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**A study of the interaction between camptothecin and
low dose rate irradiation during concomitant application to
human melanoma cells**

by

Daron G. Owen, BSc

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

Master of Science

**Ottawa-Carleton Institute for Physics
Department of Physics
Carleton University
Ottawa, Ontario
July 12, 2000**

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Chair, Department of Physics

Thesis Supervisor

Carleton University
August 18, 2000

Abstract

Chemotherapeutic drugs have greater potential for an increased therapeutic gain when used in conjunction with brachytherapy (i.e., low dose rate irradiation, LDRI) treatments than when combined with external beam radiation therapy. This is because more effective targeting of the radiation to the tumour tissue occurs during brachytherapy, avoiding unwanted toxicity towards normal tissue otherwise caused by the combined effects of the radiation and the drug.

This thesis examines the effects of the interaction between LDRI and camptothecin (CPT), a well known chemotherapeutic agent, on a radioresistant human tumour cell line. LDRI, delivered at 0.53 Gy h^{-1} , allows for complete sublethal damage repair (SLDR) during irradiation. CPT is a good candidate to combine with LDRI since it is known to inhibit SLDR. The results are evaluated in terms of clonogenic survival and residual DNA damage in the form of double strand breaks (DSBs) as determined by two standard assays (comet, CHEF). Survival data are further evaluated using isobologram analysis.

The concentrations of CPT investigated are $1 \text{ }\mu\text{M}$, $10 \text{ }\mu\text{M}$, and $25 \text{ }\mu\text{M}$. The results show that at the higher concentrations, CPT is effective at potentiating LDRI in its killing of these tumour cells. Unexpectedly, this is accompanied by large increases in α rather than in β . This suggests that potentiation of LDRI by CPT in this study may be more related to a modification of intrinsic radiosensitivity of the cells rather than inhibition of SLDR. At $25 \text{ }\mu\text{M}$, the reduction in survival after the concomitant application of CPT and LDRI is related to an increase in the number of residual DNA DSBs, lending support to the notion that the unrejoined DSB is a lethal cellular lesion for combined CPT and LDRI treatments..

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Table of Contents

Abstract	iii
Acknowledgments	iv
Table of Contents	v
List of Abbreviations	viii
List of Tables	ix
List of Figures	x
1 Introduction	1
1.1 Low Dose Rate Irradiation	2
1.2 Camptothecin	5
1.3 Combining LDRI and CPT	8
1.4 Linear Quadratic Model	13
1.5 CHEF Electrophoresis	16
1.6 Comet Assay	18

1.7	DNA DSBs and Clonogenic Survival	22
1.8	Hypothesis and Specific Aims	26
2	Materials and Methods	28
2.1	Experimental Procedure	28
2.1.1	The Cell Line	28
2.1.2	Cell Culture Conditions	29
2.1.3	LDRI, CPT, and Combined LDRI/CPT Procedures	30
2.1.4	Clonogenic Assay	31
2.1.5	CHEF DNA double strand break Assay	33
2.1.6	Comet Assay	37
2.2	Data Analysis	39
2.2.1	Curve Fitting	39
2.2.2	Interpretation of Isobolograms	40
2.2.3	Construction of Isobolograms	43
3	Results	49
3.1	LDRI, CPT, and Concomitant Exposures	49
3.2	Isobologram Analysis	61
3.3	CHEF Electrophoresis	70
3.4	Comet Assay	74

4	Discussion	87
4.1	LDRI, CPT, and Concomitant Exposures	88
4.2	Isobologram Analysis	94
4.3	CHEF Electrophoresis	97
4.4	Comet Assay	98
5	Conclusions	101
5.1	Future Research	102
	Appendix	105
	References	115

List of Abbreviations

CHEF	Clamped Homogeneous Electric Field (electrophoresis)
CPT	Camptothecin
CPT-11	Irinotecan
DNA	Deoxyribonucleic acid
DSB	double strand break
FAR	Fraction of Activity Released
Gy	Gray, SI unit for absorbed dose [$1 \text{ Gy} = 1 \text{ J kg}^{-1}$]
HDR	High Dose Rate
HDRI	High Dose Rate Irradiation
LDR	Low Dose Rate
LDRI	Low Dose Rate Irradiation
LQ	Linear Quadratic (model)
PLDR	Potentially Lethal Damage Repair
SER	Sensitisation Enhancement Ratio
SLDR	Sub-Lethal Damage Repair
TBE	Tris /Boric Acid/ EDTA (buffer)
TL	Tail Length
TM	Tail Moment
TPT	Topotecan

List of Tables

Table 3.1.1 Comparison of LQ fits for CPT alone	50
Table 3.1.2 Comparison of LQ fits for LDR alone and combined LDR and CPT	53
Table 3.1.3 Statistical comparison of LDR and combined LDR and CPT	57
Table 3.1.4 Statistical comparison of LDR and normalized combined LDR and CPT ..	59
Table 3.1.5 Change in normalized combined LDR and CPT α , β relative to LDR	60
Table 3.4.1 Comparison of comet parameters between two experiments	79

List of Figures

Figure 1.2.1 Mechanism of cell killing by CPT	7
Figure 1.4.1 Sample survival curve	14
Figure 2.1.5.1 CHEF electrophoresis chamber and electric field vectors	35
Figure 2.2.2.1 Sample isobologram	41
Figure 2.2.3.1 Graphical representation of isobologram construction	46
Figure 2.2.3.2 Determination of fractional additivity	47
Figure 3.1.1 Survival curves after 1 μ M, 10 μ M, and 25 μ M CPT	51
Figure 3.1.2 Survival curves after LDR and 1 μ M CPT combined	54
Figure 3.1.3 Survival curves after LDR and 10 μ M CPT combined	55
Figure 3.1.4 Survival curves after LDR and 25 μ M CPT combined	56
Figure 3.2.1 Predicted survival for LDR and 1 μ M CPT combined	64
Figure 3.2.2 Predicted survival for LDR and 10 μ M CPT combined	65
Figure 3.2.3 Predicted survival for LDR and 25 μ M CPT combined	66
Figure 3.2.4 Fractional additivity for LDR and 1 μ M CPT combined	67
Figure 3.2.5 Fractional additivity for LDR and 10 μ M CPT combined	68
Figure 3.2.6 Fractional additivity for LDR and 25 μ M CPT combined	69

Figure 3.3.1 FAR vs dose for LDR and 10 μ M CPT alone and combined	71
Figure 3.3.2 FAR vs dose for LDR and 25 μ M CPT alone and combined	72
Figure 3.3.3 Sample CHEF gel electrophoresis run	73
Figure 3.4.1 Typical comet images	76
Figure 3.4.2 Tail length vs dose for LDR and 25 μ M CPT alone and combined	77
Figure 3.4.3 Tail moment vs dose for LDR and 25 μ M CPT alone and combined	78
Figure 3.4.4 Survival vs normalized TM for LDR and 25 μ M CPT combined	81
Figure 3.4.5 Survival vs normalized TL for LDR and 25 μ M CPT combined	82
Figure 3.4.6 Survival vs normalized TM for LDR alone	83
Figure 3.4.7 Survival vs normalized TL for LDR alone	84
Figure 3.4.8 Survival vs normalized TM for 25 μ M CPT alone	85
Figure 3.4.9 Survival vs normalized TL for 25 μ M CPT alone	86
Figure A-1 Isobolograms for LDR and 1 μ M CPT	106
Figure A-2 Isobolograms for LDR and 10 μ M CPT	107
Figure A-3 Isobolograms for LDR and 25 μ M CPT	108

Chapter 1

Introduction

The need for combined modality therapy in the treatment of cancer arises from the inability of radiation therapy and surgery to consistently cure intermediate stage solid tumours. This is due either to sub-populations of very radioresistant cells within the irradiated field, or to the early spread of the disease throughout the body as the primary tumour sheds its cells into the bloodstream (Vokes, 1998). Sequential combined modality therapy has been widely investigated and used already with some success. Concomitant chemoradiotherapy is a newer idea in which radiation and chemotherapeutic agents are given at the same time. This still involves the systemic application of the drug, while also allowing for a radiation sensitizing effect within the irradiated field. This study investigates the concomitant application of low dose rate irradiation and the chemotherapeutic drug camptothecin using human tumour cells *in vitro*.

1.1 Low Dose Rate Irradiation

The range of dose rates of importance in radiotherapy is from about 0.1 Gy/h to several Gy/min. In this range, the fraction of cells killed by radiation is reduced as the dose rate is reduced. Clinical radiotherapy is concerned with high dose rate (HDR), on the order of 1 Gy/min, for external beam therapy, and low dose rate (LDR) for brachytherapy, which can range from about 0.4 to 2 Gy/h (Hall & Brenner, 1991). The term “brachytherapy” is derived from the Greek word *bracchio*, meaning “short”, and refers to “treatment with a radioisotope at a short distance.” A brachytherapy treatment is described as either intracavitary or interstitial. In an intracavitary treatment, the radioactive source is placed in a body cavity in close proximity to the tumour. In an interstitial treatment, the source is implanted directly into the tumour volume. Brachytherapy was developed in the early 1900s due to the availability of purified radium, which was the first source to be used. It is believed that the first person to suggest the treatment of cancer by direct implantation of radioactive sources was Alexander Graham Bell, as described in an article he published in 1903 (Bell, 1903). If brachytherapy is the sole treatment, a typical schedule would be 50 to 70 Gy in 5 to 9 days. In any treatment, the total dose should be adjusted for dose rate. Clinical studies show that late effects on normal tissues as well as tumour control vary with dose rate for a given total dose (Hall, 1994). Brachytherapy is often used as a boost to external beam treatments, or as an adjunct to chemotherapy.

There are three key advantages of LDR brachytherapy over external beam therapy: (1) the source is close to or in the tumour, resulting in good dose distributions; (2) the short

overall treatment time counters the repopulation of the tumour; (3) the low dose rate results in an increased therapeutic advantage between tumour control and damage to late responding tissues (Brenner, 1997). In moving to fractionated HDR brachytherapy, the advantage of source positioning is retained, but treatment time is longer, and the dose rate is high. In brachytherapy, the dose is targeted to the tumour tissue much more so than in external beam therapy. This minimizes the radiobiological damage to nearby normal tissues which can be early responding (responsible for extending external beam treatments over more than a month) as well as late responding, which, in external beam radiotherapy, often represent the dose limiting endpoint.

Long treatment times can be a significant cause of local failure of radiotherapy. The repopulation of tumour cells is accelerated during the treatment and the cells grow faster than they can be killed (Brenner, 1993; Slevin *et al.*, 1992; Withers *et al.*, 1988). The optimal treatment time is a compromise between short times to minimize repopulation, and long times to prevent early complications. LDR brachytherapy limits early complications so that no compromise needs to be made. Relative to external beam radiotherapy, the treatment time is much shorter for a comparable effective dose to the tumour.

It has been known for a long time that, in general, lowering the dose rate results in a reduction in radiobiological damage (Hall & Bedford, 1964). For a given dose, increasing the dose rate will increase late effects much more than it will increase tumour control. In the same way, decreasing the dose rate will decrease late effects much more than it will decrease tumour control. Therefore, the therapeutic ratio (ratio of tumour control to complications) will increase as the dose rate decreases (Brenner, 1997). This phenomenon, known as the

dose-rate effect, is one of the main factors that determines the biological consequence of a given absorbed dose due to X or γ -rays, and it is explained by sublethal damage repair (SLDR). Cells that have sustained sublethal damage (SLD) can repair it if given sufficient time. If more SLD occurs before repair and interacts with the earlier SLD, it becomes lethal damage (Dale & Jones, 1998). SLDR is observed as an increase in survival when a radiation dose is split into two fractions separated by a time interval, or when the dose rate is lowered. SLDR occurs in tumours and in normal tissues, both in vivo and in vitro.

The dose-rate effect and SLDR are best observed on the cell survival curve, which is the relationship between the fraction of cells retaining their reproductive integrity and absorbed dose. The surviving fraction is plotted on a logarithmic scale on the ordinate against dose on the abscissa. For acute treatments with sparsely ionizing radiation such as X and γ -rays, the curve starts out straight at low doses with a finite initial slope. At higher doses the curve bends, then tends to straighten out again at even higher doses. The bending region extends over a few Gy and is referred to as the shoulder of the survival curve. The shoulder reflects the accumulation of SLD and it is generally true that a large shoulder indicates a substantial dose-rate effect, and vice versa (Hall & Brenner, 1991).

As the dose rate is reduced, the slope of the survival curve becomes progressively shallower and the shoulder begins to disappear as a greater proportion of SLD is repaired. This was first predicted by Lea and Catcheside (Lea & Catcheside, 1942). At a low enough dose rate a limiting slope is reached and the entire survival curve is simply an extension of the initial slope of the acute response curve. This limiting slope reflects the complete repair of sublethal damage, which is assumed to be taking place in a low dose rate treatment. At the

limiting slope the dose rate is still sufficient to arrest cells in their cycle and prevent progression (Hall & Brenner, 1991).

1.2 Camptothecin

Camptothecin (CPT) is a derivative of an alkaloid (nitrogen-containing organic base obtained from plants) isolated from the wood, bark, and fruit of the Chinese tree *Camptotheca acuminata*. Preparations from the tree have been used in traditional Chinese medicine for the treatment of various illnesses, including tumours (Liu, 1995). CPT was discovered and analyzed by Monroe Wall and Mansukh Wani in 1966 (Wall *et al.*, 1966). Others demonstrated its ability to inhibit DNA and RNA synthesis, and, in mammalian cells, to produce reversible single-stranded DNA breaks (Liu, 1995). A more water-soluble sodium salt formation of the drug showed promise in early clinical trials, but testing was discontinued because of toxic side effects (Gupta *et al.*, 1995). Interest in CPT was renewed in the late 1980s when the nuclear enzyme topoisomerase I was identified as its target (Liu, 1995). Less toxic, more water-soluble semi-synthetic and totally synthetic analogues such as CPT-11 (Irinotecan) and Topotecan were produced, and began clinical trials in the early 1990s.

Topoisomerases are nuclear enzymes present in all malignant and normal eukaryotic cells. They are also present in bacteria, plants, and some viruses. The helical structure and length of cellular DNA make it difficult for certain cellular processes (transcription, replication, repair) to take place (Pommier, 1996). Topoisomerases facilitate these processes by changing the topology of the DNA, releasing the torsional tension therein. The

topoisomerase enzyme accomplishes this by binding directly to the DNA and creating an enzyme-linked DNA break (cleavable complex) in either one strand (topoisomerase I) or both strands (topoisomerase II). In the case of topoisomerase I (the target of CPT), the single strand break creates a transient gate through which the other strand can pass, thereby unwinding and relaxing the DNA. The enzyme then re-ligates the DNA without base loss or change in the DNA sequence (Pommier, 1996).

Camptothecin is a topoisomerase I inhibitor that binds to the DNA-topoisomerase I cleavable complex and stabilizes it (Rich & Kirichenko, 1998). The model for the mechanism of cell kill by CPT is based on the notion of its apparent specificity towards cells in the S-phase of the cell cycle, during which DNA synthesis is taking place (Ryan *et al.*, 1991; Del Bino *et al.*, 1990; Del Bino *et al.*, 1991). One study found that S-phase cells were 1000 times more sensitive to CPT than cells in G₁ or G₂ (Del Bino *et al.*, 1991), but the levels of topoisomerase I and CPT-stabilized cleavable complexes remain relatively constant throughout the cell cycle (Heck *et al.*, 1988; Chow & Ross, 1987). This suggests that DNA synthesis is required in order for the cytotoxicity of CPT to manifest itself. The accepted model, as illustrated in Fig. 1.2.1, is that by binding to the DNA-topoisomerase I cleavable complex and stabilizing it, CPT prevents the rejoining of the broken DNA strand. During S-phase or unscheduled replication after genomic stress, the reversible, stabilized cleavable complex collides with the advancing replication fork and converts the DNA single strand break into an irreversible DNA double strand break (DSB) (Rich & Kirichenko, 1998). The repair of these double strand breaks is very slow, and it is thought that the sensitivity of various cell types can be attributed to differences in the level of induction of these double

Figure 1.2.1 Mechanism of cell killing by CPT. CPT binds to the noncleavable topoisomerase I - DNA complex forming a reversible cleavable complex. If not reversed, this cleavable complex will collide with the replication fork, causing a DNA DSB.

strand breaks and the ability of the cell to carry out their repair (Ng *et al.*, 2000; Ryan *et al.*, 1991). In addition, the higher the levels of topoisomerase I, the greater the frequency of cleavable complexes. The trapping of cleavable complexes by CPT is what leads to cell death, therefore, CPT will cause greater DNA damage in cells with higher levels of topoisomerase I (Pommier, 1996). One of the reasons for the effectiveness of CPT as an anti-cancer agent is that, when compared to normal cells, higher levels of topoisomerase I enzyme have been reported to exist in several different cancers (colon, ovarian, esophageal, prostate, stomach, breast, lung, melanoma) (Husain *et al.*, 1994; Crespi *et al.*, 1988; Pommier, 1996; Potmesil, 1994).

There may be more about the mechanism of cell kill by CPT that remains unknown. The drug is active in human tumour xenografts, which typically have low S-phase fractions (Dancey & Eisenhauer, 1996). Some studies have shown that the amount of cell kill by CPT involves more than the S-phase fraction of the cell population and that non-proliferating cells are also killed (Slichenmayer *et al.*, 1993; Ng *et al.*, 1995; Holm *et al.*, 1989; O'Connor *et al.*, 1991). Therefore, other cellular effects of camptothecin may be linked to its cytotoxicity.

1.3 Combining LDR and CPT

There are two basic reasons for combining radiation and drugs: (1) to improve the local regional control of the tumour, and (2) to increase the control of distant metastases. Two important ways in which combined radiation and drugs can give an improved therapeutic strategy are spatial cooperation and toxicity independence (Steel & Peckham, 1979). Spatial

cooperation is achieved when the disease in one anatomical site missed by one therapeutic agent is dealt with adequately by the other. When two agents can be combined without having to reduce their dose levels much due to toxicity, a better therapeutic response can be expected. The clinical relevance of additive effects in chemo-radiation treatments depends on having either one or both of spatial cooperation and toxicity independence. When combining radiation and drugs, however, it is hoped that there will be some interaction between the two, leading to an enhancement of tumour cell kill and greater than additive (supra-additive or synergistic) effects.

There have been several studies combining CPT and its analogues with HDR in which some sort of synergism was observed. In one such study using confluent cells of the RT112 bladder carcinoma cell line (one of the most resistant of tumour cell lines to ionizing radiation), CPT, added for 5 hours after irradiation, appeared to sensitize the cells to radiation to some degree, showing a sensitisation enhancement ratio (SER) of 1.10 at 10% survival. The SER is defined as the dose required to reduce survival to a certain level in the absence of the drug to that in its presence. Split-dose experiments in the same study in which two doses of 4 Gy were separated by increasing intervals of time from one to four hours, during which CPT was present, showed a marked decrease in the recovery ratio (RR) when CPT was present, as compared to when it was not. The recovery ratio is the ratio of the surviving fraction measured at each time interval to that seen with no dose separation. This indicates that CPT inhibited the repair of sublethal damage between dose fractions (Musk & Steel, 1990).

A study of the effects of the CPT analogue Topotecan on confluent, extremely

radioresistant human malignant melanoma (U1-Mel) cells when combined with HDR showed enhanced radiation lethality at clinically feasible drug concentrations. This effect was only seen when the drug was present during or shortly after irradiation. The effect was irreversible, dose dependent, and caused by the inhibition of SLDR and potentially lethal damage repair (PLDR) (Lamond *et al.*, 1996). Potentially lethal damage (PLD) is the component of radiation damage that can be modified by changing the conditions in the environment of the cells after irradiation. PLDR can occur if the cells are prevented from dividing for 6 hours or more after irradiation and is manifest as an increase in survival (Hall, 1994). In a study from our own lab it was shown that CPT effectively inhibited SLDR in confluent cultures of Sk Mel-3 cells treated with HDR (Ng *et al.*, 1994).

An active metabolite of CPT-11 called SN-38 sensitized exponential cultures of Chinese hamster ovary (CHO) cells to HDR when it was present 30 minutes before and during irradiation. Additive effects were seen in confluent cultures. Similar effects were observed in DU-145 (human prostatic carcinoma) cells and EMT-6/KU (mouse mammary carcinoma) cells (Sasai *et al.*, 1998).

Another study with CHO cells used the induction of chromosome aberrations instead of survival as the measured quantity. Synergistic effects in the number of induced aberrations were observed in exponential CHO cells treated post-irradiation with CPT. Higher levels of synergism were observed for exchange type aberrations, suggesting that the interaction of unrepaired radiation induced and CPT induced lesions during replication may be involved in the observed synergism (Zanier *et al.*, 1996).

Confluent cultures of U1-Mel cells were sensitized to radiation when CPT was

administered either during or immediately after HDR irradiation, but not when CPT was given prior to irradiation. The enhanced cell killing was proportional to the initial extent of damage caused by ionizing radiation (Boothman *et al.*, 1992). The conclusions of these studies suggest that the camptothecins can be effective sensitizers to HDR in both exponential and confluent cells, and that they are proficient inhibitors of DNA repair, whether SLDR or PLDR.

Combining CPT and low dose rate irradiation is a relatively new idea that has undergone little study. It is known that CPT inhibits SLDR (Rich & Kirichenko, 1998; Musk & Steel, 1990; Lamond *et al.*, 1996; Ng *et al.*, 1994), and, by definition, LDR allows SLDR to occur during treatment. Thus CPT is a good candidate for combination with LDR because of its ability to inhibit the SLDR that LDR allows.

Since topoisomerase I appears to be the sole cytotoxic target of CPT, it may be possible to selectively enhance the response of tumour cells to LDR since, in many cases, they have higher levels of topoisomerase I than do normal cells (Husain *et al.*, 1994; Crespi *et al.*, 1988; Pommier, 1996; Potmesil, 1994). We are aware of only three studies that have examined the effects of CPT on LDR irradiation (Hennequin *et al.*, 1994; Roffler *et al.*, 1994; Musk & Steel, 1990). Roffler *et al.* investigated the interaction between camptothecin and γ -ray irradiation on cultured rat hepatoma (AS-30D) cells, for the potentiation of radioimmunotherapy. Irradiation with γ -rays from a ^{60}Co source, followed by a 2 hour exposure to CPT, was found to act additively at low doses (less than 2 Gy) and synergistically at higher radiation doses (shown by isobologram analysis with 20 % survival as an endpoint). An 8 hour pretreatment with CPT resulted in sub-additive effects at 4 Gy, and supra additive

effects at 2 Gy (Roffler *et al.*, 1994). Musk and Steel combined CPT with LDR irradiation from a ^{60}Co source, at a rate of 3 cGy/min (1.8 Gy/h), and observed the effects on the very radioresistant bladder carcinoma RT112 cell line. Treatments were carried out with the cells at confluence. They found that the presence of CPT completely abolished low dose rate sparing and had a greater sensitizing effect than it did when combined with HDR. CPT, added for 5 hours after irradiation produced an SER of 1.32 at 10 % survival. A topoisomerase II inhibitor, VP16 (etoposide) was also tested. It was found that it did not significantly affect cell survival at non-toxic doses at either high or low dose rate irradiation. Assuming each agent is truly specific to its target enzyme, this suggests that topoisomerase I, but not topoisomerase II, is involved in the recovery of cells from damage induced by ionizing radiation (Musk & Steel, 1990).

Finally, Hennequin et al investigated the interaction of γ -rays from a ^{137}Cs source (1 Gy/h) and camptothecin (3 μM). The studies were done *in vitro* using exponential phase HeLa-S3 cells and Chinese hamster V-79 fibroblasts. Concomitant exposure to CPT and LDR consistently produced supra-additive cell killing in both cell lines. The sensitization was attributed to accumulation of the cells in the camptothecin-sensitive S-phase, by irradiation in the case of HeLa cells, and by the combination of irradiation and CPT in the case of V-79 cells (Hennequin *et al.*, 1994).

1.4 Linear Quadratic Model

Many mathematical models can adequately fit survival data. Such models include the Incomplete-Repair (IR) model (Thames, 1984), the Repair/Interaction model (Harder & Virsik-Peuckert, 1984), the Repair-Misrepair model (Tobias, 1985), the Saturable Repair model (Goodhead, 1985), the Lethal and Potentially Lethal (LPL) model (Curtis, 1986), and the Linear Quadratic (LQ) model (Hall, 1994). Most are quite similar, and the survival data in the literature are not precise enough to discriminate between models. In fact, one publication (Brenner *et al.*, 1998), has demonstrated that the LQ model and most other common radiobiological models result in similar predictions of time-dose relationships. The Linear Quadratic model has become the model of choice for radiotherapy because it is a mechanistic, biologically based model, and it has only two adjustable parameters, making it very practical. It adequately represents survival data for doses used as single fractions in radiotherapy (Hall, 1994).

The Linear Quadratic model was developed by Chadwick and Leenhouts (Chadwick & Leenhouts, 1973). Their model was based on the assumption that a double strand break (DSB) in the DNA is the critical damage that will induce cell death. It assumes that there are two modes of action by which ionizing radiation can produce a DSB in the DNA. These are represented by the two adjustable parameters in the LQ equation:

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

where S.F. is the surviving fraction, D is the accumulated dose of radiation, and α and β are constants that determine the initial slope and the degree of downward curvature of the cell survival curve (Figure 1.4.1).

Figure 1.4.1 Sample survival curve

The linear term, α , is proportional to the dose, and the quadratic term, β , is proportional to the square of the dose (Hall, 1994). The linear component accounts for DSBs produced by a single ionizing event, and is independent of dose rate. The quadratic component accounts for DSBs produced via the interaction of two single strand breaks (SSB), each of which is the result of an independent ionizing event. If one of the SSBs is repaired before the other occurs, then the DSB will not be produced. The quadratic component is, therefore, dependent on dose rate and the repair half time of the cells in question (Brown, 1996). The ratio of α to β , (α/β), is the dose at which cell killing by the linear and quadratic components

is equal. Late-responding tissues (most normal tissues) usually have lower α/β ratios than early-responding tissues (most tumours). When the α/β ratio is small the shape of the dose-response relationship is more curved, indicative of the influence of the β term at low doses. A large β component is evidence that a large proportion of the DNA DSBs are being produced via the interaction of independently produced SSBs. Given more time to repair, there will be fewer SSBs able to interact in such a manner. When the α/β ratio is large, the α component (which is independent of the dose rate and the amount of repair taking place) dominates at low doses. This means that as the dose rate, or dose per fraction, is decreased, tissues with lower α/β ratios will be preferentially spared, relative to those with higher values of α/β . Therefore, modulation of the dose rate is a powerful tool for changing the therapeutic ratio (Dale & Jones, 1998). Survival curves for low dose rate treatments generally have very small β components since the dose rate is usually low enough to allow the repair of SSBs before the second break occurs.

The underlying principles of the LQ model were first elucidated by Lea and Catcheside in 1942 (Lea & Catcheside, 1942) in their study of the induction by radiation of chromosomal aberrations in *Tradescantia* spores. Some prefer to interpret the LQ model in terms of chromosomal aberrations instead of DNA double strand breaks. By this interpretation, cells in which there is an exchange-type aberration (two separate prereplication chromosomes, each with a DSB in its DNA, exchange their broken pieces to form a dicentric and an acentric fragment) lose their reproductive integrity (Hall, 1994). Aberrations are formed when pairs of nearby DSBs wrongly rejoin to one another (Brenner & Herbert, 1997). The exchange of broken DNA fragments replicates during DNA synthesis resulting in a distorted chromosome

with two centromeres (dicentric) and a fragment with no centromere (acentric). The other lethal type of chromosome aberration involves the formation of a ring. At low doses, the two breaks are caused by a single electron set in motion by the absorption of an x- or γ -ray. The probability that the two breaks will interact to form a lethal exchange-type aberration is proportional to dose. At higher doses, the two breaks may be caused by two separate electrons. The probability of an interaction between the two breaks is then proportional to the square of the dose (Hall, 1994). As before, the linear term, α , is dose rate independent, while the quadratic term, β , is dose rate dependent. Regardless of which interpretation one chooses to accept, the Linear Quadratic model is a very useful tool for radiotherapy. It allows the prediction of the results for various fractionation patterns, where the dose is protracted over different amounts of time. One can use the LQ model to calculate iso-effect doses, which, besides providing the basis for the examination of various fractionation schemes, allow oncologists to compensate appropriately for missed radiotherapy treatments (Brenner & Herbert, 1997). The LQ model has been used to fit all survival data in this study.

1.5 CHEF Electrophoresis

Gel electrophoresis is a method used to separate charged biological molecules of different sizes, such as proteins, RNA, and DNA. To separate DNA using conventional electrophoresis techniques, the DNA is embedded in a solid gel matrix, and a constant electric field is applied across the gel. The DNA, being negatively charged, migrates toward the anode through the pores in the gel. Factors determining the velocity with which the DNA

migrates are the coefficient of viscosity of the gel, the radius of the molecule, and the net force on the molecule (defined as the product of the electric field and the net charge on the molecule) (Brown, 1996). Conventional electrophoresis is limited to the separation of DNA fragments less than 0.75 megabase pairs (Mbp) due to what is known as “reptation”. This is when larger DNA fragments orient themselves in the direction of the electric field and move irrespective of their molecular weight. In order to migrate, the DNA must straighten out, since the diameter of the pores in the gel through which it must pass are smaller than a random coil of DNA. Beyond a certain length, once straightened out, the DNA will all migrate at the same rate, regardless of differences in their size (Blocher & Kunhi, 1990). The solution to this problem is pulsing of the electric field, which will allow the separation of larger fragments. This development in electrophoretic technique termed Pulsed Field Gel Electrophoresis (PFGE) was first introduced by Schwartz and Cantor in 1984 (Schwartz & Cantor, 1984). In its simplest version, pulses of an electric field across the gel are separated by pauses during which the electric field is removed. During this time the elongated DNA molecules will relax back towards their equilibrium conformation. Separation of higher molecular weight molecules can be achieved by choosing the appropriate times for the on and off phases (Blocher *et al.*, 1989).

A further improvement came with the advent of Asymmetric Field Inversion Gel Electrophoresis (AFIGE). In this variation of the PFGE technique, two electric fields of different field strength and opposite polarity are applied alternately for different pulse intervals. AFIGE allowed the separation of DNA up to 9 Mbp in size (Stamato & Denko, 1990). Stamato and Denko applied the AFIGE method to the detection of DNA DSBs in

mammalian cells in 1990. Mammalian chromosomes vary in size between 50-250 Mbp, and are therefore too large to migrate through a gel. If, however, the DNA is damaged, then smaller fragments caused by DSBs will be able to migrate in the presence of an electric field. Thus, the fraction of DNA that migrates into the gel is a measure of the induced DNA DSBs (Stamato & Denko, 1990).

Separation of DNA is further improved when two homogeneous electric fields are applied alternately at an angle of 120° , forcing the DNA to zig-zag through the gel. This is Clamped Homogeneous Electric Field (CHEF) electrophoresis. The DNA migrates along the resulting net vector of the two electric fields. CHEF electrophoresis allows the separation of DNA molecules up to 10 Mbp. DNA fragments from irradiated cells fall into this range, and the fraction of fragments up to this size is correlated with the number of radiation induced DSBs. The smallest amount of damage that can be measured using CHEF electrophoresis is equivalent to about 1 Gy of γ -rays (Blocher & Kunhi, 1990). CHEF electrophoresis was used in this study to evaluate the amount of DNA damage sustained by the cells due to the various treatments they underwent.

1.6 Comet Assay

The comet assay is a convenient and sensitive method for looking at DNA damage in individual cells. In 1984, Ostling and Johanson put forth the idea of performing gel electrophoresis on individual cells embedded in agarose and lysed to form nucleoids (Ostling & Johanson, 1984). The main advantage of this type of assay is that by looking at individual

cells, one can measure the heterogeneity within a treated population of cells. The comet assay can reveal whether all cells within a population show equal amounts of DNA damage, and whether all cells within a population repair DNA damage at the same rate (Olive *et al.*, 1990). A slowly repairing population of cells may be so because all cells repair slowly, or because some rejoin breaks rapidly while others fail to repair at all. In this way, sub-populations of resistant and sensitive cells can be identified using the comet assay.

There are also practical advantages to the comet assay over some of the more traditional methods used to measure DNA damage. Few cells are required (only a few thousand per sample), there is no need for radiolabelling of cells, and the slides can be dried and stored for later analysis (Olive, 1999). The current study benefits from the fact that only a small number of cells are required, considering the limitations of the LDR apparatus in this regard.

There are two versions of the comet assay: alkaline and neutral. The alkaline assay is for the detection of DNA SSBs. In this assay, the cells are lysed and electrophoresed under alkaline conditions, which causes the denaturation of the DNA, separating each pair of strands and allowing the individual strands to migrate independently. Replication forks are seen as breaks, and DSBs represent less than 5 % of the total damage measured (Olive & Banath, 1993; Olive, 1999). In this study the neutral comet assay was used, which allows the detection of DNA DSBs. In order to detect DSBs, the cells must be lysed and electrophoresed in a neutral (non-denaturing) solution, able to remove proteins (Olive *et al.*, 1991). Higher doses and longer lysis times are required to detect DSBs since they occur much less frequently (25-40 times) than do SSBs. Damage after doses as low as 5 Gy from

x-rays can be detected using the neutral comet assay (Olive *et al.*, 1991), but this limit can be improved by using non-proliferating cells (Olive, 1999). Gel electrophoresis techniques applied to proliferating cells are complicated by the effects of “S-phase retardation”, which denotes the much slower migration of DNA from cells in the S-phase, thought to be caused by the presence of replication forks. During replication, DNA is transiently present as “bubble” and “Y” shaped structures, making its passage through the pores of a gel much more difficult (Olive & Banath, 1993; Olive *et al.*, 1994). Therefore, lower levels of DNA damage can be measured in non-proliferating cells, which have a small S-phase fraction.

The name “comet” assay was introduced by Peggy Olive in 1989 (Olive, 1989). Each damaged cell, after having been stained with a DNA binding dye and viewed under any fluorescent microscope with the appropriate filter, has the appearance of a comet, with a bright fluorescent head, and a tail with an intensity related to the amount of damage sustained by the cell. As the cells accumulate more damage, the electric field draws out more broken and relaxed DNA from the head. The tail then becomes longer and contains more DNA. The distance travelled by the DNA is related to the size of the broken pieces, and the intensity of the tail is an indication of the number of pieces able to migrate (Olive *et al.*, 1990).

With the comet assay, repair of DNA damage will manifest itself as “shrinkage” of the comet tail. However, the assay has no way of determining whether the repair was performed correctly. Therefore, misrepair may lead to a rapid shrinkage of the comet, but these cells will probably not be clonogenic survivors.

The two most common parameters used to assess DNA damage in this assay are tail length (TL) and tail moment (TM). Tail length is defined as follows:

$$TL = |cm_{head} - \Omega_{tail}| \quad (2)$$

where cm_{head} is the position of the centre of mass (mean position) of the comet head, and Ω_{tail} is the position of the end of the comet tail. Tail length is measured in microns. The expression for tail moment is:

$$TM = \Phi_{tail} \times |cm_{head} - cm_{tail}| \quad (3)$$

where Φ_{tail} is the percentage of DNA in the tail, and cm_{tail} is the position of the centre of mass (mean position) of the tail. The tail moment parameter is a more comprehensive measurement of the DNA damage sustained by the cell since it not only considers the distance the DNA migrates, but also the amount of DNA that migrates from the head of the comet. Tail moment is also measured in microns. At low doses, little damage is visible, and tail length increases with dose. However, at higher doses, the length of the tail saturates, and as more dose is given, DNA continues to enter the tail without increasing its length (Kent *et al.*, 1995). In their study, Kent et al (1995) observed a linear increase in tail length as a function of dose in Chinese hamster ovary (CHO-K1) cells subjected to ^{60}Co irradiation at a dose rate of 9 Gy min^{-1} up to 40 Gy. At this point there was a plateau such that no further increase in tail length was observed. The plateau region observed by Kent et al (1995) begins at a dose well above the maximum doses of radiation used in the current work. In the same study they observed that tail moment increased linearly with dose over a larger range than did tail length,

and there was no tendency to plateau. The neutral comet assay was used in the current study to assess the amount of DNA damage, in the form of DSBs, sustained by the cells under different treatment conditions.

1.7 DNA DSBs and Clonogenic Survival

The evidence suggesting the nucleus of cells as the sensitive site for radiation induced lethality is abundant (Hall, 1994). Early studies with algae, amoebae, and the eggs of frogs, using a microbeam of radiation to selectively irradiate either the nucleus or the cytoplasm, showed that the nucleus was the much more sensitive of the two. Later, similar studies were conducted with mammalian cells. This time, Chinese hamster fibroblasts were irradiated with short-range α -particles from a Polonium microneedle source (Munro, 1970). It was found that 250 Gy delivered only to the cytoplasm had no effect on the proliferative ability of the cell. However, a few α -particles penetrating 1 or 2 μm into the nucleus could prove to be lethal (Hall, 1994).

The evidence implicating chromosomal DNA as the target of radiation within the nucleus is circumstantial but overwhelming. Tritiated thymidine incorporated into DNA kills cells. The radiation is in the form of short-range β -particles, and is therefore very localized. The radiation type, oxygen concentration, and dose rate affect the amount of chromosomal damage in a way that is qualitatively and quantitatively similar to that in which they affect cell killing. Also, in plants, the larger the mean chromosome volume (defined as the ratio of nuclear volume to chromosome number) the greater the radiosensitivity (Hall, 1994). If DNA

is the primary target for radiation induced lethality, then there must exist a relationship between clonogenic survival and some type of DNA damage.

The DNA double strand break is believed to be the most important lesion produced in chromosomes by radiation (Hall, 1994). DNA DSBs are generally considered to be of greater consequence to biological systems than SSBs since they can lead directly to chromosome aberrations (Olive *et al.*, 1991). However, the existence of a correlation between cell survival and DSBs is controversial. It has been shown that, in yeast, one unrepaired DSB is lethal (Frankenberg *et al.*, 1981). Current methods used to measure DSBs in mammalian cells are not sensitive enough to be able to relate one unrepaired DSB directly to a lethal lesion. There has been some success in correlating the level of DSB induction with survival or radiosensitivity. Radford found that DNA DSB induction varied markedly among the different cell types examined (normal and transformed fibroblasts of rodent and human origin) and that the level of initial DSBs reflected the radiosensitivity of the cells (Radford, 1986). Similar findings have been reported in other tumour cell lines (Nunez *et al.*, 1996; Kelland *et al.*, 1988). Others have found no such relationship (Muller *et al.*, 1994; Cassoni *et al.*, 2000; Dikomey *et al.*, 1998; Marples *et al.*, 1998; Iliakis & Okayasu, 1988).

It seems more likely that there would be a relationship between the level of residual DSBs (those left unrepaired after sufficient time for complete repair to take place) and radiosensitivity as measured by survival. One study was able to correlate cell killing and non-repaired DSBs ($r^2 = 0.87$) measured 24 h after x-ray irradiation (Dikomey *et al.*, 1998). This correlation held for both the repair proficient and deficient cell lines. The nine cell lines used did not vary in the number of induced DSBs. In a study of four human lung carcinoma cell

lines given LDR irradiation (0.02 Gy/min), there was a correlation between radiosensitivity and levels of DSBs measured after irradiation (Cassoni *et al.*, 2000). Such a correlation did not exist for HDR treatment.

One problem in radiotherapy is the existence of very radioresistant cells within a tumour that often cause the treatment to fail. The resistance of these cells is explained by their greater capacity to effect the repair of DNA damage. In a study with three tumour cell lines given doses in a range comparable to the dose per fraction in typical radiotherapy schemes (about 2 Gy), the difference in radiosensitivity could not be explained by the amount of initial DNA damage directly after exposure to x-rays (Muller *et al.*, 1994). There was, however, a correlation with residual damage, and although all three cell lines showed the same extent of initial damage, the study concluded that radiation sensitivity depends on both the amount of initial damage and the repair capacity of the cells in question. Another study, using seven cervical tumour cell lines, was unable to find a correlation between initial or residual DSBs and SF₂ (surviving fraction at 2 Gy) after either HDR or LDR irradiation (Marples *et al.*, 1998). Interestingly, a significant correlation ($r = -0.86$) was found between LDR SF₂ values and the ratio of the slopes of the initial and residual DSBs dose-response curves, which represents the fraction of DNA damage remaining.

As has been shown, there are many studies in which a correlation between radiosensitivity and residual DNA DSBs has been observed. However, this is not always the case. Olive *et al.* (1994) found that DSB rejoining capacity did not correlate well with radioresistance in the six human tumour cell lines examined (Olive *et al.*, 1994). One possible explanation for this lack of correlation has to do with the fidelity of repair. Cells with greater

rejoining capacities are not necessarily always rejoining DSBs correctly. In some instances it is possible that they might even be producing chromosome aberrations during rejoining (Britten *et al.*, 1999).

In addition to ionizing radiation, there are many chemotherapeutic agents that produce DNA damage in cells including SSBs and DSBs. Camptothecin is only one of them. In one study, multicell spheroids were exposed to several DNA-damaging drugs (including etoposide, a topoisomerase-2 inhibitor, derived from CPT, that selectively damages external proliferating cells of spheroids) with the goal of determining whether prompt DNA damage could be predictive for cell killing and drug resistance (Olive & Banath, 1997). It was found that with most of the drugs tested, it was the number of lesions per cell, not repair efficiency, that determined survival after treatment of the spheroids. The authors further state that for radiation, and probably any agent that produces equal amounts of DNA damage in all cells of spheroids, repair proficiency is the major determinant of survival. The fact that ionizing radiation produces the same number of breaks in almost all cell types for a given dose means that it should not be possible to correlate initial DNA damage with cell killing. By the same token, due to this very fact, it should be possible to correlate residual DNA damage with cell killing since residual damage is then a direct measure of the repair capacity of the cells in question. One can only postulate the relationship that may exist between survival and DNA damage when ionizing radiation is combined with chemotherapeutic agents.

1.8 Hypothesis and Specific Aims

The hypothesis of this thesis is that camptothecin can significantly potentiate the response of the radioresistant Sk Mel-3 human tumour cell line to low dose rate irradiation, as measured by clonogenic surviving fraction, and that this potentiation is a result of an increase in the number of residual DNA double strand breaks in the cells.

Specific aims are:

- Establish the response of Sk Mel-3 cells to low dose rate irradiation as well as to three concentrations (1 μ M, 10 μ M, 25 μ M) of camptothecin. Fit the responses with the linear quadratic model.
- Establish the response of Sk Mel-3 cells to low dose rate irradiation in combination with each of the aforementioned concentrations of camptothecin. Fit the responses with the linear quadratic model.
- Use isobologram analysis to determine the effects of the interaction between low dose rate irradiation and camptothecin (i.e. additive, sub-additive, supra-additive) in terms of clonogenic survival.
- Where a positive interaction between low dose rate irradiation and camptothecin is observed, evaluate the amount of residual DNA damage, in the form of double strand breaks, using the CHEF and comet assays.
- Determine if the residual DNA double strand breaks as measured by the comet assay are correlated with survival for the combination of low dose rate irradiation with camptothecin at a concentration of 25 μ M.

This thesis is comprised of five chapters and an Appendix. The first chapter is the introduction, followed by materials and methods, results, discussion, and conclusions. A discussion of the interpretation and construction of isobolograms is found in the materials and methods section. The Appendix contains the individual isobolograms as well as the code for the C program written to calculate the values necessary for plotting them.

Chapter 2

Materials and Methods

2.1 Experimental Procedure

2.1.1 The Cell Line

To test the hypothesis of this study, a human melanoma (Sk-Mel 3) cell line was used. Melanoma is a type of cancer that develops when melanocytes, the cells that produce melanin pigment, undergo a malignant transformation. This type of cancer occurs most often in the skin (Fleming *et al.*, 1995). The Sk Mel-3 cell line was chosen as the tumour model since it is one of the most resistant tumour cell lines to both ionizing radiation and CPT, when either is given alone (Ng *et al.*, 1998; Ng *et al.*, 1994; Raaphorst *et al.*, 1994). Therefore, if CPT can potentiate radiation effects in such a resistant cell line, then a similar, if not greater effect, could be expected in other types of tumour cell lines of lower resistance. Note that it is not a claim of this study that melanoma is a good candidate for brachytherapy treatment with CPT

in the clinic. The Sk Mel-3 cell line was isolated from tumour cells released by trypsinization of the lymph node metastases. The cells are grown as a monolayer and have a fibroblast-like morphology. They are hypertetraploid human cells, meaning that, on average, each contains two times the amount of DNA present in a normal human diploid cell. Samples of Sk Mel-3 cells were obtained from the American Type Culture Collection (ATCC) (ATCC, 1994).

2.1.2 Cell Culture Conditions

The cells were cultured in medium consisting of Dulbecco's modified Eagle's medium (DMEM) [(Nutrient Mixture Ham's F12 (1:1) (Gibco BRL) supplemented with 10 % (v/v) Fetal Bovine Serum (Wisent), 1 mM MEM non-essential amino acids (Gibco BRL), 10 mM sodium bicarbonate (Fisher), and 20 mM Hepes (Wisent)]. Cells were incubated at 37°C in a humidified atmosphere of 2.5 % carbon dioxide and 97.5 % air.

The cells were seeded on day 0 at a concentration of 3×10^4 cells per vial. The medium was changed on day 5, and the experiments were performed on day 8. The total amount of medium in each vial was 500 μ L. It was previously determined that by the day of the experiment, the cells had grown to confluence in a monolayer on the surface of the vials (Raaphorst *et al.*, 1994). All experiments were performed while the cells were in plateau phase in order to minimize cell cycle effects (Wilkins *et al.*, 1996).

2.1.3 LDR, CPT, and Combined LDR/CPT Procedures

For continuous low dose rate (LDR) irradiation, the cells were irradiated in glass scintillation vials, each having a surface area of 1.3 cm² on which the cells could attach and grow. Only 14 vials could be placed in the irradiator at any one time. This put a strict limitation on the number of cells available for each dose point. The irradiator itself consisted of a circular array of eight 100 mCi ²²⁶Ra sources, encased in lead, and contained in an incubator to allow for control of temperature (Raaphorst *et al.*, 1994). All irradiations were carried out at 37°C. The dose rate was 0.88 cGy/min (or 0.53 Gy/h), a clinically relevant dose rate for brachytherapy (Hall & Brenner, 1991). This dose rate was determined by TLD dosimetry relative to a ⁶⁰Co reference protocol (Heller, 1993).

Cells to be treated with CPT alone were grown in the same glass vials in order to maintain consistency between experiments, with respect to the growth conditions. In these experiments, the drug was added to the medium in each vial, and remained there for the duration of the radiation treatment. Vials not requiring radiation treatment were kept at 37°C in the incubator. For the combined LDR and CPT treatments, the drug was added in the same manner, immediately before placing the cells in the irradiator.

2.1.4 Clonogenic Assay

The effects of each treatment on cell survival were quantified using the clonogenic surviving fraction assay. According to this assay, a cell has survived a treatment if it has retained its reproductive integrity (i.e., it is able to proliferate to form a large colony of cells). Such a cell is said to be clonogenic. Reproductive death is a relevant endpoint in cancer treatment because in order to eradicate a tumour, it is only necessary to stop the tumour cells from proliferating and repopulating the tumour after treatment.

To do this, cells are released from the vials following treatment by using the enzyme trypsin ((0.2 % w/v in citrate saline)(Gibco BRL)). The medium was aspirated, and the cells in each vial were washed with 200 μ L citrate saline (134 mM KCl (BDH), 17.6 mM citric acid trisodium salt (Sigma)), which was then aspirated as well. The citrate saline wash removed any medium left in the vials that would have inhibited the effect of the trypsin. Trypsin dissolved the protein bonds binding the cells to the vials and allowed the cells to be released. 100 μ L of trypsin per vial was added. The cells were returned to the incubator (37°C) for 5-8 minutes to allow trypsinization to occur. Once the majority of cells had detached from the surface of the vial, at least 500 μ L of fresh medium with 10 % FBS was used to resuspend the cells into a single cell suspension, stopping the action of the trypsin. The cells in the suspension were counted using an electronic cell counter (Particle Data Elzone 80) which had been calibrated by a Bright-Line haemocytometer (Reichert). The necessary dilutions were performed, and the appropriate number of cells needed to give about 50 colonies per plate was seeded into 60 mm petri dishes containing 4 mL of medium. The

plates were then incubated at 37°C for 11 days. At the end of the incubation period, the media was removed and the cells were stained for at least one half hour with Methyl Blue (0.2 % w/v Methylene Blue (Sigma) in 70 % ethanol (BDH)). The plates were rinsed in water and allowed to dry. Colonies containing 50 or more cells were counted.

For each dose point, the average count of three plates was used for subsequent calculations. The control plates, which received no treatment, were used to calculate the plating efficiency (PE), according to the following equation:

$$PE = \frac{\text{colonies counted}}{\text{cells plated}} \quad (4)$$

Plating efficiency accounts for the death of cells due to factors other than the treatment itself. With the use of the plating efficiency, the surviving fraction (SF) was calculated for each dose point according to the formula below.

$$SF = \frac{\text{colonies counted}}{\text{cells plated} \times PE} \quad (5)$$

Each experiment was done three times. The mean and standard error of the mean were calculated for each set of three experiments. The surviving fraction was plotted on semi-logarithmic paper as a function of dose and/or exposure time to CPT.

2.1.5 CHEF DNA double strand break Assay

Cells used for the Clamped Homogenous Electric Field (CHEF) assay were grown to confluence in medium containing $0.02 \mu\text{Ci/mL}$ ^{14}C -thymidine ($[2\text{-}^{14}\text{C}]$ Thymidine, 0.1 mCi/mL (Mandel)). The dose to the cells from such a small amount of ^{14}C was too low to kill the cells. Having reached confluence, the cells were treated with LDR irradiation and/or CPT as described above. In preparing to quantify the amount of DNA damage, in the form of double strand breaks (DSBs), sustained by the cells, a single cell suspension was prepared in the same manner as described in the previous section. A volume of the cell suspension containing 1×10^6 cells was centrifuged at 4°C and 1200 rpm for 5 minutes. After removing the supernatant, leaving the pellet of cells intact, the pellet was re-suspended in $500 \mu\text{L}$ of warm phosphate buffered saline (PBS) (Sigma). To this cell suspension was added $500 \mu\text{L}$ of 2 % low melting point (LMP) agarose (Gibco BRL) at 40°C . The two were mixed together gently, creating a 1 % agarose solution at a concentration of 1×10^6 cells/mL.

Plugs were made by casting samples of this solution in CHEF disposable plug molds (Bio-Rad), and allowing them to solidify on ice. The embedding of the cells in agarose gel was to prevent mechanical shearing of DNA during lysis (Blocher & Kunhi, 1990). Once solidified, the plugs were placed in lysis buffer (0.5 mM EDTA (disodium dihydrate) (Sigma), 1.0 % w/v Sarkosyl (Sigma), $1.0 \mu\text{g/ml}$ Proteinase K (Sigma)) for 24 hours at 50°C . Lysing the cells breaks the cell membrane and removes the protein from the DNA. The next day, the lysis buffer was removed, and the plugs were washed four times in 1 mL of TBE (Tris/Boric Acid/EDTA) buffer (Bio-Rad) for half an hour to one hour each, under gentle agitation. The

plugs were then stored at 4°C until they were to be run in a gel. At 4°C the plugs would be stable for three months to one year (Bio-Rad, 1992).

The gel was made of 0.8 % Pulsed Field Certified Agarose (Bio-Rad) in 100 mL of 1x TAE (Tris/Acetic Acid/EDTA) buffer (Bio-Rad). The agarose was heated in a microwave oven until it dissolved completely. The 14 cm wide by 13 cm long casting stand provided with the CHEF-DR 11 system was leveled in advance. Two ten-well combs were inserted, to a height of 2 mm above the surface of the platform. Once the temperature of the agarose had dropped below 60°C, it was poured into the casting stand, and was allowed to solidify at room temperature for approximately half an hour. Once solidified, the combs were removed, and one third of a plug was placed in each well. The DNA standard *Saccharomyces cerevisiae* (*S. Cerevisiae*) (Bio-Rad) was placed in at least one well of each gel as a control for the proper running of the gel. Each well was filled and covered with 0.8% LMP agarose, and allowed to solidify.

Prior to placing the gel and its platform into the electrophoresis cell, 2 L of 1 x TAE buffer was poured into the cell. The variable speed pump was turned on, allowing the buffer to circulate through the system and be cooled by the Model 1000 Mini Chiller (Bio-Rad). A setting of 70 on the pump regulator was used, corresponding to a flow rate of approximately 0.75 L/min. The temperature of the buffer in the cell was allowed to equilibrate to 14°C, and the gel was placed in the electrophoresis cell.

Three gels were run, each representing several experiments. The voltage gradient was 1.5 V/cm for all three gels, but the run time and switch time varied slightly. The gels were run for 24.4 h with a 11000 s switch time. The switch time is the length of time the electric

field is in one direction before switching back to the other. The CHEF system, with its hexagonal array of 24 electrodes, produces two electric field vectors (Figure 2.1.5.1).

Figure 2.1.5.1 CHEF electrophoresis chamber showing the two electric field vectors at 120° to each other.

When the $+60^\circ$ vector is activated, the net field vector is from NW to SE. When the -60° vector is activated, the net field vector is from NE to SW. During a run, the electric field vector switches between these two orientations, resulting in a 120° included field angle. For an even number of switch intervals the net direction of DNA migration is from N to S. When the electrophoresis was finished, the gel was removed from the cell and placed into a plastic container for staining in a solution of 100 μL of 10 mg/mL ethidium bromide (EtBr) (Gibco BRL) in 1 L of ddH₂O. The gel was stained for 1 h under gentle agitation. This allowed the

EtBr to bind directly to the DNA. The gel was then destained for 0.5 h in ddH₂O in order to remove any non-specific binding of EtBr to the gel itself. The gel was placed on a 312 nm variable intensity transilluminator (Fisher) which fluoresces the EtBr so that the stained DNA can be viewed. A digital image of each gel was captured.

To quantify the amount of DNA that had migrated out of the plug and into the gel, the gel was cut into lanes. For each lane, the well containing the plug was separated from the rest of the lane. For each dose point, the pieces of the gel corresponding to the well and those corresponding to the lane were placed in separate scintillation vials. To each vial was added 50 μ L of 10 M HCl. The vials were then heated on a hot plate to melt the gel. The HCl prevented the gel from solidifying once it was removed from the hot plate and allowed to cool. Once melted, 10 mL of Ecolite scintillation fluid (ICN) was added to each vial. Each vial was then shaken vigorously to mix the melted gel with the fluid. The vials were labeled appropriately and placed in a liquid scintillation counter which counted each sample for ¹⁴C for 8 minutes. The count per minute (cpm) was determined for each sample, and the fraction of activity released (FAR) from the well was calculated using the following equation:

$$FAR = \frac{cpm \text{ in lane}}{cpm \text{ in well} + cpm \text{ in lane}} \quad (6)$$

Each experiment was done three times. The mean and standard error of the mean were calculated for each set of three experiments. The FAR was plotted as a function of dose and/or equivalent exposure time to CPT, and all points were normalized to zero by

subtracting from them the FAR of the control samples.

2.1.6 Comet Assay

Having reached confluence, the cells were treated with LDR irradiation and/or CPT in the same manner as for the clonogenic and CHEF assays. A strip of Gelbond (Mandel) film was cut to size and three or four 2-well Supercell Lab-Tek II chamber slides (Nalge Nunc) were attached to the hydrophilic side of the strip. The cells were then trypsinized, counted, and diluted in medium to a concentration of 4×10^5 cells/mL. A 1:10 dilution in agarose of this cell suspension was made by mixing 50 μ L of the cell suspension with 450 μ L of 1 % LMP agarose (Gibco BRL) in PBS (Sigma) at 40°C in a 1 mL Eppendorf tube. For each dose point, 120 μ L of this solution was aliquoted to an exposure well on the Gelbond strip. Once the agarose had cooled, the Supercell chambers were removed from the Gelbond strips, and the strips were placed in 50 mL of final lysis buffer solution, consisting of: 2.5 M NaCl (BDH), 100 mM Tetrasodium EDTA (Sigma), 10 mM Tris base (Gibco BRL), 1 % Sodium Lauryl Sarcosine (Sigma), 1 % Triton X-100 (Fisher), and 10% DMSO (BDH). The Triton X-100 and DMSO were added to the required volume of lysis buffer on the day of the experiment.

The strips were maintained in lysis buffer and incubated at 37°C for 1 hour. After 1 hour, the lysis buffer was poured off, the strips were rinsed with ddH₂O, placed in 50 mL of 1 x TBE (Bio-Rad) and left at room temperature for 20 minutes under gentle agitation. At the end of 20 minutes the strips were removed from the TBE. Each was then placed in a mini

horizontal submarine gel electrophoresis chamber (Hoefer) covered with 220 mL of 1 x TBE buffer and electrophoresed for 40 minutes at 1 V/cm. After electrophoresis, the strips were rinsed with ddH₂O then covered with 70 % ethanol (BDH). The strips were left in the ethanol for 2 hours, at which point they were removed and allowed to air dry overnight.

The gels were stained, two at a time, with SYBR Gold stain (5 µL stock in 50 mL dH₂O), for 10 minutes at room temperature in the dark. They were then removed from the stain and rinsed in dH₂O for 1 minute. Each strip of two gels was then placed on a microscope slide, covered with a large coverslip, and viewed under oil using the 40x objective lens with the “NB” (narrow blue) -cube filter. Samples were viewed using an Olympus BX60 microscope with a KP-D 581 (Hitachi) digital colour camera attached. Cells were illuminated with 495 nm light excitation from a 100 W mercury burner. DNA damage parameters were quantified using the ALKOMET v3.1 image analysis system (Richard Brancker Research Ltd., Ottawa, Ontario). Individual images were digitized into a pixel matrix. For each comet, head and tail regions were defined, and the total intensity, tail length and comet length, as well as the percentage of DNA in the head and tail regions were determined. Each experiment was done two times. Normalized tail length and tail moment, calculated as follows:

$$TL_{xGy(norm)} = \left(\frac{TL_{xGy}}{TL_{0Gy}} - 1 \right) \times 100 \% \quad (7)$$

$$TM_{xGy(norm)} = \left(\frac{TM_{xGy}}{TM_{0Gy}} - 1 \right) \times 100 \% \quad (8)$$

were plotted as a function of dose and/or equivalent exposure time to CPT.

2.2 Data Analysis

2.2.1 Curve Fitting

Survival curves were fit to the linear quadratic model, equation (1), using SigmaPlot version 4.00 (1997 SPSS Inc.). The SigmaPlot curve fitter uses the Marquardt-Levenberg algorithm to find the coefficients of the independent variables that give the best fit between the equation and the data. The algorithm seeks the values of the parameters that minimize the sum of the squared differences between the values of the observed and predicted values of the dependent variable:

$$SS = \sum_{i=1}^n (y_i - \hat{y})^2 \quad (9)$$

where y_i is the observed value of the dependent variable, and $\hat{y}$ is the value of the dependent variable as predicted by the equation to which the data is being fit. It is an iterative process in which the algorithm continually makes better guesses at the values of the parameters until the difference between the residual sum of squares no longer decreases significantly. The parameters, which are α and β in the case of a linear quadratic fit, are obtained with their estimated standard errors, based in part on the value of the residual sum of squares.

The plots of the comet assay parameters tail moment and tail length versus dose were

fit using the same algorithm, but to a different equation:

$$y = a(1 - e^{-bx}) \quad (10)$$

Unlike the linear quadratic model, this equation has no biological significance. It was chosen only because it approximated the data fairly well.

2.2.2 Interpretation of Isobolograms

Whenever agents are combined, the question arises as to whether the effects of the combination are greater or less than what would be expected on the basis of the effects of each agent on its own. Isobologram analysis, as described by Steel and Peckham (1979), was developed to answer this question, and has since been used by several authors to evaluate the effectiveness of treatments combining radiation and drugs (Marchesini *et al.*, 1996; Roffler *et al.*, 1994; Giocanti *et al.*, 1993; Hennequin *et al.*, 1994). For each dose point on the two-agent combined dose response curve there is, of course, a corresponding level of survival that was observed experimentally. Each of these is a potential isoeffect level to be examined by isobologram analysis. This analysis uses the single-agent dose response curves to generate isoeffect lines representing all possible combinations of the two agents that would mathematically result in the reduction of survival to the isoeffect level in question. Basically, given two agents, A and B, the isobologram calculation involves determining how much dose of agent B is required to reduce survival to the isoeffect level assuming the cells have already

received a dose of agent A, which has reduced the survival to a certain level, obviously less than the isoeffect level. This addition of doses to achieve a fixed level of survival is performed twice under two different assumptions. The first is that each agent acts completely independently of the other, and this corresponds to the isoeffect line referred to as *mode 1*. Conversely, the other assumption is that the two agents act in a dependent manner, such that there is an exact complementation of the effect of each treatment by the other (Hennequin *et al.*, 1994). This corresponds to the *mode 2* isoeffect line. The mode 1 and mode 2 lines delineate the region referred to as the envelope of additivity, within which all responses are considered additive.

Figure 2.2.2.1 Sample isobologram

In the case where each single-agent dose response curve is non-linear, two mode 2 lines are required because the shape changes depending on which agent is given first in the calculation.

Referring to figure 2.2.2.1, points to the left of the envelope of additivity represent a supra-additive effect (i.e. a response greater than would be expected based on any analysis of the individual single-agent dose response curves). Points within the envelope represent an additive effect, but require careful interpretation. A point near the mode 2 line implies that the agents are acting by the same mechanism, while a point near the mode 1 line implies that the agents are acting by independent mechanisms. Points to the left of the mode 1 line always imply a positive interaction between the agents. Within the envelope it can be said that there is an enhancement of effect, but not to an extent that would be unexpected based on some interpretation of the single agent dose response curves. A sub-additive effect is represented by a point lying to the right of the envelope of additivity, implying inhibition or antagonism. A point lying outside the box in figure 2.2.2.1, above or to the right, would indicate that one agent allowed the other to be given at a higher level than it could have been given on its own, for the same effect, implying protection.

In this study, isobologram analysis was used to determine whether or not synergistic potentiation occurred when CPT was given concomitantly with low dose rate irradiation. The isobologram corresponding to each isoeffect level examined was constructed by computer calculation.

2.2.3 Construction of Isobolograms

For a particular level of isosurvival, an isobologram is constructed from the fits to the single-agent survival data. From these fits can be determined all possible combinations of doses from each agent on its own that will result in the level of survival chosen as isosurvival. In the present study, all survival data were fit to the linear quadratic model, equation (1). This equation can be rearranged into a second order polynomial with a right hand side equal to zero by taking the natural logarithm of each side, as shown in equation (11).

$$\beta D^2 + \alpha D + \ln(sf) = 0 \quad (11)$$

Equation (11) has the form of the quadratic equation shown in equation (12)

$$ax^2 + bx + c = 0 \quad (12)$$

where $a = \beta$, $b = \alpha$, $c = \ln(sf)$, and $x = D$. Equation (13) gives the solution to the quadratic equation, assuming a , b , and c are real.

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (13)$$

Substituting the variables shown in equation (11) and solving for the dose D, equation (13) becomes:

$$D = \frac{-\alpha + \sqrt{\alpha^2 - 4\beta \ln(sf)}}{2\beta} \quad (14)$$

The positive root of the discriminant has been taken so that values of D will always be positive. Thus, the dose of each single agent required to achieve a particular level of survival was determined from equation (14), using the α and β parameters from the linear quadratic fit to the survival data for that agent.

Figure 2.2.3.1 illustrates diagrammatically how the appropriate doses of each agent are calculated so that their sum reduces survival to the chosen level of isosurvival. It shows the single agent survival curves for two agents, A and B, that have also been applied concomitantly. The summation of doses can be performed by either mode 1 or mode 2. Given a dose of agent A, D_A , which reduces survival to sf_A , a further reduction in survival, equal to the amount denoted by x, is needed in order to reach the desired level of isosurvival, represented as isosf. The mode 1 summation argues that the agents act by independent mechanisms, such that the cell killing already produced by agent A has no effect on the killing to be produced by agent B. Therefore, the reduction in survival, x, needed to be produced by agent B, must begin from 100 % survival as if no dose of agent A had been given. The dose of agent B, D_B , reduces survival by the amount x from 100 % to the level denoted by sf_B . Assuming independence of action, the product of the surviving fraction after each agent

describes the sum of their individual effects, as shown in equation (15).

$$sf_A \times sf_B = isosf \quad (15)$$

Therefore, according to mode 1, D_A is calculated first from equation (14), substituting sf_A for sf . Then, D_B is calculated solving equation (15) for sf_B and substituting that value for sf in equation (14). Calculation according to mode 1 can be summarized by the following equations:

$$\begin{aligned} D_A &= D(sf_A) \\ D_B &= D(sf_B = isosf / sf_A) \end{aligned} \quad (16)$$

The mode 2 summation argues that the two agents act by similar mechanisms, such that there is an exact complementation of the effect of each agent by the other. Therefore, the reduction in survival, x , needed to be produced by agent B, begins at sf_A , and reduces survival directly to $isosf$. Therefore, according to mode 2, D_A is calculated as it is in mode 1, while the dose of agent B, D'_B , is equal to the difference between the dose of agent B alone required to reduce survival to $isosf$ and the dose of agent B required to reduce survival to sf_A . The mode 2 calculations are summarized as follows:

$$\begin{aligned} D_A &= D(sf_A) \\ D'_B &= D_B(isosf) - D_B(sf_A) \end{aligned} \quad (17)$$

Figure 2.2.3.1 Graphical representation of isobologram construction.

For both mode 1 and mode 2, calculations are performed for values of sf_A from 1 to isosf, and therefore, for values of sf_B from isosf to 1, for a chosen increment in survival after each iteration. As was mentioned in the previous section, the mode 2 calculation is performed twice when both of the single-agent response curves are non-linear. One calculation is done assuming agent A is given first, and the other is done assuming agent B is given first. Plotting the doses of agent B versus the corresponding doses of agent A required to reach the chosen level of isosurvival results in an isobologram as shown in Figure 2.2.2.1.

In this study, 5 isobolograms were constructed for the combination of LDRI with each concentration of CPT. The 5 isosurvival levels were chosen as those resulting from concomitant exposure to LDRI doses of 2 Gy, 4 Gy, 8 Gy, 12 Gy, and 16 Gy, and either 1 μ M, 10 μ M, or 25 μ M CPT. These individual isobolograms can be found in the Appendix.

Figure 2.2.3.2 Determination of fractional sub and supra additivities from individual isobolograms.

A more effective way to present the isobologram data is by the method described by Hennequin et al (1994). Figures 3.2.4, 3.2.5, and 3.2.6 were plotted in this manner. Figure 2.2.3.2 shows how these plots were constructed.

The single-agent survival curves in the present work were such that the mode 2 lines of the isobolograms constructed from them were always to the left of the corresponding mode 1 line. Therefore, for supra additive points, the relative supra additivity was determined as the ratio of the dose corresponding to the leftmost mode 2 line, $(x_1 + x_2)$ relative to agent B and $(y_1 + y_2)$ relative to agent A, on one hand, and the difference in dose between this line and the point of interest, either x_1 or y_1 , on the other. The relative supra additivity relative to either dose of agent B, $x_1 / (x_1 + x_2)$, or to dose of agent A, $y_1 / (y_1 + y_2)$, was then plotted as a function of dose as in Figures 3.2.4, 3.2.5, and 3.2.6.

The same procedure was applied to sub additive points except that doses were measured relative to the mode 1 line and the maximum dose used for each agent. For points within the envelope of additivity, doses were measured relative to the mode 1 line and the leftmost mode 2 line. For purposes of normalization, the mode 1 line was defined as 0 % additivity and the left most mode 2 line was defined as 100 % additivity.

Chapter 3

Results

3.1 LDRI, CPT, and Concomitant Exposures

The purpose of these experiments was to determine the effects on the clonogenic survival of Sk Mel-3 cells of combining LDRI and CPT in a concomitant exposure. To this end, the dose response of the cells to LDR irradiation alone, and to each concentration (1 μ M, 10 μ M, 25 μ M) of CPT alone were established. Subsequently, exposure to CPT was combined with LDR irradiation, producing a dose response curve for the concomitant exposure at each concentration of CPT. In an attempt to use clonogenic survival analysis to quantify the effects of an interaction between CPT and LDR irradiation during the concomitant exposures, the effect of the drug was then normalized out of these exposures. This was done by dividing the surviving fraction of each dose point on the concomitant dose response curve by the surviving fraction of the corresponding dose point on the CPT alone dose response curve. This is a technique based on the same principle that is applied when

dividing all points of a dose response curve by the plating efficiency of the cells in order to normalize the data to the zero dose. The normalized concomitant dose response curves could then be compared directly to the LDR irradiation alone curve. Student's t-tests were performed on pairs of corresponding dose points between the LDR irradiation alone curve and each of the normalized and unnormalized concomitant exposure curves to determine the level of significance of any differences observed.

Figure 3.1.1 shows the dose response curves for each concentration (1 μM , 10 μM , 25 μM) of CPT alone. These curves were fit with the linear quadratic model and the estimated parameters α and β with their estimated standard errors and the coefficient of determination, R^2 , for each fit are shown in Table 3.1.1.

Table 3.1.1 Comparison of the linear quadratic fits to the dose response curves of Sk Mel-3 cells to 1 μM , 10 μM , and 25 μM concentrations of CPT.

Parameter	1 μM	10 μM	25 μM
α (h^{-1})	0.04 ± 0.01	0.03 ± 0.01	0.031 ± 0.008
β (h^{-2})	$(0.4 \pm 5) \times 10^{-4}$	$(9 \pm 5) \times 10^{-4}$	$(2 \pm 4) \times 10^{-4}$
R^2	0.92	0.95	0.95

The α and β parameters of Table 3.1.1 can be compared directly to those in Table 3.1.2 by dividing by $(0.53 \text{ Gy}\cdot\text{h}^{-1})$ and $(0.53 \text{ Gy}\cdot\text{h}^{-1})^2$ respectively, where $0.53 \text{ Gy}\cdot\text{h}^{-1}$ is the dose rate of the LDR irradiator. From Figure 3.1.1, it is seen that the 10 μM concentration of CPT is the one that produced the greatest amount cell killing. Curiously, the cells had the highest survival after exposure to the highest concentration, 25 μM , of CPT. Still, the three curves

Figure 3.1.1 Survival curves for the Sk Mel-3 cell line after exposure to 1 μM , 10 μM and 25 μM CPT. Each point represents the average of three experiments, with the error bars representing SEM between experiments.

appear quite similar, and after point by point comparisons using a two-tailed student's t-test, the differences between them were found to be not statistically significant ($P > 0.05$).

Throughout the clonogenic experiments the Sk Mel-3 cells exhibited an average plating efficiency of about 75 %. It was also found to be consistent, whether measured at the beginning or the end of the experiment. In Figures 3.1.2, 3.1.3, 3.1.4, and in subsequent figures where DNA damage parameters are plotted as a function of dose, there are two x-axes. One in terms of radiation dose in Gy, and the other in terms of CPT and LDR exposure time in hours. The CPT and LDR exposure times are the same because, whether given alone or in combination with LDR irradiation, the CPT was left on the cells for durations equal to the amounts of time required to give each of the radiation doses employed, using the LDR irradiator. This is obvious in the case of the combined treatments since the drug is on the cells while they are being irradiated. It was done in the CPT alone treatment for the purpose of normalization.

Figures 3.1.2, 3.1.3, and 3.1.4 show the survival curves resulting from the concomitant exposures to LDR irradiation and each of 1 μM , 10 μM , and 25 μM concentrations of CPT, respectively. The concomitant exposure curve normalized to remove the effects of the CPT is shown for direct comparison to the LDR alone curve. The HDR dose response curve for Sk Mel-3 has been generated previously (Ng *et al.*, 1994). Represented as a dashed line, it is included in these figures as a reference. The curves in all three figures were fit with the linear quadratic model and the estimated parameters α and β with their estimated standard errors and the coefficient of determination, R^2 , for each fit are shown in Table 3.1.2.

Table 3.1.2. Comparison of the linear quadratic fits to the dose response curves of Sk Mel-3 cells to LDR as well as to concomitant exposure to LDR and each of 1 μM , 10 μM , and 25 μM concentrations of CPT.

Experiment	α (Gy^{-1})	β (Gy^{-2})	R^2
LDR	0.05 ± 0.02	0.005 ± 0.002	0.98
LDR + CPT 1 μM	0.09 ± 0.01	0.007 ± 0.002	0.99
LDR + CPT 1 μM normalized	0.02 ± 0.03	0.005 ± 0.003	0.9
LDR + CPT 10 μM	0.15 ± 0.01	0.006 ± 0.002	0.99
LDR + CPT 10 μM normalized	0.09 ± 0.03	0.004 ± 0.004	0.94
LDR + CPT 25 μM	0.19 ± 0.05	0.004 ± 0.008	0.95
LDR + CPT 25 μM normalized	0.14 ± 0.04	0.003 ± 0.005	0.9

From the survival curves in Figures 3.1.2, 3.1.3, and 3.1.4 it is seen that with each concentration of CPT, the concomitant exposure of the cells to CPT and LDR irradiation resulted in considerably more cell killing than either agent alone. Also, LDR irradiation seems to be more cytotoxic to the cells than is CPT when given alone at any of the concentrations used here. A one-tailed Student's t-test was applied to the unnormalized concomitant exposure data in order to determine if the surviving fraction values were significantly lower at each dose point than those achieved with either agent on its own. The results of this test are shown in Table 3.1.3.

Figure 3.1.2 Survival curves for the Sk Mel-3 cell line after LDRI, 1 µM CPT, and concomitant exposure to LDRI and 1 µM CPT (both normalized and unnormalized). Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.1.3 Survival curves for the Sk Mel-3 cell line after LDRI, 10 µM CPT, and concomitant exposure to LDRI and 10 µM CPT (both normalized and unnormalized). Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Figure 3.1.4 Survival curves for the Sk Mel-3 cell line after LDRI, 25 μ M CPT, and concomitant exposure to LDRI and 25 μ M CPT (both normalized and unnormalized). Each point represents the average of three experiments, with the error bars representing SEM between experiments.

Table 3.1.3 P-values of a one-tailed Student's t-test comparing survival after concomitant exposure to CPT and LDR irradiation and either agent alone. Values of P less than 0.05, indicating a statistically significant reduction in survival, are shown in bold type.

	LDR + 1 μ M CPT		LDR + 10 μ M CPT		LDR + 25 μ M CPT	
	1 μ M CPT	LDR	10 μ M CPT	LDR	25 μ M CPT	LDR
1 Gy	0.2835	0.2396	0.0571	0.0506	0	0.0334
2 Gy	0.3988	0.2441	0.4867	0.16	0.0155	0.0282
4 Gy	0.057	0.0714	0.0171	0.0262	0.0191	0.0461
6 Gy	0.312	0.3256	0.0205	0.0123	0.0055	0.0109
8 Gy	0.0609	0.062	0.146	0.0421	0.0042	0.0125
10 Gy	0.0323	0.0195	0.0706	0.0081	0.0264	0.0129
12 Gy	0.0051	0.0023	0.0223	0	0.0336	0
14 Gy	0.0852	0.0126	0.0269	0.0027	0.0219	0.0093
16 Gy	0.001	0.0261	0.0032	0.0152	0.0308	0.0154

The addition of 1 μ M CPT to LDR irradiation was found to significantly decrease survival with respect to 1 μ M CPT alone at doses of 10 Gy, 12 Gy, and 16 Gy. When compared to LDR alone, the same effect was seen at doses of 10 Gy and higher. The shape of the LDR curve shows the dose rate sparing effect. As the dose is delivered over a longer period of time at a lower rate, the cells are given the opportunity to repair damage during the treatment. The addition of 1 μ M CPT did not abolish this sparing effect. Had it done so, the resulting survival curve would follow closely the dashed line of the HDR response. Upon normalizing away the effect of the CPT, the resulting curve moved above the LDR alone

curve, indicating a reduction in the effectiveness of the radiation to kill cells when combined with CPT at 1 μ M, compared to when it is given alone.

Adding 10 μ M CPT to LDR irradiation significantly reduced the survival as compared to 10 μ M CPT alone at doses of 4 Gy, 6 Gy, and at 12 Gy and above. A significant reduction when compared to LDR alone occurred at all doses of 4 Gy and above. The combination of 10 μ M CPT and LDR irradiation resulted in greater cell killing than did the combination of 1 μ M CPT and LDR irradiation, though there was still a visible dose rate sparing effect. The normalized concomitant exposure curve fell below the LDR alone curve, indicating a positive interaction between the two agents and an enhancement of radiation cytotoxicity. Based on this curve, an SER of 1.14 at 15 % survival was calculated. The endpoint most commonly used for SER calculations is 10 %, however, survival after LDR irradiation alone did not reach this level within the dose range employed.

The concomitant exposure of 25 μ M CPT and LDR irradiation reduced the survival level of the cells by a statistically significant amount at all dose points when compared to either 25 μ M CPT given alone, or LDR irradiation alone. Based on the survival curves, the combination of 25 μ M CPT and LDR irradiation was more effective at reducing survival than were the combinations at the other two concentrations of CPT. However, the difference is very small in the case of 10 μ M CPT. The dose rate sparing effect was not abolished at this concentration of CPT either, though the linear quadratic fit to the concomitant exposure data falls below the HDR curve up to about 5 Gy. When the effects of the CPT were normalized away, the curve remained below that for LDR irradiation alone, once again, indicating a positive interaction between the two agents and an enhancement of the ability of the radiation

to kill cells. An SER of 1.35 was calculated at 15 % survival.

Qualitatively, upon observing the survival curves, CPT has some potentiating effect on LDR irradiation at concentrations of 10 μ M and 25 μ M. The normalized curves for the concomitant exposures to CPT and LDR irradiation at CPT concentrations of 10 μ M and 25 μ M remained below that for LDR alone. At these two concentrations, a one-tailed Student's t-test was applied to the data in order to determine if the difference was significant. The results of this test are shown in Table 3.1.4.

Table 3.1.4 P-values of a one-tailed Student's t-test comparing normalized survival after concomitant exposure to CPT and LDR irradiation and LDR alone. Values of P less than 0.05, indicating a statistically significant reduction in survival, are shown in bold type.

	LDR + 10 μ M CPT	LDR + 25 μ M CPT
1 Gy	0.0902	0.0248
2 Gy	0.368	0.0468
4 Gy	0.0452	0.1846
6 Gy	0.0617	0.095
8 Gy	0.2112	0.1592
10 Gy	0.1715	0.0247
12 Gy	0.0298	0.0036
14 Gy	0.0723	0.012
16 Gy	0.0358	0.0165

From Table 3.1.4 it is seen that, based on the analysis of survival curves, the addition of 10 μ M CPT significantly potentiates LDR irradiation at doses of 4 Gy, 12 Gy, and 16 Gy.

There is also significant potentiation at doses of 1 Gy, 2 Gy, 10 Gy and above when 25 μM CPT is combined with LDR irradiation.

Survival data fit to the linear quadratic model are described by two parameters, α and β . Therefore, any change in the shape of the curve will be reflected in one or both of these parameters. It is instructive to examine changes in these parameters when a treatment is modified, for example, by the addition of a chemotherapeutic drug such as CPT to LDR irradiation. Table 3.1.5 shows the percentage change in the values of α and β , relative to those for LDR irradiation alone, when CPT is combined with LDR irradiation and the effects of the drug are normalized away.

Table 3.1.5 Percentage change in the α and β values of the normalized concomitant exposure curves relative to those for LDR alone.

	$(\alpha_{\text{concomitant}}/\alpha_{\text{LDR}} - 1) \times 100\%$	$(\beta_{\text{concomitant}}/\beta_{\text{LDR}} - 1) \times 100\%$
1 μM	-63.9	11.4
10 μM	83.1	-18.8
25 μM	190.8	-46.3

In each case, adding CPT to LDR irradiation caused a much larger change in α than in β . The addition of 1 μM CPT to LDR irradiation resulted in an α value reduced by 63.9 % and a β value increased by 11.4 %. For the 10 μM and 25 μM cases, the values of α increased by 83.1 % and 190.8 %, while the values of β decreased by 18.8 % and 46.3 % respectively. The higher the concentration of CPT, the greater the change in both α and β . Clearly, any potentiation, or lack thereof, is due more to changes in α than to changes in β . The β

component actually decreased when 10 μ M and 25 μ M CPT were combined with LDR irradiation.

3.2 Isobologram Analysis

The clonogenic results showed that depending on the concentration of CPT used, and the dose of radiation given, CPT could significantly potentiate the effects of LDR irradiation. The response of the cells to the concomitant exposure to CPT and LDR irradiation can also be compared to the predicted survival, given the response of the cells to each agent on its own. Predicted survival levels are calculated for each dose point by multiplying the survival observed with CPT alone by the survival observed with LDR alone. This type of calculation assumes independence of action between the two agents. Predicted survival curves compared to what was actually observed after concomitant exposure to CPT and LDR irradiation are shown in Figures 3.2.1, 3.2.2, and 3.2.3 for 1 μ M, 10 μ M, and 25 μ M CPT respectively. However, in these types of experiments, where two agents are being combined, the most stringent test is the isobologram technique. This technique is considered the gold standard for evaluating the nature of the interaction between two agents in combined modality experiments. Individual isobolograms were generated at levels of survival resulting from LDR irradiation doses of 2 Gy, 4 Gy, 8 Gy, 12 Gy, and 16 Gy combined with the corresponding time-equivalent exposure to CPT, for each concentration of CPT used. These individual isobolograms are found in the Appendix. The five isobolograms generated for each concentration of CPT have been combined into one graph according to the method described

by Hennequin et al (1994). Briefly, the fractional additivity relative to radiation dose or to CPT exposure time, whether sub (infra) additive, supra additive, or simply additive, is a function of the distance of the point from the mode 1 and mode 2 lines of each isobologram. Plots of fractional additivity as a function of radiation dose or CPT exposure time are shown in Figures 3.2.4, 3.2.5, and 3.2.6 for CPT concentrations of 1 μM , 10 μM , and 25 μM respectively.

It can be seen from Figure 3.2.1 that most of the predicted values of survival fall below the actual observed values. This means that the combination of LDR irradiation and 1 μM CPT is less effective than would be predicted based on the effects of each agent alone. This agrees with what was seen in Figure 3.1.2, comparing the normalized LDR + CPT survival curve to that for LDR alone. At a concentration of 10 μM , adding CPT to LDR irradiation produces a slightly greater than predicted amount of cell killing, as shown in Figure 3.2.2. For all but two of the dose points evaluated, the actual survival is less than the predicted survival. This verifies the observed difference of the normalized LDR + CPT survival curve from that for LDR alone, shown in Figure 3.1.3. The 25 μM CPT produced the same effect as the 10 μM , but to a larger extent. The actual survival is lower than the predicted survival at every dose point examined, and the difference is, on average, greater than with the 10 μM CPT. This agrees with the results as shown in Figure 3.1.4.

The analysis described above revealed that at 10 μM and 25 μM concentrations of CPT, the survival after concomitant treatment was lower than would be predicted by the survival due to each agent alone, assuming independence of action. The mode 1 line of the isobologram assumes independence of action between the two agents. The more difficult test

to pass in order to achieve supra additivity is the mode 2 line, which assumes dependence of action. Here, killing of one agent is affected by the killing of the other. From Figures 3.2.4, 3.2.5, and 3.2.6 it is seen that few combinations of radiation dose and CPT exposure were able to pass this test. Figure 3.2.4 shows that at all but one dose point examined, the effects of the combination of 1 μ M CPT with LDR irradiation were sub additive. The one additive point (12 Gy) was just barely so, and was very close to the mode 1 line, implying that the agents were acting by independent mechanisms.

Upon combining 10 μ M CPT with LDR irradiation, all but one of the dose combinations examined were additive according to isobologram analysis. Supra additivity of about 18 % was achieved at the 4 Gy point, relative to both the radiation dose and the time of exposure to 10 μ M CPT. The 2 Gy point fell directly on the lowest mode 2 line, while the three other points were all near the centre of the envelope of additivity.

Figure 3.2.6 shows that the concomitant exposure of Sk Mel-3 cells to 25 μ M CPT and LDR irradiation resulted in additive effects at the 4 Gy, 12 Gy, and 16 Gy dose points, and supra additive effects at the 2 Gy and 8 Gy points. At the 2 Gy point the level of supra additivity was about 72 % relative to CPT exposure time and 68 % relative to radiation dose. The effect was much less at 8 Gy, with 16 % supra additivity relative to CPT exposure time, and 9 % relative to radiation dose. The 4 Gy point was close to the middle of the envelope of additivity, while the 12 Gy and 16 Gy points were closer to the mode 2 line, implying that the agents were acting by the same mechanism.

Figure 3.2.1 Survival curves for the concomitant exposure of Sk Mel-3 cells to LDR and 1 μ M CPT, both actual (unnormalized) and that predicted by survival after each agent alone.

Figure 3.2.2 Survival curves for the concomitant exposure of Sk Mel-3 cells to LDRI and 10 μ M CPT, both actual (unnormalized) and that predicted by survival after each agent alone.

Figure 3.2.3 Survival curves for the concomitant exposure of Sk Mel-3 cells to LDR and 25 μ M CPT, both actual (unnormalized) and that predicted by survival after each agent alone.

Figure 3.2.4 Plots of the fractional infra/supra additivities relative to the radiation dose or to the length of exposure to 1 μ M CPT. The hatched area is the normalized envelope of additivity.

Figure 3.2.5 Plots of the fractional infra/supra addivities relative to the radiation dose or to the length of exposure to 10 μ M CPT. The hatched area is the normalized envelope of additivity.

Figure 3.2.6 Plots of the fractional infra/supra addivities relative to the radiation dose or to the length of exposure to 25 μ M CPT. The hatched area is the normalized envelope of additivity.

3.3 CHEF Electrophoresis

The clonogenic results and the isobologram analysis determined that the effect of combining CPT with LDR irradiation was at least additive at CPT concentrations of 10 μ M and 25 μ M. In light of this, these two concentrations of the drug were used to test the hypothesis that the observed differences in survival would manifest themselves as differences in the qualitative amount of DNA DSBs measured after each treatment. CHEF electrophoresis was used to measure the level of DNA DSBs, and the results are shown in Figures 3.3.1 and 3.3.2 for the 10 μ M and 25 μ M concentrations of CPT respectively. The fraction of activity released (FAR) has been plotted as a function of the radiation dose as well as the CPT and LDR exposure time. Figure 3.3.3 shows a sample gel run on the CHEF under the conditions specified in section 2.1.5 of the Materials and Methods.

From Figures 3.3.1 and 3.3.2 it is seen that only a very small fraction of the total activity was released from the well at any dose. In some cases, such as the 8 Gy and 12 Gy points of the LDR alone response, negative values of FAR were measured. Since all the data were normalized to the control FAR at zero dose, this means that the 8 Gy and 12 Gy points had a lower FAR than that of the control. The error bars overlapped one another at each dose point such that there was no significant difference between any of the treatments. In fact, a two-tailed Student's t-test comparing the FAR of all dose points for each treatment to a FAR of zero showed that there was no significant difference between any of the FAR values measured and a FAR of zero. From the sample gel shown in Figure 3.3.3 it is seen by the amount of fluorescence in each lane that hardly any DNA has left the well. As is

Figure 3.3.1 Fraction of activity released for the Sk Mel-3 cell line after LDRI, 10 μ M CPT, and concomitant exposure to LDRI and 10 μ M CPT (both normalized and unnormalized). Each point represents the average of three experiments, with the error bars representing SEM between experiments. The cell concentration is 1×10^6 cells/mL.

Figure 3.3.2 Fraction of activity released for the Sk Mel-3 cell line after LDR, 25 μ M CPT, and concomitant exposure to LDR and 25 μ M CPT (both normalized and unnormalized). Each point represents the average of three experiments, with the error bars representing SEM between experiments. The cell concentration is 1×10^6 cells/mL.

Figure 3.3.3 Sample of a CHEF gel electrophoresis run on Sk Mel-3 cells after exposure to doses of either LDRI, 10 μ M CPT, or LDRI and 10 μ M CPT combined. The numbers represent the radiation dose in Gy and/or the equivalent exposure time to CPT. The cells were at a concentration of 1×10^6 cells/mL.

evidenced in Figures 3.3.1 and 3.3.2, the amount of radioactively labeled DNA released into the lanes was too small to quantify accurately in terms of radioactivity.

3.4 Comet Assay

Analysis of DNA damage using the CHEF electrophoresis system did not reveal any relationship between clonogenic survival and the number of residual DNA DSBs. There was not even a relationship between increasing dose of LDR irradiation, CPT exposure time, or the two combined, and the number of residual DNA DSBs in the cells. For these reasons it was necessary to look elsewhere for the answers to these questions.

Another assay able to quantify DNA damage in the form of DSBs, the comet assay, was used for the same purpose as the CHEF assay. Residual DNA DSBs were measured with the comet assay after Sk Mel-3 cells were treated with each of LDR irradiation alone, 25 μ M CPT alone, and the two applied concomitantly. Digital images of three typical cells, or comets, are shown in Figure 3.4.1. Image A is that of an untreated cell, and little of the DNA has left the nucleus. The cell in image B has been exposed to 8 Gy of LDRI and 25 μ M CPT, while that in image C has been exposed to 16 Gy of LDRI and 25 μ M CPT. Qualitatively it is seen that as the dose was increased, more DNA left the nucleus. Increasing doses also caused the DNA to travel a greater distance. In order to quantify these observations, the comet parameters of tail length and tail moment, as defined in equations (2) and (3) respectively, were calculated then normalized by the control case according to equations (9) and (10). Normalized tail length is plotted in Figure 3.4.2 and normalized tail moment is

plotted in Figure 3.4.3, each as a function of radiation dose as well as CPT and LDR exposure time. The comet assay data is from two individual experiments. However, each value of tail moment (TM) and tail length (TL) from either experiment is an average of 50 measurements from one sample of cells. Therefore, there is an error associated with each experimental value. If the results of the two experiments were similar enough, the data could be combined and an overall error obtained and plotted as error bars in Figures 3.4.2 and 3.4.3. However, as is seen in Table 3.4.1, in most cases, the mean values of TM and TL between experiments for corresponding dose points do not overlap within their associated standard errors. The variation between experiments is larger than the variation within one experiment, such that the data cannot be pooled together. In order to obtain some idea of the error involved, each experiment was considered as a single point. The average of the two experiments was taken, and the error on this value was expressed as half of the difference between the averages of the two experiments.

From Figure 3.4.2 it is seen that all three curves rise steeply from 0 Gy to 4 Gy. Beyond 4 Gy, both the LDR alone and CPT alone curves begin to level off, and then plateau between 12 Gy and 16 Gy. The LDR + CPT curve continues to rise steeply above 4 Gy. The beginnings of a plateau can be observed at about 16 Gy. At doses larger than 4 Gy the combination of LDR irradiation and 25 μ M CPT produces comets with considerably longer tails than either LDR or CPT on their own. Nearly the same effect is seen with the normalized tail moment in Figure 3.4.3. At 4 Gy it has approximately the same value for three treatments. The slopes of the curves for LDR alone and CPT alone begin to decrease after 4 Gy, but a plateau is never reached, up to 16 Gy. The LDR + CPT curve continues

Figure 3.4.1 Typical images of comets viewed at 40x magnification . Broken DNA is drawn out of the nucleus after electrophoresis at 1 V/cm for 40 minutes. The images are of (A) a control cell, and cells exposed to (B) 8 Gy LDRI and 25 μM CPT, and (C) 16 Gy LDRI and 25 μM CPT.

Figure 3.4.2 DNA damage expressed as normalized tail length after LDRI, 25 μ M CPT, and concomitant exposure to LDRI and 25 μ M CPT. Each point represents the average of two experiments. Error bars represent half of the difference between the averages of the two experiments.

Figure 3.4.3 DNA damage expressed as normalized tail moment after LDR, 25 μ M CPT, and concomitant exposure to LDR and 25 μ M CPT. Each point represents the average of two experiments. Error bars represent half of the difference between the averages of the two experiments.

increasing almost linearly with dose out to 16 Gy. Above 4 Gy, and especially at 8 Gy and 16 Gy, the combination of LDR irradiation and 25 μ M CPT produces comets with notably greater tail moments than either LDR or CPT on their own.

Table 3.4.1 Mean value and associated standard error of the 50 unnormalized tail moment (TM) and tail length (TL) measurements for each of the two comet experiments. In the Dose column, X denotes the presence of LDRI, D the presence of the drug, CPT, and the number is the dose in Gy and/or equivalent time of exposure to CPT.

Dose	Experiment 1		Experiment 2	
	TM (μ m)	TL (μ m)	TM (μ m)	TL (μ m)
0	9.3 $\pm$ 0.2	48 $\pm$ 2	11.2 $\pm$ 0.2	55 $\pm$ 2
4X	11.7 $\pm$ 0.1	72 $\pm$ 2	14.5 $\pm$ 0.3	77 $\pm$ 1
4D	11.9 $\pm$ 0.3	79 $\pm$ 3	11.9 $\pm$ 0.3	70 $\pm$ 2
4XD	12.3 $\pm$ 0.3	79 $\pm$ 2	11.5 $\pm$ 0.3	66 $\pm$ 2
8X	10.8 $\pm$ 0.3	74 $\pm$ 2	12.2 $\pm$ 0.2	78 $\pm$ 3
8D	10.0 $\pm$ 0.2	66 $\pm$ 3	12.0 $\pm$ 0.3	81 $\pm$ 3
8XD	12.5 $\pm$ 0.5	86 $\pm$ 3	14.3 $\pm$ 0.5	94 $\pm$ 3
12X	11.1 $\pm$ 0.3	77 $\pm$ 2	13.0 $\pm$ 0.4	89 $\pm$ 3
12D	12.3 $\pm$ 0.4	83 $\pm$ 3	12.7 $\pm$ 0.4	88 $\pm$ 3
12XD	12.6 $\pm$ 0.4	90 $\pm$ 3	14.1 $\pm$ 0.5	104 $\pm$ 3
16X	12.4 $\pm$ 0.3	83 $\pm$ 2	13.7 $\pm$ 0.3	72 $\pm$ 2
16D	12.0 $\pm$ 0.3	73 $\pm$ 3	12.8 $\pm$ 0.4	78 $\pm$ 3
16XD	16.4 $\pm$ 0.6	105 $\pm$ 2	14.8 $\pm$ 0.6	93 $\pm$ 2

The results in Figures 3.4.2 and 3.4.3 indicate that greater numbers of residual DNA DSBs are produced in Sk Mel-3 cells when LDR irradiation and 25 μ M CPT are applied concomitantly than when either is applied alone. If the DNA DSB is the critical lesion leading

to cell death, then there should exist some type of correlation between the decreased survival and the increased number of DNA DSBs when the two agents are combined. Normalized tail moment and normalized tail length were correlated linearly with survival for concomitant exposure to LDR irradiation and 25 μ M CPT in Figures 3.4.4 and 3.4.5 respectively. The negative correlations observed were quite strong. Values of the correlation coefficient were $r = -0.92$ for tail moment, and $r = -0.99$ for tail length. Figures 3.4.6 and 3.4.7 show plots of surviving fraction versus normalized tail moment and normalized tail length, respectively, for exposure to LDR irradiation alone. The correlation coefficients were $r = -0.89$ for tail moment, and $r = -0.81$ for tail length. Exposure to 25 μ M CPT alone resulted in a moderate negative correlation between surviving fraction and tail moment, $r = -0.69$, and a fairly strong negative correlation between surviving fraction and tail length, $r = -0.86$. Plots of surviving fraction versus normalized tail moment and normalized tail length for exposure to 25 μ M CPT alone are shown in Figures 3.4.8 and 3.4.9 respectively.

Figure 3.4.4 Normalized tail moment correlated with survival for concomitant exposure to LDRI and 25 μM CPT. Each survival point represents the average of three experiments. Each tail moment point represents the average of two experiments. The value of the correlation coefficient was $r = -0.92 \pm 0.01$.

Figure 3.4.5 Normalized tail length correlated with survival for concomitant exposure to LDRI and 25 μ M CPT. Each survival point represents the average of three experiments. Each tail length point represents the average of two experiments. The value of the correlation coefficient was $r = -0.99 \pm 0.00$.

Figure 3.4.6 Normalized tail moment correlated with survival for exposure to LDRI. Each survival point represents the average of three experiments. Each tail moment point represents the average of two experiments. The value of the correlation coefficient was $r = -0.89 \pm 0.02$.

Figure 3.4.7 Normalized tail length correlated with survival for exposure to LDRI. Each survival point represents the average of three experiments. Each tail length point represents the average of two experiments. The value of the correlation coefficient was $r = -0.81 \pm 0.05$.

Figure 3.4.8 Normalized tail moment correlated with survival for exposure to 25 μ CPT. Each survival point represents the average of three experiments. Each tail moment point represents the average of two experiments. The value of the correlation coefficient was $r = -0.69 \pm 0.09$.

Figure 3.4.9 Normalized tail length correlated with survival for exposure to 25 μM CPT. Each survival point represents the average of three experiments. Each tail length point represents the average of two experiments. The value of the correlation coefficient was $r = -0.86 \pm 0.03$.

Chapter 4

Discussion

Evidence that inhibitors of topoisomerase I can be effective sensitisers of ionizing radiation has been accumulating for some time. In particular, CPT and its analogues have been shown to potentiate the effects of x-irradiation on several different types of mammalian tumour cell lines. Potential benefits of combining CPT with LDR irradiation are less evident because to date, such a combination has not been as widely investigated. In theory, a radiosensitiser could be very useful in conjunction with brachytherapy since the radiation dose is targeted to the tumour tissue. This helps to avoid unwanted sensitisation of surrounding normal tissue. The present work investigated this idea by studying the concomitant exposure of a radioresistant human melanoma cell line to CPT and LDR irradiation. The results are discussed below, and, where applicable, comparisons to previous findings have been made.

4.1 LDRI, CPT, and Concomitant Exposures

The response of the cells to LDR irradiation alone was determined first. These cells are known from previous studies to be resistant to both radiation and CPT (Ng *et al.*, 1998; Ng *et al.*, 1994). Under LDR irradiation conditions, the α and β parameters of the linear quadratic fit to the survival data were found to be 0.05 Gy^{-1} and 0.005 Gy^{-2} respectively. The presence of the non-zero β value is representative of the curvature of the fit, and indicates that perhaps not all of the repairable sublethal damage was repaired during the treatment. Under the same conditions, Raaphorst *et al* (1994) were able to fit the survival data for Sk Mel-3 cells after LDR irradiation with a straight line, indicating a β value of 0. However, the fit of the present study and that of Raaphorst *et al* (1994) is each representative of similar amounts of cell kill, each having reduced survival to approximately 10 % after a dose of 16 Gy.

The response of Sk Mel-3 cells to CPT was determined by exposing them to three different concentrations of the drug (1 μM , 10 μM , and 25 μM), each for increasing amounts of time corresponding to the time required to give the LDR irradiation doses used. The linear quadratic model describes the effects of ionizing radiation on cells, and is typically reserved for cases where ionizing radiation is the cytotoxic agent being used. It was used in the present study also to fit the survival data when CPT was the lone cytotoxic agent to which the cells were exposed. In this case it can be thought of as simply a second-order polynomial fit. Cell killing by CPT is also the result of DNA damage, but not by the same mechanism as that for ionizing radiation, which is described by the linear quadratic model. However,

although the mechanisms are different, the lethal lesion, the DNA DSB, is likely the same in both cases. The response of the cells to CPT was similar for each concentration of the drug. After comparing all three curves by the use of a Student's t-test, it was found that there was no significant difference between any two of the curves. This is not totally unexpected in light of results published by Ng *et al* (1998). In their study, confluent cultures of Sk Mel-3 cells were exposed to increasing concentrations of CPT, each for 8 hours at 37°C. A 6 µM concentration of CPT reduced survival to roughly 80 %. Beyond this concentration, up to 20 µM, there was no further reduction in survival. This plateau effect indicated that for a given length of exposure, increasing the concentration of the drug beyond 6 µM had no further effect. One can then hypothesize that above this threshold concentration, increasing the length of exposure is more relevant than further increases in drug concentration if greater amounts of cell kill are to be achieved.

Consistent with the three other studies known to have examined the effects of combining CPT with some form of low dose rate irradiation (Hennequin *et al.*, 1994; Roffler *et al.*, 1994; Musk & Steel, 1990), the addition of CPT to LDR irradiation reduced the level of survival of the cells considerably with respect to LDR irradiation alone. Musk and Steel (1990) used a ^{60}Co source with a dose rate of 3 cGy min⁻¹, while Roffler *et al* (1994) quoted no dose rate for the ^{60}Co source used in their experiments. Hennequin *et al* used a ^{137}Cs source with a dose rate of 1.7 cGy min⁻¹. The ^{226}Ra source used in the present work had a lower dose rate of 0.88 cGy min⁻¹, but all the dose rates mentioned fall within the clinically relevant range (Hall & Brenner, 1991). However, since each of the aforementioned sources is a γ-ray emitter, the exact isotope used is not a factor when comparing between

experiments. All γ -rays have the same relative biological effectiveness (RBE) of 1 with respect to 250 kV x-rays. The present study showed a significant reduction at 10 Gy and above for 1 μ M CPT, 4 Gy and above for 10 μ M CPT, and at all doses used for 25 μ M CPT. This apparent concentration dependence regarding the effectiveness of CPT when combined with LDR irradiation is not evident from the three previous studies. Each of them used only a single concentration of CPT when combining the drug with radiation. It is an interesting effect considering the aforementioned similarity between the responses of the cells to each concentration of CPT alone for increasing amounts of time. Although Ng *et al* (1998) observed a plateau above 6 μ M with respect to survival in Sk Mel-3 cells subjected to increasing concentrations of CPT, perhaps when combined with LDR irradiation the interaction between the two is such that higher concentrations are more effective. One possible explanation is that beyond the level of killing CPT is capable of on its own, the more of it that is present in the cells, the more of it that is available to interact with sublethal radiation damage, rendering it lethal and killing the cell.

Comparing the normalized LDR + CPT survival curves to that of LDR alone, after having normalized away the effects of the drug, revealed a positive interaction between the two agents for the 10 μ M and 25 μ M concentrations. The normalized LDR + CPT 1 μ M survival curve fell above that for LDR alone, indicating a less than additive response, or a negative interaction. This is not protection however. By definition, protection exists only when the unnormalized concomitant exposure produces less kill than exposure to LDR irradiation alone. At 10 μ M and 25 μ M, the normalized LDR + CPT survival curves fell below that for LDR irradiation alone, indicating a positive interaction and a greater than

additive effect. Based on the analysis of the survival data, it is reasonable to assume that concentrations of CPT below 1 μM will have no sensitising effect on the cells to LDR irradiation, and that concentrations between 10 μM and 25 μM , and perhaps above, will have a sensitising effect. The findings of the present work say nothing regarding the effectiveness of CPT concentrations between 1 μM and 10 μM . However, two of the other three studies published previously did use concentrations of CPT in this range. Hennequin *et al* (1994) reported sensitisation of Chinese hamster V-79 fibroblasts and HeLa-S3 cells to γ -rays from a ^{137}Cs source using a concentration of 3 μM CPT. These results cannot be directly compared though, since the cells treated in these experiments were in the exponential phase of growth.

Musk and Steel (1990) showed a sensitising effect of CPT on one of the most radioresistant tumour cells lines ever established. Confluent cultures of the human bladder carcinoma cell line RT112 were irradiated with ^{60}Co γ -rays and exposed to CPT at a concentration of 0.87 $\mu\text{g ml}^{-1}$ (2.5 μM). The results of these two studies are consistent with what was found in the present work. Surprisingly, Roffler *et al* (1994) reported sensitisation of AS-30D rat hepatoma cells to γ -rays from a ^{60}Co source using a concentration of CPT below 1 μM . This would not have been expected based on the results of the present work. However, it may be attributable to differences between cell lines or irradiation conditions.

In determining at which dose points there was a significant reduction in survival, indicative of a sensitising effect, after concomitant exposure to LDR irradiation and either 10 μM or 25 μM CPT, the effect of the drug was first normalized away. Subsequent t-tests showed significant reductions in survival at 4 Gy, 12 Gy, and 16 Gy when CPT was used at a concentration of 10 μM , and at 1 Gy, 2 Gy, as well as 10 Gy and above at the 25 μM

concentration of CPT. Intuitively, significant differences would be expected at the higher doses where the curves are more separated one from the other. It is less evident that there would be significant differences at the lower doses. However, the logarithmic scale is slightly deceptive in this respect. The relative error associated with each value also plays a role in that a large error on each number of a pair of numbers that differ greatly in their actual value may cause the difference between the two not to be significant. Conversely, two numbers with less of a difference between their actual values may be found to be significantly different if the associated error of each value is sufficiently small. The apparent sensitisation at certain low doses of LDR irradiation is also interesting in relation to the changes in α and β from LDR alone to the normalized concomitant exposures. With the addition of 10 μM CPT, the value of α increased by 83.1 %. When LDR irradiation was complemented by 25 μM CPT, there was a 190.8 % increase in the value of α . The α -component of the linear quadratic model dominates at low doses and is responsible for determining the initial slope of the survival curve. Perhaps these changes in α and the corresponding increase in the initial slope of each curve are responsible for the significant reductions in survival at 4 Gy for 10 μM , and at 1 Gy and 2 Gy for 25 μM CPT.

According to the linear quadratic model, changes in α represent changes in the intrinsic radiosensitivity of the cells. Changes in β represent changes in the amount of sublethal damage repair taking place. CPT has previously been shown to be an inhibitor of SLDR. Musk and Steel (1990) showed this in confluent cultures of human bladder carcinoma cells. In a split dose experiment where two doses of 4 Gy were separated by increasing intervals of time, the recovery ratio was reduced from 2.60 ± 0.37 to 1.78 ± 0.24 when CPT

was present between doses, compared to when it was not. Under conditions of LDR irradiation, the addition of CPT completely abolished low dose rate sparing, indicating SLDR during the exposure. Ng *et al* (1994) showed that, after correction for drug toxicity, CPT inhibited SLDR in confluent cultures of Sk Mel-3 cells, the same as those used in the current study. Therefore, it was expected that, in the present work, CPT would act to sensitise Sk Mel-3 cells to LDR irradiation by inhibiting the repair of sublethal damage during treatment. If this had occurred, it would have manifested itself as a rather large increase in the value of β . The β component accounts for cell killing by the interaction of two SSBs before repair can take place. Given sufficient time to repair, the β component will go to 0. Therefore, under LDRI conditions, which allow for complete SLDR, the addition of another agent which causes the β component to increase is not allowing complete repair to occur. However, at the two concentrations of CPT where some sensitisation was observed, the values of β decreased. With the addition of 10 μ M CPT, the value of β decreased by 18.8%. A decrease of 46.3 % was observed when LDR irradiation was complemented by 25 μ M CPT.

The changes in α and β observed here seem to indicate that CPT has modified the response of the cells to LDR irradiation by changing their intrinsic radiosensitivity, rather than by affecting the repair of sublethal damage. This contradicts previous findings as described above, and there is no simple explanation for this observation. It can be hypothesized that perhaps certain proteins such as histones, which are associated with DNA, are affected by the presence of CPT. If the presence of these histones protects the DNA to a certain extent from single-event radiation damage, and if the introduction of CPT affects the histones in such a way as to render the DNA more susceptible or accessible to this mode of radiation action,

then sensitisation due to changes in α could be observed. A similar idea was postulated in order to explain findings that clonogenic radiosensitivity was related to the levels of initial radiation induced DNA damage in human tumour cells. It was thought that this relationship might be related to differences in the chromatin conformation that affect the accessibility of scavenging molecules to damaged DNA or radical attack (Oleinick *et al.*, 1984).

4.2 Isobologram Analysis

The present study used isobologram analysis to determine the nature of the effects of interactions between CPT and LDR irradiation on Sk Mel-3 cells more rigorously than is possible by examination of cell survival curves alone. Curves of equal cytotoxic effect were generated from the survival curves of LDR irradiation alone and CPT exposure alone. This type of analysis allowed the prediction of the effects of combining the two agents based on opposing assumptions of independence and dependence of action (Steel & Peckham, 1979).

The comparisons between the unnormalized survival curves for the concomitant exposure to LDR irradiation and CPT and the predicted responses based on the individual dose response curves and independence of action between the two agents agreed very well with the observations made by comparing the normalized LDR + CPT curve to that for LDR alone. This was expected since both ways of identifying sensitisation are similar. However, the comparison between the predicted response and the observed response is a more logical approach, and one that required no normalization of the data.

After analysis of the data by the isobologram technique, the combination of LDR

irradiation and CPT resulted in very few experimental points that were deemed as representing a supra additive effect. The only such points were the 4 Gy point with 10 μ M CPT, and the 2 Gy and 8 Gy points with 25 μ M CPT. These results demonstrate the increased rigor of isobologram analysis and the need for its use whenever it is necessary to determine whether the effects of a combination of agents are greater or less than what would be expected on the basis of the effects of each agent on its own. Based solely upon examination of and comparisons between survival curves, it would have been concluded that supra additive effects existed for the dose points corresponding to the bold type entries in Table 3.1.4. It was for these dose points that a significant reduction in survival from the LDR irradiation alone curve was found to have occurred after normalization of the LDR + CPT curve. Isobologram analysis prevented the false conclusion of supra additivity at many doses.

The sensitisation observed at low radiation doses was discussed earlier with respect to survival curves. This effect is confirmed by isobologram analysis. The only supra additive point for the combination of LDR irradiation and 10 μ M CPT was at a low dose of 4 Gy. Of the two supra additive points observed when LDR irradiation was combined with 25 μ M CPT, 2 Gy and 8 Gy, the one with the higher percentage supra additivity by far was the 2 Gy point. As postulated before, the change in the value of α is most likely responsible for this. The mode 1 and mode 2 isoeffect lines used to delineate the envelope of additivity of an isobologram are constructed directly from the mathematical fits to the single agent survival data, which in this case, are described by the linear quadratic parameters α and β . Therefore, a linear quadratic fit to the concomitant exposure data in which the value of α has increased by a substantial amount is more likely to mean that supra additivity is occurring at lower

doses.

In the study by Hennequin *et al* (1994), plots of fractional additivities as a function of radiation dose or length of drug exposure increased as a function of dose and exposure time from additive to greater and greater supra additivity. The results of the present work, plotted in the same manner in Figures 3.2.4, 3.2.5, and 3.2.6, did not reveal the same trend.

The concentration dependence was also observable after isobologram analysis. As the concentration of CPT used was increased, greater additive effects and more supra additive effects were observed. In some cases at the 25 μ M concentration of CPT, the experimental point lay to the left of one of the mode 2 lines, but still to the right of the other, thus remaining in the envelope of additivity. If, for example, the point lay to the left of the mode 2 line calculated when the LDR irradiation dose is given first, then one may deduce supra additive effects if radiation were given first in the concomitant exposure. However, this scenario is irrelevant to the present study since for each concomitant exposure, the radiation and the drug were present simultaneously for equal amounts of time. Therefore, only those points to the left of both mode 2 lines were considered to be representative of supra additive effects. Overall, the 25 μ M concentration of CPT produced the greatest sensitising effects when added to LDR irradiation, as determined by isobologram analysis. This general result is in good agreement with what was found by examination of the survival curves.

4.3 CHEF Electrophoresis

As mentioned earlier, CHEF electrophoresis was used to test the hypothesis that the increased cell killing after concomitant exposure of Sk Mel-3 cells to LDR irradiation and CPT is a result of an increase in the level of residual DNA DSBs in the cells. Experiments were done at the 10 μ M and 25 μ M CPT concentration levels, but the results were inconclusive. All values of FAR observed were very small and not significantly different from zero. Others, however, have found CHEF electrophoresis to be quite sensitive, with a resolution limit equivalent to the effect of a γ -ray dose of about 1 Gy (Blocher *et al.*, 1989). As explained previously, the CHEF requires a much greater number of cells to run a sample than does the comet assay. The nature of the LDR irradiator used in this study requires that the cells to be irradiated are grown in vials with a small (1.3 cm²) growing surface. This makes it difficult to obtain a large number of cells for a sample. Perhaps if samples could have been run at a higher cell concentration, CHEF would have been able to detect more DNA DSBs (Blocher & Kunhi, 1990). Also, the fact that the DNA standards ran properly suggests that the levels of DNA damage resulting from the treatments to which CHEF electrophoresis was applied were simply not great enough to be detected with this assay.

4.4 Comet Assay

The inconclusive results of the CHEF assay necessitated the use of another method for measuring the level of DNA DSBs in a treated population of cells. The single cell gel electrophoresis technique, known as the comet assay, was employed. The comet parameters of tail length and tail moment were plotted as normalized tail length and normalized tail moment. These are unitless values, each normalized to the effects of the corresponding control dose. Normalization of these parameters has been done in another study (Hu & Hill, 1996). This same study used the neutral comet assay to evaluate the DNA damage in CHO cells after exposure to LDR irradiation at 9 cGy min^{-1} . The dose rate used in the present study, also termed low dose rate, is lower by a factor of about 10, at $0.88 \text{ cGy min}^{-1}$. It is not known whether the neutral comet assay has been used to evaluate residual DNA DSBs in cells exposed to ionizing radiation at such a low dose rate. The dose range used in the study by Hu and Hill (1996) was from 20 Gy to 100 Gy. The lowest dose in this range is higher than the highest dose used in the present study, making a direct comparison of results less meaningful. However, the results agreed to the extent that, in both studies, normalized tail moment increased with increasing dose of radiation. Normalized tail length was not used in that study.

As is seen from Table 3.4.1, there was some variability between the two experiments. The experiments were performed on different days, and the gels for each experiment were also run on different days. Therefore, there is a possibility that the results were influenced by a systematic error. For all but three dose points, the 4 Gy and 16 Gy points from the

concomitant exposure to LDRI and CPT, and the 4 Gy point for the CPT alone exposure, the values of TM and TL in the second experiment were larger than those observed in the first. However, this held true even for the control comets, to which the rest of the data was normalized.

In a study by Olive and Banath (1997), the DNA damaging effects of several drugs on SiHa human cervical carcinoma cell spheroids were evaluated using the neutral comet assay. Included in these drugs was the topoisomerase II poison etoposide. DNA damage increased with increasing drug concentration and time of exposure for all drugs tested. These results agree with the trend for Sk Mel-3 cells exposed to 25 μ M CPT. Normalized tail moment and normalized tail length increased with increasing time of exposure.

The shape of the curves fitted to the normalized tail moment and tail length data, and in particular those for the concomitant exposure to LDR irradiation and CPT, are similar to the shape described by Kent *et al* (1995) after CHO-K1 cells were subjected to HDR irradiation. Tail length was found to increase linearly with dose, but had a tendency to plateau at higher doses. Tail moment increased linearly with dose and had no tendency to plateau. However, in this study the dose range extended to 100 Gy, and tail length reached a plateau only after a dose of 40 Gy. In spite of different treatment conditions, the results agree in their finding that tail length, as a measure of DNA damage, is useful only at the lower end of the radiation dose range. The dose at which tail length begins to plateau and is no longer a good measure of the amount of DNA damage sustained by the cells will depend on the exact type of radiation treatment being used, and any modifications to this treatment, such as the addition of a second cytotoxic agent.

The increased values of normalized tail length and moment for the concomitant exposure to LDR irradiation and CPT indicate the presence of more residual DNA DSBs relative to either LDR irradiation alone or CPT alone. Olive and Banath (1997) correlated tail moment and survival in order to evaluate the strength of the relationship between the two. The same was done here for both normalized tail length and normalized tail moment. The strongest negative correlation obtained was between survival and normalized tail length for the concomitant exposure to LDR irradiation and CPT. The next strongest correlation was between survival and normalized tail moment for the same exposure. The fact that the strongest negative correlations between survival and DNA damage in the form of DSBs were for this concomitant exposure suggests that perhaps most of the damage sustained was in the form of unrejoined DSBs. Cells whose DNA DSBs are repaired, that is to say, rejoined correctly, will survive and reproduce to form a colony of cells. Correlations between cell survival and DNA DSBs will be strong if most of the rejoined DSBs were rejoined correctly. A high frequency of misrepair could lead to weak correlations between survival and DNA DSBs since cells with misrepaired DSBs will most likely not be clonogenic survivors, but neither does their DNA contain DSBs to account for the loss of reproductive integrity.

Chapter 5

Conclusions

The main conclusions of this work are as follows:

1. Camptothecin is effective at potentiating low dose rate irradiation in its killing of radioresistant Sk Mel-3 human melanoma cells.
2. Based on the results of the clonogenic assay, potentiation of LDR irradiation killing occurs to a larger extent the higher the concentration of CPT used. Isobologram analysis confirmed this with mostly sub additive effects at 1 μ M, mostly additive effects at 10 μ M, and some supra additive as well as additive effects at 25 μ M. This concentration dependence with respect to the effects of the interaction between LDR irradiation and CPT is a new result not apparent from previous work. The other studies known to have examined the combination of LDRI and CPT used only one concentration of CPT.

3. Large increases in the α component of the normalized concomitant exposure survival curves relative to the value of α for LDR irradiation alone showed that the potentiation at 10 μM and 25 μM CPT is not due to the inhibition of sublethal damage repair. The corresponding β components even decreased. This new and unexpected result has not been found in previous studies.
4. Strong negative correlations ($r < -0.9$) between clonogenic surviving fraction and normalized comet tail length and tail moment for concomitant exposure to LDR irradiation and 25 μM CPT revealed another new result, that the potentiation of LDR irradiation by CPT was related to an increase in the number of residual DNA double strand breaks.
5. CPT analogues may, therefore, be useful as adjuncts to LDR irradiation since the targeting of LDR irradiation to tumour tissue may minimize undesired potentiation of LDR irradiation to normal tissue.

5.1 Future Research

The Sk Mel-3 cell line used in this work was chosen because of its high level of radioresistance and cross resistance to CPT, not because melanoma is a type of cancer particularly suited to treatment by brachytherapy. A logical next step would be to test other types of tumour cells such as colorectal (Martinez-Monge *et al.*, 1999) and ovarian (Dent *et al.*, 2000), for the same effect that was seen with the Sk Mel-3 cells. Many tumours and tumour cell lines, including colorectal and ovarian, have been shown to possess elevated levels

of topoisomerase I. Because of this, there is a potential for a differential sensitizing effect between the tumour and surrounding normal tissue (Lamond *et al.*, 1996). Also, the Food and Drug Administration (USA) has recently approved the use of CPT analogues as second-line treatments for cancer in these two sites, although not in combination with any form of radiation treatment.

The present study showed, through analysis of changes in the α and β parameters, that the sensitising effect of CPT was not due to the inhibition of SLDR. Whether or not the same effect is seen in other tumour cell types under conditions of LDR irradiation should also be investigated. The extent to which a population of cells is able to perform SLDR is stated in terms of the recovery ratio. It is measured by performing split dose experiments under conditions of HDR irradiation, and, therefore, perhaps the results of such experiments cannot be translated directly to what is happening under conditions of LDR irradiation.

Since the present study was done using confluent Sk Mel-3 cells, it would be instructive to perform the same experiments with exponential phase (actively cycling) cultures of the same cells in order to investigate the effects of cell cycle on the potentiating effects of CPT. Solid tumours consist of both non-actively cycling (confluent) and actively cycling (exponential phase) cells. It would also be beneficial to measure topoisomerase I content and activity in Sk Mel-3 cells, both confluent and actively cycling, before, during, and after treatment. Topoisomerase I is the target of CPT, and changes in the levels of its content and activity during treatment may affect the final outcome. At least one study has shown that topoisomerase I activity was down-regulated in mammalian cells following ionizing HDR irradiation (Boothman *et al.*, 1992).

Finally, since CPT itself is not used in the clinic, similar studies to the present work should be undertaken using some of the more clinically relevant topoisomerase I inhibitors and analogues of CPT such as Topotecan and CPT-11, two of those approved by the Food and Drug administration (USA) for clinical use.

Appendix

The plots of fractional additivity relative to radiation dose or to CPT exposure time, whether sub (infra) additive, supra additive, or simply additive, represent data from individual isobolograms. In this study, each such plot shows the combined data of 5 isobolograms. The individual isobolograms are shown here in Figures A-1, A-2, and A-3, for CPT concentrations of 1 μM , 10 μM , and 25 μM respectively. The unit of the y-axes marked LDRI is dose in Gy. The same unit is used on the x-axes marked CPT. In this instance, however, it represents only time of exposure. It was used for convenience since the CPT was left on the cells for durations equal to the amounts of time required to give each of the radiation doses employed using the LDR irradiator. The values can be converted into units of time by dividing by the dose rate of the irradiator. The single point in each isobologram is a survival point for the concomitant exposure produced by the doses of LDRI and CPT that intersect at the point. The position of each point with respect to the mode 1 and mode 2 lines generated for the same level of survival, termed isosurvival, determines its classification as either sub-additive, additive, or supra-additive.

The code for the C program written to calculate the values necessary for plotting the individual isobolograms follows Figures A-1, A-2, and A-3. *Italicized text is commentary identifying the different components of the program.* The code is based on the theory of section 2.2.3 and employs the equations found therein.

Figure A-1 Individual isobolograms for the combination of LDRI and 1 μ M CPT. (A), (B), (C), (D), and (E) were generated for isosurvival levels equal to the survival after concomitant exposure to 1 μ M CPT and 2 Gy, 4 Gy, 8 Gy, 12 Gy, and 16 Gy respectively.

Figure A-2 Individual isobolograms for the combination of LDRI and 10 μM CPT. (A), (B), (C), (D), and (E) were generated for isosurvival levels equal to the survival after concomitant exposure to 10 μM CPT and 2 Gy, 4 Gy, 8 Gy, 12 Gy, and 16 Gy respectively.

Figure A-3 Individual isobolograms for the combination of LDRI and 25 μ M CPT. (A), (B), (C), (D), and (E) were generated for isosurvival levels equal to the survival after concomitant exposure to 25 μ M CPT and 2 Gy, 4 Gy, 8 Gy, 12 Gy, and 16 Gy respectively.

A program to calculate the values necessary for plotting an isobologram:

Solve for dose D from the L-Q model equation:

$$SF = \exp(-(\alpha * D + \beta * D^2))$$

To put this into the form of a simple quadratic equation with a zero RHS, take the natural logarithm of both sides:

$$\beta * D^2 + \alpha * D - \ln(SF) = 0$$

Therefore,

$$D = ((-\alpha + \sqrt{\alpha^2 - 4 * \beta * \ln(SF)}) / (2 * \beta))$$

```
#include <stdio.h>
#include <math.h>
#include <stddef.h>
#include <stdlib.h>
#include "VectorF.h"
```

```
void nrerror(char error_text[]);
float *vector(long nl, long nh);
void free_vector(float *v, long nl, long nh);
```

```
int main(void)
{
```

FILE	*fp;	<i>output file</i>
float	*doseA1st;	<i>vector of doses of agent A (radiation), A given first</i>
float	*doseB2nd;	<i>vector of doses of agent B (drug) Mode 1, A given first</i>
float	*doseBprime;	<i>vector of doses of agent B (drug) Mode 2, A given first</i>
float	*doseA2nd;	<i>vector of doses of agent A (radiation), B given first</i>
float	*doseB1st;	<i>vector of doses of agent B (drug) Mode 1, B given first</i>
float	*doseAprime;	<i>vector of doses of agent A (radiation) Mode 2, B given first</i>

```

float  isodoseA;      dose of agent A alone required to reach isosurvival
float  isodoseB;      dose of agent B alone required to reach isosurvival
float  step;          increment in survival after each iteration
float  sum;           (1.0 + isosf) used to calculate sfB values for output
float  sf;            surviving fraction at which doses A, B, and B' are
                     calculated

float  sfA;           surviving fraction at which dose A was calculated
float  sfB;           surviving fraction at which dose B was calculated

int    i;             counter
int    numpts;        number of survival points to evaluate
int    totalpts;      (numpts+ 1)

float  aone, atwo;     alpha from LQ fits to radiation and drug curves
                     respectively

float  bone, btwo;     beta from LQ fits to radiation and drug curves respectively
float  isosf;          level of survival under investigation

char   answer;         user response to prompt for correct data entry

fp =fopen("isobologram.xls", "w");      open output file

dataentry:    {

    enter the coefficients

    printf("Enter the alpha and beta values for the fit to the radiation alone curve:\n");
    printf("alpha 1 = ");
    scanf("%g", &aone);
    printf("beta 1 = ");
    scanf("%g", &bone);

    printf("Enter the alpha and beta values for the fit to the drug alone curve:\n");
    printf("alpha2 = ");
    scanf("%g", &atwo);
    printf("beta2 = ");
    scanf("%g", &btwo);

```

enter the desired level of isosurvival

```
printf("Enter the desired level of isosurvival:\n");
printf("isosurvival = ");
scanf("%f", &isof);
```

enter the number of points used to plot each isobologram curve

```
printf("Enter the desired number of points to calculate each isobologram
      curve:\n");
printf("numpts = ");
scanf("%d", &numpts);
```

```
}
```

display the values input for confirmation

```
printf("\nalpha 1 = (%g)\n", aone);
printf("\nbeta 1 = (%g)\n", bone);
printf("\nalpha 2 = (%g)\n", atwo);
printf("\nbeta 2 = (%g)\n", btwo);
printf("\nisosurvival = (%f)\n", isof);
printf("\nThere will be %d points of data, in addition to the initial\n", numpts);
printf("data point at isosurvival, giving %d points in total.\n", numpts+1);
```

data input corrections

```
printf("\nIf you would like to change any of the values entered press 'y'.");
printf("\nTo continue press any other key, then <Return>.\n");
printf("\nAnswer = ");
scanf(" %c", &answer);
```

```
printf("\nAnswer = (%c)\n", answer);
```

```
if (answer == 'y') goto dataentry;
```

```
else
```

Initialization of Vectors and Preliminary Calculations

```

step = (float)((1.0 - isosf)/numpts);
totalpts = numpts + 1;

doseA1st = vector(1, totalpts+1);
doseB2nd = vector(1, totalpts+1);
doseBprime = vector(1, totalpts+1);

doseA2nd = vector(1, totalpts+1);
doseB1st = vector(1, totalpts+1);
doseAprime = vector(1, totalpts+1);

isodoseA = (float)((-aone + sqrt((aone*aone) - 4*bone*log(isosf)))/(2*bone));
isodoseB = (float)((-atwo + sqrt((atwo*atwo) - 4*btwo*log(isosf)))/(2*btwo));

printf("\n\t\tIsodose for radiation alone = (%lf)\n", isodoseA);
printf("\n\t\tIsodose for drug alone = (%lf)\n", isodoseB);

```

Mode 1: Plot the doses of each agent that give log survival values that add up to the chosen level of isosurvival. For each agent, start from $sf = 1$ when determining the dose required. (ie. do not take into account the kill from agent A when determining the additional dose of agent B required to bring the level of survival down to the isosurvival level.)

$$sfA * sfB = isosf$$

*Mode 2: This method of addition takes into account the cell kill resulting from agent A when determining what dose of agent B is required to reduce survival to the level of isosurvival. Therefore, if A has reduced survival to 0.6, and isosurvival is 0.3, then the additional dose of B required to reach isosurvival is equal to the dose of B required to reach isosurvival minus the dose of B alone to reach 0.6.
(ie. $Db' = Db(sf=0.3) - Db(sf=0.6)$)*

Determine Mode 1 and Mode 2 values for Isobologram plots

```

i = 1;
sf=isosf;
for (i=1; i<=totalpts; i++)

```

```
{
```

Mode 1 and Mode 2 for agent A (radiation) given before agent B (drug)

```
doseA1st[i] = (float)((-aone + sqrt((aone*aone) - 4*bone*log(sf)))/(2*bone));
```

```
doseB2nd[i] = (float)((-atwo + sqrt((atwo*atwo) -  
4*btwo*log(isosf/sf)))/(2*btwo));
```

```
doseBprime[i] = isodoseB - (float)((-atwo + sqrt((atwo*atwo) -  
4*btwo*log(sf)))/(2*btwo));
```

Mode 2 for agent B (drug) given before agent A (radiation)

```
doseB1st[i] = (float)((-atwo + sqrt((atwo*atwo) - 4*btwo*log(sf)))/(2*btwo));
```

```
doseA2nd[i] = (float)((-aone + sqrt((aone*aone) -  
4*bone*log(isosf/sf)))/(2*bone));
```

```
doseAprime[i] = isodoseA - (float)((-aone + sqrt((aone*aone) -  
4*bone*log(sf)))/(2*bone));
```

```
sf=sf+step;
```

```
}
```

display the results:

*columns 1-4: Mode 1
columns 5-7: Mode 2*

*col.1 sfA
col.2 doseA(first)
col.3 doseB(second)
col.4 sfB
col.5 doseBprime
col.6 doseB(first)
col.7 doseA(prime)*

```

i = 1;
sum = (float)(1.0 + isosf);

sf=isosf;

for (i=1; i<=totalpts; i++)
{
    sfA = sf;
    sfB = sum - sf;

    fprintf(fp, "%2f\t%f\t%f\t%2f\t%f\t%f\t%f\n", sfA, doseA1st[i],
        doseB2nd[i], sfB, doseBprime[i], doseB1st[i], doseAprime[i]);

    printf("%2f\t%f\t%f\t%2f\t%f\t%f\t%f\n", sfA, doseA1st[i], doseB2nd[i],
        sfB, doseBprime[i], doseB1st[i], doseAprime[i]);

    sf=sf+step;
}

printf("\n\t\tIsodoseA = (%f)\n", isodoseA);
printf("\n\t\tIsodoseB = (%f)\n", isodoseB);

fprintf(fp, "\nstep = (%f)\n", step);

free_vector(doseA1st, 1, totalpts+1);
free_vector(doseB2nd, 1, totalpts+1);
free_vector(doseBprime, 1, totalpts+1);
free_vector(doseA2nd, 1, totalpts+1);
free_vector(doseB1st, 1, totalpts+1);
free_vector(doseAprime, 1, totalpts+1);

fclose(fp);
printf("\n");

return 0;
}

#undef NRANSI

```


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