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**A comparison of pulsed dose rate to low dose rate irradiation
with/without mild hyperthermia using three human cell lines**

by

Malgorzata Niedbala, BSc

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of

Master of Science

Department of Physics
Carleton University
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Ottawa, Ontario
November 9, 1998
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acceptance of the thesis,

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Chair, Department of Physics

Thesis Supervisor

Carleton University
December 14, 1998

ABSTRACT

Computer-controlled radiotherapy technology has motivated the investigation of pulsed dose rate as a replacement for conventional low dose rate treatment. To determine whether different pulsed dose rate schemes could simulate low dose rate irradiation, radiosensitive and radioresistant ovarian carcinoma cells, and normal human fibroblasts were irradiated *in vitro* using different fraction sizes, 0.53 Gy, 1.1 Gy, 1.6 Gy, 2.1 Gy, and 3.2 Gy, and their corresponding time intervals, 1h, 2h, 3h, 4h, and 6h. Selected fraction sizes, 0.53 Gy and 1.6 Gy, in combination with mild hyperthermia were also investigated to determine if radiosensitization due to hyperthermia occurred. The survival of the cells was assessed using the clonogenic assay. The data showed that for the resistant cell line the shorter fractionation schemes were equivalent to low dose rate, while larger fractionation schemes had lower survivals than that for low dose rate, suggesting incomplete repair of radiation damage. The sensitive cell line showed this equivalence only with the 1.6 Gy fraction size. The normal cell line showed no equivalence between any of its pulsed dose rate schemes and low dose rate. For the pulsed dose rate experiments with mild hyperthermia, it was determined that hyperthermia did not radiosensitize the cells for the 0.53 Gy given every 1h fractionation scheme used, while it radiosensitized the carcinoma cell lines when using the 1.6 Gy fraction size, but not the normal cell line. This resulted in a potential clinical advantage for using the 1.6 Gy fraction size with mild hyperthermia.

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LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
Gy	Gray, SI unit for absorbed dose [1 Gy = 1 J/kg]
HCl	Hydrochloric acid
HDR	High dose rate
LDR	Low dose rate
LQ	Linear-quadratic (model)
mM	Millimolar
PBS	Phosphate buffered saline
PDR	Pulsed dose rate
PE	Plating efficiency
RNA	Ribonucleic acid
SEM	Standard error of the mean
SLD	Sublethal damage
SF2	Surviving fraction after 2 Gy
T _{1/2}	Repair half-time

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1.0 INTRODUCTION

A wide range of dose-rates has been used in radiation biology and radiation therapy, extending from a few cGy per day to hundreds of Gy in a fraction of a second. The dose rate of importance in radiotherapy extends from about 0.1 Gy/h to several Gy/min [1]. Clinical radiotherapy is concerned largely with either high dose rate (HDR) for external beam therapy or low dose rate (LDR) for interstitial and intracavitary therapy. This study investigates both types of dose rates and a new type of treatment, called pulsed dose rate (PDR), using human cells *in vitro*.

1.1 Low Dose Rate

Low dose rate (LDR) is the dose rate used in clinical brachytherapy. Brachytherapy (from Greek “short range” therapy) is a technique in which radioactive sources are placed either in body cavities in close proximity to the tumour (intracavitary) or they are implanted directly into the tumour volume (interstitial). This has a great advantage over external beam irradiation in that the malignant tissue receives a very high dose while allowing normal tissue sparing. A commonly used dose in brachytherapy is 50 to 70 Gy in 5 to 9 days. Total dose should be adjusted for dose rate, because clinical studies show that both tumour control and effects on normal tissues vary with dose rate for a given total dose [2]. A conventional brachytherapy treatment protocol uses typically 0.4 to 2 Gy/h dose rate, the limiting factor being normal tissue tolerance, which should be fully used to maximize the possibility of tumour control. However, the maximum dose that can be delivered with acceptable damage to surrounding normal tissues depends not only

on dose rate, but also on the difference in the rate of repair of sublethal damage between malignant and normal tissues [1]. Sublethal damage (SLD) is damage that under normal circumstances can be repaired unless additional sublethal damage is added (from a second dose of radiation) with which it can interact to produce lethal damage, which is irreparable [2-4]. Sublethal damage repair occurs during low dose rate irradiation and between fractions of a pulsed dose rate treatment. This SLD repair results in the dose-rate effect, which is very important in radiotherapy. The dose-rate effect and hence SLD and SLD repair are best observed on the cell survival curve, which describes the relationship between the radiation dose and the proportion of cells that survive (Figure 1.1).

Figure 1.1. Sample survival curve

Qualitatively, the shape of the survival curve can be described in relatively simple terms. At low doses for sparsely ionizing radiation such as x-rays as in our case, the survival curve starts out straight (from zero to a few Gy in dose range) on the log-linear

plot with a finite initial slope; the surviving fraction is an exponential function of dose. At higher doses (3-6 Gy) the curve bends and then straightens out again at very high doses (over 8 Gy) [2]. It appears to be generally true that a large shoulder in the acute response (HDR irradiation) is associated with a substantial dose-rate effect, whereas a smaller shoulder is associated with a modest dose-rate effect. Therefore the shoulder on the acute dose response curve reflects the accumulation of sublethal damage, and the dose-rate effect reflects the repair of sublethal damage [1]. When the dose-rate is progressively reduced, the slope of the survival curve becomes progressively shallower as a greater proportion of SLD is repaired [1,5,6]. A limiting slope is reached, reflecting complete repair of sublethal damage, which is an extension of the initial slope of the acute dose survival curve. The dose-rate effect caused by repair of sublethal damage is most dramatic between 0.01 and 1 Gy/min, and its magnitude varies enormously between different types of cells [2].

1.2 Pulsed Dose Rate

Progress in the application of ionizing radiation to cancer therapy has often been a consequence of technical innovation [7]. Such is the case with pulsed dose rate (PDR) brachytherapy technique. With the advent of remote afterloaders, pulsed dose rate can be achieved by a machine which steps a single high-activity source through the catheters of an implant, with dwell positions and time adjusted under computer control to achieve the required dose distribution. The clinical advantages of using pulsed dose rate brachytherapy over conventional brachytherapy would be:

- 1) Easily controlled dose distribution – dose can be more precisely applied, and the dose distribution can be altered during the course of the treatment.
- 2) Radiation protection – reduced source manipulation, and the use of a single source instead of an inventory of sources, which would also add the benefit of decreased shielding and costs.
- 3) Equipment sharing – unlike low dose rate brachytherapy, where the sources are limited to one patient at a time (usually for at least a full day), pulsed dose rate would allow patients to share the source during their off intervals.
- 4) Convenience for the patient – more mobility during the off periods, during which visiting can be safely accomplished.

Pulsed dose rate consists of a series of short high dose rate pulses separated by equally spaced time intervals, such that the overall treatment time and average dose rate would be the same as that used in conventional low dose rate brachytherapy. This would, therefore, offer the radiobiological advantages of low dose rate (dose-rate effect and normal tissue sparing) if a combination of radiation pulse widths and time intervals that would result in equivalence to low dose rate could be found. However, there is need for caution, because late responding tissues (e.g. oligodendrocytes in the central nervous system, renal tubule cells in the kidney, etc., tissues in which radiation damage might not be evident until at least six months after treatment) are more sensitive to changes in fractionation patterns [8]. Also, although studies are directed at finding equivalence of PDR to LDR, ultimately, the treatment goal is to deliver as high a dose to the tumour as possible, while minimizing the damage to surrounding normal tissues.

The theoretical basis for comparison of LDR and PDR exposure schemes was originally proposed by Brenner and Hall [9]. In their 1991 paper they analyzed data from 36 cell lines of human origin and suggested that a pulsed regimen consisting of 0.5 Gy given in a 10-min pulse (using a dose rate of 3 Gy/h) and repeated every hour would be functionally equivalent to continuous low dose rate irradiation of 0.5 Gy/h. Another study that also used the linear quadratic parameters to predict optimum PDR schedules suggested that good equivalence can still be maintained even if pulses of 0.5 Gy are given in 10 minutes to half an hour, as often as once an hour [10]. This study implied that pulse repetition frequencies up to about 2 hours would meet the conditions for equivalence, even up to dose-rates of 3 Gy/h. A recent study agrees with these predictions, and it also suggests that longer pulse intervals of up to 3 hours could be used with PDR, still keeping the overall average dose rate at 0.5 Gy/h [11]. A longer time interval between fractions would offer a greater clinical advantage in terms of the number of patients being treated at one time with equipment sharing. *In vitro* data to verify these predictions experimentally has been published using different dose rates and different cell lines [7,12-14]. These studies were done mostly with rodent cell lines, and only one study compared responses to PDR using human cell lines. An early study with rat cells showed PDR-LDR equivalence using an overall average dose rate of 0.5 Gy/h for pulses repeated from half an hour up to 6 hours [7]. The dose rate of the pulses was 66 Gy/h, therefore the shortest pulse was given in less than one minute, not in 10 minutes as proposed in the original study. Increased cytotoxicity to the tumour cells was observed when increasing the fraction size and interfraction interval to 6 Gy given every 12 hours. This early study showed encouraging results for the clinical application, since a large interfraction period

of 6 hours would allow many patients to be treated at one time, and also it used a higher dose rate for the pulses, shortening the length of each pulse while still maintaining the equivalence. Another study with rodent cell lines compared the responses of cells in a monolayer and spheroids, which would better simulate an actual tumour [12]. This study used an overall dose rate of 0.94 Gy/h given in pulses of 0.94 Gy every 1 hour (pulse width duration of 4.4–5.2 min), and 3.76 Gy given every 4 hours. For the spheroid system the hourly scheme was indistinguishable from the low dose rate protocol, but for the PDR treatment every 4 hours more cytotoxicity was observed. The monolayer system, however, showed increased sensitivity to both PDR schedules. The difference in response is due to the fact that the cells used are less radiosensitive when grown as spheroids than when in a monolayer culture. Another *in vitro* study with a rodent cell line used a higher overall dose rate of 1.12 Gy/h [13]. This study investigated PDR regimens consisting of hourly radiation pulses of 5, 10, or 20 minutes and a 20-minute radiation pulse every 2 hours, resulting in greater mean cell kills than the LDR regimen, although not statistically different. From these last two studies, therefore, it does not appear that doubling the dose rate originally proposed would have significant effect on the PDR-LDR equivalence. Human tumour *in vitro* data using 0.6 Gy/h with varying pulse widths and time intervals (from every 1 hour up to 12 hours) given in 3.8 to 45.6 minute pulses shows that there is variability between cell lines in the PDR-LDR equivalence [14]. This variability, therefore, supports the initial conservative suggestion that hourly, or probably two hourly, pulses would be expected to result in effective equivalence between PDR and LDR for a wide spectrum of cell lines of human origin. *In vivo* studies with animal systems were used to investigate the PDR-LDR equivalence

with acute (jejunal stem cells) and late (cataract induction) responding tissues [15,8]. Data from the acute responding system shows that for an average dose rate of 0.7 Gy/h, the dose given in a 1-min hourly pulse was more effective than the 10-min pulse previously shown to be equivalent to the continuous LDR regimen. Increasing the dose rate in the pulse in the clinical situation would shorten the length of each pulse and therefore allow treatment of a greater number of patients. In the late responding tissue it was found that 10-min pulses each hour, 10-min pulses every 4 hours, and 100-s pulses every hour showed no significant differences from the LDR regimen, using an overall dose rate of 0.6 Gy/h. These *in vivo* studies, therefore, give credence to the notion that practical PDR treatments can be designed that are biologically equivalent to conventional LDR, not only in terms of early responses such as tumour control, but also in terms of late effects on normal tissues, which often are more serious.

1.3 Hyperthermia

Hyperthermia involves heating the body or a localized part of the body to temperatures above the normal 37°C. Regarding cancer therapy, based on a considerable body of data, it is generally agreed that local hyperthermia alone has no role in the curative treatment of tumours [2]. However, the use of hyperthermia in combination with radiation in the treatment of local tumours has demonstrated its effectiveness as a radiosensitizer [16-18]. One of the mechanisms of radiosensitization may be through the inhibition of sublethal damage repair [19-23]. In brachytherapy, where low dose rates are used, extensive repair of SLD occurs, therefore it is logical to predict that the effectiveness of low dose rate irradiation would be enhanced by the simultaneous

application of heat. In fact, some *in vitro* studies (primarily on rodent cells) show that hyperthermia is a more effective radiosensitizer when combined with LDR than with HDR [18,24-25]. Studies in animal tumours also show that there is potential for hyperthermia combined with brachytherapy, but with hyperthermia usually administered before or after irradiation [18]. One study showed that hyperthermia at the midpoint of the brachytherapy treatment was most effective, indicating the possible potential for simultaneous treatments [18]. Although many studies have demonstrated the effectiveness of hyperthermia when given at temperatures $> 42-43^{\circ}\text{C}$ [16-18,26], difficulties were experienced when heating many types of tumours above temperatures of 43°C and maintaining temperature uniformity throughout the tumour. However, recently it has been shown that mild hyperthermia ($39-41^{\circ}\text{C}$) can completely inhibit the sparing effect usually seen with low dose rate irradiation. Simultaneous mild hyperthermia and brachytherapy was shown to be very effective in several *in vitro* studies [18,25,27], and in recent human cancer therapy studies [19,28,29]. One study with human cells suggests that the degree of thermoradiosensitization may be inversely related to radiosensitivity. In this study, it was demonstrated that the most radioresistant cell line became the most radiosensitive when subjected to the combined treatment of mild hyperthermia and low dose rate irradiation, while the most radiosensitive cell line was the most unaffected by the combined treatments [18]. This shows a good potential for clinical application of such combined treatments.

From the evidence of the clinical potential of using mild hyperthermia in combination with low dose rate irradiation, the next step is to investigate the potential of these combined modalities on pulsed dose rate irradiation with a possible application to

clinical brachytherapy. If PDR demonstrates similar radiobiological advantages to LDR irradiation, especially the dose rate sparing effect and repair of sublethal damage, then in theory mild hyperthermia combined with PDR would also show the inhibition of repair of sublethal damage that is seen as with LDR. In the literature, there is only one such study using rodent cell lines [7]. It shows equivalence between LDR and PDR (0.5 Gy given every hour) at 37°C, and then it shows that 41°C hyperthermia combined with LDR and PDR maintains this equivalence, but at much lower survival levels. Therefore from this study, there is some evidence that mild hyperthermia may sensitize cells to pulsed dose rate irradiation in a similar way to that of mild hyperthermia combined with low dose rate irradiation.

1.4 The Linear Quadratic Model

In the past many models have been proposed to predict the total dose required to produce tolerable normal tissue injury. Empirical models such as nominal standard dose (NSD) and related formulas such as cumulative radiation effect (CRE) and time, dose, and fractionation (TDF) have been replaced by mechanistic models of radiation effect on DNA that better account for the differences between tumour and normal tissue reactions [30]. The literature contains a number of these mechanistic models, such as the Linear Quadratic Model [2], the Lethal and Potentially Lethal Model [31], the Saturable Repair Model [32], the Repair-Misrepair Model [33], etc. Although there is no widespread agreement on which of the numerous models of survival is best, the model most used in the literature to describe cell survival is the Linear Quadratic Model. On the other hand, a recent publication [34] has demonstrated that the LQ model and most other common

radiobiological models such as those mentioned above, result in similar predictions of time-dose relationships. However, in part because of the least number of parameters involved compared with the other models, and because the LQ formalism is now used almost universally in radiotherapy for calculating isoeffect doses for different fractionation/protraction schemes, the LQ model is the model used in the experiments in this study.

The LQ model was presented by Chadwick and Leenhouts to explain the effects of radiation on cell survival. It is based on the assumption that a double strand break in the DNA is the critical target for radiation damage. It assumes there are two components of cell killing by radiation, one of which is proportional to the dose (linear component, α), and the other proportional to the square of the dose (quadratic component, β) [2]. The linear component accounts for double strand breaks from a single ionizing event, and is considered to be irreparable. The quadratic component accounts for double strand breaks from two separate ionizing events, and is considered repairable if the dose is split into two fractions with enough time between them for repair of the first break to take place before the second break occurs [2]. A large β component therefore usually implies accumulation of sublethal damage and hence potential for repair if the dose is fractionated. This repairable component is also usually very small or nonexistent in low dose rate irradiation survival curves where ionizing events are spaced enough so that repair of single strand breaks takes place before a second break occurs.

The mathematical expression for the Linear Quadratic Model is as follows:

$$S.F. = \exp(-\alpha D - \beta D^2) \quad (1)$$

where S.F. is surviving fraction, D is the accumulated dose. At very low doses the linear term in the dose dominates, whereas at the higher doses the survival is dominated by the quadratic component. This model is proven to describe adequately high dose rate and low dose rate survival curves.

A very useful quantity to help calculate the contribution of unrepaired sublethal damage is the repair half-time ($T_{1/2}$), which describes the rate at which repair takes place [35]. The half-time for sublethal damage repair is defined as the time necessary to complete 50% of the maximally attainable repair, assuming mono-exponential kinetics. This parameter is used in the LQ formalism as described by Lea and Catcheside [6,9]. Their approach involves the Linear Quadratic Model as described by equation (1). This formalism states that if some time elapses between the passage of the first and second ionizing events, there exists a possibility of the first damaged site being repaired before interacting with the second. This repair will result in a reduction of the second, quadratic term in equation (1) by a factor denoted “G” by Lea and Catcheside [6,9]:

$$S.F. = \exp(-\alpha D - G\beta D^2) \quad (2)$$

This factor G depends on the temporal distribution of the dose, as well as on the time dependence of the function describing the repair of the damaged species:

$$G = \int_0^T h(t)\tau(t)dt \quad (3)$$

where T is the total time of the irradiation. Here h(t) describes the distribution of energy depositions in time, being the probability that two energy depositions are separated by a

time interval between t and $t+dt$. $\tau(t)$ is the recovery function for the elementary damaged sites; τ is taken as exponentially decreasing with time:

$$\tau(t) = \exp(-t/t_0) \quad (4)$$

where t_0 is a characteristic repair time (related to the repair half time as $T_{1/2} = 0.693t_0$).

The function $h(t)$ is related to the time dependent dose rate, $I(t)$ or $I(s)$ in the formula, by

$$h(t) = \frac{2}{D^2} \int_0^{T-t} I(s) I(s+t) ds \quad (5)$$

where D is total dose. Equation 5 and thus G can be calculated by a variety of mathematical methods. The simplest case is a constant dose rate, where

$$G = 2(t_0/T)^2 [T/t_0 - 1 + e^{-T/t_0}] \quad (6)$$

where T is total treatment time. This can be used with low dose rate data.

For a general fractionation case of N equal fractions each of duration c and period b between fractions, the function G becomes:

$$G = \frac{2}{(Nc)^2} \left[Nt_0c + Nt_0^2 \left(1 - \frac{1}{x} \right) + t_0^2 \left(x + \frac{1}{x} - 2 \right) \left\{ \frac{N}{1-y} - \frac{y(1-y^N)}{(1-y)^2} \right\} \right] \quad (7)$$

where $x = \exp(-c/t_0)$ and $y = \exp(-b/t_0)$. This formalism, therefore, was used in fitting pulsed dose rate survival curves to determine the parameters α , β , and t_0 . A limitation of this model to note is that it assumes no change in radiosensitivity between fractions, and no cell cycle redistribution effects.

1.4.1 Radiosensitivity Parameters

Estimated parameters play an important role in comparing radiosensitivities between cell types and treatment modalities, especially those parameters derived from the Linear Quadratic model [36]. To measure radiosensitivity, the following endpoints have been used: the initial slope of the survival curve, α , from the linear quadratic model, and the surviving fraction after 2 Gy (SF2). With the exception of highly radiosensitive cell lines, the parameter α tends to have values ranging from 0.2 Gy^{-1} for the more radioresistant cell lines to 0.6 Gy^{-1} for the more radiosensitive, with a mean of about 0.35 Gy^{-1} [37,38]. The SF2 is a useful parameter since it has been found that the *in vitro* radiosensitivity of human tumour cells correlates with the clinical response characteristics of the various tumour types. This parameter has values ranging from 0.5 for the more radioresistant cell lines to 0.15 for the most radiosensitive. Therefore the radiosensitivities of the three cell lines (A270s, A2780cp, and AG1522) have been compared using the SF2 for the high dose rate and low dose rate irradiations.

1.5 Hypothesis and Specific Aims

The hypothesis of this thesis is that pulsed dose rate can be equivalent to conventional low dose rate, in combination with or without mild hyperthermia, as determined from cell survival curves using clonogenic assay.

Specific aims are:

- Determine the intrinsic radiosensitivities of three cell lines using high dose rate and low dose rate irradiation. Fit the responses with the linear quadratic model and analyze the radiosensitivity parameters and the sublethal damage component.

- Establish the response of the three cell lines to pulsed dose rate irradiation using five different pulse widths and time intervals. Use the linear quadratic model to fit the responses, and compare the results from the fits to the fit for low dose rate. Using a statistical test, determine for which pulse widths and interfraction intervals the equivalence between low dose rate and pulsed dose rate irradiation responses will hold.
- Combine hyperthermia with the pulsed dose rate modalities and assess the degree of sensitization resulting from this treatment.
- Determine if any of the treatments studied would present possible advantages in the clinic, by comparing the responses of the normal cells to the malignant.

This thesis is comprised of five chapters and an Appendix. The first chapter is the introduction, followed by materials and methods, then results, discussion, and conclusions. The Appendix deals with statistical methods and analysis used in the results section.

2.0 MATERIALS AND METHODS

2.1 The cell lines

Three cell lines were used in all the experiments; two ovarian carcinoma cell lines and normal human fibroblasts. Fibroblasts are actively growing cells that form the fibres of connective tissues [40], and since they usually constitute a component of any normal tissue, including those surrounding a tumour, they are a good representation of contact inhibited normal cells. The normal human diploid (of normal chromosome number) fibroblasts that were used in our experiments, AG1522, were obtained from the Coriell Cell Repository, Camden, NJ, and were considered to be of normal radiosensitivity.

Two ovarian carcinoma cell lines were used for the tumour model. One of the ovarian carcinomas was a parental cell line, A2780s, considered to be sensitive to radiation. The variant A2780cp was established by intermittent (100 μ M) exposure to cisplatin, and it is considered to be radiation and cisplatin resistant compared to the parental cell line [41,42]. These two cell lines were used because they may represent a good model of tumour tissues having similar origins but with different intrinsic radiosensitivities.

All cell lines were grown in medium which is a combination of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco) supplemented with 10% Fetal Bovine serum (Gibco), 1mM MEM non-essential amino acids (Gibco), 10 mM sodium bicarbonate (Sigma), and 20 mM Hepes (Boehringer Mannheim). Cells were incubated at 37°C in a humidified atmosphere of 2% CO₂ and 98% air.

For the ovarian carcinoma cell lines, cells were plated at 10^5 cells per flask or 10^4 and 3×10^4 per vial for the A2780cp and A2780s, respectively. The reason for the difference between plating in vials for the two cell lines is that the A2780s did not attach as well to the glass surface. The medium in flasks or vials was changed on day 5, and the experiments were performed on day 8. With the normal cells, 2×10^5 cells were plated per flask or 10^4 cells per vial. The medium was changed on day 5, and the experiments were performed on day 8. All the experiments were performed at plateau phase because the cell densities and growth kinetics were more representative of tissues than exponentially growing cells [43].

2.2 LDR, HDR, and PDR procedures

For continuous low dose rate (LDR) irradiation, cells were irradiated in 1.3 cm^2 glass vials by an array of ^{226}Ra sources (total activity 800 mCi) contained in a temperature controlled incubator. The dose rate used was 0.88 cGy/min (or 0.53 Gy/h). This dose rate was determined by TLD dosimetry relative to a ^{60}Co reference protocol [44]. The irradiations were carried out at 37°C , except for the hyperthermia experiments, allowing repair to occur during the irradiation.

For high dose rate (HDR) and pulsed dose rate (PDR) irradiation, cells were irradiated in 25 cm^2 flasks with a 250 KVP x-ray unit at a dose rate of 142 cGy/min (or 85.2 Gy/h). The irradiations were carried out on ice ($\sim 4^\circ\text{C}$) in order to minimize the amount of repair occurring during the irradiation procedure [45]. However, for PDR, cells were kept in a temperature controlled water bath between fractions. The dose to the cells was measured by using an ion chamber [46].

For the hyperthermia experiments, the temperature of the low dose rate irradiation incubator was increased to 40°C for the duration of the radiation exposures. For the PDR experiments, the flasks with the cells were kept in a water bath at 40°C and the cells were initially irradiated on ice. However, an experiment was performed by irradiating the cells without ice to verify that ice does not affect the radiosensitivity of cells to the treatment.

2.3 The Clonogenic Assay

After irradiation, cell survival was determined by the colony forming assay, a method originally described by Puck et al [2,47,48]. The clonogenic assay is an *in vitro* assay, and it is a measure of the reproductive integrity of cells. When referring to cell death, it means not just the immediate death of the cell, but also the inability of the cell to reproduce further. A cell that has retained its reproductive integrity is able to proliferate and produce a colony.

Following radiation treatment, the depleted medium was aspirated, and the cell monolayer was rinsed with citrate saline (134 mM KCl (BDH), 17.6 mM citric acid (Sigma)) to neutralize the trypsin inhibiting effect of any traces of medium. Trypsin (0.2% w/v in citrate saline) (Gibco), which is an enzyme that releases cells from the monolayer, was then rinsed over the cells. After the cells started detaching from the surface of the flask or vial, fresh medium was used to resuspend the cells into a single cell suspension. The cells in the suspension were counted using an electronic cell counter (Particle Data Elzone 80, calibrated by haemocytometer) or a Bright-Line haemocytometer (Reichert). Both methods produced comparable counts. The cells were diluted to the desired concentration and plated such that about 50 colonies per plate

would be achieved. For low cell concentrations 60 mm petri dishes were used with 4 ml of medium. For concentrations where more cells needed to be plated ($>10^5$), 100 mm plates were used with 10 ml of medium per plate. After an incubation period of fourteen days for A2780s and A2780cp or eleven days for AG1522, the cells were stained with crystal violet (0.2% w/v in 70% ethanol) and colonies of 50 or more cells were scored.

Typically three dishes per dose for each experiment were plated plus three plates for a control point, 0 Gy, which was used to calculate the plating efficiency (PE).

The plating efficiency was calculated as follows:

$$PE = \frac{\text{colonies counted}}{\text{cells seeded}} \quad (8)$$

Therefore the surviving fraction was calculated according to the formula:

$$S.F. = \frac{\text{colonies counted}}{\text{cells seeded} \times PE} \quad (9)$$

Each experiment was repeated at least twice (once for most of the hyperthermia experiments due to time constraints) and the mean and the standard error of the mean (SEM) were calculated for each set of experiments. The surviving fraction data points were plotted on a semi-logarithmic scale as a function of dose.

2.4. Curve Fitting

Survival curves were fitted with the linear quadratic model using the Grapher version 1.24 (Golden Software Inc) plotting program. The fits were obtained by using Origin 5.0, Microcal Software. This software uses the Levenberg-Marquardt method involving weighted non-linear least squares fit method (chi-square minimization).

2.4.1 Statistical Analysis

As mentioned above, the linear quadratic fits were obtained by the least squares method, which involves estimating parameters by the minimization of the following [49]:

$$Q(p_1, p_2, \dots) = \frac{1}{n^{\text{eff}} - p} \sum_{i=1}^N w_i (y_i - f(x_i; p_1, p_2, \dots))^2 \quad (10)$$

where n^{eff} is the total number of experimental points used in the fitting, p is the total number of adjustable parameters used in the fitting, and the difference between the two is the number of degrees of freedom. The independent and dependent variables are x and y , respectively, and $f(x)$ is the fitting function. The quantity w is the weighting factor (in this case the inverse of the squared standard error was used).

Once the acceptable minimum value for Q has been found by iteration, the alpha and beta parameter values are obtained with their estimated standard errors (which are based on the value of Q and on the covariance matrix).

To test the equivalence between the fits of low dose rate and pulsed dose rate responses, a statistical test using the F-distribution was used, and the procedure is described in detail in the Appendix.

3.0 RESULTS

3.1 High Dose Rate and Low Dose Rate Irradiations

The purpose of these experiments was to compare the radiosensitivities of the three cell lines using both high dose rate (HDR) and low dose rate (LDR) irradiations. Several studies have shown that differences in radiosensitivities among cell lines, both for fibroblasts [39,50,51] and tumours [37,39,52-55], can be measured *in vitro* using the clonogenic assay, and that these radiosensitivities represent the intrinsic sensitivities of the cells.

Figure 3.1.1 shows the survival curves after HDR irradiation for the three cell lines A2780s, A2780cp, and AG1522. These curves were fit with the linear quadratic model and the estimated parameters α and β with their estimated standard errors are shown in table 3.1.1 along with the SF2 and its standard error. From Figure 3.1.1, the A2780cp is seen to have the highest survival and a considerable shoulder, while the A2780s has the lowest survival and a very small shoulder. The survival of the AG1522 cell line is very similar to that of the A2780cp. The shoulder in the survival curve can be described by the β parameter. For the A2780s the value for β is 0.0195 Gy^{-2} , which is smaller than the 0.0580 Gy^{-2} value for A2780cp or 0.0307 Gy^{-2} for AG1522. The differences in survival of the three cell lines can also be observed when looking at their sensitivity parameters. From Table 3.1.1, the α value for the A2780s is 1.08 Gy^{-1} , which is very high, while the A2780cp has the lowest α value of 0.320 Gy^{-1} and the AG1522 has an intermediate value of 0.521 Gy^{-1} . The SF2 for the A2780s is 0.119, which is almost 5 times lower than the 0.523 SF2 value for the A2780cp.

Figure 3.1.1. Survival curves of A2780s, A2780cp and AG1522 cell lines after high dose rate irradiation on ice. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

The SF2 for the AG1522 cell line is 0.302, which is higher than that for the A2780s and slightly lower than that for the A2780cp cell lines.

Table 3.1.1 Comparison of radiosensitivity parameters and β values between the A2780s, A2780cp, and AG1522 cell lines for high dose rate irradiation.

Parameter	A2780S	A2780CP	AG1522
SF2	0.119 ± 0.057	0.523 ± 0.178	0.302 ± 0.011
α (Gy ⁻¹)	1.08 ± 0.03	0.320 ± 0.071	0.521 ± 0.022
β (Gy ⁻²)	0.0195 ± 0.0033	0.0580 ± 0.0087	0.0307 ± 0.0032

Table 3.1.2a Comparison of radiosensitivity parameters and β values between the A2780s, A2780cp, and AG1522 cell lines for low dose rate irradiation.

PARAMETER	A2780S	A2780CP	AG1522
SF2	0.203 ± 0.0539	0.501 ± 0.069	0.606 ± 0.023
α (Gy ⁻¹)	0.600 ± 0.047	0.333 ± 0.00635	0.245 ± 0.005
β (Gy ⁻²)	0.00548 ± 0.00857	-0.0007 ± 0.0012	0.00088 ± 0.00100

Figure 3.1.2. Survival curves for the A2780s, A2780cp, and AG1522 cell lines after low dose rate irradiation. Each point represents the average of three to five experiments, and the error bars represent the SEM between experiments.

Figure 3.1.2 shows the survival curves for the three cell lines given low dose rate irradiation. All cell lines show a dose rate sparing effect. This is because when the irradiation is delivered over a long period of time, the cells have the opportunity to repair damage during the irradiation and subsequently have a higher survival. Table 3.1.2a shows the SF2 with its standard error, and the linear quadratic parameters and their estimated standard errors for the three cell lines with low dose rate irradiation. Table 3.1.2b shows the LQ model parameters and the results of the statistical test that compares the full LQ model to a linear exponential model (reduced model). This was done to determine the statistical significance of the beta parameter. F^* is the calculated value, and it is compared to the F-distribution value for a given number of degrees of freedom (See Appendix for more details). From the table it can be seen that the quadratic components for the three cell lines are not statistically different from zero, and therefore the data can be fitted by linear exponential curves. Table 3.1.3 shows the results of the student's t-test of the SF2 values when comparing low dose rate to high dose rate (a p-value smaller than 0.1 is considered to be statistically significant). The SF2 for the A2780s cell line for low dose rate is 0.203, which is the smallest value for all the cell lines. The A2780cp cell line has a SF2 of 0.5011. The AG1522 SF2 of 0.606, on the other hand, is the highest among the cell lines. From the table, the α value for the AG1522 cell line is 0.245 Gy^{-1} , which is the smallest for the cell lines, and it is a big decrease from the α value for high dose rate. The A2780cp α value, however, does not seem to have changed from its high dose rate value, while the A2780s low dose rate α is smaller than its high dose rate value.

Table 3.1.2b. Statistical significance of the quadratic term.

Cell Line	Full Model	Reduced Model	F*(Full v Red.)
A2780s	$\alpha = 0.600 \pm 0.047 \text{ Gy}^{-1}$	$\alpha = 0.628 \pm 0.013 \text{ Gy}^{-1}$	0.0171 (< 4.20)
	$\beta = 0.00548 \pm 0.00857 \text{ Gy}^{-2}$	$\beta = 0$	
A2780cp	$\alpha = 0.333 \pm 0.006 \text{ Gy}^{-1}$	$\alpha = 0.331 \pm 0.014 \text{ Gy}^{-1}$	0.00180 (< 4.50)
	$\beta = -0.0007 \pm 0.0012 \text{ Gy}^{-2}$	$\beta = 0$	
AG1522	$\alpha = 0.245 \pm 0.005 \text{ Gy}^{-1}$	$\alpha = 0.248 \pm 0.013 \text{ Gy}^{-1}$	0.106 (< 4.75)
	$\beta = 0.000880 \pm 0.001000 \text{ Gy}^{-2}$	$\beta = 0$	

Table 3.1.3 The results of the student's t-test after testing low dose rate and high dose rate SF2.

	A2780S	A2780CP	AG1522
SF2	p=0.0844	p=0.746	p=0.0024

3.2 Pulsed Dose Rate Irradiation

Five different fractionation schemes were used with the three cell lines. The pulses and interfraction intervals used are displayed in table 3.2.1, as well as the total doses. These values were used with all the pulsed dose rate irradiations, including those with hyperthermia.

Table 3.2.1 PDR pulse heights, pulse widths, interfraction intervals, and total doses. All dose fractions were given at 12.5 mA except where indicated.

Pulse Height (Gy)*	Pulse Width (seconds)	Interfraction Interval (h)	Total Dose (Gy)
0.53	60 at 5 mA	1	9.54
1.1	50	2	9.9
1.6	78	3	9.6
2.1	113	4	10.5
3.2	150	6	9.6

* the dose fractions were rounded off after the 0.53 Gy fraction size because of the imprecision of the time-measuring instrument, but the 0.53 Gy was not rounded off to show that the values are its multiples.

3.2.1 A2780cp

Figure 3.2.1.1 shows the survival curve for the 0.53 Gy given every hour (0.53 Gy/1h) and the low dose rate curve. The 1.1 Gy/2h, 1.6 Gy/3h, 2.1 Gy/4h, and 3.2 Gy/6h fractionation schemes are shown in Figures 3.2.1.2, 3.2.1.3, 3.2.1.4, and 3.2.1.5, respectively, with the low dose rate curve for comparison. Figure 3.2.1.6 shows the fits of the linear quadratic model for each fraction size along with LDR and HDR fits. When evaluating these curves, it can be seen that the 0.53 Gy/1h, 1.1 Gy/2h, and 1.6 Gy/3h

seem to have similar survival to the low dose rate curve, and similar among themselves. On the other hand, the 2.1 Gy/4h and 3.2 Gy/6h have survivals also similar to one another, but lower than that of the low dose rate. After obtaining the α and β parameters and their estimated standard errors, the G factor was calculated, and equation 7 was used to calculate the repair half-time for each fractionation scheme. Results are displayed in Tables 3.2.1.1a-3.2.1.1e.

Table 3.2.1.1a. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 0.53 Gy/1h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 0.53 Gy/1h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.303 ± 0.054	0.308 ±0.006	0.353 (< 4.35)	0.706 (< 4.12)
β (Gy ⁻²)	0.0004 ± 0.0065	0		
G-factor	0.00697		N/A	
T _{1/2}	1.04 s		N/A	

From Table 3.2.1.1a it can be seen that the beta component is not statistically different from zero, meaning that the data can be fitted by a linear exponential curve. This fraction size is not statistically different from the low dose rate data, as determined by a small F* value.

Figure 3.2.1.1. Survival curves for the A2780cp cell line after 0.53 Gy/1h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.1.2. Survival curves for the A2780cp cell line after 1.1 Gy/2h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.1.3. Survival curves for the A2780cp cell line after 1.6 Gy/3h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.1.4. Survival curves for the A2780cp cell line after 2.1 Gy/4h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.1.5. Survival curves for the A2780cp cell line after 3.2 Gy/6h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.1.6. Survival curve LQ fits for the A2780cp cell line after the each pulsed dose rate scheme, along with high dose rate, and low dose rate irradiations.

Table 3.2.1.1b. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 1.1 Gy/2h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 1.1 Gy/2h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.307 ± 0.009	0.312 ± 0.009	0.250 (< 4.43)	1.06 (< 4.14)
β (Gy ⁻²)	0.0007 ± 0.0012	0		
G-factor	0.0128		N/A	
T _{1/2}	1.98 s		N/A	

Table 3.2.1.1b and Table 3.2.1.1c show similar results to those of Table 3.2.1.1a.

The beta components are not statistically different from zero for both 1.1 Gy/2h and 1.6 Gy/3h and both are determined to be statistically equivalent to LDR.

Table 3.2.1.1c. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 1.6 Gy/3h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 1.6 Gy/3h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.332 ± 0.011	0.358 ±0.007	0.0190 (< 4.39)	2.67 (< 4.13)
β (Gy ⁻²)	0.0034 ± 0.0014	0		
G-factor	0.00593		N/A	
T _{1/2}	10.8 s		N/A	

Tables 3.2.1.1d and 3.2.1.1e have different results from the other three tables, but their results are similar to each other. Both have statistically significant beta values

(therefore a reduced model was not calculated), and both 2.1 Gy/4h and 3.2 Gy/6h are not statistically equivalent to LDR.

Table 3.2.1.1d. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 2.1 Gy/4h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 2.1 Gy/4h and LDR, and the statistical significance of beta are also shown.

Parameter	Full Model	F*	
		β significance	LDR equivalence
α (Gy ⁻¹)	0.277 ± 0.007	5.64 (> 4.50)	12.0 (> 4.14)
β (Gy ⁻²)	0.0162 ± 0.0007		
G-factor	0.291	N/A	
T _{1/2}	1.78 h	N/A	

Table 3.2.1.1e. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 3.2 Gy/6h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 3.2 Gy/6h and LDR, and the statistical significance of beta are also shown.

Parameter	Full Model	F*	
		β significance	LDR equivalence
α (Gy ⁻¹)	0.328 ± 0.004	8.52 (> 4.96)	9.08 (> 4.20)
β (Gy ⁻²)	0.0147 ± 0.0005		
G-factor	0.262	N/A	
T _{1/2}	1.78 min	N/A	

The experimental value for repair half-time for the A2780cp cell line has been determined to be between 2.5 to 3 hours when using low dose rate irradiation [56]. In comparison, the $T_{1/2}$ values calculated from the model are much smaller, ranging from

about a second to almost two hours. This is because if the repair constant is shorter than the fractionation interval, it will not lead to precise estimation [9]. To overcome this, equation 6 was used to find the repair half-time for the low dose rate regimen, and this value was used to determine β and G-factor values. Then the β value was fixed when performing the least squares regression to determine a new α value. Results are displayed in Table 3.2.1.2.

Table 3.2.1.2. Linear Quadratic parameters as determined from repair half-time calculated for low dose rate, which was 3.40 minutes.

Fraction size (Gy)	G-factor $T_{1/2}=3.40$ min	α (Gy ⁻¹)	β (Gy ⁻²)
0.53	0.0542	0.283	0.00314
1.1	0.105	0.273	0.00612
1.6	0.155	0.291	0.00897
2.1	0.181	0.329	0.0105
3.2	0.288	0.310	0.0167

The $T_{1/2}$ value estimated for low dose rate irradiation was 3.40 minutes. That is still not in agreement with the experimental value, however, it corresponds well with the small $T_{1/2}$ values for other cell lines proposed by the original study that developed this model for pulsed dose rate [9]. The α and β values obtained from the low dose rate repair half-time fit the data reasonably well, and are comparable to the original values within the errors (no errors were estimated on the calculated values because they were fixed during the minimization). This is not the case when the experimental value is used.

This shows that the model fits the data well, even though there is a discrepancy between the calculated and the experimental values. This discrepancy could possibly be due to the fact that a beta value is not an accurate estimator of repair half-time.

3.2.2 A2780s

The results obtained with the 0.53 Gy/1h fraction size are shown in Figure 3.2.2.1, while the 1.1 Gy/2h, 1.6 Gy/3h, 2.1 Gy/4h, and 3.2 Gy/6h fraction sizes are shown in Figures 3.2.2.2, 3.2.2.3, 3.2.2.4, and 3.2.2.5, respectively, along with the low dose rate survival curve. Figure 3.2.2.2.6 shows the linear quadratic fits for all the PDR schemes along with the LDR and HDR fits. It can be observed that the shape of the fractionation survival fits for the A2780s is different than that for the previous cell line. The curves start out straight, but then they level off, giving a “ski-slope” effect. This effect can be quantified by the β parameter taken from the linear quadratic fit using equation (3). Table 3.2.2.1 shows the resulting parameters from fitting the curves with the linear quadratic model along with their estimated standard errors, and the F-test for LDR-PDR equivalence. From this table, it can be observed that while the α values remain positive, the β values for all the fractionation fits are negative. Another observation is that the α values seem to increase with increasing fraction size, implying change in the intrinsic radiosensitivity of the cells, ranging between the α values for high dose rate and low dose rate. Because these changes are not accounted for in the linear quadratic model, the same analysis as with the previous cell line using the G-factor and $T_{1/2}$ was not possible for the A2780s. From the table, it can be seen that only the 1.6 Gy/3h fractionation scheme is statistically equivalent to LDR. However, a qualitative analysis of the surviving curves

shows that the 0.53 Gy/1h and 1.1 Gy/2h also seem to be equivalent to low dose rate curve, but only up until about 6 Gy, then the pulsed dose rate curves start diverging upwards from the low dose rate curve. The 2.1 Gy/4h and 3.2 Gy/6h, on the other hand, have a lower survival at lower doses and then their survival at higher doses (close to 10 Gy) approaches the same survival level of low dose rate due to this leveling-off effect. This effect observed in the pulsed dose rate curves cannot be described by the linear quadratic model, which assumes no change in radiosensitivity between treatment modalities, and β values that are equal to or greater than zero. This effect could be due to a number of different possibilities. Several possible explanations were investigated, including feeder cell effect, cell cycle redistribution and cell growth, and adaptive response and/or a radiosensitive sub-population (see Discussion).

Table 3.2.2.1. Linear quadratic parameters from the fits of the pulsed dose rate experiments with their estimated standard errors, and the F-test for LDR-PDR equivalence.

Fraction Size (Gy)	α (Gy ⁻¹)	β (Gy ⁻²)	F*
0.53	0.718 ± 0.004	-0.0237 ± 0.0006	13.2 (> 4.01)
1.1	0.843 ± 0.037	-0.0370 ± 0.0046	4.48 (> 4.01)
1.6	0.711 ± 0.022	-0.00842 ± 0.00343	0.309 (< 4.07)
2.1	1.01 ± 0.02	-0.0344 ± 0.0021	6.68 (> 4.08)
3.2	1.03 ± 0.01	-0.0370 ± 0.0014	5.27 (> 4.12)

Figure 3.2.2.1. Survival curves for the A2780s cell line after 0.53 Gy/1h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.2.2. Survival curves for the A2780s cell line after 1.1 Gy/2h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.2.3. Survival curves for the A2780s cell line after 1.6 Gy/3h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.2.4. Survival curves for the A2780s cell line after 2.1 Gy/4h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.2.5. Survival curves for the A2780s cell line after 3.2 Gy/6h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.2.6. Survival curve LQ fits for the A2780s cell line after the each pulsed dose rate scheme, along with high dose rate, and low dose rate irradiations.

3.2.3 AG1522

Figures 3.2.3.1-3.2.3.5 show the survival curves for the 0.53 Gy given every 1 hour, 1.1 Gy/2h, 1.6 Gy/3h, 2.1 Gy/4h, and 3.2 Gy/6h, along with low dose rate and high dose rate curves for comparison. A plot of the linear quadratic fits is shown on figure 3.2.3.6. Unlike pulsed dose rate curves for the other two cell lines, none of the fractionation schemes studied approach the low dose rate survival level. Also, none of the curves have a very large shoulder, even though a shoulder was observed in the A2780cp cell line at larger fraction sizes. This would imply that sublethal damage is being repaired.

Tables 3.2.3.1a-3.2.3.1e show the LQ model parameters with their estimated standard errors, and the repair half-time values along with the G-factors.

Table 3.2.3.1a. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 0.53 Gy/1h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 0.53 Gy/1h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.421 ± 0.015	0.414 ± 0.007	0.634 (< 4.43)	18.1 (> 4.16)
β (Gy ⁻²)	-0.00116 ± 0.00252	0		
G-factor	0		N/A	
T _{1/2}	N/A		N/A	

From Table 3.2.3.1a it can be seen that the beta component is negligible, but that the PDR scheme is not equivalent to LDR. This can be seen in the other fractionation schemes as well, except for the 3.2 Gy/6h, which has a significant beta component.

Figure 3.2.3.1. Survival curves for the AG1522 cell line after 0.53 Gy/1h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.3.2. Survival curves for the AG1522 cell line after 1.1 Gy/2h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.3.3. Survival curves for the AG1522 cell line after 1.6 Gy/3h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.3.4. Survival curves for the AG1522 cell line after 2.1 Gy/4h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.3.5. Survival curves for the AG1522 cell line after 3.2 Gy/6h pulsed dose rate, high dose rate, and low dose rate. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.2.3.6. Survival curve LQ fits for the AG1522 cell line after the each pulsed dose rate scheme, along with high dose rate, and low dose rate irradiations.

Figure 3.2.3.7. Survival curve for the three cell lines following 0.53 Gy/1h fractionation scheme.

Table 3.2.3.1b. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 1.1 Gy/2h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 1.1 Gy/2h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.359 ± 0.028	0.392 ±0.005	0.0239 (< 4.43)	20.2 (> 4.16)
β (Gy ⁻²)	0.00723 ± 0.00588	0		
G-factor	0.236		N/A	
T _{1/2}	1.55 h		N/A	

Table 3.2.3.1c. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 1.6 Gy/3h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 1.6 Gy/3h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.447 ± 0.040	0.448 ±0.009	0.0147 (< 4.75)	12.6 (> 4.23)
β (Gy ⁻²)	0.00032 ± 0.00513	0		
G-factor	0.0104		N/A	
T _{1/2}	1.51 s		N/A	

Table 3.2.3.1d. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 2.1 Gy/4h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 2.1 Gy/4h and LDR, and the statistical significance of beta are also shown.

Parameter	Model		F*	
	Full	Reduced	β significance	LDR equivalence
α (Gy ⁻¹)	0.524 ± 0.048	0.538 ± 0.008	0.629 (< 4.96)	13.0 (> 4.26)
β (Gy ⁻²)	0.00176 ± 0.00574	0		
G-factor	0.0580		N/A	
T _{1/2}	10.8 s		N/A	

Table 3.2.3.1e. Linear quadratic parameters from the fit of the pulsed dose rate experiments for the 3.2 Gy/6h fractionation scheme with the estimated standard errors, the G-factor, and $T_{1/2}$. The results from the statistical analysis for the equivalence between 3.2 Gy/6h and LDR, and the statistical significance of beta are also shown.

Parameter	Full Model	F*	
		β significance	LDR equivalence
α (Gy ⁻¹)	0.568 ± 0.004	7.31 (> 5.32)	6.34 (> 4.35)
β (Gy ⁻²)	0.00555 ± 0.00125		
G-factor	0.182	N/A	
T _{1/2}	43.2 s	N/A	

It can be seen from the tables that the $T_{1/2}$ values are inconsistent, varying from a few seconds to over an hour, likely because the beta values are not statistically different from zero for the most part, which could lead to imprecise estimation of the repair half-times. As before, the repair half-time is then calculated from low dose rate parameters, and used in the linear quadratic fit. Results are shown in table 3.2.3.2.

Table 3.2.3.2. Linear Quadratic parameters as determined from repair half-time calculated for low dose rate, which was 11.5 minutes.

Fraction size (Gy)	G-factor $T_{1/2}=11.5$ min	α (Gy ⁻¹)	β (Gy ⁻²)
0.53	0.0580	0.405	0.00178
1.1	0.110	0.376	0.00336
1.6	0.163	0.412	0.00500
2.1	0.194	0.491	0.00596
3.2	0.319	0.554	0.00978

The $T_{1/2}$ value for low dose rate was calculated to be 12 minutes. The α and β values obtained from this value fit the data reasonably well, and are comparable to the original values (within the errors of the original values). This calculated repair half-time value is slightly lower than the values of 16 and 23 minutes calculated in the original study that proposed this model [9]. However, taking into consideration errors due to difference in technique among experimenters and variation between experiments, our values compare favourably with the ones in the study, showing that the model is fairly consistent. On the other hand, the calculated values would have to be compared to the experimental value of repair half-time, which was not available.

Figure 3.2.3.7 shows the plot of the 0.53 Gy/1h response of the three cell lines for comparison. Although none of the PDR survival levels approach the LDR survival for the normal cell line (figures 3.2.3.1-3.2.3.6), from this plot it can be seen that its survival for the 0.53 Gy/1h is still higher than that for the sensitive cell line. This would be clinically beneficial, since it would allow normal cell sparing. The resistant cell line, on

the other hand, has a survival level that is higher than that of the normal cell line for the 0.53 Gy/1h. Although this would seem detrimental in a clinical situation, the normal cells would not receive the same high levels of radiation as the tumour cells because as mentioned in the introduction, the dose distribution can be controlled by computer quite accurately, minimizing the dose to normal tissues.

3.3 Pulsed Dose Rate with 40°C hyperthermia

3.3.1 A2780cp

Figure 3.3.1.1 shows the survival curves for the 0.53 Gy/1h fractionation scheme given in combination with 40°C hyperthermia, and 0.53 Gy/1h without hyperthermia. Figure 3.3.1.2 shows a similar comparison for the 1.6 Gy/3h. As mentioned in the Materials and Methods section, some of the PDR plus hyperthermia experiments were performed by irradiating on ice, which could have changed their response due to shock. To verify this, another experiment was performed without irradiating on ice, and the results showed that the ice had no significant effect on the cell response to hyperthermia. Currently there are no mathematical models to fit the pulsed dose rate combined with hyperthermia treatment, but the linear quadratic model was used because its parameters can be used to test the equivalence to the treatments without hyperthermia. However, the parameters cannot be interpreted as described by the linear quadratic model, therefore they are only used for the purposes of equivalence testing. Table 3.3.1.1 shows the F-test between the linear quadratic parameters of the fits. From Figure 3.3.1.1, by inspection, it can be seen that there is no sensitization due to 40°C hyperthermia with the pulsed dose data. Figure 3.3.1.2, however, shows that there is some sensitization when using the 1.6

Gy/3h fractionation scheme with hyperthermia. The statistical test confirms this; there is equivalence for the 0.53 Gy/h fractionation scheme with and without hyperthermia. The 1.6 Gy/3h on the other hand, shows no equivalence between the two treatments.

Table 3.3.1.1. Results of the F-test between the α and β parameters for treatments with and without hyperthermia

	0.53 Gy/1h	1.6 Gy/3h
0.53 Gy + 40°C	$F^* = 1.73 (< 3.20)$	N/A
1.6 Gy + 40°C	N/A	$F^* = 9.14 (> 3.30)$

Figure 3.3.1.1. Survival curves for the A2780cp cell line after 0.53 Gy/1h pulsed dose rate with and without 40°C mild hyperthermia.

Figure 3.3.1.2. Survival curves for the A2780cp cell line after 1.6 Gy/3h pulsed dose rate with and without 40°C mild hyperthermia.

3.3.2 A2780s

Figure 3.3.2.1 shows the plots of 0.53 Gy/1h with hyperthermia, along with 0.53 Gy/1h without hyperthermia. Figure 3.3.2.2 shows a similar comparison for the 1.6 Gy/3h fraction size. Table 3.3.2.1 shows the F test between the α and β parameters between the different fits. Qualitatively, from looking at the graphs, there is no sensitization for 0.53 Gy/1h as compared to the results obtained without hyperthermia, while 1.6 Gy/3h with hyperthermia shows some sensitization. The F-test confirms this; there is no sensitization for the 0.53 Gy/1h, but there is some for the 1.6 Gy/3h.

Table 3.3.2.1. F-test between the α and β parameters for treatments with and without hyperthermia

	0.53 Gy/1h	1.6 Gy/3h
0.53 Gy + 40°C	F* = 1.14 (< 3.38)	N/A
1.6 Gy + 40°C	N/A	F* = 9.47 (> 3.32)

Figure 3.3.2.1. Survival curves for the A2780s cell line after 0.53 Gy/1h pulsed dose rate with and without 40°C mild hyperthermia.

Figure 3.3.2.2. Survival curves for the A2780s cell line after 1.6 Gy/3h pulsed dose rate with and without 40°C mild hyperthermia.

3.3.3 AG1522

Figure 3.3.3.1 shows the AG1522 response to 0.53 Gy given every hour in combination with 40°C hyperthermia. As with the previous cell lines, there is no obvious difference in survival whenever the treatment is combined with hyperthermia or not. Figure 3.3.3.2 shows a similar result for 1.6 Gy every 3 hours PDR plus hyperthermia. There is no significant difference whether treatment is combined with hyperthermia or not. Table 3.3.3.1 confirms this equivalence. This is unlike the results with the other two cell lines, where both cell lines showed sensitization to this PDR treatment with hyperthermia. To observe the effects of this, the 1.6 Gy/3h PDR plus hyperthermia survival curves were plotted on the same graph in Figure 3.3.3.3. Survival for the AG1522 cell line is still greater than that for the A2780s as before. When comparing the normal cells to the resistant carcinoma, however, at the level of 10 Gy the survivals for the two cell lines are close together. More importantly, if the survival fits were extended past the 10 Gy level to 12 Gy, for example, then the survival for the normal cell would be greater than that for the tumour cells. Therefore a therapeutic advantage would be gained from this combination treatment.

Table 3.3.3.1. F-test between the α and β parameters for treatments with and without hyperthermia

	0.53 Gy/1h	1.6 Gy/3h
0.53 Gy + 40°C	F* = 0.815 (< 3.30)	N/A
1.6 Gy + 40°C	N/A	F* = 2.12. (< 3.40)

Figure 3.3.3.1. Survival curves for the AG1522 cell line after 0.53 Gy/1h pulsed dose rate with and without 40°C mild hyperthermia.

Figure 3.3.3.2. Survival curves for the AG1522 cell line after 1.6 Gy/3h pulsed dose rate with and without 40°C mild hyperthermia.

Figure 3.3.3.3. Survival curve for the three cell lines following 1.6 Gy/3h fractionation scheme with 40°C mild hyperthermia.

4.0 DISCUSSION

Three cell lines with different intrinsic radiosensitivities were chosen for this study. Two ovarian carcinoma cell lines, one sensitive and the other one resistant, were used as a model of two tumour cell lines of different radiosensitivities, but of similar genetic background. In the clinic, when treating a tumour, there is a possibility of radiation damage to surrounding normal tissues. It was important, therefore, to determine the response of normal cells to the various treatments, as well. The normal human fibroblasts used in this study usually constitute a component of any normal tissue, including those surrounding a tumour, hence this cell line was a good model to assess the normal tissue tolerance to radiation damage from the treatments studied.

4.1 High Dose Rate and Low Dose Rate Irradiations

The radiosensitivity of the three cell lines was determined by comparing their high dose rate and low dose rate survival curves. The high dose rate survival curve for the A2780cp cell line was observed to have a large shoulder region, which was quantified by the largest β value (0.0580 Gy^{-2}) among the three cell lines. This indicates that at higher doses the damage is lethal due to sublethal damage accumulation and the survival decreases more rapidly, therefore the accumulated sublethal damage could potentially be repaired when using a fractionated regimen. The range of β values found in the literature for several cell lines following acute irradiation is $0.02\text{-}0.06 \text{ Gy}^{-2}$ with a mean of 0.032 Gy^{-2} (37,38). The 0.0580 Gy^{-2} β value for the A2780cp cell line lies, therefore, in the upper range of the β values. The A2780s cell line, on the other hand, had no visible

shoulder on the survival curve indicating that, even at low doses, there is little sublethal damage accumulated before the damage becomes lethal. Its β value was the smallest of the three cell lines, and its value of 0.0195 Gy^{-2} lies in the lower end of the β values. The AG1522 cell line had an intermediate shoulder and a β value of 0.307 Gy^{-2} , which was closer to the average β value found in the literature [37,38].

The radiosensitivities of the three cell lines were determined by the radiosensitivity parameters α and SF2. It has been established that a positive correlation exists between the steepness of the initial slope of the survival curve (described by the α parameter) for human tumour cells and their clinical response [38,39]. The range of α values found in the literature for acute irradiation lies in the $0.2\text{-}0.6 \text{ Gy}^{-1}$ range, where the lower end indicates radioresistance, while the upper end indicates radiosensitivity, with a mean of about 0.35 Gy^{-1} [37,38]. The α value for the A2780s was found to be 1.08 Gy^{-1} , which is much higher than the upper radiosensitive limit of the cell lines found in the literature, indicating that the cell line is very radiosensitive. The A2780cp cell line was found to have a α value of 0.320 Gy^{-1} , which is close to the average of α values found in the literature. This average α value and the fact that the cell line is called radioresistant may seem inconsistent. However, the cell is referred to as radioresistant only in comparison to its parental strain, which as seen from its α value is very radiosensitive. The α value for the AG1522 was 0.521 Gy^{-1} , which is slightly higher than the average, however, the survival levels for this cell line were similar to those for the A2780cp, and this cell line is considered to be of normal radiosensitivity.

The HDR α and β values obtained in this study for the three cell lines compare well with those determined previously in our laboratory. The averages of α and β values

obtained for the A2780s cell line by four other experimenters are $0.9 \pm 0.2 \text{ Gy}^{-1}$ and $0.03 \pm 0.01 \text{ Gy}^{-2}$, respectively. These same experimenters determined the α and β values for the A2780cp cell line to be $0.3 \pm 0.2 \text{ Gy}^{-1}$ and $0.04 \pm 0.03 \text{ Gy}^{-2}$, respectively. Published values for α and β for the AG1522 cell line are 0.49 Gy^{-1} and 0.52 Gy^{-1} for α , and 0.035 Gy^{-2} and 0.026 Gy^{-2} for β [43,57].

The SF2 is another radiosensitivity parameter. A 2 Gy dose is also a typical dose per fraction used in clinical radiotherapy, and there is evidence that the radiosensitivity of human tumour cell lines to low radiation doses correlates with clinical experience [37]. Although the SF2 ranges widely, for acute irradiation it averages about 0.5 for the least responsive tumours (radioresistant) to 0.15 for the most responsive (radiosensitive). The SF2 for the A2780cp was 0.523, and for A2780s it was 0.119, indicating the cell lines are radioresistant and radiosensitive, respectively. The SF2 for the AG1522 was 0.302, suggesting that this cell line had a normal response to acute irradiation.

Low dose rate survival curves were also plotted to compare the radiosensitivities of the cells. The SF2 for the A2780s cell line was 0.203, which was the smallest value for all the cell lines. Although this SF2 value is not quite statistically different from the SF2 for the high dose rate, which is 0.119 (p-value from the t-test close to 0.1), the increase in survival from high dose rate to low dose rate is more dramatic at higher doses. The A2780cp cell line has a SF2 of 0.5011, which is also not significantly different from the high dose rate value (p=0.844). The AG1522 SF2 of 0.606, however, is statistically very different from the high dose rate value (p=0.0024). All cell lines show low dose rate recovery, especially the AG1522 cell line, which shows an increase in SF2 by a factor of 2.7 from high dose rate to low dose rate. For the A2780cp cell line the α value for low

dose rate irradiation is within the error range when compared to the α value for high dose rate irradiation, which indicates that the intrinsic radiosensitivity of the cells has not changed. However, the same is not true for the other cell lines; both show a two-fold increase in their low dose rate irradiation α values when compared to their high dose rate values. For the AG1522 cell line, its α value of 0.245 is comparable to the values 0.21, 0.19, and 0.215 found in the literature [9,58], and this cell line especially shows an increase in survival that surpasses that of the A2780cp cell line. The β values for all the cell lines are as expected with low dose rate irradiation; statistically not different from zero, which means that sublethal damage is being repaired due to the dose rate sparing effect.

4.2 Pulsed Dose Rate Irradiation

As mentioned previously, with low dose rate irradiation sublethal damage is being repaired as the treatment progresses because ionizing events are spaced far enough so that repair of single strand breaks takes place before a second break occurs. Therefore in order to be equivalent to low dose rate irradiation, a pulsed dose rate treatment would require interfraction intervals spaced wide enough compared to the size of the dose fractions, that this repair of sublethal damage would take place, assuming no change in radiosensitivity between fractions. The dose rate effect is also an important consideration, and the low dose rate used in these experiments, 0.53 Gy/h, is within the range (0.5-0.7 Gy/h) used in most publications on PDR [7-11,14,15].

4.2.1 A2780cp

The survival curve fit for the A2780cp cell line with the 0.53 Gy/1h fraction size showed that this fractionation scheme was statistically equivalent to low dose rate. The results showed no apparent change in radiosensitivity between fractions (similar α values), and a negligible β component, implying complete repair. This equivalence between the two treatments was determined by the F-test, and it is in agreement with mathematical predictions [9-11], as well as with several *in vitro* studies [7,12-14], and two *in vivo* studies [8,15].

The 1.1 Gy/2h fraction size was also shown to be statistically equivalent to LDR. Here too, the α value was similar to the HDR and LDR values, and the β value was statistically not different from zero. So with this fraction size, as with the 0.53 Gy/1h, there was no change in radiosensitivity and repair of SLD was completed between interfraction intervals. This equivalence is supported by studies that used a similar fractionation scheme in mathematical predictions [10,11], as well as in some *in vitro* studies [7,13,14]. No *in vivo* data that used this fractionation scheme was available.

Another fractionation scheme that proved to be statistically equivalent to LDR was 1.6 Gy given every three hours. This fraction size also had a similar α value to those of the other treatments, and its β component was not different from zero either. This equivalence is supported by a study that used mathematical modeling with this fraction size [11], however, there are only a few *in vitro* studies that used this fractionation scheme, one that supports the equivalence [7], and one that does not [14].

The last two fraction sizes used, 2.1 Gy/4h and 3.2 Gy/6h, showed no equivalence to LDR. Although their α values were also similar to the other values, the β components

for both treatments were significant, implying incomplete repair of sublethal damage. One *in vitro* study supports this finding [14], but another does not [7]. Also, an *in vivo* study using a late effects model found equivalence between LDR and the 2.1 Gy/4h [8].

The results from the PDR experiments with the A2780cp cell line therefore indicate that the most appropriate fraction size to replace LDR while maintaining equivalence would be 1.6 Gy/3h. This is because in a clinical situation this fraction size with a three-hour interval would allow more patients being treated than if the time intervals were one hour or two. The only limitation would be the availability of equipment that could be shared. Another fraction size to be considered would be the 2.1 Gy/4h. Although this fraction size showed no equivalence to PDR, this cell line is a malignant cell line, after all, and therefore maximum killing would be desirable, as well as normal tissue sparing. This normal tissue sparing was observed in the late responding *in vivo* system that used this fraction size [8]. However, more *in vivo-in vitro* systems would have to be compared before this fraction size is considered for the clinic.

4.2.2. A2780s

The results obtained with this cell line were different in many aspects from its resistant derivative. First of all, the shape of the PDR survival curves was different, with a negative beta component for each fractionation scheme. This makes the results impossible to interpret in view of sublethal damage repair, therefore the results were not described by the linear quadratic model. Another difference is that the α values varied among the fraction sizes. A trend was observed of increasing alpha values with increasing fraction size. To determine a possible cause for the PDR results with the

A2780s cell line, several effects were investigated such as the feeder cell effect, cell cycle redistribution and cell growth, and adaptive response and/or a radiosensitive sub-population.

4.2.2.1 Feeder Cell Effect

At high doses, especially with sensitive cell lines, high numbers of cells (up to a million cells) are plated in order to give the desired number of about 50 colonies because there is a lot of cell kill as a result of radiation damage. Lethal damage to a cell does not necessarily mean immediate cell death, but it could also mean the loss of the cell's ability to divide [2]. Many such cells may provide growth factors and contact inhibition, which could potentially stimulate cell division and damage repair, respectively, therefore aiding in the survival of cells that have retained their reproductive integrity. This could potentially lead to an increase in survival at higher doses such as that observed in the survival curves shown in Figures 3.2.2.1-3.2.2.5.

A feeder cell effect experiment was designed to test such possibility. Feeder cells in this experiment have been given a single dose of 30 Gy. Different aliquots of these cells were plated along with fixed numbers of untreated cells. After fourteen days the colonies were scored and plotted as a function of the number of feeder cells plated as shown in Figure 4.2.2.1.1. It can be seen from the figure that the number of colonies does not follow an upward trend with an increase in the number of feeder cells. This suggests, therefore, that the leveling-off effect of pulsed dose rate irradiation curves is not likely due to the feeder cell effect.

Figure 4.2.2.1.1. Feeder cell effect curves. Number of colonies is a function of the number of feeder cells added to each petri dish. Each point represents the average of three petri dishes, with the error bars representing SEM between each set of dishes.

4.2.2.2. Cell cycle redistribution and cell growth

As stated in the Materials and Methods section, all experiments were performed at plateau phase. This means that theoretically the cells were contact-inhibited and therefore not dividing. However, in tumour cells there is cell turnover and thus there exists a possibility that a fraction of cells was dividing and hence progressing through the cell cycle. Therefore it is possible that a significant part of these cells could have been in a more radioresistant phase of the cell cycle, usually late S-phase and early G₁ in cells with longer cell cycle times [2], while being irradiated. This could then contribute at least in part to the shape of the PDR survival curves. To verify this, the progression of the cells through the cell cycle was monitored using flow cytometry. Cells were treated with propidium iodide, which stains the DNA and allows the amount of DNA in the nucleus to be measured. The amount of DNA in a cell is related to the phase of the cell cycle at the time of treatment. Cells in G₂ or mitosis (M) will have twice as much DNA as those in G₁. Cells in the DNA synthesis phase (S-phase, between G₁ and G₂) will have an amount of DNA between the amounts found in G₁ and G₂/M [45]. To identify cells in the S-phase that were actively cycling from those that were quiescent (non-cycling), 5-bromodeoxyuridine (BrdU) was added to the cells. BrdU is a halogenated pyrimidine and when fed to the cells, it is incorporated into DNA in place of thymidine and its presence can be detected by an appropriate stain. In this case, anti-BrdU FITC was used. Anti-BrdU is an antibody against BrdU and it can identify cells that are in S-phase and that have incorporated BrdU [2].

Replicate samples from one experiment were used for analysis. After treatment, BrdU was added directly to the medium of the cells, and then the cells were

incubated for 30 minutes at 37°C to allow sufficient time for incorporation. Following incubation, the cells were dissociated into a single cell suspension using the method described in section 2.3. After obtaining a good cell harvest with minimal clumping, the cells were resuspended in BSA/PBS (bovine serum albumin, a protein to keep cells from clumping, in phosphate buffered saline, which stabilizes osmolarity and the pH). After centrifuging and aspirating the solution, the pellet was resuspended in citrate saline, and then in cold ethanol, which fixes the cells and keeps proteins from breaking down. HCl was added to aggregate the cells, and then the acid was neutralized with sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$). The cells were eventually resuspended in 5 ml of 70% cold ethanol, vigorously vortexing the cells as the ethanol was added to prevent cell clumping. The samples could be stored at -20°C for several weeks. On the day of the analysis, the cells were centrifuged at 2000 rpm for 10 min and the ethanol was removed. The cells were resuspended in 50 µl of Tween 20/BSA/PBS solution (Tween-20 is a detergent that lyses the cell membrane to allow stain to get into the nucleus). Then 100 µl of RNAase solution was added to break down the RNA in the cells along with 20 µl of Anti-BrdU FITC per 10^6 cells, and the cells were incubated for 30 min at room temperature. Following incubation the cells were resuspended in 1 ml of Sheath Fluid containing 5 µg/ml of propidium iodide. The samples were then promptly run using a Coulter Epics XL flow cytometer at the Health Sciences Centre, University of Ottawa. The cell cycle distribution was analyzed using the Multicycle program (Phoenix Flow Systems) which determines the percent of cells in each phase of the cell cycle.

Figure 4.2.2.2.1. shows a sample plot of the cell cycle distribution for one of the flasks analyzed. This is a plot of number of cells versus DNA content, therefore the cells

with the most DNA content will be in the G2/M phase, represented by the smaller peak starting around channel 64. The cells with the least DNA content, as shown in the tall sharp peak around channel 32, are in the G1 phase. Cells in the S-phase are distributed in between the G1 and G2/M peaks. The distribution of the cells was optimized using chi-squared minimization built into the software used. Table 4.2.2.2.1 shows the different percentages of cells in the various parts of the cell cycle, comparing effects of a time interval between two doses (six hour interval between 6.4 and 9.6 Gy), and the distribution at the beginning of the experiment (T=0h) of untreated cells versus that determined at the end of the experiment (T=12h).

Table 4.2.2.2.1. Cell cycle distribution measured at the beginning and the end of the experiment, as well as at two different doses. There is an error of about 10% on each percentage value.

Cell cycle distribution	0 Gy T = 0h	0 Gy T = 12h	6.4 Gy T = 6h	9.6 Gy T = 12h
%G1	79.8	81.9	79.2	78.1
%G2/M	7.2	7.6	12.3	10.1
%S	13.0	10.5	8.5	11.9

From the table, it can be observed that the cells are indeed in a true plateau phase; there is no cell growth or cell cycling. The respective cell cycle distributions do not change significantly during the course of the experiment (no change between values at the start and at the end of the experiment), and also there is no change when giving the cells a second dose of radiation after an interval of six hours.

DNA content is measured by propidium iodide, which determines the proportion of cells in the S-phase. However, this technique does not distinguish cells in the S-phase that are not cycling (quiescent) from those that are actively cycling. Non-cycling cells in the S-phase are arrested in that phase and therefore would not contribute to the radioresistance of the cell population. Those that are cycling, however, could contribute. From the table, the percentage of cells in the S-phase is between 8.5 to 13, and this includes cycling and quiescent cells. To distinguish between them, cells were stained with BrdU. Analysis showed that the percentage of cycling S-phase cells in the whole S-phase population was less than one, therefore cell cycle and cell growth are not likely factors that would contribute to the increase in survival when using pulsed dose rate irradiation.

Figure 4.2.2.2.1. Sample figure of cell cycle distribution. The first sharp peak represents the proportion of cells in the G1 phase of the cell cycle, and the second peak represents the proportion of cells in the G2/M phase of the cell cycle.

4.2.2.3 Change in Radiosensitivity Between Fractions

From Table 3.2.1.1 it was observed that the value of the α parameter for the different fraction sizes ranges between the LDR and HDR values, increasing with increasing fraction size. This could indicate a change in the radiosensitivity of the cells due to either an adaptive response or the killing of a sub-population of radiosensitive cells, or a combination of both. It is possible that each fraction size used was able to protect the cells against subsequent exposures to radiation, resulting in an adaptive response, the mechanisms of which are yet unknown [46]. Another possibility is that there exists a sub-population of cells that is more radiosensitive than the rest of the cells in the culture, and the killing of these cells even at small doses could result in a decrease in survival curve slope. A third possibility is that the shape of the curves observed in the pulsed dose rate experiments with the A2780s cell line could be due to a combination of both. Distinguishing between these possibilities is beyond the scope of this study. However, an experiment was designed to verify if any changes in radiosensitivity are occurring. This experiment involved irradiating the cells with an “inducing dose”, repeating for a range of inducing doses (0.5, 1, 1.5, and 2 Gy) which were selected because they were similar to the fraction sizes used. One group of irradiated cells was delayed plating for 6 hours, while the other group was irradiated with 6 Gy after 6 hours and plated immediately. The surviving fraction was calculated as the following ratio:

$$SF = \frac{SF(\text{inducing dose} + 6 \text{ Gy})}{SF(\text{inducing dose})} \quad (13)$$

This was done to eliminate the effect of the inducing dose. Note that the inducing dose would also eliminate most of (or all of) the effects of a radiosensitive sub-population of cells. Figure 4.2.2.3.1 shows the results of this experiment plotted along with the high dose rate curve. It can be seen that there is an increase in survival up to about six-fold from that of the high dose rate when using inducing doses. This would suggest, therefore, that a fractionated treatment could potentially have an effect on the radiosensitivity of the cells due to adaptive response, with possibly a very small contribution from a radiosensitive sub-population of cells. Therefore, since the linear quadratic model assumes no changes in radiosensitivity in the cells between fractions, then it cannot describe the A2780s pulsed dose rate survival curves. This experiment was repeated for the A2780cp cell line as well to verify that there is no change in radiosensitivity between fractions in the PDR experiments, and therefore that the results can be interpreted by the linear quadratic model. The results from this experiment are shown in Figure 4.2.2.3.2, and it can be seen that in fact there is no increase in survival from that of the high dose rate.

Therefore this possibility of adaptive response observed with the A2780s cell line means that each fraction causes the cells to become increasingly resistant to subsequent fractions, which could have serious consequences in the clinic for tumour control if this phenomenon could be determined to exist in actual patients.

Figure 4.2.2.3.1. Adaptive response experiment for the A2780s cell line, along with the high dose rate curve.

Figure 4.2.2.3.2. Adaptive response experiment for the A2780cp cell line, along with the high dose rate curve.

For this cell line, the only fraction size that was equivalent to LDR for this cell line was 1.6 Gy/3h. The other fraction sizes would not be recommended for the clinic, as they demonstrate large adaptive response effects. The 1.6 Gy/3h, however, supports the previous suggestion with the A2780cp cell line of being appropriate for clinical application.

4.2.3. AG1522

The PDR experiments for the normal cell line show no equivalence to LDR for any of the PDR treatments. The PDR alpha values also seem to follow a trend of increasing α values with increasing fraction size so that survival levels for PDR range between those for LDR and HDR. The beta values are not negative, but they are statistically not different from zero for the most part. One possible explanation for these observations is potentially lethal damage, which is damage that can be repaired by post-irradiation conditions [2], and this type of damage could affect the alpha component. Another possibility is that in fitting the data there is a trade-off between alpha and beta values, meaning that slightly increasing or decreasing the alpha values results in corresponding decreases or increases in beta values, respectively, which would result in similarly good fits. Adaptive response is also a possible explanation since the decreasing α values with decreasing fraction size can be interpreted as changes in radiosensitivity. This was also observed in the A2780s cell line, and it was experimentally determined that adaptive response was the most likely cause for this trend. However, these possibilities were not investigated.

Although none of the PDR schedules were found to be statistically equivalent to LDR, this does not mean that PDR would be a disadvantage in the clinic in terms of normal tissue sparing as compared to LDR. As mentioned in the introduction, the dose distribution with computer-controlled remote afterloaders is quite accurate, so there would still be minimal damage to the surrounding normal tissue. However, even if there was some exposure to normal cells (which would still be lower than the dose to the tumour cells), as seen from the survival curves, normal tissues can still repair considerable damage. The PDR response of the normal cells should also be compared to that of the tumour cells. As seen from figure 3.2.3.7, after a fractionation scheme of 0.53 Gy/1h, the survival of the normal cells is higher than that of the sensitive A2780s, even if it is lower than that of the resistant A2780cp. This shows that PDR could at least be used in the clinic to treat sensitive tumours, and still get a higher survival in the normal tissues.

Repair half-times were found for the A2780cp and AG1522 cell lines. When these values were derived from the beta values, the results were inconsistent, showing half-time values that ranged from a few seconds to just over an hour. This could be due in part to the fact that most of the beta values for both cell lines were not statistically different from zero, therefore the beta values for PDR were not good estimators of repair half-time. Instead, LDR values were used to find repair half-times. However, the value obtained for the resistant cell line was much lower than the actual value determined by experiment (3.4 min versus 2-3 hours), but as with PDR, the beta component for LDR was not statistically different from zero. There were no $T_{1/2}$ values for this cell line in the original paper that proposed the model that was used in determining the half-times [9]. The repair half-time calculated from LDR data for the normal cell line (12 minutes),

however, compared well to the values in the study (16 and 23 minutes) for this cell line using LDR [9]. No experimental value was available. However, the beta component for the LDR data was also negligible, therefore the proposed model of calculating repair half-times using the G-factor in the LQ model might not be accurate.

4.3 Pulsed Dose Rate With 40°C Hyperthermia

Although several studies have demonstrated the effectiveness of mild hyperthermia as a radiosensitizer [18,25,27], mild 40°C hyperthermia administered between fractions was found to be ineffective in terms of sensitization to radiation for the 0.53 Gy/1h for all three cell lines. They all showed that 0.53 Gy/1h at 37°C was not statistically significant from 0.53 Gy/1h at 40°C. This is in contrast to the only PDR study that used 41°C hyperthermia with 0.5 Gy/1h fractionation scheme [7]. However, this difference could be due to the fact that in this study a rodent cell line was used and rodent cell lines tend to be more sensitive to hyperthermia, and to the fact that a slightly higher temperature was used.

The 1.6 Gy/3h, on the other hand, when combined with hyperthermia showed sensitization with the two carcinoma cell lines. Even though this sensitization seemed slight on the survival curve, nonetheless it was found to be statistically significant for both cell lines. The normal cell line, however, showed no significant sensitization. This was a very important result in terms of clinical application, therefore it was further investigated by comparing the response of the three cell lines to 1.6 Gy/3h with hyperthermia on the same graph. From the plot, it was observed that the normal cell line still had a higher survival than the sensitive A2780, and although at first the A2780cp had

a higher survival due to its shoulder (possibly due to SLD repair inhibition by hyperthermia), at higher doses the survival for the two cell lines was very close. But most importantly, if the trend of the fit was to be extended past the 10 Gy dose, then the survival for the normal cell line would be higher than that for the resistant carcinoma cells. This would especially be important in the clinic, where doses of 30 Gy or more are used. The 1.6 Gy/3h would therefore present a radiobiological advantage in favour of using PDR in the clinic, especially in combination with mild hyperthermia.

5.0 CONCLUSIONS

From the radiosensitivity parameters and accumulation of sublethal damage, therefore, it can be observed that the three cell lines have different responses to acute irradiation. The A2780s is the most radiosensitive of all the three cell lines according to α and SF2 values, and has the smallest shoulder on the survival curve, which corresponds to a small β . The A2780cp cell line is the most radioresistant according to the radiosensitivity parameters, and has the largest shoulder on the survival curve, or the largest β . The AG1522 cell line has an intermediate radiosensitivity compared to the other two cell lines, and its shoulder on the survival curve is also in between the other two cell lines, therefore this cell line is of normal radiosensitivity.

For the PDR experiments, the A2780cp showed equivalence for the 0.53 Gy/1h, 1.1 Gy/2h, and 1.6 Gy/3h fractionation schemes compared to LDR. The A2780s showed equivalence only between 1.6 Gy/3h and LDR. This cell line showed a possibility of adaptive response in all its PDR treatments, therefore caution is needed when applying PDR to the clinic. The AG1522 cell line showed no equivalence between any of its PDR schemes and LDR. In the clinic, however, dose is optimized to maximize tumour kill and minimize damage to surrounding normal tissues. Repair half-times were calculated using a linear quadratic model with a G-factor [9], however it was found that this method was not an accurate way of estimating these values.

For the PDR experiments with mild hyperthermia, it was determined that hyperthermia at 40°C did not provide a statistically significant thermoradiosensitization for all three cell lines with the 0.53 Gy/1h fractionation scheme. The 1.6 Gy/3h,

however, showed sensitization due to hyperthermia for the two tumour cell lines, but none for the normal cell line. At higher doses, survival for the normal cells would be the highest among all three cell lines if this treatment was used, which could potentially be an advantage in the clinic.

From the PDR treatments investigated, the 1.6 Gy/3h would be the recommended fraction size to be further investigated in clinical PDR brachytherapy, especially in combination with mild hyperthermia.

5.1 Future Research

From this study and several other *in vitro* studies, it was observed that the equivalence between PDR and LDR is cell-line dependent [7,10,12]. More work is therefore needed in this area, especially with human fibroblasts of different radiosensitivities. Also, some cell lines suggested adaptive response, which could have serious implications in the clinic. Therefore further study of adaptive response that could potentially arise from the PDR experiments should be investigated. In particular, adaptive response experiments should be done with the A2780s cell line with the same inducing doses, but for a range of challenge doses (from 2 Gy to 10 Gy). Adaptive response should also be investigated with the AG1522 cell line, to determine if it explains the trend in the PDR alpha values.

The 40°C hyperthermia used with the PDR experiments showed no sensitization in some cases, and in others there was some significant sensitization, but it was still relatively small. A study using 41°C hyperthermia showed a very large sensitization with

PDR [7], therefore PDR with hyperthermia at different temperatures should be investigated.

Recent work described in the literature investigates the effects of modified PDR treatments both using mathematical modeling [11,59,60] and one study has clinical data [61]. A modified PDR treatment drops the constraint that the overall dose and treatment time must be the same as the corresponding conventional brachytherapy treatment, and by allowing somewhat longer overall times [11,62]. This would be an advantage in the clinic from a practical point of view because treatments could be designed with daytime schedules only, which would be convenient both for the patient and for the staff. However, there is very little *in vitro* data with this type of treatment. First, this treatment should be designed on cell lines in which the alpha and beta parameters and the repair half-time are known. Then based on these values, an experiment could be designed in which 2 Gy fraction sizes are given in four fractions per day, with an interval of 3h, for example [11].

Experiments should be designed to further test the model that was used in the PDR experiments [9]. This model showed discrepancies between estimated and experimental values, therefore careful experimentation should be performed using low dose rate irradiation to determine both the experimental and calculated values of the repair half-time. An experiment would involve splitting a dose into two separated by a time interval, usually six hours long, during which recovery takes place. The alpha and beta parameters can be estimated from low dose rate survival curves. Also, it is possible that there are more than one component to the repair, one fast and one slow, and this should also be investigated [44].

APPENDIX A

The linear quadratic parameters estimation was done using the method of least squares (equation 10). For the statistical analysis, unweighted least squares were used. This was done for all the experiments, and here the LDR and 1.6 Gy/3h experiments for A2780cp are used as an example. To be able to use this statistical analysis, the negative natural logarithm of all the surviving fractions was taken and modeled as follows:

$$S_i = \alpha D_i + \beta D_i^2 + \varepsilon_i \quad (\text{A-1})$$

where S_i is the value of the response (transformed surviving fraction) of the i th trial, D_i is the value of the independent variable (dose) in the i th trial, ε_i is the random error term, and α and β are the LQ parameters [63]. This model is then used in the least squares method (equation 10) to estimate the values of the α and β parameters.

For the LDR with A2780cp case, the estimated parameters with their standard errors were found to be:

$$\hat{\alpha} = 0.3313 \pm 0.0439 \text{ Gy}^{-1}$$

$$\hat{\beta} = 0.0004 \pm 0.0052 \text{ Gy}^{-2}$$

It can be observed that the standard error on the beta value is larger than the actual value. To determine the significance of the quadratic component, the full LQ model was tested against a reduced model with only the linear component, and significance was measured using the F-statistic, which compares two population variances [64]. This was done for all experiments with betas smaller than their error:

$$\text{Full model: } f(D_i) = \alpha D_i + \beta D_i^2$$

$$\text{Reduced model: } f(D_i) = \alpha D_i$$

From the reduced model,

$$\hat{\alpha} = 0.333 \pm 0.163 \text{ Gy}^{-1}$$

To use the statistic to test the hypothesis that the quadratic component is not statistically significant from zero, first some values had to be calculated for both the full and the reduced models:

Sum of squares:

$$SSE = \sum_{i=1}^n (S_i - \hat{f}(D_i))^2 \quad (\text{A-2})$$

Where S_i is the transformed surviving fraction, and $f(D_i)$ is the negative natural logarithm of the linear quadratic model.

$$\text{Mean square: } MSE = SSE/(n-2) \quad (\text{A-3})$$

Where $n-2$ is the number of degrees of freedom (total number of data points minus the two parameters, alpha and beta).

The test statistic is then calculated:

$$F^* = (SSE_r - SSE_f)/(2 * MSE_f) \quad (\text{A-4})$$

Where the subscripts r and f stand for reduced and full model, respectively.

If $F^* < F(1-\alpha; 1, n-2)$, then the quadratic component is not statistically different from zero (Note: the α used here is not the alpha parameter of the LQ model, but it is used to calculate confidence coefficients).

If $F^* \geq F(1-\alpha; 1, n-2)$, then the quadratic component is significant.

F distribution is a distribution based on the number of degrees of freedom and the $(1-\alpha)100$ percentile, i.e. $F(1-\alpha; v_1, v_2)$, where v_1 and v_2 are the respective numbers of degrees of freedom. In all the comparisons, the test was estimated at a 95% level of significance. The v_1 was the number of adjustable parameters in the reduced model (usually equal to 1 or 2, depending on the model), and v_2 was taken to be n -(number of adjustable parameters in the full model).

For the LDR case, F^* was calculated to be 0.00180, which was much smaller than the F value of about 4.5 (from tables), therefore the quadratic component was found to be statistically not significant from zero.

To establish PDR-LDR equivalence, the data for LDR and the particular fraction size, 1.6 Gy/3h, for example, were pooled together. The full and reduced models become:

$$\text{Full model: } f(D_i) = \alpha_{\text{PDR+LDR}}D_i + \beta_{\text{PDR+LDR}}D_i^2$$

$$\text{Reduced model: } f(D_i) = \alpha_{\text{LDR}}D_i$$

where the data is fitted by a model that is a combination of the LDR and PDR alpha's and beta's. The number of degrees of freedom in this case is $n-2$ for the full model, and $n-1$ for the reduced model since there is only one parameter.

The $SSE_f = 2.32$, and the $MSE_f = 0.0627$ for the full model. The $SSE_r = 2.657$ for the reduced model, and therefore the F^* statistic was calculated according to equation (A-4), and it was found to be 2.53, which is smaller than $F(.95; 1, 37) = 4.15$ (from tables), therefore the two treatments were determined to be equivalent.

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IMAGE EVALUATION TEST TARGET (QA-3)

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