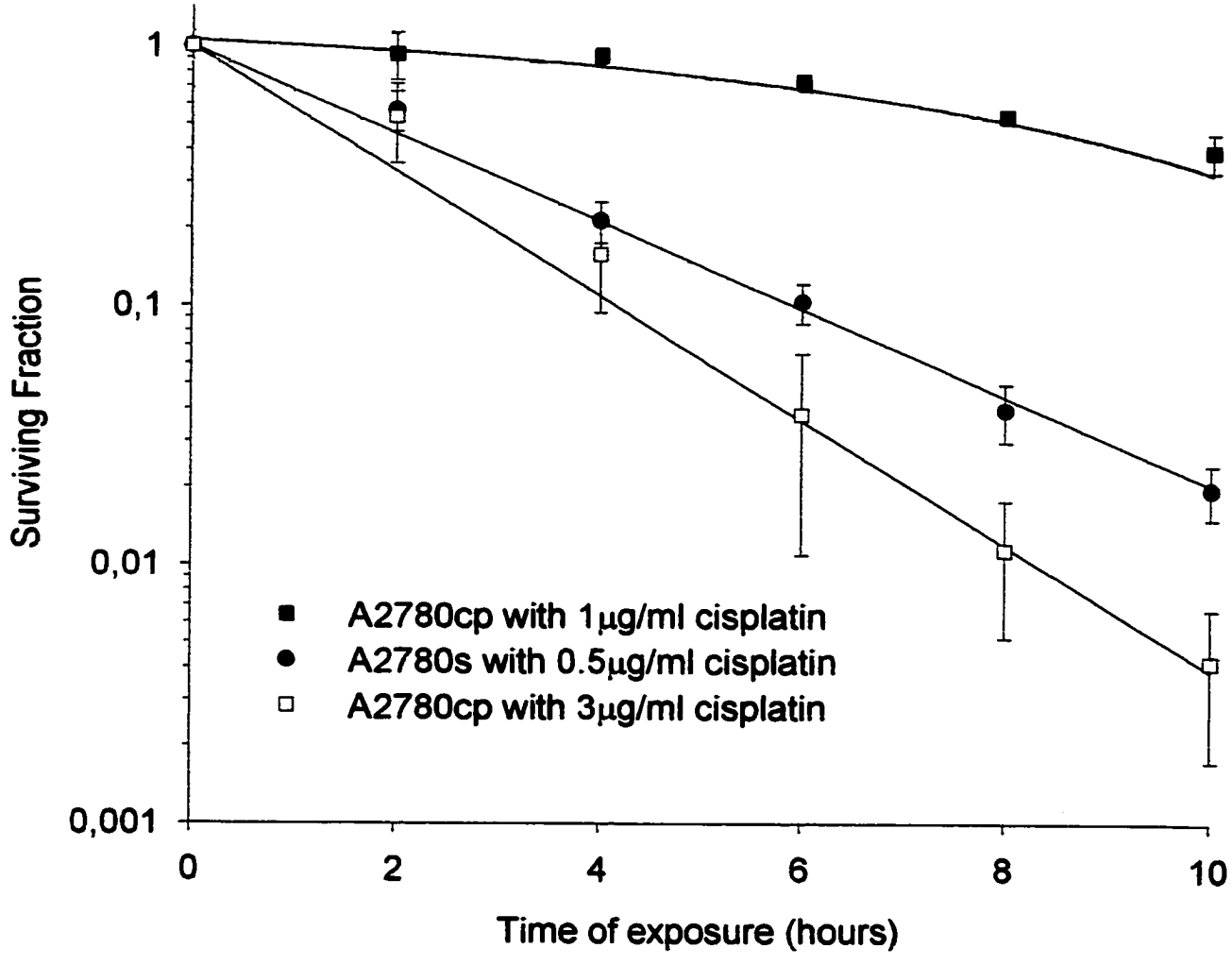

1
0,1
0,01
0,001
Surviving Fraction
A2780cp with 3μg/ml cisplatin
A2780s with 2μg/ml cisplatin
0
1
2
3
4
Cisplatin exposure (hours)

Figure 3.1.5. Survival after 2 to 10 hours treatments with 1 μg/ml and 3 μg/ml cisplatin for the 2780cp cells and 0.5 μg/ml cisplatin for the A2780s cells. Each point represents the average of three experiments, and the error bars represent the SEM between experiments. The 1 μg/ml and the 3 μg/ml cisplatin survival curves for the A2780cp are significantly different.


1
0,1
0,01
0,001
Surviving Fraction
0
2
4
6
8
10
Time of exposure (hours)
A2780cp with 1μg/ml cisplatin
A2780s with 0.5μg/ml cisplatin
A2780cp with 3μg/ml cisplatin

lower than 10%.

The A2780s cells showed a cross-sensitivity to cisplatin and radiation (Fig. 3.1.6). The resistant variants were not only more resistant to cisplatin, but to radiation as well, exemplifying their cross-resistance to both agents. At lower doses, such as 2 Gy, often used in the clinic, the A2780cp cell line was three times more resistant than the A2780s. At a higher dose of 6 Gy, there was an order of magnitude difference in survival between the two cell lines.

The survival curve for each cell line possessed a shoulder, indicating their capacity to accumulate sublethal radiation damage. Delaying plating experiments showed the effects of allowing repair to take place during 6 hours of incubation, after irradiation (Fig. 3.1.6).

For a level of survival after immediate plating that equals 0.01, the A2780cp had about the same increase in survival as the A2780s when time for repair was allowed before plating. Even at the same radiation dose of 6 Gy the A2780cp showed the same amount of repair as the A2780s. Therefore, the A2780cp has the same repair capacity as the A2780s. As far as the A2780cp cell line is concerned, all the points were significantly different between the HDR with and without 6 hours delayed plating ($p<0.05$). For the A2780s, the difference is only seen at 4 and 6 Gy ($p<0.05$).

Fig. 3.1.7. shows two pulse dose rate (PDR) experiments (1 Gy/h and 2 Gy/h) and an HDR experiment for the A2780s. Since the total dose is divided into pulses during PDR, the cells had time to repair between each radiation pulse and for one hour after the last radiation pulse.

Figure 3.1.6. High dose rate (HDR) irradiation survival curves with and without 6 hours delayed plating. Each point represents the average of 3 experiments for the delayed plating survival curves and 3 to 7 experiments for the HDR survival curves. The error bars represent the SEM between experiments. For the A2780cp cells, the difference between HDR points and the delayed plating points are all significantly different. For the A2780s cells a significant difference is only seen after 2 Gy.

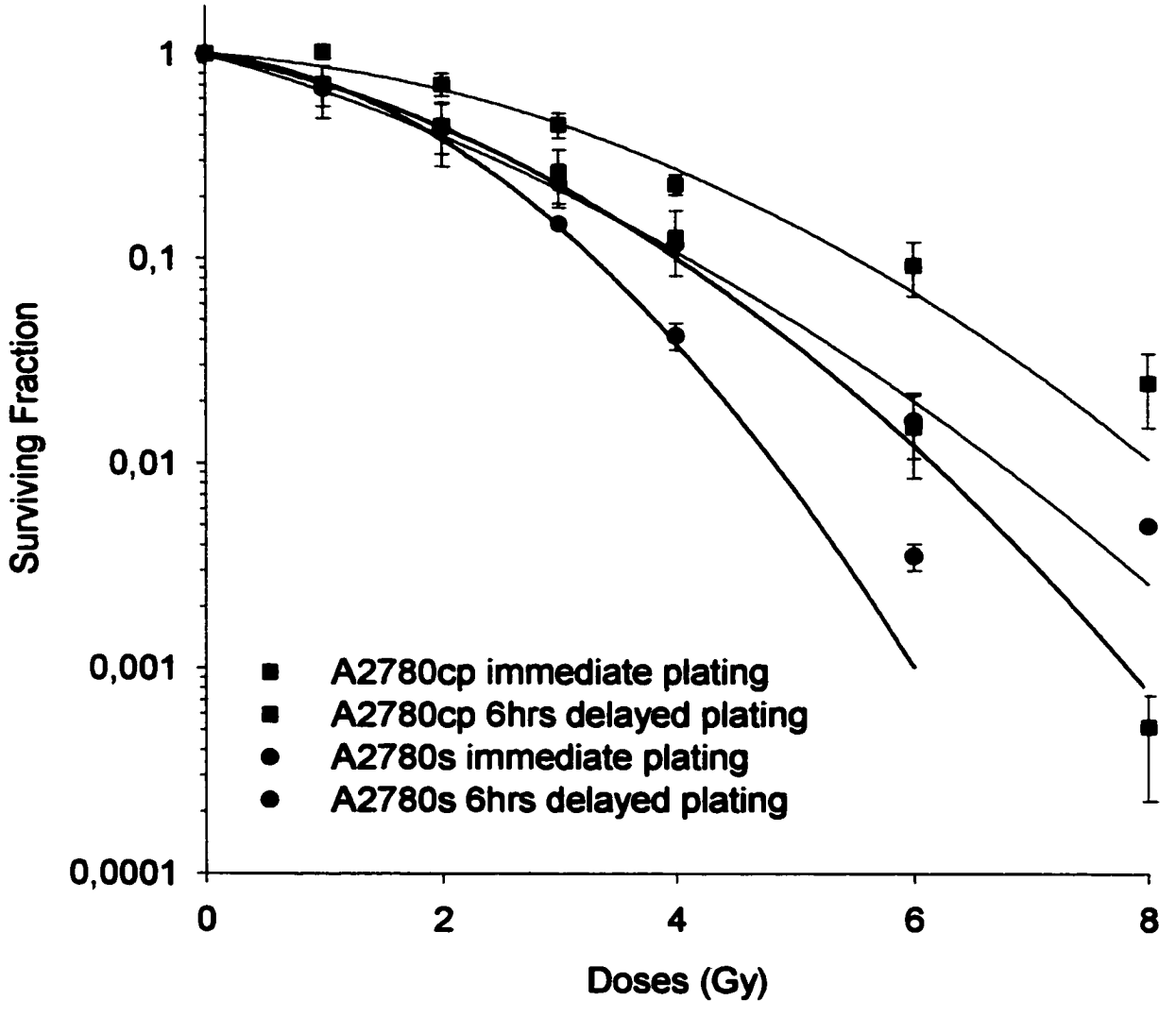
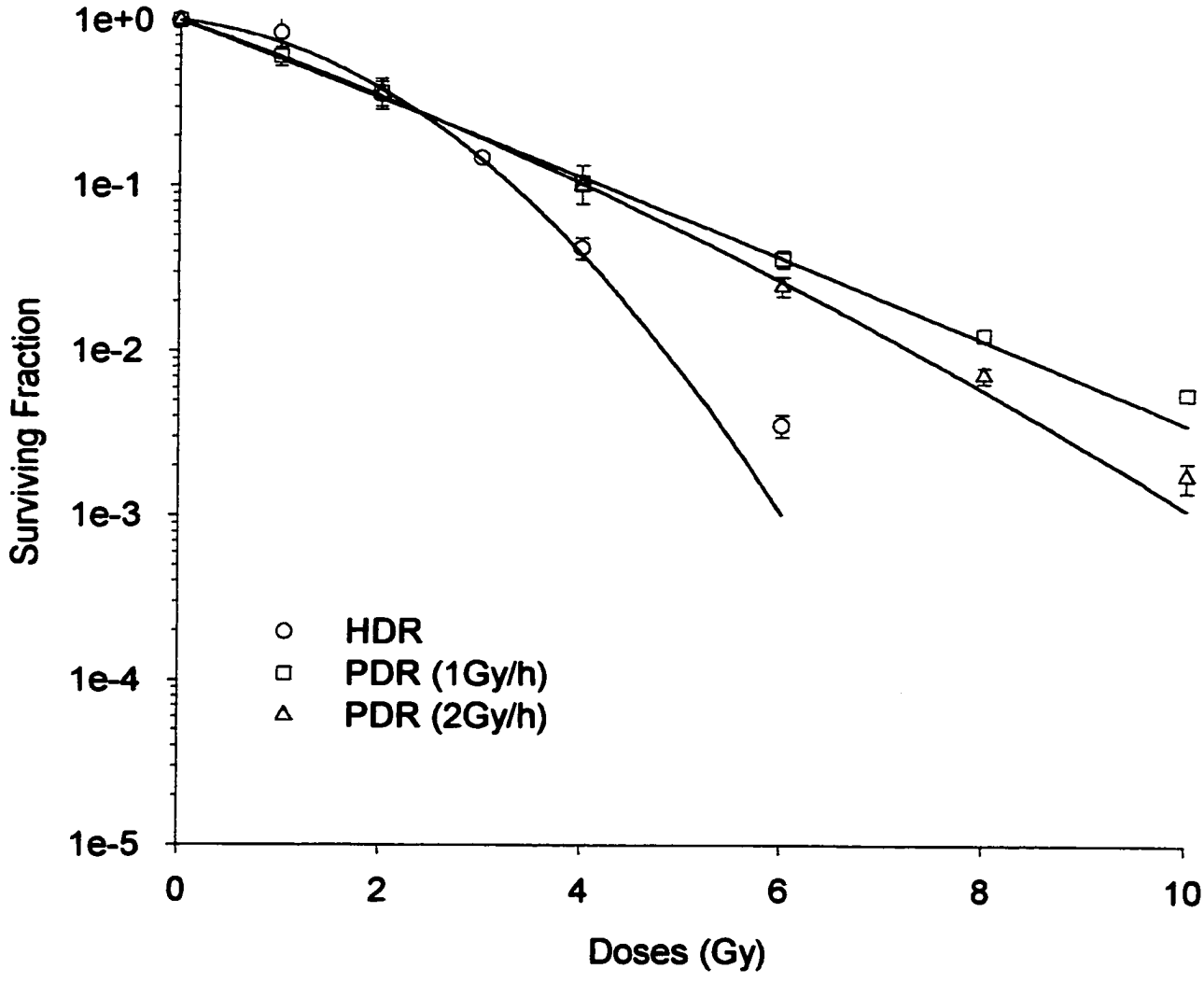

1
0,1
0,01
0,001
0,0001
Surviving Fraction
A2780cp immediate plating
A2780cp 6hrs delayed plating
A2780s immediate plating
A2780s 6hrs delayed plating
0
2
4
6
8
Doses (Gy)

Figure 3.1.7. HDR and pulse dose rate (PDR) irradiation survival curves for the A2780s cells. Plotted are mean ± SEM values from 3 independent experiments for the PDR experiments and 3 to 7 experiments for the HDR points. The difference between HDR and the PDR survival curves is significant at 4 Gy and 6 Gy. The PDR (1 Gy/h) and the PDR (2 Gy/h) show a significant difference after 4 Gy.


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
HDR
PDR (1Gy/h)
PDR (2Gy/h)
0
2
4
6
8
10
Doses (Gy)

The 1 Gy/h experiment consisted of giving 1 Gy every hour for a total of 10 Gy. The length of the treatment was 10 hours. For the 2 Gy/h experiment, 2 Gy was given every hour up to a total of 10 Gy, the length of the treatment being 5 hours. The 1 Gy/h experiment not only had a smaller dose per pulse, but also a longer time available for the cells to repair than in the 2 Gy/h experiment. This is why the survival was consistently higher after 4 Gy for the 1 Gy/h experiment compared to the 2 Gy/h experiment ($p<0.05$). The HDR exposure produced the lowest survival; as the cells were given no time to repair.

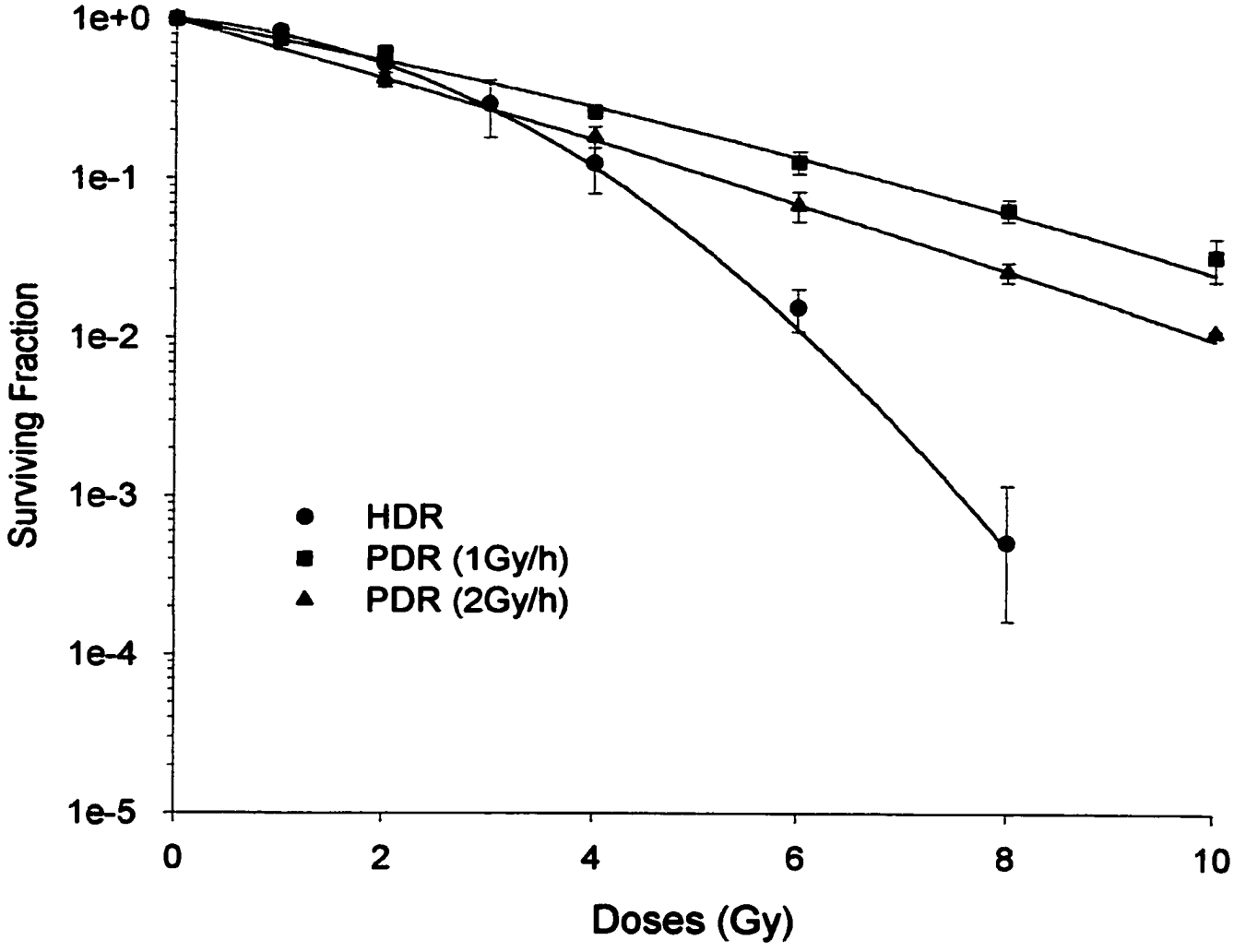
In the case of the parental cell line, a difference was seen between the 1 Gy/h and 2 Gy/h PDR experiments and the HDR curve at the 4 Gy dose point and higher ($p<0.05$) (Fig. 3.1.7). At this dose, the cells had 4 hours to repair in the 1 Gy/h scheme and 2 hours in the 2 Gy/h scheme.

The linear quadratic model does not completely fit the curve for the A2780s 1 Gy/h PDR experiment. This has been shown previously (Niedbala *et al.*, 2001a). At 8 Gy and 10 Gy, the curves rise, meaning that the cells are becoming more resistant. This effect was not seen with the 2 Gy/h regimen.

The same experiments were done with the A2780cp (Fig. 3.1.8.). For the 1 Gy/h experiment, the difference in survival with respect to the HDR was only seen at doses of 4 Gy and higher ($p<0.05$). For the 2 Gy/h, the difference was seen at doses of 6 Gy and above ($p<0.05$). An increase in survival is consistently present after 2 Gy when the 2 Gy/h and the 1 Gy/h PDR experiments are compared.

The time allowed for the repair of radiation damage needed to be

Figure 3.1.8. HDR and PDR irradiation survival curves for the A2780cp cells. Plotted are mean ± SEM for 4 independent experiments for the 1 Gy/h treatment, 3 independent experiments for the 2 Gy/h treatment and 3 to 4 experiments for the HDR points. The difference between HDR and the PDR survival curves is significant at 6 Gy and 8 Gy for the 2 Gy/h and at 4,6 and 8 Gy for the 1 Gy/h. The PDR (1 Gy/h) and the PDR (2 Gy/h) are significantly different after 2 Gy.


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
HDR
PDR (1Gy/h)
PDR (2Gy/h)
0
2
4
6
8
10
Doses (Gy)

sufficiently long for the cells to accumulate enough repair in order for a difference in survival to be observed. For the 2 Gy/h and 1 Gy/h experiments, the A2780s cells repaired more damage than the A2780cp. This may be explained by the fact that the A2780s cells accumulated more damage after each pulse, which may have led to an increase in the total possible repair.

3.2 Cisplatin added before radiation

Fig. 3.2.1A shows the normalized survival of the A2780s when 2 μg/ml cisplatin was added for 1 to 3 hours immediately before a 4 Gy dose. To show the interaction between cisplatin and radiation, the effects of the drug were normalized by taking the surviving fraction after cisplatin treatment alone as 100% survival. When 2 μg/ml cisplatin was added for 1 to 3 hours before radiation it did radiosensitize the A2780s cells ($p<0.05$ for the 1 hour, 2 hour and 3 hour points). The unnormalized data showed more than additivity when cisplatin and radiation combined (Fig. 3.2.1B). The longer the cisplatin exposure, the lower the final survival levels.

Fig. 3.2.1C demonstrates cisplatin alone survival for this experiment (shown previously with Fig. 3.1.4 in the form of a survival curve). Each of these survival levels were normalized in Fig. 3.2.1A.

To confirm that there is radiosensitization in the A2780s, we had to add cisplatin to the entire dose range of HDR survival curve. In Figs. 3.2.2 and 3.2.3, the cisplatin exposure time and concentration were kept constant and the

Figure 3.2.1A. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours before a 4 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are significantly different from the 4 Gy alone point.

B. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours before a 4 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are significantly different.

C. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours. The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are significantly different.

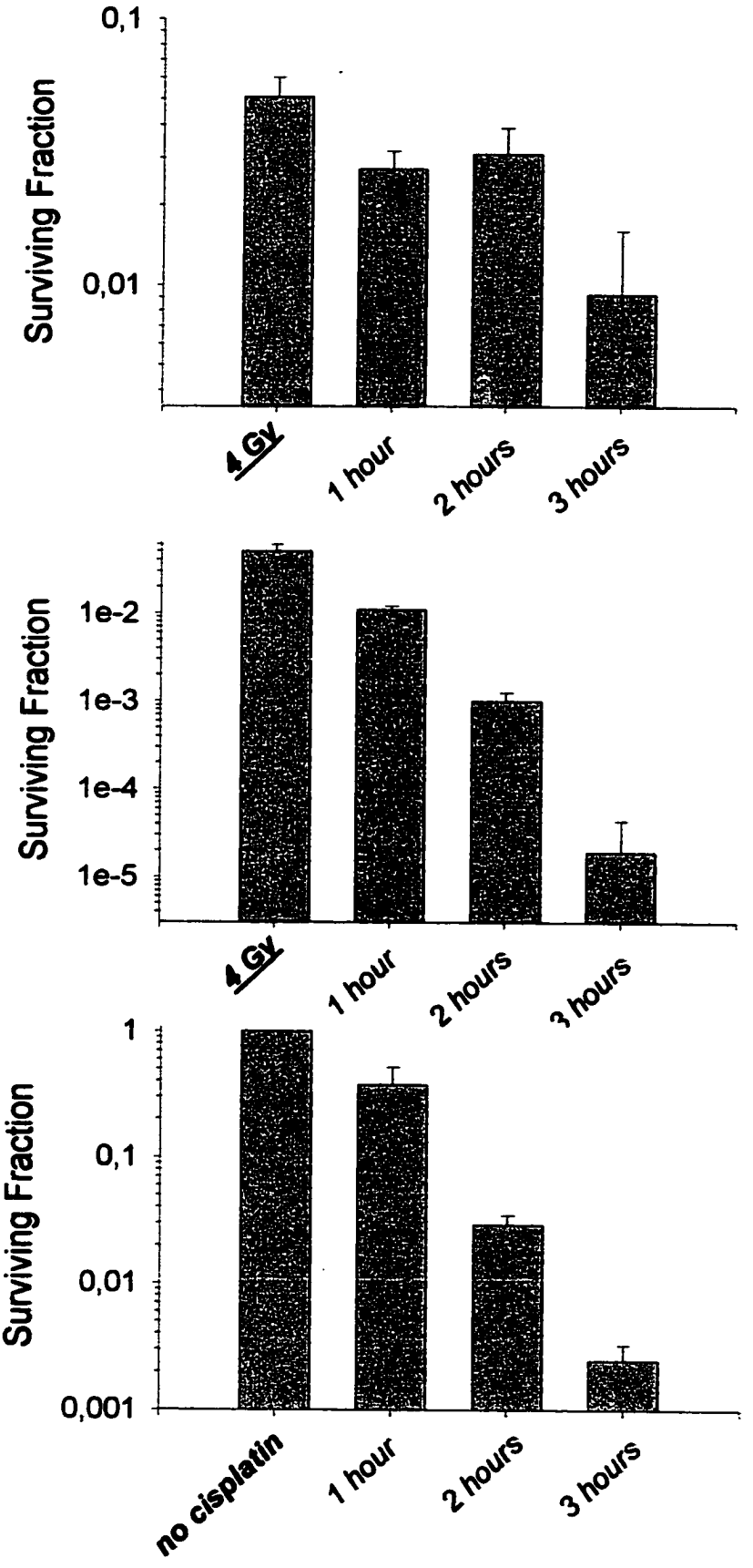

0,1
0,01
Surviving Fraction
4 Gy
1 hour
2 hours
3 hours
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
4 Gy
1 hour
2 hours
3 hours
1
0,1
0,01
0,001
Surviving Fraction
no cisplatin
1 hour
2 hours
3 hours

Figure 3.2.2. Survival of the A2780s cells after exposure to 2 μg/ml cisplatin for 1 hour before an HDR irradiation treatment. Normalized and unnormalized data. Plotted are mean ± SEM values from 3 independent experiments. There is only a significant decrease in survival after normalization when cisplatin is added to the 2 Gy alone point.

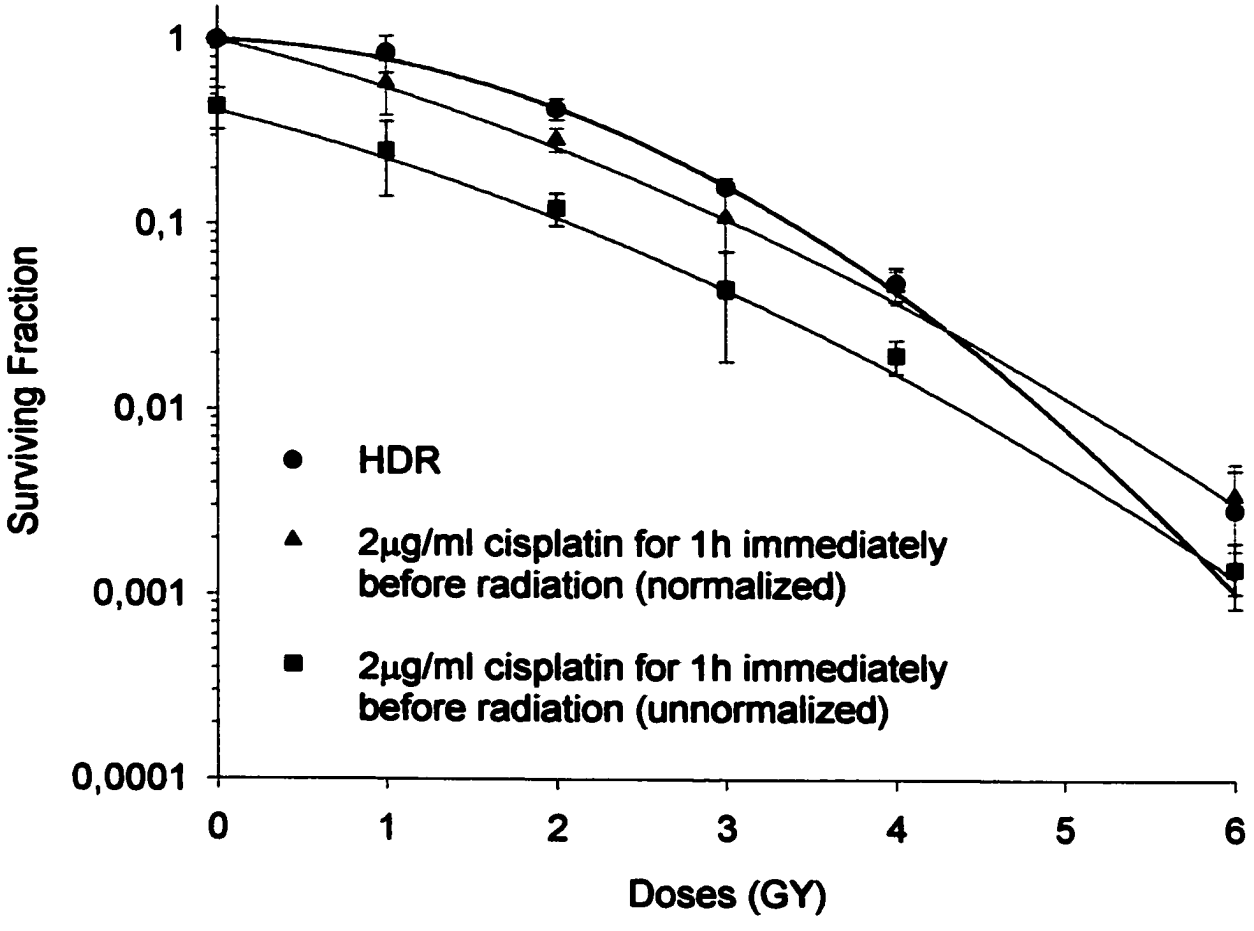
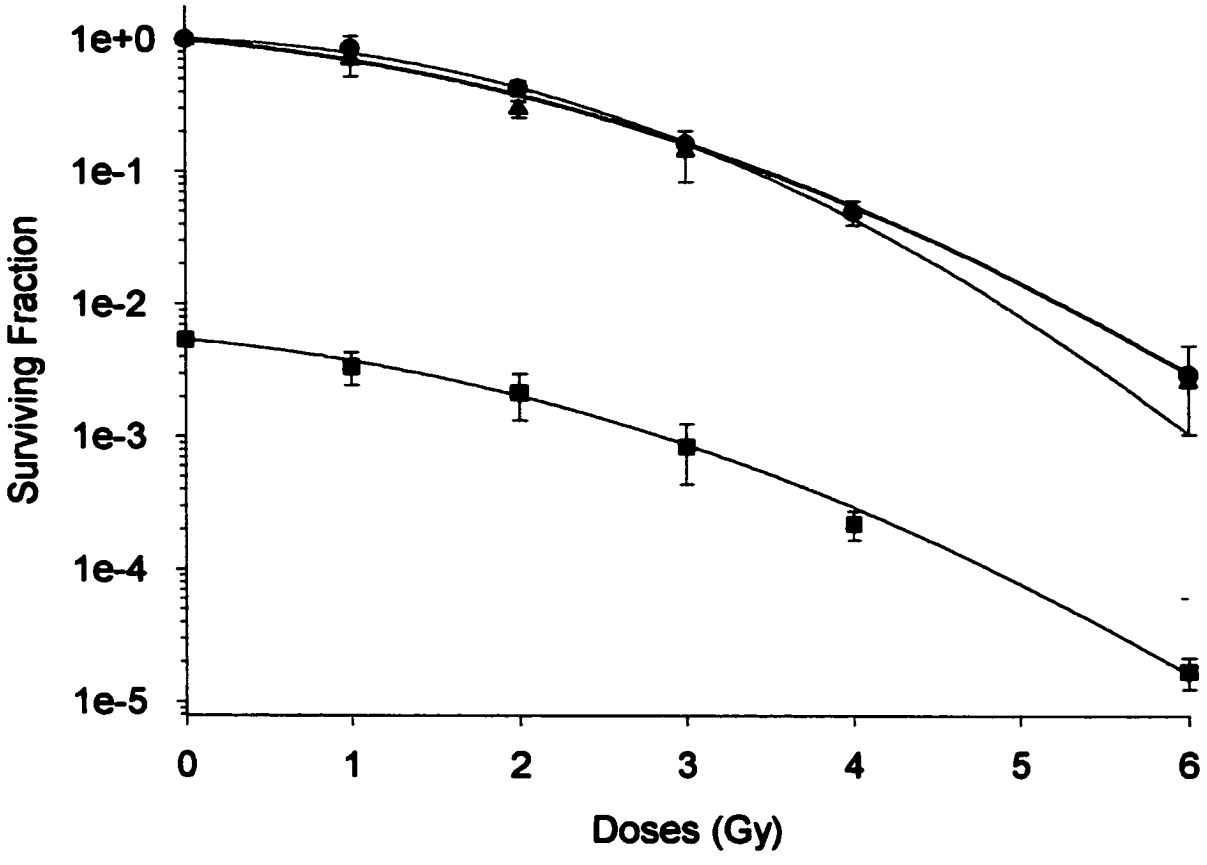

Surviving Fraction
1
0,1
0,01
0,001
0,0001
HDR
2µg/ml cisplatin for 1h immediately before radiation (normalized)
2µg/ml cisplatin for 1h immediately before radiation (unnormalized)
0
1
2
3
4
5
6
Doses (GY)

Figure 3.2.3. Survival of the A2780s cells after exposure to 2 μg/ml cisplatin for 3 hours immediately before an HDR irradiation treatment. Normalized and unnormalized data. Plotted are mean ± SEM values from 3 independent experiments. There is no significant difference between the HDR-alone curve and the unnormalized data for the combined treatment.



- ● HDR
- ▲ 2μg/ml cisplatin for 3 hours immediately before radiation (normalized)
- ■ 2μg/ml cisplatin for 3 hours immediately before radiation (unnormalized)

survival was assessed for different radiation doses. For Fig. 3.2.2, radiosensitization is seen at 2 Gy when 2 μg/ml is added for 1 hour before the radiation dose. For the rest of the radiation doses there is no radiosensitization. Fig. 3.2.3 normalized data showed no radiation sensitization when 2 μg/ml cisplatin was added for 3 hours before radiation. Additivity always remained, as demonstrated by the unnormalized survival curves, at both cisplatin concentrations. When experimenting the entire HDR survival curve with and without cisplatin, we were not able to confirm the cisplatin sensitization shown in Fig. 3.2.1A.

For the A2780cp, the cisplatin dose of 3 μg/ml did not significantly radiosensitize the cells, when added 2 and 4 hours immediately before the 6 Gy radiation ($p>0.05$). On the other hand, radiation sensitization was significant when cisplatin is added for 1 and 3 hours before a 6 Gy radiation dose ($p<0.05$). The effects of the drug, were normalized for a direct comparison with the radiation alone data treatment (Fig. 3.2.4).

There was a smaller decrease in survival for the unnormalized data for the A2780cp when compared to the A2780s (Fig. 3.2.4B). The A2780cp was far more resistant to the cisplatin dose of 3 μg/ml (Fig. 3.2.4C) than the A2780s with a cisplatin dose of 2 μg/ml (Fig. 3.2.4C). Furthermore, the resistant variant shows a smaller additive effect for the combined treatment.

Again to verify if the sensitization is seen when the entire survival curve is done, we looked at the effect of 3 μg/ml cisplatin added for 4 hours immediately before the HDR treatment. The survival curve also showed additivity for the

Figure 3.2.4A. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours before a 6 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. Only the 1 hour and the 3 hour points are significantly different from the 6 Gy alone point.

B. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours before a 6 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are significantly different.

C. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours. The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are significantly different from each other except for the 3 hour and the 4 hour cisplatin treatment points.

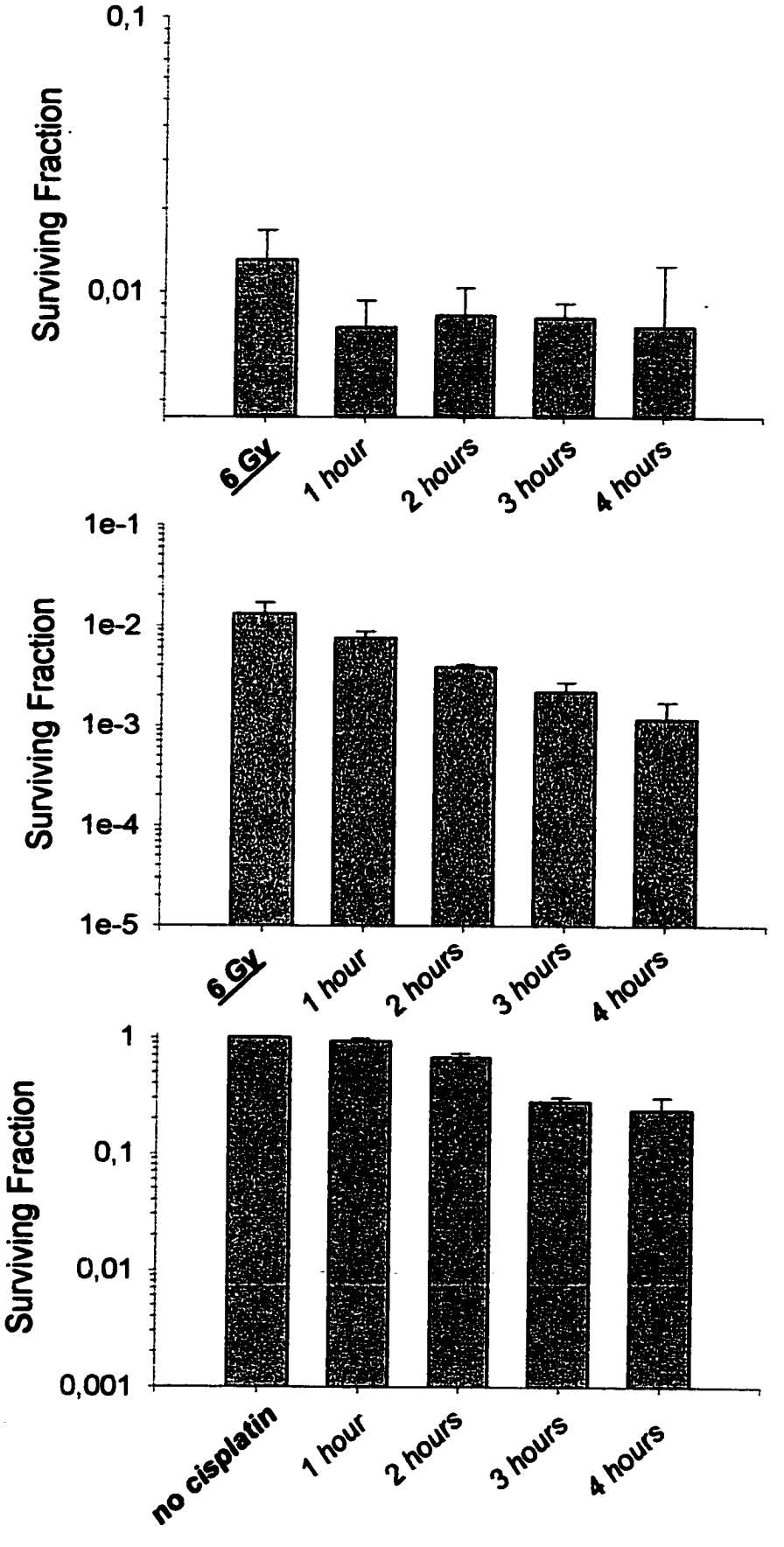
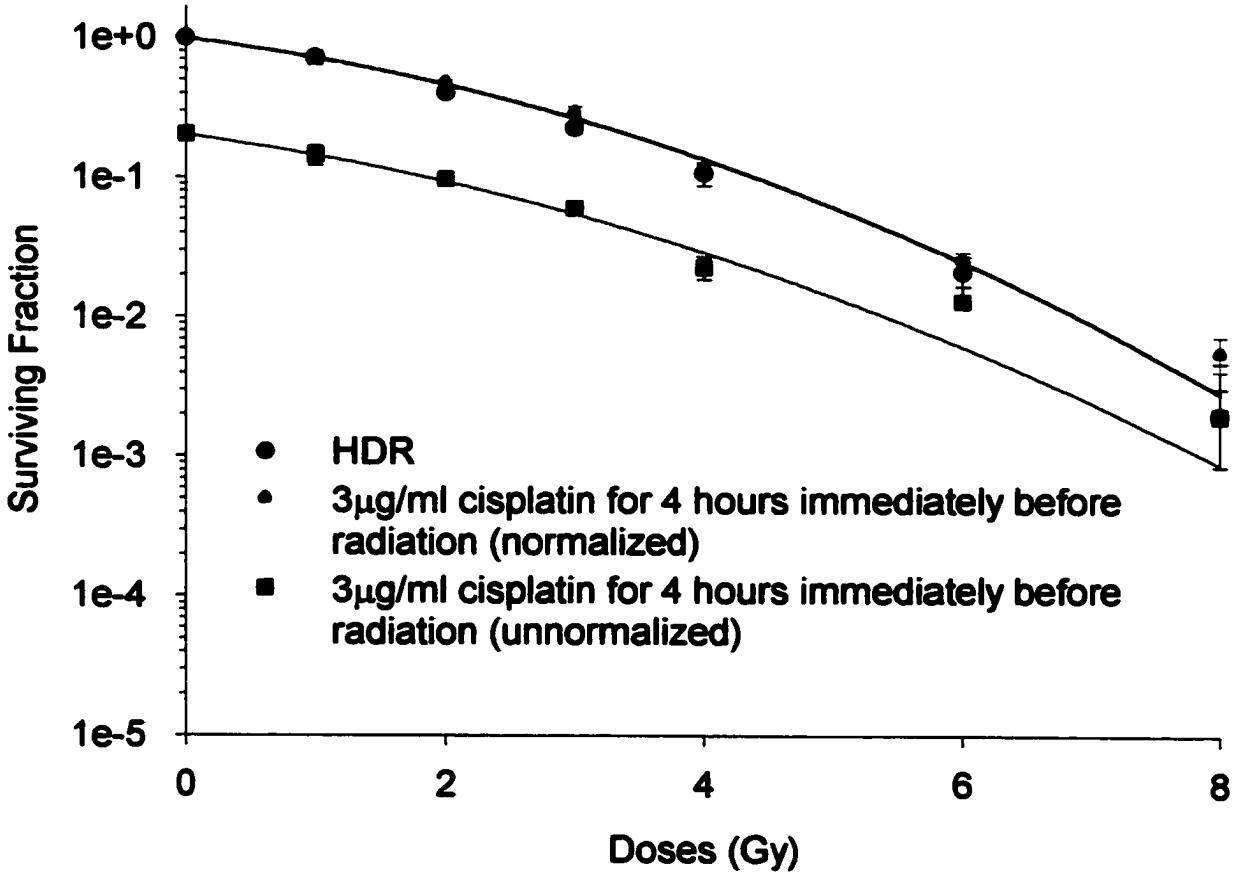

0,1
0,01
Surviving Fraction
6 Gy
1 hour
2 hours
3 hours
4 hours
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
6 Gy
1 hour
2 hours
3 hours
4 hours
1
0,1
0,01
0,001
Surviving Fraction
no cisplatin
1 hour
2 hours
3 hours
4 hours

Figure 3.2.5 Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 4 hours immediately before an HDR irradiation treatment. Normalized and unnormalized data. Plotted are mean ± SEM values from 3 independent experiments. There is no significant difference between the HDR alone curve and the normalized data for the combined treatment.


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
0
2
4
6
8
Doses (Gy)
HDR
3μg/ml cisplatin for 4 hours immediately before radiation (normalized)
3μg/ml cisplatin for 4 hours immediately before radiation (unnormalized)

A2780cp cells (Fig. 3.2.5, unnormalized data). Sensitization was not seen when the A2780s and A2780cp were treated with cisplatin immediately before HDR radiation. Consequently, the action of the drug and the radiation did interact with each other.

To verify if cisplatin added 24 hours before a radiation dose would increase repair mechanisms and make the cells more resistant to the subsequent dose of radiation, different doses of cisplatin were added for 24 hours before radiation and the resulting survival levels were compared. Fig. 3.2.6A represents the screening experiment, where cisplatin doses of 0.05, 0.1 and 1 μg/ml were added 24 hours prior to a dose of 4 Gy, in an attempt to induce resistance in the A2780s cells. There was no difference between the radiation-alone and the cisplatin with radiation survival levels for the normalized data ($p>0.05$) and the unnormalized data (Fig. 3.2.6B).

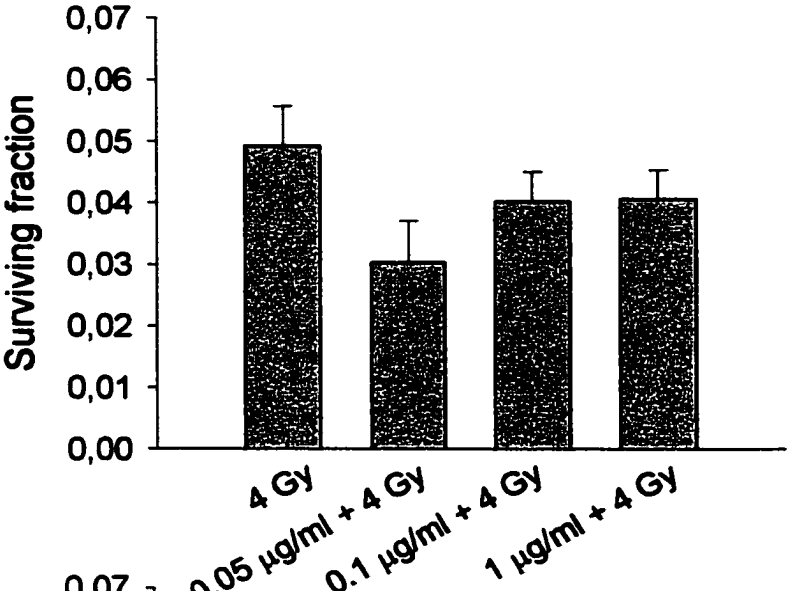
Fig. 3.2.6C demonstrates that, with delaying plating for 24 hours, the survival of the cisplatin treated cells was approximately the same as the untreated cells. This allowed repair of cisplatin damage to occur. There was no real additivity except for the highest cisplatin dose point (1 μg/ml showing $p<0.05$) since cisplatin damage was repaired by the time radiation treatment was given. This is not surprising given that the survival, after immediate plating, was very high, leaving little damage to repair.

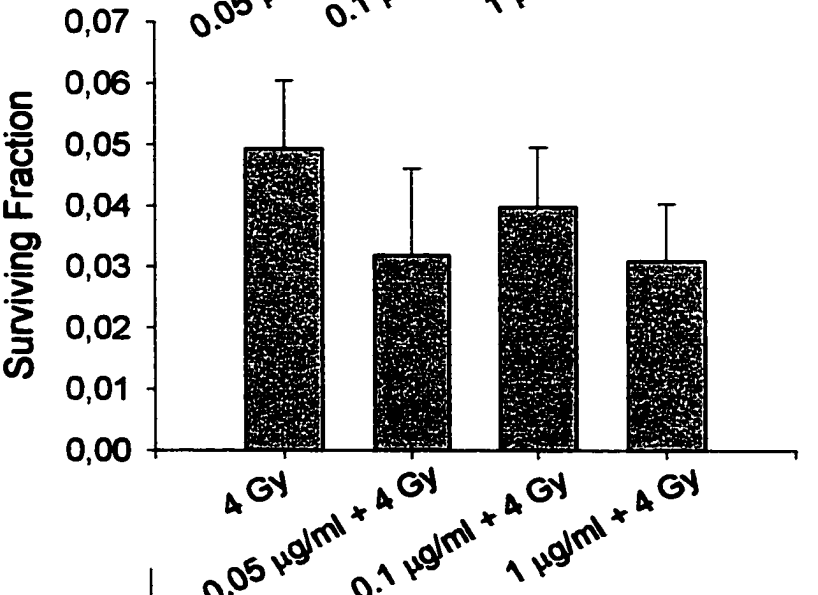
Figs. 3.2.7A and 3.2.7B represent the screening experiment done on the A2780cp where different cisplatin doses (0.01 μg/ml to 10 μg/ml) were given for

Figure 3.2.6A. Survival of the A2780s cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before a 4 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are not significantly different from each other except for the 0.05 μg/ml point which is significantly different from the 4 Gy alone point.

B. Survival of the A2780s cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before a 4 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are not significantly different.

C. Survival of the A2780s cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before plating. The experiments are done in triplicate and the SEM is calculated to give the error bars. For all the points, there is no significant decrease in survival after cisplatin treatment except for the 1 μg/ml cisplatin dose point.


Surviving fraction
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
4 Gy
0.05 µg/ml + 4 Gy
0.1 µg/ml + 4 Gy
1 µg/ml + 4 Gy


Surviving Fraction
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
4 Gy
0.05 µg/ml + 4 Gy
0.1 µg/ml + 4 Gy
1 µg/ml + 4 Gy

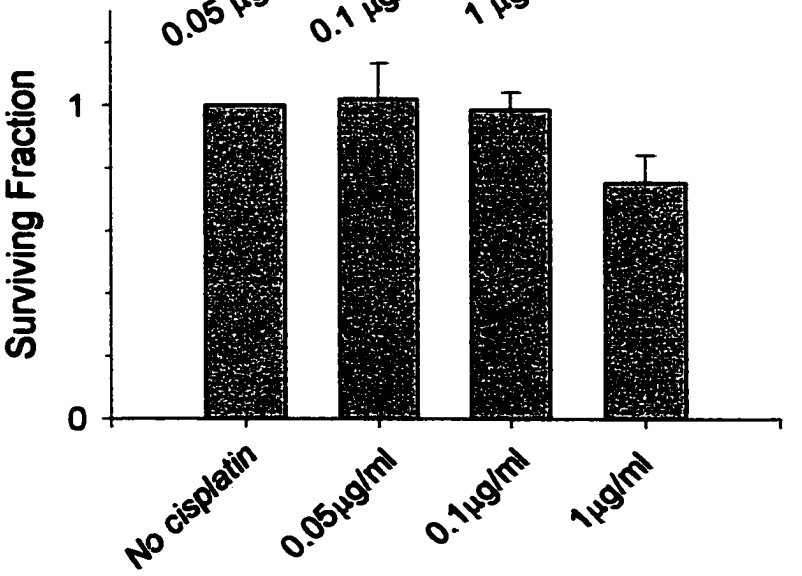
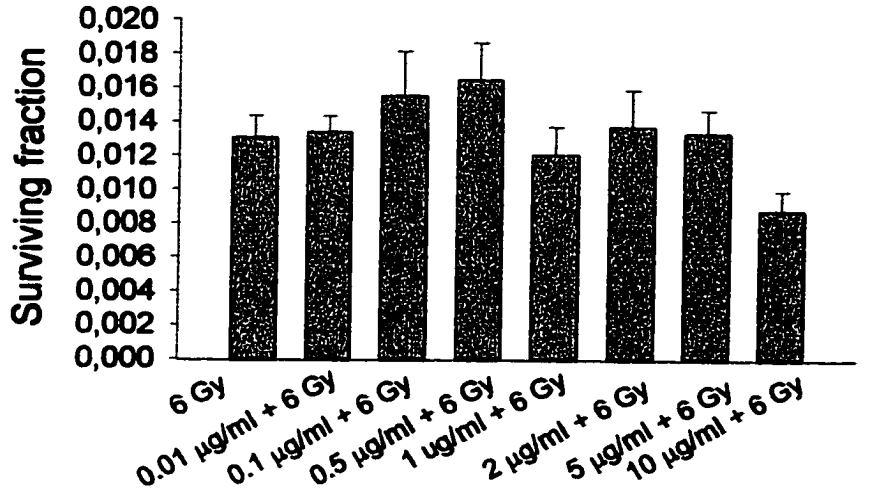
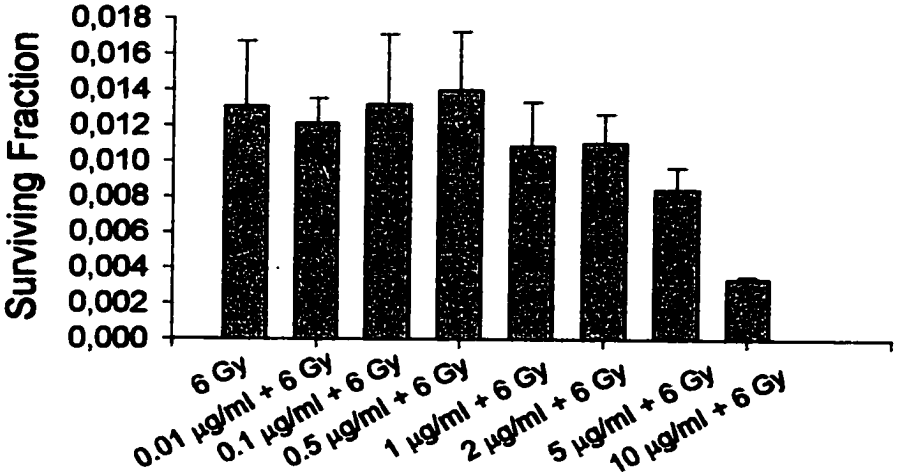

Surviving Fraction
1
0
No cisplatin
0.05µg/ml
0.1µg/ml
1µg/ml

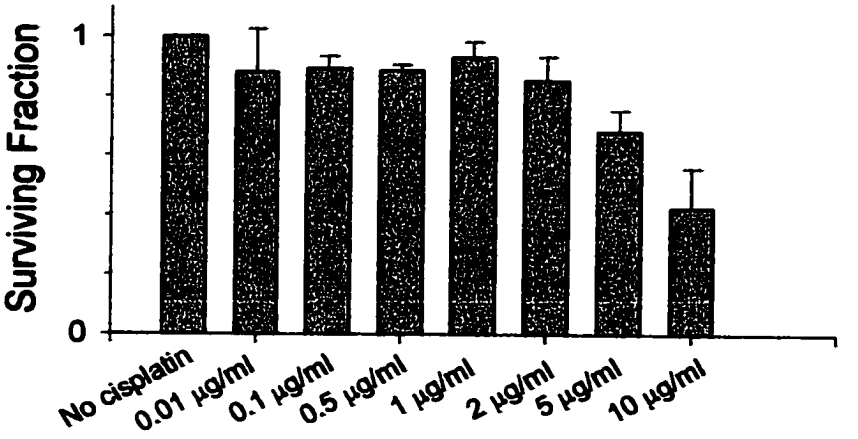
Figure 3.2.7A. Survival of the A2780cp cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before a 6 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are not significantly different from the 6 Gy alone point except for the 10 μg/ml dose point.

B. Survival of the A2780cp cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before a 6 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All the points are not significantly different from the 6 Gy alone point except for the 10 μg/ml dose point.

C. Survival of the A2780cp cells exposed to the indicated cisplatin doses for 1 hour, 24 hours before plating. The experiments are done in triplicate and the SEM is calculated to give the error bars. For all the points, there is no significant decrease in survival after cisplatin treatment except for the 10 μg/ml dose point.


Surviving fraction
0,020
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
6 Gy
0.01 µg/ml + 6 Gy
0.1 µg/ml + 6 Gy
0.5 µg/ml + 6 Gy
1 ug/ml + 6 Gy
2 µg/ml + 6 Gy
5 µg/ml + 6 Gy
10 µg/ml + 6 Gy


Surviving Fraction
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
6 Gy
0.01 µg/ml + 6 Gy
0.1 µg/ml + 6 Gy
0.5 µg/ml + 6 Gy
1 µg/ml + 6 Gy
2 µg/ml + 6 Gy
5 µg/ml + 6 Gy
10 µg/ml + 6 Gy


Surviving Fraction
1
0
No cisplatin
0.01 µg/ml
0.1 µg/ml
0.5 µg/ml
1 µg/ml
2 µg/ml
5 µg/ml
10 µg/ml

1 hour, 24 hours prior to the 6 Gy irradiation dose. There was no significant induction of radioresistance at any of the concentrations of cisplatin (Fig. 3.2.7A). An additive effect was only seen at the 10 μg/ml cisplatin dose (Fig. 3.2.7B). Survival of the A2780cp, after the cisplatin-alone treatment, decreased only significantly at the 10 μg/ml dose (Fig. 3.2.7C). Once again, when plating was delayed 24 hours, it allowed time for repair of cisplatin damage.

If the different cisplatin doses are not inducing radioresistance, when added 24 hours prior to radiation, perhaps the time interval between the two treatments is not ideal. Appendix 1 shows an additional experiment with the A2780cp, where a dose of 1 μg/ml cisplatin was added for 1 hour, at different time intervals before the dose of 6 Gy. Again, no induction of radioresistance was observed. There was a small increase in survival after the 39 and 48 hours time intervals, but the experiment was not repeated and more statistical analysis is required.

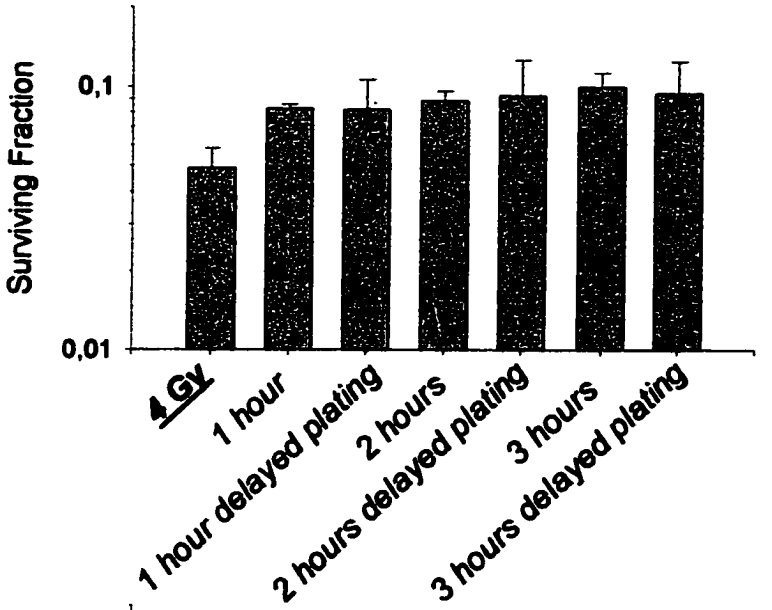
3.3 Cisplatin added after radiation

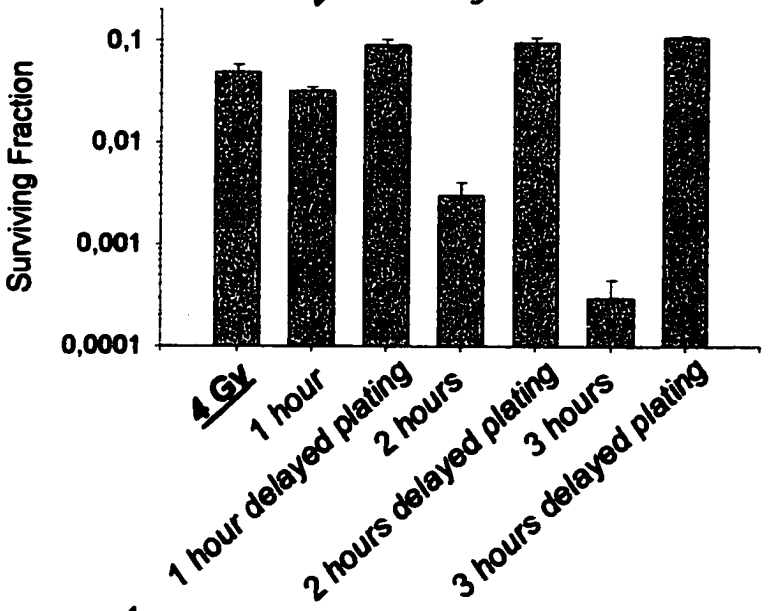
Fig. 3.3.1A illustrates the normalized survival fraction of the A2780s, when 2 μg/ml cisplatin were added, from 1 to 3 hours after 4 Gy. When the drug was added after radiation, there was an increase in survival due to the repair of radiation damage during the cisplatin treatment. This indicated that the cisplatin did not inhibit repair in the A2780s. The longer the cisplatin exposure, the longer

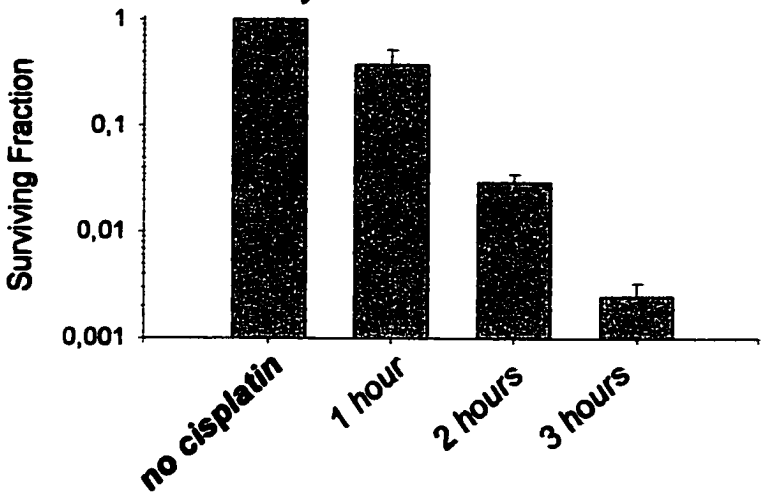
Figure 3.3.1A. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours after a 4 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All points show a significant difference from the 4 Gy alone point. There is also no significant difference between the points with and without cisplatin for the same incubation time post-irradiation.

B. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours after a 4 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All points are significantly different from the 4 Gy alone point.

C. Survival of the A2780s cells exposed to 2 μg/ml cisplatin for 1 to 3 hours. The experiments are done in triplicate and the SEM is calculated to give the error bars. There is a significant decrease in survival for all points.


Surviving Fraction
0,1
0,01
4 Gy
1 hour
1 hour delayed plating
2 hours
2 hours delayed plating
3 hours
3 hours delayed plating


Surviving Fraction
0,1
0,01
0,001
0,0001
4 Gy
1 hour
1 hour delayed plating
2 hours
2 hours delayed plating
3 hours
3 hours delayed plating


Surviving Fraction
1
0,1
0,01
0,001
no cisplatin
1 hour
2 hours
3 hours

the cells have to repair the radiation damage. When the effect of the cisplatin-alone treatment was removed (Fig. 3.3.1A normalized data), the resulting increase in survival was similar to the control, where the plating was delayed after radiation ($p<0.05$). The unnormalized data (Fig. 3.3.1B) shows a decrease in survival proportional to the cisplatin dose, when it is added after radiation. The Fig. 3.3.1C shows the decrease in survival in the A2780s cell line after the cisplatin-alone treatment.

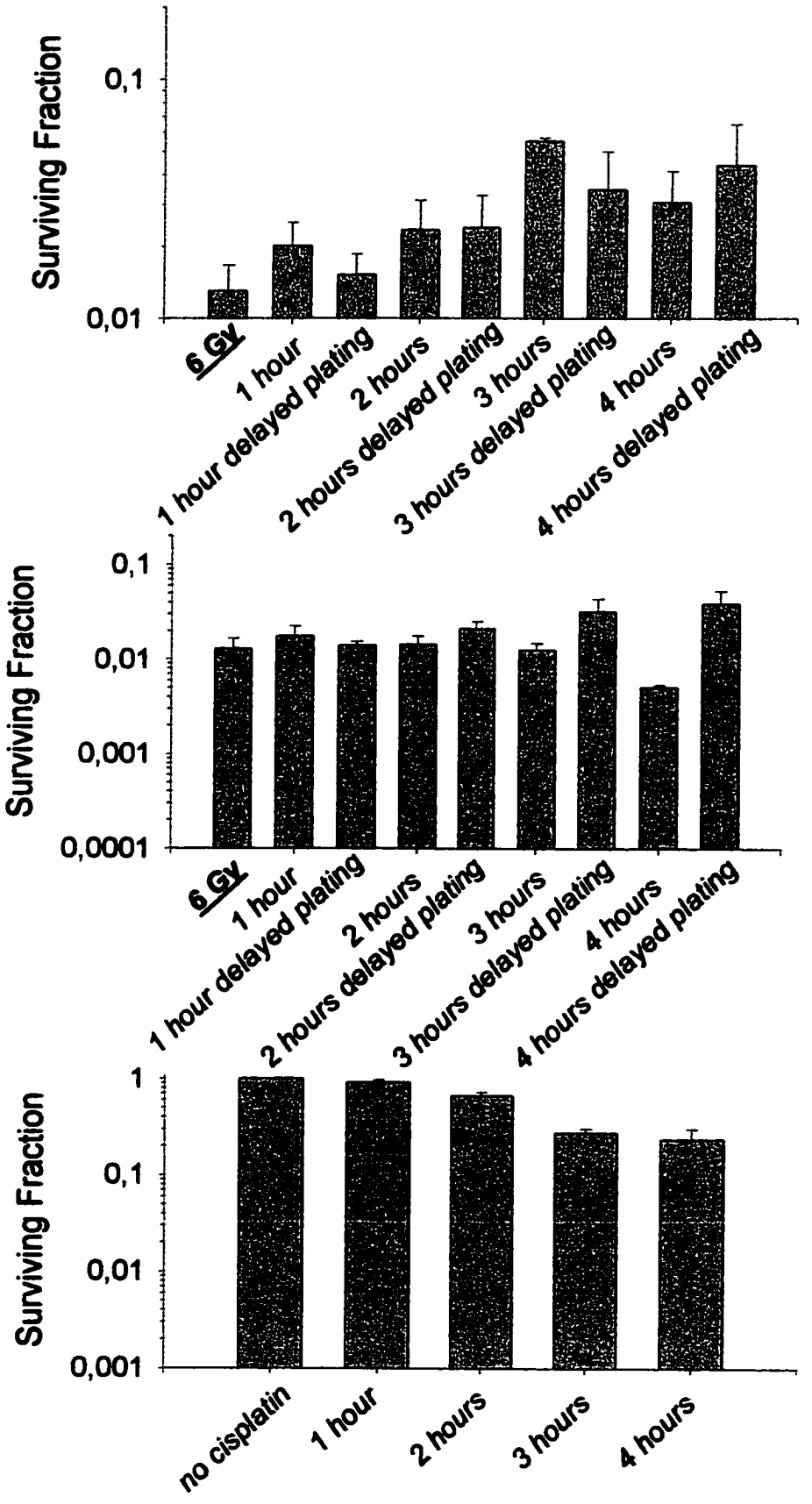
Similarly, when the drug was added after radiation on the A2780cp cell line, there was an increase in survival due to the repair of radiation damage during the cisplatin treatment (Fig. 3.3.2A normalized data, $p<0.05$ except for the 1 hour point without cisplatin). This indicates that cisplatin did not inhibit repair in the A2780cp cells, as seen with A2780s cells. This increase in survival was proportional to the incubation time after radiation for each set of data points. There is no difference between the control point without cisplatin and the normalized cisplatin points ($p>0.05$).

The unnormalized cisplatin with radiation data (Fig. 3.3.2B) shows a decrease in survival compared to the delayed plating controls at the 3 and 4 hours treatment times ($p<0.05$). The A2780cp was again more resistant to the 3 μg/ml cisplatin dose (Fig. 3.3.2C) than the A2780s with the 2 μg/ml cisplatin dose (Fig. 3.3.1C). When cisplatin was added, along with radiation, to the A2780cp (Fig. 3.3.2B), the overall killing effect was less than it was for the A2780s (Fig. 3.3.1B).

Figure 3.3.2A. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours after a 6 Gy dose (normalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. All points show a significant difference from the 6 Gy alone point except for the 1 hour delayed plating point. There is no significant difference within each pair of data points with and without cisplatin for the same post-irradiation incubation time.

B. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours after a 6 Gy dose (unnormalized data). The experiments are done in triplicate and the SEM is calculated to give the error bars. The 2 hour delayed plating point, the 3 hour delayed plating point, the 4 hour delayed plating point and the 4 hour cisplatin treatment point are significantly different from the 6 Gy alone point. There is a significant difference within each pair of data points with and without cisplatin for the same post-irradiation time.

C. Survival of the A2780cp cells exposed to 3 μg/ml cisplatin for 1 to 4 hours. The experiments are done in triplicate and the SEM is calculated to give the error bars. There is no significant difference between the 3 and 4 hour points.


Surviving Fraction
0,1
0,01
0,001
0,0001
1
6 Gy
1 hour
1 hour delayed plating
2 hours
2 hours delayed plating
3 hours
3 hours delayed plating
4 hours
4 hours delayed plating
no cisplatin

3.4 Cisplatin added during pulsed irradiation

In an attempt to look at cisplatin and radiation interaction, in terms of radioresistance or radiosensitization, the next logical step was to apply the two treatments simultaneously. We decided to study PDR radiation therapy, a new brachytherapy technique being evaluated in certain clinics.

Cisplatin was present during the entire PDR irradiation treatment. Experiments on the A2780s were then performed to screen for the effect of different doses of cisplatin added to a dose of 6 Gy (1 Gy/h) PDR irradiation treatment.

An increase in radiation resistance was observed for the A2780s (Fig. 3.4.1A normalized data). The interaction between radiation and 0.5 μg/ml, 0.75μg/ml and 1 μg/ml cisplatin resulted in more than 50% increase in survival, when it was present during the PDR (1Gy/h) treatment ($p<0.05$). Even in the presence of sub-additivity, there was still a large decrease in survival, when cisplatin was added to the PDR treatment (Fig. 3.4.1B).

After 6 hours of the 1 Gy/h for the A2780s, only 4% of the cells survived (Figs. 3.4.1A and 3.4.1B)(6 Gy control). The 6 hours cisplatin treatment at doses of 0.5 μg/ml, also left 4% of the cells viable (Fig. 3.4.1C). When the two treatments were combined, 0.16% of the cells should remain viable, but in fact, more than 0.75% of the cells were found remaining (Fig. 3.4.1B).

Fig. 3.4.2 shows the whole survival curve for the 1 Gy/h PDR experiments for the A2780s, with and without the presence of 0.5 μg/ml cisplatin during the

Figure 3.4.1A. Survival of the A2780s cells after PDR (1Gy/h up to 6Gy) with the indicated cisplatin doses present during the entire radiation treatment (normalized data). The experiments are done 4 times and the SEM is calculated to give the error bars. All points show a significant increase in survival compared to the 6 Gy (1 Gy/h) alone point.

B. Survival of the A2780s cells after PDR (1 Gy/h up to 6 Gy) with the indicated cisplatin doses present during the entire radiation treatment (unnormalized data). The experiments are done 4 times and the SEM is calculated to give the error bars. All points show a significant decrease in survival compared to the 6 Gy (1 Gy/h) alone point.

C. Survival of the A2780s cells after exposure to the indicated cisplatin doses. The experiments are done 4 times and the SEM is calculated to give the error bars. All the points show a significant decrease in survival after cisplatin treatment.

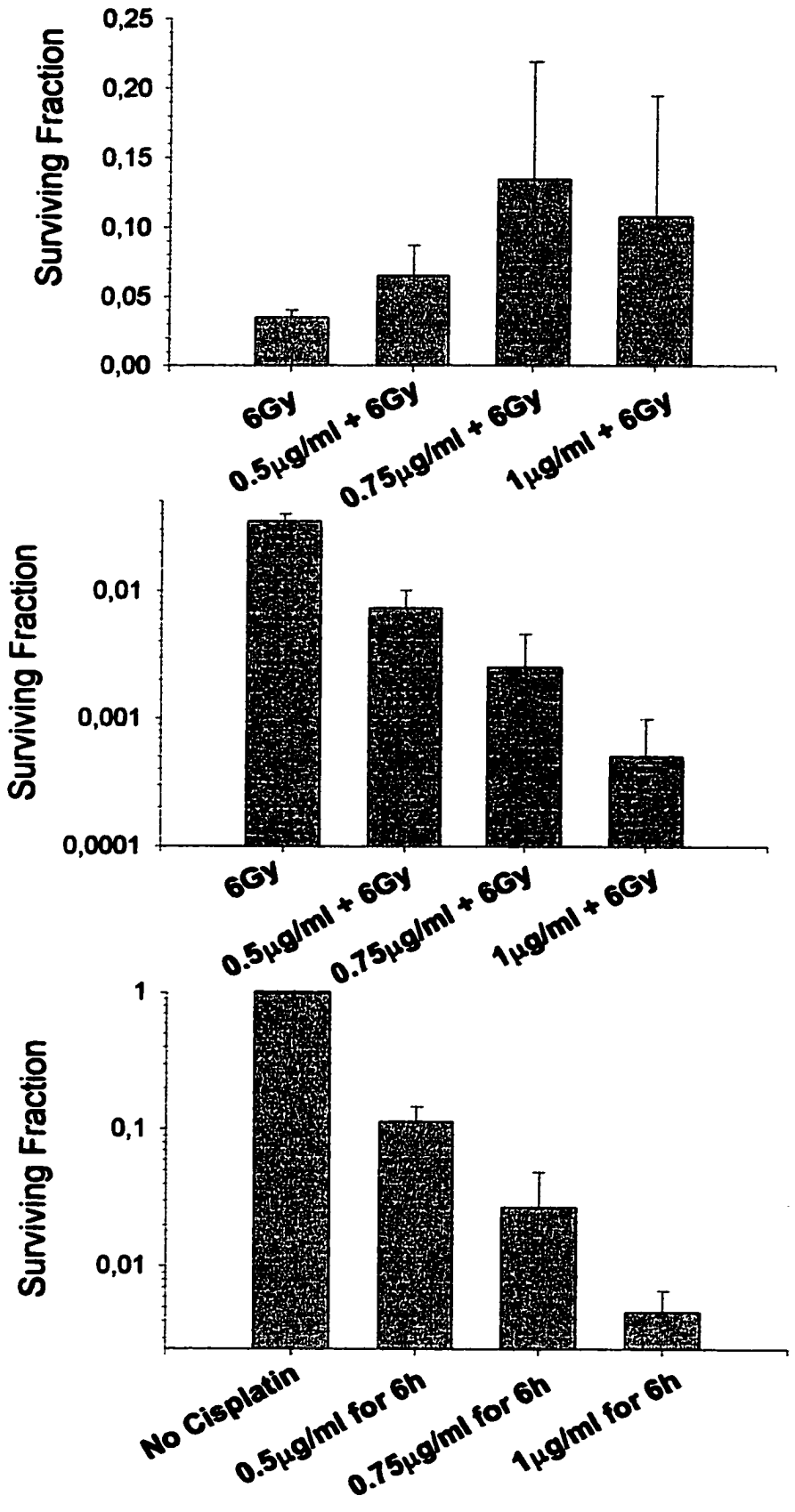
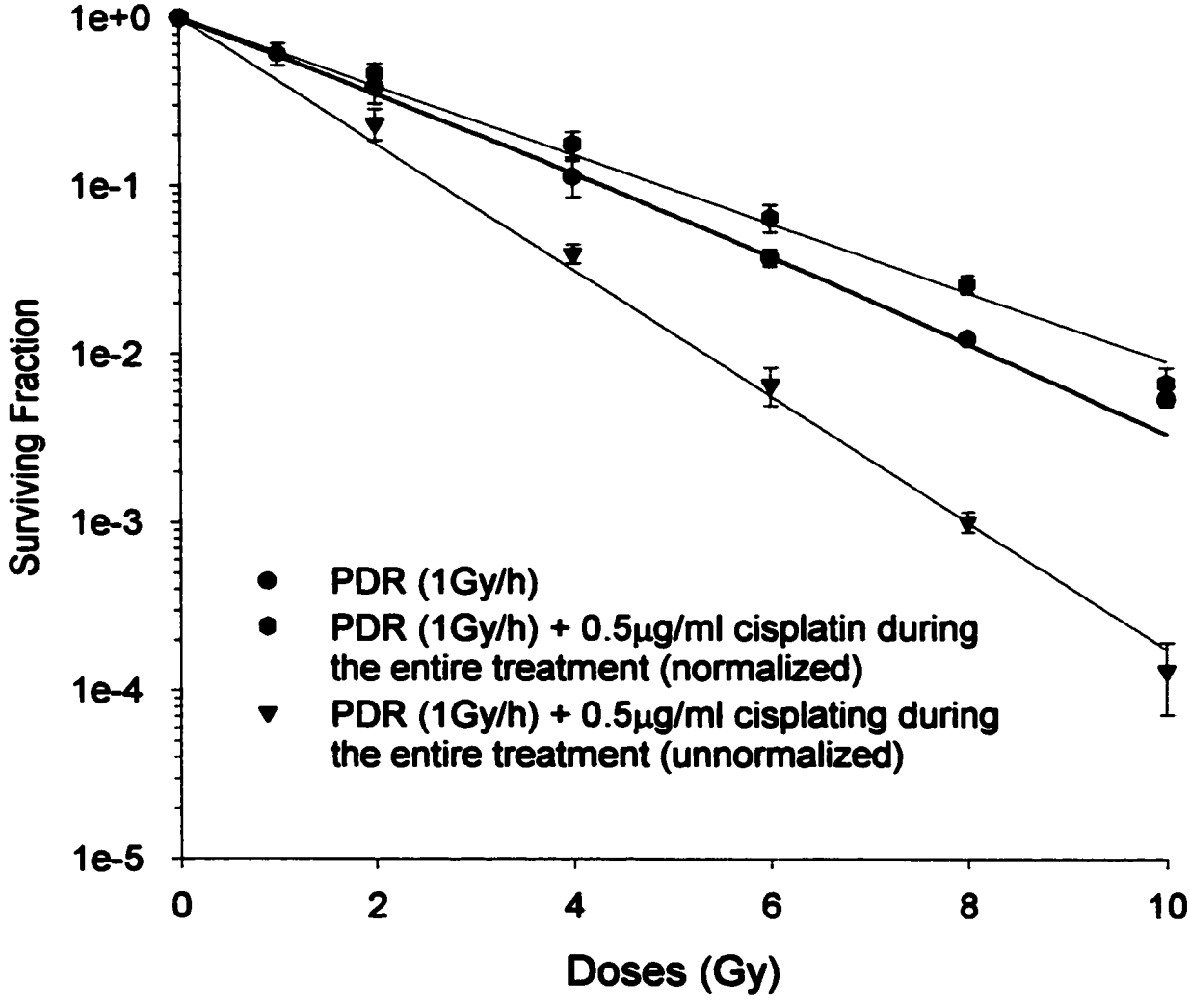

Surviving Fraction
0,25
0,20
0,15
0,10
0,05
0,00
6Gy
0.5µg/ml + 6Gy
0.75µg/ml + 6Gy
1µg/ml + 6Gy
Surviving Fraction
0,01
0,001
0,0001
6Gy
0.5µg/ml + 6Gy
0.75µg/ml + 6Gy
1µg/ml + 6Gy
Surviving Fraction
1
0,1
0,01
No Cisplatin
0.5µg/ml for 6h
0.75µg/ml for 6h
1µg/ml for 6h

Figure 3.4.2. Survival of the A2780s cells after PDR (1 Gy/h) treatment with and without 0.5 μg/ml cisplatin present during the entire radiation treatment. Both the normalized and unnormalized data are shown. Plotted are mean ± SEM values from 3 independent experiments. The PDR with cisplatin (normalized data) and PDR alone curves are significantly different at doses between 2 Gy and 10 Gy. The combined treatments unnormalized curve is significantly different from the PDR (1 Gy/h) alone curve.


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
0
2
4
6
8
10
Doses (Gy)
PDR (1Gy/h)
PDR (1Gy/h) + 0.5μg/ml cisplatin during the entire treatment (normalized)
PDR (1Gy/h) + 0.5μg/ml cisplating during the entire treatment (unnormalized)

entire 6 hour treatment. When cisplatin was present, there was an increase in resistance to pulsed radiation. After normalization, the dose response curve for the combination of radiation and cisplatin extends above the curve for radiation-alone, indicating a sub-additive interaction between the two agents.

Fig. 3.1.5 shows the survival curve for the A2780s, after exposure to 0.5 μg/ml cisplatin for increasing amounts of time. At this concentration, the significantly induced resistance only occurred for the 4, 6 and 8 Gy points (Fig. 3.4.2). At the 10 Gy point the radiation-alone curve is not significantly different from the radiation in combination with the cisplatin curves. When cisplatin was present during the PDR treatment, the upward bending of the curve was no longer seen.

The same cisplatin dose applied to the 2 Gy/h PDR regimen produced survival curves, as shown in Fig. 3.4.3. In this case, no increase in resistance was observed. Since the dose per fraction was larger (2 Gy/h), and the time allowed for repair was shorter in Fig. 3.4.3, it was possible to conclude that the less than additive effect of cisplatin seen in Fig. 3.4.2 could be linked to the longer time allowed for repair during the radiation treatment.

For the A2780cp cells, there was also a significant increase in resistance (Fig. 3.4.4A, normalized data, $p<0.05$). Higher cisplatin concentrations were used for the A2780cp, since they are more resistant. The cisplatin doses, needed to induce resistance in the A2780cp cells, were higher. There was a 3-fold increase in survival, when 5 μg/ml cisplatin was present during the PDR

Figure 3.4.3. Survival of the A2780s cells after PDR (2 Gy/h) treatment with and without 0.5 μg/ml cisplatin present during the entire radiation treatment. Both the normalized and unnormalized data are shown. Plotted are mean ± SEM values from 3 independent experiments. There is no significant difference between the PDR with cisplatin treatment (normalized data) and PDR alone. The combined treatments unnormalized curve is significantly different from the PDR (1 Gy/h) alone curve.

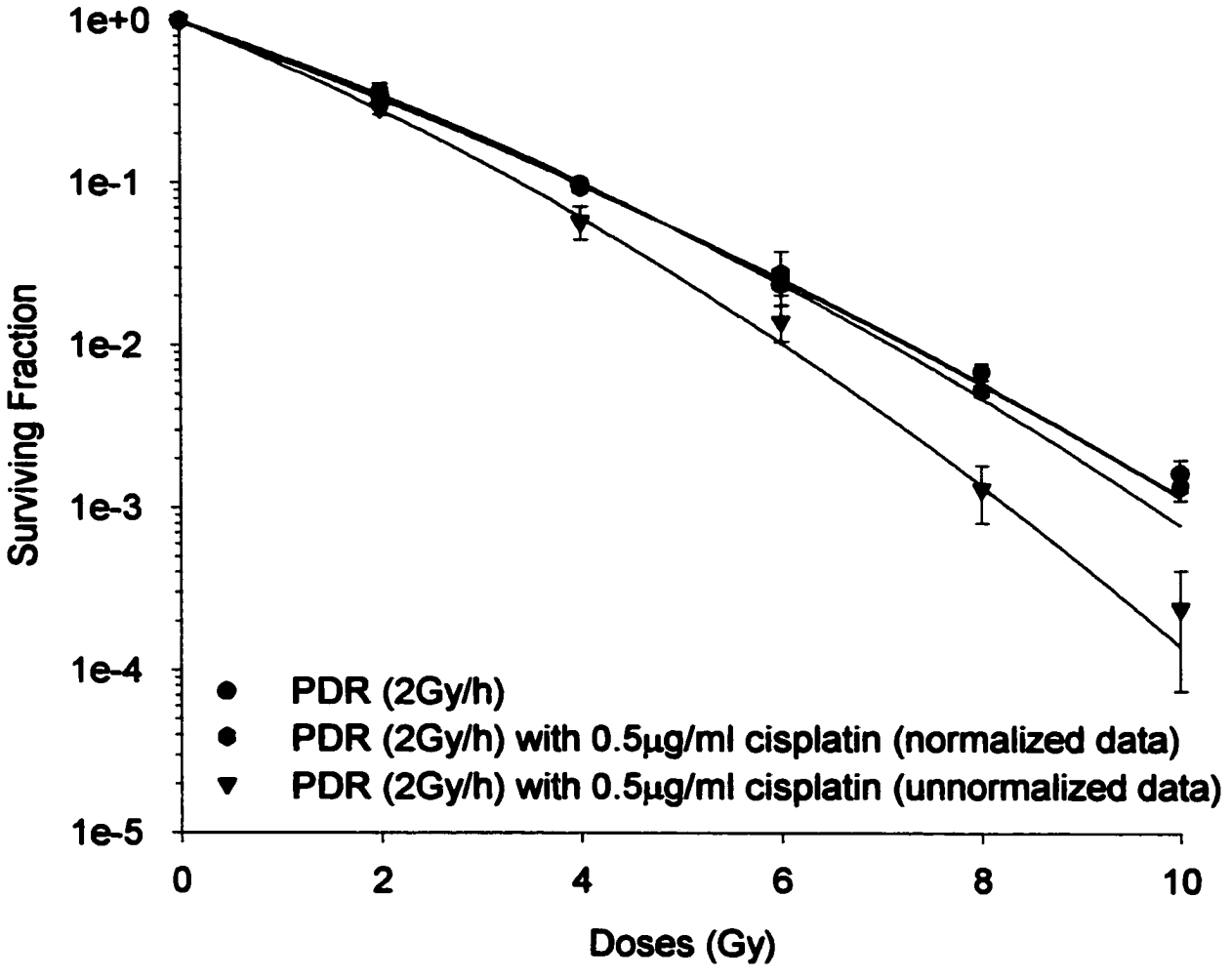
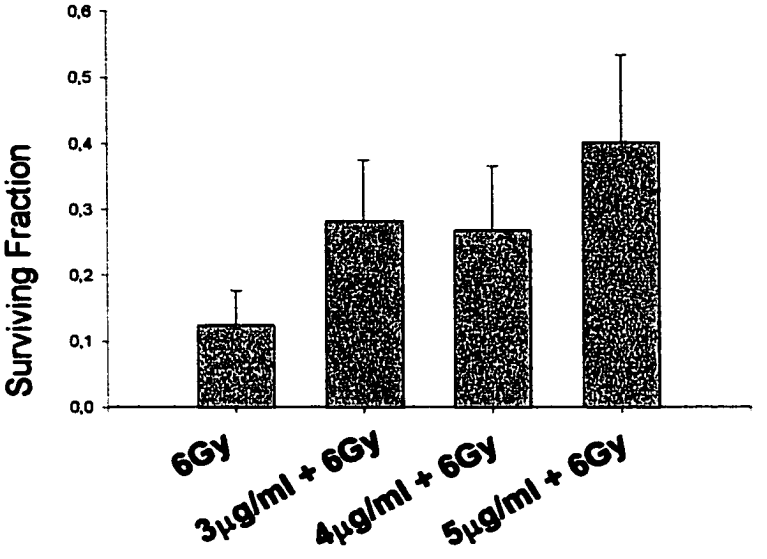
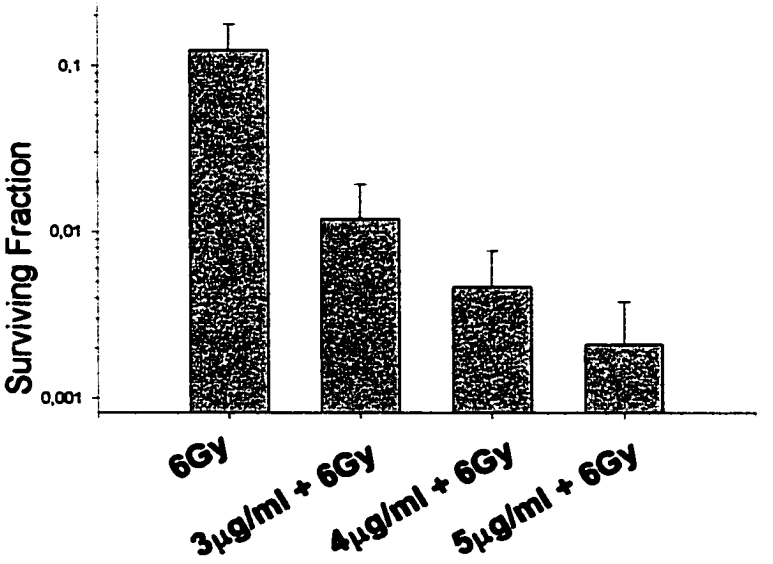

1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
0
2
4
6
8
10
Doses (Gy)
PDR (2Gy/h)
PDR (2Gy/h) with 0.5μg/ml cisplatin (normalized data)
PDR (2Gy/h) with 0.5μg/ml cisplatin (unnormalized data)

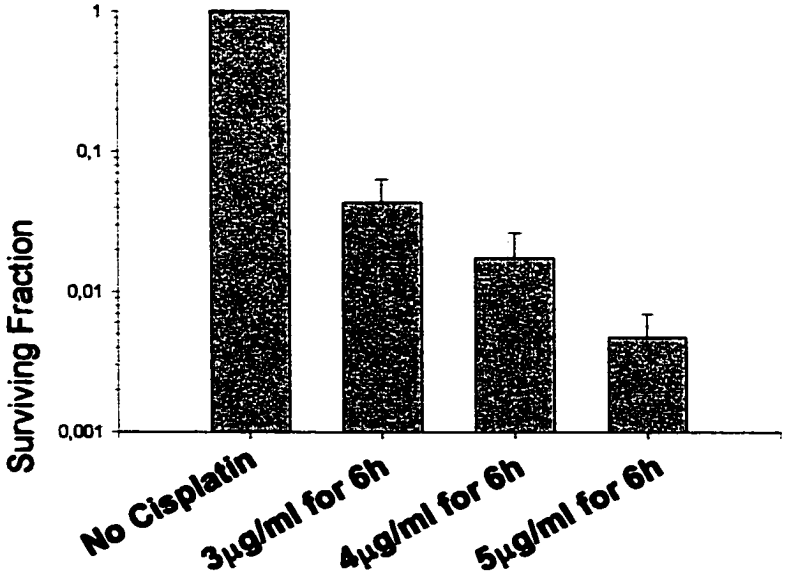
Figure 3.4.4A. Survival of the A2780cp cells after PDR (1 Gy/h up to 6 Gy) with the indicated cisplatin doses present during the entire radiation treatment (normalized data). The experiments are done 4 times and the SEM is calculated to give the error bars. All the points show a significant increase in survival compared to the 6 Gy (PDR) alone point.

B. Survival of the A2780cp cells after PDR (1 Gy/h up to 6 Gy) with the indicated cisplatin doses present during the entire radiation treatment (unnormalized data). The experiments are done 4 times and the SEM is calculated to give the error bars. All the points show a significant decrease in survival compared to the 6 Gy (PDR) alone point.

C. Survival of the A2780cp cells after exposure to the indicated cisplatin doses. The experiments are done 4 times and the SEM is calculated to give the error bars. All the points show a significant decrease in survival after cisplatin treatment.


Surviving Fraction
0,6
0,5
0,4
0,3
0,2
0,1
0,0
6Gy
3µg/ml + 6Gy
4µg/ml + 6Gy
5µg/ml + 6Gy


Surviving Fraction
0,1
0,01
0,001
6Gy
3µg/ml + 6Gy
4µg/ml + 6Gy
5µg/ml + 6Gy


Surviving Fraction
1
0,1
0,01
0,001
No Cisplatin
3µg/ml for 6h
4µg/ml for 6h
5µg/ml for 6h

(1 Gy/h) treatment ($p<0.05$).

The induced radioresistance was successfully seen in both cell lines when different doses of cisplatin were added during a 6 Gy PDR (1 Gy/h) treatment (Figs. 3.4.1 and 3.4.4). Fig. 3.1.5 shows the cisplatin survival curves for the experiments for both cell lines.

The A2780cp experiment (Fig. 3.4.4) for 6 Gy was compared to the entire survival curve for the PDR treatment, with and without cisplatin. Figs. 3.4.5 and 3.4.6 respectively compare the 1 Gy/h PDR treatment, with and without 1 μg/ml and 3 μg/ml cisplatin, during the entire radiation treatment. For the 1 μg/ml cisplatin treatment with PDR, there was no radioresistance, when the curve was normalized to the radiation-alone treatment (Fig. 3.4.5), but resistance was seen for the normalized curve of the 3 μg/ml cisplatin treatment with PDR (Fig. 3.4.6) at doses higher than 2Gy ($p<0.05$).

There was a smaller additive effect at longer cisplatin exposure times for the 1 μg/ml experiment (additivity only significant at 8 and 10 Gy respectively (Fig. 3.4.5)) compared to the 3 μg/ml experiment (Fig. 3.4.6). The cells were highly resistant to long cisplatin exposure times, as shown in Fig. 3.1.5. For the 2 Gy/h experiment (Fig. 3.4.7), the cisplatin was only present for 5 hours. Therefore, the cisplatin exposure was too short to make any contribution to the PDR treatment in terms of cell killing. It is surprising to see that, as was the case for the A2780s, cisplatin did not inhibit repair between pulses. However, inhibition of repair may occur under other fractionation patterns that leave more time for repair. To test whether the A2780s cells were made permanently more

Figure 3.4.5. Survival of the A2780cp cells treated with PDR (1 Gy/h) with and without 1 μg/ml cisplatin. Both normalized and unnormalized data are shown for the combined treatment. There is no significant difference between the normalized data for the combined treatment and the PDR alone curve. There is only a significant difference at the 8 Gy and 10 Gy points between the unnormalized data and the PDR (1 Gy/h) points.

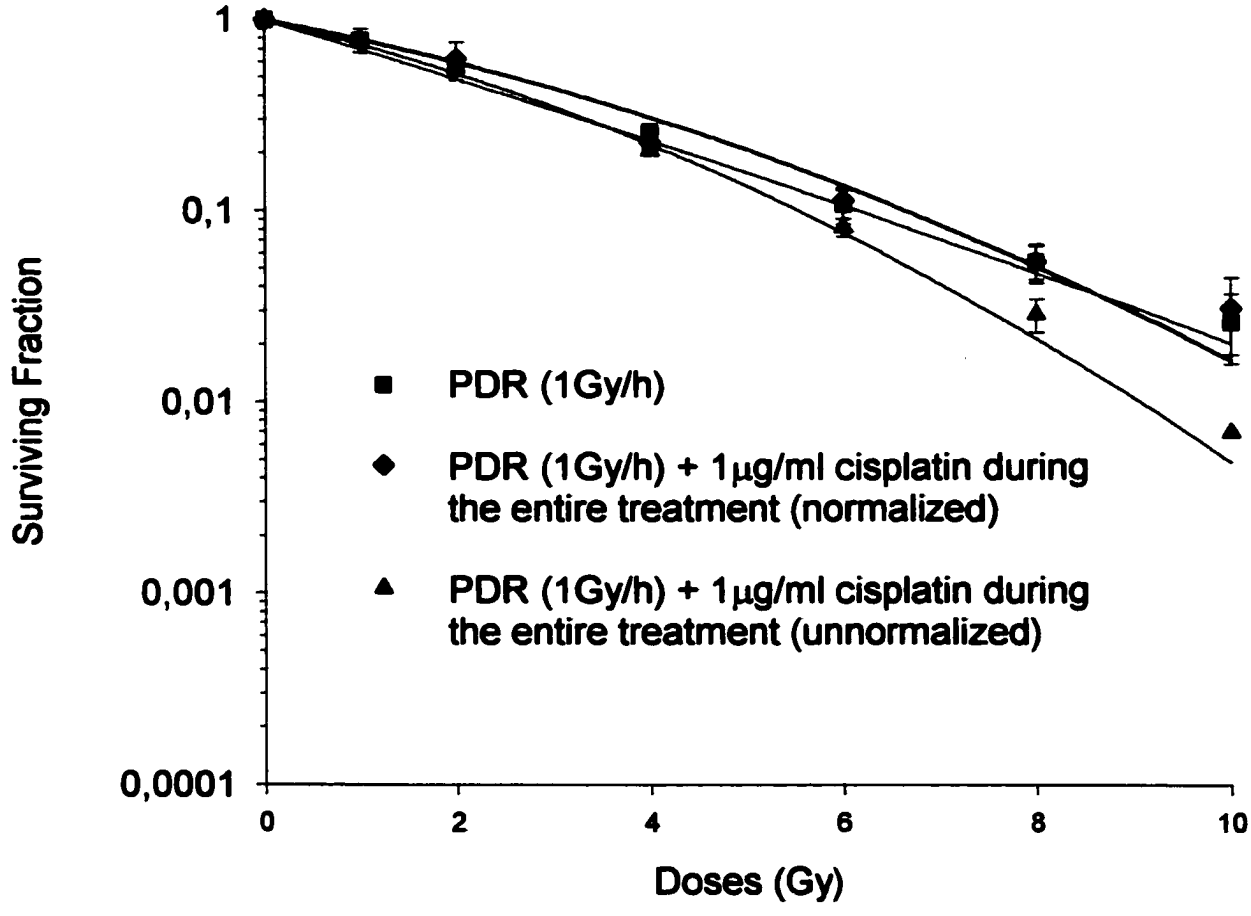

1
0,1
0,01
0,001
0,0001
Surviving Fraction
0
2
4
6
8
10
Doses (Gy)
PDR (1Gy/h)
PDR (1Gy/h) + 1μg/ml cisplatin during the entire treatment (normalized)
PDR (1Gy/h) + 1μg/ml cisplatin during the entire treatment (unnormalized)

Figure 3.4.6. Survival of the A2780cp cells treated with PDR (1 Gy/h) with and without 3 μg/ml cisplatin. Both normalized and unnormalized data are shown for the combined treatment. Plotted are mean ± SEM values from 3 independent experiments. The PDR with cisplatin (normalized data) and PDR alone curve are significantly different. The unnormalized curve for the combined treatment is significantly different from the PDR alone curve.

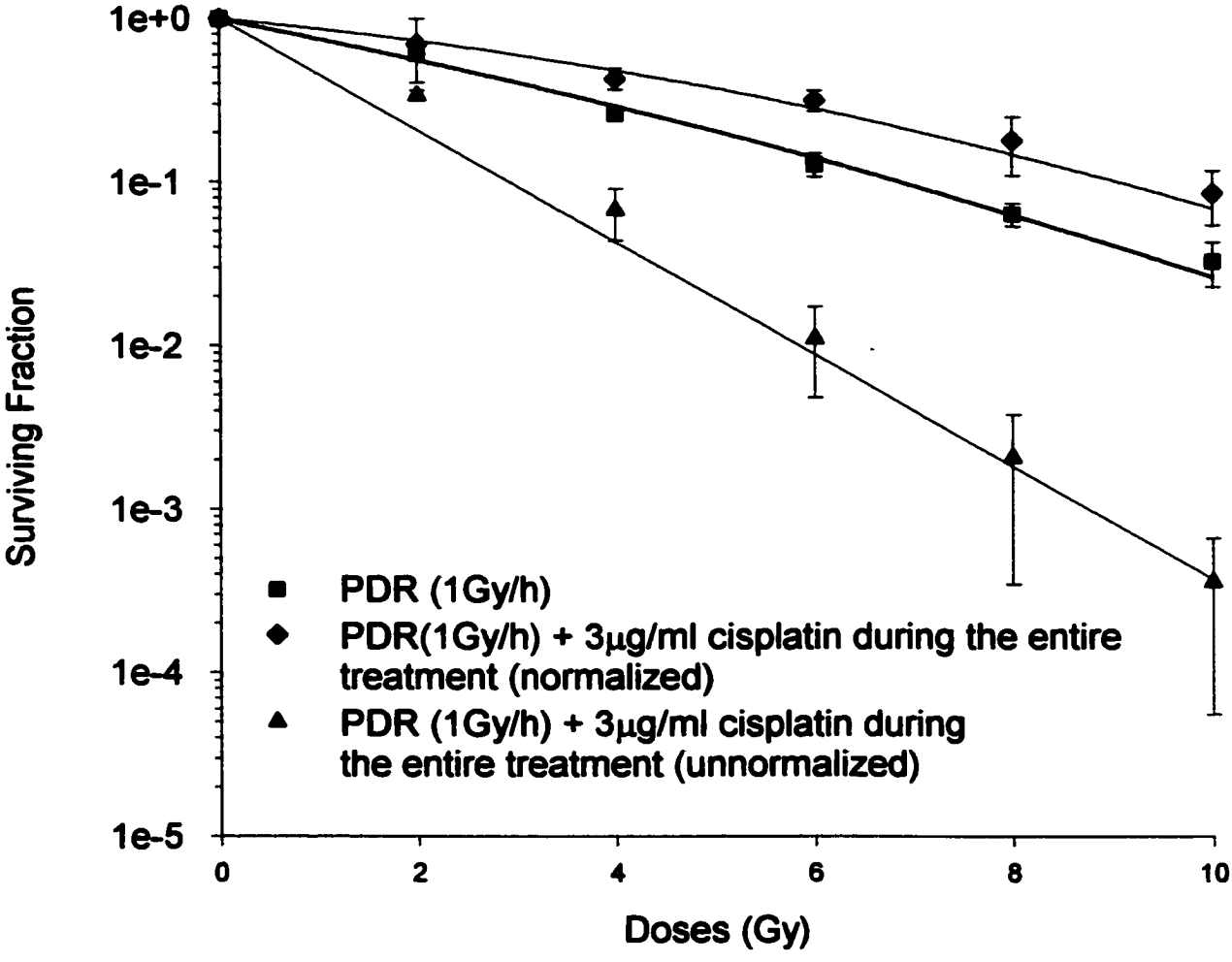
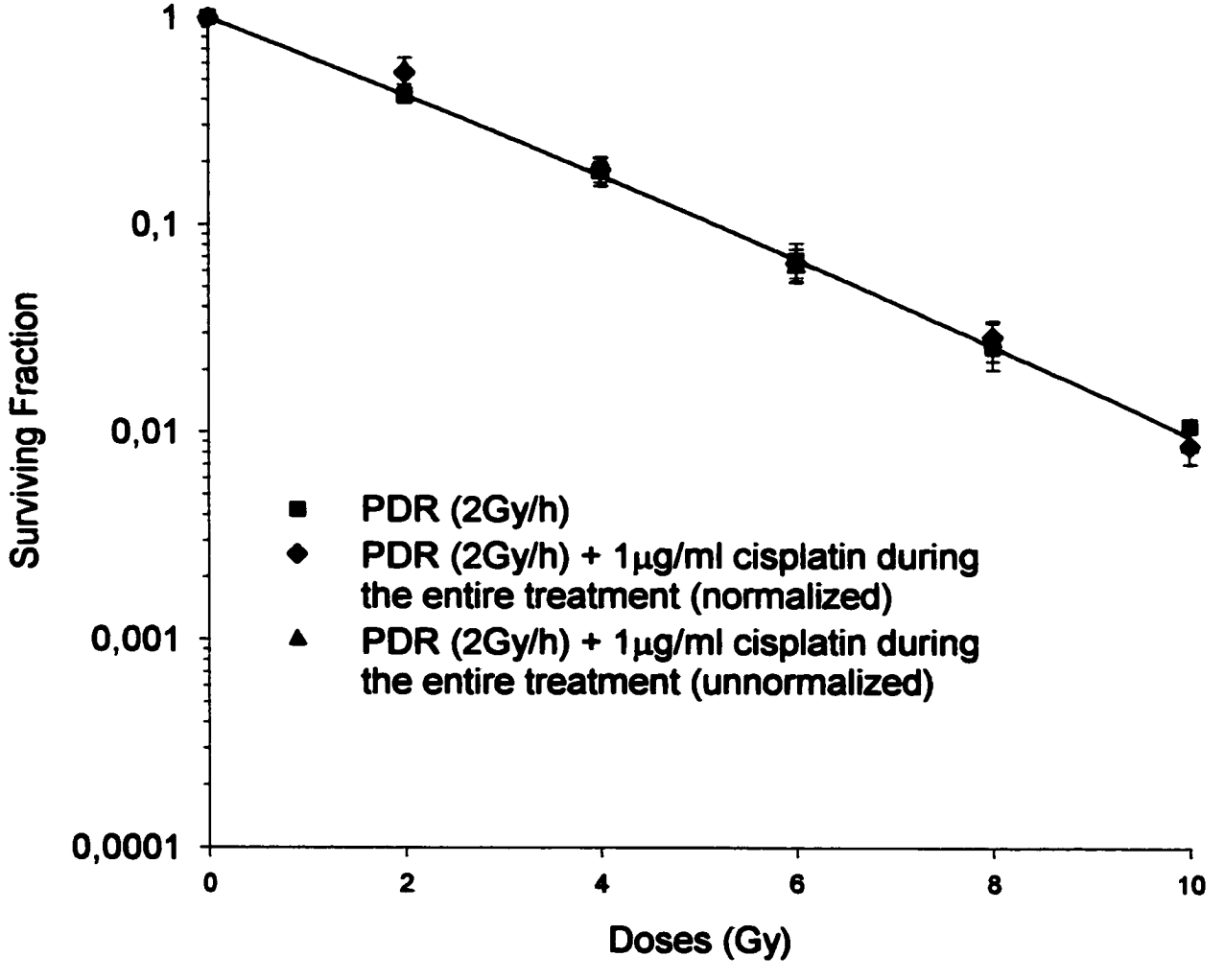

1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Surviving Fraction
0
2
4
6
8
10
Doses (Gy)
PDR (1Gy/h)
PDR(1Gy/h) + 3μg/ml cisplatin during the entire treatment (normalized)
PDR (1Gy/h) + 3μg/ml cisplatin during the entire treatment (unnormalized)

Figure 3.4.7. Survival of the A2780cp cells treated with PDR (2 Gy/h) with and without 1 μg/ml cisplatin. Both the normalized and unnormalized data are shown for the combined treatment. All the curves are not significantly different.


1
0,1
0,01
0,001
0,0001
Surviving Fraction
0
2
4
6
8
10
Doses (Gy)
PDR (2Gy/h)
PDR (2Gy/h) + 1µg/ml cisplatin during the entire treatment (normalized)
PDR (2Gy/h) + 1µg/ml cisplatin during the entire treatment (unnormalized)

resistant to radiation after treatment with PDR alone (1 Gy/h), cisplatin-alone (0.5 μg/ml), and the two combined, HDR survival curves were done on these cells three days after each of the above treatments. These survival curves are shown in Appendix 2.

The results reveal that the A2780s cells, previously exposed to PDR (1 Gy/h) with 0.5 μg/ml cisplatin, were no longer resistant to radiation. As expected, cells previously exposed to 0.5 μg/ml cisplatin, for 10 hours, showed no increased resistance, compared to untreated cells.

4.0 DISCUSSION

4.1 Characterization of the cell lines

The A2780cp cisplatin-resistant phenotype used for our experiments was established by giving intermittent stepwise increase concentrations of cisplatin up to 70 μM to the A2780s cells and is properly identified as the A2780cp70 cell line (Behrens *et al.*, 1987a;Lai *et al.*, 1988b). Other series of cisplatin-resistant cell lines were developed and named according to the maximum dose reached at the end of the increasing cisplatin exposure of the A2780s. In the case of the cp series, A2780cp20 and A2780cp70, the cisplatin exposure was intermittent and reached a maximum dose of 20 μM and 70 μM respectively. In the case of the c series, A2780c8, A2780c30, A2780c50, A2780c80, A2780c100 and A2780c200, the exposure was continuous with increasing concentration of cisplatin. For these cell lines, the doses reached at the end of the cisplatin exposure are 8 μM, 30 μM, 50 μM, 80 μM, 100μM and 200μM respectively (Johnson *et al.*, 1994;Ferry *et al.*, 2000;Hamaguchi *et al.*, 1993). These cell lines are isogenic in origin but have different intrinsic cisplatin and radiation sensitivities. Therefore these cell lines are good models to use in one study of sensitization or resistance seen with different cisplatin and radiation regimens.

Figs. 3.1.1 and 3.1.2 show the growth of ovarian carcinoma parental A2780s and resistant cells A2780cp70. Both cell lines grow at similar rates in DMEM/F12 medium, with a doubling time of approximately 24 hours. Previous

studies have found slightly different doubling times for the A2780c8 (25.3 ± 1 hours) and the A2780s (22.1 ± 0.7 hours), using RPMI 1640 medium. These studies have also shown lower cloning or plating efficiencies for the A2780s (~27%) and the A2780c8 (~36%) (Behrens *et al.*, 1987b). For our experiments, the plating efficiency was high (~65% for the A2780s and ~75% for the A2780cp70). Since both cell lines had similar plating efficiencies and similar doubling times, it was possible to compare their survival after different treatments. The cells, not fed on day 6, showed no further proliferation due to a lack of space and nutrients.

The A2780cp showed cross-resistance to both cisplatin (Figs. 3.1.3, 3.1.4 and 3.1.5) and HDR irradiation treatment (Fig. 3.1.6). Even in the PDR experiment, the A2780cp showed resistance to the 1 Gy/h and the 2 Gy/h compared to the A2780s (Figs. 3.1.7. and 3.1.8). Our experiments further demonstrated a modest difference in radiation sensitivity between the A2780s and the A2780cp, compared to the difference in the cisplatin sensitivity (Figs.3.1.3, 3.1.6, 3.1.7 and 3.1.8).

Radiation induced resistance does not necessarily make the cells cross-resistant to cisplatin. Studies showed this when resistance to radiation was induced in the ovarian cancer cells, AovC-O, with 1.5 Gy, every 48 hours for 6 months. Surprisingly, the radiation resistance induced cisplatin sensitivity. The resistance was lost, however, after 6 months of culturing the cells without radiation. At that time, the cisplatin sensitivity was also lost. Resistance to cisplatin might have unfavorable consequences for ionizing radiation, but the

reverse is not true (De Pooter *et al.*, 1991).

The A2780cp was also found resistant to cisplatin analogues (carboplatin, iproplatin and tetraplatin) (Behrens *et al.*, 1987b). It was also determined that the A2780 cisplatin-resistant series (CP20, CP70, C30, C50, C80, C100 and C200) showed cross-resistance to radiation and to a variety of drugs (carboplatin, epipodophyllotoxin, adriamycin, mitoxantrone and taxol). The cross-resistance to radiation was, however, modest (2-fold). In that particular study, the level of resistance to these drugs was proportional to the primary resistance to cisplatin. This could be explained by the levels of glutathione in the series being 4 to 50-fold the level found in the drug-sensitive parental cell lines (Hamaguchi *et al.*, 1993). Glutathione is binding cisplatin and is inhibiting its cytotoxicity. The increase in glutathione levels in the resistant cell lines is associated with increased expression of γ-glutamylcysteine synthase and γ-glutamyl transpeptidase involved in its synthesis (Godwin *et al.*, 1992).

As far as repairing is concerned, in our study the A2780cp showed the same increase in survival compared to the A2780s when plating was delayed by 6 hours (Fig. 3.1.6). In contrast, increased DNA repair was found in the A2780cp70 compared to their parental cell lines (Lai *et al.*, 1988a). We also did not find, in the course of our experiments, any noticeable difference in the potentially lethal damage (PLDR) repair between the two cell lines.

Even if our experiments did not show any difference in the level of PLDR repair after radiation treatment in the A2780s and the A2780cp cell lines, the intrinsic sensitivity of these cells lines depends strongly on the proficiency to

repair the radiation and the cisplatin damage.

In the case of cisplatin, the resistance is often linked to increase repair of the cisplatin adducts. Studies showed that there was an increase in the removal of platinum from genomic DNA in the resistant cell lines (cp70, c30, c80 and c200) proportional to the level of resistance (Johnson *et al.*, 1994). The A2780c200 cisplatin-resistant ovarian cancer cell line showed a 3-fold increase in nucleotide excision repair (NER). Additionally, the cisplatin resistance was associated with UV resistance. NER is an important UV repair mechanism. The A2780c200 overexpressed the ERCC1 gene involved in the NER pathway and a 2-fold increase in NER was seen in the A2780s, when ERCC1-XPF protein was added to the whole cell extract using the single lesion excision assay. Adding the ERCC1 in the whole cell extract of the A2780c200 had little effect on the NER (Ferry *et al.*, 2000).

More studies showed a correlation between radiosensitivity of the different cell lines and the ability of their whole cell extract to properly rejoin EcoRI-induced dsbs in the cell-free plasmid reactivation assay. Nuclear protein extracts derived from radiosensitive tumour cells were less capable of properly rejoining EcoRI-induced dsbs in plasmids compared to nuclear protein extracts derived from radioresistant tumour cells (Britten *et al.*, 1997). The dsb rejoining capacity of human tumour cells does not correlate favourably with their relative radioresistance (Olive *et al.*, 1994). On the other hand, radiation-induced chromosomal aberration correlates well with radiosensitivity in tumours (Sasai *et al.*, 1994).

It was previously demonstrated that the level of dsbs induced by radiation is the same for the A2780s and A2780cp70. And after 240 minutes (Britten *et al.*, 1999b) or 360 minutes (Abbot *et al.*, 2002) of post-irradiation time for repair, dsb levels were the same. The difference between the two cell lines resides in the cell fidelity to repair the dsbs and not in the quantity of dsbs repaired. The A2780s have shown high levels of illegitimate recombination of dsbs compared to the A2780cp70. One explanation would be that the A2780cp70 had a higher level of homologous repair (HR) or a suppressed non-homologous end-joining (NHEJ) repair pathway (Britten *et al.*, 1999b) (Abbot *et al.*, 2002).

Our experiment demonstrated that the difference between the A2780 and its resistant variant resides solely in the cisplatin and radiation sensitivities since they both show similar growth and repair of PLDR after radiation treatment.

4.2 Cisplatin added before or after radiation

Our results showed that the interaction between cisplatin and radiation is essentially additive when a cisplatin dose of 2 μg/ml is added for 1 and 3 hours to the A2780s before irradiation and a cisplatin dose of 3 μg/ml is added to the A2780cp70 for 4 hours before irradiation (Figs. 3.2.2, 3.2.3, 3.2.5, 3.3.1 and 3.3.2). The additivity was seen when the drug was added either immediately before or after radiation. When the experiments were done for the 4 Gy point only for the A2780s cells (Fig. 3.2.1) and at 6Gy for the A2780cp (Fig. 3.2.4) there is a significant decrease in survival when cisplatin was added before

radiation at certain cisplatin dose points. This could be interpreted as radiosensitization but the results could not be reproduced when the entire radiation survival curves were performed. It seems that the error bars are small for the screening experiments and are misleadingly showing a difference between the points. It could also mean that the radiosensitization effect is very small and is hard to detect depending on the way the experiment is carried out.

Our results do not confirm completely a previous study done in our laboratory. That study used the A2780s and A2780cp70 cell lines in the plateau phase. It showed that radiosensitization was only achieved when a cisplatin dose of 2 μg/ml was added for a period of 1 hour immediately after radiation (HDR treatment). According to this study, when 2 μg/ml was added before the radiation treatment, antagonistic interaction was observed in both cell lines. When 4 μg/ml cisplatin was added immediately before radiation, sensitization was only seen at higher doses for the A2780s and at all doses for the A2780cp. When 2 μg/ml or 4 μg/ml cisplatin were added after radiation, they induced the same levels of radiosensitization in both cell lines. And when 2 μg/ml cisplatin was added for 2 hours, with radiation given in the middle of the dose exposure, sensitization was seen in the A2780cp and resistance was seen in the A2780s. When 2 μg/ml cisplatin was added for 1 hour, with radiation treatment given after the first half-hour, sensitization was seen in both cell lines (Raaphorst *et al.*, 1995b;Raaphorst *et al.*, 1995a).

Our results consistently showed no cisplatin radiosensitization for both cell lines. Another researcher in our laboratory also noticed additivity in the A2780s

and A2780cp cell lines using the same growth conditions. These results were repeated during the same time period as our results (Dong Ping Yang, unpublished data).

The fact that radiosensitization was no longer seen by cisplatin with these cell lines could reside in the methodology of the experiment. In our experiments, the media used to treat cells with cisplatin was the same media covering the cells in the plateau phase. The cisplatin-containing media was removed before the radiation treatment and replaced with depleted media, recovered from the plateau phase. It is possible that the cisplatin effect could be different if, in lieu of depleted media, fresh media was used to treat the cells with cisplatin. Different results may also be obtained if different lots of fetal bovine serum were used in the experiment. In our study, 10% fetal bovine serum was employed, instead of 7.5% fetal bovine serum and 7.5% new-born calf serum.

Our experiments were performed over a period of 2 years. Most of them were performed three times with a time interval of more than three months between each of the trials. The error bars remain relatively small and this time interval between each of the experiment allows us to take into consideration any change in the experimental conditions.

Additivity between cisplatin and radiation was also observed in another ovarian cancer cell line grown to the plateau phase. In that particular study, the parental ovarian cancer cell line AovC-O and the derived cisplatin-resistant cell line AovC-CDDP/Ro were both cross-resistant to radiation just the same as our cell lines. Cisplatin doses of 1, 2.5 and 5 μM were given to sensitive AovC-O and

the AovC-CDDP/Ro cell lines for the period of 16 hours and for the period 4 hours immediately before or after HDR treatment. For this combination treatment, only additivity was found (the dose modifying factor (DMF) was around 1). The DMF for the combined treatments was calculated by dividing the radiation dose required to obtain a certain survival level by the dose required to obtain the same survival level when cisplatin is combined with radiation. Cisplatin doses of 0.5,1 and 2.5 μM given to the AovC cell for 16 hours, or 4 hours, immediately before or after HDR, also resulted in additivity of the two treatments (DMF varying between 0.89 and 1.08). Analysis of the normalized survival curves showed no difference in the α and β parameters for both cell lines (Scalliet *et al.*, 1999). Compared to our study these doses are smaller. Doses of 0.5 μM are equivalent to 0.066 μg/ml, 1 μM to 0.13 μg/ml, 2.5 μM to 0.33 μg/ml and 5 μM to 0.66 μg/ml. Also, cisplatin times of exposure in the above study are longer (4 hours and 16 hours before and after radiation) than the cisplatin times of exposure in our experiments (1 to 4 hours before and after radiation). Doses of 2 μg/ml for the A2780s, for 1 to 3 hours, and 3 μg/ml, for 1 to 4 hours immediately before or after radiation, were used in our experiments. The toxicity of the cisplatin doses used in Scalliet *et al* experiments is comparable to the toxicity of the cisplatin doses used in our experiments if we take into consideration the longer time of exposure (16 hours) that they used for the 1,2.5 and 5 μM cisplatin treatments. Our study confirms the results of this particular study. In both cases, no interaction was found when cisplatin was added to the ovarian cancer cells immediately before or after radiation.

The AovC-O / AovC-CDDP/Ro cell lines are also a good model to study the combination of radiation and cisplatin analogues on ovarian cancer cells. In these cell lines, it was shown that carboplatin radiosensitized the cells at a dose of 5 μM for the AovC-O and 10 μM for the AovC-CDDP/Ro cell line (Scalliet *et al.*, 1999). It would have been interesting to see if carboplatin would also radiosensitized the A2780s and the A2780cp.

Additivity between cisplatin and radiation was shown in other cellular models. Additivity effects of cisplatin and radiation were observed in four human tumour cell lines: MRI-186 (adenocarcinoma of the cervix), Caski (squamous cell carcinoma of the cervix), LX-1 (adenocarcinoma of the lung) and Hela-S3 (adenocarcinoma of the cervix). The first three cell lines were established at the time of the experiments.

The treated cells were exponentially grown in oxic conditions. Cisplatin was given for one hour. The HDR treatment was given after the first half-hour of the cisplatin treatment. Cisplatin doses of 2 and 5 μg/ml were used for the Caski cells, 0.5 and 2.5μg/ml for the Hela-S3 cells and 0.5, 1, and 2.5 μg/ml for the MRI-186 cells.

For the three cell lines, cisplatin did not sensitize the cells to radiation. Recovery between 2 split doses of 3 Gy was studied in the LX-1 cells, each in the presence of 5 μg/ml cisplatin for 1 hour, with radiation given in the first half-hour, and 0 to 16 hours to allow for repair between the radiation doses. In the LX-1 cells, cisplatin did not influence the SLDR of the cells (Flentje *et al.*, 1992).

Another interesting study demonstrated an additive effect with cisplatin and radiation in glioma cell cultures. These cultures were derived from human glioblastoma multiforme biopsy tissues from 4 different patients, and the U373 glioma cell line. Doses of 0.5 μg/ml and 1μg/ml of cisplatin were used to treat the cells for 2 hours before HDR irradiation. For each of the individual cell cultures, a parallel shift of the radiation survival curve occurred with the use of cisplatin, indicating an additional cell killing effect (Fehlauer *et al.*, 2000).

Even though a large number of experiments were showing additivity when radiation was combined with cisplatin, it was established that cisplatin is a modest radiation sensitizer in mammalian cells. In most experiments showing sensitization by cisplatin, DMFs were usually between 1 and 1.3 (Dewit, 1987) even if a DMF of 2.2 was seen in RIF-1 tumours cells (Begg *et al.*, 1986).

The Chinese hamster V79 lung cells, using 10 μM cisplatin, had a modest radiosensitization effect under hypoxia in the plateau phase cells (Douple & Richmond, 1978) and exponential phase cells (Stratford *et al.*, 1980). There was also inhibition of PLDR and SLDR (Dritschilo *et al.*, 1979). As well, radiosensitization, using 2.5 μM cisplatin, was observed in rat hepatoma cells (H_4 cells). The level of sensitization to radiation was more pronounced when the cells were treated at the plateau phase, compared to the exponential phase. Hypoxia increased even more the radiosensitizing effect of cisplatin on the plateau phase cells (Carde *et al.*,1981).

In the latter study, both PDR and SLDR were largely inhibited by the presence of cisplatin. To measure inhibition of SLDR, the 2.5 μM cisplatin was

added before the first pulse, or between the two pulses of radiation. PLDR was assessed by delaying plating of cells by 0 to 24 hours. Cisplatin was added after radiation until the cells were plated.

In our experiment, cisplatin did not inhibit potentially lethal damage repair (PLDR) in the A2780s (Fig.3.3.1A) and the A2780cp (Fig.3.3.2A). When the drug was given after radiation, there was an increase in survival due to the repair of radiation damage during the cisplatin treatment. The level of repair was the same in the non-treated and treated cells after normalization. What could be interpreted as sub-additivity, or increased resistance to radiation in the presence of cisplatin, is only the repair taking place during the cisplatin treatment.

It is important to take into account the appropriate controls, that is the radiation treatment and the time it takes for the following cisplatin exposure to unfold. The longer the cisplatin exposure, the more time the cells have to repair their radiation damage, thus conferring them an increased survival. Longer cisplatin exposure times, at smaller doses, would have been useful to evaluate inhibition of PLDR as long as 24 hours after radiation treatment.

Previous studies showed that cisplatin in the A2780s and the A2780cp cell lines inhibits sublethal damage repair (SLDR). To study SLDR, doses of 2 and 4 μg/ml of cisplatin were added before or after the first dose of 4 Gy, followed by 1 to 8 hours to allow for repair before a second dose of 4 Gy. Survival was assessed at that point. In both A2780s and A2780cp cell lines, SLDR was inhibited with 4 μg/ml of cisplatin given before or after the first dose (Raaphorst *et al.*, 1995a). For the purpose of our study, we did not carry out any experiments

to see if SLDR was inhibited by cisplatin in the A2780s and A2780cp. This would still need to be confirmed.

Some studies were done on radiation-induced resistance with cisplatin. It is known that repeated cisplatin exposure in the A2780s renders the cells more resistant to radiation by a cross-resistance mechanism. It is also known that cisplatin induces radioprotection in human T98 glioma cells. When the cells were treated 5 times with cisplatin doses of 1 μM, for 24 hours during a period of three months, the pre-treated cells became more resistant to both radiation and cisplatin. The phenomenon was independent of glutathione cisplatin inhibition (Poppenborg *et al.*, 1997).

On the other hand, sublethal cisplatin doses have been shown to make yeast cells resistant to radiation for a short period of time. Studies in yeast reported that sublethal doses of cisplatin, given 2 hours before radiation, increased resistance to cell killing (Dolling *et al.*, 1999). SK-OV-3 human ovarian tumour cells, exposed *in vitro* to 2 Gy, twice a day for two weeks, generated resistance to cisplatin (Hill *et al.*, 2000). To verify the radioresistance theory, different doses of cisplatin were added for one hour, 24 hours prior to radiation, and the resulting survival levels were compared. There was no induced resistance in either cell line.

4.3 Cisplatin added during pulsed irradiation

There are no recent experiments on the effects of chemotherapeutic drugs

given in conjunction with a pulse dose rate (PDR) irradiation treatment. Most chemoradiation experiments have been carried out using low dose rate (LDR) irradiation with chemotherapy. The LDR used in brachytherapy is often used in the clinical settings. The progression of LDR brachytherapy to PDR brachytherapy has led us to the investigation of the use of cisplatin during the PDR irradiation treatment.

Hyperthermia, a repair inhibitor, is known to sensitize A2780s and A2780cp to PDR (Niedbala *et al.*, 2001b). Concurrent cisplatin, also known as a repair inhibitor, and LDR treatments radiosensitized the A2780s and the A2780cp. The DMF ranged from 1.6 to 5.8 and greater radiosensitization was achieved in the A2780s (Raaphorst *et al.*, 1996).

Since there is repair between each pulse of radiation, and cisplatin is a known inhibitor of PLDR and SLDR, radiation sensitization would be expected if the drug is present during the entire treatment. Surprisingly, after normalizing the effect of cisplatin, resistance was observed that increased with the number of radiation pulses given to the cells.

In both the A2780s and the A2780cp cell lines, the same effect was seen at the 1 Gy/h PDR regimen. The effect of resistance was no longer seen when the 2 Gy/h pulses were used with the cisplatin treatment. At smaller pulses of radiation, it is possible that cisplatin selectively kills the more sensitive cells and leaves the more resistant cells that no longer respond to the radiation treatment. Since the cells are cross-resistant to both cisplatin and radiation, it is possible that resistant cells in the non-homogeneous population of cells reveal

themselves, after the sensitive cells are eliminated by the cisplatin treatment. The cells were plated after the PDR and cisplatin treatments and were grown for 3 days after which they were given an HDR irradiation treatment. The cells did not show any increase in radiation resistance. This would suggest that the radiation resistance was present for a short period of time and could not be seen three days after the treatment. It could also mean that there is no selection of a resistant subpopulation of cells.

Higher doses of cisplatin resulted in higher radiation resistance after 1 Gy/h PDR for up to 6 Gy total dose. To verify the selectivity of resistant cells hypothesis, it would be interesting to isolate A2780cp and A2780s cells one by one for cloning. After obtaining enough clones from each cell, a PDR (1 Gy/h) experiment, with concurrent cisplatin during treatment, could be performed. If the resistance was seen only in certain clones, it could be concluded that the response of the cells varies according to their genetic characteristics. However, if the response was the same, then the resistance may be attributed to cellular protection by cisplatin.

5.0 CONCLUSIONS

In summary, cisplatin did not radiosensitize the A2780s and A2780cp cells when given immediately before or after a radiation dose. The combination of cisplatin and radiation in both cell lines resulted in an additive effect. This additive effect could still be a valuable therapeutic gain in the treatment of cancer.

The radioresistance hypothesis was not confirmed in our study. When added 24 hours before radiation, cisplatin did not up-regulate the repair mechanisms of the cells, and therefore, did not increase the cell resistance to radiation at the time of the radiation dose.

The only observed radioresistance was for the fractionation scheme of 1 Gy/h, with cisplatin given simultaneously. This induced-radiation resistance may have a genetic origin and selection of a subpopulation of resistant cells could have occurred. It may also be the result of molecular protection by the cisplatin. For example, the cisplatin inter- and intrastrand links can stabilize the DNA, thereby facilitating the repair of radiation damage. Further studies are thus required in order to determine the actual cause of the observed radioresistance.

In conclusion, our experiments clearly demonstrate the many possible ways in which cisplatin and radiation can interact, and they confirm the importance of *in vitro* studies in the optimization of clinical treatments.

6.0 FUTURE WORKS

Further investigations should be conducted to see if there is selection by the cisplatin treatment of a resistant cell population that becomes enriched and resists radiation. It would be necessary to clone cells and derive a population of cells from one single cell. If the resistance is no longer seen for certain clones when cisplatin is added during PDR with the homogeneous population of the clone cells that would mean that the resistance seen with the heterogeneous population of cells is caused by selection of the resistant cells. If the resistance to radiation is observed with all the clones that would mean that other resistance mechanisms could be involved. One mechanism could be the interaction between the repair mechanisms. If cisplatin adduct repair mechanisms are up-regulated, it could prepare the cells for the subsequent radiation doses of radiation and render the cells more tolerant to that radiation treatment. Cisplatin could also hold the DNA together and keep the dsbs closer together for repair.

REFERENCES

Abbot, H. E., Raaphorst, P. G. (2002). Comparison of Repair and Rejoining Fidelity Between Two Idogenic Human Ovarian Carcinoma Cell Lines. *Int. J. Radiat. Bio.* In press.

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* **15**, 2177-2196.

Akaboshi, M., Kawai, K., Maki, H., Akuta, K., Ujeno, Y., & Miyahara, T. (1992). The number of platinum atoms binding to DNA, RNA and protein molecules of HeLa cells treated with cisplatin at its mean lethal concentration. *Jpn.J.Cancer Res.* **83**, 522-526.

Barlow, C., Brown, K. D., Deng, C. X., Tagle, D. A., & Wynshaw-Boris, A. (1997). Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. *Nat.Genet.* **17**, 453-456.

Bedford, J. S. (1991). Sublethal damage, potentially lethal damage, and chromosomal aberrations in mammalian cells exposed to ionizing radiations. *Int.J.Radiat.Oncol.Biol.Phys.* **21**, 1457-1469.

Begg, A. C. (1990). Cisplatin and radiation: interaction probabilities and therapeutic possibilities. *Int.J.Radiat.Oncol.Biol.Phys.* **19**, 1183-1189.

Begg, A. C., van der Kolk, P. J., Dewit, L., & Bartelink, H. (1986). Radiosensitization by cisplatin of RIF1 tumour cells in vitro. *Int.J.Radiat.Biol.Relat Stud.Phys.Chem.Med.* **50**, 871-884.

Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., & Ozols, R. F. (1987a). Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.* **47**, 414-418.

Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., & Ozols, R. F. (1987b). Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.* **47**, 414-418.

Britten, R. A., Kuny, S., & Perdue, S. (1999a). Modification of non-conservative double-strand break (DSB) rejoining activity after the induction of cisplatin resistance in human tumour cells. *Br.J.Cancer* **79**, 843-849.

Britten, R. A., Kuny, S., & Perdue, S. (1999b). Modification of non-conservative double-strand break (DSB) rejoining activity after the induction of cisplatin resistance in human tumour cells. *Br.J.Cancer* **79**, 843-849.

Britten, R. A., Liu, D., Kuny, S., & Allalunis-Turner, M. J. (1997). Differential level of DSB repair fidelity effected by nuclear protein extracts derived from radiosensitive and radioresistant human tumour cells. *Br.J.Cancer* **76**, 1440-1447.

Brizel, D. M., Albers, M. E., Fisher, S. R., Scher, R. L., Richtsmeier, W. J., Hars, V., George, S. L., Huang, A. T., & Prosnitz, L. R. (1998). Hyperfractionated irradiation with or without concurrent chemotherapy for locally advanced head and neck cancer. *N.Engl.J.Med.* **338**, 1798-1804.

Brown, R., Hirst, G. L., Gallagher, W. M., McIlwrath, A. J., Margison, G. P., van der Zee, A. G., & Anthoney, D. A. (1997). hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents. *Oncogene* **15**, 45-52.

Brown, S. J., Kellett, P. J., & Lippard, S. J. (1993). Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. *Science* **261**, 603-605.

Carde, P. & Laval, F. (1981) Effect of cis-dichlorodiammine platinum II and x rays on mammalian cell survival. *Int. J. Radiat. Oncol. Biol. Phys.* **7**, 929-933.

Cardenes, H. & Randall, M. E. (2000). Integrating radiation therapy in the curative management of ovarian cancer: current issues and future directions. *Semin.Radiat.Oncol.* **10**, 61-70.

Chadwick, K. H., Leenhouts, H. P., Szumiel, I., & Nias, A. H. (1976). An analysis of the interaction of a platinum complex and radiation with CHO cells using the molecular theory of cell survival. *Int.J.Radiat.Biol.Relat Stud.Phys.Chem.Med.* **30**, 511-524.

Chougule, P. B., Suk, S., Chu, Q. D., Leone, L., Nigri, P. T., McRae, R., Lekas, M., Barone, A., Bhat, D., & Bellino, J. (1994). Cisplatin as a radiation sensitizer in

the treatment of advanced head and neck cancers. Results of a phase II study. *Cancer* **74**, 1927-1932.

Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985). In vivo effects of cis- and trans-diamminedichloroplatinum(II) on SV40 chromosomes: differential repair, DNA-protein cross-linking, and inhibition of replication. *Biochemistry* **24**, 7533-7540.

Cohen, S. M. & Lippard, S. J. (2001b). Cisplatin: from DNA damage to cancer chemotherapy. *Prog.Nucleic Acid Res.Mol.Biol.* **67**, 93-130.

Cohen, S. M. & Lippard, S. J. (2001a). Cisplatin: from DNA damage to cancer chemotherapy. *Prog.Nucleic Acid Res.Mol.Biol.* **67**, 93-130.

Cromie, G. A., Connelly, J. C., & Leach, D. R. (2001). Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. *Mol.Cell* **8**, 1163-1174.

Crul, M., Schellens, J. H., Beijnen, J. H., & Maliepaard, M. (1997). Cisplatin resistance and DNA repair. *Cancer Treat.Rev.* **23**, 341-366.

Dabholkar, M., Vionnet, J., Bostick-Bruton, F., Yu, J. J., & Reed, E. (1994). Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J.Clin.Invest* **94**, 703-708.

De Pooter, C. M., Scalliet, P. G., Elst, H. J., Huybrechts, J. J., Gheuens, E. E., Van Oosterom, A. T., Fichtinger-Schepman, A. M., & De Bruijn, E. A. (1991). Resistance patterns between cis-diamminedichloroplatinum(II) and ionizing radiation. *Cancer Res.* **51**, 4523-4527.

de Pree, C., Popowski, Y., Weber, D., Nouet, P., Rouzaud, M., & Kurtz, J. M. (1999). Feasibility and tolerance of pulsed dose rate interstitial brachytherapy. *Int.J.Radiat.Oncol.Biol.Phys.* **43**, 971-976.

Dewit, L. (1987). Combined treatment of radiation and cisdiamminedichloroplatinum (II): a review of experimental and clinical data. *Int.J.Radiat.Oncol.Biol.Phys.* **13**, 403-426.

Dijt, F. J., Fichtinger-Schepman, A. M., Berends, F., & Reedijk, J. (1988).

Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. *Cancer Res.* **48**, 6058-6062.

Dolling, J. A., Boreham, D. R., Brown, D. L., Raaphorst, G. P., & Mitchel, R. E. (1999). Cisplatin-modification of DNA repair and ionizing radiation lethality in yeast, Saccharomyces cerevisiae. *Mutat.Res.* **433**, 127-136.

Douple, E. B. & Richmond, R. C. (1978). Platinum complexes as radiosensitizers of hypoxic mammalian cells. *Br.J.Cancer Suppl* **37**, 98-102.

Douple, E. B. The Use of Platinum Chemotherapy to Potentiate Radiotherapy. *Platinum Metals Review* **29**, 118-125. 1985.

Dritschilo, A., Piro, A. J., & Kelman, A. D. (1979). The effect of cis-platinum on the repair of radiation damage in plateau phase Chinese hamster (V-79) cells. *Int.J.Radiat.Oncol.Biol.Phys.* **5**, 1345-1349.

Eguchi-Kasai, K., Kosaka, T., Sato, K., & Kaneko, I. (1991). Reparability of DNA double-strand breaks and radiation sensitivity in five mammalian cell lines. *Int.J.Radiat.Biol.* **59**, 97-104.

Eliopoulos, A. G., Kerr, D. J., Herod, J., Hodgkins, L., Krajewski, S., Reed, J. C., & Young, L. S. (1995). The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene* **11**, 1217-1228.

Fehlauer, F., Barten-Van Rijbroek, A. D., Stalpers, L. J., Leenstra, S., Lindeman, J., Tjahja, I., Troost, D., Wolbers, J. G., van, d., V, & Sminia, P. (2000). Additive cytotoxic effect of cisplatin and X-irradiation on human glioma cell cultures derived from biopsy-tissue. *J.Cancer Res.Clin.Oncol.* **126**, 711-716.

Ferry, K. V., Hamilton, T. C., & Johnson, S. W. (2000). Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF. *Biochem.Pharmacol.* **60**, 1305-1313.

Fink, D., Aebi, S., & Howell, S. B. (1998). The role of DNA mismatch repair in drug resistance. *Clin.Cancer Res.* **4**, 1-6.

Fink, D., Zheng, H., Nebel, S., Norris, P. S., Aebi, S., Lin, T. P., Nehme, A., Christen, R. D., Haas, M., MacLeod, C. L., & Howell, S. B. (1997). In vitro and in

vivo resistance to cisplatin in cells that have lost DNA mismatch repair. *Cancer Res.* **57**, 1841-1845.

Flentje, M., Eble, M., Haner, U., Trinh, S., & Wannenmacher, M. (1992). Additive effects of cisplatin and radiation in human tumour cells under oxic conditions. *Radiother.Oncol.* **24**, 60-63.

Fountzilas, G., Athanassiadis, A., Nikolaou, A., Kalogera-Fountzila, A., Tzitzikas, J., Samantas, E., Skarlos, D., Zamboglou, N., & Daniilidis, J. (1997). Accelerated bifractionated radiation with concurrent cisplatin administration in locally advanced head and neck cancer: a feasibility study. *Tumouri* **83**, 735-739.

Fowler, J. F. (2001). Biological factors influencing optimum fractionation in radiation therapy. *Acta Oncol.* **40**, 712-717.

Frankenberg-Schwager, M. (1989). Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. *Radiother.Oncol.* **14**, 307-320.

Frankenberg-Schwager, M. & Frankenberg, D. (1990). DNA double-strand breaks: their repair and relationship to cell killing in yeast. *Int.J.Radiat.Biol.* **58**, 569-575.

Freshney R.I. (1994) Culture of Animal Cells 3rd Edition. New York, Wiley-Liss. 153-154.

Furuse, K., Fukuoka, M., Kawahara, M., Nishikawa, H., Takada, Y., Kudoh, S., Katagami, N., & Ariyoshi, Y. (1999). Phase III study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine, and cisplatin in unresectable stage III non-small-cell lung cancer. *J.Clin.Oncol.* **17**, 2692-2699.

Gallagher, W. M., Cairney, M., Schott, B., Roninson, I. B., & Brown, R. (1997). Identification of p53 genetic suppressor elements which confer resistance to cisplatin. *Oncogene* **14**, 185-193.

Gandara, D. R., Edelman, M., Lara, P., Roberts, P., & Leigh, B. (2000). Evolution of combined modality therapy for stage III non-small-cell lung cancer. *Oncology (Huntingt)* **14**, 35-41.

Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., & Anderson, M. E. (1992). High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc.Natl.Acad.Sci.U.S.A* **89**, 3070-3074.

Hall, E. J. (1994). Radiobiology for the radiobiologist. J. B. Lippincott Company, Philadelphia. p1-13; p16-26; p29-43.

Hamaguchi, K., Godwin, A. K., Yakushiji, M., O'Dwyer, P. J., Ozols, R. F., & Hamilton, T. C. (1993). Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. *Cancer Res.* **53**, 5225-5232.

Harrison, L. B., Raben, A., Pfister, D. G., Zelefsky, M., Strong, E., Shah, J. P., Spiro, R. H., Shaha, A., Kraus, D. H., Schantz, S. P., Carper, E., Bodansky, B., White, C., & Bosl, G. (1998). A prospective phase II trial of concomitant chemotherapy and radiotherapy with delayed accelerated fractionation in unresectable tumours of the head and neck. *Head Neck* **20**, 497-503.

Henkels, K. M. & Turchi, J. J. (1997). Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cell lines. *Cancer Res.* **57**, 4488-4492.

Henkels, K. M. & Turchi, J. J. (1999). Cisplatin-induced apoptosis proceeds by caspase-3-dependent and - independent pathways in cisplatin-resistant and - sensitive human ovarian cancer cell lines. *Cancer Res.* **59**, 3077-3083.

Hill, B. T., Moran, E., Etievant, C., Perrin, D., Masterson, A., Larkin, A., & Whelan, R. D. (2000). Low-dose twice-daily fractionated X-irradiation of ovarian tumour cells in vitro generates drug-resistant cells overexpressing two multidrug resistance-associated proteins, P-glycoprotein and MRP1. *Anticancer Drugs* **11**, 193-200.

Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J., & Sancar, A. (1994a). HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc.Natl.Acad.Sci.U.S.A* **91**, 10394-10398.

Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J., & Sancar, A. (1994b). HMG-domain proteins specifically inhibit the repair of the major DNA adduct of

the anticancer drug cisplatin by human excision nuclease. *Proc.Natl.Acad.Sci.U.S.A* **91**, 10394-10398.

Jeggo, P. A. (1998). DNA breakage and repair. *Adv.Genet.* **38**, 185-218.

Johnson, S. W., Perez, R. P., Godwin, A. K., Yeung, A. T., Handel, L. M., Ozols, R. F., & Hamilton, T. C. (1994). Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. *Biochem.Pharmacol.* **47**, 689-697.

Jordan, P. & Carmo-Fonseca, M. (2000). Molecular mechanisms involved in cisplatin cytotoxicity. *Cell Mol.Life Sci.* **57**, 1229-1235.

Keys, H. M., Bundy, B. N., Stehman, F. B., Muderspach, L. I., Chafe, W. E., Suggs, C. L., III, Walker, J. L., & Gersell, D. (1999). Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. *N.Engl.J.Med.* **340**, 1154-1161.

King, L. A., Downey, G. O., Potish, R. A., Adcock, L. L., Carson, L. F., & Twiggs, L. B. (1991). Concomitant whole-abdominal radiation and intraperitoneal chemotherapy in advanced ovarian carcinoma. A pilot study. *Cancer* **67**, 2867-2871.

Kirchgessner, C. U., Patil, C. K., Evans, J. W., Cuomo, C. A., Fried, L. M., Carter, T., Oettinger, M. A., & Brown, J. M. (1995). DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science* **267**, 1178-1183.

Lai, G. M., Ozols, R. F., Smyth, J. F., Young, R. C., & Hamilton, T. C. (1988a). Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. *Biochem.Pharmacol.* **37**, 4597-4600.

Lai, G. M., Ozols, R. F., Smyth, J. F., Young, R. C., & Hamilton, T. C. (1988b). Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. *Biochem.Pharmacol.* **37**, 4597-4600.

Landis, S. H., Murray, T., Bolden, S., & Wingo, P. A. (1998). Cancer statistics, 1998. *CA Cancer J.Clin.* **48**, 6-29.

Le Chevalier, T., Arriagada, R., Quoix, E., Ruffie, P., Martin, M., Tarayre, M.,

Lacombe-Terrier, M. J., Douillard, J. Y., & Laplanche, A. (1991). Radiotherapy alone versus combined chemotherapy and radiotherapy in nonresectable non-small-cell lung cancer: first analysis of a randomized trial in 353 patients. *J.Natl.Cancer Inst.* **83**, 417-423.

Lee, S. E., Mitchell, R. A., Cheng, A., & Hendrickson, E. A. (1997). Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. *Mol.Cell Biol.* **17**, 1425-1433.

Lehnert, S. & Chow, T. Y. (1997). Low doses of ionizing radiation induce nuclear activity in human tumour cell lines which catalyzes homologous double-strand recombination. *Radiat.Environ.Biophys.* **36**, 67-70.

Leskov, K. S., Criswell, T., Antonio, S., Li, J., Yang, C. R., Kinsella, T. J., & Boothman, D. A. (2001). When X-ray-inducible proteins meet DNA double strand break repair. *Semin.Radiat.Oncol.* **11**, 352-372.

Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J., & McKay, B. C. (1999). Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene* **18**, 583-592.

Marples, B. & Joiner, M. C. (1993). The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. *Radiat.Res.* **133**, 41-51.

Mello, J. A., Lippard, S. J., & Essigmann, J. M. (1995). DNA adducts of cis-diamminedichloroplatinum(II) and its trans isomer inhibit RNA polymerase II differentially in vivo. *Biochemistry* **34**, 14783-14791.

Morris, M., Eifel, P. J., Lu, J., Grigsby, P. W., Levenback, C., Stevens, R. E., Rotman, M., Gershenson, D. M., & Mutch, D. G. (1999). Pelvic radiation with concurrent chemotherapy compared with pelvic and para-aortic radiation for high-risk cervical cancer. *N.Engl.J.Med.* **340**, 1137-1143.

Mymryk, J. S., Zaniewski, E., & Archer, T. K. (1995). Cisplatin inhibits chromatin remodeling, transcription factor binding, and transcription from the mouse mammary tumour virus promoter in vivo. *Proc.Natl.Acad.Sci.U.S.A* **92**, 2076-2080.

Niedbala, M., Alsbeih, G., Ng, C. E., & Raaphorst, G. P. (2001a). Equivalence of

pulsed-dose-rate to low-dose-rate irradiation in tumour and normal cell lines. *Radiat.Res.* **155**, 297-303.

Niedbala, M., Ng, C. E., & Raaphorst, G. P. (2001b). Response to pulsed dose rate irradiation with and without mild hyperthermia using tumour and normal cell lines. *Int.J.Hyperthermia* **17**, 536-544.

Olive, P. L., Banath, J. P., & MacPhail, H. S. (1994). Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumour cell lines. *Cancer Res.* **54**, 3939-3946.

Perego, P., Giarola, M., Righetti, S. C., Supino, R., Caserini, C., Delia, D., Pierotti, M. A., Miyashita, T., Reed, J. C., & Zunino, F. (1996). Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res.* **56**, 556-562.

Pomp, J., Woudstra, E. C., & Kampinga, H. H. (1999). Pulsed-dose-rate and low-dose-rate brachytherapy: comparison of sparing effects in cells of a radiosensitive and a radioresistant cell line. *Radiat.Res.* **151**, 449-453.

Poppenborg, H., Munstermann, G., Knupfer, M. M., Hotfilder, M., Hacker-Klom, U., & Wolff, J. E. (1997). Cisplatin induces radioprotection in human T98G glioma cells. *Anticancer Res.* **17**, 1131-1134.

Prestayko A.W., Crooke S.T., & Carter S.K. (1980) Cisplatin Current Status and New Developments. New York, Academic Press. 1-527.

Raaphorst, G. P., Miao, J., & Ng, C. E. (1997). Cisplatin and mild hyperthermia in radiosensitization to low dose rate irradiation in human ovarian carcinoma cells. *Anticancer Res.* **17** , 3469-3472.

Raaphorst, G. P., Ng, C. E., & Shahine, B. (1999). Comparison of radiosensitization by 41 degrees C hyperthermia during low dose rate irradiation and during pulsed simulated low dose rate irradiation in human glioma cells. *Int.J.Radiat.Oncol.Biol.Phys.* **44**, 185-188.

Raaphorst, G. P., Wang, G., Stewart, D., & Ng, C. E. (1996). Concomitant low dose-rate irradiation and cisplatin treatment in ovarian carcinoma cell lines sensitive and resistant to cisplatin treatment. *Int.J.Radiat.Biol.* **69**, 623-631.

Raaphorst, G. P., Wang, G., Wilkins, D., Davis, L., Bussey, A., Stewart, D., & Ng, C. E. (1994). Evaluation of cisplatin response in three radiation resistant and three radiation sensitive cell lines. *Anticancer Res.* **14**, 1939-1942.

Raaphorst, G. P., Wang, G., & Ng, C. E. A study of cisplatin-radiosensitization through inhibition of repair of sublethal radiation damage in ovarian carcinoma cells sensitive and resistant to cisplatin. *Int.J.Oncol.* **7**, 1373-1378. 1995a

Raaphorst, G. P., Wang, G., & Ng, C. E. Radiosensitization by cisplatin treatment in cisplatin resistant and sensitive human ovarian carcinoma cell lines. *Int.J.Oncol.* **7**, 325-330. 1995b.

Robbins, K. T., Kumar, P., Regine, W. F., Wong, F. S., Weir, A. B., III, Flick, P., Kun, L. E., Palmer, R., Murry, T., Fontanesi, J., Ferguson, R., Thomas, R., Hartsell, W., Paig, C. U., Salazar, G., Norfleet, L., Hanchett, C. B., Harrington, V., & Niell, H. B. (1997). Efficacy of targeted supradose cisplatin and concomitant radiation therapy for advanced head and neck cancer: the Memphis experience. *Int.J.Radiat.Oncol.Biol.Phys.* **38**, 263-271.

Rose, P. G., Bundy, B. N., Watkins, E. B., Thigpen, J. T., Deppe, G., Maiman, M. A., Clarke-Pearson, D. L., & Insalaco, S. (1999). Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N.Engl.J.Med.* **340**, 1144-1153.

Rosen, E. M., Fan, S., Rockwell, S., & Goldberg, I. D. (1999). The molecular and cellular basis of radiosensitivity: implications for understanding how normal tissues and tumours respond to therapeutic radiation. *Cancer Invest* **17**, 56-72.

Sasai, K., Evans, J. W., Kovacs, M. S., & Brown, J. M. (1994). Prediction of human cell radiosensitivity: comparison of clonogenic assay with chromosome aberrations scored using premature chromosome condensation with fluorescence in situ hybridization. *Int.J.Radiat.Oncol.Biol.Phys.* **30**, 1127-1132.

Scalliet, P., De Pooter, C., Hellemans, P. W., De Bruijn, E. A., & Van Oosterom, A. T. (1999). [Interactions of carboplatin, cisplatin, and ionizing radiation on a human cell line of ovarian cancer]. *Cancer Radiother.* **3**, 30-38.

Sharma, V. M. & Wilson, W. R. (1999). Radiosensitization of advanced squamous cell carcinoma of the head and neck with cisplatin during concomitant radiation therapy. *Eur.Arch.Otorhinolaryngol.* **256**, 462-465.

Shiloh, Y. (2001). ATM (ataxia telangiectasia mutated): expanding roles in the DNA damage response and cellular homeostasis. *Biochem.Soc.Trans.* **29**, 661-666.

Skov, K. A., MacPhail, H. S., & Marples, B. (1994). The effect of radiosensitizers on the survival response of hypoxic mammalian cells: the low X-ray dose region, hypersensitivity and induced radioresistance. *Radiat.Res.* **138**, S113-S116.

Smith, G. C. & Jackson, S. P. (1999). The DNA-dependent protein kinase. *Genes Dev.* **13**, 916-934.

Stratford, I. J., Williamson, C., & Adams, G. E. (1980). Combination studies with misonidazole and a cis-platinum complex: cytotoxicity and radiosensitization in vitro. *Br.J.Cancer* **41** , 517-522.

Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., & Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J.* **17**, 5497-5508.

Thomas, G. M. & Dembo, A. J. (1993). Integrating radiation therapy into the management of ovarian cancer. *Cancer* **71**, 1710-1718.

Vikhanskaya, F., Colella, G., Valenti, M., Parodi, S., D'Incalci, M., & Broggini, M. (1999). Cooperation between p53 and hMLH1 in a human colocarcinoma cell line in response to DNA damage. *Clin.Cancer Res.* **5**, 937-941.

Vikhanskaya, F., Marchini, S., Marabese, M., Galliera, E., & Broggini, M. (2001). P73a overexpression is associated with resistance to treatment with DNA-damaging agents in a human ovarian cancer cell line. *Cancer Res.* **61**, 935-938.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature* **408**, 307-310.

Wallace, S. S. (1998). Enzymatic processing of radiation-induced free radical damage in DNA. *Radiat.Res.* **150**, S60-S79.

Weinfeld, M., Rasouli-Nia, A., Chaudhry, M. A., & Britten, R. A. (2001). Response

of base excision repair enzymes to complex DNA lesions. *Radiat.Res.* **156**, 584-589.

Wendt, T. G., Grabenbauer, G. G., Rodel, C. M., Thiel, H. J., Aydin, H., Rohloff, R., Wustrow, T. P., Iro, H., Popella, C., & Schalhorn, A. (1998). Simultaneous radiochemotherapy versus radiotherapy alone in advanced head and neck cancer: a randomized multicenter study. *J.Clin.Oncol.* **16**, 1318-1324.

Wilkins, D. E., Ng, C. E., & Raaphorst, G. P. (1997). Cell cycle perturbations in cisplatin-sensitive and resistant human ovarian carcinoma cells following treatment with cisplatin and low dose rate irradiation. *Cancer Chemother.Pharmacol.* **40**, 159-166.

Wong, R., Milosevic, M., Sturgeon, J., Pintilie, M., Fyles, A., Levin, W., Rosen, B., Depetrillo, D., Oza, A., Manchul, L., Murphy, J., & Chapman, W. (1999). Treatment of early epithelial ovarian cancer with chemotherapy and abdominopelvic radiotherapy: results of a prospective treatment protocol. *Int.J.Radiat.Oncol.Biol.Phys.* **45**, 657-665.

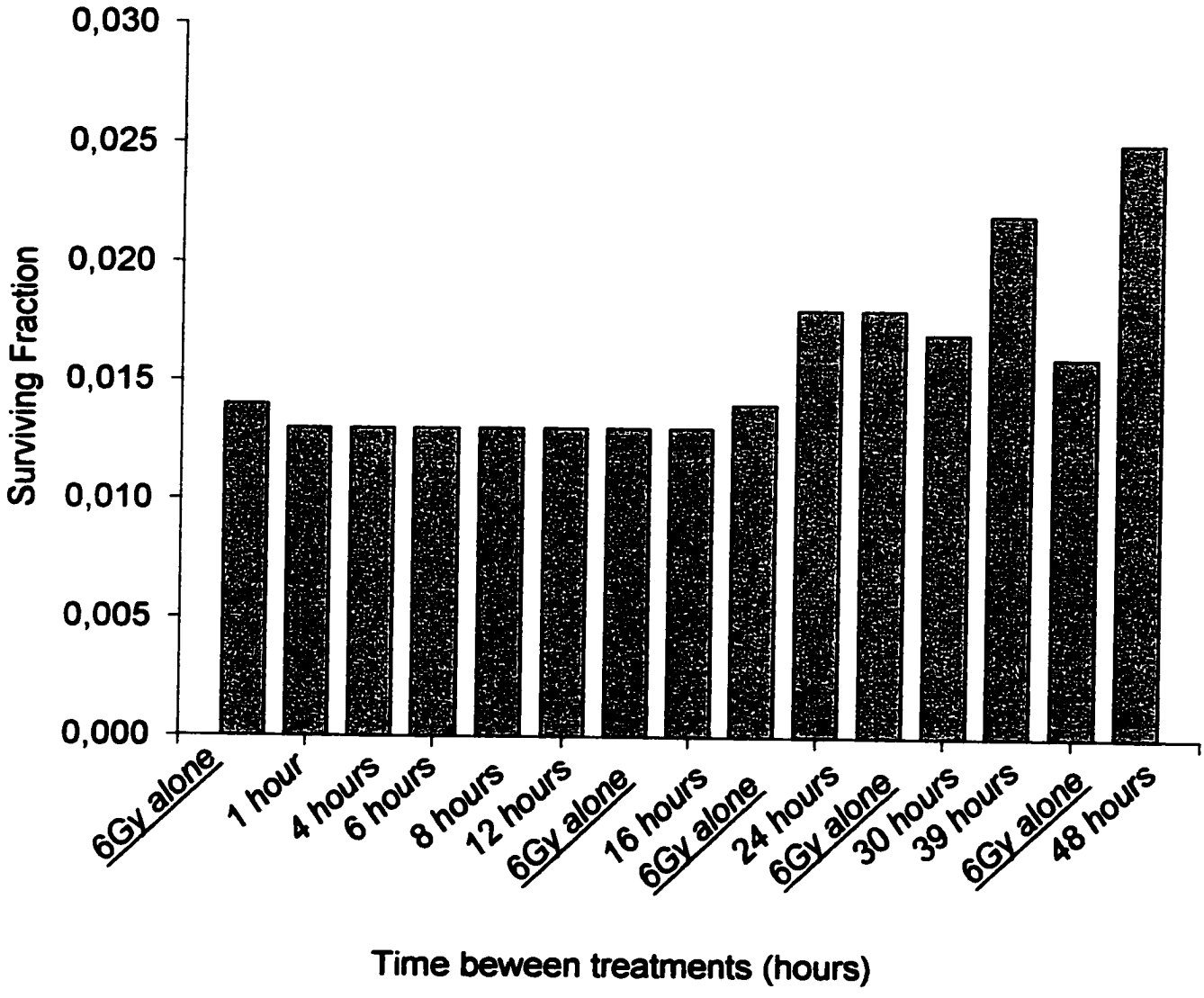
Xu, Y. & Baltimore, D. (1996). Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev.* **10**, 2401-2410.

Yapp, D. T., Lloyd, D. K., Zhu, J., & Lehnert, S. M. (1998). The potentiation of the effect of radiation treatment by intratumoural delivery of cisplatin. *Int.J.Radiat.Oncol.Biol.Phys.* **42**, 413-420.

Zamble, D. B., Mu, D., Reardon, J. T., Sancar, A., & Lippard, S. J. (1996a). Repair of cisplatin--DNA adducts by the mammalian excision nuclease. *Biochemistry* **35**, 10004-10013.

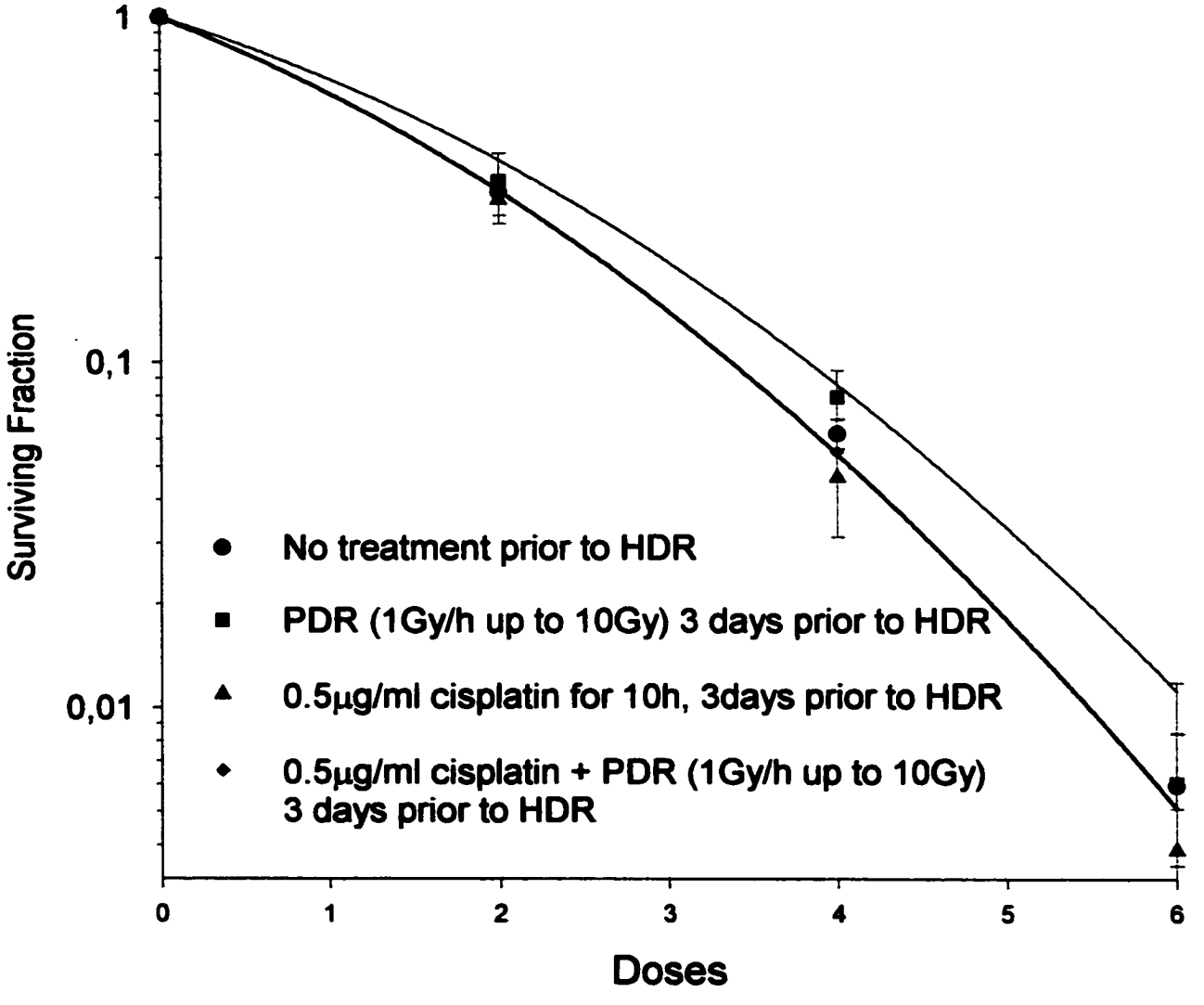
Zamble, D. B., Mu, D., Reardon, J. T., Sancar, A., & Lippard, S. J. (1996b). Repair of cisplatin--DNA adducts by the mammalian excision nuclease. *Biochemistry* **35**, 10004-10013.

APPENDIX 1



The A2780cp cells were exposed to 1μg/ml cisplatin for 1 hour at different time intervals before a 6 Gy doses. The experiment was done once.

APPENDIX 2



A2780s HDR survival 3 days after treatment with 0.5 µg/ml cisplatin during PDR (1 Gy/h up to 10 Gy), PDR (1 Gy/h up to 10 Gy) alone and 0.5 µg/ml cisplatin for 10 hours. The HDR survival curve represents an average of 2 experiments, and the error bars represent the SEM between the two experiments.