INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

**ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600**

UMI[®]

**Examining the role of the Non-Homologous End-Joining
repair pathway in Cisplatin Radiosensitization
and Sub-Lethal Damage Repair**

by

Winston Kenji Myint

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
Ottawa-Carleton Institute of Physics
Ottawa, Ontario
January, 2001
© copyright
2001 Kenji Myint

**National Library
of Canada**

**Acquisitions and
Bibliographic Services**

**395 Wellington Street
Ottawa ON K1A 0N4
Canada**

**Bibliothèque nationale
du Canada**

**Acquisitions et
services bibliographiques**

**395, rue Wellington
Ottawa ON K1A 0N4
Canada**

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-66914-9

Canada

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis

**Examining the role of the Non-Homologous End-Joining
repair pathway in Cisplatin Radiosensitization
and Sub-Lethal Damage Repair**

submitted by

Winston Kenji Myint

in partial fulfillment of the requirements
for the degree of Master of Science

Thesis Supervisor

Chair of Department of Physics
for J. C. Armitage

Carleton University

ABSTRACT

The application of cisplatin in combined chemo-radiation treatments may be limited by the lack of understanding of the underlying mechanism in cisplatin radiosensitization. It is thought that this process is linked to the inhibition of repair of DNA double-strand breaks, though it is uncertain of which repair pathway is involved in mammalian cells. Ku80^{-/-} cells are deficient in the non-homologous DNA double-strand break repair process and were consequently used to assess the relation of this pathway with the mechanism of cisplatin radiosensitization and repair of sub-lethal damage. Both Ku80-deficient cells and the wild-type MEF cells were exposed to clinically applicable doses of cisplatin for 1 hour immediately prior to exposure to 250 kV x-rays.

Radiosensitization was observed in the wild-type cells but none in the Ku80^{-/-} cells, implying that the non-homologous end-joining pathway may play a large role in cisplatin radiosensitization. Concurrent administration of cisplatin with radiation produced similar levels of radiosensitization. Conversely, higher concentrations of cisplatin applied immediately prior to radiation revealed an increasing resistance to radiation in both cell lines. This trend is not thought to be tied to the NHEJ process but rather to resistant sub-populations of cells remaining after subsequently lethal doses of cisplatin. Further experiments revealed that the high concentrations of cisplatin did not alter cell cycle distribution. Finally, split dose experiments revealed that the NHEJ pathway also plays a significant role in sub-lethal damage repair.

ACKNOWLEDGEMENTS

There are many individuals whom I've had the privilege of working with to the completion of this thesis and I'd like to express my appreciation for each and everyone of them. First and foremost, I'd like to thank my supervisor Dr. Peter Raaphorst for his knowledge, encouragement, and ongoing support. It was a great benefit to feed off of his love of science and optimistic nature. I would also like to thank Dr. Cheng Ng for his knowledge and assistance in the lab.

It was a pleasure to work with and learn from the many students and technicians in the physics lab at the Ottawa Regional Cancer Center. In particular, I would like to thank the lab manager Sheryl Cybulski for sharing her experience and knowledge. I'd also like to express my gratitude to Daron Owen for his knowledge and more importantly, for his friendship.

Finally, the never-ending support and encouragement from my parents has helped me more than they will ever know. Last but not least, I would like to thank Christine Payant for her knowledge and patience throughout this entire study, and for her strength and support when things seemed most difficult.

TABLE OF CONTENTS

	Page
Acceptance Form.....	ii
Abstract.....	iii
Acknowledgements.....	iv
Table of Contents.....	v
Key Abbreviations.....	vii
List of Figures.....	viii
List of Tables.....	x
1.0 INTRODUCTION.....	1
1.1 X-radiation.....	1
1.1.1 Linear-Quadratic (LQ) Model.....	3
1.2 Cisplatin.....	5
1.3 Cisplatin and X-Radiation.....	9
1.3.1 <i>In vitro</i> Studies.....	9
1.3.2 Clinical Studies.....	10
1.4 DNA DSB Repair.....	12
1.4.1 NHEJ (Non-Homologous End-Joining).....	13
1.4.2 Ku80.....	16
1.5 Hypothesis.....	17
2.0 MATERIALS AND METHODS.....	19
2.1 Cell Lines.....	19
2.2 HDR Irradiation and Cisplatin Treatments.....	20
2.3 Clonogenic Assay.....	21
2.4 Curve Fitting.....	23
2.5 Split Dose Recovery.....	24
2.6 Flow Cytometry.....	25

	Page
3.0 RESULTS.....	27
3.1 Cisplatin Radiosensitization.....	27
3.1.1 HDR Irradiation.....	27
3.1.2 Cisplatin Response.....	30
3.1.3 Cisplatin and HDR Irradiation Experiments.....	32
3.1.4 Optimizing Radiosensitization.....	45
3.1.5 Flow Cytometry.....	49
3.2 Split Dose Experiments.....	53
4.0 DISCUSSION.....	57
4.1 Cisplatin Radiosensitization.....	57
4.1.1 HDR Irradiation.....	57
4.1.2 Cisplatin Response.....	59
4.1.3 Cisplatin and HDR Irradiation Experiments.....	60
4.1.4 Optimizing Radiosensitization.....	66
4.1.5 Flow Cytometry.....	68
4.2 Split Dose Experiments	69
5.0 CONCLUSIONS.....	74
5.1 Future Research.....	77
6.0 REFERENCES.....	79

KEY ABBREVIATIONS

Cisplatin	<i>cis</i> -diamminedichloroplatinum (II)
CHO	chinese hamster ovary
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PK _{cs}	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
HDR	High dose rate
HR	Homologous recombination
IMRT	Intensity-modulated radiotherapy
LDR	Low dose rate
LQ	Linear-Quadratic
MEF	Murine embryonic fibroblast
NHEJ	Non-homologous end-joining
SF	Surviving fraction
SIR	Silent information regulator
SLD	Sub-lethal damage
SSB	Single-strand break
Transplatin	<i>trans</i> -diamminedichloroplatinum (II)

LIST OF FIGURES

	Page
Figure 1.1	A schematic diagram demonstrating the direct and indirect action of x-radiation interacting with the DNA helix. 2
Figure 1.2	Schematic representation of cisplatin forming reactive species and reacting with DNA to form monofunctionally and bifunctionally bound adducts. 8
Figure 1.3	A proposed model for NHEJ in mammalian cells 15
Figure 3.1	Comparing the responses of the wild-type MEF's and the Ku80 ^{-/-} cell line to a range of HDR x-radiation. 29
Figure 3.2	The response of the MEF and Ku80 ^{-/-} cell lines to a range of cisplatin concentrations for a 1 hour treatment period. 31
Figure 3.3	Comparing the HDR radiation response of the MEF cell line to the response from a combined treatment of cisplatin and HDR radiation (1 µg/mL cisplatin for 1 hour immediately prior to irradiation). 35
Figure 3.4	Comparing the HDR radiation response of the Ku80 ^{-/-} cell line to the response from a combined treatment of cisplatin and HDR radiation (1 µg/mL cisplatin for 1 hour immediately prior to irradiation). 36
Figure 3.5	The unnormalized response of the MEF cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 µg/mL of cisplatin for a 1 hour period immediately prior to irradiation. 41
Figure 3.6	The normalized response of the MEF cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 µg/mL of cisplatin for a 1 hour period immediately prior to irradiation. 42
Figure 3.7	The unnormalized response of the Ku80 ^{-/-} cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 µg/mL of cisplatin for a 1 hour period immediately prior to irradiation. 43
Figure 3.8	The normalized response of the Ku80 ^{-/-} cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 µg/mL of cisplatin for a 1 hour period immediately prior to irradiation. 44

	Page
Figure 3.9	Comparing the HDR response of the MEF cell line to the combined treatment of cisplatin and HDR radiation given in two different protocols. In the first, the cisplatin is given for 1 hour immediately prior to irradiation. In the second, the irradiation is given halfway through the 1 hour cisplatin treatment.
	46
Figure 3.10	Comparing the HDR response of the Ku80 ^{-/-} cell line to the combined treatment of cisplatin and HDR radiation given in two different protocols. In the first, the cisplatin is given for 1 hour immediately prior to irradiation. In the second, the irradiation is given halfway through the 1 hour cisplatin treatment.
	47
Figure 3.11	The surviving fraction of the MEF cell line in response to 6 µg/mL of cisplatin for 1 hour followed by 4 Gy of HDR radiation, given 0, 4, and 8 hours between the cisplatin and radiation treatments.
	50
Figure 3.12	The split dose recovery (4 Gy per dose) of the MEF cell line grown to confluency, given 0, 1, and 3 µg/mL of cisplatin for 1 hour immediately prior to the initial dose of radiation. A cisplatin split dose recovery (3 µg/mL cisplatin per dose) was also measured.
	55
Figure 3.13	The split dose recovery (4 Gy per dose) of the Ku80 ^{-/-} cell line grown to confluency, given 0, 1, and 3 µg/mL of cisplatin for 1 hour immediately prior to the initial dose of radiation. A cisplatin split dose recovery (3 µg/mL cisplatin per dose) was also measured.
	56

LIST OF TABLES

	Page
Table 3.1	Comparison of the radiosensitivity parameters, α , β , and SF_2 , between the parental MEF and mutant Ku80 ^{-/-} cell lines given high dose rate irradiation.
Table 3.2	P-values from a student t-test comparing individual dose points between the MEF and Ku80 ^{-/-} radiation responses.
Table 3.3	P-values from a student t-test comparing individual dose points between the MEF and Ku80 ^{-/-} cisplatin responses.
Table 3.4	Comparing the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line between the HDR radiation response and the response from a combination of 1 μ g/ml cisplatin for 1 hour immediately prior to HDR radiation.
Table 3.5	P-values from a student t-test comparing individual dose points between the MEF cell responses from radiation treatment alone and from the combination of 1 μ g/mL of cisplatin with radiation.
Table 3.6	Comparing the radiosensitivity parameters, α , β , and SF_2 , of the Ku80-deficient cell line from the HDR radiation response and the response from a combination of 1 μ g/ml cisplatin for 1 hour immediately prior to HDR radiation.
Table 3.7	P-values from a student t-test comparing individual dose points between the Ku80-deficient cell responses from radiation treatment alone and from the combination of 1 μ g/mL of cisplatin with radiation.
Table 3.8	A summary of the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line for the HDR radiation response and the response from a combination of 1, 3, and 6 μ g/ml of cisplatin for 1 hour immediately prior to HDR radiation.
Table 3.9	A summary of the radiosensitivity parameters, α , β , and SF_2 , of the Ku80 ^{-/-} cell line for the HDR radiation response and the response from a combination of 1, 3, and 6 μ g/ml of cisplatin for 1 hour immediately prior to HDR radiation.

		Page
Table 3.10	Comparing the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line when given 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR radiation and when treated with HDR during (halfway through) the same 1 hour treatment of cisplatin.	48
Table 3.11	Comparing the radiosensitivity parameters, α , β , and SF_2 , of the Ku80 ^{-/-} deficient cell line when given 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR radiation and when treated with HDR during (halfway through) the same 1 hour treatment of cisplatin.	49
Table 3.12	Cell cycle distributions obtained from flow cytometry techniques for the parental MEF cell line given treatments of 0, 1, and 3 $\mu\text{g/mL}$ of cisplatin for a 1 hour period.	52
Table 3.13	Cell cycle distributions obtained from flow cytometry techniques for the Ku80 deficient cell line given treatments of 0, 1, and 3 $\mu\text{g/mL}$ of cisplatin for a 1 hour period.	52

1.0 INTRODUCTION

Radiotherapy has established itself as an effective treatment for a variety of cancers and has progressed in quality and accuracy as technological advances have been made. In recent years, the combined treatment of chemotherapy and radiotherapy have been explored extensively in clinical studies, revealing promising results for an assortment of cancers [Brattstrom, 2000, Montana, 2000, Santin, 2000]. Not only does this combination modality treatment have the potential to increase the killing of tumour cells and inhibit repair of radiation damage, it has demonstrated an increase in survival and local control rates as well.

Certain chemotherapeutic agents, such as cisplatin, have the potential to radiosensitize tumour cells, a benefit which is continually being developed in chemo-radiation treatments. Though this radiosensitizing effect has been observed in numerous studies [Geldof, 1999, Glass, 1997, Raaphorst, 1994], the underlying mechanism is not well understood. A greater understanding of this mechanism would increase the potential of combined chemo-radiation therapy modalities as well as enable the optimization of such a treatment process.

1.1 X-Radiation

The significant interactions between x-radiation and biological matter are those that produce molecular or atomic ionizations. The resultant ionizations can alter the molecular structure and function. Exposure to x-radiation causes a variety of lesions in

the DNA including single-strand breaks (SSB), double-strand breaks (DSB), base alterations, as well as intra- and inter-strand crosslinks. Ionizing radiation produces approximately 1000 SSBs and 25 to 40 DSBs per cell per gray for most cell types, with a few exceptions such as hypoxic cells [Olive, 1998]. If this damage is left unrepaired, there is a possibility that the cellular structure and function will also be affected. These changes, in turn, can lead to cell transformations, somatic mutations, or cell death.

Figure 1.1 A schematic diagram demonstrating the indirect and direct action of x-radiation interacting with the DNA helix. [Adapted from Hall, 1994].

In biological matter, the critical target is the DNA (deoxyribonucleic acid) of cells [Hall, 1994]. When the energy from x-radiation is absorbed in the cells, there is a probability that it will interact with the DNA either directly or indirectly (Figure 1.1). When the atoms of the DNA are ionized by radiation, the radiation is interacting directly with the critical target. The indirect action occurs when ionizations form in surrounding molecules, such as water, in the nearby environment of the DNA strands. These ionized molecules form highly reactive free radicals (molecules which contain an unpaired electron in their outer shell, eg. hydroxyl radical) which can subsequently interact and cause damage to the DNA helix. It is generally believed that the lethal lesion is a double-strand break in the DNA of cells [Douple, 1990].

1.1.1 Linear-Quadratic (LQ) Model

The response of a cell line to radiation is often fitted to a survival model, not only to develop a further understanding of the data, but also for clinical applications. Radiobiological cell survival models are aimed at quantitatively describing the physical, biological, and chemical effects of radiation action. Hence, as our understanding of the mechanisms associated with radiation action have developed, the mathematical models representing these theories have subsequently been modified.

Cell survival models have undergone many stages of development, but the one common trait among them all is the endpoint, cell death. Early theories such as the single-hit multitarget model [Lea, 1955], the multi-hit multitarget model [Puck, 1956], and the two-component multitarget model [Bender, 1962] were based on target theory

and so the dose-dependence was arbitrarily formulated. Furthermore, the mathematical parameters involved in their models had no logical link to cellular mechanisms. In 1973, Chadwick and Leenhouts explained the effect of radiation on cells by assuming that a double-strand break in the DNA-helix was the critical damage leading to cell death while also assuming that certain repair processes were taking place [Chadwick, 1973]. The model was based on a theory of dual radiation action proposed by Kellerer and Rossi in the early 1970's. The main assumptions considered were that: (i) low- and high-dose levels produce different modes of radiation action (ii) radiation produces sublesions within the cell, directly proportional to the absorbed dose and (iii) a biological lesion occurs when two sublesions within the cell combine with one another, each lesion having a fixed probability to give a biological effect, and all pairs of sublesions within a sensitive site have an equal probability of combining. On the basis of this theory, they presented the linear-quadratic model:

$$S(D) = \exp(-\alpha D - \beta D^2) \quad (\text{Eqn.1})$$

which emphasizes that the effect of radiation dose depends on various biological and physical phenomena, and not only on the simple kinetics of dual lesions. D represents the single dose fraction and S represents the survival fraction, the fraction of total cells which retain reproductive integrity. The linear (α) component represents the double strand breaks which are caused by a single ionizing event, and the quadratic (β) component represents two independent ionizing events. The independent events are considered to be two individual single-strand breaks which occur in relatively close

proximity as well as within the same time frame. This damage can often be repaired more easily than double-strand breaks if given sufficient time and is located at break sites far enough apart from each other. Thus, the quadratic term is also considered to be representative of the level of sub-lethal damage repair which occurs.

Since the development of the LQ model, other more specific models have been created, focussing on stochastic processes in their formulation. Examples of such models are the repair-misrepair model [Tobias, 1985], the incomplete-repair model [Thames, 1985], the lethal-potentially lethal model [Curtis, 1986], and the saturable repair model [Goodhead, 1985]. Even though these models have expanded on the LQ model, it's interesting to note that the LQ model still succeeds as the popular choice. The newer models have shown greater correlation to cellular mechanisms, but in doing so have gained many more mathematical parameters and terms, complicating the dose prescriptions of complex radiotherapy treatments [Brenner, 1997, Fowler, 1989]. Thus, the LQ model retains a simplistic beauty while still keeping good experimental fit with data and agreeing with biological theory.

1.2 Cisplatin

Cis-diamminedichloroplatinum(II) (cisplatin) is one of the most widely used chemotherapeutic agents in cancer therapy. It is very effective in treating certain types of cancer including testicular and ovarian cancers. The cytotoxic effect of cisplatin arises from the formation of cisplatin-DNA covalent adducts which cause cell cycle arrest, inhibition of DNA replication and transcription, and eventually apoptosis [Turchi, 2000].

It has become evident that cells lacking the ability to repair these adducts are rendered extremely sensitive to this particular damage to the DNA. This has been established for xeroderma pigmentosum cells, Fanconi's anaemia fibroblasts, and repair-deficient Chinese hamster ovary cells [Fichtinger-Schepman, 1995].

The cisplatin molecule itself is small, neutral, and platinum based, with two amine groups and two chloride atoms arranged in a square-planar *cis*- geometry. When placed within the cellular environment, the molecule undergoes hydrolysis where one or both of the chloride atoms are replaced with water molecules (Figure 1.2). Two different reactive species can be formed, which are both able to bind to the DNA to form either monofunctional or bifunctional adducts. With bi-functionally bound adducts, either inter-strand or intra-strand crosslinks can be formed.

The cisplatin molecule mainly reacts with the N7 position of either the guanine or adenine nucleotide of the DNA backbone. The majority (approximately 70 %) of the adducts formed are intra-strand crosslinks between adjacent guanines [Yang, 1996]. A smaller portion (approximately 15%) are intra-strand crosslinks between adjacent adenine and guanine molecules. The remaining adducts consist of N1 to N3 intra-strand crosslinks between guanines separated by another nucleotide, inter-strand guanine to guanine crosslinks where the DNA sequence is a guanine followed by cytosine, and finally monofunctionally bound adducts with guanine or adenine. It has been suggested that the biological activity of cisplatin is a result of the intra-strand crosslinks between adjacent guanines or adjacent guanine and adenine pairs. It is believed that these

crosslinks form a structural distortion within the DNA strand, resulting in harmful effects on transcription and replication [Yang, 1996]. The manner in which these distortions affect various cycles of the cell are not well understood. This is exemplified by the isomer of the platinum (II) compound, *trans*-diamminedichloroplatinum (II) (transplatin), which differs structurally from cisplatin in that the lower chloride atom is switched with the lower amine molecule, placing the two chloride atoms at opposite diagonals. This isomer can form adducts with DNA much like cisplatin, but in contrast is not an effective anticancer drug.

Figure 1.2 Schematic representation of cisplatin forming reactive species and reacting with DNA to form monofunctionally and bifunctionally bound adducts [Adapted from Dewitt, 1987].

1.3 Cisplatin and X-Radiation

Individually, each agent has established itself as a potent treatment modality in the battle against cancer. Each modality has been extensively studied, and it has become apparent that the underlying mechanisms for their cytotoxic abilities vary greatly.

Cisplatin acts to damage and kill cells from the formation of cisplatin-DNA adducts, whereas x-radiation interacts with the DNA by forming ionizations and free radicals. An *in vitro* study examined the cisplatin response from three radiation sensitive mutant cell lines paired with normally responding or radiation resistant cell lines of parental or similar origin [Raaphorst, 1994]. The radiation sensitive cell lines were V3 (Chinese hamster ovary), AT5BI (human fibroblast), and HT144 (melanoma), all showing reduced ability to repair sublethal radiation damage. In addition, V3 was incapable of repairing radiation induced DNA DSBs and AT5BI was deficient in nucleotide excision repair. Each of the cell lines revealed cisplatin responses which were similar to those of the corresponding parental cell line with the exception of AT5BI which revealed an increased resistance to cisplatin, indicating no cross sensitivity between radiation and cisplatin. This supports the theory that the mechanisms of action from the two agents are not the same for these cell lines.

1.3.1 *In vitro* Studies

When the cisplatin and x-radiation treatments were combined together, many *in vitro* and *in vivo* studies had revealed very promising results, though not always without complications. One study investigated the effect of combining cisplatin with radiotherapy treatments on a prostate tumour cell line, comparing the combined treatment

survival response with that of the radiation response [Geldof, 1999]. The cisplatin was applied to the cells concomitantly with irradiation in one experimental protocol, and 3 hours prior to irradiation in a second protocol. In both conditions, the combination of cisplatin with irradiation demonstrated synergistic treatment effects, implying that the cytotoxic effect was higher than the sum of the individual treatment cytotoxicities (supradditivity). Another *in vitro* study on human cervical carcinoma cells evaluated the effect of clinically relevant levels of cisplatin on radiation sensitivity [Britten, 1996]. This experimental procedure also involved the concomitant administration of cisplatin with irradiation, but showed different results. In this set of experiments, 19 cloned human cervical tumour cell lines exposed to the combined treatment resulted in additive toxicities, but not supradditivity. The group suggested that the higher level of tumour cell kill would still enhance local control, via additivity and not radiosensitization. The results from many different cell lines revealed enough potential for many future groups to pursue *in vivo* and eventually, clinical studies.

1.3.2 Clinical Studies

In recent years, cisplatin/radiotherapy treatments have been incorporated into many clinical trials to investigate its effect on an assortment of different cancers and under a variety of different conditions. Many studies demonstrated greater local control rates, a good benefit for metastatic carcinomas [Brattstrom, 2000, Glass, 1997, Dunkel, 2000, Eifel, 2000]. However, the toxicity of cisplatin, including nausea, vomiting, nephrotoxicity, neurotoxicity, and ototoxicity, were of concern to the patients, so much that it prevented certain patients from continuing with particular studies [Brahmer, 1998].

A study examining the effect of three-dimensional intensity-modulated radiotherapy (IMRT) in the treatment of nasopharyngeal carcinoma applied concurrent chemotherapy (cisplatin) with the therapy and observed increased levels of local-regional control [Sultanem, 2000]. The group was also hoping to demonstrate that the combined treatment would result in a decrease in the number of distant metastases in their patients. Unfortunately, the distant metastasis-free rate was the same as was observed with IMRT alone. Another study involving patients with carcinoma of the vulva also looked to combining modalities for higher local control rates [Montana, 2000]. In addition, the group determined that the patients could undergo resection of the lymph nodes and primary tumour following preoperative chemo-radiation. A third interesting application of concurrent administration of cisplatin with radiation therapy was investigated on the immune function of patients with locally advanced cervical cancer [Santin, 2000]. Santin et al found that the treatment did not alter the magnitude and the characteristics of radiation-induced immunosuppression. It is evident that the radiosensitizing potential of cisplatin is apparent for *in vitro* studies, but the extent to which the drug can be used clinically is still left uncertain.

In order for a drug to be clinically useful as a radiation sensitizer, it must have a greater effect on tumour response than on the response of the critical normal tissue. The treatment conditions, such as time of delivery and applied concentration, must be optimized to maximize the synergistic effect with radiation. Additionally, the drug can not interfere with the radiation treatment schedule. For example, if hematological or gastrointestinal toxicities were to delay the course of radiation, then the sensitizing effect

is wasted as residual cancer cells are given time to repopulate [Eifel, 2000]. To aid cisplatin in fulfilling these criteria, it would be helpful to understand the mechanism of action in which the drug sensitizes cells to radiation. This mechanism is not well understood, though studies have suggested that it is connected to the inhibition of repair of radiation-induced DNA damage [Douple, 1990]. This was demonstrated in studies which observed a reduction in the “shoulder” region of the chemo-radiation response [Douple, 1990], reduced levels of potentially lethal damage recovery [Wilkins, 1993], and finally, decreased levels of sub-lethal damage repair when compared with radiation treatments alone [Schwachofer, 1991].

1.4 DNA DSB Repair

Unrepaired DSBs are likely to result in cell death and if incorrectly repaired, will likely cause mutagenesis and possibly carcinogenesis. Therefore, it is important for the cell to have multiple mechanisms for the repair of these lesions. Although there are many forms of DNA repair, two major pathways are thought to be responsible for the repair of DSBs in eukaryotes. The first process is homologous recombination (HR) and results in the precise repair of the DNA lesion but requires the presence of homologous sequences (ie. homologous chromosome) found in the genome. This is the predominant repair mechanism of DSBs in yeast cells, while mammalian cells show a low frequency of homologous recombination activity [Jeggo, 1998, Marcon, 2000]. Instead, the mechanism which plays the major role for repair of DSBs in mammalian cells requires little or no homology, a process known as non-homologous end-joining (NHEJ) or non-homologous recombinational repair. Both repair pathways are evident in yeast and

mammalian cells, but the extent to which each plays a role in the repair of DSB differs. A Recent study indicated that the HR and NHEJ may be complementary to each other for DSB repair in mammalian cells [Takata, 1998]. The study showed that the two repair mechanisms may be cell cycle dependent in mammalian cells. The extent to which HR plays a role in the response of mammalian cells to radiation is still unclear. Studies show that cells derived from *rad54* (an essential gene in the HR process) knockout mice reveal a sensitivity to radiation, though the sensitivity is not very drastic [Taylor, 1998] . Furthermore, cells recently constructed from a *rad52* (another essential gene involved with HR) knockout mouse show no sensitivity to radiation at all. This implies that mammalian cells have alternative routes for repairing DNA double-strand breaks.

1.4.1 NHEJ

The first gene involved with NHEJ for DNA DSB repair was identified in 1994, triggering the determination of an assortment of components related to the process in the following years [Taccioli, 1994]. The recent identification of all of the genes involved with this repair pathway has led to several proposed models for the NHEJ process.

The principal component of the NHEJ pathway is the Ku70/Ku80 heterodimer, a pair of proteins 70 kDa and 86 kDa in mass, respectively [Wu, 1996]. Both the Ku70 and Ku80 proteins are present in all eukaryotic cells and have a high affinity for binding to DNA DSBs [Jin, 1997, Lustig, 1999, Boulton, 1996]. The component often affiliated with the Ku complex is the DNA-dependent protein kinase (DNA-PK), consisting of a 465 kDa catalytic subunit (DNA-PK_{cs}) [Critchlow, 1998, Cary, 1997] and the Ku

complex as the other subunit [Yaneva, 1997]. DNA-PK_{cs} can also bind to DNA ends in the absence of Ku, though it is thought that Ku is required for stability.

Two other components involved in NHEJ are DNA ligase IV and XRCC4. XRCC4 is a nuclear phosphoprotein and is an effective substrate for DNA-PK *in vitro*. Studies have shown that it has a strong and specific interaction with DNA ligase IV and may act to target it to the break site and stimulate its DNA end-joining activity. There are also several proteins whose functions are still a matter of speculation but are suspected to play a role in NHEJ. These are the RAD50 and MRE11 proteins in mammalian cells, which are joined by XRS2 in yeast cells [Lustig, 1999]. It is speculated that these proteins act in a multi-protein complex for either meiotic or mitotic recombination and for NHEJ in yeast cells. It has not been determined if these proteins play a role in NHEJ for mammalian cells. Finally, the last set of proteins involved in this pathway are silent information regulator (SIR) genes, Sir2p, Sir3p, and Sir4p, functioning as transcriptional silencing genes.

A model for NHEJ in mammalian cells is shown in Figure 1.3 (proposed by Jeggo, 1998). The first stage of the model begins with the Ku complex binding to the DSB site at the DNA ends, after which it recruits DNA-PK_{cs}. The Ku protein is thought to translocate along the DNA and associate with the Sir proteins (along with factors interacting with these proteins) to condense the chromatin in the region of the strand break. The purpose of this condensation may be to prevent nucleolytic degradation of the free DNA ends, to prevent the free ends from errant rejoining, and to inhibit transcription

Figure 1.3 A proposed model for NHEJ in mammalian cells [Adapted from Jeggo, 1998]. Step 1: Ku binds to double-strand DNA ends, recruits DNA-PK_{cs}, and activates DNA-PK activity. Step 2: Further proteins are recruited (Sir2p, 3p, and 4p) and result in condensation of chromatin, reducing accessibility of DNA ends. Step 3: Recruitment of further proteins results in release of Sir proteins and chromatin decondenses. DNA ligase IV interacting with XRCC4 facilitates rejoining of the break.

and replication in the area of the double strand break. The Ku complex further interacts with XRCC4, and together recruit the aid of DNA Ligase IV which reassembles the strand break [Ramsden, 1998]. Once the necessary repair proteins have been recruited, the accessibility of the site may be increased by decondensation of the chromatin. Recent reports show that Mre11p, Rad50p, and Xrs2p affect chromatin structure during meiosis [Lustig, 1999]. Therefore, it is possible that these proteins may also play a similar role in the NHEJ pathway.

1.4.2 Ku80

Ku was originally discovered as an autoantigen in patients with polymyositis-scleroderma overlap syndrome. The term Ku was taken from the first two letters of the original patients name. It has become evident that the Ku protein complex plays a vital role in the repair of ionizing radiation-induced DNA DSBs through aiding in the mechanism of NHEJ. It is one of the most abundant DNA end-binding proteins in the human cell [Featherstone, 1999] and is still being researched for its role in a variety of pathways [Osipovich, 1999]. Ku's role involves protection of DNA ends from degradation, bridging of DNA ends prior to joining, and recruitment and activation of proteins that repair DSBs [Ramsden, 1998].

The determination of the genes involved in NHEJ have enabled the production of mutant cell lines deficient in Ku80 gene, XRCC5 [Jeggo, 1998]. The functional potential of the Ku80 protein has been examined by studies that have utilized such mutant cell lines and mice [Marangoni, 2000]. Ku80^{-/-} mutants not only have low or undetectable levels of the Ku80 protein, but also lack double-strand DNA end-binding and DNA-PK activities [Singleton, 1997]. An *in vitro* study involving Ku80-deficient mouse fibroblasts revealed that the cells were sensitive to ionizing radiation and that the increased radiosensitivity was possibly due to the deficiency in DSB rejoining [Wachsberger, 1999].

Further studies have verified these results with other Ku80^{-/-} mutant cell lines, and have also demonstrated that transfection of these cells with Ku80 cDNA restores the

radiation resistance and DSB repair efficiency [Burgman, 1997; Ross, 1995; Stevens, 1999]. Another study involving Ku80^{-/-} mouse cells showed that these cells have a significantly increased number of chromosomal aberrations (eg. DNA strand breaks), indicating that Ku80 may also be responsible for maintaining genomic stability [Difilippantonio, 2000]. An *in vivo* study investigated the effects of ionizing radiation on Ku80-deficient mice. These experiments showed similar results to those found from most *in vitro* studies, displaying a hypersensitivity to gamma radiation characterized by loss of hair pigmentation, severe injury to the gastrointestinal tract, and enhanced mortality [Nussenzweig, 1997].

1.5 Hypothesis

The clinical applications of cisplatin in combined chemotherapy and radiotherapy treatments may be limited by the lack of understanding of the underlying mechanism in cisplatin radiosensitization. Studies have shown that this mechanism may be linked to the inhibition of repair of ionizing radiation-induced DNA double-strand breaks. This study aimed to take this a step further by specifying the mechanism of repair. Therefore, it is hypothesized that the mechanism of radiosensitization by cisplatin is linked to the non-homologous end-joining pathway of DNA DSB repair in eukaryotic cells.

The study was initially organized with three main objectives to accomplish, though each objective was highly dependent upon the results of the previous objective. The first objective of this study was to determine whether cisplatin could radiosensitize murine cells using various clinically applicable concentrations of cisplatin. The second

objective was to examine the role of NHEJ in the radiosensitizing mechanism by utilizing a mutant murine cell line deficient in the Ku80 protein. This objective was expected to branch into several supporting experiments whose goals would be to optimize and stabilize the treatment conditions during the second objective. Finally, the third objective was to elucidate any correlation of sub-lethal damage repair with non-homologous end-joining and the mechanism of cisplatin radiosensitization.

The experimental procedures and apparatus utilized within this study are described in the Materials and Methods section, including the cell lines used, the type of treatments applied, and the assays and experiments performed. The data from the cisplatin radiosensitization and split dose experiments are listed and plotted in the Results section, including radiosensitivity parameters corresponding to the responses from various treatments. These data are then subsequently described in the Discussion section. Finally, a summary of the thesis along with comments on future work are covered in the Conclusions section.

2.0 MATERIALS AND METHODS

2.1 Cell Lines

Experiments were performed on two cell lines in this study, both of which were donated by P. Burgman and G. Li from the Memorial Sloan-Kettering Cancer Center in New York, NY. The first cell line was a murine embryonic fibroblast (MEF) and was used to demonstrate the response of normal cells to drug and radiation treatments. The second cell line was derived from the MEF cells (by the aforementioned donors) and is referred to as the Ku80^{-/-} cell line. They are deficient in the protein Ku80, a key element in the process of non-homologous recombinational repair. As a result, this cell line is considered to be incapable of performing this repair process and is known to be extremely sensitive to x-radiation. The Ku80^{-/-} cell line was used to examine the role of the non-homologous recombinational repair pathway in cisplatin radiosensitization and sub-lethal damage repair.

The cell lines were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (F-12), both obtained from Gibco. The media was supplemented with 10% Fetal Bovine Serum (Gibco) and 0.1mM MEM non-essential amino acids (Gibco). The pH was buffered using 20 mM HEPES (Boehringer Mannheim) and 10 mM sodium bicarbonate (Sigma), and the cells were incubated at 37°C in a humidified atmosphere of 2% CO₂ and 98% air.

All experiments were prepared by inserting 5×10^5 cells into 25 cm² tissue culture flasks in a solution of 4mL of medium. After 3 days, the depleted medium was aspirated and fresh medium was added allowing further growth of the cells. The experiments were performed on the 5th day when the cells had reached confluency. Cells are known to be confluent when they completely fill their growing area (ie. tissue culture flask or dish). This level of cell growth within the dishes was selected due to the instability of the mutant cell line to various treatments when experiments were originally performed at plateau phase. Due to the relative comparisons being made during this study, this should not affect the analysis of results.

2.2 HDR Irradiation and Cisplatin Treatments

The confluent cells were treated with high dose rate (HDR) irradiation, an exposure to cisplatin, or a combination of the two treatments. The cells were irradiated in 25 cm² tissue culture flasks at room temperature with a Pantak Bipolar Series, model HF320 x-ray unit operating at 250 kVp with a 1.87 mm base aluminum filter and a customized aluminum flattening filter, giving a dose rate of 168 cGy per minute (calibrated with a Farmer ion chamber).

Drug treatments involved treatment with cisplatin for clinical intravenous injection (Faulding Canada Inc.), consisting of 1 mg cis-diaminedichloroplatinum (II), 1 mg mannitol, and 9 mg NaCl, dissolved in 1mL of water for injection. The concentrated cisplatin solution (1mg/mL) was diluted with isotonic citrate saline (134 mM KCl (BDH) and 17.6 mM citric acid trisodium salt (Sigma, Canada)) to obtain the

desired quantities of cisplatin needed for a particular experiment. When placed in aqueous solutions, the chloride atoms of cisplatin are substituted with water molecules and so there is a degradation of the cisplatin solution (Sigma Product Information). The stability in aqueous solutions is enhanced in the presence of sodium chloride, possibly due to the presence of excess chloride ions, and hence the use of the saline solution for the dilution.

The cells were treated by applying a measured quantity of cisplatin directly to the culture media covering the cells. At the end of the exposure period (one hour), the medium was aspirated and new medium was added. In sequential experiments, depleted medium was added instead of new medium to limit any cell cycling effects. The subsequent x-ray treatment was then given within 5 minutes of the cisplatin treatment. It should be noted that in some particular experiments, the secondary treatment was given during the initial cisplatin treatment. Once all treatments were complete, the cells were processed with the clonogenic assay.

2.3 Clonogenic Assay

The clonogenic or colony forming assay gives an indication of cell survival levels after being given an experimental treatment. It is an *in vitro* process that measures whether or not a cell has maintained its reproductive integrity or its ability to sustain proliferation [Hall, 1994]. When a cell maintains these qualities after the experimental treatment, it will be able to proliferate indefinitely until a large colony is formed, and hence it is considered to be clonogenic.

Once the cells had been treated with either cisplatin, radiation, or a combination of the two, the media was aspirated from the tissue culture flask and the cells were washed with isotonic citrate saline. Once they were washed, the cells were trypsinized (0.2% w/v trypsin (Gibco) in citrate saline for 7 minutes of incubation at 37°C), a process which utilizes enzymes to release the cells from the monolayer until a single cell suspension is achieved. The citrate saline was used to rinse away certain remnant proteins left behind by the medium and which inhibit the enzymatic action of the trypsin. The single cell suspension was counted with an electronic cell counter (Particle Data Elzone 80) and plated with fresh medium in 60 mm or 120 mm tissue culture dishes depending on the number of cells plated. When less than 10^5 cells were plated, the 60 mm dishes were used with 4 mL of medium, and when plating anything greater than 10^5 cells, the 120 mm dishes were used with 12 mL of medium.

Usually, a low, medium, and high concentration of cells were plated for each experimental flask to ensure that a sufficient number of colonies could form from at least one of the concentrations. Furthermore, three dishes were plated with cells for each concentration to allow for sufficient statistical averaging. After plating, the cells were incubated for 10 to 13 days depending on the treatment involved in the experiment, allowing time for the viable cells to proliferate and form sufficiently large colonies. After the incubation period was complete, the media was removed from the dishes and the colonies were fixed and stained (methylene blue (Sigma)) in preparation for counting. Viable colonies were scored when a minimum of fifty cells were present after the allotted incubation period.

Each experiment required at least one control point (0 Gy, 0 µg/mL cisplatin), the number of control points depending on the duration of the experiment. Control points were often performed at the beginning and at the end of the experiment to ensure the stability of the conditions throughout the experimental period. The controls allowed for the calculation of the plating efficiency (PE) of the cells, defined as the percentage of cells seeded that grow into colonies [Hall, 1994]. It was calculated by utilizing the following formula:

$$PE = \frac{\text{Colonies counted}}{\text{Cells seeded}} \quad (\text{Eqn.2})$$

Once the PE was determined from cells not treated with any agents, the survival level of the cells given a specific treatment was represented by the surviving fraction (SF) and was calculated as follows:

$$SF = \frac{\text{Colonies counted}}{(\text{Cells seeded}) \times (PE)} \quad (\text{Eqn.3})$$

All experiments were repeated a minimum of three times (unless otherwise indicated in the Results Section) and the mean and the standard error in the mean were calculated for each point.

2.4 Curve Fitting

All radiation response survival curves were fit to the linear-quadratic formula with the graphing software Sigmaplot for Windows Version 4.00. This software utilizes the Levenberg-Marquardt algorithm to determine the coefficients or parameters of the

independent variables (ie. α and β values) that give the “best fit” between the linear-quadratic equation and the experimental data.

2.5 Split Dose Recovery

Sublethal damage repair is observed when there is an increase in cell survival when a given radiation dose is split into two fractions, separated by a time interval [Hall, 1994]. Hence, split dose experiments allowed the determination of levels of SLDR given various combinations of cisplatin and x-radiation treatments. Cells were irradiated with a first dose and then incubated at 37°C for various times and then given a second equivalent dose of radiation. Radiation doses that resulted in relatively lethal iso-survival levels in both of the cell lines were selected (refer to Figure 3.1). The parental MEF cell line received two fractions of 4 Gy while the much more radiosensitive mutant Ku80^{-/-} cell line received two fractions of 1.5 Gy. Cisplatin treatments were given for 1 hour immediately prior to the initial radiation treatment. Once the entire treatment had been completed, the cells were processed with the clonogenic assay. The resultant survival levels were then used to form the recovery ratio (RR), a quantitative measure of the amount of sublethal damage repair given a specific interval of time between doses. It is represented by the ratio of the surviving fraction resulting from two-dose fractionation to the survival from a single equivalent dose [Hall, 1994]. The recovery ratio was calculated as follows:

$$RR = \frac{(SF \text{ from Split Dose})}{(SF \text{ from Single Dose})} \quad (\text{Eqn.4})$$

2.6 Flow Cytometry

The cell cycle analysis was performed on a flow cytometer at the University of Ottawa Health Sciences building. In preparation for use of the flow cytometer, at least 10^6 cells were required for each assay. Once sufficient cells were grown in T-25 flasks with 4 mL of medium, they were trypsinized, resuspended in medium, and centrifuged at 1500 rpm for 7 minutes. The supernatant was then aspirated and the remaining cell pellet was resuspended in 100 μ L of phosphate buffered saline (PBS). The cell suspension was added to 5 mL of cold (-20°C) 70% ethanol in a 15 mL falcon tube while placing the ethanol on a vortex to ensure a relatively even distribution. This sample was stored overnight at -20°C for fixing.

When the samples were ready to be analyzed, they were centrifuged at 1500 rpm for 10 minutes. Once again, the supernatant was then subsequently aspirated but this time the cell pellet was resuspended in 2 mL of PBS. The sample was centrifuged a second time at 2000 rpm for a 10 minute period, after which the supernatant was aspirated and the cell pellet was resuspended in 100 μ L of RNAase (Sigma) solution. It was important to obtain a single cell suspension during this resuspension process to reduce the risk of clotting the flow cytometer. The RNAase solution was prepared by dissolving the RNAase in PBS to make a stock solution with a concentration of 100 $\mu\text{g/mL}$, and subsequently aliquoting the solution into 1 mL Eppendorf tubes which were stored at -20°C .

Immediately after the cells were resuspended in RNAase solution, the mixture was incubated for 20 minutes in a 37°C water bath. Once complete, the solution was removed from the water bath and 900 µL of 25 µg/mL propidium iodide (Sigma) solution was added. The propidium iodide (PI) solution was prepared with a concentration of 1 mg per mL of PBS, stored in an eppendorf tube. The tube was covered with aluminum foil to protect the solution from light and refrigerated at 4°C until required for use in preparation of the flow cytometry analysis. The cell solution was stained with the PI solution for 20 minutes while kept on ice in a covered ice bucket. The cells were then ready to be inserted into the flow cytometer.

The flow cytometry data were analyzed with Multicycle AV (Phoenix Flow Systems), a software package developed to examine the DNA content and cell cycle analysis of flow cytometry data.

3.0 RESULTS

3.1 Cisplatin Radiosensitization

These experiments were performed to determine whether the MEF and Ku80^{-/-} cell lines would demonstrate an increased radiosensitivity to radiation when treated with clinically applicable doses of cisplatin. These data were produced from *in vitro* experiments utilizing the colony forming assay, and calculated from the average of three experiments with the error bars represented by the standard error of the mean.

3.1.1 HDR Irradiation

The HDR radiation responses of the parental MEF and mutant Ku80^{-/-} cell lines are shown in Figure 3.1. Both cell lines were treated with a range of HDR radiation, plated, incubated, and subsequently analyzed for their level of survival using equations (2) and (3). The data were then fit to the linear-quadratic equation (Eqn.1) for each cell line, with the survival plotted as a function of radiation dose. The radiosensitivity of the cell lines can be characterized by several parameters, such as the α and β parameters obtained from the linear-quadratic model. Another parameter which can describe the level in which radiation affects a cell line is the SF₂ value, representing the surviving fraction resulting from a dose of 2 Gy. Values for these parameters are shown in Table 3.1 for both the MEF and Ku80 deficient cell lines. The parental cell line revealed a broad shoulder region, characteristic of a large β parameter ($0.0443 \pm 0.0025 \text{ Gy}^{-2}$), followed by a linear component that is characterized by the α parameter ($0.145 \pm 0.011 \text{ Gy}^{-1}$).

Table 3.1 Comparison of the radiosensitivity parameters, α , β , and SF_2 , between the parental MEF and mutant Ku80^{-/-} cell lines given high dose rate irradiation.

Cell Line	α (Gy ⁻¹)	β (Gy ⁻²)	SF_2
MEF	0.145 ± 0.011	0.0443 ± 0.0025	0.627 ± 0.059
Ku80^{-/-}	2.03 ± 0.05	-----	0.0180 ± 0.0056

In contrast to the MEF's, the Ku80 deficient cells demonstrate a much greater HDR radiation sensitivity, evident through the increase in slope and lack of shoulder. This was verified with the radiosensitivity parameters listed in Table 3.1, showing a very large α value of 2.03 ± 0.05 Gy⁻¹ and a very minute β parameter (0.00187 ± 0.0162 Gy⁻²) contribution relative to the alpha value. In fact, the β value had an uncertainty that was larger than the actual value and thus, was considered to be negligible. It should be noted that β values were omitted from tables when they were found to be not statistically different from zero, for all radiation response data fitted to the linear-quadratic formula. The SF_2 values for the parental and mutant cell lines are 0.627 ± 0.059 and 0.0180 ± 0.0056 , respectively. This implies that the cell survival of the Ku80^{-/-} cell line at 2 Gy is approximately a factor of 35 times less than that of the MEF cell line.

Table 3.2 P-values from a student t-test comparing individual dose points between the MEF and Ku80^{-/-} radiation responses. A p-value less than 0.05 is considered to be statistically significant.

Dose (Gy)	p-value
1	0.0382
2	0.0085
3	0.0016

Figure 3.1 Comparing the responses of the wild-type MEF's and the Ku80-/- cell line to a range of HDR x-radiation. Each point is the average of three experiments, with the error bars represented by the standard error of the mean.

The radiation responses from the two cell lines were examined to see if their data points were statistically significantly different from one another. This was accomplished by performing a student t-test to produce the p-values listed in Table 3.2. Any p-value less than 0.05 is considered to be statistically significant. The survivals at the same dose values from the two trends were compared, each corresponding to p-values calculated to be less than 0.05.

3.1.2 Cisplatin Response

In this experiment, the cells were treated with a range of clinically applicable doses of cisplatin, varying from 0 $\mu\text{g/mL}$ to 10 $\mu\text{g/mL}$, given for a treatment period of 1 hour. These responses are shown in Figure 3.2. The data from both cell lines was approximated to a linear fit (although the MEF data appears to follow a non-linear trend), revealing the similarities in their sensitivities to cisplatin. Both the MEF and the Ku80^{-/-} cell lines show that their trends lie almost directly upon each other within the given clinical range of cisplatin concentrations examined. The two responses were examined for their statistical difference – the p-values for several dose points shared between the two trends are listed in Table 3.3. These values reveal that the cisplatin responses from the two cell lines are not statistically different.

Figure 3.2 The response of the MEF and Ku80^{-/-} cell lines to a range of cisplatin concentrations for a 1 hour treatment period. Each point represents the average of three experiments, with the error bars represented by the standard error of the mean.

Table 3.3 P-values from a student t-test comparing individual dose points between the MEF and Ku80-/- cisplatin responses. A p-value greater than 0.05 is not considered to be statistically significant.

Dose ($\mu\text{g/mL}$)	p-value
1	0.8810
3	0.0933
6	0.6982

3.1.3 Cisplatin and HDR Irradiation Experiments

The response of the MEF cell line to a combination of cisplatin and HDR radiation is shown in Figure 3.3 and is compared to the previously acquired radiation response alone (Figure 3.1). Each point has been normalized to omit the cytotoxic effect of the cisplatin. It should be noted that although there is an inherent uncertainty affiliated with the 0 Gy points of all response curves, these points have been fit to 1 to allow comparison of different treatment curves. The cells were treated with 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to irradiation, with doses ranging from 0 Gy to 8 Gy. When compared to the response from HDR radiation alone, the combined treatment demonstrates an increased radiosensitivity, evident from the steeper slope of the survival curve. The α , β , and SF_2 radiosensitivity parameters for this cell line are listed in Table 3.4. The SF_2 value for the HDR radiation response is 0.627 ± 0.057 , compared to a value of 0.498 ± 0.014 for the response from a combination treatment of 1 $\mu\text{g/mL}$ of cisplatin plus HDR radiation. This shows that after 2 Gy of x-radiation, the level of cell killing is increased by 13% when utilizing a 1 $\mu\text{g/mL}$ cisplatin pre-treatment without considering the cytotoxicity of the cisplatin. The degree of radiosensitization observed in Figure 3.3 was statistically tested by comparing the response from the radiation treatment to the

response from the combined cisplatin and radiation treatment. The results are shown in Table 3.5. These values reveal that the two responses are statistically different.

Table 3.4 Comparing the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line between the HDR radiation response and the response from a combination of 1 $\mu\text{g/ml}$ cisplatin for 1 hour immediately prior to HDR radiation.

Treatment	α (Gy^{-1})	β (Gy^{-2})	SF_2
HDR	0.145 ± 0.011	0.0443 ± 0.0024	0.627 ± 0.059
1 $\mu\text{g/mL}$ Cisplatin and HDR	0.222 ± 0.046	0.0634 ± 0.0123	0.498 ± 0.014

Table 3.5 P-values from a student t-test comparing individual dose points between the MEF cell responses from radiation treatment alone and from the combination of 1 $\mu\text{g/mL}$ of cisplatin with radiation. A p-value less than 0.05 is considered to be statistically significant.

Dose (Gy)	p-value
1	0.0744
2	0.0470
3	0.0408
4	0.0219
5	0.0385
6	0.0085
8	0.0099

The response of the Ku80^{-/-} cell line to the combination of cisplatin and HDR radiation is shown in Figure 3.4 and is also compared to the previously acquired radiation alone response (Figure 3.1). The cells were treated with the same protocol as was done

with the parental cell line, with the exception of a much more limited range of radiation dose (0 Gy to 3 Gy) due to the hypersensitivity of the mutant cell line. Unlike the response seen with the parental MEF cells, the Ku80 deficient cells revealed no significant difference in slope or curvature in the response, indicating no apparent increase in radiosensitivity. This is verified by the radiation sensitivity parameters, α , β , and SF_2 , of the mutant cell line shown in Table 3.6. There is relatively little difference between the parameters, regardless of the treatment used. Student t-tests were also performed on these two responses to statistically examine the significance between the two sets of data (Table 3.7). These values show that the radiation response alone and the combined treatment response are not statistically different.

Figure 3.3 Comparing the HDR radiation response of the MEF cell line to the response from a combined treatment of cisplatin and HDR radiation (1 ug/mL cisplatin for 1 hour immediately prior to radiation).

Figure 3.4 Comparing the HDR radiation response of the Ku80^{-/-} cell line to the response from a combined treatment of cisplatin and HDR radiation (1 ug/mL cisplatin for 1 hour immediately prior to radiation).

Table 3.6 Comparing the radiosensitivity parameters, α , β , and SF_2 , of the Ku80-deficient cell line from the HDR radiation response and the response from a combination of 1 $\mu\text{g/ml}$ cisplatin for 1 hour immediately prior to HDR radiation.

Treatment	α (Gy^{-1})	β (Gy^{-2})	SF_2
HDR	2.03 ± 0.05	-----	0.0180 ± 0.0056
1 $\mu\text{g/mL}$ Cisplatin and HDR	1.94 ± 0.06	-----	0.0180 ± 0.0060

Table 3.7 P-values from a student t-test comparing individual dose points between the Ku80-deficient cell responses from radiation treatment alone and from the combination of 1 $\mu\text{g/mL}$ of cisplatin with radiation. A p-value less than 0.05 is considered to be statistically significant.

Dose (cGy)	p-value
25	0.7365
50	0.7439
75	0.8260
100	0.6164
300	0.9000

In order to determine whether the MEF's would experience a greater level radiosensitization with higher concentrations of cisplatin, the cells were pre-treated with 3 and 6 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR irradiation. The results from these experiments are shown in Figures 3.5 and 3.6, and compared to the previously acquired radiosensitization data (Figure 3.3). Figure 3.5 reveals the total combined cytotoxicity of the cisplatin and the HDR radiation, whereas Figure 3.6 shows the response curves with the cisplatin cytotoxicity normalized for each case to enable direct comparison of the effect on the radiation sensitivity of the cells.

As expected, the unnormalized data showed an increase in the initial overall cell killing, which was related to the increase in the treatment of cisplatin. However, as the radiation dose was increased, the cells treated with 3 and 6 $\mu\text{g/mL}$ of cisplatin revealed an increased resistance to radiation. This was evident from their shallower slopes and loss of the characteristic shoulders in the survival curves, supported by the negligible β parameter contribution in these trends. Figure 3.5 accentuates the overall level of cell killing from the combination of cisplatin and radiation, but the response of the cells to radiation is more evident when the cisplatin cytotoxicity is normalized, as shown in Figure 3.6. The normalized data enables direct comparison of the cellular responses to radiation.

Table 3.8 A summary of the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line for the HDR radiation response and the response from a combination of 1, 3, and 6 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR radiation.

Treatment	$\alpha \text{ (Gy}^{-1}\text{)}$	$\beta \text{ (Gy}^{-2}\text{)}$	SF_2
HDR	0.145 ± 0.011	0.0443 ± 0.0025	0.627 ± 0.059
1 $\mu\text{g/mL}$ Cisplatin and HDR	0.222 ± 0.046	0.0634 ± 0.0123	0.498 ± 0.142
3 $\mu\text{g/mL}$ Cisplatin and HDR	0.545 ± 0.047	-----	0.335 ± 0.050
6 $\mu\text{g/mL}$ Cisplatin and HDR	0.300 ± 0.049	-----	0.528 ± 0.123

of the survival curves when treated with 0, 1, 3 and 6 $\mu\text{g/mL}$ of cisplatin. The parental cells surviving 3 and 6 $\mu\text{g/mL}$ of cisplatin show that they become more radioresistant (at radiation doses of approximately 5 and 4 Gy, respectively) when compared to cells not treated with any cisplatin prior to the experiment. The radiosensitivity parameters of the parental cells for all treatment conditions are summarized in Table 3.8. It is evident that the β component of the LQ model decreases with increasing application of cisplatin concentration.

Figures 3.7 and 3.8 show the response of the Ku80^{-/-} cell line to a combination of cisplatin and HDR radiation. The 3 and 6 $\mu\text{g/mL}$ cisplatin treatment radiation responses were compared to the previously acquired radiosensitization data (Figure 3.4) and displayed in these figures. The protocol used for these cells was the same as was used for the parental cell line, using cisplatin concentrations of 0, 1, 3, and 6 $\mu\text{g/mL}$. As was performed with the MEF cell line, the data are displayed both in an unnormalized format, and in a normalized format respectively for Figure 3.7 and 3.8, which accounted for the level of cisplatin cell killing. Once again, it is apparent that the overall level of cytotoxicity increased with greater concentrations of cisplatin, but with 3 and 6 $\mu\text{g/mL}$ of cisplatin there is a decrease in the slope and α values (Table 3.9) of the survival curves (Figure 3.7). The level of this radioresistance is more evident in Figure 3.8, where a direct comparison of the effect of the radiation is displayed. The radioresistant effect is observed at every level of radiation dose due to the lack of shoulder in the survival curve of the HDR radiation response, and subsequently all other combination cisplatin and radiation responses. The radiosensitivity parameters for the Ku80^{-/-} cisplatin and

radiation experiments are summarized in Table 3.9. The values show little change with increasing cisplatin application, in agreement with trends observed in the response curves (Figure 3.8).

Figure 3.5 The response of the MEF cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 ug/mL of cisplatin for a 1 hour period immediately prior to irradiation. The unnormalized curves represent the combined cytotoxic effect of both HDR radiation and cisplatin.

Figure 3.6 The response of the MEF cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 ug/mL of cisplatin for a 1 hour period immediately prior to irradiation. The effect of all cisplatin cytotoxicities have been normalized for this data.

Figure 3.7 The response of the Ku80^{-/-} cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 ug/mL of cisplatin for a 1 hour period immediately prior to irradiation. The unnormalized curves represent the combined cytotoxic effect of both HDR radiation and cisplatin.

Figure 3.8 The response of the Ku80^{-/-} cell line to HDR radiation given pre-treatment with 0, 1, 3, and 6 ug/mL of cisplatin for a 1 hour period immediately prior to irradiation. The effect of all cisplatin cytotoxicities have been normalized for this data.

Table 3.9 A summary of the radiosensitivity parameters, α , β , and SF_2 , of the Ku80-/- cell line for the HDR radiation response and the response from a combination of 1, 3, and 6 $\mu\text{g/ml}$ of cisplatin for 1 hour immediately prior to HDR radiation.

Treatment	α (Gy^{-1})	β (Gy^{-2})	SF_2
HDR	2.03 ± 0.05	-----	0.0180 ± 0.0056
1 $\mu\text{g/mL}$ Cisplatin and HDR	1.94 ± 0.06	-----	0.0180 ± 0.0060
3 $\mu\text{g/mL}$ Cisplatin and HDR	1.77 ± 0.04	-----	0.0229 ± 0.0083
6 $\mu\text{g/mL}$ Cisplatin and HDR	1.45 ± 0.07	-----	0.0662 ± 0.0113

3.1.4 Optimizing Radiosensitization

Although experiments with higher concentrations of cisplatin showed no increase in radiosensitization, it was possible that the manner in which the cisplatin and radiation were given to the cells would produce a greater or lesser radiosensitizing effect. Figure 3.9 reveals the survival of the parental cell line when the HDR radiation dose was given to the cells halfway through the 1 hour treatment of 1 $\mu\text{g/mL}$ of cisplatin. Due to time constraints, data from the full range of radiation doses was not obtained, but it is evident that the response shows no significant difference from the data where the cells were treated with 1 $\mu\text{g/mL}$ of cisplatin for 1 hour prior to the radiation. The time constraints also limited the number of experiments performed in this manner, increasing the level of

Figure 3.9 Comparing the HDR response of the MEF cell line to the combined treatment of cisplatin and HDR radiation given in two different protocols. In the first, the cisplatin is given for 1 hour immediately prior to irradiation. In the second, the irradiation is given halfway through the 1 hour cisplatin treatment.

Figure 3.10 Comparing the HDR response of the Ku80^{-/-} cell line to the combined treatment of cisplatin and HDR radiation given in two different protocols. In the first, the cisplatin is given for 1 hour immediately prior to irradiation. In the second, the cells are irradiated during the 1 hour cisplatin treatment.

fluctuation in the data points and having no standard error of the mean calculated for this data. The radiosensitivity parameters from the two protocols are shown in Table 3.10.

Table 3.10 Comparing the radiosensitivity parameters, α , β , and SF_2 , of the parental MEF cell line when given 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR radiation and when treated with HDR during (halfway through) the same 1 hour treatment of cisplatin.

Treatment	α (Gy^{-1})	β (Gy^{-2})	SF_2
Cisplatin before HDR	0.222 ± 0.046	0.0634 ± 0.0123	0.498 ± 0.142
HDR during Cisplatin	0.293 ± 0.040	0.0604 ± 0.012	0.453 ± 0.103

This experiment was then performed with the mutant cell line and displayed in Figure 3.10. As expected, there was no further radiosensitization, but rather a response whose trend showed a very similar slope to that of the HDR radiation response and the response from a treatment of cisplatin given prior to the HDR radiation. This experiment was also only performed once due to time constraints and hence, has no calculated values for the standard error of the mean. The limited number of experiments is evident in the this data which reveals high levels of fluctuation above and below the fitted trend. The radiosensitivity parameters for this cell line are listed in Table 3.11.

Table 3.11 Comparing the radiosensitivity parameters, α , β , and SF_2 , of the Ku80-/- deficient cell line when given 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to HDR radiation and when treated with HDR during (halfway through) the same 1 hour treatment of cisplatin.

Treatment	α (Gy^{-1})	β (Gy^{-2})	SF_2
Cisplatin before HDR	1.94 ± 0.06	-----	0.0180 ± 0.0060
HDR during Cisplatin	2.00 ± 0.06	-----	0.0176 ± 0.0092

To examine the effect of varying the time between the cisplatin treatment and the HDR radiation, the parental cells were treated with 6 $\mu\text{g/mL}$ of cisplatin for 1 hour and then placed in a 37°C incubator for up to 8 hours before treating with 4 Gy of x-radiation. The surviving fraction was measured with 0, 4, and 8 hour periods of incubation and plotted in Figure 3.11. The data were normalized to the 0 hour incubation period, which represented the standard protocol from the previous radiosensitization experiments (Figures 3.3 to 3.8). The survival of the cells drastically decreased with increasing incubation time between cisplatin and radiation treatments. With 4 and 8 hours of incubation, the surviving fraction reduced to (0.44 ± 0.03) and (0.09 ± 0.05) , respectively.

3.1.5 Flow Cytometry

Flow cytometry is a powerful aid in the determination of DNA content and cell cycle analysis. Entire studies have been devoted to using this technique, and similarly, numerous informative flow cytometry experiments could have been designed for this

Figure 3.11 The surviving fraction of the MEF cell line in response to 6 ug/mL of cisplatin for 1 hour followed by 4 Gy of HDR radiation, given 0, 4, and 8 hours between the cisplatin and radiation treatments. All surviving fractions have been normalized to the 0 hour point.

study. In order to limit the experiments performed with flow cytometric methods, specific goals were set. The purpose of these experiments was to determine whether the addition of varying concentrations of cisplatin to the parental MEF and mutant Ku80-deficient cell line would affect the cell cycle distribution.

Cells were treated with various concentrations of cisplatin for 1 hour, after which they were fixed and prepared for analysis in the flow cytometer. This cisplatin treatment simulated the treatment given prior to radiation treatments in the cisplatin radiosensitization experiments to verify whether the drug treatment would alter the cell cycle distribution within. The flow cytometry cell cycle distribution data is summarized and listed in Tables 12 and 13 for the MEF and Ku80^{-/-} cells, respectively. The uncertainty in the values was calculated from the square root of the number of cells counted within the flow cytometer sample. It is evident that the distribution of cells throughout the cell cycle is not altered by treatment with various concentrations of cisplatin. The uncertainty in these values was also calculated from the square root of the number cells counted within the flow cytometer sample. Though the relative percentages are slightly different from that of the parental cell line, the distribution remains relatively constant regardless of the cisplatin treatment given to the cells.

Table 3.12 Cell cycle distributions obtained from flow cytometry techniques for the parental MEF cell line given treatments of 0, 3, and 6 $\mu\text{g/mL}$ of cisplatin for a 1 hour period.

Treatment	S-phase (%)	G1-phase (%)	G2-phase (%)
0 $\mu\text{g/mL}$ Cisplatin	28.8 ± 0.3	68.5 ± 0.7	2.70 ± 0.03
3 $\mu\text{g/mL}$ Cisplatin	32.3 ± 0.3	61.7 ± 0.6	6.00 ± 0.06
6 $\mu\text{g/mL}$ Cisplatin	29.3 ± 0.3	66.3 ± 0.7	4.40 ± 0.04

Table 3.13 Cell cycle distributions obtained from flow cytometry techniques for the Ku80 deficient cell line given treatments of 0, 3, and 6 $\mu\text{g/mL}$ of cisplatin for a 1 hour period.

Treatment	S-phase (%)	G1-phase (%)	G2-phase (%)
0 $\mu\text{g/mL}$ Cisplatin	43.1 ± 0.4	50.3 ± 0.5	6.60 ± 0.07
3 $\mu\text{g/mL}$ Cisplatin	48.2 ± 0.5	49.2 ± 0.5	2.60 ± 0.03
6 $\mu\text{g/mL}$ Cisplatin	43.1 ± 0.4	50.9 ± 0.5	6.00 ± 0.06

3.2 Split Dose Experiments

The goal of these experiments was to determine the level the MEF and Ku80^{-/-} cell lines were capable of repairing sub-lethal DNA damage. This was accomplished by allowing varying amounts of incubation periods between two fractions of radiation or cisplatin. The survival data was then utilized to calculate a recovery ratio for each incubation period using equation (4). The overall trend observed is referred to as the split dose or Elkind recovery [Hall, 1994] and represents the level of sub-lethal damage repair with respect to incubation time. These data are plotted in Figures 3.12 and 3.13, corresponding to the responses of the parental and mutant cell lines, respectively, with all curves approximated to a linear fit. Due to time constraints, these experiments were not able to be repeated and hence, lack statistical support and error bars.

Figure 3.12 shows that the MEF cell recovery from a split dose of HDR radiation alone was quite substantial, reaching a recovery ratio of approximately 4 after 8 hours of incubation between doses. When the cells were treated with 1 $\mu\text{g/mL}$ of cisplatin for 1 hour immediately prior to the initial dose of radiation, the recovery was diminished, reaching about 2. Finally, when the cells were treated with 3 $\mu\text{g/mL}$ of cisplatin, the cells showed no apparent recovery, but rather a gradual decrease in the recovery ratio. A similar trend was observed when a cisplatin split dose experiment was performed. It should be noted that although the data were fitted to a linear trend line, there were consistently lower levels of recovery observed in each of the responses when given approximately 2 hours of incubation time between doses.

The Ku80^{-/-} cell line response to various split dose experiments did not show the variation of results seen with the parental cell line. Figure 3.13 shows the level of recovery when the cells were given an HDR radiation split dose, given a combination of cisplatin and HDR radiation, and with a cisplatin split dose. Regardless of the treatment selected, the mutant cell line revealed no increase in recovery ratio, indicating a lack of ability to repair sub-lethal damage. As was seen with the parental cell line, when no recovery was observed, there was a gradual decrease in the recovery ratio. The decrease in the level of repair after 2 hours of incubation observed with the parental cell line was not as noticeable with the mutant cells.

Figure 3.12 The split dose recovery (4 Gy per dose) of the MEF cell line grown to confluency, given 0, 1, and 3 ug/mL of cisplatin for 1 hour immediately prior to the initial dose of radiation. A cisplatin split dose recovery (3 ug/mL cisplatin per dose) was also measured.

Figure 3.13 The split dose recovery (1.5 Gy per dose) of the Ku80^{-/-} cell line grown to confluency, given 0, 1, and 3 ug/mL of cisplatin for 1 hour immediately prior to the initial dose of radiation. A cisplatin split dose recovery (3 ug/mL cisplatin per dose) was also measured.

4.0 DISCUSSION

4.1 Cisplatin Radiosensitization

The use of genetically mutated cell lines deficient in specific cellular mechanisms has become a powerful tool for developing knowledge on the mechanistic behaviour of various processes in radiobiology and molecular biology. This study utilized one such mutant cell line, Ku80-deficient cells, to determine the involvement of the NHEJ pathway in cisplatin radiosensitization and sub-lethal damage repair. The second cell line, MEF cells, played an equally important role in these experiments to demonstrate the normal cellular response to the applied treatments. MEF cells are the parental cell line to the Ku80^{-/-} cells, but even though the two may share similar genetic backgrounds, their characterization in response to damage differs greatly.

4.1.1 HDR Irradiation

The initial experiments involved determining the inherent radiation sensitivities of the two cell lines and comparing them with one another. As expected, the wild-type MEF cell line demonstrated an ability to repair sub-lethal damage, evident in the large shoulder region of the radiation response. This was equally evident from the α and β values which showed that the quadratic component of the LQ model was quite substantial relative to the linear component. In this manner, it is often more useful to consider the α/β ratio rather than their individual quantities to characterize a survival curve.

However, as α goes to zero, the ratio tends towards zero, and as β goes to zero, the ratio approaches infinity. Hence, there are also limitations to the utilization of the α/β ratio.

Conversely, the Ku80-deficient cells revealed a hyper-sensitivity to radiation, showing no shoulder region in the survival curve. The relatively drastic increase in radiosensitivity is evident in the SF₂ values which were 0.627 ± 0.057 and 0.0228 ± 0.0056 for the parental and mutant cells, respectively. The α component was in complete dominance relative to the β component, stressing the linear shape of the response. The degree to which the two cell lines varied in radiosensitivities was supported by the student t-test for statistical significance which demonstrated that each dose point shared between the two responses was significantly different from one another.

The radiation responses of the MEF and Ku80^{-/-} cell lines agree with published data that also shows the extreme increase in radiation sensitivity when the Ku80 protein is depleted from an embryonic murine cell line [Wachsberger, 1999]. Due to the relatively recent genetic development of mutant cell lines such as Ku80^{-/-}, there is currently minimal published data regarding these radiation experiments for these particular cell lines. However, there is published radiation response data on other cell lines which contain mutants deficient in the Ku80 protein, and are hence, similarly defective in their NHEJ activities. One such example is the Chinese ovarian hamster (CHO) – K1 cell line from which the extensively studied xrs mutants were derived. These published materials may differ in their parental cell radiation responses (though not significantly – SF₂ value of approximately 0.5 ± 0.1), but the relative increase in the radiation sensitivity of the mutant cell lines is very similar to that observed with the MEF mutants [Ross, 1995, Singleton, 1997, Stevens, 1999]. The same trend is also observed

in another published article involving a different Chinese hamster cell line, V79B [Errami, 1998].

Furthermore, all of the published data mentioned above reveal that the characteristic shoulder in the parental cell line radiation survival was essentially eliminated in the Ku80-deficient radiation response. The data obtained from this study is supported by the theory that radiation sensitivity is linked to inhibited repair of DNA damage. More specifically, it agrees with the theory that this inhibited repair is via a lack of non-homologous end-joining activity, the dominant process of DSB repair in mammalian cells.

4.1.2 Cisplatin Response

The response of the two cell lines to various concentrations of cisplatin permits the examination of the relative cytotoxic effect of the chemotherapeutic agent. The level of cell-killing observed with clinically relevant concentrations of cisplatin is comparable to the damaging effect of radiation. Therefore, the additive effect of clinical treatments involving combined cisplatin and radiotherapy could potentially double the tumour kill without even considering any radiosensitizing effects which might occur. Due to the nature of the DNA damaging agent, it is not necessarily suitable to fit these responses with the linear-quadratic model, which was specifically developed for damage from radiation action.

Regardless of the fit, the importance of this data is the relative difference between the two responses. It is evident that the parental and mutant cell lines share similar reactions to the cytotoxic effects of cisplatin, a drastic difference to the relative radiation responses of the two cells. Though the initial assumption of treating a cell line hypersensitive to radiation with cisplatin would be that of an equally acute sensitivity to this DNA damaging agent, a closer look at the mechanisms of action behind the two agents shows that this is not the case. As described in the introduction, radiation damages the DNA through direct or indirect ionization events which lead to breaks in the DNA double strand [Hall, 1994]. However, cisplatin cytotoxicity is regulated by the formation of cisplatin-DNA adducts which inhibit further vital DNA processes [Dewitt, 1987]. Since the structure of the DNA is consistent in both cell lines, formation of adducts should not be significantly affected by the presence or absence of the Ku complex and it can be assumed that there would be approximately equal amounts of adducts in each of the cell lines. Therefore, this would imply that both the parental MEF and the Ku80^{-/-} cell lines should have similar responses to cisplatin, agreeing with data observed in this study. These data were also supported by statistical analysis testing, which revealed that there was no significant difference between several of the dose points examined between the two cell lines (all p-values were calculated to be greater than 0.05).

4.1.3 Cisplatin and HDR Irradiation Experiments

When exposed to a combination of cisplatin and HDR irradiation, the parental MEF cells revealed an increased radiosensitivity when compared to the response from a

treatment of radiation alone. The cytotoxic effect of cisplatin was normalized out of these survival responses so that a direct comparison of the response to radiation could be observed. The SF_2 values reveal that there is an enhanced radiosensitization in the combined treatment, increasing the level of cell killing by 13%. Clinically, the cytotoxicity of the cisplatin would also be considered, further increasing the net benefit of combined modality treatments. If a multi-fraction radiotherapy treatment were being considered in the clinic, then a 13% increase in the radiosensitivity of the cells being attacked would accumulate substantially with each fraction. Statistically, all values produced by the student t-test support the significant increase in the slope of the trend, with the exception of the very first dose point.

It is hypothesized in this study that the NHEJ plays a role in cisplatin radiosensitization of mammalian cells. If this is the case, then it would be expected that cells deficient in this pathway would not be further radiosensitized by this chemotherapeutic agent. The results in this study agree with this hypothesis, evident from the radiation response of the Ku80^{-/-} cells to a combined treatment of cisplatin and HDR irradiation. The similarity between the radiation alone response and the combined treatment response demonstrates that no radiosensitizing effects occurred with these cells. The statistical p-values for these responses were all greater than 0.05, supporting the minimal difference between the two responses. This suggests that the mechanism of cisplatin radiosensitization is linked to the NHEJ repair pathway and that the hypothesis of this thesis is correct.

The extent to which cisplatin radiosensitization depends on NHEJ requires further investigation, though due to the complexity of repair systems observed in mammalian cells, it can be speculated that other processes may be involved. If NHEJ were the sole pathway responsible for cisplatin radiosensitization and if the cisplatin dose were sufficient to completely inhibit the NHEJ pathway, then theoretically it would be expected that the combined treatment response of the MEF cell line would essentially overlap the Ku80^{-/-} radiation response. If another distinct repair pathway were involved in cisplatin radiosensitization, then the response of the Ku80^{-/-} cell line to a combined cisplatin and radiation treatment would experience further radiosensitization.

Regardless, it seems that the Ku80 protein is playing an important role in specific repair processes related to cisplatin radiosensitization. This is supported by recently published studies which shows evidence of a relationship between cisplatin-DNA adducts and Ku80 activity [Turchi, 2000]. Turchi et al demonstrated that the Ku protein complex displayed a reduced ability to translocate on DNA containing cisplatin-DNA adducts when compared to a control, undamaged DNA. The decreased rates of translocation resulted in decreased interaction between Ku and DNA-PK_{cs}, as well as reduced catalytic rates. It was also found that the position of the adducts affected the kinase activity, with maximum inhibition occurring when the adducts were formed closest to the DNA strand-break site.

Further support of the involvement of Ku80 in the mechanism of cisplatin and radiation interaction comes from published data on murine leukemic cells showing cross-

resistance between radiation and cisplatin [Frit, 1999]. Frit et al selected and characterized a murine cisplatin resistant cell line that exhibits cross-resistance to ionizing radiation due to an increased capacity to repair DSBs when compared with the parental cell line. They linked this increased DNA-end binding activity to the Ku protein complex and their data strongly suggests that the phenotype of cross-resistance to ionizing radiation is due to an increase in Ku activity. Britten et al also supported the notion that the radiosensitivity of DSB repair proficient human tumour cell lines was partly determined by the predisposition of these cell lines to activate non-conservative DSB rejoining pathways [Britten, 1999]. These studies further elucidate the connection between cisplatin and radiation interactions with the Ku protein complex and NHEJ.

Once the radiosensitizing effect of cisplatin was observed in the parental cell line, it was necessary to verify whether higher concentrations of cisplatin would result in a greater level of radiosensitization. These data were first plotted in an unnormalized fashion, representing the total cytotoxic effect of both the cisplatin and the radiation. For the range of radiation dose considered (0Gy to 8Gy) for the MEF cells, the combined cytotoxicity generally increased with higher cisplatin concentrations. However, the shape of the survival responses altered dramatically when 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ of cisplatin were applied prior to the radiation dose. These curves lacked the shoulder previously observed in the radiation alone response and the 1 $\mu\text{g/mL}$ + radiation response, and additionally tended to decrease in slope with increasing concentrations of cisplatin.

This trend is accentuated when the data is normalized such that the effect of the cisplatin cytotoxicity is eliminated from the plot. As before, this enables the direct comparison of the radiation on the cells. The normalized data reveals that the cells treated with 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ of cisplatin appear to develop an increasing resistance to radiation. In fact, the cells pre-treated with 6 $\mu\text{g/mL}$ of cisplatin becomes more radiation resistant than cells treated with no cisplatin for radiation doses of approximately 4.5 Gy and higher. The trend was also evident in the radiosensitivity parameters which revealed a greater α/β ratio for the 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ cisplatin treatments. Before any speculations could be made regarding this peculiar effect, it was necessary to perform the same experiments with the Ku80-deficient cell line.

The response of the Ku80^{-/-} cell line to a combined treatment of cisplatin and radiation was also initially plotted in an unnormalized fashion to account for the total cytotoxicity of the two agents. This data showed that the overall cell killing effect increased relative to the concentration of cisplatin applied to the cells, similar to that observed with the parental cells. The alteration in the survival curves was not as pronounced with this mutant cell line because of the lack of shoulder in the original radiation response curve. However, the decreasing slope for the cisplatin pre-treatments of 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ was equally obvious to that seen with the MEFs, once again accentuated by the normalized curves. The Ku80^{-/-} cells demonstrated increased radiation resistance in both the 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ concentrations of cisplatin.

It appears that this increasing radioresistant effect is equally prominent in both cells lines, implying that the mechanism is likely unrelated to the NHEJ pathway. The loss of the characteristic shoulder in the parental cell line response is usually indicative of a loss of ability to repair SLD. However, if this were the case, it would likely be complemented by an increased slope attributed to enhanced radiosensitization, and not the shallower slope observed in this data (Figure 3.6 and 3.8). Another circumstance in which there is essentially no shoulder observed in survival curves occurs when repair is saturated, such as in low dose rate (LDR) irradiation treatments. This is also not applicable to this study since HDR irradiation was applied in single fractions.

It is postulated that this trend may be due to one of several reasons. One possibility is that the high concentrations of cisplatin are triggering a cell resistant mechanism which inhibits damage caused by radiation. Another possibility is that the 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ cisplatin concentrations are sufficient to eliminate most of the cells which lie in the more sensitive portion of the cell cycle, thus leaving behind only those in the more resistant stage, the S phase cells.

The most plausible reason became evident upon inspection of the cisplatin responses of the two cell lines. The 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ concentrations of cisplatin result in the death of approximately 90% and 99% of the cell populations, respectively. In the 6 $\mu\text{g/mL}$ pre-treatment of cisplatin, the experimental population is based upon the remaining 1% of the cells which were able to survive the harsh chemotherapy agent onslaught. It is very likely that this remaining sub-population is inherently resistant to

such DNA damaging agents, and is thus not well representative of the normal distribution of cells. These presumptions were not investigated within this study, but leave much room for future research.

4.1.4 Optimizing Radiosensitization

In order to maximize the level of cisplatin radiosensitization, studies were done with altered treatment conditions in hopes of producing enhanced effects [Brattstrom, 2000, Geldof, 1999, Raaphorst, 1997, Wilkins, 1993]. Previous yeast studies involving cisplatin and radiation have shown that the greatest level of radiosensitization was observed when the cells were treated immediately prior to or during the irradiation procedure. Hence, combined treatment experiments were performed where the parental and mutant cells were irradiated during (halfway through) a 1 hour treatment of 1 $\mu\text{g/mL}$ of cisplatin (Figure 3.9). This data demonstrated that cisplatin treatments applied immediately prior to or during irradiation treatments result in similar levels of cisplatin radiosensitization with MEF cells. This was also verified with the mutant cell line even though no radiosensitization was observed when treated with cisplatin prior to irradiation (Figure 3.10). The results confirmed that no radiosensitization was evident under any of the conditions examined within this study.

The time between the cisplatin treatment and the HDR irradiation was also varied to investigate the effect this delay would have on the survival fraction of the parental cells. This experiment also permitted the examination of possible cell resistant mechanisms that might have developed using high concentrations of cisplatin as a pre-

treatment to radiation. In section 3.1.3, the 3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$ pre-treatments of cisplatin resulted in an increased radioresistance in both the parental and mutant cell lines. If this trend were due to the development of cell resistant mechanisms from the harsh cytotoxicity of the cisplatin, then this pathway would require time to grow. Hence in this experiment, it was decided to pre-treat with the concentration of cisplatin that resulted in the largest degree of radiation resistance, 6 $\mu\text{g/mL}$. The cells were then permitted time for any mechanisms to develop, after which the 4 Gy dose of HDR radiation was applied (Figure 3.11). As the resistance to radiation was observed similarly in both cell lines, this experiment was performed solely on the parental cells with the assumption that its results regarding cell resistant mechanisms would be indicative of both the MEF and the Ku80^{-/-} cells.

If a cell resistant mechanism was actually being developed within the cells, then the surviving fraction would be expected to increase with time. The results demonstrated something entirely different, rather a decrease in the total survival of the cells. Though it is possible that this was due to an increased level of radiosensitization, it is unlikely because of the degree to which the total cytotoxicity was increased. A more conceivable explanation would be that even though the cisplatin-DNA adduct formation occurs during the one-hour treatment period, the cytotoxic mechanism resulting from these adducts continues to harm cells long after the application has been removed. In other words, when *in vitro* cells are treated with high concentrations of cisplatin, the cytotoxicity may not end when the treatment is removed.

4.1.5 Flow Cytometry

In this study, flow cytometry was utilized to examine the stability of the experimental conditions for the combined cisplatin and radiation experiments. More specifically, this technique was applied to determine whether pre-treatment with various concentrations of cisplatin would alter the cell cycle distribution. The 0 $\mu\text{g/mL}$ results verify that the cells were relatively confluent from the substantial percentage of S-phase cells ($28.8\% \pm 0.3\%$ for the MEF cells, and $43.1\% \pm 0.4\%$ for the Ku80^{-/-} cells) evident within both cell lines. The greater percentage of S-phase cells in the mutant cell line is attributed to its slightly slower growth rate compared to the parental cells.

Both cell lines show that relatively consistent cell cycle distributions regardless of the concentration of cisplatin applied to the cells before the analysis. This verifies that the cell cycle distribution was not altered between the different pre-treatments applied in the cisplatin radiosensitization experiments. Therefore, the cisplatin treatments applied throughout this study did not change the cell cycle distribution. Each sample was prepared from a single flask of cells, hence, representing a single population. The relatively small uncertainty in the values do not account for the variance in the values within a specific phase. Perhaps this could be remedied in future experiments by sampling three different populations of cells for each treatment of cisplatin, and calculating the uncertainty from the standard error of the mean.

4.2 Split Dose Experiments

These experiments were designed to measure the degree to which a cell population is capable of repairing sub-lethal DNA damage. The focus of this data (Figures 3.12 and 3.13) was on the relative differences and the observation of varying trends between the different treatments and cell lines, and hence, specific repair rates were not considered in this study. The radiation split dose was formulated by referring to the radiation responses of both cell lines and acquiring lethal doses which produce approximately similar levels of cell kill. Due to the hypersensitivity of the mutant cell line, two doses of 1.5 Gy separated by time were given to the Ku80^{-/-} cells, while a more substantial 4 Gy dose was applied twice to the parental MEF cells.

As expected, the greatest level of sub-lethal damage repair was observed in the parental cell line treated with HDR radiation alone, agreeing with the data from the radiation response curve which demonstrated a large shoulder region. Both the rate and total degree of repair were reduced when the MEFs were pre-treated with 1 µg/mL of cisplatin. The cisplatin radiosensitization experiments revealed minimal or undetectable shoulders for parental cells treated with 3 µg/mL and 6 µg/mL of cisplatin prior to HDR irradiation. These split dose experiments show agreement with those results, demonstrating no increase in the recovery ratio at all when pre-treated with 3 µg/mL of cisplatin. The Ku80-deficient cells revealed no observable shoulders in any of the radiation response curves. This data was verified by the lack of increase in the recovery ratio for all of the mutant split dose experiments.

Sub-lethal damage under normal circumstances can be repaired in hours unless additional sublethal damage is added with which it interacts to form lethal damage [Hall, 1994]. This term was formed for purely operational reasons since the mechanisms involved in repair are not completely understood. These experiments provide insight into some of these mechanisms and may help to define the pathway that is known as sub-lethal damage repair. The MEF split dose experiments reveal that cisplatin inhibits the cell's ability to recover, implying that a possible connection may lie between cisplatin-DNA adduct formation and sub-lethal damage repair. The cisplatin and radiation experiments revealed that there lies a connection between Ku80, and subsequently NHEJ, to cisplatin radiosensitization. Therefore, it can be postulated that the NHEJ pathway plays a major role in sub-lethal damage repair. The lack of repair observed with the Ku80^{-/-} cells provides additional support of NHEJ's role in sub-lethal damage repair. If further DNA repair mechanisms were involved in SLDR, then an increase in the recovery ratio would be expected in the Ku80 split dose experiments. This is supported by published data investigating the cellular recovery from radiation damage of several radiosensitive cell lines. Thacker and Wilkinson showed that several of their recovery-defective cell lines had measurable deficiencies in DNA DSB repair, and concluded that the recovery pathway was likely to be associated with the rejoining of DNA DSBs [Thacker, 1995].

Experiments were also performed on both cell lines involving a split dose of cisplatin. In this case, the similar cisplatin sensitivities between the two cell lines allowed for the use of the same split dose of cisplatin to be applied during the

experiment. In both cell lines, no recovery was observed, indicating that higher concentrations of cisplatin not only inhibit sub-lethal damage repair between radiation doses, but also between cisplatin treatments.

The split dose experimental data (Figures 3.12 and 3.13) revealed that the recovery ratio after 2 to 3 hours of incubation time between fractions were consistently lower than would be expected from a consistently increasing trend. The cells in this study were grown to confluency before any treatments or experiments were performed. The lengthy nature of this experiment permits the cells to continue redistribution throughout the cell cycle, allowing more cells to enter a more sensitive portion after 2 or 3 hours. More specifically, the survivors of the first radiation dose are predominantly in the most resistant phase of the cell cycle, late S phase. If a second dose of radiation is applied to the cells after several hours, these cells progress to a more sensitive phase (G_2 or M) resulting in a decrease in the recovery ratio [Hall, 1994].

It is interesting to note the gradual decrease in the recovery ratio for MEF cells treated with 3 $\mu\text{g/mL}$ of cisplatin, and for all treatments on Ku80 $^{-/-}$ cells. This downward trend is also observed in the published data of a radiosensitive human malignant glioma cell line [Allalunis-Turner, 1995]. In the parental cell line, it is possible that this effect may be due to increased levels of cisplatin-DNA bifunctional adduct formation with time. Mono-functional adducts formed between cisplatin and DNA can evolve into bifunctional adducts if given sufficient time to progress to such a stage. It has been shown that short exposure to cisplatin results in prevalently mono-functional adducts, but

as time is extended, the number of mono-functional adducts decreases and the number of bifunctional adducts increases [Eastman, 1986]. Furthermore, a study involving several cell lines revealed that the number of bifunctional adducts continues to increase for many (12 to 24) hours subsequent to the cisplatin treatment [Plooy, 1985].

Therefore, it is possible that the delayed increase in cytotoxicity in the parental cell line is a result of the delayed formation of cisplatin-DNA bifunctional adducts. This behaviour was also observed in the results of the MEF cell resistant mechanism experiments where the parental cells were treated with a high concentration of cisplatin, and given varying amounts of time before treatment with HDR radiation. Studies show that cisplatin-DNA adducts are repaired via excision repair, though there is potential for a combination of excision and recombinational repair [Calsou, 1993]. This removal process is a slow one, depending on the type and cell cycle stage of cells, and ranging from one to four days of repair time [Hill, 1994, Larminat, 1993]. Thus, the adduct formation period is much quicker than the removal period. In the mutant cell line, such a postulate fails to explain the trends observed in the split dose recovery curves. In this case, the decreasing effect is not only observed in cells treated with cisplatin but also in Ku80^{-/-} cells which were treated with only HDR radiation. It is possible that the mechanism responsible for the decreasing trend is entirely different in the mutant cell line when compared to the parental, and thus, continued research is still necessary.

The most plausible explanation for this decreasing trend is that it is not actually decreasing with increased incubation time but only appears to given the range of times

permitted within this study. It is likely that there is an initial drop in recovery due to cell cycle redistribution, but after this time the data is relatively steady. It may have been wiser to normalize this stabilized line to a recovery ratio of 1. This may have been more prevalent in studies involving greater levels of incubation time (approximately 24 to 48 hours). Therefore, the decreasing trends observed within this study are unlikely to be attributed to any specific mechanisms other than cell cycle redistribution.

5.0 CONCLUSIONS

The application of cisplatin in conjunction with radiation can enhance radiotherapy treatments by providing greater local control rates. In order to optimize such combined modality treatments, it is necessary to understand the mechanism responsible for the radiosensitizing benefits of cisplatin. Previous studies have shown that it is related to the inhibition of repair of radiation-induced DNA DSBs. Yeast studies have shown that this mechanism involves the homologous recombinational repair pathway, the dominant DSB repair pathway in yeast cells. However, in mammalian cells, the pathway responsible for repairing DSBs is mainly the NHEJ process, though there are possibilities that HR may also play a complementary role.

Based on the findings of these previous studies, this study focussed on the NHEJ pathway and its involvement in cisplatin radiosensitization and sub-lethal damage repair. The radiation responses of the parental mammalian MEF cell line revealed a large shoulder in the survival curve, supported by a corresponding large β value from the LQ model. The radiation response of the mutant Ku80-deficient cell line revealed much greater sensitivity to radiation, and essentially a complete lack of ability to repair DNA DSBs, evident from the lack of shoulder and high α -value. The drastic differences in responses between the two cell lines was attributed to the genetic NHEJ DSB repair deficiency of the mutant cell line.

The cisplatin responses were observed to be the same in both cell lines with further statistical support which showed insignificant differences between the two sets of data. This similarity is supported by the cytotoxic mechanism of cisplatin, which is to form cisplatin-DNA adducts that inhibits DNA replication and transcription, eventually leading to apoptosis. Therefore, the NHEJ deficiency of the Ku80 mutant cells would not be expected to affect the degree of cisplatin cytotoxicity.

Parental cells treated with 1 µg/mL of cisplatin for one hour immediately prior to irradiation revealed an increased sensitivity to radiation. However, this supra-additive effect was not observed in the Ku80^{-/-} cell line. This supports the hypothesis of this study that the NHEJ pathway does indeed play a role in the mechanism of cisplatin radiosensitization in eukaryotic cells. This is further supported by studies which reveal that cisplatin-DNA adducts reduce the ability of Ku to translocate along the DNA, decreasing the level of Ku's catalytic rate and interaction with DNA-PK_{cs} [Turchi, 2000]. Further experimentation with both the MEF and Ku80^{-/-} cells showed that altering the treatment protocol to the concurrent administration of 1 µg/mL cisplatin and x-radiation did not result in any further degree of radiosensitization.

Although there are no direct clinical implications of developing an understanding of the mechanism behind cisplatin radiosensitization, this information can still lead to enhanced treatment techniques. With more knowledge of the process involved, it is possible to investigate methods to increase levels of radiosensitization by inhibiting the NHEJ pathway in target sites. Further optimization of combined modality treatments

could also be investigated since knowledge of the responsible pathway for cisplatin radiosensitization might bring insight into the temporal relation between cisplatin and radiation.

When higher concentrations (3 $\mu\text{g/mL}$ and 6 $\mu\text{g/mL}$) of cisplatin were applied with the same protocol, both cell lines revealed an increasing resistance to radiation and the loss of DNA repair ability in the parental cell line. As this trend was equally prominent in both the parental and the mutant cells, it was likely not related to Ku80 and the NHEJ pathway. Though there are several possible explanations for this effect, it is most due to the high-level of cytotoxicity from high concentrations of cisplatin, leaving behind largely resistant sub-populations of cells for the remainder of the experimental treatment. Cell cycle analysis from flow cytometry studies demonstrated that the application of 0, 3, or 6 $\mu\text{g/mL}$ of cisplatin to both the parental and mutant cell lines did not affect the cell cycle distribution significantly.

Split dose experiments demonstrated that cisplatin inhibits the repair of sub-lethal damage in the parental cells, agreeing with the trends observed in the cisplatin radiosensitization experiments. As no SLD repair was observed with the Ku80^{-/-} cells regardless of the concentration of cisplatin treatment applied (0, 1, or 3 $\mu\text{g/mL}$), no further repair of damage could be expected. This lack of recovery observed in the Ku80-deficient cells implies that the NHEJ pathway may also play a role in the repair of sub-lethal DNA damage.

5.1 Future Work

This study has focused on the response of Ku80-deficient cells and its wild-type parental cells to various cisplatin and x-radiation treatment protocols. The extent to which HR plays a role in DNA DSB repair in mammalian cells is still under current investigation. Therefore, cisplatin radiosensitization experiments could also be performed on a HR-deficient mammalian cell line, permitting direct comparison with NHEJ-deficient cell responses. Current technology has also permitted the genetic generation of mammalian cells deficient in both NHEJ and HR, which would provide another interesting cell line to investigate similar cisplatin and radiation experiments. Double-strand break experiments, such as the utilization of CHEF (clamped homogeneous electric field) techniques, would also provide additional data on the direct involvement of NHEJ and HR on DSBs when treated with combinations of cisplatin and radiation.

The resultant increase in radiation resistance observed in both cell lines after treatment with 3 and 6 $\mu\text{g/mL}$ of cisplatin prior to irradiation could be investigated by examining the cells which retain clonogenic ability after the high concentrations of cisplatin treatment. In other words, both the MEF and Ku80-deficient cells could be treated with a high concentration of cisplatin, plated, and given sufficient time to form colonies. These colonies should represent the radiation resistant cells and can be selected and grown to confluency in flasks. A series of cisplatin and radiation experiments could verify whether these cells are indeed more resistant to radiation than their predecessors (cells not treated with high concentrations of cisplatin).

Combined modality radiotherapy treatments not only incorporate chemotherapeutic agents into the protocol, but also thermal treatments such as hyperthermia. A much broader scope for future work would be to investigate the response of NHEJ-deficient cells, HR-deficient cells, and NHEJ/HR-deficient cells to combination hyperthermia, cisplatin, and radiation treatments. This would further develop our understanding of the mechanisms behind cisplatin and hyperthermia radiosensitization, and enable the enhancement of such combined modality treatments.

6.0 REFERENCES

1. Allalunis-Turner, M. J., Zia, P.K.Y., Barron, G.M., Mirzayans, R., Day, R.S. (1995). "Radiation-induced DNA damage and repair in cells of a radiosensitive human malignant glioma cell line." Radiation Research **144**: 288-293.
2. Bender, M. A., Gooch, P.C. (1962). "The kinetics of x-ray survival of mammalian cells in vitro." Int. J. Radiat. Biol. **5**: 133-45.
3. Boulton, S. J., Jackson, S.P. (1996). "Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways." EMBO Journal **15**(18): 5093-5103.
4. Brahmer, J. R., Ettinger, D.S. (1998). "Carboplatin in the treatment of small cell lung cancer." The Oncologist **3**: 143-154.
5. Brattstrom, D., Bergqvist, M., Hesselius, P., Wagenius, G., Brodin, O. (2000). "Different fraction schedules and combinations with chemotherapy in radiation treatment of non-small cell lung cancer." Anticancer Research **20**: 2087-2090.
6. Brenner, D. J., Herbert, D.E. (1997). "The use of the linear-quadratic model in clinical radiation oncology can be defended on the basis of empirical evidence and theoretical argument." Am. Assoc. Phys. Med **24**(8): 1245-48.
7. Britten, R. A., Evans, A.J., Allalunis-Turner, M.J., Pearcey, R.G. (1996). "Effect of cisplatin on the clinically relevant radiosensitivity of human cervical carcinoma cell lines." Int. J. Radiat. Oncol. Biol. Phys. **34**(2): 367-374.
8. Britten, R. A., Kuny, S., Perdue, S. (1999). "Modification of non-conservative double-strand break (DSB) rejoining activity after the induction of cisplatin resistance in human tumour cells." Brit. J. of Cancer **79**(5/6): 843-849.
9. Calsou, P., Salles, B. (1993). "Role of DNA repair in the mechanisms of cell resistance to alkylating agents and cisplatin." Cancer Chemother. Pharmacol. **32**: 85-89.
10. Cary, R. B., Peterson, S.R., Wang, J., Bear, D.G., Bradbury, E.M., Chen, D.J. (1997). "DNA looping by Ku and the DNA-dependent protein kinase." Proc. Natl. Acad. Sci. **94**: 4267-4272.
11. Chadwick, K. H., Leenhouts, H.P. (1973). "A molecular theory of cell survival." Phys. Med. Biol. **18**: 78-87.
12. Critchlow, S. E., Jackson, S.P. (1998). "DNA end-joining: From yeast to man." Trends in Biochem. Sciences **23**: 394-399.

13. Curtis, S. B. (1986). "Lethal and potentially lethal lesions induced by radiation - a unified repair model." Radiation Research **106**: 252-270.
14. Dewitt, L. (1987). "Combined treatment of radiation and cisdiamminedichloroplatinum (II): A review of experimental and clinical data." Int. J. Radiat. Oncol. Biol. Phys. **13**: 403-426.
15. Difilippantonio, M. J., Zhu, J., Chen, H.T., Meffre, E., Nussenzweig, M.C., Max, E.E., Ried, T., Nussenzweig, A. (2000). "DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation." Nature **404**(30): 510-514.
16. Double, E. B. (1990). Interactions between platinum coordination complexes and radiation. Boca Raton, Florida, CRC Press.
17. Dunkel, I. J., Aledo, A., Kernan, N.A., Kushner, B., Bayer, L., Gollamudi, S.V., Finlay, J.L., Abramson, D.H. (2000). "Successful treatment of metastatic retinoblastoma." Cancer **89**(10): 2117-2121.
18. Eastman, A. (1986). "Reevaluation of interaction of cis-dichloro(ethylenediammine)platinum (II) with DNA." Biochemistry **25**: 3912-3915.
19. Eifel, P. J. (2000). "Chemoradiation for carcinoma of the cervix: advances and opportunities." Radiation Research **154**: 229-236.
20. Errami, A., Finnie, N.J., Morolli, B., Jackson, S.P., Lohman, P.H.M., Zdzienicka, M.Z. (1998). "Molecular and biochemical characterization of new X-ray-sensitive hamster cell mutants defective in Ku80." Nucleic Acids Research **26**(19): 4332-4338.
21. Featherstone, C., Jackson, S.P. (1999). "Ku, a DNA repair protein with multiple cellular functions?" Mutation Research **434**: 3-15.
22. Fichtinger-Schepman, A. M. J., van Dijk-Knijnenburg, H.C.M., van der Velde-Visser, S.D., Berends, F., Baan, R.A. (1995). "Cisplatin- and carboplatin-DNA adducts: is Pt-AG the cytotoxic lesion?" Carcinogenesis **16**(10): 2447-2453.
23. Fowler, J. F. (1989). "The linear-quadratic formula and progress in fractionated radiotherapy." British Journal of Radiology **62**: 679-94.
24. Frit, P., Canitrot, Y., Muller, C., Foray, N., Calsou, P., Marangoni, E., Bourhis, J., Salles, B. (1999). "Cross-resistance to ionizing radiation in a murine leukemic cell line resistant to cis-dichlorodiammineplatinum(II): Role of Ku autoantigen." Molecular Pharmacology **56**: 141-146.
25. Geldof, A. A., Kruit, A., Newling, D.W.W., Slotman, B.J. (1999). "Synergism between cisplatin and radiotherapy in an in vitro prostate tumor cell line." Anticancer Research **19**: 505-508.

26. Glass, J., Silverman, C.L., Axelrod, R., Corn, B.W., Andrews, D.W. (1997). "Fractionated Stereotactic Radiotherapy with cis-platinum radiosensitization in the treatment of recurrent, progressive, or persistent malignant astrocytoma." Amer. J. Clin. Oncol. **20**(3): 226-229.
27. Goodhead, D. T. (1985). "Saturable repair models of radiation action in mammalian cells." Radiation Research **104**: S58-S67.
28. Hall, E. J. (1994). Radiobiology for the Radiobiologist. Philadelphia, J.B. Lippencott Company.
29. Hill, B. T., Shellard, S.A., Fichtinger-Schepman, A.M.J., Schmoll, H.J., Harstrick, A. (1994). "Differential formation and enhanced removal of specific cisplatin-DNA adducts in two cisplatin-selected resistance human testicular teratoma sublines." Anti-Cancer Drugs **5**: 321-328.
30. Jeggo, P. A. (1998). "Identification of genes involved in repair of DNA double-strand breaks in mammalian cells." Radiation Research **150**: S80-S91.
31. Jin, S., Weaver, D.T. (1997). "Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions." EMBO **16**(22): 6874-6885.
32. Larminat, F., Zhen, W., Bohr, V.A. (1993). "Gene-specific DNA repair of interstrand cross-links induced by chemotherapeutic agents can be preferential." J. Biol. Chem. **268**: 2649-2654.
33. Lea, D. E. (1955). Actions of radiation on living cells: The target theory. Cambridge, Cambridge University Press.
34. Lustig, A. J. (1999). "The Kudos of non-homologous end-joining." Nature Genetics **23**: 130-131.
35. Marangoni, E., Foray, N., O'Driscoll, M., Douc-Rasy, S., Bernier, J., Bourhis, J., Jeggo, P. (2000). "A Ku80 fragment with dominant negative activity imparts a radiosensitive phenotype to CHO-K1 cells." Nucleic Acids Research **28**(23): 4778-4782.
36. Marcon, F., Boei, J.J.W.A., Natarajan, A.T. (2000). "Recombination between homologous chromosomes does not play a dominant role in the formation of radiation-induced chromosomal aberrations." Int. J. Radiat. Biol. **76**(10): 1343-1348.
37. Montana, G. S., Thomas, G.M., Moore, D.H., Saxer, A., Mangan, C.E., Lentz, S.S., Averette, H.E. (2000). "Preoperative chemo-radiation for carcinoma of the vulva with N2/N3 nodes: A gynecologic oncology group study." Int. J. Radiat. Oncol. Biol. Phys. **48**(4): 1007-1013.

38. Nussenzweig, A., Sokol, K., Burgman, P., Li, L., Li, G.C. (1997). "Hypersensitivity of Ku80-deficient cell lines and mice to DNA damage: The effects of ionizing radiation on growth, survival, and development." Proc. Natl. Acad. Sci. **94**: 13588-13593.
39. Olive, P. L. (1998). "The role of DNA single- and double-strand breaks in cell killing by ionizing radiation." Radiation Research **150**((Suppl.)): S42-S51.
40. Osipovich, O., Duhe, R.J., Hasty, P., Durum, S.K., Muegge, K. (1999). "Defining functional domains of Ku80: DNA end binding and survival after radiation." Biochem. and Biophys. Res. Comm. **261**: 802-807.
41. Plooy, A. C. M., van Dijk, M., Berends, F., Lohman, P.H.M. (1985). "Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with cis-diamminedichloroplatinum (II)." Cancer Research **45**: 4178-4184.
42. Puck, T. T., Marcus, P.I. (1956). "Actions of X-rays on mammalian cells." Journal Exp. Med. **103**: 653-66.
43. Raaphorst, G. P., Wang, G., Wilkins, D., Davis, L., Bussey, A., Stewart, D., Ng, C.E. (1994). "Evaluation of cisplatin response in three radiation resistant and three radiation sensitive cell lines." Anticancer Research **14**: 1939-42.
44. Raaphorst, G. P., Miao, J., Ng, C.E. (1997). "Cisplatin and mild hyperthermia in radiosensitization to low dose rate irradiation in human ovarian carcinoma cells." Anticancer Research **17**: 3469-3472.
45. Ramsden, D. A., Gellert, M. (1998). "Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks." EMBO Journal **17**(2): 609-614.
46. Ross, G. M., Eady, J.J., Mithal, N.P., Bush, C., Steel, G.G., Jeggo, P.A., McMillan, T.J. (1995). "DNA strand break rejoining defect in xrs-6 is complemented by transfection with the human Ku80 gene." Cancer Research **55**: 1235-1238.
47. Santin, A. D., Hermonat, P.L., Ravaggi, A., Bellone, S., Roman, J., Pecorelli, S., Cannon, M., Parham, G.P. (2000). "Effects of concurrent cisplatin administration during radiotherapy vs. radiotherapy alone on the immune function of patients with cancer of the uterine cervix." Int. J. Radiat. Oncol. Biol. Phys. **48**(4): 997-1006.
48. Schwachofer, J. H. M., Crooijmans, R.P.M.A., Hoohenhout, J., Kal, H.B., Theeuwes, A.G.M. (1991). "Effectiveness in inhibition of recovery of cell survival by cisplatin and carboplatin: Influence of treatment sequence." Int. J. Radiat. Oncol. Biol. Phys. **20**: 1235-1241.

49. Singleton, B. K., Priestley, A., Steingrimsdottir, H., Gell, D., Blunt, T., Jackson, S.P., Lehmann, A.R., Jeggo, P.A. (1997). "Molecular and biochemical characterization of xrs mutants defective in Ku80." Molecular and Cellular Biology 17(3): 1264-1273.
50. Stevens, C. W., Stamato, T.D., Mauldin, S.K., Getts, R.C., Zeng, M., Cerniglia, G.J. (1999). "Radiation-Induced Recombination Is Dependent on Ku80." Radiation Research 151: 408-413.
51. Sultanem, K., Shu, H., Xia, P., Akazawa, C., Quivey, J.M., Verhey, L.J., Fu, K.K. (2000). "Three-dimensional intensity-modulated radiotherapy in the treatment of nasopharyngeal carcinoma: The university of California-San Francisco experience." Int. J. Radiat. Oncol. Biol. Phys. 48(3): 711-722.
52. Taccioli, G. E., Gottlieb, T.M., Blunt, T., Priestley, A., Demengeot, J., Mizuta, R., Lehmann, A.R., Alt, F.W., Jackson, S.P., Jeggo, P.A. (1994). "Ku80: Product of the XRCC5 gene. Role in DNA repair and V(D)J recombination." Science 265: 1442-1445.
53. Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., Takeda, S. (1998). "Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells." EMBO Journal 17(18): 5497-5508.
54. Taylor, E. M., Lehmann, A.R. (1998). "Review: Conservation of eukaryotic DNA repair mechanisms." Int. J. Radiat. Biol. 74(3): 277-286.
55. Thacker, J., Wilkinson, R.E. (1995). "The genetic basis of cellular recovery from radiation damage: Response of the radiosensitive irs lines to low-dose-rate irradiation." Radiation Research 144(294-300).
56. Thames, H. D. (1985). "An 'incomplete-repair' model for survival after fractionated and continuous irradiations." Int. J. Radiat. Biol. 47: 319-339.
57. Tobias, C. A. (1985). "The repair-misrepair model in radiobiology: Comparison to other models." Radiation Research 104: S77-S95.
58. Turchi, J. J., Henkels, K.M., Zhou, Y. (2000). "Cisplatin-DNA adducts inhibit translocation of the Ku subunits of DNA-PK." Nucleic Acids Research 28(23): 4634-4641.
59. Wachsberger, P. R., Li, W-H., Guo, M., Chen, D., Cheong, N., Ling, C.C., Li, G., Iliakis, G. (1999). "Rejoining of DNA double-strand breaks in Ku80-deficient mouse fibroblasts." Radiation Research 151: 398-407.

60. Wilkins, D. E., Heller, D.P., Raaphorst, G.P. (1993). "Inhibition of potentially lethal damage recovery by cisplatin in a brain tumor cell line." Anticancer Research **13**: 2137-2142.
61. Wu, X., Lieber, M.R. (1996). "Protein-protein and protein-DNA interaction regions within the DNA end-binding protein Ku70-Ku86." Molecular and Cellular Biology **16**(9): 5186-5193.
62. Yaneva, M., Kowalewski, T., Lieber, M.R. (1997). "Interaction of DNA-dependent protein kinase with DNA and with Ku: Biochemical and atomic-force microscopy studies." EMBO **16**(16): 5098-5112.
63. Yang, D., Wang, A.H.J. (1996). "Structural studies of interactions between anticancer platinum drugs and DNA." Prog. Biophys. Molec. Biol. **66**(1): 81-111.